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Inflammatory Monocyte Effector Mechanisms

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Abstract

Monocytes are blood-derived mononuclear phagocytic cells that traffic throughout the body and can provide rapid innate immune effector responses in response to microbial pathogen infections. Amongst blood monocytes, the most abundant subset in mice is represented by inflammatory $Ly6C^+$ $CCR2^+$ monocytes and is the functional equivalent of the $CD14^+$ monocytes in humans. Herein we focus on published evidence describing the exquisite functional plasticity of these cells, and we extend this overview to their multiples roles *in vivo* during host immune defenses against microbial pathogen infections, as antigen-presenting cells, inflammatory cells or Trojan horse cells.

Keywords

Inflammatory $Ly6C^+$ monocytes; Microbial pathogens; Effector functions; Antigen presentation; CCR2

Introduction

Monocytes are blood-derived myeloid cells that belong to the mononuclear phagocytic system (MPS), a specialized system of phagocytic cells localized throughout the body (1, 2). The cells of this system provide innate immune responses, support the adaptive immune response and play a role in the maintenance of tissue homeostasis. Monocytes are a critical component of the MPS and are important in many diseases with an inflammatory component, such as infection, cardiovascular disease, type I diabetes and cancer. Circulating monocytes, like most dendritic cells (DCs) and some tissue-associated macrophages, originate *in vivo* from hematopoietic stem cell-derived progenitors with myeloid-restricted potential. In the bone-marrow, the successive commitment steps toward monocyte differentiation include common myeloid progenitors (CMPs), granulocyte-macrophage precursors (GMPs) and the macrophage/DC progenitors (MDPs). Finally, the MDPs give

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rise to the common DC progenitor (CDP) and the common monocyte progenitor (cMoP) found in bone marrow and spleen (3, 4). The cMoP was suggested to be restricted to monocytes and monocyte-derived macrophages (4).

In mice, at least two major subsets of blood monocytes have been defined: the Ly6C$^+$ monocytes (CX3CR1^{int}CCR2$^+$) also called 'inflammatory' monocytes and the Ly6C$^-$ monocytes (CX3CR1^{hi}CCR2$^-$) also known as 'patrolling' monocytes that both express the M-CSF receptor (M-CSFR/CD115) (5, 6). While inflammatory monocytes are most crucial during acute inflammation, and undergo CCR2-dependent bone-marrow mobilization, the patrolling subset has mostly been defined by its ability to survey blood vessels, a behavior qualified as ‘patrolling’. In humans, complete understanding of monocyte subsets remains to be investigated. Today, three of them have been proposed, i.e., the CD16$^+$CD14$^+$ and the CD14$^+$CD16$^{int/low}$ monocytes which functionally resemble Ly6C$^+$ inflammatory murine monocytes, and the CD14^{dim}CD16$^+$ which are equivalent to the murine Ly6C$^-$ subset and exhibit a patrolling behavior (7). Some of the overlapping functional features of these subsets of monocytes in mice and humans include important molecules involved in trafficking (CX3CR1, CCR2, CD62L, LFA1), cellular functions (phagocytosis, innate sensing, antigen presentation) as well as the expression of antimicrobial and cytokine effector functions upon activation (TNFα, IL-1β, NO/RO). A detailed overview and comparison of mouse and human monocyte subsets is the subject of another review of this volume.

The current review focuses on the multiple 'effector mechanisms' expressed by murine monocytes that are essential in host immune defenses and microbial pathogen elimination. We ***first*** provide an overview of the known functional features of these cells allowing for their rapid sensing of microbial pathogens, mobilization and microbicidal effector mechanisms. ***Second***, we discuss how these characteristics are linked to either protective immunity or deleterious immune responses. We only summarize *in vivo* evidence provided from mouse models and when possible, we refer to human studies.

1. Cell-intrinsic functional characteristics of Ly6C$^+$ monocytes

Ly6C$^+$ monocytes are blood circulating mononuclear leucocytes with short half-life (~19 hours, (8)) which represent >80% of the blood monocytes at steady state (2). Both under sterile and microbial inflammatory conditions, Ly6C$^+$ monocytes can differentiate into a progeny of distinct functional subtypes of effector cells, commonly defined as 'inflammatory Ly6C$^+$ monocytes'. Acquisition of multiple functional features by these cells is largely regulated by inflammatory cues from the environment. Seminal studies from the Pamer lab using the mouse model of infection by the intracellular bacterium *Listeria monocytogenes (Lm)*, had defined differentiating Ly6C$^+$ monocytes based on their functions, i.e., their ability to rapidly produce the microbicidal molecule nitric oxid (NO) and secrete the potent inflammatory mediator TNF-α. This led to defining these cells as 'Tip-DCs' for TNF-α and NO producing dendritic cells (9, 10). While such denomination was functionally very relevant, specifically in the context of *Lm* clearance in mice, it did not account for the complexity of effector functions that may be expressed by these cells and their very high plasticity (See Figure). For these reasons, and to discriminate Ly6C$^+$ monocyte-derived cells

from conventional DCs (cDCs) inside tissues, subsequent studies have attempted to define more universal cell-surface markers such as the C-type lectin CD209/DC-SIGN (11) and the high-affinity IgG receptor FcγRI CD64 (12). Recently too, Jung and colleagues have proposed an interesting model to incorporate different fates of activated Ly6C$^+$ monocytes into either effector monocytes, monocyte-derived DCs (Mo-DCs) or monocytederived macrophages (Mo-MP), that is largely based on the nature and timing of inflammatory cues (13). Yet the fate of Ly6C$^+$ monocytes is beyond the scope of this section, in which we are focusing on intrinsic functional features of 'undifferentiated' / 'differentiating' Ly6C$^+$ monocytes accounting for their ability to express distinct effector functions after microbial pathogen-mediated activation *in vivo*.

1.1. Sensing machineries

How to sense danger signals?—Ly6C$^+$ monocytes are equipped with sets of innate scavenging and sensing receptors and functional activation pathways that allow for efficient detection of microbial-pathogen derived molecules as well as molecules released as a result of tissue injury and cell death. Unraveling the molecular basis for this exceptional plasticity is the subject of intense investigations. Important studies from the Geissmann lab and the Randolph lab have conducted in depth genetic expression profiling comparisons between mouse and human monocyte subsets yet these were performed at homeostasis with the major focus on defining developmental pathways of these cells (7, 14). Through the study of many murine models of infection that include viruses, bacteria, fungi and parasites, series of reports discussed below have nonetheless established specific pattern recognition receptors (PRRs) and activation pathways that can be expressed by Ly6C$^+$ monocytes, and involved in orchestrating their functional differentiation.

TLRs and MyD88: Early studies from the Pamer lab using *Lm* in mice, suggested that Ly6C$^+$ monocytes differentiated into Tip-DCs in a MyD88-dependent manner, supporting the functional involvement of MyD88 in their ability to secrete TNFα and express iNOS (10). Interesting work from the Muraille group using mice infected with *Brucella melitensis* bacteria, the causing agent of Brucellosis in humans, as a relevant model of gram$^-$ bacterial infection, added further support to the functional importance of MyD88 but not TRIF in promoting Ly6C$^+$ monocytes differentiation into iNOS$^+$ cells (15). This study involved TLR4 as initiating sensor, yet only partially accounted for the MyD88$^{-/-}$ phenotype. Investigating further the role of Ly6C$^+$ monocytes during infection of mice with the intracellular parasite *Leishmania major*, the same group also reported the key role of MyD88, in part via TLR9, in generating iNOS$^+$ inflammatory monocytes (16). In a recent work, the Steinman lab found that upon injection of purified LPS or *Escherichia coli*, Ly6C$^+$ monocytes rapidly differentiated into antigen-presenting cells (APCs) exhibiting comparable potency as conventional dendritic cells (cDCs), through TLR4 and TRIF but not MyD88 (11). Of note, while all these studies clearly demonstrate the presence of specific TLRs and functional activation pathways intrinsically present in Ly6C$^+$ monocytes and involved in regulating their differentiation into iNOS$^+$ and/or TNFα$^+$ cells, and potent APCs, none of them formally established their cell-intrinsic requirements. In line with this interpretation, a very recent report by the Sparwasser group utilized elegant gain of function experiments in which a 'floxed-STOP' cassette was inserted upstream of the MyD88 adaptor, preventing its

expression, unless when the Cre recombinase was expressed (17). Functional MyD88 expression in CD11c$^+$ DCs only, with lack of MyD88 in all other cells, was sufficient to orchestrate Ly6C$^+$ monocytes differentiation into Tip-DCs during *Lm* infection, establishing that cell-intrinsic expression of MyD88 by Ly6C$^+$ monocytes may not be required. In addition to becoming Tip-DCs, multiple reports have provided compelling evidence that inflammatory monocytes can secrete important amounts of bioactive IL-12 following various microbial pathogen infections in mice, that include *Lm* (18) and *Citrobacter rodentium* bacteria (19, 20), *Leishmania major* and *Toxoplasma gondii* parasites, influenza virus {Leon, 2007 #1560}{Goldszmid, 2012 #1163;Nakano, 2009 #206}, though the exact sensing pathway has not been elucidated.

Barton and colleagues also reported that influenza virus derived products specifically trigger Ly6C$^+$ monocytes to secrete type I interferon in a Toll-like receptor 2 (TLR2) dependent manner, an unexpected finding given that type I interferon during viral infection is usually produced via recognition of nucleic acids by TLR7 and TLR9 in plasmacytoid DCs (pDCs), a subset of DCs that produces high levels of type I IFN (24). While evidence for TLR2 involvement in early sensing of viruses and subsequent non-type I IFN proinflammatory response was documented (25, 26), the connection between TLR2 and type I IFN secretion by activated Ly6C$^+$ monocytes suggested the uniqueness of this pathway inside these cells. Along similar lines, Jung and colleagues have also found that Ly6C$^+$ monocytes can express TLR2 in a model of induced colitis (27).

Nonetheless, and even though inefficient maturation of inflammatory monocytes was reported in MyD88$^{-/-}$ mice in most of these infections (15, 16, 28), except in the case of *T. gondii* for which MyD88 was not important (22), both the sensor(s) and activation pathways would remain to be formally defined using adoptive transfers of knockout Ly6C$^+$ monocytes or mice bearing cellspecific knocked out pathways.

Cytosolic sensors

-DDX41 and STING: Independent works from the Scheu and the Lienenklaus groups using *Lm* as a model system and IFNβ-reporter or cell-specific IFNβ-knockout mice suggested that activated Ly6C$^+$ monocytes represent the major source of type I IFN during this infection (29, 30). Multiple *in vitro* and *in vivo* studies over the years, in particular from the Portnoy lab, provided compelling evidence that cyclic di-nucleotides, c-di-AMP and c-di-GMP, secreted by this bacterium is the major trigger of type I IFN production, a process that occurs inside infected cell cytosol when *Lm* escapes from early phagosomes to cell cytoplasm (31, 32). Cyclic dinucleotide sensing inside myeloid cells required DDX41, a cytosolic DNA binding helicase that binds cyclic dinucleotides, and further associates with the endoplasmic reticulum sensor STING to induce TBK1/IRF3-mediated transcription of type I IFN (33, 34). Elegant *in vivo* experiments using STING$^{-/-}$ mice, mutants of *Lm* secreting variable amounts of c-di-AMP and co-immunization with synthetic cyclic di-AMPs as adjuvant, further confirmed that STING represented the major pathway of type I IFN induction during *Lm* infection (35). While not yet formally proven, all these data collectively suggest that Ly6C$^+$ monocytes indeed possess unique pathways of activation involving specific sets of cytosolic nucleic acid sensors leading to type I IFN production.

-Inflammasomes: The functional expression of different inflammasomes complexes by Ly6C$^+$ monocytes and human CD14$^+$ monocytes is supported by numerous studies from several groups, both in mice challenged with bacteria (*Lm*, *Francisella tularensis*, *Mycobacterium tuberculosis (Mtb)*), viruses (Vaccinia, murine cytomegalovirus), fungi *(Candida albicans)* (36–38) and on freshly isolated human blood monocytes (36, 39). These studies provided evidence for the presence of both precursor and/or bioactive IL-1 and/or IL-18 effector cytokines as a result of expression of functional inflammasome complexes inside inflammatory monocytes.

-Nod1 and Nod2: Lastly, Jung and colleagues reported that Ly6C$^+$ monocytes expressed Nod2 and could contribute to colitis through this sensing pathway (27). The cytosolic PRRs Nod1 and Nod2 may have evolved to enable the detection of cytosolic invasive bacteria, and in particular bacterial cell wall peptidoglycans (PGNs) compounds such as d-glutamyl-mesodiaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), as shown by many studies using model cell lines and bone-marrow macrophages *in vitro* (40). However, Nod2$^{-/-}$ mice increased susceptibility to oral but not intravenous *Lm* and to other intestinal bacteria such as *Helicobacter pilori* or *Citrobacter rodentium* seems to be accounted for by both impaired detection of invading bacteria by non-hematopoietic cells and altered intestinal myeloid cell responsiveness and homeostasis (19). This altogether suggested that, at least *in vivo*, the importance of Nod1 and Nod2 sensing inside monocytes is complex and requires further investigations.

In summary, Ly6C$^+$ monocytes exhibit tremendous plasticity in their multiple possible functional fates, which allows them to act as highly efficient sensing cells of the innate immune system. This is accounted for by their constitutive or induced expression of impressive sets of sensors and pathways of activation. Further studies will be important to continue characterizing the full potential of these cells and precisely understand how they integrate distinct pattern recognition receptors (PRRs) signals.

Activating cytokines and chemokines: Inflammatory cytokines and chemokines are usually secreted as a consequence of early PRRs triggering and can act both in an autocrine and a paracrine manner. Work from the Locksley lab for instance provided evidence that the expression of iNOS by inflammatory monocytes is dependent on lymphocyte-derived IFNγ during mouse listeriosis (41). Similarly, in mice inoculated with cysts of the parasite *T. gondii*, Sher and colleagues documented that IFNγ from NK cells orchestrates Ly6C$^+$ monocyte differentiation into F4/80$^+$ macrophages and IL-12-producing Mo-DCs (22). Along these lines, we found that memory T cell-derived MIP1α/CCL3 acted as a key signal to induce Ly6C$^+$ monocytes to secrete high levels of TNFα, which could act in an autocrine manner to promote their own reactive oxygen species (ROS) production (42). Recently, we also reported that IFNγ, which is massively produced by memory T cells independently of cognate antigen recognition, indeed represents a key ignition signal for triggering a robust microbicidal effector program inside Ly6C$^+$ monocytes *in vivo* (43). Finally, Ly6C$^+$ monocytes were shown to respond to type I IFN and respectively *trans*-present bioactive IL-15 (37) or turn off IL-1 secretion (44) during murine *Lm* or *Mtb* infections. Thus altogether the current body of evidence suggests that other cytokines and chemokines are

likely to shape monocyte differentiation, and further studies will be required to formally establish such possibility.

1.2. Trafficking machineries

How to traffic to injured tissues? Chemokine receptors and adhesion molecules—Chemokines represent essential orchestrators of cell mobilization and relocation inside tissues as was recently reviewed (45). Because monocytes constantly traffic inside tissues, they can be rapidly mobilized during microbial or sterile inflammation.

CCR2: Ly6C$^+$ monocytes express high levels of the chemokine receptor CCR2 (9). The major mechanism involved in the rapid mobilization of Ly6C$^+$ monocytes from their site of production, i.e., the bone-marrow, to the blood involves the chemokine receptor CCR2, and its ligands, mostly CCL2, CCL7 and CCL8 (46). Induced by various inflammatory signals, MCP-1/CCL2, is quickly secreted by bone marrow mesenchymal stem cells and their progeny, including CXC chemokine ligand (CXCL)12-abundant reticular cells (47), and this enables for massive release of Ly6C$^+$ monocytes to the blood, a necessary step for these cells to access injured tissues. Hematopoietic cells can also participate to CCL2 production through TLR and type I interferon-dependent cytosolic pathways (48). Triggering of cytosolic Nod2 via bacterialderived MDP can also induce the secretion of type I IFN and CCL2, and the mobilization of Ly6C$^+$ monocytes from the bone-marrow (19, 49).

Original studies from the Luster, Karpus and Ransohoff labs suggested that CCR2$^{-/-}$ and CCL2$^{-/-}$ (the major ligand for CCR2) mice were resistant to the development of experimental autoimmune encephalitis (EAE) induced upon injection of the myelin oligodendrocyte glycoprotein (MOG) derived peptide 35–55 (50, 51). Protection against disease progression correlated to the lack of mononuclear cell infiltrates in the central nervous system (CNS) of MOG-injected mice, altogether suggesting that CCR2$^+$ monocytes, at least in part via CCL2, contributed to pathogenesis. This interpretation was further confirmed in recent work using combinations of irradiated WT or CCR2$^{-/-}$ recipient or donor bone-marrow chimeras and elegant parabiont experiments (52). A report by the Malat group documented that Ly6C$^+$ monocytes could also be recruited to the cardiac endothelium via B cell-derived CCL7 (MCP-3), another ligand of CCR2, which contributes to tissue injury and acute myocardial infarction (53).

In murine models of acute viral infections of the CNS, using either lymphochorionmeningitis virus or West Niles virus, studies from the McGavern and the King labs, supported the importance of the CCR2/CCL2 axis for Ly6C$^+$ monocyte-mediated brain damages (54, 55). Even though these reports provided strong evidence that CCR2 may also regulate Ly6C$^+$ monocytes access to inflamed tissues, its 'dominant' function is likely to regulate CCR2$^+$ monocyte egress from bone-marrow to the blood. Thus, in summary, whether retention of monocytes inside the bone-marrow is mostly accounting for increased resistance of CCR2$^{-/-}$ or CCL2$^{-/-}$ mice to EAE or viral CNS infections, was not formally assessed.

CX3CR1: In the blood, CX3CR1 in particular contributes to the access of Ly6C$^+$ monocytes to the spleen during *Lm* infection where they undergo differentiation into

inflammatory monocytes/Tip-DCs (56). Consistent with these observations, the CX3CR1 ligand, e.g., the fractalkin CX3CL1, is found expressed in marginal/T cell zones of the spleen. CX3CR1 was also reported by the Randolph group to be involved in $Ly6C^+$ monocytes accumulation from blood to atherosclerotic plaques found in $ApoE^{-/-}$ mice, a model of increased plasma cholesterol levels (57). Of note, the patrolling $Ly6C^{low}$ monocytes express high levels of CX3CR1 compared to $Ly6C^+$ monocytes, and this is required for their endothelium scanning behavior (6).

CCR1 and CCR5: Early studies found low constitutive expression of CCR1 and CCR5 on mouse and human monocytes (58, 59). While we and others have found evidence of CCR1 and CCR5 (42, 43, 57, 60) cell-surface upregulation during activation, the precise contribution of these receptors in promoting or preventing pathology during antimicrobial responses requires further studies. In this context, an elegant study by the Cerf-Bensoussan group in mice orally infected with cysts of the parasite *T. gondii* showed that the chemokine CCL3 produced by a subset of IL-18 activated NK cells, promoted intestinal recruitment of $Ly6C^+$ monocytes via CCR1, which contributed to early parasite killing but subsequently induced devastating intestinal ileitis (61). However, most evidence of the involvement of CCR1 and CCR5 on monocytes/macrophages are related to atherosclerosis/cardiovascular diseases and autoimmune pathologies such as rheumatoid arthritis, colitis, multiple sclerosis and transplant rejection (45, 60). Because levels of CCL3 and CCL5 in particular, two major ligands of both chemotactic receptors, increase in the course of distinct microbial infection, it seems likely that they participate to $Ly6C^+$ monocyte recruitment to infected tissues/foci (42, 43).

Adhesion molecules: $Ly6C^+$ monocytes utilize adhesion mechanisms to access infected tissues from peripheral blood. During *Lm* infection, $Ly6C^+$ monocytes, use the integrins CD11b and CD44 as well as ICAM-1 to access liver infected foci (62). This process appears largely independent of G-protein mediated chemotaxis since pertussis toxin treatment of purified monocytes -which inhibits G-protein receptor signal transduction- prior to transfer did not prevent them to access infected livers with comparable efficiency as untreated monocytes. Of note, these results do not rule out the implication of other mechanisms in the context of different infections, in the liver as well as in other organs (see above). For instance PLGS-1 or L-selectin/CD62L expressed by $Ly6C^+$ monocytes respectively contribute to adhesion to dermal venules during *Leishmania major* infection and to endothelia during atherosclerosis or thioglycolate-induced peritonitis (45). Interestingly, in West Nile Virus induced encephalitis, $Ly6C^+$ monocyte accumulation in the CNS is mediated through the integrin/ligand pair VLA-4/VCAM-1, with antibodies against ICAM-1 or LFA-1 having little impact on this process (63). Thus altogether the cited studies suggest the existence of multiple adhesion mechanisms utlized by activated $Ly6C^+$ monocytes to access infected tissues.

1.3. Microbicidal machineries

Monocytes are exquisite phagocytes exhibiting high phagocytic capacity, though the patrolling subset in humans ($CD14^{dim}CD16^+$) was shown to have lower phagocytic ability than the functional equivalents of the inflammatory subsets (7). However the formal

comparison in mice still needs to be done. Ly6C$^+$ monocytes are also equipped with the machinery to produce high levels of reactive oxygen and nitrogen species during bacterial (9, 42), fungal (64), parasitic (16, 65) and viral infections (66), some of which being essential for microbial killing. Of note, we have further linked the production of reactive oxygen species to antimicrobial autophagy during *Lm* infection (67), yet this did not appear essential during candidiasis (68). Still relying on mice immunized with *Lm* as a model, we recently reported potent expression by Ly6C$^+$ monocytes of a set of genes encoding the guanylate binding proteins (Gbp), Gbp1-11 (43), that belong to the IFN-γ-inducible GTPases superfamily. These were implicated in cell-autonomous host defense against intracellular bacteria through the activation of the phagocyte oxidase, antimicrobial peptides and autophagy effectors (69, 70).

2. Orchestrating host antimicrobial protective responses

As discussed in the first part of this review, Ly6C$^+$ monocytes can express sets of functions that make them highly fit as essential responders against microbial infections. Many studies have established the importance of these cells in mice models of bacterial, viral, parasitic and fungal infections, and this has been largely reviewed elsewhere (45, 71). Thus we decided to focus below on published evidence that support a role for inflammatory monocytes as key orchestrators of innate and adaptive responses. We propose to subdivide their roles according to their requirement to present cognate antigen to T cells or alternately, to provide other functions such as inflammatory cytokines and transportation as Trojan horses.

2.1. Mechanisms that involve cognate antigen presentation

Ly6C$^+$ monocytes upregulate surface MHC molecules as well as costimulatory receptors in the course of activation with microbial pathogens or pathogen-derived molecules such as LPS. While this capacity to present antigen does not seem to impact the course of infection in most infection models, in some instances, it has been shown that activated Ly6C$^+$ monocytes are able to contribute to T cell priming. The Webster group provided strong evidence along these lines by showing that Ly6C$^+$ monocytes contributed to antigen-dependent activation of influenza-specific CD8$^+$ T cells in mice airways, although, while required for ultimate control of viral infection, the immediate consequences of such CD8$^+$ T cell activation were largely deleterious to the host (66). The Ardavin lab provided indirect evidence that Ly6C$^+$ monocyte-derived DCs may present peptide-MHC complexes to prime protective Th1 CD4$^+$ T cells during *Leishmania major* infection in resistant C57BL/6 mice, yet these results were conflicting with later analyses showing that *L. major*-derived peptide-MHC complexes could only be found on DCs but not on activated Ly6C$^+$ monocytes {Leon, 2007 #1560;Muraille, 2010 #1561}. Another example of the importance of Ly6C$^+$ monocytes for antigen-specific T cell priming was reported in studies from the Pamer lab using a mouse model of invasive aspergillosis, with the mold *Aspergillus fumigatus*. *A. fumigatus* can cause lethal infections in immunocompromised humans, and priming of *Aspergillus*-specific CD4$^+$ T cells requires the presence of Ly6C$^+$ monocytes and their progeny, by a process that involves transportation of fungal spores to dLNs (73). Whether Ly6C$^+$ monocytes directly prime T cells in this model still needs to be investigated. A report

from the Iwasaki lab in the HSV2 infection model also suggested that Ly6C$^+$ monocytes derived cells in the vaginal mucosa could contribute to protective Th1 CD4$^+$ T cell-activation, yet the requirement for cognate T cell antigen stimulation was not determined (74). Overall, the cited studies suggest that pathogen-activated Ly6C$^+$ monocytes certainly acquire the ability to present pathogen-derived antigenic peptides to further activate or restimulate effector T cells, yet they are unlikely to initiate naive T cell priming *in vivo*.

2.2. Mechanisms that involve cytokines and/or transportation

A body of work from independent laboratories has established that Ly6C$^+$ monocytes and their activated progeny contribute to host immune defenses against microbial pathogens independently of antigen-mediated activation of lymphocytes. In the first part of this review, we have listed many intrinsic functional features that are or can be expressed by activated Ly6C$^+$ monocytes, and which represent the basis of their functional plasticity. Production of inflammatory cytokines and chemokines, expression of microbicidal molecules and transport of pathogens are amongst some of these most prominent roles.

Cytokine-dependent mechanisms: Inflammatory mediators—Monocytes express multiple sensors of microbial-derived products as extensively discussed in the first part of this review. Triggering of these pathways by microbial-derived products leads to different outcomes that largely depend on the pathogen. One of the first consequences of their ability to sense pathogens and activate innate pathways is the secretion of proinflammatory cytokines (in particular TNF-α, IL-1, IL-18, IL-12) and chemokines (CCL3, CXCL9) that directly contribute to the rapid amplification of the immune response and pathogen clearance. Since the production of these mediators may require complex multi-step processes and does not necessarily occur directly downstream innate sensing, we discuss the literature in light of these evidences.

Importance of cytokine-driven functional maturation of Ly6C$^+$ monocytes: In the well-studied model of infection by *Lm*, both TNF-α and production of nitric oxid (NO) are important for resistance to the primary infection, and Ly6C$^+$ monocytes represent by far the major producers of these mediators, which collectively supports their essential role in *Lm* killing through these mechanisms. Neutrophils and tissue-macrophages however can also produce these mediators and possibly could contribute to host protection in this model (75), although only to limited extent. In fact, retention of Ly6C$^+$ monocytes in the bone-marrow of CCR2$^{-/-}$ mice and therefore lack of these cells -but not of neutrophils or macrophages- impairs mice resistance to *Lm* infection (76). While the formal demonstration that TNF-α and/or NO production by Ly6C$^+$ monocytes indeed represents the mechanism of *Lm* clearance, the current body of work does provide conclusive evidence for this interpretation. Investigating further the mechanism of production of these key mediators, the recent data from the Sparwasser group have suggested that Ly6C$^+$ monocytes can differentiate into Tip-DCs with no cell-intrinsic requirement for MyD88 (17). Results from the Locksley lab have proposed that NK-cell derived IFN-γ - themselves activated in response to cDCs-derived IL-12- is essential for Ly6C$^+$ monocyte maturation into Tip-DCs (41), a result in agreement with our own data establishing the key function of IFN-γ in promoting Ly6C$^+$ monocyte differentiation during recall infection with *Lm* (43). In this setting though, MyD88 was

required for TNF-α secretion while expression of iNOS and many other functional markers - costimulation, chemokines and cytokine and their receptors-only required IFN-γ that was derived from the memory T cells. Discrepancy with the Sparwasser study could be that requirements are different during the primary and the recall infection. During mouse *Brucella mellitensis* infection, Muraille and colleagues also suggested a comparable mechanism in which iNOS expression by activated $Ly6C^+$ monocytes, an essential mechanism of host protection, appears to depend on IFN-γ, with possible implication of MyD88 - though the study did not assess the monocyte-intrinsic requirements (15).

As an interesting and complex counter regulation mechanism involved in the inhibition of bone-marrow derived $Ly6C^+$ monocytes differentiation into Tip-DCs in the spleen, LNs and liver of mice infected with the protozoan parasite *Trypanosoma brucei brucei* -the cause of sleeping sickness in humans-, the Beschin lab established the importance of IL-10 signals received by $Ly6C^+$ monocytes through elegant gain and loss of function experiments (65, 77). While Tip-DCs production in this model was dependent on cell-intrinsic MyD88 and IFN-γ signals, IL-10 could limit this process and associated tissue damages, providing further evidence of the importance of cytokines in regulating their possible fates.

Work from the Sher group in mice inoculated with *Toxoplasma gondii* cysts, again supported the idea that $Ly6C^+$ monocytes functional maturation is primarily driven by cytokines, by showing that NK-cell derived IFN-γ promoted the functional differentiation of $Ly6C^+$ monocytes at the sites of infection into IL-12 producing cells and in a MyD88-independent manner. IL-12 is a critical cytokine in driving Th1 T cell responses and host resistance to this infection, yet this remained largely independent of iNOS expression (78). In another model of parasitic infection, the blood stage non-lethal murine *Plasmodium chabaudi (Pc)* that exhibits features reminiscent of the chronic human infection with *P. falciparum*, the Langhorne group has reported that $Ly6C^+$ monocytes contributed to more efficient elimination of blood parasites, mostly during the later stage of the disease during which rebounds of parasitemia are observed over several weeks (79). Consistent with such result, patients with acute uncomplicated malaria exhibited higher numbers of $CD14^+CCR2^+CX3CR1^+$ monocytes in the peripheral blood compared to malaria-exposed uninfected controls (80). While conclusions of the mouse study were based on the use of $CCR2^{-/-}$ mice in which $Ly6C^+$ monocytes are retained in the bone-marrow of mice, further analysis suggested robust functional differentiation of these cells with increased expression of reactive oxygen and nitrogen species, proinflammatory cytokines and capacity to phagocytose parasites, overall consistent with the impaired parasite clearance observed in $CCR2^{-/-}$ mice. Which innate and/or cytokines sensing pathways control these processes in the monocytes will require further studies, though it is known that IFN-γ is protective in this parasitic infection and comparable mechanisms as described for *Lm* or *T. gondii* may apply (81).

As another example related to a different cytokine and infection, Sher and colleagues reported the differentiation of a population of $Ly6C^+$ mononuclear cells that is likely to be the progeny of $Ly6C^+$ monocytes, and which produces important amounts of IL-1α and β during murine *Mtb* lung infection, two essential cytokines for controlling this infection (44). In this model, the production of IL-1 unexpectedly did not require a functional

inflammasome cytosolic sensing pathway (82), yet it was negatively regulated by type I interferon, and to some extent $CD4^+$ T cell-derived IFN-γ.

In a very recent study utilizing the chronic Th2-polarizing helminth parasite *Schistosoma mansoni* and adoptive transfers of $Ly6C^{hi}$ monocytes, Loke and colleagues provided good evidence that these cells may differentiate inside infected livers into cells expressing markers (YM1, PD-L2) reminiscent of alternatively activated macrophages (83). The data further suggested this process to be dependent on $CD4^+$ T cells, most likely involving the Th2 cytokines such as IL-4, IL-5 and IL-13 secreted by parasite-specific $CD4^+$ T cells. Together, these results revealed another potential cytokine-driven functional fate of activated $Ly6C^+$ monocytes during a parasitic worm infection.

Thus, altogether these reports underline the importance of cytokine-mediated functional differentiation of $Ly6C^+$ monocytes that is taking place during infections, and which may occur independently from their ability to sense pathogen-derived molecules.

Importance of innate sensing-driven functional maturation of $Ly6C^+$ monocytes: During *Lm* infection for instance, monocyte-intrinsic sensing occurs and this involves the cytosolic DDX41/STING pathway which largely accounts for type I IFN production (33–35). We found that type I IFN can act in a paracrine manner to promote *trans*-presentation of bioactive IL-15 by activated $Ly6C^+$ monocytes (37). Concomitant stimulation of the inflammasome pathway via multiple cytosolic sensors (see section 1), leads to secretion of bioactive IL-18. Both IL-15 and IL-18 from activated monocytes drive rapid NK and memory $CD8^+$ T cell activation and differentiation into cytolytic and IFN-γ-secreting effector cells with no need of cognate antigen recognition (37). While type IFN is acting as a secondary messenger, the initiating pathways are intrinsic to $Ly6C^+$ monocytes. Overall such mechanism of non-cognate cytokine-mediated activation of memory $CD8^+$ T cells can contribute to modest but measurable levels of protection against heterologous, non-related microbial pathogens, adding to existing host innate immune defense mechanisms. A study from the Iwasaki group documented the importance of $Ly6C^+$ monocytes in eliciting protective IFN-γ-dependent effector T cell mucosal responses during primary infection with herpes simplex virus 2, possibly via a similar mechanism (74).

Along similar lines, work from the Kuchler lab in a model of murine *Candida albicans* infection suggested the importance of type I IFN signals in the functional maturation of $Ly6C^+$ monocytes, ultimately promoting lethal, sepsis-like outcomes. However these studies neither addressed the cellular source of type I IFN, nor whether the observed defects were intrinsic to monocytes and involved type I IFN signals to these cells in particular (84).

Several groups utilizing models of viral infections such as Vaccinia, Influenza or herpes simplex viruses, also suggested that triggering of cell-intrinsic innate sensing pathways leads to rapid production of antiviral type I IFN or polarizing cytokines such as IL-12. The Barton group provided strong yet unexpected evidence that $Ly6C^+$ monocytes could produce important amounts of type I IFN via a TLR2/MyD88 mechanism *in vitro*, though formal demonstration of the cellular origin of type I IFN produced during the viral infection *in vivo* was lacking (24). Reports by the Iwasaki and by the Gunn labs highlighted $Ly6C^+$

monocytes as key producers of Th1 polarizing cytokines during HSV2 and Influenza infections, in particular IL-12, but the cell-intrinsic pathways were not defined (23, 74). A further study suggested the importance of concomitant expression of TLR7 and MAVS innate sensors in hematopoietic cells for IL-12 and type I IFN production during influenza infection, however cell-intrinsic requirements would need to be determined (85).

Transport-dependent mechanisms: the Trojan horses—While often essential for direct host protective immune defenses, Ly6C$^+$ monocytes have also been implicated in the transport of live pathogens to tissues and evidence for such a role are discussed below. In a mouse model of invasive fungal infection with *Aspergillus fumigatus*, an important cause of invasive disease in immunocompromised patients, Pamer and colleagues elegantly demonstrated that Ly6C$^+$ monocytes transported fungal spores (conidia) from infected lungs to draining lymph nodes, which was essential for subsequent clearance of fungi from infected lungs (73, 86, 87). The same group recently documented that Ly6C$^+$ monocytes major role during pulmonary *Mtb* infection was indeed to carry *Mtb* bacteria to infected dLNs and this was an essential step in efficient priming of *Mtb*-specific CD4$^+$ T cells by resident cDCs (88). During murine leishmaniasis too, Muraille and colleagues reported the presence of live *L. major* parasites inside Ly6C$^+$ monocytes present in the lesions and the dLNs, also suggesting a possible role as a vehicle (16). In humans, CD14$^+$CD16$^+$ inflammatory monocytes were found to harbor HIV virus and can be infected *in vitro*, and some studies suggest that they may transport the virus to the central nervous system (89, 90).

Conclusions and Perspectives

Inflammatory monocytes, Ly6C$^+$ in mice or CD14$^+$/CD16$^{int/low}$ in humans, can express a variety of functional molecules and pathways. Their roles during microbial infections and inflammatory pathologies have been the subject of many studies over the past decade, largely in mouse models but also, to some extent in humans. These studies have revealed a plethora of functions exerted by these cells, witnessing their tremendous functional plasticity. However, the precise cues and most importantly the combinations of these cues especially those that determine their functional fates remain unclear. While defining their origin during homeostasis and whether master transcription factors regulate their development are subjects of intense investigations, providing a comprehensive understanding of their possible fates in inflammatory conditions and the potential roles of transcriptional regulators will require much further work. This is an essential task, both in terms of basic scientific knowledge and for future medical applications. Studies using siRNA approaches as well as immune modifying nanoparticles that specificaly target inflammatory monocytes are helpful in examining monocyte biology (91, 92). Systematic analyses harnessing micro-RNA and long non-coding RNA to examine transcriptional regulatory mechanisms associated with monocyte differentiation will likely further inform not only the complexity surrounding these cells, but potential therapeutic targets for future examination.

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Highlights

1. Ly6C^{hi} 'inflammatory' monocytes use the chemokine receptor CCR2 to egress from bone marrow.
2. Ly6C^{hi} monocytes possess multiple functional pathogen sensing mechanisms and innate activation pathways.
3. The fate of activated Ly6C^{hi} monocytes largely depends on T and NK lymphocyte derived cytokines and chemokines.
4. Ly6C^{hi} monocytes can differentiate into effector cells, tissue-macrophages and monocyte-derived dendritic cells.

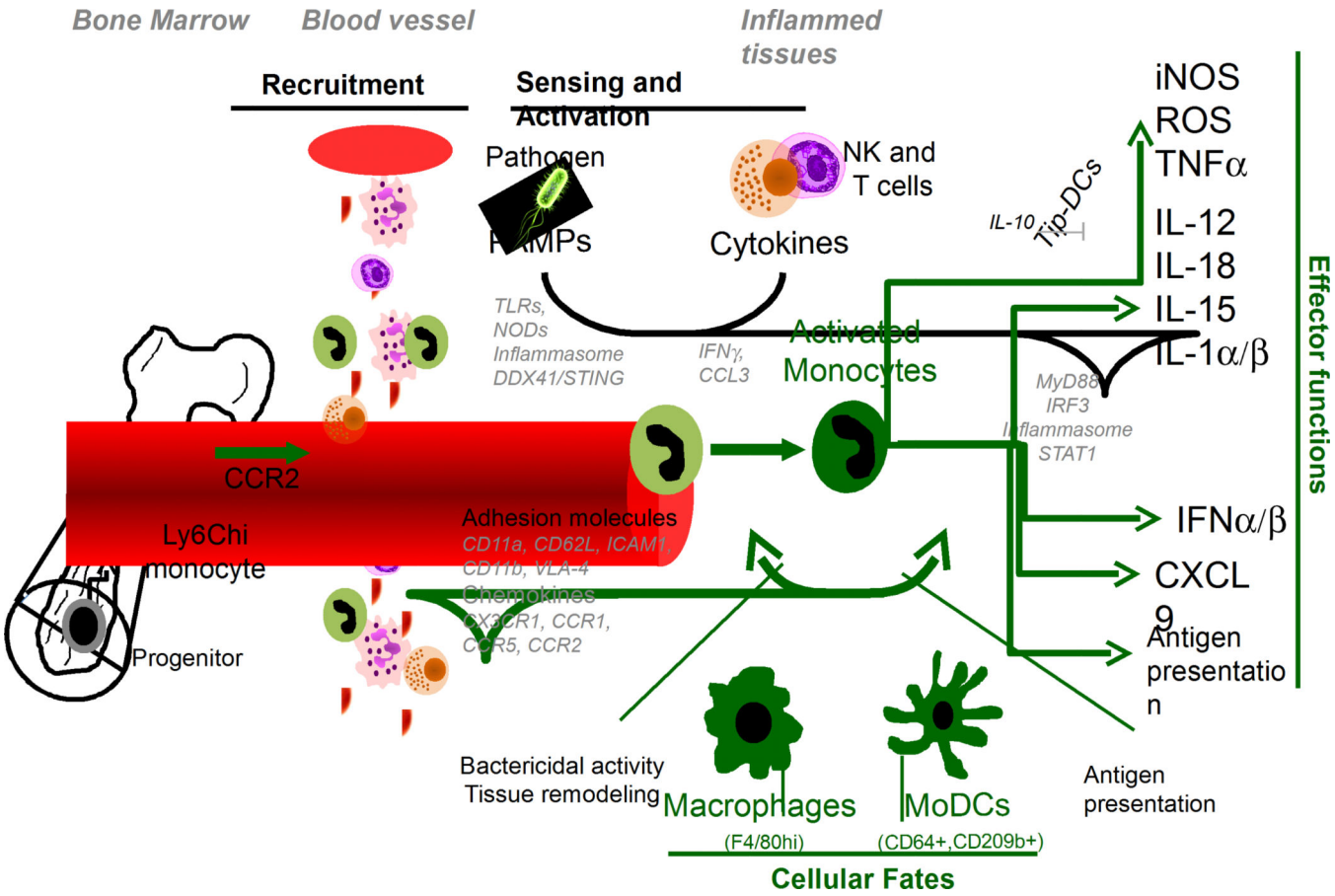

Figure. The Multi-Potency of Ly6Chi Monocytes

Upon microbial infection, Ly6Chi monocytes are rapidly mobilized from the bone-morrow to the blood. Via chemokine and adhesion processes, they reach infected tissues. Through sensing of microbial products and lymphoyte-derived cytokines and chemokines, they can differentiate into multi-effector cells, macrophages and/or monocyte-derived DCs (MoDCs).