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UNIVERSITY

**The role of the CXCL12/CXCR4 axis in
monocyte tissue compartmentalisation
and homeostasis**

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August 2016

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TISSUE COMPARTMENTALISATION AND HOMEOSTASIS**

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Videos are made available at the following url:

<https://www.dropbox.com/sh/c1w27yuxaii75ug/AADQkkLhxfhRjCBKRecTvFQLa?dl=0>

ABBREVIATIONS

A

ALI: Acute lung injury

APC: Antigen presenting cell

B

BM: Bone marrow

BrdU: 5-bromo-2-deoxyuridine

C

cDC: Conventional dendritic cell

CAR cells: Cxcl12-abundant reticular cells

CDP: Common dendritic cell progenitor

C/EBP α : CCAAT/enhancer-binding protein alpha

CLP: Caecum ligation and puncture

CFSE : 5,6-carboxyfluorescein diacetate succinimidyl ester

cMoP: Common monocyte progenitor

D

DAMPs: Danger associated molecular patterns

DC: Dendritic cell

DSS: Dextran sodium sulfate

E

EAE: Experimental autoimmune encephalomyelitis

F

FITC: Fluorescein isothiocyanate

G

G-CSF: Granulocyte colony stimulating factor

GFP: Green fluorescent protein

GM-CSF: Granulocyte-macrophage colony stimulating factor

H

HSC: Haematopoietic stem cell

I

IL-3: Interleukin 3

iNOS: Inducible nitric oxide synthase

IRF8: Interferon regulatory factor 8

K

KLF4: Kruppel-like factor 4

L

LIF: Leukaemia inhibitory factor

M

M-CSF: Macrophage colony stimulating factor

MC: Monocyte-derived cell

MDP: Monocyte-dendritic cell progenitor

MHCII: Major Histocompatibility Complex Class II

MPS: Mononuclear phagocyte system

N

NOD: Nucleotide oligomerization domain

P

PAMPs: pathogen-associated molecular patterns

PD-L1: Programmed death-ligand 1

PD-L2: Programmed death-ligand 2

pDC: plasmacytoid dendritic cell

R

RFP: Red fluorescent protein

S

SCF: Stem cell factor

SDF-1: Stromal-derived factor 1

SHG: Second harmonic generation

SPF: Specific pathogen free

T

TAM: Tumour associated macrophages

TED: Transepithelial dendrites

Th1: T helper cell type 1

Th2: T helper cell type 2

TLR: Toll-like receptor

TNF- α : Tumour necrosis factor α

Tip-DC: TNF- α /iNOS-producing dendritic cell

TpMo: Transitional pre-monocyte

V

VEGF-A: Vasoactive endothelial growth factor A

W

WHIM: Warts, Hypogammaglobulinemia, Infections and Myelokathexis

ABSTRACT

Monocytes are integral to innate immune responses against microbial and sterile tissue injury. However, mechanisms that regulate their compartmentalisation in distinct tissues and their rapid mobilisation following inflammatory signals remain poorly understood. Using bone marrow (BM) intravital imaging and BrdU pulsing assays, we found that while all monocytes respond to mobilisation cues, only a minority is able to enter the peripheral circulation, suggesting a heterogeneity among monocytes. Using computational analysis approaches to interpret multi-dimensional datasets, we demonstrate that BM Ly6C^{hi} monocytes consist of two distinct subpopulations (CXCR4^{hi} and CXCR4^{lo} subpopulations) in both mice and humans. Transcriptome studies and *in vivo* assays revealed functional differences between the two subpopulations. Notably, the CXCR4^{hi} subset proliferates and is immobilized in the BM for the replenishment of functionally mature and mobilisable CXCR4^{lo} monocytes. We propose that the CXCR4^{hi} subset represents a "transitional pre-monocyte" population and this sequential step of maturation serves to maintain a stable pool of BM monocytes. Additionally, reduced CXCR4 expression on monocytes, upon their exit into the circulation, does not reflect its diminished role in monocyte biology. Specifically, CXCR4 regulates monocyte peripheral cellular activities by governing their circadian oscillations, homing to BM and splenic reservoirs and pulmonary margination. Importantly, we showed that reduced monocyte margination through CXCR4 inhibition resulted in decreased occurrence of lung injury and mortality in mouse models of sepsis. Altogether, our study demonstrates the multifaceted role of CXCR4 in defining BM monocyte heterogeneity and in regulating their function in peripheral tissues.

Keywords: monocytes, immune system development, chemokines, trafficking, intravital imaging, multiphoton microscopy

1 Introduction

1.1 The mononuclear phagocyte system (MPS)

The mononuclear phagocyte system (MPS) consists of a group of immune cells that are highly phagocytic in nature. These cells include monocytes, dendritic cells (DCs) and macrophages that which have the capacity to ingest potentially harmful particles and constitute the first line of defence. While macrophages participate in tissue defence and homeostasis by engulfing pathogens, apoptotic bodies and promoting the recruitment of other immune cells during inflammation, DCs sample antigens present in the tissue environment and subsequently migrate to draining lymph nodes to mount an adaptive immune response. In contrast to these cells, monocytes were solely considered as circulating precursors for the replenishment of mature tissue resident macrophages. Moreover, during inflammatory conditions, it was conceived that monocytes could also give rise to dendritic cells. However, an increasing amount of evidence indicates that this initial assumption was potentially incorrect, and that monocytes could have origins and effector functions of their own.

1.1.1 The discovery of mononuclear phagocytes

At the end of the 19th century, Metchnikoff was the first to propose a classification of phagocytic cells through a series of seminal experiments. Specifically, he noticed through intravital imaging that the insertion of a rose thorn into starfish larvae led to an accumulation of cells that surrounded the foreign body in an attempt to eliminate it. More

importantly, he discovered that these cells could be distinguished into two populations known as macrophages (large eaters) and microphages (which will be known later on as granulocytes) (Gordon, 2008).

Consequently, analogous populations of macrophages were discovered in vertebrates and humans. These cells were identified in tissue preparations through their ability to ingest colouring agents, leading to the subsequent discovery of a vast number of tissue macrophages, such as microglia, Kupffer cells and histiocytes. However, these discoveries also led to a substantial amount of confusion as differing names were given to the same cell population depending on who discovered it (Yona and Gordon, 2015).

Therefore, in order to formalise a common origin for all tissue macrophages, van Furth and Cohn introduced the concept of the MPS in 1968, where monocytes and macrophages were classified as a differentiating continuum. This lineage tracing was demonstrated using radioactive isotopes, which revealed that monocytes progenitors begin their journey in the BM and enter the blood where they serve to replenish tissue macrophages. Importantly, macrophages were then seen as terminally differentiated cells that had lost the ability to self-renew (van Furth and Cohn, 1968; van Furth et al., 1972).

1.1.2 Inclusion of dendritic cells in the mononuclear phagocytes family

Shortly after the establishment of the MPS, Steinman, who was then working in the laboratory of Cohn, observed a novel cell type that was

present in the mouse spleen and lymphoid organs, which he subsequently named them as dendritic cells (DCs). Compared to macrophages, DCs displayed substantial differences in morphology by displaying long dendritic processes and were less efficient at phagocytosis (Steinman and Cohn, 1973; 1974). However, DCs showed high expression of MHCII, which was correlated to a superior ability to prime naive T cells in a mixed leukocyte reaction as to any other antigen presenting cells (APCs) known at that time. Subsequently, DCs were also included among the mononuclear phagocyte family as a distinct population of cells that differed from macrophages (Steinman and Nussenzweig, 1980; Yona and Gordon, 2015).

Since their initial discovery, DCs are now found to be a highly heterogeneous population of cells. Currently, two main families of DCs are described: plasmacytoid DCs (pDCs) and conventional DCs (cDCs). In mice, pDCs can be distinguished from other DCs by their expression of Siglec-H, BST2 and B220 (Swiecki and Colonna, 2015). Unlike cDCs that mainly reside in secondary lymphoid organs and peripheral tissues, pDCs display a round morphology and can be found in the blood where they are functionally equipped to produce type I interferons during viral infections.

On the other hand, cDCs, for which the nomenclature has recently been standardised, can be divided into 2 main subsets: cDC1 and cDC2 (Guilliams et al., 2014) **[Figure 1.1]**. Phenotypically, cDC1s are defined by the expression of XCR1 and CD8 in lymphoid organs, while their expression of the integrin CD103 distinguishes these cells in non-lymphoid tissues. In contrast, cDC2s are defined by the expression of SIRP α and CD4 in lymphoid organs, as well as the integrin CD11b in

non-lymphoid tissues. While cDC1s excel at cross-presenting antigens to CD8⁺ T cells, cDC2s display higher ability to present antigens to CD4⁺ T cells (Merad et al., 2013).

Besides their difference in term of function, DCs can also be distinguished from macrophages through their growth factor requirements during homoeostasis. While M-CSF is absolutely necessary for macrophage development (Dai et al., 2002; Pixley and Stanley, 2004), a deficiency in *Flt3* or its ligand *Flt3L* drastically reduces the number of all DC subsets in every organ (Liu and Nussenzweig, 2010; Liu et al., 2009). Furthermore, it is now well established that DCs arise from progenitors that are distinct from those of monocytes and macrophages in the steady state. In particular, DCs arise from the common DC progenitor (CDP) (Auffray et al., 2009; Naik et al., 2007) that are generated in the BM. Consequently, CDPs were found to give rise to pDCs and pre-cDCs, which will ultimately give rise to cDC1s and cDC2s that infiltrate tissues (Merad et al., 2013; Schlitzer et al., 2015). Notably, DC subsets also require distinct transcription factors for their full maturation. For instance, the development of pDCs critically involves *E2-2* and *Irf8* while cDC1s depend on *Batf3* and *Irf8*, and cDC2s require *Irf4*, *Klf4* and *Notch2* (Merad et al., 2013).

Figure 1.1 Mononuclear phagocytes development

While tissue-resident macrophages derive from pre-natal progenitors and self-renew throughout the whole life of an individual, monocytes and DCs are short-lived and need constant replenishment from MDPs in the BM. MDPs can give rise to progenitors committed towards monocytic lineage (cMoP) or DC lineage (CDP). CDPs can give rise to pDCs or pre-DCs that will replenish cDCs in tissues. In contrast, cMoPs give rise to Ly6C^{hi} monocytes that can be mobilised from the BM. Once in the peripheral circulation, Ly6C^{hi} monocytes can differentiate into Ly6C^{lo} monocytes, repopulate certain populations of tissue-resident macrophages, or can infiltrate tissues during inflammation to complement the function of tissue-resident macrophages and cDCs.

1.1.3 Rediscovery of macrophage ontogeny

Based on the MPS conceptualised by van Furth and Cohn in 1968, monocytes and macrophages represent a differentiation continuum, whereby tissue resident macrophages are constantly being replenished by bone marrow-derived monocytes in the steady state (van Furth and Cohn, 1968; van Furth et al., 1972). However, this dogma has now been questioned through discrepancies in several experiments. Firstly, primitive macrophages in the yolk sac were observed to appear during embryogenesis before the apparition of fetal monocytes (Takahashi et al., 1989). Secondly, genetically-modified mice that had monocytopenia displayed relatively normal tissue resident macrophages numbers (Kuziel et al., 1997). Thirdly, the experiments conducted by van Furth and Cohn involved radioactive isotopes and BM irradiation (van Furth and Cohn, 1968). These factors are mediators of inflammation, which could have triggered the infiltration of monocytes into tissues (Hashimoto et al., 2013).

Subsequently, it became increasingly clear that tissue macrophages could be derived from progenitors other than monocytes. Notably, a study on microglia that employed cell-tracing techniques revealed that tissue macrophages could self-renew without the input of circulating monocytes (Ginhoux et al., 2010). This observation was the first to be reported and involved a strategy that labels yolk sac macrophages but not fetal or adult monocytes. In this study, they found that macrophages could infiltrate the brain rudiment at 9.5, proliferate, and self-maintain throughout their entire life (Ginhoux et al., 2010). This phenomenon was further substantiated by other groups using similar strategies, whereby most resident macrophages were reported to seed the tissues during

embryonic/fetal life without the input of circulating monocytes (Hashimoto et al., 2013; Schulz et al., 2012).

While it is now established that most tissue macrophages may derive from progenitors other than monocytes, the origin of tissue macrophages in each tissue was found to be highly dependent on the organ's microenvironment. In particular, the gut, dermis, heart, pancreas and male peritoneal cavity were found to display a strong monocyte chimerism that increases throughout life (Bain et al., 2016; Calderon et al., 2015; Molawi et al., 2014; Tamoutounour et al., 2013; Zigmond et al., 2012). These findings suggest that the replacement of tissue macrophages by circulating monocytes in these organs could result from a persistent low-grade inflammation that involved the exposure of microbiota-derived factors (gut, dermis, peritoneum), or local organ remodelling (heart, pancreas).

Taken together, monocytes and macrophages are now increasingly being recognised as separate entities based on their ontogeny in the steady state. More importantly, recent efforts in nomenclature definition have now classified tissue resident macrophages as cells with an embryonic origin (Guilliams et al., 2014) **[Figure 1.1]**.

1.2 Monocyte development and trafficking

Although monocytes, macrophages and DCs are characterised within the same family in the MPS, an increasing amount of evidence indicates that monocytes are not mere precursors of macrophages and DCs

during inflammation. Instead, monocytes possess distinct ontogeny, trafficking patterns and effector functions.

1.2.1 Monocyte subsets in mouse and human

Monocytes are a heterogeneous population that can be categorised into two main subsets that include classical and non-classical monocytes (Geissmann et al., 2003; Ziegler-Heitbrock et al., 2010). Notably, monocytes express CD115 (also known as M-CSFR or CSF1R), CD11b (also called integrin α M) and CX3CR1 in both mice and humans. However, classical monocytes can be distinguished from non-classical monocytes through their high expression of CCR2 and CD62L, and their low expression of CD43 (Ingersoll et al., 2010; Wong et al., 2011). In addition to these markers, mouse monocytes are usually distinguished by the expression of Ly6C, with classical monocytes being commonly termed as Ly6C^{hi} monocytes, while non-classical monocytes are known as Ly6C^{lo} monocytes. In comparison, human monocytes are characterised by the expression of CD14 and CD16, with classical monocytes being CD14⁺CD16⁻ and non-classical monocytes being CD14⁻CD16⁺. Since this thesis mainly focuses on mouse monocytes, classical and non-classical monocytes will be referred to as Ly6C^{hi} and Ly6C^{lo} monocytes respectively. **[Table 1.1]**

Upon the discovery of monocytes as a heterogeneous population (Geissmann et al., 2003), Sunderkotter *et al.* subsequently discovered that a developmental relationship existed between the two monocytes subsets. By depleting monocytes with clodronate liposomes followed by their in vivo tagging using a fluorescent dye, Ly6C^{hi} monocytes that

appeared in the blood were found to give rise to Ly6C^{lo} monocytes over time (Sunderkötter et al., 2004). This phenomenon was further confirmed by Jung's group through an endogenous fate-mapping model. Furthermore, they determined that Ly6C^{hi} monocytes were short-lived with a half-life of 20 hours while Ly6C^{lo} monocytes exhibited a longer half-life of 48 hours in the steady state (Yona et al., 2013). Notably, a similar relationship seems to exist in human whereby CD14⁺ classical monocytes gives rise to CD16⁺ non-classical monocytes (Wong et al., 2011).

	Human and Mouse	Mouse	Human
Classical monocyte	CD115 ⁺ CD11b ⁺ CCR2 ⁺ CD43 ⁻ CD62L ⁺	Ly6C ^{hi}	CD14 ⁺
Non-classical monocyte	CD115 ⁺ CD11b ⁺ CCR2 ⁻ CD43 ⁺ CD62L ⁻	Ly6C ^{lo}	CD16 ⁺

Table 1.1 Monocytes phenotype in mice and humans.

1.2.2 Identification of BM monocyte committed progenitors

Following the discovery of a committed progenitor that can give rise to all myeloid cells known as the granulocyte-monocyte progenitor (GMP) in the early 2000's (Akashi et al., 2000), Geissmann's group subsequently identified the monocyte-DC progenitor (MDP), which specifically replenishes DCs and cells from the monocyte lineage (Fogg et al., 2006). Subsequently, the common DC progenitor (CDP) was discovered based on the expression of Flt3, which is an important mediator for the generation of all DC subsets (Auffray et al., 2009; Naik et al., 2007). Since CDPs are unable to produce monocytes, these findings further substantiate the notion that DCs and monocytes form distinct lineages due to their distinguished ontogenies. More recently, a

committed monocyte progenitor has now been identified, known as the common monocyte progenitor (cMoP), which only possesses the ability to form monocytes (Hettinger et al., 2013) **[Figure 1.1]**.

1.2.3 Growth factors and monocyte development

Monocyte development relies critically on the growth factor M-CSF, as *Csf1^{op/op}* (a mutation that inactivates *Csf1*) and *Csf1r^{-/-}* mice displayed profound monocytopenia and reduced tissue resident macrophages (Dai et al., 2002). Nevertheless, each monocyte subset is affected by *Csf1r*-signalling in varying degrees. In particular, antibody blockade or conditional deletion of *Csf1r* leads to the depletion of Ly6C^{lo} monocytes but marginally affects the Ly6C^{hi} subset. These findings hence suggest that CSF1R-signalling functions as a survival factor mainly for terminally differentiated Ly6C^{lo} monocytes (Greter et al., 2012; Hashimoto et al., 2011; Yona et al., 2013). More importantly, *Csf1r*-signalling is unlikely to promote monopoiesis on its own, but may synergise with other growth factors such as stem cell factor (SCF), leukaemia inhibitory factor (LIF), interleukin-3 (IL-3) and IL-6 (Hettinger et al., 2013; Pixley and Stanley, 2004).

Besides M-CSF, the mechanistic action of other growth factors on monocytes has also been investigated. Among them, GM-CSF (also called *Csf2*) is a crucial growth factor for the development of certain subsets of macrophages and DCs, such as alveolar macrophages (Shibata et al., 2001) or cDC1s (Greter et al., 2012). However, at the steady-state, GM-CSF is dispensable for the development of both monocyte subsets (Greter et al., 2012; Italiani and Boraschi, 2014).

Nevertheless, the effect of G-CSF (also called *Csf3*) on monocyte development remains elusive. While G-CSF treatment in mice leads to the loss of monocytes, it is unclear whether this occurs as a direct effect or is the result of a hematopoietic shift towards granulopoiesis (Christopher et al., 2011).

1.2.4 Transcriptional control of monocyte development

While the transcriptomic programming of tissue resident macrophages and DC subsets are well described (Gautier et al., 2012; Miller et al., 2012), little is known about how monocyte development is transcriptionally controlled. Nevertheless, interferon regulatory factor 8 (IRF8) has been reported to be a key factor in promoting the differentiation of early myeloid progenitor towards the monocytic lineage. Specifically, IRF8 physically interacts with CCAAT/enhancer-binding protein alpha (C/EBP α), which results in the inhibition of C/EBP α activity. Hence, while granulocyte development remains unaffected in the absence of IRF8, Ly6C^{hi} monocyte differentiation is greatly impaired (Kurotaki et al., 2014). Furthermore, Kruppel-like factor 4 (KLF4) has also been reported to be essential for monocyte differentiation, as its absence results in impaired Ly6C^{hi} monocyte numbers (Alder et al., 2008; Feinberg et al., 2007). Specifically, *Irf8*^{-/-} myeloid progenitors do not express KLF4 and overexpression of KLF4 in *Irf8*^{-/-} myeloid progenitors does not fully restore monocyte differentiation, suggesting that KLF4 possibly acts downstream of IRF8 (Kurotaki et al., 2013). Notably, cMoPs were found to differentiate into Ly6C^{hi} monocytes before giving rise to Ly6C^{lo} monocytes (Hettinger et al., 2013). However, *Irf8*^{-/-} and *Klf4*^{-/-} deficient mice display relatively normal Ly6C^{lo} monocyte

numbers, suggesting the existence of an alternative developmental pathway for this subset (Alder et al., 2008; Kurotaki et al., 2014).

1.2.5 Ly6C^{lo} monocyte development

A significant advancement in the understanding of Ly6C^{lo} monocyte biology arose from the discovery of NR4A1 as a critical regulator of their development. Specifically, Ly6C^{lo} monocytes numbers in the blood, BM and spleen were drastically reduced despite normal Ly6C^{hi} monocyte numbers in *Nr4a1*^{-/-} mice (Hanna et al., 2011). In particular, the remaining Ly6C^{lo} monocytes in *Nr4a1*^{-/-} were found to be arrested in the S phase, suggesting that NR4A1 is required for the full differentiation and maturation of Ly6C^{lo} monocytes from Ly6C^{hi} monocytes (Hanna et al., 2011). Furthermore, human non-classical monocytes also express high levels of NR4A1, which suggests a similar importance of this pathway in human monocyte development (Hanna et al., 2012).

1.2.6 Monocyte life-cycle and trafficking patterns

1.2.6.1 Monocyte egress from the bone marrow

Monocytes arise from cMoPs in the BM and develop into mature Ly6C^{hi} monocytes before being released into the blood (Hettinger et al., 2013). In comparison to other myeloid cells, monocytes have an exceedingly short transit time through the BM and are rapidly released into the circulation after their last division (Goto et al., 2003). Therefore, it is essential that the immune system tightly regulates the retention/release of BM monocytes such that only mature cells are allowed to access the

circulation. In particular, CCR2 is a critical factor that regulates BM monocyte egress, as *Ccr2*^{-/-} mice displayed a profound reduction in circulating Ly6C^{hi} monocytes that was accompanied by an increase in their BM numbers (Tsou et al., 2007).

Besides the role of CCR2-signaling in mediating monocyte egress in the steady-state, a wide range of mediators promotes the mobilisation of Ly6C^{hi} monocytes from the BM during inflammatory conditions. These factors include Toll-like receptor (TLR) ligands (Shi et al., 2011) and type I interferons (Jia et al., 2009). Despite the identification of these factors as BM monocyte mobilisers, it was not well understood how an infection in distant tissue compartments could trigger the mobilisation of these cells from the BM. To this end, Pamer's group addressed this question through an elegant study, which demonstrated BM stromal cells as a relay point in the integration of inflammatory stimuli and promotion of myeloid cell release into the circulation. Specifically, they showed that Nestin⁺ stromal cells are able to sense small doses of LPS through TLR4, resulting in a CCL2 production that promotes the egress of CCR2-expressing Ly6C^{hi} monocytes (Shi et al., 2011). While this study had focused only on the expression of CCL2 from LPS-stimulated stromal cells, it is likely that other danger associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) could lead to similar effects, as BM stromal cells are known to express a wide range of TLRs (Shi et al., 2011).

While CCR2-signaling serves as the main mechanistic pathway for monocyte mobilisation, a few chemokine receptors have also been reported to fine-tune the release of Ly6C^{hi} monocytes from the BM. Among them, CX3CR1 was found to influence the accumulation of

monocytes in the BM after cyclophosphamide-induced myeloablation (Jacquelin et al., 2013). More importantly, it is known that the CXCR4/CXCL12 axis functions as a key BM retention signal for a wide range of leukocyte populations, including haematopoietic stem cells (HSCs), B cells or neutrophils (Nagasawa, 2014). Furthermore, the inhibition of CXCR4 is thought to increase the mobilisation of Ly6C^{hi} monocytes from the BM (Liu et al., 2015). However, it remains unclear how CX3CR1 and CXCR4 may cooperate with CCR2 for monocyte mobilization in the steady state, and whether they act similarly in the context of an infectious stimulus

Unlike Ly6C^{hi} monocytes, mechanisms underlying the egress of Ly6C^{lo} monocytes from the BM are not well documented. Since Ly6C^{lo} monocytes do not express CCR2, their exit from the BM seems largely independent of CCR2. Nevertheless, a recent study reported that S1PR5 mediates the BM release of Ly6C^{lo} monocytes (Debien et al., 2013). However, how CX3CR1 and CXCR4 act on the trafficking of Ly6C^{lo} monocytes remain poorly defined.

1.2.6.2 The fate of circulating monocytes

1.2.6.2.1 Infiltration of Ly6C^{hi} monocytes into tissues

Ly6C^{hi} monocytes are highly versatile cells that survey the peripheral circulation for inflammatory sites. During the steady-state, Ly6C^{hi} monocytes rarely infiltrate tissues to replace tissue resident macrophage populations (Ginhoux and Guilliams, 2016). However, upon sensing inflammatory stimuli, they infiltrate rapidly into these sites and can differentiate into monocyte-derived macrophages. While most tissue

resident macrophages are not derived from monocytes in the steady-state, certain organs that experience a chronic low-grade inflammation allow the infiltration of Ly6C^{hi} monocytes to replenish the resident tissue macrophage population. In particular, gut tissue macrophages display a relatively short half-life of 3 weeks and hence require Ly6C^{hi} monocyte infiltration to maintain their numbers (Zigmond et al., 2012). Furthermore, Ly6C^{hi} monocytes also infiltrate the heart or pancreas albeit with a slower kinetics during the steady-state (Calderon et al., 2015; Molawi et al., 2014).

Notably, the infiltration of leukocytes into tissues have also been found to follow circadian patterns. These trafficking patterns may arise from a concept of anticipatory inflammation and could represent an evolutionary adaptation to pathogens that are encountered at certain time of the day (Scheiermann et al., 2012). In agreement with this hypothesis, the expression of CCL2 by endothelial cells was found to be at its highest during the active phase (which correspond to night time for rodents and day time for humans), when circulating CCR2⁺ Ly6C^{hi} monocyte numbers are at its lowest (Nguyen et al., 2013; Scheiermann et al., 2012).

1.2.6.2.2 Ly6C^{lo} monocytes survey vascular integrity

Early work on Ly6C^{lo} monocytes suggests that these cells could act as an early responder during inflammatory responses. Specifically, Ly6C^{lo} monocytes were found to infiltrate the peritoneum before granulocytes and Ly6C^{hi} monocytes, and acted as the main producer of TNF- α during the early phase of infection after intraperitoneal inoculation of *Listeria*

monocytogenes (Auffray et al., 2007). However, apart from this initial study, little is known about the capacity of Ly6C^{lo} monocytes to extravasate out of blood vessels and their function upon entering into tissues. Instead, Ly6C^{lo} monocytes are now seen as a terminally differentiated population, whose main role is to survey and maintain blood vessel integrity. To perform these roles, Ly6C^{lo} monocytes constitutively "patrol" peripheral tissues by exhibiting a long-range crawling onto the blood endothelium (Auffray et al., 2007; Carlin et al., 2013). In particular, Ly6C^{lo} monocytes are able to remove vascular amyloid- β in a mouse model of Alzheimer's disease (Michaud et al., 2013) and can scavenge oxidised low-density lipoproteins during atherosclerosis (Wu et al., 2009). In addition, Ly6C^{lo} monocytes can recruit neutrophils to remove damaged endothelial cells upon sensing vascular insults (Carlin et al., 2013).

1.2.6.2.3 Monocyte contribution to the marginal pool

While Ly6C^{hi} monocytes are adapted towards infiltrating sites of inflammation, they were also found to rapidly disappear from the peripheral circulation in the absence of inflammation (Varol et al., 2007). Several phenomena may account for their disappearance: (1) their accumulation as a marginal pool, (2) their homing back to the BM or (3) their entry into the spleen reservoir.

Early kinetic studies and the establishment of mouse blood volume in the steady state revealed that the blood vascular system consisted of two different pools of cells: a circulating pool and a marginal pool (van Furth and Sluiter, 1986). In particular, the marginal pool contains

leukocytes that are temporarily withdrawn from the circulation. Two main phenomena account for the margination of leukocytes: 1) the sequestration of leukocytes into small diameter microvessels such as the lung vascular bed, and 2) collisions between "soft" red blood cells (RBCs) and "rigid" leukocytes that push the latter onto vessel walls in large diameter veins (Fay et al., 2016). While the majority of leukocytes are proportionally divided between the marginal and circulating pools in the steady state, an early study by van Furth determined that around 60% of total blood monocytes in mice are margined (van Furth and Sluiter, 1986). Furthermore, the existence of a marginal pool of monocytes has also been demonstrated in humans. In this study, radio-isotope labelling of monocytes in humans revealed that about 75% of monocytes belong to the marginal pool (Meuret and Hoffmann, 1973), which is close to the size of the marginating pool of granulocytes (Athens et al., 1961). Granulocytes have also been found to freely exchange between the circulating and marginating pools, suggesting that this phenomenon may occur for monocytes too.

While the concept of margination is not recent, mechanisms underlying this phenomenon are not fully understood. Initial work using the infusion of vasoactive catecholamines such as epinephrine in humans has led to significant leukocyte demargination (BIERMAN et al., 1952). In such conditions, demargination has been associated with the downregulation of adhesion molecules that include selectins (Weber et al., 2004). However, it is unclear whether other molecules such as integrins and chemokine receptors may affect lung margination. Furthermore, biophysical factors are involved in the margination process, as epinephrine treatment on leukocytes was found to reorganise their actin cytoskeleton, resulting in enhanced pliability and a reduced likelihood for

these cells to be sequestered within the microcirculation (Fay et al., 2016).

1.2.6.2.4 Monocyte homing back to the BM

In the absence of inflammation, Ly6C^{hi} monocytes were found to home back constitutively to the BM (Varol et al., 2007). However, the underlying reasons for this trafficking pattern remain unclear. It was suggested that the BM could act as a site for the conversion of Ly6C^{hi} to Ly6C^{lo} monocytes (Varol et al., 2008). However, while it is clear that Ly6C^{lo} monocytes in the periphery are generated from blood Ly6C^{hi} monocytes, recent data have suggested that Ly6C^{lo} monocytes can be generated in the BM *in situ* and may thus constitute a different population from their blood counterparts (Yona et al., 2013).

Alternatively, the homing of Ly6C^{hi} monocytes to the BM may arise due to the need of these cells to act as a local APC. For instance, infected neutrophils were found to home back to the BM and prime CD8⁺ T cells alongside other myeloid APCs during vaccinia infection (Duffy et al., 2012). In support of this proposition, Ly6C^{hi} monocytes have been shown to have the capacity to directly present blood-borne antigens and activate BM CD8⁺ T cells (Milo et al., 2013).

1.2.6.2.5 Monocyte trafficking in the spleen

While the BM has been traditionally held as the only organ that contains bona fide monocytes, this notion was subsequently challenged by

seminal studies conducted by Swirski and colleagues, who discovered a secondary reservoir of monocytes in the spleen. Notably, the population of monocytes in the spleen was relatively large and equivalent to the number of mature monocytes that were found in one tibia of the mouse (Swirski et al., 2009). Furthermore, both Ly6C^{hi} and Ly6C^{lo} subsets of monocytes were equivalently represented in numbers and are located in the red pulp. More importantly, Ly6C^{hi} monocytes, and to a lesser extent, Ly6C^{lo} monocytes can be mobilised from the spleen into the blood through Angiotensin II/AGTR1A signalling. Interestingly, the mobilisation of monocytes from the spleen, in contrast to the BM, is CCR2-independent (Swirski et al., 2009). Moreover, the production of Angiotensin II during myocardial infarction results in the infiltration of Ly6C^{hi} monocytes into the infarct that originated from the spleen and the peripheral circulation, but not from the BM (Swirski et al., 2009). Nevertheless, it remains unclear if infectious agents could also induce the mobilisation of monocytes from the splenic reservoir.

While the importance of Ly6C^{lo} monocytes in the spleen is still relatively unclear, it is likely that Ly6C^{lo} monocytes may complement the function of red pulp macrophages, since these cells are known to specialise in the engulfment of cellular debris. Furthermore, Ly6C^{lo} monocytes may help to promote antigen tolerance as they can cross-present antigens derived from apoptotic bodies to CD8⁺ T cells and suppress their activation in a PDL1-dependent fashion (Peng et al., 2009).

1.2.7 Monocytes and monocyte-derived cells in health and disease

As circulating cells that constitutively patrol tissue compartments for vascular breaches and inflammation, monocytes play critical roles in health and diseases by ensuring that pathogens are efficiently eliminated and inflammation subsided back to homeostasis. While monocytes possess unique functions as effector cells in their own right, their role in inflammatory diseases often relies on their differentiated state, either as monocyte-derived dendritic cells or macrophages (Mildner et al., 2013).

1.2.7.1 Monocyte-derived cells as a “emergency squad” during inflammation

It is well established that Ly6C^{hi} monocytes minimally replenish tissue resident macrophages (Ginhoux and Guillemins, 2016) and do not give rise to cDCs, as these cells possess their own committed progenitor (Naik 2007, Auffray 2009), during the steady state. However, tissue resident macrophages and cDCs often die of apoptosis/necrosis or migrate out of the tissues during inflammation. Therefore, the absence of these cells during inflammation is often replenished by Ly6C^{hi} monocytes that typically serve as an "emergency squad" to fill their absence (Mildner et al., 2013). Notably, Ly6C^{hi} monocytes are highly plastic cells that augment the immune response according to the environmental stimuli. They also display enhanced abilities at detecting inflammation, performing effector functions (phagocytosis, pro-inflammatory cytokine production, local antigen presentation) and participating in tissue remodelling and resolution (degradation of the extra-cellular matrix, efferocytosis) (Mildner et al., 2013; Segura and Amigorena, 2013).

As Ly6C^{hi} monocytes enter the inflamed tissues to replace and complement the function of tissue resident macrophages and cDCs, it is not surprising that the resulting monocyte-derived cells (MCs) share common macrophage and DC markers during their differentiation. These shared phenotypic markers, which includes F4/80, CD11c and MHCII, results in a substantial amount of difficulty in discriminating MCs from tissue-resident macrophages and cDCs (León et al., 2007; Nakano et al., 2009; Serbina et al., 2003b). Therefore, a wide variety of names has been given to MCs depending on where and how they were discovered and these names include inflammatory macrophages, exudate macrophages, inflammatory DCs, monocyte-derived DCs, TNF- α /iNOS-producing (Tip)-DCs (Geissmann et al., 2010).

To address the confusion in MC discrimination from tissue-resident macrophages and cDCs, the Immunological Genome (ImmGen) Project was recently started (Heng et al., 2008). In this project, prototypical macrophages, DCs and MCs populations were processed and sorted for microarray before comparative analysis. Notably, studies from the ImmGen project has helped to delineate core transcriptomic signatures and markers for tissue-resident macrophages (MerTK, CD64) (Gautier et al., 2012) and cDCs (CD26, BTLA, *Zbtb46*) (Miller et al., 2012). Despite this initiative, the study and tracking of MCs from other phagocytes during inflammation still remains considerably challenging. Therefore, fate-mapping models using novel transgenic mice have recently been generated (including *Cx3cr1*^{creERT2} and *Ccr2*^{creERT2}), and these tools allow enhanced targeting of MCs for a better understanding of their involvement during inflammation (Croxford et al., 2015; Yona et al., 2013).

1.2.7.2 Monocyte-derived cells function

While monocyte-derived DCs and macrophages have been widely described in previous literature, their involvement and function during infection remain unclear. To this end, Pamer's group were the first to describe the involvement of an MC population during infection in a seminal study. They named this population Tip-DCs, as these cells were the major producer of these pro-inflammatory mediators during *Listeria monocytogenes* infection (Serbina et al., 2003b). More importantly, Tip-DCs were absolutely vital for the control of disease progression, as their absence was associated with increased bacteria load and mortality (Kurihara et al., 1997; Serbina et al., 2003b; 2003a). Subsequently, MCs in other contexts were described as key players not only during bacterial infection, but also during viral (Aldridge et al., 2009), fungal (Hohl et al., 2009) and parasitic (León et al., 2007) infections.

During the course of an infection, the infiltration of MCs and their associated functions are generally beneficial for the host and is in agreement with their supposed synergistic action with tissue resident macrophages and cDCs (Mildner et al., 2013). For instance, MCs can act as an effector cell which produce pro-inflammatory cytokines *in situ* (Serbina et al., 2003b; Soudja et al., 2012) and perform pathogen phagocytosis (Espinosa et al., 2014; Hohl et al., 2009). Secondly, they can function as a local APC (Aldridge et al., 2009), or transport antigens to the draining lymph node where they prime naive T cells (Rivera et al., 2011; Samstein et al., 2013). Thirdly, they are able to dampen inflammation and protect the host against excessive damage (Grainger et al., 2013; Nascimento et al., 2014).

However, it is noteworthy that the differentiation and function of MCs are highly dependent on the context, timing and tissue location. For example, while MCs are required to diminish the bacteria burden, they are dispensable in priming CD4⁺ and CD8⁺ T cells, during *Listeria monocytogenes* infection (Serbina et al., 2003b). In contrast, MCs were found to promote Th1 response by producing IL-12 and presenting antigens to specific CD4⁺ T cells during *Leishmania major* infection (León et al., 2007). Therefore, further work would be required to determine the factors that stimulate the specific functions of MCs.

1.2.7.3 Detrimental effects of monocyte derived cells

While MCs are remarkable in their ability to combat infections, their plasticity and associated function also result in substantial collateral damage during immunopathologies such as allergies, autoimmune diseases, inflammatory bowel disease and cancer.

It has been reported that MCs act as pro-inflammatory effector cells during experimental autoimmune encephalomyelitis (EAE) where they produce pro-inflammatory cytokines and initiate demyelination (Yamasaki et al., 2014). Similarly, during DSS-induced colitis, infiltrating CX3CR1^{int} MCs are key drivers of the pathology as they produced high amounts of IL-6 and IL-1 β and do not adopt a tolerogenic profile such as CX3CR1^{hi} lamina propria macrophages (Bain et al., 2012; Zigmond et al., 2012).

MCs have also been shown to perform DC-like functions and contribute to house dust mite related asthma. As such, they participate in the

priming of Th2 mediated immunity by transporting the allergen into the draining lymph node where they prime CD4⁺ T cells *in situ* (Plantinga et al., 2013).

Finally, MCs can display macrophage-like features during cancer and metabolic disorders. For instance, MCs (which are classically called tumour associated macrophages or TAM) are essential players of cancer progression on multiple aspects. Firstly, they promote tumour intravasation and metastasis through the production of tissue remodelling factors. Secondly, they support angiogenesis via the production of VEGF-A, and the suppression of anti-tumoural responses via the recruitment of Tregs or the direct inhibition of T cell responses through the PD-L1/PD-L2 pathway (Noy and Pollard, 2014).

1.2.7.4 Monocyte-derived cells development

Unlike tissue resident macrophages and cDCs, the developmental requirements of MCs are still poorly understood. In a seminal study, Sallusto and Lanzavecchia generated the first MCs in vitro displaying DC-like properties using GM-CSF and IL-4 (Sallusto et al., 1994). Similarly, GM-CSF was used on mouse BM cells in vitro to generate MCs with DC-like features (Helft et al., 2015; Inaba et al., 1992; Lutz et al., 1999).

Since then, the implication of GM-CSF in the development of MCs have been extensively researched. Evidence have suggested that GM-CSF plays an important role in a wide range of animal models during infection (Zhan et al., 1998) or autoimmune diseases (Campbell et al., 1997;

McQualter et al., 2001). Specifically, *Csf2*^{-/-} mice displayed increased pathogen burden during *Listeria monocytogenes* infection, and are completely protected from EAE, which is in line with the involvement of MCs in these diseases. However, since multiple myeloid cell types are responsive towards GM-CSF-signalling (Croxford et al., 2015), the precise implication of this signalling on MCs remains to be formally demonstrated. Nevertheless, several inflammatory models using competitive BM chimaeras (WT vs GM-CSF-signalling deficient) have provided some insights into their regulatory function (Greter et al., 2012). Surprisingly, M-CSF, but not GM-CSF, was required by MCs for their development (Greter et al., 2012). However, GM-CSF appeared to be necessary for the priming of their effector phenotype, as MCs that were deficient for GM-CSF-signalling in a novel genetic model showed full protection against EAE. In particular, GM-CSF was necessary to "license" MCs for a pathogenic phenotype in the central nervous system (Croxford et al., 2015). Therefore, it seems that MCs require different factors for their development and function, with M-CSF regulating their survival and differentiation while GM-CSF controls the execution of their effector function.

However, certain questions still require further investigation. In particular, it is still not clear whether all Ly6C^{hi} monocytes show the same potential to give rise to MCs skewed towards a more DC-like or macrophage-like phenotype, or if they show a certain degree of commitment before entering the tissues. Several pieces of evidence suggest that this could be the case, as Ly6Chi monocytes can be primed in the BM through local IFN- γ production by NK cells towards immunoregulatory functions (Askenase et al., 2015).

1.2.8 Chemokine receptors and monocyte trafficking

Among mononuclear phagocytes, a key feature of monocytes lies in their ability to promptly navigate between distinct tissue compartments. For instance, monocytes need to leave the BM where they are generated to access the peripheral circulation. Once in the circulation, monocytes can enter inflamed tissues, accumulate in the spleen as a reservoir or home back to the BM. Thus, checkpoints are put in place to ensure proper host defence while avoiding self-damage. Chemokine receptors and adhesion molecules play a central role to maintain those checkpoints, and orchestrate monocyte migratory properties. Hence, in the following part of the introduction, we are going to review the major chemokines and chemokine receptors involved in the maintenance of monocytes compartmentalisation.

1.2.8.1 Chemokines and chemokine receptors

Chemokines are a superfamily of small cytokines (~8-10kDa) that help coordinate cell migration *in vivo* and are present in all vertebrates. According to its etymology, chemokines promote chemotaxis, which defines as a chemical that promotes migration. In addition to chemotaxis, chemokines are now increasingly being recognised for their role in cell activation and mediating tissue development during embryogenesis (Zlotnik and Yoshie, 2012).

Chemokines present cysteine residues that are essential for the formation of tertiary protein folding through disulphide bonds. Their structural formations are widely varied, which results in different

numbers and arrangement of their cysteine residues that are employed towards their classification. In particular, 4 groups of chemokines exist based on the spacing between cysteine residues: XC, CC, CXC, CX3C (where X represent a non-cysteine amino acid). Apart from the XC family which contains only 2 chemokines (XCL1 and XCL2), all other chemokines are known to contain 4 cysteine residues.

Historically, chemokines have been categorised into homeostatic and inflammatory chemokines (Zlotnik and Yoshie, 2012). Homeostatic chemokines are involved mainly in basal cell migration such as T-cell migration into lymphoid organs. In contrast, inflammatory chemokines are mainly expressed in response to pro-inflammatory stimuli, such as LPS, TNF- α or IL-1 β . However, this distinction is now currently challenging to define as an increasing number of chemokines are discovered to harbour functions that can be considered to be both homeostatic and inflammatory.

Chemokines bind to 7-trans membrane domain chemokine receptors and rely on G-proteins to mediate their signal transduction. To promote cell migration, the conventional signal transduction results in the successive generation of phosphatidylinositol 4,5-bisphosphate (PIP₂), diacylglycerol (DAG) and inositol triphosphate (IP₃), which ultimately leads to a Ca²⁺ flux from internal organelles. Notably, this Ca²⁺ flux is typically used as a readout for chemokine-mediated signal transduction (Pawig et al., 2015). Following signal transduction, β -arrestin mediates desensitisation and is recruited at the chemokine receptor site to promote its internalisation through clathrin-mediated endocytosis. This so-called desensitisation process is required to prevent chemokine receptor "over-signalling" (Bennett et al., 2011).

However, not all chemokine receptors present signalling properties. In particular, atypical chemokine receptors (ACKRs) (Nibbs and Graham, 2013) typically function as scavengers and sinks that help to buffer chemokine bioavailability (ACKR1, ACKR2) (Ford et al., 2014; Peiper et al., 1995). They can also help to shape gradients that indirectly promote cell migration during and after neonatal development (ACKR3, ACKR4) (Abe et al., 2014; Ulvmar et al., 2014).

Nevertheless, there is a high degree of redundancy between chemokines that further contributes towards their complexity. Although an estimated 50 chemokines exist in human, only around 25 chemokine receptors have been identified. Furthermore, several chemokines usually bind to the same receptor or one chemokine may bind to multiple receptors. For example, CXCL12 binds to CXCR4 and ACKR3, whereas ACKR3 can bind CXCL11 and CXCL12. In the context of monocytes, several chemokine receptors, namely CCR2 and CX3CR1, have been found to be crucial for their trafficking in steady and disease state. However, emerging candidates, such as CXCR4, have also been noted to be increasingly involved in pathology, which prompts for further studies in order to understand its role.

1.2.8.2 CCR2 and its ligands

CCR2 and its cognate ligands have been closely associated with monocyte/macrophage trafficking and function. Interestingly, the identification of CCL2 and its subsequent cloning was based on an initial study that involved the observation of monocytes/macrophage infiltration

into tumours. As such, Leonard and colleagues determined the molecular basis underlying this infiltration and successively isolated CCL2 from human glioma cell lines (Yoshimura et al., 1989). A few years later, Charo and colleagues characterised two human receptors that demonstrated a high binding ability for CCL2: CCR2A and CCR2B (Charo et al., 1994). Notably, the main difference between these two isoforms lies in their cell sub-localisation: while CCR2B is mainly expressed on the cell surface, CCR2A remains mostly cytosolic, and is hence largely uninvolved in the binding of CCL2 *in vivo* (Wong et al., 1997). Furthermore, only one CCR2 isoform is present in mouse, and display ~80% homology with human CCR2B, which suggests a conserved function between these two species (Kurihara and Bravo, 1996).

Although additional ligands for CCR2 were subsequently being discovered, CCL2 and CCL7 remain the most important ligands in both mice and humans. Of note, a high degree of redundancy and complexity exist between CCR2 and its ligands. For instance, CCL2 can bind to CCR4, while CCL7 does not bind to CCR4 but can bind to CCR1 and CCR3 [Table 1.2].

Ligands	Host	Receptors
CCL2	human, mouse	CCR2, CCR4, ACKR1, ACKR2
CCL7	human, mouse	CCR1, CCR2, CCR3, ACKR1, ACKR2
CCL8	human, mouse	CCR1, CCR2, CCR5, CCR8, ACKR1, ACKR2
CCL12	mouse	CCR2
CCL13	human	CCR1, CCR2, CCR3, ACKR1, ACKR2
CCL16	human	CCR1, CCR2, CCR5, ACKR2

Table 1.2 CCR2 and its ligands in mice and humans.

Among immune cells, monocytes classically display the highest expression of CCR2, followed by cDCs and subsets of NK and T cells (Bachelier et al., 2014). Furthermore, the expression of CCR2 differs depending on the subset of monocyte, with Ly6C^{hi} monocytes expressing high levels of CCR2, while Ly6C^{lo} monocytes are CCR2⁻ (Geissmann et al., 2003). In addition, this differential expression is functionally relevant as CCR2-deficient mice display ~90% decrease in circulating Ly6C^{hi} monocyte numbers, while Ly6C^{lo} monocytes remain unaffected (Qu, 2004; Serbina and Pamer, 2006; Tsou et al., 2007). Mechanistically, these low circulating Ly6C^{hi} monocyte numbers in *Ccr2*^{-/-} are a result of their inability to leave the BM, while splenic Ly6C^{hi} monocyte numbers mirror those of the blood (Serbina and Pamer, 2006). Notably, while *Ccl2*^{-/-} and *Ccl7*^{-/-} mice display a similar phenotype, *Ccl8*^{-/-} and *Ccl12*^{-/-} do not display any similarities, suggesting that CCL2 and CCL7 are the two main chemokines guiding CCR2-expressing Ly6C^{hi} monocytes out of the BM (Tsou et al., 2007). Nevertheless, *Ccl7*^{-/-} mice display larger Ly6C^{hi} monocytes numbers in the BM compared to *Ccl2*^{-/-} mice (Tsou et al., 2007), suggesting a perhaps more important role of CCL7 in mediating BM egress.

Upon entering the circulation, Ly6C^{hi} monocytes depend critically on CCR2-signalling to enter the tissues. As such, tissue macrophage populations that require monocytes for their replenishment during the steady state, such as gut and dermal macrophages, are greatly reduced in *Ccr2*^{-/-} mice (Bain et al., 2012; Tamoutounour et al., 2013). A similar observation can also be drawn during thioglycollate-induced peritonitis, whereby Ly6C^{hi} monocytes require CCR2 to access the peritoneum (Serbina and Pamer, 2006).

While CCL2 and CCL7 are thought to display similar expression patterns during infections and play an equally important role in the peripheral recruitment of Ly6C^{hi} monocytes (Jia et al., 2008), the effects of CCL2 is substantially well documented compared to CCL7. Unlike chemokines such as CX3CL1, CCL3, CCL5, CXCL1 and CXCL2, CCL2 display circadian rhythmic oscillations in tissues during the steady-state (Scheiermann et al., 2012). Specifically, higher levels of CCL2 are expressed by endothelial cells at night and are correlated with increased leukocyte extravasation. During inflammation, such as *Listeria monocytogenes* infection, the recruitment of Ly6C^{hi} monocytes into tissues is increased in concomitant to rising levels of Ccl2 (Nguyen 2013). Furthermore, *Ccr2*-deficiency broadly abolishes Ly6C^{hi} monocyte recruitment to tissues (Serbina and Pamer, 2006). In contrast, Ly6C^{lo} monocytes appeared less affected by circadian oscillations and this is in agreement with their low CCR2 expression (Nguyen et al., 2013). Of note, CCL2 expression is induced by a wide range of pro-inflammatory stimuli such as TLRs and NOD ligands. Moreover, CCL2 is expressed during inflammation by an important number of stromal cell populations and monocytes/macrophages that can amplify their own recruitment (Serbina et al., 2008).

Since the CCR2/CCL2 axis is restricted mostly to monocytes and the expression CCL2 is highly notable during inflammation, there has been increasing interest in targeting its signalling to correct monocyte-associated immunopathologies. In particular, at least four patent applications that propose to target CCR2, in order to modulate monocyte recruitment, have been submitted since 2009, with some applications displaying encouraging results (Jackson and Woollard,

2014). For instance, a peptide blocking CCL2-dependent chemotaxis is able to diminish the progression of EAE (Auvynet et al., 2016) and this is in agreement with findings that *Ccr2*^{-/-} mice are protected from EAE (Fife et al., 2000). A more direct targeted approach using CCR2 inhibition can also be achieved using lipid nanoparticles containing a siRNA directed against CCR2. Specifically, monocytes that ingest such nanoparticles displayed decreased CCR2, which led to reduced Ly6C^{hi} monocyte infiltration and a better prognosis/lesser clinical signs during myocardial infarction, atherosclerosis and lymphoma (Leuschner et al., 2011). However, while these results are highly promising, caution needs to be taken when targeting the CCR2/CCL2 axis in therapy. In particular, studies have shown that the withdrawal of anti-CCL2 treatment leads to increased metastasis and mortality in a mouse model of breast cancer (Bonapace et al., 2014). A possible explanation may be due to the fact that MCs may acquire self-renewal capacity during their differentiation and do not need constant replenishment from Ly6C^{hi} monocytes even though their initial recruitment into tumours is CCR2-dependent (Franklin et al., 2014). Furthermore, a similar mechanism occurs during atherosclerosis (Robbins et al., 2013), which highlights potential applications in other chronic pathologies.

1.2.8.3 The CX3CR1-CX3CL1 axis

Unlike CCR2 that has multiple ligands, CX3CR1 and CX3CL1 form a "monogamous" pair, as CX3CL1 is the sole ligand for CX3CR1 while this is also reciprocally true. Historically, CX3CR1 was discovered to be highly expressed in the gut and tissues of the rat central nervous system, where it was subsequently cloned (Harrison et al., 1994). Interestingly, its ligand CX3CL1 also exists as a membrane-bound form.

Hence, expressed of CX3CL1 by the endothelium under flow conditions can also act as an adhesion molecule (Fong et al., 1998).

A major progress in the understanding of CX3CR1 in leukocyte trafficking was aided by the generation of the *Cx3cr1^{gfp/+}* mouse by Jung and colleagues (Jung et al., 2000). Expression of *Cx3cr1* in this fluorescent reporter was initially found to be restricted to monocytes, subsets of T and NK cells, splenic DCs as well as certain macrophages such as the microglia (Jung et al., 2000), which is in line with a previous report (Harrison et al., 1994). While more recent findings indicate that all macrophages express *Cx3cr1* during their development (Lavin et al., 2014; Yona et al., 2013), *Cx3cr1* expression in adult macrophages is highly restricted to certain tissues such as the microglia, kidney and gut macrophages (Yona et al., 2013). Interestingly, *Cx3cr1* expression patterns closely correspond to the expression patterns of *Cx3cl1* in the fluorescent reporter mouse that was generated by Jung's group (Kim et al., 2011). Specifically, *Cx3cl1* is restricted to stromal populations and is mainly expressed by neurones, kidney tubular epithelium and glomeruli, as well as intestine epithelial cells, where CX3CR1⁺ macrophages were found to be in close proximity with *Cx3cl1*-expressing cells. Those interactions indicate a functional relevance as *Cx3cr1*-deficient microglia show defective synaptic pruning (Paolicelli et al., 2011). In addition, CX3CR1/CX3CL1 interactions in the gut are responsible for the extensions of transepithelial dendrites (TEDs), which are pseudopods emitted by CX3CR1⁺ macs through the epithelium to sample bacteria in the lumen (Niess et al., 2005).

Among monocytes, *Cx3cr1* is differentially expressed between both subsets, as Ly6C^{lo} monocytes are CX3CR1^{hi} whereas Ly6C^{hi} monocytes

are CX3CR1^{int} (Geissmann et al., 2003). Notably, similar expression patterns have been reported in humans, as high and low levels of CX3CR1 were found on CD16⁺ and CD14⁺ monocytes respectively (Geissmann et al., 2003). The high expression of CX3CR1 in Ly6C^{lo} monocytes is also functionally relevant, as *Cx3cr1*-deficient monocytes display diminished patrolling behaviour in the blood vasculature (Auffray et al., 2007; Carlin et al., 2013). Moreover, membrane-bound CX3CR1-signalling within the endothelium is important to promote Ly6C^{lo} monocyte survival, as they displayed increased cell death by apoptosis in *Cx3cr1*-deficient mice (Landsman et al., 2009). Similarly, a human polymorphism for CX3CR1 (named CX3CR1-M280 due to a threonine substitution for a Methionine in position 280) is associated with impaired monocyte chemotaxis and adhesive properties (McDermott et al., 2003). Of note, since CX3CR1 is expressed only at intermediate levels in Ly6C^{hi} monocytes, this may suggest a diminished role of CX3CR1-signalling in their trafficking. Nevertheless, *Cx3cr1*^{-/-} mice showed impaired recruitment of Ly6C^{hi} monocytes in the spleen during *Listeria monocytogenes* infection, which was linked to increased bacterial burden (Auffray et al., 2009). A similar phenotype can be observed during systemic Candidiasis whereby *Cx3cr1*^{-/-} mice displayed reduced survival, as well as reduced infiltration of MCs in kidneys and increased organ damage (Lionakis et al., 2013).

Since CX3CR1/CX3CL1-signalling can promote the chemotaxis and adhesion of monocytes, it is perhaps not surprising to that this axis is implicated in a high number of monocyte-associated immunopathologies. Indeed, *Cx3cr1*-deficient Ly6C^{hi} monocytes showed reduced infiltration into atherosclerotic plaques (Combadière et al.,

2003), and is correlated with a reduced risk of atherosclerosis in CX3CR1-M280 patients (McDermott et al., 2003).

However, while CX3CR1-inhibition/deficiency may ameliorate certain monocyte-associated diseases, its absence also results in unexpected phenotypes. For instance, in a model of age macular degeneration, *Cx3cr1*^{-/-} displayed increased accumulation of MCs in the retina, which was associated with an increased damage of photoreceptors (Combadière et al., 2007; Sennlaub et al., 2013). This was further correlated with an increased risk of age macular degeneration in CX3CR1-M280 individuals (Combadière et al., 2007). More recently, in a cancer model, *Cx3cr1*-deficient Ly6C^{lo} monocytes showed an impaired arrest in lung microvasculature, which ultimately led to a failure in NK cell recruitment and increased tumour burden, suggesting a protective role for CX3CR1 in this aspect (Hanna et al., 2015).

Unlike CCR2 that promises certain therapeutic effect in the clinics, pre-clinical data collected from mouse models targeting CX3CR1 show more contrasting results. Furthermore, CX3CR1 inhibition has yielded little clinical applications thus far, despite the generation of a blocking peptide (Dorgham et al., 2009). This failure could possibly be attributed to the complexity of the CX3CR1/CX3CL1-signalling axis. In particular, CX3CR1 not only promotes chemotaxis, but also adhesive and anti-apoptotic effects. Moreover and unlike CCR2, several tissue macrophages express high levels of CX3CR1, which can be a major confounding factor during inflammation. Therefore, further studies are needed to understand the full complexity and mechanistic actions of the CX3CR1-CX3CL1 axis.

1.2.8.4 The CXCR4-CXCL12 axis

CXCL12 was initially identified from mouse stromal cell lines by Honjo and colleagues, where it was originally named SDF-1 for stromal derived factor 1 (Tashiro et al., 1993). Soon thereafter, it was independently cloned by Nagasawa and colleagues, who described it as a pre-B cell growth factor that could promote their development in synergy with IL-7 (Nagasawa et al., 1994). A strong interest in CXCL12 subsequently developed when it was found to block HIV entry, as its receptor CXCR4 was used to promote the virus entry (Bleul et al., 1996; Oberlin et al., 1996). Since these early discoveries, many other roles have been assigned to the CXCL12/CXCR4 axis. Besides being critical for the recruitment of leukocytes into inflamed tissues, CXCL12 also plays pivotal roles in immune responses and the cellular organisation of organs (Nagasawa, 2014).

CXCR4 and CXCL12 show the highest conservation for chemokines and chemokine receptors in vertebrates with more than 95% homology between mice and human. Structurally, CXCL12 is produced by alternative splicing in mice to form 3 isoforms, with CXCL12 α being the predominant form, which is produced by a wide range of stromal cells during embryonic and adult life.

Furthermore, CXCR4/CXCL12 play a crucial role during embryogenesis: in the absence of either *Cxcr4* and *Cxcl12*, mice die prenatally due to defects in hematopoiesis, heart, brain and blood vessel development. Specifically, CXCL12 plays a leading role in the heart development through the formation of the cardiac ventricular septum (Nagasawa, 2014). It also modulates neurogenesis by regulating the appropriate

assembly of neurones, and guiding primordial germ cells to the gonads (Nagasawa, 2014). Moreover, it is implicated in vasculature development by mediating blood vessels branching in many organs. Apart from CXCR4 and CXCL12, ACKR4 (also known as CXCR7) also plays a major role during embryogenesis in shaping CXCL12 gradients and modulating CXCR4-signalling (Sierro et al., 2007; Wang et al., 2011).

In addition to organogenesis, CXCR4/CXCL12 axis modulates a number of hematopoietic processes, including the seeding and the maintenance of HSCs in the BM, as well as B cells (Nagasawa et al., 1996), NK cells (Noda et al., 2011) and pDCs development (Kohara et al., 2007). Specifically, CXCL12 is expressed by a wide range of BM stromal cells that include endothelial cells, osteoblasts, and heterogeneous and overlapping populations of perivascular cells such as *Cxcl12*-abundant reticular (CAR) cells, *LepR*⁺ cells, *Nestin*⁺ cells (Ding and Morrison, 2013; Kunisaki et al., 2013; Omatsu et al., 2014). Consequently, selective deletion of *Cxcl12* in these populations further highlights its importance, as these *Cxcl12*-expressing BM stromal cells are necessary to form microenvironmental niches to regulate HSC self-renewal, proliferation and differentiation.

Apart from the regulation of HSCs, the CXCR4-CXCL12 axis is also known to be important for the BM retention of a wide variety of leukocytes, in particular B cells and neutrophils. To illustrate this aspect, a human immunodeficiency caused by a gain-of-function in CXCR4 results in the WHIM syndrome (for Warts, Hypogammaglobulinemia, Infections and Myelokathexis). Specifically, the majority of WHIM patients carry a CXCR4 protein with a truncated C-terminal cytoplasmic

tail. This leads to impaired recruitment of the β -arrestin, and thus impaired desensitisation that leads to increased CXCR4-signalling. Among other clinical aspects, WHIM patients have profound peripheral neutropenia and suffer from HPV-related warts due to a defect in pDCs and cDCs development (Tassone et al., 2010). Unlike other types of congenital neutropenia, WHIM neutrophils develop normally but are excessively retained in the BM due to increased CXCR4-signalling (McDermott et al., 2011). A CXCR4 inhibitor (AMD3100) is currently in phase I clinical trial in WHIM patients, which restores neutrophil counts and alleviates symptoms (McDermott et al., 2014).

While most studies on WHIM patients have been focused on neutrophils and other leukocytes, little attention has been paid to monocytes. However, a study on WHIM patients showed decreased monocyte blood counts that correlated with increased BM cellularity (Siedlar et al., 2008). Another report showed that monocyte blood counts in WHIM patients could be corrected by AMD3100 administration (McDermott et al., 2011). More recently, a mouse study reported an increase in circulating monocytes that correlated with a mild decrease in BM numbers (Liu et al., 2015). Together, these studies implicate the involvement of CXCR4 in the regulation of monocyte blood counts together with increased monocyte retention in the BM in humans. However, while the involvement of the CXCR4/CXCL12 axis in BM leukocyte retention is well characterised, its implication outside the BM remains largely elusive.

Clinically, the CXCR4/CXCL12 axis is also known to be involved in several human conditions. For instance, accumulating evidences suggest that cancer cells employ multiple aspects of CXCR4/CXCL12

signalling during tumour progression, metastasis and angiogenesis. CXCR4 overexpression in tumour cells has also been demonstrated in at least 23 different cancer types (Teicher and Fricker, 2010). Furthermore, CXCR4-expressing tumour cells have been shown to infiltrate CXCL12-rich organs such as BM, lymph nodes or lungs (Price et al., 2016; Teicher and Fricker, 2010). Hence, disruption of the CXCR4/CXCL12 axis appears as a promising cancer therapeutic option, and is already showing positive outcomes in mouse models of lymphoma (Pitt et al., 2015).

While extensive studies have been performed to understand the involvement of CXCR4/CXCL12-signalling on tumour cells, the precise involvement of this pathway in the recruitment and survival of leukocytes is not fully understood. Of note, the hypoxic tumour microenvironment is known to induce the expression of CXCL12, which can promote the recruitment of MCs (Lewis et al., 2016). In a mouse model of breast cancer, depletion of MCs or inhibition of CXCR4 in conjunction with a hypoxia-inducing agent was also found to limit tumour growth (Welford et al., 2011).

1.2.8.5 Other chemokine receptors and adhesion molecules

While CCR2 and CX3CR1, and to a lesser extent CXCR4, have received the most attention with regards to monocyte trafficking, the involvement of other chemokine receptors and adhesion molecules have also been reported. Among them, CCR1 and CCR5 are expressed on monocytes and induce chemotaxis in both subsets of monocytes. However, studies have been hampered due to the complexity of these

receptors with regards to cell expression and ligand specificity. For instance, while CCR1 and CCR5 both bind CCL3 and CCL5 with strong affinity, CCR1 and CCR5 bind a multitude of other ligands with considerable overlap [Table 1.3]. Furthermore, expression of CCR1 and CCR5 were not restricted to monocytes, as a wide range of other immune and stromal cells were also found to express these receptors.

Receptors	Ligands
CCR1	CCL3, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23, CCL26
CCR5	CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL16, CCL26

Table 1.3 The complexity of CCR1 and CCR5 interaction with their ligands.

It was found that CCR1 and CCR5 on monocytes do not play redundant roles in response to CCL5 under shear flow conditions *in vitro*. In particular, while both receptors mediate endothelium transmigration, CCR1 triggers firm arrest of monocytes, while CCR5 promotes its spreading (Weber et al., 2001). *In vivo*, both receptors play a role in monocyte-associated diseases and infections. Specifically, *Ccr5*-deficiency is linked to reduced clearance of *Listeria monocytogenes* (Zhou et al., 1998), and a decreased recruitment of both monocytes subsets into atherosclerotic lesions (Combadière et al., 2008; Tacke et al., 2007). Furthermore, *Ccr1*^{-/-} mice displayed reduced clinical signs during EAE (Rottman et al., 2000), and decreased infarct size during experimental myocarditis (Liehn et al., 2008).

In the steady-state, both subsets of monocytes do not express *Ccr7* (Geissmann et al., 2003). Although monocytes were found to be present

in LNs, they usually access LNs through blood HEVs and not the afferent lymph. During inflammation such as in experimental atopic dermatitis models, MCs that have infiltrated the skin can migrate into draining LNs through CCR7, albeit with a lesser efficiency than cDCs (Tamoutounour et al., 2013). Moreover, during DSS colitis, a population of MCs can also acquire CCR7-dependent migration to mesenteric LNs where they prime naive T cells (Zigmond et al., 2012). Thus, the acquisition of CCR7 appears downstream of MCs during inflammation, and is in line with their complementary role with cDCs.

Apart from chemokines, other molecules have been reported to be essential for cell extravasation, adhesion to vessels wall and the eventual recruitment of other immune cells into tissues (Nourshargh and Alon, 2014). Among them, selectins and integrins play a major role. Specifically, selectins mediate the slow rolling of leukocytes on activated venular walls, while integrins promote the subsequent firm adhesion of cells onto blood vessels that eventually leads to interstitial migration within tissues.

The L-selectin (also called CD62L) is differentially expressed between monocyte subsets (Sunderkötter et al., 2004). Specifically, Ly6C^{lo} monocytes do not express L-selectin and are thus not affected by its loss. On the other hand, Ly6C^{hi} monocytes crucially depend on L-selectin for their rolling and tethering onto inflamed blood vessels (Tedder et al., 1995; Xu et al., 2008). In addition, Ly6C^{hi} monocytes require L-selectin for their efficient recruitment into the peritoneum (Tedder et al., 1995), the skin, or their direct entry into LNs through HEVs during inflammation (Jakubzick et al., 2013; León and Ardavín, 2008).

Lastly, two major integrins play major functions in mediating monocyte adhesion, recruitment and tissue migration: MAC-1 and LFA-1. MAC-1 is a heterodimer formed from by a CD11b molecule (integrin α M) and a CD18 molecule (integrin β 2). On the other hand, LFA-1 is a heterodimer formed by a CD11a molecule (integrin α L) and a CD18 molecule. Although these integrins can bind several different ligands, ICAM-1 serves as a common ligand. Functionally, blocking MAC-1 or ICAM-1 inhibits the recruitment of Ly6C^{hi} monocytes during *Listeria monocytogenes* infection, and is linked to increased mortality (Rosen et al., 1989; Shi et al., 2010). On the other hand, blocking LFA-1 or ICAM-1/2 completely abrogates the patrolling behaviour of Ly6C^{lo} monocytes (Auffray et al., 2007; Carlin et al., 2013).

1.3 Imaging monocytes through intravital multiphoton microscopy

1.3.1 Features of intravital microscopy

The immune system consists of a dynamic network of interconnecting compartments that guards the host from pathogen invasion at specific points of entry. In particular, monocytes are circulating cells that traffic constantly through blood vessels to patrol tissue organs. However, apart from several pioneer studies that include the "rose thorn" experiment from Metchnikoff (Gordon, 2008), most of our fundamental understanding of immunology relies on static experimental methods. These methods include the use of histology that allows the visualisation of several markers in a tissue section or flow cytometry whereby ~10 parameters from a single cell suspension may be analysed, however at the expense

of the loss of spatial information. Since immune cells such as monocytes are highly dynamic and rely heavily on their trafficking behaviour to execute their effector functions, it is therefore critically important that their behaviour and functions be studied in a spatiotemporal manner. While subsequent studies have retrieved cells, such as lymphocytes, from organ homogenates and imaged their behaviour *in vitro*, these approaches have failed to factorise the native tissue microenvironment into their experimental conditions that could have potentially led to artefacts (Germain et al., 2006). However, these constrain have now been addressed with the recent progress in light microscopy techniques together with the development of novel fluorescent probes and proteins that allow the direct observation of immunological processes in live animals (Germain et al., 2006).

Since the early 2000's, multiphoton microscopy, and to a lesser extent confocal laser scanning microscopy, has shed new lights on our understanding of immune dynamics *in vivo* (Germain et al., 2012). From its origins as a tool employed for the study of cellular interactions occurring within lymphoid organs, the use of intravital imaging has now greatly expanded towards many other aspects of the immune response, such as host cell-pathogen interactions (Gebhardt et al., 2011; Müller et al., 2012), immune cell activation (Moreau et al., 2012) and immune cell development (Moran et al., 2011). In addition, this technique allows careful delineation of discrete cellular events in both lymphoid and non-lymphoid organs, including the migration of dendritic cells (DCs) out from the skin (Tal et al., 2011) and the egress of immune cells from the BM (Devi et al., 2013; Kohler et al., 2011).

1.3.2 Principle and advantages of multiphoton microscopy

While both confocal and multiphoton can be used to image immune cells at high resolution in a living mouse, there are several important differences between these two techniques. First of all, different mechanisms of excitation are used between confocal and multiphoton microscopy. Specifically, conventional microscopy such as the confocal uses a single photon excitation to increase the energy state of a fluorochrome. Fluorescence occurs when the excited fluorochrome diminishes back to its basal state, and at the same time emit a lower energy photon. For instance, a fluorescein molecule is excited by absorbing a 494nm photon (higher energy) and emits a 521nm photon (lower energy) when it goes back to its basal state. In contrast, two-photon excitation will require the fluorochrome to simultaneously absorb two photons carrying half the energy that is required to attain excitation (Ishikawa-Ankerhold et al., 2012). Using the same example, a fluorescein molecule hence needs to absorb two 988nm photons in order to be excited. Of note, the fluorescein molecule still emits a 521nm photon when relaxing back to the basal state.

Taken together, these different excitation properties results in distinct consequences when imaging a biological sample. In practice, the "two-photon" effect is a rare event as fluorochromes absorb two photons simultaneously and is confined to the focal plane. In contrast, single photon techniques such as confocal microscopy require the whole sample to be illuminated, which restricts their use for prolonged imaging sessions as this process increases the likelihood of photodamage and phototoxicity to tissues.

More importantly, the multiphoton technology uses low-energy infrared light (typically in a range of ~780-1300nm) to excite fluorochromes. As such, infrared light can penetrate deeper into tissues and allows imaging at much higher depth. For instance, while a depth of ~80µm can be achieved by confocal microscopy, multiphoton imaging allows imaging at a depth of up to 200-400µm. Consequently, the use of confocal microscopy for intravital imaging is mostly restricted to transparent (cremaster muscle) or superficial organs (liver, skin), while multiphoton imaging has been used to image almost every mouse tissues at a high depth (Germain et al., 2012).

Finally, multiphoton microscopy enables the imaging of tissue structures without the need of a fluorescent label by using a second harmonic generation (SHG) signal. Specifically, SHG signal is generated through certain proteins displaying crystalline structures such as collagen that can emit fluorescent light at half the excitation wavelength (Zipfel et al., 2003). For example, a 950nm excitation wavelength can be used to excite collagen fibres, which will emit a fluorescent signal at 475nm. In practice, SHG is particularly useful when imaging certain organs. For example, SHG helps to distinguish the epidermis (SHG negative) from the dermis (SHG rich) in the skin and bone structures in the BM (Devi et al., 2013; Li et al., 2012).

1.3.3 Current tools to visualise the immune system through intravital imaging

Several approaches are commonly used to follow individual immune cells through intravital imaging. This includes the (1) use of cytosolic

dyes, (2) antibody labelling and (3) genetically engineered mice that carry fluorescent reporters for specific cell types. Cytosolic dyes are widely used in lymphoid cell biology, and consist of dyes such as CFSE (for 5,6-carboxyfluorescein diacetate succinimidyl ester) that allows the labelling of targeted cells *ex vivo* and transferring the labelled population back into an animal. This approach allows the tracing of highly purified populations *in vivo*, provided the labelled population has the capacity to home/infiltrate into the desired organ. For example, T cells with a specific TCR can be isolated from the spleen, stained *ex vivo*, and transferred into another mouse before imaging the lymph nodes for these cells (Bousso, 2008). However, this approach also carries disadvantages. Specifically, this method may potentially introduce a substantial amount of artefacts, such as cell differentiation/maturation or cell death, since cells have to be manipulated *ex vivo*. Furthermore, cytosolic dyes are known to be toxic at high concentrations and may lead to the impairment of cell migration *in vivo* (Germain et al., 2006).

While the use of fluorescently conjugated antibodies for cell labelling is a widespread approach to study the behaviour of intravascular leukocytes (Sreeramkumar et al., 2014; Wong et al., 2013), antibodies classically do not diffuse into organs, such as the skin and lymph nodes, which have a tight endothelial barrier. Furthermore, antibodies may directly activate cells (ex: anti CD3), or bind in an unspecific manner to Fc receptors, depending on the target. In particular, Fabs may be utilised to circumvent Fc-mediated effects (Moreau et al., 2012). However, they are currently not commercially available, which hence limits their usage.

The last approach consists of transgenic animals that express fluorescent proteins under the control of a cell-specific promoter (e.g.

Foxp3^{gfp/+} to detect regulatory T cells). In this approach, no prior staining steps are required, thus minimising the potential artefacts introduced through ex vivo cell manipulation/antibody injection.

Mononuclear phagocytes are highly plastic in nature and hence, carry characteristics that are highly influenced by their immediate environment. Due to their adaptation to the local microenvironment, their phenotype hence significantly changes from one tissue to another. To address this issue, a number of fluorescent reporter mice that target genes essential for their development (MacGreen/MacBlue/MaFIA which all target *Csf1r*, *NR4A1-GFP*), cell trafficking (*Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}*), or markers classically used for cell identification (*hCD68-GFP*, *Langerin-GFP*, *CD11c-YFP* or *DPE-GFP* that encodes a modified CD4-promoter) have been generated (McArdle et al., 2016).

To date, no fluorescent reporter system is truly specific for monocytes. While *Cx3cr1^{gfp/+}* and *Ccr2^{rfp/+}* mice have been used to identify monocytes *in vivo*, a substantial amount of macrophages, DCs, NK and T cells are labeled at the same time. While it is possible to sort monocytes from these mice by flow cytometry and adoptively transfer them to study their behaviour *in vivo*, this method potentially introduces artefacts, as previously discussed. While the use of endogenous reporters significantly prevents these artefacts, the specificity of each reporter in a given tissue needs to be tested. For instance, the specificity of a mouse strain in a given organ can be assessed using these criteria: 1) the cell type of interest should account for the majority of the labeled cells, and 2) the cell type of interest should display the highest fluorescence intensity to distinguish them from the background.

1.4 Objective of the study

Monocytes are central players in innate defence. As a highly plastic population of cells, monocytes can complement the function of cDCs and tissue resident macrophages by promoting the initiation and/or resolution of immune responses, during inflammatory situations.

However, uncontrolled activation and trafficking of monocytes may be harmful to the host and this process has been linked to inflammatory diseases, such as atherosclerosis and cancer progression. Under physiological conditions, the BM is the primary site for monocyte production, where they reside until they mature and are mobilised into the circulation. Thus, the mobilisation of monocytes from the BM represents a major checkpoint in their trafficking to ensure efficient microbial immunosurveillance without compromising host integrity.

Current knowledge of monocyte trafficking between distinct tissue compartments is mainly restricted to the involvement of CCR2 and CX3CR1. Specifically, while CX3CR1 participates in the adhesion and crawling of Ly6C^{lo} monocytes on endothelial surfaces as well as the tissue recruitment of Ly6C^{hi} monocytes under certain conditions, CCR2 is mainly involved in the egress from the BM and the infiltration into tissues of Ly6C^{hi} monocytes. However, it is unclear whether a "safeguard" mechanism exists to retain monocytes in the BM and prevent their uncontrolled release.

As a well-known BM retention factor for other immune cell types, CXCR4 is thus likely to be a potential candidate in retaining monocytes

in the BM. This hypothesis is supported by evidence that WHIM patients who carry a gain-of-function for CXCR4 display increased retention of leukocytes in the BM and peripheral monocytopenia. However, the precise involvement CXCR4 in monocyte trafficking between distinct tissue compartments remains elusive. In this study, we hence propose the following objectives:

- 1) To establish an intravital imaging method to follow the behaviour of monocytes in the BM at the steady state and under inflammatory conditions
- 2) To address the role of CXCR4 in the mobilisation/retention of monocytes in the BM
- 3) To determine the implication of CXCR4 in the trafficking of monocytes in the periphery

2 Materials and Methods

2.1 Mice

C57BL/6 mice (6-10 weeks old) were bred and maintained under specific pathogen-free conditions in the Biological Resource Centre (BRC) of A*STAR, Singapore. *Lyz2^{cre/cre}* (B6.129P2-*Lyz2^{tm1(cre)lfo}/J*), *Ccr2^{rfp/+}* (B6.129(Cg)-*Ccr2^{tm2.1lf}/J*), *Cx3cr1^{gfp/+}* (B6.129P-Cx3cr1^{tm1Litt}/J), *Cxcr4^{fl/fl}* (B6.129P2-Cxcr4^{tm2Yzo}/J), *Cxcl12^{Dsred/+}* (STOCK *Cxcl12^{tm2.1Sjm}/J*), MaFIA (C57BL/6-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J), NOD-*scid Il2rg^{-/-}* (NSG; NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ*), *Rosa26^{mT/mG}* (STOCK *Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Lo}/J*) and CD45.1 mice were obtained from the Jackson Laboratory. *Lyz2^{cre/cre}* mice were cross bred in-house with *Cxcr4^{fl/fl}* mice to generate progeny that were CXCR4-deficient in the myeloid lineage only (termed *Lyz2^{cre}Cxcr4^{fl}*) (Eash et al., 2009). *Flt3L^{-/-}* (C57BL/6-*flt3L^{tm1Imx}*) mice were obtained from Taconic. *Tlr4^{-/-}* mice were obtained from Oriental BioService. Gain-of-function *Cxcr4^{1013/+}* mice (termed *Cxcr4^{WHIM}*) were kindly provided by Françoise Bachelierie (Balabanian et al., 2012). For fate mapping purposes, *Lyz2^{cre}Cxcr4^{fl}* were crossed to *Rosa26^{mT/mG}* mice (termed *Rosa^{mT/mG}:Lyz2^{cre}Cxcr4^{fl}*). Humanised mice were generated by Qingfeng Chen's group as described (Chen et al., 2013). All transgenic mice were maintained on a C57BL/6 background and experiments were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of the BRC, in accordance with the guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore.

2.2 Buffers

FACS consisted of 3% FBS (Biowest) + 2 mM EDTA (pH = 8) in 1X PBS. Blocking buffer was prepared from FACS buffer supplemented with 2% mouse and rat serum (Sigma).

2.3 Treatments

To mobilise monocytes from the BM, mice were treated with 10ng LPS derived from E.coli (serotype O55:B5, Alexis Biochemicals) or 300ng CCL2 (R&D systems) administered via the intravenous route. To determine the effect of CXCR4 inhibition on monocyte trafficking, mice were treated with 5mg/kg AMD3100 (plerixafor; Sigma-Aldrich) via subcutaneous injection. All experiments were carried out between ZT4 to ZT8 unless otherwise stated.

2.4 Tissue preparation for flow cytometry and cell sorting

2.4.1 Blood

Blood was collected via an incision in the sub-mandibular region into Eppendorf tubes filled containing 20 μ L of EDTA (0.5 M, pH = 8). Red blood cell (RBC) lysis was performed by treating samples with 10 times the amount of commercial-grade 1X RBC lysis buffer (eBioscience) (e.g. 1mL of lysis buffer was added to 100 μ L of blood) for 4 minutes at room temperature. 2 mL of FACS buffer was added to the samples and centrifuged at 1350 rpm for 5 minutes. RBC lysis was repeated for an

additional 2 minutes, washed and centrifuged again at 1350 rpm for 5 minutes.

2.4.2 Bone marrow

Mice femurs were flushed into 2 mL of FACS buffer using a 21G needle that was attached to a 1 mL syringe to obtain a single cell suspension. RBC lysis was performed by adding 1 mL of RBC lysis buffer into samples and incubating at room temperature for 3 minutes. 5 mL of FACS buffer was then added into samples and the effluent was passed through a 70 mm nylon mesh sieve before centrifugation at 1350 rpm for 5 minutes.

2.4.3 Spleen

Spleens were finely minced in a 1.5 mL Eppendorf tube before homogenising into a single cell suspension through 70 mm nylon mesh sieves with syringe plungers and FACS buffer. Samples were centrifuged at 1350 rpm for 5 minutes before conducting 5 min of RBC lysis with 1 mL of RBC lysis buffer. Cell suspensions were passed through a 70mm nylon mesh sieve again before centrifugation at 1350 rpm for 5 minutes.

2.4.4 Lung

Lungs were removed *en bloc* and finely minced before enzymatic digestion with 1 mL of a mixture containing 154 U/mL Collagenase IV (Sigma), 500 U/mL DNase I (Roche) and 10% FBS in RPMI for 45 min in a 37°C water bath. 2 mL of cold FACS buffer was subsequently added and samples were homogenised into a single cell suspension using a 19G needle attached to a 3 mL syringe. Samples were next passed through 70mm nylon mesh sieves with syringe plungers and centrifuged for 5 min at 1350 rpm. RBCs in lung samples were lysed by adding 1 mL of 1X RBC lysis buffer and incubating samples for 3 min. Finally, samples were washed with 5 mL of FACS buffer and centrifuged at 1350 rpm for 5 min.

2.4.5 Flow cytometry and cell sorting

All samples were incubated in 100 µL of blocking buffer with fluorescent antibodies for 20 min at 4°C. Straining was stopped by adding 2 mL of FACS and samples were centrifuged at 1350 rpm for 5 min. DAPI (1µg/mL, Thermo Fisher) was added to exclude dead cells. Antibodies were purchased from eBioscience, Biolegend, BD Bioscience or R&D systems. Mouse cells were stained with the following antibodies: CCR2 (475301), CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD16/32 (93), CD19 (eBio1D3), CD31 (390), CD43 (S7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD45R/B220 (RA3-6B2), CD115 (AFS598), cKit (2B8), CXCR4 (2B11), CX3CR1 (SA011F11), F4/80 (Cl:A3-1 or BM8), Flt3 (A2F10), IA-IE (M5/114.152), Ly6C (HK1.4), Ly6G (1A8), NK1.1 (PK136) and Ter119 (TER-119). Human cells were stained with the following antibodies: CD3 (UCHT1), CD10 (eBioCB-CALLA), CD11b (ICRF44), CD11c (B-ly6), CD14 (322A-1), CD16 (3G8), CD19 (HIB19), CD20 (2H7), CD34 (581), CD45 (HI30),

CD56 (MEM188), CD123 (6H6), CXCR4 (12G5) and HLA-DR (L243). Dead cells were identified and excluded using DAPI staining. Lung, blood and BM monocytes were identified as CD45⁺CD115⁺cKit⁻Ly6G⁻ Lineage (CD3, CD19, CD45R, NK1.1) negative and Ly6C^{hi}CD43⁻ or Ly6C^{lo}CD43⁺ according to the subset. Cells were acquired on a BD LSR II flow cytometer (Becton Dickinson) using FACSDiva Software, and data were analysed using Flowjo Software (Tree Star). Total number of cells collected from blood or each tissue/organ was quantified using count beads (CountBright™, Life Technologies) according to the manufacturer's protocol. CXCR4 expression on monocytes was quantified as median fluorescent intensity (MFI) and normalised by subtracting the fluorescence minus one (FMO) control. For cell sorting, BM cells were sorted using a FACS ARIA II (Becton Dickinson) to achieve > 98% purity.

2.5 Chemotaxis assay

Femurs and tibias were harvested from C57BL/6 mice and flushed with ~5 mL of PBS containing 3% FCS. RBCs were lysed and the resultant cells were re-suspended in sterile PBS for labeling with antibodies against CD11b, CD45, CD115, CXCR4, Flt3, cKit and Ly6C, as well as an exclusion cocktail of antibodies against unwanted lineages (CD3, CD45R/B220, Ly6G and NK1.1). BM monocytes were identified as Lin⁻Flt3⁻cKit⁻CD115⁺Ly6C^{hi} cells that displayed either a CXCR4^{hi} or CXCR4^{lo} phenotype. The CXCR4^{hi} and CXCR4^{lo} subsets were then sorted at high speed using a BD FACS Aria III apparatus (Becton Dickinson). A total of 1×10^5 sorted monocytes were transferred onto the upper layer of a polycarbonate membrane with 3 µm pore size (Transwell; Corning),

before being placed into lower chambers containing 0-50 ng recombinant CCL2 (R&D systems) and incubated at 37°C for 2 hours. After incubation, the cells in the bottom chamber were harvested and analysed by flow cytometry to determine the percentage of migrated monocytes (expressed as a proportion of the total initially loaded into the upper chamber).

2.6 BrdU pulsing

For *in vivo* assays, mice were administered 1.5 mg BrdU (BD pharmingen) via intraperitoneal injection at indicated time points. To detect BrdU incorporation into monocytes, cells were surface-stained, fixed, permeabilised, and subjected to intracellular staining with FITC-conjugated anti-BrdU antibody according to the manufacturer's protocol (BrdU Flow kit; BD Biosciences) prior to analysis by flow cytometry.

2.7 Adoptive transfer of BM monocytes

Femurs and tibias were harvested from CD45.1 mice and flushed with ~5mL PBS containing 3% FCS. RBCs were lysed and the remaining cells were re-suspended in sterile PBS. A total of 10 million CD45.1 donor cells were adoptively transferred into CD45.2 recipient mice that were simultaneously treated with AMD3100 or PBS. Recipient mice were euthanised at the time points indicated in the respective figures, and the perfused femurs were harvested for flow-cytometric identification of donor monocyte (CD45.1) sequestration into the BM of recipient animals (CD45.2). For intra-femoral transfer of BM Ly6C^{hi}

monocyte subsets, sorted 5×10^5 CD45.1⁺ CXCR4^{hi} were re-suspended in PBS and were transferred into the tibia of CD45.2⁺ mice via an insulin syringe with a short needle (Becton Dickinson). At 8 and 24 hours after cell transfer, tibias were collected and the resulting cell suspensions were analysed by flow cytometry to establish the phenotype of the CD45.1⁺ progeny of the transferred cell populations.

2.8 Dimensionality reduction using t-SNE and automatic clustering

BM Ly6C^{hi} monocytes were manually gated from multi-color flow cytometry data as shown in **[Figure 3.11a]**, and were exported in a FCS file. In this study, t-Distributed Stochastic Neighbor Embedding (t-SNE) dimensionality reduction was performed using `bh_tsne`, an implementation of t-SNE via Barnes-Hut approximations. R was used as an interface to execute `bh_tsne` as previously described (Becher et al., 2014). K-means automatic clustering was performed using CYT in Matlab. 90000 events were used for t-SNE dimensionality reduction.

2.9 Transcriptomic analysis

Total RNA was extracted using Arcturus PicoPure RNA Isolation kit according to manufacturer's protocol. All mouse RNAs were analysed on Perkin Elmer Labchip GX system for quality assessment with RIN > 7.9. cDNA libraries were prepared using 2 ng of total RNA and 1 μ L of a 1:50000 dilution of ERCC RNA Spike in Controls (Ambion) using SMARTSeq v2 protocol (Picelli et al., 2014) except for the following

modifications: 1) Use of 20 μ M TSO, 2) Use of 250 pg of cDNA with 1:5 reaction of Illumina Nextera XT kit. The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip. All 6 samples were subjected to an indexed PE sequencing run of 2 x 51 cycles on an Illumina HiSeq 2500 Rapid mode (14 samples per lane).

RNA-Seq data in the form of FASTQ files were subsequently mapped to the mouse genome build mm10 using the STAR alignment software. The mapped reads were then counted using featureCounts (part of Subread package) based on the GENCODE M9 annotations. The raw counts were then used for a differential gene expression analysis (DEG) using edgeR (R version 3.1.2) with adjusted p value < 0.05 and fold change greater than 2 to identify genes differentially regulated in monocyte subsets. A filter for genes having an average count of at least 10 was done to eliminate low expressing and non-expressing genes prior to DEG analysis. Principal Component Analysis (PCA) of samples was done using log2CPM (cpm: count per million reads) values of all detected genes. PCA, volcano plot and heat maps were done in R version 3.1.2.

2.10 Computational inference of development path

Wanderlust was executed using CYT in Matlab as previously published (Bendall et al., 2014). Wanderlust was run on 6 phenotypic markers: CXCR4, CD31, CD16/32, CX₃CR1, CCR2 and CD11b using the following parameters: number of neighbors $l = 30$, $k = 5$, number of landmarks $nl = 20$, number of graphs to generate $ng = 25$, distance metric = angular. The starting point (early events) consisted of selected

Ly6C^{hi} cells that expressed high levels of CXCR4, CD31 and CD16/32 as observed on cMoPs, which is the most direct progenitor of BM Ly6C^{hi} monocytes. 90000 events were used for Wanderlust analysis.

2.11 Parabiosis

Female mice were anaesthetised using 2.5% Avertin (15 mL/kg), shaved at the corresponding lateral sides of the body, and then surgically joined as previously described (Hashimoto et al., 2013). After surgery, the mice were treated with Baytril (0.05-2 mg/kg s.c.) and Buprenorphine (5-20 mg/kg s.c.) and allowed to recover for 8 weeks. Since *Lyz2^{cre}Cxcr4^{fl}* and *Cxcr4^{WHIM}* mice exhibit steady-state monocytosis and monocytopenia respectively, parabiosis with WT animals results in unequal exchange of circulating leukocytes [Figure 3.21e]; the results of these experiments were hence represented as a homing ratio to control for differences in absolute cell numbers between these mice. To determine the number of cells that had infiltrated a given organ, the number of non-host cells present in that organ was divided by the total number of non-host cells present in the host circulation (expressed as the 'homing ratio'). To determine the total number of circulating cells, the blood volume present in an individual mouse was calculated using the formula: $y = 0.0715 x$, where y and x represent the blood volume (mL) and body weight (g) respectively as previously described (van Furth and Sluiter, 1986).

2.12 Bone marrow chimeric mice

To generate BM chimeric mice, 6 weeks old WT and *Tlr4*^{-/-} mice were irradiated with two doses of 550 rad spaced 3 hours apart and reconstituted with 4 x 10⁶ cells BM from WT or *Tlr4*^{-/-} mice under sterile conditions. Recipient mice were analysed 6 weeks after irradiation and number of Ly6C^{hi} monocytes quantified 1 hour after administration of LPS.

2.13 Wright-Giemsa staining

Sorted BM Ly6C^{hi} monocyte subsets were cytopun onto glass slides, dried for 20 minutes, and stained with the Hema 3 System according to the manufacturer's protocol (Fisher Diagnostics). Images were analysed with an Olympus IX73 microscope with a 100x oil immersion objective (Olympus).

2.14 Scanning electron microscopy

Sorted CXCR4^{hi} and CXCR4^{lo} monocytes were seeded onto coverslips and incubated at 37°C, 5% CO₂ for 3hrs to allow cell adhesion onto coverslips. Cells were fixed with 2.5% Glutaraldehyde (Sigma) in PBS, washed and post-fixed in 1% Osmium tetroxide (Electron Microscopy Services) in PBS, dehydrated in ethanol and processed by critical point drying (Leica EM CPD030). Finally, samples were sputter-coated with gold using Leica EM SCD050 and imaged on the JSM-6701F, JEOL scanning electron microscope at 4000X magnification. 60-70 cells were acquired for each tested group and representative images of a typical phenotype were presented in the figure.

2.15 Intravital multiphoton microscopy

2.15.1 Intravital multiphoton imaging of the skull bone marrow

2.15.1.1 Experimental setup

In our study we used the skull BM imaging model (Mazo et al., 1998), since it does not require extensive invasive surgery unlike the thinned long bone model (Kohler et al., 2011). Skull BM imaging was performed as previously described (Devi et al., 2013; Evrard et al., 2015). Specifically, mice were anaesthetised by intra-peritoneal injection of ketamine (150 mg/kg) and xylazine (10mg/kg). The jugular vein was cannulated and 50 µg Evans Blue (in 50µL sterile PBS) was delivered to label blood vessels. Mice were subsequently immobilised on a heated custom-made stage to maintain the body temperature at 37°C, and a small area of skin on the skull was excised to expose the BM. The exposed skull was superfused with sterile PBS and covered with a glass coverslip. Images measuring 450 x 450 µm were acquired every 20 seconds using a 4µm z-step size with an approximate depth of 45µm. Imaging was conducted using a multiphoton LaVision TriM Scope II microscope (LaVision BioTec) equipped with a 20x 1.0 NA water immersion objective lens and a Coherent Chameleon pulsed infrared laser (Ti:Sa and OPO). GFP and RFP excitation were conducted at a wavelength of 950nm and 1100nm respectively. GFP fluorescence was detected with a 525/50 longpass mirror (Chroma) while RFP fluorescence was detected with a 595/40 longpass mirror. For both excitation wavelengths, Evans blue fluorescence was detected through a 655/40 longpass mirror. After acquisition, images were transformed into time series movies using Imaris (Bitplane).

2.15.1.2 Treatments and *in vivo* MHCII staining

Mice were imaged for an initial period of 30 minutes (baseline) prior to intravenous injection with LPS or CCL2, or subcutaneous injection of AMD3100, followed by imaging for a further 3 hours. *Cx3cr1^{gfp/+}Flt3L^{+/+}* or *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice were injected i.v. with 4 µg of a PE-conjugated MHCII antibody (IA-IE; clone M5/114.15.2, Biolegend) where indicated. One hour after injection, mice were prepared for skull BM intravital imaging and were subsequently imaged for 5 min/field of view (450 x 450 µm). When used together, GFP and PE were excited at a wavelength of 990 nm and fluorescence was detected with 525/50 and 579/34 longpass mirrors (Chroma) respectively for GFP and PE. A minimum of 5 fields of view was imaged per mouse.

2.15.1.3 Image analysis

Snapshots were stitched together by use of Fiji where indicated. Stitched images or snapshots were processed by use of Imaris software (Bitplane) to adjust threshold values for the different fluorescence channels. Where necessary, drifts occurring during imaging were corrected using FIJI. Tracking of GFP⁺ cells was performed semi-automatically using Imaris spot-tracking algorithms, and the mean velocity and displacement length were extracted.

2.15.1.4 Cell morphology analysis

CX3CR1-GFP⁺ objects were selected by use of the Imaris surface tool. Cell volume and sphericity were calculated by Imaris and subsequently plotted into Prism (GraphPad Software). Objects with a volume < 100 mm³ were excluded from analysis. Where indicated, GFP⁺ objects were further distinguished based on their MHCII-PE positivity.

2.15.2 Intravital multiphoton imaging of the lungs

2.15.2.1 Experimental setup

Imaging of the lung was performed as previously described (Looney et al., 2010). Specifically, mice were anaesthetised with a cocktail of ketamine and xylazine before cannulation of the trachea to allow connection to a mechanical ventilator (tidal volume ~8-10 μ L/g body weight, respiratory rate ~120 breaths per minute) (MiniVent 845; Hugo Sachs Elektronik). Mice were then placed onto a heat pad to maintain body temperature at 37°C, followed by the removal of skin, muscle and two rib bones to expose the lung. A custom-made vacuum window ring was used to immobilise the lung via application of a negative pressure vacuum (~40mmHg). To label the pulmonary vasculature, 70kDa TRITC-conjugated dextran (250 μ g in 100 μ L saline; Sigma-Aldrich) or Evans blue (50 μ g in 50 μ L sterile PBS) was administered intravenously. Experiments were performed at 880nm or 990nm excitation respectively when using TRITC-dextran or Evans blue vascular labelling. For both excitation wavelengths, GFP fluorescence was detected with a 525/50 longpass mirror. TRITC fluorescence was detected with a 574/39 longpass mirror while Evans blue fluorescence was detected through a 655/40 longpass mirror. 364 μ m x 364 μ m images were

acquired at an imaging speed of 2 fps over a period of 30 minutes using a 4µm z-step size with an approximate depth of 20µm.

2.15.2.2 Treatments and *in vivo* Ly6B.2 staining

Experimental groups consisted of mice administered with AMD3100 subcutaneously, LPS intravenously, or a combination of AMD3100 and LPS together administered via the different routes. Imaging of the lung began 45 minutes after single treatment with AMD3100 or LPS, whereas co-treatment of both AMD3100 and LPS were imaged at 2 hours into AMD3100 and 45 minutes into LPS simultaneously. For the visualisation of monocyte subsets, 4µg of Ly6B.2-PE (7/4, Novus Biologicals) was administered intravenously in *Cx3cr1^{gfp/+}* mice and was detected using the same settings than MHCII-PE labelling (**Figure 23C and D**).

2.15.2.3 Image analysis

After acquisition, 24 frames were averaged to match respiratory movements and drifts during imaging were corrected where necessary using FIJI. Images were subsequently transformed into time series movies using Imaris. Tracking of GFP⁺ cells was performed semi-automatically using Imaris spot-tracking algorithms and mean velocity was extracted. The duration of GFP⁺ cell adherence in the lung vasculature was tabulated manually.

2.16 Localisation of intravascular lung monocytes

Intravascular staining of lung monocytes was performed *in vivo* accordingly to a previously described protocol (Anderson et al., 2014). Specifically, mice were anaesthetised by isoflurane inhalation and biotin-conjugated anti-CD45.2 (clone 104, eBioscience) was administered intravenously (4 µg antibody in 200 µL saline). The antibody was allowed to circulate for 3 min before the mice were euthanised. Lungs were removed *en bloc* and processed immediately in an excess volume of PBS containing 3% FCS and 2mM EDTA to dilute any excess antibody. FITC-conjugated anti-CD45.2 (clone 104, eBioscience) was included in the antibody staining panel in order to identify extravascular cells.

2.17 Lung efflux assay

2.17.1 Lung efflux assay in mice

The method used for tandem blood sample collection from the carotid artery and vena cava was carried out as published (Devi et al., 2013). Briefly, mice were anaesthetised with isoflurane and a midline incision was performed on the neck to expose the underlying carotid artery. A microvascular clamp was applied to the artery, which was then cannulated by insertion of EDTA-filled polyethylene 10 tubing (inner diameter: 0.28mm; outer diameter: 0.61mm). After securing the cannula with a surgical suture, the clamp was released and ~40 µL of arterial blood was collected into an Eppendorf tube. Blood was simultaneously drawn from the inferior vena cava using a 26G needle attached to a pre-filled EDTA syringe. Samples were collected 1 hour after LPS injection, or 2 hours after AMD3100 treatment. For mice that were co-treated with AMD3100 and LPS, the CXCR4 inhibitor was administered 1 hour prior

to injection of LPS, and blood sample collection took place a further 1 hour later. Both arterial and venous blood samples were subsequently analysed by flow cytometry.

2.17.2 Lung efflux assay in non-human primates

For lung efflux assays done on non-human primates, all experiments were performed on adult female *Macaca fascicularis* at the Singhealth Experimental Medicine Center (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care) under approval #2012/SHS/692 from the IACUC of Singapore as previously described (Devi et al., 2013). Briefly, mid-line laparotomy was performed under general anesthesia and ~1 mL of blood was each collected from the abdominal aorta and inferior vena cava at baseline and hourly intervals after s.c. administration of AMD3100 (0.5 mg/kg). Monocyte numbers were determined using a Hematology Analyser (Beckman-Coulter). The number of monocytes leaving the pulmonary circulation (termed net release) was calculated by subtracting the number of monocytes present in the venous blood sample from the number of monocytes present in the arterial blood sample.

2.18 Lung injury and pulmonary vascular permeability assay

To assess the influence of monocytes or CXCR4-signaling on pulmonary vascular permeability, we used a LPS-Zymosan induced model of acute lung injury (ALI) (O'Dea et al., 2009). For the induction of

ALI, mice were administered intravenously with 10ng of LPS for 2 hours. Subsequently, 150mg in 100mL of Zymosan-A (Sigma-Aldrich) was re-suspended with 5 μ L per g of 10 mg/mL of Evans blue (Sigma-Aldrich) and injected intravenously. Mice were sacrificed 1 hour later and lungs were perfused with 10 mL of PBS using a peristaltic pump (MINIPULS 3, Gilson). Lungs were weighed and Evans blue was extracted from the lung by incubating samples in 1 mL of N,N-dimethylformamide (Sigma-Aldrich) at 37°C overnight. The supernatant was separated by centrifugation at 5000g for 30 min. The concentration of Evans blue in lung homogenate supernatants was quantified by a dual wavelength spectrophotometric method at absorptions of 620 and 740 nm, which allows for correction of contaminating heme pigments using the following formula: $E_{620} \text{ (corrected)} = E_{620} - (1.426 \times E_{740} + 0.030)$

2.19 Caecum ligation and puncture (CLP)-induced sepsis

Experimental procedures were carried out as previously described (Rittirsch et al., 2009) . Specifically, the peritoneal cavity was exposed under ketamine/xylazine anesthesia and the cecum was exteriorised. ~80% of the cecum was ligated distal of the ileo-cecal valve using a non-absorbable 7-0 suture. A 23G needle was used to perforate the distal end of the cecum through and through and a small drop of feces was extruded through the puncture before being relocated into the peritoneal cavity. For sham controls, the cecum was exteriorised but relocated back into the peritoneal cavity without any further manipulation. Finally, the peritoneum was closed using sutures (5/0, 1mm, Silkam, Braun) and subsequently treated with saline and

Buprenorphine (5-20 mg/kg s.c.) via s.c. injection. Age-matched controls were included for all procedures.

2.20 Statistical analysis

Statistical analyses were performed using Prism software (GraphPad). Data were tested using either Student's t-test (normal distribution) or ANOVA (1-way or 2-way as appropriate) as indicated in the respective figure legends. P-values < 0.05 were considered significant.

3 Results and Discussion

3.1 Understanding the mobilisation of BM monocytes through an intravital imaging model

The mobilisation of monocytes from the BM represents a crucial step in their homeostasis. In particular, an optimal number of monocytes are required in the circulation at any one point in time, as a dysregulation in monocyte numbers may lead to detrimental outcomes, such as inefficient immune responses or collateral damage, to the host. Thus far, reported mechanisms on monocyte mobilisation have been mainly investigated through cell population based assays such as flow cytometry. While these approaches allow quantitative investigation of cell numbers and localisation in distinct body compartments, such assays lack the spatiotemporal resolution to examine subtle dynamic events. Therefore, we utilised an intravital imaging model of the BM to complement our end-point observations obtained through flow cytometry, as well as to unveil a deeper mechanistic understanding of their mobilisation activities.

3.1.1 Comparative analysis of *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA BM

The use of fluorescent reporter mice for intravital imaging studies is paramount for the understanding of cell trafficking events. While an extensive amount of fluorescent reporter animals are available for studying mononuclear phagocytes, each model carries specific advantages and disadvantages that affect the outcome of intravital imaging studies (Hume, 2011; McArdle et al., 2016). We shortlisted 3 fluorescent reporter mice that carry genes expressed by monocytes and

are known to be essential for their biology: *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA. CX3CR1 and CCR2 are two chemokine receptors crucially involved in the trafficking of monocytes. On the other hand, MaFIA is a mouse line that carries a transgene reporting for *Csf1r* expression.

Next, we identify the optimal reporter mouse for studying monocyte behaviour within the intact BM by implementing the following strategy: (1) we first determined the fluorescent labelling pattern of each reporter mice through flow cytometry; (2) we next imaged the BM of these mice through intravital multiphoton microscopy and correlate these information with flow cytometry data.

To identify the various fluorescently-labelled immune cell populations in these mice, we used a multicolour antibody panel and gated our cells according to the strategy shown in **[Figure 3.1]**. Ly6C^{hi} and Ly6C^{lo} monocytes were found to be fluorescently labeled in all three reporter mice strains **[Figure 3.2a]**. However, we noticed that a variety of other immune cells were also labeled at varying degrees according to each reporter strain. In the MaFIA mouse, myeloid cells such as neutrophils, macrophages and DCs were labeled **[Figure 3.2a]**. On the other hand, macrophages, DCs, and NK, and/or T cells, but not neutrophils, present varying levels of RFP fluorescence intensity in *Ccr2^{rfp/+}* mice **[Figure 3.2a]**. In contrast, *Cx3cr1^{gfp/+}* labels DCs but labels fewer other immune populations as compared with MaFIA and *Ccr2^{rfp/+}* mice **[Figure 3.2a]**.

Figure 3.1 Characterisation of BM immune cells in fluorescent reporter mice

BM was analyzed by flow cytometry with indicated gating strategies in *Cx3cr1^{gfp/+}* **(a)**, *Ccr2^{rfp/+}* **(b)** and MaFIA **(c)** mice for the following immune cells: Ly6C^{hi} monocytes (*red*), Ly6C^{lo} monocytes (*blue*), macrophages (*indigo*), DCs (*violet*), neutrophils (*green*) and T/NK cells (*yellow*).

Next, we aim to understand the proportion of monocytes that are fluorescently labelled compared to other immune cells in each reporter strain. To address this, we compared the numbers of each immune cell type in relation to all fluorescently labelled cells. Using this methodology, we found that the MaFIA reporter mouse was not suitable for monocyte study as neutrophils, but not monocytes, represent the major GFP⁺ population in the BM of these mice **[Figure 3.2b]**. In contrast, Ly6C^{hi} monocytes represent more than 60% of labeled cells, on average, in both *Cx3cr1^{gfp/+}* and *Ccr2^{rfp/+}* mice **[Figure 3.2b]**. However, upon further analysis, *Ccr2^{rfp/+}* mice substantially differed from *Cx3cr1^{gfp/+}* mice in regards to the fluorescence intensity among labeled cells. Specifically, monocytes display similar levels of RFP as myeloid cells, NK and/or T cells in *Ccr2^{rfp/+}* mice. On the other hand, *Cx3cr1^{gfp/+}* mice displayed high GFP intensity that was restricted to myeloid cells. In particular, Ly6C^{lo} monocytes and DCs expressed the highest levels of GFP, followed by Ly6C^{hi} monocytes.

Notably, we found that as high as 25% of fluorescently labelled cells in *Cx3cr1^{gfp/+}* and *Ccr2^{rfp/+}* reporter mice could not be identified by our flow cytometry analysis **[Figure 3.1b]**. Whereas these undetermined cells express intermediate to high levels of RFP in *Ccr2^{rfp/+}* mice, their levels of GFP in *Cx3cr1^{gfp/+}* animals are relatively much lower. As such, unlike

with *Ccr2^{rfp/+}* mice, these undetermined GFP⁺ cells in *Cx3cr1^{gfp/+}* animals are likely to fall below the detection threshold of multiphoton microscopy. Together, these points further illustrate the incompatibility of *Ccr2^{rfp/+}* mice but highlight the efficiency of *Cx3cr1^{gfp/+}* animal model for the purpose of BM monocytes studies.

Figure 3.2 Determination of fluorescence intensity and proportion of fluorescence positive cells in *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA reporter mice

(a) Histograms demonstrating the relative GFP/RFP intensity in different leukocyte populations among *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA reporter mice. **(b)** Pie charts representing the proportion of specific leukocyte populations among the GFP/RFP⁺ fraction in *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA reporter mice.

Now that we have identified the proportion of monocytes that are fluorescently labelled and their intensity through flow cytometry in each reporter mouse, we next validated the suitability of each strain for *in vivo*

imaging. We examined each of the *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA reporter mice by multiphoton intravital fluorescence microscopy. To obtain a comprehensive view of the skull BM architecture, we acquired stitched images for our analysis **[Figure 3.3a,b]**. Evans blue was administered to highlight BM sinusoids while the SHG signal was used to identify bone/collagen structures. Of note, the BM parenchyma which contains the hematopoietic cells is devoid of SHG signal **[Figure 3.3b,c]**. We found that the majority of parenchymal space contained GFP⁺ cells in the MaFIA mouse **[Figure 3.3b]**. At a higher magnification, GFP⁺ cells presented heterogeneous morphology, with the majority of these cells displaying a low cell volume and a spherical morphology, which is consistent with the fact that the majority of the labeled cells are neutrophils **[Figure 3.2b, 3.3c]**. On the other hand, *Ccr2^{rfp/+}* mice exhibited lesser cells in the parenchymal space than the MaFIA mouse **[Figure 3.3b]**. However, RFP⁺ cells displayed heterogeneous morphologies, which include large and stellate to small and spherical, as well as <1µm diameter objects **[Figure 3.3b,c]**. Such apparent morphological heterogeneity might be explained by the presence of multiple RFP⁺ immune cells types, as revealed by our flow cytometry analysis **[Figure 3.1a]**. Finally, *Cx3cr1^{gfp/+}* mice displayed the least amount of fluorescently labelled BM cells **[Figure 3.3b]**. Among these GFP⁺ cells, we observed two types of morphology: large and stellate cells with protruding dendrites, as well as small and spherical cells **[Figure 3.3b,c]**. These data are hence in agreement with our flow cytometry analysis, whereby monocytes and DCs represent the two predominantly labelled immune populations and displayed the highest GFP intensity among CX3CR1⁺ cells **[Figure 3.1b]**.

Of note, we found that as high as 25% of fluorescently labeled cells in *Cx3cr1^{gfp/+}* and *Ccr2^{rfp/+}* reporter mice could not be identified by our flow cytometry analysis **[Figure 3.1b]**. Whereas these undetermined cells express intermediate to high levels of RFP in *Ccr2^{rfp/+}* mice, their levels of GFP in *Cx3cr1^{gfp/+}* animals are relatively much lower. As such, unlike with *Ccr2^{rfp/+}* mice, these undetermined GFP⁺ cells in *Cx3cr1^{gfp/+}* animals are like to fall below the detection threshold of multiphoton microscopy. Together, these points further illustrate the incompatibility of *Ccr2^{rfp/+}* mice, but highlight the efficiency of *Cx3cr1^{gfp/+}* animal model for the purpose of BM monocytes studies.

Figure 3.3 Visualisation of the skull BM in *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA reporter mice via multiphoton intravital microscopy.

(a) An overview of steps involved during intravital imaging of the skull calvarium BM. Skin was first removed carefully to expose the BM. Images were acquired via stitching snapshots of the BM to form a final image of 800 X 1200 µm or as a single snapshot sized at 450 X 450 µm. To label sinusoidal vessels, mice were injected i.v. with Evans blue (n= 3-5/ reporter strain). **(b)** A representative stitched image of the skull BM in *Cx3cr1^{gfp/+}*, *Ccr2^{rfp/+}* and MaFIA reporter mice. Original scale bars,

100 μm . **(c)** (*Upper*) snapshot of the skull BM, whereby single plane images were acquired at different depths ranging from 0 to 36 μm along the axis. Original scale bars, 50 μm . (*Lower*) In addition, magnified single-plane images at z-positions 12 μm and 24 μm are shown. Original scale bar, 20 μm . GFP/RFP⁺ cells, *Green*; sinusoidal blood vessels, *red*; and bone/collagen structures (through SHG), *blue*.

Collectively, our flow cytometry and multiphoton imaging analysis suggest that the *Cx3cr1^{gfp/+}* mouse may represent the most suited reporter mouse for the study of BM monocyte behaviour among the three common reporter strains. As shown, CX3CR1⁺ monocytes account for the majority of GFP-labelled cells and expressed the highest fluorescence intensity among BM GFP⁺ cells. However, our flow cytometry analysis also revealed that DCs displayed similar levels of GFP expression as monocytes while intravital imaging revealed large and stellate GFP⁺ cells that in close proximity with monocytes in the BM. These findings hence suggest that these cells in the *Cx3cr1^{gfp/+}* mouse may interfere with the accuracy of monocyte tracking *in vivo*.

3.1.2 CX3CR1⁺ cells carrying a dendritic morphology are DCs and share a similar location with monocytes

While the *Cx3cr1^{gfp/+}* mouse appears to be the most suitable reporter animal, we wanted to confirm the identity of the large and stellate cells that were GFP-bright and located in the BM parenchyma. Our flow cytometry results suggest that these cells could be DCs **[Figure 3.2a]**. Furthermore, BM DCs have been previously reported to be CX3CR1⁺MHCII⁺CD11c⁺ (Sapozhnikov et al., 2008), while monocytes in the BM do not express MHCII. Therefore, we administered a fluorescent anti-MHCII antibody to *Cx3cr1^{gfp/+}* mice to identify DCs in situ **[Figure**

3.4a]. We found that CX3CR1⁺MHCII⁺ cells were generally larger, with protruding and motile dendrites, whereas CX3CR1⁺MHCII⁻ were generally smaller and spherical **[Figure 3.4a, video 1]**. To confirm these observations, we set up an image analysis workflow to correlate cellular morphology and MHCII expression **[Figure 3.4b]**. We first selected cells based on GFP expression, followed by their positivity for MHCII; finally, we plotted MHCII⁺ and MHCII⁻ cells according to morphologic criteria that include cell volume and sphericity. We defined cells with a high cell volume and low sphericity as cells displaying a "DC-like" morphology, whereas cells that consist of a low cell volume and high sphericity have a "monocyte-like" morphology **[Figure 3.4b]**. Using these morphologic criteria, we found that the majority of CX3CR1⁺MHCII⁺ cells have a "DC-like" morphology while the majority of CX3CR1⁺MHCII⁻ have a "monocyte-like" morphology.

Figure 3.4 CX3CR1⁺ cells that possess a DC-like morphology in the BM of *Cx3cr1^{gfp/+}* mice are DCs.

Cx3cr1^{gfp/+} reporter mice were administered i.v. with PE-conjugated MHCII antibody and Evans blue to detect for MHCII⁺ cells and blood vessels, respectively. **(a)** Snapshots of 450 X 450 mm demonstrating the individual fluorescent channels that detects for CX₃CR1⁺ cells, MHCII⁺ cells, and blood sinusoids, with a final merged image of all channels in *Cx3cr1^{gfp/+}* reporter mice. (Lower) Magnified region of the individual snapshots. Original scale bars, 20 mm. **(b)** An outline of the process involved during image analysis. Objects were first "gated" for a GFP⁺ signal before CX₃CR1⁺ objects were separated based on their

MHCII expression. CX₃CR1⁺MHCII⁺ and CX₃CR1⁺MHCII⁻ objects were subsequently analyzed for cell volume and sphericity and represented on a scatter plot. Objects with a volume of >1000 μm^3 and sphericity <0.65 were considered to display a DC-like morphology, whereas monocyte-like cells possessed volumes of <2000 μm^3 and sphericity >0.65. MFI, Mean fluorescence intensity. (c) Percentage of cells displaying a DC-like or monocyte-like morphology, are shown in red and green, respectively. (d) Scatter plot demonstrating a merged view of the total amount of CX₃CR1⁺MHCII⁺ and CX₃CR1⁺MHCII⁻ cells present in *Cx3cr1^{gfp/+}* reporter mice. Data are pooled from 4 fields of view from the same mouse (n = 3 mice).

Taken together, our results confirm our hypothesis that the large and stellate GFP⁺ cells in the BM parenchymal space were DC-like. Furthermore, this method provides further evidence that morphologic criteria can be used as a plausible and inexpensive approach to distinguish monocytes from DC-like cells in the BM of *Cx3cr1^{gfp/+}* mice.

Together, this method provides further evidence that morphologic criteria can be used as a plausible and inexpensive approach to distinguish DCs from monocytes in the BM of *Cx3cr1^{gfp/+}* mice.

3.1.3 *Flt3L*-deficiency reduces DC numbers and improve the identification of monocytes in the BM of *Cx3cr1^{gfp/+}* mice

Our results indicate that DCs can be discriminated from monocytes morphologically. To track monocyte activities in the BM accurately and efficiently, we next sought to deplete CX₃CR1⁺ DCs from the BM specifically. It has been previously reported that CX₃CR1⁺ DCs can be ablated by administration of DT in CD11c-DTR mice, which express the diphtheria toxin receptor under the control of the CD11c promoter (Milo

et al., 2013; Sapoznikov et al., 2008). However, this system lacks specificity for DC depletion, as other CD11c-expressing cells are shown to be targeted upon DT administration (Jung et al., 2002; Probst et al., 2005). In addition, DT-induced cell depletion in CD11c-DTR mice is known to result from the induction of cell death, which consequently, may lead to neutrophilia and monocytosis (Tittel et al., 2012). Thus, this approach precluded the use of these mice to study BM monocyte mobilisation under physiologic conditions.

Flt3 signalling plays a crucial and non-redundant role in DC homeostasis, as the lack of Flt3 or its ligand Flt3L affects DC proliferation, differentiation and the maintenance of DC numbers (Merad et al., 2013). We thus took advantage of this property and generated a crossbred *Cx3cr1^{gfp/+}Flt3L^{-/-}* reporter mouse. To validate this mouse model, we first analysed the frequency of DCs, monocytes and macrophages in *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice as compared to *Cx3cr1^{gfp/+}Flt3L^{+/+}* controls by flow cytometry **[Figure 3.5a-f]**. We found a decrease in BM cellularity in *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice, which is consistent with the known involvement of Flt3L in general hematopoiesis (Onai et al., 2007). Thus, to ensure an unbiased comparison of the cellular distribution in *Flt3L^{-/-}* and WT controls, we expressed the cell count in each immune populations as a fraction of GFP⁺ cells. Based on this readout, we show that CX3CR1⁺ DCs are significantly reduced in *Flt3L^{-/-}* animals, whereas no difference was observed in CSF1R-dependent macrophages and monocytes **[Figure 3.5c-f]**, as previously reported (Dai et al., 2002). Having shown that DCs are efficiently depleted in *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice, we next aimed to confirm this finding by intravital imaging. We acquired stitched images and performed a semi-automated quantification of GFP⁺ cells which were separated

according to their morphology: DC-like and monocyte-like **[Figure 3.6a]**. Accordingly, we found a significant reduction in cells harbouring a DC-like morphology in *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice as compared to *Cx3cr1^{gfp/+}Flt3L^{+/+}* controls **[Figure 3.6a,b]**. Of note, we detected a modest but significant decrease of DC-like cells by imaging **[Figure 3.6b]**, as compared with a sharp decrease of DCs by flow cytometry (1.53- vs 2.43-fold decrease) **[Figure 3.5a,c]**. Conceivably, this discrepancy could be attributed to a small population of CX3CR1⁺ BM macrophages that were not affected by the loss of Flt3L. These CX3CR1⁺ BM macrophages could be heterogeneous for MHCII and may possess a DC-like morphology according to multiphoton imaging, resulting in the inability to separate these cells from DCs in the earlier stage of our analysis.

Figure 3.5 Flt3L deficiency in *Cx3cr1^{gfp/+}Flt3L^{-/-}* reporter mice results in impaired DC development in the BM.

Flow cytometry plots identifying BM DCs (CX₃CR1⁺CD11c^{hi}MHCII^{hi}) (a) and monocytes (CX₃CR1⁺CD11b⁺CD115⁺Ly6C^{hi} or Ly6C^{lo}, according to the subset) (b). Comparison of percentages of DCs (c), macrophages (d), Ly6C^{hi} monocytes (Mo) (e) and Ly6C^{lo} monocytes (f) in *Cx3cr1^{gfp/+}Flt3L^{+/+}* and *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice. Cell percentages are expressed as a ratio of the cell count over the total GFP⁺ cell number. (n

= 5 mice/reporter strain). Data are shown as mean $\pm$ SEM and are pooled from 2 different experiments. n.s., Non-significant; * $P < 0.05$ (unpaired t-test).

In summary, we showed that *Flt3L*-deficiency leads to the reduction of DC-like CX3CR1⁺ cells in the BM, and this reduction is correlated with a significant reduction in BM DC frequency, thereby facilitating the tracking and study of BM monocyte behaviour in *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice. While our imaging data suggests that BM macrophages possess a DC-like morphology, monocytes could be clearly distinguished from these cells through our morphologic criteria, suggesting that this methodology can still be applied to specifically "segment" out monocytes in *Cx3cr1^{gfp/+}* mice.

Figure 3.6 Discriminating DCs from monocytes using morphology analysis in *Cx3cr1^{gfp/+}Flt3L^{-/-}* reporter mice.

(a) Representative stitched images of the skull BM of *Cx3cr1^{gfp/+}Flt3L^{+/+}* and *Cx3cr1^{gfp/+}Flt3L^{-/-}* reporter mice. Mice were administered with Evans blue i.v. to label sinusoidal vessels ($n = 3/\text{strain}$). Original scale bars (*left panel*), 100 μm . A white box on a stitched image displays a magnified version of an indicated region. Single plane images for $z = 12$ and 24 μm are illustrated. Original scale bars (*right panel*), 20 μm . DC-like cells, Magenta (*arrowheads*); monocyte-like cells, *green*; and BM sinusoids, *gray*. **(b)** CX3CR1⁺ cells were distinguished according to their morphology; i.e., cells with a volume >1000 μm^3 and sphericity >0.65 and volume <2000 μm^3 and sphericity >0.65 were considered to display a DC-like or monocyte-like morphology, respectively. The frequencies of DC-like or monocyte-like cells present in *Cx3cr1^{gfp/+}Flt3L^{+/+}* and *Cx3cr1^{gfp/+}Flt3L^{-/-}* reporter mice were analyzed and represented as a pie chart ($n = 3$ mice/reporter strain).

3.1.4 *Flt3L*-deficiency does not impair monocyte mobilisation from the BM

Among monocytes, the shorter-lived, pro-inflammatory Ly6C^{hi} subset represents the majority of monocytes that egress from the BM in a CCR2-dependant manner upon responding to peripheral challenges such as LPS (Shi et al., 2011).

Since our current results have demonstrated the *Cx3cr1^{gfp/+}Flt3L^{-/-}* mouse to be an ideal model for the visualisation of BM monocytes, we next ensured that the absence of Flt3L in this reporter mouse does not impair the migratory behaviour of BM monocytes. To this end, we subjected *Cx3cr1^{gfp/+}* mice to subclinical doses of LPS and analysed their BM and circulating Ly6C^{hi} monocyte numbers over time by flow cytometry [Figure 3.7a-c]. We observed that Ly6C^{hi} circulating numbers peaked 4h after LPS treatment [Figure 3.7b], while their BM numbers significantly decreased within 2h [Figure 3.7c]. We next compared the ability of monocytes to egress from the BM after LPS challenge in *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice versus *Cx3cr1^{gfp/+}Flt3L^{+/+}* controls. Similar to control mice, Ly6C^{hi} monocytes in *Cx3cr1^{gfp/+}Flt3L^{-/-}* mice showed increased blood counts and decreased BM numbers 2h after LPS treatment [Figure 3.7d,e]. Collectively, these data provide evidence that the lack of Flt3L does not significantly impair the ability of Ly6C^{hi} monocytes to egress from the BM in response to pro-inflammatory stimuli, such as LPS.

Figure 3.7 Monocyte mobilisation by LPS is not affected by the loss of Flt3L.

(a) Gating strategy to identify blood (*upper panel*) and BM (*lower panel*) Ly6C^{hi} and Ly6C^{lo} monocytes in *Cx3cr1^{gfp/+}* mice. (b-c) Kinetics of Ly6C^{hi} monocytes in blood (b) and BM (c) after i.v. injection of LPS (10 ng) in *Cx3cr1^{gfp/+}Flt3L^{+/+}* mice (*n* = 4 mice/ group/time-point). Data are shown as mean $\pm$ SD and are representative of 2 independent experiments. ***P* < 0.01 (unpaired t-test). (d-e) Comparison of the frequencies of Ly6C^{hi} monocytes (expressed as a ratio of the cell count over the total GFP⁺ cell number) in the blood (d) or BM (e), 2 h after LPS administration in *Cx3cr1^{gfp/+}Flt3L^{+/+}* and *Cx3cr1^{gfp/+}Flt3L^{-/-}* reporter mice. In both tissues, Ly6C^{hi} monocytes were considered Lin⁻ (CD3, NK1.1, Siglec-H) CD11b⁺SSC^{lo}Ly6G⁻CX₃CR1⁺ (*n* = 6–10 mice/group/condition). Data are shown as mean $\pm$ SEM and are pooled from 2 different experiments. *****P* < 0.0001 (unpaired t-test).

To further validate this finding, we analysed the behaviour of BM Ly6C^{hi} monocytes following LPS challenge through intravital imaging. Since monocytes exit the BM between 1 to 2 hours following LPS treatment, it is likely that we could detect a significant change in their behaviour at these time points. Indeed, while most GFP⁺ monocytes remain in the BM parenchyma at baseline [Figure 3.8a; video 2], they steadily increased their motility 1h following LPS administration, and gradually egressed from the parenchymal space into sinusoidal vessels within 2h [Figure 3.8a; video 2]. Furthermore, this increase in cell motility was accompanied by increased cellular displacement, which is in agreement with monocyte departure from the BM parenchyma [Figure 3.8b,c].

Of note, our tracking analysis indicated that BM monocytes, in *Cx3cr1^{gfp/+}Flt3L^{-/-}* and *Cx3cr1^{gfp/+}Flt3L^{+/+}* mice, displayed similar motility patterns 1h after LPS administration but *Cx3cr1^{gfp/+}Flt3L^{-/-}* monocytes exhibited a slight but significant increase in their velocity and displacement 2h after LPS treatment as compared to controls [Figure 3.8b,c; video 2,3]. Despite this difference, we believe that the overall functional mobilisation of BM monocytes is not significantly altered in

Flt3L-deficient mice, as there is no detectable difference in the number of circulating monocytes compared to controls in response to LPS **[Figure 3.7d]**.

Furthermore, using our morphologic identification method, we analysed the behaviour of BM CX3CR1⁺ DC-like cells over time. These cells were sessile during baseline conditions, with occasional probing through dendritic processes, akin to the behaviour of DCs in other organs (Ng et al., 2008). However, unlike monocytes, DC-like cells did not increase their motility following LPS treatment **[Figure 3.8d,e]**.

Figure 3.8 Intravital imaging of LPS-induced monocyte mobilisation in *Cx3cr1^{gfp/+} Flt3L^{-/-}* reporter mice is not affected by the loss of Flt3L.

(a) (Left) Maximum z-projected images of the skull BM in the *Cx3cr1^{gfp/+}* mouse at baseline. White dotted areas represent the BM parenchyma. (Right) Thirty-minute duration tracks of individual GFP⁺ monocyte-like cells at baseline and 1 and 2 h after LPS treatment in *Cx3cr1^{gfp/+} Flt3L^{+/+}* and *Cx3cr1^{gfp/+} Flt3L^{-/-}* reporter mice. Red dotted areas represent the BM parenchyma. Original scale bars, 20 μm . GFP⁺ cells, Green; sinusoidal blood vessels, red. **(b-c)** The mean velocity **(b)** and displacement **(c)** of GFP⁺ monocyte-like cells were measured at baseline and 1 and 2 h after LPS treatment in *Cx3cr1^{gfp/+} Flt3L^{+/+}* and *Cx3cr1^{gfp/+} Flt3L^{-/-}* reporter mice ($n = 3$ mice/group/treatment). **** $P < 0.0001$ (1-way ANOVA). **(d)** Mean velocity of GFP⁺ DC-like cells at baseline and 1 and 2 h after LPS treatment in *Cx3cr1^{gfp/+} Flt3L^{+/+}* mice ($n = 3$ mice/treatment). **(e)** A

representative end-point image illustrating the tracks of GFP⁺ DC-like from baseline to 2 h after LPS treatment. Original scale bar, 20 μ m.

Taken together, we provide a novel approach, through the combinatorial use of a *Cx3cr1^{gfp/+}* reporter mouse, multiphoton intravital microscopy and subsequent image analysis based on cell morphology, to study the mobilisation of BM monocytes. More importantly, these results obtained by multiphoton microscopy support and complement our flow cytometry data by providing in site information that includes morphological identification of DCs and monocytes, and the migratory status of these cells.

3.1.5 Discussion

In the first part of our study, we present a novel approach that allows the study of monocyte mobilization from the BM in vivo. Specifically, we achieved this through a combination of the *Cx3cr1^{gfp/+}Flt3L^{-/-}* reporter mouse, multiphoton intravital microscopy and subsequent image analysis based on cell morphology. Our current study also provides a significant conceptual advance by illustrating the use of a *Cx3cr1^{gfp/+}* based, dual functionality fluorescence reporter line for improving the specificity of monocyte imaging in the BM. More importantly, these multiphoton imaging results support and complement our flow cytometry data by providing in situ information that include morphological identification of DCs and monocytes and the migratory status of these cells.

The release of monocytes from the BM represents a major checkpoint in monocyte homeostasis as this process has a significant impact on the number of circulating monocytes. Thus, it is imperative to characterize the spatiotemporal regulation of monocyte mobilization from the BM to attain a deeper understanding of monocyte function. In this chapter, we propose the use of a *Cx3cr1^{gfp/+}Flt3L^{-/-}* mouse as a tool for analysing and tracking the cellular behaviour of monocytes in the BM *in vivo*. In particular, we showed that the absence of DCs in this mouse model allows efficient tracking of monocyte egress without affecting monocyte trafficking activities. Of note, it remains uncertain if the loss of DCs may influence other effector aspects of monocyte function. We also detected a decrease in monocyte cellularity, as well as other leukocytes in the absence of Flt3L, consistent with its involvement in early hematopoietic development (Onai et al., 2007). We however believe the absence of Flt3L is unlikely to affect monocytes directly since cMoPs or mature Ly6C^{hi} monocytes do not express Flt3 (Hettinger et al., 2013) (as discussed later in thesis) **[Figure 3.11a]**. Our study nevertheless demonstrates the *Cx3cr1^{gfp/+}Flt3L^{-/-}* mouse as a suitable model for the purpose of *in vivo* monocyte tracking in the BM through intravital imaging.

As part of the mononuclear phagocyte family, macrophages usually share in certain organs similar phenotypic characteristics with DCs through the expression of common surface markers (Gautier et al., 2012; Miller et al., 2012). These factors result in substantial difficulty in delineating macrophages from the DC lineage distinctively, especially since current technology does not allow a high number parameters to be available during intravital/immunofluorescence imaging. Since a monocyte specific reporter mouse is also currently unavailable, we

hence believe that our proposed *Cx3cr1^{gfp/+}* based combinatorial approach serves as the most suitable approach in deciphering monocyte trafficking activities *in vivo* as these mice are readily available. Furthermore, we believe that this approach would also be beneficial towards understanding the dynamic behavior and development of mononuclear phagocytes in other organs.

It is likely that the easy access to public microarray datasets generated by the Immgen project (Gautier et al., 2012; Miller et al., 2012), and the reduced cost of whole genome gene expression analysis will allow the discovery of genes with high cell specificity which will allow establishment of more specific transgenic models (Scott et al., 2016). More importantly, we believe that the recent advances in genome editing techniques such as CRISPR/Cas9 technology will allow the efficient and rapid generation of a novel fluorescent reporter mouse strains to improve the specificity of monocyte or other phagocytes tagging for future studies (Jain et al., 2016).

3.2 BM Ly6C^{hi} monocytes are functionally heterogeneous

3.2.1 Only a small fraction of BM monocytes are mobilised at a time

Upon analysis of our intravital imaging data, we noticed that while all monocytes respond to LPS by increasing their motility and displacement, only a small fraction could leave the parenchymal space and access the blood circulation [video 2,3]. To confirm these findings, we followed BM monocytes that were pulsed with the thymidine analog 5-bromo-2'deoxyuridine (BrdU) during LPS mobilization (Yona et al.,

2013) . Using this method, we detected newly egressed BrdU⁺ monocytes in the circulation after LPS treatment [Figure 3.9a]. However, this increase in monocyte blood counts was correlated with a rather minor 30% decrease in BM monocytes [Figure 3.9a]. Together, this implies that a limited pool of monocytes can be mobilised at one point in time, suggesting that BM monocytes may not consist of a homogenous population and could display different maturation states.

Figure 3.9 LPS treatment mobilises only a small fraction of BrdU⁺ Ly6C^{hi} monocytes into the bloodstream.

Mice were administered 1.5mg of BrdU i.p. and analyzed for BrdU⁺ Ly6C^{hi} monocytes in the blood (*left*) and BM (*right*) after 8 hours of pulsing. Results are represented as mean $\pm$ SD and representative of one out of three experiments. (n = 5 mice per group). *P< 0.05; **P< 0.01 (unpaired t-test).

3.2.2 Multi dimensional analysis of flow cytometry data reveals BM monocyte heterogeneity

To determine if potential heterogeneity exists among BM monocytes, we established a multicolour flow cytometry panel and analysed the data using a dimensional reduction algorithm. We used the t-distributed stochastic linear embedding (t-SNE) algorithm, which has been recently

employed to analyse multidimensional datasets such as those obtained from CyTOF (a.k.a. mass cytometry) experiments, but can also be applied to virtually any other forms of datasets, including flow cytometry data (Maaten and Hinton, 2008) [Figure 3.10a, more information at: <https://lvdmaaten.github.io/tsne/>]. Briefly, this algorithm allows the visualisation of multidimensional datasets into a 2D or 3D scatter plot called the t-SNE map. In the t-SNE map, cells that display high similarities in term of protein expression will be closely located and form distinct clusters, thus allowing the identification of discrete populations that could easily be omitted by conventional flow cytometry gating (Amir et al., 2013; Becher et al., 2014).

Figure 3.10 Example of a t-SNE algorithm implementation.

Pictures of objects clustered using t-SNE algorithm. Photos displaying similar characteristics will be share similar locations on the t-SNE map. Adapted from <https://lvdmaaten.github.io/tsne/>

After excluding committed progenitors such as cMoPs and other contaminating cells, we subjected BM Ly6C^{hi} monocytes to t-SNE dimensional reduction algorithm **[Figure 3.11a]**. Visualisation of BM Ly6C^{hi} monocytes on the t-SNE map revealed heterogeneity among these cells, which can be categorised into two main through automated clustering through the k-means method **[Figure. 3.11a]**. Upon examination of the markers included in our analysis, we found that CXCR4 delineates Ly6C^{hi} monocytes into two subsets that closely represent the outcome generated through automated clustering **[Figure 3.11b]**. In addition, CXCR4 segmentation resulted in the highest fold-change in protein expression between the two clusters **[Figure 3.11c,d]**, suggesting that CXCR4 can serve as a suitable marker in delineating BM Ly6C^{hi} monocyte heterogeneity.

Figure 3.11 BM Ly6C^{hi} monocytes are heterogeneous.

(a) BM Ly6C^{hi} monocytes were gated accordingly (*left*) and subjected to t-SNE dimensional reduction (*middle*). Automatic clustering performed using k-means, and clusters plotted into the t-SNE map (*right*). (b) Indicated markers are color mapped from blue (low expression) to red (high expression) into the t-SNE map. (c-d) Overlaid histograms (c) and tabulated table (d) of indicated markers constructed from data obtained from the k-means of cluster1 (*red*) and cluster 2 (*blue*) that was generated from automated clustering.

3.2.3 CXCR4 allows the delineation of BM classical monocytes into two distinct subsets in mouse and human

Using the same gating strategy as indicated [Figure 3.11a], we tested whether CXCR4 could distinguish BM Ly6C^{hi} monocyte heterogeneity.

Indeed, we found two distinct populations of Ly6C^{hi} monocytes in the BM that consist of a CXCR4^{hi} and CXCR4^{lo} subset **[Figure 3.12a]**. We next sorted these cells to determine if these cells were morphologically distinct. Scanning electron microscopy revealed morphological differences between these two subsets, with only the CXCR4^{lo} subset spreading its cytoplasm upon adhering to coverslips **[Figure 3.12b]**. Furthermore, Giemsa staining revealed the presence of cytoplasmic vacuoles and a more condensed and "bean" shaped nucleus that was present only in the CXCR4^{lo} subset **[Figure 3.12c]**.

We also determined if human BM monocytes displayed similar CXCR4 heterogeneity as mice BM monocytes. Indeed, we discovered that human BM monocytes can be distinguished into CXCR4^{hi}CD14^{lo} and CXCR4^{lo}CD14^{hi} subsets **[Figure 3.12d]**. Taken together, our results identify heterogeneity in the currently established BM Ly6C^{hi} monocyte pool and the presence of a distinct subpopulation that can be delineated through CXCR4.

Figure 3.12 CXCR4 delineates the heterogeneity between BM classical monocytes in mice and humans.

(a) Representative FACS plots depicting CXCR4 expression on Ly6C^{hi} monocytes in blood (*top*) and BM (*bottom*). Division of BM Ly6C^{hi} monocytes into CXCR4^{hi} (*blue*) and CXCR4^{lo} (*red*) populations based on CXCR4 and CD11b. (b) CXCR4^{hi} (*top*) and CXCR4^{lo} (*bottom*) subsets seeded onto coverslips and analyzed by scanning electron microscopy. Scale bars: 1µm. (c) CXCR4^{hi} (*top*) and CXCR4^{lo} (*bottom*) subsets were cytopun and stained with Giemsa coloration. (d) Gating strategy for human BM monocytes (pre-gated on DAPI^{neg}/singlets/Lin^{neg} (CD3/CD10/CD19/CD20/CD56/CD123)). Classical monocytes are defined as Lin⁻CD14⁺HLA-DR⁺CD34⁻CD16⁻CD11b⁺ and are subsequently subdivided into CXCR4^{hi} and CXCR4^{lo} populations.

3.2.4 Transcriptome profiling reveals distinct gene expression signatures between BM Ly6C^{hi} CXCR4^{hi} and CXCR4^{lo} monocytes

To determine if CXCR4^{hi} and CXCR4^{lo} monocyte subsets may be functionally distinct, we performed whole transcriptome sequencing using next generation sequencing (NGS) to compare their genome-wide RNA expression profiles. Principal component analysis (PCA) of all expressed genes revealed two distinct and highly separated transcriptomic profiles **[Figure 3.13a]**, implying that the CXCR4^{hi} subpopulation is clearly distinct from the CXCR4^{lo} subpopulation at the transcriptome level. Among the 10535 genes that expressed in these cells, we identified 3630 genes differentially expressed between these two subsets, with a particular skewing of genes that are overexpressed in the CXCR4^{hi} subpopulation **[Figure 3.13b]**. Notably, we found a significantly large enrichment of cell cycle-dependent genes such as *Ccnf*, *Top2a*, *Mki67* and *E2f7* in the CXCR4^{hi} subpopulation **[Figure 3.13b]**. In contrast, the CXCR4^{lo} subset is enriched in genes such as *Clec4a3*, *Lyz1*, *Sirpb1b*, *Siglec1* and *Il1b* that were generally associated with monocyte effector function **[Figure 3.13b]**. Analysis of genes according to their biological function revealed distinct expression signatures that distinguish the CXCR4^{hi} from the CXCR4^{lo} subset **[Figure 3.13c-i]**. Besides the large number of cell-cycle related genes that are significantly enriched in CXCR4^{hi} subpopulation as compared to the CXCR4^{lo}, we also found an enrichment of genes associated with DNA metabolism and repair **[Figure 3.13d]**. On the other hand, genes associated with cellular activation, antigen presentation, phagocytosis, response to inflammation and cell motility are significantly enrichment in the CXCR4^{lo} subpopulation **[Figure 3.13e-i]**.

Figure 3.13 Transcriptomics reveal distinct expression signatures between the CXCR4^{hi} and CXCR4^{lo} Ly6C^{hi} monocyte populations.

Next generation sequencing (NGS) was performed on RNA extracted from sorted BM CXCR4^{hi} and CXCR4^{lo} subsets. **(a)** Principle component analysis (PCA) of gene expression. **(b)** Volcano plots depicting the log₂ fold change between the CXCR4^{hi} and CXCR4^{lo} subset versus the –log₁₀FDR. **(c-i)** Heat maps showing genes that are significantly differentially expressed between the CXCR4^{hi} and CXCR4^{lo} subset that are involved in cell cycle **(c)**, DNA metabolic processes **(d)**, cell activation and antigen presentation **(e)**, phagocytosis **(f)**, chemokines and inflammation **(g)**, cell motility and adhesion **(h)** and development and differentiation **(i)**.

Taken together, our transcriptome analysis identifies key genome expression differences that delineate functional heterogeneity between the CXCR4^{hi} and CXCR4^{lo} subsets. In particular, the enrichment of cell cycle genes in the CXCR4^{hi} subpopulation contrasts with the enrichment of genes involved in monocyte function in the CXCR4^{lo} subpopulation and hence suggest a potential developmental relationship between these two subsets.

3.2.5 Identification of a developmental relationship between subpopulations of BM Ly6C^{hi} monocytes

To determine if the newly identified subpopulations of BM Ly6C^{hi} are developmentally related, we used the Wanderlust algorithm, which orders single cells in a constructed trajectory that correspond to their most likely developmental path within the high-dimensional space. This *in silico* approach has been used previously to recapitulate the development of human B cells using CyTOF (Bendall et al., 2014). The output of the Wanderlust algorithm corresponds to a predicted trajectory parameter, for which low and high values of Wanderlust represent early and late stages respectively, within a developmental continuum. Using the wanderlust algorithm, we found that early events (i.e. immature

cells) are represented by CXCR4^{hi}CD11b^{lo}CCR2^{lo}CX3CR1^{lo} cells, whereas late events (i.e. mature cells) consist of CXCR4^{lo}CD11b^{hi}CCR2^{hi}CX3CR1^{hi} cells [Figure 3.14a]. Furthermore, we found that during the course of the predicted monocyte developmental trajectory, CXCR4, CD31 and CD16/32 were downregulated, while CCR2, CX3CR1 and CD11b were upregulated [Figure 3.14b]. These results hence suggest that the CXCR4^{hi} subset may act as the precursor of the CXCR4^{lo} subset.

Figure 3.14 Identification of a developmental relationship between subpopulations of BM Ly6C^{hi} monocytes.

(a) BM Ly6C^{hi} monocytes subjected to the Wanderlust algorithm. Events were plotted against the wanderlust parameter and gated for low and high values, representing “early” and “late” predicted events respectively. Early and late events were next plotted for CXCR4 expression against CD11b, CCR2 and CX3CR1. **(b)** Wanderlust traces

showing decrease in CXCR4, CD31 and CD16/32 during the early trajectory (0-0.5), and increase in CX3CR1, CCR2 and CD11b during the late trajectory (0.5-1). **(c)** Intra-femoral transfer of sorted CD45.1⁺ CXCR4^{hi} subset into CD45.2 recipients. Blue dots represent transferred cells after 36 hours. **(d)** Representative FACS plots of BrdU⁺ (*blue*) and BrdU⁻ (*red*) Ly6C^{hi} monocytes after BrdU administration.

To confirm these findings *in vivo*, we adoptively transferred sorted CD45.1 CXCR4^{hi} BM Ly6C^{hi} monocytes into recipient mice and observed that the CXCR4^{hi} subset differentiated into the CXCR4^{lo} subset **[Figure 3.14c]**. Moreover, administration of the thymidine analog BrdU into mice, which allows us to label and trace dividing BM monocyte progenitors, further confirmed that the CXCR4^{hi} subset acts as an immediate precursor of the CXCR4^{lo} subset **[Figure 3.14d]**. Taken together, our results reveal a previously undefined developmental pathway of monocytes, and that the CXCR4^{hi} subset acts as an immediate precursor of mature BM Ly6C^{hi} monocytes.

3.2.6 CXCR4 delineates functional heterogeneity in BM Ly6C^{hi} monocytes

Our current results demonstrate that CXCR4 defines the phenotypic heterogeneity of BM Ly6C^{hi} monocytes. We next determined how this CXCR4 heterogeneity influences their mobilisation from the BM by tracking CXCR4 expression levels on BrdU-labeled BM Ly6C^{hi} monocytes and the appearance of BrdU⁺ Ly6C^{hi} monocytes in the circulation. Specifically, a higher CXCR4 expression is observed on immature/differentiating BrdU⁺ BM Ly6C^{hi} monocytes as compared to the mature/developed BrdU⁻ population at early time points **[Figure 3.15a,b]**. Furthermore, the appearance of BrdU⁺ Ly6C^{hi} monocytes in

the blood occurred when the CXCR4 expression on BM Ly6C^{hi} monocytes was low, thereby confirming that only the mature CXCR4^{lo} subset is able to egress the BM during homeostasis [Figure 3.15b,c].

Figure 3.15 CXCR4 identifies the mobilisable pool of BM Ly6C^{hi} monocytes.

(a) BrdU⁺ (red) and BrdU⁻ (blue) cells were gated (top) and overlayed onto histograms (bottom) to compare their expression of CXCR4 at

indicated time points after BrdU administration *in vivo*. **(b)** Alteration in CXCR4 expression level over time among BM Ly6C^{hi} monocytes (*left*) and percentage of BrdU⁺ Ly6C^{hi} monocytes in the blood at various time points (*right*). Δ MFI was calculated by subtracting the CXCR4 MFI of BrdU⁻ Ly6C^{hi} monocytes from that of BrdU⁺ Ly6C^{hi} monocytes. Results are expressed as mean $\pm$ SEM ($n=5$). **(c)** Graph showing correlation between blood BrdU⁺ Ly6C^{hi} monocytes and Δ MFI of CXCR4 in the BM. Significance was determined using a Pearson correlation test. **(d)** CXCR4^{hi} (blue; *left*) and CXCR4^{lo} (red; *right*) subset counts after LPS administration. Results expressed as mean $\pm$ SD ($n=5$). n.s. non-significant, ** $P < 0.01$, *** $P < 0.001$ (One-way ANOVA). **(e)** BrdU⁺ CXCR4^{hi} and CXCR4^{lo} subset counts after 2 hours of LPS administration. Results expressed as mean $\pm$ SD ($n=5$) n.s. non-significant, *** $P < 0.001$ (Student's t-test).

While it is clear that the CXCR4^{lo} subset represents mature Ly6C^{hi} monocytes that egress into the circulation during the steady-state, it is unclear if both CXCR4^{hi} and CXCR4^{lo} subsets may be mobilised during inflammatory conditions. To address this, we administered LPS, which is a potent stimulus of their CCR2-mediated egress [Figure 3.7, 3.8]. Intriguingly, we observed that LPS administration results only in the egress of CXCR4^{lo}, but not CXCR4^{hi} BM Ly6C^{hi} monocytes [Figure 3.15d]. Importantly, CXCR4 delineation of BrdU-labeled BM Ly6C^{hi} monocytes uncovered the specific mobilisation of the CXCR4^{lo} but not the CXCR4^{hi} subset [Figure 3.15e], thereby further confirming its expression as a key marker in identifying the mobilisable pool of mature BM Ly6C^{hi} monocytes. Furthermore, these results provide an explanation towards the smaller than expected number of monocytes that can egress the BM at one point of time [Figure 3.9].

Since CXCR4-CXCL12 signalling is essential for retaining Ly6C^{hi} monocytes in the BM [Figure 3.15d], we hypothesised that a stronger retention force in response to CXCR4-signalling may account for the inability of the CXCR4^{hi} subset to egress in response to LPS. To this

end, we examined whether CXCR4 inhibition could mobilise BM monocytes through multiphoton intravital microscopy. However, to our surprise and in contrast to the work of others (Jung et al., 2015; Liu et al., 2015), we observed that, unlike LPS or CCL2 administration, CXCR4-inhibition failed to promote any monocyte egress from the BM **[Figure 3.16a,b; video 4,5,6]**. Furthermore, while LPS and CCL2 treatments drastically increased monocyte motility in the BM, CXCR4-inhibition with AMD3100 led to decreased motility **[Figure 3.16c,d]**. These results hence suggest that CXCR4-signalling may be involved in the basal movements of monocytes in the BM. In addition, CX3CR1⁺ monocytes were found to co-localise with CAR cells in the BM **[video 7]**. More importantly, we did not detect any difference in BM monocyte numbers, even after their segregation into CXCR4^{hi} and CXCR4^{lo} subpopulations, after AMD3100 treatment **[Figure 3.16e]**. Together, these data hence suggest that the inhibition of CXCR4 alone does not result in any active migratory egress of BM monocytes.

Figure 3.16 Mobilisation of BM monocytes towards LPS and CCR2-signalling in comparison to inhibition of CXCR4-signalling.

(a) Maximum z-projected image of skull BM of *Cx3cr1^{gfp/+}* mouse during resting state. To delineate blood sinusoids, mice were injected with

Evans Blue i.v. and BM parenchyma where hematopoietic cells reside is delineated by white dotted lines (scale bar: 50µm. *green*: CX3CR1⁺ cells; *red*: Evans Blue). **(b)** Tracks of individual GFP⁺ monocytes taken for a duration of thirty minutes at baseline or 1 to 2 hours post-LPS treatment (*top*), CCL2 (*middle*) or AMD3100 (*bottom*) treatments (1 hour for CCL2 and 2 hours for LPS and AMD3100 treatments). Red dotted areas represent the BM parenchyma and white lines represent cell tracks. Scale bar: 50µm. Quantitative analysis for the mean velocity **(c)** and displacement length **(d)** of GFP⁺ monocytes at baseline and after LPS (left), CCL2 (middle) and AMD3100 (right) treatments. Results are representative of 1 mouse from 3 independent experiments per treatment. N.s. non significant; ****P< 0.0001 (One-way ANOVA). **(e)** CXCR4^{hi} (*left*) and CXCR4^{lo} (*right*) monocyte counts in BM 2 hours after indicated treatments. For mice co-treated with AMD3100 and LPS, AMD3100 was administered 30min prior to LPS. *P<0.05 (One-way ANOVA)

While CXCR4 inhibition was not sufficient to result in any BM monocyte egress, we noticed that AMD3100 treatment before LPS administration resulted in the further mobilisation of the CXCR4^{lo} subset, but not the CXCR4^{hi} subset **[Figure 3.16e]**. These results hence suggest that the retention force provided by CXCR4-signalling is not the sole factor involved in the immobilisation of the CXCR4^{hi} subset. Specifically, these results further indicate a possible limitation in their intrinsic response towards mobilisation cues. One possibility would be the lack of appropriate receptor(s) to promote mobilisation. Indeed, consistent with the results from our Wanderlust analysis **[Figure 3.14a]**, we observed that the immature CXCR4^{hi} subset displayed lower surface expression of CCR2 **[Figure 3.17a]**. This was correlated functionally in a transwell migration assay, whereby the CXCR4^{hi} subset displayed poor migration towards the CCR2 cognate ligand CCL2 **[Figure 3.17b]**. Taken together, these results hence indicate that the diminished response towards CCR2-signaling accounts for the immobilisation of the CXCR4^{hi} subset in the BM. More importantly, our data also highlights CXCR4 as

a key marker in the identification of the mature active pool of BM Ly6C^{hi} monocytes.

Figure 3.17 CXCR4^{hi} monocytes are inferior in their migration towards the cognate ligand of CCR2, CCL2.

(a) Representative histogram of CCR2 expression levels of the CXCR4^{hi} and CXCR4^{lo} monocyte subsets compared with the appropriate fluorescence minus one control (*left*). CCR2 MFI (arbitrary units) of CXCR4^{hi} and CXCR4^{lo} monocytes (*right*). Results are expressed as mean $\pm$ SEM ($n=15$). **** $P<0.0001$ (Student's t-test). **(b)** Percentage of BM CXCR4^{hi} and CXCR4^{lo} monocytes undergoing chemotactic migration towards varying concentrations of CCL2. Results are expressed as mean $\pm$ SD and are representative of 2 independent experiments. * $P<0.05$, ** $P<0.01$ (2-way ANOVA).

3.2.7 Discussion

In the second part of our study, we report the discovery of heterogeneity among BM Ly6C^{hi} monocytes. Specifically, we found that BM Ly6C^{hi} monocytes are not a homogeneous population as previously thought. Instead, they consist of two subsets (CXCR4^{hi} and CXCR4^{lo}) that are functionally distinct in their immunological roles. Furthermore, we extend the role of CXCR4 beyond its reported function as a mere BM anchoring signal by demonstrating its involvement in the control of monocyte tissue trafficking activities.

Using a combination of computational and flow cytometry analysis, we observed that cMoP-derived BM Ly6C^{hi} monocytes consist of two distinct subpopulations as defined by their CXCR4 expression. Isolation and further analysis of these cells revealed that the CXCR4^{hi} and CXCR4^{lo} subpopulations are transcriptionally distinct. In particular, the CXCR4^{hi} subpopulation was highly enriched in genes associated with cell cycle, cell division and DNA repair. In contrast, the CXCR4^{lo} subpopulation showed a profound enrichment in genes associated with cell activation, phagocytosis, motility and pattern recognition. These results hence suggest that a considerable amount of functional maturation continues to occur within the CXCR4^{hi} subpopulation. Consistent with this notion, results from our functional studies validated that the CXCR4^{hi} subset gives rise to mature CXCR4^{lo} Ly6C^{hi} monocytes over time in the BM, and that this CXCR4^{hi} subpopulation remains immobilised in the BM even under inflammatory conditions. Hence, based on our cellular phenotypic analysis, transcriptional profiling and functional assays of the CXCR4^{hi} subpopulation of BM Ly6C^{hi} monocytes, we propose that this newly identified population represents a "transitional pre-monocyte" (TpMo), that serves as an immediate precursor of mature Ly6C^{hi} monocytes **[Figure 3.18]**.

Figure 3.18 CXCR4 identifies a transitional pre-monocyte population in the BM.

Monocytes arise from cMoPs and undergo a developmental pathway that ends with Ly6C^{lo} monocytes as the terminally differentiated form of monocytes. In this study, we propose that cMoP-differentiated Ly6C^{hi} monocytes undergo a developmental transition, which involves a transitional pre-monocyte (TpMo) stage, before giving rise to mature Ly6C^{hi} monocytes. TpMos are identified based on their CXCR4^{hi} expression compared to mature Ly6C^{hi} monocytes that are CXCR4^{lo}. Furthermore, TpMos can be distinguished from cMoPs based on their negative expression for c-kit. Functionally, TpMos are immobilized in the BM to serve as an immediate precursor for the active pool of mature CXCR4^{lo} Ly6C^{hi} monocytes.

We believe that the existence of the TpMo population forms a regulatory checkpoint that precludes from the uncontrolled release of BM monocytes and allows for the rapid replenishment of functionally mature monocytes. Our results also suggest a new working model whereby

monocyte programs their intrinsic mobilising capacity according to their functional maturity, which in turn ensures that only functionally mature monocytes are being mobilised into the peripheral circulation. While our current data seems to suggest that TpMos are fully committed to form mature Ly6C^{hi} monocytes, further work would be required to determine if TpMos may also give rise to Ly6C^{lo} monocytes as an alternative pathway. Furthermore, the transcription factors that are necessary for their development may also be explored. It is noteworthy that our studies also revealed that TpMos differ from cMoPs based on their loss of expression of c-kit. However, it is still unclear how TpMos may differ from cMoPs functionally and if there are other phenotypic markers that allows further discrimination of these two populations.

Overall, classical Ly6C^{hi} monocytes are increasingly recognised as important therapeutic targets in various inflammatory diseases. We thus envision that the identification of the TpMo in our study may form the basis for future enhanced targeted treatment for monocyte-mediated diseases. While it is now certain that TpMos remain immobilised in the BM under LPS stimulation, it remains unclear if these cells may be mobilised into the circulation under high amounts of inflammatory stimuli or pathogen load, such as in the case of sepsis. Since our transcriptomic data suggests that TpMos are functionally less mature than CXCR4^{lo} Ly6C^{hi} monocytes, their mobilisation into the circulation during such disease states may lead to undesirable consequences in the host. In light of this possibility, the mechanistic action of other chemokine receptors, integrins and sympathetic nerve receptors that affect their trafficking behaviour during these inflammatory conditions may be explored.

Furthermore, preliminary evidence suggests that TpMos exhibit a high amount of proliferative activity that is comparable to that of cMoPs during the steady-state. It is therefore unclear how the proliferative activity of cMoPs and TpMos are distinctively driven in response to various forms of inflammatory stimuli or infectious agents in order to replenish monocytes that have left the BM. It is also uncertain how their proliferative activity may be implicated in diseases. Specifically, it would be interesting to determine whether certain infectious agents may hijack their proliferative activity, possibly by entry through the CXCR4 receptor, which may lead to uncontrolled proliferation and unintended collateral damage to the host. In support of this hypothesis, the role and proliferative activity of TpMos in diseases such as cancer/leukaemia should also be explored to determine if the proliferative capacity of TpMos might be a likely candidate/marker of disease progression.

Besides the discovery of CXCR4 as a key marker in the delineation of BM Ly6C^{hi} monocyte heterogeneity, we found that TpMos and mature Ly6C^{hi} monocytes also displayed profound differences in response to mobilisation cues. CXCR4-signaling has been shown to be an important mediator in retaining immune cells, including monocytes, in the BM (Griffith et al., 2014; Ma et al., 1999; Martin et al., 2003). Notably, a previous study proposed that the anchoring signal of CXCR4 is desensitised when CCR2 is activated through inflammatory stimuli (Jung et al., 2015). However, our results revealed that only mature CXCR4^{lo}, but not the immature CXCR4^{hi}, Ly6C^{hi} monocytes were able to leave the BM under CCR2-dependent inflammatory stimuli, indicating that this proposed mechanism should be interpreted with caution. Furthermore, we showed that CXCR4^{hi} TpMos remained unresponsive to mobilisation cues due to a combined effect of CXCR4-mediated retention and

reduced responsiveness to CCR2-agonists. For technical reasons, we could not confirm these findings using intravital imaging. Specifically, while CXCR4^{hi} TpMos express lower CX3CR1 as compared to mature CXCR4^{lo} monocytes at the protein level, this is not correlated at the gene expression level, and hence these subsets cannot be distinguished using *Cx3cr1*^{gfp/+} reporter mice. Together, our results suggest that monocytes must switch from a functionally "BM-residing" phenotype into a "mobilisable" phenotype as they mature in order to enter the circulation. We believe that this functional heterogeneity forms a "two-step checkpoint": (1) to ensure that not all Ly6C^{hi} BM monocytes are mobilised into the circulation at one point of time, even in response to inflammatory cues, and (2) to have a precursor population that differentiates and replenishes mature mobilisable monocytes.

It is also noteworthy that our data, including our imaging experiments, demonstrated that inhibiting CXCR4 alone is insufficient to trigger active migratory egress of mature BM Ly6C^{hi} monocytes. This is in contrast to two recent studies, which demonstrated a reduction of BM monocyte numbers after CXCR4 inhibition and in the absence of mobilising signals (Jung et al., 2015; Liu et al., 2015). One possible explanation for this discrepancy may be due to differing TLR agonist/endotoxin levels that are present in different animal facilities. For instance, C57BL/6 SPF mice co-housed with pet-store mice displayed a profound increase in circulating monocytes and neutrophils numbers, likely because pathogenic bacteria derived products promote the active egress of myeloid cells from the BM (Beura et al., 2016). This hypothesis is also supported by our results, whereby CXCR4 inhibition promoted further mobilisation of monocytes from the BM after LPS exposure **[Figure 3.16E]**. We would also like to highlight that our findings are not in

contradiction with the notion that CXCR4 is important for monocyte retention in the BM as reported by previous studies (Griffith et al., 2014), but rather our data suggest that CXCR4 inhibition lowers the mobilisation threshold for monocytes to egress from the BM.

3.3 CXCR4 regulates monocyte homeostasis through their shuttling between the BM and blood

3.3.1 CXCR4 regulates monocyte circulating numbers

Our results suggest that the immune system selectively regulates monocyte entry into the circulation by enforcing a secondary checkpoint in the BM compartment, which allows only the mature CXCR4^{lo} subset to be responsive to mobilisation cues. While it seems that their ability to respond to CCR2-signalling is a key factor in determining their mobilisation capacity [Figure 3.17a,b], it remains unclear how CXCR4 functions to fine-tune the release of these cells into the circulation.

To address this question, we used a conditional knockout mouse for CXCR4 under the control of the *Lyz2* promoter since the full deficiency in CXCR4 is embryonic lethal (Nagasawa, 2014). In the blood of this conditional knockout mouse (termed *Lyz2^{cre}Cxcr4^{fl}*), myeloid cells such as neutrophils, Ly6C^{hi} and Ly6C^{lo} monocytes were found to be deficient of CXCR4 [Figure 3.19a-c]. However, cells that do not express the *Lyz2* promoter such as B cells (Abram et al., 2014; Clausen et al., 1999), did not show alterations in their CXCR4 expression [Figure 3.19b].

Figure 3.19 CXCR4 is efficiently deleted in the myeloid compartment of *Lyz2^{cre}Cxcr4^{fl}* mice.

(a) Gating strategy for blood monocytes (pre-gated on DAPI^{neg}/singlets). Monocytes are defined as Lin (CD3/B220/NK1.1)⁻CD115⁺Ly6G⁻CD11b⁺ and Ly6C^{hi}CD43⁻ or Ly6C^{lo}CD43⁺ according to the subset. **(b)** Comparison of blood leukocyte population in *Rosa^{mT/mG}·Lyz2^{cre}* mice and *Rosa^{mT/mG}·Lyz2^{cre}Cxcr4^{fl}* mice depicting the percentage of *Lyz2^{cre}* mediated recombination (GFP⁺) at the *Rosa26* locus (*left*) and the normalized CXCR4 median fluorescence intensity (MFI, arbitrary units) of circulating leukocyte subsets (*right*). Normalized MFI was calculated as follows: $MFI^{CXCR4} - MFI^{FMO}$ (FMO: fluorescence minus one). Mean $\pm$ SD (n=4-5 mice per group). N.s. non significant, *P< 0.05, **P< 0.01, ****P<0.0001 (Two-way ANOVA). **(c)** Representative FACS plot depicting the expression of CXCR4 in *Ly6C^{hi} Mo* (*red*) as compared to *Cxcr4^{fl}* controls.

To complement this approach, we used a knock-in mouse strain that carried a CXCR4 gain-of-function mutation found in patients with WHIM syndrome (termed *Cxcr4^{WHIM}* or WHIM mouse). In this mouse, all cells displayed increased CXCR4-signalling with impaired desensitisation, akin to that observed in WHIM patients (Balabanian et al., 2012).

Using these CXCR4 genetic-modified mice, we detected a 10-fold increase in monocytes circulating monocyte numbers in *Ly2^{cre}Cxcr4^{fl}* mice as compared to *Cxcr4^{fl}* control animals [Figure 3.20a,b]. In contrast, WHIM mice exhibit monocytopenia, with an average 5-fold reduction in circulating Ly6C^{hi} monocytes, and 10-fold lower numbers in Ly6C^{lo} monocytes relative to WT controls, which can be reversed by inhibiting CXCR4 with AMD3100 [Figure 3.20a-c].

In addition, BrdU-pulsing experiments revealed that BrdU⁺ Ly6C^{hi} monocytes appear in the blood just 4h later in *Ly2^{cre}Cxcr4^{fl}* mice, but not until 8-12h in WT mice, and 12-24h in WHIM mice [Figure 3.20d]. These results hence suggest that CXCR4 fine-tunes the release of mature monocytes into the blood by regulating their rate of release into the circulation.

Figure 3.20 CXCR4 controls circulating numbers.

(a) Representative FACS plots of Ly6C^{hi} (red) and Ly6C^{lo} (blue) monocytes in *Cxcr4*^{WHIM} mice, *Lyz2*^{cre}*Cxcr4*^{fl} mice and *Cxcr4*^{fl} control mice. **(b)** Monocyte numbers in *Cxcr4*^{fl} control mice vs *Lyz2*^{cre}*Cxcr4*^{fl} mice. Results are expressed as mean $\pm$ SEM. ($n=5$) **** $P<0.0001$ (Student's t-test) **(c)** Circulating Ly6C^{hi} (left) and Ly6C^{lo} (right) monocyte counts after AMD3100 administration in WT mice at indicated time points. Results are representative of three independent experiments and expressed as mean $\pm$ SEM ($n= 3-5$). n.s. non-significant, * $P<0.05$, *** $P<0.001$, **** $P<0.0001$ (multiple Student's t-test). **(d)** Kinetics of BrdU⁺ Ly6C^{hi} monocyte release into the circulation of WT, *Lyz2*^{cre}*Cxcr4*^{fl} and *Cxcr4*^{WHIM} mice. Results are expressed as mean $\pm$ SEM ($n=5$). *** *Lyz2*^{cre}*Cxcr4*^{fl} versus wild-type control, *** *Cxcr4*^{WHIM} versus wild-type control, $P<0.001$ (multiple Student's t-test).

3.3.2 CXCR4 controls the homing of monocytes back to the reservoirs

Our current data indicates that an inefficient release of BM monocytes into the circulation accounts for the dysregulated circulating numbers observed in CXCR4-genetically modified mice. However, circulating monocyte homeostasis may also depend on the rate of monocytes that exit from the peripheral circulation to infiltrate tissue compartments, or home to reservoirs such as the spleen or the BM. Since the BM represents the largest source of CXCL12, it is likely that circulating monocyte numbers may be influenced by their continuous homing to the BM during homeostasis.

To address this hypothesis, we adoptively transferred CD45.1 BM monocytes into CD45.2 recipient mice, and observed that grafted monocytes rapidly decline in numbers in the blood and accumulate in the BM via a mechanism that was disrupted by CXCR4 inhibition through AMD3100 [Figure 3.21a,b].

Figure 3.21 CXCR4 controls the homing of monocytes into tissue reservoirs.

(a-b) Total number of donor (CD45.1⁺) monocytes with a Ly6C^{hi} (*left*) or Ly6C^{lo} phenotype (*right*) detected in blood **(a)** or BM **(b)** in recipient CD45.2⁺ mice that had been pre-treated with or without AMD3100. Results are expressed as mean $\pm$ SD ($n=4$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (2-way ANOVA). **(c-d)** Homing capacity (calculated as a ratio) of monocytes or indicated immune cells of wild-type and *Lyz2^{cre}Cxcr4^{fl}* **(c)** or *Cxcr4^{WHIM}* **(d)** into the BM or spleen of their respective partners in parabiotic mice. Monocyte, neutrophil and T-cell numbers were quantified in blood, BM and spleen prior to calculation of the respective homing ratios to normalize for unequal exchange in circulating leukocytes ($n=4$). n.s. non-significant, * $P<0.05$, ** $P<0.01$ (Student's t-test). **(e)** Percentage of non-host cells present in blood of wildtype and *Lyz2^{cre}Cxcr4^{fl}* mice (*left*), and wildtype and *Cxcr4^{WHIM}* parabiotic mice (*right*). Results are expressed as mean $\pm$ SD ($n=4-5$) and representative of one out of two independent experiments.

To further validate these findings, we established parabiosis between CD45.1 WT mice, and either CD45.2 *Lyz2^{cre}Cxcr4^{fl}* or CD45.2 *Cxcr4^{WHIM}* partners. As the *Lyz2^{cre}Cxcr4^{fl}* and WHIM mice exhibit monocytosis and monocytopenia at the steady-state respectively **[Figure 3.20a-c]**, *Lyz2^{cre}Cxcr4^{fl}* cells repopulate the blood of their WT partners **[Figure 3.21e]**, while WT cells repopulated the blood of their WHIM partners **[Figure 3.21e]**. This results in an unequal exchange of cell numbers between the parabionts. To address this issue, we formulated a homing ratio such that cells that home to their respective partner (non-host) organs were normalised to the circulating cells that traffic into the non-host **[Figure 3.21c]**. This approach allows us to determine how a genetic loss- or gain-of-function in CXCR4 affects monocyte homing patterns *in vivo*. Using this approach, we observed a significant decrease in the homing of CXCR4-deficient monocytes to the BM and spleen **[Figure 3.21c]**. This trend was similarly observed with neutrophils **[Figure 20c]**, and is consistent with the known requirement of CXCR4 in mediating their homing to the BM (Devi et al., 2013; Martin et al., 2003; Suratt et al., 2004). In contrast, no significant difference in

T-cell homing was observed between *Lyz2^{cre}Cxcr4^{fl}* and WT parabionts, since T-cells are not targeted by the *Lyz2^{cre}*-mediated deletion of CXCR4 (Abram et al., 2014) **[Figure 3.21c]**, and confirms that our earlier observations on monocytes are cell intrinsic and specifically mediated by CXCR4. In contrast, WHIM mice exhibit a corresponding increase in monocyte homing to the BM in their WT partners **[Figure 3.21d]**. While differences in WHIM Ly6C^{hi} monocyte homing to the spleen are not statistically significant, there is a trend towards increased homing of these cells to the spleen **[Figure 3.21d]**. Furthermore, WHIM Ly6C^{lo} monocytes exhibited an increased homing to the spleen of their WT partners **[Figure 3.21d]**. Together, these data underline the importance of CXCR4 in mediating the homing of monocytes to the BM and splenic reservoirs.

3.3.3 CXCR4 regulates the circadian oscillation in circulating monocyte numbers

Our current results indicate that CXCR4 regulates monocyte homeostasis in a bidirectional manner between the BM and blood compartments. These findings hence suggest that blood monocyte numbers can be controlled through changes in their CXCR4 expression. A previous study has shown that Ly6C^{hi} monocytes exhibit diurnal variation in numbers that is controlled by the circadian rhythm (Nguyen et al., 2013). Based on our current results, we hence hypothesise that CXCR4-signalling may play a role in this process.

To this end, we found that Ly6C^{hi} monocytes exhibit fluctuations in CXCR4 expression according to the circadian rhythm **[Figure 3.22a]**. In

particular, we observed that CXCR4 expression on monocytes is significantly lower at ZT5 (zeitgeber 5, i.e. 5h after the onset of light) as compared to ZT13 (i.e. 1h after the offset of light), which corresponds to the high and low number of circulating monocytes at ZT5 and ZT13 respectively **[Figure 3.22a]**. More importantly, this change in monocyte numbers under the influence of the circadian rhythm is abrogated in the absence of CXCR4 **[Figure 3.22a]**. Interestingly, we also observed that the circadian oscillations had no impact on the expression of CXCR4 and numbers of the immature CXCR4^{hi} BM Ly6C^{hi} subset **[Figure 3.22b]**. On the other hand, we detected an increased expression of CXCR4 and numbers of the mature CXCR4^{lo} BM Ly6C^{hi} subset at ZT13 **[Figure 3.22c]**, which coincided with low Ly6C^{hi} monocytes counts in the blood at this timepoint **[Figure 3.22a]**. These results hence suggest that monocytes may selectively home to the BM at night through CXCR4. Taken together, these data hence indicate a regulatory relationship between CXCR4 and the circadian control of circulating monocyte numbers that was specific towards mature monocytes.

Figure 3.22 CXCR4 regulates circadian rhythmic oscillations in circulating Ly6C^{hi} monocyte numbers.

(a) Blood Ly6C^{hi} monocyte values of normalized CXCR4 MFI (value obtained after subtraction of FMO control) on *Cxcr4^{fl}* control mice (*left*) and counts between *Cxcr4^{fl}* control and *Lyz2^{cre}Cxcr4^{fl}* mice (*right*) were quantified at ZT5 (zeitgeber 5, 5 hours after the onset of light) and ZT13 (at the beginning of the active phase). **(b-c)** BM Ly6C^{hi} monocytes that are CXCR4^{hi} (immature) **(b)** and CXCR4^{lo} (mature) **(c)** were quantified for their numbers (*left*) and their normalized CXCR4 MFI (*right*) at ZT5 and ZT13. Results are expressed as mean $\pm$ SD ($n=4-5$). n.s. non-significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Student's t-test).

3.3.4 Discussion

While it is well established that CXCR4 is a crucial signal to retain monocytes in the BM, it remains unclear if reduced CXCR4 expression upon their egress into the circulation indicates a diminished function in monocyte biology. In contrast to this assumption, we observed that

CXCR4 functions as a key factor in the spatiotemporal localisation of monocytes in peripheral compartments.

Interestingly, a lower CXCR4 expression in mature Ly6C^{hi} monocytes was not associated with a decreased importance of CXCR4-signalling in monocyte biology. Indeed, we found that CXCR4 circadian oscillation is associated with changes in Ly6C^{hi} monocytes circulating numbers. While the numbers of Ly6C^{hi} monocytes in tissues such as the spleen and the lungs are in phase with those of the blood (data not shown), they are in antiphase with those of the BM, suggesting Ly6C^{hi} monocytes home to the BM in a circadian fashion.

Specifically, Varol et al. have previously demonstrated that monocytes could shuttle back to the BM through an unknown mechanism in the steady state (Varol et al., 2007). Using parabiosis and adoptive transfer approaches, we provide the first experimental evidence to demonstrate that CXCR4-mediated tissue homing of monocytes represents a key mechanism in regulating their circulating monocyte numbers. Furthermore, we showed that circulating monocytes constitutively home back to the BM in a CXCR4-dependent manner, which is in line with the postulation discussed by Wang et al. in their study (Wang et al., 2009). However, future work would be required to determine the reasons for their active homing back to the BM. Besides homing back to the BM as a homeostatic requirement to maintain circulating monocyte numbers, it is also possible that monocytes may bring back antigens that were scavenged in the blood to directly present to T cells, or transfer antigens to other BM APCs such as DCs or macrophages. Alternatively, Ly6C^{hi} monocytes that home back to the BM may differentiate into BM Ly6C^{lo} monocytes with distinct patrolling/immunosurveillance properties, which could have been “educated” during their transit in the circulation.

Notably, we found a similar trend in CXCR4-dependent homing to the spleen, which further supports the idea that the spleen contains a reservoir of bona fide monocytes that is not simply synonymous with the blood pool. While we believe that the BM remains the major compartment where circulating monocytes home to as the size of the splenic reservoir (Swirski et al., 2009) and its CXCL12 expression (Inra et al., 2015) are much smaller than that of the BM. Splenic monocytes are mobilised during vascular and ischemic injury and increase in numbers through extramedullary hematopoiesis (Leuschner et al., 2012; Robbins et al., 2012). Therefore, future work would be required to determine the extent of monocyte homing to the spleen in these scenarios and how the absence of their homing through CXCR4 inhibition affects disease outcome.

It is important to point out that this bi-directional regulation of monocyte trafficking between the blood and BM compartments through an identical CXCR4 signal suggests a functional outcome that is highly contextual. It is perceived that the high levels of CXCL12 in the BM serve as an anchoring force towards CXCR4-expressing monocytes. In line with this idea, we observed decreased monocyte motility in the BM parenchyma following CXCR4-inhibition. Consequently, mature monocytes that have downregulated CXCR4 may be found at perisinusoidal spaces where they are ready to respond to CCR2 mobilising signals. After entering the bloodstream, CXCR4-expressing monocytes may sense CXCL12 as they traffic past BM niche cells, which result in their sequestration into the BM parenchymal space. Furthermore, our data highlights the temporal regulation of CXCR4 by demonstrating its role in governing the circadian rhythmic oscillation of mature monocyte numbers.

It is noteworthy that while the inhibition of CXCR4 results in a 10-fold increase in circulating monocyte numbers, our preliminary data revealed that monocyte-derived CX3CR1^{hi} macrophages in the gut did not increase in numbers in the steady state. This phenomenon does not seem attributed to reduced infiltration capacity of monocytes into tissues in the absence of CXCR4, as *Cxcr4*-deficient monocytes were found to home efficiently into the skin in an ischemia/reperfusion model. Therefore, it is likely that tissue compartments can only support a finite number of macrophages at any one point in time due to the competition for survival and growth factors (Scott et al., 2016; Theurl et al., 2016).

3.4 CXCR4 regulates the margination of monocyte in the pulmonary circulation

3.4.1 Endotoxemia promotes the retention of monocytes in the pulmonary circulation

It is well established that LPS administration is a potent mediator that mobilises monocytes from the BM, and consequently results in increased circulating monocyte numbers (Shi et al., 2011) **[Figure 3.7b,c]**. However, we consistently observed a phase of monocytopenia in the blood that occurred 1h after LPS administration **[Figure 3.7b, Figure 3.23a]**, which corresponded to a timepoint with no significant changes in BM monocyte numbers **[Figure 3.7c]**. These results hence suggest that blood monocytes are possibly withdrawn temporarily from the circulation into other tissues other than the BM.

To address this hypothesis, we first adopted an intravascular staining protocol **[Figure 3.23b]**, which allowed discrimination between intravascular and extravascular cell distributions in tissues *in vivo* (Anderson et al., 2014; Ng et al., 2011). In the lungs, this approach confirmed the extravascular location of alveolar macrophages, whereas monocytes and neutrophils were identified as being intravascular **[Figure 3.23c]**, which is in agreement with the work of others (Anderson et al., 2014; Barletta et al., 2012; Rodero et al., 2015). Using this method, we found that the disappearance of circulating monocytes after LPS administration was accompanied by a gradual accumulation of these cells in the intravascular compartment of the lung, with limited monocyte sequestration into other major organs such as the spleen, liver and kidney **[Figure 3.23e,f]**. Furthermore, this phenomenon was mediated by the intrinsic sensing of LPS by monocytes, and not by vascular stromal cells, as our BM chimera data showed that *Tlr4*-deficient monocytes are unable to accumulate in the lung vasculature **[Figure 3.23g]**. Therefore, our data suggests that circulating monocytes are transiently withdrawn from the peripheral circulation and are sequestered within the lung vasculature in response to systemic inflammatory stimuli.

Figure 3.23 Endotoxemia promotes monocyte retention in the pulmonary circulation.

(a) Blood Ly6C^{hi} (top) and Ly6C^{lo} (bottom) monocyte counts after intravenous treatment with 10ng LPS. Results are representative of one out of three independent experiments. (b) Illustration showing the intravascular labeling of leukocytes with anti-CD45 antibodies. (c) Flow cytometry plots showing the extravascular and intravascular localization of lung immune cells. Results are representative of one out of three independent experiments. (d-e) Quantification of Ly6C^{hi} (left) and Ly6C^{lo} (right) monocytes in blood (d) and lung tissues (e) from LPS-treated

mice. Results are expressed as mean $\pm$ SEM ($n=4$) and representative of one out of four experiments. n.s. non-significant, $*P<0.05$, $**P<0.01$, $***P<0.001$ (One-way ANOVA). **(f)** Fold increase in Ly6C^{hi} (*left*) and Ly6C^{lo} (*right*) monocytes between baseline and after 1 hour of LPS administration in lungs, spleen, liver and kidneys. Results are expressed as mean $\pm$ SD ($n=4-5$) and representative of one out of three experiments. $****P<0.0001$ (One-way ANOVA) **(g)** WT and *Tlr4*^{-/-} chimeric mice were analyzed for Ly6C^{hi} monocyte numbers after 1h of LPS administration. Each data point represents one individual mouse and results are representative of two individual experiments. n.s. non-significant, $**P<0.01$ (One-way ANOVA).

3.4.2 Intravital visualisation of lung monocytes

To understand the mechanism underlying LPS-induced monocyte accumulation in the lung, we utilised intravital two-photon microscopy to visualise the pulmonary microvasculature. To allow stable imaging of monocytes with minimal perturbation of the lung physiology, mice were anaesthetised and placed under a mechanical respirator. The rib cage was subsequently opened, and a vacuum chamber equipped with a coverslip was applied to stabilise the lung and minimise their respiratory movements (Looney et al., 2010). Notably, while the alveolar compartment can be imaged at high resolution using this model, larger bronchioles cannot be visualised without additional *ex vivo* manipulations (Thornton et al., 2012).

To select the most suitable reporter mouse for the imaging and identification of lung monocytes, we compared the fluorescence intensity and specificity for monocytes in the *Cx3cr1*^{gfp/+} and *Ccr2*^{gfp/+} mouse. Synonymous to results obtained with the BM **[Figure 3.1,3.2,3.3]**, *Cx3cr1*^{gfp/+} reporter mice delineated monocytes with appropriate sensitivity and specificity, as all monocytes were labelled and

constituted the majority of labelled cells **[Figure 3.24a]**. In contrast, while all monocytes were labelled in the *Ccr2^{rfp/+}* mouse, the majority of lung RFP⁺ cells were identified as T cells **[Figure. 3.24a]**. Hence, we chose the *Cx3cr1^{gfp/+}* reporter mouse to study the behaviour of lung monocytes.

Figure 3.24 Multiphoton intravital imaging reveals monocytes margination in the pulmonary circulation.

(a) Pie chart depicting the proportion of immune cells that are fluorescent for GFP in the *Cx3cr1^{gfp/+}* mouse (*top*) and RFP in the

Ccr2^{flp/+} mouse (*bottom*). (**b**) A representative GFP⁺ monocyte (arrowhead) alters its morphology through pulmonary capillaries. (Scale bar: 15µm; time: h:mm:ss. *green*: CX₃CR1⁺ cells; *red*: TRITC-dextran). (**c**) Snapshots of GFP⁺ monocytes in the lung of a *Cx3cr1^{gfp/+}* reporter mouse, before (*top*) and after (*bottom*) i.v. administration of a Ly6B.2-PE antibody (4µg). A representative GFP⁺ monocyte is depicted migrating through pulmonary capillaries (white track), becomes PE⁺ after antibody administration (scale bar: 15µm; time: h:mm:ss; *green*: CX₃CR1⁺ cells, *red*: Ly6B.2⁺ cells, *blue*: Evans blue). (**d**) FACS plots of lungs from *Cx3cr1^{gfp/+}* mice i.v. injected with Ly6B.2-PE. Ly6C^{hi} (*red*), Ly6C^{lo} monocytes (*orange*) and neutrophils (*green*) were plotted in relation to Ly6B.2 expression. Results are representative of one out of three independent experiments.

At basal conditions, we found that monocytes interacted frequently with the small diameter lung capillaries that surround the alveoli. Furthermore, monocytes were found to decrease their velocity and often alter their morphology upon travelling through these capillaries [**Figure 3.24b**], which are characteristic features of margination (Kuebler and Goetz, 2002; Looney and Bhattacharya, 2014).

Since it is well known that Ly6C^{hi} monocytes display lower levels of GFP in *Cx3cr1^{gfp/+}* mice (Geissmann et al., 2003), we ensured that our approach was able to detect Ly6C^{hi} monocytes by administering an anti-Ly6B.2 antibody (also known as 7/4) to stain these cells *in vivo*. Within 5 minutes after antibody administration, we could detect Ly6B.2⁺GFP⁺ cells interacting with the lung vasculature by intravital imaging [**Figure 3.24c**; **video 8**] and these cells were identified as Ly6C^{hi} monocytes through flow cytometry [**Figure 3.24d**]. Together, these data confirm that our imaging approach accounted for both subsets of monocytes.

3.4.3 CXCR4 inhibition prevents monocyte retention in the pulmonary circulation during endotoxemia in mice

While monocytes typically slow down and arrest as they pass through the lung vasculature at basal state [Figure 3.24b, 3.25a,b], we found that LPS exposure exacerbates this behaviour, leaving to a more prolonged arrest and active crawling of monocytes on the lung endothelium [Figure 3.25a,b; video 9].

Figure 3.25 Intravital imaging reveals increased monocyte margination in the pulmonary circulation after LPS treatment.

(a) Schematic of treatments during lung imaging experiments. **(b)** Monocyte mean velocity and duration of adherence after indicated treatments. Results expressed as mean ± SEM ($n=3$). n.s. non-significant, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (One-way ANOVA).

Since lung endothelial cells express CXCL12 (Devi 2013), we speculated that the CXCL12/CXCR4-signalling axis might be involved in monocyte margination in the lung. To test this hypothesis, we treated *Cx3cr1^{gfp/+}* mice with AMD3100 and observed that monocytes travelled through the lung microcirculation at a higher velocity and with significantly reduced adherence to the endothelium, even in mice treated with the “pro-margination” stimulus LPS [Figure 3.25a,b]. Of

note, the sole inhibition of CXCR4 with AMD3100 promoted the demargination of lung monocytes **[Figure 3.25a,b]**.

We next sought to determine whether CXCR4 regulates monocyte margination equally in both Ly6C^{hi} and Ly6C^{lo} subsets. To this end, we employed our previously published "lung efflux assay", to quantify monocyte release from the pulmonary circulation without interference from interposing organs (BIERMAN et al., 1952; Devi et al., 2013). Briefly, we collected blood from the carotid artery and from the vena cava in tandem and quantified monocytes in both samples. We next subtracted monocyte numbers in carotid blood from monocytes present in the vena cava to obtain the net release of cells from the pulmonary circulation **[Figure 3.26a]**. Using this assay, we observed that CXCR4-inhibition through AMD3100 increased the net release of both Ly6C^{hi} and Ly6C^{lo} monocytes from the lungs **[Figure 3.26b]**. However, only the Ly6C^{hi} subset displayed a significant increase in lung retention following LPS, and this process can be prevented by AMD3100 treatment **[Figure 3.26b]**. Notably, these results were similar to our observations obtained through intravital imaging **[Figure 3.25a,b]**. We also substantiate these findings by utilising genetically modified mice that had a gain- or a loss-of-function in CXCR4-signalling. We found that LPS challenge resulted in a greater accumulation of Ly6C^{hi}, but not Ly6C^{lo} monocytes in the lungs of the CXCR4 gain-of-function WHIM mouse. In contrast, a CXCR4 loss-of-function in *Lyz2^{cre}Cxcr4^{fl}* mice totally abrogated this accumulation **[Figure 3.26c]**. Together, these data indicates that endotoxemia promotes the retention of Ly6C^{hi} monocytes within the lung microcirculation and this process can be prevented through CXCR4 inhibition.

Figure 3.26 CXCR4 mediates monocyte margination in the pulmonary circulation.

(a) Schematic of the lung efflux assay. **(b)** Monocyte numbers released from the lung vasculature following the indicated treatments. Results are expressed as mean ($n=5-7$). n.s. non-significant, $*P<0.05$, $**P<0.01$, $***P<0.001$ (Student's t-test). **(c)** LPS-induced fold-increase in lung Ly6C^{hi} (left) and Ly6C^{lo} (right) monocytes in $Lyz2^{cre} Cxcr4^{fl}$ mice, $Cxcr4^{WHIM}$ mice, and $Cxcr4^{fl}$ control mice assessed 1 hour post-treatment. Results are expressed as mean $\pm$ SEM ($n=4-7$). n.s. non-significant, $*P<0.05$, $***P<0.001$ (One-way ANOVA).

3.4.4 CXCR4 inhibition promotes the demargination of classical monocytes from the lungs of humanised mice and non-human primates

To extend these findings in the context of humans, we found that CXCR4-inhibition through AMD3100 also markedly increased circulating CD14⁺CD16⁻ classical monocytes in humanised mice [Figure 3.27a]. In

addition, this increase in circulating monocytes was associated with an increase net release of these cells from the pulmonary circulation under resting conditions, suggesting that CXCR4 inhibition promotes the demargination of human monocytes from the lungs [Figure 3.27b]. This phenomenon was similarly observed in an experimental model of non-human primates [Figure 3.27c]. Collectively, these results hence suggest that CXCR4 regulation of monocyte margination is likely to be physiologically relevant in humans.

Figure 3.27 CXCR4 mediates monocyte margination in humanized mice and non-human primates.

(a-b) Frequency of blood **(a)** and pulmonary release **(b)** of CD14⁺CD16⁻ classical monocytes in humanized mice at baseline and 2 hours after AMD3100 treatment ($n=8-11$). Results are representative of one out of two independent experiments. ****** $P<0.01$ (Student's t-test). **(c)** Net

monocyte release from the lungs of two individual non-human primates (*Macaca fascicularis*) over a 4h observation period.

3.4.5 Functional relevance of CXCR4-mediated monocyte margination in the pulmonary microcirculation

The accumulation of myeloid cells in the lung is a hallmark of injury in pulmonary diseases. It is thus likely that an increase in margination and adherence to the lung endothelium may predispose towards tissue injury (MacNee and Selby, 1993). To test this hypothesis, we utilised a model of acute lung injury (ALI) (O'Dea et al., 2009), and found that *Lyz2^{cre}Cxcr4^{fl}* mice display a significant decrease in vascular leakage compared to *Cxcr4* controls [Figure 3.28a]. Furthermore, in a high-grade model of sepsis (caecum ligation and puncture; CLP), CXCR4-deficient Ly6C^{hi} monocytes accumulated at a significantly lower extent in the lungs [Figure 3.28b]. This decreased accumulation was accompanied by an improved survival outcome as compared to *Cxcr4^{fl}* controls [Figure 3.28c]. Together, these data suggest that CXCR4 continues to play important roles in regulating monocyte margination in the pulmonary microcirculation, while disruption of its signalling ameliorates lung injury and sepsis mortality.

Figure 3.28 Inhibition of CXCR4 reduces lung vascular leakage and prevents sepsis mortality.

Lyz2^{cre}Cxcr4^{fl} mice were compared to *Cxcr4^{fl}* control mice for lung vascular leakage during acute lung injury (**a**) ($n=5$) $**P<0.01$ (Student's t-test) or fold-increase accumulation of lung $Ly6C^{hi}$ (*left*) and $Ly6C^{lo}$ (*right*) monocytes (**b**) ($n=3-5$) $***P<0.001$ (Student's t-test) and their mortality using the Kaplan-Meier survival curve (**c**) ($n=10$) $****P<0.0001$ (Mantel-Cox) in the CLP sepsis model. Results are representative of one out of three independent experiments.

3.4.6 Discussion

Leukocytes rely heavily on the circulatory system to transport themselves to various tissues/organs. During this trafficking process, leukocytes may interact with endothelial cells for prolonged periods of time, which determines their transit time in organs. However, the functional outcomes of these leukocytes in vascular activities have been underappreciated. It has been proposed that the margined pool is caused by the need for leukocytes to alter their morphology as they transit and crawl through small-caliber microvessels (Kuebler and Goetz, 2002). In particular, the lung represents a major site of leukocyte margination as it has exclusive vascular arrangements that consist of 40-100 interconnected capillary segments among several alveolar units that can be as small as $2\mu m$ (Hogg, 1987; Staub and Schultz, 1968). In addition, due to anatomical constraints, the lungs are the only organ to

receive the full cardiac output via the pulmonary artery. More importantly, monocytes and neutrophils constitute the largest proportion of the margined pool of leukocytes due to their larger diameter (Downey et al., 1990). Furthermore, monocytes are retained in the capillaries to a much larger extent than neutrophils due to their increased stiffness and less ability to deform. Coupled by their increased adhesive properties, it is plausible that a change in monocyte transit time in the lung may have a significant impact on total circulating monocyte numbers. In agreement with this hypothesis, we observed that low levels of endotoxin were sufficient to cause a distinct phase of circulating monocytopenia that is associated with increased margination in the lung.

Previous studies have shown that leukocyte margination depends on a balance between the propelling force executed by the shear stress of the blood flow, and a retaining force that delays monocyte transit (Martin et al., 1982). Data from our intravital imaging experiments suggest that CXCR4 interactions partly constitute the retention force in the lung vasculature, which could be mediated through adhesion with CXCL12-expressing endothelial cells. Based on these results, it is likely that CXCR4 regulates the margination of monocytes in more ways than one. CXCR4 may regulate the propelling force of the margination process as CXCL12 has been previously shown to act as a vasoconstrictor that may alter the cardiac output during CXCR4 inhibition with AMD3100 (Mieno et al., 2006). Alternatively, CXCR4 may also affect the retaining force directly through adhesion with CXCL12-expressing endothelial cells or indirectly through integrins. We found that CXCL12 is expressed by the lung endothelium (Devi et al., 2013) while changes in the actin cytoskeleton for trafficking and motility can be modulated by G-protein

coupled receptors (Ganguly et al., 2011). Additionally, *in vitro* studies with human monocytes revealed that LPS causes cytoskeletal changes in monocytes that results in stiffness as well as upregulation of CD18 (Doherty et al., 1994). We found that monocytes margined and accumulated similarly in *Itgam*^{-/-} (CD11b-deficient) and WT mice (data not shown) in response to LPS, suggesting that CD18 is not the only factor in the increased margination process after endotoxemia exposure *in vivo*. Therefore, it is plausible that both CXCR4-CXCL12 signalling and β 2-integrins play distinct but co-operative roles in the margination of monocytes, such that CXCR4 precludes the margination course by forcing cells to alter their morphology and decrease their speed before the integrins take control over the crawling and adhesion process. In support of this hypothesis, we found that CXCR4 inhibition before, but not after, exposure to endotoxemia prevents the accumulation of cells (data not shown), thereby strongly suggesting that CXCR4 influences the upstream pathway of the margination process.

We have shown that CXCR4 expression on monocytes oscillates throughout the day and peaks at night. While we did not find an increase in lung monocytes numbers at night (data not shown), it remains unclear whether monocytes may display increased margination at this timing. Further studies using lung intravital imaging will be needed to address whether monocyte margination occurs in a circadian manner.

While leukocyte margination is a fundamental physiological process, their dysregulated intravascular migration and uncontrolled aggregation are associated with several human inflammatory diseases. Here, we show that LPS triggers increased accumulation of Ly6C^{hi} monocytes in the intravascular space of the lung that may lead to injury. However, it is unclear if monocyte margination occurs only as a result of pathogenic stimuli and if this process is organ-specific. In particular, studies by

Takata's group have also reported the presence of lung margination in sterile conditions, such as upon ventilator (Wilson et al., 2009) and acid aspiration induced lung injury (Patel et al., 2012). Notably, prior LPS exposure worsens the outcome of ventilation-induced lung injury (Wilson et al., 2009), suggesting that monocyte margination may occur through sensing of both pathogen associated molecule patterns (PAMPs) and damage associated molecular pattern molecules (DAMPs). Although the phenomenon of monocyte margination in these studies were reported in lung-specific injuries, studies have also documented the association between liver damage and subsequent lung injury (Blackwell et al., 1999; Colletti and Green, 2001), suggesting a high possibility of monocyte margination to occur in the pulmonary vasculature as a secondary effect upon a distal organ injury. It is conceived that this may occur through the sensing of DAMPs by circulating monocytes in the intravascular compartment of the liver, which may reduce their ability to alter their morphology upon trafficking through the very narrow capillaries of the lung (Doherty et al., 1994). Consequently, this increases their transit time in the pulmonary vasculature and their likelihood to interact with CXCL12 expressing endothelial cells, resulting in their margination and accumulation in the lung. Since CXCR4 inhibition was found to prevent their margination and reduce the incidence of vascular leakage in acute lung injury, we believe that our study also provides a mechanistic understanding of how CXCR4 antagonism may attenuate lung inflammatory diseases as described by previous studies (Drummond et al., 2015; Lukacs et al., 2010). While it appears that CXCR4 doesn't play a significant role in Ly6C^{lo} monocyte margination during endotoxemia, it is likely that these cells may utilise a different array of molecules to interact with the endothelium, such as the LFA-1 integrin, as supported by a previous study (Carlin et al., 2013).

So far, we have shown that CXCR4 inhibition can prevent the margination of monocytes to the lung microcirculation, under inflammatory and non-inflammatory condition. However, it is still unclear whether CXCR4-inhibition through AMD3100 can reverse monocyte accumulation in lungs once they are already margined. While CXCL12-CXCR4 interaction can enhance the chemotaxis of various cell types, it can also promote conformational changes of integrins from low to high affinity (Alon and Ley, 2008). Preliminary experiments suggest the involvement of the LFA-1 integrin downstream of CXCR4 in lung monocyte margination after LPS. While such data could imply that monocyte margination may be irreversible, additional work using lung intravital imaging will provide a definitive answer.

Notably, while monocytes are essential for bacteria clearance, they are also major contributors of the cytokine storm that leads to organ damage and mortality (Weber et al., 2015). In agreement with this statement, monocyte depletion during acute lung injury has been shown to improve the outcome of vascular injury (Dhaliwal et al., 2012; O'Dea et al., 2009). Thus, our study also shows that CXCR4-mediated monocyte compartmentalisation may serve as a major biological regulatory mechanism that underlies immune homeostasis to prevent excessive damage to the host **[Figure 3.29]**.

Figure 3.29 CXCR4 mediates monocyte margination and pulmonary injury.

Upon entering the circulation, CXCR4 plays a significant role in monocyte margination in the pulmonary vasculature. Under resting conditions, inhibition of CXCR4 alone using AMD3100 leads to monocyte demargination. However, exposure to subclinical LPS doses triggers increased pulmonary monocyte margination, leading to a transient state of monocytopenia and lung damage. AMD3100 treatment before inflammatory stimuli prevented the accumulation of monocytes in the lung microcirculation. Disruption in CXCR4 signalling also attenuated lung injury and prevented sepsis mortality.

4 Conclusion

In summary, our study provides new insight into the current framework of monocyte development and homeostasis by identifying a previously unknown population of transitional pre-monocytes among BM Ly6C^{hi} monocytes. Furthermore, our results extend the role of CXCR4 beyond its function as a mere BM retention factor, to an important regulator in peripheral tissue trafficking with implications in pulmonary inflammation. Hence, we envision that our results will provide a new framework for understanding monocyte biology and may lead to the engineering of improved monocyte targeting therapeutic strategies.

5 References

- Abe, P., Mueller, W., Schutz, D., MacKay, F., Thelen, M., Zhang, P., and Stumm, R. (2014). CXCR7 prevents excessive CXCL12-mediated downregulation of CXCR4 in migrating cortical interneurons. *Development* *141*, 1857–1863.
- Abram, C.L., Roberge, G.L., Hu, Y., and Lowell, C.A. (2014). Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. *Journal of Immunological Methods* *408*, 89–100.
- Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* *404*, 193–197.
- Alder, J.K., Georgantas, R.W., Hildreth, R.L., Kaplan, I.M., Morisot, S., Yu, X., McDevitt, M., and Civin, C.I. (2008). Kruppel-like factor 4 is essential for inflammatory monocyte differentiation in vivo. *The Journal of Immunology* *180*, 5645–5652.
- Aldridge, J.R., Moseley, C.E., Boltz, D.A., Negovetich, N.J., Reynolds, C., Franks, J., Brown, S.A., Doherty, P.C., Webster, R.G., and Thomas, P.G. (2009). TNF/*i*NOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proceedings of the National Academy of Sciences* *106*, 5306–5311.
- Alon, R., and Ley, K. (2008). Cells on the run: shear-regulated integrin activation in leukocyte rolling and arrest on endothelial cells. *Current Opinion in Cell Biology* *20*, 525–532.
- Amir, E.-A.D., Davis, K.L., Tadmor, M.D., Simonds, E.F., Levine, J.H., Bendall, S.C., Shenfeld, D.K., Krishnaswamy, S., Nolan, G.P., and Pe'er, D. (2013). viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nature Biotechnology* *31*, 545–552.
- Anderson, K.G., Mayer-Barber, K., Sung, H., Beura, L., James, B.R., Taylor, J.J., Qunaj, L., Griffith, T.S., Vezys, V., Barber, D.L., et al. (2014). Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat Protoc* *9*, 209–222.
- Askenase, M.H., Han, S.-J., Byrd, A.L., da Fonseca, D.M., Bouladoux, N., Wilhelm, C., Konkelt, J.E., Hand, T.W., Lacerda-Queiroz, N., Su, X.-

- Z., et al. (2015). Bone-Marrow-Resident NK Cells Prime Monocytes for Regulatory Function during Infection. *Immunity* 1–36.
- Athens, J.W., Haab, O.P., Raab, S.O., Mauer, A.M., Ashenbrucker, H., Cartwright, G.E., and Wintrobe, M.M. (1961). LEUKOKINETIC STUDIES. IV. THE TOTAL BLOOD, CIRCULATING AND MARGINAL GRANULOCYTE POOLS AND THE GRANULOCYTE TURNOVER RATE IN NORMAL SUBJECTS*. *J. Clin. Invest.* 40, 989–995.
- Auffray, C., Fogg, D.K., Narni-Mancinelli, E., Senechal, B., Trouillet, C., Saederup, N., Leemput, J., Bigot, K., Campisi, L., Abitbol, M., et al. (2009). CX3CR1⁺ CD115⁺ CD135⁺ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. *Journal of Experimental Medicine* 206, 595–606.
- Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007). Monitoring of Blood Vessels and Tissues by a Population of Monocytes with Patrolling Behavior. *Science* 317, 666–670.
- Auvynet, C., Baudesson de Chanville, C., Hermand, P., Dorgham, K., Piesse, C., Pouchy, C., Carlier, L., Poupel, L., Barthelemy, S., Felouzis, V., et al. (2016). ECL1i, d(LGTFLKC), a novel, small peptide that specifically inhibits CCL2-dependent migration. *The FASEB Journal* 30, 2370–2381.
- Bachelier, F., Ben-Baruch, A., Burkhardt, A.M., Combadiere, C., Farber, J.M., Graham, G.J., Horuk, R., Sparre-Ulrich, A.H., Locati, M., Luster, A.D., et al. (2014). International Union of Pharmacology. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacological Reviews* 66, 1–79.
- Bain, C.C., Scott, C.L., Uronen-Hansson, H., Gudjonsson, S., Jansson, O., Grip, O., Williams, M., Malissen, B., Agace, W.W., and Mowat, A.M. (2012). Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunology* 6, 498–510.
- Bain, C.C., Hawley, C.A., Garner, H., Scott, C.L., Schridde, A., Steers, N.J., Mack, M., Joshi, A., Williams, M., Mowat, A.M.I., et al. (2016). Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. *Nat Comms* 7, 1–14.
- Balabanian, K., Brotin, E., Biajoux, V., Bouchet-Delbos, L., Lainey, E.,

- Fenneteau, O., Bonnet, D., Fiette, L., Emilie, D., and Bachelier, F. (2012). Proper desensitization of CXCR4 is required for lymphocyte development and peripheral compartmentalization in mice. *Blood* 119, 5722–5730.
- Barletta, K.E., Cagnina, R.E., Wallace, K.L., Ramos, S.I., Mehrad, B., and Linden, J. (2012). Leukocyte compartments in the mouse lung: Distinguishing between marginated, interstitial, and alveolar cells in response to injury. *Journal of Immunological Methods* 375, 100–110.
- Becher, B., Schlitzer, A., Chen, J., Mair, F., Sumatoh, H.R., Teng, K.W.W., Low, D., Ruedl, C., Riccardi-Castagnoli, P., Poidinger, M., et al. (2014). High-dimensional analysis of the murine myeloid cell system. *Nat. Immunol.*
- Bendall, S.C., Davis, K.L., Amir, E.-A.D., Tadmor, M.D., Simonds, E.F., Chen, T.J., Shenfeld, D.K., Nolan, G.P., and Pe'er, D. (2014). Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development. *Cell* 157, 714–725.
- Bennett, L.D., Fox, J.M., and Signorel, N. (2011). Mechanisms regulating chemokine receptor activity. *Immunology* 134, 246–256.
- Beura, L.K., Hamilton, S.E., Bi, K., Schenkel, J.M., Odumade, O.A., Casey, K.A., Thompson, E.A., Fraser, K.A., Rosato, P.C., Filali-Mouhim, A., et al. (2016). Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* 532, 512–516.
- BIERMAN, H.R., KELLY, K.H., CORDES, F.L., BYRON, R.L., POLHEMUS, J.A., and RAPPOPORT, S. (1952). The release of leukocytes and platelets from the pulmonary circulation by epinephrine. *Blood* 7, 683–692.
- Blackwell, T.S., Debelak, J.P., Venkatakrishnan, A., Schot, D.J., Harley, D.H., Pinson, C.W., Williams, P., Washington, K., Christman, J.W., and Chapman, W.C. (1999). Acute lung injury after hepatic cryoablation: Correlation with NF- κ B activation and cytokine production. *Surgery* 126, 518–526.
- Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T.A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 382, 829–833.
- Bonapace, L., Coissieux, M.-M., Wyckoff, J., Mertz, K.D., Varga, Z., Junt, T., and Bentires-Alj, M. (2014). Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis.

Nature *515*, 130–133.

Bousso, P. (2008). T-cell activation by dendritic cells in the lymph node: lessons from the movies. *Nature Reviews Immunology* *8*, 675–684.

Calderon, B., Carrero, J.A., Ferris, S.T., Sojka, D.K., Moore, L., Epelman, S., Murphy, K.M., Yokoyama, W.M., Randolph, G.J., and Unanue, E.R. (2015). The pancreas anatomy conditions the origin and properties of resident macrophages. *Journal of Experimental Medicine* *212*, 1497–1512.

Campbell, I.K., Bendele, A., Smith, D.A., and Hamilton, J.A. (1997). Granulocyte-macrophage colony stimulating factor exacerbates collagen induced arthritis in mice. *Ann. Rheum. Dis.* *56*, 364–368.

Carlin, L.M., Stamatiades, E.G., Auffray, C., Hanna, R.N., Glover, L., Vizcay-Barrena, G., Hedrick, C.C., Cook, H.T., Diebold, S., and Geissmann, F. (2013). Nr4a1-Dependent Ly6C(low) Monocytes Monitor Endothelial Cells and Orchestrate Their Disposal. *Cell* *153*, 362–375.

Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J., and Coughlin, S.R. (1994). Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc. Natl. Acad. Sci. U.S.A.* *91*, 2752–2756.

Chen, Q., Khoury, M., Limmon, G., Choolani, M., Chan, J.K.Y., and Chen, J. (2013). Human Fetal Hepatic Progenitor Cells Are Distinct from, but Closely Related to, Hematopoietic Stem/Progenitor Cells. *Stem Cells* *31*, 1160–1169.

Christopher, M.J., Rao, M., Liu, F., Woloszynek, J.R., and Link, D.C. (2011). Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. *Journal of Experimental Medicine* *208*, 251–260.

Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Förster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* *8*, 265–277.

Colletti, L.M., and Green, M. (2001). Lung and liver injury following hepatic ischemia/reperfusion in the rat is increased by exogenous lipopolysaccharide which also increases hepatic TNF production in vivo and in vitro. *Shock* *16*, 312–319.

Combadière, C., Feumi, C., Raoul, W., Keller, N., Rodero, M., Pezard,

- A., Lavalette, S., Houssier, M., Jonet, L., Picard, E., et al. (2007). CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration. *J. Clin. Invest.* *117*, 2920–2928.
- Combadière, C., Potteaux, S., Gao, J.-L., Esposito, B., Casanova, S., Lee, E.J., Debré, P., Tedgui, A., Murphy, P.M., and Mallat, Z. (2003). Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. *Circulation* *107*, 1009–1016.
- Combadière, C., Potteaux, S., Rodero, M., Simon, T., Pezard, A., Esposito, B., Merval, R., Proudfoot, A., Tedgui, A., and Mallat, Z. (2008). Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation* *117*, 1649–1657.
- Croxford, A.L., Lanzinger, M., Hartmann, F.J., Schreiner, B., Mair, F., Pelczar, P., Clausen, B.E., Jung, S., Greter, M., and Becher, B. (2015). The Cytokine GM-CSF Drives the Inflammatory Signature of CCR2(+) Monocytes and Licenses Autoimmunity. *Immunity*.
- Dai, X.-M., Ryan, G.R., Hapel, A.J., Dominguez, M.G., Russell, R.G., Kapp, S., Sylvestre, V., and Stanley, E.R. (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* *99*, 111–120.
- Debien, E., Mayol, K., Biajoux, V., Daussy, C., De Agüero, M.G., Taillardet, M., Dagany, N., Brinza, L., Henry, T., Dubois, B., et al. (2013). S1PR5 is pivotal for the homeostasis of patrolling monocytes. *Eur. J. Immunol.* *43*, 1667–1675.
- Devi, S., Wang, Y., Chew, W.K., Lima, R., A-González, N., Mattar, C.N.Z., Chong, S.Z., Schlitzer, A., Bakocevic, N., Chew, S., et al. (2013). Neutrophil mobilization via plerixafor-mediated CXCR4 inhibition arises from lung demargination and blockade of neutrophil homing to the bone marrow. *Journal of Experimental Medicine* *210*, 2321–2336.
- Dhaliwal, K., Scholefield, E., Ferenbach, D., Gibbons, M., Duffin, R., Dorward, D.A., Morris, A.C., Humphries, D., MacKinnon, A., Wilkinson, T.S., et al. (2012). Monocytes Control Second-Phase Neutrophil Emigration in Established Lipopolysaccharide-induced Murine Lung Injury. *Am J Respir Crit Care Med* *186*, 514–524.
- Ding, L., and Morrison, S.J. (2013). Haematopoietic stem cells and early

lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**, 231–235.

Doherty, D.E., Downey, G.P., Schwab, B., Elson, E., and Worthen, G.S. (1994). Lipopolysaccharide-induced monocyte retention in the lung. Role of monocyte stiffness, actin assembly, and CD18-dependent adherence. *The Journal of Immunology* **153**, 241–255.

Dorgham, K., Ghadiri, A., Hermand, P., Rodero, M., Poupel, L., Iga, M., Hartley, O., Gorochoy, G., Combadiere, C., and Deterre, P. (2009). An engineered CX3CR1 antagonist endowed with anti-inflammatory activity. *Journal of Leukocyte Biology* **86**, 903–911.

Downey, G.P., Doherty, D.E., Schwab, B., Elson, E.L., Henson, P.M., and Worthen, G.S. (1990). Retention of leukocytes in capillaries: role of cell size and deformability. *J. Appl. Physiol.* **69**, 1767–1778.

Drummond, S., Ramachandran, S., Torres, E., Huang, J., Hehre, D., Suguihara, C., and Young, K.C. (2015). CXCR4 Blockade Attenuates Hyperoxia-Induced Lung Injury in Neonatal Rats. *Neonatology* **107**, 304–311.

Duffy, D., Perrin, H., Abadie, V., Benhabiles, N., Boissonnas, A., Liard, C., Descours, B., Reboulleau, D., Bonduelle, O., Verrier, B., et al. (2012). Neutrophils Transport Antigen from the Dermis to the Bone Marrow, Initiating a Source of Memory CD8⁺ T Cells. *Immunity* **37**, 917–929.

Eash, K.J., Means, J.M., White, D.W., and Link, D.C. (2009). CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood* **113**, 4711–4719.

Espinosa, V., Jhingran, A., Dutta, O., Kasahara, S., Donnelly, R., Du, P., Rosenfeld, J., Leiner, I., Chen, C.-C., Ron, Y., et al. (2014). Inflammatory Monocytes Orchestrate Innate Antifungal Immunity in the Lung. *PLoS Pathog* **10**, e1003940.

Evrard, M., Evrard, M., Chong, S.Z., Chong, S.Z., Devi, S., Devi, S., Chew, W.K., Chew, W.K., Lee, B., Lee, B., et al. (2015). Visualization of bone marrow monocyte mobilization using Cx3cr1gfp/+Flt3L^{-/-} reporter mouse by multiphoton intravital microscopy. *J. Leukoc. Biol.* **97**, 611–619.

Fay, M.E., Myers, D.R., Kumar, A., Turbyfield, C.T., Byler, R., Crawford, K., Mannino, R.G., Laohapant, A., Tyburski, E.A., Sakurai, Y., et al. (2016). Cellular softening mediates leukocyte demargination and

trafficking, thereby increasing clinical blood counts. *Proc. Natl. Acad. Sci. U.S.A.* 201508920–13.

Feinberg, M.W., Wara, A.K., Cao, Z., Lebedeva, M.A., Rosenbauer, F., Iwasaki, H., Hirai, H., Katz, J.P., Haspel, R.L., Gray, S., et al. (2007). The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation. *Embo J* 26, 4138–4148.

Fife, B.T., Huffnagle, G.B., Kuziel, W.A., and Karpus, W.J. (2000). CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *Journal of Experimental Medicine* 192, 899–905.

Fogg, D.K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D.R., Cumano, A., and Geissmann, F. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311, 83–87.

Fong, A.M., Robinson, L.A., Steeber, D.A., Tedder, T.F., Yoshie, O., Imai, T., and Patel, D.D. (1998). Fractalkine and CX 3CR1 Mediate a Novel Mechanism of Leukocyte Capture, Firm Adhesion, and Activation under Physiologic Flow. *Journal of Experimental Medicine* 188, 1413–1419.

Ford, L.B., Cerovic, V., Milling, S.W.F., Graham, G.J., Hansell, C.A.H., and Nibbs, R.J.B. (2014). Characterization of Conventional and Atypical Receptors for the Chemokine CCL2 on Mouse Leukocytes. *The Journal of Immunology* 193, 400–411.

Franklin, R.A., Liao, W., Sarkar, A., Kim, M.V., Bivona, M.R., Liu, K., Pamer, E.G., and Li, M.O. (2014). The Cellular and Molecular Origin of Tumor-Associated Macrophages. *Science*.

Ganguly, S., Saxena, R., and Chattopadhyay, A. (2011). Reorganization of the actin cytoskeleton upon G-protein coupled receptor signaling. *BBA - Biomembranes* 1808, 1921–1929.

Gautier, E.L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., Helft, J., Chow, A., Elpek, K.G., Gordonov, S., et al. (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat. Immunol.* 13, 1118–1128.

Gebhardt, T., Whitney, P.G., Zaid, A., Mackay, L.K., Brooks, A.G., Heath, W.R., Carbone, F.R., and Mueller, S.N. (2011). Different patterns of peripheral migration by memory CD4⁺ and CD8⁺ T cells. *Nature* 477,

216–219.

Geissmann, F., Jung, S., and Littman, D.R. (2003). Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties. *Immunity* 19, 71–82.

Geissmann, F., Gordon, S., Hume, D.A., Mowat, A.M., and Randolph, G.J. (2010). Unravelling mononuclear phagocyte heterogeneity. *Nature Reviews. Immunology* 10, 453–460.

Germain, R.N., Miller, M.J., Dustin, M.L., and Nussenzweig, M.C. (2006). Dynamic imaging of the immune system: progress, pitfalls and promise. *Nature Reviews. Immunology* 6, 497–507.

Germain, R.N., Robey, E.A., and Cahalan, M.D. (2012). A decade of imaging cellular motility and interaction dynamics in the immune system. *Science* 336, 1676–1681.

Ginhoux, F., and Guilliams, M. (2016). Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity* 44, 439–449.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R., et al. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330, 841–845.

Gordon, S. (2008). Elie Metchnikoff: Father of natural immunity. *Eur. J. Immunol.* 38, 3257–3264.

Goto, Y., Hogg, J.C., Suwa, T., Quinlan, K.B., and van Eeden, S.F. (2003). A novel method to quantify the turnover and release of monocytes from the bone marrow using the thymidine analog 5³-bromo-2³-deoxyuridine. *AJP: Cell Physiology* 285, C253–C259.

Grainger, J.R., Wohlfert, E.A., Fuss, I.J., Bouladoux, N., Askenase, M.H., Legrand, F., Koo, L.Y., Brenchley, J.M., Fraser, I.D.C., and Belkaid, Y. (2013). Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat Med* 19, 713–721.

Greter, M., Helft, J., Chow, A., Hashimoto, D., Mortha, A., Agudo-Cantero, J., Bogunovic, M., Gautier, E.L., Miller, J., Leboeuf, M., et al. (2012). GM-CSF Controls Nonlymphoid Tissue Dendritic Cell Homeostasis but Is Dispensable for the Differentiation of Inflammatory Dendritic Cells. *Immunity* 36, 1031–1046.

- Griffith, J.W., Sokol, C.L., and Luster, A.D. (2014). Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu. Rev. Immunol.* 32, 659–702.
- Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B.U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat. Rev. Immunol.* 14, 571–578.
- Hanna, R.N., Shaked, I., Hubbeling, H.G., Punt, J.A., Wu, R., Herrley, E., Zaugg, C., Pei, H., Geissmann, F., Ley, K., et al. (2012). NR4A1 (Nur77) Deletion Polarizes Macrophages Toward an Inflammatory Phenotype and Increases Atherosclerosis. *Circulation Research* 110, 416–427.
- Hanna, R.N., Carlin, L.M., Hubbeling, H.G., Nackiewicz, D., Green, A.M., Punt, J.A., Geissmann, F., and Hedrick, C.C. (2011). The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat. Immunol.* 12, 778–785.
- Hanna, R.N., Cekic, C., Sag, D., Tacke, R., Thomas, G.D., Nowyhed, H., Herrley, E., Rasquinha, N., McArdle, S., Wu, R., et al. (2015). Patrolling monocytes control tumor metastasis to the lung. *Science* 350, 985–990.
- Harrison, J.K., Barber, C.M., and Lynch, K.R. (1994). cDNA cloning of a G-protein-coupled receptor expressed in rat spinal cord and brain related to chemokine receptors. *Neuroscience Letters* 169, 85–89.
- Hashimoto, D., Chow, A., Greter, M., Saenger, Y., Kwan, W.-H., Leboeuf, M., Ginhoux, F., Ochando, J.C., Kunisaki, Y., van Rooijen, N., et al. (2011). Pretransplant CSF-1 therapy expands recipient macrophages and ameliorates GVHD after allogeneic hematopoietic cell transplantation. *Journal of Experimental Medicine* 208, 1069–1082.
- Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M.B., Leboeuf, M., Becker, C.D., See, P., Price, J., Lucas, D., et al. (2013). Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes. *Immunity* 38, 792–804.
- Helft, J., Böttcher, J., Chakravarty, P., Zelenay, S., Huotari, J., Schraml, B.U., Goubau, D., and Sousa, C.R.E. (2015). GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c+MHCII+ Macrophages and Dendritic Cells. *Immunity* 42, 1197–1211.

- Heng, T.S.P., Painter, M.W., Elpek, K., Lukacs-Kornek, V., Mauermann, N., Turley, S.J., Koller, D., Kim, F.S., Wagers, A.J., Asinowski, N., et al. (2008). The Immunological Genome Project: networks of gene expression in immune cells. *Nat. Immunol.* 9, 1091–1094.
- Hettinger, J., Richards, D.M., Hansson, J., Barra, M.M., Joschko, A.-C., Krijgsveld, J., and Feuerer, M. (2013). Origin of monocytes and macrophages in a committed progenitor. *Nat. Immunol.* 14, 821–830.
- Hogg, J.C. (1987). Neutrophil kinetics and lung injury. *Physiological Reviews* 67, 1249–1295.
- Hohl, T.M., Rivera, A., Lipuma, L., Gallegos, A., Shi, C., Mack, M., and Pamer, E.G. (2009). Inflammatory Monocytes Facilitate Adaptive CD4 T Cell Responses during Respiratory Fungal Infection. *Cell Host and Microbe* 6, 470–481.
- Hume, D.A. (2011). Applications of myeloid-specific promoters in transgenic mice support in vivo imaging and functional genomics but do not support the concept of distinct macrophage and dendritic cell lineages or roles in immunity. *Journal of Leukocyte Biology* 89, 525–538.
- Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *Journal of Experimental Medicine* 176, 1693–1702.
- Ingersoll, M.A., Spanbroek, R., Lottaz, C., Gautier, E.L., Frankenberger, M., Hoffmann, R., Lang, R., Haniffa, M., Collin, M., Tacke, F., et al. (2010). Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* 115, e10–e19.
- Inra, C.N., Zhou, B.O., Acar, M., Murphy, M.M., Richardson, J., Zhao, Z., and Morrison, S.J. (2015). A perisinusoidal niche for extramedullary haematopoiesis in the spleen. *Nature* 1–19.
- Ishikawa-Ankerhold, H.C., Ankerhold, R., and Drummen, G.P.C. (2012). Advanced Fluorescence Microscopy Techniques—FRAP, FLIP, FLAP, FRET and FLIM. *Molecules* 17, 4047–4132.
- Italiani, P., and Boraschi, D. (2014). From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Front Immunol* 5, 514.

- Jackson, W.D., and Woollard, K.J. (2014). Targeting monocyte and macrophage subpopulations for immunotherapy: a patent review (2009 – 2013). *Expert Opinion on Therapeutic Patents* 24, 779–790.
- Jacquelin, S., Jacquelin, S., Licata, F., Licata, F., Dorgham, K., Dorgham, K., Hermand, P., Hermand, P., Poupel, L., Poupel, L., et al. (2013). CX3CR1 reduces Ly6Chigh-monocyte motility within and release from the bone marrow after chemotherapy in mice. *Blood* 122, 674–683.
- Jain, R., Tikoo, S., and Weninger, W. (2016). Recent advances in microscopic techniques for visualizing leukocytes in vivo. *F1000Res* 5, 915–10.
- Jakubzick, C., Gautier, E.L., Gibbings, S.L., Sojka, D.K., Schlitzer, A., Johnson, T.E., Ivanov, S., Duan, Q., Bala, S., Condon, T., et al. (2013). Minimal Differentiation of Classical Monocytes as They Survey Steady-State Tissues and Transport Antigen to Lymph Nodes. *Immunity*.
- Jia, T., Jia, T., Serbina, N., Serbina, N.V., Brandl, K., Brandl, K., Zhong, M., Zhong, M.X., Leiner, I.M., Charo, I.F., et al. (2008). Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during *Listeria monocytogenes* infection. *The Journal of Immunology* 180, 6846–6853.
- Jia, T., Leiner, I., Dorothee, G., Brandl, K., and Pamer, E.G. (2009). MyD88 and Type I Interferon Receptor-Mediated Chemokine Induction and Monocyte Recruitment during *Listeria monocytogenes* Infection. *The Journal of Immunology* 183, 1271–1278.
- Jung, H., Mithal, D.S., Park, J.E., and Miller, R.J. (2015). Localized CCR2 Activation in the Bone Marrow Niche Mobilizes Monocytes by Desensitizing CXCR4. *PLoS ONE* 10, e0128387.
- Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., and Littman, D.R. (2000). Analysis of Fractalkine Receptor CX3CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion. ... *And Cellular Biology*.
- Jung, S., Unutmaz, D., Wong, P., Sano, G.-I., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., et al. (2002). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* 17, 211–220.
- Kim, K.-W., Vallon-Eberhard, A., Zigmond, E., Farache, J., Shezen, E., Shakhar, G., Ludwig, A., Lira, S.A., and Jung, S. (2011). In vivo

structure/function and expression analysis of the CX3C chemokine fractalkine.

Kohara, H., Omatsu, Y., Sugiyama, T., Noda, M., Fujii, N., and Nagasawa, T. (2007). Development of plasmacytoid dendritic cells in bone marrow stromal cell niches requires CXCL12-CXCR4 chemokine signaling. *Blood* 110, 4153–4160.

Kohler, A., De Filippo, K., Hasenberg, M., van den Brandt, C., Nye, E., Hosking, M.P., Lane, T.E., Mann, L., Ransohoff, R.M., Hauser, A.E., et al. (2011). G-CSF-mediated thrombopoietin release triggers neutrophil motility and mobilization from bone marrow via induction of Cxcr2 ligands. *Blood* 117, 4349–4357.

Kuebler, W.M., and Goetz, A.E. (2002). The marginated pool. *Eur Surg Res* 34, 92–100.

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., et al. (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* 502, 637–643.

Kurihara, T., and Bravo, R. (1996). Cloning and Functional Expression of mCCR2, a Murine Receptor for the C-C Chemokines JE and FIC. *J. Biol. Chem.* 271, 11603–11606.

Kurihara, T., Warr, G., Loy, J., and Bravo, R. (1997). Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *Journal of Experimental Medicine* 186, 1757–1762.

Kurotaki, D., Osato, N., Nishiyama, A., Yamamoto, M., Ban, T., Sato, H., Nakabayashi, J., Umehara, M., Miyake, N., Matsumoto, N., et al. (2013). Essential role of the IRF8-KLF4 transcription factor cascade in murine monocyte differentiation. *Blood* 121, 1839–1849.

Kurotaki, D., Yamamoto, M., Nishiyama, A., Uno, K., Ban, T., Ichino, M., Sasaki, H., Matsunaga, S., Yoshinari, M., Ryo, A., et al. (2014). IRF8 inhibits C/EBPα activity to restrain mononuclear phagocyte progenitors from differentiating into neutrophils. *Nat Comms* 5, 4978.

Kuziel, W.A., Morgan, S.J., Dawson, T.C., Griffin, S., Smithies, O., Ley, K., and Maeda, N. (1997). Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc. Natl. Acad. Sci. U.S.A.* 94, 12053–12058.

Landsman, L., Bar-On, L., Zerneck, A., Kim, K.-W., Krauthgamer, R.,

- Shagdarsuren, E., Lira, S., Weissman, I., Weber, C., and Jung, S. (2009). CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood* 113, 963–972.
- Lavin, Y., Winter, D., Blecher-Gonen, R., David, E., Keren-Shaul, H., Merad, M., Jung, S., and Amit, I. (2014). Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment. *Cell* 159, 1312–1326.
- León, B., and Ardavín, C. (2008). Monocyte migration to inflamed skin and lymph nodes is differentially controlled by L-selectin and PSGL-1. *Blood* 111, 3126–3130.
- León, B., López-Bravo, M., and Ardavín, C. (2007). Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against *Leishmania*. *Immunity* 26, 519–531.
- Leuschner, F., Rauch, P.J., Ueno, T., Gorbатов, R., Marinelli, B., Lee, W.W., Dutta, P., Wei, Y., Robbins, C., Iwamoto, Y., et al. (2012). Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *Journal of Experimental Medicine* 209, 123–137.
- Leuschner, F., Dutta, P., Gorbатов, R., Novobrantseva, T.I., Donahoe, J.S., Courties, G., Lee, K.M., Kim, J.I., Markmann, J.F., Marinelli, B., et al. (2011). Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nature Biotechnology* 29, 1005–1010.
- Lewis, C.E., Harney, A.S., and Pollard, J.W. (2016). The Multifaceted Role of Perivascular Macrophages in Tumors. *Cancer Cell* 30, 18–25.
- Li, J.L., Goh, C.C., Keeble, J.L., Qin, J.S., Ben Roediger, B., Jain, R., Wang, Y., Chew, W.K., Weninger, W., and Ng, L.G. (2012). Intravital multiphoton imaging of immune responses in the mouse ear skin. *Nat Protoc* 7, 221–234.
- Liehn, E.A., Merx, M.W., Postea, O., Becher, S., Djalali-Talab, Y., Shagdarsuren, E., Kelm, M., Zerneck, A., and Weber, C. (2008). Ccr1 deficiency reduces inflammatory remodelling and preserves left ventricular function after myocardial infarction. *J Cellular Mol Med* 12, 496–506.
- Lionakis, M.S., Swamydas, M., Fischer, B.G., Plantinga, T.S., Johnson, M.D., Jaeger, M., Green, N.M., Masedunskas, A., Weigert, R., Mikelis, C., et al. (2013). CX3CR1-dependent renal macrophage survival

promotes *Candida* control and host survival. *J. Clin. Invest.* **123**, 5035–5051.

Liu, K., and Nussenzweig, M.C. (2010). Development and homeostasis of dendritic cells. *Eur. J. Immunol.* **40**, 2099–2102.

Liu, K., Vitoria, G.D., Schwickert, T.A., Guernonprez, P., Meredith, M.M., Yao, K., Chu, F.-F., Randolph, G.J., Rudensky, A.Y., and Nussenzweig, M. (2009). In vivo analysis of dendritic cell development and homeostasis. *Science* **324**, 392–397.

Liu, Q., Li, Z., Gao, J.-L., Wan, W., Ganesan, S., McDermott, D.H., and Murphy, P.M. (2015). The CXCR4 antagonist AMD3100 redistributes leukocytes from primary immune organs to secondary immune organs, lung and blood in mice. *Eur. J. Immunol.* **45**, 1855–1867.

Looney, M.R., and Bhattacharya, J. (2014). Live Imaging of the Lung. *Annu. Rev. Physiol.* **76**, 431–445.

Looney, M.R., Thornton, E.E., Sen, D., Lamm, W.J., Glenney, R.W., and Krummel, M.F. (2010). Stabilized imaging of immune surveillance in the mouse lung. *Nature Methods* **8**, 91–96.

Lukacs, N.W., Berlin, A., Schols, D., Skerlj, R.T., and Bridger, G.J. (2010). AMD3100, a CXCR4 Antagonist, Attenuates Allergic Lung Inflammation and Airway Hyperreactivity. *The American Journal of Pathology* **160**, 1353–1360.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rössner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *Journal of Immunological Methods* **223**, 77–92.

Ma, Q., Jones, D., and Springer, T.A. (1999). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* **10**, 463–471.

Maaten, L., and Hinton, G. (2008). Visualizing data using t-SNE. *Journal of Machine Learning Research*.

MacNee, W., and Selby, C. (1993). New perspectives on basic mechanisms in lung disease. 2. Neutrophil traffic in the lungs: role of haemodynamics, cell adhesion, and deformability. *Thorax* **48**, 79–88.

Martin, B.A., Wright, J.L., Thommasen, H., and Hogg, J.C. (1982). Effect

of pulmonary blood flow on the exchange between the circulating and marginating pool of polymorphonuclear leukocytes in dog lungs. *J. Clin. Invest.* **69**, 1277–1285.

Martin, C., Martin, C., Burdon, P.C.E., Burdon, P.C.E., Bridger, G., Bridger, G., Gutierrez-Ramos, J.C., Gutierrez-Ramos, J.C., Williams, T.J., Williams, T.J., et al. (2003). Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity* **19**, 583–593.

Mazo, I.B., Gutierrez-Ramos, J.C., Frenette, P.S., Hynes, R.O., Wagner, D.D., and Andrian, von, U.H. (1998). Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. *Journal of Experimental Medicine* **188**, 465–474.

McArdle, S., Mikulski, Z., and Ley, K. (2016). Live cell imaging to understand monocyte, macrophage, and dendritic cell function in atherosclerosis. *Journal of Experimental Medicine* **213**, 1117–1131.

McDermott, D.H., Liu, Q., Ulrick, J., Kwatema, N., Anaya-O'Brien, S., Penzak, S.R., Filho, J.O., Priel, D.A.L., Kelly, C., Garofalo, M., et al. (2011). The CXCR4 antagonist plerixafor corrects panleukopenia in patients with WHIM syndrome. *Blood* **118**, 4957–4962.

McDermott, D.H., Fong, A.M., Yang, Q., Sechler, J.M., Cupples, L.A., Merrell, M.N., Wilson, P.W.F., D'Agostino, R.B., O'Donnell, C.J., Patel, D.D., et al. (2003). Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. *J. Clin. Invest.* **111**, 1241–1250.

McDermott, D.H., Liu, Q., Velez, D., Lopez, L., Anaya-O'Brien, S., Ulrick, J., Kwatema, N., Starling, J., Fleisher, T.A., Priel, D.A.L., et al. (2014). A phase 1 clinical trial of long-term, low-dose treatment of WHIM syndrome with the CXCR4 antagonist plerixafor. *Blood* **123**, 2308–2316.

McQualter, J.L., Darwiche, R., Ewing, C., Onuki, M., Kay, T.W., Hamilton, J.A., Reid, H.H., and Bernard, C.C. (2001). Granulocyte macrophage colony-stimulating factor: a new putative therapeutic target in multiple sclerosis. *Journal of Experimental Medicine* **194**, 873–882.

Merad, M., Sathe, P., Helft, J., and Miller, J. (2013). The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting - Annual Review of Immunology, 31(1):563. Annual Review of

- Meuret, G., and Hoffmann, G. (1973). Monocyte Kinetic Studies in Normal and Disease States. *Br J Haematol* 24, 275–285.
- Michaud, J.-P., Bellavance, M.-A., Préfontaine, P., and Rivest, S. (2013). Real-Time In Vivo Imaging Reveals the Ability of Monocytes to Clear Vascular Amyloid Beta. *Cell Reports* 5, 646–653.
- Mieno, S., Boodhwani, M., Ramlawi, B., Li, J., Feng, J., Bianchi, C., Laham, R.J., Li, J., and Sellke, F.W. (2006). Human Coronary Microvascular Effects of Cardioplegia-Induced Stromal-Derived Factor-1 α . *The Annals of Thoracic Surgery* 82, 657–663.
- Mildner, A., Yona, S., and Jung, S. (2013). A close encounter of the third kind: monocyte-derived cells. *Adv. Immunol.* 120, 69–103.
- Miller, J.C., Brown, B.D., Cohain, A., Pandey, G., Leboeuf, M., Elpek, K.G., Helft, J., Hashimoto, D., Chow, A., Price, J., et al. (2012). Deciphering the transcriptional network of the dendritic cell lineage. *Nat. Immunol.* 13, 888–899.
- Milo, I., Sapoznikov, A., Kalchenko, V., Tal, O., Krauthgamer, R., van Rooijen, N., Dudziak, D., Jung, S., and Shakhar, G. (2013). Dynamic imaging reveals promiscuous cross-presentation of blood-borne antigens to naive CD8⁺ T cells in the bone marrow. *Blood*.
- Molawi, K., Wolf, Y., Kandalla, P.K., Favret, J., Hagemeyer, N., Frenzel, K., Pinto, A.R., Klapproth, K., Henri, S., Malissen, B., et al. (2014). Progressive replacement of embryo-derived cardiac macrophages with age. *Journal of Experimental Medicine* 10, 1538.
- Moran, A.E., Holzapfel, K.L., Xing, Y., Cunningham, N.R., Maltzman, J.S., Punt, J., and Hogquist, K.A. (2011). T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *Journal of Experimental Medicine* 208, 1279–1289.
- Moreau, H.D., Lemaître, F., Terriac, E., Azar, G., Piel, M., Lennon-Dumenil, A.-M., and Bousso, P. (2012). Dynamic in situ cytometry uncovers T cell receptor signaling during immunological synapses and kinapses in vivo. *Immunity* 37, 351–363.
- Müller, A.J., Filipe-Santos, O., Eberl, G., Aebischer, T., Späth, G.F., and Bousso, P. (2012). CD4⁺ T cells rely on a cytokine gradient to control intracellular pathogens beyond sites of antigen presentation. *Immunity* 37, 147–157.
- Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S.,

- Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 382, 635–638.
- Nagasawa, T., Kikutani, H., and Kishimoto, T. (1994). Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2305–2309.
- Nagasawa, T. (2014). CXC chemokine ligand 12 (CXCL12) and its receptor CXCR4. *J Mol Med* 92, 433–439.
- Naik, S.H., Sathe, P., Park, H.-Y., Metcalf, D., Proietto, A.I., Dakic, A., Carotta, S., O'Keeffe, M., Bahlo, M., Papenfuss, A., et al. (2007). Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat. Immunol.* 8, 1217–1226.
- Nakano, H., Lin, K.L., Yanagita, M., Charbonneau, C., Cook, D.N., Kakiuchi, T., and Gunn, M.D. (2009). Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nat. Immunol.* 10, 394–402.
- Nascimento, M., Huang, S.C., Smith, A., Everts, B., Lam, W., Bassity, E., Gautier, E.L., Randolph, G.J., and Pearce, E.J. (2014). Ly6Chi Monocyte Recruitment Is Responsible for Th2 Associated Host-Protective Macrophage Accumulation in Liver Inflammation due to Schistosomiasis. *PLoS Pathog* 10, e1004282–15.
- Ng, L.G., Hsu, A., Mandell, M.A., Roediger, B., Hoeller, C., Mrass, P., Iparraguirre, A., Cavanagh, L.L., Triccas, J.A., Beverley, S.M., et al. (2008). Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS Pathog* 4, e1000222.
- Ng, L.G., Qin, J.S., Roediger, B., Wang, Y., Jain, R., Cavanagh, L.L., Smith, A.L., Jones, C.A., de Veer, M., Grimbaldston, M.A., et al. (2011). Visualizing the neutrophil response to sterile tissue injury in mouse dermis reveals a three-phase cascade of events. *J. Invest. Dermatol.* 131, 2058–2068.
- Nguyen, K.D., Fentress, S.J., Qiu, Y., Yun, K., Cox, J.S., and Chawla, A. (2013). Circadian Gene Bmal1 Regulates Diurnal Oscillations of Ly6Chi Inflammatory Monocytes. *Science* 341, 1483–1488.
- Nibbs, R.J.B., and Graham, G.J. (2013). Immune regulation by atypical chemokine receptors. *Nature Reviews. Immunology* 13, 815–829.

- Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G., et al. (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307, 254–258.
- Noda, M., Omatsu, Y., Sugiyama, T., Oishi, S., Fujii, N., and Nagasawa, T. (2011). CXCL12-CXCR4 chemokine signaling is essential for NK-cell development in adult mice. *Blood* 117, 451–458.
- Nourshargh, S., and Alon, R. (2014). Leukocyte Migration into Inflamed Tissues. *Immunity* 41, 694–707.
- Noy, R., and Pollard, J.W. (2014). Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity* 41, 49–61.
- O'Dea, K.P., Wilson, M.R., Dokpesi, J.O., Wakabayashi, K., Tatton, L., van Rooijen, N., and Takata, M. (2009). Mobilization and margination of bone marrow Gr-1^{high} monocytes during subclinical endotoxemia predisposes the lungs toward acute injury. *The Journal of Immunology* 182, 1155–1166.
- Oberlin, E., Amara, A., Bachelier, F.O., Bessia, C., Virelizier, J.-L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D.F., et al. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* 382, 833–835.
- Omatsu, Y., Seike, M., Sugiyama, T., Kume, T., and Nagasawa, T. (2014). *Foxc1* is a critical regulator of haematopoietic stem/progenitor cell niche formation. *Nature* 1–16.
- Onai, N., Obata-Onai, A., Schmid, M.A., and Manz, M.G. (2007). *Flt3* in regulation of type I interferon-producing cell and dendritic cell development. *Annals of the New York Academy of Sciences* 1106, 253–261.
- Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., et al. (2011). Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. *Science* 333, 1456–1458.
- Patel, B.V., Wilson, M.R., and Takata, M. (2012). Resolution of acute lung injury and inflammation: a translational mouse model. *Eur. Respir. J.* 39, 1162–1170.
- Pawig, L., Klasen, C., Weber, C., Bernhagen, J., and Noels, H. (2015).

Diversity and Inter-Connections in the CXCR4 Chemokine Receptor/Ligand Family: Molecular Perspectives. *Front Immunol* 6, 1–23.

Peiper, S.C., Wang, Z.X., Neote, K., and Martin, A.W. (1995). The Duffy antigen/receptor for chemokines (DARC) is expressed in endothelial cells of Duffy negative individuals who lack the erythrocyte receptor. *The Journal of*

Peng, Y., Latchman, Y., and Elkon, K.B. (2009). Ly6Clow Monocytes Differentiate into Dendritic Cells and Cross-Tolerize T Cells through PDL-1. *The Journal of Immunology* 182, 2777–2785.

Picelli, S., Faridani, O.R., rklund, A.S.K.B.O., Winberg, G.O.S., Sagasser, S., and Sandberg, R. (2014). Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* 9, 171–181.

Pitt, L.A., Tikhonova, A.N., Hu, H., Trimarchi, T., King, B., Gong, Y., Sanchez-Martin, M., Tsirigos, A., Littman, D.R., Ferrando, A.A., et al. (2015). CXCL12-Producing Vascular Endothelial Niches Control Acute T Cell Leukemia Maintenance. *Cancer Cell* 27, 755–768.

Pixley, F.J., and Stanley, E.R. (2004). CSF-1 regulation of the wandering macrophage: complexity in action. *Trends in Cell Biology* 14, 628–638.

Plantinga, M., Guilliams, M., Vanheerswyngheles, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., et al. (2013). Conventional and Monocyte-Derived CD11b+ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. *Immunity* 38, 322–335.

Price, T.T., Burness, M.L., Sivan, A., Warner, M.J., Cheng, R., Lee, C.H., Olivere, L., Comatas, K., Magnani, J., Kim Lyerly, H., et al. (2016). Dormant breast cancer micrometastases reside in specific bone marrow niches that regulate their transit to and from bone. *Sci Transl Med* 8, 340ra73–340ra73.

Probst, H.C., Tschannen, K., Odermatt, B., Schwendener, R., Zinkernagel, R.M., and Van Den Broek, M. (2005). Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells. *Clin. Exp. Immunol.* 141, 398–404.

Qu, C. (2004). Role of CCR8 and Other Chemokine Pathways in the Migration of Monocyte-derived Dendritic Cells to Lymph Nodes. *Journal of Experimental Medicine* 200, 1231–1241.

- Rivera, A., Hohl, T.M., Collins, N., Leiner, I., Gallegos, A., Saijo, S., Coward, J.W., Iwakura, Y., and Pamer, E.G. (2011). Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. *Journal of Experimental Medicine* 208, 369–381.
- Robbins, C.S., Chudnovskiy, A., Rauch, P.J., Figueiredo, J.L., Iwamoto, Y., Gorbato, R., Etzrodt, M., Weber, G.F., Ueno, T., van Rooijen, N., et al. (2012). Extramedullary Hematopoiesis Generates Ly-6Chigh Monocytes That Infiltrate Atherosclerotic Lesions. *Circulation* 125, 364–374.
- Robbins, C.S., Hilgendorf, I., Weber, G.F., Theurl, I., Iwamoto, Y., Figueiredo, J.-L., Gorbato, R., Sukhova, G.K., Gerhardt, L.M.S., Smyth, D., et al. (2013). Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med*.
- Rodero, M.P., Poupel, L., Loyher, P.-L., Hamon, P., Licata, F., Pessel, C., Hume, D.A., Combadière, C., and Boissonnas, A. (2015). Immune surveillance of the lung by migrating tissue monocytes. *Elife* 4, e07847.
- Rosen, H., Gordon, S., and North, R.J. (1989). Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. Absence of monocytes at infective foci allows *Listeria* to multiply in nonphagocytic cells. *Journal of Experimental Medicine* 170, 27–37.
- Rottman, J.B., Slavin, A.J., Silva, R., Weiner, H.L., Gerard, C.G., and Hancock, W.W. (2000). Leukocyte recruitment during onset of experimental allergic encephalomyelitis is CCR1 dependent. *Eur. J. Immunol.* 30, 2372–2377.
- Sallusto, F., Sallusto, F., Lanzavecchia, A., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *Journal of Experimental Medicine* 179, 1109–1118.
- Samstein, M., Schreiber, H.A., Leiner, I.M., Susac, B., Glickman, M.S., and Pamer, E.G. (2013). Essential yet limited role for CCR2⁺ inflammatory monocytes during *Mycobacterium tuberculosis*-specific T cell priming. *Elife* 2, e01086.
- Sapozhnikov, A., Pewzner-Jung, Y., Kalchenko, V., Krauthgamer, R., Shachar, I., and Jung, S. (2008). Perivascular clusters of dendritic cells

provide critical survival signals to B cells in bone marrow niches. *Nature Immunology* 9, 388–395.

Scheiermann, C., Kunisaki, Y., Lucas, D., Chow, A., Jang, J.-E., Zhang, D., Hashimoto, D., Merad, M., and Frenette, P.S. (2012). Adrenergic nerves govern circadian leukocyte recruitment to tissues. *Immunity* 37, 290–301.

Schlitzer, A., Sivakamasundari, V., Chen, J., Sumatoh, H.R.B., Schreuder, J., Lum, J., Malleret, B., Zhang, S., Larbi, A., Zolezzi, F., et al. (2015). Identification of cDC1- and cDC2-committed DC progenitors reveals early lineage priming at the common DC progenitor stage in the bone marrow. *Nat. Immunol.* 16, 718–728.

Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E.W., Pollard, J.W., et al. (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336, 86–90.

Scott, C.L., Zheng, F., De Baetselier, P., Martens, L., Saeys, Y., De Prijck, S., Lippens, S., Abels, C., Schoonooghe, S., Raes, G., et al. (2016). Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Comms* 7, 10321.

Segura, E., and Amigorena, S. (2013). Inflammatory dendritic cells in mice and humans. *Trends Immunol.*

Sennlaub, F., Auvynet, C., Calippe, B., Lavalette, S., Poupel, L., Hu, S.J., Dominguez, E., Camelo, S., Levy, O., Guyon, E., et al. (2013). CCR2(+) monocytes infiltrate atrophic lesions in age-related macular disease and mediate photoreceptor degeneration in experimental subretinal inflammation in Cx3cr1 deficient mice. *EMBO Mol Med* 5, 1775–1793.

Serbina, N.V., and Pamer, E.G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 7, 311–317.

Serbina, N.V., Kuziel, W., Flavell, R., Akira, S., Rollins, B., and Pamer, E.G. (2003a). Sequential MyD88-Independent and -Dependent Activation of Innate Immune Responses to Intracellular Bacterial Infection. *Immunity* 19, 891–901.

Serbina, N.V., Salazar-Mather, T.P., Biron, C.A., Kuziel, W.A., and Pamer, E.G. (2003b). TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection. *Immunity* 19, 59–70.

- Serbina, N.V., Jia, T., Hohl, T.M., and Pamer, E.G. (2008). Monocyte-Mediated Defense Against Microbial Pathogens. *Annual Review of Immunology* 26, 421–452.
- Shi, C., Jia, T., Mendez-Ferrer, S., Hohl, T.M., Serbina, N.V., Lipuma, L., Leiner, I., Li, M.O., Frenette, P.S., and Pamer, E.G. (2011). Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. *Immunity* 34, 590–601.
- Shi, C., Velázquez, P., Hohl, T.M., Leiner, I., Dustin, M.L., and Pamer, E.G. (2010). Monocyte trafficking to hepatic sites of bacterial infection is chemokine independent and directed by focal intercellular adhesion molecule-1 expression. *The Journal of Immunology* 184, 6266–6274.
- Shibata, Y., Berclaz, P.Y., Chroneos, Z.C., Yoshida, M., Whitsett, J.A., and Trapnell, B.C. (2001). GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity* 15, 557–567.
- Siedlar, M., Rudzki, Z., Strach, M., Trzyna, E., Pituch-Noworolska, A., Błaut-Szlósarczyk, A., Bukowska-Strakova, K., Lenart, M., Grodzicki, T., and Zembala, M. (2008). Familial occurrence of warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome. *Arch. Immunol. Ther. Exp.* 56, 419–425.
- Sierro, F., Biben, C., Martínez-Muñoz, L., Mellado, M., Ransohoff, R.M., Li, M., Woehl, B., Leung, H., Groom, J., Batten, M., et al. (2007). Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14759–14764.
- Soudja, S.M., Ruiz, A.L., Marie, J.C., and Lauvau, G. (2012). Inflammatory Monocytes Activate Memory CD8⁺ T and Innate NK Lymphocytes Independent of Cognate Antigen during Microbial Pathogen Invasion. *Immunity* 37, 549–562.
- Sreeramkumar, V., Adrover, J.M., Ballesteros, I., Cuartero, M.I., Rossaint, J., Bilbao, I., Nácher, M., Pitaval, C., Radovanovic, I., Fukui, Y., et al. (2014). Neutrophils scan for activated platelets to initiate inflammation. *Science* 346, 1234–1238.
- Staub, N.C., and Schultz, E.L. (1968). Pulmonary capillary length in dogs, cat and rabbit. *Respir Physiol* 5, 371–378.
- Steinman, R.M., and Cohn, Z.A. (1973). Identification of a novel cell type

in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *Journal of Experimental Medicine* 137, 1142–1162.

Steinman, R.M., and Cohn, Z.A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *Journal of Experimental Medicine* 139, 380–397.

Steinman, R.M., and Nussenzweig, M.C. (1980). Dendritic Cells: Features and Functions. *Immunol. Rev.* 53, 127–147.

Sunderkötter, C., Nikolic, T., Dillon, M.J., van Rooijen, N., Stehling, M., Drevets, D.A., and Leenen, P.J.M. (2004). Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *The Journal of Immunology* 172, 4410–4417.

Suratt, B.T., Petty, J.M., Young, S.K., Malcolm, K.C., Lieber, J.G., Nick, J.A., Gonzalo, J.-A., Henson, P.M., and Worthen, G.S. (2004). Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis. *Blood* 104, 565–571.

Swiecki, M., and Colonna, M. (2015). The multifaceted biology of plasmacytoid dendritic cells. *Nat. Rev. Immunol.* 1–15.

Swirski, F.K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., Figueiredo, J.-L., Kohler, R.H., Chudnovskiy, A., Waterman, P., et al. (2009). Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325, 612–616.

Tacke, F., Alvarez, D., Kaplan, T.J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J., Mack, M., van Rooijen, N., et al. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* 117, 185–194.

Takahashi, K., Yamamura, F., and Naito, M. (1989). Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *Journal of Leukocyte Biology* 45, 87–96.

Tal, O., Lim, H.Y., Gurevich, I., Milo, I., Shipony, Z., Ng, L.G., Angeli, V., and Shakhar, G. (2011). DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *Journal of Experimental Medicine* 208, 2141–2153.

Tamoutounour, S., Williams, M., Montanana Sanchis, F., Liu, H., Terhorst, D., Malosse, C., Pollet, E., Ardouin, L., Lucie, H., Sanchez,

- C., et al. (2013). Origins and Functional Specialization of Macrophages and of Conventional and Monocyte-Derived Dendritic Cells in Mouse Skin. *Immunity* 39, 925–938.
- Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T. (1993). Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science* 261, 600–603.
- Tassone, L., Moratto, D., Vermi, W., De Francesco, M., Notarangelo, L.D., Porta, F., Lougaris, V., Facchetti, F., Plebani, A., and Badolato, R. (2010). Defect of plasmacytoid dendritic cells in warts, hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome patients. *Blood* 116, 4870–4873.
- Tedder, T.F., Steeber, D.A., and Pizcueta, P. (1995). L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *Journal of Experimental Medicine* 181, 2259–2264.
- Teicher, B.A., and Fricker, S.P. (2010). CXCL12 (SDF-1)/CXCR4 Pathway in Cancer. *Clinical Cancer Research* 16, 2927–2931.
- Theurl, I., Hilgendorf, I., Nairz, M., Tymoszyk, P., Haschka, D., Asshoff, M., He, S., Gerhardt, L.M.S., Holderried, T.A.W., Seifert, M., et al. (2016). On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat Med* 1–11.
- Thornton, E.E., Krummel, M.F., and Looney, M.R. (2012). Live imaging of the lung. *Curr Protoc Cytom Chapter 12*, Unit12.28.
- Tittel, A.P., Heuser, C., Ohliger, C., Llanto, C., Yona, S., Hämmerling, G.J., Engel, D.R., Garbi, N., and Kurts, C. (2012). Functionally relevant neutrophilia in CD11c diphtheria toxin receptor transgenic mice. *Nat Meth* 9, 385–390.
- Tsou, C.-L., Peters, W., Si, Y., Slaymaker, S., Aslanian, A.M., Weisberg, S.P., Mack, M., and Charo, I.F. (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *Journal of Clinical Investigation* 117, 902–909.
- Ulvmar, M.H., Werth, K., Braun, A., Kelay, P., Hub, E., Eller, K., Chan, L., Lucas, B., Novitzky-Basso, I., Nakamura, K., et al. (2014). The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat. Immunol.* 1–10.
- van Furth, R., and Cohn, Z.A. (1968). The origin and kinetics of mononuclear phagocytes. *Journal of Experimental Medicine* 128, 415–

435.

van Furth, R., and Sluiter, W. (1986). Distribution of blood monocytes between a marginating and a circulating pool. *Journal of Experimental Medicine* 163, 474–479.

van Furth, R., Cohn, Z.A., Hirsch, J.G., Humphrey, J.H., Spector, W.G., and Langevoort, H.L. (1972). The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bulletin of the World Health Organization* 46, 845–852.

Varol, C., Landsman, L., Fogg, D.K., Greenshtein, L., Gildor, B., Margalit, R., Kalchenko, V., Geissmann, F., and Jung, S. (2007). Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *Journal of Experimental Medicine* 204, 171–180.

Varol, C., Yona, S., and Jung, S. (2008). Origins and tissue-context-dependent fates of blood monocytes. *Immunology and Cell Biology* 87, 30–38.

Wang, Y., Li, G., Stanco, A., Long, J.E., Crawford, D., Potter, G.B., Pleasure, S.J., Behrens, T., and Rubenstein, J.L.R. (2011). CXCR4 and CXCR7 Have Distinct Functions in Regulating Interneuron Migration. *Neuron* 69, 61–76.

Wang, Y., Cui, L., Gonsiorek, W., Min, S.-H., Anilkumar, G., Rosenblum, S., Kozlowski, J., Lundell, D., Fine, J.S., and Grant, E.P. (2009). CCR2 and CXCR4 regulate peripheral blood monocyte pharmacodynamics and link to efficacy in experimental autoimmune encephalomyelitis. *J Inflamm (Lond)* 6, 32.

Weber, C., Weber, K.S.C., Klier, C., Gu, S., Wank, R., Horuk, R., and Nelson, P.J. (2001). Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and TH1-like/CD45RO⁺T cells. *Blood* 97, 1144–1146.

Weber, G.F., Weber, G.F., Chousterman, B.G., Chousterman, B.G., He, S., He, S., Fenn, A.M., Fenn, A.M., Nairz, M., Nairz, M., et al. (2015). Interleukin-3 amplifies acute inflammation and is a potential therapeutic target in sepsis. *Science* 347, 1260–1265.

Weber, P.S.D., Toelboell, T., Chang, L.-C., Tirrell, J.D., Saama, P.M., Smith, G.W., and Burton, J.L. (2004). Mechanisms of glucocorticoid-induced down-regulation of neutrophil L-selectin in cattle: evidence for effects at the gene-expression level and primarily on blood neutrophils. *Journal of Leukocyte Biology* 75, 815–827.

- Welford, A.F., Biziato, D., Coffelt, S.B., Nucera, S., Fisher, M., Pucci, F., Di Serio, C., Naldini, L., De Palma, M., Tozer, G.M., et al. (2011). TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. *J. Clin. Invest.* **121**, 1969–1973.
- Wilson, M.R., O'Dea, K.P., Zhang, D., Shearman, A.D., van Rooijen, N., and Takata, M. (2009). Role of Lung-marginated Monocytes in an In Vivo Mouse Model of Ventilator-induced Lung Injury. *Am J Respir Crit Care Med* **179**, 914–922.
- Wong, C.H.Y., Jenne, C.N., Petri, B., Chrobok, N.L., and Kubes, P. (2013). Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance. *Nat. Immunol.* **14**, 785–792.
- Wong, K.L., Tai, J.J.-Y., Wong, W.-C., Han, H., Sem, X., Yeap, W.-H., Kourilsky, P., and Wong, S.-C. (2011). Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood* **118**, e16–e31.
- Wong, L.M., Myers, S.J., Tsou, C.L., Gosling, J., Arai, H., and Charo, I.F. (1997). Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene. Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *J. Biol. Chem.* **272**, 1038–1045.
- Wu, H., Gower, R.M., Wang, H., Perrard, X.Y.D., Ma, R., Bullard, D.C., Burns, A.R., Paul, A., Smith, C.W., Simon, S.I., et al. (2009). Functional Role of CD11c⁺ Monocytes in Atherogenesis Associated With Hypercholesterolemia. *Circulation* **119**, 2708–2717.
- Xu, H., Manivannan, A., Crane, I., Dawson, R., and Liversidge, J. (2008). Critical but divergent roles for CD62L and CD44 in directing blood monocyte trafficking in vivo during inflammation. *Blood* **112**, 1166–1174.
- Yamasaki, R., Lu, H., Butovsky, O., Ohno, N., Rietsch, A.M., Cialic, R., Wu, P.M., Doykan, C.E., Lin, J., Cotleur, A.C., et al. (2014). Differential roles of microglia and monocytes in the inflamed central nervous system. *Journal of Experimental Medicine* **10**, 1538.
- Yona, S., and Gordon, S. (2015). From the Reticuloendothelial to Mononuclear Phagocyte System – The Unaccounted Years. *Front Immunol* **6**, 1–7.

- Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Williams, M., Misharin, A., et al. (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 38, 79–91.
- Yoshimura, T., Robinson, E.A., Tanaka, S., Appella, E., Kuratsu, J., and Leonard, E.J. (1989). Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *Journal of Experimental Medicine* 169, 1449–1459.
- Zhan, Y., Lieschke, G.J., Grail, D., Dunn, A.R., and Cheers, C. (1998). Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of *Listeria monocytogenes*-infected mice. *Blood* 91, 863–869.
- Zhou, Y., Kurihara, T., Ryseck, R.P., Yang, Y., Ryan, C., Loy, J., Warr, G., and Bravo, R. (1998). Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. *The Journal of Immunology* 160, 4018–4025.
- Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J.M., Liu, Y.J., MacPherson, G., Randolph, G.J., et al. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood* 116, e74–e80.
- Zigmond, E., Varol, C., Farache, J., Elmaliah, E., Satpathy, A.T., Friedlander, G., Mack, M., Shpigel, N., Boneca, I.G., Murphy, K.M., et al. (2012). Ly6C^{hi} monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity* 37, 1076–1090.
- Zipfel, W.R., Williams, R.M., Christie, R., Nikitin, A.Y., Hyman, B.T., and Webb, W.W. (2003). Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7075–7080.
- Zlotnik, A., and Yoshie, O. (2012). The Chemokine Superfamily Revisited. *Immunity* 36, 705–716.