Phenotypic Polarization of Macrophages in Atherosclerosis

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Abstract—Macrophages orchestrate the inflammatory response in inflamed tissues, and recent work indicates that these cells can alter their phenotypes and functions accordingly in response to changes in the microenvironment. Initial work in models of cardiovascular disease used immunologic markers to characterize macrophage phenotypes present in atherosclerotic plaque, and these studies have lately been extended through the use of markers that are more specific for atherosclerosis and metabolic disease. Together, these studies have led to a novel view of the function of macrophages in the development of atherosclerosis that suggests dynamic plasticity. Understanding this plasticity and the ensuing macrophage heterogeneity could lead to novel strategies of pharmacological intervention to combat chronic inflammation in metabolic diseases. Most importantly, revealing the functional characteristics of individual macrophage phenotypes will lead to a better understanding of their contribution to lesion development and plaque stability. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: atherosclerosis ▪ chemokines ▪ cytokines ▪ macrophages

Overview of Macrophage Polarization Phenotypes in Atherosclerosis

Despite major advances in our understanding of the various functions of macrophages in atherosclerotic lesions, what actually determines macrophage phenotypic polarization during lesion development remains enigmatic. It is generally accepted that the tissue microenvironment determines macrophage phenotypic polarization. That is, macrophages specifically respond to extracellular cues ranging from bacterial components to oxidatively modified molecules, translating information by using a range of cell surface receptors and their associated intracellular signaling cascades. On the contrary, macrophages also respond to changes in their intracellular milieu, such as cholesterol loading or endoplasmic reticulum stress, by inducing highly specified adaptive mechanisms and reactions, using intracellular sensors, such as redox-dependent transcription factors or nuclear hormone receptors. Although the response to inflammatory and anti-inflammatory stimuli is quite well understood, the mechanisms that are evoked by other stimuli are just beginning to be unraveled.

Classical and Alternative Activation

A continuum of pro- and anti-inflammatory macrophages, with extreme polarization phenotypes M1 and M2 (or classically and alternatively activated) macrophages, can be found in atherosclerotic lesions. Although it is unlikely that bacterial components activate toll-like receptor (TLR) signaling to produce M1 macrophages in atherosclerotic lesions, endogenously formed TLR2 and TLR4 ligands, such as free fatty acids, oxidized lipids, and high mobility group 1 protein, as well as interferon γ, likely contribute to classical activation of lesion macrophages. The nuclear factor (NF)κB pathway is important for upregulation of inflammatory gene expression in M1 macrophages, and myeloid deletion of IkB kinase results in decreased inflammation and reduced lesion formation in low-density lipoprotein receptor−deficient mice. In addition, activation of the NODlike receptor family, pyrin domain containing 3 (NLRP3) inflammasome was shown to contribute to inflammatory gene expression and M1 phenotypic polarization in lesion macrophages. Moreover, decreased migratory capability and thus prolonged residence in a tissue could contribute to an inflammatory phenotype. In that regard, oxidized phospholipids inhibited migration of macrophages, and oxidized low-density lipoprotein (LDL) was shown to prolong the residence of macrophages in atherosclerotic lesions via CD36-induced signaling.

Microenvironmental factors that contribute to M2 polarization in atherosclerotic lesions are primarily thought to be not only Th2-type cytokines (interleukin [IL]-4, IL13, IL10) but also immunocomplexes or certain lipid products, all of which have been shown to drive M2 polarization by inducing different signaling and transcriptional programs. Moreover, engulfment of apoptotic cells leads to an M2-like phenotype, characterized by the release of not only anti-inflammatory mediators, transforming growth factor−β and IL10, but also prostaglandin E2, which, depending on relative prostaglandin E2 receptor subtype expression, could have both pro- or anti-inflammatory activity.

Several transcription factors, including peroxisome proliferator activated receptor (PPAR)γ and Kruepplike factor-4, were shown to promote M2 polarization in macrophages. In support of a protective role of M2 macrophages in atherosclerosis, myeloid-specific deficiency of either PPARγ or Kruepplike factor-4 resulted in accelerated...
lesion formation in apolipoprotein E–deficient\textsuperscript{19} or low-density lipoprotein receptor knockout\textsuperscript{20} mice. Moreover, application of PPAR\textgamma\ ligands resulted in a preferential M2 polarization in mice and in humans.\textsuperscript{27} The nuclear orphan receptor (NR)4A was recently shown to be involved in macrophage differentiation, and although one study demonstrated NR4A to be involved in macrophage phenotypic polarization, showing that myeloid deletion of NR4A led to increase inflammatory gene expression in macrophages, resembling an M1 phenotype, and increased atherosclerotic lesion formation in low-density lipoprotein receptor knockout mice,\textsuperscript{21} another study demonstrated that myeloid NR4A does not play a role in these processes.\textsuperscript{22}

Macrophage Phenotypic Changes in Response to Tissue Damage

Lipid accumulation and oxidative tissue damage are hallmarks of atherosclerotic lesions. Macrophages sense a variety of lipid classes, which induces highly specific transcriptional programs and phenotypic changes.\textsuperscript{4} Oxidized phospholipids are lipid oxidation products that accumulate during atherosclerosis and thus are characteristic of the immunologic environment in atherosclerotic lesions. Oxidized phospholipids induce a transcriptional program in macrophages that is driven by the redox-dependent transcription factor, nuclear factor erythroid-2 related factor (Nrf2).\textsuperscript{11} As a consequence, cells respond with upregulation of a battery of redox-regulating genes, including heme-oxygenase, glutathione-reductase and -synthase, as well as thioredoxin reductase and sulfiredoxin, which results in the formation of the Mox phenotype. Mox macrophages are abundant in advanced mouse lesions, comprising \textasciitextsuperscript{\approx}30\% of all CD68-positive cells\textsuperscript{11} and may play an atheroprotective role, because myeloid deficiency of Nrf2 results in accelerated atherogenesis in low-density lipoprotein receptor-deficient mice.\textsuperscript{23} However, expression of certain inflammatory genes, including cyclooxygenase-2 and IL\textsubscript{1}\beta, was also upregulated in Mox macrophages. Oxidized phospholipids have the potential to modulate TLR-dependent gene expression, and we have recently shown that macrophages respond to oxidized phospholipids by upregulation of inflammatory gene expression in wild-type, but not in TLR2-deficient murine bone marrow–derived macrophages.\textsuperscript{24} However, the potency of expression was several fold lower than that induced by conventional TLR2 ligands, such as lipoteichoic acid.

Intraplaque hemorrhage is a serious event that may significantly contribute to lesion destabilization. Therefore, it is plausible that repair mechanisms come into play that involve macrophages. Macrophages responding to heme and hemo-globin in areas of plaque hemorrhage have been called Mhem, a phenotype that is characterized by increased HO-1 expression\textsuperscript{25,26} that is induced via CD163 and involves activation of Nrf2.\textsuperscript{27} Mhem macrophages have reduced capacity for foam cell formation, potent anti-inflammatory, and tissue regenerative function, and thus may play an important role in plaque stabilization.\textsuperscript{25,28}

Chemokines, cytokines, and growth factors all can contribute to phenotypic polarization of macrophages within atherosclerotic lesions.\textsuperscript{29} Treatment of macrophages with granulocyte-macrophage colony stimulating factor induces a CD11c\textsuperscript{*} phenotype that resembles M1 macrophages,\textsuperscript{30} and granulocyte-macrophage colony stimulating factor–deficient mice have reduced atherosclerosis.\textsuperscript{31} The platelet-derived chemokine CXCL4 is abundant in atherosclerotic lesions\textsuperscript{32,33} and was shown to induce a transcriptional profile in macrophages that was strikingly different from that seen in M1 or M2 macrophages and were termed M4.\textsuperscript{34} These M4 macrophages showed a decreased phagocytotic capacity\textsuperscript{35} and, interestingly, CXCL4 treatment drastically downregulated the expression of the hemoglobin receptor CD163, resulting in a decreased ability to express HO-1,\textsuperscript{35} thereby potentially counteracting the development of the Mhem phenotype. Whether M4 macrophages are involved in regulating repair mechanisms remains to be shown.

Impact of Lipid Loading on Phenotypic Polarization

An important function of lesion macrophages is to remove excess lipid, mostly in the form of LDL, and the lipid-laden macrophages that appear in the plaque are referred to as foam cells.\textsuperscript{7} Transcriptional profiling of foam cells generated by oxidized LDL treatment revealed a dendritic cell-like phenotype, including upregulation of CD11c and MHC-II.\textsuperscript{36} Proteomic analysis of foam cells generated by acetyl-LDL loading revealed changes in expression of genes involved not only in lipid metabolism but also in complement activation and lysosomal proteolysis, often by post-transcriptional mechanisms.\textsuperscript{7}

Foam cells have a remarkably long survival time within lesions, despite the huge toxic load and stressful environment. The mechanisms that protect foam cells and increase their resistance to stress-induced damage are still poorly understood. Similarly, little is known about the propensity of individual macrophage phenotypes to become foam cells. For example, both M4 and Mhem were shown to have a reduced capacity to take up cholesterol and increased liver X receptor (LXR)-dependent reverse cholesterol transport,\textsuperscript{26,34} which would imply that M1 or M2 macrophages are the main foam cell precursors.

Traditionally, foam cell formation has been linked to a proinflammatory phenotype.\textsuperscript{38} Nevertheless, the mechanisms that link cholesterol loading to inflammatory reactions in macrophages have not yet been well defined. Both LXRs and PPARs are important lipid sensors that not only regulate expression of genes involved in lipid metabolism but also were shown to suppress inflammatory signaling in macrophages.\textsuperscript{16} Saturated and polyunsaturated fatty acids were recently shown to differentially control PPARγ-dependent inflammatory gene expression.\textsuperscript{39} Moreover, PPARδ activation reduced very low density lipoprotein-mediated foam cell formation and inflammatory gene expression in macrophages.\textsuperscript{40} A recent study from by Spann et al\textsuperscript{41} demonstrated that cholesterol loading coincided with a general LXR-dependent downregulation of inflammatory pathways and gene expression in macrophages. These authors identify desmosterol, a precursor of cholesterol, as an intrinsic regulator of lipid and inflammatory homeostasis in foam cells. Together, these findings imply that the extracellular milieu is mainly responsible for proinflammatory activation of lipid-laden lesion macrophages, whereas intracellular lipid sensing can inhibit inflammatory responses.
Localization of Macrophage Phenotypes Within Atherosclerotic Plaques

In light of the coexistence of various macrophage phenotypes in atherosclerotic lesions, the question arises whether individual phenotypes are localized to specific regions within atherosclerotic plaques. A recent study explored this question and analyzed the spatial distribution of macrophage phenotypes in human plaques at different stages of atherosclerosis development. These authors found that both M1 and M2 macrophage numbers increased during atherogenesis and were equally distributed at the fibrous cap region. On the contrary, M1 macrophages were the predominant phenotype in rupture-prone shoulder regions, whereas in the adventitia M2 markers were predominant. Finally, in areas of plaque hemorrhage, CD163 expression was pronounced, likely representing the Mhem phenotype. Interestingly, foam cells demonstrated no distinct M1 or M2 phenotype. Similarly, Chinetti-Gbaguidi et al. have localized M2 macrophages in human atherosclerotic lesions in more stable cell-rich areas of plaque away from the lipid core. The authors show that these same regions of plaque have high levels of IL-4, an M2 polarizing cytokine. This macrophage population seems to be relatively resistant to foam cell development based on staining for lipids. In vitro studies indicate that IL-4 polarized macrophages exhibit decreased cholesterol uptake and increased ability to store esterified cholesterol esters compared with M1 or resting macrophages.

A comparison of human carotid with femoral atherosclerotic plaques revealed that M1 macrophage markers were increased in carotid, whereas M2 markers were predominant in femoral artery atherosclerosis, indicating that M1 accumulation may be a characteristic of symptomatic lesions. In support of this hypothesis, it was shown that several matrix metalloproteinases upregulated in human-derived macrophages polarized to the M1 phenotype in vitro are also colocalized with M1 macrophages in atherosclerotic plaques. A better understanding of the relationship between localization of macrophage phenotypes and their function will lead to the discovery of useful markers for plaque growth and stability.

Dynamic Plasticity of Macrophages in Atherosclerosis

As described above, several distinct macrophage phenotypes or states have been defined and, importantly, macrophages exhibiting properties characteristic of these different phenotypes have been shown to reside within atherosclerotic plaques (Table). Although several studies have suggested potential roles for different macrophage phenotypes in cardiovascular disease, our knowledge a coherent description of the relationship among the different phenotypes and how these different phenotypes come to coexist within the same location has not yet been provided.

We suggest that there are 4 underlying features that define lesion macrophage phenotypic polarization. First, we hypothesize that lesional macrophages explore a continuum among different states with what have been described as polarized states (eg, M1 and M2) existing at the far extremes, characterized by expression markers and defined by assuming different functions. As discussed in more detail below, existing data suggest that plaque-associated macrophages also express features that are intermediate between the extremes. Whether those macrophages are in a transition state between phenotypes or whether they have defined functions is not known. Second, this continuum of phenotypes is defined at the molecular level by interactions and cross-talk among an overlapping set of transcription factors. Third, macrophages can dynamically shift their position in the phenotypic continuum in response to local changes in environmental signals. Finally, we suggest this dynamic plasticity most likely evolved to respond to acute changes in lipid levels and reactive oxygen species that occur during tissue damage or the phagocytosis of dying cells.

Is Phenotypic Polarization Reversible?

During an acute inflammatory reaction, for instance in response to bacterial infection, a tissue macrophage can contribute to boosting the inflammatory response by polarizing into an M1 phenotype. Once bacteria are cleared, however, the change in the immunologic microenvironment allows the same cell to contribute to resolution and tissue regeneration by first switching into an M2 and possibly a pro-resolving phenotype (Mres). Although the ability of M1, M2, and Mox to switch between phenotypes has been demonstrated in vitro, polarization to the M4 phenotype seems to be irreversible. This ability to switch phenotypes in response to environmental changes leads to the important question as to why inflammation in chronically inflamed tissues, such as atherosclerotic lesions, is not resolved. Could it be that appropriate stimuli are not present, or is the ability of cells to alter their phenotypes blocked or compromised? An important aspect in proper resolution is the phagocytosis of dead cells, known as effecrotosis. Engagement of apoptotic cells is immunologically quiet and usually accompanied by anti-inflammatory mechanisms. Compromised efccrotosis leads to prolonged inflammation, and we have shown that the Mox phenotype exhibits reduced engulfment capacity, compared with M1 and M2 macrophages. Interestingly, feeding mice a diet rich in polyunsaturated fatty acids and fish oil resulted in a remarkable improvement of efccrotosis and reversal of macrophage phenotypes from M1 to M2.

Phenotypic Changes of Plaque Macrophages in Response to Lipids: Role for LXRs and PPARs

It is now well recognized that high levels of LDL cholesterol and chronic inflammation are 2 of the major forces driving the pathogenesis of atherosclerosis. In macrophages, inflammatory signals acting through NFκB, activator-protein-1, and interferon regulatory factors (IRFs) promote the transcription of proinflammatory cytokines and metalloproteases that function to recruit additional immune cells to blood vessel walls, alter the function of smooth muscle cells, and increase the susceptibility of atherosclerotic plaque to thrombotic events. In contrast, the accumulation of oxidized and other modified forms of cholesterol by macrophages has recently been shown to limit inflammation by activating a second set of transcription factors, LXRα and LXRβ. The LXRs are members of the nuclear hormone receptor superfamily of ligand-activated...
transcription factors, and both LXRα and LXRβ are expressed in macrophages. Importantly, the endogenous ligands for LXR are derivatives of cholesterol that increase coordinately with intracellular cholesterol levels, thus allowing these receptors to regulate gene expression in conjunction with changes in intracellular cholesterol. Thus, because macrophages accumulate cholesterol, the transcriptional activity of the LXRs increases (Figure). Importantly, binding sites for LXR-retinoic X receptor heterodimers have been identified in the promoter of the ABCA1 gene. ABCA1 is required for the process of reverse cholesterol transport, whereby cells efflux internal cholesterol to acceptor proteins to form nascent high-density lipoprotein particles. Treatment of primary macrophages or macrophage cell lines with LXR agonists results in induction of the ABCA1 gene, increased levels of ABCA1 protein, and an increase in cholesterol efflux. LXR also binds directly to the promoters of genes encoding other proteins involved in reverse cholesterol transport, whereby cells efflux internal cholesterol to acceptor proteins to form nascent high-density lipoprotein particles. Treatment of animal cells with synthetic LXR agonists increases ABCA1 expression, and decreases cholesterol efflux. Recent work indicates that exposure of macrophages to hemoglobin or heme induces both an antioxidant pathway, similar to that observed with oxidized phospholipids, and the LXR signaling pathway. Heme-dependent phosphorylation of the activating transcription factor ATF1 promotes binding of ATF1 to the promoters of both the HO-1 and LXRβ genes. Increased LXRβ expression leads to activation of the LXR-dependent cholesterol efflux and anti-inflammatory pathways. In human cells, the LXRα gene contains a LXR binding site in the promoter allowing further amplification of the heme-ATF1 signal via upregulation of LXRα by LXRβ. As seen with cholesterol loaded cells, heme-treated macrophages have reduced expression of proinflammatory cytokines and increased expression of IL10. Interestingly, both LXRβ and LXR induce genes encoding proteins that inhibit apoptosis. Because we propose that both pathways can be partially active in the same cells, it could be the combination of these signaling pathways that prolongs macrophage life-span during the early stages of atherosclerosis facilitating lesion development (Figure). These observations suggest that in the context of chronic inflammation and elevated cholesterol levels associated with atherosclerosis, macrophages dynamically integrate opposing pro- and anti-inflammatory signals and adjust gene expression accordingly.

The nuclear receptors, PPARγ and PPARδ, regulate transcription in response to the direct binding of fatty acids. Importantly, increasing the transcriptional activity of either
by monounsaturated fatty acids was also shown to drive macrophages toward an anti-inflammatory M2 phenotype. M2 macrophages preferentially oxidize fat to generate energy, whereas M1 macrophages favor glycolysis, and the PPARs regulate genes that encode proteins that mediate the uptake of fatty acids, for instance CD36, and the oxidation of fatty acids. Activation of PPARδ by monounsaturated fatty acids was also found to synergize with IL-4 to enhance the expression of M2 marker genes, such as Arg1. The estrogen-related receptor alpha (ERRα) is an additional member of the nuclear receptor superfamily that seems to contribute to macrophage function. In contrast to the LXRs and PPARs, ERRα does not directly bind a small molecule or metabolic intermediate, and thus this receptor is not formally a metabolic sensor. In macrophages, ERRα transcriptional activity is controlled by the levels of the transcriptional coactivator protein, the PPARγ coactivator 1β (PGC-1β). Macrophage expression of PGC-1β is induced by signal transducer and activator of transcription 6 in response to IL-4 and ERRα/PGC-1β contributes to the shift toward fat oxidation that occurs in M2 macrophages by inducing genes involved in mitochondrial function and biogenesis. Similarly, mitochondrial-generated reactive oxygen species in macrophages is required for the efficient clearance of infected cells, and ERRα/PGC-1β is also required for the oxidative burst that generates reactive oxygen species in macrophages in response to IFN-γ treatment. Clearly, macrophages have evolved several mechanisms to sample their environment and sense intracellular lipids to modulate gene expression and phenotype accordingly.

### Summary

It is clear during the development and progression of atherosclerosis that macrophages are exposed to many different environmental cues, including pro- and anti-inflammatory cytokines, cholesterol, oxidized lipids, and heme that influence gene expression and regulate macrophage function. The level and intensity of these different signals changes constantly during the progression of atherosclerosis, and we imagine that the intensity of the various signaling molecules may vary regionally within plaque with macrophages near the surface of the lesion exposed to a different environment than macrophages located near the necrotic core or at the shoulder.

Cholesterol, oxidized lipids, and heme all seem to counteract or modulate the activity of pro- and anti-inflammatory cytokines, suggesting that macrophages may be searching for optimal equilibrium that balances their ability to mount and sustain potentially destructive inflammatory responses, with repair pathways associated with the ability to process and excrete the cholesterol, oxidized lipids, and heme that accumulates at sites of cell death and tissue damage. The phenotypic plasticity of macrophages described in this review most likely evolved to allow dynamic responses to acute environmental changes supporting the homing to sites of injury and the subsequent clearance of dead cells and repair of damaged tissue. Atherosclerosis, on the contrary, is a pathological response to chronic inflammation and elevated cholesterol levels. Interestingly, NFκB, LXRs, PPARs, and Nrf2, all induce genetic networks that facilitate macrophage survival. In the face of chronic stimulation, these survival networks may in fact be counterproductive, fostering atherosclerosis by favoring the retention of macrophages at sites of disease in the blood vessel wall.

### Outlook

In response to the changing environment in developing atherosclerotic plaques and competition between different transcription networks, macrophages can shift transiently between phenotypes. These phenotypic shifts can be localized, for instance depending on where a single cell is positioned in plaque, and change over time. Phenotypic polarization is not an all-or-nothing event, suggesting that macrophages most likely exist in a continuum among multiple reversible phenotypes that reflect the activity of different transcriptional networks (NFκB, LXR, PPAR, Nrf2, etc) and crosstalk between these networks. The current classification of macrophage phenotypes is based mostly on the expression of a small number of markers. Large-scale expression profiling and systems genetics approaches will ultimately lead to a more accurate assignment of biological functions to the different macrophage phenotypes. Several critical questions remain to be addressed as follows: (1) Is the foam cell a distinct phenotypic state or are their multiple types of foam cell phenotypes? (2) Can the essential signaling nodes that control phenotype switching/plasticity be identified? (3) Can...
pharmacological and genetic interventions be developed that can manipulate macrophage phenotype in plaque in ways that will benefit patients therapeutically?

Sources of Funding
This work was supported by National Institutes of Health (NIH) grant R01 HL084422 and an American Heart Association grant in aid to N. Leitinger, and the NIH grant 1R01HL096864-01A1 to I.G. Schulman.

Disclosures
None.

References
Leitinger and Schulman

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