Physiological Reviews

Immunotherapy for Atherosclerosis

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Abstract

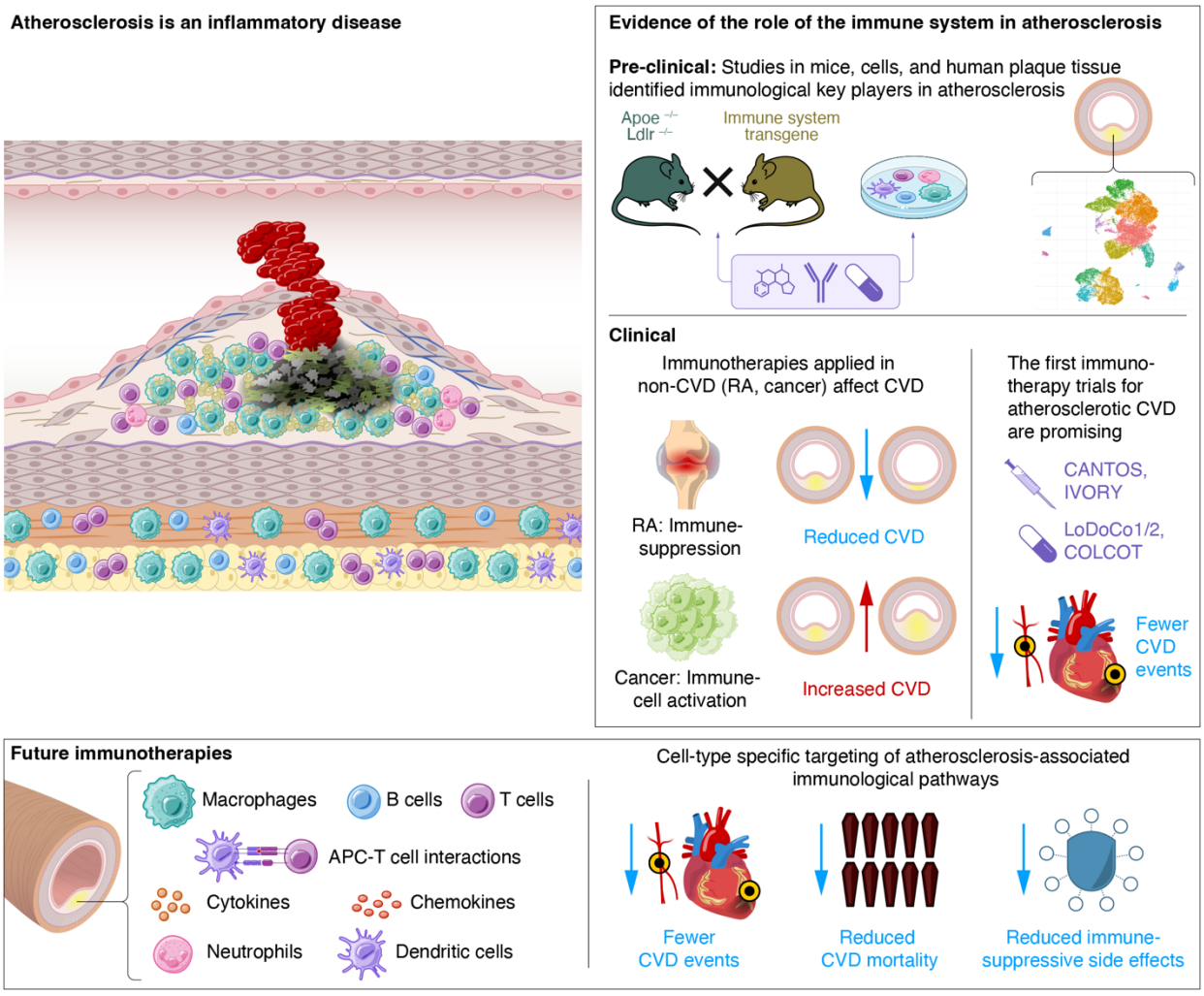
Cardiovascular disease is the global #1 cause of mortality and morbidity. The majority of cardiovascular diseases is caused by atherosclerosis, a lipid-driven, inflammatory disease of the middle- and large-sized arteries. The disease is characterized by the formation of atherosclerotic plaques throughout the arterial tree. Over the years, insights into the pathogenesis of atherosclerosis have shifted from a 'lipid-driven' model to a 'response-to-injury' perspective, and more recently to a 'lipid-driven inflammatory disease' viewpoint.

We are now aware that a network of multiple immune cell-types and -subsets of the innate and adaptive immune system inhabit our arteries. Intricate interactions between these immune cell-subsets, non-immune cells, and local environmental substances such as lipids, cell debris and calcium cause a fluidic balance of pro-inflammatory and regulatory responses. A dysregulation of this balance towards a pro-inflammatory milieu drives atherosclerotic disease progression.

Although we have acknowledged that atherosclerosis is an inflammatory disease, state-of-the-art treatments are still based on lipid lowering, anti-hypertensive and lifestyle-changing strategies. In the past decade, clinical phase I, II and III trials targeting the immune system revealed that patients tolerate immunotherapy, show decreased inflammation and/or had a reduction in cardiovascular endpoints. However, the search for novel immunotherapeutic targets and treatment regimens as well as stratification of patients who would benefit from such treatments to combat atherosclerotic cardiovascular disease is only just beginning. In this review article, we will highlight the newest insights on the different cell subsets and components of the immune system in atherosclerosis and elaborate on current and future immunotherapeutics to treat atherosclerotic cardiovascular disease.

Call out box for clinicians

Although we have acknowledged for decades that atherosclerosis is an inflammatory disease, state-of-the-art treatments are still based on lipid-lowering, anti-hypertensive and lifestyle-changing strategies, and not on modulating inflammation. Recent clinical trials targeting the immune system revealed that CVD patients tolerate immune therapy, show decreased levels of inflammation and/or had a reduction in cardiovascular endpoints. We here describe the intricate network of immune cell interactions relevant for atherosclerosis and discuss potential immunotherapeutic approaches to treat cardiovascular disease.



Summary Figure. The current concept of atherosclerosis is that it is a lipid-driven immune disease, in which both the innate and adaptive immune system play a major role. In atherosclerotic plaques, a plethora of innate and adaptive immune cell subsets inhabit the atherosclerotic plaque, as well as the underlying adventitia and perivascular adipose tissue (left). *Pre-clinical* studies in cell culture models and atherosclerotic mouse models have provided major insights into immune cell subsets as well as inflammatory pathways that drive atherosclerosis. Using single cell technologies on human atherosclerotic plaque specimens, novel (non-)immune cell subsets and their interactions have been identified and detailed. Clinical trials using immunotherapies for autoimmune diseases such as rheumatoid arthritis (RA), revealed that RA patients treated with immunotherapy had decreased CVD. Patients receiving immunotherapy in oncology, that boost T cell activation to facilitate tumor killing, has aggravated cardiovascular disease (CVD). The first clinical trials targeting inflammation in atherosclerotic CVD in humans showed a reduction in (vascular) inflammation and/or CVD events (right). Current immunotherapies in CVD are promising, but need optimization as some induce side effects, and do not decrease overall mortality. Future immunotherapeutics aim to target atherosclerosis-associated immunological and inflammatory pathways in a cell-type specific manner to reduce CVD and overall mortality without causing side-effects.

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1. ATHEROSCLEROSIS: EPIDEMIOLOGY, RISK FACTORS AND CLINICAL MANIFESTATIONS

1.1 Epidemiology

Atherosclerosis, a lipid-driven inflammatory disease characterized by a buildup of atherosclerotic plaques of middle- and large-sized arteries, is a major underlying cause of cardiovascular disease (CVD). Clinically, atherosclerosis frequently presents as ischemic heart disease, ischemic stroke, and peripheral arterial disease. CVD are the #1 global cause of mortality and a significant contributor to disability. In 2019 alone, CVD claimed 17.9 million lives worldwide, comprising 32% of all recorded deaths (https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)). The prevalence of CVD persists in an upward trajectory across most nations, with age-standardized CVD rates witnessing a concerning resurgence (1). Eastern Europe bears the highest total CVD mortality rate, standing at 553 deaths per 100,000 individuals, whereas Australasian countries exhibit the lowest rate at 123 deaths per 100,000 (Figure 1). The escalation in global CVD deaths from 12.4 million in 1990 to 19.8 million in 2022 not only reflects population growth and aging, but also worsening of preventable metabolic, environmental, and behavioral risk factors (2).

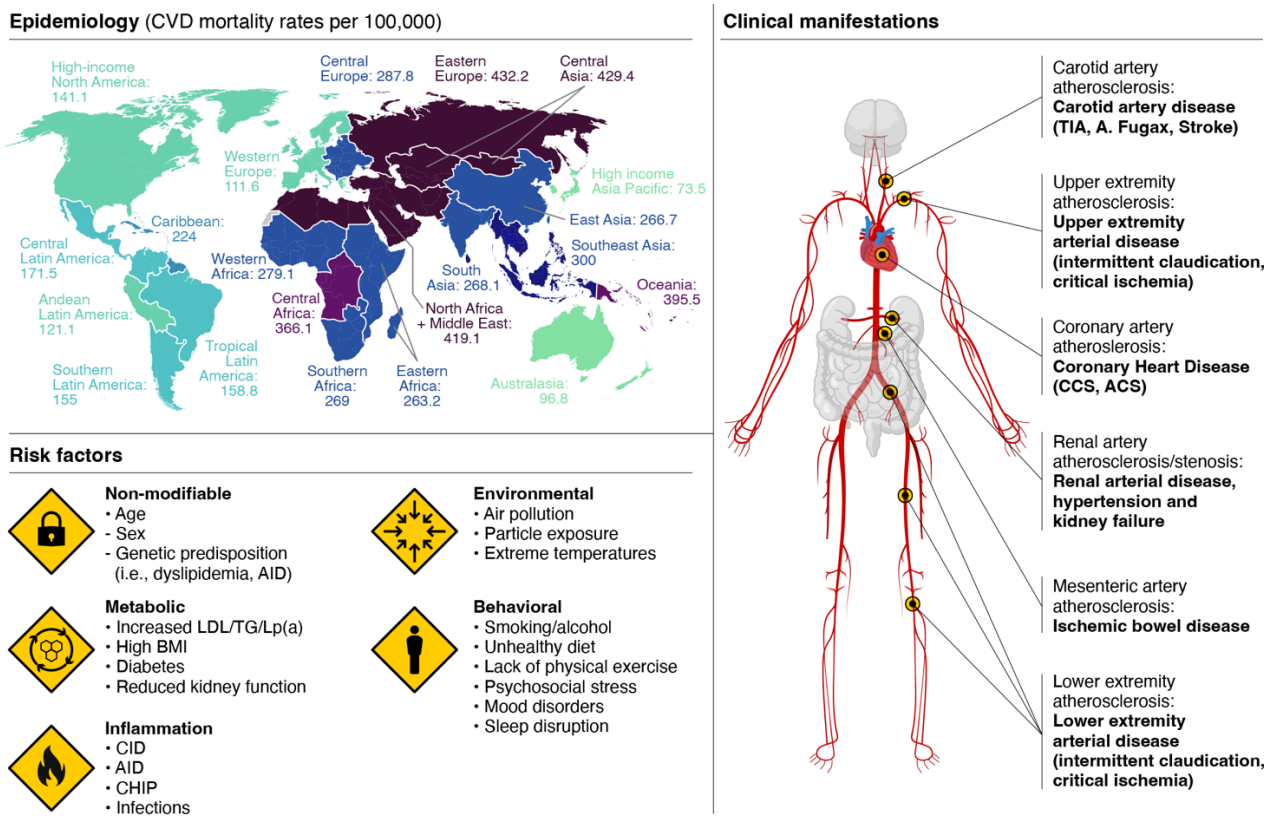

Figure 1. Epidemiology, risk factors and clinical manifestations of atherosclerosis

CVD associated mortality is on the rise, especially in eastern Europe, the Middle East, North Africa and Central Asia, where mortality rates are above 400 per 100,000 (source: https://www.jacc.org/global-burden-cvd/interactive/21-region). Major risk factors for atherosclerotic CVD can be divided in non-modifiable risk factors (i.e., age and sex); metabolic risk factors (i.e., hyperlipidemia); inflammation (chronic inflammatory disease (CID), acute inflammatory disease (AID), Clonal hematopoiesis of Indeterminate Potential (CHIP), and infections); environmental risk factors (i.e., air pollution) and behavioral risk factors (i.e., lack of physical exercise). Atherosclerosis can result in a wide range of clinical symptoms, including coronary artery disease, cerebral artery disease and peripheral artery disease, depending on the arterial bed affected.

1.2 Risk factors for atherosclerotic cardiovascular disease

The latest update of the 'Global Burden of Diseases, Injuries, and Risk Factors' Study (GBD), involving over 10,000 collaborators worldwide, underscored the main risk factors for CVD. These include *environmental factors* such as (household) air pollution, lead exposure, and extreme temperatures; *metabolic factors* including systolic blood pressure, LDL cholesterol levels, body mass index, fasting plasma glucose, and kidney dysfunction; and *behavioral factors* encompassing dietary habits, smoking, exposure to secondhand smoke, alcohol consumption, and physical activity levels. Additionally, non-modifiable risk factors like sex, age, and family history of cardiovascular events have been extensively documented (2). In a 2023 meta-analysis on the prevalence of cardiovascular risk factors, the global prevalence of 8 of the most important cardiovascular risk factors was high, with insufficient sleep quality (38.5%), no adherence to a healthy diet (34.1%), dyslipidemia (34.1%), hypertension (29.4%) and insufficient physical activity (26.4%) scoring the highest, followed by obesity (17.3%), nicotine exposure (15.4%) and diabetes (12.1%), stressing the need to implement prevention strategies involving lifestyle-changes and adequate treatment strategies to keep hypertension, dyslipidemia and diabetes under control (3). An additional important risk factor that increases the prevalence of (atherosclerotic) CVD is inflammation. Patients suffering from chronic inflammatory diseases (including COPD or asthma) (4), auto-immune diseases (including systemic lupus erythematosus and rheumatoid arthritis) (5, 6), acute inflammation following injury (7), as well as infections, such as influenza, HIV or SARS-CoV-2 (8-10), are all at an increased risk at developing cardiovascular disease. Another disorder that stresses the importance of inflammation as a risk factor, and driver for CVD is Clonal hematopoiesis of Indeterminate Potential (CHIP). CHIP is characterized by the presence of somatic mutations in blood leukocytes with a clonal size of at least 2% allele frequency. These somatic mutations occur during ageing (11), and most frequently affected genes are the epigenetic regulators Tet methylcytosine dioxygenase 2 (tet2) and DNA (cytosine-5) methyltransferase 3 alpha (DNMT3A). Genes that are less frequently affected are sex combs like 1 (ASXL1), serine/arginine-rich splicing factor 2 (SRSF2), or signaling proteins such as Janus kinase 2 (JAK2). Mouse models in which tet2 and JAK2 were modified exhibited an increase in atherosclerosis, by aggravating macrophage inflammation (12-15). In humans, CHIP was shown to nearly double the cardiovascular risk compared to healthy individuals establishing CHIP as a new cardiovascular risk factor (12, 16) (Figure 1).

1.3 Clinical manifestations of atherosclerosis

The clinical repercussions of atherosclerosis hinge on two key factors: the arterial territory primarily affected at a given time, and the speed of arterial occlusion. Given its systemic nature, atherosclerosis can concurrently affect multiple territories, thus patients experiencing a myocardial infarction face heightened stroke risks and vice versa. Notably, within one month of a myocardial infarction, 0.9% of patients may suffer an ischemic stroke, escalating to 3.7% within a year, with a doubled one-year mortality compared to those unafflicted by stroke (17, 18). Conversely, a meta-analysis from 2018 indicated a myocardial infarction risk of up to 3.6% annually following a stroke (19). Atherosclerosis impacts diverse arterial systems: it can affect coronary arteries, precipitating coronary artery disease (CAD), arteries irrigating the brain culminating in cerebrovascular disease, and peripheral arteries, compromising circulation to extremities and visceral organs (Figure 1).

Coronary artery disease syndromes are classified as acute or chronic, depending on the speed of arterial occlusion. Typical clinical presentations include chest discomfort, often described as pain, pressure, or tightness (*angina pectoris*), as well as chest pain-equivalents such as dyspnea, epigastric pain, and pain in the left or right arm or neck and/or jaw. While some patients may exhibit few symptoms, others may experience fainting due to circulatory collapse, cardiogenic shock, acute heart failure, arrhythmias, or cardiac arrest.

Acute coronary syndromes (ACS) encompass patients with recent changes in clinical symptoms or signs, with or without alterations on the 12-lead electrocardiogram (ECG), and with or without acute elevations in cardiac troponin (cTn) concentrations. This spectrum includes myocardial infarction, marked by troponin C (cTn) release due to acute cardiomyocyte injury/necrosis, in contrast with unstable angina, defined as myocardial ischemia at rest or with minimal exertion without acute cardiomyocyte injury/necrosis. Unstable angina is characterized by prolonged (>20 min) angina at rest, new-onset severe angina (angina increasing in frequency, duration, or threshold) or angina following a recent myocardial infarction episode. Currently, in acute scenarios, ECG and cTn, in addition to clinical manifestations, guide the initial therapeutic management. The presence of ST-segment elevation defines ST-elevation myocardial infarction (STEMI), indicating total luminal occlusion, whereas ST-elevation absence with cardiomyocyte necrosis is a non-ST-elevation myocardial infarction (NSTEMI) (20).

In chronic coronary syndromes, various clinical presentations may occur, ranging from asymptomatic to *angina pectoris* (with or without obstructive disease in the epicardial coronary arteries), often associated with physical or psychological exertion, or chest pain-equivalent symptoms such as dyspnea and new onset of heart failure or left ventricular dysfunction (21).

Stroke is the second most common cause of death following coronary artery disease (CAD). Projections from Eurostat, the statistical office of the European Union, predict a 34% increase in the total number of strokes in Europe between 2015 and 2035 (https://strokeeurope.eu/executive-summary/Stroke Alliance for Europe: The burden of stroke in Europe) . Of all strokes, 80% are ischemic, with one-third of them related to extracranial carotid disease. Atherosclerosis in the carotid artery affects approximately 21% of people aged 30–79 years, resulting in carotid plaque rupture in around 816 million individuals and carotid stenosis in about 58 million individuals (22). Among the ischemic strokes, only 20% are vertebrobasilar, and these are also primarily caused by atherosclerosis. A recent symptomatic >50% vertebral artery stenosis may be associated with a 30% risk of stroke over a 5-year period (23).

A stroke is a sudden-onset focal neurological dysfunction, with symptoms lasting more than 24 hours (or resulting in death in less than 24 hours), typically from non-traumatic, vascular origins (24). Stroke in evolution is either a fluctuating neurological deficit without full recovery or a progressively worsening neurological deficit over a 24-hour period (24). In contrast, a transient ischemic attack (TIA) is an episode of focal brain, retinal, or spinal cord dysfunction lasting less than 24 hours, also of non-traumatic, vascular origin (25). Crescendo TIAs refer to multiple TIAs in a short time frame, usually more than two TIAs in 24 hours, or at least three events in one week, with full recovery between each episode (26). The clinical manifestations of stroke vary widely and may include contralateral motor or sensory deficits, slurred speech, cranial nerve deficits, limb weakness, or visual disturbances such as amaurosis fugax. Hemodynamic symptoms may be less predictable and atypical, including limb tremor, retinal claudication, headache, syncope, and generalized fatigue. Symptoms may arise from artery occlusion or embolization to

the cerebral circulation and depend on hemodynamic conditions and the presence and/or patency of collaterals.

Patients with lower extremity artery atherosclerotic disease (LEAD) can be asymptomatic, and clinical signs can vary widely. Assessing walking capacity is crucial for detecting clinically silent LEAD. Walking difficulties may stem from an inability to walk enough to reveal symptoms (e.g., due to heart failure) and/or reduced pain sensitivity (e.g., diabetic neuropathy), both common co-morbidities. The ankle-brachial index serves as a first-line test for screening and diagnosis, alongside duplex ultrasound, a non-radiation imaging method. Intermittent claudication, ranging from mild to severe or disabling, is a classical symptom akin to angina but affecting the lower extremities. Critical limb ischemia is characterized by ischemic rest pain, with or without tissue loss (ulceration or gangrene) or infection. It often represents generalized, severe atherosclerosis, carrying a three-fold increased risk of myocardial infarction, stroke, and vascular death compared to patients with intermittent claudication (27, 28).

Upper extremity artery disease due to atherosclerosis is rare and poorly studied, typically involving the brachiocephalic trunk, subclavian, and axillary arteries (29). Possible clinical manifestations include arm claudication, Raynaud's syndrome, rest pain, ischemic ulcerations, or gangrene. Another organ affected by atherosclerosis is the kidney, contributing to 1-2% of all cases of hypertension in the general population (30). Atherosclerotic plaques typically form at the origin of the renal artery, often associated with areas of flow turbulence (31, 32). Many lesions have minimal hemodynamic effects and remain clinically silent until they progress to a "critical" level, which triggers pressor mechanisms or inflammatory and ischemic injury (33). Atherosclerotic renovascular disease can manifest as renal artery stenosis and renal ischemia. It may be a discreet process, as blood pressure does not rise until the stenosis reaches 60% or greater, and the kidney can tolerate moderate flow reduction. It may be incidentally discovered by abdominal murmurs or during vascular imaging for other reasons. In addition to hypertension, sodium and water retention may contribute to circulatory congestion and pulmonary edema in patients with left ventricular deterioration, leading to ischemic nephropathy, which involves both large and small blood vessels and results in a decline in glomerular filtration rate.

Like CAD in the heart, intestinal ischemia**,** caused by atherosclerosis in the superior and/or inferior mesenteric artery/ies can be categorized based on the timing of onset into acute and chronic forms. Collateral circulation in the gastrointestinal tract can compensate for a 75% acute reduction in mesenteric perfusion for up to 12 hours without causing significant injury (34). In chronic mesenteric ischemia, atherosclerosis is the underlying cause in 95% of cases. The etiologies of acute intestinal ischemia are largely associated with atherosclerosis, such as mesenteric arterial embolism (50%), intestinal hypoperfusion or nonocclusive mesenteric ischemia (20%-30%), and mesenteric arterial thrombosis (15%-25%) (35). Symptoms and signs may vary, often including recurrent abdominal pain after eating, which can lead to weight loss in chronic cases. In acute cases, abdominal pain and tenderness may be accompanied by rectal bleeding or bloody diarrhea. Mesenteric ischemia accounts for only 0.1% of all hospital admissions, making it a rare medical condition, despite its high mortality rates ranging from 24% to 94% (36) (Figure 1).

Atherosclerosis is thus the underlying cause of a wide range of cardiovascular diseases. Although a significant reduction in cardiovascular mortality and morbidity has been achieved using LDL-

cholesterol lowering therapies, antihypertensives and lifestyle-changing strategies, atherosclerotic CVD still poses a major global health burden (21, 37, 38) (Figure 1).

2. THE ATHEROSCLEROTIC PLAQUE

The name *atherosclerosis* refers to thickening of the arterial wall (*sclerosis* means 'hardening') and accumulation of lipids, cells, extracellular matrix, and calcium (*athere* means 'gruel') that characterize the atherosclerotic plaque (39). Atherosclerotic plaques selectively form in middle- and large-sized arteries and can be found in every human being to a greater or lesser extent. The formation of atherosclerotic plaques starts in early childhood, where it remains clinically silent (40), and progresses with age, when atherosclerotic CVD becomes evident (41-43).
Although considered a major health problem of modern age, atherosclerotic CVD has been bothering mankind for millennia. Atherosclerotic plaques have been found in aorta, coronary arteries, and peripheral arteries of mummies from all over the world dated as early as 3000 BC (44-47). Remarkably, mummies carrying SNPs in their ancient DNA that are currently associated with a predisposition to atherosclerotic CVD, were found to have higher atherosclerotic plaque burden than those who with a low polygenic risk score for ASCVD (48).
The macroscopical and microscopical characterization of the atherosclerotic plaque forms the basis of many of our current theories and insights into the pathogenesis of atherosclerosis. A detailed understanding of the histological and morphological features of the progressing atherosclerotic plaque is key to understanding pathophysiological mechanisms underlying the disease.

2.1 The non-diseased artery

The non-diseased human artery contains four layers: the intima, the media, the adventitia and the peri-vascular adipose tissue (PVAT) (Figure 2). The intima is the innermost part of the artery and enclosed by the lumen-facing endothelium and the internal elastic lamina, that demarks the arterial media. The intima contains delicate connective tissue with a few vascular smooth muscle cells (VSMCs) (49). Throughout the arterial tree, the intima varies in thickness and often undergoes eccentric or diffuse adaptive intimal thickening. These intimal thickenings are not due to atherosclerosis but are physiological adaptations of the intima caused mechanical stresses, such as changes is pulse rate, blood pressure, arterial geometry, flow rate, and flow resistance (41).
The media is the muscular arterial wall and is confined by the internal and external elastic laminae. The media is composed of fenestrated elastic laminae of VSMCs, interspersed with collagen fibrils and proteoglycans. Its main function is to support the arterial structure and change vessel diameter to regulate blood flow and blood pressure (50).
The adventitia is the outermost layer of the artery and comprised of a dense collagen-rich matrix containing fibroblast, VSMCs, vasa vasorum, nerve fibers and, in a non-diseased artery, immune regulatory immune cells (mostly resident macrophages). The role of the adventitia is not completely understood but is considered an essential regulator of vascular wall structure and function (reviewed in (51)). The adventitia is surrounded by perivascular adipose tissue, a layer

of adipocytes that contains vasa vasorum, nerve fibers, and (resident) immune cells (52, 53) (Figure 2).

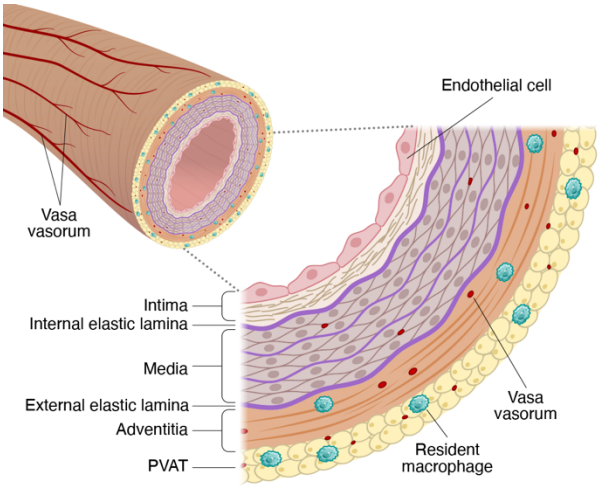

Figure 2. Structure of a non-diseased artery. The non-diseased arterial wall consists of 4 layers: the intima, media and adventitia and the perivascular adipose tissue (PVAT). The intima consists of an endothelial cell layer, a subendothelial layer with some connective tissue and is separated from the media by the internal elastic lamina. The media predominantly contains multiple layers of vascular smooth muscle cells, with dispersed elastic laminae, and other extracellular matrix components. The media is separated from the adventitia by the external elastic lamina. The adventitia is composed of a collagen rich matrix, with dispersed VSMCs, nerve fibers, resident and a few recruited immune cells are found, and vasa vasorum, that can extend into the media and intima. The PVAT contains adipocytes and similar components as the adventitia.

2.2 Histology of the atherosclerotic plaque

2.2.1 The first descriptions of atherosclerotic plaques

The first reports on the characteristics of the atherosclerotic plaque date from the 19th century. In 1855, Carl von Rokitansky (1804-1878) described atherosclerosis as an excessive formation of plaques on the interior of arteries. According to von Rokitansky, these plaques start as thickening of the vascular inner layer, which becomes covered by a pseudo membrane: fibrin. This pseudo membrane develops either in an atheromatous process with debris and cholestearine, or into vascular ossifications (54). In 1858, Virchow (1821-1902), the founder of cellular pathology, presented a lecture on the 'atheromatous affection of arteries. He described atherosclerosis as a process of *simply fatty metamorphosis*, which is characterized by the transformation of the existing histological elements into a state of degeneration. Second, he claimed that atherosclerotic plaques transfer to a *stage of irritation*, comparable to the stage of swelling an enlargement seen in inflammatory processes. Lastly, he described a stage of '*atheromatous degeneration'* where plaques consist of cholestearine plates, granule cells and fat granules, and hard lumps of softened substance resulting in the *pultaceous* character of the atheromatous matter (55).

Although we now have a more pronounced knowledge of processes and features of the different stages of atherosclerosis, many aspects of current histological classifications are based on the observations of our predecessors in the 1850s-1950s (56).

2.2.2 Classifications of atherosclerosis

After Virchow and von Rokitansky described the morphological features of atherosclerotic plaques, several histological classifications of atherosclerosis have been developed. The classifications that are currently in use are the AHA classification of atherosclerosis, developed on behalf of the 'Committee on Vascular Lesions' of the Atherosclerosis of the American heart Association in 1992-1995 (41-43) and an updated more refined classification of human atherosclerosis as proposed by Dr Virmani in 2000 (49).

The AHA classification of atherosclerosis recognizes 6 different stages of atherosclerotic plaque development (41-43), but starts out with a detailed description of a normal intima. Type I lesions, the first atherosclerotic lesions, are characterized by an increased number of intimal macrophages of which some have a foamy appearance. Type II lesions, or fatty streak lesions are the first macroscopically visible lesions and look like small lipid dots. They consist of layers of macrophage foam cells, lipid-droplet containing VSMCs, and occasional extracellular lipid droplets. Type III lesions are characterized by extracellular lipid pools and represent the transition from early to and advanced lesion type: the pre-atheroma (42). The first type of advanced atherosclerotic plaque is the type IV lesion, which contains a necrotic core with extracellular lipids and cholesterol crystals, the atheroma, but no increase in extracellular matrix. The type V lesion is characterized by prominent new extracellular matrix formation, that form a fibrous cap: the fibro-atheroma. Both type IV and type V lesions can develop fissures, hematoma or thrombi, and are then considered a type VI lesion, i.e., plaque rupture or intraplaque hemorrhage (43). Type VI lesions have the highest association to clinical symptoms resulting from atherosclerosis, as the forming thrombus can rapidly occlude the lumen and cause ischemia or infarction, such as myocardial infarction or stroke, depending on the arterial bed affected (43, 57).

The classification as proposed by Dr Virmani is more refined, and better reflects the complexity of the atherosclerotic plaque (49), as has been detailed in Figure 3. The first stages of atherosclerosis are defined as *intimal thickenings* and *intimal xanthomas*. Intimal thickenings consist of VSMCs embedded in a proteoglycan rich matrix, whereas *Intimal xanthomas* reflect the accumulations of lipid loaden macrophages into the intima. *Pathological intimal thickenings* are poorly qualified intermediate lesions, with no apparent necrosis, no cellular debris, and dispersed lipids in the deeper layers that are overgrown by a SMC-rich fibrous caps and scattered sparse lymphocytes and macrophages. The *fibrous cap atheroma* contains a true necrotic core containing cholesterol esters, phospholipids, free cholesterol and triglycerides, The fibrous cap consists of smooth muscle cells in a proteoglycan-collagen matrix, with a variable number of macrophages and lymphocytes. Fibrous cap atheromata can progress toward vulnerable atherosclerotic lesions and then become thin fibrous cap atheromata that show signs of loss of SMCs, overlay a large necrotic core, and exhibit features of inflammation (49) (Figure 3).

Lesions with thrombi are considered responsible for clinical symptoms of CVD, as thrombi can occlude arterial beds (57). However, the presence of plaque rupture is not always associated with clinical events, as non-fatal lesions were shown to exhibit features of plaque rupture (57). Vice versa, many plaques show luminal thrombi, without evidence for plaque rupture (58). Therefore, the designation of a single category to atherothrombotic lesions is too simplistic. Dr Virmani's classification distinguishes two types of atherothrombotic lesions: *plaque rupture* and *plaque erosion*. Plaque rupture is characterized by the presence of a fissure, where the thrombus is in direct contact with the necrotic core. Plaque erosion is characterized by absence of a fissure, and

the endothelium is often absent at the site of thrombus. Additional subtypes of lesions containing thrombi are plaques with intraplaque hemorrhage and, plaques with healed ruptures or erosions (49) (Figure 3).

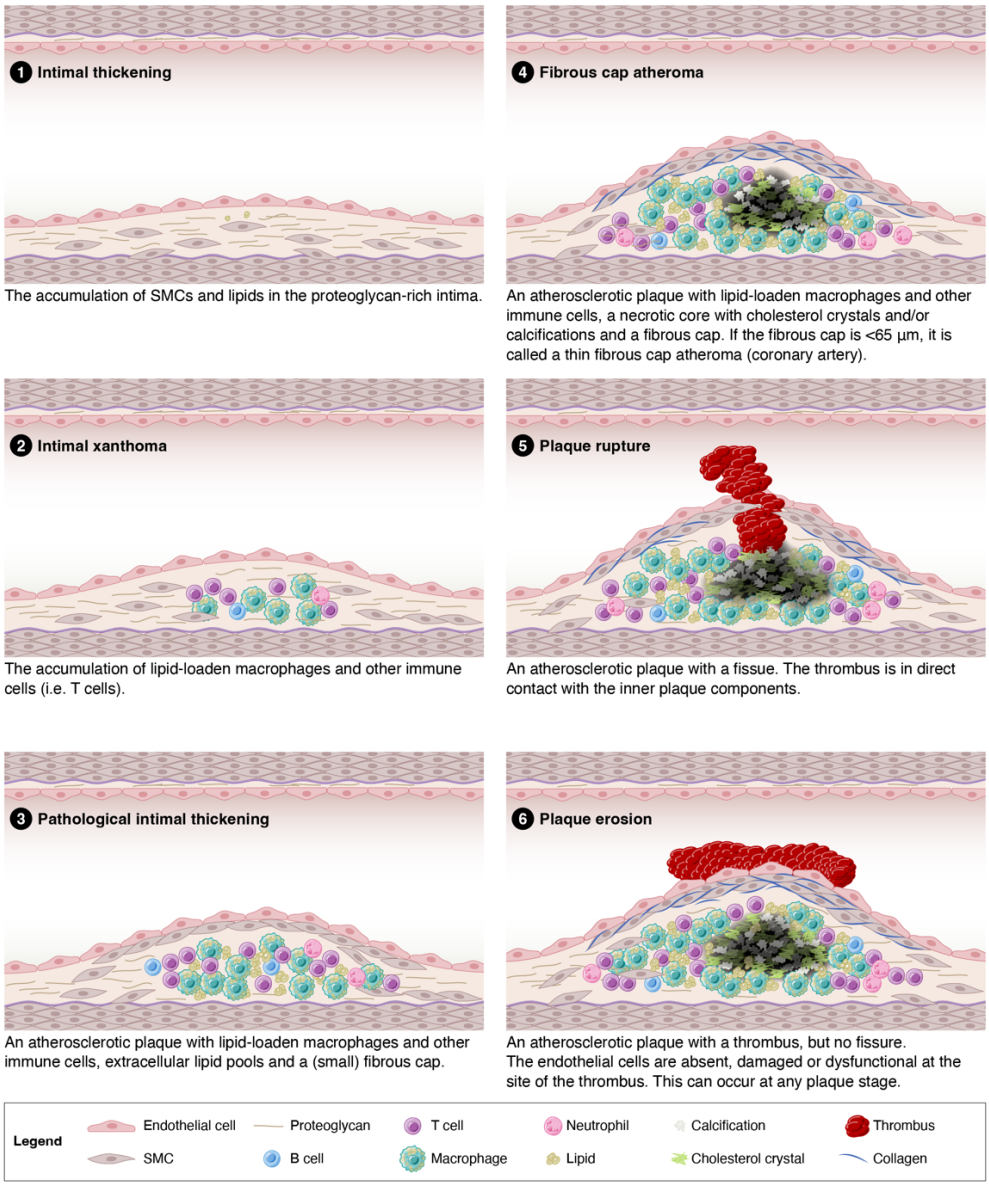

Figure 3: Histological features of the pathogenesis of atherosclerosis 1.*Intimal thickening*: the process of atherosclerosis starts when VSMCs and lipids accumulate in the proteoglycan-rich intima. 2. *Intimal xanthoma*: excess uptake of lipids attracts the first immune cells into the intima, i.e., (lipid laden) macrophages and T cells. 3. *Pathological intimal thickening*: Immune cells keep accumulating, more and more macrophages obtain a foam cell rich appearance, and the first extracellular lipid pools become visible. A fibrous cap, composed of VSMCs, fibroblasts and extracellular matrix components is forming. 4. *Fibrous cap atheroma*: immune cells keep accumulating and a fibrous cap has fully developed. The core of the plaque shows necrosis and extracellular lipids, often in the forms of cholesterol crystals. Calcium deposits form. This area is called the necrotic core. The fibrous cap starts thinning in some areas. 5. *Plaque rupture*: a fissure in the fibrous cap exposes the thrombogenic contents of the plaque to the blood. A thrombus forms on top of the plaque and may narrow or occlude the arterial lumen. 6. *Plaque erosion*: Thrombus on top of an intact atherosclerotic plaque. The endothelium can be intact, damaged or absent.

Plaque formation can take several years, depending on various risk factors. The biological age of different components of advanced human plaques has been analyzed using the ^{14}C bomb-peak dating method, employing accelerator mass spectrometry (59)—a technique used in archaeology and forensic medicine. Human plaque tissue turnover time in living patients has been demonstrated to vary and can be very long, with calcified areas found to be up to 20 years old. Significant differences in the age of various plaque regions have been observed, with the cap being the youngest part (59). These differing biological ages seem to be independent of symptoms, but using the same technique, apoptosis (mainly in the cap and core of plaques) was identified as the most significant mechanism associated with a younger plaque age, reflecting rapid plaque progression in living patients (60). Atherosclerosis can thus remain asymptomatic for extended periods before becoming suddenly unstable, typically due to an acute atherothrombotic event caused by plaque rupture (~65% in coronaries from patients suffering sudden coronary death), plaque erosion (~30%) or calcified nodule (~5%) (49, 61).

2.3 Animal models of atherosclerosis

Animal models of atherosclerosis have been invaluable to the understanding of the pathogenesis of atherosclerosis. The first animals to be used were non-human primates and rabbits, who develop atherosclerosis after being fed a high cholesterol containing diet (62-65). In non-human primates, the first fatty streak lesions can be observed within one month, and fibrous cap atheromas occur within 1 year (62-64). Rabbits also develop atherosclerosis upon a being fed a high cholesterol diet. Several models are in use, ranging from New Zealand white rabbits, Watanabe rabbits, and rabbits that have been genetically modified (65, 66). Rabbits develop large, foam cell rich lesions within weeks, and fibrous cap atheromas within months (66).

Nowadays, the mouse is the most common experimental animal model used for atherosclerosis research, due to the ability to easily introduce and mutate genes. However, mice do not develop atherosclerosis spontaneously. The strain that is most susceptible to develop atherosclerosis is the C57Bl6 mouse strain. When fed a high cholesterol diet, these mice develop fatty streak lesions, but not fibrous cap atheromas (67). This strain has been used as background for the gene-deficient models that are more prone to atherosclerosis. The most used mouse models for atherosclerosis today are the *Apoe*$^{-/-}$ and the *Ldlr*$^{-/-}$ mouse models. The *Apoe*$^{-/-}$ mouse model, that lacks apolipoprotein E, is hyperlipidemic, and develops atherosclerosis when fed either a normal chow or a high cholesterol diet (68). *Apoe*$^{-/-}$ mice develop all stages of atherosclerosis, from fatty streak lesions to advanced fibrous cap atheromata (69), to intraplaque hemorrhage and rupture (70-72), although the latter is still under debate (73). *Ldlr*$^{-/-}$ mice lack the low- density lipoprotein receptor and display low levels of hyperlipidemia when fed a normal chow diet, and severe hyperlipidemia when fed a diet high in cholesterol, and develop atherosclerosis comparable to the *Apoe*$^{-/-}$ mouse model (74). Inducible models of atherosclerosis include the injection of an adeno-associated virus vector containing the D374Y gain-of-function mutant form of PCSK9 (75), and administration of an siRNA directed against the LDL receptor (76).

Pig models of atherosclerosis have become popular for late pre-clinical studies, as the pig's cardiovascular system more closely resembles human cardiovascular physiology than the mouse. Pig models used for atherosclerosis research are pigs susceptible to hyperlipidemia (77), such as the Yucatan minipig (78), FBM (Familial Hypercholesterolemia Bretoncelles Meishan) pigs (79) or

Göttingen pig (80), as well as genetically modified pig models such as the Yucatan minipig containing the human gain of function D374Y-PCSK9 mutant (81).

3. EMERGING THEORIES ON THE PATHOGENESIS OF ATHEROSCLEROSIS (1850-NOW): A TALE OF THROMBOSIS, LIPIDS, INJURY AND INFLAMMATION

In the past two centuries, our insights into the pathogenesis of atherosclerosis have gone through many theories and concepts which are still evolving. The evolution of the pathobiological insights on how plaques form and progress closely mirror the advancements of cellular and molecular biology. Our scientific insights into cell-types and molecules involved in the initiation, progression and regression of atherosclerotic plaques go hand in hand with major technical discoveries in histology, flow cytometry, development of mutant mouse models and genomics, all the way to the single cell (spatial) technologies that we use nowadays. The following section will give a short overview on the emergence and development of the 5 fundamental theories on the pathogenesis of atherosclerosis: **1.** the thrombosis model; **2.** the infiltration model; **3.** the lipid model; **4.** the response to injury model and **5**. the inflammation model (Figure 4). All 5 models are derived from observations and experimental data and have contributed insights into the pathogenesis of atherosclerosis as we know it today.

3.1 Atherosclerosis: the thrombosis model

In the 1850s, von Rokitansky was one of the first trying to explain the pathogenesis of atherosclerosis. Based on his observations that atherosclerotic plaques often contained mural thrombi or fibrin depositions inside the plaque, he theorized that atherosclerotic plaques were formed by organized thrombi (the so called *"encrustation"* hypothesis) (54). In 1949, Duguid postulated that an encrustation of small mural thrombi existed at the sites of arterial injury. These thrombi would subsequently undergo organization by the growth of SMC and fibrous tissue into them, and they would become incorporated into vascular lesions, causing luminal narrowing (82) (Figure 4). Over the years, this theory has been significantly refined but is still considered to be one of the contributing mechanisms to growth of atherosclerotic plaques (83).

3.2 Atherosclerosis: the infiltration model

Around the same time (1856), Virchow observed lipid deposits in the atherosclerotic plaque(55). Based on these findings, Virchow proposed the *'infiltration'* theory. He suggested that a low-grade injury to the arterial wall resulted in inflammatory infiltration, causing an increased passage and accumulation of plasma constituents in the intima (Figure 4). He dismissed von Rokitansky's 'encrustation' hypothesis because the localized intimal thickenings (atherosclerotic plaques) were subendothelial and 'hence could not be derived from surface deposits' (56).

3.3 Atherosclerosis: the lipid model

Based on the observations of Virchow and others, the Russian scientist Ignatowski tried to develop an animal model of atherosclerosis by inducing hyperlipidemia. He fed rabbits a diet of milk and egg yolk and was able to induce atherosclerosis (84). In 1913, Anitschkow reproduced this model, by adding cholesterol to the normal rabbit diet (85). This gave rise to the lipid theory

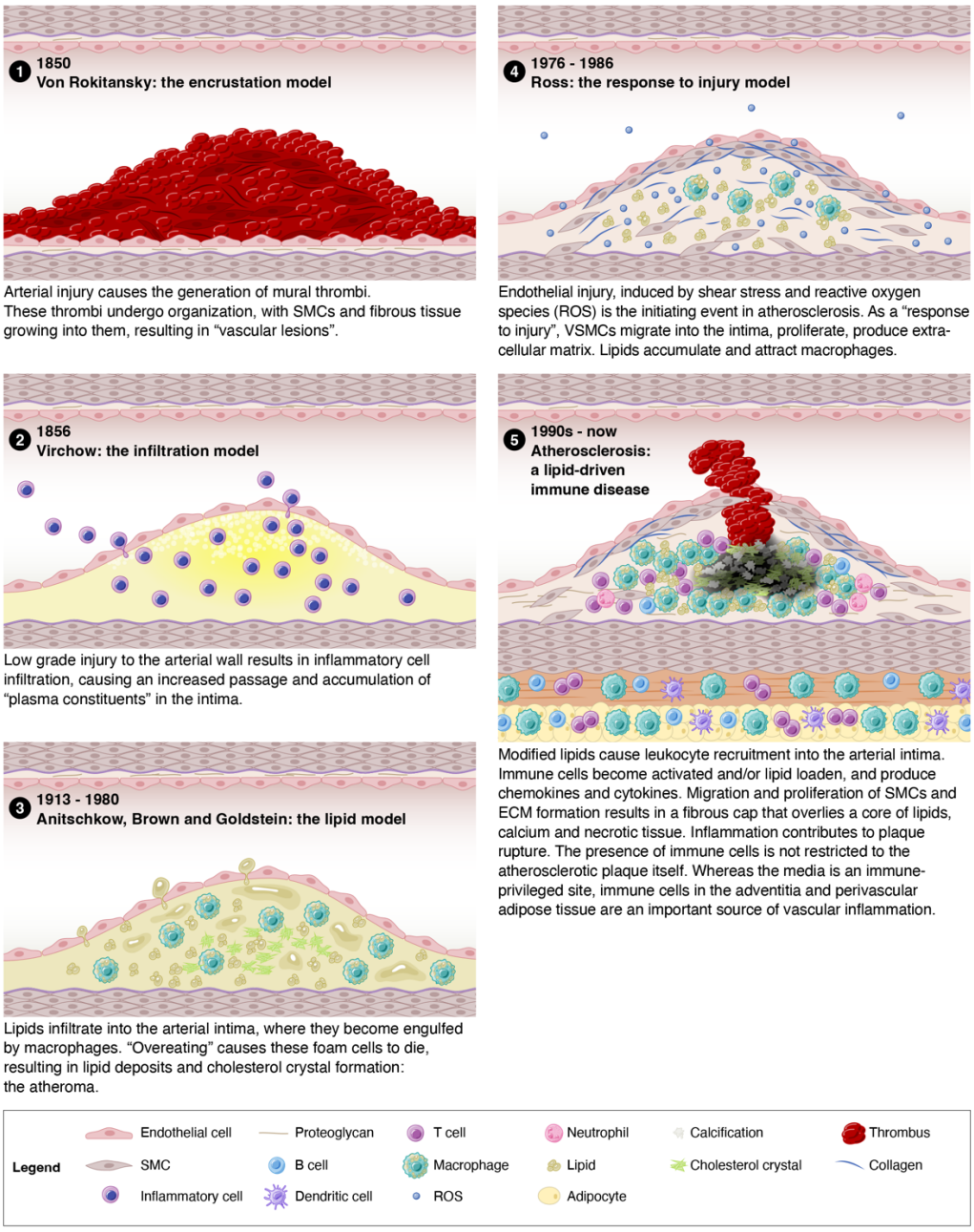

Figure 4: Emerging theories on the pathogenesis of atherosclerosis. In the 1850s, von Rokitansky proposed the '*encrustation*' model. He theorized that thrombi would adhere to the arterial wall and undergo reorganization, thereby forming a plaque (1). Around the same time, Virchow viewed atherosclerotic plaques to be composed as an inflammatory cell infiltrate embedded in plasma constituents, including cholesterol (2). Between 1900 and the 1980s, atherosclerosis was merely seen as a lipid driven disease. Lipids infiltrate into the arterial wall where they accumulate and are being engulfed by macrophages. The continuous lipid loading causes these macrophages to die. Extracellular lipid accumulation and cell death results in the formation of a necrotic core full of cholesterol crystals (3). In the 1970s, Ross and Glomset developed new theory. Atherosclerosis is caused by a 'response to injury', induced by shear stress and reactive oxygen species. As a response, VSMCs migrate into the intima, proliferate and produce extracellular matrix. Modified lipids can induce injury and are being taken up by macrophages (4). Nowadays, atherosclerosis is considered a lipid driven immune disease. It was discovered that (modified) lipid infiltration cause innate and adaptive immune cells to infiltrate into the arterial wall, including the adventitia and PVAT where they are activated, and cause a chronic inflammatory response, thereby progressing the disease (5).

of atherosclerosis that remained the dominant theory on the origin of atherosclerosis until the 1990s and forms a major part of our understanding of atherogenesis today.
In this classical model, which is still accepted today, an excess of lipids caused lipid accumulation in the intima of the arterial wall. The excess of intimal lipids results in an infiltration of macrophages that were believed to play a crucial role in phagocytosing lipids. Macrophages were primarily viewed as scavenger cells, working to remove infiltrated fat particles within the intima. It was acknowledged that these macrophages would "overeat," leading to cell death and the spillover of lipids, ultimately resulting in the formation of atheromas (Figure 4). Consequently, the pathogenesis of atherosclerosis was predominantly considered a lipid disorder, characterized by an excessive influx of lipids into the arterial wall (86). The success of the theory was reinforced by the discovery of the LDL Receptor by Goldstein and Brown. They found that the LDL receptor was able to regulate the systemic levels in the blood of the LDL via its rate of uptake through the hepatic LDL receptor pathway. This was evidenced by the fact that lack of functional LDL receptors is responsible for the massive accumulation of LDL in patients with homozygous familial hypercholesterolemia (87, 88).

3.4 Atherosclerosis: the response to injury model

As insights into the pathogenesis of atherosclerosis progressed, and as it was observed that besides lipids, plaques were composed of different cell types that were affected by hyperlipidemia, novel insights emerged. In 1976, Ross and Glomset postulated that endothelial cell injury, particularly by oxidative LDL and shear stress was the initiating event in atherosclerosis (89, 90). The "*response to injury*" hypothesis of atherosclerosis proposed that endothelial denudation was the first step in atherosclerosis and evolved over the years. In 1986, novel insights lead to an update of this hypothesis and highlighted the possible interaction between endothelial cells, macrophages and VSMCs (91). This version emphasized that endothelial dysfunction and/or endothelial activation rather than denudation caused atherosclerosis. The injury can be mechanical, chemical, or immunological. The response of the local arterial wall is characterized by migration of VSMCs from the media into the intima, rapid proliferation of these VSMCs within the intima, massive production of extracellular matrix and accumulation of lipids (91) (Figure 4).

3.5 Atherosclerosis: the inflammation hypothesis

In the 1990s, it became clear that other factors besides lipids and endothelial dysfunction were driving atherosclerosis. Despite changes in lifestyle and lipid lowering pharmacological approaches, atherosclerotic cardiovascular disease still ranked #1 as cause of death (92). Furthermore, it was observed that atherosclerosis was not a slow-developing process per se, and that plaque rupture and thrombosis were responsible for acute cardiovascular events such as myocardial infarction or stroke (93). None of these phenomena could be explained by current theories, and the search for new mechanisms was initiated.
The observation that inflammation plays a role in atherosclerosis was already proposed in the 19th century (55), but firm evidence was provided in the 1980s, when immunohistochemistry enabled precise identification of cell types that were present in atherosclerotic plaques. Not only macrophages (94), but also T cells were observed to be present in atherosclerotic plaques (95), and over the years, almost every immune cell was found to be represented within an

atherosclerotic plaque (96, 97). Moreover, a wide range of pro-inflammatory cytokines, such as tumor necrosis factor α, IL1β and IL6, and chemokines such as MCP-1, were observed in atherosclerotic plaques (98, 99) (Figure 4). In clinical studies, elevated levels of CRP and its main inducer IL6 were associated with adverse prognosis in unstable angina patients (100, 101). Elevated levels of CRP, IL6 and TNFα were associated with an adverse cardiovascular prognosis in larger population studies (102, 103).

Not only the atherosclerotic lesion itself, but also the adventitia and peripheral adipose tissue were found to contribute to vascular inflammation. When atherosclerosis progresses, both the adventitia and PVAT not only house resident immune cells, but become infiltrated with both innate and adaptive immune cells, which, in addition to the adipocytes, secrete inflammatory mediators contributing to the progression of atherosclerosis(53, 96, 104).

These, and many more experimental findings set the stage for the hypothesis of inflammation as the pathogenic mechanism for atherosclerosis. In a 1999 review by Ross, atherosclerosis was defined as "an inflammatory disease" (105). "*It is well established that lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that can be best described, in aggregate, as an inflammatory disease. In fact, key cell types and mediators of the inflammatory and immune response are main regulators of the evolution of an atherosclerotic plaque. The response is mediated by monocyte-derived macrophages and specific subsets of T lymphocytes, which both migrates from the blood and multiply within the lesion. Activation of these cells leads to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors, which can induce further damage and eventually leading to focal necrosis. Thus, cycles of accumulation of mononuclear cells, migration and proliferation of smooth muscle cells, and formation of fibrous tissue leads to further enlargement and remodeling of the lesion, so that it become covered by a fibrous cap that overlies a core of lipid and necrotic tissue. Furthermore, a consensus is emerging that inflammation plays a role in the pathophysiology of acute complications of atherosclerotic process, such as thrombosis*" (Figure 4).

4. THE CURRENT PARADIGM: ATHEROSCLEROSIS: A LIPID-DRIVEN, CHRONIC INFLAMMATORY DISEASE

In the past 35 years, our insights into how immune cells and their inflammatory mediators drive and modulate atherosclerosis have increased exponentially (96, 106). Novel single cell technological developments have provided the field with novel tools to map the many cell subsets that are present in the atherosclerotic plaque, the adventitia, and perivascular adipose tissue (107). We have only just begun to determine their characteristics, functions, interactions, and roles in atherosclerosis. The capability to quickly generate conditional knockout mice, lineage tracing mice and the advancement of analysis methods has provided novel insights on the effects of the immune system and inflammation in an in vivo setting. Lastly, the concept that inflammation, besides lipids, is an important driver of atherosclerotic cardiovascular disease has also entered the clinical arena. Plasma biomarkers, including hsCRP and IL6 are now commonly used to determine individuals at risk for atherosclerotic cardiovascular disease (108). Advances in PET technology and tracer synthesis technologies made it possible to detect atherosclerotic plaques that show high levels of inflammation, with ^{18}F-FDG PET being used in a clinical (trial) setting (109). Using the Fat

Attenuation Index during CT imaging can detect changes in PVAT induced by inflammation, that correlate with atherosclerotic plaque inflammation in patients (110). Lastly, clinical trials using anti-inflammatory drugs such as anti-IL1β and colchicine, were successful and showed that immunotherapeutics can be effective in combatting ASCVD (96).

4.1 A short pathogenesis of atherosclerosis: the current view

The current view of atherosclerosis is that of a lipid-driven chronic inflammatory disease of the medium-sized and large arteries, that involves an intricate interaction between immune cells of the innate and adaptive immune system and non-immune cells within the atherosclerotic plaque and the arterial wall (summarized in Figure 5), but also in blood and secondary lymphoid organs.

Atherosclerotic plaques are located in non-laminar flow regions, often at the opposite flow-divider walls. These areas are prone to shear stress induced endothelial cell activation and exhibit increased permeability, and leukocyte recruitment (111). Circulating lipids, especially LDL cholesterol starts entering the subendothelial space, where LDL, containing apolipoprotein B, becomes modified, thereby eliciting danger signals that are recognized by the innate immune system present in the arterial intima. These 'Danger Associated Molecular Patterns" (DAMPs) activate Toll Like Receptor signaling as well as the inflammasome in innate immune cells, thereby initiating the inflammatory cascade. Monocytes, macrophages, and neutrophils start releasing chemokines and cytokines, neutrophils release their neutrophil extracellular traps (NETs), resulting in a perpetuating cascade of leukocyte recruitment and activation. In parallel, VSMCs and fibroblasts become activated, and migrate from the media to form the fibrous cap. Activated VSMCs and fibroblasts produce extracellular matrix, further contributing to fibrous cap formation. Macrophages, but also VSMCs phagocytose excessive lipids, and become foam cells. Excess lipid uptake, as well as inadequate efferocytosis results in cell death and extracellular lipid accumulation in the plaque as well as the formation of cholesterol crystals: the beginning of the necrotic core.

At the same time, antigens, including peptides from apolipoprotein B, are being presented in the in the immunological synapse by antigen presenting cells, including macrophages, dendritic cells and B cells, and drain to secondary lymphoid organs. In the plaque, but especially in secondary lymphoid organs, antigens are recognized by iNKT- cells (particularly lipid antigens) and/or T cells. If antigens are presented to T cells with support of co-stimulation, this will provoke an adaptive immune response, resulting in antigen specific T cell proliferation and antibody production. Innate type B cells, producing only anti-OSE IgM antibodies are also found in the plaque. The continued inflammatory response results in worsened endothelial dysfunction, increased lipid uptake, recruitment and influx of more immune cells (reviewed in (96)).

During atherogenesis, the adventitia and PVAT that mostly house resident immune cells, becomes inhabited with activated innate and adaptive immune cells, that form clusters and lymphoid aggregates, that facilitate immune cell interactions, as well as APC-T cell interactions. The perivascular adipocytes underlying the atherosclerotic plaque are avid producers of chemokines and cytokines (52, 53, 96, 104). Ultimately, the chronic inflammatory response induces breakdown of the fibrous cap as well as thrombogenicity of the endothelium, resulting in plaque rupture or erosion, i.e., atherothrombosis (reviewed in (96)) (Figure 5).

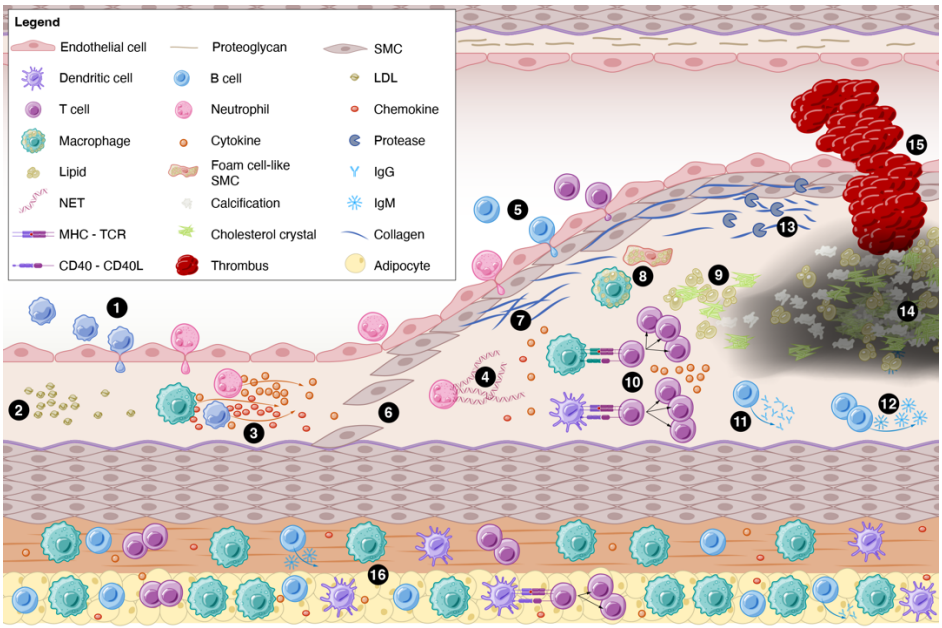

Figure 5: The Pathogenesis of Atherosclerosis Atherosclerotic plaques mostly form at sites where laminar flow is disturbed. This, combined with oxidative stressors, results in the activation of the endothelium, increased permeability, upregulation of leukocyte adhesion molecules and leukocyte recruitment (1). Circulating lipids, especially LDL cholesterol starts entering the subendothelial space, where LDL, containing apolipoprotein B, becomes modified (2), thereby eliciting danger signals that are recognized by the innate immune system present in the arterial intima. Monocytes, macrophages, and neutrophils start releasing chemokines and cytokines (3), and neutrophils form neutrophil extracellular traps (NETs) (4), resulting in a perpetuating cascade of leukocyte recruitment and activation. This includes the recruitment of adaptive immune cells, including T and B cells, into the plaque (5). VSMCs and fibroblasts migrate from the media to form the fibrous cap (6). Activated VSMCs and fibroblasts produce collagen and other extracellular matrix molecules, further contributing to fibrous cap formation (7). Macrophages, but also VSMCs phagocytose excessive lipids, and become foam cells (8). Excess lipid uptake, as well as inadequate efferocytosis results in cell death and extracellular lipid accumulation in the plaque as well as the formation of cholesterol crystals: the beginning of the necrotic core (9). In parallel, antigens, including peptides from apolipoprotein B, are being presented in the in the immunological synapse (TCR-MHC complex and co-stimulatory/co-inhibitory complex) by antigen presenting cells, resulting in antigen specific T cell proliferation and cytokine production (10), and antibody production (IgG) by B cells (11). Innate type B cells, producing only anti-OSE IgM antibodies are also found in the plaque (12). Ultimately, the chronic inflammatory response induces breakdown of the fibrous cap (13), development of a necrotic core (14), resulting in plaque rupture and atherothrombosis (15). During atherosclerosis, the adventitia and perivascular adipose tissue, originally house resident immune cells become inhabited by a plethora of immune cells, similar to those in the atherosclerotic plaque, thereby actively contributing to vascular inflammation and progression of atherosclerosis (16).

4.2 Immune cells populating our non-diseased and atherosclerotic arteries

Immune cells are common inhabitants of both the non-diseased arterial wall as well as the atherosclerotic plaque, and a large variability of immune cell types and subsets populate our non-diseased and atherosclerotic arteries. The immune cells enter and sometimes exit the atherosclerotic plaque and are capable of aggravating and regulating local inflammation, and therefore are crucial in the progression, stability, and regression of atherosclerotic plaques. Numerous experimental and preclinical studies have proven a key role for both the innate and the adaptive immune system in atherosclerosis (52, 96, 104). In the next section, we will summarize the contribution of the most important immune cell types and their subsets in atherosclerosis.

5. CELLS OF THE INNATE IMMUNE SYSTEM IN ATHEROSCLEROSIS

The innate immune system serves as the body's first line of defence against pathogens, providing a rapid, nonspecific response to potential threats. Unlike the adaptive immune system, which develops specific responses tailored to specific antigens, the innate immune system relies on evolutionarily conserved mechanisms to recognize and combat a wide array of invaders. Key components of the innate immune system include skin and mucosal barriers, soluble components, and cellular responses. The innate immune system relies on soluble components, such as the complement system, antimicrobial peptides, and acute-phase proteins. The complement system, for example, facilitates pathogen clearance by promoting opsonization (a process that helps identify and destroy old cells or pathogens, by coating them with opsonins, such as antibodies and complement proteins), membrane attack complex (MAC) formation, and recruitment of phagocytic cells. The cellular components are mediated by innate immune cells, including macrophages, monocytes, neutrophils, dendritic cells, and natural killer (NK) cells.

5.1 Macrophages

Macrophages are mononuclear phagocytes that are integral to every organ, where they support tissue homeostasis through functional specialization (112). Tissue resident macrophages are identifiable by their characteristic expression of a handful of pan-macrophage markers (e.g. CD11b, F4/80, MertK and CD64). They are endowed with core macrophage functions and related gene signatures that underpin their survival (Csf1 and Maf), non-opsonic phagocytosis (CD14, CD36, Clec7a and Mrc1), opsonic receptor-dependent phagocytosis (fcgr1, 3, 4 and Itgam), complement pathway (C1qb, c and C3ar1) and efferocytosis (Timd4, Mertk and Sirpa). The core macrophage signature points to a specialized role in immune surveillance. Macrophages as well as dendritic cells express several opsonic (i.e. antibody and complement) and non-opsonic pattern recognition receptors that recognize a range of related ligands and result in their phagocytosis and endocytosis. Different Fc receptors are involved in uptake and destruction of pathogens as well as the negative regulation of effector functions, whereas various complement receptors such as CR3 mediate macrophage phagocytosis, cell migration and activation. The engulfed microorganisms are subjected to a wide range of reactive oxygen species (ROS), including superoxide anion, hydroxyl radicals, hypochlorous acid, nitric oxide, antimicrobial cationic proteins and peptides, and lysozyme (113).

Non-opsonic pattern recognition receptors include the mannose receptor (CD206 or MR1), the scavenger receptors SR-A, and MARCO, which mediate phagocytosis and endocytosis of a wide range of microorganisms (114), and members of a family of molecules called toll-like receptors (115). These pattern recognition receptors on dendritic cells and macrophages are involved in the recognition of yeast cell wall pathogen associated molecular patterns (PAMPs) such as mannans, lipopolysaccharides on the surface of Gram-negative bacteria, teichoic acids present on Gram positive bacteria, lipoproteins and zymosan, as well as endogenous damage associated molecular patterns (DAMPs) (116).

Macrophages in tissue orchestrate the complex processes involved in tissue development, wound healing, tissue repair, and remodeling. They recognize and engulf dying, senescent and abnormal cells, as well as bacteria and immune complexes. They engage in a dynamic interplay

Text box 1:

Macrophage polarization

A common example of macrophage plasticity is their existence in two primary activation states: the classical (M1) and the alternative (M2) polarization. Stein et al. described for the first time that macrophages could be alternatively activated via the Th2 cytokine interleukin (IL)-4 to tailor their responses towards fungal infection and allergies (118). The alternate response was phenotypically and functionally distinct from classical priming with the Th1 cytokine interferon (IFN) γ.

Macrophage activation is associated with distinct metabolic phenotypes. Mills et al. identified the differential activation of metabolic pathways in macrophages derived from two mouse strains, leading to the identification of iNOS and arginase 1 as markers for pro-inflammatory (M1) and alternatively activated (M2) macrophages, that are still being used today(119). Classically activated (M1) macrophages, undergo a metabolic shift towards glycolysis, which supports their pro-inflammatory and microbicidal functions. Conversely, alternatively activated (M2) macrophages, rely more on oxidative metabolism, including fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS), to support their tissue repair and immunoregulatory functions.

Mantovani proposed an overarching model of macrophage polarization that encompassed further heterogeneity in polarization responses, whereby M2 macrophages can be further subdivided into different subsets with distinct functions, such as M2a, M2b, and M2c, depending on the activating signals and the microenvironmental context (117, 120). M2a Macrophages are activated by interleukin-4 (IL-4) and interleukin-13 (IL-13), typically produced during type 2 immune responses, and promote tissue repair and remodeling and enhance extracellular matrix deposition and fibrosis. M2b Macrophages are activated by immune complexes in combination with Toll-like receptor (TLR) or IL-1 receptor ligands and they promote resolution of inflammation and tissue repair. M2b macrophages may express a unique set of markers, including CD86 and CD206, and produce interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1Ra). M2c Macrophages are activated by anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), as well as glucocorticoids. Exhibit immunosuppressive and anti-inflammatory properties, participate in tissue remodeling and wound healing, engage in phagocytosis of apoptotic cells (efferocytosis). M2c macrophages often express CD163, a scavenger receptor involved in clearance of hemoglobin-haptoglobin complexes and produce high levels of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β). Mosser conceptualized macrophage plasticity as a spectrum of macrophage transcriptional states, where single or combined stimuli drive various transcriptome signatures (121, 122).

Gordon et al. proposed a useful paradigm of macrophage activation that includes 4 stages: differentiation by growth factors such as CSF1 and CSF2, priming or polarization by IFN-γ or IL-4 and IL-13, activation into an effector phenotype (e.g. by TLRs), and finally resolution or deactivation to enable repair functions by anti-inflammatory mediators like IL-10 and TGF-β(123). Colony-stimulating factor 1 (CSF1) and colony-stimulating factor 2 (CSF2), also known as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) respectively, are two key cytokines that play important roles in the regulation of macrophage maturation and polarization. CSF1 promotes the differentiation and survival of monocytes and macrophages, as well as the development and maintenance of tissue-resident macrophage populations. CSF1 has been associated with the generation of M2-like macrophages, which are typically involved in tissue repair, wound healing, and immunoregulation (124). On the other hand, CSF2, stimulates the production and differentiation of granulocytes and macrophages from bone marrow progenitor cells. GM-CSF has been shown to induce the differentiation of macrophages towards an M1-like phenotype, which is associated with pro-inflammatory functions.

The functional state of macrophages is influenced by individual or combined signals, which leads transcriptional and epigenetic modification in macrophages facilitating the activation of several transcription factors (125). Pro-inflammatory signals, IFNγ, lipopolysaccharide (LPS), granulocyte-macrophage colony-stimulating factor (GMCSF) and virus-derived molecules, exert responses through transcription factors including signal transducer and activator of transcription 1/2, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), STAT5 and interferon regulatory factor (IRF5), respectively (125, 126). Anti-inflammatory or anti-parasitic infection responses are induced by IL-4, IL-10, glucocorticoids and macrophage colony-stimulating factor (MCSF) through STAT6, peroxisome proliferator-activated receptors (PPARγ), jumonji domain-containing protein (JMJD) 3 and IRF4 (125, 126). Pathological conditions generate diverse and dynamic microenvironments in which both M1 and M2 stimulatory factors often co-exist(127). Recent work has identified that macrophages challenged by IFN-γ and IL-4 simultaneously display both M1 and M2 gene transcriptome signatures while each stimulus mutually inhibits its counterpart's activation at transcriptional and epigenomic levels (127).

with stromal and structural cells within each organ by producing growth factors and other signaling molecules underpinning host defense and organ development and function.
Macrophages can dynamically adapt their functional characteristics and phenotypes in response to changes in their microenvironment or stimuli, a feature termed *macrophage plasticity* (117) (Textbox 1: Macrophage polarization). Macrophages can transition between different activation states or phenotypes, exhibiting a spectrum of diverse functions that allow them to effectively participate in various physiological processes, including immune responses, tissue repair, and homeostasis maintenance.

5.1.1 Macrophages in the arterial wall: atherosclerosis

Our understanding of myeloid cell ontogeny, tissue specification and activation has benefitted from advancements in fate mapping, single cell biology and parabiosis (when the circulation of two mice of distinct genotype are joined) techniques. For instance, while monocytes, DCs and granulocytes originate by a multipotent stem cell the hematopoietic stem cell (HSC), new evidence was found that tissue macrophages, originally thought to be derived from bone marrow derived HSCs and continually replenished (128), originate from embryonic precursors during fetal development (129-133). We will discuss the fundamental aspects of these models and their application and relevance to atherosclerosis below.

5.1.1.1 Resident vascular macrophages

In the aorta, two macrophage niches are available both at the steady state and during disease development. Resident vascular macrophages are detected in both intimal and adventitial layers in the steady state (134-136) (Figure 2, Figure 5). Lyve1^{+}CD206^{+} resident arterial macrophages are typically located in the adventitia and depend on CSF1 (colony-stimulating factor 1 or MSCF) and CX3CR1 for their survival. Their origin is consistent with successive waves of arterial colonization, entailing yolk sac erythron-myeloid progenitors and fetal liver monocytes during the embryonic period, followed by influx of bone marrow-derived monocytes in the postnatal period. A sharp decline in yolk sac labeling from 60% at birth to 20% in the days following birth indicates a discrete window of replacement of embryonic precursor by hematopoietic stem cells postnatally (134)
Adventitial macrophages have a topographic and functional interaction with smooth muscle cells, contributing to the homeostasis, optimal arterial diameter, and extracellular matrix (ECM) content of the arterial wall (137). Lyve1^{+} resident adventitial macrophages ensure arterial homeostasis under physiological conditions by clearing excessive collagen deposition by smooth muscle cells (SMCs), preventing unfavorable vessel wall remodeling and dilatation (137). Our work showed that resident-like macrophages constitute most of the macrophage pool in the murine atherosclerotic aorta and that their representation decreases during atherogenesis (138, 139). Recent work showed that early exposure to western diet leads to a loss of resident Lyve1+ macrophages within the aorta, exacerbating atherogenesis progression and CVD risk in lifespan (140).
Intimal CD11c^{+} myeloid cells been consistently recognized as crucial contributors to atherogenesis. Deletion of these cells via CD11c-DTR (diphtheria toxin receptor) has been shown to reduce the atherogenic burden (136, 141). CD11c^{+} macrophages can be differentiated from intimal dendritic cells using multi-analyte approaches in both human and mouse studies (138,

142). $CD11c^+$ aortic intima resident macrophages, referred to as MacAir, are dependent on CSF-1 (colony-stimulating factor 1) and derive from bone-marrow derived progenitors and are seeded at birth (136). $CD11c^+$ arterial macrophages originate from the ductus arteriosus and migrate within the aorta, particularly in the inner curvature of the aorta (a predilection site for atherosclerosis) in the immediate post-natal period. These events coincide with systemic circulatory changes. At birth, the closure of the ductus arteriosus, driven by increased oxygen levels, reduced prostaglandins, and rising systemic vascular resistance, redirects blood flow from the fetal shunt to the pulmonary circulation. Simultaneously, pressure in the aorta increases due to the cessation of placental circulation and the increased output from the left ventricle. The $CD11c^+$ arterial intimal resident macrophages play a role in preventing thrombosis via the plasminogen receptor (143). In the early stages of atherogenesis, embryonic MacAir cells accumulate lipids and undergo proliferation, contributing to early lesion formation (136).
Parabiosis studies, which involve surgically joining two living organisms to share a common circulatory and hematopoietic system between the paired organisms, have demonstrated that macrophage chimerism in the aorta remains low (approximately 5%). This finding indicates that once the arterial macrophage niche is established postnatally, these cells renew primarily through local proliferation, with only a minimal contribution from circulating monocytes under steady-state conditions (134, 144). Additionally, parabiosis studies have provided key insights into the dynamics of macrophage origin during plaque development. In early atherogenesis, there is a significant wave of monocyte recruitment, as evidenced by similar macrophage chimerism levels in the blood and aorta, supporting previous findings that monocytes contribute to lesion progression (145-148). However, during late atherogenesis, aortic chimerism is low, suggesting that macrophage pools in the aorta are mostly sustained by local proliferation, with relatively limited input from circulating monocytes (144). Macrophage behavior may also be subset- and location- dependent. Further experiments also revealed that under steady-state conditions, resident intimal arterial macrophages maintain their population through a low rate of local proliferation without significant contribution from bone marrow-derived monocytes. As atherosclerosis progresses, intimal resident macrophages are gradually replaced by bone marrow-derived counterparts (136). Thus, macrophage origin in the healthy vessel wall and in atherosclerotic plaques is complex and its importance in vascular biology and atherogenesis require further investigation.

5.1.1.2 Lipid associated macrophages

Traditionally, phagocytosis of lipids has been seen as the principal role of macrophages in atherosclerosis (149). Macrophages present in atherosclerotic plaque contain substantial amounts of cholesterol esters (150) and assume a foamy or bubbly appearance due to a high content of lipid droplet in their cytoplasm, hence the name “foam cells” (Figure 5, Figure 6). Foam cells are the hallmark of atherosclerosis (151).
The initial step in foam cell formation involves the uptake of LDL cholesterol by macrophages in the arterial wall. In 1979, Brown and Goldstein proposed that macrophages express specific receptors, named scavenger receptors (SRs) (151), primarily the LDL receptor (LDLR), as well as scavenger receptor class A (SR-A) and CD36 (152), LDL particles are then degraded into amino acids and free cholesterol within lysosomes (149). Free cholesterol is released from the

lysosomes into the cytosol, whereas excess of cholesterol esters is accumulated in lipid droplets by catalyzing with acyl coenzyme A:cholesterol acyltransferase-1 (ACAT-1) (153).
The LDL receptor (LDLR) enables internalization of LDL via endocytosis, triggering a cascade eventually causing LDL degradation in the lysosomes and downregulation of LDLR expression (154). Individuals with a mutant LDL receptor suffer from familial hypercholesterolemia, elevated LDL particles in their bloodstream and myocardial infarction at a young age (155). The expression of LDLR by macrophages is important for foam cell formation (156).
Apoe$^{-/-}$*MSR-A* mice show a 60% reduction in atherosclerosis(157). The CD36 and SR-A scavenger receptors enable lipid uptake by macrophages which is important for clearing oxLDL, but unregulated oxLDL uptake creates lipid-loaded foam cells(158). Deleting SR-A in *Ldlr*$^{-/-}$ mice reduces lesion area to a considerable extent (159). However, deleting both SR-A and CD36 in *Apoe*$^{-/-}$ mice has no effect on lesion area or foam cell formation but it achieves a reduction in inflammation, macrophage apoptosis and plaque necrosis (160), suggesting a key role in immune sensing.
As macrophages cannot limit cholesterol uptake through scavenger receptors, they depend on cholesterol and lipid efflux pathways to prevent transforming into foam cells, as demonstrated in *Ldlr*$^{-/-}$ mice deficient in sphingomyelin synthase (161). In *Ldlr*$^{-/-}$ mice, defective ABCA1-mediated cholesterol and lipid efflux causes unlimited lipid accumulation in macrophages (162). IFN-γ affects scavenger receptor expression and hampers cholesterol efflux by macrophages from obese C57BL/6 mice and generally promotes hyperglycemia and fat inflammation(163).
Several transcription factors are involved in foam cell formation. Liver X Receptors (LXRs) are nuclear receptors that play a central role in regulating cholesterol homeostasis. Activation of LXRs promotes the expression of genes involved in cholesterol efflux, such as ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), which facilitate the removal of excess cholesterol from macrophages. Sterol Regulatory Element-Binding Proteins (SREBPs): SREBPs are transcription factors that control the expression of genes involved in lipid synthesis and uptake. Activation of SREBPs promotes the expression of genes involved in fatty acid and cholesterol biosynthesis, contributing to lipid accumulation in foam cells. Foam cells are not only derived from macrophages, but they have been shown to derive also from smooth muscle cells undergoing transdifferentiation (164).
Lipid droplets (reviewed in (165)) are dynamic organelles found in most cell types, ranging from adipocytes (fat cells) to hepatocytes (liver cells) and macrophages. They serve as key sites for storing and mobilizing neutral lipids, primarily triglycerides (TGs) and cholesterol esters (CEs). Lipid droplets are composed of a hydrophobic core of neutral lipids surrounded by a monolayer of phospholipids, free cholesterol, and associated proteins. The core contains triglycerides, cholesterol esters, and other hydrophobic molecules. Proteins such as perilipins and members of the PAT (perilipin, ADRP, TIP47) protein family coat the surface of LDs, regulating their formation, stability, and interactions with other cellular components. The primary function of LDs is to store excess energy in the form of neutral lipids. They can interact with various organelles such as the endoplasmic reticulum, mitochondria, and peroxisomes to exchange lipids and regulate cellular lipid homeostasis. Autophagy, a cellular process involved in the degradation and recycling of cellular components, plays a role in regulating foam cell formation (166, 167). Dysregulation of autophagy can lead to the accumulation of lipid droplets and exacerbate foam cell formation (168).

Endoplasmic reticulum (ER) stress is emerging as a significant contributor to foam cell biology and atherosclerosis development. The ER is a crucial organelle involved in protein folding, lipid synthesis, and calcium storage. Disruption of ER function leads to ER stress, triggering a cellular response known as the unfolded protein response (UPR) (169). High levels of cholesterol can alter the lipid composition of the ER membrane and impair its integrity and function (170). ER stress activates the UPR, a signaling pathway aimed at restoring ER homeostasis by reducing protein synthesis, enhancing protein folding capacity, and degrading misfolded proteins. The UPR is mediated by three main transmembrane proteins: inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (171), and activating transcription factor 6 (ATF6) (169). Once ER stress is triggered, foam cells undergo apoptotic or necroptotic cell death (170, 172, 173). ER stress can also trigger inflammatory signaling pathways, exacerbating monocyte recruitment and atherosclerosis progression. Foam cells drive necrotic core formation owing to their uptake of intraplaque lipids leading to increased ER stress and cell death (174).

Accumulation of desmosterol in lipid-associated macrophages lead to activation of LXR target genes, inhibition of SREBP target genes, selective reprogramming of fatty acid metabolism, and suppression of inflammatory-response genes, promoting lipid homeostasis (175). Desmocholesterol suppresses inflammasome activation and protects against atherogenesis and athero-inflammation (176). This evidence suggests that lipid-loading pathways are not directly linked to initiation of inflammation and that other signaling events are required to induce inflammation.

OxLDL and other modified forms of LDL have been shown to exert inflammatory effects through several different pattern recognition receptors, including Toll-like receptors (TLR) (171, 177, 178). Increasing evidence supports roles for TLR 2 and 4 in promoting inflammation and atherosclerosis in mouse models and human lesions (179-181). In addition, macrophage uptake of cholesterol crystals has been shown to result in activation of the inflammasome (181). Antioxidant therapies did not achieve the desired clinical efficacy in CVD, indicating that the main driver of atherogenesis is not oxidation.

Nuclear Factor-kappa B (NF-κB) is a key transcription factor involved in inflammatory signaling. Activation of NF-κB in response to inflammatory stimuli promotes the expression of pro-inflammatory cytokines and chemokines, which contribute to the recruitment of immune cells and exacerbation of inflammation within the arterial wall. In addition to NF-κB, other signaling pathways involved in inflammation, such as the mitogen-activated protein kinase (MAPK) pathway and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, also contribute to foam cell formation by promoting the expression of inflammatory mediators and adhesion molecules. NFkB is involved in macrophage survival (182).

5.1.2.2 Macrophage polarization in atherosclerosis

Macrophages exhibit great plasticity (*see text box*). In murine atherosclerotic lesions, M2 macrophages accumulate initially in early lesions, while during lesion progression M1 macrophages predominate (183). Deletion of Arginase-2 in *Apoe*$^{-/-}$ mice reduces lesion size, macrophage inflammation and necrotic core formation (184). Hypoxia in mice is shown to enhance the expression of M1 macrophage markers while reducing M2 macrophage markers, contributing to necrotic core formation (185). In human atherosclerotic plaques, M1 macrophages are more frequently found in rupture-prone shoulder regions of the plaque, while

M2 macrophages are more common in the adventitia (186). Human symptomatic carotid plaques, which have a greater content of lipid and leukocytes, have increased expression of M1 macrophage markers while femoral plaques, with more fibroconnective tissue, collagen, and calcification, have greater M2 marker expression (187). M2 macrophages have also been observed in stable regions of human plaques (188). Normalizing plasma HDL levels upregulates M2 macrophage markers and downregulates pro-inflammatory markers (189).
Oxidized phospholipids within murine atherosclerotic plaques have been shown to downregulate M1 and M2 gene expression, phagocytic and chemotactic capabilities and induce an Mox macrophage phenotype associated with Nrf2-mediated expression of redox-regulatory genes, such as heme oxygenase-1, thioredoxin reductase 1 and sulfiredoxin (190).
Platelet-derived chemokine CXCL4 has been shown to produce a separate M4 human macrophage phenotype, which is less efficient at phagocytosis, has reduced expression levels of CD163 and HO-1 and is a terminal state in that it not able to transform into other macrophage phenotypes once polarized (191). CXCL4-induced M4 macrophages downregulate scavenger receptor expression, and upregulate cholesterol efflux transporters, in comparison to M-CSF-induced macrophages (191), thus rendering M4 macrophages less able to take up cholesterol and become foam cells. Their presence in atheromatous lesions is enriched in vulnerable plaques (191), where they express MMP7 and S100A8.
Hemoglobin-haptoglobin (Hb:Hp) complexes or Heme – both found in intraplaque hemorrhage – induce hemorrhage-associated or Mhem macrophage phenotypes. Exposure to Hb:Hp or Heme stimulate ATF-1-mediated HO-1 expression, subsequently increasing expression of the Hb:Hp scavenger receptor CD163 and IL-10 while suppressing MHC-II expression by human macrophages (192, 193). This macrophage phenotype is resistant to foam cell formation due to the promotion of LXRalpha and ABCA1 expression and cholesterol efflux (194). Their role is, however, not wholly protective because CD163+ CD206+ macrophages in the plaque can promote angiogenesis and vascular permeability and inflammatory cell recruitment (195). They are associated with a vulnerable plaque phenotype (196), suggesting that they can become activated and promote atherogenesis.

5.1.2.3 Macrophage heterogeneity at the single cell level

Three main macrophage populations with different inflammatory properties were identified in human (197-200) and murine single cell studies (201). The first is an inflammatory macrophage population expressing high levels of IL-1β, a well-recognized immune-target in atherosclerosis, further highlighting its relevance for atherosclerosis progression. The second is a vascular resident-like phenotype that is enriched in genes involved in antigen presentation and endocytosis. Finally, Trem2^{hi} (Triggering Receptor Expressed on Myeloid Cells 2) macrophages, a subset previously identified in obesity as a protective one, highly express genes associated with lipid handling and metabolism and mouse studies have confirmed that these cells might be foamy macrophages, a hallmark of atherosclerosis (202). Trem2^{hi} macrophages express high levels of genes associated with lipid metabolism, cholesterol efflux and oxidative phosphorylation including *Trem2*, *Cd9*, *Lpl*, *Fabp5*, *Plin2*, *Abcg1*, *Spp*1 and *Itgax* and do not express inflammatory gene signatures suggesting a homeostatic lipid handling role in atherosclerosis(202) . Recently, two subsets of *Trem2* macrophages have been defined using scRNAseq(203): *Gpnmb*^{hi}*Trem2*^{hi} macrophages displayed the known homeostatic lipid handling signature, while Slamf9^{hi}*Trem2*^{hi}

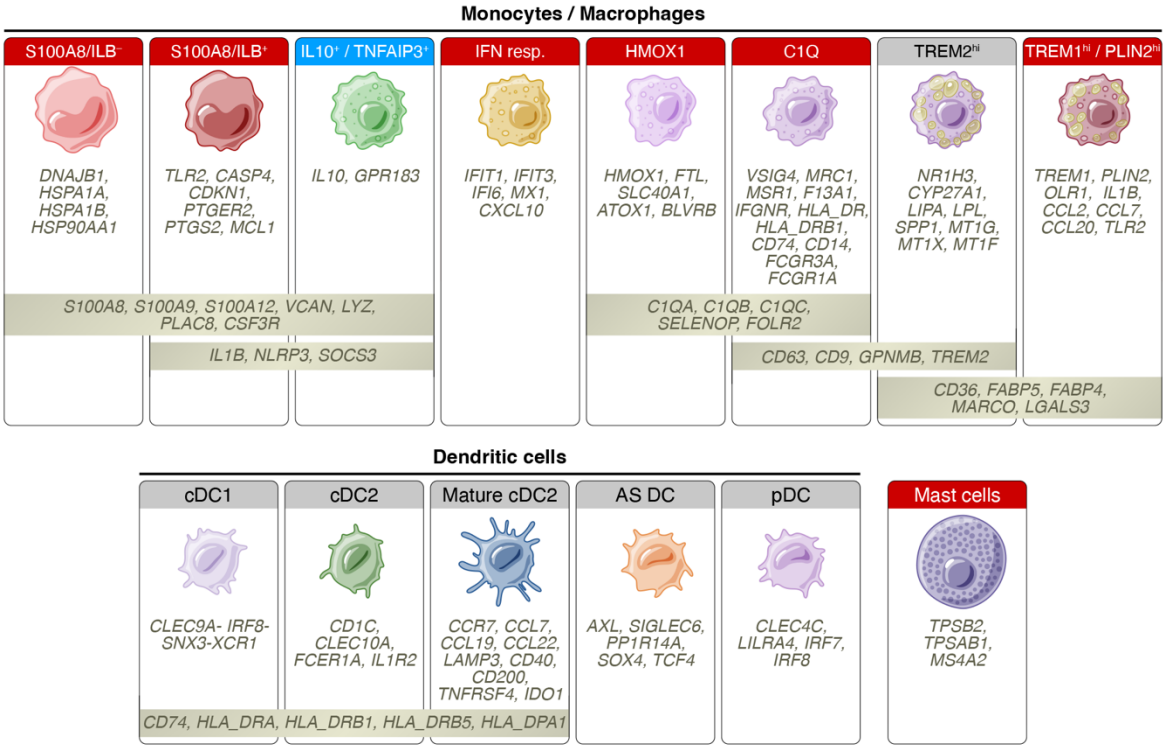

Figure 6. Myeloid cell subsets in atherosclerosis

The immune landscape of the human carotid atherosclerotic plaque has been recently resolved through the use of the single cell biology techniques. Fourteen distinct myeloid populations were identified; a mast cell population (*TPSB2, TPSAB1, MS4A2*), a plasmacytoid dendritic cell population (pDC; *CLEC4C, IRF7, IRF8, JCHAIN, LILRA4*), 4 conventional DCs (cDCs) and 7 monocytes/macrophage populations. The main subsets are further downstream classified as pro (red) or anti (blue) – atherogenic role through analysis of the top differentially expressed genes (DEGs).

All cDCs populations express of HLA-DR genes as well as individual gene signature; cDC2 cells express *CD1C, CLEC10A, FCER1A* while cDC1 cluster is identified by the expression of *CLEC9A, IRF8* and *SNX3.* Mature cDC2s are recognized by high expression of the egress chemokine receptor *CCR7* and CCR7 specific chemokines (*CCL17, CCL19*, and *CCL22*) as well as maturation genes (*LAMP3, IDO1*) and immunoregulatory genes such as *CD40*, *CD200*, *TNFRSF4* (gene coding OX40) and *CD274* (gene coding PDL-1). Finally, a small population of *AXL* and *SIGLEC6* (AS) expressing DC is identified, however, its role in disease is still unknown.

Monocytes/ macrophages in the human carotid plaque segregate into 7 distinct populations. S100A8/IL1B^{+} and S100A8/IL1B^{-} populations have a less differentiated phenotype compared to the rest of the macrophages found in the plaque and are sometimes referred to as monocytes or mono-macs. They share a high expression of calgranulins *S100A8/S1009/S10012*. However, they differ in other genes: Cluster S100A8/IL1B^{-} is characterised by a stress response gene signature including several heat shock protein genes coding for HSP70 (*HSPA1A, HSPA1B, HSP6*), HSP90 (*HSP90AA1*) and HSP40 (*DNAJB1*). The S100A8/IL1B^{+} cluster is characterised by expression of genes in the inflammasome pathway; *IL1B, NLRP3, CASP4*, pro-inflammatory cytokine/chemokines*; TNF, CCL3, CCL4, CCL3L3, CCL4L2, CCL20*, pro-inflammatory transcription factors; *CEBPB* and *NFKB1* and receptors; *TLR2, TREM1*. In line with a highly inflammatory profile, S100A8/IL1B^{+} expresses senescence genes (*CDKN1*, *PTGER2, PTGS2)* as well as genes involved in cell death, proliferation and survival (*BCL2A1, BTG1, GOS2*). This suggests S100A8/IL1B^{+} as a terminal and a highly pro-inflammatory and pro-atherogenic cluster. The IL10^{+}/TNAIP3^{+} population shares a high *IL1B* expression with S100A8/IL1B^{+}, however, in addition to expressing pro-inflammatory genes, IL10^{+}/TNAIP3^{+} has a clear anti-inflammatory signature, exclusively expressing the anti-inflammatory cytokine *IL10*, the NFκb inhibitor *TNFAIP3* and the anti-inflammatory receptor *GPR183,* highlighting the potential role of this subset in rebalancing the immune responses in plaques.

C1Q and HMOX1 clusters are defined by high expression of the complement C1q family (*C1QA, C1QB, C1QC*), genes common to resident-like macrophages, suggesting an homeostatic role in the atherosclerotic plaque environment.
TREM2^{hi} and a PLIN2^{hi}/TREM1^{hi} are 2 distinct lipid-associated macrophages (LAMs) clusters. They share a lipid-associated transcriptional signature including fatty acid-binding proteins (FABP4, FABP5) and lipid scavenger receptors (CD36, MARCO). TREM2^{hi} expresses genes involved in regulating cholesterol metabolism, transport, and efflux (*ANXA2, APOC1, APOC2, APOE, CYP27A1* and *NR1H3*-the gene encoding the transcription factor LXRa) as well as genes involved in lysosomal degradation (*LIPA, LGMN, PSAP*) and a strong antioxidant signature related to metallothionein *MT1G, MT1X, MT1E, MT1F*, a group of antioxidant proteins expressed in response to injury. This gene signature along with a lack of inflammatory genes suggests TREM2^{hi} to be highly competent in lipid uptake, cholesterol metabolism and efflux.
PLIN2^{hi}/TREM1^{hi} macrophages expresses a high level of the perilipin gene *PLIN2,* a *bona fide* lipid storage and foam cells marker, coding for a structural component of lipid droplets. Unlike TREM2^{hi} cluster, PLIN2^{hi}/TREM1^{hi} lacks expression of genes involved in lysosomal degradation and most genes involved in cholesterol efflux suggesting that the lipid uptake is not paralleled by an increase in efflux leading to marked cholesterol storage. PLIN2^{hi}/TREM1^{hi} expresses genes in the inflammatory and inflammasome pathway *TREM1, TNF, CEBPB, OLR1* and *IL1B* as well as genes involved in ER unfolding protein response such as *ATF3, CALR, ACADVL, HSPA5* and *HSP90B1* and genes involved in apoptosis, anti-proliferation and survival such as *GOS2, BTG1, BCL2A1, IER3*, *EGR1, BNIP3L* and *MCL1.* Finally, the phenotype of TREM1 foam cells is completed by a high chemokine secretory signature, PLIN2^{hi}/TREM1^{hi} cells selectively express *CCL2* and *CCL7* in the plaque as well as high levels of *CCL20, CXCL3, CXCL2* and *CXCL8*. This strong and specific chemokine signature suggest that these inflammatory foam cells might propagate the disease by playing a major role in the recruitment of immune cells to the plaque.

macrophages expressed a more inflammatory gene signature (*Tnf*, *Ccl3*, *Ccl4*, *Nfkbiz*) and less lipid handling gene signature when compared to *Gpnmb*^{hi} macrophages.
Recent studies have highlighted the multifaceted role of TREM2 in atherosclerosis. Conditional deletion of TREM2 in CX3CR1-expressing cells revealed a reduction in foam cell formation and survival, accompanied by decreased atherosclerotic lesion size, but with no observable effect on monocyte recruitment (204). In contrast, studies involving global or hematopoietic deletion of TREM2 demonstrated its critical role in promoting efferocytosis (the phagocytic clearance of apoptotic cells) and controlling plaque necrosis, underscoring its contribution to lesion stability (205). Interestingly, therapeutic TREM2 agonism has been shown to reverse atherosclerotic lesion progression and necrotic core formation (205, 206). This suggests that while TREM2-dependent foam cell survival contributes to plaque growth under certain conditions, it also plays a protective role by mitigating the development of the necrotic core, a hallmark of advanced and unstable plaques. These findings point to a dual role for TREM2 in balancing foam cell functions, lesion stability, and progression. Further research is needed to determine whether these distinct effects of TREM2 arise from macrophage heterogeneity within atherosclerotic lesions, such as differences between resident arterial macrophages and recruited monocyte-derived macrophages, or whether they reflect context-dependent mechanisms of TREM2 signaling in foam cells.
A new subset of lipid-associated macrophages that exhibit both a high lipid signature and inflammatory score has been recently identified in human carotid plaques. This subset, termed inflammatory lipid-associated macrophages or iLAMs was referred to as PLIN2^{hi}/TREM1^{hi} due to the highest expression of lipid-droplet associated gene, perilipin (PLIN2) and the triggering receptor expressed on myeloid cells 1 (TREM1) known to be the inflammatory “antagonist” of TREM2 (198) (Figure 6). The transcriptional signature of this subset is consistent with murine

studies where loss of TREM1 was linked to significant reduction of atherosclerotic lesions, reduction of foam cell formation and recruitment of monocytes (207, 208). Computational and functional experiments demonstrate that PLIN2^{hi}/TREM1^{hi} iLAMs derive from TREM2^{hi} macrophages through loss of lipid handling genes, including cholesterol efflux transporters and acquisition of inflammatory transcriptional signature (e.g. IL1beta, CCL2, NALP3, NFκB) consistent with a pathogenic transition (198).

5.2 Monocytes

Monocytes are mononuclear phagocytes that are produced in the bone marrow and circulate in the bloodstream. They are derived from macrophage-dendritic cell precursors (MDPs) in the bone marrow, which express CD115, c-Kit, CX_3CR1 and Flt3 (209). Monocytes rely on various chemokine receptors and adhesion molecules to traffic from the bone marrow and blood to tissues. There are two main populations of monocytes: classical (mouse-Ly6C^{hi} CCR2^{hi}CX_3CR1^{lo}, human CD14^{hi}CD16^{-}) and nonclassical monocytes (mouse-Ly6C^{lo} CCR2^{lo}CX_3CR1^{hi}, human-CD14^{lo}Cd16^{+})(210). CD14 is a co-receptor for TLR-4 enabling lipopolysaccharide detection while CD16 (or FCγRIII) belongs to the Fcγ receptor family that bind to antibodies during immune responses such as antibody-dependent cell-mediated cytotoxicity (ADCC). Ly6C^{high}CCR2^{high}CX_3CR1^{low} monocytes are recruited to inflamed sites, depend on CCR2 to mobilize from the bone marrow (210, 211). Ly-6C^{low}CCR2^{low}CX_3CR1^{high} monocytes – resident or non-classical monocytes - patrol the endothelium to maintain homeostasis, depend on LFA-1 and CX_3CR1 for crawling and extravasation (212). In steady state, patrolling monocytes promote vascular endothelial cell homeostasis; they remove damaged cells and debris from the vasculature and are associated with healthy vascular remodeling (213, 214). Nonclassical monocytes derive from classical monocytes in a Nur77-dependent manner (215, 216). Conversion of classical into non classical monocytes has been demonstrated also in humans (129).

5.2.1 Monocytes in atherosclerosis

Studies in both mice and humans have demonstrated that increased severity of atherosclerosis is associated with an expansion of the blood monocyte pool. Ly6C^{hi} monocytes represent 90% of monocytes accumulating in murine atherosclerotic lesions (146). Hypercholesterolemia increases the proliferation and differentiation of Ly-6C^{hi} monocytes, impairs conversion to resident subsets (which can be reversed by statins) and elevates macrophage foam cell formation (146).

On the other hand, Ly6C^{lo} monocytes, also referred to as patrolling monocytes, preferentially differentiated into disease associated CD11c^{+} myeloid cells (147). In agreement with this finding, a subset of non-classical monocytes was found to be enriched in patients with coronary artery disease using mass cytometry (217). Deficiency of Nur77, the transcription factor involved in Ly6C^{lo} monocyte development, has been shown to increase atherosclerosis and obesity, suggesting an atheroprotective role of patrolling monocytes in line with their homeostatic functions described (214, 218-220). Lyn kinase enhances atherogenesis by impairing the atheroprotective function of Ly6C^{lo} monocytes (220). Depletion of CD11b^{+} cells using a diphtheria toxin system reduces atherosclerotic plaque area and the amount of lipid, macrophages, SMCs, collagen, and necrotic core size, however there is no effect on established plaques (221).

In regression models of atherosclerosis, either by normalizing plasma HDL levels or transplanting the aortic arch from *Apoe*$^{-/-}$ mice into wild-type mice, Ly6C^{hi} monocytes emigrate from regressing plaques, and the expression of M2 macrophage markers is increased in bone marrow-derived macrophages and plaque macrophages (189, 222). Atherosclerotic regression is dependent on the recruitment of monocytes from the blood via CCR2 (223). Loss of arginase 1 and ornythin decarboxylases impairs regression by interfering with MertK dependent efferocytosis (224, 225), suggesting that the establishment of M2 polarization is inherent to atherosclerosis regression and the re-establishment of effective efferocytosis.
The amount of pro-inflammatory CD14^{+}CD16^{+} monocytes and serum TNF-α levels is elevated in patients with coronary artery disease (226) and this monocyte subset negatively correlates with fibrous cap thickness (227). Flow cytometry analysis showed CD14^{++}CD16^{+} monocytes, particularly those from patients who have suffered a myocardial infarction, upregulate their expression of CD11c in response to VLDL uptake, high fat consumption or elevated circulating triglycerides (following high fructose consumption), and the upregulation is particularly pronounced in patients with large necrotic cores (228). Active and stabilized CD11c increases VLA-4 expression on monocytes and thus enables it to form clusters and bind to endothelial VCAM-1, strengthening the adhesion of recruited monocytes (228).

5.3 Dendritic Cells

Dendritic cells (DCs) are the most capable antigen presenting innate immune cells. DCs come in 3 subsets, i.e., plasmacytoid DCs (pDCs), and 2 conventional DC subsets, cDC1 and cDC2. Plasmacytoid DCs are the main players in viral immunity, and secrete large amounts of interferons, whereas cDCs are responsible for antigen presentation and priming of naïve T cells in the T-cell zones (229). cDC1 cells are involved in cytotoxic immune responses, can cross-present antigens, and are crucial in the response to intracellular pathogens and anti-tumor responses, whereas cDC2 cells have a multitude of functions including responses to extracellular pathogens and directing CD4+ T cell responses (230).

5.3.1 Dendritic Cells in Atherosclerosis

Dendritic cells are present in the healthy arterial wall and are predominantly located in the intima in atherosclerosis prone areas (141, 231). When atherosclerosis progresses, the number of arterial DCs increases. DCs are located within the atherosclerotic plaque (232), in the adventitia and PVAT and in arterial tissue lymphoid organs (ATLOs) (233). In atherosclerosis, ApoB100, HSP60, HSP65, and beta2-glycoprotein are considered auto-antigens that are being presented by DCs (234). Following uptake by antigens, DCs mature, produce pro-inflammatory cytokines and naïve T cells become activated, thereby driving atherosclerosis (97). Dendritic cells present atherosclerosis prone areas or atherosclerotic plaques can have a foamy appearance and were shown to be able to take up lipids via scavenger receptors such as SR-A, CD36 and LOX1(235). Lipid loaden DCs are very well capable of activating naïve T cells (236). The highest DC content is found in vulnerable plaques. Plaque and adventitial DCs that interact with exhibit a mature phenotype, expressing co-stimulatory molecules like CD86 and CD40 (237, 238).
The healthy intima predominantly contains CD11c^{+}CD11b^{+}CD103^{-} DCs (141), which are also prominent in atherosclerotic plaques (239). This CD11b^{+} cDC subset can exit atherosclerotic plaques and migrate to draining lymph nodes via CCL19/CCL21 and their receptor CCR7 (240).

$CD11c^+CD11b^+CD103^-$ DCs in atherosclerotic plaques are inversely correlated with plaque inflammation in humans and mice, suggesting that $CD11b^+$ cDCs are protective in atherosclerosis (239).

Elimination of CD11c+ DCs in *Apoe*$^{-/-}$ mice decreased lipid content in early atherosclerotic plaques (141). DCs are capable of modulating lipid homeostasis. Absence of CD11c+ DCs in *Apoe*$^{-/-}$ mice raises VLDL and LDL cholesterol levels(241). When the lifespan of DCs was prolonged in CD11c-BCL2 transgenic *Apoe*$^{-/-}$ mice, VLDL and LDL cholesterol levels had decreased(242). The underlying mechanism is still unclear, although it gut DCs may affect lipid absorption(243).

$CD103^+$ conventional DCs (cDC1), which make up 20-30% of the aortic DC population require fms-like tyrosine kinase 3 (Flt3L) signalling for survival and development (241). Deficiency in Flt3 reduces the representation of $CD103^+$ DCs and regulatory T cells and increases the expression of IFN-γ and TNF-α causing the exacerbation of atherosclerosis, suggesting that Flt3-dependent $CD103^+$ DCs protect against atherosclerosis (244).

Another DC subset important in atherosclerosis are the $CCL17^+$ DCs, which are an alternative CD11b+ cDC subset. $CCL17^+$ DCs accumulate in the intima and adventitia of atherosclerotic vessels in mice and express CD11c, MHC-II, CD11b, CCR7 and the co-stimulatory molecules CD40, CD80 and CD86. Absence of this CCL17+ DC population resulted in a decrease in atherosclerosis, by causing an expansion of Tregs (245).

pDCs are a heterogeneous DC subset, that can be beneficial and detrimental in atherosclerosis (246). pDCs promote atherosclerosis through activation of T_H1 cell responses and secretion of interferon (IFN)-α (247), but can protect against atherosclerosis by inducing a Treg response (248).

Recent single cell RNAseq studies aiming to better phenotype cell subsets present in atherosclerotic plaques showed that there were 4 major DC subsets in murine atherosclerotic plaques: cDC1, cDC2, pDC and mature DCs (201). CyTOF studies showed similar subsets, with clear populations of cDC1 and cDC2 cells, as well as pDCs (138, 249). Studies from human atherosclerotic plaques reveal the presence of up to 5 dendritic cell subsets: cDC1, cDC2, mature cDC2, AS DCs and pDCs (197-199) (Figure 6). The exact function and relevance of these subsets in the pathogenesis of atherosclerosis is still under investigation.

5.4 Neutrophils

Neutrophils constitute the predominant immune cell population in the blood stream, representing approximately 40%–70% of all circulating leukocytes in humans. Elevated levels of circulating neutrophils and neutrophil-derived biomarkers, such as myeloperoxidase (224) and neutrophil gelatinase-associated lipocalin (NGAL), have been associated with adverse outcomes in patients with ACS (250). These biomarkers serve as indicators of ongoing inflammation and neutrophil activation, aiding in risk stratification and prognostication. Despite their abundance in circulation, neutrophils comprise a minor fraction of immune cells within atherosclerotic plaques, where macrophages and T cells predominate.

Recent studies have shed light on the potential significance of targeting neutrophil activation and neutrophil effector mechanisms, including the production of reactive oxygen species (ROS), release of cytotoxic enzymes through degranulation, and formation of neutrophil extracellular traps (NETs). These emerging therapeutic strategies hold promise for ameliorating atherosclerotic burden and enhancing plaque stability, particularly at the preclinical level.

Despite advancements in understanding the molecular machinery underlying neutrophil functions, the upstream signaling pathways and transcriptional regulators governing these processes in neutrophils remain poorly elucidated. Further research is warranted to unravel these complexities and identify novel therapeutic targets for managing atherosclerosis.

Neutrophils are involved in all stages of atherosclerosis (reviewed in (251)). In mice, neutrophil depletion reduces atherogenesis, and increased levels of circulating neutrophils exacerbate plaque formation, suggesting a role in lesion development (252). Neutrophils contribute to atherogenesis by adhering to activated endothelial cells and transmigrating into the arterial intima, where they release pro-inflammatory mediators and reactive oxygen species (ROS). This promotes endothelial dysfunction and facilitates the recruitment of other immune cells, fostering plaque formation and progression (253). Neutrophil Extracellular Traps (NETs), web-like structures based of DNA, histones, and antimicrobial proteins released by activated neutrophils, have been implicated in atherosclerosis (254). NETs promote thrombosis, exacerbate inflammation, and contribute to endothelial dysfunction, macrophage activation and plaque destabilization, thereby enhancing the risk of acute cardiovascular events (255). Neutrophils release NETs that degrade the fibrous cap of atherosclerotic lesions, predisposing them to rupture. NETs contain histone H4, which binds and exert a direct cellular lytic action onto smooth muscle cells resulting in plaque destabilization (256). Moreover, neutrophils can promote platelet activation and aggregation, further exacerbating thrombus formation(257). In addition, NETs induce plaque erosion leading to thrombosis (258). Targeting neutrophil recruitment, activation, or NET formation may represent potential therapeutic strategies for mitigating atherosclerosis progression and reducing cardiovascular events. Recent studies showed that intermittent exposure to high fat diet accelerates atherogenesis via L-1β-dependent neutrophil progenitor reprogramming (259).

5.5 Mast Cells

Mast cells, traditionally known for their role in allergic reactions and immune responses, have been implicated in the pathophysiology of cardiovascular diseases (CVD). Mast cells are found in connective tissues, mostly submucosal tissues and the dermis and are important for alerting the immune system to local infection. Derived from common myeloid progenitors, they play an important role in allergic reactions by degranulation, releasing proteins such as histamine, which is followed by sustained inflammation (260).

Mast cells accumulate within atherosclerotic plaques as they progress, and their numbers are associated with acute cardiovascular events, indicating their involvement in plaque destabilization. They have been observed in coronary and carotid artery plaques; specifically localized at sites of plaque erosion, hemorrhage or rupture, while the presence of adventitial mast cells correlates with cardiovascular events (261). Within plaques, they release a range of mediators, including proteases such as cathepsin G. These proteases can degrade low-density lipoproteins (LDL) and facilitate their binding to proteoglycans (PGs), thus promoting plaque progression. They secrete serine proteases such as chymase which can activate MMPs promoting plaque instability, and the conversion of angiotensinogen to angiotensin (262) to promote vasoconstriction possibly restricting remodeling of the arterial wall. A deficiency in kit – which depletes mast cells – reduces atherosclerotic disease (263).

Mast cells are involved in regulating vascular permeability. Increased permeability can contribute to intraplaque hemorrhage, vascular leakage, and the recruitment of neutrophils to murine atherosclerotic plaques via the CXCR2/VLA-4 axis (264). They produce chemokines like CXCL1, which recruit inflammatory cells such as CXCR2+ neutrophils to the plaque site, exacerbating inflammation within the plaque environment.
The mast cell-derived pro-inflammatory cytokines IL-6 and IFN-γ advance atherogenesis by elevating the production of matrix-degrading proteases(263). Antagonizing CCR3 inhibits perivascular mast cell recruitment, subsequently reduces plaque and necrotic core size, thus stabilizing plaques, and inhibiting mast cell chymase had the same effect (265). Through these mechanisms, mast cells actively contribute to plaque destabilization, erosion, rupture, and thrombosis, all of which are critical events leading to acute cardiovascular syndromes

6. CELLS OF THE ADAPTIVE IMMUNE SYSTEM IN ATHEROSCLEROSIS

The adaptive immune system reacts in a highly specific way. Adaptive immune responses are initiated by the innate immune system and require antigen presentation by antigen presenting cells. Adaptive immunity includes humoral as well as cell-mediated mechanisms, which are executed by B and T lymphocytes respectively. Important features of the adaptive immune response are antigen recognition, co-stimulation, clonal expansion and differentiation of T and B cells into effector, cytotoxic, plasma or memory cells. Therefore, adaptive immune responses are slower than innate responses. Upon exposure to a previously encountered antigen, the appropriate memory cells will generate faster, stronger, and more effective immune responses.
The first evidence that the adaptive immune system was crucial in atherosclerosis emerged in the 1980s. T cells, B-cells and other HLA^+ antigen presenting cells were found to be present in human atherosclerotic plaques (95, 266, 267). In *Apoe*$^{-/-}$ mice, absence of T- and B-cells decreased atherosclerosis when fed a normal chow diet (268), which was also observed in *ldlr*$^{-/-}$ mice on a high cholesterol diet(269). Splenectomized mice developed more atherosclerosis, and transfer of B cells ameliorated atherosclerotic disease (270). Transfer of activated CD4+ T cells aggravated atherosclerosis in mice(271). These early studies form the basis of our current knowledge on adaptive immune responses in atherosclerosis.

6.1 T cells

T cells are observed in all stages of atherosclerosis and can progress or regulate inflammation in atherosclerosis (266, 272). Both $CD4^+$ and $CD8^+$ T cells initiate immune responses after presentation of peptides on major histocompatibility type II (APCs) and type I (all cells) complexes, respectively. Binding of an antigen to a cognate T cell receptor (TCR), together with a co-stimulatory signal, activates T cells and can cause their (clonal) proliferation (229, 273). In patients with CVD, the number of $CD4^+$ T cells is increased (197). Mice develop less atherosclerosis when $CD4^+$ T cells are being depleted (274), yet MHCII deficiency increased atherosclerosis (275), showing CD4 T cells also contains atheroprotective subsets such as Tregs (276).
Human carotid atherosclerotic plaques contain an increased amount of $CD4^+CD69^+$ T cells compared to that same individual's PBMCs. CD69 becomes rapidly upregulated after TCR-HLA

engagement, indicating an antigen specific T cell response within the atherosclerotic plaque (277). Comparing blood vs carotid plaque scTCR sequencing revealed plaque specific clonal expansion of CD4+ T cells, including a small percentage of the Treg population, whereas $CD8^+$ T cells only showed little plaque specific clonal expansion (277).

These data suggest that atherosclerosis has an autoimmune component driven by antigen presentation and recognition. The most intensively studied atherosclerosis specific self-antigen is apolipoprotein B, the core protein of LDL. Ex vivo studies showed that plaque $CD4^+$ T cells can recognize oxLDL that was processed and presented by APCs (267). More recent work has shown that apoB100 specific $CD4^+$ T cells are increased in plasma of patients suffering from ASCVD (278, 279),(280, 281). Antigens are not restricted to apoB100. Other proteins, such as MDA, and PC, beta2 glycoprotein 1, collagen 6A6 (282), and heat shock protein 60, as well and the mitochondrial dehydrogenase ALDH4 (283), have been identified as potential antigens, but whether they cause antigen-induced clonal expansion of T cells needs to be investigated further (234, 284).

$CD4^+$ T cells polarize into distinct Th cells or Treg cell subsets, which can either activate or dampen the response of other immune cells, exert pro-or anti-inflammatory effects on tissue resident cells, help B-cells produce IgG antibodies or become cytolytic (285). Each $CD4^+$ T cell subset has specific transcriptional programs and patterns of cytokine secretion that can accelerate or attenuate atherosclerosis. These distinct CD4+ T cell subsets are Th1, Th2, Th9, Th17, Th22, TFH, $CD28^-$ cells, and Treg T cells.

6.1.1 CD4+ T cell subsets in atherosclerosis

Th1 cells promote atherosclerosis and is the most prominent Th cell subset found in the atherosclerotic plaque (286-288). Th1 *cells* are driven by the transcription factor Tbet, express CXCR3 and CCR5 and produce IFNγ. In addition to IFNγ, Th1 cells in the plaque also express other pro-atherogenic cytokines including IL2, IL3 and TNFα, which activate macrophages, T cells, VSMCs and ECs and accelerate plaque inflammation (272). Deficiency of Tbet, IFNγ or its receptor protects mice from atherosclerosis (286, 289, 290), whereas administration of IFNγ aggravates atherosclerosis (291). IFNγ was found to inhibit VSMC proliferation (98) and affect macrophage polarization (292).

Th2 cells are involved in the defense against parasites and are pivotal in asthma and allergies, but its role in atherosclerosis is less clear. Their determining transcription factor is GATA3, and they produce IL4. In mouse atherosclerotic plaques, T cells express the Th2 associated cytokines IL4, IL5, IL10 and IL13 (249). However, whether these cells are pro- or anti-atherogenic remains unclear. Patients with more peripheral Th2 cells have lower atherosclerosis burden(293), and IL4 release from activated leukocytes negatively correlates with atherosclerosis (294). However, in a study among 2104 adults from the Multi-Ethnic Study of Atherosclerosis, an increase in circulating Th2 cells was found to be at an increased risk for incident stroke (295).

IL4 can antagonize Th1 responses and diminishes atherosclerosis in mice (296). Other studies show the opposite, i.e, that deficiency of IL4 is atheroprotective (297). Immunization of $ApoE^{-/-}$ mice fed a HCD with and apoB100 peptide increased IL4 expression in T cells but did not show any effect on atherosclerosis (298). Other Th2 associated cytokines were clearly athero-protective. In humans, plasma IL5 levels correlate with CVD (299, 300). Immunization of $Ldlr^{-/-}$ mice with modified LDL is atheroprotective, inducing Th2 skewed response with high production

of IL5 and IL13 (301). IL13 administration increases plaque collagen content and reduces plaque macrophage infiltration (302). IL33 induces the production of Th2 cytokines, reduces atherosclerosis in *Apoe*$^{-/-}$ mice by increasing IL4, IL5 and Il13 levels (303). Absence of IL33 had no effects on atherosclerosis (304, 305).

Th9 cells are poorly characterized. They produce IL9 after stimulation by TGFβ and IL4, and inhibition by IFNγ (305). IL9 levels are increased in patients with CAD, and higher in human atherosclerotic plaques than in the non-atherosclerotic vessel wall (306). IL9 administration in *Apoe*$^{-/-}$ mice exerted pro-atherogenic effects, by inducing VCAM1 expression on endothelial cells, which mediated inflammatory cell infiltration into atherosclerotic lesions (307). Recent work suggests fatty acids such as oleic acid promote differentiation of T cells towards a Th9 phenotype, providing a potential rational for their increased numbers in CAD patients (308).

Th17 cells are IL17 producing T-cells, that are defined by the transcription factor nuclear receptor RORγt(309). Th17 cells are activated by IL23 (310). In immune cells, and several non-immune cells, IL17 induces the secretion of IL6, gmCSF, and chemokines, all of which are pro-atherogenic (309). IL6 and TGFβ can induce a subtype of Th17 cells that produce both IL10 and IL17 (311, 312). The role of IL17 in atherosclerosis is still under debate (313). IL17A, the most well-known member of the IL17 cytokine family, has been reported to increase (314-316), decrease (317, 318) (319) or not affect (318) atherosclerosis burden. Other studies indicate that IL17 can promote plaque stability by increasing collagen 1 production in VSMCs (320). Both IL17A and its receptor have been reported to affect atherosclerosis dependent on the location within the arterial tree: IL17A- and IL17R- deficient atherosclerotic mice had less atherosclerosis in the aortic arch and aortic roots, but did not affect atherosclerosis in the thoracic aorta (321). Work with antigen (ApoB) specific $CD4^+$ T-cells suggests that IL-17 production by these cells increases with disease progression and comes at the expense of regulatory effects of T cells (280).

Also in clinical studies, the role of IL17 in atherosclerotic CVD is still unclear. Although 2 small studies showed that patients with unstable angina or acute MI have higher circulating IL17 levels, compared with patients with stable angina or healthy individuals (322, 323), this could not be confirmed in larger studies(324, 325). In these studies, IL17 levels did not associate with CAD (324), and low IL17 levels were associated with a higher risk of subsequent cardiovascular events in patients with MI (325). IL17 expression in human carotid atherosclerotic plaques was associated with a stable plaque phenotype with a lower macrophage content and a higher VSMC content (319, 326).

Th22 cells are characterized by the production of IL22, and their lack of IL17 and IFNγ (327), and use AHR as AHR as transcription factor. IL22 levels were found increased in patients with symptomatic atherosclerotic disease (328), and patients with acute coronary events have more Th22 cells in their circulation(329, 330). In *Apoe*$^{-/-}$ mice, IL22 stimulates medial VSMC migration, and transforms them into a synthetic phenotype, thereby aggravating atherosclerosis (331). IL22 can repress pro-atherogenic gut microbiota, which reduces atherosclerosis (332).

TFH (follicular helper) cells reside in B cell follicles and, together with B cells, maintain and form germinal centers. There, they interact with germinal center B cells and mediate anti-body isotype switching(333). TFH cells are defined by the transcription factor B-cell lymphoma 6 (BCL6).

TFH cells in atherosclerotic mice exhibit increased autoimmune responses compared to non-atherosclerotic mice (334). Marginal zone B cells inhibit the response of TFH cells, and limit atherosclerosis development and progression (335). TFH cell activity in germinal centers can be

regulated by a subset of $CD8^+$ T cells with regulatory function, which was considered defective in *Apoe*$^{-/-}$ mice (336). TFH cells are being maintained using the co-stimulatory ICOS (inducible T-cell co-stimulator)-ICOSL signaling. Absence of ICOS–ICOSL signalling in *Apoe*$^{-/-}$ mice reduces atherosclerosis burden, due to reduced numbers of T_{FH} cells in secondary lymphoid organs (337). Ageing increases the proportion of TFH cells in *Apoe*$^{-/-}$ mice, but not in wild-type mice (336). Interestingly, T_{FH} cells can derive from T_{reg} cells. These 'switched' T_{FH} cells are pro-atherogenic and their depletion reduces atherosclerosis in *Apoe*$^{-/-}$mice (337).

CD4$^+$*CD28*$^-$ *T cells* lack CD28 expression, the main co-stimulatory receptor of naive $CD4^+$ T cells. $CD28^-$ T cells are only found in humans and non-human primates and are pro-inflammatory and cytotoxic (338). Patients with acute coronary syndrome, but also patients with early atherosclerosis(339) have a higher number of circulating $CD28^-$ T cells than healthy individuals (340-342), and $CD28^-$ T cells from these patients are resistant to apoptosis (343). ScRNAseq also identified a cluster of CD4+CD28- cells in human atherosclerotic plaques, which had a cytotoxic transcriptional profile and contained increased levels of granzyme B in culture (199). Moreover, human unstable atherosclerotic plaques are invaded by clonally expanded T cells, including a large monoclonal population of $CD28^-$ T cells (342). Recent work suggests these cytotoxic CD4 T-cells may be derived from former regulatory T cells (Treg) which may indicate a self-reactive profile of these cells (344).

6.1.2 Regulatory T cells in atherosclerosis

Regulatory T cells are strong modulators of the immune system. They are essential for maintaining peripheral tolerance, preventing autoimmunity and limiting chronic inflammatory disease (345). Regulatory T cells are defined by the transcription factor FoxP3 and the ILR2A (CD25). Tregs have been shown to play a protective role in atherosclerosis. In *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mouse models, depletion of Tregs increases atherosclerosis (276, 346), whereas treatment with IL-2 complexes, as well as anti-CD3 antibodies increased Treg numbers and reduced atherosclerosis (347)(348-350). The protective effects of Tregs have been attributed to their secretion of TGFβ and IL10, both of which are able to reduce atherosclerosis and/or induce plaque stability in mice (351-353). In patients, low numbers of Tregs are correlated with coronary artery disease (354-356), albeit not in all studies (348), and not with carotid artery disease (348, 356).

Tregs are plastic and can acquire pro-inflammatory features, thereby becoming exTregs. In late-stage experimental atherosclerosis, Tregs start expressing both FoxP3 and T-bet and lose their immune-regulatory phenotype, while still retaining phenotypic similarities with Tregs (288, 357, 358). In a recent study, where Tregs and exTregs from Treg/exTreg lineage tracker ($FoxP3^{eGFP\text{-}Cre\text{-}ERT2}ROSA26^{GAG\text{-}fl\text{-}Stop\text{-}fl\text{-}tdTomato}$) $ApoE^{-/-}$ mice were submitted to RNAseq, exTregs were found to have a distinct gene signature compared to Tregs, characterized by upregulation of *Tbx21*, *Gzmk*, *Prf1*, *Nkg7*, *ifng* and *ccl4*. Based on this gene signature and Cite-seq data obtained from PBMCs from coronary artery disease patients, human exTregs could be identified as $CD3^+CD4^+CD16^+CD56^+$ cells and were found to be distinct from Tregs and NK cells. $CD3^+CD4^+CD16^+CD56^+$ cells were found to express inflammatory cytokines, chemokines, chemokine receptors and cytotoxic mediators. Human exTregs lost their suppressive capacities and acquired cytotoxic properties. Interestingly, human exTregs of patients suffering from

coronary artery disease express higher levels of inflammatory and cytotoxic genes that patients without coronary artery disease (344).

The activity of Tregs increases upon binding of their TCR to a cognate antigen. In atherosclerosis, a subpopulation of human and mouse T_{reg} cells was found to react with the ApoB-peptide (278). In early atherosclerosis in *Apoe*$^{-/-}$ mice, apoB-reactive T cells were found to have a Treg-like transcriptome, suggesting the most of the apoB reactive T cells are Tregs. However, throughout atherogenesis, these apoB-reactive Tregs gain mix Th cell signatures overlapping multiple lineage phenotypes with transcripts of Th1, Th2, Th17 and TFH cells. Transfer of these apoB reactive exTregs failed to protect from atherosclerosis(280). In patients without cardiovascular disease, >60% of ApoB-reactive CD4$^+$ T cells were true ApoB-reactive T_{reg} cells (278). In patients with subclinical atherosclerotic disease, the number of true ApoB-reactive T_{reg} cells decreased to 30% and the remaining FoxP3+ cells acquired Th1 and Th17 characteristics (278).

6.1.3 CD8$^+$ T cells in atherosclerosis

CD8$^+$ T cells recognize antigenic peptides that are presented by MHC-I on any nucleated cell. CD8$^+$ T cells mature into cytotoxic T cells that can kill virus-infected and abnormal cells, including tumor cells, using cytotoxic pathways. Patients with coronary artery disease have increased levels of cytotoxic CD8$^+$ T cells in blood compared with healthy individuals (359, 360) and CD8$^+$ T cells are abundant in atherosclerotic plaques in humans and mice (361-363) . TCR sequencing of T cells from human atherosclerotic plaques identified a subpopulation of CD8$^+$ T cells with an increased frequency, suggesting clonal expansion in the plaque (197, 364). In human coronary atherosclerotic plaques, clonal expansion of CD8$^+$ T cells was observed, with TCRs specific for viral epitopes, including influenza and corona virus epitopes (364), which have the potential to cross react with human protein. However, whether this expansion is indeed driven by atherosclerotic plaque antigens remains to be determined, as virus specific CD8$^+$ T cells are not phenotypically different from other CD8 T cells in the plaque (365). In another study, circulating CD8$^+$ T cells from CAD patients were found to have enrichment of TCR signaling pathways, as well as enrichment of cytotoxic and exhaustion pathways compared to controls without CAD (366). In contrast to CD4+ T-cells, antigen specificity of CD8+ T-cells in the lesion remains to be determined. In experimental studies, CD8$^+$ T cells have been shown to target a wide range of cell types in the plaque affecting smooth muscle cell differentiation (367) and killing endothelial cells and foam cells (368), but also T-cells and dendritic cells. Their wide range of effects has both pro-atherogenic and atheroprotective consequences. CD8$^+$ T cell depletion with antibodies reduced atherosclerosis in atheroprone mice (368-370) suggesting that CD8$^+$ T cells are pro-atherogenic. Other studies suggest that CD8$^+$ T cells can also have an atheroprotective role, for instance by reducing the number of Th1 cells accumulating in the lesion (371) or by eliminating Tfh cells in lymphoid organs (336). Induction of CD8$^+$ T cells through immunization may also mediate atheroprotective effects in atherosclerosis. Adoptive transfer of CD8$^+$ T cells mediate the atheroprotective effects of immunization with an ApoB-related peptide (p210) in *Apoe*$^{-/-}$mice (372). Adoptive transfer of CD8$^+$ T cells from p210-immunized mice reduced atherosclerosis in *Apoe*$^{-/-}$ mice compared with transfer of CD8$^+$ T cells from control mice, primarily through a reduction in dendritic cells (372). Aged atherosclerotic mice exhibit an increase in CD8$^+$GranzymeK$^+$ T cells (373). Depletion of CD8$^+$ T cells attenuated atherosclerosis in aged mice and transfer of aged CD8+ T cells significantly enhanced atherosclerosis in recipient mice lacking

$CD8^+$ T cells. Within the mouse atherosclerotic plaques, age associated $CD8^+$ T cells, including a granzyme K effector memory subset, had accumulated, and clonally expanded. These had a pro-atherogenic transcriptomic signatures of T cell activation, migration, cytotoxicity, and exhaustion, suggesting that aged $CD8^+$ T cells, and not young $CD8^+$ T cells are detrimental for atherosclerosis (374).

6.1.5 Natural killer T cells in atherosclerosis

Natural killer T (NKT) cells are a unique subset of T cells that recognize lipid antigens presented by the non-classical MHCI molecule CD1d (375). They can be divided into type I or invariant NKT (iNKT) cells, which have few variant TCRs, and type II NKT cells, which have more variable TCRs. Activation of iNKT cells results in the rapid release of T_H1, T_H2 and T_H17 cytokines, depending on the expression of the same transcription factors that define the corresponding T_H cell subtypes: T-bet, GATA3 and RORγt, respectively (376). Like $CD8^+$ T cells, iNKT cells can also express the cytotoxic proteins perforin and granzyme B (376). Most studies using *Apoe*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice suggest that iNKT cells are pro-atherogenic (376-384). iNKT cells are thought to promote atherosclerosis by secreting cytokines, which can activate other immune cells present in the atherosclerotic lesion (376-384). These results could not be reproduced in human CVD disease. High levels of iNKT cells were associated with a decreased risk of incident coronary events in patients (385). The role of type II NKT cells in atherosclerosis is unknown.

6.1.6 γδT cells in atherosclerosis

γδT cells, in contrast to the αβ T cell, do not recognize specific antigens (386). Increased amounts of γδT cells are present in the human and mouse atherosclerotic plaque (387, 388), and produce IL17 and IL23 (388, 389). Hyperlipidemic mice deficient in γδT cells mice showed a decrease in early plaque formation (388, 389). Another study failed to see an effect of γδT cells in early atherosclerosis (390). In patients with CAD, the number of γδT cells, as well as its activation markers had decreased (391). However, in the MESA and Cardiovascular Health Study, the amount of γδT cells was not correlated with angina pectoris or myocardial infarction (392). The exact role of this T cell subset in atherosclerosis is unclear.

6.1.7 T-cells in the single cell era

The emergence of single cell analyses has resulted in an increased interest in the role of T cells of atherosclerosis. T cells were found to represent >50% of all immune cells in both mouse and human atherosclerotic plaques and were found to outnumber the plaque macrophages in these analyses. The T cell repertoire, based multi-parameter surface markers, as well as single cell transcriptomes, is very heterogeneous, and has changed the demarcations between our well-defined subsets such as Th1/2/9/17/22, Tregs and cytotoxic T cells.

Single cell RNAseq and CyTOF analysis of atherosclerotic mouse aortas revealed 3-10 different T cell subsets (138, 201, 249, 393), and can generally be subdivided into multiple activating states of CD4+ and CD8+ T cells, depending on the granularity of the analysis. Next to these subsets, murine plaques contain thymocyte like cells ($CD4^+/CD8^+$ cells) and a Treg cluster (201). Further analysis revealed that peripheral T cell tolerance had deteriorated during atherosclerosis, which was most pronounced within the plaque, as well as the artery tertiary lymphoid organs. Clonal expansion of CD4+, CD8+ and Tregs could be observed, as well as transcripts associated with T cell exhaustion, Treg-Th17 conversion, and dysfunctional antigen presentation (393). In aged *Ldlr*$^-$

$^{-/-}$ mice, whose plaques show more similarities with human plaques, then number of T-cells exceeded that found in young, high cholesterol fed ldlr$^{-/-}$ mice, and show more diversity, including the appearance GrzK^{+}CD8^{+} T-cells and a more prominent representation of exhausted T cells (373).

In human atherosclerotic plaques, the plaque T cell population was even more diverse reporting three (394), four (198), five(199) and thirteen (197) CD4^{+} T cell subsets and two (394), three, four (198, 199) and eight (197) CD8^{+} T cell subsets, depending on the numbers of cells required and analysis methods. In plaques, CD8^{+} T cells are more abundant than CD4^{+} T cells, virtually all T cells are of an effector and/or memory type, and a significant proportion of T cells display signs of exhaustion. Based on transcriptional profiling and clustering, one can define CD4^{+} Tregs, CD4^{+} central memory cells, CD4^{+} effector memory cells, exhausted CD4^{+} T cells, and terminally differentiated CD45RA expressing T cells (Temra) (277). In the CD8^{+} population, one can define effector memory cells, IFN response T cells, cytotoxic effector T cells, Temra, and a T cell population with a 'mucosal associated invariant T cell' signature(198) (Figure 7). These novel data have provided great insights into the heterogeneity of the T cell, characteristics of T cell subsets and their putative functions.

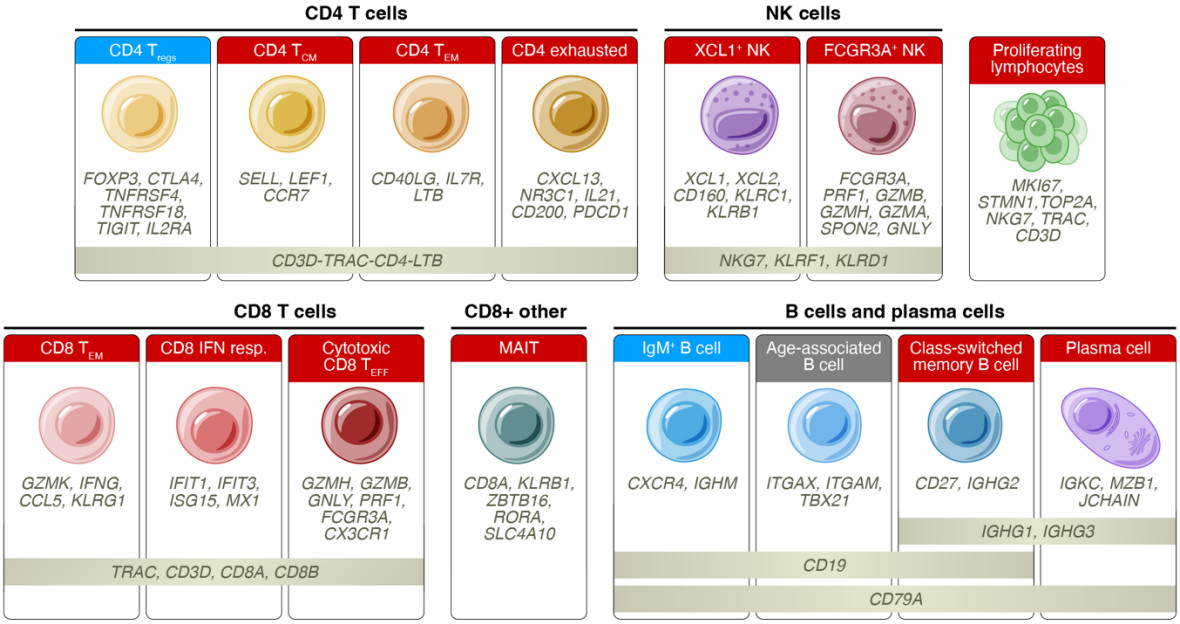

Figure 7. Lymphoid cell subsets in atherosclerosis

Lymphoid cells in the atherosclerosis plaque can be broadly divided into CD4 T-cells, CD8 T-cells, B cells and NK cells, however single cell sequencing has revealed a variety of subsets within these lineages. Apart from regulatory T-cells (T_{regs}), CD4 T cells are not subdivided along the classic T-helper paradigm, but appear to exist in various stages of activation; ranging from a naïve like- central memory CD4 T-cells (T_{CM}) to CD40L+ effector memory CD4 T cells (T_{EM}) and ultimately, exhausted CD4 T cells. CD8^{+} T cells consist of highly cytotoxic effector cells (T_{EFF}), which express cytolysis related genes encoding Granzyme B (*GZMB*), Granulysin (*GNLY*) and perforin (*PRF1*), but also contain terminal differentiation (*GZMK*) or senescence associated subsets (T_{EM} and CD8 IFN response). Mucosal-associated Invariant T-cells (MAIT) and Natural Killer (NK) cells make up minor populations, although 2 distinct NK cell population can be distinguished, with one population bearing markers of cytolysis (FCGR3A+ NK cells) and a second population expressing chemotaxis related genes (XCL1^{+} NK). Finally, B-cells make up about 10% of the lymphocyte population in the atherosclerotic plaque, but present various stages of B cell maturation, including unswitched IgM^{+} B-cells, class switched (IgG^{+}) memory B cells and antibody producing plasma cells. The enigmatic age-associated B cell is defined by co-expression of genes translating CD11c (*ITGAX*), CD11b (*ITGAM*) and T-bet (*TBX21*).

The first data have come out showing an association of several of these subsets with the occurrence of cerebrovascular disease. Within the $CD4^+$ T cell cluster, Fernandez et al. identified 4 transcription-based clusters that showed differential expression between plaques of symptomatic and asymptomatic patients (197). Remarkably, $CD4^+$ T cells in plaques from asymptomatic patients were highly enriched for Type I IFN and IFNG transcripts, and the IL1 and IL6 signaling pathway. The $CD8^+$ T cells in plaques of asymptomatic patients had a similar activation profile than the $CD4^+$ T cells but were also characterized by increase expression of chemotactic and cytotoxicity genes, as well as exhaustion genes (197). In another study, $CD4^+FoxP3^+$ Tregs were found to have acquired Th17 and Th9 features and were, together with $CD4^+NR4A1^+$ and $CD8^+SLC4A10^+$ T cells, associated with cerebrovascular events (395).
Additional studies investigating the relevance of these subsets in atherogenesis, their association with clinical features, as well as their drug-targetability are needed.

6.2 B cells

B cells are mostly known for their role in the adaptive immune system through secretion of antibodies, cytokines and their contribution to T cell activation, but also have innate immune cell type functions. As such, B cells play a key role in responses to pathogens but can also mediate chronic autoimmunity. Over the past few decades, we have gained important insights in B cell heterogeneity and function in atherosclerosis. Using preclinical atherosclerosis models and CVD patient material, different B cell subtypes have been identified, with each a distinct role in the progression of atherosclerosis. The surface marker expression profile, location and functionality of B cells show both similarities as well as some diversity between mice and human.

6.2.1 Murine B cell subsets

Murine B cells are generally categorized into B1 and B2 subsets, based on unique characteristics and cell surface markers(396). B1 cells develop in the fetal liver, predominantly inhabit serosal cavities, and undergo self-renewal in the periphery (397). Some B1 cells reside in spleen and bone marrow where they primarily produce IgM antibodies in a T cell-independent (TI) manner (398, 399) (400), serving as the innate arm of adaptive immunity. Murine B1 cells are subdivided into B1a and B1b cells, based on CD5 expression with B1a cells being $CD5^+$ and B1b cells $CD5^-$ (401, 402), and can be activated by TI-1 antigens (i.e., microbial TLR ligands) and TI-2 antigens (i.e.,. bacterial antigens that crosslink BCRs). B2 cells are more abundant, and originate in bone marrow, where they develop into immature B cells through Ig heavy and light chain rearrangement (403). Immature B cells then exit the BM and inhabit secondary lymphoid organs (SLOs), where they differentiate into marginal zone (MZ) B cells or follicular (FO) B cells where they complete their maturation (403, 404). The majority of B2 cells are FO B cells that are activated in an antigen-specific T cell dependent manner in the germinal center (GC), where they undergo clonal expansion and somatic hypermutation. This enables them to produce highly antigen-specific antibodies and generate IgG, IgE or IgA antibodies by class switching. GC B cells differentiate into antibody-secreting plasma cells or memory B cells (403). Other B cell subtypes regulate inflammatory reactions through cytokine production. Regulatory B cells (Breg) produce cytokines such as IL10, which suppresses T helper cells, inhibits macrophage antigen presentation and pro-inflammatory cytokine production (405).

6.2.2 B cells and atherosclerosis in mice

Pre-clinical studies revealed that B cells regulate disease progression in a subset-dependent manner (270, 406-411) (Figure 8). Caligiuri et al. showed that splenectomized *Apoe*$^{-/-}$ mice display exacerbated atherosclerosis, a phenotype that could be rescued by reintroducing of B cells (270). Accordingly, Major et al. demonstrated that genetically-induced B cell deficiency in *Ldlr*$^{-/-}$ mice resulted in a loss of anti-oxidized LDL antibodies and a subsequent 30-40% increase in atherosclerotic lesion area (406). These two studies suggested a potential protective role for B cells in atherosclerosis. In line with these findings, it was shown that mice that had deficient IgM secretion had increased atherosclerosis (412-414). B cell depletion studies with an anti-CD20 antibody or B-cell activating factor receptor deletion (BAFFR$^{-/-}$), that mostly affect the B2 cell resulted in decreased atherosclerosis compared to control mice despite comparable serum cholesterol levels (407, 409, 410, 415). Mechanistic studies revealed that B2 cells promoted atherosclerosis via production of pathogenic IgG, activation of T cells, and induction of pro-inflammatory cytokines such as IFNγ, TNFα, IL1β and chemokines such as MCP-1 (407, 409, 410, 415). Furthermore, murine B2 B cells produce IgG antibodies that bind oxidation specific epitopes (OSE) and stimulate inflammation, at least in part through the Fc-γ receptor (416-418).

Adoptive transfer experiments using different B cell subsets confirmed a subset dependent role of B cells on atherogenesis. B2 cells drive progression of atherosclerosis (418, 419) whereas B1 cells provide protection (419, 420). B1 cells exert their protective function through multiple mechanisms. B1 cells secrete IgM that binds OSE on LDL, preventing lipid uptake and inflammatory cytokine production by macrophages. This reduces the formation of foam cells and inflammation involved in atherosclerosis development. In addition, B1 cell derived IgM facilitates clearance of apoptotic cells by binding to epitopes on their cell surface, hereby limiting inflammation (421). Remarkably, innate response activator (IRA) B cells, derived from B1a cells, are considered pro-atherogenic. IRA B cells are induced by LPS to produce GM-CSF, promoting extramedullary hematopoiesis in the spleen and atherosclerosis (400, 422-424) .

Aging induces functional impairment in the B-cell compartment(425, 426). Aging of *Ldlr*$^{-/-}$ mice on standard laboratory diet showed a significant accumulation of age-associated B cells (ABC) which are characterized by expression of CD11c and/or CD11b, in the atherosclerotic aorta (373, 427). Similarly, CD11c expressing B cells were increased in spleen, blood and bone marrow of *Apoe*$^{-/-}$ mice upon aging, concomitant with an increase in aortic plaque size (428). In vitro assays and single cell RNAseq analysis on atherosclerotic plaques from aged *Ldlr*$^{-/-}$ mice demonstrated that ABCs are highly potent antigen-presenting cells and are enriched in genes involved in co-stimulation, inflammation and plasma cell differentiation(373, 427). The presence of ABCs has been associated with several autoimmune diseases, including rheumatoid arthritis (429), multiple sclerosis (430), and systemic lupus erythematosus (431), where they may contribute to disease progression by production of pro-inflammatory cytokines and high levels of autoantibodies. Although the exact contribution of ABCs to atherosclerosis remains to be elucidated, they could potentially drive inflammation and produce atherogenic IgG. Besides ABCs, aged atherosclerotic mice showed an increase in Bregs (373), whose role in atherosclerosis is still not clear. Multiple studies have shown that Bregs can exert a protective role in atherosclerosis via secretion of IL10 (432-435). However, chimeric *Ldlr*$^{-/-}$ mice with a B cell-specific deficiency in IL10 did not display altered atherosclerosis development(408).

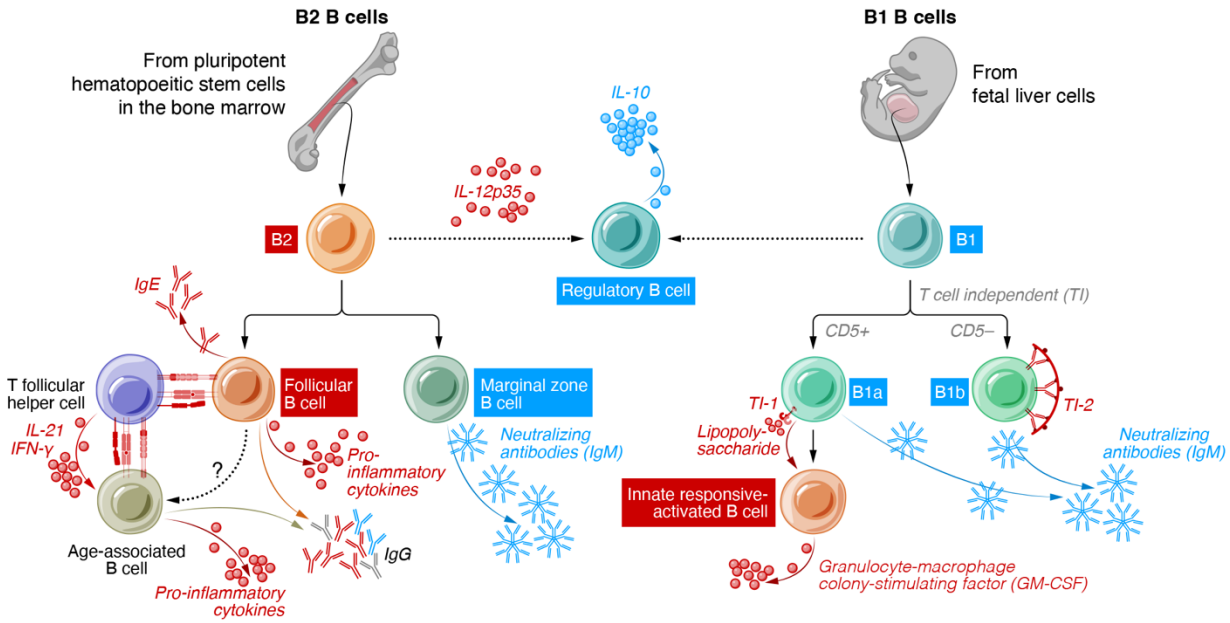

Figure 8. Murine B cell subsets. Conventional B2 B cells develop in the bone marrow and reside in follicles or in the marginal zone. B2 cells can differentiate into marginal zone B cells which can rapidly produce antibody, particularly IgM in response to circulating antigens. B2 cells can also become follicular B cells and produce high affinity, class switched, immunoglobulins in a follicular T cell (Tfh) dependent manner. In addition, Tfh cells can contribute to the activation of age-associated B cells (ABCs), characterized by the expression of CD11b, CD11c and the transcription factor T-bet. ABCs are potent antigen-presenting cells and can secrete cytokines and antibodies that may contribute to disease development. B1 B cells develop in the fetal liver and are subdifferentiated by surface CD5 expression into $CD5^+$ B1a cells and $CD5^-$ B1b cells. B1a cells are activated by T cell independent type 1 (TI-1) antigen through toll like receptor (TLR) signaling secreting IgM natural antibodies (NAb). B1b cells are additionally activated by T cell independent type 2 (TI-2) antigen through the B cell receptor. Activated B1a cells under inflammatory conditions can become innate response activating (IRA) B cells that secrete GM-CSF. Regulatory B cells (Bregs) can be derived from B2, B1 or possibly a unique B10 lineage. They are defined by IL-10 secretion and immune suppression.

6.2.3 B cells and atherosclerosis in humans

Several B cell subsets are considered to play a major role in human atherosclerosis (Figure 7). Analysis of blood gene expression profiles, single nucleotide polymorphisms (SNP) and Bayesian network analysis constructed from data of 188 coronary heart disease cases and 188 age- and sex-matched controls from the Framingham Heart Study (FHS) identified B cell genes and B cell-centered immune function to predominate in coronary artery disease (436).

Likewise, many studies report an association between coronary artery disease (CAD) and B cell-derived antibodies to OSE on LDL (437-442). Consistent with murine studies demonstrating B1 cell-derived IgM attenuated, and B2-derived IgG promotes atherosclerosis, IgM to OSE was inversely and IgG to OSE was positively associated with coronary stenoses >50% in a cohort of 504 patients (439). Moreover, IgM and IgG autoantibodies and immune complexes could modify risk prediction for CVD (438), predicted incident CVD (ischemic stroke, myocardial infarction, new-onset unstable angina, acute coronary interventions, and vascular death) (440), independently associated with time to incident MACE (441) and were associated with unstable plaque features on intravascular ultrasound IVUS (442).

Likewise, low IgM to native and MDA-modified versions of a specific ApoB100 peptide (p210) were associated with CAD events. However, these studies reported low levels of IgG to p210 and

p45 in CAD events (443, 444). Low levels of IgG to native p210 but not IgM to MDA-modified p210 were inversely associated with severity of CAD and MI risk (445), suggesting a role for different isotypes and/or idiotypes in atherosclerosis. As in murine models, evidence for a protective role for IgM to OSE in human atherosclerosis is more consistent than the evidence for IgG.

Identification of the human B cell that produces antibodies that impact on atherosclerosis has been challenging. While the B cells producing these antibodies in mice have been well described, translation of these murine findings to humans has been challenging (446-449) likely due to differences in surface markers and functional responses (450). Recent advances in single cell analytics, has allowed better characterization of B cell subtypes. An integrated multi-omics single cell atlas of human B cells identified 12 unique circulating human B cell clusters revealing cluster-specific metabolic, biosynthetic and immune signaling activity (451). Multi-omics characterization of B cell subtypes in lymphoid and mucosal tissues revealed spatiotemporal aspects of human memory B cells and marginal zone (MZB) B cells, both of which can transit through germinal centers, acquiring somatic mutations to foster antigen specificity (452-454). Characterization of human B cell subtypes that produce antibodies that modulate atherosclerotic disease is needed to allow the development of B cell-targeted therapeutic strategies.

Although B1 cells have been defined to be the major source of IgM in mice, identification of the human equivalent has been elusive. A putative human B1 cell has been identified by sort-purifying B cell fractions and testing for key murine B1 cell characteristics (spontaneous IgM secretion, efficient T cell stimulations, and tonic intracellular signaling) (455). B cells expressing $CD20^{+}CD27^{+}CD43^{+}$ fulfilled these criteria. $CD20^{+}CD27^{+}CD43^{+}$ "B1" cells produced IgM to modified phospholipids linked to atherosclerosis (455, 456).

A higher frequency of $CD27^{+}CD43^{+}$ B1-like cells in circulation was associated with fewer secondary cardiovascular events, defined as cardiovascular death, stroke, MI, coronary intervention, and peripheral intervention following carotid endarterectomy (457). While IgM production and reduced can be linked to these $CD27^{+}CD43^{+}$ B1-like cells, study of the chemokine receptor CXCR4 demonstrated that the amount of CXCR4 on the surface of these cells provided stronger correlation with atheroprotective IgM antibodies specific for malondialdehyde (MDA)-modified LDL. Moreover, the amount of CXCR4 on $CD20^{+}CD27^{+}CD43^{+}$ B1-like cells was inversely associated with plaque burden and unstable features as measured by intravascular ultrasound (IVUS)(414). In support of these associative human findings, gain and loss of function studies in mice demonstrated that CXCR4 expression on B1a cells induced migration to bone marrow, enhanced production of IgM to MDA-LDL (414) and loss of CXCR4 on B cells increased atherosclerosis in female mice (458).

B2 cells develop in the bone marrow, move to lymphoid organs and transition into mature naïve B cells in follicular regions, or differentiate to become MZB, memory B cells or antibody producing plasma cells. In germinal centers within secondary lymphoid organs, antigen stimulated B2 cells undergo affinity maturation through somatic hypermutation, and isotype switch to produce highly specific isotype class switched antibodies. In the early antibody response, plasmablasts are rapidly produced but are short lived (459). In humans, increased plasmablasts are associated with atherosclerosis (460). The plasmablasts then further develop into plasma cells, which secrete much higher levels of antibodies, including high-affinity IgG (461). This IgG has been shown to correlate with coronary artery stenosis in some human studies (439, 462, 463).

In addition to IgG antibodies, B2 cells can also class switch to IgE. Evidence linking IgE to human atherosclerosis includes the finding of increased coronary atherosclerosis in patients with hyper IgE syndrome (464, 465), studies showing association of serum IgE levels with coronary artery disease (466, 467). Murine studies have shown that IgE promotes atherosclerosis (468-470). The precise identification of an allergen inducing the IgE response has been more elusive. IgE to the mammalian oligosaccharide galactose-α-1,3-galactose (a-gal) is associated with increased atheroma volume and plaques with unstable characteristics as measured by intravascular ultrasound (IVUS) in a cohort of 118 patients (471). IgE to a-gal is induced by tick bites and can lead to allergy to mammalian products like beef, a syndrome called the a-gal syndrome (AGS). IgE sensitization to a-gal was independently associated with noncalcified plaque burden and obstructive CAD in their cohort of 1056 patients and occurs at higher frequency in patients with STEMI than those with stable or no CAD. Plasma levels of IgE to a-gal are associated with an increased frequency in CCR6+ switched memory B cells both in subjects with increased CAD (472) and those with AGS (473). IgE to common food allergens was found to be associated with cardiovascular mortality in the National Health and Examination Survery (NHANES) and the Multi-Ethnic Study of Atherosclerosis (MESA)(474). These intriguing findings need murine validation for causality but may represent a new tick-induced risk factor for CVD.

B2 cells can also differentiate into memory B and MZB cells. An unbiased approach to identify the atheroprotective IgM-MDA-LDL-producing B cell in humans resulted in the identification of $CD27^{+}IgM^{+}CD24^{hi}$ cells, that were identified a circulating MZB cells (428). Consistent with these human findings, Nus et.al. had shown murine MZB to be a source of IgM to OSE (335, 475). Unlike mice, B2-derived MZB are a major source of IgM-MDA-LDL antibodies.

ABCs, identified by low cel surface expression of CD21, and high expression of CD11c, CD11b and co-expression of the transcription factor T-bet, are present in the circulation and carotid plaques of CVD patients (373), and subsets of $CD11c^{+}$ B cells showed an association with severe atherosclerosis in humans (428), warranting further research into this B cell subset.

Although cell surface markers for murine Bregs have been identified, markers to identify human Bregs remain unclear (476, 477). Although decreased serum levels of IL10 have long been associated with human cardiovascular disease (478, 479), it is unclear whether IL10 produced Bregs cells is sufficient to attenuate atherosclerosis, although patients with a history of atherosclerotic events had lower levels of $IL10^{+}$ B cells (460).

7. THE IMMUNOLOGICAL SYNAPSE

The innate and adaptive immune system are joint in the immunological synapse (Figure 9). Antigen presenting cells, except for the B-cell, are part of the innate immune system and include dendritic cells and macrophages. APCs express antigenic peptides on their MHCI or MHCII complex, that are recognized by T cell receptors (TCRs) of $CD4^{+}$ and $CD8^{+}$ T cells. Antigens known to play a role in atherosclerosis include ApoB, HSP60, MDA, and PC (234). The MHC-TCR signal is known as signal 1 induces both T cell and APC activation. Following recognition of an antigen by the T cell receptor (TCR) a second signal, 'signal 2' is required to propagate the MHC-TCR response. 'Signal 2' is provided by co-stimulation and boosts (and in some cases dampen) T cell proliferation, activation, and polarization, whereas co-inhibitory molecules counteract these responses(273, 480). A third signal provided by cytokines present in the microenvironment can

influence skewing of naïve T cells towards different T-helper cell subsets. Following successful APC-T cell activation, a plethora of chemokines and cytokines are released to drive or dampen the initiated immune response.

The immunological synapse is a strong regulator of atherosclerosis development and progression. The main players in the synapse, such as MHCI and MHCII, TCR as well as co-stimulatory and coinhibitory molecules are present in both human and murine atherosclerotic plaque tissue, and lymphoid organs (481). In a recent study, Duran et al. found that almost all members of the co-stimulatory and co-inhibitory immune checkpoint family can be detected on few or multiple immune cell subsets that reside within the atherosclerotic plaque (481). Both plaque specific and well as lymphoid organ-based actions of the immunological synapse have proven to play an important role in atherosclerosis (96).

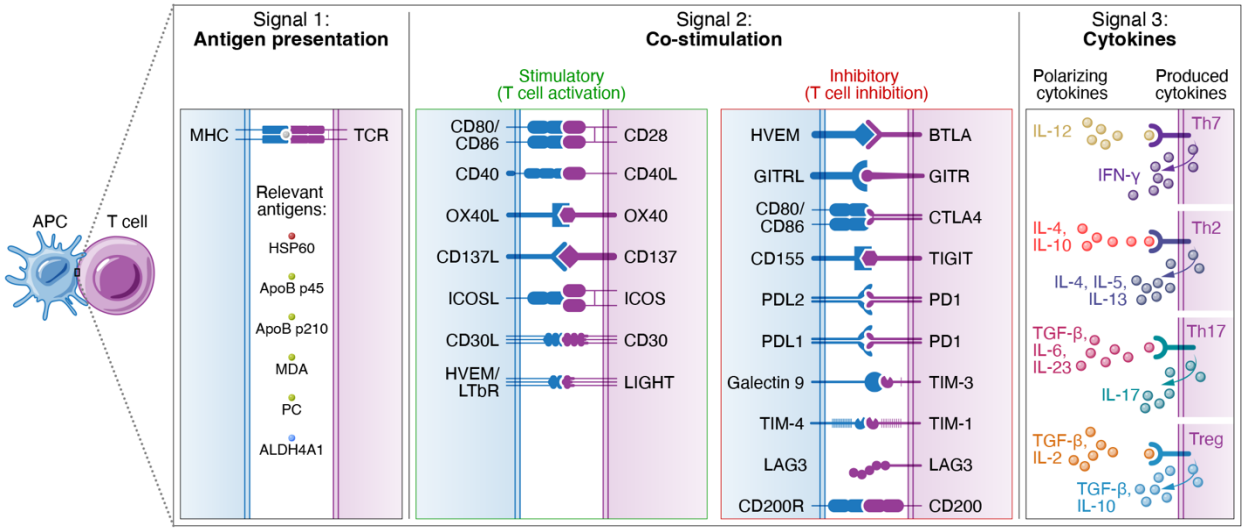

Figure 9. The immunological synapse in atherosclerosis. Antigen-presenting cells (APCs) can mediate T cell activation, proliferation and polarization through three different signals. Signal 1 consists of presentation of atherosclerosis-relevant antigens, including HSP60, ApoB, MDA-LDL, PC, ALDH4A1, on MHC molecules on APCs to the T cell receptor (TCR) on T cells. In addition to antigen recognition, T cells also require co-stimulation for subsequent activation. Such co-stimulatory signals are provided by immune checkpoint proteins, which alternatively may also elicit inhibitory signals that can halt T cell proliferation or induce skewing towards anti-inflammatory immune cells. A third signal is provided by cytokines present in the atherosclerotic microenvironment, which can influence T cell polarization towards for instance IFNγ-secreting Th1 cells. HSP = heat-shock protein, MDA = malondialdehyde, PC=phosphocholine, ALDH= aldehyde dehydrogenase.

7.1 Co-stimulation and co-inhibition in atherosclerosis: immune checkpoints

Immune checkpoints are categorized into 2 major families: the Immunoglobulin Superfamily, which includes both co-stimulatory and coinhibitory immune checkpoint dyads, and the tumor-necrosis factor (TNF)-receptor super family, which primarily exist of co-stimulatory immune checkpoint proteins, though some exhibit co-inhibitory functions (482, 483). The Immunoglobulin Superfamily encompasses CD28-CD80/CD86, CTLA4-CD80/86, PDL1/2-PD1, LAG3, ICOS-ICOSL, BTLA, CD200, CD155, TIM3, and VISTA. The TNF Receptor Superfamily includes

pairs such as CD40L-CD40, OX40-OX40L, CD27-CD70, GITR-GITRL, CD137-CD137L, CD30-CD30L, LTβR-LTα/β, RANK-RANKL, Fn14-TWEAK, and LIGHT-HVEM/LTβR. While each immune checkpoint receptor-ligand pair has a distinct, often cell-type-specific function, a general rule is that co-stimulatory checkpoints enhance inflammation, whereas co-inhibitory checkpoints suppress it. Numerous preclinical studies have demonstrated the involvement of both types of immune checkpoints in various aspects of atherogenesis (481, 484).

7.1.1 Co-stimulation

On of the most investigated co-stimulatory dyads in cardiovascular disease is the *CD40-CD40L* dyad, members of the TNF(R) superfamily. CD40L is primarily expressed on T cells and platelets but is also found on endothelial cells, vascular smooth muscle cells, mast cells, and natural killer cells. CD40 is present on antigen-presenting cells (APCs) such as B cells, dendritic cells, and macrophages, but can also be found on neutrophils, eosinophils, basophils, platelets, endothelial cells, vascular smooth muscle cells, fibroblasts, adipocytes, and epithelial cells. The binding of CD40L to CD40 during T cell-APC interactions drives T cell effector responses, dendritic cell maturation, and B cell antibody production with Ig-isotype switching (485), while in other cell types, this interaction promotes platelet-leukocyte aggregation (486), leukocyte adhesion to the endothelium (487), and macrophage activation (488). In patients, increased levels of soluble CD40L and (soluble) CD40, and/or single nucleotide polymorphisms in the CD40 gene correlate with the presence of CVD, including recurrent myocardial infarction and stroke (489-491). In murine models, Genetic and/or antibody mediated inhibition of CD40L or CD40 effectively reduces atherosclerosis. Plaques were not only smaller, but also rich in collagen and housed a limited number of immune cells, resembling stable, clinically safe, atherosclerotic lesions (487, 492-495). The actions of CD40 and CD40L are cell-divergent, also in atherosclerosis. Selective depletion of CD40L on all T cells significantly reduces plaque load by diminishing (Th)1 responses, resulting in reduced IFNγ levels (496). Besides T cells, CD40L is abundantly expressed on platelets. When platelets are CD40L deficient, no effects on atherosclerosis can be observed (496). However, deficiency of platelet CD40L ameliorates atherothrombosis induced plaque growth (496), and prevents acceleration of atherosclerosis that is induced by thrombin-induced platelet activation (486). Deficiency of CD40 on dendritic cells mirrors T-cell CD40L deficiency, with a reduction in atherosclerosis due to a deficient Th1 response (496). Macrophage CD40-deficiency reduces atherosclerosis and necrotic core formation. CD40-deficient macrophages have anti-inflammatory properties and exhibit enhanced efferocytosis, without affecting Th1 responses (488). Deficiency of macrophage CD40 has no effect on fibrous cap formation (488). Deficiency of endothelial cell CD40 increases atherosclerotic plaque stability (497), and deficiency of adipocyte CD40 decreased atherosclerotic plaques size, but increased the number of lymphoid and myeloid progenitors in the bone marrow, and resulted in an increase in T-cell numbers in the plaques, and increased necrotic core sizes (498). Absence of platelet CD40 reduces atherosclerosis, by affecting platelet induced leukocyte recruitment (499). These data highlight the cell-divergent function of CD40L-CD40 interactions in atherosclerosis. These findings highlight the complex, cell-type-specific roles of CD40L-CD40 signaling in atherogenesis, underscoring its potential as a therapeutic target for modulating plaque composition and disease progression.

Other TNF-(receptor) family members that have been shown to drive atherosclerosis are *GITR-GITRL*, *CD27-CD70*, *CD30-CD30L* and *OX40-OX40L*. GITR is a marker for regulatory T cells, but it exerts different functions. It can enhance Treg's suppressive capacities and facilitate Treg proliferation(500, 501), but it also can enhance effector T cell function (502). Overexpression of T cell GITR, induced by GITRL-overexpressing B cells reduces atherosclerosis by Treg expansion (503). GITR is also expressed by monocytes and macrophages (504-507). Activation of GITR in human and murine macrophage cell lines as well as primary monocytes/macrophages induces release of cytokines (TNFα, IL8, MCP1), and MMP9 (504-506). In atherosclerotic plaques, GITR is expressed on macrophages, T cells, endothelial cells and SMCs. The abundance of GITR expression in the plaque and circulating sGITR levels are associated with plaque vulnerability and the occurrence of cerebrovascular events (508). Global deficiency of GITR in a mouse model of atherosclerosis reduced monocyte recruitment to the arterial wall and prevented macrophage activation and necrotic core formation, thereby reducing atherosclerosis (508). *CD27*, expressed on many immune cell types, has a protective effect on atherosclerosis, due to its role in Treg function(509). The CD30-CD30L co-stimulatory pair is involved in activation and proliferation of adaptive immune cells. Blockade of this pathway in Ldlr-/- mice reduced atherosclerosis development (510). Interruption of OX40L, the ligand of OX40, which was found the be present in the CVD risk locus, reduces atherosclerosis and facilitates plaque regression by affecting both T and B cell responses(511, 512).Moreover, scRNAseq data revealed high expression of OX40 on effector and memory T cells, including Tregs, residing in the atherosclerotic plaque of CVD patients (199).
An important co-stimulatory dyad of the immunoglobulin superfamily is CD28-CD80/CD86, which is in an intricate balance with the coinhibitory CTLA4-CD80/86 dyad. CD80 and CD86 are expressed on antigen presenting cells, whereas CD28 and CTLA4 are restricted to T cells. CD28-CD80/86 interactions propagate T cell responses and promote survival and memory cell formation of $CD4^+$ and $CD8^+$ T cells. CTLA4 limits T cell activation, and promotes Treg expansion and suppression(513). CD80/86-deficient $ApoE^{-/-}$ mice show diminished T effector cell responses and a decrease in plaque area (514). Chimeric *CD80/CD86*$^{-/-}$*LDLr*$^{-/-}$ mice show aggravated atherosclerosis, due to a reduction in regulatory T cell numbers (276). The CD28/CTLA4-CD80/CD86 system thus has divergent ways to modulate immune responses and determine atherosclerosis progression or reduction. Overexpression of the co-inhibitory CTLA4 reduced atherosclerotic burden and plaque inflammation, and aneurysm formation in *Apoe*$^{-/-}$ mice, due to a decrease in effector T cells(515, 516). Antibody mediated inhibition of CTLA4 aggravated atherosclerosis and, with plaques that were enriched in T-cells and had large necrotic cores (517). Expression of CD80/CD86 in human plaques is correlates with a vulnerable plaque phenotype (518) and a bioinformatic study links CD86 to immune cell activation in human atherosclerosis (519). Patients suffering from coronary artery disease and those at risk of stroke contain higher expression of CD80 and CD86 on monocyte-derived dendritic cells and B cells (520).

7.1.2 Co-inhibition

One of the most powerful co-inhibitory receptors is PD1, which binds to PDL1/2. Both belong to the immunoglobulin superfamily, and they are important immunotherapeutic targets to induce $CD8^+$ mediated killing of tumor cells in cancer. PD1 is expressed on T cells, whereas its ligands, PD-L1 and PD-L2 can be found on many cell-types including APCs, endothelial cells and tumor cells (521). PD1 and its ligands can modulate atherogenesis. Absence of PD-L1/2 as well as antibody

treatment with PD1 antagonists in atherosclerotic mouse models increases atherosclerosis. The arterial wall contained excessive amounts of $CD4^+$ and $CD8^+$ T cells into the arterial wall (522, 523). Combination therapy of a PD1 inhibitor, and a CTLA4 inhibitor in *Ldlr*$^{-/-}$ mice, resulted in a vulnerable, pro-inflammatory atherosclerotic plaque phenotype. This phenotype was caused by an excess of effector T-cells that resulted in endothelial activation and increased recruitment of $CD8^+$ T-cells within the atherosclerotic lesion (524). *Ldlr*$^{-/-}$ mice that were treated with an agonistic PD1 antibody had less atherosclerosis. PD1 agonism caused a beneficial inflammatory milieu with less IFNγ-producing $CD4^+$ effector cells and cytotoxic $CD8^+$ T cells, an increase in regulatory $IL10^+$ CD4 T-cells and Bregs, supporting the potential of stimulating PD1 as a strategy to reduce atherosclerosis (525). In addition, adoptive transfer of PD-L1 expressing B cells inhibited collar-induced atherosclerosis in *Apoe*$^{-/-}$ mice by inhibition of T follicular helper cell responses (526).
PD1 and PD-L1 was reduced on T-cells and dendritic cells in patients with CVD, which was associated with increased T cell activity (527). High levels of PD1 were observed on plaque T-cells, which was associated with T-cell cell exhaustion pathways, occurring after T-cell activation (197). sPD-L1 levels are increased in patients with coronary artery disease (528, 529).
CD200-CD200R interactions protect against atherosclerosis. CD200R limits monopoiesis, monocyte recruitment and macrophage activation in experimental atherosclerosis. CD200R is expressed on classical monocytes of ASCVD patients and associates with a lower burden of coronary artery disease and a stable plaque phenotype (530).
Like any other co-inhibitory molecules, also TIM-1, TIM-3, TIM4, as well as BTLA can protect against atherosclerosis (435, 531-533). TIM proteins are structurally characterized by an extracellular immunoglobulin V domain and a mucin-like domain, in addition to a transmembrane domain and an intracellular cytoplasmic tail. While TIM-1 and TIM-3 are expressed on B- and/or T cells, TIM-4 is mostly expressed on macrophages and dendritic cells. TIM-1 can interact with TIM-4, while galectin-9 is the binding partner of TIM-3. TIM proteins can also recognize phosphatidylserine (PS) on apoptotic cells, aiding in their clearance. Monoclonal antibody blockade of TIM-1 (clone 3D10), TIM-3 (clone RMT3-23) or TIM-4 (clone 21H12) in *Ldlr*$^{-/-}$ mice significantly enhanced atherosclerosis development and associated inflammation (531, 532). In contrast, treatment of *ApoE*$^{-/-}$ mice with another TIM-1 antibody (RMT1-10) attenuated atherosclerosis via expansion of protective IgM^+ B1a cells (534). TIM-1 can thus exert both a co-inhibitory and co-stimulatory function, and studies have suggested this may be strongly linked to the ligand density (535) and the extent of TIM-1 engagement by the monoclonal antibodies (536). Mice with a genetic deficiency in TIM-1 signaling (*Tim-1*$^{\Delta mucin}$ mice) show exacerbated atherosclerosis with reduced $IL10^+$ Bregs and Th2 cells (532), illustrative of a coinhibitory role for TIM-1 in atherosclerosis. Activation of BTLA reduces early plaque formation and induces a collagen rich phenotype in advanced plaques. The mechanisms of action include a decrease in follicular B-cell and the increase in regulatory T- and B cells upon BTLA activation (537). Coinhibitory protein Tigit was significantly elevated on T cells isolated from *Ldlr*$^{-/-}$ mice fed a Western-type diet, but stimulation of this inhibitory signaling pathway in vivo did not alter atherosclerosis development, despite successful inhibition of T cell proliferation (533). In human carotid plaques, Tigit expressing $CD4^+$ T cells are detected, particularly in the Treg compartment (277).

8. INFLAMMATION INDUCED BY NON-IMMUNE CELLS IN ATHEROSCLEROSIS

Over the years, it has become clear that not only immune cells, but also non-immune cells are key in mediating inflammation in the arterial wall, thereby driving atherogenesis. In this review, we will highlight the contribution of 2 non-immune cell-types that play a key role in the 'inflammatory' pathogenesis of atherosclerosis: endothelial cells and vascular smooth muscle cells.

8.1 Endothelial cells in atherosclerosis

Endothelial cells (ECs) form the innermost lining of all blood vessels and fulfill numerous crucial functions in vascular physiology, extending beyond mere separation of blood from the vessel wall. They serve as guardians of vascular equilibrium by managing vascular tone and permeability, facilitating leukocyte adhesion and extravasation, and regulating hemostasis and thrombosis (538). Their response to various stressors, including shear stress, varies depending on their location within the vasculature, impacting their morphology, intracellular signaling, and gene expression, thereby influencing their functionality.

ECs play a pivotal role in regulating vascular reactivity through the secretion of three key vasoactive peptides: nitric oxide, endothelin-1 and prostacyclin (539). Nitric oxide's association with mechanisms underlying hypertension and atherosclerosis has been recognized since the 1980s(540) marking the initial identification of ECs' significant involvement in pathological processes related to vascular tone regulation (541).

The risk of (athero)thrombosis escalates with increased endothelial expression of tissue factor (TF) and the release of von Willebrand factor (vWF). Additionally, endothelial apoptosis contributes to an augmented presence of circulating CD146+ endothelial cells (ECs) in the bloodstream (538). Moreover, endothelial apoptosis and localized endothelial denudation can instigate thrombus formation through plaque erosion, a phenomenon implicated in approximately 20% of acute coronary syndrome cases (542).

Plaques prone to rupture exhibit a distinctive metabolic profile characterized by increased glycolysis, more utilization of amino acids, and reduced fatty acid oxidation (543). Changes in endothelial cell (EC) metabolism are recognized both as consequences of and contributors to endothelial dysfunction in atherosclerosis. Notably, EC activation is mediated through YAP/tafazzin (TAZ) signaling (544, 545). Studies have demonstrated that YAP/TAZ signaling induces glycolysis in ECs, and conversely, heightened glycolytic activity can upregulate the YAP/TAZ pathway (546), potentially leading to a cyclical and sustained pro-inflammatory response in the compromised endothelium (547). In animal models, inhibition of glycolysis has shown promise in mitigating these effects, although further human data is required to validate these findings (548).

8.1.1 Flow and shear stress

EC serve as the interface between blood and vessel walls, sensing shear stress magnitude and patterns through mechanosensory proteins and organelles. They then translate these signals into intracellular changes via mechano-transduction pathways, with alterations occurring at transcriptional, epigenetic, and protein levels, significantly impacting atherosclerosis.

Exposure of endothelial cells to disturbed flow triggers turnover and senescence, elevates oxidative stress (marked by reactive oxygen species, ROS), and compromises barrier function.

Key genes involved in thrombosis, inflammation, and vascular tone regulation are influenced by the flow pattern experienced by endothelial cells (549, 550). Especially Krüppel-like factor (KLF2) KLF2 and -4, highly expressed during laminar flow, are highly responsive to hemodynamic stimuli. They are upregulated in athero-resistant arterial regions and by statin treatment (551), where they maintain and anti-inflammatory program. Disturbed flow lowers KLF2 and -4 expression. Recently, γ proto-cadherins, which are upregulated in the endothelium during ASCVD, were identified as key suppressors of KLF2 and -4, and their genetic deletion protects mice from developing atherosclerosis, and are therefore a potent EC specific target to lower ASCVD (552).
While unidirectional, laminar shear stress is considered atheroprotective, pulsatile disturbed shear stress, such as oscillatory flow typically encountered at arterial branches and vessel wall irregularities, fosters chronic inflammation, thereby promoting atherogenesis (553). Shear stress is detected by various mechanosensors, including integrins (e.g., integrin α5—annexin A2 interaction), CD31/PECAM-1, VE-cadherin, and vascular endothelial growth factor receptor 2 (VEGFR2). The interaction between the mechanosensitive cation channel Piezo1, the purinergic P2Y2 receptor, and Gq/G11-mediated signaling has been identified as mediating inflammatory signaling and atherosclerosis under disturbed flow conditions, but not under laminar shear stress (554). Moreover, plexin D1 (PLXND1) is essential for endothelial cell response to shear stress and regulates the site-specific distribution of atherosclerosis (555). However, disturbed flow also induces anti-inflammatory feedback loops, such as the endothelial adrenomedullin-calcitonin-like receptor (CALCRL) axis, which signals through cAMP, reducing endothelial inflammation and lesion formation (556).

8.1.2 Endothelial cell barrier function

Endothelial cell (EC) barrier integrity relies on tight and adherens junction complexes (557), while transmigration is governed by specific receptor-ligand interactions in conjunction with substrate stiffness and intrinsic endothelial cell stiffness (558). Activation of ECs results in diminished vascular barrier function, heightened paracellular and transcellular transport, and increased deposition of fatty streaks, initially, particularly in regions of disturbed flow.
Endothelial ICAM-1 and VCAM-1 clusters generate cellular protrusions to facilitate leukocyte transmigration. The expression levels of ICAM-1 and VCAM-1 on the cell surface are modulated in response to signals from TNFα or IL-1β, dictating whether paracellular or transcellular routes are favored for diapedesis (559). During diapedesis, the paracellular space is sealed through RhoA activation and the formation of a contractile actin ring. Pathways leading to heightened endothelial permeability involve targets of HIF1-α and NF-κB, such as vascular endothelial growth factors, IL-1β, IL-6, tumor necrosis factor α (TNFα), as well as factors like TGFβ and ROS.
The endothelial layer is covered by a dense glycocalyx—a gel-like matrix composed of proteoglycans and extracellular matrix components, crucial for facilitating transendothelial transport, including lipoproteins(560). Degradation of the glycocalyx by plasminogen activator inhibitor (Serpine1) leads to increased endothelial permeability through downregulation of junctional proteins like occludin and claudin-5. Recent insights have unveiled endothelial transcytosis as a mechanism for LDL transportation, an active process mediated by various endothelial receptors, notably scavenger receptors class B type 1 (SR-B1) and activin-like kinase 1 (ALK1), both localized within caveolae, specialized membrane microdomains involved in endocytic processes (561, 562). The absence of caveolin-1, the primary protein constituent of

caveolae, in *Ldlr*$^{-/-}$ mice, diminishes LDL transport across the endothelium and reduces vascular inflammation in early-stage atherosclerosis, irrespective of eNOS activity (563, 564). Although binding of LDL and oxidized LDL (oxLDL) to CD36 and oxLDL receptor 1 (LOX-1) has been observed, none of these receptors influence endothelial LDL transcytosis.
In addition to endothelial transcytosis, studies have revealed endothelial breaches and hemorrhage in the intima of carotid arteries in *Apoe*$^{-/-}$ mice at sites of local flow perturbation. These sites attract leukocytes and foster the formation of fatty streaks (565).

8.1.3 Endothelium – immune system interactions

The involvement of ECs in atherosclerosis encloses their role in inflammation, where they interact with immune cells and respond to LDL and pathogen-associated molecular patterns. ECs directly secrete pro-inflammatory cytokines like IL-1β, monocyte chemoattractant protein-1 (MCP-1), and IL-8, contributing to atherosclerosis progression (566). EC inflammation promotes leukocyte recruitment by upregulating surface adhesion molecules such as E-selectin, vascular cellular adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1). Activation of NF-κB, further stimulated by high mobility group box 1 (HMGB1), triggers assembly of the EC inflammasome, leading to further increased interleukin 1β (IL-1β) release. ECs expressing these ligands actively facilitate leukocyte transport or favor diapedesis across the endothelial barrier (567). Local immune cell activation perpetuates tissue inflammation, enhancing endothelial permeability and allowing entry of plasma proteins into the interstitial space. Disrupted endothelial homeostasis promotes lipoprotein particle permeation and trapping in the subendothelial space, exacerbating inflammation and plaque progression.
In recent years, the role of extracellular vesicles, varying in size and contents, in mediating vascular cell-cell communication has emerged as a possible modulator of vascular function in atherogenesis(568). These vesicles facilitate intercellular signaling among ECs and between ECs and other immune and non-immune cell types (569). Such communication pathways might hold promise as potential biomarkers or therapeutic targets for future applications.

8.1.4 Endothelial cell plasticity

ECs are renowned for their remarkable plasticity, capable of transitioning between various cell identities, including adopting a mesenchymal phenotype, and then potentially reverting back to an EC state.
Endothelial-to-mesenchymal transition (EndMT) describes the molecular process wherein an endothelial cell undergoes a series of events leading to a shift in phenotype toward a mesenchymal cell, such as a myofibroblast or smooth muscle cell (570). However, there is no universally agreed-upon molecular criteria for defining EndMT. Typically, criteria include reduced expression of endothelial genes/proteins, increased expression of mesenchymal genes/proteins, or ideally, both (571). Examples of endothelial markers include CD31, VE-Cadherin, and endothelial nitric oxide synthase (NOS3), while mesenchymal markers may include alpha-smooth muscle actin (α-SMA), calponin, SM22a, and versican. Additional features, such as upregulation of EndMT-associated transcription factors like TWIST, SMAD3, ZEB2, SNAI1, and SNAI2, are also considered (571). Functionally, EndMT cells display diminished ability to form tubules, inhibit thrombin formation, and uptake LDL cholesterol—characteristics typical of ECs—while exhibiting enhanced mesenchymal cell traits like matrix invasion, migration, contraction, and collagen

production (571-575). However, robust human data confirming the extent and causality of EndMT in adult cardiovascular disease remains scarce.

EndMT is induced by various signaling cascades, including TGF-β, Notch, and Wnt/β-catenin pathways. Transcriptionally regulated by factors like snail, slug, and twist, EndMT is facilitated by hypoxia, inflammation, and oxidative stress. Signaling through TGFβR1/2 promotes the expression of inflammatory and mesenchymal genes while suppressing conventional EC identity genes, ultimately activating ECs to undergo EndMT and contribute to atherogenic lesion formation. Although TGFβ2 within plaques may aid in maintaining stability by reducing inflammation and matrix degradation (572), targeted inhibition, such as delivering short interfering RNAs specifically to ECs, could potentially induce atherosclerotic plaque regression in mouse models (576). Research into endogenous antagonists of the EndMT program is nascent. Interestingly, FGF signaling shows promise as a potent inhibitor of EndMT and potentially the atherosclerotic process (577, 578).

In addition to inducing endothelial inflammation and EndMT, disturbed flow, termed FIRE (disturbed Flow-Induced Reprogramming of EC)(579), also promotes endothelial-to-immune-cell-like transition (EndIT) during atherogenesis (580, 581). Supporting the EndIT concept, a meta-analysis of 28 microarray datasets from ECs exposed to various stimuli suggests its validity (582), though further confirmation via endothelial cell lineage-tracing studies is warranted (583).

8.1.5 Endothelial heterogeneity

Recent advancements in single-cell RNA-sequencing have unveiled significant heterogeneity among ECs, with its few human studies demonstrating variability across identified clusters (199, 584, 585) (Figure 10). In one of the most recent studies, four distinct clusters were delineated (199): three representing activated endothelium (marked by CD34, PECAM1, and TIE1), which actively exacerbates inflammation in advanced lesions through cell adhesion, neovascularization, and mediating leukocyte extravasation, and one potentially indicating endothelial-to-mesenchymal transition (identified by ACTA2, NOTCH3, and MYH11, typical markers for smooth muscle cells) (199).

The first cluster exhibited unique expression of ACKR1, associated with venous ECs and the vasa vasorum in mice(586) , along with PRCP involved in angiogenesis and regeneration of damaged endothelium. E.1 and E.2 both expressed VCAM1 but were distinguished by differential expression of extracellular matrix genes in E.1 and cell mobility markers such as FGF18 and HEG1 in E.2(586).

In another, even more recent study examining carotid plaques from only three patients, inflammatory ECs (characterized by IL6, ACKR1, HLA-DQA1) were predominant in proximal plaque regions, while distal ECs displayed profiles suggestive of intimal repair (marked by ITLN, DKK2) and extracellular matrix modulation (including FN1)(584). Further investigations involving larger human sample sizes and more comprehensive functional analyses are imperative to deepen our understanding of EC heterogeneity and its implications.

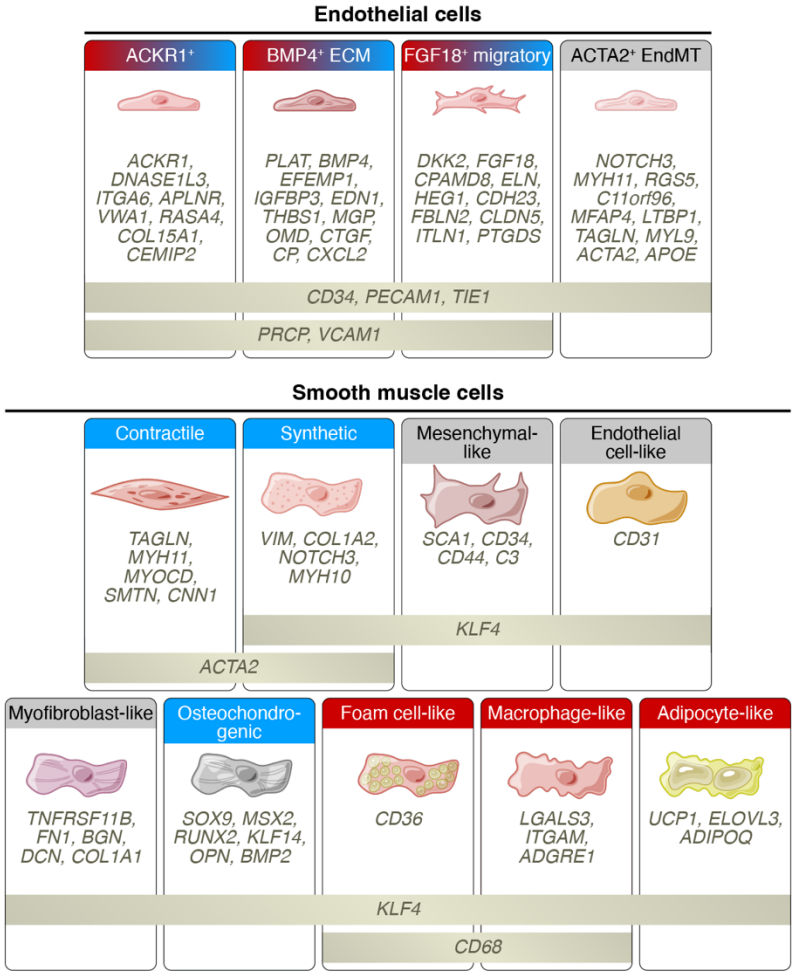

Figure 10. Endothelial cell and VSMC subsets in atherosclerosis

Endothelial cells and VSMC in human atherosclerotic plaques can be categorized into various groups, though no absolute consensus exists. As more studies emerge, newer classifications may arise. These cells undergo phenotypic transitions that are potentially dependent on the stage of the disease and the specific region within the plaque. Their roles range from athero-protective, tentatively mostly anti-atherosclerotic (labeled in blue), to athero-promoting, tentatively mostly pro-atherosclerotic (labeled in red). Some phenotypes may exhibit both functions depending on the context (shaded red and blue), while others remain largely unknown (gray). To simplify this complexity, endothelial cells, all of which express markers such as CD34, PECAM1, and TIE1, can be classified into at least four phenotypes. The ACKR1+ phenotype is involved in inflammation, tissue repair, angiogenesis, and vascular integrity maintenance. The BMP4+ phenotype is particularly associated with extracellular matrix (ECM)-related mechanisms. The FGF18+ phenotype plays a key role in angiogenesis, cell migration, and responses to mechanical stress. The ACTA2+ phenotype appears to represent a mesenchymal-like endothelial cell, possibly corresponding to a transitional state, engaging in fibrosis, injury response, and ECM remodeling. This phenotype also exhibits high expression of several genes that are also prominently expressed in VSMC.

VSMCs traditionally perform a contractile function, primarily characterized by ACTA2 expression. However, this expression decreases markedly when VSMC transition into alternative phenotypes in which KLF4 becomes more dominantly expressed. One such phenotype is the synthetic state, in which VSMC adopt a cobblestone morphology, produce ECM, and exhibit reduced expression of contractile proteins. Another phenotype involves VSMC acquiring endothelial-like characteristics, with increased expression of CD31. Some VSMC exhibit markers associated with progenitor cells, such as SCA1 and CD34, developing a mesenchymal-like phenotype. Others take on characteristics of myofibroblast-like cells, which exhibit features between fibroblasts and VSMCs and are highly prolific in

synthesizing collagen type I and fibronectin. In some cases, VSMCs undergo osteogenic and chondrogenic differentiation, expressing markers such as RUNX2 and OPN in osteogenic pathways or SOX9 in chondrogenic pathways, contributing to vascular calcification through mechanisms involving BMP2.
Further illustrating their plasticity, VSMC can also transdifferentiate into foam-cell-like, macrophage-like, and adipocyte-like states. Foam-cell-like VSMCs express CD36, a fatty acid transporter and scavenger receptor of oxidized LDL, playing a role in lipid accumulation. Macrophage-like VSMCs exhibit high levels of CD68, CD11b, galectin-3, and ADGRE1, contributing to immune and inflammatory responses. Adipocyte-like VSMCs express markers initially described in brown adipose tissue, including UCP1, as well as ELOVL3, involved in fatty acid synthesis, and ADIPOQ, which encodes adiponectin. These transitions highlight the remarkable plasticity of both endothelial cells and VSMCs in atherosclerotic plaques, emphasizing their diverse roles in disease progression and their potential as therapeutic targets.

8.2 Vascular smooth muscle cells in atherosclerosis

Under steady-state conditions, arterial smooth muscle cells (SMCs) exhibit a dense, spindle-shaped morphology, tightly packed with microfilaments and featuring characteristic contractile markers such as smooth muscle alpha actin 2 (ACTA2), transgelin (TAGLN), and myosin heavy chain 11 (MYH11). These markers facilitate the exertion of contractile properties crucial for regulating vascular tone and blood pressure. The transcriptional regulation of SMC contractile properties is orchestrated by serum response factor (SRF) and its coactivator myocardin (MYOCD) (587). SRF's binding to MYOCD is essential for the expression of contractile genes, as this interaction enables the specific expression of contractile genes in SMCs.
SMCs are present in the early stages of human pre-atherosclerotic intima and play a crucial role in both the retention of lipoproteins and, later in the formation of the fibrous cap (588). While there are differing viewpoints, prevailing theories propose that intimal SMCs likely originate from a subset of medial SMCs. This occurs through oligoclonal migration from the media to the intima, followed by proliferation within the intima. However, only a fraction of these clones survives, leaving the exact origin of SMCs within human plaques still unclear (589, 590). In general, SMCs are known to migrate into the intima and subsequently proliferate, contributing to the formation of the fibrous cap. The migration of medial SMCs may be preceded by proliferation, and both dividing and non-dividing SMCs can participate in plaque formation (591). However, lineage-tracing studies in various models suggested that SMC migration occurs independently of proliferation. Additionally, evidence indicates that SMC proliferation begins within the media even before migration occurs. Thus far, there is no evidence supporting the notion that intimal SMCs can migrate back to the media. Several studies propose more sources of SMCc within the vessel wall, such as adventitial mesenchymal stem cells (MSCs), pericytes, fibroblasts, or endothelial cells (ECs).(574, 577, 592-594). Some plaque-derived SMCs may originate from ECs through endothelial-to-mesenchymal transition (EndMT), which is induced by factors like transforming growth factor beta signaling, oscillatory shear stress, oxidative stress, and hypoxia (as mentioned in the endothelial cells section).
In advanced atherosclerotic plaques, many SMCs exhibit low proliferative rates, with only a select few clones displaying high proliferation (595). These highly proliferative SMCs are believed to form the fibrous cap and infiltrate the core (596). Apart from reduced proliferation, plaque SMCs demonstrate early senescence, characterized by the up-regulation of cell-cycle arrest markers and shorter telomeres compared to medial SMCs (597). Senescent SMCs contribute to atherosclerosis and plaque instability in mice, promoting calcification by initiating a transition to an osteoblastic phenotype *in vitro* (598). Various forms of SMC death occur within the plaque,

including apoptosis and necrosis such as necroptosis and ferroptosis (599). Apoptosis of medial contractile SMCs or myofibroblast-like SMCs in the intima appears to be detrimental, as it is associated with plaque vulnerability, calcification, and media degeneration (600). However, much remains unknown regarding SMC phenotypes and cell death mechanisms in atherosclerosis.

In the 1950s, Atschul observed through light microscopy that foam cells might originate from smooth muscle cells (601). Building on this, Geer, in the 1960s, noted that "the lipid lay almost exclusively in smooth muscle," and “smooth muscle cells often contained so many vacuoles that they appeared to be in a transitional stage between smooth muscle and foam cells”(602). Ross, in the 1970s, proposed that the accumulation of smooth muscle cells (SMCs) was essential for lipid deposition (603). He argued that "the lipid deposits occur either within smooth muscle cells or outside them in association with connective tissue matrix components, which are secretory products of smooth muscle cells." As SMCs accumulate lipid, they adopt a morphology resembling inefficient foam cells (604). Cholesterol-loaded SMCs exhibit reduced capacities for phagocytosis and efferocytosis compared to bone marrow-derived macrophages (605). These foam cells derived from SMCs also display lower levels of ABCA1, the rate-limiting exporter of excess intracellular cholesterol. This suggests impaired foam cell function and compromised ability to export cholesterol.

Another feature of SMCs is their ability to secrete bioactive molecules, including matrix proteins and pro-inflammatory mediators, which can potentially influence other cells in a paracrine manner. Some of these molecules are encapsulated into vesicles and are shed from the cell surface to facilitate signal transmission between cells. The secretome of phenotypically switched SMCs may contain critical information from the donor cell, and it can even determine the fate of the recipient cell. The identification of specific molecules within this secretome holds promise for clinical applications as potential biomarkers for cardiovascular disease (CVD) in the future. Examples of matrix proteins that could be significant include osteomodulin (606), endotrophin (607), and mimecan (608). Protein groups such as MMP9, S100A8/S100A9, cathepsin D, and galectin-3-binding protein have shown potential to differentiate between symptomatic and asymptomatic carotid plaques, suggesting their utility for risk stratification in cardiovascular diseases (609, 610).

There is a robust bidirectional interaction between SMCs and the extracellular matrix (ECM). The composition of the ECM is influenced by the delicate balance between its production and breakdown. During the early stages of plaque formation, matrix metalloproteinases (MMPs) play a pivotal role in facilitating SMC migration by degrading the surrounding connective tissue. Concurrently, the type and quantity of ECM produced vary depending on the phenotype of the SMCs (611). Particularly, SMCs exhibiting a synthetic phenotype release various inflammatory molecules, leading for instance to cell death in adjacent cells (612). Pro-inflammatory cytokines such as IL-1β, IL-6, and MCP-1 play significant roles in promoting atherosclerosis by attracting monocytes and inducing cell death. Additionally, these cytokines induce the expression of adhesion molecules and receptors that facilitate monocyte recruitment and regulate inflammatory signaling. While synthetic SMCs are recognized for their role in forming ECM in the fibrous caps, they can also exacerbate inflammation, calcification, cell aging, and plaque instability depending on the local microenvironment and disease stage (613). Specifically, synthetic SMCs release exosomes that may induce calcification when they contain components

such as phosphatidylserine (PS), Annexin A6, and low levels of calcification inhibitors. These exosomes may also promote vascular coagulation by exposing PS and tissue factor on their surface (614). Furthermore, matrix vesicles derived from apoptotic SMCs can be sites for calcification in plaques, while osteochondrocyte-like SMCs secrete calcifying vesicles, contributing to the progression of calcification (615).

Arterial calcification is another mechanism involving SMCs. In calcified arteries, these cells elevate the expression of bone-related transcription factors such as Msx2, Sox9, and Runx2, along with mature chondrogenic markers including collagen X and MMP13 (616). SMCs activate cellular programs aimed at facilitating the deposition of bone matrix within the arterial wall and give rise to the majority of chondrocyte-like precursors (98%) within plaques (617). Deletion of Runx2 prevents calcification in mouse models by reducing the presence of SMC-derived mature osteochondrogenic-like cells, as well as calcium deposition and mineralization within the deep intima layers (618).

During vascular injury or atherosclerosis, SMC undergo phenotypic switching, initially marked by the downregulation of contractile genes (619). Lineage tracing studies have provided robust evidence indicating that SMC-derived cells constitute a significant proportion (30%–70%) of all plaque cells and over 60% of SMCs in atherosclerotic plaques dedifferentiate to a "pioneer" state, capable of transitioning to several multipotent states (620, 621). Within plaques, SMCs *in vivo* exhibit diverse phenotypes, some of which may have detrimental effects. These cells downregulate contractile markers during atherosclerosis while adopting alternative phenotypes, including macrophage-like, foam cell-like, adipocyte-like, osteochondrogenic-like, myofibroblast-like, and mesenchymal stem cell-like phenotypes, briefly described below (591, 622).

A pivotal molecular factor driving the differentiation of SMCs from a contractile to a dedifferentiated transitional state is the transcription factor Kruppel-like factor 4 (KLF4)(585). KLF4 regulates the transition of SMCs to multiple phenotypes in advanced atherosclerotic lesions. MicroRNAs (miRNAs)(623), epigenetic modifications (e.g., histone acetylations, methylations such as histone modification H3K4me2)(624, 625) , and various environmental stimuli (such as integrins, cytokines, and growth factors like Platelet-derived growth factor (PDGF)-BB and TGF-beta) regulate the SMC phenotype(622).

Finally, another intriguing aspect of SMCs is their plasticity, manifested through phenotype switching, which contributes to the extensive heterogeneity of profiles observed(612). Phenotype switching encompasses any phenotypic alteration leading to an alternative non-contractile state in SMCs. Despite the various phenotype switches summarized below, the histone modification H3K4me2 appears to persist and can serve as a possible specific marker for SMCs (626).

A burgeoning body of literature, employing diverse nomenclatures, offers a rich and complex array of information. However, despite some efforts towards consensus, a universal agreement on the classification and categorization of SMC phenotypes remains elusive (107, 627). The discrepancies among reports can be attributed to factors such as the limited number of studies, particularly in humans, variations in arterial beds analyzed, in experimental methodologies, and data analysis approaches.

Nevertheless, the following SMC phenotypes are frequently described, especially in human studies, here presented from those that might contribute mostly to plaque stability to less stability, from a mostly hypothetical perspective (Figure 10).

Contractile SMCs have the capacity to undergo transdifferentiation into fibroblast-like cells or myofibroblast-like SMCs, characterized by an increased expression of fibronectin 1, osteoprotegerin, collagen type 1a1, and various small proteoglycans, along with the upregulation of fibroblast-specific pathways. The transcription factor 21 (TCF21) plays a pivotal role in promoting this phenotypic signature, which is commonly observed in the protective fibrous cap. Interestingly, elevated expression of TCF21 has been associated with a decreased risk of coronary artery disease in humans (628).

Another type of modulated or switched SMCs are the mesenchymal-like SMCs. These cells express the stem cell activating antigen 1 (Sca1) within atherosclerotic plaques and have the capability to differentiate into contractile SMCs (593, 621).

The transition from a contractile to a synthetic phenotype is characterized by the loss of contractile markers such as ACTA2, MYH11, TAGLN, and calponin, coupled with the acquisition of synthetic organelles and increased migratory and proliferative properties (612). While most ACTA2+ cells are typically found in the fibrous cap, ACTA2- cells derived from SMCs accumulate within the core, challenging the old notion that the core is predominantly occupied by macrophages(620, 626). The synthetic phenotype serves as a hallmark of vascular repair, driven by the persistent exposure to stimuli inducing phenotypic switching within the plaque. Notably, SMCs retaining the synthetic phenotype may probably still retain the ability to revert back to their contractile phenotype (629).

Intermediate cells derived from SMCs, termed "SEM" cells (stem cell, endothelial cell, monocyte), represent an intermediate state in VSMC phenotypic switching that may lead to various ultimate fates (629). These cells exhibit multipotent characteristics, capable of differentiating into other SMC signatures or reverting to a contractile SMC phenotype. Retinoic acid, known for its anticancer properties, has been identified as a regulator of the transition from SMCs to SEM cells. Dysregulation of retinoic acid signaling has been observed in symptomatic human atherosclerosis. An interesting finding for future potential applications is that the activation of retinoic acid signaling inhibits the transition of SMCs to SEM cells, reduces atherosclerotic burden, and promotes fibrous cap stability.

SMCs may undergo a transformation to an osteochondrogenic phenotype in response to elevated levels of calcium and phosphate (630). Osteochondrogenic SMCs exhibit an upregulation of osteochondrogenic markers such as runt-related transcription factor 2 (RUNX2), SRY-related HMGbox (SOX9), and osteopontin. They deposit a matrix conducive to calcification, including collagen type II and X, and secrete calcifying vesicles (630). Concurrently, they downregulate calcification inhibitors such as vitamin K-dependent matrix Gla-protein and fetuin A. In human coronary arteries, this SMC phenotype is associated with plaque destabilization (585).

The SMC-derived osteochondrogenic population can be manipulated through SMC-specific ablation of specific genes, such as Ahr or Klf4 (585, 631). Notably, SMC-specific ablation of Ahr or Klf4 also alters lesion size, fibrous cap morphology, and the presence of intermediate SMC clusters, suggesting that the regulatory function of these genes in SMC transition go beyond the osteochondrogenic phenotype.

Thioredoxin-interacting protein (TXNIP) is another regulator of the osteochondrogenic SMC switch and appears to be significant in human plaque calcification (632). Microcalcifications (<50 μm) are associated with increased inflammation and are predominantly observed in the fibrous cap of human lesions, making them detrimental to plaque stability. In contrast,

macrocalcifications (>200 µm) tend to accumulate in the deep intima or necrotic core in organized structures and may promote plaque stability (633). Despite their nomenclature, osteochondrogenic SMCs are more susceptible to apoptosis and differ functionally from mature, bone-forming osteoblasts (634).
Contractile SMCs have the capability to transdifferentiate into an endothelial cell (EC)-like phenotype, characterized by the expression of EC markers such as CD31, von Willebrand factor, and VE-cadherin (635). In human SMCs, this conversion into ECs can occur in culture through an intermediate progenitor state induced by stem cell reprogramming factors. Further differentiation into ECs is regulated via Notch signaling pathways (636).
Interestingly, these SMC-derived ECs exhibit full endothelial cell functionality, as evidenced by the expression of CD31, and have been proposed to contribute to intraplaque hemorrhage and neovascularization, phenomena commonly observed in vulnerable plaques (637).
SMCs can also transform into foam cells in humans when exposed to aggregated or oxidized low-density lipoprotein. Intriguingly, it appears that a significant proportion of foam cells may originate from SMCs, at least in mice. The uptake of lipids typically occurs through specialized scavenger receptors such as SR-A and CD36, but alternative pathways such as pinocytosis, phagocytosis and autophagy may also be involved (638-640) .
Foam cell-like SMCs exhibit reduced expression of the key cholesterol exporter ABCA1 and lower levels of lysosomal acid lipase, which is the primary enzyme responsible for hydrolyzing lipoprotein-derived and intracellular cholesteryl esters (641). Unlike macrophages, SMCs tend to retain lipoprotein-derived cholesteryl esters in lysosomes rather than storing excess cholesterol in cytosolic droplets, potentially contributing to the reduced likelihood of lesion regression. In addition to their increased lipid uptake, lipid-loaded SMCs in culture can secrete pro-inflammatory mediators and may undergo apoptosis (642).
Furthermore, besides the increased uptake of lipids and potentially impaired efflux or degradation of lipoproteins, SMCs can synthesize fatty acids de novo through peroxisome proliferator-activated receptors gamma and the liver X receptor pathway (643). Although they do not fully differentiate into mature adipocytes and their role remains unclear, these adipocyte-like SMCs express high levels of adipsin and genes associated with lipogenesis in human plaques (644).
Another phenotype transformation induced by lipid accumulation is the transition into macrophage-like SMCs (621, 640). These cells exhibit elevated expression of markers typically associated with macrophages, including LGALS3, CD68, CD11b, and pro-inflammatory cytokines such as CCL2 (640). However, it is important to note that not all lipid-loaded SMCs *in vivo* undergo this macrophage-like transformation, and not all plaque SMCs expressing LGALS3 acquire a macrophage-like phenotype(585, 628). Instead, LGALS3 is detected in over 60% of plaque SMC during a transitional phase towards a non-macrophage state yet characterized by a pro-inflammatory and osteochondrogenic phenotype. These cells may represent some of the earliest inhabitants of the plaque (585). Although the presence of macrophage markers in SMCs suggests an inflammatory response, these cells may not fully function like typical macrophages but are hypothetical quite deleterious for the plaques.
In summary, the current literature on SMCs offers only a partial glimpse into their phenotype, function, interactions, temporal dynamics, and spatial distribution. Recent advancements in spatial transcriptomics and proteomics in atherosclerosis research are contributing additional

pieces to this intricate puzzle (394, 610). However, comprehensive information regarding the true nature of atheroprotective and atheropromoting SMC phenotypes in human arterial territories, as well as within individual plaques, and the mechanisms governing these phenotypes, remains elusive.
Further investigations aimed at delineating the biological trajectory of SMCs, their diverse profiles, spatial distributions, and functional roles, incorporating unbiased various omics approaches alongside functional studies, are imperative. Such efforts are essential to ascertain the potential of SMCs as novel therapeutic targets for advancing the prevention and treatment of atherosclerosis.

9. TARGETING THE IMMUNE SYSTEM IN ATHEROSCLEROSIS

9.1 Targeting inflammation in atherosclerotic CVD: proof of concept trials

9.1.1 Clinical Trials: targeting innate immunity in atherosclerotic CVD

For decades, managing dyslipidemia has been the primary focus in controlling atherosclerosis. However, despite remarkable advancements in lowering circulating lipoproteins, a residual risk for cardiovascular events persists. This led to exploring novel targets beyond LDL reduction, particularly focusing on modulators of inflammation. Despite efforts with various antioxidants (645-647), losmapimod (a p38 mitogen-activated protein kinase inhibitor) (648), and inclacumab (a P-selectin antagonist) (649), clinical trials have not yielded success.
At the forefront of the inflammatory cascade lie several potential targets, such as the NLRP3 (NOD-like receptor family, pyrin domain-containing protein 3) – IL1β – IL6 pathway. Drug repurposing from adjacent therapeutic areas also involving inflammation has emerged as an intuitive strategy (Figure 12).
Methotrexate is a first-line anti-inflammatory agent commonly used in rheumatoid arthritis, psoriasis and other forms of inflammatory arthritis. It has also been linked to a decrease in CV events in patients with RA (650). Methotrexate has several mechanisms of action inhibit aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC) enzyme, leading to intracellular accumulation of AICAR and increased the release of adenosine release. Methotrexate inhibits activation of nuclear factor-κB (NF-κB) by increasing both adenosine release and activation of adenosine receptor A_{2a} and by inhibiting the reduction of BH2 to BH4. It also affects T cells (651). It has also been shown to suppress the activation of NF-κB and NLRP3/Caspase-1 pathways in joint tissue (652). Its efficacy was explored in the Cardiovascular Inflammation Reduction Trial (CIRT), where low doses were administered to patients with stable coronary artery disease (CAD) alongside diabetes mellitus (DM) or metabolic syndrome, or both(653). Contrary to expectations, patients receiving low-dose methotrexate did not experience fewer cardiovascular events, nor exhibit lower levels of IL-1β, IL-6, hs-CRP. Instead, they experienced a higher incidence of side effects such as transaminitis, leucopenia, anemia, and infections. This negative trial had no requirements of high residual inflammation among enrolled patients, which underscored the importance of focusing on patients with sufficient inflammatory burden to be worth the benefit of an intervention (653).

This consideration was pivotal in the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) trial (654). In this randomized, double-blind, prospective study, 10,061 patients, at least 30 days post-myocardial infarction, with a hs-CRP level ≥ 2 mg/L (despite optimal guideline-directed medical treatment), received canakinumab—a monoclonal antibody neutralizing IL-1beta—subcutaneously, every 3 months, compared to placebo. Without significantly reducing lipid levels, the 150mg canakinumab dose met the prespecified primary and secondary endpoints, demonstrating a 15% reduction in myocardial infarction, stroke, or cardiovascular death. This effect size was comparable to that achieved with approved PCSK9 inhibitors (655), despite no impact on lipoprotein levels. Canakinumab did reduce the overall CV mortality rate by about 30% in patients that responded with a reduction in hsCRP and IL6 after the first injection. Non-responders were identifiable at the follow-up hsCRP measurement at 3 months (656). However, patients receiving canakinumab experienced an increased incidence of fatal infections, sepsis, and cancer mortality, aligning with theoretical expectations given the role of IL-1β in host defense mechanisms. CANTOS stands as a landmark trial, illustrating the efficacy of targeting inflammation in atherosclerosis and paving the way for exciting advancements in anti-inflammatory interventions.

Another widely known and cheap agent is colchicine, traditionally used for decades in gout and various other inflammatory conditions. Colchicine operates by inhibiting components of the NLRP3 inflammasome, a mechanism akin to its action in response to the deposition of sodium urate crystals in soft tissue—a phenomenon that bears resemblance to the process observed in atherosclerosis following the deposition of cholesterol crystals in the intima(657). The first outcome trial evaluating colchicine in atherosclerotic cardiovascular disease dates back to 2013 with the Low Dose Colchicine for Secondary Prevention of Cardiovascular Disease (LoDoCo) study (658). This prospective, randomized study involved 532 patients with stable coronary disease, comparing colchicine treatment (0.5 mg/day) to no treatment over a 3-year follow-up period. The composite primary endpoint included acute coronary syndrome, out-of-hospital cardiac arrest or non-cardioembolic ischemic stroke. Despite limitations such as the absence of placebo control and blinding, the small patient cohort, and a short follow-up duration, low-dose colchicine demonstrated efficacy, prompting the subsequent larger LoDoCo2 randomized-controlled, double-blind trial. In LoDoCo2, involving 5522 patients with chronic coronary disease (only 15% of whom were female), daily administration of 0.5 mg colchicine or placebo showcased benefits as early as 6 months into the study, with a composite endpoint comprising cardiovascular death, myocardial infarction, ischemic stroke, or coronary revascularization(659). In a recent study, however, a prespecified subgroup analysis identified a difference in magnitude of treatment effect of colchicine by region (Australia: HR 0.51; 95% CI 0.39-0.67 vs The Netherlands: HR 0.92; 95% CI 0.71-1.20), painting a mixed picture (659, 660).

Colchicine was further investigated in the context of acute coronary syndromes in the COLCOT (Colchicine Cardiovascular Outcomes Trial) trial, which randomized 4745 patients in a double-blind fashion, with only 19% being female, and with a slightly shorter follow-up duration (23 months compared to LoDoCo2's 29 months)(661). Once again, colchicine (0.5 mg daily) exhibited a lower risk of cardiovascular events compared to placebo. Both trials could have benefited from a more comprehensive monitoring of biomarkers to provide insights into mechanistic perspectives. Neither trial demonstrated a reduction in mortality with colchicine; instead, an increased frequency of diarrhea was noted in colchicine-treated patients. Regarding the potential

for elevated infection rates, only COLCOT reported a slight increase in pneumonias (0.9% vs. 0.4%)(661).

Furthermore, an intriguing small-scale prospective, nonrandomized, observational study involving 80 patients with acute coronary syndrome, undergoing coronary computed tomography, compared colchicine 0.5 mg/day with optimal medical therapy alone or in combination with colchicine, with a follow-up period of 1 year. Despite the modest cohort size, the study revealed that low-dose colchicine favorably impacted coronary plaque morphology (662).

The "CLEAR SYNERGY" trial, a recent large-scale clinical study, included 3,528 patients randomized to receive colchicine 0.5 mg and 3,534 assigned to placebo after percutaneous coronary intervention (PCI), with a preference for the Boston Scientific SYNERGY everolimus-eluting platinum chromium stent system. Initially, the dosing of colchicine was weight-based (once daily for patients under 70 kg and twice daily for those 70 kg or above), but it was modified to a once-daily regimen during the trial due to higher discontinuation rates with twice-daily dosing. The study demonstrated no significant difference in major adverse cardiovascular events between the colchicine and placebo groups when added to standard post-MI treatment and PCI. These results differ from earlier studies like COLCOT and LoDoCo2, which showed that colchicine reduced cardiovascular events in similar populations (663). Additional research is needed to explore the reasons for these differing outcomes across trials.

Another alternative target in the inflammatory cascade is interleukin-6 (IL-6), positioned downstream from IL-1β in the NLRP3 inflammasome pathway. The ASSAIL-MI (ASSessing the effect of Anti-IL-6 treatment in Myocardial Infarction) trial, conducted in a randomized, double-blind, placebo-controlled manner, examined the impact of the interleukin-6 receptor inhibitor tocilizumab on myocardial salvage in acute ST-elevation myocardial infarctions, involving nearly 200 patients. The trial demonstrated an increase in myocardial salvage by magnetic resonance imaging within 3 to 7 days post-treatment (664).

Concurrently, investigations into targeting the IL-6 ligand with monoclonal antibodies like ziltivekimab are underway. Notably, the ZEUS (Effects of ziltivekimab versus placebo on cardiovascular outcomes in participants with established atherosclerotic cardiovascular disease, chronic kidney disease, and systemic inflammation) randomized trial is of particular interest (NCT05021835). Its objective is to establish the superiority of subcutaneous ziltivekimab (15 mg) once monthly in reducing the risk of cardiovascular events compared to placebo, both in conjunction with standard of care. The trial includes participants with high-sensitivity C-reactive protein levels equal to or greater than 2mg/L, established atherosclerosis, chronic kidney disease (stage 3-4), and systemic inflammation. Commenced in 2021, it aims to enroll 6200 participants and is anticipated to conclude in 2025.

9.1.2 Clinical Trials: targeting adaptive immunity in atherosclerotic CVD

Efforts to modulate the adaptive immune system's response to atherosclerosis and its clinical manifestations are emerging, albeit with considerably less progress compared to autoimmune diseases and cancer (Figure 12).

Observations have revealed that IgM antibodies, known as natural antibodies, recognize epitopes associated to oxidized LDL, prompting numerous strategies aimed at harnessing endogenous antibodies to mitigate atherosclerosis(665). Several groups have pursued strategies involving the

immunization of mice, either actively or passively, using for instance, defined immunogenic epitopes of apolipoprotein B (666-669). Recently, psoriasis patients were randomly assigned to receive a 50-mL infusion of either 1245 mg of orticumab (n = 52) or placebo (n = 25) for 12 weeks In the subgroup with elevated coronary inflammation, the CaRi-Heart risk score indicated a potential for an almost 50% relative risk reduction for CVD in response to orticumab, suggesting that reducing oxLDL-dependent inflammation might beneficial in patients with psoriasis and possibly the general population(670).

Regarding the adaptive immune system, preclinical evidence suggests the involvement of mature B-cells in the recruitment of inflammatory monocytes to the site of myocardial infarction. Depletion of B-cells through a single injection of CD20 antibody has reduced infarct size and enhanced myocardial contractility (671). Moreover, in patients with myocardial infarction, B-cell activation upon admission was associated with a heightened risk of death and recurrent myocardial infarction over a two-year follow-up period (671). Intriguingly, B-cell depletion using anti-CD20 antibody in murine models has also shown a reduction in the development of atherosclerosis(407, 409).

Monoclonal antibodies like Rituximab, which target human B-cells, have been extensively used in autoimmune disorders, inflammatory conditions, and cancer. Recently, there has been an attempt to repurpose them for cardiovascular diseases. In the prospective, open-label, single-arm, dose-escalation phase 1/2a clinical trial known as RITA-MI (Rituximab in patients with acute ST-elevation myocardial infarction), Rituximab was administered as a single intravenous dose to patients who experienced acute ST-elevation myocardial infarction and underwent successful primary percutaneous coronary intervention within 24 hours of the onset of cardiac chest pain(672). Despite being a small, single-center study involving only 24 patients (mostly white males, lacking a control group) with a follow-up duration of only 6 months, Rituximab was found to be safe (the primary endpoint) and well-tolerated. It effectively depleted 96% of circulating mature B-cells within 30 minutes of infusion initiation, even at low doses (200mg). B-cell repopulation at 6 months was observed to be dose-dependent, while immunoglobulin levels remained unaffected during the follow-up period. These promising findings have led to the initiation of the ongoing RITA-MI2 multinational randomized, double-blind, placebo-controlled clinical trial. Funded by the European Commission, this trial aims to investigate the impact of B-cell depletion with Rituximab (at doses of 200mg, 1000mg, and placebo) on left ventricular systolic function, assessed by cardiac magnetic resonance at 6 months (the primary endpoint). The trial involves 558 patients with acute anterior ST-elevation myocardial infarction. Results regarding cardiac remodeling are anticipated by 2026 (DOI:10.3030/899991).

In addition to the modulation of B-cells, ongoing research explores the manipulation of other immune mechanisms. Preclinical models have demonstrated that increasing regulatory T-cells (Tregs, $CD4^+CD25^+FOXP3^+$) can decrease plaque inflammation, slow progression, and even regress atherosclerosis (276, 673). Low levels of circulating $CD4^+FoxP3^+$ T cells have been associated with an increased risk of acute coronary events during a 15-year follow-up period (356). Moreover, Tregs have shown to promote myocardial wound healing (674), attenuate adverse left ventricular remodeling (675), and improve cardiac function (676). Tregs express the interleukin IL-2(IL-2) receptor, IL-2Ra, which binds to IL-2 with high affinity, rendering this subset highly sensitive to IL-2 (677). IL-2 appears to be the most crucial cytokine in Tregs development and stability (678).

Inspired by its success in other immune diseases (679-682), subcutaneous low doses of IL-2 have been administered to patients with ischemic heart disease, both in stable coronary disease (trial Part A) and acute coronary syndromes (trial Part B), to assess safety and demonstrate the mechanism of Tregs expansion. This trial, known as LILACS (Low-dose InterLeukin 2 in patients with stable ischaemic heart disease and Acute Coronary Syndromes), utilized Aldesleukin (Proleukin, Novartis), a commercially available recombinantly produced IL-2 licensed for the treatment of metastatic renal cell carcinoma in the UK (683). LILACS not only provided safety data (no related serious adverse events), but also determined the dose required to increase Tregs by 75%, without significantly affecting T effector cells. Single-cell RNA sequencing techniques were employed, allowing for ligand-receptor expression analyses that provided insights into possible mechanisms underlying aldesleukin's Tregs expansion, such as activating co-stimulatory signals through CD28-CD86. Intriguingly, aldesleukin led to dose-dependent decreases in B cells and CD8+ T cells, and was shown to increase the number of Bregs, prompting the need for further investigation, and was shown to (683, 684).
The subsequent IVORY (Low-dose interleukin 2 for the reduction of vascular inflammation in acute coronary syndromes) trial (685), involved 60 patients with acute coronary syndromes and hsC-reactive protein levels >2mg/L, who were randomized to receive either low-dose IL-2 or placebo for 8 weeks. ^{18}F-FDG (2-deoxy-2-[Fluorine-18] fluoro-D-glucose) positron emission tomography-computed tomography showed a significant decrease in vascular inflammation in the aorta and carotid arteries, and an increase in circulating Tregs. A trend towards a reduction in major cardiovascular events could be observed after 2.5 years of follow up (https://doi.org/10.1161/circ.150.suppl_1.4144964), showing that increasing Tregs is a promising strategy in treating atherosclerotic CVD.

9.2 Future Immunotherapeutics for atherosclerosis: lessons from approved immunotherapies

Over the past 20 years, treatments using monoclonal antibodies specifically targeting the immune system have been developed to treat autoimmune diseases as well as cancer. The cardiovascular impact of these therapies, although infrequently reported, allows us to validate the clinical relevance of the knowledge acquired from experimental studies about the role of these immunotherapeutic targets in atherosclerosis (686) (Figure 13).

9.2.1 Lessons from autoimmune diseases

9.2.1.1 Anti-TNFα treatment

Anti-TNFα treatment is standard of care for many autoimmune diseases, including rheumatoid arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, psoriasis, and inflammatory bowel disease (687). These auto-immune diseases are associated with an increased risk of CVD(5, 6), which is frequently monitored in clinical trials and databases. Therefore, studying CVD in these patient groups can provide us with valuable information on the potential of anti-TNFα treatment to combat CVD. Rheumatoid arthritis (RA) patients that were treated with anti-TNFα vs other therapies and followed for 2 years, had a lower incidence of cardiovascular events (688). In another study, RA patients that were non- and moderate- responders to anti-TNFα treatment had a >2 times increase in risk of acute coronary syndromes, whereas patients that responded well to anti-TNFα treatment had a risk of acute coronary syndromes comparable to that of the general population (689). The *Consortium of Rheumatology Researchers of North America*

Targeting innate immunity in atherosclerosis					
Trial name	**Agent**	**Target**	**Patient cohort**	**Primary end point**	**Main CVD outcomes**
CIRT	Methotrexate	Broad anti-inflammatory effect	4,786 patients with previous MI and DMII or metabolic syndrome	Composite of nonfatal MI, nonfatal stroke, or cardiovascular death	No beneficial effects
CANTOS	Canakinumab	IL1β	10,061 patients with previous MI and elevated plasma CRP levels	Non-fatal MI, non-fatal stroke or death from cardiovascular causes	The 150-mg dose of canakinumab reduced cardiovascular events compared with placebo, independent of lipid level reductions
COLCOT	Colchicine	Inflammasome	4,745 patients with MI within 30 days before enrolment	Death from cardiovascular causes, resuscitated cardiac arrest, MI, stroke, or hospitalization for angina leading to coronary revascularization	Colchicine decreased the risk of the composite end point compared with placebo
LoDoCo2	Colchicine	Inflammasome	5,522 patients with chronic coronary artery disease	Death from cardiovascular causes, spontaneous MI, ischemic stroke or ischemia-driven coronary revascularization	Colchicine decreased the risk of the composite end point compared with placebo
CLEAR SYNERGY	Colchicine	Inflammasome	7,062 patients after PCI (SYNERGY stent) treatment	Composite of death from cardiovascular causes, recurrent myocardial infarction, stroke, or unplanned ischemia-driven coronary revascularization	No beneficial effects
Targeting adaptive immunity in atherosclerosis					
Trial name	**Agent**	**Target**	**Patient cohort**	**Primary end point**	**Main CVD outcomes**
Orticumab	Anti-MDA-ApoB100	MDA-LDL	77 psoriasis patients with myocardial inflammation	Coronary inflammation (fat attenuation index)	Reduction of coronary inflammation
IVORY	Low dose IL2	Treg expansion	60 high risk ACS patients	Vascular inflammation (18FdG-PET/CT)	Reduction of arterial inflammation

Figure 11. Overview of a selection of clinical trials using immunotherapeutics to target the innate or adaptive immune system.
Trials targeting the innate immune system are CIRT (Cardiovascular Inflammation Reduction Trial), CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study), COLCOT (COLchicine Cardiovascular Outcomes Trial), LoDoCo2 (Low Dose Colchine 2 trial) and the CLEAR SYNERGY TRIAL. Trials targeting the adaptive immune system use orticumab, and anti-MDA-LDL antibody targeting MDA modified LDL, and IVORY (low dose Interleukin 2 for the reduction of Vascular inflammation in acute coronaRY syndromes). Methotrexate: okay to put with innate? Or make a general category?? Shall we add the CD47 study of Nick Leeper to innate??

rheumatoid arthritis registry that followed more than 10,000 patients for 24-months showed a reduction of non-fatal cardiovascular events when RA patients received anti-TNFα treatment compared to other treatments (690). In patients with psoriatic arthritis, anti-TNFα treatment was also shown to reduce plaque burden compared to patients treated with non-selective immunomodulators in a 4-year period (691). Although these studies predict that anti-TNFα therapy is a promising strategy to treat atherosclerotic cardiovascular disease, anti-TNFα therapy in CVD patients had no effect on heart failure (692) or even worsened left ventricular function in heart failure patients (693).

9.2.1.2 CTLA4-Ig treatment

A CTLA4-Ig analogue, called Abatacept has been applied successfully in patients with rheumatoid arthritis, most of them refractive to anti-TNFα therapy. Until now, only limited data are available on the effects of Abatacept on cardiovascular disease in humans. However, most data point towards a beneficial effect on ASCVD outcomes (694-696). Abatacept significantly reduced ASCVD in a study comparing biologics with synthetic disease-modifying antirheumatic drugs (DMARDs) (694) and reduced the incidence of MACE in TNFα non-responders in a 2-year follow-

up compared to Rituximab users (695). Similar results were found in a study where rheumatoid arthritis patients using TNFα inhibitors were compared to Abatacept users using Medicare and MarketScan databases. Abatacept was associated with a 20% reduced risk of MACE (696). However, when Abatacept treatment was compared with Tociluzumab in anti-TNFα treatment-resistant rheumatoid arthritis patients, no beneficial effects on ASCVD outcomes were found (697). In experimental settings, Abatacept successfully decreases atherosclerosis in *Ldlr*$^{-/-}$ mice neointima formation in C57Bl/6J mice(698), and blocks age-related heart failure in mice (699). Well-designed clinical trials testing the effects Abatacept in CVD setting are still being awaited.

9.2.1.3 Anti-IL12/Anti-IL23-p40 treatment

Anti-IL12/IL23p40 therapy showed promising effects on ameliorating psoriatic disease. IL12 is known to be pro-atherogenic (700), and IL23 seems to be anti-atherogenic (332). Treatment with anti-IL12/23p40 antibodies significantly increased the risk of cardiovascular events in patients suffering from psoriasis (701, 702), so does not seem a favorable target to treat CVD.

9.2.1.4 Anti-IL17 treatment

Anti-IL17 antibodies are FDA approved and effective in psoriasis, psoriatic arthritis, and ankylosing spondylitis. The effects on CVD in treated patients are unknown. As described in *section 6*, the effects of IL17 on atherosclerosis in experimental models is still unclear (314-318, 321). Similarly, correlations of plasma IL17 levels and its association with CVD is contradictory. Plasma IL17 levels increase in patients with unstable angina or acute MI compared with patients with stable angina and healthy individuals (322, 323). In other studies IL17 was not associated with CAD, and low IL17 levels (324) were associated with a higher risk of cardiovascular events (325). Anti-IL17 treatment in patients with psoriasis did improve endothelial function, but does not affect cardiovascular biomarkers (703, 704), and is believed to positively affect psoriasis-associated cardiovascular disease (705). Older studies, although underpowered for cardiovascular disease, report that anti-IL17 treatment may increase atherothrombotic events (706).

9.2.2 Lessons from oncology

Co-inhibitory immune checkpoint therapy is associated with an increased risk for cardiovascular events in oncology patients (707). In a systematic review, that included 10,106 patients treated with anti-CTLA4 and/or anti-PD1 antibodies, the incidence of arterial thrombotic events (myocardial infarction or stroke) was 1.1% (708). In another study, involving 2842 cancer patients, the incidence of atherosclerotic cardiovascular events, had increased 4.7-fold in patients that were treated with coinhibitory immune checkpoint inhibitors (709). Anti-PD1 treatment increased the risk for venous and arterial thrombo-embolism, suggesting that PD1 blockage results in a pro-thrombotic state, although mechanisms are still unknown (710, 711). These studies not only increase awareness to the fact that inhibiting co-inhibitory immune checkpoints can increase the risk for cardiovascular events in cancer patients (712), but also suggest the importance of co-inhibition, and PD1-PDL1 interactions in particular, in keeping atherosclerotic plaque inflammation in control (713). Additional studies detailing the cell-type specific mechanisms of PD-PDL1/2 interactions in atherosclerosis are needed before applying PD(L1/2) based targeted immunotherapeutics in CVD.

9.3 New immunotherapeutic targets: preclinical studies in atherosclerosis with high translational potential

Our understanding of the role of immune cell networks and its mediators in atherosclerosis has increased significantly over the past few decades, as can be read in sections 3 and 4. Based on these data, attractive, new immunotherapeutic targets have been identified. The complexity of the immune system allows the investigation of a myriad of therapeutic targets. The next section will provide a snapshot of current immunotherapeutic targets for atherosclerosis that have a high potential to enter or proceed in the translational pipeline of drug development (Figure 13).

9.3.1 Targeting the innate immune system

9.3.1.1 Bone marrow

Inadequate resolution of low-grade inflammation is integral to all stages of atherosclerosis (96, 106), with macrophages playing key roles in disease initiation, progression, aggravation and acceleration. Swirski, Nahrendorf and others found that atherosclerotic lesions grow through the continuous recruitment of circulating monocytes, and that recruitment is proportional to the severity of the disease (145). In mouse models, they found that hypercholesterolemia triggers progressive monocytosis (146). In the slipstream of these findings, key mechanistic links between accelerated hematopoiesis and lipid metabolism, and the role of extramedullary hematopoiesis in expanding the leukocyte pool have been uncovered (714-716). Besides macrophage expansion through myelopoiesis, local macrophage proliferation as a mechanism that further contributes to lesion growth(144). Most of these insights were acquired in preclinical mouse models of cardiovascular disease, but substantial clinical evidence shows a consistent association between leukocytosis and cardiovascular disease (717).

For the past two decades, stressors that potentially accelerate atherosclerosis progression have been increasingly studied and linked to altered hematopoiesis. Among the different correlations studied, atherothrombotic events themselves, psychosocial stress, sleep and lifestyle factors, and intermitted exposure to high fat diet, have been mechanistically linked to atherosclerosis aggravation in preclinical models and in patients.

In mouse models, macrophage dynamics have been clearly mapped, from monocyte production in the bone marrow, monocyte egress from the spleen (718) and recruitment to the vessel wall to macrophage accumulation in the plaque (146, 716). Importantly, it is becoming increasingly evident, from seminal preclinical work (719) and studies in patients (720), that atherothrombotic events, such as myocardial infarction and stroke, induce monocytosis and aggravate the disease process.

Although our understanding of the immune system's involvement in acute stress-induced cardiovascular events is still in its early stages, its role in the progression of chronic stress-related cardiovascular disease (CVD) is well-established (721-723). Clinical evidence indicates that stress-related disorders, such as post-traumatic stress disorder (PTSD) in combat veterans, significantly elevate the long-term risk of atherosclerosis and related events. Chronic psychosocial stress can increase the risk of myocardial infarction (724-726). A PET imaging study by Tawakol and colleagues showed that increased activity in the amygdala, a brain region central to fear and stress responses, is associated with heightened hematopoietic activity and a greater risk of

cardiovascular events (727). Studies in mouse models show that psychosocial stress and atherosclerosis progression are mechanistically linked by hematopoiesis (728-731).
Leukocyte dynamics and dietary habits influence cardiovascular disease risk. For example, fasting leads to systemic suppression of CCL2 production (732) and an increase in CXCR4 expression on circulating monocytes, triggered by elevated corticosterone levels (733). Interestingly, fasting also promoted the re-entry of monocytes from the blood into the bone marrow, increasing their lifespan (734, 735). In a mouse model of atherosclerosis, intermittent fasting decreased the number of circulating Ly6C^{high} monocytes compared to continuous feeding, resulting in smaller aortic lesions While intermitted fasting has shown clear anti-inflammatory benefits, a recent study in the *Ldlr*$^{-/-}$ mouse model showed that intermitted exposure to a high fat diet causes atherosclerosis acceleration (736). Using bone marrow transplantation and pharmacological interventions, the authors uncovered that myeloid progenitor reprogramming during the first four weeks of high fat diet exposure underlaid the emergency production of neutrophils when re-exposed after an 8-week period of conventional diet (259).
Mediated by chronic, low-grade inflammation, age is among the most critical risk factors for developing atherosclerotic cardiovascular disease. As we age, immune function becomes broadly dysregulated, with innate immune dysfunction increasing systemic inflammation, a process known as inflammaging (737). Accumulating evidence indicates that atherosclerosis accelerates inflammaging. We and others have shown that epigenetic programming of hematopoietic bone marrow stem and progenitor cells (HSPCs)(738) is crucial for immune cell function and drives this premature immune aging process (739).
A landmark immune aging study, published in 2021, shows that transfer of splenocytes from aged mice drives systemic aging, driving senescence of solid organs (740). In human subjects, scoring age based on the frequency of immune cell populations is a much better indicator of immune system function than chronological age and predictive of all-cause mortality in the Framingham Heart Study (741). In line with these observations, the inflammatory age clock correlates with immune senescence, frailty, co-morbidities and cardiovascular aging (741).
As our understanding of the hematopoietic system's central role in atherosclerosis development, leukocyte dynamics, a variety of physical and psychosocial stressor grows, a model in which the bone marrow serves as a central hub that integrating these processes emerges (Figure 12). Continuous research in this space will expand, improve and refine this framework, to aid in designing interventions, which can be focused on lifestyle or diet, but also for developing cardiovascular immunotherapy strategies.

9.3.1.2 Targeting the macrophage

9.3.1.2.1 Macrophage maturation, survival and proliferation

CSF1 is a key cytokine that sustains the differentiation and survival of macrophages. Csf1op/op mice have a naturally occurring gene mutation that results in loss of CSF1, and they develop 70% less lesions than control apo E KO mice despite very high cholesterol levels (1300 mg/dL) (742), indicating that maturation and survival of macrophages is a condition *sine qua non* for the establishment of atherosclerotic lesions. Macrophage maturation has been described in detail in an *in situ* immunohistochemical analysis of surface markers expression by macrophages in human aortic lesions at post-mortem (743). Subtyping of macrophages with antibody against CD14 (LPS receptor), anti CD11b, CD36 (scavenger receptor) revealed a distribution pattern. Classical

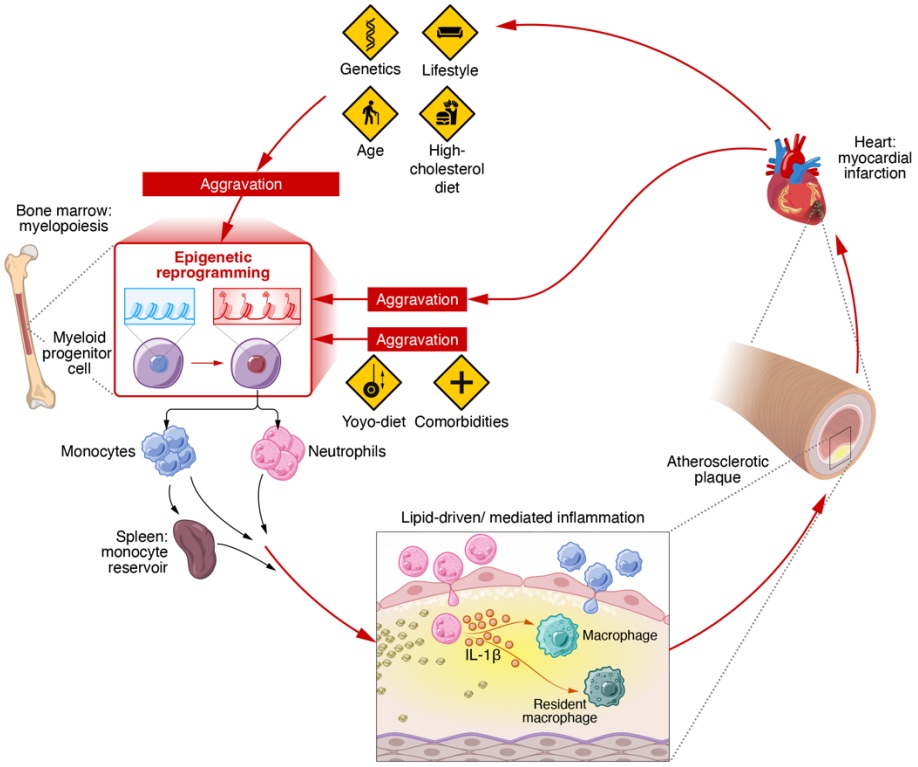

Figure 12: Innate immune cell dynamics: role of the bone marrow
Atherosclerosis is a lipid-driven inflammatory disease that causes macrophage accumulation in the vessel wall by the recruitment of monocytes from the bone marrow, a process that is regulated by enhanced proliferation of myeloid progenitor cells. With genetics and age being uncontrollable factors, several stressors have been identified that accelerate hematopoiesis through the accumulation of epigenetic modifications in bone marrow progenitors, which in turn reduce hematopoietic diversity and cause a myeloid bias that aggravates the inflammatory process. These stressors include, but are not limited to lifestyle and sleep, lack of exercise, a Western type diet and intermitted exposure to this diet, as well as co-morbidities such as diabetes and obesity. Ultimately, inflammatory atherosclerotic plaque rupture may cause myocardial infarction or stroke, events that themselves cause bone marrow progenitor rewiring, but also indirectly contribute through other stressors, creating a positive feedback loop and an elevated risk of recurrent atherothrombotic events.

atheromatous plaques showed a gradual shift in phenotypic expression towards the center of the lesion. Cells in the superficial layers were positive only for CD14 (a monocyte marker), while deeper localized cells were gradually losing the expression of CD14, to become $CD68^+$, $CD11b^+$ and $CD36^+$ cells, with positivity for phosphatase and oil red O, in part exhibiting the features of foam cells. This observation showed a gradual change of phenotype of macrophages, which coincides with developmental stages of the atherosclerotic lesions. In successive studies, also HLA-DP+ cells were found in a study of aortic tissue and coronary arterial specimens (743). Lesional macrophages express also ICAM-1, CD11a, and CD11c (744).
Other growth factors also contribute to macrophage survival and activation. CSF2 or GM-CSF is a cytokine that acts as a growth factor for granulocytes (neutrophils, eosinophils, and basophils) and macrophages. GMCSF promotes the survival and proliferation of CD11c+ myeloid cells in the intima at the site of nascent lesion formation (745). In advanced atherosclerotic lesions GM-CSF–

mediated production of interleukin-23 increases apoptosis susceptibility in macrophages by promoting proteasomal degradation of the cell survival protein Bcl-2 (B-cell lymphoma 2) and by increasing oxidative stress (746).
Macrophages from atherosclerotic plaque are able to proliferate (144, 747, 748). Robbins et al showed that macrophage proliferation is important at the steady state to maintain resident vascular macrophage pools but also in advanced atherogenesis (144). Macrophage proliferation in atherogenesis is driven by scavenger receptor Msr1 (144).Moreover, CSF1 could be important in the maturation process from monocytes to macrophages. Targeting mediators of macrophage maturation, survival and proliferation would be beneficial to reduce atherosclerosis progression.

9.3.1.2.2 Macrophage mediated matrix degradation and thrombosis
Lesional macrophages constitutively express matrix degrading proteinases (MMP-1 interstitial collagenase, MMP2 (72 kDa gelatinase), MMP3 stromelysin, and the 92 kDa gelatinase (MMP-9) (749, 750). Macrophages in the plaque express tissue factor (751), another mechanism leading to atherosclerotic disease phases of instability. Cytokines, such as TNFα and IL-1 are key mediators in metalloproteinase upregulation in macrophages (752), such as in tissue factor expression (753).
In rabbits, foam cells downregulate arginase-1, while upregulating MMP-12 and nitric oxide production, particularly near the lipid core (754). MMP-14^{+} foam cells express the M1 marker COX-2 while TIMP3 (an MMP inhibitor which inhibits foam cell invasion) co-localizes with CD206 (755). MMP-14^{high}TIMP3^{low} foam cells – induced by pro-inflammatory cytokines - proliferate and invade tissues more easily but can also undergo apoptosis following challenge by LPS or starvation (755). TIMP3^{+} foam cells surround clusters of TIMP3^{-} foam cells in the plaque's shoulder regions (755). Therefore, MMP upregulation and TIMP downregulation may facilitate collagen degradation in the core of advanced plaques.
Macrophages are an important producer of MMPs in the plaque. NFκB can activate the expression of MMP-1, -3 and -9 by human macrophages and rabbit foam cells (756). Numerous MMPs have been found in macrophage-rich regions of human plaques, also co-localizing with cleaved collagen, and these MMPs are particularly abundant in inflamed lipid-rich plaques in comparison to fibrous plaques (757). Classical macrophage activation *in vitro* selectively upregulates several MMPs, phospho-JNK and NFκB, and reduces TIMP3, while alternative macrophage activation upregulates a separate group of MMPs and TIMP3 in human macrophages (758). Targeting matrix degradation is a good therapeutic option to prevent plaque growth and destabilization.

9.3.1.2.3 Macrophage cytokine production
Numerous cytokines that have pivotal roles in the pathogenesis of atherosclerosis are produced by macrophages. Macrophages are very important in immunoregulation as they secrete products such as cytokines and chemokines that influence and modulate the immune response. First, they produce IL-12 and IL-18 that direct naïve T cells towards the T_{H1} subtype and induce cell-mediated immunity (759). IL-12 and IL-18, in turn, induce IFNγ production from T cells that activates macrophages further. This process can be blocked by the T_{H2} products IL-4, IL-10 and IL-13 that inhibit the generation of IL-12 by human monocytes and macrophages.

Macrophages secrete GM-CSF, TNFα and IL-1 that enhance dendritic cell antigen-presenting function by up-regulating the expression of MHC, CD80, CD86, CD40 and other costimulatory molecules. IL-10 produced by macrophages and T_{H2} cells down-regulates dendritic cell antigen-presentation and can block this enhancement of antigen presentation. IL-1 has multiple effects on T cells and especially T_{H0} and T_{h2}. It enhances IL-2 and IFNγ production, induces IL-2 receptors on T cells and augments cytotoxic T lymphocyte activity. TNFα also affects T cells. It augments T cell responsiveness to antigen directly or via stimulation of other cytokines such as IL-6, and it up-regulates the expression of the IL-2R. In combination with IL-2 and IL-6, TNFα can also induce T cell activation in the absence of T cell receptor engagement (760, 761). Chronic exposure, however, of T cells to TNFα, however, has been shown to down-regulate T cell responsiveness and proliferation, suggesting that an autoregulatory mechanism is taking place (762).

TNF-α is primarily produced by macrophages in the plaque and its deletion reduces atherosclerotic disease in *Apoe*$^{-/-}$ mice (763). In humans, TNFα is present in plaques and levels of TNFα in the peripheral blood predict future coronary events in post-MI patients (103, 764).

IL-1 is a pro-inflammatory cytokine that drives inflammation in atherosclerosis (reviewed here (765). Both isoforms of IL-1, IL-1α and IL-1β are involved in atherosclerosis. Recent studies in mice showed that IL-1α plays a role in the remodeling of arteries during early atherogenesis, whereas IL-1β is mainly drives vascular inflammation in later stages of atherosclerosis (766). However, IL-1β showed to have a protective role in mice in advanced atherosclerosis as well, via promotion and maintenance of a smooth muscle cells and collagen in the fibrous cap (767). Additionally, IL-1α forms a link between the immune system and coagulation through the activation of IL-1α by thrombin, underscoring its importance of the pathogenesis of CV events (768). In humans, IL-1 is expressed in coronary arteries of patients with CAD and is considered therapeutically tractable (769).

IL-6 is a pro-inflammatory cytokine of innate immunity, that serves as a secondary downstream mediator of cytokines such as IL-1. It is a central stimulus for the acute phase response. IL-6 stimulates the production of CRP, amongst other acute phase reactants, in hepatocytes (770). IL-6 signaling contributes to atherosclerosis and plaque destabilization in mice (771). Human data showed that elevated IL-6 is associated with an increased risk of MI, and genetic studies provided evidence of a causal role for IL-6 receptor signaling in CVD (772-774).

Although anti-IL1β therapy has been shown to ameliorate atherosclerotic CVD (654), and trials blocking IL6 in ASCVD are ongoing, targeting other macrophage cytokines may have better/additional effects on ASCVD.

9.3.1.2.4 Macrophage efferocytosis

Macrophages are main contributors to the formation of the necrotic core in atherosclerotic plaques by undergoing apoptosis (775). Several pathways contribute to these processes. Free cholesterol loading of murine peritoneal macrophages activates Fas ligand which subsequently leads to Fas-mediated apoptosis (776). Activation of endoplasmic reticulum (ER) stress pathway through C/EBP-homologous protein (CHOP) triggers macrophage apoptosis (777). Lipoprotein(a), a carrier of oxidized phospholipids triggers apoptosis of ER-stressed macrophages via CD36/TLR2, has been shown to increase the size of the necrotic core in rabbits (778). Deleting type I IFN signaling (779), TLR2 and TLR4 signaling (780), or the scavenger receptors SR-A and CD36 reduce the progression to advanced necrotic lesions.

Development of the necrotic core can also occur due to defective efferocytosis (clearance of apoptotic cells by macrophages) in advanced lesions (781), through downregulation or cleavage of efferocytosis receptors (782, 783), and dysregulation of expression of "eat me" signals (784, 785). MerTK, an efferocytosis receptor expressed by macrophages, has a non-redundant role in efferocytosis, and its loss causes pro-inflammatory immune responses, accelerates atherosclerosis and increases plaque necrosis (783, 786). LRP-1 (LDLR-related protein 1) mediates cell death and impairment of efferocytosis worsening necrotic core formation (787). Blocking CD47 with a neutralising antibody improved efferocytosis and ameliorated atherosclerosis in *Apoe*$^{-/-}$ mice (785) via reintegration of 'eat me" signals on macrophages, and was shown to reduce arterial inflammation in patients with ASCVD (788). Drugs targeting CD47 (Hu5F9-G4, TTI-621) are currently being tested in clinical studies as cancer therapies (789, 790).

9.3.1.2.5 Enhancing resident vascular macrophages

Microenvironmental factors have huge importance in tissue-specific macrophage programming. During disease, "stress" pathways will also exert their influence (791). Pattern recognition receptors including toll-like receptor (TLRs) and C-type lectin receptor (CLRs) pathways are well placed to sense the environmental cues in macrophages (792). We and others have shown that TLRs have a role in atherogenesis (reviewed in (793)). CLRs are preferentially expressed by the myeloid lineage and recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (794). CLRs carry either an immunoreceptor tyrosine-based activation motif or immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic domain(795),providing a balance in cellular activation in the immune system. ITAM-bearing CLRs such as CLEC4E and CLEC9A promotes atherogenesis by modulating myeloid functions (796, 797). The role of CLRs with an ITIM, such as CLEC4A2, in atherosclerosis was unknown. We recently revealed the protective role of resident vascular macrophages in atherosclerosis, via a CLR with an ITIM, CLEC4A2 (139). Using single cell technologies and conditional ablation, we demonstrated that the expression of the C-type lectin receptor CLEC4A2, also known as dendritic cell immunoreceptor 1 (DCIR1), is a distinguishing feature of vascular resident macrophages endowed with protective properties against atherosclerosis (139). CLEC4A2 is one of the top genes that distinguishes resident macrophages from the activated resident ones at the gene and protein level. Ablation of CLEC4A2^{+} macrophages via a *LysM*$^{Cre+}$*Clec4a2*$^{flox/DTR}$ mouse strain aggravates lesion formation in response to atherosclerosis. Moreover, we showed that CLEC4A2 maintains the vascular resident macrophage pool by licensing monocytes to join the resident pool. Through genetic deletion and competitive bone marrow chimera experiments, we identified CLEC4A2 as an intrinsic regulator of macrophage tissue adaptation by promoting monocyte-to-macrophage differentiation via colony stimulating factor 1 (CSF1) over CSF2 in vascular health and disease.

CLEC4A senses endogenous glycoproteins and HIV1-derived glycoproteins by its carbohydrate recognition domain and signals through its ITIM domain by recruiting tyrosine phosphatases SHP1 and SHP2 (798).Several polymorphisms in the *CLEC4A* gene are strongly associated with susceptibility to rheumatoid arthritis, systemic lupus erythematosus and primary Sjogren's syndrome (799, 800). Mice deficient in the murine homologue, *Clec4a2*, develop worse collagen-induced arthritis and experimental autoimmune encephalomyelitis (801, 802). We showed that during atherogenesis, CLEC4A2 deficiency results in loss of resident vascular macrophages and

their homeostatic properties (139), leading to enhanced lesion formation. Using scRNA-seq and functional assays, we demonstrated that CLEC4A2 limits TLR2- and TLR4-dependent pro-inflammatory cytokine production in murine macrophages. Moreover, *Clec4a2*$^{-/-}$ macrophages accumulate higher levels of intracellular lipid when exposed to oxLDL relative to their WT counterpart, due to downregulation of cholesterol efflux gene expression (*Abca1* and *Abcg1*), ultimately leading to dysregulated reverse cholesterol transport (RCT). SHP1 inhibition replicates the CLEC4A2-dependent lipid dysregulation in WT macrophages (139).

9.3.1.3 Targeting monocytes in atherosclerosis

Chemokine/chemokine receptor mediated mechanisms as well as leukocyte adhesion molecules are crucial in recruiting monocytes into the arterial wall. Blocking these systems result in significant reduction of atherosclerosis in murine models (803). LDL receptor deficient mice that carry targeted deletions of the gene for CCL2 or its receptor CCR2 exhibit significantly less atherosclerosis than LDL receptor KO with intact CCL2 signaling (804-806). A deficiency in adhesion molecules such as P-selectin, ICAM-1 and VCAM-1, or blockage of their interactions with their respective ligands and integrins can reduce monocyte recruitment and atherosclerotic lesion size (807, 808). *In vivo* cell tracking studies showed that inflammatory monocytes use chemokine receptors CX3CR1, CCR2 and CCR5 to enter plaques while resident monocytes only use CCR5 (147). Murine knockout studies showed that Ly-6C^{hi} monocytes depend CCR2 for their mobilization from the bone marrow and tissue infiltration, as a deficiency in CCR2 reduces their numbers in the circulation and tissues while maintaining high levels in the bone marrow (148, 809). Treatment with a CCR2-antagonists in CVD patients decreased hsCRP levels (810). A deficiency in CX_3CR1 reduces lesion size and macrophage accumulation leading to morphological features of a stable plaque (811) suggesting Ly-6C^{hi} monocytes may promote plaque instability. Combined deletion of CCL2, CX_3CR1 and CCR5 virtually eliminates atherosclerosis, monocyte accumulation, and halts bone marrow and blood monocytosis (148). Thus, targeting integrin or chemokine signaling is a therapeutic option to reduce monocyte and macrophage accumulation in atherogenesis (803).

When considering targeting monocytes in atherosclerosis, it is crucial to examine changes in the early stages of hematopoiesis of hematopoietic stem and progenitor cells (HSPCs). These progenitor cells differentiate into and renew the myeloid, lymphoid, and erythrocytic lineages in the bone marrow. More recently, attention has broadened to extra-medullary anatomical sites—locations outside of the bone marrow where HSPCs can produce monocytes (731, 812, 813). Monocytes produced within these extra-medullary sites, similar to those derived from the bone marrow, have been shown to infiltrate atherosclerotic lesions and contribute to atherogenesis (146, 716). Conditions such as hypercholesterolemia, stress, inflammation, and other risk factors associated with atherosclerosis can induce emergency hematopoiesis. This includes extramedullary hematopoiesis, particularly in the spleen, and contributes to disease progression by influencing the differentiation of hematopoietic stem and progenitor cells in the bone marrow towards myeloid lineages. Preclinical murine studies have shown that Ly6Chi splenic monocytes not only contribute to the growth of atheromas but also play a role in plaque instability (716, 718).

Not only classical monocytes are involved in atherogenesis. A recent study identified heterogeneity in nonclassical monocytes in subjects with CAD. An expansion of a Slan^{+}CXCR6^{+} nonclassical monocyte subset in CAD subjects was positively correlated with CAD severity. This nonclassical subset can migrate towards CXCL16 and shows an increased efferocytosis capacity, indicating it may play an atheroprotective role (217). Moreover, intermediate monocytes subsets could be distinguished in human PBMC, by the expression of HLA-DR, CXCR3, and CD206. The subset expressing HLA-DR^{+}CXCR3^{+}CD206^{+} was associated with CAD severity, and it positively correlated with the Gensini Score of CAD severity (814).

9.3.2 Targeting the adaptive immune system

9.3.2.1 Targeting T cells in atherosclerosis

9.3.2.1.1 Vaccination strategies

Immune recognition of antigens in atherosclerosis, especially of (ox)LDL containing peptides results in the generation of autoantibodies and oxLDL-reactive T cells(234). Immunization with ApoB-derived antigens was protective against atherosclerosis via diverse mechanisms including the induction of a humoral antibody response, Treg activation, suppression of CD4^{+} T cells and CD8+ T-cell mediated reduction of dendritic cell numbers in the plaque (815). These findings have yet not translated into vaccination strategies in humans. The Glacier study, conducted in patients with stable atherosclerotic CVD, showed that passive immunization with MLDL1278A, an anti-oxLDL antibody, added to lipid-lowering therapies did not reduce cardio- vascular events or arterial inflammation (816). A caveat of this clinical trial is that in patients with stable atherosclerotic disease, plaque inflammation may be too limited for anti-oxLDL treatment to have an effect. Also, the 3-month follow-up period may be too short to prove effectiveness. The strategy of passive immunization, followed in the Glacier study, bypasses the cellular arm of the immune system and will not lead to protective CD4+ and CD8+ T cell responses. Finally, the focus on one single antigen (oxLDL) may have been too limited in scope as multiple other antigens have been implicated in the pathogenesis of atherosclerosis.

Recent advancements in this field, have set the first steps to better identify the antigen. In silico prediction models have helped to identify which ApoB peptides can bind to the diverse repertoire of major compatibility complex II variants, resulting in the detection of 30 ApoB peptides that were able to induce a human T cell response in vitro (817). Moreover, several studies using scTCR in atherosclerotic plaques identified clonally expanded CD4^{+} and CD8^{+} T cell in atherosclerotic plaques (277). Resolving the antigen specificity of TCR may reveal new antigen of specify existing ones.

Next to the antigen, the vaccine formulation is critical for the type of immune response that is evoked. Formulations that are specifically designed to induce Treg are particularly appealing and have been shown to reduce atherosclerosis in preclinical models (818-820). Detailed characteristics of variants of antigenic peptides presented in atherosclerosis, in combination with for a tailored vaccination formulation will enable more precise vaccination strategies.

9.3.2.1.2 Targeting T cell recruitment

T cells, just like myeloid cells, use chemokines and chemokine receptors to traffic towards and into the arterial wall (803). The CXCR6 receptor is expressed on T cells and promotes T cell homing

and IFNγ production. Mice lacking CXCR6 show a reduction in atherosclerosis (821). CXCR3 is also expressed on T cells, and mainly interacts with CXCL10, but can also bind CXCL9 and CXCL11. Blocking CXCR3 reduces the number of Th1 cells, thereby reducing atherosclerosis (822), and inhibiting CXCR10 shows similar effects (823). Homo-arginine supplementation decreased CXCR3 on T cells and showed a reduction in atherosclerosis (824). Recently, a non-canonical chemokine receptor pathway was discovered, where CCL17, that binds CXCR4 on Tregs to reduce their suppressive effects, was found to bind CCR8 as well, thereby inducing CCL3 and suppressing Treg functions. Deletion of both CCL3 and CCR8 in T cells increased Treg numbers and led to a reduction in atherosclerosis (825). Although we still do not fully understand the intricate chemokine/chemokine receptor system in atherosclerosis, experimental studies have revealed that these are promising targets.

9.3.2.2 Targeting B cells in atherosclerosis

The advent of single cell analytics and other advanced technologies have allowed unprecedented discovery of B cell subtypes and functions linked to atherosclerosis in humans, providing novel immunotherapeutic targets to counter CVD.

B cell targeted therapies are widely used in a variety of disease states including B cell leukemias, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), and multiple myeloma (MM) to treat patients (826-828). Although B cell-targeted therapies are not currently used for atherosclerosis, the association of B cell subtypes and B cell-derived antibodies in human atherosclerosis coupled with mechanistic studies of subset-specific effects of B cells on atherosclerosis in pre-clinical models, suggests that B cell targeted therapies may impact human atherosclerosis. In the following sections, we review different therapeutic strategies and their potential implications for atherosclerosis.

9.3.2.2.1 B Cell Reduction

Drugs that deplete B cells and plasma cells include rituximab-CD20, ofatumumab-CD20, ocrelizumab-CD20, blinatomumab-CD19, inebilizumab-CD19, inotuzumab-, ozogamicin-CD22, elotuzumab-SLAMF7, daratumumab-CD38, GSK2857916-BCMA, AMG420/BI836909-BCMA (826, 827). Given B cell subset-specific effects on atherosclerosis, the impact of these drugs on atherosclerosis would likely be dependent on which B cell subset is most affected.

CD20-specific monoclonal antibody depletion of B cells in mice preferentially depleted B2 cells, caused a greater reduction in IgG than IgM, resulting in less atherosclerosis (407, 409). The attenuated impact on IgM is likely due to the fact that despite both B1 and B2 cells expressing CD20, the niches where they are predominantly found may not be equally accessible to the delivered antibody. For example, 95-98% of B cells in the bone marrow, blood, spleen, lymph nodes, and gut-associated lymphoid tissues were depleted by anti-CD20 antibody, while only 30-43% of B1 cells and 43-78% of B2 cells were depleted in the peritoneal cavity (the homeostatic niche for B1 cells) (829). Rheumatoid arthritis (RA) patients that take rituximab demonstrate decreased inflammation, improved endothelial function, and decreased carotid intima-media thickness (cIMT) (828, 830), suggesting that rituximab can be beneficial for CVD. In kidney transplant patients treated with rituximab for desensitization of ABO-incompatible organs, there was a lower cumulative rate of atherosclerotic cardiovascular disease especially if the subjects had diabetes mellitus, pretransplant dialysis or older age (831). Yet, most clinical trials of

rituximab for autoimmune diseases do not provide convincing evidence for reduction of major adverse cardiovascular events (832). The RITA-MI study showed that rituximab could be applied safely in CVD patients (672), and follow up studies are eagerly awaited (672).

B cell numbers can also be reduced by agents that target factors that promote B cell survival such as B-cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL). Binding of these ligands to receptors such as BAFFR and TACI, in conjunction with antigen to the BCR, induces activation of NF-κB to increase B cell survival. Clinical uses for therapies targeting BAFF/BAFFR and APRIL include treating graft-versus-host disease (GVHD), RA, SLE, and myasthenia gravis (833, 834). Blocking BAFF-BAFFR interaction in mice reduces B2 B cell survival and *Ldlr*$^{-/-}$ mice with BAFFR deficiency had decreased B2 but not B1a B cells and less atherosclerosis (835). Similar findings were reported in BAFFR-deficient *Apoe*$^{-/-}$ mice (410). However, BAFF has been shown to increase anti-oxLDL IgM production and reduce atherosclerosis in hyperlipidemic mice (836) and to have effects on other immune cells such as regulatory T cells (Tregs) which expand in response to BAFFR activation (837). Whether these agents could have a role in reducing atherosclerosis in humans remains to be seen but addressing this may be complex given the myriad effects of the BAFF family members.

9.3.2.2.2 Plasma Cell Depletion

Therapies aimed at depleting plasma cells are frequently applied to treat multiple myeloma (GSK2857916-BCMA, AMG420/BI836909-BCMA, elotuzumab-SLAMF7, daratumumab-CD38) (838), although these therapies have been associated with vascular thrombosis in humans (839). The potential of plasma cell depletion to treat atherosclerosis is debatable. Plasma cell depletion has been shown to reduce atherosclerotic lesion size (840), but to increase plaque instability (841). A similar phenotype was observed in a murine model of deficient antibody production, which had smaller plaques, but showed large necrotic cores (415). These findings raise concern for applying this treatment strategy for atherosclerosis and underscores the importance of evaluating subjects receiving these therapies for untoward cardiovascular effects.

9.3.2.2.3 B Cell Receptor (BCR) Modulation

Several therapies have been developed to modulate BCR activation to treat cancers such as chronic lymphocytic leukemia (842). As BCR activation can results in release of chemokines such as CCL3 and CCL4 that enhance infiltration of monocytes and T cells into local tissues (843), blocking BCR activation with Bruton's tyrosine kinase (BTK) inhibitors, such as Ibrutinib and acalabrutinib, and Epratuzumbab, an agonist of CD22, would decrease chemokines (844). Perhaps BCR signaling inhibitors could be atheroprotective due to diminished pro-inflammatory chemokine release, although this is not known.

In summary, B cells are important regulators of diet-induced atherosclerosis in mice, exerting their effects in a subset-dependent manner. Evidence in humans, while largely associative, supports these findings. Together, they lay a foundation for potential B cell targeted immunotherapy approaches for atherosclerosis. Yet, more work is needed to develop, implement and evaluate the effects of B cell therapies in human atherosclerosis. Attention to utilizing current clinical studies and clinical practice in oncology or rheumatology that employ antibody or small molecules that modulate B cell numbers, function and T cell interactions on subclinical and clinical cardiovascular events could be an important and powerful first step.

9.3.3 Targeting the immunological synapse in atherosclerosis: co-stimulation & co-inhibition

Co-stimulatory and co-inhibitory members of the immune checkpoint family are important regulators of immune responses. Pre-clinical, as well as limited clinical data have proven the potential of co-stimulatory and co-inhibitory immune checkpoint molecules as immunotherapeutic target for atherosclerotic CVD (845).

9.3.3.1 Targeting co-stimulation in atherosclerosis: the CD40-CD40L dyad

Inhibition of CD40 or CD40L results in a reduction of atherosclerosis and atherosclerotic plaque stability. The CD40-CD40L dyad exerts cell divergent functions, that all affect atherosclerosis via different mechanisms as has been described in *section 8*.

CD40's cell divergent functions are also reflected in its signal transduction pathways. CD40 has no intrinsic signaling, but uses adaptor molecules, the TNF Receptor Associated Factors (TRAF), which are involved in signaling of many members of the TNFR family. In B-cells, CD40 signaling is executed by TRAF2 and TRAF3, which often are counteractive, thereby carefully regulating B cell function (846) (847). In endothelial cells, CD40-TRAF1, -3 and -6 interactions seem to hamper inflammation, whereas CD40-TRAF2 and -TRAF5 interactions aggravate inflammation (848). Monocyte and macrophage CD40 favor TRAF6 as main signaling molecule to induce activation (849). In atherosclerosis, blocking CD40-TRAF6 but not CD40-TRAF2/3/5 signaling reduces atherosclerosis burden, which was mostly due to a reduction in monocyte recruitment and macrophage activation (487). Administration of a small molecule inhibitor that was designed to block CD40-TRAF6 signaling (850, 851) reduced atherosclerosis, even when given to mice with established atherosclerosis (852). The CD40-TRAF6 SMI reduced monocyte and macrophage activation, as it lowered canonical NFkB activation (852). This immunotherapy was safe and selective to macrophage function, as treated *Apoe*$^{-/-}$ mice did not show any immune suppressive side effects: antibody production and Ig isotype switching was still intact, and antigen dependent T cell proliferation was not affected (852). Incorporation of the CD40-TRAF6 inhibitor into HDL nanobiologics, enabling macrophage targeted delivery of the inhibitor stabilized atherosclerotic plaques in *Apoe*$^{-/-}$ mice (853), and was deemed safe in non-human primates (853). Blocking CD40-TRAF6 interactions were also found beneficial to treat obesity- and hypertension-induced vascular inflammation and oxidative stress (854-856), as well as graft vs host disease by modulating trained immunity (857).

CD40L can bind to other receptors than CD40. The interaction between CD40L and Mac-1 plays a significant role in leukocyte recruitment. A peptide that specifically blocks CD40L at its Mac-1 interaction site (cM7) significantly reduces leukocyte recruitment in peritonitis and atherosclerosis (858), and a monoclonal antibody against CD40L's interaction site at Mac1 (anti-M7) was highly effective in reducing myeloid cell recruitment in a murine model of sterile sepsis (859).

Clinical trials that block CD40 or CD40L with CVD parameters as primary outcomes have not yet been performed. Until now, we have not fully grasped the mechanisms of actions of the CD40L-CD40 dyad in atherogenesis. Our preclinical data have revealed that cell-targeted anti-CD40(L) therapies, therapies targeting CD40-signaling intermediates or therapies targeting non-classical CD40L interactions are preferred to complete blockage of CD40(L) to circumvent immune suppressive side-effects. As many trials are currently running that are testing the clinical effects of blocking CD40L or CD40 in human auto-immune diseases, we hope that cardiovascular

readouts of these trials will be assessed and become available. Meanwhile, further development of targeted CD40(L) based immunotherapeutics for ASCVD, including anti-M7 antibodies (859), and CD40-TRAF6 SMIs (852, 853) for 'in human use' are eagerly being awaited.

9.3.3.2 Targeting co-inhibition in atherosclerosis

Inhibitory Immune Checkpoint inhibitors (ICIs) such as nivolumab (anti-PD-1), pembrolizumab (anti-PD-L1/L2), atezolizumab (anti-PD-L1), durvalumab (anti-PD-L1), avelumab (anti-PD-L1), and ipilimumab (anti-CTLA4) (860) are widely used in oncology and mostly act via inhibition of PD1 or PDL1/2. Although very successful in treating cancer, these immunotherapies are associated with cardiotoxicity (i.e., myocarditis, acute myocardial infarction, and vasculitis) and increased atherosclerosis-driven cardiovascular events (707, 861). These studies revealed the importance of the PD1-PDL1/2 axis in atherogenesis. Agonizing PD1/PD-L1 interactions or activating other co-inhibitory molecules, such as CTLA4, Tim3, and Tigit in atherosclerosis would potentially be of interest. However, additional research in both pre-clinical models and in clinical trials using ICIs is sorely needed.

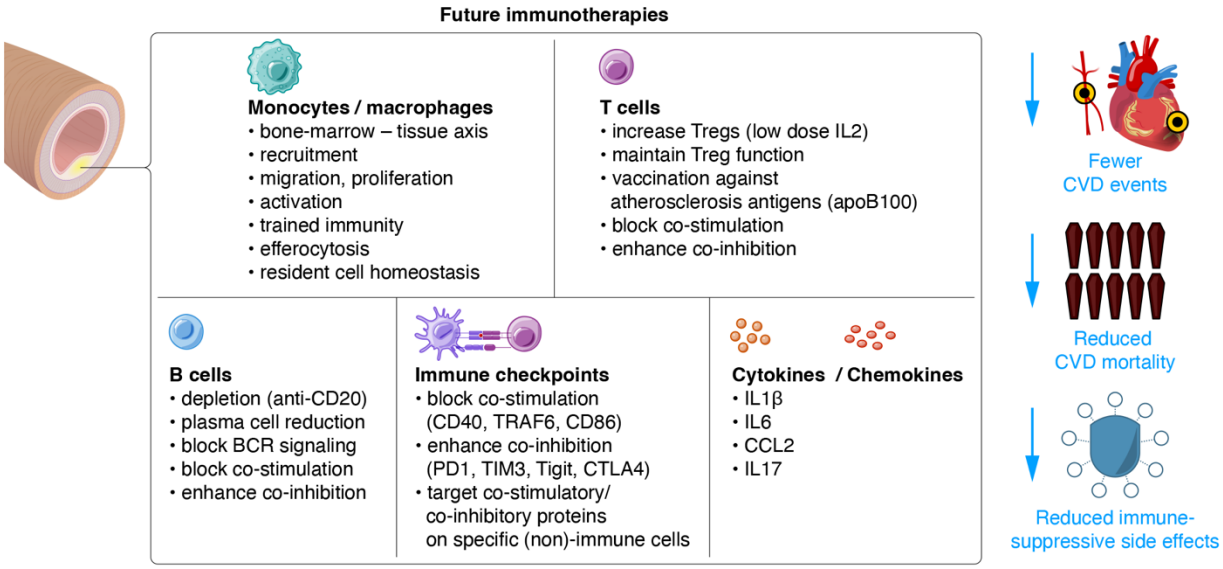

Figure 13: Future immunotherapeutic strategies for the treatment of atherosclerosis. Current immunotherapies in CVD are promising, but need optimization as some induce side effects, and do not decrease overall mortality. Future immunotherapeutics aim to target atherosclerosis-associated immunological and inflammatory pathways in a cell-type specific manner to reduce CVD and overall mortality without causing side-effects.
Add more chemokines/cytokines?: CCL17..

10. IMMUNOTHERAPIES FOR ATHEROSCLEROTIC CVD: AN OUTLOOK

Immune cell activation and its resulting inflammation play an important role in the pathogenesis of atherosclerosis. We are obtaining more and more insights on the cell-subsets that are involved in atherogenesis and on the intricate networks and mechanisms of immune cell communication that drive plaque progression. The immune system is distributed in its architecture, and it operates through a network of cells, tissues, and organs, collectively defending against pathogens

while at the same time maintain homeostasis. This is a lot of moving pieces. To choose the most effective immunotherapy for ASCVD, we need to tackle simultaneously all the key components of this decentralized system including: i) the immune cell diversity that allows for specialized functions; ii) the vascular tissue-based immunity tailored to atherosclerosis; iii) the communication networks that coordinate immune responses across different locations in the body. This is more critical now than ever because we witnessed a recent shift in the whole CV *exposome*, with and better management of lipid metabolism and a major epidemic of diabetes and obesity, linked to cardiometabolic inflammation.

The clinical trials so far have highlighted the importance of selecting patients with biomarker evidence of inflammation (653, 656). However, other options for trial design will have a transformative effect on the field. When designing clinical trials to test immunotherapeutics in cardiovascular disease, the current read-out remains the occurrence of a cardiovascular event. This requires large populations, and an extensive trial period, as the percentage of cardiovascular events is rather low. It is therefore of utmost importance to develop novel read outs that can detect (a reduction in) cardiovascular inflammation, such as novel blood biomarkers or CT or PET imaging markers(110, 862). Finally, biomarkers should be used also to monitor the drug response early. For instance, in a CANTOS sub-study, lack of effect on CRP at 3 months indicated lack of efficacy on outcomes (656). Non-responders can be identified early and numbers needed to treat can be refined, as well as risk of loss of homeostasis. Targeting canakinumab or other immunotherapies to responders might improve clinical effectiveness and cost-effectiveness.

We need to widen our scope of immunotherapeutic targets by sorting the pieces of the cardiovascular immunology puzzle and building a cardiovascular immunology-driven pipeline of therapeutic targets. Numerous pre-clinical studies targeting a myriad of cells, molecules and processes involved in atherogenesis have been shown to reduce atherosclerosis in mouse models, which makes selection of novel therapeutic targets, relevant for human ASCVD difficult. We probably have most pieces of the puzzle, but no framework for their hierarchical spatio-temporal placement and, thus, knowledge on how to target them in the right place, at the right time.

In the last three decades, we in the biomedical community have seen the advent and dramatic rise of genomic and functional genomic approaches driven by high throughput sequencing technologies. These have had a dramatic impact on the discovery process including helping generate hypotheses for further downstream mechanistic studies. Along with enabling investigator-initiated studies, these technologies have catalyzed large-scale genomics consortia including those working on the cellular molecular drivers of cardiovascular disease which have helped uncover the critical role the immune system is playing in disease etiology as well as providing valuable data resources. These datasets are increasingly pointing to the importance of the Immune system in human CVD. In a meta-analysis of genome-wide association studies, the CARDIoGRAMplusC4D consortium assessed 6.7 million common and 2.7 million low-frequency variants (863). Along with confirming known CAD-associated loci, they identified ten new loci which included genes involved in leukocyte migration and anti-inflammatory effects. Another large-scale program, the Trans-Omics for Precision Medicine (TOPMed) works toward elucidating the genetic architecture and biology of heart, lung, blood and sleep disorders and includes a number of CVD-related consortia including Multi-Ethnic Study of Atherosclerosis (MESA) and Cardiovascular Health Study (CHS)(864). TOPMed has generated over 180,000 whole-genome

sequences as well as RNA-seq, DNA methylation, protein, and metabolic profiles for thousands to tens of thousands of samples (https://topmed.nhlbi.nih.gov). Notably, many of these profiles are derived from human PBMCs. Additionally, the following search of the Gene Expression Omnibus (GEO) database (865) "cardiovascular disease AND immune" yielded 1431 studies which contained some kind of omics data. Many of the datasets generated by these consortia as well as individual investigators contain bulk RNA-seq data which represents an effective average of gene expression levels over cell types. Fortunately, increasingly powerful deconvolution methods are capable of deriving relatively accurate estimates of cell type proportions as well as estimates of average gene expression levels for each cell type (866-868) (869) (870, 871). Along with the results of these and many other studies, the data that they've generated and made publicly available together with many powerful tools enable reanalysis that will yield further insights into the role of the immune system in cardiovascular disease.

The recent rise in single cell technologies is enabling the field to obtain a detailed view on immune cell subsets that are present in human atherosclerotic plaque, how they interact with each other, and which pathways they employ to exert their function. Although many more studies, data analyses and validation studies need to be performed, these results will certainly facilitate selection of novel targets that are relevant for human disease. In a recent study, single cell data of PBMCs of patients, suffering from CVD, were used to find a drug combatting inflammation in the LINC database, a drug repurposing database. The drug saracatinib was found the reduce inflammation and successfully reduced atherosclerosis and macrophage activation in pre-clinical models (872). The growing number of single cell studies are revealing the role of the immune system in atherosclerosis at various stages of disease progression. However, a serious limitation of single cell omics approaches is their cost. Machine learning based frameworks are beginning to emerge that are effectively attempting to reduce the cost (873). They leverage existing cohort-based studies where bulk RNA-seq was performed for all patients and single cell RNA-seq was performed for a few. They select the most informative patients for further scRNA-seq and generate representative scRNA-seq for the remaining patients (873). The combination of growing omics assays, available data and powerful analytical frameworks holds great promise for further revealing the critical role that the immune system is playing in cardiovascular disease and identifying immune-based therapeutic targets.

Immunotherapies are not without risks. Targeting the immune system may result in immune suppressive side effects, that are, in a slowly developing disease as atherosclerosis, not acceptable. Recent insights have shown that many pathways in the immune system show cell-divergent effects. Cytokines may be pro-inflammatory for one cell-type, and anti-inflammatory for the other cell type (874), which is also true for other molecules, including costimulatory and co-inhibitory immune checkpoints (845). Therefore, cell-targeted immunotherapeutic approaches are preferred. Cell targeting can be accomplished using cell-preferential nanoparticles or nanobiologics (875), bi-specific antibodies or targeting unique cell-specific protein-protein interactions. Nucleic acid based immunotherapeutics, in the form of siRNA, antisense oligonucleotides and mRNAs have been proven powerful ways of intervening with the immune system. Recently, a new platform has been developed that enables specific delivery of nucleic acid-based therapeutics to myeloid cells and its progenitors, using apolipoprotein-based nanoparticles (876). This approach was proven successful in a syngeneic tumor model in mice

(876), and has great potential for delivering myeloid-cell restricted immunotherapeutics in atherosclerosis.

The atherosclerosis field is entering a new and exciting era, in which we will obtain more detailed insights into the immunological pathways that are so relevant in atherosclerotic cardiovascular disease. Although we will face significant challenges to design and implement (Figure 12) immunotherapeutics to combat atherosclerotic CVD in the next decades, immunotherapies will become part of the standard of care to treat cardiovascular diseases.

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