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Lipopolysaccharide-induced activation of haematopoietic stem cells *in vivo*

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

Isaac Asimov

I dedicate this work to my grandfather, **Günter Thamm**.
You are the strongest and kindest person I know.

Abstract

Haematopoietic stem cells (HSCs) are the best-characterised adult stem cells and ensure life-long maintenance of the blood system. Under homeostatic conditions most of the HSCs are found in a quiescent state, but can be activated in response to stress (Wilson *et al.*, 2008). Infection is a major source of natural stress to the haematopoietic system, leading to exhaustion of large amounts of immune cells during the defence against invading pathogens. Reestablishment of homeostasis following infection depends on the replacement of immune cells by haematopoietic progenitors. Interestingly, recent studies have indicated that HSCs themselves can be activated in response to infection (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010; Takizawa *et al.*, 2011). However, whether HSCs are able to directly sense the infectious state remains unclear. In this work, we have investigated the *in vivo* short-term response of HSCs to lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria. Low dose administration of LPS to mice induces a rapid TLR4 dependent proliferation of even the most dormant HSCs, without impairing their long-term repopulating potential. This activation is accompanied by upregulation of stem cell antigen 1 (Sca-1) and dependent on the expression of Sca-1 on HSCs. Both *in vitro* and *in vivo* data demonstrate that activation of HSCs via LPS is not a direct effect of LPS on HSCs. Instead, LPS treatment stimulates myeloid cells in the BM, most probably inflammatory monocytes, to produce inflammatory cytokines, which in turn induce the activation of HSCs. Furthermore, our results suggest that the activation of HSCs is mediated by initial cell–cell-interaction of these myeloid cells with HSCs. Using genetic mouse models we show that the activation of HSCs is regulated by combined IFNα, IFNγ and TNFα signalling. Moreover, TNFα alone induces a Sca-1 dependent activation of HSCs *in vivo*, making these cells susceptible to chemotherapy. In addition to IFN and TNFα signalling also IL-1β signalling is necessary to mediate the LPS-induced activation of HSCs. Interestingly, IL-1β itself leads to increased proliferation of HSCs, most likely through direct activation of IL-1 signalling in these cells. Thus, our work provides novel cellular and genetic insights into the identity of LPS induced cytokine production, leading to HSC activation. Thus, providing a way to rapidly induce the production of mature immune cells upon infection, leading to a fast restoration of homeostasis upon bacterial infections. Furthermore, our work provides new insights into the role of the pro-inflammatory cytokines TNFα and IL-1β in the regulation of HSC function.

Zusammenfassung

Hämatopoetische Stammzellen (HSCs) gehören zu den am besten charakterisierten adulten Stammzellen und ermöglichen die lebenslange Aufrechterhaltung des Blutsystems. Unter homöostatischen Bedingungen befinden sich die meisten HSCs in einem ruhenden Zustand, können jedoch als Reaktion auf Stress aktiviert werden (Wilson *et al.*, 2008). Infektionen sind eine wesentliche Quelle natürlicher Stresssituationen für das hämatopoetische System, da sie während der Abwehr gegen die eindringenden Pathogene zu einer Erschöpfung großer Mengen von Immunzellen führen. Die auf eine Infektion folgende Wiederherstellung der Homöostase hängt vom Ersatz der Immunzellen durch hämatopoetische Vorläuferzellen ab. Interessanterweise haben jüngste Studien gezeigt, dass HSCs selbst als Reaktion auf Infektionen aktiviert werden können (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010; Takizawa *et al.*, 2011). Ob HSCs jedoch in der Lage sind den infektiösen Zustand direkt wahrzunehmen, bleibt unklar. In dieser Arbeit haben wir die kurzzeitige Reaktion von HSCs auf Lipopolysaccharid (LPS), einen Hauptbestandteil der Zellwand Gram(-) Bakterien, *in vivo* untersucht. Die Verabreichung geringer Mengen von LPS an Mäuse führt zu einer schnellen TLR4-abhängigen Proliferation ruhender Stammzellen, ohne dabei das Langzeit-Besiedelungspotential dieser Zellen zu beeinträchtigen. Diese Aktivierung wird von einer Hochregulierung von stem cell antigen 1 (Sca-1) begleitet und ist gleichzeitig abhängig von der Expression von Sca-1 auf HSCs. Sowohl *in vitro* als auch *in vivo* Ergebnisse zeigen, dass die Aktivierung der HSCs kein direkter Effekt von LPS auf die HSCs ist. Stattdessen führt LPS-Behandlung zu einer Stimulierung myeloider Zellen im Knochenmark, höchtwahrscheinlich von inflammatorischen Monozyten. Die myeloiden Zellen produzieren dann inflammatorische Zytokine, die wiederum die HSCs aktitivieren. Des Weiteren, weisen unsere Ergebnisse darauf hin, dass die Aktivierung der HSCs durch anfängliche Zell-Zell-Interaktion zwischen diesen myeloiden Zellen und den HSCs vermittelt wird. Zusätzlich können wir mit genetischen Modellen aufzeigen, dass die Aktivierung der HSCs durch eine Kombination aus IFNα-, IFNγ- und TNFα-Signalgebung reguliert wird. Außerdem zeigen wir, dass *in vivo* TNFα alleine eine Sca-1–abhängige Aktivierung von HSCs auslösen kann, wodurch diese anfällig für Chemotherapie werden. Zusätzlich zu IFN und TNFα-Signalgebung konnten wir zeigen, dass IL-1β-Signalgebung für die LPS-induzierte Aktivierung von HSCs notwendig ist und dass IL-1β selbst zu einer erhöhten Proliferation von HSCs führt, die sehr wahrscheinlich durch eine direkte Aktivierung von IL-1β-Signalgebung in HSCs ausgelöst wird.

Unsere Arbeit liefert folglich neue zelluläre und genetische Einsicht in die Identität der LPS-induzierten Zytokin-Produtkion, die eine Aktivierung von HSCs auslöst. Dadurch kommt es tu einer zur raschen Produktion differenzierter Immunzellen, die zu einer schnellen Wiederherstellung der Homöostase nach einer bakteriellen Infektion führt. Des Weiteren, bietet unsere Arbeit neue Erkenntnisse über die Rolle der pro-inflammatorischen Zytokine TNFα und IL-1β bei der Regulierung der Funktion von HSCs.

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Nomenclature

5-FU	5-Fluorouracil
a-HSC	Activated haematopoietic stem cell
Ang-1	Angiopoietin-1
BM	Bone marrow
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony-forming unit
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMRP	Common myeloid repopulating progenitor
CXCL	Chemokine CXC-motif ligand
CXCR3	CXC-motif chemokine receptor 3
d-HSC	Dormant haematopoietic stem cell
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
G-SCF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GMP	Granulocyte/macrophage lineage-restricted progenitor
HSC	Haematopoietic stem cell
HSPCs	Haematopoietic stem and progenitor cells
i.f.	Intra-femoral
i.p.	Intraperitoneal
i.v.	Intravenous
IκB	Inhibitor of κB
IFNα	Interferon alpha

IFNγ	Interferon gamma
Ifnar	Interferon alpha receptor
Ifngr	Interferon gamma receptor
IKK	I$\varkappa$B kinase
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-1RA	Interleukin-1 receptor antagonist
IL-1RI	Type I Interleukin-1 receptor
IL-1RII	Type II Interleukin-1 receptor
IL-6	Interleukin 6
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
JAK	Janus kinase
LBP	LPS-binding protein
LCMV	Lymphocytic choriomeningitis virus
lin	Lineage
lin depl	Lineage depleted
LMPP	Lymphoid-biased multipotent progenitor
LPS	Lipopolysaccharide
LRC	Label retaining cell
LT-HSC	Long-term haematopoietic stem cell
LTA	Lipoteichoic acid
M.avium	Mycobacterium avium
MAPKKK	Mitogen-activated protein kinase kinase kinase
MegE	Megakaryocyte/erythroid
MEP	Megakaryocyte/erythrocyte lineage-restricted progenitor
MERP	Megakaryocyte-erythroid repopulating progenitor
MkEP	Megakaryocyte/erythrocyte progenitors
MkRP	Megakaryocyte repopulating progenitor
MPP	Multipotent progenitor
MyD88	Myeloid differentiation primary response gene 88
NF-$\varkappa$**B**	Nuclear factor 'kappa-light-chain-enhancer' of activated B cells
ODN	Oligodeoxynucleotide
OPP	Oligopotent progenitor
PAMP	Pathogen-associated molecular pattern

PBL	Peripheral blood leukocytes
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PDTC	Pyrrolidine dithiocarbamate
PGN	Peptidoglycan
polyI:C	Polyinosinic:polycytidylic acid
PRR	Pattern-recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RIP1	Receptor interacting protein-1
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
ST-HSC	Short-term haematopoietic stem cell
STAT	Signal transducer and activator of transcription
TIR	Toll/Interleukin 1 receptor
TIRAP	TIR-domain containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TPO	Thrombopoietin
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain containing adaptor-inducing interferon-β
wt	Wildtype

1. Introduction

1.1. Haematopoietic stem cells and the haematopoietic system

1.1.1. An early history of HSC research

The first evidence of HSCs arose shortly after the bombings of Hiroshima and Nagasaki, when physicians observed that those people who died over a prolonged period from lower doses of irradiation suffered from haematopoietic failure. Their haematopoietic system was unable to regenerate sufficient white blood cells to cope with infections, as well as enough platelets to clod their blood (reviewed in Domen *et al.*, 2006). A few years later, scientists were able to show that mice exposed to a lethal dose of irradiation died from similar symptoms, but could be rescued by bone marrow transfer from non-irradiated animals. As this rescue did not only occur when irradiated mice were transplanted with mouse bone marrow, but also when they were transplanted with bone marrow from rats or guinea pigs, 'humoral' or chemical factors rather than cells where suggested to be the source of the recovery (Lorenz *et al.*, 1951). In 1956, however, two groups were able to show that the recovery after bone marrow transplantation into lethally irradiated animals was due to a repopulation of the recipient by the transplanted cells. By using cells with chromosomal translocations or rat cells transplanted into mice, it was proven that the donor cells survived and repopulated the recipient (Ford *et al.*, 1956; Nowell *et al.*, 1956). With this observation the search for the haematopoietic stem cell began.

The first experiments proving that specific cells of the bone marrow are able to differentiate into various cell types where completed in the 1960s. It was shown that colonies formed on the spleen after transplantation of bone marrow cells were of clonal nature, with all cells in one colony emerging from one single transplanted cell (Becker *et al.*, 1963). Subsequently, it was demonstrated that single colony-forming cells were able to differentiate along more than one pathway, because the colonies consisted of different cell types, such as granulocytes and erythrocytes (Wu *et al.*, 1967). Nevertheless, these spleen colonies consist of cells of myeloid origin only. Thus, these experiments could indeed show the existence of single cells able to differentiate into various cell types of

the myeloid lineage, however if these cells were simultaneously able to reconstitute the lymphoid lineage was not clear. The proof for the existence of single haematopoietic cells with the potential to differentiate into all cell types of the myeloid as well as the lymphoid lineage was demonstrated in 1977. Chromosomal aberrations were used to identify single cell clones and prove that some of the transplanted cells were able to re-constitute the myeloid as well as the lymphoid lineage. Furthermore, the existence of cells able to form cells of either the myeloid or the lymphoid lineage, but not both, was shown (Abramson *et al.*, 1977). These results were the first evidence for the existence of a HSC, with the ability to differentiate into all cell types of the blood system, as well as the existence of restricted progenitors, which were only capable of differentiation into cells from the myeloid or the lymphoid lineage, today known as common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs).

1.1.2. Functional HSC assays

Functional HSC assays are necessary to analyse the stem cell potential of different cell populations, such as differentiation and self-renewal. These functional assays can be subdivided into *in vitro* assays or *in vivo* short-term and long-term repopulation assays. The *in vitro* and short-term assays asses the potential of progenitors, while long-term repopulation assays can be used to asses the self-renewal potential, quan-tity and quality of HSCs. An important *in vitro* assay is the the colony-forming unit (CFU) or colony-forming cell (CFC) assay, a method based on the ability of HSPCs to form colonies in semi-solid methylcellulose medium in the presence of cytokines (Cashman *et al.*, 1983). These colonies can be counted and characterised, according to their lineage restriction as erythrocyte (CFU-E or erythroid-restricted burst-forming units, BFU-E), megakaryocyte (CFU-Mk), granulocyte/monocyte (CFU-GM) or mixed colonies (CFU-GEMM) (Cashman *et al.*, 1983; Coulombel *et al.*, 1983; Purton and Scadden, 2007). The measurement of B and T lymphocyte potential with this assay is more difficult and re-quires specialised culture conditions (reviewed in Purton and Scadden, 2007). Among the long-term *in vitro* assays are the long-term culture-initialising cell (LTC-IC) assay (Dexter *et al.*, 1977; Whitlock and Witte, 1982; Sutherland *et al.*, 1989) and the Cobble-Stone Area Forming Cell (CAFC) assay (Ploemacher *et al.*, 1989). Both of these assays are based on the assumption that only HSCs can retain their clonogenic potential in cul-ture over a long period. In both assays the bone marrow cells are cultured for several weeks with a stromal feeder layer. In the LTC-IC assay the frequency of LT-initiating cells is estimated through limiting dilutions (Sutherland *et al.*, 1989). The CAFC assay is based on the observation, that HSPCs creep into the stromal layer and settle there as

clusters of tightly packed cells, the cobblestone areas. The CAFCs represent primitive haematopoietic cells and their frequency in different subsets within the haematopoietic hierarchy can be quantified by the frequency of CAFCs over time (Ploemacher *et al.*, 1989). In both assays the clonogenic potential of long-term cultured cells is assessed by a CFU assay. However, all of these method measure the *in vitro* colony forming potential of progenitors, rather than HSCs (reviewed in Purton and Scadden, 2007). The colony forming potential of transplanted bone marrow cells can be analysed by the Colony-Forming Unit-Spleen (CFU-S) assay. For this assay, bone marrow cells are transplanted into lethally irradiated mice and the formation of colonies on the spleen is analysed between 10 and 14 days after transplantation (Till and McCulloch, 1961).

Nevertheless, these methods cannot be used to evaluate the actual stem cell potential within a fraction of bone marrow cells. For this purpose long-term repopulation assays are used. For all of these assays, bone marrow cells are transplanted into lethally irradiated mice and the repopulation of the recipient with the donor cells is assessed after 4 month or later. All of these assays are based on the original bone marrow transplantation experiments (Lorenz *et al.*, 1951; Ford *et al.*, 1956; Nowell *et al.*, 1956), but enable a more refined readout. In competitive repopulation assays, a defined number of bone marrow cells from the source to be studied are transplanted together with a defined number of HSCs from a source of known repopulation potential. The frequency of repopulating units (RU) can then be assessed in comparison to a control transplantation (Harrison, 1980). This assay is, for example, used to asses the engraftment potential of HSCs from transgenic mice in comparison to wildtype cells. However, this assay cannot distinguish between differences in the quality of HSCs and differences in the number of HSCs. This assay is also critically dependent on the number of HSCs in the analysed population. To analyse the frequency of HSCs in a given bone marrow sample, limiting-dilution assays are used. In these assays, cells are transplanted in a series of dilutions, together with competing or rescue bone marrow cells. The number of cells in the sample can then be calculated from the number of repopulated recipients (Taswell, 1981; Szilvassy *et al.*, 1990). This method is costly, because many mice have to be transplanted with different dilutions of cells, and is therefore usually used after a difference in the engraftment potential of a given sample has been detected with the competitive repopulation assay. Furthermore, this assay may not accurately detect HSCs at a single cell level (reviewed in Purton and Scadden, 2007) To asses the self-renewal and long-term reconstitution potential of a fraction of bone marrow cells, serial transplantations are used. This is the only method to assess the actual stem cell potential, as progenitors are also have the capacity to provide multilineage reconstitution to a primary recipient, while only

HSCs are able to multilineage reconstitute a secondary or tertiary recipient. Cells from a primary recipient are used as donor cells for a secondary recipient and cells from the secondary recipient may then be used as donor cells for a tertiary recipient (Lemischka *et al.*, 1986). However, the results of this assay may be influenced by impaired homing or engraftment, rather than impaired self-renewal (reviewed in Purton and Scadden, 2007). In addition to these assays, repopulating assays are performed, using single cells to analyse the repopulation capacity of single cells rather than a population of cells (Osawa *et al.*, 1996; Wagers *et al.*, 2002; Camargo *et al.*, 2006; Dykstra *et al.*, 2007; Yamamoto *et al.*, 2013).

Transplanted HSCs reach the bone marrow and find their niche by a process termed "homing". The traditional way of transplanting bone marrow cells is through tail–vein or retro–orbital (intravenous) injection into the blood. However, many HSCs become trapped in organs such as lung and liver, and the recovery of cells in the bone marrow and spleen may therefore be rather low (Szilvassy *et al.*, 1999). Using this method to study the engraftment potential of HSCs is therefore not sufficient to differentiate between defects in engraftment or homing. To circumvent the necessity of HSCs to home to the bone marrow, cells can be directly injected intra–femorally into the bone cavity (Kushida *et al.*, 2001).

1.1.3. HSC markers and the haematopoietic hierarchy

HSCs can be identified through their cell surface phenotype

After it was clear that HSCs exist, the main goal was to isolate these cells to gain a better understanding of their properties and behaviour. The main problems for the isolation of HSCs were the rarity of the stem cells in the bone marrow and their similar morphology with other white blood cells. Therefore many tried to identify surface markers to enable isolation of HSCs by fluorescence activated cell sorting (FACS). This method, in combination with the functional assays mentioned above, facilitates the identification and isolation of single cells marked with fluorescent antibodies. The first steps in the identification of markers suitable to isolate HSCs were made in the 1980s. It was first found that HSCs reside within a population negative for the lineage markers B220, CD4/CD8, Gr-1 and CD11b (Lin^-) and positive for Thy1 (Müller–Sieburg *et al.*, 1986). After showing that mice transplanted with $Thy1^+Lin^-$ cells survived lethal irradiation, while mice transplanted with $Thy1^-$ cells died, it was subsequently revealed that the actual stem cell properties reside within the Thy1 low population ($Thy1^{lo}$) of Lin^- cells (Müller–Sieburg *et al.*, 1988). As this population of $Thy1^{lo}Lin^-$ cells was still heterogeneous, the marker set to isolate HSCs was further refined. In 1988, stem cell antigen 1 (Sca-1) was established as

one of the markers further refining the definition of HSCs. Only 30 Thy1^{lo}Lin$^-$Sca-1$^+$ cells were sufficient to rescue a lethally irradiated mouse and provide long-term reconstitution to the recipient. Although it was shown that the Sca-1$^+$ fraction of the bone marrow contained long-term repopulation potential, Spangrude *et al.* suggested that this fraction contained all stem cell activity of the bone marrow (Spangrude *et al.*, 1988). This hypothesis was later proven, by showing that neither Thy1$^-$, nor Lin$^+$ or Sca-1$^-$ cells can repopulate a recipient mouse. However, the study showed that mice transplanted with Thy1$^-$, Lin$^+$ or Sca-1$^-$ cells survived longer in comparison to mice which were not transplanted with any bone marrow cells, suggesting that these cells contained short-term reconstitution potential (Uchida and Weissman, 1992). Another marker to refine the definition of HSCs was identified by proving that only the c-Kit$^+$ fraction of Thy1^{lo}Lin$^-$Sca-1$^+$ cells had long-term multi-lineage reconstitution potential (Ikuta and Weissman, 1992). In the 1990s various groups contributed to the further refinement of the HSC marker set. In 1996, it was shown that within the pool of Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells, only the CD34$^-$ cells were able to long-term repopulate a recipient, while the CD34$^+$ cells only had short-term repopulation potential (Osawa *et al.*, 1996). In the meantime, other markers were proposed, such as CD43, CD38, CD27 and CD135 (Moore *et al.*, 1994; Randall *et al.*, 1996; Wiesmann *et al.*, 2000; Adolfsson *et al.*, 2001). Of these markers only CD135 (Flt3) is currently used as a marker for HSCs, where multi-lineage reconstitution potential was shown to reside within the Flt3$^-$ population of LSK cells, while Flt3$^+$ LSK cells only reconstituted the lymphoid lineage (Adolfsson *et al.*, 2001). In 2005 another set of markers, the SLAM markers, was identified, that helped to enhance the definition of HSCs markedly. It was shown that HSCs expressed high levels of CD150, but low levels of CD48 and CD244, compared to MPPs and whole bone marrow. Furthermore, the engraftment potential of a single sorted cell was improved from 21% for Thy1^{lo}Lin$^-$Sca-1$^+$c-Kit$^+$ cells to 47% by adding CD150$^+$CD48$^-$ to the sorting scheme. A further enhancement of the purity of HSCs can be reached by excluding CD41$^+$ cells, which represent megakaryocytes and megakaryocyte progenitors (Kiel *et al.*, 2005). Adding CD34 to the LK CD150$^+$CD48$^-$ gating scheme is the most robust currently in use and was therefore used for most of the *in vivo* analysis performed in this thesis.

The role of Sca-1 in HSC function

Although most of the HSC markers have been identified quite a while ago, their functional role in HSCs is still under debate. Sca-1 is one of these candidates. Knockout mice for Sca-1 (*Sca-1$^{-/-}$*) display a relatively mild haematopoietic phenotype. It was shown that the T cells in these mice develop normally, but are hyperresponsive to antigen stimu-

lation (Stanford *et al.*, 1997). Furthermore, these mice display a mild thrombocytopenia as well as a decrease in megakaryocytes and their precursors. Additionally, they show a decrease granulocyte, erythrocyte, macrophage and megakaryocyte progenitor activity. *Sca-1$^{-/-}$* HSCs have a competitive disadvantage compared to wildtype HSCs and fail to repopulate one third of lethally irradiated secondary recipients. This data suggests that Sca-1 is required for the regulation of HSC self-renewal and the development of committed progenitor cells, megakaryocytes and platelets (Ito *et al.*, 2003).

The haematopoietic hierarchy
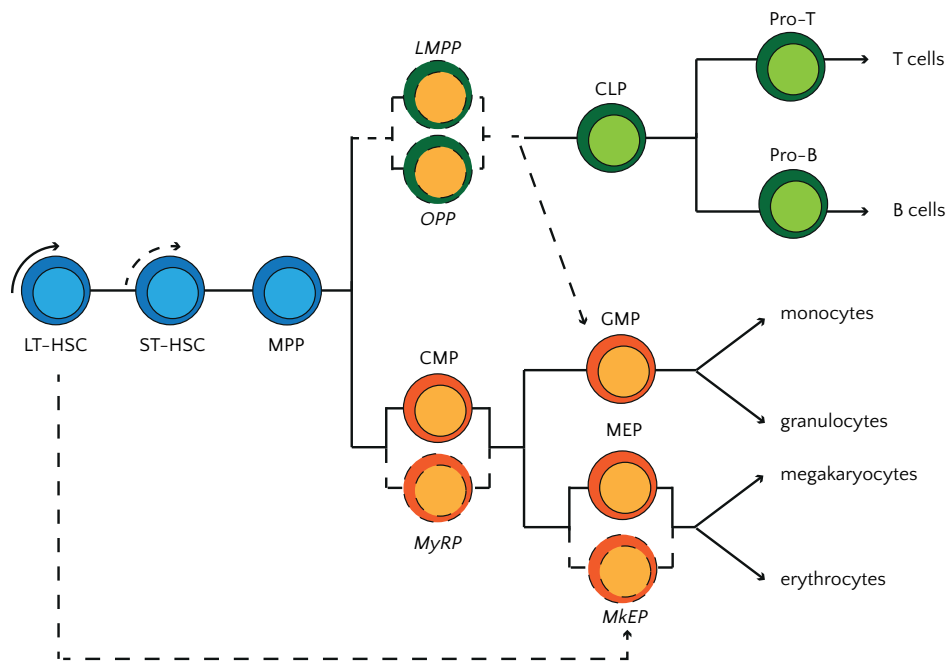
Figure 1.1.: The model of haematopoietic differentiation is changing
The classical model (solid lines) of HSC differentiation proposes the long-term HSC (LT-HSC) at the top of the haematopoietic hierarchy, subsequently differentiating into a short-term HSC (ST-HSC) and a multi-potent progenitor (MPP) (Morrison and Weissman, 1994; Morrison *et al.*, 1997). The first decision of commitment between the lymphoid and the myeloid lineage is made when the MPP differentiates into either a common lympoid progenitor (CLP) (Kondo *et al.*, 1997) or a common myeloid progenitor (CMP) (Akashi *et al.*, 2000; Reya *et al.*, 2001). The revised model (dashed lines) of the haematopoietic hierarchy proposes the first lineage choice between myeloid-restricted and lympho-myeloid progenitors (LMPP or oligopotent progenitors, OPP), with the LMPPs/OPPs unable to differentiate into megakaryocytes or erythrocytes (MkE). Another type of progenitor cells corresponds to the earlier proposed CMPs (myeloid repopulating progenitors, MyRPs) and is able to differentiate into all cells of the myeloid lineage, including MkE. Furthermore, the model proposes a direct short-cut from LT-HSCs to megakaryocyte/erythrocyte progenitors (MkEPs) (Adolfsson *et al.*, 2005; Yamamoto *et al.*, 2013).

With the establishment of markers to identify multipotent haematopoietic stem and progenitor cells, scientists started to investigate the hierarchy of the haematopoietic system and the chronology of differentiation from the HSC to mature haematopoietic cells. At first, a differentiation was made between CD34⁻ long-term repopulating HSCs (LT-HSCs), capable of multi-lineage reconstitution over a long period, CD34⁺ short-term HSCs (ST-HSCs), capable of short-term self-renewal and reconstitution of a recipient for less than 8 weeks, and multipotent progenitors (MPPs), with the potential to form the different haematopoietic lineages but without self-renewal capacity (Morrison and Weissman, 1994). Thereafter, it was shown that the LT-HSCs reside at the top of the haematopoietic hierarchy, subsequently differentiating first into ST-HSCs and then into MPPs (Morrison *et al.*, 1997). Hereafter, a cell population with lineage restriction for the lymphoid lineage was identified as Lin⁻IL-7R⁺Thy1⁻Sca-1^lo c-Kit^lo, termed common lymphoid progenitor (CLP). This population showed a restriction toward lymphoid differentiation *in vitro* as well as *in vivo*. Furthermore, a single cell was able to reconstitute B cells as well as T cells (Kondo *et al.*, 1997). After the identification of a CLP an equivalent cell type able to differentiate into the myeloid lineage within the IL-7R⁻ fraction of the bone marrow was identified, the common myeloid progenitors (CMP). These cells are able to differentiate into granulocyte/macrophage lineage-restricted progenitors (GMPs) or megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs) (Akashi *et al.*, 2000). These findings led to the proposal of the classical model of haematopoietic lineage commitment, where MPPs arise from ST-HSCs and in turn differentiate into CLPs or CMPs, as the first line of commitment to either the lymphoid or the myeloid lineage. The CLPs subsequently differentiate into B or T cell progenitors, while the CMPs differentiate further into GMPs or MEPs (Figure 1.1, solid lines) (Akashi *et al.*, 2000; Reya *et al.*, 2001). The classical model was challenged when a population of progenitor cells within the Flt3⁺ fraction of LSK cells with lympho-myeloid potential, but without the ability to form megakaryocytes or erythrocytes, was identified (Adolfsson *et al.*, 2005). These progenitors were termed lymphoid-biased multipotent progenitors (LMPP). The discovery of those cells challenged the prediction of the classical model - that the separation of the lymphoid and myeloid lineage is the first step in differentiation - as it suggests an earlier separation of cells capable of erythro-megakaryocytic differentiation (megakaryocyte/erythroid progenitors, MkEPs) from the rest of the progenitors. The MkEPs reside within the Flt3⁻ fraction of LSK cells and it was postulated that those cells emerge in a differentiation step from HSCs to either Flt3⁻ MkEPs or Flt3⁺ LMPPs (Adolfsson *et al.*, 2005). This observation was strengthened by a study, showing that Flt3⁻PU.1^lo cells are highly enriched for committed MEPs (Nutt *et al.*, 2005) and a publication proving an

early separation of progenitors with a restricted myeloid potential and progenitors with lympho-myeloid potential (Lai and Kondo, 2006). Contradicting to this newly proposed model, Forsberg *et al.* showed that the Flt3⁺, as well as the Flt3⁻ cells, retain the potential to differentiate into megakaryocytes and erythrocytes (Forsberg *et al.*, 2006). As none of these studies was performed using single cell techniques to specifically determine the differentiation potential of single stem and progenitor cells, the question of which model holds true remained a controversial subject.

In 2013 two groups were able to provide data from large-scale single cell experiments, leading to a revised model of haematopoietic differentiation. The Nakauchi lab subdivided the CD34⁻ LSK pool of cells into three fractions, dependent on their expression of CD150 and CD41, and transplanted approximately 300 single cells into primary and secondary recipients. This enabled the investigation of the long-term differentiation potential of a single cell of each of these populations (Yamamoto *et al.*, 2013). Progenitors with differentiation potential equivalent to CMPs, MEPs and MkPs were identified in all of the transplanted fractions. These progenitors were thereafter termed common myeloid repopulating progenitors (CMRPs), megakaryocyte-erythroid repopulating progenitors (MERPs) or megakaryocyte repopulating progenitors (MkRPs) respectively. Furthermore, it was shown that these cells have a higher repopulation capacity than CD34⁺ LSK cells (Yamamoto *et al.*, 2013). After the establishment of the existence of a MkRP population, the question of whether these megakaryocyte-primed progenitors could directly emerge from a HSC, arose. Therefore, single cells from all three fractions were sorted and allowed to divide once in culture before transplanting each of the daughter cells separately. This allowed assesment of the fate of the individual daughter cells. With this experiment, together with the above mentioned findings, evidence for a direct differentiation from LT-HSCs to MkRPs was provieded (Yamamoto *et al.*, 2013). These findings, along with the results from Adolfsson *et al.*, identifying a LMPP capable of differentiation into the lymphoid and the myeloid lineage, but not into erythrocytes or megakaryocytes (Adolfsson *et al.*, 2005), led to the proposal of a revised model of HSC differentiation. In this model the first lineage choice is made between myeloid-restricted and lympho-myeloid progenitors, whereby the lympho-myeloid progenitors lose the ability to produce cells of the megakaryocyte/erythrocyte lineage. The lympho-myeloid progenitors (LMPP or oligopotent progenitors, OPP) are able to produce cells of the lymphoid lineage as well as cells of the granulocyte/macrophage lineage (Akashi *et al.*, 2000). Another type of progenitor corresponds to the earlier proposed CMPs (termed MyRPs by Yamamoto *et al.*) and is able to produce all cells of the myeloid lineage. Furthermore, this model proposes a direct short-cut from LT-HSCs to MkEPs (Figure 1.1, dashed lines)

(Adolfsson *et al.*, 2005; Yamamoto *et al.*, 2013). This model was further supported by another large-scale single-cell analysis published in 2013 by the group of Stuart Orkin. Here, single cells from different bone marrow populations were used for large-scale expression profiling by qRT-PCR. Similarities between MegE primed cells and HSCs in respect to their gene expression profiles was shown. Furthermore, the study identified two types of CMPs; one closely linked to MegEs and one more closely related to the lympho-myeloid lineage (Guo *et al.*, 2013). This data provides further proof for the revised model of haematopoietic differentiation, with a close relationship between HSCs and the MkE lineage and two pathways of myeloid differentiation. Further proof of the MkE priming of HSCs was given by data showing a hierarchical relationship between von Willebrand factor positive (vWF⁺) platelet-primed HSCs at the apex of the HSC hierarchy with a downstream differentiation to vWF⁻ lymphoid-biased HSCs lacking platelet-priming (Sanjuan-Pla *et al.*, 2013). Taken together these studies clearly demonstrate that the markers used to identify individual populations of haematopoietic cells are still not refined enough and that these populations are still very heterogeneous. Hence, single cell analysis will be crucial to gain a full understanding of the haematopoietic hierarchy and the differentiation potential of individual populations. Furthermore, single cell analysis will further refine the understanding of the function of haematopoietic stem and progenitor cells and help to find new markers for the identification of different HSPC populations.

1.1.4. Dormant haematopoietic stem cells

A fraction of HSCs reside in a dormant state

Most of the HSCs (70%) are found in the G_0 state of the cell cycle under homeostasis at any given time point (Wilson *et al.*, 2008). This low cycling kinetic ensures life long production of blood cells and reduces the risk of leukaemia, as mutations mainly occur in fast cycling cells (Wang and Dick, 2005). Furthermore, it protects the HSCs from exhaustion (Orford and Scadden, 2008). The experiments showing that most of the HSCs are quiescent were, however, a snapshot of a given time point and the question arose how the cycling behaviour of HSCs changes over a longer period. First experiments treating mice with BrdU over a period of time led to the conclusion, that all HSCs are cycling regularly, as during a period of one week most of the primitive HSCs had incorporated BrdU and therefore had undergone at least one cell cycle (Bradford *et al.*, 1997). This result was further supported by the finding that after 6 months of continuous BrdU treatment, 99% of all LT-HSCs had divided and incorporated BrdU (Cheshier *et al.*, 1999). The first results of a label-retaining assay in HSCs were published in 2007 (Kiel *et al.*, 2007). Mice

were treated with BrdU for 10 days, followed by a BrdU-free chase period of 70 days and subsequent analysis of the presence of BrdU in HSCs by microscopy. From these experiments it was concluded that a dormant population of HSCs does not exist, as all HSCs were labeled within 10 days and only 6% of HSCs retained the label after the chase period (Kiel *et al.*, 2007). However, the existence of a fraction of dormant HSCs within the pool of LSK CD150$^+$CD48$^-$CD34$^-$ cells was demonstrated by similar label-retaining experiments (Wilson *et al.*, 2008). Mice were treated with BrdU for 10-13 days followed by a BrdU-free chase for up to 306 days. After 13 days almost all cells within the LSK fraction were labelled with BrdU, as BrdU itself induced cycling of these cells. After a chase of 70 days around 30% of HSCs retained the BrdU label and after 306 days, there were still approximately 5% BrdU$^+$ label-retaining cells (LRCs) detectable. These results could be reproduced in another mouse model, expressing a histone H2B-GFP fusion protein under the control of a tetracycline-responsive regulatory element. Using mathematical modelling two populations of HSCs were predicted: a dormant population (d-HSCs), cycling only every 145 days and representing around 15% of all HSCs and an active population (a-HSCs), cycling approximately every 36 days. Furthermore, it was shown that only the dormant fraction of HSCs was able to ensure long-term multi-lineage engraftment in transplantation experiments (Wilson *et al.*, 2008). A more thorough mathematical analysis of the data produced by Wilson *et al.* revealed an even higher proportion of dormant HSCs, namely 30-45%, which are dividing every 149-193 days (van der Wath *et al.*, 2009). Another study, however, suggests that dormant as well as cycling HSCs are able to long-term reconstitute lethally irradiated recipients to the same extend (Takizawa *et al.*, 2011). In this study, LSK cells were isolated and labeled with CFSE, to track single cell divisions, before transplanting them into non-irradiated recipients. Analysis of the divisional history of the transplanted cells revealed a population of cells that did not divide within 21 weeks. This population of cells that had not divided was compared to ≥5x-divided cells that had divided five times or more within 7 or 12-14 weeks, in transplantation assays. Surprisingly the ≥5x-divided cells were able to long-term repopulate the recipients in serial transplantations. Furthermore, these cells were able to give rise to a population of non-cycling HSCs after transplantation (Takizawa *et al.*, 2011). These results suggest that not only the dormant HSCs are able to long-term reconstitute a lethally irradiated recipient. However, the initial isolation and labelling of the LSK cells before transplantation may alter their cycling behaviour and therefore the results may not reflect the actual homeostatic conditions. A recently published study, shows that the divisional history of HSCs is a better measure of HSC function than their cell-surface phenotype (Qiu *et al.*, 2014). This study suggests that HSCs loose their functional activity after a few

cell divisions and might not return to full dormancy after they have been activated. Furthermore, a fraction of $CD34^+$ cells, with robust self-renewal potential, was identified within the population of dormant HSCs. Nevertheless, this fraction was outperformed by $CD34^-$ dormant HSCs in secondary transplantations, suggesting a hierarchical structure within the population of dormant HSCs with the LSK $CD150^+CD48^-CD135^-CD34^-$ cells at the top of the hierarchy (Qiu *et al.*, 2014). The balance between quiescence and cycling is a critical factor, to ensure the maintenance of long-term HSCs able to self-renew and replenish the system during the whole life of an animal.

Regulation of HSC cycling

The balance between the dormant and the activated state of HSCs needs to be tightly regulated, not only to prevent HSC exhaustion, but to enable fast replenishment of the system upon injury. Many intrinsic and extrinsic factors have been proposed to have a role in the maintenance of HSC quiescence or in the activation of dormant HSCs into cycle. The decision, for a cell to progress into an active cell cycle or to return to quiescence is made during early G_1 phase and is influenced by growth factors (reviewed in Sherr and Roberts, 1999). These growth factors activate the expression of D cyclins, which form a complex with the cyclin dependent kinases (CDK) 4 or 6. This complex phosphorylates the transcriptional repressor Retinoblastoma (Rb), leading to an activation of the transcription factor E2F and the progression from early G_1 to late G_1 phase. A further phosphorylation of Rb by a cyclinE-CDK2 complex leads to an additional transcriptional activation of genes necessary for the progression into S phase. This process is regulated by antagonising CDK inhibitors (CDKi) of two families: the INK4 and the Cip/Kip family. The INK4 family consists of $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$ and targets the CDK4 and CDK6 complexes. The Cip/Kip family targets the CDK2 complex, as well as the CDK4/6 complexes, and contains the CDKis p21, p27 and p57 (reviewed in Sherr and Roberts, 1999). The role of these regulators in HSC quiescence has been investigated in various knockout models. Mice deficient for the three cyclinD kinases or CDK4 and CDK6 die during embryogenesis due to haematopoietic failure and the fetal liver HSCs from these mice show severe defects in self-renewal (Kozar *et al.*, 2004; Malumbres *et al.*, 2004; Pietras *et al.*, 2011). These studies indicate that the D cyclins, as well as CDK4/6 are necessary for active cycling of HSCs during embryogenesis. The role of these proteins in adult HSCs, however, remains to be further elucidated. Many studies have been performed to investigate the role of the CDKis in establishing and maintaining HSC quiescence. While p21 seems so have a role in the stress response of HSCs, rather than during homeostasis, p57 has been strongly implicated to be a main

player in HSC quiescence. Apart from high expression in HSCs, deletion studies have revealed a necessity for p57 in the establishment of HSC quiescence (Matsumoto *et al.*, 2011; Zou *et al.*, 2011). Conditional knockout of p57 using the MxCre model, led to a significant reduction in HSC and progenitor numbers, increased apoptosis in these cells as well as defects in the self-renewal capacity of HSCs (Matsumoto *et al.*, 2011). An impaired repopulating activity of fetal liver HSCs from p57-deficient mice has been shown (Zou *et al.*, 2011). Furthermore, it was shown that p57 inhibits the cyclinD/CDK4/6 complex, by binding cyclin D and inhibiting its transport into the nucleus. Only following release from p57, cyclin D can be transported into the nucleus, were it forms a complex with CDK4/6, leading to an active cell cycle (Zou *et al.*, 2011). In both of these studies, p57-deficiency led to a defect in HSC quiescence and self-renewal (Matsumoto *et al.*, 2011; Zou *et al.*, 2011). However, the question remains whether p57 is only necessary to establish quiescence after cycling of HSCs or whether it is also required to maintain the quiescent state. As the results were obtained in cycling HSCs, after induction of Cre expression with pIC or in fetal liver HSCs, these studies point toward a role of p57 in the establishment of quiescence after cycling of HSCs. If p57 also has a role in maintaining the HSCs in their quiescent state needs to be further investigated (reviewed in Tesio and Trumpp, 2011). In addition to the CDKis, the transcriptional repressors of the Rb family, Rb, p107 and p130, known to play a role in the transition from early to late G_1 phase have also been implicated in the maintenance of HSC quiescence. While deficiency in either of the three family members does not lead to a severe phenotype, conditional knockout of all three led to increased HSC proliferation, as well as self-renewal defects. This data shows that the Rb family members collectively have a role in maintaining HSC quiescence (Viatour *et al.*, 2008; Pietras *et al.*, 2011). Apart from these intrinsic factors, many extrinsic factors have been proposed to have a role in maintaining HSC quiescence.

1.1.5. Maintenance of HSC self-renewal and reconstitution potential

The self-renewal ability of HSCs is closely linked to their dormant state, while differentiation is accompanied by increased proliferation. Many factors have been proposed to be involved in the maintenance of HSC functions ranging from cytokines to signalling pathways. The role of three signalling pathways has been extensively studied: the TGF-β pathway, the Pten/Akt pathway and the Wnt signalling pathway (reviewed in Rossi *et al.*, 2012). Deletion of different components of the TGF-β signalling pathway have varying effects on HSCs. While knockout of the TGF-β type I receptor led to embryonal lethality of mice, no effects on haematopoiesis were detected in fetal or in adult HSCs (Larsson *et al.*, 2001, 2003), suggesting that signalling through this receptor is not essential for

haematopoiesis and HSC maintenance. In constrast, the conditional deletion of *Smad4*, a downstream signalling component of the TGF-β pathway, resulted in self-renewal defects of adult HSCs (Karlsson *et al.*, 2007). Furthermore, deletion of *TGF-β2* led to a decrease in HSC repopulating ability of adult HSCs and a defect in fetal liver HSCs upon serial transplantation (Langer *et al.*, 2004). In addition, niche secreted TGF-β has recently been shown to regulate HSC dormancy (Yamazaki *et al.*, 2009). All of these findings suggest that TGF-β is a positive regulator of HSC function. The role of the Akt signalling has been studied using various knockout models. Double-knockout of *Akt1* and *Akt2* significantly impairs HSC function in competitive transplants and maintenance of LT-HSCs. Furthermore, the HSCs in these mice displayed increased quiescence (Juntilla *et al.*, 2010). In accordance with these findings, the deletion of a negative regulator of Akt signalling, *Pten*, led to increased cycling of HSCs, loss of LT-HSCs and development of myeloproliferative disorders, ultimately resulting in leukaemia (Yilmaz *et al.*, 2006; Zhang *et al.*, 2006b; Guo *et al.*, 2008). A recent study suggests that these effects are due to an increased production of G-CSF by myeloid cells, leading to the mobilisation of HSCs, splenomegaly and myeloproliferative disease and do not display a direct effect of *Pten*-loss in HSCs (Tesio *et al.*, 2013). Furthermore, mice deficient for FoxO proteins, which are negatively regulated by Akt signalling, display a phenotype very similar to *Pten*-deficient mice. These mice show decreased repopulating activity due to increased cycling, but do not develop leukaemia (Tothova *et al.*, 2007). Deletion of FoxO3a alone already leads to loss of reconstitution ability, manifested in serial transplantation, suggesting a regulatory role of FoxOs in HSC self-renewal (Miyamoto *et al.*, 2007). Controversial results have been obtained during the investigation of the role of Wnt signalling in HSC maintenance. While Mx-Cre-mediated β-*catenin* deletion resulted in normal HSC repopulation and self-renewal (Cobas *et al.*, 2004), the Vav-Cre-induced deletion of β-*catenin* led to decreased long-term repopulating ability (Zhao *et al.*, 2007). As Vav-Cre deletion takes place during fetal haematopoiesis, this result might reflect a differential requirement for β-*catenin* in haematopoiesis during embryogenesis and in adult mice. Interestingly, constitutive active β-catenin in adult mice led to a severe disruption of HSC function and a block in differentiation (Kirstetter *et al.*, 2006; Scheller *et al.*, 2006). In accordance with these results, *Wnt3a*-null mice have reduced numbers of fetal liver HSCs, with a severe impairment of reconstitution in serial transplantations (Luis *et al.*, 2009). Apart from the direct signalling components of the Wnt signalling pathway, some studies analysed the role of inhibitors of Wnt signalling in HSC maintenance. Loss of the transcription factor Sox17 led to a decrease in fetal liver HSCs, while having minimal impact on adult HSCs (Kim *et al.*, 2007). Furthermore, the conditional

deletion of *Apc*, another negative regulator of Wnt signalling, led to increased cycling of adult HSCs and progenitors, leading to bone marrow failure, as well as exhaustion of the pool of myeloid progenitors and LMPPs (Qian *et al.*, 2008). Two other studies analysed the effects of overexpression of two inhibitors of Wnt signalling in osteoblasts. Overexpression of *Dkk1* led to increased cycling and reduced regeneration potential of HSCs (Fleming *et al.*, 2008). Similarly, overexpression of *Wif1* led to loss of HSC quiescence and decreased self-renewal (Schaniel *et al.*, 2011). In summary, these studies suggest differential roles for Wnt signalling in the fetal and the adult haematopoiesis, but clearly show that a tight regulation of Wnt signalling is essential to preserve HSC function. Apart from these signalling pathways, a number of cytokines have been suggested to be involved in the regulation of HSC function. Stem Cell factor (SCF), Thrombopoietin (TPO), Flt3–ligand, IL–3, IL–6, IL–11 and granulocyte colony–stimulating factor (G–CSF) have been initially identified to promote the expansion of human HSCs in culture (Bhatia *et al.*, 1997; Conneally *et al.*, 1997; Miller and Eaves, 1997; Gammaitoni *et al.*, 2003), a method used in the clinics to increase the number of HSCs prior to bone marrow transplantation. SCF and TPO have been shown to have a role in the maintenance of HSCs *in vivo*, where the HSCs of *Scf*-deficient Steel mice are unable to maintain long-term repopulating activity (McCarthy *et al.*, 1977). Furthermore, mice lacking the receptor for SCF, *c-Kit*, show reduced numbers of LT-HSCs, as well as a progressive depletion of HSCs in competitive as well as non-competitive transplantations, due to increased cycling of HSCs (Thoren *et al.*, 2008). The phenotype of *kit*-mutant mice can be of use for transplantation assays. A mouse line was generated, which lacks T and B cells (*Rag2$^{-/-}$*) as well as NK cells (referred to as $\gamma_c{}^{-/-}$) and is HSC-deficient due to a *kit*-mutation (*Kit$^{W/Wv}$*). This mouse was called *Rag2$^{-/-}\gamma_c{}^{-/-}$Kit$^{W/Wv}$* and enables the transplantation of HSCs without irradiation (Waskow *et al.*, 2009). As irradiation is a major source of bone marrow stress, this mouse line will help to investigate the behaviour and function of transplanted HSCs under conditions that more closely resemble the homeostatic situation. Loss of the receptor for TPO, *cMPL*, led to defects in reconstitution potential and self-renewal of HSCs (Kimura *et al.*, 1998). Reduced expansion of HSCs following transplantation and accelerated cycling kinetics of HSCs was the consequence of loss of TPO itself, implicating a role for TPO signalling in HSC expansion upon transplantation as well as the maintenance of quiescence (Qian *et al.*, 2007). Another cytokine suggested to have a role in the maintenance of HSCs is Angiopoietin–1 (Ang-1). It has been shown that signalling of Ang-1 through the receptor Tie2 is required for adult hematopoiesis, while it is dispensable for fetal haematopoiesis (Takakura *et al.*, 1998; Puri and Bernstein, 2003). More specifically, Tie2 has been shown to mark a quiescent subset of HSCs, which can be found in

close contact to osteoblasts in the bone marrow niche (Arai *et al.*, 2004). Furthermore, Ang-1 has been shown to enhance the ability of HSCs to become quiescent and adhere to the bone, suggesting a role for Tie2/Ang-1 signalling in the maintenance of HSCs in a quiescent state in the bone marrow niche (Arai *et al.*, 2004). Cxcl12 has been suggested as a master regulator of HSC migration and retention in the bone marrow and seems to be especially important for the homing of HSCs to fetal and adult haematopoietic sites (McGrath *et al.*, 1999). Loss of *Cxcr4*, the receptor of Cxcl12, leads to hyperproliferation (Nie *et al.*, 2008) and mobilisation (Christopher *et al.*, 2009) of HSCs. These studies show that the regulation of HSC self-renewal and differentiation is critically mediated by several pathways and factors. Furthermore, this regulation may be dependent on the localisation of the HSCs within the bone marrow niche.

1.1.6. HSCs reside in specialised niches within the bone marrow
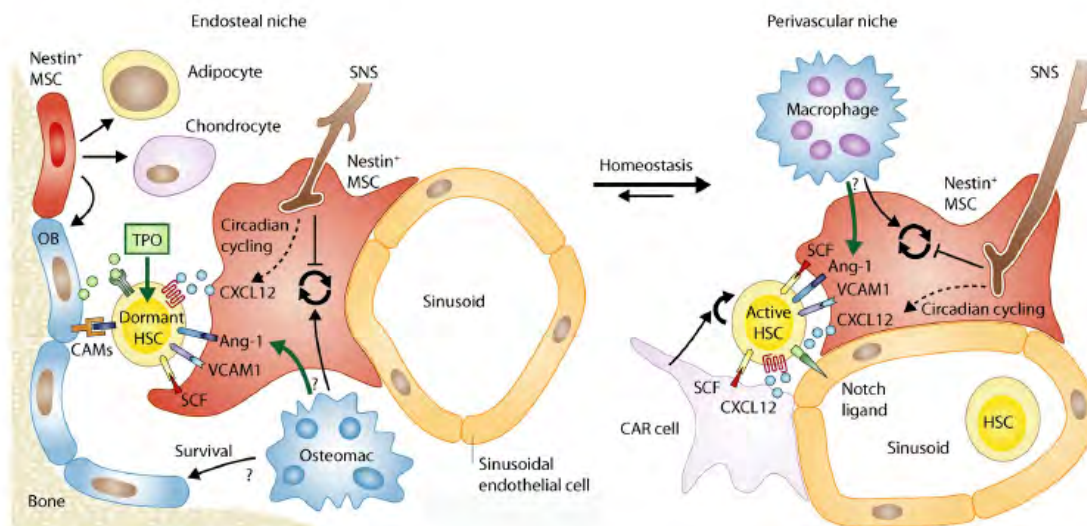
Figure 1.2.: HSCs may reside within two distinct niches
Two HSC niches have been proposed: the endosteal niche, most likely harbouring the quiescent (dormant) HSCs and the perivascular niche, suggested to harbour the homeostatic HSCs. Different cell types have been implied to make up the bone marrow niche, including osteoblasts, macrophages, CAR cells, nestin+ MSCs and endothelial cells. The cells of the bone marrow niche interact with HSCs through soluble factors, such as CXCL12 and TPO, as well as through cell–cell-interaction via for example Ang-1/Tie2 or SCF/c–Kit. (modified from Ehninger and Trumpp, 2011)

The bone marrow environment, the so called BM niche, is essential for the maintenance of HSC quiescence and self-renewal. HSCs in the bone marrow are found near the endosteum and/or near the sinusoidal endothelium. Therefore, two niches have been

proposed: the endosteal niche, harbouring quiescent HSCs with high repopulation activity (Zhang *et al.*, 2003; Grassinger *et al.*, 2010) and the perivascular niche, harbouring most of the LSK CD150$^+$ cells (Kiel *et al.*, 2005), however this concept is still under discussion. Over the years, several cell types have been proposed to make up these niches and thereby maintain HSCs. Osteoblastic cells were suggested to be part of the HSC niche, as numbers of osteoblasts strongly correlate with HSC numbers in the bone marrow (Zhang *et al.*, 2003; Calvi *et al.*, 2003; Visnjic *et al.*, 2004). These cells are mainly found in the endosteal niche and produce factors, like CXCL12 (Ponomaryov *et al.*, 2000), that support HSC maintenance, as well as adhesion molecules that may facilitate HSC–niche interactions. These data support the hypothesis that osteoblasts are part of the niche, how they regulate HSC maintenance is however not completely understood (reviewed in Mercier *et al.*, 2012). Interestingly, G–CSF-induced mobilisation of HSCs from the bone marrow to the blood stream is accompanied by loss of osteoblasts and a decrease in factors required for HSC maintenance (Christopher *et al.*, 2009; Winkler *et al.*, 2010). This effect has been furthermore shown to be due to loss of trophic endosteal macrophages (osteomacs), as depletion of macrophages led to decreased osteoblast numbers and HSC mobilisation comparable to G–CSF treatment (Winkler *et al.*, 2010). Further evidence for this finding was provided by specific depletion of CD169$^+$ macrophages from the bone marrow, which also led to mobilisation of HSCs (Chow *et al.*, 2011). Additionally, it was shown that G–CSF signalling in myeloid cells alone is sufficient to induce osteoblast suppression and HSC mobilisation (Christopher *et al.*, 2011). All of these findings support the hypothesis that macrophages have a role in the HSC niche. Another type of immune cell has recently been suggested to be part of the HSC niche. Another type of immune cell, regulatory T cells are found in co–localisation with HSCs in the bone marrow. These cells seem to participate in the formation of a zone where HSPCs reside and might be protected from immune attack (Fujisaki *et al.*, 2011). Furthermore, CXCL12-abundant reticular (CAR) cells have been proposed to be part of the HSC niche. These cells are found in close proximity to HSCs and are located in the perivascular as well as in the endosteal region of the bone marrow (Sugiyama *et al.*, 2006). Depletion of CAR cells leads to reduced numbers of HSCs and increased HSC quiescence (Omatsu *et al.*, 2010). A population of cells very similar to these CAR cells, but less abundant, are Nestin$^+$ mesenchymal stem cells (MSCs). These cells are spatially associated with HSCs and adrenergic nerve fibres, which have been shown to play a role in the retention of HSCs in the bone marrow (Katayama *et al.*, 2006; Mendez–Ferrer *et al.*, 2008). Depletion of Nestin$^+$ MSCs leads to reduced numbers of HSCs in the bone marrow as well as reduced homing of transplanted HSCs (Mendez–Ferrer *et al.*, 2010). Whether these

Nestin[+] MSCs represent a subpopulation of CAR cells is not clear yet. The contribution of these Nestin[+] MSCs to the HSC niche has been challenged by two studies, showing that deletion of *Cxcl12* in these cells does not impair HSC numbers and frequency (Ding and Morrison, 2013; Greenbaum *et al.*, 2013). However, it has to be further investigated whether this excludes a role for these cells in the BM niche (Hanoun and Frenette, 2013). The conditional deletion of *Cxcl12* in several other cell types revealed a role for CXCL12 expressed by endothelial and stromal cells in the maintenance of HSCs in the bone marrow, suggesting that HSCs are mainly residing in the perivascular niche (Ding and Morrison, 2013; Greenbaum *et al.*, 2013). As these studies only assessed the number of LSK CD150^{+}CD48^{-} cells, this does not exclude that quiescent HSCs are located in the endosteal niche. Nevertheless, there are multiple lines of evidence suggesting a vascular contribution to the HSC niches. First, generation of HSCs occurs in association with vascular areas and blood flow during embryonic development. Second, extramedullary haematopoiesis in humans occurs in perivascular areas during haematopoietic stress. Third, HSCs have been observed in the vicinity of sinusoids (Kiel *et al.*, 2005) and endothelial cells have been shown to express molecules that facilitate HSC homing and mobilisation (reviewed in Mercier *et al.*, 2012). Endothelial cells have been implicated to support long-term HSCs through direct cellular contact as well as through the production of factors that promote haematopoiesis and adhesion molecules that are thought to facilitate HSC homing (Rafii *et al.*, 1997; Li *et al.*, 2004; Sipkins *et al.*, 2005; Butler *et al.*, 2010; Kobayashi *et al.*, 2010). A recent study provided more evidence for the hypothesis, that quiescent and proliferating HSCs reside in distinct niches (Kunisaki *et al.*, 2013). This study identified arterioles, lined by a rare subset of Nestin[+] MSCs, called periarteriolar Nestin cells (Nes[peri]), to be found in close proximity to quiescent HSCs. In addition, the study provided insight into the behaviour of HSCs upon activation, as treatment of mice with polyinosinic-polycytidylic acid (polyI:C) or G-CSF led to a significant reduction of the number of HSCs found in proximity to these arterioles. Furthermore, it was shown that depletion of a subset of these Nes[peri] cells expressing the pericyte marker NG2, led to a significant increase in HSC cycling, as well as a reduction of HSC number and frequency, observed in the bone marrow as well as the spleen, suggesting that depletion of NG2^{+} Nes[peri] cells led to a loss of HSCs, rather than to their mobilisation. This study suggests that arterioles form niches that are indispensable for the maintenance of HSC quiescence (Kunisaki *et al.*, 2013). Nonetheless, the existence of two distinct niches as well as the contributing cell types to the bone marrow niche are still controversially discussed.

1.2. The response of HSCs to infection

During infection, mature immune cells are lost, either due to mobilisation to the infected tissue or due to cell death (reviewed in King and Goodell, 2011a). To ensure fast replenishment of the system haematopoiesis is induced by haematopoietic stem and progenitor cells upon infection. The mechanisms how HSPCs can sense the infection have been widely discussed. As HSPCs are usually not directly infected, a direct infection of HSCs is unlikely to be the cause of increased haematopoiesis (Johns *et al.*, 2009; Baldridge *et al.*, 2010; King and Goodell, 2011a). More likely, however, is a direct sensing of pathogens by HSPCs or an alteration of HSPC behaviour in response to cytokines produced upon infection. These two possibilities have been subject of numerous studies and evidence has been provided for both scenarios.

1.2.1. The role of TLR signalling in innate immunity

The immune system of mammals consists of two major subsystems, the innate immunity, which builds the first line of defence against invading pathogens, and the adaptive immunity, which is responsible for the elimination of pathogens in the late phase of the immune response and the generation of an immunological memory (reviewed in Mogensen, 2009). The innate immune system is based on physical and chemical barriers to infection as well as cell types recognising pathogens through pattern-recognition receptors (PRRs) (reviewed in Mogensen, 2009; Lester and Li, 2014). Toll-like receptors (TLRs) are the best studied PRRs and are involved in the response to various pathogen-associated molecular patterns (PAMPs). TLRs are type I transmembrane proteins with an ectodomain containing leucin-rich repeats that mediate the recognition of PAMPs and an intracellular Toll/Interleukin 1 (IL-1) receptor (TIR) domain, which is conserved among TLRs, as well as in the IL-1 receptor (reviewed in Kawai and Akira, 2010; Akira and Takeda, 2004). In mice, twelve different TLRs are known, which can be categorised into extracellular (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) and intracellular (TLR3, TLR7, TLR8 and TLR9) TLRs. The extracellular TLRs mainly recognise microbial membrane components, while the intracellular TLRs, which are localised in the endoplasmatic reticulum (ER), endosomes, lysosomes or endolysosomes, recognise nucleic acids (reviewed in Kawai and Akira, 2010). Specifically, TLR2 forms complexes with either TLR1 or TLR6, which bind to components of the microbial cell walls and membranes, like lipoproteins and peptidoglycans (Ozinsky *et al.*, 2000; Takeuchi *et al.*, 2001, 2002; Alexopoulou *et al.*, 2002; Travassos *et al.*, 2004). TLR4 is the main receptor of lipopolysaccharide (LPS) recognition (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999; Hoshino *et al.*, 1999), while

TLR5 binds flagellin (Hayashi *et al.*, 2001), a component of bacterial flagella. Of the intracellular TLRs, TLR3 binds double-stranded RNA (dsRNA) (Alexopoulou *et al.*, 2001), like polyI:C, while TLR7 and TLR8 bind single-stranded RNA (ssRNA) (Heil *et al.*, 2004; Diebold *et al.*, 2004). Furthermore, TLR9 binds unmethylated CpG DNA (Hemmi *et al.*, 2000), which is mimicked by synthetic CpG oligodeoxynucleotides (CpG ODNs). These unmethylated CpG motifs are highly abundant in microbial genomes, but very rare in the mammalian genome. Activation of TLRs leads to the recruitment of different adaptor proteins, namely myeloid differentiation primary response gene 88 (MyD88), TIR-domain containing adapter-inducing interferon-β (TRIF), TIR-domain containing adaptor protein (TIRAP) or TRIF-related adaptor molecule (TRAM). These adaptors induce two different pathways of TLR signalling: the MyD88-dependent pathway, involving MyD88 and TIRAP, and the MyD88-independent pathway, involving TRIF and TRAM (reviewed in Kawai and Akira, 2010). All TLRs, except for TLR3, use the MyD88-dependent pathway. The MyD88-independent pathway, is only used by TLR3 and TLR4. Activation of the MyD88-dependent pathway mainly leads to the production of inflammatory cytokines, whereas the activation of the MyD88-independent pathway leads to production of type I IFNs and inflammatory cytokines (reviewed in Kawai and Akira, 2010). Upon PAMP recognition MyD88 is recruited to the TIR domain of the TLR, leading to an association with interleukin-1 receptor associated kinase (IRAK) 4, which in turn recruits and phosphorylates IRAK1. The phosphorylation of IRAK1 leads to a subsequent autophosphorylation and the binding of TNF receptor associated factor 6 (TRAF6). Through activation of TAK1, a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, TRAF6 induces the activation of IκB kinase (IKK) complex, leading to a phosphorylation of inhibitors of κB (IκBs) and subsequent activation of nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NFκB) signalling and the production of inflammatory cytokines (reviewed in Akira and Takeda, 2004). Upon TLR3 and TLR4 signalling another pathway is activated by recruitment of TRIF to the TLR. TRIF subsequently recruits TRAF6 and TAK1 for NFκB activation, as well as TBK1–IKKi through TRAF3, which catalyses the phosphorylation of interferon regulatory factor 3 (IRF3) (reviewed in Kawai and Akira, 2010). IRF3 translocates to the nucleus upon phosphorylation, where it binds the co-activators p300 and CBP, leading to the activation of type I IFN gene expression. The IFNs in turn activate IFN inducible genes like *cxcl10* and *irg1*. Furthermore, IRF3 activates IRF7, which amplifies the activation of type I IFNs (reviewed in Akira and Takeda, 2004). Apart from activating IRF3, TRAF3 also inhibits the MyD88-dependent pathway (reviewed in Kawai and Akira, 2010). TLR4 is the only TLR, that uses both the MyD88-dependent, as well as the MyD88-independent pathway. TLR4 binds

LPS in a complex with MD2, involving the LPS-binding protein (LBP), that binds soluble LPS, and CD14, which binds the LBP-LPS complex and delivers it to the TLR4–MD2 complex. TLR4 initially recruits MyD88 and triggers activation of NFϰB and MAPKs. Subsequently, TLR4 undergoes endocytosis, trafficking to endosomes, where it recruits TRAM and TRIF, leading to IRF3 and late-phase NFϰB activation. Interestingly, MyD88 as well as TRIF activation is necessary for the TLR4 induced production of inflammatory cytokines, while the other TLRs can induce inflammatory cytokines by solely using the MyD88- or the TRIF-dependent pathway (reviewed in Kawai and Akira, 2010). In summary, TLR signalling leads to the production of inflammatory cytokines and interferons, which are important to mediate the innate immune response to an infection, as well as the transition from the innate to the adaptive immunity.

1.2.2. The effects of TLR signalling on HSC behaviour and function

It has been shown that HSCs and haematopoietic progenitors express TLRs, like TLR2 and TLR4, and can directly respond to stimulation with TLR ligands (Nagai *et al.*, 2006; Megías *et al.*, 2012; Zhao *et al.*, 2014). Many studies have provided insights into the effects of TLR signalling on HSPCs. Common to most of these studies is the increased cycling of HSPCs in response to infections or direct TLR stimulation, as well as an increase in mobilisation of HSPCs to the periphery and differentiation towards the myeloid lineage (Zhang *et al.*, 2008; Yanez *et al.*, 2009, 2010; Takizawa *et al.*, 2011; Esplin *et al.*, 2011). A skewing of HSPC differentiation towards the myeloid lineage, as well as the production of cytokines in response to treatment with TLR ligands or pathogens *in vitro* has also been shown for human HSPCs (Kim *et al.*, 2004; Sioud *et al.*, 2006; Singh *et al.*, 2008), suggesting that human HSPCs might react similar to infections as murine HSPCs. Another common alteration of HSC behaviour is a reduced repopulation capacity of HSCs that have been exposed to chronic infection or chronic treatment with TLR ligands (Rodriguez *et al.*, 2009; Chen *et al.*, 2010; Takizawa *et al.*, 2011; Esplin *et al.*, 2011). More specifically, it has been shown that LPS stimulation *in vivo* leads to an expansion of the HSC pool, due to increased cycling (Zhang *et al.*, 2008; Rodriguez *et al.*, 2009; Takizawa *et al.*, 2011; Esplin *et al.*, 2011), accompanied by a loss of repopulation potential upon chronic TLR4 activation (Rodriguez *et al.*, 2009; Takizawa *et al.*, 2011; Esplin *et al.*, 2011). Furthermore, several studies have suggested that LPS treatment *in vitro* as well as *in vivo* leads to a myeloid skewing of HSC differentiation, leading to an increased production of myeloid cells (Nagai *et al.*, 2006; Esplin *et al.*, 2011; Megías *et al.*, 2012). In addition, it has been proposed that the increased differentiation towards macrophages upon LPS stimulation is due to a direct activation of TLR4 signalling in the HSCs (Megías *et al.*, 2012). Together,
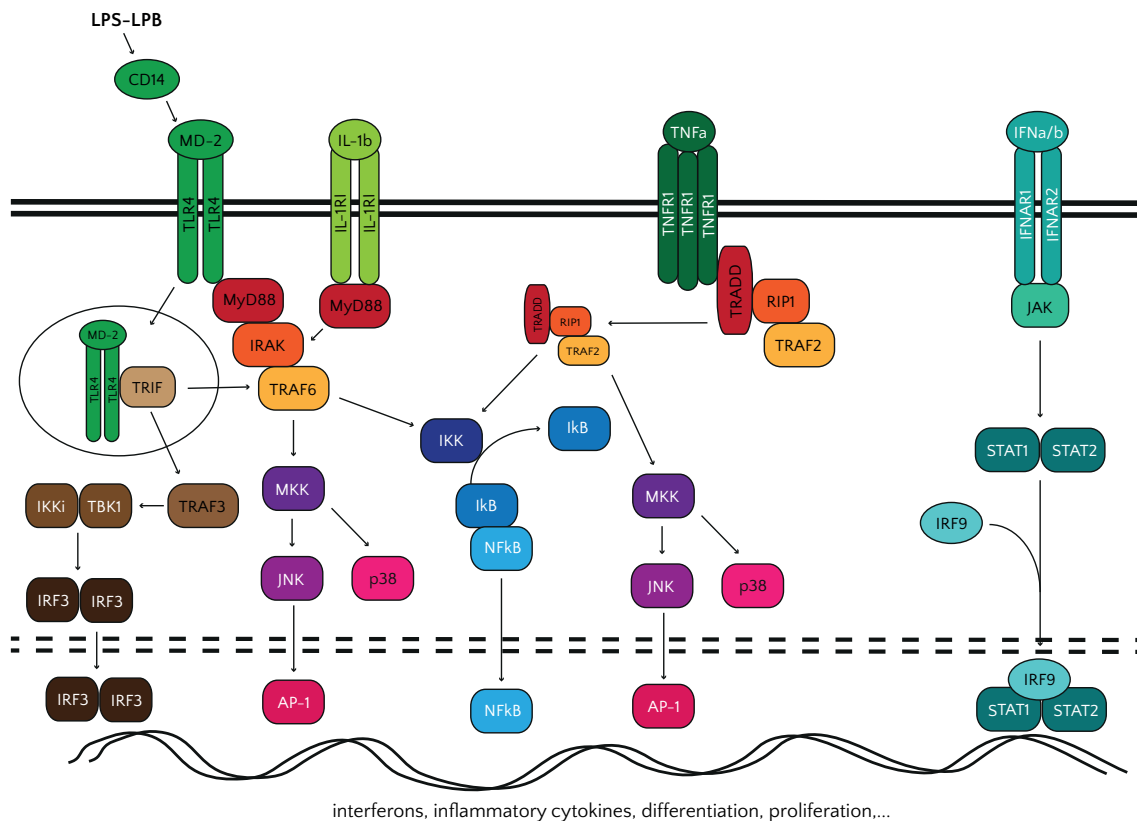
Figure 1.3.: Overview of different signalling pathways
The signalling pathways of TLRs, the IL–1RI and the TNFR1 show high similarities. TLR as well as IL–1RI use MyD88 as an adaptor molecule leading to the recruitment of IRAKs and TRAF6 and a subsequent activation of MAPK and NFϰB signalling. TNFR1 does not utilise MyD88, but also activates MAPK and NFϰB signalling through RIP1 and TRAF2. Apart from the MyD88-dependent pathway TLR4 can signal via TRIF, leading to the activation of IRF3 and subsequent production of interferons, which in turn lead to the induction of IFN inducible genes, like *cxcl10*. All of these pathways ultimately lead to the regulation of genes involved in the production of inflammatory cytokines, cell proliferation and differentiation (reviewed in Akira and Takeda, 2004; Borden *et al.*, 2007; Bradley, 2008; Weber *et al.*, 2010; Kawai and Akira, 2010).

these studies suggest that LPS activates HSCs into cycle, leading to their differentiation towards myeloid cells, likely through direct activation of TLR4 signalling in HSCs. However, most of these studies do not rule out a indirect effect of cytokines produced by other cells due to TLR signalling as the cause of the effects on HSC behaviour. Indeed, many studies have provided evidence for the role of inflammatory cytokines in the regulation of HSC proliferation, differentiation and self-renewal.

1.2.3. The role of inflammatory cytokines in immunity and their effects on HSCs

The activation of TLR signalling, as well as other pathways activated during the innate immune response, lead to the production of several cytokines, performing various

functions during infection and inflammation. The role of these cytokines in the promotion of the immune response has been intensively studied. More recently, some of these cytokines have also been implicated to have a role in the regulation of HSC function.

Interferons (IFNs)

IFNs are mainly produced by plasmacytoid dendritic cells (pDCs) (Perussia *et al.*, 1985; Asselin-Paturel *et al.*, 2003) upon activation of the MyD88-independent pathway of TLR signalling, through the activation of IRF3 (reviewed in Kawai and Akira, 2010). Although IFNs have been initially identified, as they are produced upon viral infections, it is known today that they also play a crucial role during bacterial infections (reviewed in Decker *et al.*, 2005). Two groups of IFNs can be distinguished: the type I interferons, including IFNα and IFNβ, and type II interferons, namely IFNγ. The type I IFNs are bound by a receptor complexed formed by heterodimerization of IFNAR1 and IFNAR2 upon ligand binding, while IFNγ binds to the IFNGR1 or IFNGR2, also leading to formation of a receptor-complex by heterodimerization. Upon activation the receptors bind different Janus kinases (JAKs), leading to their phosphorylation and subsequent activation of signal transducers and activators of transcription (STATs). Upon activation of IFNAR, STAT1 and STAT2 dimerize and are released from the receptor. Subsequently the STAT1-STAT2 complex translocates to the nucleus, where it binds IRF9, leading to the formation of the IFN-stimulated gene factor 3 (ISGF3), which binds to IFN-stimulated response elements in the promoter regions of IFN-stimulated genes (ISGs), leading to their transcription. Apart from the inhibition of viral reproduction, IFNs lead to the expression of MHC class I and class II molecules, the production of adhesion molecules and chemokines, to induce recruitment of leukocytes, and mediate the activation of cytotoxic effector functions among cells of the innate and the adaptive immune system (reviewed in Borden *et al.*, 2007; Ivashikiv and Donlin, 2014). In more detail, various ISGs lead to the inhibition of virus replication, translation of viral genes and can ultimately induce apoptosis of infected cells. An example of an ISG is *Mx1* (Horisberger *et al.*, 1983; Staeheli *et al.*, 1986). Mx proteins interfere with intracellular trafficking and activity of viral polymerases, thus inhibiting replication of many RNA viruses (reviewed in Haller *et al.*, 2007). The IFN-inducible Mx-promotor in combination with a Cre-element is furthermore, used in various mouse models, to induce conditional deletion of target genes (Kuhn *et al.*, 1995).

Interferons have been shown to directly induce proliferation of HSCs (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010). Short-term treatment of mice with the TLR ligand pIC, inducing the production of IFNα, or direct IFNα treatment led to an increased proliferation of dormant HSCs dependent on Stat1 signalling (Essers *et al.*, 2009). Apart

from an increase in cycling of HSCs, an increased expression of Sca-1 could be detected on the surface of HSPCs. Interestingly, the activation of HSCs with IFNα was not only accompanied by an upregulation of Sca-1, but also dependent on the presence of Sca-1, as has been shown using *Sca-1*$^{-/-}$ mice (Essers *et al.*, 2009). In contrast to the increased cycling of HSCs in response to short-term pIC treatment, long-term treatment led to reduced repopulation and self-renewal capacity in competitive transplants (Essers *et al.*, 2009; Sato *et al.*, 2009). Similar effects have been shown for IFNγ, identified to be the cause of HSC alterations in *M. avium* infections. The HSCs of *M. avium* infected mice show increased mobilisation to the periphery, as well as reduced engraftment potential, dependent on IFNγ , but not IFNα , signalling. Furthermore, direct stimulation of mice with IFNγ led to an increased cycling of HSCs, as well as their mobilisation to the periphery (Baldridge *et al.*, 2010). These findings are further supported by two studies showing that loss of the IFN-inducible immunity related p47 GTPase Irgm1 leads to HSC defects, due to a dysregulation of IFN signalling (Feng *et al.*, 2008; King *et al.*, 2011b). HSCs of *Irgm1*$^{-/-}$ mice show increased expression of IFN-inducible genes, leading to increased cycling and impaired engraftment (Feng *et al.*, 2008; King *et al.*, 2011b). These effects can be rescued by additional deletion of Stat1, leading to impaired IFN signalling, showing that the HSC defects are caused by the increased IFN signalling in *Irgm1*$^{-/-}$ mice (King *et al.*, 2011b). A recently published study has challenged these findings, proposing that IFNγ has anti-proliferative effects on HSCs, however the findings of this study are based on reduced numbers of LSK cells after *in vitro* treatment with IFNγ and reduced engraftment potential of these cells (de Bruin *et al.*, 2013). Therefore, the findings are actually in agreement with the earlier studies, showing that excessive IFN signalling leads to exhaustion of the HSC pool. Furthermore, the finding that IFNγ treatment *in vitro* leads to decreased cycling of HSCs might be due to a requirement of the niche environment to induce activation of HSCs (King *et al.*, 2013; Prendergast and Essers, *unpublished data*).

Tumour necrosis factor alpha (TNFα)

TNFα is a pro-inflammatory cytokines produced upon infection, in part through TLR signalling. TNFα can be produced by various types of cells, including endothelial cells, fibroblasts and osteoclasts, but mainly monocytes and macrophages. TNFα is initially expressed as pro-TNFα in a membrane-bound form, but can be released through cleavage, mediated by the TNFα converting enzyme (TACE), resulting in a soluble form of TNFα. TACE can not only mediate the cleavage of pro-TNFα , but also the cleavage of its receptor, leading to a neutralisation of TNFα action. TNFα can bind to two distinct receptors, TNFR1 (TNFRSF1A, p55) mainly mediates the pro-inflammatory and the apoptosis

pathway, while binding to TNFR2 (TNFRSF1B, p75) promotes tissue repair and angiogenesis. Upon TNFα binding, TNFR1 recruits TNFR-associated death domain (DD) protein (TRADD), which recruits receptor interacting protein-1 (RIP1) and TRAF2. The TRADD-RIP1-TRAF2 complex is subsequently released from the receptor and leads to activation of IκB, through recruitment of MEKK-3 and TAK1, ultimately leading to the activation of NFκB signalling. Furthermore the TRADD-RIP1-TRAF2 complex recruits apoptosis-signalling kinase-1 (ASK-1), which activates the MAP2Ks MEK-4 and MEK-6, leading to the phosphorylation of JNK and p38 MAPK. Phosphorylated JNK subsequently phosphorylates c-Jun, a subunit of the transcription factor AP-1, necessary for the interaction with CBP/p300 and gene activation. Many pro-inflammatory effects of TNFα can be explained by TNFα signalling in endothelial cells. In response to TNFα endothelial cells express adhesion molecules, including ICAM-1, VCAM-1 and E-selectin, and release chemokines, such as IL-8, MCP-1 and IP-10, subsequently leading to the recruiting of leukocytes to the site of infection. In addition, TNFα has been shown to be the cause of lethal sepsis, as mice immunised with rabbit-anti serum to TNFα were protected from lethal effects of *E. coli*-derived LPS (reviewed in Bradley, 2008).

The effects of TNFα on the behaviour of HSCs have been controversially reported. TNFα has been shown to have inhibitory as well as stimulatory effects on HSCs, dependent on the mechanism of interaction (Jacobsen *et al.*, 1992). Many studies report a reduction in colony-formation as well as in reconstitution potential of HSPCs in response to TNFα, for murine as well as for human cells (Broxmeyer *et al.*, 1986; Selleri *et al.*, 1995; Dybedal *et al.*, 2001; Bryder *et al.*, 2001). Conversely, it has been shown that HSCs of older p55$^{-/-}$ (TNFR1) mice are decreased in numbers and have a reduced self-renewal capacity, indicating that signalling through p55 is essential for the regulation of haematopoiesis at the stem cell level (Rebel *et al.*, 1999). However, a recently published study using double-knockout mice for both TNF receptors (*Tnfrsf1 dKO*) showed an enhanced repopulation capacity of HSCs from these mice in competitive transplants (Pronk *et al.*, 2011). Furthermore, it has been shown that repeated TNFα treatment led to a reduced bone marrow cellularity as well as a reduced repopulation potential, comparable to the effects of long-term IFN treatment (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010). Therefore, the authors of this study proposed that TNFα has an anti-proliferative effect on HSCs *in vivo* (Pronk *et al.*, 2011). Nevertheless, many of these studies have been performed *in vitro* and none provides information about the direct effect of short-term TNFα treatment on the cycling behaviour of HSCs.

CXCL9, CXCL10 and CXCL11

CXCL10, along with CXCL9 and CXCL11, are mainly produced in response to IFN signalling (reviewed in Borden *et al.*, 2007; Groom and Luster, 2011). CXCL10, as well as CXCL9 and CXCL11, binds to the CXC chemokine receptor 3 (Cxcr3). Upon activation Cxcr3 coordinates the inflammation, by regulating the generation of effector T cells and their subsequent migration into the periphery, where the Cxcr3 ligands regulate the interactions of effector T cells in the inflamed tissue (reviewed in Groom and Luster, 2011). A role for CXCL9, CXCL10 or CXCL11 in regulation of HSC function has so far not been shown.

Interleukin-6 (IL-6)

The expression of IL-6 is triggered by TNFα, IL-1, LPS, viral infections or products released by necrotic cells, whereby TLR induced production of IL-6 is one of the earliest events. The primary source of IL-6 are monocytes and macrophages, but T cells can also produce IL-6 during chronic inflammation. IL-6 has been proposed as a key signal for the transition from innate to adaptive immunity, as it regulates various functions of B and T cells (reviewed in Naugler and Karin, 2008), like B cell growth and antibody production (Tanner and Tosato, 1992) and differentiation of T(H)-17 cells (Zhou *et al.*, 2007). IL-6 has furthermore been suggested to be involved in the regulation of HSPCs upon TLR stimulation as well as during autoimmune disease (Zhang *et al.*, 2008; Chen *et al.*, 2010). IL-6 has been shown to be involved in the expansion of the LSK population upon *E. coli* infection, due to increased Sca-1 expression (Zhang *et al.*, 2008). Furthermore, IL-6 has been shown to be involved in mTOR mediated HSC defects, observed in autoimmune disease (Chen *et al.*, 2010).

Interleukin-1 beta (IL-1β) and IL-1α

IL-1β belongs to the IL-1 family, which comprises 11 proteins, with IL-1α and IL-1β being the founding members. While IL-1α is primarily membrane bound, IL-1β is secreted. Both IL-1α and IL-1β bind to the type I IL-1 receptor (IL-1RI), which is ubiquitously expressed. The activation of IL-1 signalling is regulated by a third ligand of IL-1RI, IL-1 receptor antagonist (IL-1RA), which binds to IL-1RI with the same affinity as IL-1α and IL-1β, but does not induce downstream signalling. Furthermore, a second receptor, the type II IL-1 receptor (IL-1RII), binds IL-1α and IL-1β, but lacks the signalling competent cytosolic part, thereby serving as a decoy receptor. Upon ligand binding, the IL-1RI undergoes a conformational change, leading to the recruitment of IL-1 receptor accessory protein (IL-1RAcP) and the subsequent recruitment of MyD88 and IRAK4, through the

TIR domain of the receptor. The phosphorylation of IRAK4 induces a signalling cascade, very similar to the MyD88-dependent signalling pathway of TLRs. Phosphorylated IRAK4 activates IRAK1 and IRAK2, leading to the recruitment of TRAF6. Subsequently, the IRAK1-IRAK2-TRAF6 complex is released from the receptor and leads to activation of the NF$\varkappa$B, JNK and p38 MAPK pathways, resulting in the activation of IL-1 inducible genes, like IL-6, MCP1, PGE2 and COX2 (reviewed in Weber *et al.*, 2010). Results of the IL-1β regulated gene expression are fever, lowered pain threshold and vasodilation. IL-1β can also lead to increased expression of adhesion molecules on endothelial cells, leading to recruitment of immune effector cells to the infected tissue. Furthermore, IL-1β has been suggested to promote the differentiation of HSCs to myeloid cells. In addition, IL-1 signalling has been shown to be essential for the development of T(H)-17 cell (reviewed in Dinarello, 2009). So far, IL-1 signalling has not been shown to have an effect on HSCs.

1.2.4. Activation of NF$\varkappa$B signalling during the innate immune response

Most of the above described cytokine-induced pathways, as well as TLR signalling, ultimately lead to the activation of NF$\varkappa$B signalling and subsequent activation of specific genes in many different cell types. NF$\varkappa$B signalling is very complex, leading to the induction of hundreds of genes and can be induced by myriads of biochemical mechanisms (reviewed in Baltimore, 2011). LPS, as well as TNFα and IL-1β, lead to the activation of the canonical pathway of NF$\varkappa$B signalling, through TRAFs and RIP1 (reviewed in Oeckinghaus *et al.*, 2011). Common to each pathway is the activation of the IKK kinase complex, consisting of IKKα ,IKKβ and IKKγ (NEMO). The activated IKK complex subsequently phosphorylates I$\varkappa$B, which is bound to NF$\varkappa$B and inhibits its translocation to the nucleus. Phosphorylation of I$\varkappa$B leads to its ubiquitylation and subsequent proteasomal degradation, releasing NF$\varkappa$B. NF$\varkappa$B then translocates to the nucleus, leading to the activation of various genes. Among these NF$\varkappa$B inducible genes is also I$\varkappa$B, serving as a negative feedback loop to regulate NF$\varkappa$B activity. Binding of I$\varkappa$B to NF$\varkappa$B in the nucleus, leads to its release from the DNA and its transport to the cytoplasm, where it is inactivated. NF$\varkappa$B activation is crucial for the induction of the innate, as well as the adaptive immunity, and regulates various cellular processes, like proliferation and differentiation (reviewed in Baltimore, 2011). Upon TLR activation NF$\varkappa$B is involved in the MyD88-induced production of inflammatory cytokines, as well as in the TRIF-mediated induction of interferons (reviewed in Akira and Takeda, 2004). A recent study has furthermore, proposed that NF$\varkappa$B signalling is required for HSC maintenance (Stein and Baldwin, 2013). In this study, a conditional deletion of p65 in the haematopoietic system led to increased cycling of HSCs, increased numbers of progenitor cells and ex-

tramedullary haematopoiesis. Furthermore, the HSCs of these mice showed a severe engraftment defect in competitive transplantation (Stein and Baldwin, 2013). This study suggests that NFκB is required for HSC maintenance during homeostasis, if NFκB is beyond that also required for the response of HSCs to infectious stimuli remains to be investigated.

1.2.5. HSCs produce cytokines upon infection

Apart from sensing cytokines produced by other immune cells, HSPCs have been recently proposed to produce a considerable amount of cytokines. Various cytokines were produced by ST-HSCs and MPPs upon treatment with TLR ligands at the single cell level, even trumping mature myeloid and lympoid cells in terms of speed, magnitude and breadth (Zhao *et al.*, 2014). As HSPCs are constantly circulating through the blood and lymph to peripheral organs (Massberg *et al.*, 2007), direct production of cytokines in response to danger signals might be an elegant way to coordinate rapid haematopoiesis at sites of infection. This production of cytokines by HSPCs might be especially important in situations, where mature cells are lost. Furthermore, it was proposed that HSPCs in the bone marrow can be subdivided into two groups, one able to sense pathogens and produce cytokines and the other group able to react to these cytokines and induce haematopoiesis. This hypothesis is supported by the finding that some LSK cells express either TLRs or cytokine receptors (Zhao *et al.*, 2014). In summary, all of the above mentioned studies provide evidence for the importance of HSPCs in the fight agains infections and the rapid replenishment of the system after the infection is cleared. The exact mechanisms how the effects of infections on HSCs are mediated, however, remain to be investigated.

2. Aim of this thesis

Many studies have been performed investigating the response of HSCs to infections mediated through TLR signalling (Kaulen *et al.*, 1983; Nagai *et al.*, 2006; Singh *et al.*, 2008; Zhang *et al.*, 2008; Yanez *et al.*, 2009, 2010; Takizawa *et al.*, 2011; Esplin *et al.*, 2011; Choi *et al.*, 2011; Megías *et al.*, 2012). Many of these studies have, however, been performed *in vitro* (Nagai *et al.*, 2006; Yanez *et al.*, 2009, 2010). Furthermore, the long-term effect of TLR activation upon infection or through repeated treatment with TLR ligands has been extensively analysed (Zhang *et al.*, 2008; Singh *et al.*, 2008; Rodriguez *et al.*, 2009; Takizawa *et al.*, 2011; Esplin *et al.*, 2011; Choi *et al.*, 2011), while the short-term effects of TLR signalling on the behaviour of HSCs *in vivo* remain unclear. We aimed to specifically investigate the role of short-term treatment with LPS, a ligand of TLR4, on the cycling behaviour of HSCs, to analyse the first response of HSCs to infections, rather than the long-term outcome of this response. This is especially important as the short-term effects might be very different from the long-term effects and may help to better understand the cause of the effects seen upon long-term treatment or chronic infections. In addition, we wanted to analyse the effects of LPS, in comparison to already published data on the effects of IFNs and other cytokines on HSCs. We have already shown that HSCs are activated in response to IFNα treatment (Essers *et al.*, 2009), a cytokine typically produced upon TLR4 signalling. We therefore asked, if this IFNα-mediated activation of HSCs might be part of a feedback loop induced upon infection. Furthermore, as the question of whether HSCs can directly respond to infection is not fully investigated to date, we wanted to analyse if the HSC response to LPS stimulation is directly mediated through TLR4 signalling within the HSCs, or indirectly mediated, through TLR4 signalling within another cell type. Thus, we aimed to investigate the response of HSCs to LPS treatment, as well as the involvement of IFNα and other cytokines in this process, using genetic mouse models, as well as *in vitro* approaches. This research gives new insights into the effects of infections on HSC behaviour and function and may therefore help to improve the treatment of chronic infections.

3. Results

3.1. Stress induced IFNα production in the bone marrow

Upon bone marrow stress, it is mainly the fast cycling progenitors that are lost and therefore have to be replenished by HSCs to ensure maintenance of the blood system. The HSCs can be subdivided into two different populations: the homeostatic and the dormant HSCs. Homeostatic HSCs are constantly but slowly cycling to ensure the maintenance of the haematopoietic system, while dormant HSCs are found in a long-term quiescent state during homeostasis, but can be rapidly activated in response to bone marrow injury (Wilson *et al.*, 2008). This activation of dormant HSCs, together with the production of progenitors by the homeostatic HSCs, ensures fast replenishment of the system. It has been shown that various forms of bone marrow stress, like 5-FU or G-CSF treatment lead to an activation of dormant HSCs (Wilson *et al.*, 2008). Interestingly, treatment with cytokines, like interferons, also leads to an effective activation of HSCs, even though interferons have an anti-proliferative effect on mature bone marrow cells (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010). As most of these mature cells are lost during the combat against the disease, the activation of HSCs might be an additional feature of the IFN production upon infection, to ensure fast replenishment of the system after blood cell loss. We therefore asked whether the production of IFNs and the following activation of quiescent HSCs might be a general feature of bone marrow injury and cell loss.

We first wanted to analyse the production of IFNα upon different forms of bone marrow stress. We used a MxCre Rosa26eYFP flox$_2$ mouse model to detect IFNα production in the bone marrow (Kuhn *et al.*, 1995; Srinivas *et al.*, 2001). These mice have a Cre element under the control of the IFNα inducible Mx promotor combined with a loxP-stop-loxP cassette followed by *eYFP* under the control of the constitutively active Rosa26 promotor (Fig. 3.1 A). The Mx promotor is turned on by IFNα signalling, leading to the expression of Cre. Cre will then excise the stop codon, thereby activating the expression of eYFP. This system enables the detection of previous IFNα production in the bone marrow, through measurement of the eYFP expression within different bone marrow cells. As a proof of principle experiment we treated MxCre Rosa26eYFP flox$_2$ mice with
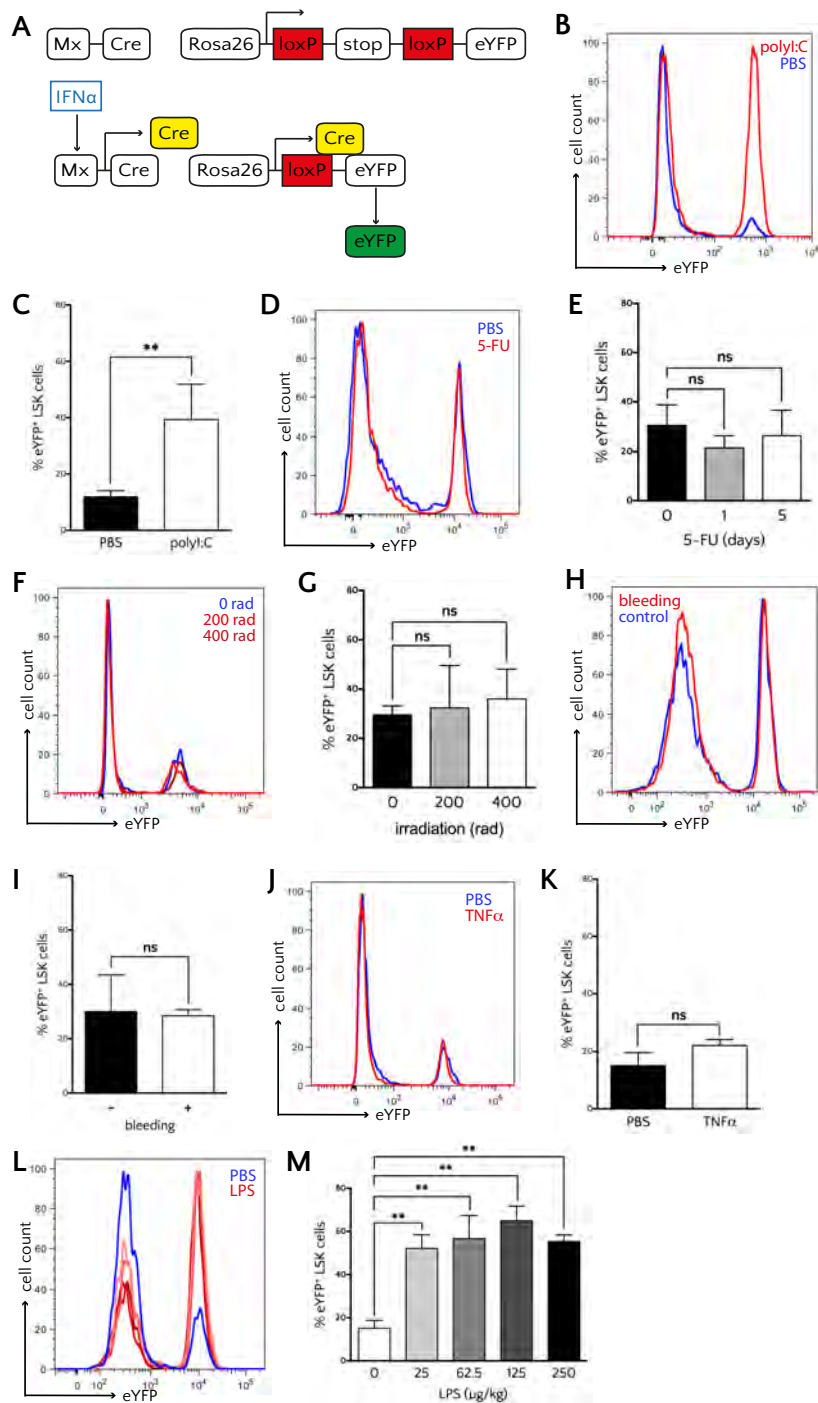
Figure 3.1.: Stress induced IFNα production in the bone marrow

(A) MxCre Rosa26 eYFP flox$_2$ mice express eYFP, after Cre expression was activated by IFNα (B–M) eYFP expression in LSK cells after different treatments of MxCre Rosa26 eYFP flox$_2$ mice. (B,C) PBS or 5 mg/kg polyI:C. (D,E) PBS or 150 mg/kg 5-FU. (F,G) Irradiation with 200 or 400 rad. (H,I) Bleeding. (J,K) PBS or 0.75 mg/kg TNFα. (L,M) PBS or 25, 62.5, 125 or 250 µg/kg LPS; representative FACS profiles (B,D,F,H,J,L) and quantitative and statistical analysis (C,E,G,I,M).

Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p ≤ 0.05, **p ≤ 0.01.

5 mg/kg polyI:C, as polyI:C leads to a strong production of IFNα in the bone marrow. We measured the expression of eYFP within bone marrow LSK cells by Flow Cytometry one week later, when cycling HSCs have returned to quiescence. Bone marrow of mice treated with PBS or polyI:C was isolated and a FACS staining was performed in order to identify LSK cells (Figure 5.1 A). While the control PBS treated cells only showed a low expression of eYFP, the expression was significantly increased upon treatment with polyI:C (Figure 3.1 B and C). This shows, that eYFP is effectively activated in LSK cells upon IFNα production in the bone marrow. As treatment with 5-FU has been shown to lead to an activation of dormant HSCs (Wilson *et al.*, 2008), we asked whether this activation might be due to IFNα production in the bone marrow upon 5-FU treatment. Although 5-FU treatment leads to an activation of HSCs, no increase in eYFP signal was detected upon treatment of 5-FU in LSK cells of MxCre Rosa26eYFP flox$_2$ mice (Figure 3.1 D and E), showing that no IFNα is produced upon 5-FU treatment in the bone marrow and the activation of HSCs upon 5-FU treatment must therefore be mediated by another mechanism. Transplantation of bone marrow cells into lethally irradiated mice is to date the experiment of choice to prove the self-renewal and repopulation capacity of HSCs. However, irradiation is a severe form of bone marrow stress to the recipient mice. To test if this form of stress leads to the production of IFNα in the bone marrow and might therefore influence the cycling behaviour of the donor cells, we irradiated MxCre Rosa26eYFP flox$_2$ mice with a sublethal dose of either 200 or 400 rad. The irradiation did, however, not lead to an increase in eYFP expression in LSK cells (Figure 3.1 F and G), demonstrating that irradiation does not lead to IFNα production in the bone marrow. Nevertheless, irradiation influences the bone marrow niche environment and therefore may alter the behaviour of transplanted HSCs in comparison to non-irradiated mice. Another form of stress to the haematopoietic system is severe blood loss. As bleeding indirectly leads to loss of bone marrow cells, because progenitor cells are activated to replenish the lost blood cells, we bled MxCre Rosa26eYFP flox$_2$ mice once, taking 5–6 drops of blood from the vena facialis. In our experiment we did not observe an increase in eYFP signal within bone marrow LSK cells after bleeding (Figure 3.1 H and I). Nevertheless, a single round of bleeding might not be severe enough to induce a stress response in the bone marrow and several rounds of bleeding might therefore lead to the production of IFNα. Whether bleeding also leads to an activation of dormant HSCs remains to be investigated. Interferons are typically produced upon infections. To investigate whether other cytokines may lead to an activation of HSCs by inducing IFNα production in the bone marrow, we treated MxCre Rosa26eYFP flox$_2$ mice with 0.75 mg/kg TNFα. Comparable to the PBS treated mice, the TNFα treated mice did not show an increased

eYFP expression in LSK cells (Figure 3.1 J and K), indicating that TNFα does not lead to IFNα production in the bone marrow. It is well established that IFNα is produced upon viral infections (reviewed in Borden *et al.*, 2007). To test if this is true for bacterial infections, MxCre Rosa26eYFP flox$_2$ mice were treated with different concentrations of lipopolysaccharide (LPS) and the eYFP expression in the bone marrow was analysed after one week. LPS is an endotoxin of the outer cell wall of Gram(-) bacteria, which is recognised by TLR4 and often used to mimic bacterial infections. The eYFP expression in LSK cells was significantly higher after treatment with LPS compared to the control PBS treated mice (Figure 3.1 L and M). These results show that also upon bacterial infection, mimicked by LPS treatment, IFNα is produced in the bone marrow.

In summary, although the baseline levels of eYFP expression are quite variable, these experiments show that IFNα production is not a general feature of bone marrow stress. However, as IFNα is produced upon LPS treatment, we wanted to investigate, if LPS leads to an activation of HSCs comparable to direct interferon treatment.

3.2. LPS leads to increased proliferation of HSCs

3.2.1. LPS induces activation of HSCs in a TLR4 dependent manner

As we could show that LPS treatment of mice leads to IFNα production in the bone marrow, we asked what effect LPS treatment would have on the behaviour of HSCs. For this purpose, wildtype mice were treated with PBS or LPS for 18 hours. The cycling behaviour of HSCs (LSK CD150$^+$CD48$^-$CD34$^-$) was assessed by staining for intracellular (ic) Ki67 combined with Hoechst33342. This staining enables the differentiation between the G_0 (icKi67^{neg}Hoechst^{low}), the G_1 (icKi67^{pos}Hoechst^{low}) and the S, G_2 and M (icKi67^{pos}Hoechst^{high}) phases of the cell cycle, as Ki67 is only expressed during an active cell cycle and Hoechst enables the identification of cells that have already doubled their chromosome set. After treatment with LPS the quiescent HSCs of wildtype mice exited the G_0 phase of the cell cycle and moved into the active G_1, S, G_2 or M phase (Figure 3.2 A and B). Whether this increase of HSCs in the G_1, S, G_2 or M phase of the cell cycle is accompanied by an active proliferation, can be determined by BrdU incorporation. Therefore, mice were treated with BrdU 14 hours before analysis. The BrdU incorporation in HSCs was then measured by Flow Cytometry. The HSCs of wildtype mice showed a significant increase in BrdU$^+$ cells after treatment with LPS (Figure 3.2 C and D), indicating that LPS leads to an increased proliferation of HSCs. In contrast to LPS treated wildtype mice, HSCs from mice lacking TLR4, one of the components of the TLR4–MD2–CD14 receptor complex detecting LPS, did not show an increase in cell cycle
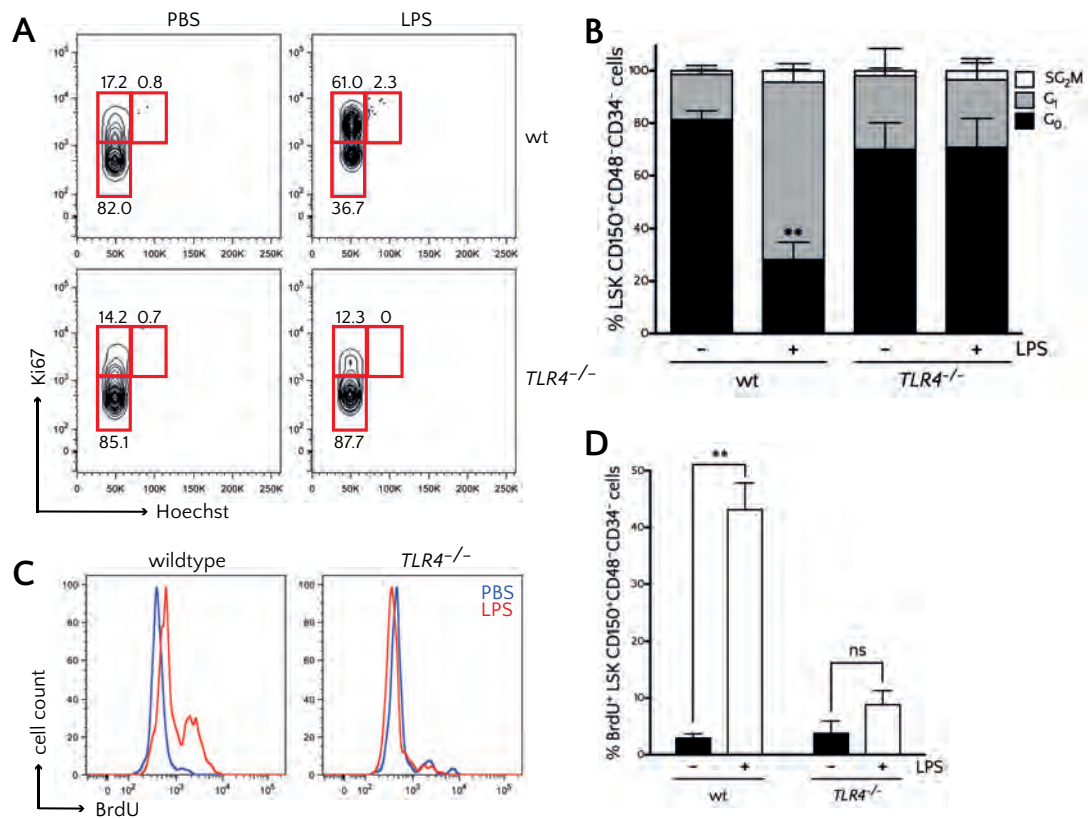
Figure 3.2.: LPS–induced activation of HSCs is dependent on TLR4 signalling
(A,B) Cell cycle analysis (G_0 cells icKi67^{neg}Hoechst^{low}) of HSCs (LSK CD150$^+$CD48$^-$CD34$^-$) from PBS or LPS (0.25 mg/kg, 18h) treated wt or *TLR4$^{-/-}$* mice. Representative FACS profiles (A) and quantitative and statistical analysis (B). (C,D) BrdU incorporation (14h) in HSCs from wt or *TLR4$^{-/-}$* mice treated with PBS or LPS (0.25 mg/kg, 18h). Representative FACS profiles (C) and quantitative and statistical analysis (D).
Data are the mean +/– SD of at least 3 mice per condition, representing similar results of 2–3 independent experiments; *$p \leq 0.05$, **$p \leq 0.01$

activity (Figure 3.2 A and B), or an increase in BrdU incorporation (Figure 3.2 C and D) upon LPS treatment, indicating that the LPS–induced activation of HSCs is dependent on TLR4 signalling.

HSCs entered an active cell cycle, even with a low dose of 12.5 µg/kg LPS (Figure 3.3 A). However, as treatment with 250 µg/kg LPS always led to a significant activation of HSCs without impairing the health of the mice, while high doses of LPS led to severe health issues, this concentration was used for further experiments. The activating effect of LPS on HSCs is transient, with a peak in between 24 and 48 hours, while the cell cycle status of HSCs was back to baseline after 72 hours. Interestingly, after 96 hours even more HSCs could be found in the G_0 phase of the cell cycle compared to the untreated
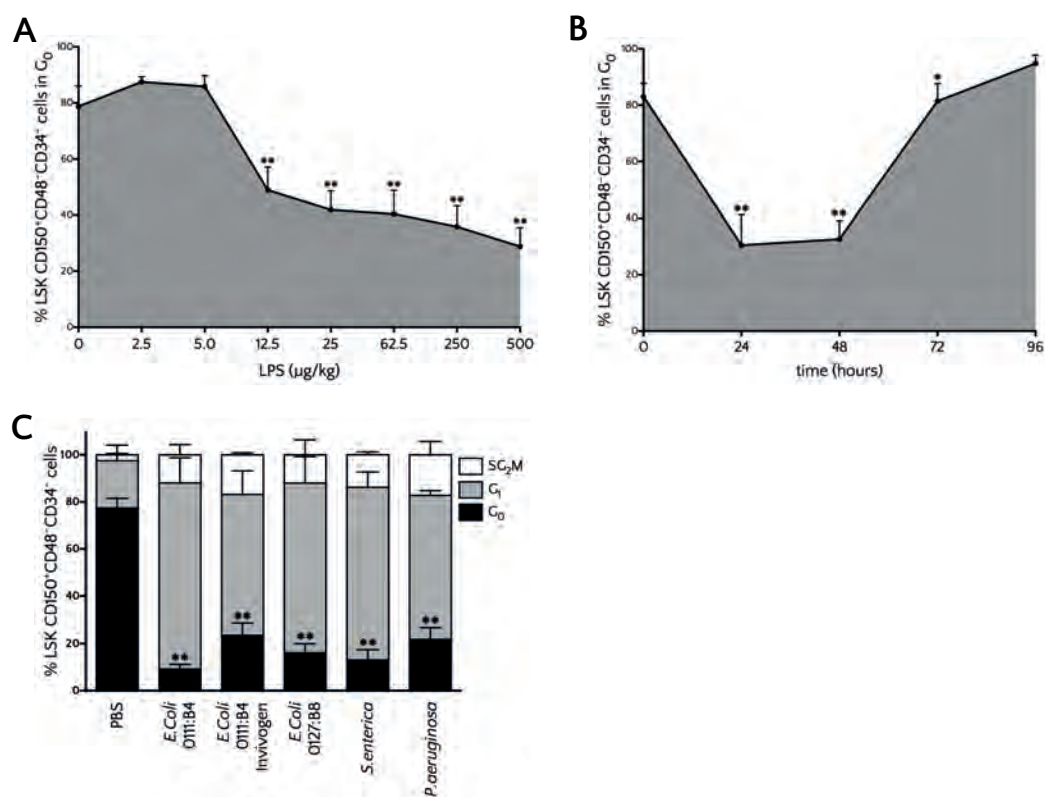
Figure 3.3.: LPS induces transient activation of HSCs
(A) HSCs in G_0 after treatment of wt mice with different concentrations of LPS for 18h. (B) HSCs in G_0 after treatment of wt mice with 0.25 mg/kg LPS at different time points. (C) Cell cycle analysis with icKi67 and Hoechst after treatment of wt mice with 0.25 mg/kg LPS from different sources (Sigma, if not indicated differently).
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

mice (Figure 3.3 B), which may reflect a short-lived increase after stimulation. All of the above experiments, as well as all following experiments were performed with the same source of LPS (*E.coli* 0111:B4, Sigma). Nevertheless, treatment of wildtype mice with different sources of LPS led to the same increase in proliferation of HSCs (Figure 3.3 C), confirming that the effect of LPS on the HSCs is not dependent on the source of LPS.

In summary, these data show that even low doses of LPS lead to a transient increase in proliferation of HSCs in the bone marrow through activation of TLR4 signalling.
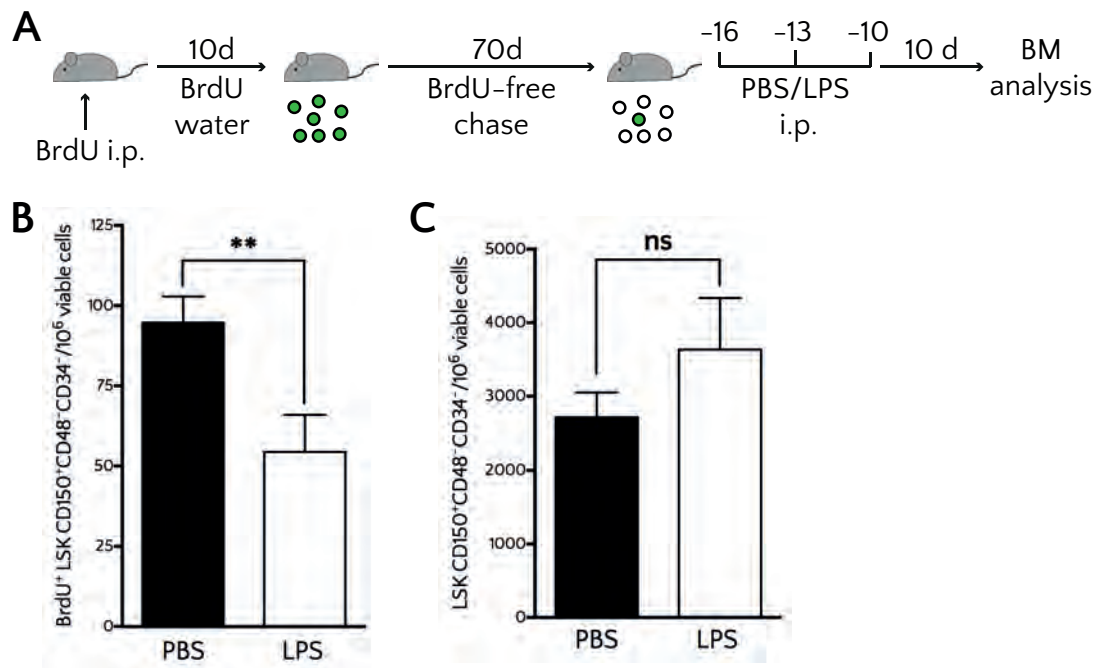
Figure 3.4.: LPS-induced activation of dormant HSCs
(A) Set-up of LRC experiment: wt mice were injected i.p. with BrdU (18 mg/kg) and given BrdU-water (1 g/l) for 10 days, leading to a labelling of all bone marrow cells with BrdU, following this the mice underwent a 70 day BrdU-free chase-period, resulting in a decreasing BrdU signal with every cell cycle, subsequently mice were injected with PBS or LPS (0.25 mg/kg, 3 times), every third day. The bone marrow was analysed 10 days later (B) Number of BrdU$^+$ LSK CD150$^+$CD48$^-$CD34$^-$ LRCs per 10^6 viable cells. (C) Number of LSK CD150$^+$CD48$^-$CD34$^-$ HSCs per 10^6 viable cells.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

3.2.2. LPS leads to the activation of dormant HSCs

To investigate whether the LPS-induced activation of HSCs shown above leads to an activation of dormant HSCs, we used a label-retaining assay with BrdU. Using BrdU to label all bone marrow cells for 10 days, followed by a BrdU-free chase period of 70 days, dormant label-retaining cells (BrdU$^+$, LRCs) can be detected. This method in combination with a FACS staining for LSK CD150$^+$CD48$^-$CD34$^-$ cells, enables the identification of dormant HSCs as BrdU$^+$ LRCs within the LSK CD150$^+$CD48$^-$CD34$^-$ population. Wild-type mice were treated as described above. At the end of the BrdU-free chase period, the mice were treated with PBS or LPS (0.25 mg/kg), three times over 7 days. The number of LRCs in these mice was analysed by Flow Cytometry 2 weeks later (Figure 3.4 A), when the HSCs have returned to quiescence. The number of LRCs is significantly decreased after LPS treatment (Figure 3.4 B), while the number of LSK CD150$^+$CD48$^-$CD34$^-$

cells is not changed (Figure 3.4 C), showing that LPS does not only lead to an activation of homeostatic HSCs, but also activates dormant HSCs to proliferate.

3.2.3. LPS treatment does not impair the long–term reconstitution potential of HSCs

Treating mice with LPS leads to an activation of dormant HSCs, which have been shown to exhibit the highest repopulation activity (Wilson *et al.*, 2008; Qiu *et al.*, 2014), into an active cell cycle. This raises the question, whether the self–renewal of those cycling cells is affected. To determine the long–term reconstitution potential of LPS–induced HSCs, CD45.2$^+$ mice were treated with PBS or LPS (0.25 mg/kg) for 24h. The bone marrow of these mice was isolated and transplanted i.f. into lethally irradiated CD45.1$^+$ mice, to exclude any effects due to impaired homing of cycling HSCs (Figure 3.5 A). We monitored the engraftment over time, by bleeding the mice every 4 weeks and determining the percentage of CD45.2$^+$ granulocytes in the blood. Granulocytes were measured, because these cells are a good indicator of active haematopoiesis, as they are short–lived (Uchida and Weissman, 1992). As shown in Figure 3.5 B, no difference in the engraftment of LPS treated cells can be observed in the primary transplant compared to the PBS treated cells. After 16 weeks the mice were killed and the engraftment was analysed in blood and bone marrow. Again no difference could be observed in the over-all engraftment of differentiated cells between LPS treated and PBS treated cells in the primary transplant (Figure 3.5 D). Furthermore, we analysed the percentage of engrafted HSCs as well as the relative number of HSCs per 10^6 viable bone marrow cells. Also here, no difference in engraftment can be detected in the primary transplant (Figure 3.5 F and G).

In addition to this analysis, 3x10^6 cells from four mice were transplanted i.f. into 3 lethally irradiated CD45.1$^+$ mice each to make secondary transplants and analyse the long–term engraftment of the LPS treated cells (Figure 3.5 A). The engraftment of the transplanted cells was investigated as described above. In the secondary transplant a minor trend of lower engraftment of LPS treated cells compared to PBS treated cells can already be observed after 4 weeks (Figure 3.5 C). The trend, of lower engraftment of LPS treated cells, already observed in the peripheral blood, could again be detected for all different cell populations, as well as for the overall engraftment, in the bone marrow 16 weeks after transplantation. (Figure 3.5 E). It is clear form analysis of the individual mice that two mice, which received LPS treated cells do not show any engraftment of CD45.2$^+$ cells, indicating a failed injection rather then an actual engraftment defect. In the other four mice, in which the transplanted LPS treated cells engrafted, no difference
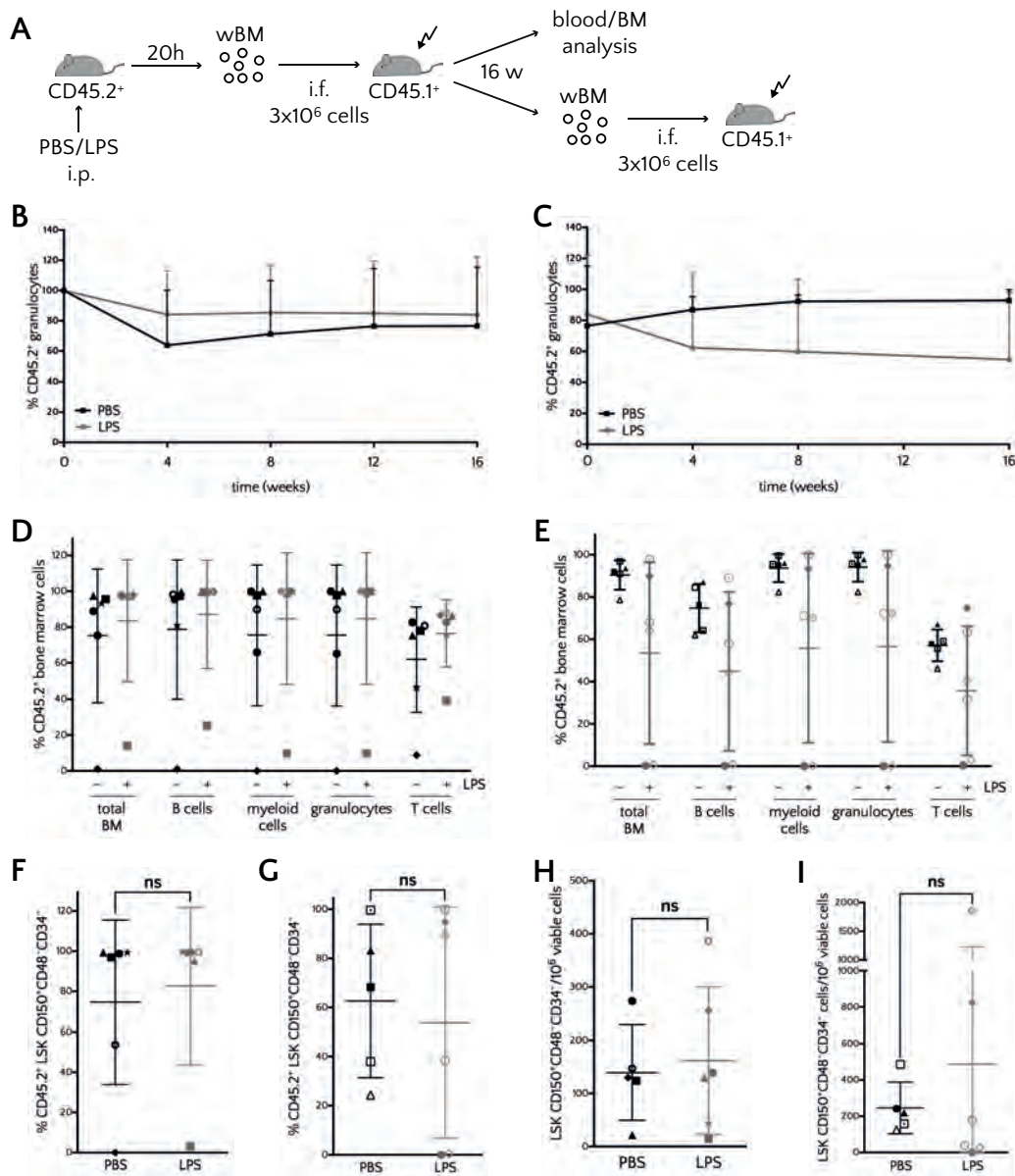
Figure 3.5.: LPS treatment does not impair the long-term reconstitution potential of HSCs
(A) Set-up of transplantation experiment: CD45.2$^+$ mice were treated with PBS or LPS (0.25 mg/kg, 24h), 3×10^6 Thy.1 depleted bone marrow cells of these mice were transplanted i.f. into lethally irradiated CD45.1$^+$ mice, the engraftment of the CD45.2$^+$ was monitored by bleeding the mice every 4 weeks, after 16 weeks the bone marrow as well as the blood was analysed for engraftment of different cell populations, furthermore 3×10^6 Thy.1 depleted bone marrow cells were transplanted i.f. into lethally irradiated secondary recipients, the engraftment in these mice was analysed as described above. (B,C) Percentage of CD45.2$^+$ granulocytes in the PBL of primary (B) and secondary (C) recipients over time. (D,E) Percentage of CD45.2$^+$ cells from different populations in the bone marrow of primary (D) and secondary (E) recipient. (F and G) Percentage (F) or relative number (G) of CD45.2$^+$ HSCs in the bone marrow of primary recipients. (H and I) Percentage (H) or relative number (I) of CD45.2$^+$ HSCs in the bone marrow of secondary recipients. Symbols indicate individual mice.
 Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

in the engraftment potential can be detected compared to the mice transplanted with PBS treated cells (Figure 3.5 E). Of most interest are the HSCs. Again, in the secondary transplant two mice did not show any engraftment of HSCs, matching the mice which likewise did not show an engraftment of the other populations (Figure 3.5 H). This finding supports the hypothesis that those two mice do not show any engraftment, because of a failed injection into the femur, as the remaining four mice show an engraftment of CD45.2$^+$ HSCs comparable to the mice transplanted with PBS treated cells (Figure 3.5 H and I).

In summary, the engraftment of LPS treated HSCs in primary recipients does not differ from the engraftment potential of PBS treated HSCs. Although, in the secondary recipients there is a trend towards a lower engraftment of the LPS treated HSCs compared to the PBS treated cells, this difference is not statistically significant. To determine whether this difference in engraftment of individual mice is due to a failed injection or reflects actual engraftment defects, further experiments have to be performed.

3.2.4. The LPS-induced activation of HSCs is dependent on Sca-1

Sca-1 has long been recognized as a marker for HSCs (Spangrude *et al.*, 1988), however, besides an increase in T lymphocyte activation upon antigen recognition (Stanford *et al.*, 1997) and a disadvantage in competitive transplantation (Ito *et al.*, 2003) of *Sca-1$^{-/-}$* mice, its function is still widely unknown. Hence, it is interesting that we can detect a Sca-1 phenotype in our model of HSC activation with LPS. Along with the increase in HSC proliferation, there is a LPS-induced shift in Sca-1 expression on HSCs and progenitor cells (Figure 3.6 A). This shift was due to both increased expression of Sca-1 on Sca-1$^+$ cells as well as an induction of Sca-1 expression on Sca-1$^-$ cells. There was a significant increase in Sca-1 expression on LK CD150$^+$ cells of LPS treated wildtype mice, while no significant increase in Sca-1 expression could be detected on cells of *TLR4$^{-/-}$* mice (Figure 3.6 B and C). These results show that the upregulation of Sca-1 after LPS is dependent on TLR4 signalling.

Interestingly, the activation of HSCs is not only accompanied by an upregulation of Sca-1, but is also dependent on the presence of Sca-1, since HSCs from mice lacking Sca-1 can no longer be activated in response to LPS. While the incorporation of BrdU was significantly increased in LK CD150$^+$CD48$^-$ cells of wildtype mice, no increase in proliferation could be detected in *Sca-1$^{-/-}$* mice (Figure 3.6 D). Taken together, these experiments show that treatment with LPS leads to an increase in Sca-1 expression on the surface of HSPCs and that the increased proliferation of HSCs after treatment with LPS is dependent on Sca-1, indicating a role for Sca-1 in the stress induced activation of HSCs.
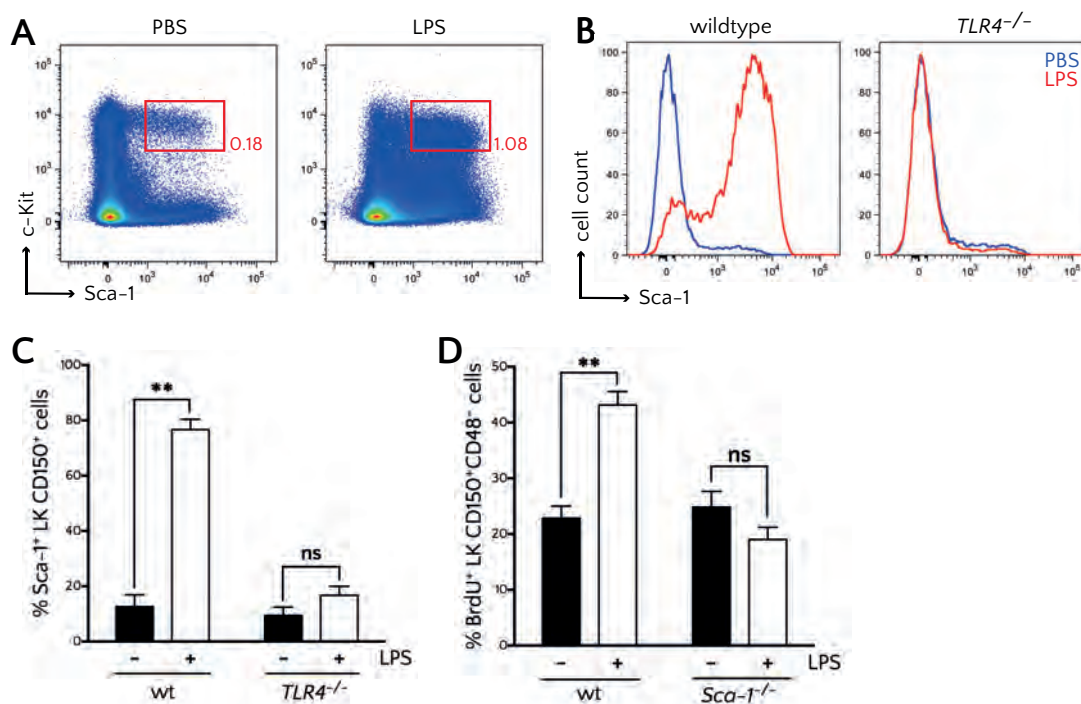
Figure 3.6.: The LPS–induced activation of HSCs is dependent on Sca-1
(A) Representative FACS plots of lineage negative cells from PBS or LPS (0.25 mg/kg, 18h) treated mice, show-ing a shift of LSK cells. (B,C) Sca-1 expression on the surface of LK CD150$^+$ cells after treatment of wt or $TLR4^{-/-}$ mice with PBS or LPS (0.25 mg/kg, 18h). Representative FACS profiles (B) and quantitative and sta-tistical analysis (C). (D) BrdU incorporation (14h) in LK CD150$^+$CD48$^-$ cells after treatment of wt or $Sca-1^{-/-}$ mice with PBS or LPS (0.25 mg/kg, 18h).
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

3.2.5. Is HSC activation a general feature of TLR signalling?

Toll–like receptors (TLRs) belong to the first line of defence against invading pathogens and therefore have an important role in the sensing of pathogens and the initiation of the immune defence (reviewed in Kawai and Akira, 2010). Hence, the activation of HSC proliferation might be a general feature of TLR signalling, ensuring a fast production of necessary immune cells for the defence, as well as a fast replenishment of the system after the infection is cleared. In addition to LPS, polyI:C, a TLR3 ligand has also been shown to activate HSCs (Essers *et al.*, 2009; Sato *et al.*, 2009).

To investigate if apart from LPS and polyI:C, other TLR ligands might also induce acti-vation of HSCs, wildtype mice were treated with ligands for different TLRs and the cell cycle activity, as well as the expression of Sca-1, was analysed by Flow Cytometry. Inter-estingly, not all of the tested TLR ligands led to an activation of HSCs (Figure 3.7 A and
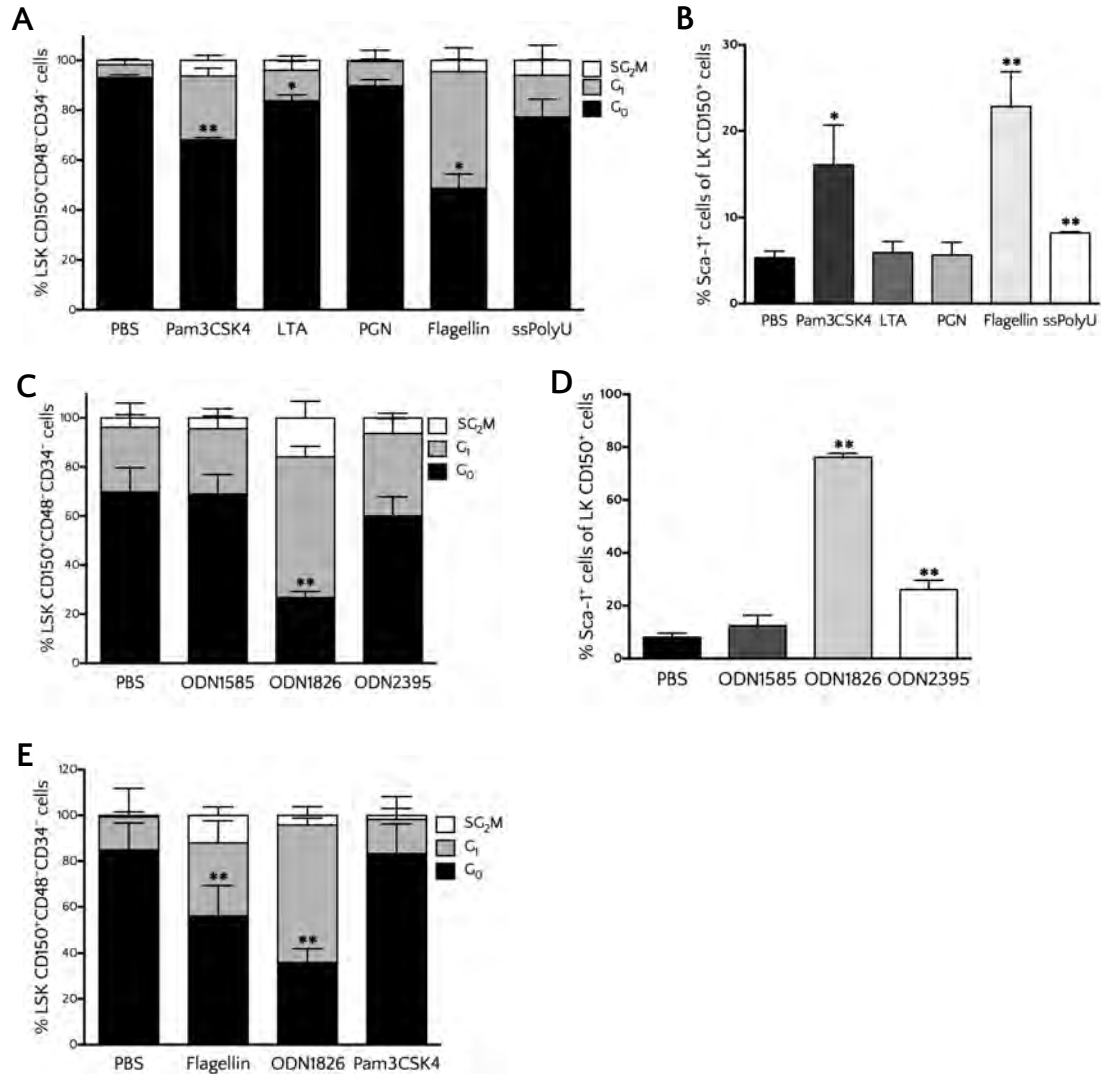
Figure 3.7.: Is HSC activation a general feature of TLR signalling?
(A) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt mice treated with Pam3CSK4 (1.5 mg/kg), Lipoteichoic acid (LTA, 2.5 mg/kg), Peptidoglycan (PGN, 2.5 mg/kg), Flagellin (1.0 mg/kg) or ssPolyU (1.0 mg/kg) for 18h. (B) Sca-1 expression of LK CD150$^+$ cells from wt mice treated with Pam3CSK4 (1.5 mg/kg), Lipoteichoic acid (LTA, 2.5 mg/kg), Peptidoglycan (PGN, 2.5 mg/kg), Flagellin (1.0 mg/kg) or ssPolyU (1.0 mg/kg) for 18h. (C) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt mice treated with ODN1585, ODN1826 or ODN2395 (1.25 mg/kg, 18h). (D) Sca-1 expression of LK CD150$^+$ cells from wt mice treated with ODN1585, ODN1826 or ODN2395 (1.25 mg/kg, 18h). (E) Cell cycle analysis (icKi67 and Hoechst) of HSCs from *TLR4*$^{-/-}$ mice treated with Flagellin (1.0 mg/kg), ODN1826 (1.25 mg/kg) or Pam3CSK4 (1.5 mg/kg) for 18h.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

C). The ligands, leading to an activation of HSCs, reflect a quite diverse set of molecules, sensed by different TLRs. An increase in cell cycle activity of HSCs could be detected after treatment with Pam3CSK4, a synthetic lipopeptide, mimicking the amino terminus of LPS, Flagellin, the major component of bacterial flagellar filaments, and ODN1826, a B type CpG ODN mimicking bacterial DNA (Figure 3.7 A and C). These ligands are sensed by TLR2, TLR5 and TLR9 respectively (Takeuchi *et al.*, 2002; Hayashi *et al.*, 2001; Hemmi *et al.*, 2000). The activation of HSCs by these ligands was again accompanied by an increase in Sca-1 expression on the surface of LK CD150$^+$ cells (Figure 3.7 B and D). The treatment with Lipoteichoic acid, Peptidoglycan, ssPolyU, ODN1585 and ODN2395 however, did not lead to an increased cell cycle activity of HSCs (Figure 3.7 A and C). As these ligands are often contaminated with LPS, we treated *TLR4$^{-/-}$* mice with Flagellin, ODN1826 and Pam3CSK4, to exclude that the effect on the proliferation of HSCs is only due to such a contamination. The treatment of *TLR4$^{-/-}$* mice with Flagellin and ODN1826 led to an increased cell cycle activity of HSCs, comparable to wildtype mice. Hence, the activation of HSCs with Flagellin and ODN1826 was not due to a contamination with LPS, but an actual effect of Flagellin and ODN1826 on the cycling behaviour of HSCs. The treatment of *TLR4$^{-/-}$* mice with Pam3CSK4, however, did not lead to an increased cell cycle activity of HSCs, indicating that the Pam3CSK4 solution was contaminated with LPS.

These experiments show that treatment with some, but not all TLR ligands leads to an increase in cell cycle activity of HSCs and an upregulation of Sca-1 on the surface of HSPCs, suggesting a rather specific effect of certain TLR ligands, instead of a general feature of TLR signalling.

3.3. Signalling pathways involved in the LPS-induced activation of HSCs

3.3.1. The LPS-induced activation of HSCs is dependent on TRIF

In contrast to other TLRs, TLR4 signalling can lead to the activation of two different pathways: the MyD88-dependent and the MyD88-independent pathway, via TRIF (Kaisho *et al.*, 2001). To investigate if only one of these pathways, or both, are involved in the LPS-induced activation of HSCs, mice lacking either MyD88 or TRIF were used. Those mice, together with wildtype mice, were treated with either 0.25 mg/kg LPS or 0.025 mg/kg LPS. We used two different doses of LPS, because 0.25 mg/kg is a relatively high dose, as treatment with 12.5 μg/kg LPS was sufficient to induce cycling of HSCs (Figure 3.3 A), and therefore leads to such a strong response that small effects in
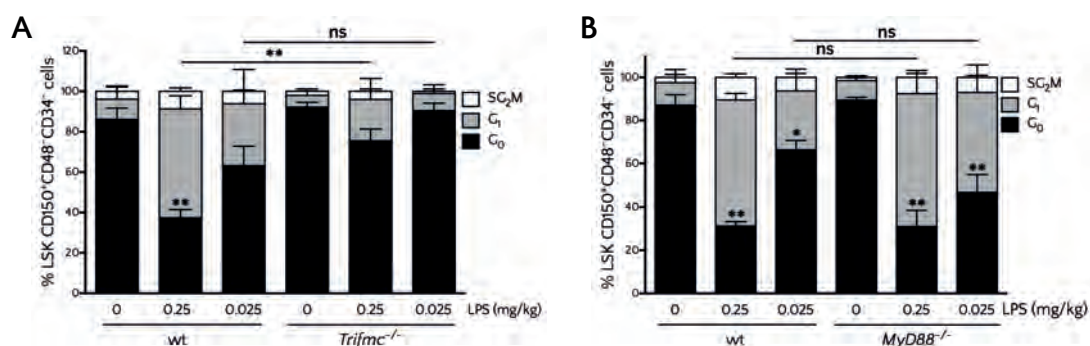
Figure 3.8.: The LPS-induced activation of HSCs is dependent on TRIF
(A) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or *Trifmc*$^{-/-}$ mice after treatment with 0.25 or 0.025 mg/kg LPS (18h) (B) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or *MyD88*$^{-/-}$ mice after treatment with 0.25 or 0.025 mg/kg LPS (18h).
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *$p \leq 0.05$, **$p \leq 0.01$

the knockout models might be not be clear. Treatment of *Trifmc*$^{-/-}$ mice with LPS did not lead to a significant activation of HSCs, independent of the LPS dose used. Nevertheless, the LPS effect on HSCs was completely rescued when the lower dose was used, while a slight, but not significant, increase in cell cycle activity could be observed with 0.25 mg/kg (Figure 3.8 A). In contrast, the treatment of *MyD88*$^{-/-}$ mice with either dose of LPS led to a clear cell cycle activation in HSCs comparable to wildtype mice (Figure 3.8 B), indicating that the LPS-induced activation of HSCs is dependent on TRIF, but not on MyD88. While MyD88 alone is sufficient to induce the production of inflammatory cytokines through most of the TLRs, activation of MyD88, as well as TRIF signalling, has been shown to be necessary for the TLR4-induced production of inflammatory cytokines (reviewed in Akira and Takeda, 2004; Kawai and Akira, 2010; Lester and Li, 2014). It can therefore not be concluded that the LPS-induced effects on HSCs are mediated through TRIF signalling alone, as TRIF signalling is necessary for the MyD88-induced production of cytokines.

3.3.2. The role of NFϰB in the LPS-induced HSC activation

It is known, that TLR4 signalling leads to an activation of NFϰB (reviewed in Kawai and Akira, 2010). To investigate the role of NFϰB in HSCs upon LPS treatment, we first analysed NFϰB activation using p65-GFP mice. These mice express p65 as a fusion protein with GFP, which can be used to detect translocation of NFϰB to the nucleus upon activation (de Lorenzi *et al.*, 2009). The localisation of p65 in LK CD150$^+$CD48$^-$ cells was
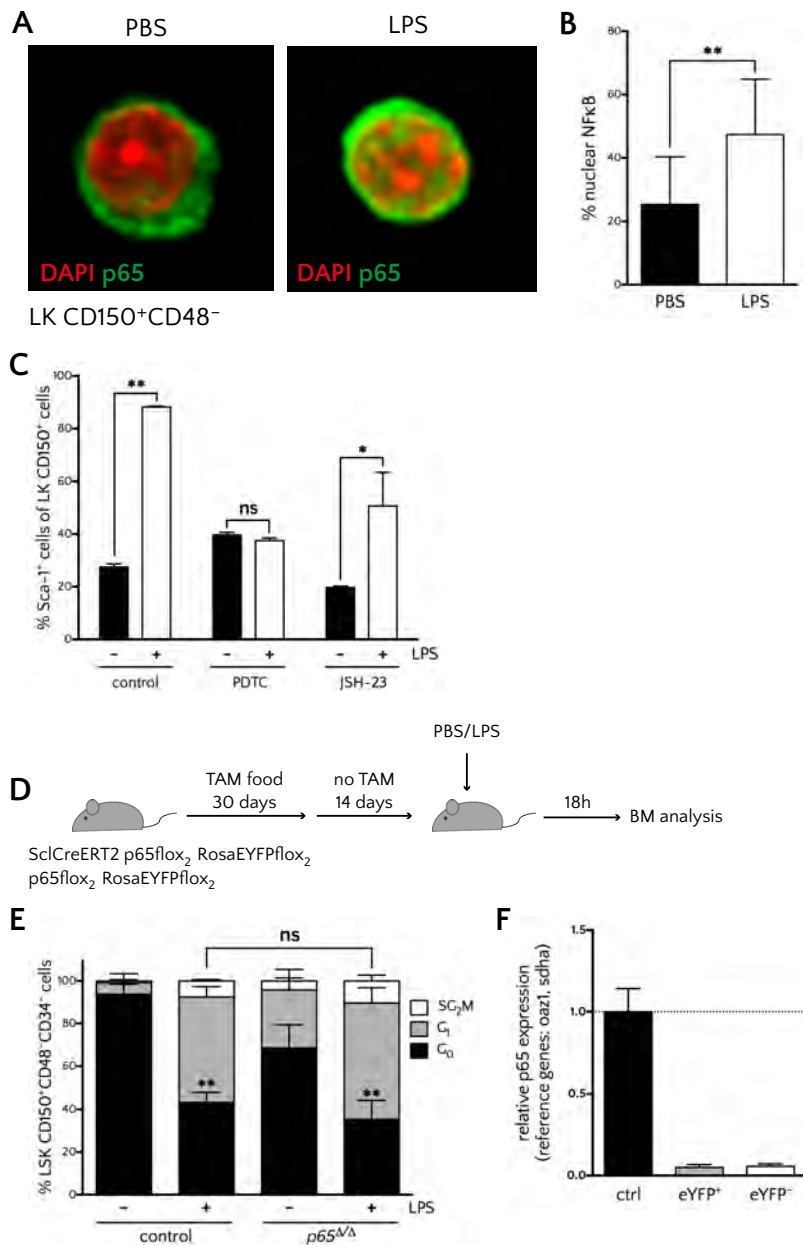
Figure 3.9.: The role of NFκB in the LPS-induced HSC activation
(A) Immunofluorescence staining of sorted LK CD150$^+$CD48$^-$ cells from p65-GFP mice after PBS or LPS (0.25 mg/kg, 16h) treatment on Poly-L-Lysine slides, stained for GFP and with DAPI, recorded with a LSM700 confocal microscope, Z-Stack of representative images made with ImageJ. (B) Quantitative and statistical analysis of colocalisation of GFP and DAPI signal from images described above, calculated with the JaCoP plugin for ImageJ using the Mander's Coefficient. (C) Sca-1 expression of LK CD150$^+$ cells after *in vitro* treatment of lin depl cells with PBS or LPS (100 ng/ml, 20h) in combination with the NFκB inhibitors PDTC or JSH-23. (D) Experimental set-up: SclCreERT2 p65 flox$_2$ RosaEYFP flox$_2$ mice or littermate controls were given Tamoxifen food for 30 days, followed by a Tamoxifen-free period of 2 weeks, after that mice were treated with PBS or LPS. (E) Cell cycle analysis (icKi67 and Hoechst) of HSCs from PBS or LPS (0.25 mg/kg, 18h) treated SclCreERT2 p65 flox$_2$ RosaEYFP flox$_2$ mice (p65$^{\Delta/\Delta}$) or littermate controls (p65 flox$_2$ RosaEYFP flox$_2$). (F) p65 expression in control, eYFP$^+$ and eYFP$^-$ LK CD150$^+$CD48$^-$ cells, measured by qRT-PCR.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

analysed by microscopy. Furthermore, the localisation of p65 was quantified using the JaCoP Plugin for Image J (Abramoff *et al.*, 2004; Bolta and Cordelieres, 2006). After LPS treatment a significantly higher colocalisation of GFP and DAPI could be detected, compared to the PBS treated cells (Figure 3.9 A and B), indicating an activation of NFϰB in HSCs upon LPS treatment.

As the activation of HSCs with LPS was accompanied by an activation of NFϰB signalling in HSCs, further experiments were performed to investigate if the activation of HSCs with LPS is also dependent on NFϰB signalling. First, two different inhibitors of NFϰB signalling were used *in vitro*. For this experiment lineage depleted cells were cultured with PDTC, JSH-23 (Liu *et al.*, 1999; Shin *et al.*, 2004) or without an inhibitor and treated with PBS or LPS (100 ng/ml, 20h). The expression of Sca-1 on the surface of LK CD150$^+$ cells was analysed. After treatment with PDTC, a clear block of the LPS-induced upregulation of Sca-1 could be detected, while the increase in Sca-1 expression was only slightly lower after treatment with JSH-23 compared to the untreated control (Figure 3.9 C). This data suggests that the LPS-induced activation of HSCs might be dependent on NFϰB signalling, however this experiment does not reflect the *in vivo* situation and from this experiment it can not be concluded, if the LPS effect is dependent on NFϰB activation in the HSCs themselves.

We next used p65 flox$_2$ mice to analyse the role of NFϰB *in vivo*. First experiments were performed with the p65 flox$_2$ mice interbred with the MxCre mouse strain. However, the treatment of those mice with polyI:C, to induce the deletion of p65, led to death of the mice within a few days. We therefore tried to transplant bone marrow cells of these mice into lethally irradiated wildtype mice, before inducing Cre expression. However, a stable engraftment of those cells was not achieved (data not shown) and this model could not be used to analyse the role of NFϰB in the LPS-induced activation of HSCs. Thus, p65 flox$_2$ mice were crossed with SclCreERT2 and the RosaEYFP flox$_2$ mouse strain. In these mice Cre is activated upon Tamoxifen treatment. However, as the Cre activation with the SclCreERT2 system is not as effective as with the MxCre model, we crossed in the RosaEYFP flox allele to identify p65-deleted cells. We treated the mice with Tamoxifen for 30 days, followed by a 2 week Tamoxifen-free period, as Tamoxifen itself has been shown to increase the proliferation of HSCs. Subsequently the mice were treated with PBS or LPS (0.25 mg/kg, 18h). Littermate controls without SclCre, were submitted to the same Tamoxifen treatment (Figure 3.9 D). In the PBS treated littermate controls, most of the HSCs could be found in the G$_0$ phase of the cell cycle, indicating that after the Tamoxifen induced activation of HSCs, the cells had returned to their normal homeostatic state of quiescence. In contrast, HSCs from the PBS treated

SclCreERT2 p65 flox$_2$ RosaEYFP flox$_2$ mice showed a slight increase in cell cycle activity, compared to the littermate controls, indicating that the deletion of p65 alone already led to an activation of HSCs. However, after LPS treatment the increase in cell cycle activity was similar between the HSCs from control and p65$^{\Delta/\Delta}$ mice (Figure 3.9 E), suggesting that the deletion of p65 does not have an effect on the LPS-induced activation of HSCs in this setting. Unfortunately, we could not use the eYFP signal to specifically analyse the HSCs, with the most effective p65 deletion, since the eYFP signal did not correspond to the p65 deletion, as p65 was efficiently deleted in eYFP$^+$ as well as eYFP$^-$ cells (Figure 3.9 F).

3.4. The LPS-induced activation of HSCs is indirectly mediated by myeloid cells

3.4.1. LPS induces activation of HSCs via an indirect mechanism

HSCs have been shown to express TLRs, like TLR2 and TLR4, and respond to stimulation with TLR ligands (Nagai *et al.*, 2006; Megías *et al.*, 2012). However, it is not clear whether the LPS-induced proliferation of HSCs is mediated by direct activation of TLR4 signalling in HSCs. Therefore mixed chimeras with 50% wildtype (CD45.1$^+$) and 50% *TLR4$^{-/-}$* (CD45.2$^+$) bone marrow were used (Figure 3.10 A and C) to test whether the LPS-induced activation of HSCs is directly mediated. When a direct activation of TLR4 signalling in HSCs is necessary for the LPS-induced proliferation, the *TLR4$^{-/-}$* cells in the chimeric setting would not respond to the LPS treatment, while the wildtype cells would start to proliferate. However, if the effect of LPS on HSCs is indirect, an activation of TLR4 on the wildtype cells would be enough to also activate the *TLR4$^{-/-}$* HSCs. The chimeras were treated with PBS or LPS for 18 hours and the proliferation of wildtype and *TLR4$^{-/-}$* HSCs was compared. As expected, the wildtype HSCs showed a decrease of cells in the G$_0$ phase after treatment with LPS (Figure 3.10 B). Similarly, the *TLR4$^{-/-}$* HSCs showed an elevated cell cycle activity after LPS treatment in this chimeric setting (Figure 3.10 B), indicating that the LPS-induced activation of HSCs is indirect. Reverse chimeras, in which wildtype bone marrow cells were transplanted into lethally irradiated *TLR4$^{-/-}$* mice, were used to determine whether the indirect effect is mediated by bone marrow or niche cells. As shown in Figure 3.10 D the wildtype HSCs started to proliferate after treatment with LPS in this setting, excluding a direct necessity to activate TLR4 signalling in cells of the bone marrow niche.

An *in vitro* approach was used to confirm the results of the *in vivo* experiments. Bone marrow cells of wildtype mice were sorted for LK CD150$^+$ cells. These cells were taken
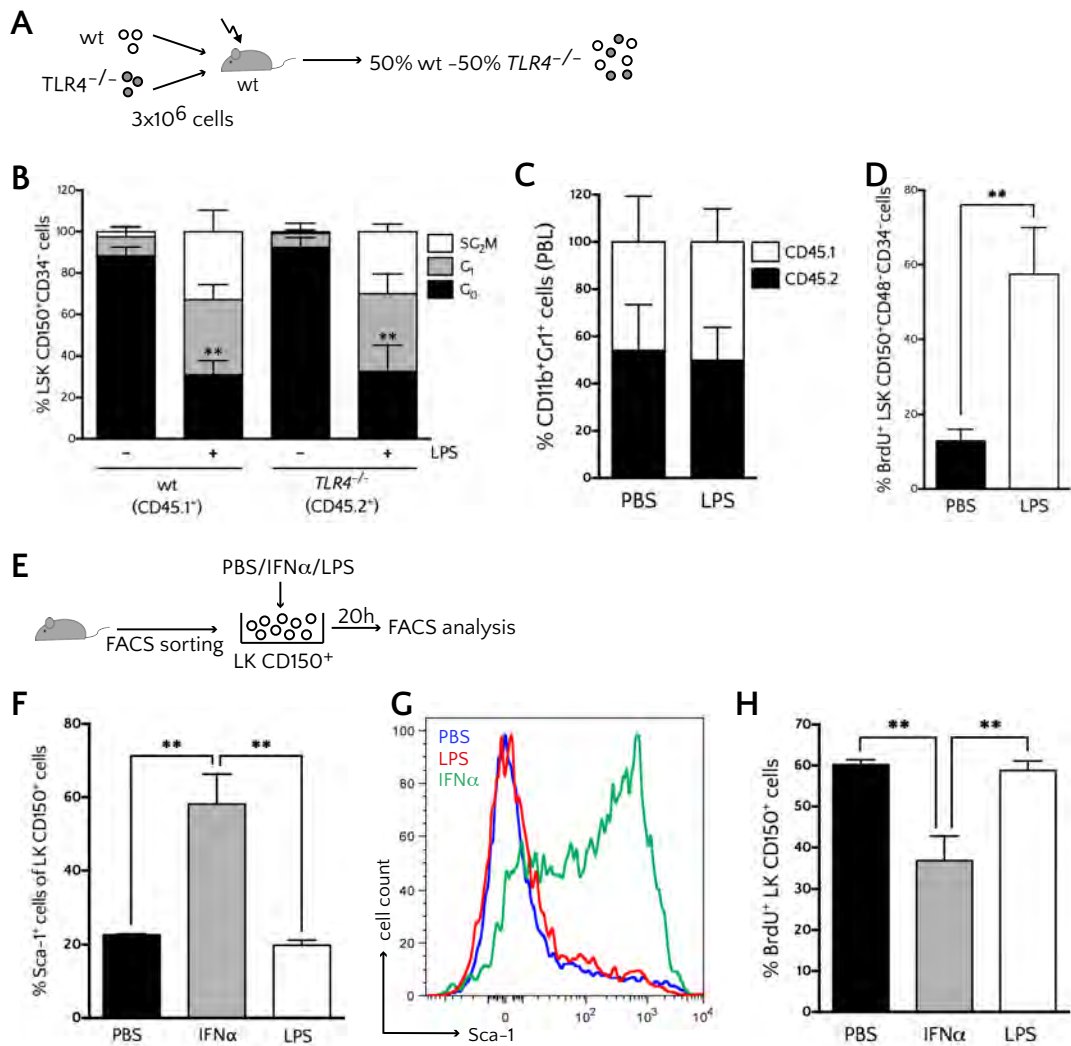
Figure 3.10.: The LPS-induced activation of HSCs is mediated by an indirect mechanism
(A) Experimental set-up of *TLR4*^-/- mixed bone marrow chimeras: a mixture of 50% wt and 50% *TLR4*^-/- bone marrow cells was transplanted into lethally irradiated wt mice. (B) Cell cycle analysis (icKi67 and Hoechst) of HSCs in mixed bone marrow chimeras (50% CD45.1^+ wt – 50% CD45.2^+ *TLR4*^-/-) after treatment with PBS or LPS (0.25 mg/kg, 18h). (C) Percentage of wt (CD45.1, white) or *TLR4*^-/- (CD45.2, black) granulocytes in peripheral blood of mixed bone marrow chimeras after PBS or LPS (0.25 mg/kg, 18h) treatment. (D) BrdU incorporation (14h) in wt HSCs of reverse *TLR4*^-/- chimeras after PBS or LPS (0.25 mg/kg, 18h) treatment. (E) Experimental set-up of *in vitro* experiments: LK CD150^+ bone marrow cells of wildtype mice were sorted, the sorted cells were treated with PBS, IFNα or LPS in culture, the cells were analysed by Flow Cytometry after 20h. (F,G) Sca-1 expression on sorted LK CD150^+ cells after *in vitro* treatment with PBS, IFNα (1000 U/ml) or LPS (100 ng/ml) for 20h. Quantitative and statistical analysis (F) and representative FACS profiles (G). (H) BrdU incorporation (3h) in LK CD150^+ cells after *in vitro* treatment of lin depleted cells with PBS, IFNα (1000 U/ml) or LPS (100 ng/ml) for 20h.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p ≤ 0.05, **p ≤ 0.01

into culture and treated with PBS, IFNα or LPS (Figure 3.10 E). It is known that IFNα has a direct effect on HSCs (Essers *et al.*, 2009), therefore IFNα was used as a positive control in this experiment. The *in vitro* treatment of sorted LK CD150$^+$ cells with IFNα led to a significant increase in Sca-1 expression on the surface of those cells, while the treatment with LPS did not have an effect on the Sca-1 expression (Figure 3.10 F and G). The up-regulation of Sca-1 on the surface of the LK CD150$^+$ cells was used as a readout for all *in vitro* experiments, as the treatment of bone marrow cells with IFNα *in vitro* did not lead to an increase in proliferation of HSPCs comparable to the *in vivo* situation, but rather led to a decrease in proliferation, probably reflecting a necessity of the HSC niche in the activation of HSCs. This decreased proliferation could not be detected after treatment of HSPCs with LPS (Figrue 3.10 H).

These experiments show that LPS-induced activation of HSCs is indirectly mediated via other cells of the bone marrow. Further experiments were performed to identify the type of bone marrow cells mediating the LPS-induced activation of HSCs.

3.4.2. The upregulation of Sca-1 in HSPCs upon LPS treatment is mediated by CD11b$^+$ cells *in vitro*

We performed *in vitro* co-culture experiments, to identify the bone marrow cell type, mediating the LPS-induced activation of HSCs. Therefore, LK CD150$^+$ cells from CD45.1$^+$ mice were sorted and taken into culture, alone or in co-culture with defined populations of bone marrow cells from CD45.2$^+$ mice. The cells were *in vitro* treated with PBS or LPS and the expression of Sca-1 on the HSPCs was used as a readout (Figure 3.11 A). To gain insight into which cell type mediates the effect of LPS on the HSPCs, LK CD150$^+$ cells were co-cultured with lineage^{low} (lin^{low}) or lineage^{high} (lin^{high}) cells. Only after co-culture with lin^{high} cells a Sca-1 upregulation on the surface of HSPCs could be detected (Figure 3.11 B), indicating that the LPS-induced effect is mediated by mature bone marrow cells. Consequently, we tested different populations of mature bone marrow cells, to determine which of these cells is involved in the mediation of the LPS-induced effect. CD11b$^+$ myeloid cells, CD4/CD8$^+$ T cells and NK1.1$^+$ NK cells were used. Sca-1 was upregulated on HSPCs after LPS treatment only in co-culture with CD11b$^+$ myeloid cells (Figure 3.11 C). To confirm that the upregulation of Sca-1 after LPS can only be mediated by CD11b$^+$ cells, LK CD150$^+$ cells were co-cultured with either CD11b$^+$ or CD11b$^-$ cells. Only co-culture with CD11b$^+$ cells led to an upregulation of Sca-1 on the surface of HSPCs (Figure 3.11 D), confirming that only CD11b$^+$, but none of the CD11b$^-$ bone marrow cells, can mediate this effect. To confirm that the LPS-induced upregulation of Sca-1 is mediated by an activation of TLR4 signalling on the CD11b$^+$ myeloid cells and not on the LK
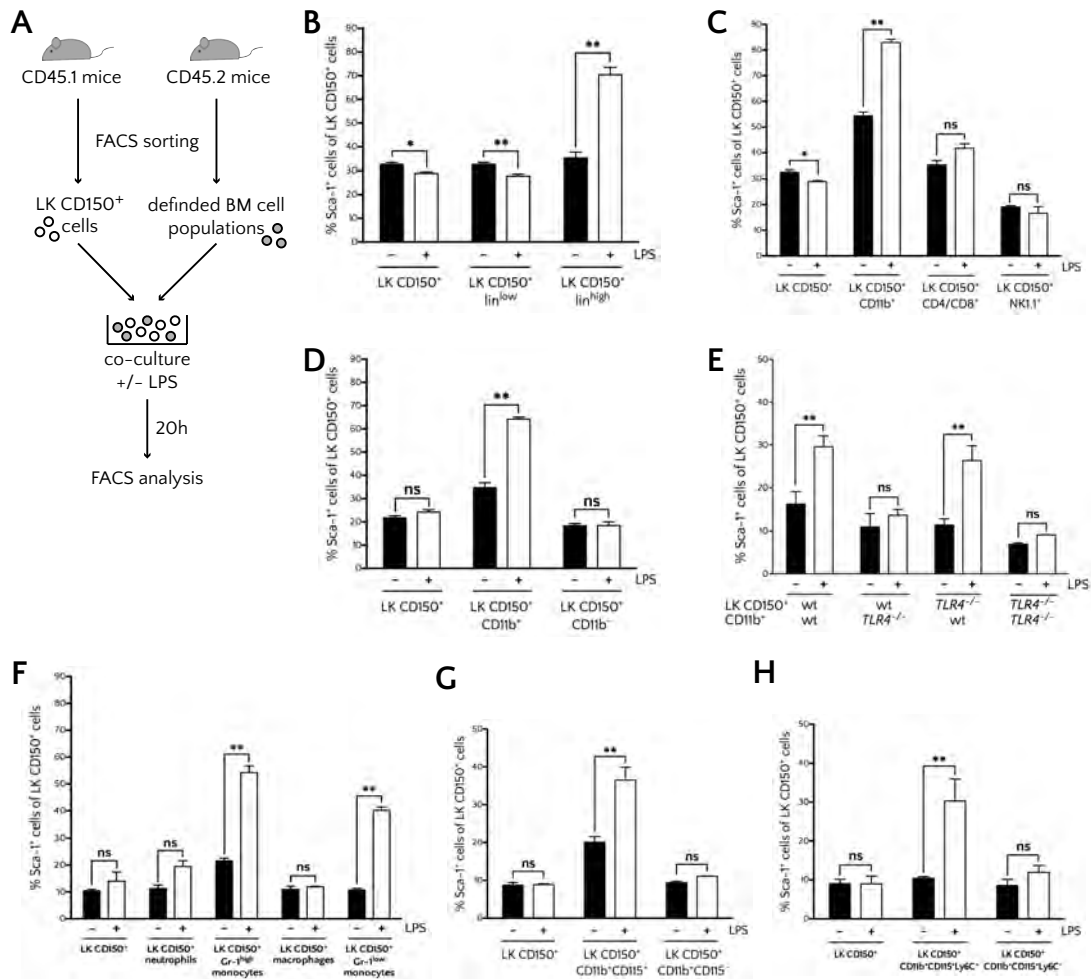
Figure 3.11.: The upregulation of Sca-1 on HSPCs upon LPS treatment is mediated by CD11b⁺ cells *in vitro*
(A) Experimental set-up of *in vitro* co-culture experiments: LK CD150⁺ (HSPCs) from CD45.1⁺ mice were sorted and co-cultured with defined populations of bone marrow cells from CD45.2⁺ mice, the cells were treated with PBS or LPS (100 ng/ml) for 20h. After 20h the cells were harvested and the Sca-1 expression was analysed by Flow Cytometry. (B-H) Sca-1 expression of sorted wt (B-H) or *TLR4*⁻/⁻ (E) LK CD150⁺ cells treated with PBS or LPS (100 ng/ml, 20h) after co-culture with (B) lin^low or lin^high cells, (C) CD11b⁺ myeloid, CD4/CD8⁺ T cells or NK1.1⁺ NK cells, (D) CD11b⁺ or CD11b⁻ cells, (E) wt or *TLR4*⁻/⁻ CD11b⁺ cells, (F) Gr-1⁻CD115⁻F4/80⁺ macrophages, Gr-1⁺CD115⁺ monocytes, Gr-1⁺CD115⁻ neutrophils or Gr-1⁻CD115⁻ monocytes, (G) CD11b⁺CD115⁺ or CD11b⁺CD115⁻ cells and (H) CD11b⁺CD115⁺Ly6C⁺ or CD11b⁺CD115⁺Ly6C⁻ cells.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p ≤ 0.05, **p ≤ 0.01

CD150$^+$ cells, a co-culture experiment with cells from *TLR4$^{-/-}$* mice was performed. An upregulation of Sca-1 could only be detected after co-culture with wildtype CD11b$^+$ cells, while co-culture with *TLR4$^{-/-}$* CD11b$^+$ cells did not have an effect on the Sca-1 expression of HSPCs (Figure 3.11 E). The upregulation of Sca-1 could also be detected when *TLR4$^{-/-}$* HSPCs are co-cultured with wildtype CD11b$^+$ cells (Figure 3.11 E), excluding a necessity of TLR4 activation on HSPCs, demonstrating that the LPS-induced effect on HSPCs is mediated by activation of TLR4 signalling in CD11b$^+$ myeloid cells.

3.4.3. Only CD11b$^+$CD115$^+$Ly6C$^+$ monocytes can mediate the increase in Sca-1 expression *in vitro*

As CD11b is expressed on several types of bone marrow cells, including monocytes, macrophages, neutrophils and NK cells (reviewed in Solovjov *et al.*, 2005), further experiments were performed to understand the LPS effect on HSPCs *in vitro*. With the data shown in Figure 3.11 C it has already been excluded that the effect is mediated by NK cells, as the co-culture of HSPCs with NK cells did not lead to an upregulation of Sca-1 in response to LPS. Therefore, the role of macrophages, monocytes and neutrophils was further evaluated. For this purpose a gating scheme established by Chow *et al.* was used to differentiate between neutrophils, macrophages, Gr-1^{high} and Gr-1^{low} monocytes (for gating scheme see Figure 5.2 A) (Chow *et al.*, 2011). In this experiment, only HSPCs that were co-cultured with monocytes, independent of their Gr-1 expression, showed an upregulation of Sca-1 (Figure 3.11 F). Since monocytes are defined as CD115$^+$ cells in the gating scheme used, it was further confirmed that only CD115$^+$, but not CD115$^-$ cells can mediate the LPS-induced upregulation of Sca-1 on the surface of HSPCs (Figure 3.11 G). These results indicate that the LPS-induced effect on HSPCs *in vitro* is mediated by CD11b$^+$CD115$^+$ monocytes. Monocytes can be further subdivided into two populations by Ly6C expression. Ly6C^{high} monocytes are defined as inflammatory monocytes, while Ly6C^{low} cells are termed resident monocytes (Geissmann *et al.*, 2003; Gordon and Taylor, 2005). To investigate, if the LPS effect is mediated by either of the two monocyte types or by both types, LK CD150$^+$ cells were co-cultured with CD11b$^+$CD115$^+$Ly6C^{high} or CD11b$^+$CD115$^+$Ly6C^{low} cells (for gating scheme see Figure 5.2 B). An increase in Sca-1 expression was only detected after co-culture with Ly6C^{high} monocytes, but not with Ly6C^{low} cells (Figure 3.11 H), suggesting that the LPS-induced effect is mediated by inflammatory monocytes *in vitro*.

In summary, these experiments show that only Ly6C^{high} inflammatory monocytes can mediate the LPS-induced upregulation of Sca-1 *in vitro*. However, these experiments

were performed *in vitro*, using Sca-1 upregulation as a readout, and this had to be further supported by *in vivo* experiments.

3.4.4. The LPS-induced activation of HSCs is mediated by myeloid cells *in vivo*

Three *in vivo* approaches were used to deplete CD11b⁺ cells from the bone marrow, to investigate whether the LPS-induced increase in proliferation of HSCs *in vivo* is also mediated by myeloid cells. A depletion of neutrophils was achieved by twice treating wildtype mice with an anti-Ly6G (1A8) antibody (Figure 3.12 A) (Casanova-Acebes *et al.*, 2013). This treatment led to a nearly complete depletion of neutrophils from the bone marrow (Figure 3.12 C). However, the depletion of neutrophils prior to the LPS treatment did not have an effect on the LPS-induced proliferation of HSCs (Figure 3.12 B). This result, together with the *in vitro* data (Figure 3.11 F), excludes a role for neutrophils in the LPS-induced activation of HSCs. To analyse the role of CD11b⁺ monocytes and macrophages in the LPS-induced activation of HSCs *in vivo* two different approaches were tested. For the first approach we used the macrophage Fas-induced apoptosis (Mafia) mouse model (Burnett *et al.*, 2004). These mice express a drug-inducible suicide gene under the control of the c-fms (CD115) promotor. Upon treatment with the homodimerizer AP20187 apoptosis is induced in all CD115 expressing cells. As *in vitro* experiments indicate a role for CD115⁺ cells in the activation of HSCs (Figure 3.11 F and G), the Mafia mouse model could be used to show if these cells are also involved in the LPS-induced activation of HSCs *in vivo*. Unfortunately, the treatment of Mafia mice with AP20187 led to mobilisation of HSCs into the bloodstream accompanied by an increased cycling of HSCs in the bone marrow (data not shown). For that reason, those mice could not be used to investigate the role of CD115⁺ cells in the LPS-induced cell cycle induction in HSCs *in vivo*. As a second model we used clodronate-loaded liposomes to deplete phagocytic cells from the bone marrow (van Rooijen *et al.*, 1997). These liposomes are not membrane-permeable and are therefore only taken up by phagocytic cells. The accumulation of clodronate within these cells leads to apoptosis (Figure 3.12 D). Thus, wildtype mice were treated once i.v. with clodronate- or PBS-loaded liposomes. The depletion of myeloid cells after clodronate treatment occurs rather fast, within 12 hours and is accompanied by a mobilisation of HSCs from the bone marrow to the periphery (Chow *et al.*, 2011). Therefore, the LPS treatment was performed 5 days after the clodronate treatment, when a significant decrease in bone marrow macrophages could still be detected, while the proliferation of bone marrow HSCs was not affected (Figure 3.12 E, F and G). The proliferation of HSCs was analysed by icKi67 and Hoechst staining. While HSCs from PBS treated mice did not show any difference in cell cycle activity
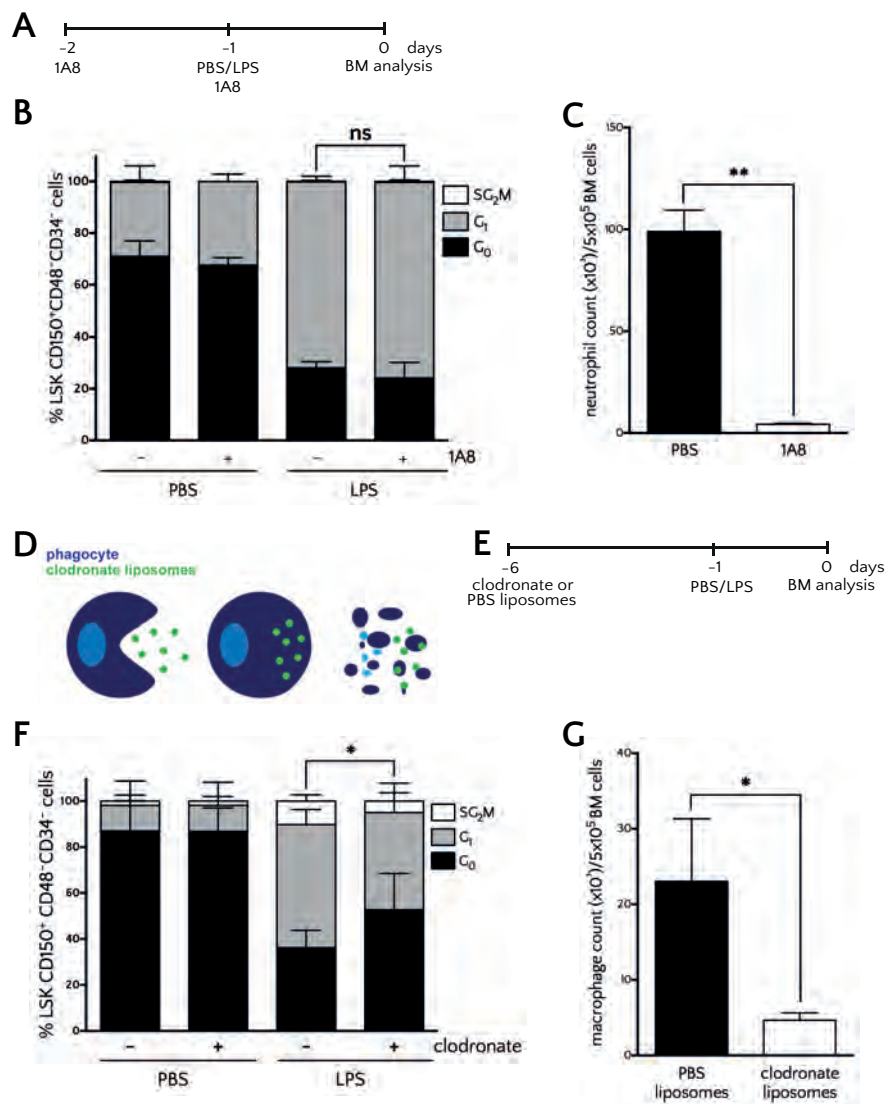
Figure 3.12.: The LPS-induced activation of HSCs is mediated by myeloid cells *in vivo*
(A) Schematic overview of neutrophil depletion experiment: mice were treated twice with the anti-Ly6G antibody 1A8 on two following days, the LPS treatment was performed 6h after the second 1A8 treatment, cycling activity of BM HSCs was analysed after 18 h. (B) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wildtype mice, treated with PBS or 1A8 to deplete neutrophils, after PBS or LPS (0.25 mg/kg, 18h) treatment. (C) Neutrophil count in the bone marrow after treatment with PBS or 1A8 (2 times, 2.5 mg/kg). (D) Effect of clodronate-loaded liposomes: the liposomes are not membrane-permeable and therefore only taken up by phagocytic cells, upon clodronate accumulation the cells undergo apoptosis. (van Rooijen *et al.*, 1997) (E) Schematic overview of clodronate treatment: mice were treated with PBS- or clodronate-loaded liposomes 5 days before the LPS treatment, BM analysis was performed 18 h after the LPS treatment (F) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt mice treated with PBS- or clodronate-loaded (3.75 g/kg) liposomes 5 days before treatment with PBS or LPS (0.25 mg/kg, 18h). (G) Macrophage count in the bone marrow after treatment with PBS- or clodronate-loaded (3.75 g/kg) liposomes 5 days after treatment. Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *$p \leq 0.05$, **$p \leq 0.01$

after clodronate treatment, a significant difference could be detected in the cell cycle activity of HSCs after LPS treatment between the PBS and clodronate liposome treated mice (Figure 3.12 F).

These data suggest a role of myeloid cells in the *in vivo* effect of LPS on the proliferation of HSCs, even though the depletion of myeloid cells with clodronate-loaded liposomes did not lead to a complete rescue of the LPS effect.

3.5. The activation of HSCs with LPS is mediated by IFN and TNFα signalling

3.5.1. Upon LPS treatment soluble factors are produced that can mediate the activation of HSCs *in vitro*

Having shown that the LPS-induced effects on HSCs are indirectly mediated by myeloid cells, we asked whether this activation is mediated through cell-cell-interaction, the production of soluble factors, or both. To investigate if cell-cell-interaction is necessary for the LPS-induced upregulation of Sca-1 *in vitro*, a transwell assay was performed (Figure 3.13 A). After 24 hours of culture the cells were harvested and the expression of Sca-1 was analysed by FACS. Only in the control setting, when the HSPCs and the myeloid cells were in contact, an upregulation of Sca-1 could be detected (Figure 3.13 B), suggesting that cell-cell-interaction is necessary to mediate the increase in Sca-1 expression upon LPS *in vitro*. Lineage depleted cells were cultured with PBS or LPS to produce conditioned medium and analyse whether soluble factors are produced upon LPS treatment *in vitro*, which can mediate the upregulation of Sca-1 on HSPCs. The resulting conditioned medium was used to treat either wildtype or *TLR4$^{-/-}$* lineage depleted cells *in vitro* (Figure 3.13 C). The *TLR4$^{-/-}$* cells were used as a control to exclude an effect solely by the LPS that might still be present in the conditioned medium. The wildtype, as well as the *TLR4$^{-/-}$* cells, showed a significant increase in Sca-1 expression after treatment with conditioned medium (Figure 3.13 D), indicating that soluble factors are produced upon LPS treatment *in vitro*, which can mediate the effect on the expression of Sca-1 on the surface of HSPCs. However, this experiment does not exclude that initial cell-cell-interaction could be necessary to promote this production of soluble factors, as indicated by the transwell experiment (Figure 3.13 B).

These experiments show that after initial cell-cell-interaction between HSPCs and myeloid cells *in vitro*, LPS leads to the production of soluble factors, which alone can mediate the increase in Sca-1 expression on the surface of HSPCs. Hence, further experiments were performed to figure out which factors are produced upon LPS treatment in
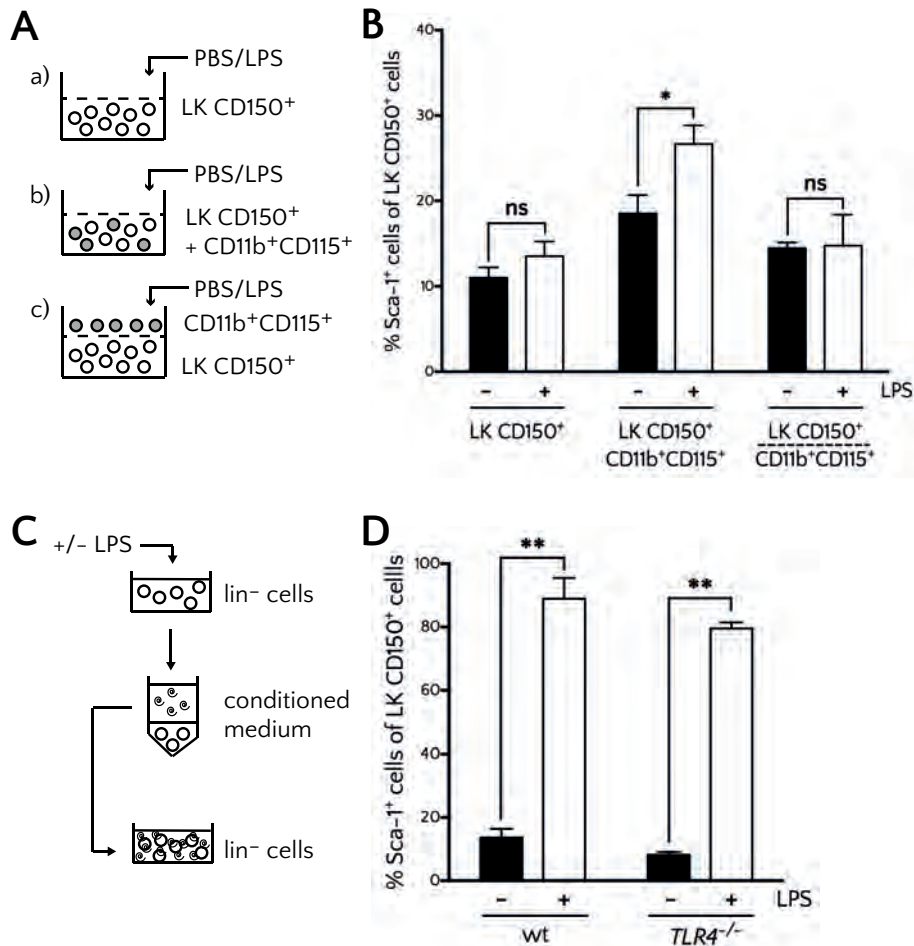
Figure 3.13.: The LPS-induced effect is mediated by soluble factors *in vitro*
(A) Experimental set-up: LK CD150+ cells were sorted into the bottom well of a transwell plate and cultured either seperately or in co-culture with CD11b+CD115+ cells either in the same well or seperated through a 0.4 μm thick filter (dotted line), cells were treated with PBS or LPS (100 ng/ml, 24h, upper well). The LPS was pipetted into the upper well, to ensure that the travelling of soluble factors through the membrane was possible. (B) Sca-1 expression of sorted LK CD150+ cells after culture in transwell plate. (C) Experimental set-up: conditioned medium was produced by treatment of lin depl cells with PBS or LPS (100 ng/ml, 18h) and subsequent isolation of supernatant, the conditioned medium was then used to treat wt or *TLR4*-/- lin depl cells for 18h. (D) Sca-1 expression of wt or *TLR4*-/- LK CD150+ cells after *in vitro* treatment of lin depl cells with conditioned medium.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p ≤ 0.05, **p ≤ 0.01

the bone marrow and which of those factors are mediating the LPS-induced activation of HSCs *in vivo*.

3.5.2. The LPS-induced activation of HSCs is in part mediated by IFN signalling

Obvious candidates for cytokines produced upon LPS treatment are IFNα and IFNγ, which have already been shown to lead to an increased proliferation of HSCs *in vivo* (Essers *et al.*, 2009; Baldridge *et al.*, 2010). Therefore, an ELISA was performed to measure the expression of IFNα and IFNγ in the bone marrow after treatment of wildtype mice with LPS (0.25 mg/kg). IFNα, as well as IFNγ were significantly increased in the bone marrow supernatant 4 hours after LPS treatment (Figure 3.14 A and B). As both IFNα and IFNγ were upregulated in the bone marrow supernatant following LPS treatment, the obvious assumption was that the LPS-induced effect on the proliferation of HSCs is mediated by IFN signalling. Therefore, mice lacking either the receptor for IFNα (*Ifnar*$^{-/-}$) or the receptor for IFNγ (*Ifngr*$^{-/-}$) were treated with 0.25 mg/kg or 0.025 mg/kg LPS for 18 hours. After treatment, the proliferation behaviour of HSCs from those mice was analysed by FACS and compared to wildtype mice. The HSCs of both knockout mice showed a significant increase in proliferation after treatment with both doses of LPS (Figure 3.14 C), indicating that neither IFNα nor IFNγ alone is mediating the LPS effect. Nevertheless, it is likely that the IFNs can compensate for each other in the knockout setting. Thus, a double-knockout mouse model for both IFN receptors (*Ifnar*$^{-/-}$*Ifngr*$^{-/-}$) was established. The experiment described above was repeated with these double-knockout mice. Upon treatment with 0.25 mg/kg of LPS, the HSCs of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ showed a cycling behaviour similar to the HSCs of wildtype mice. However, after treatment with 0.025 mg/kg LPS, the effect on the proliferation of HSCs could be completely rescued (Figure 3.14 D). Interestingly, the upregulation of Sca-1 in the *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice was lower compared to wildtype mice after treatment with 0.25 mg/kg of LPS, although the cell cycle induction is comparable (Figure 3.14 E). These results imply that the LPS-induced activation is not completely dependent on, but in part mediated by IFN signalling. Hence, there might be other cytokines produced upon LPS that can mediate the effects on HSCs. To show the production of cytokines, apart from IFNs, the conditioned medium experiment, described above, was repeated using wildtype and *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ cells (Figure 3.13 C). Although the increase in Sca-1 expression was lower after *in vitro* LPS treatment of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ lin depl cells compared to wildtype cells, the increase in Sca-1 expression after treatment with conditioned medium was comparable to the control setting, were the cells were directly treated with LPS (Figure 3.14 F), suggesting cytokines apart from IFNs, able to mediate the effects of LPS on HSPCs.
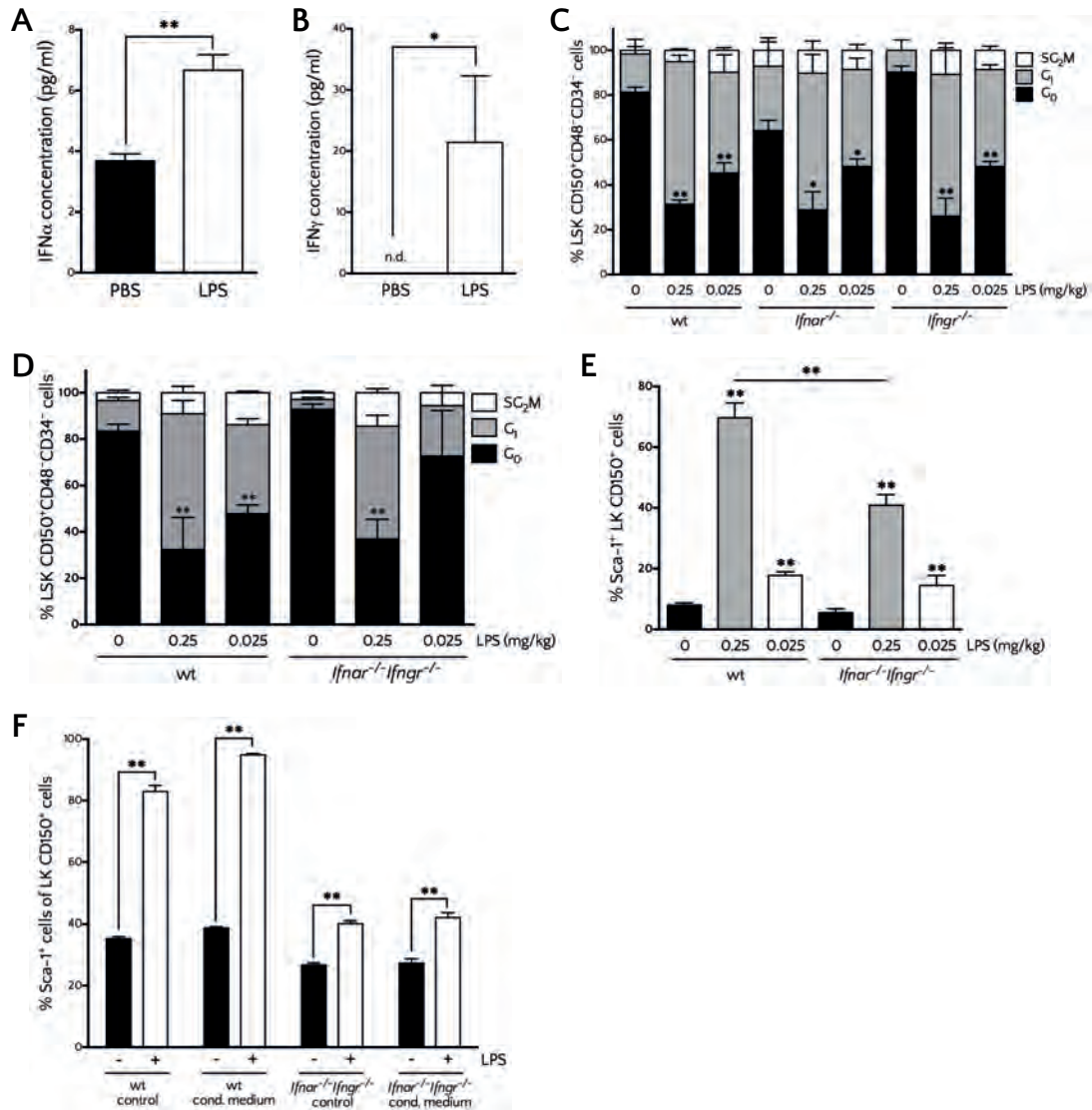
Figure 3.14.: The LPS-induced activation of HSCs is in part mediated by IFN signalling

(A) IFNα and (B) IFNγ levels (ELISA) in BM supernatant after *in vivo* treatment with PBS or LPS (0.25 mg/kg, 4h). (C) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt, *Ifnar*$^{-/-}$ or *Ifngr*$^{-/-}$ mice after treatment with PBS or LPS (0.25 mg/kg or 0.025 mg/kg, 18h). (D) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice after treatment with PBS or LPS (0.25 mg/kg or 0.025 mg/kg, 18h). (E) Sca-1 expression of LK CD150$^+$ cells from wt or *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice after treatment with PBS or LPS (0.25 mg/kg or 0.025 mg/kg, 18h). (F) Sca-1 expression of wt or *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ LK CD150$^+$ cells after *in vitro* treatment of lin depl cells with conditioned medium, obtained from wt cells, or direct treatment with PBS or LPS (control).

Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

3.5.3. The LPS-induced activation of HSCs *in vivo* is not mediated by IL-6 or CXCL10

To investigate which cytokines, apart from IFNs, are involved in the LPS-induced activation of HSCs, we analysed the expression of LPS inducible cytokines in the bone marrow of LPS treated wildtype mice (Björkbacka *et al.*, 2004). The expression of Interleukin 6 (IL-6) was highly induced upon treatment with LPS (Figure 3.15 A) and treatment of wildtype mice with IL-6 led to a slight increase in Ki-67 expression in HSCs (Figure 3.15 B), indicating that IL-6 might be a cytokine involved in the LPS-induced activation of HSCs. Nevertheless, treatment of *IL-6$^{-/-}$* mice with LPS did not lead to a reduced cell cycle induction in HSCs compared to wildtype mice, if anything the *IL-6$^{-/-}$* HSCs seemed to cycle even more compared to the wildtype HSCs (Figure 3.15 C). These data indicate that IL-6 is most likely not involved in the LPS effect on HSCs.

Another cytokine typically produced upon LPS treatment is C-X-C motif chemokine 10 (CXCL10) (Björkbacka *et al.*, 2004). We therefore performed an ELISA, analysing the bone marrow supernatant of LPS treated wildtype mice for CXCL10 expression. CXCL10 was highly upregulated in the bone marrow supernatant upon LPS treatment (Figure 3.15 D). Again, we treated mice with CXCL10 and analysed the BrdU incorporation of HSCs. Although, there seemed to be a slight increase in proliferation of HSCs upon CXCL10, this increase was not significant (Figure 3.15 E). Nevertheless, we analysed the cell cycle induction in Cxcr3-deficient (*Cxcr3$^{-/-}$*) mice after LPS treatment, to fully exclude a role of CXCL10 in the LPS-induced activation of HSCs. Apart form CXCL10, Cxcr3 serves as a receptor for CXCL9 and CXCL11. Treatment of *Cxcr3$^{-/-}$* mice with either 0.25 or 0.025 mg/kg LPS led to a significant increase in cell cycle activity of HSCs comparable to wildtype mice (Figure 3.15 F), excluding a role of CXCL10, CXCL9 and CXCL11 in the LPS-induced activation of HSCs.

3.5.4. The LPS-induced activation of HSCs is dependent on TNFα

Tumor necrosis factor alpha (TNFα) is typically produced upon LPS treatment (Björkbacka *et al.*, 2004). In the bone marrow supernatant of LPS treated mice the expression of TNFα was induced, as determined by ELISA (Figure 3.16 A). To investigate whether the LPS-induced activation of HSCs is dependent on TNFα, we treated wildtype and *Tnfrsf1 dKO* mice, lacking both receptors for TNFα (Pronk *et al.*, 2011), with 0.25 or 0.025 mg/kg of LPS and analysed the cell cycle behaviour of HSCs. After treatment with the relatively high dose of 0.25 mg/kg the HSCs of *Tnfrsf1 dKO* mice show a cell cycle induction comparable to wildtype HSCs. However, after reducing the dose to 0.025 mg/kg of LPS, the effect on the cycling of HSCs was rescued in the *Tnfrsf1 dKO* mice (Figure 3.16 B), indicat-
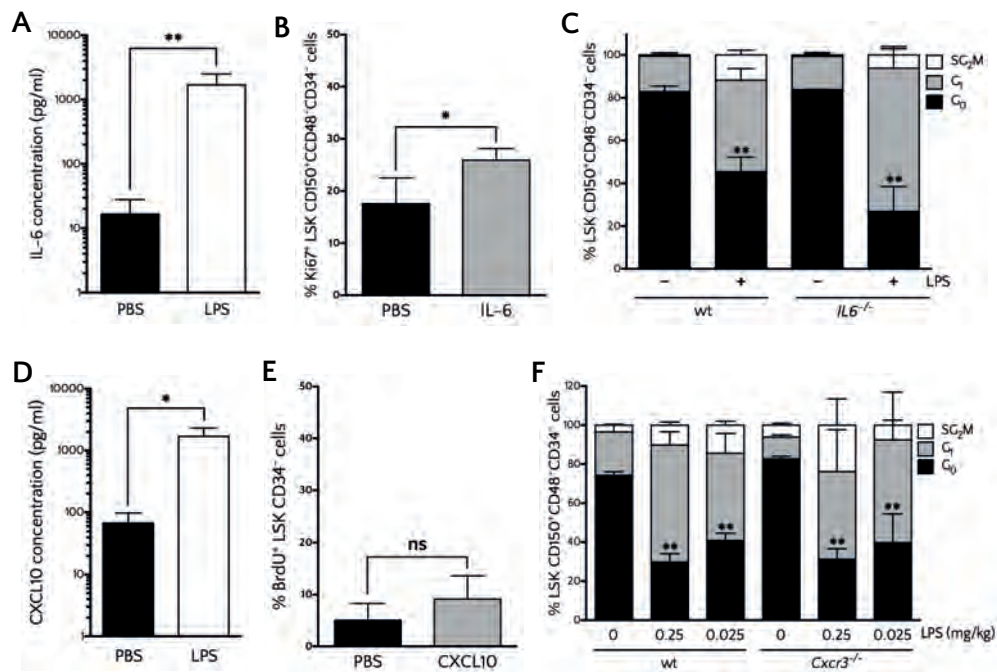
Figure 3.15.: The LPS-induced activation of HSCs *in vivo* is not mediated by IL-6 or CXCL10
(A) IL-6 levels (ELISA) in BM supernatant after *in vivo* treatment with PBS or LPS (0.25 mg/kg, 4h). (B) Ki-67 expression in HSCs after treatment of wt mice with PBS or IL-6 (0.05 mg/kg, 18h). (C) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or *IL-6$^{-/-}$* mice after treatment with PBS or LPS (0.25 mg/kg, 18h). (D) CXCL10 levels (ELISA) in BM supernatant after *in vivo* treatment with PBS or LPS (0.25 mg/kg, 4h). (E) BrdU incorporation (14h) in wt HSCs after PBS or CXCL10 (0.5 mg/kg, 18h) treatment. (F) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or *Cxcr3$^{-/-}$* mice after treatment with PBS or LPS (0.25 mg/kg or 0.025 mg/kg, 18h).
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *$p \leq 0.05$, **$p \leq 0.01$

ing that the LPS-induced activation of HSCs is in part dependent on TNFα. Comparable to *Ifar$^{-/-}$Ifngr$^{-/-}$* (Figure 3.14 E), the increase in Sca-1 expression upon LPS, was lower in *Tnfrsf1 dKO* mice in comparison to wildtype mice (Figure 3.16 C).

3.6. TNFα-induced activation of HSCs

3.6.1. TNFα activates HSCs to enter an active cell cycle

As the LPS-induced activation of HSCs is impaired in *Tnfrsf1 dKO* mice, we asked whether TNFα alone would lead to an activation of HSCs. Wildtype and *Tnfrsf1 dKO* mice were treated with 0.75 mg/kg of TNFα and the cell cycle activity of HSCs as well as the incorporation of BrdU was analysed. Upon treatment with TNFα wildtype HSCs
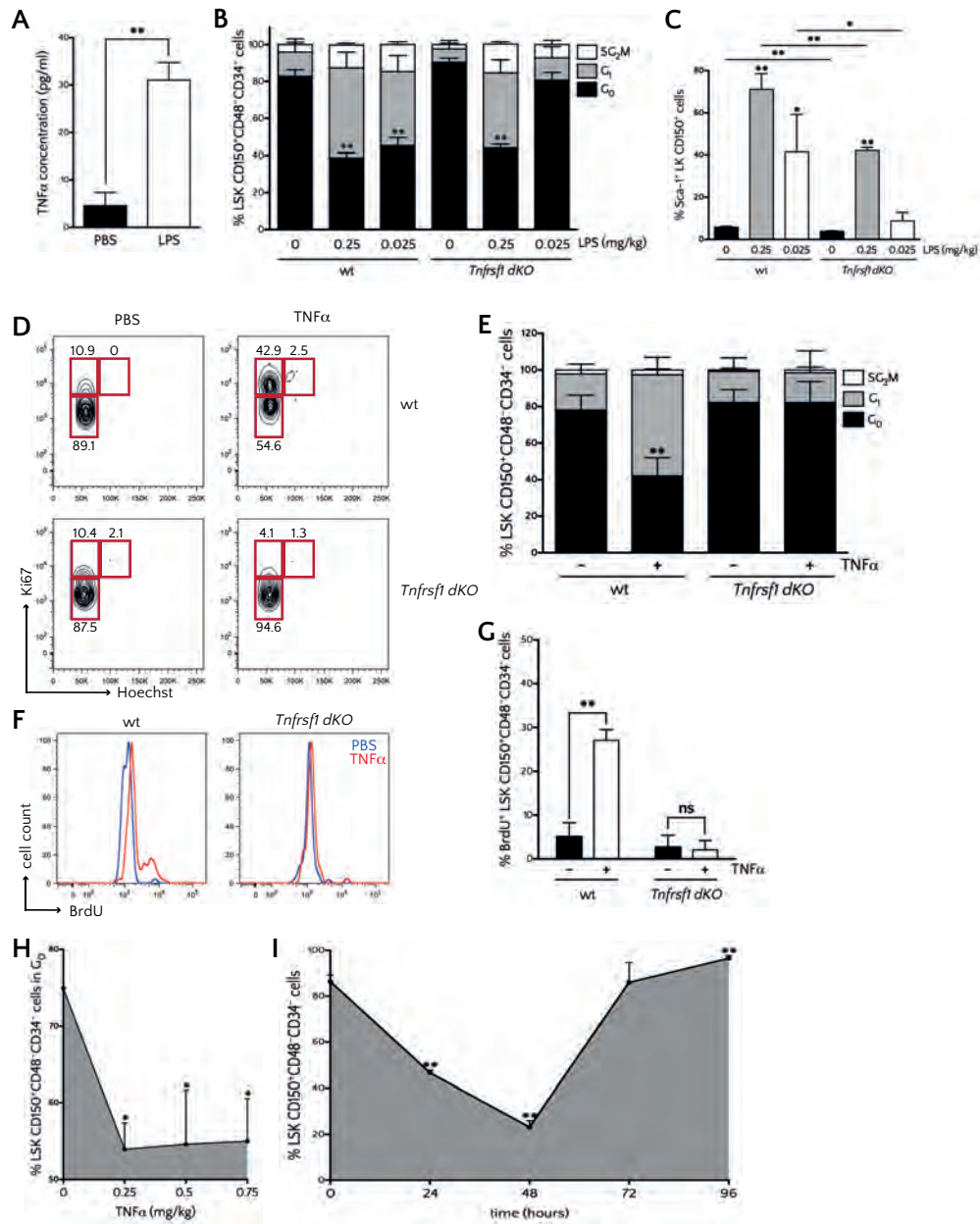
Figure 3.16.: TNFα-induced activation of HSCs *in vivo*
(A) TNFα levels (ELISA) in BM supernatant after *in vivo* treatment with PBS or LPS (0.25 mg/kg, 4h). (B) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or *Tnfrsf1 dKO* mice after treatment with PBS or LPS (0.25 mg/kg or 0.025 mg/kg, 18h). (C) Sca-1 expression on LK CD150$^+$ cells after treatment of wt or *Tnfrsf1 dKO* mice with PBS or LPS (0.25 mg/kg or 0.025 mg/kg, 18h). (D,E) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or *Tnfrsf1 dKO* mice after treatment with PBS or TNFα (0.75 mg/kg, 18h). Representative FACS profiles (D) and quantitative and statistical analysis (E). (F,G) BrdU incorporation of HSCs from wt or *Tnfrsf1 dKO* mice after treatment with PBS or TNFα (0.75 mg/kg, 18h). Representative FACS profiles (F) and quantitative and statistical analysis (G). (H) HSCs in G_0 after treatment of wt mice with different doses of TNFα (18h). (I) HSCs in G_0 after treatment of wt mice with 0.75 mg/kg TNFα at different time points.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

exited the G_0 phase of the cell cycle and entered the G_1, S, G_2 or M phase (Figure 3.16 D and E). HSCs were also actively proliferating, shown by an increased BrdU incorporation when compared to PBS treated mice (Figure 3.16 F and G). As expected, this increased cell cycle activity of HSCs was dependent on the expression of the TNF receptors and was therefore inhibited in the *Tnfrsf1 dKO* mice (Figure 3.16 D-G). This activation of HSCs with TNFα can be induced with doses of 0.25 mg/kg (Figure 3.16 H). Comparable to the treatment with LPS (Figure 3.3 B), the activation of HSCs with TNFα was transient, with a peak at 24 hours and the return to homeostasis after 72 hours (Figure 3.16 I). In summary, these results show that TNFα treatment leads to a TNF receptor-dependent transient activation of HSCs into an active cell cycle.

3.6.2. The TNFα-induced activation of HSCs is dependent on Sca-1

The activation of HSCs with LPS as well as IFNα is accompanied by upregulation of Sca-1 and dependent on the presence of Sca-1. To ascertain if this is also the case for the activation of HSCs with TNFα, expression of Sca-1 on the surface of TNFα treated wildtype and *Tnfrsf1 dKO* LK CD150$^+$ cells was analysed. Treatment of wildtype mice with TNFα led to a significant increase of Sca-1 expression on LK CD150$^+$ cells, while an increase could not be detected in *Tnfrsf1 dKO* mice (Figure 3.17 A and B). The increase in Sca-1 expression was lower when compared to the treatment with LPS (Figure 3.6 B and C). To investigate whether the activation of HSCs with TNFα is also dependent on Sca-1, we treated wildtype and *Sca-1$^{-/-}$* mice with TNFα and analysed the proliferation of HSCs. LK CD150$^+$CD48$^-$ cells of wildtype mice showed a significant increase in proliferation upon TNFα treatment. However, an increase in BrdU incorporation could not be detected in *Sca-1$^{-/-}$* LK CD150$^+$CD48$^-$ cells (Figure 3.17 C), indicating that the activation of HSCs with TNFα is dependent on Sca-1, despite a lower increase in Sca-1 expression upon TNFα treatment.

3.6.3. TNFα activates dormant HSCs through an indirect mechanism

TNFα alone can activate HSCs to proliferate in a TNF receptor and Sca-1 dependent manner. Thus, we asked whether this activation is mediated by direct TNFα signalling in the HSCs or via an indirect mechanism. Wildtype (CD45.1) and *Tnfrsf1 dKO* (CD45.2) bone marrow cells were transplanted into lethally irradiated wildtype mice in a 50:50 ratio (Figure 3.18 A). It has already been shown that the *Tnfrsf1 dKO* cells have a competitive advantage over wildtype cells in transplantation assays (Pronk *et al.*, 2011) and therefore the ratio between wildtype and *Tnfrsf1 dKO* HSCs was approximately 20:80 at the time of analysis (Figure 3.18 C). We treated these mixed chimeras with TNFα and analysed the
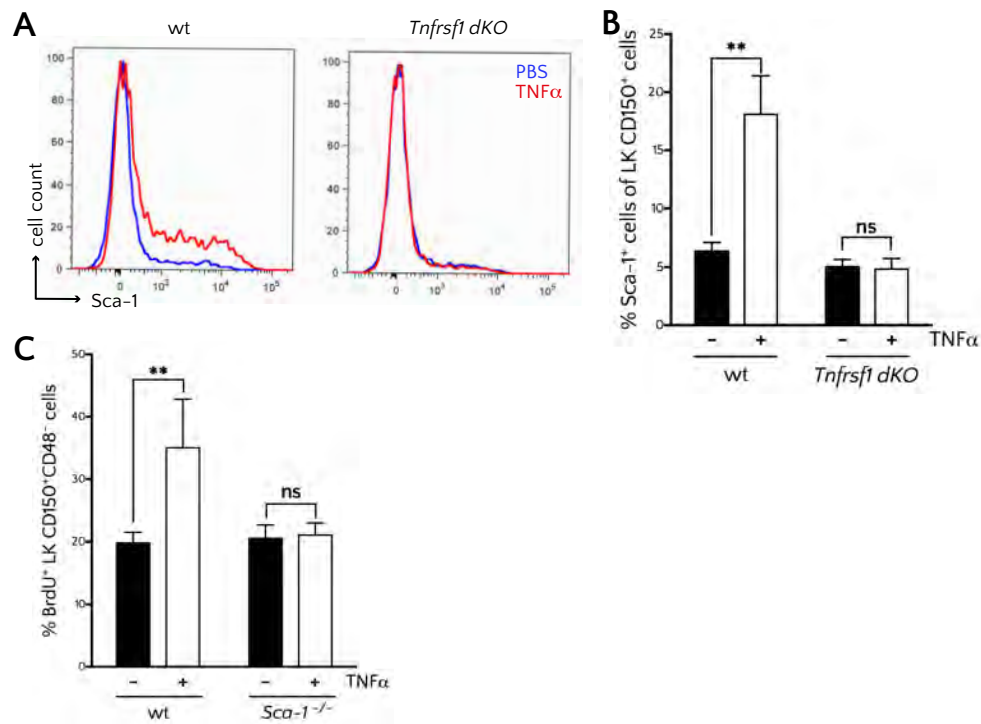
Figure 3.17.: The TNFα-induced activation of HSCs is dependent on Sca-1
(A,B) Sca-1 expression on the surface of LK CD150$^+$ cells after treatment of wt or *Tnfrsf1 dKO* mice with PBS or TNFα (0.75 mg/kg, 18h). Representative FACS profiles (A) and quantitative and statistical analysis (B). (C) BrdU incorporation (14h) in LK CD150$^+$CD48$^-$ cells after treatment of wt or *Sca-1$^{-/-}$* mice with PBS or TNFα (0.75 mg/kg, 18h).
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

cell cycle behaviour of wildtype and *Tnfrsf1 dKO* HSCs. As expected, the wildtype HSCs exited the G$_0$ state of the cell cycle upon treatment with TNFα. However, the *Tnfrsf1 dKO* HSCs also showed an increased cell cycle activity upon TNFα in this setting (Figure 3.18 B), suggesting that the TNFα-induced activation of HSCs is at least in part mediated via an indirect mechanism. To investigate the role of the bone marrow niche in the TNFα-induced activation of HSCs, wildtype bone marrow cells were transplanted into lethally irradiated *Tnfrsf1 dKO* mice, giving reverse chimeras. After 16 weeks these mice were treated with PBS or TNFα and the cell cycle activity of HSCs was analysed by icKi67 and Hoechst staining. In this setting, wildtype cells had a normal cell cycle induction (Figure 3.18 D), excluding a direct necessity of TNFα signalling in niche cells for the activation of HSCs. The finding that TNFα-induced activation of HSCs is at least in part mediated by an indirect mechanism raises the question of which factors are directly acting on the
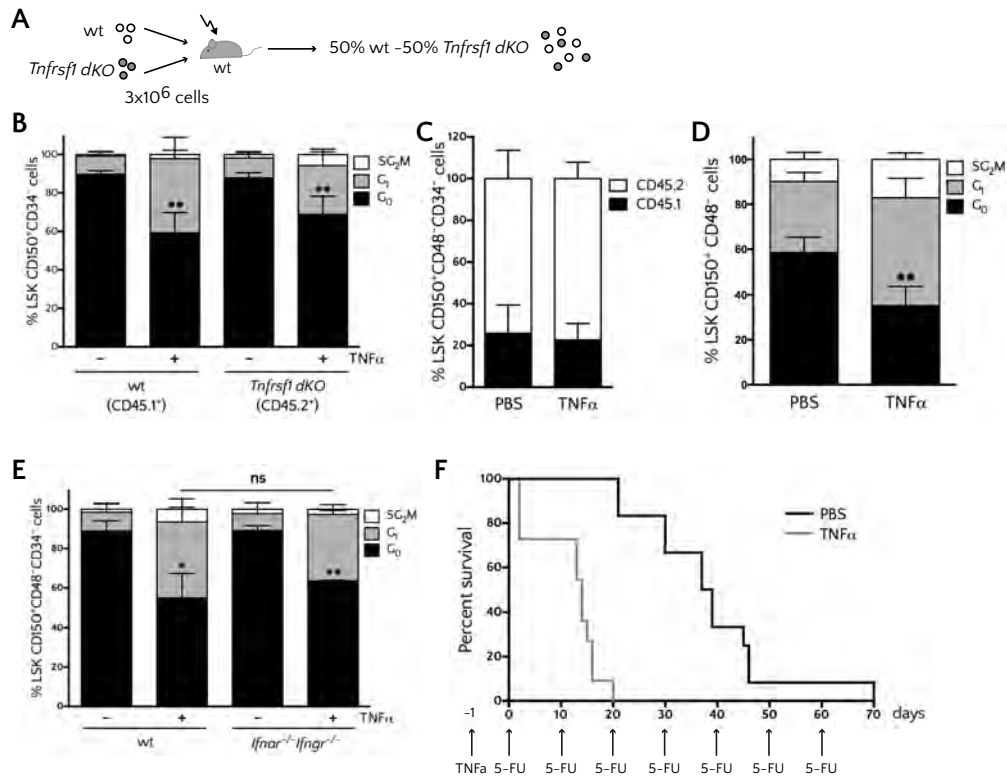
Figure 3.18.: TNFα activates dormant HSCs in part through an indirect mechanism
(A) Experimental set-up of mixed chimeras: bone marrow cells of wt (CD45.1) and *Tnfrsf1 dKO* (CD45.2) mice were transplanted into lethally irradiated wt mice in a 50:50 ratio. (B) Cell cycle analysis (icKi67 and Hoechst) of wt and *Tnfrsf1 dKO* HSCs in the chimeric setting after treatment with PBS or TNFα (0.75 mg/kg, 18h). (C) Chimerism of wt (CD45.1, black) and *Tnfrsf1 dKO* (CD45.2, white) HSCs in mixed bone marrow chimeras at time of analysis (16 weeks after transplantation) (D) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt mice transplanted into lethally irradiated *Tnfrsf1 dKO* mice after treatment with PBS or TNFα (0.75 mg/kg, 18h). (E) Cell cycle analysis (icKi67 and Hoechst) of wt and *Ifnar$^{-/-}$ Ifngr$^{-/-}$* mice after treatment with PBS or TNFα (0.75 mg/kg, 18h). (F) Survival curve of PBS or TNFα pre-treated mice after repeated 5-FU treatments: wildtype mice were treated with PBS or TNFα (0.75 mg/kg) 24h hours before repeated 5-FU treatments were started, mice were treated with 5-FU (150 mg/kg) every ten days.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p ≤ 0.05, **p ≤ 0.01

HSCs to induce proliferation upon TNFα treatment. We therefore treated wildtype and *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice with TNFα to investigate if the TNFα-induced activation of HSCs is mediated by IFN signalling. The HSCs of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice responded to TNFα with an increase in cell cycle activity comparable to the wildtype HSCs (Figure 3.18 E). This results, together with the data from the MxCre Rosa26eYFPflox$_2$ mouse model where we did not see an increase in IFNα production in the bone marrow upon TNFα treatment (Figure 3.1 J and K), excludes involvement of IFN signalling in the TNFα-induced activation of HSCs. Thus far, the analysis of LSK CD150$^+$CD48$^-$CD34$^-$ HSCs after treatment with TNFα does not prove an activation of quiescent HSCs with TNFα. Activation of quiescent HSCs has been shown to make mice susceptible to repeated 5–FU treatment (Essers *et al.*, 2009), thus we tested whether TNFα pre-treatment would sensitise mice to 5–FU (Figure 3.18 F). Indeed, mice treated with TNFα 24 hours before the first treatment with 5–FU died significantly earlier compared to PBS treated mice after repeated 5–FU treatments. While all PBS treated mice survived at least 3 treatments with 5–FU and half of the mice even survived more than 4 treatments, all of the TNFα treated mice died after the second 5–FU treatment (Figure 3.18 F), suggesting that TNFα sensitises the mice to repeated 5–FU treatments by activating the quiescent HSCs.

In summary, these experiments show that TNFα activates quiescent HSCs, however the mechanism seems to be at least in part indirectly mediated by another cell type of the bone marrow.

3.7. Gene expression profiling of activated HSCs

3.7.1. Gene expression analysis of LPS treated wildtype and *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice

As the LPS-induced effects on HSCs are in part mediated by IFN signalling, we wanted to get a better understanding of the mechanisms involved in the activation of the HSCs in wildtype and *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice. Mice were treated with PBS or LPS for 16 hours and LK CD150$^+$CD48$^-$ were sorted from bone marrow. From these cells RNA was isolated and gene expression analysis was performed using the Ilumina BeadChip® Sentrix Array. Reverse transcription, as well as the expression analysis were performed by the DKFZ Genomics and Proteomics Core Facility (Figure 3.19 A). After normalisation, data was analysed using R. Figure 3.19 B shows a heat map after non-supervised hierarchical clustering using the 5000 most variable genes of all four sample groups. The tree showed a clear separation between the wildtype and the *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ samples, indicating that the gene expression pattern of the *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ HSCs was different to wildtype HSCs at baseline. This clustering can also be shown by a principle compo-

nent analysis, again using the 5000 most variable genes (Figure 3.19 C). Although the clustering showed high variations between the wildtype and the *Ifnar$^{-/-}$Ifngr$^{-/-}$* HSCs, commonly regulated genes could be identified (Figure 3.19 D and E). Among the upregulated genes were mainly genes involved in the immune response – Saa3, CCL5, Ffar2, Plac8, CD74 and Psmb10 – as well as genes that might be associated with an increased metabolic activity due to activation – Pgls, Bcs1l or Pgd. Furthermore, there were some genes upregulated that might indicate a differentiation induced due to the activation, such as S100a9, Erdr1 or Sdc3 (Figure 3.19 D). More interestingly, among the downregulated genes was Cdkn1c (p57), which has been shown to be essential for the maintenance of HSC quiescence (Matsumoto *et al.*, 2011; Zou *et al.*, 2011). To validate the results of the array, we analysed the expression of selected genes by qRT-PCR and compared their regulation upon LPS treatment with the data of the array. We were able to confirm the upregulation of Serpina3g, Socs3 and CCL5, as well as the downregulation of Apbb1, Foxa3 and CXCL4 (Figure 3.19 F and G). Although the fold changes between the array and the qPCR data are not comparable, this shows that the direction of regulation in the array data resembles the results of the q-RT-PCR, demonstrating that the microarray data reliably reflects the expression of the analysed genes.

3.7.2. Gene expression analysis after treatment with IFNα, LPS or TNFα

The activation of HSCs can not only be achieved by treatment with IFNα and LPS, but also with TNFα. Therefore, another MicroArray analysis was performed, treating wildtype mice with TNFα and comparing this data to the data from the previous array, where we treated wildtype mice with PBS, LPS or IFNα (Figure 3.20 A). With this analysis we aimed to identify a common mechanism that is regulating the increase in proliferation after the different treatments. As outlined in section 3.7.1, we performed non-supervised hierarchical clustering as well as a principle component analysis using the 5000 most regulated genes. In this analysis a clear similarity between the IFNα and the LPS treated samples was detected, while the TNFα treated samples clustered separately from the other treatments (Figure 3.20 B and C), indicating that the LPS-induced activation of HSCs is very similar to the IFNα induced activation, while the TNFα-induced activation seems to use another mechanism. Nevertheless, this does not exclude a common mechanism inducing the activation of HSCs at a downstream point of the signalling pathways. Also genes that are similarly regulated in all treatments could be identified (Figure 3.20 D and E). These include Oas2, Plac8, Prg2, Ffar2 and CD74, which were most likely upregulated as a consequence of the immune response, as well as Fbox39, Aars and Uqcrb, which again point towards increased metabolic activity after activation. Among
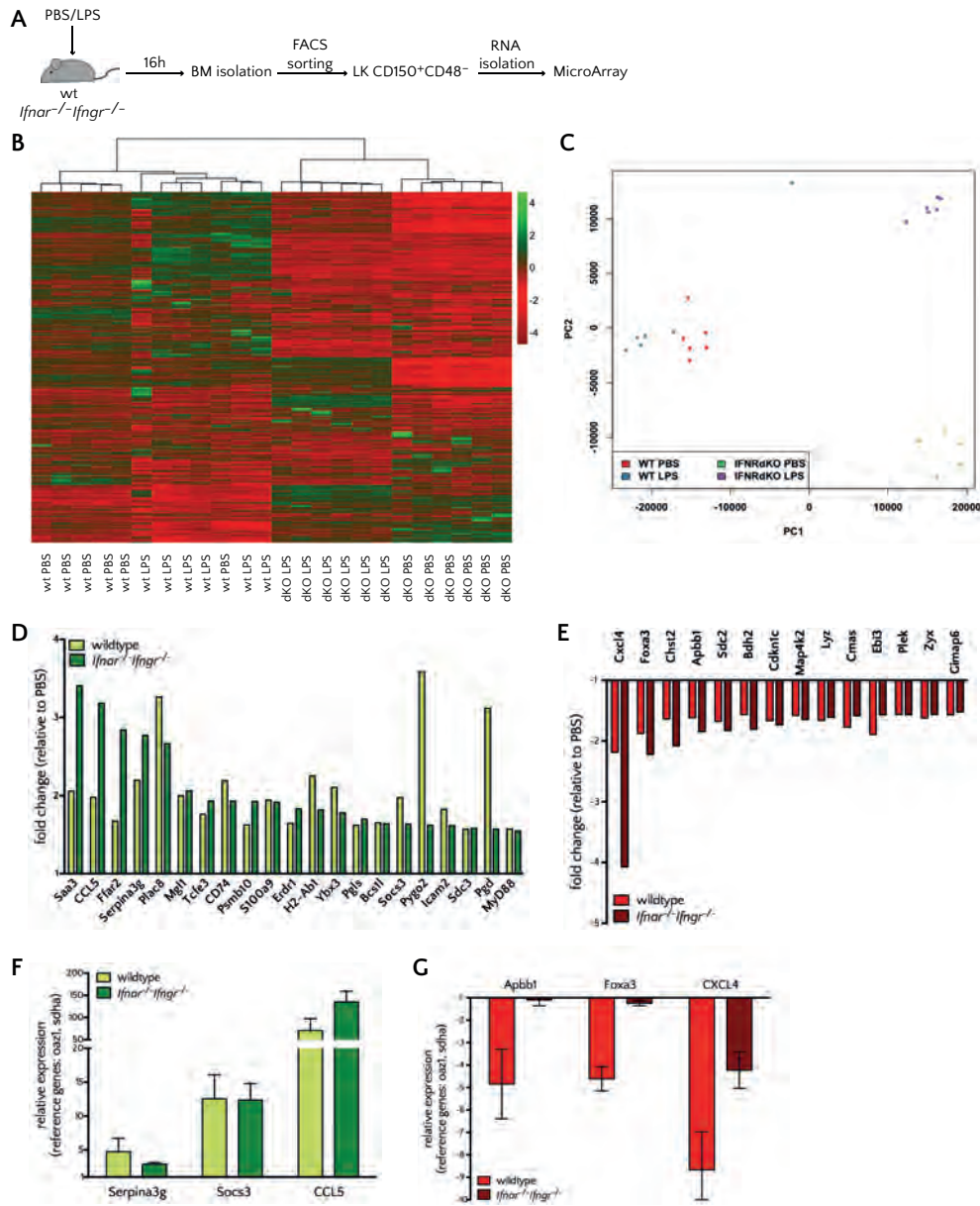
Figure 3.19.: Gene expression analysis of LPS treated wildtype and *Ifnar⁻/⁻ Ifngr⁻/⁻* mice
(A) Experimental set-up: LK CD150⁺CD48⁻ cells from wt or *Ifnar⁻/⁻ Ifngr⁻/⁻* mice treated with PBS or LPS
(0.25 mg/kg, 16h) were sorted and RNA was used for expression analysis with the Ilumina BeadChip® Sen-
trix Array. (B) Unsupervised hierarchical clustering of the 5000 most variable genes. (C) Principle com-
ponent analysis of the 5000 most variable genes. (D,E) Fold change of selected genes regulated in wt and
Ifnar⁻/⁻ Ifngr⁻/⁻ mice after LPS treatment. (F,G) Validation of expression of selected genes by qRT-PCR.

the downregulated genes was Matn4, an extracellular matrix protein, which seems to a have a role in haematopoiesis (Uckelmann and Essers, *unpublished data*). Furthermore, some genes that have been implicated to have a role in HSC self-renewal or have been shown to be highly expressed in HSCs compared to progenitors, were downregulated upon activation. These include Ptn, Fhl1 and Cadps2. The downregulation of these genes might again point towards an increased differentiation of the activated cells. The third group of downregulated genes, included genes that have been suggested to have anti-proliferative effects (Rprm, Marcks and Sdc2), however a role of these genes in HSC regulation has not been shown so far (Figure 3.20 E).

3.7.3. Gene expression analysis of IFNα, LPS or TNFα treated wildtype and LPS treated *Ifnar$^{-/-}$Ifngr$^{-/-}$* mice

The LPS-induced activation of HSCs is mediated in part by IFN and in part by TNFα signalling (Figures 3.14 and 3.16), suggesting that the LPS-induced activation of *Ifnar$^{-/-}$Ifngr$^{-/-}$* HSCs is mainly mediated by TNFα signalling. Hierarchical clustering was performed, as well as a principle component analysis, using the 5000 most regulated genes in the wildtype samples, comparing the gene expression data of wildtype HSCs treated with IFNα, LPS or TNFα to *Ifnar$^{-/-}$Ifngr$^{-/-}$* HSCs treated with LPS. A clear separation of wildtype and *Ifnar$^{-/-}$Ifngr$^{-/-}$* could be detected in the hierarchical clustering as well as in the principle component analysis (Figure 3.21 A and B). Interestingly, the TNFα treated samples formed a separate group, with low similarities to the wildtype treated samples, as well as to the *Ifnar$^{-/-}$Ifngr$^{-/-}$* samples, indicating that the LPS-induced activation of *Ifnar$^{-/-}$Ifngr$^{-/-}$* HSCs might not be solely mediated by TNFα (Figure 3.21 A and B). However, the gene regulation of *Ifnar$^{-/-}$Ifngr$^{-/-}$* HSCs after TNFα was not analysed.

The only cell cycle regulator noted in the analysis of the array data, p57, has already been implicated to have a role in the maintenance of HSC quiescence (Matsumoto *et al.*, 2011; Zou *et al.*, 2011; Tesio and Trumpp, 2011). Expression of p57 was analysed by qRT-PCR after treatment of wildtype mice with polyI:C, LPS or TNFα and *Ifnar$^{-/-}$Ifngr$^{-/-}$* after treatment with LPS. There was a decrease of p57 expression in all samples (Figure 3.21 C), suggesting a common regulatory mechanism leading to the activation of HSCs. However, the decrease in p57 expression is likely a consequence of the LPS-induced activation of HSCs. Identification of the signalling pathways leading to this decrease in p57 expression might therefore lead to a better understanding of the regulation of HSC proliferation upon infection. The MicroArray data allows the identification of some common feature, like the decreased expression of p57 among all treatments, however it is
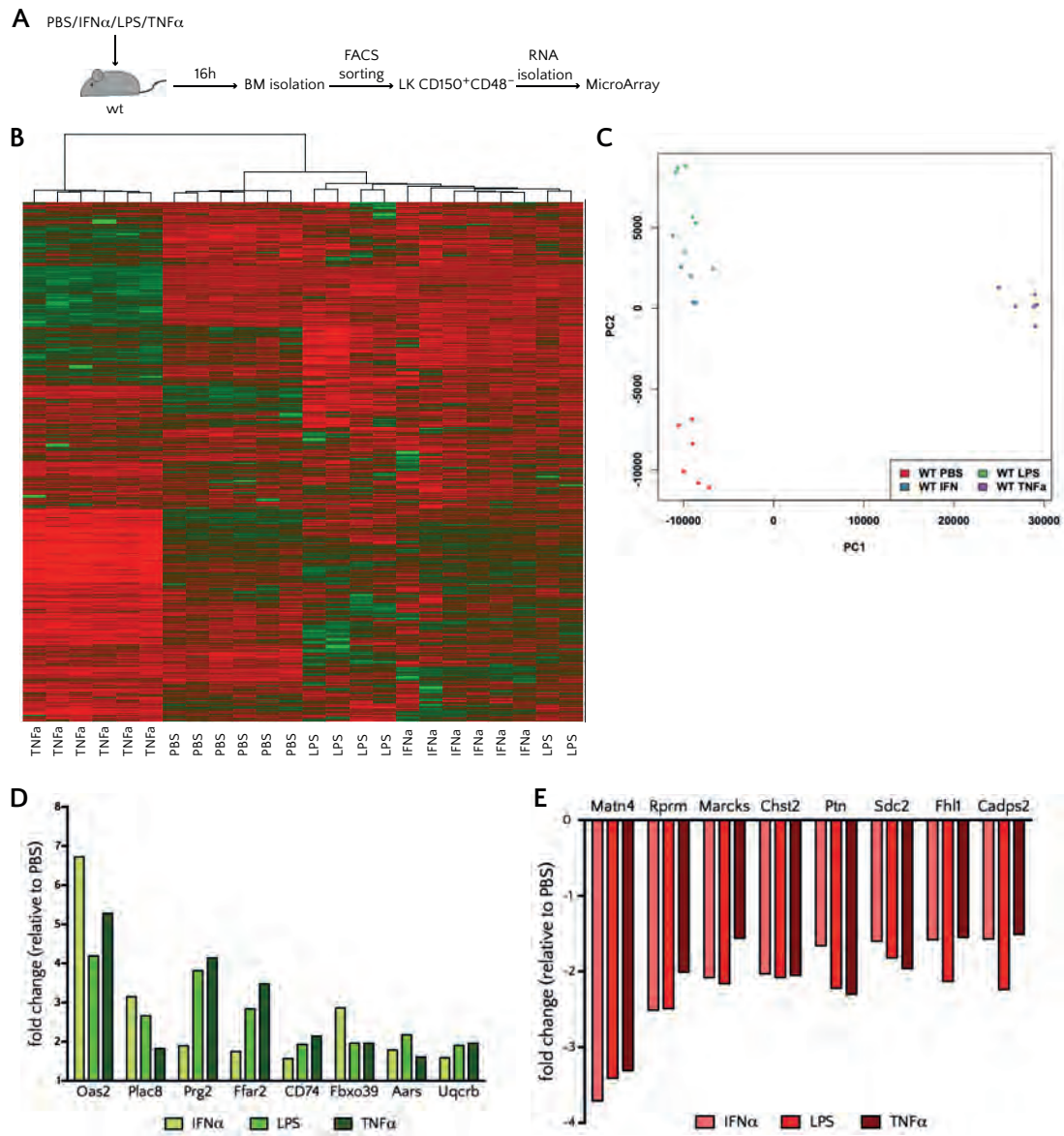
Figure 3.20.: Gene expression analysis after treatment with IFNα, LPS or TNFα
(A) Experimental set-up: LK CD150⁺CD48⁻ cells from wt mice treated with PBS, IFNα (50×10^5 U/kg), LPS (0.25 mg/kg) or TNFα (0.75 mg/kg) for 16h were sorted and RNA was used for expression analysis with the Ilumina BeadChip® Sentrix Array. (B) Unsupervised hierarchical clustering of the 5000 most variable genes. (C) Principle component analysis of the 5000 most variable genes. (D,E) Fold change of selected genes similarly regulated after all treatments.
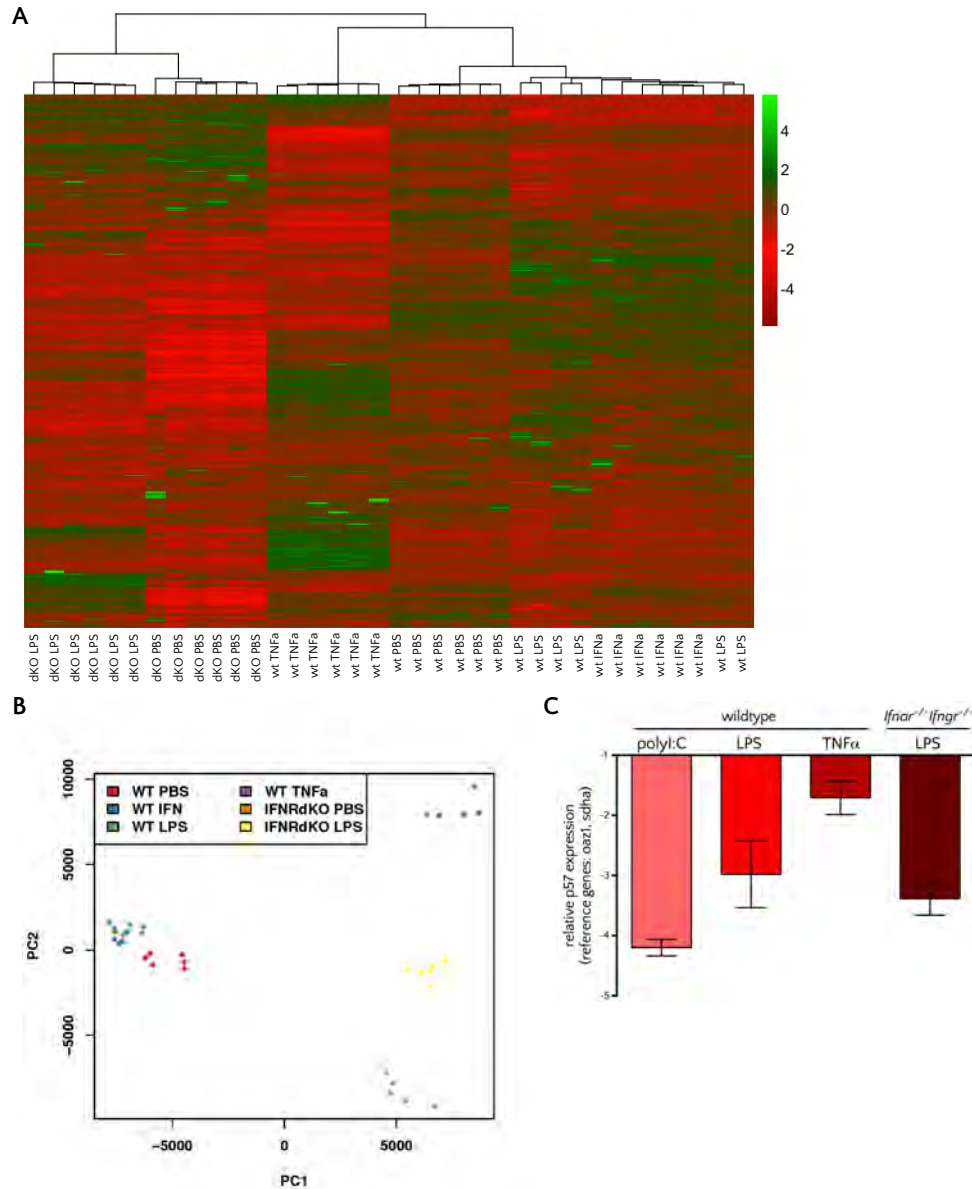
Figure 3.21.: Gene expression analysis of IFNα, LPS or TNFα treated wildtype and LPS treated *Ifnar⁻ᐟ⁻ Ifngr⁻ᐟ⁻* mice

(A) Unsupervised hierarchical clustering of the 5000 most variable genes. (C) Principle component analysis of the 5000 most variable genes. (C) p57 expression of LK CD150⁺CD48⁻ cells from wildtype mice treated with polyI:C (5 mg/kg), LPS (0.25 mg/kg) or TNFα (0.75 mg/kg) or *Ifnar⁻ᐟ⁻ Ifngr⁻ᐟ⁻* mice treated with LPS, relative to PBS treated samples, analysed by qRT-PCR.

Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments.

difficult to find a common mechanism leading to the increased cycling of HSCs upon different stimuli.

3.8. The role of IL–1β in the activation of HSCs

3.8.1. IL–1β treatment leads to increased proliferation of HSCs in a MyD88 dependent manner

Our data suggests that the TNFα–induced activation of HSCs is indirectly mediated (Figure 3.18 B) and that IFN signalling is not involved in the TNFα–induced activation of HSCs (Figures 3.1 J and K, 3.18 E), implying that there are other cytokines mediating the LPS and TNFα–induced activation of HSCs. As IL–1β is typically produced upon LPS signalling (Björkbacka *et al.*, 2004), the production of IL–1β in the bone marrow was analysed after treatment of wildtype mice with LPS or TNFα by ELISA. LPS, as well as TNFα treatment, led to an increase of IL–1β expression in the bone marrow supernatant (Figure 3.22 A). We therefore hypothesised that IL–1β might be a potential activator of HSCs in the context of LPS and TNFα treatment. To investigate the effects of IL–1β on HSCs, wildtype mice were treated with PBS or IL–1β and cells were analysed for cell cycle behaviour as well as the Sca–1 expression of HSPCs. After treatment with IL–1β the HSCs of wildtype mice exited the G_0 phase and entered an active cell cycle (Figure 3.22 B and D). Furthermore, the HSCs started to actively proliferate upon IL–1β (Figure 3.22 C and E), indicating that IL–1β leads to an activation of HSCs comparable to treatment with other cytokines or LPS. Apart from the cell cycle induction, the expression of Sca–1 on LK CD150$^+$ was analysed, as Sca–1 has been shown to be involved in the activation of HSCs with polyI:C, LPS and TNFα (Essers *et al.*, 2009) (Figures 3.6 D and 3.17 C). The HSPCs of IL–1β treated mice showed a slight, but not significant, increase in Sca–1 expression (Figure 3.22 F and G). Interestingly, treatment of *Sca–1$^{-/-}$* mice with IL–1β still led to a rescue of the IL–1β–induced increase in proliferation of HSCs, although the upregulation of Sca–1 on protein as well as on mRNA level is negligible in comparison to the Sca–1 increase seen with polyI:C or LPS (Figure 3.23 A and B), indicating that the activation of HSCs might not be directly dependent on an increase in Sca–1 expression. The IL–1β signalling pathway is mediated by the adaptor MyD88, *MyD88$^{-/-}$* mice were treated with IL–1β and the cell cycle induction and Sca–1 expression was compared to wildtype mice. As expected, the HSCs of *MyD88$^{-/-}$* mice did not respond to IL–1β treatment with increased proliferation or Sca–1 expression (Figure 3.22 B–G). To investigate if IL–1β directly activates HSCs, we sorted LK CD150$^+$ cells and treated them with PBS, IFNα, LPS or IL–1β in culture. Because the upregulation of Sca–1 with IL–1β is very low, Sca–1 can not be used as a readout for this
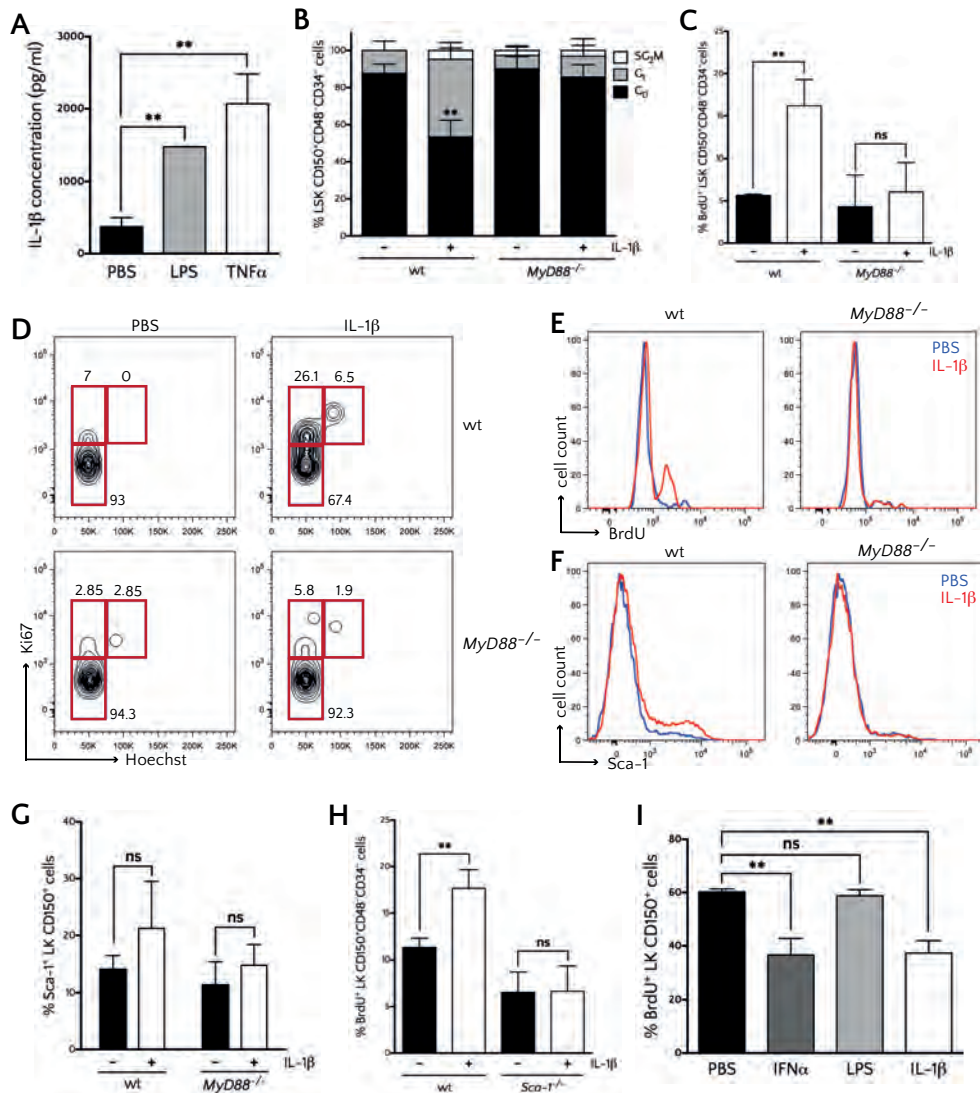
Figure 3.22.: IL-1β treatment leads to increased proliferation of HSCs in a MyD88 dependent manner
(A) IL-1β levels (ELISA) in the bone marrow supernatant of PBS, LPS (0.25 mg/kg, 4h) or TNFα (0.75 mg/kg, 4h) treated wt mice. (B,D) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wt or $MyD88^{-/-}$ mice after treatment with PBS or IL-1β (0.125 mg/kg, 18h). Quantitative and statistical analysis (B) and representative FACS profiles (D). (C,E) BrdU incorporation (14h) in HSCs from wt or $MyD88^{-/-}$ mice after treatment with PBS or IL-1β (0.125 mg/kg, 18h). Quantitative and statistical analysis (C) and representative FACS profiles (E). (F,G) Sca-1 expression on LK CD150+ cells from wt or $MyD88^{-/-}$ mice after treatment with PBS or IL-1β (0.125 mg/kg, 18h). Quantitative and statistical analysis (G) and representative FACS profiles (F). (H) BrdU incorporation (14h) in HSCs from wt or $Sca-1^{-/-}$ mice after treatment with PBS or IL-1β (0.125 mg/kg, 18h). (I) BrdU incorporation (3h) after *in vitro* treatment of sorted LK CD150+ cells with PBS, IFNα (1000 U/ml), LPS (100 ng/ml) or IL-1β (100 ng/ml) for 20 h.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p ≤ 0.05, **p ≤ 0.01

experiment. We therefore analysed BrdU incorporation. Treatment of sorted LK CD150$^+$ with IL-1β *in vitro* led to a decreased cycling of these cells, comparable to the treatment with IFNα, while the treatment with LPS did not lead to a decrease in BrdU incorporation (Figure 3.22 I). Nevertheless, this is indicative of, not proof of, a direct activation of HSCs with IL-1β and thus it has to be further investigated, if the *in vivo* activation of HSCs is also mediated by a direct mechanism. For that purpose a set of mixed bone marrow chimeras with bone marrow from wildtype and IL-1 receptor (IL-1R) deficient mice have been prepared, to analyse the activation of the *IL-1R$^{-/-}$* HSCs in this setting.
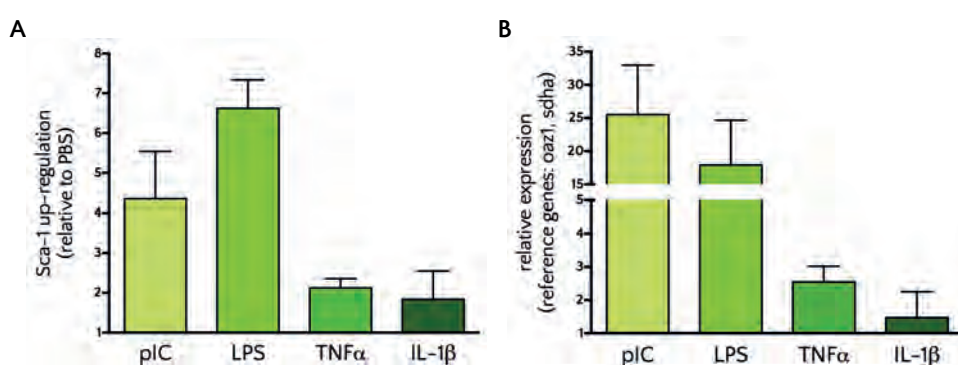
Figure 3.23.: Sca-1 expression on protein and mRNA level after different treatments
(A) Increase in Sca-1 expression on LK CD150$^+$ cells after treatment with polyI:C (5 mg/kg), LPS (0.25 mg/kg), TNFα (0.75 mg/kg) or IL-1β (0.125 mg/kg) for 18h, measured by Flow Cytometry, relative to PBS treated cells. (B) Increase in Sca-1 mRNA levels of sorted LK CD150$^+$CD48$^-$ from wt mice treated with polyI:C (5 mg/kg), LPS (0.25 mg/kg), TNFα (0.75 mg/kg) or IL-1β (0.125 mg/kg) for 16h, measured by qRT-PCR, relative to PBS treated samples.
Data are the mean +/- SD of at least 3 mice per condition, representing similar results of 2-3 independent experiments; *p ≤ 0.05, **p ≤ 0.01

3.8.2. The LPS-induced activation of HSCs is dependent on IFN, TNFα and IL-1β signalling

IFNα, TNFα as well as IL-1β can mediate activation of HSCs (Essers *et al.*, 2009) (Figures 3.16 D-G and 3.22 B-E) and that all of these cytokines are produced upon LPS treatment (Figures 3.14 A and B, **??** A and 3.22 A). To further investigate the role of IFN, TNFα and IL-1β signalling in the LPS-induced activation of HSCs, two inhibitors were used. Etanercept is a synthetic TNFα inhibitor, used in the clinics to treat for example rheumatic diseases (reviewed in Bradley, 2008), while the IL-1 receptor antagonist (IL-1RA) is a naturally-occurring protein secreted by immune cells, to regulate the activity of IL-1α and IL-1β (reviewed in Weber *et al.*, 2010). Wildtype mice were treated with Etanercept for ten
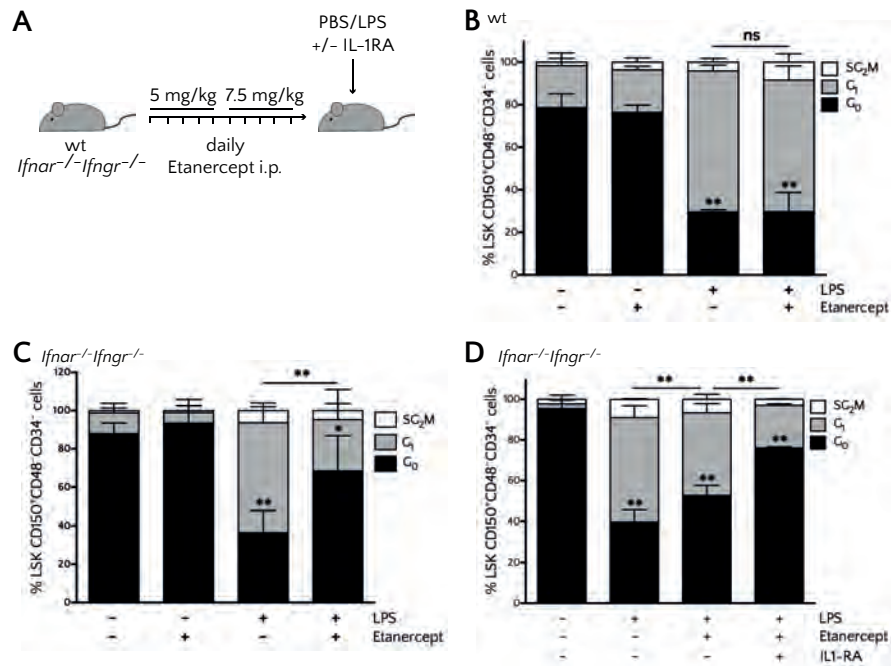
Figure 3.24.: The LPS–induced activation of HSCs is dependent on IFN, TNFα and IL–1β signalling
(A) Experimental set-up: wt or *Ifnar⁻/⁻ Ifngr⁻/⁻* mice were treated with Etanercept (days 1–5 5 mg/kg, days 6–10 7.5 mg/kg) or PBS for 10 days, at day 10 the mice were treated with IL-1RA or PBS 1h before the treatment with PBS or LPS (0.25 mg/kg, 18h). (B) Cell cycle analysis (icKi67 and Hoechst) of HSCs from wildtype mice treated with PBS or Etanercept for 10 days and PBS or LPS (0.25 mg/kg) for 18h. (C) Cell cycle analysis (icKi67 and Hoechst) of HSCs from *Ifnar⁻/⁻ Ifngr⁻/⁻* mice treated with PBS or Etanercept for 10 days and PBS or LPS (0.25 mg/kg) for 18h. (D) Cell cycle analysis (icKi67 and Hoechst) of HSCs from *Ifnar⁻/⁻ Ifngr⁻/⁻* mice treated with PBS or Etanercept for 10 days and PBS or LPS (0.25 mg/kg) for 18h, as well as PBS or IL-1RA 1h before the LPS treatment.
Data are the mean +/– SD of at least 3 mice per condition, representing similar results of 2–3 independent experiments; *p $\leq$ 0.05, **p $\leq$ 0.01

days, to ascertain the effects of blocking TNFα alone. Mice were subsequently treated with PBS or 0.25 mg/kg LPS (Figure 3.24 A) and the cell cycle induction of HSCs was analysed. The Etanercept treated wildtype HSCs showed an increased cell cycle activity upon LPS, comparable to untreated controls (Figure 3.24 B). This was expected, as the treatment of *Tnfrsf1 dKO* mice with 0.25 mg/kg of LPS also led to a normal cell cycle induction of HSCs. Treatment of *Ifnar⁻/⁻ Ifngr⁻/⁻* with 0.25 mg/kg LPS led to an increased cell cycle of HSCs, while treatment with a lower dose of LPS led to a rescue of the LPS effect in *Tnfrsf1 dKO* as well as *Ifnar⁻/⁻ Ifngr⁻/⁻* mice. The Etanercept treatment was therefore repeated in *Ifnar⁻/⁻ Ifngr⁻/⁻* mice, to achieve a combined blocking of IFN and TNFα signalling, before treating those mice with PBS or LPS. The increase in cell

cycle activity of HSCs upon LPS treatment, was significantly reduced compared to the untreated controls, however we could not achieve a complete rescue of the effect (Figure 3.24 C), indicating that the LPS–induced effect on HSCs is not mediated by IFN and TNFα signalling alone. To analyse the LPS–induced effects on HSCs, IFN, TNFα and IL–1β signalling was blocked at the same time. *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice were treated with Etanercept for 10 days and additionally with IL–1RA, 1 hour before the LPS treatment. Again, a significant reduction in the increase in cell cycle activity upon treatment with Etanercept alone was observed, which can be further enhanced by combined treatment with Etanercept and IL–1RA (Figure 3.24 D). Nevertheless, the effect of LPS on HSCs still cannot be completely rescued, indicating that there might be some other cytokines involved in the LPS–induced activation of HSCs or that the blocking effects of Etanercept and IL–1RA are not complete.

In summary, this data shows that the LPS–induced activation of HSCs is mainly mediated by a combination of IFN, TNFα and IL–1β signalling, however we could not exclude the involvement of other cytokines produced upon LPS treatment.

4. Discussion

4.1. The short-term effect of LPS on HSCs differs from the long-term effect

We have investigated the *in vivo* short-term effects of LPS treatment on the cycling behaviour of HSCs. With this research we aimed to understand the first response of HSCs to infections and how this might be linked to already published data on the long-term effects of infections or treatment with TLR ligands on HSCs (Zhang *et al.*, 2008; Singh *et al.*, 2008; Rodriguez *et al.*, 2009; Takizawa *et al.*, 2011; Esplin *et al.*, 2011; Choi *et al.*, 2011). Furthermore, we wanted to link our results to previously published data on HSC behaviour in response to cytokines, such as IFNs and TNFα. As we had already shown that IFNα treatment leads to an increase in proliferation of HSCs, we asked whether this might be part of a feedback-loop induced upon infection. We were able to show that *in vivo*, short-term treatment of mice with LPS led to an increase in proliferation of even the most dormant HSCs (Figures 3.2 and 3.4). This is in accordance with previously published data showing increased cycling of HSCs in response to long-term LPS treatment or infection (Kaulen *et al.*, 1983; Zhang *et al.*, 2008; Yanez *et al.*, 2009; Takizawa *et al.*, 2011; Esplin *et al.*, 2011). In contrast to long-term LPS effects, we show that the short-term effect of LPS on HSCs was transient, with a return of the HSCs to quiescence after 72 hours (Figure 3.3 B). This finding is in keeping with previously published work, showing that HSCs can enter cycle upon stress, but rapidly return to their dormant state when the situation is resolved (Wilson *et al.*, 2008; Essers *et al.*, 2009; Takizawa *et al.*, 2011). With these findings we can now explain the loss of repopulation potential of HSCs seen in long-term studies, as chronic activation of TLR signalling leads to constant cycling of HSCs, resulting in their exhaustion, as shown in various studies (Rodriguez *et al.*, 2009; Takizawa *et al.*, 2011; Esplin *et al.*, 2011). This effect of constant cycling of HSCs on their engraftment potential has also been shown in response to activation of HSCs with other stimuli, such as IFNs (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010). A more recent study has, however, challenged the hypothesis that IFN treatment leads to an increased proliferation of HSCs, by showing that IFNγ treatment of HSCs in culture leads to

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reduced cell numbers and that infection of mice with LCMV led to impaired engraftment of HSCs upon competitive transplantations (de Bruin *et al.*, 2013). From these results it was concluded that IFNs have an anti-proliferative effect on HSCs and that the results obtained in previous studies are due to a contamination of the population analysed, as progenitor cells start to express Sca-1 upon IFN stimulation (Essers *et al.*, 2009; Baldridge *et al.*, 2010; de Bruin *et al.*, 2013). However, these results support the results obtained in the previous studies, as the engraftment defect upon LCMV infection is very likely due to constant cycling of HSCs in this setting (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010; de Bruin *et al.*, 2013; King *et al.*, 2013). It might be true that the induction of Sca-1 expression on progenitor cells leads to an increase of progenitors in the LSK population, however, the use of further markers, like CD150, CD48 and CD34, nonetheless, enables the identification of real HSCs also upon Sca-1 upregulation (King *et al.*, 2013). Furthermore, for IFNα as well as for LPS treatment we were able to show that even the most dormant LRCs are activated into a state of active proliferation (Essers *et al.*, 2009) (Figure 3.4). Similarly, differences in long-term and short-term effects of TNFα on HSCs can be observed. We have shown that TNFα treatment of mice leads to an increased cell cycle activity of HSCs, as well as an activation of quiescent HSCs into proliferation (Figures 3.16 D-G and 3.18 F). These findings could explain the results of a recently published study on HSCs of mice lacking both TNF receptors (*Tnfrsf1 dKO*). This study shows an enhanced repopulation activity of HSCs from *Tnfrsf1 dKO* mice compared to HSCs from wildtype mice in competitive transplantations, concluding that TNFα has an anti-proliferative effect on HSCs (Pronk *et al.*, 2011). Nevertheless, these studies do not show direct cell cycle analysis of HSCs from *Tnfrsf1 dKO* mice. Therefore, these findings can be explained by our findings that TNFα treatment leads to an increased proliferation of HSCs. This increased cycling upon TNFα signalling might lead to a reduced engraftment potential of wildtype HSCs in competition with *Tnfrsf1 dKO* HSCs, which do not respond to TNFα and are therefore more quiescent, as quiescent HSCs have been shown to exhibit an engraftment advantage over cycling HSCs (Spangrude and Johnson, 1990; Passegue *et al.*, 2005). TNFα induced activation of wildtype HSCs in this setting might be due to TNFα production upon irradiation, which represents major bone marrow stress, or due to small amounts of TNFα that might be constantly produced in the bone marrow. In contrast to long-term activation of HSCs in response to infections, the repopulation potential of HSCs treated with a single dose of LPS was not impaired (Figure 3.5). This is in accordance with our finding that the LPS induced proliferation of HSCs is transient (Figure 3.3 B) and findings by others that HSCs are able to reestablish quiescence and successfully repopulate a recipient after they have been activated with LPS (Takizawa

et al., 2011). In summary, the short–term as well as the long–term effects of LPS exposure on HSCs seen by us and others can be explained by increased cycling of HSCs, leading to loss of quiescence as well as exhaustion of the HSC pool upon chronic LPS signalling. Furthermore, increased cycling of HSCs might be a cause of bone marrow failure seen in patients suffering from chronic infections.
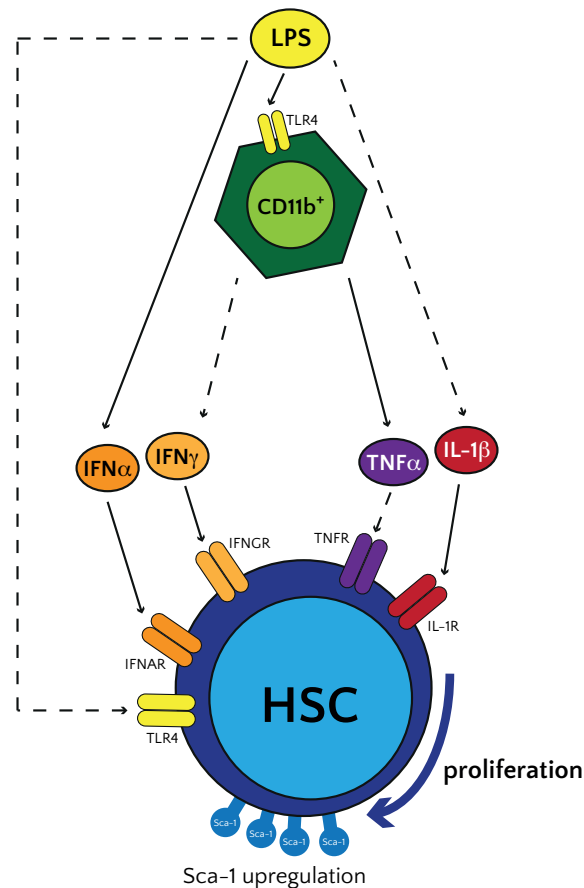
Figure 4.1.: The LPS–induced activation of HSCs
The LPS–induced activation of HSCs is indirectly mediated through the production of inflammatory cytokines by CD11b$^+$ cells and possibly other cells. These cytokines include IFNα, IFNγ, TNFα and IL–1β. The IFNs and IL–1β lead to a direct activation of HSCs, while the effect of TNFα is most likely indirect. The HSCs start to proliferate and upregulate Sca–1 upon LPS–induced stimulation.

4.2. The LPS–induced activation of HSCs is indirectly mediated

We have shown that the LPS–induced activation of HSCs is indirectly mediated (Figure 3.10) and that myeloid cells, more specifically inflammatory monocytes are involved in this activation (Figures 3.11, 3.12 and 4.1). These findings suggest that at least in terms

of HSC cycling the effects are not induced by direct activation of TLR signalling within the HSCs. This is in contrast to a study proposing that differentiation of HSPCs upon LPS treatment is directly mediated (Megías *et al.*, 2012). In this study, LSK cells from wildtype mice were transplanted into non-irradiated *TLR4*$^{-/-}$ recipients and directly treated with LPS, leading to a differentiation of the transplanted cells into macrophages (Megías *et al.*, 2012). However, LSK cells are a rather heterogenous population and tend to rapidly differentiate upon transplantation. Therefore, these experiments are a hint of direct sensing of LPS by LSK cells, but no proof for a direct activation of TLR signalling in HSCs. We demonstrated an indirect activation of HSCs with LPS using mixed bone marrow chimeras, in which also *TLR4*$^{-/-}$ HSCs showed increased proliferation upon LPS treatment (Figure 3.10 B). However, with this experiment we cannot rule out a direct effect of LPS on HSCs, that might be overshadowed by the indirectly induced proliferation of HSCs. To reduce the indirect effects upon LPS, one could analyse the cycling behaviour of *TLR4*$^{-/-}$ HSCs in a chimeric setting with very few wildtype HSCs in relation to *TLR4*$^{-/-}$ HSCs. However, it is very difficult to set up such chimeras, as the *TLR4*$^{-/-}$ HSCs show an engraftment advantage over wildtype HSCs (reviewed in Baldrige *et al.*, 2011), and will therefore outcompete the wildtype HSCs in such a setting over time. Assuming that the proliferation of HSCs upon LPS is linked to the increased expression of Sca-1, our *in vitro* results point towards a solely indirect effect of LPS on HSCs. Although the LPS treatment of HSPCs in culture does not lead to an increased cycling of HSCs, we can compare the *in vitro* effects of LPS treatment to effects of IFNα treatment, which has been shown to induce a direct activation of HSCs (Essers *et al.*, 2009; Sato *et al.*, 2009). IFNα treatment of HSPCs in culture leads to an increased expression of Sca-1 on HSPCs, as well as a reduced proliferation of these cells, while these effects cannot be detected upon *in vitro* LPS treatment (Figure 3.10 F-H), again pointing towards a solely indirect activation of HSCs with LPS.

This difference between the *in vivo* and the *in vitro* treatment of HSCs has been observed in various studies. While the *in vivo* treatment of mice with IFN leads to increased proliferation of HSCs, *in vitro* studies have reported an opposite effect (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010; de Bruin *et al.*, 2013). Similarly, the *in vitro* treatment of HSPCs with TNFα led to inhibition of HSC cycling in various studies (Jacobsen *et al.*, 1992; Rusten *et al.*, 1994; Jacobsen *et al.*, 1994; Dybedal *et al.*, 2001), while we showed an increased proliferation of HSCs upon *in vivo* treatment with TNFα (Figure 3.16 D-G). These differences are likely due to the absence of the bone marrow niche environment in culture (reviewed in Prendergast and Essers, 2014). Although, our results exclude a direct necessity of TLR4 activation in niche cells to induce the activation of

HSCs (Figure 3.10 D), it is likely that indirect effects alter the niche environment, promoting the increased proliferation of HSCs, as the interaction of HSCs with their niches has been shown to be essential for the maintenance of HSC quiescence. It is possible that factors produced by mature immune cells or even by the HSCs themselves as response to infections, alter the secretion of factors that mediate the retention of HSCs in the niche. It has for example been shown that G-CSF, a cytokine produced upon infection, leads to a reduced secretion of CXCL12 by osteoblasts, resulting in release of HSCs from their niches (Winkler *et al.*, 2010; Christopher *et al.*, 2011). It can therefore be assumed that other cytokines produced upon infection, such as IFNs, TNFα or others, might have similar effects. It has been shown that activation of HSCs with polyI:C leads to a relocation of these cells within the bone marrow niche (Kunisaki *et al.*, 2013), indicative of changes in the bone marrow microenvironment induced upon infections. A typical effect of inflammatory cytokines is the induction of adhesion molecule expression on endothelial cells, leading to the recruitment of immune effector cells to the sites of infection (reviewed in Mogensen, 2009). These adhesion molecules "mark the way" from the bone marrow, where for example monocytes and B cells are located during homeostasis, to the infected tissue. It is therefore likely that similar to mature immune cells, HSCs also adhere to the endothelial cells of the bone marrow vasculature upon increased expression of adhesion molecules, leading to a relocation of HSCs from the endosteum to the vasculature. This could in turn enable a fast release of newly produced progenitors into the periphery. As there is no increased mobilisation of HSCs upon a single dose of LPS (data not shown), it is likely that the HSCs are retained within the bone marrow, moving only to the periphery after they have differentiated into progenitors or mature cells. In addition, immune cells, like macrophages and T cells, have been suggested to be part of the HSC niche (Winkler *et al.*, 2010; Chow *et al.*, 2011; Fujisaki *et al.*, 2011). As these cells are clearly affected by immune stimulatory mechanisms, it is very likely that these effects will also lead to an alteration of the HSC niche, resulting in changes in HSC behaviour.

Our finding that myeloid cells can mediate the LPS-induced effects on HSCs (Figures 3.11 and 3.12), sheds new light on the role of these myeloid cells in the bone marrow during infection. Our *in vitro* results suggest that the LPS-induced effects are mainly mediated by inflammatory monocytes (Figure 3.11), which are found in the bone marrow during homeostasis but can be rapidly recruited to the infected tissue where they differentiate to macrophages or dendritic cells (Gordon and Taylor, 2005; Robbins and Swirski, 2010; Shi and Pamer, 2011). This recruitment of monocytes and their subsequent loss from the bone marrow leads to the necessity to produce new monocytes to fight in-

fection and to restore homeostatic conditions. Therefore, it is possible that monocytes produce factors that mediate re-production through direct activation of HSPCs or indirect induction of changes in the BM microenvironment, when they are activated and leave the bone marrow. Thus, the activation of monocytes by LPS would indirectly lead to a subsequent activation of HSCs to proliferate and differentiate towards the myeloid lineage. This is supported by findings, that LPS treatment actually leads to differentiation of HSPCs to myeloid cells (Nagai *et al.*, 2006; Luca *et al.*, 2009; Yanez *et al.*, 2010; Esplin *et al.*, 2011; Megías *et al.*, 2012). Nevertheless, our findings are based on *in vitro* experiments alone and may therefore not correspond to the actual *in vivo* situation. Our *in vivo* results, however, further support the *in vitro* findings. There is a decrease in cell cycle induction of HSCs from clodronate treated mice in comparison to the control mice (Figure 3.12 F), indicating a role for myeloid cells in the LPS-induced activation of HSCs *in vivo*. This difference is, however, low and the pre-treatment of mice with clodronate did not lead to a complete rescue of the LPS-induced effects. This could be for several reasons, one being that we treated the mice with clodronate 5 days before the LPS treatment, to avoid the initial wave of HSC mobilisation upon loss of myeloid cells. After five days many of the initially depleted cells, like for example monocytes (Chow *et al.*, 2011), have already re-emerged (data not shown), while the number of bone marrow macrophages remains significantly lower compared to the control treated mice (Figure 3.12 G). This could, however, mean that the cells that are actually mediating the LPS-induced activation of HSCs have already re-emerged and are therefore at least in part able to induce the increased proliferation of HSCs upon LPS. This would support the *in vitro* finding that only monocytes, but not macrophages, can mediate the increase in Sca-1 expression on HSPCs upon LPS treatment. Another reason for the incomplete rescue of the LPS effect could be the involvement of several cell types in the LPS-induced activation of HSCs, as cytokines are produced by different cell types upon LPS signalling (Akira and Takeda, 2004; Kawai and Akira, 2010). For example, interferons are mainly produced by plasmacytoid dendritic cells (pDCs) (Perussia *et al.*, 1985; Asselin-Paturel *et al.*, 2003), which are not depleted upon clodronate treatment (data not shown). Therefore, the production of interferons by pDCs might be responsibly for the increased cycling of HSCs in this experiment. Furthermore, the depletion of myeloid cells with clodronate does not lead to a complete loss of these cells and the remaining cells might be enough to induce the cycling of HSCs upon LPS. It is unlikely that myeloid cells do not have any role in the LPS-induced activation of HSCs, as they have been shown to be the main producers of inflammatory cytokines in response to LPS (reviewed in Kawai and Akira, 2010) and macrophages have been shown to be in close contact with HSCs in their niche (Win-

kler *et al.*, 2010; Chow *et al.*, 2011). It is therefore likely, that cells of the myeloid lineage, like monocytes or macrophages, are at least in part producing the cytokines that are responsible for LPS-induced activation of HSCs. Nevertheless, there might be several cell types, like other cells that have been proposed to make up the HSC niche as well as other immune cells, involved in the process of HSC activation and further experiments need to be performed to get a full understanding of this process.

4.3. The LPS-induced activation of HSCs is mediated by cytokines

We have shown that the LPS-induced activation of HSCs is mediated by IFNs, TNFα and IL-1β (Figures 3.14, 3.16, 3.22 and 3.24). These are cytokines typically produced upon TLR stimulation (reviewed in Akira and Takeda, 2004; Borden *et al.*, 2007; Bradley, 2008; Kawai and Akira, 2010; Weber *et al.*, 2010) and the role of these cytokines during immunity has been extensively studied. Furthermore, a role for IFNs and TNFα in the regulation of HSC functions has been proposed. IFNα, as well as IFNγ, have been shown to directly induce the proliferation of HSCs (Essers *et al.*, 2009; Sato *et al.*, 2009; Baldridge *et al.*, 2010). In combination with our findings that IFNα and IFNγ are produced upon LPS treatment (Figures 3.1 and 3.14 A and B), it is not surprising that these IFNs are also involved in the LPS-mediated activation of HSCs. We furthermore showed that TNFα alone can induce activation of HSCs (Figure 3.16) and that IFN, as well as TNFα signalling, are involved in the LPS-induced activation of HSCs. However, the increase in proliferation of HSCs is not completely dependent on IFN or TNFα signalling alone upon a high dose of LPS (Figures 3.14 and 3.16). Furthermore, even upon combined blocking of IFN and TNFα signalling treatment of mice with a high dose of 0.25 mg/kg LPS still led to a significant increase in cycling HSCs (Figure 3.24 C). It is therefore likely that the activation of HSCs upon LPS is mediated by several cytokines, rather than just by IFNs and TNFα, as LPS leads to the production of many different cytokines (Björkbacka *et al.*, 2004) and all of these cytokines could have a possible effect on the cycling behaviour of HSCs. We excluded involvement of CXCL9, CXCL10 and CXCL11 in the LPS-induced activation of HSCs to proliferate (Figure 3.15 F), although these cytokines might be involved in the regulation of other responses of HSCs to infection, such as differentiation. A role for IL-6 in this effect of LPS on HSCs can, however, not be completely excluded with our results. Treatment with a higher dose of IL-6 might actually lead to a clearer activation of HSCs and treatment of *IL-6$^{-/-}$* mice with a low dose of LPS could very well have an effect on the induced cycling of HSCs. Interestingly, we were able to show for the first time that IL-1β treatment of mice led to an increased cycling of HSCs (Figure 3.22 B-E). As IL-1β is highly

upregulated upon LPS treatment (Figure 3.22 A) (Björkbacka *et al.*, 2004) and combined blocking of IFN, TNFα and IL-1β signalling led to a further reduction of the cell cycle activation of HSCs upon LPS (Figure 3.24 D), we propose that IL-1β, as well as IFNs and TNFα, are involved in the LPS-induced activation of HSCs. Furthermore, our data provides a first indication that the IL-1β induced activation of HSCs might be directly induced by IL-1β signalling within the HSCs, as HSPCs respond to IL-1β treatment *in vitro* similar to IFNα treatment. IL-1β might therefore be the missing link in the LPS-induced activation of HSCs in *Ifnar^{-/-}Ifngr^{-/-}* mice as well as in the TNFα -induced activation of HSCs, directly affecting the cycling behaviour of HSCs. This hypothesis is further supported by the finding that IL-1β is not only upregulated upon LPS treatment, but is also highly induced upon TNFα treatment (Figure 3.22 A), suggesting that IL-1β is one of the factors leading to the TNFα-induced activation of HSCs. Our results, however, do not exclude the involvement of further cytokines in the LPS-induced effects on HSCs, as even upon combined blocking of IFN, TNFα and IL-1β signalling, a significant increase in cycling HSCs upon LPS could still be detected (Figure 3.24 D). It is therefore likely that the LPS induced effects on HSCs can be mediated by several cytokines, excluding a dependency on only one cytokine upon stimulation with a high dose of LPS. This observation makes the identification of single cytokines that are involved in the LPS-induced activation of HSCs more complex. Hence, it is necessary to perform further experiments to investigate the effects of IL-6 and other cytokines, like CCL5, on the cycling behaviour of HSCs, as well as their role in the LPS-induced activation of HSCs.

MicroArray analysis of activated HSCs upon different stimuli potentially allowed us to investigate the common mechanisms leading to increased cycling of HSCs. However, from this data we could not convincingly conclude a common mechanism activated in HSCs upon all different treatments. Nevertheless, a common feature of the signalling pathways of LPS, as well as IFNs, TNFα and IL-1β, is NFϰB activation. Furthermore, we could show that NFϰB is translocated to the nucleus in HSCs upon activation with LPS (Figure 3.9 A and B), indicative of activation of the NFϰB pathway. In addition, we showed that the increase of Sca-1 expression *in vitro* was dependent on NFϰB signalling (Figure 3.9 C). These findings, suggest that NFϰB could be mediating the activation of HSCs upon different stimuli. In contrast, treatment of mice with a conditional deletion of p65 led to a normal activation of HSCs (Figure 3.9 E), although the deletion efficiency of p65 was relatively high (Figure 3.9 F). Therefore, it will be necessary to perform further experiments, to analyse whether activation of HSCs upon different stimuli is dependent on NFϰB activation within the HSCs. The use of different mouse models and inhibition of NFϰB signalling *in vivo* might be of use to answer this question.

4.4. The role of HSC activation in the immune response

The activation of TLR signalling upon LPS or polyI:C treatment leads to an increased cycling of HSCs (Essers *et al.*, 2009; Sato *et al.*, 2009) (Figure 3.2). As TLR signalling is important in the first line against all kinds of infections, it is likely that activation of HSCs is a general feature of TLR signalling, initiating a fast production of immune cells to fight the infection and replenish the system after the infection is cleared. An effect of other TLR ligands on HSCs has been shown. Stimulation of HSPCs with the TLR2 ligand Pam3CSK4, the TLR9 ligand ODN1585 as well as LPS led to an increased differentiation of HSPCs towards the myeloid lineage (Nagai *et al.*, 2006; Megías *et al.*, 2012), indicating that LPS and polyI:C are not the only TLR ligands regulating HSC functions. The activation of HSCs into an active cycle, however, appears thus far to be a specific feature of some TLR ligands, rather than a general effect of TLR activation, as only ODN1826 and Flagellin led to a increased cell cycle of HSCs independent of TLR4 signalling (Figure 3.7). However, we only limited our testing to TLR ligands from one company, therefore the treatment of mice with these TLR ligands from another source could actually lead to increased cycling of HSCs.

Nevertheless, our data provide new insight into the role of HSCs in the fight against infection. We could show that LPS treatment, mimicking a bacterial infection, leads to effective activation of dormant HSCs into proliferation, without impairing their self-renewal potential (Figures 3.2, 3.4 and 3.5). LPS signalling leads to a huge burst of cytokine production in the bone marrow and many of these cytokines are probably involved in the regulation of HSC function upon infection. The activation of proliferation of HSCs in this process is most likely only one initial step in the response of HSCs to infection, subsequently leading to differentiation and production of immune effector cells, as well as activation of effector function, that might be exhibited by the HSCs themselves. Furthermore, we can show that cytokines, that have long been known to play a role in the regulation of mature immune cells (reviewed in Borden *et al.*, 2007; Bradley, 2008; Weber *et al.*, 2010), can also influence the cycling behaviour of primitive HSCs and progenitor cells (Figures 3.14, 3.16 and 3.22). This may provide a clever mechanism to simultaneously induce immune effector cells to fight the infection and HSCs to proliferate and subsequently differentiate, saving time and energy. Interestingly, we could show that the pro-inflammatory cytokines TNFα and IL–1β, which are characterised by their ability to induce inflammation and make infections worse (reviewed in Dinarello, 2000), as well as IFNs, that have been shown to inhibit the spread of viruses by blocking infected cells (reviewed in Borden *et al.*, 2007), exhibit very similar effects on the cycling behaviour of HSCs. Future research will be necessary to investigate the functional links between

HSC activation upon pro-inflammatory cytokines and IFNs, as well as the mechanistic differences that might underly these two processes. A common mechanism of action of these cytokines is the initiation of expression of adhesion molecules and chemokines by endothelial cells, leading to the recruitment of immune effector cells to the sites of infection (reviewed in Mogensen, 2009). It is possible that these chemokines and adhesion molecules have similar effects on the behaviour of HSCs, leading to the release of these cells from the endosteal niche to the perivascular niche (Kunisaki *et al.*, 2013), where they start to proliferate and produce progenitor cells. The proximity to the vasculature might then enable the fast release of the produced cells into the periphery, although HSCs themselves are not mobilised upon short-term LPS treatment (data not shown). Furthermore, many cytokines have been shown to regulate proliferation and differentiation of mature immune cells, such as B cells, it is therefore possible that they can exhibit similar functions on HSCs, directly inducing increased proliferation and differentiation.

Our results furthermore suggest that LPS-induced activation of HSCs is mediated by myeloid cells, more specifically, inflammatory monocytes (Figure 3.11) that require cell-cell-interactions with HSCs in order to induce the production of cytokines mediating the activation of HSCs (Figure 3.13). This is a likely scenario, as inflammatory monocytes are the main type of monocytes present in the bone marrow and can be mobilised to the periphery upon infection (Gordon and Taylor, 2005; Robbins and Swirski, 2010; Shi and Pamer, 2011). This mobilisation may lead to an interaction between the HSCs and the monocytes, before the monocytes leave the bone marrow. The monocytes might then be activated to produce cytokines mediating the activation of HSCs to produce more monocytes and perhaps even other immune cells to fight the infection, as well as for the replenishment of the system after the infection is cleared. It has already been shown that HSCs are found in close proximity to macrophages in the bone marrow (Winkler *et al.*, 2010; Chow *et al.*, 2011), it is therefore likely that monocytes can also be found near HSCs. It is consequential that only monocytes that are actually located close to HSCs produce factors to induce activation of HSCs, as these inflammatory cytokines mediate the immune response and their production should thus be tightly regulated. It has furthermore been shown that HSCs themselves produce cytokines in response to infections (Zhao *et al.*, 2014). This might be a way to carry on the production of immune cells in the bone marrow, after the immune effector cells have been mobilised to the periphery. After the infection is cleared, the HSCs return to their dormant state and therefore retain their self-renewal and multilineage reconstitution potential, as long as they are not chronically induced to cycle, ensuring life-long production of blood cells.

4.5. Concluding remarks and future perspective

Our research provides new insights into the regulation of HSC quiescence and proliferation upon infection and the role of inflammatory cytokines in this regulation. This will help to further current understanding of the role of HSCs during infection, as well as the effects of infections on HSC function. We propose a mechanism inducing HSC exhaustion and eventually even BM failure upon chronic infection, due to a constant increase in the cycling activity of HSCs. Future experiments will have to be performed to investigate how the activation of HSCs upon different stimuli is mechanistically linked, as well as the subsequent effects of LPS- and cytokine-induced activation of HSCs on their behaviour and function. Furthermore, it will be interesting to analyse the relationship of different cytokines in the activation of HSCs, as many cytokines can induce production of each other. In addition, we would like to investigate which of these cytokines are actually directly regulating HSCs and which are only leading to indirect effects on HSCs. It will also be worthwhile to analyse if LPS might exhibit direct effects on HSCs, in addition to the indirect effects on HSC activation. It will be of great benefit to identify the common mechanisms leading to HSC activation upon different stimuli, as this will help to gain further insight into the regulation of HSC quiescence. For this purpose, it will be helpful to further investigate the role of NFκB signalling, as well as Sca-1, in the activation of HSCs. Another future research interest is the role of cytokines during homeostatic conditions, as most of the inflammatory cytokines are constantly present in small concentrations and might also influence the behaviour of HSCs during homeostasis. Moreover, a thorough understanding of the effects induced by single cytokines as well as combinations of cytokines, may help to improve the treatment of patients suffering from bone marrow failure due to chronic infections or defective DNA repair mechanism, by blocking certain cytokines. Furthermore, this research can give insight into the behaviour of human HSCs as, in contrast to mice which are kept in a pathogen-free environment, humans are exposed to various pathogens every day. This raises the question, whether a quiescent or dormant HSC actually exists in humans, or whether human HSCs might constantly cycle due to a constant stimulation by infectious particles. Indeed, the constant cycling of HSCs due to the daily exposure with pathogens, might be a cause for HSC defects occurring upon ageing. Therefore, blocking of cytokines leading to HSC activation might also be a treatment route for patients suffering from immune defects or bone marrow failure due to ageing. Hence, our research not only provides new insight into the behaviour of HSCs upon infection, but may also help to improve treatment of patients suffering from immune defects and bone marrow failure.

5. Materials and Methods

5.1. Mouse strains

All mice were housed in individually ventilated cages at the DKFZ animal facility. Animal procedures were performed according to protocols approved by the Regierungspräsidium Karlsruhe (no. G–181/08, G–266/12 and G–157/13). If not indicated differently, the mice are on a C57Bl/6 background.

C57Bl/6 J. C57Bl/6 J mice are referred to as (CD45.2$^+$) wildtype (wt) mice. These mice were purchased from Harlan Laboratories.

B6.SJL–Ptprca–Pep3b–/BoyJ. B6.SJL–Ptprca–Pep3b–/BoyJ mice are referred to as CD45.1$^+$ wt mice. These mice were purchased from Charles River Laboratories, Italy.

B6.Tlr4lps–del/JthJ. B6.Tlr4lps–del/JthJ mice are referred to as $TLR4^{-/-}$ mice. These mice show a defective response to LPS due to a mutation in the $Tlr4$ gene (Vogel $et\ al.$, 1979; Poltorak $et\ al.$, 1998).

B6.Ly6atm1Pmf. B6.Ly6atm1Pmf mice are referred to as $Sca-1^{-/-}$ mice. These mice lack the HSC marker stem cell antigen 1 (Sca–1) and show an engraftment defect of HSCs in competitive transplantations. (Stanford $et\ al.$, 1997; Ito $et\ al.$, 2003).

B6.Cg Ifnar1tm1Agt, B6.Cg–Ifngr1tm1Agt and B6.Cg.Ifnar1tm1Agt Ifngr1tm1Agt. B6.Cg Ifnar1tm1Agt are referred to as $Ifnar^{-/-}$, B6.Cg–Ifngr1tm1Agt are referred to as $Ifngr^{-/-}$ and B6.Cg.Ifnar1tm1Agt Ifngr1tm1Agt are referred to as $Ifnar^{-/-}Ifngr^{-/-}$ mice. The mice lack the receptors for IFNα, IFNγ or both, leading to a defect in the response to these cytokines. (Huang $et\ al.$, 1993; Müller $et\ al.$, 1994).

Stock Tg(Mx1–cre)1Cgn Gt(ROSA)26Sortm1.1(EYFP)Cos.
Stock Tg(Mx1–cre)1Cgn Gt(ROSA)26Sortm1.1(eYFP)Cos mice are referred to as MxCre Rosa–26eYFP flox$_2$ mice. This mouse line was established by crossing the MxCre strain with

the Rosa26eYFP strain. These mice were used to monitor IFNα production in the bone marrow. The mice are on a mixed background as described (Kuhn *et al.*, 1995; Srinivas *et al.*, 2001).

B6.CgTicam1tm1Aki. B6.CgTicam1tm1Aki mice are referred to as *Trifmc*$^{-/-}$ mice. These mice lack the TRIF-adaptor of the TLR4 and TLR3 signalling pathways, leading to defective IFN production upon activation (Yamamoto *et al.*, 2003).

B6.Cg Myd88tm1Aki. B6.Cg Myd88tm1Aki mice are referred to as *Myd88*$^{-/-}$ mice. They lack the MyD88-adaptor, which is part of most TLR signalling pathways. Lack of MyD88 leads to a defective production of cytokines upon TLR signalling (Adachi *et al.*, 1998).

B6;129S-Tnfrsf1atm1Imx Tnfrsf1btm1Imx. B6;129S-Tnfrsf1atm1Imx Tnfrsf1btm1Imx mice are referred to as *Tnfrsf1 dKO* mice. They lack both receptors for TNFα, the TNFR1 and the TNFR2, leading to a defective response to TNFα. Those mice are on a mixed C57Bl/6 and 129S7/SvEvBrd background (Peschon *et al.*, 1998; Pronk *et al.*, 2011).

B6-Tg(Tal1-cre/ERT)42-056Jrg Relatm1Mpa Gt(ROSA)26Sortm1(EYFP)Cos. B6-Tg(Tal1-cre/ERT)42-056Jrg Relatm1Mpa Gt(ROSA)26Sortm1(EYFP)Cos mice are referred to as Scl-CreERT2 p65 flox$_2$ RosaEYFP flox$_2$ mice. These mice were obtained by crossing the SclCreERT2 mouse strain to the p65 flox$_2$ and the RosaEYFP flox$_2$ mouse strain. Through activation of Cre expression by the Tamoxifen inducible Scl-promotor, p65, part of the NF-ϰB molecule, is deleted, resulting in a defect in the canonical NFϰB signalling pathway. Furthermore, the activation of Cre leads to the expression of EYFP, enabling the identification of cells with active Cre expression (Srinivas *et al.*, 2001; Göthert *et al.*, 2005; Luedde *et al.*, 2008).

B6.Relatm2.1Mpa. B6.Relatm2.1Mpa mice are referred to as p65-GFP mice. The cells of these mice express a fusionprotein of GFP and p65 from the endogenous p65 genomic locus, enabling the analysis of NFϰB activation through translocation to the nucleus (de Lorenzi *et al.*, 2009).

B6.129P2-Cxcr3tm1Dgen/J. B6.129P2-Cxcr3tm1Dgen/J mice are referred to as *Cxcr3*$^{-/-}$ mice. Those mice lack Cxcr3, the receptor for CXCL9, CXCL10 and CXCL11 (Deltagen, 2005).

B6.Il6tm1Kopf. B6.Il6tm1Kopf mice are referred to as *IL-6$^{-/-}$* mice. These mice lack the cytokine IL-6, leading to an impaired immune response (Kopf *et al.*, 1994).

B6.Tg(Csf1r–EGFP–NGFR/FKBP1A/TNFRSF6)2Bck. B6.Tg(Csf1r–EGFP–NGFR/ FKBP1A/TNFRSF6)2Bck mice are referred to as Mafia mice. These mice express a drug-inducible suicide gene under the control of the c-fms (CD115) promotor. Upon treatment with the homodimerizer AP20187 apoptosis is induced in all CD115 expressing cells (Burnett *et al.*, 2004).

5.2. *In vivo* treatment of mice

For treatment of HSCs, 8-10 week old mice were injected intraperitoneally (i.p.) with 0.25 mg/kg LPS (*E.coli* 0111:B4, Sigma), 0.75 mg/kg TNFα (Peprotech), 0.125 mg/kg IL–1β (Peprotech), 5 mg/kg polyI:C (Peprotech), 0.5 mg/kg CXCL10 (R&D Systems), 0.05 mg/kg IL-6 (R&D Systems) in 200 µl PBS or subcutaneous (s.c.) with 50x10^5 U/kg IFNα (Miltenyi Biotec) in 100 µl for 18 hours, unless indicated otherwise. Control mice were injected with an equal volume of PBS.

For studies with different TLR ligands, mice were injected i.p. with 1.5 mg/kg Pam3CSK4, 2.5 mg/kg LTA, 2.5 mg/kg PGN, 1.0 mg/kg Flagellin, 1.0 mg/kg ssPolyU, 1.25 mg/kg ODN1585, ODN1826 or ODN2395 (Invivogen) in 200 µl for 18 hours.

For analysis of IFNα production in MxCre Rosa26eYFP flox$_2$ mice, mice were irradiated with 200 or 400 rad or 5-6 drops of blood were taken from the vena facialis.

For p65 deletion in SclCreERT2 p65 flox$_2$ RosaEYFP flox$_2$ mice, mice were treated with Tamoxifen (1 g/kg) in the food for 30 days, followed by 14 days without Tamoxifen.

5.2.1. Depletion of cells from the bone marrow

For depletion of myeloid cells mice were injected i.v. with 3.75 g/kg (300 µl) clodronate-loaded liposomes (van Rooijen *et al.*, 1997). Control mice were injected with an equal volume of PBS-loaded liposomes.

For fas-induced deletion of myeloid cells Mafia mice were injected i.p. with 0.5 mg/kg AP20187 (Clontech) in 200 µl PBS on five consecutive days (Burnett *et al.*, 2004).

To deplete neutrophils mice were injected i.p. with 2.5 mg/kg anti-Ly6G antibody (clone: 1A8) in 200 µl PBS on 2 consecutive days (Casanova–Acebes *et al.*, 2013).

5.2.2. LRC assay

For LRC assays, cells were labeled by one i.p injection of 18 mg/kg BrdU (Sigma) and a 10 day pulse-period with BrdU (1 mg/ml, glucose) in the drinking water, followed by a 70-day BrdU free chase period (Wilson *et al.*, 2008). After the chase period, mice were injected with 0.25 mg/kg LPS or PBS, three times, every third day. Bone marrow analysis was performed 2 weeks after the last LPS injection.

5.2.3. 5-FU treatments

For analysis of IFNα production upon 5-Fluoro-Uracil (5-FU) treatment, mice were injected i.p. with 150 mg/kg 5-FU (300 µl).

For long-term 5-FU treatment mice were initially injected with 0.75 mg/kg TNFα or PBS. The following day, repeated 5-FU treatments with 150 mg/kg 5-FU (300 µl) i.p. were started. The 5-FU treatment was repeated every 10 days until the mice died.

5.2.4. Etanercept and IL-1RA

For inhibition of TNFα Etanercept (Enbrel®,FDA 1998) was injected i.p. for 10 days (5 mg/kg in 100 µl PBS on day 1-5; 7.5 mg/kg in 150 µl PBS on day 6-10); LPS was injected on day 10.

For inhibition of IL-1β signalling 2.5 mg/kg IL-1 receptor antagonist (IL-1RA) (Peprotech) was injected i.p. in 200 µl PBS 1 hour before the LPS treatment.

5.3. Isolation of bone marrow and peripheral blood

To collect bone marrow cells, bones of legs, hips and spines were isolated and crushed in RPMI-1640 medium supplemented with 2% fetal bovine serum (FBS). Cell suspensions were filtered with a 40 µm filter before use.

For isolation of peripheral blood (PBL), 4-5 drops of blood were collected from the vena facialis into an EDTA containing tube. Erythrocytes were lysed with 1 ml of ACK lysing buffer. Cells were washed and used for further analysis.

5.4. Lineage depletion

For depletion of lineage positive (lin⁺) cells, bone marrow cells were incubated with lineage antibodies against CD4, CD8, CD11b, B220, Gr-1 and TER119 for 30 min. After antibody incubation lin⁺ cells were depleted using Dynabeads® Magnetic Beads (Invit-

rogen). Cells were incubated with the beads for 20 min, before non-bound cells were isolated using a magnet.

5.5. Generation of chimeras

Bone marrow was isolated as described. BM cells were Thy1 depleted: Cells were incubated with an anti-Thy1 antibody (AT83) for 20 min, before rabbit complement and Dnase 1 were added. Subsequently cells were incubated for 20 min at 37 °C. For transplantation $3x10^6$ cells were diluted in 100-200 µl of PBS and intravenously (i.v.) injected into lethally irradiated (2x500 rad) mice. For mixed bone marrow chimeras cells were mixed prior to injection in a 1:1 ratio and a FACS analysis of the injected cells was performed to determine the input-ratio. PBL chimerism was assessed by FACS analysis after 4-6 weeks. For long-term reconstitution assay of LPS treated HSCs mice were injected with PBS or LPS 24 hours before bone marrow isolation. Bone marrow was Thy1 depleted, as described. $3x10^6$ cells were diluted in 20 µl PBS and injected intrafemorally (i.f.) into lethally irradiated (2x500 rad) mice. After 4, 8 and 12 weeks PBL chimerism was determined by FACS analysis. After 16 weeks mice were sacrificed. Reconstitution in PBL and BM was analysed and $3x10^6$ whole BM cells from the primary recipients were transplanted i.f. into lethally irradiated (2x500 rad) secondary recipients. Again PBL chimerism was determined after 4, 8 and 12 weeks and reconstitution of PBL and BM was analysed after 16 weeks. All mice were kept on antibiotic (Cotrim) containing water for 3 weeks post transplantation. To enable identification of recipient and donor cells, mice with different CD45 isoforms were used. Identification of donor cells is then possible by FACS staining for CD45.1 and CD45.2.

5.6. FACS analysis

FACS antibodies were purchased from several suppliers (table 5.13.1). All antibodies were titrated with whole bone marrow prior to use in experiments. The antibodies were used in concentration according to this titration, to ensure optimal staining and avoid unspecific signals. Antibodies were diluted in staining buffer (PBS/2% FBS and 24G2, 1:1), to prevent unspecific binding. 100 µl of antibody solution were used for $5-20x10^6$ cells. BM cells were stained for HSC and progenitor subsets using lineage antibodies (CD4, CD8, CD11b, Gr-1, B220 and TER119), cKit, Sca-1, CD150, CD48 and CD34 (Figure 5.1). BM cells were stained for myeloid cells with antibodies against CD11b, Gr-1, CD115, Ly-6C and F4/80 (Figure 5.2). For staining of BM cells for other mature haematopoietic cells anti-

bodies against CD4, CD8, CD41, B220 and TER119 were used. All cell suspensions were filtered through a nylon mesh filter (70 µm) prior to FACS analysis to prevent clumping.

For flow cytometric data analysis the BD™ LSRII or BD LSRFortessa™ (Becton Dickinson™, San Jose, CA), equipped with a 350 nm, 405 nm, 488 nm, 561nm, and 640 nm laser, or the 9 color Cyan™ ADP Analyzer (Beckman Coulter GmbH, Germany), equipped with a 407 nm, 488 nm, and 635 nm laser, were used. Prior to analysis of multicolor samples, OneComp eBeads stained with single antibodies were used to perform compensation using the auto-compensation tool of the BD FACSDiva™ software. Analysis of flow cytometric data was performed using the TreeStar FlowJo software version 9.6.4 or version 8.8.7.
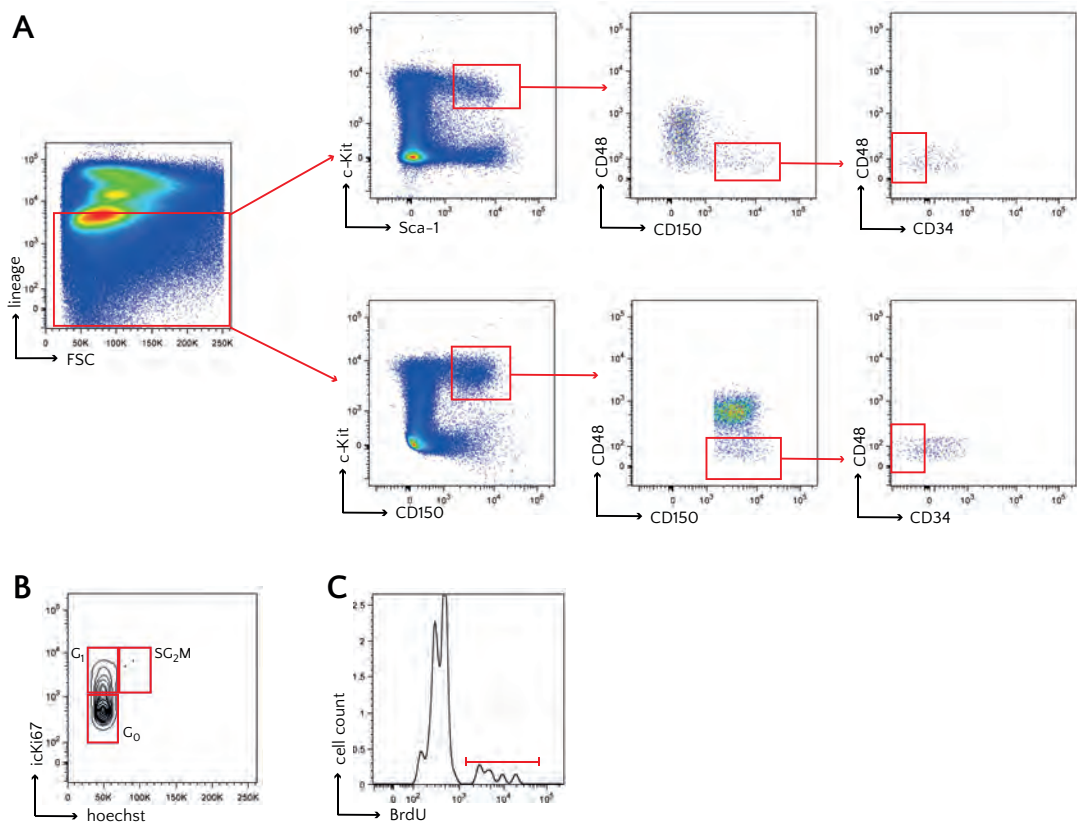

Figure 5.1.: HSC Flow Cytometry gating scheme
(A) Gating scheme for FACS analysis of LSK CD150$^+$CD48$^-$CD34$^-$ (HSCs, upper panel) and LK CD150$^+$CD48$^-$CD34$^-$ (lower panel) cells. (B) Representative FACS profile of HSCs stained for icKi67 and Hoechst. (C) Representative FACS profile of BrdU incorporation in HSCs.
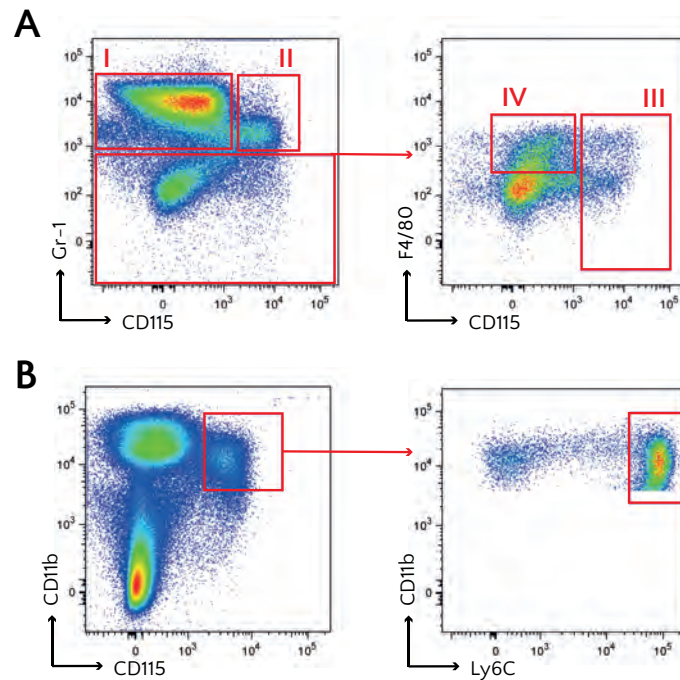
Figure 5.2.: Gating scheme for *in vitro* co-culture experiments
(A) Gating scheme for neutrophils (I), Gr-1^{high} monocytes (II), Gr-1^{low} monocytes (III) and macrophages (V) (Chow *et al.*, 2011). (B) Gating scheme for CD11b$^+$CD115$^+$Ly6C^{high} inflammatory monocytes.

5.7. FACS sorting

To isolate HSCs and progenitor cells, BM was isolated and cells were lineage depleted. For *in vitro* cultures lineage negative cells were stained with antibodies against the lineage markers, c-Kit and CD150. Lin$^-$c-Kit$^+$CD150$^+$ (LK CD150$^+$) cells were sorted. To sort different populations of myeloid cells for *in vitro* culture BM cells were stained with antibodies against CD11b, CD115, Gr-1, F4/80 and Ly-6C. For qRT-PCR or Immunofluorescence lineage negative cells were stained with antibodies against the lineage markers, c-Kit, CD150 and CD48. LK CD150$^+$CD48$^-$ cells were sorted. Cell sorting was performed on a FASC Aria I, FACS Aria II or FACS Aria III (Becton Dickinson™, San Jose, CA) at the DKFZ Flow Cytometry Service Unit.

5.8. Cell cycle analysis and BrdU staining

For cell cycle analysis cell surface staining was performed in combination with intracellular Ki67-Hoechst staining. After cell surface staining BM cells were fixed with BD Cytofix/Cytoperm™ Buffer, washed and stained with anti-Ki67-FITC or -APC for 30 min

or over night. Cells were co-stained with Hoechst 33342 (Molecular Probes) at 25 µg/ml for the final 10 min.

For proliferation assays with BrdU, mice were injected i.p with BrdU (18 mg/kg, Sigma) 14 hours prior to analysis. BM cells were stained with antibodies against surface markers. For BrdU staining the BD Pharmigen™ BrdU Flow Kit was used. Fixation and DNase treatment were performed according to manufacturer's instructions.

5.9. mRNA expression anaylsis

5.9.1. RNA isolation

RNA isolation was performed using the Arcturus PicoPure™ RNA Isolation Kit or the RNeasy Micro Kit (Qiagen). Cells were collected in 50 µl Extraction Buffer and lysed for 30 min at 42°C. Protocol was performed according to manufacturer's instructions, including DNA digestion by RNase-free DNase (Qiagen). RNA was either directly transcribed into cDNA or stored at -80°C for further use.

5.9.2. Reverse transcription

Reverse transcription was performed using the SuperScript®VILO™Synthesis Kit according to manufacturer's instructions. cDNA was diluted in RNase-free water and stored at -20°C until further use.

5.9.3. MicroArray

For expression profiling LK CD150$^+$CD48$^-$ cells from wildtype mice treated with PBS, LPS, IFNα or TNFα or *Ifnar$^{-/-}$Ifngr$^{-/-}$* mice treated with PBS or LPS were sorted directly into 50 µl Extraction Buffer. RNA isolation was performed as described, using the RNeasy Micro Kit. The RNA was frozen at -80°C and further processed by the DKFZ Genomics and Proteomics Service Unit. Quality control of the RNA was performed using a Agilent Bioanalyzer 2100. Samples with a RIN score below 7 were excluded from analysis. Samples were amplified using the Ovation PicoSL WTA system and labeled with the BiotinIL Module from NuGEN. For expression analysis the samples were hybridised to the Illumina MouseWG-6 v2.0 Expression BeadChip®.

5.9.4. Analysis of expression data

After normalisation the expression data was analysed using R. As the MicroArray includes several probes for some genes the data was first aggregated. Subsequently the means were calculated for all replicates of one treatment. These means were then used

to calculate the fold changes between different treatments. Only the genes with a fold change higher than 1.5 or lower than 0.66 were considered as regulated. The genes similarly regulated upon different treatments were identified using Microsoft Office™ Excel. Furthermore, R was used to construct a heat map as well as perform principle component analysis (PCA). For that, the variance was calculated for all genes and the 5000 genes with the highest variance were used for the calculation of the heat map and the principle component analysis. The heat map was constructed using the pheatmap function, the principle component analysis was conducted using the prcomp function. Before these calculations were performed not assigned expression values were replaced by 0.

Examples of R code used

```
data <- read.table(file = "file-location", header=T, dec = ".", sep ="\t")


#Aggregation of data
agg.data <- aggregate(data[,-1], by = data["Symbol"], FUN = mean)


#Fold change calculation
PBS <- data.frame(agg.data[,1], Means = rowMeans(agg.data[,2:7]))
IFN <- data.frame(agg.data[,1], Means = rowMeans(agg.data[,8:13]))
FC.PBS.IFN <- data.frame(agg.data[,1], IFN[,2]/PBS[,2])


#Calculation of variance
variance<-apply(expression[,4:ncol(agg.data)],1,var)
agg.data<-cbind(agg.data, variance)
agg.data<-agg.data[order(agg.data[,"variance"], decreasing=T),]


#Replacement of n.a. expression values by 0
agg.data<-agg.data[which(rowSums(is.na(agg.data))==0),]


#Assigment of matrix
agg.data_selected<-expression[,-1:-3]
agg.data_selected<-apply(agg.data_selected,2,as.numeric)


#Generation of heatmap
pheatmap(agg.data_selected[1:5000,1:ncol(agg.data_selected)-1],
```

```
scale="row",clustering_distance_cols="euclidean", clustering_method="ward",
color=colorRampPalette(c("darkblue","blue","yellow","orange"))(256),
treeheight_row=0)

#PCA and PCA plot
pca<-prcomp(t(agg.data_selected[1:5000, 1:ncol(agg.data_selected)-1]))
IDstype<-IDstype<-substring(colnames(agg.data_selected)
[-ncol(agg.data_selected)],4,99)
colors<-brewer.pal(6,"Set1")
IDstoEdge<-c("wt.PBS"=colors[1],"wt.IFN"=colors[2],"wt.LPS"=colors[3],
"wt.TNFa"=colors[4], "IFNRdKO.PBS"=colors[5], "IFNRdKO.LPS"=colors[6])
colors<-IDstoEdge[IDstype]
ar(font=2, font.axis=2, font.lab=2)
plot(pca$x[,1:2], col=colors, pch=20)
legend("topleft", legend=c("WT PBS", "WT IFN", "WT LPS", "WT TNFa",
"IFNRdKO PBS", "IFNRdKO LPS"), fill=brewer.pal(6, "Set1"), ncol=2)
```

5.9.5. Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR) RNA isolation, using the Arcturus PicoPure™ RNA Isolation Kit, and reverse transcription were performed as described. 384-well plates were used to set up qRT-PCR reactions, with primer (table 5.1) concentrations of 0.5 μM and a 2x-DNA-polymerase-SYBR-Green master-mix from Applied Biosystems. qRT-PCR was performed using the Applied Biosystems ViiA™ 7 Real-Time PCR System with an initial denaturation step of 95°C for 10 min followed by 40 cycles of 15 s 95°C and 60 seconds 60°C. Expression values were normalized to *sdha* and *oaz1* levels. Relative gene expression was calculated by comparative $\Delta\Delta$CT-method.

5.10. Protein expression analysis

5.10.1. Immunofluorescence

For immnuofluorescence stainings cells were sorted into PBS/2% FBS. Subsequently cells were coated on Polysine™ slides for 20 min. Fixation of cells was performed using BD Cytofix/Cytoperm™ Buffer for 10 min. After washing with PBS slides were stored at -20°C. Cells were blocked with PBS/10% BSA/0.3% Triton X-100 for 1 hour. Primary antibodies were diluted in PBS/1% BSA/0.3% Triton X-100 and staining was performed

gene	primer	sequence	product [nt]
sdha	fwd	AAGTTGAGATTTGCCGATGG	74
sdha	rev	TGGTTCTGCATCGACTTCTG	74
oaz1	fwd	TTTCAGCTAGCATCCTGTACTCC	77
oaz1	rev	GACCCTGGTCTTGTCGTTAGA	77
p57	fwd	TCTCGGGGATTCCAGGAC	136
p57	rev	ACGTTTGGAGAGGACACC	136
serpina3g	fwd	CAGGAACCAAGGATCTGAGAGT	104
serpina3g	rev	GACACCTGCCATTCCTGTG	104
socs3	fwd	CACACAAGGAGCCAAACACA	59
socs3	rev	TAGCCACCTGGGTGAATCC	59
ccl5	fwd	TGCAGAGGACTCTGAGACAGC	149
ccl5	rev	GAGTGGTGTCCGAGCCATA	149
apbb1	fwd	CCTGTTGGGGTAGACGTGAT	60
apbb1	rev	ACGGCTACTGGAAGACAGGA	60
foxa3	fwd	CCAAACCACCATATTCCTACATC	77
foxa3	rev	ACTCAGGGTCAGCATCTTGC	77
cxcl4	fwd	TGGGATCCATCTTAAGCACA	93
cxcl4	rev	CCATTCTTCAGGGTGGCTAT	93
p65	fwd	TGGCGAGAGAAGCACAGATA	135
p65	rev	CCCACAAGTTCATGTGGATG	135

Table 5.1.: List of qRT-PCR primers

at 4°C over night or for 2 hours at RT. After washing 3 times with PBS, cells were again blocked with PBS/10% BSA/0.3% Triton X-100 for 15 min. Secondary antibodies were diluted in PBS/1% BSA/0.3% Triton X-100 and staining was performed at RT for 2 hours. Before slides were covered with ProLong® Gold Antifade Reagent with DAPI, they were washed 3 times with PBS. Microscopy was performed using a Zeiss LSM 700 confocal microscope. Analysis of microscopic images was performed using the ImageJ software (Abramoff *et al.*, 2004). Co-localisation analysis was performed using the JaCop plugin for ImageJ. This plugin enables the measurement of the co-localisation of two signals using the Mender's coefficient (Bolta and Cordelieres, 2006).

5.10.2. ELISA

For ELISA, bones of legs and hips were flushed or crushed in 750 µl RPMI/2% FBS. ELISAs were performed according to manufacturer's instructions using different ELISA kits (5.13.2). Absorbance was measured using the SpectraMax M5 Microplate Reader from Molecular Devices.

5.11. *In vitro* cultures

Lineage depleted or sorted cells were cultured in StemPro®-34 SFM medium supplemented with L-Glutamine, Penicillin/Streotpmycin, Flt3 (50 ng/ml), mTPO (50 ng/ml) and mSCF (50 ng/ml) for 18-20 hours at 37°C, 5% CO_2. Cells were treated with 100 ng/ml LPS or 1000 U/ml IFNα. For cell cycle analysis BrdU (10 μM) was added to the medium for the final 3 hours of culture. Subsequently cells were harvested and stained for FACS analysis.

For co-culture experiments sorted LK CD150$^+$ cells (HSPCs) were cultured with different subsets of mature haematopoietic cells in a 2:1 ratio. To enable identification of sorted HSPCs by Flow Cytometry, HSPCs were sorted from CD45.1$^+$ mice, while differentiated cells were sorted from CD45.2$^+$ mice or vice versa.

For NF$\varkappa$B inhibition lineage depleted cells were cultured with 10 μg/μl JSH-23 or 100 μg/ml PDTC and treated with PBS or LPS (100 ng/ml) for 18 hours.

For transwell experiments, LK CD 150$^+$ cells were sorted into the lower well of a transwell plate. The cells were then culture either alone, in co-culture with CD11b$^+$CD115$^+$ cells in the same well or separated through a 4 μm thick filter, to avoid cell-cell-contact. The cells were treated with LPS (100 ng/ml) or PBS, by pipetting the LPS or PBS into the upper well.

Conditioned medium was obtained by culturing lineage depleted cells, treated with PBS or LPS. After 18 hours cells were harvested and pelleted. Supernatant was used to treat freshly isolated lineage depleted cells for another 18 hours in culture.

5.12. Statistics

For statistical analysis and graphical representation of data the GraphPad Prism® software version 6.0 was used. Statistical analysis was performed using an unpaired *t-test*. Statistical significance is indicated by * $p < 0.05$ or ** $p < 0.01$. Error bars indicate the standard deviation. Each graph represents similar results of two to three independent experiments with at least three mice per condition.

5.13. Materials

5.13.1. Antibodies

Antigen	Label	Clone	Company
B220	PECy5, PECy7	RA3–6B2	eBioscience
B220	biotin	RA3–6B2	BioLegend
CD4	PECy7	GK1.5	eBioscience
CD4	biotin	GK1.5	BioLegend
CD8a	PECy7	53–6.7	eBioscience
CD8a	biotin	53–6.7	BioLegend
CD11b	biotin	M1/70	BioLegend
CD11b	PECy7	M1/70	eBioscience
CD34	Alexa700, FITC	RAM34	eBioscience
CD45.1	FITC	A20.1	eBioscience
CD45.2	Pacific Blue	104	BioLegend
CD45.2	Alexa700	104	BD Pharmigen
CD48	PE	HM48-1	eBioscience
CD48	Pacific Blue	HM48-1	BioLegend
CD115	APC	AFS98	eBioscience
CD150	PECy5, APC	TC15-12F12.2	BioLegend
c–Kit	PE, APC, eFluor780	2B8	eBioscience
F4/80	FITC	CI:A3-1	AbD Serotec
F4/80	eFluor450	BM8	eBioscience
GFP	unlabelled	–	Invitrogen
Gr–1 (Ly6G)	PE, PECy7	RB6–8C5	eBioscience
Gr–1	biotin	RB6–8C5	BioLegend
Ki67	FITC, APC	B56	BD Pharmigen
Ly–6C	APCCy7	HK1.4	BioLegend
NK1.1	PE	PK136	eBioscience
Sca–1	APCCy7	D7	BD Pharmigen
Streptavidin	PETexasRed	–	BD Pharmigen
TER119	PECy7, APCCy7	TER119	eBioscience
TER119	biotin	TER119	BioLegend

Table 5.2.: List of FACS antibodies

5.13.2. Kits

Name	Company	Order no.
FITC BrdU Flow Kit	BD Pharmigen	559619
APC BrdU Flow Kit	BD Pharmigen	552598
SuperScript® VILO™ cDNA Synthesis Kit	Life Technologies	11754250
Arcturus® PicoPure® RNA Isolation Kit	Life Technologies	KIT0204
RNeasy Micro Kit	Qiagen	74004
Mouse TNF–alpha Quantikine ELISA Kit	R&D Systems	MTA00B
Mouse IL–1 beta/Il–1F2 Quantikine ELISA Kit	R&D Systems	MLB00C
Mouse CXCL10/Ip–10/CRG–2 Quantikine ELISA Kit	R&D Systems	MCX100
Mouse IFN–gamma Quantikine ELISA Kit	R&D Systems	MIF00
VeriKine™ Mouse Interferon Alpha ELISA Kit	PBL Assay Science	42120–1
VeriKine™ Mouse Interferon Beta ELISA Kit	PBL Assay Science	42400–1
Mouse IL–6 ELISA Kit	Thermo Scientific	EM2IL6

Table 5.3.: List of kits

Bibliography

Abramoff, M. D., Magalhaes, P. J. and Ram, S. J. (2004) Image processing with ImageJ. *Biophotonics International* **11**(7):36–42.

Abramson, S., Miller, R. G. and Phillips, R. A. (1977) The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. *The Journal of Experimental Medicine* **145**(6):1567–1579.

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. and Akira, S. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**(1):143–150.

Adolfsson, J., Borge, O. J., Bryder, D., Theilgaard-Mönch, K., Åstrand-Grundström, I., Sitnicka, E., Sasaki, Y. and Jacobsen, S. E. W. (2001) Upregulation of Flt3 expression within the bone marrow Lin⁻Sca-1⁺c-kit⁺ stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity* **15**(4):659–669.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O.-J., Thoren, L. A. M., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M. and Jacobsen, S. E. W. (2005) Identification of Flt3⁺ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: a revised road map for adult blood lineage commitment. *Cell* **121**(2):295–306.

Akashi, K., Traver, D., Miyamoto, T. and Weissman, I. L. (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**(6774):193–197.

Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. *Nature Reviews Immunology* **4**(7):499–511.

Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A. (2001) Recognition of double-stranded RNA and activaiton of NF-kappaB signalling by Toll-like receptor 3. *Nature* **413**(6857):732–738.

Alexopoulou, L., Thomas, V., Schnare, M., Lobet, Y., Anguita, J., Schoen, R. T., Medzhitov, R., Fikrig, E. and Flavell, R. A. (2002) Hyporesponsiveness to vaccination with Borrelia

burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. *Nature Medicine* **8**(8):878–884.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G. Y. and Suda, T. (2004) Tie2/Angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**(2):149–161.

Asselin-Paturel, C., Brizard, G., Pin, J.-J., Briere, F. and Trinchieri, G. (2003) Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *The Journal of Immunology* **171**(12):6466–6477.

Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C. and Goodell, M. A. (2010) Quiescent haematopoietic stem cells are activated by IFNγ in response to chronic infection. *Nature* **465**(7299):793–797.

Baldrige, M. T., King, K. Y. and Goodell, M. A. (2011) Inflammatory signals regulate hematopoietic stem cells. *Trends in Immunology* **32**(2):57–65.

Baltimore, D. (2011) NF-κB is 25. *Nature Immunology* **12**(8):683–685.

Becker, A. J., McCulloch, E. A. and Till, J. E. (1963) Cytolocical demonstation of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197**(4866):452–454.

Bhatia, R., McGlave, P. B., Miller, J. S., Wissink, S., Lin, W. N. and Verfaillie, C. M. (1997) A clinically suitable ex vivo expansion culture system for LTC-IC and CFC using stroma-conditioned medium. *Experimental Hematology* **25**(9):980–991.

Björkbacka, H., Fitzgerald, K. A., Huet, F., Li, X., Gregory, J. A., Lee, M. A., Ordija, C. M., Dowley, N. E., Golenbock, D. T. and Freeman, M. W. (2004) The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. *Physiological Genomics* **19**(3):319–330.

Bolta, S. and Cordelieres, F. P. (2006) A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy* **224**(3):213–232.

Borden, E. C., Sen, G. C., Uze, G., Silverman, R. H., Ransohoff, R. M., Foster, G. R. and Stark, G. R. (2007) Interferons at age 50: past, current and future impact on biomedicine. *Nature Reviews Drug Discovery* **6**(12):975–990.

Bradford, G. B., Williams, B., Rossi, R. and Bertoncello, I. (1997) Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Experimental Hematology* **25**(5):445–453.

Bradley, J. R. (2008) TNF-mediated inflammatory disease. *Journal of Pathology* **214**(2):149–160.

Broxmeyer, H. E., Williams, D. E., Lu, L., Cooper, S., Anderson, S. L., Beyer, G. S., Hoffman, R. and Rubin, B. Y. (1986) The suppressive influences of human tumor necrosis factros on bone marrow hematopoietic progenitor cells from normal donors and patients with leukemia: syergism of tumor necrosis factor and interferon-γ *The Journal of Immunology* **136**(12):4487–4495.

de Bruin, A. M., Demirel, Ö., Hooibrink, B., Brandts, C. H. and Nolte, M. A. (2013) Interferon-γ impairs proliferation of hematopoietic stem cells in mice. *Blood* **121**(18):3578–3585.

Bryder, D., Remsfjell, V., Dybedal, I., Theilgaard-Mönch, K., Högerkorp, C.-M., Adolfsson, J., Borge, O. J. and Jacobsen, S. E. W. (2001) Self-renewal of multipotent long-term repopulating hematopoietic stem cells is negatively regulated by Fas and Tumor Necrosis Factor Receptor Activation. *The Journal of Experimental Medicine* **194**(7):941–952.

Burnett, S., Kershen, E., Zhang, J., Zhang, L., Straley, S., Kaplan, A. and Cohen, D. (2004) Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. *Journal of Leukocyte Biology* **75**(4):612–623.

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. and Rafii, S. (2010) Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* **6**(3):251–264.

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. and Scadden, D. T. (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**(6960):841–846.

Camargo, F. D., Chambers, S. M., Drew, E., McNagny, K. M. and Goodell, M. A. (2006) Hematopoietic stem cells do not engraft with absolute efficiencies. *Blood* **107**(2):501–507.

Casanova-Acebes, M., Pitaval, C., Weiss, L. A., Nombela-Arrieta, C., Chèvre, R., A-González, N., Kunisaki, Y., Zhang, D., van, N., Silberstein, L. E., Weber, C., Nagasawa, T., Frenette, P. S., Castrillo, A. and Hidalgo, A. (2013) Rhytmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* **153**(5):1025–1035.

Cashman, J., Henkelman, D., Humphries, K., Eaves, C. and Eaves, A. (1983) Individual BFU-E in polycythemia vera produce both erythropoietin dependent and independent progeny. *Blood* **61**(5):876–884.

Chen, C., Liu, Y., Liu, Y. and Zheng, P. (2010) Mammalian target of rapamycin activation underlies HSC defects in autoimmune disease and inflammation in mice. *The Journal of Clinical investigation* **120**(11):4091–4101.

Cheshier, S. H., Morrison, S. J., Liao, X. and Weissman, I. L. (1999) *In vivo* proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proceedings of the National Academy of Sciences* **96**(6):3120–3125.

Choi, H.-H., Kim, K.-K., Kim, K. D., Kim, H.-J., Jo, E.-K. and Song, C.-H. (2011) Effects of mycobacterial infection on proliferation of hematopoietic precursor cells. *Microbes and Infection* **13**(14-15):1252–1260.

Chow, A., Lucas, D., Hidalgo, A., Méndez-Ferrer, S., Hashimoto, D., Scheiermann, C., Battista, M., Leboeuf, M., Prophete, C., van Rooijen, N., Tanaka, M., Merad, M. and Frenette, P. S. (2011) Bone marrow CD169$^+$ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *The Journal of Experimental Medicine* **208**(2):261–271.

Christopher, M. J., Liu, F., Hilton, M. J., Long, F. and Link, D. C. (2009) Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization. *Blood* **114**(7):1331–1339.

Christopher, M. J., Rao, M., Liu, F., Woloszynek, J. R. and Link, D. C. (2011) Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematipoietic progenitor mobilization by G-CSF in mice. *The Journal of Experimental Medicine* **208**(2):251–260.

Cobas, M., Wilson, A., Ernst, B., Mancini, S. J. C., MacDonald, H. R., Kemler, R. and Radtke, F. (2004) β-catenin is dispensable for hematopoiesis and lymphopoiesis. *The Journal of Experimental Medicine* **199**(2):221–229.

Conneally, E., Cashman, J., Petzer, A. and Eaves, C. (1997) Expansion *in vitro* of transplantable human cord blood stem cells demonstrated using a quantitative assay of

their lympho-myeloid repopulating activity in nonobese diabetic–*scid/scid* mice. *Proceedings of the National Academy of Sciences* **94**(18):9836–9841.

Coulombel, L., Eaves, A. C. and Eaves, C. J. (1983) Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hematopoietic progenitors in the adherent layer. *Blood* **62**(2):291–297.

Decker, T., Müller, M. and Stockinger, S. (2005) The yin and yang of type I interferon activity in bacterial infection. *Nature Reviews Immunology* **5**(9):675–687.

Deltagen, I. (2005) NIH initiative supporting placement of Deltagen, Inc. mice into public repositories. *MGI Direct Data Submission* .

Dexter, T. M., Allen, T. D. and Lajtha, L. G. (1977) Conditions controlling the proliferation of hematopoietic stem cells in vitro. *Journal of Cellular Physiology* **91**(3):335–344.

Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. and e Sousa, C. R. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**(5663):1529–1531.

Dinarello, C. A. (2000) Proinflamamtory Cytokines. *CHEST Journal* **118**(2):503–508.

Dinarello, C. A. (2009) Immunological and inflammatory functions of the interleukin-1 family. *Annuel Reviews of Immunology* **27**:519–550.

Ding, L. and Morrison, S. J. (2013) Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**(7440):231–235.

Domen, J., Wagers, A. and Weissman, I. L. (2006) *Regenerative Medicine (Chapter 2)*. Deparment of Health and Human Services.

Dybedal, I., Bryder, D., Fossum, A., Rusten, L. S. and Jacobsen, S. E. W. (2001) Tumor necrosis factor (TNF)–mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. *Blood* **98**(6):1782–1791.

Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., Lee, S.-J., Brinkman, R. and Eaves, C. (2007) Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell* **1**(2):218–229.

Ehninger, A. and Trumpp, A. (2011) The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *The Journal of Experimental Medicine* **208**(3):421–428.

Esplin, B. L., Shimazu, T., Welner, R. S., Garrett, K. P., Nie, L., Zhang, Q., Humphrey, M. B., Yang, Q., Borghesi, L. A. and Kincade, P. W. (2011) Chronic Exposure to a TLR Ligand Injures Hematopoietic Stem Cells. *The Journal of Immunology* **186**(9):5367–5375.

Essers, M. A. G., Offner, S., Blanco-Bose, W. E., Waibler, Z., Kalinke, U., Duchosal, M. A. and Trumpp, A. (2009) IFNα activates dormant haematopoietic stem cells *in vivo. Nature* **458**(7240):904–909.

Feng, C. G., Weksberg, D. C., Taylor, G. A., Sher, A. and Goodell, M. A. (2008) The p47 GTPase Lrg-47 (Irgm1) links host defense and hematopoietic stem cell proliferation. *Cell Stem Cell* **2**(1):83–89.

Fleming, H. E., Janzen, V., Celso, C. L., Guo, J., Leahy, K. M., Kronenberg, H. M. and 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**(3):274–283.

Ford, C. E., Hamerton, J. L., Barnes, D. W. H. and Loutit, J. F. (1956) Cytological identification of radiation-chimeras. *Nature* **177**(4506):452–454.

Forsberg, E. C., Serwold, T., Kogan, S., Weissman, I. L. and Passegue, E. (2006) New evidence supporting megakaryocyte-erythrocyte potential of Flk2/Flt3$^+$ multipotent hematopoietic progenitors. *Cell* **126**(2):415–426.

Fujisaki, J., Wu, J., Carlson, A. L., Silberstein, L., Putheti, P., Larocca, R., Gao, W., Saito, T. I., Celso, C. L., Tsuyuszaki, H., Sato, T., Cote, D., Sykes, M., Strom, T. B., Scadden, D. T. and Lin, C. P. (2011) In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* **474**(7350):216–219.

Gammaitoni, L., Bruno, S., Sanavio, F., Gunetti, M., Kollet, O., Cavalloni, G., Falda, M., Fagioli, F., Lapidot, T., Aglietta, M. and Piacibello, W. (2003) Ex vivo expansion of human adult stem cells capable of primary and secondary hemopoietic reconstitution. *Experimental Hematology* **31**(3):261–270.

Geissmann, F., Jung, S. and Littman, D. R. (2003) Blood monocytes consist of two principle subsets with distinct migratory properties. *Immunity* **19**(1):71–82.

Gordon, S. and Taylor, P. R. (2005) Monocyte and macrophage heterogeneity. *Nature Reviews Immunology* **5**(12):953–964.

Göthert, J., Gustin, S., Green, M., Göttgens, B., Izon, D. and Begley, C. (2005) In vivo fate-tracing studies using the Scl stem cell enhancer: embryonic hematopoietic stem cells significantly contribute to adult hematopoiesis. *Blood* **105**(7):2724–2732.

Grassinger, J., Haylock, D. N., Williams, B., Olsen, G. H. and Nilsson, S. K. (2010) Phenotyp-ically identical hematopoietic stem cells isolated from different regions of the bone marrow have different biologic potential. *Blood* **116**(17):3185–3196.

Greenbaum, A., Hsu, Y.-M. S., Day, R. B., Schuettpelz, L. G., Christopher, M. J., Borgerding, J. N., Nagasawa, T. and Link, D. C. (2013) CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* **495**(7440):227–230.

Groom, J. R. and Luster, A. D. (2011) CXCR3 ligands: redundant, collaborative and antag-onistic functions. *Immunology and Cell Biology* **89**(2).

Guo, G., Luc, S., Marco, E., Lin, T.-W., Peng, C., Kerenyi, M. A., Beyaz, S., Kim, W., Xu, J., Das, P. P., Neff, T., Zou, K., Yuan, G.-C. and Orkin, S. H. (2013) Mapping cellular hierarchy by single-cell analysis of the cell surface repertoire. *Cell* **13**(4):492–505.

Guo, W., Lasky, J. L., Chang, C. J., Mosessian, S., Lewis, X., Xiao, Y., Yeh, J. E., Chen, J. Y., Iruela-arispe, M. L., Varella-Garcia, M. and Wu, H. (2008) Multi-genetic events collabo-ratively contribute to Pten-null leukaemia stem-cell formation. *Nature* **453**(7194):529–533.

Haller, O., Staeheli, P. and Kochs, G. (2007) Interferon-induced Mx proteins in antiviral host defense. *Biochimie* **89**(6-7):812–818.

Hanoun, M. and Frenette, P. S. (2013) This niche is a maze: an amazing niche. *Cell Stem Cell* **12**(4):391–392.

Harrison, D. E. (1980) Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood* **55**(1):77–81.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M. and Aderem, A. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**(6832):1099–1103.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H. and Bauer, S. (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**(5663):1526–1529.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Matsumoto, H., Hoshino, K., Wag-ner, H., Takeda, K. and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**(6813):740–745.

Horisberger, M. A., Staeheli, P. and Haller, O. (1983) Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. *Proceedings of the National Academy of Sciences* **80**(7):1910–1914.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Hyporesponsive to Lipopolysaccharide: Evidence for TLR4 as the Lps Gene Product. *The Journal of Immunology* **162**(7):3749–3752.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R. M. and Aguet, M. (1993) Immune response in mice that lack the interferon-gamma receptor. *Science* **259**(5102):1742–1745.

Ikuta, K. and Weissman, I. L. (1992) Evidence that hematopoietic stem cells express mouse *c-kit* but do not depend on steel factor for their generation. *Proceedings of the National Academy of Sciences* **89**(4):1502–1506.

Ito, C. Y., Li, C. Y. J., Bernstein, A., Dick, J. E. and Stanford, W. L. (2003) Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. *Blood* **101**(2):517–523.

Ivashikiv, L. B. and Donlin, L. T. (2014) Regulation of type I interferon respsonses. *Nature Reviews Immunology* **14**(1):36–49.

Jacobsen, F. W., Rothe, M., Rusten, L., Goeddel, D. V., Smeland, E. B., Veiby, O. P., Slordal, L. and Jacobsen, S. E. W. (1994) Role of the 75-kDa tumor necrosis factor receptor: Inhibition of early hematopoiesis. *Proceedings of the National Academy of Sciences* **91**(22):10695–10699.

Jacobsen, S. E. W., Ruscetti, F. W., Dubois, C. M. and Keller, J. R. (1992) Tumor Necrosis Factor α directly and indirectly regulates hematopoietic progenitor cell proliferation: role of colony-stimulating factor receptor modulation. *The Journal of Experimental Medicine* **175**(6):1759–1772.

Johns, J. L., MacNamara, K. C., Walter, N. J., Winslow, G. M. and Borjesson, D. L. (2009) Infection with *Anaplasma phagocytophilum* induces multilineage alterations in hematopoietic progenitor cells and peripheral blood cells. *Infection and Immunity* **77**(9):4070–4080.

Juntilla, M. M., Patil, V. D., Calamito, M., Joshi, R. P., Birnbaum, M. J. and Koretzky, G. A. (2010) AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. *Blood* **115**(20):4030–4038.

Kaisho, T., Takeuchi, O., Hoshino, K. and Akira, S. (2001) Endotoxin-induced maturation of MyD88-deficient dendritic cells. *The Journal of Immunology* **166**(9):5688–5694.

Karlsson, G., Blank, U., Moody, J. L., Ehninger, M., Singbrant, S., Deng, C. X. and Karlsson, S. (2007) Smad4 is critical for self-renewal of hematopoietic stem cells. *The Journal of Experimental Medicine* **204**(3):467–474.

Katayama, Y., Battista, M., Kao, W.-M., Hidalgo, A., Peired, A. J., Thomas, S. A. and Frenette, P. S. (2006) Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* **124**(2):407–421.

Kaulen, D. R., Sanin, A. V., Khorobrykh, V. V., Malikov, V. E. and Dranovskaia, E. A. (1983) [Stimulating action of Brucella melitensis lipopolysaccharide on hemopoiesis in mice]. *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii* **8**:84–87.

Kawai, T. and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology* **11**(5):373–384.

Kiel, M. J., He, S., Ashkenazi, R., Gentry, S. N., Teta, M., Kushner, J. A., Jackson, T. L. and Morrison, S. J. (2007) Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. *Nature* **449**(7159):238–242.

Kiel, M. J., Yilmaz, Ö. H., Iwashita, T., Yilmaz, O. H., Terhorst, C. and Morrison, S. J. (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches from stem cells. *Cell* **121**(7):1109–1121.

Kim, I., Saunders, T. L. and Morrison, S. J. (2007) Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell* **130**(3):470–483.

Kim, J., Oh, Y.-K., Youn, J. and Ahn, M.-J. (2004) *Escherichia coli* up-regulates proinflammatory cytokine expression in granulocyte/macrophage lineages of CD34$^+$ stem cells via p50 homodimeric NF-$\varkappa$B. *Clinical and Experimental Immunology* **137**(2):341–350.

Kimura, S., Roberts, A. W., Metcalf, D. and Alexander, W. S. (1998) Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proceedings of the National Academy of Sciences* **95**(3):1195–1200.

King, K. Y., Baldridge, M. T., Weksberg, D. C., Chambers, S. M., Lukov, G. L., Wu, S., Boles, N. C., Jung, S. Y., Qin, J., Liu, D., Songyang, Z., Eissa, N. T., Taylor, G. A. and Goodell, M. A. (2011b) Irgm1 protects hematopoietic stem cells by negative regulation of IFN signaling. *Blood* **118**(6):1525–1533.

King, K. Y., Essers, M., Trumpp, A. and Goodell, M. A. (2013) Interferons promote cell division and inhibit self-renewal of HSCs in vivo. *Blood* **eLetter**.

King, K. Y. and Goodell, M. A. (2011a) Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. *Nature Reviews Immunology* **11**(10):685–692.

Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E. and Nerlov, C. (2006) Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nature Immunology* **7**(10):1048–1056.

Kobayashi, H., Butler, J. M., O'Donnell, R., Kobayashi, M., Ding, B. S., Bonner, B., Chiu, V. K., Nolan, D. J., Shido, K., Benjamin, L. and Rafii, S. (2010) Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature Cell Biology* **12**(11):1046–1056.

Kondo, M., Weissman, I. L. and Akashi, K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**(5):661–672.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H. and Köhler, G. (1994) Impaired immune and acute-phase response in interleukin-6-deficient mice. *Nature* **368**(6469):339–342.

Kozar, K., Ciemerych, M. A., Rebel, V. I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Genh, Y., Yu, Q., Bhattacharya, S., Bronson, R. T., Akashi, K. . and Sicinski, P. (2004) Mouse development and cell proliferatoin in the absence of D-cyclins. *Cell* **118**(4):477–491.

Kuhn, R., Schwenk, F., Aguet, M. and Rajewski, K. (1995) Inducible gene targeting in mice. *Science* **269**(5229):1427–1429.

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., Mar, J. C., Bergman, A. and Frenette, P. S. (2013) Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* **502**(7473):637–643.

Kushida, T., Inaba, M., Hisha, H., Ichioka, N., Esumi, T., Ogawa, R., Iida, H. and Ikehara, S. (2001) Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune disease in MRL/lpr mice. *Blood* **97**(10):3292–3299.

Lai, A. Y. and Kondo, M. (2006) Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *The Journal of Experimental Medicine* **203**(8):1867–1873.

Langer, J. C., Henckaerts, E., Orenstein, J. and Snoeck, H. W. (2004) Quantitative trait analysis reveals transforming growth factor-beta2 as a positive regulator of early hematopoietic progenitor and stem cell function. *The Journal of Experimental Medicine* **199**(1):5–14.

Larsson, J., Blank, U., Helgadottir, H., Björnsson, J. M., Ehninger, M., Goumans, M. . J., Fan, X., Leveen, P. and Karlsson, S. (2003) TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. *Blood* **102**(9):3129–3135.

Larsson, J., Goumans, M. J., Sjörstrand, L. J., van Rooijen, M. A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C. L. and Karlsson, S. (2001) Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *The EMBO Journal* **20**(7):1663–1673.

Lemischka, I. R., Raulet, D. H. and Mulligan, R. C. (1986) Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* **45**(6):917–927.

Lester, S. N. and Li, K. (2014) Toll-like receptors in antiviral innate immunity. *Journal of Molecular Biology* **426**(6):1246–1264.

Li, W., Johnson, S. A., Shelley, W. C. and Yoder, M. C. (2004) Hematopoietic stem cell repopulating ability can be maintained in vitro by some primary endothelial cells. *Experimental Hematology* **32**(12):1226–1237.

Liu, S. F., Ye, X. and Malik, A. B. (1999) Inhibition of NF-$\varkappa$B activation by pyrrolidine dithiocarbamate prevents in vivo expression of proinflammatory genes. *Circulation* **100**(12):1330–1337.

Lorenz, E., Uphoff, D., Reid, T. R. and Shelton, E. (1951) Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *Journal of the National Cancer Institute* **12**(1):197–201.

de Lorenzi, R., Gareus, R., Fengler, S. and Pasparakis, M. (2009) GFP-p65 Knock-in Mice as a Tool to Study NF-$\varkappa$B Dynamics In Vivo. *Genesis* **47**(5):323–329.

Luca, K. D., Frances-Duvert, V., Asensio, M.-J., Ihsani, R., Debien, E., Taillardet, M., Verhoeyen, E., Bella, C., Lantheaume, S., Genestier, L. and Defrance, T. (2009) The TLR1/2 agonist PAM_3CSK_4 instructs commitment of human hematopoietic stem cells to a myeloid cell fate. *Leukemia* **23**(11):2063–2074.

Luedde, T., Heinrichsdorff, J., de Lorenzi, R., de Vos, R., Roskams, T. and Pasparakis, M. (2008) IKK1 and IKK2 cooperate to maintain bile duct integrity in the liver. *Proceedings of the National Academy of Sciences* **105**(28):9733–9738.

Luis, T. C., Weerkamp, F., Naber, B. A. E., Baert, M. R. M., de Haas, E. F. E., Nikolic, T., s. Heuvelmans, Krijger, R. R. D., van Dongen, J. J. M. and Staal, F. J. T. (2009) Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood* **113**(3):546–554.

Malumbres, M., Sotillo, R., Santamaria, D., Galan, J., Cerezo, A., Ortega, S., Dubus, P. and Barbacid, M. (2004) Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. *Cell* **118**(4):493–504.

Massberg, S., Schaerli, P., Knezevic-Maramica, I., Köllnberger, M., Tubo, N., Moseman, E. A., Huff, I. V., Junt, T., Wagers, A. J., Mazo, I. B. and von Andrian, U. H. (2007) Immuno-surveillance by hematopoietic progentior cells trafficking through blood, lymph, and peripheral tissues. *Cell* **131**(5):994–1008.

Matsumoto, A., Takeishi, S., Kanie, T., Susaki, E., Onoyama, I., Tateishi, Y., Nakayama, K. and Nakayama, K. I. (2011) p57 is required for quiescence and maintenance of adult hematopoietic stem cells. *Cell Stem Cell* **9**(3):262–271.

McCarthy, K. F., Ledney, G. D. and Mitchell, R. (1977) A deficiency of hematopoietic stem cells in steel mice. *Cell and Tissue Kinetics* **10**(2):121–126.

McGrath, K. E., Koniski, A. D., Maltby, K. M., McGann, J. K. and Palis, J. (1999) Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Developmental Biology* **213**(2):442–456.

Megías, J., Yáñez, A., Moriano, S., O'Connor, J.-E., Gozalbo, D. and Gil, M.-L. (2012) Direct Toll like Receptor-Mediated Stimulation of Hematopoietic Stem and Progenitor Cells Occurs *in vivo* and Promotes Differentiation towards Macrophages. *Stem Cells* **30**(7):1486–1495.

Mendez-Ferrer, S., Lucas, D., Battista, M. and Frenette, P. S. (2008) Haematopoietic stem cell release is regulated by circadian oscillation. *Nature* **452**(7186):442–447.

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. and Frenette, P. S. (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**(7308):829–834.

Mercier, F. E., Ragu, C. and Scadden, D. T. (2012) The bone marrow at the crossroads of blood and immunity. *Nature Reviews Immunology* **12**(1):49–60.

Miller, C. L. and Eaves, C. J. (1997) Expansion *in vitro* of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proceedings of the National Academy of Sciences* **94**(25):13648–13653.

Miyamoto, K., Araki, K. Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., Chen, C., Hosokawa, K., Nakauchi, H., Nakayama, K., Nakayama, K. I., Harada, M., Motoyama, N., Suda, T. and Hirao, A. (2007) Foxo3a is essential for maintenance of the hematopoietic stem cell pool. *Cell Stem Cell* **1**(1):101–112.

Mogensen, T. H. (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews* **22**(2):240–263.

Moore, T., Huang, S., Terstappen, L. W. M. M., Bennett, M. and Kumar, V. (1994) Expression of CD43 on murine and human pluripotent hematopoietic stem cells. *The Journal of Immunology* **153**(11):4978–4987.

Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E. and Weissman, I. L. (1997) Identification of a lineage of multipotent hematopoietic progenitors. *Development* **124**(10):1929–1939.

Morrison, S. J. and Weissman, I. L. (1994) The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1**(8):661–673.

Müller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. and Aguet, M. (1994) Functional role of type I and type II interferons in antiviral defense. *Science* **264**(5167):1918–1921.

Müller-Sieburg, C. E., Townsend, K., Weissman, I. L. and Rennick, D. (1988) Proliferation and differentiation of highly enriched mouse hematopoietic stem cells and progenitor cells in response to defined growth factors. *The Journal of Experimental Medicine* **167**(6):1825–1840.

Müller-Sieburg, C. E., Whitlock, C. A. and Weissman, I. L. (1986) Isolation of two early B lymphocyte progenitos form mouse marrow: a committed pre-pre-B cell and a clonogenic Thy-1$^{\text{lo}}$ hematopoietic stem cell. *Cell* **44**(4):653–662.

Nagai, Y., Garrett, K. P., Ohta, S., Bahrun, U., Kouro, T., Akira, S., Takatsu, K. and Kincade, P. W. (2006) Toll-like Receptors on Hematopoietic Progenitor Cells Stimulate Innate Immune System Replenishment. *Immunity* **24**(6):801–812.

Naugler, W. E. and Karin, M. (2008) The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. *Trends in Molecular Medicine* **14**(3):109–119.

Nie, Y., Han, Y.-C. and Zou, Y.-R. (2008) CXCR4 is required for the quiescence of primitive hematopoietic cells. *The Journal of Experimental Medicine* **205**(4):777–783.

Nowell, P. C., Cole, L. J., Habemyer, J. G. and Roan, P. L. (1956) Growth and continued function of rat marrow cell in x-irradiated mice. *Cancer Research* **16**(3):258–261.

Nutt, S. L., Metcalf, D., D'Amico, A., Polli, M. and Wu, L. (2005) Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. *The Journal of Experimental Medicine* **201**(2):221–231.

Oeckinghaus, A., Hayden, M. S. and Ghosh, S. (2011) Crosstalk in NF-ϰB signaling pathways. *Nature Immunology* **12**(8):695–708.

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K. and Nagasawa, T. (2010) The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* **33**(3):387–399.

Orford, K. W. and Scadden, D. T. (2008) Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nature Reviews Genetics* **9**(2):115–128.

Osawa, M., Hanada, K.-I., Hamada, H. and Nakauchi, H. (1996) Long-term lymphohematopoietic reconstitution by single CD34-low/negative hematopoietic stem cell. *Science* **273**(242-245).

Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L. and Aderem, A. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proceedings of the National Academy of Sciences* **97**(25):13766–13771.

Passegue, E., Wagers, A. J., Giuriato, S., Anderson, W. C. and Weissman, I. L. (2005) Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *The Journal of Experimental Medicine* **202**(11):1599–1611.

Perussia, B., Fanning, V. and Trinchieri, G. (1985) A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses. *Natural Immunity and Cell Growth Regulation* **4**(3):120–137.

Peschon, J., Torrance, D. S., Stocking, K. L., Glaccum, M. B., Otten, C., Willis, C. R., Charrier, K., Morrissey, P. J., Ware, C. B. and Mohler, K. M. (1998) TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *The Journal of Immunology* **160**(2):943–952.

Pietras, E. M., Warr, M. R. and Passegue, E. (2011) Cell cycle regulation in hematipoietic stem cells. *The Journal of Cell Biology* **195**(5):709–720.

Ploemacher, R. E., van der Sluijs, J. P., Voerman, J. S. A. and Brons, N. H. C. (1989) In vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. *Blood* **74**(8):2755–2763.

Poltorak, A., He, X., Smirnova, I., Liu, M.-Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. (1998) Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in *Tlr4* Gene. *Science* **282**(5396):2085–2088.

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. and Lapidot, T. (2000) Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *The Journal of Clinical Investigation* **106**(11):1331–1339.

Prendergast, A. and Essers, M. A. G. () personal communication.

Prendergast, A. M. and Essers, M. A. (2014) Hematopoietic stem cells, infection, and the niche. *Annals of the New York Academy of Sciences* **1310**(1):51–57.

Pronk, C. J. H., Veiby, O. P., Bryder, D. and Jacobsen, S. E. W. (2011) Tumor necrosis factor restricts hematopoietic stem cell activity in mice: involvement of two distinct receptors. *The Journal of Experimental Medicine* **208**(8):1563–1570.

Puri, M. C. and Bernstein, A. (2003) Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. *Proceedings of the National Academy of Sciences* **100**(22):12753–12758.

Purton, L. E. and Scadden, D. T. (2007) Limiting factors in murine hematopoietic stem cell assays. *Cell Stem Cell* **1**(3):263–270.

Qian, H., Buza-Vidas, N., Hyland, C. D., Jensen, C. T., Antonchuk, J., Mansson, R., Thoren, L. A., Ekblom, M., Alexander, W. S. and Jacobsen, S. E. W. (2007) Critical role of thrombopoietin in mainteining adult quiescent hematopoietic stem cells. *Cell Stem Cell* **1**(6):671–684.

Qian, Z., Chen, L., Fernald, A. A., Williams, B. O. and Beau, M. M. L. (2008) A critical role for Apc in hematopoietic stem and progenitor cell survival. *The Journal of Experimental Medicine* **205**(9):2163–2175.

Qiu, J., Papatsenko, D., Niu, X., Schniel, C. and Moore, K. (2014) Divisional history and hematopoietic stem cell function during homeostasis. *Stem Cell Reports* **2**(4):473–490.

Qureshi, S. T., Larivière, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P. and Malo, D. (1999) Endotoxin-tolerant Mice Have Mutations in Toll-like Receptor 4 (Tlr4). *The Journal of Experimental Medicine* **189**(4):615–625.

Rafii, S., Mohle, R., Shapiro, F., Frey, B. M. and Moore, M. A. (1997) Regulation of hematopoiesis by microvascular endothelium. *Leukemia and Lymphoma* **27**(5-6):375–386.

Randall, T. D., Lund, F. E., Howard, M. C. and Weissman, I. L. (1996) Expression of murine CD38 defines a population of long-term reconstituting hematopoietic stem cells. *Blood* **87**(10):4057–4067.

Rebel, V. I., Hartnett, S., Hill, G. R., Lazo-Kallanian, S. B., Ferrara, J. L. M. and Sieff, C. A. (1999) Essential role for the p55 tumor necrosis factor receptor in regulating hematopoiesis at the stem cell level. *The Journal of Experimental Medicine* **190**(10):1493–1503.

Reya, T., Morrison, S. J., Clarke, M. F. and Weissman, I. L. (2001) Stem cells, cancer and cancer stem cells. *Nature* **414**(6859):105–111.

Robbins, C. D. and Swirski, F. K. (2010) The multiple roles of monocyte subsets in steady state and inflammation. *Cellular and Molecular Life Sciences* **67**(16):2685–2693.

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. and Carlesso, N. (2009) Dysfunctional expansion of hematopoietic stem cells and block of myeloid differentiation in lethal sepsis. *Blood* **114**(19):4064–4076.

van Rooijen, N., Bakker, J. and Sanders, A. (1997) Transient suppression of macrophage functions by liposome-encapsulated drugs. *Trends in Biotechnology* **15**(5):178–185.

Rossi, L., Lin, K. K., Boles, N. C., Yang, L., King, K. Y., Jeong, M., Mayle, A. and Goodell, M. A. (2012) Less is more: Unveiling the functional core of hematopoietic stem cells through knockout mice. *Cell Stem Cell* **11**(3):302–317.

Rusten, L. S., Jacobsen, F. W., Lesslauer, W., Loetscher, H., Smeland, E. B. and Jacobsen, S. E. W. (1994) Bifunctional effects of tumor necrosis factor α (TNFα) on the growth of mature and primitive human hematopoietic progenitor cells: Involvement of p55 and p75 TNF receptors. *Blood* **83**(11):3152–3159.

Sanjuan-Pla, A., Macaulay, I. C., Jensen, C. T., Woll, P. S., Luis, T. C., Mead, A., Moore, S., Carella, C., Matsuoka, S., Jones, T. B., Chowdhury, O., Stenson, L., Lutteropp, M., Green, J. C. A., Facchini, R., Boukarabila, H., Grover, A., Gambardella, A., Thonjuea, S., Carrelha, J., Tarrant, P., Atkinson, D., Clark, S.-A., Nerlov, C. and Jacobsen, S. E. W. (2013) Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature* **502**(7470):232–235.

Sato, T., Onai, N., Yoshihara, H., Arai, F., Suda, T. and Ohteki, T. (2009) Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. *Nature Medicine* **15**(6):696–700.

Schaniel, C., Sirabella, D., Qiu, J., Niu, Y., Lemischka, I. R. and Moore, K. A. (2011) Wnt-inhibitory factor 1 dysregulation of the bone marrow niche exhausts hematopoietic stem cells. *Blood* **118**(9):2420–2429.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M. M., Birchmeier, W., Tenen, D. G. and Leutz, A. (2006) Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nature Immunology* **7**(10):1037–1047.

Selleri, C., Sato, T., Anderson, S., Young, N. S. and Maciejewski, J. P. (1995) Interferon-γ and Tumor Necrosis Factor-α suppress both early and late stage hematopoiesis and induce programmed cell death. *Journal of Cellular Physiology* **165**(3):538–546.

Sherr, C. J. and Roberts, J. M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes and Development* **13**(13):1501–1512.

Shi, C. and Pamer, E. G. (2011) Monocyte recruitment during infection and inflammation. *Nature Reviews Immunology* **11**(11):762–774.

Shin, H.-M., Kim, M.-H., Kim, B. H., Jung, S.-H., Kim, Y. S., Park, H. J., Hong, J. T., Min, K. R. and Kim, Y. (2004) Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-𝜘B without affecting I𝜘B degradation. *FEBS letters* **571**(1-3):50–54.

Singh, P., Yao, Y., Weliver, A., Broxmeyer, H. E., Hong, S.-C. and Chang, C.-H. (2008) Vaccinia virus infection modulates the hematopoietic cell compartments in the bone marrow. *Stem Cells* **26**(4):1009–1016.

Sioud, M., Floisand, Y., Forfand, L. and Lund-Johansen, F. (2006) Signaling through Toll-like receptor 7/8 induces the differentiation of human bone marow CD34+ progenitor cells along the myeloid lineage. *Journal of Molecular Biology* **364**(5):945–954.

Sipkins, D. A., Wei, X., Wu, J. W., Runnels, J. M., Cote, D., Means, T. K., Luster, A. D., Scadden, D. T. and Lin, C. P. (2005) In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* **435**(7044):969–973.

Solovjov, D. A., Pluskota, E. and Plow, E. F. (2005) Distinct roles for the α and β subunits in the functions of integrin $\alpha^M\beta^2$. *The Journal of Biological Chemistry* **280**(2):1336–1345.

Spangrude, G. J., Heimfeld, S. and Weissman, I. L. (1988) Purification and characterization of mouse hematopoietic stem cells. *Science* **241**(4861):58–62.

Spangrude, G. J. and Johnson, G. R. (1990) Resting and activated subsets of mouse mulit-potent hematopoietic stem cells. *Proceedings of the National Academy of Sciences* **87**(19):7433–7437.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Developmental Biology* **1**(4).

Staeheli, P., Haller, O., Boll, W., Lindenmann, J. and Weissmann, C. (1986) Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. *Cell* **44**(1):147–158.

Stanford, W. L., Haque, S., Alexander, R., Liu, X., Latour, A. M., Snodgrass, H. R., Koller, B. H. and Flood, P. M. (1997) Altered Proliferative Response by T Lymphocytes of Ly-6A (Sca-1) Null Mice. *The Journal of Experimental Medicine* **185**(5):705–717.

Stein, S. J. and Baldwin, A. S. (2013) Deletion of the NF-𝜘B subunit p65/RelA in the hematopoietic compartment leads to defects in hematopoietic stem cell function. *Blood* **121**(25):5015–5024.

Sugiyama, T., Kohara, H., Noda, M. and Nagasawa, T. (2006) Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 cheomokine signaling in bone marrow stromal cell niches. *Immunity* **25**(6):977–988.

Sutherland, H. J., Eaves, C. J., Eaves, A. C., Dragowska, W. and Landsdorp, P. M. (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* **74**(5):1563–1570.

Szilvassy, S. J., Bass, M. J., Zant, G. V. and Grimes, B. (1999) Organ-selective homing defines engraftment kinetics of murine hematopoieitc stem cells and is compromised by ex vivo expansion. *Blood* **93**(5):1557–1566.

Szilvassy, S. J., Humphries, R. K., Lansdrop, P. M., Eaves, A. C. and Eaves, C. J. (1990) Quantitative assay for totipotent reconstituting hematopoietic stem cells by competitive repopulation strategy. *Proceedings of the National Academy of Sciences* **87**(22):8736–8740.

Takakura, N., Huang, X. L., Naruse, T., Hamaguchi, I., Dumont, D. J., Yanocopoulos, G. D. and Suda, T. (1998) Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* **9**(5):677–686.

Takeuchi, O., Kawai, T., Muhlradt, P. F., Morr, M., Radolf, J. D., Zychlinsky, A., Takeda, K. and Akira, S. (2001) Discrimination of bactrial lipoproteins by Toll-like receptor 6. *International Immunology* **13**(7):933–940.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R. L. and Akira, S. (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *The Journal of Immunology* **169**(1):10–14.

Takizawa, H., Regoes, R. R., Boddupalli, C. S., Bonhoeffer, S. and Manz, M. G. (2011) Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. *The Journal of Experimental Medicine* **208**(2):273–284.

Tanner, J. E. and Tosato, G. (1992) Regulation of B-cell growth and immunoglobulin gene transcription by interleukin-6. *Blood* **79**(2):452–459.

Taswell, C. (1981) Limiting dilution assays for the determination of immunocompetent cell frequencies. *The Journal of Immunology* **126**(4):1614–1619.

Tesio, M., Oser, G. M., Baccelli, I., Blanco-Bose, W., Wu, H., Göthert, J. R., Kogan, S. C. and Trumpp, A. (2013) Pten loss in the bone marrow leads to G-CSF-mediated HSC mobilization. *The Journal of Experimental Medicine* **210**(11):2337–2349.

Tesio, M. and Trumpp, A. (2011) Breaking the Cell Cycle of HSCs by p57 and Friends. *Cell Stem Cell* **9**(3):187–192.

Thoren, L. A., Liuba, K., Bryder, D., Nygren, J. M., Jensen, C. T., Qian, H., Antonchuk, J. and Jacobsen, S. E. W. (2008) Kit regulates maintenance of quiescent hematopoietic stem cells. *The Journal of Immunology* **180**(4):2045–2053.

Till, J. E. and McCulloch, E. A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Research* **14**(2):213–222.

Tothova, Z., Kollipara, R., Huntly, B. J., Lee, B. H., Castrillon, D. H., Cullen, D. E., McDowell, E. P., Lazo-Kallanian, S., Williams, I. R., Sears, C., Armstrong, S. A., Passegue, E., DePinho, E. A. and Gilliland, D. G. (2007) FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* **128**(2):325–339.

Travassos, L. H., Giardin, S. E., Philpott, D. J., Blanot, D., Nahori, M. A., Werts, C. and Boneca, I. G. (2004) Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Reports* **5**(10):1000–1006.

Uchida, N. and Weissman, I. L. (1992) Searching for hematopoietic stem cells: evidence that thy1.1^{lo} Lin$^-$ Sca-1$^+$ cells are the only stem cells in C57BL/Ka-Thy1.1 bone marrow. *The Journal of Experimental Medicine* **175**(1):175–184.

Uckelmann, H. and Essers, M. A. G. () personal communication.

Viatour, P., Somervaille, T. C., Venkatasubrahmanyam, S., Kogan, S., McLaughlin, M. E., Weissman, I. L., Butte, A. J., Passegue, E. and Sage, J. (2008) Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. *Cell Stem Cell* **3**(4):416–428.

Visnjic, D., Kalajzic, Z., Rowe, D. W., Katavic, V., Lorenzo, J. and Aguila, H. K. (2004) Hematipoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* **103**(9):3258–3264.

Vogel, S. N., Hansen, C. T. and Rosenstreich, D. L. (1979) Characterization of a Congenitally LPS-Resistant, Athymic Mouse Strain. *The Journal of Immunology* **122**(2):619–622.

Wagers, A. J., Sherwood, R. I., Christensen, J. L. and Weissman, I. L. (2002) Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* **297**(5590):2256–2259.

Wang, J. C. Y. and Dick, J. E. (2005) Cancer stem cells: lessons from leukemia. *Trends in Cell Biology* **15**(9):494–501.

Waskow, C., Madan, V., Bartels, S., Costa, C., Blasig, R. and Rodewald, H. R. (2009) Hematopoietic stem cell transplantation without irradiation. *Nature Methods* **6**(4):267–269.

van der Wath, R. C., Wilson, A., Laurenti, E., Trumpp, A. and Liò, P. (2009) Estimating Dormant and Active Hematopoietic Stem Cell Kinetics through Extensive Modeling of Bromodeoxyuridine Label-Retaining Cell Dynamics. *PLOS ONE* **4**(9):e6872.

Weber, A., Wasiliew, P. and Kracht, M. (2010) Interleukin-1 (IL-1) Pathway. *Science Signalling* **3**(105):cm1.

Whitlock, C. A. and Witte, O. N. (1982) Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proceedings of the National Academy of Sciences* **79**(11):3608–3612.

Wiesmann, A., Phillips, R. L., Mojica, M., Pierce, L. J., Searles, A. E., Spangrude, G. J. and Lemischka, I. (2000) Expression of CD27 on murine hematopoietic stem and progenitor cells. *Immunity* **12**(2):193–199.

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., Liò, P., MacDonald, H. R. and Trumpp, A. (2008) Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell* **135**(6):1118–1129.

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. and Levesque, J.-P. (2010) Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* **116**(23):4815–4828.

Wu, A. M., Till, J. E., Siminovitch, L. and McCulloch, E. A. (1967) A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *Journal of Cellular Physiology* **69**(2):177–184.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. and Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301**(5633):640–643.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K. L., Ema, H. and Nakauchi, H. (2013) Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* **154**(5):1112–1126.

Yamazaki, S., Iwama, A., Takayanagi, S., Eto, K., Ema, H. and Nakauchi, H. (2009) TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood* **113**(6):1250–1256.

Yanez, A., Flores, A., Murciano, C., O'Connor, J.-E., Gozalbo, D. and Gil, M. L. (2010) Signalling through TLR2/MyD88 induces differentiation of murine bone marrow stem and progenitor cells to functional phagocytes in response to *Candida albicans*. *Cellular Microbiology* **12**(1):114–128.

Yanez, A., Murciano, C., O'Connor, J.-E., Gozalbo, D. and Gil, M. L. (2009) *Candida albicans* triggers proliferation and differentiation of hematopoietic stem and progenitor cells by MyD88-dependent signaling. *Microbes and Infection* **11**(4):531–535.

Yilmaz, O. H., Valdez, R., Theisen, B. K., Guo, W., Ferguson, D. O., Wu, H. and Morrison, S. J. (2006) Pten dependence distinguishes haemtopoietic stem cells from leukaemia-initiating cells. *Nature* **441**(7092):475–482.

Zhang, C. C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C. and Lodish, H. F. (2006b) Angiopoietin-like proteins stimulate *ex vivo* expansion of hematopoietic stem cells. *Nature Medicine* **12**(2):240–245.

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. and Li, L. (2003) Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**(6960):836–841.

Zhang, P., Nelson, S., Bagby, G. J., II, R. S., Shellito, J. E. and Welsh, D. A. (2008) The lineage-c-Kit+Sca-1+ cell response to *Escherichia coli* bacteremia in Balb/c mice. *Stem Cells* **26**(7):1778–1786.

Zhao, C., Blum, J., Chen, A., Kwon, H. Y., Jung, S. H., Cook, J. M., Lagoo, A. and Reya, T. (2007) Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell* **12**(6):528–541.

Zhao, J. L., Ma, C., O'Connell, R. M., Mehta, A., DiLorento, R., Heath, J. R. and Baltimore, D. (2014) Conversion of danger signals into cytokine signals by hematopoietic stem

and progenitor cells for regulation of stress-induced hematopoiesis. *Cell Stem Cell* **14**(4):445–459.

Zhou, L., Ivanov, I. I., Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D. E., Leonard, W. J. and Littman, D. R. (2007) IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nature Immunology* **8**(9):967–974.

Zou, P., Yoshihara, H., Hosokawa, K., Tai, I., Shinmyozu, K., Tsukahara, F., Maru, Y., Nakayama, K., Nakayama, K. I. and Suda, T. (2011) p57^{Kip2} and p27^{Kip1} cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. *Cell Stem Cell* **9**(3):247–261.

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Uta Demel, an MD student who joined the lab in 2013, performed all the IL-1β experiments, under my supervision.

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