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REVIEW

Inflammation and immune system interactions in atherosclerosis

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Abstract Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for 16.7 million deaths each year. The underlying cause of the majority of CVD is atherosclerosis. In the past, atherosclerosis was considered to be the result of passive lipid accumulation in the vessel wall. Today's picture is far more complex. Atherosclerosis is considered a chronic inflammatory disease that results in the formation of plaques in large and mid-sized arteries. Both cells of the innate and the adaptive immune system play a crucial role in its pathogenesis. By transforming immune cells into pro- and anti-inflammatory chemokine- and cytokine-producing units, and by guiding the interactions between the different immune cells, the immune system decisively influences the propensity of a given plaque to rupture and cause clinical symptoms like myocardial infarction and stroke. In this review, we give an overview on the newest insights in the role of different immune cells and subtypes in atherosclerosis.

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Introduction

The most common underlying cause of cardiovascular diseases, such as myocardial infarction or stroke, is atherosclerosis [1, 2]. Atherosclerosis is a slowly progressing disease in which lesions (plaques) are formed in large and mid-sized arteries. Risk factors are hypertension, diabetes, smoking, and excessive food intake, but also previous infections (influenza, oral pathogens) or underlying (auto) immune diseases like lupus, Wegener's granulomatosis or rheumatoid arthritis [3–6]. Although plaques can grow to a sufficiently large size to compromise blood flow, most of its clinical complications are attributable to arterial occlusion due to plaque erosion or rupture [7]. Plaques form at predisposed regions characterized by disturbed blood flow dynamics, such as curvatures and branch points [7]. In the past two to three decades, experimental and patient studies have fueled the notion that atherosclerosis is a lipid-driven chronic inflammatory disease of the arterial wall in which several components of both the innate and adaptive immune system play a pivotal role.

The development of atherosclerosis is initiated by activation, dysfunction and structural alterations of the endothelium leading to subendothelial retention of lipid components from the plasma, such as low-density lipoprotein (LDL). Here, lipids are susceptible to modification by oxygen radicals (like reactive oxygen species) and enzymes (such as myeloperoxidase and lipoxygenases) initiating the inflammatory process. The endothelium becomes activated, secretes chemokines such as CCL2, and starts expressing adhesion molecules, such as E-selectin and VCAM-1, thereby promoting the

adhesion of leukocytes and activated platelets to the endothelium. Activated platelets secrete additional chemokines (like CCL5 and CXCL4) and undergo interactions with leukocytes to further boost immune cell infiltration [8]. Monocytes, T cells and dendritic cells (DCs) are the first cell types present in the lesions. In the intima, monocytes differentiate into macrophages (or DCs). Subsequently, these phagocytes start to ingest (modified) lipids and become 'foam cells'. T cells are recruited in parallel with macrophages and also produce atherogenic mediators. DCs are already present in normal arteries but are actively recruited during atherosclerosis [9].

Most of our recent insights are derived from experiments performed in atherosclerotic mouse models, i.e. the ApoE$^{-/-}$ mouse and the LDLr$^{-/-}$ mouse, which have slightly different characteristics. ApoE$^{-/-}$ mice have a spontaneously hyperlipidemic profile, and develop atherosclerosis without dietary intervention, whereas LDLr$^{-/-}$ mice only develop atherosclerosis when fed a high fat diet. By varying the amount of cholesterol and fat in the diet, atherosclerotic plaque progression in both mouse models can be modulated, and atherosclerotic plaque burden, activation of the immune system and lipid levels are thus dependent on the setting and model in which the experiment has been performed [10]. These factors can potentially influence the outcome of the results. Therefore, the findings listed in this review should be interpreted with some caution. Moreover, atherosclerosis is not a homogeneous disease, but can differ in its progression in the different sites of the arterial tree. Data obtained from one site are therefore not necessarily true for the other sites, although in most cases, the effects of an intervention are similar at different sites [10–12].

In this review, we discuss the newest insights on the role of the individual immune cell types and their interactions during innate and the adaptive immune responses in atherosclerosis. The review is based on data that are obtained from, and confirmed by multiple experiments performed by different laboratories in humans and mouse models of atherosclerosis.

Innate immune cells in atherosclerosis (Fig. 1)

The innate or non-specific immune system is the first line of defense in the body and includes anatomical (e.g., the skin) and humoral barriers (e.g., complement), as well as cellular components (e.g., phagocytes). In contrast to the adaptive immune system, the innate immune system has no memory, but recognizes, responds to, and combats pathogenic substances fast and in a non-specific manner.

Monocytes and macrophages

Monocytes are short-lived mononuclear phagocytes of myeloid origin that represent about 3–8 % of total leukocytes in the blood [13]. In mice, two monocyte subsets have been identified [14]: The inflammatory monocyte, which is preferentially recruited to inflamed tissues and has a Ly6C^{high}CX3CR1^{low}CCR2^{+} profile, and the resident or patrolling monocyte, which is characterized by CX3CR1-dependent homing to non-inflamed tissues and has a Ly6C^{low}CX3CR1^{high}CCR2^{-} profile [13–16]. Both subsets can differentiate into macrophages and dendritic cells, and Ly6C^{+} cells are able to convert to Ly6C^{-} cells in vivo [13, 15]. In humans, three major monocyte subsets exist [17, 18]. The "classical" CD14^{++}CD16^{-} subset resembles the mouse Ly6C^{high} inflammatory subset and also highly expresses CCR2, whereas the "non-classical" CD14^{+}CD16^{++} monocytes are a possible counterpart of mouse Ly6C^{-} cells, expressing high levels of CX3CR1 and CCR5 but low levels of CCR2 [19]. Additionally, an "intermediate" CD14^{++}CD16^{+}CCR2^{+} subset can be distinguished [20].

Monocyte recruitment and adhesion to atherosclerotic plaques

Hypercholesterolemia induces monocytosis in ApoE$^{-/-}$ mice and especially increases inflammatory Ly6C^{high} monocyte counts [21], which are more prone to enter the atherosclerotic plaque [21, 22]. The increase in monocytes is due to an increase in hematopoietic stem and progenitor cells (HSPCs) in the bone marrow, which are outsourced to the spleen and exert extra-medullary hematopoiesis, thereby generating a splenic reservoir of monocytes that are also able to 'feed' the atherosclerotic plaque [23, 24]. Interestingly, proteins involved in cholesterol efflux pathways tightly regulate proliferation and migration of HSPCs. The ATP binding cassette transporters A1 and G1, as well as apolipoprotein E, are strong inhibitors of myelopoiesis in the bone marrow, and their inhibition induces increased proliferation and mobilization of HSPCs, resulting in monocytosis and neutrophilia, and increased atherosclerosis [25, 26].

Besides a rise in monocyte numbers, chemokine-dependent monocyte recruitment and survival is also increased in atherosclerosis [16, 22, 27]. Tracking of blood monocytes in mice indicates their continuous recruitment to plaques, which increases proportionally with lesion size [28]. Chemokines and their receptors direct cells towards sites of inflammation via interactions with glycosaminoglycans (GAGs) [29]. Blocking CCR2, CX3CR1, or CCR5, or deficiency in their ligands CCL2, CX3CL1, or CCL5, invariably leads to a reduction of monocyte influx in the plaque (both Ly6C^{high} and Ly6C^{low}) and an attenuation of atherosclerosis [27, 30–34]. Cheng et al. reported an increase in CX3CL1 expression in advanced plaques. Other studies report only a minor effect of CCR2 blockade or bone marrow deficiency at later stages of atherosclerosis, suggesting that Ly6C^{hi} are mainly important at earlier stages, whereas Ly6C^{lo} or $^{-}$ are

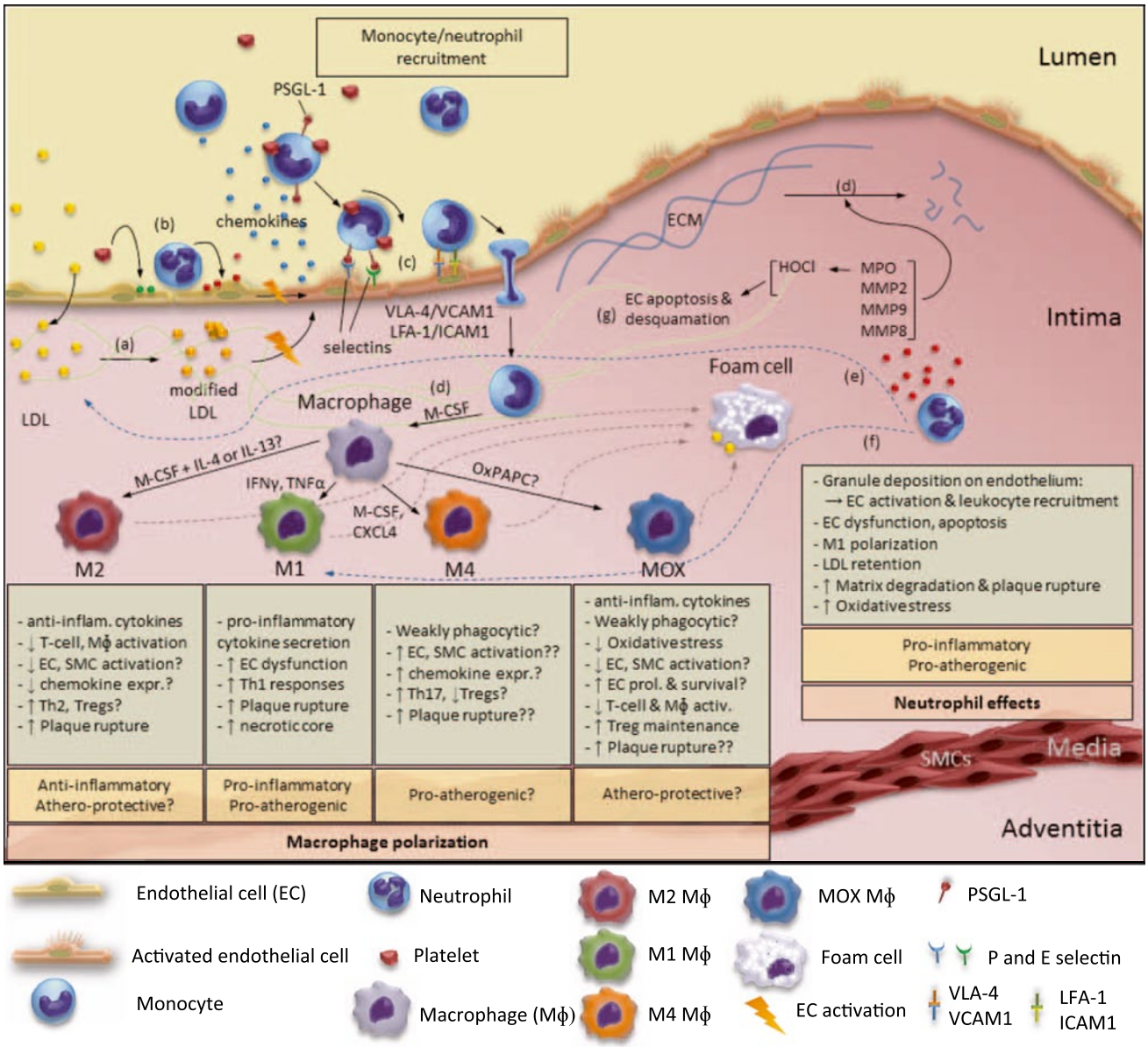

Fig. 1 Role of monocytes and neutrophils in atherosclerosis. **a** Lipoproteins enter the intima, bind to proteoglycans, accumulate, become modified and activate the endothelium. **b** Platelets deposit C–C motif chemokine ligand 5 (CCL5) on the endothelium, promoting neutrophil recruitment to the vessel wall. Activated neutrophils secrete granule proteins such as myeloperoxidase, azurocidin, and proteinase-3 that will enhance endothelial activation and dysfunction by inducing adhesion molecule expression, permeability changes and limiting the bioavailability of nitric oxide. Moreover, granule proteins secreted or deposited on the endothelium induce adhesion and recruitment of inflammatory monocytes, but can also modify chemokines, enhancing their ability to attract monocytes. **c** Activated endothelial cells release chemokines, such as MCP-1, that attract circulating monocytes. Monocytes bind to P and E selectin on endothelial cells, roll and finally come to arrest by adherence of their adhesion molecules (VLA-4, LFA-1) to VCAM-1 and ICAM1 on the endothelium. Platelets promote monocyte-endothelial interactions by expression of P-selectin, but can also form monocyte-platelet aggregates that further promote recruitment. Eventually, monocytes enter the intima through trans-endothelial diapedesis. **d** Infiltrated monocytes differentiate to macrophages, involving M-CSF, after which they polarize into various macrophage subsets (Ml, M2, M4 or MOX) that exert numerous effects and can become foams cells. Subset functions reviewed in Butcher et al. **e** Plaque neutrophils trap LDL in the vessel wall by secretion of α-defensin that binds LDL. **f** Neutrophils promote Ml polarization of macrophages. **g** Neutrophil-derived MMPs and MPO-dependent oxidative stress induces apoptosis of endothelial cells and degradation of basement membrane, leading to endothelial desquamation. **h** Neutrophil MMPs can also degrade ECM components affecting plaque stability. *ECM* extracellular matrix, *MMP* matrix metalloproteinase, *MPO* myeloperoxidase, *LDL* low-density lipoprotein, *M-CSF* macrophage colony stimulating factor, *IFN* interferon, *TNF* tumor necrosis factor, *OxPAPC* oxidation products of l-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphatidylcholine, *EC* endothelial cell, *HOCI* hypochlorous acid, *PSGL-1* P-selectin glycoprotein ligand-1, *VLA-4* very late antigen-4, *VCAM-1* vascular cell adhesion molecule-1, *LFA-1* leukocyte function-associated molecule 1, *ICAM-1* intercellular adhesion molecule, *SMC* smooth muscle cell

particularly prominent at later stages of plaque development [35–37].

Following chemokinesis, monocytes adhere to and roll on endothelial cells through interaction with selectins (such as E- and P-selectin) [38, 39]. During rolling, monocytes upregulate integrins, like $\alpha_4\beta_1$, leading to firm adhesion, arrest, and subsequent diapedesis. Within the intima, monocytes secrete lipoprotein-binding proteoglycans resulting in increased accumulation of modified LDL, which sustains inflammation [40, 41]. The endothelial cell itself also becomes activated and expresses chemokines and proteases, thereby perpetuating the inflammatory response [42–44].

Platelets can promote monocyte–endothelial cell interactions by their expression of P-selectin [8]. Repeated injections of P-selectin-deficient platelets into ApoE$^{-/-}$ mice resulted in smaller lesions compared to mice injected with P-selectin-expressing platelets [8]. Platelet P-selectin is important in the formation of platelet–leukocyte aggregates, which promote the release of chemokines, such as CCL2, CCL5, and cytokines, like IL-1β, enhancing endothelial activation, leukocyte recruitment, rolling, and transmigration [45, 46]. In addition, platelets can deposit chemokines, like CCL5, on activated endothelium, which enhances monocyte recruitment and adhesion to the vascular wall [8].

An alternative route for inflammatory cells to enter the arterial wall is via the adventitia through the vasa vasorum [47, 48]. However, the relative contribution of this process to atherosclerotic plaque development and progression is still under debate.

Macrophages and atherosclerosis

Once in the intima, differentiation factors like macrophage colony-stimulating factor (M-CSF) differentiate monocytes into macrophages [39, 49]. Macrophages are phagocytic cells, but can also instruct other immune cells by producing various immune effector molecules and by acting as antigen-presenting cells (APCs).

Osteopetrotic (op/op) mice, which are deficient in M-CSF and lack macrophages, are extremely resistant to atherosclerosis [50, 51]. CD11b-DTR mice, in which monocytes/macrophages are selectively depleted by diphtheria toxin, show a profound reduction in early plaque development. However, when macrophages are depleted when established plaques have formed, the reduction in atherosclerosis is less clear, suggesting a more important role for macrophages in the initiation of atherosclerosis [52].

Foam cell formation and cholesterol efflux Once macrophages start to ingest and process LDL, they acquire lipid droplets in their cytoplasm. When uptake exceeds efflux, or efflux is disturbed, lipids accumulate and macrophages become ‘foam cells’. Scavenger receptors SRA and CD36 mediate LDL uptake, and gene-deletion or bone-marrow transplantation experiments emphasize their function in (ox)LDL uptake and atherosclerosis [53–56]. However, other studies indicate that SRA and CD36 deficiency do not completely abolish foam cell formation [57, 58], and therefore additional mechanisms, like macropinocytosis or other classes of scavenger receptors, may also play a role.

Once taken up, lipoproteins release entrapped cholesterol, which downregulates the expression of LDL receptors and decreases endogenous cholesterol synthesis. Intracellular free cholesterol undergoes re-esterification by ACAT (acyl-CoA cholesterol ester transferase) [39, 59], but can also traffic to the plasma membrane to become available for efflux [39, 60]. Impairment of efflux or ACAT function leads to cytotoxicity and macrophage death [60]. Removal of cholesterol from the cell occurs at the plasma membrane by passive diffusion or transfer to apolipoprotein A1 and HDL, a process involving ATP-binding cassette (ABC) transporters, in particular ABCA1 and ABCG1 [60]. Deficiency of ABCA1 or both ABCA1 and ABCG1 in bone marrow-derived cells enhances atherosclerosis, and mice expressing the human ApoA-1 transgene, which increases HDL and cholesterol efflux, have reduced leukocytosis and atherosclerosis [60–62].

Macrophages mediate plaque inflammation Macrophages express a myriad of receptors including pattern recognition receptors (PRRs; e.g., TLRs, CLRs, NLRs, scavenger receptors) and cytokine receptors (e.g., TNFRs, interleukin receptors, growth factor receptors) through which they scan their environment for activation or polarization signals (e.g., PAMPs, pathogen-associated molecular patterns; DAMPS, danger-associated molecular patterns; cytokines; and growth factors) [63–66]. Upon activation, macrophages/foam cells produce inflammatory cytokines and chemokines that enhance inflammation and further regulate monocyte/T cell infiltration [67–70]. Macrophages in the atherosclerotic plaque are capable of releasing a large repertoire of proinflammatory cytokines including IL-1, IL-6, IL-12, IL-15, IL-18, TNF family members (such as TNFα), and MIF, as well as anti-inflammatory cytokines like IL-10 and TGF-β family members (TGF-β1, BMPs, GDFs) [71, 72]. In particular, TLR 2 and 4 have been shown to be important stimulators of macrophage cytokine production in an atherosclerotic context [73–76].

Macrophage exposure to crystalline material, like cholesterol crystals that form in the macrophage foam cell after massive uptake of (modified) lipids, but also increased oxidative stress within plaques, can lead to the formation of an inflammasome complex affecting protein maturation and secretion [77]. Inflammasome formation leads to activation of caspase-1 that rapidly cleaves pro-IL1β and pro-IL18 into

their mature forms, which are both pathogenic inflammatory cytokines. Transplantation of Nlrp3, ASC and IL-1 (essential components of the inflammasome complex)-deficient bone marrow in $LDLr^{-/-}$ mice revealed a crucial involvement of the inflammasome in atherosclerosis as both plaque size and serum IL-18 were significantly reduced [77].

Within the atherosclerotic plaque, sustained inflammation, growth factor deprivation, and oxidative stress accompanied by prolonged activation of endoplasmic reticulum (ER) stress pathways result in macrophage apoptosis and necrosis. The unfolded protein response (UPR) [78], with factors like C/EBP homologous protein, $Ca2^{+}$/calmodulin-dependent protein kinase II, STAT1, and NOX, plays a major role in this process [79–82]. Necrosis and apoptosis, and the subsequent defective efferocytosis of macrophage cell debris result in the formation of a necrotic lipid core within the plaque, and can induce a vulnerable plaque [83].

Besides producing inflammatory mediators, macrophages, as well as SMCs and neutrophils, produce proteases, such as matrixmetalloproteases, tPA, uPA, elastases, and cathepsins [84], capable of degrading extracellular matrix components. These proteases contribute significantly to thinning of the fibrous cap, making atherosclerotic plaques more vulnerable to rupture.

Macrophage heterogeneity in plaques Macrophages are a heterogeneous population that can be divided into classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages are induced by TLR ligands (such as LPS) or IFNγ [39]. They enhance and sustain inflammatory responses via production of TNFα, IL-6, IL-1β, and IL-12 [39], and produce killing agents like iNOS. Continuous M1 activation results in tissue damage and eventually impaired wound healing. M2 macrophages are stimulated by cytokines such as IL-4 or IL-13, but also by immune complexes and parasitic antigens [39], and secrete IL-10 and TGFβ. M2 macrophages promote tissue repair and healing, stimulate angiogenesis, scavenge debris, and dampen immune responses [85, 86]. M1/M2 macrophages can switch phenotype depending on their microenvironment [87].

The concept of M1 and M2 macrophages in atherosclerosis is not so clear-cut. Both M1 and M2 subsets are present in human atherosclerotic plaques [88] in all plaque stages [89], with M1 macrophages present at sites of plaque rupture, and M2 macrophages far from the lipid core [90] and in the adventitia [92]. M2 macrophage foam cells contain smaller lipid droplets than M1 macrophages, suggesting less lipid uptake than M1 macrophages [90]. However, other reports show that ER stress promotes M2 polarization and that M2 macrophages contain a higher expression of SR-A and CD36 [91, 92]. In $ApoE^{-/-}$ mice, early plaques predominantly contained M2 (arginase I^{+}) macrophages. With plaque progression, a phenotypic switch towards an M1 (arginase II^{+}) dominant profile was observed [93]. Upon plaque regression macrophages reduce the expression of M1 markers (i.e. MCP-1, TNF) and exhibit more M2 markers (i.e. Arg I, MNR) [94]. These data indicate that the microenvironment at later stages of atherosclerosis promotes M1 polarization, and thus atherosclerotic plaque progression. Interestingly, when macrophages in $ApoE^{-/-}$ mice were polarized towards M2 by schistosoma infection, circulating cholesterol levels decreased and plaque size was reduced or not affected [95–97].

Kadl et al. [98] described a new macrophage subset, Mox, present in advanced murine atherosclerotic plaques. Mox are macrophages stimulated with oxidized phospholipids and are characterized by an anti-oxidant response (through NRF2). They have low phagocytic and chemotactic capacity, and typically express Heme oxygenase-1 (HO-1). Whether Mox macrophages are atheroprotective needs further investigation. Gleissner et al. [99] introduced M4 macrophages, being human macrophages differentiated by CXCL4. This subset is weakly phagocytic, and shows lower expression of scavenger receptors, but increased levels of cholesterol efflux transporters.

In conclusion, macrophages, as the most abundant cell type in atherosclerotic plaques, strongly affect plaque formation and progression through a profound effect on intraplaque cholesterol homeostasis, inflammation, and necrotic core formation as well as extracellular matrix degradation. Affecting atherosclerosis on multiple levels makes macrophages an interesting cell type for the development of therapeutic strategies.

Neutrophils

Neutrophils are among the first cell types to respond to invading micro-organisms or tissue damage by inducing rapid neutralization and clearance of pathogens via endocytosis of foreign material and production of reactive oxygen species, myeloperoxidase (MPO), and proteolytic enzymes. In humans, an association between intra-plaque neutrophil numbers and features of unstable plaques (large lipid core, low collagen, and smooth muscle cell content) [100] has been reported. In $ApoE^{-/-}$ mice, neutrophils interact with endothelial cells and accumulate in regions of high inflammatory activity [101–103]. In early atherosclerotic mouse plaques, neutrophils localize in the sub-endothelial space, while in more advanced to rupture-prone plaques, they can be found in the shoulder region, fibrous cap, adventitia, and in areas of intra-plaque bleeding [101, 102, 104].

Neutrophil granule proteins and atherosclerosis

Much of the neutrophil proinflammatory activity can be attributed to the release of granule proteins. MPO, azurocidin, LL-37, α-defensins, and NGAL have been identified

inside human atherosclerotic lesions [105–109] and are also secreted into the plasma upon neutrophil activation.

Recently, Soehnlein and colleagues [110] reported that Cramp$^{-/-}$ ApoE$^{-/-}$ mice had smaller plaques with reduced macrophage numbers compared to ApoE$^{-/-}$ mice. This effect was attributed to the lack of endothelial CRAMP deposition by neutrophils, resulting in reduced adhesion of classical monocytes and neutrophils. α-defensin, another granule protein, is able to trap LDL in the vessel wall, leading to the accumulation of LDL that will be oxidized and eventually contribute to local inflammation and plaque growth [111, 112].

Neutrophils also affect advanced atherosclerosis by secretion and activation of different matrix metalloproteinases and elaborate MMP8 and a few cathepsins amongst others, which in turn degrade the basement membrane as well as components of the extracellular matrix, leading to plaque fragility and eventually erosion or rupture [113, 114].

In conclusion, various cell types of the innate immune system play important roles in both initiation and progression of atherosclerosis, either reducing or aggravating disease burden. However, as the local inflammation of the arterial wall sustains, many of the immunomodulatory agents secreted by innate immune cells have the capacity to tune or even activate adaptive immune responses, directly or by recruiting key players in adaptive immunity to inflammatory foci.

The adaptive immune system in atherosclerosis (Fig. 2)

The adaptive immune system comprises highly specialized cell types that respond to both microbial as well as non-microbial substances in a very specific way. Adaptive immune responses are slow, are initiated by the innate immune system and require antigen presentation by APCs. 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, clonal expansion and differentiation of lymphocytes to effector or memory cells. Upon exposure to a previously encountered antigen, the appropriate memory cells will generate faster, stronger and more efficient immune responses.

Dendritic cells

Dendritic cells (DCs) are professional APCs that play a critical role in innate, but also in regulation of adaptive, immune responses [9]. DCs originate from DC precursors, coming from the bone marrow, or from monocytes. They can be found in both lymphoid and non-lymphoid tissues throughout the body, where they form sophisticated and complex networks allowing them to interact with different lymphocyte populations. DCs provide an important link between innate and adaptive immune responses and play a critical role in host defense to pathogens and cancer, but also in tolerance to self and prevention of autoimmunity [9].

DC heterogeneity

The dendritic cell population is heterogeneous and can be divided into four major categories [115]: conventional DCs (cDCs), plasmacytoid DCs (pDCs), monocyte-derived DCs, and Langerhans cells. Conventional DCs predominate in a steady state and are specialized for antigen processing and presentation. Two main classes of cDCs exist: migratory DCs (mDCs) and lymphoid tissue resident DCs (rDCs). mDCs are antigen sampling sentinels originating from early precursors in peripheral tissues, are restricted to lymph nodes, and cannot be found in the spleen. rDCs are found in lymph nodes, spleen, and thymus. They can be subdivided into CD4^{+}DCs, CD8α^{+}DCs, and CD4^{-}CD8α^{-} DCs. CD8α^{+} DCs are professional cross-presenting cells and play a major role in priming cytotoxic CD8^{+} T cell responses, whereas CD4^{+}DCs and CD4^{-} CD8α^{-} DCs are more efficient at presenting MHC class II-associated antigens to CD4^{+}T cells. rDCs do not traffic from other tissues but develop from local lymphoid tissue precursor DCs.

During inflammation and in response to growth factors like GM-CSF or TLR4 ligands, monocytes fully differentiate into monocyte-derived DCs. Similar to cDCs, these cells express CD11c, MHC II, CD24, and SIRPα, but also MAC3. Monocyte-derived DCs have an antigen-presenting capacity, including the ability to cross-present antigens.

Acting at the interface of innate and adaptive immunity, pDCs have the unique ability to rapidly produce large amounts of type I interferons, but have only poor antigen-presenting capacity. pDCs are broadly distributed over the body and, at least in mice, express SIGLEC-H, BST2, and CD45RA. Human pDCs are also CD45RA^{+}, but also express BDCA-2 and LILRA4 (ILT7).

DCs and macrophages One of the major problems in studying the role of DCs in non-lymphoid tissues, and especially in the aorta, is that the distinction between macrophages and DCs is not so clear. There is little agreement about the utility of specific markers for identifying distinct cell types in tissues. In a recent paper by Becker et al. [116], a proteomic approach was applied to find membrane markers specific for macrophages, M1 and M2 macrophages, and dendritic cells. Although unique membrane signatures for (M1 vs. M2) macrophages versus DCs could be detected, some frequently used markers disproved to be cell type-specific. One of those common markers to distinguish DCs in mice is CD11c [116]. In atherosclerosis, this problem is even more relevant, since macrophage foam cells in the plaque

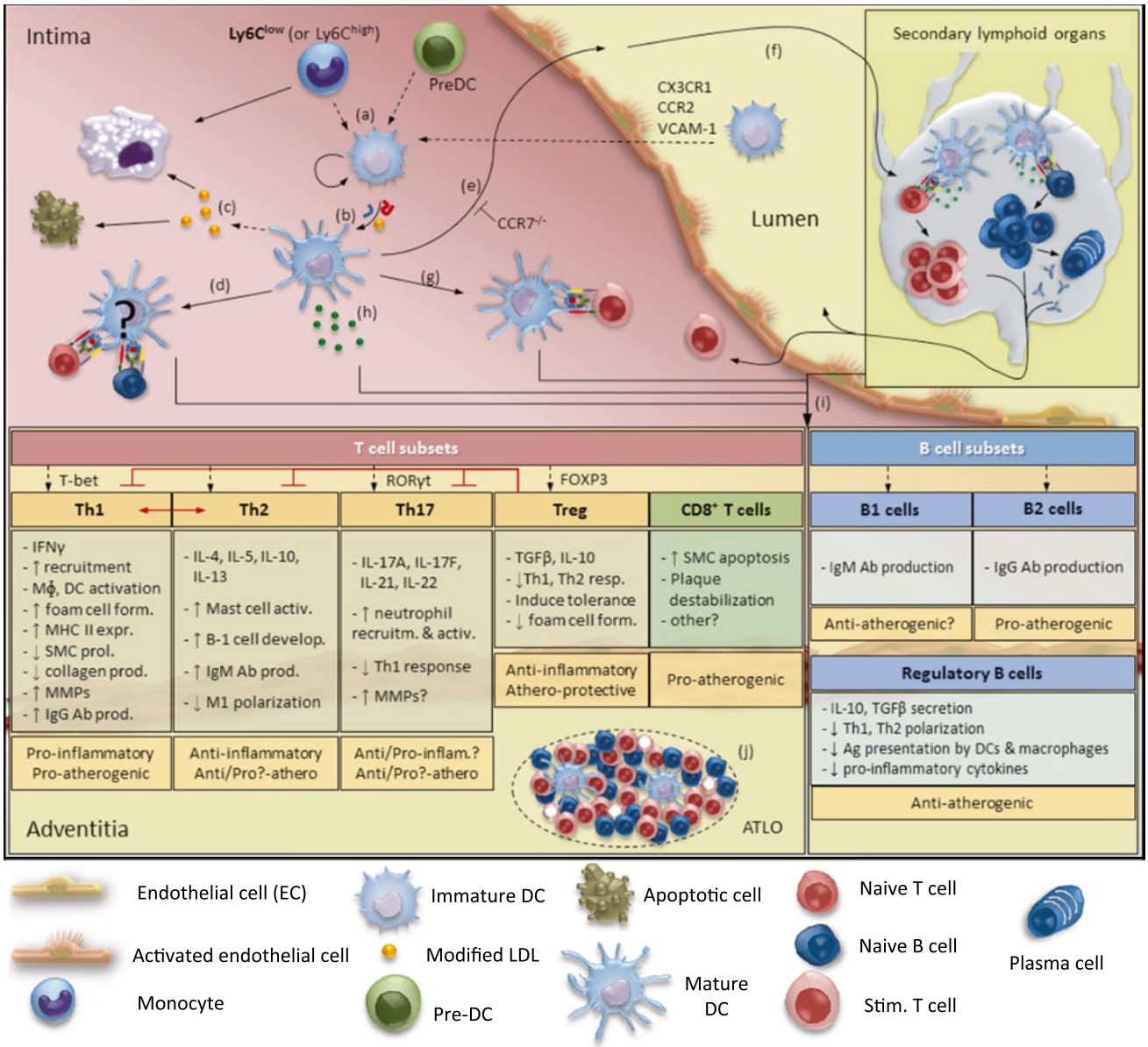

Fig. 2 Dendritic cell functions in atherosclerosis. **a** Dendritic cells (DC) accumulate in the plaque through direct recruitment from the lumen, local proliferation and differentiation from either monocytes (preferentially Ly6C^{low}) or DC precursors. Recruitment of DCs from the plaque to the lumen is CX3CR1, CCR2 and VCAM-1 dependent. **b** Plaque DCs take up (atherosclerosis-specific) antigens, become activated and mature. **c** DCs take up oxLDL and can become foam cells. OxLDL induces DC maturation, but can also trigger DC apoptosis that might contribute to necrotic core formation. **d** Mature DCs are professional antigen presenting cells, whether direct antigen presentation occurs in the plaque is not known. **e** Dendritic cells can emigrate from the plaque into the lumen, a process that is inhibited by CCR7 deficiency and dyslipidemia. Dendritic cells can also emigrate from the plaque via lymphatics. **f** Emigrated DCs migrate towards secondary lymphoid organs (spleen and lymph nodes), where they present antigens to T and B lymphocytes. T cells become activated and clonally expand, after which they enter the blood stream and migrate to the plaque. After DC antigen presentation B cells divide and eventually differentiate into plasma cells. Plasma cells produce various types of immunoglobulin antibodies that affect immune responses. Stimulated T (and B cells) can enter the plaque where they exert different effector functions, either promoting or reducing atherosclerosis. **g** Dendritic cells inside the plaque can restimulate primed T cells entering the plaque, boosting immune responses. **h** Dendritic cells secrete several chemokines that influence leukocyte recruitment to the plaque. Most DC-derived chemokines, like CCL17 and CCL22, are involved in T cell recruitment. Dendritic cells also secrete various pro-inflammatory (e.g., TNFa, IFNγ, IL-6, IL-12) and anti-inflammatory (e.g., IL-10) cytokines that either stimulate or dampen immune responses. **i** DC antigen presentation and cytokine production directly activate various B and T cell subsets that all affect atherosclerosis in specific ways. **j** DCs also contribute to the formation of arterial tertiary lymphoid organs (ATLOs), that affect plaque development remotely. *MMP* matrix metalloproteinase, *LDL* low-density lipoprotein, *EC* endothelial cell, *VCAM-1* vascular cell adhesion molecule-1, *pre-DC* DC precursor, *Ig* immunoglobulin, *SMC* smooth muscle cell, *Mcj* macrophage, *MHC* major histocompatibility, *TGF* transforming growth factor

and lipid-filled DCs, both show an abundant expression of CD11c [117]. However, macrophages and DCs also have unique membrane expression profiles, morphological different features, and exert specific functions, and are therefore truly different cell types.

DCs in atherosclerosis

Although DCs were discovered in 1973 by Steinman and Cohn [118], it took until 1995 before DCs were described in the aorta [119]. Few DCs are present in the normal aorta of healthy mice, where they preferentially reside in the adventitia, apart from a few scattered intimal DCs [120]. DCs are mainly found at sites prone to develop atherosclerosis, such as the lesser curvature and branch points of the aortic arch [121, 122]. $CD11c^+$ DC numbers dramatically increase in both intima and adventitia during atherosclerosis [123–125]. In advanced lesions, DCs cluster with T cells and localize in the plaque shoulder and rupture-prone regions of plaques [121, 126, 127]. In patients with angina pectoris or acute myocardial infarction, blood-derived DC precursors are reduced, while in CAD patients blood DC numbers are down, which might be explained by increased recruitment to plaques [128–130].

Dendritic cells are central to atherogenesis as they are directly implicated in both cholesterol homeostasis and the immune response. Selective ablation of DCs or extension of their lifespan were found to result in an increase or decrease in plasma cholesterol levels, respectively [131]. However, increasing DC lifespan did not affect atherosclerosis progression since the protective effects of cholesterol lowering were counterbalanced by enhanced Th1- and Th17-mediated autoantibody responses. Transfer of DCs pulsed with atherosclerosis-specific antigens results in either protection or aggravation of atherosclerosis depending on environmental signals during DC pulsing and the animal model used [132, 133]. Moreover, vaccination strategies with oxLDL-pulsed DCs before atherosclerosis induction showed a promising reduction in plaque size and overall amelioration of immune–inflammatory responses [134].

Two papers were published recently showing opposing roles for pDCs in atherosclerosis. Daissormont et al. [135] reported a protective role for pDCs, as depletion of these cells in $LDLr^{-/-}$ mice using an anti-PDCA-1 antibody resulted in enhanced T cell accumulation and CD4+ T cell activation and exacerbation of plaque development. In the $ApoE^{-/-}$ mouse, Döring [136] as well as Macritchie et al. [137] recently observed decreases in early plaque formation upon treatment with an antibody against PDCA-1, an effect that was attributed to a TLR9-dependent IFNα release upon pDC activation by the neutrophil-derived DNA/CRAMP complexes [136]. These divergent findings might be explained by the different methodologies used, such as the kind of depletion antibodies and administration regimens.

DC accumulation in plaques DC accumulation in plaques can result from three different events: direct recruitment, local proliferation, and/or impaired egress. Different immune cells in the aorta can attract preDCs and monocytes by expression and secretion of different receptors and cytokines. Absence of CX3CR1, CCR2, or VCAM-1 reduces atherosclerosis not only by an effect on monocyte recruitment but also correlates with decreased DC accumulation [27, 125, 138, 139]. Accumulation of DCs in the arterial wall can also be influenced by interactions with platelets, for example, through P-selectin for rolling and mac1 for firm adhesion [8]. DCs might predominantly differentiate from $Ly6C^{low}$ monocytes that act as precursors for inflammatory DCs [22]. Recruited or resident DCs can proliferate locally, as was recently demonstrated in the aorta and secondary lymphoid organs [140, 141], contributing to increased numbers of DCs. In early atherosclerotic lesions, monocyte-derived DCs can emigrate from lesions; however, in hyperlipidemic mice, the egress from developed plaques might be impaired [142, 143].

DCs and lipid uptake In addition to macrophages, DCs can accumulate lipids and contribute to disease initiation and progression [144]. After only a few days of high fat diet feeding, lipid-loaded $CD11c^+$ DCs can be detected in the aorta of $LDLr^{-/-}$ mice. OxLDL promotes differentiation of macrophages into DCs [145]. Uptake of lipids induces DC maturation markers and enhances antigen presentation to NKT and T cells [146], but does not affect the antigen-presenting capacity of monocyte-derived macrophages [142], and impairs CD40- or TLR-induced dendritic cell maturation [147].

DCs and antigen presentation In atherosclerotic plaques, T cells are found in close proximity with DCs, implying DC–T cell interactions [127, 148]. Several studies have indicated that oxLDL induces several changes that are characteristic for DC maturation, including enhanced expression of co-stimulatory molecules and increased ability to stimulate T cells [146, 149]. Moreover, deficiency of co-stimulatory molecules involved in antigen loading, immunological synapse formation, and T cell activation (CD80, CD86, CD40) all led to reduced atherosclerosis [150, 151]. Several studies using DC transfer, depletion, or modulation, have indicated that DCs are capable of skewing immune responses in atherosclerosis either towards an athero-protective or -promoting profile [131–134]. It is likely that, under atherosclerotic conditions, DCs take up atherosclerosis-specific antigens [152], become locally activated, and migrate out of the plaque towards either local draining or distant lymph

nodes, where they induce T cell activation and proliferation. Indeed, DCs sorted from the aorta have the capacity to induce antigen-specific proliferation of T cells [122, 124, 153]. Moreover, aortic DC were reported to take up injected OVA from the blood, cross-present it to $CD8^+$ TCR transgenic OT-I T cells, and subsequently induce OT-I T cell proliferation after isolation [122], while another study showed that OVA-loaded bone marrow-derived DCs induced OT-I T cell proliferation in the adventitia of OT-I $Rag2^{-/-}$ mice [123]. It is also possible that T cells, originally primed in secondary lymphoid organs, migrate into the plaque to be re-stimulated by DCs locally, which may be more important at later stages of atherosclerosis where DC egress is reduced [154]. Overall, these processes perpetuate local inflammation and increase plaque growth.

DCs and cytokine production Dendritic cells have the ability to produce various anti- and pro-inflammatory cytokines. TLR engagement, for example, can lead to the production of pro-inflammatory cytokines, including TNF, IL-6, and IL-12, all of which have been shown to be atherogenic [152, 155–160], but TLR induction can also lead to IL-10 production, which is atheroprotective [161]. $IL12p40^{-/-}ApoE^{-/-}$ mice have smaller lesions [157], whereas recombinant IL-12 injection increases lesion size [158]. IL-12 affects atherosclerosis by driving Th1 polarization and T cell recruitment [160]. Dendritic cells also produce many other cytokines, like IL-23 or IL-27, for which the role in atherosclerosis remains unclear [162]. pDCs typically produce high amounts of IFNα and β upon TLR9 activation, of which the latter has been shown to promote atherosclerosis by stimulation of macrophage recruitment [163].

Some cytokines produced by DCs in an atherosclerotic environment are chemokines that influence immune cell recruitment into the lesion. Most DC chemokines are involved in T cell recruitment. For example, CCL17 (TARC) and CCL22 (MDC) [164] are expressed in the plaque and attract T cells by interaction with the CCR4 receptor. Recently, Weber et al. [124] described CCL17-expressing cDCs in the aorta of $ApoE^{-/-}$ mice. These cells arev associated with T cell recruitment; however, Treg accumulation was decreased combined with restrained Treg homeostasis in lymph nodes, contributing to atherosclerosis. Secretion of CCL2 by DCs was shown to play a role in the recruitment of monocytes, memory T cells, and DCs to the site of inflammation [165]. In addition, DCs also produce CCL4 that attracts NK cells, monocytes, and some other immune cells [166].

DCs and tolerance

Under homeostatic conditions, DCs are known to have a tolerogenic effect [167]. In the normal artery wall, resident DCs are thought to promote tolerance to antigen by silencing T cells. However, the inflammatory atherosclerotic microenvionment can activate DCs to switch from tolerance to activation of the immune system [168, 169]. Interestingly, Hermansson et al. [133] recently showed that this switch can be reversed, as injection of DCs pulsed with ApoB100 in the presence of the immunosuppressive cytokine IL-10 conferred protection against atherosclerosis in $ApoB100^{tg}LDLr^{-/-}$ mice. Therefore, inducing tolerance to atherosclerosis-specific antigens might be a promising therapeutic target for the treatment of atherosclerosis.

In conclusion, dendritic cells influence atherosclerosis by production of chemokines and cytokines, antigen presentation, and lipid uptake either promoting inflammation or inducing tolerance. However, the exact role of dendritic cells in directing different T and B cell subsets during atherosclerosis is not yet fully understood.

T cells

T cells are lymphocytes that are characterized by the presence of a T cell receptor (TCR) on their cell surface. They originate from hematopoietic stem cells in the bone marrow that give rise to progenitors which migrate to the thymus for further development, maturation, and selection to become T cells. After maturation, T cells are released from the thymus and are present in the blood and lymph nodes, where they play a central role in adaptive immunity. However, subsets of T cells, such as the $CD4^+$ T cells, also exert innate immune cell functions by activating various innate immune cells and helping macrophages to kill intracellular pathogens [170]. When T cells encounter an antigen-presenting cell (APC) that presents a peptide specific for their TCR, an efficient T cell response can be initiated.

T cells were first detected in human plaques in 1985 [171], followed by the observations that HLA/MHCII and T cell cytokines, such as IFNγ, were also present. The detection of antibodies and T cells specific for oxLDL, combined with the presence of oligoclonal T cell populations in lesions, confirmed a role for adaptive immunity in atherosclerosis [171–176]. $Rag2^{-/-}ApoE^{-/-}$ mice that were fed a normal chow diet showed a decrease in atherosclerosis. However, when fed a high cholesterol diet, the effects were less clear [177]. Similar results were obtained in the $LDLR^{-/-}$ model. When fed a Western-type diet for a short period, $Rag1^{-/-}LDLR^{-/-}$ mice showed a decrease in atherosclerosis. However, when the diet was prolonged for 12 or 16 weeks, the effects were negligible [178]. In a different study, where 2 strains of $Rag2^{-/-}LDLR^{-/-}$ mice were fed a 'milk fat' or Western-type diet for 12 weeks, $Rag2^{-/-}LDLR^{-/-}$ that were 93 or 96 % backcrossed to C57Bl6 mice showed a significant reduction in atherosclerosis in the aortic sinus. However, in the innominate

artery, only the Rag2$^{-/-}$LDLR$^{-/-}$ mice that were 93 % on a C57Bl6 background, showed a reduction in atherosclerosis [179]. These somewhat ambivalent data show that lack of T and B cells decreases atherosclerosis, but that this effect is dependent on diet (duration), the site of the arterial tree, and the genetic background.

T cells are recruited to the vessel wall in parallel with macrophages, but in less quantity. Mechanisms involved are similar to monocyte recruitment [180]. In the arterial wall, T cells become activated in response to antigens and start to produce pro-inflammatory mediators (e.g., IFNγ), which further amplify the inflammatory response, aggravating disease progression [180, 181]. Different T cell subsets exist that can influence atherosclerosis in various ways, both at early plaque stages as well as advanced lesions. CD4$^+$ T cells and to a lesser extent CD8$^+$ and γδ T cells are present in plaques of atherosclerotic mice. Knockout depleting antibodies and cell transfer experiments suggest an overall pro-atherogenic role for CD4$^+$ T cells starting early during atherosclerotic disease progression [182–184]. However, in one report, CD4$^{-/-}$ApoE$^{-/-}$ females exhibited an increased load of atherosclerosis, predominantly at the lower aorta [11]. This increase could be due to the absence of CD4$^+$ Tregs and a compensatory increase in CD8$^+$ cells in this mouse model [11]. The role of CD8$^+$ T cells in atherogenesis is still controversial [185, 186].

Classically, T cell responses are initiated by APCs (DCs, macrophages, and B cells), but can also be antigen-independent. After antigen presentation, T cell activation occurs through simultaneous engagement of the TCR with peptide antigen on MHC class complexes and co-stimulatory molecules with their ligands. In atherosclerosis, the antigen that triggers the immune response and induces T cell proliferation and polarization is still not completely identified. However, recent evidence points towards atherosclerosis-specific antigens such as (the ApoB100 part of) LDL, and postulate that intimal DCs present these in draining or even distant lymph nodes [126, 187]. As the plaque itself contains classical as well as non-classical APCs (e.g., SMCs and endothelial cells), effector T cells immigrating into the lesion can be (re)activated by antigen presentation inside lesions [171, 172, 187]. In line with this, oligoclonal T cell populations have been identified inside the plaque [176, 188, 189].

CD4+ T cell subsets in atherosclerosis

Th1 response in atherosclerosis The majority of T cells in atherosclerosis are of the Th1 profile, characterized by the production of high levels of IFNγ. IFNγ promotes the recruitment of T cells and macrophages to the plaques contributing to plaque growth, augments macrophage uptake of lipids leading to the formation of foam cells, increases the activation of APCs, enhances their MHC II expression, and enhances the secretion of Th1-promoting cytokines [67, 190, 191]. These events lead to an expansion of atherosclerotic plaque burden and perpetuation of the pathogenic Th1 response. IFNγ also contributes to plaque vulnerability and rupture by inhibition of SMC infiltration, proliferation, and collagen production, but also by increasing the production of matrix metalloproteinases [67, 192–194]. Studies deleting IFNγ or its receptors report reduced atherosclerosis, while injection of recombinant IFNγ leads to increased lesion size [195–198]. Besides their role in T cell activation by antigen presentation, DCs and macrophages are instrumental in Th1 differentiation through secretion of IL-12. IL-12 activates Th1 transcription factors (such as STAT4 and T-bet) and upregulates IFNγ expression, while downregulating IL-4 and IL-5 in T cells. Patients with coronary artery disease (CAD) show increased STAT4 levels in CD4$^+$ T lymphocytes [199]. Moreover, a study on cytokine expression in advanced human atherosclerotic plaques confirmed the dominance of pro-inflammatory Th1 cytokines [200]. In addition, Zhao et al. [201] reported Th1 and Th17 activation in patients with CAD. Intervention in IL-12 or IL-18 gene or receptor function was found to reduce plaque development in mice, while administration of these cytokines accelerated disease progression, suggesting atherosclerosis is affected by an imbalance in T cell subsets [157, 158, 202–206]. Collectively, these data point towards a pro-atherogenic function of Th1 responses.

Th2 response in atherosclerosis Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and provide help for antibody production by B cells [207]. Th2 cells are rare in atherosclerotic lesions, although their number is increased in hyperlipidemia. IL-4 drives Th2 cell differentiation by activation of the transcription factor GATA3 (through STAT6), leading to an increase in IL-4 and IL-5 production and a decrease in IFNγ [207]. Th2 cells were thought to be atheroprotective as they oppose the pro-atherogenic Th1 differentiation. However, the role of Th2 cells in atherosclerosis is still controversial and depends on the site and stage of the lesions as well as on the experimental model used. Studies on IL-4, the prototypic Th2 cytokine, report either no (in ApoE$^{-/-}$ mice given angiotensin II) [208] or pro-atherogenic (in LDLR$^{-/-}$ mice) effects [209]. Possible pro-atherogenic effects of IL-4 might include activation of mast cells. Administration of IL-13, another prominent Th2 cytokine favorably affects atherosclerotic plaque morphology by reducing plaque inflammation and inducing plaque fibrosis in LDLR$^{-/-}$ mice, and inducing a protective M2 macrophage phenotype [210]. Accordingly, IL13$^{-/-}$LDLR$^{-/-}$ mice have accelerated atherosclerosis [210]. IL-5 and IL-33 appear to exhibit overt anti-atherogenic properties [211, 212]. IL-5 protects against atherosclerosis by promoting B-1 cell development and, ensuing production of protective antibodies [213],

while IL-33 may at least in part exert its effect through induction of IL-5 [211].

Treg response in atherosclerosis Natural regulatory T cells (Tregs) are characterized by expression of CD4, CD25, and the transcription factor FoxP3. Tregs maintain self-tolerance and prevent autoimmunity by suppression of immune responses, such as Th1 and Th2 responses. Natural Tregs (Th3) develop in the thymus and recognize specific self-antigens. However, Treg cells can also be generated in the periphery in the presence of TGFβ or IL-10, the so-called induced Tregs (iTregs, Tr3).

Regulatory T cells are present in plaques [214, 215], and depletion using anti-CD25 antibodies in atherosclerotic mice results in increased lesion size [216]. Furthermore, transfer of bone marrow cells from $CD80^{-/-}CD86^{-/-}$ or $CD28^{-/-}$ mice (which do not contain T regs) in $LDLr^{-/-}$ mice resulted in increased lesion size, whereas transfer of Tr1 cells, regulatory T cells that produce high levels of IL-10 and low levels of TGFβ, or natural $CD4^{+}CD25^{+}$ Tregs, significantly reduced atherosclerosis [216, 217], showing a protective role for regulatory T cells in atherosclerosis.

Regulatory T cells are known to produce large amounts of TGFβ and IL-10. Although TGFβ has an atheroprotective role [216], it is not clear whether Tregs exert their protective function directly through secretion of TGFβ or through other immunosuppressive mechanisms [218, 219]. Interestingly, DCs are able to induce Treg formation and play a role in the maintenance of Treg function through production of TGFβ [220, 221]. Production of IL-10 by regulatory T cells may also contribute to their athero-protective effects, as IL-10 has been shown to repress atherosclerotic development [222, 223].

Regulatory T cells play an important role in the development of atherosclerosis by repressing immune function and provide an interesting target for the modulation of the disease.

Th17 cells IL-17-producing helper T cells (Th17 cells) are protective against fungal and bacterial infections, but are also involved in the development of some autoimmune diseases [224]. Th17 cells mainly produce IL-17A and IL-17F as well as IL-21 and IL-22. In mice, both TGFβ and IL-6 are necessary for Th17 differentiation [225], whereas IL-21 and IL-23 are, respectively, required for Th17 proliferation and maintenance.

Although Th17 cells are present in both murine and human atherosclerotic lesions [226–228], their role remains controversial, as both atherogenic as well as atheroprotective effects have been reported. Both Th17 cells and IL-17 protein accumulate in lesions. Increased IL-17 expression in human lesions has been associated with lower macrophage numbers, higher SMC content, and an overall more fibrotic phenotype, suggesting that IL-17 promotes plaque stability [229]. However, others report increased IL-17 mRNA expression in symptomatic plaques compared to non-symptomatic ones, with a correlation between IL-17 expression and complicated, unstable, and lipid-rich lesions [227]. Many studies have interfered with IL-17 signaling in atherosclerosis [226, 230–232]: transplantation of IL17 receptor-deficient bone marrow into $LDLr^{-/-}$ mice, as well as antibody treatment against IL17A reduced plaque size [226, 231, 232]. $IL17A^{-/-}ApoE^{-/-}$ mice show a profound reduction in atherosclerosis, and a decreased recruitment of immune cells in the aortic arch region, but not in the abdominal aorta, suggesting a site-specific effect [233]. In contrast, Taleb et al. [234] found a protective role for Th17 cells in atherosclerosis by using T cell-specific SOCS3 deletion in $LDLr^{-/-}$ mice. A suppressor of cytokine signaling 3 (SOCS3) is a major negative feedback regulator of STAT3, a transcription factor crucial for Th17 differentiation. In this same study, administration of an anti-IL17A antibody accelerated atherosclerosis, indicating that Th17 cells may be protective [236].

The interplay and imbalances between the different T cell subsets are important in the pathogenesis of atherosclerosis. An imbalance in Th1/Th2 towards the Th1 response promotes the progression of atherosclerosis, whereas prominent Th2 and Treg responses are anti-inflammatory and result in a reduction of atherosclerosis and/or a more favorable plaque morphology. How Th17 cells affect atherogenesis still needs to be determined.

CD8+ T cells in atherosclerosis

$CD8^{+}$ T cells are important in cell-mediated immunity, capable of inducing death in infected or dysfunctional somatic cells. $CD8^{+}$ T cells express T cell receptors that recognize specific antigens presented on MHC class I molecules, present on all nucleated cells. As MHCI molecules mainly present cytosolic peptides, this represents an effective mechanism for clearing viruses and other intracellular pathogens. Once activated, $CD8^{+}$ T cells induce apoptosis in their target cells by releasing cytotoxins, like perforin, granzymes, and granulysin. However, $CD8^{+}$ T cells also secrete cytokines such as IFNγ and TNFα.

$CD8^{+}$ T cells are present in both murine and human plaques [235, 236]. Although $CD8^{+}$ T cells are only present in low numbers in early lesions, they appear to be the dominating T cell type in advanced human lesions [236]. While no effects on plaque size are observed in $CD8^{+}$T cell-deficient $ApoE^{-/-}$ mice, atherosclerosis is reduced in MHC class I-deficient C57Bl/6 mice on a high-fat diet. In addition, stimulation of $CD8^{+}$ T cells responses with a CD137 agonist resulted in increased lesion size accompanied by enhanced $CD8^{+}$ T cell recruitment to the lesions, suggesting

a proatherogenic role for this T cell subset [186]. Kolbus et al. [237] recently reported activation of $CD8^+$ T cells after feeding $ApoE^{-/-}$ mice a high-fat diet. Interestingly, these cells were detected in plaque-draining lymph nodes and preceded $CD4^+$ T cell activation, suggesting a role for $CD8^+$ T cells in early atherogenesis.

NKT cells in atherosclerosis

Unlike conventional T cells, which recognize peptide antigens presented by MHC molecules, NKT cells recognize a variety of (glyco)lipid antigens presented by a unique TCR on CD1d molecules APCs. Upon activation, NKT cells secrete both pro-inflammatory cytokines, such as IFN, and anti-inflammatory cytokines, like IL-4, IL-10, and IL-13 [238]. Activated NKT cells can interact in a CD1d-dependent manner with other immune cells, promoting DC maturation and monocyte activation [238], and can induce tolerance by communicating with Tregs [239].

NKT cells are present in the shoulder region of human carotid artery plaques, and in abdominal aortic aneurysms [240]. Both $CD1d^{-/-}$ mice (lacking NKT cells) on a high-fat diet or $CD1d^{-/-}$ mice on $ApoE^{-/-}$background show decreased atherosclerosis [241–243]. Moreover, repeated exogenous activation of NKT cells by α-GalCer in $ApoE^{-/-}$ mice, or adoptive transfer of unstimulated NKT cells in $Rag1^{-/-}LDLR^{-/-}$ mice, aggravate atherosclerosis [241–244]. Other studies have shown that invariant V alpha 14 NKT cells are responsible for increasing early plaque formation [245], that the $CD4^+$NKT cell subset is responsible for the pro-atherogenic activity of NKT cells [246], and that the contribution of NKT cells in atherosclerosis is restricted to early lesion development [247].

B cells

B cells originate from the bone marrow and play an important role in humoral immune responses. They are characterized by the presence of a B cell receptor and are classically known for their ability to produce antibodies important for the clearance of antigens. B cells possess antigen-presenting capacities, activating both $CD4^+$ and $CD8^+$ T cells. In addition, they can also secrete a variety of cytokines (e.g., IFN-γ, IL-2, IL-12, IL-4, IL-6, and IL-10) and promote chemokine production (e.g., CXCL12, CXCL13, CCL19, and CCL21), key players in modulating chronic immune responses by promoting leukocyte recruitment and polarizing T cells [248, 249].

According to their surface antigens, mature B cells can be categorized into B1, conventional B2, or marginal zone B cells [248]. B1 cells reside in serosal cavities and participate in innate immunity by T cell-independent production of the majority of natural IgM antibodies. Conventional B2 cells are present in bone marrow and lymphoid organs and are the B cells important in adaptive immunity by production of specific IgG antibodies to their cognate antigen. Marginal zone B cells can be found in the spleen, where they play a role in the first-line defense against blood-borne antigens. Upon antigen recognition, all mature B cells can differentiate into plasma cells. However, only B2 cells have the ability to become memory B cells.

Although B cells are only occasionally detected in the atherosclerotic intima [250], early plaques contain large amounts of IgM and IgG [251]. Furthermore, both IgM and IgG antibodies have been described in plaques at all stages of lesion development [252].

Recent studies have evaluated the role of B cells in the immune response during atherosclerosis. Splenectomy in mice resulted in larger plaques, which could be prevented by adoptive transfer of unfractionated splenic B cells [253]. Furthermore, transfer of B cell-deficient bone marrow (μMT) into $LDLr^{-/-}$ mice resulted in increased lesion size in parallel with reduced antigen presentation and antibody and cytokine production in both early and late atherosclerosis [254]. These data indicate that atheroprotective immunity develops during atherosclerosis progression with B cells playing a beneficial role. Paradoxically, some studies have also reported detrimental effects for B cells. CD20-targeted B cell depletion in mouse models of atherosclerosis reduced lesion size [255, 256]. Furthermore, deficiency or adoptive transfer of B2 B cells revealed this B cell subtype to be pro-atherogenic [257]. These findings not only imply that B cells have both pro and anti-atherogenic roles in atherosclerosis but also indicate that different B cell subtypes are involved in atherosclerosis immunity, complicating the role of B cells in the disease. However, these studies do not discriminate between cellular B cell functions and production of antibodies.

OxLDL is highly immunogenic, and anti-oxLDL antibodies can be detected in atherosclerotic plaques as well as in the circulation of mice and men [258, 259]. OxLDL-specific antibody IgG titers correlate with atherosclerosis [260–262], while oxLDL-specific IgM titers are associated with atheroprotection [263, 264]. Accordingly, Binder et al. [265] showed that pneumococcal vaccination of $LDLr^{-/-}$ mice reduced atherosclerosis by expanding T15 (anti-oxLDL) natural IgM antibodies. In addition, the same group indicated that the atheroprotective effect seen after immunization with MDA-LDL was due to increased T15 antibody titers that resulted from IL-5 production by Th2 T cells [212]. This was confirmed as a deficiency in bone marrow IL-5, a cytokine important in non-cognate maturation and Ig secretion of B1 cells, which reduces oxLDL-reactive IgM levels and accelerates atherosclerosis [212]. In addition, Lewis et al. [266] reported a dramatic increase in atherosclerosis in mice lacking IgM in their serum, again

supporting a protective role for IgM in atherosclerosis. Similar conclusions were drawn from some well-powered human clinical studies [260, 267, 268]. IgM antibodies are therefore considered anti-atherogenic, while antigen-driven IgG responses are considered to be pro-atherogenic.

As with T cells, the B cell population also contains B cell subsets capable of dampening immune responses. These regulatory B cells modulate the immune response through mechanisms similar to T cells, via secretion of IL-10 and TGFβ [269], or via their Ag-presentation ability or interactions with other immune cells via their secretion of Abs [269]. This way, regulatory B cells might suppress both Th1 and Th2 polarization and reduce antigen presentation and pro-inflammatory cytokine production by dendritic cells and macrophages. Regulatory B cells may act on atherosclerotic lesions either remotely (LNs or ATLOs) or within lesions. However, their functions and impact on atherosclerosis remains to be investigated.

In conclusion, we can state that B cell subtypes, exerting both pro and anti-atherogenic effects, are important in atherosclerosis and provide some interesting therapeutic options. However, there is still much to learn about B cell subsets and their mechanisms influencing atherosclerosis.

Costimulatory/coinhibitory interactions

The interaction between the different immune cells, and the (consequent) secretion of immune-regulatory and activating cytokines and chemokines determines the progression of atherosclerosis.

Key players in modulating these complex immune interactions and responses are the group of co-stimulatory and co-inhibitory molecules belonging to the CD28/B7 family and the tumor necrosis factor (TNF)/TNF-receptor family. Classically, co-stimulatory molecules provide the signal for proliferation and polarization of T cells and thereby also regulate the phenotype of the APC upon interaction of a T cell (TCR) with an antigen-presenting cell (MHCII/HLA). However, expression of co-stimulatory molecules is ubiquitous, and we know now that most of them are not only present on the majority of immune cells but also on platelets, endothelial cells, and vascular smooth muscle cells where they regulate inflammation [270].

In atherosclerosis, co-stimulatory molecules play a major, but diverse, role in atherosclerosis [270]. In the B7/CD28 family, genetic deficiency or inhibition of B7-1, B7-2, ICOS, and PD-L1/2 affected atherosclerosis. Deficiency of B7-1 and B7-2 in LDLR$^{-/-}$ mice has been shown to inhibit early atherosclerotic lesion development, and reduce the amount of MHCII expression in atherosclerotic plaques, and their CD4^{+} T cells produced less IFNγ [151]. However, different results were obtained when B7-1/B7-2$^{-/-}$ or CD28$^{-/-}$ bone marrow was given to irradiated LDLR$^{-/-}$ mice. These chimeric mice developed more atherosclerosis, and this was attributed to their impaired Treg development [271]. Similar contradictory results were obtained by studying inhibition of ICOS, a positive co-stimulatory molecule for CD4^{+} cells. Instead of the expected reduction in atherosclerosis, both immunization with ICOS and bone marrow transplantation of ICOS$^{-/-}$ bone marrow into LDLR$^{-/-}$ mice showed an aggravation of atherosclerosis, which was also due to an impaired Treg function [272, 273]. Moreover, deficiency of PD-PD-L1/2 interactions, a co-inhibitory dyad, aggravated atherosclerosis, and induced a pro-inflammatory plaque phenotype [274]. These studies with sometimes opposing results illustrate the complexity of co-stimulatory and co-inhibitory pathways which can influence functions of both pro-inflammatory effector T cells and Treg suppression.

For the TNF and TNF-R family members, the results are more consistent. Inhibition of Ox40-Ox40L signaling results in an impaired atherosclerosis development, while mice over-expressing Ox40L have accelerated atherosclerosis [275, 276]. The same is true for CD137-CD137L (4-1BB/4-1BBL), where treatment with an agonistic CD137 antibody results in accelerated atherosclerosis and the development of an inflammatory, vulnerable plaque phenotype [186].

One of the most elaborately studied co-stimulatory molecules in atherosclerosis is the CD40L-CD40 dyad. Inhibition of CD40L not only decreased atherosclerotic plaque burden but also induced plaques with a beneficial plaque phenotype that were rich in collagen and only contained a limited amount of immune cells [277, 278]. Blocking of CD40L when atherosclerotic plaques had established was even capable of transforming vulnerable plaques with a high level of inflammation and a low level of collagen towards the inflammatory-poor beneficial plaque phenotype [279, 280]. CD40L antagonists are therefore known as the most potent plaque reducers and plaque stabilizers in a laboratory setting. For CD40, the results are somewhat divergent. In one study, CD40$^{-/-}$ApoE$^{-/-}$ mice, as well as the CD40$^{-/-}$ bone marrow chimeras, showed a clear decrease in atherosclerosis [281], while in another study, CD40$^{-/-}$LDLr$^{-/-}$ mice showed no reduction in atherosclerosis [282].

The actions of CD40 and CD40L are rather cell type-specific. Bone marrow transplantation of CD40$^{-/-}$, but not CD40L$^{-/-}$ bone marrow results in a decrease in atherosclerosis, suggesting that bone marrow-derived CD40, but not CD40L, is crucial in atherosclerosis [281, 283, 284]. Transfer of CD40L$^{-/-}$ platelets prevented the platelet-induced increased in atherosclerosis, by impairing leukocyte–platelet interactions and inducing a transient increase in Tregs [285].

Interestingly, different cell type-specific CD40 signal transduction pathways tightly regulate atherosclerosis. CD40 does not have intrinsic signal capabilities, but needs adaptor molecules, the TNF-receptor associated factors

(TRAFs), to exert signaling. By using *CD40*$^{-/-}$ mice that carried chimeric human/murine CD40 transgenes with mutations in the TRAF2/3/5 or TRAF6 binding domains or both under MHCII, we found that mice deficient in the CD40–TRAF2/3/5 binding site develop normal atherosclerosis and have more CD4^{+} effector T cells, but also more regulatory T cells. Mice deficient in CD40–TRAF6 interactions hardly develop any atherosclerosis and their plaques contain only few inflammatory cells [281], which is also true for neointima formation [286]. Systemically, the different CD40-TRAF interactions induce several immunological patterns in blood, spleen, and lymph nodes. A deficiency of CD40–TRAF6 interactions results in low numbers of CD4^{+} effector T cells and pDCs, and a switch towards Ly6C^{low} monocytes and an M2 macrophage phenotype, whereas a deficiency of CD40–TRAF2/3/5 interactions induces increased Treg numbers and a change in DC phenotype [281].

The family of co-stimulatory molecules is very powerful in mediating immune cell interactions and immune cell phenotypes in atherosclerosis. However, most of the actions of co-stimulatory molecules are cell type-specific, and dependent on a variety of signaling pathways. Although the first pathways of co-stimulation in atherosclerosis have been unraveled, many more of these pathways will be discovered in the upcoming years.

Conclusions

Over the past few years, new immune cell subsets, among which are several that have immune-modulating properties, have been discovered to play an important role in atherosclerosis.

Skewing the vascular immune response towards an anti-inflammatory profile would be beneficial for patients suffering from atherosclerosis, and immune-based cell therapies are therefore of interest. Dendritic cells, as potent regulators of immune responses, represent an important cell type in this view. Several studies using vaccination strategies in animals already show promising results for such techniques. M2 macrophages, regulatory T and B cells and B1 cells are other cell types with an immune regulatory function, which should be exploited as potential therapy options for atherosclerosis.

A major challenge is to tweak immune responses to avoid compromising the patient's host defense. An interesting therapeutic option is therefore modulation of the immune system by co-stimulatory molecules.

However, the precise functions, and the interactions of these immune(modulatory) cells with other immune cells within the plaque, still need to be unraveled. Only then will we be successful in developing immunemodulatory strategies to treat atherosclerosis safely and effectively.

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