Mast cells in advanced atherosclerosis: from human plaque stability to new therapeutic targets

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Chapter 1

General introduction

Cardiovascular disease

Cardiovascular diseases (CVDs) are responsible for high mortality rates worldwide, representing 32% of all global death[1]. CVD refers to a group of diseases affecting the heart and the vasculature, including coronary artery disease, heart failure and peripheral artery disease. The underlying pathology of most CVDs is atherosclerosis, which is characterized by the gradual build-up of atherosclerotic plaques in the subendothelial layer of medium to large arteries[2,3]. These plaques are composed of lipids, inflammatory cells and fibrous elements, such as collagen and elastin. The progression of atherosclerotic plaques causes arterial narrowing, called stenosis, which obstructs blood flow and leads to oxygen and nutrient deprivation in the affected area[4]. Plaque development is typically clinically silent until the artery is significantly narrowed (over 70% stenosis), leading to the onset of symptoms, such as chest pain. More severe symptoms occur when a destabilized plaque ruptures, resulting in thrombus formation. The thrombus can partially or completely block the artery and lead to (fatal) clinical events, such as a stroke in the brain or a heart attack[4].

Several risk factors contribute to the development and progression of CVD, encompassing both modifiable and non-modifiable risk factors. Major modifiable risk factors that increase the risk for developing CVD include lifestyle and behavioral factors, such as smoking, high cholesterol levels due to unhealthy diet and lack of physical activity. Therefore, adopting a healthy lifestyle can significantly reduce CVD risk. Studies have demonstrated that individuals who maintain a healthy lifestyle experience nearly an 80% reduction in the incidence of heart attacks[5,6]. Non-modifiable risk factors include age, sex and genetic predisposition, such as familial hypercholesterolemia[7]. Moreover, individuals with chronic inflammatory or autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, are at an increased risk of developing CVD[8]. Current therapeutic strategies to prevent acute cardiovascular syndromes are primarily focused on lowering plasma cholesterol levels by using, for example, statins and, more recently, by inhibiting PCSK9[9]. Once a cardiovascular event has occurred, anti-thrombotic treatment and/or surgical interventions aimed at revascularization are often applied. Although these approaches effectively reduce CVD risk, the high mortality rates suggest that current treatments/interventions remain inadequate for a large proportion of high-risk individuals, highlighting a significant need for new therapeutic strategies.

Atherosclerosis has long been considered as a primarily lipid-driven disease. More recently, however, inflammation has emerged as another key driver of atherosclerosis. In the past decade, several clinical trials have proven that targeting the immune system is a promising strategy to reduce cardiovascular risk. In 2017, the CANTOS trial was the first randomized placebo-controlled clinical trial specifically targeting inflammation in atherosclerosis[10]. In this trial, coronary artery disease patients with a history of myocardial infarction and elevated

high-sensitivity C-reactive protein (hsCRP) were treated with canakinumab, a monoclonal antibody neutralizing interleukin (IL)-1β. Treatment with canakinumab significantly lowered plasma levels of hsCRP and IL-6 without affecting lipid levels, resulting in a reduced risk of cardiovascular events by 15%[10]. Since the CANTOS trial, numerous other trials have been designed targeting the immune system. For instance, the safety of low-dose IL-2 administration and ability to induce an anti-inflammatory T cell response in atherosclerosis patients was tested in the LILACS trial[11,12], which led to the currently ongoing IVORY trial to measure the effects of low-dose IL-2 on major cardiovascular events (MACE)[13]. Furthermore, the RESCUE and RESCUE-2 trials focused on targeting IL-6 in chronic kidney disease patients, who are at increased risk of developing atherosclerotic cardiovascular disease (ASCVD)[14,15]. Targeting IL-6 using ziltivekimab showed a profound dose-dependent reduction in inflammatory biomarker hsCRP in these patients[14,15]. Based on these promising results, a follow-up trial (ZEUS) is currently ongoing to examine whether IL-6 targeting can lower the incidence of MACE[16]. Altogether, these data further support that the immune system is a promising target. However, these clinical trials are not without severe adverse events, as systemic immune suppression was associated with a higher risk of fatal infections[10]. Therefore, there is an urgent need to discover new targets for possible immunomodulatory therapies that directly treat atherosclerosis with limited adverse events.

Development of atherosclerosis

In healthy state, arteries consist of three main layers: the intima, media, and adventitia. The intima is the innermost layer composed of endothelial cells that acts as a selective barrier between the blood and the arterial wall[17]. The media primarily consists of vascular smooth muscle cells (VSMCs) and elastic lamina and the adventitial layer is composed of connective tissue, *e.g.* collagen- and nerve fibers and fibroblasts[17]. In unbranched areas of the artery, circulating blood generates a frictional force, known as laminar shear stress, which is necessary to maintain vascular physiology[18]. However, in areas with bifurcation, branch points or major curvature, blood flow patterns are disturbed and exposed to oscillatory shear stress (OSS), which contributes to endothelial dysfunction by upregulating adhesion molecules and inducing an inflammatory response[18]. Endothelial dysfunction also leads to impairment of tight junctions, which regulate cell permeability, leading to vascular leakage. In addition to OSS, endothelial dysfunction is initiated by pro-atherogenic risk factors, such as hypercholesterolemia, diabetes mellitus, smoking and aging[19,20].

Atherosclerosis initiation

Endothelial dysfunction allows lipoproteins to infiltrate the intimal layer, which is a characteristic of atherosclerotic plaque formation. Lipoproteins are complex molecules consisting of lipids and proteins and are important regulators of lipid levels in the circulation. Moreover, lipoproteins facilitate the transportation of hydrophobic particles, such as

cholesterol and triglycerides. Thereby, lipoproteins ensure that cells receive cholesterol, which is an essential component for cell membranes and steroid hormone synthesis, and triglycerides, that serve as a primary energy source for the body[21]. Lipoproteins are classified based on their size, density and apolipoprotein content, including chylomicrons, lipoprotein(a) (Lp(a)), very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL)[22]. Among these, HDL is often described as 'good cholesterol', because it removes excess cholesterol from the bloodstream and transports it back to the liver, a process known as reverse cholesterol transport[23]. In contrast, LDL is considered as 'bad cholesterol', since elevated circulating LDL levels can lead to increased cholesterol deposition and accumulation in the arterial wall, contributing to atherosclerotic plaque formation[24]. In the arterial wall, LDL particles are prone to undergo oxidative modifications, resulting in oxidized and aggregated LDL particles (oxLDL). Accumulation of oxLDL in the intima stimulates the expression of adhesion molecules on the surface of activated endothelial cells, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Simultaneously, endothelial cells release chemokines, such as C-C motif Chemokine Ligand 2 (CCL2) and CCL5, into their environment, which attracts circulating monocytes to the site of inflammation[25]. These monocytes firmly attach to the endothelium by binding their integrins to the upregulated adhesion molecules on the endothelial surface and transmigrate into the intima. Once migrated, monocytes differentiate into macrophages in response to locally produced macrophage colony-stimulating factor (M-CSF) and other cytokines[2,3]. Macrophages recognize modified lipoproteins, primarily oxLDL, as foreign and facilitate their uptake via scavenger receptors or phagocytosis. The excessive uptake of modified lipoproteins by macrophages transforms them into lipid-laden foam cells. Cholesterol efflux from foam cells is mediated by ATP-binding cassette transporters ABCA1 and ABCG1, which facilitate the removal of excess cholesterol to HDL and thereby reducing lipid accumulation in these cells. Impairment of this cholesterol efflux process results in enhanced foam cell formation. The buildup of these foam cells in the intimal layer of the arterial wall leads to the formation of fatty streaks, which represent the earliest stage of atherosclerotic lesions[26]. Fatty streak formation begins in early adolescence and typically remains stable for years without causing clinical symptoms. However, over time, these fatty streaks can progress into more advanced plaques and could eventually lead to severe clinical events. **Figure 1** summarizes the development of fatty streaks.

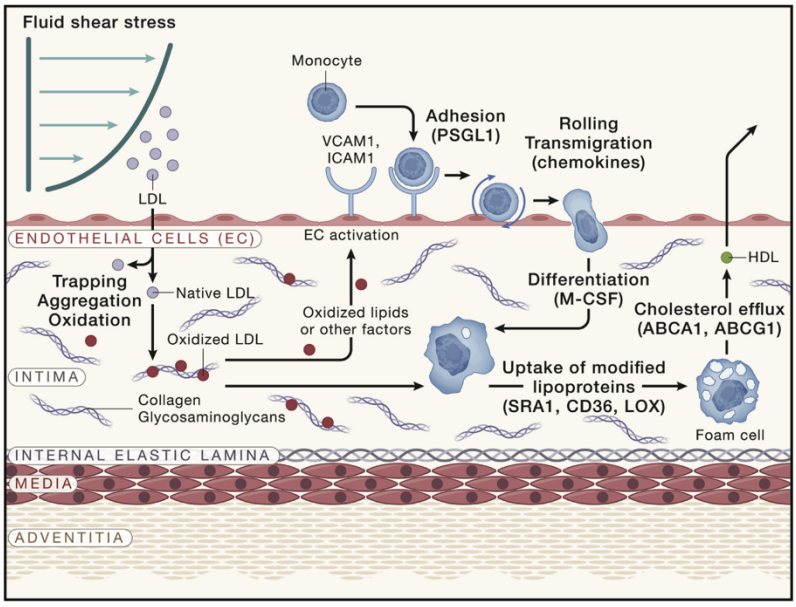

Figure 1. Fatty streak formation. Shear stress induces endothelial dysfunction, contributing to increased permeability of the vessel wall. Lipoproteins, primarily low-density lipoprotein (LDL), enter the intima and become oxidized (oxLDL). Endothelial cells are activated and express adhesion molecules on their cell surface. Circulating monocytes are recruited to the site of inflammation, attach to the adhesion molecules, and transmigrate into the intima. In response to M-CSF, migrated monocytes differentiate in macrophages. Macrophages take up the oxidized lipoproteins and become foam cells. *Adapted from Björkegren et al. (2022). Cell, 185(10), 1630–1645.*

Advanced atherosclerosis

Following plaque initiation, fatty streaks progress into more advanced plaques through continuous accumulation of lipids and foam cells, and infiltration of additional leukocytes, primarily T cells. Interaction of T cells with antigen-presenting cells, like macrophages, leads to T cell activation and the secretion of pro-inflammatory cytokines, such as interferon(IFN)-γ[2,3]. Cytokine and chemokine secretion by foam cells and T cells enhances the recruitment of other leukocytes to the plaque, including neutrophils, B cells and mast cell progenitors. The sustained influx of immune cells contributes to a chronic immune response in the plaque, driving plaque progression. The progression of fatty streaks to more advanced plaques also involves the migration of VSMCs from the media into the intima layer, where they can undergo phenotypic switching[27–30]. In healthy arteries, VSMCs exhibit a contractile phenotype, regulating vascular tone. However, in diseased arteries, VSMCs shift towards a proliferative and migratory phenotype, covering the subendothelial layer of the plaque. Here, these migratory VSMCs form a fibrous cap over the lipid- and immune cell-rich core of the plaque by producing extracellular matrix components, such as collagen, elastin and proteoglycans[31]. Upon phenotypic switching, VSMCs could also differentiate into a macrophage-like phenotype, enabling them to take up lipids and become foam cells[28]. Lipid overload in the cytoplasm of macrophages and VSMCs leads to lipotoxicity and causes these cells to go in apoptosis. The subsequent clearance of apoptotic cells mediated by phagocytes, a process

called efferocytosis, is important for the tissue homeostasis[32]. In advanced plaques, however, the efferocytosis capacity of phagocytes becomes impaired resulting in secondary necrosis and associated inflammation[33]. Together, exacerbated cell apoptosis and impaired efferocytosis lead to the formation of a lipid-rich necrotic core, a hallmark of plaque vulnerability[34]. Moreover, failure of macrophages to clear apoptotic bodies leads to secretion of pro-inflammatory cytokines, like TNFα, which induces osteogenic gene expression in VSMCs[35]. This, together with the release of calcifying extracellular vesicles by VSMCs, initiates microcalcification in highly inflamed plaques, which has been linked to plaque instability. Over time, accumulation of calcified nodules leading to macrocalcification stabilizes the advanced plaque[35,36]. In addition to chronic inflammation, calcification and a lipid-rich necrotic core, advanced plaques are characterized by neovascularization and intraplaque hemorrhage (IPH)[4,37,38]. In response to hypoxia and inflammation in the plaque environment, angiogenic factors are released that stimulate the development of new microvessels[37]. Neovascularization increases inflammatory burden in the plaque by facilitating the infiltration of circulating immune cells. Moreover, leakiness or rupture of immature microvessels can cause intraplaque hemorrhage, contributing to plaque destabilization[38]. Advanced atherosclerotic plaque destabilization is further aggravated by thinning of the fibrous cap. Local inflammatory cells, including mast cells and macrophages, produce matrix metalloproteinases (MMPs), which degrade extracellular matrix components, thus reducing fibrous' cap structural integrity[34,39]. Subsequent plaque rupture can trigger thrombus formation. Additionally, thrombotic events may arise from superficial erosion, a condition characterized by an abundant presence of VSMCs and extracellular matrix with minimal lipid and foam cell accumulation[40]. This type of thrombosis differs from thrombosis due to fibrous cap rupture and is initiated by endothelial cell apoptosis, but the exact mechanism is still unclear[40]. Both plaque rupture and erosion can result in complete arterial occlusion and severe clinical complications, such as myocardial infarction. **Figure 2** demonstrates the processes involved in the development of advanced plaques.

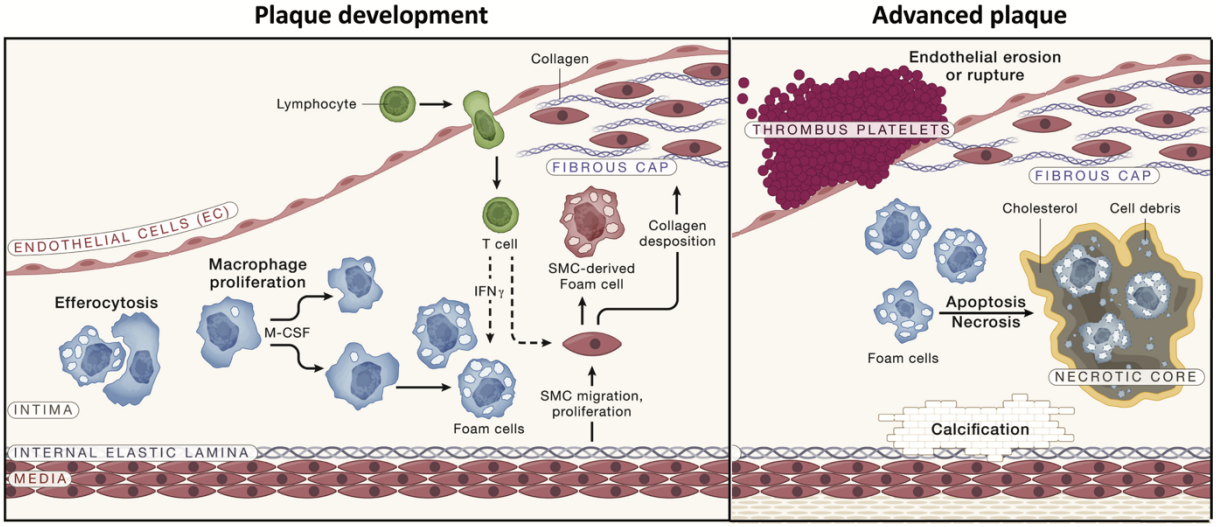

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Figure 2. Development of advanced plaques. Macrophages proliferate in the plaque and facilitate the clearance of apoptotic foam cells, a process called efferocytosis. Vascular smooth muscle cells (VSMCs) migrate from the media to the intima, where they cover the endothelial layer. VSMCs proliferate and produce extracellular matrix proteins forming a fibrous cap. T cells, and other leukocytes, are recruited to the plaque environment, exacerbating the chronic immune response. In advanced plaques, efferocytosis is impaired and apoptotic foam cells undergo secondary necrosis, forming a lipid-rich necrotic core. Plaque destabilization is further aggravated by thinning of the fibrous cap, which ultimately leads to plaque rupture and thrombus formation. *Adapted and modified from Björkegren et al. (2022). Cell, 185(10), 1630–1645.*

The immune system in atherosclerosis

The immune system protects the body against infection and tissue damage and is divided into two main components: innate and adaptive immunity[41]. Innate immunity acts as the first non-specific immunological mechanism of defense that responds rapidly to pathogens and tissue damage. The innate immune response is mediated by various cells including monocytes, macrophages, dendritic cells, neutrophils, natural killer cells and granulocytes like mast cells and basophils. These cells express pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), allowing them to process them and present small peptide fragments, also known as antigens, on their surface. Adaptive immune cells, involving T cells and B cells, act in an antigen-specific manner by recognizing specific antigens presented by innate immune cells. Unlike the innate immune response, adaptive immunity develops slowly. However, an important feature of the adaptive immune response is the development of immunological memory, leading to a faster and stronger immune response upon subsequent exposure to the same antigen[42]. In the etiology of atherosclerosis, both the innate and adaptive immune response play an essential role. The contribution of immune cells to atherosclerosis that are relevant for this thesis will be discussed below.

<u>Monocytes and macrophages</u>

Monocytes are innate immune cells that originate from hematopoietic stem cells in the bone marrow and circulate in the blood. Monocytes serve homeostatic and immunomodulatory functions depending on their subtype. However, the versatile properties and substantial degree of heterogeneity of this cell lineage makes it difficult to assign clear-cut phenotypes and functional properties. Historically, three subtypes of monocytes are commonly described in humans based on the surface expression of CD14 and CD16: non-classical, intermediate and classical monocytes[43]. In mice, two main monocytes subtypes are identified based on Ly6C expression, where $Ly6C^{hi}$ monocytes correspond classical monocytes and $Ly6C^{lo}$ monocytes correspond non-classical monocytes[44]. The most abundant monocyte subset in the blood is the classical monocyte, characterized by high expression of CD14 and low expression of CD16 ($CD14^{++}CD16^{-}$). Classical monocytes are mainly involved in phagocytosis and the secretion of

pro-inflammatory cytokines, like TNFα, exacerbating local inflammation. In atherosclerosis, classical monocytes are recruited to the site of inflammation, where they migrate from the blood into the plaque and differentiate in macrophages or dendritic cells. Non-classical monocytes are characterized as $CD14^{lo}CD16^{++}$ in humans. At steady state, non-classical monocytes patrol the endothelium of the vasculature and rapidly respond to injury. Intermediate monocytes ($CD14^{+}CD16^{+}$) have been shown to express high levels of major histocompatibility complex II (MHCII) and exhibit antigen presenting capacities[43,45]. Recently, novel human monocytes subsets have been described in individuals with common CVD risk factors based on extensive transcriptome and protein analysis, including an IFN-responsive subset and $MHCII^{hi}$ subset[46]. Circulating monocyte numbers and their activation status are increased in hypercholesterolemic individuals[47]. Moreover, multiple clinical studies described an association between monocytes and CVD risk, highlighting their critical involvement in atherosclerosis development[48,49].

Upon entering the plaque, monocytes differentiate into macrophages, which can further polarize in different subtypes in response to environmental stimuli. Macrophages are typically classified into two main subtypes, M1 and M2 macrophages, representing opposite ends the polarization spectrum[50]. M1 macrophages are polarized in response to T helper 1 (Th1) produced cytokines like IFNγ, while M2 macrophages are polarized by exposure to Th2 cytokines like IL-4 and IL-13. In the atherosclerotic plaque, M1 macrophages are the most abundant subtype producing high levels of pro-inflammatory cytokines and thereby enhancing plaque progression. In contrast, M2 macrophages counteract inflammation by secretion of IL-10 and transforming growth factor (TGF)β and are considered anti-inflammatory. Similar to monocytes, macrophages are versatile cells and macrophage polarization is a dynamic process, resulting in many intermediate subsets. Recent advances in the development of technologies like cytometry by time-of-flight (CyTOF) and single-cell RNA sequencing led to an extended classification of macrophage into four common subpopulations in human and mouse atherosclerotic plaques: resident-like, foamy/$Trem2^{hi}$, inflammatory and interferon-inducible (IFNIC) macrophages[51–56]. Resident-like macrophages originate from embryonic precursors and are characterized by *Lyve1* expression. This macrophage subtype resides predominantly in the adventitia of both healthy and diseased arteries, where they inhibit the production of collagen by VSMCs[57]. $Trem2^{hi}$ macrophages are lipid-laden foam cells that accumulate in the plaque, but are not found in healthy arteries. They express genes that are associated with lipid-handling and pathway analysis of intraplaque $TREM2^{hi}$ macrophages indeed showed enrichment in lipid metabolism and regulation of cholesterol efflux[51]. Recent single-cell RNA sequencing analysis of human carotid plaques demonstrated that these homeostatic $TREM2^{hi}$ macrophages can transition into inflammatory $PLIN2^{hi}/TREM1^{hi}$ lipid-associated macrophages through a TLR2-dependent mechanism[58]. Inflammatory macrophages represent the largest macrophage subset in the plaque and are considered as the main drivers of inflammation. This subset is enriched for pro-inflammatory genes, such as

Il1b, Cxcl2 and *Tnf*, and resemble the M1 macrophage phenotype[56,59]. IFNIC macrophages are a small cluster of cells that have an enriched gene expression pattern characteristic of a type 1 IFN response. Their exact function in atherosclerosis is still unclear and remains to be elucidated[56,60].

Neutrophils

Neutrophils are short-lived granulocytes that originate from hematopoietic stem cell origin in the bone marrow. Neutrophils are the most prevalent type of leukocyte in human blood and are one of the first cells to be recruited to the site of inflammation upon infection or tissue damage. Upon arrival, activated neutrophils produce and release reactive oxygen species (ROS) in their environment. In addition, degranulation lead to the release preformed granular content, containing proteases such as myeloperoxidase (MPO), cathepsin G and neutrophil elastase (NE)[61]. Eventually, neutrophils undergo apoptosis, which is tightly regulated to prevent tissue damage. Clearance of apoptotic cells by macrophages via efferocytosis results in the release of anti-inflammatory signals. Besides apoptosis, a specialized form of cell death is identified for neutrophils, called NETosis, that results in the formation and release of neutrophil extracellular traps (NETs). NETs are web-like structures composed of DNA-histone complexes and granular proteins. The process of NETosis can be induced by various stimuli, including inflammatory signals and ROS, leading to chromatin decondensation in the nucleus and mixing with cytoplasmic granular proteins. Subsequent rupture of the cell membrane releases the NETs in the extracellular environment to capture pathogens. In contrast to apoptosis, NETosis is a pro-inflammatory process and can lead to tissue damage[62,63].

Neutrophils contribute to all stages of atherosclerosis. In the early stage of atherosclerosis development, chemokine signalling promotes neutrophil recruitment to the plaque. At the luminal site, activated neutrophils facilitate recruitment, firm adhesion and extravasation of monocytes via the release of chemokines and cathepsin G[64]. Immunohistochemical staining using Ly6G and CD66b antibodies allowed identification of neutrophils in early and advanced murine and human plaques, respectively, providing evidence that neutrophils also migrate into the plaque[65,66]. In addition to regulating monocyte entry in the plaque, neutrophils also accelerate foam cell formation by releasing MPO, which leads to oxidation of LDL[64,67]. In advanced atherosclerosis, neutrophils mainly contribute to plaque destabilization through the secretion of MMPs, in particular MMP-2 and MMP-9, which degrade the extracellular matrix components that leads to thinning of the fibrous cap[68]. In support of their contribution to advanced atherosclerosis, high intraplaque neutrophils numbers associated with hallmarks of rupture-prone atherosclerotic plaques[66].

T cells

Recent mapping of the immune cell landscape in the atherosclerotic plaque revealed that T cells are the predominant leukocyte population[54]. T cells are part of the adaptive immune

system that originate from hematopoietic stem cells in the bone marrow, where they differentiate into common lymphoid precursors (CLPs). CLPs migrate to the thymus to undergo a process of maturation and selection to become functional naïve $CD4^+$ or $CD8^+$ T cells[69]. The activation of naïve T cells is tightly regulated and requires three signals. The first signal includes antigen recognition in peripheral lymphoid organs. T cell receptors (TCRs) expressed on the surface of naïve T cells bind to a specific antigen presented by MHC-molecules on antigen presenting cells (APCs), like macrophages and dendritic cells. $CD4^+$ T cells recognize antigens presented by MHC class II molecules, while $CD8^+$ T cells recognize antigens presented by MHC class I molecules. Upon MHC-TCR interaction, naïve T cells clonally expand and differentiate into effector T cells. Simultaneously, the second signal is provided by ligand-receptor interaction of costimulatory or coinhibitory molecules on T cells and APCs. Costimulatory signaling activates T cells, whereas coinhibitory signaling suppresses T cell effector function. Finally, the third signal is provided by cytokines, which regulates the differentiation of naïve T cells into specific subtypes, including $CD4^+$ T helper cells (Th) or cytotoxic $CD8^+$ T cells (CTLs)[70].

In the atherosclerotic plaque, multiple $CD4^+$ Th subsets have been identified, including Th1, Th2, Th17 and regulatory T cells (Treg), which all have distinct roles in disease progression. Th1 cells, characterized by the expression of T-box transcription factor TBX21 (T-bet), are the most prominent subset in the plaque and have been shown to exert a pro-atherogenic role via the secretion of IFNγ and TNFα[70]. Experimental studies showed that T-bet or IFNγ deficiency protected mice from atherosclerosis[71,72], whereas administration of IFNγ enhanced plaque development in mice[73]. The exact function of Th2 and Th17 cells in atherosclerosis remains controversial and depends on the disease stage and experimental mouse model. For instance, the main Th2 cytokine IL-4 has been shown to suppress the Th1 response and ameliorate atherosclerosis in *Apoe*$^{-/-}$ mice[74], whereas IL-4 deficiency in *Ldlr*$^{-/-}$ mice that were fed a high-fat diet was also found to reduce plaque formation[75]. Similar to IL-4, conflicting results have been reported on the role of Th17-specific cytokine IL-17 in atherosclerosis. Some experimental studies suggest that IL-17 may have a pro-atherogenic function[76,77], whereas other studies indicate it could be atheroprotective[78] or have no significant effect[79]. Another key subset of $CD4^+$ T cells are Tregs, which are protective against atherosclerosis via the secretion of anti-inflammatory cytokines IL-10 and TGFβ. Moreover, Tregs have the capacity to directly inhibit the proliferation of effector T cells, especially Th1 and Th17 cells[80]. Additional subsets such as Th9, Th22 and follicular T helper (Tfh) cells have been identified, but their exact role in atherosclerosis development remains to be elucidated.

Upon interaction with antigen-MHC I complexes, $CD8^+$ T cells generally differentiate into a cytotoxic subset (CTL), which is traditionally recognized for its ability to kill infected cells. CTLs exert three main functions, including the secretion of large amounts of IFNγ and TNFα to induce inflammation, Fas-FasL signaling to induce apoptosis, and the release of perforin and granzymes to lyse target cells. Recent single-cell studies confirmed that $CD8^+$ T cells constitute

a substantial proportion in human and mouse atherosclerotic plaques[52,54–56], but their function in atherosclerosis is controversial. For instance, experimental studies indicated that $CD8^+$ T cell depletion in atherosclerotic-prone mice can result in both pro-atherogenic and atheroprotective outcomes[81–83]. These conflicting findings could possibly be attributed to heterogeneity of $CD8^+$ T cell subsets, consisting of pro-inflammatory $CD8^+$ T cells and anti-inflammatory regulatory $CD8^+$ T cells[70]. In depth analysis of $CD8^+$ T cells in human atherosclerotic plaques revealed various phenotypes, including effector-memory cells, terminally differentiated effector cells, and central-memory cells[55,84]. Clinical studies showed that coronary artery disease patients have increased levels of $CD8^+$ T cells in their blood[85,86]. However, the specific roles of the various $CD8^+$ T cell subsets and their association with atherosclerosis development requires further investigation.

B cells

In addition to T cells, the adaptive immune system consists of B cells. B cells mediate humoral immunity by the production of antibodies, also called immunoglobulins (Ig). B cells are generally divided in two main populations: B1 cells and B2 cells, which have distinct origins and functions.

B1 cells develop from precursors in the fetal liver and their long-term maintenance mainly relies on their self-renewing capacity. The B1 cell population primarily resides in pleural and peritoneal cavities, but smaller populations are also present in the spleen and bone marrow. Here, B1 cells spontaneously secrete high amounts of natural IgM antibodies in a T cell-independent manner[87,88]. In mice, B1 cells can be further divided into B1a ($CD5^+$) and B1b ($CD5^-$) cells based on the expression of CD5[89]. Since B1 cells are involved in early, non-specific immune responses, they can be considered as innate-like B cells. In atherosclerosis, B1 cells exert an atheroprotective role via the production of IgM antibodies directed against oxidation specific epitopes (OSE) expressed on oxLDL particles, reducing foam cell formation and inflammation. B1-derived IgM further limits inflammation in the plaque by binding epitopes on apoptotic cells enhancing their clearance[90]. Adoptive transfer of B1 cells in atherosclerosis-prone mice significantly reduced atherosclerotic plaque formation[91,92]. In clinical studies, OSE-specific IgM levels were shown to negatively correlate with atherosclerosis stage and cardiovascular outcomes, supporting the protective role in atherosclerosis[93,94].

In contrast to B1 cells, B2 cells develop in the bone marrow. Here, CLPs differentiate into immature B cells through rearrangement of the immunoglobulin heavy and light chains resulting in the formation of unique B cell receptors that are expressed on the B cell surface. Immature B cells, also referred to as transitional B cells, exit the bone marrow and enter peripheral lymphoid tissues to become mature naïve B cells, which further differentiate into marginal zone B cells (MZ) or follicular B cells (FO). FO B cells represent the majority of mature B cells and reside in lymphoid follicles of the spleen and lymph nodes. FO B cells require

antigen stimulation and interaction with T cells to become fully activated and migrate to the germinal center (GC). Upon interaction with Tfh cells in the GC, activated B cells undergo class-switching, somatic hypermutation and affinity maturation, leading to the production of high affinity antibodies. Ultimately, GC B cells differentiate into plasma cells, which secrete high-affinity antibodies, or memory B cells, ensuring long-term immune protection[88,90]. Differentiation of FO B cells into IgG-producing plasma cells has been shown to promote atherosclerosis[95]. Moreover, a reduction of FO B cells in high fat diet-fed atherosclerosis-prone mice treated with an agonistic BTLA antibody attenuated atherosclerosis, supporting a pro-atherogenic role for FO B cells in atherosclerosis[96]. In contrast, MZ B cells are a distinct subset of B2 cells that mainly reside in the marginal zones of the spleen, where they rapidly respond to blood-borne antigens. Like B1 cells, MZ B cells are considered innate-like cells that produce high amounts of IgM antibodies in a T cell independent manner. As mentioned before, IgM antibodies protect against atherosclerosis by limiting inflammation. In addition to IgM production, MZ B cell act atheroprotective by limiting the Tfh response and thereby inhibiting FO B cell activation[97].

Mast cells

In 1878, Paul Ehrlich was the first to describe 'Mastzellen', meaning well-fed cells, as connective tissue cells containing cytoplasmic granula[98]. Nowadays, mast cells are still recognized as a type of granulocyte that is part of the innate immune system. Traditionally, mast cells are known for their contribution to the first line of defence against pathogens, but are also associated with allergic responses. Mast cells originate from hematopoietic stem cells in the bone marrow that differentiate via common myeloid progenitors into granulocyte-macrophage progenitors, that can give rise to all types of granulocytes. After lineage commitment, mast cell progenitors (MCp) enter the circulation and migrate to peripheral tissues[99]. Upon tissue entrance, MCp develop into mature mast cells, which can be characterized by the expression of c-Kit (CD117) and the Fc epsilon receptor I (FcεRI). Mature mast cell are also defined by the development of cytoplasmic granules containing proteases like tryptase and chymase, and other inflammatory mediators[100]. Mast cells are widely distributed in several tissues, *e.g.* the skin and the lungs, but are also present in low numbers in healthy arterial tissue[101]. In mice, mast cells are broadly divided in two main subtypes based on their tissue location, including connective tissue mast cells (CTMC) and mucosal mast cell (MMC). Human mast cells are divided based on their protease content, including tryptase only mast cells (MCT) and tryptase-chymase mast cells (MCTC)[102].

Mast cells can be activated via various routes (**Figure 3**), highlighting their central role in immune responses. The most common activation route is via the IgE-dependent mechanism, where antigens cross-link IgE bound to the high-affinity receptor FcεRI on the mast cell surface. Besides, also IgE-independent mechanisms can activate mast cells and may lead to degranulation, such as through complement proteins, toll-like receptors (TLRs), and via

neuropeptides. Upon activation, mast cells excrete a plethora of mediators in their direct environment, such as proteases that are stored in their granules, *e.g.* tryptase and chymase, and *de novo* synthesized cytokines and chemokines, like tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6)[103].

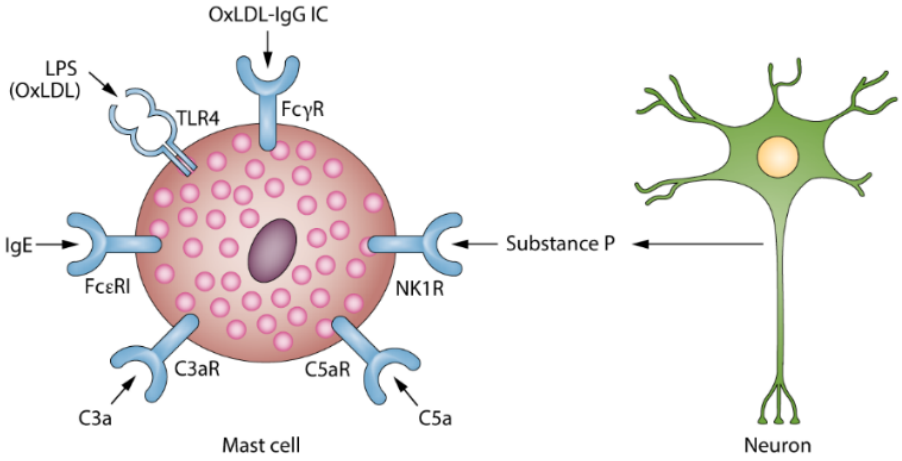

Figure 3. Possible mast cell activation routes that are implicated in atherosclerosis. The most common route for mast cell activation is via crosslinking of antigen-IgE complexes to the Fc ε receptor I (FcεRI). Other routes involve activation via toll-like receptors (TLR), neuropeptides (NK1R), Fcγ receptor, and complement receptors (C3aR and C5aR). *Adapted from Shi et al. (2015). Nat. Rev. Cardiol, 12(11), 643–658[103].*

Experimental studies in atherosclerosis-prone mice provided evidence of a pro-atherogenic role of mast cells. Excessive mast cell activation in mice was shown to exacerbate plaque development and result in an increased incidence of intraplaque haemorrhages[104,105]. In contrast, mast cell-deficient mice developed smaller plaques with a more stable phenotype[106]. In human atherosclerotic plaques, mast cell numbers elevate upon plaque progression and these intraplaque mast cells positively associated with plaque vulnerability hallmarks, such as intraplaque haemorrhage (IPH) and microvessel density[107]. Moreover, mast cell numbers were increased in plaques from patients that suffered a clinical cardiovascular event during follow-up[107]. In addition, patients suffering from systemic mastocytosis, a rare disorder characterized by an abnormal accumulation of mast cells, were shown to display increased CVD events[108]. Mast cells primarily drive plaque progression and destabilization via the release of pro-atherogenic mediators. For example, the secretion of cytokines and chemokines by activated mast cells promote infiltration and activation of other immune cells in the plaque. Activated mast cells also enhance foam cell formation by releasing heparin, which facilitates the formation of heparin-LDL complexes and subsequent phagocytosis by macrophages. Moreover, mast cells contribute to plaque destabilization by releasing histamine and the proteases tryptase and chymase in their environment, which facilitate extracellular matrix degradation by activating matrix metalloproteinases (MMPs)[109]. Inhibition of chymase activity in *Apoe*$^{-/-}$ mice reduced atherosclerotic plaque size and increased plaque collagen content, indeed indicating the destabilizing effect of chymase[110]. The release of angiogenic factors by

mast cells promote intraplaque neovascularization, which is a hallmark of plaque instability[111,112]. Together, these data strongly indicate that mast cells actively contribute to plaque destabilization and subsequent cardiovascular events. In this thesis, the role of mast cells in atherosclerosis and their potential as therapeutic target will be discussed.

Thesis outline

In this thesis, the role of mast cells in atherosclerosis and novel therapeutic strategies to inhibit atherosclerosis progression will be discussed. The first part of the thesis specifically focuses on the relation between mast cells and advanced human atherosclerotic plaque characteristics. The second part of the thesis focuses on identifying and examining new potential therapeutic targets to treat atherosclerosis.

In **chapter 2**, we implemented a flow-cytometry-based method to identify mast cells and its activation status in advanced human carotid artery plaques. We evaluated the relation between mast cells and key features of human plaque stability. Moreover, we studied the association between total IgE plasma levels and plaque stability. In **chapter 3**, we further investigated the link between mast cells and plaque stability in a large patient cohort from the BiKe biobank. In particular, we examined the role of mast cells in plaque calcification via vascular smooth muscle cells.

In the second part of the thesis, we used single-cell RNA sequencing data of human carotid plaques to identify novel therapeutic targets in atherosclerosis. Potential targets were evaluated in a mouse model for atherosclerosis. In **chapter 4,** we examined the therapeutic potential of Bruton's Tyrosine Kinase in atherosclerosis by treating *Ldlr*$^{-/-}$ mice with a small molecule inhibitor in early and advanced experimental atherosclerosis. **Chapter 5** explores Leukemia Inhibitory Factor Receptor (LIFR) signaling as novel therapeutic target in the treatment of atherosclerosis.

Finally, all data reported in this thesis is summarized and discussed in **chapter 6**, including future perspectives of this research.

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