Single-cell immune profiling of atherosclerosis: from omics to therapeutics

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Chapter 1

General Introduction

Cardiovascular disease

Cardiovascular diseases (CVDs) comprise all diseases affecting the heart or blood vessels and are responsible for the majority of mortality globally. On average, 17.9 million people die as a consequence of CVD each year worldwide.[1,2] Atherosclerotic cardiovascular disease (ASCVD) accounts for the majority (85%) of CVD-related deaths.[3] ASCVD refers to coronary heart disease, cerebrovascular diseases, peripheral artery disease and abdominal and descending thoracic aortic aneurysm.[4] All these conditions have atherosclerosis as common underlying cause. Atherosclerosis is characterized by chronic, lipid-driven vascular inflammation that induces atherosclerotic plaque formation in the intima of medium to large arteries.[5] The continuously advancing intimal plaques are eventually prone to rupture, resulting in thrombus formation and clinical manifestations due to ischemia of surrounding tissues, such as a myocardial infarction (MI) or stroke. ASCVD has multiple risk factors, including age, a sedentary lifestyle, an unhealthy diet, smoking, hypertension, dyslipidemia, obesity, diabetes mellitus and genetic predisposition, such as a mutation in the LDL receptor (LDLr) leading to familiar hypercholesterolemia.[6]

CVD has a major impact on the social-economic burden as it was estimated by the European Heart Network that CVD-related health care costs the European Union economy €210 billion on average. Currently, lifestyle interventions such as increased physical activity, a healthy diet, reduced smoking and alcohol consumption, are considered as the fundament of ASCVD treatment.[7] By alleviating multiple major risk factors at once, the risk of all-cause mortality can theoretically be reduced by 66%.[8] Furthermore, a reduced risk ratio was observed for both cardiovascular mortality and disease upon achieving improved cardiovascular health metrics (as defined by the American Heart Association).[9,10] Despite these efforts, additional pharmacological and surgical interventions are often still required.

For long, atherosclerosis has primarily been considered as lipid-driven disease. Reduction of circulating LDL cholesterol by means of statin treatment has led to great advances in cardiovascular disease treatment. Nevertheless, recurrent events have occurred in over 20% of patients that have suffered from acute coronary heart disease and received high dose statin treatment.[11,12] Currently, upon the occurrence of a cardiovascular event, therapies are mainly directed at revascularization. These include antithrombotic treatment and/or several surgical methods.[13] Percutaneous transluminal coronary angioplasty with or without stent placement is generally performed after MI incidence to reopen the coronary artery.[14] However, upon severe occlusion of multiple arteries or presence of diabetes and/or heart failure,

coronary artery bypass surgery is the preferred choice of surgical intervention.[15] For cerebrovascular ASCVD, carotid endarterectomy surgery is recommended for patients with ≥70–99% symptomatic stenosis in the carotid artery to reduce the risk of stroke.[16] Carotid endarterectomy surgery is solely performed if the overall benefit of surgery outweighs the potential peri-operative complications.[17]

Nowadays, it has become evident that apart from disturbed lipid metabolism, there is a prominent role for immune cells in the development of atherosclerosis. In the last decade, a handful of clinical trials have supported a pivotal role for the immune system in the treatment of atherosclerosis.[18] The CANTOS trial was the first to show that treatment with an anti-inflammatory monoclonal antibody against Interleukin (IL)-1β reduced the risk of cardiovascular events by 15%.[19] This study provided clear evidence that intersecting with the immune system ameliorates disease. Negative side-effects of general immune suppression were however also observed. Since the CANTOS trial, several other trials have been designed targeting the immune system in ASCVD. In the CIRT trial, patients with previous myocardial infarction and type 2 diabetes or metabolic syndrome were treated with methotrexate, but with no effect on cardiovascular outcomes. In the COLCOT, LoDoCo and LoDoCo2 trials patients with recent myocardial infarction were treated with colchicine and showed a significantly reduced risk of ischemic cardiovascular events.[20–22] The RESCUE trial has focused on the treatment of patients with elevated levels of high-sensitive C-Reactive Protein (hsCRP) and chronic kidney disease (CKD), which significantly increases cardiovascular disease risk. These patients were treated with Ziltivekimab, targeting IL-6, and showed a dose-dependent reduction in hsCRP levels at the end of the trial.[23] The RESCUE trial is at present being followed up by the ZEUS trial, in which a similar set up is executed, but additionally cardiovascular events will be monitored.[24] Finally, LILACS is a the second current ongoing clinical trial in which patients are treated with a low dose of IL-2 to induce an anti-inflammatory T cell response.[25] Altogether, these data further support the notion that the immune system is a promising target for future therapeutic strategies.

Atherosclerosis

Early lesion development

Arteries and veins consist of three layers: the intima, the smooth muscle cell-rich media and the adventitia. In the healthy vasculature, these layers are protected by a non-permeable endothelial cell layer. The endothelium acts as a selective barrier that allows the exchange of molecules between blood and tissues. It

consists of a continuous monolayer of endothelial cells linked by different types of adhesive structures or cellular junctions.[26] Furthermore, through the secretion of vasoconstrictor and vasorelaxant molecules endothelial cells modify smooth muscle cells thereby managing vascular tone.[27] Differences in blood flow result in a variety of hemodynamic forces that directly impact the endothelium. Whereas in unbranched areas of the artery a relatively uniform laminar blood flow occurs, a disturbed flow pattern is often observed in areas of bifurcation, branch points or major curvature.[28] Shear stress is necessary to maintain a proper vascular physiology. Through local mechanotransduction mechanisms, it is capable of modifying endothelial cell phenotype and barrier function.[29] However, at areas with disturbed flow patterns, oscillatory shear stress occurs, which causes endothelial damage and upregulation of adhesion molecules. In addition, endothelial dysfunction is in part mediated by pro-atherogenic factors like dyslipidemia and pro-inflammatory cytokines, altogether being the initiation trigger for atherosclerosis development. Endothelial cells are activated and upregulate the expression of leukocyte adhesion molecules, amongst which intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin.[30,31]

Simultaneously, the endothelium becomes more permeable allowing the transmigration of lipoproteins. Circulating lipoproteins facilitate the transport of hydrophobic particles, *e.g.* cholesterol and triglycerides. Thereby they play an essential role in the distribution of respectively structural components for cell membranes and steroid hormones, and a key source for energy for the body.[32] There is a variety of lipoproteins that are characterized based on size, density and their associated apolipoproteins, including chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoproteins (HDL) and lipoprotein (a) (Lp(a)).[33] Hyperlipidemia has been associated with atherosclerosis, in which elevated VLDL and primarily LDL have been described to promote atherosclerosis development. Apolipoprotein B100 (ApoB100), which is a part of LDL, binds to proteoglycans in the extracellular matrix of the damaged endothelial cell layer, thereby reducing retention of LDL.[34-36] These bound cholesterol-rich LDL particles are subsequently prone to chemical modification by *e.g.* oxidation into oxidized LDL (oxLDL).[37]

Concurrently, oxLDL stimulates the endothelial cells to secrete chemokines, such as C-C motif Chemokine Ligand 5 (CCL5) and CCL2, which leads to the recruitment of monocytes to atherosclerosis-prone sites. These monocytes crawl over the vessel wall and subsequently adhere to the endothelium by binding of integrins very late antigen-4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1) to the upregulated

leukocyte adhesion molecules and infiltrate in the intima.[38,39] Secretion of macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) by endothelial cells stimulates monocytes to differentiate into macrophages. Macrophages are phagocytes, meaning that they engulf debris, pathogens and dead cells.[40] Apart from monocyte-derived macrophages, there is also a population of resident macrophages that are of embryonic origin and reside in amongst others the arteries to act in the first line of defense.[41] In the intima, oxLDL is phagocytosed via scavenger receptors, such as CD36 and scavenger receptor class A, by resident macrophages at first and by monocyte-derived macrophages in later stages of disease development.[42–44] The continuous uptake of oxLDL by macrophages results in excessive lipid storage in the cells as seen by accumulating lipid droplets. These lipid-rich macrophages are called foam cells and are a hallmark for early atherosclerotic plaque development. Consequently, foam cells further contribute to atherosclerotic plaque progression by the secretion of multiple proinflammatory cytokines and chemokines thereby amplifying local inflammation.[45] The intimal thickening that is a result of intimal oxLDL, foam cell formation and initial immune infiltration is called a 'fatty streak'. If plasma cholesterol levels are sufficiently lowered, fatty streaks can almost completely regress.[46] However, fatty streak formation occurs in the subclinical phase of disease development and progress into more advanced lesions that induce clinical manifestations. In **Figure 1** the processes contributing to atherosclerosis development are summarized.

Advanced atherosclerosis

A sustained hyperlipidemic and pro-inflammatory environment increasingly exacerbates plaque development. After initial foam cell development, other immune cells, including T cells, also enter the fatty streak. By the secretion of proinflammatory cytokines, *e.g.* Interferon (IFN)-γ, T cells are capable of regulating both innate immune cells and smooth muscle cells.[47] Upon plaque progression, medial smooth muscle cells migrate into the intima and acquire different phenotypes. Whereas in the healthy medial layer smooth muscle cells have a quiescent contractile phenotype that is important to maintain vascular tone, they are capable of dedifferentiation into a synthetic state with advancing disease. Synthetic smooth muscle cells regain proliferative capacity and migrate into the intimal layer. This is a consequence of loss of expression of genes that encode for cytoskeletal proteins such as α-Smooth Muscle Actin (αSMA) and smooth muscle myosin heavy chains (MYH11). Instead, synthetic smooth muscle cells upregulate genes that express extracellular matrix-related proteins.[48–51] Consequently, these cells are a primary source of extracellular matrix in the atherosclerotic plaque.[52] Furthermore, synthetic smooth muscle cells are well known to accumulate underneath the breached endothelial layer thereby forming a so-called fibrous cap. This cap has a stabilizing function due to its collagen

and proteoglycan-rich matrix, which prevents the plaque from rupture and releasing its contents.[48,53] There is also accumulating evidence suggesting that smooth muscle cells can undergo a phenotypic switch towards a foam cell-like phenotype. Similar scavenger receptors as seen on foam cells, such as CD36 and SR-A1, are highly expressed on synthetic smooth muscle cells and concurrent phagocytic capacity of cholesterol-rich lipoproteins has been shown.[52,54,55] Krüppel-like factor 4 (KLF4) is an essential factor involved in the gain of macrophage-like properties by smooth muscle cells.[56] Interestingly, lineage tracing has shown that the majority of foam cells in the atherosclerotic plaques may have a smooth-muscle cell origin.[57]

The continuous lipid uptake by macrophages and smooth muscle cells becomes unsustainable as the plaque further expands. Although these cells express cholesterol efflux transporters such as ATP-binding cassette transporter A1 (ABCA1) and ABCG1, the excessive lipid uptake outweighs the efflux capacities at a certain stage. As a consequence, foam cells will go in apoptosis due to lipotoxicity. Efferocytosis is a highly regulated process that is initiated by phagocytes, such as macrophages, to take up apoptotic cells. Clearing of apoptotic cells is vital in maintaining tissue homeostasis in disease in order to avoid secondary necrosis and related induced inflammation.[58,59] With atherosclerosis progression, however, this process becomes impaired as the efferocytic capacity of phagocytes is reduced, leading to an increased number of apoptotic bodies and subsequent secondary necrosis.[60–62] This leads to formation of a necrotic core, which is characterized by the accumulation of apoptotic (immune) cells, cellular debris and lipid deposition.[63] Eventually, the growing necrotic core results in increased arterial stenosis and is associated with plaque instability.

Another key process in advanced atherosclerosis is the degradation of extracellular matrix and simultaneous fibrous cap thinning. The continuous cycle of lipoprotein infiltration, foam cell formation and necrotic core expansion occurs with a congruent chronic inflammatory response. Over time, the local inflammation in the plaque aggravates and significantly contributes to plaque destabilization. IFN-γ secreted by T cells for instance inhibits interstitial collagen production by smooth muscle cells[47,64] Furthermore, multiple immune cells that accumulate in the lesion are responsible for the secretion and activation of a variety of proteases that actively degrade the extracellular matrix. Matrix metalloproteases (MMPs) are commonly described to contribute to this process, of which primarily MMP-2 and MMP-9 that are secreted by activated macrophages and neutrophils.[65,66] Moreover, with atherosclerosis progression mast cells accumulate in the lesion as well.[67] Mast cells are well known to secrete proteases chymase and tryptase that independently degrade several collagen types, fibronectin and elastin. However, both chymase and tryptase amplify

the conversion pro-MMP-1 and pro-MMP-3 to their active forms, which subsequently activate *e.g.* MMP-2 and MMP-9, thereby further aggravating matrix degeneration in the atherosclerotic plaque and thus promote plaque instability.[68]

Altogether these processes will eventually destabilize the plaque to such an extent that the fibrous cap ruptures. Hereto, the necrotic content of the plaque which contains a large amount of thrombogenic factors, such as tissue factor, gets released in the vessel lumen and initiate thrombus formation and subsequent occlusion of the artery. Additionally, thrombus formation could also occur as a consequence of plaque erosion. Eroded plaques are often characterized by a less inflamed and more extracellular matrix-rich phenotype, which is more often found in patients treated with lipid-lowering drugs and is also more prevalent in women. Due to endothelial apoptosis, underlying collagen is exposed and initiates the accumulation of neutrophils and platelets and subsequent thrombus formation.[47,69,70] Both with plaque rupture and erosion, thrombus formation can lead to full occlusion of surrounding arteries, thereby inducing ischemia of the surrounding tissues and subsequent clinical events, such as stroke and myocardial infarction.

Experimental models of atherosclerosis

Due to the complex etiology of the atherosclerotic plaque, experimental studies are still largely dependent on *in vivo* atherosclerosis models. Despite recent advances in *in vitro* models of the healthy vasculature[71], to date these models are not applicable to model the advanced atherosclerotic plaque in culture. Atherosclerosis has been examined in a large variety of animal models, including rats, rabbits, pigs, and non-human primates. Nevertheless, mice remain the most common animal model for atherosclerosis.[72] Since the murine lipoprotein profile differs substantially from that in humans, atherosclerosis mouse models generally require a high fat and cholesterol diet, often referred to as western type diet, to accelerate disease development.[73] Nevertheless, wild type (C57BL/6) mice only developed small areas with fatty streaks after 14 weeks fed with a cholate containing high fat diet.[74] Instead, genetically modified murine models are used that specifically target the lipoprotein metabolism to increase circulating VLDL and LDL concentrations. The *apoE* and *Ldlr* deficient mouse models are most commonly used. ApoE is a constituent of VLDL and chylomicrons and is the main ligand for the clearance of these lipoproteins by the liver via several receptor systems, including the LDLr and the LDL receptor-related protein (LRP).[75] Deficiency of this gene already significantly elevates plasma cholesterol levels and this largely increases with a western type diet.[76] *ApoE*$^{-/-}$ mice naturally develop atherosclerotic plaques, which quickly become advanced upon diet feeding.[77] On the contrary, *Ldlr*$^{-/-}$ mice, which lack the LDLr required for clearance of VLDL and LDL particles by the liver, are characterized by modestly elevated levels of both VLDL and LDL in the blood. The advantage of this model is that the plasma cholesterol is largely

transported by LDL particles, which more closely resembles the human lipoprotein profile as compared to the *apoE*$^{-/-}$ mouse. Moreover, *LDLR* deficiency in humans is the underlying cause of familial hypercholesterolemia.[78,79] On a regular diet, these mice develop small atherosclerotic lesions in the first few months. Yet, with western type diet feeding they rapidly develop advanced atherosclerotic plaques. Of note, it has recently been shown that aged *Ldlr*$^{-/-}$ on a regular diet for two years, which reflects the average age of symptomatic cardiovascular patients, these mice develop advanced atherosclerosis as well.[80] Finally, atherosclerosis can also be induced in C57BL/6 mice via adeno-associated virus (AAV)-mediated overexpression of pro-protein convertase subtilisin/kexin type 9 (PCSK9)[81,82] PCSK9 is involved in hepatic LDLR recycling. It induces endocytosis and lysosomal degradation of the receptor, which results in reduced LDL uptake by the liver. Overexpression of this gene thus elevates circulating LDL and promotes atherosclerosis development. Nowadays, PCSK9 inhibitors have been introduced as treatment for both cardiovascular disease and familial hypercholesterolemia.[83]

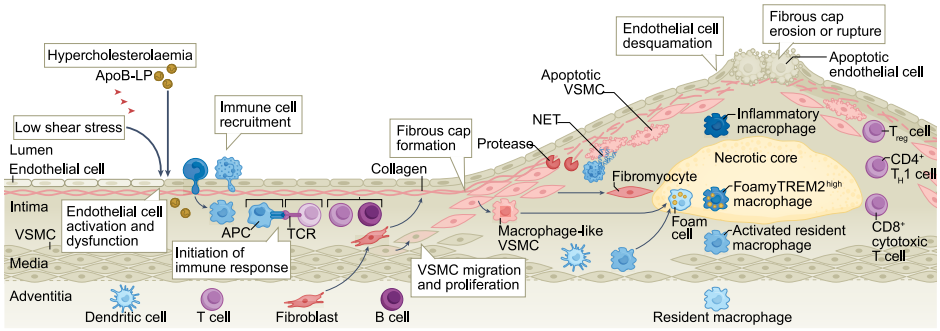

Figure 1. Development of atherosclerosis. Hypercholesterolemia and low shear stress damage the endothelial cell layer of the vessel wall resulting in endothelial dysfunction and activation. The endothelial cell layer becomes permeable and allows ApoB-containing lipoproteins (ApoB-LP) to enter the arterial wall. These ApoB-LPs get oxidized and further induce endothelial cell activation. Subsequently, monocytes are recruited, transmigrate and differentiate into macrophages. Both these macrophages and recruited vascular smooth muscle cells (VSMCs) in turn take up the oxLDL particles and differentiate into foam cells. This forms the fatty streak. As atherosclerosis progresses, the continuous uptake of lipoproteins by foam cells induces lipotoxicity and induces apoptosis. This is the foundation of the necrotic core that develops with aggravating disease. Furthermore, adaptive immune cells are also recruited to the atherosclerotic lesion. Antigen presentation by APCs induces T cell and B cell activation which will subsequently contribute to the pro-inflammatory environment in the lesion. Migration of VSMCs to the breached endothelial layer will induce fibrous cap formation. Over time, the plaque becomes unstable due to increased necrotic core formation alongside extracellular matrix degradation and apoptosis of VSMCs in the cap. As a consequence, the plaque will rupture and induce thrombosis leading to subsequent clinical manifestations. *Adapted and modified with permission from Engelen et al. (2022) Nat. Rev. Cardiol. 19(8):522-542.*[18]

The immune system in atherosclerosis

Although dyslipidemia has a pivotal role in atherosclerosis development, it has become evident that inflammation is a crucial process for disease progression. The immune system is responsible for protecting the body from infection and tissue injury.[84] Hereto, it employs two different arms: the innate and the adaptive immune system, otherwise referred to as respectively 'non-specific' and 'specific'.[85] The innate immune response is the first line of defense and becomes activated by the expression of pattern recognition receptors (PRRs).[86] These PRRs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are molecular structures not found in the host organism and therefore essential for innate immune cells to protect from pathogens. DAMPs on the other hand are endogenous molecular structures produced upon tissue damage and will be recognized as 'self' to activate natural immunity.[87] Multiple innate immune cells are capable of processing these PAMPs and/or DAMPs, which will induce a specific response based on the received signal. Subsequently, these cells are capable of presenting small peptide fragments of the pathogen on their surface to initiate the adaptive immune system. These fragments are called antigens or epitopes. Each adaptive immune cell has its own unique receptor that specifically binds the antigen presented by innate immune cells. Importantly, adaptive immune cells gain immunological memory after the first antigen encounter, which allows a rapid activation of these cells if they are rechallenged with the same antigen.[88] Therefore, the adaptive immune system is an important regulator for the resolution of inflammation. Both the innate and adaptive immune system are involved in atherosclerosis. The contribution of the different immune cells involved will be discussed below.

Innate immunity

The innate immune system plays a pivotal role in the initiation of atherosclerosis. The innate immune cells that are involved in disease development include monocytes, macrophages, dendritic cells, mast cells, neutrophils[89], natural killer cells[90] and innate lymphoid cells[91]. The cells that are relevant for this thesis are described more in detail below.

Monocytes and macrophages

Monocytes develop and mature from a hematopoietic stem cell origin in the bone marrow. There are two commonly described subsets: non-classical and classical monocytes.[92] Mon-classical monocytes are characterized as $Ly6C^{-}CCR2^{-}CX3CR1^{+}$ in mice and $CD14^{low}CD16^{+}$ in humans.[93] Their function is to patrol the vasculature to rapidly respond to, amongst others, endothelial damage. To do so, they recruit neutrophils that induce focal endothelial necrosis, and subsequently clear the

remaining cellular debris.[94,95] This subset of monocytes is often considered anti-inflammatory. Classical monocytes are defined as Ly6C^{hi}CCR2^{+}CX3CR1^{-} in mice and CD14^{+}CD16^{-} in humans and have a pro-inflammatory phenotype.[93] In contrast to non-classical monocytes, classical monocytes are specifically recruited to sites of inflammation or tissue remodeling. Here, they can extravasate from the blood and differentiate into monocyte-derived macrophages or dendritic cells.[92,96,97] As described above, monocytes are recruited upon endothelial damage and intimal (ox) LDL accumulation. Indeed, a positive correlation has been described between aortic monocytes and atherosclerotic lesion area.[98] Furthermore, in mice it was confirmed that classical monocytes develop into macrophages in the atherosclerotic aorta.[99] In line, the high expression of CCR2 rapidly redirects monocytes to the CCL2 secreting endothelial cells. Consequently, depletion of *Ccr2* in *ApoE*$^{-/-}$ mice significantly reduced atherosclerotic lesion size by three-fold. [100]

When entering the vascular intima, monocytes differentiate into macrophages upon stimulation with various cytokines and growth factors present in the plaque. Macrophages have a multitude of different phenotypes in health and disease. For years, a classical division was commonly used to distinguish two types of macrophages: M1 and M2 macrophages. The M1 phenotype is a pro-inflammatory subset that is polarized by either T helper 1 (Th1) secreted cytokines such as IFN-γ of TNFα or by recognition bacterial lipopolysaccharide (LPS) through the Toll-like receptor 4 (TLR4).[101] Their main function is phagocytosis of pathogens, after which M1 macrophages subsequently secrete a plethora of pro-inflammatory cytokines, including IL-1β, IL-6 and TNFα.[102] In atherosclerosis, oxLDL is also capable of inducing TLR4-associated macrophage activation, thereby promoting a pro-inflammatory environment in the plaque by the secretion of amongst others IL-6 and IL-1β secretion, of which the latter has been specifically targeted in the CANTOS trial.[19,103] M2 macrophages on the other hand have been generally considered anti-inflammatory and are polarized by secretion of *e.g.* IL-4 and IL-13 by Th2 cells. Generally, M2 macrophages are considered anti-inflammatory due to their increased secretion of IL-10 and TGFβ.[104,105] They play an important role in angiogenesis, wound healing and tissue remodeling and are potent scavenging cells.[104,106] Furthermore, they have pro-fibrotic capacities.[107] Based on these capacities, M2 macrophages have been described to resolve plaque inflammation.[108]

This distinction is largely based on *in vitro* assays assessing phenotypic changes of macrophages. However, at least in atherosclerosis, *in vivo* work has shown that the variety of macrophage phenotypes is way beyond just the pro- and anti-inflammatory subtypes. In the last decade, new techniques, such as cytometry by time-of-flight (CYTOF) and single-cell RNA sequencing, have shed new light on macrophage diversity

in the atherosclerotic plaque. Within the murine atherosclerotic aorta, five different subclasses of macrophages have been identified.[109,110] An inflammatory macrophage subset, which is characterized by expression of pro-inflammatory cytokines and genes that are involved in the inflammasome-induced conversion of pro-IL-1β into its active form. Furthermore, another pro-inflammatory subset of macrophage has been identified as type-I interferon inducible cells (IFNIC), which upregulate interferon-induced genes such as *Ifit3* and *Irf7*.[111] Both subsets are likely to be monocyte-derived. An anti-inflammatory foamy macrophage subset has mainly been characterized by the expression of *Trem2* (Triggering receptor expressed on myeloid cells 2).[112] These foamy macrophages were detected at different stages of atherosclerosis, yet not in healthy arteries. Expression of *Trem2* on BODIPY$^+$ lipid laden cells confirmed their foam cell-like phenotype, which additionally also expressed other genes associated with foam cell formation, including *Abca1*, *Abcg1* and *Cd36*. [41,113] Lastly, two resident macrophage subtypes have been characterized in murine atherosclerotic aortas. These macrophages reside both in healthy and diseased arteries. Lymphatic vessel endothelial hyaluronan receptor 1 (*Lyve1*)$^+$ resident macrophages are of embryonic origin and inhibit collagen synthesis by smooth muscle cells.[114,115] Recently, MAC^{AIR} (aortic-intima resident macrophage) macrophages were characterized. This subset of resident macrophages originates from blood monocytes shortly after birth and contribute to early lesion development by secretion of *Il1b*. They maintain by local proliferation in the aorta and are involved in the initial lipid uptake with atherosclerosis initiation. Yet, they encounter limited proliferation with plaque progression.[116] In human atherosclerosis macrophages have currently been generally divided in four subtypes, consisting of two types of pro-inflammatory macrophages, a TREM2$^+$ macrophage subset and resident-like macrophage population.[117–119] The pro-inflammatory macrophages are either characterized by expression of *IL1B* and associated genes that are involved in IL1β production or by expression of *TNF*. Of note, Fernandez *et al*. mainly detected *IL1B* expression in macrophages of asymptomatic patients.[119] The TREM2$^+$ macrophage subset is likely involved in lipid metabolism as supported by expression of *ABCA1*, *ABCG1*, *OLR1* and little proinflammatory genes. Furthermore, they express *CD9*, which has been associated to fibrosis in other diseases.[120] Finally, similar to murine atherosclerosis, a *LYVE1*$^+$ resident macrophage subset has been identified that may also affect antigen presentation and complement activation.[117]

Dendritic cells

Besides macrophages, dendritic cells (DCs) are classified as a distinct lineage of mononuclear phagocytes. The DC is the most potent antigen presenting cell (APC) and forms a bridge between the innate and adaptive immune system.[121] DCs regulate either antigen-specific T cell responses or tolerogenic responses to self-antigens. They reside

in both lymphoid and non-lymphoid tissues.[122] For long, a clear phenotypic distinction between macrophages and DCs has been a matter of debate as in inflammatory conditions they share expression of surface markers, such as CD11c.[123,124] Especially monocyte-derived cells have been shown to exhibit a very plastic phenotype as they acquire functional properties from both macrophages and dendritic cells (moDC) based on the microenvironment.[125–127] Nevertheless, the common DC progenitor (CDP) has been identified as unique precursor for both classical (cDC) and plasmacytoid DCs (pDC).[127,128] CDPs are localized in the bone marrow and either mature into pDCs within the bone marrow, or give rise to pre-DCs that enter the circulation, become immature DCs and mature into cDCs in lymphoid and non-lymphoid tissues. cDCs are divided in cDC1s and cDC2s, dependent on respectively BATF3 and IRF4 expression.[129,130]

Immature DCs patrol peripheral tissues to identify potential antigens. Upon internalization of antigens at inflammatory sites, DCs mature and migrate through the afferent lymphatic vessels to adjacent draining lymph nodes.[131] In this process, DCs downregulate their phagocytic capacity and gain antigen presenting properties by upregulation of CCR7 to enhance migration, and costimulatory molecules such as CD40, CD80 and CD86. Furthermore, they upregulate major histocompatibility complex (MHC) I or II, which are required for antigen presentation.[132,133] To induce an antigen-specific T cell response, the APC requires three signals: an interaction between the MHC and T cell receptor, a costimulatory or coinhibitory stimulation and finally cytokine secretion that altogether define whether the naive T cell gains an effector or an immunosuppressive function.[134,135] Of note, antigen presentation is not restricted to DCs, but could also be performed by other immune cells, such as macrophages, B cells and mast cells.[135,136]

DCs are present in the adventitia of healthy arteries. With atherosclerosis progression, they accumulate in advanced lesions, particularly in the rupture prone shoulder regions. [124,137,138] Furthermore, DCs are also found in adventitial tertiary lymphoid organs (ATLOs), which also significantly contribute to atherosclerosis progression.[139,140] Several studies have aimed to identify a causal contribution of DCs to atherosclerosis.[141] In early atherosclerosis, DCs have been shown to play an important role in cholesterol metabolism as upon dyphteria-toxin induced deletion of CD11c reduced plaque lipid content of *Ldlr*$^{-/-}$ mice.[142] In line, induction of a prolonged lifespan of DCs by overexpression of *Bcl2* resulted in an atheroprotective reduction of plasma cholesterol levels.[143] Interestingly, vaccination with *ex vivo* oxLDL-pulsed DCs significantly reduced atherosclerotic lesion size and increased plaque stability.[144] Additionally, adoptive transfer of ApoB100-loaded dendritic cells also significantly reduced atherosclerotic lesion size and reduced effector T cell activation, indicative of

a tolerogenic DC response.[145] These studies both underline the therapeutic potential of vaccination with tolerogenic DCs (tolDCs) for the treatment of atherosclerosis. Amongst others in rheumatoid arthritis, this strategy has been shown to effectively reduce disease burden and has resulted in the start of multiple clinical trials.[146,147] Nevertheless, a well-defined epitope to induce this tolerogenic response in atherosclerosis still remains to be identified.

Mast cells

Another prominent innate immune cell in atherosclerosis is the mast cell. This pro-inflammatory effector cell is widely distributed in several tissues and is predominantly found near surfaces exposed to the environment, such as the blood vessels, the skin, the airways and the gastrointestinal tract, allowing these cells to act as a first line of defense against pathogens.[148] Mast cells develop from hematopoietic stem cells, in particular a subset of granulocyte-monocyte progenitors, in the bone marrow.[149] These subsequently enter the circulation as mast cell progenitors.[150,151] Similar to macrophages, mast cells only mature within peripheral tissues. Mature mast cells are characterized by expression of c-Kit (CD117) and the Fcε receptor I (FcεRI).[152] Furthermore, they have a granule-rich cytoplasm that contains a plethora of inflammatory mediators. Upon activation, mast cells degranulate and release their mast-cell specific proteases tryptase and chymase, histamine and depending on the strength of the interaction they release amongst other cytokines, chemokines, leukotrienes and growth factors.[153,154] Mast cells are activated through a variety of mechanisms (**Figure 2**). Binding of an antigen-sensitized IgE to its high affinity receptor FcεRI results in a rapid degranulation and the concurrent release of proteases and lipid mediators, whereafter cytokines, chemokines and growth factors can be secreted as well.[154–157] This IgE-mediated activation is the most commonly known route of activation, however mast cells can also be activated via toll-like receptors, the FcγR, complement receptors and a number of neuropeptide receptors.[158] The secretion of mast cell mediators could have both pro- and anti-inflammatory functions on the surrounding cells. Additionally, mast cells have also been described as atypical antigen presenting cells by upregulation of MHC II molecules and could hereto induce T cell responses.[135,159,160]

Although mast cells are mostly known for their contribution to allergic reactions and airway diseases like asthma, they also play a significant role in atherosclerosis development. Mast cells are found in all stages of plaque development and their numbers have been shown to increase with disease progression.[161–163] Furthermore, a positive correlation has been observed between intraplaque mast cell numbers

and future cardiovascular events.[67] Multiple experimental atherosclerosis studies further outline the functional importance of mast cells in atherosclerosis. Systemic activation of mast cells exacerbates atherosclerosis development in *Apoe*$^{-/-}$ mice, which was resolved upon treatment with the mast cell stabilizer cromolyn.[164] Moreover, depletion of mast cells in *Ldlr*$^{-/-}$ mice significantly reduced atherosclerotic lesion size, which was restored after repopulation with bone-marrow derived mast cells.[165] The pro-atherogenic effects of mast cells are mainly attributed to the proteases they secrete upon activation. Chymase has been shown to induce apoptosis of both vascular smooth muscle cells and endothelial cells[166–169] and both chymase and tryptase activate matrix metalloproteases (MMPs) inducing matrix degradation and subsequent plaque remodeling.[170] Indeed, treatment with a chymase inhibitor not only reduced plaque size, but it also increased collagen content and reduced necrotic core size and the frequency and size of intraplaque hemorrhages, indicative of improved plaque stability.[171] Furthermore, in both *in vitro* and *in vivo* settings, mast cells can induce foam cell formation as heparin binds to LDL particles, resulting in complex formation and phagocytosis by plaque macrophages.[172–175] The secretion of pro-inflammatory cytokines also affects other surrounding immune cells thereby contributing to plaque progression.[176] Targeting mast cell activation or their migration to the atherosclerotic plaque are thus promising therapeutic approaches to improve disease outcome.

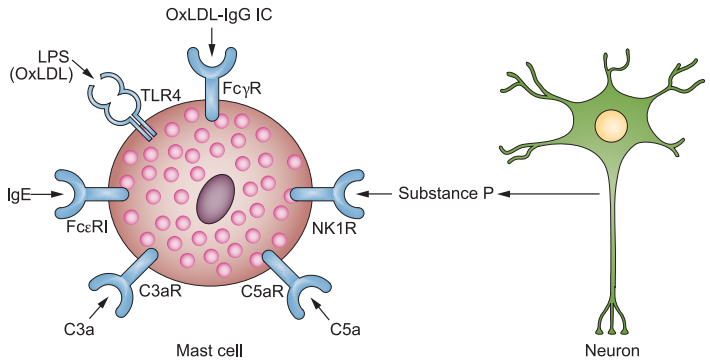

Figure 2. Different pathways of mast cell activation that occur during atherosclerosis development. Mast cell activation is considered pro-atherogenic. Multiple pathways have been described to induce mast cell activation with the progression of atherosclerosis. The most commonly known route of activation is upon binding and sensitization of IgE to the FcεRI, leading to subsequent degranulation of the mast cell. Furthermore, activation via the complement receptors C3aR and C5aR, TLR4, FcγR and NK1R result in degranulation and/or cytokine secretion by the mast cell. All pathways have been shown to independently contribute atherosclerosis. *Adapted with permission from Shi et al. (2015) Nat. Rev. Cardiol. 12(11):643-58.*[158]

Adaptive immunity

The adaptive immune response is initiated as secondary line of defense and it is mainly characterized by its immunological memory. The adaptive immune system consists of two different cell types: T and B cells. Both cell types play a prominent role in atherosclerosis.[177-181]

T cell development and activation

T cells originate from hematopoietic stem cells and migrate to the thymus to undergo maturation and selection before they enter the circulation. Within the thymus, $CD4^+CD8^+$ T cell precursors develop into two lineages depending on the T cell receptor chains they obtain: αβ or γδ T cells.[182,183] Each TCR has an antigen-binding site which is determined through V(D)J recombination. The α and γ chains are assembled from Variable (V) and Joining (J) segments, whereas the β and δ chain also have an additional diversity (D) segment.[184] Through somatic DNA recombination, a variable region is generated from these segments on the TCR that is unique for each individual T cell and specific for an antigen-MHC complex. Subsequently, the T cell precursors are selected based on their affinity for self-peptides and the strength of the TCR signal.[185] The majority of the T cell precursors are subject to death by neglect when they fail to sufficiently recognize a self-peptide-MHC complex or are unable to generate TCR signal that allows activation and differentiation of the T cell. T cell precursors that have too high affinity for self-peptides are negatively selected and go into apoptosis to avoid an autoimmune response. Finally, the positively selected T cell precursors have low self-reactivity and sufficient TCR signaling for maturation.[186,187] These cells will further differentiate into $CD4^+$ and $CD8^+$ T cells, dependent on their affinity for respectively MHCII or MHCI, and exit the thymus into the periphery. As a result, there is a large and diverse pool of different TCRs that are capable of responding to pathogen-derived antigens as well as preserving self-tolerance.

Naïve T cells get activated after encountering their cognate antigen presented by an APC. Upon antigen presentation, the naïve T cells will clonally expand and differentiate into a large pool of effector T cells with the same TCR. Activated T cells then migrate to the site of inflammation, such as the atherosclerotic plaque, and be primed again by local antigen presenting cells (**Figure 3**).[188] When the inflammation is resolved, the majority of the effector T cells go into apoptosis. However, a part of the effector T cells become circulating memory T cells that can elicit a quick response upon subsequent engagement with the same antigen.[189]

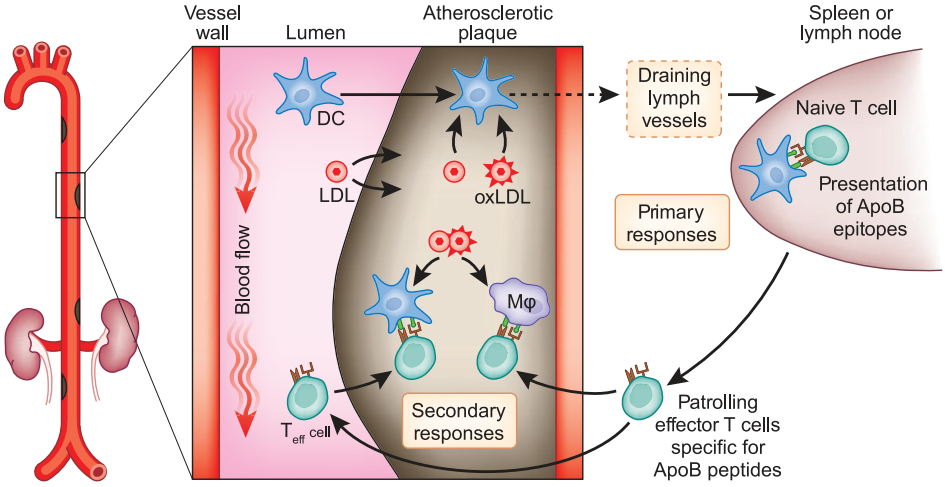

Figure 3. T cell activation in atherosclerosis. APCs, such as DCs patrol the circulation for antigens to present on their surface, for example peptides from ApoB100. APCs enter the plaque to take up antigens and subsequently migrate to draining lymphoid organs to present the antigen to naïve T cells. These will be activated and go back into the circulation as effector T cell. When they are recruited to the atherosclerotic plaque, they will undergo secondary activation by APCs residing in the lesion and exert their effector function. *Adapted with permission from Hansson et al. (2011) Nat. Immunol. 12, 204-212.*[188]

T cells play a prominent role in atherosclerosis development. A reduction in fatty streak formation was observed in *Ldlr*$^{-/-}$ mice lacking both B and T cells.[190] Similarly, a significant reduction in atherosclerotic plaque size was observed in immunodeficient *Apoe*$^{-/-}$ mice, yet upon reconstitution with CD4^{+} T cells atherosclerosis progression was accelerated.[191] Both CD4^{+} and CD8^{+} T cells have been detected in human atherosclerotic plaques[192] and contribute to multiple aspects of atherosclerosis progression.

CD4^{+} T cells

CD4^{+} T cells have a multifaceted role in atherosclerosis. Naïve CD4^{+} T cells develop into different T helper (Th) subsets dependent on the cytokines secreted with antigen presentation. Multiple CD4^{+} Th subsets have been described to affect disease progression. Th1, Th2, Th17 and regulatory T cells (T_{reg}) are most commonly studied in atherosclerotic plaques and will be discussed in more detail below. Apart from the beforementioned Th subsets, Th9[193,194], Th22[194,195], follicular T helper (Tfh) cells[196-198] and a subset of cytotoxic CD4^{+} T cells[199,200] have also been described in the context of atherosclerosis yet it remains elusive if they exert pro- or anti-atherogenic functions.

Th1 cells differentiate from naïve CD4+ T cells upon stimulation with IL-12. They are characterized by expression of the transcription factor T-bet and secretion of IFN-γ, IL-2 and TNFα.[178] Hereto, they contribute to the local pro-inflammatory environment in the atherosclerotic plaque. Indeed, T-bet deficient *Ldlr*$^{-/-}$ mice developed significantly smaller atherosclerotic lesions.[201] Furthermore, depletion of both IFN-γ and its receptor both resulted in a substantial reduction in plaque size[202,203], whereas treatment with IFN-γ concurrently aggravated disease.[204] Through secretion of IFN-γ, Th1 cells also promote plaque vulnerability as this cytokine inhibits smooth muscle cell proliferation, induces foam cell formation and promotes a pro-inflammatory phenotype for macrophages.[205-207] Altogether, these data provide clear evidence for the pro-atherogenic role of Th1 cells in atherosclerosis.

In contrast to Th1 cells, the function of Th2 cells in atherosclerosis is less evident. Th2 cells are defined by the expression of transcription factor GATA-3 and the secretion of IL-4, IL-5 and IL-13.[208] Th2 cells are well-known for their role in the protection of helminth infections and their contribution to asthma and allergic diseases.[209] In atherosclerosis however, conflicting results have been observed for these T cells. Since IL-4 was shown to inhibit Th1 responses, Th2 cells were initially thought to be atheroprotective.[210] However, whereas depletion of IL-4 ameliorated atherosclerosis in both *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice[211,212], induction of an ApoB100-specific Th2 response did not alter disease progression.[213] On the contrary, IL-4 released from mononuclear leukocytes were associated with reduced risk of CVD and circulating Th2 cells were negatively associated with common carotid intima-media thickness.[214] Plasma IL-5 was shown to inversely correlate with carotid intima-media thickness in women.[215] Furthermore, immunization against modified LDL initiated a Th2-response, resulting in secretion of IL-5 which mitigated atherosclerosis development.[216] The cytokine IL-13 was reported to be anti-atherogenic as well.[217] Collectively, how Th2 exactly contribute to atherosclerosis progression remains ambiguous.

Differentiation of Th17 cells requires IL-23 and results in upregulation of the transcription factor RORγT and secretion of IL-17.[218] Like Th2 cells, there is still some discrepancy regarding the role of Th17 cells in atherosclerosis. Deficiency of IL-17 has been described to be either atheroprotective[219], pro-atherogenic[220] or not affecting atherosclerosis at all.[221] Yet, administration of IL-17 or anti-IL-17 antibodies demonstrated that this cytokine promotes atherosclerosis development.[222-224] Deficiency of the IL-23 receptor (IL-23R) reduced IL-17 production by CD4+ T cells but did not affect atherosclerosis in both a full-body knockout and upon adoptive transfer of IL-23R deficient CD4+ T cells into atheroprone immunodeficient mice.[225] In humans, a similar disparity is observed regarding how Th17 act on disease progression. Patients

with acute coronary syndrome exhibited higher numbers of peripheral Th17 cells as well as increased levels of IL-17 and IL-23 compared to controls.[226] Plasma IL-17 was also associated with patients with acute myocardial infarction and it was elevated in patients that had a complex lesion defined by angiographic analysis.[227] However, IL-17 has also been shown to promote collagen production by human vascular smooth muscle cells *in vitro* and *IL17A* and *RORC* (encoding for RORγT) expression were both positively associated with expression of *ACTA2* (α-SMA) and *COL1A1* (Procollagen 1α1) indicating that IL-17 promotes plaque stability.[228] This was further supported by another study that also found increased levels of IL-17 expression in plaques with a stable phenotype.[229] The complexity in which Th17 cells affect atherosclerosis could in part be explained by their plasticity.[230] Upon pro- stimulation with TGFβ3, Th17 cells could also upregulate IFN-γ[231], whereas stimulation with TGFβ1 and IL-6 could promote Th17 to secrete the anti-inflammatory cytokine IL-10.[232,233] Taken together, it appears that Th17 exert no clear pro- or anti-atherogenic role and that their function is largely dependent on the microenvironment they reside in.

T_{regs} are involved in preserving self-tolerance and resolving inflammation.[234] They are characterized by expression of forkhead box protein P3 (FOXP3), IL-2RA (CD25) and lack of CD127. Their differentiation is induced by TGF-β and IL-2 or by weak TCR interactions.[235,236] Upon activation they secrete the anti-inflammatory cytokine IL-10. Although the number of T_{regs} in atherosclerotic plaques are limited[237], several experimental atherosclerosis studies have determined an atheroprotective role for these cells. Depletion of T_{regs} significantly increased atherosclerotic lesion size[238,239], and adoptive transfer of T_{regs} correspondingly attenuated disease progression.[238,240] Furthermore, expansion of T_{regs} using an IL-2 complex ameliorated atherosclerosis and increased plaque stability.[241] A different approach for T_{reg} expansion even resulted in regression of existing atherosclerotic lesions.[242] In line, vaccination against FOXP3 reduced the percentage of T_{regs} and exacerbated atherosclerosis.[243] The suppressive function of T_{regs} in atherosclerosis is in part mediated by secretion IL-10 as lack of this gene promotes disease progression.[244-246] In brief, T_{regs} have an important atheroprotective function. Induction of T_{regs}, as examined in the LILACS trial, is therefore a promising strategy to treat CVD patients.

$CD8^+$ T cells

$CD8^+$ are commonly known to act in the defense against infectious pathogens, such as bacteria and viruses, and contribute to anti-tumor immunity.[247,248] The majority of $CD8^+$ T cells differentiate into a cytotoxic subset after encountering an antigen-MHC I complex. These cytotoxic $CD8^+$ T cells are subsequently capable of killing the infected cells that carry the presented antigen in three ways: (1) through secretion of IFN-γ and TNF-α, that

respectively promote the inflammatory response and induce apoptosis, (2) by interaction of Fas with the Fas receptor on the target cell to induce apoptosis and (3) by the secretion of granzymes and perforin which are responsible for lysis of the target cell.[249,250]

The role of $CD8^+$ T cells in atherosclerosis remains inconclusive. Interference with MHC-I-related antigen presentation significantly reduced $CD8^+$ T cell numbers, but did not affect atherosclerosis.[251] Furthermore, depletion of *Cd8a* in *Apoe*$^{-/-}$ mice did not alter atherosclerotic lesion size either.[252] Nevertheless, both pro-atherogenic and atheroprotective roles for $CD8^+$ T cells have been described in mice as well. High-fat diet rapidly induced $CD8^+$ T cell activation in *Apoe*$^{-/-}$ mice as measured by their increased IFN-γ production in draining lymph nodes of the aortic root.[253] Moreover, administration of monoclonal antibodies against CD8α and CD8β significantly reduced atherosclerotic plaque size and reduced necrotic core size in *Apoe*$^{-/-}$ mice.[254] In line, the same study showed that adoptive transfer of $CD8^+$ T cells aggravated atherosclerosis and induced plaque vulnerability. This was attributed to the secretion of perforin, granzyme B and TNFα, as reconstitution of $CD8^+$ T cells deficient for these proteins did not affect atherosclerosis in immunodeficient mice. In contrast, treatment with a CD8α-depleting antibody in *Ldlr*$^{-/-}$ mice with advanced atherosclerosis increased plaque vulnerability as seen by increased necrotic core area.[255] This protective role for $CD8^+$ T cells was further supported in another study in which immunization with the ApoB100-derived peptide p210 resulted in expansion of $CD8^+$ T cells and a subsequent reduction in atherosclerotic plaque size.[256]

It has been hypothesized that these opposing effects of $CD8^+$ T cell depletion in are due to the heterogeneity of $CD8^+$ T cells. A subset referred to as Tc17 cells, characterized by RORγT expression and IL-17 secretion, was shown to increase in atherosclerotic lesions compared to the spleen. Yet, transfer of Tc17 cells into *Cd8a*$^{-/-}$*Apoe*$^{-/-}$ mice did not directly affect plaque size.[257] On the other hand, regulatory $CD8^+$ T cells, defined by expression of CD25 and FOXP3, have been described to be atheroprotective.[258,259]

In humans, mainly a pro-atherogenic role for $CD8^+$ T cells has been described. Increased levels of circulating cytotoxic $CD8^+$ T cells were detected in patients with coronary artery disease.[260,261] Furthermore, $CD8^+$ T cells are found in large numbers in the human atherosclerotic plaque, particularly in the shoulder regions and fibrous caps.[192,261,262]

B cells

B cells play an important role in humoral and cellular immunity. They are well known for the production of antibodies, but also contribute to T cell activation through antigen presentation or the cytokines they secrete.[263] In atherosclerosis, B cells

were first described to have a protective function, as adoptive transfer of B cells into splenectomized mice and B cell deficiency resulted in respectively a reduction and an increase in plaque size[264,265] Since then, several subsets of B cells have been associated with atherosclerosis.[179,180] The B1 subset is characterized by the secretion of natural antibodies and protects from atherosclerosis.[266] B1 cells can be further subdivided in B1a and B1b cells. A prominent anti-atherogenic function is denoted for B1a cells, as they are the main producers of IgM antibodies.[267,268] Deletion of secreted IgM indeed significantly increased atherosclerotic lesion size, concurrent with an increase in IgE and a corresponding increase in mast cell activation.[269] IgM antibodies that resolve atherosclerosis by clearing oxLDL and necrotic debris are of specific interest.[270] The secretion of oxLDL-targeted IgM antibodies was found to be mediated through secretion of IL-5 by Th2 cells. In addition, an atheroprotective function for B1b cells was also described as they also produce IgM antibodies targeting oxidation specific epitopes on LDL.[271] B2 cells however are generally considered pro-atherogenic.[272] B2 cells can be divided in follicular (FO) and marginal zone (MZ) B cells. After encountering their cognate antigen, FO B cells migrate towards the T cell area in lymphoid organs and upon interaction with Th cells that recognize the same antigen, they can proliferate and undergo class switching.[273] FO B cells are subsequently capable of secreting amongst others IgG and IgE, which are both pro-atherogenic.[181,274] MZ B cells can secrete antibodies independent of T cell activation and can hereto rapidly produce IgM and IgG antibodies.[275] However, MZ B cells were also shown to be protective as they regulate Tfh cells which subsequently reduces FO B cell activation.[276] Finally, a subset of IL-10^{+} regulatory B cells has also been identified in atherosclerosis, yet their precise role in atherosclerosis is not fully elucidated yet.[277-279] Collectively, proper targeting of the humoral immune response in atherosclerosis could provide a beneficial therapeutic strategy against atherosclerosis.

Single-cell transcriptomics in atherosclerosis research

The introduction of single-cell transcriptomics has revolutionized biological research. Single-cell RNA sequencing was first applied in 2009, in which the mRNA transcriptome was uncovered from a manually isolated single cell.[280] Since then, the field of single-cell RNA sequencing has rapidly evolved, allowing for the analysis of single cell transcriptomes of thousands of cells per sample.[281] The great advantage of this technology lies in the fact that it provides an unbiased analysis of the different cells present in heterogenous tissue samples, whereas conventional methods like immunohistochemistry and flow cytometry rely on pre-defined markers. Furthermore, single cell transcriptomics are likely to identify small cellular populations that would

otherwise be diluted when using bulk RNA sequencing.[282] By now, the field of single-cell multi-omics has been complemented with several modalities to extend the single cell analysis.[283] This gives the possibility to include antibodies for proteomic characterization of for instance certain lineage markers necessary for proper cell annotation (CITE-seq). Furthermore, the epigenome can be assessed using single-cell ATAC sequencing and both TCR and BCR clones can be detected per single cell to assess clonal expansion for extensive immune profiling.

The application of single-cell transcriptomics has significantly advanced the field of atherosclerosis in the past decade. In 2018, single-cell RNA sequencing was first applied in both *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice to map the cells present in atherosclerotic plaques.[112,284] This uncovered amongst others the presence of TREM2^{+} macrophages in atherosclerosis. Since then, a body of literature has been generated in which single-cell transcriptomics has been applied in both murine and human atherosclerosis. This has significantly enhanced our knowledge on the different cells present in (human) atherosclerosis. We will further elaborate on these findings in chapter 2 of this thesis.

Thesis outline

In this thesis, single-cell multi-omics were applied to generate a cellular atlas of the human atherosclerotic plaque. These data were subsequently further analyzed to identify and examine new potential targets to prevent atherosclerotic disease progression.

In **chapter 2** we provide an overview of how single-cell RNA sequencing has improved our knowledge in atherosclerosis and aneurysm formation. We describe the different cell populations identified in studies performed in diseased tissues of both murine and human origin. Finally, we elaborate on overlapping cellular subsets potentially contributing to both diseases. In **chapter 3** we unbiasedly mapped the cells present in human atherosclerotic plaques by using single-cell RNA and ATAC sequencing and discovered a large T cell population as well as cellular plasticity and intercellular communication pathways. Since we detected a large population of T cells in the advanced human plaque, we further investigated whether these T cells underwent clonal expansion indicative of an antigen-induced response. Hereto, in **chapter 4**, we applied single-cell TCR sequencing to assess T cell clonality in atherosclerosis. We identified a plaque-enriched clonally expanded CD4^{+} T cell subset, suggesting an autoimmune component in atherosclerosis. In **chapter 5** we developed a flow cytometry method to characterize mast cell phenotype in human atherosclerosis.

Here, we showed that the majority of mast cells express the activation marker CD63 in the human plaque. Moreover, a high percentage of these activated mast cells had IgE bound to their surface, indicating that the FcεRI-IgE pathway is of importance in mast cell activation in atherosclerosis. We elaborate on the mast cell in **chapter 6** in which we examined how aging affects mast cell phenotype, since this is a prominent risk factor for atherosclerosis. We describe that the aging microenvironment in the plaque increases mast cell activation in the atherosclerotic aorta and promotes the antigen-presenting capacities of these cells. Finally, we applied the human single-cell RNA sequencing data set to identify two genes that potentially affect atherosclerosis. In **chapter 7** we blocked BLT1 receptor to inhibit leukotriene B4-mediated mast cell migration to the plaque. This did neither affect atherosclerosis progression nor mast cell migration towards the plaque in *Ldlr*$^{-/-}$ mice. In **chapter 8** we used a small molecule to inhibit IL4I1, which was uniquely present on TREM2^{+} macrophages. Although this did induce a clear pro-inflammatory T cell response, atherosclerosis development was not altered. In **chapter 9** all data in this thesis will be summarized and discussed, including concluding remarks and future perspectives.

References

1. World Health Organization. Noncommunicable diseases. https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases (2022).
2. Timmis, A. *et al.* European Society of Cardiology: cardiovascular disease statistics 2021. *Eur Heart J* **43**, 716–799 (2022).
3. World Health Organization. Cardiovascular diseases (CVDs). https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (2021).
4. Grundy, S. M. *et al.* 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA Guideline on the Management of Blood Cholesterol: Executive Summary. *J Am Coll Cardiol* **73**, (2019).
5. Bäck, M., Yurdagul, A., Tabas, I., Öörni, K. & Kovanen, P. T. Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. *Nature Reviews Cardiology 2019 16:7* **16**, 389–406 (2019).
6. Bays, H. E. *et al.* Ten things to know about ten cardiovascular disease risk factors. *Am J Prev Cardiol* **5**, 100149 (2021).
7. Libby, P. *et al.* Atherosclerosis. *Nature Reviews Disease Primers 2019 5:1* **5**, 1–18 (2019).
8. Loef, M. & Walach, H. The combined effects of healthy lifestyle behaviors on all cause mortality: A systematic review and meta-analysis. *Prev Med (Baltim)* **55**, 163–170 (2012).
9. Fang, N., Jiang, M. & Fan, Y. Ideal cardiovascular health metrics and risk of cardiovascular disease or mortality: A meta-analysis. *Int J Cardiol* **214**, 279–283 (2016).
10. Lloyd-Jones, D. M. *et al.* Defining and Setting National Goals for Cardiovascular Health Promotion and Disease Reduction. *Circulation* **121**, 586–613 (2010).
11. Cannon, C. P. *et al.* Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N Engl J Med* **350**, 1495–1504 (2004).
12. Libby, P., Ridker, P. M. & Hansson, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature* **473**, 317–325 (2011).
13. Collet, J.-P. *et al.* 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevationThe Task Force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). *Eur Heart J* **42**, 1289–1367 (2021).
14. Braunwald, E. The treatment of acute myocardial infarction: the Past, the Present, and the Future. *Eur Heart J Acute Cardiovasc Care* **1**, 9–12 (2012).
15. Michaels, A. D. & Chatterjee, K. Angioplasty Versus Bypass Surgery for Coronary Artery Disease. *Circulation* **106**, (2002).
16. Bonati, L. H. *et al.* European Stroke Organisation guideline on endarterectomy and stenting for carotid artery stenosis. *Eur Stroke J* **6**, I–XLVII (2021).
17. Calvillo-King, L., Xuan, L., Zhang, S., Tuhrim, S. & Halm, E. A. Predicting Risk of Perioperative Death and Stroke After Carotid Endarterectomy in Asymptomatic Patients: Derivation and Validation of a Clinical Risk Score. *Stroke; a journal of cerebral circulation* **41**, 2786 (2010).
18. Engelen, S. E., Robinson, A. J. B., Zurke, Y. X. & Monaco, C. Therapeutic strategies targeting inflammation and immunity in atherosclerosis: how to proceed? *Nat Rev Cardiol* **19**, 522–542 (2022).
19. Ridker, P. M. *et al.* Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *New England Journal of Medicine* **377**, 1119–1132 (2017).

20. Nidorf, S. M. *et al.* Colchicine in Patients with Chronic Coronary Disease. *New England Journal of Medicine* **383**, 1838–1847 (2020).
21. Tardif, J.-C. *et al.* Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. *New England Journal of Medicine* **381**, 2497–2505 (2019).
22. Opstal, T. S. J. *et al.* Long-Term Efficacy of Colchicine in Patients With Chronic Coronary Disease: Insights From LoDoCo2. *Circulation* **145**, 626–628 (2022).
23. Ridker, P. M. *et al.* IL-6 inhibition with ziltivekimab in patients at high atherosclerotic risk (RESCUE): a double-blind, randomised, placebo-controlled, phase 2 trial. *The Lancet* **397**, 2060–2069 (2021).
24. Ridker, P. M. From RESCUE to ZEUS: will interleukin-6 inhibition with ziltivekimab prove effective for cardiovascular event reduction? *Cardiovasc Res* **117**, e138–e140 (2021).
25. Zhao, T. X. *et al.* Low-dose interleukin-2 in patients with stable ischaemic heart disease and acute coronary syndromes (LILACS): protocol and study rationale for a randomised, double-blind, placebo-controlled, phase I/II clinical trial. *BMJ Open* **8**, (2018).
26. Michiels, C. Endothelial cell functions. *J Cell Physiol* **196**, 430–443 (2003).
27. Peiró, C. *et al.* Influence of Endothelium on Cultured Vascular Smooth Muscle Cell Proliferation. *Hypertension* **25**, 748–751 (1995).
28. Gimbrone, M. A., Topper, J. N., Nagel, T., Anderson, K. R. & Garcia-Cardeña, G. Endothelial Dysfunction, Hemodynamic Forces, and Atherogenesisa. *Ann N Y Acad Sci* **902**, 230–240 (2000).
29. Cunningham, K. S. & Gotlieb, A. I. The role of shear stress in the pathogenesis of atherosclerosis. *Laboratory Investigation 2005 85:1* **85**, 9–23 (2004).
30. Cybulsky, M. I. & Gimbrone, M. A. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* **251**, 788–791 (1991).
31. Libby, P. The changing landscape of atherosclerosis. *Nature 2021 592:7855* **592**, 524–533 (2021).
32. Lu, Y. *et al.* The Functional Role of Lipoproteins in Atherosclerosis: Novel Directions for Diagnosis and Targeting Therapy. *Aging Dis* **13**, 491 (2022).
33. Bhargava, S., de la Puente-Secades, S., Schurgers, L. & Jankowski, J. Lipids and lipoproteins in cardiovascular diseases: a classification. *Trends in Endocrinology & Metabolism* **33**, 409–423 (2022).
34. Borén, J. *et al.* Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding. *Journal of Clinical Investigation* **101**, 2658 (1998).
35. Flood, C. *et al.* Molecular Mechanism for Changes in Proteoglycan Binding on Compositional Changes of the Core and the Surface of Low-Density Lipoprotein-Containing Human Apolipoprotein B100. *Arterioscler Thromb Vasc Biol* **24**, 564–570 (2004).
36. Tabas, I., Williams, K. J. & Borén, J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* **116**, 1832–1844 (2007).
37. Alique, M., Luna, C., Carracedo, J. & Ramírez, R. LDL biochemical modifications: a link between atherosclerosis and aging. *Food Nutr Res* **59**, (2015).
38. Moore, K. J. & Tabas, I. The Cellular Biology of Macrophages in Atherosclerosis. *Cell* **145**, 341 (2011).
39. Moore, K. J., Sheedy, F. J. & Fisher, E. A. Macrophages in atherosclerosis: a dynamic balance. *Nature Reviews Immunology 2013 13:10* **13**, 709–721 (2013).
40. Shapouri-Moghaddam, A. *et al.* Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol* **233**, 6425–6440 (2018).

41. Willemsen, L. & de Winther, M. P. J. Macrophage subsets in atherosclerosis as defined by single-cell technologies. *J Pathol* **250**, 705–714 (2020).
42. Goldstein, J. L., Ho, Y. K., Basu, S. K. & Brown, M. S. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A* **76**, 333 (1979).
43. Kunjathoor, V. V. *et al.* Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem* **277**, 49982–49988 (2002).
44. Williams, J. W. *et al.* Limited proliferation capacity of aortic intima resident macrophages requires monocyte recruitment for atherosclerotic plaque progression. *Nat Immunol* **21**, 1194–1204 (2020).
45. Wilson, H. M. Macrophages heterogeneity in atherosclerosis – implications for therapy. *J Cell Mol Med* **14**, 2055–2065 (2010).
46. Björkegren, J. L. M. *et al.* Plasma Cholesterol-Induced Lesion Networks Activated before Regression of Early, Mature, and Advanced Atherosclerosis. *PLoS Genet* **10**, e1004201 (2014).
47. Libby, P. *et al.* Atherosclerosis. *Nature Reviews Disease Primers 2019 5:1* **5**, 1–18 (2019).
48. Allahverdian, S., Chaabane, C., Boukais, K., Francis, G. A. & Bochaton-Piallat, M. L. Smooth muscle cell fate and plasticity in atherosclerosis. *Cardiovasc Res* **114**, 540 (2018).
49. Owens, G. K., Kumar, M. S. & Wamhoff, B. R. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* **84**, 767–801 (2004).
50. Rensen, S. S. M., Doevendans, P. A. F. M. & van Eys, G. J. J. M. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. *Neth Heart J* **15**, 100–8 (2007).
51. Gomez, D. & Owens, G. K. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res* **95**, 156–164 (2012).
52. Doran, A. C., Meller, N. & McNamara, C. A. Role of Smooth Muscle Cells in the Initiation and Early Progression of Atherosclerosis. *Arterioscler Thromb Vasc Biol* **28**, 812–819 (2008).
53. Bentzon, J. F., Otsuka, F., Virmani, R. & Falk, E. Mechanisms of Plaque Formation and Rupture. *Circ Res* **114**, 1852–1866 (2014).
54. Rong, J. X., Shapiro, M., Trogan, E. & Fisher, E. A. Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *Proceedings of the National Academy of Sciences* **100**, 13531–13536 (2003).
55. Li, H., Freeman, M. W. & Libby, P. Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and in vitro by cytokines. *J Clin Invest* **95**, 122–133 (1995).
56. Shankman, L. S. *et al.* KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nat Med* **21**, 628–637 (2015).
57. Wang, Y. *et al.* Smooth Muscle Cells Contribute the Majority of Foam Cells in ApoE (Apolipoprotein E)-Deficient Mouse Atherosclerosis. *Arterioscler Thromb Vasc Biol* **39**, 876–887 (2019).
58. Doran, A. C., Yurdagul, A. & Tabas, I. Efferocytosis in health and disease. *Nature Reviews Immunology 2019 20:4* **20**, 254–267 (2019).
59. Björkegren, J. L. M. & Lusis, A. J. Atherosclerosis: Recent developments. *Cell* **185**, 1630–1645 (2022).
60. Schrijvers, D. M., De Meyer, G. R. Y., Herman, A. G. & Martinet, W. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. *Cardiovasc Res* **73**, 470–480 (2007).
61. Tabas, I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol* **25**, 2255–2264 (2005).

62. Geng, Y. J. & Libby, P. Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme. *Am J Pathol* **147**, 251 (1995).
63. Weber, C. & Noels, H. Atherosclerosis: current pathogenesis and therapeutic options. *Nature Medicine 2011 17:11* **17**, 1410–1422 (2011).
64. Hansson, G. K., Hellstrand, M., Rymo, L., Rubbia, L. & Gabbiani, G. Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med* **170**, 1595–1608 (1989).
65. Moore, K. J. & Tabas, I. Macrophages in the pathogenesis of atherosclerosis. *Cell* **145**, 341–355 (2011).
66. Soehnlein, O. Multiple Roles for Neutrophils in Atherosclerosis. *Circ Res* **110**, 875–888 (2012).
67. Willems, S. *et al*. Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur Heart J* **34**, 3699–3706 (2013).
68. Johnson, J. L., Jackson, C. L., Angelini, G. D. & George, S. J. Activation of Matrix-Degrading Metalloproteinases by Mast Cell Proteases in Atherosclerotic Plaques. *Arterioscler Thromb Vasc Biol* **18**, 1707–1715 (1998).
69. Partida, R. A., Libby, P., Crea, F. & Jang, I. K. Plaque erosion: a new in vivo diagnosis and a potential major shift in the management of patients with acute coronary syndromes. *Eur Heart J* **39**, 2070–2076 (2018).
70. Libby, P. & Pasterkamp, G. Requiem for the 'vulnerable plaque'. *Eur Heart J* **36**, 2984–2987 (2015).
71. Moses, S. R., Adorno, J. J., Palmer, A. F. & Song, J. W. Vessel-on-a-chip models for studying microvascular physiology, transport, and function in vitro. *Am J Physiol Cell Physiol* **320**, C92–C105 (2021).
72. Daugherty, A. *et al*. Recommendation on Design, Execution, and Reporting of Animal Atherosclerosis Studies: A Scientific Statement From the American Heart Association. *Circ Res* **121**, e53–e79 (2017).
73. Getz, G. S. & Reardon, C. A. Diet and Murine Atherosclerosis. *Arterioscler Thromb Vasc Biol* **26**, 242–249 (2006).
74. Paigen, B., Morrow, A., Brandon, C., Mitchell, D. & Holmes, P. Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis* **57**, 65–73 (1985).
75. Van Eck, M. *et al*. Essential role for the (hepatic) LDL receptor in macrophage apolipoprotein E-induced reduction in serum cholesterol levels and atherosclerosis. *Atherosclerosis* **154**, 103–112 (2001).
76. Meir, K. S. & Leitersdorf, E. Atherosclerosis in the Apolipoprotein E–Deficient Mouse. *Arterioscler Thromb Vasc Biol* **24**, 1006–1014 (2004).
77. Zhang, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. Spontaneous Hypercholesterolemia and Arterial Lesions in Mice Lacking Apolipoprotein E. *Science (1979)* **258**, 468–471 (1992).
78. Getz, G. S. & Reardon, C. A. Animal Models of Atherosclerosis. *Arterioscler Thromb Vasc Biol* **32**, 1104–1115 (2012).
79. Emini Veseli, B. *et al*. Animal models of atherosclerosis. *Eur J Pharmacol* **816**, 3–13 (2017).
80. Smit, V. *et al*. Single-cell profiling reveals age-associated immunity in atherosclerosis. *Cardiovasc Res* (2023) doi:10.1093/CVR/CVAD099.
81. Roche-Molina, M. *et al*. Induction of sustained hypercholesterolemia by single adeno-associated virus-mediated gene transfer of mutant hPCSK9. *Arterioscler Thromb Vasc Biol* **35**, 50–59 (2015).

82. Bjørklund, M. M. *et al.* Induction of atherosclerosis in mice and hamsters without germline genetic engineering. *Circ Res* **114**, 1684–1689 (2014).
83. Punch, E., Klein, J., Diaba-Nuhoho, P., Morawietz, H. & Garelnabi, M. Effects of PCSK9 Targeting: Alleviating Oxidation, Inflammation, and Atherosclerosis. *J Am Heart Assoc* **11**, 23328 (2022).
84. Medzhitov, R. Origin and physiological roles of inflammation. *Nature 2008 454:7203* **454**, 428–435 (2008).
85. Vivier, E. & Malissen, B. Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nature Immunology 2005 6:1* **6**, 17–21 (2004).
86. Li, D. & Wu, M. Pattern recognition receptors in health and diseases. *Signal Transduction and Targeted Therapy 2021 6:1* **6**, 1–24 (2021).
87. Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* **81**, 1–5 (2007).
88. Netea, M. G., Schlitzer, A., Placek, K., Joosten, L. A. B. & Schultze, J. L. Innate and Adaptive Immune Memory: an Evolutionary Continuum in the Host's Response to Pathogens. *Cell Host Microbe* **25**, 13–26 (2019).
89. Silvestre-Roig, C., Braster, Q., Ortega-Gomez, A. & Soehnlein, O. Neutrophils as regulators of cardiovascular inflammation. *Nature Reviews Cardiology 2020 17:6* **17**, 327–340 (2020).
90. Palano, M. T. *et al.* When a Friend Becomes Your Enemy: Natural Killer Cells in Atherosclerosis and Atherosclerosis-Associated Risk Factors. *Front Immunol* **12**, 5718 (2022).
91. Engelbertsen, D. & Lichtman, A. H. Innate lymphoid cells in atherosclerosis. *Eur J Pharmacol* **816**, 32–36 (2017).
92. Ginhoux, F. & Jung, S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature Reviews Immunology 2014 14:6* **14**, 392–404 (2014).
93. Ingersoll, M. A. *et al.* Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* **115**, (2010).
94. Auffray, C. *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* **317**, 666–670 (2007).
95. Carlin, L. M. *et al.* Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell* **153**, 362–375 (2013).
96. Geissmann, F., Jung, S. & Littman, D. R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71–82 (2003).
97. Buscher, K., Marcovecchio, P., Hedrick, C. C. & Ley, K. Patrolling Mechanics of Non-Classical Monocytes in Vascular Inflammation. *Front Cardiovasc Med* **4**, 80 (2017).
98. Swirski, F. K. *et al.* Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease. *Proc Natl Acad Sci U S A* **103**, 10340–10345 (2006).
99. Swirski, F. K. *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest* **117**, 195–205 (2007).
100. C. Dawson, T., A. Kuziel, W., A. Osahar, T. & Maeda, N. Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis* **143**, 205–211 (1999).
101. Chen, Y. J. *et al.* Eps8 protein facilitates phagocytosis by increasing TLR4-MyD88 protein interaction in lipopolysaccharide-stimulated macrophages. *J Biol Chem* **287**, 18806–18819 (2012).
102. Shapouri-Moghaddam, A. *et al.* Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol* **233**, 6425–6440 (2018).
103. Geng, H. *et al.* The effects of ox-LDL in human atherosclerosis may be mediated in part via the toll-like receptor 4 pathway. *Mol Cell Biochem* **342**, 201–206 (2010).

104. Sica, A., Erreni, M., Allavena, P. & Porta, C. Macrophage polarization in pathology. *Cell Mol Life Sci* **72**, 4111–4126 (2015).
105. Wang, N., Liang, H. & Zen, K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Front Immunol* **5**, (2014).
106. Jetten, N. *et al.* Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. *Angiogenesis* **17**, 109–118 (2014).
107. Braga, T. T., Agudelo, J. S. H. & Camara, N. O. S. Macrophages During the Fibrotic Process: M2 as Friend and Foe. *Front Immunol* **6**, (2015).
108. Moore, K. J., Sheedy, F. J. & Fisher, E. A. Macrophages in atherosclerosis: a dynamic balance. *Nature Reviews Immunology 2013 13:10* **13**, 709–721 (2013).
109. Roy, P., Orecchioni, M. & Ley, K. How the immune system shapes atherosclerosis: roles of innate and adaptive immunity. *Nature Reviews Immunology 2021 22:4* **22**, 251–265 (2021).
110. Zernecke, A. *et al.* Meta-Analysis of Leukocyte Diversity in Atherosclerotic Mouse Aortas. *Circ Res* **127**, 402–426 (2020).
111. King, K. R. *et al.* IRF3 and type I interferons fuel a fatal response to myocardial infarction. *Nat Med* **23**, 1481–1487 (2017).
112. Cochain, C. *et al.* Single-Cell RNA-Seq Reveals the Transcriptional Landscape and Heterogeneity of Aortic Macrophages in Murine Atherosclerosis. *Circ Res* **122**, 1661–1674 (2018).
113. Kim, K. *et al.* Transcriptome Analysis Reveals Nonfoamy Rather Than Foamy Plaque Macrophages Are Proinflammatory in Atherosclerotic Murine Models. *Circ Res* **123**, 1127–1142 (2018).
114. Ensan, S. *et al.* Self-renewing resident arterial macrophages arise from embryonic CX3CR1(+) precursors and circulating monocytes immediately after birth. *Nat Immunol* **17**, 159–168 (2016).
115. Lim, H. Y. *et al.* Hyaluronan Receptor LYVE-1-Expressing Macrophages Maintain Arterial Tone through Hyaluronan-Mediated Regulation of Smooth Muscle Cell Collagen. *Immunity* **49**, 326-341. e7 (2018).
116. Williams, J. W. *et al.* Limited proliferation capacity of aortic intima resident macrophages requires monocyte recruitment for atherosclerotic plaque progression. *Nature Immunology 2020 21:10* **21**, 1194–1204 (2020).
117. de Winther, M. P. J. *et al.* Translational opportunities of single-cell biology in atherosclerosis. *Eur Heart J* **44**, 1216–1230 (2023).
118. Depuydt, M. A. C. *et al.* Microanatomy of the Human Atherosclerotic Plaque by Single-Cell Transcriptomics. *Circ Res* **127**, 1437–1455 (2020).
119. Fernandez, D. M. *et al.* Single-cell immune landscape of human atherosclerotic plaques. *Natuere Medicine* **25**, 1576–1588 (2019).
120. Ramachandran, P. *et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level*. *Nature* vol. 575 (Springer US, 2019).
121. Subramanian, M. & Tabas, I. Dendritic cells in atherosclerosis. *Semin Immunopathol* **36**, 93–102 (2014).
122. Subramanian, M. & Tabas, I. Dendritic cells in atherosclerosis. *Semin Immunopathol* **36**, 93–102 (2014).
123. Satpathy, A. T. *et al.* Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *Journal of Experimental Medicine* **209**, 1135–1152 (2012).
124. Zernecke, A. Dendritic Cells in Atherosclerosis. *Arterioscler Thromb Vasc Biol* **35**, 763–770 (2015).
125. Tamoutounour, S. *et al.* Origins and Functional Specialization of Macrophages and of Conventional and Monocyte-Derived Dendritic Cells in Mouse Skin. *Immunity* **39**, 925–938 (2013).

126. Varol, C. *et al.* Intestinal Lamina Propria Dendritic Cell Subsets Have Different Origin and Functions. *Immunity* **31**, 502–512 (2009).
127. Guilliams, M. *et al.* Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature Reviews Immunology 2014 14:8* **14**, 571–578 (2014).
128. Gil-Pulido, J. & Zernecke, A. Antigen-presenting dendritic cells in atherosclerosis. *Eur J Pharmacol* **816**, 25–31 (2017).
129. Hildner, K. *et al.* Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* **322**, 1097–1100 (2008).
130. Tamura, T. *et al.* IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol* **174**, 2573–2581 (2005).
131. Liu, J., Zhang, X., Cheng, Y. & Cao, X. Dendritic cell migration in inflammation and immunity. *Cellular & Molecular Immunology 2021 18:11* **18**, 2461–2471 (2021).
132. Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C. & Amigorena, S. Antigen Presentation and T Cell Stimulation by Dendritic Cells. *https://doi.org/10.1146/annurev.immunol.20.100301.064828* **20**, 621–667 (2003).
133. Bobryshev, Y. V. Dendritic cells in atherosclerosis: current status of the problem and clinical relevance. *Eur Heart J* **26**, 1700–1704 (2005).
134. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature 1998 392:6673* **392**, 245–252 (1998).
135. Kambayashi, T. & Laufer, T. M. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nature Reviews Immunology 2014 14:11* **14**, 719–730 (2014).
136. Ait-Oufella, H., Sage, A. P., Mallat, Z. & Tedgui, A. Adaptive (T and B Cells) Immunity and Control by Dendritic Cells in Atherosclerosis. *Circ Res* **114**, 1640–1660 (2014).
137. Yilmaz, A. *et al.* Emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques. *Atherosclerosis* **176**, 101–110 (2004).
138. Erbel, C. *et al.* Functional profile of activated dendritic cells in unstable atherosclerotic plaque. *Basic Res Cardiol* **102**, 123–132 (2007).
139. Mohanta, S. K. *et al.* Artery Tertiary Lymphoid Organs Contribute to Innate and Adaptive Immune Responses in Advanced Mouse Atherosclerosis. *Circ Res* **114**, 1772–1787 (2014).
140. Yin, C., Mohanta, S. K., Srikakulapu, P., Weber, C. & Habenicht, A. J. R. Artery tertiary lymphoid organs: Powerhouses of atherosclerosis immunity. *Front Immunol* **7**, 217268 (2016).
141. Zhao, Y., Zhang, J., Zhang, W. & Xu, Y. A myriad of roles of dendritic cells in atherosclerosis. *Clin Exp Immunol* **206**, 12–27 (2021).
142. Paulson, K. E. *et al.* Resident Intimal Dendritic Cells Accumulate Lipid and Contribute to the Initiation of Atherosclerosis. *Circ Res* **106**, 383–390 (2010).
143. Gautier, E. L. *et al.* Conventional Dendritic Cells at the Crossroads Between Immunity and Cholesterol Homeostasis in Atherosclerosis. *Circulation* **119**, 2367–2375 (2009).
144. Habets, K. L. L. *et al.* Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc Res* **85**, 622–630 (2010).
145. Hermansson, A. *et al.* Immunotherapy With Tolerogenic Apolipoprotein B-100–Loaded Dendritic Cells Attenuates Atherosclerosis in Hypercholesterolemic Mice. *Circulation* **123**, 1083–1091 (2011).
146. Vigario, F. L., Kuiper, J. & Slütter, B. Tolerogenic vaccines for the treatment of cardiovascular diseases. *EBioMedicine* **57**, 102827 (2020).
147. Jansen, M. A. A. *et al.* Targeting of tolerogenic dendritic cells towards heat-shock proteins: a novel therapeutic strategy for autoimmune diseases? *Immunology* **153**, 51 (2018).

148. Galli, S. J., Gaudenzio, N. & Tsai, M. Mast Cells in Inflammation and Disease: Recent Progress and Ongoing Concerns. *https://doi.org/10.1146/annurev-immunol-071719-094903* **38**, 49–77 (2020).
149. St. John, A. L., Rathore, A. P. S. & Ginhoux, F. New perspectives on the origins and heterogeneity of mast cells. *Nature Reviews Immunology 2022 23:1* **23**, 55–68 (2022).
150. Dahlin, J. S. & Hallgren, J. Mast cell progenitors: Origin, development and migration to tissues. *Mol Immunol* **63**, 9–17 (2015).
151. Dahlin, J. S. *et al.* Lin− CD34hi CD117int/hi FcεRI+ cells in human blood constitute a rare population of mast cell progenitors. *Blood* **127**, 383–391 (2016).
152. El-Agamy, D. S. Targeting c-kit in the therapy of mast cell disorders: Current update. *Eur J Pharmacol* **690**, 1–3 (2012).
153. Krystel-Whittemore, M., Dileepan, K. N. & Wood, J. G. Mast cell: A multi-functional master cell. *Front Immunol* **6**, 165675 (2016).
154. Galli, S. J., Borregaard, N. & Wynn, T. A. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nature Immunology 2011 12:11* **12**, 1035–1044 (2011).
155. Nagata, Y. & Suzuki, R. FcεRI: A Master Regulator of Mast Cell Functions. *Cells* **11**, 622 (2022).
156. Sibilano, R., Frossi, B. & Pucillo, C. E. Mast cell activation: A complex interplay of positive and negative signaling pathways. *Eur J Immunol* **44**, 2558–2566 (2014).
157. Kalesnikoff, J. & Galli, S. J. New developments in mast cell biology. *Nature Immunology 2008 9:11* **9**, 1215–1223 (2008).
158. Shi, G. P., Bot, I. & Kovanen, P. T. Mast cells in human and experimental cardiometabolic diseases. *Nat Rev Cardiol* **12**, 643–658 (2015).
159. Dimitriadou, V. *et al.* Expression of functional major histocompatility complex class II molecules on HMC-1 human mast cells. *J Leukoc Biol* **64**, 791–799 (1998).
160. Poncet, P., Arock, M. & David, B. MHC class II-dependent activation of CD4+ T cell hybridomas by human mast cells through superantigen presentation. *J Leukoc Biol* **66**, 105–112 (1999).
161. Kaartinen, M., Penttilä, A. & Kovanen, P. T. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* **90**, 1669–1678 (1994).
162. Atkinson, J. B., Harlan, C. W., Harlan, G. C. & Virmani, R. The association of mast cells and atherosclerosis: a morphologic study of early atherosclerotic lesions in young people. *Hum Pathol* **25**, 154–159 (1994).
163. Kovanen, P. T., Kaartinen, M. & Paavonen, T. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation* **92**, 1084–1088 (1995).
164. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115**, 2516–2525 (2007).
165. Sun, J. *et al.* Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat Med* **13**, 719–724 (2007).
166. Leskinen, M. J., Lindstedt, K. A., Wang, Y. & Kovanen, P. T. Mast cell chymase induces smooth muscle cell apoptosis by a mechanism involving fibronectin degradation and disruption of focal adhesions. *Arterioscler Thromb Vasc Biol* **23**, 238–243 (2003).
167. Leskinen, M. J. *et al.* Mast cell chymase induces smooth muscle cell apoptosis by disrupting NF-kappaB-mediated survival signaling. *Exp Cell Res* **312**, 1289–1298 (2006).

168. Mäyränpää, M. I., Heikkilä, H. M., Lindstedt, K. A., Walls, A. F. & Kovanen, P. T. Desquamation of human coronary artery endothelium by human mast cell proteases: implications for plaque erosion. *Coron Artery Dis* **17**, 611–621 (2006).
169. Heikkilä, H. M. *et al.* Activated mast cells induce endothelial cell apoptosis by a combined action of chymase and tumor necrosis factor-alpha. *Arterioscler Thromb Vasc Biol* **28**, 309–314 (2008).
170. Johnson, J. L., Jackson, C. L., Angelini, G. D. & George, S. J. Activation of Matrix-Degrading Metalloproteinases by Mast Cell Proteases in Atherosclerotic Plaques. *Arterioscler Thromb Vasc Biol* **18**, 1707–1715 (1998).
171. Bot, I. *et al.* Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. *Cardiovasc Res* **89**, 244–252 (2011).
172. Kokkonen, J. O. & Kovanen, P. T. Low-density-lipoprotein binding by mast-cell granules. Demonstration of binding of apolipoprotein B to heparin proteoglycan of exocytosed granules. *Biochem J* **241**, 583–589 (1987).
173. Kokkonen, J. O. & Kovanen, P. T. Stimulation of mast cells leads to cholesterol accumulation in macrophages in vitro by a mast cell granule-mediated uptake of low density lipoprotein. *Proc Natl Acad Sci U S A* **84**, 2287–2291 (1987).
174. Kokkonen, J. O. Stimulation of rat peritoneal mast cells enhances uptake of low density lipoproteins by rat peritoneal macrophages in vivo. *Atherosclerosis* **79**, 213–223 (1989).
175. Kaartinen, M., Penttilä, A. & Kovanen, P. T. Extracellular mast cell granules carry apolipoprotein B-100-containing lipoproteins into phagocytes in human arterial intima. Functional coupling of exocytosis and phagodytosis in neighboring cells. *Arterioscler Thromb Vasc Biol* **15**, 2047–2054 (1995).
176. Bot, I., Shi, G. P. & Kovanen, P. T. Mast cells as effectors in atherosclerosis. *Arterioscler Thromb Vasc Biol* **35**, 265 (2015).
177. Gisterå, A. & Hansson, G. K. The immunology of atherosclerosis. *Nature Reviews Nephrology 2017 13:6* **13**, 368–380 (2017).
178. Saigusa, R., Winkels, H. & Ley, K. T cell subsets and functions in atherosclerosis. *Nat Rev Cardiol* **17**, 387 (2020).
179. Douna, H. & Kuiper, J. Novel B-cell subsets in atherosclerosis. *Curr Opin Lipidol* **27**, 493–498 (2016).
180. Sage, A. P., Tsiantoulas, D., Binder, C. J. & Mallat, Z. The role of B cells in atherosclerosis. *Nature Reviews Cardiology 2018 16:3* **16**, 180–196 (2018).
181. Mallat, Z. & Binder, C. J. The why and how of adaptive immune responses in ischemic cardiovascular disease. *Nature Cardiovascular Research 2022 1:5* **1**, 431–444 (2022).
182. Anderson, G. & Jenkinson, E. J. Lymphostromal interactions in thymic development and function. *Nature Reviews Immunology 2001 1:1* **1**, 31–40 (2001).
183. Kumar, B. V, Connors, T. J. & Farber, D. L. Human T Cell Development, Localization, and Function throughout Life. (2018) doi:10.1016/j.immuni.2018.01.007.
184. Bassing, C. H., Swat, W. & Alt, F. W. The Mechanism and Regulation of Chromosomal V(D)J Recombination. *Cell* **109**, S45–S55 (2002).
185. Moran, A. E. & Hogquist, K. A. T-cell receptor affinity in thymic development. *Immunology* **135**, 261–267 (2012).
186. Hogquist, K. A. Signal strength in thymic selection and lineage commitment. *Curr Opin Immunol* **13**, 225–231 (2001).
187. Lutes, L. K. *et al.* T cell self-reactivity during thymic development dictates the timing of positive selection. *Elife* **10**, (2021).

188. Hansson, G. K. & Hermansson, A. The immune system in atherosclerosis. *Nat Immunol* **12**, 204–212 (2011).
189. Marshall, J. S., Warrington, R., Watson, W. & Kim, H. L. An introduction to immunology and immunopathology. *Allergy, Asthma and Clinical Immunology* **14**, 1–10 (2018).
190. Song, L., Leung, C. & Schindler, C. Lymphocytes are important in early atherosclerosis. *J Clin Invest* **108**, 251–259 (2001).
191. Zhou, X., Nicoletti, A., Elhage, R. & Hansson, G. K. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation* **102**, 2919–2922 (2000).
192. van Dijk, R. A. *et al.* A change in inflammatory footprint precedes plaque instability: a systematic evaluation of cellular aspects of the adaptive immune response in human atherosclerosis. *J Am Heart Assoc* **4**, (2015).
193. Zhang, W. *et al.* IL-9 aggravates the development of atherosclerosis in ApoE-/- mice. *Cardiovasc Res* **106**, 453–464 (2015).
194. Lin, Y. Z. *et al.* Circulating Th22 and Th9 levels in patients with acute coronary syndrome. *Mediators Inflamm* **2013**, (2013).
195. Zhang, L. *et al.* Elevated frequencies of circulating Th22 cell in addition to Th17 cell and Th17/Th1 cell in patients with acute coronary syndrome. *PLoS One* **8**, (2013).
196. Nus, M. *et al.* Marginal zone B cells control the response of follicular helper T cells to a high-cholesterol diet. *Nat Med* **23**, 601–610 (2017).
197. Ryu, H. *et al.* Atherogenic dyslipidemia promotes autoimmune follicular helper T cell responses via IL-27. *Nat Immunol* **19**, 583–593 (2018).
198. Douna, H. *et al.* IFNγ-Stimulated B Cells Inhibit T Follicular Helper Cells and Protect Against Atherosclerosis. *Front Cardiovasc Med* **9**, 781436 (2022).
199. Liuzzo, G. *et al.* Unusual CD4+CD28null T Lymphocytes and Recurrence of Acute Coronary Events. *J Am Coll Cardiol* **50**, 1450–1458 (2007).
200. Tomas, L. *et al.* Low Levels of CD4+CD28null T Cells at Baseline Are Associated With First-Time Coronary Events in a Prospective Population-Based Case-Control Cohort. *Arterioscler Thromb Vasc Biol* **40**, 426–436 (2020).
201. Buono, C. *et al.* T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proc Natl Acad Sci U S A* **102**, 1596–1601 (2005).
202. Buono, C. *et al.* Influence of interferon-gamma on the extent and phenotype of diet-induced atherosclerosis in the LDLR-deficient mouse. *Arterioscler Thromb Vasc Biol* **23**, 454–460 (2003).
203. Gupta, S. *et al.* IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest* **99**, 2752–2761 (1997).
204. Whitman, S. C., Ravisankar, P., Elam, H. & Daugherty, A. Exogenous Interferon-γ Enhances Atherosclerosis in Apolipoprotein E−/− Mice. *Am J Pathol* **157**, 1819–1824 (2000).
205. Hansson, G. K., Hellstrand, M., Rymo, L., Rubbia, L. & Gabbiani, G. Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med* **170**, 1595–1608 (1989).
206. Yu, X. H., Zhang, J., Zheng, X. L., Yang, Y. H. & Tang, C. K. Interferon-γ in foam cell formation and progression of atherosclerosis. *Clin Chim Acta* **441**, 33–43 (2015).
207. Orecchioni, M., Ghosheh, Y., Pramod, A. B. & Ley, K. Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages. *Front Immunol* **10**, (2019).

208. Walker, J. A. & McKenzie, A. N. J. TH2 cell development and function. *Nature Reviews Immunology 2017 18:2* **18**, 121–133 (2017).
209. Sun, L., Su, Y., Jiao, A., Wang, X. & Zhang, B. T cells in health and disease. *Signal Transduction and Targeted Therapy 2023 8:1* **8**, 1–50 (2023).
210. Wurtz, O., Bajénoff, M. & Guerder, S. IL-4-mediated inhibition of IFN-gamma production by CD4+ T cells proceeds by several developmentally regulated mechanisms. *Int Immunol* **16**, 501–508 (2004).
211. King, V. L., Szilvassy, S. J. & Daugherty, A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. *Arterioscler Thromb Vasc Biol* **22**, 456–461 (2002).
212. Davenport, P. & Tipping, P. G. The Role of Interleukin-4 and Interleukin-12 in the Progression of Atherosclerosis in Apolipoprotein E-Deficient Mice. *Am J Pathol* **163**, 1117 (2003).
213. Engelbertsen, D. *et al*. Induction of T helper 2 responses against human apolipoprotein B100 does not affect atherosclerosis in ApoE-/- mice. *Cardiovasc Res* **103**, 304–312 (2014).
214. Engelbertsen, D. *et al*. T-helper 2 immunity is associated with reduced risk of myocardial infarction and stroke. *Arterioscler Thromb Vasc Biol* **33**, 637–644 (2013).
215. Silveira, A. *et al*. Plasma IL-5 concentration and subclinical carotid atherosclerosis. *Atherosclerosis* **239**, 125–130 (2015).
216. Binder, C. J. *et al*. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Invest* **114**, 427–437 (2004).
217. Cardilo-Reis, L. *et al*. Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype. *EMBO Mol Med* **4**, 1072–1086 (2012).
218. Peters, A., Lee, Y. & Kuchroo, V. K. The many faces of Th17 cells. *Curr Opin Immunol* **23**, 702–706 (2011).
219. Usui, F. *et al*. Interleukin-17 deficiency reduced vascular inflammation and development of atherosclerosis in Western diet-induced apoE-deficient mice. *Biochem Biophys Res Commun* **420**, 72–77 (2012).
220. Danzaki, K. *et al*. Interleukin-17A deficiency accelerates unstable atherosclerotic plaque formation in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **32**, 273–280 (2012).
221. Madhur, M. S. *et al*. Role of interleukin 17 in inflammation, atherosclerosis, and vascular function in apolipoprotein e-deficient mice. *Arterioscler Thromb Vasc Biol* **31**, 1565–1572 (2011).
222. Gao, Q. *et al*. A critical function of Th17 proinflammatory cells in the development of atherosclerotic plaque in mice. *J Immunol* **185**, 5820–5827 (2010).
223. Erbel, C. *et al*. Inhibition of IL-17A attenuates atherosclerotic lesion development in apoE-deficient mice. *J Immunol* **183**, 8167–8175 (2009).
224. Smith, E. *et al*. Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **121**, 1746–1755 (2010).
225. Engelbertsen, D. *et al*. IL-23R deficiency does not impact atherosclerotic plaque development in mice. *J Am Heart Assoc* **7**, (2018).
226. Cheng, X. *et al*. The Th17/Treg imbalance in patients with acute coronary syndrome. *Clin Immunol* **127**, 89–97 (2008).
227. Hashmi, S. & Zeng, Q. T. Role of interleukin-17 and interleukin-17-induced cytokines interleukin-6 and interleukin-8 in unstable coronary artery disease. *Coron Artery Dis* **17**, 699–706 (2006).
228. Gisterå, A. *et al*. Transforming growth factor-β signaling in T cells promotes stabilization of atherosclerotic plaques through an interleukin-17-dependent pathway. *Sci Transl Med* **5**, (2013).

229. Taleb, S. *et al.* Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *Journal of Experimental Medicine* **206**, 2067–2077 (2009).
230. Taleb, S., Tedgui, A. & Mallat, Z. IL-17 and Th17 cells in atherosclerosis: subtle and contextual roles. *Arterioscler Thromb Vasc Biol* **35**, 258–264 (2015).
231. Hirota, K. *et al.* Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* **12**, 255–263 (2011).
232. McGeachy, M. J. *et al.* TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell–mediated pathology. *Nature Immunology 2007 8:12* **8**, 1390–1397 (2007).
233. Lee, Y. *et al.* Induction and molecular signature of pathogenic Th17 cells. *Nat Immunol* **13**, 991–999 (2012).
234. Vignali, D. A. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. *Nature Reviews Immunology 2008 8:7* **8**, 523–532 (2008).
235. Fu, S. *et al.* TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. *Am J Transplant* **4**, 1614–1627 (2004).
236. De Rosa, V. *et al.* Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. *Nat Immunol* **16**, 1174 (2015).
237. de Boer, O. J., van der Meer, J. J., Teeling, P., van der Loos, C. M. & van der Wal, A. C. Low Numbers of FOXP3 Positive Regulatory T Cells Are Present in all Developmental Stages of Human Atherosclerotic Lesions. *PLoS One* **2**, e779 (2007).
238. Ait-Oufella, H. *et al.* Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med* **12**, 178–180 (2006).
239. Klingenberg, R. *et al.* Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis. *J Clin Invest* **123**, 1323–1334 (2013).
240. Mor, A. *et al.* Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* **27**, 893–900 (2007).
241. Foks, A. C. *et al.* Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis* **218**, 53–60 (2011).
242. Kita, T. *et al.* Regression of atherosclerosis with anti-CD3 antibody via augmenting a regulatory T-cell response in mice. *Cardiovasc Res* **102**, 107–117 (2014).
243. van Es, T. *et al.* Vaccination against Foxp3(+) regulatory T cells aggravates atherosclerosis. *Atherosclerosis* **209**, 74–80 (2010).
244. Mallat, Z. *et al.* Protective role of interleukin-10 in atherosclerosis. *Circ Res* **85**, (1999).
245. Pinderski Oslund, L. J. *et al.* Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler Thromb Vasc Biol* **19**, 2847–2853 (1999).
246. Caligiuri, G. *et al.* Interleukin-10 Deficiency Increases Atherosclerosis, Thrombosis, and Low-density Lipoproteins in Apolipoprotein E Knockout Mice. *Molecular Medicine* **9**, 10 (2003).
247. Wong, P. & Pamer, E. G. CD8 T cell responses to infectious pathogens. *Annu Rev Immunol* **21**, 29–70 (2003).
248. Mittrücker, H. W., Visekruna, A. & Huber, M. Heterogeneity in the Differentiation and Function of CD8+ T Cells. *Arch Immunol Ther Exp (Warsz)* **62**, 449–458 (2014).
249. Andersen, M. H., Schrama, D., Thor Straten, P. & Becker, J. C. Cytotoxic T Cells. *Journal of Investigative Dermatology* **126**, 32–41 (2006).
250. Van Duijn, J., Kuiper, J. & Slutter, B. The many faces of CD8+ T cells in atherosclerosis. *Curr Opin Lipidol* **29**, 411–416 (2018).
251. Kolbus, D. *et al.* TAP1-Deficiency Does Not Alter Atherosclerosis Development in Apoe −/− Mice. *PLoS One* **7**, (2012).

252. Elhage, R. *et al.* Deleting TCRαβ+ or CD4+ T Lymphocytes Leads to Opposite Effects on Site-Specific Atherosclerosis in Female Apolipoprotein E-Deficient Mice. *Am J Pathol* **165**, 2013 (2004).
253. Kolbus, D. *et al.* CD8+ T cell activation predominate early immune responses to hypercholesterolemia in Apoe-/- mice. *BMC Immunol* **11**, 58 (2010).
254. Kyaw, T. *et al.* Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. *Circulation* **127**, 1028–1039 (2013).
255. Van Duijn, J. *et al.* CD8+ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4+ T-cell responses. *Cardiovasc Res* **115**, 729–738 (2019).
256. Chyu, K. Y. *et al.* CD8+ T cells mediate the athero-protective effect of immunization with an ApoB-100 peptide. *PLoS One* **7**, (2012).
257. Van Duijn, J. *et al.* Tc17 CD8+ T cells accumulate in murine atherosclerotic lesions, but do not contribute to early atherosclerosis development. *Cardiovasc Res* **117**, 2755 (2021).
258. Zhou, J. *et al.* CD8(+)CD25(+) T cells reduce atherosclerosis in apoE(-/-) mice. *Biochem Biophys Res Commun* **443**, 864–870 (2014).
259. Clement, M. *et al.* Control of the T follicular helper-germinal center B-cell axis by $CD8^+$ regulatory T cells limits atherosclerosis and tertiary lymphoid organ development. *Circulation* **131**, 560–570 (2015).
260. Bergström, I., Backteman, K., Lundberg, A., Ernerudh, J. & Jonasson, L. Persistent accumulation of interferon-γ-producing CD8+CD56+ T cells in blood from patients with coronary artery disease. *Atherosclerosis* **224**, 515–520 (2012).
261. Hwang, Y. *et al.* Expansion of CD8(+) T cells lacking the IL-6 receptor α chain in patients with coronary artery diseases (CAD). *Atherosclerosis* **249**, 44–51 (2016).
262. Gewaltig, J., Kummer, M., Koella, C., Cathomas, G. & Biedermann, B. C. Requirements for CD8 T-cell migration into the human arterial wall. *Hum Pathol* **39**, 1756–1762 (2008).
263. Lebien, T. W. & Tedder, T. F. B lymphocytes: how they develop and function. *Blood* **112**, 1570–1580 (2008).
264. Caligiuri, G., Nicoletti, A., Poirierand, B. & Hansson, G. K. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest* **109**, 745–753 (2002).
265. Major, A. S., Fazio, S. & Linton, M. F. B-Lymphocyte Deficiency Increases Atherosclerosis in LDL Receptor–Null Mice. *Arterioscler Thromb Vasc Biol* **22**, 1892–1898 (2002).
266. Binder, C. J. & Silverman, G. J. Natural antibodies and the autoimmunity of atherosclerosis. *Springer Semin Immunopathol* **26**, 385–404 (2005).
267. Kyaw, T. *et al.* B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res* **109**, 830–840 (2011).
268. Hosseini, H. *et al.* Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyreactive IgM producing B1a lymphocytes. *Cardiovasc Res* **106**, 443–452 (2015).
269. Tsiantoulas, D. *et al.* Increased Plasma IgE Accelerate Atherosclerosis in Secreted IgM Deficiency. *Circ Res* **120**, 78–84 (2017).
270. Kyaw, T., Tipping, P., Bobik, A. & Toh, B. H. Protective role of natural IgM-producing B1a cells in atherosclerosis. *Trends Cardiovasc Med* **22**, 48–53 (2012).
271. Rosenfeld, S. M. *et al.* B-1b Cells Secrete Atheroprotective IgM and Attenuate Atherosclerosis. *Circ Res* **117**, e28 (2015).
272. Kyaw, T. *et al.* Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol* **185**, 4410–4419 (2010).

273. Crotty, S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* **41**, 529–542 (2014).
274. Tay, C. *et al.* Follicular B Cells Promote Atherosclerosis via T Cell-Mediated Differentiation Into Plasma Cells and Secreting Pathogenic Immunoglobulin G. *Arterioscler Thromb Vasc Biol* **38**, e71–e84 (2018).
275. Cerutti, A., Cols, M. & Puga, I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nature Reviews Immunology 2013 13:2* **13**, 118–132 (2013).
276. Douna, H. *et al.* B- and T-lymphocyte attenuator stimulation protects against atherosclerosis by regulating follicular B cells. *Cardiovasc Res* **116**, 295–305 (2020).
277. Sage, A. P. *et al.* Regulatory B cell-specific interleukin-10 is dispensable for atherosclerosis development in mice. *Arterioscler Thromb Vasc Biol* **35**, 1770–1773 (2015).
278. Strom, A. C. *et al.* B regulatory cells are increased in hypercholesterolaemic mice and protect from lesion development via IL-10. *Thromb Haemost* **114**, 835–847 (2015).
279. Douna, H. *et al.* Bidirectional effects of IL-10+ regulatory B cells in Ldlr-/- mice. *Atherosclerosis* **280**, 118–125 (2019).
280. Tang, F. *et al.* mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods 2009 6:5* **6**, 377–382 (2009).
281. Svensson, V., Vento-Tormo, R. & Teichmann, S. A. Exponential scaling of single-cell RNA-seq in the past decade. *Nature Protocols 2018 13:4* **13**, 599–604 (2018).
282. Olsen, T. K. & Baryawno, N. Introduction to Single-Cell RNA Sequencing. *Curr Protoc Mol Biol* **122**, (2018).
283. Baysoy, A., Bai, Z., Satija, R. & Fan, R. The technological landscape and applications of single-cell multi-omics. *Nature Reviews Molecular Cell Biology 2023* 1–19 (2023) doi:10.1038/s41580-023-00615-w.
284. Winkels, H. *et al.* Atlas of the Immune Cell Repertoire in Mouse Atherosclerosis Defined by Single-Cell RNA-Sequencing and Mass Cytometry. *Circ Res* **122**, 1675–1688 (2018).