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Review

Review

Behind the monocyte's mystique: uncovering their developmental trajectories and fates

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Summary

Monocytes are circulating myeloid cells that are derived from dedicated progenitors in the bone marrow. Originally thought of as mere precursors for the replacement of tissue macrophages, it is increasingly clear that monocytes execute distinct effector functions and may give rise to monocyte-derived cells with unique properties from tissue-resident macrophages. Recently, the advent of novel experimental approaches such as single-cell analysis and fate-mapping tools has uncovered an astonishing display of monocyte plasticity and heterogeneity, which we believe has emerged as a key theme in the field of monocyte biology in the last decade. Monocyte heterogeneity is now recognized to develop as early as the progenitor stage through specific imprinting mechanisms, giving rise to specialized effector cells in the tissue. At the same time, monocytes must overcome their susceptibility towards cellular death to persist as monocyte-derived cells in the tissues. Environmental signals that preserve their heterogenic phenotypes and govern their eventual fates remain incompletely understood. In this review, we will summarize recent advances on the developmental trajectory of monocytes and discuss emerging concepts that contributes to the burgeoning field of monocyte plasticity and heterogeneity.

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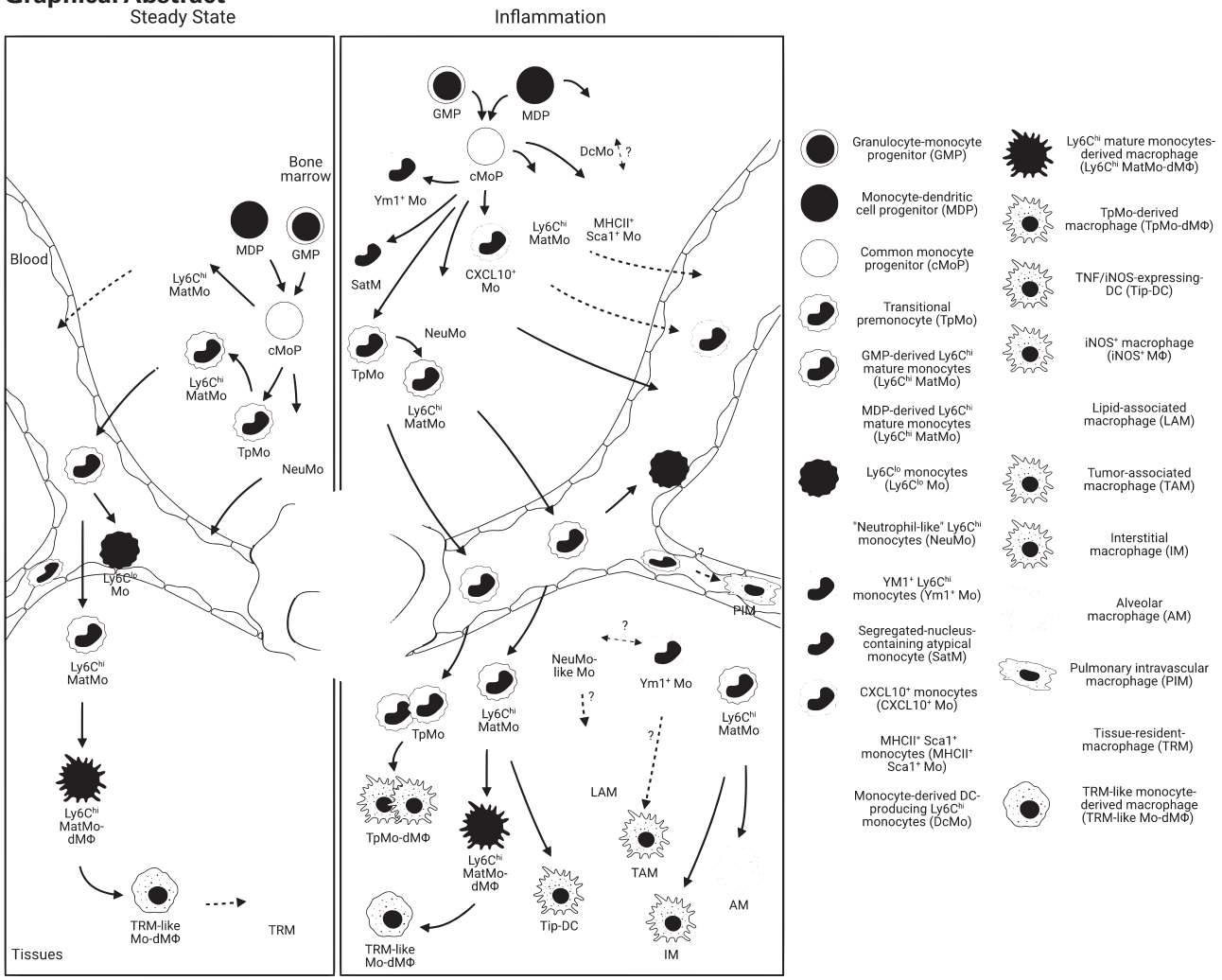
Graphical Abstract



Keywords: monocyte heterogeneity, monocyte-derived cells, macrophages, bone marrow progenitors and precursors, monopoiesis

Abbreviations: Ang II: angiotensin II; AT-1: angiotensin type 1; BAM: border-associated macrophage; BCG: Bacillus Calmette-Guerin; BM: bone marrow; cDC: conventional dendritic cell; CDP: common dendritic cell progenitor; cMoP: common monocyte progenitor; CMP: common myeloid progenitor; COMMD10: copper metabolism MURR1 domain 10; DAP12: DNAX activating protein of 12 kD; DC: dendritic cell; DCMo: moDC-producing Ly6C^{hi} monocyte; EAE: experimental autoimmune encephalomyelitis; EMP: erythro-myeloid progenitor; GMP: granulocyte-monocyte progenitor; HNF: hepatic nuclear factor; HSC: haematopoietic stem cell; HSPC: haematopoietic stem and progenitor cell; LAM: lipid-associated macrophage; LPS: lipopolysaccharide; MatMo: mature Ly6C^{hi} monocyte; MDP: monocyte and dendritic cell progenitor; MGC: multinucleated giant cell; MI: myocardium ischaemia; Mo-dMΦ: monocyte-derived macrophage; moDC: monocyte-derived dendritic cell; NeuMo: neutrophil-like Ly6C^{hi} monocyte; PIM: pulmonary intravascular macrophage; SatMs: segregated-nucleus-containing atypical monocyte; TAM: tumour-associated macrophage; TF: transcription factor; TIP-DC: TNF/iNOS-expressing-dendritic cell; TLR: toll-like receptor; TpMo: transitional premonocyte; TREM1: Triggering Receptor Expressed On Myeloid Cells 1; TRM: tissue-resident macrophages.

Introduction

Monocytes were first discovered in human white blood cells as large mononucleated leukocytes with kidney-shaped nuclei by Paul Ehrlich in 1880 [1]. Originally thought of as phagocytic cells [2] or mere precursors for macrophages [3], these monocyte concepts have been significantly transformed in distinct settings in the last few decades. In particular, it is now well established that tissue-resident macrophages (TRMs) and conventional dendritic cells (cDCs) have defined precursors with the former originating in the embryo before the onset of bone marrow (BM) haematopoiesis [4–6]. Consequently, these cells were found to maintain themselves independently from incoming monocytes and are only replaced by the latter through specific tissue cues [7]. Monocytes constitute ~10% and ~4% of peripheral leukocytes in humans and mice respectively [7]. They can be categorized broadly as 'classical' monocytes, exemplified by CD14^{+}CD16^{+} expression in humans and Ly6C^{hi}CX$_3$CR1^{int}CCR2^{+}CD43^{lo} in mice; and 'non-classical' monocytes, depicted as CD14^{lo}CD16^{+} expression in humans and Ly6C^{lo}CX$_3$CR1^{hi}CCR2^{lo}CD43^{+} in mice [7, 8]. In humans, CD88 and CD89 further delineate monocytes from circulating inflammatory DCs in the blood [9]. Importantly, the advent of novel technologies has uncovered an astonishing display of monocyte plasticity and heterogeneity, which we believe has surfaced as one of the essential themes in the field of monocyte biology in recent years. Emerging evidence suggests that monocytes can be imprinted as early as in the progenitor or precursor stage by the tissue microenvironment to adopt specialized roles that alter the fate of immune responses, revealing a previously under-appreciated complexity in their plasticity [10]. At the same time, monocytes are also recognized as short-lived cells [10]. Consequently, how the immune system ensures

a continuity of imprinted or 'trained' functional responses beyond the monocyte stage will rely on tissue/niche-specific signals that prolong their viability and function upon their differentiation. In this article, we will present an overview of the developmental trajectory of monocytes (Fig. 1) and focus our discussion on the latest findings of how tissue and cellular signals imprint and alter their eventual fates. Specifically, we will review current evidence of monocyte phenotypes, revisit competing dogmas, and discuss how the ontogeny of these phenotypes is unique to distinct disease settings. Finally, we will explore how these concepts contribute together towards monocyte-derived heterogeneity in tissues and offer perspectives on their transitional states.

Monocyte development: Different fates before birth

Fetal versus Adult monocytes

The earliest form of monocytes begins its development in the embryo and are derived from erythro-myeloid progenitors that migrate and seed the fetal liver at E9.5 of gestation in mice [11, 12]. These fetal monocytes subsequently emerge in the fetal liver at around E12.5 and upon entering the blood circulation at E13.5 [13, 14], colonize the open niches of all the organs apart from those in the brain [15] to give rise to steady-state TRMs. In contrast to these fetal monocytes that are found in the developing embryo, adult monocytes that are found in the circulation are derived from hematopoietic stem cells (HSCs) located within the BM. These HSCs first generate a population of common myeloid progenitors that subsequently differentiate into granulocyte-monocyte progenitors (GMPs) and the monocyte and dendritic cell (DC) progenitors (MDPs) [10]. While MDPs were first believed to be the only monocyte progenitor, it is now recognized that GMPs and MDPs both give rise to monocytes [10]. Current findings indicate a disparity between the developmental lineage among GMPs, MDPs, and common monocyte progenitors (cMoPs) [16–18]. In particular, cMoPs, which represent the first stage of monocyte commitment, can be derived from both GMPs and MDPs albeit disparities in their transcriptomes and

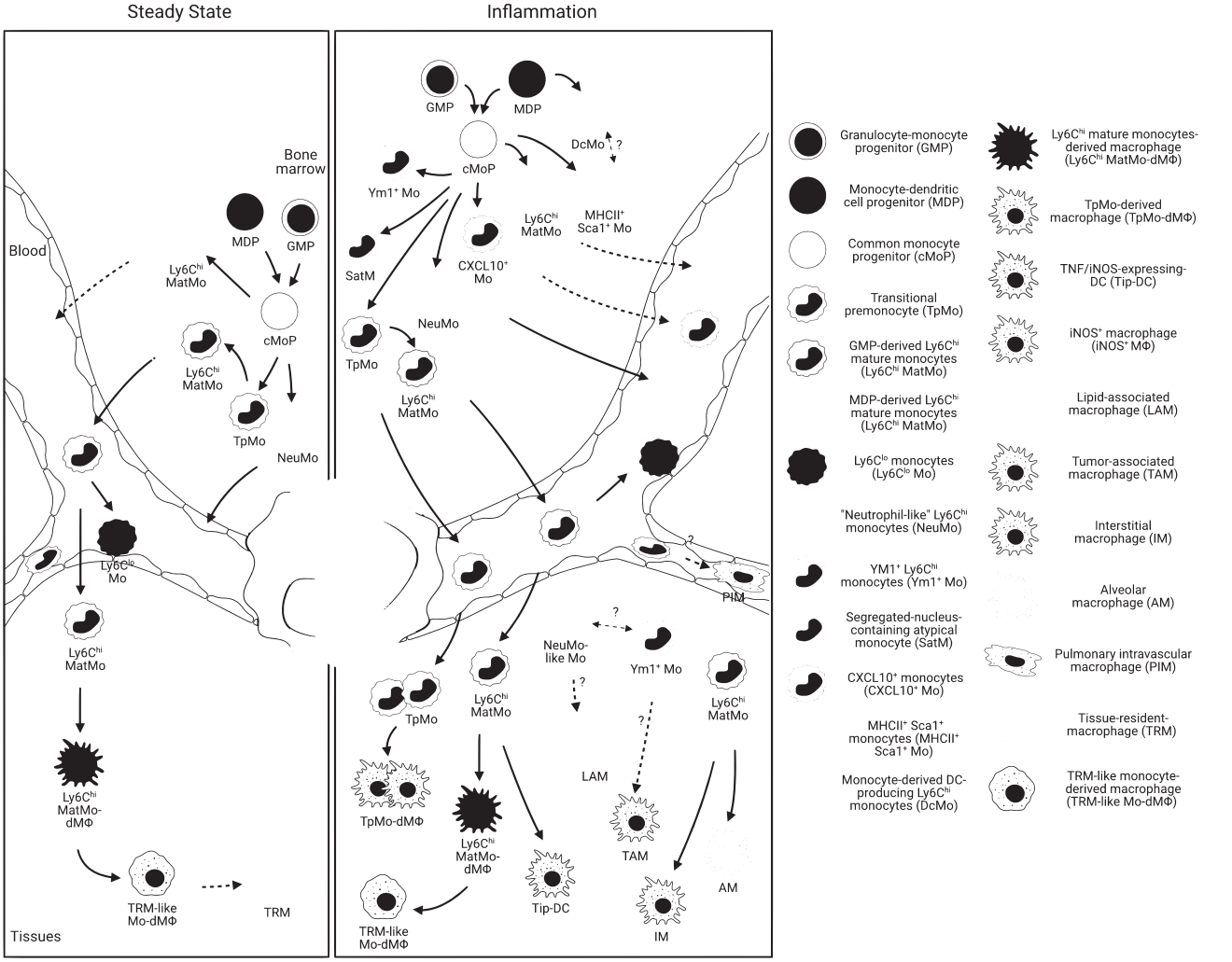


Figure 1: Emerging complexity in the expanding monocyte universe. Monocyte heterogeneity is now recognized to occur at distinct stages of development and locations. During the steady-state, classical Ly6C^{hi} monocytes may originate from two distinct BM progenitors (GMP or MDP) and differentiate along a continuum to give rise to either non-classical Ly6C^{lo} monocytes in the endothelium or TRMs in organs that require TRM replenishment. During inflammatory states, GMPs or MDPs may be primed in the BM to give rise to unique monocyte subsets with different functional states. Monocytes and their precursors/progenitors may also undergo additional modifications upon entering the circulation or tissues, resulting in further complexity in their heterogeneity. The list of monocyte subsets and monocyte-derived cells is not exhaustive but is illustrated to the best of our knowledge to depict the emerging complexity of monocyte heterogeneity.

function [19, 20]. From the cMoPs, monocyte-committed development then commences, first differentiating into transitional premonocytes (TpMos) (CXCR4^{hi}Ly6C^{hi}CCR2^{+}) before downregulating CXCR4 into mature classical Ly6C^{hi} monocytes (CXCR4^{lo}Ly6C^{hi}CCR2^{+}) that exit into the circulation [21]. Alternatively, Ly6C^{hi} monocytes were also found to convert into non-classical Ly6C^{lo} monocytes within the BM in mice [17, 22] although these Ly6C^{lo} monocytes cells were not found in BM biopsies in humans [23].

While studies have revealed that fetal monocytes share many similarities with adult monocytes, fetal monocytes appear to be functionally immature as they poorly express pathogen recognition or antigen presentation genes and do not rely on CCR2-signalling to migrate into tissues [13]. More importantly, unlike adult monocytes that are terminally differentiated and do not proliferate actively in the blood, fetal monocytes retain a high capacity to proliferate in tissues even in the absence of the CSF-1 receptor [14], thereby displaying a more progenitor-like phenotype compared to their adult counterparts. It is likely that these disparities are needed for monocytes to carry out their roles in different stages of life—fetal monocytes require the proliferative capacity as their main function is to colonize tissues and give rise to TRMs efficiently while the higher expression levels of effector function genes in adult monocytes are needed for immunosurveillance in the periphery. Consequently, genes required for differentiation in adult monocytes are only activated when they enter the tissues during times of need or when inflammation occurs.

Emergency monopoiesis

During inflammatory states, an increase in monocyte demand is significantly required, resulting in a process known as emergency monopoiesis that may also occur for other myeloid cell types [24]. Emergency monopoiesis occurs when an increase in committed monocyte progenitors in the BM occurs at the expense of expansion of other lineage-committed progenitors [25]. For example, DC-lineage progenitors were found to be reduced in order to enhance monocyte production for pathogen control during systemic bacterial infection [26]. Importantly, these severe inflammation states that result in emergency monopoiesis may generate monocytes with alternative phenotypes. Recently, the existence of distinct monocyte trajectories was demonstrated by Weinreb *et al.* using a combination of lineage tracing and single-cell RNA-seq [19]. Evidence of imprinting in the early stages of monocyte development in these studies is in line with earlier work by Yáñez *et al.*, where they demonstrated that GMPs and MDPs expressed contrasting transcription factors (TFs) typically seen in neutrophils (*Gfi1, Cebpa*) and monocytes/DCs (*Irf8, Klf4*) respectively [20]. Upon exposure to CSF-1 *in vitro*, novel differentiated BM monocyte subsets were seen to emerge from GMPs and MDPs. These GMP- and MDP-derived mature monocytes were subsequently reported to be functionally unique and responded distinctly to independent microbial agents. In particular, Yanez *et al.* demonstrated that *in vivo* activation of IFN-γ signalling via CpG triggered the emergence of MDP-derived monocytes labelled as monocyte-derived DC (moDC)-producing Ly6C^{hi} monocytes (DCMos) [20]. DCMos were found to express *Cd74*, *Cd209, Flt3, H2-Aa, and H2-Ab1* genes involved in antigen presentation [10, 20]. Furthermore, DCMo-like cells have been described by Askenase *et al.* in an infection model of *T. gondii* [27]. These monocytes with DCMo gene signatures were primed by IFN-γ producing natural killer cells in the BM. Furthermore, these cells expressed Sca-1 and MHCII, as well as inflammatory mediators such as PGE2 and IL-10 that contribute to the inflammatory resolution process. Recently, Barman *et al.* also identified DCMos and described its expansion from MDPs within the BM of old aging mice [28]. It was outlined that this observation was not representative of improved antigen presentation functions with aging but instead an inflammatory state coupled with underlying deficiencies.

On the other hand, Yáñez *et al.* demonstrated that *in vivo* LPS predominantly stimulated the differentiation of GMPs into monocytes termed as 'neutrophil-like' Ly6C^{hi} monocytes (NeuMos), characterized by their expression of genes such as *Elane, Ctsg, Chil3, Mpo,* and *Lcn2* [20]. A study by Ikeda *et al.* later identified a subset of Ym1^{+}(*Chil3*) Ly6C^{hi} monocytes (Ym1^{+} Mos) that displayed NeuMo properties and these cells were derived from GMP but not MDP-differentiated cMoPs under LPS challenge [29]. Ym1^{+} Mos were deemed crucial for inflammation resolution, simultaneously producing higher levels of anti-inflammatory IL-10 and lower pro-inflammatory cytokines (IL-6, IL-12) than Ym1^{-} monocytes. NeuMos were also discovered to be present in the steady-state BM and blood [30, 31], perhaps suggesting the presence of a background signal that maintains a basal level differentiation of GMPs into NeuMos in the BM. On the other hand, a deviation of GMP differentiation into cMoPs into a unique progenitor state could also occur in particular diseases such as bleomycin-induced fibrosis, whereby cells termed segregated-nucleus-containing atypical monocytes (SatMs) were discovered [32]. These SatMs were derived from a progenitor downstream of GMPs but distinct from cMoPs and MDPs [32]. Notably, these cells also displayed neutrophil-like properties such as expression of lipocalin, cathepsin G, neutrophil elastase, and S100a8. Interestingly, both DCMos (*Cd74, H2-Aa, and H2-Ab1*) [33] and NeuMos (*S100a9, Retnlg, Mmp8, Saa3*) [30] were reported separately in two studies using an experimental autoimmune encephalomyelitis (EAE) model albeit with different protocols, suggesting that the generation of these cells might involve an intricate play of mechanisms for their appearance.

Trained immunity

While it is clear that distinct BM monocyte subsets are generated through the conditioning of upstream progenitors, the duration and mechanism that these unique subsets are maintained have often been debated. This question has now been addressed through the concept of 'trained immunity', whereby innate immune cells undergo long-term functional reprogramming through epigenetic modifications in their progenitors, resulting in an altered response towards a secondary challenge [33]. These findings were observed as early as in the 1970s, whereby glucan pre-treated mice were protected against subsequent septic *S. aureus* infection [34]. Trained immunity was also observed in the study conducted by Askenase *et al.*, whereby DCMos isolated from IFN-γ stimulated mice were reportedly heightened, producing more PGE2 and IL-10 upon secondary infection compared to naïve mice [27]. Recently, it has also been shown that exposure to LPS at low versus high doses results in distinct epigenomic landscapes, with key implications for pathology in chronic diseases [35]. Furthermore, Bacillus Calmette-Guerin (BCG) resulted in trained immunity in both mice and human studies, leading to improved monocyte functions

[36–38]. Interestingly, BCG-vaccinated individuals displayed protective monocyte responses against non-related infections such as an experimental infection with an attenuated yellow fever virus vaccine strain through upregulation of IL-1β [37]. Studies by Cirovic *et al.* have also shown that hepatic nuclear factor family members 1α and 1β are crucial regulators that underlie the transcriptional shift, resulting in an activated transcriptional signature of peripheral CD14^{+} monocytes 3 months after BCG vaccination [38]. In addition, Katzmarski *et al.* have demonstrated that trained immunity can be retained across generations by showing that prior parental infection imparts resistance towards future infections in their offspring in mice [39]. Observations of early life perturbations that result in life-long diseases later in adulthood have also attributed these effects to possible imprinting of EMP-derived cells such as fetal monocytes [40]. However, how these epigenetic modifications in embryonic or BM progenitors can be translated into TRM and monocyte-derived cells upon differentiation remains to be explored. Future studies exploring the manipulation of epigenetic modifications resulting in such persistent trained memory might be beneficial for combating chronic pathologies.

Monocyte reservoirs in the periphery: Strategically positioned to await cues

Upon leaving the BM, monocytes spend the majority of their lifetime in circulation in the blood. Classical or Ly6C^{hi} monocytes are the first monocytic cells that leave the BM and have a half-life of approximately 22 h before they constitutively differentiate into an intermediate state that expresses intermediate levels of Ly6C or CD14 before giving rise to Ly6C^{lo} or non-classical monocytes that have a longer half-life of about 48 h in mice [22, 41]. These patterns were similarly observed in humans albeit with longer circulating lifespans for intermediate and non-classical monocytes [23]. Their differentiation process is governed by Nr4a1 and C/EBPβ signalling [42–44] while CSF-1 signalling [22] and the long non-coding RNA *Morrbid* [45] regulate their half-lives. Briefly, Ly6C^{hi} monocytes play a critical role in surveying the circulation for inflammatory sites and are the main cells that extravasate into these inflamed tissues to give rise to monocyte-derived cells. In contrast, Ly6C^{lo} monocytes serve as gatekeepers of the blood endothelium by patrolling blood vessels through an LFA-1/ICAM interaction with endothelial cells, which allows them to scavenge luminal microparticles and maintain the endothelial integrity. They are thought to be terminally differentiated and have been proposed to represent blood macrophages [10, 41].

Spleen monocytes

Besides the BM and circulation, monocytes have also been discovered to reside in other peripheral tissues (Fig. 2). In the spleen, a population of monocytes was identified by Swirski *et al.* within the subcapsular red-pulp region [46] and their numbers and function were recently found to be regulated by gut microbial products [47]. Based on morphology and transcriptome profiles, these splenic monocytes originated from the migration of their blood analogues first produced in the BM [46] and have been shown to be sequestered within the spleen where they clustered mostly in the reticular fibre-rich pulp cords. In the event of certain pathological events such as myocardium ischaemia, the increase of angiotensin II (Ang II) serum levels triggered splenic monocyte egress into the inflamed heart via angiotensin type 1 (AT-1) receptor binding where they differentiated specifically into cardiac macrophages [48, 49]. Mobilization of splenic monocytes into peripheral tissues has also been described in Alzheimer's disease and in muscular dystrophy [50]. These studies mainly incorporated the use of *Ccr2*$^{-/-}$ mice (whereby BM monocytes are unable to egress into the periphery), as well as splenectomy, CD45.1 to CD45.2 splenic transplantation and intravital imaging experiments to distinguish splenic from BM-derived circulating monocytes in these mobilization events. While it is unclear why splenic monocytes are preferentially mobilized in these pathological conditions, it is evident that the splenic reservoir of monocytes may serve as an important source of cells for immediate immune defense within the spleen. Specifically, a study done by Hoffman *et al.* on *Salmonella enterica* serovar Typhimurium (*S. Tm*) infection demonstrated that *S. Tm* pathogens characteristically invade the spleen and activate the differentiation of Ly6C^{hi} and Ly6C^{lo} monocytes into distinct macrophage populations that highly express iNOS and CD9 respectively with the latter replacing red-pulp macrophages [47]. Through the course of infection, these splenic monocyte populations are further maintained alongside *S. Tm* pathogens in their local environment, suggesting that the monocyte reservoir in the spleen may serve as a ready source of precursor cells that can be rapidly differentiated to control the invasion of pathogens in the spleen [47]. Given the appearance of new monocyte-derived macrophage populations, it is also probable that splenic monocyte–pathogen interactions could also prime monocytes towards atypical developmental fates, and further studies would be needed to determine these outcomes.

Besides the spleen, blood Ly6C^{hi} monocytes have also been identified to settle in other peripheral organs such as the skin, lungs, and lymph nodes. Specifically, Jakubzick *et al.* demonstrated that these Ly6C^{hi} monocytes remain nominally differentiated in comparison to their blood counterparts after entering these tissues, but undoubtedly retain their capability to differentiate into macrophages [51], perhaps in response to appropriate stimuli.

Lung marginal pool of monocytes

We have previously shown that the lung consists of a marginal pool of monocytes that are regulated by CXCR4-signalling [21]. This marginal pool was formed due to the multiple small-calibre microvessels (<5 μm in diameter) specifically in the lung, which necessitate larger leukocytes such as monocytes (6–8 μm) to slow down and deform from a spherical into an elongated shape for their transit [52]. Consequently, the lung harbours more monocytes within its vasculature than any other organ [53, 54] and while this marginal pool of monocytes is sequestered from the circulating blood, they can still be rapidly mobilized within the lung parenchyma in times of need [55]. Upon endotoxin sensing, Ly6C^{hi} monocytes increased their lung transit time by adhering to the endothelium [23, 56], resulting in an increased predisposition towards lung injury that can be reversed with CXCR4 inhibition [21]. In particular, livestock animals such as sheep are known to be highly susceptible to acute lung injury induced by such endotoxin exposure [57, 58]. It was postulated that these animals possess a resident lung macrophage population known as pulmonary intravascular macrophages (PIMs) [59, 60], which are seemingly absent in humans and mice. However, O'Dea *et*

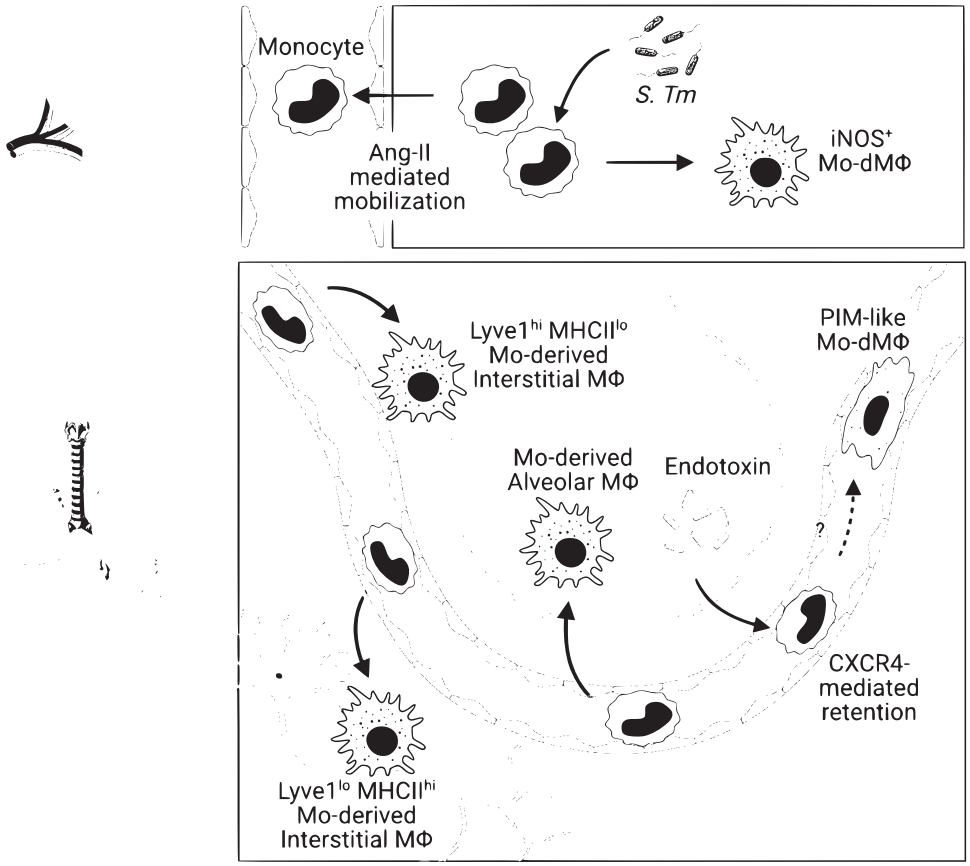


Figure 2: Monocyte reservoirs serve as rapid responders during inflammation. *[Top]* During MI, splenic monocytes enter the circulation in response to increased serum Angiotensin II (Ang II) levels. They subsequently infiltrate the heart and differentiate into cardiac macrophages. Exposure to *Salmonella typhimurium* (*S. Tm*) triggers splenic monocyte differentiation into iNOS-expressing macrophages. *[Bottom]* Monocytes in the lung differentiate into interstitial and alveolar macrophages with distinct phenotypes according to the environmental signals they encounter. CXCR4-signalling regulates monocyte retention in lung microvessels. Upon sensing endotoxins, monocytes adhere to the endothelium and prolong their lung transit time, possibly resembling PIM-like monocyte-derived macrophages over chronic exposure.

al [56]. have discussed that the accumulation of monocytes in the lungs under chronic systematic inflammation may eventually give rise to a population with a PIM-like phenotype [61, 62]. Future studies that involve time kinetics and single-cell sequencing analysis of the lung marginal pool of monocytes after endotoxin exposure would be needed to clarify these intriguing questions. Considering the significant damage that these marginal pools of monocytes exert towards acute lung injury, its physiological relevance remains enigmatic and further research would be required to understand its protective role in various conditions. Future studies exploring specific markers to distinguish monocytes from the spleen versus the lung reservoir and BM-derived circulating monocytes would also be beneficial to provide further insight into their different roles in disease settings.

Timely waves of monocytes with alternative functions

As earlier discussed, monocyte progenitors can be primed in the BM to give rise to monocyte phenotypes with unique functions. Since systemic exposure to pathogens and inflammation may occur before these signals reach the BM, it is conceived that circulatory monocytes may adopt their own unique trajectory after such stimuli. Using single-cell transcriptomic analysis, Lawlor *et al.* demonstrated that upon exposure to LPS, classical blood monocytes displayed an early activation state characterized by the expression of chemoattractant such as CXCL7, CCL7, and CCL8, followed by a late activation state characterized by the expression of effector molecules such as IL-6, IL-8, and IL-1β [63]. These data suggest that the first wave of monocytes may function mainly as an emergency squad to recruit other immune cells during inflammation, whereas the second wave of monocytes was primed for effector functions such as pathogen clearance. However, many questions still remain: How do these circulating phenotypes differ from monocytes that arise through the priming of BM progenitors such as NeuMos and DCMos? In a recent study, Rigamonti *et al.* have attempted to address this question by demonstrating nine distinct functional populations of human circulating monocytes in healthy donors [31]. Specifically, there were five populations related either to inflammatory, neutrophil-like, interferon-related, or platelet-related pathways among the classical subset of monocytes. On the other hand, non-classical monocytes consisted of two populations, one of which had elevated expression of complement components. In particular, they identified one cluster of cells in both classical and non-classical subsets that had a strong cytotoxic signature that was markedly increased during COVID-19 infection. Furthermore, patients with advanced gastrointestinal cancer displayed a selective increase of monocytes with platelet-associated pathways while immunotherapy increased the population of interferon-related

monocytes. These findings hence suggest that circulating monocytes may indeed derive from distinct developmental trajectories. However, it is unclear if these circulating monocyte phenotypes described in the human settings may represent the same kind of cells that have been primed in the BM or are a unique phenotype that has been primed in the blood. It is also uncertain what happens to NeuMos and DcMos when they are further exposed to circulatory inflammatory signals. Do these cells continue to perform their dedicated functions that were conveyed to them in the BM or do they eventually develop into a tolerance state [64, 65]? Finally, do NeuMos and DcMos eventually differentiate into Ly6C^{lo} monocytes that carry distinct functions from that of Ly6C^{lo} monocytes that were differentiated from circulating unprimed Ly6C^{hi} monocytes? Taken together, these studies highlight numerous ontogeny possibilities that can contribute towards monocyte heterogeneity in the circulation and future studies that address these questions will provide fascinating insights into their plasticity and trajectories.

Monocyte differentiation in tissues: Shapeshifting according to environmental signals

As monocytes infiltrate into the tissue, they gradually acquire unique signatures under the influence of distinct tissue local cues, resulting in TFs that direct tissue-specific differentiation of macrophages [66, 67]. Nevertheless, monocyte differentiation into macrophages occurs in a step-wise process through specific cues as summarized below.

Phenotypic and functional changes of monocytes during differentiation

As Ly6C^{hi} monocytes begin their differentiation, they have been commonly described to undergo a loss of Ly6C while increasing their expression of CD11c, CX_3CR1, and CD64 [68–70], which complicates the discrimination of these cells from Ly6C^{lo} monocytes that might have potentially migrated into the tissue. Consequently, it remains a challenge to truly differentiate these two cell types considering the derivation of Ly6C^{lo} monocytes from Ly6C^{hi} monocytes. While it has been proposed that Ly6C^{lo} monocytes are generally constrained to residency in the vasculature where they represent vasculature-resident macrophages [22, 71], their ability to differentiate into tissue-resident macrophages have been reported in some settings through adoptive transfer experiments [72–74] and in the *Nr4a1*$^{-/-}$ mice [75]. Therefore, developing improved tools to fate-map and track the development of Ly6C^{hi} separately from Ly6C^{lo} monocytes would be needed in the future to resolve this challenge.

Besides the downregulation of Ly6C expression, the differentiation process of Ly6C^{hi} monocytes into macrophages relies on the entry of these non-proliferating cells into the cell cycle [76, 77]. In a mouse model of skeletal muscle injury, inflammatory Ly6C^{hi} monocytes were discovered to lose their expression of Ly6C and adopted a proliferative state before giving rise to macrophages that assist in resolution and regeneration [78]. A similar developmental monocytic fate was described in retinal pigment epithelial cell injury where Ly6C^{hi} monocytes infiltrated the retina and upregulated their proliferative capacity to replenish the loss of endogenous microglia and thereafter acquired microglia-like morphologies [79]. These findings were similarly described in other inflamed tissues such as during cutaneous wound healing [80], urinary tract infection [81], and after helminth infection in the liver [82], where monocytes were found to become proliferative after a few days in the tissue. Deficiency in DNAX activating protein of 12 kD (DAP12) resulted in reduced proliferation of monocytes upon CSF-1 stimulation, suggesting that CSF-1 may potentially be important for inducing their proliferative state [77]. Nevertheless, Ly6C^{hi} monocytes in the blood are non-proliferative yet constitutively absorb CSF-1 [22]. Therefore, further research would be required to decipher the exact signals required for entry and exit of Ly6C^{hi} monocytes in the cell cycle. Specific blockage of proliferation only in monocyte-derived cells and not TRMs would also provide further insight into the specific reasons for the proliferative activity of monocytes upon their differentiation.

Niche signals that influence the differentiation outcome of monocytes

Since tissue-derived signals are critical for the subsequent fate of monocyte-derived cells, it is conceived that the absence or presence of inflammatory signals in the tissue would also alter their differentiation outcome. Furthermore, basic homeostatic cues such as neuronal, circadian rhythms, sleep, diet, and the microbiome have also been recently found to have a significant impact on monocyte fate [83, 84]. In particular, the gut is constantly exposed to a low-grade inflammation caused by commensal microbiota and their products, resulting in monocytes undergoing a loss of Ly6C and concomitant gain in MHCII expression, which has been described as the 'monocyte waterfall' [68, 85] that have also been described in other inflammatory settings [86]. In particular, major transcriptomic changes were already found between Ly6C^{hi} monocytes and TRMs early after engraftment [87]. Using an adoptive monocyte transfer model with transcriptional profiling in the small intestine, Desalegn and Pabst further showed that donor monocytes altered their transcriptional profile as early as day 1 after transfer in a Triggering Receptor Expressed On Myeloid Cells 1 (TREM1)-dependent manner, indicating that monocytes are highly sensitive to tissue cues and modify their gene expression as soon as they enter the tissue [88]. Interestingly, monocytes infiltrating the inflamed small intestine adopted an alternative differentiation program from the earliest time point, characterized by higher expression of pro-inflammatory mediators as compared to their counterparts in the normal gut [88]. These distinct transcriptional profiles were documented despite monocytes displaying a superficially similar Ly6C^{+}MHCII^{-} surface phenotype in both homeostasis and inflamed conditions, indicating that improved surface phenotyping would be needed in future studies to correlate their functional profiles. These studies also emphasize the importance of the timing and tissue-specific cues in determining whether monocytes differentiate into functionally similar or different cells from their TRM counterparts. Interestingly, monocytes also give rise to distinct macrophage subtypes in the same tissue depending on their interaction with the microniche [89, 90]. For example, Chakarov *et al.* demonstrated two distinct monocyte-derived interstitial macrophage populations expressing differential levels of Lyve1, MHCII, and CX_3CR1 that coexist in specific subtissular niches across tissues [91]. Monocyte-derived macrophages were also found to mimic the transcriptomic signatures of embryo-derived TRMs in the steady-state or after resolution of inflammation, suggesting that tissue signals are highly critical in providing

instructions for TRM identities, be it from monocytes or from embryonic progenitors [7, 15]. For example, Hoeffel *et al.* demonstrated a role for sensory neurons in influencing monocyte recruitment, resulting in the differentiation of monocytes into dermal-resident macrophages that are functionally similar to the embryo-derived Tim4$^+$ macrophages with healing properties [92].

Persistence of imprinted monocytes into monocyte-derived cells

While it is evident that the tissue provides critical signals to modulate the outcome of monocyte-derived cells, Ly6C^{hi} monocytes often adopt distinct phenotypic and effector functions that may be imprinted as early as in the BM [20, 29, 33]. Therefore, whether these phenotypes may be further modified or continue to persist in the tissue remains unclear. During EAE, Ly6C^{hi} monocytes are mobilized into the inflamed tissue where they utilize CSF-2 from T cells to generate inflammatory cytokines and chemokines for pathology [30]. Interestingly, these CNS infiltrating monocytes did not integrate into the tissue-resident microglia pool even after resolution of clinical symptoms [93] although monocytes to MHCII$^+$ microglia transformation was reported after experimental bacterial meningitis [94]. While it is unclear why these findings may occur, it is important to highlight the consideration of heterogeneity among brain macrophages that include border-associated macrophages (BAMs), which unlike microglia in the brain parenchyma, continue to harbour monocyte and inflammatory signatures after resolution [95]. Similarly, monocytes that are recruited into the liver in a model of viral hepatitis [96] and as part of a VEGF-initiated angiogenic program [97] did not differentiate into macrophages. However, on the other hand, infection with gammaherpesvirus generated IL-10-producing MHCII^{hi}Sca-1^{hi} monocytes within the BM, which resulted in engraftment of these regulatory monocytes in the alveolar macrophage pool that self-maintained for months after [98]. Therefore, it seems that differing models of inflammation may provide varying levels of survival factors to retain monocyte-derived cells in distinct anatomical sites of the tissue. Studies have also shown that BM-imprinted monocytes such as NeuMos and DcMos are distinct from mature Ly6C^{hi}-derived monocytes [20, 29, 33] but how these cells behave in the periphery depends on distinct models of inflammation. In particular, Giladi *et al.* have shown that CXCL10$^+$ monocytes that appear during EAE [33] were exclusively derived from BM monocyte progenitors while mature unprimed Ly6C^{hi} monocytes that enter the CNS gave rise to another subset of cells characterized by iNOS$^+$Arg1$^+$ expression, which seemed similar to TNF/iNOS-expressing (Tip)-DCs that were originally described by Pamer *et al.* [99]. These findings support a previous study [100] that depicted two distinct CCR2-dependent Ly6C^{hi} monocyte subsets: classical Ly6C$^+$MHCII$^-$CD209a$^-$ monocytes that gave rise to Tip-DCs and Ly6C$^+$MHCII$^+$CD209a$^+$ cells that could be primed in the BM. Intriguing, IFN-γ has also been shown to be a key modulator for the appearance of Tip-DCs [101–103], similar to the IFN-γ-driven mechanism of CXCL10$^+$ monocytes [33]. Therefore, further fate-mapping studies would be required to confirm the origin of Ly6C$^+$MHCII$^+$CD209a$^+$ cells and tissue outcome of DcMos that are primed in the BM. It is also intriguing to note that NeuMo-like populations may possibly give rise to distinct monocyte-derived cells in the tissue. In Cohen *et al.*'s investigation of the effects of copper metabolism MURR1 domain 10 (COMMD10) protein deficiency during liver injury-induced inflammation, the developmental fate of COMMD10-deficient Ly6C^{hi} monocytes in the BM was skewed towards a NeuMo phenotype [104]. This NeuMo-like monocyte population was then recruited to the inflamed liver and further found to mirror the gene expression profile of lipid-associated macrophages (LAMs) [104]. NeuMo-like monocyte populations have also been recently described in the tumour, where they adopt a Ym1$^+$Ly6C^{hi} or Chil3$^+$ phenotype that drives a pro-tumoural response [105, 106]. However, how these cells may integrate into the tumour-associated macrophage (TAM) population remains unclear. Finally, while it seems that BM-imprinted monocytes may have little ability to be further modified in peripheral tissues, Desalegn and Pabst showed that an adoptive transfer of BM monocytes from donors with established small intestinal inflammation developed into a phenotype characteristic of the homeostatic condition when transferred into healthy host tissues [88]. These findings hence indicate that local environmental cues may surpass initial priming phenotypes of monocytes to alter their functional outcome, similar to studies conducted by Lavin *et al* [107]. whereby macrophages were able to alter their phenotypes when transferred to other tissue environments. Nevertheless, an in-depth understanding of precise tissue- and context-specific factors that regulate monocyte plasticity would be required to clarify these observations.

Mobilization of monocyte progenitors into the periphery: Rising up to the occasion

Monocytes and TRMs require specific growth factors for their differentiation and survival [7, 10, 15]. As a result, only a dedicated number of residential cells can be supported by the tissue at any one point in time. Indeed, Guilliams *et al.* have proposed a paradigm whereby macrophages and monocyte-derived cells occupy individual 'niches' in the tissue and these niche sites are limited by the amount of trophic factors required for survival [108, 109]. In particular, monocyte-derived cells and TRMs rely heavily on stromal cell-derived CSF-1 for their proliferation and survival [110], and therefore, it has been proposed that the levels of CSF-1 in the tissue dictate the number of niches and residential cells in the tissue. Interestingly, this phenomenon closely resembles a model of 'quorum sensing' [111, 112], which is the ability to detect and respond to cell population density similarly to that described in bacteria. According to this model, monocytes and macrophages readily consume tissue-derived CSF-1, which allows them to regulate the number of TRMs or monocyte-derived cells in the tissue by functioning as a CSF-1 sink [22, 113]. It is likely that the 'quorum sensing' model is partially responsible for allowing a pool of undifferentiated monocytes to reside in tissues, although more studies would be needed to determine if there are other factors that allow monocytes to remain in an undifferentiated state.

Death of TRMs and the monocyte conundrum

TRMs are long-lived cells that have the ability to self-renew in the absence of monocyte input during the steady-state [5]. Since CSF-1 levels govern the availability of niche spaces in the tissue [114–116], it is conceived that monocytes are only able to begin their differentiation process when there is an excess of niche spaces that cannot be fulfilled by the proliferation of

remaining TRMs. For example, this might occur when tissues such as the uterus [117, 118] and mammary gland [119] undergo an expansion in stromal network and size as seen during pregnancy; or in the event of TRM cellular death in inflammatory settings [120–124] that impedes the expansion of neighbouring TRMs. In particular, TRMs are well established to carry out homeostatic functions in the tissue but poorly adapt to inflammatory stimuli, which leads to a replacement of TRMs by monocyte-derived cells. Indeed, Ginhoux *et al.* have proposed that the death of TRMs is an altruistic and necessary process to counteract infections [125]. However, this process may be inappropriately executed in non-infectious settings whereby the death of TRMs resulted in exacerbated liver damage during non-alcoholic steatohepatitis due to less efficient hepatic triglyceride storage by monocyte-derived cells [126]. While TRM death necessitates the infiltration of monocyte-derived cells, the urgency for monocyte replacement in these distinct settings differs significantly. Specifically, the temporary absence of TRMs would likely result in minimal consequences in the steady-state or non-pathogenic inflammatory settings while the lack of TRMs in the presence of pathogens leads to dire consequences associated with increased morbidity if these empty niches are not replaced rapidly to reinstate the peripheral immune defense [127–129]. To complicate matters, active monocyte differentiation is incompatible with simultaneous anti-pathogen effector functions as monocytes are highly susceptible to cellular death after exhaustion of their effector functions. Monocytes from COVID-19-infected patients had activated NLRP3 and AIM2 inflammasomes leading to increased pyroptosis cell death, especially in severe cases [130]. Furthermore, in response to bacterial pathogens or toll-like receptor (TLR) ligand stimulation, monocytes produce pro-inflammatory cytokines and their activation triggers phagocytosis-induced cell death [131]. Similarly, inflammasome activation occurs and monocytes are subjected to various cell death pathways [132]. Consequently, whether the host has evolved strategies to balance the need of monocytes for immunosurveillance versus the replenishment of the TRM niche to sustain immune defense against invading pathogens remains unclear.

Mobilization of monocyte precursors for TRM replenishment

Recently, we have shown that the host is able to overcome the monocyte effector versus replenishment conundrum in an acute bacterial infection model and sepsis setting by mobilizing TpMos, a constitutive proliferating immediate precursor of mature Ly6C^{hi} monocytes (MatMos) located in the BM, into the periphery in a CCR2-independent manner [133]. Upon migrating into the periphery, TpMos were found to serve as an important source of proliferative monocytes that can readily replenish the macrophage pool. This contrasts with MatMos, which are terminally differentiated cells, which were earlier described to enter the cell cycle only after a few days in the presence of local proliferative cues in the tissue site. Indeed, we discovered that TpMos not only gave rise to more macrophages at a much faster rate than MatMos, TpMo-derived macrophages were also more resistant to cellular death. These findings are also reminiscent of the proliferative and progenitor-like features of fetal monocytes that give rise to TRMs rapidly in the embryo [13, 14], suggesting that TpMos may be recruited due to the similar qualities that they possess with fetal monocytes for TRM replenishment.

More importantly, the mobilization of TpMos into the periphery may represent a unique form of 'quorum-sensing', whereby the host is able to sense an urgent need for macrophage replenishment at the peripheral site during an infectious setting. This is because TpMos were mobilized from the BM into the periphery only when TRMs were depleted in the presence of an ongoing bacterial infection and their numbers in the periphery correlated with an increasing bacteria burden. In contrast, TpMos were not found in the circulation in the steady state or when TRMs were depleted in the absence of infection. Consequently, these findings highlight an evolutionary mechanism that enables the host to balance the competing demands of monocytes specifically under a pathogen threat, such that the bactericidal and macrophage replacement tasks can be specialized by MatMos and TpMos in the early stages of infection, respectively.

The mobilization of BM TpMos into the periphery suggests that TpMos and other progenitors might provide critical effector functions beyond their roles in the BM. Indeed, while hematopoietic stem and progenitor cells (HSPCs) reside mostly in specialized niches in the BM, these cells are not entirely sessile even in adulthood [134, 135]. It is well established that some HSPCs may recirculate between BM and blood and it is thought that their recirculation fosters the local production of tissue-resident innate immune cells in both steady-state and inflammatory settings [136]. A dysregulation in their circulatory numbers has also been associated with disease states [137]. Besides HSPCs, committed progenitors such as common DC progenitors (CDPs) [138], as well as GMPs and MDPs [139], have been described to migrate into the circulation during infectious settings, with the latter study demonstrating their role in suppressing inflammation. In support of these findings, our research revealed that TpMos gave rise to macrophages that were less inflammatory and transcriptionally distinct from MatMo-derived macrophages, leading to improved sepsis survival [133]. Since TpMos are the direct precursors of MatMos and the differentiation of these cells occurs along a continuum, it remains challenging to delineate TpMo-derived macrophages from MatMo-derived macrophages through specific markers and future studies would be needed to identify and determine their functions in other inflammatory settings. Nonetheless, our results highlight the distinct outcomes of monocyte-derived cells when priming occurs in monocyte precursors/progenitors versus terminally differentiated monocytes, reminiscence of how NeuMos and DcMos are exclusive phenotypes from priming of monocyte progenitors and not mature Ly6C^{hi} monocytes themselves [20, 29, 33]. In further support of these notions, Losslein *et al.* have also highlighted monocyte progenitors that have been mobilized into the periphery as key contributors towards multinucleated giant cells (MGCs), a unique macrophage type found in granulomas towards mycobacteria [140]. They showed that *Mycobacterium tuberculosis* (*M. tb*) programmed cMoPs to accumulate cholesterol and lipids, which are prerequisites for giant cell transformation but may also favour intracellular mycobacterial survival since cholesterol serves as an energy source for *M. tb* [140]. Their findings also suggest that the adaption towards MGC formation requires progenitor traits, which include high proliferative activity and commitment towards the monocyte lineage [140]. Taken together, these findings indicate a deep level of plasticity in the monocyte hierarchal system and that the host has evolved strategies

to exploit the traits of monocyte progenitors for effector functions in the periphery (Fig. 3).

Monocyte-derived cells in tissues: Revisiting transitional inflammatory states

In response to an injury or infectious insult, the tissue often undergoes two distinct phases: an inflammatory phase followed by a resolution phase, which coincides with macrophages or monocyte-derived cells exhibiting either an inflammatory or anti-inflammatory phenotype, respectively [7, 10, 84, 141].

Debunking the M1/M2 classification of TRMs

The concept that macrophages or monocyte-derived cells can be grouped into two such phenotypes was initiated in the early 1980s and 1990s, whereby a paradigm of classically or alternatively activated macrophage subset was established [142–144]. Classically activated macrophages had an overall pro-inflammatory phenotype while alternatively activated displayed immunosuppression activities [142, 145]. Subsequently, Mills *et al.* described a similar dogma with the M1/M2 nomenclature whereby M1 macrophages were induced by IFN-γ or LPS that primed Th1/Th17 responses while M2 macrophages were polarized through IL-4 and generated Th2 responses [146].

Gradually, these concepts were employed in various settings, which led to substantial confusion among the field of myeloid heterogeneity. Besides the fusion and interchangeable use of the M1/M2 nomenclature with the classical and alternative macrophage polarization concept over time, markers linked to inflammatory processes and resolution of inflammation were beginning to be considered M1 and M2 markers respectively [147]. However, the M1/M2 concept is based on an *in vitro* construction, and it has been shown that TRMs lose their tissue-specific gene expressions after being incubated in culture for a period of time [148]. Furthermore, a comparison of *in vitro* gene lists to *in vivo* M1 and M2 macrophages by Orecchioni *et al.* revealed little similarity in these two settings [142]. Consequently, it seems that the macrophage polarization dogma is the result of an IL-12/arginase ratio with M1 and M2 signatures at the extreme ends and forcing macrophage and monocyte-related data into M1/M2 groups limits potential data discovery. More importantly, these studies often highlight the ability of M1 and M2 phenotypes to be highly dynamic, suggesting that *in vitro* macrophages have the potential to switch from an M1 to M2 phenotype interchangeably [149–151].

While the M1/M2 concept has provided some functional insight under specific conditions, it is increasingly clear that these theories do not provide a comprehensive understanding of the actual dynamics of macrophages and monocyte-derived

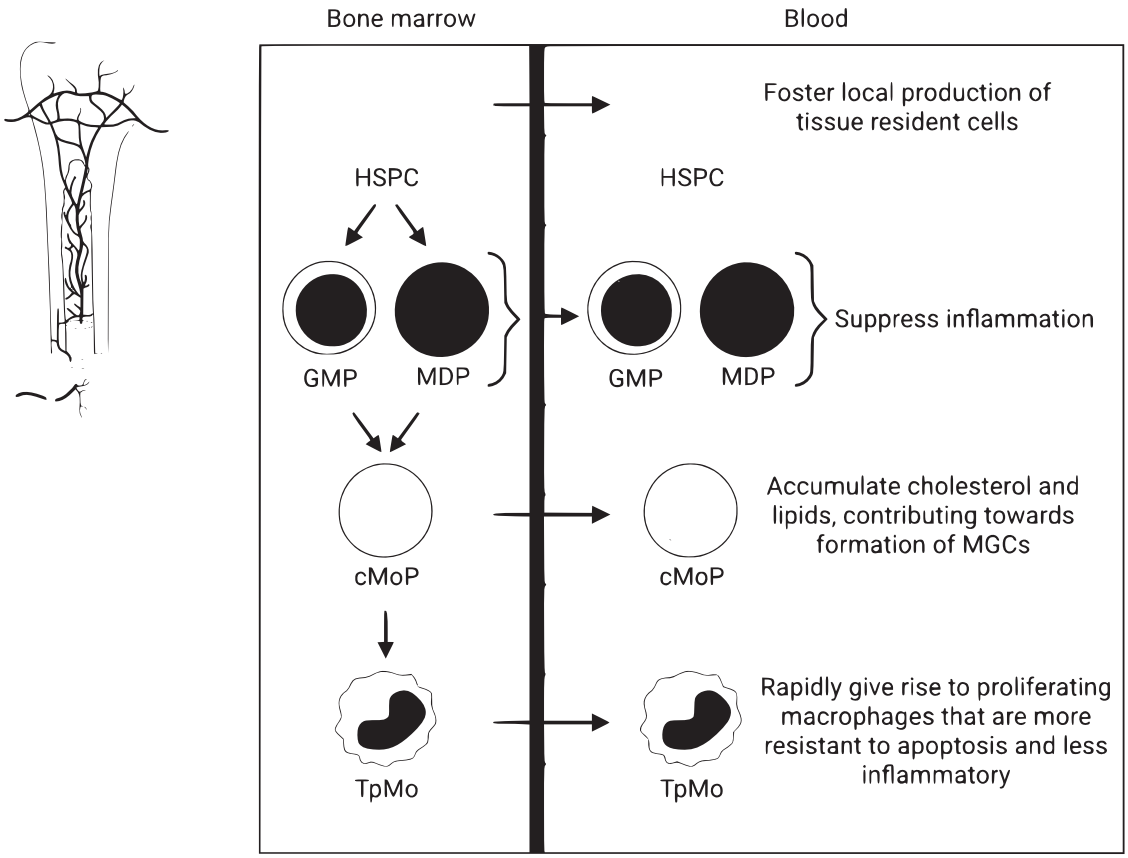


Figure 3: Mobilization of monocyte progenitors from the BM into the periphery during inflammation. Although hematopoietic stem and progenitor cells (HSPCs) and their progenies are usually immobilized in the BM to generate mature monocytes, they have also been found to have distinct roles in the periphery. HSPCs recirculate between the BM and blood to foster local production of tissue-resident immune cells. GMPs and MDPs migrate into the circulation during infections to suppress inflammation. cMoPs exposed to mycobacteria accumulate cholesterol and lipids, inducing the formation of MGCs against mycobacteria. TpMos are mobilized into circulation and infiltrate tissues during bacterial infections to generate proliferating macrophages with attenuated inflammatory response and apoptotic resistance, improving sepsis survival.

cells that occur *in vivo*. A pertinent question of the M1-M2 phenotype switch remains: is it the same cell that switches from a pro-inflammatory M1 to an anti-inflammatory M2 phenotype through the course of inflammation? In response to this question, it is increasingly clear that recent work combining fate-mapping, single-cell transcriptomics and epigenetics have revealed that macrophages are less plastic than we thought [152]. In particular, Guilliams and Svedberg have proposed that prolonged tissue residency restricts the plasticity of macrophages in order to allow steady-state tissues to imprint a consistent transcriptomic identity on different types of progenitors [152]. Consequently, this process includes silencing of inflammatory signatures on incoming monocytes, in order to safeguard tissue homeostasis [152]. Indeed, it is now evident that TRMs often undergo cellular death upon exposure to inflammatory signals [120–124] and even if they do survive, these cells clearly carry out non-inflammatory roles compared to infiltrating monocyte-derived cells [15], suggesting that an M1 to M2 switch in TRMs would rarely occur *in vivo*.

Switching of monocyte-derived cell phenotypes *in vivo*

In contrast to TRMs, monocytes that infiltrate during inflammation are imprinted with both tissue and inflammation signatures and may disappear upon cellular death or convert into cells that represent TRMs upon tissue resolution [152]. Studies that have utilized fate-mapping and adoptive transfer experiments have indicated that the same monocyte-derived cell may undergo a phenotypic switch under certain circumstances [153]. Specifically, Chen *et al.* utilized a *Ccr2*-driven fate-mapping strategy with tamoxifen-inducible Cre-loxP recombination in a stroke model and found that monocyte-derived cells gradually lost their inflammatory signature and increase their restorative phenotype [154]. While the mechanism for this is unclear, Arnold *et al.* have labelled circulating monocytes with fluorescent latex beads in a model of skeletal muscle injury and discovered that these monocytes differentiated into inflammatory macrophages that switched into a restorative phenotype only upon phagocytosis of apoptotic cells from muscle cell debris [78]. These findings suggest that monocyte-derived cells may require certain specific cues for them to switch their phenotypes. Furthermore, in a recent study by Kratofil *et al.* that utilized a similar *Ccr2*-driven fate-mapping approach to Chen *et al.*, monocytes were not found to contribute towards bacterial clearance but instead converted to ghrelin-producing monocyte-derived macrophages that were important for would healing and persisted for weeks after infection [155]. Since the expression of ghrelin was only detected 14 days post-infection in their study, whether ghrelin expression is restricted only to monocytes that have differentiated into a restorative macrophage phenotype and if its expression is absent in inflammatory monocytes would be an intriguing avenue for further research. Furthermore, it is likely that the avoidance of anti-bacterial functions by these monocytes preserves their viability by avoiding phagocytosis-induced cell death [131], which allows these cells to differentiate and persist in the environment. Since neutrophils were the main immune cells involved in bacterial clearance [155], it would be interesting to investigate the impact of neutrophil depletion on monocyte differentiation and its subsequent phenotypes. In light of recent literature that documents distinct BM-imprinted monocytes upon inflammation and mobilization of BM monocyte precursors [20, 29, 33, 133, 140], it also remains unclear if these cell types may give rise to

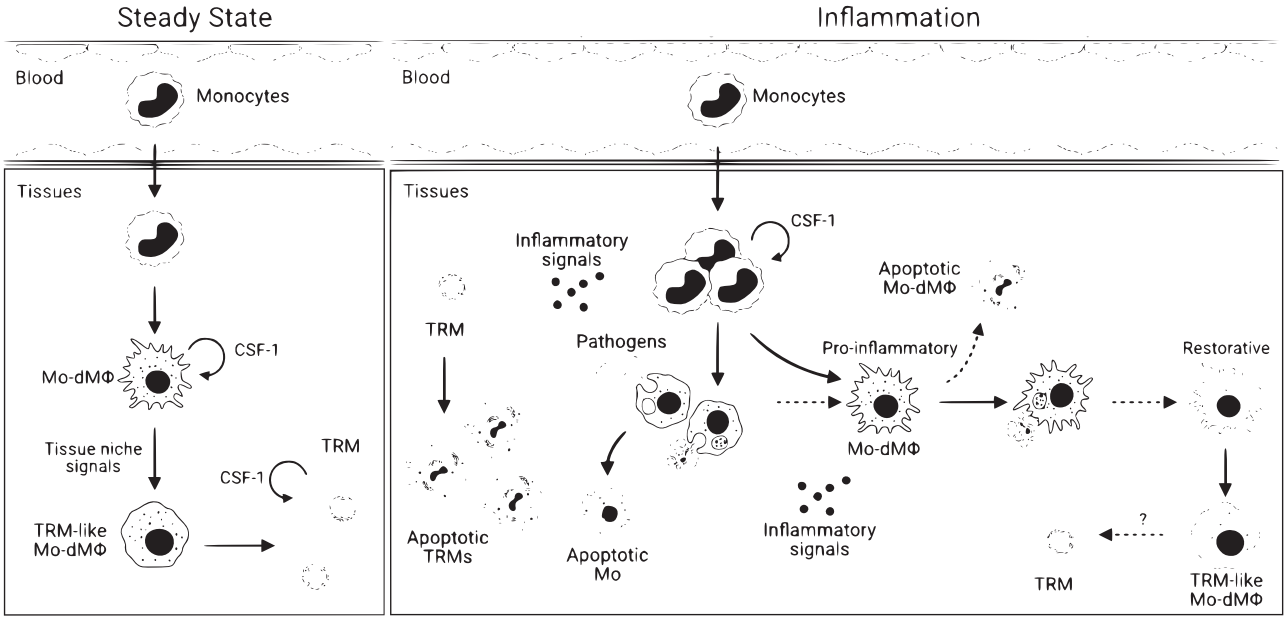


Figure 4: Monocyte-derived cells undergo distinct transitional states during tissue inflammation. During the steady state, Ly6C^{hi} monocytes infiltrate tissues like the heart, pancreas, intestines, and dermis, producing monocyte-derived macrophages (Mo-dMΦs) that resemble TRMs. CSF-1 regulates Mo-dMΦ and TRM proliferation, survival and numbers in the tissue niche. During inflammation, pathogens and inflammatory mediators trigger the death of TRMs. Ly6C^{hi} monocytes, and Ly6C^{lo} monocytes to some extent, infiltrate inflamed tissues and proliferate through CSF-1. They either execute effector functions and undergo phagocytosis-induced cell death, or differentiate into pro-inflammatory Mo-dMΦs. Mo-dMΦs that manage to survive inflammatory signals and phagocytose apoptotic debris consequently adopt a restorative phenotype. Restorative Mo-dMΦs could persist weeks after inflammation and gradually transform into TRMs.

different macrophages with inflammatory and anti-inflammatory properties over time. Taken together, the persistence of monocyte-derived cells and their ability to undergo a phenotypic switch over the course of disease relies on a combination of tissue signals (Fig. 4) and future studies combining spatiotemporal methods, fate-mapping, transcriptomic, and epigenetic analysis will provide further insight into these mechanisms.

Conclusion

Monocytes are unique cells that can function both as a precursor and effector cell in the periphery. It is now increasingly clear that these cells embark on different fates based on cues from distinct environmental signals. In particular, recent advances and tools that allow us to tease out nuance signals have highlighted how basic environmental factors such as neuronal signals and diet have a much larger impact on monocyte development and function than we would have expected. It is anticipated that the advent of improved technological methods in the future would allow us to converge all these datasets into one unity in order to appreciate the immense depth of plasticity they are capable of and identify coordinating mechanisms between these signals under disease conditions. While emerging evidence suggests that monocyte plasticity begins way earlier than expected, many questions remain for future investigation. In particular, the signals that allow monocyte phenotypes to persist or change with differentiation in the tissue remain unclear. Furthermore, what factors allow some monocyte-derived cells to persist for long periods after tissue resolution while some conditions prevent the engraftment of these cells into the macrophage pool? Given the rapid advancement of research tools that are increasingly available for answering these questions, a systems immunology and in-depth comprehension of monocyte biology should provide important insight for the future development of novel therapeutic strategies.

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Ethical approval

Ethical approval is not applicable to this article as no human or animal research was performed for data generation in this paper.

Conflicts of interest

The authors declare no conflict of interest.

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Data availability

Data sharing is not applicable to this article as no datasets were generated or analysed in this paper.

Author contributions

Writing, Y.C.T, M.Y.C, and S.Z.C; Visualization, Y.C.T., and S.Z.C.; Funding Acquisition, S.Z.C.; Supervision, S.Z.C.

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Clinical trial registration

The clinical trial registration is not applicable to this article as no clinical trial was carried out in this paper.

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