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Monocyte and Macrophage Dynamics during Atherogenesis

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Abstract

Vascular inflammation is associated with and in large part driven by changes in the leukocyte compartment of the vessel wall. Here, we focus on monocyte influx during atherosclerosis, the most common form of vascular inflammation. Although the arterial wall contains a large number of resident macrophages and some resident dendritic cells, atherosclerosis drives a rapid influx of inflammatory monocytes (Ly-6C$^+$ in mice) and other monocytes (Ly-6C$^-$ in mice, also known as patrolling monocytes). Once in the vessel wall, Ly-6C$^+$ monocytes differentiate to a phenotype consistent with inflammatory macrophages and inflammatory dendritic cells. The phenotype of these cells is modulated by lipid uptake, Toll-like receptor ligands, hematopoietic growth factors, cytokines and chemokines. In addition to newly recruited macrophages, it is likely that resident macrophages also change their phenotype. Monocyte-derived inflammatory macrophages have a short half-life. After undergoing apoptosis, they may be taken up by surrounding macrophages or, if the phagocytic capacity is overwhelmed, can undergo secondary necrosis, a key event in forming the necrotic core of atherosclerotic lesions. In this review, we discuss these and other processes associated with monocytic cell dynamics in the vascular wall and their role in the initiation and progression of atherosclerosis.

Introduction

Monocytes, macrophages and dendritic cells are key cells in the initiation and progression of atherosclerosis. Here, we review the available evidence on how monocytes reach atherosclerotic lesions, how they differentiate into inflammatory macrophages, the possible relation between resident and inflammatory macrophages, and the fate of these cell types as the atherosclerotic lesions progress. We also refer the reader to several excellent previous reviews on the subject [1-6]. Because of space constraints, we will consider dendritic cells only insofar as they are monocyte-derived. Reviews on dendritic cells in atherosclerosis can be found elsewhere [7-9].

The healthy mouse aorta contains a significant number of Mac-1$^+$ cells, most of which are resident macrophages [10-12]. In addition to CD11b (the α chain of the Mac-1 integrin), these cells also express the phagocyte marker CD68 and the macrophage marker F4/80. Although some macrophages in lymphoid tissues like the spleen derive from blood monocytes [13-15], the origin and lineage of resident vascular macrophages is unknown. Microglia [16] and Langerhans cells [17] have been shown to proliferate in situ. Since there was no evidence of

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microglia progenitor recruitment from the circulation [16], microglia cells may arise from embryonic precursor cells. It is possible that resident vascular macrophages share a similar nature, although this has not been investigated.

Monocytes

Under conditions of atherosclerosis, monocytes are rapidly recruited into the vessel wall. Mouse monocytes develop from a bone marrow precursor cell, the monocyte-dendritic cell precursor (MDP) [18]. MDPs give rise to all blood monocytes and the common dendritic cell precursor (CDP) [19], but MDPs do not give rise to granulocytes. Under conditions of acute inflammation, hematopoietic progenitor cells can differentiate to dendritic cells outside the bone marrow [20]. The growth factor M-CSF and its receptor CD115 are critical for MDP differentiation into monocytes [21, 22]. The cell fate decisions that occur in the bone marrow for monocyte differentiation require a number of specific transcription factors. Expression of PU.1, a member of the Ets transcription factor family, is induced during early myeloid differentiation and is high in mature monocytes and granulocytes [23, 24]. Studies of mice in which PU.1 has been deleted show a complete defect in production of monocytes, granulocytes and B and T lymphocytes. These mice are either embryonic lethal or die from sepsis shortly after birth [25, 26]. PU.1 stimulates the Egr family of transcription factors that play a role in monocyte development [27, 28]. Interferon regulatory factor-8 (IRF-8) also known as interferon consensus sequence binding protein or ICSBP, is a member of the IRF family of transcription factors. Studies with IRF8 knockout mice (*Irf8*$^{-/-}$) have demonstrated that IRF8 inhibits granulocyte differentiation, while promoting monocyte proliferation from progenitor bone marrow cells [29, 30]. JunB is a component of the AP-1 transcriptional complex, and junB is highly expressed in granulocytes and in myeloid precursors [31, 32]. JunB is a negative regulator of cell proliferation through inhibition of cyclin D1 and activation of the Cdk inhibitor p16 [33, 34]. Moreover, several studies have shown that mice lacking myeloid expression of junB develop a myeloproliferative disorder similar to chronic myeloid leukemia [35, 36]. JunB expression was reduced in bone marrow of *Nr4a1*$^{-/-}$*Nr4a3*$^{-/-}$ knockout mice [37]. Taken together, these data suggest that junB is an essential component of myeloid differentiation, and that the orphan nuclear receptors Nr4a1 and/or Nr4a3 may regulate junB expression and monocyte differentiation in bone marrow. Other transcription factors, including KLF4 [38] and the Maf family [39] have also been implicated in monocyte development and differentiation. Once mouse monocytes egress from the bone marrow, they circulate in the blood with a half-life of about 17 hours [40-42]. Many of these measurements were made before monocyte subsets were known, and the circulatory half-life may well differ for different monocyte subsets. Mature monocytes reside in the subcapsular red pulp of the spleen, where they are rapidly deployed in response to inflammatory signals [15]. These spleen monocytes show a gene expression profile that is very similar to that of blood monocytes.

In mice and humans, several monocyte subsets have been described. Originally, Ly-6C^{+} monocytes were described as “inflammatory” and Ly-6C^{-} as “resident” [43]. The Ly-6C^{+} monocytes also express the MCP-1 receptor CCR2, the adhesion molecule L-selectin, and low levels of the chemokine receptor CX3CR1. Conversely, Ly-6C^{-} monocytes express high levels of CX3CR1 and of the $\alpha_L\beta_2$ integrin LFA-1 [43, 44]. In humans, CD14^{high} and CD14^{+}CD16^{+} monocytes were described [45]. More recently, a CD14^{dim} human monocyte subset was found to be the subset containing the population that patrols blood vessels [46]. Human CD14^{high} monocytes also express CCR2, L-selectin and the Fc receptor CD64. Among human monocytes, CD14^{+}CD16^{+} cells lack CCR2, but express CD32 and higher levels of MHC-II. The differential function of CD14^{+}CD16^{+} and CD14^{dim} monocytes was recently described [46]. Since the definition of the monocyte subsets is currently based on surface markers, it is unclear and controversial whether they can interconvert [43, 47]. Lineage

tracking studies will be needed to understand the lineage relationship between these monocyte subsets and their differentiation into resident and inflammatory vascular macrophages.

Circulating monocytes of patients with cardiovascular disease display high levels of surface receptors that may be involved in inflammatory responses. Monocyte TLR4 expression has been reported to increase in patients with coronary arteriosclerosis and acute coronary syndrome [48-50]. TLR4 is a signaling co-receptor of CD14. Both $CD14^{+}CD16^{+}$ and $CD14^{high}CD16^{-}$ monocytes secrete proinflammatory cytokines in response to LPS, the bacterial TLR4 ligand. $CD14^{dim}$ monocytes do not respond to LPS, but respond to viruses and endogenous nucleic acids via TLR7 and TLR8 [46]. In asymptomatic hypercholesterolemic subjects, TLR4 is expressed on $CD14^{+}CD16^{+}$ and at lower levels on $CD14^{high}CD16^{-}$ monocytes [51]. However, it is unclear whether expansion of TLR4-positive monocytes in patients with clinical manifestations of atherosclerosis is associated with the $CD14^{+}CD16^{+}$ or the $CD14^{high}CD16^{-}$ population.

At sites of inflammation, monocytes are recruited to lymphoid and non-lymphoid tissues [52], where they phagocytose microbes, apoptotic cells and host-derived damage-associated molecules and complexes, such as oxidized or otherwise modified low density lipoprotein (LDL) [53, 54]. These monocyte-derived cells have variably been described as inflammatory dendritic cells [55, 56] or inflammatory macrophages. In the context of atherosclerosis, these cells can give rise to foam cells and initiate formation of fatty streaks [57], but resident dendritic cells have also been shown to produce foam cells [56]. Interestingly, increased numbers of inflammatory macrophages and DCs are found even in the normal mouse aorta at sites that are predisposed to develop atherosclerotic lesions [55]. It is presently unclear to what extent these cells differentiate (lineage commitment) or whether they show plasticity (phenotypic changes in response to their environment) [58].

Ly-$6C^{+}$ monocytes are known to give rise to $CD11b^{+}CD11c^{+}$ inflammatory macrophages in the intestinal lamina propria [59, 60]. In a model of *Listeria monocytogenes* infection, monocyte-derived cells have been described as TNF and iNOS-producing Tip-DCs [61]. These cells express high levels of MHC-II, CD80 and CD86, and are efficient at presenting antigens [62]. They phagocytose microbes, promote inflammation by secreting cytokines and degrade tissue by proteolytic enzymes including matrix metalloproteinases. Although Tip-DCs migrate to secondary lymphoid organs in listeria-infected mice [61], their migration in atherosclerosis has not been studied. These Ly-$6C^{+}$ monocyte-derived cells have been compared to M1 (see below) macrophages [1], but it is unclear whether these cells fully recapitulate the phenotype of classical M1 macrophages [63, 64], which were originally defined based on human blood monocyte-derived macrophages grown in vitro in the presence of M-CSF, IFN-γ and LPS [65]. It is presently not clear whether Tip-DCs and inflammatory macrophages are distinct or overlapping subsets among $CD11b^{+}CD11c^{+}$ cells.

The Ly-$6C^{-}$ monocytes patrol the inside of blood vessels in mice [66]. Their interaction with the vascular endothelium requires LFA-1 ($\alpha_L\beta_2$ integrin) and the chemokine receptor CX3CR1 [66]. CX3CR1 has been shown to be critical for the survival of the Ly-$6C^{-}$ subset [40]. Human $CD14^{dim}$ monocytes also show this patrolling behavior when infused into mice [46]. Although the patrolling behavior is well described to cover long distances and large areas of the endothelial surface of blood vessels, the kinetics of recruitment, migration into the extravascular space and survival of Ly-$6C^{-}$ cells in the tissue have not been investigated yet. The Ly-$6C^{-}$ monocytes can differentiate into macrophages that produce chemokines like CXCL9 and CXCL10, pro-angiogenic factors like VEGF, participate in tissue remodeling and phagocytosis [1, 64]. The relation between the monocyte subsets and M1, M2 and other macrophages (see below) remains unclear.

Monocyte recruitment

Monocytes are thought to reach the arterial wall by transmigrating through the luminal endothelium. However, the evidence for this is circumstantial and monocyte transmigration into atherosclerotic lesions has not been observed directly. Morphologic studies show that monocytes can be found adherent to the luminal endothelium [10]. Ex vivo perfusion studies show that monocytes can roll and adhere to the luminal endothelium of atherosclerotic, but not normal arteries [67, 68], but in these experiments the wall shear stress was lower than in vivo, and did not follow the normal cardiac cycle. In vivo observations of leukocyte adhesion in mouse abdominal aorta have been reported [69], but the nature of the leukocytes (monocytes or granulocytes) was not identified. In a recent intravital microscopic study, the chemokine receptor requirement for neutrophil, but not monocyte recruitment into the carotid artery was reported [70].

The traditional leukocyte adhesion cascade involves capture, selectin-dependent rolling, activation by an endothelial surface-bound chemokine, integrin-mediated adhesion, and transendothelial migration [71, 72]. The main selectin responsible for monocyte rolling in atherosclerotic mouse arteries appears to be P-selectin [67]. L-selectin appears to be involved in lymphocyte homing to mouse aorta [12], but not in monocyte recruitment. Most monocyte firm adhesion is dependent on the $\alpha_4\beta_1$ integrin, also known as VLA-4 [68, 73], which can bind to certain isoforms of fibronectin and to VCAM-1, an immunoglobulin family molecule highly expressed in endothelial cells near atherosclerotic lesions [74]. Although monocytes express other integrins including $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1), their role in recruitment to large arteries has not been documented. A list of adhesion molecules relevant to monocyte recruitment can be found in [75].

The immobilized chemokines responsible for monocyte arrest probably include CCL5, also known as RANTES, CXCL1, also known as KC in the mouse or GRO-α in humans, and IL-8 in humans [76]. Whether CCL2, also known as MCP-1, can trigger monocyte adhesion is controversial [73, 76], although CCL2 binding to endothelial cell glycosaminoglycans may provide a reservoir for retaining high local concentrations of CCL2 [77].

Blocking experiments have suggested that the Ly-6C$^+$ and Ly-6C$^-$ monocyte subsets may use different chemokines to access atherosclerotic lesions [78]. Ly-6C$^-$ monocyte recruitment was reduced by 40% when CCR5 was blocked, whereas Ly-6C$^+$ monocyte recruitment to sites of atherosclerosis was reduced by 40-50% when CCR5, CCR2 or CX3CR1 were targeted [78]. However, in these experiments, only the net effect of blocking these chemokines or their receptors was recorded, but the monocyte recruitment was not observed directly. Consistent with intravital microscopic and flow chamber experiments, CCL5 might be expected to be involved in arrest, and CCL2 perhaps in transendothelial migration. The importance of chemokines in recruiting both Ly-6C$^+$ and Ly-6C$^-$ monocytes is emphasized by the finding that combined inhibition of CX3CR1, CCL2and CCR5 almost abolishes atherosclerosis in the *Apoe*$^{-/-}$ mouse model of atherosclerosis [79].

Among the monocyte subsets, Ly-6C^{hi} monocytes express significantly more functional PSGL-1 than Ly-6C$^-$ cells [80]. Consistent with this observation, flow chamber assays showed that Ly-6C$^+$ monocytes adhere more avidly to P-selectin and E-selectin [80]. This would suggest that Ly-6C$^+$ monocytes may preferentially enter into atherosclerotic lesions, perhaps through a platelet-dependent mechanism. In vivo, Ly-6C$^+$ monocytes were indeed shown to bind to activated endothelium and infiltrate atherosclerotic lesions better than Ly-6C$^-$ monocytes [81]. Moreover, Ly-6C$^+$ monocytes accumulate in the blood of mice fed a high-fat high-cholesterol “western” diet [81]. Ex vivo imaging showed that Ly-6C$^+$ monocytes preferentially localized to lesion-prone sites like the lesser curvature of the aortic arch and

arterial branch points [82]. Since Ly-6C$^+$ monocytes are known to give rise to CD11b$^+$CD11c$^+$ inflammatory macrophages and Tip-DCs in other models [60, 61], it is tempting to speculate that many of the "macrophages" accumulating in atherosclerotic lesions may actually be Tip-DCs. Interestingly, most CD11b$^+$CD11c$^+$ cells in the atherosclerotic mouse aorta co-express high levels of MHC-II, which is responsible for peptide antigen presentation to CD4 T cells, and F4/80, a classical macrophage marker (Koltsova and Ley, unpublished results, 2011). Similar cells have recently been called resident intimal dendritic cells [56]. However, unlike dendritic cells in lymphoid organs, these cells are probably monocyte-derived.

Like other blood cells, monocytes must transmigrate through the endothelium to reach their destination. In vitro systems in the absence of flow show a prominent role of the endothelial cell surface immunoglobulin-like adhesion molecule CD31 in monocyte transendothelial migration [83]. Genetic absence of CD31 reduces monocyte transmigration in most mouse strains [84], but not in C57BL/6 mice, which are most commonly used in atherosclerosis studies. Other molecules that have been implicated in monocyte transendothelial migration include ICAM-1, VCAM-1, JAM-A, JAM-C, ESAM, ICAM-2 and CD99 [72]. However, these molecules are also used by other leukocytes, and no direct in vivo evidence exists that these mechanisms specifically regulate monocyte recruitment to atherosclerotic lesions in vivo. With the exception of VCAM-1, which is also a key adhesion molecule for monocytes [85], the differential effect of blocking or knocking out these molecules on the recruitment of monocyte subsets has not been studied.

Role of platelets

Platelets have long been known to participate in atherogenesis [86], and atherosclerotic lesions express detectable levels of platelet antigens like CD41 and P-selectin. Although it is unlikely that intact platelets survive in lesions, platelets greatly facilitate and accelerate monocyte accumulation [87, 88]. Platelets form bridges between monocytes and endothelial cells [88-91]. Consistent with this role, infusing activated platelets accelerates atherosclerotic lesion formation [89]. Platelet-monocyte adhesion is often dependent on platelet P-selectin and monocyte P-selectin Glycoprotein Ligand-1 (PSGL-1). Indeed, selectively eliminating P-selectin expression from platelets only (and not from endothelial cells) strongly protects mice from atherosclerosis [92]. Also, removing *Pf4*, the gene encoding the platelet chemokine CXCL4, from the mouse genome results in reduced atherosclerosis [93]. PF4 is only expressed in platelets and their precursor cells. Heterodimers between CXCL4 (PF4) and CCL5 (RANTES) have been implicated in atherogenesis, because they can promote monocyte arrest [94].

Blocking the main platelet adhesion molecule for von Willebrand factor, GP1bα, also delays atherosclerosis [95]. This is probably due to interrupting platelet binding to endothelial vWF, thus preventing immobilized platelets from nucleating monocyte adhesion (figure 1). Platelets bind to circulating monocytes in patients [90] and mice with atherosclerosis [89]. They can even promote the formation of heteroaggregates. However, platelets bind neutrophils [96] as avidly as monocytes. It is not known why monocytes, but not neutrophils seem to depend on platelets for recruitment to atherosclerotic lesions.

In addition to facilitating monocyte recruitment, platelets also secrete factors that influence the monocyte-macrophage phenotype. An important modifier of macrophage phenotype is platelet factor 4 (PF4 or CXCL4), which induces a unique transcriptome in human blood monocyte-derived macrophages [97] devoid of the hemoglobin-haptgoglobin receptor CD163 [98]. Consistent with a pro-atherosclerotic role of PF4, *Pf4*$^{-/-}$ mice show reduced atherosclerotic lesion sizes [93].

Atherosclerotic lesion macrophages

Macrophages are a major cell type of early atherosclerotic lesions and play important roles at all stages of lesion progression. As we noted earlier, macrophage phenotypes in atherosclerotic lesions are likely the result of both lineage commitment and phenotypic changes in response to their environment [58, 99]. Because current technology for interrogating macrophage lineages and functions in atherosclerotic lesions has serious limitations, most studies addressed mechanisms of macrophage differentiation in vitro.

In vitro, human monocytes can differentiate into various macrophage subsets. The most common growth factor used to grow macrophages in vitro is M-CSF, generating unpolarized (M0) macrophages. If these macrophages are treated with IFN-γ followed by LPS, they polarize to an M1 phenotype with characteristic expression of TNF-α and IL-12 [64]. M2 macrophages were originally described as human blood monocyte-derived macrophages differentiated in the presence of M-CSF and IL-4 or IL-13 (M2a), immune complexes and IL-1β or LPS (M2b) or in the presence of IL-10, TGF-β or glucocorticoids (M2c) [100]. M2c cells produce the pro-angiogenic growth factor VEGF [100]. Monocyte-derived macrophages can also be grown with PF4 without the requirement of M-CSF, resulting in M4 macrophages [97]. M4 macrophages have a unique transcriptome that is closer to M2 than M1. In the presence of the oxidized phospholipid oxPAPC, mouse bone marrow-derived macrophages express a small and unique transcriptome (119 characteristic genes) that includes the Hox1 gene encoding the anti-inflammatory enzyme heme oxygenase-1 [101]. This macrophage phenotype has been called Mox [101] and shares poor phagocytic capacity with M4 macrophages. However, Mox cells are clearly different from M4 cells in many other aspects. The sphingolipid sphingosine-1-phosphate has also been shown to generate a macrophage phenotype closer to a M2 than M1, with increased expression of arginase-I and reduced expression of TNFα [102]. Taken together, it is likely that modified LDL and inflammatory mediators like IFN-γ, TNF-α, TGF-β and IL-10 can trigger phenotypic modulation of macrophages in the artery wall during atherogenesis.

Macrophages can be isolated from the peritoneal cavity of mice or grown from bone marrow precursor cells. It is not known whether resident peritoneal macrophages are monocyte-derived. Among other features, resident peritoneal macrophages express high levels of 12/15-lipoxygenase and are extremely efficient efferocytes that take up apoptotic cells [103]. By contrast, bone marrow harbors monocyte precursors including MDPs and CDPs, which can give rise to various lineages. Mouse bone marrow-derived macrophages proliferate in vitro and can be differentiated to M1, M2 and a regulatory “Mreg” phenotype [104], which is phenotypically similar to the human M2c phenotype.

Atherosclerotic lesions contain macrophages with M1, M2, M4 and Mox markers [6, 98, 101], but it is unlikely that the in vitro phenotypes in their pure form exist in diseased arteries in vivo. Flow cytometric analysis of aortas from *Ldlr*$^{-/-}$ mice fed an atherogenic diet for 30 weeks revealed that 39% of the aortic macrophages expressed the M1 marker CD86, 21% the M2 marker CD206 (mannose receptor), 45% the Mox marker heme oxygenase-1, where 10% co-expressed CD86 [101]. M4 macrophages were not investigated in this mouse study, but in human atherosclerotic coronary arteries, many macrophages are CD163^{-}, a hallmark of the M4 phenotype [98].

The phenotype of lesional macrophages is incompletely understood. Laser capture microdissection has yielded limited numbers of cells and small amounts of mRNA, which has been interrogated for a few gene products [105]. Genome-wide analysis of lesional macrophages has not been reported, although the transcriptome of the mixed cell populations contained in atherosclerotic human coronary artery segments has been

analyzed [106]. Many lesional cells express the macrophage marker F4/80 and the integrins $\alpha_M\beta_2$ (CD11b CD18) and $\alpha_x\beta_2$ (CD11c CD18). Because CD11c is also expressed on many dendritic cells, lesional macrophages, likely derived from recently immigrated monocytes, have been called DCs [56105]. Under atherogenic conditions, these inflammatory macrophages accumulate in the aorta and show reduced emigration from lesions [107]. During atherosclerosis progression, these cells appear not only in the neointima, but also in the adventitia [12], where they may participate in antigen presentation [9] and cytokine production.

Foam cell formation

An initiating event in the formation of atherosclerotic plaques is excessive lipid accumulation in vascular wall macrophages. Macrophages in atherosclerotic arteries eventually become lipid-laden foam cells [108] through a process regulated by the balance between the uptake of modified LDL and efflux of cholesterol and other lipids [109]. In 1913, Nikolai Anitchkow described these cells as 'Cholesterinesterphagozyten' observed in the aorta of cholesterol-fed rabbits [110]. In the early 1980s, Ross Gerrity was the first to document the early entry of monocytes into the susceptible areas of the vessel wall in cholesterol-fed animals [111]. Gerrity described a "monocyte clearance system" in which large numbers of circulating monocytes invade the intima of lesion-prone areas in arteries, become phagocytic, and accumulate lipid [10]. It is indeed likely that most of the foam cells differentiate from newly recruited monocytes, but the details of this process are not known. Alternatively or in addition, resident intimal DCs may accumulate lipid and become foam cells [56]. Foam cells can migrate back into the circulation by crossing the aortic endothelium in reverse direction [11]. It is not known whether this is a major clearance pathway for foam cells.

Upon differentiation, macrophages display high levels of surface expression of scavenger receptors (SR-A, LOX-1, CXCL16 and CD36), which have the ability to take up modified lipoproteins, such as copper-oxidized, acetylated and malondialdehyde (MDA)-modified LDL [112-119] (figure 2). Atherosclerosis studies with CD36 (and SR-A) knockout mice performed in different laboratories have been contradictory. The Febbraio/Silverstein group demonstrated that CD36 deficiency reduced atherosclerosis [116, 120], but the Moore/Freeman group reported that knocking out CD36 had no effect on atherosclerotic lesion size or even increased aortic root lesions [121]. It is possible that scavenger receptors have a differential effect early and late in atherosclerosis, which could reconcile these different findings [116, 117, 121, 122].

Fluid-phase uptake of native and modified LDL is another endocytic pathway that generates macrophage foam cells during atherogenesis [123]. This macropinocytosis occurs constitutively in human monocyte-derived macrophages differentiated in vitro with M-CSF. Further studies from the same group recognized that both macropinocytosis and micropinocytosis of native LDL lead to foam cell formation [124]. In mouse macrophages, minimally oxidized LDL is recognized by the TLR4 MD-2 complex and induces Syk-dependent membrane ruffling and robust macropinocytosis, resulting in uptake of native and modified LDL and foam cell formation [125, 126]. Fluid-phase pinocytosis of fluorescent nanoparticles has been demonstrated in macrophages of mouse atherosclerotic lesions [127], and lipid accumulation and foam cell formation in early lesions of *Tlr4*$^{-/-}$ *apoE*$^{-/-}$ mice was reduced by 70-80% compared to *apoE*$^{-/-}$ controls [128]. Under certain dietary conditions, whole body and macrophage TLR4-deficient mice have less atherosclerosis than their *Tlr4*$^{+/+}$ counterparts [129, 130]. Carotid atherosclerotic plaques dissected from symptomatic patients have higher levels of TLR4 expression compared to the lesions from asymptomatic patients [131], and individuals with inactivating SNPs in the *Tlr4* gene have lower risk of atherosclerosis and cardiovascular events, although not all studies agree (reviewed in [132]).

Excess cholesterol accumulated in macrophages via scavenger receptor-mediated and/or fluid-phase uptake is removed from macrophages by ATP binding cassette (ABC) transporters. ABCA1 and ABCG1 are upregulated during macrophage differentiation, and these transporters function to regulate cholesterol efflux and reverse cholesterol transport [133-137]. PPARγ and LXR agonists function in part to regulate macrophage foam cell formation in atherogenesis [138-142]. PPARγ agonists can inhibit foam-cell formation in vivo through ABCA1-dependent [141] and ABCA1-independent pathways [143, 144]. Activation of PPARγ reduces cholesterol esterification and induces expression of ABCG1 [143]. LXR activation in macrophages reduces foam cell formation via induction of both ABCA1 [145] and ABCG1 [146, 147]. Fisher and colleagues recently found that both LXR isoforms were important in atherosclerosis regression. These investigators transplanted aortic arches from atherosclerotic *Apoe*$^{-/-}$ mice with or without LXRα or LXRβ deficiency into WT recipients. Plaques from both LXRα and LXRβ-deficient *Apoe*$^{-/-}$ mice exhibited impaired regression and reduced emigration of macrophages from plaques [148]. Thus, both LXR and PPARγ signaling inhibit macrophage foam cell formation in atherosclerotic plaques.

During atherogenesis, several eicosanoid-generating enzymatic pathways are induced in macrophages, including 5-lipoxygenase and 12/15-lipoxygenase (5-LO, 12/15-LO) [149-154]. Both enzymes have been linked to atherogenesis [151, 155-157]. Oxidized fatty acids produced in macrophages contribute to formation of minimally-modified and oxidized LDLs. IL-13, an important cytokine for the alternative activation of macrophages, induces 12/15-LO and CD36 in macrophages [158-160]. Engagement of the αMβ2 integrin significantly inhibited IL-13-mediated foam cell formation [161]. There is some controversy as to whether the 12/15-LO pathway is pro-atherogenic. Although 12/15LO can clearly generate oxidized lipids in LDL and is pro-atherogenic in mice [156, 157, 162], human 15-LO has been reported to be atheroprotective in rabbits [163-165]. This may relate to the concept that the 12/15-LO enzyme can also generate lipoxins and resolvins [166-168], which are important for the resolution of inflammatory responses. Thus, depending on the artery microenvironment, the 12/15-LO enzyme may confer either an atheroprotective or pro-atherogenic role.

Fatty acids and eicosanoids regulate expression of both the ABCA1 and ABCG1 transporters [169-171]. Arachidonic acid and 12-S-hydroxyeicosatetraenoic acid (12-S-HETE), produced by 12/15-LO, cause reduced ABCA1 and ABCG1 protein expression in macrophages. Both mice [172] and humans [169] with type 2 diabetes have reduced expression and functional activity of ABCG1 in macrophages. As it is known that subjects with type 2 diabetes have increased 12-S-HETE production through an induction in 12/15LO activity [173], it is plausible to speculate that these elevated levels of eicosanoids in type 2 diabetic subjects contribute to loss of ABCG1.

Recently, important links have been made between ABC transporters and vascular inflammation. The interaction of apoA-I with ABCA1 activates signaling molecules, such as Janus kinase 2 (JAK2) [174]. ABCA1-mediated activation of JAK2 activates STAT3 independently of the lipid transport function of ABCA1. ABCA1-expressing macrophages suppressed the induction of inflammatory genes in macrophages in response to LPS. LPS-treated macrophages from macrophage-specific ABCA1-deficient mice exhibited enhanced expression of pro-inflammatory cytokines and increased activation of NFκB, which could be inhibited by silencing MyD88 [175]. This was normalized when excess free cholesterol was removed from macrophages with cyclodextrin, which suggests that increased inflammatory TLR signaling through lipid rafts occurs when ABCA1 is absent. Similar findings have been reported for ABCG1, where macrophages deficient in ABCG1 showed increased cholesterol accumulation and enhanced TLR signaling in response to LPS [176]. ABC transporters may also play a role in myeloid proliferation. Tall and colleagues recently reported that proliferation of hematopoietic stem cell precursors is regulated by cholesterol efflux

mechanisms involving HDL, ABCG1 and ABCA1. Mice deficient in both ABCA1 and ABCG1 displayed leukocytosis and an expansion of the $Lin^{-}Sca\text{-}1^{+}Kit^{+}$ hematopoietic progenitor cell population in the bone marrow [177]. Thus, new evidence is emerging that strongly links cholesterol efflux mechanisms with inflammatory processes in macrophages.

Apoptosis and efferocytosis

As atherosclerosis has been deemed an inflammatory disease [178], inflammatory factors are expected to be involved in both the progression and resolution of atherosclerosis. One important function of macrophages is the clearance of apoptotic cells by phagocytes (a process called 'efferocytosis') [179]. Efferocytosis is one function of alternatively activated (M2) macrophages (discussed above) [180]. As macrophages engulf oxidized lipids and other cellular debris in the arterial wall during early stages of atherogenesis, many of these macrophages undergo apoptosis. In early atherogenesis, macrophage apoptosis is associated with reduced atherosclerosis progression [181, 182]. This is most likely due to effective efferocytosis by neighboring phagocytes, which, in turn, reduces pro-inflammatory mediators present in the artery wall [183]. Efficient efferocytosis has been shown to induce anti-inflammatory mediators, such as IL-10 and TGFβ [184]. However, uptake of excessive apoptotic cells by macrophages induces endoplasmic reticulum (ER) stress and the unfolded protein response. Oxidized phospholipids, free cholesterol and oxysterols can also trigger ER stress [185]. Oxidized phospholipids and oxidized lipoproteins trigger apoptosis in ER-stressed macrophages through a mechanism requiring both CD36 and TLR2 [186]. As atherosclerosis progresses, efferocytosis is thought to become impaired [187]. A failure of efferocytosis leads to 'secondary necrosis', in which macrophages die and release their cellular contents, including debris, oxidized lipids and pro-inflammatory mediators. Secondary necrosis amplifies the inflammatory response and leads to the development of a necrotic core in the plaque. The possible effects of defective efferocytosis are detailed in a recent review [179]. One possibility, among many, may be a change in macrophage phenotype that occurs in the artery wall during atherogenesis, leading to an accumulation of poorly phagocytic macrophages in the artery wall [188]. Minimally oxidized LDL prolongs the survival of foam cells loaded with free cholesterol or extensively oxidized LDL, but also inhibits efferocytosis [125, 189], and thereby may exacerbate vascular inflammation. Thus, the balance between apoptosis, efferocytosis and secondary necrosis determines atherosclerosis progression and severity.

In summary, monocytes play important roles in the initiation, progression and complications of atherosclerosis. Their recruitment to the artery wall, their differentiation to macrophages, and their phenotypes can be modulated by factors present within the microenvironment of the artery wall, including oxidized lipids, TLR ligands, hematopoietic growth factors, cytokines, and chemokines. Within atherosclerotic plaques, the dynamic modulation of macrophage phenotypes impacts atherosclerosis progression by modulating ongoing inflammatory responses within the vessel wall, by regulating apoptotic cell clearance within the developing plaque, and by egress mechanisms. Thus, the dynamic roles that macrophages play in early and advanced atherosclerotic plaques make macrophage phenotype modulation an attractive therapeutic targets for the prevention and treatment of cardiovascular disease.

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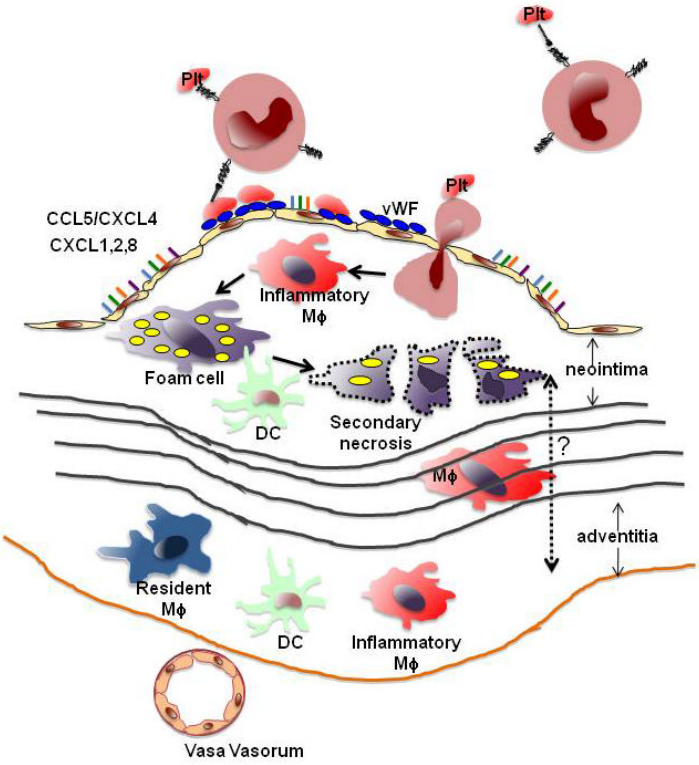

Figure 1. Monocyte recruitment to atherosclerotic lesions

Blood monocytes may be decorated with platelets through P-selectin-PSGL-1 interactions. They can also roll and adhere to platelets bound to von Willebrand Factor (vWF, blue multimers) secreted from endothelial cells. Monocyte arrest can be triggered by chemokines (colored bars) including CCL5/CXCL4 heterodimers, CXCL1, CXCL2 and (in humans) CXCL8. Arrested monocytes are thought to transmigrate into the lesion, where they differentiate to inflammatory macrophages (Mφ) and foam cells, which can undergo secondary necrosis to form the necrotic core in the neointima. Inflammatory macrophages may also migrate through the media (black lines represent laminae elasticae) to the adventitia. Monocytes may also be recruited to the adventitia through vasa vasorum. Resident macrophages (blue) and dendritic cells (green) are constitutively found in the adventitia and may be able to migrate between these two compartments (dashed arrow).

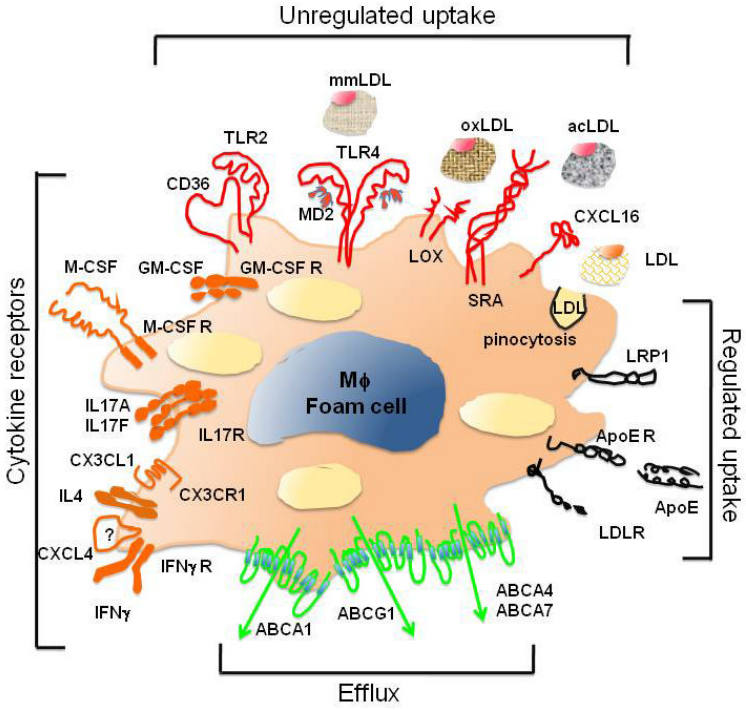

Figure 2. Factors that determine macrophage phenotype(s) in atherosclerotic lesions

Macrophages in atherosclerotic lesions display markers characteristic of several in vitro-differentiated macrophage phenotypes, which suggest possible factors and receptors that determine their differentiation from monocytes and their function in vivo. The growth factor M-CSF is almost universally required for the monocyte to macrophage differentiation (except M4 macrophages), whereas GM-CSF appears to promote a highly pro-inflammatory phenotype. IFN-γ and LPS polarize macrophages into an M1 phenotype, whereas IL4 (and IL13) and a number of other factors discussed in this review lead to M2 polarization. CXCL4 acts through an unidentified chondroitin sulfate proteoglycan receptor, while IL-17 promotes proliferation of mouse macrophages in vitro and macrophage accumulation in vivo [190]. CX3CL1 binding to its receptor CX3CR1 can promote macrophage survival. Macrophages in atherosclerotic lesions accumulate excessive amounts of lipid, and numerous intracellular lipid droplets make the neointimal macrophages look like “foam cells.” Regulated uptake of native LDL via the family of LDL receptors (LDLR, ApoER and LRP1) plays a limited role in foam cell formation. The major mechanism of excessive lipid accumulation is via unregulated uptake of oxidized (oxLDL), minimally modified (mmLDL), acetylated (acLDL) or otherwise modified LDL. This uptake is mediated by CD36 (alone or as heterodimer with TLR2), SRA, LOX-1, CXCL16, TLR4/MD-2 and a number of other receptors. Unmodified LDL also enters macrophages by micro- and macro-pinocytosis. The ABC transporters ABCA1, ABCG1 and ABCA4/7 mediate reverse transport (efflux) of cholesterol, oxysterols and phospholipids, but the presence of foam cells in the lesions indicates that the efflux mechanisms become eventually overwhelmed by unregulated LDL uptake. Lipid accumulation has profound effects on the macrophage gene expression, adhesion, apoptosis, efferocytosis and other characteristics and functions.