# Role of chemokine receptors in inflammation-induced haematopoietic progenitor cell mobilisation

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# Abstract

**Background**: There is a high cellular turnover in the haematopoietic system which necessitates that new cells are continuously produced to replace old and senescent ones. The haematopoietic stem cells and its progenitors meet this requirement. In adults, haematopoietic stem and progenitor cells (HSPCs) generally occupy a unique microenvironment in the bone marrow called the niche. However, recent findings have shown that approximately 0.06% of HSPCs circulate between bone marrow and periphery in steady state. Evidence shows that HSPC express cytokine receptors and pathogen recognition receptors [e.g. Toll-like receptors (TLRs)], which suggest that HSPCs may directly respond to inflammation and infection. The capacity of HSPCs to directly respond to infection is demonstrated in models of bacterial infection using LPS injection that significantly increases the number of circulating HSPCs. However, the underlying mechanism is not clearly understood. As chemokines orchestrate *in vivo* cellular migration, it was **hypothesised** that HSPC inducibly express inflammatory chemokine receptors that enable them to respond to circulating chemokines during infection and inflammation. **Methods and results**: Here, the impact of systemic or peripheral inflammation on HSPC of mice was investigated using LPS injection and topical imiquimod cream/TPA treatment respectively. Using haematopoietic progenitor colony-forming assays, RT-QPCR on isolated progenitors, gene-knockout mice, flow cytometric analysis and *in vivo* antibody-mediated neutralisation experiments, data are provided showing that HSPC inducibly express chemokine receptors in response to inflammation. Critically, the topical imiquimod inflammation model required functional chemokine receptor 2 (Ccr2) for HSPC mobilisation in contrast to both systemic LPS and topical TPA models, which were Ccr2-independent. Furthermore, dermal inflammation was necessary for imiquimod-mediated HSPC mobilization, as subcutaneously administered imiquimod did not result in significant HSPC mobilization. **Conclusion**: The data suggest that, in addition to the established CXCR4-CXCL12 axis that regulates homeostatic HSPC trafficking, the inflammatory chemokine-chemokine receptor axes may also be crucial in modulating HSPC functions during infection and inflammation.

# Table of Contents

# List of Tables

# List of Figures

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# Author’s Declaration

I declare that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution. Wherever the work of others has been cited, it is dully acknowledged and referenced.

Signature:…….

Name: Patrick Adu

# Definitions/Abbreviations

| | |
|---|---|
| **A** | |
| ACKR | Atypical Chemokine Receptor |
| AGM | Aorta-Gonad-Mesonephrous |
| **B** | |
| BFU-E | Burst-Forming Unit, Erythroid |
| **C** | |
| CAR | CXCL12 Abundant Reticular cells |
| CFU-E | Colony-Forming Unit, Erythroid |
| GEMM | Granulocyte, Erythrocyte, Macrophage, Megakaryocyte |
| GM | Granulocyte-Macrophage cell |
| CFU-S | Colony-Forming Unit, Spleen |
| CIA | Collagen-Induced Arthritis |
| CLP | Common Lymphoid Progenitor |
| CMP | Common Myeloid Progenitor |
| **D** | |
| DRY | Aspartic acid-Arginine-Tyrosine motif |
| DAG | Diacylglycerol |
| dHSC | Definitive Haematopoietic Stem Cell |
| **E** | |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EPO | Erythropoietin |
| EPOR | Erythropoietin Receptor |
| **F** | |
| FDA | Food and Drug Administration |
| FMO | Fluorescence-Minus-One |
| **G** | |
| GAGs | Glycosaminoglycans |
| G-CSF | Granulocyte Colony-Stimulating Factor |
| G-CSFR | Granulocyte Colony-Stimulating Factor Receptor |
| GMP | Granulocyte-Macrophage Progenitor |
| GRO | Growth Related Protein |
| **H** | |
| HPC | Haematopoietic Progenitor Cell |
| HSPC | Haematopoietic Stem and Progenitor Cell |
| HSC | Haematopoietic Stem Cell |
| **I** | |
| IMQ | Imiquimod |
| I.P. | Intraperitoneal injection |
| I.V. | Intravenous injection |
| **J** | |
| JAK | Janus Kinase |
| **L** | |
| LMPP | Lymphoid-Primed Multipotent Progenitors |
| LPS | Lipopolysaccharide |
| LT-HSC | Long-Term Haematopoietic Stem Cell |
| **M** | |
| MEP | Megakaryocyte-Erythrocyte Progenitors |
| MkP | Megakaryocyte Progenitors |
| MMP | Matrix Metalloproteinase |
| MPP | Multipotent Progenitors |
| MSC | Mesenchymal Stromal Cell |
| **P** | |
| PKC | Protein Kinase C |
| PreCFU-E | Precursor Colony-Forming Unit, Erythroid |
| PreGM | Precursor Granulocyte-Macrophage cell |
| PreMegE | Precursor Megakaryocyte-Erythrocyte cell |
| PRR | Pattern Recognition Receptor |
| **S** | |
| S.C. | Subcutaneous injection |
| Sca-1 | Stem Cell Antigen 1 |
| SLAM | Signalling Lymphocytic Activated Molecule |

# Chapter 1: General introduction

# 1.1 Introduction

Trafficking of cells is a fundamental physiological process essential not only for proper development during embryogenesis but also for proper function of the immune system in organisms. This directed cell migration to specific anatomical sites is essential for such processes as cardiogenesis, angiogenesis and development of the CNS (Adler et al., 2005, Baggiolini, 1998). Cellular trafficking is however, kept under tight control as dysregulation frequently leads to pathology. Amongst the key regulators of cellular trafficking are chemotactic cytokines called chemokines. The cells of the haematopoietic system are one such system whose migratory properties are controlled by these chemokines. It is estimated that the haematopoietic system generates $3 \times 10^5$ red blood cells and $3 \times 10^4$ white blood cells per second in a 70-Kg human in steady state. This means that haematopoietic system have a high turnover rate and thus constantly needs to be replenished to sustain the life of an individual in steady-state haematopoiesis (Takizawa et al., 2012, Hirayama et al., 1999). This long-term cell production is made possible by the presence of a rare population of cells called haematopoietic stem cells (HSCs) that, through symmetric/asymmetric division, are able to self-renew and differentiate respectively, to progenitors with increasingly refined lineage commitment. In vertebrates, blood stem cells are produced in a variety of tissues depending on the developmental stage of the organism (Galloway and Zon, 2003).

## 1.1.1 Chemokines and their receptors

Chemokines are small chemoattractant cytokines, 8-12 kDa, secreted by immune as well as stromal cells, which are involved in regulating directional cell migration *in vivo* (Rot and von Andrian, 2004). These chemokines signal through their cognate G-protein coupled receptors (GPCRs) and, function in concert, with adhesion molecules as well as glycosaminoglycans (Springer, 1994, Dwir et al., 2004). Approximately, 50 chemokines and 18 chemokine receptors have been discovered in humans suggesting redundancy in the ligand:receptor interaction in the chemokine system (Rot and von Andrian, 2004, Charo and Ransohoff, 2006).

### 1.1.2 Classification of chemokines

Traditionally, chemokines have been classified based either on functional, or structural, criteria. The functional classification is based on the context in which the chemokine is secreted. Specifically, chemokines are described as being either homeostatic or inflammatory. Inflammatory chemokines are generally not expressed in resting tissues but are inducibly expressed upon leucocyte and stromal cell activation in response to pro-inflammatory mediators such as tumour necrosis factor, interferon gamma and pathogen associated molecular pattern. They are fundamental in directional cell migration to sites of infection, trauma and inflammation. Although most inflammatory chemokines are regulated at the transcriptional level by pro-inflammatory mediators, some (e.g. CXCL1, CXCL4, CXCL8 and CCL5) are also produced by platelets, and stored as preformed proteins in their $\alpha$-granules, but released upon platelet activation (Power et al., 1995, Weiss et al., 1979, Brandt et al., 2000). Homeostatic chemokines, on the other hand, are continuously expressed in specific tissue locale and are vital for such processes as stem cell trafficking during embryogenesis, immune surveillance, as well as homing of leukocytes to lymph nodes and also HSC to the bone marrow after transplantation (Moser, 2003, Moser and Willimann, 2004, Mantovani, 1999). It must be noted however that this functional classification is not exclusive as some homeostatic chemokines show inducible expression in certain circumstances.

Structural classification of chemokines is based on the presence of a well-conserved cysteine (C) motif in the N-terminus of their structure. Under this system of classification, the chemokines are grouped into four main sub-families: **CC chemokines** have the first two cysteine groups juxtaposed. The CC chemokine subfamily constitutes the most abundant chemokine class and is mainly encoded by a multigene cluster on chromosome 17q11.2. **CXC chemokines** have a non-cysteine amino acid separating the first two cysteine groups. Based on the presence, or absence, of an ELR (Glu-Leu-Arg) motif just prior to the first cysteine residue, the CXC chemokine subfamily may be further grouped into angiogenic and angiostatic chemokines respectively. The angiogenic CXC chemokines mainly attract neutrophils and are primarily encoded by a gene cluster on chromosome 4q12-q13; the angiostatic CXC chemokines mainly attract T-lymphocytes and are encoded by a gene cluster on chromosome 4q21.21. The

**CX3C chemokine** has three non-cysteine amino acids that separate the first two cysteine groups. The CX3C chemokine is encoded on chromosome 16q13 and mainly attracts T-lymphocytes and monocytes. The CX3C chemokine family has only one member and exists as a transmembrane protein; proteolytic cleavage leads to secretion of soluble CX3C chemokine. The **C chemokine** has only two cysteine residues, selectively attracts T-lymphocytes and is encoded by a gene on chromosome 1q23 (Locati et al., 2005, Zlotnik and Yoshie, 2012).

### 1.1.3 Classification of chemokine receptors

Chemokine receptors are seven-transmembrane spanning G-protein coupled receptors (GPCRs) with their N-termini exposed outside the cell while their C-termini are intracellular. The chemokine receptors are classified into four main groups based on the structural sub-family of chemokines they bind (see figure 1-1 for an overview of chemokine-chemokine receptor pairing). Thus, we define CXCR (cognate receptors for CXC chemokines), CCR (cognate receptors for CC chemokine), CX3CR (cognate receptor for CX3C chemokine) and XCR (cognate receptor for XC chemokine) receptors. The N-terminus, and the three extracellular loops, of the chemokine receptors have been shown to be necessary for ligand binding, whereas the C-terminus and the three intracellular loops are required for signal transduction (Mayer and Stone, 2000, Booth et al., 2002, Govaerts et al., 2003).

### 1.1.4 Chemokine receptor signalling

It is widely accepted that chemokine receptor binding and activation follows a two-step process: firstly, the core domains of the chemokine ligand bind to the N-terminus and the extracellular loops of the chemokine receptor; and secondly, this is followed by penetration of the receptor's helical structure by the N-terminus of the ligand (Gupta et al., 2001). Ligand occupancy of the requisite chemokine receptor leads to conformational change, receptor dimerization (hetero- or homo-dimerization), as well as phosphorylation of the conserved serine and threonine residues in the short intracytoplasmic tails of the chemokine receptor (Youn et al., 2001, Han et al., 1999). Subsequent to this activation, there is dissociation of the hetero-trimeric G-protein (Gαβγ) into the GTP-associated Gαi subunit and Gβγ subunit. The Gαi subunit is recruited to the

chemokine receptor through one of its intracellular loops, and induces activation of Src family proteins and a cascade of signalling events [e.g. mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) cascades] leading to cytoskeletal rearrangement, and degranulation (Arai and Charo, 1996, Mellado et al., 1998, Damaj et al., 1996, Myers et al., 1995). The Gßγ subunit also activates phospholipase C leading to the formation of inositol triphosphate and diacylglycerol (DAG) (Jiang et al., 1996). The inositol triphosphate mobilizes calcium ions from intracellular stores in the endoplasmic reticulum whereas DAG activates protein kinase C and eventually mediates receptor desensitization through receptor internalization (Wu et al., 1998). It must be noted that it is the Gßγ subunit that mediate the chemotactic responses associated with chemokine receptor signalling (Moratz et al., 2004)

Other events that are generated downstream of chemokine receptor signalling includes trafficking and differentiation of haematopoietic precursors, leukocyte activation of multiple integrins for firm endothelial adhesion as well as integrin recycling at the leading edge of migrating leukocytes (Vulcano et al., 2003, Pribila and Shimizu, 2003, Lawson and Maxfield, 1995).

There are many cellular processes that regulate chemokine receptor signalling. Amongst these are the adaptor proteins called arrestins. These arrestins (e.g. ß-arrestins) regulate chemokine receptor signalling via receptor desensitization in one of two ways: (1) binding of the arrestins to the activated receptor may stearically hinder receptor-G-protein interaction (Vroon et al., 2006); (2) arrestin binding may target the receptor to the endosomal pathway for degradation, dephosphorylation or recycling back to the cell membrane (Kelly et al., 2008, Wolfe and Trejo, 2007). Other mechanisms that regulate chemokine receptor signalling such as ligand-induced receptor internalisation and G-protein coupled receptor kinases (Ferguson, 2001).

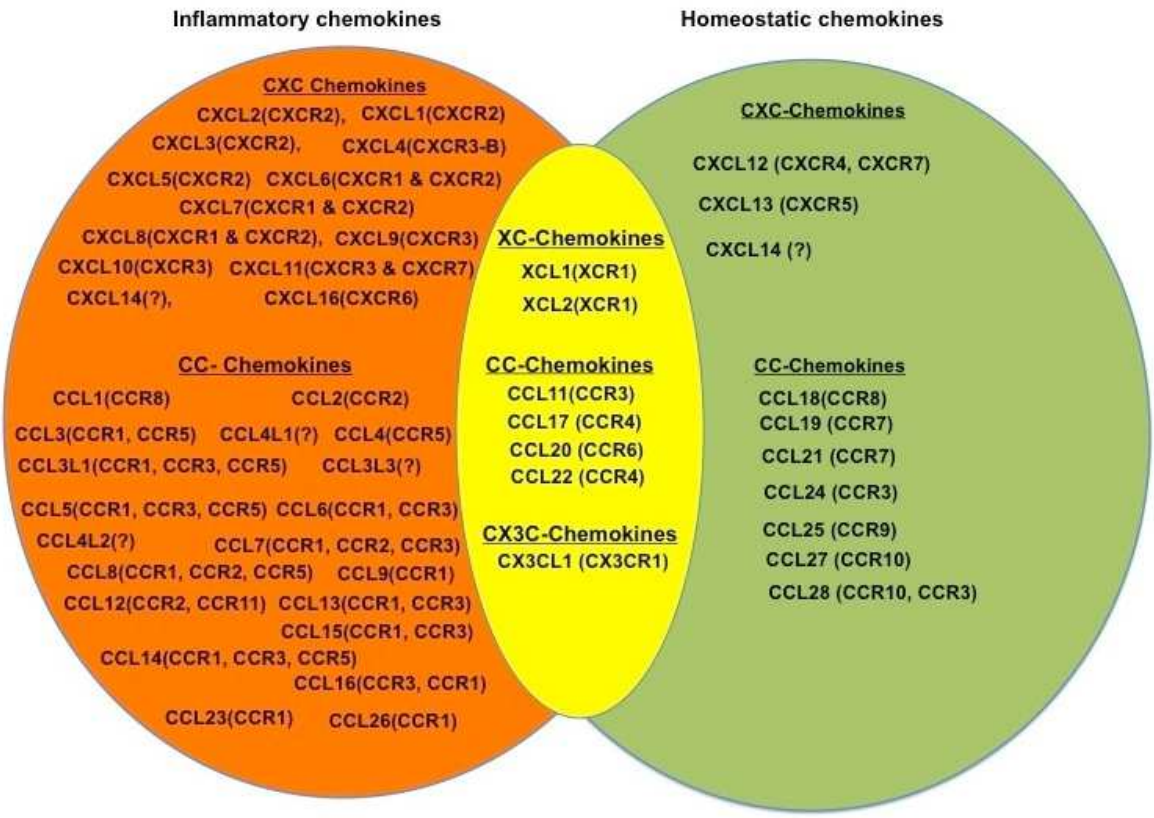



**Figure 1-1 An overview of the chemokine-chemokine receptor interaction.**
The intersection of the Venn diagram indicates dual chemokines; chemokine receptors are shown in bracket beside the chemokine they interact, whereas question mark in bracket indicates chemokines with unknown receptor. Adapted from (Zlotnik and Yoshie, 2012, Locati et al., 2005)

### 1.1.5 Chemokine receptor expression on early haematopoietic progenitors

Although the expression of chemokine receptors on terminally differentiated haematopoietic cells is well established, data on the expression of chemokine receptors on haematopoietic stem and progenitors are only now emerging. Interestingly, although certain chemokines like CXCL1 (GROα), CXCL2 (GROß), CXCL8 and CCL3 (MIP-1α) have been demonstrated to be actively haematopoietic stem and progenitor (HSPC) mobilizing agents, there has not been conclusive evidence about the expression of their respective chemokine receptors on the HSPCs. In fact, it has been speculated that the HSPC mobilizing actions of CXCL1, CXCL2, and CXCL8 are due to an indirect effect on neutrophils, as CXCR2, the cognate receptor for these chemokines, has not been shown to be expressed on HSPCs (Pruijt et al., 2002, Pelus et al., 2004). In a thioglycollate model of aseptic inflammation, it was recently shown that haematopoietic stem and progenitor cells use CCR2 to migrate to sites of inflammation in mice (Si et al., 2010). Using flow cytometry as well as other *in vitro* and *in vivo* migration assays, this paper demonstrated that murine haematopoietic progenitor cells

(defined as $Lin^{-}Kit^{+}$) express functional CCR2. In addition, signals received through CCR2 were also previously demonstrated to be critical in limiting haematopoietic progenitor cell proliferation as well as haematopoietic progenitor cell survival in mice (Reid et al., 1999). However, another study detected CCR3, CCR9 and CXCR4 in long-term haematopoietic stem cell (LT-HSC) and short-term HSC (ST-HSC) at the mRNA level; although with the exception of CXCR4, these progenitor populations did not migrate in response to the respective chemokine ligands in *in vitro* chemotaxis assay (Wright et al., 2002). Other chemokine receptors like CCR1, CCR2, CCR4, CCR5 and CXCR2 were neither consistently detected nor were the haematopoietic progenitors responsive in chemotaxis assay using cognate ligands for these receptors. In addition, studies that tracked chemokine receptor expression in human ontogeny (i.e., foetal liver, mobilised peripheral blood and adult bone marrow) demonstrated that although $Lin^{-}$ progenitors expressed CXCR1, CXCR2, CXCR4, and CCR5; only $CD34^{-}CD38^{-}Lin^{-}$ and $CD34^{+}CD38^{-}Lin^{-}$ cells expressed CXCR4 (Rosu-Myles et al., 2000). Moreover, primitive cord blood haematopoietic progenitors defined in that study as $CD34^{-}CD38^{-}Lin^{-}$ and $CD34^{+}CD38^{-}Lin^{-}$ were also shown to express CXCR1, CXCR2, CXCR4, CCR5, CCR7 and CCR8.

Perhaps the only chemokine receptor that has been unequivocally demonstrated to be expressed in HSPC (both mouse and human studies), as well as differentiated haematopoietic cells, is CXCR4. The CXCR4-CXCL12 axis has been demonstrated to be vital in embryogenesis as it is required for proper neurogenesis, cardiogenesis, vasculogenesis and haematopoiesis (Nagasawa et al., 1996). In addition, seeding of the bone marrow by foetal HSC during foetal development is inhibited by disruption of the CXCR4-CXCL12 axis (Zou et al., 1998, Ma et al., 1998a). Moreover, the CXCR4-CXCL12 axis is vital in adult haematopoiesis as it is necessary for homing and retention of haematopoietic stem and progenitor cells to the bone marrow as well as maintenance of the HSPC pool size and quiescence (Nie et al., 2008, Hundt et al., 2006). As discussed below (section 1.13.1, 1.13.4.1.3-5), the indispensable role of the CXCR4-CXCL12 axis is exploited to induce HSPC mobilization for clinical stem cell transplantation.

In spite of the apparent discrepancies in data on the expression of chemokine receptors on HSPCs, it has been shown that chemokine-mobilised HSPCs have

superior repopulation kinetics, and competitive engraftment potential, compared to those mobilized by the more traditional HSPC mobilizing agent, granulocyte colony-stimulating factor (G-CSF) (Broxmeyer et al., 2005, Pelus and Fukuda, 2006, Fukuda et al., 2007). Therefore, proper understanding of this chemokine-chemokine receptor axis in HSPC biology may be a key component in discovery of new therapeutic targets for improved clinical HSPC mobilization as well as for defining the mechanisms involved in HSPC trafficking in health and disease.

### 1.1.6 Chemokines and diseases

During the initial phase of injury and infection, innate immune cells, notably neutrophils, are recruited to these sites to help resolve the acute inflammatory response. In later time points, monocytes are also recruited to the inflamed sites as well and subsequently differentiate to macrophages to begin the process of tissue repair. However, in cases where this initial cellular response fails to resolve the acute inflammation and/or injury, the persistence of macrophages as well as recruitment of other cells of the adaptive immune system like lymphocytes leads to chronic inflammation (Nathan and Ding, 2010). Since chemokines are known to orchestrate recruitment of both innate and adaptive immune cells to sites of infection and inflammation, it is not surprising that these chemotactic inducers have been implicated in a host of inflammatory diseases. Data from both *in vitro* and transgenic animal models have provided evidence to establish chemokines and their receptors as crucial players in a number of diseases. The role of chemokine receptors in two specific human diseases [HIV (human immunodeficiency virus) infection and rheumatoid arthritis] have been reviewed here as an example. Table 1-1 gives a brief summary of some transgenic mouse models that have shed light on the role of specific chemokine receptors in specific disease or inflammatory contexts as well as the human diseases in which these chemokine receptors have been shown to play crucial roles. Others have previously published detailed explorations concerning the roles of specific chemokine, and chemokine receptors, in the pathogenesis of inflammatory, infectious and autoimmune diseases (White et al., 2013, Romagnani et al., 2004, Strieter et al., 2007).

**Table 1-1 Chemokine receptors in diseases and disease models**

| Receptor KO | Developmental defects | Pathophysiologic roles [disease models (mouse strain)] | Human diseases where receptor plays a role |
|---|---|---|---|
| CCR1-/- | None | 1. Increased mortality [*A. fumigatus* infection (C57Bl/6 mice)]<br><br>2. Reduced granuloma formation; excessive Th1 response [*S. mansoni* infection (C57BL/6 mice)]<br><br>3. Less Th2 response; failure to clear parasite [*Leishmania* infection (Sv129xC57BL/6 mice)] | Rheumatoid arthritis, multiple sclerosis, endometriosis associated with pelvic pain |
| CCR2-/- | None | 1. Failed bacterial clearance [*L. monocytogenes* infection (Sv129xICR)]<br><br>2. Increased lethality; absence of monocytosis [West Nile Virus (C57BL/6)]<br><br>3. Reduced macrophage recruitment [thioglycollate peritonitis (Sv129xC57BL/6] | Multiple sclerosis, rheumatoid arthritis, atherosclerosis, bone metastases |
| CCR4-/- | None | 1. Decreased mortality; enhanced bacterial clearance [polymicrobial sepsis (C57Bl/6)]<br><br>2. Reduced lethality & tissue damage [Dengue virus infection (C57BL/6)] | Adult T-cell leukaemia/lymphoma |
| CCR5-/- | None | 1. Reduced survival; defective $T_{reg}$ recruitment (Graft versus host disease (Sv129xC57BL/6)]<br><br>2. Reduced survival; defective leucocyte recruitment to the brain [*Cryptococcus neoformans* infection (Sv129xC57BL/6)] | HIV infection, rheumatoid arthritis |

| | | |
|---|---|---|
| CXCR4-/- | Embryonic lethal | HIV infection, cancer metastasis |

### 1.1.6.1 Human immunodeficiency virus (HIV) infection

HIV is a retrovirus and can be broadly divided into two tropic forms; M-tropic (infects primary macrophages), and T-tropic (primarily infects transformed T-cells). HIV infection or entry into a given cell occurs through a two-step process: firstly, the attachment of the virus glycoprotein 120 (gp120) to the host CD4 antigen (Sattentau and Weiss, 1988). This is then followed by fusion of the virus with host cell surface co-receptors in order to deposit the viral genome into the host cytoplasm (Alkhatib et al., 1996, Choe et al., 1996, Doranz et al., 1996). The co-receptors have been identified as belonging to the chemokine receptor family i.e. CCR5 and CXCR4. The M-tropic virus has been shown to preferentially utilise CCR5 as the co-receptor, whereas the T-tropic virus uses CXCR4 as the co-receptor. Thus, the HIV variants are also either called R5 (virus using CCR5 as co-receptor) or X4 (virus using CXCR4 as co-receptor) in recognition of which co-receptor is used for viral entry. The R5 variants generally predominate in earlier HIV infection; however, a switch from the R5 to X4 phenotype, later in the course of the infection, leads to the progression to acquired immunodeficiency syndrome (AIDS) (Connor and Ho, 1994, Schuitemaker et al., 1992).

Liu et al. provided evidence for the fundamental role of CCR5 in the initiation of HIV infection when they demonstrated that individuals with homozygous deletion of 32-base pairs (CCR5Δ32) in the second extracellular loop of CCR5 gene were resistant to HIV infection (Liu et al., 1996). This deletion was subsequently shown to prevent functional cell surface expression of CCR5. It is estimated that the prevalence of the CCR5Δ32 mutation is about 1% in the Caucasian population of European origin, although the mutation could be higher in northern European populations (Novembre et al., 2005). Not surprisingly, the Food and Drug Administration (FDA) recently approved a CCR5 blocker, called Maraviroc, for the treatment of HIV (Dorr et al., 2005, Wilkin and Gulick, 2012).

Although, the CXCR4 antagonist, AMD3100, also strongly prevents HIV infection, due to its strong ability to mobilise haematopoietic stem and progenitor cells (HSPC) to blood, it is currently used to mobilise HPSC for autologous stem cell

transplantation (Devine et al., 2004, Devine et al., 2008). The use of AMD3100 as an HSPC mobilising agent is extensively discussed later in this chapter.

#### 1.1.6.2 Rheumatoid arthritis

Rheumatoid arthritis is a chronic, autoimmune and inflammatory disease that affects about 0.5-1% of people in western countries (Symmons et al., 2002). The disease is characterised by persistent inflammation of the lining of synovial tissues (synovitis), formation of fibrovascular tissue (pannus formation) in the affected joint, and ultimately bone, cartilage and ligament destruction. Overproduction of pro-inflammatory cytokines such as TNF, IL-6 and IL-1, from fibroblast-like synoviocytes and macrophage-like synoviocytes, seems to be a major driver of the chronic inflammation as well as bone destruction observed in rheumatoid arthritis (Feldmann et al., 1996, Muller-Ladner et al., 1996, Choy et al., 2002). Other research findings also suggest that activation of osteoclasts partly accounts for the characteristic bone destruction (Cohen et al., 2008). It has also been estimated that more than 50% of rheumatoid arthritis patients have the classic autoantibody called rheumatoid factor or autoantibodies directed against citrullinated peptides (van der Linden et al., 2009). Moreover, other findings showed genetic predisposition as a major risk factor in developing rheumatoid arthritis (van der Woude et al., 2009).

Studies using animal models have helped to shape our understanding of the basic mechanisms underlying human rheumatoid arthritis as well as the development of new therapeutic strategies. Among these animal models are the adjuvant-induced arthritis model [e.g. complete Freund's adjuvant (CFA)], and the antigen/proteoglycan immunisation-induced arthritis [e.g. collagen-induced arthritis (CIA)] model (Courtenay et al., 1980, Hopkins et al., 1984).

Many chemokines have been shown to be important in the pathophysiology of rheumatoid arthritis. For example, Koch et al. showed that *in vitro* stimulation of synovial fibroblast with TNF-$\alpha$, or IL-1, or IFN$\gamma$ (each of which is known to be a major driver of rheumatoid arthritis) led to increased CCL2 and CCL3 production. In agreement with these *in vitro* data, they also showed that CCL2 and CCL3 concentrations were elevated in synovial fluid from rheumatoid arthritis patients (Koch et al., 1992, Matsui et al., 2001, Ruth et al., 2003). Other studies in

human rheumatoid arthritis patients that profiled the expression of chemokine receptors, showed that CCRs1-7 were expressed in leukocytes from peripheral blood, synovial fluid and tissues (Szekanecz et al., 2006, Szekanecz et al., 2010, Katschke et al., 2001, Ruth et al., 2001).

In spite of the many studies that have demonstrated fundamental involvement the chemokine-chemokine receptor axes in the pathology of rheumatoid arthritis, therapeutic interventions aimed at interfering with these axes have not been met with much success. For example, Maraviroc (CCR5 antagonist) was unable to show clinical efficacy in a recent randomized, double-blind placebo-controlled, Phase IIa, clinical trial in rheumatoid arthritis patients (Fleishaker et al., 2012). Similarly, in another previous clinical trial in rheumatoid arthritis patients that involved monoclonal antibody-mediated neutralisation of CCL2 (CCR2 ligand), patients did not demonstrate any clinical improvement (Haringman et al., 2006).

## 1.2 Atypical chemokine receptors (ACKRs)

These are a group of 7-transmembrane receptors that are structurally related to the chemokine receptor subfamily but are unable to orchestrate G-protein-coupled signalling events. Members of the ACKR family include ACKR1 (DARC, Duffy antigen receptor for chemokines), ACKR2 (D6), ACKR3 (CXCR7) and ACKR4 (CCRL1) (Bachelerie et al., 2014). Evidence indicates that one of the fundamental differences between the ACKRs and the conventional chemokine receptors is the alteration of the DRYLAIV consensus sequence in the second intracellular loop of the ACKRs (Galliera et al., 2004, Graham et al., 2012). These structural modifications lead to uncoupling of G-proteins from the ACKRs and a selective bias towards β-arrestin signalling, in contrast to the combined β-arrestin and G-protein-coupled signalling, described for the conventional chemokine receptors in section 1.1.4. Interestingly, two of the conventional chemokine receptors, XCR1 and CXCR6, have been shown to have modifications in the DRYLAIV consensus sequence (Chandrasekar et al., 2004, Yoshida et al., 1998), suggesting that such modifications alone does not account for the lack of G-αi-mediated signalling of the ACKRs. Recently, data from crystallographic analysis of seven transmembrane receptors suggested that three elements i.e. the DRY motif [transmembrane domain 3 (TM3)], the CWXP motif (TM6) and the

NPXXY$^{5-6}$F motif (TM7) are the most crucial elements for a seven transmembrane receptor to activate Gαi signalling (Nygaard et al., 2009). Further research on the structure-functional relationship of the ACKRs is thus warranted to fully elucidate the mechanistic details underlining their inability to activate Gαi signalling.

Functionally, the ACKRs have been shown to modulate the actions of chemokines by binding, internalising, and degrading chemokines (ACKR2-4) or by transcytosis of chemokine ligands (ACKR1). ACKR1 is expressed by erythrocytes, and vascular endothelial cells, and has been proposed to act as a "sink" for inflammatory chemokines (Kashiwazaki et al., 2003, Darbonne et al., 1991). ACKR2 selectively scavenge inflammatory CC chemokines, and is expressed by the syncytiotrophoblastic tissue of the placenta, lymphatic endothelial cells of the gut, lungs and skin, as well as by some leukocytes (Nibbs et al., 1997, Nibbs et al., 2001, Madigan et al., 2010). ACKR3 (CXCR7) on the other hand, scavenges CXCL12 and CXCL11, and is expressed in some haematopoietic, mesenchymal and neuronal cells (Balabanian et al., 2005, Su et al., 2002). In addition, studies from genetic deletion of ACKR3 in mice have implicated a role for this receptor in cardiogenesis (Sierro et al., 2007). ACKR4 selectively scavenges homeostatic chemokine ligands, CCL19, CCL21 and CCL25, and has been suggested to have a role in regulating chemokine-mediated adaptive immune responses (Townson and Nibbs, 2002, Gosling et al., 2000, Graham et al., 2012).

## 1.3 Pattern recognition receptors

The presence of invading pathogens is typically detected through ligation of pattern recognition receptors (PRR) by specific conserved pathogen-associated molecular patterns (PAMPs) on the invading organism. These PRRs are diverse and include Toll-like receptors (TLRs), nucleotide-binding oligomerisation protein domains receptors (NLRs), and purinergic receptors. Expression of PRRs has been demonstrated not only on innate immune effector cells like dendritic cells, monocytes, macrophages and neutrophils, but also on endothelial cells and haematopoietic progenitor cells (Nagai et al., 2006, Esplin et al., 2011).

### 1.3.1 Toll-like receptors (TLRs)

One of the fundamental studies underpinning the biology of toll-like receptors came in 1996 when it was demonstrated that Drosophila carrying a mutation in the "Toll" gene failed to mount effective anti-fungal immunity (Lemaitre et al., 1996). Later studies identified the human and mouse homologues of the "Toll" receptor and murine loss of function mutational studies established the fundamental role of the Toll-like receptors in innate immunity (Medzhitov et al., 1997, Poltorak et al., 1998, Hoshino et al., 1999). Most of the TLRs (TLR1-9) identified thus far are well conserved between human and mouse; TLR10 is functional only in humans whereas TLR11-13 are only present in mice (Akira et al., 2006). TLR-1, -2, -4, -5, -6, and -11 are expressed as cell surface receptors whereas TLR-3, -7, -8, and -9 are expressed in intracellular vesicles and can detect nucleic acids.

Structurally, the cell surface TLRs are type I transmembrane receptors with an N-terminal extracellular domain (required for PAMP recognition), a transmembrane domain and a leucine-rich repeat intracellular domain (required for adaptor protein recruitment and downstream signalling). The cytoplasmic domain is also called the TIR [Toll-interleukin-1 (IL-1) receptor] domain in recognition of its high homology to the cytoplasmic domain of the IL-1 receptor. Signalling through the TLRs can be either myeloid differentiation primary response gene 88 (MyD88)-dependent, or MyD88-independent, depending on the TLR involved and the adaptor proteins recruited. Ultimately, activation of the TLRs leads to induction of expression of cytokines, chemokines and interferons depending on the TLR involved.

### 1.3.2 MyD88-dependent TLR signalling

The MyD88 pathway is adopted by all the TLRs identified to date. The C-terminal domain of the MyD88 protein has a TIR domain whereas the N-terminal portion contains a death domain. This allows the C-terminal domain of MyD88 protein to form a homotypic association with the TIR domain of TLRs. Upon ligand binding, MyD88 recruits IRAK (IL-1 receptor-associated kinase) through its death domain and consequently activates the Janus Kinase (JNK) and nuclear factor kappa B (NF-kB) pathways.

### 1.3.3 MyD88-independent TLR signalling

The MyD88-independent pathway is used in TLR4 signalling as well as TLR3 signalling pathways (Kawai et al., 2001, Kaisho et al., 2001, Alexopoulou et al., 2001). Numerous findings indicate that MyD88-independent TLR4 signalling leads to the induction of interferon regulatory genes through the interferon regulatory factor-3 (IRF-3)-IFN-ß-Stat1 pathway (Doyle et al., 2002, Toshchakov et al., 2002, Hoshino et al., 2002). TLR3 signalling has also been shown to induce NF-*k*B as well as IFN-inducible genes through IRF-3 using two adaptor proteins TANK-binding kinase 1 (TBK1) and inhibitory kappa kinase (IKK*ε*/IKK*ι*) (Sharma et al., 2003, Fitzgerald et al., 2003).

### 1.3.4 Toll-like receptor 4 (TLR4)

TLR4 belongs to the class of cell surface TLRs that also includes TLR-1, -2, -4, -5, and -6. They detect presented microbial products such as lipids (TLR1), lipoprotein (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5) and lipoproteins (TLR6). Most of these TLRs have been demonstrated to homodimerise, or heterodimerise, upon ligand binding so as to recruit the requisite adaptor proteins for subsequent downstream signalling. TLR4 was the first member to be discovered as the receptor for lipopolysaccharide (LPS), which precipitated septic shock in Gram-negative bacterial infection (Akira et al., 2006). Expression of TLR4 has been demonstrated on most leukocytes, platelets, epithelial, parenchymal endothelial and other endothelial cells (Visintin et al., 2001, Muzio et al., 2000, Menzies-Gow et al., 2002, Sabroe et al., 2002, Abreu et al., 2003). TLR4 signalling in response to LPS requires formation of a complex with serum LPS-binding protein (LPB), membrane-bound CD14 and MD-2 (Shimazu et al., 1999, Viriyakosol et al., 2001, da Silva Correia et al., 2001). Downstream TLR4 signalling may be either MyD88-dependent or MyD88-independent.

### 1.3.5 Toll-like receptor 7 (TLR7)

TLR7, along with TLR-3, -8 and -9 are expressed intracellularly and are therefore important for detection of viral nucleic acids that are presented via the endosomal pathway. TLR7 is expressed in both mice and humans in the spleen, lung and placenta. TLR8, which is structurally similar to TLR7, is however only functionally expressed in humans in lungs and monocytes (Jurk et al., 2002).

Both TLR7 and TLR8 naturally recognize single stranded RNA (ssRNA) viruses such as influenza, Sendai, and Coxsackie B, usually after the virus (either replicating or non-replicating viruses) has been internalized into endosomes (Heil et al., 2004, Diebold et al., 2004, Lund et al., 2004, Triantafilou et al., 2005). Interaction of TLR7 and/or TLR8 with ligand generates MyD88-dependent signalling leading to either induction of pro-inflammatory cytokines and chemokines through the nuclear factor-kB (NF-kB) pathway, or interferons through the IRF7 pathway.

It has also been shown in both TLR7 deficient mice, and HEK 293 cells transfected with human TLR7, that TLR7 recognizes the imidazoquinoline compounds, imiquimod and resiquimod as well as loxoribine (a guanosine analog) (Hemmi et al., 2002, Lee et al., 2003).

## 1.4 Pattern recognition receptor (PRR) expression on Haematopoietic stem and progenitor cells

It is an established fact that innate immune cells such as neutrophils, monocytes, dendritic cells and macrophages sense the presence of invading pathogens through intracellular or extracellular PRRs. The timing for the TLR acquisition during haematopoietic stem and progenitor cell differentiation, and any effect they may exert on lineage specification, was however not investigated until recently. Recent data, from both human and murine models, have demonstrated that HSPCs also express TLRs at both gene and protein levels. Haematopoietic stem cells (which were defined as $Lin^-Sca^+Kit^+Flk^-$), MPP ($Lin^-Sca^+Kit^+Flk^+$) and other progenitors were demonstrated to express functional, cell surface TLR2, and TLR4 as well as TLR-related molecules CD14 and MD-2 by flow cytometry (Nagai et al., 2006). Nagai et al. further demonstrated in *in vitro* cultures that LPS and $Pam_3CSK_4$ (ligands for TLR4 and TLR1/2 respectively) were able to stimulate MyD88-dependent differentiation of haematopoietic progenitors [i.e., common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs) and granulocyte-macrophage progenitors (GMPs)] in the absence of haematopoietic growth factors. Additionally, *in vivo* LPS administration was demonstrated to bind TLR4 on bone marrow HSPCs. There has also been another report showing constitutive expression of functional TLR9 in human CD34+ haematopoietic progenitors that could mediate MAPK-induced

signalling upon CpG oligodinucleotide ligation (Kim et al., 2005). Moreover, another recent report also demonstrated the expression of functional TLR2, TLR4 and TLR9 on common dendritic progenitors (CDP) and that TLR9 ligation led to down-regulation of CXCR4, but up-regulation of CCR7, resulting in increased CDP migration to lymph nodes and subsequent differentiation into dendritic cells (Schmid et al., 2011). Another report demonstrated that human HSPCs express functional TLR-1, -2, -3, -4 and -6 and further showed that $Pam_3CSK_4$ (TLR1/2 ligand) instructed HSC commitment to myeloid differentiation (while inhibiting B-lymphoid fate commitment) through upregulation of the transcription factors GATA-1, C/EBPα and PU.1 (De Luca et al., 2009). A more elaborate study published recently provided unequivocal evidence that TLRs on HSPCs directly sense their respective ligands (Megias et al., 2012). The experimental design involved transplantation of WT HSPC cells into $TLR2^{-/-}$, $TLR4^{-/-}$, or $MyD88^{-/-}$ mice prior to injection of soluble $Pam_3CSK_4$, LPS, or ODN respectively. Data from that report indicated that TLR ligands stimulated the production of inflammatory macrophages from the transplanted cell demonstrating that the TLR receptors on the transplanted HSPC directly recognized their injected ligands.

In addition to TLRs, human bone marrow $CD34^+$ haematopoietic progenitor cells were demonstrated to express NOD2 receptors. Subsequently, muramyl dipeptide induced the formation of $CD11c^+$ myeloid cells from human $CD34^+$ HPCs (Sioud and Floisand, 2009). These and many other findings suggest that, in the context of inflammation, TLR ligation may replace and/or act in concert with endogenous cytokines in mediating HSPC proliferation and differentiation to meet the immediate immunological need by mechanisms that have yet to be unravelled. It is also reasonable to hypothesise that HSPC may be directly involved in the inflammatory response to infection through their functional PRRs.

## 1.5 Haematopoiesis during development

The emergence of the haematopoietic system in vertebrates occurs in two distinct waves: primitive haematopoiesis, largely concerned with production of erythroid cells to provide oxygen to the developing embryo as well as myeloid cells, and definitive haematopoiesis capable of producing all myeloerythroid and lymphoid cells of the adult haematopoietic system (Palis, 2008). Though the site

of primitive haematopoiesis is well established, the anatomical site of emergence of definitive haematopoiesis is very much debated.

### 1.5.1 Primitive haematopoiesis

Primitive haematopoiesis in the mouse begins between embryonic day (E) 7 and E7.5 as a result of an ingression of the extra-embryonic mesoderm through the posterior primitive streak and ultimately haematopoietic differentiation in the yolk sac (Silver and Palis, 1997). Using *in vitro* assays, it was experimentally shown that the yolk sac indeed contained clonogenic myeloid progenitors (colony forming units-culture, CFU-C), adult type definitive haematopoietic stem cells (dHSC) and colony forming units-splenic (CFU-S) (Moore and Metcalf, 1970). However, later work using transplantation of yolk sac derived cells to irradiated murine hosts found that yolk sac lacked CFU-S prior to E9.5 and dHSC prior to E11.5 (Muller et al., 1994, Medvinsky et al., 1993, Medvinsky et al., 1996).

### 1.5.2 Definitive haematopoiesis

It has been proposed that dHSC originate *de novo* from an intra-embryonic tissue region called the aorta-gonad-mesonephros (AGM) region at E9.5 (Muller et al., 1994, Medvinsky et al., 1993, Medvinsky et al., 1996). This supposition, that adult haematopoiesis has an intra-embryonic origin, is also supported by earlier findings in avian models (Dieterlen-Lievre, 1975, Cormier and Dieterlen-Lievre, 1988). Using *in vivo* transplantation assays, another study has demonstrated that the *de novo* AGM HSC induction is restricted to the ventral tissues of the AGM (Peeters et al., 2009). That study further implicated Hedgehog proteins as being critical in this dHSC generation from the AGM. In contrast to the studies cited above, one recent report that adopted lineage-tracing through induction of stage/tissue specific recombination events questioned the intra-embryonic origins of dHSC and suggested yolk sac origins of dHSC (Samokhvalov et al., 2007). Interestingly, another subsequent report that used the same mutant mouse failed to corroborate that finding but suggested shear stress in the AGM, caused by heart beat-induced blood flow, as a requirement for the *de novo* generation of dHSC in the AGM (Adamo et al., 2009). The Adamo et al. study evaluated the effect of fluid shear stress on the gene expression profile and haematopoietic potential of *in vitro* cultured embryoid body-derived cells. They

demonstrated that fluid shear stress increased the expression of *Runx1* [essential for haematogenic endothelium cell specification towards haematopoietic lineage (Lancrin et al., 2009)], Flk1$^{+}$(indicating the formation of haemangioblast), *Myb* [a marker for haemogenic endothelium (North et al., 2002)], and *Klf2* [a mechano-activated gene known to stimulate erythropoiesis (Basu et al., 2005)]. Subsequent *in vitro* haematopoietic colony-forming assays substantiated these findings that abrogation of fluid shear stress perturbed the haematopoietic potential of the AGM.

There has also been an on-going question regarding the lineage of the cells within the AGM from which dHSC are derived. Initially, it was proposed that haematopoietic stem cells and endothelial cells both originated from a unique bi-potential cell population called the haemangioblast. Several lines of evidence from *in vitro* blast colony assay using ES-cells, which identified haemangioblasts, supported this concept (Choi et al., 1998, Kennedy et al., 2007, Keller, 2005). Later studies however, led to the proposition of haematogenic endothelium as the common ancestral cell for both haematopoietic and endothelial cells. This concept proposes that haematopoietic stem cells originate from intra-aortic endothelial cells in the embryo. Evidence for this concept came from experiments that showed that the first dHSC expressed both haematopoietic and endothelial cell surface markers (North et al., 1999, Sanchez et al., 1996, North et al., 2002). Studies using live imaging in Zebrafish embryos, and slices of mouse AGM, have provided further unequivocal evidence of the haematopoietic stem cell emergence from the haematogenic endothelium (Bertrand et al., 2010a, Boisset et al., Kissa and Herbomel, 2010). Recent publications have however reconciled the two concepts, i.e. the haemangioblast and haematogenic endothelial origins of haematopoietic stem cells, by demonstrating that under the transcriptional influence of the stem cell leukaemia (*Scl*) gene, Flk$^{+}$ haemangioblasts generate haematogenic endothelial cells which subsequently differentiate into haematopoietic stem cells, or structural endothelial cells, under the transcriptional regulation of Runx1 and HoxA3 respectively (Iacovino et al., 2010, Lancrin et al., 2009).

Thus, the currently accepted dogma is that dHSC originate *de novo* in the AGM even prior to the establishment of circulation since *in vitro* cultured E8.0 para-aortic splanchnopleura (which later forms the AGM) generates multilineage

progenitors, although the yolk sacs of both mouse and human generates only myeloerythroid progenitors (Tavian et al., 2001, Cumano et al., 1996). In addition, transplantation of *in vitro* cultured E8.0 mouse para-aortic splanchnopleura cells into Rag2γC$^{-/-}$ recipients mice generated long-term multilineage reconstitution (lymphomyeloid cells), whereas the same transplantation assay using cultured yolk sac cells generated only short term myeloerythroid cells (Cumano et al., 2001). A summary of the developmental pathway leading to specification of adult type HSCs is illustrated in figure 1-2 below.

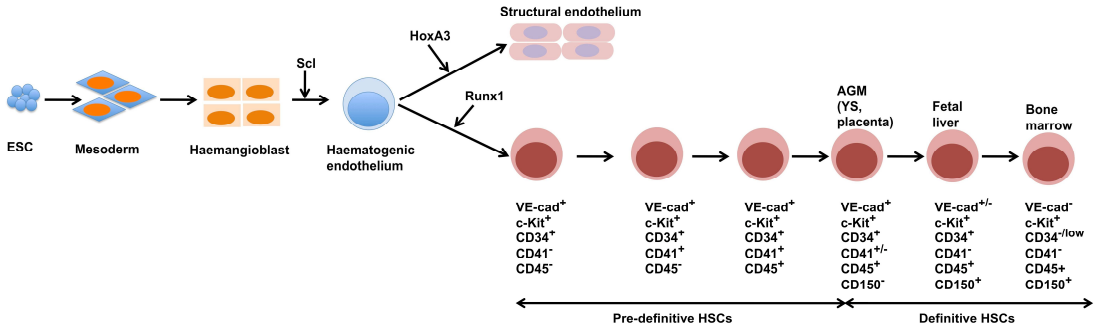



**Figure 1-2: A schematic representation of the developmental pathway towards the emergence of definitive HSC from embryonic stem cells.**
Shown are some of the critical transcription factors and cell surface antigenic expression that mark specific stages of haematopoietic stem cell ontogeny. Scl; stem cell leukaemia: ESC; embryonic stem cell. [Adapted from: (Medvinsky et al., 2011, Lancrin et al., 2009, Iacovino et al., 2010, North et al., 2002)].

## 1.5.3 Sites for embryonic haematopoiesis

In addition to the yolk sac and the AGM that are haematopoietic in the embryo, several other organs have been shown to harbour haematopoietic stem cells and to have haematopoietic activity. These include the placenta, umbilical cord, liver and the spleen. There is an on-going debate as to whether the placenta and the umbilical cord are also sites for *de novo* dHSC generation. What has been established is that dHSC from the AGM (and possibly the placenta and the umbilical cord) colonize the foetal liver between E11.5-E12.5 in the mouse (Medvinsky et al., 2011). Although foetal liver HSC eventually seed the bone marrow at ~E16-17 (Morrison et al., 1995), there are some inherent differences between the two HSC populations. Murine foetal liver HSC express distinct cell surface markers such as Mac-1 and AA4.1 (Jordan et al., 1995, Morrison et al., 1995), and generate other unique cell populations such as Ly-1$^{+}$ B-1a B cells (that form most of the B cells in the newborn) and CD4$^{+}$CD3$^{-}$ lymphotoxin β$^{+}$

integrin α4β7$^{+}$ which are most likely follicular dendritic cell precursors (Ikuta et al., 1990, Havran and Allison, 1988, Mebius et al., 1997). In addition, unlike adult HSCs, foetal HSCs have been shown to be actively dividing, with foetal liver providing the microenvironment for these expanding HSCs, and these cells show robust reconstitution potential in irradiated hosts compared to bone marrow counterparts (Holyoake et al., 1999, Harrison et al., 1997). Moreover, whereas seeding of foetal liver with HSC does not require a functional CXCR4-CXCL12 axis, this axis is a necessity for bone marrow colonization by adult HSC (Kawabata et al., 1999).

In the human foetus, however, the yolk sac forms at about day 13 post gestation and haematopoiesis begins in the yolk sac by day 16 (Prindull, 1992). HSPC later seed the liver after blood vessel formation and, beginning at 5-6 weeks of gestation, undergo an exponential increase in numbers as a result of intense proliferation with minimal differentiation in the foetal liver (Migliaccio et al., 1986, Hann et al., 1983). Beginning from the 6$^{th}$ week of gestation, the liver predominantly becomes a site of erythropoiesis, although evidence suggests that myelopoiesis, lymphopoiesis and megakaryocyte production are also detectable (Prindull, 1992). Between 4-5 months of pregnancy, the foetal bone marrow becomes a functional haematopoietic tissue (Golden-Mason and O'Farrelly, 2002).

#### 1.5.3.1 Foetal spleen and thymus

In mice, HSC from the foetal liver colonize the foetal spleen and thymus at about E13-14 and initiate haematopoietic activity in these organs until birth (Godin et al., 1999). After birth, the spleen maintains minimal haematopoietic activity in steady state; however, during conditions of stress, its haematopoietic activity can increase considerably. The thymus on the other hand, remains the site of T-cell maturation.

### 1.5.4 Sites for adult haematopoiesis

Beginning from birth, and throughout life in vertebrates, the bone marrow becomes the main site for haematopoiesis in steady state. Other organs like the spleen and the liver retain minimal residual haematopoietic activity in steady

state but become extra haematopoietic sites during conditions of stress on the bone marrow. The ability of the bone marrow to sustain the life-long haematopoietic requirements of organisms is due to a unique class of cells called haematopoietic stem cells (HSC) equipped with inherent self-renewal and differentiation potential.

## 1.6 Haematopoietic stem cells (HSCs).

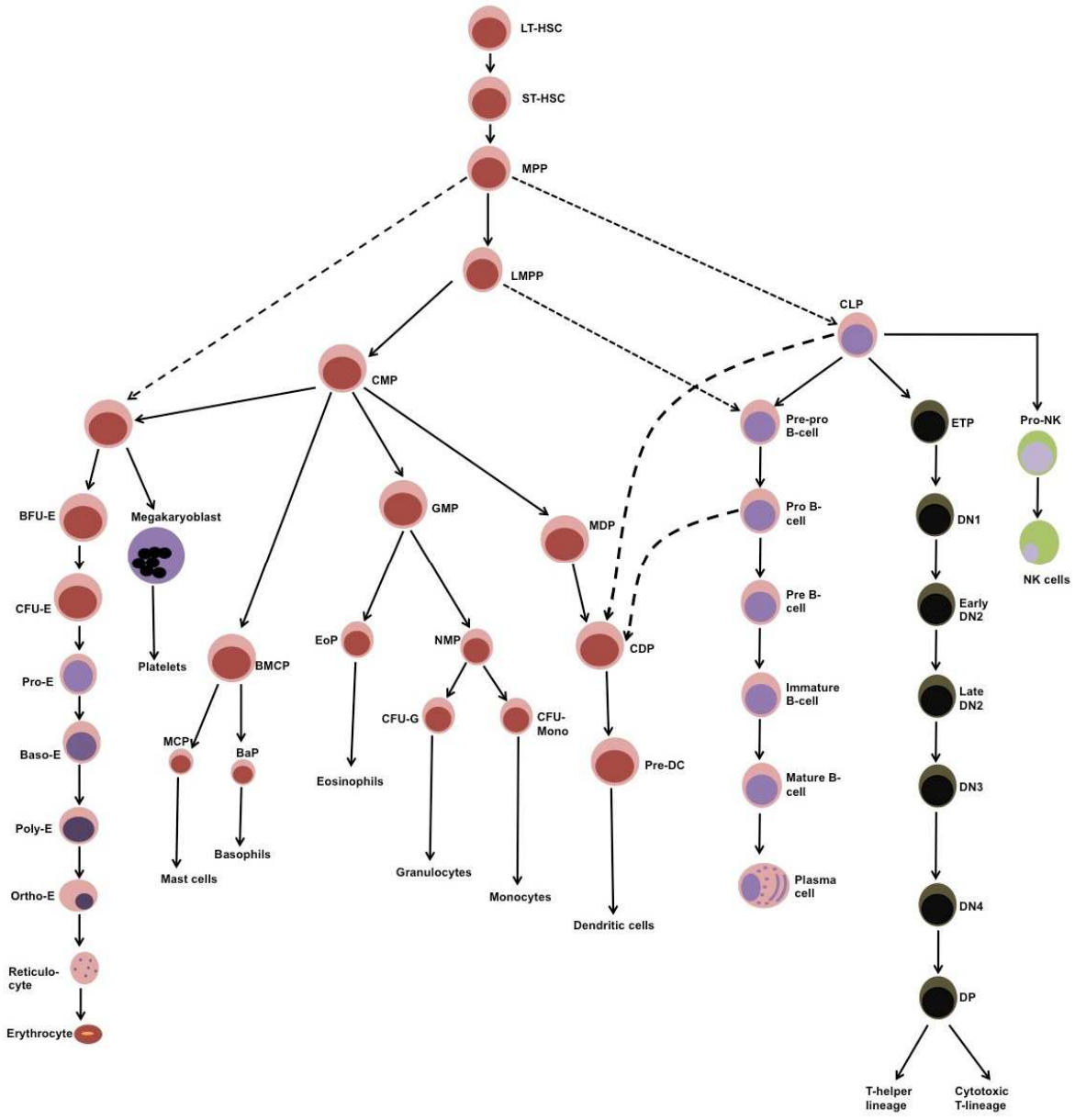



**Figure 1-3: A schematic of the haematopoietic stem and progenitor cell differentiation and lineage commitment pathways.**
Shown are the various lineage choices from the LT-HSC to series of multipotent progenitors which subsequently differentiate to effector cells. Adapted from (Iwasaki and Akashi, 2007, Nutt and Kee, 2007, Naito et al., 2011, Socolovsky, 2007, Nagasawa, 2006, Perry and Soreq, 2002, Friedman, 2007).

In adult mice, HSCs have been detected in bone marrow, spleen and liver whereas in the adult human, they have been detected in the spleen and bone marrow (Wolber et al., 2002, Dor et al., 2006). Functionally, HSC are defined by their ability to reconstitute the entire repertoire of haematopoietic cells i.e., the myeloerythroid and lymphoid lineages, when transplanted into an irradiated host. Symmetric cell division of HSC leads to self-renewal of the HSC. However, the HSC can also undergo asymmetric cell division to give one HSC and short-term haematopoietic stem cells (ST-HSC) and a series of multipotent progenitors (MPP) that finally differentiate to functional terminally differentiated haematopoietic cells (see figure 1-3 above). Whereas the ST-HSC and MPP can sustain haematopoiesis only for a short term (up to 8 weeks), the long term HSC sustain life-long haematopoiesis. Although HSC numbers vary with age, sex and strain of mice as well as the purification protocol adopted, HSCs generally account for about 0.001-0.01% of the nucleated cells in the bone marrow of mice (Challen et al., 2009, Kiel et al., 2005). In spite of the inherent ability of HSCs to undergo proliferation and differentiation to sustain haematopoiesis, it must be emphasized that under homeostatic conditions HSCs are mainly quiescent (Wilson and Trumpp, 2006). Moreover, it has been proposed that the dormant HSC have a high reconstitution potential and divide every 145 days, or 175-350 days, in mouse and human respectively, whereas the active HSC have lower reconstitution ability and divide every 36 days (Catlin et al., 2011, Wilson et al., 2008). In fact, studies have shown that haematopoietic stress caused by infection, cytokines, or chemical agents, may lead to premature exhaustion of HSCs as they push the dormant HSC to exit its quiescent/$G_o$ phase of the cell cycle (Cheng et al., 2000, Hock et al., 2004).

What is evident from the literature is that the current immunophenotypic protocols for identifying HSCs are inadequate because recent findings have demonstrated heterogeneity within the isolated HSC. For example, it was recently shown that based on the SLAM (signalling lymphocytic activation molecule) phenotype ($Lin^-Kit^+Sca\text{-}1^+CD34^-CD48^-CD150^+$), the population previously identified as true HSCs (Kiel et al., 2005) could still be separated based on CD150 expression with the $CD150^{high}$ population representing the most primitive HSC population (Morita et al., 2010). In addition, two recent publications have further demonstrated, through functional repopulation assays,

that within the SLAM phenotype, HSCs are still heterogeneous and can be separated based on c-Kit expression with the c-Kit$^{low/int}$ population having the true stem cell phenotype (Shin et al., 2014, Grinenko et al., 2014). Therefore, more research is needed to fully characterize the HSC phenotype so as to identify the unique population with 'stemness' potential and provide a better understanding of the determinants of the switch from self-renewal to differentiation.

Although, haematopoietic stem and progenitor cells (HSPCs) reside in specialized niches in the bone marrow of adults, it has been estimated that physiologically, ~100-400 HSPCs are in the circulation at any time point in mice (Wright et al., 2001, Goodman and Hodgson, 1962). These HSPCs egress from the bone marrow to blood in response to sphingosine 1-phosphate, circulate to several tissue and re-enter the blood through the thoracic duct and subsequently home back to the bone marrow (Massberg et al., 2007). It is further suggested that these circulating HSPCs upregulate CD47 expression to evade phagocytic activity of innate immune cells such as macrophages (Jaiswal et al., 2009). Other reports also implicate induced CD274 expression by HSPCs as being important in their ability to evade the adaptive arm of the immune system during homeostatic HSPC trafficking (Zheng et al., 2011). Moreover, it must be stated that the homeostatic trafficking of HSPC is under circadian control by the release of noradrenaline, with peak levels being achieved 5 hours after the initiation of light and trough levels 5 hours after the onset of darkness (Mendez-Ferrer et al., 2008). Mendez-Ferrer et al. demonstrated that the fluctuation occurred as a result of noradrenaline regulation of CXCL12 protein levels in the bone marrow niche; bone marrow CXCL12 secretion decreasing to lowest levels 5 hour after light initiation (in anti-phase with noradrenaline levels) and peaking 5 hours post darkness initiation.

### 1.6.1 Identification of HSPCs

Haematopoietic stem and progenitor cell identification is increasingly based on a variety of cell surface markers, and biochemical features as described briefly below.

#### 1.6.1.1 Multi-colour flow cytometry

In the mouse, this typically adopts staining protocols that exclude markers for lineage specific cells (CD4, CD5, CD8, B220, Ter119, Mac-1 and Gr-1) but include positivity for c-Kit and/ Sca-1 antigens depending on which primitive population is of interest. It should be noted however that although most of the cell surface markers in mice and human haematopoietic progenitor cell populations are well conserved, there are fundamental differences in the markers used for the phenotypic isolation of mouse and human HSC (see table 1-2). For example, whereas mouse HSCs are CD150+ and CD34- (Kiel et al., 2005), human HSCs are CD150-, but CD34+ (Novelli et al., 1998, Goodell et al., 1997).

**Table 1-2: Immunophenotypic markers used for identifying haematopoietic stem and progenitor cells in mice and humans.**
[Adapted from (Wilson et al., 2007, Doulatov et al., 2012, Adolfsson et al., 2005)

| Marker | Cell type |
|---|---|
| ***Mouse*** | |
| Lin-Sca-1+Kit+ | Haematopoietic stem and progenitor cells (LSK) |
| Lin-Sca-1+Kit+CD34+CD135+CD150- | Multipotent progenitors (MPP) |
| Lin-Sca-1+Kit+CD34+CD48+CD135-CD150+ | MPP1 |
| LSK CD34+CD48+CD135-CD150- | MPP2 |
| LSK CD34+CD48+CD135+CD150- | MPP3 |
| LSK CD34-CD41-CD135+CD150+ | Short-term haematopoietic stem cells |
| LSK IL7rα- | Myeloerythroid progenitors |
| LSK IL7rα+ | Common lymphoid progenitors |
| LSK CD34+CD135+ | Lymphoid-primed MPP (LMPP) |
| LSK IL7rα-CD34+CD16/32- | Common myeloid progenitors (CMP) |
| LSK IL7rα-CD34-CD16/32- | Megakaryocyte-erythrocyte progenitors (MEP) |
| LSK IL7rα-CD34+CD16/32+ | Granulocyte-macrophage progenitors (GMP) |
| LSK IL7rα-CD48-CD150+ | Long-term haematopoietic stem cells (HSC) |
| LSK CD34-CD48-CD135-CD150+ | Dormant HSC |
| LSK CD34+CD48-CD135-CD150+ | Activated HSC |
| **Human** | |
| Lin-CD34+CD38-CD45RA-CD90+CD49f+ | Long-term haematopoietic stem cells |
| Lin-CD34+CD38-CD45RA-CD90-CD49f+ | Multipotent progenitors (MPP) |
| Lin-CD34+CD38-CD45RA+CD10+CD7- | Immature lymphoid progenitors (MLP) |
| Lin-CD34+CD38-CD45RA-CD135+CD10-CD7- | Common myeloid progenitors (CMP) |
| Lin-CD34+CD38-CD45RA-CD135-CD10-CD7- | Megakaryocyte-erythrocyte progenitors (MEP) |

| | |
|---|---|
| Lin-CD34+CD38-CD45RA+CD135+CD10-CD7- | Granulocyte-macrophage progenitors (GMP) |

#### 1.6.1.2 Dye efflux properties

This method of HSC identification is based on the unique ability of HSC to efflux fluorescent vital dyes such as Hoechst 33342. This high dye effluxing activity has been shown to be due to the endowment of the HSCs with higher amount of multi-drug resistant ATP binding cassette transporters (Goodell et al., 1996, Camargo et al., 2006, Zhou et al., 2001). Thus the haematopoietic progenitor population appears as a "side population" i.e., a separate population of cells that are negative for the Hoechst 33342 dye. This protocol is combined with other strategies to identify the HSC population. Species-specific differences in HSPC biology are also manifest concerning the side population protocol since HSPCs in humans are devoid of the side population pattern seen in mice (Pearce and Bonnet, 2007).

#### 1.6.1.3 Functional identification of HSC

*In vitro* clonal culture assays can be used to assess the presence of haematopoietic progenitors in a given cell population as well as their differentiation potential. These *in vitro* assay systems include the liquid and semi-liquid-based media containing combinations of cytokines and growth factors that allow the growth of the progenitor population of interest. Since the permissive conditions allowing the *in vitro* culture of HSC have not been discovered, the many numerous *in vitro* culture systems only give an indication of the presence of haematopoietic progenitors. Some *in vivo* assays such as radioprotection and spleen colony forming unit day-12 (d-12 CFU-S) have also been used to assess progenitor cell function and number (Pallavicini et al., 1997, Kondo et al., 2003). The gold standard for functional assessment of LT-HSC population has been the long-term repopulation assay that assesses the haematopoietic system reconstitution potential of a particular test cell (Harrison, 1980, Harrison et al., 1993, Bhattacharya et al., 2008, Kondo et al., 2003). In this assay, the cell population whose functionality is being tested is intravenously injected into a conditioned host, and then peripheral blood samples are withdrawn at regular intervals to quantify the relative contribution of the transplanted HSC to the lympho-myeloid lineages in the reconstituted

haematopoietic system. The conditioning is assumed to provide “space” in the bone marrow niche for the transplanted HSC (Metcalf, 2007).

## 1.7 Haematopoietic niche

It is generally accepted that HSCs are housed in a unique anatomical microenvironment called the stem cell niche that integrates both intrinsic and extrinsic signals to regulate such processes as self-renewal, differentiation and storage of cycling and quiescent stem cells. The niche concept was proposed by Schofield when he observed that contact between prospective haematopoietic stem cells and cells within their microenvironment was required to sustain stem cell behaviour (Schofield, 1978). Research has substantiated the niche concept for all somatic stem cells. Via secretion of soluble factors such as cytokines, chemokines and extracellular matrix proteins, the bone marrow stromal component of the niche provides the critical microenvironment necessary for sustenance of HSC functions.

Two main niches are proposed for the HSC in the adult bone marrow; the endosteal and vascular niches. It must be stated beforehand that the two niches are probably not mutually exclusive, as more findings seem to suggest that a dynamic interaction takes place in the bone marrow (Kiel and Morrison, 2008). The principal difference between the two proposed niches might be the oxygen content; higher in the vascular niche hypothesized to house mostly active HSCs whereas the endosteal/osteoblastic niche is hypoxic and retains mostly dormant HSCs (Parmar et al., 2007, Venezia et al., 2004).

### 1.7.1 Endosteal niche

Morphological evidence supports the notion that the more dormant HSCs localize to the endosteum in trabecular regions of long bones, whereas more differentiated haematopoietic progenitors are found mainly in the central regions of the bone marrow close to vascular sinusoids (Foudi et al., 2009, Zhang et al., 2003, Lo Celso et al., 2009, Xie et al., 2009). The high $Ca^{2+}$ ion concentration in the bone marrow endosteal surface has been shown to be critical for proper homing and lodging of HSCs to the endosteal niche (Adams et al., 2006) as the HSCs were shown to express Calcium ion receptors (CaR). Cells

lining the endosteal surface of bone, called osteoblasts, have been implicated as an essential component of the HSC niche (Deguchi et al., 1999, Ducy et al., 2000). Other subsequent studies provided evidence for the direct involvement of osteoblasts in maintenance of the HSC niche. For example, in one study, increased constitutive synthesis of osteoblasts was achieved by expressing parathyroid hormone under the type 1 collagen α1 (Col1α1) promoter (Calvi et al., 2003). Since parathyroid hormone is involved in calcium homeostasis, the constitutive increase in calcium levels resulted in increased bone formation and hence increased osteoblasts with a concomitant increase in HSC numbers demonstrating a direct correlation between osteoblasts and HSC numbers. Other studies that conditionally depleted osteoblasts reported impairment of bone marrow haematopoiesis with apparent extramedullary haematopoiesis in the liver and spleen (Visnjic et al., 2004, Zhu et al., 2007). Additional evidence implicating the essential role of osteoblasts in supporting HSCs came from *in vitro* studies that identified many haematopoietic supporting cytokines as being secreted by osteoblastic cell lines (Taichman, 2005, Taichman and Emerson, 1998). Moreover, *in vivo*, osteoblasts express angiopoietin 1 (Ang-1) which interacts with the tyrosine receptor kinase, Tie2, expressed on HSC, in a mechanism that maintains HSC in a quiescent state in the bone marrow (BM) niche (Arai et al., 2004).

In addition, several receptors known to interact with osteopontin (secreted by osteoblasts), like CD44, α4 and α5β1 integrins, have been demonstrated to be expressed on HSC (Scott et al., 2003, Schmits et al., 1997). Gain and loss of function mutation experiments have established that several transmembrane or secreted factors from the osteoblasts such as CXCL12, kit ligand (Zsebo et al., 1990, Broxmeyer et al., 1991), bone morphogenetic protein-4 (Goldman et al., 2009), and annexin II (Jung et al., 2007) are important for proper HSC function *in vivo*. The osteoblasts further secrete other essential haematopoietic growth factors such as GM-CSF, G-CSF, hepatocyte growth factor and IL-6 (Taichman et al., 2001, Taichman et al., 1996).

Recent findings however cast doubt on the exact identity of the cells of the osteogenic lineage involved in HSC niche function. Using conditional deletion of the ribonuclease Dicer 1 in both osteoprogenitors and osteocalcin-positive mature osteoblasts in mice, it was demonstrated that osteoprogenitors, but not

osteocalcin-positive osteoblast, are the main osteogenic niche component (Raaijmakers et al., 2010). In addition, other findings implicate other stromal cells called CXCL12 abundant reticular (CAR) cells as the principal cell type that associate directly with HSC in the niche (Sugiyama et al., 2006). These CAR cells were shown to produce high amounts of CXCL12 that interacts with CXCR4 on HSPCs to retain them in the niche.

Further findings also implicate a rare cell population called nestin$^+$ mesenchymal stem cells, which were shown to have osteogenic, chondrogenic and adipogenic potential as a critical component of the bone marrow niche (Mendez-Ferrer et al., 2010). Colocalization experimental data from Mendez-Ferrer et al. indicated that within the bone marrow, HSC physically associate with nestin$^+$ MSC and that HSC home near these nestin$^+$ MSC upon transplantation. These nestin$^+$ MSC were shown to produce high levels of CXCL12, kit ligand, and angiopoietin, all of which are known to be required for the retention and maintenance of HSC quiescence and self-renewal. Conditional ablation of these nestin$^+$ MSC resulted in ~50% reduction in multipotent progenitors and mobilization of HSCs to the spleen. Further research is thus needed to clarify the lineage relationship between these osteoblasts, CAR, and nestin$^+$ MSC to pinpoint which one(s) is/are the key endosteal niche components.

### 1.7.2 Vascular niche

It is well documented that during bone marrow stress, haematopoiesis occurs in extramedullary sites, such as liver and spleen, which are rich in vascular sinusoids but devoid of osteogenic cells. Numerous experimental findings are shedding light on the role of the vascular compartment in haematopoiesis. Immunofluorescence techniques based on SLAM markers indicated that HSCs (Lin- CD41- CD48- CD150+) localized to sinusoidal endothelial linings in both the bone marrow and spleen (Kiel et al., 2005). This provided evidence that at least the vascular cell compartment provided a microenvironment that harbours HSC. To be recognised as a valid niche, the vascular endothelial cells must demonstrate the ability to independently support the long-term self-renewal potential of HSC. Using both *in vitro* culture, and *in vivo* serial transplantation assays, Butler et al. showed that bone marrow endothelial cells elaborate angiogenesis-related factors that support the expansion of HSC (defined as Lin$^-$

Sca-1$^{+}$Kit$^{+}$CD34$^{-}$CD135$^{-}$), as well as maintenance of its self-renewal potential in a mechanism that involved direct HSPC-endothelial cell contact and Notch signalling (Butler et al., 2010). Remarkably, inhibition of the angiogenic pathway through antibody-mediated blockade of vascular endothelial growth factor receptor 2 (anti-VEGFR-2) and vascular endothelial cadherin (anti-VE-cadherin), led to defective recovery of haematopoiesis in sublethally irradiated hosts (Butler et al., 2010); this series of experiments demonstrated the essential role of vascular endothelial cell-induced angiocrine signalling in the maintenance of HSPC function. These *in vivo* antibody neutralisation data from Butler et al. corroborated the findings from a prior study that phenotypically characterised the bone marrow vascular niche sinusoidal endothelial cells as VE-cadherin$^{+}$VEGFR2$^{+}$VEGFR3$^{+}$Sca-1$^{-}$ (Hooper et al., 2009). In another seminal work in which stem cell factor was deleted in bone marrow endothelial and perivascular cells, HSCs were preferentially depleted (Ding et al., 2012). This suggested two possible scenarios; that in steady state, these cells may secrete SCF that is critical in HSC lodging and/or HSC reside in the perivascular niche.

In addition, stromal cell lines, which have demonstrable support for HSC maintenance, have been isolated from both liver and the spleen (O'Neill et al., 2004, Wineman et al., 1996, Moore et al., 1997). These stromal cells have been shown to have an endothelial phenotype and are used for *in vitro* cultures. Since the liver and the spleen are known to contain HSC and are devoid of osteogenic precursors, these findings indicate that the endothelial compartment can also provide a microenvironment that supports haematopoiesis.

## 1.8 Adult extramedullary haematopoiesis

Extramedullary haematopoiesis refers to all non-bone marrow haematopoiesis and may involve single or multiple lineages. Technically, these include all the haematopoietic activities that occur in the yolk sac, AGM, placenta, liver and spleen during foetal development. In adults, the main sites for extramedullary haematopoiesis are the spleen, lymph nodes and the liver as well as, in some exceptional cases, the skin (Corella et al., 2008). It may thus be a safe assumption to say that extramedullary haematopoiesis principally involves reactivation, in adulthood, of microenvironments that were involved in foetal haematopoiesis. Factors mediating extramedullary haematopoiesis are variable

and include hypoxia-induced increased liver and/or splenic erythropoiesis as a consequence of increased circulating erythropoietin, primary myelofibrosis as well as infection-mediated haematopoietic activity in extramedullary sites (Bozzini et al., 1970, Thiele et al., 1990). In the broader sense, extramedullary haematopoiesis could also refer to processes involved in maturation of haematopoietic progenitor cells that migrate from the bone marrow to organs such as the thymus, spleen and other lymphoid organs.

Recent findings are shedding light on the potential mechanisms that may underlie extramedullary haematopoiesis. One such experiment using FOXP3-null mice implicated GM-CSF and IL-3 from overly activated T-cells as the cause of the increased myelopoietic activity in the spleen and liver (Lee et al., 2009). This experiment thus established that the functions of $T_{regs}$ are important in controlling extramedullary haematopoiesis in steady state. Others have also shown through antibody-based depletion of NK cells, that NK cells may be a negative regulator of extramedullary myelopoiesis (Hansson et al., 1988). In addition others, using infection models, have also shown that lipopolysaccharide (TLR4 ligand) and $Pam_3CSK_4$ (TLR2 ligand) can directly signal through their cognate receptors to mediate extramedullary haematopoietic activities (Nagai et al., 2006). Interestingly, findings from that paper indicated that these TLRs could reprogramme lymphoid progenitors to differentiate into myeloid cells.

### 1.8.1 Spleen

Historically, the adult spleen is known to contribute to erythropoiesis, but also become fully haematopoietic in conditions of bone marrow stress (Yanai et al., 1991). The spleen in adult mice has been shown to contain a rare population of HSC that are capable of long-term reconstitution potential in lethally irradiated host (Tan and O'Neill, 2009, Wolber et al., 2002). Similarly to the medullary hypoxic endosteal niche, the splenic sinusoidal endothelium in the red pulp is estimated to provide the hypoxic microenvironment that supports HSC expansion and proliferation during extramedullary haematopoiesis (Tavassoli and Weiss, 1973). Recent findings suggest that the endogenous splenic HSPC may differentiate to give a distinct tissue specific antigen presenting cell type called L-DC (dendritic-like cells) (Tan and O'Neill, 2009) during homeostatic conditions. It has further been suggested that interaction of stroma of the spleen with this

haematopoietic precursor may be critical in influencing its differentiation potential as bone marrow HSCs seeded on splenic stroma in *in vitro* cultures also give rise to L-DC (Tan et al., 2010).

### 1.8.2 Liver

The adult liver harbours HSC (Taniguchi et al., 1996, Watanabe et al., 1996), and maintains minimal erythropoietic and myelopoietic activities in steady state. Some studies have shown that the quantities of HSC in adult human liver are comparable to those of the bone marrow although similar studies in mice found murine liver to contain about 50% of murine bone marrow HSC (Crosbie et al., 1999, Taniguchi et al., 1996). Again, in humans, it has been shown that the majority of differentiating bone marrow HSCs ($CD34^+CD38^+$ HSC) expresses the myeloid marker CD33 in contrast to only about 5% of hepatic HSCs (Golden-Mason et al., 2000). Thus it is not surprising that the adult liver has been found to be the maturation site for some populations of unconventional T-cell such as $CD4^-CD8^-$ double negative cells and γδT cells (Golden-Mason and O'Farrelly, 2002). Others have also demonstrated that the adult liver is an active lymphopoietic organ in both humans and mice (Kawamura et al., 1999, Doherty et al., 1999, Norris et al., 1998). Taken together with other findings that the adult liver produces IL-7 (Golden-Mason et al., 2001) and IL-15 (Golden-Mason and O'Farrelly, 2002); two cytokines which have been demonstrated to be indispensable for extra-thymic T-cell development (Ohteki et al., 1997, Laky et al., 1998), it is fair to say that the adult liver provides a microenvironment capable of sustaining haematopoietic activity.

## 1.9 Not all adult haematopoietic cells are from bone marrow HSC

Increasingly, there is evidence showing that some tissue-specific macrophages are endowed with stem cell-like self-renewal capabilities and are able to proliferate to meet local demands. These tissue macrophages have been shown to originate from embryonic progenitors in the yolk sac or foetal liver, have stem cell-like quiescence in homeostasis and self-renew independently of bone marrow HSC in demand-driven conditions (Schulz et al., 2012, Ginhoux et al., 2010, Hoeffel et al., 2012). Most of the experimental evidence supporting the

alternative developmental origin of these tissue macrophages came from lineage tracing experiments where genetic approaches were used to trace the fate of progenitors using specific lineage markers. In one such experiment, Cre-recombinase was expressed under the control of the chemokine receptor, CX3CR1, which is expressed in monocytic progenitors, but not most mature macrophages, and thus allowed the labelling and subsequent tracking of all such progenies. (Yona et al., 2013). It became clear that although some peritoneal macrophages were labelled, others like Kuppfer cells, Langerhans, alveolar or splenic macrophages were not labelled indicating that these had origins other than bone marrow derived monocytic progenitors. In other experiments that used pulse-labelling of primitive haematopoietic cells at E7.5 (mainly macrophages and erythroid cells) with Runx-Cre also demonstrated that ~30% of adult brain microglia cells were labelled, thus indicating their embryonic origin, since adult type HSC are established after E8-8.5 in the AGM (Ginhoux et al., 2010). Other data came from disruption of definitive haematopoiesis through genetic inactivation of the transcription factor c-Myb, which although is absolutely necessary for definitive haematopoiesis, it is dispensable for primitive haematopoiesis (Schulz et al., 2012). It was demonstrated in those experiments that besides adult brain microglia, other tissue macrophages emerge despite the abrogation of definitive haematopoiesis.

The consensus from these and many other experiments is that, these tissue specific macrophages are derived from the embryonic/primitive haematopoiesis, migrate to these tissues where they undergo extensive proliferative expansion during embryonic development but switch into quiescence after birth (Chorro et al., 2009, Murphy et al., 2008, Merad et al., 2002, Kierdorf et al., 2013).

## 1.10 Haematopoietic lineage specification

As shown in figure 1-4, haematopoietic stem cells undergo differentiation into multipotent, and a series of increasingly lineage-restricted, progenitors and ultimately terminally differentiated mature cells. This occurs under the influence of both extrinsic (growth factors, cytokines and other signalling effector molecules) and intrinsic (cell-intrinsic transcriptional, chromatin remodelling and other epigenetic regulatory mechanisms) cues that may be selectively activated, or de-activated, to favour a specific lineage. It has been

shown that HSCs express low levels of key master myeloerythroid transcription factors (but not lymphoid-priming factors) which were suggested to induce a myeloerythroid priming state in the HSCs and whose enforced expression may skew the lineage fate/differentiation pathway adopted (Traver and Akashi, 2004, Laiosa et al., 2006, Miyamoto et al., 2002). In line with its proposed B, T and GM, but not MkE (megakaryocyte erythrocyte progenitors), potential the LMPP (lymphoid-primed multipotent progenitor) population has also been shown to co-express genes specific for lymphoid lineage (Rag1 and IL-7) as well as GM lineage (Mansson et al., 2007, Adolfsson et al., 2005) differentiation. Numerous genetic approaches that used loss-of-function, or gain-of-function, mutation have increased our appreciation of the unique roles played by specific transcription factors. It must be noted however that evidence suggests that even committed progenitors still retain some level of lineage plasticity as they can be reprogrammed into a different lineage through forced expression of another lineage specific transcription factor. In line with this it has been demonstrated that enforced C/EBPα, or GATA-1, or GATA-2, expression can reprogramme CLP (common lymphoid progenitors) into GM, MkE and mast cell lineage cells respectively (Iwasaki et al., 2006, Hsu et al., 2006, Iwasaki et al., 2003). This perhaps offers some insight into the molecular mechanisms that skew haematopoietic output during inflammatory and diseased states.

### 1.10.1 Transcriptional control of lineage specification

The influence that a transcription factor exerts on lineage fate of HSPCs depends on its dosage, quantity (up-regulated or down-regulated) as well as the context (i.e. presence and/or absence of antagonistic transcription factors) and timing of its production (DeKoter and Singh, 2000, Iwasaki et al., 2005a, Iwasaki et al., 2006). The LT-HSCs have been shown to express low levels of myeloerythroid transcription factors such as PU.1, C/EBPα (CCAAT/enhancer-binding protein α), and GATA-2 (Tsai and Orkin, 1997, Miyamoto et al., 2002). These transcription factors have been suggested to be important for self-renewal of HSCs since post-natal disruption of PU.1, or C/EBPα, led to rapid loss, and expansion, of HSCs respectively (Zhang et al., 2004a, Iwasaki et al., 2005b). A brief overview of some of the major transcription factors regulating aspects of haematopoietic stem and progenitor cell differentiation is depicted in figure 1-4

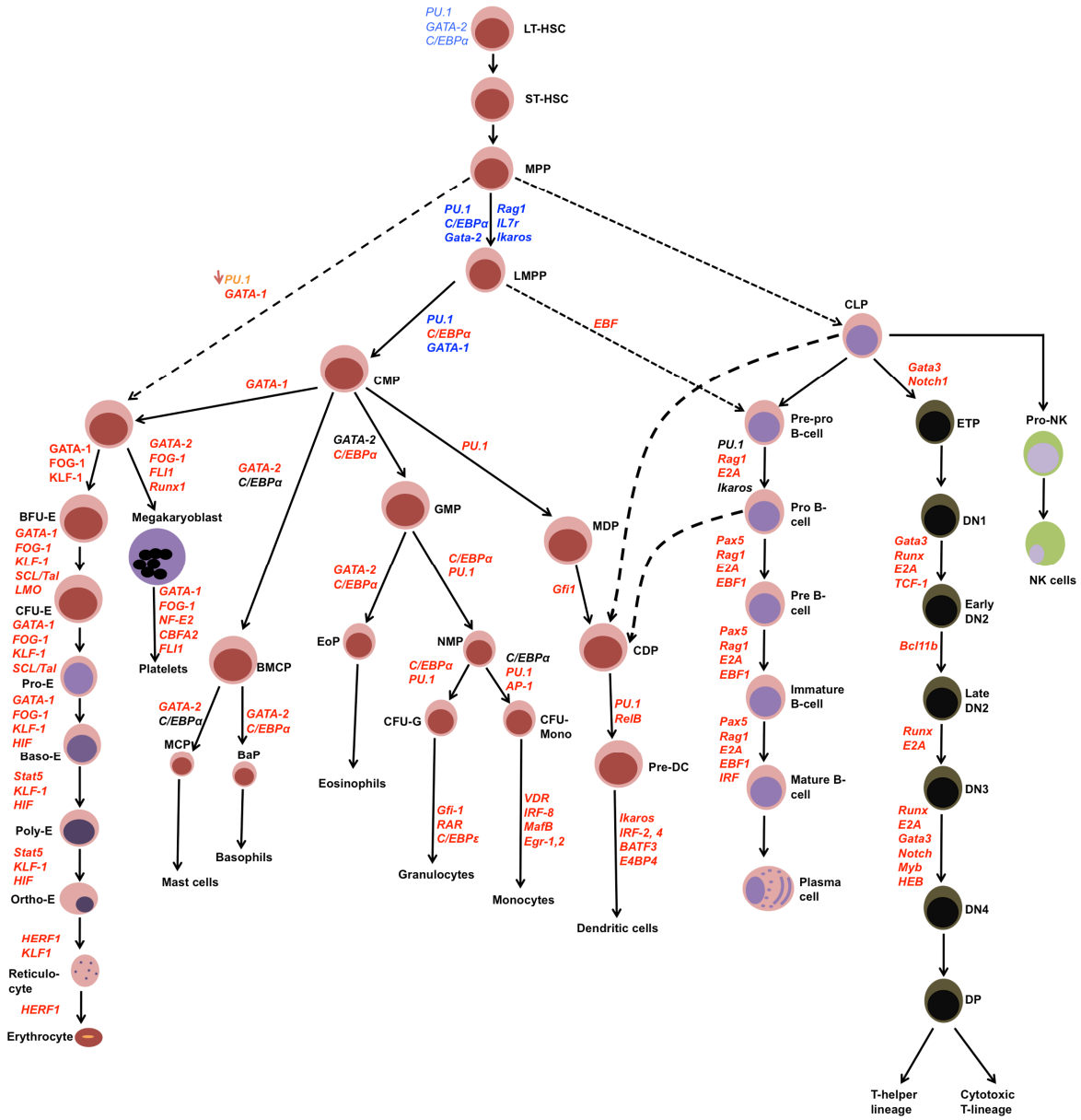



**Figure 1-4 Haematopoietic stem and progenitor cell differentiation and lineage commitment pathway.**
Indicated are some of the transcription factors that prime (in blue), induce (in red) or repress (in black) lineage commitment at specific stages in the pathway. Adapted from (Iwasaki and Akashi, 2007, Nutt and Kee, 2007, Naito et al., 2011, Socolovsky, 2007, Nagasawa, 2006, Perry and Soreq, 2002, Friedman, 2007).

### 1.10.1.1 Granulocyte and monocyte transcription factors

Co-operativity between the transcription factors PU.1 and C/EBPα is necessary for inducing CMP specification from the LMPP. Traver et al. demonstrated C/EBPα expression in HSC (low priming levels), CMP and GMP but not CLP and MEP progenitors (Traver et al., 2001). Conditional deletion of C/EBPα in adult mice, as well as gene knockout mice ($C/EBP\alpha^{-/-}$), both led to a block of CMP to GMP transition thus substantiating the importance of C/EBPα for myelopoiesis (Zhang et al., 2004a, Heath et al., 2004). The expression of C/EBPα has been demonstrated to increase in downstream myeloid progenitors to preferentially

specify granulocytic lineage over monocytic lineage differentiation (Radomska et al., 1998, Cheng et al., 1996). Interestingly, recent findings suggest that the emergency granulopoietic response mounted against infectious and inflammatory conditions is under the transcriptional control of CEBP/ß (Hirai et al., 2006, Satake et al., 2012). Clearly, a model thus emerges in which CEBP/α transcriptionally regulates steady state granulopoiesis, whereas CEBP/ß regulates emergency granulopoiesis (Manz and Boettcher, 2014). Expression of C/EBPε and Gfi-1 (growth factor independent 1) are necessary for terminal granulocyte differentiation and development of secondary granules (Yamanaka et al., 1997a, Yamanaka et al., 1997b, Lekstrom-Himes et al., 1999).

PU.1 on the other hand is more broadly expressed in HSC (low priming levels), and shows higher levels in MPP, LMPP, granulocytic and monocytic cells. It is also expressed in CLP, B- and T-lymphoid progenitors but present at lower levels in MEP (Klemsz et al., 1990, Chen et al., 1995). Studies using PU.1 null mice have shown that these mice have markedly reduced CLP and GMP but increased MEP at the progenitor level as well as lack of B cells and monocytes at the effector cell level (Iwasaki et al., 2005b, Scott et al., 1994). Downstream effects of increasing PU.1 levels include preferential induction of monopoiesis (over granulopoiesis) in cooperation with the Egr-2 transcription factor as well as repression of GATA-1 and thus erythroid specification (Dahl et al., 2003, Rekhtman et al., 1999, Rekhtman et al., 2003, Stopka et al., 2005).

In another study, conditional deletion of PU.1 in haematopoietic progenitors showed it to be absolutely required for dendritic cell (DC) generation, probably through its regulation of Flt3 expression (Carotta et al., 2010). The emergence of DC is unique, as it has been shown that DC can be generated from both CMP and CLP as shown in figure 1-4.

#### 1.10.1.2 Megakaryocyte and erythrocyte lineage commitment transcription factors

Increased expression of GATA-2 and its subsequent interaction with Friend of GATA-1 (FOG-1) induce repression of PU.1 resulting in emergence of MEP (megakaryocyte-erythrocyte progenitors) progenitors from multipotent progenitors (Stachura et al., 2006). Up-regulation of GATA-1 expression leads to

displacement of GATA-2 from its binding to FOG-1 and differentiation of MEP to Burst forming unit, erythroid, (BFU-E) (Welch et al., 2004). The N-terminal region of GATA-1 has been shown to be necessary for binding of coactivators such as FOG-1 (Trainor et al., 1996, Crispino et al., 1999). The GATA-1:FOG-1 complex is necessary for the induction of an open chromatin loop, ß-globin gene synthesis and c-kit downregulation in downstream erythroblast differentiation (Welch et al., 2004, Vakoc et al., 2005). GATA-1 has also been shown to induce proliferation, and survival, of erythroblasts through up-regulation of the Bcl-$_{xL}$ anti-apoptotic gene (Gregory et al., 1999, Weiss et al., 1994).

In addition, microarray studies have demonstrated a critical role for KLF1 (Kruppel-like transcription factor 1) in repressing FLI-1 (friend leukaemia integration 1) and other critical megakaryocytic lineage genes in MEP and thus specifying an erythroid differentiation programme in MEP (Frontelo et al., 2007, Siatecka et al., 2007). KLF-1 has been shown to be necessary for both primitive and definitive erythropoiesis as well as for regulating genes required for haeme, and erythrocyte cytoskeletal protein biosynthesis (Drissen et al., 2004, Hodge et al., 2006, Nuez et al., 1995, Nilson et al., 2006). Additionally, KLF-1 has been demonstrated to be necessary for γ-(foetal) to ß-(adult) haemoglobin switching during erythropoietic ontogeny (Donze et al., 1995, Wijgerde et al., 1996).

Sustained GATA-2 and FOG-1 expression in MEP together with up-regulation of FLI-1 (Friend leukaemia integration 1) has been shown to induce megakaryocytic lineage fate choice. Gain-of-function and loss-of-function studies have demonstrated that KLF-1 and FLI-1 act as mutually antagonistic fate determinants for MEP precursors (Bouilloux et al., 2008, Klimchenko et al., 2009, Tallack and Perkins, 2009, Starck et al., 2010). FLI-1 is required for the expression of critical megakaryocyte genes such as glycoprotein (Gp) 1ba, Gp9, Mpl (thrombopoietin receptor) and platelet factor 4 (Wang et al., 2002, Pang et al., 2006). It has been shown that GATA-1 is required for downstream megakaryoblast terminal differentiation to platelets as GATA-1 null mice, and humans with GATA-1 point mutations, have thrombocytopenia and defective platelets (Shivdasani et al., 1997, Nichols et al., 2000, Freson et al., 2001).

In addition, the transcription factor nuclear factor erythroid 2 (NF-E2) has been implicated as being necessary for inducing pro-platelet formation and platelet

release during megakaryoblast terminal differentiation (Shivdasani et al., 1995, Lecine et al., 1998).

#### 1.10.1.3 Lymphoid transcription factors

Transcription factors mediating MPP to LMPP transition have yet to be defined. However, the emergence of CLP from LMPP has been demonstrated to coincide with increased IL7rα expression on the LMPP population (Kang and Der, 2004). Evidence from published data suggest that expression of the E2A transcription factor and induction of IL7r-STAT5 signalling results in the induction of EBF1 (early B-cell factor 1) expression in CLP and thus skews its differentiation towards the B-lineage precursor (Roessler et al., 2007, Dias et al., 2005, Kikuchi et al., 2005, Seet et al., 2004). EBF1 then induces expression of the B-lineage commitment factor, Paired box 5 protein (Pax5), leading to the differentiation of Pro-pre-B cells to the Pre-B cells (Roessler et al., 2007). Both Pax5 and EBF1 were shown to establish positive feedback regulatory loops that amplify their combined effect to sustain B cell differentiation. Thus, both EBF1, and E2A, act synergistically to induce a B-lineage specification programme in CLP, whereas Pax5 acts as the B-lineage commitment factor (see figure 1-4). Pax5 is stably expressed from the pro-B cell stage until terminal differentiation and is finally down-regulated in plasma cells (Fuxa and Busslinger, 2007). Pax5 has been demonstrated, through global transcriptional profiling approaches, to promote B-lineage commitment by upregulating B-cell differentiation genes such as Aiolos, inhibitor of DNA binding 3 (Id3), IRF4, IRF8, CD19 and CD79a, while repressing non-B cell promoting genes such as MCSF-R and Notch1 (Delogu et al., 2006, Cobaleda et al., 2007).

Whereas the development of all B lymphoid cells occurs mainly in the bone marrow, T lymphocyte development occurs in the thymus. Among the most prominent transcription factors necessary for the emergence of early T-cell progenitors (ETP) from the LMPP are Notch1 and T cell factor 1 (TCF-1). Inhibition of Notch signalling, or TCF-1, has been demonstrated to result in loss of ETP with no apparent effect on the LMPP population (Sambandam et al., 2005, Weber et al., 2011). The TCF-1 was demonstrated to be a downstream target of Notch signalling. Other transcription factors such as GATA-binding protein 3 (GATA-3), E2A, and RUNT-related transcription factor (Runx) have all

been demonstrated to be necessary at various stages in T-lymphoid progenitor specification as shown in figure 1-4 (Hosoya et al., 2009, Dias et al., 2008, Talebian et al., 2007, Bain et al., 1997). However, it has been shown that cells at the early DN2 (Double Negative 2, as they express neither CD4 nor CD8 antigen) stage still retain DC, NK and macrophage potential with T-lineage commitment occurring in the late DN2 stage under the influence of the B-cell leukaemia 11b (Bcl11b) transcription factor (Masuda et al., 2007, Ikawa et al., 2010, Li et al., 2010). It has been proposed that Bcl11b induces T-lineage commitment at the DN2 to DN3 transition by repressing the essential myeloid lineage transcription factor PU.1.

At the DN3 stage, these αβT-cell precursors undergo β-selection under the control of T cell receptor β gene (*Tcrb*) as well as VDJ (Variable, Diverse and Joining) rearrangement to express functional TCRβ chain. Several gene knockout, or conditional deletion, studies have demonstrated the essential roles played by transcription factors such as E2A, HEB (basic helix-loop helix protein related to E2A), Notch1, Myb (myeloblastosis viral oncogene homolog), Runx1 and GATA-3 in TCRβ chain rearrangement (Wojciechowski et al., 2007, Wolfer et al., 2002, Lieu et al., 2004, Pai et al., 2003, Egawa et al., 2007). DN3 thymocytes that have successfully undergone β-selection develops into double positive (DP) cells co-expressing CD4 and CD8 receptors. Due to differential affinity of αβTCR of DP cells to MHCI and MHCII self-antigen, DP thymocytes undergo positive selection to become one of two mature single positive subsets; MHCII-selected CD4+CD8- helper lineage or MHCI-selected CD4-CD8+ cytotoxic lineage (Germain, 2002).

## 1.11 Microenvironment mediated signalling regulating haematopoiesis.

### 1.11.1 Notch

Notch is a single transmembrane receptor that depends on intercellular contact for activation. Mammalian cells that are responsive to Notch ligands (Jagged 1-2, and Delta 1, 3, 4) usually express one or more Notch receptors (Notch 1-4). The Notch receptors 1, -2 and -3 are expressed in haematopoietic cells (haematopoietic progenitors, erythroid precursors and monocytes), whereas

Notch receptor 4 is expressed in vascular endothelial cells; the Notch ligands are however expressed by antigen presenting cells, bone marrow stromal cells and thymic epithelial cells (Ohishi et al., 2003, Ohishi et al., 2002, Milner et al., 1994, Felli et al., 1999). Notch ligands are generally internalized and processed in endosomes through the activity of E3-ubiquitin ligases Mindbomb and Neuralised before presentation on plasma membrane for efficient receptor activation (Le Borgne et al., 2005). This ubiquitin ligase activity has been found to be a fundamental regulatory step in Notch activation as Mindbomb deficiency has been demonstrated to result in defective Notch activation (Itoh et al., 2003, Koo et al., 2005). Notch ligand-Notch receptor interaction leads to ADAM (A Disintegrin And Metalloproteinase) mediated cleavage of the intracellular Notch domain from the transmembrane subunit that translocates to the nucleus and interact with co-activators (e.g. Mastermind) to induce target gene expression like HES (Hairy/Enhancer of Split) family of transcription factors.

A large body of evidence from both mutant mouse and Zebrafish models suggests that Notch signalling is dispensable for the emergence of primitive haematopoiesis during embryonic development (Bertrand et al., 2010b, Burns et al., 2005, Robert-Moreno et al., 2005, Robert-Moreno et al., 2007). However, there is growing evidence that Notch1 signalling is required for the emergence of definitive haematopoiesis from the AGM. This is not surprising considering that Notch1 has unequivocally been shown to be required for establishment of an arterial programme in the AGM (Koo et al., 2005, Duarte et al., 2004, Domenga et al., 2004). Interestingly, the Notch signalling pathway has been shown not to be necessary for homeostatic HSC maintenance in adults. Conditional deletion experiments have established a critical role for Notch1 in the generation of T-cell progenitors that seed the thymus and their subsequent differentiation (Mancini et al., 2005, Kim et al., 2008, Radtke et al., 1999).

Clinically, Notch pathway dysfunction has been implicated in about 50% of human T-cell acute lymphoblastic leukaemia (T-ALL) (Weng et al., 2004).

### 1.11.2 Wingless (Wnt) signalling pathway

The Wnt signalling pathway has been categorized into three main pathways: canonical Wnt pathway, planar cell polarity pathway and Wnt-$Ca^{2+}$ (non-

canonical Wnt) pathway (Staal et al., 2008). Most of the experiments assessing the role of Wnt signalling on haematopoiesis have targeted the canonical Wnt pathway which is mediated through ß-catenin and T-cell factor (Tcf)/Lymphocyte-enhancer binding factor (Lef) transcription factors. There are about 19 Wnt proteins that signal through Frizzled (Fz) proteins that serve as receptors. Fizzled proteins may function in complex with low-density lipoprotein receptor-related protein (LRP). In the absence of Wnt ligand binding to Fz, ß-catenin is phosphorylated at multiple sites by glycogen synthase kinase (GSK)3ß through its sequestration to the destruction complex composed of axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and GSK3ß. This phosphorylation process targets ß-catenin for proteasomal degradation through the ß-Trcp (ß-transducin repeat containing protein) ubiquitin protein. However, binding of Wnt ligands to Fz/LRP5 complex leads to disassembly of the destruction complex, stabilization and accumulation of ß-catenin and its subsequent translocation into the nucleus to stimulate transcriptional activation of Tcf/Lef family of transcription factors (Staal et al., 2002, Li et al., 2007). Another level of regulation of the canonical Wnt signalling pathway is the existence of naturally occurring soluble decoy receptors such as secreted frizzled-related protein (sFRP), Wnt inhibitory factor-1 (WIF-1) and Dickkopf-related proteins (DKK) that bind and block the LRP5/6 co-receptor (Mao et al., 2001, Bafico et al., 2001, Hsieh et al., 1999, Bafico et al., 1999).

There is an ongoing debate on the exact role of the Wnt signalling pathway in haematopoiesis as previous studies have given conflicting results. Several *in vitro* studies in both mice and humans have indicated that exposure of haematopoietic progenitor cells to Wnt ligands lead to expansion and increased colony-forming abilities several fold greater than those of non-treated controls (Van Den Berg et al., 1998, Austin et al., 1997). Reports using *In vivo* models have also implicated the canonical Wnt signalling as being necessary for HSC function. For example, Fleming et al. used transgenic mice expressing the Wnt inhibitor DKK1 in osteoblasts to demonstrate that inhibition of Wnt function in the niche led to irreversible loss of HSC repopulating potential as a result of reduced p21Cip1 expression and increased cycling (Fleming et al., 2008). Also, Luis et al. demonstrated the critical need of Wnt3a in both foetal and adult haematopoiesis through the use of $Wnt3a^{-/-}$ mice (Luis et al., 2009). They

showed, through *in vitro* cultures and transplantation assays, that Wnt3a was required for HSC fate decisions as $Wnt3a^{-/-}$ mice had intact B-lymphoid but impaired myeloid progenitor differentiation potential, impaired thymocyte development and impaired HSC long-term repopulation ability.

However, gain-of-function mutation studies have provided conflicting reports in the literature. For example, groups using the same retroviral induced expression of constitutively active ß-catenin in HSC in transgenic mice have reported contrasting findings; some studies reporting an expanded HSC compartment with increased repopulation ability as against the finding of multilineage lymphoid and myeloid differentiation potential with no attending HSC expansion in other studies (Willert et al., 2003, Reya et al., 2003, Baba et al., 2005, Baba et al., 2006). Others have also excluded the relevance of Wnt signalling in haematopoiesis and lymphopoiesis as mice with deletion of ß-catenin and γ-catenin had no demonstrable haematopoietic phenotypes (Jeannet et al., 2008, Koch et al., 2008). Perhaps, the exception to the ongoing debate concerning the role of Wnt signalling in haematopoiesis is in immature T-cell development in the thymus that has been consistently shown to require Notch signalling.

### 1.11.3 Thrombopoietin (TPO)/myeloproliferative leukaemia virus oncogene (MPL) signalling pathway

Thrombopoietin (TPO) is an acidic glycoprotein, constitutively produced mainly in the liver; bone marrow stromal cells, kidney and spleen are also known to produce locally acting TPO (Lok et al., 1994, Qian et al., 1998). It has been known to be primarily concerned with megakaryopoiesis by signalling through the Mpl receptor. In addition to the expression of Mpl in megakaryocytes, platelets, haemangioblasts and HSC in adults, Mpl mRNA has also been detected in the yolk sac, AGM and foetal liver in mouse embryos at E10.5 (Debili et al., 1995, Forsberg et al., 2005, Methia et al., 1993). Interestingly, transplantation assays undertaken with E14.5 foetal liver haematopoietic progenitors that were enriched using phenotypic Mpl expression ($AA4^{+}Sca\text{-}1^{+}Mpl^{+}$) demonstrated superior repopulation ability compared with $Mpl^{-}$ ($AA4^{+}Sca\text{-}1^{+}Mpl^{-}$) counterparts (Petit-Cocault et al., 2007, Solar et al., 1998).

The main stimulus regulating circulating TPO levels is megakaryocyte and platelet mass in the circulation as TPO is removed from the circulation through its binding to Mpl receptors on platelets. Higher platelet counts are thus associated with lower TPO and hence reduced megakaryopoietic output, as there will be a correspondingly higher Mpl to absorb TPO from circulation. Although, TPO mRNA in liver and kidneys are unresponsive to thrombocytopenia, the TPO mRNA in the bone marrow stromal cells increases in response to thrombocytopenia suggesting that different regulatory mechanisms may be at play in different organs (Stoffel et al., 1996, Sungaran et al., 2000, McCarty et al., 1995).

Recent elegant studies using gene knockout mice have shown that the TPO/Mpl signalling pathway may not be necessary for pre-natal haematopoiesis as $TPO^{-/-}$ mice were born normally (Qian et al., 2007). However, it became apparent that the TPO/Mpl pathway is critical in maintenance of postnatal HSC quiescence as these $TPO^{-/-}$ HSCs demonstrated significant age-related decline with concomitant increased cycling and reduction in cyclin-dependent kinase inhibitors $p57^{kip2}$ and $p19^{ink4D}$. Yoshihara and colleagues have also provided additional data showing that bone marrow osteoblasts express TPO and that quiescent long-term repopulating HSC expressing Mpl localize preferentially towards $TPO^{+}$ osteoblasts (Yoshihara et al., 2007). Moreover, they showed that whereas TPO treatment increased the quiescent HSC fraction, neutralizing anti-Mpl antibody treatment mediated entry of HSC into cell cycle and release from their microniches. Furthermore, de Laval and colleagues have also demonstrated that TPO/Mpl pathway is required for the non-homologous end-joining DNA repair mechanisms in HSC (de Laval et al., 2013). These findings demonstrate the unique roles that TPO/Mpl pathway has in regulating *in vivo* HSC quiescence and maintenance of its genomic integrity.

### 1.11.4 CXCL12-CXCR4 axis

The unique contribution of the CXCL12-CXCR4 axis in regulating HSPC behaviour has been described in section 1.13.1.

## 1.12 Haematopoietic growth factors

These are glycoproteins that directly, or indirectly, regulate haematopoiesis by acting in concert with the niche components to control haematopoietic stem and progenitor cell survival, proliferation and differentiation as well as haematopoietic progenitor lineage commitment and function of terminally differentiated cells. The haematopoietic progenitors, and terminal differentiated cells, have been shown to express receptors for one or more of these growth factors.

### 1.12.1 Granulocyte colony stimulating factor (G-CSF)

Murine G-CSF was characterised by Nicola et al as a 24Kd or 25Kd hydrophilic glycoprotein with a neuraminic acid moiety (Nicola et al., 1983). Later, human G-CSF was purified from the human bladder carcinoma cell line 5637 and squamous carcinoma cell line (CHU-2) with a molecular weight of 18kDa or 19kDa. Cloning studies identified cDNAs coding for a human G-CSF protein of 174 and 177 amino acid residues (Welte et al., 1985, Nomura et al., 1986, Nagata et al., 1986, Souza et al., 1986). However, these studies identified the 174 amino acid version as having three amino acids deleted at the N-terminus, O-glycosylated on Thr133, as well as being the most active and abundant form of the protein.

G-CSF is produced by endothelial cells, fibroblasts, stromal cells, mesothelial cells, monocytes and macrophages, to mainly regulate neutrophilic granulopoiesis (Demetri et al., 1989, Zsebo et al., 1988). Inflammatory mediators such as TNF-α, IL-1, IL-6, LPS, 12-O-tetradecanoylphorbol 13-acetate (TPA) and IFN-γ may also stimulate G-CSF production from activated immune cells like monocytes during infection or inflammation (Zsebo et al., 1988, Koeffler et al., 1987, Herrmann et al., 1986, Vellenga et al., 1988, Ernst et al., 1989). G-CSF exerts its biologic effect mostly through its cognate receptor called G-CSFR which is expressed on neutrophils and their precursors (Demetri and Griffin, 1991). Expression of G-CSFR on haematopoietic progenitors such as MPP, CMP and GMP (McKinstry et al., 1997, Onai et al., 2006, Mansson et al., 2009) has also been demonstrated. G-CSFR expression has also been demonstrated on human placenta, vascular endothelial cells and activated T-lymphocytes;

although the functional relevance of G-CSFRs on these cells is not clear (Uzumaki et al., 1989, Bussolino et al., 1989).

The use of G-CSF as haematopoietic stem and progenitor cell mobilization agent is discussed in section 1.13.2.1. Moreover, G-CSF has also been employed with chemotherapy to treat and prevent neutropenia (Heuser et al., 2007, Roberts, 2005, Ozer et al., 2000).

### 1.12.2 Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF is generally produced, upon stimulation, by a number of cells including mast cells, macrophages, endothelial cells, fibroblasts as well as activated T- and B-cells (Cousins et al., 1994, Nimer and Uchida, 1995). Depending on the degree of its N- and O-glycosylation, the molecular weight may vary between 18-30kD. Unlike erythropoietin (EPO) however, the degree of glycosylation is not critical for biological activity.

The effect of GM-CSF is dependent on its concentration; at lower doses, it stimulates macrophage progenitors but also stimulates multipotent progenitors as well as progenitors of erythrocytes, granulocytes, eosinophils and megakaryocytes at higher concentrations (Burgess and Metcalf, 1980). Despite the effect of GM-CSF on haematopoietic progenitors, GM-CSF deficient mice develop normally with apparently normal homeostatic haematopoiesis. However, these mice demonstrated the critical need for GM-CSF in pulmonary resistance to infection and inflammation by bacteria and fungi (Stanley et al., 1994, Deepe et al., 1999). In addition to its role in inducing differentiation of specific haematopoietic progenitors, GM-CSF has been shown to exert immune stimulating effects on antigen presenting cells (APCs) by inducing maturation of APCs, as well as skewing the Th1/Th2 cytokine balance (Wada et al., 1997, Gonzalez-Juarrero et al., 2005).

GM-CSF exerts its effect by signalling through the cell surface GM-CSF receptor, which is composed of α (GM-CSFRα) and ßc (GM-CSFRßc) subunits that are expressed on haematopoietic cells (monocytes, macrophages, granulocytes, lymphocytes and haematopoietic progenitors) as well as non-haematopoietic

cells such as endothelial and alveolar epithelial cells (Griffin et al., 1990, Miyajima, 1992). Interestingly, studies in mice homozygous for the GM-CSF gene revealed that these mice had normal haematopoiesis, but had features characteristic of the human disease called alveolar proteinosis (Stanley et al., 1994). A later study supported those findings by showing that autoantibodies directed against human GM-CSF was the primary cause of human pulmonary alveolar proteinosis (Uchida et al., 2007).

Clinically, GM-CSF has been used for the treatment of neutropenia subsequent to bone marrow transplantation, or chemotherapy (Gerhartz et al., 1993, Lieschke et al., 1989).

### 1.12.3 SCF/KITL

Stem cell factor is widely expressed constitutively by many cell types in the body including endothelial cells, stromal cells, keratinocytes and fibroblasts (Heinrich et al., 1993, Longley et al., 1993). In both humans and mice, SCF is produced in two biologically active forms; soluble and transmembrane forms, as a result of alternative splicing that may, or may not, include the proteolytic cleavage site at exon 6 (Anderson et al., 1991, Anderson et al., 1990, Du et al., 1993, Rolink et al., 1991). Although agents that activate protein kinase C, or increase the concentration of cytosolic calcium, have been shown to cleave cell surface SCF to produce the soluble form (Huang et al., 1992), evidence suggests that the two isoforms of SCF play non-redundant roles as the soluble form is unable to completely compensate for deficiency in the transmembrane isoform (Russell, 1979). Unlike G-CSF and GM-CSF where inflammatory stimuli like TNF profoundly increases their production by bone marrow stromal cells, only modest increases in SCF production are detected with these inflammatory stimuli in the same cells (Broudy et al., 1987).

SCF functions by binding, and signalling, through a 145kDa glycoprotein receptor called c-kit/CD117 which is a member of the type III receptor tyrosine kinase family (Yarden et al., 1987, Qiu et al., 1988, Besmer et al., 1986). The receptor is composed of an N-terminal extracellular domain with five Ig-like motifs, a single transmembrane domain and a cytoplasmic C-terminal domain with tyrosine kinase activity that has both an ATP-binding region and a

phosphotransferase region. Evidence suggests that whereas the fourth Ig-like motif of the N-terminal domain is required for receptor homodimerisation upon SCF binding, the first three Ig-like motifs are required for ligand binding (Blechman et al., 1993, Lev et al., 1993, Blechman et al., 1995). Two naturally occurring isoforms exist in normal tissues due to alternative splicing at codon 510 within the extracellular domain, leading to the presence (kit A), or absence (kit), of the four amino acids Gly-Asn-Asn-Lys (Reith et al., 1991). The ratio of the two isoforms varies between tissues as well as in different physiological or pathological states (Piao et al., 1994).

The c-kit receptor is expressed in several cells and tissues including spermatogonia, endothelial cells, cerebellum, as well as being broadly expressed within the haematopoietic hierarchy (Yoshinaga et al., 1991, Smith et al., 1994, Broudy et al., 1994, Manova et al., 1992). SCF has been shown to have pleiotropic effects such as supporting spermatogenesis, as well as melanocyte and mast cell development (Vincent et al., 1998, Broudy, 1997). It has also been shown that by signalling through its cognate receptor, c-kit, SCF regulates self-renewal of both foetal and adult HSCs (Sharma et al., 2007, Thoren et al., 2008, Waskow et al., 2009). In addition, it induces cell cycle entry and survival of haematopoietic progenitor cells possibly through anti-apoptotic effects (Li and Johnson, 1994, Carson et al., 1994). Through direct interaction of their cognate receptors, SCF has been shown to synergise with EPO to regulate erythroid development (Wu et al., 1997). SCF also synergises with other growth factors like G-CSF, GM-CSF, thrombopoietin, IL-3, and IL-6 to support the formation of colonies from CFU-E, CFU-GEMM, CFU-GM and CFU-Meg in *in vitro* cultures (Bernstein et al., 1991, Nocka et al., 1990, Hendrie et al., 1991, McNiece et al., 1991, Broudy et al., 1995, Briddell et al., 1991).

### 1.12.4 Erythropoietin (EPO)

EPO is a 30.4kDa glycoprotein that regulates erythropoiesis and tissue protection during injury in mammals (Bahlmann et al., 2004, Brines et al., 2000, Junk et al., 2002). The *in vivo* biological activity of EPO is governed by its four glycosylation sites; the higher the glycosylation, the longer the half-life and hence biological activity (Takeuchi et al., 1990, Lukowsky and Painter, 1972). EPO induces erythropoiesis predominantly by its anti-apoptotic effect on late

erythroid precursors; Burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E) and erythroblast to stimulate survival, maturation and red blood cell production. During foetal development, the liver is the main site for EPO production. However, in adulthood, EPO is produced by the liver (Koury et al., 1991, Zanjani et al., 1977) and by the peritubular cells of the renal cortex (Jacobson et al., 1957, Fisher and Birdwell, 1961, Koury et al., 1988) in response to hypoxia. Other organs with detectable EPO mRNA include spleen, lung, and testis; however, their ability to produce functional EPO protein is debatable since they are not able to compensate for EPO production in cases of renal dysfunction. EPO mRNA is also detectable in the brain where it has been demonstrated to exert a neuroprotective action in inflammatory- and hypoxia-induced injury (Sakanaka et al., 1998, Brines et al., 2000).

Several other growth factors including G-CSF, GM-CSF, SCF, IL-1, IL-3, IL-6, IL-11, and insulin growth factor-1 (IGF-1) function synergistically with EPO in erythropoiesis by acting on upstream haematopoietic progenitor cells to induce lineage commitment to the erythroid lineage. EPO exerts its effect by signalling through its cognate receptor, EPOR, which is expressed on erythroid precursors. EPOR expression begins on BFU-E and increases as these progenitors differentiate towards CFU-E. The receptor expression subsequently decreases as these progenitors undergo further differentiation leading to the lack of EPOR on reticulocytes and erythrocytes (Sawada et al., 1990, Wickrema et al., 1992). Figure 5 shows some of the cytokines that act during the differentiation of HSC to erythrocytes and the specific precursor stages at which EPO is crucially required.

EPO binding to EPOR induces conformational changes that result in dimerization of two EPORs and subsequently, phosphorylation of its cytoplasmic Janus Kinase-2 (JAK2) (Remy et al., 1999, Witthuhn et al., 1993). In addition, the tyrosine kinase residues on the intracellular domains of the EPOR also becomes phosphorylated and serve as docking sites for SRC homology 2 containing domains (Tauchi et al., 1995, Barber et al., 2001), thus leading to the activation of several signalling pathways including PI-3K/Akt, STAT5, protein kinase C (PKC) and MAP kinase (Constantinescu et al., 2001, Klingmuller, 1997). However, others have shown the Jak2/STAT5 pathway to be the principal signalling transducer for EPOR (Klingmuller et al., 1996, Socolovsky et al., 1999). In fact,

the enhanced survival of erythroid progenitors was shown to be due to Jak2/STAT5 signalling in which activated STAT5 translocated into the nucleus to induce the expression of the anti-apoptotic gene $Bcl_{XL}$ (Socolovsky et al., 1999, Socolovsky et al., 2001). Inhibition of the effects of EPO may occur through the suppressor of cytokine signaling-3 (SOCS3) that acts as a negative feedback to inhibit EPO action, or by de-phosphorylation of JAK-2 by haematopoietic cell phosphatase, to terminate the action of EPO (Sasaki et al., 2000, Yi et al., 1995).

At the transcriptional level, the production of EPO is under the regulation of hypoxia inducible factor 2α (HIF-2α) (Warnecke et al., 2004). During normoxic conditions, the EPO promoter is suppressed by GATA-2; however, in hypoxic conditions, GATA-2 levels decrease with an increase in GATA-1 activity (Tsuchiya et al., 1997, Imagawa et al., 2003). At the same time, there is stabilization of HIF-2α, which interacts with the hypoxia response element in the promoter region of EPO to induce EPO synthesis. Generally, the level of EPO is low in steady state, thus ensuring that most erythroid progenitors undergo apoptosis with only a few developing into the terminal erythrocytic stage. However, in conditions of hypoxia, or anaemia, EPO production increases significantly resulting in survival of erythroid committed progenitors and ultimately an increase in erythrocyte production (Lacombe et al., 1988, Koury et al., 1989).

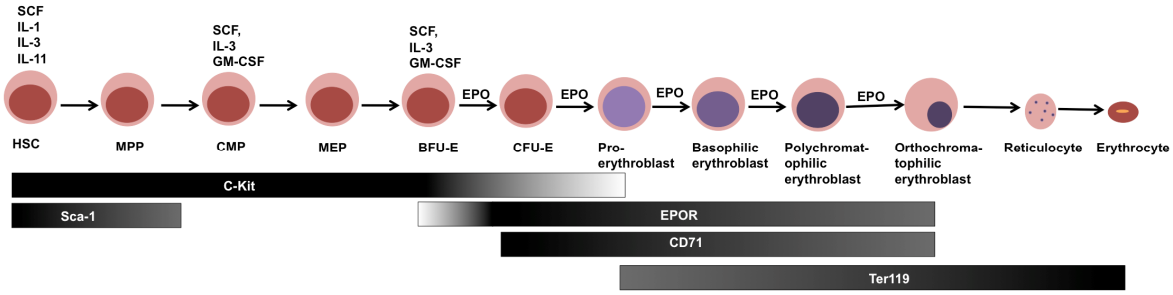



**Figure 1-5 Murine erythrocyte differentiation pathway from haematopoietic stem cell (HSC) showing the stages responsive to erythropoietin (EPO).**
Shown are some of the antigens expressed at different stages of the differentiation pathway and some common cytokines that play critical roles at various stages. Gray indicates low expression, and dark indicate high expression of antigens. MPP, multipotent progenitors; CMP, common myeloid progenitors; MEP, Megakaryocyte-erythroid progenitor; BFU-E, burst forming unit, erythroid; CFU-E, colony forming unit, erythroid; EPO, erythropoietin; EPOR, erythropoietin receptor. Adapted from (Sawada et al., 1990, Socolovsky, 2007).

### 1.12.5 The interleukins (ILs).

Although there are several interleukins that affect haematopoiesis either directly or indirectly, most of them are not clinically employed as haematopoietic growth factors because of their adverse effects. These include IL-1 which is produced in two isoforms (α and ß) mostly from cells of the monocytes/macrophage lineage and acts on early haematopoietic progenitors.

### 1.12.6 Interleukin 3 (IL-3)

IL-3 (also called multi-lineage CSF) is a 20-26kD, pleiotropic and monomeric haematopoietic growth factor that stimulates HSC self-renewal as well as acting synergistically with other growth factors to support differentiation of downstream haematopoietic progenitors. The major sources of IL-3 include activated eosinophils, $CD4^+$ T cells, NK cells and mast cells (Ihle et al., 1983, Metcalf, 1989, Pierce, 1989). Signalling by IL-3 is induced in responsive cells through the IL-3 receptor (IL-3R) composed of α (IL-Rα/CD123) and ß (IL-3Rß) subunits (Miyajima et al., 1992). IL-3R is expressed on HSPCs, haematopoietic effector cells, testis, placenta and brain (Morikawa et al., 1996). IL-3 binding induces hetero-dimerisation of the α and ß subunits through disulphide bonding for optimal receptor activation (Orban et al., 1999). This conformational change in IL-3R leads to recruitment and activation of Janus Kinase 2 (JAK2) through association of the membrane proximal region of IL-3R and the N-terminal region of JAK2 and ultimately recruitment of multiple STAT (signal transducer and activation of transcription) proteins in haematopoietic cells (Quelle et al., 1994, Jaster et al., 1997). One consequence of the JAK2/STAT signalling is MAPK- and PI3K/AKT-induced anti-apoptotic effects (mediated through Bcl-2 and Bcl-XL) that confer growth and survival advantages in haematopoietic cells (Datta et al., 1997, Kinoshita et al., 1995, Sakai and Kraft, 1997).

In a study that used *Runx1* haploinsufficient mice, it was demonstrated that IL-3 may be required for the *de novo* emergence of HSC from the AGM during embryogenesis by acting as a survival and proliferation factor (Robin et al., 2006). Another study also found increased expression of CD123 (IL-3Rα) expression in various haematopoietic malignancies and postulated that this receptor expression could be used as a diagnostic criterion (Munoz et al., 2001).

The clinical utility of IL-3 is however limited due to its considerable side effects possibly as a consequence of IL-3R expression on many cell types (Eder et al., 1997, Ottmann et al., 1990).

## 1.13 Haematopoietic stem and progenitor cell circulation.

### 1.13.1 CXCL12-CXCR4 axis

CXCL12 is a homeostatic chemokine that is produced in six different isoforms (SDF-1α, ß, γ, δ, ε and φ) due to alternative splicing of the CXCL12 gene leading to differences in the exon 4 (attached to the C-terminus) in the variant isoforms (Janowski, 2009, Ho et al., 2012, Yu et al., 2006). This affects the stability in blood and hence duration of biological activity as well as tissue distribution of CXCL12 isoforms. For example, CXCL12α is ubiquitously expressed by many organs but is rapidly degraded in blood, whereas CXCL12ß has improved stability and is mainly restricted to highly vascularised organs like kidney, liver and spleen (Yu et al., 2006, Janowski, 2009). Due to the presence of numerous basic amino acids, CXCL12 proteins are positively charged thus endowing them with a strong attachment to glycosaminoglycans (GAGs) and increased stability (Rueda et al., 2008). Endothelial cells, and bone marrow stromal cells, have been shown to constitutively produce CXCL12 (Ponomaryov et al., 2000). The N-terminus of CXCL12 is important for receptor binding and signalling which explains why N-terminal cleavage abrogates chemotactic responses.

The cognate receptors of CXCL12 are CXCR4 and ACKR3 (CXCR7). Expression of functional CXCR4 supports CXCL12 chemotaxis as has been demonstrated on a host of stem cells (e.g. haematopoietic stem cells, primordial germ cells, satellite cells, neural and endothelial stem cells), haematopoietic cells and malignant cells (Aiuti et al., 1997, Ma et al., 1998a, Kucia et al., 2004a, Kucia et al., 2005, Ratajczak et al., 2003, Day et al., 2010). ACKR3, on the other hand, has been proposed as a decoy receptor and is expressed in organs such as the heart, brain, testes and placenta (Singh et al., 2012, Sun et al., 2010, Nibbs and Graham, 2013).

CXCL12, and its receptor CXCR4, have been demonstrated to be fundamentally required during embryogenesis as targeted disruption of CXCL12 in mutant mice

was shown to result in embryonic lethality (Nagasawa et al., 1996, Nagasawa, 2000). CXCL12 has also been found to be required for trafficking of several organ-type progenitor cells during foetal development as well as migration of foetal liver HSC to colonize bone marrow (Nagasawa et al., 1996, Kucia et al., 2004a, Kucia et al., 2004b). Proper development of the heart, cerebellum, and the gastrointestinal tract, has all been shown to require the presence of CXCL12 (Zou et al., 1998, Nagasawa, 2001). In the adult haematopoietic system, the CXCL12/CXCR4 axis has been shown to be critical for HSPC retention, quiescence and homing to the bone marrow (Lapidot et al., 2005). CXCR4 has been found to be co-receptor for T-tropic HIV infection, important for metastases of various cancer types as well a marker for poor prognosis in these cancers (Kucia et al., 2005, Li et al., 2004, Libura et al., 2002, Scala et al., 2005).

ACKR3 on the other hand appears to bind CXCL11 as a ligand in addition to CXCL12. There is still an ongoing debate as to whether ACKR3 signals upon ligand binding. In the haematopoietic system, there is still no evidence concerning the expression of ACKR3 on HSPC, although its expression on B-cells, neutrophils and monocytes has been reported (Infantino et al., 2006, Sierro et al., 2007). With the exception of heart defects, CXCR7 KO mice were shown to develop normally with no haematopoietic abnormalities (Sierro et al., 2007, Gerrits et al., 2008). Although further work needs to be done concerning the role of ACKR3 in HSPC biology, it is unlikely that ACKR3 will have profound effects on HSPC functions.

### 1.13.2 Haematopoietic stem and progenitor mobilization

Research has shown that very low numbers of haematopoietic stem and progenitor cells circulate in the periphery at any time point under steady state (Wright et al., 2001, Lapidot and Petit, 2002). In mice it has been estimated that ~ 400 HSPCs circulate at any given moment in the peripheral blood (Goodman and Hodgson, 1962, Wright et al., 2001, McCredie et al., 1971, Chervenick and Boggs, 1971). Moreover, it has been shown that these circulating HSPCs may enter tissues and return to the peripheral blood via the lymphatic vasculature and thoracic duct (Massberg et al., 2007). Although the process of homeostatic HSPC egress from the bone marrow is not completely understood, evidence suggests that both humoral (chemokines and sphingosine 1-phosphate) and neural (sympathetic) inputs may play a role. Enforced release of HSPC from the

bone marrow, called mobilization, was first shown in the 1980s in response to myeloablative agents (To et al., 1984, To et al., 1989). Exercise, psychological anxiety, bleeding and infection models (lipopolysaccharide) have also been shown to mediate increased HSPC egress from the bone marrow (Barrett et al., 1978, Cline and Golde, 1977, Kollet et al., 2006, Elsenbruch et al., 2006). Many other pharmacological agents that generally disrupt the factors that mediate HSPC-niche attachments have subsequently been identified as HSPC mobilisers and are discussed below. Mobilised peripheral blood haematopoietic stem cells (PBHSC) have now become the preferred source of stem cells for transplantation. For rapid, and optimal, reconstitution in autologous transplantation, a $CD34^+$ cell count of $\geq 5x10^6$ cells/kg is generally required, although a minimum of $2x10^6$ cells/kg body weight may still be used in poor mobilisers (Reiffers et al., 1994, Bensinger et al., 1995, Weaver et al., 1995).

#### 1.13.2.1 Cytokines

Among the cytokines employed for HSPC mobilization are G-CSF, GM-CSF, SCF and IL-1. However, G-CSF remains the prototypical agent employed for HSPC mobilization in the clinic. G-CSF is generally administered at a dose of 5-10$\mu gKg^{-1}day^{-1}$ S.C. over 4-6 consecutive days. Interestingly, it has been demonstrated that G-CSF preferentially mobilizes quiescent haematopoietic stem cells into the circulation (Roberts and Metcalf, 1995). Several mechanisms have been proposed to contribute to the ability of G-CSF to mobilize HSPC to the periphery. Accumulating evidence shows that the haematopoietic stem cell mobilization effect of G-CSF is indirect, as HSCs do not express the G-CSF receptor. Some have postulated that G-CSF induces activation of neutrophils that release matrix metalloproteinase-9, cathepsin G and elastases, which cause proteolytic cleavage of niche retention factors like CXCL12. In addition, other reports found that G-CSF-induced haematopoietic progenitor cell mobilization occurred in response to neutrophil elastase-mediated degradation of bone marrow CXCL12 and upregulation of CXCR4 expression on immature human CD34+ and $CD38^{-/low}$ cells (Petit et al., 2002). Others have used genetic approaches, involving G-CSFR-deficient mice, to underscore the critical need for functional neutrophils in orchestrating the G-CSF-mediated HSPC release (Liu et al., 1997, Liu et al., 2000). Compared to bone marrow derived HSPC, G-CSF-mobilised HSPC have enhanced engraftment potential and this has been attributed partly to their

lower expression of the CXCL12-degrading enzyme, CD26, and their consequent enhanced migration towards CXCL12 (Bonig et al., 2007). Others have implicated down-regulation of CXCL12 at the mRNA level in both osteoblast and bone marrow stromal cells as being important in HSPC-induced mobilization by G-CSF (Semerad et al., 2005).

Although G-CSF mobilized haematopoietic progenitors offer numerous advantages over bone marrow derived counterparts, it has been observed that 25% of patients with lymphoma or multiple myeloma, as well as those with Fanconi anaemia, or previous chemotherapy, fail to mobilize with G-CSF (Moskowitz et al., 1998, Bensinger et al., 1994, Croop et al., 2001). In addition, recent findings suggest that the 10-20% (Anderlini et al., 1997, Holm, 1998) of healthy volunteers who fail to mobilize with G-CSF may be the result of polymorphism in the G-CSF receptor (Bogunia-Kubik et al., 2012). For these reasons, G-CSF is generally used mostly in combination with chemotherapy in these poor mobilizers; a protocol that increases the subsequent dose of G-CSF required with its consequent toxicity as well as increased incidence of post-treatment malignancies (Dreger et al., 1995, Anderlini et al., 1996, Pusic et al., 2008). Among the side effects observed with G-CSF administration are sickle cell crises in individuals with Haemoglobin S or C genotype, bone pain (probably resulting from granulocytic marrow hyperplasia), splenic rupture, myocardial infarction, anaphylactoid reaction, intracranial haemorrhage and flare-up in rheumatoid arthritis (Bensinger et al., 1996, Adler et al., 2001, Fortanier et al., 2002, Nuamah et al., 2006).

#### 1.13.2.2 Chemokines ligands/agonists

In contrast to cytokines like G-CSF that require multiple dosing over several days to mobilize HPCs to optimal levels, chemokine-based mobilizing agents rapidly mobilize haematopoietic progenitors within minutes to hours per single dose (Pelus and Fukuda, 2008). In addition, when compared to G-CSF-mobilised HSPC, there are indications of a superior engraftment of chemokine-mobilised HSPC, judged by contribution to donor chimerism, in both primary and secondary transplantation assays (King et al., 2001, Pelus and Fukuda, 2006, Fukuda et al., 2007). Both CC- and CXC-chemokines have been shown to be haematopoietic stem cell mobilizing agents as discussed below.

#### 1.13.2.2.1 CXC-chemokine mobilization agents

Among the CXC-chemokines employed for haematopoietic progenitor cell mobilization are the CXCR2 ligands [CXCL2 (GROß), or $CXCL8_{\Delta4}$ ($GRO\beta_{\Delta4}$)], CXCL8, as well as CXCL12 peptide analogs (CTCE-0021 and CTCE-0214).

#### 1.13.2.2.2 Growth-regulated (GRO) proteins (GROβ/CXCL2 and $GRO\beta\Delta_4$/$CXCL2\Delta_4$)

The GRO proteins (CXCL2 or $CXCL2_{\Delta4}$) have been the most studied agents and are employed at $2.5mgKg^{-1}$ S.C. (Pelus and Fukuda, 2006, Fukuda et al., 2007). This induces rapid mobilization that peaks at 15 minutes post injection and returns to baseline in 60 minutes. In trying to unravel the mechanistic details in mouse models, it has been suggested that the rapid mobilization occurs as a result of indirect effects on bone marrow neutrophils as CXCR2 expression was absent on HSPCs (Pruijt et al., 2002, Pelus et al., 2004). However, in studies that profiled the chemokine receptor expression on lineage depleted haematopoietic progenitors during ontogeny in humans, CXCR2 (together with CXCR1, CXCR4, and CXCR5) was detected on bone marrow, mobilized blood, cord blood and foetal blood by flow cytometry (Rosu-Myles et al., 2000). That study further revealed that whereas the most primitive adult progenitors (defined in the study as $Lin^-CD34^+CD38^-$) did not express CXCR2, the most primitive cord blood cell expressed higher levels of CXCR2. Further work thus needs to be done to reconcile the discrepancy between the human and mouse data.

The experience with CXCR2 ligands has shown that a single dose of CXCL2 or $CXCL2_{\Delta4}$ mobilizes HSPC to similar levels as the multiple daily dosing of G-CSF and indeed, is more predictable in its mobilization action, causes less apoptosis and a more primitive phenotype of the mobilised cells compared to G-CSF. Although either agent could be used as stand-alone mobiliser, a strong synergy has been demonstrated between G-CSF ($50\mu gKg^{-1}$ twice daily S.C. for 4 days) and $CXCL2_{\Delta4}$ ($2.5mgKg^{-1}$ S.C. given on the last dose of G-CSF), as combined therapy showed a 7-10-fold increase in long-term haematopoietic progenitor colony-forming potential compared to G-CSF given alone (Pelus et al., 2002).

#### 1.13.2.2.3 CXCL8 (IL-8)

Pro-inflammatory cytokines such as TNFα, IL-1, IL-3 and GM-CSF induce the production of CXCL8 from such cells as monocytes, neutrophils, keratinocytes, endothelial cells and mast cells (Strieter et al., 1989, Matsushima and Oppenheim, 1989). CXCL8 signals through both CXCR1 and CXCR2 which are highly expressed on neutrophils (Holmes et al., 1991, Murphy and Tiffany, 1991). Not surprisingly, CXCL8 induces chemotaxis of neutrophils, release of metalloproteinases (e.g. MMP-9), shedding of L-selectin as well as up-regulation of leukocyte function antigen-1 expression (LFA-1). Single injection of CXCL8 has been shown to mediate a rapid haematopoietic progenitor cell mobilization in both mice (30μg I.P. per mouse) and rhesus monkeys (10-100μgKg$^{-1}$ I.V.) (Laterveer et al., 1995, Laterveer et al., 1996). The number of mobilized haematopoietic progenitors peaked in 15-30 minutes and offered radioprotection as well as long-term lymphomyeloid reconstitution in lethally ablated hosts.

Mechanistically, evidence has been provided demonstrating that the CXCL8-induced haematopoietic progenitor cell mobilization is critically dependent on neutrophils as antibody-mediated depletion of neutrophils inhibited HPC mobilization (Hestdal et al., 1991). Other evidence points to roles of LFA-1 and metalloproteinase-9, probably from activated neutrophils, as being critical as blocking of either substantially ablates the mobilization observed in mice and rhesus monkeys (Pruijt et al., 1999, Czuprynski et al., 1994).

#### 1.13.2.2.4 CXCL12 (SDF-1α) peptide

In studies that investigated the potential of exogenously administered CXCL12 to mobilize HSPC, an Adenoviral delivery system (AdCXCL12) was adopted to sustain adequate plasma levels of CXCL12 since the native CXCL12 was rapidly degraded in blood (Hattori et al., 2001). In that report, I.V. AdCXCL12, but not recombinant CXCL12, significantly mobilized leukocytes, platelets and haematopoietic progenitor cells to the circulation in severe combined immunodeficient mice. The mobilized haematopoietic progenitor cells demonstrated repopulating potential in lethally irradiated recipients, indicating the presence of long-term stem cells in this population. Others using the same AdCXCL12 model have also shown that endothelial progenitor cells are mobilized

in addition to the HSPC as endothelial cells express CXCR4 (Moore et al., 2001, Molino et al., 2000).

Other studies that investigated the CXCL12-CXCR4 axis in HSPC mobilization used the CXCL12 peptide analogues, CTCE-0021 and CTCE-0214 (Pelus et al., 2005, Zhong et al., 2004). CTCE-0021 was administered intravenously at 25mg/Kg in a single dose and demonstrated a dose-dependent effect on haematopoietic progenitor mobilization within 60 minutes post injection. When given in combined therapy with G-CSF (50μg/kg/day b.i.d x 4 days S.C.), CTCE-0021 (given at 25mg/kg 16 hours after last G-CSF dose) exhibited synergistic HSPC mobilization activity. Interestingly, it was observed that although multi-dosing sustained an increased peripheral blood polymorphonuclear count, HPC mobilization could not be re-stimulated after the first dose. Mechanistically, it was demonstrated that CTCE-0021-induced down-regulation of CXCR4 expression on haematopoietic progenitors with concomitant alteration in the CXCL12 gradient but had no effect on proteases such as MMP-9, neutrophil elastase and cathepsin G.

The other CXCL12 analog, CTCE-0214, has been employed at a similar dose with similar HSPC mobilization activity as CTCE-0021 (Zhong et al., 2004).

#### 1.13.2.2.5 CXCR4 agonist (ATI-2341)

ATI-2341, is a pepducin derived from the first intracellular loop of human CXCR4 and has been shown to have CXCR4 agonist properties (Tchernychev et al., 2010). In both mouse and non-human primate models, ATI-2341 was given intravenously at a dose of 0.2-0.66μmol/Kg and demonstrated time-dependent HSPC mobilisation potential comparable to the CXCR4 antagonist, AMD3100. Interestingly, unlike AMD3100 that mobilises lymphomyeloid progenitors, ATF-2341 was shown to have a unique ability to mobilize granulocytes and myeloid-biased HSPCs, but not lymphocytes. This may be particularly important as a means to prevent Graft-versus-host reactions. A recent study has proposed that the functional selectivity of ATI-2341, compared to CXCL12, may be due to its preferential activation of Gαi (but not β-arrestins) leading to biased CXCR4 agonist effects (Quoyer et al., 2013).

#### 1.13.2.2.6 Chemokine receptor antagonist- AMD3100

The bicyclam molecule 1,1'- [1,4-phenylene-bis(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane also known as AMD3100 is a selective, competitive CXCL12 receptor (CXCR4) inhibitor which was initially investigated for its ability to block T-tropic HIV infection (Schols et al., 1997a, Schols et al., 1997b, Donzella et al., 1998, Hendrix et al., 2004). Noticeably, significant leucocytosis, and increased CD34+ cell numbers, were observed in peripheral blood harvest. These observations, coupled with the importance of the CXCL12/CXCR4 axis in HSPC homing, prompted investigation geared towards HSPC mobilization. AMD3100 is rapidly absorbed after subcutaneous administration with 87% bioavailability and a $t_{1/2}$ of 3.6 hours. Subsequent murine models demonstrated a 40-fold increase in circulating haematopoietic progenitor cells with subcutaneous AMD3100 given at 5mg/kg as a single dose (Broxmeyer et al., 2005). In addition to inducing significant haematopoietic progenitor cell mobilisation in healthy volunteers (Liles et al., 2003), AMD3100 was also able to orchestrate mobilization in patients suffering from multiple myeloma, or non-Hodgkin lymphoma, when combined with G-CSF (Devine et al., 2004, Flomenberg et al., 2005). Other studies also suggested that the level of CD34+ haematopoietic progenitor cell mobilisation peaked at about 10-14 hours (Liles et al., 2003). More importantly, AMD3100 mobilised HSPC had a more quiescent and primitive phenotype, higher cell surface CXCR4 and very late antigen-4 (VLA-4) expression translating into better and faster engraftment with fewer doses compared to G-CSF mobilized products (Fruehauf et al., 2009, Donahue et al., 2009). Unlike the many side effects associated with G-CSF, only minor side effects like mild gastrointestinal tract disturbance, headache and injection site erythema have been reported with AMD3100 (Devine et al., 2004, Liles et al., 2003). Clinically, AMD3100 is used at a dose of 240μg/kg S.C. per single dose as an HSPC mobiliser with or without G-CSF (Stewart et al., 2009).

#### 1.13.2.2.7 CC-chemokine mobilizing agents

Of the CC-chemokines, only recombinant human CCL3 (macrophage inflammatory protein-1, MIP-1α) has been demonstrated to have HSPC mobilizing effects. Due to its inhibitory action on cell cycling, CCL3 has been shown to protect multipotent haematopoietic progenitors from chemotherapy-induced

myelosuppression (Dunlop et al., 1992, Lord et al., 1992). However, due to the tendency of human CCL3 to form high molecular weight polymers, a genetic variant of the native CCL3 molecule, called BB-10010, has been evaluated for its HSPC mobilising properties (Hunter et al., 1995). BB-10010 was produced by a single amino acid substitution (Asp26>Ala) and has a reduced tendency to form aggregates at physiological pH. Just like the native CCL3, BB-10010 was demonstrated to inhibit bone marrow haematopoietic progenitor cell cycling, and mobilise HSPC into the peripheral circulation (Lord et al., 1995). However, for additive HSPC mobilisation effect, BB-10010 may be used in combination with G-CSF, or AMD3100, to achieve optimal haematopoietic progenitor harvest for transplantation (Broxmeyer et al., 2007). Although CCL3 signals through both CCR1 and CCR5, the HPC mobilization response is presumed to be due to CCR1 (Broxmeyer et al., 1999, Hunter et al., 1995, Lord et al., 1995).

#### 1.13.2.3 VLA-4 antagonist

In humans, research findings indicate that VLA-4 (α4ß1) is expressed on CD34+ HSPC and is upregulated by IL-3 and SCF, but downregulated by G-CSF (Prosper et al., 1998, Bellucci et al., 1999, Lichterfeld et al., 2000, Yamaguchi et al., 1998). In fact, G-CSF-induced HSPC mobilization was associated with decreased VLA-4 expression. Further studies using chimeric, or conditional genetic ablation, of α4- or ß1-integrin in mice have shed more light on the role of VLA-4 in the haematopoietic system during ontogeny. $\beta1^{-/-}$ HSPCs failed to support haematopoiesis as there was defective HSPC colonization of foetal liver and also spleen, as well as liver and bone marrow in adult mice (Fassler and Meyer, 1995, Hirsch et al., 1996, Potocnik et al., 2000). However, α4 integrin was only absolutely necessary to support myeloerythroid and lymphoid differentiation in adult mice (Arroyo et al., 1996, Arroyo et al., 1999). Conditional deletion of α4 integrin resulted in HSPC mobilization and sequestration in the spleen and an inability to home and competitively repopulate a lethally irradiated host when the $\alpha4^{-/-}$ HSPC were transplanted to wild type recipients (Scott et al., 2003, Priestley et al., 2006, Priestley et al., 2007). Antibody-mediated inhibition experiments provided additional evidence for the role of the VLA-4/VCAM-1 axis in HSPC homing to haematopoietic sites. For example, pre-treatment of lethally ablated hosts with anti-VCAM-1 antibody prevented wild type HSPC from homing to the bone marrow whereas treatment of HSPC with anti-VLA-4 antibody prior

to transplantation had the same inhibitory effect on homing (Papayannopoulou et al., 1995, Vermeulen et al., 1998).

Other studies also adopted the use of a small molecule antagonist of VLA-4, BIO5192, to evaluate its HSPC mobilizing effect in mice. It was demonstrated that BIO5192 administered intravenously at 1mg/kg mobilized HSPC 30-fold over baseline levels within 60 minutes. Interestingly, BIO5192 exhibited additive effects with either AMD3100 (three fold above AMD3100 alone), or G-CSF (five-fold over G-CSF alone), as well as when used with both in combined therapy (17-fold compared with G-CSF alone) (Ramirez et al., 2009). It is interesting to note that the HSPC mobilization induced by the VLA-4 antagonist occurs independently of the CXCL12/CXCR4 axis, as it was unaffected in $CXCR4^{-/-}$ chimeras (Christopher et al., 2009). It will be interesting to see how poor mobilisers will respond to HSPC mobilization using either VLA-4 antagonist alone, or in a combined therapy with AMD3100 and/or G-CSF.

#### 1.13.2.4 Elements of Innate immunity

Innate immunity is naturally present and non-specifically functions to protect an organism from infection. It develops with the organism during embryogenesis and may thus form a closely-knit unit with components in the niche harbouring various stem cells. Not surprisingly, signalling through receptors, or disruption of the function of several elements of the immune system, have been implicated in the retention and mobilization of HSPC from the bone marrow (Nagai et al., 2006). These include components of the complement cascade, bioactive lipids, neutrophils, macrophages and Toll-like receptors as briefly reviewed below.

##### 1.13.2.4.1 Components of the complement system

Evidence for the implication of the complement system in HSPC retention and subsequent mobilization came from studies on C3a- and C5a-deficient mice. Whereas C3a deficient mice showed enhanced mobilisation with G-CSF, C5a deficient mice were poor mobilisers with G-CSF. These series of experiments established that the C3a complement product is required for HSPC retention in the bone marrow whereas C5a may induce HSPC trafficking from the bone marrow (Molendijk et al., 1986, Ratajczak et al., 2004a, Reca et al., 2007). It has been suggested that complement activation via the classical pathway, due to

naturally occurring antibodies (NAs) that become activated as a consequence of G-CSF-induced tissue damage, may be the major mechanism underlying the HSPC modulatory effect. This hypothesis is supported by the findings that immunodeficient humans (Sekhsaria et al., 1996) and immunodeficient mice (Reca et al., 2007) are poor mobilisers due to lack of NAs since supplementation of wild-type immunoglobulins in mice significantly improved HSPC mobilization (Reca et al., 2007). Critically important findings on the C3a pro-homing effect on HSPC were provided by co-localisation experiments that established that C3a increases incorporation of CXCR4 and Rac-1 in lipid rafts and hence increases HSPC binding affinity to CXCL12 (Ratajczak et al., 2004b, Ratajczak et al., 2006). Others have also suggested that G-CSF-induced activation of the coagulation cascade, and thrombin production, may also have roles in HSPC mobilization (Huber-Lang et al., 2006, Bonig et al., 2001, Canales et al., 2002, Markiewski and Lambris, 2007).

#### 1.13.2.4.2 Granulocytes

Several lines of experimental evidence have implicated granulocytes as playing major roles in HSPC mobilization although the exact mechanism(s) underlying such roles are still being elucidated. It has been proposed that G-CSF induces enhanced granulopoiesis and activation of granulocytes leading to release of proteolytic enzymes (e.g. MMP-9, cathepsin G and elastase) that cleave the HSPC niche adhesive factors such as VLA-4, CXCL12, and kit ligand in the bone marrow (Levesque et al., 2003b, Levesque et al., 2003a, Levesque et al., 2004). The proteolytic environment induced by activated granulocytes has also been suggested as being vital in generation of activated complement product C5a. Granulocytes have been proposed to “pave the way” for subsequent HSPC release as they have been demonstrated to be the first group of cells to egress from the bone marrow in most HSPC mobilization protocols (King et al., 2001, Sato et al., 1994, Glaspy et al., 1997).

It is interesting to note that other models that used mice genetically deficient in several proteases demonstrated normal mobilization with G-CSF, thus suggesting that the role of granulocytes may not be an absolute requirement for HSPC mobilization (Levesque et al., 2004, Cramer et al., 2008).

#### 1.13.2.4.3 Macrophages

Recent findings have strongly suggested a role for macrophages in the retention of HSPC in the bone marrow niche. Two groups of recent publications, adopting depletion of specific macrophage populations, particularly stand out. In the first publication, bone marrow Gr-1$^-$F4/80$^+$CD169$^+$ macrophages were depleted using clodronate-loaded liposomes, or an inducible c-fms macrophage Fas-induced apoptosis model in mice (MAFIA model) or transgenic mice in which DTR is under the control of the CD169 promoter. In all cases, depletion of bone marrow CD169$^+$ macrophages was associated with reduced CXCL12 protein in bone marrow extracellular fluid (~40% decrease) as well as HSPC mobilization to blood and spleen (Chow et al., 2011). The authors further demonstrated that the reduction in CXCL12 protein secretion occurred preferentially in Nestin+ MSC instead of osteoblasts, although the factor(s) that mediate the Nestin+ MSC-CD169 macrophage interaction were not identified. The second set of publications also used clodronate-loaded liposomes or the MAFIA model to demonstrate that depletion of bone marrow F4/80$^+$ osteomacs (Chang et al., 2008b, Winkler et al., 2010b) was associated with mobilization of HSPC with lympho-myeloid reconstitution potential in lethally ablated host. Further work still needs to be done to evaluate whether osteomac and CD169$^+$ macrophage populations are the same or different populations as well as the exact effects these populations exert on niche retention factors in homeostatic conditions.

#### 1.13.2.4.4 TLR ligands

LPS has been shown to induce HSPC mobilization to the periphery in murine models of endotoxaemia (Zhang et al., 2005, Vos et al., 1972, Quesenberry et al., 1973). It was also observed that CFU-GM counts were significantly increased in the bone marrow, blood, and spleen, 48 hours post I.V. LPS injection. In addition, in vivo *E. coli*-induced bacteraemia stimulated haematopoietic progenitor cell mobilization into the blood as well as phenotypic inversion of Lin$^-$ Sca$^-$Kit$^+$ to Lin$^-$Sca$^+$Kit$^+$ (Zhang et al., 2008). It is increasingly becoming clear that inflammatory responses affect phenotypic expression of antigens in the HSPC compartment. For example, it was recently shown that the rate of haematopoietic progenitor cell differentiation towards the pro-erythroid lineage was inversely correlated to Sca-1 expression (Chang et al., 2008a).

## 1.14 Aims and Objectives

To investigate the modulation in the HSPC expression of inflammatory chemokine receptors in steady states and inflammatory states.

To investigate the role that inflammatory chemokine-chemokine receptor axes play in the mobilization and/or migration of HSPC during inflammation.

To further explore the molecular mechanisms underlying the inflammation-induced HSPC mobilisation.

# Chapter 2: Materials and Methods

## 2.1 General solutions and consumables

**Plastics:** All plastics used were purchased from either Corning laboratories (Loughborough, UK) or Gibco (Invitrogen, Paisley, UK). Plates for quantitative polymerase chain reaction (QPCR) and filter tips were purchased from StarLab (Milton, Keynes, UK).

**1% Acid Alcohol:** 990mL of 70% ethanol plus 10 mL of concentrated HCL per litre of solution

**Complete medium:** 500mL IMDM [Iscove's Modified Dulbecco's Medium, (Invitrogen, Paisley, UK)] containing 4%$^{v}/_{v}$ foetal calf serum (FCS), penicillin (100 units/mL), streptomycin (100µg/mL), and 2mM L-Glutamate.

**Dulbecco's Phosphate Buffered Saline, 1x (1x DPBS) without $CaCl_2$ & $MgCl_2$:** 1x DPBS was purchased from Life Technologies (Paisley, UK).

**Eosin Y solution:** Putts eosin stain was purchased from CellPath (Newtown, Powys, UK) and diluted 1:2 with distilled water before being used.

**Fluorescence activated cell sorting (FACS) buffer:** 500ml of 1x DPBS containing 4%$^{v}/_{v}$ FCS, 2mM EDTA and 0.09%$^{w}/_{v}$ Sodium Azide.

**FACS sorting buffer:** 500ml 1x DPBS containing 1%$^{v}/_{v}$ FCS.

**Chemokine receptor FACS staining buffer:** 500ml 1x DPBS containing 4%$^{v}/_{v}$ FCS, 2mM EDTA and 0.2%$^{w}/_{v}$ Sodium Azide

**Chemotaxis buffer:** 0.5%$^{w}/_{v}$ Bovine Serum Albumin (BSA) in IMDM

**Haematoxylin Z solution:** Haematoxylin 'Z' solution was purchased from CellPath (Newtown, Powys, UK) and filtered before being used.

**Hanks' Balanced Salt Solution 1x with $CaCl_2$ and $MgCl_2$ (1x HBSS):** 1x HBSS was purchased from Life Technologies (Paisley, UK).

**0.2X Primer mix:** 0.8µL each of 20X Taqman probe + TE buffer up to 80µL

**2X Reverse Transcriptase Specific Target Amplification mix per reaction:** 1.4 µL of 0.2X primers mix, 2.8 µL of CellsDirect™ 2X reaction mix (Invitrogen, Paisley, UK), 0.056 µL of SUPERase-In (Ambion, Paisley, UK), 0.112 µL of Superscript® III RT/Platinium® Taq Mix (Invitrogen, Paisley, UK), and 0.672 µL of Resuspension buffer (Invitrogen, Paisley, UK).

**PureLink DNase digestion cocktail (per 80µL):** 8µL DNase digestion buffer (Life Technologies, Paisley, UK), 10µL of 3U/µL DNase (Life Technologies, Paisley, UK), and 64µL RNase-free water.

**Lipopolysaccharide (LPS)**
Ultra-pure LPS from *E. coli* 0111:B4 strain was purchased from InvivoGen (Toulouse, France), reconstituted at 1mg/mL in 1x DPBS (endotoxin-free) and kept frozen at -20C until used.

**12-O-tetradecanoylphorbol-13-acatate (TPA)**
TPA was purchased from Sigma-Aldrich (Irvine, UK), reconstituted to a concentration of 100µM in acetone and kept at -20°C in the dark until used. 100µL of this reconstituted TPA was used per treatment in the TPA peripheral inflammation model.

**Aldara™ (imiquimod) 5% cream**
Aldara™ 5% cream was purchased from Meda AB (Solna, Sweden). Each sachet of the imiquimod cream contains 250mg cream equivalent to 12.5mg active imiquimod.

**Collagenase IV**
Collagenase from *Clostridium histolyticum* (sterile-filtered, Type IA-S, 0.5-5.0 FALGPA units/mg solid, >125 CDU/mg solid) was purchased from Sigma-Aldrich (Irvine, UK) and reconstituted in 1X HBSS to a concentration of 10mg/mL. 300µL of the reconstituted collagenase IV was used per 1mL of bone digestion to achieve a final concentration of 3mg/mL.

## 2.2 Mice

All wild type (WT) mice used for the studies were 8-12 week old, female C57BL/6 and were purchased from Harlan laboratories (Bichester, UK). CCR2 null

mice were 8-12 week old females, on a C57BL/6 background, and were originally purchased from Jackson's laboratory (Maine, U.S.A). Mice used were kept under specific pathogen free conditions at the Central Research Facility, CRF (University of Glasgow, UK). All procedures were carried out in accordance with the United Kingdom Home Office License regulations.

### 2.2.1 Topical imiquimod cream or topical TPA-induced peripheral inflammation model.

The dorsal skin of 8-12 week old, female mice was shaved at least a day prior to application of inflammatory agents to prevent direct systemic access of the topically applied agent through any skin abrasions. For TPA application, acetone was used as a control as the TPA was reconstituted in acetone; for topical imiquimod cream application, aqueous cream application was used as a control. 100µL of 100µM TPA, or 62.5mg Aldara™ cream (equivalent to 3.125mg imiquimod active ingredient), was daily applied to the shaved dorsal skin for 3 consecutive days, after which tissues were harvested for the various experimental procedures described below. In all cases, other groups of mice received 62.5mg aqueous cream, or 100µl acetone, treatment to the shaved dorsal skin as controls for the imiquimod cream and TPA treatments respectively.

### 2.2.2 Intraperitoneal LPS injection model

In order to ensure that effects detected in the model were due specifically to TLR4 signalling and not due to contaminating TLR2 that occurs with other LPS products, Ultra-pure LPS was purchased from InvivoGen (Toulouse, France). This was reconstituted to a concentration of 1mg/mL in endotoxin-free DPBS. In all the LPS injection models, mice (WT or CCR2 KO mice) were each given a single dose of intraperitoneal (I.P.) 100µg LPS (in 100µL PBS), or 100µL I.P. PBS injection as a control. 18-24 hours after the injection, the mice were culled by $CO_2$ asphyxiation and tissues were harvested and used for subsequent experiments as described in the relevant sections below.

# 2.3 Tissue processing, embedding and sectioning

## 2.3.1 Tissue processing

WT or CCR2 null mice received the topical imiquimod cream/TPA treatment model exactly as described in section 2.2.1, after which mice were euthanized in a $CO_2$ chamber, and perfused with 20mL of 1x DPBS (Invitrogen, Paisley, UK). The dorsal skins that had directly received the inflammatory agent were subsequently harvested and fixed in 10% neutral buffered formalin for 24 hours. The fixed skin tissues were then dehydrated through a series of dehydrating conditions using an automated tissue processor (Shandon Citadel 1000, Thermo Scientific, Loughborough, UK) with a programming condition as detailed below. These processing conditions ensured the complete dehydration of the tissues to enable paraffin infiltration (embedding) as described in section 2.3.1.1

| | |
|---|---|
| 70% Alcohol | 1 hour |
| 90% Alcohol | 1 hour |
| 95% Alcohol | 1 hour |
| 100% Alcohol | 1x1 hour, 1x 2 hours, 1x 2.5 hours |
| Xylene | 2x 1 hour, 1x 1.5 hours |
| Molten paraffin wax | 2x 4 hours at 60-65°C |

### 2.3.1.1 Embedding

After the skin had been processed as described above, it was then placed in plastic moulds and embedded in paraffin wax blocks (Histocentre 3, Thermo Scientific). The embedded tissues were then cooled for several hours on the integral cold plate. When the block was set, it was stored at room temperature until sectioning.

### 2.3.1.2 Tissue sectioning

Prior to sectioning, the paraffin blocks were placed on crushed ice to harden the wax as well as to equilibrate the densities across the surface of the block. Skin tissues were cut to 6-8μm and floated in a water bath set to a temperature of 40°C to smoothen out any creases in the sections. Next, the sections were lifted out onto Superfrost$^R$ plus slide (Thermo Scientific), and placed on a histology hot plate (Raymond A Lamb Hotplate, Thermo Scientific) set at 55ºC to dry the

sections. The slides were then filed and kept at room temperature until required for staining.

## 2.4 Haematoxylin and Eosin (H&E) staining of skin sections.

The H&E staining was employed to examine the tissue morphology. Haematoxylin is a dark purplish dye that, by its basic nature, stains the acidophilic/negatively charged components like nuclear chromatin of cells and tissues. Eosin on the other hand, is a reddish-acidophilic dye that preferentially stains the basophilic cytoplasmic components. Therefore, the use of these two dyes with contrasting properties and staining characteristics enabled a better examination of tissue morphology. As described below, the regressive H&E staining technique in which sections are initially stained in stronger haematoxylin solution and subsequently differentiated in acid-alcohol solution was employed.

### 2.4.1 H&E staining procedure

The slides were de-waxed in Xylene 1 for 10 minutes and then re-hydrated through decreasing alcohol concentrations; i.e. absolute Alcohol, 95% alcohol, and 70% alcohol respectively (10 dips each). Next, the slides were washed in running water for a minute, and then stained in haematoxylin Z for 7 minutes. After this the slides were washed in running water (until water ran clear), and taken through 12 dips in 1% Acid Alcohol to allow differentiation of the staining reaction. Next, the slides were washed in running water for 60 seconds and incubated in Scotts Tap Water for 2 minutes, washed in running water again, and then stained for 4 minutes in Eosin Y that had been diluted 1:2. After washing in running water for 2 minutes the slides were taken through 70% Alcohol, 95% alcohol and absolute alcohol (10 dips each). The sections were then taken through three changes of Xylene i.e. Xylene 2, 3 and 4 (1 minute each), mounted in dibutyl phthalate xylene (DPX) and a coverslip (using 0.13-0.17mm cover glass) was then applied. The mounted slides were allowed to dry at room temperature before visualization by bright-field microscopy.

## 2.5 Fluorescent activated cell sorting (FACS)

Details of the monoclonal antibodies used for flow cytometry are listed in table 2-1 below. The antibodies were directly conjugated to one of the following: Allophycocyanin (APC), APC conjugated to Cyanine (APC-Cy7), R-Phycoerythrin (PE), PE conjugated to Cyanine 7 (PE-Cy7), PE-Cy5, Pacific Blue, Peridinin-Chlorophyll-Protein (PerCP), PerCP conjugated to Cyanine 5.5 (PerCP-Cy5.5), PE conjugated to CF594 (PECF594), AlexaFluor 488 or Fluorescein (FITC). Antibodies for staining of CXC-chemokine receptor 4, CXCR4, as well as differentiated haematopoietic lineage markers cocktail (CD4, CD5, CD8a, CD11b, CD45R/B220, Gr-1 and Ter119) were biotin conjugated. Therefore, an indirect staining procedure that required secondary staining with Streptavidin conjugated to a flourochrome was employed. Also, the antibody for staining mouse CCR2 was derived in goat; this therefore required secondary staining using anti-goat antibody. All antibody-staining reactions were done in 1.5mL Eppendorf tubes and on ice unless indicated otherwise. All samples were re-suspended in FACS buffer and filtered through a 70μm cell strainer into 12 x 75 mm polystyrene tubes (Beckman Coulter Inc., High Wycombe, UK) for flow cytometric data acquisition on CyAn$^{TM}$ ADP Analyzer (Beckman Coulter Inc., High Wycombe, UK).

**Table 2-1 List of antibodies used for flow cytometry**

| Mouse antibody | Fluorochrome | Clone | Supplier | Concentration |
|---|---|---|---|---|
| CD16/CD32 (Fc Block | | | Miltenyi Biotech | 1/100 |
| CD4 | Biotin | H129.19 | BD Biosciences | 1/1600 |
| CD5 | Biotin | 53-7.3 | BD Biosciences | 1/800 |
| CD8a | Biotin | 53-6.7 | BD Biosciences | 1/800 |
| CD11b | Biotin | M1/70 | BD Biosciences | 1/200 |
| CD45R/B220 | Biotin | RA3-6B2 | BD Biosciences | 1/200 |
| Ter119 | Biotin, rat anti-mouse | | BD Biosciences | 1/50 |
| Gr-1/Ly-6G/C | Biotin | RB6-8C5 | BD Biosciences | 1/200 |
| Streptavidin | Pacific Blue | | Molecular Probes | 1/100 |
| CD117/c-Kit | APC-Cy7 | 2B8 | BioLegend | 1/200 |
| CD117/c-Kit | APC | | Miltenyi Biotec | 1/200 |
| Sca-1/Ly6-A/E | PE-Cy7 | E13-161.7 | BioLegend | 1/200 |
| CD135 | PE Cy5 | A2F10 | BioLegend | 1/25 |

| | | | | |
|---|---|---|---|---|
| CD48 | FITC | HM48-1 | eBioscience | 1/600 |
| CD150 SLAM | APC | TC15-12F12.2 | BioLegend | 1/200 |
| CD150 SLAM | PE | TC15-12F12.2 | BioLegend | 1/200 |
| CD127/IL7rα | PE-Cy7 | A7R34 | BioLegend | 1/100 |
| CD127/IL7rα | PE | A7R34 | BioLegend | 1/100 |
| CD16/32 | APC-Cy7 | 93 | BioLegend | 1/25 |
| CD16/32 | PerCP Cy5.5 | 93 | eBioscience | 1/25 |
| CD105 | PE-CF594 | MJ7/18 | BD Bioscience | 1/100 |
| CD34 | FITC | RAM34 | BD Biosciences | 1/25 |
| Ter119 | FITC | TER-119 | BioLegend | 1/25 |
| CD41 | APC | MWReg30 | BioLegend | 1/100 |
| CD41 | FITC | MWReg30 | BioLegend | 1/100 |
| CD71 | PE Cy7 | R17217 | BioLegend | 1/25 |
| Goat anti-mouse CCR2 | anti-Goat AlexaFluor488 | | Abcam | 1/100 |
| CXCR4 | Biotin | 2B11 | eBioscience | 1/100 |
| Rat IgG2bκ Iso control | Biotin | eB149/10H5 | eBioscience | 1/100 |
| CD31 | APC | MEC13.3 | BioLegend | 1/100 |
| CD51 | PE | RMV-7 | BioLegend | 1/100 |
| CD115 | APC | AFS98 | BioLegend | 1/50 |
| F4/80 | FITC | BM8 | BioLegend | 1/25 |
| Gr-1 | Brilliant violet | RB6-8C5 | BioLegend | 1/25 |
| CD11b | PE Cy5.5 | M1/70 | BioLegend | 1/25 |
| CD169 | PE | 3D6.112 | BioLegend | 1/100 |
| Live/Dead Aqua | | | Molecular Probes | 1/100 |
| CD45 | FITC | 30-F11 | BioLegend | 1/50 |
| Streptavidin | APC | | BioLegend | 1/100 |

### 2.5.1 Bone marrow and peripheral blood HSPC flow cytometry procedure

Mice received the topical imiquimod cream/TPA treatment or I.P. LPS injection model exactly as described in sections 2.2.1 & 2.2.2 respectively. After the treatment, the mice were euthanized by $CO_2$ asphyxiation and peripheral blood was taken from the aorta into EDTA microtainer tubes (BD Biosciences, Oxford,

UK). Full haematology profiles were obtained from these blood samples using HEMAVET 950FS automated blood counter (DREW Scientific Inc., Dallas, U.S.A).

The bones [2x (femur + tibia +hip bones)] were also harvested from these same mice, and cleared of muscle. Following this, the bones were crushed in a mortar and pestle to isolate the bone marrow. Next, the crushed bone/bone marrow samples were re-suspended in FACS buffer, filtered through a 70μm cell strainer and spun down at 300xg (4°C) for 5 minutes. After discarding the supernatant, the cell sediments were re-suspended at $10^7$ cells/mL in Ammonium Chloride solution (StemCell Technologies, Vancouver, Canada), to lyse the red cells. Next, the cells were washed in FACS buffer and then enriched for haematopoietic stem and progenitor cells using the CD117/c-Kit Microbeads and the LS columns according to manufacturer's instructions (Miltenyi Biotec, GmbH, Germany); see section 2.5.2 below for details.

### 2.5.2 c-Kit enrichment and HSPC staining protocols

As haematopoietic stem and progenitor cells all express the c-Kit antigen, the c-Kit-enrichment protocol allowed for substantial depletion of differentiated haematopoietic cells. For the c-Kit enrichment, 2.5μL of c-Kit Microbeads was added per $10^7$ cells (peripheral blood or bone marrow cells) per 100μL FACS buffer and incubated for 20 minutes on a rotating platform at 4°C (in the dark). The samples were then washed and run through the LS column fitted to its magnetic stand to passively select for c-Kit+ cells. After washing the columns with about 10mL of FACS buffer, the columns were taken out of the magnetic stand and the positively selected cells (positive fraction) were eluted with 8mL of FACS buffer. Next, the positive fractions were then centrifuged at 300xg (4°C) for 5 minutes and stained for flow cytometric analysis according to one of the following:

(A) For SLAM staining, the c-kit enriched cells were initially stained with anti-mouse FcR blocker (Miltenyi Biotec, GmbH, Germany) for 5 minutes, and then washed in 1mL of FACS buffer at 300xg (for 5 minutes at 4ºC). Next, the cells were stained with c-Kit-APC-Cy7, Sca-1-PE Cy7, CD48-FITC, CD150-APC/PE and a cocktail of biotinylated lineage markers (CD4, CD5, CD8a, CD11b, Ter119, CD45R/B220 and Gr-1) anti-mouse antibodies for 15 minutes on ice (in the dark).

The cells were washed in 1mL ice-cold FACS buffer and subsequently stained with Streptavidin Pacific Blue on ice for 15 minutes in the dark.

(B). For myeloerythroid progenitor cell staining, the enriched cells were stained with biotin lineage cocktail, c-Kit-APC-Cy7, IL7Rα-PE Cy7, CD41-FITC, CD150-PE, CD105 PECF594, and CD16/32 PerCP Cy5.5 anti-mouse antibodies for 15 minutes on ice. After washing the cells in FACS buffer, the cells were next stained with Streptavidin Pacific Blue as described in protocol A above.

(C). For chemokine receptor (CXCR4 or CCR2) staining, the c-Kit enriched cells were stained according to protocols A or B above, and then followed by staining for the chemokine receptor in chemokine receptor FACS staining buffer. The primary chemokine receptor antibody was added (see table for staining concentrations) and incubated on ice for 30 minutes. The cells were then washed in ice-cold chemokine receptor FACS staining buffer, spun at 300g (4°C), cell pellets then re-suspended in chemokine receptor FACS staining buffer and secondary antibody was added and subsequently incubated on ice for another 20 minutes. After the final staining step, the cells were washed in 1mL chemokine receptor staining buffer, and then re-suspended in chemokine receptor FACS staining buffer for FACS data acquisition on CyAn ADP (Beckman Coulter Inc., High Wycombe, UK).

In all staining protocols, unstained cells, single colour-stained samples and fluorescence-minus-one (FMO) samples were included as controls for gating and compensation settings. Moreover, dead cells were excluded by staining with Live/Dead Aqua fixable dye (Life Technologies, Paisley, UK) for 30 minutes on ice as the final staining step. All flow cytometric data were acquired on CyAn™ ADP Analyzer (Beckman Coulter Inc., High Wycombe, UK) and the data were subsequently analysed using FlowJo (Tree Star Inc., Ashland, USA)

#### 2.5.2.1 Bone marrow macrophage (Mϕ) staining

In order to understand how each of the systemic (i.e. by LPS injection) and peripheral inflammation models affected the medullary mononuclear phagocytic cells, the bone marrow cells were immunophenotypically stained to delineate monocytes and the macrophage populations. The protocol is essentially as

previously published (Chow et al., 2011). WT mice were treated with either topical imiquimod cream/TPA, or the I.P. LPS injection exactly as described in sections 2.2.1 and 2.2.2 respectively. Following this, the femur and tibia were harvested and crushed in a mortar and pestle to isolate the marrow cells. The bone marrow/bone fragments were next filtered through a 70μm cell strainer and the filtrate was spun at 300xg (4°C) for 5 minutes. The cell pellets were re-suspended in 500μL ice-cold Ammonium Chloride solution per $10^6$ cells for 60 seconds, to lyse red blood cells. Next, the cells were washed in 1mL of FACS buffer and spun at 300g for 5 minutes. The cells were then treated with FcR blocking antibody at a concentration of 1μl per $10^6$ cells at 4°C for 10 minutes, and then washed in 1mL of FACS buffer at 300xg (4°C) for 5 minutes. Following this, the cells were stained with a cocktail antibody consisting of CD11b, CD169, F4/80, CD115, and Gr-1 antibodies, at 4°C for 20 minutes in the dark. The cells were next washed in 1mL FACS buffer and spun down at 300xg (4°C) for 5 minutes. After discarding the supernatants, the cells were next stained with Live/Dead Aqua (1μl per $10^6$ cells) at room temperature for 20 minutes. Following this, the cells were washed in 1mL of FACS buffer at 300xg (4°C) for 5 minutes, and re-suspended in FACS buffer for flow cytometric data acquisition on CyAn™ ADP flow cytometer (Beckman Coulter, High Wycombe, UK).

#### 2.5.2.2 Bone marrow stromal cell staining

The femora, tibiae and hipbones of mice that had been taken through the LPS injection model (section 2.2.2) or imiquimod/TPA treatment model (section 2.2.1) were flushed several times with 1X DPBS to remove the marrow. The resulting bones were minced with a scalpel, and then incubated in 3mg/mL collagenase IV (Sigma-Aldrich, Irvine, UK) at 37°C on a shaking platform for 40 minutes (Semerad et al., 2005). Next, the bone fragments were crushed in a mortar and pestle and then depleted of residual haematopoietic cells using the lineage using the mouse lineage cell depletion kit (Miltenyi Biotec, GmbH, Germany) according to manufacturer's instructions. Firstly, the cells were re-suspended in 40μL FACS buffer per $10^7$ total cells, and then stained with 10μL biotin-antibody cocktail for 10 minutes at 4°C (in the dark). Next, 30μL of FACS buffer and 20μL anti-biotin Microbeads were added and incubated at 4°C for an additional 20 minutes. After washing the cells in 1mL FACS buffer, the non-

haematopoietic cells were sorted through negative selection using LS columns (Miltenyi Biotech, GmbH, Germany). The resultant negative fraction was spun down at 300xg for 5 minutes and stained for 20 minutes at 4°C with a cocktail of lineage antibodies (CD4, CD5, CD8a, CD11b, Ter119, B220, Gr-1), CD31, CD45, Sca-1 and CD51 antibodies as well as Live/Dead Aqua for dead cell exclusion. The staining protocol is essentially as described previously (Winkler et al., 2010a, Semerad et al., 2005). The cells were then washed and flow cytometric data acquired using CyAn$^{TM}$ ADP Analyzer (Beckman Coulter Inc., High Wycombe, UK).

## 2.6 Primers for quantitative polymerase chain reaction (QPCR)

Primers used for QPCR were purchased from Applied Biosystems (Paisley, UK), and their respective catalogue numbers and names are as published in table 2-2. Table 2-3 also details the primers used for the TLDA assay.

**Table 2-2 Taqman probes used for RT-QPCR**

| Gene | Probe | Catalogue number |
|---|---|---|
| Ccr1 | FAM | Mm01216147_m1 |
| Ccr2 | FAM | Mm00438270_m1 |
| Ccr3 | FAM | Mm01216172_m1 |
| Ccr4 | FAM | Mm04207878_m1 |
| Ccr5 | FAM | Mm01216171_m1 |
| Ccr6 | FAM | Mm01700298_m1 |
| Ccr7 | FAM | Mm01301785_m1 |
| Ccr8 | FAM | Mm01351703_m1 |
| Ccr9 | FAM | Mm02528165_s1 |
| Ccr10 | FAM | Mm01292449_m1 |
| Cxcr1 | FAM | Mm00731329_s1 |
| Cxcr2 | FAM | Mm00438258_m1 |
| Cxcr3 | FAM | Mm00438259_m1 |
| Cxcr4 | FAM | Mm01292123_m1 |
| Cxcr5 | FAM | Mm00432086_m1 |
| Cxcr6 | FAM | Mm00472858_m1 |
| Cxcr7 | FAM | Mm00432610_m1 |

| | | |
|---|---|---|
| Cx3cr1 | FAM | Mm01301785_m1 |
| Ccrl1 | FAM | Mm01232441_m1 |
| Actb (Beta-actin) | FAM | Mm01205647_g1 |
| B2M (beta 2-microglobulin) | FAM | Mm00437762_m1 |
| DARC | FAM | Mm04207950_g1 |
| Foxo3 | FAM | Mm01185722_m1 |
| Hprt | FAM | Mm01545399_m1 |
| Itga4 | FAM | Mm01277951_m1 |
| c-Kit | FAM | Mm00445212_m1 |
| Vwf | FAM | Mm00550376_m1 |

**Table 2-3 Genes used for Taqman Low Density Array**

| Gene | Probe | Catalogue number |
|---|---|---|
| Il1b | FAM | Mm00434228_m1 |
| Il4 | FAM | Mm00445259_m1 |
| Il10 | FAM | Mm00439616_m1 |
| Il12b | FAM | Mm01288990_m1 |
| Il13 | FAM | Mm00434204_m1 |
| Il17a | FAM | Mm00439619_m1 |
| Il22 | FAM | Mm00444241_m1 |
| Tnfa | FAM | Mm00443258_m1 |
| Ifng | FAM | Mm00801778_m1 |
| Tgfb1 | FAM | Mm00441724_m1 |
| 18S | FAM | Hs99999901_s1 |
| Tbp | FAM | Mm00446971_m1 |
| Ccl2 | FAM | Mm00441242_m1 |
| Ccl3 | FAM | Mm00441258_m1 |
| Ccl4 | FAM | Mm00443111_m1 |
| Ccl5 | FAM | Mm01302427_m1 |
| Ccl6 | FAM | Mm01302419_m1 |
| Ccl7 | FAM | Mm00443113_m1 |
| Ccl17 | FAM | Mm00516136_m1 |
| Ccl19 | FAM | Mm00839967_g1 |
| Ccl20 | FAM | Mm00444228_m1 |
| Ccl21 | FAM | Mm03646971_gH |
| Ccl22 | FAM | Mm00436439_m1 |
| Ccl27a | FAM | Mm01215829_m1 |
| Cxcl2 | FAM | Mm00436450_m1 |
| Cxcl5 | FAM | Mm00436451_g1 |
| Cxcl10 | FAM | Mm00445235_m1 |
| Cxcl11 | FAM | Mm00444662_m1 |
| Cxcl12 | FAM | Mm00445553_m1 |
| Cxcl13 | FAM | Mm00444534_m1 |
| Cxcl14 | FAM | Mm00444699_m1 |
| Cx3cl1 | FAM | Mm00436454_m1 |

## 2.7 RNA extraction from tissues

WT mice received topical imiquimod cream or TPA treatment exactly as described in section 2.2.1, after which mice were culled by $CO_2$ asphyxiation. The shaved dorsal skin tissues (that directly received the treatment) were harvested into RNAlater (Qiagen) and kept overnight at 4°C. From these same mice, spleen and bones [2x (tibia +femur + hip bone)] were also harvested into RNAlater (Qiagen) and kept overnight at 4°C. On the next day, skin tissue was each placed in a 2mL microcentrifuge tube, and 1mL of TRIzol® reagent (Ambion) was added to each tube. Two 5mm stainless steel beads (Qiagen) were added to each tube and then placed in TissueLyser LT machine (Qiagen) set at 500Hz, to homogenize the tissues, for 10 minutes. The TRIzol/tissue lysate mixture was then incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. 200μL of chloroform was next added to each tube and shaken vigorously (by hand) for 15 seconds followed by 3 minutes incubation at room temperature. Following this, each sample was centrifuged for 15 minutes at 12000xg (4°C). After the centrifugation, the upper phase containing the RNA (~600μL) was transferred to a fresh 1.5mL microcentrifuge tube. Next, an equal volume of 70% ethanol was added to each sample, and then thoroughly mixed by vortexing. Next, the RNA samples were added to the PureLink™ RNA mini kit Spin cartridge columns, washed and eluted in accordance with manufacturer's instructions (Ambion, Paisley, UK) so as to purify the RNA. Genomic DNA contamination was removed by incubating each column with 80μL of PureLink DNase digestion cocktail for 15 minutes at room temperature. Afterwards, the columns were washed respectively with 500μL of Buffer I and II and then eluted with 50μL of RNase free water. The RNA concentrations were subsequently measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Loughborough, UK). The stock RNA samples were kept at -80°C until required for downstream processing such as RNA quality verification and cDNA synthesis.

### 2.7.1 Measurement of RNA quality and degradation

Although the NanoDrop 2000 spectrophotometric technique was able to give the RNA concentration of the skin samples, it was deemed necessary to verify the quality of the RNA before using it for downstream gene profiling assay. This was

to ensure that the samples were devoid of contaminants or degraded products that could inadvertently affect the gene expression data. Therefore, the Agilent 2100 Bioanalyzer (Agilent Technologies) was employed to determine the quality and integrity of RNA samples. The advantages of the Agilent 2100 Bioanalyzer technology include not only the ability to accurately measure the RNA concentration of these samples from low cell numbers, but also the RNA Integrity Number (RIN) that gives an indication of low molecular weight impurities/contaminants. The RIN is a classification system that assigns a number 1 (most degraded) through to 10 (non-degraded) to total eukaryotic RNA based on a software algorithm, and thereby removing all forms of user-dependent bias. The output results from the Agilent 2100 Bioanalyzer technology also include an electropherogram plot and gel images that enables one to identify the cause of a poor RIN, i.e. contaminants and/or degradation. For skin RNA samples used for TLDA gene analysis, the RNA Nano LabChip kit (Agilent technology, Edinburgh, UK) was used to determine the RIN and also validate the NanoDrop 2000 spectrophotometric RNA concentration.

### 2.7.2 Complementary DNA (cDNA) synthesis for RNA isolated from LK cells

The high capacity RNA-to-DNA kit (Applied Biosystems, Paisley, UK) was used for the cDNA synthesis in accordance with manufacturers' protocol. A volume of RNA isolate equivalent to 30ng was added to 1μL of 20X enzyme mix [contains MultiScribe MuLV (Moloney murine leukaemia virus) reverse transcriptase enzyme and RNase inhibitor protein], 10μL of 2x reverse transcriptase buffer (contains dNTPs, random octamers and Oligo dt-16) and made up to a total of volume of 20μL with Nuclease-free water for the cDNA synthesis. The resultant mixture was spun down at 300xg for 1 minute and incubated on a Veriti® thermocycler 2 (Applied Biosystems, Paisley, UK) with the following cycling condition:

| | |
|---|---|
| 37°C | 60 minutes |
| 95°C | 5 minutes |
| 4°C | ∞, hold |

Each cDNA sample was diluted 1:5 with nuclease-free water and kept at -80°C until required for QPCR.

### 2.7.3 cDNA synthesis from LSK cells

WT mice subjected to topical imiquimod cream/TPA treatment exactly as described in section 2.2.1. 200 bone marrow LSK (lineage marker negative, c-Kit+ and Sca-1+), or 100 peripheral blood LSK cells, were sorted into respective wells of a 96-well plate containing 5μL of 2X Reverse Transcriptase Specific Target Amplification (RTSTA) master mix reaction buffer to enrich samples for genes of interest. The plate was vortexed to facilitate the cell lysis process, centrifuged at 300xg for 60 seconds and then pre-amplified on a Veriti® thermocycler 2 (Applied Biosystems, Paisley, UK) according to the following cycling conditions:

| | | |
|---|---|---|
| 50ºC | 15 minutes | |
| 96ºC | 2 minutes | |
| 95ºC | 15 seconds | ⎫ |
| 65ºC | 4 minutes | ⎭ 22 cycles |

Next, the plate was centrifuged at 300xg for 60 seconds, the samples were diluted 1:5 with DNA re-suspension buffer (Invitrogen, Paisley, UK) and kept at -20°C until required for QPCR. To check for genomic DNA contamination, other reaction mixes were also set up exactly as above, but with Taq polymerase replacing the Superscript III RT/Platinium Taqmix in the 2X RTSTA buffer so as to obtain reverse transcriptase (RT) controls. The QPCR reactions were undertaken using the Fluidigm 48.48 Dynamic array platform as described in section 2.7.6.

### 2.7.4 cDNA synthesis from LT-HSC, MPP1, MPP2, and MPP3 populations

To profile the HSPC for chemokine receptor gene expression in mice were treated with I.P. LPS or I.P. PBS injection exactly as described in section 2.2.2. The cells were next stained for differential expression of Lin, Live/Dead Aqua, c-Kit, Sca-1, CD48 and CD150 antigens in accordance with the signalling lymphocyte antigen molecule (SLAM) staining protocol described in section 2.5.3 (Kiel et al., 2005). The LT-HSC, MPP1, MPP2 and MPP3 populations were immunophenotypically defined as $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD150^+$, $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^+CD150^+$, $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^+CD150^-$, and $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD150^-$ respectively. 200 cells from each of the LT-HSC, MPP1, MPP2 and MPP3

populations were FACS sorted on FACSAria II (BD Biosciences, Oxford, UK) into 96-well plate containing 5μL of 2X Reverse Transcriptase Specific Target Amplification (RTSTA) master mix reaction buffer. The cDNA syntheses were undertaken essentially as described in section 2.7.4 above. The QPCR was undertaken using the Fluidigm 48.48 Dynamic array as described in section 2.7.6 below.

### 2.7.5 Fluidigm 48.48 Dynamic Array IFC chip run

The Fluidigm 48.48 Dynamic Array Integrated Fluidic Circuit (IFC) was primed for use in accordance with manufacturers' instructions (Fluidigm BioMark™ HD System). The QPCR was performed in accordance with the protocol for the BioMark System. The cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes (Hot start), and then 40 cycles of 95°C (denaturation) and 60°C for 60 seconds (annealing). Data were then processed using the BioMark Real-time PCR analysis software (version 3.0.4). The RT-QPCR data were next analysed using Microsoft Office Excel (2011). The data were first normalised to *Hprt* gene expression to obtain the comparative Ct values, and subsequently relative to their respective calibrator sample to obtain the $2^{-\Delta\Delta CT}$.

### 2.7.6 Complementary DNA (cDNA) synthesis for Taqman Low Density Array (TLDA)

All cDNA for downstream TLDA assay were synthesised according to the Precision nanoScript Reverse Transcriptase kit protocol (PrimerDesign Ltd.). A total volume of 20μl cDNA was synthesised per each sample in separate reaction tubes as per reaction cocktails below:

*Reaction cocktail 1*

| **Component** | **1 Reaction** |
|---|---|
| RNA template | 1μg-1.5μg |
| Random nanomer primer | 1μL |
| Oligo-dT primer | 1μL |
| Nuclease-free water | Up to 10μL |

*Reaction cocktail 2*

| Component | 1 Reaction |
|---|---|
| NanoScript 10X buffer | 2μL |
| dNTP Mix 10nM of each | 1μL |
| DTT 100nM | 2μL |
| NanoScript enzyme | 1μL |
| Nuclease-free water | 4μL |
| Final volume | 10μL |

Approximately 1.5μg RNA was added to reaction cocktail 1 (see above) and heated to 65˚C for 5 minutes and then cooled immediately on ice. Following this, 10μL of reaction cocktail 2 was added per reaction tube, spun down and subjected to the following cycling conditions:

| | |
|---|---|
| 25°C | 5 minutes |
| 55°C | 20 minutes |
| 75°C | 15 minutes |
| 4°C | ∞, hold |

Each reaction tube was spun down at 300xg for 1 minute and diluted 1:5 with nuclease-free water and kept at -80°C until required for TLDA gene profiling.

## 2.7.7 Taqman QPCR

| Component | Volume per reaction |
|---|---|
| 2X Taqman Universal Master Mix | 5.0μL |
| cDNA template | 1.5μL |
| Nuclease-free water | 3.5μL |

To avoid intra-sample variability, a master-mix consisting of all the components listed above was prepared, vortexed and spun down to ensure thorough mixing of all components. 9μL of the master-mix was pipetted into each well and 1μL Taqman primer probe was added per well to make a 10μL total reaction volume. Each sample reaction was carried out in triplicate to validate the reproducibility of results. The QPCR reactions were carried out in a 384-well plate on the 7900HT Fast Real-time PCR system (Applied Biosystems) programmed according to the following cycling conditions:

95ºC 2 minutes

95ºC 15 seconds
65ºC 60 seconds } 40 cycles

Data were analysed by RQ-manager version 1.2.2 (Applied Biosystems, Paisley, UK).

Non-amplification in the RT and water controls indicated the absence of genomic DNA contamination and contamination from the reagents respectively.

To allow for plate-to-plate comparison, the same automatically generated threshold fluorescence number (Ct) was used to compensate for background fluorescence signals. This Ct values represent the amplification cycle number at which the recorded fluorescence values is significantly above this pre-set background fluorescence in each sample. The recordings of the Ct values always occur during the exponential phase of the PCR amplification. For relative RT-QPCR assays, the Ct values of a gene of interest in experimental samples was compared directly to the Ct values of that same gene in the relevant control samples (calibrator) to generate the relative quantities; the results were then normalized to that of the relative quantities of a reference gene to normalize for differences in RNA templates and efficiencies of the reverse transcription reactions. These results were then reported as fold changes that gives an indication of how more or less a gene is expressed in experimental groups as compared to its expression in the calibrator (control) sample.

## 2.7.8 Normalisation of QPCR data

In quantifying the gene expression levels in different samples, variation between the gene expression levels between experimental and calibrator groups could be due to actual biological differences or variations introduced as a result of experimental techniques. Several measures were thus taken to compensate for any variations introduced as a result of variability in experimental techniques. For the LSK relative RT-QPCR, equal cell numbers were used as the starting template material. In all other RT-QPCR reactions, equal amounts of starting RNA templates were used. In addition to the above measures, a normalizer/reference gene was selected to compensate for any other sources of variations such as varying PCR amplification efficiencies. Preliminary

experiments showed that TATA Binding Protein (TBP) and HPRT (hypoxanthine-guanine phosphoribosyltransferase) were more robust to the experimental conditions and were thus selected.

## 2.8 Mouse *in vitro* methylcellulose colony-forming assays

As discussed in the general introductory chapter, the gold standard for assessing the functionality of HSC is the competitive repopulation assay in lethally ablated hosts. However, the haematopoietic colony-forming assay offers the opportunity to test the presence of haematopoietic progenitor cell (HPC) population in a given sample as it provides the necessary growth nutrients that permit the in vitro multiplication of these progenitor cells. The choice of the media selected determines which progenitor cell population could be detected as a single media that permits the growth of all lymphomyeloid progenitors was yet to be manufactured.

### 2.8.1 *In vitro* mouse HPC colony-forming assay.

#### 2.8.1.1 Introduction

All haematopoietic colony-forming assays involving tissues from mice were undertaken using myeloerythroid growth supporting media, Methocult™ GF M3434 (StemCell Technologies, Vancouver, Canada). This is a semi-solid methylcellulose-based media containing supplements of recombinant human (rh) insulin, rhErythropoietin, fetal bovine serum, ß-mercaptoethanol, recombinant murine (rm) stem cell factor, rmIL-3 and rmIL-6, thereby allowing the identification and enumeration of myeloerythroid colonies. 1x $10^5$ leucocytes from peripheral blood or spleen (see below) were seeded into 1.1mL of Methocult™ GF M3434 and incubated for 7-10 days at 37°C in a humidified chamber in the presence of 5% $CO_2$. After the incubation period, haematopoietic progenitor cell-derived colonies were enumerated using an inverted microscope.

#### 2.8.1.2 Inflammation-induced HPC mobilisation assay

To investigate the potential of peripheral inflammation inducing the mobilisation of haematopoietic stem and progenitor cells from the bone marrow to the

circulation, WT mice received topical imiquimod cream/TPA treatment exactly as described in section 2.2.1. Other groups of mice were given I.P. LPS injection as described in section 2.2.2 to investigate the ability of systemic inflammation to mobilise HSPC to the circulation. At the end of each model, mice were culled by $CO_2$ asphyxiation and peripheral blood harvested into EDTA anti-coagulated tubes. Next, the red blood cells were lysed with ice-cold Ammonium Chloride solution at a ratio of 10 parts Ammonium chloride solution: 1 part of blood. Next, $1x10^5$ leucocytes were seeded into MethocultTM GF and incubated exactly as described in section 2.8.1.1.

In other experiments, CCR2 KO mice were used instead of WT mice; however, the experimental details were essentially the same as described above.

#### 2.8.1.3 Ligand-induced haematopoietic stem and progenitor cell mobilisation assay

**Table 2-4 Dosage of ligands used for HPC mobilisation assay**

| Ligand | Dosage & route | Duration |
|---|---|---|
| rmCCL2 | 50µg/kg or 75µg/kg body weight S.C. | 30-60 minutes |
| rmCCL4 | 50µg/kg or 75µg/kg body weight S.C. | 30-60 minutes |
| rmCCL11 | 50µg/kg or 75µg/kg body weight S.C. | 30-60 minutes |
| rhCCL3 | 75µg/kg body weight S.C. | 30 minutes |
| rhCCL7 | 75µg/kg body weight S.C. | 30 minutes |
| AMD3100 | 5mg/kg body weight | 60 minutes |
| Soluble imiquimod | S.C 100µg soluble imiquimod per mouse per day | 3 days |

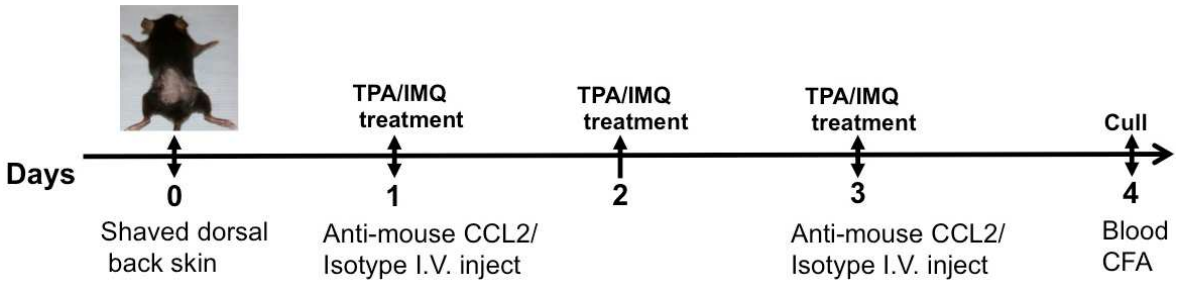
Recombinant murine (rm) CCl2, rmCCL4, rmCCL11, rhCCL3, and rhCCL7 were purchased from Peprotech (London, UK). These chemokine ligands were initially dissolved in minimal volume of sterile water and made up to 1mg/mL final concentration with 0.1%$^{w}/_{v}$ BSA (Bovine Serum Albumin) to prevent adsorption of the chemokines onto the tubes. Imiquimod was purchased from

InvivoGen(Toulouse, France) and reconstituted in 1X DPBS at 1mg/mL. AMD3100 was purchased from Sigma-Aldrich (Irvine, UK) and reconstituted in 1X DPBS at 1mg/mL. WT mice were given doses of one of the ligands for duration indicated in table 2-4 above. For the chemokine ligand mobilisation assay, equivalent volume of 0.1%$^{w}/_{v}$ BSA was used as a control, whereas in AMD3100 or soluble imiquimod injections, equivalent volumes of 1X DPBS were injected into mice as controls. At the end of the specific treatment protocol, the mice were culled by $CO_2$ asphyxiation and peripheral blood samples were taken into EDTA microtainer tubes (BD Biosciences, Oxford, UK). After lysing RBCs with 10x volumes of Ammonium chloride solution (StemCell Technologies, Vancouver, Canada), $1x10^5$ leucocytes were plated in 1.1mL Methocult$^{TM}$ GF M3434 (StemCell Technologies, Vancouver, Canada), incubated and HPC subsequently enumerated exactly as described in section 2.8.1.1.

In other experiments, CCR2 KO mice were used instead of WT mice; however, the details of the experimental protocols were exactly as described above.

### 2.8.1.4 *In vivo* Ccl2 neutralisation assay

A schematic of the *in vivo* Ccl2 neutralisation experimental procedure is shown below:




For Ccl2 in vivo neutralisation experiment, anti-mouse CCL2 affinity purified polyclonal antibody (catalogue no: AF-479-NA) was purchased from R&D systems (Abingdon, UK) and reconstituted in 1X DPBS to 1μg/mL. Normal goat IgG control antibody (catalogue no.: AB-108-C) was also purchased from R&D systems (Abingdon, UK) as a negative control. Three groups of WT mice labelled A, B and C, were set up for these experiments. Mice in groups A and B each received 62.5mg imiquimod cream application on shaved dorsal skin for three consecutive

days. However, mice in group A each received a single intravenous dose of 15μg anti-mouse CCL2 affinity purified polyclonal antibody on day 1, and day 3; on the other hand, mice in group B, each received 15μg normal goat IgG control antibody also on day 1 and day 3. Mice in group C received topical aqueous cream treatment for three consecutive days. Mice in all three groups i.e. A, B and C were euthanized in a $CO_2$ chamber on day 4 and peripheral blood taken for haematopoietic colony-forming assay as described in section 2.8.1.1.

## 2.9 Enzyme-linked immunosorbent assay (ELISA)

### 2.9.1 Plasma samples

WT mice were given either the topical imiquimod cream/TPA treatment or the I.P. LPS injection exactly as described in section 2.2.1 and 2.2.2 respectively. After the duration of the treatment elapsed, mice were culled by $CO_2$ asphyxiation and blood taken into EDTA anti-coagulated tubes. Blood was spun at >8000g and plasma was taken and kept at -80ºC until required for ELISA.

### 2.9.2 Extraction of bone and bone marrow extracellular fluid proteins

WT mice were treated with either topical imiquimod cream/TPA or I.P. LPS injection exactly as described in sections 2.2.1 & 2.2.2 respectively. The mice were culled by $CO_2$ asphyxiation and femur and tibia were harvested from these mice and cleared of muscle tissue. After cutting the ends of each bone, 1mL of PBS was used to repeatedly flush out the marrow. The marrow washout was then pipetted up and down several times to loosen the cell aggregates and then spun at 400xg for 10 minutes. The bone marrow supernatant, henceforth called bone marrow extracellular fluid was kept frozen at -80ºC until needed for CXCL12 ELISA. Next, the hollow cortical bones were homogenised in 400μL of mammalian protein extraction reagent (M-PER, Thermo-Scientific, Pearce, UK) using a TissueLyser LT machine (Qiagen, Manchester, UK) set at 500Hz (for 10 minutes). Afterwards, the cortical bone homogenates were centrifuged at >10,000xg for 10 minutes. The supernatants were kept frozen at -80ºC until needed for CXCL12 ELISA.

In some experiments, whole bone marrow proteins (i.e. bone marrow + bone tissue proteins) were extracted by homogenising whole tibia and femur bones in 400µL mammalian protein extraction reagent (M-PER, Thermo-Scientific, Pearce, UK) using the TissueLyser LT machine (Qiagen) set at 500Hz (for 10 minutes). The whole bone marrow homogenates were spun at 10,000 xg for 10 minutes and the supernatants were kept frozen at -80ºC until required for CXCL12 ELISA.

#### 2.9.2.1 CCL2 ELISA experimental procedure

For quantitative estimation of mouse peripheral blood CCl2, mouse CCL2 (MCP-1) ELISA Ready-SET-Go (Catalogue no. 88-7391) was purchased from eBiosciences (Hatfield, UK) and used in accordance with manufacturers' protocol. A Corning Costar 9018 ELISA plate was coated with 100µL/well of capture antibody (reconstituted in coating buffer) and the plate was sealed and incubated overnight at 4°C. On the next day, each well was washed 5x with 300µL washing buffer (0.05%v/v Tween 20 in 1x DPBS; pH 7.2-7.4). After blotting the plate with absorbent paper, each well was blocked with 200µL of 1X assay diluent and incubated at room temperature for 1 hour. Next, each well was washed (3x) with ~300µL washing buffer, blotted with absorbent paper, and 100µL of the top standard and serial dilutions of the standards were added to the respective wells in duplicates. 100µL of neat, as well as 1:5 and 1:10 dilutions of samples (dilutions made with 1X ELISA diluent), were next added to their respective wells and incubated at room temperature for 2 hours. After washing each well 5 times and blotting with absorbent paper, 100µL of detection antibody was added to each well and incubated for 1 hour at room temperature. Afterwards, each well was washed 5x with washing buffer, and blotted thoroughly, 100µL of Avidin-HRP was added to each well, sealed and incubated at room temperature for 30 minutes. Each well was next washed 7x with washing buffer and blotted with absorbent paper, 100µL of 1X TMB substrate solution was added to each well and then incubated at room temperature for another 15 minutes. 50µL of stop solution (2N $H_2SO_4$) was then added to each well and the optical densities of each well in the plate was immediately read firstly, at 450nm, and then at 550nm, on a Tecan Sunrise™ microplate reader (Tecan, Maennedorf, Switzerland). Subtracting optical density readings at 550nm from each result corrected for background optical density interferences. The corrected optical

densities of the top standard and its serial dilutions were used to generate a standard curve and the data analysed by Magellan™ software (Tecan).

#### 2.9.2.2 Mouse CXCL12 ELISA

The mouse Quantikine® CXCL12/SDF-1α ELISA kit (catalogue no. MCX120; R & D Systems) was used to determine the quantities of Cxcl12 in plasma, bone and bone marrow extracellular fluid in accordance with manufacturer's instruction. 0.1mg of total protein from whole bone marrow or bone marrow extracellular fluid or bone protein extract was used for the assay. Sufficient volume of Assay Diluent was added to volumes of samples equivalent to 0.1mg to make up to 50μL and subsequently transferred to wells of a 96-well microplate. 50μL of serially diluted mouse CXCL12 standard, or mouse CXCL12 control were also added to the relevant wells. The plate was then sealed with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. The subsequent washing of the plate and further processing were essentially as stated in the manufacturer's protocol. The optical density of each well of the plate was determined using a Tecan Sunrise™ microplate reader (Tecan, Maennedorf, Switzerland) set to 450 nm wavelength, with wavelength correction set at 570 nm.

## 2.10 Mouse Bone marrow and plasma proteome array

### 2.10.1 Sample preparation

Plasma and whole bone marrow protein was harvested from mice (WT or CCR2 KO mice) treated with topical imiquimod cream/TPA (see section 2.2.1) exactly as described in sections 2.9.1 and 2.9.2. In order to obtain representative data, plasma was pooled from three mice per treatment and 150μL was used for each array. Also, bone marrow protein extracts were pooled from three mice per treatment and ~300μg used for the proteome profile array described below.

### 2.10.2 Bone marrow and plasma angiogenesis proteome array

The plasma and bone marrow angiogenesis proteome profiling was done in accordance with manufacturer's instructions using the mouse angiogenesis array

kit (Cat. No. ARY015; R&D Systems, Abingdon, UK). The 4 array membranes were each placed in separate wells containing 2.0mL of Array Buffer 6 to block the membrane against non-specific binding. Next, the membranes were incubated at room temperature on a rocking platform shaker for 60 minutes. Simultaneously, sample/antibody mixture was constituted by adding the requisite volumes of samples (bone marrow homogenate or plasma protein) to 500µL of array buffer 4 in separate tubes. After adjusting the sample:array buffer 4 volume to 1.5mL with array buffer 6, 15µL of detection antibody cocktail was added and incubated for 60 minutes at room temperature. When the incubation period elapsed, the blocking buffer (array buffer 6) was aspirated from each well, the respective sample/antibody mixture was added to the wells, covered and incubated overnight at 4°C on a rocking platform. The next day, each membrane was washed three times in 20mL of 1X wash buffer on a rocking platform for 10 minutes each. Afterwards, each membrane was incubated in Streptavidin-HRP on a shaking platform for 30 minutes at room temperature. Each membrane was then washed 3X in 20mL of 1X wash buffer on a rocking platform for 10 minutes each. Next, each membrane was completely blotted on absorbent paper, placed on a plastic sheet protector with the identification number facing up. One millilitre of Chemi-Reagent Mix was added to each membrane, covered with a plastic sheet protector and subsequently smoothed out to ensure uniform distribution to all corners of the blot. Next, the membranes were incubated for 1 minute and then thoroughly blotted with absorbent paper. Afterwards, the membranes were placed in an autoradiography film cassette with their identification numbers facing up, exposed to X-ray film for 1-10 minutes and the film developed. Developed films were scanned with Epson Perfection 3490 Photo and signal intensities of developed films were analysed with ImageJ version 1.46r (National Institute of Health, USA).

### 2.10.3 Plasma cytokine proteome array

The cytokine proteome array kit (catalogue no: ARY006) was purchased from R & D Systems (Abingdon, UK). The procedure for the array was essentially as described for the angiogenesis array described in section 2.10.2 above, except that plasma as well as antibody cocktail detecting cytokine/chemokines were used.

## 2.11 CCL2 chemokine transmigration assay

To investigate the functional relevance of Ccr2 expressed on haematopoietic stem and progenitor cells, an *in vitro* chemotaxis assay utilising CCl2 was employed. Recombinant murine CCL2 was purchased from PeproTech (London, UK), reconstituted in sterile water and made up to 1ng/mL in chemotaxis buffer. 24-well transwell plates (6.5mm diameter inserts, 5.0μm pore size) were purchased from Corning Incorporated (Radnor, USA). Bone marrow cells from mice given topical Imiquimod cream/TPA treatment or LPS injection (as described in sections 2.2.1 and 2.2.2 respectively) were processed for FACS (as described in section 1.5.1) and stained with biotinylated lineage antibody cocktail, c-Kit-APC-Cy7 and Live/Dead Aqua exactly as described in section 1.5.2. $2x10^5$ LK cells (Lin-, c-Kit+ haematopoietic progenitor cells) were sorted on FACS Aria II (BD Biosciences) into FACS sorting buffer, and then washed in 1mL of chemotaxis buffer. The cell were spun down at 300xg (for 5 minutes at 4ºC) and re-suspended in 100μl of chemotaxis buffer. 50ng of CCL2 was added to 600uL chemotaxis buffer in the lower well chamber. The inserts were placed back into each well with chemokine-chemotaxis buffer and incubated at 37°C for 10 minutes to allow equilibration. The 100ul cell suspension ($2x10^5$) was then added to the inserts and incubated for 4-5 hours at 37°C, in the presence of 5% $CO_2$. After the incubation period, the inserts were carefully removed with forceps and transferred into a 24-well plate. 400μL of chemotaxis buffer in the lower chamber containing migrated cells were cultured in Methocult™ GF M3434 for 7-10 days, and colonies were visualised and enumerated using an inverted bright-field microscope.

## 2.12 Statistical analyses

Data analyses were undertaken using Prism 5 software (GraphPad, San Diego, USA). Unless otherwise indicated, results are presented as mean ± standard error of mean (SEM). Prior to analysis, all data were first tested for normality using the D'Agostino and Pearson omnibus normality test and Kolmogorov-Smirnov test to verify if they were normally distributed. For samples that passed the normality test, a 2-tailed unpaired t-test was used to compare each treatment group with its respective control; for samples not passing the normality test, two groups were compared using the 2-tailed Mann-Whitney U test. Data comparing

more than two groups were analysed using 2-tailed Kruskal-Wallis test with Dunn's post-test, unless the data passed the normality test in which case they were compared using One-Way ANOVA with Tukey's post-test. Statistical significance were defined as *$p<0.05$, **$p< 0.01$, ***$p< 0.0001$.

# Chapter 3: Systemic inflammation model

## 3.1 Aims and introduction

Lipopolysaccharide (LPS) is one of the conserved molecular patterns in Gram-negative bacteria that are recognized by the mammalian TLR4. Accordingly, LPS has been used as a model of Gram-negative bacterial infection (Haziot et al., 1996, Rietschel et al., 1994). In the context of haematopoietic stem and progenitor cell (HSPC) biology, LPS has been shown to induce a significant mobilisation of HSPC to the circulation when administered through intra-peritoneal or intravenous routes (Vos et al., 1972, Quesenberry et al., 1973). Although it was shown recently that HSPC express TLR4 (Nagai et al., 2006), the mechanisms that orchestrate this LPS-induced HSPC mobilisation are still unclear. Chemokines are small chemotactic cytokines, and one of these chemokines, CXCL12, orchestrates migration of stem cells to specific anatomical sites during embryonic development. In addition, other chemokines also play fundamental roles in orchestrating the migration of leucocytes to sites of infection and inflammation in adult organisms. Due to the crucial roles played by chemokines in orchestrating *in vivo* cellular migration, I **hypothesised** that HSPC inducibly express chemokine receptors in response to LPS injection and that manipulation of the chemokine-chemokine receptor axis was crucial in the LPS-induced HSPC mobilisation and subsequent migration to inflamed sites. Additionally, as the retention of HSPC in the bone marrow niche is aided by specific HSPC-niche cell interactions, and/or HSPC interactions with soluble factors elaborated by these niche cells, I further **hypothesised** that the HSPC mobilisation induced by systemic LPS injection is also related to modulation of these niche cells and/or their secreted soluble factors. To test these hypotheses, I aimed to address the following questions:

1. Does TLR4 ligation/LPS injection orchestrate HSPC mobilisation?

2. Does systemic LPS injection alter the HSPC compartment in the bone marrow?

3. Does systemic LPS injection alter the HSPC niche in the bone marrow?

4. What cytokines and chemokines are induced in response to systemic LPS injection?

By addressing these questions, I sought to obtain a comprehensive understanding of the roles for the chemokine-chemokine receptor axis, and bone marrow niche, in HSPC biology during LPS mobilisation.

## 3.2 Does TLR4 ligation induce HSPC mobilisation?

### 3.2.1 Introduction

To address this question, the intraperitoneal (I.P.) LPS injection was employed to investigate how *in vivo* TLR4 ligation impacted HSPC migration or localisation in C57BL/6 mice. LPS is a product of Gram-negative bacteria and is a known TLR4 ligand. To ensure that any subsequent observations made were purely due to TLR4 signalling, Ultra-pure LPS, which is devoid of contaminating TLR2 ligands, was purchased from InvivoGen. The *in vitro* haematopoietic progenitor cell (HPC) growth-supporting medium selected to enumerate circulating HPC was Methocult™ GF M3434. This M3434 medium is a cytokine-enriched, methylcellulose-based medium that supports the *in vitro* growth of myeloerythroid progenitor cells. Although this assay does not allow the estimation of haematopoietic stem cells (HSC) and lymphoid progenitors, it allows the enumeration of other multipotent haematopoietic progenitors (HPC) such as CFU-GEMM (colony forming unit (CFU)- with granulocyte, erythrocyte, megakaryocyte and macrophage potential), CFU-GM (CFU with granulocyte-macrophage potential), and BFU-E (Burst-forming unit, erythroid), which are downstream of the HSC in the haematopoietic hierarchy.

Mice were given a single dose of either I.P. LPS (100μg in 100μL PBS) or 100μL I.P. PBS as a control. 18-24 hours after the injection, mice were culled by $CO_2$ asphyxiation and peripheral blood was taken through cardiac puncture. After lysing red blood cells with Ammonium Chloride solution, $1x10^5$ cells were seeded into 1.1mL of Methocult™ GF M3434 (StemCell Technologies) and incubated at 37°C in 5% $CO_2$ to enumerate haematopoietic progenitor cells mobilised to the circulation. After 7-10 days of incubation, HPC colonies were visualised and counted using an inverted microscope.

### 3.2.2 Intraperitoneal LPS injection induces HSPC mobilisation.

As summarised in the scatter plot in figure 3-1A, I.P. LPS significantly induced HPC mobilisation to the peripheral circulation in mice compared to PBS treated controls (***p=0.0001; 2-tailed unpaired T-test). On average, LPS injection induced approximately, a 4-fold (22.85/5.6) increase in total circulating progenitor cell numbers compared to PBS treated controls. Further analysis of the colony morphology showed that LPS injection induced increased circulating levels of CFU-GEMM, CFU-GM and BFU-E compared to PBS controls; although the majority of the progenitors were CFU-GM (figure 3-1B).

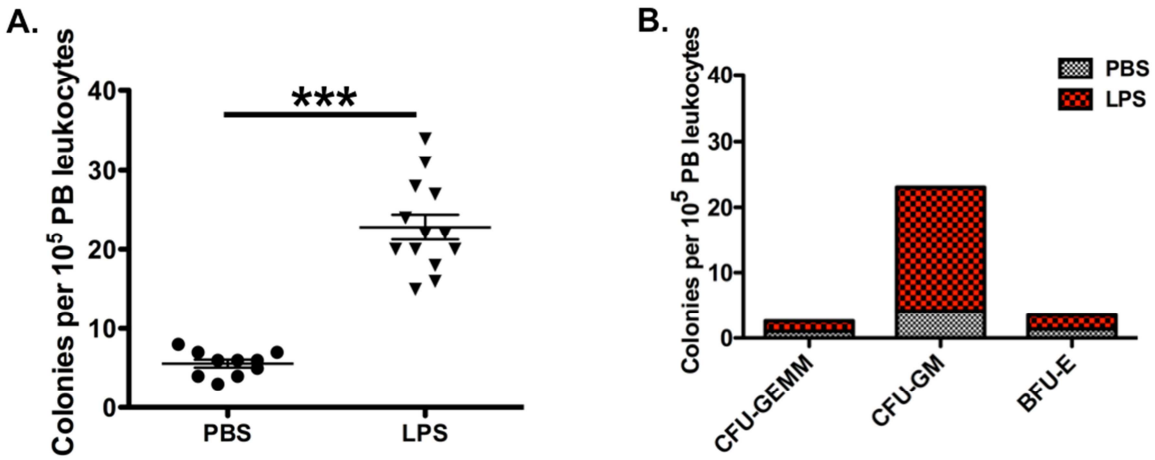



**Figure 3-1 LPS induces haematopoietic progenitor cell mobilisation to peripheral blood.** C57BL/6 mice were either given a single dose of intraperitoneal LPS (100µg in 100µL PBS), or 100µL I.P. PBS injection as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and peripheral blood taken for haematopoietic colony-forming assay. After lysing red blood cells with Ammonium chloride, $1x10^5$ leucocytes were seeded into 1.1mL of Methocult$^{TM}$ GF M3434 medium (StemCell Technologies) in duplicates, and incubated for 7-10 days in a 5% $CO_2$ humidified chamber. Colonies representing haematopoietic progenitor cell were then enumerated using an inverted microscope. In A, the total colonies formed were compared for the LPS treated groups and PBS treated controls. In B, the different myeloerythroid progenitor colonies were compared. [***p=0.0001; 2-tailed, unpaired t-test, n=5-7 mice in each experiment (5 independent experiments); data points are a representation of two independent experiments pooled together; Data were plotted as mean ±SEM].

Taken together, the data presented in this section indicate that LPS injection induces HPC mobilisation to peripheral blood.

## 3.3 Does systemic LPS injection alter the HSPC compartment in the bone marrow?

### 3.3.1 LPS injection significantly reduces bone marrow cellularity.

After establishing that LPS injection mobilised HPC to the circulation, the wider effects of systemic LPS injection on the HSPC compartment (including HSC), was

further explored through fluorescence associated cell sorting (FACS). Mice were given a single dose of 100μg I.P. LPS (or 100μL I.P. PBS as a control) and were culled 24 hours after the injection. Bones [2x (tibia + femur + hip bones) were harvested from each mouse, cleared of muscle and crushed in a mortar and pestle. Red blood cells were lysed with Ammonium chloride solution and total bone marrow cellularity was calculated using a haemocytometer with Trypan Blue dead cell exclusion. As shown in figure 3-2, LPS injection significantly reduced the total bone marrow cellularity compared to PBS treated controls (**p= 0.0014; 2-tailed Mann-Whitney U test). On average, LPS injection reduced the total bone marrow cellularity by approximately 33% compared to PBS treated control [(111.8-75.24)/111.8]*100%.

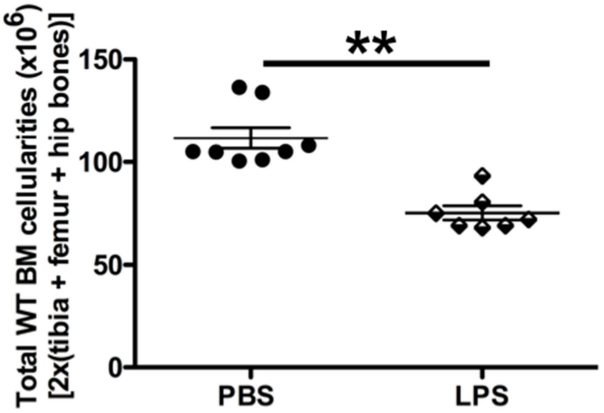



**Figure 3-2 Intraperitoneal LPS injection significantly reduces bone marrow cellularity.** C57BL/6 mice were either given a single dose of I.P. LPS (100μg in 100μL PBS) or 100μL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and bones [2x (tibia + femur + hip bones)] were taken for cell count. Total bone marrow cellularity was estimated using haemocytometer and trypan blue dead cell exclusion. Data were plotted as mean±SEM [**p=0.0014, 2-tailed Mann-Whitney U test; n=7-8 (3 independent experiments)].

### 3.3.2 LPS injection causes an apparent increase in bone marrow HSPC frequency.

In terms of abundance, HSC have been estimated to account for about 0.001-0.01% of total nucleated bone marrow cells in mice (Challen et al., 2009, Kiel et al., 2005). Therefore, to increase the chances of detecting measurable HSC numbers through flow cytometric analysis, the bone marrow cells were c-Kit enriched using the CD117 Microbeads and LS columns (Miltenyi Biotec, Surrey, UK) according to manufacturer's instructions. All HSPC have been shown to express the c-Kit antigen (Zhao et al., 2000) and therefore this enrichment technique allowed for a substantial depletion of cells not expressing this antigen. c-Kit enriched bone marrow cells were subsequently stained for

differential expression of haematopoietic lineage-specific markers (CD4, CD5, CD8a, CD11b, B220, Ter119 and Gr-1), as well as Sca-1, c-Kit, CD150 and CD48 antigens to analyse the HSPC compartment in accordance with the CD150+ SLAM (signalling lymphocyte activation molecule) antibody staining protocol (Kiel et al., 2005). A representative gating strategy used to analyse the HSPC compartment is shown in figure 3-3. Dead cells were excluded using a combination of singlet selection and then Live/dead Aqua fixable dye retention to gate out dead cells. Previous reports have established that HSPC are negative for antigens that identify terminally differentiated haematopoietic cells (Lin$^-$), but are positive for both the stem cell antigen (Sca-1$^+$) and CD117 (c-Kit$^+$) antigens, i.e. the LSK gate in figure 3-3 (Zhao et al., 2000, van de Rijn et al., 1989, Spangrude and Brooks, 1993). Therefore the cells in the LSK gate represent most of the HSPC; these LSK cells were thus selected for subsequent analysis.

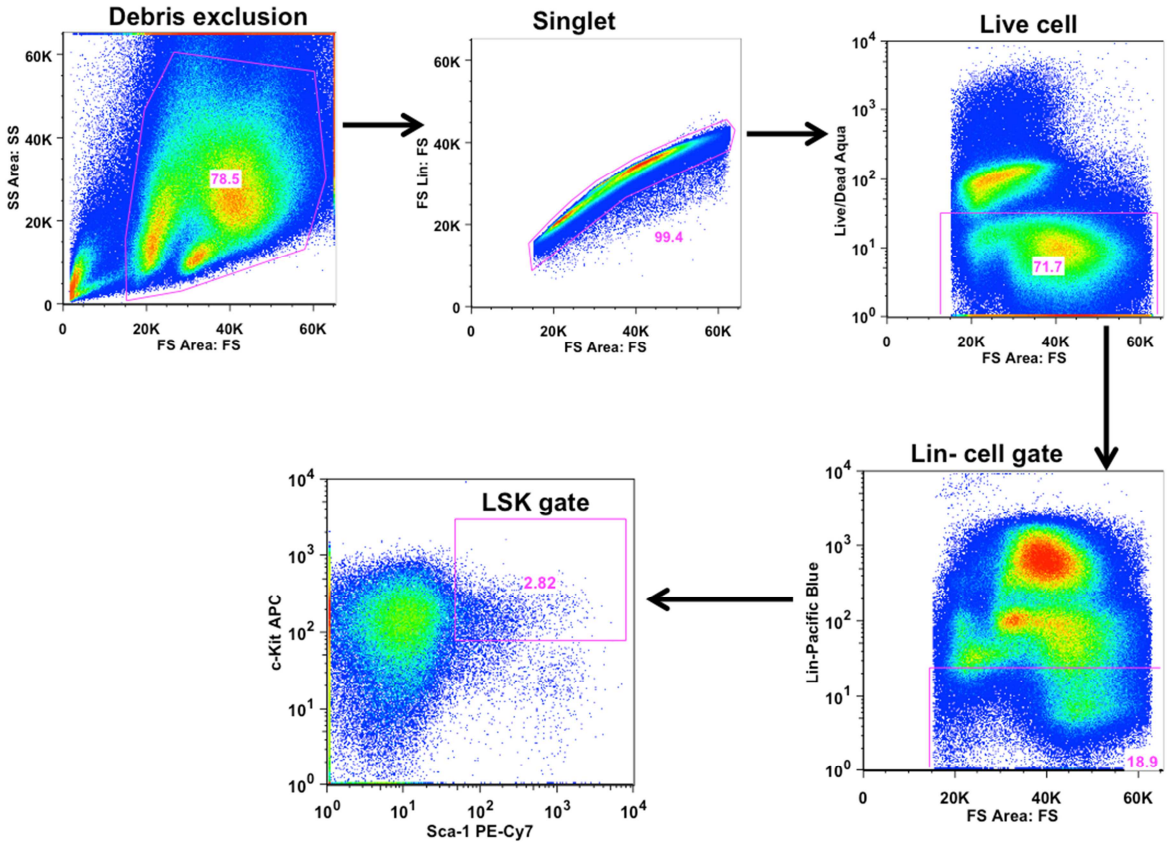



**Figure 3-3 Gating strategy used for haematopoietic stem and progenitor (LSK) cell identification**

C57BL/6 mice were either given a single dose of I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec). The c-Kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), c-Kit, Sca-1, CD48 and CD150 to evaluate the HSPC compartment. After excluding dead cells through doublet exclusion and

Live/Dead Aqua fixable dye retention, cells positive for markers of terminal differentiation were also gated out. Next, cells co-expressing the c-Kit antigen and Sca-1 antigens were selected as the haematopoietic stem and progenitor cells (LSK gate).

A comparison of the proportion of cells in the LSK gate between the LPS and PBS treated mice revealed an apparent expansion of the LSK compartment in response to LPS injection (compare figures 3-4 A & B). Consistent with this apparent increase in the proportion of bone marrow LSK cells, there was a statistically significant increase in the frequency of the LSK cells in the bone marrow of LPS treated mice compared to PBS treated controls (figure 3-4C: p=0.0001; 2-tailed, unpaired t test). The apparent increase in the proportion of LSK cells also reached statistical significance (figure 3-4D: p=0.0001; 2-tailed, unpaired t test). Moreover, on average, the cells in the LSK compartment of the bone marrow of LPS-treated mice were ~6-fold more frequent (1.555/0.273) when compared to the bone marrow of PBS-treated controls.

Altogether, the dot plots of the flow cytometric data show that LPS injection causes a pseudo re-acquisition of Sca-1 expression by some $Kit^{+}Sca\text{-}1^{-}$ cells (compare figures 3-4 A & B) resulting in an apparent expansion of the LSK compartment.

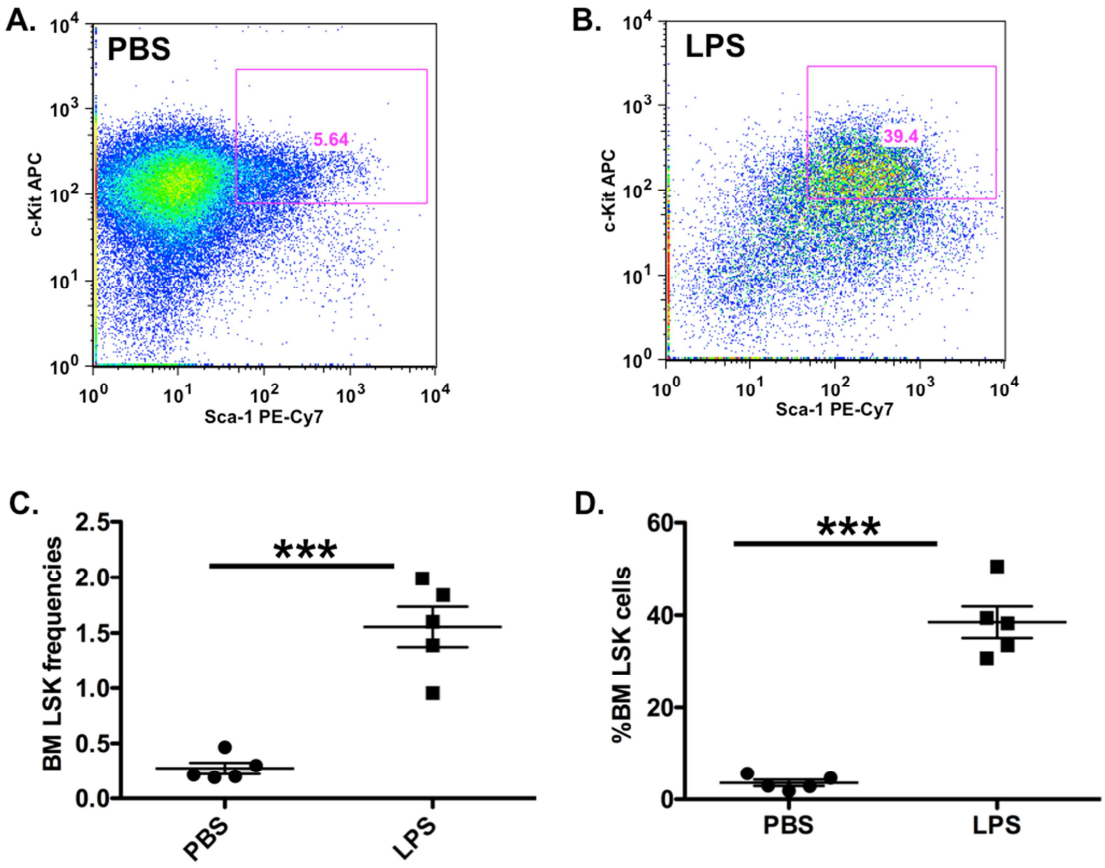


**Figure 3-4 LPS injection causes an apparent expansion of the bone marrow LSK compartment.**
Figures A & B are representative plots of the LSK compartment in the I.P. LPS (100μg in 100μL PBS) or 100μL I.P. PBS injected mice based on the gating strategy shown in figure 3-3. Figure C is a scatter plot comparing the frequency of the cells in the LSK compartment per $1x10^6$ total c-Kit enriched bone marrow cells (***p=0.0001; 2-tailed unpaired t-test; n=5 (three independent experiments)]. Figure D compares the percentages of the LSK cells in the I.P. LPS or I.P. PBS treated mice (***p=0.0001, 2-tailed unpaired t-test; n=5 (three independent experiments)].

### 3.3.3 LPS injection depletes the bone marrow HSPC populations.

The flow cytometric analysis of the HSPC compartment (LSK cells) presented in **section 3.3.2 (figure 3-3A-C)** showed that LPS injection significantly increased Sca-1 expression. This phenomenon of pseudo increase in Sca-1 expression has been reported in other inflammation models (Zhang et al., 2008, Melvan et al., 2011, Shi et al., 2013). Therefore, in order to avoid potential bias introduced by pseudo Sca-1 expression, a new gating strategy that excludes the Sca-1 antigen was adopted to analyse the HSPC compartment. Previously, Adolfsson et al. published a protocol that allowed the immunophenotypic characterisation of the HPC compartment into lymphoid-primed multipotent progenitor cells (LMPP) and common lymphoid progenitor (CLP) cells based on differential expression of CD34 and FLT3 antigens (Adolfsson et al., 2005). This protocol was employed with minor modifications: (1) due to the LPS-induced increase in Sca-1 antigen expression, Sca-1 was excluded from the gating strategy; (2) CD150 expression was included to permit the identification of the LT-HSC population in accordance with Kiel et al. who showed that expression of CD150 antigen identified the LT-HSC population from the haematopoietic progenitor cells (Kiel et al., 2005). Therefore, in the staining protocol employed in the studies below, LMPP, CLP, ST-HSC and multipotent progenitor populations were defined as $Lin^-Kit^+CD34^+FLT3^+$, $Lin^-Kit^+CD34^-FLT3^+$, $Lin^-Kit^+CD34^+FLT3^-$ and $Lin^-Kit^+CD34^-FLT3^-$ respectively in accordance with previously published protocols that were modified on the basis of pseudo Sca-1 expression (Adolfsson et al., 2005, Yang et al., 2005, Osawa et al., 1996).

Figure 3-5 is a representative plot showing the gating strategy used to select the haematopoietic stem and progenitor cells ($Lin^-Kit^+$ cells, henceforth called LK) for further analysis. After excluding cellular debris through forward-side scatter plots, dead cells were excluded using a combination of doublet exclusion and retention of the Live/Dead Aqua fixable dye. After excluding terminally

differentiated haematopoietic cell i.e. $Lin^+$ cells, c-$Kit^{high}$ expressing cells were selected as the haematopoietic progenitor cells, i.e. LK cells.

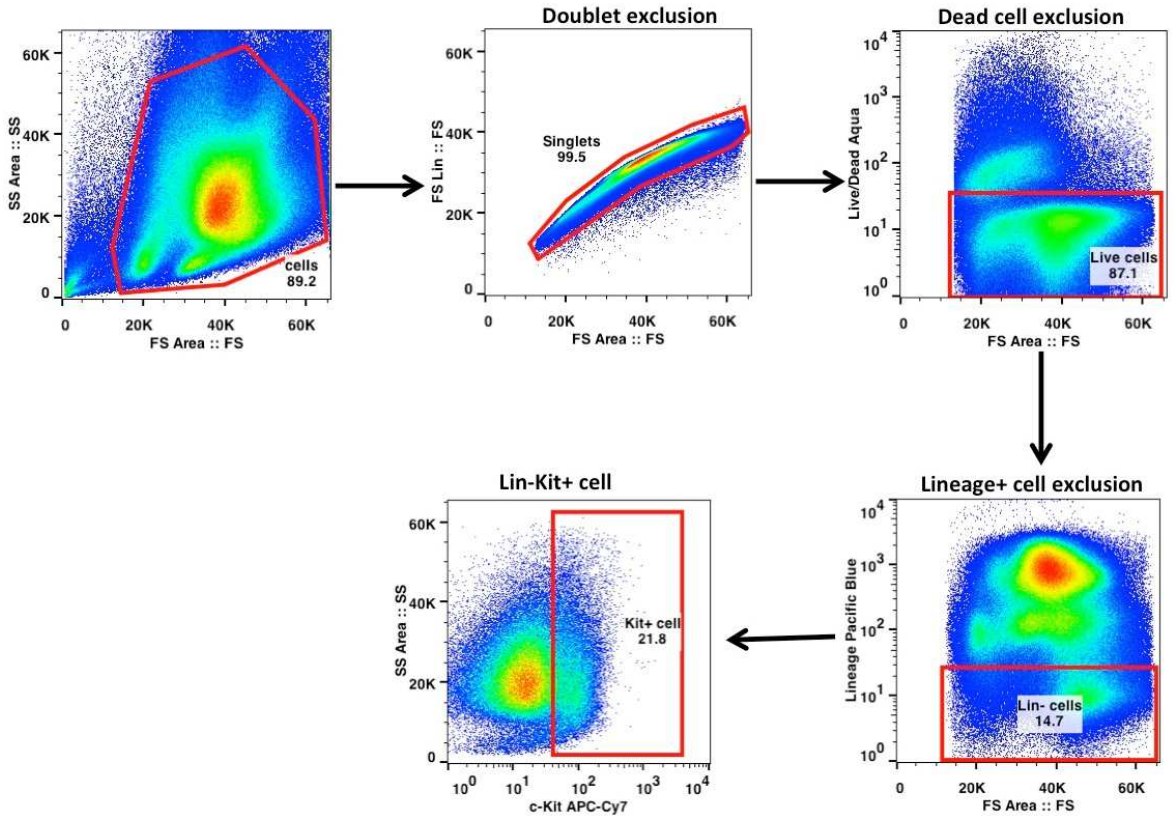



**Figure 3-5 Gating strategy used for haematopoietic progenitor (LK) cell identification**
C57BL/6 mice were either given a single dose of I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec). The c-Kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), as well as c-Kit, Sca-1, CD34, CD150 and FLT3 antibodies to evaluate the HSPC compartment. After excluding dead cells through doublet exclusion and Live/Dead Aqua fixable dye retention, cells positive for markers of terminal differentiation of haematopoietic cells were also gated out. Next, cells expressing high levels of c-Kit antigen were selected as the haematopoietic progenitor cells (LK gate).

Figures 3-6 A and B are representative respective dot plots showing LK cells from PBS or LPS treated mice, re-gated for differential expression of CD34 and FLT3 antigens. As summarised in the bar graph in figure 3-6 C, LPS injection significantly reduced the bone marrow HPC populations. Specifically, LPS injection caused significant reduction in the bone marrow CLP population when compared to PBS treated controls (figure 3-6C, $p<0.0001$; 2-tailed unpaired t-test). In addition, LPS injection significantly reduced the bone marrow LMPP population compared to PBS treated controls (figure 3-6C, $p<0.0001$; 2-tailed unpaired t-test). Moreover, LPS injection also reduced the ST-HSC population compared to PBS treated controls (figure 3-6C, $p<0.0001$; 2-tailed unpaired t-test).

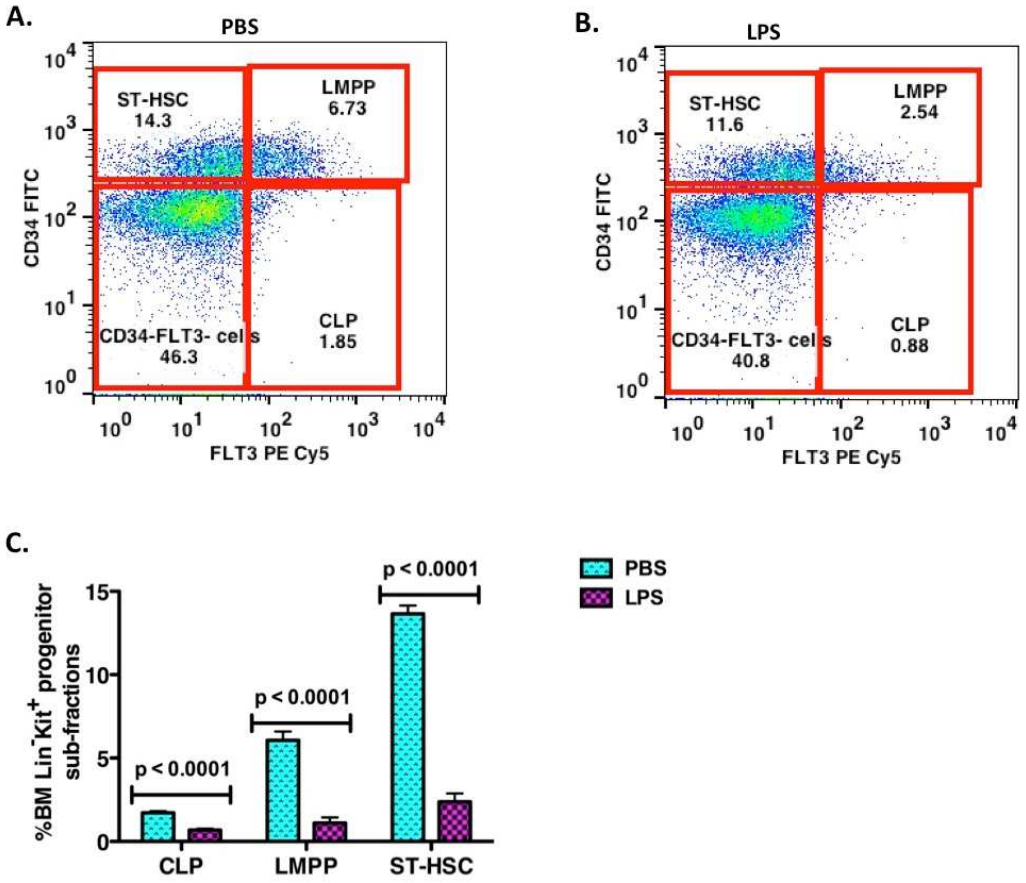



**Figure 3-6 Intraperitoneal LPS-induced inflammation decreases the proportion of HPC with lymphoid potential.**
C57BL/6 mice received a single dose of Ultra-pure I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS. 18-24 hours after the injection, mice were culled by $CO_2$ asphyxiation and bone marrow cells [2x (tibia + femur + hip bones)] were harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer's instructions (Miltenyi Biotec). The c-Kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), as well as c-Kit, CD34, CD150, and FLT3 antibodies to evaluate the HSPC compartment. After excluding dead cells (see gating strategy in figure 3-5), c-Kit+ cells were re-gated for expression of CD34 and FLT3 antigens to delineate CLP, LMPP, ST-HSC and $CD34^-FLT3^-$ multipotent cells. Figures A & B are respective representative dot plots showing the sub-gating of the bone marrow LK cells from PBS and LPS treated mice based on CD34 and FLT3 expression. Figure C is a bar graph summarising the changes in the proportions of the LMPP, ST-HSC, CLP and $LKCD34^-FLT3^-$ multipotent progenitors in response to the systemic LPS-induced inflammation. Statistical significance was estimated using the unpaired t-test (2-tailed) in all cases. (n=5, 2-independent experiments)].

## 3.3.4 LPS injection significantly reduces bone marrow LT-HSC population.

The Lin-Kit+CD34-FLT3- cell population (see gating strategy in section 3.3.3, figure 3-6 **above**) was re-gated for the differential expression of c-Kit and CD150 expression to examine the effect of systemic LPS injection on the long-term haematopoietic stem cell population (LT-HSC). Previously, Kiel et al. published a protocol intimating that CD150 antigen expression enriches for LT-HSC population within the HSPC cells. Figures 3-7 A & B are representative dot plots showing CD150 expression within the $Lin^-Kit^+CD34^-FLT3^-$ multipotent progenitor

cells in the bone marrow from I.P. PBS or I.P. LPS treated mice respectively. As summarised in the scatter plot in figure 3-7 C, LPS injection caused significant reduction in the proportion of the LT-HSC cells (defined as $Lin^{-}Kit^{+}CD34^{-}FLT3^{-}CD150^{high}$ cells) in the bone marrow of C57BL/6 mice compared to PBS treated controls (figure 3-7C, p=0.0005; 2-tailed unpaired t-test).

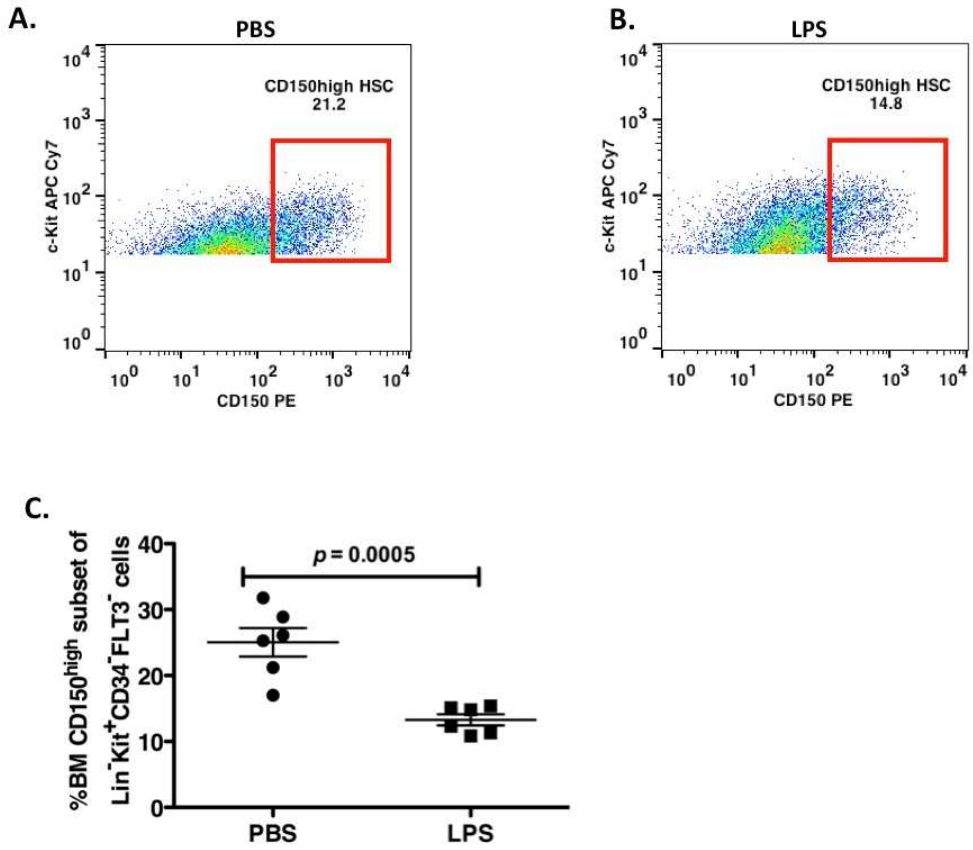



**Figure 3-7 LPS injection significantly reduces the bone marrow LT-HSC population in C57BL/6 mice.**
The $CD34^{-}FLT3^{-}$ cells (see gating strategy in figure 3-6 above) from I.P. PBS treated mice (A) or I.P. LPS treated mice (B), were re-gated for the expression of c-Kit and CD150 antigens to characterise the LT-HSC population. Figure C is a representative scatter plot summarising the effect of the I.P. LPS injection on the LT-HSC population in the bone marrow of C57BL/6 mice. Statistical significance was calculated using the 2-tailed unpaired t-test. [N=6; 2 independent experiments].

In summary, the data presented in this section show that LPS injection altered the HSPC compartment by significantly reducing the bone marrow cellularity that was reflected in significant reductions in the CLP, LMPP, ST-HSC and LT-HSC populations.

## 3.4 Does systemic LPS injection alter the HSPC niche in the bone marrow?

As reviewed extensively in the general introductory chapter, and elsewhere (Ehninger and Trumpp, 2011), the bone marrow niche comprises cellular

components and their secreted soluble products that interact with HSPC to retain them in the bone marrow. Thus, disruption of one or more of these interactions has been shown to be fundamental in influencing HSPC localisation and migration. To obtain a comprehensive picture of how LPS-induced systemic inflammation impacted the HSPC niche, this section of the study sought to address:

1. How LPS injection affected the CXCR4/CXCL12 axis

2. How LPS injection affected the bone marrow stromal cell compartment

3. How LPS injection affected the mononuclear phagocytic cells of the bone marrow.

In this way, I sought to obtain a comprehensive understanding of how LPS injection affected the bone marrow HSPC niche. Ultimately, the main objective was to begin the process of elucidating the basic mechanisms underpinning the HSPC mobilisation seen in the LPS injection model.

## 3.4.1 LPS-induced systemic inflammation modulates the CXCR4-CXCL12 axis in HSPC

### 3.4.1.1 Introduction

In line with the crucial roles played by the CXCR4/CXCL12 axis in the retention of HSPC in the bone marrow niche (Lapidot et al., 2005, Lapidot and Kollet, 2002, Lapidot and Petit, 2002), many reports have shown that perturbation of the CXCR4/CXCL12 signalling axis is one of the fundamental mechanisms underpinning the ability of agents like G-CSF and AMD3100 to mobilise HSPC to the circulation (Christopher et al., 2009, Semerad et al., 2005). As I.P. LPS injection induced haematopoietic progenitor cell mobilisation [as demonstrated in haematopoietic colony forming assay (**figure 3-1, section 3.2.2**)], I sought to understand how the LPS-induced systemic inflammation modulated Cxcr4 expression in the haematopoietic progenitor cells as well as CXCL12 protein concentration in the bone marrow and peripheral blood.

#### 3.4.1.2 LPS injection decreases haematopoietic progenitor cell surface CXCR4 expression.

Flow cytometry was employed to determine cell surface Cxcr4 expression in the haematopoietic progenitor cell population. As shown in **section 3.3** of this study, and by others (Shi et al., 2013, Zhang et al., 2008), the inflammation models altered Sca-1 expression in the haematopoietic progenitor cells. Therefore, to obtain unbiased CXCR4 expression data in the HSPC of C57BL/6 mice, the LK ($Lin^{-}Kit^{+}$ cells) that encompasses the entire lymphomyeloid progenitor HSPC compartment was investigated for CXCR4 expression in the LPS injection model. Mice received a single I.P. LPS/PBS injection exactly as described in section 3.3.1. The gating strategy for LK cells was essentially as described in **section 3.3.3.** The dot plots in **figures** 3-8 A & B show the LK cell gating of the bone marrow of mice treated with either I.P. PBS or I.P. LPS respectively. Figure 3-8 C shows the histogram overlay plots for CXCR4 expression in LK cells from mice treated with I.P. PBS or LPS. The CXCR4 specific isotype-control staining pattern (**figure 3-8C**) shows the specificity of the CXCR4 antibody staining. As the histogram plots showed two distinct peaks for CXCR4 expression in the haematopoietic progenitor cells, only the $CXCR4^{high}$ expressing cells were further analysed in figure 3-8D. As summarised in the scatter plot in **figure 3-8D**, LPS-induced systemic inflammation significantly reduced the LK CXCR4 cell surface expression compared to PBS treated controls (figure 3-8 D, $p < 0.0001$; 2-tailed unpaired t-test).

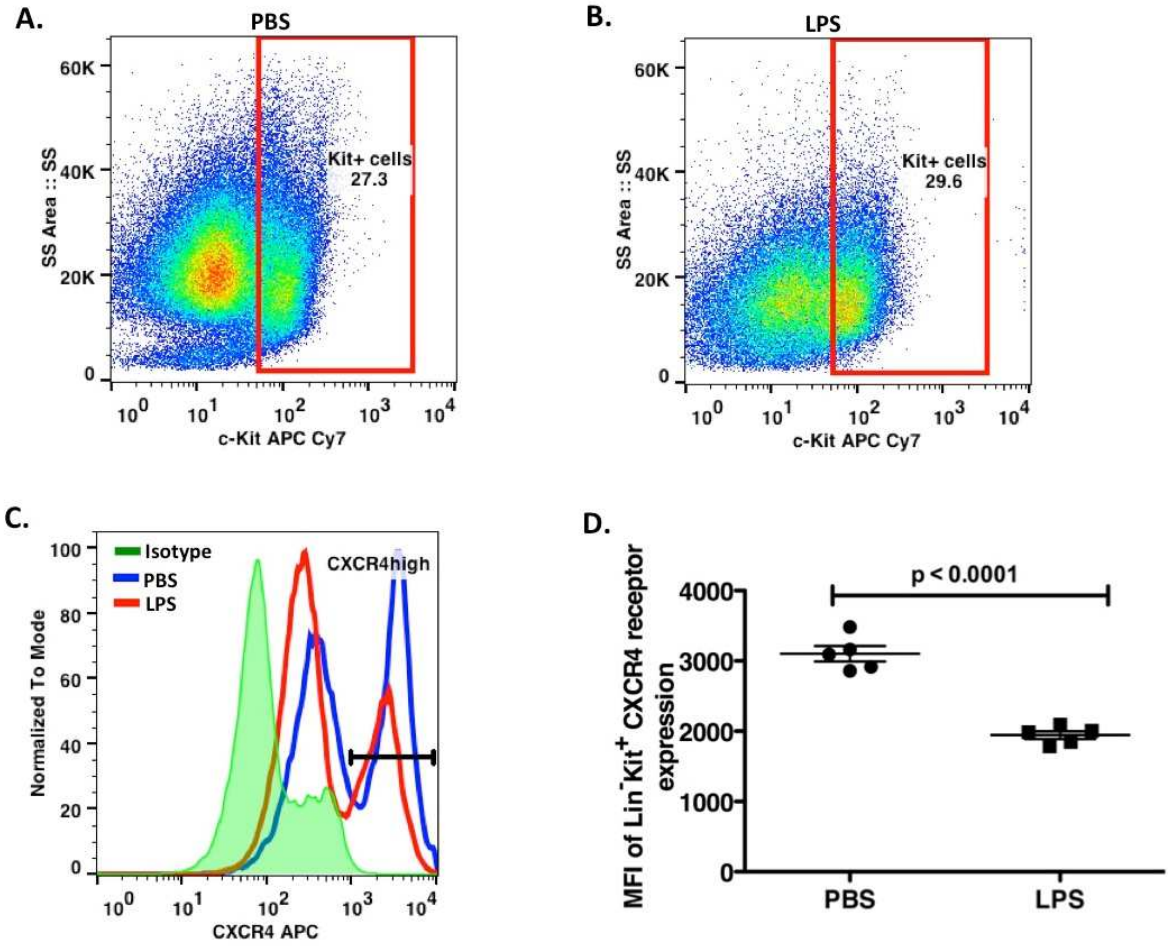



**Figure 3-8 LPS induced systemic inflammation reduces CXCR4 expression on HSPC.** C57BL/6 mice were either given a single dose of I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and bone marrow cells [2x (tibia + femur + hip bones)] were harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer's instructions (Miltenyi Biotec). The c-Kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), as well as c-Kit, CXCR4, and Live/Dead Aqua fixable dye to analyse the HSPC (LK) compartment for cell surface CXCR4 receptor expression. After gating on live cells through dead cell exclusion using the Live/Dead Aqua fixable dye, $Lin^-$ cells were selected for further analysis (see section 3.3.3 for example of gating strategy). Figures A & B are representative dot plots showing the LK (HSPC) gate for PBS and LPS treated bone marrow cells respectively. Figure C is a representative histogram plot showing the overlay of LK CXCR4 receptor expression for LPS and PBS treated mice (showing two distinct peaks representing $CXCR4^{low}$ and $CXCR4^{high}$ cells) as well as CXCR4 isotype-matched control. Figure D is a scatter plot summarising the MFI of the surface CXCR4 expression of the LK compartment for LPS and PBS treated controls. Statistical significance was calculated using the 2-tailed, unpaired t-test [N=5-6 (2 independent experiments)].

Taken together, the reduced CXCR4 receptor expression of the HSPC population, observed with LPS-induced inflammation, possibly play a contributory role to the increased numbers of HSPC released in response to the LPS injection.

#### 3.4.1.3 LPS-induced systemic inflammation significantly reduces bone marrow CXCL12 concentration.

Having examined surface Cxcr4 expression in the LK population, I next sought to understand how LPS-induced systemic inflammation also affected the production

of CXCL12, which is the ligand for CXCR4. An enzyme-linked immunosorbent assay (ELISA) was employed to measure concentrations of CXCL12 in the plasma, bone marrow, bone marrow extracellular fluid, and bone protein extract. As discussed in the general introductory chapter, exogenously administered CXCL12 mobilises HSPC from the bone marrow as it induces a sustained peripheral blood CXCL12 level, thus creating a CXCL12 gradient between the blood and bone marrow. Circulating CXCL12 levels were estimated by measuring the CXCL12 concentration in the plasma. The aim was to determine whether LPS-induced systemic inflammation was able to create a similar CXCL12 concentration gradient that favoured the exit of HSPC from the bone marrow. Figure 3-9A shows the calibration curve used to establish the limit of detection of the assay with reliability ($R^2$=0.997) between the CXCL12 concentration and the optical density of the measurements. The results from the plasma CXCL12 measurement showed comparable CXCL12 levels in PBS-treated and LPS-injected groups (figure 3-9B). This suggests that LPS-induced systemic inflammation did not increase plasma CXCL12 concentration that might have led to the HSPC mobilisation.

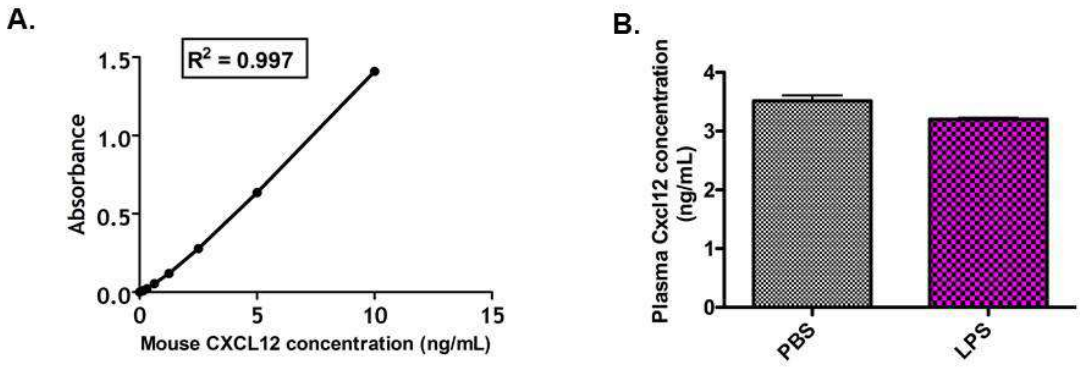



**Figure 3-9 LPS induced systemic inflammation does not significantly change plasma CXCL12 concentration.**
C57BL/6 mice were either given a single dose of I.P. LPS (100µg in 100µL PBS), or 100µL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation, peripheral blood was taken into EDTA anti-coagulation tubes, spun at >10,000xg for 10 minutes and plasma taken for CXCL12 ELISA. Figure A is the calibration curve showing a good fit ($R^2$=0.997) for the concentration of CXCL12 and optical density and the detection limit of the assay. Figure B summarises the measured CXCL12 concentration in the plasma of PBS and LPS treated mice. (n=5).

To measure the total bone marrow CXCL12 concentration, whole bones [2x (femur + tibia)] were homogenised in 400µL of mammalian protein extraction reagent (M-PER, Thermo Scientific) to extract the total bone marrow protein. Also, to examine the separate contribution from the bone tissue and the bone marrow extracellular fluid towards the measured total bone marrow CXCL12, bone marrow extracellular fluid, and bone protein extract, CXCL12 levels were

also measured. Extracellular bone marrow proteins were extracted in accordance with previously published protocols (Semerad et al., 2005, Levesque et al., 2001). Measurement of total bone marrow CXCL12 concentration revealed that the LPS-induced systemic inflammation significantly decreased the total bone marrow CXCL12 concentration (**figure 3-10A, p= 0.0286; 2-tailed, Mann-Whitney U test**). In addition, the LPS-induced inflammation also significantly reduced the bone marrow extracellular fluid CXCL12 concentration (**figure 3-10B, p= 0.0286; 2-tailed, Mann-Whitney U test**). However, the CXCL12 concentration in the bone protein extract, showed no difference between LPS- and PBS-treated groups **(figure 3-10C)**.

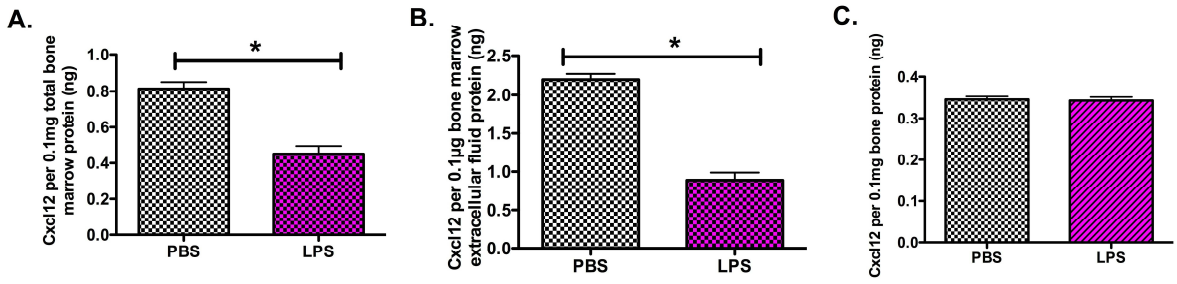



**Figure 3-10 Systemic LPS-induced inflammation significantly reduces bone marrow CXCL12 levels.**

C57BL/6 mice were either given a single dose of I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and bones [2x (tibia +femur)] were harvested for further processing. In some cases (figure A), whole bone were homogenised in 400µL of mammalian protein extraction reagents (MPER, Thermo Scientific) to obtain total bone marrow protein. In other cases (figure B), the marrow was flushed repeatedly with 1mL of PBS, and the resulting PBS-bone marrow suspension was spun at >10,000xg to harvest the bone marrow extracellular fluid protein. The hollow bone tissues were also homogenised in 400µL of mammalian protein extraction reagent, spun at 10,000xg and supernatants harvested to obtain bone protein extract (figure C). About 0.1mg of the respective proteins (from LPS, or PBS, treated mice) was used for CXCL12 quantification using the Quantikine mouse CXCL12 ELISA kit (R&D Systems). Figures A, B and C show the respective plots of CXCL12 measured in total bone marrow protein, bone marrow extracellular fluid, and bone proteins respectively for LPS and PBS treated groups. [*p=0.0286; 2-tailed Mann-Whitney U test, (n=5)].

Altogether, the results from the ELISA data suggest that LPS-induced systemic inflammation could have created a perturbation of the CXCL12 gradient favouring the egress of CXCR4-expressing cells, including HSPCs, from the bone marrow. This argument is supported by the findings that although circulating CXCL12 levels [as estimated by the plasma CXCL12 (figure 3-9B)] remained unchanged between LPS and PBS treated mice, the extracellular bone marrow CXCL12 concentration was significantly reduced [(reduced to ~half the level in PBS treated control (see figure 3-10B)] by LPS-induced systemic inflammation. Therefore, perturbation of the CXCL12 gradient may be another significant component of the mechanism underpinning the LPS-induced HSPC mobilisation.

## 3.4.2 The impact of LPS-induced inflammation on the bone marrow stromal cells.

### 3.4.2.1 Introduction.

The role of the bone marrow microenvironment, or niche, in regulating haematopoietic stem and progenitor cell behaviour has been discussed at length in the general introductory chapter. Previous reports have shown that the cellular components of the bone marrow stroma that include endothelial cells, nestin$^{+}$ mesenchymal stromal cells, CXCL12-abundant reticular cells, and osteoblasts, as well as their elaborated soluble factors such as CXCL12, stem cell factor, angiopoietin-1 and osteopontin, interact with HSPC to mediate their retention in the bone marrow. Not surprisingly, many previous findings have shown that most HSPC mobilising agents modulate the functions of these cellular niche components (Chow et al., 2011, Christopher et al., 2009). For example, G-CSF has been shown to reduce Cxcl12 secretion in the bone marrow by inhibiting osteoblast activity (Semerad et al., 2005).

In order to begin evaluating the role of the bone marrow stromal cells in LPS-induced haematopoietic progenitor cell mobilisation, flow cytometry was employed to assess the endothelial cell, mesenchymal stromal cell and osteoblast populations in the endosteal bone surface. Mice were given I.P. LPS/PBS injection exactly as described in section 3.3.1, and bones were harvested as described in section 3.3.3. After flushing out the marrow, the bones were incubated in digestion buffer consisting of 3mg/mL collagenase IV (Sigma-Aldrich, Irvine, UK) in 1xHBSS at 37°C for 45 minutes (Semerad et al., 2005). The bones were then crushed in a mortar and pestle, and filtered through a 70μm cell strainer (BD Biosciences, Oxford, UK) to remove bone fragments. The resulting suspension was spun at 300xg and the bone stromal cells were depleted of haematopoietic cells using the mouse lineage cell depletion kit and LS magnetic columns in accordance with manufacturer's protocol (Miltenyi Biotec, Surrey, UK). The resulting cells were next stained for differential expression of lineage antibody cocktail (Lin), CD45, CD31, CD51, and Sca-1 antigens in accordance with previously published protocol (Winkler et al., 2010a). Dead cells were excluded using Live/Dead Aqua (Life Technologies). To ensure the exclusion of haematopoietic cells, cells staining positive for either

CD45 and/or the cocktail of haematopoietic lineage detection antibody (Lin) were excluded as shown in the gating strategy (figure 3-11). Bone marrow endothelial cells, mesenchymal stromal cells and osteoblasts were defined as $Lin^{-}CD45^{-}Sca\text{-}1^{+}CD31^{+}$, $Lin^{-}CD45^{-}CD31^{-}Sca\text{-}1^{+}CD51^{+}$ and $Lin^{-}CD45^{-}CD31^{-}Sca\text{-}1^{-}CD51^{+}$ respectively (Winkler et al., 2010a).

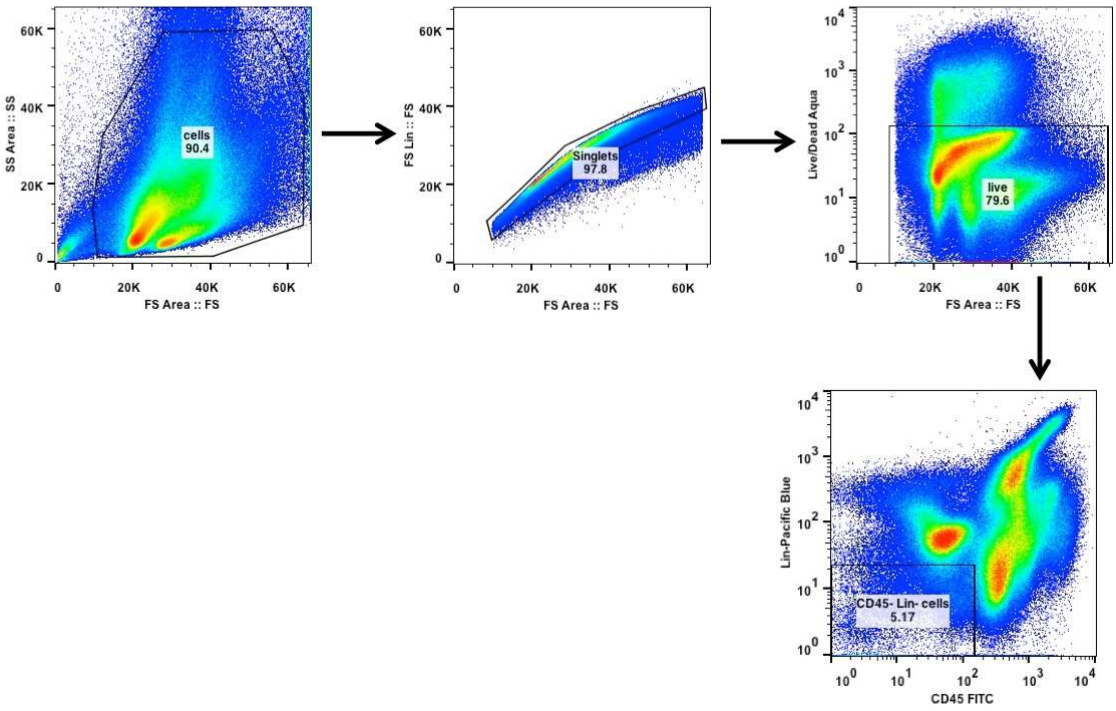



**Figure 3-11 Gating strategy used to select the bone marrow stromal cells.**
C57BL/6 mice were either given a single dose of I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation, and bones [2x (tibia + femur + hip bones)] were harvested, and the marrow was subsequently flushed with PBS. The remaining bone tissue was incubated in 3mg/mL collagenase IV in 1xHBSS buffer at 37°C for 45 minutes. The bones were then crushed using a mortar and pestle, filtered to exclude bone fragment and then depleted of haematopoietic cells using biotinylated lineage antibody cocktail, anti-biotin Microbeads and LS magnetic columns (Miltenyi Biotec). The resultant cells were stained for differential expression of haematopoietic lineage markers [Lin cocktail antibody (CD4, CD5, CD8a, CD11b, CD45R, Ter119 and Gr-1)], CD45, CD31, Sca-1 and CD51. The gating strategy used to exclude both dead cells and cells of the haematopoietic lineages from the subsequent analysis of the bone marrow stromal cell compartment is shown.

### 3.4.2.2 LPS-induced systemic inflammation increases bone marrow endothelial cells

The $CD45^{-}Lin^{-}$ cell gate (shown in the gating strategy in section 3.4.2.1 above; figure 3-11) was sub-gated for differential expression of Sca-1 and CD31 antigens. The $Lin^{-}CD45^{-}Sca\text{-}1^{+}CD31^{-}$ population was further analysed as discussed in section 3.4.2.3 below. The $Lin^{-}CD45^{-}Sca\text{-}1^{+}CD31^{+}$ population was referred to as endothelial cells in agreement with previously published reports (Winkler et al., 2010a, Winkler et al., 2010b). As shown in figure 3-12 (compare figures A & B), LPS-induced systemic inflammation caused an expansion in the proportion of the

bone marrow endothelial cell compartment. This increase in the endothelial cell proportion reached statistical significance (figure 3-12 C; **p=0.001; 2-tailed, Unpaired t-test).

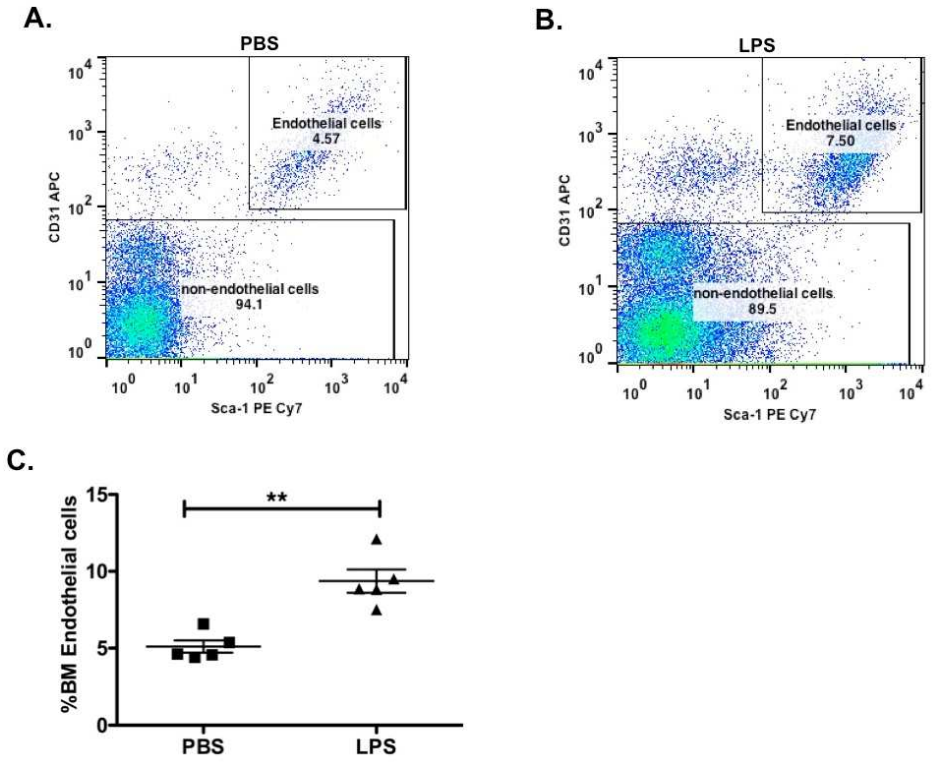



**Figure 3-12 LPS injection increases the proportion of bone marrow endothelial cells.** The $CD45^{-}Lin^{-}$ non-haematopoietic cells (see figure 3-11 for gating strategy), from bone stromal cells of either PBS, or LPS, treated mice, were re-gated for differential expression of CD31 and Sca-1 antigens to identify endothelial cells ($CD45^{-}Lin^{-}CD31^{+}Sca\text{-}1^{+}$) and non-endothelial cells ($CD45^{-}Lin^{-}CD31^{-}Sca\text{-}1^{-/+}$) for further analysis. Figures A and B are representative dot plots for the re-gating of the $CD45^{-}Lin^{-}$ non-haematopoietic cells from PBS and LPS treated mice respectively. C is a scatter plot summarising the effects of the LPS injection on the bone marrow endothelial cell compartment as compared to PBS treated controls. Data presented in the scatter plots are mean±SEM [**p=0.001; 2-tailed, unpaired t-test; n=5 (2 independent experiments)]

### 3.4.2.3 LPS injection significantly increases MSC but reduces bone marrow osteoblast population.

The $Lin^{-}CD45^{-}Sca\text{-}1^{-/+}CD31^{-}$ cells (indicated as non-endothelial cells in figure 3-12 A & B), were next re-gated for the differential expression of CD51 and Sca-1 antigens. Cells that co-expressed CD51 and Sca-1 (i.e. $Lin^{-}CD45^{-}Sca\text{-}1^{+}CD31\text{-}CD51^{+}$) were subsequently referred to as mesenchymal stromal cells (MSC), whereas cells that expressed the CD51, but not Sca-1 antigens, (i.e. $Lin^{-}CD45^{-}Sca\text{-}1^{-}CD31^{-}CD51^{+}$) were referred to as osteoblasts in agreement with previous reports (Semerad et al., 2005, Short et al., 2009, Winkler et al., 2010b). As shown in figures 3-13 A & B, and summarised in figure 3-13C, I.P. LPS-induced systemic inflammation orchestrated a statistically significant increase in the proportion of MSC in the total bone marrow stromal population (figure 3-13C,

p<0.0001; 2-tailed, Unpaired t-test). In addition, systemic LPS led to a statistically significant reduction in the osteoblast population (figure 3-13C, p=0.0017; 2-tailed, unpaired t-test).

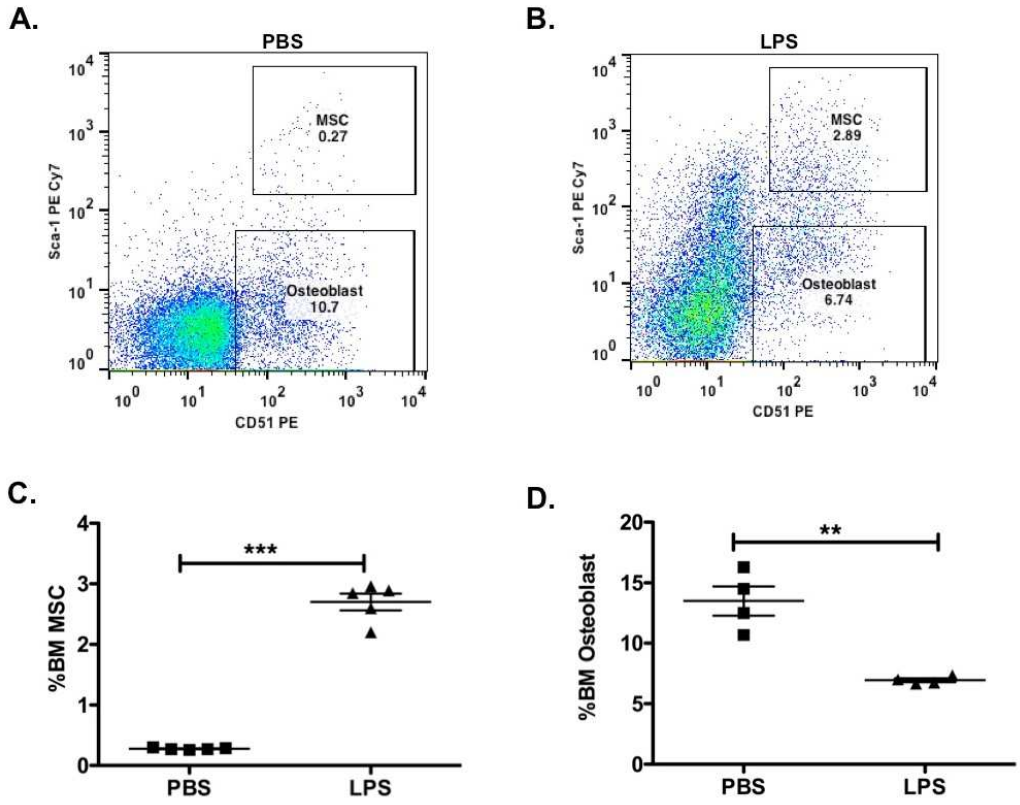



**Figure 3-13 LPS injection significantly alters the osteoblasts and MSC of the HSPC niche.** The non-endothelial cells ($CD45^-Lin^-CD31^-Sca\text{-}1^{-/+}$) (see figure 3-12 A & B for gating strategy), from bone stromal cells of either I.P. PBS, or I.P. LPS, treated mice, were re-gated for differential expression of CD51 and Sca-1 antigen to identify osteoblasts ($CD45^-Lin^-CD31^-Sca\text{-}1^-CD51^+$) and mesenchymal stromal cells [MSC, $CD45^-Lin^-CD31^-Sca\text{-}1^+CD51^+$). Figures A & B are representative plots from the re-gating of the $CD45^-Lin^-CD31^-Sca\text{-}1^{-/+}$ non-endothelial cells from PBS and LPS treated mice respectively. C is a scatter plot comparing the proportion of MSC cells in PBS and LPS treated mice. D is a scatter plot comparing the proportions of osteoblasts in PBS and LPS treated mice. [***p<0.0001; **p=0.0017; 2-tailed Unpaired t-test, n=4 (2 independent experiments)].

Taken together, systemic LPS-induced inflammation led to significant modulation of the bone marrow stromal cell population that has established roles as critical components of the bone marrow niche.

### 3.4.3 The effects of LPS-induced inflammation on bone marrow mononuclear phagocytic cells.

Previous studies have indicated that cellular components of the innate immune system are important in the retention of haematopoietic stem and progenitor cells in the bone marrow (Chow et al., 2011, Winkler et al., 2010b). To begin to address how these cells were impacted by systemic LPS injection, bone marrow cells from I.P. LPS, or PBS, injected mice were analysed by flow cytometry in

accordance with a previously published protocol (Chow et al., 2011). Figure 3-14 shows the gating strategy used to sub-set the mononuclear phagocytic cells. After excluding dead cells through positive staining for Live/Dead Aqua fixable dye, the live cells were gated for differential expression of the granulocytic marker, Gr-1, and the monocytic marker, CD115. This enabled the exclusion of Gr-$1^{high}CD115^{+}$ cells (mainly neutrophils and monocytes), to allow for the Gr-$1^{low}CD115^{+/-}$ cells to be further analysed (see sections 3.4.3.1 & 3.4.3.2 below).

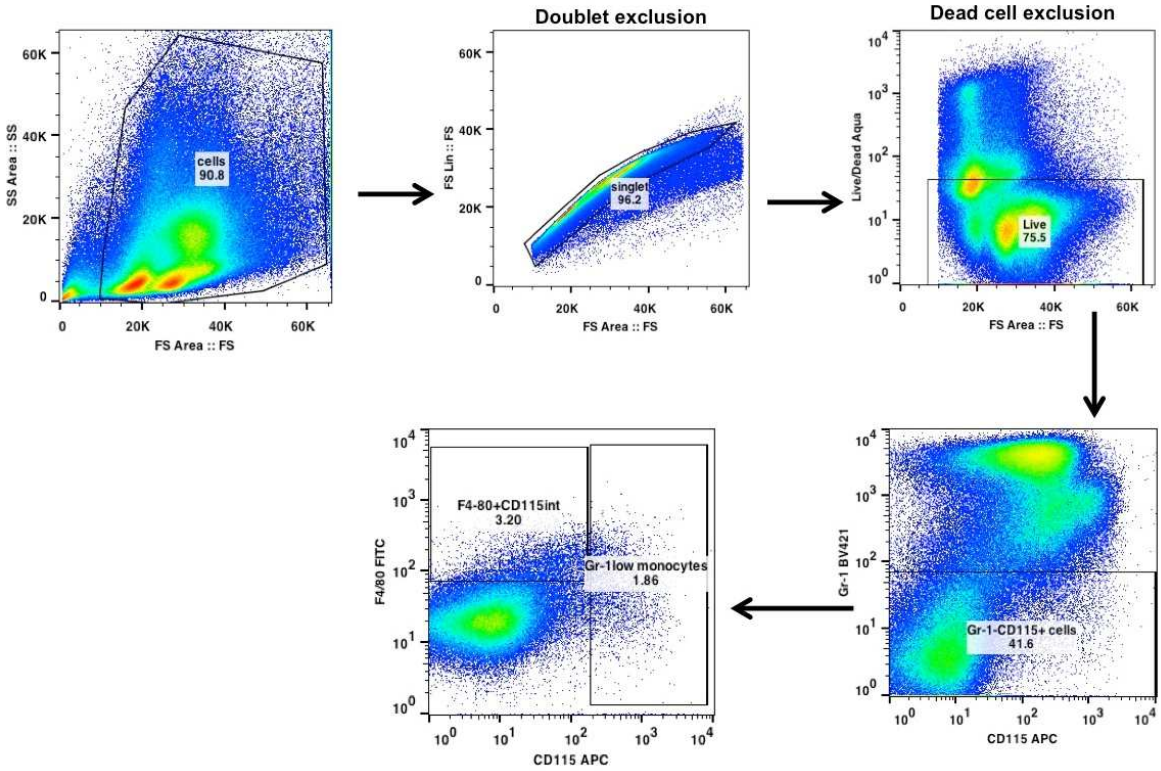



**Figure 3-14 Gating strategy used to characterise the bone marrow mononuclear phagocytic cells.**
C57BL/6 mice were either given a single intraperitoneal injection of Ultra-pure LPS (100µg in 100µL PBS) or 100µL PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation, and bones [2x (tibia + femur)] were harvested and the marrow flushed with FACS buffer. After lysing red blood cells with Ammonium chloride, the cells were stained with Live/Dead Aqua fixable dye, F4/80, CD115, Gr-1, CD11b and CD169 antibodies to evaluate the mononuclear phagocytic cells of the bone marrow. Figure 3-14 is a representative plot illustrating the gating strategy used to exclude dead cells through doublet exclusion and retention of Live/Dead Aqua fixable dye. The live cells were then sub-gated for differential expression of Gr-1 and CD115 antigen to select Gr-$1^{-/low}CD115^{-/+}$ mononuclear cells. These Gr-$1^{-/low}CD115^{-/+}$ mononuclear cells were then re-gated for the differential expression of F4/80 and CD115 antigens to identify the Gr-$1^{low}CD115^{+}$ monocytic cells and Gr-$1^{-/low}CD115^{int}$ mononuclear cells.

### 3.4.3.1 LPS injection reduces the bone marrow Gr-$1^{low}CD115^{+}$ monocytic population.

The Gr-$1^{low}CD115^{+/-}$ cells were subsequently re-gated for differential expression of CD115 and F4/80 antigens to delineate two cellular populations: $F4/80^{+}$ but $CD115^{int}$ (CD115 intermediate expressing cells), and F4/80+ but $CD115^{high}$

monocytic cells. When these Gr-1$^{low}$F4/80$^{+}$CD115$^{high}$ monocytic cell populations from bone marrow of LPS and PBS treated mice were compared, it was apparent that systemic LPS injection significantly reduced this population (figure 3-15, p = 0.0289; 2-tailed, Mann-Whitney U test).

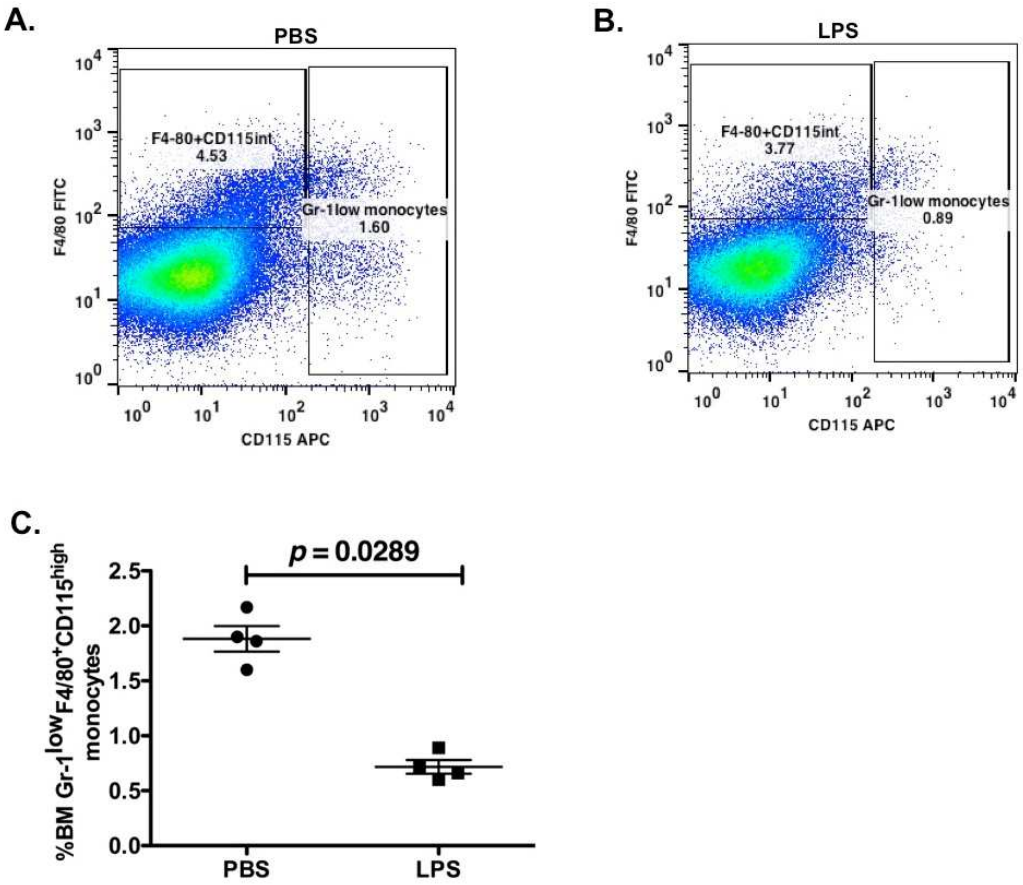



**Figure 3-15 LPS injection reduces the bone marrow Gr-1$^{low}$CD115$^{high}$ monocytic population.** A & B are the respective representative plots comparing the bone marrow mononuclear cells (Gr-1$^{low}$CD115$^{high}$ monocytic) of PBS and LPS injected mice (see figure 3-14 for gating strategy). C is a scatter plot summarising the changes in the Gr-1$^{low}$CD115$^{high}$ monocytic cells in response to either PBS or LPS intraperitoneal injection. [p=0.0289; 2-tailed, Mann-Whitney U test, n=4 (three independent experiments).

### 3.4.3.2 LPS injection significantly reduces the bone marrow macrophage population.

The Gr-1$^{low}$F4/80$^{+}$CD115$^{int}$ population (see gating strategy from section 3.4.3.1) was sub-gated for differential expression of CD169 and CD11b expression to delineate the macrophage population (Gr-1$^{-/low}$F4/80$^{+}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$) as defined by Chow et al. (Chow et al., 2011). Chow et al. demonstrated that clodronate liposome depletion of this CD169$^{+}$ macrophage population led to mobilisation of HSPC to the circulation. The aim was therefore to try to understand whether systemic LPS-induced inflammation affected this macrophage population. As shown in figures 3-16 A &B, and summarised in figure

3-16C, systemic LPS-induced inflammation led to a marginal, but significant, reduction of this bone marrow CD169$^{+}$ macrophage population when compared to PBS treated control (p = 0.046; 2-tailed Unpaired t-test).

Taken together, the data presented suggest that LPS induced systemic inflammation led to significant reductions in the bone marrow monocytic and macrophage populations.

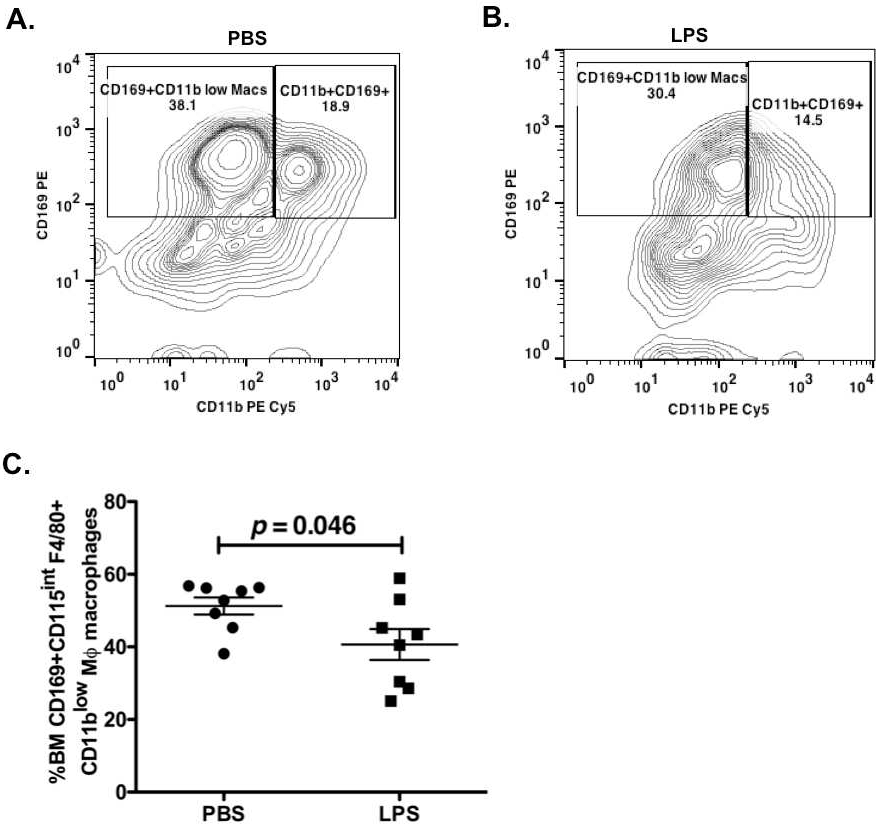



**Figure 3-16 Intraperitoneal LPS injection reduces the bone marrow CD169$^{+}$ macrophage population.**
The F4/80$^{+}$CD115$^{int}$ mononuclear cells (see figure 3-15 gating strategy) were then re-gated for the differential expression of F4/80 and CD11b antigens to identify the CD169$^{+}$ macrophage population (Gr-1$^{-}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$ mononuclear cells) and Gr-1$^{-}$CD115$^{int}$CD169$^{+}$CD11b$^{+}$ mononuclear cells. [p=0.046; 2-tailed unpaired t-test (n=4; 2 independent experiments pooled together).

In summary, data presented in this section suggest that LPS injection altered the HSPC niche by:

A. Significantly altering the CXCR4/CXCL12 axis in the HSPC niche by reducing the HSPC surface expression of CXCR4 and reducing bone marrow CXCL12 concentration.

B. Significantly reducing the bone marrow stromal osteoblast population while significantly increasing the MSC and endothelial cell populations.

C. Significantly altering the bone marrow mononuclear phagocytic cells by significantly reducing the Gr-$1^{low}$CD$115^{+}$ monocytic cell and CD$169^{+}$ macrophage populations.

## 3.5 What chemokines and cytokines are produced in response to systemic LPS injection?

### 3.5.1 Introduction

The data presented thus far provide evidence that LPS-induced systemic inflammation resulted in a significant mobilisation of haematopoietic progenitor cells into the circulation. As cellular migration in multicellular organisms occurs in response to molecular cues such as chemokines and cytokines, it was interesting to understand how the LPS-induced systemic inflammation comprehensively affected chemokine and cytokine production as well as chemokine receptor expression in the HSPC. This section thus set out to address the following questions:

A. What chemokines and cytokines are expressed in the bone marrow in response to LPS injection?

B. What chemokines and cytokines are expressed in the plasma in response to LPS injection?

C. What chemokine receptors are expressed in HSPC in response to LPS injection?

In order to obtain a comprehensive picture of the inflammatory chemokines and cytokines that are expressed in the bone marrow and the peripheral blood in response to LPS injection, proteome arrays were employed (see sections 3.5.1-3.5.2 below). These arrays are basically nitrocellulose membranes that have been pre-spotted with specific capture antibodies by the manufacturer (R&D Systems). Prior to adding samples to the membranes, the samples are pre-mixed with a cocktail of biotinylated antibodies, and the resultant antibody/cytokine

complexes are then incubated with the membranes to allow binding to cognate antibodies immobilised on the membranes. Following this, the membranes are washed and Streptavidin-HRP, as well as chemiluminescent detection reagents, are added sequentially to enable detection of bound cytokines (see materials and methods for details). Images are then acquired with X-ray films, the images are scanned and then analysed using ImageJ software (NIH, USA) to determine pixel intensity of each spot.

Moreover, in order to determine what chemokine receptors are expressed in the HSPC in response to LPS injection, RT-QPCR was employed as explained in section 3.5.3 below.

## 3.5.2 Profiling of the bone marrow proteome in response to LPS injection.

As the bone marrow provides the niche for the retention of HSPC, and its perturbation generally leads to HSPC release to the circulation, the bone marrow cytokine milieu was firstly evaluated to assess what cytokines are expressed in the bone marrow niche in response to LPS-induced systemic inflammation. The proteome array kit (ARY015; R&D Systems) was used for this purpose. As shown in **figure 3-17** and **table 3-1**, this proteome array kit detects 53 different proteins, some of which are chemokines, cytokines, growth factors, and other angiogenesis related proteins.

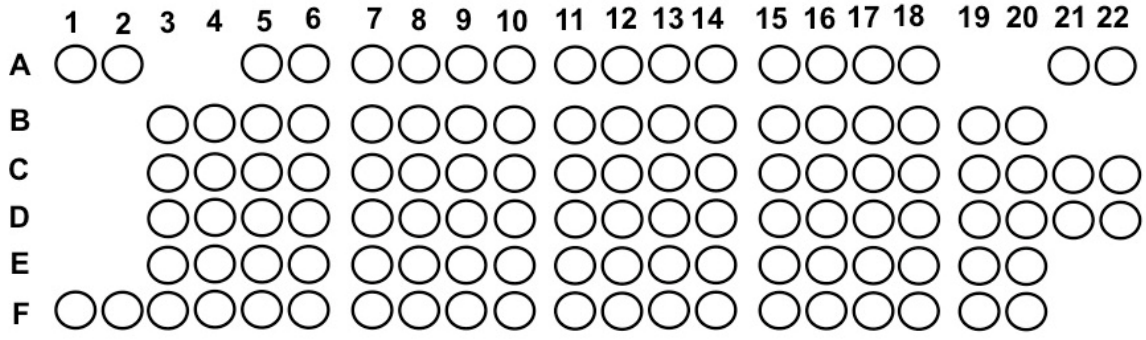



**Figure 3-17 A schematic of the Mouse Angiogenesis Array overlay**
Shown is the Mouse Angiogenesis Array overlay indicating the design of the array and how analytes are pre-spotted on the membranes.

**Table 3-1 Enumeration of the array coordinates and the analytes they represent on the mouse angiogenesis proteome array**

| Coordinate | Analyte | Coordinate | Analyte | Coordinate | Analyte |
|---|---|---|---|---|---|
| A1, A2 | Reference spots | C5, C6 | CX3CL1 | E3, E4 | Osteopontin |

| | | | | | |
|---|---|---|---|---|---|
| A5, A6 | ADAMTS1 | C7, C8 | GM-CSF | E5, E6 | PD-ECGF |
| A7, A8 | Amphiregulin | C9, C10 | HB-EGF | E7, E8 | PDGF-AA |
| A9, A10 | Angiogenin | C11, C12 | HGF | E9, E10 | PDGF-AB/ PDGF-BB |
| A11, A12 | Angiopoietin-1 | C13, C14 | IGFBP-1 | E11, E12 | Pentraxin-3 |
| A13, A14 | Angiopoietin-3 | C15, C16 | IGFBP-2 | E13, E14 | Platelet factor 4 |
| A15, A16 | Coagulation factor III | C17, C18 | IGFBP-3 | E15, E16 | PIGF-2 |
| A17, A18 | CXCL16 | C19, C20 | IL-1$\alpha$ | E17, E18 | Prolactin |
| A21, A22 | Reference spots | C21, C22 | IL-1$\beta$ | E19, E20 | Proliferin |
| B3, B4 | Cyr61 | D3, D4 | IL-10 | F1, F2 | Reference spots |
| B5, B6 | DLL4 | D5, D6 | CXCL10 | F3, F4 | CXCL12 |
| B7, B8 | CD26 | D7, D8 | CXCL1 | F5, F6 | Serpin E1 |
| B9, B10 | EGF | D9, D10 | Leptin | F7, F8 | Serpin F1 |
| B11, B12 | Endoglin | D11, D12 | CCL2 | F9, F10 | Thrombospondin-2 |
| B13, B14 | Endostatin | D13, D14 | CCL3 | F11, F12 | TIMP-1 |
| B15, B16 | Endothelin-1 | D15, D16 | MMP-3 | F13, F14 | TIMP-4 |
| B17, B18 | FGF acidic | D17, D18 | MMP-8 (pro form) | F15, F16 | VEGF |
| B19, B20 | FGF basic | D19, D20 | MMp-9 (pro & active) | F17, F18 | VEGF-B |
| C3, C4 | FGF-7 | D21, D22 | IGFBP-9 | F19, F20 | Negative control |

To evaluate the bone marrow inflammatory proteome milieu, C57BL/6 mice received a single I.P. injection of either Ultra-pure LPS or PBS as described in section 3.3.1, and bones harvested exactly as described in section 3.3.3. The bones were cleared of muscle, and then homogenised in mammalian protein extraction reagent (M-PER, Thermo Scientific). As they were expensive, the arrays were run only once; however, to obtain representative data, bone marrow homogenates from three mice were pooled and 300$\mu$g of the resultant whole bone marrow homogenates used for the subsequent proteome array. A scanned image of the membranes with the signal intensities representing analytes on a developed X-ray film (after 4 minutes exposure) is shown in **figure 3-18.**

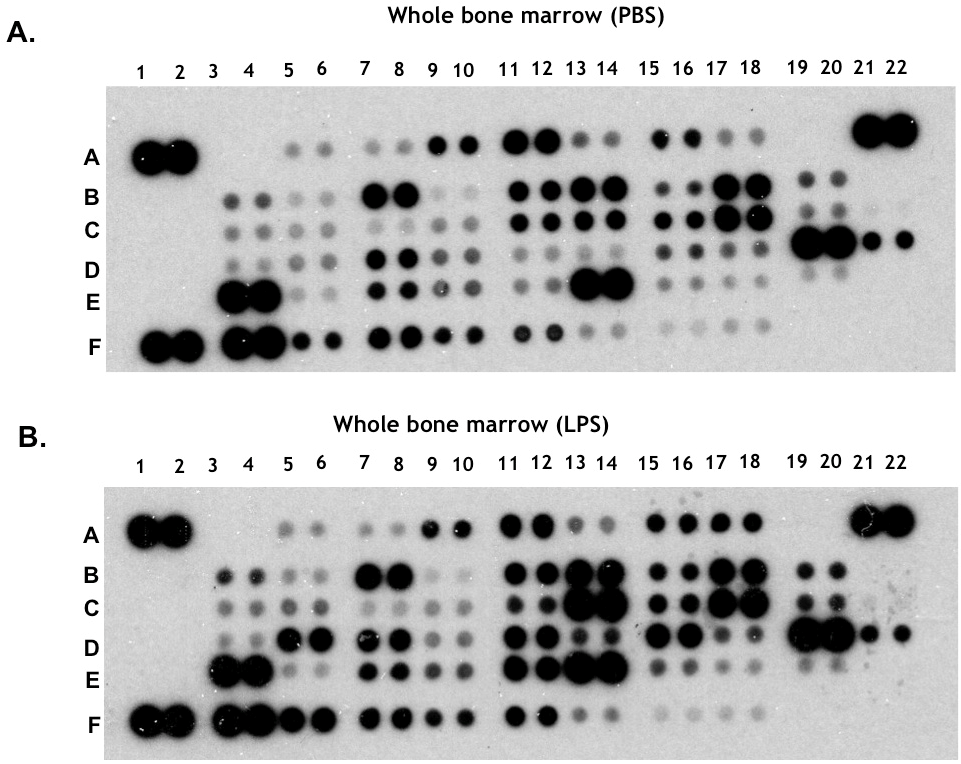



**Figure 3-18 Representative bone marrow angiogenesis array blots showing developed signal intensities of spots.**
C57BL/6 mice were either given a single dose of intraperitoneal LPS (100µg in 100µL PBS) or 100µL PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation, bones [2x (tibia + femur+ hip bones)] were harvested and homogenised in 400µL of mammalian protein extraction reagent. The total bone marrow homogenates were spun at >10,000xg for 10 minutes and supernatants taken for proteome array profiling. In order to obtain a representative proteome profile for each treatment group, protein lysates from three mice were pooled together for each treatment group (LPS or PBS treated controls). After estimating the pooled lysate protein concentration using the BCA method, ~300µg was used for the proteome profiling using the Mouse angiogenesis proteome array (R&D systems). A & B are the respective scanned images of the blots from PBS and LPS treated mice, after developing on X-ray films.

The signal intensities were measured with ImageJ 1.48 (NIH, USA) software. As each analyte is represented in duplicate, the signal intensities of the duplicate spots were then averaged to get the mean signal intensities. Next, the mean signal intensity of the spots for negative controls was subtracted from that of the calculated mean signal intensity of each analyte to correct for background signals. Fold change in mean signal intensity for each analyte was calculated by dividing the mean signal intensity of the LPS treated group by the mean signal intensity of the respective analyte in the PBS treated group. To increase the stringency of the assay, only analytes demonstrating a ≥2-fold modulation were further analysed.

### 3.5.2.1 LPS injection modulates bone marrow chemokine expression

The bar graph in figure 3-19 shows the CC- and CXC-chemokines that demonstrated >2-fold change in expression levels in response to the I.P. LPS injection.

For the inflammatory CC-chemokines, there was increased expression of bone marrow CCL2 (8-fold increase) and CCL3 (~4-fold increase) in response to the I.P. LPS injection. In addition, the expression of CXC-chemokines was also modulated. Specifically, there were >8-fold increases in the expression of bone marrow CXCL10 and CXCL16 in response to systemic LPS injection compared to PBS treated controls.

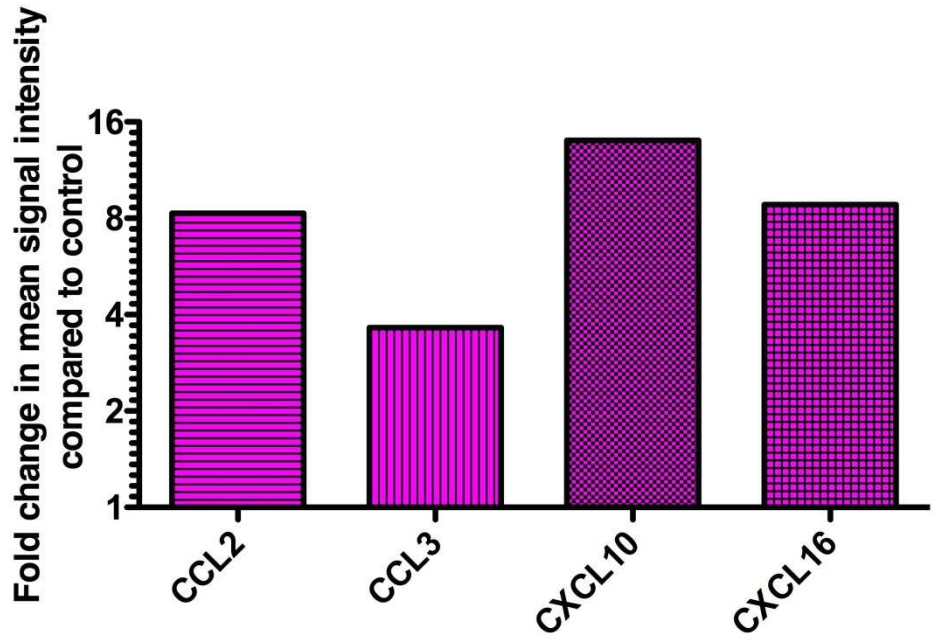



**Figure 3-19 I.P. LPS injection modulates the expression of chemokines in the bone marrow.** The signal intensity of each spot on the developed array blots (see figure 3-18) was estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represented each cytokine, the mean of the signal intensity of each chemokine was calculated, and the corrected signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control from the mean signal intensity of each chemokine. Next, the fold change in the corrected mean signal intensities were estimated for each analyte by dividing the corrected mean signal intensity of that analyte in the LPS treated group by the corrected mean signal intensity of that same analyte in the PBS treated group. Figure 3-19 is a bar graph showing the fold change in chemokines in the bone marrow of C57BL/6 mice in response to the LPS injection. Fold change >1, means increased production of the analyte in response to LPS.

### 3.5.2.2 Other inflammatory mediators produced in the bone marrow in response to I.P. LPS injection.

The other protein changes detected in the proteome array profiler are summarized in the bar graph in figure 3-20. As stated in the previous section, only analytes meeting the cut-off threshold of ≥2-fold modulation are presented

in figure 3-20. The systemic LPS-induced inflammation led to >3-fold increase in the expression of coagulation factor III in the bone marrow. As sepsis is known to induce a pro-coagulant phenotype, LPS-induced increase in coagulation factor III was not a surprising finding. Interestingly, although endoglin and endostatin have opposing roles in angiogenesis, i.e. angiogenic and angiostatic respectively, the expression of both proteins was increased by >2-fold.

Matrix metalloproteinases (MMP) are known to degrade extracellular tissue matrix, and have been shown to promote angiogenesis, cancer metastasis and cellular migration. Generally, they are secreted in latent (inactive/pro) forms that require proteolytic cleavage to become the active forms that have functional activity. The proteome array data showed that LPS-induced inflammation led to ~2-fold increase in the expression MMP-8 in the bone marrow. In contrast, LPS-induced inflammation led to a 32-fold reduction in the bone marrow MMP-9 (pro- and active forms). This profound reduction in bone marrow MMP-9 activity is interestingly considering that previous reports have implicated increased MMP-9 activity as being crucial for HSPC mobilisation (Heissig et al., 2002, Jalili et al., 2010).

The pentraxin family consists of pro-inflammatory proteins including C-reactive proteins and pentraxin-3. Increased production of pentraxin-3 is seen in various cells such as endothelial cells, fibroblast, monocytes and macrophages in response to inflammatory mediators (Han et al., 2005, Introna et al., 1996). In addition, pre-formed pentraxin-3 is also known to be stored in the specific granules of neutrophils and is released upon neutrophil activation (Jaillon et al., 2007). As shown in figure 3-20, LPS systemic injection led to >3-fold increase in the bone marrow secretion of pentraxin-3 suggesting an inflammatory bone marrow milieu.

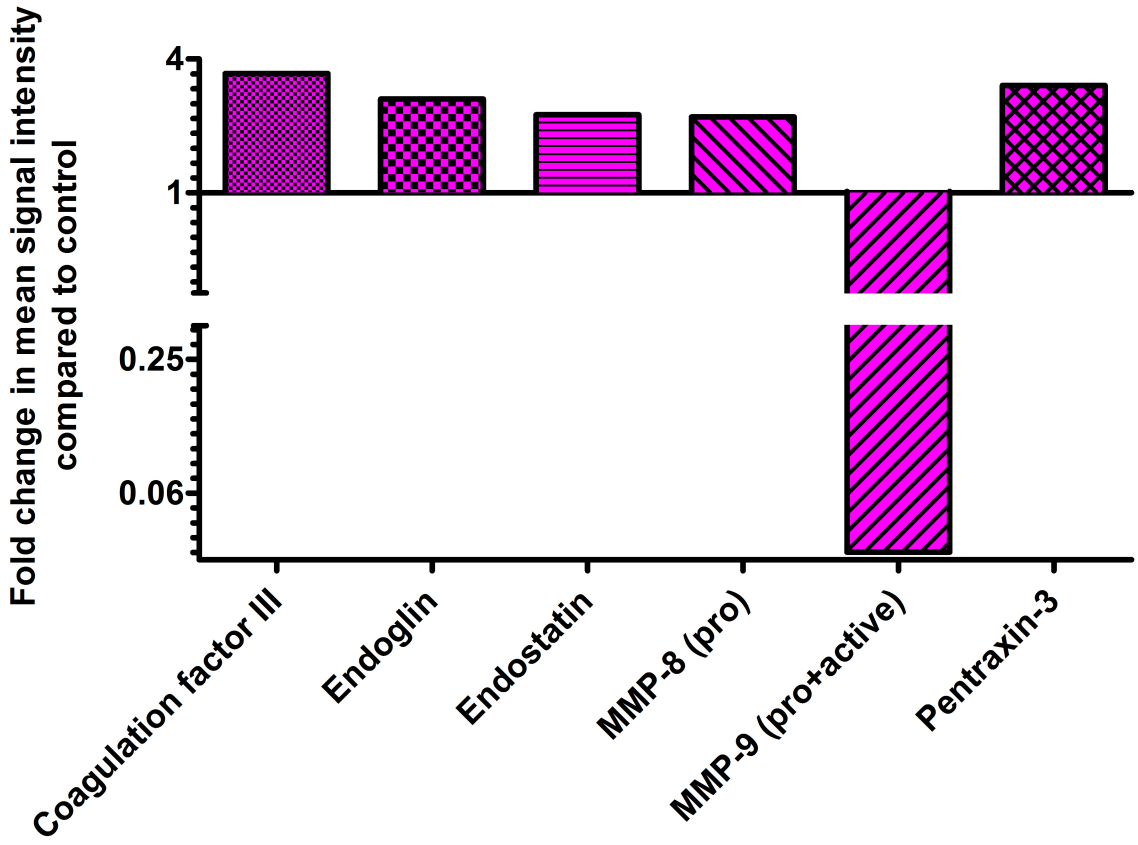



**Figure 3-20 LPS injection modulates bone marrow cytokine expression.**
The signal intensities of each spot on the developed array blots (see figure 3-18) were estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each cytokine, the mean of the signal intensity of each cytokine was calculated, and the corrected signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control from the mean signal intensity of each cytokine. Next, the fold change in the corrected mean signal intensities were estimated for each analyte by dividing the corrected mean signal intensity of that analyte in the LPS treated sample by the corrected mean signal intensity of that same analyte in the PBS treated group. Figure 3-20 is a bar graph showing the fold change in the analytes in response to the LPS injection.

### 3.5.3 Profiling of the circulating plasma cytokines and chemokines in response to I.P. LPS injection.

The proteome array, presented in section 3.5.1-3.5.2 above, profiled the inflammatory context in the bone marrow. As I.P. LPS injection resulted in increased circulating haematopoietic progenitors, it was also deemed important to analyse the proteome profile in peripheral blood. As cellular migration is not stochastic, but an instructive process in response to specific cues, the aim was to define the repertoire of cytokines and chemokines in the plasma (post LPS-induced inflammation) and then compare these data to the bone marrow proteome (presented in section 3.5.1) to identify possible differences that could reveal potential candidates involved in orchestrating the increased numbers of mobilised haematopoietic progenitors seen in the systemic LPS-induced

inflammation model. To begin to do this, the mouse cytokine array panel A (ARY006; R&D Systems) was employed. This proteome array allowed the parallel detection of the relative levels of a total of 40 proteins (cytokines and chemokines). It consists of nitrocellulose membranes on which capture antibodies for specific proteins are pre-spotted (duplicate for each analyte). The principle underlying the assay is essentially as described for the proteome array ARY015 in section 3.5.1 above. Figure 3-21 and table 3-2, respectively, show the cytokine array overlay and the coordinates corresponding to the analytes in the mouse cytokine array panel A.

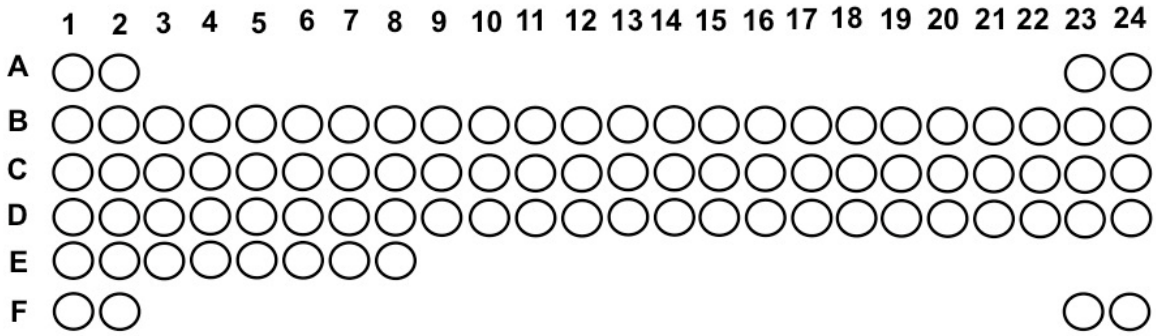



**Figure 3-21 Mouse Cytokine Array Panel A coordinates**
Shown is the Mouse Cytokine Array Panel A overlay indicating the design of the array and how analytes are pre-spotted on the membranes.

**Table 3-2 Enumeration of the array coordinates and the analytes they represent on the Mouse Cytokine Array Panel A**

| Coordinate | Target | Coordinate | Target | Coordinate | Target |
|---|---|---|---|---|---|
| A1, A2 | Reference spot | C3, C4 | IL-4 | D9, D10 | CCL2 |
| A23, A24 | Reference spot | C5, C6 | IL-5 | D11, D12 | CCL12 |
| B1, B2 | CXCL13 | C7, C8 | IL-6 | D13, D14 | CXCL9 |
| B3, B4 | C5/C5a | C9, C10 | IL-7 | D15, D16 | CCL3 |
| B5, B6 | G-CSF | C11, C12 | IL-10 | D17, D18 | CCL4 |
| B7, B8 | GM-CSF | C13, C14 | IL-13 | D19, D20 | CXCL2 |
| B9, B10 | CCL1 | C15, C16 | IL-12p70 | D21, D22 | CCL5 |
| B11, B12 | CCL11 | C17, C18 | IL-16 | D23, D24 | CXCL12 |
| B13, B14 | sICAM-1 | C19, C20 | IL-17 | E1, E2 | CCL17 |
| B15, B16 | IFN-$\gamma$ | C21, C22 | IL-23 | E3, E4 | TIMP-1 |
| B17, B18 | IL-1$\alpha$ | C23, C24 | IL-27 | E5, E6 | TNF-$\alpha$ |
| B19, B20 | IL-1$\beta$ | D1, D2 | CXCL10 | E7, E8 | TREM-1 |
| B21, B22 | IL-1r$\alpha$ | D3, D4 | CXCL11 | F1, F2 | Reference spot |
| B23, B24 | IL-2 | D5, D6 | CXCL1 | F23, F24 | Negative control |
| C1, C2 | IL-3 | D7, D8 | M-CSF | | |

To evaluate the circulating inflammatory proteome milieu, mice were treated with I.P. injection of either Ultra-pure LPS or PBS exactly as described in section

3.3.1. Peripheral blood was taken from these mice into EDTA microtainer tubes (BD Biosciences, Oxford, UK). The blood was centrifuged at 8000xg for 10 minutes and plasma was harvested for the subsequent proteome array. Plasma from three mice was pooled together and 150µL of the resultant pooled plasma was used for the subsequent proteome array in order to obtain representative data. As platelets are known to contain chemokines that are released upon activation, the rational for using plasma, instead of serum, was to avoid the possibility of activated platelets artificially increasing the amounts of cytokines detected in each sample. Figure 3-22 shows scanned images of the blots with spot intensity representing analytes on a developed X-ray film (after 8 minutes exposure).

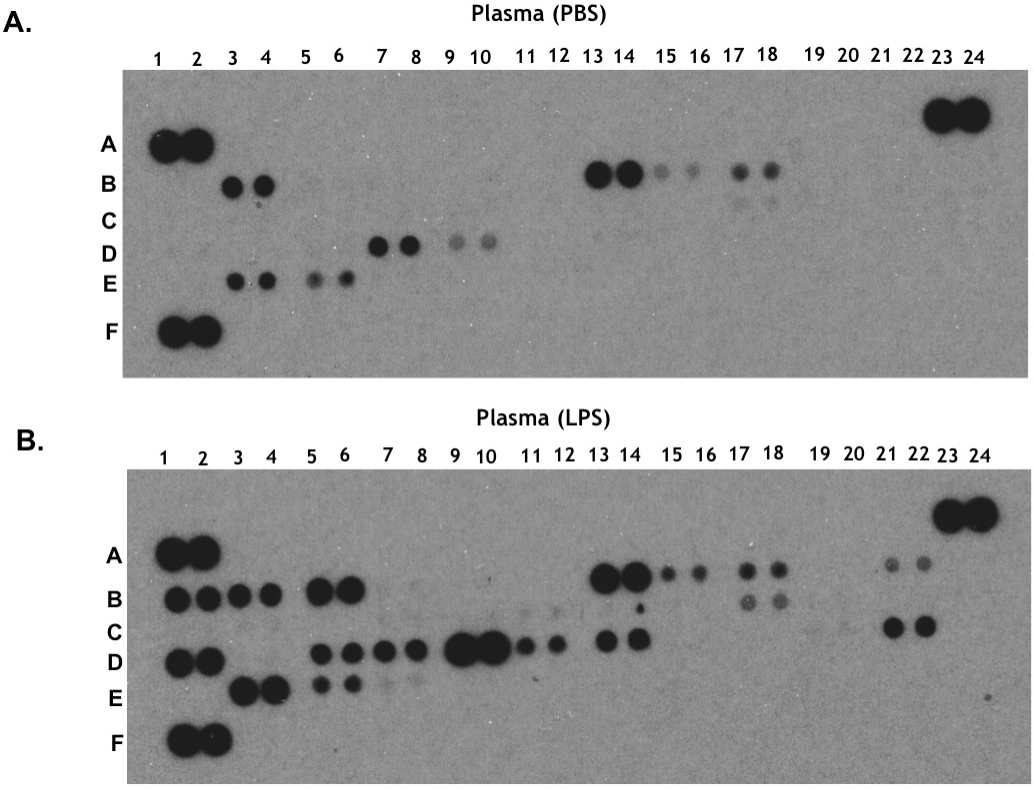



**Figure 3-22 Array blots showing cytokines detected in the plasma I.P. PBS or I.P. LPS treated mice.**
C57BL/6 mice were either given a single dose of intraperitoneal LPS (100µg in 100µL PBS) or 100µL PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation, peripheral blood was taken into EDTA anti-coagulated tube, spun at >10,000xg for 10 minutes and plasma taken for cytokine proteome array. 150µL of plasma from either LPS, or PBS, injected mice was used for the cytokine proteome profiling using the Mouse Cytokine Array panel A (ARY006; R&D Systems). A & B are the respective scanned images of the blots from PBS and LPS treated mice, after developing on X-ray films. In each case plasma was pooled from three mice and 150µL of the resultant plasma was used to obtain a representative proteome profile for each treatment.

The signal intensity of each spot was quantified using ImageJ 1.42 software (NIH, USA). Mean spot intensity was next calculated from respective duplicate spots (representing each cytokine). The mean signal of the spots corresponding to the negative control was then subtracted from the calculated mean signal of each analyte to obtain the corrected mean signal intensities. As some cytokines were inducibly expressed in response to systemic LPS injection, but not in PBS treated plasma, the mean signal intensities (corrected for background) of the LPS- and PBS-treated samples were plotted side-by-side for comparison, rather than fold changes as presented in figure 3-19 & 3-20.

#### 3.5.3.1 Intraperitoneal LPS injection mediates increased circulating plasma chemokine levels.

The bar graph in figure 3-23 shows the differentially expressed CC and CXC chemokines detected in the plasma of I.P. PBS or I.P. LPS treated mice plotted side by side. For CC chemokines, whereas CCL2 was highly expressed in the plasma of LPS treated mice, it was barely detectable in plasma of PBS treated mice. In addition, both CCL5 and CCL12 were highly expressed in the plasma of LPS treated mice; however, the levels of these chemokines (i.e. CCL5 and CCL12) in the plasma of PBS treated mice were below the limit of detection of this assay. For CXC chemokines, CXCL1, CXCL9, CXCL10 and CXCL13 were all highly expressed in the plasma of LPS treated mice, although they were not detectable in the plasma of PBS controls. Considering that LPS injection is a model of Gram-negative bacterial infection, the increased inflammatory chemokine levels detected in the plasma of LPS treated mice is not a surprising finding.

Taken together with the bone marrow proteome array, it is interesting to point out that both CCL2 and CXCL10 were highly expressed both in the bone marrow, and the circulation, in response to I.P. LPS injection. However, some chemokines such as CCL3, CCL5, CXCL9 were differentially expressed between the bone marrow and the plasma.

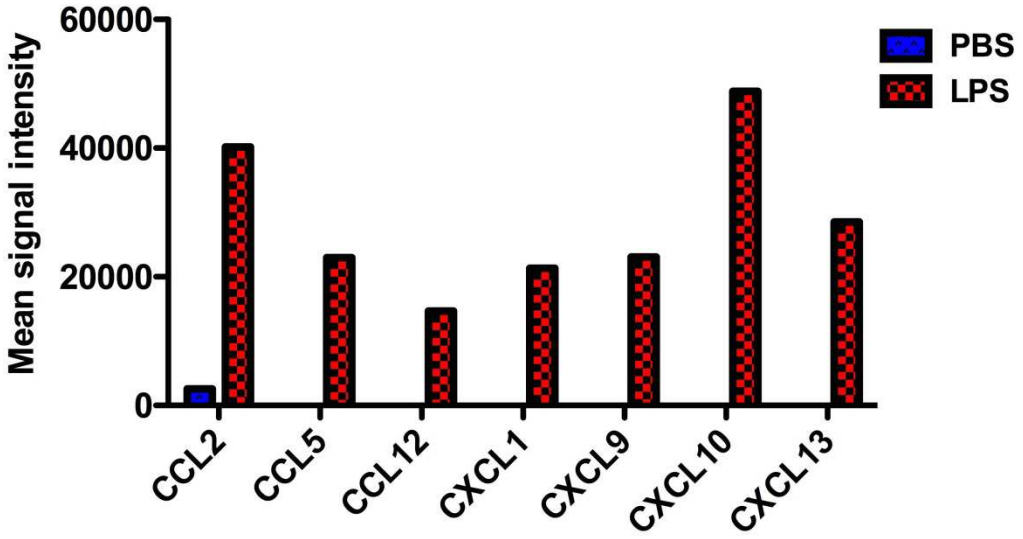



**Figure 3-23 LPS injection induces inflammatory & homeostatic chemokine expression in the plasma**
The signal intensities of each spot on the developed array blots (see figure 3-22) were estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each cytokine, the mean of the signal intensity of each cytokine was calculated, and the corrected signal intensities were calculated by subtracting the mean signal intensity of the spots for the negative control from the mean signal intensity of each cytokine. Figure 3-23 is a bar graph showing the corrected mean signal intensities of the chemokines in the plasma of LPS and PBS treated mice plotted side-by-side.

### 3.5.3.2 Cytokines and growth factors produced in response to I.P. LPS injection.

The bar graph in figure 3-24 summarises the comparison of the cytokines and growth factors that were differentially detected in the plasma of LPS and PBS treated mice. The expression of G-CSF (granulocyte-colony stimulating factor) was highly increased in response to LPS injection, in contrast to PBS controls where the level of G-CSF was below the detection limit of the cytokine array. Considering that I.P. LPS injection models Gram-negative bacterial infection, the increased expression of plasma G-CSF is not a surprising finding as emergency granulopoiesis generally occurs in response to most bacterial infections (Selig and Nothdurft, 1995, Watari et al., 1989). In addition, recognition of LPS by the TLR4 receptor involves CD14 on myelomonocytic cells that ultimately leads to the activation of these cells (Nioche et al., 1988). Thus, the increased G-CSF could be considered as a physiological response as G-CSF is produced from activated mononuclear cells (see the general introductory chapter). However, as G-CSF is a known HSPC mobilising agent, the LPS injection-induced increase in its plasma levels could also partly account for the HSPC mobilisation observed in the I.P. LPS injection model.

The immunodulatory and antiviral cytokine, interferon-gamma (IFN-γ) was approximately 3-fold more highly expressed in the plasma of LPS treated mice compared to controls. Not surprisingly LPS, which is a component of the cell wall of Gram-negative bacteria, induced increased expression of the pro-inflammatory cytokine, IL-1α (interleukin-1 alpha), compared to PBS treated controls. As the proper balance between IL-1α and IL-1rα (IL-1 receptor antagonist) has been shown to be important in the pathophysiology of many diseases (Ma et al., 1998b, Piguet et al., 1993), it is also not surprising that I.P. LPS injection also induced increased expression of IL-1rα (~1500 fold), in contrast to PBS treated controls where the level of IL-1rα was below the limit of detection of the cytokine array. This is in agreement with a previous study that found approximately 100 fold greater IL-1rα, in comparison with IL-1α, in the plasma during an experimental endotoxaemia (Granowitz et al., 1991). Moreover, IL-16, which is also a pro-inflammatory cytokine, was highly expressed in the plasma of LPS treated mice, even though IL-16 was not detectable in the plasma of PBS mice.

Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) was approximately 2-fold more highly expressed in the plasma of LPS treated mice when compared to controls. Another pro-inflammatory cytokine that was ~2-fold more expressed in the plasma of LPS treated mice compared to PBS treated controls was tumour necrosis factor-alpha (TNF-α). Taken together, systemic LPS inflammation induced an inflammatory milieu in the plasma.

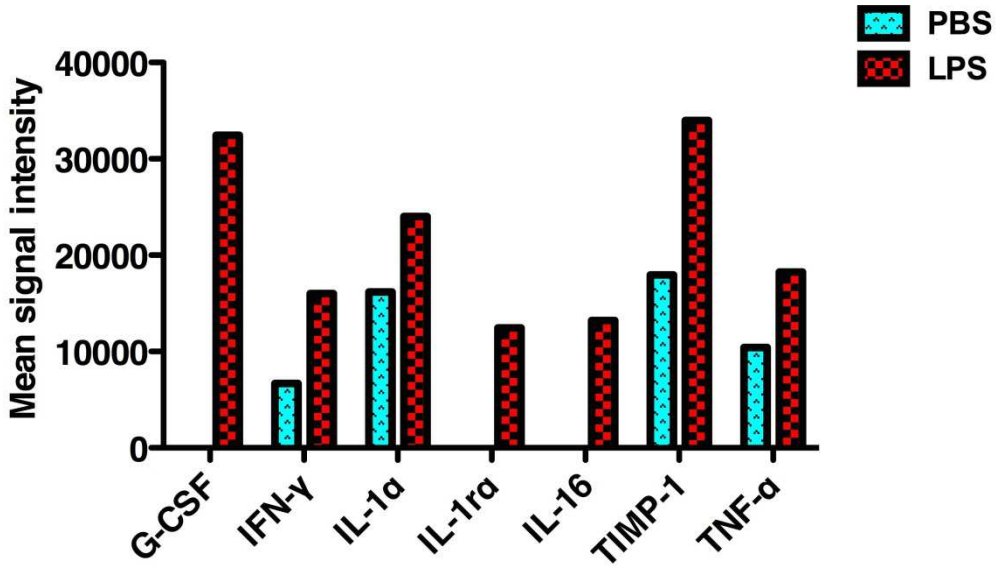



**Figure 3-24 LPS injection modulates the plasma cytokine expression**

The signal intensities of each spot on the developed array blots (see figure 3-20) were estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each chemokine, the mean of the signal intensity of each chemokine was calculated, and the corrected signal intensities were calculated by subtracting the mean signal intensity of the spots for the negative control from the mean signal intensity of each cytokine. Figure 3-24 is a bar graph showing the corrected mean intensities of the LPS and PBS treated mice plotted side-by-side for the cytokines and growth factors expressed in response to the LPS, or PBS, treatment.

### 3.5.4 Profiling of bone marrow HSPC for chemokine receptors and ACKR expression.

Having shown that LPS injection induced HPC mobilisation to the circulation in mice, and that inflammatory chemokines were elaborated in the bone marrow and peripheral blood in response to the LPS injection, it was interesting to examine the HSPC to determine whether they inducibly expressed chemokine receptors in response to LPS injection. Chemokine-chemokine receptor interaction is one of the fundamental axes that mediate cellular migration. Therefore, the hypothesis that HSPC alter chemokine receptor expression in response to the LPS-induced systemic inflammation model was tested. To do this, mice were treated with I.P. LPS/PBS exactly as described in section 3.3.1, and bone marrow was harvested and processed exactly as described in section 3.3.3. The bone marrow cells were stained in accordance with CD150 SLAM antibody staining protocol (Kiel et al., 2005) for enumeration of the HSC ($Lin^-$ $Sca\text{-}1^+Kit^+CD150^+CD48^-$), and MPP1 ($Lin^-Sca\text{-}1^+Kit^+CD150^+CD48^+$), MPP2 ($Lin^-Sca\text{-}1^+Kit^+CD150^-CD48^+$), and MPP3 ($Lin^-Sca\text{-}1^+Kit^+CD150^-CD48^-$) cells. 200 cells from each of the HSC, MPP1, MPP2, or MPP3, populations were sorted on FACS Aria II (BD Biosciences) into 5μL of reverse transcriptase (RT)-specific target amplification master-mix (Fluidigm Technologies) and pre-amplified using thermal cycling conditions as described in the materials and methods chapter. Sorting exactly the same number of cells enabled the normalisation of starting material for the downstream gene profiling. Additionally, the pre-amplification step enabled the reverse transcription of target genes [chemokine receptors and atypical chemokine receptors (ACKR)] to cDNA for quantitative polymerase chain reaction (QPCR). The QPCR was undertaken using the Fluidigm 48.48 Dynamic Chip array platform in accordance with manufacturer's protocol (Fluidigm Technologies), and target gene expression was normalised to Hprt levels. In all cases, the gene expression in the LT-HSC from PBS treated group was used as the calibrator (baseline) for subsequent analysis. Additionally, to increase the stringency of the gene expression data, a fold change ≥2 (upregulated gene

expression), or ≤2 (downregulated gene expression), was set as the threshold for assuming the modulation in gene expression to be above background.

#### 3.5.4.1 HSC express chemokine receptors and ACKRs

As shown in the bar graph in figure 3-25, the analysis of the chemokine receptor gene expression profiling in LT-HSC revealed that in the non-inflamed state, both homeostatic and inflammatory chemokine receptors as well as atypical chemokine receptors could be detected. More importantly, LPS-induced systemic inflammation led to up-regulation of the expression of the inflammatory CC-chemokine receptors Ccr1 (>7-fold increase), ccr2 (~8-fold increase), Ccr3 (2-fold increase) and Ccr5 (>2-fold increase) in the LT-HSC (LSK CD48-CD150+) population compared to the LT-HSC from PBS treated controls (figure 3-25). In addition, of the CXC-chemokine receptors, there was an approximately 4-fold increase in the expression of the inflammatory chemokine receptor, Cxcr2.

Atypical chemokine receptors (ACKR) have been shown to modulate the function of chemokines to prevent exaggerated immune responses and modulate cell movement. Therefore, in the context of inflammation-induced chemokine gene expression, it is possible that expression of these atypical receptors may also be modulated. As shown in the bar graph in figure 3-25, the LPS-induced systemic inflammation caused up-regulated expression of Ackr3 [Cxcr7 (~4 fold)], and ACKR4 [Ccrl1 (>4 fold)] in the LT-HSC population. The expression of the c-kit antigen was included as a positive control, to validate the gene expression in the fluidigm data, as HSPC are known to express c-kit antigen. In addition, the gene for Von Willibrand factor (VWF) was consistently detected in the LT-HSC population in basal state (i.e. LT-HSC from PBS treated groups); the expression of this gene was also downregulated by approximately 6-fold in response to the LPS-induced systemic inflammation.

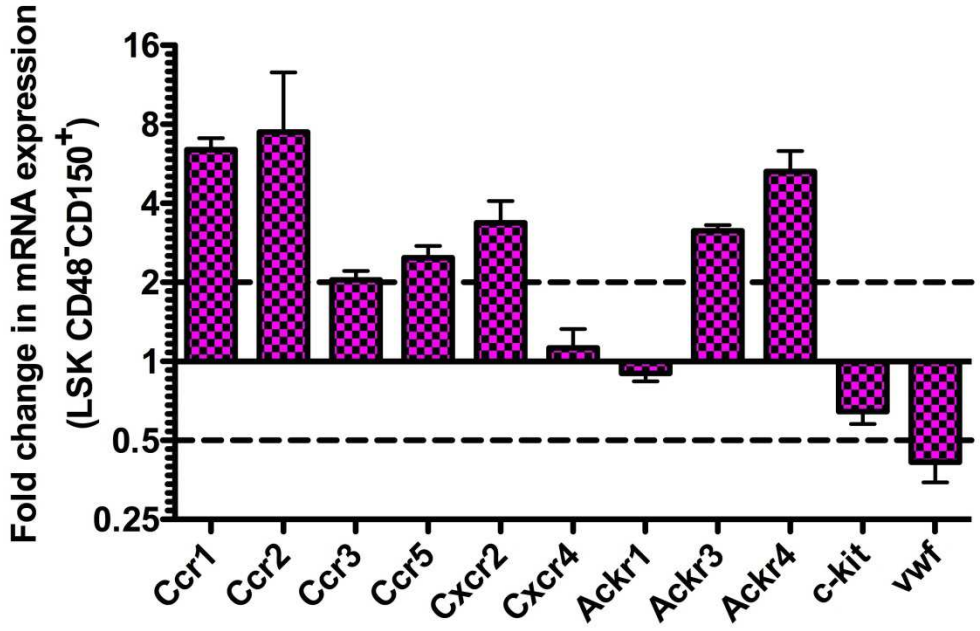



**Figure 3-25 Changes in chemokine receptors and ACKRs in LT-HSC in response to LPS**
C57BL/6 mice were either given a single dose of intraperitoneal LPS (100µg in 100µL PBS), or 100µL I.P. PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer's instructions (Miltenyi Biotec). The c-kit enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), c-Kit, Sca-1, CD48 and CD150 to evaluate HSPC compartment. 200 LT-HSC (defined as $Lin^-Sca\text{-}1^+Kit^+CD48^-CD150^+$) cells from either LPS or PBS treated mice were sorted on FACS Aria II for RT-QPCR using the Fluidigm microfluidic 48.48 Dynamic array platform. Gene expression was normalised to Hprt expression levels and fold change in gene expression calculated relative to the gene expression in LT-HSC population from PBS treated controls; (N=8).

### 3.5.4.2 LPS injection modulates the chemokine receptor and ACKR expression in MPP populations.

LT-HSC sits atop the haematopoietic stem and progenitor cell hierarchy and is the cell population from which all downstream cells are derived. Therefore, the expression of the chemokine receptors and ACKRs in the LT-HSC of PBS-treated mice was next set as the baseline (calibrator population) to allow for the comparison of the chemokine receptor and ACKR expression in the haematopoietic progenitor cell in the PBS and LPS treated mice. This analysis permitted two comparisons to be made: how the gene expression in each progenitor population varied from the expression of the same gene in the PBS treated LT-HSC population; and also how the LPS-induced inflammation modulated the gene expression in the MPP1, MPP2, and MPP3 populations compared to the respective PBS treated MPP1, MPP2, and MPP3 populations. As stated above (see section 3.5.4), to increases the stringency of the data, a threshold of fold change of ≥2 (upregulated gene expression), or ≤2

(downregulated gene expression), was set as a criterion for assuming the modulation in gene expression to be above background.

The data from the chemokine receptor and ACKR gene expression profiling of the MPP1, MPP2 and MPP3 populations are as summarised in the bar graphs in figures 3-26 A, B & C respectively. For the CC-chemokine receptors, only CCR1 demonstrated a consistent >2-fold upregulated expression in response to the I.P. LPS injection in MPP1, MPP2, and MPP3 populations. Although CCR2 transcript was also consistently detected in all the MPP populations, it was only in the MPP1 population that it showed a significant upregulation in response to the I.P. LPS injection (figure 3-26A, p=0.0001; 2-tailed unpaired t-test). The expression of CCR2 was comparable between the PBS and LPS treated MPP2 and MPP3 populations (figure 3-26B & C). The expression of CCR3 in the MPP1-3 populations, were also modulated by I.P. LPS injection; 8-fold downregulation in MPP1 population, but a >4-fold increase in MPP3 population. However, it was only in the MPP2 population that LPS treatment led to a >4-fold increase in CCR5 expression.

For CXC chemokine receptors, CXCR2 was significantly downregulated in the MPP1 population in response to LPS injection when compared to the MPP1 population from PBS treated controls (figure 3-26A, p=0.0238, 2-tailed unpaired t-test). However, the expression of the CXCR2 transcript was comparable between the PBS treated and LPS treated MPP2 populations (figure 3-26B). For the atypical chemokine receptor gene expression, only ACKR4 consistently demonstrated a >2-fold upregulated expression in response to LPS injection (figure 3-26 A, B & C). Whereas ACKR1 expression was downregulated in both MPP1 and MPP2 population in response to LPS injection, ACKR3 was only consistently detected in the MPP3 population. Interestingly, vwf gene expression was consistently downregulated in all the MPP populations (figure 3-26 A, B & C).

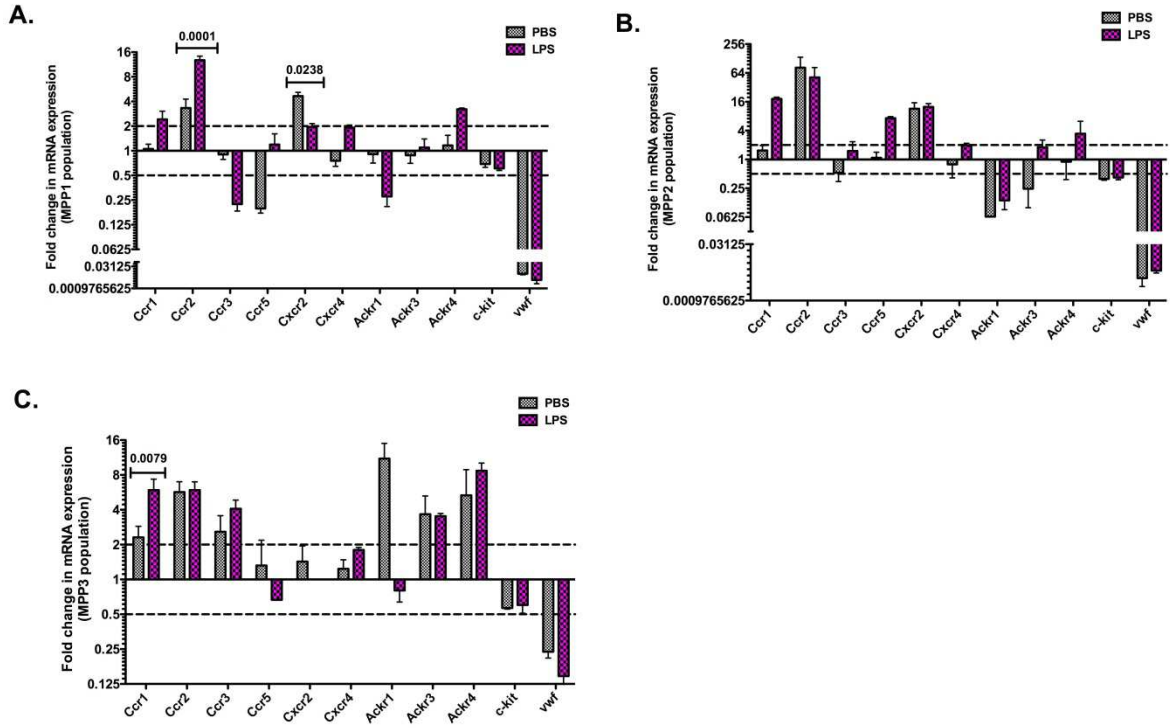



**Figure 3-26 I.P. LPS injection modulates chemokine receptor and atypical chemokine receptor expression in the HSPC compartment.**
C57BL/6 mice were either given a single dose of intraperitoneal LPS (100μg in 100μL PBS) or 100μL PBS as a control. 18-24 hours after the injection, mice were euthanized by $CO_2$ asphyxiation and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec). The c-kit enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), c-Kit, Sca-1, CD48 and CD150 to evaluate HSPC compartment. Dead cells were excluded using Live/Dead Aqua. 200 cells from the MPP1 (A), MPP2 (B) and MPP3 (C) population from either LPS or PBS treated mice were sorted on FACS Aria II for QPCR using the Fluidigm microfluidic 48.48 Dynamic array platform. Gene expression was firstly normalised to Hprt expression levels, and fold change in gene expression calculated relative to the gene expression in the LT-HSC population from PBS treated controls. Figure 3-26 A, B, & C summarises the plot of the fold changes in the respective gene expression in response to LPS, or PBS, treatment plotted side-by-side. [Statistical significance was calculated using 2-tailed unpaired t-test to compare the fold change in the gene expression in LPS treated sample to the fold change in the expression of that same gene in PBS treated mice; (N=8)].

## 3.6 Discussion of the LPS-induced systemic inflammation model

### 3.6.1 LPS-induced inflammation significantly reduces the bone marrow HSPC.

In this chapter I sought to obtain a comprehensive understanding of the mechanism underlying the increased HSPC mobilisation from bone marrow into the circulation caused by I.P. LPS injection. LPS, which is a component of the cell wall of Gram-negative bacteria, has been used in models of sepsis. Although previous reports have established the ability of systemic LPS injection to orchestrate release of HSPC from the bone marrow into the circulation, the

mechanistic details still remain to be elucidated. In agreement with previous reports, the experiments reported here also found that I.P. LPS injection induced significant increases in the numbers of circulating haematopoietic progenitor cells as measured by haematopoietic colony-forming assay of peripheral blood (Vos et al., 1972, Quesenberry et al., 1973). In accordance with LPS-induced mobilisation of bone marrow HSPC to the circulation, the LPS injection significantly reduced the bone marrow cellularity by approximately 33% when compared to PBS treated controls. Furthermore, this reduced bone marrow cellularity was also reflected in significantly reduced CLP, LMPP, ST-HSC and LT-HSC populations in the bone marrow. As reported in other inflammation models (Zhang et al., 2008, Shi et al., 2013), this study also identified an increase in expression of the Sca-1 antigen resulting in an apparent expansion of the proportion of the HSPC (LSK cells) in the LPS treated mice as revealed by flow cytometric analysis. Zhang et al. demonstrated that this apparent expansion of the HSPC was the result of a phenotypic inversion of $Lin^{-}Sca\text{-}1^{-}Kit^{+}$ into $Lin^{-}Sca\text{-}1^{+}Kit^{+}$ as a result of re-expression of Sca-1 antigen in response to LPS (Zhang et al., 2008). In the light of these findings, Sca-1 was omitted from subsequent antibody staining panels.

### 3.6.2 Role of the bone marrow niche in LPS-induced HSPC mobilisation

The interactions of the HSPC and the niche cells, and/or soluble products from these cells are crucial in the retention of the HSPC in their native bone marrow microenvironment. Many previous studies have established that disruption of this HSPC-niche interaction is a critical component of the mechanism underlying most HSPC mobilising agents such as G-CSF and AMD3100 (Levesque et al., 2003a, Levesque et al., 2003b, Liles et al., 2003, Broxmeyer et al., 2005). In order to understand how the I.P. LPS-induced systemic inflammation affected some of these factors, proteome array, ELISA, and flow cytometric analysis were employed to evaluate these factors. Firstly, I wanted to understand how CXCR4 expression was affected at the protein level as the CXCR4-CXCL12 receptor interaction has been established as perhaps the most critical niche component. A previous report proposed that constitutive receptor internalisation and subsequent recycling, independent of ligand binding, was the mechanism regulating homeostatic cell surface CXCR4 expression in human $CD34^{+}$

haematopoietic progenitors (Zhang et al., 2004b). It is therefore possible that the LPS-induced reduction in cell surface Cxcr4 expression may be the result of interference in receptor recycling, or increased degradation of the internalised Cxcr4 or induction of processes that are inhibitory to effective translation of the Cxcr4 transcript.

The ELISA data presented in this study also showed a significant reduction in bone marrow CXCL12 in response to I.P. LPS induced systemic inflammation. This reduction in bone marrow CXCL12 levels may not be surprising considering the increased circulating/plasma G-CSF as revealed in the proteome array data. Previous studies found that reduction in bone marrow CXCL12 was a significant component of the exogenously administered G-CSF-mediated HSPC mobilisation (Christopher et al., 2009, Semerad et al., 2005). However, as G-CSF-induced mobilisation takes a matter of days, in contrast to the 24-hour time point used for the I.P. LPS systemic inflammation model, other factors may contribute to the significant HSPC mobilisation observed with the systemic LPS inflammation model.

The data presented in this chapter also argue for a role of osteoblasts in the HSPC mobilisation observed in the I.P. LPS systemic inflammation model. Previous studies have shown that cells of the osteoblastic lineage, mesenchymal stromal cells (MSC) and bone marrow endothelial cells are critical components of the bone marrow endosteal niche. These cells have been proposed to be the major producers of critical soluble niche factors such as CXCL12, angiopoietin-1, stem cell factor, and osteopontin (Ducy et al., 2000, Calvi et al., 2003, Arai et al., 2004, Mendez-Ferrer et al., 2010). Therefore, a disruption in the function of one of these cellular niche components (i.e. MSC, osteoblasts and endothelial cells) indirectly leads to perturbation of concentration of one or more of these soluble niche products and hence results in HSPC egress from the bone marrow into the peripheral blood. The data presented in this chapter revealed a significant increase in the proportion of bone marrow MSC and endothelial cell populations in response to the I.P. LPS-induced systemic inflammation; in contrast, the I.P. LPS-induced systemic inflammation led to a significant reduction in the proportion of the bone marrow osteoblast population. Interpreting these data in the light of the significant reduction in CXCL12 protein in the bone marrow, it is reasonable to suggest that, in the I.P. LPS-induced

HSPC mobilisation, the critical cellular niche component responsible for CXCL12 reduction may be the osteoblastic cell population.

Interestingly, Heissig et al. previously implicated a role for MMP-9 in HSPC mobilisation by showing that myeloablation-induced increase in bone marrow CXCL12 levels resulted in a corresponding increase in MMP-9 activity that favoured HSPC mobilisation as a consequence of cleavage of c-Kit ligand in the bone marrow (Heissig et al., 2002). As shown in section 3.4.1.3 (see figure 3-10), LPS injection resulted in reduced bone marrow CXCL12 levels as well as reduced MMP-9 levels (see figure 3-20) making it unlikely that the LPS-induced HSPC mobilisation depended on increased MMP-9 activity.

Finally, the phagocytic mononuclear cells of the innate arm of the immune system have also been shown to have roles in retention of HSPC in the bone marrow niche. In line with this, Chow et al. found that clodronate-mediated depletion of bone marrow $CD169^{+}$ macrophage populations led to significant mobilisation of bone marrow HSPC to the circulation (Chow et al., 2011). In addition, Winkler et al. also demonstrated that G-CSF-induced HSPC mobilisation occurred partly due to a depletion of the bone marrow macrophage population, which they called osteomac (Winkler et al., 2010b). Others have also reported that depletion of bone marrow granulocytic cell population accounted in part for the HSPC mobilisation observed with CXCL2, CXCL8 and G-CSF (Levesque et al., 2003b, Pruijt et al., 2002). In order to understand the potential roles played by these mononuclear phagocytic cells in the I.P. LPS-induced HSPC mobilisation, the bone marrow of LPS, or PBS, treated mice was analysed by flow cytometry. These analyses revealed two interesting findings concerning the cells of the mononuclear phagocytic system: firstly, I.P. LPS significantly reduced the bone marrow $Gr\text{-}1^{low}F4/80^{+}CD115^{high}$ monocytic cell population compared to PBS treated controls. Secondly, the I.P. LPS induced inflammation reduced the $CD169^{+}$ macrophage population (defined as $Gr\text{-}1^{-}F4/80^{+}CD115^{int}CD169^{+}CD11b^{low}$) (Chow et al., 2011). Therefore, the I.P. LPS-induced HSPC mobilisation may also have a significant contribution from perturbation of cells of the mononuclear phagocytic system in agreement the previously published data described above.

### 3.6.3 Potential role of the inflammatory chemokine/chemokine receptor axes in LPS-induced HSPC mobilisation.

The chemokine expression profiling data showed that many inflammatory chemokines i.e. CCL2, CCL5, CXCL1, CXCL9 and CXCL10 were inducibly expressed in the plasma of LPS treated mice although these chemokines were below the level of detection in PBS treated control mice. This inducible expression of inflammatory chemokines in both the plasma and bone marrow is not surprising considering that a critical component in LPS-induced inflammation comes from activated monocytic cells (Nioche et al., 1988, Wright et al., 1990). Thus these elaborated inflammatory mediators could be products released from the activated cells. Moreover, the plasma levels of the homeostatic chemokine, CXCL13, increased in response to the I.P. LPS injection. Interestingly, the chemokine receptor profiling data showed that CCR1, CCR2 and CXCR2 could be consistently detected in the bone marrow HSPC. In addition, I.P. LPS injection induced upregulated expression of the transcripts of these chemokine receptors, suggesting a potential instructive role of the inflammatory milieu. As chemokine-chemokine receptor interactions are one of the key molecular cues orchestrating cellular migration, these findings suggest that these inflammatory chemokine receptors may have a role in the I.P. LPS-induced HSPC mobilisation.

## 3.7 Chapter summary

From the data presented in this chapter, the following findings are important:

1. LPS injection causes significant mobilisation of HSPC to the circulation leading to significantly reduced bone marrow cellularity as well as reduced CLP, LMPP, ST-HSC and LT-HSC populations in the bone marrow.

2. The mechanism underlying the I.P. LPS inflammation-induced HSPC mobilisation is potentially multi-factorial involving many players i.e. disruption of the CXCR4/CXCL12 axis by downregulation of HSPC surface CXCR4 and bone marrow CXCL12 protein expression; increased secretion of G-CSF that might be involved in cleavage of CXCL12 and decreased osteoblasts population; depletion of the bone marrow mononuclear phagocytic cell population i.e. monocytes and $CD169^{+}$ macrophage; and inducible expression of chemokine receptors that may enable them to respond to circulating inflammatory chemokines.

# Chapter 4: Peripheral inflammation model

## 4.1 Aims and introduction

Data presented in the previous chapter provides evidence that HPC are mobilised to the circulation in response to systemic LPS injection. In addition, the chemokine receptor gene expression profiling, and the proteome array data, were suggestive of a potential role for the chemokine/chemokine receptor axis in systemic inflammation-induced HSPC mobilisation. However there are many peripheral pathological processes, like psoriasis and dermatitis, that also have inflammatory underpinnings and therefore it was also important to assess the effects of peripheral inflammation on the HSPC compartment to see if it could indirectly induce HSPC mobilisation via release of inflammatory mediators into the circulation. In addition, the data presented in the previous chapter did not determine whether the inflamed context alone, or TLR signalling, or both were critical in orchestrating HSPC exit from the bone marrow. In order to understand whether the inflammatory context alone and/or TLR signalling-induced inflammation was critical for inducing HSPC mobilisation, I set out to use sterile and TLR driven peripheral inflammation models. These involve cutaneous 12-O-tetradecanoylphorbol-13-acetate (TPA), and imiquimod cream, application respectively. Imiquimod cream contains a TLR7 ligand (Hemmi et al., 2002, Lee et al., 2003), whereas TPA is a purely inflammatory agent with no associated TLR signal induction (Blumberg et al., 1984a, Blumberg et al., 1984b, Sharkey et al., 1984). TPA has been used extensively to induce sterile inflammation (Lee et al., 1994, Chiba et al., 1984). It is known to induce inflammation by activating protein kinase C through its mimicry of diacylglycerol (Blumberg et al., 1984a, Blumberg et al., 1984b). Recently, imiquimod cream application to the dorsal skin of mice has also been proposed as a model of human psoriasis (van der Fits et al., 2009). The dose of imiquimod cream employed in this study is in accordance with the dose used to model human psoriasis as published by van der Fits et al. Imiquimod mimics viral infection as it act as a TLR7/8 ligand (in humans) and TLR7 ligand (in mouse). T**he hypothesis** that was tested was that HSPCs may inducibly express inflammatory chemokine receptors in response to peripheral inflammatory mediators, and that the inflammatory chemokine/chemokine receptor axis influences the migratory properties of HSPC. The fundamental questions I aimed to address in this part of the study were:

1. Does peripheral tissue inflammation orchestrate HSPC mobilisation?

2. Does peripheral tissue inflammation impact the bone marrow HSPC compartment?

3. Does peripheral tissue inflammation alter the HSPC niche?

4. What chemokines and cytokines are produced in response to peripheral tissue inflammation?

In this way, I sought to obtain a comprehensive understanding of the role of the inflammatory chemokine/chemokine receptor axis in HSPC biology in peripheral tissue inflammation models associated with infectious components as well as purely inflammatory stimuli with no infectious surrogates.

## 4.2 Does peripheral inflammation orchestrate HSPC mobilisation

### 4.2.1 Introduction

In the previous chapter, the ability of systemic inflammation to orchestrate HSPC mobilisation was evaluated using the I.P. LPS injection model. In this chapter, the ability of peripheral inflammation to influence HSPC biology was also assessed using topical imiquimod cream, or topical TPA application, to the shaved dorsal skin of mice. To elicit the peripheral inflammation, mice received a single daily application of either ~62.5mg imiquimod cream (equivalent to 3.125mg active TLR7 ligand), or 100μM TPA, on shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received topical application of either aqueous cream or acetone as controls for imiquimod cream and TPA treatments respectively. After the treatment, mice were culled by $CO_2$ asphyxiation and peripheral blood, bone marrow and spleen harvested for further analysis (see details described in relevant sections below).

Histological sections of skin that directly received inflammatory agent application were also examined. Also, the cellularity of the bone marrow and spleen was assessed to understand how the peripheral inflammation models affected haematopoietic activity in each of these organs. Additionally, the

ability of the peripheral inflammation models to induce HSPC mobilisation from the bone marrow to the circulation and spleen were investigated using HPC colony-forming assays.

### 4.2.2 Histology

Before investigating the systemic effects produced by the topical imiquimod cream, or TPA, treatment, I sought to examine the morphology of the skin to determine the local effects produced by each of these inflammatory agents. The dorsal skin that directly received the topical imiquimod cream/TPA treatment (see section 4.2.1 for description) was harvested, embedded in paraffin, sectioned to 8μm and stained by haematoxylin and eosin. Figures 4-1A-D are the representative micrographs of the skin sections of mice treated with aqueous cream (A), imiquimod cream (B), acetone (C), and TPA (D). Topical imiquimod cream, or TPA, treatment induced a profound inflammatory response in the dorsal skin of mice as shown by the epidermal thickening [indicated by the arrows in figures 4-1B (imiquimod cream treatment) and 4-1D (TPA treatment)].

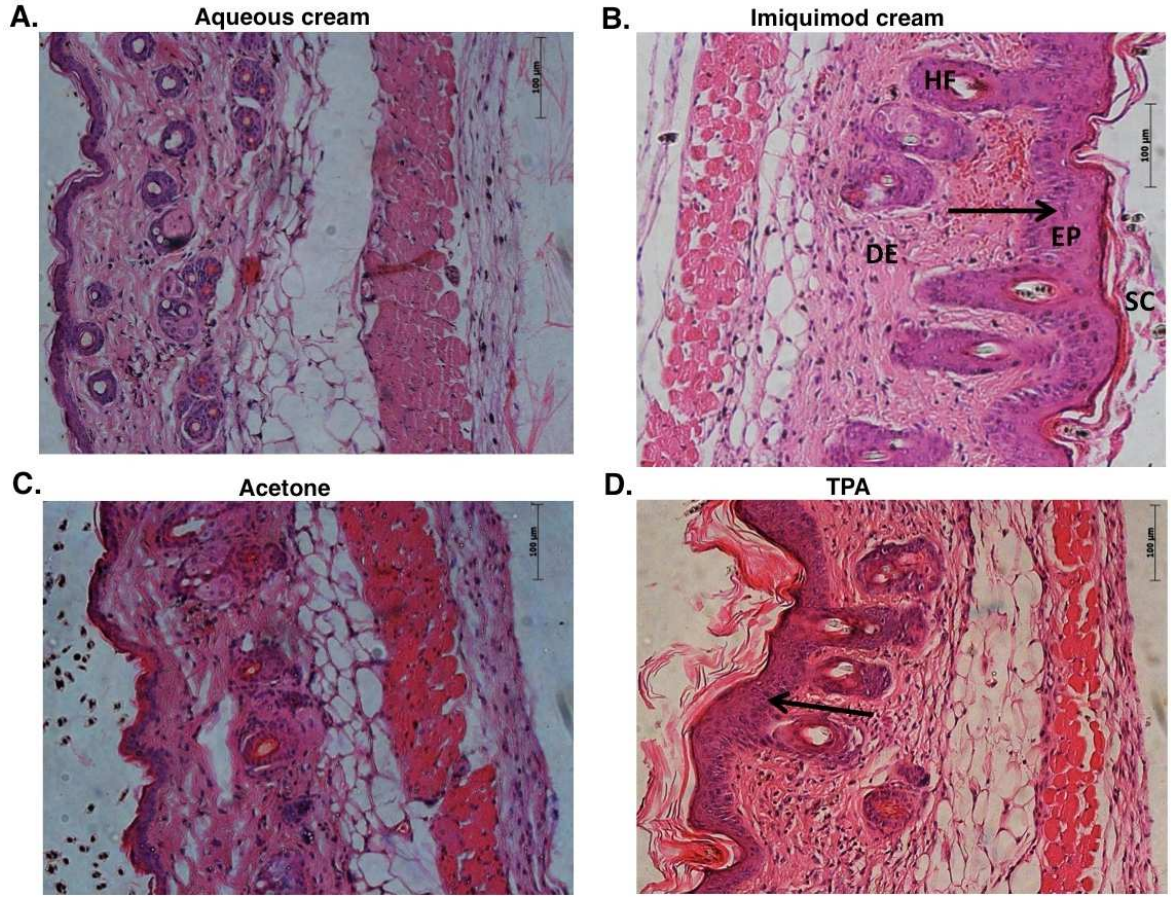



**Figure 4-1 Topical imiquimod cream, or TPA, treatment causes epidermal thicknening in the dorsal skin of mice.**

The dorsal skin of mice that had directly received aqueous cream (A), or topical imiquimod cream (B), or acetone (C), or TPA (D), treatment was harvested, embedded in paraffin, sectioned to 8µm and stained with haematoxylin and eosin for morphological examination. Images were acquired using 20X magnification on Zeiss Axiostar Plus Microscope fitted with AxioVision software; arrow points to epidermal thickening in response to imiquimod cream (B), or TPA (D), treatment. [DE, dermis; EP, epidermis; HF, hair follicle; SC, stratum corneum]

### 4.2.3 Topical imiquimod cream, or TPA, application induces HSPC mobilisation

The number of haematopoietic progenitors in peripheral blood and spleen were assessed using colony-forming assays as a measure of how the different peripheral inflammatory stimuli affected haematopoietic progenitor cell mobilisation. As explained in the previous chapter, the medium employed for the haematopoietic colony-forming assay was Methocult$^{TM}$ GF M3434 (StemCell Technologies). 1 x$10^5$ peripheral blood or splenic leucocytes from the topical imiquimod cream or TPA treated mice (or their respective control groups) were seeded into 1.1mL of Methocult$^{TM}$ GF M3434 in duplicate, and incubated for 7-10 days at 37°C in a humidified chamber, after which colonies were identified and enumerated using an inverted microscope.

As shown in the scatter plot in **figure 4-2A**, both imiquimod cream, and TPA, treatment induced statistically significant increases in the numbers of circulating myeloerythroid progenitor colonies, predominantly CFU-GM ($p<0.0001$ in each case; 2-tailed unpaired t test in both cases). On average, imiquimod cream treatment induced >4-fold increase (19.33/4.444) in circulating myeloerythroid progenitors; whereas, TPA-induced inflammation resulted in >5-fold (25.56/4.667) increase in the number of circulating myeloerythroid progenitors. Figures 4-2 B, C & D are representative pictures of colonies counted as CFU-GEMM, CFU-GM and CFU-E respectively.

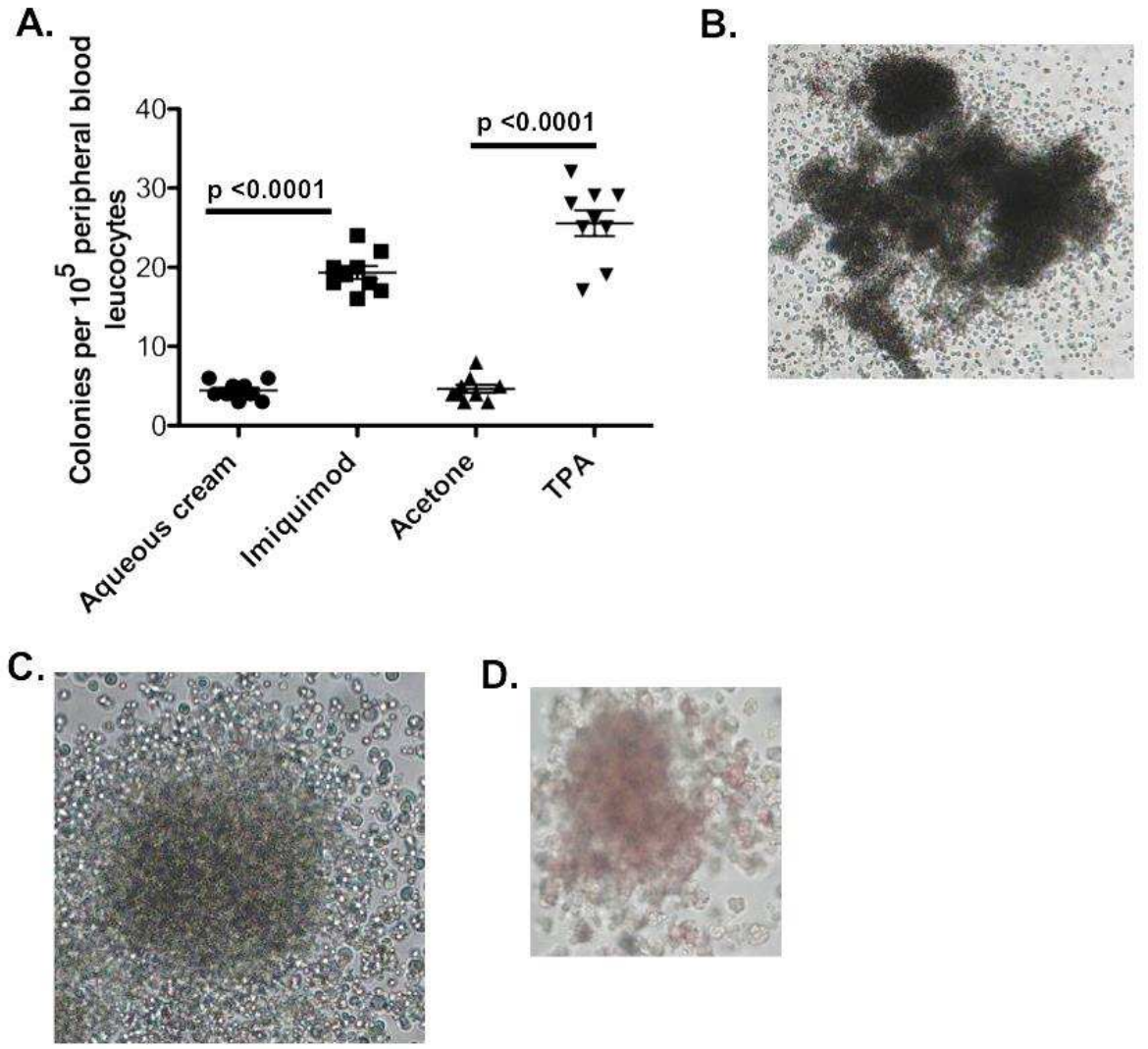



**Figure 4-2 Topical imiquimod cream or TPA application mobilises HPC to the circulation.** C57BL/6 mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100μM in 100μL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and peripheral blood was taken for haematopoietic colony-forming assay. After lysing red blood cells with Ammonium chloride, $1x10^5$ leucocytes were seeded into 1.1mL of Methocult™ GF M3434 medium (StemCell Technologies) in duplicate, and incubated for 7-10 days at 37°C, in a 5% $CO_2$ humidified chamber. Colonies representing haematopoietic progenitor cells were then identified and enumerated using an inverted microscope. In figure 4-2 A, the total colonies formed were compared for each treatment group and its respective control i.e. imiquimod cream with aqueous cream and TPA with acetone treated controls. [p<0.0001; 2-tailed, unpaired t-test, n=4-5 mice per experiment; (5 independent experiments)]. Data were plotted from mean ±SEM]. Figures B, C, and D, are respective representative colonies identified as CFU-GEMM, CFU-GM and CFU-E.

In addition, imiquimod cream, or TPA, induced peripheral inflammation led to increased numbers of total myeloerythroid progenitors in the spleen (figure 4-3, p<0.0001; 2-tailed unpaired t-test in each case). Topical imiquimod cream-induced peripheral inflammation resulted in >5-fold (53.38/9.250) increase in total splenic myeloerythroid progenitors, whereas TPA-induced peripheral inflammation led to >4-fold (44.56/10.44) increase in splenic myeloerythroid progenitor cell numbers.

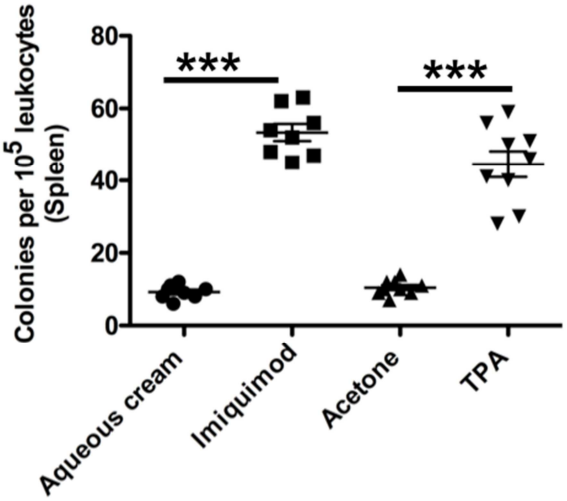



**Figure 4-3 Topical imiquimod cream or TPA application significantly increases HPC in the spleen.**
C57BL/6 mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100μM in 100μL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and spleens were taken for haematopoietic colony-forming assay. After mincing and filtering the spleen through a 70μm cell strainer, red blood cells were lysed with Ammonium chloride solution. Next, $1x10^5$ leucocytes were seeded into 1.1mL of Methocult™ GF M3434 medium (StemCell Technologies) in duplicate, and incubated for 7-10 days at 37°C, in a 5% $CO_2$ humidified chamber. Colonies representing haematopoietic progenitor cell were then enumerated using an inverted microscope. In figure 4-3, the total colonies formed were compared for each treatment group and its respective control i.e. imiquimod cream with aqueous cream and TPA with acetone treated controls. [***p<0.0001; 2-tailed, unpaired t-test, n=4-5 mice per experiment; 5 independent experiments). Data were plotted from mean ±SEM].

### 4.2.4 Topical imiquimod cream, or TPA, treatment significantly reduces bone marrow cellularity.

Bones [2x (tibia + femur + hip bone)] harvested from mice treated with topical imiquimod cream, or TPA, as described in section 4.2.1, were crushed in a mortar and pestle, re-suspended in FACS buffer and filtered through 70μm cell strainer (BD Biosciences) to eliminate bone fragments. After lysing red blood cells with Ammonium chloride solution, total cells were counted using a haemocytometer with trypan blue dead cell exclusion. As summarised in the scatter plot in figure 4-4, peripheral inflammation induced by either imiquimod cream or TPA treatment significantly reduced the bone marrow cellularity compared to their respective controls (**figure 4-4,** p = 0.0014; 2-tailed Mann-Whitney U test). On average, the topical imiquimod cream application reduced the total bone marrow cellularity by 47.8% [(111.8-58.41)/111.8*100%], whereas the topical TPA treatment reduced the total bone marrow cellularity by 27.1% [(110.7-80.68)/110.7*100%].

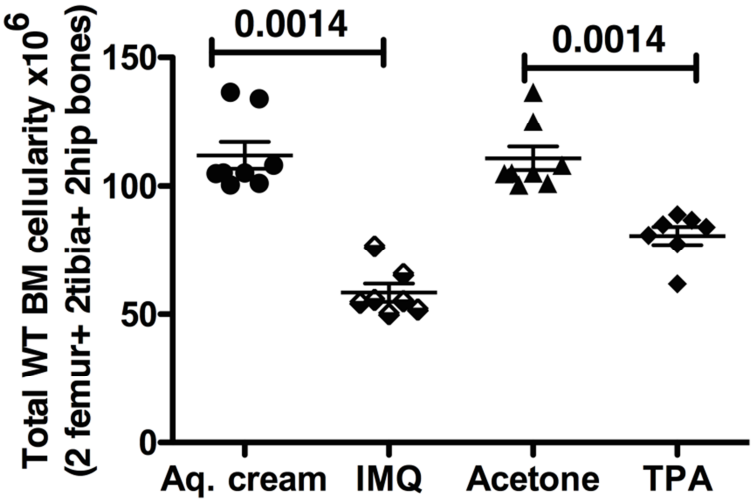



**Figure 4-4 Topical imiquimod cream, or TPA, application significantly reduces the bone marrow cellularity.**
A single, daily dose of topical imiquimod cream [IMQ (62.5mg)], or TPA (100μM in 100μL acetone) was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream, or acetone, application as controls for topical imiquimod cream and TPA application respectively. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and bone marrow [2x (tibia + femur + hip bones)] harvested for total bone marrow cellularity estimation. Total bone marrow cells were counted using haemocytometer and the trypan blue dead cell exclusion method. Statistical significance was calculated by comparing each treatment group with its respective control group. [2-tailed Mann-Whitney U test; n=7-8 (3 independent experiments)].

### 4.2.5 Topical imiquimod cream, or TPA, application significantly increases spleen cellularity

Spleen harvested from mice treated with the topical imiquimod cream or TPA (see section 4.2.1) was minced to isolate haematopoietic cells (see materials and methods for details). Total spleen cellularity was determined using a haemocytometer with trypan blue dead cell exclusion. As summarised in the scatter plot in figure 4-5, peripheral inflammation following topical imiquimod cream, or TPA, application, significantly increased splenic cellularity when compared to their respective control groups (figure 4-5, $p = 0.0002$ (imiquimod cream treatment) or $p < 0.0001$ (TPA treatment); 2-tailed Mann-Whitney U test in both cases). On average, the topical imiquimod cream treatment increased the splenic cellularity by 2.7-fold (213.7/80.62) compared to aqueous cream treated controls, whereas topical TPA treatment increased the splenic cellularity by ~1.9-fold (147.3/78.52) compared to acetone treated controls.

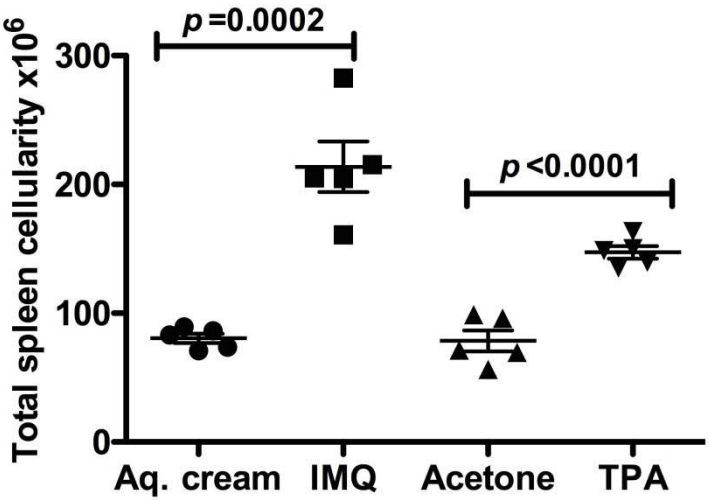



**Figure 4-5 Topical imiquimod cream, or TPA, application significantly increases the splenic cellularity.**
A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or TPA (100μM in 100μL acetone) was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and spleens harvested for cellularity estimation. Total spleen cells were counted using a haemocytometer with the trypan blue dead cell exclusion method. Statistical significance was calculated by comparing each treatment group with its respective control group. [2-tailed Mann-Whitney U test; n=5 (3 independent experiments)].

In summary, data presented in this section suggest that the topical imiquimod cream and TPA treatments:

(A) Significantly mobilised HPC to the circulation, leading to significant reductions in bone marrow cellularity.

(B) Significantly increased the splenic cellularity with an accompanying increase in haematopoietic progenitor cells in the spleen, which may be suggestive of infiltration of the spleen by mobilised HSPC and/or increased haematopoietic activity in the spleen in response to each of these peripheral inflammation-inducing agents.

## 4.3 Does the peripheral inflammation impact the HSPC compartment?

### 4.3.1 Immunophenotypic characterisation of HSPC

As stated in the previous chapter, one of the drawbacks with the haematopoietic colony-forming assay is its inability to support the growth of LT-HSC. In addition, the medium employed in the haematopoietic colony-forming assays, described in the previous sections, does not support the growth of lymphoid progenitor cells.

In order to obtain a comprehensive understanding of how the different peripheral inflammation models impacted subsets of cells in the HSPC compartment, flow cytometry was employed to immunophenotypically characterised these cells. Mice received topical imiquimod cream, or TPA, application as described in **section 4.2.1 above**. Femur, tibia and hipbones were next harvested from these mice and processed exactly as described in **section 4.2.4** to isolate the marrow cells. After enriching the marrow cells for haematopoietic stem and progenitor cells (see previous chapter for details), the c-Kit enriched cells were subsequently stained using the CD150+ SLAM staining protocol as described in the previous chapter (Kiel et al., 2005).

### 4.3.2 Topical imiquimod cream, or TPA, treatment causes an apparent LSK/HSPC expansion

The gating strategy used to select the HSPC/LSK was exactly as described in the previous chapter (section 3.2). In line with previously published data suggesting that Sca-1 expression is modulated by inflammation (Zhang et al., 2008), the Sca-1 protein expression was observed to be significantly upregulated by each of the peripheral inflammatory stimuli resulting in apparent expansion of the LSK compartment (compare figures 4-6 A & B for aqueous cream and imiquimod cream treatment respectively; or figures 4-6 C & D for topical acetone and TPA application respectively). As summarised in the scatter plot in figure 4-6E, the increased Sca-1 expression was reflected in apparently increased frequency of the HSPC/LSK cells in the bone marrow of topical imiquimod cream or TPA treated mice compared to relevant controls ($p<0.001$; 2-tailed unpaired t-test in each case).

Altogether, by comparing figures 4-6 A & B, to C & D, it is evident that this phenomenon of pseudo Sca-1 re-expression by $Kit^{+}Sca\text{-}1^{-}$ cells is a mainly a consequence of TLR-driven inflammation (see also chapter 3).

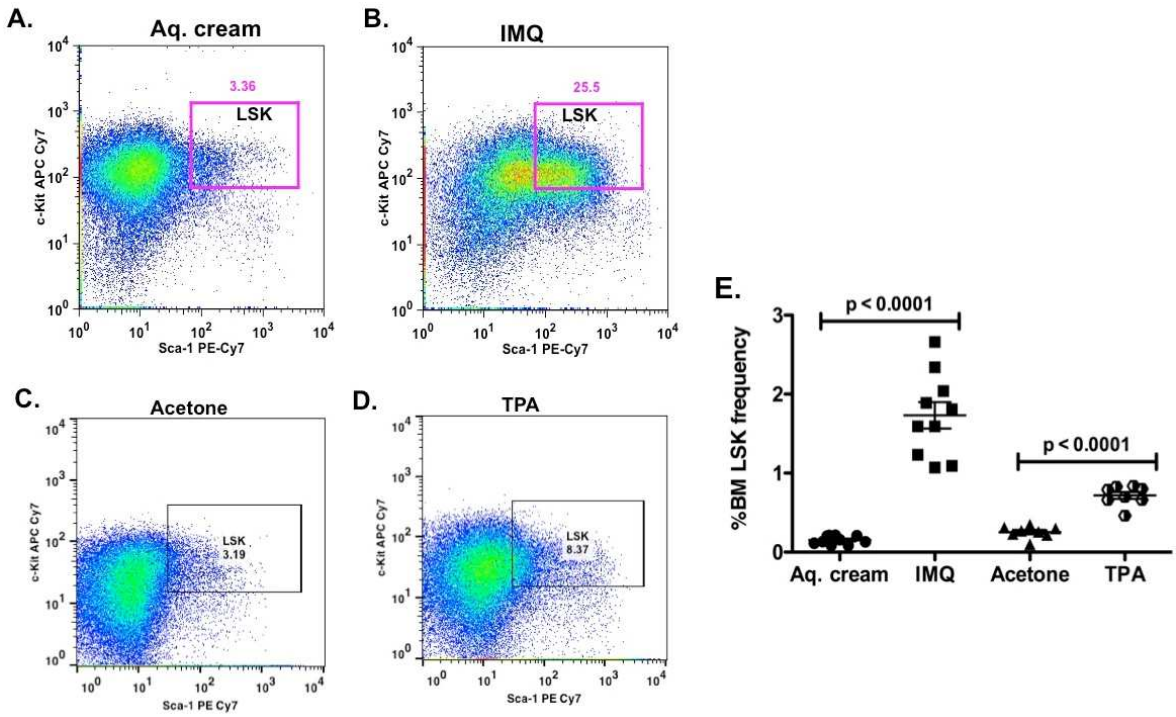



**Figure 4-6 Topical imiquimod cream (IMQ), or TPA, treatment leads to apparent increase in bone marrow LSK cells.**
Figures 4-6 A & B are respective representative dot plots showing the gating of the bone marrow LSK cells in mice treated with either topical aqueous cream or imiquimod cream. Similarly, figures 4-6 C & D are respective representative dot plots showing the gating of the bone marrow LSK cells in mice treated with either topical acetone, or TPA. Figure 4-6F compares the frequency of the cells in the LSK compartment per $1x10^6$ total c-Kit enriched bone marrow cells between each treatment group and its respective control [***p<0.001; 2-tailed unpaired t-test; n=4-5 (two independent experiments pooled together)].

### 4.3.3 Topical imiquimod cream, or TPA, application alters the cellular composition of the HSPC compartment.

Although the data presented in section 4.2.2 showed that topical imiquimod cream or TPA treatment significantly reduced total bone marrow cellularity, the data presented in section 4.3.2 was, paradoxically, suggestive of increased HSPC numbers in response to each of the peripheral inflammation inducing agents. This was most probably due to the increased Sca-1 expression as shown above (and also in the previous chapter). In order to obtain an unbiased immunophenotypic characterisation of the HSPC compartment, the method published by Adolfsson et al. (modified as explained in the previous chapter) was employed to stain bone marrow cells from mice treated with either topical imiquimod cream or TPA (Adolfsson et al., 2005). The strategy used for gating the LK cells was exactly as explained in the previous chapter. The dot plots in figures 4-7 A & B are the respective immunophenotypic characterisation of the bone marrow LK cells from mice treated with topical aqueous cream or imiquimod cream. Figures 4-7 C & D are also the respective dot plots of the

immunophenotypic characterisation of LK cells from mice treated with topical acetone or TPA. As summarised in the bar graph in figure 4-7E, whereas topical imiquimod cream treatment significantly increased the bone marrow CLP population (p=0.004; 2-tailed unpaired t-test), topical TPA treatment significantly reduced this population (p=0.0011; 2-tailed unpaired t test). Also, each of the peripheral inflammatory agents significantly reduced the LMPP population when compared to their respective controls (p=0.0006, or p=0.0027 for imiquimod cream and TPA treatment respectively; 2-tailed unpaired t-test). In addition, topical TPA application significantly reduced the bone marrow ST-HSC population compared to acetone treated controls (p=0.0197; 2-tailed unpaired t test). Although there was a trend towards reduction in the ST-HSC population in response to the topical imiquimod cream treatment, this did not reach statistical significance.

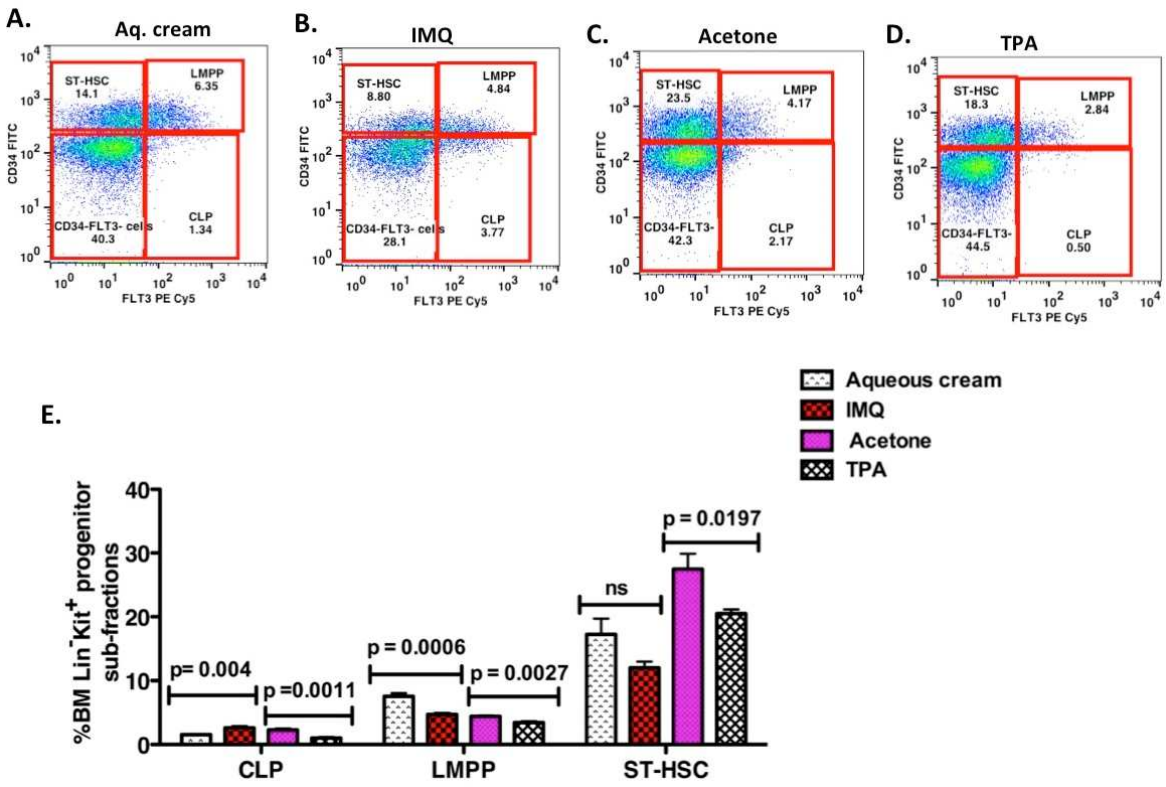



**Figure 4-7 Topical imiquimod cream or TPA treatment modulates cellular composition of the HSPC compartment.**

A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or TPA (100µM in 100µL acetone) was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec). The

c-Kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), as well as c-Kit, CD34, CD150, and FLT3 antibodies to evaluate the HSPC compartment. After excluding dead cells, c-Kit+ cells were re-gated for expression of CD34 and FLT3 antigens to identify CLP, LMPP, ST-HSC and $CD34^{-}FLT3^{-}$ multipotent cells. Figures A & B are respective representative dot plots showing the sub-gating of the bone marrow LK cells from topical aqueous cream and imiquimod cream treated mice based on CD34 and FLT3 expressions; similarly, figures C & D are respective representative dot plots showing the sub-gating of the bone marrow LK cells from acetone and TPA based on CD34 and FLT3 expressions. Figure E is a bar graph summarising the changes in the proportions of the LMPP, ST-HSC, CLP and $LKCD34^{-}FLT3^{-}$ multipotent progenitors in response to the either topical imiquimod cream or TPA treatment. Statistical significance was estimated using the unpaired t-test (2-tailed) in all cases. (n=5, 2-independent experiments)].

### 4.3.4 Topical imiquimod cream application significantly reduces bone marrow phenotypic LT-HSC population.

As explained elsewhere (Kiel et al., 2005) and in the previous chapter, within the HSPC population, CD150 antigenic expression enriches for cells with long-term repopulation potential (LT-HSC). As LT-HSC expresses neither CD34 nor FLT3 antigens (Yang et al., 2005, Osawa et al., 1996), the $CD34^{-}FLT3^{-}$ multipotent cells (see gating in figure 4-7 above) were re-gated for differential expression of c-Kit and CD150 antigens. As shown in figures 4-8 A & B and summarised in figure 4-8E, topical imiquimod cream treatment significantly reduced the bone marrow $Lin^{-}Kit^{+}CD34^{-}FLT3^{-}CD150^{high}$ population ($p<0.0001$; 2-tailed unpaired t-test). However, the topical TPA application did not significantly alter the bone marrow LT-HSC population (compare figures 4-8 C & D).

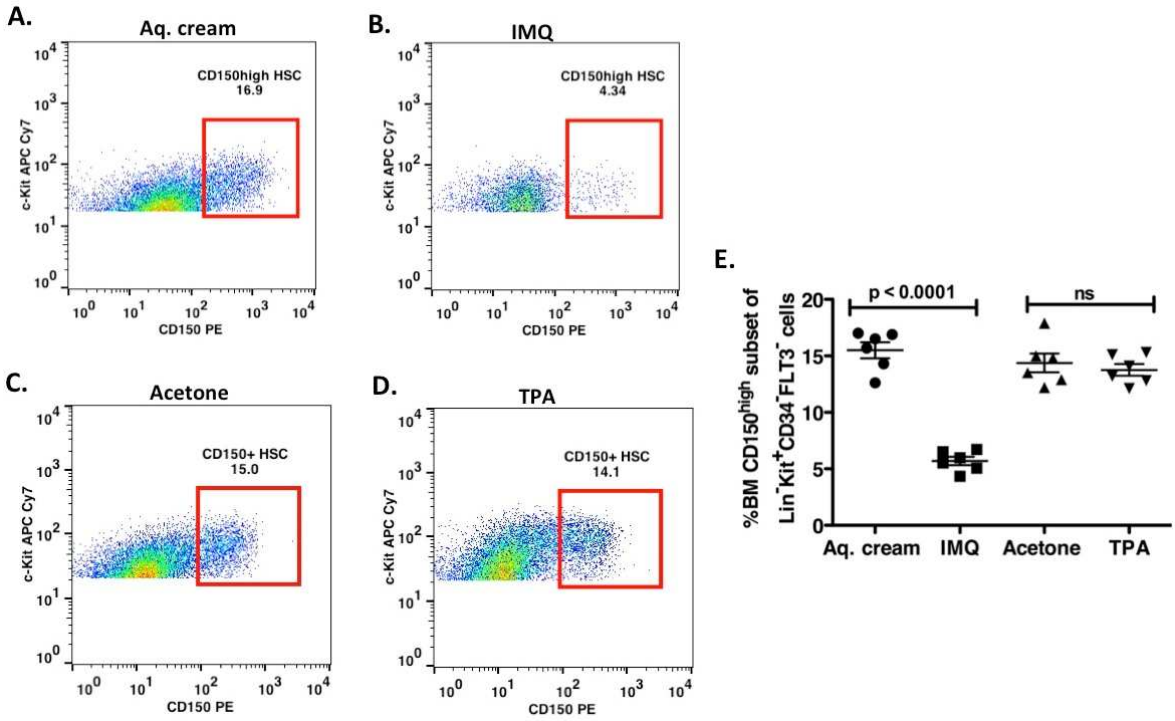



**Figure 4-8 Topical imiquimod cream treatment significantly reduces the bone marrow Lin$^-$ Kit$^+$CD34$^-$FLT3$^-$CD150$^{high}$ population in mice.**
The CD34$^-$FLT3$^-$ cells (see gating strategy in figure 4-7 above) from aqueous cream (A) or imiquimod cream (B) or acetone (C) or TPA (D) treated mice were re-gated for differential expression of c-Kit and CD150 antigens to identify the LT-HSC population (Lin$^-$Kit$^+$CD34$^-$FLT3$^-$ CD150$^{high}$ cells). Figure E is a scatter plot summarising the impact of the topical imiquimod cream or TPA treatment on the LT-HSC population in the bone marrow of mice. Statistical significance was calculated using the 2-tailed unpaired t-test. [N=6; 2 independent experiments].

In summary, data presented in this section suggest that:

(A) Whereas peripheral inflammation induced by topical imiquimod cream treatment causes significantly increased Sca-1 expression in HSPC population, TPA-induced peripheral inflammation only caused a moderate increase in Sca-1 re-expression on HSPC population.

(B) Whereas topical imiquimod cream treatment significantly increased CLP and reduced the LT-HSC and LMPP population, TPA treatment significantly reduced the CLP, LMPP, and ST-HSC populations without significantly altering the LT-HSC population in the bone marrow of mice.

## 4.4 Does the peripheral inflammation alter the bone marrow niche?

As extensively reviewed by others (Ehninger and Trumpp, 2011) and as outlined in the general introductory chapter, the haematopoietic stem cell niche has cellular component (e.g. macrophages, osteoblasts, endothelial cells,

mesenchymal stromal cells) and their elaborated soluble products (e.g. CXCL12, stem cell factor, angiopoietin-1, etc.). The interactions between these niche cells, or their secreted products, and the HSPC are critical in ensuring HSPC retention and proper function in the organism. Therefore, to understand the extent to which the peripheral inflammation models impacted the bone marrow niche, the following questions were addressed:

1. How do the peripheral inflammation models affect the CXCR4/CXCL12 axis in the bone marrow?

2. How do the peripheral inflammation models affect the bone marrow stromal cell compartment (i.e. MSC, endothelial cells and osteoblasts)?

3. How do the peripheral inflammation models affect the mononuclear phagocytic cells of the bone marrow?

The overall objective was to begin the process of elucidating the mechanistic details that resulted in the egress of bone marrow HSPC to the circulation as reported in previous sections of this study.

### 4.4.1 How do the peripheral inflammation models affect the CXCR4/CXCL12 axis in the bone marrow?

The essential role of the CXCR4/CXCL12 axis in the retention of HSPC in the bone marrow has been extensively reviewed in the general introductory chapter. Others have shown that perturbation of this CXCR4/CXCL12 signalling axis is one of the fundamental mechanisms used by most HSPC mobilising agents (Christopher et al., 2009, Semerad et al., 2005). It has been shown that HSPC express CXCR4 that interacts with CXCL12 secreted from bone marrow stromal cells such as osteoblasts and MSC (Aiuti et al., 1997, Peled et al., 2000). In this section of the study the main objectives were: firstly, to use flow cytometry to analyse HSPC for CXCR4 expression in mice treated with topical imiquimod cream or TPA; and secondly, to measure the plasma and bone marrow CXCL12 concentration by ELISA to understand how each of the peripheral inflammation models affected the CXCL12 levels. Ultimately, I hoped to understand whether a perturbation of the CXCR4/CXCL12 axis contributed to the increased circulating HSPC seen in the topical imiquimod cream, or TPA application, models.

#### 4.4.1.1 Topical imiquimod cream, or TPA, application does not significantly alter HSPC CXCR4 expression.

Mice received daily topical imiquimod cream, or TPA, application for three consecutive days (see section 4.2.1 for details). Bone marrow cells were enriched for HSPC by the c-Kit enrichment protocol described in previous sections, and stained with Live/Dead aqua fixable dye as well as haematopoietic lineage (Lin) cell markers, c-Kit, and CXCR4 antibodies to assess CXCR4 cell surface expression in HSPC (LK cells). The gating strategy for the LK cells was essentially as described in the previous chapter. Figures 4-9 A & B are the respective histogram overlays of the CXCR4 expression in the LK/HSPC population from mice treated with either topical imiquimod cream or topical TPA. As shown by the two distinct peaks in figures 4-9 A & B, there were two patterns of HSPC CXCR4 expression; $CXCR4^{low}$ and $CXCR4^{high}$ cells. The scatter plot in figure 4-9C summarises the impact of topical imiquimod cream, or TPA, treatment on $CXCR4^{high}$ expressing HSPC, showing that neither treatment significantly altered the CXCR4 expression (ns = not significant; 2-tailed unpaired t-test). The CXCR4 specific isotype-control staining pattern (figures 4-9 A and B) shows the specificity of the CXCR4 staining.

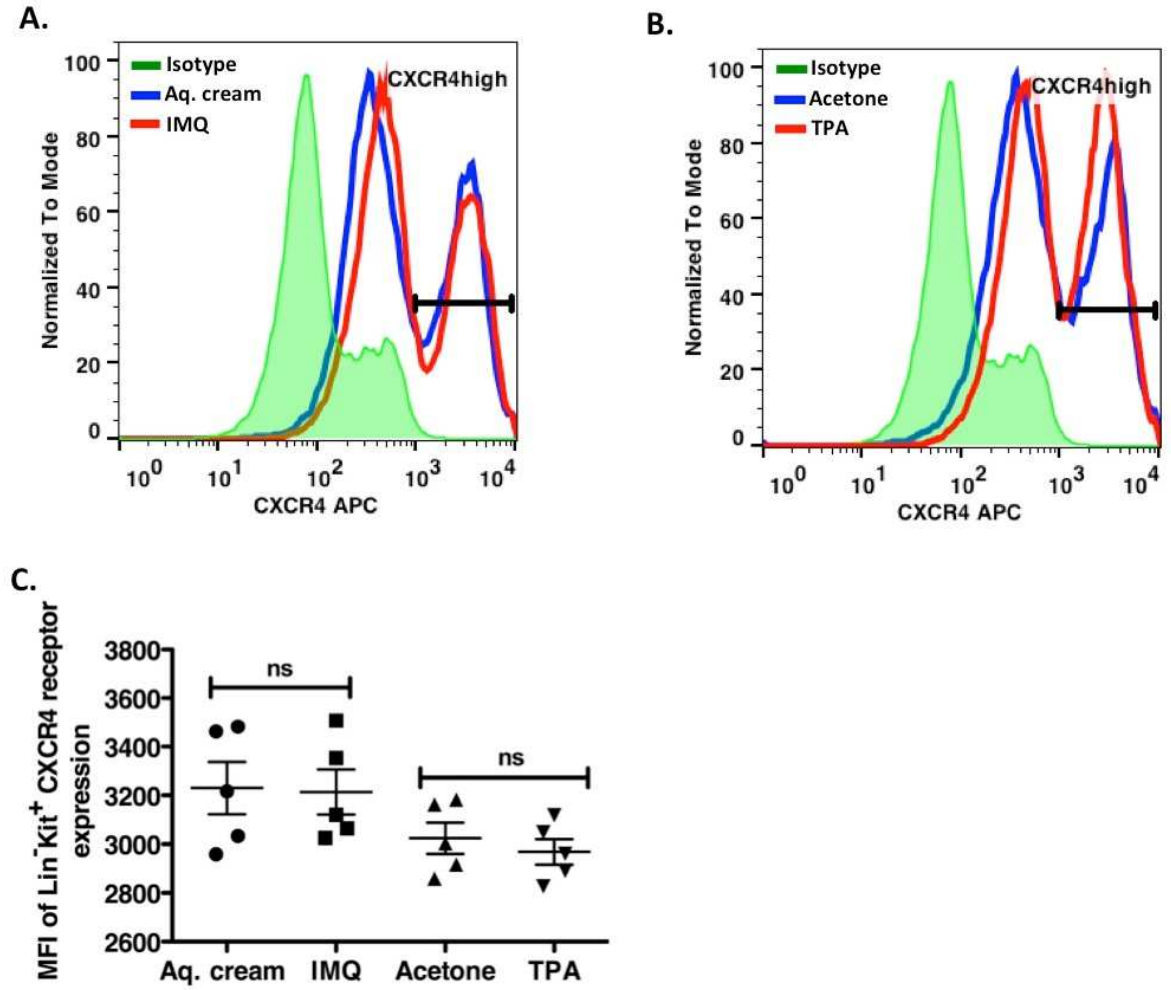



**Figure 4-9 Neither topical imiquimod cream, nor TPA, application significantly alters HSPC surface CXCR4 expression.**
C57BL/6 mice were either given a single daily dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application for three consecutive days. On day four, the mice were euthanized by $CO_2$ asphyxiation and bone marrow cells [2x (tibia + femur + hip bones)] were harvested, crushed in a mortar and pestle, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec, UK). The c-Kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), c-Kit, CXCR4, and Live/Dead Aqua fixable dye to analyse the HSPC (LK) compartment for cell surface CXCR4 receptor expression. After gating on live cells through dead cell exclusion using the Live/Dead Aqua fixable dye, Lin- cells were selected for further analysis. Figure A is a histogram plot showing the overlay of the CXCR4 expression in HSPC from imiquimod cream or aqueous cream treated mice and isotype control. Similarly, figure B is a histogram overlay showing the CXCR4 expression in bone marrow HSPC of mice treated with either topical acetone or TPA. Figure C is a scatter plot summarising of the comparison of the MFI for the impact of imiquimod cream/TPA treatment on HSPC surface $CXCR4^{high}$ expressing cells.. Statistical significance was calculated using the 2-tailed unpaired t-test. (n=5; 2 independent experiments).

#### 4.4.1.2 Topical imiquimod cream or TPA treatment significantly reduces bone marrow CXCL12 concentration.

Having addressed the effects of the peripheral inflammation on LK/HSPC cell surface CXCR4 expression, I next sought to investigate the bone marrow CXCL12 concentrations. CXCL12 is the ligand for CXCR4, and it is the interaction of this ligand with CXCR4 that aids the retention of HSPC in the bone marrow. The amount of CXCL12 in the peripheral blood was also quantified. The goal was to

firstly investigate whether the bone marrow CXCL12 concentration was affected by any of the peripheral inflammation inducing agents. Secondly, by measuring the peripheral blood CXCL12 concentration, I also wanted to understand if any perturbation in the bone marrow CXCL12 created a CXCL12 gradient that favoured the exit of HSPC from the bone marrow. Mice were treated with topical imiquimod cream or TPA as described in the previous sections. Peripheral blood and total bone marrow proteins were harvested as described in chapter 3. Total CXCL12 concentration in plasma, or total bone marrow protein extract, from mice treated with topical imiquimod cream (or aqueous cream treated controls), or TPA (or acetone treated controls), was estimated using ELISA as described in section 3.4.1.3 (see chapter 3).

The calibration curve used to establish the correlation between the optical density and CXCL12 concentration as well as the assay detection range (0-10ng) was as described in the previous chapter (section 3.4.1.3, figure 3-9A). As shown in figure 4-10A, neither topical imiquimod cream, nor TPA, application significantly affected the amount of CXCL12 in the circulation of mice compared to their respective controls. However, topical imiquimod cream-, or TPA-induced, peripheral inflammation significantly reduced the concentration of total bone marrow CXCL12 (figure 4-10B: p=0.0286; 2-tailed Mann-Whitney U test).

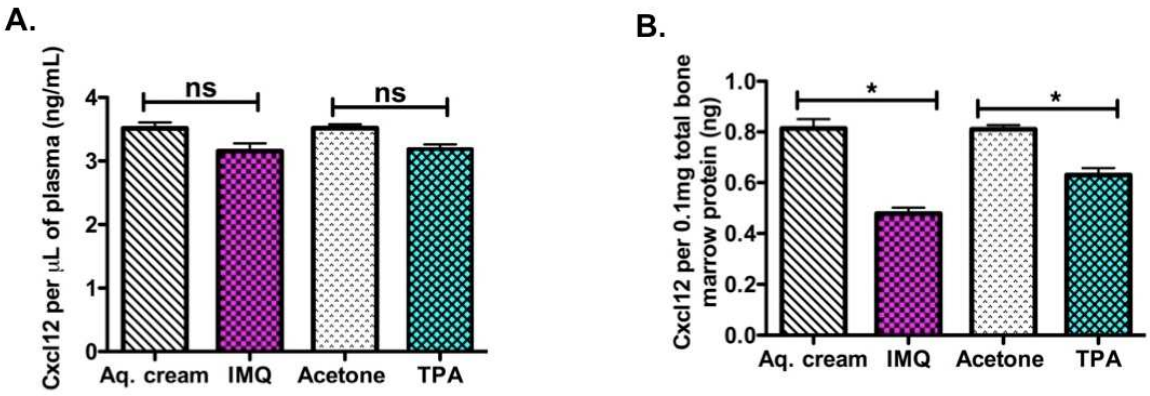



**Figure 4-10 Topical imiquimod or TPA application significantly reduce bone marrow CXCL12 concentration.**
C57BL/6 mice were given a single daily topical application of either 62.5mg imiquimod cream, or 100µM TPA (in 100µL acetone), for three consecutive days. On day four, mice were euthanized by $CO_2$ asphyxiation, peripheral blood was taken into EDTA anti-coagulation tubes, spun at >10,000xg for 10 minutes and plasma harvested for ELISA. Simultaneously, bones [2x (tibia + femur + hip bones)] were also harvested, and total bone marrow protein extracted for CXCL12 ELISA. Figure A summarises the measured CXCL12 concentration in the plasma of imiquimod cream (or aqueous cream) or TPA (or acetone) treated mice. Figure B summarises the estimated CXCL12 concentration in the total bone marrow protein extract of imiquimod cream (or aqueous cream) or TPA (or acetone) treated mice. [*p=0.0286; 2-tailed Mann-Whitney U test, (n=5)].

Taken together, data presented in this section suggest that the topical imiquimod cream or TPA treatment-induced HSPC mobilisation may have a significant contribution from a perturbed CXCR4/CXCL12. This suggestion is supported by the findings that although the HSPC CXCR4 expression was not significantly altered, there were significant reductions in bone marrow CXCL12 concentration. As neither the topical imiquimod cream, nor TPA, treatment altered the plasma CXCL12 concentration, it is reasonable to suggest that maintained plasma CXCL12 levels coupled with a reduced bone marrow CXCL12 levels, might not only have disrupted the HSPC-niche interaction, but also might have created a gradient that favoured HSPC migration towards the plasma CXCL12.

### 4.4.2 How do the peripheral inflammation models affect the bone marrow stromal cell compartment?

In this section of the study, the aim was to understand how these peripheral inflammation models (i.e. topical imiquimod cream, or topical TPA, application) affected the bone marrow stromal cell compartment, i.e. endothelial cells, osteoblasts, mesenchymal stromal cells (MSC). The critical roles played by these bone marrow stromal cellular components have been extensively reviewed in the general introductory chapter. Flow cytometry was employed to analyse the bone marrow stromal compartment to investigate how the peripheral inflammation models affected these bone marrow stromal cells. Mice received topical imiquimod cream, or TPA, application as described in section 4.2.1. Bones [2x (tibia + femur + hip bones)] were harvested, flushed with PBS to eliminate haematopoietic cells and processed exactly as described in section 3.4.2.1 of the previous chapter (Semerad et al., 2005). The bone stromal cells were stained for differential expression of lineage antibody cocktail (Lin), CD45, CD31, CD51, and Sca-1 antigen as previously published (Winkler et al., 2010a). The gating strategy used for characterising the bone marrow endothelial cells, MSC and osteoblasts is exactly as described in section 3.4.2.1 of the previous chapter. As explained in the previous chapter, bone marrow endothelial cells, mesenchymal stromal cells and osteoblasts were defined as $Lin^-CD45^-Sca\text{-}1^+CD31^+$, $Lin^-CD45^-CD31^-Sca\text{-}1^+CD51^+$ and $Lin^-CD45^-CD31^-Sca\text{-}1^-CD51^+$ respectively (Winkler et al., 2010a).

#### 4.4.2.1 Peripheral inflammation models differentially modulate bone marrow endothelial cell populations.

The non-haematopoietic/bone marrow stromal cells (defined as $CD45^{-}Lin^{-}$ cells, see previous chapter for gating strategy) were sub-gated for differential expressions of Sca-1 and CD31 antigens. Figures 4-11 A & B are the respective dot plots showing the re-gating of the stromal cells from the bone marrow of mice treated with topical aqueous cream or imiquimod cream. Figure 4-11 C & D are also the respective dot plots showing the differential expression of CD31 and Sca-1 by the bone stromal cells from mice treated with either topical acetone or TPA respectively. The $Lin^{-}CD45^{-}Sca\text{-}1^{+}CD31^{+}$ stromal cell population was referred to as endothelial cells in agreement with previously published reports (Winkler et al., 2010a, Winkler et al., 2010b). As summarised in figure 4-11 E, whereas topical imiquimod cream treatment significantly increased the bone marrow endothelial cell population (p=0.0066; 2-tailed unpaired t-test), topical TPA treatment modestly, but significantly reduced the bone marrow stromal endothelial cell population (p=0.0445; 2-tailed unpaired t-test).

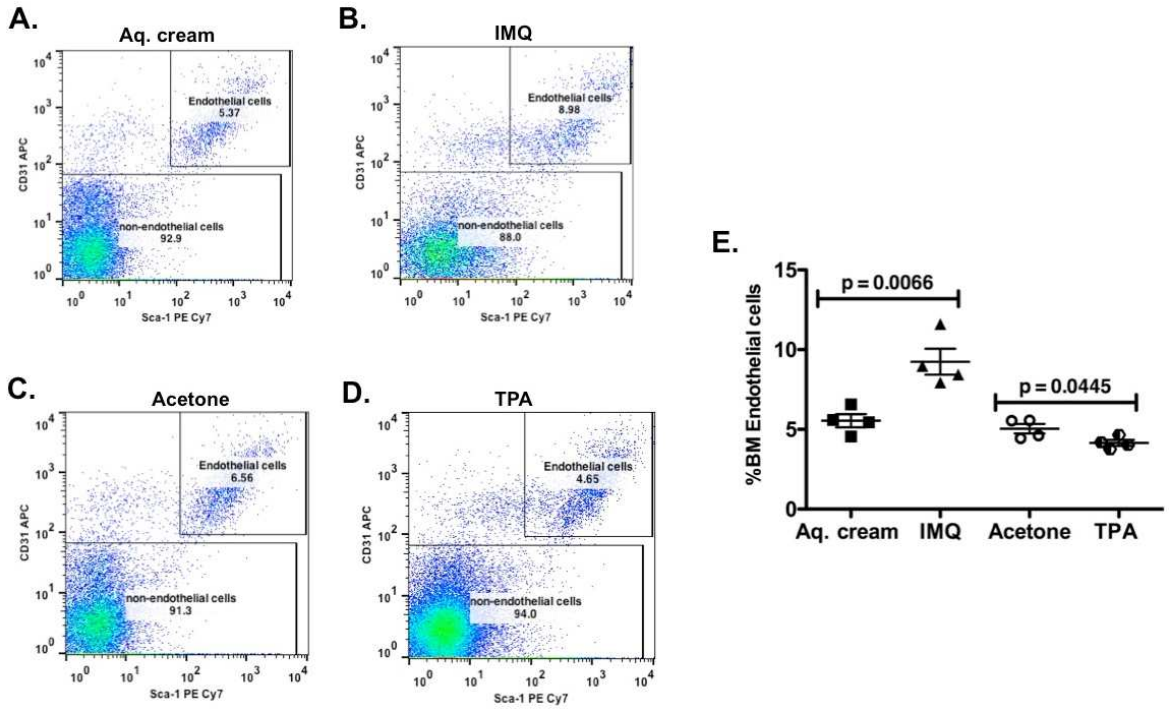



**Figure 4-11 Bone marrow stromal endothelial cells are differentially modulated by topical imiquimod cream, or TPA, treatment.**
The $CD45^{-}Lin^{-}$ non-haematopoietic cells from bone stromal cells were re-gated for differential expression of CD31 and Sca-1 to identify endothelial cells ($CD45^{-}Lin^{-}CD31^{+}Sca\text{-}1^{+}$) and non-endothelial cells ($CD45^{-}Lin^{-}CD31^{-}Sca\text{-}1^{-/+}$) for further analysis. Figures A and B are the respective representative dot plots for the gating of the $CD45^{-}Lin^{-}$ non-haematopoietic cells from topical aqueous and imiquimod cream treated mice respectively. Similarly, figures C & D are also the respective representative dot plots of non-haematopoietic bone stromal cells from mice treated with either topical acetone or TPA. Figure E is a scatter plot summarising the effects of the imiquimod

cream or TPA treatment on the bone marrow endothelial cell compartment as compared to respective control groups. Statistical significance was calculated using the 2-tailed unpaired t-test. Data presented in scatter plot are mean±SEM [n=4 (2 independent experiments)].

#### 4.4.2.2 Topical imiquimod cream or TPA treatment modulates bone marrow osteoblast and mesenchymal stromal cells.

The $Lin^-CD45^-Sca\text{-}1^+CD31^-$ cells (indicated as non-endothelial cells in 4-11 A-D **above**), were next, re-gated for the differential expression of CD51 and Sca-1. Cells that co-expressed CD51 and Sca-1 (i.e. $Lin^-CD45^-Sca\text{-}1^+CD31^-CD51^+$) were subsequently referred to as mesenchymal stromal cells (MSC); cells that expressed CD51, but not Sca-1, (i.e. $Lin^-CD45^-Sca\text{-}1^-CD31^-CD51^+$) were referred to as osteoblasts in agreement with previous reports (Semerad et al., 2005, Short et al., 2009, Winkler et al., 2010b). Figures 4-12 A & B are the respective representative dot plots showing the gating strategy for MSC and osteoblasts from mice treated with topical aqueous cream or imiquimod cream; figures 4-12 C & D are similar representative plots from mice treated with topical acetone, and TPA respectively. As summarised in the scatter plot in figure 4-12E, although topical TPA treatment did not significantly alter the bone marrow MSC population, imiquimod cream application significantly increased the bone marrow MSC population ($p=0.0014$; 2-tailed unpaired t-test). In addition, each of the topical inflammatory agents significantly reduced the bone marrow osteoblast population (figure 4-12F, $p=0.0011$ or $p=0.0273$ for imiquimod cream and TPA treatment respectively; 2-tailed unpaired t-test in each case).

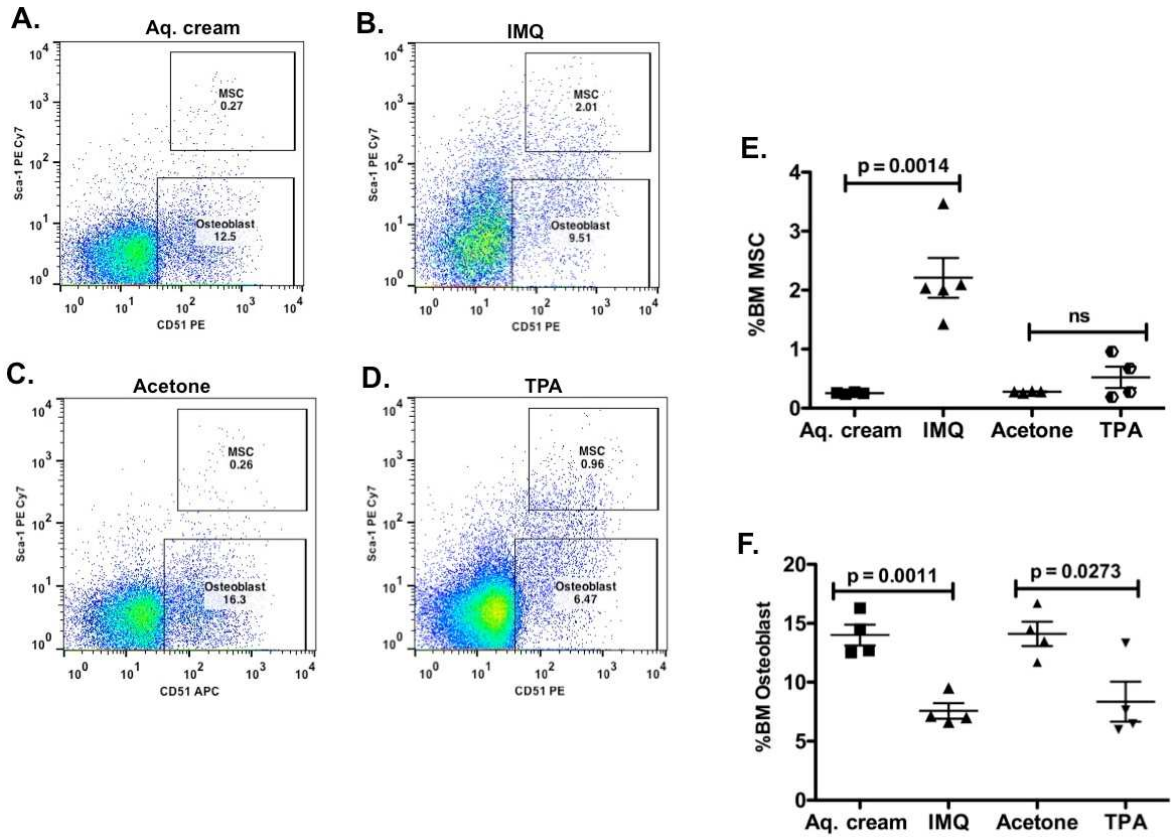



**Figure 4-12 Bone stromal osteoblasts and MSC populations are differentially modulated by topical imiquimod cream or TPA treatment.**
The non-endothelial cells ($CD45^-Lin^-CD31^-Sca\text{-}1^{-/+}$) (see figures 4-11 for gating strategy), from bone stromal cells were re-gated for differential expression of CD51 and Sca-1 antigen to identify osteoblasts ($CD45^-Lin^-CD31^-Sca\text{-}1^-CD51^+$) and mesenchymal stromal cells [MSC, $CD45^-Lin^-CD31^-Sca\text{-}1^+CD51^+$). Figures A & B are the respective representative plot for the re-gating of the $CD45^-Lin^-CD31^-Sca\text{-}1^{-/+}$ non-endothelial cells from aqueous cream and imiquimod cream treated mice; figures C & D are similar respective plots of non-endothelial cells from mice treated with topical acetone or TPA. Figure E is a scatter plot summarising the impact of the topical imiquimod cream or TPA treatment on the bone stromal MSC population. Figure F is also a scatter plot summarising the impact of topical imiquimod cream or TPA treatment on the bone stromal osteoblasts. Statistical significance was calculated using the 2-tailed unpaired t-test. Data in scatter plots are presented as mean ±SEM (n=4; 2 independent experiments)].

Taken together, each of the peripheral inflammation models led to significant modulation of the bone marrow stromal cell compartment and these may have impacted the HSPC-retention ability of the bone marrow niche.

### 4.4.3 How do the peripheral inflammation models affect the mononuclear phagocytic cells of the bone marrow?

Numerous cells in the innate arm of the immune system have been demonstrated to have essential roles in the retention of HSPC in the bone marrow niche (Levesque et al., 2003a, Levesque et al., 2003b, Chow et al., 2011). Various reports have shown that not only does depletion of these innate immune cells lead to HSPC mobilisation, but also that most HSPC mobilising agents act indirectly by activating some of these innate cells to release hydrolytic enzymes

that degrade HSPC niche retention factors. In this section of the study, the aim was to begin to elucidate the potential roles played by cells of the mononuclear phagocytic system in peripheral inflammation-induced HSPC mobilisation. Two specific mononuclear cell populations were investigated in the bone marrow of mice treated with either topical imiquimod cream, or TPA, namely, Gr-$1^{low}$F4/$80^{+}$$CD115^{high}$ monocytic cells and CD169+ macrophages. The staining protocol used was in accordance with a previously published protocol (Chow et al., 2011) and the gating strategy was exactly as described in section 3.4.3 of the previous chapter.

#### 4.4.3.1 Topical imiquimod cream application significantly increases bone marrow Gr-$1^{low}$$CD115^{high}$ monocytic cell population.

The bone marrow Gr-$1^{low}$$CD115^{+/-}$ cells from either imiquimod cream treated, or TPA treated mice, or their respective controls, were re-gated for differential expression of CD115 and F4/80 antigens to delineate two cellular populations: F4/$80^{+}$ but $CD115^{int}$ (CD115 intermediate expressing cells), and F4/$80^{+}$ but $CD115^{high}$ monocytic cells. Figures 4-13 A & B are the respective representative dot plots of F4/80 and CD115 differential expression by bone marrow Gr-$1^{low}$$CD115^{+/-}$ cells from aqueous cream, or imiquimod cream, treated mice; figures 4-13 C & D are similar plots for mice treated with either topical acetone, or TPA, respectively. As summarised in figure 4-13E, topical imiquimod cream application significantly increased the Gr-$1^{low}$F4/$80^{+}$$CD115^{high}$ monocytic cell population compared to aqueous cream treated controls (figure 4-13E, $p = 0.0006$; 2-tailed, Unpaired t-test). On the other hand, topical TPA application did not significantly alter the bone marrow Gr-$1^{low}$F4/$80^{+}$$CD115^{high}$ monocytic cell population.

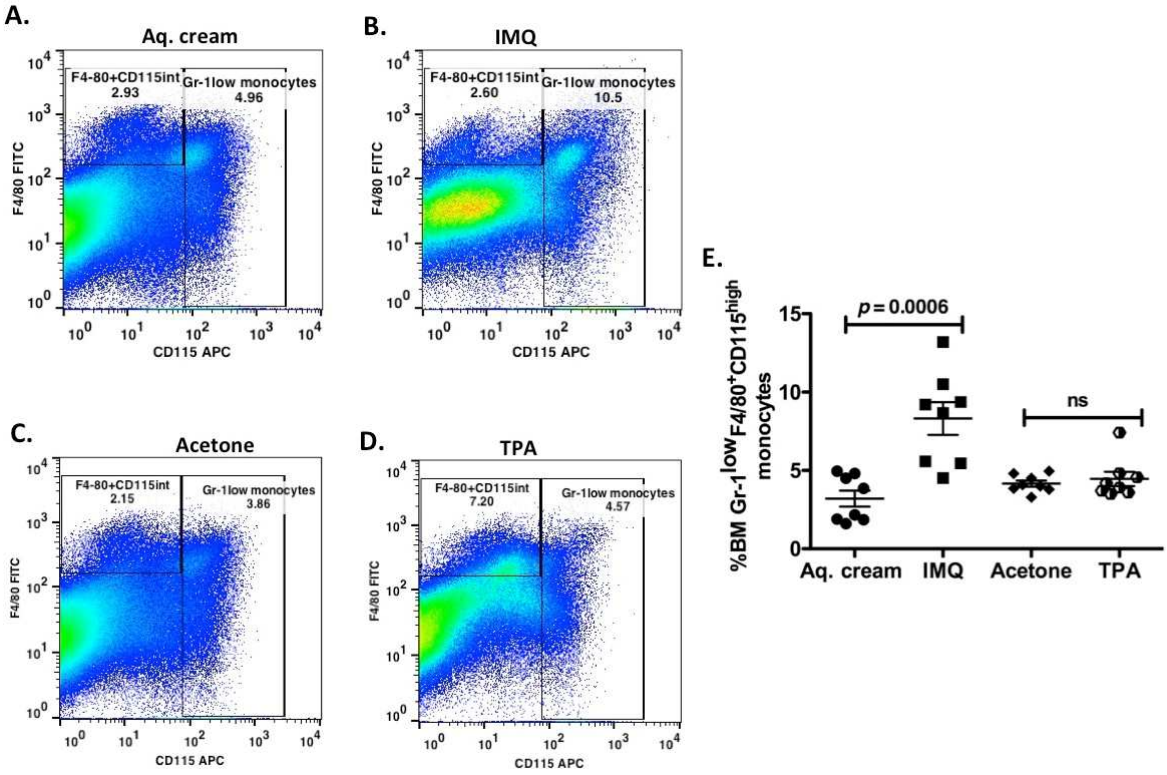



**Figure 4-13 Topical imiquimod cream application significantly increases the bone marrow Gr-1$^{low}$CD115$^{high}$ monocytic population.**
Bone marrow cells were gated for differential expression F4/80 and CD115 antigens to investigate the Gr-1$^{low}$CD115$^{high}$ monocytic cells. A & B are the respective representative plots showing the bone marrow mononuclear cells (Gr-1$^{low}$CD115$^{high}$ monocytic) of aqueous cream and imiquimod cream treated mice; similarly, C & D are the respective representative dot plots of Gr-1$^{low}$CD115$^{high}$ monocytic cells from the bone marrow of mice treated with topical acetone or TPA. E is a scatter plot summarising the changes in the Gr-1$^{low}$CD115$^{high}$ monocytic cells in response to either imiquimod cream or TPA treatment. Statistical significance was calculated using the 2-tailed unpaired t-test. [Ns; means non-significant, n=4 (two independent experiments pooled together)].

### 4.4.3.2 Topical imiquimod cream or TPA treatment differentially modulates the bone marrow CD169+ macrophage population.

Previously, Chow et al. published a protocol that used differential expression of CD169 and CD11b antigens by cells of the Gr-1$^{low}$F4/80$^{+}$CD115$^{int}$ population (see section 4.4.3.1 for gating strategy) to identify a macrophage population (defined as Gr-1$^{-/low}$F4/80$^{+}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$) that was crucial for the retention of HSPC in the bone marrow niche (Chow et al., 2011). They established this by demonstrating that clodronate-liposome-induced depletion of this macrophage population led to mobilisation of HSPC to the circulation. To understand how much of the HSPC mobilisation seen with the peripheral inflammation models adopted for this study impacted this macrophage population, the Gr-1$^{low}$F4/80$^{+}$CD115$^{int}$ from mice treated with either topical imiquimod cream or TPA application (or their respective control groups), were re-gated for differential expression of CD169 and CD11b. Figures 4-14 A & B are the respective representative dot plots showing the CD169 and CD11b differential

expression for the Gr-1$^{low}$F4/80$^{+}$CD115$^{int}$ population from the bone marrow of mice treated with either topical aqueous cream or imiquimod cream; figures 4-14 C & D are similar representative plots from mice treated with either topical acetone, or TPA. As summarised in figure 4-14E, topical imiquimod cream treatment significantly reduced the bone marrow Gr-1$^{-/low}$F4/80$^{+}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$ macrophage population when compared to aqueous cream treated control ($p<0.0001$; 2-tailed Unpaired t-test). In contrast, topical TPA treatment modestly, but significantly increased the Gr-1$^{-/low}$F4/80$^{+}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$ macrophage population when compared to acetone treated controls ($p<0.0001$; 2-tailed Unpaired t-test).

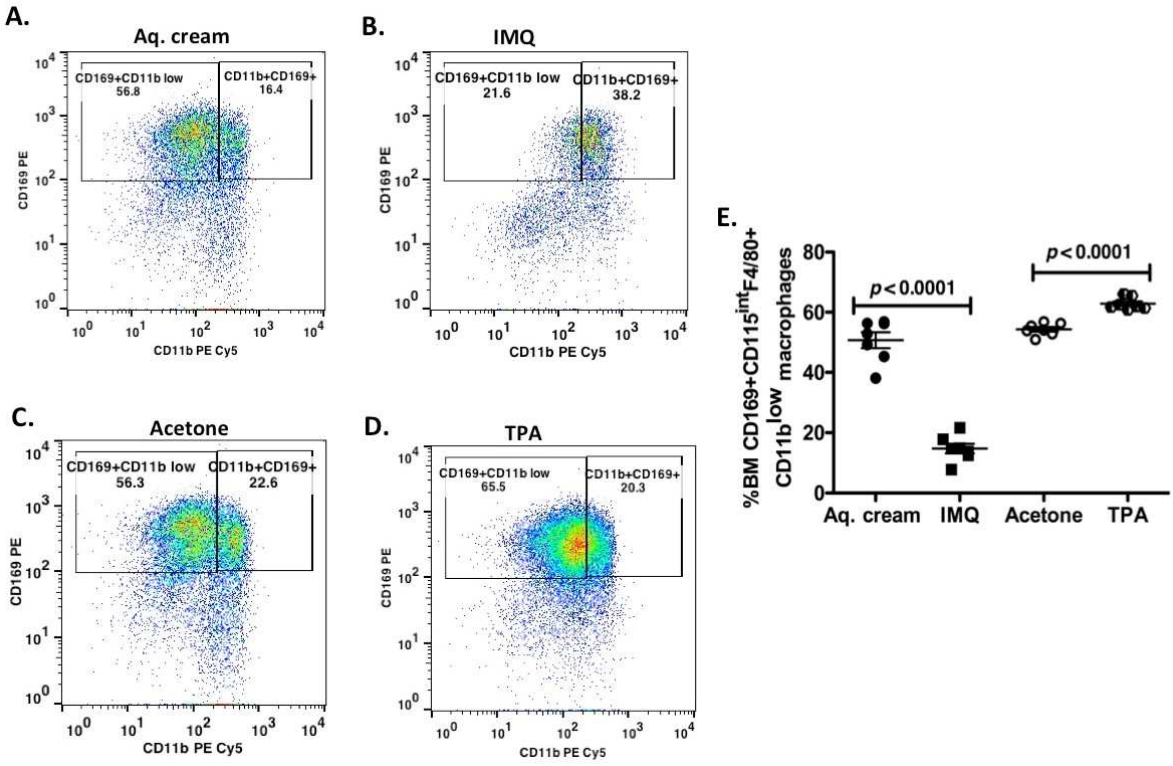



**Figure 4-14 Bone marrow CD169+ macrophage population is differentially affected by topical imiquimod cream or TPA treatment.**
The F4/80$^{+}$CD115$^{int}$ mononuclear cells (see figure 4-13 for gating strategies) from the bone marrow were re-gated for the differential expressions of CD169 and CD11b antigens to identify the Gr-1$^{-}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$ macrophage population and Gr-1$^{-}$CD115$^{int}$CD169$^{+}$CD11b$^{+}$ mononuclear cells. A & B are the respective representative dot plots of the bone marrow of mice treated with either aqueous cream or imiquimod cream, showing the gating of the Gr-1$^{-}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$ macrophage population and Gr-1$^{-}$CD115$^{int}$CD169$^{+}$CD11b$^{+}$ mononuclear cells; C & D are similar plots from the bone marrow of mice treated with acetone and TPA respectively. E is a scatter plot summarising the impact of topical imiquimod cream or TPA treatment on the bone marrow Gr-1$^{-}$CD115$^{int}$CD169$^{+}$CD11b$^{low}$ macrophage population. Statistical significance was calculated using the 2-tailed unpaired t-test. (n=6; 2 independent experiments).

In summary, data presented in this section suggest that the peripheral inflammation agents altered the HSPC niche by:

A. Significantly altering the CXCR4/CXCL12 axis in the HSPC niche by reducing the bone marrow CXCL12 concentration in both topical imiquimod cream, and TPA, treatment models.

B. In the bone marrow stromal cell compartment, whereas topical imiquimod cream treatment significantly reduced the bone marrow stromal osteoblasts population, it also significantly increased the MSC and endothelial cell populations. On the other hand, although topical TPA treatment did not significantly alter the MSC population, it significantly reduced both osteoblasts and endothelial cell populations.

C. With bone marrow mononuclear phagocytic cells, whereas topical imiquimod cream treatment significantly reduced the Gr-$1^{-/low}$F4/$80^{+}$CD$115^{int}$CD$169^{+}$CD$11b^{low}$ macrophage population, it also significantly increased the Gr-$1^{low}$CD$115^{high}$ monocytic cells. Topical TPA treatment on the other hand, significantly increased the bone marrow Gr-$1^{-/low}$F4/$80^{+}$CD$115^{int}$CD$169^{+}$CD$11b^{low}$ macrophage population, even though it did not significantly alter the Gr-$1^{low}$CD$115^{high}$ monocytic cell population.

## 4.5 What chemokines and cytokines are produced in response to the peripheral inflammation?

The data presented thus far have shown that topical imiquimod cream, or TPA, treatment mobilised HSPC to the circulation. As the topical imiquimod cream or TPA was applied directly to shaved dorsal skin of mice, the implication is that the inflammatory processes elicited by these peripheral inflammatory stimuli were communicated to the bone marrow niche. To obtain an understanding of the inflammatory milieu elicited in response to the topical imiquimod cream, or TPA, treatment and how this is reflected in the plasma, this section of the study sought to answer the following questions:

1. What cutaneous chemokines and cytokines are produced in response to topical imiquimod cream or TPA treatment?

2. What chemokines and cytokines are released into the plasma in response to the topical imiquimod cream or TPA treatment?

3. What chemokines and cytokines are produced in the bone marrow in response to the topical imiquimod cream or TPA treatment?

4. What chemokine receptors are expressed by bone marrow and peripheral blood HSPC in response to the topical imiquimod cream, or TPA, treatment?

As the imiquimod cream, or the TPA, was directly applied to the dorsal skin of the mice, the overall objective of this section of the study was to profile the skin, peripheral blood, bone marrow and HSPC for inflammatory signatures and then determine whether any of these mediators could have orchestrated the HSPC mobilisation reported in the previous sections.

## 4.5.1 What cutaneous chemokines and cytokines are produced in response to the topical imiquimod cream, or TPA, treatment?

Before determining the inflammatory mediators in the circulation and bone marrow niche, the level of inflammation in the skin (direct point of stimuli application) elicited by imiquimod cream, or TPA, painting was first determined by RT-QPCR. Mice received topical imiquimod cream, or TPA, treatment exactly as described in section 4.2.1. After the treatment, the mice were euthanized by $CO_2$ asphyxiation and dorsal skin harvested and analysed by Taqman Low Density Array (TLDA) for chemokine and cytokine gene expression in comparison with respective control mice, i.e. aqueous cream or acetone treatment for imiquimod cream and TPA respectively.

### 4.5.1.1 Assessment of the quality of skin RNA isolates.

Before proceeding to profile the inflammatory gene signatures in the skin, the quality of the RNA isolates from the dorsal skin was assessed with nanochips using the Agilent 2100 Bioanalyzer platform. The Bioanalyzer provides an RNA integrity number (RIN), ratio of 28s/18s, as well as a graphical output that gives information on the degree of noise and low molecular weight contamination, as shown in **figures 4-15A-D**. Only samples with RIN >8.0 were used for downstream TLDAs.

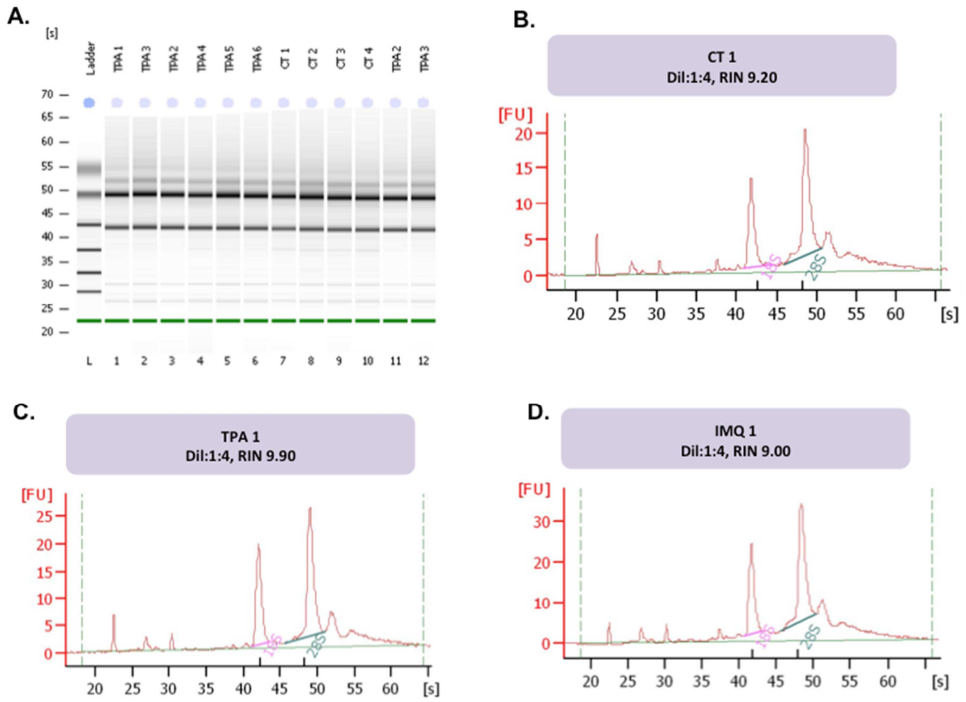



**Figure 4-15 Assessment of the quality of skin RNA isolates used for the inflammatory gene signature assay**

62.5mg imiquimod cream (IMQ) or 100μM TPA was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days, after which skin was harvested for total RNA isolation. A-D shows verification of RNA integrity as assessed by the Agilent Bioanalyser 2100. Figure A shows a representative gel image (A), and B-D are the representative electropherogram plots for samples (CT= representative plot for control samples). The x-axis represents the amplicon size (nt) whereas the y-axis represents fluorescence units (FU) for the electropherogram plots; the distinct 18S and 28S peaks demonstrate lack of noise.

Approximately, 1500ng of total RNA was used for complementary DNA (cDNA) synthesis and subsequent downstream TLDA plate analysis. The TLDA is a customised 384-well microfluidic plate (Applied Biosystems, UK) containing primers for pre-selected CCL, CXCL, CX3CL and XCL chemokines as well as other inflammatory cytokines. In all, the format selected allowed the profiling of a total of 32 genes implicated in inflammatory responses (**see materials and methods for a list of the genes)**. All gene expression levels were firstly normalised to cutaneous TATA binding protein expression levels, and secondly, related to their respective controls (i.e. aqueous cream or acetone for imiquimod cream and TPA treatment respectively) to obtain the fold change ($\Delta\Delta$Ct). Therefore, a fold change of >1 or <1, means that the topical imiquimod cream/TPA application induced upregulation or downregulation, in that gene's expression compared to control samples. To enable a direct comparison of the modulation in gene expression in response to each of the peripheral inflammatory agents to be made, the fold change of each gene expression, in response to imiquimod cream, or TPA, treatment were plotted on the same axis.

Statistical significance for differences in the fold change in expression of each gene was calculated by using the 2-tailed Mann-Whitney U test.

#### 4.5.1.2 Cutaneous CC-chemokines expressed in response to topical imiquimod cream, or TPA, treatment.

A representative plot for the cutaneous CC-chemokines expressed in response to either topical imiquimod cream, or TPA, treatment is shown in figure 4-16. Whereas topical imiquimod cream induced a modest increase in Ccl2 expression, TPA treatment induced almost a 32-fold increase in expression. Not surprisingly, the fold change in Ccl2 gene expression was significantly different between the two treatments ($p<0.05$; 2-tailed Mann-Whitney U test). For Ccl3 expression in the skin, whereas topical imiquimod cream application induced a >32-fold increase in expression, topical TPA application induced a >1000-fold increase. The fold change in the Ccl3 gene expression was significantly different between the two treatments ($p<0.05$; Mann-Whitney U, 2-tailed test). For Ccl4 expression, whereas topical imiquimod cream application induced an approximately 8-fold increase in expression, topical TPA application induced a >1000-fold increase ($p<0.001$; Mann-Whitney U, 2-tailed test). Also, topical imiquimod cream or TPA application induced ~4-fold increase in Ccl5 expression. In addition, cutaneous Ccl7 gene expression was significantly increased in TPA application (>8-fold increase) compared to imiquimod (~2-fold increase) application ($p=0.029$; 2-tailed Mann-Whitney U test).

Interestingly, there were significant, but differential modulations of cutaneous Ccl6 ($p=0.029$), Ccl17 ($p=0.0002$), Ccl19 ($p=0.028$), Ccl20 ($p=0.028$), Ccl21 ($p=0.0294$) and Ccl22 ($p=0.0286$) gene expression in response to topical imiquimod cream and TPA application (2-tailed Mann-Whitney U test in all cases); whereas, topical imiquimod cream application down-regulated the expression of these genes, TPA application up-regulated the expression of these same genes. On the other hand, cutaneous Ccl27 gene expression was down-regulated by both topical imiquimod cream and TPA application. The difference in the down-regulated Ccl27 expression in response to topical imiquimod cream or TPA treatment reached statistical significance ($p=0.0286$; 2-tailed Mann-Whitney U test).

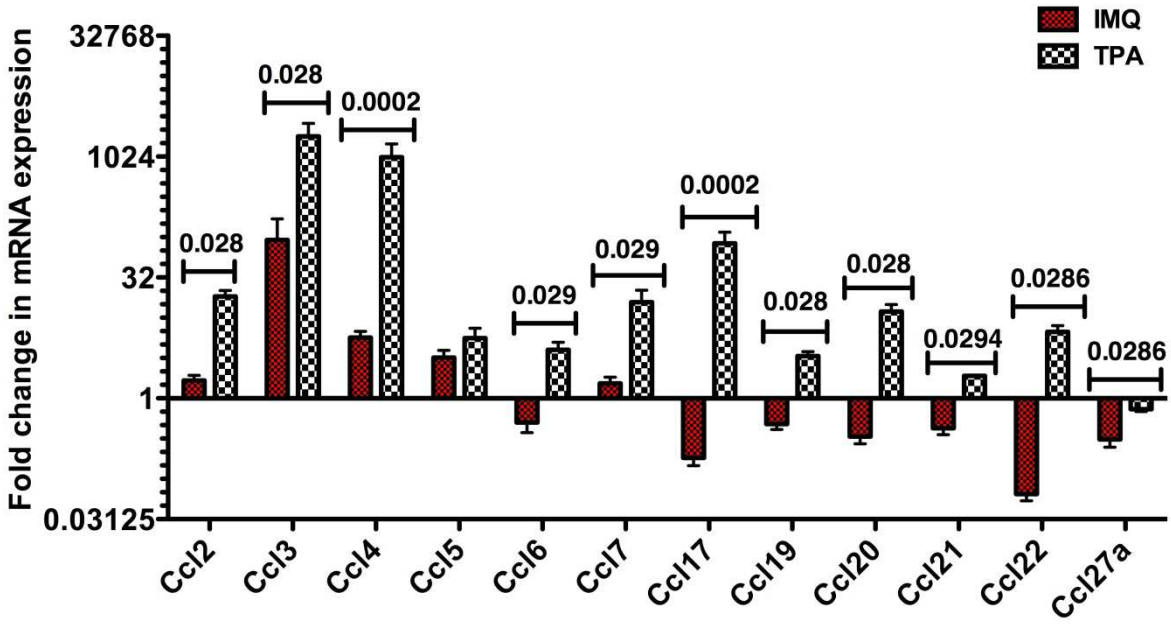



**Figure 4-16 Cutaneous CC-chemokine gene expression is modulated by topical imiquimod cream or TPA application.**

A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or TPA (100μM in 100μL acetone) was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days and skin harvested for total RNA isolation. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. 1.5μg of total RNA was used for cDNA synthesis, diluted 1:5 and then used for chemokine and cytokine gene analysis using Taqman Low Density Analysis. Gene expression was firstly, normalised to the cutaneous TBP gene expression, and then secondly to the respective gene expression in the relevant controls to obtain the fold changes (ΔΔCt) in gene expression. The bar graph in figure 4-16 summarises the modulation of CC-chemokine gene expression in the dorsal skin in response to either topical imiquimod cream or TPA application. Statistical analysis compared the fold change in gene expression in response to topical imiquimod cream application to the fold change in the same gene expression induced by topical TPA application using 2-tailed Mann-Whitney U test. [(n=4; 2 independent experiments pooled together)].

#### 4.5.1.3 Cutaneous CXC- and CX3C-chemokines expressed in response to topical imiquimod cream, or TPA, treatment.

A summary of the topical imiquimod cream and TPA application-induced modulation of cutaneous CXC and CX3C gene expression is shown in figure 4-17. Imiquimod cream application induced a ≥32-fold increase in cutaneous Cxcl2 and Cxcl5 expression; TPA application, on the other hand, induced >1000-fold increase in expression of both genes. The differences in the fold changes in the expression of Cxcl2 and Cxcl5 in response to either topical imiquimod cream or TPA treatments reached statistical significance ($p<0.001$ or $p<0.01$ for Cxcl2 and Cxcl5 respectively; 2-tailed Mann-Whitney U test in both cases). The expression of cutaneous Cxcl11 was significantly, but differentially modulated by topical imiquimod cream and TPA ($p=0.0079$; 2-tailed Mann-Whitney U test); whereas topical imiquimod cream treatment caused a >32-fold down-regulation, TPA treatment caused ~32-fold increased expression. Also, there was ~32-fold

increase in expression of Cxcl13 in response to topical imiquimod cream, in contrast to TPA treatment which led to ~4-fold reduction in expression. The fold change in expression was significantly different between the two treatment groups (p=0.006; 2-tailed Mann-Whitney U test). The cutaneous expression of Cxcl10, Cxcl12, Cxcl14 and Cx3cl1 were not significantly different from baseline levels (i.e. almost the same as control samples).

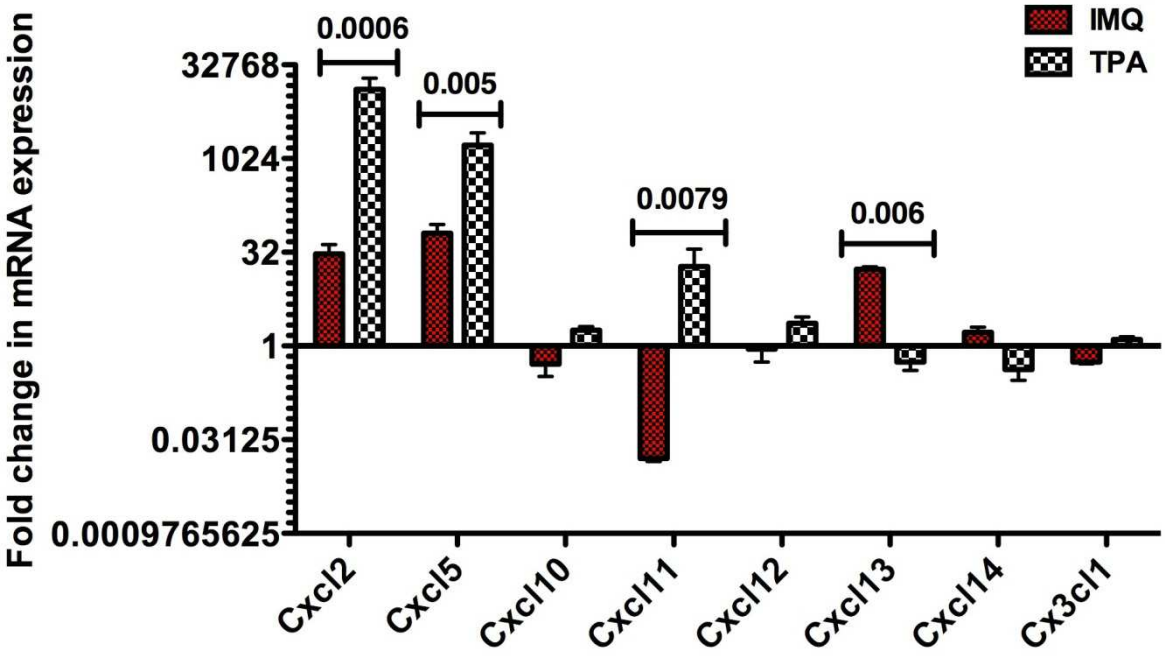



**Figure 4-17 Cutaneous CXC- and CX3C- chemokine gene expression is modulated by topical imiquimod cream or TPA application.**

A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or TPA (100μM in 100μL acetone) was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days and skin harvested for total RNA isolation. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. 1.5μg of total RNA was used for cDNA synthesis, diluted 1:5 and then used for chemokine and cytokine gene analysis using Taqman Low Density gene Analysis. Gene expression was firstly, normalised to the cutaneous TBP gene expression, and then secondly to the respective gene expression in the relevant controls to obtain the fold changes (ΔΔCt) in gene expression. Figure 4-17 summarises the modulation of CXC-chemokine gene expression in the dorsal skin in response to either imiquimod cream or TPA application. Statistical analysis compared the fold change in gene expression in response to topical imiquimod cream application to the fold change in the same gene expression induced by topical TPA application using 2-tailed Mann-Whitney U test. [(n=4; 2 independent experiments pooled together)].

#### 4.5.1.4 Cutaneous cytokines expressed in response to topical imiquimod cream or TPA treatment.

The cytokine signatures also followed the same differential regulation pattern shown by the CC and CXC chemokines (see **figure 4-18**). The only cytokine genes that were up-regulated in both models were interferon-gamma (IFN-γ) and interleukin-1β (IL-1β), whereas IL-4 was only up-regulated in response to topical imiquimod cream treatment. Topical imiquimod cream treatment induced ~4-

fold down-regulation of TNF-α expression in dorsal skin, but topical TPA treatment induced ~32-fold up-regulation of expression. This differential modulation of cutaneous TNF-α expression was statistically significant (p=0.0286; 2-tailed Mann-Whitney U test). There was a modest up-regulation in expression of interferon-γ (IFN-γ) in response to topical imiquimod cream, or the topical TPA, treatment. The difference in the increased IFN-γ expression in the dorsal skin treated with either topical imiquimod cream or TPA was statistically significant (p=0.0286; 2-tailed Mann-Whitney U test). The expression of TGFβ1 was increased by ~4-fold in response to TPA treatment; the expression of TGFβ1 was only slightly down-regulated in response to topical imiquimod treatment.

Although IL-1ß expression was increased in dorsal skin in response to each of topical imiquimod cream and TPA treatments, the difference in the fold changes in IL-1ß expression, in response to the two treatments, was statistically significant (p=0.0286; 2-tailed Mann-Whitney U test). Interestingly, IL-4 gene expression was only up-regulated (32-fold) in response to topical imiquimod treatment. In addition, whereas, IL-10 gene expression was slightly down-regulated in response to the topical imiquimod cream application, there was a >8-fold up-regulation in expression in response to topical TPA application. The difference in IL-10 expression in response to each of topical imiquimod cream or TPA application was statistically significant (p=0.0286; 2-tailed Mann-Whitney U test). IL-13 was significantly, but differentially, modulated by the two treatments (p=0.0286; 2-tailed Mann-Whitney U test); whereas it was ~16-fold up-regulated in response to the topical TPA application, there was ~16-fold down-regulation in response to topical imiquimod cream application model. IL-17a was only moderately expressed in TPA treated skin. In addition, IL-22 expression was >4-fold down-regulated in response to topical imiquimod cream treatment, although its expression was not altered by the topical TPA treatment.

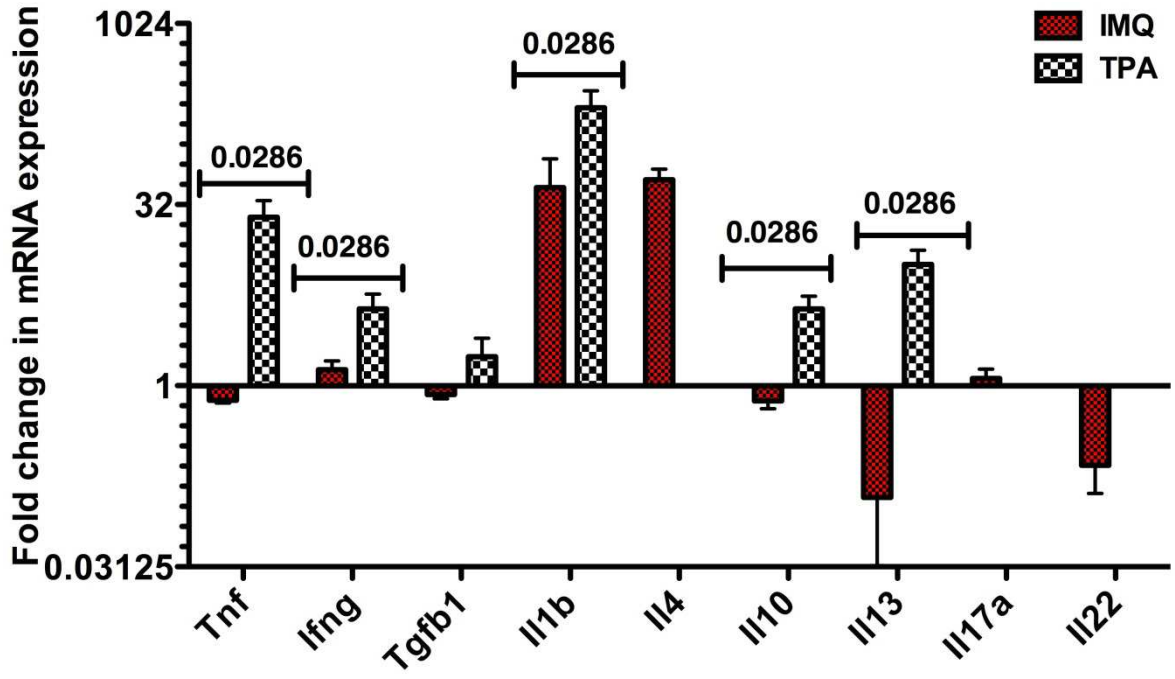



**Figure 4-18 Cutaneous cytokine gene expression is modulated by topical imiquimod cream or TPA application.**
A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or TPA (100μM in 100μL acetone) was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days and skin harvested for total RNA isolation. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. 1.5μg of total RNA was used for cDNA synthesis, diluted 1:5 and then used for chemokine and cytokine gene analysis using Taqman Low Density Analysis. Gene expression was firstly, normalised to the cutaneous TBP gene expression, and then secondly to the respective gene expression in the relevant controls to obtain the fold changes (ΔΔCt) in gene expression. Figure 4-18 summarises the modulation of cytokine gene expression in the dorsal skin in response to either cream or TPA application. Statistical analysis compared the fold change in gene expression in response to topical imiquimod cream application to the fold change in the same gene expression induced by topical TPA application using 2-tailed Mann-Whitney U test, [(n=4; 2 independent experiments pooled together)].

## 4.5.2 What chemokines and cytokines are released into the plasma in response to the topical imiquimod cream or TPA treatment?

### 4.5.2.1 Introduction

The cutaneous gene expression data presented above suggest that the topical imiquimod cream or TPA treatment elicited upregulation of inflammatory gene expression in the skin. However, for the inflammatory gene signatures to effectively reach the bone marrow, and thus induce HSPC mobilisation, it is imperative that the genes are effectively translated to functional proteins that enter the circulation. In order to examine the inflammatory proteome profile in the peripheral blood, the aim in this section of the study was to profile the same cytokines studied in the previous chapter and compare the repertoire of cytokines and chemokines in the plasma (in the systemic and peripheral

inflammation models) for potential candidates that might orchestrate the increased numbers of mobilised haematopoietic progenitors. Therefore, the same mouse cytokine array panel A (ARY006; R&D Systems), as used for the plasma inflammatory proteome profiling in the LPS-induced inflammation model was employed. As explained in the previous chapter, this proteome array allowed the parallel detection of the relative levels of a total of 40 proteins (cytokines and chemokines). It principally consists of nitrocellulose membranes on which are pre-spotted capture antibodies for specific proteins (duplicate for each analyte). The principle underlying the assay procedure is essentially as described for the proteome array ARY015 in section 3.5.1 in the previous chapter.

To evaluate the circulating inflammatory proteome milieu, peripheral blood was taken from mice treated with topical imiquimod cream, or TPA, (as described in section 4.2.1) into EDTA microtainer tubes (BD Biosciences). The blood was centrifuged at 8000xg for 10 minutes and plasma was harvested for the subsequent proteome array. As the arrays were expensive, the array was run once for each treatment; however, plasma from three mice (for each treatment group) was pooled together and 150μL of the resultant pooled plasma was used for the subsequent proteome array in order to obtain representative data.

#### 4.5.2.2 Quantification of plasma proteome analytes

Figure 4-19 shows scanned images of the blots, with the signal intensities representing plasma analytes on a developed X-ray film from aqueous cream (A), or imiquimod (IMQ) cream (B), acetone (C), or TPA (D), treated mice (after 8 minutes exposure).

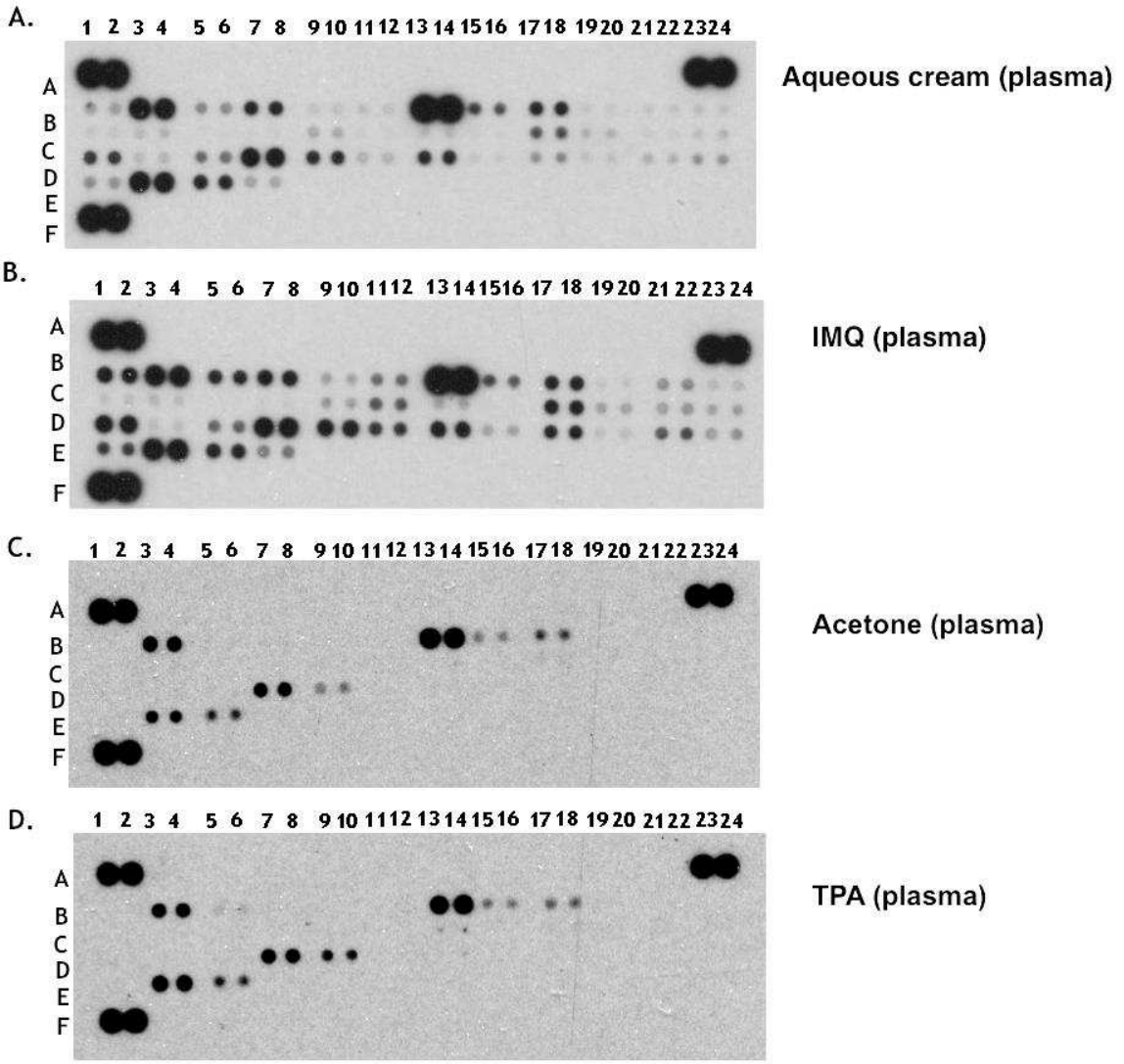



**Figure 4-19 Mouse cytokine proteome profiling array blots showing signal intensities of analytes for plasma from imiquimod cream or TPA treated mice.**
A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or TPA (100µM in 100µL acetone) was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of aqueous cream or acetone as controls for imiquimod cream and TPA treatment respectively. On the fourth day, mice were euthanized by $CO_2$ asphyxiation, and plasma taken from the respective mice for cytokine proteome assay. In order to obtain a representative proteome profile for each treatment group, plasma from three mice was pooled together for each treatment group (i.e. topical imiquimod cream or aqueous cream). 150µL of the respective plasma samples was used for the proteome profiling using the Mouse cytokine proteome array kit (R&D systems). A & B are the respective scanned images of the blots from the plasma of mice treated with aqueous cream or imiquimod cream; C & D are also the respective scanned images of blots from the plasma of mice treated with acetone or TPA.

The spot coordinates and the analytes they represent are exactly as described in section 3.5.2 of the previous chapter. The signal intensities were estimated with ImageJ 1.48 (NIH, USA) software. As each analyte is represented by duplicate spots, the signal intensities of the duplicate spots were then averaged to get the mean signal intensities. Next, the mean signal intensity of the spots for negative controls was subtracted from that of the calculated mean signal intensity of each analyte to correct for background signals. Then, fold change in mean signal intensity for each analyte was calculated by dividing the mean signal intensity of

the imiquimod cream/TPA treated group by the mean signal intensity of the respective analyte in the aqueous cream/acetone treated group to obtain a fold change in that analyte concentration. As explained in the previous sections, the objective of this analysis was to set the mean signal intensity of each analyte in the aqueous cream (control for imiquimod cream) or acetone (control group for TPA treatment) to baseline (i.e. 1 in each case). Therefore, the fold change in mean signal intensity plotted for each analyte (see below) gives an indirect measure of how much the circulating levels of that particular protein was increased (fold change >1), or decreased (fold change <1), in response to the topical imiquimod cream/TPA-induced peripheral inflammation compared to respective controls. To increase the stringency of the assay, only analytes demonstrating a >2-fold change in expression were considered to merit further consideration.

#### 4.5.2.3 Imiquimod cream or TPA treatment modulates chemokine levels in the circulation.

Figure 4-20 summarises the fold changes seen in the expression of CC- and CXC-chemokines in the plasma of mice treated with either topical imiquimod cream or TPA. Topical imiquimod cream treatment caused increased circulating CC-chemokines; CCL2 (>7-fold increase), CCL3 (>2-fold increase), CCL4 (>4-fold increase), CCL5 (~7-fold increase), CCL11 (~6-fold increase), CCL12 (~8-fold increase) and CCL17 (>2fold increase). However, the only CC chemokine that showed a >2-fold increased expression in response to topical TPA treatment was CCL2.

For CXC-chemokines, whereas topical imiquimod cream application caused reduced expression of circulating CXCL2 (>4-fold reduction), and CXCL11 (>2-fold reduction), it also caused approximately 4-fold increase in CXCL13 levels. In the plasma of TPA treated mice, none of the CXC-chemokines was expressed above the background, i.e. all were below the cut-off threshold fold change of 2.

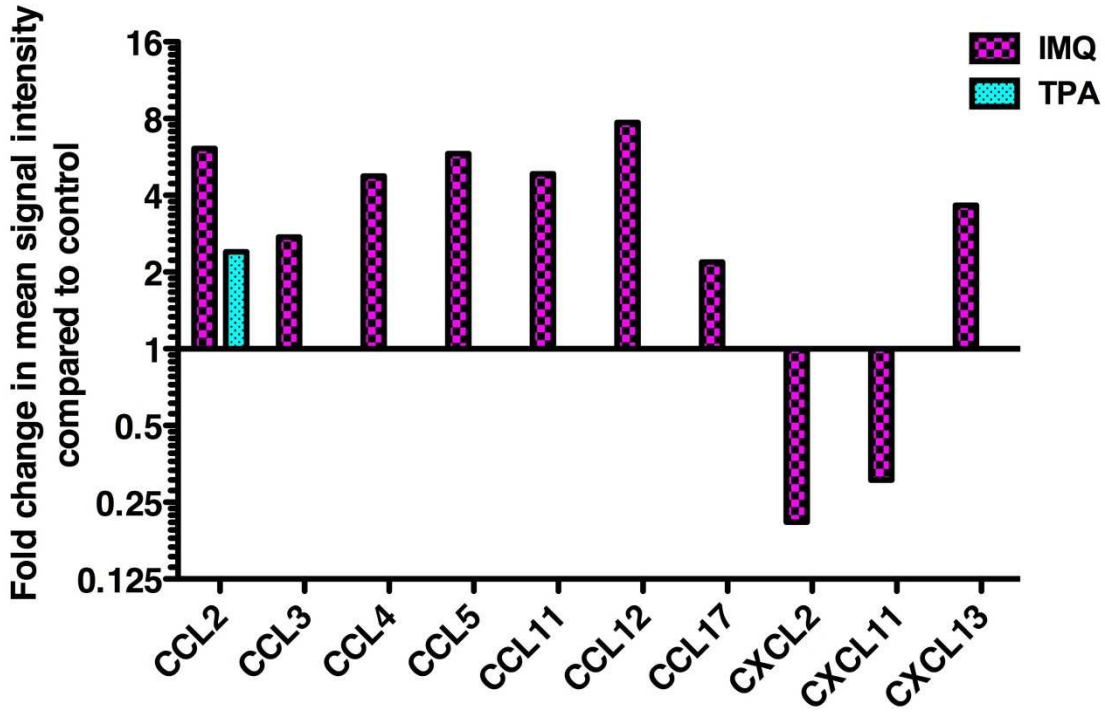



**Figure 4-20 Topical imiquimod cream application modulates chemokine expression in the plasma.**
The pixel intensity of each spot on the developed array blots (see figure 4-19) was estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each chemokine, the mean of the signal intensity of each chemokine was calculated, and the corrected signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control, from the mean signal intensity of each chemokine. Next, the fold change in the corrected mean signal intensities were estimated for each analyte by dividing the corrected mean signal intensity of that analyte in the imiquimod cream/TPA treated sample by the corrected mean signal intensity of that same analyte in the aqueous cream/acetone treated group. Figure 4-20 is a bar graph showing the fold change in the plasma chemokines in response to topical imiquimod cream or TPA application. Fold change >1, means increased production of the chemokine in response to the treatment; fold change <1, indicates reduced chemokine levels in response to the treatment.

#### 4.5.2.4 Topical imiquimod cream or TPA treatment modulates circulating cytokine expression.

Figure 4-21 summarises the expression of various growth factors and cytokines in response to the topical imiquimod cream/TPA-induced peripheral inflammation. Topical imiquimod cream application led to a 4-fold increase in the level of the myelopoietic cytokine G-CSF in the plasma. Topical imiquimod cream treatment also caused increased circulating levels of a host of pro-inflammatory cytokines, namely, TNF-$\alpha$ (>2-fold increase), IL-16 (>2-fold increase), IL-17 (>4-fold increase), IL-23 (~6-fold increase) and IL-27 (~4-fold increase). It is interesting to note that topical imiquimod cream treatment also led to approximately a 4-fold increase in circulating levels of IL-1$\alpha$ antagonist, IL-1r$\alpha$, even though the fold change in IL-1$\alpha$ itself was not altered. In addition, the topical imiquimod cream application led to ~32-fold increase in the expression of the anti-

inflammatory cytokine, IL-10, in the plasma compared to aqueous cream treated controls.

Unlike the topical imiquimod cream treatment that elicited increases in many inflammatory mediators, the only analyte that showed a >2-fold increase in circulating levels in response to TPA treatment was tissue inhibitor of matrix metalloproteinase 1 (TIMP-1).

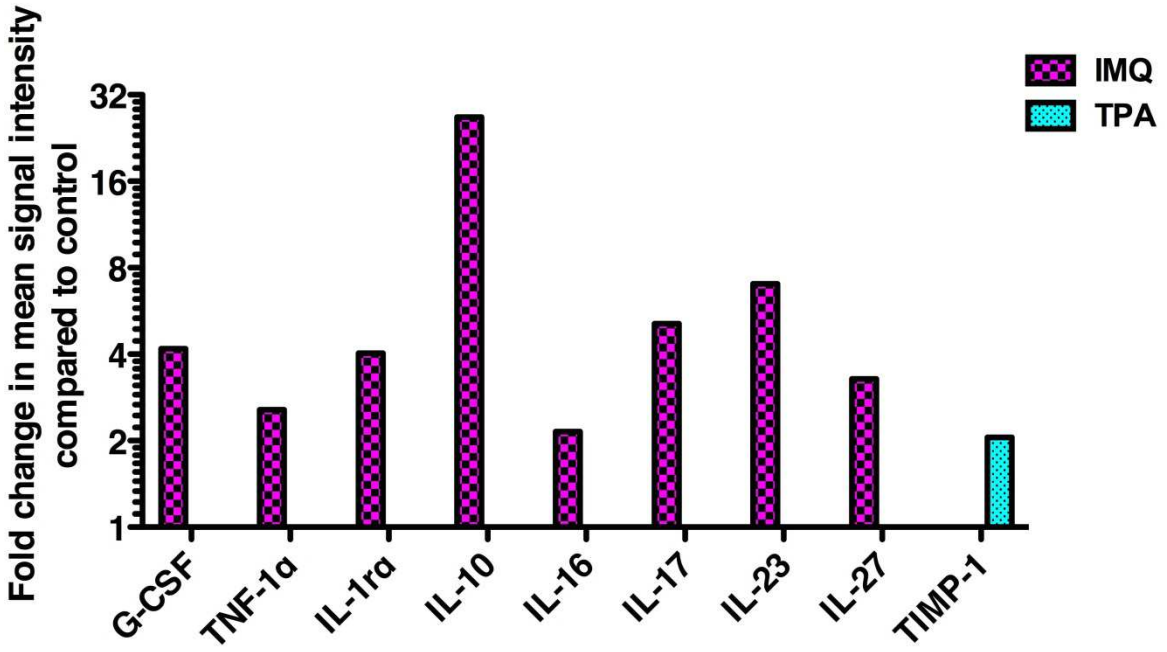



**Figure 4-21 Topical imiquimod cream application modulates cytokine expression in the plasma.**
The pixel intensity of each spot on the developed array blots (see figure 4-19) were estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each cytokine, the mean of the signal intensity of each cytokine was calculated, and the corrected signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control, from the mean signal intensity of each cytokine. Next, the fold change in the corrected mean signal intensities were estimated for each analyte by dividing the corrected mean signal intensity of that analyte in the imiquimod cream/TPA treated sample by the corrected mean signal intensity of that same analyte in the respective control group. Figure 4-21 is a bar graph showing the fold change in the plasma cytokines in response to the topical imiquimod cream or TPA application. Fold change >1, means increased production of the cytokine in response to the treatment.

Taken together, it is reasonable to state that compared to topical TPA application, the topical imiquimod cream application induced a strong pro-inflammatory milieu in the plasma. The strong IL-10 production in the imiquimod cream treated mice could therefore be a negative feedback mechanism to prevent exaggerated/deregulated immune response. It is possible that the TPA treatment induces production of inflammatory mediators that peaked at an earlier time point than the 72 hours used for the plasma proteome profiling. The only inflammatory chemokine that was consistently highly expressed in the

plasma of both imiquimod cream and TPA treated mice was CCL2 suggesting a potential role for CCL2 in the observed effects (this is explored further in the next chapter).

## 4.5.3 What chemokines and cytokines are produced in the bone marrow in response to the topical imiquimod cream or TPA application?

### 4.5.3.1 Introduction

The overall objective of this thesis was to try to obtain a comprehensive understanding of how different inflammatory contexts affected HSPC migratory properties. The cutaneous gene expression data presented in section 4.5.1 suggested that topical imiquimod (TLR7 ligand) and TPA (inflammatory agent acting through PKC) application elicited different inflammatory signatures. In addition, the plasma proteome profiling data presented above (see section 4.5.2) also suggested that, at least in the imiquimod cream application model, many inflammatory chemokines and cytokines were released into the circulation. To understand how these cutaneous inflammatory gene signatures, and the circulating inflammatory milieu, reflected in the bone marrow niche, proteome arrays were again employed to profile for differences and/or similarities in bone marrow proteins between imiquimod cream- and TPA-treated mice. Through these arrays, I hoped to identify differences in proteome factors between the bone marrow and the circulation that may offer possible explanation(s) for the increased HSPC egress from the bone marrow in response to each treatment. The mouse angiogenesis array kit (ARY015) was selected for this purpose (see previous chapter for details). As the arrays are expensive, each array was run once; however, to make it representative, bone marrow protein extracts from three mice were pulled together and used for the array.

Approximately 300μg of whole bone marrow protein extract was used for each array. **The experimental details are exactly as described in section 3.5.4 in the previous chapter.** Figures 4-22 shows the array blots from total bone marrow protein extract of aqueous cream (A), or imiquimod (B), or acetone (C), or TPA (D) treated mice.

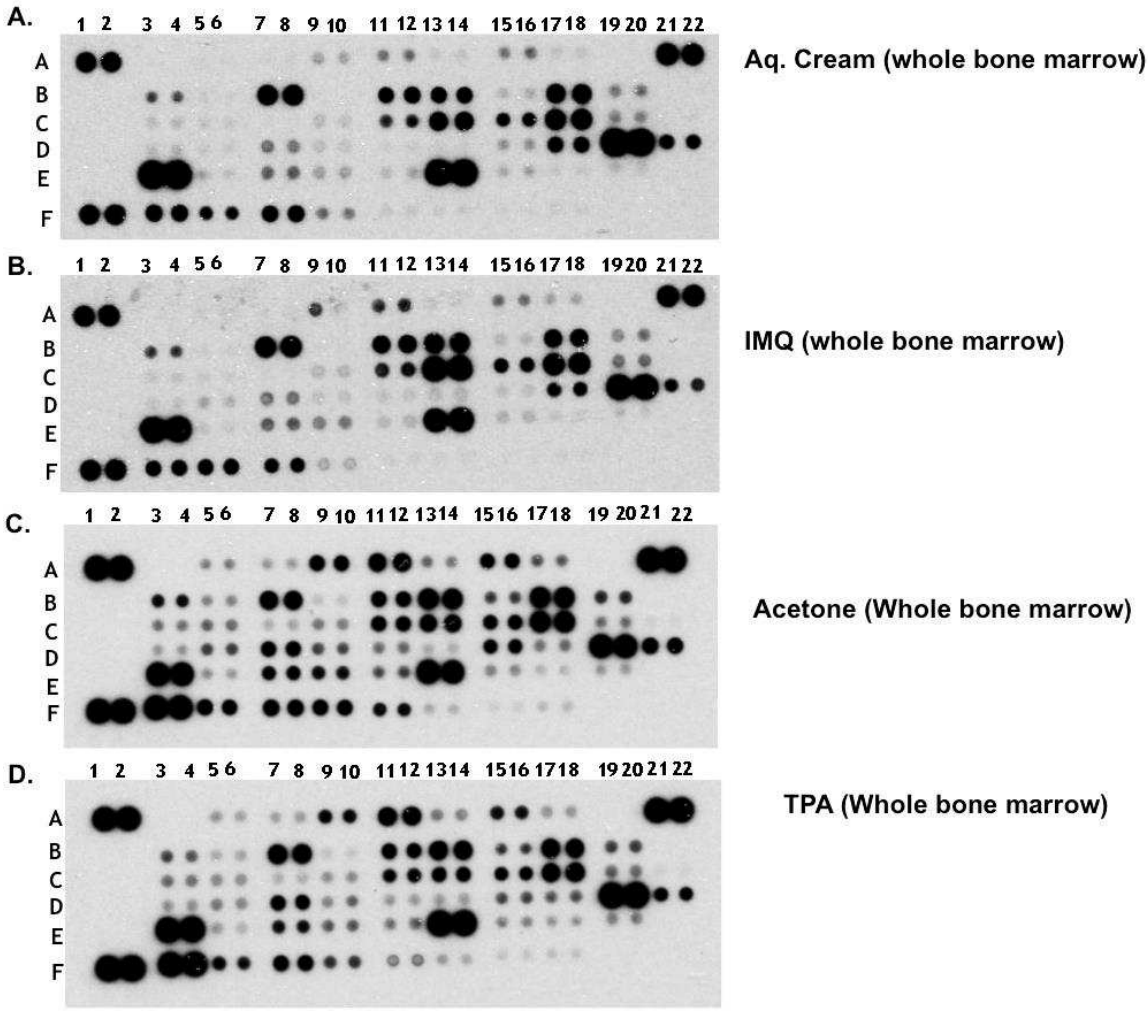



**Figure 4-22 Array blots for bone marrow proteome profiling using the mouse angiogenesis proteome array kit.**
A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or TPA (100µM 100µL acetone), was applied to the shaved dorsal skin of C57BL/6 mice for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of aqueous cream or acetone as controls for imiquimod cream and TPA treatment respectively. On the fourth day, mice were euthanized by $CO_2$ asphyxiation, bones [2x (tibia + femur+ hip bones)] were harvested, and homogenised in 400µL of mammalian protein extraction reagent (MPER, Thermo Scientific). The total bone marrow homogenates were spun at >10,000xg for 10 minutes and supernatants taken for proteome array profiling. In order to obtain a representative proteome profile for each treatment group, bone marrow protein lysates from three mice were pooled together for each treatment group. After estimating the pooled lysate protein concentration using the BCA method, ~300µg of the respective samples was used for the proteome profiling using the Mouse angiogenesis proteome array kit (R&D systems). A & B are the respective scanned images of the blots of the bone marrow proteome from aqueous cream (aq. cream) and imiquimod cream; C & D are the respective scanned image of the blots of the protein extracts from the bone marrow of acetone and TPA treatment.

After estimating the signal intensity of each spot using ImageJ 1.48 (NIH, USA), similar assumptions, as explained in section 4.5.2, were used to calculate the fold change in signal intensities. To increase the stringency of the assay, only analytes demonstrating >2-fold modulation were considered to merit further discussion.

### 4.5.3.2 Chemokines and cytokines produced in the bone marrow in response to topical imiquimod cream or TPA treatment.

As summarised in the bar graph in figure 4-23, topical imiquimod cream application led to a >2-fold reduction in the level of bone marrow osteopontin (OSPN), and CX3CL1 compared to aqueous cream treated controls. In addition, topical imiquimod cream treatment led to increases in the bone marrow IL-1α (2-fold increase) and IL-1β (>4-fold increase) when compared to aqueous cream treated controls.

Interestingly, the only chemokine that showed a >2-fold reduced expression in the bone marrow of TPA treated mice was CXCL16. No other cytokines or chemokines were detected in this proteome analysis.

It is also interesting to note that no significant alteration in bone marrow CXCL12 was found in spite of the significant reductions as determined by ELISA in section 4.4.1.2, thus highlighting the limitations of the proteome arrays.

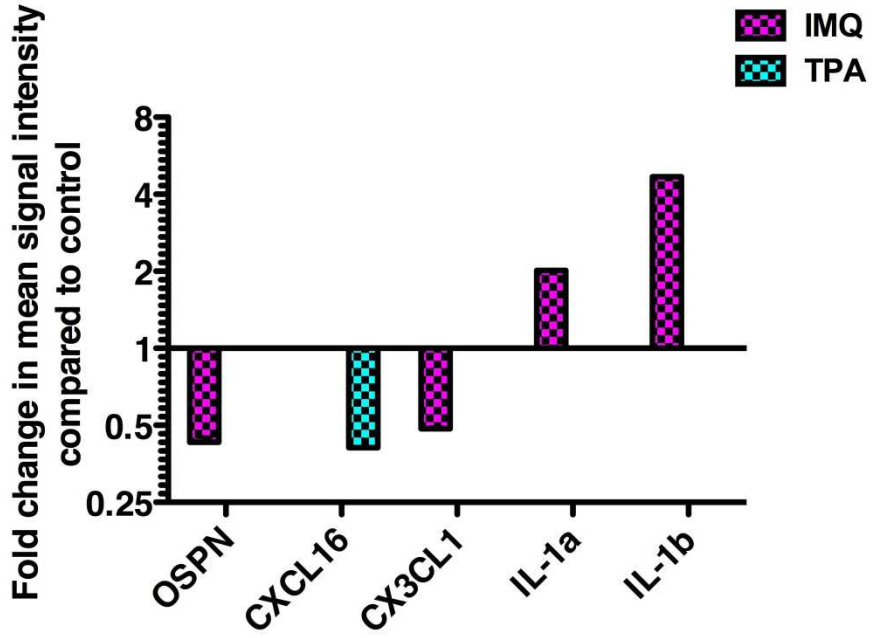



**Figure 4-23 Topical imiquimod cream application modulates chemokine and cytokine expression in the bone marrow.**
The signal intensities of each spot on the developed array blot (see figure 4-22) was estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each cytokine, the mean of the signal intensity of each cytokine/chemokine was calculated, and the corrected signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control, from the mean signal intensity of each cytokine. Next, the fold change in the corrected mean signal intensities were estimated for each analyte by dividing the corrected mean signal intensity of that analyte in the imiquimod cream/TPA treated sample by the corrected mean signal intensity of that same analyte in the respective control groups. Figure 4-23 is a bar graph showing the fold change in the bone marrow analytes in response to the topical imiquimod cream or TPA treatment. Fold

change >1, means increased production of the analyte in response to the treatment; fold change <1, indicates reduced analyte concentration in response to the treatment.

#### 4.5.3.3 Other angiogenesis related proteins released in response to topical imiquimod cream or TPA treatment.

The other analytes also detected by the angiogenesis array are presented in figure 4-24 below. Topical imiquimod cream application induced a 2-fold or >4-fold increased bone marrow secretion of platelet-derived growth factor-AA (PDGF-AA) and PDGF-AB respectively. However, topical TPA treatment led to >2-fold reduction in the bone marrow PDGF-AB levels. Also, topical imiquimod cream application induced an ~8-fold increase in pentraxin-3 secretion in the bone marrow and a >2-fold increased expression of insulin-like growth factor binding protein-3 (IGFBP-3) compared to aqueous cream treated controls.

Topical TPA treatment on the other hand led to a >2-fold increase in the expression of MMP-8 in the bone marrow. However, whereas TPA treatment caused a >2-fold reduction in the TIMP-1 levels in the bone marrow, it caused a >2-fold increase in the bone marrow TIMP-4 levels.

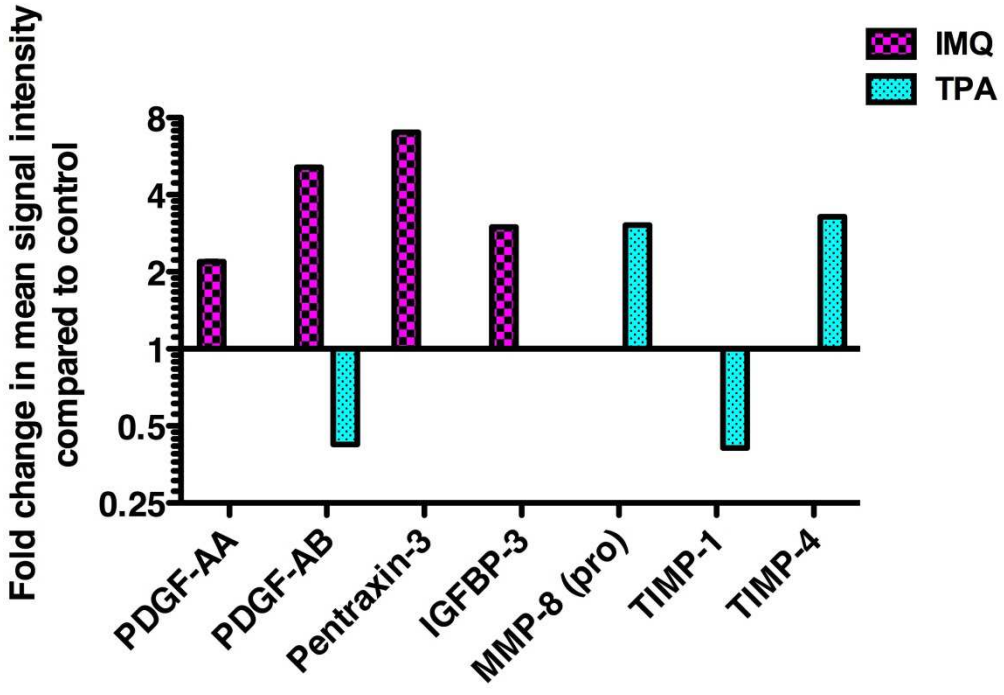



**Figure 4-24 Topical imiquimod cream or TPA treatment modulates the expression of angiogenesis-related factors in the bone marrow.**
The signal intensities of each spot on the developed array blot (see figure 4-22) was estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each cytokine, the mean of the signal intensity of each cytokine was calculated, and the corrected signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control, from the mean signal intensity of each cytokine. Next, the fold change in the corrected mean signal intensities were estimated for each analyte by dividing the corrected mean density of that analyte in the imiquimod cream/TPA treated sample by the corrected mean signal intensity of that same

analyte in the respective control groups. Figure 4-24 is a bar graph showing the fold change in the bone marrow analytes in response to the topical imiquimod cream or TPA treatment. Fold change >1, means increased production of the analyte in response to the treatment; fold change <1, indicates reduced analyte concentration in response to the treatment.

In summary, the data presented in this section suggest that at the 72 hour time point where samples were taken, topical imiquimod cream, or TPA, treatment affected the chemokine and cytokine expression by:

(A) Induction of strong inflammatory chemokine and cytokine gene expressions in the skin in response to each of topical imiquimod cream or TPA.

(B) Topical imiquimod cream treatment comparatively induced the expression of inflammatory chemokines in the plasma with a corresponding strong IL-10 response. Most of the circulating inflammatory mediators were not altered in the bone marrow milieu.

(C) Interestingly, although TPA treatment induced a strong inflammatory gene expression in the dorsal skin at the 72 hour time point employed for the gene and proteome array assays, there were no corresponding increases in inflammatory mediators in the circulation in these mice.

(D). Altogether, the only inflammatory chemokine that was highly expressed in the plasma of both imiquimod cream and TPA treated mice was CCL2. This is further explored in the next chapter.

## 4.5.4 What chemokine receptors are produced by bone marrow and peripheral blood HSPC population in response to the topical imiquimod cream, or TPA, application?

### 4.5.4.1 Introduction

Having established that topical imiquimod cream or TPA application induced mobilisation of haematopoietic progenitor cells into the circulation (section 4.2.4) and that these peripheral inflammation models orchestrated increased elaboration of inflammatory chemokines in the plasma, with CCL2 the common chemokine expressed in both imiquimod cream and TPA treatments (section 4.5.3), it was deemed important to assess how these changes impacted chemokine receptor expression in HSPC. As chemokines are amongst the key players involved in cellular migration, I hypothesised that HSPC inducibly express

inflammatory chemokine receptors in response to these peripheral inflammatory agents (i.e. topical imiquimod cream or TPA application), that enabled them to migrate in response to the increased circulating inflammatory chemokines especially CCL2.

To do this, mice received topical imiquimod cream or TPA treatment exactly as described in section 4.2.1. Bone marrow from these mice was harvested and processed for flow cytometry exactly as described in section 4.3.1. In order to obtain an overview of the entire repertoire of chemokine receptors expressed in the HSPC in response to each of the topical imiquimod cream or TPA treatment, LSK cells from both the peripheral blood and bone marrow were sorted by flow cytometry for RT-QPCR. 200 bone marrow or 100 peripheral blood LSK cells were sorted into respective wells of 96-well plates and then pre-amplified using the specific target pre-amplification protocol in accordance with manufacturer's instructions (Fluidigm Technologies; see materials and methods for details). The QPCR was undertaken using the Fluidigm 48.48 Dynamic Chip array platform in accordance with the manufacturer's protocol (see materials and methods for details).

The assumptions made in the analyses of the Fluidigm data to calculate the fold change in gene expression were as described in the previous chapter. Only genes showing fold change of 2 or more were considered to merit further discussion.

#### 4.5.4.2 Bone marrow HSPC (LSK) expresses chemokine receptors

The bar graph in figure 4-25 summarises the fold changes in bone marrow HSPC chemokine receptor gene expressions in response to either topical imiquimod cream or TPA treatment. For CC-chemokine receptor expression, CCR2, CCR3, CCR5, and CCR9 genes were expressed and modulated by the two topical inflammatory agents. Specifically, whereas imiquimod cream treatment induced a >32-fold increase in CCR2 expression, TPA treatment caused a 16-fold increase in expression in the bone marrow HSPC. The difference in the inducible CCR2 expression in the bone marrow HSPC was significant between the two treatments ($p=0.01$; 2-tailed Mann-Whitney U test). Topical imiquimod cream treatment also led to ~16-fold reduction in CCR3 expression. However, the fold change in CCR3 expression in response to TPA treatment was below the cut-off threshold of 2.

Topical imiquimod cream or TPA application induced up-regulation in the expression of CCR5; both topical imiquimod cream application and topical TPA application induced >8-fold increase in CCR5 gene expression. Also, whereas topical imiquimod cream application induced ~8-fold down-regulation of CCR7 expression, the fold change in CCR7 expression in response to topical TPA treatment was below the cut-off used to screen out background noise. Moreover, there was a >2-fold down-regulated expression of CCR9 in response to the topical imiquimod cream treatment.

The two topical inflammation models also modulated the expression of CXC-chemokine genes in bone marrow HSPC, i.e., CXCR2, CXCR3, and CXCR5, as well as the gene for CX3CR1. Specifically, for CXCR2 expression, topical imiquimod cream or topical TPA application induced ~8-fold up-regulation in CXCR2 expression. The expression of the CXCR3 was >2-fold down-regulated in response to topical imiquimod cream treatment; the expression of CXCR3 in response to TPA treatment was however below the cut-off threshold established to screen against background noise. In addition, topical imiquimod cream application induced ~2-fold up-regulation in CXCR4 expression. Furthermore, whereas topical TPA application induced an ~4-fold down-regulation in CXCR5 expression, the expression of CXCR5 in response to topical imiquimod cream application was not above background. There was also inducible up-regulation in the CX3CR1 gene in response to the topical imiquimod cream application (16-fold increase) or topical TPA application (>6-fold increase). The difference in the fold change in CX3CR1 expression in response to topical imiquimod cream and TPA treatment was statistically significant ($p=0.0003$; 2-tailed Mann-Whitney U test).

Finally, the c-kit and cited-2 genes were included in the assay as positive controls to validate the data as these two genes have demonstrable expression in cells of the HSPC compartment. Interestingly, both transcripts were consistently detected in the bone marrow HSPC, but neither topical imiquimod cream nor TPA treatment modulated their expression.

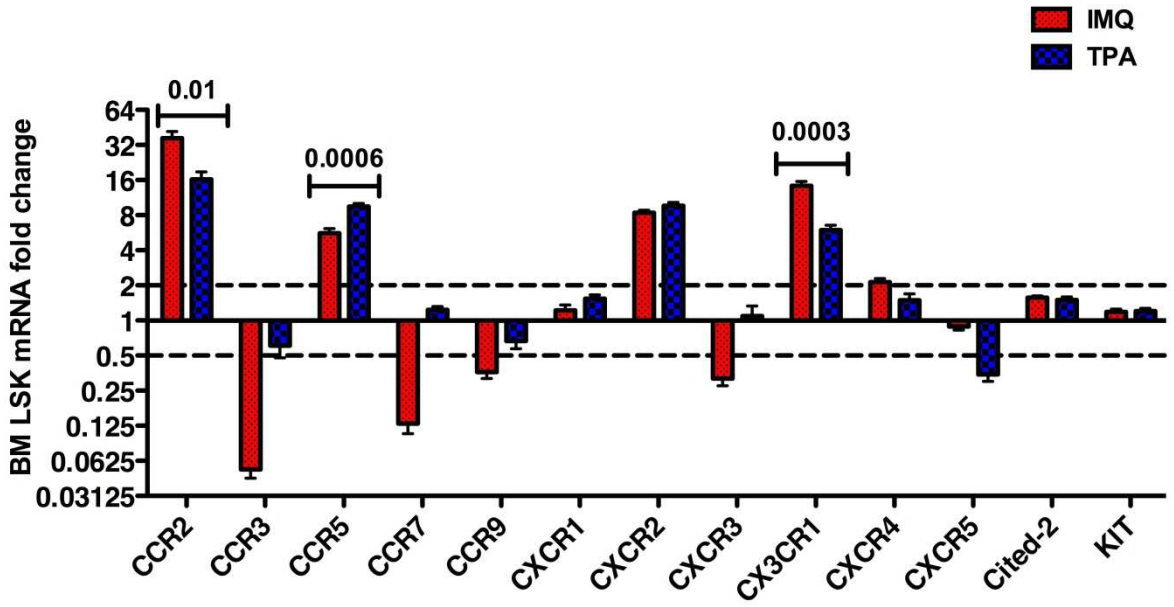



**Figure 4-25 Topical imiquimod cream or TPA application modulates chemokine receptor gene expression in the bone marrow HSPC**
C57BL/6 mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100μM in 100μL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec). The c-kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), as well as c-Kit, and Sca-1, to evaluate HSPC compartment. After excluding dead cells through Live/Dead Aqua fixable dye retention, cells positive for markers for terminal differentiation of haematopoietic cells were also gated out. Next, 200 bone marrow HSPC/LSK cells were sorted on FACSAria II for RT-QPCR using the Fluidigm 48.48 Dynamic array platform (Fluidigm Technologies). Gene expression was firstly normalised to Hprt levels and then secondly, to the respective control treated sample i.e. aqueous cream or acetone for imiquimod cream and TPA treatment respectively to obtain the fold change in expression (ΔΔCt). The fold changes in the bone marrow HSPC chemokine receptor expression induced by either topical imiquimod cream application or topical TPA application were plotted on the same axis to enable direct comparison. Statistical analyses were all computed using the 2-tailed, Mann-Whitney U test. (N=8).

### 4.5.4.3 Peripheral blood LSK/HSPC express chemokine receptors

The calculations for the fold change (ΔΔCt) in the gene expression in blood HSPC (LSK), was done using similar assumptions as explained for the ΔΔCt calculations for bone marrow HSPC (see section 4.5.4.1 above). The results of the analyses of peripheral blood HSPC chemokine receptor expression are summarised in the bar graph in figure 4-26. For CC-chemokines, the expression of CCR2, CCR5, CCR7 and CCR9, were modulated in response to the topical imiquimod cream or TPA application. Specifically, although topical TPA treatment caused ~16 fold down-regulation of CCR2 expression, the fold change in CCR2 expression in response to imiquimod cream treatment was below the cut-off threshold. The expression of CCR5 was also differentially, but significantly, modulated in response to topical

imiquimod cream or TPA application (p=0.004, 2-tailed Mann-Whitney U test). Specifically, whereas topical imiquimod cream induced ~6-fold up-regulation of CCR5 expression, topical TPA application induced ~2-fold down-regulation. In addition, the topical imiquimod cream, or topical TPA, application induced down-regulation of CCR7 expression in the peripheral blood HSPC population; a >2-fold or a >32-fold down-regulation in CCR7 gene expression in response to topical imiquimod cream and TPA application respectively. The difference in the CCR7 down-regulation was statistically significant between the two treatment groups (p=0.0007; 2-tailed Mann-Whitney U test). Moreover, topical imiquimod cream, or TPA, application induced CCR9 gene down-regulation; topical imiquimod cream application induced a >16-fold down-regulation, whereas topical TPA application induced ~2-fold reduction. The difference in the induced CCR9 gene down-regulation between the two treatment groups reached statistical significance (p=0.0001; 2-tailed, Mann-Whitney U test).

CXC-chemokine receptor gene expression in peripheral blood HSPC, i.e. CXCR1, CXCR2, CXCR4 and CXCR5 was also modulated in response to each of the two peripheral inflammation models. The expression of CXCR1 was differentially, but significantly, modulated in response to the topical imiquimod or TPA application (p=0.004; 2-tailed Mann-Whitney U test). Specifically, whereas topical imiquimod cream induced a >2-fold down-regulation in expression, the topical TPA treatment induced ~4-fold upregulation. Also, each of the peripheral inflammation models induced up-regulation of CXCR2. Specifically, whereas topical imiquimod cream application caused ~8-fold up-regulation, topical TPA application induced >32-fold up-regulation. The difference in the induced CXCR2 gene expression in response to the topical imiquimod cream and TPA application reached statistical significance (p=0.0007; 2-tailed Mann-Whitney U test). Interestingly, topical imiquimod cream treatment caused ~2-fold up-regulation in CXCR4 gene expression; although the fold change in CXCR4 expression in response to topical TPA treatment was below the cut-off threshold. Moreover, the two peripheral inflammation models significantly, but differentially, modulated the expression of the CXCR5 gene in peripheral blood HSPC (p=0.004; 2-tailed, Mann-Whitney U test). Whereas topical imiquimod cream application caused a 4-fold up-regulation in CXCR5 expression, topical TPA application induced a >2-fold downregulation.

As stated in the previous section, the Cited-2 and c-Kit genes were included as positive controls to validate the gene expression data. However, it is interesting to note that TPA treatment caused ~4-fold increase in expression of both c-Kit and cited-2 transcripts in peripheral blood HSPC.

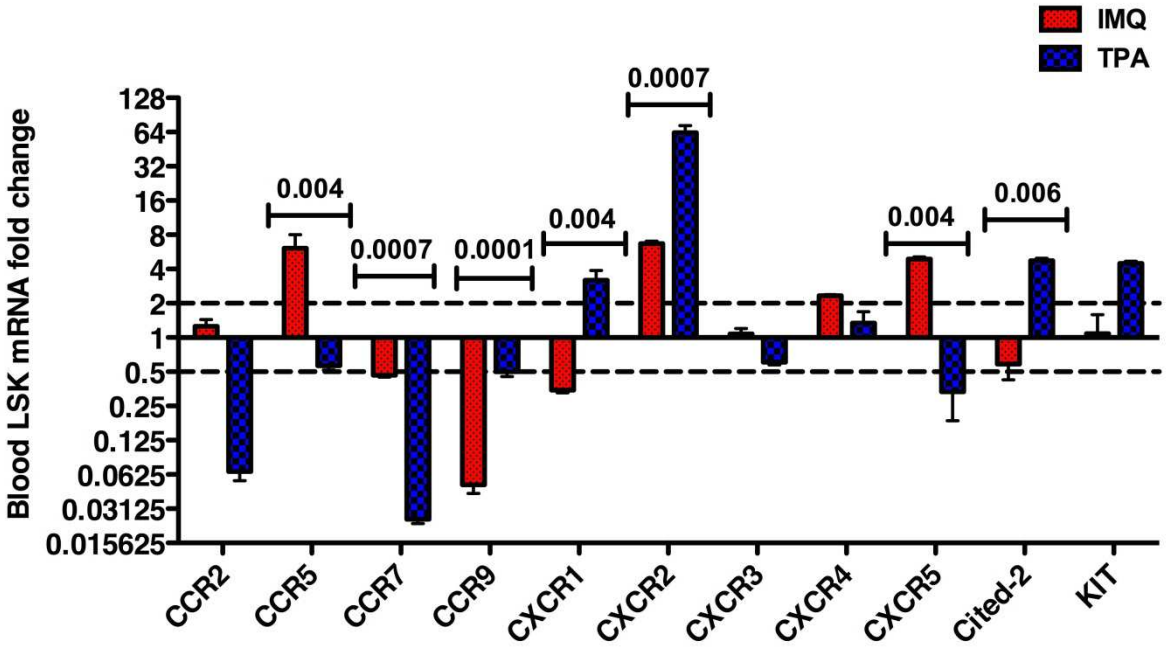



**Figure 4-26 Topical imiquimod cream or TPA application modulates chemokine receptor gene expression in the peripheral blood HSPC**

C57BL/6 mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream or TPA application respectively. On day 4, the mice were euthanized by $CO_2$ asphyxiation, peripheral blood was harvested into EDTA anticoagulation tubes. After lysing red blood cells, the cells were enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotech, UK). The c-kit-enriched cells were next stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), as well as c-Kit, and Sca-1, to evaluate HSPC compartment. After excluding dead cells through Live/Dead Aqua fixable dye retention, HSPC/LSK cells were sorted on FACSAria II for RT-QPCR using the Fluidigm 48.48 Dynamic array platform. Gene expression was firstly, normalised to Hprt levels, and secondly, to the respective control treated sample i.e. aqueous cream or acetone for imiquimod cream or TPA treatment respectively to obtain the fold change in gene expression ($\Delta\Delta$Ct). The fold changes in the peripheral blood HSPC chemokine receptor expression induced by either topical imiquimod cream application or topical TPA application were plotted on the same axis to enable direct comparison. Statistical analyses were all computed using the 2-tailed, Mann-Whitney U test. (N=8).

## 4.6 Discussion of the data from the peripheral inflammation model

### 4.6.1 Topical imiquimod cream or TPA treatment induced peripheral inflammation mobilises HSPC to the periphery.

In the previous chapter, evidence was provided showing that systemic inflammation induced by LPS injection caused significant alterations in the bone

marrow niche leading to HSPC mobilisation to the circulation. This chapter sought to broaden this understanding by investigating the effects of peripheral inflammation on HSPC mobilisation and localisation in mice. Topical imiquimod cream and TPA treatment models of peripheral inflammation were employed to elucidate whether inflammatory signals alone and/or TLR ligation were crucial in inflammation-induced HSPC mobilisation. In this chapter, evidence is provided showing that topical imiquimod cream, or TPA, treatment mobilised significant numbers of HSPC from the bone marrow to the circulation (see figure 4-2). This demonstrates that TLR ligation was not obligatory in the inflammation-induced HSPC mobilisation as TPA does not act through TLRs. The HSPC mobilisation in response to topical imiquimod cream treatment resulted in ~48% reduction in bone marrow cellularity compared to aqueous cream treated controls (see figure 4-4). This reduction in bone marrow cellularity was further reflected in the reductions in LMPP, ST-HSC and LT-HSC populations (figures 4-7 & 4-8). Similarly, topical TPA treatment also led to ~27% reduction in bone marrow cellularity that was reflected in significant reductions in bone marrow CLP, LMPP, and ST-HSC populations (figures 4-4, 4-7 & 4-8). As reported elsewhere (Shi et al., 2013, Zhang et al., 2008), and in the previous chapter, topical imiquimod cream treatment also led to apparent expansion of the HSPC compartment as a result of re-expression of Sca-1 on $Kit^{+}Sca\text{-}1^{-}$ HSPC. TPA-induced inflammation, on the other hand, only caused a slight increase in this pseudo Sca-1 re-expression, suggesting a TLR-driven effect as the underlying cause of this Sca-1 phenotypic inversion i.e. the re-acquisition of Sca-1 antigens by previously Sca-1 negative cells.

Interestingly, each of the peripheral inflammatory agents caused a significant increase in spleen cellularity, i.e., 2.7-fold or 1.9-fold increased cellularity in response to topical imiquimod cream, and TPA, treatment respectively. This was suggestive of either increased extramedullary haematopoietic activity in the spleen and/or increased sequestration of haematopoietic cells into the spleen.

### 4.6.2 Role of the bone marrow niche in the peripheral inflammation induced HSPC mobilisation

The interactions of the HSPC and the niche cells, and/or soluble products from these cells are crucial in the retention of the HSPC in their native bone marrow

microenvironment. Many previous studies have established that disruption of these HSPC-niche interactions are a critical component of the mechanism underlying most HSPC mobilising agents such as G-CSF, and AMD3100 (Levesque et al., 2003a, Levesque et al., 2003b, Liles et al., 2003, Broxmeyer et al., 2005). The data presented herein suggest that peripheral inflammation, induced by either topical imiquimod cream, or topical TPA, application perturbed the CXCR4/CXCL12 axis in the bone marrow niche. Each of the peripheral inflammation-inducing agents (topical imiquimod cream or TPA) caused significant reduction in the bone marrow CXCL12 concentration (as measured by ELISA). In contrast, neither topical imiquimod cream nor TPA treatment significantly altered the HSPC CXCR4 expression. It is interesting to note that neither of the peripheral inflammation models significantly altered the peripheral blood CXCL12 concentration. Therefore, it is reasonable to suggest that the sustained plasma CXCL12 concentration, with a concomitant reduced bone marrow CXCL12, could have created a CXCL12 gradient that favoured HSPC egress to the circulation.

The data presented in this chapter also argue strongly for a role for osteoblasts in the HSPC mobilisation observed with each of the peripheral inflammation models. The bone marrow stromal cell compartment, i.e. osteoblastic lineage cells, mesenchymal stromal cells (MSC) and bone marrow endothelial cells, has been demonstrated to be a critical component of the bone marrow endosteal niche. These cells have been proposed to be the major producers of critical soluble niche factors such as CXCL12, angiopoietin-1, stem cell factor, and osteopontin (Ducy et al., 2000, Calvi et al., 2003, Arai et al., 2004, Mendez-Ferrer et al., 2010). Interfering with the function of these stromal niche cells will therefore potentially lead to increased egress of HSPC from the bone marrow niche. The data presented in this chapter showed that topical imiquimod cream application led to significant increases in the bone marrow endothelial cell and MSC populations. In contrast, the topical TPA application led to significant reduction in the bone marrow endothelial cell population, although the MSC population was not significantly altered. It is reasonable to speculate that these increases in MSC and endothelial cells in response to topical imiquimod cream treatment, but not TPA treatment, could have resulted in the release of inflammatory mediators in the bone marrow of topical imiquimod

treated mice (in contrast to the few inflammatory mediators detected in the bone marrow of topical TPA treated mice). In addition, both peripheral inflammatory agents led to significant reduction in the proportion of the bone marrow osteoblast populations. Interpreting these data in the light of the significant reduction in bone marrow CXCL12 concentration, it is reasonable to suggest that, in both peripheral inflammation models, the most probable critical cellular niche component involved in the secretion of CXCL12 might have been the osteoblast cell population as it was the only stromal niche cellular component that was significantly reduced in the bone marrow of both topical imiquimod cream and TPA treated mice (see figures 4-11 & 4-12) (Christopher et al., 2009, Semerad et al., 2005, Christopher and Link, 2008).

Finally, the phagocytic mononuclear cells of the innate arm of the immune system have also been shown to have roles in the retention of HSPC in the bone marrow niche. Many previous reports have used depletion experiments to provide evidence for specific cell populations such as $CD169^{+}$ macrophages (Chow et al., 2011), osteomacs (Winkler et al., 2010b) and granulocytic cells (Levesque et al., 2003b, Pruijt et al., 2002) as being crucial in HSPC retention in the bone marrow. Interestingly, whereas topical imiquimod cream treatment led to a significant increase in the bone marrow monocytic cell population (in agreement with the findings reported in the systemic inflammation model in the previous chapter), topical TPA application did not significantly alter this cell population. Moreover, and in agreement with data published by Chow et al., topical imiquimod cream treatment led to significant reduction in the $Gr\text{-}1^{-}$ $F4/80^{+}CD115^{int}CD169^{+}CD11b^{low}$ macrophage population (Chow et al., 2011). In stark contrast, the topical TPA application significantly increased the $Gr\text{-}1^{-}$ $F4/80^{+}CD115^{int}CD169^{+}CD11b^{low}$ macrophage population in the bone marrow. Therefore, whereas the topical imiquimod cream-induced HSPC mobilisation may have a significant contribution from a perturbation of the cells of the mononuclear phagocytic system (in agreement the previously published macrophage depletion data), the topical TPA application model rather increased the macrophage population, suggesting that there may be fundamental differences in the mechanisms involved.

### 4.6.3 Inflammatory mediators may play a role in the inflammation-induced HSPC mobilisation.

Chemokine ligand/chemokine receptor interactions are fundamental in cellular migration in multicellular organisms. The HSPC chemokine receptor transcript data presented in this chapter showed that topical imiquimod cream or TPA treatment upregulated the expression of CCR2, CCR5, CXCR2 and CX3CR1 in bone marrow HSPC. In addition, topical imiquimod cream treatment also caused increased expression of CCL2, CCL3, CCL4, CCL5, and CCL11 in the circulation, thus raising the possibility that chemokine/chemokine receptor interaction may have a role in the HSPC mobilisation to the circulation in response to topical imiquimod cream treatment. Interestingly, the only chemokine that was increased in the circulation of TPA treated mice was CCL2, which perhaps may be suggestive of a role of CCR2/CCL2 axis in the observed HSPC egress from the bone marrow in response to topical imiquimod cream or TPA treatment (see next chapter). Surprisingly, the only chemokine receptor gene that was upregulated in peripheral blood HSPC in response to each of the two peripheral inflammation models was CXCR2. It is also interesting to note that although expression of many inflammatory cytokine and chemokine genes was upregulated in the dorsal skin of TPA treated mice, the corresponding protein products of these same genes could not be detected in the plasma of the mice. This may suggest that either the genes were not efficiently translated or that their protein products did not enter the circulation.

Also, the topical imiquimod cream application induced a strong increase in the expression of pro-inflammatory cytokines such as, TNF-1$\alpha$, IL-16, IL-17, IL-23 and IL-27. This agrees with a previous publication that established the topical imiquimod cream application as a model of human psoriasis (van der Fits et al., 2009). In that paper, Van der Fits et al. implicated the IL-23/IL-17 axis as the critical determinant of the psoriasis phenotype. Moreover, others have found that in topical imiquimod cream treated mice, as well as in human psoriasis patients, neutrophils and pDCs infiltrated the skin lesions and that IL-17, IL-22, and IL-23 were important for the observed disease pathology (Cai et al., 2011, Stanley, 2002, Van Belle et al., 2012). It is therefore possible that cells that infiltrated the lesion sites produced some of the circulating cytokines detected in the cytokine array. However, the higher IL-10 (~32-fold increase) levels

detected in the plasma of mice treated with topical imiquimod cream suggest that feedback mechanisms were invoked to modulate the inflammatory response in these mice. Previous reports have suggested that direct TLR ligation on immune cells such as macrophages and myeloid derived dendritic cells led to increased IL-10 secretion (Agrawal et al., 2003, Dillon et al., 2004, Boonstra et al., 2006). Thus, it is possible that the increased IL-10 concentration detected in the plasma may be the result of a direct TLR7 ligation on some of these recruited and resident skin immune cells. However, none of these inflammatory cytokines were detectable in the plasma of the mice treated with the topical TPA. Therefore, it is reasonable to suggest that because the TPA application has no inherent TLR signalling properties, the inflammation is probably more localised. In contrast, due to the expression of TLR7 in some leucocytes (Renn et al., 2006, Kadowaki et al., 2001, Jarrossay et al., 2001), the initial inflammatory stimulus elicited by the topical imiquimod cream application might have been amplified by the subsequent TLR7 signalling in the recruited leucocyte populations. This, therefore, sets up an enhanced inflammatory context that requires a negative feedback response (in this case a strong IL-10 induction) to prevent an exaggerated inflammatory response. Since most of these leucocyte populations are motile, it is reasonable to speculate that this skin-induced inflammation, in the topical imiquimod cream model, is likely to be communicated to other organs in the body.

As an alternative explanation, one can argue that as the vehicle for the TPA treatment, i.e. acetone, is volatile, it evaporated quickly and therefore did not allow sufficient time for the TPA to penetrate the epidermal skin layer. In contrast, as the imiquimod was in the form of a cream that remained on the skin surface for longer period of time, imiquimod could have had sufficient time to penetrate the epidermal and dermal skin layers and ultimately entered the systemic circulation to initiate a comparatively stronger inflammatory response. Therefore, the differences in the durations for which the topical imiquimod cream and TPA remained in contact with the dorsal skin might have been a contributory factor as to why TPA produced a more localised inflammatory response whereas topical imiquimod cream produced a more generalised and systemic effect.

## 4.7 Chapter summary

Both topical imiquimod cream and TPA application modulate inflammatory chemokine receptor gene expression in the HSPC. These modulations of the chemokine receptor gene expression may have a role in the observed increased numbers of HSPC in the circulating blood as increased levels of inflammatory mediators were released into the circulation. In addition, both treatments caused significantly increased splenic cellularity and haematopoietic progenitor cell numbers in the spleen suggesting increased haematopoietic activity in the spleen. The following should also be noted:

1. The topical imiquimod cream application-induced peripheral inflammation orchestrated HSPC mobilisation through a multi-faceted mechanism that probably involves one or more of the following: significant reduction in the bone marrow CXCL12 concentration without altering peripheral blood CXCL12 levels, and thus generating a CXCL12 gradient that favours the exit of HSPC from the bone marrow; depletion of the $CD169^{+}$ macrophage population, as well as depletion of the stromal bone osteoblast population, leading to decreased bone marrow CXCL12. All these might have combined to favour HSPC egress from the bone marrow to the circulation.

2. Topical TPA application induced inflammation also orchestrated HSPC mobilisation that may involve: reduction in the bone marrow CXCL12 concentration that favours a positive gradient towards the peripheral blood CXCL12; significant reduction in the stromal bone osteoblast population that might have contributed to the reduction in the bone marrow CXCL12 as osteoblasts are a major source of CXCL12.

3. The only inflammatory chemokine that was highly expressed in the plasma in both topical imiquimod cream, and TPA, treatment models was CCL2, suggesting that if a common chemokine was responsible for the HSPC egress, then the CCL2/CCR2 axis was worth consideration (this is explored further in chapter 5).

# Chapter 5: Role of CCR2 in the topical imiquimod cream-induced HPC mobilisation.

## 5.1 Introduction and aims

In the previous two chapters, evidence was provided that both LPS-induced systemic inflammation and peripheral inflammation (due to either topical imiquimod cream or TPA application) resulted in the mobilisation of increased numbers of haematopoietic progenitor cells to the circulation. Efforts were made to provide some insights into the basic mechanisms underpinning the increased egress of the HSPC from the bone marrow. Among these, it was found that inflammatory chemokine receptor expression by HSPC was modulated in both the systemic and the peripheral inflammation models. As a matter of fact, CCR2 was highly upregulated in bone marrow HSPC during the systemic inflammation model; in the peripheral inflammation models too, CCR2 was the most highly upregulated (>16-fold increase over baseline) chemokine receptor. In addition, although the CCR2 ligand, CCL2, was highly expressed in both the bone marrow and peripheral blood during LPS-induced systemic inflammation, CCL2 was consistently highly expressed in the peripheral blood in the peripheral inflammation models (i.e. topical imiquimod cream or TPA application). In this chapter, I hypothesised that the CCR2/CCL2 axis may have a role in the observed HSPC mobilisation during the LPS-induced systemic inflammation model and/or the peripheral inflammation model. In order to test this hypothesis, I aimed to address the following questions:

1. Does the inflammation-induced CCR2 gene expression in bone marrow HSPC translate to functional cell surface CCR2 protein expression?

2. How does disruption of the CCR2/CCL2 axis (i.e. CCR2 knock out or CCL2 neutralisation) impact the observed HSPC mobilisation during the inflammation?

3. How does the peripheral inflammation model in WT compare to CCR2 KO mice?

4. How do exogenously administered chemokine ligands affect the numbers of HSPC in peripheral blood?

In this way I sought to begin a detailed exploration of the role of the inflammatory chemokine-chemokine receptor axis in HSPC biology during inflammation.

## 5.2 Does the inflammation-induced CCR2 gene expression in HSPC translate to functional cell surface protein?

### 5.2.1 Introduction

In the previous chapters, data were provided that both the LPS-induced systemic inflammation, and peripheral inflammation models (i.e. topical imiquimod cream or topical TPA application) inducibly modulated the expression of some inflammatory chemokine receptors in HSPC. In addition, it was also shown that the most highly upregulated inflammatory chemokine receptor transcript, particularly in the bone marrow HSPC during the peripheral inflammation models, was CCR2. Previous reports have indicated that mRNA abundance does not necessarily translate into functional cellular protein abundance as many post-transcriptional regulatory processes could affect the downstream translational processes (de Sousa Abreu et al., 2009, Maier et al., 2009). Thus it was necessary to establish that the upregulated Ccr2 mRNA in the bone marrow HSPC was translated into functional, cell surface protein in the haematopoietic progenitors. Flow cytometric analysis was employed for this purpose. More importantly, as CCR2 was the most highly upregulated inflammatory chemokine receptor in the bone marrow HSPC, I aimed to address the following questions:

(A) Do HSPC express cell surface CCR2 in response to inflammation?

(B) Does such CCR2 expression affect migration of HSPC towards an *in vitro* gradient of CCL2?

By addressing these questions, I hoped to understand whether during inflammation the CCR2/CCL2 axis influenced HSPC biology and thus played a role in the observed increased numbers of HSPC exiting the bone marrow in response to any of the inflammation models used in this study.

## 5.2.2 Do the HSPCs express cell surface CCR2 in response to inflammation?

As stated in previous chapters, the haematopoietic colony-forming assay employed in this study i.e. the Methocult™ GF 3434 medium, allowed the detection of myeloerythroid progenitors. Therefore, the objective in this part of the study was to characterise the surface CCR2 expression in these myeloerythroid progenitors in order to build upon the data reported in the previous chapters. Previously, Pronk et al. published a protocol that used differential cell staining for CD41, CD105, CD150, Sca-1, c-Kit, and CD16/32 (FcgRII/III) antigens, to characterise the hierarchy of myeloerythroid progenitors (Pronk et al., 2007). This protocol was employed, but with a slight modification. As shown in the previous chapters and by others (Zhang et al., 2008), the inflammatory context affected the expression of the Sca-1 antigen and therefore had the potential to inadvertently bias subsequent analysis. Therefore, IL-7Rα antigen expression was used, instead of Sca-1 antibody, to exclude lymphoid-biased progenitors from the subsequent downstream analysis of the myeloerythroid progenitors. Thus, the various myeloerythroid progenitor cells were defined as: MkP, megakaryocyte progenitors ($Lin^-c\text{-}Kit^+IL\text{-}7R\alpha^-CD41^+CD150^+$); GMP, granulocyte-macrophage progenitors ($Lin^-c\text{-}Kit^+IL\text{-}7R\alpha^-CD41^-CD150^-CD16/32^+$); PreGM, precursors of granulocyte-macrophage progenitors ($Lin^-c\text{-}Kit^+IL\text{-}7R\alpha^-CD41^-CD150^-CD16/32^-CD105^-$); CFU-E, colony-forming unit-erythroid ($Lin^-c\text{-}Kit^+IL\text{-}7R\alpha^-CD41^-CD150^-CD16/32^-CD105^+$); preCFU-E, precursors of CFU-E ($Lin^-c\text{-}Kit^+IL\text{-}7R\alpha^-CD41^-CD150^+CD16/32^-CD105^+$); PreMegE, precursors of megakaryocyte-erythroid progenitors ($Lin^-c\text{-}Kit^+IL\text{-}7R\alpha^-CD41^-CD150^+CD16/32^-CD105^-$).

### 5.2.2.1 Gating strategy used for the CCR2 receptor staining in myeloerythroid progenitors.

WT mice were treated with either systemic LPS injection, or with topical imiquimod cream or TPA application, exactly as described in the previous chapters. Femur, tibia as well as hipbones were harvested from these mice and processed for FACS by enriching the bone marrow cell isolates for HSPC using the c-Kit Microbeads and LS magnetic columns (see previous chapters for details). The cells were then stained for lineage (Lin) marker cocktail (CD4, CD5, CD8a,

CD11b, B220, Ter119 and Gr-1), c-Kit and Live/dead aqua, CD41, CD105, CD150 CD16/32 and IL7Rα antibodies to analyse the myeloerythroid progenitor compartment. To ensure the specificity of the CCR2 antibody staining, FMO (fluorescent minus-one) controls in which all the antibodies in the staining cocktail were used except the CCR2 antibody were included in all experiments. To further increase the stringency of the assay, bone marrow cells from CCR2 KO mice were also stained for CCR2, and compared with the WT mice samples to further check for non-specific staining.

The dot plots in figure 5-1 show representative plots illustrating the gating strategy used to firstly, exclude debris, dead cells and terminally differentiated haematopoietic cells ($Lin^+$ cells) from further analysis. The myeloerythroid progenitors, defined herein as $Lin^-c\text{-}Kit^+IL7R\alpha^-CD41^-CD150^{+/-}$ cells, were then selected and re-gated in subsequent analysis to characterise the GMP, PreGM, CFU-E, PreCFU-E and PreMegE populations (see **sections 5.2.2.2-5.3.2.7 below**).

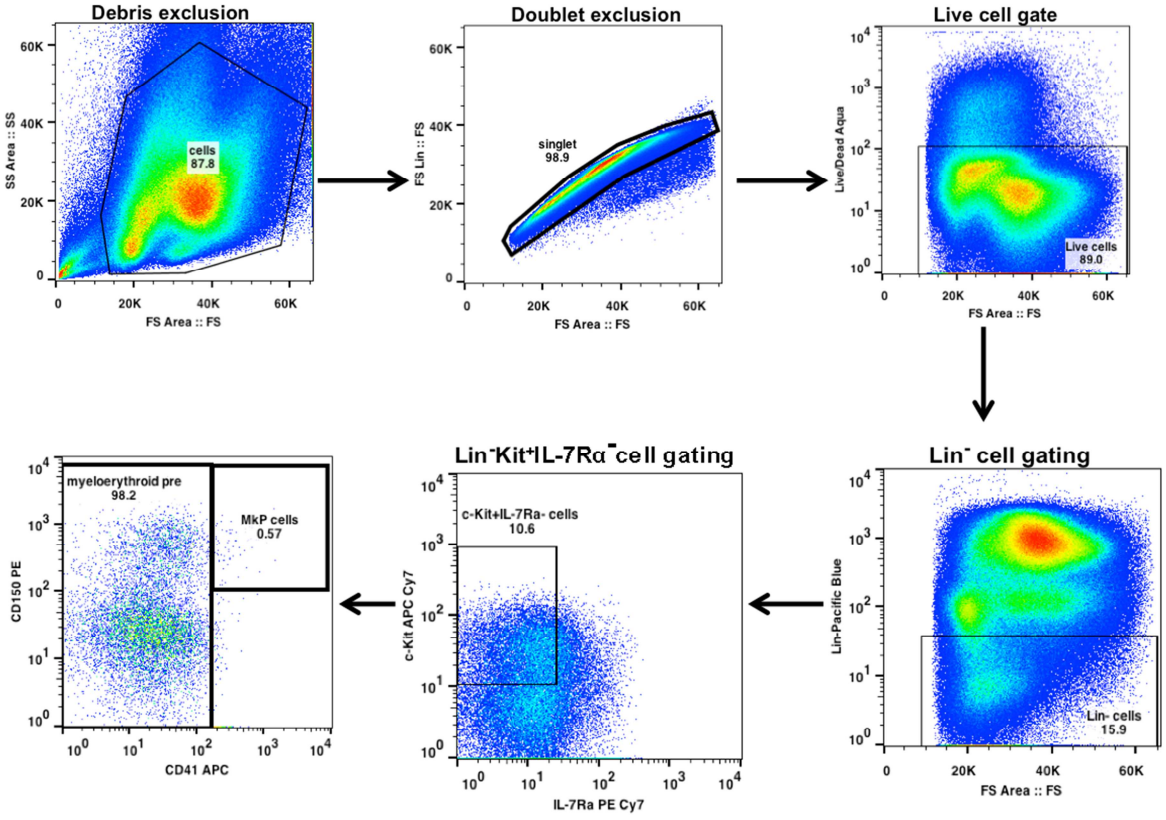



**Figure 5-1 Gating strategy used to characterise bone marrow myeloerythroid progenitor cell populations.**
C57BL/6 mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On

day three of the model, other groups of mice received a single I.P. injection of 100μL LPS or 100μL I.P. PBS injection as a control. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec). The c-kit-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), c-Kit, IL-7Rα, CD41, CD150, CD16/32, CD105, and Live/Dead Aqua antibodies to evaluate myeloerythroid compartment of the bone marrow. Debris and dead cells were excluded using forward-side scatter characteristics, doublet exclusion, and Live/Dead Aqua fixable cell retention. Next, cells positive for markers of terminal differentiation of haematopoietic cells were also gated out. Next, cells expressing the c-Kit antigen, but negative for the IL-7Rα antigens were selected so as to exclude the lymphoid-biased progenitors from the subsequent analysis. Re-gating on this population for the differential expression of CD41 and CD150 enabled the selection of the myeloerythroid progenitors, i.e. $Lin^-Kit^+IL\text{-}7R\alpha^-CD41^-CD150^{-/+}$ cells, that were re-analysed as described below.

#### 5.2.2.2 LPS-induced inflammation significantly upregulates CCR2 expression in GMP cells

The $Lin^-c\text{-}Kit^+IL7R\alpha^-CD41^-CD150^{+/-}$ cells i.e. the myeloerythroid progenitor cells, (see section 5.3.2.1 and figure 5-1 for gating strategy) were next re-gated for differential expression of CD150 and CD16/32 antigens to define the GMP cells ($CD16/32^+CD150^-$ cells), and non-GMP cells ($CD150^+CD16/32^-$ cells). Figures 5-2 A & B show representative plots for the GMP cell delineation from bone marrow of mice treated with I.P. PBS and I.P. LPS respectively. The histogram overlay in figure 5-2 **C** shows expression of CCR2 on GMP cells from mice treated with either LPS or PBS injection. As summarised by the scatter plot in figure 5-2D, I.P. LPS injection led to significantly increased CCR2 expression on the bone marrow GMP cells when compared to I.P. PBS treated controls (figure 5-2 D; $p=0.0286$, 2-tailed Mann-Whitney U test).

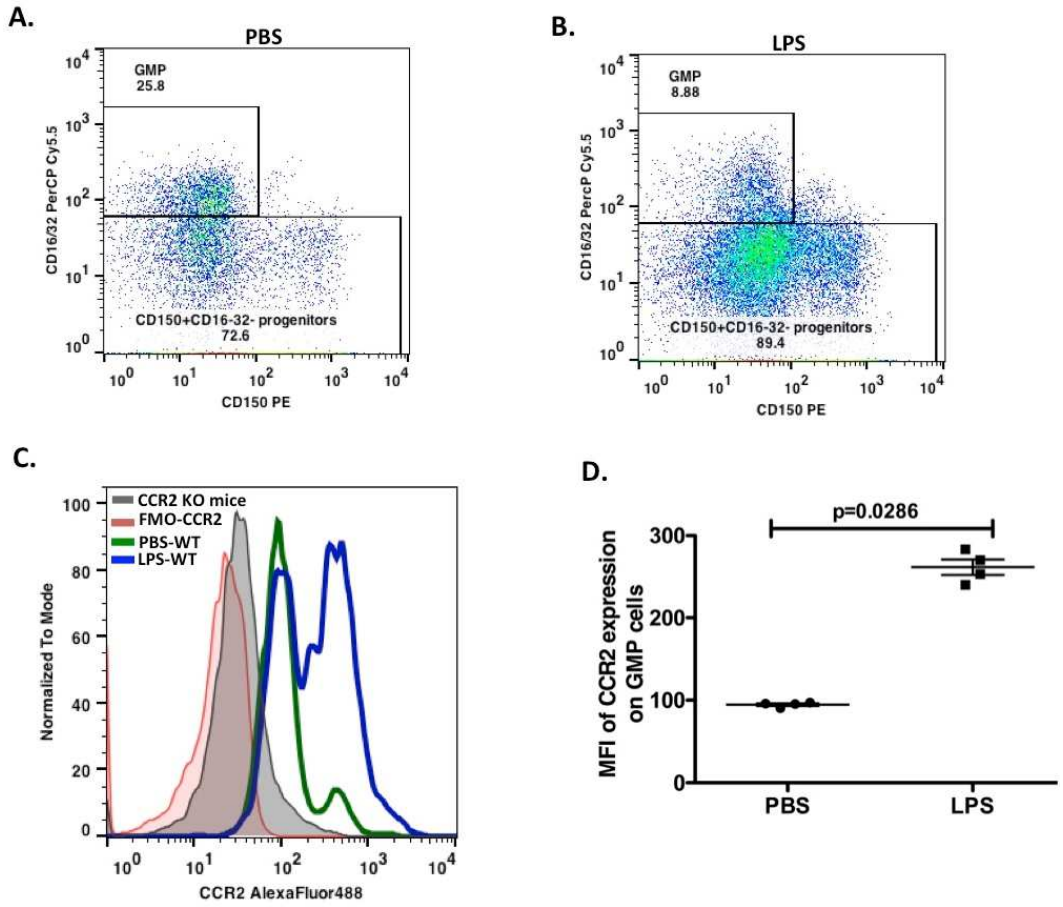



**Figure 5-2 LPS-induced systemic inflammation increases CCR2 expression on GMP cells.** The myeloerythroid progenitor cell, i.e. $Lin^-Kit^+IL\text{-}7R\alpha^-CD41^-CD150^{-/+}$ population from I.P. PBS (A) or I.P. LPS (B), were re-gated for differential expression of CD16/32 and CD150 antigens to identify the GMP cells (i.e. $Lin^-Kit^+IL\text{-}7R\alpha^-CD41^-CD150^-CD16/32^+$ cells) and non-GMP cells (i.e. $Lin^-Kit^+IL\text{-}7R\alpha^-CD41^-CD150^{-/+}CD16/32^-$ cells). C shows the histogram overlay of the CCR2 expression in the GMP cells from the bone marrow of PBS (in green) or LPS (in blue) or CCR2 KO mice or fluorescent-minus-CCR2 antibody controls. D is a scatter plot summarising the comparison of the MFI of the CCR2 expression in the GMP from the bone marrow of I.P. PBS or I.P. LPS treated WT C57BL/6 mice. Statistical significance was calculated by using the 2-tailed, Mann-Whitney U test to compare the MFI of CCR2 expression on bone marrow GMP cells from I.P. PBS and I.P. LPS treated mice. (N=4).

### 5.2.2.3 LPS-induced inflammation significantly upregulates CCR2 expression on PreGM cells.

The non-GMP cells i.e. $CD150^+CD16/32^-$ cells, (see section 5.3.2.2) were re-gated for the differential expression of the CD105 and CD150 antigens to delineate the PreGM, CFU-E, PreCFU-E, and PreMegE populations. Figures 5-3 A & B are the respective representative plots showing the characterisation of these myeloerythroid progenitor population from the bone marrow of WT mice treated with either I.P. PBS or I.P. LPS injection. Each of these myeloerythroid sub-populations was then re-gated for the expression of surface CCR2. Figure 5-3 C is a representative histogram overlay plot for the expression of surface CCR2 in the PreGM cells. This analysis showed that the PreGM cells also express CCR2 that was upregulated by the I.P. LPS injection. This I.P. LPS-induced increase in CCR2

expression was statistically significant when compared to I.P. PBS treated controls (figure 5-3 D, p=0.0286; 2-tailed, Mann-Whitney U test).

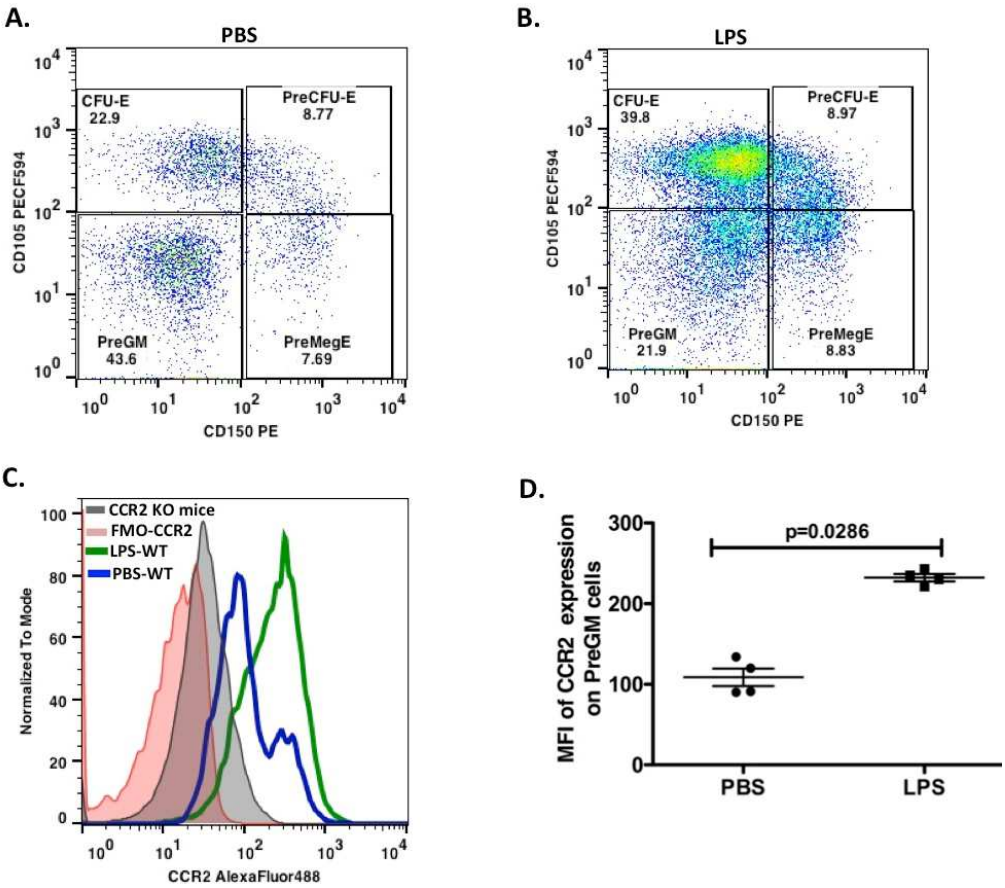



**Figure 5-3 LPS-induced systemic inflammation significantly increases CCR2 expression on PreGM cells.**
The non-GMP cells i.e. $Lin^{-}Kit^{+}IL\text{-}7R\alpha^{-}CD41^{-}CD150^{-/+}CD16/32^{-}$ cells from I.P. PBS or I.P. LPS treated WT C57BL/6 mice were re-gated for differential expression of the CD105 and CD150 antigens to characterise the PreGM, CFU-E, PreCFU-E, and PreMegE populations. A and B are representative plots showing the PreGM, CFU-E, PreCFU-E, and PreMegE subsets from bone marrow of I.P. PBS or I.P. LPS treated mice respectively. C shows the histogram overlay of the CCR2 expression in the PreGM cells from the bone marrow of I.P. PBS (in blue) or I.P. LPS (in green) or CCR2 KO mice or fluorescent-minus-CCR2 antibody controls. D is a scatter plot summarising the comparison of the MFI of the CCR2 expression in the PreGM cells from the bone marrow of I.P. PBS and I.P. LPS treated WT mice. Statistical significance was calculated by using the 2-tailed, Mann-Whitney U test to compare the MFI of PreGM CCR2 expression from I.P. PBS and I.P. LPS treated mice. (N=4).

#### 5.2.2.4 CCR2 expression on CFU-E and PreCFU-E are not significantly modulated by LPS-induced inflammation.

Erythroid-biased progenitors were also analysed for CCR2 expression. However, as shown in figures 5-4 A & B, although the expression of CCR2 could be detected in the CFU-E and PreCFU-E populations, the I.P. LPS injection did not significantly alter CCR2 expression in these populations.

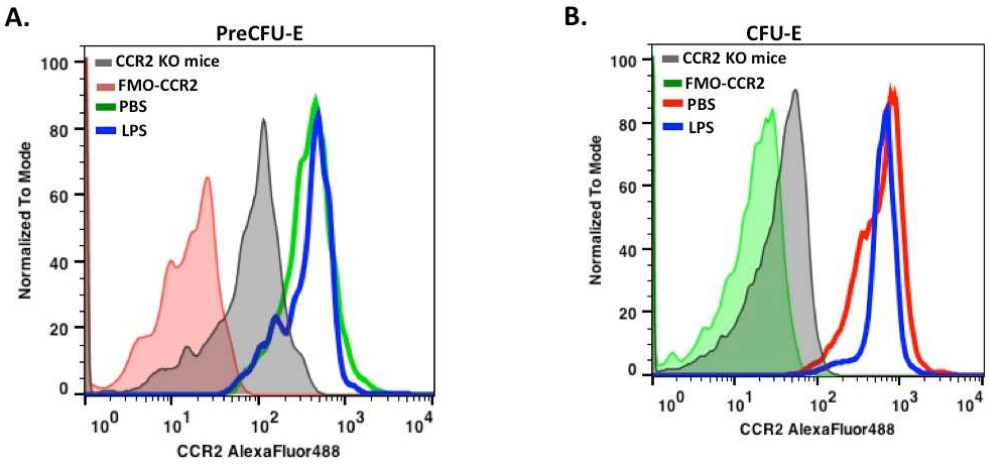



**Figure 5-4 LPS-induced systemic inflammation does not significantly alter CCR2 expression on CFU-E or PreCFU-E.**
The CFU-E and PreCFU-E were also re-analysed for CCR2 expression. A is the representative histogram overlay for the CCR2 expression in PreCFU-E from the bone marrow of WT mice treated with I.P. PBS injection (in green), or I.P. LPS injection (in blue). B is the representative histogram overlay for the CCR2 expression in CFU-E from the bone marrow of WT mice treated with either I.P. PBS injection (in red), or I.P. LPS injection (in blue) application.

### 5.2.2.5 Topical imiquimod cream or TPA treatment significantly increases CCR2 expression on GMPs.

A similar gating strategy described in section 5.2.2.1 was used to select the myeloerythroid progenitor cell population, i.e. $Lin^{-}c\text{-}Kit^{+}IL7R\alpha^{-}CD41^{-}CD150^{+/-}$ cells, from mice treated with topical imiquimod cream or TPA. The representative dot plots in figure 5-5 A shows a histogram overlay illustrating CCR2 expression in bone marrow GMP cells of mice treated with either topical aqueous cream or imiquimod cream; similarly, figure 5-5B is a representative histogram overlay for CCR2 expression in bone marrow GMP cells of mice treated with either acetone or TPA. As summarised in the scatter plot in figure 5-5C, topical imiquimod cream or TPA treatment significantly increased CCR2 expression on GMP cells ($p=0.0286$, 2-tailed Mann-Whitney U test in both cases).

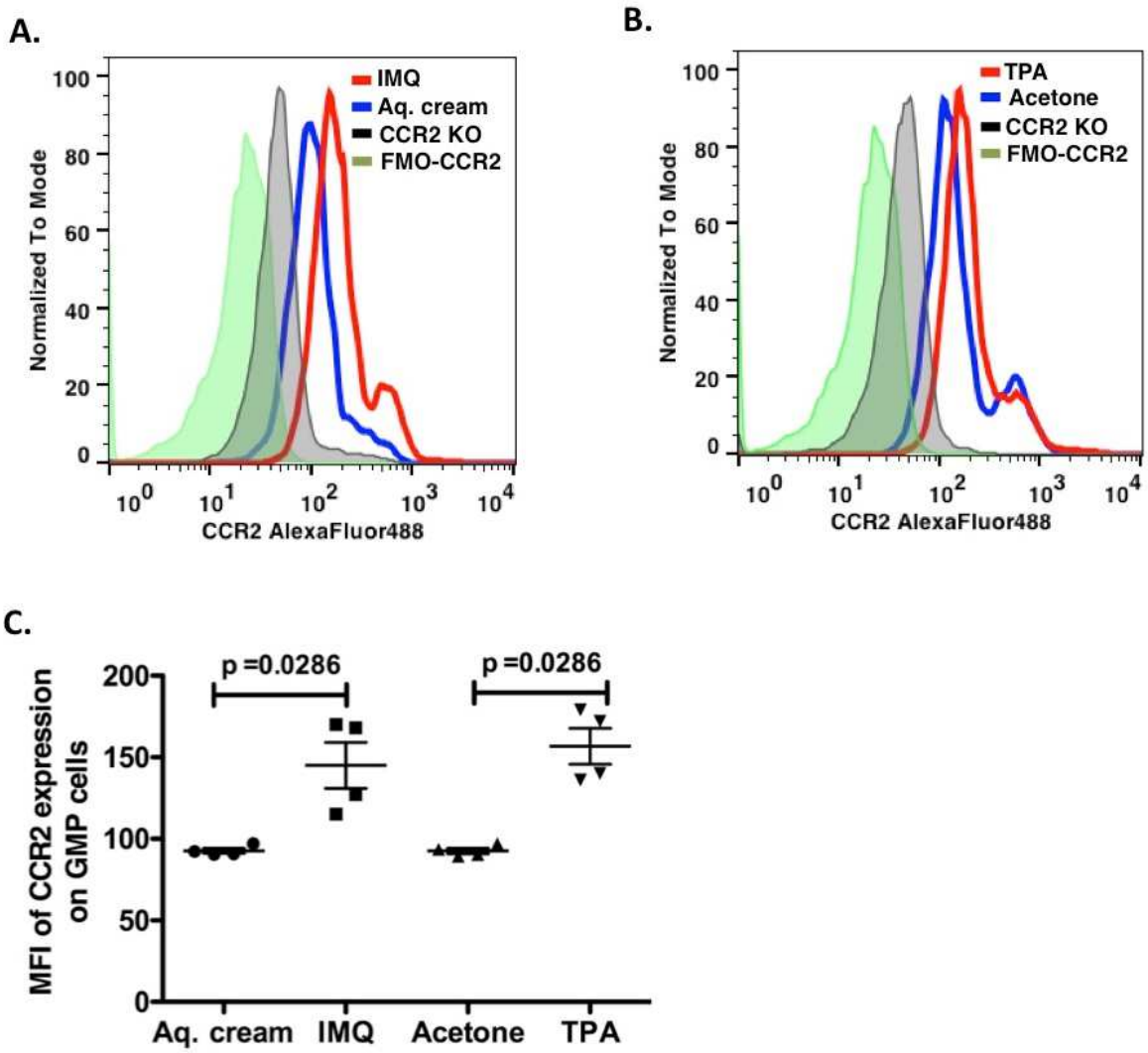



**Figure 5-5 Topical imiquimod cream or TPA treatment significantly increases CCR2 expression on bone marrow GMP cells.**

The myeloerythroid progenitor cells, i.e. $Lin^{-}Kit^{+}IL\text{-}7R\alpha^{-}CD41^{-}CD150^{-/+}$ population from topical aqueous cream treated mice or topical imiquimod cream treated mice (or their respective controls), were re-gated for differential expression of CD16/32 and CD150 antigens to identify the GMP cells (i.e. $Lin^{-}Kit^{+}IL\text{-}7R\alpha^{-}CD41^{-}CD150^{-}CD16/32^{+}$ cells) and non-GMP cells (i.e. $Lin^{-}Kit^{+}IL\text{-}7R\alpha^{-}CD41^{-}CD150^{-/+}CD16/32^{-}$ cells). A shows the histogram overlay of the CCR2 expression on GMP cells from the bone marrow of topical aqueous cream (in blue) or topical imiquimod cream (in red) treated WT mice, or CCR2 KO mice, or cells from WT mice stained with fluorescent-minus-CCR2 antibody control; similarly, figure B is the histogram overlay showing the expression of CCR2 on the GMP cells of mice treated with either acetone or TPA. As summarised in the scatter plot in figure C, both topical imiquimod cream and TPA treatment significantly increase the expression of CCR2 on bone marrow GMP cells [2-tailed, Mann-Whitney U test (N=4)].

### 5.2.2.6 Topical imiquimod cream or TPA treatment significantly increases CCR2 expression on PreGM cells.

The characterisation of bone marrow PreGM, CFU-E, PreCFU-E, and PreMegE populations of mice treated with topical imiquimod cream or TPA (and their respective controls) was exactly as described in section 5.2.2.3. Figure 5-6A is a representative histogram overlay showing the effect of topical imiquimod cream treatment on CCR2 expression in the PreGM population; similarly, figure 5-6B is a representative histogram overlay showing the impact of TPA treatment on

CCR2 expression in the PreGM cells. As summarised in the scatter plot in figure 5-6C, topical imiquimod cream, or TPA, treatment significantly increased the CCR2 expression on the PreGM cells (p=0.0286; 2-tailed Mann-Whitney U test in both cases).

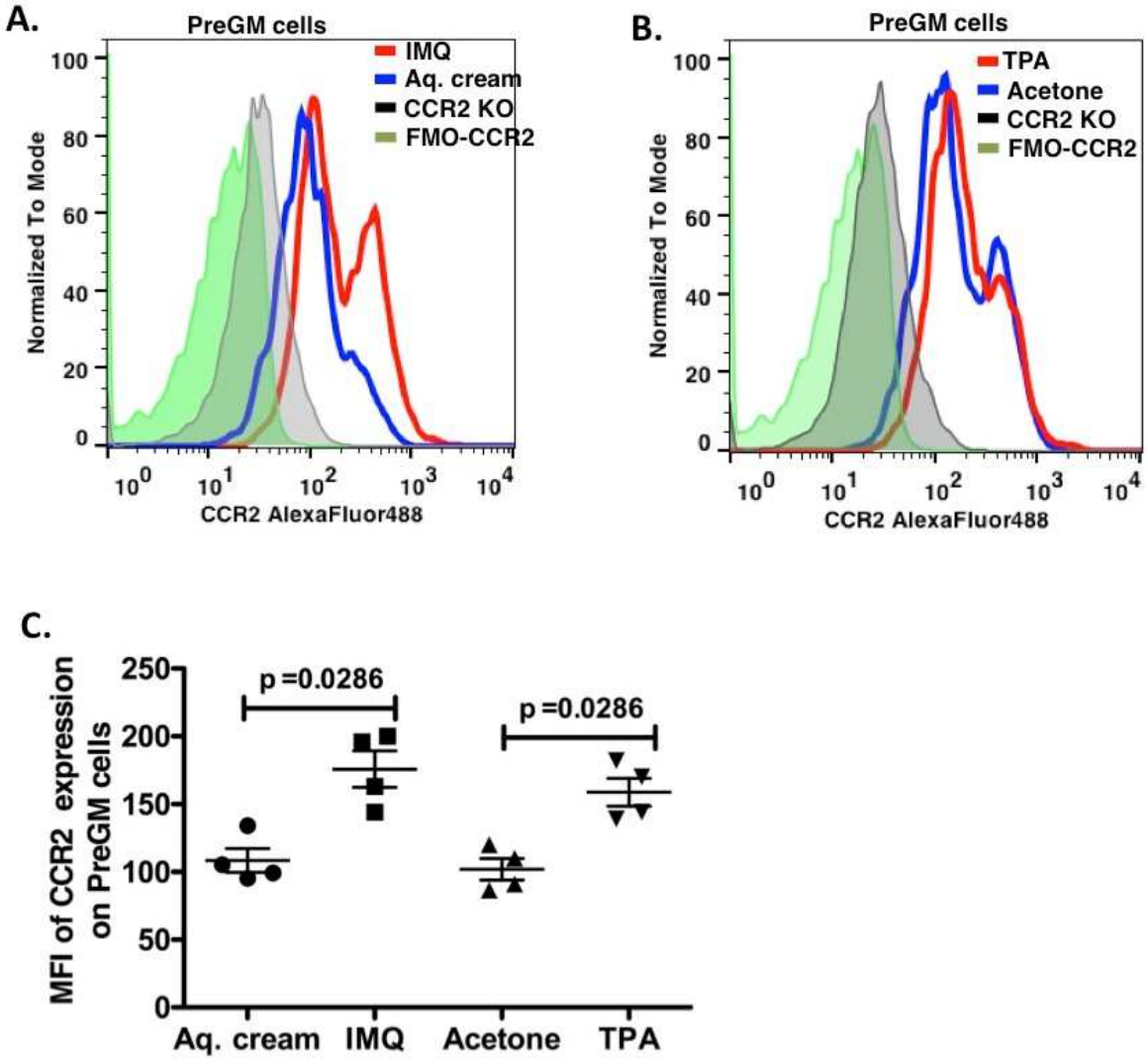



**Figure 5-6 Topical imiquimod cream- or TPA-induced inflammation significantly increases CCR2 expression on PreGM cells.**
The non-GMP cells i.e. $Lin^{-}Kit^{+}IL\text{-}7R\alpha^{-}CD41^{-}CD150^{-/+}CD16/32^{-}$ cells (see section 5.2.2.3 for gating strategy) from topical imiquimod cream/TPA treated WT mice, were re-gated for differential expression of the CD105 and CD150 antigens to delineate the PreGM, CFU-E, PreCFU-E, and PreMegE populations. A shows the histogram overlay illustrating CCR2 expression in the PreGM cells of the bone marrow of topical aqueous cream treated (in blue) or topical imiquimod cream (in red) treated WT mice, or CCR2 KO mice or bone marrow cells from WT mice stained with fluorescent-minus-CCR2 antibody control. Similarly, B is also a histogram overlay showing CCR2 expression on PreGM cells of mice treated with either acetone (in blue) or TPA (in red). C is a scatter plot summarising the impact of topical imiquimod cream or TPA treatment on CCR2 expression in PreGM cells [2-tailed, Mann-Whitney U test in each (N=4)].

#### 5.2.2.7 Topical imiquimod cream or TPA treatment does not significantly alter the CCR2 expression in CFU-E or PreCFU-E cells.

The scatter plots in figures 5-7 A & B respectively summarise CCR2 expression in PreCFU-E and CFU-E in response to either topical imiquimod cream or TPA treatment. Both populations expressed CCR2, although neither imiquimod cream nor TPA treatment significantly altered the receptor expression in these populations.

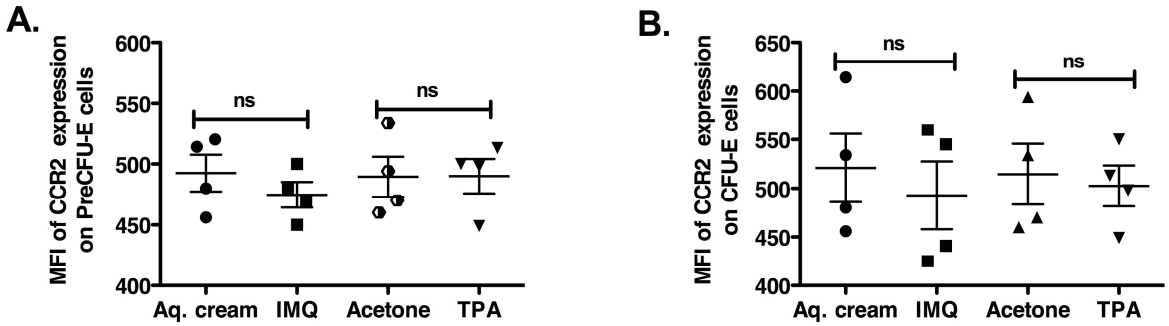



**Figure 5-7 Topical imiquimod cream-induced inflammation does not alter CCR2 expression in CFU-E or PreCFU-E.**
The CFU-E and PreCFU-E of mice treated with either topical imiquimod cream, or TPA, were also re-analysed for CCR2 expression. A is a scatter plot summarising the impact of topical imiquimod cream or TPA treatment on CCR2 expression in PreCFU-E cells. B also summarises the impacts of topical imiquimod cream or TPA treatment on CCR2 expression on CFU-E cells. All statistical significances were calculated using the 2-tailed Mann-Whitney U test (n=4).

Taken together, these data suggest that GMP and PreGM cells significantly increase their expression of CCR2 in response to inflammation.

## 5.2.3 Does increased CCR2 expression affect migration of HSPC towards an *in vitro* gradient of CCL2?

### 5.2.3.1 Introduction

After establishing that the HSPC, particularly the GMP and the preGMP populations, inducibly express increased surface CCR2 in response to peripheral or systemic inflammation, I next sought to understand the functional relevance of CCR2 in the HSPC. To do this, I employed *in vitro* chemotaxis assays, using the 5.0μm pore size polycarbonate membrane Transwell® chamber (Corning Incorporated, USA), to test the ability of the HSPC to migrate towards CCL2. Mice received systemic LPS injection or topical imiquimod cream/TPA treatment exactly as described in the previous chapters. Femur, tibia as well as hipbones were harvested from these mice, crushed in a mortar and pestle to isolate the bone marrow cells, which were then enriched for HSPC using the c-Kit

Microbeads and LS magnetic columns (see previous chapters for details). The cells were then stained for lineage marker cocktail (CD4, CD5, CD8a, CD11b, B220, Ter119 and Gr-1), c-Kit and Live/dead aqua antibodies. After excluding dead cells and debris, cells negative for markers for differentiated haematopoietic lineage cells (Lin-), but positive for the c-Kit antigen (Kit+), henceforth called LK cells, were FACS sorted on a FACSAria II into chemotaxis buffer (IMDM containing 1% BSA). The LK cells were sorted, instead of the LSK cells, because of the sensitivity of Sca-1 expression to TLR-driven inflammation as reported in the previous chapters.

#### 5.2.3.2 Set up of the CCL2 chemotaxis assay

All the chambers that included CCL2 are henceforth, called +CCL2 chamber, whereas the chambers that excluded CCL2 are called –CCL2 chamber. The concentration of CCL2 (20ng/mL) used for the chemotaxis assay, i.e. in the lower Transwell® chamber, was in accordance with a previous publication (Si et al., 2010) who also used LK cells for their chemotaxis assay to study the functional relevance of CCR2 in HSPC. 2 x $10^5$ LK cells were added to the upper Transwell® chamber, and incubated for 3-5 hours at 37°C in a humidified incubator in the presence of 5% $CO_2$ plus CCL2 in the lower chamber (i.e. +CCL2 chamber). For each treatment group, other chambers were also set up with $2x10^5$ LK cells in the upper chamber but with no CCL2 in the lower chamber (i.e. -CCL2 chamber) to account for spontaneous cellular migration. After the incubation period, 400μL of the medium in the lower chamber, containing LK cells that had migrated in response to the CCL2, were seeded into 3mL of Methocult™ GF M3434 medium for duplicate 1.1mL cultures in 35mm plates. The plates were incubated and haematopoietic progenitor colonies enumerated exactly as described in the previous chapters.

#### 5.2.3.3 Haematopoietic progenitor cells from I.P. LPS or topical imiquimod/TPA treated mice have enhanced *in vitro* migration towards CCL2.

As summarised in **figure 5-8**, LK cells from naïve WT mice (i.e. no treatment) migrated towards CCL2 suggesting the presence of functional CCR2 on these cells ($p<0.0001$; 2-tailed paired t-test). LK cells from LPS treated mice also migrated in significant numbers in response to CCL2 compared to the –CCL2 control

(p=0.0015; 2-tailed t-test). In addition, significantly higher numbers of LK cells from topical imiquimod cream treated mice migrated towards CCL2 (p<0.0001; 2-tailed paired t-test). Moreover, significantly higher numbers of haematopoietic progenitors from topical TPA treated mice also migrated in response to the CCL2 (p=0.0001; 2-tailed paired t-test). Furthermore, when topical imiquimod cream and TPA treatment groups were compared, significantly higher numbers of LK cells from TPA treated mice migrated towards CCL2 than LK cells from imiquimod cream treated mice (p<0.0001; 2-tailed unpaired t-test).

Taken together, these data argue strongly that the CCR2 expression in the HSPC has functional relevance.

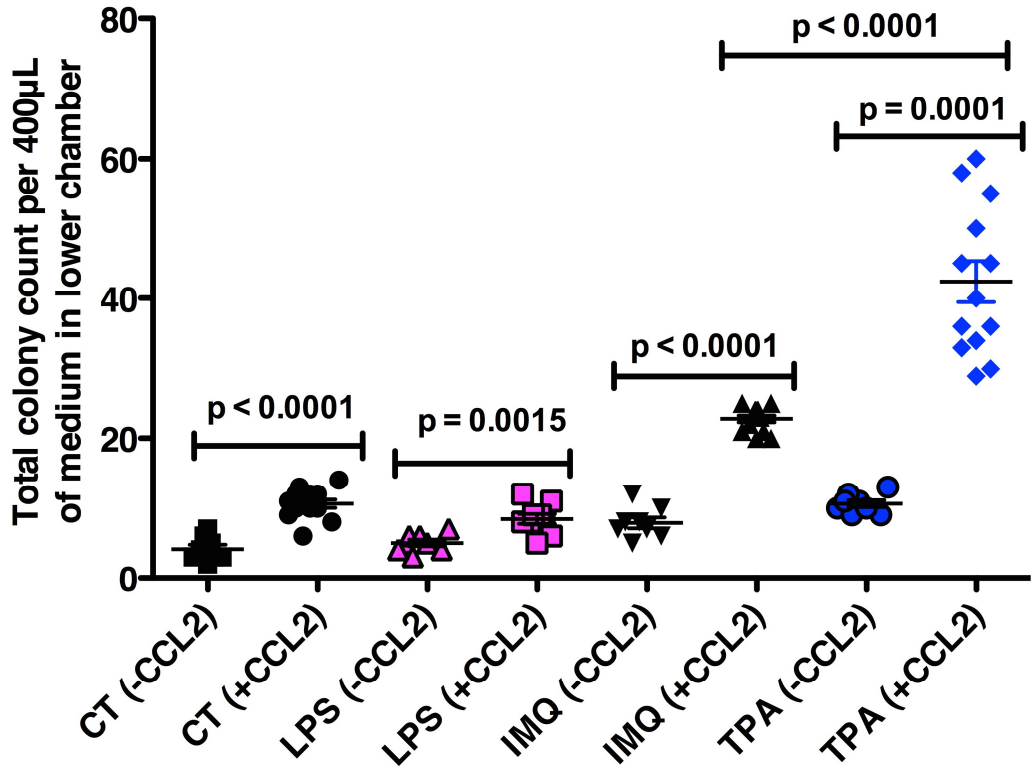



**Figure 5-8 Enhanced in vitro chemotaxis of haematopoietic progenitors, from I.P. LPS or topical imiquimod cream/TPA treated mice, towards CCL2.**
C57BL/6 mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day three of the model, other groups of mice received a single I.P. injection of 100µg LPS or 100µL I.P. PBS as a control. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and bone marrow [2x (tibia + femur + hip bones)] harvested, and enriched for haematopoietic progenitor cells using the CD117 (c-Kit) Microbeads and LS magnetic columns according to manufacturer instructions (Miltenyi Biotec, UK). The HSPC-enriched cells were then stained with lineage antibody cocktail (CD4, CD5, CD8a, Mac-1, B220, Ter119 and Gr-1), c-Kit, and Live/Dead Aqua antibodies. Debris and dead cells were excluded using forward-side scatter characteristics, doublet exclusion, and Live/Dead Aqua fixable dye retention. Next, cells positive for markers of terminal differentiation of haematopoietic cells were also gated out, and $2x10^5$ LK cells were FACS sorted into chemotaxis

buffer. The LK cells were washed once, and were subsequently seeded to the upper Transwell® chamber of 5μm pore size, with 20ng/mL CCL2 in the lower chamber and incubated for 3-5 hours. After the incubation period, 400μL of the chemotaxis buffer in the lower Transwell® chamber was subsequently seeded into 3mL of Methocult™ GF M3434 for duplicate 1.1mL culture for 7-10 days at 37°C in a 5% $CO_2$ incubator. Total colonies were counted using an inverted microscope, and statistical significance calculated using the 2-tailed paired t-test in all cases except the comparison between topical imiquimod cream and TPA LK cells where 2-tailed unpaired t-test was used. (N=3-4; 2-independent experiments pooled together).

In summary, the following findings should be noted from the data presented in this section:

(A) Myeloerythroid progenitors express functional CCR2.

(B) CCR2 expression particularly on GMP and PreGM cells is upregulated in response to inflammatory stimuli.

## 5.3 Does disruption of the CCR2/CCL2 axis disturb the inflammation-induced HSPC mobilisation?

### 5.3.1 Introduction

The data presented thus far show that myeloerythroid progenitors express CCR2 that is increased in response to inflammation. Having demonstrated the function of the increased CCR2 expression in *in vitro* chemotaxis assays, it was deemed important to also confirm the functional relevance of the CCR2/CCL2 axis in *in vivo* settings. In this section of the study, I hypothesised that the CCR2/CCL2 axis was critical in the inflammation-induced HSPC mobilisation and therefore its disruption would abrogate the inflammation-orchestrated HSPC mobilisation. To test this hypothesis, I sought to address the following questions:

(1) How does systemic or peripheral inflammation affect plasma CCL2 concentration?

(2) Does the disruption of the CCR2/CCL2 axis, i.e. in CCR2 KO mice, or by CCL2 neutralisation in WT mice, abrogate the systemic or peripheral inflammation-induced HSPC mobilisation?

### 5.3.2 How does LPS injection or topical imiquimod cream/TPA-induced peripheral inflammation affect plasma CCL2 concentration?

The proteome array data presented in the previous chapters suggested that the systemic or peripheral inflammation models led to an increase in the plasma CCL2 levels when compared to their respective controls. However, the nature of the array data precluded quantitative protein measurements. In line with the hypothesis that the CCR2/CCL2 axis might play a role in the inflammation orchestrated HSPC mobilisation, I sought to further validate the CCL2 array data by quantitatively measuring the plasma CCL2 concentration using ELISA. A mouse CCL2 ELISA kit was purchased from EBiosciences (Hatfield, UK) and used to measure the plasma CCL2 concentration in accordance with the manufacturer's specifications. WT mice received systemic LPS injection, or imiquimod cream/TPA treatment, exactly as described in the previous chapters. Peripheral blood was harvested from these mice into EDTA microtainer tubes (BD Biosciences). The blood was spun at 10,000xg for 10 minutes, after which the plasma was harvested and used for the CCL2 ELISA measurements. The results of the plasma CCL2 quantification by ELISA are shown in **figure 5-9**.

The CCL2 calibration curve (**figure 5-9 A**) established a good fit ($R^2$=0.995) for the relationship between the CCL2 concentration and the absorbance or optical density of analytes. In addition, it also helped establish the detection limits of the ELISA assay. In agreement with the proteome array data presented in the previous chapters (see chapters 3 & 4), topical imiquimod cream or topical TPA application-induced peripheral inflammation each caused a statistically significant increase in the concentration of CCL2 in the plasma (figure 5-9B; p=0.0079, 2-tailed, Mann-Whitney U test in each case). In addition, LPS-induced systemic inflammation also significantly increased the concentration of circulating CCL2 (figure 5-9B; p=0.0079; 2-tailed Mann-Whitney U test).

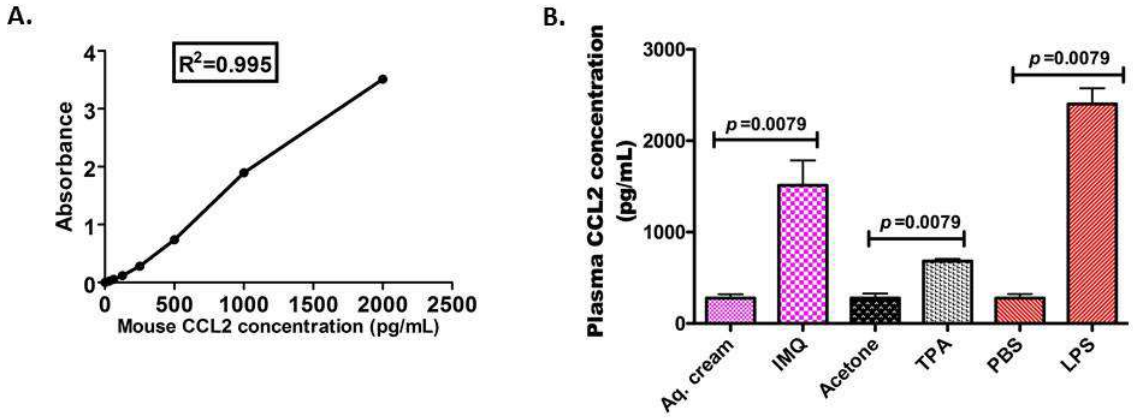



**Figure 5-9 Peripheral and systemic inflammations significantly increased plasma CCL2 concentration.**
C57BL/6 mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100μM in 100μL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day three of the model, other groups of mice received a single I.P. injection 100μL LPS or 100μL I.P. PBS injection as a control. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and peripheral blood was taken into EDTA anti-coagulation tubes, spun at >10,000xg for 10 minutes and plasma harvested for ELISA. Figure A is the calibration curve showing a good fit ($R^2$=0.995) for the concentration of CCL2 and absorbance (optical density) and the detection limit of the assay. Figure B summarises the measured CCL2 concentration in the plasma of imiquimod cream (or aqueous cream) or TPA (or acetone) treated mice or LPS (or PBS controls) treated mice. Statistical significance was estimated for each treatment by using the 2-tailed, Mann-Whitney U test to compare the CCL2 concentration in the plasma of topical imiquimod cream or topical TPA treated mice or I.P. LPS treated mice with their respective controls. (N=5).

## 5.3.3 Does disruption of the CCR2/CCL2 axis abrogate the systemic or peripheral inflammation-induced HSPC mobilisation?

This question was addressed in two parts: firstly, by repeating the inflammation-induced HSPC mobilisation models using CCR2 KO mice, and secondly, by neutralisation of CCL2 using anti-CCL2 antibodies in WT mice.

### 5.3.3.1 Topical imiquimod cream application is unable to induce HSPC mobilisation in CCR2 KO mice.

To begin to assess how disruption of the CCR2/CCL2 axis impacted the inflammation induced HSPC mobilisation, $CCR2^{-/-}$ KO mice were used. The caveat with the use of these mice is that these mice carry a complete CCR2 gene deletion; hence both haematopoietic and non-haematopoietic cells carry this deletion. Thus, the findings presented below should be interpreted in the context that the observations may be directly due to the absence of CCR2 in the haematopoietic cells, and/ or, an indirect effect due to the absence of CCR2 in the non-haematopoietic cells. Besides, others have also demonstrated

phenotypes such as monocytopenia and defective monocyte/macrophage recruitment in these mice (Boring et al., 1997).

CCR2 KO mice received systemic LPS injection, or imiquimod cream/TPA treatment, exactly as described in the previous chapters. Peripheral blood was harvested from these mice to measure HSPC numbers using Methocult™ GF M3434 exactly as described in the previous chapters. After the incubation period, total colonies, representing haematopoietic progenitors mobilised in response to the various treatments, were enumerated using an inverted microscope. Figure 5-10 is a representative scatter plot summarising the total colonies counted from such cultures.

In agreement with the data presented for WT mice in the previous chapters, I.P. LPS injection significantly mobilised increased haematopoietic progenitors to the circulation in CCR2 KO mice, in comparison to the I.P. PBS treated groups (figure 5-10, $p < 0.0001$; 2-tailed unpaired t-test). However, in direct contrast to the data presented for WT mice (see previous chapter), topical imiquimod cream application failed to mobilise increased numbers of haematopoietic progenitors into the circulation in CCR2 KO mice. Topical TPA application also induced significant mobilisation of increased haematopoietic progenitors into the circulation in CCR2 KO mice ($p < 0.0001$; 2-tailed unpaired t-test).

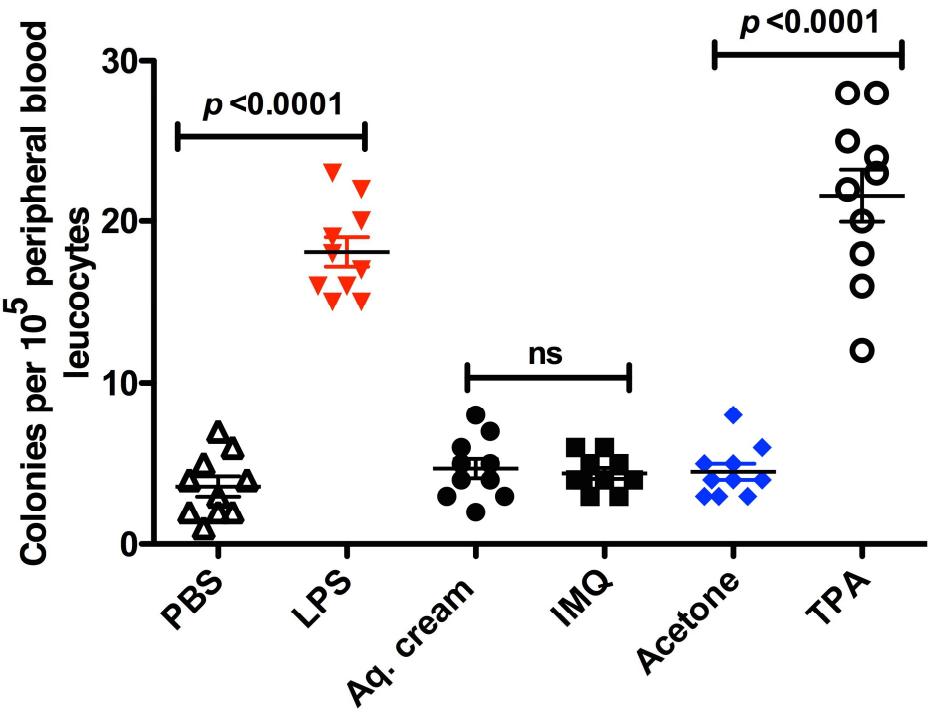



**Figure 5-10 Topical imiquimod cream application is unable to mobilise HPC in CCR2 KO mice.**
CCR2 KO mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day three of the model, other groups of mice received a single I.P. injection of 100µL LPS or 100µL I.P. PBS injection as a control. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and peripheral blood was harvested into EDTA anti-coagulation tubes. After lysing red blood cells with Ammonium chloride solution, $1x10^5$ peripheral blood leucocytes were seeded into 1.1mL of Methocult™ GF M3434 in duplicates, and incubated for 7-10 days at 37°C in a humidified incubator containing 5% $CO_2$. After the incubation period, the total haematopoietic progenitor colonies were counted using an inverted microscope. Statistical significance was calculated using the unpaired t-test to compare the total colony count of each treatment group with its respective controls. [N=5; (3 independent experiments)].

Taken together, these data suggest that CCR2 may play an important role in HSPC mobilisation observed with the topical imiquimod cream application model. However, the HSPC mobilisation observed with either systemic LPS injection or topical TPA application would appear not to have any major dependence on the presence of CCR2.

### 5.3.4 Does *in vivo* CCL2 neutralisation abrogate imiquimod cream-induced HSPC mobilisation in WT mice?

The data presented thus far suggest that CCR2 may play a role in the topical imiquimod cream induced HSPC mobilisation. However, as stated earlier, the CCR2 KO mouse used in this assay was a complete gene knock-out in which the expression of CCR2 was absent in all tissues. To further clarify the role of the

CCR2/CCL2 axis in topical imiquimod cream-induced HSPC mobilisation in WT mice, *in vivo* CCL2 neutralisation with anti-CCL2 antibodies was carried out.

To do this, WT mice received a single, daily topical application of 62.5mg imiquimod cream application for 3 consecutive days. However, on days 1 and 3 during the model, one group received intravenous (I.V.) injection of 15μg anti-mouse polyclonal CCL2 antibody (R&D Systems, Abingdon, UK) or I.V. injection of an equivalent amount of polyclonal goat IgG (R&D Systems, Abingdon, UK) as isotype-matched control. The dose of the anti-CCL2 neutralising antibody used was in agreement with previously published data (Fujimoto et al., 2009). On day 4 of the model, mice were euthanized by $CO_2$ asphyxiation and $1x10^5$ peripheral blood leucocytes were cultured in Methocult$^{TM}$ GF M3434 medium exactly as described in the previous chapters.

The scatter plot in figure 5-11 summarises the impact of CCL2 neutralisation on the topical imiquimod cream treatment-induced HSPC mobilisation. The comparison of the total haematopoietic colony counts showed that the neutralising anti-CCL2 antibody significantly reduced the number of haematopoietic progenitor cells mobilised to peripheral blood in WT mice compared to isotype-matched controls ($p<0.0001$, One-way ANOVA, with Tukey's post-test). These data thus show that in vivo neutralisation of CCL2 in WT mice recapitulates the defective HSPC mobilisation seen in CCR2-/- mice in response to topical imiquimod cream treatment.

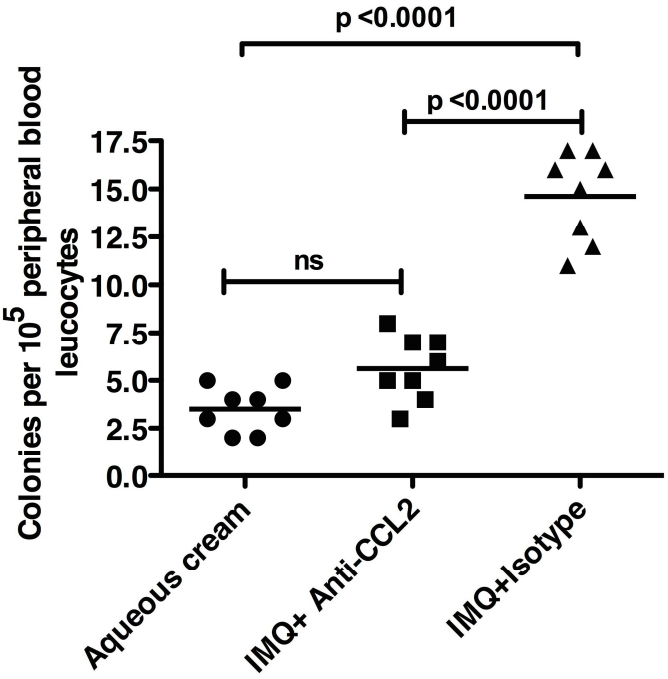



**Figure 5-11 Anti-CCL2 neutralising antibody significantly reduces the HPC mobilised in response to topical imiquimod cream application.**
Two groups of C57BL/6 mice each received a single, daily application of topical 62.5mg imiquimod cream on the shaved dorsal skin for 3 consecutive days; of these, one group received simultaneous intravenous injection of anti-mouse CCL2 antibody, on days 1 and 3 of the model, whilst the other group received an equivalent volume of isotype-matched control. Another group of mice received topical aqueous cream application to provide estimate of peripheral blood basal HPC colony count. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and peripheral blood was harvested into EDTA microtainer tubes. After lysing red blood cells with Ammonium chloride solution, $1x10^5$ peripheral blood leucocytes were seeded into 1.1mL of Methocult$^{TM}$ GF M3434 in duplicate, and incubated for 7-10 days at 37°C in a humidified incubator containing 5% $CO_2$. After the incubation period, the total haematopoietic colonies were counted using an inverted microscope. Statistical significance was calculated using the One-Way ANOVA with Tukey multiple comparison to compare all treatment groups. [N = 4; 2-independent experiments)].

# 5.4 How does the peripheral inflammation model in CCR2 KO mice compare to WT mice?

## 5.4.1 Introduction

After establishing that topical imiquimod cream failed to mobilise HSPC in CCR2 KO mice, I next sought to investigate whether there were major differences in the cutaneous responses of WT mice and CCR2 KO mice to the peripheral inflammation models that might explain this phenomenon. The main questions I sought to answer in this part of the study were:

(A) How different are the responses of the dorsal skins of WT and CCR2 KO mice to the imiquimod cream or TPA treatment?

(B) Which chemokines and cytokines were released into the circulation of CCR2 KO mice in response to topical imiquimod cream treatment?

In this way I sought to gain further insight into what could have been the underlying cause of the differences in response to topical imiquimod cream application in WT and CCR2 KO mice.

## 5.4.2 How different are the responses of the dorsal skins of WT and CCR2 KO mice to the imiquimod cream treatment?

### 5.4.2.1 Introduction

Having found that topical imiquimod cream application was able to induce HSPC mobilisation in WT mice, but not in CCR2 KO mice, I next sought to understand the differential response to the topical imiquimod cream application in the two mouse strains. Firstly, as the imiquimod cream and TPA were directly applied to the dorsal skin, it was interesting to verify whether the dorsal skin of both mouse strains responded similarly to the peripheral inflammation-inducing agents. To do this, haematoxylin and eosin staining was employed to examine the morphology of the imiquimod cream/TPA treated skin. Although the primary focus in this section of the study was topical imiquimod cream treatment, TPA treatment model was also included as a comparator.

WT mice or CCR2 KO mice received the imiquimod cream/TPA (or controls) application to shaved dorsal skin exactly as described in the previous chapters. The dorsal skin was then harvested into 10% neutral buffered formalin (10%NBF) and kept overnight at 4°C. The skin was next processed and embedded in paraffin (see materials and methods for details). Blocks were sectioned to 8μm, and then stained using haematoxylin and eosin. To enable a direct comparison of the effect of the peripheral inflammation-inducing agents on the dorsal skins of WT and CCR2 KO mice, the thickness of the epidermis was measured in each mouse strain as a quantitative index of the inflammatory response.

#### 5.4.2.2 Topical imiquimod cream application induces a significantly greater inflammatory response in the dorsal skin of WT compared to CCR2 KO mice

Figures 5-12 A & B are the respective representative photomicrographs of the dorsal skins from topical aqueous cream (A) or imiquimod, IMQ cream (B) treated mice from WT mice; figures 5-12 C & D are also the respective representative micrographs of the dorsal skins from topical aqueous cream (C) or imiquimod cream (D) treated CCR2 KO mice. By comparing A & B, it was apparent that the topical imiquimod cream induced a severe inflammatory response in the dorsal skin as evidenced by the thickened epidermis in B compared to A. However, compared to the aqueous cream treated control (figure 5-12 C), topical imiquimod cream application (figure 5-12 D) induced only a moderate inflammatory response in the dorsal skin of the CCR2 KO mice. When the epidermal thickness was measured as a quantitative index of the inflammatory response (figure 5-12E), it was apparent that the topical imiquimod cream application induced a significant inflammatory response in the dorsal skin of WT mice compared to aqueous cream treated controls ($p<0.0001$; 2-tailed unpaired t-test). Also, the topical imiquimod cream application induced a significant inflammatory response in the dorsal skin of CCR2 KO mice when compared to aqueous cream treated controls ($p<0.0001$; 2-tailed unpaired t-test), although this was significantly less than that seen in WT mice ($p=0.0001$; 2-tailed unpaired t-test). This suggests that the CCR2 KO mice responded less well to the topical imiquimod cream treatment.

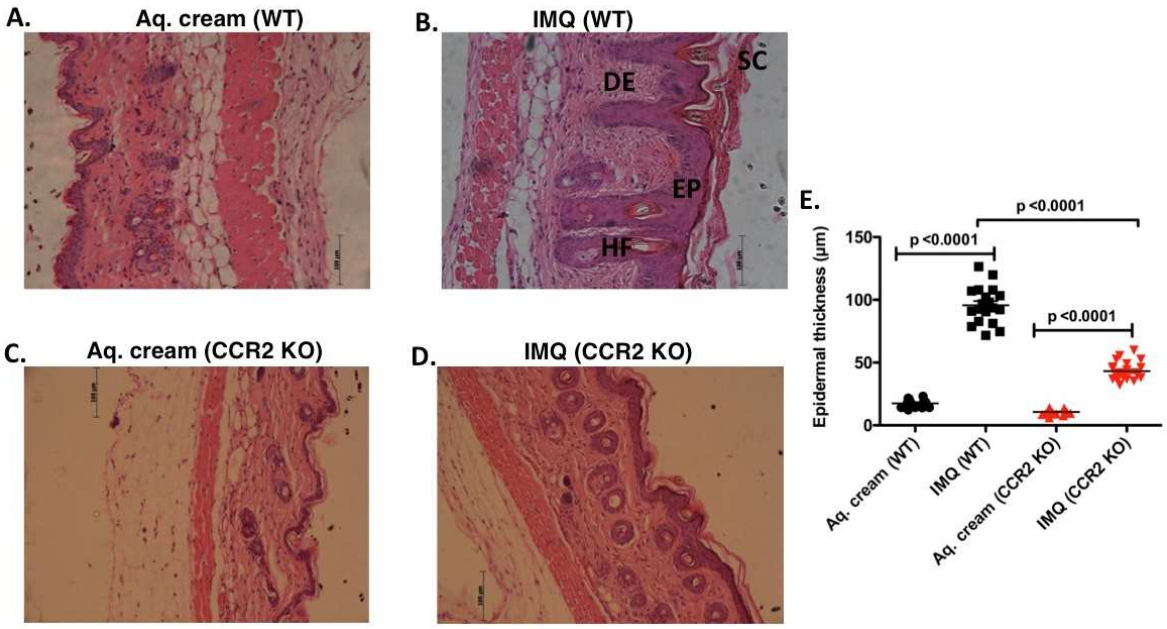



**Figure 5-12 CCR2 KO mice have reduced cutaneous inflammation in response to topical imiquimod cream application.**
WT mice or CCR2 KO mice received a single, daily dose of topical imiquimod cream (62.5mg), or equivalent amount of topical aqueous cream application, to the shaved dorsal skin for three consecutive days. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and the dorsal skins that directly received the topical imiquimod cream were harvested into 10% neutral buffered formalin. 24-hours later, the skin tissues were paraffin embedded in a tissue processor, sectioned to 8µm and stained with haematoxylin and eosin for morphological examination. A and B are respective representative micrographs of dorsal skin of WT mice treated with either aqueous cream or topical imiquimod cream (20X magnification). C and D are the micrographs of the dorsal skin of CCR2 KO mice treated with either topical aqueous cream or topical imiquimod cream (20X magnification). In E, the epidermal thickness was measured using AxioVision software on Zeiss Axiostar Plus Microscope (Carl Zeiss, Welwyn, Garden City, UK) and compared between treatment groups, in the respective strains, by the unpaired t-test. [(N=8), DE, dermis; EP, epidermis; HF, hair follicle; SC, stratum corneum].

### 5.4.2.3 Topical TPA application induces comparable inflammatory response in both WT and CCR2 KO mice

Figures 5-13 A & B are the respective representative photomicrographs of the dorsal skins from either topical acetone (A) or TPA (B) treated skin from WT mice; figure 5-13 C & D are also the respective representative micrographs of the dorsal skins from either topical acetone (C) or TPA (D) treated CCR2 KO mice. By comparing A & B, it was apparent that the topical TPA application induced a severe inflammatory response in the dorsal skin of WT mice as evidenced by the thickened epidermis in B, compared to A. Also, compared to the acetone treated control (figure 5-13 C), the topical TPA application (figure 5-13 D) induced a severe inflammatory response in the dorsal skin of the CCR2 KO mice. When the epidermal thickness was measured as a quantitative index of the inflammatory response (figure 5-13E), it was apparent that the topical TPA application induced a significant inflammatory response in the dorsal skin of WT mice ($p<0.0001$; 2-tailed unpaired t-test). The topical TPA application also

induced a significant inflammatory response in the dorsal skin of CCR2 KO mice ($p<0.0001$; 2-tailed unpaired t-test). Unlike the topical imiquimod cream model in which there was a significant difference in the inflammatory response between the WT and CCR2 KO mice, the topical TPA application induced comparable inflammatory responses in the two strains of mice.

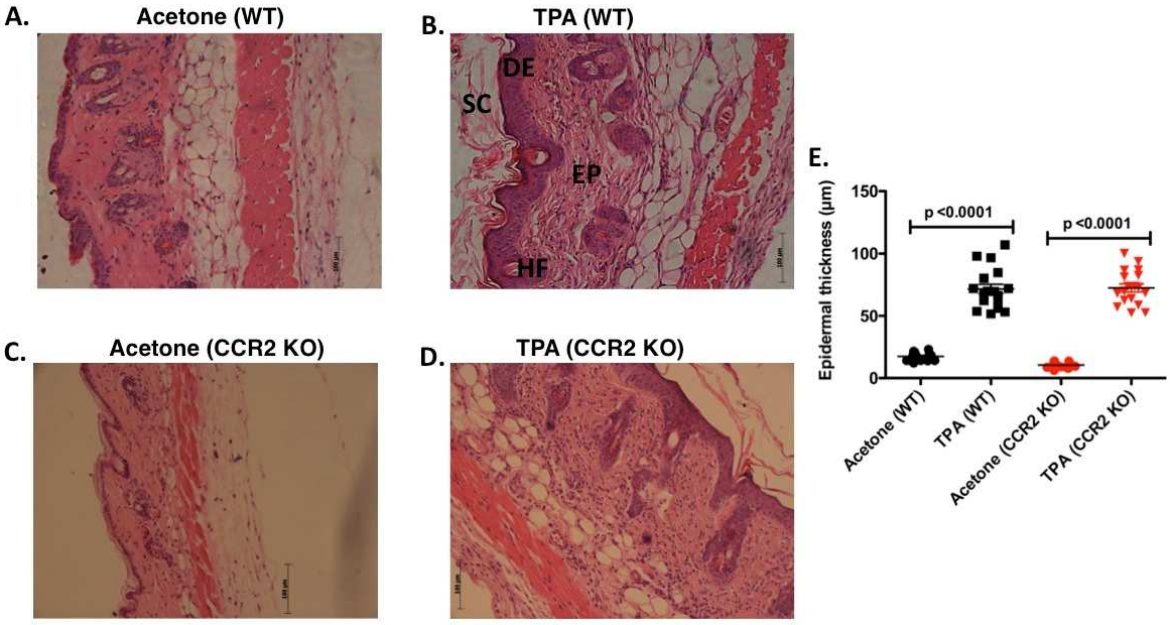



**Figure 5-13 Topical TPA application induces comparable inflammatory responses in the epidermis of WT and CCR2 KO mice.**
WT mice or CCR2 KO mice received a single, daily dose of topical TPA (100µM in 100µL acetone), or topical application of 100µL acetone as a control, to the shaved dorsal skin for three consecutive days. On day 4, the mice were euthanized by $CO_2$ asphyxiation, and the dorsal skins that directly received the topical acetone or TPA application were harvested into 10% neutral buffered formalin. 24-hours later, the skin tissues were paraffin embedded in a tissue processor, sectioned to 8µm and stained with haematoxylin and eosin for morphological examination. A and B are the respective representative micrographs from dorsal skin of WT mice treated with either topical acetone or topical TPA (20X magnification). C and D are the respective micrographs from the dorsal skin of CCR2 KO mice treated with either topical acetone or topical TPA (20X magnification). In E, the epidermal thickness was measured using AxioVision software on Zeiss Axiostar Plus Microscope (Carl Zeiss, Welwyn, Garden City, UK) and compared between treatment groups, in the respective strains, by the unpaired t-test. [(N=8), DE, dermis; EP, epidermis; HF, hair follicle; SC, stratum corneum].

### 5.4.3 Which chemokines and cytokines are released into the circulation in response to topical imiquimod cream treatment?

In the previous chapter, the plasma of mice treated with topical imiquimod cream was profiled for circulating cytokines and chemokines using the mouse cytokine array panel A (ARY006; R&D Systems). This same array kit was employed to characterise the plasma of CCR2 KO mice treated with the topical imiquimod cream model (see previous chapter for details). The objective was to compare the inflammatory proteome of the CCR2 KO mice with that of WT mice

to verify whether the differential response of the dorsal skin to the topical imiquimod cream was also reflected in the inflammatory mediators in the plasma. Ultimately, the aim was to probe for any candidate molecule(s) that could be explored as a potential ligand involved in the HSPC mobilisation seen in the topical imiquimod cream model in WT mice. The principle of the mouse cytokine array kit and the inflammatory mediators they detect, have been described in previous chapters. Just as explained in the previous chapters, 150µL of plasma, pooled from three mice, was used for the proteome array in order to obtain representative data.

#### 5.4.3.1 Quantifying the inflammatory mediators detected by the cytokine array.

Figures 5-14 A & B show scanned images of the array blots from the topical aqueous cream and imiquimod cream treated CCR2 KO mice respectively.

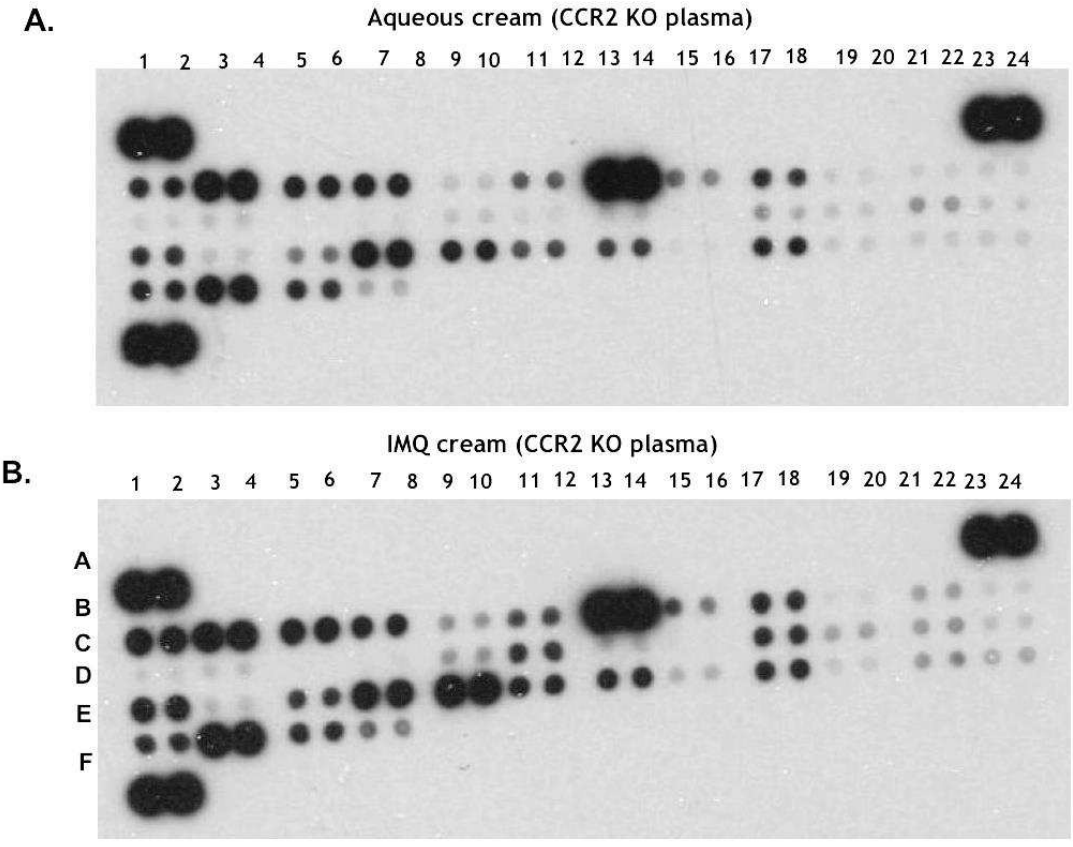



**Figure 5-14 Array blots of plasma proteome detected by mouse cytokine proteome array**
A single, daily dose of topical imiquimod cream [IMQ (62.5mg)] or equivalent amount of aqueous cream was applied to the shaved dorsal skin of CCR2 KO mice for three consecutive days. On the fourth day, mice were euthanized by $CO_2$ asphyxiation, and plasma was taken from the respective mice for cytokine proteome assay. In order to obtain a representative proteome profile for each treatment group, plasma from three mice was pooled together for each treatment group (i.e. topical imiquimod cream or aqueous cream). 150µL of the respective plasma samples was used for the proteome profiling using the Mouse cytokine array panel A kit (R&D systems). A & B are the

respective scanned images of the blots of the plasma from aqueous cream or imiquimod cream treated CCR2 KO mice.

As explained previously, the signal intensities were estimated with ImageJ 1.48 (NIH, USA) software. The fold changes in analyte expression levels were calculated using similar assumptions as outlined in the previous chapters. Only analytes demonstrating fold changes of 2 or more were considered to be sufficiently above background to merit further consideration.

#### 5.4.3.2 Estimating the plasma chemokines in CCR2 KO mice in response to topical imiquimod application.

Figure 5-15 summarises the topical imiquimod cream-induced modulations in production of both CC- and CXC-chemokines in the plasma of CCR2 KO mice. For the CC-chemokines, the expression levels of CCL1, CCL2, CCL3 and CCL5 were all increased in the plasma of CCR2 KO mice compared to aqueous cream treated controls. Specifically, topical imiquimod cream application led to 2-fold, ~4-fold, >12-fold or ~8-fold increases in the levels of circulating CCL1, CCL2, CCL3 and CCL5 respectively. The increases in the expression levels of these chemokines compared favourably to the WT data (see section 4.5.2.3 of the previous chapter). However, there were some differences; whereas topical imiquimod cream treatment caused increased circulating CCL11 (6-fold increase), CCL12 (8-fold increase) and CCL17 (>2-fold increase) in WT mice, the circulating levels of these chemokines were below the threshold set to screen background noise. In addition, whereas CCL1 was ~2-fold more expressed in CCR2 KO plasma in response to topical imiquimod cream treatment, it was not expressed above background in WT mice.

For CXC-chemokines, topical imiquimod cream application induced a >2-fold increase in circulating CXCL1 and CXCL13 in CCR2 KO mice when compared to aqueous cream treated controls. Although there were similarly increased CXCL13 expression levels in WT mice in response to topical imiquimod cream treatment, there was no increased CXCL1 expression in WT mice. In addition, there was also a >2-fold reduced levels of CXCL2 and CXCL11 expression in the plasma of WT mice in response to topical imiquimod cream treatment; these reduced levels of CXCL2 and CXCL11 were not detected in CCR2 KO mice.

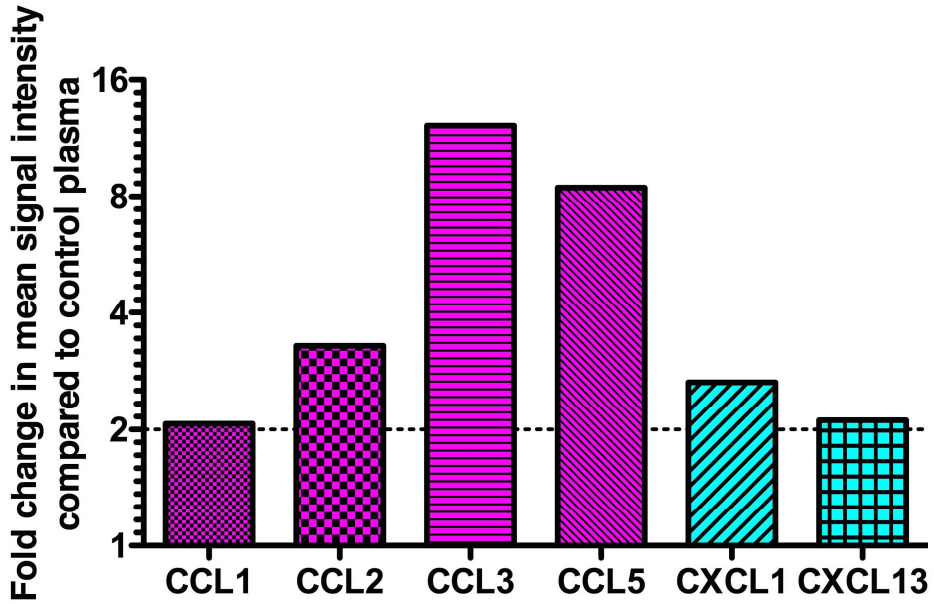



**Figure 5-15 Topical imiquimod cream application modulates chemokine expression in the plasma of CCR2 KO mice.**
The signal intensities of each spot on the developed array blots (see section 5.4.3.1) were estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each chemokine, the mean of the pixel density of each chemokine was calculated, and the corrected signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control, from the mean signal intensity of each chemokine. Next, the fold change in the corrected mean signal intensities was estimated for each analyte by dividing the corrected mean signal intensity of that analyte in the imiquimod cream treated sample by the corrected mean signal intensity of that same analyte in the aqueous cream treated group. Figure 5-15 is a bar graph showing the fold change in the plasma chemokines in response to the topical imiquimod cream application. Fold change >1, means increased production of the cytokine in response to topical imiquimod cream application.

### 5.4.3.3 Topical imiquimod cream application modulates plasma cytokines in CCR2 KO mice

The bar graph in figure 5-16 summarises the impact of the topical imiquimod cream application on circulating plasma cytokines in CCR2 KO mice. Interestingly, topical imiquimod cream application resulted in ~28-fold increase in triggering receptor expressed on myeloid cells 1 (TREM-1) levels in the circulation of CCR2 KO mice. This contrasts to the data from WT mice (see previous chapter), in which TREM-1 was not expressed above background.

The plasma levels of interleukins (ILs) in CCR2 KO mice were also modulated by the topical imiquimod cream treatment. Specifically, topical imiquimod cream led to a >2-fold increase in circulating IL-1$\alpha$ levels in CCR2 KO mice. In addition, topical imiquimod cream treatment also led to >2-fold increase in the level of the natural antagonist for IL-1$\alpha$, the IL-1r$\alpha$, in the plasma of CCR2 KO mice. This increase in the levels of both IL-1$\alpha$, and IL-1r$\alpha$ is understandable considering that a delicate balance is kept between IL-1$\alpha$ and IL-1r$\alpha$ to maintain cellular

homeostasis (Arend, 2002). Also, there was a strong increase (~28-fold) in the plasma levels of the anti-inflammatory cytokine, IL-10, in the circulation of CCR2 KO mice. Moreover, there were also increases in plasma levels of IL-16 (~8-fold increase) and IL-17 (~4-fold increase) in CCR2 KO mice. These topical imiquimod cream treatment-induced increases in the IL-1rα, IL-10, IL-16 and IL-17 were comparable to the imiquimod cream treatment-induced changes in WT mice (see previous chapter). However, there were many differences; whereas topical imiquimod cream treatment led to increased circulating levels of G-CSF (4-fold increase), TNF-1α (>2-fold increase), IL-23 (8-fold increase) and IL-27 (4-fold increase) in WT mice, the expression of these cytokines was not above background in CCR2 KO mice.

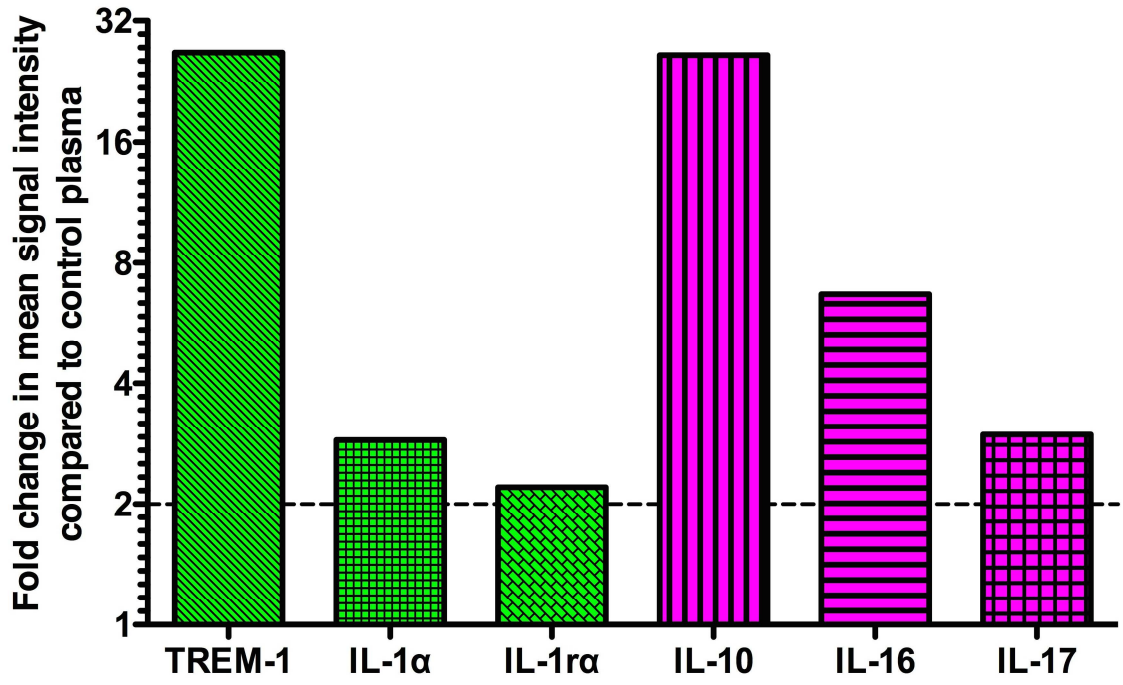



**Figure 5-16 Topical imiquimod cream application modulates cytokine expression in the plasma of CCR2 KO mice.**
The signal intensity of each spot on the developed array blots (see figure 5-14) were estimated with ImageJ 1.42 software (NIH, USA). As duplicate spots represent each cytokine, the mean of the signal intensity of each cytokine was calculated, and the corrected mean signal intensities were next calculated by subtracting the mean signal intensity of the spots for the negative control, from the mean signal intensity of each cytokine. Next, the fold change in the corrected mean signal intensities was estimated for each analyte by dividing the corrected mean signal intensity of that analyte in the imiquimod cream treated sample by the corrected mean signal intensity of that same analyte in the aqueous cream treated group. Figure 5-16 is a bar graph showing the fold change in the plasma cytokines in response to the topical imiquimod cream application. Fold change >1, means increased production of the cytokine in response to topical imiquimod cream application.

### 5.4.4 HPC mobilisation is not just the result of soluble factors released from the skin

To determine whether soluble factors from the skin was the cause of the HPC mobilisation action seen with the topical imiquimod cream or TPA painting, protein skin homogenates were administered I.P. to assess their ability to recapitulate the imiquimod cream- or TPA-induced HPC mobilisation. To do this, WT mice received the topical imiquimod cream/TPA treatment exactly as described in the previous chapters. After the treatment, mice were culled by $CO_2$ asphyxiation and the dorsal skin was harvested into PBS. ~150mg of the skin from each mouse was minced with a scalpel, placed in 1.5mL microfuge tubes, two stainless steel beads were added and the mixture homogenised in 1mL PBS (see Materials and Methods for details). After centrifugation of the homogenate at 10,000xg for 10 minutes to get rid of hair and skin fragments, 150μL of the supernatant was injected I.P. per mouse. 60 minutes after the injection, mice were culled by $CO_2$ asphyxiation, and peripheral blood harvested for myeloerythroid colony-forming assay in Methocult™ GF m3434 as described in previous sections. 60 minutes was chosen as the time point for this experiment in accordance with previous data showing that chemokine ligands are able to induce HSPC mobilisation within this time frame (Lord et al., 1992, Hunter et al., 1995).

As shown in **figure 5-17**, skin homogenates from neither the imiquimod cream-inflamed skin nor TPA-inflamed skin could induce mobilisation of HPC compared to controls. Thus, within the limits of the time frame used for this assay, the data suggest that although the inflammation initiated in the cutaneous skin was fundamental in the HSPC mobilisation seen in the peripheral inflammation models, other innate or adaptive cellular elements may be required to communicate these initial stimuli to the HSPC niche to mobilise HSPC.

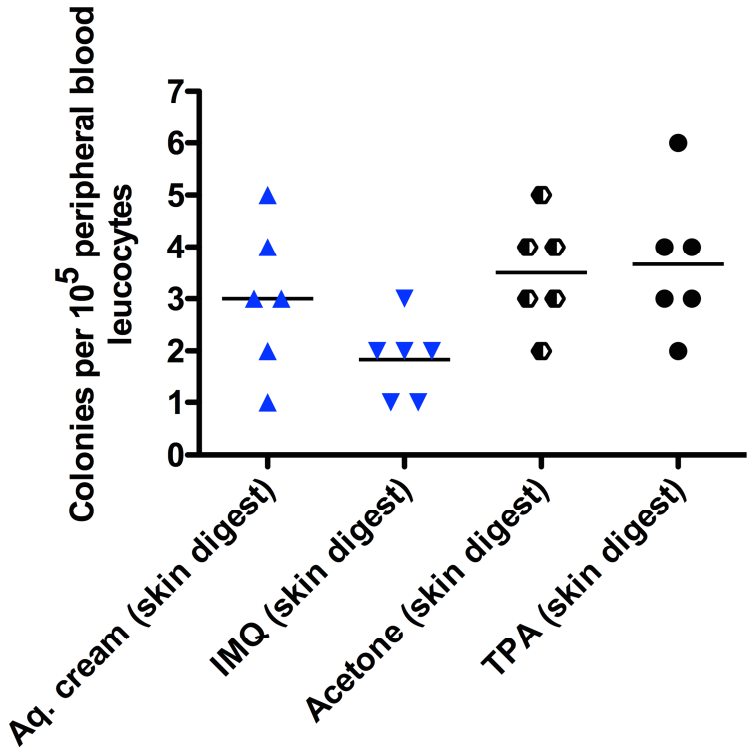



**Figure 5-17 Homogenates from digested skin of either topical imiquimod cream or TPA treated mice are unable to mobilise HSPC.**
C57BL/6 mice were either given a single dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application on the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice were given a topical application of equivalent amounts of either aqueous cream or acetone application on the shaved dorsal skin as controls for topical imiquimod cream and topical TPA application respectively. On the fourth day, the mice were euthanized by $CO_2$ asphyxiation, and the shaved dorsal skin was harvested. 150mg of the skin from each mouse was homogenised in 1mL PBS, spun at 10,000xg and supernatants harvested in each case. 150µL of skin homogenates were injected I.P. per mouse per treatment group. 60 minutes after the injection, the mice were euthanized by $CO_2$ asphyxiation, and peripheral blood harvested for haematopoietic progenitor colony-forming assay. After lysing the red blood cells, $1x10^5$ peripheral blood leucocytes were seeded into 1.1mL of m3434 in duplicates, and colonies counted after 7-10 days culture at 37°C in 5% $CO_2$ humidified chamber. (N=3, 2 independent experiments).

In summary, the data presented in this section suggest that:

1. CCR2 expression is required for the full spectrum of responses to topical imiquimod cream treatment.

2. Topical imiquimod cream treatment is characterised by a strong IL-10 and TREM-1 expression in the plasma of $CCR2^{-/-}$ mice.

## 5.5 How do exogenously administered chemokines affect the HSPC numbers in peripheral blood?

### 5.5.1 Introduction

The chemokine receptor gene expression data presented in the previous chapter for both peripheral inflammation models (see also chapter 3 for similar findings in the LPS-induced systemic inflammation model) show that HSPC express chemokine receptors and that these receptors are modulated by inflammatory stimuli. Interestingly, prior reports have established that exogenous administration of some chemokines such as CXCL2, CXCL8, and CCL3 significantly mobilise HSPC to circulation. Given the number of chemokine receptors that were detectable by QPCR and the detection of their respective ligands in the plasma, I set out to test the hypothesis that in a non-inflamed context, HSPC may be mobilised in response to exogenously administered ligands of the chemokine receptors that were detected on the HSPC. Among the chemokine receptors that were highly modulated in the bone marrow HSPC were CCR2 (>16-fold increase in both imiquimod and TPA-induced inflammation), CCR3 (>8-fold decrease in imiquimod cream-induced inflammation) and CCR5 (>4-fold increase in both imiquimod cream and TPA-induced inflammation). Surprisingly, of the CC-chemokine receptors, only CCR5 expression was ~8-fold upregulated in peripheral blood HSPC in imiquimod cream treated mice.

### 5.5.2 Exogenously administered CCL2, CCL4 or CCL11 fail to mobilise HSPC in resting mice.

The data presented in this chapter showed that HSPC express CCR2 and that this CCR2 expression was fundamental for imiquimod cream-induced HSPC mobilisation. However, one question that remains unanswered is whether the HSPC surface expression of CCR2 in resting mice was sufficient to allow these cells to respond to exogenously administered ligands. I therefore set out to use exogenous recombinant murine chemokines, namely CCL2 (CCR2 ligand), CCL4 (CCR5 ligand) and CCL11 (CCR3 ligand) to assess their potential to induce HSPC mobilisation in a non-inflamed context. As the HSPC chemokine receptor gene profiling (see previous chapters) showed that these cells expressed CCR2, CCR3 and CCR5, I surmised that with the relatively specific receptor affinities of CCL2, CCL4 and CCL11, the results of these mobilisation assays would provide insight as

to which chemokine receptor(s) was/were sufficiently expressed on the HSPC surface in the resting sate to impact on *in vivo* chemotactic response.

Previously published data (Broxmeyer et al., 1998, Lord et al., 1995), showed that CC-chemokines could mobilise HSPC at a dose of 50μg/kg in 60 minutes. Therefore, a dosage of either 50μg/kg or 75μg/kg body weight given as a single S.C. injection for either 30 minutes or 60 minutes was employed to titrate the probable dose that could induce HPC mobilisation. However, as summarised in **figures 5-18 A, B and C**, irrespective of the dose (50μg/kg or 75μg/kg) or time (30 minutes or 60 minutes), none of the chemokines, CCL2, CCL4 or CCL11, could induce increased numbers of circulating HPC, although AMD3100 that was used as a positive control mobilised increased numbers of circulating HPC.

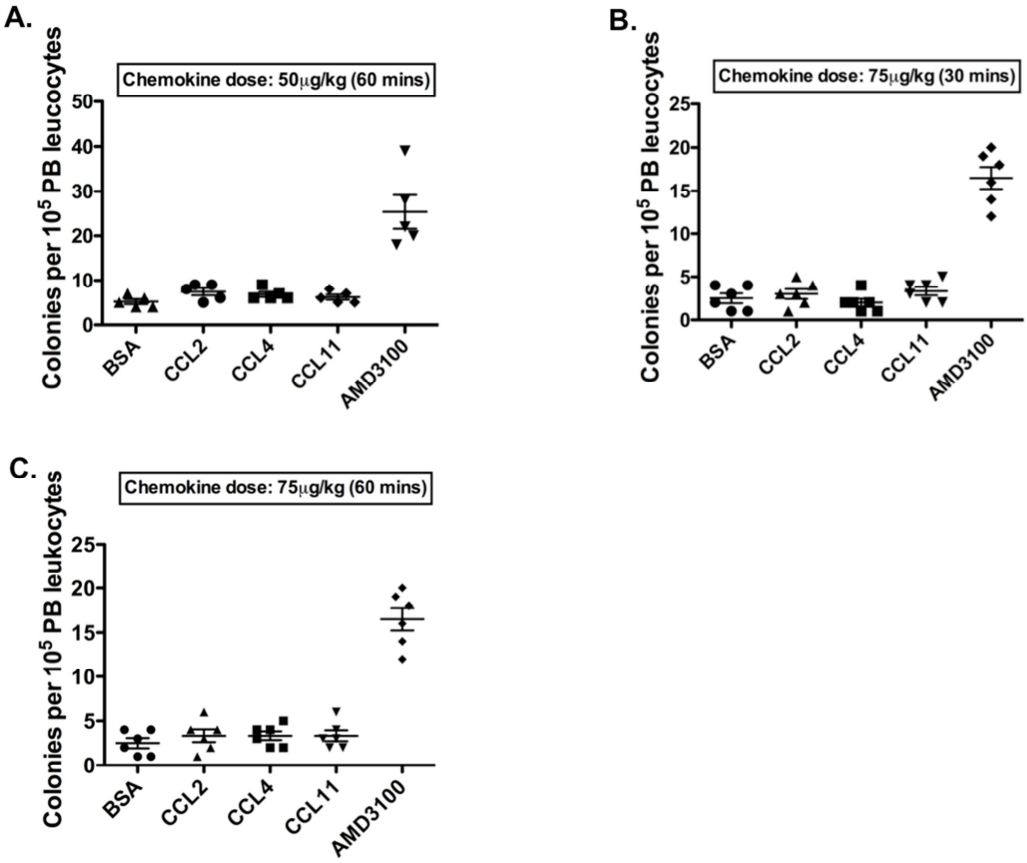



**Figure 5-18 Exogenously administered CCL2, CCL4, or CCL11 are unable to orchestrate HSPC mobilisation to the circulation.**

C57BL/6 mice received a single S.C. injection of 50μg/kg or 75μg/kg body weight of one of recombinant murine CCL2, CCL4, or CCL11. Simultaneously, other groups of mice received a single dose of either AMD3100 (5mg/kg body weight) or an equivalent volume of 0.1%$^{w}/_{v}$ BSA as positive and negative controls respectively. 30 or 60 minutes after the injection, the mice were euthanized through $CO_2$ asphyxiation and peripheral blood harvested for haematopoietic progenitor colony-forming assay. After lysing red blood cells, 1x$10^5$ peripheral blood leucocytes were seeded into 1.1mL of Methocult$^{TM}$ GF M3434 in duplicates, and colonies counted using an inverted microscope, after 7-10 days culture at 37°C in 5% $CO_2$ in a humidified chamber. [(n = 3), three independent experiments].

In summary, data presented in this section suggest that in the non-inflamed context, exogenously administered CCL2, CCL4 or CCL11 are unable to mobilise HSPC to the circulation suggesting the expression of their cognate receptors on the HSPC surface may not be sufficient to permit them to respond to these ligands.

## 5.6 Discussion and summary

This chapter sought to understand the roles played by the CCR2/CCL2 axis in the LPS-induced systemic inflammation or the topical imiquimod cream or topical TPA inflammation mediated egress of HSPC from the bone marrow. The objective was to begin to explore one of the candidates emerging from the chemokine receptor gene expression and chemokine ligand proteome array data presented in the previous chapters. The data presented herein show that myeloerythroid progenitors express functional CCR2 that is increased in response to inflammation. The data further argue that although HSPC may express chemokine receptors, the role of these chemokine receptors in HSPC biology may be context-dependent. For example, data shown in this chapter demonstrated that exogenously administered CCL2, or CCL4, or CCL11, failed to mobilise HSPC into the circulation in resting mice, i.e. in non-inflamed context. However, the CCR2 KO mice and CCL2 neutralisation experiments argue strongly that in an inflamed context, induced by topical imiquimod cream treatment, the CCR2/CCL2 axis was crucial in mediating HSPC egress from the bone marrow. Thus, an inflamed context may be required to induce the expression of inflammatory chemokine receptors on the HSPC that enable them to respond to chemotactic cues.

### 5.6.1 CCR2 is required for the full spectrum of responses to the topical imiquimod cream.

The expression of CCR2 appears to be a critical requirement in eliciting the full spectrum of responses to topical imiquimod cream. Firstly, although topical TPA application induced a comparable morphological change in the dorsal skin of WT mice and CCR2 KO mice, topical imiquimod cream could not induce comparable inflammatory responses in WT mice and CCR2 KO mice. Specifically, whereas the topical imiquimod cream caused exaggerated dorsal skin inflammatory response

noticeable by hyperkeratosis, and acanthosis, it only induced a modest inflammatory response in the dorsal skin of CCR2 KO mice. Previously, Vestergaard et al. showed that monocytes in peripheral blood of psoriatic and atopic dermatitis patients significantly up-regulate their CCR2 expression (Vestergaard et al., 2004). Others have also shown that keratinocytes from the skin of psoriatic patients produce increased levels of CCL2 (Giustizieri et al., 2001, Deleuran et al., 1996, Kaburagi et al., 2001). As the topical imiquimod cream application model has been proposed to model human psoriatic diseases, it is reasonable to argue that the exaggerated inflammatory response observed in WT, but absent in CCR2 KO mice, was the result of a functional CCR2 that enabled the influx of inflammatory cells like monocytes into the cutaneous skin in response to CCL2 released from keratinocytes and mast cells (Drobits et al., 2012).

These differences in the response of WT and CCR2 KO mice to the topical imiquimod cream was also reflected in the circulating chemokine and cytokine in the plasma. In a paper published by van der Fits et al that established topical imiquimod application in mice as a model of human psoriasis, they implicated the IL-23/IL-17 axis as the critical determinant in the induction of the psoriasis phenotype (van der Fits et al., 2009). Interestingly, IL-23 was barely detectable in the CCR2 KO plasma, in contrast to its high expression in the plasma of WT mice (as reported in the previous chapter). In addition, IL-17 was also expressed at lower levels in CCR2 null mice in comparison to WT mice. This differential IL-17 and IL-23 expression may, in part, mechanistically explain the reduced level of inflammation observed in CCR2 null mice seen with reduced epidermal inflammation. Interleukin-10 secretion was however, highly upregulated in both WT and CCR2 null mice perhaps as a negative feedback mechanism to modulate the inflamed state. Others have reported that direct TLR ligation (e.g. TLR-2, -3, -4 and -9) on immune cells like macrophages, and myeloid derived dendritic cells, leads to increased IL-10 secretion (Agrawal et al., 2003, Dillon et al., 2004, Boonstra et al., 2006). Thus, it is possible that the massive increase in IL-10 detected in the plasma may be the result of a direct TLR7 ligation on some of these immune cells. Whatever the exact source of the IL-10 production, it is clear that it was independent of CCR2 expression as both WT mice and CCR2 KO mice exhibited comparable levels of IL-10 in the plasma.

It is also interesting to note that there was high expression of TREM-1 in the plasma of CCR2 null mice (~28-fold increase above the baseline) treated with topical imiquimod cream application; this was in contrast to the very low level of TREM-1 in WT mice (i.e. not above background noise) reported in the previous chapter. Human neutrophils, monocytes and macrophages have been shown to express TREM-1 (Bouchon et al., 2000, Colonna and Facchetti, 2003). In granulocytes, it has been shown that TLR ligation leads to up-regulation of TREM-1 expression, which then acts to amplify the innate immune response by mediating the expression of pro-inflammatory chemokines such as CXCL8, CCL2, CCL3 and CCL7 (Bleharski et al., 2003). This TREM-1 mediated amplification of inflammatory chemokine expression has also been shown to be crucial in recruiting T helper 1 ($T_H1$) cells and thus linking the innate and the adaptive immune systems (Bleharski et al., 2003). Therefore, the differential expression of TREM-1 in topical imiquimod cream-treated CCR2 null mice, compared to its expression in topical imiquimod cream-treated WT mice, offers some insights concerning the elevated CCL3 expression in CCR2 null mice compared to WT mice. Given that both WT and CCR2 null mice were given the same dose of topical imiquimod cream treatment for the same duration, and yet, gave different pattern of TREM-1 expression, it will be interesting to investigate the potential role that CCR2 plays in the induction of TREM-1 expression. It is also important to interpret these data in the light of other previous finding that TREM-1 was not upregulated in non-infectious inflammatory diseases like psoriasis and ulcerative colitis (Bouchon et al., 2001). Therefore, the fact that TREM-1 is highly upregulated in CCR2 null mice, but not WT mice, may be an indication that, in the absence of functional CCR2 receptor, topical imiquimod cream is unable to induce a psoriasis phenotype in the dorsal skin of mice.

## 5.7 Chapter summary

In this chapter, data has been presented to show that:

1. HSPC particularly the myeloerythroid progenitors, i.e. GMP, PreGM, CFU-E and PreCFU-E, express CCR2.

2. CCR2 expression is fundamental to the full spectrum of the inflammatory responses mediated by the topical imiquimod cream application.

3. CCR2/CCL2 axis is fundamental the topical imiquimod cream application-induced HSPC mobilisation.

# Chapter 6: General discussion

## 6.1 Introduction

Anaemia of chronic disease has been known to complicate the prognosis of many inflammatory disease processes such as rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel diseases (Maury et al., 2003, Gasche et al., 2001, Weiss and Goodnough, 2005). Although the impact of inflammatory mediators on erythropoiesis has been extensively investigated, the same cannot be said of the HSPC compartment. Evidence is now emerging that many inflammatory processes mobilise increased numbers of haematopoietic progenitors into the circulation. However, the underlying mechanisms remain to be explored. In this study, I sought to use mouse models of systemic and peripheral inflammation to investigate the mechanisms driving the inflammation-induced HSPC mobilisation. As chemokines are one of the key players orchestrating *in vivo* cellular migration in multicellular organisms, I also sought to examine whether inflammation-driven HSPC mobilisation was the consequence of inducible expression of inflammatory chemokine receptors on HSPC, which would enable them to migrate in response to elaborated inflammatory chemokines. LPS (TLR4 ligand) injection and topical imiquimod cream treatment (TLR7 ligand) were employed to study the roles of TLR signalling in HSPC mobilisation, whereas the sterile inflammatory agent, TPA, was employed to investigate the role played by non-TLR-driven inflammatory responses to mobilise HSPC. Moreover, this study also sought to understand whether the mode of delivery of the inflammatory process, i.e. systemic via I.P., or peripheral via topical application, has a bearing on HSPC mobilisation from the bone marrow.

In this chapter, the basic mechanistic concepts that emerged from the data presented in the previous chapters are discussed as well as conclusions and future directions presented.

## 6.2 HSPC mobilisation with or without TLR signalling

Using haematopoietic progenitor colony-forming assays as an indirect measure of the number of haematopoietic progenitors in circulation, data presented in this thesis show that LPS (TLR4 ligand), imiquimod cream (TLR7 ligand), or TPA (PKC stimulant with no TLR signalling effect), treatments each mobilise HSPC to the

circulation. This therefore demonstrates that TLR ligation was not obligatory for the HSPC mobilisation, but that the crucial component was the inflamed context. Although HSPC have not been traditionally proposed as directly involved in inflammation and infection resolution, recent evidence suggests otherwise. For example, not only have haematopoietic progenitors been shown to express TLRs, but also ligation of TLR4 in these progenitors was shown to lead to increased generation of common myeloid progenitor cells as well as differentiation of lymphoid progenitors into dendritic cells (Nagai et al., 2006, Rodriguez et al., 2009). The data presented in this thesis that showed that TPA-driven sterile inflammation also mobilises increased HSPC numbers to the circulation therefore broaden our understanding and suggests that HSPC might play crucial roles in the resolution of both TLR-driven and non-TLR-driven inflammatory pathologies.

Previously, others have reported that inflammatory models led to significant increases in Sca-1 expression in the HSPC compartment (Zhang et al., 2008, Melvan et al., 2011). Using LPS injection as a model of infection in TLR4 null mice, Shi et al. demonstrated that this increased Sca-1 expression was a TLR-driven effect (Shi et al., 2013). The flow cytometric data presented in this thesis support that conclusion as both LPS and imiquimod cream treatment significantly increased Sca-1 expression in HSPC whereas TPA treatment did not. Interestingly, using various *in vivo* and *in vitro* infection models, Snapper et al. showed that increased Sca-1 expression in lymphocytes was dependent on the production of IFN-α/β and /or –γ (Snapper et al., 1991). Whilst a >2-fold increase in IFN-γ levels were detected in the plasma of LPS treated mice, the expression of IFN-γ in response to imiquimod cream treatment was not above the levels in control groups. As different time points were employed in the two models, i.e. 24 hours and 72 hours for LPS and imiquimod cream treatment respectively, it is possible that elevated IFN-γ levels were also responsible for the increased Sca-1 expression on HSPC, but that the window for detecting elevated IFN-γ plasma levels was missed in the imiquimod cream model. However, this does not rule out the possibility that different mechanisms might have been responsible for the increased Sca-1 expression in the LPS injection, and topical imiquimod cream, treatments.

The flow cytometry data presented in this thesis also showed that both TLR-driven inflammatory processes [i.e. LPS injection (TLR4) or topical imiquimod cream treatment (TLR7)] significantly reduced the proportion of bone marrow LT-HSC population in contrast to the non-TLR driven inflammatory process, i.e. TPA treatment, which did not significantly alter the LT-HSC population. Although this study did not examine the long-term effects of these inflammation models on HSPC biology, it is reasonable to speculate that chronic TLR-driven inflammation could potentially lead to HSC exhaustion whereas chronic, non-TLR driven, inflammation may not have such a direct detrimental effect on the LT-HSC. In this light, a population study in Sweden found an association between chronic infection/inflammation and haematopoietic malignancies in humans, suggesting that chronic exposure to inflammatory mediators may even trigger neoplastic transformation (Kristinsson et al., 2011). It was also reported that long term exposure of mice to low dose of LPS impaired HSC self-renewal and skewed HSC differentiation towards the myeloid lineage (Esplin et al., 2011). Others also showed that IFN-γ significantly impairs the self-renewal capacity of LT-HSC and ultimately led to bone marrow failure (de Bruin et al., 2013). Therefore, although the inflammation-induced HSPC mobilisation may be physiologically important for the survival of organisms in acute inflammatory situations, unresolved inflammation may overwhelm the ability of the haematopoietic system to restore homeostasis in the long-term and lead to pathology.

## 6.3 Inflammation modulates the HSPC niche

The bone marrow niche provides a safe haven that houses haematopoietic stem and progenitor cells. Within the niche, interactions between the HSPC, and cellular components as well as their secreted soluble products, ensure the maintenance of self-renewal, proliferation, differentiation and retention of HSPC. Many agents employed for mobilisation of HSPC for clinical use have been shown to disrupt one or more of these HSPC-niche interactions. Although LPS injection has been shown to mobilise HSPC to the circulation, the mechanism underlying this mobilising activity has not been fully elucidated. The data presented in this thesis show that both TLR-driven inflammation (LPS injection or imiquimod cream treatment), and non-TLR-driven inflammation, significantly reduce bone marrow CXCL12 concentration, suggesting a role for a perturbed

CXCR4/CXCL12 axis in the inflammation-induced HSPC mobilisation. However, flow cytometric data showed that whereas LPS injection led to significant reduction in HSPC surface CXCR4 expression, neither topical imiquimod cream, nor TPA, application significantly altered HSPC surface CXCR4 expression. Many previous reports have provided evidence indicating that cellular components of the bone marrow niche are being responsible for CXCL12 production in the niche. Amongst the prime candidates have been osteoblasts, endothelial cells and mesenchymal stromal cells (Ding et al., 2012, Mendez-Ferrer et al., 2010, Calvi et al., 2003, Jung et al., 2006). Through flow cytometry, evidence is provided in this thesis arguing for osteoblasts as the prime CXCL12 producing cells (at least amongst the three cellular niche components investigated in this study, i.e. osteoblast, endothelial cells and MSC). This conclusion stems from the fact that in spite of the reduced bone marrow CXCL12 concentration, both LPS injection and topical imiquimod cream treatment significantly increased bone marrow stromal MSC and endothelial cell fractions. The only stromal bone niche cellular element that was significantly reduced in each of the LPS injection and topical imiquimod cream/TPA treatment was osteoblasts, suggesting that osteoblast may be the major CXCL12 secreting cells. While this conclusion is contrary to other published data that implicates immature MSC and endothelial cells as the CXCL12 producing cells (Greenbaum et al., 2013, Sugiyama et al., 2006), it is important to note that these studies were undertaken using conditional deletion of CXCL12 in different niche cell types in non-inflamed context. It is possible that processes regulating CXCL12 production in steady state may not necessarily be the same processes that pertain in stressed conditions.

It is also interesting to note that both I.P. LPS and topical imiquimod cream treatment models led to high expression of G-CSF and significantly reduced bone marrow CD169+ macrophage cells. Previously, Levesque et al. proposed that G-CSF mediated HSPC mobilisation was partly due to its activation of neutrophils to release proteolytic enzymes (Levesque et al., 2001). In addition, it has also been shown that CD169+ macrophages are fundamental niche cells that release soluble factors which stimulate nestin+ MSCs to release CXCL12 (Chow et al., 2011). Therefore, the combined impact of a reduced CD169+ macrophage population, and increased circulating G-CSF levels, may have also significantly

contributed to the reduced bone marrow CXCL12 in both TLR-driven inflammations. However, in spite of the significant reduction in bone marrow CXCL12 in TPA treated mice, there were increased bone marrow CD169+ macrophage cell numbers as well as similar G-CSF levels in control and TPA treated groups. These suggest the possibility that different mechanisms may be involved in the TLR and non-TLR driven HSPC mobilisation.

## 6.4 Identification of chemokine and cytokine expression in response to LPS injection, or topical imiquimod cream/TPA treatment.

Using proteome array profiling of the plasma, the chemokines that were highly expressed in response to either LPS injection, or topical imiquimod cream treatment were CCL2, CCL5, CCL12 and CXCL13. However, of these chemokines, the only one that was also highly expressed in response to topical TPA treatment was CCL2, suggesting that if a common inflammatory chemokine expression played a role in orchestrating HSPC mobilisation, CCL2 could be such a candidate. The RT-QPCR data showed that inflammatory stimuli modulated the chemokine receptor expression on bone marrow HSPC, with CCR2 being the most highly expressed in the topical imiquimod cream/TPA treatment model. Using flow cytometry, data were also provided showing that haematopoietic progenitors expressed cell surface CCR2, which was upregulated, particularly in GMP and PreGM cells, in response to inflammatory stimuli. In testing the *in vivo* functional relevance of the inducible CCR2 expression in orchestrating the inflammation-induced HSPC mobilisation, CCR2 KO mice were used. These data showed that any role played by the inflammatory chemokine/chemokine receptor axis may be context dependent as each of LPS injection and topical TPA treatment, mobilised HSPC in these mice, whereas topical imiquimod cream could not. Targeted *in vivo* neutralisation of CCL2 using intravenous administration of anti-CCL2 neutralising antibodies during the imiquimod cream treatment abrogated the HSPC mobilisation suggesting the essential requirement for a functional CCR2/CCL2 axis in the topical imiquimod cream model. This is in agreement with previous data that demonstrated the requirement for the CCR2/CCL2 axis in inflammation-induced HSPC mobilisation (Si et al., 2010).

Additional evidence supporting the essential requirement for the expression of CCR2 in mediating the full spectrum of imiquimod cream treatment model also came from the cytokine proteome array and skin histology data. Whereas the topical imiquimod cream treatment induced a strong increase in the expression of the pro-inflammatory cytokines IL-17, IL-23 and IL-27 in WT mice, it induced increased expression of IL-17 (moderate) and very high TREM-1 levels in CCR2 KO mice. Van der fits et al. who established topical imiquimod cream application as a model of human psoriasis (van der Fits et al., 2009) implicated the IL-23/IL-17 axis as the critical determinant of the psoriasis phenotype. However, in the absence of CCR2, topical imiquimod cream was unable to elicit a psoriasiform phenotype in CCR2 KO mice as evidenced by the significantly reduced epidermal thickening in CCR2 KO mice. Previously, Bouchon et al. showed that TREM-1 was not upregulated in non-infectious inflammatory diseases like psoriasis and ulcerative colitis (Bouchon et al., 2001). Therefore, the fact that TREM-1 is highly upregulated in CCR2 null mice, but not WT mice, may be an indication that, in the absence of functional CCR2 receptor, topical imiquimod cream is unable to induce a psoriasis phenotype in the dorsal skin of mice.

## 6.5 Ability of inflammatory chemokine ligands to mobilise HSPC is context dependent.

Haematopoietic stem cell mobilisation is an important component of the clinical therapy for treatment of malignancies. Although G-CSF has been the mainstay in the clinic for mobilising HSPC, due to the many drawbacks such as side effects, multiple dosing, as well as its inability to mobilise in some patients and healthy donors, the drive has been towards safer agents that mobilises HSPC faster with minimal inconvenience to the donor (Holm, 1998, Croop et al., 2001). Chemokine-based mobilising agents have shown immense promise in this area. For example AMD3100, a CXCR4 antagonist, mobilises HSPC within 60 minutes upon a single administration (Liles et al., 2003, Broxmeyer et al., 2005). Also CXCL2 and CXCL8 have also been shown to mobilise HSPC in a matter of hours (Pelus et al., 2004, Pruijt et al., 2002). The RT-QPCR data and proteome array data demonstrated chemokine/chemokine receptor axes may have significant impact on HSPC mobilisation during inflammation. In addition, the CCR2 KO mice, and *in vivo* CCL2 neutralisation, data demonstrated the importance of the

CCR2/CCL2 axis in imiquimod cream-induced HSPC mobilisation. As clinical HSPC mobilisation is conducted in the non-inflamed context, exogenous administration of chemokine ligands was tested to examine their abilities to mobilise HSPC in mice. However, administration of CCL2 (CCR2 ligand), or CCL4 (CCR5 ligand), or CCL11 (CCR3, & CCR5 ligand), showed that none of these chemokines was able to mobilise HSPC into the circulation in mice. Altogether, these data show that, in the non-inflamed context, the expression of inflammatory chemokine receptors on the cell surface of HSPC may be insufficient to enable them to respond to exogenously administered ligand. This is in contrast to CCL3 that was shown to mobilise HSPC in the non-inflamed context (Broxmeyer et al., 1999). Employing CCR1 null mice in a series of ligand-induced HSPC mobilisation assays, Broxmeyer et al. concluded that CCR1 may be the dominant receptor orchestrating the HPC mobilising effects that occur in response to exogenously administered CCL3. Interestingly, CCR1 was not detected on the HSPC.

Taken together, the ability of chemokine ligands to mobilise HSPC into circulation differs in two ways: (1) In steady state some inflammatory chemokines mobilise HSPC into circulation through indirect action on terminal differentiated haematopoietic cells (Pruijt et al., 2002, Pelus et al., 2004, Broxmeyer et al., 1999); (2) In the inflamed context, HSPC inducibly express inflammatory chemokine receptors which enable them to migrate towards elaborated inflammatory chemokines in the circulation. Data presented in this thesis, particularly in the topical imiquimod cream treatment model, provide evidence for the second scenario. The long-term repercussion of this phenomenon of inflammation-induced chemokine receptor expression, and subsequent HSPC mobilisation, on the HSPC biology might be particularly important in chronic inflammatory diseases. Such inflammatory chemokine receptor expression on HSPC and subsequent egress from the bone marrow may ultimately lead to HSPC depletion in the bone marrow as well as bone marrow and peripheral blood cytopenia. This may be another contributory factor to the anaemia associated with chronic inflammatory diseases.

# 6.6 Proposed models for inflammation-induced HSPC mobilisation

## 6.6.1 Proposed model for LPS-induced HSPC mobilisation

Figure 6-1 summarises the proposed model of HSPC mobilisation occurring in response to I.P. LPS injection. As endothelial cells express TLR4, the circulating LPS stimulate these cells to produce G-CSF that enters the circulation (Boettcher et al., 2012). Both the elaborated G-CSF and LPS reaches the bone marrow niche through the circulation. As HSPC express TLR4 (Nagai et al., 2006) and G-CSFR (McKinstry et al., 1997), the presence of LPS and G-CSF stimulates emergency granulopoiesis that depletes GMP cell numbers (see **appendix 2**). In addition, there is a reduction in bone marrow $CD169^{+}$ macrophage cells, perhaps as a result of mobilisation to other tissues. HSPC also reduce surface CXCR4 expression in response to LPS probably as a result of interference with the re-expression internalised CXCR4. The combined effects of a reduced osteoblast numbers and increased G-CSF lead to reduced bone marrow CXCL12, thereby creating a CXCL12 gradient in the circulation. Ultimately, this peripheral blood CXCL12 gradient mobilises neutrophils [resulting in peripheral blood Neutrophilia (see **appendix 13B**) and HSPC to the circulation.

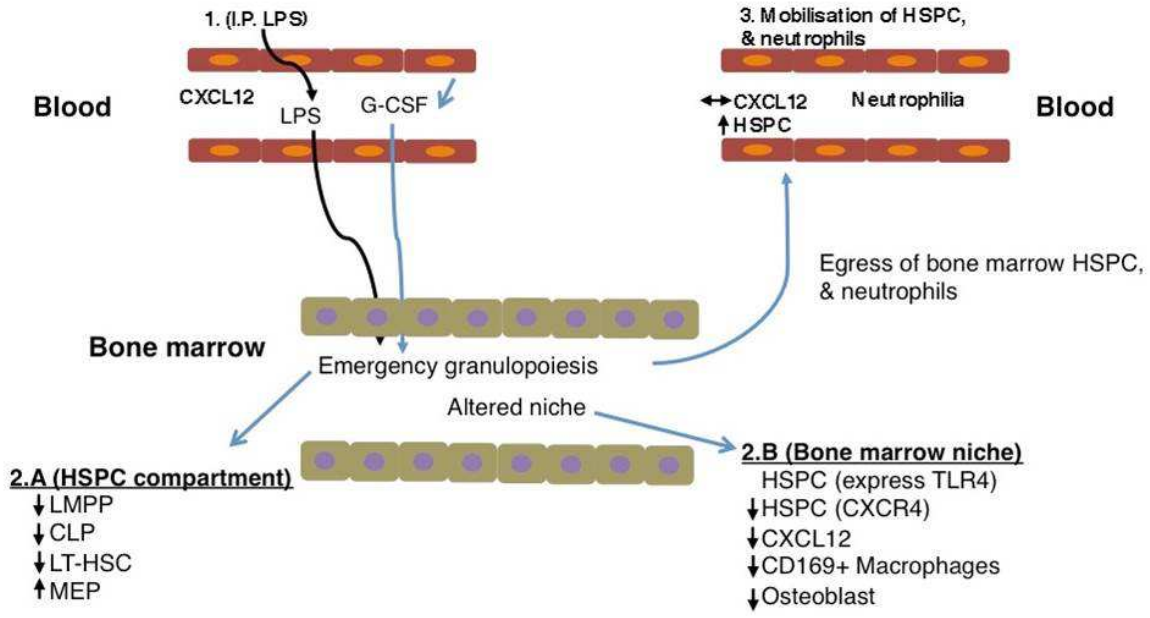



**Figure 6-1 Proposed mechanism of I.P. LPS orchestrated HSPC mobilisation.**
**1.** Endothelial cells sense LPS and produce G-CSF that enters the circulation (Boettcher et al., 2012): **2.** Both G-CSF and LPS reaches the bone marrow niche through the circulation; As HSPC express TLR4 (Nagai et al., 2006) and G-CSFR (McKinstry et al., 1997), the presence of LPS and G-CSF stimulates emergency granulopoiesis; There is reduction in bone marrow CD169 macrophage cells in addition to a concomitant downregulation of HSPC surface CXCR4

expression; Combined effect of reduced osteoblasts and increased G-CSF leads to reduced bone marrow CXCL12, thereby creating a CXCL12 gradient in the circulation: **3.** This CXCL12 gradient mobilises neutrophils (peripheral blood neutrophilia) and HSPC to the circulation. [Arrow pointing down indicates reduction in cell population or receptor expression; Arrow pointing upwards indicates increased cell population; Double-headed horizontal arrow indicates no change in expression]

### 6.6.2 Proposed model for imiquimod cream treatment-induced HSPC mobilisation

Figure 6-2 summarises the proposed model for the topical imiquimod cream treatment-induced HSPC mobilisation. Resident skin cells like keratinocytes and mast cells perceive the imiquimod cream treatment and release CCL2 (Drobits et al., 2012). The CCL2, and G-CSF (most likely produced by skin endothelial cells as well as vascular endothelial cells), enter the circulation. In response to the circulating CCL2, HPC (and monocytes and neutrophils?) upregulates surface CCR2 expression resulting in mobilisation of HPC, monocytes and neutrophils to the periphery (see appendix 13 A & B). In addition, the increased G-CSF stimulates emergency granulopoiesis (see appendix 6) in bone marrow (leading to reduction in LMPP, CLP & LT-HSC) and reduction in CXCL12 levels. Significant reduction in bone marrow CD169+ macrophage numbers and increased osteoclast activity further reduce bone marrow CXCL12 levels and helps HPC to exit the bone marrow. Induction of a strong IL-10 secretion from innate immune cells is invoked to prevent dysregulated inflammatory reaction as a consequence of continual TLR7 ligation.

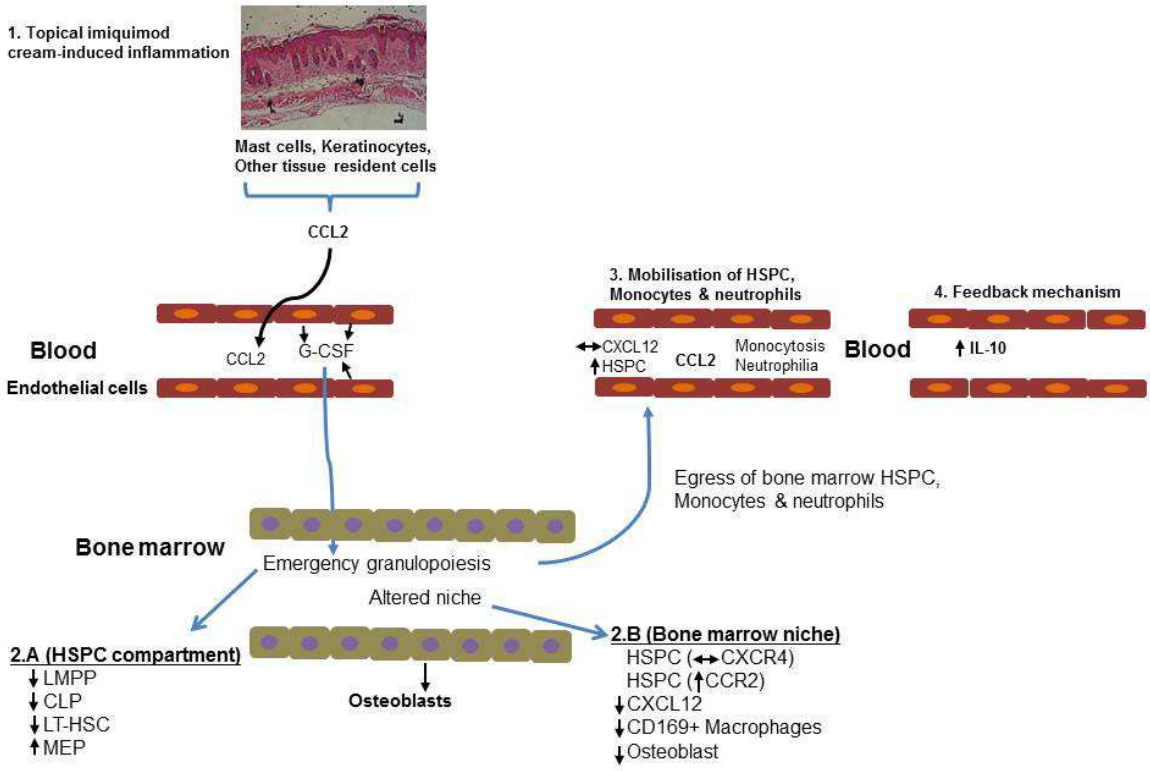



**Figure 6-2 Proposed mechanism for topical imiquimod cream-induced HPC mobilisation** **1**. Resident skin cells like keratinocytes and mast cells perceive the imiquimod cream treatment and release CCL2; The CCL2, and G-CSF (probably from skin endothelial cells as well as vascular endothelial cells), enter the circulation: **2**. Upregulated surface CCR2 expression on MEP (and monocytes and neutrophils?) enable them to respond to the circulating CCL2 and results in mobilisation of HPC, monocytes and neutrophils; G-CSF causes emergency granulopoiesis in bone marrow (leading to reduction in LMPP, CLP & LT-HSC) and reduction in CXCL12: **3**. Significant reduction in bone marrow CD169+ macrophages and increased osteoclast activity further helps HPC to exit the bone marrow: **4**. Induction of a strong IL-10 secretion to prevent dysregulated inflammatory reaction.

## 6.7 Future direction

In this study I was able to define some mechanistic details explaining the inflammation-induced HSPC mobilisation. However, certain fundamental questions remain unanswered. These include: (1) what happens to the mobilised HSPC, i.e. do they home back to the bone marrow or do they migrate to the point of stimuli application, and if they do, what fate awaits them? (2) What epigenetic changes occur in these mobilised HSPC and are they reprogrammed after the infection? In future experiments, it will be interesting to explore these questions. Future studies using adoptive transfer of genetically marked or fluorescently labelled HSPC into congenic mouse strains, and subsequent harvesting of the skin tissues for analysis, will be critical in providing answers to these questions. The harvested skin tissues could be examined by a combination of techniques such as IVIS imaging and flow cytometry to shed light on whether

the mobilised HSPC reach the inflamed skin and whether they differentiate to specific haematopoietic lineages.

In chapter 5, I showed that in the absence of CCR2 expression, topical imiquimod cream treatment significantly increased TREM-1 expression instead of IL-17/IL-23 expression as occurred in WT mice. However, due to time constraints, I was not able to undertake an in-depth exploration of the role of CCR2 in the production of TREM-1. As upregulation in TREM-1 has been implicated in the pathophysiology of sepsis and other inflammatory disease conditions (Bouchon et al., 2001, Gibot et al., 2005, Buckland et al., 2011, Lagler et al., 2009, Wu et al., 2011), it would be interesting to examine how targeting CCR2 could be a means of modulating TREM-1 expression for therapeutic benefit to patients.

Some previous data showed that increased osteoclast activity lead to the degradation of HSPC-niche factors resulting in HSPC mobilisation to the circulation (Kollet et al., 2006). However, other recent data have challenged this by showing that HSPC mobilisation was unaffected in osteoclast deficient mice and even reduced in mice with increased osteoclast activity (Miyamoto et al., 2011). Thus, the exact role of osteoclasts in HSPC biology remains an open question. In both systemic and peripheral inflammation models, I showed that there were significant increases in bone marrow Gr-$1^{low}$CD$115^{+}$ monocytes. As osteoclasts are derived from the monocytic lineage, it would be interesting to investigate whether osteoclast formation and activity were affected by any of the inflammatory models and whether these contributed to the inflammation-induced HSPC formation.

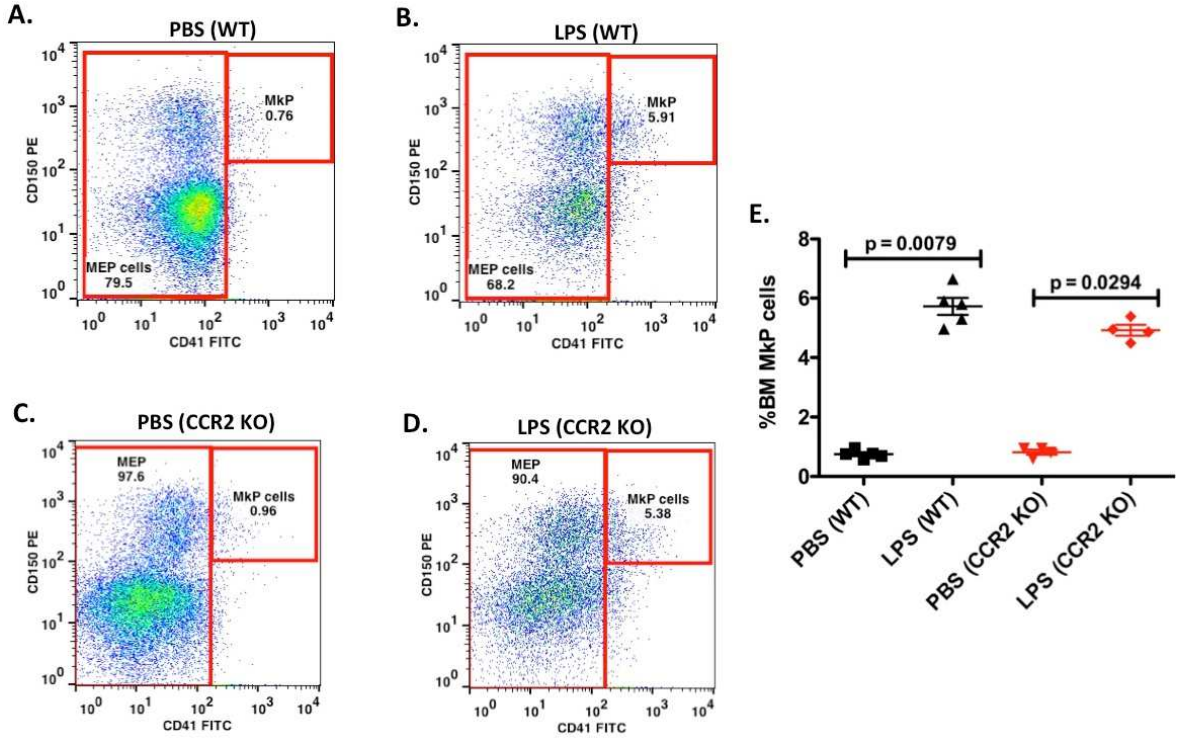
Although data presented in this thesis show that TPA treatment also mobilises HSPC to the circulation, there were a lot of unknowns in mechanism driving the TPA-induced HSPC mobilisation. This might have been due the time points at which data were collected. It would be interesting to further explore the TPA treatment model using earlier time points to try to understand the processes that led to HSPC egress from the bone marrow.

Lastly, in this thesis, snapshots were provided in connection with the impacts of inflammation on the bone marrow niche. It would be interesting to investigate how the inflammatory signals affected the distribution of the HSC in the niche.

For example, Kiel et al. used immunofluorescence technique to investigate the localisation of HSC in frozen bone sections of naïve/unmanipulated mice (Kiel et al., 2005). It would be interesting to use similar techniques to try to localise the HSC in relation to the endosteal and endothelial niches post inflammatory models to shed light on whether inflammation impacts the niche preference of HSC.

# Appendices

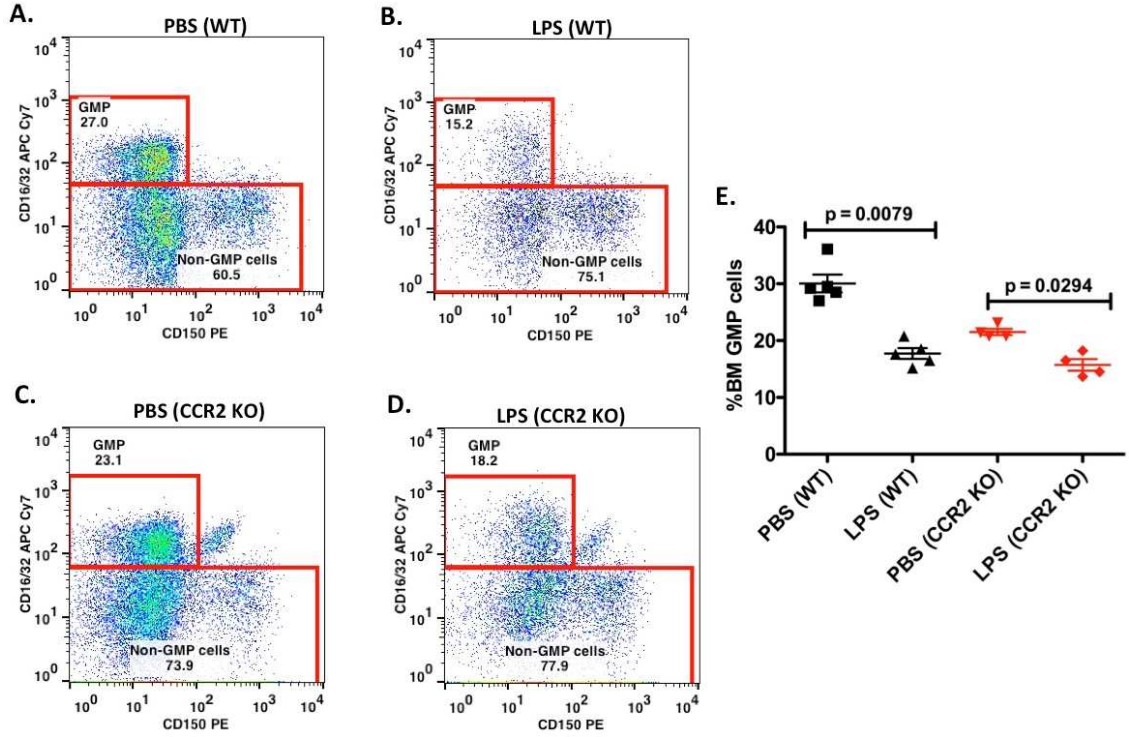
## Appendix 1




**Appendix 1 LPS injection significantly increases the bone marrow MkP cell numbers**

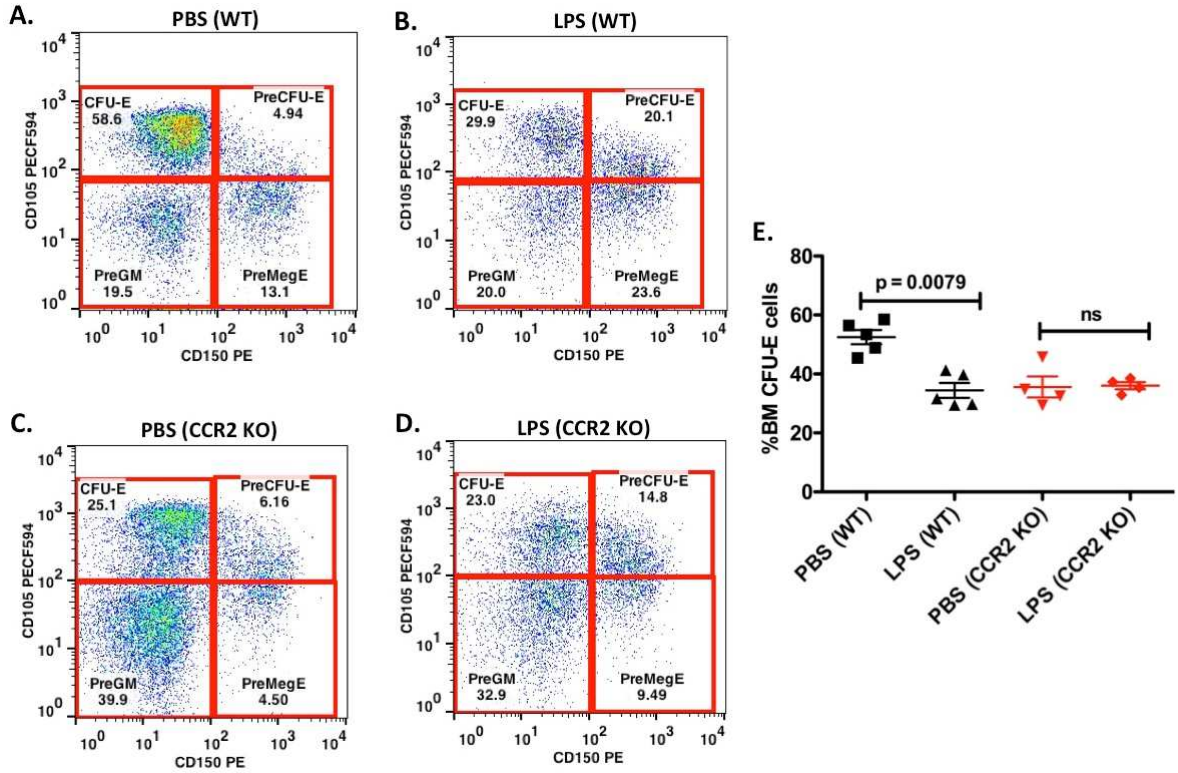
The $Kit^{+}IL\text{-}7R\alpha^{-}$ cell populations from the bone marrow of WT or CCR2 KO mice treated with either I.P. PBS or I.P. LPS were re-gated for the differential expression of CD41 and CD150 to elucidate MkP and MEP cell populations. A & B are the representative dot plots for MkP and MEP characterisation in the bone marrow of WT mice treated with I.P. PBS and I.P. LPS respectively. C & D are the representative dot plots for the MkP and MEP characterisation in the bone marrow of CCR2 KO mice treated with I.P. PBS and I.P. LPS respectively. E is a scatter plot summarising the impact of the I.P. PBS or I.P. LPS on the MkP population in both WT and CCR2 KO mice. Statistical significance was calculated using the Mann-Whitney U test (2-tailed) to compare the MkP population in I.P. PBS treated mice to the MkP population in I.P. LPS treated mice. [N= 4-5; 2-independent experiments].

## Appendix 2




**Appendix 2 LPS injection significantly reduces the bone marrow GMP cell numbers.** The MEP cell population from the bone marrow of WT or CCR2 KO mice treated with either I.P. PBS or I.P. LPS were re-gated for the differential expression of CD16/32 and CD150 antigens to characterise the GMP and non-GMP cell population. A & B are the representative dot plots for the GMP and non-GMP cell characterisation in the bone marrow of WT mice treated with I.P. PBS and I.P. LPS respectively. Similarly, C & D are the representative dot plots for the GMP and non-GMP cell characterisation in the bone marrow of CCR2 KO mice treated with either I.P. PBS (C) or I.P. LPS (D). E is a scatter plot summarising the impact of the I.P. PBS or I.P. LPS on the bone marrow GMP population in both C57B/L6 and CCR2 KO mice. Statistical significance was calculated using the Mann-Whitney U test (2-tailed) to compare the proportions of the GMP cells from I.P. PBS treated groups to that of I.P. LPS treated groups. [N= 4-5; 2-independent experiments].

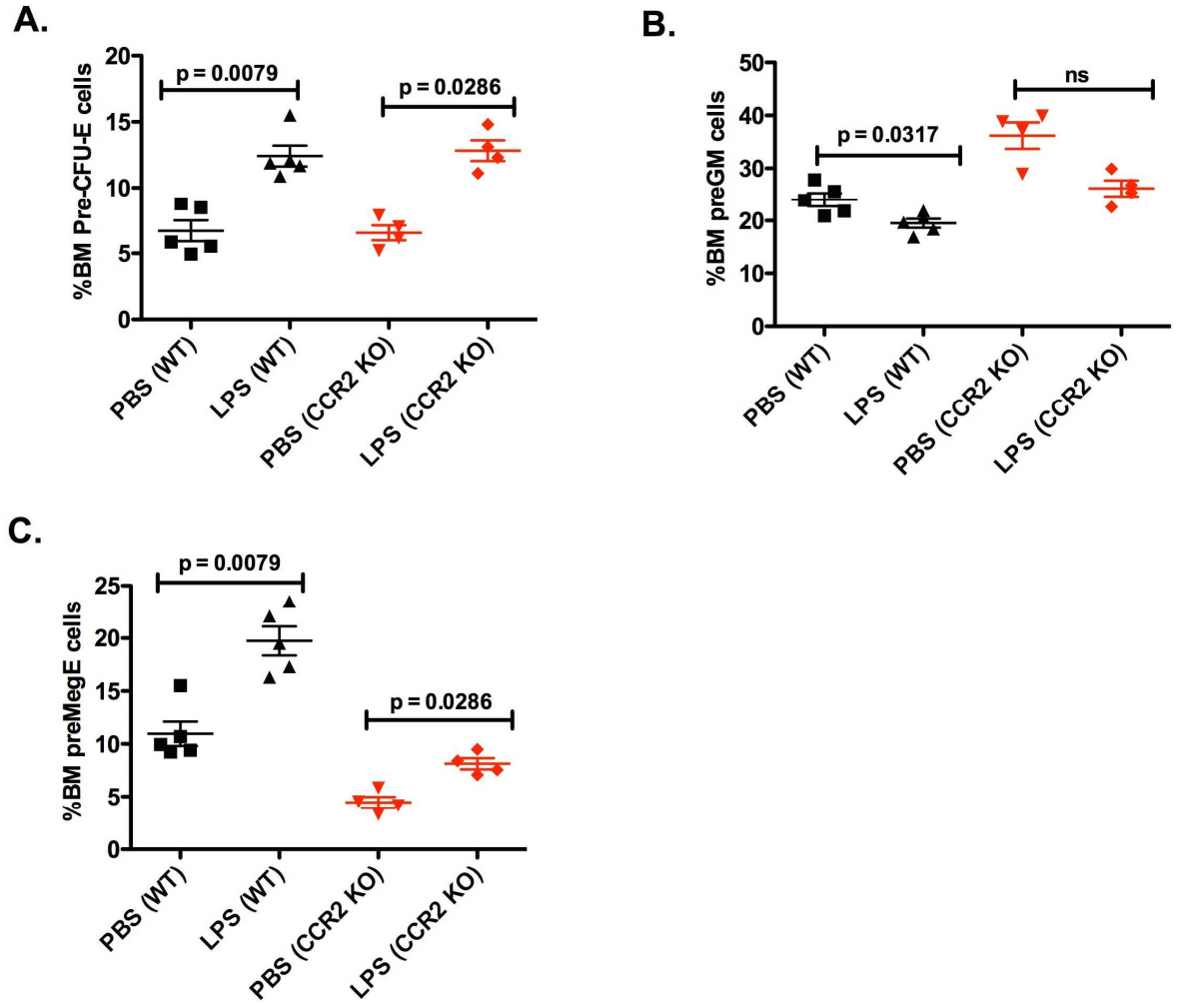
## Appendix 3




**Appendix 3 LPS injection significantly reduces the bone marrow CFU-E cell population in WT mice.**

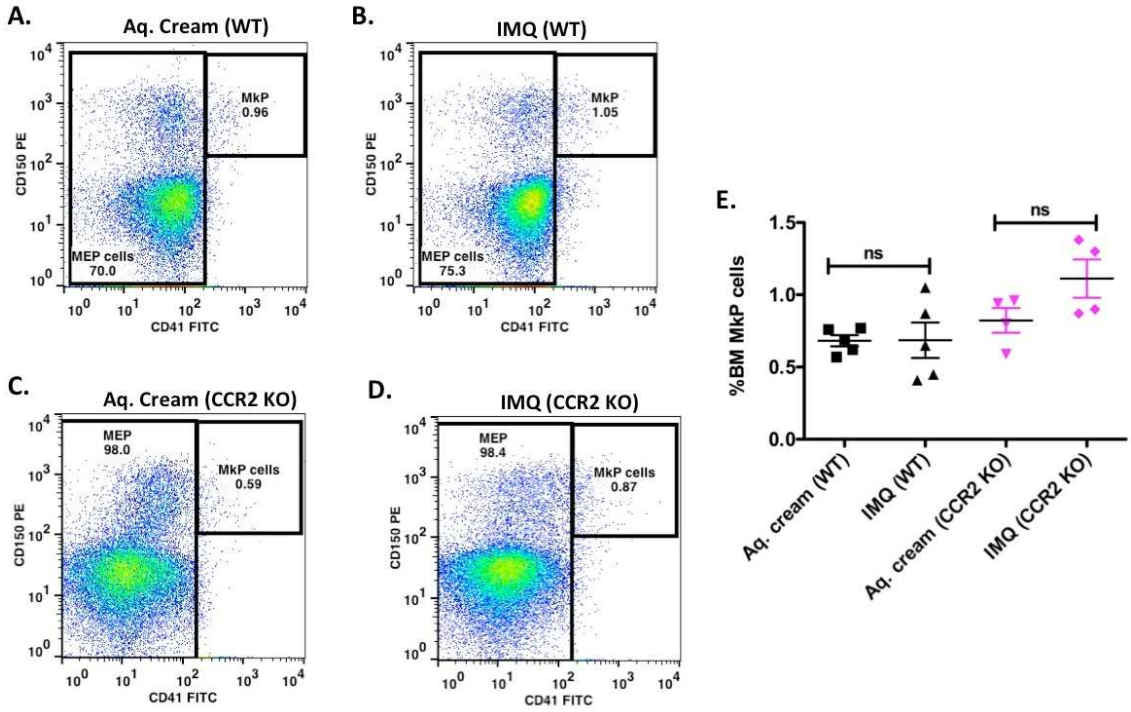
The bone marrow non-GMP cell populations of WT or CCR2 KO mice treated with either I.P. PBS or I.P. LPS were re-gated for the differential expression of CD105 and CD150 antigens to characterise the CFU-E, PreCFU-E, PreGM and the PreMegE cell populations. A & B are the representative dot plots showing the CFU-E, PreCFU-E, PreGM and the PreMegE cell characterisation in the bone marrow of WT mice treated with either I.P. PBS (A) or I.P. LPS (B). Similarly, C & D are the representative dot plots showing the CFU-E, PreCFU-E, PreGM and the PreMegE cell characterisation in the bone marrow of CCR2 KO mice treated with either I.P. PBS (D) or I.P. LPS (E). E is a scatter plot summarising the impact of the I.P. PBS or I.P. LPS on the CFU-E population in both WT and CCR2 KO mice. Statistical significance was calculated using the Mann-Whitney U test (2-tailed) to compare the proportions of the CFU-E cells from I.P. PBS treated groups to that of I.P. LPS treated groups. [N= 4-5; 2-independent experiments].

Appendix 4




**Appendix 4 LPS injection significantly modulates the bone marrow PreCFU-E, PreGM, and PreMegE cell populations.**
The bone marrow non-GMP cell population in WT or CCR2 KO mice treated with either I.P. PBS or I.P. LPS were re-gated for the differential expression of CD105 and CD150 antigens to characterise the CFU-E, PreCFU-E, PreGM and the PreMegE cell population. A is a scatter plot summarising the impact of the I.P. PBS or I.P. LPS injection on bone marrow PreCFU-E cells in WT and CCR2 KO mice. B is a scatter plot summarising the impact of the I.P. PBS or I.P. LPS injection on the bone marrow PreGM cells in WT and CCR2 KO mice. C is a scatter plot summarising the impact of the I.P. PBS or I.P. LPS injection on bone marrow PreMegE cells in WT and CCR2 KO mice. [Statistical significances were calculated in all cases by using the Mann-Whitney U test (2-tailed) to compare the proportions of the given progenitor population from the I.P. PBS treated groups to the respective population in the I.P. LPS treated groups. N= 4-5; (2-independent experiments in all cases)].

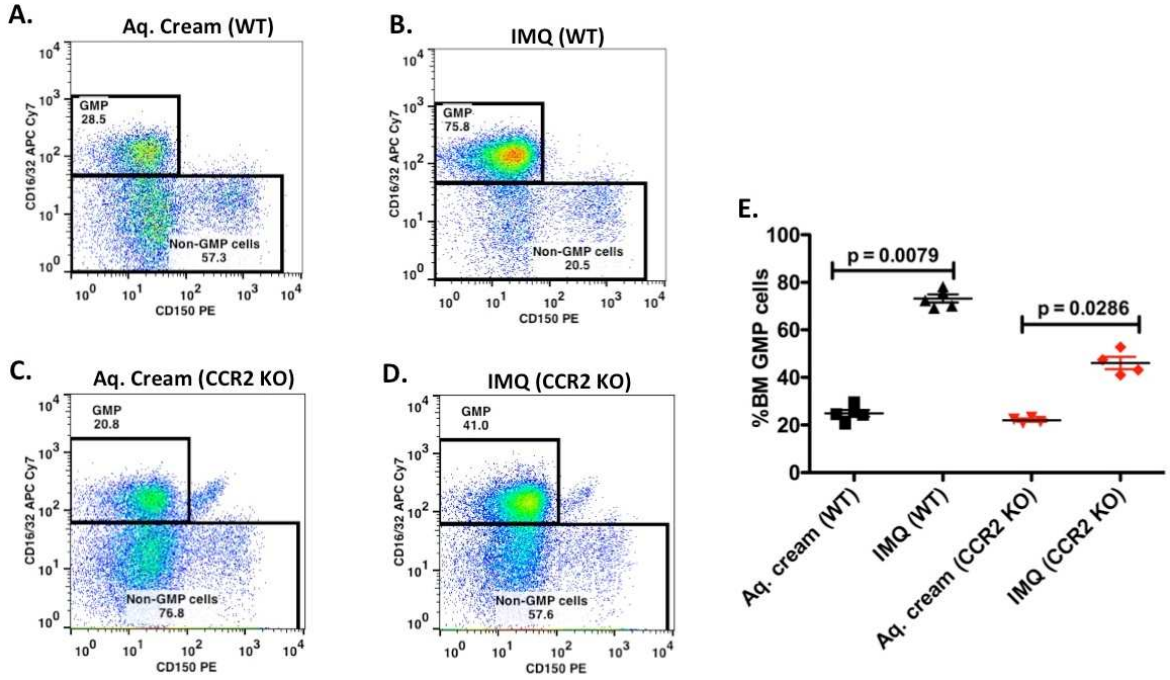
# Appendix 5




**Appendix 5 Topical imiquimod cream application does not significantly change the bone marrow MkP cell numbers.**

The $Kit^{+}IL\text{-}7R\alpha^{-}$ cell populations from the bone marrow of WT or CCR2 KO mice treated with either topical aqueous cream or topical imiquimod cream application were re-gated for the differential expression of CD41 and CD150 antigens to characterise the MkP and the MEP cell population. A & B are the representative dot plots for bone marrow MkP and MEP characterisation in WT mice treated with either topical aqueous cream (A) or topical imiquimod cream (B). C & D are the representative dot plots for bone marrow MkP and MEP characterisation in CCR2 KO mice treated with either topical aqueous cream (C) or topical imiquimod cream (D). E is a scatter plot summarising the impact of the topical aqueous cream or topical imiquimod cream application on the bone marrow MkP cells in both WT and CCR2 KO mice. Statistical significance was calculated by using the Mann-Whitney U test (2-tailed) to compare the proportions of the MkP cells in the aqueous cream treated groups to the proportion of MkP cells in the imiquimod cream treated groups. [N= 4-5; 2-independent experiments].

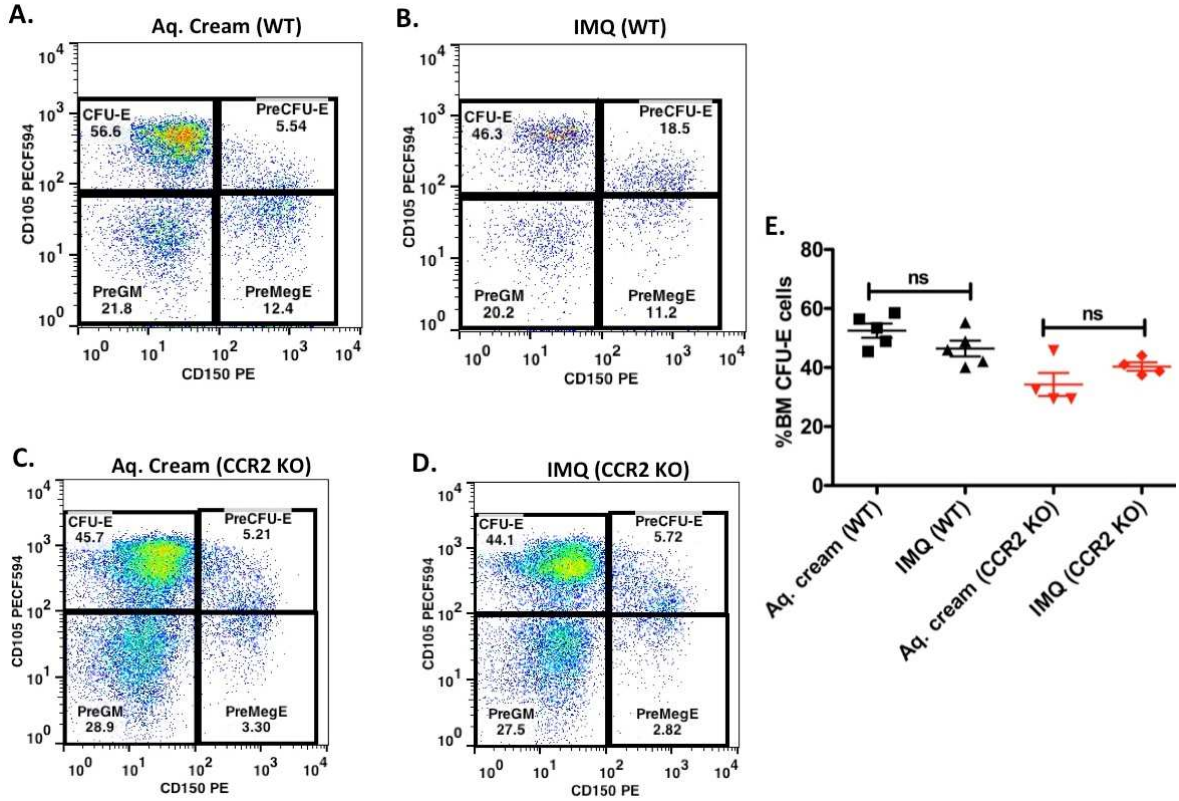
# Appendix 6




**Appendix 6 Topical imiquimod cream application significantly increases the bone marrow GMP cell numbers.**

The MEP cell population from the bone marrow of WT or CCR2 KO mice treated with either topical aqueous cream or topical imiquimod cream were re-gated for the differential expression of CD16/32 and CD150 antigens to characterise the GMP and the non-GMP cell population. A & B are the representative dot plots of bone marrow GMP and non-GMP cell characterisation in WT mice treated with either topical aqueous cream (B) or topical imiquimod cream (B). C & D are the representative dot plots for bone marrow GMP and non-GMP cell characterisation in CCR2 KO mice treated with either topical aqueous cream (C) or topical imiquimod cream (D). E is a scatter plot summarising the impact of the topical aqueous cream or topical imiquimod cream application on the bone marrow GMP population in both WT and CCR2 KO mice. Statistical significance was calculated using the Mann-Whitney U test (2-tailed) to compare the proportion of the GMP cells in the topical aqueous cream treated groups to the proportion of GMP cells in the topical imiquimod cream treated groups. [N= 4-5; 2-independent experiments].

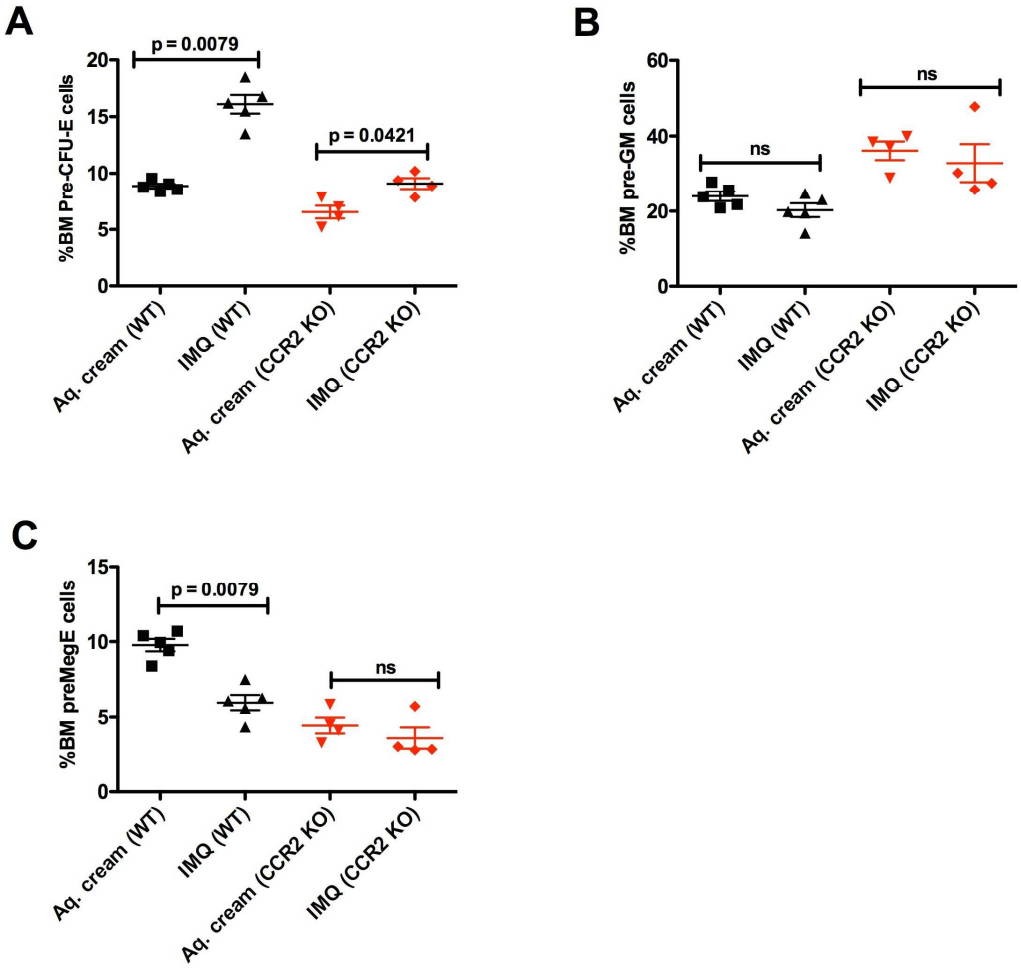
# Appendix 7




**Appendix 7 Topical imiquimod cream application does not significantly change the bone marrow CFU-E cell numbers.**

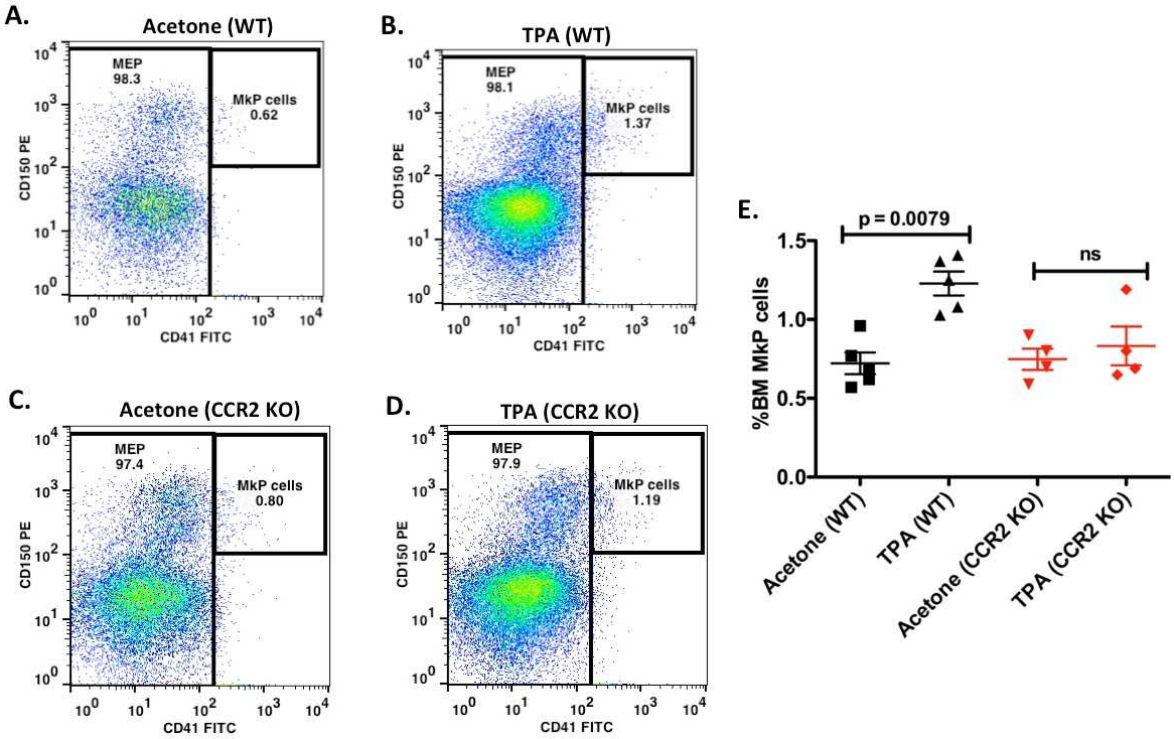
The bone marrow non-GMP cell population in WT or CCR2 KO mice treated with either topical aqueous cream, or topical imiquimod cream, were re-gated for the differential expression of CD105 and CD150 antigens to characterise the CFU-E, PreCFU-E, PreGM and the PreMegE cell populations. A & B are the representative dot plots for the CFU-E, PreCFU-E, PreGM and the PreMegE cell characterisation in WT mice treated with either topical aqueous cream (A) or imiquimod cream (B). C & D are the representative dot plots for the CFU-E, PreCFU-E, PreGM and the PreMegE cell characterisation in CCR2 KO mice treated with either topical aqueous cream (C) or imiquimod cream (D). E is a scatter plot summarising the impact of the topical aqueous cream or topical imiquimod cream application on the bone marrow CFU-E population in both WT and CCR2 KO mice. Statistical significance was calculated using the Mann-Whitney U test (2-tailed) to compare the proportions of the CFU-E cells from topical aqueous cream treated groups to that of topical imiquimod cream treated groups. [N= 4-5; 2-independent experiments].

Appendix 8




**Appendix 8 Topical imiquimod cream treatment modulates the bone marrow PreCFU-E, PreGM, and PreMegE cell numbers.**
The bone marrow non-GMP cell population in WT or CCR2 KO mice treated with either topical aqueous cream or topical imiquimod cream were re-gated for the differential expression of CD105 and CD150 antigens to characterise the CFU-E, PreCFU-E, PreGM and the PreMegE cell populations. A is a scatter plot summarising the impact of the topical aqueous cream or topical imiquimod cream treatment on the bone marrow PreCFU-E in WT and CCR2 KO mice. B is a scatter plot summarising the impact of the topical aqueous cream or topical imiquimod cream treatment on bone marrow PreGM cells in WT and CCR2 KO mice. C is a scatter plot summarising the impact of the topical aqueous cream or topical imiquimod cream application on bone marrow PreMegE cells in WT and CCR2 KO mice. [Statistical significances were calculated in all cases by using the Mann-Whitney U test (2-tailed) to compare the proportions of the given progenitor population from topical aqueous cream treated groups to the respective population in the topical imiquimod cream treated groups. N= 4-5; (2-independent experiments in all cases)].

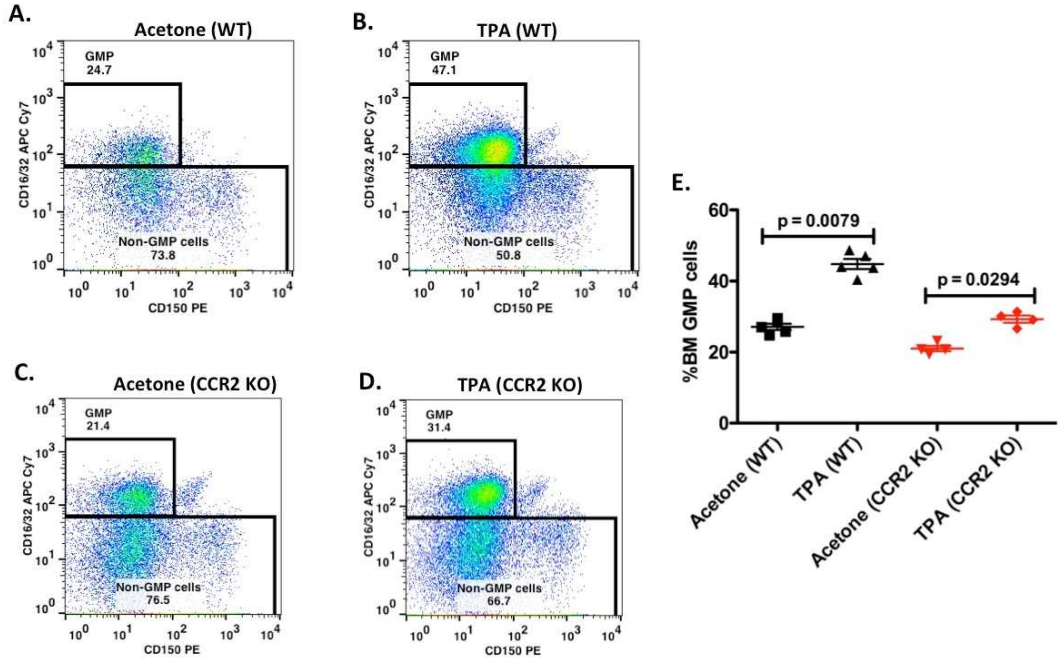
# Appendix 9




**Appendix 9 Topical TPA application significantly increases the bone marrow MkP cell numbers in WT mice.**

The $Kit^{+}IL\text{-}7R\alpha^{-}$ cell populations from the bone marrow of WT or CCR2 KO mice treated with either topical acetone or TPA application were re-gated for the differential expression of CD41 and CD150 antigens to characterise the MkP and the MEP cell populations. A & B are the representative dot plots for the bone marrow MkP and MEP characterisation in WT mice treated with either topical acetone (A) or TPA (B). Similarly, C & D are the representative dot plots for the bone marrow MkP and MEP characterisation in CCR2 KO mice treated with either topical acetone (C) or TPA (D). E is a scatter plot summarising the impact of the topical acetone or TPA application on the MkP population in both WT and CCR2 KO mice. Statistical significance was calculated by using the Mann-Whitney U test (2-tailed) to compare the proportions of the MkP cells in the topical acetone treated groups to the proportion of the MkP cell from the topical TPA treated groups. [N= 4-5; 2-independent experiments].

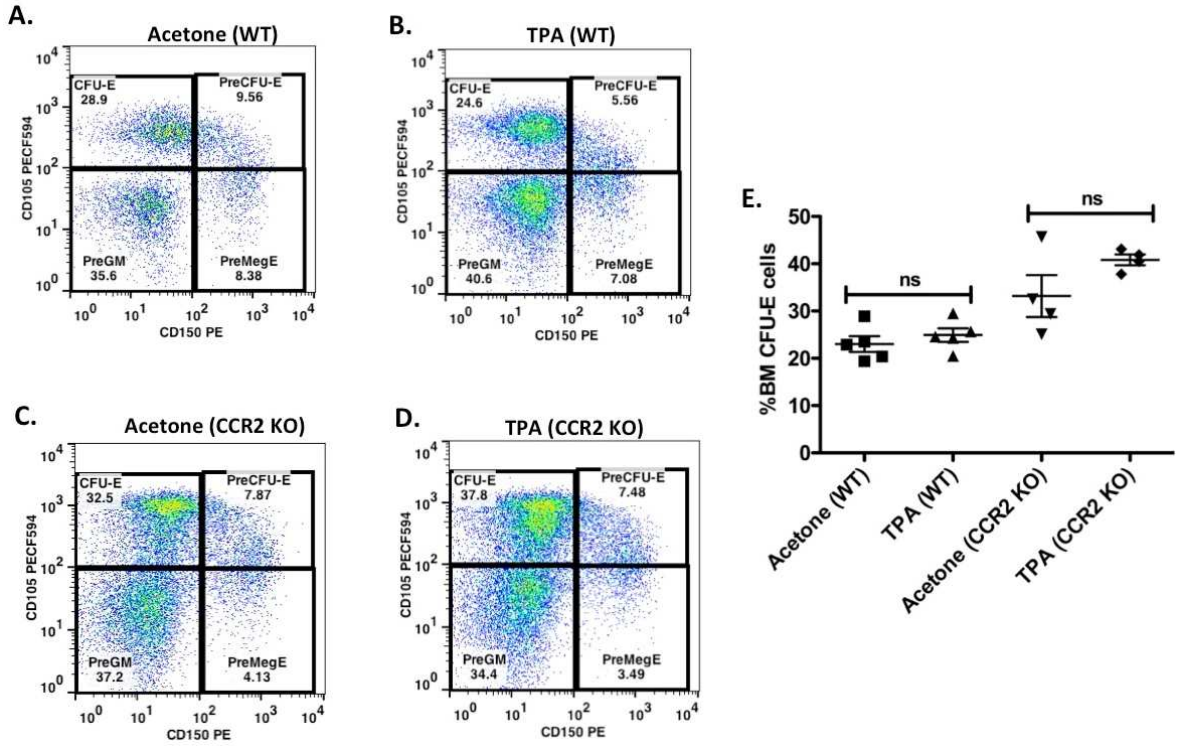
# Appendix 10




**Appendix 10 Topical TPA application significantly increases the bone marrow GMP cell numbers.**

The bone marrow MEP cell populations in WT or CCR2 KO mice treated with either topical acetone, or TPA, application were re-gated for the differential expression of CD16/32 and CD150 to characterise the GMP and the non-GMP cell populations. A & B are the representative dot plots showing the bone marrow GMP and non-GMP cell characterisation in WT mice treated with either topical acetone (A), or TPA (B). C & D are the representative dot plots for the bone marrow GMP and non-GMP cell characterisation in CCR2 KO mice treated with either topical acetone (C), or TPA (D). E is a scatter plot summarising the impact of the topical acetone, or TPA treatment on the GMP population in both WT and CCR2 KO mice. Statistical significance was calculated using the Mann-Whitney U test (2-tailed) to compare the proportion of the GMP cells from the bone marrow of topical acetone treated groups to the proportion of GMP cells from the bone marrow of topical TPA treated groups. [N= 4-5; 2-independent experiments].

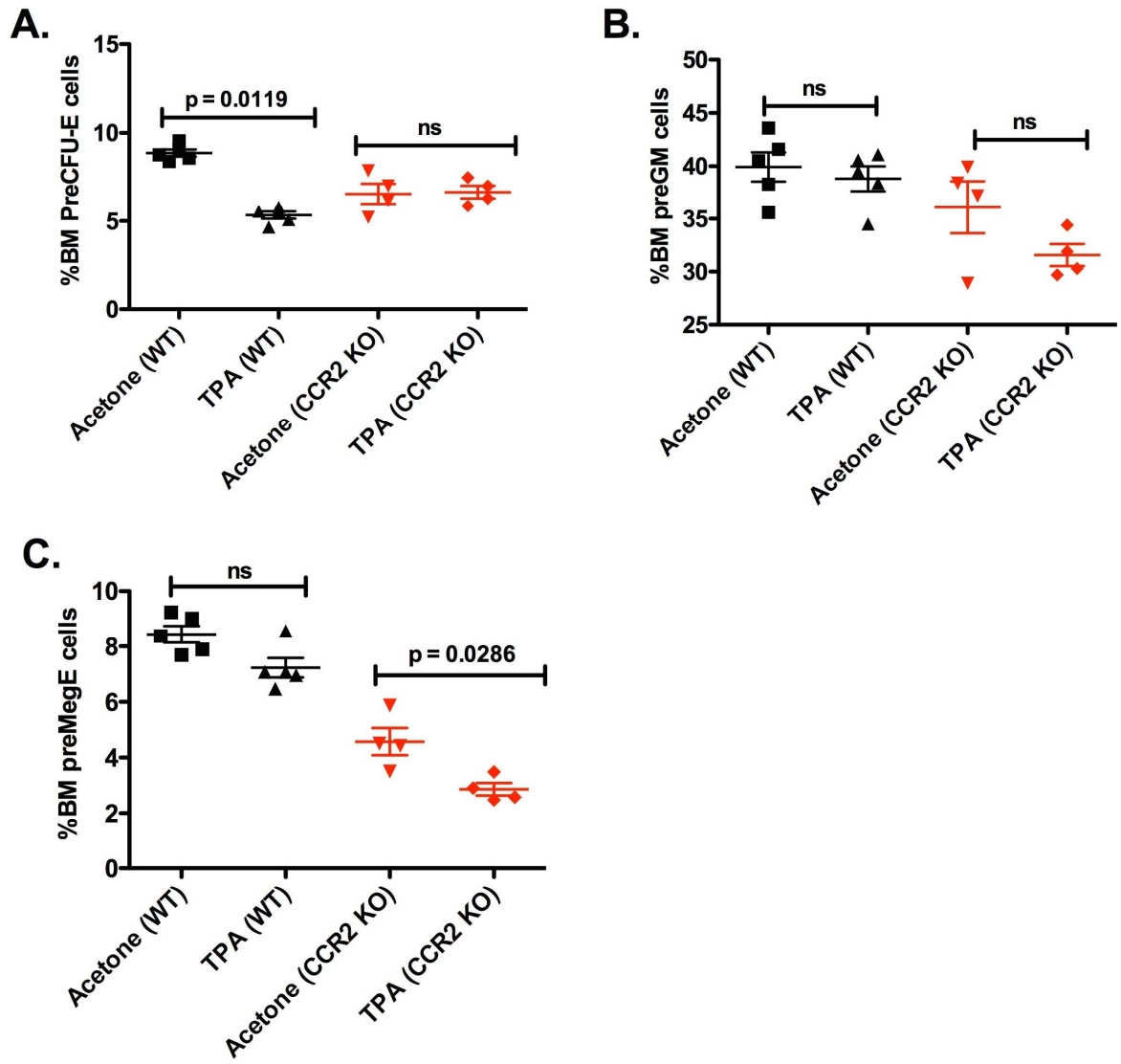
# Appendix 11




**Appendix 11 Topical TPA application does not significantly change the bone marrow CFU-E cell numbers.**

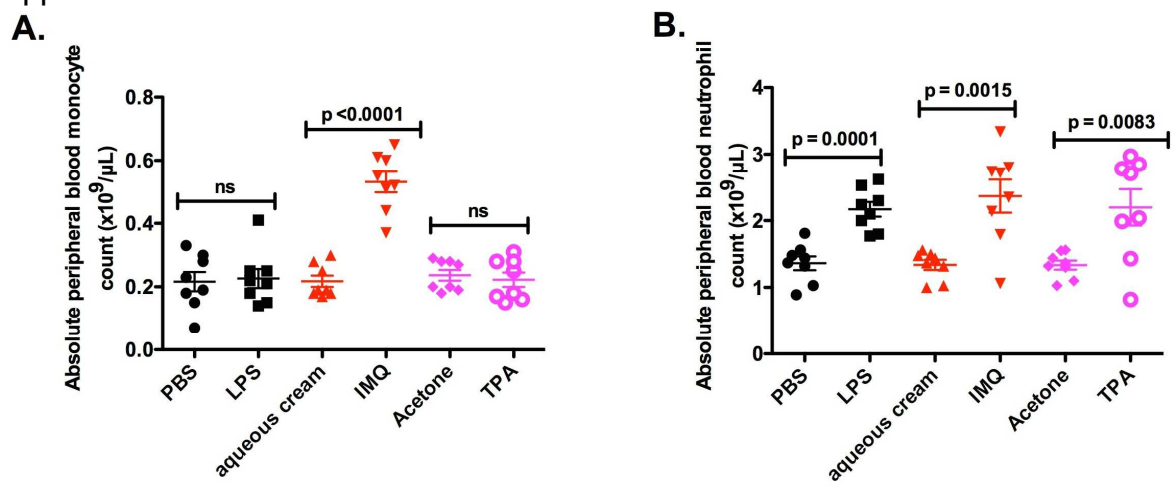
The bone marrow non-GMP cell populations in WT or CCR2 KO mice treated with either topical acetone, or TPA were re-gated for the differential expression of CD105 and CD150 antigens to characterise the CFU-E, PreCFU-E, PreGM and the PreMegE cell populations. A & B are the representative dot plots for the CFU-E, PreCFU-E, PreGM and the PreMegE cell characterisation in the bone marrow of WT mice treated with either topical acetone (A), or TPA (B). C & D are the representative dot plots of the CFU-E, PreCFU-E, PreGM and the PreMegE cell characterisation in CCR2 KO mice treated with either topical acetone (C), or TPA (D). E is a scatter plot summarising the impact of the topical acetone, or TPA, treatment on the CFU-E population in both WT and CCR2 KO mice. Statistical significance was calculated using the Mann-Whitney U test (2-tailed) to compare the proportions of the CFU-E cells from topical acetone treated groups to that of topical TPA treated groups. [N= 4-5; 2-independent experiments].

Appendix 12




**Appendix 12 Topical TPA application modulates the bone marrow PreCFU-E, PreGM, and PreMegE cell population.**
The bone marrow non-GMP cell populations in WT or CCR2 KO mice treated with either topical acetone, or TPA were re-gated for the differential expressions of CD105 and CD150 antigens to characterise the CFU-E, PreCFU-E, PreGM and the PreMegE cell populations. A is a scatter plot summarising the impact of the topical acetone, or TPA, application on the bone marrow PreCFU-E cells in WT and CCR2 KO mice. B is a scatter plot summarising the impact of the topical acetone, or TPA, application on the bone marrow PreGM cells in WT and CCR2 KO mice. C is a scatter plot summarising the impact of the topical acetone, or TPA, application on the bone marrow PreMegE cells in WT and CCR2 KO mice. [Statistical significances were calculated in all cases by using the Mann-Whitney U test (2-tailed) to compare the proportions of the given progenitor population from topical acetone treated groups to the respective population in the topical TPA treated groups. N= 4-5; (2-independent experiments in all cases)].

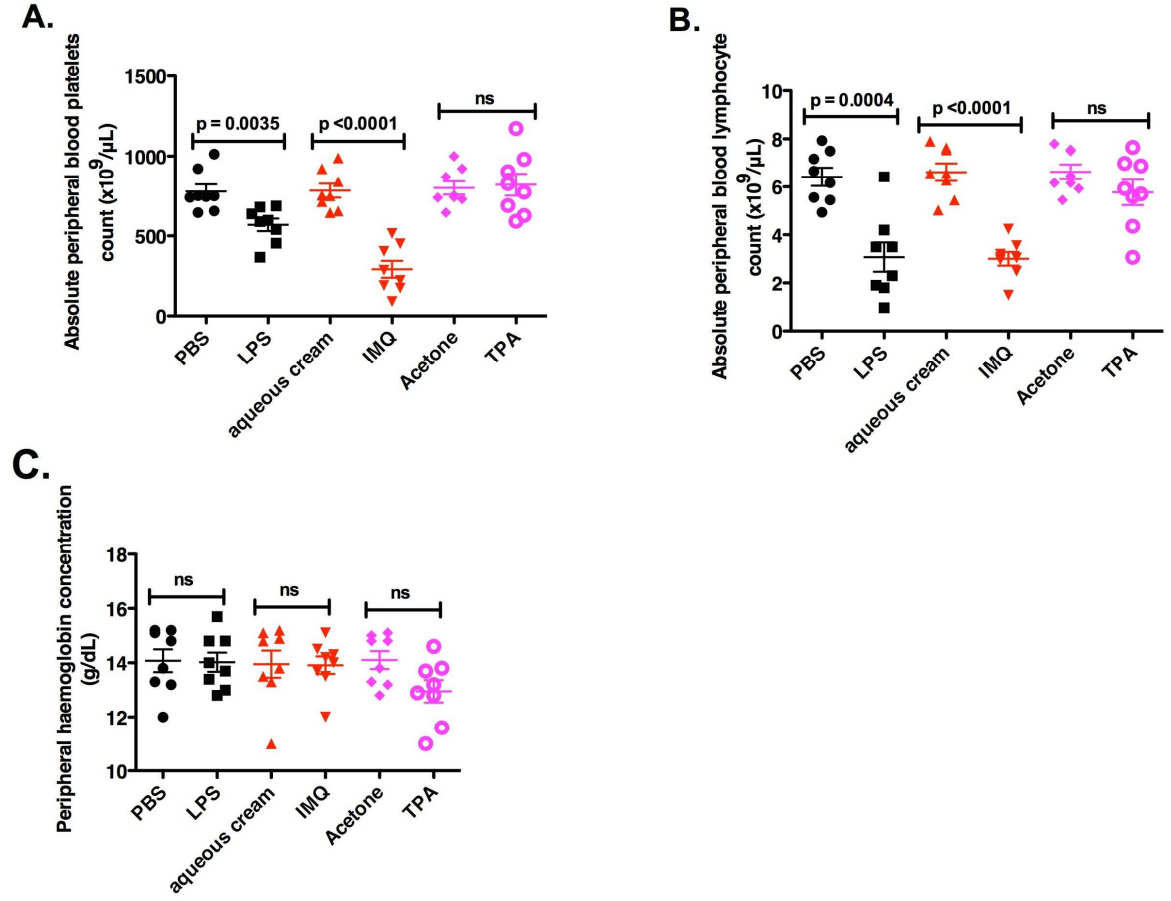
Appendix 13




**Appendix 13 Modulation of peripheral blood monocyte and neutrophil counts in WT mice treated with I.P. LPS or topical imiquimod cream/TPA.**

WT mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application to shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day 3 of the model, other groups of mice received a single dose of I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS as a control. On the fourth day, the mice were euthanized by $CO_2$ asphyxiation, peripheral blood was taken into EDTA microtainer tubes and full blood counts assessed by the automated veterinary haematology analyser, HEMAVET 950FS (Drew Scientific Inc., USA). A & B are scatter plots summarising the peripheral blood monocyte and neutrophil counts respectively mice treated with I.P. LPS injection or topical imiquimod cream or TPA (or their respective controls). Each treatment group was compared to its respective control group using the unpaired t-test (2-tailed) to determine the statistical significance. [n=8).

Appendix 14




**Appendix 14 Peripheral blood platelet, lymphocyte counts and haemoglobin concentration in mice treated with I.P. LPS or topical imiquimod cream/TPA.**
WT mice received a single, daily dose of topical imiquimod cream (62.5mg) or TPA (100µM in 100µL acetone) application to the shaved dorsal skin for three consecutive days. Simultaneously, other groups of mice received equivalent amounts of topical application of either aqueous cream or acetone application as controls for topical imiquimod cream and TPA application respectively. On day 3 of the model, other groups of mice received a single dose of I.P. LPS (100µg in 100µL PBS) or 100µL I.P. PBS as a control. On the fourth day, the mice were euthanized by $CO_2$ asphyxiation, peripheral blood was taken into EDTA microtainer tubes and full blood counts assessed by the automated veterinary hematology analyser, HEMAVET 950FS (Drew Scientific Inc., USA). A, B & C are scatter plots summarising the peripheral blood platelet counts, lymphocyte counts and haemoglobin concentration respectively in WT mice treated with I.P. LPS injection or topical imiquimod cream or TPA (or their respective controls). Each treatment group was compared to its respective control group using the unpaired t-test (2-tailed) to determine the statistical significance. [n=8).

# List of References

ABREU, M. T., THOMAS, L. S., ARNOLD, E. T., LUKASEK, K., MICHELSEN, K. S. & ARDITI, M. 2003. TLR signaling at the intestinal epithelial interface. *J Endotoxin Res,* 9**,** 322-30.

ADAMO, L., NAVEIRAS, O., WENZEL, P. L., MCKINNEY-FREEMAN, S., MACK, P. J., GRACIA-SANCHO, J., SUCHY-DICEY, A., YOSHIMOTO, M., LENSCH, M. W., YODER, M. C., GARCIA-CARDENA, G. & DALEY, G. Q. 2009. Biomechanical forces promote embryonic haematopoiesis. *Nature,* 459**,** 1131-5.

ADAMS, G. B., CHABNER, K. T., ALLEY, I. R., OLSON, D. P., SZCZEPIORKOWSKI, Z. M., POZNANSKY, M. C., KOS, C. H., POLLAK, M. R., BROWN, E. M. & SCADDEN, D. T. 2006. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature,* 439**,** 599-603.

ADLER, B. K., SALZMAN, D. E., CARABASI, M. H., VAUGHAN, W. P., REDDY, V. V. & PRCHAL, J. T. 2001. Fatal sickle cell crisis after granulocyte colony-stimulating factor administration. *Blood,* 97**,** 3313-4.

ADLER, M. W., GELLER, E. B., CHEN, X. & ROGERS, T. J. 2005. Viewing chemokines as a third major system of communication in the brain. *AAPS J,* 7**,** E865-70.

ADOLFSSON, J., MANSSON, R., BUZA-VIDAS, N., HULTQUIST, A., LIUBA, K., JENSEN, C. T., BRYDER, D., YANG, L., BORGE, O. J., THOREN, L. A., ANDERSON, K., SITNICKA, E., SASAKI, Y., SIGVARDSSON, M. & JACOBSEN, S. E. 2005. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell,* 121**,** 295-306.

AGRAWAL, S., AGRAWAL, A., DOUGHTY, B., GERWITZ, A., BLENIS, J., VAN DYKE, T. & PULENDRAN, B. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol,* 171**,** 4984-9.

AIUTI, A., WEBB, I. J., BLEUL, C., SPRINGER, T. & GUTIERREZ-RAMOS, J. C. 1997. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med,* 185**,** 111-20.

AKIRA, S., UEMATSU, S. & TAKEUCHI, O. 2006. Pathogen recognition and innate immunity. *Cell,* 124**,** 783-801.

ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* 413**,** 732-8.

ALKHATIB, G., COMBADIERE, C., BRODER, C. C., FENG, Y., KENNEDY, P. E., MURPHY, P. M. & BERGER, E. A. 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science,* 272**,** 1955-8.

ANDERLINI, P., PRZEPIORKA, D., SEONG, C., SMITH, T. L., HUH, Y. O., LAUPPE, J., CHAMPLIN, R. & KORBLING, M. 1997. Factors affecting mobilization of CD34+ cells in normal donors treated with filgrastim. *Transfusion,* 37**,** 507-12.

ANDERLINI, P., PRZEPIORKA, D., SEONG, D., MILLER, P., SUNDBERG, J., LICHTIGER, B., NORFLEET, F., CHAN, K. W., CHAMPLIN, R. & KORBLING, M. 1996. Clinical toxicity and laboratory effects of granulocyte-colony-stimulating factor (filgrastim) mobilization and blood stem cell apheresis from normal donors, and analysis of charges for the procedures. *Transfusion,* 36**,** 590-5.

ANDERSON, D. M., LYMAN, S. D., BAIRD, A., WIGNALL, J. M., EISENMAN, J., RAUCH, C., MARCH, C. J., BOSWELL, H. S., GIMPEL, S. D., COSMAN, D. & ET AL. 1990. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell,* 63**,** 235-43.

ANDERSON, D. M., WILLIAMS, D. E., TUSHINSKI, R., GIMPEL, S., EISENMAN, J., CANNIZZARO, L. A., ARONSON, M., CROCE, C. M., HUEBNER, K., COSMAN, D. & ET AL. 1991. Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24. *Cell Growth Differ,* 2**,** 373-8.

ARAI, F., HIRAO, A., OHMURA, M., SATO, H., MATSUOKA, S., TAKUBO, K., ITO, K., KOH, G. Y. & SUDA, T. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell,* 118**,** 149-61.

ARAI, H. & CHARO, I. F. 1996. Differential regulation of G-protein-mediated signaling by chemokine receptors. *J Biol Chem,* 271**,** 21814-9.

AREND, W. P. 2002. The balance between IL-1 and IL-1Ra in disease. *Cytokine Growth Factor Rev,* 13**,** 323-40.

ARROYO, A. G., YANG, J. T., RAYBURN, H. & HYNES, R. O. 1996. Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. *Cell,* 85**,** 997-1008.

ARROYO, A. G., YANG, J. T., RAYBURN, H. & HYNES, R. O. 1999. Alpha4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. *Immunity,* 11**,** 555-66.

AUSTIN, T. W., SOLAR, G. P., ZIEGLER, F. C., LIEM, L. & MATTHEWS, W. 1997. A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. *Blood,* 89**,** 3624-35.

BABA, Y., GARRETT, K. P. & KINCADE, P. W. 2005. Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. *Immunity,* 23**,** 599-609.

BABA, Y., YOKOTA, T., SPITS, H., GARRETT, K. P., HAYASHI, S. & KINCADE, P. W. 2006. Constitutively active beta-catenin promotes expansion of multipotent hematopoietic progenitors in culture. *J Immunol,* 177**,** 2294-303.

BACHELERIE, F., GRAHAM, G. J., LOCATI, M., MANTOVANI, A., MURPHY, P. M., NIBBS, R., ROT, A., SOZZANI, S. & THELEN, M. 2014. New nomenclature for atypical chemokine receptors. *Nat Immunol,* 15**,** 207-8.

BAFICO, A., GAZIT, A., PRAMILA, T., FINCH, P. W., YANIV, A. & AARONSON, S. A. 1999. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. *J Biol Chem,* 274**,** 16180-7.

BAFICO, A., LIU, G., YANIV, A., GAZIT, A. & AARONSON, S. A. 2001. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol,* 3**,** 683-6.

BAGGIOLINI, M. 1998. Chemokines and leukocyte traffic. *Nature,* 392**,** 565-8.

BAHLMANN, F. H., SONG, R., BOEHM, S. M., MENGEL, M., VON WASIELEWSKI, R., LINDSCHAU, C., KIRSCH, T., DE GROOT, K., LAUDELEY, R., NIEMCZYK, E., GULER, F., MENNE, J., HALLER, H. & FLISER, D. 2004. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. *Circulation,* 110**,** 1006-12.

BAIN, G., ENGEL, I., ROBANUS MAANDAG, E. C., TE RIELE, H. P., VOLAND, J. R., SHARP, L. L., CHUN, J., HUEY, B., PINKEL, D. & MURRE, C. 1997. E2A

deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol,* 17**,** 4782-91.

BALABANIAN, K., LAGANE, B., INFANTINO, S., CHOW, K. Y., HARRIAGUE, J., MOEPPS, B., ARENZANA-SEISDEDOS, F., THELEN, M. & BACHELERIE, F. 2005. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem,* 280**,** 35760-6.

BARBER, D. L., BEATTIE, B. K., MASON, J. M., NGUYEN, M. H., YOAKIM, M., NEEL, B. G., D'ANDREA, A. D. & FRANK, D. A. 2001. A common epitope is shared by activated signal transducer and activator of transcription-5 (STAT5) and the phosphorylated erythropoietin receptor: implications for the docking model of STAT activation. *Blood,* 97**,** 2230-7.

BARRETT, A. J., LONGHURST, P., SNEATH, P. & WATSON, J. G. 1978. Mobilization of CFU-C by exercise and ACTH induced stress in man. *Exp Hematol,* 6**,** 590-4.

BASU, P., MORRIS, P. E., HAAR, J. L., WANI, M. A., LINGREL, J. B., GAENSLER, K. M. & LLOYD, J. A. 2005. KLF2 is essential for primitive erythropoiesis and regulates the human and murine embryonic beta-like globin genes in vivo. *Blood,* 106**,** 2566-71.

BELLUCCI, R., DE PROPRIS, M. S., BUCCISANO, F., LISCI, A., LEONE, G., TABILIO, A. & DE FABRITIIS, P. 1999. Modulation of VLA-4 and L-selectin expression on normal CD34+ cells during mobilization with G-CSF. *Bone Marrow Transplant,* 23**,** 1-8.

BENSINGER, W., APPELBAUM, F., ROWLEY, S., STORB, R., SANDERS, J., LILLEBY, K., GOOLEY, T., DEMIRER, T., SCHIFFMAN, K., WEAVER, C. & ET AL. 1995. Factors that influence collection and engraftment of autologous peripheral-blood stem cells. *J Clin Oncol,* 13**,** 2547-55.

BENSINGER, W. I., BUCKNER, C. D., ROWLEY, S., STORB, R. & APPELBAUM, F. R. 1996. Treatment of normal donors with recombinant growth factors for transplantation of allogeneic blood stem cells. *Bone Marrow Transplant,* 17 Suppl 2**,** S19-21.

BENSINGER, W. I., LONGIN, K., APPELBAUM, F., ROWLEY, S., WEAVER, C., LILLEBY, K., GOOLEY, T., LYNCH, M., HIGANO, T., KLARNET, J. & ET AL. 1994. Peripheral blood stem cells (PBSCs) collected after recombinant granulocyte colony stimulating factor (rhG-CSF): an analysis of factors correlating with the tempo of engraftment after transplantation. *Br J Haematol,* 87**,** 825-31.

BERNSTEIN, I. D., ANDREWS, R. G. & ZSEBO, K. M. 1991. Recombinant human stem cell factor enhances the formation of colonies by CD34+ and CD34+lin- cells, and the generation of colony-forming cell progeny from CD34+lin- cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. *Blood,* 77**,** 2316-21.

BERTRAND, J. Y., CHI, N. C., SANTOSO, B., TENG, S., STAINIER, D. Y. & TRAVER, D. 2010a. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature,* 464**,** 108-11.

BERTRAND, J. Y., CISSON, J. L., STACHURA, D. L. & TRAVER, D. 2010b. Notch signaling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. *Blood,* 115**,** 2777-83.

BESMER, P., MURPHY, J. E., GEORGE, P. C., QIU, F. H., BERGOLD, P. J., LEDERMAN, L., SNYDER, H. W., JR., BRODEUR, D., ZUCKERMAN, E. E. & HARDY, W. D. 1986. A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. *Nature,* 320**,** 415-21.

BHATTACHARYA, D., EHRLICH, L. I. & WEISSMAN, I. L. 2008. Space-time considerations for hematopoietic stem cell transplantation. *Eur J Immunol,* 38**,** 2060-7.

BLECHMAN, J. M., LEV, S., BARG, J., EISENSTEIN, M., VAKS, B., VOGEL, Z., GIVOL, D. & YARDEN, Y. 1995. The fourth immunoglobulin domain of the stem cell factor receptor couples ligand binding to signal transduction. *Cell,* 80**,** 103-13.

BLECHMAN, J. M., LEV, S., BRIZZI, M. F., LEITNER, O., PEGORARO, L., GIVOL, D. & YARDEN, Y. 1993. Soluble c-kit proteins and antireceptor monoclonal antibodies confine the binding site of the stem cell factor. *J Biol Chem,* 268**,** 4399-406.

BLEHARSKI, J. R., KIESSLER, V., BUONSANTI, C., SIELING, P. A., STENGER, S., COLONNA, M. & MODLIN, R. L. 2003. A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J Immunol,* 170**,** 3812-8.

BLUMBERG, P. M., JAKEN, S., KONIG, B., SHARKEY, N. A., LEACH, K. L., JENG, A. Y. & YEH, E. 1984a. Mechanism of action of the phorbol ester tumor promoters: specific receptors for lipophilic ligands. *Biochem Pharmacol,* 33**,** 933-40.

BLUMBERG, P. M., KONIG, B., SHARKEY, N. A., LEACH, K. L., JAKEN, S. & JENG, A. Y. 1984b. Analysis of membrane and cytosolic phorbol ester receptors. *IARC Sci Publ***,** 139-56.

BOETTCHER, S., ZIEGLER, P., SCHMID, M. A., TAKIZAWA, H., VAN ROOIJEN, N., KOPF, M., HEIKENWALDER, M. & MANZ, M. G. 2012. Cutting edge: LPS-induced emergency myelopoiesis depends on TLR4-expressing nonhematopoietic cells. *J Immunol,* 188**,** 5824-8.

BOGUNIA-KUBIK, K., GIERYNG, A., GEBURA, K. & LANGE, A. 2012. Genetic variant of the G-CSF receptor gene is associated with lower mobilization potential and slower recovery of granulocytes after transplantation of autologous peripheral blood progenitor cells. *Cytokine,* 60**,** 463-7.

BOISSET, J. C., VAN CAPPELLEN, W., ANDRIEU-SOLER, C., GALJART, N., DZIERZAK, E. & ROBIN, C. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature,* 464**,** 116-20.

BONIG, H., BURDACH, S., GOBEL, U. & NURNBERGER, W. 2001. Growth factors and hemostasis: differential effects of GM-CSF and G-CSF on coagulation activation--laboratory and clinical evidence. *Ann Hematol,* 80**,** 525-30.

BONIG, H., PRIESTLEY, G. V., OEHLER, V. & PAPAYANNOPOULOU, T. 2007. Hematopoietic progenitor cells (HPC) from mobilized peripheral blood display enhanced migration and marrow homing compared to steady-state bone marrow HPC. *Exp Hematol,* 35**,** 326-34.

BOONSTRA, A., RAJSBAUM, R., HOLMAN, M., MARQUES, R., ASSELIN-PATUREL, C., PEREIRA, J. P., BATES, E. E., AKIRA, S., VIEIRA, P., LIU, Y. J., TRINCHIERI, G. & O'GARRA, A. 2006. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J Immunol,* 177**,** 7551-8.

BOOTH, V., KEIZER, D. W., KAMPHUIS, M. B., CLARK-LEWIS, I. & SYKES, B. D. 2002. The CXCR3 binding chemokine IP-10/CXCL10: structure and receptor interactions. *Biochemistry,* 41**,** 10418-25.

BORING, L., GOSLING, J., CHENSUE, S. W., KUNKEL, S. L., FARESE, R. V., JR., BROXMEYER, H. E. & CHARO, I. F. 1997. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest,* 100**,** 2552-61.

BOUCHON, A., DIETRICH, J. & COLONNA, M. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol,* 164**,** 4991-5.

BOUCHON, A., FACCHETTI, F., WEIGAND, M. A. & COLONNA, M. 2001. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature,* 410**,** 1103-7.

BOUILLOUX, F., JUBAN, G., COHET, N., BUET, D., GUYOT, B., VAINCHENKER, W., LOUACHE, F. & MORLE, F. 2008. EKLF restricts megakaryocytic differentiation at the benefit of erythrocytic differentiation. *Blood,* 112**,** 576-84.

BOZZINI, C. E., BARRIO RENDO, M. E., DEVOTO, F. C. & EPPER, C. E. 1970. Studies on medullary and extramedullary erythropoiesis in the adult mouse. *Am J Physiol*, 219**,** 724-8.

BRANDT, E., LUDWIG, A., PETERSEN, F. & FLAD, H. D. 2000. Platelet-derived CXC chemokines: old players in new games. *Immunol Rev,* 177**,** 204-16.

BRIDDELL, R. A., BRUNO, E., COOPER, R. J., BRANDT, J. E. & HOFFMAN, R. 1991. Effect of c-kit ligand on in vitro human megakaryocytopoiesis. *Blood,* 78**,** 2854-9.

BRINES, M. L., GHEZZI, P., KEENAN, S., AGNELLO, D., DE LANEROLLE, N. C., CERAMI, C., ITRI, L. M. & CERAMI, A. 2000. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci U S A,* 97**,** 10526-31.

BROUDY, V. C. 1997. Stem cell factor and hematopoiesis. *Blood,* 90**,** 1345-64.

BROUDY, V. C., KAUSHANSKY, K., HARLAN, J. M. & ADAMSON, J. W. 1987. Interleukin 1 stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor. *J Immunol,* 139**,** 464-8.

BROUDY, V. C., KOVACH, N. L., BENNETT, L. G., LIN, N., JACOBSEN, F. W. & KIDD, P. G. 1994. Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor. *Blood,* 83**,** 2145-52.

BROUDY, V. C., LIN, N. L. & KAUSHANSKY, K. 1995. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. *Blood,* 85**,** 1719-26.

BROXMEYER, H. E., COOPER, S., HANGOC, G., GAO, J. L. & MURPHY, P. M. 1999. Dominant myelopoietic effector functions mediated by chemokine receptor CCR1. *J Exp Med,* 189**,** 1987-92.

BROXMEYER, H. E., HANGOC, G., COOPER, S., CAMPBELL, T., ITO, S. & MANTEL, C. 2007. AMD3100 and CD26 modulate mobilization, engraftment, and survival of hematopoietic stem and progenitor cells mediated by the SDF-1/CXCL12-CXCR4 axis. *Ann N Y Acad Sci,* 1106**,** 1-19.

BROXMEYER, H. E., MAZE, R., MIYAZAWA, K., CAROW, C., HENDRIE, P. C., COOPER, S., HANGOC, G., VADHAN-RAJ, S. & LU, L. 1991. The kit receptor and its ligand, steel factor, as regulators of hemopoiesis. *Cancer Cells,* 3**,** 480-7.

BROXMEYER, H. E., ORAZI, A., HAGUE, N. L., SLEDGE, G. W., JR., RASMUSSEN, H. & GORDON, M. S. 1998. Myeloid progenitor cell proliferation and mobilization effects of BB10010, a genetically engineered variant of human macrophage inflammatory protein-1alpha, in a phase I clinical trial in patients with relapsed/refractory breast cancer. *Blood Cells Mol Dis,* 24**,** 14-30.

BROXMEYER, H. E., ORSCHELL, C. M., CLAPP, D. W., HANGOC, G., COOPER, S., PLETT, P. A., LILES, W. C., LI, X., GRAHAM-EVANS, B., CAMPBELL, T. B., CALANDRA, G., BRIDGER, G., DALE, D. C. & SROUR, E. F. 2005. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med,* 201**,** 1307-18.

BUCKLAND, K. F., RAMAPRAKASH, H., MURRAY, L. A., CARPENTER, K. J., CHOI, E. S., KUNKEL, S. L., LUKACS, N. W., XING, Z., AOKI, N., HARTL, D. & HOGABOAM, C. M. 2011. Triggering receptor expressed on myeloid cells-1 (TREM-1) modulates immune responses to Aspergillus fumigatus during fungal asthma in mice. *Immunol Invest,* 40**,** 692-722.

BURGESS, A. W. & METCALF, D. 1980. The nature and action of granulocyte-macrophage colony stimulating factors. *Blood,* 56**,** 947-58.

BURNS, C. E., TRAVER, D., MAYHALL, E., SHEPARD, J. L. & ZON, L. I. 2005. Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev,* 19**,** 2331-42.

BUSSOLINO, F., WANG, J. M., DEFILIPPI, P., TURRINI, F., SANAVIO, F., EDGELL, C. J., AGLIETTA, M., ARESE, P. & MANTOVANI, A. 1989. Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. *Nature,* 337**,** 471-3.

BUTLER, J. M., NOLAN, D. J., VERTES, E. L., VARNUM-FINNEY, B., KOBAYASHI, H., HOOPER, A. T., SEANDEL, M., SHIDO, K., WHITE, I. A., KOBAYASHI, M., WITTE, L., MAY, C., SHAWBER, C., KIMURA, Y., KITAJEWSKI, J., ROSENWAKS, Z., BERNSTEIN, I. D. & RAFII, S. 2010. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell,* 6**,** 251-64.

CAI, Y., SHEN, X., DING, C., QI, C., LI, K., LI, X., JALA, V. R., ZHANG, H. G., WANG, T., ZHENG, J. & YAN, J. 2011. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity,* 35**,** 596-610.

CALVI, L. M., ADAMS, G. B., WEIBRECHT, K. W., WEBER, J. M., OLSON, D. P., KNIGHT, M. C., MARTIN, R. P., SCHIPANI, E., DIVIETI, P., BRINGHURST, F. R., MILNER, L. A., KRONENBERG, H. M. & SCADDEN, D. T. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature,* 425**,** 841-6.

CAMARGO, F. D., CHAMBERS, S. M., DREW, E., MCNAGNY, K. M. & GOODELL, M. A. 2006. Hematopoietic stem cells do not engraft with absolute efficiencies. *Blood,* 107**,** 501-7.

CANALES, M. A., ARRIETA, R., GOMEZ-RIOJA, R., DIEZ, J., JIMENEZ-YUSTE, V. & HERNANDEZ-NAVARRO, F. 2002. Induction of a hypercoagulability state and endothelial cell activation by granulocyte colony-stimulating factor in peripheral blood stem cell donors. *J Hematother Stem Cell Res,* 11**,** 675-81.

CAROTTA, S., DAKIC, A., D'AMICO, A., PANG, S. H., GREIG, K. T., NUTT, S. L. & WU, L. 2010. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity,* 32**,** 628-41.

CARSON, W. E., HALDAR, S., BAIOCCHI, R. A., CROCE, C. M. & CALIGIURI, M. A. 1994. The c-kit ligand suppresses apoptosis of human natural killer cells through the upregulation of bcl-2. *Proc Natl Acad Sci U S A,* 91**,** 7553-7.

CATLIN, S. N., BUSQUE, L., GALE, R. E., GUTTORP, P. & ABKOWITZ, J. L. 2011. The replication rate of human hematopoietic stem cells in vivo. *Blood,* 117**,** 4460-6.

CHALLEN, G. A., BOLES, N., LIN, K. K. & GOODELL, M. A. 2009. Mouse hematopoietic stem cell identification and analysis. *Cytometry A,* 75**,** 14-24.

CHANDRASEKAR, B., BYSANI, S. & MUMMIDI, S. 2004. CXCL16 signals via Gi, phosphatidylinositol 3-kinase, Akt, I kappa B kinase, and nuclear factor-kappa B and induces cell-cell adhesion and aortic smooth muscle cell proliferation. *J Biol Chem,* 279**,** 3188-96.

CHANG, H. H., HEMBERG, M., BARAHONA, M., INGBER, D. E. & HUANG, S. 2008a. Transcriptome-wide noise controls lineage choice in mammalian progenitor cells. *Nature,* 453**,** 544-7.

CHANG, M. K., RAGGATT, L. J., ALEXANDER, K. A., KULIWABA, J. S., FAZZALARI, N. L., SCHRODER, K., MAYLIN, E. R., RIPOLL, V. M., HUME, D. A. & PETTIT, A. R. 2008b. Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo. *J Immunol,* 181**,** 1232-44.

CHARO, I. F. & RANSOHOFF, R. M. 2006. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med,* 354**,** 610-21.

CHEN, H., RAY-GALLET, D., ZHANG, P., HETHERINGTON, C. J., GONZALEZ, D. A., ZHANG, D. E., MOREAU-GACHELIN, F. & TENEN, D. G. 1995. PU.1 (Spi-1) autoregulates its expression in myeloid cells. *Oncogene,* 11**,** 1549-60.

CHENG, T., RODRIGUES, N., SHEN, H., YANG, Y., DOMBKOWSKI, D., SYKES, M. & SCADDEN, D. T. 2000. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science,* 287**,** 1804-8.

CHENG, T., SHEN, H., GIOKAS, D., GERE, J., TENEN, D. G. & SCADDEN, D. T. 1996. Temporal mapping of gene expression levels during the differentiation of individual primary hematopoietic cells. *Proc Natl Acad Sci U S A,* 93**,** 13158-63.

CHERVENICK, P. A. & BOGGS, D. R. 1971. In vitro growth of granulocytic and mononuclear cell colonies from blood of normal individuals. *Blood,* 37**,** 131-5.

CHIBA, M., SLAGA, T. J. & KLEIN-SZANTO, A. J. 1984. A morphometric study of dedifferentiated and involutional dark keratinocytes in 12-O-tetradecanoylphorbol-13-acetate-treated mouse epidermis. *Cancer Res,* 44**,** 2711-7.

CHOE, H., FARZAN, M., SUN, Y., SULLIVAN, N., ROLLINS, B., PONATH, P. D., WU, L., MACKAY, C. R., LAROSA, G., NEWMAN, W., GERARD, N., GERARD, C. & SODROSKI, J. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell,* 85**,** 1135-48.

CHOI, K., KENNEDY, M., KAZAROV, A., PAPADIMITRIOU, J. C. & KELLER, G. 1998. A common precursor for hematopoietic and endothelial cells. *Development,* 125**,** 725-32.

CHORRO, L., SARDE, A., LI, M., WOOLLARD, K. J., CHAMBON, P., MALISSEN, B., KISSENPFENNIG, A., BARBAROUX, J. B., GROVES, R. & GEISSMANN, F. 2009. Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. *J Exp Med,* 206**,** 3089-100.

CHOW, A., LUCAS, D., HIDALGO, A., MENDEZ-FERRER, S., HASHIMOTO, D., SCHEIERMANN, C., BATTISTA, M., LEBOEUF, M., PROPHETE, C., VAN ROOIJEN, N., TANAKA, M., MERAD, M. & FRENETTE, P. S. 2011. Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med,* 208**,** 261-71.

CHOY, E. H., ISENBERG, D. A., GARROOD, T., FARROW, S., IOANNOU, Y., BIRD, H., CHEUNG, N., WILLIAMS, B., HAZLEMAN, B., PRICE, R., YOSHIZAKI, K., NISHIMOTO, N., KISHIMOTO, T. & PANAYI, G. S. 2002. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. *Arthritis Rheum,* 46**,** 3143-50.

CHRISTOPHER, M. J. & LINK, D. C. 2008. Granulocyte colony-stimulating factor induces osteoblast apoptosis and inhibits osteoblast differentiation. *J Bone Miner Res,* 23**,** 1765-74.

CHRISTOPHER, M. J., LIU, F., HILTON, M. J., LONG, F. & LINK, D. C. 2009. Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization. *Blood,* 114**,** 1331-9.

CLINE, M. J. & GOLDE, D. W. 1977. Mobilization of hematopoietic stem cells (CFU-C) into the peripheral blood of man by endotoxin. *Exp Hematol,* 5**,** 186-90.

COBALEDA, C., SCHEBESTA, A., DELOGU, A. & BUSSLINGER, M. 2007. Pax5: the guardian of B cell identity and function. *Nat Immunol,* 8**,** 463-70.

COHEN, S. B., DORE, R. K., LANE, N. E., ORY, P. A., PETERFY, C. G., SHARP, J. T., VAN DER HEIJDE, D., ZHOU, L., TSUJI, W., NEWMARK, R. & DENOSUMAB RHEUMATOID ARTHRITIS STUDY, G. 2008. Denosumab treatment effects on structural damage, bone mineral density, and bone turnover in rheumatoid arthritis: a twelve-month, multicenter, randomized, double-blind, placebo-controlled, phase II clinical trial. *Arthritis Rheum,* 58**,** 1299-309.

COLONNA, M. & FACCHETTI, F. 2003. TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses. *J Infect Dis,* 187 Suppl 2**,** S397-401.

CONNOR, R. I. & HO, D. D. 1994. Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J Virol,* 68**,** 4400-8.

CONSTANTINESCU, S. N., HUANG, L. J., NAM, H. & LODISH, H. F. 2001. The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif. *Mol Cell,* 7**,** 377-85.

CORELLA, F., BARNADAS, M. A., BORDES, R., CURELL, R., ESPINOSA, I., VERGARA, C. & ALOMAR, A. 2008. [A case of cutaneous extramedullary hematopoiesis associated with idiopathic myelofibrosis]. *Actas Dermosifiliogr,* 99**,** 297-300.

CORMIER, F. & DIETERLEN-LIEVRE, F. 1988. The wall of the chick embryo aorta harbours M-CFC, G-CFC, GM-CFC and BFU-E. *Development,* 102**,** 279-85.

COURTENAY, J. S., DALLMAN, M. J., DAYAN, A. D., MARTIN, A. & MOSEDALE, B. 1980. Immunisation against heterologous type II collagen induces arthritis in mice. *Nature,* 283**,** 666-8.

COUSINS, D. J., STAYNOV, D. Z. & LEE, T. H. 1994. Regulation of interleukin-5 and granulocyte-macrophage colony-stimulating factor expression. *Am J Respir Crit Care Med,* 150**,** S50-3.

CRAMER, D. E., WAGNER, S., LI, B., LIU, J., HANSEN, R., RECA, R., WU, W., SURMA, E. Z., LABER, D. A., RATAJCZAK, M. Z. & YAN, J. 2008. Mobilization of hematopoietic progenitor cells by yeast-derived beta-glucan requires activation of matrix metalloproteinase-9. *Stem Cells,* 26**,** 1231-40.

CRISPINO, J. D., LODISH, M. B., MACKAY, J. P. & ORKIN, S. H. 1999. Use of altered specificity mutants to probe a specific protein-protein interaction in differentiation: the GATA-1:FOG complex. *Mol Cell,* 3**,** 219-28.

CROOP, J. M., COOPER, R., FERNANDEZ, C., GRAVES, V., KREISSMAN, S., HANENBERG, H., SMITH, F. O. & WILLIAMS, D. A. 2001. Mobilization and collection of peripheral blood CD34+ cells from patients with Fanconi anemia. *Blood,* 98**,** 2917-21.

CROSBIE, O. M., REYNOLDS, M., MCENTEE, G., TRAYNOR, O., HEGARTY, J. E. & O'FARRELLY, C. 1999. In vitro evidence for the presence of hematopoietic stem cells in the adult human liver. *Hepatology,* 29**,** 1193-8.

CUMANO, A., DIETERLEN-LIEVRE, F. & GODIN, I. 1996. Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell,* 86**,** 907-16.

CUMANO, A., FERRAZ, J. C., KLAINE, M., DI SANTO, J. P. & GODIN, I. 2001. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity,* 15**,** 477-85.

CZUPRYNSKI, C. J., BROWN, J. F., MAROUSHEK, N., WAGNER, R. D. & STEINBERG, H. 1994. Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to Listeria monocytogenes infection. *J Immunol,* 152**,** 1836-46.

DA SILVA CORREIA, J., SOLDAU, K., CHRISTEN, U., TOBIAS, P. S. & ULEVITCH, R. J. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *J Biol Chem,* 276**,** 21129-35.

DAHL, R., WALSH, J. C., LANCKI, D., LASLO, P., IYER, S. R., SINGH, H. & SIMON, M. C. 2003. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol,* 4**,** 1029-36.

DAMAJ, B. B., MCCOLL, S. R., NEOTE, K., SONGQING, N., OGBORN, K. T., HEBERT, C. A. & NACCACHE, P. H. 1996. Identification of G-protein binding sites of the human interleukin-8 receptors by functional mapping of the intracellular loops. *FASEB J,* 10**,** 1426-34.

DARBONNE, W. C., RICE, G. C., MOHLER, M. A., APPLE, T., HEBERT, C. A., VALENTE, A. J. & BAKER, J. B. 1991. Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J Clin Invest,* 88**,** 1362-9.

DATTA, S. R., DUDEK, H., TAO, X., MASTERS, S., FU, H., GOTOH, Y. & GREENBERG, M. E. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell,* 91**,** 231-41.

DAY, C. E., GUILLEN, C., WILLARS, G. B. & WARDLAW, A. J. 2010. Characterization of the migration of lung and blood T cells in response CXCL12 in a three-dimensional matrix. *Immunology,* 130**,** 564-71.

DE BRUIN, A. M., DEMIREL, O., HOOIBRINK, B., BRANDTS, C. H. & NOLTE, M. A. 2013. Interferon-gamma impairs proliferation of hematopoietic stem cells in mice. *Blood,* 121**,** 3578-85.

DE LAVAL, B., PAWLIKOWSKA, P., BARBIERI, D., BESNARD-GUERIN, C., CICO, A., KUMAR, R., GAUDRY, M., BAUD, V. & PORTEU, F. 2013. Thrombopoietin promotes NHEJ DNA repair in hematopoietic stem cells through specific activation of Erk and NF-kappaB pathways and their target, IEX-1. *Blood,* 123**,** 509-19.

DE LUCA, K., FRANCES-DUVERT, V., ASENSIO, M. J., IHSANI, R., DEBIEN, E., TAILLARDET, M., VERHOEYEN, E., BELLA, C., LANTHEAUME, S., GENESTIER, L. & DEFRANCE, T. 2009. The TLR1/2 agonist PAM(3)CSK(4)

instructs commitment of human hematopoietic stem cells to a myeloid cell fate. *Leukemia,* 23**,** 2063-74.

DE SOUSA ABREU, R., PENALVA, L. O., MARCOTTE, E. M. & VOGEL, C. 2009. Global signatures of protein and mRNA expression levels. *Mol Biosyst,* 5**,** 1512-26.

DEBILI, N., WENDLING, F., COSMAN, D., TITEUX, M., FLORINDO, C., DUSANTER-FOURT, I., SCHOOLEY, K., METHIA, N., CHARON, M., NADOR, R. & ET AL. 1995. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. *Blood,* 85**,** 391-401.

DEEPE, G. S., JR., GIBBONS, R. & WOODWARD, E. 1999. Neutralization of endogenous granulocyte-macrophage colony-stimulating factor subverts the protective immune response to Histoplasma capsulatum. *J Immunol,* 163**,** 4985-93.

DEGUCHI, K., YAGI, H., INADA, M., YOSHIZAKI, K., KISHIMOTO, T. & KOMORI, T. 1999. Excessive extramedullary hematopoiesis in Cbfa1-deficient mice with a congenital lack of bone marrow. *Biochem Biophys Res Commun,* 255**,** 352-9.

DEKOTER, R. P. & SINGH, H. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science,* 288**,** 1439-41.

DELEURAN, M., BUHL, L., ELLINGSEN, T., HARADA, A., LARSEN, C. G., MATSUSHIMA, K. & DELEURAN, B. 1996. Localization of monocyte chemotactic and activating factor (MCAF/MCP-1) in psoriasis. *J Dermatol Sci,* 13**,** 228-36.

DELOGU, A., SCHEBESTA, A., SUN, Q., ASCHENBRENNER, K., PERLOT, T. & BUSSLINGER, M. 2006. Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. *Immunity,* 24**,** 269-81.

DEMETRI, G. D. & GRIFFIN, J. D. 1991. Granulocyte colony-stimulating factor and its receptor. *Blood,* 78**,** 2791-808.

DEMETRI, G. D., ZENZIE, B. W., RHEINWALD, J. G. & GRIFFIN, J. D. 1989. Expression of colony-stimulating factor genes by normal human mesothelial cells and human malignant mesothelioma cells lines in vitro. *Blood,* 74**,** 940-6.

DEVINE, S. M., FLOMENBERG, N., VESOLE, D. H., LIESVELD, J., WEISDORF, D., BADEL, K., CALANDRA, G. & DIPERSIO, J. F. 2004. Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J Clin Oncol,* 22**,** 1095-102.

DEVINE, S. M., VIJ, R., RETTIG, M., TODT, L., MCGLAUCHLEN, K., FISHER, N., DEVINE, H., LINK, D. C., CALANDRA, G., BRIDGER, G., WESTERVELT, P. & DIPERSIO, J. F. 2008. Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. *Blood,* 112**,** 990-8.

DIAS, S., MANSSON, R., GURBUXANI, S., SIGVARDSSON, M. & KEE, B. L. 2008. E2A proteins promote development of lymphoid-primed multipotent progenitors. *Immunity,* 29**,** 217-27.

DIAS, S., SILVA, H., JR., CUMANO, A. & VIEIRA, P. 2005. Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. *J Exp Med,* 201**,** 971-9.

DIEBOLD, S. S., KAISHO, T., HEMMI, H., AKIRA, S. & REIS E SOUSA, C. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science,* 303**,** 1529-31.

DIETERLEN-LIEVRE, F. 1975. On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol,* 33**,** 607-19.

DILLON, S., AGRAWAL, A., VAN DYKE, T., LANDRETH, G., MCCAULEY, L., KOH, A., MALISZEWSKI, C., AKIRA, S. & PULENDRAN, B. 2004. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *J Immunol,* 172**,** 4733-43.

DING, L., SAUNDERS, T. L., ENIKOLOPOV, G. & MORRISON, S. J. 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature,* 481**,** 457-62.

DOHERTY, D. G., NORRIS, S., MADRIGAL-ESTEBAS, L., MCENTEE, G., TRAYNOR, O., HEGARTY, J. E. & O'FARRELLY, C. 1999. The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J Immunol,* 163**,** 2314-21.

DOMENGA, V., FARDOUX, P., LACOMBE, P., MONET, M., MACIAZEK, J., KREBS, L. T., KLONJKOWSKI, B., BERROU, E., MERICSKAY, M., LI, Z., TOURNIER-LASSERVE, E., GRIDLEY, T. & JOUTEL, A. 2004. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes Dev,* 18**,** 2730-5.

DONAHUE, R. E., JIN, P., BONIFACINO, A. C., METZGER, M. E., REN, J., WANG, E. & STRONCEK, D. F. 2009. Plerixafor (AMD3100) and granulocyte colony-stimulating factor (G-CSF) mobilize different CD34+ cell populations based on global gene and microRNA expression signatures. *Blood,* 114**,** 2530-41.

DONZE, D., TOWNES, T. M. & BIEKER, J. J. 1995. Role of erythroid Kruppel-like factor in human gamma- to beta-globin gene switching. *J Biol Chem,* 270**,** 1955-9.

DONZELLA, G. A., SCHOLS, D., LIN, S. W., ESTE, J. A., NAGASHIMA, K. A., MADDON, P. J., ALLAWAY, G. P., SAKMAR, T. P., HENSON, G., DE CLERCQ, E. & MOORE, J. P. 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat Med,* 4**,** 72-7.

DOR, F. J., RAMIREZ, M. L., PARMAR, K., ALTMAN, E. L., HUANG, C. A., DOWN, J. D. & COOPER, D. K. 2006. Primitive hematopoietic cell populations reside in the spleen: Studies in the pig, baboon, and human. *Exp Hematol,* 34**,** 1573-82.

DORANZ, B. J., RUCKER, J., YI, Y., SMYTH, R. J., SAMSON, M., PEIPER, S. C., PARMENTIER, M., COLLMAN, R. G. & DOMS, R. W. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell,* 85**,** 1149-58.

DORR, P., WESTBY, M., DOBBS, S., GRIFFIN, P., IRVINE, B., MACARTNEY, M., MORI, J., RICKETT, G., SMITH-BURCHNELL, C., NAPIER, C., WEBSTER, R., ARMOUR, D., PRICE, D., STAMMEN, B., WOOD, A. & PERROS, M. 2005. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob Agents Chemother,* 49**,** 4721-32.

DOULATOV, S., NOTTA, F., LAURENTI, E. & DICK, J. E. 2012. Hematopoiesis: a human perspective. *Cell Stem Cell,* 10**,** 120-36.

DOYLE, S., VAIDYA, S., O'CONNELL, R., DADGOSTAR, H., DEMPSEY, P., WU, T., RAO, G., SUN, R., HABERLAND, M., MODLIN, R. & CHENG, G. 2002. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity,* 17**,** 251-63.

DREGER, P., KLOSS, M., PETERSEN, B., HAFERLACH, T., LOFFLER, H., LOEFFLER, M. & SCHMITZ, N. 1995. Autologous progenitor cell transplantation: prior exposure to stem cell-toxic drugs determines yield and engraftment of peripheral blood progenitor cell but not of bone marrow grafts. *Blood,* 86**,** 3970-8.

DRISSEN, R., PALSTRA, R. J., GILLEMANS, N., SPLINTER, E., GROSVELD, F., PHILIPSEN, S. & DE LAAT, W. 2004. The active spatial organization of the beta-globin locus requires the transcription factor EKLF. *Genes Dev,* 18**,** 2485-90.

DROBITS, B., HOLCMANN, M., AMBERG, N., SWIECKI, M., GRUNDTNER, R., HAMMER, M., COLONNA, M. & SIBILIA, M. 2012. Imiquimod clears tumors in mice independent of adaptive immunity by converting pDCs into tumor-killing effector cells. *J Clin Invest,* 122**,** 575-85.

DU, X. X., KELLER, D., MAZE, R. & WILLIAMS, D. A. 1993. Comparative effects of in vivo treatment using interleukin-11 and stem cell factor on reconstitution in mice after bone marrow transplantation. *Blood,* 82**,** 1016-22.

DUARTE, A., HIRASHIMA, M., BENEDITO, R., TRINDADE, A., DINIZ, P., BEKMAN, E., COSTA, L., HENRIQUE, D. & ROSSANT, J. 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev,* 18**,** 2474-8.

DUCY, P., SCHINKE, T. & KARSENTY, G. 2000. The osteoblast: a sophisticated fibroblast under central surveillance. *Science,* 289**,** 1501-4.

DUNLOP, D. J., WRIGHT, E. G., LORIMORE, S., GRAHAM, G. J., HOLYOAKE, T., KERR, D. J., WOLPE, S. D. & PRAGNELL, I. B. 1992. Demonstration of stem cell inhibition and myeloprotective effects of SCI/rhMIP1 alpha in vivo. *Blood,* 79**,** 2221-5.

DWIR, O., GRABOVSKY, V. & ALON, R. 2004. Selectin avidity modulation by chemokines at subsecond endothelial contacts: a novel regulatory level of leukocyte trafficking. *Ernst Schering Res Found Workshop***,** 109-35.

EDER, M., GEISSLER, G. & GANSER, A. 1997. IL-3 in the clinic. *Stem Cells,* 15**,** 327-33.

EGAWA, T., TILLMAN, R. E., NAOE, Y., TANIUCHI, I. & LITTMAN, D. R. 2007. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J Exp Med,* 204**,** 1945-57.

EHNINGER, A. & TRUMPP, A. 2011. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med,* 208**,** 421-8.

ELSENBRUCH, S., LUCAS, A., HOLTMANN, G., HAAG, S., GERKEN, G., RIEMENSCHNEIDER, N., LANGHORST, J., KAVELAARS, A., HEIJNEN, C. J. & SCHEDLOWSKI, M. 2006. Public speaking stress-induced neuroendocrine responses and circulating immune cell redistribution in irritable bowel syndrome. *Am J Gastroenterol,* 101**,** 2300-7.

ERNST, T. J., RITCHIE, A. R., DEMETRI, G. D. & GRIFFIN, J. D. 1989. Regulation of granulocyte- and monocyte-colony stimulating factor mRNA levels in human blood monocytes is mediated primarily at a post-transcriptional level. *J Biol Chem,* 264**,** 5700-3.

ESPLIN, B. L., SHIMAZU, T., WELNER, R. S., GARRETT, K. P., NIE, L., ZHANG, Q., HUMPHREY, M. B., YANG, Q., BORGHESI, L. A. & KINCADE, P. W. 2011. Chronic exposure to a TLR ligand injures hematopoietic stem cells. *J Immunol,* 186**,** 5367-75.

FASSLER, R. & MEYER, M. 1995. Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev,* 9**,** 1896-908.

FELDMANN, M., BRENNAN, F. M. & MAINI, R. N. 1996. Rheumatoid arthritis. *Cell,* 85, 307-10.

FELLI, M. P., MARODER, M., MITSIADIS, T. A., CAMPESE, A. F., BELLAVIA, D., VACCA, A., MANN, R. S., FRATI, L., LENDAHL, U., GULINO, A. & SCREPANTI, I. 1999. Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development. *Int Immunol,* 11, 1017-25.

FERGUSON, S. S. 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev,* 53, 1-24.

FISHER, J. W. & BIRDWELL, B. J. 1961. The production of an erythropoietic factor by the in situ perfused kidney. *Acta Haematol,* 26, 224-32.

FITZGERALD, K. A., MCWHIRTER, S. M., FAIA, K. L., ROWE, D. C., LATZ, E., GOLENBOCK, D. T., COYLE, A. J., LIAO, S. M. & MANIATIS, T. 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol,* 4, 491-6.

FLEISHAKER, D. L., GARCIA MEIJIDE, J. A., PETROV, A., KOHEN, M. D., WANG, X., MENON, S., STOCK, T. C., MEBUS, C. A., GOODRICH, J. M., MAYER, H. B. & ZEIHER, B. G. 2012. Maraviroc, a chemokine receptor-5 antagonist, fails to demonstrate efficacy in the treatment of patients with rheumatoid arthritis in a randomized, double-blind placebo-controlled trial. *Arthritis Res Ther,* 14, R11.

FLEMING, H. E., JANZEN, V., LO CELSO, C., GUO, J., LEAHY, K. M., KRONENBERG, H. M. & SCADDEN, D. T. 2008. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell,* 2, 274-83.

FLOMENBERG, N., DEVINE, S. M., DIPERSIO, J. F., LIESVELD, J. L., MCCARTY, J. M., ROWLEY, S. D., VESOLE, D. H., BADEL, K. & CALANDRA, G. 2005. The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood,* 106, 1867-74.

FORSBERG, E. C., PROHASKA, S. S., KATZMAN, S., HEFFNER, G. C., STUART, J. M. & WEISSMAN, I. L. 2005. Differential expression of novel potential regulators in hematopoietic stem cells. *PLoS Genet,* 1, e28.

FORTANIER, C., KUENTZ, M., SUTTON, L., MILPIED, N., MICHALET, M., MACQUART-MOULIN, G., FAUCHER, C., LE CORROLLER, A. G., MOATTI, J. P. & BLAISE, D. 2002. Healthy sibling donor anxiety and pain during bone marrow or peripheral blood stem cell harvesting for allogeneic transplantation: results of a randomised study. *Bone Marrow Transplant,* 29, 145-9.

FOUDI, A., HOCHEDLINGER, K., VAN BUREN, D., SCHINDLER, J. W., JAENISCH, R., CAREY, V. & HOCK, H. 2009. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol,* 27, 84-90.

FRESON, K., DEVRIENDT, K., MATTHIJS, G., VAN HOOF, A., DE VOS, R., THYS, C., MINNER, K., HOYLAERTS, M. F., VERMYLEN, J. & VAN GEET, C. 2001. Platelet characteristics in patients with X-linked macrothrombocytopenia because of a novel GATA1 mutation. *Blood,* 98, 85-92.

FRIEDMAN, A. D. 2007. Transcriptional control of granulocyte and monocyte development. *Oncogene,* 26, 6816-28.

FRONTELO, P., MANWANI, D., GALDASS, M., KARSUNKY, H., LOHMANN, F., GALLAGHER, P. G. & BIEKER, J. J. 2007. Novel role for EKLF in megakaryocyte lineage commitment. *Blood,* 110, 3871-80.

FRUEHAUF, S., VELDWIJK, M. R., SEEGER, T., SCHUBERT, M., LAUFS, S., TOPALY, J., WUCHTER, P., DILLMANN, F., ECKSTEIN, V., WENZ, F., GOLDSCHMIDT, H., HO, A. D. & CALANDRA, G. 2009. A combination of granulocyte-colony-stimulating factor (G-CSF) and plerixafor mobilizes more primitive peripheral blood progenitor cells than G-CSF alone: results of a European phase II study. *Cytotherapy,* 11**,** 992-1001.

FUJIMOTO, H., SANGAI, T., ISHII, G., IKEHARA, A., NAGASHIMA, T., MIYAZAKI, M. & OCHIAI, A. 2009. Stromal MCP-1 in mammary tumors induces tumor-associated macrophage infiltration and contributes to tumor progression. *Int J Cancer,* 125**,** 1276-84.

FUKUDA, S., BIAN, H., KING, A. G. & PELUS, L. M. 2007. The chemokine GRObeta mobilizes early hematopoietic stem cells characterized by enhanced homing and engraftment. *Blood,* 110**,** 860-9.

FUXA, M. & BUSSLINGER, M. 2007. Reporter gene insertions reveal a strictly B lymphoid-specific expression pattern of Pax5 in support of its B cell identity function. *J Immunol,* 178**,** 8222-8.

GALLIERA, E., JALA, V. R., TRENT, J. O., BONECCHI, R., SIGNORELLI, P., LEFKOWITZ, R. J., MANTOVANI, A., LOCATI, M. & HARIBABU, B. 2004. beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *J Biol Chem,* 279**,** 25590-7.

GALLOWAY, J. L. & ZON, L. I. 2003. Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. *Curr Top Dev Biol,* 53**,** 139-58.

GASCHE, C., WALDHOER, T., FEICHTENSCHLAGER, T., MALE, C., MAYER, A., MITTERMAIER, C., PETRITSCH, W. & AUSTRIAN INFLAMMATORY BOWEL DISEASES STUDY, G. 2001. Prediction of response to iron sucrose in inflammatory bowel disease-associated anemia. *Am J Gastroenterol,* 96**,** 2382-7.

GERHARTZ, H. H., ENGELHARD, M., MEUSERS, P., BRITTINGER, G., WILMANNS, W., SCHLIMOK, G., MUELLER, P., HUHN, D., MUSCH, R., SIEGERT, W. & ET AL. 1993. Randomized, double-blind, placebo-controlled, phase III study of recombinant human granulocyte-macrophage colony-stimulating factor as adjunct to induction treatment of high-grade malignant non-Hodgkin's lymphomas. *Blood,* 82**,** 2329-39.

GERMAIN, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol,* 2**,** 309-22.

GERRITS, H., VAN INGEN SCHENAU, D. S., BAKKER, N. E., VAN DISSELDORP, A. J., STRIK, A., HERMENS, L. S., KOENEN, T. B., KRAJNC-FRANKEN, M. A. & GOSSEN, J. A. 2008. Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. *Genesis,* 46**,** 235-45.

GIBOT, S., LE RENARD, P. E., BOLLAERT, P. E., KOLOPP-SARDA, M. N., BENE, M. C., FAURE, G. C. & LEVY, B. 2005. Surface triggering receptor expressed on myeloid cells 1 expression patterns in septic shock. *Intensive Care Med,* 31**,** 594-7.

GINHOUX, F., GRETER, M., LEBOEUF, M., NANDI, S., SEE, P., GOKHAN, S., MEHLER, M. F., CONWAY, S. J., NG, L. G., STANLEY, E. R., SAMOKHVALOV, I. M. & MERAD, M. 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science,* 330**,** 841-5.

GIUSTIZIERI, M. L., MASCIA, F., FREZZOLINI, A., DE PITA, O., CHINNI, L. M., GIANNETTI, A., GIROLOMONI, G. & PASTORE, S. 2001. Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine

production profile in response to T cell-derived cytokines. *J Allergy Clin Immunol,* 107**,** 871-7.

GLASPY, J. A., SHPALL, E. J., LEMAISTRE, C. F., BRIDDELL, R. A., MENCHACA, D. M., TURNER, S. A., LILL, M., CHAP, L., JONES, R., WIERS, M. D., SHERIDAN, W. P. & MCNIECE, I. K. 1997. Peripheral blood progenitor cell mobilization using stem cell factor in combination with filgrastim in breast cancer patients. *Blood,* 90**,** 2939-51.

GODIN, I., GARCIA-PORRERO, J. A., DIETERLEN-LIEVRE, F. & CUMANO, A. 1999. Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *J Exp Med,* 190**,** 43-52.

GOLDEN-MASON, L., CURRY, M. P., NOLAN, N., TRAYNOR, O., MCENTEE, G., KELLY, J., HEGARTY, J. E. & O'FARRELLY, C. 2000. Differential expression of lymphoid and myeloid markers on differentiating hematopoietic stem cells in normal and tumor-bearing adult human liver. *Hepatology,* 31**,** 1251-6.

GOLDEN-MASON, L., KELLY, A. M., TRAYNOR, O., MCENTEE, G., KELLY, J., HEGARTY, J. E. & O'FARRELLY, C. 2001. Expression of interleukin 7 (IL-7) mRNA and protein in the normal adult human liver: implications for extrathymic T cell development. *Cytokine,* 14**,** 143-51.

GOLDEN-MASON, L. & O'FARRELLY, C. 2002. Having it all? Stem cells, haematopoiesis and lymphopoiesis in adult human liver. *Immunol Cell Biol,* 80**,** 45-51.

GOLDMAN, D. C., BAILEY, A. S., PFAFFLE, D. L., AL MASRI, A., CHRISTIAN, J. L. & FLEMING, W. H. 2009. BMP4 regulates the hematopoietic stem cell niche. *Blood,* 114**,** 4393-401.

GONZALEZ-JUARRERO, M., HATTLE, J. M., IZZO, A., JUNQUEIRA-KIPNIS, A. P., SHIM, T. S., TRAPNELL, B. C., COOPER, A. M. & ORME, I. M. 2005. Disruption of granulocyte macrophage-colony stimulating factor production in the lungs severely affects the ability of mice to control Mycobacterium tuberculosis infection. *J Leukoc Biol,* 77**,** 914-22.

GOODELL, M. A., BROSE, K., PARADIS, G., CONNER, A. S. & MULLIGAN, R. C. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med,* 183**,** 1797-806.

GOODELL, M. A., ROSENZWEIG, M., KIM, H., MARKS, D. F., DEMARIA, M., PARADIS, G., GRUPP, S. A., SIEFF, C. A., MULLIGAN, R. C. & JOHNSON, R. P. 1997. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med,* 3**,** 1337-45.

GOODMAN, J. W. & HODGSON, G. S. 1962. Evidence for stem cells in the peripheral blood of mice. *Blood,* 19**,** 702-14.

GOSLING, J., DAIRAGHI, D. J., WANG, Y., HANLEY, M., TALBOT, D., MIAO, Z. & SCHALL, T. J. 2000. Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol,* 164**,** 2851-6.

GOVAERTS, C., BONDUE, A., SPRINGAEL, J. Y., OLIVELLA, M., DEUPI, X., LE POUL, E., WODAK, S. J., PARMENTIER, M., PARDO, L. & BLANPAIN, C. 2003. Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3. *J Biol Chem,* 278**,** 1892-903.

GRAHAM, G. J., LOCATI, M., MANTOVANI, A., ROT, A. & THELEN, M. 2012. The biochemistry and biology of the atypical chemokine receptors. *Immunol Lett,* 145**,** 30-8.

GRANOWITZ, E. V., SANTOS, A. A., POUTSIAKA, D. D., CANNON, J. G., WILMORE, D. W., WOLFF, S. M. & DINARELLO, C. A. 1991. Production of interleukin-

1-receptor antagonist during experimental endotoxaemia. *Lancet,* 338**,** 1423-4.

GREENBAUM, A., HSU, Y. M., DAY, R. B., SCHUETTPELZ, L. G., CHRISTOPHER, M. J., BORGERDING, J. N., NAGASAWA, T. & LINK, D. C. 2013. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature,* 495**,** 227-30.

GREGORY, T., YU, C., MA, A., ORKIN, S. H., BLOBEL, G. A. & WEISS, M. J. 1999. GATA-1 and erythropoietin cooperate to promote erythroid cell survival by regulating bcl-xL expression. *Blood,* 94**,** 87-96.

GRIFFIN, J. D., SPERTINI, O., ERNST, T. J., BELVIN, M. P., LEVINE, H. B., KANAKURA, Y. & TEDDER, T. F. 1990. Granulocyte-macrophage colony-stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes, and their precursors. *J Immunol,* 145**,** 576-84.

GRINENKO, T., ARNDT, K., PORTZ, M., MENDE, N., GUNTHER, M., COSGUN, K. N., ALEXOPOULOU, D., LAKSHMANAPERUMAL, N., HENRY, I., DAHL, A. & WASKOW, C. 2014. Clonal expansion capacity defines two consecutive developmental stages of long-term hematopoietic stem cells. *J Exp Med*.

GUPTA, S. K., PILLARISETTI, K., THOMAS, R. A. & AIYAR, N. 2001. Pharmacological evidence for complex and multiple site interaction of CXCR4 with SDF-1alpha: implications for development of selective CXCR4 antagonists. *Immunol Lett,* 78**,** 29-34.

HAN, B., MURA, M., ANDRADE, C. F., OKUTANI, D., LODYGA, M., DOS SANTOS, C. C., KESHAVJEE, S., MATTHAY, M. & LIU, M. 2005. TNFalpha-induced long pentraxin PTX3 expression in human lung epithelial cells via JNK. *J Immunol,* 175**,** 8303-11.

HAN, K. H., GREEN, S. R., TANGIRALA, R. K., TANAKA, S. & QUEHENBERGER, O. 1999. Role of the first extracellular loop in the functional activation of CCR2. The first extracellular loop contains distinct domains necessary for both agonist binding and transmembrane signaling. *J Biol Chem,* 274**,** 32055-62.

HANN, I. M., BODGER, M. P. & HOFFBRAND, A. V. 1983. Development of pluripotent hematopoietic progenitor cells in the human fetus. *Blood,* 62**,** 118-23.

HANSSON, M., PETERSSON, M., KOO, G. C., WIGZELL, H. & KIESSLING, R. 1988. In vivo function of natural killer cells as regulators of myeloid precursor cells in the spleen. *Eur J Immunol,* 18**,** 485-8.

HARINGMAN, J. J., GERLAG, D. M., SMEETS, T. J., BAETEN, D., VAN DEN BOSCH, F., BRESNIHAN, B., BREEDVELD, F. C., DINANT, H. J., LEGAY, F., GRAM, H., LOETSCHER, P., SCHMOUDER, R., WOODWORTH, T. & TAK, P. P. 2006. A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis. *Arthritis Rheum,* 54**,** 2387-92.

HARRISON, D. E. 1980. Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood,* 55**,** 77-81.

HARRISON, D. E., JORDAN, C. T., ZHONG, R. K. & ASTLE, C. M. 1993. Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Exp Hematol,* 21**,** 206-19.

HARRISON, D. E., ZHONG, R. K., JORDAN, C. T., LEMISCHKA, I. R. & ASTLE, C. M. 1997. Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term. *Exp Hematol,* 25**,** 293-7.

HATTORI, K., HEISSIG, B., TASHIRO, K., HONJO, T., TATENO, M., SHIEH, J. H., HACKETT, N. R., QUITORIANO, M. S., CRYSTAL, R. G., RAFII, S. & MOORE, M. A. 2001. Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. *Blood,* 97**,** 3354-60.

HAVRAN, W. L. & ALLISON, J. P. 1988. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature,* 335**,** 443-5.

HAZIOT, A., FERRERO, E., KONTGEN, F., HIJIYA, N., YAMAMOTO, S., SILVER, J., STEWART, C. L. & GOYERT, S. M. 1996. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity,* 4**,** 407-14.

HEATH, V., SUH, H. C., HOLMAN, M., RENN, K., GOOYA, J. M., PARKIN, S., KLARMANN, K. D., ORTIZ, M., JOHNSON, P. & KELLER, J. 2004. C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development in vitro and in vivo. *Blood,* 104**,** 1639-47.

HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H. & BAUER, S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science,* 303**,** 1526-9.

HEINRICH, M. C., DOOLEY, D. C., FREED, A. C., BAND, L., HOATLIN, M. E., KEEBLE, W. W., PETERS, S. T., SILVEY, K. V., EY, F. S., KABAT, D. & ET AL. 1993. Constitutive expression of steel factor gene by human stromal cells. *Blood,* 82**,** 771-83.

HEISSIG, B., HATTORI, K., DIAS, S., FRIEDRICH, M., FERRIS, B., HACKETT, N. R., CRYSTAL, R. G., BESMER, P., LYDEN, D., MOORE, M. A., WERB, Z. & RAFII, S. 2002. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell,* 109**,** 625-37.

HEMMI, H., KAISHO, T., TAKEUCHI, O., SATO, S., SANJO, H., HOSHINO, K., HORIUCHI, T., TOMIZAWA, H., TAKEDA, K. & AKIRA, S. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol,* 3**,** 196-200.

HENDRIE, P. C., MIYAZAWA, K., YANG, Y. C., LANGEFELD, C. D. & BROXMEYER, H. E. 1991. Mast cell growth factor (c-kit ligand) enhances cytokine stimulation of proliferation of the human factor-dependent cell line, M07e. *Exp Hematol,* 19**,** 1031-7.

HENDRIX, C. W., COLLIER, A. C., LEDERMAN, M. M., SCHOLS, D., POLLARD, R. B., BROWN, S., JACKSON, J. B., COOMBS, R. W., GLESBY, M. J., FLEXNER, C. W., BRIDGER, G. J., BADEL, K., MACFARLAND, R. T., HENSON, G. W. & CALANDRA, G. 2004. Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J Acquir Immune Defic Syndr,* 37**,** 1253-62.

HERRMANN, F., CANNISTRA, S. A. & GRIFFIN, J. D. 1986. T cell-monocyte interactions in the production of humoral factors regulating human granulopoiesis in vitro. *J Immunol,* 136**,** 2856-61.

HESTDAL, K., RUSCETTI, F. W., IHLE, J. N., JACOBSEN, S. E., DUBOIS, C. M., KOPP, W. C., LONGO, D. L. & KELLER, J. R. 1991. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J Immunol,* 147**,** 22-8.

HEUSER, M., GANSER, A. & BOKEMEYER, C. 2007. Use of colony-stimulating factors for chemotherapy-associated neutropenia: review of current guidelines. *Semin Hematol,* 44**,** 148-56.

HIRAI, H., ZHANG, P., DAYARAM, T., HETHERINGTON, C. J., MIZUNO, S., IMANISHI, J., AKASHI, K. & TENEN, D. G. 2006. C/EBPbeta is required for 'emergency' granulopoiesis. *Nat Immunol,* 7**,** 732-9.

HIRAYAMA, F., AIBA, Y., IKEBUCHI, K., SEKIGUCHI, S. & OGAWA, M. 1999. Differentiation in culture of murine primitive lymphohematopoietic progenitors toward T-cell lineage. *Blood,* 93**,** 4187-95.

HIRSCH, E., IGLESIAS, A., POTOCNIK, A. J., HARTMANN, U. & FASSLER, R. 1996. Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. *Nature,* 380**,** 171-5.

HO, T. K., SHIWEN, X., ABRAHAM, D., TSUI, J. & BAKER, D. 2012. Stromal-Cell-Derived Factor-1 (SDF-1)/CXCL12 as Potential Target of Therapeutic Angiogenesis in Critical Leg Ischaemia. *Cardiol Res Pract,* 2012**,** 143209.

HOCK, H., HAMBLEN, M. J., ROOKE, H. M., SCHINDLER, J. W., SALEQUE, S., FUJIWARA, Y. & ORKIN, S. H. 2004. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature,* 431**,** 1002-7.

HODGE, D., COGHILL, E., KEYS, J., MAGUIRE, T., HARTMANN, B., MCDOWALL, A., WEISS, M., GRIMMOND, S. & PERKINS, A. 2006. A global role for EKLF in definitive and primitive erythropoiesis. *Blood,* 107**,** 3359-70.

HOEFFEL, G., WANG, Y., GRETER, M., SEE, P., TEO, P., MALLERET, B., LEBOEUF, M., LOW, D., OLLER, G., ALMEIDA, F., CHOY, S. H., GRISOTTO, M., RENIA, L., CONWAY, S. J., STANLEY, E. R., CHAN, J. K., NG, L. G., SAMOKHVALOV, I. M., MERAD, M. & GINHOUX, F. 2012. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med,* 209**,** 1167-81.

HOLM, M. 1998. Not all healthy donors mobilize hematopoietic progenitor cells sufficiently after G-CSF administration to allow for subsequent CD34 purification of the leukapheresis product. *J Hematother,* 7**,** 111-3.

HOLMES, W. E., LEE, J., KUANG, W. J., RICE, G. C. & WOOD, W. I. 1991. Structure and functional expression of a human interleukin-8 receptor. *Science,* 253**,** 1278-80.

HOLYOAKE, T. L., NICOLINI, F. E. & EAVES, C. J. 1999. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. *Exp Hematol,* 27**,** 1418-27.

HOOPER, A. T., BUTLER, J. M., NOLAN, D. J., KRANZ, A., IIDA, K., KOBAYASHI, M., KOPP, H. G., SHIDO, K., PETIT, I., YANGER, K., JAMES, D., WITTE, L., ZHU, Z., WU, Y., PYTOWSKI, B., ROSENWAKS, Z., MITTAL, V., SATO, T. N. & RAFII, S. 2009. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell,* 4**,** 263-74.

HOPKINS, S. J., FREEMONT, A. J. & JAYSON, M. I. 1984. Pristane-induced arthritis in Balb/c mice. I. Clinical and histological features of the arthropathy. *Rheumatol Int,* 5**,** 21-8.

HOSHINO, K., KAISHO, T., IWABE, T., TAKEUCHI, O. & AKIRA, S. 2002. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. *Int Immunol,* 14**,** 1225-31.

HOSHINO, K., TAKEUCHI, O., KAWAI, T., SANJO, H., OGAWA, T., TAKEDA, Y., TAKEDA, K. & AKIRA, S. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol,* 162**,** 3749-52.

HOSOYA, T., KUROHA, T., MORIGUCHI, T., CUMMINGS, D., MAILLARD, I., LIM, K. C. & ENGEL, J. D. 2009. GATA-3 is required for early T lineage progenitor development. *J Exp Med,* 206**,** 2987-3000.

HSIEH, J. C., KODJABACHIAN, L., REBBERT, M. L., RATTNER, A., SMALLWOOD, P. M., SAMOS, C. H., NUSSE, R., DAWID, I. B. & NATHANS, J. 1999. A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature,* 398**,** 431-6.

HSU, C. L., KING-FLEISCHMAN, A. G., LAI, A. Y., MATSUMOTO, Y., WEISSMAN, I. L. & KONDO, M. 2006. Antagonistic effect of CCAAT enhancer-binding protein-alpha and Pax5 in myeloid or lymphoid lineage choice in common lymphoid progenitors. *Proc Natl Acad Sci U S A,* 103**,** 672-7.

HUANG, E. J., NOCKA, K. H., BUCK, J. & BESMER, P. 1992. Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2. *Mol Biol Cell,* 3**,** 349-62.

HUBER-LANG, M., SARMA, J. V., ZETOUNE, F. S., RITTIRSCH, D., NEFF, T. A., MCGUIRE, S. R., LAMBRIS, J. D., WARNER, R. L., FLIERL, M. A., HOESEL, L. M., GEBHARD, F., YOUNGER, J. G., DROUIN, S. M., WETSEL, R. A. & WARD, P. A. 2006. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med,* 12**,** 682-7.

HUNDT, M., TABATA, H., JEON, M. S., HAYASHI, K., TANAKA, Y., KRISHNA, R., DE GIORGIO, L., LIU, Y. C., FUKATA, M. & ALTMAN, A. 2006. Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. *Immunity,* 24**,** 513-22.

HUNTER, M. G., BAWDEN, L., BROTHERTON, D., CRAIG, S., CRIBBES, S., CZAPLEWSKI, L. G., DEXTER, T. M., DRUMMOND, A. H., GEARING, A. H., HEYWORTH, C. M., LORD, B. I., MCCOURT, M., VARLEY, P. G., WOOD, L. M., EDWARDS, R. M. & LEWIS, P. J. 1995. BB-10010: an active variant of human macrophage inflammatory protein-1 alpha with improved pharmaceutical properties. *Blood,* 86**,** 4400-8.

IACOVINO, M., CHONG, D., SZATMARI, I., HARTWECK, L., RUX, D., CAPRIOLI, A., CLEAVER, O. & KYBA, M. 2010. HoxA3 is an apical regulator of haemogenic endothelium. *Nat Cell Biol,* 13**,** 72-8.

IHLE, J. N., KELLER, J., OROSZLAN, S., HENDERSON, L. E., COPELAND, T. D., FITCH, F., PRYSTOWSKY, M. B., GOLDWASSER, E., SCHRADER, J. W., PALASZYNSKI, E., DY, M. & LEBEL, B. 1983. Biologic properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, p cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. *J Immunol,* 131**,** 282-7.

IKAWA, T., HIROSE, S., MASUDA, K., KAKUGAWA, K., SATOH, R., SHIBANO-SATOH, A., KOMINAMI, R., KATSURA, Y. & KAWAMOTO, H. 2010. An essential developmental checkpoint for production of the T cell lineage. *Science,* 329**,** 93-6.

IKUTA, K., KINA, T., MACNEIL, I., UCHIDA, N., PEAULT, B., CHIEN, Y. H. & WEISSMAN, I. L. 1990. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell,* 62**,** 863-74.

IMAGAWA, S., NAKANO, Y., OBARA, N., SUZUKI, N., DOI, T., KODAMA, T., NAGASAWA, T. & YAMAMOTO, M. 2003. A GATA-specific inhibitor (K-7174) rescues anemia induced by IL-1beta, TNF-alpha, or L-NMMA. *FASEB J,* 17**,** 1742-4.

INFANTINO, S., MOEPPS, B. & THELEN, M. 2006. Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells. *J Immunol,* 176**,** 2197-207.

INTRONA, M., ALLES, V. V., CASTELLANO, M., PICARDI, G., DE GIOIA, L., BOTTAZZAI, B., PERI, G., BREVIARIO, F., SALMONA, M., DE GREGORIO, L., DRAGANI, T. A., SRINIVASAN, N., BLUNDELL, T. L., HAMILTON, T. A. & MANTOVANI, A. 1996. Cloning of mouse ptx3, a new member of the pentraxin gene family expressed at extrahepatic sites. *Blood,* 87**,** 1862-72.

ITOH, M., KIM, C. H., PALARDY, G., ODA, T., JIANG, Y. J., MAUST, D., YEO, S. Y., LORICK, K., WRIGHT, G. J., ARIZA-MCNAUGHTON, L., WEISSMAN, A. M., LEWIS, J., CHANDRASEKHARAPPA, S. C. & CHITNIS, A. B. 2003. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev Cell,* 4**,** 67-82.

IWASAKI, H. & AKASHI, K. 2007. Myeloid lineage commitment from the hematopoietic stem cell. *Immunity,* 26**,** 726-40.

IWASAKI, H., MIZUNO, S., ARINOBU, Y., OZAWA, H., MORI, Y., SHIGEMATSU, H., TAKATSU, K., TENEN, D. G. & AKASHI, K. 2006. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes Dev,* 20**,** 3010-21.

IWASAKI, H., MIZUNO, S., MAYFIELD, R., SHIGEMATSU, H., ARINOBU, Y., SEED, B., GURISH, M. F., TAKATSU, K. & AKASHI, K. 2005a. Identification of eosinophil lineage-committed progenitors in the murine bone marrow. *J Exp Med,* 201**,** 1891-7.

IWASAKI, H., MIZUNO, S., WELLS, R. A., CANTOR, A. B., WATANABE, S. & AKASHI, K. 2003. GATA-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. *Immunity,* 19**,** 451-62.

IWASAKI, H., SOMOZA, C., SHIGEMATSU, H., DUPREZ, E. A., IWASAKI-ARAI, J., MIZUNO, S., ARINOBU, Y., GEARY, K., ZHANG, P., DAYARAM, T., FENYUS, M. L., ELF, S., CHAN, S., KASTNER, P., HUETTNER, C. S., MURRAY, R., TENEN, D. G. & AKASHI, K. 2005b. Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood,* 106**,** 1590-600.

JACOBSON, L. O., GOLDWASSER, E., FRIED, W. & PLZAK, L. 1957. Role of the kidney in erythropoiesis. *Nature,* 179**,** 633-4.

JAILLON, S., PERI, G., DELNESTE, Y., FREMAUX, I., DONI, A., MOALLI, F., GARLANDA, C., ROMANI, L., GASCAN, H., BELLOCCHIO, S., BOZZA, S., CASSATELLA, M. A., JEANNIN, P. & MANTOVANI, A. 2007. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J Exp Med,* 204**,** 793-804.

JAISWAL, S., JAMIESON, C. H., PANG, W. W., PARK, C. Y., CHAO, M. P., MAJETI, R., TRAVER, D., VAN ROOIJEN, N. & WEISSMAN, I. L. 2009. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell,* 138**,** 271-85.

JALILI, A., SHIRVAIKAR, N., MARQUEZ-CURTIS, L. A., TURNER, A. R. & JANOWSKA-WIECZOREK, A. 2010. The HGF/c-Met axis synergizes with G-CSF in the mobilization of hematopoietic stem/progenitor cells. *Stem Cells Dev,* 19**,** 1143-51.

JANOWSKI, M. 2009. Functional diversity of SDF-1 splicing variants. *Cell Adh Migr,* 3**,** 243-9.

JARROSSAY, D., NAPOLITANI, G., COLONNA, M., SALLUSTO, F. & LANZAVECCHIA, A. 2001. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur J Immunol,* 31**,** 3388-93.

JASTER, R., ZHU, Y., PLESS, M., BHATTACHARYA, S., MATHEY-PREVOT, B. & D'ANDREA, A. D. 1997. JAK2 is required for induction of the murine DUB-1 gene. *Mol Cell Biol,* 17**,** 3364-72.

JEANNET, G., SCHELLER, M., SCARPELLINO, L., DUBOUX, S., GARDIOL, N., BACK, J., KUTTLER, F., MALANCHI, I., BIRCHMEIER, W., LEUTZ, A., HUELSKEN, J. & HELD, W. 2008. Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. *Blood,* 111**,** 142-9.

JIANG, H., KUANG, Y., WU, Y., SMRCKA, A., SIMON, M. I. & WU, D. 1996. Pertussis toxin-sensitive activation of phospholipase C by the C5a and fMet-Leu-Phe receptors. *J Biol Chem,* 271**,** 13430-4.

JORDAN, C. T., ASTLE, C. M., ZAWADZKI, J., MACKAREHTSCHIAN, K., LEMISCHKA, I. R. & HARRISON, D. E. 1995. Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. *Exp Hematol,* 23**,** 1011-5.

JUNG, Y., WANG, J., SCHNEIDER, A., SUN, Y. X., KOH-PAIGE, A. J., OSMAN, N. I., MCCAULEY, L. K. & TAICHMAN, R. S. 2006. Regulation of SDF-1 (CXCL12) production by osteoblasts; a possible mechanism for stem cell homing. *Bone,* 38**,** 497-508.

JUNG, Y., WANG, J., SONG, J., SHIOZAWA, Y., HAVENS, A., WANG, Z., SUN, Y. X., EMERSON, S. G., KREBSBACH, P. H. & TAICHMAN, R. S. 2007. Annexin II expressed by osteoblasts and endothelial cells regulates stem cell adhesion, homing, and engraftment following transplantation. *Blood,* 110**,** 82-90.

JUNK, A. K., MAMMIS, A., SAVITZ, S. I., SINGH, M., ROTH, S., MALHOTRA, S., ROSENBAUM, P. S., CERAMI, A., BRINES, M. & ROSENBAUM, D. M. 2002. Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proc Natl Acad Sci U S A,* 99**,** 10659-64.

JURK, M., HEIL, F., VOLLMER, J., SCHETTER, C., KRIEG, A. M., WAGNER, H., LIPFORD, G. & BAUER, S. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat Immunol,* 3**,** 499.

KABURAGI, Y., SHIMADA, Y., NAGAOKA, T., HASEGAWA, M., TAKEHARA, K. & SATO, S. 2001. Enhanced production of CC-chemokines (RANTES, MCP-1, MIP-1alpha, MIP-1beta, and eotaxin) in patients with atopic dermatitis. *Arch Dermatol Res,* 293**,** 350-5.

KADOWAKI, N., HO, S., ANTONENKO, S., MALEFYT, R. W., KASTELEIN, R. A., BAZAN, F. & LIU, Y. J. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med,* 194**,** 863-9.

KAISHO, T., TAKEUCHI, O., KAWAI, T., HOSHINO, K. & AKIRA, S. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol,* 166**,** 5688-94.

KANG, J. & DER, S. D. 2004. Cytokine functions in the formative stages of a lymphocyte's life. *Curr Opin Immunol,* 16**,** 180-90.

KASHIWAZAKI, M., TANAKA, T., KANDA, H., EBISUNO, Y., IZAWA, D., FUKUMA, N., AKIMITSU, N., SEKIMIZU, K., MONDEN, M. & MIYASAKA, M. 2003. A high endothelial venule-expressing promiscuous chemokine receptor DARC can bind inflammatory, but not lymphoid, chemokines and is dispensable for lymphocyte homing under physiological conditions. *Int Immunol,* 15**,** 1219-27.

KATSCHKE, K. J., JR., ROTTMAN, J. B., RUTH, J. H., QIN, S., WU, L., LAROSA, G., PONATH, P., PARK, C. C., POPE, R. M. & KOCH, A. E. 2001. Differential expression of chemokine receptors on peripheral blood,

synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. *Arthritis Rheum,* 44**,** 1022-32.

KAWABATA, K., UJIKAWA, M., EGAWA, T., KAWAMOTO, H., TACHIBANA, K., IIZASA, H., KATSURA, Y., KISHIMOTO, T. & NAGASAWA, T. 1999. A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. *Proc Natl Acad Sci U S A,* 96**,** 5663-7.

KAWAI, T., TAKEUCHI, O., FUJITA, T., INOUE, J., MUHLRADT, P. F., SATO, S., HOSHINO, K. & AKIRA, S. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol,* 167**,** 5887-94.

KAWAMURA, H., KAWAMURA, T., KOKAI, Y., MORI, M., MATSUURA, A., OYA, H., HONDA, S., SUZUKI, S., WEERASHINGHE, A., WATANABE, H. & ABO, T. 1999. Expansion of extrathymic T cells as well as granulocytes in the liver and other organs of granulocyte-colony stimulating factor transgenic mice: why they lost the ability of hybrid resistance. *J Immunol,* 162**,** 5957-64.

KELLER, G. 2005. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev,* 19**,** 1129-55.

KELLY, E., BAILEY, C. P. & HENDERSON, G. 2008. Agonist-selective mechanisms of GPCR desensitization. *Br J Pharmacol,* 153 Suppl 1**,** S379-88.

KENNEDY, M., D'SOUZA, S. L., LYNCH-KATTMAN, M., SCHWANTZ, S. & KELLER, G. 2007. Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood,* 109**,** 2679-87.

KIEL, M. J. & MORRISON, S. J. 2008. Uncertainty in the niches that maintain haematopoietic stem cells. *Nat Rev Immunol,* 8**,** 290-301.

KIEL, M. J., YILMAZ, O. H., IWASHITA, T., TERHORST, C. & MORRISON, S. J. 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell,* 121**,** 1109-21.

KIERDORF, K., ERNY, D., GOLDMANN, T., SANDER, V., SCHULZ, C., PERDIGUERO, E. G., WIEGHOFER, P., HEINRICH, A., RIEMKE, P., HOLSCHER, C., MULLER, D. N., LUCKOW, B., BROCKER, T., DEBOWSKI, K., FRITZ, G., OPDENAKKER, G., DIEFENBACH, A., BIBER, K., HEIKENWALDER, M., GEISSMANN, F., ROSENBAUER, F. & PRINZ, M. 2013. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nat Neurosci,* 16**,** 273-80.

KIKUCHI, K., LAI, A. Y., HSU, C. L. & KONDO, M. 2005. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med,* 201**,** 1197-203.

KIM, J. M., KIM, N. I., OH, Y. K., KIM, Y. J., YOUN, J. & AHN, M. J. 2005. CpG oligodeoxynucleotides induce IL-8 expression in CD34+ cells via mitogen-activated protein kinase-dependent and NF-kappaB-independent pathways. *Int Immunol,* 17**,** 1525-31.

KIM, Y. W., KOO, B. K., JEONG, H. W., YOON, M. J., SONG, R., SHIN, J., JEONG, D. C., KIM, S. H. & KONG, Y. Y. 2008. Defective Notch activation in microenvironment leads to myeloproliferative disease. *Blood,* 112**,** 4628-38.

KING, A. G., HOROWITZ, D., DILLON, S. B., LEVIN, R., FARESE, A. M., MACVITTIE, T. J. & PELUS, L. M. 2001. Rapid mobilization of murine hematopoietic stem cells with enhanced engraftment properties and evaluation of hematopoietic progenitor cell mobilization in rhesus monkeys by a single

injection of SB-251353, a specific truncated form of the human CXC chemokine GRObeta. *Blood,* 97**,** 1534-42.
KINOSHITA, T., YOKOTA, T., ARAI, K. & MIYAJIMA, A. 1995. Regulation of Bcl-2 expression by oncogenic Ras protein in hematopoietic cells. *Oncogene,* 10**,** 2207-12.
KISSA, K. & HERBOMEL, P. 2010. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature,* 464**,** 112-5.
KLEMSZ, M. J., MCKERCHER, S. R., CELADA, A., VAN BEVEREN, C. & MAKI, R. A. 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell,* 61**,** 113-24.
KLIMCHENKO, O., MORI, M., DISTEFANO, A., LANGLOIS, T., LARBRET, F., LECLUSE, Y., FERAUD, O., VAINCHENKER, W., NOROL, F. & DEBILI, N. 2009. A common bipotent progenitor generates the erythroid and megakaryocyte lineages in embryonic stem cell-derived primitive hematopoiesis. *Blood,* 114**,** 1506-17.
KLINGMULLER, U. 1997. The role of tyrosine phosphorylation in proliferation and maturation of erythroid progenitor cells--signals emanating from the erythropoietin receptor. *Eur J Biochem,* 249**,** 637-47.
KLINGMULLER, U., BERGELSON, S., HSIAO, J. G. & LODISH, H. F. 1996. Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5. *Proc Natl Acad Sci U S A,* 93**,** 8324-8.
KOCH, A. E., KUNKEL, S. L., HARLOW, L. A., JOHNSON, B., EVANOFF, H. L., HAINES, G. K., BURDICK, M. D., POPE, R. M. & STRIETER, R. M. 1992. Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. *J Clin Invest,* 90**,** 772-9.
KOCH, U., WILSON, A., COBAS, M., KEMLER, R., MACDONALD, H. R. & RADTKE, F. 2008. Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. *Blood,* 111**,** 160-4.
KOEFFLER, H. P., GASSON, J., RANYARD, J., SOUZA, L., SHEPARD, M. & MUNKER, R. 1987. Recombinant human TNF alpha stimulates production of granulocyte colony-stimulating factor. *Blood,* 70**,** 55-9.
KOLLET, O., DAR, A., SHIVTIEL, S., KALINKOVICH, A., LAPID, K., SZTAINBERG, Y., TESIO, M., SAMSTEIN, R. M., GOICHBERG, P., SPIEGEL, A., ELSON, A. & LAPIDOT, T. 2006. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med,* 12**,** 657-64.
KONDO, M., WAGERS, A. J., MANZ, M. G., PROHASKA, S. S., SCHERER, D. C., BEILHACK, G. F., SHIZURU, J. A. & WEISSMAN, I. L. 2003. Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol,* 21**,** 759-806.
KOO, B. K., LIM, H. S., SONG, R., YOON, M. J., YOON, K. J., MOON, J. S., KIM, Y. W., KWON, M. C., YOO, K. W., KONG, M. P., LEE, J., CHITNIS, A. B., KIM, C. H. & KONG, Y. Y. 2005. Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development,* 132**,** 3459-70.
KOURY, S. T., BONDURANT, M. C. & KOURY, M. J. 1988. Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. *Blood,* 71**,** 524-7.
KOURY, S. T., BONDURANT, M. C., KOURY, M. J. & SEMENZA, G. L. 1991. Localization of cells producing erythropoietin in murine liver by in situ hybridization. *Blood,* 77**,** 2497-503.
KOURY, S. T., KOURY, M. J., BONDURANT, M. C., CARO, J. & GRABER, S. E. 1989. Quantitation of erythropoietin-producing cells in kidneys of mice by in situ

hybridization: correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration. *Blood,* 74**,** 645-51.
KRISTINSSON, S. Y., BJORKHOLM, M., HULTCRANTZ, M., DEROLF, A. R., LANDGREN, O. & GOLDIN, L. R. 2011. Chronic immune stimulation might act as a trigger for the development of acute myeloid leukemia or myelodysplastic syndromes. *J Clin Oncol,* 29**,** 2897-903.
KUCIA, M., JANKOWSKI, K., RECA, R., WYSOCZYNSKI, M., BANDURA, L., ALLENDORF, D. J., ZHANG, J., RATAJCZAK, J. & RATAJCZAK, M. Z. 2004a. CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *J Mol Histol,* 35**,** 233-45.
KUCIA, M., RATAJCZAK, J., RECA, R., JANOWSKA-WIECZOREK, A. & RATAJCZAK, M. Z. 2004b. Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury. *Blood Cells Mol Dis,* 32**,** 52-7.
KUCIA, M., RECA, R., MIEKUS, K., WANZECK, J., WOJAKOWSKI, W., JANOWSKA-WIECZOREK, A., RATAJCZAK, J. & RATAJCZAK, M. Z. 2005. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. *Stem Cells,* 23**,** 879-94.
LACOMBE, C., DA SILVA, J. L., BRUNEVAL, P., FOURNIER, J. G., WENDLING, F., CASADEVALL, N., CAMILLERI, J. P., BARIETY, J., VARET, B. & TAMBOURIN, P. 1988. Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. *J Clin Invest,* 81**,** 620-3.
LAGLER, H., SHARIF, O., HASLINGER, I., MATT, U., STICH, K., FURTNER, T., DONINGER, B., SCHMID, K., GATTRINGER, R., DE VOS, A. F. & KNAPP, S. 2009. TREM-1 activation alters the dynamics of pulmonary IRAK-M expression in vivo and improves host defense during pneumococcal pneumonia. *J Immunol,* 183**,** 2027-36.
LAIOSA, C. V., STADTFELD, M. & GRAF, T. 2006. Determinants of lymphoid-myeloid lineage diversification. *Annu Rev Immunol,* 24**,** 705-38.
LAKY, K., LEFRANCOIS, L., VON FREEDEN-JEFFRY, U., MURRAY, R. & PUDDINGTON, L. 1998. The role of IL-7 in thymic and extrathymic development of TCR gamma delta cells. *J Immunol,* 161**,** 707-13.
LANCRIN, C., SROCZYNSKA, P., STEPHENSON, C., ALLEN, T., KOUSKOFF, V. & LACAUD, G. 2009. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature,* 457**,** 892-5.
LAPIDOT, T., DAR, A. & KOLLET, O. 2005. How do stem cells find their way home? *Blood,* 106**,** 1901-10.
LAPIDOT, T. & KOLLET, O. 2002. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. *Leukemia,* 16**,** 1992-2003.
LAPIDOT, T. & PETIT, I. 2002. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp Hematol,* 30**,** 973-81.
LATERVEER, L., LINDLEY, I. J., HAMILTON, M. S., WILLEMZE, R. & FIBBE, W. E. 1995. Interleukin-8 induces rapid mobilization of hematopoietic stem cells with radioprotective capacity and long-term myelolymphoid repopulating ability. *Blood,* 85**,** 2269-75.
LATERVEER, L., LINDLEY, I. J., HEEMSKERK, D. P., CAMPS, J. A., PAUWELS, E. K., WILLEMZE, R. & FIBBE, W. E. 1996. Rapid mobilization of hematopoietic progenitor cells in rhesus monkeys by a single intravenous injection of interleukin-8. *Blood,* 87**,** 781-8.

LAWSON, M. A. & MAXFIELD, F. R. 1995. Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature,* 377**,** 75-9.

LE BORGNE, R., REMAUD, S., HAMEL, S. & SCHWEISGUTH, F. 2005. Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. *PLoS Biol,* 3**,** e96.

LECINE, P., VILLEVAL, J. L., VYAS, P., SWENCKI, B., XU, Y. & SHIVDASANI, R. A. 1998. Mice lacking transcription factor NF-E2 provide in vivo validation of the proplatelet model of thrombocytopoiesis and show a platelet production defect that is intrinsic to megakaryocytes. *Blood,* 92**,** 1608-16.

LEE, J., CHUANG, T. H., REDECKE, V., SHE, L., PITHA, P. M., CARSON, D. A., RAZ, E. & COTTAM, H. B. 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. *Proc Natl Acad Sci U S A,* 100**,** 6646-51.

LEE, J. H., WANG, C. & KIM, C. H. 2009. FoxP3+ regulatory T cells restrain splenic extramedullary myelopoiesis via suppression of hemopoietic cytokine-producing T cells. *J Immunol,* 183**,** 6377-86.

LEE, W. Y., LOCKNISKAR, M. F. & FISCHER, S. M. 1994. Interleukin-1 alpha mediates phorbol ester-induced inflammation and epidermal hyperplasia. *FASEB J,* 8**,** 1081-7.

LEKSTROM-HIMES, J. A., DORMAN, S. E., KOPAR, P., HOLLAND, S. M. & GALLIN, J. I. 1999. Neutrophil-specific granule deficiency results from a novel mutation with loss of function of the transcription factor CCAAT/enhancer binding protein epsilon. *J Exp Med,* 189**,** 1847-52.

LEMAITRE, B., NICOLAS, E., MICHAUT, L., REICHHART, J. M. & HOFFMANN, J. A. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell,* 86**,** 973-83.

LEV, S., BLECHMAN, J., NISHIKAWA, S., GIVOL, D. & YARDEN, Y. 1993. Interspecies molecular chimeras of kit help define the binding site of the stem cell factor. *Mol Cell Biol,* 13**,** 2224-34.

LEVESQUE, J. P., HENDY, J., TAKAMATSU, Y., SIMMONS, P. J. & BENDALL, L. J. 2003a. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *J Clin Invest,* 111**,** 187-96.

LEVESQUE, J. P., HENDY, J., WINKLER, I. G., TAKAMATSU, Y. & SIMMONS, P. J. 2003b. Granulocyte colony-stimulating factor induces the release in the bone marrow of proteases that cleave c-KIT receptor (CD117) from the surface of hematopoietic progenitor cells. *Exp Hematol,* 31**,** 109-17.

LEVESQUE, J. P., LIU, F., SIMMONS, P. J., BETSUYAKU, T., SENIOR, R. M., PHAM, C. & LINK, D. C. 2004. Characterization of hematopoietic progenitor mobilization in protease-deficient mice. *Blood,* 104**,** 65-72.

LEVESQUE, J. P., TAKAMATSU, Y., NILSSON, S. K., HAYLOCK, D. N. & SIMMONS, P. J. 2001. Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. *Blood,* 98**,** 1289-97.

LI, C. L. & JOHNSON, G. R. 1994. Stem cell factor enhances the survival but not the self-renewal of murine hematopoietic long-term repopulating cells. *Blood,* 84**,** 408-14.

LI, J., SUTTER, C., PARKER, D. S., BLAUWKAMP, T., FANG, M. & CADIGAN, K. M. 2007. CBP/p300 are bimodal regulators of Wnt signaling. *EMBO J,* 26**,** 2284-94.

LI, P., BURKE, S., WANG, J., CHEN, X., ORTIZ, M., LEE, S. C., LU, D., CAMPOS, L., GOULDING, D., NG, B. L., DOUGAN, G., HUNTLY, B., GOTTGENS, B., JENKINS, N. A., COPELAND, N. G., COLUCCI, F. & LIU, P. 2010. Reprogramming of T cells to natural killer-like cells upon Bcl11b deletion. *Science,* 329**,** 85-9.

LI, Y. M., PAN, Y., WEI, Y., CHENG, X., ZHOU, B. P., TAN, M., ZHOU, X., XIA, W., HORTOBAGYI, G. N., YU, D. & HUNG, M. C. 2004. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell,* 6**,** 459-69.

LIBURA, J., DRUKALA, J., MAJKA, M., TOMESCU, O., NAVENOT, J. M., KUCIA, M., MARQUEZ, L., PEIPER, S. C., BARR, F. G., JANOWSKA-WIECZOREK, A. & RATAJCZAK, M. Z. 2002. CXCR4-SDF-1 signaling is active in rhabdomyosarcoma cells and regulates locomotion, chemotaxis, and adhesion. *Blood,* 100**,** 2597-606.

LICHTERFELD, M., MARTIN, S., BURKLY, L., HAAS, R. & KRONENWETT, R. 2000. Mobilization of CD34+ haematopoietic stem cells is associated with a functional inactivation of the integrin very late antigen 4. *Br J Haematol,* 110**,** 71-81.

LIESCHKE, G. J., MAHER, D., CEBON, J., O'CONNOR, M., GREEN, M., SHERIDAN, W., BOYD, A., RALLINGS, M., BONNEM, E., METCALF, D. & ET AL. 1989. Effects of bacterially synthesized recombinant human granulocyte-macrophage colony-stimulating factor in patients with advanced malignancy. *Ann Intern Med,* 110**,** 357-64.

LIEU, Y. K., KUMAR, A., PAJEROWSKI, A. G., ROGERS, T. J. & REDDY, E. P. 2004. Requirement of c-myb in T cell development and in mature T cell function. *Proc Natl Acad Sci U S A,* 101**,** 14853-8.

LILES, W. C., BROXMEYER, H. E., RODGER, E., WOOD, B., HUBEL, K., COOPER, S., HANGOC, G., BRIDGER, G. J., HENSON, G. W., CALANDRA, G. & DALE, D. C. 2003. Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. *Blood,* 102**,** 2728-30.

LIU, F., POURSINE-LAURENT, J. & LINK, D. C. 1997. The granulocyte colony-stimulating factor receptor is required for the mobilization of murine hematopoietic progenitors into peripheral blood by cyclophosphamide or interleukin-8 but not flt-3 ligand. *Blood,* 90**,** 2522-8.

LIU, F., POURSINE-LAURENT, J. & LINK, D. C. 2000. Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF. *Blood,* 95**,** 3025-31.

LIU, R., PAXTON, W. A., CHOE, S., CERADINI, D., MARTIN, S. R., HORUK, R., MACDONALD, M. E., STUHLMANN, H., KOUP, R. A. & LANDAU, N. R. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell,* 86**,** 367-77.

LO CELSO, C., FLEMING, H. E., WU, J. W., ZHAO, C. X., MIAKE-LYE, S., FUJISAKI, J., COTE, D., ROWE, D. W., LIN, C. P. & SCADDEN, D. T. 2009. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature,* 457**,** 92-6.

LOCATI, M., BONECCHI, R. & CORSI, M. M. 2005. Chemokines and their receptors: roles in specific clinical conditions and measurement in the clinical laboratory. *Am J Clin Pathol,* 123 Suppl**,** S82-95.

LOK, S., KAUSHANSKY, K., HOLLY, R. D., KUIJPER, J. L., LOFTON-DAY, C. E., OORT, P. J., GRANT, F. J., HEIPEL, M. D., BURKHEAD, S. K., KRAMER, J. M. & ET AL. 1994. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature,* 369**,** 565-8.

LONGLEY, B. J., JR., MORGANROTH, G. S., TYRRELL, L., DING, T. G., ANDERSON, D. M., WILLIAMS, D. E. & HALABAN, R. 1993. Altered metabolism of mast-

cell growth factor (c-kit ligand) in cutaneous mastocytosis. *N Engl J Med,* 328**,** 1302-7.

LORD, B. I., DEXTER, T. M., CLEMENTS, J. M., HUNTER, M. A. & GEARING, A. J. 1992. Macrophage-inflammatory protein protects multipotent hematopoietic cells from the cytotoxic effects of hydroxyurea in vivo. *Blood,* 79**,** 2605-9.

LORD, B. I., WOOLFORD, L. B., WOOD, L. M., CZAPLEWSKI, L. G., MCCOURT, M., HUNTER, M. G. & EDWARDS, R. M. 1995. Mobilization of early hematopoietic progenitor cells with BB-10010: a genetically engineered variant of human macrophage inflammatory protein-1 alpha. *Blood,* 85**,** 3412-5.

LUIS, T. C., WEERKAMP, F., NABER, B. A., BAERT, M. R., DE HAAS, E. F., NIKOLIC, T., HEUVELMANS, S., DE KRIJGER, R. R., VAN DONGEN, J. J. & STAAL, F. J. 2009. Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood,* 113**,** 546-54.

LUKOWSKY, W. A. & PAINTER, R. H. 1972. Studies on the role of sialic acid in the physical and biological properties of erythropoietin. *Can J Biochem,* 50**,** 909-17.

LUND, J. M., ALEXOPOULOU, L., SATO, A., KAROW, M., ADAMS, N. C., GALE, N. W., IWASAKI, A. & FLAVELL, R. A. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A,* 101**,** 5598-603.

MA, Q., JONES, D., BORGHESANI, P. R., SEGAL, R. A., NAGASAWA, T., KISHIMOTO, T., BRONSON, R. T. & SPRINGER, T. A. 1998a. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A,* 95**,** 9448-53.

MA, Y., THORNTON, S., BOIVIN, G. P., HIRSH, D., HIRSCH, R. & HIRSCH, E. 1998b. Altered susceptibility to collagen-induced arthritis in transgenic mice with aberrant expression of interleukin-1 receptor antagonist. *Arthritis Rheum,* 41**,** 1798-805.

MADIGAN, J., FREEMAN, D. J., MENZIES, F., FORROW, S., NELSON, S. M., YOUNG, A., SHARKEY, A., MOFFETT, A., GRAHAM, G. J., GREER, I. A., ROT, A. & NIBBS, R. J. 2010. Chemokine scavenger D6 is expressed by trophoblasts and aids the survival of mouse embryos transferred into allogeneic recipients. *J Immunol,* 184**,** 3202-12.

MAIER, T., GUELL, M. & SERRANO, L. 2009. Correlation of mRNA and protein in complex biological samples. *FEBS Lett,* 583**,** 3966-73.

MANCINI, S. J., MANTEI, N., DUMORTIER, A., SUTER, U., MACDONALD, H. R. & RADTKE, F. 2005. Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood,* 105**,** 2340-2.

MANOVA, K., BACHVAROVA, R. F., HUANG, E. J., SANCHEZ, S., PRONOVOST, S. M., VELAZQUEZ, E., MCGUIRE, B. & BESMER, P. 1992. c-kit receptor and ligand expression in postnatal development of the mouse cerebellum suggests a function for c-kit in inhibitory interneurons. *J Neurosci,* 12**,** 4663-76.

MANSSON, R., HULTQUIST, A., LUC, S., YANG, L., ANDERSON, K., KHARAZI, S., AL-HASHMI, S., LIUBA, K., THOREN, L., ADOLFSSON, J., BUZA-VIDAS, N., QIAN, H., SONEJI, S., ENVER, T., SIGVARDSSON, M. & JACOBSEN, S. E. 2007. Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. *Immunity,* 26**,** 407-19.

MANSSON, R., ZANDI, S., WELINDER, E., TSAPOGAS, P., SAKAGUCHI, N., BRYDER, D. & SIGVARDSSON, M. 2009. Single-cell analysis of the common lymphoid progenitor compartment reveals functional and molecular heterogeneity. *Blood,* 115**,** 2601-9.

MANTOVANI, A. 1999. The chemokine system: redundancy for robust outputs. *Immunol Today,* 20**,** 254-7.

MANZ, M. G. & BOETTCHER, S. 2014. Emergency granulopoiesis. *Nat Rev Immunol,* 14**,** 302-14.

MAO, B., WU, W., LI, Y., HOPPE, D., STANNEK, P., GLINKA, A. & NIEHRS, C. 2001. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature,* 411**,** 321-5.

MARKIEWSKI, M. M. & LAMBRIS, J. D. 2007. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am J Pathol,* 171**,** 715-27.

MASSBERG, S., SCHAERLI, P., KNEZEVIC-MARAMICA, I., KOLLNBERGER, M., TUBO, N., MOSEMAN, E. A., HUFF, I. V., JUNT, T., WAGERS, A. J., MAZO, I. B. & VON ANDRIAN, U. H. 2007. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell,* 131**,** 994-1008.

MASUDA, K., KAKUGAWA, K., NAKAYAMA, T., MINATO, N., KATSURA, Y. & KAWAMOTO, H. 2007. T cell lineage determination precedes the initiation of TCR beta gene rearrangement. *J Immunol,* 179**,** 3699-706.

MATSUI, T., AKAHOSHI, T., NAMAI, R., HASHIMOTO, A., KURIHARA, Y., RANA, M., NISHIMURA, A., ENDO, H., KITASATO, H., KAWAI, S., TAKAGISHI, K. & KONDO, H. 2001. Selective recruitment of CCR6-expressing cells by increased production of MIP-3 alpha in rheumatoid arthritis. *Clin Exp Immunol,* 125**,** 155-61.

MATSUSHIMA, K. & OPPENHEIM, J. J. 1989. Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF. *Cytokine,* 1**,** 2-13.

MAURY, C. P., LILJESTROM, M., LAIHO, K., TIITINEN, S., KAARELA, K. & HURME, M. 2003. Tumor necrosis factor alpha, its soluble receptor I, and -308 gene promoter polymorphism in patients with rheumatoid arthritis with or without amyloidosis: implications for the pathogenesis of nephropathy and anemia of chronic disease in reactive amyloidosis. *Arthritis Rheum,* 48**,** 3068-76.

MAYER, K. L. & STONE, M. J. 2000. NMR solution structure and receptor peptide binding of the CC chemokine eotaxin-2. *Biochemistry,* 39**,** 8382-95.

MCCARTY, J. M., SPRUGEL, K. H., FOX, N. E., SABATH, D. E. & KAUSHANSKY, K. 1995. Murine thrombopoietin mRNA levels are modulated by platelet count. *Blood,* 86**,** 3668-75.

MCCREDIE, K. B., HERSH, E. M. & FREIREICH, E. J. 1971. Cells capable of colony formation in the peripheral blood of man. *Science,* 171**,** 293-4.

MCKINSTRY, W. J., LI, C. L., RASKO, J. E., NICOLA, N. A., JOHNSON, G. R. & METCALF, D. 1997. Cytokine receptor expression on hematopoietic stem and progenitor cells. *Blood,* 89**,** 65-71.

MCNIECE, I. K., LANGLEY, K. E. & ZSEBO, K. M. 1991. Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages. *Exp Hematol,* 19**,** 226-31.

MEBIUS, R. E., RENNERT, P. & WEISSMAN, I. L. 1997. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity,* 7**,** 493-504.

MEDVINSKY, A., RYBTSOV, S. & TAOUDI, S. 2011. Embryonic origin of the adult hematopoietic system: advances and questions. *Development,* 138**,** 1017-31.

MEDVINSKY, A. L., GAN, O. I., SEMENOVA, M. L. & SAMOYLINA, N. L. 1996. Development of day-8 colony-forming unit-spleen hematopoietic progenitors during early murine embryogenesis: spatial and temporal mapping. *Blood,* 87**,** 557-66.

MEDVINSKY, A. L., SAMOYLINA, N. L., MULLER, A. M. & DZIERZAK, E. A. 1993. An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature,* 364**,** 64-7.

MEDZHITOV, R., PRESTON-HURLBURT, P. & JANEWAY, C. A., JR. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature,* 388**,** 394-7.

MEGIAS, J., YANEZ, A., MORIANO, S., O'CONNOR, J. E., GOZALBO, D. & GIL, M. L. 2012. Direct Toll-like receptor-mediated stimulation of hematopoietic stem and progenitor cells occurs in vivo and promotes differentiation toward macrophages. *Stem Cells,* 30**,** 1486-95.

MELLADO, M., RODRIGUEZ-FRADE, J. M., ARAGAY, A., DEL REAL, G., MARTIN, A. M., VILA-CORO, A. J., SERRANO, A., MAYOR, F., JR. & MARTINEZ, A. C. 1998. The chemokine monocyte chemotactic protein 1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. *J Immunol,* 161**,** 805-13.

MELVAN, J. N., SIGGINS, R. W., BAGBY, G. J., STANFORD, W. L., WELSH, D. A., NELSON, S. & ZHANG, P. 2011. Suppression of the stem cell antigen-1 response and granulocyte lineage expansion by alcohol during septicemia. *Crit Care Med,* 39**,** 2121-30.

MENDEZ-FERRER, S., LUCAS, D., BATTISTA, M. & FRENETTE, P. S. 2008. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature,* 452**,** 442-7.

MENDEZ-FERRER, S., MICHURINA, T. V., FERRARO, F., MAZLOOM, A. R., MACARTHUR, B. D., LIRA, S. A., SCADDEN, D. T., MA'AYAN, A., ENIKOLOPOV, G. N. & FRENETTE, P. S. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature,* 466**,** 829-34.

MENZIES-GOW, A., YING, S., SABROE, I., STUBBS, V. L., SOLER, D., WILLIAMS, T. J. & KAY, A. B. 2002. Eotaxin (CCL11) and eotaxin-2 (CCL24) induce recruitment of eosinophils, basophils, neutrophils, and macrophages as well as features of early- and late-phase allergic reactions following cutaneous injection in human atopic and nonatopic volunteers. *J Immunol,* 169**,** 2712-8.

MERAD, M., MANZ, M. G., KARSUNKY, H., WAGERS, A., PETERS, W., CHARO, I., WEISSMAN, I. L., CYSTER, J. G. & ENGLEMAN, E. G. 2002. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol,* 3**,** 1135-41.

METCALF, D. 1989. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature,* 339**,** 27-30.

METCALF, D. 2007. On hematopoietic stem cell fate. *Immunity,* 26**,** 669-73.

METHIA, N., LOUACHE, F., VAINCHENKER, W. & WENDLING, F. 1993. Oligodeoxynucleotides antisense to the proto-oncogene c-mpl specifically inhibit in vitro megakaryocytopoiesis. *Blood,* 82**,** 1395-401.

MIGLIACCIO, G., MIGLIACCIO, A. R., PETTI, S., MAVILIO, F., RUSSO, G., LAZZARO, D., TESTA, U., MARINUCCI, M. & PESCHLE, C. 1986. Human embryonic

hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac----liver transition. *J Clin Invest,* 78**,** 51-60.

MILNER, L. A., KOPAN, R., MARTIN, D. I. & BERNSTEIN, I. D. 1994. A human homologue of the Drosophila developmental gene, Notch, is expressed in CD34+ hematopoietic precursors. *Blood,* 83**,** 2057-62.

MIYAJIMA, A. 1992. Molecular structure of the IL-3, GM-CSF and IL-5 receptors. *Int J Cell Cloning,* 10**,** 126-34.

MIYAJIMA, A., KITAMURA, T., HARADA, N., YOKOTA, T. & ARAI, K. 1992. Cytokine receptors and signal transduction. *Annu Rev Immunol,* 10**,** 295-331.

MIYAMOTO, K., YOSHIDA, S., KAWASUMI, M., HASHIMOTO, K., KIMURA, T., SATO, Y., KOBAYASHI, T., MIYAUCHI, Y., HOSHI, H., IWASAKI, R., MIYAMOTO, H., HAO, W., MORIOKA, H., CHIBA, K., KOBAYASHI, T., YASUDA, H., PENNINGER, J. M., TOYAMA, Y., SUDA, T. & MIYAMOTO, T. 2011. Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization. *J Exp Med,* 208**,** 2175-81.

MIYAMOTO, T., IWASAKI, H., REIZIS, B., YE, M., GRAF, T., WEISSMAN, I. L. & AKASHI, K. 2002. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell,* 3**,** 137-47.

MOLENDIJK, W. J., VAN OUDENAREN, A., VAN DIJK, H., DAHA, M. R. & BENNER, R. 1986. Complement split product C5a mediates the lipopolysaccharide-induced mobilization of CFU-s and haemopoietic progenitor cells, but not the mobilization induced by proteolytic enzymes. *Cell Tissue Kinet,* 19**,** 407-17.

MOLINO, M., WOOLKALIS, M. J., PREVOST, N., PRATICO, D., BARNATHAN, E. S., TARABOLETTI, G., HAGGARTY, B. S., HESSELGESSER, J., HORUK, R., HOXIE, J. A. & BRASS, L. F. 2000. CXCR4 on human endothelial cells can serve as both a mediator of biological responses and as a receptor for HIV-2. *Biochim Biophys Acta,* 1500**,** 227-40.

MOORE, K. A., EMA, H. & LEMISCHKA, I. R. 1997. In vitro maintenance of highly purified, transplantable hematopoietic stem cells. *Blood,* 89**,** 4337-47.

MOORE, M. A., HATTORI, K., HEISSIG, B., SHIEH, J. H., DIAS, S., CRYSTAL, R. G. & RAFII, S. 2001. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. *Ann N Y Acad Sci,* 938**,** 36-45; discussion 45-7.

MOORE, M. A. & METCALF, D. 1970. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol,* 18**,** 279-96.

MORATZ, C., HARRISON, K. & KEHRL, J. H. 2004. Regulation of chemokine-induced lymphocyte migration by RGS proteins. *Methods Enzymol,* 389**,** 15-32.

MORIKAWA, Y., TOHYA, K., HARA, T., KITAMURA, T. & MIYAJIMA, A. 1996. Expression of IL-3 receptor in testis. *Biochem Biophys Res Commun,* 226**,** 107-12.

MORITA, Y., EMA, H. & NAKAUCHI, H. 2010. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med,* 207**,** 1173-82.

MORRISON, S. J., HEMMATI, H. D., WANDYCZ, A. M. & WEISSMAN, I. L. 1995. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci U S A,* 92**,** 10302-6.

MOSER, B. 2003. Chemokines: role in immune cell traffic. *Eur Cytokine Netw,* 14**,** 204-10.

MOSER, B. & WILLIMANN, K. 2004. Chemokines: role in inflammation and immune surveillance. *Ann Rheum Dis,* 63 Suppl 2**,** ii84-ii89.

MOSKOWITZ, C. H., GLASSMAN, J. R., WUEST, D., MASLAK, P., REICH, L., GUCCIARDO, A., COADY-LYONS, N., ZELENETZ, A. D. & NIMER, S. D. 1998. Factors affecting mobilization of peripheral blood progenitor cells in patients with lymphoma. *Clin Cancer Res,* 4**,** 311-6.

MULLER-LADNER, U., KRIEGSMANN, J., FRANKLIN, B. N., MATSUMOTO, S., GEILER, T., GAY, R. E. & GAY, S. 1996. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. *Am J Pathol,* 149**,** 1607-15.

MULLER, A. M., MEDVINSKY, A., STROUBOULIS, J., GROSVELD, F. & DZIERZAK, E. 1994. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity,* 1**,** 291-301.

MUNOZ, L., NOMDEDEU, J. F., LOPEZ, O., CARNICER, M. J., BELLIDO, M., AVENTIN, A., BRUNET, S. & SIERRA, J. 2001. Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies. *Haematologica,* 86**,** 1261-9.

MURPHY, J., SUMMER, R., WILSON, A. A., KOTTON, D. N. & FINE, A. 2008. The prolonged life-span of alveolar macrophages. *Am J Respir Cell Mol Biol,* 38**,** 380-5.

MURPHY, P. M. & TIFFANY, H. L. 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science,* 253**,** 1280-3.

MUZIO, M., BOSISIO, D., POLENTARUTTI, N., D'AMICO, G., STOPPACCIARO, A., MANCINELLI, R., VAN'T VEER, C., PENTON-ROL, G., RUCO, L. P., ALLAVENA, P. & MANTOVANI, A. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol,* 164**,** 5998-6004.

MYERS, S. J., WONG, L. M. & CHARO, I. F. 1995. Signal transduction and ligand specificity of the human monocyte chemoattractant protein-1 receptor in transfected embryonic kidney cells. *J Biol Chem,* 270**,** 5786-92.

NAGAI, Y., GARRETT, K. P., OHTA, S., BAHRUN, U., KOURO, T., AKIRA, S., TAKATSU, K. & KINCADE, P. W. 2006. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity,* 24**,** 801-12.

NAGASAWA, T. 2000. A chemokine, SDF-1/PBSF, and its receptor, CXC chemokine receptor 4, as mediators of hematopoiesis. *Int J Hematol,* 72**,** 408-11.

NAGASAWA, T. 2001. Role of chemokine SDF-1/PBSF and its receptor CXCR4 in blood vessel development. *Ann N Y Acad Sci,* 947**,** 112-5; discussion 115-6.

NAGASAWA, T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol,* 6**,** 107-16.

NAGASAWA, T., HIROTA, S., TACHIBANA, K., TAKAKURA, N., NISHIKAWA, S., KITAMURA, Y., YOSHIDA, N., KIKUTANI, H. & KISHIMOTO, T. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature,* 382**,** 635-8.

NAGATA, S., TSUCHIYA, M., ASANO, S., YAMAMOTO, O., HIRATA, Y., KUBOTA, N., OHEDA, M., NOMURA, H. & YAMAZAKI, T. 1986. The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor. *EMBO J,* 5**,** 575-81.

NAITO, T., TANAKA, H., NAOE, Y. & TANIUCHI, I. 2011. Transcriptional control of T-cell development. *Int Immunol,* 23**,** 661-8.

NATHAN, C. & DING, A. 2010. Nonresolving inflammation. *Cell,* 140**,** 871-82.

NIBBS, R. J. & GRAHAM, G. J. 2013. Immune regulation by atypical chemokine receptors. *Nat Rev Immunol,* 13**,** 815-29.
NIBBS, R. J., KRIEHUBER, E., PONATH, P. D., PARENT, D., QIN, S., CAMPBELL, J. D., HENDERSON, A., KERJASCHKI, D., MAURER, D., GRAHAM, G. J. & ROT, A. 2001. The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol,* 158**,** 867-77.
NIBBS, R. J., WYLIE, S. M., YANG, J., LANDAU, N. R. & GRAHAM, G. J. 1997. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *J Biol Chem,* 272**,** 32078-83.
NICHOLS, K. E., CRISPINO, J. D., PONCZ, M., WHITE, J. G., ORKIN, S. H., MARIS, J. M. & WEISS, M. J. 2000. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. *Nat Genet,* 24**,** 266-70.
NICOLA, N. A., METCALF, D., MATSUMOTO, M. & JOHNSON, G. R. 1983. Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. *J Biol Chem,* 258**,** 9017-23.
NIE, Y., HAN, Y. C. & ZOU, Y. R. 2008. CXCR4 is required for the quiescence of primitive hematopoietic cells. *J Exp Med,* 205**,** 777-83.
NILSON, D. G., SABATINO, D. E., BODINE, D. M. & GALLAGHER, P. G. 2006. Major erythrocyte membrane protein genes in EKLF-deficient mice. *Exp Hematol,* 34**,** 705-12.
NIMER, S. D. & UCHIDA, H. 1995. Regulation of granulocyte-macrophage colony-stimulating factor and interleukin 3 expression. *Stem Cells,* 13**,** 324-35.
NIOCHE, S., TAZI, A., LECOSSIER, D. & HANCE, A. J. 1988. Production of granulocyte colony-stimulating factor (G-CSF) by human cells: T lymphocyte-dependent and T lymphocyte-independent release of G-CSF by blood monocytes. *Eur J Immunol,* 18**,** 1021-6.
NOCKA, K., BUCK, J., LEVI, E. & BESMER, P. 1990. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J,* 9**,** 3287-94.
NOMURA, H., IMAZEKI, I., OHEDA, M., KUBOTA, N., TAMURA, M., ONO, M., UEYAMA, Y. & ASANO, S. 1986. Purification and characterization of human granulocyte colony-stimulating factor (G-CSF). *EMBO J,* 5**,** 871-6.
NORRIS, S., COLLINS, C., DOHERTY, D. G., SMITH, F., MCENTEE, G., TRAYNOR, O., NOLAN, N., HEGARTY, J. & O'FARRELLY, C. 1998. Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. *J Hepatol,* 28**,** 84-90.
NORTH, T., GU, T. L., STACY, T., WANG, Q., HOWARD, L., BINDER, M., MARIN-PADILLA, M. & SPECK, N. A. 1999. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development,* 126**,** 2563-75.
NORTH, T. E., DE BRUIJN, M. F., STACY, T., TALEBIAN, L., LIND, E., ROBIN, C., BINDER, M., DZIERZAK, E. & SPECK, N. A. 2002. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity,* 16**,** 661-72.
NOVELLI, E. M., RAMIREZ, M. & CIVIN, C. I. 1998. Biology of CD34+CD38- cells in lymphohematopoiesis. *Leuk Lymphoma,* 31**,** 285-93.
NOVEMBRE, J., GALVANI, A. P. & SLATKIN, M. 2005. The geographic spread of the CCR5 Delta32 HIV-resistance allele. *PLoS Biol,* 3**,** e339.
NUAMAH, N. M., GOKER, H., KILIC, Y. A., DAGMOURA, H. & CAKMAK, A. 2006. Spontaneous splenic rupture in a healthy allogeneic donor of peripheral-blood stem cell following the administration of granulocyte colony-

stimulating factor (g-csf). A case report and review of the literature. *Haematologica,* 91, ECR08.
NUEZ, B., MICHALOVICH, D., BYGRAVE, A., PLOEMACHER, R. & GROSVELD, F. 1995. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature,* 375, 316-8.
NUTT, S. L. & KEE, B. L. 2007. The transcriptional regulation of B cell lineage commitment. *Immunity,* 26, 715-25.
NYGAARD, R., FRIMURER, T. M., HOLST, B., ROSENKILDE, M. M. & SCHWARTZ, T. W. 2009. Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol Sci,* 30, 249-59.
O'NEILL, H. C., WILSON, H. L., QUAH, B., ABBEY, J. L., DESPARS, G. & NI, K. 2004. Dendritic cell development in long-term spleen stromal cultures. *Stem Cells,* 22, 475-86.
OHISHI, K., KATAYAMA, N., SHIKU, H., VARNUM-FINNEY, B. & BERNSTEIN, I. D. 2003. Notch signalling in hematopoiesis. *Semin Cell Dev Biol,* 14, 143-50.
OHISHI, K., VARNUM-FINNEY, B. & BERNSTEIN, I. D. 2002. The notch pathway: modulation of cell fate decisions in hematopoiesis. *Int J Hematol,* 75, 449-59.
OHTEKI, T., HO, S., SUZUKI, H., MAK, T. W. & OHASHI, P. S. 1997. Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development. *J Immunol,* 159, 5931-5.
ONAI, N., OBATA-ONAI, A., TUSSIWAND, R., LANZAVECCHIA, A. & MANZ, M. G. 2006. Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. *J Exp Med,* 203, 227-38.
ORBAN, P. C., LEVINGS, M. K. & SCHRADER, J. W. 1999. Heterodimerization of the alpha and beta chains of the interleukin-3 (IL-3) receptor is necessary and sufficient for IL-3-induced mitogenesis. *Blood,* 94, 1614-22.
OSAWA, M., HANADA, K., HAMADA, H. & NAKAUCHI, H. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science,* 273, 242-5.
OTTMANN, O. G., GANSER, A., SEIPELT, G., EDER, M., SCHULZ, G. & HOELZER, D. 1990. Effects of recombinant human interleukin-3 on human hematopoietic progenitor and precursor cells in vivo. *Blood,* 76, 1494-502.
OZER, H., ARMITAGE, J. O., BENNETT, C. L., CRAWFORD, J., DEMETRI, G. D., PIZZO, P. A., SCHIFFER, C. A., SMITH, T. J., SOMLO, G., WADE, J. C., WADE, J. L., 3RD, WINN, R. J., WOZNIAK, A. J. & SOMERFIELD, M. R. 2000. 2000 update of recommendations for the use of hematopoietic colony-stimulating factors: evidence-based, clinical practice guidelines. American Society of Clinical Oncology Growth Factors Expert Panel. *J Clin Oncol,* 18, 3558-85.
PAI, S. Y., TRUITT, M. L., TING, C. N., LEIDEN, J. M., GLIMCHER, L. H. & HO, I. C. 2003. Critical roles for transcription factor GATA-3 in thymocyte development. *Immunity,* 19, 863-75.
PALIS, J. 2008. Ontogeny of erythropoiesis. *Curr Opin Hematol,* 15, 155-61.
PALLAVICINI, M. G., REDFEARN, W., NECAS, E. & BRECHER, G. 1997. Rescue from lethal irradiation correlates with transplantation of 10-20 CFU-S-day 12. *Blood Cells Mol Dis,* 23, 157-68.
PANG, L., XUE, H. H., SZALAI, G., WANG, X., WANG, Y., WATSON, D. K., LEONARD, W. J., BLOBEL, G. A. & PONCZ, M. 2006. Maturation stage-specific regulation of megakaryopoiesis by pointed-domain Ets proteins. *Blood,* 108, 2198-206.

PAPAYANNOPOULOU, T., CRADDOCK, C., NAKAMOTO, B., PRIESTLEY, G. V. & WOLF, N. S. 1995. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. *Proc Natl Acad Sci U S A,* 92**,** 9647-51.

PARMAR, K., MAUCH, P., VERGILIO, J. A., SACKSTEIN, R. & DOWN, J. D. 2007. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci U S A,* 104**,** 5431-6.

PEARCE, D. J. & BONNET, D. 2007. The combined use of Hoechst efflux ability and aldehyde dehydrogenase activity to identify murine and human hematopoietic stem cells. *Exp Hematol,* 35**,** 1437-46.

PEETERS, M., OTTERSBACH, K., BOLLEROT, K., ORELIO, C., DE BRUIJN, M., WIJGERDE, M. & DZIERZAK, E. 2009. Ventral embryonic tissues and Hedgehog proteins induce early AGM hematopoietic stem cell development. *Development,* 136**,** 2613-21.

PELED, A., KOLLET, O., PONOMARYOV, T., PETIT, I., FRANITZA, S., GRABOVSKY, V., SLAV, M. M., NAGLER, A., LIDER, O., ALON, R., ZIPORI, D. & LAPIDOT, T. 2000. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood,* 95**,** 3289-96.

PELUS, L. M., BIAN, H., FUKUDA, S., WONG, D., MERZOUK, A. & SALARI, H. 2005. The CXCR4 agonist peptide, CTCE-0021, rapidly mobilizes polymorphonuclear neutrophils and hematopoietic progenitor cells into peripheral blood and synergizes with granulocyte colony-stimulating factor. *Exp Hematol,* 33**,** 295-307.

PELUS, L. M., BIAN, H., KING, A. G. & FUKUDA, S. 2004. Neutrophil-derived MMP-9 mediates synergistic mobilization of hematopoietic stem and progenitor cells by the combination of G-CSF and the chemokines GRObeta/CXCL2 and GRObetaT/CXCL2delta4. *Blood,* 103**,** 110-9.

PELUS, L. M. & FUKUDA, S. 2006. Peripheral blood stem cell mobilization: the CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Exp Hematol,* 34**,** 1010-20.

PELUS, L. M. & FUKUDA, S. 2008. Chemokine-mobilized adult stem cells; defining a better hematopoietic graft. *Leukemia,* 22**,** 466-73.

PELUS, L. M., HOROWITZ, D., COOPER, S. C. & KING, A. G. 2002. Peripheral blood stem cell mobilization. A role for CXC chemokines. *Crit Rev Oncol Hematol,* 43**,** 257-75.

PERRY, C. & SOREQ, H. 2002. Transcriptional regulation of erythropoiesis. Fine tuning of combinatorial multi-domain elements. *Eur J Biochem,* 269**,** 3607-18.

PETIT-COCAULT, L., VOLLE-CHALLIER, C., FLEURY, M., PEAULT, B. & SOUYRI, M. 2007. Dual role of Mpl receptor during the establishment of definitive hematopoiesis. *Development,* 134**,** 3031-40.

PETIT, I., SZYPER-KRAVITZ, M., NAGLER, A., LAHAV, M., PELED, A., HABLER, L., PONOMARYOV, T., TAICHMAN, R. S., ARENZANA-SEISDEDOS, F., FUJII, N., SANDBANK, J., ZIPORI, D. & LAPIDOT, T. 2002. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol,* 3**,** 687-94.

PIAO, X., CURTIS, J. E., MINKIN, S., MINDEN, M. D. & BERNSTEIN, A. 1994. Expression of the Kit and KitA receptor isoforms in human acute myelogenous leukemia. *Blood,* 83**,** 476-81.

PIERCE, J. H. 1989. Oncogenes, growth factors and hematopoietic cell transformation. *Biochim Biophys Acta,* 989**,** 179-208.

PIGUET, P. F., VESIN, C., GRAU, G. E. & THOMPSON, R. C. 1993. Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. *Cytokine,* 5**,** 57-61.

POLTORAK, A., HE, X., SMIRNOVA, I., LIU, M. Y., VAN HUFFEL, C., DU, X., BIRDWELL, D., ALEJOS, E., SILVA, M., GALANOS, C., FREUDENBERG, M., RICCIARDI-CASTAGNOLI, P., LAYTON, B. & BEUTLER, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science,* 282**,** 2085-8.

PONOMARYOV, T., PELED, A., PETIT, I., TAICHMAN, R. S., HABLER, L., SANDBANK, J., ARENZANA-SEISDEDOS, F., MAGERUS, A., CARUZ, A., FUJII, N., NAGLER, A., LAHAV, M., SZYPER-KRAVITZ, M., ZIPORI, D. & LAPIDOT, T. 2000. Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J Clin Invest,* 106**,** 1331-9.

POTOCNIK, A. J., BRAKEBUSCH, C. & FASSLER, R. 2000. Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. *Immunity,* 12**,** 653-63.

POWER, C. A., CLEMETSON, J. M., CLEMETSON, K. J. & WELLS, T. N. 1995. Chemokine and chemokine receptor mRNA expression in human platelets. *Cytokine,* 7**,** 479-82.

PRIBILA, J. T. & SHIMIZU, Y. 2003. Signal transduction events regulating integrin function and T cell migration: new functions and complexity. *Immunol Res,* 27**,** 107-28.

PRIESTLEY, G. V., SCOTT, L. M., ULYANOVA, T. & PAPAYANNOPOULOU, T. 2006. Lack of alpha4 integrin expression in stem cells restricts competitive function and self-renewal activity. *Blood,* 107**,** 2959-67.

PRIESTLEY, G. V., ULYANOVA, T. & PAPAYANNOPOULOU, T. 2007. Sustained alterations in biodistribution of stem/progenitor cells in Tie2Cre+ alpha4(f/f) mice are hematopoietic cell autonomous. *Blood,* 109**,** 109-11.

PRINDULL, G. 1992. Cell trafficking and early ontogeny of human lymphopoietic progenitor cells. *Bone Marrow Transplant,* 9 Suppl 1**,** 36-8.

PRONK, C. J., ROSSI, D. J., MANSSON, R., ATTEMA, J. L., NORDDAHL, G. L., CHAN, C. K., SIGVARDSSON, M., WEISSMAN, I. L. & BRYDER, D. 2007. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem Cell,* 1**,** 428-42.

PROSPER, F., STRONCEK, D., MCCARTHY, J. B. & VERFAILLIE, C. M. 1998. Mobilization and homing of peripheral blood progenitors is related to reversible downregulation of alpha4 beta1 integrin expression and function. *J Clin Invest,* 101**,** 2456-67.

PRUIJT, J. F., FIBBE, W. E., LATERVEER, L., PIETERS, R. A., LINDLEY, I. J., PAEMEN, L., MASURE, S., WILLEMZE, R. & OPDENAKKER, G. 1999. Prevention of interleukin-8-induced mobilization of hematopoietic progenitor cells in rhesus monkeys by inhibitory antibodies against the metalloproteinase gelatinase B (MMP-9). *Proc Natl Acad Sci U S A,* 96**,** 10863-8.

PRUIJT, J. F., VERZAAL, P., VAN OS, R., DE KRUIJF, E. J., VAN SCHIE, M. L., MANTOVANI, A., VECCHI, A., LINDLEY, I. J., WILLEMZE, R., STARCKX, S., OPDENAKKER, G. & FIBBE, W. E. 2002. Neutrophils are indispensable for hematopoietic stem cell mobilization induced by interleukin-8 in mice. *Proc Natl Acad Sci U S A,* 99**,** 6228-33.

PUSIC, I., JIANG, S. Y., LANDUA, S., UY, G. L., RETTIG, M. P., CASHEN, A. F., WESTERVELT, P., VIJ, R., ABBOUD, C. N., STOCKERL-GOLDSTEIN, K. E., SEMPEK, D. S., SMITH, A. L. & DIPERSIO, J. F. 2008. Impact of mobilization

and remobilization strategies on achieving sufficient stem cell yields for autologous transplantation. *Biol Blood Marrow Transplant,* 14**,** 1045-56.

QIAN, H., BUZA-VIDAS, N., HYLAND, C. D., JENSEN, C. T., ANTONCHUK, J., MANSSON, R., THOREN, L. A., EKBLOM, M., ALEXANDER, W. S. & JACOBSEN, S. E. 2007. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell,* 1**,** 671-84.

QIAN, S., FU, F., LI, W., CHEN, Q. & DE SAUVAGE, F. J. 1998. Primary role of the liver in thrombopoietin production shown by tissue-specific knockout. *Blood,* 92**,** 2189-91.

QIU, F. H., RAY, P., BROWN, K., BARKER, P. E., JHANWAR, S., RUDDLE, F. H. & BESMER, P. 1988. Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family--oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. *EMBO J,* 7**,** 1003-11.

QUELLE, F. W., SATO, N., WITTHUHN, B. A., INHORN, R. C., EDER, M., MIYAJIMA, A., GRIFFIN, J. D. & IHLE, J. N. 1994. JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. *Mol Cell Biol,* 14**,** 4335-41.

QUESENBERRY, P. J., MORLEY, A., RYAN, M., HOWARD, D. & STOHLMAN, F., JR. 1973. The effect of endotoxin on murine stem cells. *J Cell Physiol,* 82**,** 239-44.

QUOYER, J., JANZ, J. M., LUO, J., REN, Y., ARMANDO, S., LUKASHOVA, V., BENOVIC, J. L., CARLSON, K. E., HUNT, S. W., 3RD & BOUVIER, M. 2013. Pepducin targeting the C-X-C chemokine receptor type 4 acts as a biased agonist favoring activation of the inhibitory G protein. *Proc Natl Acad Sci U S A,* 110**,** E5088-97.

RAAIJMAKERS, M. H., MUKHERJEE, S., GUO, S., ZHANG, S., KOBAYASHI, T., SCHOONMAKER, J. A., EBERT, B. L., AL-SHAHROUR, F., HASSERJIAN, R. P., SCADDEN, E. O., AUNG, Z., MATZA, M., MERKENSCHLAGER, M., LIN, C., ROMMENS, J. M. & SCADDEN, D. T. 2010. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature,* 464**,** 852-7.

RADOMSKA, H. S., HUETTNER, C. S., ZHANG, P., CHENG, T., SCADDEN, D. T. & TENEN, D. G. 1998. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol,* 18**,** 4301-14.

RADTKE, F., WILSON, A., STARK, G., BAUER, M., VAN MEERWIJK, J., MACDONALD, H. R. & AGUET, M. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity,* 10**,** 547-58.

RAMIREZ, P., RETTIG, M. P., UY, G. L., DEYCH, E., HOLT, M. S., RITCHEY, J. K. & DIPERSIO, J. F. 2009. BIO5192, a small molecule inhibitor of VLA-4, mobilizes hematopoietic stem and progenitor cells. *Blood,* 114**,** 1340-3.

RATAJCZAK, J., RECA, R., KUCIA, M., MAJKA, M., ALLENDORF, D. J., BARAN, J. T., JANOWSKA-WIECZOREK, A., WETSEL, R. A., ROSS, G. D. & RATAJCZAK, M. Z. 2004a. Mobilization studies in mice deficient in either C3 or C3a receptor (C3aR) reveal a novel role for complement in retention of hematopoietic stem/progenitor cells in bone marrow. *Blood,* 103**,** 2071-8.

RATAJCZAK, M. Z., MAJKA, M., KUCIA, M., DRUKALA, J., PIETRZKOWSKI, Z., PEIPER, S. & JANOWSKA-WIECZOREK, A. 2003. Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the presence of both muscle progenitors in bone marrow and hematopoietic stem/progenitor cells in muscles. *Stem Cells,* 21**,** 363-71.

RATAJCZAK, M. Z., RECA, R., WYSOCZYNSKI, M., KUCIA, M., BARAN, J. T., ALLENDORF, D. J., RATAJCZAK, J. & ROSS, G. D. 2004b. Transplantation studies in C3-deficient animals reveal a novel role of the third complement component (C3) in engraftment of bone marrow cells. *Leukemia,* 18**,** 1482-90.

RATAJCZAK, M. Z., RECA, R., WYSOCZYNSKI, M., YAN, J. & RATAJCZAK, J. 2006. Modulation of the SDF-1-CXCR4 axis by the third complement component (C3)--implications for trafficking of CXCR4+ stem cells. *Exp Hematol,* 34**,** 986-95.

RECA, R., CRAMER, D., YAN, J., LAUGHLIN, M. J., JANOWSKA-WIECZOREK, A., RATAJCZAK, J. & RATAJCZAK, M. Z. 2007. A novel role of complement in mobilization: immunodeficient mice are poor granulocyte-colony stimulating factor mobilizers because they lack complement-activating immunoglobulins. *Stem Cells,* 25**,** 3093-100.

REID, S., RITCHIE, A., BORING, L., GOSLING, J., COOPER, S., HANGOC, G., CHARO, I. F. & BROXMEYER, H. E. 1999. Enhanced myeloid progenitor cell cycling and apoptosis in mice lacking the chemokine receptor, CCR2. *Blood,* 93**,** 1524-33.

REIFFERS, J., FABERES, C., BOIRON, J. M., MARIT, G., FOURES, C., FERRER, A. M., CONY-MAKHOUL, P., PUNTOUS, M., BERNARD, P., VEZON, G. & ET AL. 1994. Peripheral blood progenitor cell transplantation in 118 patients with hematological malignancies: analysis of factors affecting the rate of engraftment. *J Hematother,* 3**,** 185-91.

REITH, A. D., ELLIS, C., LYMAN, S. D., ANDERSON, D. M., WILLIAMS, D. E., BERNSTEIN, A. & PAWSON, T. 1991. Signal transduction by normal isoforms and W mutant variants of the Kit receptor tyrosine kinase. *EMBO J,* 10**,** 2451-9.

REKHTMAN, N., CHOE, K. S., MATUSHANSKY, I., MURRAY, S., STOPKA, T. & SKOULTCHI, A. I. 2003. PU.1 and pRB interact and cooperate to repress GATA-1 and block erythroid differentiation. *Mol Cell Biol,* 23**,** 7460-74.

REKHTMAN, N., RADPARVAR, F., EVANS, T. & SKOULTCHI, A. I. 1999. Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev,* 13**,** 1398-411.

REMY, I., WILSON, I. A. & MICHNICK, S. W. 1999. Erythropoietin receptor activation by a ligand-induced conformation change. *Science,* 283**,** 990-3.

RENN, C. N., SANCHEZ, D. J., OCHOA, M. T., LEGASPI, A. J., OH, C. K., LIU, P. T., KRUTZIK, S. R., SIELING, P. A., CHENG, G. & MODLIN, R. L. 2006. TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response. *J Immunol,* 177**,** 298-305.

REYA, T., DUNCAN, A. W., AILLES, L., DOMEN, J., SCHERER, D. C., WILLERT, K., HINTZ, L., NUSSE, R. & WEISSMAN, I. L. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature,* 423**,** 409-14.

RIETSCHEL, E. T., KIRIKAE, T., SCHADE, F. U., MAMAT, U., SCHMIDT, G., LOPPNOW, H., ULMER, A. J., ZAHRINGER, U., SEYDEL, U., DI PADOVA, F. & ET AL. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J,* 8**,** 217-25.

ROBERT-MORENO, A., ESPINOSA, L., DE LA POMPA, J. L. & BIGAS, A. 2005. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development,* 132**,** 1117-26.

ROBERT-MORENO, A., ESPINOSA, L., SANCHEZ, M. J., DE LA POMPA, J. L. & BIGAS, A. 2007. The notch pathway positively regulates programmed cell death during erythroid differentiation. *Leukemia,* 21**,** 1496-503.

ROBERTS, A. W. 2005. G-CSF: a key regulator of neutrophil production, but that's not all! *Growth Factors,* 23**,** 33-41.

ROBERTS, A. W. & METCALF, D. 1995. Noncycling state of peripheral blood progenitor cells mobilized by granulocyte colony-stimulating factor and other cytokines. *Blood,* 86**,** 1600-5.

ROBIN, C., OTTERSBACH, K., DURAND, C., PEETERS, M., VANES, L., TYBULEWICZ, V. & DZIERZAK, E. 2006. An unexpected role for IL-3 in the embryonic development of hematopoietic stem cells. *Dev Cell,* 11**,** 171-80.

RODRIGUEZ, S., CHORA, A., GOUMNEROV, B., MUMAW, C., GOEBEL, W. S., FERNANDEZ, L., BAYDOUN, H., HOGENESCH, H., DOMBKOWSKI, D. M., KARLEWICZ, C. A., RICE, S., RAHME, L. G. & CARLESSO, N. 2009. Dysfunctional expansion of hematopoietic stem cells and block of myeloid differentiation in lethal sepsis. *Blood,* 114**,** 4064-76.

ROESSLER, S., GYORY, I., IMHOF, S., SPIVAKOV, M., WILLIAMS, R. R., BUSSLINGER, M., FISHER, A. G. & GROSSCHEDL, R. 2007. Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. *Mol Cell Biol,* 27**,** 579-94.

ROLINK, A., STREB, M., NISHIKAWA, S. & MELCHERS, F. 1991. The c-kit-encoded tyrosine kinase regulates the proliferation of early pre-B cells. *Eur J Immunol,* 21**,** 2609-12.

ROMAGNANI, P., LASAGNI, L., ANNUNZIATO, F., SERIO, M. & ROMAGNANI, S. 2004. CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trends Immunol,* 25**,** 201-9.

ROSU-MYLES, M., KHANDAKER, M., WU, D. M., KEENEY, M., FOLEY, S. R., HOWSON-JAN, K., YEE, I. C., FELLOWS, F., KELVIN, D. & BHATIA, M. 2000. Characterization of chemokine receptors expressed in primitive blood cells during human hematopoietic ontogeny. *Stem Cells,* 18**,** 374-81.

ROT, A. & VON ANDRIAN, U. H. 2004. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol,* 22**,** 891-928.

RUEDA, P., BALABANIAN, K., LAGANE, B., STAROPOLI, I., CHOW, K., LEVOYE, A., LAGURI, C., SADIR, R., DELAUNAY, T., IZQUIERDO, E., PABLOS, J. L., LENDINEZ, E., CARUZ, A., FRANCO, D., BALEUX, F., LORTAT-JACOB, H. & ARENZANA-SEISDEDOS, F. 2008. The CXCL12gamma chemokine displays unprecedented structural and functional properties that make it a paradigm of chemoattractant proteins. *PLoS One,* 3**,** e2543.

RUSSELL, E. S. 1979. Hereditary anemias of the mouse: a review for geneticists. *Adv Genet,* 20**,** 357-459.

RUTH, J. H., ROTTMAN, J. B., KATSCHKE, K. J., JR., QIN, S., WU, L., LAROSA, G., PONATH, P., POPE, R. M. & KOCH, A. E. 2001. Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthritis Rheum,* 44**,** 2750-60.

RUTH, J. H., SHAHRARA, S., PARK, C. C., MOREL, J. C., KUMAR, P., QIN, S. & KOCH, A. E. 2003. Role of macrophage inflammatory protein-3alpha and its ligand CCR6 in rheumatoid arthritis. *Lab Invest,* 83**,** 579-88.

SABROE, I., JONES, E. C., USHER, L. R., WHYTE, M. K. & DOWER, S. K. 2002. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol,* 168**,** 4701-10.

SAKAI, I. & KRAFT, A. S. 1997. The kinase domain of Jak2 mediates induction of bcl-2 and delays cell death in hematopoietic cells. *J Biol Chem,* 272**,** 12350-8.

SAKANAKA, M., WEN, T. C., MATSUDA, S., MASUDA, S., MORISHITA, E., NAGAO, M. & SASAKI, R. 1998. In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc Natl Acad Sci U S A,* 95**,** 4635-40.

SAMBANDAM, A., MAILLARD, I., ZEDIAK, V. P., XU, L., GERSTEIN, R. M., ASTER, J. C., PEAR, W. S. & BHANDOOLA, A. 2005. Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat Immunol,* 6**,** 663-70.

SAMOKHVALOV, I. M., SAMOKHVALOVA, N. I. & NISHIKAWA, S. 2007. Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature,* 446**,** 1056-61.

SANCHEZ, M. J., HOLMES, A., MILES, C. & DZIERZAK, E. 1996. Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. *Immunity,* 5**,** 513-25.

SASAKI, A., YASUKAWA, H., SHOUDA, T., KITAMURA, T., DIKIC, I. & YOSHIMURA, A. 2000. CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2. *J Biol Chem,* 275**,** 29338-47.

SATAKE, S., HIRAI, H., HAYASHI, Y., SHIME, N., TAMURA, A., YAO, H., YOSHIOKA, S., MIURA, Y., INABA, T., FUJITA, N., ASHIHARA, E., IMANISHI, J., SAWA, T. & MAEKAWA, T. 2012. C/EBPbeta is involved in the amplification of early granulocyte precursors during candidemia-induced "emergency" granulopoiesis. *J Immunol,* 189**,** 4546-55.

SATO, N., SAWADA, K., TAKAHASHI, T. A., MOGI, Y., ASANO, S., KOIKE, T. & SEKIGUCHI, S. 1994. A time course study for optimal harvest of peripheral blood progenitor cells by granulocyte colony-stimulating factor in healthy volunteers. *Exp Hematol,* 22**,** 973-8.

SATTENTAU, Q. J. & WEISS, R. A. 1988. The CD4 antigen: physiological ligand and HIV receptor. *Cell,* 52**,** 631-3.

SAWADA, K., KRANTZ, S. B., DAI, C. H., KOURY, S. T., HORN, S. T., GLICK, A. D. & CIVIN, C. I. 1990. Purification of human blood burst-forming units-erythroid and demonstration of the evolution of erythropoietin receptors. *J Cell Physiol,* 142**,** 219-30.

SCALA, S., OTTAIANO, A., ASCIERTO, P. A., CAVALLI, M., SIMEONE, E., GIULIANO, P., NAPOLITANO, M., FRANCO, R., BOTTI, G. & CASTELLO, G. 2005. Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. *Clin Cancer Res,* 11**,** 1835-41.

SCHMID, M. A., TAKIZAWA, H., BAUMJOHANN, D. R., SAITO, Y. & MANZ, M. G. 2011. Bone marrow dendritic cell progenitors sense pathogens via Toll-like receptors and subsequently migrate to inflamed lymph nodes. *Blood,* 118**,** 4829-40.

SCHMITS, R., FILMUS, J., GERWIN, N., SENALDI, G., KIEFER, F., KUNDIG, T., WAKEHAM, A., SHAHINIAN, A., CATZAVELOS, C., RAK, J., FURLONGER, C., ZAKARIAN, A., SIMARD, J. J., OHASHI, P. S., PAIGE, C. J., GUTIERREZ-RAMOS, J. C. & MAK, T. W. 1997. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood,* 90**,** 2217-33.

SCHOFIELD, R. 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells,* 4**,** 7-25.

SCHOLS, D., ESTE, J. A., HENSON, G. & DE CLERCQ, E. 1997a. Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. *Antiviral Res,* 35**,** 147-56.

SCHOLS, D., STRUYF, S., VAN DAMME, J., ESTE, J. A., HENSON, G. & DE CLERCQ, E. 1997b. Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J Exp Med,* 186**,** 1383-8.

SCHUITEMAKER, H., KOOT, M., KOOTSTRA, N. A., DERCKSEN, M. W., DE GOEDE, R. E., VAN STEENWIJK, R. P., LANGE, J. M., SCHATTENKERK, J. K., MIEDEMA, F. & TERSMETTE, M. 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J Virol,* 66**,** 1354-60.

SCHULZ, C., GOMEZ PERDIGUERO, E., CHORRO, L., SZABO-ROGERS, H., CAGNARD, N., KIERDORF, K., PRINZ, M., WU, B., JACOBSEN, S. E., POLLARD, J. W., FRAMPTON, J., LIU, K. J. & GEISSMANN, F. 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science,* 336**,** 86-90.

SCOTT, E. W., SIMON, M. C., ANASTASI, J. & SINGH, H. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science,* 265**,** 1573-7.

SCOTT, L. M., PRIESTLEY, G. V. & PAPAYANNOPOULOU, T. 2003. Deletion of alpha4 integrins from adult hematopoietic cells reveals roles in homeostasis, regeneration, and homing. *Mol Cell Biol,* 23**,** 9349-60.

SEET, C. S., BRUMBAUGH, R. L. & KEE, B. L. 2004. Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. *J Exp Med,* 199**,** 1689-700.

SEKHSARIA, S., FLEISHER, T. A., VOWELLS, S., BROWN, M., MILLER, J., GORDON, I., BLAESE, R. M., DUNBAR, C. E., LEITMAN, S. & MALECH, H. L. 1996. Granulocyte colony-stimulating factor recruitment of CD34+ progenitors to peripheral blood: impaired mobilization in chronic granulomatous disease and adenosine deaminase--deficient severe combined immunodeficiency disease patients. *Blood,* 88**,** 1104-12.

SELIG, C. & NOTHDURFT, W. 1995. Cytokines and progenitor cells of granulocytopoiesis in peripheral blood of patients with bacterial infections. *Infect Immun,* 63**,** 104-9.

SEMERAD, C. L., CHRISTOPHER, M. J., LIU, F., SHORT, B., SIMMONS, P. J., WINKLER, I., LEVESQUE, J. P., CHAPPEL, J., ROSS, F. P. & LINK, D. C. 2005. G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow. *Blood,* 106**,** 3020-7.

SHARKEY, N. A., LEACH, K. L. & BLUMBERG, P. M. 1984. Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc Natl Acad Sci U S A,* 81**,** 607-10.

SHARMA, S., TENOEVER, B. R., GRANDVAUX, N., ZHOU, G. P., LIN, R. & HISCOTT, J. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science,* 300**,** 1148-51.

SHARMA, Y., ASTLE, C. M. & HARRISON, D. E. 2007. Heterozygous kit mutants with little or no apparent anemia exhibit large defects in overall hematopoietic stem cell function. *Exp Hematol,* 35**,** 214-220.

SHI, X., SIGGINS, R. W., STANFORD, W. L., MELVAN, J. N., BASSON, M. D. & ZHANG, P. 2013. Toll-like receptor 4/stem cell antigen 1 signaling promotes hematopoietic precursor cell commitment to granulocyte development during the granulopoietic response to Escherichia coli bacteremia. *Infect Immun,* 81**,** 2197-205.

SHIMAZU, R., AKASHI, S., OGATA, H., NAGAI, Y., FUKUDOME, K., MIYAKE, K. & KIMOTO, M. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med,* 189**,** 1777-82.

SHIN, J. Y., HU, W., NARAMURA, M. & PARK, C. Y. 2014. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J Exp Med*.

SHIVDASANI, R. A., FUJIWARA, Y., MCDEVITT, M. A. & ORKIN, S. H. 1997. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J,* 16**,** 3965-73.

SHIVDASANI, R. A., ROSENBLATT, M. F., ZUCKER-FRANKLIN, D., JACKSON, C. W., HUNT, P., SARIS, C. J. & ORKIN, S. H. 1995. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell,* 81**,** 695-704.

SHORT, B. J., BROUARD, N. & SIMMONS, P. J. 2009. Prospective isolation of mesenchymal stem cells from mouse compact bone. *Methods Mol Biol,* 482**,** 259-68.

SI, Y., TSOU, C. L., CROFT, K. & CHARO, I. F. 2010. CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. *J Clin Invest,* 120**,** 1192-203.

SIATECKA, M., XUE, L. & BIEKER, J. J. 2007. Sumoylation of EKLF promotes transcriptional repression and is involved in inhibition of megakaryopoiesis. *Mol Cell Biol,* 27**,** 8547-60.

SIERRO, F., BIBEN, C., MARTINEZ-MUNOZ, L., MELLADO, M., RANSOHOFF, R. M., LI, M., WOEHL, B., LEUNG, H., GROOM, J., BATTEN, M., HARVEY, R. P., MARTINEZ, A. C., MACKAY, C. R. & MACKAY, F. 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**,** 14759-64.

SILVER, L. & PALIS, J. 1997. Initiation of murine embryonic erythropoiesis: a spatial analysis. *Blood,* 89**,** 1154-64.

SINGH, A. K., ARYA, R. K., TRIVEDI, A. K., SANYAL, S., BARAL, R., DORMOND, O., BRISCOE, D. M. & DATTA, D. 2012. Chemokine receptor trio: CXCR3, CXCR4 and CXCR7 crosstalk via CXCL11 and CXCL12. *Cytokine Growth Factor Rev,* 24**,** 41-9.

SIOUD, M. & FLOISAND, Y. 2009. NOD2/CARD15 on bone marrow CD34+ hematopoietic cells mediates induction of cytokines and cell differentiation. *J Leukoc Biol,* 85**,** 939-46.

SMITH, F. O., BROUDY, V. C., ZSEBO, K. M., LAMPKIN, B. C., BUCKLEY, C. V., BUCKLEY, J. D., OPIE, T., WOODS, W. G., HAMMOND, G. D. & BERNSTEIN, I. D. 1994. Cell surface expression of c-kit receptors by childhood acute myeloid leukemia blasts is not of prognostic value: a report from the Childrens Cancer Group. *Blood,* 84**,** 847-52.

SNAPPER, C. M., YAMAGUCHI, H., URBAN, J. F., JR. & FINKELMAN, F. D. 1991. Induction of Ly-6A/E expression by murine lymphocytes after in vivo immunization is strictly dependent upon the action of IFN-alpha/beta and/or IFN-gamma. *Int Immunol,* 3**,** 845-52.

SOCOLOVSKY, M. 2007. Molecular insights into stress erythropoiesis. *Curr Opin Hematol,* 14**,** 215-24.

SOCOLOVSKY, M., FALLON, A. E., WANG, S., BRUGNARA, C. & LODISH, H. F. 1999. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell,* 98**,** 181-91.

SOCOLOVSKY, M., NAM, H., FLEMING, M. D., HAASE, V. H., BRUGNARA, C. & LODISH, H. F. 2001. Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. *Blood,* 98**,** 3261-73.

SOLAR, G. P., KERR, W. G., ZEIGLER, F. C., HESS, D., DONAHUE, C., DE SAUVAGE, F. J. & EATON, D. L. 1998. Role of c-mpl in early hematopoiesis. *Blood,* 92**,** 4-10.

SOUZA, L. M., BOONE, T. C., GABRILOVE, J., LAI, P. H., ZSEBO, K. M., MURDOCK, D. C., CHAZIN, V. R., BRUSZEWSKI, J., LU, H., CHEN, K. K. & ET AL. 1986.

Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science,* 232**,** 61-5.
SPANGRUDE, G. J. & BROOKS, D. M. 1993. Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. *Blood,* 82**,** 3327-32.
SPRINGER, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell,* 76**,** 301-14.
STAAL, F. J., LUIS, T. C. & TIEMESSEN, M. M. 2008. WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol,* 8**,** 581-93.
STAAL, F. J., NOORT MV, M., STROUS, G. J. & CLEVERS, H. C. 2002. Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. *EMBO Rep,* 3**,** 63-8.
STACHURA, D. L., CHOU, S. T. & WEISS, M. J. 2006. Early block to erythromegakaryocytic development conferred by loss of transcription factor GATA-1. *Blood,* 107**,** 87-97.
STANLEY, E., LIESCHKE, G. J., GRAIL, D., METCALF, D., HODGSON, G., GALL, J. A., MAHER, D. W., CEBON, J., SINICKAS, V. & DUNN, A. R. 1994. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A,* 91**,** 5592-6.
STANLEY, M. A. 2002. Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential. *Clin Exp Dermatol,* 27**,** 571-7.
STARCK, J., WEISS-GAYET, M., GONNET, C., GUYOT, B., VICAT, J. M. & MORLE, F. 2010. Inducible Fli-1 gene deletion in adult mice modifies several myeloid lineage commitment decisions and accelerates proliferation arrest and terminal erythrocytic differentiation. *Blood,* 116**,** 4795-805.
STEWART, D. A., SMITH, C., MACFARLAND, R. & CALANDRA, G. 2009. Pharmacokinetics and pharmacodynamics of plerixafor in patients with non-Hodgkin lymphoma and multiple myeloma. *Biol Blood Marrow Transplant,* 15**,** 39-46.
STOFFEL, R., WIESTNER, A. & SKODA, R. C. 1996. Thrombopoietin in thrombocytopenic mice: evidence against regulation at the mRNA level and for a direct regulatory role of platelets. *Blood,* 87**,** 567-73.
STOPKA, T., AMANATULLAH, D. F., PAPETTI, M. & SKOULTCHI, A. I. 2005. PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. *EMBO J,* 24**,** 3712-23.
STRIETER, R. M., GOMPERTS, B. N. & KEANE, M. P. 2007. The role of CXC chemokines in pulmonary fibrosis. *J Clin Invest,* 117**,** 549-56.
STRIETER, R. M., KUNKEL, S. L., SHOWELL, H. J., REMICK, D. G., PHAN, S. H., WARD, P. A. & MARKS, R. M. 1989. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta. *Science,* 243**,** 1467-9.
SU, A. I., COOKE, M. P., CHING, K. A., HAKAK, Y., WALKER, J. R., WILTSHIRE, T., ORTH, A. P., VEGA, R. G., SAPINOSO, L. M., MOQRICH, A., PATAPOUTIAN, A., HAMPTON, G. M., SCHULTZ, P. G. & HOGENESCH, J. B. 2002. Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci U S A,* 99**,** 4465-70.
SUGIYAMA, T., KOHARA, H., NODA, M. & NAGASAWA, T. 2006. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity,* 25**,** 977-88.
SUN, X., CHENG, G., HAO, M., ZHENG, J., ZHOU, X., ZHANG, J., TAICHMAN, R. S., PIENTA, K. J. & WANG, J. 2010. CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression. *Cancer Metastasis Rev,* 29**,** 709-22.

SUNGARAN, R., CHISHOLM, O. T., MARKOVIC, B., KHACHIGIAN, L. M., TANAKA, Y. & CHONG, B. H. 2000. The role of platelet alpha-granular proteins in the regulation of thrombopoietin messenger RNA expression in human bone marrow stromal cells. *Blood,* 95**,** 3094-101.

SYMMONS, D., TURNER, G., WEBB, R., ASTEN, P., BARRETT, E., LUNT, M., SCOTT, D. & SILMAN, A. 2002. The prevalence of rheumatoid arthritis in the United Kingdom: new estimates for a new century. *Rheumatology (Oxford),* 41**,** 793-800.

SZEKANECZ, Z., SZUCS, G., SZANTO, S. & KOCH, A. E. 2006. Chemokines in rheumatic diseases. *Curr Drug Targets,* 7**,** 91-102.

SZEKANECZ, Z., VEGVARI, A., SZABO, Z. & KOCH, A. E. 2010. Chemokines and chemokine receptors in arthritis. *Front Biosci (Schol Ed),* 2**,** 153-67.

TAICHMAN, R., REILLY, M., VERMA, R., EHRENMAN, K. & EMERSON, S. 2001. Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. *Br J Haematol,* 112**,** 438-48.

TAICHMAN, R. S. 2005. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood,* 105**,** 2631-9.

TAICHMAN, R. S. & EMERSON, S. G. 1998. The role of osteoblasts in the hematopoietic microenvironment. *Stem Cells,* 16**,** 7-15.

TAICHMAN, R. S., REILLY, M. J. & EMERSON, S. G. 1996. Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. *Blood,* 87**,** 518-24.

TAKEUCHI, M., TAKASAKI, S., SHIMADA, M. & KOBATA, A. 1990. Role of sugar chains in the in vitro biological activity of human erythropoietin produced in recombinant Chinese hamster ovary cells. *J Biol Chem,* 265**,** 12127-30.

TAKIZAWA, H., BOETTCHER, S. & MANZ, M. G. 2012. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood,* 119**,** 2991-3002.

TALEBIAN, L., LI, Z., GUO, Y., GAUDET, J., SPECK, M. E., SUGIYAMA, D., KAUR, P., PEAR, W. S., MAILLARD, I. & SPECK, N. A. 2007. T-lymphoid, megakaryocyte, and granulocyte development are sensitive to decreases in CBFbeta dosage. *Blood,* 109**,** 11-21.

TALLACK, M. R. & PERKINS, A. C. 2009. Megakaryocyte-erythroid lineage promiscuity in EKLF null mouse blood. *Haematologica,* 95**,** 144-7.

TAN, J. K. & O'NEILL, H. C. 2009. Haematopoietic stem cells in spleen have distinct differentiative potential for antigen presenting cells. *J Cell Mol Med,* 14**,** 2144-50.

TAN, J. K., PERIASAMY, P. & O'NEILL, H. C. 2010. Delineation of precursors in murine spleen that develop in contact with splenic endothelium to give novel dendritic-like cells. *Blood,* 115**,** 3678-85.

TANIGUCHI, H., TOYOSHIMA, T., FUKAO, K. & NAKAUCHI, H. 1996. Presence of hematopoietic stem cells in the adult liver. *Nat Med,* 2**,** 198-203.

TAUCHI, T., FENG, G. S., SHEN, R., HOATLIN, M., BAGBY, G. C., JR., KABAT, D., LU, L. & BROXMEYER, H. E. 1995. Involvement of SH2-containing phosphotyrosine phosphatase Syp in erythropoietin receptor signal transduction pathways. *J Biol Chem,* 270**,** 5631-5.

TAVASSOLI, M. & WEISS, L. 1973. An electron microscopic study of spleen in myelofibrosis with myeloid metaplasia. *Blood,* 42**,** 267-79.

TAVIAN, M., ROBIN, C., COULOMBEL, L. & PEAULT, B. 2001. The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: mapping multipotent hematopoietic cell fate in intraembryonic mesoderm. *Immunity,* 15**,** 487-95.

TCHERNYCHEV, B., REN, Y., SACHDEV, P., JANZ, J. M., HAGGIS, L., O'SHEA, A., MCBRIDE, E., LOOBY, R., DENG, Q., MCMURRY, T., KAZMI, M. A., SAKMAR, T. P., HUNT, S., 3RD & CARLSON, K. E. 2010. Discovery of a CXCR4 agonist pepducin that mobilizes bone marrow hematopoietic cells. *Proc Natl Acad Sci U S A,* 107**,** 22255-9.

THIELE, J., STEINBERG, T., HOEPPNER, B., WIENHOLD, S., WAGNER, S., DIENEMANN, D. & FISCHER, R. 1990. Histo- and immunomorphometry of megakaryopoiesis in chronic myeloid leukemia with myelofibrosis and so-called primary (idiopathic) osteo-myelofibrosis/-sclerosis. *Anal Cell Pathol,* 2**,** 215-27.

THOREN, L. A., LIUBA, K., BRYDER, D., NYGREN, J. M., JENSEN, C. T., QIAN, H., ANTONCHUK, J. & JACOBSEN, S. E. 2008. Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol,* 180**,** 2045-53.

TO, L. B., HAYLOCK, D. N., KIMBER, R. J. & JUTTNER, C. A. 1984. High levels of circulating haemopoietic stem cells in very early remission from acute non-lymphoblastic leukaemia and their collection and cryopreservation. *Br J Haematol,* 58**,** 399-410.

TO, L. B., HAYLOCK, D. N., THORP, D., DYSON, P. G., BRANFORD, A. L., HO, J. Q., DART, G. D., ROBERTS, M. M., HORVATH, N., BARDY, P. & ET AL. 1989. The optimization of collection of peripheral blood stem cells for autotransplantation in acute myeloid leukaemia. *Bone Marrow Transplant,* 4**,** 41-7.

TOSHCHAKOV, V., JONES, B. W., PERERA, P. Y., THOMAS, K., CODY, M. J., ZHANG, S., WILLIAMS, B. R., MAJOR, J., HAMILTON, T. A., FENTON, M. J. & VOGEL, S. N. 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol,* 3**,** 392-8.

TOWNSON, J. R. & NIBBS, R. J. 2002. Characterization of mouse CCX-CKR, a receptor for the lymphocyte-attracting chemokines TECK/mCCL25, SLC/mCCL21 and MIP-3beta/mCCL19: comparison to human CCX-CKR. *Eur J Immunol,* 32**,** 1230-41.

TRAINOR, C. D., OMICHINSKI, J. G., VANDERGON, T. L., GRONENBORN, A. M., CLORE, G. M. & FELSENFELD, G. 1996. A palindromic regulatory site within vertebrate GATA-1 promoters requires both zinc fingers of the GATA-1 DNA-binding domain for high-affinity interaction. *Mol Cell Biol,* 16**,** 2238-47.

TRAVER, D. & AKASHI, K. 2004. Lineage commitment and developmental plasticity in early lymphoid progenitor subsets. *Adv Immunol,* 83**,** 1-54.

TRAVER, D., MIYAMOTO, T., CHRISTENSEN, J., IWASAKI-ARAI, J., AKASHI, K. & WEISSMAN, I. L. 2001. Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. *Blood,* 98**,** 627-35.

TRIANTAFILOU, K., ORTHOPOULOS, G., VAKAKIS, E., AHMED, M. A., GOLENBOCK, D. T., LEPPER, P. M. & TRIANTAFILOU, M. 2005. Human cardiac inflammatory responses triggered by Coxsackie B viruses are mainly Toll-like receptor (TLR) 8-dependent. *Cell Microbiol,* 7**,** 1117-26.

TSAI, F. Y. & ORKIN, S. H. 1997. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood,* 89**,** 3636-43.

TSUCHIYA, T., OKADA, M., UEDA, M. & YASUKOCHI, Y. 1997. Activation of the erythropoietin promoter by a point mutation from GATA to TATA in the -30 region. *J Biochem,* 121**,** 193-6.

UCHIDA, K., BECK, D. C., YAMAMOTO, T., BERCLAZ, P. Y., ABE, S., STAUDT, M. K., CAREY, B. C., FILIPPI, M. D., WERT, S. E., DENSON, L. A., PUCHALSKI, J. T., HAUCK, D. M. & TRAPNELL, B. C. 2007. GM-CSF autoantibodies and neutrophil dysfunction in pulmonary alveolar proteinosis. *N Engl J Med,* 356**,** 567-79.

UZUMAKI, H., OKABE, T., SASAKI, N., HAGIWARA, K., TAKAKU, F., TOBITA, M., YASUKAWA, K., ITO, S. & UMEZAWA, Y. 1989. Identification and characterization of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells. *Proc Natl Acad Sci U S A,* 86**,** 9323-6.

VAKOC, C. R., LETTING, D. L., GHELDOF, N., SAWADO, T., BENDER, M. A., GROUDINE, M., WEISS, M. J., DEKKER, J. & BLOBEL, G. A. 2005. Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol Cell,* 17**,** 453-62.

VAN BELLE, A. B., DE HEUSCH, M., LEMAIRE, M. M., HENDRICKX, E., WARNIER, G., DUNUSSI-JOANNOPOULOS, K., FOUSER, L. A., RENAULD, J. C. & DUMOUTIER, L. 2012. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. *J Immunol,* 188**,** 462-9.

VAN DE RIJN, M., HEIMFELD, S., SPANGRUDE, G. J. & WEISSMAN, I. L. 1989. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. *Proc Natl Acad Sci U S A,* 86**,** 4634-8.

VAN DEN BERG, D. J., SHARMA, A. K., BRUNO, E. & HOFFMAN, R. 1998. Role of members of the Wnt gene family in human hematopoiesis. *Blood,* 92**,** 3189-202.

VAN DER FITS, L., MOURITS, S., VOERMAN, J. S., KANT, M., BOON, L., LAMAN, J. D., CORNELISSEN, F., MUS, A. M., FLORENCIA, E., PRENS, E. P. & LUBBERTS, E. 2009. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol,* 182**,** 5836-45.

VAN DER LINDEN, M. P., VAN DER WOUDE, D., IOAN-FACSINAY, A., LEVARHT, E. W., STOEKEN-RIJSBERGEN, G., HUIZINGA, T. W., TOES, R. E. & VAN DER HELM-VAN MIL, A. H. 2009. Value of anti-modified citrullinated vimentin and third-generation anti-cyclic citrullinated peptide compared with second-generation anti-cyclic citrullinated peptide and rheumatoid factor in predicting disease outcome in undifferentiated arthritis and rheumatoid arthritis. *Arthritis Rheum,* 60**,** 2232-41.

VAN DER WOUDE, D., HOUWING-DUISTERMAAT, J. J., TOES, R. E., HUIZINGA, T. W., THOMSON, W., WORTHINGTON, J., VAN DER HELM-VAN MIL, A. H. & DE VRIES, R. R. 2009. Quantitative heritability of anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis. *Arthritis Rheum,* 60**,** 916-23.

VELLENGA, E., RAMBALDI, A., ERNST, T. J., OSTAPOVICZ, D. & GRIFFIN, J. D. 1988. Independent regulation of M-CSF and G-CSF gene expression in human monocytes. *Blood,* 71**,** 1529-32.

VENEZIA, T. A., MERCHANT, A. A., RAMOS, C. A., WHITEHOUSE, N. L., YOUNG, A. S., SHAW, C. A. & GOODELL, M. A. 2004. Molecular signatures of proliferation and quiescence in hematopoietic stem cells. *PLoS Biol,* 2**,** e301.

VERMEULEN, M., LE PESTEUR, F., GAGNERAULT, M. C., MARY, J. Y., SAINTENY, F. & LEPAULT, F. 1998. Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. *Blood,* 92**,** 894-900.

VESTERGAARD, C., JUST, H., BAUMGARTNER NIELSEN, J., THESTRUP-PEDERSEN, K. & DELEURAN, M. 2004. Expression of CCR2 on monocytes and

macrophages in chronically inflamed skin in atopic dermatitis and psoriasis. *Acta Derm Venereol,* 84**,** 353-8.
VINCENT, S., SEGRETAIN, D., NISHIKAWA, S., NISHIKAWA, S. I., SAGE, J., CUZIN, F. & RASSOULZADEGAN, M. 1998. Stage-specific expression of the Kit receptor and its ligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. *Development,* 125**,** 4585-93.
VIRIYAKOSOL, S., TOBIAS, P. S., KITCHENS, R. L. & KIRKLAND, T. N. 2001. MD-2 binds to bacterial lipopolysaccharide. *J Biol Chem,* 276**,** 38044-51.
VISINTIN, A., MAZZONI, A., SPITZER, J. H., WYLLIE, D. H., DOWER, S. K. & SEGAL, D. M. 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J Immunol,* 166**,** 249-55.
VISNJIC, D., KALAJZIC, Z., ROWE, D. W., KATAVIC, V., LORENZO, J. & AGUILA, H. L. 2004. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood,* 103**,** 3258-64.
VOS, O., BUURMAN, W. A. & PLOEMACHER, R. E. 1972. Mobilization of haemopoietic stem cells (CFU) into the peripheral blood of the mouse; effects of endotoxin and other compounds. *Cell Tissue Kinet,* 5**,** 467-79.
VROON, A., HEIJNEN, C. J. & KAVELAARS, A. 2006. GRKs and arrestins: regulators of migration and inflammation. *J Leukoc Biol,* 80**,** 1214-21.
VULCANO, M., STRUYF, S., SCAPINI, P., CASSATELLA, M., BERNASCONI, S., BONECCHI, R., CALLERI, A., PENNA, G., ADORINI, L., LUINI, W., MANTOVANI, A., VAN DAMME, J. & SOZZANI, S. 2003. Unique regulation of CCL18 production by maturing dendritic cells. *J Immunol,* 170**,** 3843-9.
WADA, H., NOGUCHI, Y., MARINO, M. W., DUNN, A. R. & OLD, L. J. 1997. T cell functions in granulocyte/macrophage colony-stimulating factor deficient mice. *Proc Natl Acad Sci U S A,* 94**,** 12557-61.
WANG, X., CRISPINO, J. D., LETTING, D. L., NAKAZAWA, M., PONCZ, M. & BLOBEL, G. A. 2002. Control of megakaryocyte-specific gene expression by GATA-1 and FOG-1: role of Ets transcription factors. *EMBO J,* 21**,** 5225-34.
WARNECKE, C., ZABOROWSKA, Z., KURRECK, J., ERDMANN, V. A., FREI, U., WIESENER, M. & ECKARDT, K. U. 2004. Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. *FASEB J,* 18**,** 1462-4.
WASKOW, C., MADAN, V., BARTELS, S., COSTA, C., BLASIG, R. & RODEWALD, H. R. 2009. Hematopoietic stem cell transplantation without irradiation. *Nat Methods,* 6**,** 267-9.
WATANABE, H., MIYAJI, C., SEKI, S. & ABO, T. 1996. c-kit+ stem cells and thymocyte precursors in the livers of adult mice. *J Exp Med,* 184**,** 687-93.
WATARI, K., ASANO, S., SHIRAFUJI, N., KODO, H., OZAWA, K., TAKAKU, F. & KAMACHI, S. 1989. Serum granulocyte colony-stimulating factor levels in healthy volunteers and patients with various disorders as estimated by enzyme immunoassay. *Blood,* 73**,** 117-22.
WEAVER, C. H., HAZELTON, B., BIRCH, R., PALMER, P., ALLEN, C., SCHWARTZBERG, L. & WEST, W. 1995. An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. *Blood,* 86**,** 3961-9.
WEBER, B. N., CHI, A. W., CHAVEZ, A., YASHIRO-OHTANI, Y., YANG, Q., SHESTOVA, O. & BHANDOOLA, A. 2011. A critical role for TCF-1 in T-lineage specification and differentiation. *Nature,* 476**,** 63-8.
WEISS, G. & GOODNOUGH, L. T. 2005. Anemia of chronic disease. *N Engl J Med,* 352**,** 1011-23.

WEISS, H. J., WITTE, L. D., KAPLAN, K. L., LAGES, B. A., CHERNOFF, A., NOSSEL, H. L., GOODMAN, D. S. & BAUMGARTNER, H. R. 1979. Heterogeneity in storage pool deficiency: studies on granule-bound substances in 18 patients including variants deficient in alpha-granules, platelet factor 4, beta-thromboglobulin, and platelet-derived growth factor. *Blood,* 54**,** 1296-319.

WEISS, M. J., KELLER, G. & ORKIN, S. H. 1994. Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes Dev,* 8**,** 1184-97.

WELCH, J. J., WATTS, J. A., VAKOC, C. R., YAO, Y., WANG, H., HARDISON, R. C., BLOBEL, G. A., CHODOSH, L. A. & WEISS, M. J. 2004. Global regulation of erythroid gene expression by transcription factor GATA-1. *Blood,* 104**,** 3136-47.

WELTE, K., PLATZER, E., LU, L., GABRILOVE, J. L., LEVI, E., MERTELSMANN, R. & MOORE, M. A. 1985. Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. *Proc Natl Acad Sci U S A,* 82**,** 1526-30.

WENG, A. P., FERRANDO, A. A., LEE, W., MORRIS, J. P. T., SILVERMAN, L. B., SANCHEZ-IRIZARRY, C., BLACKLOW, S. C., LOOK, A. T. & ASTER, J. C. 2004. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science,* 306**,** 269-71.

WHITE, G. E., IQBAL, A. J. & GREAVES, D. R. 2013. CC chemokine receptors and chronic inflammation--therapeutic opportunities and pharmacological challenges. *Pharmacol Rev,* 65**,** 47-89.

WICKREMA, A., KRANTZ, S. B., WINKELMANN, J. C. & BONDURANT, M. C. 1992. Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells. *Blood,* 80**,** 1940-9.

WIJGERDE, M., GRIBNAU, J., TRIMBORN, T., NUEZ, B., PHILIPSEN, S., GROSVELD, F. & FRASER, P. 1996. The role of EKLF in human beta-globin gene competition. *Genes Dev,* 10**,** 2894-902.

WILKIN, T. J. & GULICK, R. M. 2012. CCR5 antagonism in HIV infection: current concepts and future opportunities. *Annu Rev Med,* 63**,** 81-93.

WILLERT, K., BROWN, J. D., DANENBERG, E., DUNCAN, A. W., WEISSMAN, I. L., REYA, T., YATES, J. R., 3RD & NUSSE, R. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature,* 423**,** 448-52.

WILSON, A., LAURENTI, E., OSER, G., VAN DER WATH, R. C., BLANCO-BOSE, W., JAWORSKI, M., OFFNER, S., DUNANT, C. F., ESHKIND, L., BOCKAMP, E., LIO, P., MACDONALD, H. R. & TRUMPP, A. 2008. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell,* 135**,** 1118-29.

WILSON, A., OSER, G. M., JAWORSKI, M., BLANCO-BOSE, W. E., LAURENTI, E., ADOLPHE, C., ESSERS, M. A., MACDONALD, H. R. & TRUMPP, A. 2007. Dormant and self-renewing hematopoietic stem cells and their niches. *Ann N Y Acad Sci,* 1106**,** 64-75.

WILSON, A. & TRUMPP, A. 2006. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol,* 6**,** 93-106.

WINEMAN, J., MOORE, K., LEMISCHKA, I. & MULLER-SIEBURG, C. 1996. Functional heterogeneity of the hematopoietic microenvironment: rare stromal elements maintain long-term repopulating stem cells. *Blood,* 87**,** 4082-90.

WINKLER, I. G., BARBIER, V., WADLEY, R., ZANNETTINO, A. C., WILLIAMS, S. & LEVESQUE, J. P. 2010a. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting

hematopoietic stem cells reside in distinct nonperfused niches. *Blood,* 116**,** 375-85.

WINKLER, I. G., SIMS, N. A., PETTIT, A. R., BARBIER, V., NOWLAN, B., HELWANI, F., POULTON, I. J., VAN ROOIJEN, N., ALEXANDER, K. A., RAGGATT, L. J. & LEVESQUE, J. P. 2010b. Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood,* 116**,** 4815-28.

WITTHUHN, B. A., QUELLE, F. W., SILVENNOINEN, O., YI, T., TANG, B., MIURA, O. & IHLE, J. N. 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell,* 74**,** 227-36.

WOJCIECHOWSKI, J., LAI, A., KONDO, M. & ZHUANG, Y. 2007. E2A and HEB are required to block thymocyte proliferation prior to pre-TCR expression. *J Immunol,* 178**,** 5717-26.

WOLBER, F. M., LEONARD, E., MICHAEL, S., ORSCHELL-TRAYCOFF, C. M., YODER, M. C. & SROUR, E. F. 2002. Roles of spleen and liver in development of the murine hematopoietic system. *Exp Hematol,* 30**,** 1010-9.

WOLFE, B. L. & TREJO, J. 2007. Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. *Traffic,* 8**,** 462-70.

WOLFER, A., WILSON, A., NEMIR, M., MACDONALD, H. R. & RADTKE, F. 2002. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity,* 16**,** 869-79.

WRIGHT, D. E., BOWMAN, E. P., WAGERS, A. J., BUTCHER, E. C. & WEISSMAN, I. L. 2002. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. *J Exp Med,* 195**,** 1145-54.

WRIGHT, D. E., WAGERS, A. J., GULATI, A. P., JOHNSON, F. L. & WEISSMAN, I. L. 2001. Physiological migration of hematopoietic stem and progenitor cells. *Science,* 294**,** 1933-6.

WRIGHT, S. D., RAMOS, R. A., TOBIAS, P. S., ULEVITCH, R. J. & MATHISON, J. C. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science,* 249**,** 1431-3.

WU, G., BENOVIC, J. L., HILDEBRANDT, J. D. & LANIER, S. M. 1998. Receptor docking sites for G-protein betagamma subunits. Implications for signal regulation. *J Biol Chem,* 273**,** 7197-200.

WU, H., KLINGMULLER, U., ACURIO, A., HSIAO, J. G. & LODISH, H. F. 1997. Functional interaction of erythropoietin and stem cell factor receptors is essential for erythroid colony formation. *Proc Natl Acad Sci U S A,* 94**,** 1806-10.

WU, M., PENG, A., SUN, M., DENG, Q., HAZLETT, L. D., YUAN, J., LIU, X., GAO, Q., FENG, L., HE, J., ZHANG, P. & HUANG, X. 2011. TREM-1 amplifies corneal inflammation after Pseudomonas aeruginosa infection by modulating Toll-like receptor signaling and Th1/Th2-type immune responses. *Infect Immun,* 79**,** 2709-16.

XIE, Y., YIN, T., WIEGRAEBE, W., HE, X. C., MILLER, D., STARK, D., PERKO, K., ALEXANDER, R., SCHWARTZ, J., GRINDLEY, J. C., PARK, J., HAUG, J. S., WUNDERLICH, J. P., LI, H., ZHANG, S., JOHNSON, T., FELDMAN, R. A. & LI, L. 2009. Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature,* 457**,** 97-101.

YAMAGUCHI, M., IKEBUCHI, K., HIRAYAMA, F., SATO, N., MOGI, Y., OHKAWARA, J., YOSHIKAWA, Y., SAWADA, K., KOIKE, T. & SEKIGUCHI, S. 1998. Different adhesive characteristics and VLA-4 expression of CD34(+)

progenitors in G0/G1 versus S+G2/M phases of the cell cycle. *Blood,* 92**,** 842-8.

YAMANAKA, R., BARLOW, C., LEKSTROM-HIMES, J., CASTILLA, L. H., LIU, P. P., ECKHAUS, M., DECKER, T., WYNSHAW-BORIS, A. & XANTHOPOULOS, K. G. 1997a. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci U S A,* 94**,** 13187-92.

YAMANAKA, R., KIM, G. D., RADOMSKA, H. S., LEKSTROM-HIMES, J., SMITH, L. T., ANTONSON, P., TENEN, D. G. & XANTHOPOULOS, K. G. 1997b. CCAAT/enhancer binding protein epsilon is preferentially up-regulated during granulocytic differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. *Proc Natl Acad Sci U S A,* 94**,** 6462-7.

YANAI, N., SATOH, T. & OBINATA, M. 1991. Endothelial cells create a hematopoietic inductive microenvironment preferential to erythropoiesis in the mouse spleen. *Cell Struct Funct,* 16**,** 87-93.

YANG, L., BRYDER, D., ADOLFSSON, J., NYGREN, J., MANSSON, R., SIGVARDSSON, M. & JACOBSEN, S. E. 2005. Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood,* 105**,** 2717-23.

YARDEN, Y., KUANG, W. J., YANG-FENG, T., COUSSENS, L., MUNEMITSU, S., DULL, T. J., CHEN, E., SCHLESSINGER, J., FRANCKE, U. & ULLRICH, A. 1987. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J,* 6**,** 3341-51.

YI, T., ZHANG, J., MIURA, O. & IHLE, J. N. 1995. Hematopoietic cell phosphatase associates with erythropoietin (Epo) receptor after Epo-induced receptor tyrosine phosphorylation: identification of potential binding sites. *Blood,* 85**,** 87-95.

YONA, S., KIM, K. W., WOLF, Y., MILDNER, A., VAROL, D., BREKER, M., STRAUSS-AYALI, D., VIUKOV, S., GUILLIAMS, M., MISHARIN, A., HUME, D. A., PERLMAN, H., MALISSEN, B., ZELZER, E. & JUNG, S. 2013. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity,* 38**,** 79-91.

YOSHIDA, T., IMAI, T., KAKIZAKI, M., NISHIMURA, M., TAKAGI, S. & YOSHIE, O. 1998. Identification of single C motif-1/lymphotactin receptor XCR1. *J Biol Chem,* 273**,** 16551-4.

YOSHIHARA, H., ARAI, F., HOSOKAWA, K., HAGIWARA, T., TAKUBO, K., NAKAMURA, Y., GOMEI, Y., IWASAKI, H., MATSUOKA, S., MIYAMOTO, K., MIYAZAKI, H., TAKAHASHI, T. & SUDA, T. 2007. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell,* 1**,** 685-97.

YOSHINAGA, K., NISHIKAWA, S., OGAWA, M., HAYASHI, S., KUNISADA, T. & FUJIMOTO, T. 1991. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. *Development,* 113**,** 689-99.

YOUN, B. S., YU, K. Y., ALKHATIB, G. & KWON, B. S. 2001. The seventh transmembrane domain of cc chemokine receptor 5 is critical for MIP-1beta binding and receptor activation: role of MET 287. *Biochem Biophys Res Commun,* 281**,** 627-33.

YU, L., CECIL, J., PENG, S. B., SCHREMENTI, J., KOVACEVIC, S., PAUL, D., SU, E. W. & WANG, J. 2006. Identification and expression of novel isoforms of human stromal cell-derived factor 1. *Gene,* 374**,** 174-9.

ZANJANI, E. D., POSTER, J., BURLINGTON, H., MANN, L. I. & WASSERMAN, L. R. 1977. Liver as the primary site of erythropoietin formation in the fetus. *J Lab Clin Med,* 89**,** 640-4.

ZHANG, J., NIU, C., YE, L., HUANG, H., HE, X., TONG, W. G., ROSS, J., HAUG, J., JOHNSON, T., FENG, J. Q., HARRIS, S., WIEDEMANN, L. M., MISHINA, Y. & LI, L. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature,* 425**,** 836-41.

ZHANG, P., IWASAKI-ARAI, J., IWASAKI, H., FENYUS, M. L., DAYARAM, T., OWENS, B. M., SHIGEMATSU, H., LEVANTINI, E., HUETTNER, C. S., LEKSTROM-HIMES, J. A., AKASHI, K. & TENEN, D. G. 2004a. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity,* 21**,** 853-63.

ZHANG, P., NELSON, S., BAGBY, G. J., SIGGINS, R., 2ND, SHELLITO, J. E. & WELSH, D. A. 2008. The lineage-c-Kit+Sca-1+ cell response to Escherichia coli bacteremia in Balb/c mice. *Stem Cells,* 26**,** 1778-86.

ZHANG, P., QUINTON, L. J., GAMBLE, L., BAGBY, G. J., SUMMER, W. R. & NELSON, S. 2005. The granulopoietic cytokine response and enhancement of granulopoiesis in mice during endotoxemia. *Shock,* 23**,** 344-52.

ZHANG, Y., FOUDI, A., GEAY, J. F., BERTHEBAUD, M., BUET, D., JARRIER, P., JALIL, A., VAINCHENKER, W. & LOUACHE, F. 2004b. Intracellular localization and constitutive endocytosis of CXCR4 in human CD34+ hematopoietic progenitor cells. *Stem Cells,* 22**,** 1015-29.

ZHAO, Y., LIN, Y., ZHAN, Y., YANG, G., LOUIE, J., HARRISON, D. E. & ANDERSON, W. F. 2000. Murine hematopoietic stem cell characterization and its regulation in BM transplantation. *Blood,* 96**,** 3016-22.

ZHENG, J., UMIKAWA, M., ZHANG, S., HUYNH, H., SILVANY, R., CHEN, B. P., CHEN, L. & ZHANG, C. C. 2011. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation. *Cell Stem Cell,* 9**,** 119-30.

ZHONG, R., LAW, P., WONG, D., MERZOUK, A., SALARI, H. & BALL, E. D. 2004. Small peptide analogs to stromal derived factor-1 enhance chemotactic migration of human and mouse hematopoietic cells. *Exp Hematol,* 32**,** 470-5.

ZHOU, S., SCHUETZ, J. D., BUNTING, K. D., COLAPIETRO, A. M., SAMPATH, J., MORRIS, J. J., LAGUTINA, I., GROSVELD, G. C., OSAWA, M., NAKAUCHI, H. & SORRENTINO, B. P. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med,* 7**,** 1028-34.

ZHU, J., GARRETT, R., JUNG, Y., ZHANG, Y., KIM, N., WANG, J., JOE, G. J., HEXNER, E., CHOI, Y., TAICHMAN, R. S. & EMERSON, S. G. 2007. Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood,* 109**,** 3706-12.

ZLOTNIK, A. & YOSHIE, O. 2012. The chemokine superfamily revisited. *Immunity,* 36**,** 705-16.

ZOU, Y. R., KOTTMANN, A. H., KURODA, M., TANIUCHI, I. & LITTMAN, D. R. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature,* 393**,** 595-9.

ZSEBO, K. M., WILLIAMS, D. A., GEISSLER, E. N., BROUDY, V. C., MARTIN, F. H., ATKINS, H. L., HSU, R. Y., BIRKETT, N. C., OKINO, K. H., MURDOCK, D. C. & ET AL. 1990. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell,* 63**,** 213-24.

ZSEBO, K. M., YUSCHENKOFF, V. N., SCHIFFER, S., CHANG, D., MCCALL, E., DINARELLO, C. A., BROWN, M. A., ALTROCK, B. & BAGBY, G. C., JR. 1988.

Vascular endothelial cells and granulopoiesis: interleukin-1 stimulates release of G-CSF and GM-CSF. *Blood*, 71, 99-103.