Abstract: The remarkable plasticity and plethora of biological functions performed by macrophages have enticed scientists to study these cells in relation to atherosclerosis for >50 years, and major discoveries continue to be made today. It is now understood that macrophages play important roles in all stages of atherosclerosis, from initiation of lesions and lesion expansion, to necrosis leading to rupture and the clinical manifestations of atherosclerosis, to resolution and regression of atherosclerotic lesions. Lesional macrophages are derived primarily from blood monocytes, although recent research has shown that lesional macrophage-like cells can also be derived from smooth muscle cells. Lesional macrophages take on different phenotypes depending on their environment and which intracellular signaling pathways are activated. Rather than a few distinct populations of macrophages, the phenotype of the lesional macrophage is more complex and likely changes during the different phases of atherosclerosis and with the extent of lipid and cholesterol loading, activation by a plethora of receptors, and metabolic state of the cells. These different phenotypes allow the macrophage to engulf lipids, dead cells, and other substances perceived as danger signals; efflux cholesterol to high-density lipoprotein; proliferate and migrate; undergo apoptosis and death; and secrete a large number of inflammatory and proresolving molecules. This review article, part of the Compendium on Atherosclerosis, discusses recent advances in our understanding of lesional macrophage phenotype and function in different stages of atherosclerosis. With the increasing understanding of the roles of lesional macrophages, new research areas and treatment strategies are beginning to emerge. (Circ Res. 2016;118:653-667. DOI: 10.1161/CIRCRESAHA.115.306256.)

Key Words: atherosclerosis ■ cholesterol ■ foam cells ■ macrophage ■ phenotype
Atherosclerosis is initiated by the subendothelial retention of apolipoprotein B–containing lipoproteins, which then triggers a maladaptive, nonresolving inflammatory process that over time drives disease progression.\textsuperscript{1–4} Although the presence of inflammatory cells in lesioned arteries was described as early as in the 1800s by Rudolph Virchow and others (for a historical perspective, see the review article by Libby\textsuperscript{5}), it took decades for the role of inflammation in the pathophysiology of atherosclerosis progression to be fully appreciated. The major immune cell in atherosclerotic lesions is the macrophage, the primary origin of which is myeloid progenitor cells in bone marrow. Myeloid progenitor cells develop into circulating monocytes, and in certain settings, the spleen acts as a reservoir for monocytes infiltrating atherosclerotic lesions, at least in the mouse.\textsuperscript{6} Interestingly, the production of monocytes in the bone marrow is stimulated by several cardiovascular risk factors, including hypercholesterolemia, leading to monocytosis (an increased number of circulating monocytes),\textsuperscript{7,8} which itself is an independent risk factor for atherosclerotic disease.\textsuperscript{9} Circulating monocytes then enter sites of arterial hemodynamic stress by adhering to the endothelial cells lining the lumen of susceptible arteries.\textsuperscript{10} Once the monocytes have entered the subendothelial space, they differentiate to lesional macrophages.\textsuperscript{11}

In addition, it is now recognized that a population of myeloid cells that have features and markers of both dendritic cells and macrophages are present in the artery wall in prelesional, susceptible arterial sites in humans, rabbits, and mice.\textsuperscript{11} It is not known if these cells help promote atherogenesis, although a proatherogenic role seems likely given that the number of these early cells correlates with susceptibility of atherosclerosis and that they can accumulate lipid and proliferate.\textsuperscript{12}

In this part of the *Compendium*, we will focus on lesion-al macrophage phenotype and function and the contribution of macrophages to the different stages of atherosclerosis. Examples of different stages of mouse and human atherosclerotic lesions and the presence of macrophages are shown in Figure 1.

### Lesional Macrophages Take on Different Phenotypes Depending on Microenvironmental Cues and Activated Intracellular Signaling Pathways

Serving to defend the organism from infection, macrophages have developed remarkable plasticity, notably, the ability to promote inflammation when needed and to turn the inflammatory response off when it is no longer needed. Thus, macrophages have the ability to assume inflammatory properties or inflammation suppressing and reparative properties that aid in the resolution phase of inflammation. Without this plasticity, an organism would either not be able to effectively fight infection or would be unable to heal after infection. In experimental systems, the classical inflammatory macrophage phenotype has been termed M1 (analogous to the T-cell nomenclature T-helper cell 1 [Th1]) and is often induced by incubating macrophages in vitro with a combination of interferon-γ (IFN-γ) and the toll-like receptor 4 (TLR4) ligand lipopolysaccharide. Lipopolysaccharide is a component of the cell wall of Gram-negative bacteria, whereas IFN-γ is produced primarily by natural killer cells and Th1 T cells after infection. When a resting macrophage encounters these stimuli, it initiates a strong inflammatory program, which includes production of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-12, and tumor necrosis factor-α (TNF-α), chemokines, such as monocyte chemoattractant protein-1 or chemokine [C–C motif] ligand 2 to attract more monocytes, and inducible nitric oxide synthase. The latter produces large quantities of nitric oxide, which helps kill pathogens. In addition, these macrophages produce reactive oxygen species through NADPH oxidase activation, which aid in pathogen destruction. Thus, the purpose of this inflammatory response is to markedly promote inflammation and kill pathogens.

Several subsets of alternatively activated macrophage populations, termed M2 (analogous to Th2 T cells) macrophages, have also been identified in vitro. The M2 phenotype can be induced by incubating macrophages with IL-4 and IL-13, both of which inhibit the M1 phenotype and prompt the macrophage to produce proresolving molecules, such as IL-10 and transforming growth factor-β. IL-4 and IL-13 are produced by Th2 T cells and are involved in tissue remodeling and repair. Moreover, IL-4 is a strong stimulus for macrophage proliferation.\textsuperscript{11}

The M1 and M2 classification of macrophage phenotypes is based on in vitro model systems with unknown relevance to in vivo states, and pure M1 and M2 macrophages almost certainly do not occur in atherosclerotic lesions, where macrophages are exposed to a plethora of stimuli that will result in different macrophage functions and cell-surface markers. Important attempts have been made to define a framework and consensus markers to describe macrophage activation,\textsuperscript{13} but more work is needed to characterize macrophage phenotypes, especially in vivo. The atherosclerotic lesional environment has been characterized as Th1-dominant based on studies with advanced human endarterectomy lesions, where IFN-γ is more abundant than IL-4.\textsuperscript{14} However, the M1/M2 paradigm is now being reassessed.\textsuperscript{16}

Several other macrophage populations have been suggested to be present in atherosclerotic lesions. These include the M(Hb) and Mhem populations,\textsuperscript{18} which are resistant to lipid loading and are induced by exposure to hemoglobin–haptoglobin complexes and haem in vitro, respectively; the Mox macrophage population, which is induced by exposure to oxidized phospholipids in vitro and is characterized by high expression of heme oxygenase-1\textsuperscript{19}; and the M4 population, which is induced by
the chemokine CXCL4. Recently, IL-17A–stimulated macrophages were suggested to constitute a new macrophage population distinct from M1, M2, and M4 macrophages.

We propose that the phenotype of lesional macrophages cannot be classified into predetermined subsets but rather is a consequence of the lesional microenvironment and the activation of specific intracellular signaling pathways. For example, activation of TLR4 can result in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, activation of ERK, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase (JNK), and IFN response genes, each of which has different downstream effects. Activation of the IL-4 receptor causes activation of signal transducer and activator of transcription 6 (STAT6), which can suppress TLR4 signaling. Cross talk among different signaling pathways is, therefore, likely to further affect macrophage phenotype. Thus, lesional macrophage phenotype can likely change rapidly as the microenvironment and intracellular signaling pathways change, for example by increased exposure to lipids or inflammatory stimuli. Indeed, laser capture microdissection of lesional CD68-positive macrophages in apolipoprotein E–deficient (Apoe−/−) mice has demonstrated that injection of lipopolysaccharide results in large increases in Ccl2, Vcam1, and Icam1 mRNA levels as early as 4 hours after injection. As such, lesional macrophages can best be viewed as representing a wide continuum of phenotypes and functions.

**Homeostatic Imbalance in Intracellular Lipids, Metabolites, and Proinflammatory Versus Proresolving Mediators Affects Macrophage Phenotype and Function**

The microenvironment in lesions is likely to be different in different areas of the lesion and in different stages of lesion development. The lesional microenvironment is also affected by systemic factors, such as dyslipidemia, low-grade inflammation associated with diabetes mellitus and autoimmune disease, and infection. We will briefly discuss 3 likely and highly interdependent contributors to macrophage phenotype:

1. **Macrophage Phenotype and Function**
2. **Proinflammatory and Proresolving Mediators**
3. **Intracellular Lipid Homeostasis**

These factors interact in complex ways to determine the phenotype of lesional macrophages, and their understanding is crucial for the development of novel therapeutic strategies to treat atherosclerosis.
cholesterol and lipid loading, metabolic state, and the balance between proinflammatory and proresolving mediators.

**Cholesterol and Lipid Loading Affect Macrophage Phenotype**

Hyperlipidemia is a well-known cause of cardiovascular disease and a strong driver of atherosclerosis in humans and animal models. One of the most commonly used mouse models of atherosclerosis—the low-density lipoprotein receptor-deficient (Ldlr−/−) mouse—takes advantage of this fact. These mice lack the ability to effectively clear LDL from the circulation and, therefore, develop large atherosclerotic lesions containing macrophage foam cells.34 Statins are currently the most effective drugs used in the prevention and treatment of cardiovascular events. The beneficial effects of statins are due primarily to their ability to lower plasma LDL cholesterol levels, although statins also exert anti-inflammatory effects that may be important in suppressing atherosclerosis in certain settings. Cholesterol-rich lipoproteins accumulate in arteries1 and are taken up by macrophages and dendritic-like cells. When macrophages take up more lipoprotein-derived cholesterol than they can excrete, the intracellular free cholesterol is converted into cholesteryl ester (CE), which accumulates in lipid droplets and results in the foam cell morphology observed by early pathologists.

Modified LDL, aggregated lipoproteins, and other substances are taken up by several different scavenger receptors (SRs) expressed by macrophages and by macrophage phagocytosis and phagocytosis35 (Figure 2A). These include SR type 1 (SR-A), CD36, SR-BI, LOX1,26 and LDL receptor–related protein 1.27 Apoe−/− mice deficient in SR-A28 or CD3629 in all tissues are largely protected from atherosclerosis. However, this effect seems to be influenced by the background of the mouse or possibly other factors, as SR-A-deficient or CD36-deficient Apoe−/− or Ldlr−/− mice backcrossed into the C57BL/6 background showed no reduction of atherosclerosis and abundant lesional macrophage foam cells.30 Recent studies have implicated SR-A in mediating proliferation of lesional macrophages,31 CD36 in inhibiting migration and promoting macrophage spreading and attachment32 and coordinating inflammasome activation,33 and SR-A and CD36 in promoting apoptosis, lesion necrotic core expansion, and inflammatory gene expression.34 The latter effects were observed in the absence of effects on lesion size and foam cell formation.34 Therefore, in addition to promoting lipid uptake, SR-A and CD36 seem to have important signaling functions that affect atherosclerosis progression.35

Whether foam cell formation per se is a proatherosclerotic event likely depends on the overall environment in which the lipid loading occurs. First, to the extent that cholesterol is stored as CE in cytoplasmic neutral lipid droplets, the membranes of the cells are protected from the cytotoxic effects of free cholesterol. Second, in a noninflammatory environment, lipid loading of macrophages may prevent inflammatory activation by activating liver X receptors (LXRs).36 The main LXR ligand in lipid-loaded macrophages was identified as desmosterol, a precursor of cholesterol in the Bloch pathway of cholesterol biosynthesis. The somewhat counterintuitive accumulation of a cholesterol precursor in lipid-loaded macrophages is because of a compensatory downregulation of cholesterol biosynthetic enzymes, and especially 24-dehydrocholesterol reductase, which is responsible for cholesterol synthesis from desmosterol.36 The physiological evolution of these protective processes may be related to a fundamental role of macrophages in clearing apoptotic cells (a process termed efferocytosis), which results in the delivery of large amounts of cholesterol to macrophages. Thus, it is plausible that cholesterol from dead cells engulfed by efferocytosis contributes to inhibition of endogenous cholesterol biosynthesis through a similar pathway, resulting in downregulation of cholesterol biosynthesis enzymes, desmosterol accumulation, and subsequent LXR activation in efferocytes.

An example where this LXR-mediated physiological process goes awry can be found with macrophages devoid of Niemann–Pick disease, type C1, the protein that is defective in patients with Niemann–Pick disease, type C1. Niemann–Pick disease, type C1 disease is a rare autosomal neurodegenerative disease resulting from the accumulation of cholesterol and glycolipids in late endosomes and lysosomes (Figure 2A). Ldlr−/− mice deficient in Niemann–Pick disease, type C1 in bone marrow–derived cells are characterized by increased atherosclerosis, and macrophages from these mice exhibit decreased synthesis of putative LXR ligands and decreased expression of cholesterol transporters (ATP-binding cassette, sub-family A, member 1 [ABCA1] and ATP-binding cassette, sub-family G, member 1 [ABCG1]), which are induced by LXRs, and impaired cholesterol efflux.37 Thus, a combination of spillover of free cholesterol or other lipid species into non-lipid droplet compartments, together with reduced production of endogenous ligands for LXR and other antiatherosclerotic transcription factors, likely explains the adverse effects of cholesterol loading under certain conditions.

Many studies have demonstrated that the balance between CE storage and efflux of free cholesterol determines the inflammatory phenotype of macrophages and that too much free cholesterol acts, at least in part, by disrupting the normal function of intracellular membranes and the plasma membrane. Thus, accumulation of unesterified free cholesterol causes inflammatory activation of macrophages by promoting endoplasmic reticulum (ER) stress38 and subsequent calcium leakage into the cytosol39; TLR4 activation by cholesterol enrichment of lipid rafts; inflammasome activation by cholesterol crystals40,41; and lysosome dysfunction.42 The lysosome is crucial in degrading LDL as lipoprotein-derived CEs in lysosomes are converted to free cholesterol by lysosomal acid lipase (Figure 2A). Lysosomal free cholesterol is then re-esterified by acyl-CoA cholesterol acyltransferase in the ER and stored in lipid droplets in the cytoplasm. Mice lacking acyl-CoA cholesterol acyltransferase 1 in bone marrow–derived cells exhibit massive xanthomatosis, and atherosclerotic lesions in these mice show a paucity of macrophage foam cells.35,43,44 While this may be due to free cholesterol-induced death,44 a recent study suggested an alternative mechanism, namely, decreased monocyte adhesion to activated endothelium.45,46

The cholesterol efflux transporters ABCA1 and ABCG1 promote cholesterol efflux from cells by transporting free cholesterol and phospholipids to free apolipoprotein A-I or...
Figure 2. Lipids and metabolism determine lesion macrophage phenotype and function. **A.** Lipoproteins affect macrophage phenotype. Lipoproteins are taken up by macrophages through macropinocytosis, phagocytosis, and scavenger receptors (SRs), including SR-A, CD36, SR-BI, and LOX1. The SRs also have signaling capacities. Because the lipoproteins are degraded in endosomes–lysosomes, cholesteryl ester (CE) is converted to free cholesterol (FC) by lysosomal acid lipase (LAL), and then redistributed to other cellular compartments through Niemann–Pick disease, type C (NPC) proteins. FC is converted back to CE by acyl-CoA cholesterol acyltransferase (ACAT) in the endoplasmatic reticulum (ER), and then again to FC before it can be effluxed from the cell through the cholesterol exporters ATP-binding cassette, sub-family A, member 1 (ABCA1) and ATP-binding cassette, sub-family G, member 1 (ABCG1) to apolipoprotein A-I (ApoA-I) or high-density lipoprotein. FC can exert detrimental effects if it is not re-esterified to CE in lipid droplets or effluxed through ABCA1 and ABCG1. For example, cholesterol crystal formation leads to inflammasome activation, and FC accumulation causes ER stress and cell death. These processes promote progression of lesions of atherosclerosis and necrotic core expansion. **B.** The metabolic state of the macrophage influences its phenotype. Increased glycolysis initiated by glucose entry into the cell through glucose transporters, such as glucose transporter 1 (GLUT1), is required for the inflammatory phenotype. Tricarboxylic acid (TCA) cycle intermediates also play important roles in inflammatory activation of macrophages. For example, the amino acid glutamine replenishes the TCA cycle to produce succinate, which in turn stabilizes the hypoxia-inducible factor 1 (HIF1) complex. Although a relative increase in fatty acid oxidation is associated with a more resolving macrophage phenotype, fatty acids and their metabolites exert several important effects in macrophages. Fatty acids are taken up by the cell through CD36 and fatty acid transport proteins (FATP) or by transport across the plasma membrane, and they are readily converted to acyl-CoAs by a group of enzymes with acyl-CoA synthetase (ACSL) activity, including long-chain ACSLs. Acyl-CoAs are channeled to different fates in the cell, including oxidative phosphorylation (OxPhos), neutral lipids, phospholipids (PL), sphingolipids, or they are used for protein modification or signaling. Sphingolipids are found in lipid rafts and are believed to promote inflammatory activation by toll-like receptors (TLRs) present in these rafts. Reduced levels of intracellular fatty acids or acyl-CoAs or reduced OxPhos lead to reduced inflammatory activation and atherosclerosis.
high-density lipoprotein (Figure 2A). Thus, ABCA1 deficiency and ABCG1 deficiency causes inflammatory activation of macrophages and promotes atherosclerosis.45–48 Importantly, the cholesterol efflux capacity of high-density lipoprotein is inversely associated with the incidence cardiovascular events in human subjects.49 A major role of ABCA1 and ABCG1 is to prevent free cholesterol accumulation in hematopoietic stem cells in the bone marrow, thereby preventing their proliferation and subsequent leukocytosis in response to fat feeding in mice,50 but, in addition, these transporters almost certainly have important local effects in lesional macrophages. Thus, an imbalance in cholesterol metabolism, rather than just increased lipid droplet formation per se, is likely to promote atherosclerosis by affecting various inflammatory and detrimental processes in macrophages. Conversely, inflammation inhibits cholesterol efflux from macrophages and reverse cholesterol transport in vivo,51 demonstrating that inflammation and reduced cholesterol efflux may participate in a detrimental amplification cycle.

Together, these findings show that the balance among uptake, intracellular processing, and efflux of cholesterol is carefully tuned and that disturbances in this complex machinery can result in macrophage dysfunction, altered activation of nuclear receptors, inflammatory activation, and atherosclerosis. Human relevance still needs to be established for much of this work. Furthermore, few of the atherosclerosis studies in this area used mouse models in which expression of the protein being studied was restricted to macrophages, leaving the possibility that some of the effects could have been mediated by other immune cells, bone marrow progenitor cells, or other cell types.

**Macrophage Metabolic State and Phenotype—A Bidirectional Relationship**

Inflammation and metabolism are inextricably linked, and the metabolic phenotype of a macrophage can determine its inflammatory phenotype and vice versa. We define macrophage metabolic phenotype as a phenotype induced by substrates and intermediates used in energy metabolism. M1 and M2 macrophages in vitro demonstrate important differences in metabolism in that M2 macrophages rely more on fatty acid oxidation, whereas M1 macrophages rely on an increase in glycolysis.52 Furthermore, fatty acid oxidation seems to be required for the M2 phenotype,53 and glycolysis is required for inflammatory activation and survival of activated macrophages.54–58 However, increased glycolysis through overexpression of the glucose transporter glucose transporter 1 (GLUT1) in myeloid cells is not sufficient to promote inflammatory activation in vivo or atherosclerosis.59 Inhibiting glycolysis in activated macrophages can result in increased apoptosis and thereby could increase necrotic core formation.

**What Factors Might Regulate Macrophage Metabolism in Lesions In Vivo?**

**Modulators of Glucose Metabolism**

Inflammatory mediators are well-known to induce several enzymes required for glucose uptake and glycolysis in macrophages (Figure 2B). It is therefore tempting to speculate that conditions associated with low-grade inflammation, such as diabetes mellitus and metabolic syndrome and several autoimmune diseases, are associated with altered metabolism in lesional macrophages. Furthermore, hypoxic conditions are believed to occur in advanced human atherosclerotic lesions. The presence of hypoxia in mouse lesions, which are much smaller than human lesions, is less certain. What is clear, however, is that hypoxia-inducible factor 1 (HIF1α and HIF1β) is present in both human and mouse atherosclerotic lesions.60–62 HIF1 is induced by hypoxia, but also by inflammatory stimuli, such as lipopolysaccharide. Interestingly, succinate generated primarily from glutamine has been shown to stabilize the HIF1 complex and induce IL-1β.63 The HIF1α and HIF1β complex induces transcription of genes involved in stimulating angiogenesis, inflammation, and metabolism.64–66 One of the genes induced by HIF1 in macrophages is GLUT1. The effect of hypoxia on atherosclerosis seems to be detrimental, with a recent study showing necrotic core expansion and defective macrophage efferocytosis in advanced mouse lesions.67 Thus, inflammatory mediators and perhaps hypoxia promote an inflammatory macrophage phenotype associated with increased glucose uptake and metabolism.

**Role of Fatty Acid Handling**

Inflammatory mediators can also increase proteins and enzymes involved in intracellular fatty acid handling. Fatty acids and their downstream lipid mediators have important effects on macrophage phenotype, and, like with cholesterol, the balance between uptake and metabolism as well as cellular localization are key. Free fatty acids enter the cell, in part through CD36 and fatty acid transport proteins, or by flip-flopping across the plasma membrane, and are quickly bound to intracellular fatty acid–binding proteins (FABPs) or are esterified into their acyl-CoA derivatives (Figure 2B). Macrophages express the FABPs, FABP4 (aP2) and FABP5 (Mal1). Macrophages deficient in FABP4 or FABP5 exhibit reduced inflammatory activation, and inhibition of FABP4 or FABP5 results in reduced atherosclerosis.61–63 This phenotype is associated with reduced inflammatory activation (NF-kB activity) and increased peroxisome proliferator–activated receptor-γ (PPARγ) activity, perhaps because free unsaturated fatty acids can act as PPARγ ligands. Conversion of free fatty acids into their acyl-CoA derivatives traps them in the cell because of the large hydrophilic CoA moiety and thereby promotes their entry into a variety of different pathways, including fatty acid oxidation, phospholipid synthesis and reacylation, diacylglycerol and triacylglycerol synthesis, esterification of free cholesterol into CEs, and sphingolipid metabolism.64 Long-chain acyl-CoA synthetases catalyze this reaction of acyl-CoA synthesis from free fatty acids and CoA. One of the acyl-CoA synthetase isofoms expressed in macrophages, acyl-CoA synthetases 1, is markedly upregulated by inflammatory mediators and is required for atherosclerosis in diabetic mice.58 Moreover, macrophages deficient in serine palmitoyltransferase subunit 2 exhibit reduced levels of sphingomyelin in lipid rafts, which results in reduced TLR4 activity and reduced atherosclerosis.65 Fatty acids might promote inflammatory processes by several different mechanisms, including altered activation of nuclear receptors, altered generation of bioactive lipid mediators, and facilitation of TLR4
activation through organization of TLR4 receptor complexes within lipid raft domains. A similar mechanism has been proposed for how free cholesterol promotes TLR4 activation.47 Finally, omega-3 fatty acids and arachidonic acid can be converted into classes of lipids called specialized proresolving mediators, that quell the inflammatory response and promote tissue repair and healing.65 Examples include lipoxins, resolvins, protections, and maresins. Inflammation resolution is defective in advanced atherosclerosis, perhaps in part because of defective specialized proresolving mediator synthesis or action, and treatment of athero-prone mice with resolving mediators suppresses plaque progression.66–70

**Role of Oxidative Phosphorylation**

Finally, excessive oxidative phosphorylation can induce mitochondrial oxidative stress (Figure 2B), which in turn can promote an inflammatory macrophage phenotype and atherosclerosis. The role of mitochondrial oxidative stress in atherosclerosis was recently addressed by taking advantage of a mouse model in which mitochondrial oxidative stress is quenched by a mitochondria-targeted catalase construct. This study demonstrated that fat-fed Ldlr−/− mice have increased mitochondrial oxidative stress in lesional macrophages and that quelling mitochondrial oxidative stress in hematopoietic cellssuppresses atherosclerosis and cytokine production by decreasing the activation of NF-κB.71 Together, these studies demonstrate that macrophage metabolism, phenotype, inflammation, and atherosclerosis are inextricably linked and that by directing flow of metabolites to specific fates in macrophages, it is possible to alter their overall phenotype.

**Balance Between Proinflammatory and Proresolving Mediators Governs Lesional Macrophage Phenotype**

The net proatherogenic effect of lesional macrophages can be understood as a delicate balance between inflammatory and proresolving processes in these cells,72 as illustrated in Figure 3.

**Proinflammatory, Proatherosclerotic Cytokines**

Macrophages can release proinflammatory cytokines and chemokines, including chemokine (C–C motif) ligand 2, IL-1, IL-6, IL-12, IL-15, IL-18, and TNF-α.73 Chemokine (C–C motif) ligand 2 and its receptor CCR2 was the first chemokine-receptor unit shown to be required for initiation of atherosclerosis in mice through promoting monocyte recruitment into lesions.74–76 Another chemokine receptor, CXCR2, was also shown early to be proatherogenic: fat-fed Ldlr−/− mice transplanted with bone marrow from CXCR2-deficient mice exhibited reduced athero-sclerosis and lesion macrophage accumulation.77 This receptor binds many chemokines of the CXC family, including CXCL1 and CXCL8 (IL-8). Both CCR2 and CXCR2 are 7-transmembrane G protein–coupled receptors that act to activate PI3 kinase, and the small G proteins, Rac and Rho, which mediate cytoskeletal rearrangements and monocyte migration into lesions. Other monocyte receptors playing a role in the recruitment of monocytes into lesions of atherosclerosis under different conditions include CCR1, CCR5, and CX3CR1.78,79

The list of cytokines and chemokines promoting atherosclerosis in mice has grown considerably. A recent addition is IL-17A, which promotes monocyte adhesion and sensitization of antigen-presenting cells to lipopolysaccharide.23 IL-17A binds to its receptor, which is composed of 2 subunits (IL-17RA and IL-17RC), and activates a signaling pathway similar to that of IL-1 and TNF-α, culminating in rapid activation of NF-κB and increased production of inflammatory mediators. These observations are in agreement with studies showing that bone marrow transplantation of TNF receptor 1 knockout mice into fat-fed Ldlr−/− mice leads to smaller lesions, smaller macrophage lesion area, and reduced chemokine (C–C motif) ligand 2 levels.80 Likewise, Apoe−/− mice deficient in IL-1β exhibit smaller lesions and reduced aortic Ccl2 mRNA.81 NF-κB is activated by IkB kinase–mediated degradation of the Ikβ subunit, which sequesters NF-κB in the cytosol. After IkB degradation, liberated NF-κB subunits translocate to the nucleus to mediate their effects on transcription. It was therefore surprising that deleting IkB kinase in myeloid cells resulted in increased atherosclerosis in Ldlr−/− mice.82 Reduced expression of IL-10, which exhibits anti-inflammatory/proresolving and antiatherosclerotic properties,83 might explain this result. Moreover, TRAF5, one of the signaling molecules downstream of TNF receptor 1 and the IL-17A receptor, has been shown to be atheroprotective by preventing monocyte recruitment.84 These findings further highlight the complexity and cross talk of intracellular signaling pathways.

IL-23 is another IL recently demonstrated to have important effects on lesions: IL-23 promotes necrotic core expansion by mediating macrophage apoptosis.85 The IL-23 receptor is composed of IL-12p40 and IL-23p19 subunits and activates a signaling pathway more similar to type I IFNs, leading to apoptosis and necrotic core expansion through STAT transcription factors, including STAT1 (Figure 3). Consistent with this notion, IFN-β in myeloid cells promotes atherosclerosis and formation of necrotic cores in mice,86 and hematopoietic STAT1 deficiency results in reduced atherosclerosis concomitant with reduced macrophage foam cell formation.87

A causative role for inflammation in promoting cardiovascular events in humans has not yet been established. However, evidence supporting a role for inflammation comes from studies demonstrating an increased risk of cardiovascular disease across a range of chronic inflammatory disorders and association between cardiovascular risk and severity of inflammation.88 Furthermore, some of the cardioprotective effects of statins may be because of their anti-inflammatory effects, particularly when administered soon after the onset of acute coronary syndromes.89

**Proresolving, Antiatherosclerotic Mediators**

Macrophages also release proresolving mediators, such as IL-10 and transforming growth factor-β70 (Figure 3). Other examples of chemokines with proresolving functions in mouse models of atherosclerosis include IL-13 and IL-27, which limit accumulation of macrophages in lesions,90,91 and CXCL5, which promotes cholesterol efflux by inducing ABCA1 in macrophages and also limits macrophage accumulation in lesions.92 Annexin A1, acting through the receptor ALX/FPR2 (formyl peptide receptor 2), has emerged as another
proresolving protein with the ability to prevent myeloid cell recruitment into lesions of atherosclerosis in mice, and some studies indicate that adenosine binding to its receptor (A<sub>2a</sub>) can be added to this growing group of proresolving molecules.

Furthermore, specialized proresolving mediators generated from arachidonic acid or omega-3 fatty acids (fish oils), including lipoxins and resolvins, mediate resolution of inflammation. The balance between the synthesis of the proinflammatory and proresolving mediators in macrophages is regulated by the subcellular localization of 5-lipoxygenase, the enzyme responsible for generation of both proatherogenic leukotriene B4 and the proresolving lipoxin A4 derived from omega-3 fatty acids. The subcellular localization of 5-lipoxygenase is, in turn, regulated by resolvin D1, which is derived from omega-3 fatty acids.

### Nuclear Receptors

Finally, the inflammatory phenotype of macrophages can be markedly suppressed by transcription factors, transcriptional transactivators and repressors, and epigenomic processes. PPARs (PPAR<sub>α</sub>, PPAR<sub>γ</sub> and PPAR<sub>δ</sub>) in macrophages are now well known to reduce inflammatory activation and atherosclerosis in mice. The PPAR<sub>γ</sub> activators thiazolidinediones, such as rosiglitazone and pioglitazone, have been used as insulin sensitizers. However, rosiglitazone was found to have adverse effects on cardiovascular end points, and other glitazones have other negative effects. Systemic PPAR<sub>γ</sub> activation is, therefore, not currently a promising strategy to reduce lesion inflammation. Fibrates, such as clofibrate and fenofibrate, act as PPAR<sub>α</sub> activators and are used for the treatment of elevated blood lipids. Like the glitazones, fibrates have shown no significant beneficial effect on cardiovascular end points. Other nuclear receptor transcription factors also suppress the inflammatory phenotype of macrophages and atherosclerosis, including LXR<sub>α</sub> and LXR<sub>β</sub>.

As discussed above, LXR<sub>α</sub> act as cholesterol sensors and induce genes involved in reverse cholesterol transport (ABCA1 and ABCG1) to promote cholesterol efflux and inhibit inflammatory activation of macrophages. The balance between proinflammatory and proresolving mediators in lesional macrophages is likely to affect their function as well as atherosclerosis.

### Transdifferentiation of Smooth Muscle Cells to Macrophage-Like Lesion Cells

In the past few years, there have been significant discoveries related to the role of smooth muscle cells and their contribution to macrophage-like foam cells in murine and human atherosclerosis. Although the origin of lesional foam cells was believed to be the smooth muscle cell by some investigators as early as in the 1950s, this concept has recently been investigated by using lineage tracing. These studies have demonstrated that smooth muscle cells make up a much larger part of lesional foam cells than previously thought and that these cells can also express macrophage markers, such as CD68. The role of these smooth muscle–derived foam cells in atherosclerosis and plaque rupture will now have to be reevaluated.

### Role of Macrophages in Lesion Initiation and Progression

Macrophage accumulation within the subendothelium or neointima constitutes one of the first steps in atherogenesis (Figure 4). Fatty streak lesions with macrophages can be observed in human fetal aortas, especially if the mother is hypercholesterolemic. These early macrophages accumulate in susceptible regions of arteries because of endothelial adhesion molecule expression and the presence of apoB lipoproteins in the subendothelium. Expression of chemokines by endothelial cells and macrophages recruit additional monocytes by interacting with monocyte receptors, including CCR2, CCR5, CX3CR1, and CXCR2, if the arterial insult, for example, hyperlipidemia, diabetes mellitus, or smoking, persists. The macrophages ingest modified lipids and other substances that accumulate in the subendothelium. The mechanisms governing atherogenesis and formation of advanced lesions are, in part, distinct. For example, macrophage apoptosis is protective...
in early lesions, resulting in reduced lesion size because of efficient efferocytosis by neighboring macrophages, but promotes lesion size and development of necrotic cores in advanced lesions because of defective efferocytosis (below). Early lesions can resolve, perhaps by macrophages leaving the lesion or, as alluded to above, by efferocytosis.

The fatty streak or pathological intimal thickening with macrophages grows primarily by accumulation of more macrophages and by expansion of macrophages foam cells (Figure 1A and 1C). Local proliferation of macrophages can also contribute to the accumulation of cells, at least in fat-fed mice and rabbits, although monocyte recruitment is likely to dominate in early lesions, whereas macrophage proliferation takes on a more important role in more advanced lesions. Arrest of macrophage proliferation results in reduced lesion size.

### Role of Macrophages in Advanced Necrotic Lesions

In advanced lesions (Figures 1B, 1D, and 4), macrophage apoptosis is increased, due in part to increased ER stress, which can be induced for example by free cholesterol or fatty acids. Macrophages undergoing ER stress are more susceptible to apoptosis induced by oxidized phospholipids or lipoproteins through pathways that involve CD36, TLR2, SR-A, and STAT1. Most importantly, advanced lesional macrophages have a defect in clearing these apoptotic cells, which contributes to plaque necrosis and increased inflammation because of release of inflammatory mediators from uncleared, postapoptotic necrotic cells.

Efferocytosis is mediated by the interaction of apoptotic cell recognition motifs, macrophage receptors, such as MerTK (MER proto-oncogene and tyrosine kinase), and molecules that bridge these 2 components. It is possible that the impaired ability of macrophages to efferocytose apoptotic cells in advanced lesions is because of impaired function of these proteins. For example, MerTK is shed from macrophages activated by TLR4 through a pathway that involves NADPH oxidase and the metalloproteinase ADAM17, and there is evidence that this process occurs in advanced human atheroma. In addition, primary necrosis of macrophages in lesions can occur through a pathway that is mediated by receptor interacting protein 3 (RIP3, a mediator of necrosis). Together, macrophage apoptosis, necrosis, and reduced efferocytosis contribute to expanding necrotic cores, which make the lesion unstable and more likely to rupture or fissure.

Macrophage autophagy (self-eating—the intracellular processes by which a cell transports cytoplasmic components,
such as damaged and dysfunctional organelles and aggregates, into the lysosomal lumen for degradation and recycling\textsuperscript{122}\textsuperscript{122} triggered by increased ER stress has been shown to protect against lesion necrosis. For example, inhibition of autophagy by silencing the E3 ubiquitin ligase ATG5 (autophagy protein 5) results in increased macrophage apoptosis and NADPH oxidase–mediated oxidative stress and, in addition, renders the apoptotic cells less well recognized by efferocytes.\textsuperscript{123}\textsuperscript{123} Furthermore, defective autophagy in macrophages is associated with proatherogenic inflammasome activation in response to cholesterol crystals and larger atherosclerotic lesions in Apoe\textsuperscript{−/−} mice\textsuperscript{124}\textsuperscript{124} and also reduced cholesterol efflux through ABCA1.\textsuperscript{125}\textsuperscript{125} Autophagy has therefore emerged as an important process that may protect lesional macrophages from apoptosis, inflammasome activation, and cholesterol accumulation.

Macrophages have also been suggested to contribute to fibrous cap thinning and plaque rupture by the secretion of matrix metalloproteinases,\textsuperscript{126}\textsuperscript{126} although this has been difficult to prove in mouse models because mice do not exhibit the type of plaque rupture that occurs in humans. Intraplaque hemorrhage is sometimes present in association with macrophage accumulation in advanced human lesions\textsuperscript{127}\textsuperscript{127} and is also seen in mice, particularly in advanced lesions of diabetic mice.\textsuperscript{128}\textsuperscript{128} It is possible that macrophages contribute to intraplaque hemorrhage in advanced lesions through the secretion of proteases.

Together, these studies demonstrate that macrophages play an important role in the development of advanced lesions, in particular necrotic core formation, which destabilizes the lesion and thereby promotes acute clinical cardiovascular events.

**Role of Macrophages in Lesion Regression and Resolution**

Regression of lesions can be induced in hyperlipidemic mouse models by aggressive lipid lowering and in diabetic mice by blood glucose lowering.\textsuperscript{129}\textsuperscript{129–132}\textsuperscript{129–132} The regression in these models is characterized by reduced lesion macrophage content (Figure 4) and altered gene expression in the remaining CD68-positive cells. CD68-positive cells from regressing lesions exhibit elevated levels of Arg1 and Cd163 (genes often used as markers of the M2 phenotype) and reduced levels of Ccl2 and Tnfa mRNA.\textsuperscript{129}\textsuperscript{129} However, there is also upregulation of the inflammatory cytokines/chemokines Cxcl2 and Il1b,\textsuperscript{129}\textsuperscript{129} suggesting that the macrophage phenotype is not entirely anti-inflammatory or possibly that CD68-positive smooth muscle cells contribute to some of the differences. It remains somewhat controversial as to whether macrophages leave the lesion during regression or whether the reduced macrophage content is due primarily to a reduced recruitment of new monocytes.\textsuperscript{11}\textsuperscript{11–133}\textsuperscript{133–135}\textsuperscript{11} The latter concept was recently supported by regression studies in diabetic mice, in which the impaired regression was because of increased recruitment rather than reduced egress.\textsuperscript{131}\textsuperscript{131} It is possible that macrophage egress and reduced monocyte recruitment both occur in regressing lesions, and that their relative contribution to lesion regression differs in different states. Improved efficiency of efferocytosis and autophagy may also contribute to the reduced macrophage content in regressing lesions. Furthermore, it is possible that macrophage proliferation is reduced in regressing lesions because of a reduced engagement of SR-A when lipid levels fall. The extent to which these mechanisms are operative in human subjects is as yet unknown.

**Novel Macrophage-Based Treatment Possibilities and Future Directions**

Lipid lowering by statins is effective in preventing cardiovascular events and would be even more effective if medication compliance and safety issues enabled earlier and more robust LDL lowering. This is particularly the case when additional cardiovascular risk factors are present, including smoking and diabetes mellitus, where low levels of LDL are usually necessary to prevent atherosclerotic disease. Perhaps the new availability of PCSK9 inhibitors will help narrow this therapeutic gap. In the meantime, however, we can ask whether therapeutic measures targeting proatherogenic processes in macrophages, particularly inflammation or defective resolution, can be additive or synergistic with lipid-lowering therapy in terms of cardiovascular risk reduction. It is possible that agents that suppress systemic inflammation will be effective,\textsuperscript{116,136}\textsuperscript{116,136} but suppression of inflammation is likely to be detrimental in the long term because of increased susceptibility of infection. Furthermore, systemic inhibition of inflammatory processes might have effects that are different from those of local inhibition of the lesional macrophage inflammatory phenotype.\textsuperscript{137}\textsuperscript{137} Increasing atherosclerosis resolution might provide novel therapeutic opportunities because proresolving mediators, unlike direct inhibitors of inflammatory cytokines or chemokines, are less likely to compromise host defense.\textsuperscript{137}\textsuperscript{137} Recent studies in mice have demonstrated the potential promise of proresolving therapy for atherosclerosis.\textsuperscript{68,93}\textsuperscript{68,93}

A more macrophage-specific strategy is to target these cells by using nanoparticles. Nanoparticles can be designed to release their cargo in lesions for activation of macrophage cell-surface receptors or for internalization by macrophages. The former was the strategy used for delivering a proresolving mediator to lesions.\textsuperscript{68}\textsuperscript{68} Other studies in mice have demonstrated beneficial effects on atherosclerosis using nanoparticles to interfere with SR-mediated oxidized lipid uptake,\textsuperscript{138}\textsuperscript{138} statin-loaded nanoparticles that have anti-inflammatory effects in lesions,\textsuperscript{139}\textsuperscript{139} and nanoparticles encapsulating CCR2-silencing short interfering RNA that prevents monocyte recruitment to lesions.\textsuperscript{140}\textsuperscript{140} Development of nanoparticles or other treatment strategies to prevent necrosis or improve efferocytosis in advanced lesions might lead to prevention of plaque rupture. Future work will be needed to determine the feasibility of treating humans with nanoparticles for long periods of time.

Although our understanding of the phenotypes and functions of macrophages in different stages of atherosclerosis has increased markedly since Virchow made his seminal observations, many important questions remain. For example, more work is needed to understand how lesional macrophages and atherosclerosis are governed by events in the bone marrow and spleen versus local factors in lesions, the role of smooth muscle transdifferentiation into macrophage-like cells in lesions, and whether targeting macrophages using new forms of therapy has promise in suppressing plaque progression and acute clinical events.
Translation of Findings From Mouse Studies to Human Disease

As we have highlighted in this review, many of the concepts that have been studied in depth in vitro and in animal models have their basis in human observations. Examples include the importance of retained apoB lipoproteins in initiating the atherogenic process and the protection against cardiovascular disease by treatments that lower those lipoproteins; the overall role of inflammatory macrophages and a maladaptive inflammatory response in lesion progression; the association of high-density lipoprotein function, that is, ability to efflux cholesterol from macrophages, with protection from cardiovascular disease; and the association of hyperoxia, ER stress, oxidative stress, cell death, and defective efferocytosis with advanced lesions. Indeed, the appreciation of the role of macrophages and inflammation in atherosclerosis has led to 2 current human trials, one using anti–IL-1β and the other low-dose methotrexate, which target the inflammatory response. Moreover, ongoing clinical trials using resolving mediator therapy in other inflammatory diseases in humans may set the stage for their use to prevent the progression of atherosclerosis. A clear challenge that lays ahead in the translational work in this area is how to assess efficacy in humans before committing to expensive and long-term end point trials. As such, parallel developments in macrophage-based imaging and biomarker studies are essential as the mechanistic and preclinical studies progress.

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Disclosures

None.

References


classical monocytes. 


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