Heterogeneous In Vivo Behavior of Monocyte Subsets in Atherosclerosis

Filip K. Swirski, Ralph Weissleder, Mikael J. Pittet

Abstract—Monocytes and macrophages play active roles in atherosclerosis, a chronic inflammatory disease that is a leading cause of death in the developed world. The prevailing paradigm states that, during human atherogenesis, monocytes accumulate in the arterial intima and differentiate into macrophages, which then ingest oxidized lipoproteins, secrete a diverse array of proinflammatory mediators, and eventually become foam cells, the key constituents of a vulnerable plaque. Yet monocytes are heterogeneous. In the mouse, one subset (Ly-6Chi) promotes inflammation, expands in hypercholesterolemic conditions, and selectively gives rise to macrophages in atheromata. A different subset (Ly-6Clo) attenuates inflammation and promotes angiogenesis and granulation tissue formation in models of tissue injury, but its role in atherosclerosis is largely unknown. In the human, monocyte heterogeneity is preserved but it is still unresolved how subsets correspond functionally. The contradistinctive properties of these cells suggest commitment for specific function before infiltrating tissue. Such commitment argues for discriminate targeting of deleterious subsets while sparing host defense and repair mechanisms. In addition to advancing our understanding of atherosclerosis, the ability to target and image monocyte subsets would allow us to evaluate drugs designed to selectively inhibit monocyte subset recruitment or function, and to stratify patients at risk for developing complications such as myocardial infarction or stroke. In this review we summarize recent advances of our understanding of the behavioral heterogeneity of monocytes during disease progression and outline emerging molecular imaging approaches to address key questions in the field. (Arterioscler Thromb Vasc Biol. 2009;29:1424-1432.)

Key Words: monocyte ▪ atherosclerosis ▪ imaging

Atherosclerosis is a complex chronic disease and a leading cause of myocardial infarction and stroke. At present, the dominant conceptual approaches to therapy involve manipulation of lipid metabolism and manipulation of inflammatory processes. Phase III clinical trials of torcetrapib, an agent that increases HDL and lowers LDL through inhibition of cholesteryl ester transfer protein (CETP), were terminated in 2006 because of increased mortality and cardiovascular events. Although numerous other inhibitors, agonists, antagonists, peptidomimetics, antisense oligonucleotides, and gene-replacement therapies aimed at targeting lipoprotein biology may prove effective as therapies for atherosclerosis or its risk factors, the experience with torcetrapib, and the fact that myocardial infarction and stroke continue to claim lives, indicate an urgent need to explore alternative treatment strategies. Targeting inflammatory processes is a prospective option; since the late 1970s, inflammation has shaped our understanding of the disease and several agents that target leukocyte recruitment and retention are currently in preclinical trials.

Pathologically, atherosclerosis is characterized by the development of lesions, or atheromata, that affect the arterial blood vessels, typically at vessel bifurcations. The mechanisms that govern the evolution of atheromata at these “sites of predilection” are complex and not yet fully understood, but they are known to involve nonlaminar blood flow, lipid accumulation and oxidation, leukocyte recruitment, mobilization of smooth muscle cells, and cell apoptosis. Their particular combination gives rise to lesions that display remarkable heterogeneity. Rupture of a “vulnerable” plaque may lead to myocardial infarction or stroke, and depends on the interplay between lesional composition and mechanical forces: “stable” lesions with a collagen-rich thick fibrous cap and small lipid core are less prone to rupture than inflammatory lesions with a thin fibrous cap and large lipid-rich core. Shifting the balance from a vulnerable to a stable plaque is an attractive therapeutic consideration that may require reprogramming of the immune system from an inflammatory state (ie, collagen breakdown, accelerated accumulation of inflammatory cells) to a regulatory or “healing” state (ie, collagen synthesis, reduced accumulation of inflammatory cells or mobilization of cells that promote resolution of inflammation). Conceptual approaches available include targeting of cell subsets or specific molecules involved during inflammatory processes.
Monocytes and macrophages are widely regarded as key cellular protagonists of atherosclerosis. Indeed, circulating monocytes efficiently adhere to activated endothelium, infiltrate atherosclerotic lesions, become lesional macrophages, and participate decisively in the development and exacerbation of atherosclerosis.4,9,10 Macrophages ingest oxidized lipoproteins via scavenger receptors, and thus as lipid-rich foam cells, they become part of the physical bulk of the disease. The cells also secrete inflammatory mediators that stimulate smooth muscle cell migration and proliferation and participate in plaque development, rupture, and thrombosis. From this perspective, it seems that monocyte/macrophages are categorically detrimental and their accumulation accelerates disease; their inhibition or ablation may seem, at first, as a clear and simple therapeutic objective. Nevertheless, monocytes are integral to the health of the organism. They are motile in the circulation, patrol the vasculature, replenish tissue with macrophages, and respond to injury, infection, and various “danger signals.”11,12 Their indiscriminate targeting would interfere with normal homeostasis and immunity and is therefore therapeutically nonviable. The discovery that monocytes comprise distinct subsets in human, mouse, and other mammals suggests specialization of function, and has stimulated interest in approaches that discriminate between “harmful” and “beneficial” subsets. Also, hints regarding diversity of macrophages that populate human atheromata have surfaced over the years,13 but it is unclear whether functional subpopulations in atheromata arise from differential stimuli encountered in regions of the plaque or reflect lineal predispositions that depend on programming before penetration into the plaque. Below we review the current knowledge on monocyte and macrophage heterogeneity, the tools that can be used to investigate the role of subtypes, and the emerging views of the role of these cells in atherosclerosis. Finally, we present possible diagnosis and treatment opportunities based on our improved understanding of monocyte and macrophage heterogeneity.

**Monocyte and Macrophage Heterogeneity**

Studies have documented monocyte heterogeneity in humans and mice. In humans, monocytes fall into at least 2 main subsets based on their expression of specific receptors, including CD14 and CD16.14–17 In mice, monocyte subsets can be divided based on expression of Ly-6C, Gr1, CC-chemokine receptor 2 (CCR2), and CX3C-chemokine receptor 1 (CX3CR1). Ly-6C⁺ monocytes are Gr1⁺CCR2⁺CX3CR1lo, whereas Ly-6C⁻ monocytes are Gr1⁻CCR2⁻CX3CR1hi. According to the relative expression of CCR2 and CX3CR1, they correspond to human CD14⁺/CD16⁻ and CD14⁻/CD16⁺ monocytes, respectively.18–20 These observations indicate that it is possible to address the in vivo relevance of human heterogeneity by studying mice.

It is currently believed that different monocyte subsets reflect developmental stages with distinct physiological functions18–33 (Table 1). Ly-6C⁺ monocytes arise in the bone marrow and enter circulation partly via CCR2.34,35 They express a number of inflammatory and proteolytic mediators, respond to inflammatory cues such as the CCR2 ligand CCL2 (also known as MCP-1), and accumulate in inflammatory sites. In the absence of inflammation, they are thought to differentiate into Ly-6C⁻ monocytes, although Ly-6C⁺ to Ly-6C⁻ conversion is still under dispute.36–38 Ly-6C⁻ monocytes appear to be antiinflammatory, support granulation tissue formation (ie, collagen deposition and healing), patrol the vasculature, and enter tissues very early after the onset of inflammation.19,22,24 In the human, an imbalance in the relative proportion of subsets has been linked to several diseases.17,39–49 However, although there may be molecular

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**Table 1. Role of Mouse Monocyte Subsets in Inflammation**

<table>
<thead>
<tr>
<th>Status</th>
<th>Behavior of Ly-6C⁺ Monocytes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>Accumulate in peritoneum in response to thioglycollate</td>
<td>Geissmann et al.19</td>
</tr>
<tr>
<td></td>
<td>Increase in No. in response to <em>Listeria monocytogenes</em> infection</td>
<td>Sunderkotter et al.20</td>
</tr>
<tr>
<td></td>
<td>Transport <em>Listeria monocytogenes</em> to the brain</td>
<td>Drevets et al.18</td>
</tr>
<tr>
<td></td>
<td>Accumulate in peritoneum in response to <em>Toxoplasma gondii</em> infection</td>
<td>Robben et al.27</td>
</tr>
<tr>
<td></td>
<td>Accumulate in hepatic lesions in response to <em>Francisella tularensis</em> infection</td>
<td>Rasmussen et al.26</td>
</tr>
<tr>
<td></td>
<td>Accumulate in injured skeletal muscle</td>
<td>Arnold et al.21</td>
</tr>
<tr>
<td></td>
<td>Increase in No. in response to thermal injury</td>
<td>Noel et al.25</td>
</tr>
<tr>
<td></td>
<td>Accumulate in injured myocardium &amp; perform inflammatory and proteolytic function</td>
<td>Nahrendorf et al.24</td>
</tr>
<tr>
<td>Chronic</td>
<td>Increase in No. in response to chronic infection with <em>Leishmania major</em></td>
<td>Sunderkotter et al.20</td>
</tr>
<tr>
<td></td>
<td>Give rise to brain microglia in microgliosis</td>
<td>Mildner et al.23</td>
</tr>
<tr>
<td></td>
<td>Increase in response to hypercholesterolemia and accumulate in atheromatia</td>
<td>Swirski et al.28</td>
</tr>
<tr>
<td></td>
<td>Accumulate in atheromata via CCR2, CCR5 and CX3CR1</td>
<td>Tacke et al.29</td>
</tr>
</tbody>
</table>

**Behavior of Ly-6C⁻ Monocytes**

<table>
<thead>
<tr>
<th>Status</th>
<th>Behavior</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Accumulate in all tissues</td>
<td>Geissmann et al.19</td>
</tr>
<tr>
<td></td>
<td>Patrol the vasculature</td>
<td>Aufray et al.22</td>
</tr>
<tr>
<td>Acute</td>
<td>Accumulate very early in tissue</td>
<td>Aufray et al.22</td>
</tr>
<tr>
<td></td>
<td>Perform antiinflammatory function, granulation tissue formation, angiogenesis</td>
<td>Nahrendorf et al.24</td>
</tr>
<tr>
<td>Chronic</td>
<td>Accumulate in atheromata via CCR5</td>
<td>Tacke et al.29</td>
</tr>
</tbody>
</table>
agreement between human CD16+ and mouse Ly-6C60 monocytes (and conversely between human CD16− and mouse Ly-6C60 monocytes), there is currently a discrepancy as to their function; CD16+ monocytes have been called “inflammatory.” It is yet to be determined whether this contradiction reflects differences between species or incomplete discrimination between subsets.

Monocytes and their progeny have long been understood as “plastic” cells, capable of adapting to their local environment.50–52 However, the existence of subsets in circulation raises the possibility that monocytes commit for specific function before tissue infiltration, and may reconcile the perhaps contradictory activities attributed to the entire monocyte repertoire. Future studies will need to determine the quality and extent of commitment, and how the local environment influences the consequent phenotype. It is conceivable that the eventual phenotype monocytes acquire in tissue depends on a sequence of ontogenically (cell dictates environment) and environmentally (environment dictates cell) integrated cues and checkpoints. The emerging picture, then, positions monocyte subsets and their progeny as active participants in a vast immune regulatory network, rather than as downstream responders of ongoing inflammation. By extension, the circulatory system is a reservoir of functionally distinct components and may be an attractive target for discriminate therapeutic intervention.

It has been known for some time that tissue macrophages are also heterogeneous.53,54 “M1” cells, or classically activated macrophages, are inflammatory and arise by stimulating their progenitors with inflammatory stimuli such as LPS or GM-CSF. “M2” cells, or alternatively activated macrophages, display a more regulatory function and arise when progenitors are cultured with IL-4 and IL-13. In vivo, these cells may be discriminated because they express a set of unique markers. It is unknown whether Ly-6C6hi monocytes selectively give rise to M1 macrophages and, conversely, whether Ly-6C6lo monocytes give rise to M2 macrophages.

**Tools to Investigate the Role of Subsets**

Most of our knowledge on the activity of monocyte populations has been obtained with ex vivo readouts (flow cytometry, histology), and has made use of a variety of genetically modified mice such as chemokine receptor knockout (CCR2−/−, CCR5−/−, CX3CR1−/−) or of mice reconstituted with modified bone marrow cells.19,23,24,27–29,32,34,35,55–59 These are useful to determine the location and phenotype of cell types at given time points. Initial investigations also focused on surrogate cell culture models that do not always reproduce the behavior of immune cells in tissues.60 Because immune processes are dynamic, new tools have been developed to track cells in real time and to inform on cellular interactions and migration and delivery of effector function (Figure 1). Cellular imaging technologies have enabled the dynamic study of labeled immune cells in intact tissue environments. The imaging approaches provide either real-time microscopic cellular resolution or quantitative whole organ information,61 and some have translational potential.62 The techniques used to label and follow the fate of monocytes and other leukocyte populations in vivo are described below and can be broadly divided into 3 categories that rely on: genetic reporters, exogenous cell trackers, and injectable imaging agents (Table 2).

Genetic reporters are used to stably express imaging agents such as fluorescent (eg, green fluorescent protein [GFP] and its derivatives),63 bioluminescent (eg, luciferases)64 or other fusion proteins (eg, herpes simplex virus-1 thymidine kinase [HSV-T]).65 The reporter genes are typically inserted under the control of a promoter of interest. For example, mice expressing GFP under the control of the Csf1r, CD11b, or Cx3cr1 promoters are available and report on monocytes and their lineage descendants.19,66 Specifically, GFP expression driven by the Cx3cr1 promoter has been used to distinguish Ly-6C6hi (Cx3cr160) from Ly-6C6lo (Cx3cr164) monocytes both ex vivo15 and in vivo.22 The genetic reporter approach is particularly useful for the study of cells at microscopic resolution and offers long-term tracking possibilities because the imaging agent is not diluted because of cell division. Multiphoton and confocal intravital microscopy (IVM) are methods of choice for the dynamic study of optically-labeled cells because they permit analysis at single cell resolution, in 3 dimensions, and at optical penetration of tissues up to

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**Figure 1.** Molecular imaging modalities that inform on monocyte/macrophage presence and function. Monocyte/macrophages and associated functions have been imaged with different molecular imaging modalities at different sensitivities and resolutions. Imaging of macrophages or function has been achieved with PET/CT (image shows uptake of64Cu targeted nanoparticles by macrophages)82; SPECT/CT (image shows accumulation of exogenously-labeled and adoptively injected111In-monocytes)83 MRI (image shows accumulation of macrophage-targeted immunomicelles)84; CT (image shows uptake of CT-contrast agent N177 by macrophages)85; FMT (image shows uptake of quenosin-nanobiotin by macrophages; unpublished); and FRI (image shows protease-activatable regions of an excised rabbit aorta).86 To gain insight into the differential in vivo behavior of monocyte subsets, promising tools involve IVM (top image shows patrolling Ly-6C6lo monocytes22; bottom image shows Ly-6C6ii-associated proteolyisis60 and the development of novel monocyte subset-targeted agents (image shows specificity of an agent for activated but not resting macrophages).81
Several different fluorophores can be excited at a single excitation wavelength, thus allowing the simultaneous detection of differentially tagged fluorescent objects. However, in the context of atherosclerosis, these methods will require the development of sophisticated tools, for example for tissue immobilization, because of the proximity of plaques to the beating heart. Also, current limitations of genetic reporter approaches include inadequate imaging in larger fields of view (e.g., whole body), at increased depths (e.g., >600 mm), or in human patients. Bioluminescence imaging is highly sensitive but detection of target signal depends on the positioning (depth) of the emitted photons and thus does not allow absolute quantification. PET imaging allows visualization of $^{131}$I– or $^{124}$I–2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil (FIAU), a radionuclide that selectively accumulates in HSV-TK expressing cells. All the agents mentioned above have been used widely and successfully in animal models, but some are potentially immunogenic and thus have limited or no clinical translatability.

Exogenous cell trackers are being used to label purified populations of monocytes. The cells are reinjected into a recipient and tracked with appropriate imaging techniques.

Exogenous trackers include a variety of optical, nuclear, and MRI agents. Optical imaging agents comprise fluorochromes that emit in the visible light range (CFSE, CMTMR) and can be detected by IVM at single cell resolution near the body surface, and far-red/near-infrared agents (VivoTag-680, VivoTag-750) that emit light at longer wavelength and allow detection of cells deeper within the whole body (at least in small animal models). Fluorescence molecular tomography (FMT) is an imaging technology that can reconstruct 3-dimensional maps of near-infrared fluorochromes in tissues based on advanced algorithms, and allows absolute quantification of target signal.

<table>
<thead>
<tr>
<th>Imaging Modality</th>
<th>Resolution</th>
<th>Genetic Reporter</th>
<th>Exogenous Cell Tracker</th>
<th>Injectable Targeting Agent</th>
<th>Fusion Imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVM</td>
<td>1 μm</td>
<td>Fluorescent proteins</td>
<td>Fluorescent agents (visible, NIR)</td>
<td>Fluorescent agents (visible, NIR)</td>
<td>No</td>
</tr>
<tr>
<td>Fiberotics</td>
<td>1 to 5 μm</td>
<td>Fluorescent proteins</td>
<td>Fluorescent agents (visible, NIR)</td>
<td>Fluorescent agents (visible, NIR)</td>
<td>No</td>
</tr>
<tr>
<td>FMT</td>
<td>1 mm</td>
<td>Fluorescent agents (NIR)</td>
<td>Isotope-labeled agents ($^{64}$Cu)</td>
<td>Isotope-labeled agents ($^{64}$Cu)</td>
<td>With CT, PET, SPECT, MRI</td>
</tr>
<tr>
<td>PET</td>
<td>1 to 2 mm</td>
<td>HS-VTK</td>
<td>Isotope-labeled agents ($^{11}$In)</td>
<td>Isotope-labeled agents ($^{11}$In)</td>
<td>With CT, FMT, PET, MRI</td>
</tr>
<tr>
<td>SPECT</td>
<td>1–2 mm</td>
<td>Isotope-labeled agents ($^{11}$In)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRI</td>
<td>10–100 μm</td>
<td>(Super)paramagnetic particles</td>
<td></td>
<td>With CT, PET, SPECT, MRI</td>
<td></td>
</tr>
</tbody>
</table>

IVM indicates intravital microscopy; fiberotics, catheter-based fiberoptic imaging; FMT, fluorescence molecular tomography; PET, positron emission tomography; SPECT, single photon emission computed tomography; MRI, magnetic resonance imaging; CT, x-ray computed tomography. Fusion imaging refers to imaging modalities that can be combined. Fusion imaging is expected to help future studies aimed at better detecting cells and other biological activities as they unfold in vivo. The advantages and limitations of the imaging modalities and labeling approaches listed here are detailed in the text.

800 μm$^{61,67–70}$ Several different fluorophores can be excited at a single excitation wavelength, thus allowing the simultaneous detection of differentially tagged fluorescent objects. However, in the context of atherosclerosis, these methods will require the development of sophisticated tools, for example for tissue immobilization, because of the proximity of plaques to the beating heart. Also, current limitations of genetic reporter approaches include inadequate imaging in larger fields of view (eg, whole body), at increased depths (eg, >600 mm), or in human patients. Bioluminescence imaging is highly sensitive but detection of target signal depends on the positioning (depth) of the emitted photons and thus does not allow absolute quantification.$^{61}$ Positron emission tomography (PET) imaging allows visualization of $^{131}$I– or $^{124}$I–2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil (FIAU), a radionuclide that selectively accumulates in HSV-TK expressing cells. All the agents mentioned above have been used widely and successfully in animal models, but some are potentially immunogenic and thus have limited or no clinical translatability.

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ability, prolonged intracellular retention, and low toxicity. They allow highly sensitive real-time monitoring of labeled cells across all resolution scales from microscopic to macroscopic and are potentially clinically translatable. However, one challenge associated with exogenous labels is to track cell fate and survival. For example, a signal does not distinguish between a viable labeled cell and an imaging agent that has been released (ie, from dying cells) and sequestered elsewhere. For fluorescent agents, flow cytometry analysis and cell sorting of labeled cells can also be considered after adoptive transfer to correlate in vivo results. For example, adoptive transfer of ex vivo-labeled monocytes has shown continuous accumulation of monocytes during atherogenesis. Accumulation can be imaged ex vivo and in vivo longitudinally and is attenuated with statin treatment. It remains unknown, however, whether defined monocyte subsets accumulate in specific lesions or at specific stages of lesional development, and whether they contribute differentially to atherogenesis.

Injectable imaging agents are compounds that can be administered into live subjects to label cells (monocyte subsets) or molecules (monocyte-associated proteases) of interest, and that can be detected by appropriate imaging modalities. Injectable agents with magnetic properties include derivitized/functionalized CLIO and USPIO particles and Gd\(^{3+}\)-loaded micelles carrying specific antibodies (eg, targeting scavenger receptors). Some agents are preferentially taken up, and in some cases phagocytosed, by endogenous monocyte and macrophage populations, and can be detected by MRI in atherosclerotic lesions. Iodine-containing contrast agents can also label lesional macrophages for detection with CT, whereas nuclear agents such as \(^{64}\)Cu-labeled nanoparticles show good avidity for lesional macrophages and efficient detection by PET imaging. Other PET tracers include \(^{18}\)F-FDG, which is taken up by all metabolically active cells and is therefore not specific to macrophages. Typically, the injectable imaging agents mentioned above label monocytes indiscriminately, and thus cannot be used to study monocyte heterogeneity. Most recently, efforts have been made to develop novel injectable agents that offer more selective targeting capabilities. The strategy uses phage libraries that are modified with various peptide affinity ligands or nanoparticle libraries derivatized with small molecules, and that are screened against cell populations of interest. For example, some small molecules “tune” nanoparticle surfaces and can redirect uptake into specific cell subpopulations. The technique has been used successfully to identify agents that are specific for activated versus resting macrophages and for M2 macrophages within the tumor-microenvironment. It is hoped that such strategies will define more agents with selectivity for monocyte or macrophage subsets. Other interesting injectable imaging agents rely on detection of molecular functions such as enzymatic activity. For example, proteases such as cathepsin B, K, S, and L and matrix metalloprotease (MMP)-2 and -12 are expressed abundantly during inflammation, and participate actively in tissue remodeling and plaque destabilisation as well as in allergic airway inflammation. Protease-targeted optical reporters are based on a polymeric scaffold that consists of near-infrared fluorochromes, specific protease peptide substrates, and partially methoxypegylated graft copolymers. The sensor is injected in its inactive state in which fluorochromes are not excitable because of auto-quenching. Proteolytic cleavage of the scaffold by a specific enzyme releases the fluorochromes and results in extensive fluorescence generation locally (dequenching). Amplification is achieved because 1 active enzyme moiety can activate multiple reporters. These probes have been used to image enzyme activity by IVM in surgically exposed carotid atherosclerotic plaques and in intact animals either by real-time near infrared catheter molecular sensing or by FMT and with cellular resolution by fluorescence microscopy in a variety of inflammatory conditions. Of note, some injectable agents confer transient background noise, and in this case it is preferable to define precise timing for imaging after injection to optimize signal-to-noise ratios. The protease-targeted optical sensors may be particularly useful to study monocyte/macrophage heterogeneity because some proteases are typically expressed at higher levels in \(\text{Ly-6}^{ci}\) monocytes when compared to their \(\text{Ly-6}^{ch}\) counterparts. Injectable imaging agents with specificity for molecular targets are under intense scrutiny because they can carry multiple reporters for imaging at different resolutions and depths, they can combine diagnostic and therapeutic interventional capabilities, and they offer the advantage of

### Table 3. Differential Expression of Biomarkers in Subsets

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cell Type (mouse)</th>
<th>Cell Type (human)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>ND</td>
<td>CD14(^{ch})CD16(^{hi})</td>
<td>Receptor for detection of bacterial lipopolysaccharide</td>
</tr>
<tr>
<td>CD16</td>
<td>ND</td>
<td>CD14(^{ch})CD16(^{hi})</td>
<td>Fc(\gamma) receptor binds to Fc portion of IgG antibodies</td>
</tr>
<tr>
<td>CGR2</td>
<td>Ly-6C(^{lo})</td>
<td>CD14(^{ch})CD16(^{hi})</td>
<td>A chemokine receptor that promotes recruitment of Ly-6C(^{lo}) monocytes to lesions in mice</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Ly-6C(^{ch})-&gt;Ly-6C(^{lo})</td>
<td>CD14(^{lo})CD16(^{hi})-&gt;CD14(^{ch})CD16(^{lo})</td>
<td>A chemokine receptor that has surprisingly been reported to promote recruitment of Ly-6C(^{lo}), but not Ly-6C(^{ch}), monocytes to lesions</td>
</tr>
<tr>
<td>Cathepsins</td>
<td>Ly-6C(^{hi})-&gt;Ly-6C(^{lo})</td>
<td>ND</td>
<td>Proteolytic enzymes that remodel the extracellular matrix and decrease stability of plaques</td>
</tr>
<tr>
<td>MMPs</td>
<td>Ly-6C(^{hi})-&gt;Ly-6C(^{lo})</td>
<td>ND</td>
<td>Proteolytic enzymes that remodel the extracellular matrix and decrease stability of plaques</td>
</tr>
<tr>
<td>MPO</td>
<td>Ly-6C(^{lo})-&gt;Ly-6C(^{lo})</td>
<td>ND</td>
<td>An oxidant-generating enzyme that promotes inflammation and plaque formation and destabilization</td>
</tr>
</tbody>
</table>

ND indicates either differences between subsets have not be determined or no clear species conservation of antigens.
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dependent manner. The accompanying Ly-6Chi monocy-

tosis requires elevated concentrations of cholesterol or lipid
derivatives, and results from increased cell survival, contin-

progression remains largely unknown.

Figure 2. Model for the role of monocyte subtypes in experimental mouse atherosclerosis. Elevated levels of cholesterol or lipid deriv-
atives ("Lipids") expand the Ly-6C\textsuperscript{hi} monocyte repertoire in circula-
tion by several mechanisms that include reduction of cell apopto-
sis, increased proliferation, and impaired Ly-6C\textsuperscript{hi} \rightarrow Ly-6C\textsuperscript{lo} conversion. Ly-6C\textsuperscript{lo} monocytes are recruited to atherosclerotic lesions via the CCR2, CCR5, and CX\textsubscript{3}CR1 chemokine receptors. The recruited cells differentiate massively into macrophages, although some cells can acquire a DC phenotype. Lesional macrophages eventually become foam cells and participate in plaque development, rupture, and thrombosis. Ly-6C\textsuperscript{lo} monocytes do not expand in periphery and infiltrate lesions less frequently than Ly-6C\textsuperscript{hi} monocytes. Their recruitment depends on CCR2, but may not require CX\textsubscript{3}CR1. Ly-6C\textsuperscript{lo} cells exhibit phagocytic and proangiogenic functions, and may preferentially mature into DC in lesions, however their participation in disease progression remains largely unknown.

being usable in various experimental animals and in humans (Table 3).

Emerging Views on the Role of Monocyte Subsets in Atherosclerosis

The combination of imaging approaches mentioned above and classical cell and molecular biology tools have revealed new insights on the roles of monocyte subsets during disease progression (Figure 2). Several reports have shown that hypercholesterolemic mice undergo a gradual and systemic increase of Ly-6C\textsuperscript{hi} monocytes. These cells adhere preferential-
tially to activated endothelium and accumulate efficiently in progressive lesions in a CCR2-, CCR5-, and CX\textsubscript{3}CR1-dependent manner.\textsuperscript{28,29} The accompanying Ly-6C\textsuperscript{hi} monocytosis requires elevated concentrations of cholesterol or lipid derivatives, and results from increased cell survival, continued proliferation, and, possibly, impaired Ly-6C\textsuperscript{hi} \rightarrow Ly-6C\textsuperscript{lo} conversion. The presence of a cholesterol-lowering drug of the statin class reduces Ly-6C\textsuperscript{hi} monocytopsis and atheroscle-
rosis.\textsuperscript{28} Thus, increased serum cholesterol levels, in addition to promoting lipid deposition to lesions\textsuperscript{95} and inducing M-CSF production for local monocyte maturation into macrophages,\textsuperscript{96,97} also trigger the expansion of circulating monocytes. These findings suggest that a cholesterol-rich diet promotes murine atherogenesis, at least in part through the development of Ly-6C\textsuperscript{hi} monocytopsis. Ly-6C\textsuperscript{hi} monocytes re-
cruited to atherosclerotic aortas differentiate into macrophages\textsuperscript{28} although some cells may acquire a DC phenotype.\textsuperscript{29} It is likely that most of the Ly-6C\textsuperscript{hi} cells acquire traits of lesional M1 cells and eventually become foam cells, although decisive experimental evidence in support of this is currently lacking.

Ly-6C\textsuperscript{lo} monocytes infiltrate atherosclerotic lesions less frequently than Ly-6C\textsuperscript{hi} monocytes. Their accumulation re-
lies on CCR5, but surprisingly not on CX\textsubscript{3}CR1 as it is the case for Ly-6C\textsuperscript{hi} monocytes.\textsuperscript{29} There exists some controversy as to how Ly-6C\textsuperscript{lo} monocytes participate in disease progress-

New Challenges and Opportunities

A number of critical questions on monocyte subsets in experimental atherosclerosis remain (Table 4). For example, do monocyte subsets accumulate in different lesions or at different stages of lesional evolution? What are the functional fates of monocyte subsets in lesions? Is ablation of specific subsets a promising treatment strategy? The technical chal-

lenges that accompany these questions stem from the size and location of lesions in the body and the frequency of mono-
cytes in the blood, and include motion artifacts, depth penetration of imaging modality, and its spatial resolution and sensitivity. The questions necessitate that the tools outlined above are optimized and validated to, eventually, specifically, and selectively target, enumerate, image and ablate candidate subsets. Future insight into the heterogeneous in vivo behav-
ior of monocyte subsets will therefore require a coordinated effort of technical and biological considerations.

Insights gained in experimental systems will require vali-
dation in humans, especially given that monocyte subsets in murine atherosclerosis appear to exhibit contradistinctive
behavior. Studies must first determine how well murine monocyte subsets correspond to their human counterparts. Further clinical studies will examine whether monocyte subsets are prospective biomarkers of disease and whether they represent treatment targets. Imaging will likely be an important tool for these purposes. For example, some exogenous cell trackers and injectable agents that selectively image subset presence or subset-associated activity may be clinically translatable. If studies show, for example, that a particular subset associates with or predicts for complications of atherosclerosis, it may be useful to use either ex vivo or in vivo imaging tools to noninvasively and rapidly assess a patient’s prognosis. The link between monocyte heterogeneity and disease also offers a number of treatment opportunities. These include inhibition of subset accumulation, inhibition of subset-associated function, subset ablation in blood or lesion, and sequestration of subsets from lesions. For example, antagonists against CCR2 may lead to better prognosis through inhibiting accumulation of CD16(lo) (CCR2(hi)) monocyte subsets. Inhibition of protease activity that associates with the inflammatory monocyte subpopulation in particular cell types from atheromata. Together, these methods may lead to better risk stratification and may pave the way for monocyte subset-based therapies.

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**Disclosures**

None.

**Table 4. Most Pressing Biological and Technical Considerations**

<table>
<thead>
<tr>
<th>Biological Questions</th>
<th>Technical Challenges</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. What is the precise fate of monocyte subsets once they have entered lesions, and do they contribute differentially to disease progression?</td>
<td>1. Validation of intravital imaging technologies with subcellular resolution to study the complexity of the host response in target sites (atheromata, draining lymph nodes)</td>
</tr>
<tr>
<td>2. Does specific ablation or inhibition of a monocyte subtype result in decreased lesion burden while sparing host defense and repair mechanisms?</td>
<td>2. Development of new agents that permit to distinguish leukocyte subtypes (monocyte, macrophage and DC subsets, and other cells present in atheromata)</td>
</tr>
<tr>
<td>3. Do the findings for mouse monocyte subsets hold true in humans?</td>
<td>3. Development of injectable agents that target cell populations of interest, can be used in humans, and have theranostic potential</td>
</tr>
</tbody>
</table>

References

Swirski et al  Heterogeneous In Vivo Behavior of Monocyte Subsets


Heterogeneous In Vivo Behavior of Monocyte Subsets in Atherosclerosis
Filip K. Swirski, Ralph Weissleder and Mikael J. Pittet

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