Developmental and functional heterogeneity of monocytes

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Running Title: Monocyte heterogeneity

Summary

Novel experimental approaches such as fate-mapping and single-cell analysis have brought fresh insight into monocyte development and function over the past decade, and redefined the monocyte field. Monocytes are now known to consist of multiple subsets generated through distinct developmental pathways with diverse functional specializations. Their fates under homeostatic conditions include the accumulation in peripheral reservoirs and the engraftment into certain resident macrophage pools. Under pathological conditions, monocytes acquire inflammatory effector functions, but can also develop regulatory properties essential for tissue repair. Importantly, monocytes recruited during inflammation are often functionally distinct from resident macrophages or conventional dendritic cells. Here we outline emerging concepts in monocyte heterogeneity, emergency monopoiesis and trained immunity and discuss how these bring new perspectives to monocyte research.

Introduction

Monocytes make up one component of the "mononuclear phagocyte system" (MPS), which they share with macrophages and conventional dendritic cells (cDC) (Guilliams et al., 2014). Monocytes are defined as circulating blood cells that constitute ~10% of peripheral leukocytes in humans and ~4% in mice. Blood monocytes are thought to develop in the adult bone marrow (BM) from a dividing common myeloid progenitor (CMP) shared with erythrocytes, platelets, cDC and granulocytes. Following their generation, monocytes are released into the peripheral circulation. Historically, monocytes were thought to represent a bridge linking BM precursors via a circulating stage with terminally differentiated macrophages and cDC in the tissue. **However, today we appreciate that in most tissues the majority of resident macrophages - but not all - have an embryonic origin, while cDC derive from distinct BM precursors (Ginhoux and Guilliams, 2016; Mildner and Jung, 2014). Therefore, monocytes have freed themselves from their exclusive image as immature siblings of macrophages or cDC. Indeed, monocytes recruited during inflammation give rise to monocyte-derived cells that are often functionally distinct from resident macrophages and cDC. Moreover, circulating monocytes consist of distinct subsets that already possess functional properties before reaching the inflamed tissues. A leading characteristic that distinguishes monocytes from other members of the MPS is that they are poised to be rapidly mobilized in large numbers to inflamed sites throughout the body, where they serve as a particularly plastic "emergency squad" to provide pro-inflammatory or resolving activities. These diverse effector functions can be further shaped in part by micro-environmental and spatial cues. Here we discuss new concepts and perspectives in monocyte research including monocyte heterogeneity, emergency monopoiesis and trained immunity.**

Monocytes lost in history

The earliest accounts of monocytes are closely related with the introduction of the microscope in the mid-nineteenth century (**Fig. 1**). Whilst still a medical student, Paul Ehrlich was developing cytological stains. Using aniline and neutral dyes in combination with nuclear morphology, he classified white blood cells into mononucleated leukocytes, some of which he termed large mononuclears with kidney shaped nuclei or so called Übergangszelle (*transitional cells*; now known as monocytes) and polymorphonuclear cells with neutrophilic (neutrophils), acidophilic (eosinophils) or basophilic granules (basophils) (Ehrlich, 1880). In 1908, Ehrlich shared the Nobel Prize in Physiology or Medicine with Ilya Metchnikoff "in recognition of their work on immunity". Metchnikoff was at this time establishing his theory of phagocytosis, giving rise to the concept of cellular innate immunity (Metchnikoff, 1887; Metchnikoff, 1892; Yona and Gordon, 2015). The term 'monocyte' was introduced -to the best of our knowledge- by Artur

Pappenheim in 1910 (Pappenheim and Ferrata, 1910). Pappenheims goal at this time was to unify a wide variety of previously described cell types into one major subgroup – the large monocyte. Over the subsequent years, the definition of monocytes became more stringent and exclusively applied to Ehrlich's *transitional cells* (Forkner, 1930; Klein, 1914; Naegeli, 1923; Sabin et al., 1925).

In 1914, Awrorow and Timofejewskij conducted the first *in vitro* cell culture experiment of human leukocytes and concluded that lymphocytes are the progenitors from which macrophages arise (Awrorow and Timofejewskij, 1914). Awrorow also observed that *in vitro* derived macrophages do not form a homogenous population, but instead vary in morphology and function, depending on the culture condition and thereby foreseeing monocyte plasticity (Awrorow and Timofejewskij, 1914). The first *in vivo* study to observe the migratory behavior of mammalian blood monocytes and differentiating into macrophages during an acute insult was performed by Ebert and Florey, using the rabbit ear chamber (Ebert and Florey, 1939). This rapid appearance of inflammatory macrophages led to the conclusion that the majority of these cells derive from circulating monocytes rather than being descendants of resident macrophage proliferation (Leder, 1967).

By the late 1960's Ralph van Furth, James Hirsch, Zanvil Cohn and colleagues introduced the term MPS established on "*similarities in morphology, function, origin and kinetics of phagocytes*" (van Furth et al., 1972). They concluded that BM pro-monocytes act as precursors to circulating monocytes, which can differentiate into macrophages in tissues. Importantly, macrophages were then described as either free or fixed. Fixed macrophages were defined, as "*probably of monocyte origin, but definitive proof has not been obtained…the morphology and functional behaviour…justify their inclusion in the MPS*". The concept that fixed macrophages would be of monocyte origin was then incorporated as textbook knowledge and the cautionary note forgotten. Subsequent technological advances permitted the experimenter to examine the relationship and turnover of monocytes and macrophages. Tissue-resident macrophages were characterized by longevity (Bouwens et al., 1986; Melnicoff et al., 1988). Furthermore, ^{89}Sr-induced monocytopenia in animals did not affect normal tissue-resident macrophage numbers (Naito and Takahashi, 1991; Oghiso et al., 1988; Sawyer et al., 1982; Yamada et al., 1990). Moreover, histological approaches demonstrated the appearance of macrophages during embryonic development before the establishment of definitive haematopoiesis (Mizoguchi et al., 1992; Naito and Takahashi, 1991; Sorokin and Hoyt, 1992). These results suggested that certain tissue-resident macrophages are independent of monocyte influx and show a self-renewal capacity, thereby posing the question: what exactly are the functions of monocytes?

Monocyte subsets

Ehrlich's *transitional cell* was described as a homogenous cell population with a kidney shaped nucleus. However, the advent of flow cytometry had an unrivalled impact on immunology and enabled the identification of monocyte heterogeneity in various species (Ziegler-Heitbrock, 2014), including humans (Passlick et al., 1989), mice (Geissmann et al., 2003), rats (Ahuja et al., 1995), pigs (Sanchez et al., 1999) and monkeys (Kim et al., 2010). Discrete populations of monocytes were first identified by morphology and differential expression of CD14 and CD16 in humans (Passlick et al., 1989), representing a milestone in monocyte biology. The combination of CD14 and CD16 on HLA-DR$^+$ cells enabled the classification of three principal human monocyte subsets: CD14$^+$CD16$^-$ monocytes, also referred to as 'classical' monocytes, make up 80-90% of the monocyte pool with the remaining 10-20% shared by CD14$^+$CD16$^+$ intermediate and CD14^{Low}CD16$^+$ 'non-classical' monocytes. A major breakthrough in the characterization of mouse monocytes arose with the generation of a mouse strain wherein a reporter protein was engineered into the *Cx3cr1* locus (CX$_3$CR1^{GFP} mice) (Jung et al., 2000), which led to the identification of two distinct CD11b$^+$CD115$^+$ monocyte subsets (Geissmann et al., 2003). In mice 'classical' monocytes are characterized by the surface marker combination Ly6C^{Hi}CX$_3$CR1^{int}CCR2$^+$CD62L$^+$CD43^{Low} **(previously termed inflammatory monocytes),** while 'non-classical' monocytes (also termed patrolling monocytes) are defined as Ly6C^{Low}CX$_3$CR1^{Hi}CCR2^{Low}CD62L$^-$CD43$^+$ (Geissmann et al., 2003; Jakubzick et al., 2013; Jung et al., 2000; Palframan et al., 2001). Transcriptional comparison between mouse and human monocytes correlated Ly6C^{Hi} monocytes with 'classical' CD14$^+$CD16$^-$ monocytes and Ly6C^{Low} monocytes with 'non-classical' CD14^{Low}CD16$^+$ monocytes, even though certain gene expression **and surface marker** differences exist. **For instance, human monocytes can be identified by their HLA-DR expression, while only a fraction of murine monocytes express MHCII.** Furthermore, mouse monocytes, but not their human equivalent, are characterized by the existence of a peroxisome proliferator-activated receptor γ (PPARγ) signature and exhibit differences in genes involved in phagocytic activity (Cros et al., 2010; Ingersoll et al., 2010).

The ratio between classical and non-classical monocytes varies between species. In humans the ratio is in favour of classical monocytes while in mouse both subsets are represented equally. However, as discussed below, recent unbiased single-cell RNA sequencing studies have broadened the heterogeneity within the intermediate population with the identification of several subsets in humans (Villani et al., 2017) and mice (Mildner et al., 2017). Their function and ontogeny **are** currently unclear.

Development of classical monocytes

Over a century ago Alexander Maximow postulated that haematopoiesis would be a highly ordered process arising from common precursors that become progressively more restricted ultimately producing diverse populations of blood cells (Maximow, 1907). This tree-like

hierarchy is modelled on the assumption of a canonical development, where a set of oligo-, bi- and unipotent progenitor cells become progressively more restricted and eventually give rise through branching decisions to all forms of circulating blood cells (**Fig. 2a**). In this model, a fraction of active haematopoietic stem cells (HSC (Busch et al., 2015)) have the ability to self-renew and generate a heterogeneous population of multipotent progenitors (MPP; (Pietras et al., 2015)) that further differentiate into two separate lineages, becoming either common myeloid progenitors (CMP; (Akashi et al., 2000)) or common lymphoid progenitors (CLP; (Kondo et al., 1997)). CLP give rise to lymphoid cells including T, B and natural killer cells but lack the potential to differentiate into the myeloerythroid lineage, while CMP have lost their lymphoid lineage capacity, but are instead able to differentiate into megakaryocyte/erythrocyte progenitor (MEP) and granulocyte/macrophage progenitors (GMP) (Akashi et al., 2000). A monocyte/DC progenitor (MDP) was subsequently identified within, and probably derived from, the GMP population. **This MDP population** was proposed to give rise to monocytes and cDC but not neutrophils in mice (Fogg et al., 2006; Olsson et al., 2016) and humans (Lee et al., 2015). However, the murine MDP population was also identified by flow cytometry within the CMP compartment and might directly emerge from Flt3-expressing CMP and not exclusively from GMP (Yanez et al., 2017). **Consequently, recent adoptive transfer experiments suggest that Ly6C^{Hi} monocytes can emerge from the GMP as well as from the MDP population (Yanez et al., 2017).** The MDP population is thought to make a binary decision to either divide into dedicated common DC precursors (CDP) or into the unipotent common monocyte progenitors (cMoP), even though it's likely that parts of the MDP population are already pre-committed into either of the two lineages (**Fig. 2b**). Adoptively transferred cMoPs gave rise to Ly6C^{Hi} and – in a time delayed fashion – to Ly6C^{Low} monocytes (Hettinger et al., 2013). cMoP have also been identified in human BM, where they were found amongst the GMP population (Kawamura et al., 2017). However, this tree-like hierarchal development is based on the isolation of fluorescent-activated cell sorting (FACS)-purified populations using limited predefined membrane markers, followed by *in vitro* colony formation experiments or adoptive transfer approaches. Although these assays have been valuable to determine lineage potential, cell fate under physiological conditions *in vivo* is most likely more complex. Indeed, advances in single-cell RNA sequencing technology has questioned the classical branching nature of haematopoietic differentiation and show that lineage commitment is already evident in supposedly oligopotent progenitors (Notta et al., 2016; Perie and Duffy, 2016) (**Fig. 2b**). Furthermore, unipotent progenitors can emerge directly from HSC without the necessity to develop sequentially through defined intermediate bipotent precursor stages (Naik et al., 2013; Velten et al., 2017), which can be in part mediated by direct contact of HSC and lineage determining cytokines such as CSF1 (Mossadegh-Keller et al., 2013). Unbiased single-cell RNA sequencing combined with indexed FACS-sorting of early myeloid precursor subsets identified cells with a monocytic transcriptional program within the

CMP as well as GMP population (Olsson et al., 2016; Paul et al., 2015), indicating the limitation of population analysis based on limited membrane markers. **However, the insufficient depth of sequencing in single-cell RNA transcriptome analysis might mask other lineage potentials, which are still biological relevant and thereby lead to premature conclusions with respect to lineage commitment. Indeed, single cell liquid cultures of various precursor stages in *in vitro* methylcellulose colony-formation assays, to measure clonal outcome, showed the existence of multipotent precursors cells (Tusi et al., 2018), even though only a limited set of cytokines and cytokine combinations were used and particular cellular-derived niche signals are absent in this experimental setup. Therefore, it is possible that precursor populations consist of a mixture of multi- or at least bi-potent as well as lineage pre-committed precursor cells (indicated in Fig. 2b by grey and lightly coloured cells, respectively).**

The commitment towards the monocyte lineage is maintained by a distinct group of haematopoietic growth and transcription factors (**Fig. 2a**). The latter includes SPI1 (encoding PU.1), IRF8, GATA2 and KLF4, as deletion of these factors perturbs BM monocytic precursor composition, resulting in reduced peripheral monocyte numbers (Alder et al., 2008; Bigley et al., 2011; Feinberg et al., 2007; Hambleton et al., 2011; Kurotaki et al., 2013; McKercher et al., 1996; Scott et al., 2016a; Sichien et al., 2016). However, IRF8 and KLF4 seem to create a regulatory circuit, since ectopic overexpression of KLF4 in *Irf8*-deficient cells partially rescues monocyte differentiation (Kurotaki et al., 2013). Furthermore, an autosomal recessive IRF8 deficiency in humans leads to reduced numbers of circulating monocytes (Hambleton et al., 2011). Also, BACH2 has been shown to contribute as a negative regulator of monopoiesis with *Bach2*-deficient mice possessing increased monocytes (Kurotaki et al., 2018). Importantly, the single-cell approach (**Fig. 2b**) provides a different tactic to study the role of transcription factors during haematopoiesis. A precursor may appear unaffected by a given mutation based on the restricted number of surface markers used for the flow cytometry identification of this precursor (**Fig. 2a**), while single-cell analysis may reveal that the mutation affects a pre-committed precursor within this pool of cells or even further upstream in the developmental tree. The MDP seems for example not strongly affected by loss of *Irf8* (Sichien et al., 2016). However, since the MDP **population** possibly contains committed monocyte- and cDC1/cDC2-precursors, it may well be that the MDP population in *Irf8*-deficient mice only consist of cDC2-committed precursors and may in fact comprise of different pool of cells as compared to **the wild-type MDP population**.

Investigations into cMoP biology has uncovered that this population possess a high proliferative capacity (Chong et al., 2016; Hettinger et al., 2013; Kawamura et al., 2017) and is characterized by Ly6C expression in mice and CD14 expression in humans (Chong et al., 2016; Hettinger et al., 2013). Further differentiation of cMoP into mature monocytes involves a transient pre-

monocyte stage, discriminated by the expression of CXCR4. Proliferative CXCR4^{+} pre-monocytes differentiate in the BM within 24 hours into Ly6C^{Hi} CXCR4^{-} monocytes, which is accompanied by the up-regulation of CCR2 (Chong et al., 2016). Therefore, CXCR4 itself is not involved in BM egression, rather CCR2 regulates BM exit of murine Ly6C^{Hi} monocytes (Serbina and Pamer, 2006). It was observed that Ly6C^{Hi} monocytes in CCR2-deficient mice become trapped within the BM, while the number of peripheral Ly6C^{Hi} monocytes are dramatically reduced (Serbina and Pamer, 2006). **Consequently, the CCR2 ligands CCL2 and CCL7, but not CCL12, are important for Ly6C^{Hi} monocyte egression from the BM, since deficiencies in these genes leads to reduced levels of circulating Ly6C^{Hi} monocytes in the circulation (Tsou et al., 2007). Under mild inflammatory conditions mesenchymal stem cells and their progeny, including CXCL12-abundant reticular cells, represent a major source of CCL2 production in the BM (Shi and Pamer, 2011), although the situation might differ under healthy conditions. Whether CCR2 also plays a role in monocyte egression in humans is unclear. Interestingly, Ly6C^{Hi} monocyte egression from the BM underlies a circadian rhythmic oscillation (Nguyen et al., 2013), which is in part CXCR4-dependent. CXCR4 also participates in the homing of classical and non-classical monocytes to central (BM) and peripheral (spleen) monocyte reservoirs (Chong et al., 2016).**

Fate of classical monocytes in healthy homeostasis

Classical monocytes represent a transient cell population with a diverse differentiation potential. In contrast to non-classical monocytes, classical monocytes are equipped with a gene-expression program that enables them to migrate into tissues under homeostatic conditions. Once classical monocytes are released into the circulation from the BM under healthy homeostasis, they remain in the circulation for approximately a day, before they either traffic to repopulate a proportion of tissue-resident macrophages in the intestine (Bain et al., 2014; Bain et al., 2013; Tamoutounour et al., 2012; Zigmond et al., 2012), dermis (Tamoutounour et al., 2013), heart (Epelman et al., 2014; Molawi et al., 2014), pancreas (Calderon et al., 2015), lung (Jakubzick et al., 2013) and testis (Mossadegh-Keller et al., 2017), or alternatively convert into non-classical monocytes (Patel et al., 2017; Yona et al., 2013). **However, classification of tissues based on the presence or absence of adult monocyte-derived macrophages is becoming increasingly complex and might vary according to age, gender and genetic background. The intestine was once considered the prototypical example of a tissue in which macrophages are short-lived and require continuous recruitment of monocytes to sustain their pool. However, the intestine also harbours TIM4^{+}CD4^{+} macrophages that self-maintain for months (Shaw et al., 2018). Importantly, these self-maintaining macrophages are required for intestinal homeostasis as depletion of these cells led to**

reduced enteric neurons, vascular leakage and delayed intestinal motility (De Schepper et al., 2018).

Under physiological conditions extravasated Ly6C^{Hi} monocytes and their descendants can be found in almost all tissues throughout the body, where they constitute **a minor yet significant fraction of** the local tissue-resident macrophage pools (Sawai et al., 2016). Striking exceptions of tissues in which little or no monocyte engraftment have been observed include the epidermis (Chorro et al., 2009; Merad et al., 2002), the central nervous system (Ajami et al., 2007; Ginhoux et al., 2010; Mildner et al., 2007) and the alveolar space (Guilliams et al., 2013; Hashimoto et al., 2013; Yona et al., 2013). These organs are probably spared from adult monocyte infiltrates due to the combination of high self-renewal potential of the tissue-resident macrophages with the restricted access to these locations for monocytes because of existing blood–brain and epithelial barriers. The appearance of monocyte-derived macrophages in tissues is accompanied by the gradual replacement of embryonic macrophages in either a quantitative fashion close to birth (Bain et al., 2014) or gradually progressing over time (Hoeffel et al., 2015; Molawi et al., 2014; Sawai et al., 2016; Schulz et al., 2012). Monocyte-derived macrophages show significant gene modifications compared to their circulating equivalent as they adapt to the local tissue environments, and acquire transcriptomic signatures that show similarities to resident macrophages of embryonic origin, even though epigenetic, transcriptional and functional differences remain (Cronk et al., 2018; Lavin et al., 2014; Scott et al., 2016b; T'Jonck et al., 2018; van de Laar et al., 2016). **These newly recruited monocyte-derived macrophages have been shown to adopt certain functions of the tissue-resident macrophages they are replacing.** Whether monocyte-derived macrophages that engraft in steady-state tissues acquire self-maintenance capacity comparable to their embryonic counterparts is debated and seems to depend on the tissue. In contrast, monocyte-derived macrophages that engraft into macrophage pools during inflammation show distinct gene signatures and often fail to self-maintain for prolonged periods (see below). It is possible that low-grade tonic inflammation is required for the continuous recruitment of monocytes into tissues, since germ-free mice show reduced numbers of monocyte-derived intestinal macrophages (Bain et al., 2014). Furthermore, continuous physical micro-trauma induced by muscle contraction in the heart could hinder survival of embryo-derived cardiac macrophages, thereby creating a niche for monocyte-derived cells (Molawi et al., 2014). A vacant cellular niche can also be induced experimentally by targeted genetic mutations (for instance *Csf1r*) or irradiation in combination with gene deletion that influence the survival/proliferation of embryonic macrophages, while transferred, gene-proficient BM cells or monocytes are spared of these effects and can successfully compete against the affected embryonic-derived population (Bennett et al., 2018; Cronk et al., 2018; Varol et al., 2017)**. Even though adult monocytes can adopt a tissue-resident macrophage**

signature, it appears that they retain a degree of their monocyte identity (e.g. *Ms4a7* expression; Bennett et al., 2018) and might respond differently during inflammation (Cronk et al., 2018).

On the other hand, Ly6C^{Hi} monocytes can maintain their monocyte-like state within tissues without differentiation into macrophages. These migrated Ly6C^{Hi} monocytes act as a local monocyte reservoir and show minimal transcriptional changes (Swirski et al., 2009).

An alternative maturation route for Ly6C^{Hi} monocytes, rather than leaving the circulation to develop into specific tissue monocyte-derived cells or to join the monocyte reservoirs, is the transition into Ly6C^{Low} monocytes. This is associated with the establishment of *de novo* enhancers and activation of poised enhancers that facilitate the transition into non-classical Ly6C^{Low} monocytes (Mildner et al., 2017; Polletti and Natoli, 2017). Mathematical modelling of the conversion rate of classical to non-classical monocytes based on pulse labelling decay in comparison to the extravasation rate into tissues indicates that vast majority of classical monocytes are recruited to peripheral tissues where they potentially differentiate into monocyte-derived cells or enter reservoirs of undifferentiated monocytes (Patel et al., 2017; Tak et al., 2017a). Interesting questions arise from these data. **Do tissue infiltrated Ly6C^{Hi} monocytes under physiological conditions sometimes convert to Ly6C^{Low} monocytes and re-enter the circulation, or do these cells always differentiate into macrophages? If the latter is the case, how is the default transition program from Ly6C^{Hi} to Ly6C^{Low} monocytes prevented to allow alternative macrophage development and how do Ly6C^{Hi}-derived macrophages transcriptionally relate to Ly6C^{Low} monocytes?** Is there a signal required to maintain monocytes in an undifferentiated stage in reservoirs?

The plastic**ity** of Ly6C^{Hi} monocytes ignites different scenarios. It is conceivable that Ly6C^{Hi} monocytes may represent a heterogeneous cell population with some cells primed for tissue infiltration and others primed for the transition into Ly6C^{Low} monocytes. Theoretically, the distinct circulating Ly6C^{Hi} monocyte subsets could even originate from different precursors. **However, single-cell RNA sequencing of murine monocytes suggests that circulating Ly6C^{Hi} and Ly6C^{Low} monocytes represent relatively homogenous populations under physiological conditions (Mildner et al., 2017), even though a higher sequencing depth and the analysis of a greater number of monocytes might uncover additional monocyte subsets. For example we do not exclude that conventional bleeding regimes might fail to retrieve some particular subsets of Ly6C^{Low} patrolling monocytes that might strongly adhere to the endothelium. In fact, a novel BM Ly6C^{Hi} monocyte subset with neutrophil-like gene expression characteristics was recently identified (Yanez et al., 2017), but whether these cells circulate under homeostatic situations needs to be clarified.**

Interestingly, the Ly6C/CD14-intermediate monocytes exhibit heterogeneity in both mice and humans (Mildner et al., 2017; Villani et al., 2017). Murine Ly6C^{int} monocytes consists of two

populations, one of which is characterized by CD209a (DC-SIGN) and MHCII expression, while the other can be interpreted as the "true" intermediate monocyte subset, since they represent a transcriptional landscape linking classical Ly6C^{Hi} to non-classical Ly6C^{Low} monocytes (Mildner et al., 2017). While CD209a^{+}MHCII^{+}Ly6C$^{Hi\text{-}to\text{-}int}$ monocytes are proposed to give rise to monocyte-derived DC under pathological conditions (Menezes et al., 2016), the origin of these cells is currently uncertain, even though these cells likely develop independently of C/EBPβ (Mildner et al., 2017). Human CD14^{+}CD16^{+} monocytes likewise show heterogeneity (Villani et al., 2017) **and are characterized in general by higher HLA gene family expression** (Gren et al., 2015; Patel and Yona, 2018; Patel et al., 2017; Schmidl et al., 2014; Zawada et al., 2011). For certain newly identified monocyte subsets we are still looking for the homologous equivalent in mice and men. One of the newly identified human monocyte subsets characterized by a cytotoxic gene signature was for example absent in murine monocyte populations (Villani et al., 2017). Taken together, Ly6C^{Hi} monocytes represent a particularly versatile biological system that even during homeostasis, provides certain tissues, including the circulation system, with monocyte-derived cells that acquire incredibly diverse functions dependent on their tissue of residence.

Development of non-classical monocytes

Classical monocytes have a relatively short lifespan in circulation of approximately one day; non-classical monocytes display a longer lifespan of around 2 days in mice and 7 days in humans, respectively (Patel et al., 2017; Yona et al., 2013). This can be indirectly observed in parabiotic mice, in which two mice are surgically joined and share a common circulation. In this system, donor-derived Ly6C^{Hi} monocytes do not reach equilibrium with their host Ly6C^{Hi} monocyte counterparts, in a similar manner to short-lived neutrophils, while lymphocytes and Ly6C^{Low} monocytes do (Liu et al., 2007). The lifespan of Ly6C^{Low} monocytes can be extended to 2 weeks, which is likely dependent upon CSF1 availability (Yona et al., 2013), a critical factor controlling monocyte/macrophage maintenance (Bartocci et al., 1987; Elmore et al., 2014). Possibly due to the increased lifespan of Ly6C^{Low} monocytes compared to their Ly6C^{Hi} counterparts, these cells are more susceptible to anti-CSF1R treatment or tamoxifen-induced deletion of the *Csf1r* gene. In both cases, the number of circulating Ly6C^{Low} monocytes decrease, while Ly6C^{Hi} monocyte numbers remain unaffected (Greter et al., 2012; Hoeffel et al., 2015; Hoeffel and Ginhoux, 2018; MacDonald et al., 2010; Mrdjen et al., 2018). The flexibility in increasing the lifespan of non-classical monocytes might secure their constant cell numbers even under pathological conditions, when the majority of classical monocytes are recruited to peripheral inflammatory lesions or when their functional transition into non-classical monocytes is blocked, e.g. due to inflammatory stimuli. This might also explain why a significant amount of non-classical monocytes remain in *Ccr2*-deficient mice, since only a minor fraction of Ly6C^{Hi} monocytes is required to replenish a long-lived population (Yona et al., 2013). Alternatively, Ly6C^{Hi}

monocytes trapped in the BM of *Ccr2*-deficient mice may convert locally into non-classical monocytes and exit the BM in a CCR2-independent manner. Interestingly, in wild-type/CCR2$^{-/-}$ mixed BM chimeras, where half the reconstituted BM is of wild-type origin and the other half of CCR2$^{-/-}$, circulating Ly6C^{Low} monocytes show the same chimerism as Ly6C^{Hi} monocytes and derive mainly from wild-type cells (Yona et al., 2013), indicating that Ly6C^{Low} monocyte are also partially dependent on CCR2. Additional factors influencing Ly6C^{Low} monocyte homeostatic survival include CX_3CL1 (Landsman et al., 2009) and TNF (Wolf et al., 2017).

The transition of classical monocytes to non-classical monocytes has been observed by the ablation of circulating monocytes using clodronate-loaded liposomes, cell transfer and pulse labelling experiments in different species including the mouse (Sunderkotter et al., 2004; Tacke et al., 2006; Varol et al., 2007; Yona et al., 2013), rat (Yrlid et al., 2006), macaque (Sugimoto et al., 2015) as well as in humans (Patel et al., 2017) and therefore presents an evolutionary conserved program. However, this does not exclude that some cells in the non-classical monocyte pool may develop without passing through a classical monocyte stage (Carlin et al., 2013).
The mechanisms driving the conversion of Ly6C^{Hi} monocytes into Ly6C^{Low} monocytes are now better understood. Delta-like 1 (Dll1) signals from endothelial cells that interact with NOTCH2 on Ly6C^{Hi} monocytes facilitates the conversion into Ly6C^{Low} cells (Gamrekelashvili et al., 2016). These data indicate that Ly6C^{Hi} and Ly6C^{Low} monocytes are biologically interconnected, a fact that is also observed on the epigenetic level since both monocyte subsets take advantage of the same promoter repertoire and only show little differences in chromatin organisation (Mildner et al., 2017). These differences are due to the establishment of *de novo* enhancers and activation of enhancers by histone acetylation (Mildner et al., 2017; Thomas et al., 2016).
On the molecular level, the conversion from classical to non-classical monocytes is accompanied by up-regulation of C/EBPβ, NR4A1 and KLF2. **Absence of NR4A1 leads to reduced Ly6C^{Low} monocyte survival and numbers (Hanna et al., 2011). Further epigenetic experiments identified one particular *Nr4a1* enhancer (E2) specific to monocytes and deletion of this enhancer leads to the absence of Ly6C^{Low} monocytes (Thomas et al., 2016). KLF2 and C/EBPβ bind to this E2 enhancer to induce *Nr4a1* expression. Subsequently, both genes were shown to be involved in Ly6C^{Low} monocyte generation (Hanna et al., 2011; Mildner et al., 2017; Tamura et al., 2017; Thomas et al., 2016).** *Csf1r* is also regulated by C/EBPβ and therefore it is likely that the absence of Ly6C^{Low} monocytes in C/EBPβ deficient mice is in part due to their inefficiency to up-regulate *Csf1r* (Mildner et al., 2017; Tamura et al., 2017). Although it remains to be experimentally demonstrated that human monocytes follow a similar molecular program during transition, *in silico* transcriptional network analysis strongly supports C/EBPβ as a central player in human monocyte biology (Schmidl et al., 2014). Moreover, C/EBPβ as well

as NR4A1 motif enrichment are evident in human monocytes by single-cell ATAC sequencing (Buenrostro et al., 2018).

The proportion of circulating classical and non-classical monocytes varies dependent on monopoiesis, tissue infiltration and their release from central (BM) or peripheral reservoirs. For instance, mice treated with the bacterial peptidoglycan muramyl dipeptide show increased conversion of Ly6C^{Hi} monocytes into Ly6C^{Low} cells in a NOD2-dependent mechanism (Lessard et al., 2017), while low concentrations of Toll-like receptor (TLR) ligands in the bloodstream of mice drive CCR2-dependent emigration of monocytes from the BM to the circulation and thereby lead to increased circulating classical Ly6C^{Hi} monocyte numbers (Shi and Pamer, 2011). In humans endotoxin induces a rapid yet transient monocytopenia during the first two hours after lipopolysaccharide (LPS) injection followed by the sequential reappearance of CD14^{+}CD16^{-} classical monocytes, followed by CD14^{+}CD16^{+} intermediate cells and finally CD14^{Low}CD16^{+} non-classical monocytes (Patel et al., 2017; Tak et al., 2017b; Thaler et al., 2016). Exercise and age can also influence the equilibrium of monocyte subsets (Heimbeck et al., 2010; Seidler et al., 2010; Verschoor et al., 2014). The number of non-classical monocytes is thus strongly linked to the physiological status of the organism and therefore represents a potential diagnostic tool (Selimoglu-Buet et al., 2015).

The function of non-classical monocytes is emerging: intravital microscopy studies have revealed that Ly6C^{Low} monocytes continuously monitor the vasculature under physiological conditions through an LFA/ICAM-dependent crawling mechanism on resting endothelial cells (Auffray et al., 2007; Carlin et al., 2013). This patrolling behaviour of Ly6C^{Low} monocytes can be observed in capillaries, arterioles and venules. Similarly, human CD14^{Low} CD16^{+} non-classical monocytes show patrolling behavior when adoptively transferred into immuno-compromised mice (Cros et al., 2010). This crawling characteristic permits Ly6C^{Low} monocytes to efficiently scavenge luminal microparticles under physiological conditions and they also play a key role in the surveillance of endothelial cell integrity.

Extramedullary haematopoiesis and monocyte reservoirs

Although the BM is considered as the *bona fide* organ for adult monocyte generation, a fraction of murine monocyte-committed precursors, e.g. MDP and cMoP, were detected in the spleen under healthy homeostasis (Hettinger et al., 2013). Since no circulating monocytic precursor could be detected in the blood (Hettinger et al., 2013), BM-independent splenic production of monocytes is plausible. The spleen serves as a significant peripheral reservoir for Ly6C^{Hi} as well Ly6C^{Low} monocytes; remarkably these monocyte pools outnumber their circulating counterparts (Swirski et al., 2009). **The lung and skin harbour undifferentiated monocytes that might serve as reservoirs as well (Jakubzick et al., 2013).** Under certain inflammatory

circumstances, these monocytes are released into the circulation and emergency extramedullary monopoiesis is augmented in an IL-1β-dependent fashion (Leuschner et al., 2012). Mobilized splenic monocytes are rapidly recruited to injured tissues as observed during atherosclerosis (Robbins et al., 2012) and ischemic myocardial injury (Swirski et al., 2009; van der Laan et al., 2014). **The precise contribution of peripheral reservoirs-released monocytes to the pool of monocytes recruited to inflamed tissues remain largely unknown and additional research is required to uncover the precise role of these reservoirs.**

Pathological conditions and emergency monopoiesis

Inflammation requires the rapid recruitment of myeloid cells to sites of injury, a process that relies on the constant generation and mobilization of BM cells. Therefore, severe inflammation can induce a state of emergency that may generate monocytes with alternative antecedents. These include monocytes that have bypassed the canonical MDP-cMoP-monocyte developmental pathway and resemble neutrophil-like Ly6C^{Hi} monocytes that derive from GMP ((Yanez et al., 2017); **Fig. 3**). Another example of a recently described monocyte subset that appears under inflammatory conditions are the segregated nucleus-containing atypical Ly6C^{Low} monocytes ((SatM) (Satoh et al., 2017)). SatM and neutrophil-like monocytes appear to represent a negligible fraction of monocytes in steady-state (Mildner et al., 2017; Satoh et al., 2017; Yanez et al., 2017), yet become conspicuous during inflammation (Satoh et al., 2017; Yanez et al., 2017). It has been proposed that LPS challenge favours GMP-derived neutrophil-like Ly6C^{Hi} monocyte production while CpG challenge favours classical MDP-derived monocyte production (Yanez et al., 2017). At present we lack reliable surface markers to distinguish neutrophil-like Ly6C^{Hi} monocytes (identified using GFI1/IRF8-reporter mice, (Yanez et al., 2017)) from classical Ly6C^{Hi} monocytes and SatM (identified as Ly6C^{Low}Ceacam1^{Hi}Msr1^{Hi}, (Satoh et al., 2017)) from non-classical Ly6C^{Low} monocytes. Furthermore, mice infected with *Toxoplasma gondii* are characterized by a shift in their monocytic phenotype. These IFN-γ induced regulatory MHCII^{Hi}Sca-1^{Hi} monocytes produce more IL-10 and prostaglandin E2 (PGE_2) (Askenase et al., 2015).

In conclusion, emergency monopoiesis does not only induce a shift in number of monocytes produced but also affects their function. Due to the functional specialization of these recently identified monocyte subsets such as the CD209a^{Hi} MHCII^{Hi} monocytes, the SatM and the neutrophil-like Ly6C^{Hi} monocytes, it will be interesting to study emergency monopoiesis at the single-cell level to assess whether it is associated to increased output of Ly6C^{Hi} monocytes with an altered function or whether it is based on the development of monocyte subsets through altered developmental pathways (e.g. neutrophil-like Ly6C^{Hi} monocytes or SatM), yielding cells that are not normally present in the steady-state and are functionally distinct from classical Ly6C^{Hi} monocytes.

Trained immunity

In the recent years it has become clear that cytokines not only directly provoke functional changes in monocytes but also influence the cellular outcome of HSCs. Emergency haematopoiesis during infections can have long-lasting effects characterized by a shift in cell fate resulting in higher production of particular immune cells at the expense of other cells, such as increased monocyte production compared to decreased cDC production (Pasquevich et al., 2015) and lymphocytes (Liu et al., 2015; Maeda et al., 2009; Pietras et al., 2016) (**Fig. 3**). This can result in increased numbers and an altered activation state of monocytes even weeks after pathogen clearance, a phenomenon termed "trained immunity" (Netea et al., 2016; Quintin et al., 2012). A number of cytokines have been proposed to play a key role in trained immunity, including IFN-γ and IL-1β, suggesting that activation of HSCs by cytokines produced by immune or non-immune cells in the BM are crucial for these long-lasting training effects (Boettcher and Manz, 2017). There is no doubt that HSCs are "trained" through epigenetic changes since HSCs isolated from animals injected with β-glucan preserved their capacity to generate more myeloid cells 12 weeks post transfer into untrained animals (Mitroulis et al., 2018). Similar findings have been reported in mice trained by Bacillus Calmette-Guérin (BCG) (Kaufmann et al., 2018). Macrophages differentiated from BCG-trained BM cells possessed an increased capacity to kill *Mycobacterium tuberculosis in vitro* and upon intratracheal transfer *in vivo*. Moreover, parabiotic partners of trained animals were better protected against *Mycobacterium tuberculosis* demonstrating that circulating cells (presumably monocytes) and not tissue resident cells from the trained partner mediate protection (Kaufmann et al., 2018). Trained immunity also occurs during metabolic diseases. When *Ldlr*$^{-/-}$ mice were fed a western diet for 4 weeks and then returned to control diet for an additional 4 weeks, serum cytokines dropped back to steady-state levels, while BM and splenic myeloid cells still produced more inflammatory cytokines upon TLR stimulation compared to control mice (Christ et al., 2018).

The concept of trained immunity has been translated to humans. BCG vaccination induced epigenetic changes in circulating monocytes of healthy volunteers 4 weeks after vaccination (Arts et al., 2018). BCG-vaccinated individuals displayed lower circulating viral titters upon subsequent experimental viral infection and their PBMCs produced higher levels of IL-1β, TNF and IL-6 upon stimulation *in vitro*. Importantly, no transcriptomic changes were observed in monocytes in these subjects, highlighting the need to study epigenetic modifications when examining trained immunity.

Taken together, these studies demonstrate that inflammation-induced emergency haematopoiesis can result in trained immunity characterized by long term epigenetic effects on HSCs to generate higher quantities of monocytes possessing increased reactivity to pathogens.

The epigenetic changes associated with monocyte training involve histone modifications

of genes encoding pro-inflammatory cytokines such as IL-6 and TNF but also genes of the mTOR pathway (Netea et al., 2016; Quintin et al., 2012). Changes in the mTOR metabolic pathway through a dectin-1/Akt/HIF1α seem essential for monocyte training since inhibition of mTOR, Akt or HIF1α reduced the increased TNF production associated with training (Cheng et al., 2014).

The particular factors involved in the epigenetic modification of HSCs and whether training of stem cells is also associated with an increased production of particular subsets of monocytes is currently unknown (**Fig. 3**). It will also be important to experimentally demonstrate the intrinsic effect of training on the function of monocyte-derived cells. Indeed, in many training experiments it is difficult to distinguish between the indirect effects of prior inflammatory reactions (effect on stromal cells, resident macrophages or memory lymphocytes) and direct effect on HSCs and their monocytic progeny.

Function of classical monocytes during inflammation and pathology

Under pathological conditions monocytes gain distinct non-redundant functions that often cannot be fulfilled by resident macrophages and cDC. These non-exclusive and sometimes overlapping effector functions comprise pro-inflammatory activities, antigen-presentation, tissue remodelling or anti-inflammatory abilities (**Fig. 4**).

The function and importance of pro-inflammatory monocytes is well documented in autoimmune diseases, such as multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). During EAE Ly6C^{Hi} monocytes are mobilised from the circulation or peripheral reservoirs, resulting in rapid influx of classical monocytes into the inflamed tissue. Initial experiments with CCR2-deficient mice that lack most circulating classical monocytes showed that these mice are virtually resistant to EAE induction (Fife et al., 2000; Izikson et al., 2002), a phenotype that could be later attributed to reduced numbers of pro-inflammatory monocytes in this mouse strain and to the pathogenic function of monocytes during disease development (Ajami et al., 2011; King et al., 2009; Mildner et al., 2009). **It was also shown that in contrast to infiltrated monocytes, microglia did not show an overt pro-inflammatory gene signature during EAE (Yamasaki et al., 2014).**

Although EAE is considered to be a T cell-mediated disease, attempts to identify classical effector T cell-secreted cytokines with encephalogenic activities like IFN-γ, IL-17, IL-22 and IL-12 failed (Becher and Segal, 2011). However, mice lacking *Csf2*, which is secreted in a RORγt-dependent manner by CD4$^+$ T cells (Codarri et al., 2011), are completely protected to EAE induction (McQualter et al., 2001). Even though CSF2 is not directly involved in monocyte generation (Hibbs et al., 2007), CSF2 determines the function of monocyte-derived cells under pathological conditions. Using the CCR2-CreERT2 mice it was demonstrated that CSF2R-signaling is needed for the production of inflammatory cytokines and chemokines by monocyte-derived cells in EAE

(Croxford et al., 2015). Furthermore, overexpression of CSF2 by T cells drives peripheral monocytes towards an MHCII^{+}CD11c^{+} phenotype leading to CNS infiltration and tissue damage in an antigen-independent manner (Spath et al., 2017). Interestingly, monocytes that infiltrated the CNS during the course of EAE did not integrate into the tissue-resident microglia pool even after clinical symptoms vanished (Ajami et al., 2011), suggesting that tissue-resident cells have a competitive advantage against peripheral infiltrated cells or that inflammatory signals might block certain developmental pathways in monocyte-derived cells.

Similar to the situation in EAE, *Ccr2*-deficient mice are protected in dextran sulphate sodium (DSS)-induced colitis, a model of ulcerative colitis (Platt et al., 2010), which indicates a similar detrimental role for monocyte-derived effector cells (Zigmond et al., 2012). However, in contrast to EAE, colitis does not rely on CSF2 signaling since *Csf2*-deficient mice are more susceptible to DSS-induced colitis (Xu et al., 2008). Effector monocytes recruited during DSS-colitis, produced less IL-10 and more TNF than resident monocyte-derived macrophages that developed before the onset of colitis (Bain et al., 2013). This lack of IL-10 production may further drive colitis although it is the IL-10 produced by regulatory T cells, but not resident monocyte-derived macrophages that is crucial to maintain homeostasis in the colon. One of the main functions of intestinal IL-10 is to suppress monocyte-derived cells themselves as lack of IL-10R on myeloid cells leads to spontaneous colitis (Zigmond et al., 2014a).

Another subset of effector monocytes are the TNF/iNOS-producing (Tip)-DC, which were originally identified during *Listeria monocytogenes* infection (Serbina et al., 2003), but were also detected during *Trypanosoma brucei* (Bosschaerts et al., 2010; Guilliams et al., 2009), *Toxoplasma gondii* (Dunay et al., 2008) or *Leishmania major* (De Trez et al., 2009) infections. TipDCs derive from Ly6C^{Hi} monocytes (Guilliams et al., 2009; Serbina et al., 2003), are CD11c^{Int}MHCII^{+}Ly6C^{Int} and produce TNF and nitric oxide. **Although these cells were classified as "DC", their contribution to CD4^{+} T cell priming during *Listeria* infection appears negligible (Serbina et al., 2003). The term TipDC is therefore misleading.** Rather these cells excel in killing pathogens as demonstrated by higher *Listeria*, *Toxoplasma* and *Leishmania* burden in mice lacking recruited monocytes (De Trez et al., 2009; Dunay et al., 2008; Serbina et al., 2003), highlighting that the resident macrophages are not sufficient to clear these pathogens alone and require assistance from recruited monocytes. Note that prolonged accumulation and activation of effector monocytes during chronic infections can also contribute to collateral tissue damage (Guilliams et al., 2009), and therefore some persistent infections might profit from the suppression of monocyte-driven inflammation to limit pathological symptoms.

Effector monocytes actively participate in fibrosis development. This involves GMP-derived C/EBPβ-dependent SatM cells that produce high levels of TNF. Mice lacking C/EBPβ were protected from bleomycin-induced fibrosis and transfer of wild type SatM restored fibrosis

development (Satoh et al., 2017). Monocytes also differentiate into pathogenic foam cells in atherosclerotic plaques (Hilgendorf et al., 2015). Anitschkow (Dock, 1958) already demonstrated that feeding rabbits purified cholesterol induced lesions and presence of cholesterol-laden cells (foam cells) similar to those detected during atherosclerosis in humans. We now know that foam cells in the atheroma originate from recruited classical monocytes. These monocytes accumulate in the growing lesion, take-up excess lipid particles and further exacerbate the disease by producing chemokines that attract additional monocytes. CCR5 and CX_3CR1 are involved in their retention within the plaques (Tacke et al., 2007) and it has also demonstrated that foam cells proliferate extensively within plaques (Robbins et al., 2013).
These examples highlight how recruited monocytes play an essential role as an emergency squad, acquiring strong microbicidal and pro-inflammatory activities. Monocytes are therefore not simply precursors that get recruited to temporarily increase the number of tissue resident macrophages but in fact give rise to functionally distinct monocyte-derived cells. Nevertheless, in certain situations, such as helminth infections, IL-4 can drive the proliferation and the immune response of resident macrophages without the aid of recruited monocytes (Jenkins et al., 2011).

Antigen presentation by monocytes

Monocytes can acquire antigen-presentation capacities. This was initially observed when human blood myeloid cells or murine BM cells were stimulated with CSF2 alone or in combination with IL-4, which promotes the differentiation into $CD11c^+MHCII^+CD11b^+$ cells with soluble antigen presenting capacity *in vitro* (Sallusto and Lanzavecchia, 1994). However, it was recently shown that CSF2-treated BM cultures consist of a heterogeneous cell population developing from distinct precursors, including cDC precursors (Helft et al., 2015). Therefore, in order to evaluate the antigen-presentation capacity of monocytes *in vitro*, it is advisable to isolate pure monocytes as a starting population. Mouse monocytes cultured in presence of IL-4 and CSF2 were shown to cross-present cell-associated antigen efficiently (Briseno et al., 2016). These monocyte-derived DC expressed high levels of BATF3 and IRF4. Interestingly, *Batf*-triple deficient cells, but not *Irf4*-deficient cells, still cross-presented antigen thereby demonstrating that cross-presentation is controlled by distinct transcriptional programs in monocyte-derived DC as compared to *Batf*-dependent cDC1, which do not require IRF4 for this function. IRF4 was also reported to be essential for the IL-4 driven development of monocyte-derived DC from human monocytes *in vitro* (Goudot et al., 2017). Comparable to the situation in mice, high-dimensional mass cytometry revealed that *in vitro* cultures of human monocytes with CSF2 and IL-4 yield cells with important phenotypic heterogeneity (Sander et al., 2017). This could potentially be the result of different human monocyte subsets present in the circulation (see above). Indeed, in mice it was found that a small subset of $CD209a^+$ monocytes possess a greater potential to

differentiate into monocyte-derived DC (Menezes et al., 2016). It is possible that this population is boosted by CSF2 treatment.

Multiple studies have demonstrated that monocytes can take up, process and present antigen *in vivo* but whether this contribution is significant to T cell priming compared to antigen-presentation by cDC is debatable and seems to depend on the inflammatory context (Jakubzick et al., 2017). Unfortunately, we currently lack reliable *in vivo* markers to distinguish activated cDC2 from monocyte-derived DC and it remains unclear whether *in vivo* monocyte-derived DC differentiate solely from CD209a^{+} monocytes or whether multiple monocyte subsets acquire antigen-presentation functions.

Contribution of monocytes during tissue remodelling

The resolution phase of the inflammatory response is not a passive process – the objective of this phase is to re-establish the tissue back to healthy homeostasis (Hunter, 1794), and if this period is disrupted, chronic inflammatory diseases can occur. Mononuclear phagocytes help orchestrate a number of aspects of this phase: for example, the safe disposal of cell debris, cytokine catabolism and the release of regulatory mediators (Newson et al., 2014). **Monocytes can already acquire a regulatory phenotype before reaching the injured or inflamed tissue whist still in the circulation (Askenase et al., 2015). In a model of resolving myocardial infraction, CCR2-expressing infiltrating monocytes are thought to promote tissue remodelling and repair via expression of VEGF that induces angiogenesis and by promoting myofibroblast accumulation** (Nahrendorf et al., 2007), and this reparative behaviour is dependent on NR4A1 (Hilgendorf et al., 2014). In paracetamol-induced liver injury infiltrating monocytes are essential for hepatic regeneration and clearance of neutrophils (Graubardt et al., 2017; Zigmond et al., 2014b). This anti-inflammatory phenotype was also adopted during CCl_4-induced liver fibrosis, where infiltrating Ly6C^{Hi} monocytes recruited to the fibrotic liver are responsible for the resolution of tissue fibrosis by degrading extracellular matrix, clearing cell debris and accelerating scar resolution (Ramachandran et al., 2012). Ly6C^{Hi} monocytes are also recruited to chronic allergic inflamed skin, where they differentiate in the presence of basophils and in an IL-4-dependent mechanism into an anti-inflammatory cell to dampen the allergic response (Egawa et al., 2013). **In this context, IL-4-activated monocyte-derived F4/80^{int}CD206^{+} macrophages convert into resident-like F4/80^{hi}CD206^{-} peritoneal macrophages (Gundra et al., 2017). A similar mechanism was also observed during the formation of liver granulomas in mice infected with *Schistosoma mansoni* (Gundra et al., 2017).** Monocytes recruited following injury to the retina play an essential role in the survival and proliferation of retinal progenitor cells by the production of IL-10 (London et al., 2011). In a model of spinal cord recovery following the centralised injury of the spinal cord, CNS-infiltrated Ly6C^{Hi} monocyte adopt an anti-inflammatory phenotype and secret IL-10, possibly

counteracting the detrimental activity of resident microglia (Shechter et al., 2009). These studies highlight the regulatory role monocyte possess, which can counteract the activation of resident macrophages. Finally, development of monocyte-derived macrophages with a tissue remodelling phenotype following injury is not always simply binary (with monocytes differentiating into either pro- or anti-inflammatory macrophages). For instance, injection of the tiger snake venom notexin, a myotoxin phospholipase that leads to the destruction of skeletal muscles, is accompanied by the infiltration of Ly6C^{Hi} monocytes that first display an early pro-inflammatory phenotype, before converting into a reparative monocyte-derived cell with the capacity to stimulate myogensis, fibre growth and restore muscle integrity (Arnold et al., 2007). Taken together, these studies highlight the plasticity of monocytes and demonstrate that these cells are essential for tissue repair and perform tasks that are not efficiently performed by resident macrophages.

Role of monocytes in cancer

Mononuclear phagocytes are located in both healthy and pathological tissue. The tumour microenvironment is no exception. The contribution of monocytes to tumour development is a multifaceted process ranging from the initiation of vessel growth to immune escape and metastasis. Tumours circumvent recognition and cell mediated elimination by suppressing the anti-tumour immune response. In this regard, tumours affect myelopoiesis in the BM and induce the expansion of myeloid cells with immunosuppressive activity, both, in animal models and in patients (Bronte et al., 2016). These cells were originally characterized by CD11b and Gr1 expression and termed 'myeloid-derived suppressor cells' (MDSC). However, CD11b and Gr1 (which recognizes both Ly6C and Ly6G) define classical monocytes as well as neutrophils and therefore both myeloid cell populations could potentially contribute to T cell suppression. Analysis of this CD11b^{+}Gr1^{+} subset as a homogenous population has caused conflicting results and confusion concerning the nature, function and ontogeny of these cells. Addition of further cell surface markers unmasked the presence of monocytic and polymorphonuclear MDSC and allowed independent analysis of both subsets (Bronte et al., 2016; Gallina et al., 2006; Huang et al., 2006; Movahedi et al., 2008). Monocytic MDSC are derive from circulating Ly6C^{Hi} monocytes, either directly from the BM or from the splenic peripheral reservoir in a CCR2-dependent manner (Cortez-Retamozo et al., 2012; Shand et al., 2014). The presence of hypoxic conditions, high concentrations of oxidative agents, release of pro-inflammatory cytokines, and limited supply of nutrients makes the tumour environment a relatively harsh milieu. Monocytes, which infiltrate the tumour tissue under these circumstances, might consequently acquire a pro-inflammatory signature that dampens lymphocyte activities, survival and proliferation in general – a phenomenon reflected in

the suppressive activities of MDSC. However, these infiltrating Ly6C^{Hi} monocytes can further differentiate into tumour-associated macrophages (TAM), which are – similar to the situation in other pathological contexts – characterized by low Ly6C expression and differential expression of MHCII, CD11c and CX_3CR1 (Movahedi et al., 2010). In general, monocyte-derived TAM represent a significant part of tissue-resident macrophages in tumours beside a fraction of TAM derive from tissue resident macrophages (Franklin et al., 2014). Differential roles for monocyte-derived TAM and tissue resident macrophage TAM have been proposed (Bowman et al., 2016; Loyher et al., 2018; Zhu et al., 2017), even though this might depend on the tumour.

Monocyte-derived TAM provide maintenance activities for tumour growth by facilitating angiogenesis, acting as pathfinders for endothelial cells, which provide oxygenation and nutrients to the growing tumour, as well as by clearing waste products. TAM exhibit negative activities on T cells and express high levels of programmed death ligand 1 (PD-L1), a ligand for immune-checkpoint receptor (Noy and Pollard, 2014). Monocyte-derived TAM therefore represents an essential component of the developing tumours by critically contributing to survival, protection and growth of tumour cells. The development of monocytic MDSC and TAM is dependent on the transcription factors IRF8 (Waight et al., 2013), STAT3 (Chalmin et al., 2010) and RBPJ (Franklin et al., 2014).

Finally, the majority of deaths from solid tumours are a result of metastasis. Monocytes promote metastasis by preparing the potential microenvironment and enable the dissemination of metastatic cells (Keirsse et al., 2018). Classical monocytes are recruited to the metastatic site in a CCL2-CCR2 dependent fashion and, once embedded within this niche, these recruited monocyte-derived cells promote the survival and growth of the metastatic tumour (Qian et al., 2011).

In conclusion, whilst it is clear that monocyte-derived cells play an important role in cancer, many confusing concepts (like the MDSC and M1 versus M2 tumour associated macrophages) plague the cancer literature (Kiss et al., 2018). Tumour mononuclear phagocytes should be analysed like any other pathological tissue with the potential of diverse roles for resident macrophages versus recruited monocytes. Fate mapping tools, single-cell sequencing, spatial transcriptomics and multiplexed microscopy will help shed light on the precise functions of monocytes during carcinogenesis and will hopefully yield novel anti-cancer strategies.

Function of non-classical monocytes during pathology

The function of Ly6C^{Low} monocytes during inflammation remains controversial. The existence of Ly6C^{Hi} and Ly6C^{Low} monocytes within the injury site was originally interpreted as evidence that both classical and non-classical monocytes can infiltrate the tissue, possibly in a sequential

manner and might contribute distinct pro- and anti-inflammatory activities. Alternatively, infiltrated Ly6C^{Hi} monocytes can lose their Ly6C expression while increasing F4/80, CD11c and CX_3CR1 during the differentiation process into effector monocyte-derived cells in the tissue, making them indistinguishable from circulating Ly6C^{Low} monocytes (Arnold et al., 2007; Avraham-Davidi et al., 2013; Zigmond et al., 2012). Examining the phenotype of infiltrating cells at the site of injury and extrapolating their origin based on surface markers has therefore caused much confusion.

Further insight into the nature of non-classical monocytes came from the observation that *Nr4a1*-deficient mice are characterized by a strong reduction of circulating Ly6C^{Low} monocytes (Hanna et al., 2011). Therefore, this mouse model in combination with the CX_3CR1^{gfp} reporter mouse (Jung et al., 2000) has become a useful tool to study the functional role of Ly6C^{Low} monocytes during pathological conditions. These patrolling Ly6C^{Low} monocytes interact with metastatic tumour cells, scavenge tumour material from the lung vasculature, and initiate NK cell recruitment and activation (Hanna et al., 2015; Plebanek et al., 2017). Ly6C^{Low} monocytes are also able to target and eliminate amyloid-β within the lumen of veins and the absence of these cells due to *Nr4a1*-deficiency increased amyloid-β deposits within the cortex and hippocampus of mice (Michaud et al., 2013).

As a word of caution, Ly6C^{Hi} monocyte function is not completely independent of the transcription factor NR4A1, since the Ly6C^{Hi} monocytes that infiltrate the infarcted myocardium show an increased expression of NR4A1 (Hilgendorf et al., 2014). Furthermore, up-regulation of this transcription factor was accompanied by reduced pro-inflammatory activities and a reparative phenotype, while absence of NR4A1 led to a more inflammatory phenotype in Ly6C^{Hi} monocyte derived cells, which results in defective healing and compromised heart function (Hilgendorf et al., 2014). Similar results were obtained in an animal model of atherosclerosis, in which *Nr4a1*-deficient monocytes and macrophages displayed enhanced toll-like receptor signalling and a pro-inflammatory phenotype (Hanna et al., 2012). Therefore, it is challenging to interpret the phenotype observed in *Nr4a1*-deficient mice as being exclusively due to the absence of Ly6C^{Low} monocytes. A new and elegant mouse model will help to resolve these uncertainties. Deletion of a monocyte-specific *Nr4a1*-enhancer element (E2) resulted in the loss of peripheral non-classical monocytes, while NR4A1-dependent inflammatory pathways were preserved in macrophages (Thomas et al., 2016).

Beside these complications in deciphering results from mice with an absence of Ly6C^{Low} monocytes, some specific functions during inflammation have been attributed to these cells. Following exposure to TLR7-mediated 'danger' signals, mimicking viral infection or local cell death, a CX_3CR1-dependent retention of Ly6C^{Low} monocytes on the affected endothelium was observed (Carlin et al., 2013). The TLR7 ligand triggered monocytes lead to the recruitment of neutrophils, which mediated focal endothelial necrosis, while subsequently Ly6C^{Low} monocytes

remove cellular debris. Recently it was shown that non-classical monocytes are superior producers of TNF following poly(I:C) injection (Garre et al., 2017). The secretion of TNF by these cells led to reduced learning-induced dendritic spine remodelling in pyramidal neurons and consequently to deficits in learning behaviour. TNF produced by non-classical monocytes was also observed in the glomerular microvasculature, where TNF supported cellular monocyte/neutrophil communication and increased neutrophilic ROS production, thereby leading to neutrophil-mediated tissue injury (Finsterbusch et al., 2016). The production of TNF in response to viruses was also shown for human non-classical monocytes (Cros et al., 2010). Finally, it was proposed that Ly6C^{Low} monocytes exhibit a protective effect during the early stages of atherogenesis by maintaining endothelial cell integrity (Quintar et al., 2017). As such, it is becoming clear that patrolling monocytes act as resident phagocytes of the circulation and thereby play a specialised role in regulating vascular inflammation.

Engraftment of monocytes in the resident macrophage pool during pathology

Another way by which inflammation-induced monocytes possessing an altered activation state will exert long-lasting effects on tissues is by integrating into the resident macrophage pool (Bonnardel and Guilliams, 2018). In some disease models, but not in others, the macrophage disappearance reaction that is often associated with inflammation (Barth et al., 1995) results in the engraftment of monocytes into the resident macrophage niche. In the case of the liver as an example, experimental diphtheria toxin induced depletion (Scott et al., 2016b), *Listeria monocytogenes* infection (Bleriot et al., 2015), paracetamol induced liver-injury (Zigmond et al., 2014b) and others (Devisscher et al., 2017) result in partial Kupffer cell loss. Subsequently, infiltrating monocytes can differentiate into Kupffer cell-like cells during all these experimental models apart from paracetamol-induced liver injury, which solely relies on the proliferation of the remaining Kupffer cells to refill this niche (Zigmond et al., 2014b). A recent report demonstrates that monocyte to Kupffer cell differentiation is suppressed by Type I IFN signalling during a model for viral hepatitis (Borst et al., 2017). The underlying mechanism is unclear and open questions remain: Is Type I IFN inducing the generation of BM inflammatory monocytes that are blocked from becoming Kupffer cells before reaching the liver? Does Type I IFN change the local microenvironment in the liver and block monocyte to Kupffer cell development?

When monocytes enter the Kupffer cell pool during inflammation it is uncertain whether these cells are functionally distinct from embryonic Kupffer cells. Indeed, it may be that these monocytic cells have been trained by the inflammation during their development and are more reactive to subsequent stimuli. In some models these monocyte-derived Kupffer cells eventually disappear from the Kupffer cell pool (Devisscher et al., 2017; Theurl et al., 2016), suggesting a diminished self-renewal capacity compared to embryonic Kupffer cells.

Gammaherpes virus infection has been associated with the generation of IL-10 producing MHCII^{Hi} Sca-1^{Hi} monocytes within the BM (Machiels et al., 2017). These monocytes resemble monocytes with a regulatory phenotype produced during *Toxoplasma gondii* infection (Askenase et al., 2015). Interestingly, gammaherpes virus infection also resulted in engraftment of regulatory monocytes in the alveolar macrophage pool. These monocyte-derived alveolar macrophages did not only self-maintain for months but retained an altered activation state that protected against allergic asthma (Machiels et al., 2017). Lung fibrosis is also associated with the differentiation of monocytes into self-maintaining alveolar macrophages with an altered gene-expression profile (Misharin et al., 2017). Whether the altered activation state of monocyte-derived alveolar macrophages in these models is imprinted locally by the virus-infected or fibrotic lung or is in part determined by emergency monopoiesis in the BM is currently unknown. Moreover, whether these models of inflammation result in the production of a particular subset of monocytes possessing the unique capacity to self-maintain or to generate functionally distinct alveolar macrophages remains to be determined. Finally, it will be interesting to understand the epigenetic modifications of resident macrophages that were present prior to the inflammatory insult and that may also have undergone inflammatory training or tolerance. Is the long-term imprinting in the resident macrophages that were present before the inflammation comparable to the long-term imprinting in macrophages that derive from monocytes differentiating during the inflammation (Bonnardel and Guilliams, 2018)?

These examples demonstrate that monocytes can integrate into resident macrophage pools during inflammation, but whether these cells can self-maintain for prolonged periods appears to depend on the tissue and the inflammatory context. New fate mapping systems that faithfully label the monocyte-derived macrophages that engraft during inflammation will be required to assess their contribution to the local immune responses.

Concluding Remarks

Recruitment of monocytes to inflamed tissues does not merely cause a temporarily increase in macrophage and DC numbers. Instead, these infiltrating monocytes will often perform functions that cannot be performed by resident macrophages or cDC and will acquire very distinct functional profiles depending on the tissue and the inflammatory context. These monocytic phenotypes include effector monocytes with inflammatory, antigen-presentation, regulatory or resident macrophage profiles, although these phenotypes can overlap. Recently new monocyte subsets have been described under inflammatory as well as healthy conditions such as the neutrophil-like Ly6C^{Hi} monocytes, the SatM and the CD209^{+} monocytes. These cells display increased pro-inflammatory, pro-fibrotic and antigen-presentation capabilities compared to steady-state Ly6C^{Hi} monocytes and future research will help to improve our understanding of their precise ontogeny, functional contribution *in vivo* and relationship to other myeloid cells.

The identification of these subsets highlights how single-cell technologies in combination with fate-mapping or *in vivo* barcoding studies will profoundly reshape our understanding of monocyte biology. Future insight into monocyte function and differentiation will provide potential therapeutic strategies that aim to support monocytic subsets with beneficial effects during pathological conditions, while hinder monocytes that contribute to disease development.

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Figures

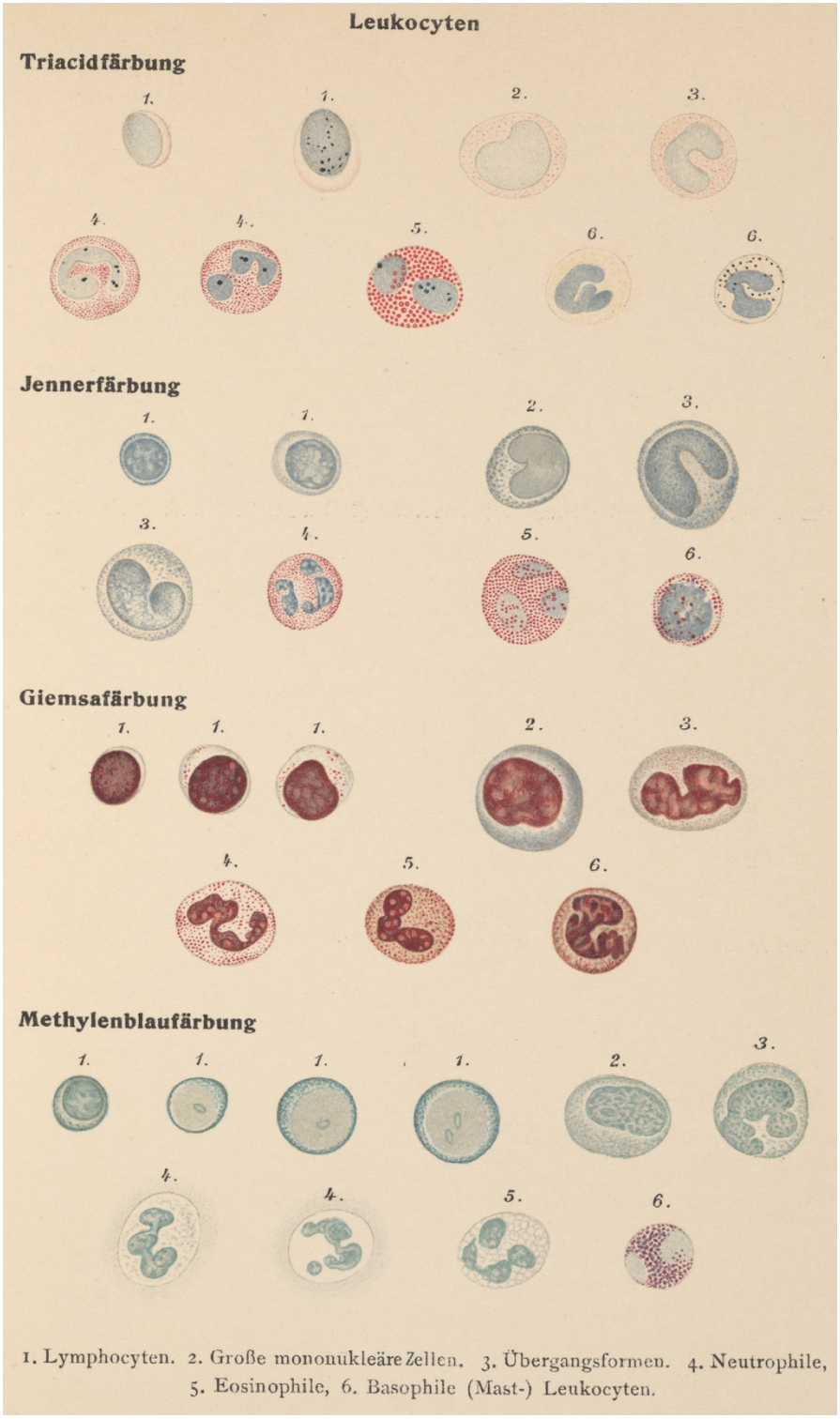

Figure 1: Historical leukocyte identification. By 1908, blood leukocytes could be stratified on the basis of their morphology and their staining properties. Different staining techniques are depicted here with 1. *Lymphocyten:* lymphocyte 2. *Grosse mononukleäre Zellen: Great* mononuclear cell 3. *Übergangsformen:* Ehrlich's transitional cell (monocyte) 4. *Neutrophile:* neutrophil 5. *Eosinophile: eosinophile* 6. *Basophile (Mast) Leukozyten:* basophil or mast cell (from (Naegeli, 1908)).

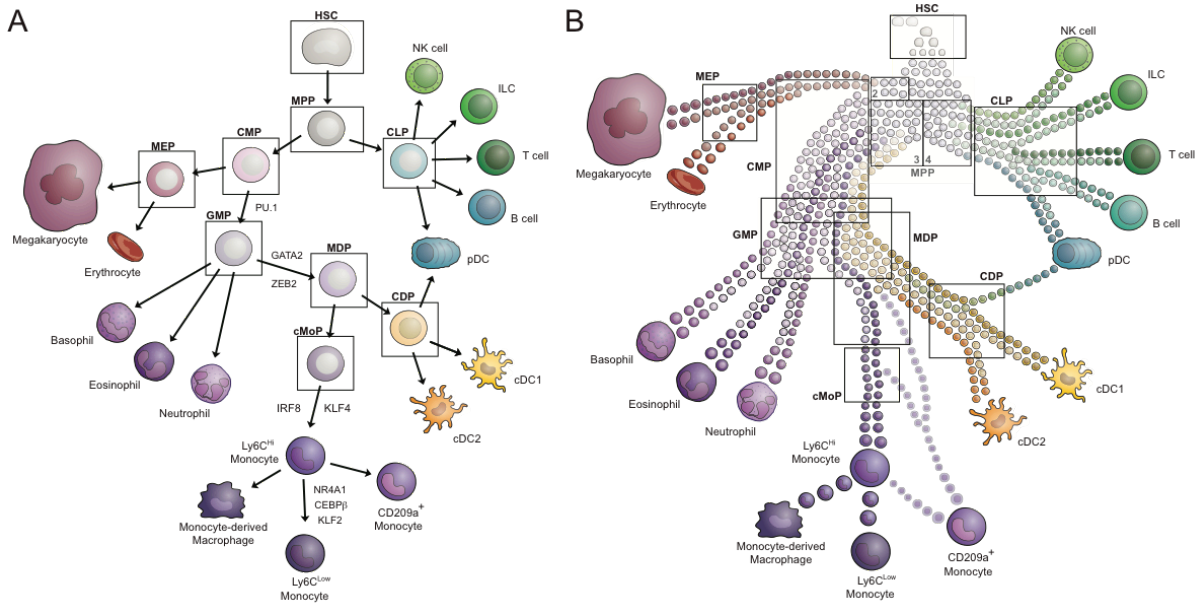

Figure. 2: Development of murine monocytes under physiological conditions. A. Depiction of the classic hierarchical representation of haematopoietic development in the bone marrow. Oligopotent precursors such as haematopoietic stem cells (HSC) or multipotent precursors (MPP) become progressively more restricted and give rise through intermediate precursor stages to all forms of circulating blood cells. In this view, defined multipotent precursor populations such as granulocyte macrophage precursors (GMP) produce either the granulocytic lineage or further differentiate into monocyte-macrophage/dendritic cell precursors (MDP). Bipotent MDPs further make binary decisions and either give rise to common dendritic cell precursors (CDP) or to common monocyte precursors (cMoP). cMoP finally differentiate into Ly6C^{Hi} cells, which are released into the circulation and can acquire different cell fates, including the transition into Ly6C^{Low} monocytes patrolling blood vessels or monocyte-derived cells in tissues. Note, that is was recently suggested that a majority of plasmacytoid DC (pDC) develop from IL-7Ra-expressing common lymphocyte precursors (CLP), while a smaller fraction derive from CDPs (Rodrigues et al., 2018). The main transcription factors involved in monocyte development are shown. CMP: common myeloid precursors. MEP: megakaryocyte/erythrocyte progenitors. **B.** Development of the haematopoietic system based on single-cell analysis. In this view, haematopoietic development starts with few active HSC, which fill a pool of MPPs. Subsets of MPPs exist (indicated by the numbers 2-4 according to (Pietras et al., 2015)) that are already primed towards certain cell lineages or even show a cellular identity comparable (but not identical) to terminal differentiated cells (indicated by light coloured circles). Further differentiated precursor populations are therefore a heterogeneous mixture of primed cells (pool of cells in the rectangles in (B)), which share a – sometimes overlapping – common surface marker profile (rectangles corresponding to the supposedly homogeneous precursors shown in (A)).

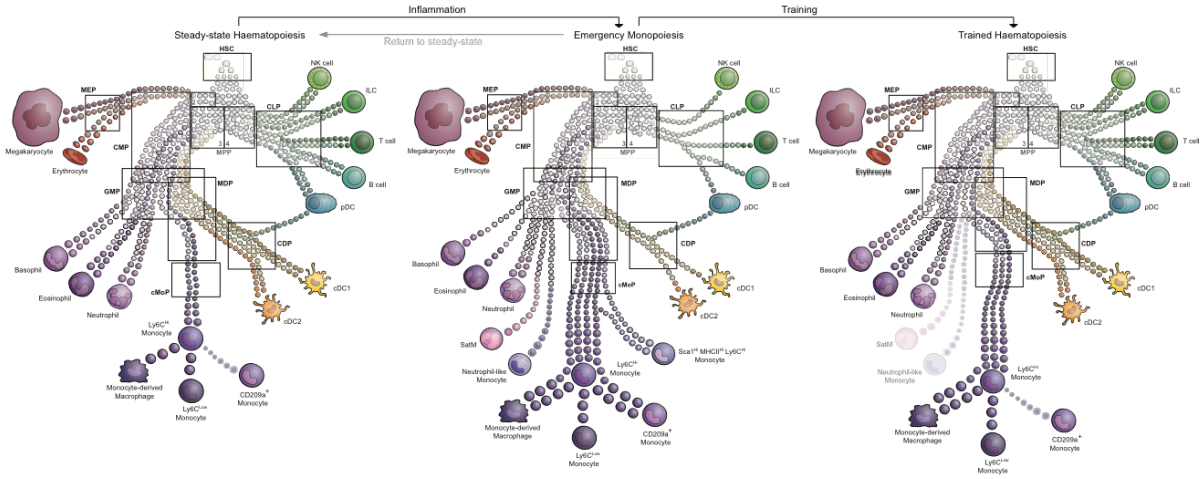

Figure. 3: Emergency monopoiesis and trained immunity. Compared to the physiological condition (left), severe inflammation can induce emergency monopoiesis leading to higher production of monocytes. Importantly, emergency monopoiesis also generates functionally distinct monocyte subsets with potentially alternative ancestry. These emergency-induced monocytes include neutrophil-like Ly6C^{Hi} monocytes and SatM that display neutrophil characteristics, but also Sca1^{Hi} monocytes with regulatory properties (middle). Emergency haematopoiesis can have long-lasting effects and yield in trained haematopoiesis characterized by a sustained increased production of monocytes that possess higher capacities to produce cytokines and superior microbicidal activities (right). The molecular mechanism that lead to the training effect and prevent haematopoiesis from returning to the steady-state after inflammation are starting to be revealed and involve IFN-γ and IL-1β-mediated signalling in HSCs.

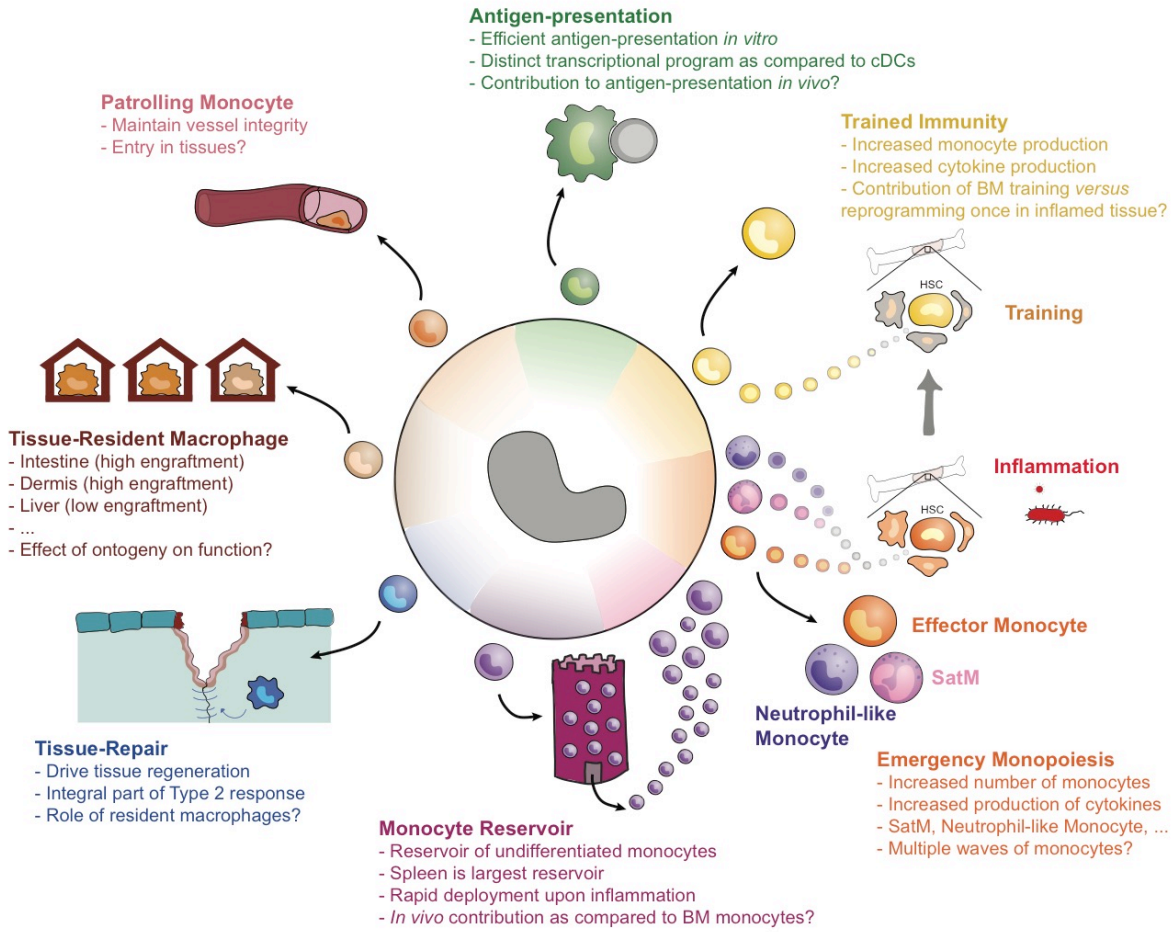

Figure 4: Diversity of monocyte functions during physiological and pathological conditions. Monocytes can engraft into peripheral monocyte reservoirs or can differentiate into non-classical patrolling monocytes or tissue-resident macrophages in the steady-state. Monocytes can also acquire inflammatory, regenerative or antigen-presentation capacities when entering inflamed tissues. Severe inflammation can lead to emergency monopoiesis with the production of novel subsets of monocytes with altered functional characteristics. Inflammation can have long-term effects on haematopoiesis and result in trained immunity associated with increased output of monocytes producing more inflammatory cytokines.

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