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EFFECTS OF INTERFERON α ON MOUSE AND HUMAN HEMATOPOIETIC STEM CELLS

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tempus fugit, amor manet
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SUMMARY

Maintenance of the hematopoietic system is dependent on hematopoietic stem cells (HSCs). During homeostasis HSCs are quiescent. However upon injury, HSCs can efficiently be activated, leading to repair of the system. Signals leading to the activation of quiescent HSCs are still largely unknown. Recently our group has shown that administration of IFNα leads to activation of mouse HSCs in vivo. This is mediated by activation of IFNAR/STAT1 signaling followed by the up-regulation of Sca-1, however the exact mechanism of cell cycle activation remains unclear. To get further insight into this process we performed microarray analysis on HSCs after treatment with IFNα. This screen identified several candidate genes, which are potentially involved in HSC activation, including cell cycle regulators like p57\textsuperscript{kip2}, Maged1 and Reprimo as well as cytokines like Ccl5 and Cxcl10, which are key regulators of inflammatory responses. Furthermore we identified interferon response genes like Ifitm1, Ifitm3, Ligp1, Ligp3 or Ddx58, which were previously linked to regulation of proliferation in different contexts. Along these studies we uncovered that Ifitm1 and Ifitm3 expression is highly enriched within hematopoietic stem and progenitor cells both on the RNA as well as on the protein level. Moreover expression is further induced by IFNα. However mice lacking the Ifitm family show normal hematopoiesis and normal HSC numbers and cycling behavior of HSCs in homeostatic conditions. Ifitm-deficient HSCs are capable to self-renew and differentiate similar to wild-type HSCs. This suggests that the Ifitm protein family is dispensable for HSCs during homeostasis. Notably Ifitm-deficient HSCs are also
efficiently activated by IFNα, similar to wild type HSCs. Microarray analysis of
HSCs from Ifitm deficient mice, both during homeostasis and after administration
of IFNα, showed no differences in the expression profiles, indicating a role for the
Ifitm family as terminal effectors rather than regulatory proteins within HSCs.
During our study it was shown by others that the Ifitm family is a potent viral
restriction factor in endothelial cells. We are currently investigating whether Ifitm
proteins have a similar role in the immune defense of HSCs.

Thus far it is still unclear whether human HSCs are similarly activated by
IFNα as mouse HSCs. To elucidate this we established a xenotransplantation
model with human cord blood cells, which allows testing of the effects of IFNα
on human HSCs in vivo. Surprisingly, unlike mouse HSCs, human HSCs are not
activated by IFNα in this model. Notably human HSCs in this model are already
less quiescent during homeostasis compared to their mouse counterparts. In the
mouse also the bacterial endotoxin LPS can induce cell cycle activation in HSCs.
Surprisingly LPS similarly activates human HSCs in our model. Gene expression
analysis showed a high overlap between the genes induced in mouse and human
HSCs after LPS treatment, while IFNα only affected cell cycle regulatory genes in
murine HSCs. One explanation for this phenotype could be an impaired
interaction of murine stromal niche cells with human HSCs and we are currently
investigating this in more detail.

Finally we examined the effect of IFNα on quiescent leukemic stem cells
(LSCs). While IFNα is known to activate normal mouse HSCs, it is unclear
whether also LSCs are similarly affected. To address this we investigated the
effects of IFNα on LSCs in a mouse model for chronic myeloid leukemia.
Surprisingly LSC were less efficiently activated compared to normal HSCs. This
can be explained by down-regulation of the IFNAR by BCR-ABL kinase activity,
which was previously described in vitro. This also highlights the importance of
exact timing of LSC activation and treatment in combination therapy approaches.
Our group is currently using this model to further identify and optimize new
possible combination therapies.

im Xeno-Transplantationsmodell die Interaktion zwischen murinen Nischenzellen und humanen HSCs gestört ist. Wir untersuchen dies derzeit genauer.

1 INTRODUCTION

1.1 Stem cells in self-renewing tissues

Stem cells are cells that have the intrinsic capability to self-renew, and therefore to produce more stem cells, as well as to differentiate, giving rise to more specialized cell types. They can be distinguished in embryonic stem cells (ES cells) and adult tissue stem cells. ES cells can only be found in the inner cell mass of the blastocyst. They are pluripotent and can give rise to cells of all three germ layers: endoderm, mesoderm and ectoderm. Contrarily adult or somatic stem cells are found in self-renewing adult tissues including the hematopoietic system, the skin and the intestinal epithelium. They are multi-potent stem cells, able to differentiate into different cells of their host tissue, and crucial for life-long tissue-maintenance.

Adult tissue stem cells are located in special microenvironments called stem cell niches. While the exact components of these niches differ for different types of stem cells, in all of them cell-cell contact with surrounding niche cells as well as secreted factors including cytokines and chemokines are crucial for the regulation of stem cell maintenance and differentiation (reviewed in [Simons and Clevers, 2011]). As adult stem cells are crucial for tissue regeneration during the whole lifetime of an organism, it is important to minimize the damage caused by environmental stress for these cells. While stem cells have efficient DNA damage repair mechanisms, they still face the risk of accumulating DNA mutations with every cell division, which can subsequently lead to cancer [Blanpain et al., 2011]. Hence many types of stem cells limit these hazardous impacts by dividing...
very infrequently. Interestingly it was shown recently for hematopoietic stem cells (HSCs), skin stem cells in the hair follicle as well as intestinal stem cells, that for each of these stem cell populations a sub-population is quiescent or long-term dormant, while the majority of these cells cycle more actively [Li and Clevers, 2010; Wilson et al., 2008]. Upon injury to the skin or to the hematopoietic system, the respective quiescent stem cells are rapidly activated and are crucial for tissue repair and regeneration, as described in more detail for the hematopoietic system in chapter 1.2.

Figure 1.1 Stem cell locations in the hair follicle, intestine and bone marrow
(A) Structure of the hair follicle and location of stem cells in the bulge and hair germ region. While the bulge area typically maintains quiescent stem cells, active stem and progenitor cells are found in the hair germ and dermal papilla. During injury to the skin bulge stem cells give rise to stem cells of the epidermis. (B) Structure of the intestinal crypt; quiescent stem cells reside in the +4 region above the paneth cells, while active Lgr5+ CBC cells are lodged between the paneth cells. (C) Quiescent HSCs are located close to the endosteme, surrounded by osteoblasts, endothelial, CAR and other cell types, while actively cycling homeostatic HSCs reside in the central marrow region more distant from osteoblasts. [Li and Clevers, 2010]
1.2 Hematopoietic stem cells

The murine hematopoietic stem cell (HSC) is to date one of the best characterized stem cells and serves as a model for other tissue stem cells. HSCs reside at the top of the hematopoietic hierarchy and are the only hematopoietic cells that have both the capability to self-renew as well as to differentiate into more committed cells (Fig 1.2). They can give rise to multi-potent progenitor cells (MPPs), which can rapidly amplify and are crucial for the expansion of the hematopoietic pool. They in turn differentiate into committed progenitor cells including common myeloid progenitor cells (CMP), megakaryocyte erythroid progenitor cells (MEP), granulocyte macrophage progenitor cells (GMP) and common lymphoid progenitor cells (CLP). These committed progenitor cells in turn give rise to the terminally differentiated cells of the hematopoietic system, including erythrocytes, platelets, granulocytes, macrophages, NK-cells or lymphocytes [Weissman and Shizuru, 2008].

Many terminally differentiated cells of the hematopoietic system, such as granulocytes, are only short-lived and need to be constantly replaced during homeostasis. Additionally, during hematopoietic stress conditions such as bleeding, viral and bacterial infections or toxic insult, the demand for production of new blood cells is further increased. In order to maintain the integrity of the system also under such conditions, hematopoietic stem and progenitor cells have to be able to sense such injuries and rapidly produce new blood cells.

The balance between the diverse differentiation pathways is tightly regulated by a network of transcription factors, which either promote or inhibit the differentiation in a specific lineage. In HSCs and multi-potent progenitor cells different transcription factors are simultaneously expressed at a low level, a state referred to as lineage priming [Ye et al., 2003]. Interestingly sub-fractions of HSCs and MPPs seem to be biased to preferentially differentiate in certain lineages, although they still possess the capability to differentiate in all lineages of the hematopoietic system [Dykstra et al., 2007; Luc et al., 2007; Mansson et al., 2007]. Contrarily many committed progenitor cells express only specific
transcription factors, e.g. C-EBPα promotes myeloid differentiation in GMPs while GATA-1 promotes differentiation towards the erythroid or megakaryocytic lineage in MEPs [Orkin and Zon, 2008].

Figure 1.2 Hierarchy of differentiation in the hematopoietic system
Hematopoietic stem cells (HSC) differentiate in a stepwise manner. During the differentiation process progenitor cells gradually lose the potential to differentiate into all lineages. Multi-potent progenitor cells (MPP) lack the self-renewal capacity of HSCs but are capable of differentiating in all hematopoietic lineages, while committed progenitor cells can only give rise to one lineage. The final differentiation of committed progenitor cells to terminally differentiated cells is a multi-step process indicated by dashed arrows. CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; LMPP, lymphoid primed multi-potent progenitor; EP, erythrocyte progenitor; MkP, megakaryocyte progenitor; GP granulocyte progenitor; MacP, macrophage progenitor; NK, natural killer cell. (adapted from [Cedar and Bergman, 2011])
1.2.1 Identification of murine and human hematopoietic stem cells

During homeostasis in adult mice, the majority of HSCs reside in the trabecular region of the bone marrow microenvironment, where they are protected from pathogens and toxic stress. In the previous decades, a combination of functional assays together with flow cytometric analysis has allowed the step-wise isolation of very pure fractions of hematopoietic stem cells [Weissman and Shizuru, 2008]. The most important assay to assess HSC function is the capability of an HSC to reconstitute the bone marrow of lethally irradiated hosts. In these assays, recipient mice are lethally irradiated with a dose that kills the majority of the hematopoietic cells of the recipient mouse, including the HSCs. Afterwards the recipient mouse is transplanted with cells from a donor mouse. The transplanted HSCs are able to rescue the host and to give life-long multi-lineage engraftment, showing both the capability of these cells to self-renew as well as to differentiate into all hematopoietic lineages. In contrast, transplantation of short-term HSCs or more committed progenitor cells only leads to a temporary reconstitution of the system, as these cells lack the self-renewal capacities of HSCs (reviewed in [Weissman and Shizuru, 2008]).

The first evidence for such bone marrow reconstitution by hematopoietic cells was already shown by Till and McCulloch, who could show efficient rescue of lethally irradiated mice by transplantation of bone marrow cells [McCulloch and Till, 1960]. Subsequently the combination of transplantation assays and advances in fluorescence activated cell sorting (FACS) techniques allowed identification of HSCs within the lineage<sup>neg</sup>Sca-1<sup>+</sup>cKit<sup>+</sup> (LSK) population of cells in the bone marrow [Uchida et al., 1994]. LSK cells do not express any cell surface markers of terminally differentiated cells (e.g. CD3, CD4 or CD8 expressed by lymphocytes or CD11b or Gr-1 expressed by granulocytes), but do express cKit as well as Sca-1 on their surface. This definition of HSCs was subsequently further refined by the addition of several newly identified markers, further enhancing the purity of long-term reconstituting stem cells. The CD34 negative fraction of LSK cells is highly enriched for HSCs, with more than one in five cells able to show long-term multi-lineage reconstitution in lethally irradiated mice [Osawa et al.,
Recently the addition of the signal-lymphocyte-activating molecule (SLAM) markers CD150 and CD48 allowed even higher purification. Within the CD150 positive and CD48 negative fraction of LSKCD34\(^+\) cells (LSKCD150\(^-\)CD48\(^-\)CD34\(^-\)) close to 50% of the cells are long-term reconstituting HSCs, as shown in limiting dilution and single cell transplantation assays [Kiel et al., 2005; Wilson et al., 2009]. The frequency of this population in the bone marrow is less than one in \(10^5\) cells of the bone marrow, illustrating the low abundance of stem cells and the steep hierarchy within the hematopoietic system [Wilson et al., 2009].

Similar to murine HSCs also human HSCs reside mainly in the marrow of long bones and the hip bone. The cell surface phenotype of human HSCs has been less well characterized so far, compared to their mouse counterparts, in part due to the lack of feasibility of transplantation assays in human patients. Human HSCs were functionally assessed both using in vitro colony formation assays as well as in vivo xenotransplantation assays [Weissman and Shizuru, 2008]. In these xenotransplantation assays human hematopoietic cells are transplanted in sub-lethally irradiated immune-compromised mice. The use of immune-compromised mice in this setting is crucial in order to avoid rejection of transplanted cells due to an immune response of the host immune system towards the foreign graft. The most frequently used immune-compromised mouse strains include NOD/SCID mice, which lack T-cells and B-cells, and more recently the highly immune compromised NOD/SCID common gamma (NSG) mice. NSG mice lack in addition to T-cells and B-cells also functional natural killer cells, and are further impaired in several signaling pathways that are important in the innate immune response, allowing the development of functional human hematolymphopoiesis in these mice [Ishikawa et al., 2005].

Transplantation of human bone marrow cells into sub-lethally irradiated NOD/SCID mice showed that the lineage negative, CD34 positive and CD38 negative population is highly enriched for hematopoietic stem cells with long term reconstitution capability [Bhatia et al., 1997a]. Notably the frequency of HSCs in the lin\(^-\)CD34\(^+\)CD38\(^-\) population of human bone marrow is lower in comparison to the purity achievable for murine HSCs using the markers described
above. Furthermore the cell surface protein Thy1 (CD90) has been shown to enrich for human HSCs in vitro as well as in transplantation experiments together with CD34 [Weissman and Shizuru, 2008]. However Thy1 was recently challenged as a marker for human HSCs as alpha-6-integrin (CD49f) has been shown to further enrich the purity of lin^{−}CD34^{+}CD38^{−} HSC irrespective of their expression of Thy1 [Notta et al., 2011]. Concluding the search for human HSC markers is still ongoing, and currently the achievable purity of human HSCs is less compared to their mouse counterparts.

1.2.2 The hematopoietic stem cell niche

Hematopoietic stem cells are preferentially located in the trabecular cavities of long bones. Immuno-histological analysis of bone sections suggest that HSCs reside in the bone marrow as individual stem cells, however their exact localization within the bone marrow is still a highly controversial topic. It is important to notice that, due to the limited number of fluorochromes that can be used simultaneously, histological techniques don’t allow identification of HSCs as accurately as flow cytometry and hence distinction between HSCs and early progenitor cells is not possible.

Putative HSCs have been identified both at the endosteum, the region located in immediate proximity to bone lining osteoblasts (OBs), as well as close to sinusoidal endothelium in more central regions of the bone marrow (Fig 1.3) [Ehninger and Trumpp, 2011]. While some sinusoids are also found in close proximity to the endosteum, the majority is located at a greater distance to the endosteal surface, suggesting the existence of two different HSC niches in the bone marrow; an endosteal niche and a perivascular niche [Kiel and Morrison, 2008; Wilson and Trumpp, 2006]. The exact relationship between these two types of HSC niches is currently still debated, and further experiments are needed to finally unravel whether they exists as distinct entities or whether HSCs might shuttle between the two niches.
The interaction of HSCs with the surrounding stromal cells of the niche is crucial for HSC quiescence, self-renewal and maintenance. This interaction takes place both through direct cell-cell contact as well as by sensing of cytokines and chemokines produced by niche cells. Amongst the most important stromal cells for HSC maintenance are osteoblasts (OBs), MSCs and CAR cells.

Bone-lining spindle-shaped osteoblasts, which can be identified by their CD45 CD31 Ter119 Sca-1 CD51+ phenotype, were the first stromal cells suggested to be a crucial component of the HSC niche. Depletion of osteoblasts leads to mobilization of HSCs to the spleen [Visnjic et al., 2004; Zhu et al., 2007]. Conversely genetically modified mice that have increased numbers of osteoblasts, show a similar increase in their HSC numbers [Calvi et al., 2003; Zhang et al., 2003]. Osteoblasts express several cell surface proteins that are known to be important for HSCs maintenance and quiescence including N-cadherin, membrane-bound SCF and VCAM1. Additionally they are a source of secreted factors involved in regulation of HSCs including TPO, Ang-1, CXCL12 and OPN.

Figure 1.3 Location of HSCs in the trabecular region of long bones
HSCs reside in the trabecular region of the bone marrow of long bones. HSCs are found both close to the endosteum, which is lined by osteoblasts, as well close to sinusoids in more central regions of the bone marrow. Perivascular nestin positive mesenchymal stem cells and CAR cells are crucial for the maintenance of HSCs. [Ehninger and Trumpp, 2011]
In addition to endosteal osteoblasts the presence of other cells, including nestin-expressing mesenchymal stem cells (nestin+ MSCs) as well as CXCL12 abundant reticular (CAR) cells was recently shown to be crucial for HSC maintenance [Mendez-Ferrer et al., 2010]. Nestin+ MSCs are mainly found around blood vessels, and in lower abundance close to the endosteum. Strikingly MSCs express higher levels of HSCs maintenance factors including SCF, Ang-1, IL7, VCAM1 and OPN, compared to all other stromal cell types including osteoblasts.
Interestingly MSCs show several similarities to CAR cells, which are mesenchymal progenitor cells, which are more abundant and mainly located in the bone marrow itself. Depletion of either of these cells impairs HSC maintenance. Due to the many similarities of these two populations it is possible that they are, at least partly, overlapping cell types [Ehninger and Trumpp, 2011].

Figure 1.5 Crosstalk between osteoblasts and HSCs
Model of the endosteal niche-HSC synapse, showing putative ligand-receptor interactions between osteoblasts and HSCs as well as intracellular pathways crucial for HSC maintenance. ANG1, angiopoietin-1; BMI1, polycomb repressor; BMP, bone morphogenetic protein; BMPR1A, BMP receptor 1A; CSL, CBF1 suppressor of Hairless and LAG1; CXCL12, CXC-chemokine ligand 12; CXCR4, CXC-chemokine receptor 4; FAK, focal adhesion kinase; HOXB4, homeobox B4; ICAM1, intercellular adhesion molecule 1; LFA1, lymphocyte function-associated antigen 1; LRP, low-density-lipoprotein-receptor-related protein; MAPK, mitogen-activated protein kinase; OPN, osteopontin; PI3K, phosphatidylinositol-3 kinase; PLC, phospholipase C; PKC, protein kinase C; PPR, PTH/PTH-related protein receptor; PTH, parathyroid hormone; SMADS, mothers against decapentaplegic-related homologue; SNO, spindle-shaped N-cadherin-expressing osteoblast; TIE2, tyrosine kinase receptor 2; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4. [Wilson and Trumpp, 2006]
In conclusion it is possible that two highly similar HSC niches exist in the bone marrow. The endosteal niche, harboring dormant HSCs close to osteoblasts and supporting dormancy by secreted factors as well as direct cell to cell contact, and the perivascular niche, harboring homeostatic HSCs close to sinusoidal endothelial cells and CAR cells, promoting HSC self-renewal (Fig 1.4). Nestin+MSCs seem to be a crucial part of both of these niches [Ehninger and Trumpp, 2011].

1.2.3 Key regulators of HSCs

HSC functions are tightly controlled by both cell-autonomous as well as cell-extrinsic factors, including the interaction with stromal cells in the bone marrow niche and response to secreted cytokines and chemokines. Extensive research in the last years allowed the identification of several signaling pathways, transcription factors, cell cycle regulators and survival factors that are critical for HSC maintenance.

Hematopoietic cytokines and secreted factors

In recent years extensive effort was focused on in vitro expansion of HSCs, as source for clinical applications. In these approaches several cytokines were identified, which promote HSC maintenance and expand HSC numbers in vitro, including stem cell factor (SCF), FMS-like tyrosine kinase 3 (Flt3)-ligand, thrombopoietin (TPO) and the interleukins IL3, IL6 and IL11 [Bhatia et al., 1997b; Conneally et al., 1997; Gammaitoni et al., 2003; Miller and Eaves, 1997; Zandstra et al., 1997]. While SCF, TPO and IL6 are also crucial for HSC maintenance in vivo, the role of IL11 is less clear as mice deficient for the IL11-receptor have no hematopoietic phenotype [Nandurkar et al., 1997]. SCF is the ligand of the c-kit receptor (CD117), which is highly expressed in hematopoietic stem and progenitor cells. Mice deficient for either SCF or c-kit are not viable and die already between embryonic day 14-16 due to severe anemia [Russell, 1979]. Mice with partial loss of c-kit show decreased numbers of LT-HSCs impaired HSC
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Quiescence [Thoren et al., 2008]. Mice that are deficient for either TPO or its receptor MPL have decreased numbers of HSCs [Carver-Moore et al., 1996; Kimura et al., 1998]. Furthermore HSC expansion post-transplantation is highly dependent on MPL and TPO, and similar to c-kit deficiency mice deficient for TPO show decreased HSCs quiescence and reduced expression of p57Kip2 and p19^INK4D [Qian et al., 2007].

Another factor secreted by osteoblasts, which is crucial for HSC maintenance is the glycoprotein osteopontin (OPN). Mice deficient for OPN have increased numbers of HSCs. Furthermore OPN deficient mice transplanted with wild-type HSCs show a similar trend, indicating that OPN might function as a negative regulator of HSC expansion [Stier et al., 2005]. Interestingly HSCs of OPN deficient mice are highly cycling, suggesting that OPN might function at least in part by inhibiting the proliferation of HSCs [Nilsson et al., 2005].

Several angiopoetin-like proteins support the in vitro expansion of mouse and human HSCs [Zhang et al., 2006; Zhang et al., 2008a]. Interestingly interaction of the Tie2 receptor on HSCs with Angiopoietin1 (Ang1) induces cobblestone formation in vitro and maintains long-term repopulating activity of HSCs in vivo. Furthermore Ang-1 enhances quiescence of HSCs and induces adhesion to bone [Arai et al., 2004]. In summary the Tie2/Ang-1 pathway plays a crucial role in maintenance of HSCs in a quiescent state.

Crucial signaling pathways

Interestingly many signaling pathways initially described during embryonic development have more recently been shown to be crucial for maintenance of adult stem cells including HSCs. The most crucial ones include the Notch pathway, the Wnt/wingless pathway and the TGFβ pathway.

Notch The notch signaling pathway controls many crucial aspects of cell fate decision and organogenesis during embryogenesis. Also in adult hematopoietic stem cells a crucial role of Notch signaling was demonstrated in both mouse and human HSCs. The Notch ligands Delta and Jagged can expand cultured mouse and human HSCs in vitro [Karanu et al., 2000; Ohishi et al.,
Furthermore constitutive activation of Notch signaling \textit{in vivo} leads to increased HSC self-renewal, while contrarily expression of a dominant negative RBP-J DNA binding factor leads to depletion of HSCs [Duncan et al., 2005; Stier et al., 2002]. However the role of Notch signaling in adult HSCs is still controversial. Deletion of Jagged1 or Jagged1/Notch1 does not affect HSC maintenance, neither during homeostasis nor during stress conditions [Mancini et al., 2005]. Furthermore also expression of dominant-negative MAML1 or deletion of RBP-J did not affect HSC maintenance. These findings could potentially be explained in part by redundancy and functional compensation by other Notch family members. Nonetheless the role of Notch in HSCs currently is controversial and needs further elucidation.

\textbf{Wnt} \hspace{1cm} The Wnt signaling pathway is a known key regulator of different kinds of stem cells as well as cancer stem cells (reviewed in [Reya and Clevers, 2005]). The first hints for a potential role of Wnt signaling in HSCs came from \textit{in vitro} cultures, where Wnt family members support the expansion of mouse and human HSCs and furthermore constitutive overexpression of activated $\beta$-catenin leads to expansion of HSCs and MPPs [Austin et al., 1997; Reya et al., 2003; Van Den Berg et al., 1998]. Surprisingly however \textit{in vivo} deletion of $\beta$-catenin alone or $\beta$-catenin and $\gamma$-catenin simultaneously has no apparent effect on HSC maintenance or transplantability [Cobas et al., 2004; Koch et al., 2008]. Therefore a role of canonical Wnt signaling in the maintenance of HSCs is unlikely. Interestingly however overexpression of the Wnt inhibitor Dkk1 in osteoblasts leads to a reduction in Wnt signaling, loss of HSC quiescence and reduced engraftment of HSCs upon transplantation [Fleming et al., 2008]. Notably Dkk1 does not only inhibit canonical Wnt signaling, which is dependent on $\beta$-catenin and $\gamma$-catenin, but also non-canonical Wnt signaling, possibly explaining the controversial results. In summary there is no evidence for a role of canonical Wnt signaling in adult HSCs while non-canonical Wnt signaling appears to play a role in the maintenance of HSC quiescence.
**Figure 1.6 Crucial signaling pathways governing HSC fate**

(A) Notch pathway. The notch receptor is activated by ligands of the Delta or Jagged families, leading to proteolytic cleavage of Notch by γ-secretase. The Notch intracellular domain (NICD) translocates to the nucleus and activates notch target genes e.g. Hes. (B) Wnt pathway. Wnt signaling is activated by binding of Wnt to Frizzled and lipoprotein-related protein (LRP) receptors. In the absence of Wnt, β-catenin is degraded by a destruction complex consisting of adenomatous polyposis coli (APC), Axin, Casein-kinase 1α (CK1α) and glycogen synthase kinase (GSK3β). Upon binding of Wnt β-catenin is stabilized and can translocate to the nucleus where it active LEF/TCF transcription factor family members. (C) TGFβ pathway. Upon binding of TGFβ, Activin or BMP to their specific receptor, SMAD proteins are phosphorylated. Phosphorylated SMAD associate with Co-Smads and translocate to the nucleus where they activate target gene transcription. (modified from [Blank et al., 2008])
**TGFβ** The transforming growth factor β superfamily, including TGFβ, activin and bone morphogenic protein (BMP), regulates a broad spectrum of processes both during embryogenesis as well as in adult organisms. TGFβ1 strongly inhibits expansion of HSCs *in vitro* and induces cell cycle arrest by inducing the cyclin-dependent kinase inhibitors p21 and p57 [Cheng et al., 2001; Scandura et al., 2004; Sitnicka et al., 1996]. However mice deficient for TGFβ type 1 receptor have no hematopoietic defects, neither during homeostasis nor during stress conditions [Larsson et al., 2003; Larsson et al., 2005]. Interestingly *in vivo* deletion of Smad4 leads to decreased repopulation ability of Smad4-deficient HSCs, suggesting a crucial role of Smad4 in HSC self-renewal [Karlsson et al., 2007]. Contrarily inhibition of Smad signaling *in vivo* by retro-viral overexpression of inhibitory Smad7 results in increased HSC self-renewal [Blank et al., 2006]. One possible explanation for this controversy might be a role of Smad4 independent of canonical Smad signaling. Interestingly Smad4 indeed was shown before to interact with Wnt as well as Notch signaling [Itoh et al., 2004; Labbe et al., 2000].

In summary the Notch, Wnt and TGFβ signaling pathways seem to be crucial regulators of HSCs. However the exact influence of these complex pathways on HSCs is still poorly understood and needs further elucidation. This is especially critical as there is both, high redundancy within each pathway, as well as crosstalk between the different pathways, which make it hard to draw final conclusions.

**Transcription factors**

Several transcription factors including Scl, Runx1, Gata1/2, Bmi1 or Myc play a crucial role in the maintenance of HSC quiescence, self-renewal, differentiation and survival. The polycomb group family member Bmi1 is crucial for HSC maintenance as HSCs from mice deficient for Bmi1 have defective self-renewal, and furthermore only show short term engraftment upon transplantation [Park et al., 2003]. Scl, Runx1 as well as Gata-2 are crucial for the specification of
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HSCs during embryogenesis [Chen et al., 2009; D’Souza et al., 2005; Ling et al., 2004]. In adult HSCs Runx1 and Gata-2 promote HSC proliferation, whereas Scl seems to be dispensable for HSC maintenance [Ichikawa et al., 2008; Ling et al., 2004; Mikkola et al., 2003]. The proto-oncogene Myc is another key regulator of HSCs. Deficiency for c-Myc results in accumulation of defective HSCs, probably due to niche-dependent defects in HSC differentiation [Wilson et al., 2004]. Strikingly deletion of both c-Myc as well as N-Myc results in pancytopenia and rapid death of mice [Laurenti et al., 2008].

Cell cycle regulators

During homeostasis the majority of HSCs are quiescent, resting in the G0 phase of the cell cycle [Wilson et al., 2008]. Active proliferation of HSCs is tightly controlled, however crucial for both self-renewal as well as differentiation. To exit the G0 phase of the cell cycle the cyclinE-CDK2 complex is required, which is negatively regulated by the CIP/KIP family of cyclin dependent kinase inhibitors (CKIs), comprising p21CIP, p27KIP1 and p57KIP2 (Fig 1.7). The same family is also crucial in controlling the G1/S-transition by inhibiting the cyclinA-CDK2 complex. Another important family of CKIs is the INK4 family, which comprises p16INK4A, p15INK4B, p18INK4C and p19INK4D (Fig 1.3). The INK4 family inhibits cyclinD-CDK4/6 complexes and thereby phosphorylation of the retinoblastoma protein (Rb). They are important regulators of cell cycle arrest in the early G1 phase, in response to cellular stress.

Loss of the CIP/KIP family member p27Kip1 has no influence on HSC numbers, cell cycle distribution or self-renewal potential, however it results in an increased hematopoietic progenitor cell compartment [Cheng et al., 2000a]. Deletion of p21CIP leads to loss of HSC quiescence, which renders HSCs more sensitive to exhaustion by cytotoxic drugs [Cheng et al., 2000b]. Strikingly loss of p57KIP2 leads to severe loss of HSC self-renewal capacity as well as a reduction of quiescence cells within the HSC compartment, which could be rescued by expression of p27KIP1 from the p57 gene locus [Matsumoto et al., 2011]. These phenotypes were even further pronounced when p27KIP1 or p21CIP were deleted.
simultaneously with p57\textsuperscript{KIP2} [Matsumoto et al., 2011; Zou et al., 2011]. In summary p57\textsuperscript{KIP2} appears to be the most crucial CKI of the CIP/KIP family in regulation of HSC maintenance and proliferation.

Contrarily to the deletion of CIP/KIP family members loss of the INK4 family member p18\textsuperscript{INK4C} results in a similar increase in HSC numbers, but surprisingly also in an increase in long-term repopulating ability upon transplantation within these cells [Yu et al., 2006; Yuan et al., 2004]. This is especially striking as in most cases increased cycling of HSCs has detrimental effects on their repopulating capability. Expression of p16\textsuperscript{INK4A} is increased during aging of mice as well as in response to cellular stress including exposure to reactive oxygen species or bone marrow transplantation [Janzen et al., 2006]. Interestingly HSCs from aged mice deficient of p16\textsuperscript{INK4A} show increased repopulating capacities comparable to young wild type mice, suggesting that p16\textsuperscript{INK4A} might negatively regulate HSC numbers during aging [Janzen et al., 2006]. In summary p16\textsuperscript{INK4A} seems to be a crucial regulator involved in the age-dependent decline in HSC functions.
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Figure 1.7 Crucial CDK inhibitors in HSCs

The cyclinD-CDK4/6 complex is crucial for phosphorylation of the retinoblastoma protein (Rb) and its subsequent degradation. Both the CIP/KIP as well as the INK4 family of CDK inhibitors targets this phosphorylation step and thereby promotes cell cycle arrest in the G0 or G1 phase. Furthermore the CIP/KIP family of CKIs is crucial for the regulation of reentry of the cell cycle from the G0 phase by inhibiting the formation of cyclinE-CDK2 complexes and for the G1/S-transition by inhibiting cyclinA-CDK2 complexes. (adapted from [Tesio and Trumpp, 2011])
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1.3 Dormancy and activation of HSCs

Unlike embryonic HSCs and adult hematopoietic progenitor cells, which are highly proliferative, the majority of adult murine HSCs are only slowly cycling during homeostasis. Cell cycle analysis showed that during homeostasis more than 70% of LSK SLAM CD34⁻ HSCs are in the G₀ phase of the cell cycle, but only about 10% of multi-potent progenitors show a similar phenotype [Wilson et al., 2008]. Notably this analysis only represents a current picture at the timepoint of analysis, but does not allow any further conclusions about the cycling behavior of these cells over time.

This important question was recently addressed by label retaining assays, similar to those performed in the identification of label retaining cells in the intestine and skin (Fig 1.1) [Fuchs, 2009]. In these assays the stem cells were labeled either chemically by the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) or genetically with a doxycycline inducible histone H2B coupled to GFP (H2B-GFP) mouse model [Foudi et al., 2009; Wilson et al., 2008]. In the following chase period, the label is effectively halved with every cell division, thus diluting the label in proliferating cells after four to five divisions. Only those cells that do not actively divide keep the label and are hence called label-retaining cells (LRCs). To gain further insight into the cycling behavior of HSCs mice were chemically labeled using a BrdU pulse for 2 weeks, followed by a BrdU free chase of up to 300 days. While the majority of LSK SLAM CD34⁻ HSCs is quiescent in Ki67/Hoechst cell cycle analysis, strikingly only a small proportion retained the label after this chase period [Wilson et al., 2008].

Mathematical modeling of the loss of the label retention of either BrdU or H2B-GFP within the HSC fraction revealed that within this stem cell compartment there are two cell populations with different cycling behaviors. Although all HSCs are quiescent, the majority of about 85% of these cells divides more frequently, while subpopulation comprising about 15% of HSCs divides only around once every 150 days under homeostatic conditions [Wilson et al., 2008]. This small subpopulation, which is almost permanently in the G₀ phase of the cell cycle, and
divides less than once in 5 months, or only about 5 times in the lifetime of a mouse is referred to as dormant HSCs, while the majority of HSCs which are quiescent but still slowly cycling is referred to as homeostatic HSCs [Foudi et al., 2009; Wilson et al., 2008]. Interestingly dormant HSCs show the highest long-term multi-lineage stem cell activity upon transplantation into lethally irradiated hosts, indicating that the most dormant HSCs are also the most potent ones and further linking dormancy to higher self-renewal capacity [Foudi et al., 2009; Wilson et al., 2008]. Notably the metabolic activity of dormant HSCs is highly reduced and their replication machinery inactive [Trumpp et al., 2010]. Dormant HSCs seem to have only a minor contribution to hematopoiesis during homeostasis, where they preserve their self-renewal capacity by avoiding cell divisions, but serve as an important reserve pool for emergency situations. Additionally their dormancy and very low metabolic profile offers them protection from various toxic substances.

Stress to the hematopoietic system including bleeding, infections, inflammation, or chemotherapeutic substances lead to loss of hematopoietic cells, especially of highly cycling populations like progenitor cells.

Treatment of mice with the chemotherapeutic agent 5-Fluoro-Uracil (5FU) efficiently kills proliferating cells of the hematopoietic system, including the highly proliferative progenitor cells, but does not harm the dormant HSC compartment. This depletion of myeloid cells leads to rapid entry of dormant HSCs into the cell cycle, which produce highly proliferative progenitor cells and finally to recovery of the hematopoietic system [Wilson et al., 2008]. A similar regulation seems to be the case also for human HSCs, as 5-FU chemotherapy also leads to rapid myelo-ablation, but the bone marrow quickly recovers after cessation of the treatment [Essers and Trumpp, 2010].

1.3.1 Activation and mobilization of HSCs by G-CSF

Granulocyte-colony-stimulating-factor (G-CSF) was one of the first agents described to affect quiescent mouse HSCs. Treatment of mice with G-CSF leads to
a rapid entry of quiescent HSCs into the cell cycle, followed by mobilization of the cells out of the bone marrow into the blood stream and to the spleen [Morrison et al., 1997]. The exact mechanism of G-CSF induced activation of HSCs is not fully understood, however recent reports shed some more light on this process. Mobilization of HSCs following G-CSF treatment is induced by the release of proteolytic enzymes such as elastase, cathepsin G or matrix metalloproteases (MMPs) by neutrophils [Christopherson et al., 2003; Heissig et al., 2002; Levesque et al., 2001; Petit et al., 2002]. Upon release these enzymes cleave several interactions between HSCs and the osteoblasts in the stem cell niche, such as the binding of SDF1 with CXCR4, the interaction of VCAM-1 and VLA-4 or the dimerization of N-Cadherins on both sides of the niche. Furthermore, treatment with G-CSF leads to depletion of endosteal macrophages as well as endosteal osteoblasts, which is accompanied by a marked reduction of HSC trophic cytokines [Christopher et al., 2011; Winkler et al., 2010]. Together, these events lead to the mobilization of the HSCs. Interestingly, once activated and mobilized by G-CSF, mouse HSCs become susceptible to chemotherapeutic treatments such as the anti-proliferative drug 5-FU or cytarabine and can then be efficiently eliminated.

1.3.2 Activation of HSCs by interferons

Interestingly recent findings showed that HSCs can also be pushed into an active cell cycle by interferon without previous myelo-ablation or subsequent mobilization. Within hours after in vivo administration of either the type I interferon IFNα or the type II interferon IFNγ in mice, quiescent HSCs enter an active cell cycle [Baldridge et al., 2010; Essers et al., 2009]. Strikingly even dormant label-retaining HSCs are efficiently activated in response to IFNα [Essers et al., 2009]. Interestingly, HSCs from mice deficient for IFNγ signaling have a decreased baseline proliferation rate, indicating a potential role of IFN signaling also during homeostasis [Baldridge et al., 2010].
Interferons were previously mainly described as important players in the activation of the immune system in the regulation of resistance to viral infections [Stark et al., 1998]. Strikingly in contrast to the effect of in vivo interferon treatment on HSCs, treatment of many cell lines as well as primary hematopoietic cells with interferon in vitro leads to an inhibition of proliferation of these cells.

IFNα-induced activation of HSCs involves several key steps. IFNα binds to the IFNα-receptor on HSCs, which leads to phosphorylation of signal transducer and activator of transcription (STAT1) as well as protein kinase B (PKB) as well as up-regulation of known interferon target genes (Fig 1.8). Furthermore, it is accompanied by up-regulation of the cell surface protein Sca-1. Interestingly the
IFNα-receptor, STAT1 as well as Sca-1 are crucial for IFNα induced activation of HSCs as mice deficient for either of those proteins do not show cell cycle induction in HSCs upon treatment with IFNα [Essers et al., 2009]. Contrarily to activation of HSCs by G-CSF treatment, HSCs activated by IFNα remain in the bone marrow and do not enter circulation [Essers et al., 2009].

Chronic treatment of mice with IFNα does not impair the function of their HSCs, since these HSCs still efficiently reconstitute upon transplantation. Interestingly however in a competitive setting chronically activated HSCs are outcompeted by HSCs that have not been previously activated [Essers et al., 2009]. Similarly HSCs from mice lacking the Interferon Response Factor-2 (IRF2), a transcriptional repressor of IFNα signaling, are highly cycling during homeostasis and outcompeted in competitive repopulation assays. [Sato et al., 2009]. While the reason for this competitive disadvantage is still unclear, one possible reason could be that the cycling HSCs are outcompeted for dormant niche space by the HSCs unresponsive to IFN signaling.

1.3.3 Other potential mechanisms of HSC activation

Beside interferons also the endotoxin lipopolysaccharide (LPS) and the cytokine tumor necrosis factor alpha (TNFα) have recently been implicated in regulation of HSC quiescence in the course of infections. Infection with the gram-negative bacterium Escherichia Coli stimulates expansion and mobilization of both human as well as mouse HSPCs via TNFα and NFκB signaling [Kim et al., 2004; Quinton et al., 2002; Shahbazian et al., 2004; Zhang et al., 2008c].

Both human as well as mouse HSPCs express a variety of Toll-like receptors, including TLR4, which is crucial for responses to LPS [Nagai et al., 2006; Sioud et al., 2006]. Indeed in vivo administration of LPS induced a transient increase in the number of LSK cells as well as an increase in self-renewal capacity and proliferation of HSCs [Scumpia et al., 2010; Takizawa et al., 2011]. The exact mechanism of HSC activation by LPS however is still not fully understood and it is likely that the observed effects are mediated by a combination of different
signaling pathways. Interestingly investigations from our laboratory showed that LPS, contrarily to IFNα, activates HSCs not directly but rather by an indirect mechanism via monocytes [Marieke Essers, personal communication].

Similar to IFNα also TNFα has anti-proliferative effects on both human as well as mouse HSCs in vitro and furthermore inhibits their colony formation as well as ability to reconstitute upon transplantation [Dybedal et al., 2001; Jacobsen et al., 1994; Jacobsen et al., 1992; Rusten et al., 1994; Zhang et al., 1995]. Mice deficient for both TNF receptors show normal proportions of HSCs during steady state, however upon transplantation TNF-receptor deficient HSCs outcompete their wildtype counterparts [Pronk et al., 2011]. Interestingly in vivo treatment of mice with TNFα leads to rapid decrease in bone marrow cellularity, similar to treatment with LPS. Moreover HSCs from TNFα treated mice were outperformed by HSCs from PBS treated mice in competitive transplantation assays [Pronk et al., 2011]. In summary the effects of TNFα on HSCs in vivo are currently still unclear, as no detailed cell cycle analysis was yet performed, however the available data indicates that TNFα could play a role similar to IFNα or LPS in activation of HSCs in the course of pathogen infections to the host.
**Figure 1.9 Inflammatory pathways involved in HSCs activation**

IFNα binds to the type I interferon receptor and activates TYK2 and JAK1 to phosphorylate STAT1 and STAT2. Upon phosphorylation STAT1 and STAT2 heterodimerize and associate with IRF9 and bind to IFN-stimulated response elements (ISREs) to activate transcription. Similarly IFNγ binds to type II interferon receptors, JAK1 and JAK2 phosphorylate STAT1, which homodimerizes and translocates to the nucleus, where it activates IFNγ activated (GAS) sequences. Binding of TNFα to the TNF-receptor causes TRADD to recruit RIP and TRAF2, which activate IKK to phosphorylate IκBα, leading to a release of NFκB. TNFα signaling also activates MEKK1, which leads to activation of AP-1 and ATF2, via JNK. AP-1 binds TPA DNA-response elements (TRE), while ATF2 binds cAMP-responsive elements (CRE). LPS binds to TLR4 and signals through Myd88 and TRIF. This recruits IRAK, which binds TRAF6 and activates NFκB and JNK pathways similar to TNF-receptor signaling. There is extensive cross-talk between TNF-receptor and TLR signaling. [Baldridge et al., 2011]
1.4 Targeting leukemic stem cells by breaking their dormancy

Similar to the normal self-renewing tissues also many leukemias and solid tumors are hierarchically organized, with only a small population of cells able to give rise to new tumors upon transplantation, while the majority of cells can not propagate the disease. These cells capable of initiating a new tumor are called tumor initiating cells or cancer stem cells (CSCs).

1.4.1 The concept of cancer stem cells

The first evidence for the existence of CSCs came from observations in acute myeloid leukemia (AML), where only a small fraction of leukemic cells is capable of transplanting the disease in immune-compromised mice [Bonnet and Dick, 1997; Lapidot et al., 1994]. Interestingly these cells show the same CD34⁺CD38⁻ cell surface phenotype as normal HSCs. In contrast, transplantation of cells from the bulk of the leukemia with either CD34⁺CD38⁺ or CD34⁻ phenotype, containing the more differentiated leukemic progeny, does not lead to development of AML [Bonnet and Dick, 1997; Lapidot et al., 1994]. After this initial observation in AML similar hierarchical organization within tumors was shown in several other forms of leukemia, for example chronic myeloid leukemia (CML), as well as many other types of solid cancers like breast cancer, colon cancer, prostate cancer and brain tumors [Cho and Clarke, 2008; Reya et al., 2001; Trumpp and Wiestler, 2008].

The abundance of these CSCs differs largely between different types of tumors, different stages of the disease as well as between individual patients. While in some malignancies the majority of cells has tumor initiating capacity, in others CSCs appear to be very rare, with less that 1 in 10,000 or 1 in 100,000 cells able to propagate the disease [Ishizawa et al., 2010; Quintana et al., 2008]. Interestingly recent experiments suggest that during tumor progression from a more benign to a more malignant and aggressive stage, the hierarchy within the different cell types of the tumor flattens, with more differentiated tumor cells
starting to show tumor initiating cell properties as well [Kelly et al., 2007; Passegue et al., 2009; Quintana et al., 2008].

Importantly the term cancer stem cell does not imply that a tumor necessarily originates from a stem cell. In fact many, especially more aggressive tumors, originate from more differentiated cells that regain self-renewing capabilities which are normally restricted to stem cells [Visvader, 2011]. Furthermore many CSCs show many other traits usually associated with stem cells, including quiescence, efficient DNA damage repair and high resistance to radiotherapy as well as anti-proliferative chemotherapy [Trumpp and Wiestler, 2008]. Therefore while many conventional therapies dramatically reduce the size of a tumor by eliminating the bulk of the tumor cells, due to their relative quiescence and increased resistance CSCs are less efficiently targeted and often survive. These residual CSCs are thought to be the main cause for relapse and metastasis formation following an initial successful therapy.

1.4.2 Chronic myeloid leukemia and leukemic stem cells

After the initial description of leukemic stem cells (LSCs) in AML, several other hematological malignancies including chronic myeloid leukemia (CML) were shown to be hierarchically organized and similarly contain rare LSCs, often with a similar phenotype as normal HSCs (reviewed in [Huntly and Gilliland, 2005]). Notably the exact phenotype of LSCs is often still controversially discussed. While LSC in AML where initially described to be exclusively found within the CD34⁺CD38⁻ population, recent development and use of more sensitive transplantation models such as NSG mice demonstrated the presence of LSCs also in other populations [Taussig et al., 2008].

Chronic myeloid leukemia is characterized by the unregulated growth of predominantly myeloid cells in the bone marrow. It was the first malignancy that was linked to a specific genetic abnormality, a single chromosomal translocation between chromosome 9 and chromosome 22, leading to the formation of the Philadelphia Chromosome and the constitutively active protein kinase BCR-ABL
[Ren, 2005]. Depending on the exact breakpoint the size of the resulting fusion protein varies, ranging from 185 to 210 kDa with p210 BCR-ABL being the clinically most important isoform. The fusion protein BCR-ABL is causative and sufficient for driving the development of the disease, and therefore became an ideal drug target [Daley et al., 1990; Ren, 2005]. This lead to the development of the tyrosine kinase inhibitor (TKI) Imatinib mesylate (also known as Gleevec), that specifically inhibits the BCR-ABL kinase, as well as to a lower degree c-kit and PDGF-receptor [Druker et al., 2001; Druker et al., 1996].

Based on clinical characteristics CML can be divided into three phases, a latent chronic phase with only mild symptoms where the majority of patients is diagnosed, an accelerated phase characterized by an increase of myeloid blasts and finally blast crisis, which rapidly progresses and resembles acute leukemia (Fig. 1.10). While the mechanisms of disease progression are not yet fully understood, one frequent event during the progression to advanced disease is the activation of β–catenin signaling in highly proliferative GMPs, giving them self-renewal potential usually restricted to HSCs [Jamieson et al., 2004; Minami et al., 2008; Zhao et al., 2007].

Imatinib mesylate efficiently targets BCR-ABL positive leukemic cells and leads to rapid hematologic and cytogenic response [O'Brien et al., 2003]. Nonetheless, even after years of Imatinib treatment, residual leukemic cells remain, which can lead to a relapse of the disease on cessation of treatment as well as due to acquisition of resistance mutations [Chu et al., 2011]. To overcome resistance to Imatinib the second generation TKI inhibitors Dasatinib and Nilotinib were developed, which show both increased potency as well as the ability to inhibit the majority of Imatinib-resistant mutations [Hantschel et al., 2008; Quintas-Cardama et al., 2007]. The reason for resistance of CML stem cells to treatment with TKIs is still highly debated and several mechanisms have been suggested.
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Within normal hematopoiesis only HSCs are capable of self-renewal as well as to give rise to progenitor cells, which in turn give rise to the terminally differentiated cells of the hematopoietic system. In Chronic Myeloid Leukemia the abundance of leukemic clones capable of self-renewing depends on the stage of the disease. In chronic phase CML LSCs give rise to progenitor cells that are slightly more proliferative compared to normal HSCs. While leukemic progenitors are still differentiating the myeloid compartment is increased due to the higher proliferation of LSCs. In advanced phase disease more differentiated granulocyte macrophage progenitors acquire self-renewal capabilities due to additional mutations, radically increase proliferation and arrest differentiation, together leading to accumulation of immature leukemic blasts. [Savona and Talpaz, 2008]

1.4.3 Mechanisms of resistance in CML stem cells

Despite the efficiency of TKIs in the treatment of CML, CML stem cells are largely resistant not only to treatment with conventional chemotherapy but also to treatment with TKIs. One potential explanation for resistance could insufficient inhibition of BCR-ABL kinase signaling within CML stem cells. Indeed CML stem cells were reported to express higher levels of BCR-ABL compared to more differentiated cells [Jiang et al., 2007]. Furthermore CML stem cells show decreased expression of the ABC transporter OCT1 which is crucial for uptake of Imatinib, as well as increased levels of MDR and ABCG2, which are both crucial in the efflux of many chemotherapeutic drugs [Engler et al., 2010; Jiang et al., 2007]. Taken together a lower uptake, increased efflux and higher levels of BCR-ABL could all lead to insufficient inhibition of BCR-ABL within CML stem cells.

Another potential explanation for the resistance of CML stem cells might be their relative quiescence, similar to the resistance of normal dormant HSCs to different toxic treatments. Interestingly quiescent CML stem cells are not only
resistant to \textit{in vitro} treatment with Imatinib but furthermore Imatinib drives these cells further into quiescence [Graham et al., 2002]. Hence breaking their dormancy and interfering with the Imatinib-induced quiescence might be a way to sensitize these cells to chemotherapy. This concept is further supported by mathematical models of the effect of drug treatment on CML clones, which suggest that one explanation for the resistance of CML stem cells might be a selective effect of Imatinib solely on proliferating leukemic cells [Roeder et al., 2006]. Interestingly these models further suggest that sustained TKI treatment might eventually lead to eradication of all leukemic clones, if no Imatinib resistance-mutations are acquired. Notably this would require an extended period of continuous treatment, until all leukemic stem cells have entered proliferation as part of normal cycling behavior and thus becoming susceptible to Imatinib [Roeder et al., 2006]. Strikingly, these models further support the hypothesis that cell-cycle-activation of leukemic stem cells in combination with Imatinib treatment might be an efficient way of eliminating CML stem cells [Roeder et al., 2006].

\textbf{1.4.4 Activating LSCs to break their resistance}

While normal HSCs are relatively resistant to treatment with the chemotherapeutic drug 5-Fluoro-Uracil (5-FU) due to their low proliferation, they are efficiently eliminated after activation by IFN$\alpha$. This combined treatment rapidly leads to death of mice due to severe pancytopenia and depletion of the HSC pool [Essers et al., 2009]. Strikingly the susceptibility of HSCs to elimination by 5-FU in this assay highly correlates with their cycling behavior, rendering them vulnerable only as long as they are actively cycling [Essers et al., 2009]. Unlike for normal HSCs it is not fully understood whether LSCs are activated by similar mechanisms, and similarly become more susceptible to elimination by chemotherapy or other treatment like TKIs.

G-CSF was one of the first agents described to induce cycling of HSCs, and several studies have been carried out to test the combination of G-CSF induced
activation and mobilization and chemotherapy to eradicate LSCs, both in AML as well as CML models (Fig 1.11).

In a xenotransplantation model for AML treatment of the mice with G-CSF in vivo efficiently activated quiescent AML stem cells. Moreover combination of G-CSF treatment with administration of the chemotherapeutic agent cytarabine lead to a pronounced reduction of AML stem cells and increased survival of the mice [Saito et al., 2010]. Notably normal HSCs were less affected by the combination treatment of G-CSF with cytarabine, suggesting a therapeutic window where toxicity is already reached for LSCs but normal HSCs survive.

Interestingly pre-treatment of CML stem cells, which are resistant to Imatinib treatment at least in part due to their relative quiescence, with G-CSF in vitro leads to cell cycle activation and renders them more susceptible to elimination by Imatinib [Jorgensen et al., 2006]. However the first clinical trial of combined administration of G-CSF and Imatinib did not lead to efficient elimination of leukemic clones in patients [Drummond et al., 2009]. A possible explanation for the failure of this trial could be sub-optimal timing of the two drugs. As Imatinib itself has anti-proliferative effects on LSCs it might counteract the activation by G-CSF and therefore prevent efficient cell cycle entry.

Another approach to eliminate CML stem cells by breaking their quiescence was suggested recently by inhibiting the tumor suppressor promyelocytic leukemia (PML) (Fig 1.11) [Ito et al., 2008]. Inhibition of PML by arsenic trioxide (As$_2$O$_3$), which leads to proteosomal degradation of PML, has previously been used successfully to treat acute promyelocytic leukemia, a form of leukemia driven by the PML-retinoic acid receptor (RAR$\alpha$) fusion protein [de The and Chen, 2010]. PML is highly expressed in mouse HSCs and loss of PML in mice induces increased activity of the mTOR pathway and increased cycling of HSCs [Ito et al., 2008]. Similarly pharmacological inhibition of PML by Arsenic trioxide (As$_2$O$_3$) leads to increased proliferation of human HSCs in vitro [Ito et al., 2008]. Interestingly loss of PML a mouse model for CML similarly resulted in a strong increase in cycling CML stem cells. Furthermore inhibition of PML by As$_2$O$_3$ treatment in combination with the chemotherapeutic drug Ara-C leads to
activation of LSCs and their elimination by Ara-C in a CML mouse model as well as in primary human AML cells in vitro [Ito et al., 2008]. Similar to the combination of G-CSF and cytarabine described above this suggests a possible therapeutic window for a combination therapy of cell cycle induction in LSCs by a block of PML in combination with chemotherapeutic drugs.

Figure 1.11 Combined therapy approaches to eliminate LSCs
(A) Both normal HSCs as well as LSCs show a significant resistance to treatment with conventional anti-proliferative chemotherapy. One possible reason for this resistance might be the relatively quiescent state of LSCs, similar to their normal HSCs counterparts. (B) HSCs can be efficiently activated by IFNα and subsequently become susceptible to elimination by 5-FU treatment. (C) LSCs can be activated by pharmacological inhibition of PML by arsenic trioxide (As2O3), which renders them vulnerable to treatment with the cytotoxic agent Ara-C. (D) Activation of LSCs by G-CSF in combination with Imatinib efficiently kills LSCs in vitro, while combination of G-CSF and cytarabine leads to pronounced reduction of LSCs in a xenograft model for AML, while normal HSCs are spared. [Essers and Trumpp, 2010]
As the effects of interferons on HSCs has only recently been discovered, it is currently unclear whether leukemic stem cells are activated in a similar manner. Interestingly IFNα has been used as standard first line treatment for CML before the introduction of Imatinib, which quickly replaced it due to its superiority in all outcome measures [Goldman, 2010]. Strikingly however despite the high efficiency of Imatinib in reducing the tumor load, patients quickly relapse after cessation of the treatment. Contrarily treatment with IFNα leads to a low but reproducible rate of curative effects in some patients [Kujawski and Talpaz, 2007].

A few clinical trials were performed testing combination treatments of IFNα and Imatinib, which showed promising initial results. These included higher major molecular response rates, which indicate a lower level of minimal residual disease, as well a the ability to overcome resistance to either of the drugs alone [Nicolini et al., 2011; Preudhomme et al., 2010; Simonsson et al., 2011]. Notably it is not yet clear whether this can be attributed to effect of IFNα on LSCs or other complimentary effects e.g. on the immune system. It will be crucial to investigate the effects of IFNα on CML stem cells in vivo to better understand the outcome of these clinical trials and to optimize the timing for the administration of the two drugs.

In summary, it is not yet clear whether LSCs can be similarly activated compared to HSCs. However several lines of evidence suggest that breaking the dormancy of LSCs might be en effective approach to break their resistance to other treatments like chemotherapy or TKIs.
2 Aim of the Thesis

It was recently shown that \textit{in vivo} administration of IFN\(\alpha\) in the mouse very efficiently leads to activation of hematopoietic stem cells [Essers et al., 2009]. The exact mechanism underlying this activation however is still unclear. One central aim of this thesis was to get further insight into the IFN\(\alpha\)-induced HSC activation and to identify and characterize potential candidate genes involved in the activation process. Moreover, so far IFN\(\alpha\) was only shown to activate HSCs in the mouse. To address the effects of IFN\(\alpha\) on human HSCs \textit{in vivo} we sought to investigate the effects of interferon on human HSCs in a xenotransplantation model in immune incompetent mice. Therefore one aim was to establish transplantation of human cord blood samples into mice and to investigate the cycling behavior of human HSC populations in the mouse in steady state as well as after treatment with IFN\(\alpha\). Furthermore combination therapies combining leukemic stem cell activation by IFN\(\alpha\) and cytotoxic treatment is a very interesting possibility to treat leukemia, however it is still unresolved whether IFN\(\alpha\) has the same effects on leukemic stem cells as on normal HSCs. To address this one aim was to investigate the effects of IFN\(\alpha\) on HSCs in a mouse model of CML, where the mice express the BCR-Abl transgene in HSPCs and develop a CML like disease. Finally another open question is whether IFN\(\alpha\) also activates stem cells outside of the hematopoietic system. Therefore the final aim of this thesis was to investigate activation of stem cells of the skin by treatment with IFN\(\alpha\).
3 RESULTS

3.1 Gene expression in HSCs following IFNα and LPS treatment

During homeostasis dormant HSCs serve as an important reserve pool, that can be rapidly activated upon injury to the hematopoietic system [Wilson et al., 2008]. The signals regulating the balance between dormant and activated HSCs are still largely unknown. Previous work from our group has shown that mouse HSCs become activated following in vivo administration of IFNα [Essers et al., 2009]. This activation is dependent on the interferon–α–receptor (IFNAR), STAT1 as well as Sca-1, as mice deficient for either of those do not show HSC activation [Essers et al., 2009]. Besides IFNα, which is mainly induced during viral infections, more recently also LPS, which is found in the outer membrane of gram-negative bacteria, was shown to drive HSCs into proliferation. In the case of LPS the activation is mediated via an indirect mechanism via monocytes rather than direct activation of HSCs [Stefanie Thamm, Raphael Lutz and Marieke Essers, personal communication]. However for both IFNα as well as LPS the exact mechanisms of activation of HSCs are still poorly understood. To gain further insight into this, we isolated LK SLAM (lineage^neg^CD117^+^CD150^+^CD48^-^) HSCs from C57BL/6 mice, 16 hours after i.p. injection of PBS, IFNα (10,000u/mouse) or LPS (5µg/mouse). From these samples we isolated RNA and performed microarray analysis. We analyzed gene expression and compared the datasets of genes differentially regulated with a fold change (FC) above 1.5 and a false discovery rate (FDR) below 5%. According to these criteria we found 600 genes to be
differentially regulated following IFNα administration and 903 following administration of LPS. The samples from each respective treatment condition were clustering together in a hierarchical cluster analysis, with the samples isolated from IFNα and LPS treated mice being more similar to each other than to the ones isolated from PBS treated mice (Fig 3.1).

Figure 3.1 Microarray analysis 16 hours after administration of PBS, IFNα or LPS
Hierarchical clustering analysis of samples after treatment with PBS, IFNα or LPS based in genes significantly changed between PBS and IFNα treated mice; false discovery rate (FDR) <5% and fold change >2. LPS 5µg per mouse, IFNα 10.000U per mouse, injected i.p. 16 hours before analysis.

3.1.1 Differentially expressed genes following IFNα treatment in vivo

As expected gene ontology analysis following in vivo IFNα treatment highlighted immune responses and responses to viral infections as the most significantly changed pathways and processes in LK SLAM HSCs (Fig 3.2 and 3.3).
RESULTS

A large proportion of the genes differentially regulated are known IFN response genes (Tab. 3.1), which play a crucial role in the regulation of the interferon response (e.g. Irf7, Irf9 or Usp18), RNA sensing (e.g. Oas genes or Ifit3) or in the host response to viral infections (e.g. Ifitm1, Ifitm3 or Mx1). Furthermore we could confirm the transcriptional up-regulation of Stat1 (2.6x up) and Stat2 (3.0x up) as well as Sca-1 (6.0x up), as already previously described [Essers et al., 2009].

Amongst the strongest induced genes we also found transcripts of the cytokines CXCL-10 (IP10, 8.6x up) and CCL5 (RANTES, 2.9x up). CXCL-10 was shown to act as a chemo-attractant for many different cell types including monocytes, lymphocytes, NK-cells as well as dendritic cells. Moreover it was shown to promote attachment of lymphocytes to endothelial cells [Dufour et al., 2002]. Similar to CXCL-10 also CCL5 was shown to be chemotactic for lymphocytes. Furthermore CCL5 is involved in activation and proliferation of NK-cells [Maghazachi et al., 1996]. The influence of these cytokines on HSCs is currently unknown. Whether they play a role in recruitment or activation of HSCs similar to lymphocytes or NK-cells needs to be investigated.

Surprisingly, we found only few cell cycle related genes to be differentially regulated. The cyclin dependent kinase inhibitor p57KIP2 (3.5x down) as well as the cell cycle regulator Reprimo (3.5x down) were down-regulated in both treatment conditions. P57KIP2 was recently shown to be a key regulator of HSCs quiescence, preventing CyclinD1 from entering the nucleus and allowing G1 progression through the cell cycle [Matsumoto et al., 2011; Zou et al., 2011]. Down-regulation of p57KIP2 might therefore be a key event following IFNα induced activation, which might be necessary in quiescent HSCs to enter the SG2M phase. The function of Reprimo has been less well described so far, however it has been proposed to be involved in p53 dependent G2 arrest of the cell cycle [Ohki et al., 2000], suggesting a link between decreased Reprimo expression and active proliferation. Interestingly p57KIP2 and Reprimo inhibit cell cycle progression at different check-points and hence down-regulation of both might be required for cell cycle induction.
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Table 3.1 Selection of differentially regulated genes in LK SLAM HSCs 16h after treatment with IFNα, in vivo
Comparison of SLAM HSCs sorted from IFNα treated C57BL/6 mice to SLAM HSCs from PBS treated C57BL/6 mice; False discovery rate (FDR) <5%; blue banded rows mark known interferon response genes; IFNα 10.000U per mouse.
### RESULTS

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Table 3.2 Selection of differentially regulated genes in LK SLAM HSCs of IFNAR\(^{−}\) mice, 16h after treatment with LPS in vivo

Comparison of SLAM HSCs sorted from LPS treated IFNAR\(^{−}\) mice to SLAM HSCs from PBS treated IFNAR\(^{−}\) mice; False discovery rate (FDR ) <5%; blue banded rows mark known interferon response genes

Taken together the up-regulation of many IFN response genes as well as IFN signaling cascades within the LK SLAM HSCs indicate a direct effect of IFN\(\alpha\) on the hematopoietic stem cells. Surprisingly we found only few genes involved in regulation of the cell cycle to be differentially regulated. This could be due to regulation of these genes on the post-transcriptional or post-translational level rather than at the transcriptional. To gain more insight into this, further analysis on the protein level – e.g. by proteomics analysis – will be needed.
Figure 3.2 Gene ontology analysis of LK SLAM HSCs 16h after *in vivo* treatment with IFNα or LPS
Most significantly changed GO pathway maps (A) and GO processes (B), analyzed with Metacore software V6.10 (Thomson Reuters). Orange bars represent p-values for LK SLAM HSCs after IFNα treatment, blue bars after LPS treatment. Fold change >1.5 and FDR<5%
3.1.2 RNA expression patterns in LPS- and IFNα activated HSC are highly overlapping

Following *in vivo* treatment with LPS we interestingly found a large overlap of differentially regulated genes in SLAM HSCs, compared to activation by IFNα. These included the induction of many interferon target genes as well as down-regulation of the cell cycle inhibitors p57KIP2 and Reprimo (Fig 3.3).

*Figure 3.3 Activation of IFN signaling in LK SLAM HSCs 16h after in vivo treatment with IFNα or LPS*
Pathway map “antiviral action of interferons” (Metacore); genes differentially regulated after treatment with IFNα (1) or LPS (2) *in vivo* are highlighted by red circles; red bars next to the respective genes indicate the level of gene induction for IFNα (1) or LPS (2). Fold change >1.5 and FDR<5%
Experiments by colleagues in the lab showed that in vivo treatment of mice with LPS leads to activation of the Mx promoter in a reporter mouse model [Marieke Essers, personal communication]. As the Mx promoter is strongly induced by IFNα this observation indicates that in vivo LPS administration leads to production of interferon. Therefore the observed similarities between the expression profiles following IFNα and LPS treatment could be explained by either production of interferon α following treatment with LPS, or through IFNα independent activation of the same pathways within the HSC.

To investigate the effects of LPS on HSCs without the influence of IFNα-signaling, we performed microarray analysis of HSCs sorted from IFNAR−/− mice, treated either with PBS, IFNα or LPS. IFNAR−/− mice lack the interferon-α-receptor, which is crucial for the binding of IFNα and the induction of the signaling cascade. As expected we found no genes to be significantly changed (FDR 5%, FC > 1.5) in SLAM HSCs of IFNAR−/− mice after treatment of IFNα. This confirms that the observed changes in wild type mice are a specific result of direct signaling of IFNα via the IFNAR without any detectable off target effects. Additionally it shows that all genes differentially regulated by LPS in IFNAR−/− mice are affected independent of IFNα.

Interestingly after treatment with LPS we found 385 genes to be significantly changed in SLAM HSCs of IFNAR−/− mice (Tab 3.2). The expression pattern was very similar to the one observed in wild type mice treated with either IFNα or LPS, including induction of Sca-1 (15.7x up) or CCL5 (5.2x up) as well as down-regulation of Reprimo (2.0x down), Pleiotrophin (2.5x down) or p57<sup>KIP2</sup> (3.1x down). Interestingly we also observed the induction of many typical IFN response genes including Igtp (8.0x up), Oasl2 (3.8x up), Irgm (3.8x up), Ifitm1 (2.2x up) and Ifitm3 (1.6x up) or Ifi44 (2.2x up) despite the lack of the IFNα signaling. Notably we did not observe induction of the RNA sensors Ifit1 and 3, the interferon regulatory genes IRF7 and IRF9 and the chemokine CXCL-10 contrary to the induction by IFNα.
The highest down-regulated gene was Matrilin4 (Matn4; 10.6x down), similar to the down-regulation after IFNα treatment of wild type mice. Matrilin 4 belongs to a family of 4 non-collagenous extracellular matrix proteins [Klatt et al., 2002]. Neither the function of Matrilin-4 in the ECM nor expression in the hematopoietic compartment has been previously described.

Taken together this data suggests that the programs triggered within the HSCs are very similar for activation by IFNα and by LPS, including the induction of typical IFN response genes after administration of both IFNα as well as LPS, suggesting a general activation mechanism. The expression changes following LPS treatment could be either totally independent of IFN signaling, or alternatively mediated by the production of type 2 or type 3 interferons after LPS administration. Notably IFNγ was recently shown to activate quiescent HSCs in the course of bacterial infections [Baldridge et al., 2010]. To ultimately rule out a role of IFNs in the LPS mediated activation of HSCs, analysis would need to be performed in IFNAR−/−IFNGR−/− double knock-out mice lacking the capability to respond to IFNα as well as to IFNγ.
3.2 Role of Sca-1 in IFNα and LPS induced HSC activation

Sca-1, also known as Ly6a (lymphocyte antigen 6 complex, locus A) is a small (14kDa) protein. It is a member of a family of type V glycophosphatidylinositol (GPI) anchored cell surface proteins. Sca-1 was one of the first cell surface proteins shown to be highly enriched in stem and progenitor cells of the hematopoietic system, and is often used in combination with lineage markers and the tyrosine-protein kinase c-kit (CD117) to characterize LSK stem and progenitor cells [Weissman and Shizuru, 2008]. Sca-1 is strongly induced upon cellular activation and interestingly expression of Sca-1 is crucial for activation of HSCs following both treatments with IFNα as well as with LPS [Essers et al., 2009]. Mice lacking Sca-1 expression show a normal hematopoietic system, but HSCs from Sca-1−/− mice perform less well than wildtype counterparts in competitive as well as serial transplantation assays, indicating impaired self-renewal capacity [Ito et al., 2003]. Furthermore BM from Sca-1−/− mice has decreased multipotential granulocyte, erythroid, macrophage and megakaryocyte colony forming units (GEMM-CFU) and colony forming unit spleen (CFU-S) activity [Ito et al., 2003].

To elucidate the role of Sca-1 in HSC activation we performed microarray analysis of LK SLAM HSCs of Sca-1−/− mice and compared the expression profile with wild type C57Bl/6 mice. Following PBS injection we detected 8 differentially expressed genes (FDR < 5%, FC > 1.5) between wild type C57Bl/6 mice and Sca-1 KO mice. Only 3 genes were changed more than 2 fold, including the transcription factor Pbx1 (pre B cell leukemia homeobox 1) which was expressed 3.13 fold higher in Sca-1−/− HSCs. Interestingly Pbx1 has been shown before to be important for maintaining the quiescence of HSCs. Conditional inactivation of Pbx1 leads to loss of long term HSCs, reduction in their quiescence as well as loss of self-renewal capacity [Ficara et al., 2008].

Following in vivo administration of IFNα we detected 213 differentially regulated genes in Sca-1−/− SLAM HSCs. The expression pattern was very similar to
the one observed in C57Bl/6 mice (Fig 3.4). Similar to wild type HSCs we detected increased expression of many IFN target genes in Sca-1−/− HSCs including lfit3 (10.9x up), Oas2 (7.7x up), lgtp (4.8x up), Oas3 (4.1x up), Irgm (3.5x up) or ifitm1 (2.2x up). Furthermore we also observed differential expression of Usp18 (6.2x up), Cxcl10 (6.2x up), Stat2 (3.2x up), Pml (2.8x up), Stat1 (2.1x up) or Cxcl4 (1.5x down).

Hierarchical clustering analysis showed a high overlap between the expression pattern of wild type and Sca-1−/− HSCs (Fig 3.4). Wild type and Sca-1 deficient HSCs from PBS treated mice clustered together with HSCs from IFNAR−/− mice treated with either PBS or IFNα. This indicates a high similarity of the expression profiles of the different genetic backgrounds during homeostasis as well as in the IFN-receptor deficient background after treatment with IFNα. Similarly HSCs from wild type and Sca-1−/− mice again clustered together after administration of IFNα, before clustering with HSCs from LPS treated wild type or IFNAR−/− mice (Fig 3.3). Taken together the expression profiles of Sca-1−/− mice are very similar to the ones from wild type mice both during homeostasis as well as after IFNα treatment, indicating that Sca-1 is not a major transcriptional regulator in HSCs in these conditions.

The similar induction of IFN response genes as well as STAT1 and STAT2 signaling was expected, as Sca-1 is known to be regulated downstream of STAT1 signaling [Essers et al., 2009]. Interestingly the cell cycle regulators p57KIP2 and Reprimo, which show decreased expression after IFNα treatment in wild type HSCs, were not significantly changed in Sca-1−/− HSCs, correlating with the lack of cell cycle induction. Furthermore also Matrilin-4, the highest down-regulated gene after both IFNα as well as LPS treatment in wild type HSCs, was not significantly changed.
RESULTS

Figure 3.4 Microarray analysis of HSCs from wild type, IFNAR^−/− and Sca-1^−/− mice treated with PBS, IFNα or LPS
Hierarchical clustering of samples according to genes significantly changed between PBS and LPS treated wildtype C57Bl/6 mice, false discovery rate (FDR) <5% and fold change >2. LPS 5µg per mouse, IFNα 10.000U per mouse, injected i.p. 16 hours before analysis.

Interestingly the transcription factor Evi1 (Ectopic viral integration site 1) was amongst the highest differentially expressed genes comparing IFNα treated HSCs from wild type mice to Sca-1^−/− mice. Evi1 expression was 3,0x lower in wild type HSCs than in HSCs of Sca-1^−/− mice. Evi1 is predominantly expresses in long term HSCs and essential for HSCs self-renewal [Kataoka et al., 2011]. It regulates proliferation of HSC as well as leukemic stem cells through GATA2 expression and is frequently mutated in myeloid leukemia [Goyama et al., 2008; Yuasa et al.,...
2005). The decrease in Evi1 following activation could indicate a loss of self-renewal capacity and induction of differentiation of a part of the activated HSC population.

While there is no role of Maged1 described in the hematopoietic system yet, it has been implicated to play a role in the regulation of differentiation and proliferation in other cell types. Maged1 is required for myoblast differentiation and muscle regeneration. Myoblasts from Maged1 knock out mice show decreased p21<sup>CIP</sup> levels and defective cell cycle exit [Nguyen et al., 2010]. Similarly also in breast cancer cells Maged1 inhibits proliferation, migration and invasion by positively regulating p53 and p21<sup>CIP</sup> expression [Du et al., 2009]. Whether Maged1 has similar functions in hematopoietic stem cells still needs to be determined. Pleiotrophin is a 18kDa growth factor which was recently shown to be involved in the regulation of HSC expansion and regeneration by activation of PI3K signaling and by β-Catenin independent activation of Cyclin D1 [Himburg et al., 2010; Istvanffy et al., 2011]. While the function of PEDF in the hematopoietic system is still elusive, it was shown be a niche-derived regulator of neural stem cells and to promote self-renewal of adult neural stem cells [Ramirez-Castillejo et al., 2006].

Overall the expression profile of wild type and Sca-1<sup>−/−</sup> HSCs share many similarities following activation by IFNa<sub>α</sub>, including expression of IFN target genes and induction of JAK/STAT signaling. The differential regulation of Evi1, Hoxa7, p57<sup>KIP2</sup> or Reprimo is the first evidence for transcription factors and cell cycle regulators being controlled downstream of Sca-1 signaling. Nonetheless the exact mechanism of HSC activation needs to be further investigated. It is possible that many changes occurring in the activation process happen not on a transcriptional level, but post transcriptionally or even at the level of posttranslational modification. To investigate further it will be interesting to imply techniques like proteomics analysis to get more insight on the events on the protein level.
3.3 The Ifitm protein family as candidate regulators of HSCs activation

One of the most interesting families of proteins, which is induced after both IFNα as well as LPS treatment in our microarray screens, is the Ifitm family of proteins. It comprises five family members in the mouse: Ifitm1, 2, 3, 5 and 6. Ifitms are short two transmembrane spanning proteins of 5 to 18 kDa (Fig 3.5). We found the expression of two family members – Ifitm1 and Ifitm3 – increased in LK SLAM HSCs following IFNα as well as LPS administration.

We decided to investigate the role of the ifitm protein family in HSCs in more detail, as there was evidence that the family might play a role in regulation of stem cells as well as proliferation.

The ifitm family was initially thought to play a role in homing and repulsion of primordial germ cells by modulating cell adhesion, however they are dispensable for germ cell development as deletion of all five family members does not impair germ line establishment [Lange et al., 2008; Tanaka et al., 2005]. Furthermore Ifitm expression has been identified as a prognostic marker in several cancers including colorectal cancer, glioma and chronic myeloid leukemia [Akyerli et al., 2005; Andreu et al., 2006; Yu et al., 2011]. Interestingly Ifitm3 binds osteopontin (Opn) in vitro, and expression of Ifitm3 in vivo leads to reduction of Opn expression [El-Tanani et al., 2010]. Osteopontin is an important component of the HSC niche and a negative regulator of HSC proliferation [Nilsson et al., 2005]. Therefore the increased expression of Ifitm3 following IFNα treatment might ultimately lead to activation of HSC proliferation by downregulation of Opn.

![Figure 3.5 Predicted structure of Ifitm3](Yount et al., 2010)
Moreover expression of Ifitm1 as well as Ifitm3 inhibits cell proliferation in vitro [Brem et al., 2003]. Notably Ifitm1 is crucial for IFNγ mediated cell cycle arrest, as suppression of Ifitm1 blocked the anti-proliferative actions of IFNγ [Yang et al., 2007].

Concluding from this data we hypothesized that the Ifitm family might play a crucial role in cell cycle activation of HSCs after in vivo treatment with IFNα.

### 3.3.1 IFNα and LPS induce expression of Ifitm family members

To get further insight into the induction of the expression of Ifitm family members and to validate our microarray expression data, we performed qPCR analysis of unfractionated total bone marrow cells as well as FACS sorted LK SLAM HSCs, 16 hours after treatment with PBS, IFNα or LPS. We observed increased expression of ifitm1 and ifitm3 in LK SLAM HSCs, both after treatment with IFNα as well as LPS. Expression of Ifitm2 was not influenced, while the expression of ifitm5 and ifitm6 was lower in IFNα and LPS treated mice (Fig 3.6 A). Interestingly contrary to the expression in HSCs, in unfractionated bone marrow cells the expression of all five ifitm family members was induced by both IFNα as well as LPS (Fig 3.6 B). This indicates a different mechanism of regulation of Ifitm expression in HSCs and more differentiated cells.

### 3.3.2 Expression of Ifitm family members is enriched in HSPCs

To elucidate the abundance of the Ifitm family of proteins in the hematopoietic system we performed qPCR analysis on FACS sorted LSKCD150°CD48 CD34− HSCs, MPPs, lineage committed progenitor cell fractions as well as differentiated cells. We detected transcripts of all five Ifitm family members in HSPCs, but only Ifitm1, 2 and 3 were expressed at levels comparable to the house keeping genes (Fig 3.7 A), while the expression levels of Ifitm 5 and 6 were very low (data not shown). Interestingly the expression of Ifitm1 and 3 was highly enriched in hematopoietic stem cells and early progenitor cells and decreasing in more committed progenitor cells. Expression was higher in common
myeloid progenitors compared to lymphoid-committed progenitors. All Ifitm family members except Ifitm 5 were expressed in CD11b^Gr1^ cells enriched for granulocytes and macrophages, with Ifitm 6 showing exclusive expression in this compartment (Fig 3.7 B). This is in concordance with a possible role of Ifitm 6 in macrophages [Han et al., 2011]. Ifitm3 was also expressed at a low level in Ter119^ erythrocytes (Fig 3.5 B).

**Figure 3.6 qPCR quantification of ifitm mRNA expression 16 hours after injections of IFNa or LPS**
Quantification of Ifitm1, 2, 3, 5 and 6 relative to the house keeping genes sdha (succinate dehydrogenase complex subunit A) and oaz1 (ornithine decarboxylase antizyme 1). All expression levels normalized to the respective PBS treated control. Dotted line indicates the expression level of PBS treated samples. (A) Expression in FACS sorted LK SLAM HSCs (LKCD150^CD48^) (B) Expression in unfractionated total bone marrow cells.
RESULTS

Figure 3.7 qPCR quantification of Ifitm mRNA expression in HSPCs and differentiated cells
Quantification of genes relative to the house keeping genes sdha (succinate dehydrogenase complex subunit A), oaz1 (ornithine decarboxylase antizyme 1) and b2m (beta-2-microglobulin). Dotted line indicates the average expression in total bone marrow samples. (A) Expression of Ifitm1, 2 and 3 in HSCs (LSKCD150^CD48^CD34^-^), MPP1/2 (LSKCD150^CD48^), MPP3/4 (LSKCD150^-^), CMPs (lin^-^CD117^-^Sca-1^-^IL7R^-^CD34^-^FcgR^-^), GMPs (lin^-^CD117^-^Sca-1^-^IL7R^-^CD34^-^FcgR^-^), MEPs (lin^-^CD117^-^Sca-1^-^IL7R^-^CD34^-^FcgR^-^) and CLPs (lin^-^CD117^-^Sca-1^-^IL7R^-^CD135^-^). (B) Expression of Ifitm family members in Ter119^+^ erythrocytes, CD11b^+^Gr1^-^ granulocytes and macrophages, B220^+^ B-cells and CD4^-^/8^-^ lymphocytes.

To get further insight on the expression of Ifitm3 in individual cells we used a mouse model, where the Ifitm3 gene was disrupted by in-frame insertion of EGFP [Lange et al., 2008]. Mice heterozygous for the Knock-in (Ifitm3EGFP^+/T^) express one allele of Ifitm3 and one allele of EGFP. EGFP expression can thus be used as an indication of Ifitm3 mRNA expression, while mice homozygous for the Knock-in (Ifitm3EGFP^T/T^) lack Ifitm3 expression (Fig 3.8 A, more detailed description of the model in the Materials and Methods Section).
RESULTS

A

B

C
**RESULTS**

Figure 3.8 EGFP expression in the hematopoietic compartment of Ifitm3EGFP Knock-in mice
(A) The Ifitm3EGFP mouse model; The Ifitm3 gene is disrupted by in-frame insertion of EGFP in the first exon of the ifitm3 gene. (B) Expression of EGFP in lineage negative cells (negative for expression of CD11b, Gr-1, CD4, CD8, B220 and Ter119), LSK cells, LSKCD150^CD48^ HSCs and LSKCD150^CD48^CD34^ HSCs. (C) Overlay of EGFP expression from lineage negative cells (green), LSK cells (blue) and LSKCD150^CD48^CD34^ HSCs (red); MFI = mean fluorescence intensity of EGFP channel. (D) Gating scheme of common myeloid (lin^neg^CD117^Sca-1^FcgR^lo^CD34^), myeloid-erythroid (lin^neg^CD117^Sca-1^FcgR^CD34^) and granulocyte-macrophage (lin^neg^CD117^Sca-1^FcgR^CD34^) progenitor cell populations and EGFP expression levels in the respective populations (E) Expression of EGFP in common lymphoid progenitor cells (CLP; lin^neg^CD117^Sca-1^-^CD135^IL7R^). (F) EGFP expression in CD4 and CD8 positive lymphocytes, B220 positive B-cells, CD11b and Gr-1 positive granulocytes and Ter119 positive erythrocytes.
We found only a small population of less than 10% of lineage negative cells (lacking expression of CD11b, Gr-1, CD4, CD8, B220 and Ter119) to express EGFP (Fig 3.8 B). Interestingly the proportion of cells positive for EGFP expression increased for earlier progenitor populations to about 40% and was highest in long-term hematopoietic stem cells (LSK SLAM CD34), with more than half of the cells expressing EGFP (Fig 3.8 B). Similarly we also noticed an increase in the mean fluorescence intensity (MFI) from more towards less differentiated populations, with HSCs again showing a six-fold higher MFI compared to lineage negative cells (Fig 3.8 C). This indicates both an increase in the frequency of cells expressing Ifitm3 within the HSC population as well as an increased expression of Ifitm3 in individual cells.

We next analyzed myeloid and lymphoid committed progenitor cells for expression of EGFP. We detected expression of EGFP in common myeloid (CMP), granulocyte-macrophage committed (GMP), myeloid-erythroid committed (MEP) as well as common lymphoid committed (CLP) progenitors at a level comparable to LSK cells (Fig 3.8 D+E). The proportion of positive cells as well as the MFI of the EGFP signal was highest in the CMP population compared to other committed progenitor populations. This was reflected also on the level of terminally differentiated cells, where only few CD4 or CD8 positive lymphocytes, B220 positive B-cells or Ter119 positive erythrocytes expressed EGFP. In contrast the expression of EGFP was very high in CD11b/Gr-1 expressing granulocytes and macrophages, confirming the high expression of Ifitm3 determined by qPCR in this population (Fig 3.8 F).

In summary the expression of EGFP in Ifitm3EGFP+T mice correlates highly with the mRNA expression quantified by qPCR. RNA expression of Ifitm1 and 3 is highly enriched in HSCs and gradually declines in more differentiated cells, with the exception of granulocytes. Interestingly the expression of Ifitm3 is enriched in HSCs in both frequency of cells expressing Ifitm3, as determined by EGFP expression in Ifitm3EGFP+T mice, as well as intensity of expression in individual cells within the HSC compartment. Strikingly however even in HSC populations enriched for Ifitm3 expression still nearly half of the cells are negative for
expression of EGFP. This raises the question whether there are functional differences between Ifitm3 positive and negative HSCs.

3.3.3 Ifitm3EGFP expression is differentially induced after IFNα and LPS treatment

Since the analysis of Ifitm3EGFP Knock-in mice showed a high heterogeneity in regards to expression of EGFP within each hematopoietic cell compartment, we next investigated whether also the stress-induced induction of Ifitm3 expression is regulated differently within each compartment. Therefore we analyzed the expression of EGFP in Ifitm3EGFP+/Tm16 hours after in vivo administration of IFNα or LPS.

Expression of EGFP was increased in LSK as well as LK SLAM HSCs after both treatments (Fig 3.9 A+B). This increase was observed both in the intensity of the EGFP signal within the EGFP-positive cells, as well as in the proportion of HSCs positive for EGFP expression. The increase in expression was stronger in HSCs of mice treated with IFNα compared to LPS. Interestingly in differentiated cells the induction of EGFP expression was different between the two treatments. Both IFNα as well as LPS lead to increased expression of EGFP in monocytes, while only IFNα induced expression of EGFP in B-cells and CD4 or CD8 positive T-cells (Fig 3.9 C). Notably expression of EGFP was induced only in a small proportion of B-cells and T-cells, while the majority of cells remained EGFP negative.

Taken together these results suggest that while both IFNα and LPS can induce expression of Ifitm3, the underlying mechanisms are different, as LPS contrarily to IFNα does not lead to induction of Ifitm3 expression in B-cells and T-cells. Furthermore the triggers leading to the induction of Ifitm3 expression is differentially regulated between hematopoietic stem and progenitor cells compared to more differentiated cells, suggesting that Ifitm3 expression is induced in HSPCs under different conditions.
Figure 3.9 Induction of EGFP-expression in the hematopoietic compartment of Ifitm3EGFP Knock-in mice

Histogram of EGFP expression in LSK and LK SLAM HSCs 16 hours after in vivo treatment with IFNα (A) or LPS (B). (C) EGFP expression 16 hours after in vivo IFNα or LPS treatment in B-cells (B220+), CD4 and CD8 positive T-cells (CD4+ and CD8+) and monocytes, granulocytes and macrophages (CD11b+Gr-1+).
3.3.4 *Ifitm3* protein expression in the hematopoietic system

As higher mRNA expression does not necessarily indicate higher protein expression of *Ifitm3* in HSCs, we next investigated the expression of *Ifitm3* protein in the hematopoietic system. Therefore we performed immune-fluorescence staining for *Ifitm3* on cytopsins of unfractionated total bone marrow cells as well as sorted LK SLAM HSCs and differentiated cells of the hematopoietic system.

Interestingly only few cells within the bone marrow stained positive for *Ifitm3*, while sorted LK SLAM HSCs were highly enriched for *Ifitm3* protein expression, with the majority of cells staining positively (Fig 3.10 A). Notably the staining pattern appeared to be on the membrane of the cells as well as within the cells organized in a clustered structure. This correlates with recent reports, showing localization of overexpressed *Ifitm3* in human HeLa cells both on the membrane as well as within the endoplasmatic reticulum [Yount et al., 2010].

To investigate whether *Ifitm3* protein expression is induced by IFNα similarly to the RNA expression, we next analyzed LK SLAM HSCs 16 hours after *in vivo* treatment with poly-(I)-(C). Similar to the up-regulation of *Ifitm3* mRNA, also the protein expression was highly induced. Moreover the clustered expression pattern was more pronounced after activation of the cells compared to homeostasis (Fig 3.10 B). Notably in concordance with the induction of EGFP expression in our *Ifitm3EGFP* mouse model, only very few B-cells and T-cells stained positive for *ifitm3* during homeostasis, while a larger proportion of cells showed expression following administration of IFNα (data not shown). Overall the expression of *Ifitm3* protein correlated highly with the expression of *Ifitm3* mRNA observed before.

In conclusion within the hematopoietic system expression of the *Ifitm* family is highly enriched in HSCs during homeostasis, both on the RNA as well as on the protein level. Moreover both RNA and protein expression is further induced by IFNα. This suggests that the *Ifitm* protein family might play a crucial role in HSCs.
Figure 3.10 Ifitm3 protein expression in HSCs and unfractoned bone marrow

(A) Immune fluorescence staining for ifitm3 (red) and DNA (DAPI, blue) in unfractoned bone marrow cells and FACS sorted LK SLAM HSCs from C57BL/6 mice at homeostasis. (B) Staining for ifitm3 (red) and DNA (DAPI, blue) in LK SLAM HSCs 16 hours after in vivo administration of PBS or pIC. Expression of Ifitm3 in LK SLAM HSCs PBS appears lower in (B) as exposure was shorter to highlight increase of signal after pIC treatment.
3.3.5 Ifitm deficient mice have no hematopoietic phenotype

To elucidate the functional role of Ifitm3 in the hematopoietic system, we isolated bone marrow from Ifitm3EGFP<sup>T/T</sup> mice, which are deficient for Ifitm3 expression, and analyzed the hematopoietic compartment. Ifitm3 deficient mice had unchanged bone marrow cellularity and normal numbers of hematopoietic stem and progenitor cells as well as B-cells, lymphocytes and granulocytes compared to heterozygous Ifitm3EGFP<sup>+</sup><sup>T</sup> littermate controls (data not shown).

As the different members of the Ifitm protein family share high similarities, it is possible that there is functional redundancy between the different family members. Therefore deletion of Ifitm3 alone might be compensated by expression of other Ifitm family members. To address the effect of absence of the complete Ifitm protein family, we analyzed the hematopoietic system of IfitmDEL<sup>−/−</sup> mice [Lange et al., 2008]. In these mice the whole Ifitm family gene cluster, including Ifitm1, Ifitm2, Ifitm3, Ifitm5 and Ifitm6, has been deleted (Fig 3.11 A). IfitmDEL<sup>−/−</sup> mice show normal development and are viable and fertile, however the number of homozygous IfitmDEL mutant animals obtained from intercrosses of heterozygous IfitmDEL mice was reported to be slightly reduced from the expected Mendelian ratio, with only 17% homozygous IfitmDel mutant mice obtained at weaning age [Lange et al., 2008]. Strikingly, we observed an even lower rate of homozygous mutant IfitmDEL animals at weaning age in our intercrosses of IfitmDEL<sup>+/−</sup> mice, with only 2% of mice having an IfitmDEL<sup>+/−</sup> genotype, while 75% were IfitmDEL<sup>−/−</sup> and 23% IfitmDEL<sup>+/+</sup> (n=139). As previous reports suggested normal development during embryogenesis [Lange et al., 2008], we reasoned that IfitmDEL<sup>−/−</sup> mice might die more frequently between birth and weaning age. To investigate this, we genotyped newly born litters from IfitmDEL<sup>−/−</sup> intercrosses at postnatal day 1. Interestingly we observed a ratio of only 3% IfitmDEL<sup>−/−</sup>, 61% IfitmDEL<sup>+/−</sup> and 36% IfitmDEL<sup>+/+</sup> (n=64) closely matching the reduced frequency observed at weaning age.
**RESULTS**

Figure 3.11 Comparison of the hematopoietic system of wild type and Ifitm family deficient mice

(A) Schematic of the targeting strategy for the deletion of the Ifitm family cluster. Example of IfitmDEL wild type and mutant mice (adapted from [Lange et al., 2008]). (B) Comparison of LSK, LSK SLAM and LSK SLAM CD34⁻ HSCs of IfitmDEL+/+ and IfitmDEL−/− mice. (C) Comparison of lymphocytes (CD4⁺ and CD8⁺), granulocytes (CD11b⁺Gr-1⁺) and B-cells (B220⁺) from IfitmDEL+/+ and IfitmDEL−/− mice.
As IfitmDEL mice develop normally until embryonic day E17.5 [Lange et al., 2008], this indicates that a large proportion of IfitmDEL\(^{-/-}\) mice likely die during or shortly after birth. Due to the very low frequency of IfitmDEL\(^{-/-}\) mice we obtained, we performed some of the following experiments with IfitmDEL\(^{-/-}\) bone marrow chimeras where specified (Fig 3.12 A). Therefore wild type C57BL/6 mice were lethally irradiated and transplanted with bone marrow from IfitmDEL\(^{-/-}\) mice. Notably IfitmDEL\(^{-/-}\) bone marrow chimeras are deficient for the Ifitm family exclusively in hematopoietic cells, while the stromal compartment originates from wild type C57BL/6 hosts.

Similar to Ifitm3 deficient mice also IfitmDEL\(^{-/-}\) mice deficient for the whole ifitm family showed normal numbers of hematopoietic stem and progenitor cells (Fig 3.11 B). Furthermore also the numbers of CD4\(^+\) and CD8\(^+\) lymphocytes, CD11\(^+\)Gr-1\(^+\) granulocytes and B220\(^+\) B-cells were not affected by the absence of the ifitm family (Fig 3.11 C).

### 3.3.6 Characterization of stem cell properties of Ifitm-deficient HSCs

We next investigated the functional characteristics of HSCs of Ifitm-deficient mice. We observed no differences in the cycling behavior between Ifitm3-deficient and wild type LSK SLAM HSCs as analyzed by Ki67/Hoechst cell cycle analysis (Fig 3.12 B). Furthermore also IfitmDEL\(^{-/-}\) LSK SLAM HSCs, isolated from IfitmDEL bone marrow chimeras, showed a similar cell cycle distribution during homeostasis compared to wild type controls (Fig 3.12 C).

Despite the large proportion of stem cells which reside in the G\(_0\) phase of the cell cycle during homeostasis, only a small proportion of these cells is long-term dormant in the mouse [Wilson et al., 2008]. To investigate whether dormant HSCs are affected by loss of Ifitm3 we performed label-retaining assays with BrdU. Mice were supplied with BrdU in the drinking water for 10 days to label all HSCs (pulse period), followed by a BrdU-free chase period of 107 days. In this assay dividing cells dilute the initial BrdU label out, while only cells that don’t undergo active cell cycle retain the label [Wilson et al., 2008].
RESULTS

We did not observe any difference in the number of label retaining cells between Ifitm3 wild type and Ifitm3 deficient mice (Fig. 3.12 D). In conclusion deletion of either Ifitm3 or the whole Ifitm-family cluster does not affect the cycling behavior of HSCs during homeostasis.

We next assessed whether the capabilities of HSCs to differentiate and self-renew are affected in the absence of Ifitm family members. Therefore we isolated bone marrow from IfitmDEL+/- and IfitmDEL-/- bone marrow chimeras and performed in vitro colony formation assays. We did not observe any effect on colony formation capacity of HSCs in the absence of the Ifitm family proteins (Fig 3.12 E).

Since in vitro colony formation does not allow any conclusion about the self-renewal capability or the lineage contribution of HSCs we next performed competitive bone marrow transplantation assays (Fig 3.12 A). In these assays bone marrow cells from IfitmDEL+/- or IfitmDEL-/- mice were isolated and mixed with an equal number of bone marrow cells from C57BL/6 Ly5.1 wild type mice (Fig 3.12 A). Hematopoietic cells from IfitmDEL mice express the CD45 isoform CD45.2, while cells from C57BL/6 Ly5.1 mice express the CD45 isoform CD45.1. Therefore the contribution of IfitmDEL-/- and wild type cells to the hematopoietic system of the host mouse can be analyzed by using antibodies specific for CD45.1 or CD45.2 in FACS analysis. This mixture of bone marrow cells was transplanted intravenously into lethally irradiated C57BL/6 Ly5.1 mice. 14 weeks later the contribution of IfitmDEL+/- and IfitmDEL-/- cells to hematopoiesis was analyzed by FACS analysis. We did not observe any difference in contribution to either myeloid (granulocytes) or lymphoid (CD4 and CD8 positive lymphocytes, B-cells) lineages between Ifitm-deficient HSCs and wild type HSCs (Fig 3.12 F).

In summary deletion of the whole Ifitm family gene cluster does not affect cycling behavior, differentiation potential, engraftment potential or self-renewal capacity of HSCs. In conclusion the Ifitm protein family is not required for hematopoiesis during homeostasis.
Figure 3.12 Characterization of Ifitm-deficient HSCs
(A) Schematic for the generation of competitive 50:50 bone marrow chimeras of wild type and IfitmDEL mutant mice. (B) Ki67/Hoechst cell cycle profile of LSK SLAM HSCs from Ifitm3EGFP heterozygous or homozygous mutant mice. (C) Ki67/Hoechst cell cycle profile of LSK SLAM HSCs from IfitmDEL mutant and wild type C57BL/6 mice. (D) Quantification of BrdU label-retaining cells (LRCs) of LSK SLAM CD34+ wild type or Ifitm3-deficient HSCs after 107 days of BrdU-free chase. (E) Quantification of colonies formed from 10,000 plated IfitmDEL wild type or mutant unfractionated bone marrow cells; in vitro MethoCult colony forming cell assay (Stem Cell Technologies). (F) Analysis of chimerism 14 weeks after competitive bone marrow transplantation of IfitmDEL (CD45.2) wild type or mutant bone marrow cells with C57BL/6 Ly5.1 (CD45.1) bone marrow cells. Quantification of the proportion of CD11b+Gr-1+ granulocytes, B220+ B-cells and CD4+/CD8+ lymphocytes derived from IfitmDEL mice (expression the CD45.2 isoform of CD45).
3.3.7 Stress induced activation of Ifitm-deficient HSCs

As previously described stress signals like IFNα lead to the activation of usually quiescent HSCs [Essers et al., 2009]. Similarly also the expression of Ifitm1 and Ifitm3 is induced upon in vivo treatment with IFNα as well as LPS. To address whether the Ifitm family plays a role in the activation of HSCs, we performed BrdU incorporation assays as well as cell cycle analysis on HSCs of Ifitm deficient mice after in vivo treatment with IFNα or LPS. Therefore mice were injected intraperitoneally with 100µg poly-(I)-(C), which leads to production of IFNα, or 5µg LPS 16 hours before analysis. Additionally mice were pulsed with 400µg BrdU 12 hours before analysis. Cells that undergo the S phase of the cell cycle and synthesize new DNA between the timepoint of BrdU injection and the timepoint of analysis, incorporate BrdU in their DNA, which can be detected with BrdU specific antibodies and analyzed by FACS.

We did not detect any difference in BrdU incorporation between Ifitm3EGFP heterozygous and mutant LSK SLAM HSCs after poly-(I)-(C) injection (Fig 3.13 A). Both wild type as well as Ifitm3 deficient HSCs showed a high increase in BrdU incorporation following poly-(I)-(C) treatment, indicating an increase in cell proliferation. Furthermore administration of poly-(I)-(C), as well as LPS, also efficiently activated LSK SLAM HSCs from IfitmDEL−/− mice (Fig 3.13 B+C).

Another crucial process during the activation of HSCs is the shutdown of the activation response and reacquisition of quiescence. To address whether the Ifitm protein family plays a role in this process we analyzed BrdU incorporation at several timepoints after injection of poly-(I)-(C) in mixed IfitmDEL bone marrow chimeras (Fig 3.12 A). Wild type and Ifitm-deficient LSK SLAM HSCs were similarly activated after poly-(I)-(C) administration (Fig 3.13 D). Furthermore the level of BrdU incorporation decreased similarly by day 5 after injection and was close to baseline at day 7 after injection (Fig 3.12 D).
RESULTS

Figure 3.13 Activation of Ifitm-deficient HSCs by IFN\(\gamma\) and LPS

(A) BrdU incorporation in LSK SLAM HSCs, 16h after in vivo administration of 100µg poly-(I):(C) (pIC) in Ifitm3EGFP wildtype and mutant mice. (B) BrdU incorporation in LSK SLAM HSCs after 2 shots of 100 µg pIC 16 hours and 3 days before analysis in wild type C57BL/6 mice and IfitmDEL\(^{-/-}\) bone marrow chimeras. (C) BrdU incorporation in LSK SLAM HSCs of IfitmDEL\(^{-/-}\) bone marrow chimeras, 24 hours after injection with 100µg pIC or 5µg LPS. (D) Timecourse of BrdU incorporation in mixed bone marrow chimeras after two shots of 100µg pIC (day -2 and day 0). (E) Analysis of CD11b\(^{+}\)Gr-1\(^{+}\) granulocyte-chimerism from peripheral blood before and after serial treatment with PBS or pIC (100µg). PBS or pIC was administered intraperitoneally every second day for a total of eight injections, and chimerism analyzed one week after the last injection.

Previous experiments from our group showed that chronically activated HSCs are outcompeted in a competitive setting by HSCs, which can not respond to the activation stimulus [Essers et al., 2009]. To address whether IfitmDEL\(^{-/-}\)
HSCs have a competitive advantage or disadvantage during chronic stimulation compared to wild type cells, we analyzed the effect of chronic IFNα stimulation on IfitmDEL 50:50 bone marrow chimeras (Fig 3.12 A). Therefore the peripheral blood chimerism of the mice was determined at the start of the experiment. Subsequently the mice were intra-peritoneally injected every second day with 100µg of poly-(I)-(C) for a total of 8 injections. One week after the last injection the chimerism was analyzed again. We did not detect any significant differences between the chimerism before and after the chronic HSC activation, indicating that IfitmDEL−/− HSCs are similarly activated by chronic IFNα exposure compared to wild type HSCs (Fig 3.13 E).

In summary the Ifitm protein family is not crucial for the activation of HSCs by IFNα or LPS, neither it is required for the reacquisition of quiescence after the activation response. Finally also in a setting of chronic activation no differences could be observed between Ifitm-deficient and wild type HSCs. This raises the question whether the Ifitm protein family might have regulatory or effector functions unrelated to maintenance and cell cycle activation in HSCs. To gain further insight into this possibility we next performed gene expression analysis in wild type and Ifitm deficient HSCs.

### 3.3.8 Gene expression profile of Ifitm-deficient HSCs

Despite previous reports, which indicated an influence of Ifitm family members on components of the HSC niche as well as on IFN induced cell cycle control [El-Tanani et al., 2010; Yang et al., 2007], we did not observe any phenotype on cycling behavior of HSCs during homeostasis, activation of HSCs by stress signals or stem cell properties of HSCs in the absence of Ifitm3 or the whole ifitm family. To get further insight in which processes the Ifitm family might be involved in hematopoietic stem cells, we performed microarray analysis of FACS sorted LK SLAM HSCs from IfitmDEL+/+ and IfitmDEL−/− bone marrow chimeras, 16 hours after in vivo treatment with PBS or IFNα.
Interestingly HSCs from PBS treated and from IFNα treated mice were clustering together, irrespective of their IfitmDEL genotype (Fig 3.14). This indicates a high similarity between the expression profiles of IfitmDEL+/+ and IfitmDEL−/− HSCs, both during homeostasis as well as after IFNα-induced HSC activation.

We found 8228 genes to be significantly changed between HSCs from PBS-treated and IFNα-treated IfitmDEL+/+ bone marrow chimeras, with 180 genes
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changed more that 1.5 fold (FDR <5%). As expected the majority of differentially regulated genes was similar to the ones observed in our previous arrays from C57BL/6 wild type mice. The strikingly higher number of significantly changed genes in this experiment can be explained by the analysis of bone marrow chimeras, which harbor HSCs originating from the same donor mice, compared to litter mate controls in previous experiments. Therefore the variability between the different samples was very low.

Interestingly only 8 genes were changed significantly (FDR<5%) between IfitmDEL+/+ and IfitmDEL−/− LK SLAM HSCs during homeostasis. Of these strikingly only three genes were changed more that 1,5 fold: Ifitm1 (3,7x down in IfitmDEL−/− HSCs), Ifitm2 (5,2x down) and Ifitm3 (8,4x down). This nicely confirms the deletion of Ifitm1, 2 and 3 in IfitmDEL−/− HSCs, as only these three family members are expressed in HSCs as described before. As expected we did not detect reduced expression of ifitm5 and 6 as they are not expressed in HSCs. Interestingly despite the large number of genes changed after administration of IFNα in IfitmDEL+/+ LK SLAM HSCs, only 11 genes were differentially expressed between IfitmDEL+/+ and IfitmDEL−/− HSCs following treatment with IFNα. Of these again only Ifitm1 (8,3x down in IfitmDEL−/− HSCs), Ifitm2 (3,5x down) and Ifitm3 (11.8x down) were changed more than 1,5 fold.

In summary we could not detect any significantly changed genes (FDR<5% and FC>1.5) between IfitmDEL wild type and mutant HSCs, neither during homeostasis nor following IFNα-induced HSC activation, with exception of the Ifitm gene family itself. This data strongly suggests that the ifitm family does not play a role in transcriptional regulation within HSCs, but rather has terminal effector functions. It cannot be excluded however that the Ifitm family regulates expression of other genes on a post transcriptional level.

In line with our study it was recently shown that Ifitm1, 2 and 3 play a crucial role as host restriction factors during viral infections with a variety of diverse viral pathogens including HCV, Dengue virus or HIV [Brass et al., 2009; Huang et al., 2011; Lu et al., 2011]. As we observed not only an induction of
Ifitm-expression in HSCs by IFNα, but also a high level of expression already at homeostasis, it is tempting to speculate that the Ifitm family could play a crucial role in protecting HSCs from viral or bacterial pathogens.

Since not only Ifitm family members but also other genes involved in immune defense mechanisms were increased in our microarray screens of IFNα induced HSCs, we decided to investigate whether expression of immune defense related genes are generally enriched within HSCs already during homeostasis, or whether this enrichment is unique for the ifitm family of proteins.
3.4 Expression of immune defense associated genes in HSPCs

In the course of viral as well as bacterial infections, IFNα is produced by host cells and inhibits spread of pathogens by a variety of mechanisms including activation of immune cells and interference with viral replication [Stark et al., 1998]. As expected, in our microarray analysis of FACS sorted HSCs we observed the induction of many genes involved in immune responses, including Ifitm1 and Ifitm3, within HSCs after treatment of mice with IFNα in vivo (Tab 3.1). Interestingly however the expression of Ifitm1 and Ifitm3 in HSCs was not only induced by IFNα, but already highly enriched for during homeostasis.

To address whether HSCs are generally enriched for the expression of viral effector proteins during homeostasis, we selected some of the proteins involved in different steps of immune responses against intracellular pathogens, which were strongly induced in our microarray analysis of HSCs after treatment with IFNα: Ifit1 and Ifit3, Ddx58 (RIG-I), Oas3 and Iigp-1. Ifit1 (Interferon-induced protein with tetratricopeptide repeats 1) and Ddx58 (DEAD box polypeptide 58; also RIG-I) play crucial roles in the recognition of viral RNA by host cells [Pichlmair et al., 2011; Pichlmair et al., 2006]. Oas3 is a member of the 2',5'-oligoadenylate synthetase family, of which several members were highly induced by IFNα. The Oas family and its downstream effector RNase L play important roles in host defense against virus infection [Lin et al., 2009]. Iigp1 (interferon inducible GTPase 1) is a member of the interferon-inducible immunity-related family of p47 GTPases, which play a crucial role as resistance factors during infections by intracellular pathogens [Liesenfeld et al., 2011; Pawlowski et al., 2011].

To verify the induction of these genes by IFNα within HSCs, we isolated RNA from LK SLAM HSCs of C57BL/6 mice, 16 hours after in vivo treatment with PBS or pIC and analyzed gene expression by quantitative Real-time PCR.
Figure 3.15 Quantification of immune defense related genes in HSPCs

(A) Expression of Ifit1, Ifit3, Ddx58, Oas3 and Iigp1 in unfractionated bone marrow cells and LK SLAM HSCs from C57BL/6 mice, 16 hours after injection of PBS or piC, quantified per qPCR. Ratio of target gene expression relative to house keeping genes Sdha and Oaz1. (B) Expression Ifit1, Ifit3, Ddx58, Oas3 and Iigp1 in different cell types of the hematopoietic system relative to expression of Sdha, Oaz1 and B2m. HSCs (LSKCD150CD48CD34), MPP1/2 (LSKCD150CD48), MPP3/4 (LSKCD150), CMPs (linnegCD117Sca-1IL7RCD34FcgRh), GMPs (linnegCD117Sca-1IL7RCD34FcgRh), MEPs (linnegCD117Sca-1IL7RCD34FcgRh), CLPs (linnegCD117Sca-1IL7RCD135), erythrocytes (Ter119), granulocytes (CD11bGr1), B-cells (B220) and lymphocytes (CD4/8).
Expression of all five genes – Ifit1, Ifit3, Ddx58, Oas3 and ligg1 – was highly induced after IFNα treatment, and to a lower extent also LPS treatment (Fig 3.15 A). Notably during homeostasis only ligg1 showed a higher expression within LK SLAM HSCs compared to unfractionated total bone marrow.

To gain further understanding on the expression of these genes within hematopoietic stem and progenitor cells during homeostasis, we analyzed FACS sorted LSK SLAM CD34− HSCs, MPPs, CMPs, GMP, MEPs, CLPs, as well as differentiated cells by qPCR. Ifit3, Ifit1, Ddx58 and Oas3 were all expressed at similar levels in HSCs compared to early multi-potent progenitor cells. Interestingly there was a striking difference between the expression levels in multi-potent (HSCs, MPP1/2 and MPP3/4) versus committed (CMP, GMP, CLP and MEPs) progenitor cells, with higher expression within the multi-potent HSPC compartment (Fig 3.15 B). Interestingly this correlates with recent findings from our laboratory, which showed enrichment of a signature of immune-defense related proteins within multi-potent LSK cells, compared to lineage committed LK Sca−1− progenitor cells in a proteomics screen [Klimmeck et al., 2012]. The expression levels of these genes in HSPCs were however low compared to the expression in differentiated cells. All four genes were expressed highly in granulocytes, Ifit3 additionally also in CD41+ megakaryocytes, and Ifit1 and Oas3 in erythrocytes (Fig 3.15 B). The only exception was ligg1, which was expressed highest in HSCs and MPPs, with low expression in differentiated cells with the exception of lymphocytes.

In summary we observed a higher expression of immune defense associated genes within multi-potent HSPCs compared to committed progenitor cells, however with the exception of ligg1 the expression level was low compared to more differentiated cells.

To gain more insight whether immune defense related genes are specifically enriched in the transcriptome of HSCs compared to all other cell types of the bone marrow, we performed microarray analysis of unfractionated bone marrow cells and FACS sorted LK SLAM HSCs, 16 hours after in vivo treatment with PBS or IFNα.
RESULTS

Figure 3.16 Comparison of the transcriptome of unfractionated bone marrow cells with LK SLAM HSCs

(A) Gene ontology analysis of significantly changed genes (FDR<5%, Fold change>2) between total bone marrow cells and FACS sorted LK SLAM HSCs, 16 hours after injection of PBS. Most significantly changed GO processes analyzed with Metacore software V6.10 (Thomson Reuters). (B) Microarray expression data from selected genes from samples of total bone marrow cells and FACS sorted LK SLAM HSCs, 16 hours after treatment with PBS or IFNα.

We found 1910 genes to be enriched in LK SLAM HSCs compared to total bone marrow (FC>2, FDR<5%) during homeostasis. Gene ontology analysis showed that the most significantly enriched processes in HSCs are related to cellular metabolism, self-renewal, cell cycle control and DNA damage repair (Fig 3.16 A). As expected we found known HSC markers highly enriched within the HSC fraction, including c-kit, Sca-1 and CD150 (Fig 3.16 B and data not shown).
Strikingly the transcript levels following administration of IFNα highly reflected the level of protein expression observed by FACS analysis, with c-kit being down-regulated, Sca-1 up-regulated and CD150 expression unaffected by IFNα. Furthermore we found several genes, identified as candidate regulators of HSC activation in our previous screen, to be highly enriched within LK SLAM HSCs during homeostasis (Tab 3.1 and Chapter 3.2). These included the cell cycle regulators Reprimo and Pleiotropin, as well as Matrilin-4 and Maged1 (Fig 3.16 B and data not shown). All of these genes were expressed at high levels within HSCs during homeostasis but are strongly down-regulated following IFNα treatment (Fig 3.16 B).

Interestingly we also found a strong enrichment for transcripts associated with blood coagulation and platelet aggregation in LK SLAM HSCs during homeostasis (Fig 3.16 A). These included expression of Von Willebrand Factor (Vwf), Platelet Factor 4 (CXCL4) and Coagulation factor II receptor (F2r). Strikingly this correlates with recent reports of enriched expression of megakaryocyte-erythrocyte genes in HSCs compared to lymphoid-primed multi-potent progenitor cells (LMPPs) [Mansson et al., 2007].

In conclusion we did not find a general enrichment of immune-defense associated transcripts within HSCs. Notable exceptions were Ifitm1, interferon-induced protein 44 (Ifi44) and interferon gamma inducible protein 47 (Ifi47 or Iigp4). Ifi44 has previously been implicated in cell cycle arrest during hepatitis C virus infections, while Ifi47 – another member of the p47 GTPase family – plays a role in clearance of protozoan and bacterial infections [Collazo et al., 2001; Hallen et al., 2007]. Thus we found no evidence that the expression of immune defense genes is generally enriched in HSCs compared to total bone marrow cells during homeostasis. Instead only the Ifitm protein family (Ifitm1, 2 and 3), as well as the family of p47 GTPases (Iigp1 and Iigp4), is enriched in LK SLAM HSCs. Whether these protein families are crucial for HSC-specific defense mechanism against viral or bacterial pathogens remains to be determined.
3.5 Activation of human HSCs in a xenotransplantation model

So far, activation of HSCs by IFNα, IFNγ as well LPS was only shown in the mouse. IFNα is widely used in the clinic to treat patients with symptoms including hepatitis B and C viral infection, multiple sclerosis, melanoma as well as several hematological malignancies including chronic myeloid leukemia, hairy cell leukemia and different kinds of lymphoma [Cooksley, 2004; Goldstein and Laszlo, 1988; Hauschild et al., 2008; Paolicelli et al., 2009; Shepherd et al., 2000]. Elucidating whether human HSCs are activated by IFNα similarly to their mouse counterparts is not only highly relevant for a better understanding of the effects as well as side effects of these treatments, but also crucial for the design of combination therapies to better target quiescent cancer stem cells in hematopoietic malignancies.

While in vitro treatment of cells with IFNα has anti-proliferative effects, only in vivo administration of IFNα leads to activation of HSCs in the mouse [Essers et al., 2009]. Thus it is crucial to investigate the effects of human IFNα on human HSCs in an in vivo model, where human HSCs reside in a bone marrow niche environment. We therefore established a xenotransplantation mouse model with human cord blood cells. As host mice for the xenotransplantation the immune compromised NSG strain was chosen, which readily accepts human grafts without rejection [Ishikawa et al., 2005; Shultz et al., 2005].

Human cord blood cells were enriched for CD34 expression by magnetic bead separation (Miltenyi Biotech). NSG mice were sub-lethally irradiated 24 hours before the transplantation to ablate mouse HSCs, and human CD34 enriched cord blood cells were transplanted by intra-femoral injection. This injection technique allows engraftment independent of an HSCs homing process and leads to better engraftment of human HSCs compared to intra-venous injection [Zhan and Zhao, 2008].
We monitored engraftment efficiency of human cells in the mouse by FACS analysis of cells isolated from the peripheral blood from eight weeks post transplantation and performed analysis of the mice after different treatments from 12 to 14 weeks after transplantation (Fig 3.17 A).

To identify human cells, antibodies specific for mouse or human CD45, the common leukocyte antigen, which is present on almost all hematopoietic cells, were used (Fig 3.17 B). Human HSCs were defined as hCD45^lin^-CD34^+CD38^- (CD34^+CD38^-) and progenitor cells as hCD45^lin^-CD34^+CD38^+ (CD34^+CD38^+). Ki67/Hoechst cell cycle analysis showed that a higher proportion of HSCs were in the G_0-phase of the cell cycle (Ki67^loHoechst^lo) compared to progenitor cells (Fig 3.17 C). Similarly a smaller fraction of HSCs was in S-phase of the cell cycle (Ki67^hiHoechst^hi) compared to progenitor cells. As Thy-1 (CD90) was proposed to enrich for human HSCs in combination with CD34 [Murray et al., 1995], we compared the cell cycle profile
of CD34^+CD38^-CD90^+ cells with CD34^+CD38^- HSCs, but did not observe any further enrichment for quiescent cells (data not shown).

Notably the proportion of quiescent human HSCs was much lower than in mouse HSCs. While about 80% of mouse LSK SLAM HSCs are in the G_0 phase of the cell cycle during homeostasis, only about 20% of human CD34^+CD38^- HSCs were found to be in G_0 in xenotransplanted mice (Fig 3.19 C). To determine whether, similar to mouse HSCs, also a proportion of human HSCs in our xenotransplantation assays is long term dormant, we performed BrdU label retaining assays. Therefore mice were supplied with BrdU in the drinking water for 10 days to label all HSCs, followed by a BrdU-free chase period of 3 weeks, in which cells dividing in this time frame loose their label. We did not detect any BrdU positive human HSCs after the chase period, indicating that there are no human HSCs in the mouse that remain long term quiescent (data not shown). Notably this absence of quiescent cells cannot be explained by the transplantation process itself, as mouse HSCs show cell cycle distributions similar to homeostatic conditions 12 weeks after transplantation (Fig 3.12 C).

3.5.1 Effect of human type I interferon on human HSCs

Currently there are more than 13 different subtypes of IFNα described in humans, with IFNα 2a and IFNα 2b being the most commonly used in the clinic [Genin et al., 2009; Woelk et al., 2007]. Therefore we decided to use IFNα 2a to elucidate the effect of type I IFN on human HSCs. To investigate whether human IFNα activates human HSCs we injected xenotransplanted mice 12-20 weeks after transplantation with PBS or 10.000 of IFNα 2a (Roche) subcutaneously, and analyzed the cell cycle distribution and BrdU incorporation by FACS analysis 16 hours later. We observed neither differences in cell cycle distribution by Ki67/Hoechst analysis nor an increase in BrdU incorporation (Fig 3.18 and data not shown). As the dosage of IFNα is critical for activation of HSCs we next administered IFNα in different doses, ranging from 10.000 to 100.000U, but did not observe any activation with any of these doses (Fig 3.18). Notably murine
HSCs are activated already by IFNα doses as low as 10U and are fully activated with 1000U [Essers et al., 2009].

Activation of HSCs in the mouse by IFNα is very rapid, with HSCs entering an active cell cycle already 16 hours after injection of IFNα. Notably kinetic analysis of BrdU uptake in mouse HSCs showed that the proportion of BrdU positive HSCs still increased until 48 hours after the injection [Essers et al., 2009]. To investigate whether human HSCs might be activated at a later time point after injection of IFNα, we performed a kinetic analysis. Therefore xenotransplanted mice were injected twice with IFNα 48 hours apart, and Ki67/Hoechst cell cycle analysis was performed 24 hours, 48 hours and 72 hours after the 2nd IFNα injection. We did not observe any decrease in the proportion of human HSCs in the G₀ phase of the cell cycle at any of these time points (Fig 3.18).

**Figure 3.18 Ki67/Hoechst analysis of quiescent human HSCs following different treatments with IFNα**

Proportion of Lin⁻⁰⁰CD3⁴⁺CD3⁸⁻ HSCs in the G₀ phase of the cell cycle in a Ki67/Hoechst FACS analysis, isolated from the bone marrow of xenotransplanted NSG mice. 16 hours after subcutaneous injection of PBS, 10.000, 50.000 or 100.000U of IFNα 2a; 48 and 72 hours after two injections of IFNα at -48 hours and 0 hour time point; 16 hours after injection of pegylated IFNα 2b (Intron A) and 16 hour after i.p. injection of poly-(l)-C or i.p. injection of poly-(l)-(C) plus additional subcutaneous injection of 10.000U of IFNα 2a.
The half-life of IFNα in human patients is very short, declining within hours after injection. Attachment of a polyethylene glycol moiety to IFNα results in a compound (pegylated IFNα) that has sustained absorption, a slower rate of clearance, and a longer half-life than unmodified IFNα [Nieforth et al., 1996; Reddy, 2004]. Due to these benefits pegylated IFNα replaced the use of recombinant IFNα in many clinical applications including treatment for hepatitis B and C viral infections [Zeuzem et al., 2000]. To address whether pegylated IFNα showed more prominent effects on human HSCs we injected xenotransplanted mice with PBS or pegylated IFNα 2b. Similarly to recombinant IFNα we did not observe cell cycle activation in human HSCs (Fig 3.18).

One major difference between mouse and human HSCs in our xenotransplantation model is the foreign mouse niche environment for human HSCs. Notably the bone marrow niche does play a crucial role in the activation of murine HSCs, however its exact contribution to the activation process is currently unknown. It is possible that human IFNα does not efficiently activate the mouse niche cells and therefore signals by niche cells are missing that are required for activation of human HSCs. To exclude this possibility, xenotransplanted mice were injected subcutaneous with human IFNα 2a and intraperitoneal with pIC, 16 hours before analysis. This additional injection of pIC activates mouse HSCs and potentially niche cells at the time point of treatment with human IFNα. Ki67/Hoechst analysis as well as BrdU incorporation showed efficient activation of mouse HSCs after treatment with pIC as well as pIC and IFNα, whereas we did not observe any effect on quiescent human HSCs (Fig 3.18 and data not shown).

Human IFNα 1 was previously reported to have cross-species activity in mouse and humans cells and was shown to induce the Mx promoter in the MxCre mouse line [Kuhn et al., 1995]. Therefore we further tested the effects of this subtype on human HSCs in our xenotransplantation model. Mice were injected subcutaneously with 100,000U of IFNα 1b and 16 hours later the cell cycle profile was analyzed by FACS analysis. Similar to treatment with other IFNα subtypes we did not observe activation of human HSCs with neither IFNα 1b nor
combination of IFNα 1b + pIC (Fig 3.19 A+C). Interestingly mouse HSCs were not only activated following treatment with IFNα 1b + pIC but also to a lower degree by IFNα 1b alone, confirming the cross species specificity of this interferon alpha subtype (Fig 3.19 B+C).

Figure 3.19 Ki67/Hoechst cell cycle analysis of human HSCs following different treatments with IFNα 1b
Cell cycle analysis of human lin<sup>−</sup>CD34<sup>−</sup>CD38<sup>−</sup> HSCs (A) and mouse LSK SLAM HSCs (B) 16 hours after treatment of xenotransplanted mice with PBS or pIC + human IFNα 1b. (C) Quantification of human lin<sup>−</sup>CD34<sup>−</sup>CD38<sup>−</sup> HSCs, lin<sup>−</sup>CD34<sup>−</sup>CD117<sup>+</sup> HSCs and mouse LSK SLAM HSCs in the G<sub>0</sub> phase of the cell cycle 16 hours after injection of PBS, IFNα 1b or pIC + IFNα 1b.
In summary in vivo administration of human IFNα did not lead to cell cycle activation of human HSCs in our xenotransplantation mouse model, neither alone nor in combination with pIC.

3.5.2 Activation of human HSCs by LPS

Administration of LPS in vivo leads to activation of mouse HSCs, at least partially independent of interferon type I and type II signaling [Marieke Essers, personal communication]. To elucidate the effects of LPS on human HSCs we injected xenotransplanted mice with LPS (5µg/mouse) and analyzed the phenotype as well as the cell cycle profile of human HSCs 24 hours later by FACS analysis. Strikingly the population of CD34⁺CD38⁻ HSCs disappeared nearly entirely, very likely due to up-regulation of CD38 expression in previously CD34⁺CD38⁻ HSCs (Fig 3.20 A). Interestingly expression of CD38 has previously been linked to activation of a variety of different hematopoietic cells types including B-cells, T-cells and Chronic lymphocytic leukemia cells [Deaglio et al., 2010; Funaro et al., 1997; Funaro et al., 1990; Sandoval-Montes and Santos-Argumedo, 2005].

Due to the induction of CD38 expression after LPS treatment, comparison of the cell cycle profile of CD34⁺CD38⁻ HSCs in homeostasis and activated state is not possible. We therefore decided to define the human HSCs as CD34⁺CD117⁺ as we observed a strong inverse correlation between expression of CD38 and expression of CD117 in CD34⁺ human HSPCs (Fig 3.20 B) and expression of CD117 was less affected (slightly decreased) following LPS treatment. Back-gating analysis confirmed that CD34⁺CD38⁻ HSCs were almost exclusively CD117⁺, while CD117⁻ cells contained nearly no CD34⁺CD38⁻ HSCs (Fig 3.20 C). As expected a larger proportion of CD34⁺CD117⁺ human HSCs was in G₀ phase of the cell cycle during homeostasis compared to CD34⁺CD117⁻ progenitor cells (data not shown).

Strikingly Ki67/Hoechst cell cycle analysis showed a marked decrease in quiescent CD34⁺CD117⁺ human HSCs following in vivo treatment with LPS (Fig.
3.20 D+E). Thus unlike IFNα, LPS can activate human HSCs in our xenotransplantation model.

Figure 3.20 Activation of human HSCs by LPS
(A) Polychromatic FACS blot of human lineage negative cells 24 hours after in vivo treatment with PBS or LPS. (B) Correlation of expression of CD38 and CD117 within lin−CD34+ (C) Back-gating analysis of CD117+ and CD117− cells on human lineage negative cells. (D) Example for cell cycle distribution of human lin−CD34+CD117− HSCs 24 hours after treatment with PBS or LPS. (E) Quantification of human lin−CD34+CD117− HSCs in G0 phase of the cell cycle, 24 hours after treatment with PBS or LPS.
3.5.3 Effects of type II and type III interferon and TNFα on human HSCs

Besides IFNα and LPS, also IFNγ has recently been shown to activate HSCs in the mouse in vivo [Baldridge et al., 2010]. Moreover also TNFα was proposed as another potential regulator of HSC activation [Baldridge et al., 2011]. While similar to IFNα and IFNγ also TNFα inhibits proliferation of HSPCs in vitro [Jacobsen et al., 1994; Jacobsen et al., 1992; Zhang et al., 1995], recent reports suggest that its functions in vivo might be different [Pronk et al., 2011; Rezzoug et al., 2008].

To elucidate the effects of type II and type III interferon as well as TNFα on human HSCs, we injected xenotransplanted mice subcutaneously with human IFNγ (100,000U/mouse), IFNλ (IL28; 5µg/mouse), TNFα (10µg/mouse) or LPS, and analyzed the cycling behavior of mouse and human HSCs 24 hours later. Mouse HSCs were activated by treatment with IFNλ, TNFα as well as LPS, while we did not observe activation by IFNγ (Fig 3.21 A). Notably the activation by LPS was much stronger than by IFNλ or TNFα. Contrarily to mouse HSCs, human HSCs were only activated by LPS while we did not observe a decrease of cells in G0 phase of the cell cycle with either IFNγ, IFNλ or TNFα.

3.5.4 Gene expression profile of activated human HSCs

As described above, we observed cell cycle activation of human HSCs in xenotransplanted mice only after treatment with LPS but not by type I, II and III interferons or TNFα, contrary to observations in mouse HSCs. One explanation for these differences between mouse and human HSCs is the foreign mouse niche environment for human HSCs in our xenotransplantation model, where critical direct cell-cell interaction or secreted factors, which are present in a human HSC niche, might be missing to achieve activation of human HSCs. As activation of HSCs by IFNα is complex and requires several signaling steps it could well be that human HSCs are not fully activated in the foreign niche, although their response to IFNα might still be partly intact.
RESULTS

To address this question and to gain more insight into the processes involved in activation of human HSCs, we performed microarray analysis of human hCD45^lin^CD34^+^CD117^+ HSCs. Xenotransplanted mice were injected with PBS, IFNα 1b (100,000U), IFNα 1b (100,000U) + pIC or LPS. 16 hours later cells were isolated from the bone marrow and lin^neg^CD34^+^CD117^+ HSCs were sorted by FACS. Next RNA was isolated from these cells and microarray analysis performed.

Comparison of gene expression data showed a higher variability between biological samples compared to the microarrays of mouse HSCs we performed earlier. Therefore we decided to accept a higher false discovery rate of 10% (5% for previous arrays) to be able to perform gene ontology analyses. We found 771

Figure 3.21 Cell cycle profile of mouse and human HSCs following treatments with IFNγ, IFNλ, TNFα and LPS

Ki67/Hoechst cell cycle profile of mouse LSK CD150+ and LSK SLAM HSCs (A) as well as of human lin^neg^CD34^+^CD117^+ HSCs (B) 24 hours after injection of IFNγ (100,000U/mouse), IFNλ (5µg/mouse), TNFα (10µg/mouse) and LPS.
genes to be differentially regulated following administration of IFNα (10% FDR; 23 genes 5% FDR), with 260 changed more than 1.5 fold, 1435 genes differentially regulated following administration of IFNα and pIC (10% FDR; 91 genes 5% FDR), with 145 changed more than 1.5 fold and 411 genes following treatment with LPS (10% FDR; 108 genes 5% FDR), 107 changed more than 1.5 fold.

We found 260 genes to be differentially regulated (FDR 10%, FC>1.5) in human CD34⁺CD117⁺ HSC following IFNα treatment. Surprisingly only 2 of those genes showed increased expression, IFN inducible protein 27 (IFI27; 2.0x up) and IFN inducible gene 6 (IFI6; 1.7x up). On the contrary we observed induction of known interferon target genes after simultaneous treatment with IFNα + pIC as well as after treatment with LPS, similar to the changes previously observed in mouse HSCs (Tab 3.3 and 3.4). We therefore only included samples from mice treated simultaneously with IFNα + pIC or LPS in our gene ontology analysis.

Interestingly STAT1 as well as several IFN target genes including IFIT3, OAS3 and other OAS family members and ISG15 were found amongst the highest induced genes after both treatment conditions. These results are highly similar to the genes up-regulated in mouse HSCs after activation by IFNα or LPS. Further similarities between mouse and human HSCs are the induction of CXCL10 after IFNα + pIC and CXCL9 after LPS stimulation. Surprisingly defensin genes were the strongest down-regulated genes on both conditions. Defensins are small proteins, which function as host defense peptides against viruses and bacteria and are induced during inflammatory processes in many tissues [Lehrer and Lu, 2012].
### RESULTS

<table>
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<th>Symbol</th>
<th>Definition</th>
<th>Synonyms</th>
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Table 3.3 Differentially regulated genes in human CD34+CD117+ HSCs 16h after treatment with IFNα+pIC in vivo

Increase of gene expression in human CD34+CD117+ HSCs FACS-sorted from xenotransplanted mice, 16 hours after treatment with IFNα + pIC compared to human HSCs from PBS treated mice; False discovery rate (FDR) <5%; blue banded rows mark known interferon response genes.

As expected we did not find any cell cycle related genes differentially regulated after IFNα + pIC treatment in human HSCs, which correlates with the lack of cell cycle induction in these cells determined by FACS analysis. Strikingly however human HSCs from LPS treated xenotransplanted mice showed increased expression of the cyclin-dependent kinase inhibitors p19 (INK4D; CDKN2D), and p21Cip1 (CDKN1A) as well as of Cyclin D1 (CCND1), all of which are involved in regulation of cell cycle progression through the G1 and S transition. The cyclin dependent kinase inhibitor p21 plays an important role in controlling cell cycle progression during the G1 phase by inhibiting cyclin-CDK2/CDK1 complexes as well as the S phase by inhibiting activity of cyclin E-CDK4 and cyclin D1-CDK4/CDK6 complexes [Harper et al., 1993; Sherr and Roberts, 1999]. Likewise p19 plays a crucial role during G1/S transition by forming complexes with CDK4.
and CDK6 and preventing activation of these kinases. Its expression oscillates in a cell cycle dependent manner and is lowest during the G1 phase and highest during the S phase [Chan et al., 1995; Hirai et al., 1995]. Finally Cyclin D1 is crucial for G1/S transition as part of a complex with CDK4/6 [De Vivo et al., 2011]. Increased expression of Cyclin D1 and p19 and p21 at the same time could be explained by a feedback loop, leading to a reacquisition of quiescence following the activation of HSCs. Notably in our analysis HSCs don’t progress through the cell cycle simultaneously. Hence the data can also be interpreted as an increased proportion of cells in either the G1 and S phase, compared to G0 phase, where a part of the cells shows elevated levels of Cyclin D1 while another part shown elevated levels of p19/p21. To elucidate this possibility, analysis would have to be performed on a single cell level.

<table>
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Table 3.4 Differentially regulated genes in human CD34+CD117+ HSCs 16h after treatment with LPS in vivo
Increase of gene expression in human CD34+CD117+ HSCs FACS-sorted from xenotransplanted mice, 16 hours after treatment with LPS compared to human HSCs from PBS treated mice; False discovery rate (FDR) <5%; blue banded rows mark known interferon response genes.
Gene ontology analysis highlighted immune responses and response to IFN as the most prominently changed processes after both IFNα + pIC as well as LPS treatment (Fig 3.22 A). Comparison of GO networks and processes showed a higher induction of processes involved in antigen presentation and responses to RNA viral infection after treatment with IFNα + pIC (Fig. 3.22 B). Strikingly, processes involved in proliferation and G1/S transition were highly induced after LPS treatment while they were not affected after administration of IFNα and pIC (Fig 3.22 B). This finding reflects the cell cycle induction by LPS, which was not observed after treatment with IFNα and pIC.

In summary the changes in gene expression patterns of human HSCs in our xenotransplantation mouse model after simultaneous treatment with IFNα + pIC or treatment with LPS reflect the changes observed in mouse HSCs. As expected in both human and mouse HSCs induction of interferon response genes and genes involved in immune responses are the most prominently changed genes. Notably the CIP/KIP family of kinase inhibitors seems to be crucial for regulation of the cell cycle after IFNα or LPS treatment, with expression of p57KIP2 affected in mouse HSCs and p21CIP in human HSCs. Interestingly the same family was recently shown to be crucial for regulation of HSC quiescence during homeostasis [Tesio and Trumpp, 2011].

As a next step it will be crucial to compare the expression profile of mouse HSCs and human HSCs after IFNα treatment in detail. While mouse HSCs are activated by IFNα, human HSCs seem to respond only partly e.g. by expression of IFNα response genes, but show no induction of the cell cycle. Investigating these differences will not only help us understand why human HSCs are not efficiently activated in this xenotransplantation model, but also help us to understand the activation process in mouse HSCs in more detail. Additionally comparison of these gene expression profiles could also provide valuable new insight into the involvement of cells of the stem cell niche, as human cells in our xenotransplantation model might miss crucial crosstalk with surrounding mouse cells.
**RESULTS**

**A. GO Processes**

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**B. Process networks**

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**Figure 3.22 Gene ontology analysis of human HSCs after treatment with IFNα + pIC or LPS**

(A) Most significantly changed GO processes in human CD34+CD117+ HSCs, 16 hours after treatment of mice with LPS, analyzed with Metacore software V6.10 (Thomson Reuters). Orange bars represent p-values for LPS treated HSCs. Fold change >1.5 and FDR<10%

(B) Comparison of process networks changed in CD34+CD117+ human HSCs after *in vivo* treatment of xenotransplanted mice with IFNα + pIC (orange bars) or LPS (blue bars). Fold change >1.5 and FDR<10%
3.6 Activation of leukemic stem cells in a mouse model of CML

Chronic myeloid leukemia (CML) is characterized by a chromosomal translocation, leading to the formation of the BCR-Abl fusion kinase [Daley et al., 1990]. Inhibition of BCR-Abl by tyrosine kinase inhibitors (TKI) like Imatinib specifically targets BCR-Abl expressing leukemic cells (LSCs) and leads to rapid hematologic and cytogenetic responses [O'Brien et al., 2003]. Nonetheless, LSCs are not efficiently eliminated by TKI treatment, and residual LSCs can lead to relapse of the disease if the treatment is discontinued [Goldman, 2009]. One possible reason for the resistance of LSCs is their dormancy, which seems to be similar to the resistance of quiescent HSCs in the mouse to cytotoxic substances. Interestingly mathematical modeling on the effects of TKI treatment in CML suggests that the resistance of leukemic clones can be explained by a selective effect of TKIs exclusively on proliferating cells [Roeder et al., 2006]. Strikingly this model further suggests that activation of leukemic stem cells in combination with TKI treatment might fully eradicate all leukemic clones. As recently shown IFNα can efficiently drive quiescent HSCs into proliferation in vivo [Essers et al., 2009]. Therefore activation of LSCs by IFNα followed by targeted therapy with TKIs might be a way to eliminate LSCs and lead to a cure of the disease.

To get further insight on the feasibility of such combination therapies we investigated the effects of IFNα on leukemic stem cells in a mouse model for chronic myeloid leukemia [Koschmieder et al., 2005]. In these SCLtTA/BCR-Abl mice the transactivator protein tTA is under the control of the murine stem cell leukemia (SCL) gene 3′ enhancer, and the P210 BCR-Abl transgene under control of a tet-responsive element (TRE). This allows to induction of human BCR-Abl expression specifically in hematopoietic stem and progenitor cells by withdrawal of Doxycycline (Fig 3.23).

To confirm the induction of a CML like disease in these mice, Dox treatment was stopped in 4 week-old mice and mice were analyzed 6 weeks later for signs of leukemia. As expected spleen size in double transgenic (dtg) mice,
RESULTS

carrying the SCLtTA and the BCR/ABL transgene, was increased compared to single transgenic (stg) littermate controls, carrying only one of the transgenes (Fig 3.24 A+B).

As reported [Koschmieder et al., 2005] the number of LSK HSPCs increased in double transgenic mice in the bone marrow as well as in the spleen (Fig 2.23). Furthermore we observed an increase in mature CD11b^Gr1^hi granulocytes as well as immature CD11b^Gr1^lo granulocytes in both bone marrow and spleen of double transgenic animals, thus confirming published data on these mice (Fig 3.24 D). Moreover the number of B-cells was decreased in double transgenic animals in bone marrow and spleen, and the number of erythrocytes decreased in the bone marrow (data not shown).

Figure 3.23 SCLtTA/BCR-ABL mouse model for chronic myeloid leukemia
Mice expressing the tet trans-activator protein tTA under control of the Stem cell leukemia gene (SCL) 3’ enhancer were crossed with mice carrying the BCR-ABL transgene under control of a tet responsive element (TRE). Upon withdrawal of Doxycycline, double transgenic litters express the BCR-ABL transgene specifically in stem and progenitor cells of the hematopoietic system.
Figure 3.24 Analysis of leukemia in SCLtTA/BCR-ABL double transgenic mice 6 weeks off Dox treatment

(A) Spleen weight of single transgenic (stg; either SCLtTA or BCR-ABL) and double transgenic (dtg; SCLtTA and BCR-ABL transgene) mice, 6 weeks after discontinuation of Dox treatment. (B) Picture of spleens from stg and dtg animals (C) FACS analysis of LSK cells in the bone marrow of stg and dtg mice and quantification of analysis in bone marrow and spleen. (D) FACS analysis of mature CD11b^Gr-1^hi and immature CD11b^Gr-1^lo granulocytes in the bone marrow of stg and dtg mice and quantification of analysis in bone marrow and spleen.

Notably we observed high variation in the onset of leukemia between different batches of mice, with some mice having string signs of leukemia 6 weeks after stop of Doxycycline, while other mice did not show signs of disease 12
weeks after Doxycycline stop. A high variation in the aggressiveness of the disease was already previously described by Koschmieder and colleagues [Koschmieder et al., 2005]. This variability in the onset of the disease could be explained due to different expression levels of - between mice homozygous or heterozygous for the transgenes as well as by different levels of expression in individual mice.

3.6.1 Establishment of a transplantation model for leukemic cells

A big caveat of the SCLtTA/BCR-ABL mouse model is that all HSC and progenitor cells in double transgenic animals express the BCR-ABL transgene and are potential leukemic stem cells. While treatment of double transgenic mice with Imatinib temporarily leads to normalization of blood counts, upon cessation of the treatment the BCR-ABL transgene is re-expressed and the leukemia relapses [Schemionek et al., 2010]. As there are no wild type HSCs which can take over the place of LSCs during treatment with the TKIs and lead to potential cure of this disease in this model, it is not suitable to investigate a combination therapy with IFNα and Imatinib.

To model chronic myeloid leukemia more closely resembling the situation in human patients, we generated mixed bone marrow chimeras, harboring wild type HSCs and CML LSCs from SCLtTA/BCR-ABL double transgenic mice. Therefore FVB/N wildtype mice were lethally irradiated with 2x 500 rad and 24 hours later transplanted with a mixture of 50% FVB/N wildtype and 50% SCLtTA/BCR-ABL single or double transgenic unfractionated bone marrow cells. Dox treatment was stopped directly after transplantation. Seven weeks after transplantation cell counts in peripheral blood were analysed.
Figure 3.25 Analysis of SCLtTA/BCR-ABL bone marrow chimeras
(A) Analysis of cell counts in peripheral blood of mixed bone marrow chimeras from SCLtTA/BCR-ABL single or double transgenic mice and FVB/N wildtype mice, 7 weeks after cessation of Doxycycline treatment; cell counts were determined with a Hemavet counter (Drew Scientific) (B) Analysis of mixed bone marrow chimeras from SCLtTA/BCR-ABL single or double transgenic mice and FVB/N wildtype mice, 11 weeks after cessation of Doxycycline treatment; Lineage negative cells, LSK cells, granulocytes and erythrocytes were analyzed by FACS analysis.
Mice transplanted with bone marrow from double transgenic mice showed increased numbers of total white blood cells, monocytes as well as neutrophils, while mice transplanted with bone marrow from single transgenic mice showed no increase (Fig 3.25 A). Four weeks later we analyzed spleen and bone marrow. Spleen weight was slightly increased in double transgenic bone marrow chimeras, but less pronounced than in SCLtTA/BCR-ABL mice at the same time point (Fig 3.25 B). Similarly the number of LSK cells was slightly increased in double transgenic mice. Interestingly we observed a strong increase in the number of undifferentiated lineage negative cells (Fig 3.25 B). This could potentially be due to inability of leukemic blasts to fully differentiate. Notably we further observed a strong increase in immature CD11b⁺Gr-1<sup>lo</sup> and mature CD11b⁺Gr-1<sup>hi</sup> granulocytes and a strong decrease of erythrocytes in bone marrow of double transgenic mice.

Taken together FVB/N mice transplanted with a mixture of wildtype cells and SCLtTA/BCR-ABL double transgenic cells develop a CML like disease within few weeks after transplantation. They show an increase of white blood cells, especially monocytes and granulocytes, as well as strong symptoms of anemia while the increase in spleen size was only modest.

3.6.2 Activation of leukemic stem cells by IFNα

While healthy HSCs are efficiently activated by IFNα <em>in vivo</em> in the mouse, the effect of IFNα on LSC is still unknown. To investigate whether IFNα activates leukemic stem cells in our model of CML, we injected SCLtTA/BCR-ABL single or double transgenic mice 8-12 weeks after cessation of Dox treatment with poly-(I)-(C) and analyzed HSPCs 16 hours later by FACS analysis.

As expected Ki67/Hoechst cell cycle analysis revealed a decrease of single transgenic LSK cells in G<sub>0</sub> phase and an increase of LSK cells in S phase of the cell cycle after treatment with poly-(I)-(C) (Fig. 3.26 C). This increase was far less pronounced in LSK cells from double transgenic animals. Notably only in a part of double transgenic mice cell cycle was induced in HSPCs, while all single transgenic mice showed a decrease of quiescent HSCs (Fig 3.26 D). Moreover,
single transgenic mice showed an up-regulation of Sca-1 expression similar to wild type FVB/N mice (Fig 3.26 A). Interestingly amongst SCLtTA/BCR-ABL double transgenic mice only few showed an increase in Sca-1 expression similar to the Sca-1 expression levels observed in single transgenic mice, while in the majority of double transgenic mice showed unchanged levels of Sca-1 expression (Fig 3.26 A). Similarly an increase of phenotypic LSK cells was only observed in a few double transgenic mice, while all single transgenic mice had increased proportions of LSK cells (Fig 3.26 B). This suggests that, unlike normal HSCs, leukemic stem cells in our CML mouse model are not uniformly activated by IFNα.

Interestingly a recent report showed that expression of BCR-ABL desensitizes CML cells to IFNα by accelerating the degradation of its receptor in vitro [Bhattacharya et al., 2011]. A similar degradation of the IFNα receptor in vivo could thus explain why LSC that express BCR-ABL are not efficiently activated.

Strikingly only a part of double transgenic SCLtTA/BCR-ABL mice did not respond, while other mice showed activation of HSPC similar to wild type mice. This is in concordance with the variation in onset of leukemia that we observed. Both phenotypes could be dependent on the level of BCR-ABL expressed in individual mice. Importantly our genotyping strategies do not currently show, whether mice are homo- or heterozygous for the BCR-ABL transgene. In future studies it will be crucial to employ such PCR strategies to set up mating strategies with less variability in BCR-ABL expression. Furthermore monitoring BCR-ABL expression in individual mice e.g. by quantitative Real-time PCR could allow even more accurate measurements.

Notably inhibition of BCR-ABL kinase activity by the TKI Imatinib increased the anti-proliferative effects of IFNα on CML cells in vitro [Bhattacharya et al., 2011]. This finding is very interesting as it suggests that treatment with TKIs might render leukemic stem cells again susceptible to activation by IFNα also in vivo, by preventing the degradation of the IFNα-receptor. Hence the exact timing
for the administration of IFNα and TKIs in combination therapies might be absolutely crucial to efficiently eliminate LSCs.

**Figure 3.26 Effects of IFNα treatment leukemic stem cells in SCLtTA/BCR-ABL mice**

(A) FACS analysis of LSK cells in single and double transgenic SCLtTA/BCR-ABL mice, 16 hours after *in vivo* treatment with PBS or poly-(I)-(C). (B) Quantification of LSK cells determined by FACS analysis in single and double transgenic SCLtTA/BCR-ABL mice, 16 hours after *in vivo* treatment with PBS or poly-(I)-(C). (C) Cells cycle profile determined by Ki67/Hoechst cell cycle analysis of LSK cells of single and double transgenic SCLtTA/BCR-ABL mice, 16 hours after *in vivo* treatment with PBS or poly-(I)-(C). (D) Quantification of LSK cells determined by FACS analysis in single and double transgenic SCLtTA/BCR-ABL mice in G₀ phase of the cells cycle, 16 hours after *in vivo* treatment with PBS or poly-(I)-(C).
3.7 Effects of IFNα on stem cells in the skin

The hematopoietic system was the first where two different pools of stem cells were described. On one hand homeostatic HSCs, which are crucial for the maintenance of the hematopoietic system, and on the other hand dormant HSCs, which are cycling very infrequently but are crucial for response to injuries [Wilson et al., 2008]. Recently similarly to the hematopoietic system different roles for populations of more actively cycling and dormant stem cells have also been proposed for tissue stem cells in the intestine and skin [Fuchs, 2009; Takeda et al., 2011; Tian et al., 2011].

The signals regulating the balance of activated and dormant tissue stem cells are still largely unknown. As IFNα can potently activate HSCs in vivo we decided to elucidate whether it has similar effects on stem cells in the skin. Therefore C57BL/6 mice were twice injected poly-(I)-(C) i.p., 3 days and 24 hours before analysis. The skin was isolated and digested overnight in trypsin to separate the epidermis from the dermis. The epidermis was scratched off, cells were suspended and used for FACS analysis. Stem cells in the skin are highly enriched within the CD34\(^+\)α6-integrin\(^{hi}\) as well as CD34\(^+\)α6-integrin\(^{low}\) population, which contain nearly all quiescent label-retaining cells of the epidermis [Blanpain et al., 2004]. As expected during homeostasis only CD34\(^-\) cells expressed Ki67, while no Ki67 expression was detectable in either CD34\(^+\)α6-integrin\(^{hi}\) or CD34\(^+\)α6-integrin\(^{low}\) skin stem cells (Fig 3.27 A). We did not observe any change of Ki67 expression following treatment with poly-(I)-(C) in skin stem cells (Fig 3.27 B). Notably as expected HSCs were highly activated and actively cycling in the same mice (Data not shown).

Besides IFNα also bacterial infections and LPS treatment activate HSCs. To elucidate the effect of LPS on stem cells in the skin mice were injected LPS and skin stem cells were isolated 24 hours later. Similarly to poly-(I)-(C) also LPS activated HSCs as expected, but CD34\(^+\)α6-integrin\(^{hi}\) and CD34\(^+\)α6-integrin\(^{low}\) skin stem cells did not show any up-regulation of Ki67 expression (Fig 3.27).
In summary systemic in vivo treatment with either poly-(l)-(C) or LPS did not activate stem cells in the skin. These findings suggest that stem cells from other regenerative organs beside the hematopoietic system are not activated by the same stimuli as HSCs.

Figure 3.27 Effects of IFNα and LPS treatment on stem cells of the skin
(A) FACS gating strategy and Ki67 expression in 7AAD CD34⁺α6-integrin⁷⁺ and 7AAD CD34⁺α6-integrin⁷⁻ stem cells from the epidermis. (B) Ki67 expression in 7AAD CD34⁺ skin stem cells from C57BL/6 mice after two i.p. injections of PBS or poly-(l)-(C) 3 days and 24 hours before analysis. (C) Ki67 expression in 7AAD CD34⁺ skin stem cells from C57BL/6 mice after a single i.p. injection of PBS or LPS 24 hours before analysis.
4 DISCUSSION

4.1 Novel insights into IFNα-induced HSC activation

In the mouse the majority of HSCs is quiescent during homeostasis, however they can be rapidly activated by IFNα \textit{in vivo}. This activation of HSCs by IFNα is dependent on the IFNα–receptor, STAT1 as well as Sca-1 [Essers et al., 2009]. Additionally the HSC bone marrow niche plays an important role in the activation of HSCs by IFNα, as isolated HSCs are not activated by IFNα \textit{in vitro}. Importantly the exact mechanism leading to cell cycle activation within HSCs is currently still unknown. Moreover while the interaction of IFNα and the IFNα-receptor on HSCs is crucial for the activation, it is still unclear how the bone marrow niche and other extra cellular factors play into this activation process.

Therefore to better understand the activation of HSCs by IFNα \textit{in vivo} we performed microarray analysis of FACS purified LK SLAM HSCs 16 hours after treatment with IFNα (Fig 3.1). As expected the most prominent gene ontology networks induced by IFNα within HSCs were known IFN response genes (Fig 3.2 and Tab 3.1). Furthermore the expression of the cytokines Cxcl10 and Ccl5 was induced, while the expression of the extracellular matrix protein Matrilin 4 and of several cell cycle regulating genes was reduced in a Sca-1 dependent manner (Tab 3.1).
4.1.1 Potential roles of interferon response genes in HSC activation

While the exact function of many of the interferon response genes is still elusive, a few of them are particularly interesting in regards to activation of HSCs by IFNα, as they have been linked to regulation of proliferation in other contexts. Amongst these genes are the RNA sensor Rig-I (Ddx58), the interferon-induced protein 44 (Ifi44) and the Lrg47 family of proteins (lgrp1 and lrgm) as well as the Ifitm family members Ifitm1 and Ifitm3, which are described in more detail in chapter 4.2 (Tab 3.1).

The DEAD-box protein 58 (DDX58), also known as retinoic acid-inducible gene 1 protein (Rig-I), is strongly induced in LK SLAM HSCs after IFNα treatment (Fig 3.15). It is a key receptor for intracellular RNA and a sensor of RNA viral infection, and further crucial in the induction of type I IFN responses as well as other inflammatory cytokines during viral infections via Irf3/7 and NF-κB pathways, respectively [Takeuchi and Akira, 2008]. Strikingly recent reports however linked Rig-I to a novel role independent of viral infections. Rig-I is a negative regulator of proliferation and survival of granulocytes during terminal granulocytic differentiation in acute myeloid leukemia and disruption of Rig-I in mice leads to development of a myeloproliferative disorder [Zhang et al., 2008b]. Furthermore Rig-I negatively regulates AML leukemia cell proliferation, by inducing activation of STAT1 [Jiang et al., 2011]. While similar to RIG-I also IFNα has anti-proliferative effects on differentiated cells, it conversely has pro-proliferative effects on HSCs in vivo. It is therefore tempting to speculate that Rig-I might also have different effects on HSCs or that even the cell cycle activation by IFNα is mediated by regulation of STAT1 signaling via Rig-I.

Similar to Rig-I also Ifi44 is associated with viral infections however its function is less well described [Hallen et al., 2007]. Interestingly Ifi44 expression is enriched in LK SLAM HSCs already during homeostasis and further highly induced after treatment with IFNα (Fig 3.16). Overexpression of Ifi44 induces an anti-proliferative state in vitro, even in cells unresponsive to IFNα [Hallen et al., 2007].
Another highly induced family of IFN response genes with a recent link to HSC regulation is the family of interferon-inducible GTPases Iigp1, Iigp2, Igtp (Irgm3) and Irgm (Iigp3). They are members of the p47 family of GTPases, which are key mediators of immune defense against intra-cellular pathogens [MacMicking, 2004; Taylor et al., 2004]. All four family members are highly induced after IFNα treatment in LK SLAM HSCs (Tab 3.1). Furthermore expression of Iigp1 was highly enriched within HSCs already during homeostasis (Fig 3.15). Strikingly Irgm is crucial for the regulation of baseline proliferation in HSCs and HSCs deficient for Irgm are hyper-proliferative and functionally impaired. Moreover HSCs deficient for Irgm do not normally respond to infectious stimuli like IFNγ. [Feng et al., 2008] Irgm is a negative regulator of IFNγ signaling, and deletion of the IFNγ-receptor in Irgm deficient mice rescues the phenotype in HSCs [King et al., 2011]. Whether other p47 family members might play similarly critical roles in regulation of IFNα induced HSC activation is still unknown.

In summary most of the IFN response genes were traditionally associated with immune defense mechanisms against intra-cellular pathogens, especially viruses. However some of these genes – including Rig-I, the p47-GTPase family or Ifi44 – might also play a role in HSCs unrelated to viral infection. While they all have anti-proliferative effects in vitro similar to IFNα, their role in vivo especially in HSCs is still elusive and needs to be further investigated.

One way to address this questions could be lenti-viral overexpression or shRNA mediated knock-down of these genes in HSCs. Notably Elisa Laurenti in our laboratory recently developed a system which allows inducible overexpression or knock-down of genes specifically in HSCs in vivo [Laurenti et al., 2010]. In this model HSCs are harvested from tTR-KRAB mice, expressing a doxycycline regulated tTR-KRAB repressor protein, and transduced by a lentiviral vector containing a cDNA or shRNA under a tet-O sequence. Subsequently administration of Doxycycline allows induction of gene expression in HSCs and their progeny [Laurenti et al., 2010].
4.1.2 Key regulators of the cell cycle in IFNα induced HSC activation

Another class of genes, which is very interesting in regards to IFNα induced activation of HSCs, are cell cycle regulators and transcription factors, including p57, Reprimo, Maged1 or Evi1. Interestingly treatment of mice with IFNα leads to down-regulation of all four of these genes in LK SLAM HSCs. Moreover strikingly the expression of none of these genes was significantly changed in LK SLAM HSCs of Sca-1-/- mice, indicating a regulation of these genes down-stream of Sca-1 (Tab 3.1 and Chapter 3.2).

The cyclin dependent kinase inhibitor p57Kip2 (Cdkn1c) belongs to the Cip/Kip family of kinase inhibitors, together with p21Cip1/Waf1 and p27KIP1, and plays an important role in the negative regulation of cell cycle progression. Interestingly recent reports showed that p57Kip2 is crucial for the maintenance of HSC dormancy during homeostasis, and loss of p57Kip2 leads to reduction of HSCs in the G0 phase of the cell cycle [Matsumoto et al., 2011; Zou et al., 2011]. Strikingly Sca-1-/- mice did not show a significant down-regulation of p57Kip2 after IFNα treatment, which suggests that expression of p57Kip2 might be regulated in a Sca-1 dependent manner.

Similarly to p57Kip2, also expression of Reprimo (Rpm) was reduced in LK SLAM HSCs after IFNα treatment. Reprimo is induced in a p53-dependent manner and loss or hyper-methylation of Reprimo has been associated with tumor progression and poor prognosis in gastric and pancreatic adenocarcinomas [Bernal et al., 2008; Luo et al., 2011; Sato et al., 2006]. Ectopic expression of Reprimo in vitro leads to G2 arrest and inhibition of nuclear translocation of cyclin B1 [Ohki et al., 2000].
Maged1 (NRAGE) is a member of the type II melanoma antigen family and was shown to block cell cycle progression and enhance apoptosis [Barker and Salehi, 2002]. While the role of Maged1 in the hematopoietic system is unknown, it plays a crucial role in skeletal muscles during wound healing. In adult muscle stem cells expression levels of Maged1 are low during homeostasis, but highly induced after injury and crucial for induction of p21\textsuperscript{Cip1/Waf1} expression and reacquisition of G\textsubscript{0} phase after muscle regeneration [Nguyen et al., 2010]. Further evidence for an important role of Maged1 in the regulation of proliferation was reported in human breast cancer cells, where ectopic expression of Maged1 induces expression of p53 and p21\textsuperscript{Cip1/Waf1} [Du et al., 2009]. Taken together lower levels of Maged1 are associated with low expression of p21\textsuperscript{Cip1/Waf1} and active progression through the cell cycle.

Figure 4.1 Potential roles of candidate genes in IFN\textalpha- induced HSC activation
IFN\textalpha binds to the IFN\textalpha-receptor (IFNAR) on the HSCs, this leads to phosphorylation of STAT1 and PKB, this in turn leads to expression of IFN\textalpha target genes and Sca-1. Together these processes lead to activation of HSCs. Genes identified in our screens, which are potentially involved in this process are shaded in grey. Red triangle indicates down-regulation by IFN\textalpha, green triangle indicates up-regulation by IFN\textalpha.
In summary down-regulation of kinase inhibitors of the CIP/KIP family could be a crucial step in the activation of HSCs by IFNα. Interestingly we observed decreased expression of p57Kip2, Reprimo as well as Maged1 following IFNα treatment only in HSCs of wild type mice, while mice deficient of Sca-1 showed no significant down-regulation. This suggests that CIP/KIP kinase inhibitors are regulated in a Sca-1⁻/⁻ dependent manner, however the exact link to Sca-1 requires further investigation.

The transcription factor Ecotropic viral integration site 1 protein homolog (Evi1; also known as Mecom and Prdm3) plays a crucial role in embryonic development and in regulation of proliferation, by interacting with both co-repressors and co-activators. Evi1 is one of the dominant oncogenes in murine and human myeloid leukemias, including acute and chronic myeloid leukemia and myelodisplastic syndrome, where it is associated with poor prognosis [Goyama and Kurokawa, 2010; Nucifora, 1997]. In the hematopoietic system Evi1 expression is highly enriched in long-term HSCs [Kataoka et al., 2011]. Heterozygosity of Evi1 in HSCs leads to impairment of self-renewal capacity, while Evi1 deficient HSCs cannot maintain hematopoiesis and lose their repopulating capacity [Goyama et al., 2008; Kataoka et al., 2011]. Contrarily overexpression of Evi1 suppresses differentiation and boosts self-renewal [Kataoka et al., 2011]. In the course of viral infections IFNα is an important mediator of cell cycle arrest in differentiated cells, while HSCs are simultaneously activated and produce new progeny. Decreased expression of Evi1 in LK SLAM HSCs of mice after IFNα treatment might indicate that in the emergency situation of a viral infection HSCs enter an active cell cycle primarily not to self-renew but rather to differentiate to quickly re-establish a pool of effector cells that was not in contact with viral pathogens. It might therefore play a crucial part not in the activation process itself, but in the fate of newly generated progeny cells. To elucidate the role of Evi1 further it will be crucial to investigate the expression of Evi1 after IFNα treatment over time and correlate it to the number of HSCs as well as
differentiated cells. Notably also the expression of Evi-1 was only significantly decreased in wild type mice, but not in Sca-1<sup>-/-</sup> mice.

### 4.1.3 Cytokines and extracellular matrix proteins in the IFN response

Another interesting classes of genes induced by IFNα in LK SLAM HSCs are cytokines and extra-cellular matrix proteins, in particular Cxcl10, Ccl5 and Matrilin4.

C-X-C-motif chemokine 10 (Cxcl10; also known as interferon induced protein 10, IP10) is a small cytokine that is secreted by many cell types after stimulation by IFNα, IFNγ or LPS [Angiolillo et al., 1995] and is also highly induced in HSCs independent of the presence of Sca-1 (Tab 3.1). Cxcl10 is crucial for efficient control of viral replication and recruitment of lymphocytes to the sites of infection [Dufour et al., 2002]. Interestingly, similar to IFNα, Cxcl10 has anti-proliferative effects on a variety of cell types including endothelial cells and reduces proliferation as well as invasiveness of melanoma cells [Antonicelli et al., 2011; Campanella et al., 2010]. The effects of Cxcl10 on HSCs are still unknown.

Chemokine ligand 5 (Ccl5; also known as RANTES) is a chemotactic cytokine that is, similar to Cxcl10, crucial for the recruitment of leukocytes to sites of inflammation [Appay and Rowland-Jones, 2001]. It has been linked to induction of proliferation in several cell types, including NK-cells and hepatic stellate cells [Maghzachi et al., 1996; Schwabe et al., 2003]. Interestingly Ccl5 was recently shown to be important for lineage choice during differentiation. Overexpression of Ccl5 in HSPCs leads to decreased output of T-cells but increased output of myeloid progenitor cells. Conversely deletion of Ccl5 leads to a decrease in myeloid-biased and increase of lymphoid-biased HSCs [Ergen et al., 2012]. Hence both, induction of proliferation of the HSCs themselves, as well a mediation of lineage choice of progenitor cells in the presence of viral pathogens are potential roles of Ccl5 during the IFN response.
Cxcl10 as well as Ccl5, could be crucial for HSC activation both intrinsically as well as by influencing cells of the niche. As both cytokines influence cell proliferation it could be possible that they are part of an autocrine activation loop, where IFNα leads to production of Cxcl10 and Ccl5 within the HSCs, which in turn further induce proliferation of HSCs. To test this hypothesis a first step could be to analyze the effects of administration of ectopic Cxcl10 and Ccl5 on HSCs in vivo. Another possibility is an effect of this cytokines on cells of the niche, which might also need to be stimulated for efficient HSC activation.

The hematopoietic stem cells niche environment is crucial not only for the maintenance of HSCs but also for their activation by IFNα, as HSCs are activated by IFNα only in vivo but not in vitro, where the HSC niche is missing [Essers et al., 2009]. One important component of this niche are extra-cellular matrix proteins. The matrilin family of non-collagenous extracellular matrix proteins has four described members and is still poorly characterized. During embryogenesis Matrilin4 is expressed in the dermis and connective tissue of internal organs, in adult mice expression was described in epithelial, muscle and nervous tissue [Klatt et al., 2002]. Surprisingly expression of Matrilin 4 in the hematopoietic system is highly enriched in LK SLAM HSCs (Fig 3.16), and strongly down-regulated after treatment with IFNα (Fig 3.16 and Tab 3.1). It is still unclear how exactly the location and lodgement of HSCs is influenced by IFNα treatment, but it is intriguing to speculate that the HSC itself could participate in relocation from a dormant niche position to a more active niche position by influencing its environment and extra-cellular matrix. To elucidate this further it will be crucial to perform immunohistochemical analysis of HSCs in the bone marrow niche after IFNα treatment.

Taken together we identified several candidate genes, which could be key players in the activation of quiescent HSCs, including a diverse set of interferon response genes, cell cycle regulators as well as cytokines and chemokines. However this analysis is based on RNA expression and hence might miss critical processes, which are regulated on a post-transcriptional or even post-translational
level. One possibility to systematically address this further would be proteomics approaches of HSCs following similar treatment schemes as investigated in this study.
4.2 Role of the Ifitm protein family in the hematopoietic system

Similar to the other interferon response genes described above also the Ifitm protein family members Ifitm1 and Ifitm3 were induced in LK SLAM HSCs after treatment with both IFNα as well as LPS (Fig 3.6, Tab 3.1 and Tab 3.2). This induction was independent of Sca-1 and similarly observed in Sca-1 deficient mice. Notably the Ifitm protein family had been linked to IFN-induced inhibition of proliferation in vitro as well as to regulation of factors crucial in the HSC niche before, as described in Chapter 3.3. We therefore decided to investigate its function within HSCs in more detail.

4.2.1 Expression of the Ifitm protein family is highly enriched in HSCs

Interestingly the expression of the Ifitm family, comprising Ifitm1, 2, 3, 5 and 6, is very heterogeneous in the hematopoietic system. Interestingly Ifitm1, 2 and 3 are strongly enriched within hematopoietic stem and progenitor cells on the RNA and at least Ifitm3 also on the protein level (Fig 3.7 and 3.10). Furthermore analysis of Ifitm3EGFP mice, heterozygous for knock-in of EGFP in the Ifitm3 locus, showed that both the frequency of cells expressing Ifitm3 as well as the level of expression within individual cells was highest in HSCs and declining in more committed progenitor cells (Fig 3.8). Immune-fluorescence analysis suggested localization of Ifitm3 on both the membrane as well as the endoplasmatic reticulum as described before in human HeLa cells (Fig 3.10) [Yount et al., 2010].

4.2.2 Induction of Ifitm expression differs between different cell types

Expression of all five Ifitm family members was induced by IFNα in unfractionated bone marrow cells (Fig 3.6). Interestingly however only Ifitm1 and Ifitm3 were induced in LK SLAM HSCs (Fig 3.6). Furthermore treatment with IFNα or LPS similarly lead to increased EGFP expression in LSK cells of Ifitm3EGFP
heterozygous mice. Contrarily only IFNα induced expression in differentiated cells, while LPS failed to do so with the exception of granulocytes (Fig 3.9). These findings suggest that expression of ifitm family members might be regulated differently in different cell types as well as following different induction signals.

4.2.3 The Ifitm family is dispensable for HSCs maintenance and activation by IFNα

Despite the enrichment of Ifitm-expression in HSCs, IfitmDEL mice lacking the whole Ifitm family cluster have normal numbers of stem and progenitor cells as well as differentiated cells compared to wild-type mice (Fig 3.11). Moreover HSCs of IfitmDEL mice showed normal cycling behavior and proportions of dormant HSCs and are capable of forming colonies as well as reconstituting lethally irradiated mice similar to wild type HSCs (Fig 3.12). Moreover HSCs deficient for Ifitm3 or the whole Ifitm family were activated by IFNα equally efficient to their wild type counterparts, similarly reacquire reacquired a quiescent state and are affected by chronic stimulation (Fig 3.13).

Taken together the Ifitm protein family is not required for maintenance of HSCs, regulation of HSC quiescence or activation of HSCs by either IFNα or LPS. While it is difficult to formally exclude that other proteins might compensate for the loss of the Ifitm family, we did not observe increased expression of other proteins in our microarray screen in HSCs of Ifitm deficient mice (Fig 3.14).

Microarray analysis further revealed that the Ifitm family is unlikely to be involved in regulation of gene expression, as we could not observe any differences in the transcriptome of Ifitm-deficient and wild type HSCs neither during homeostasis nor during IFNα induced activation. This findings strongly suggests that the Ifitm proteins do not have regulatory functions within HSCs, at least on the transcriptional level, but are rather terminal effectors.
4.2.4 The Ifitm family as protection mechanism for HSCs during viral infections

Interestingly such an effector function has recently been shown. Particularly the Ifitm family is a key player in restricting entry and spread of many different enveloped viruses. These include viruses as diverse as influenza H1N1, Dengue virus, SARS corona virus, Hepatits C virus or human immunodeficiency virus (HIV) [Brass et al., 2009; Huang et al., 2011; Lu et al., 2011; Raychoudhuri et al., 2011]. Moreover Ifitm3 also in vivo restricts the morbidity and mortality associated with influenza infections in mice [Everitt et al., 2012].

Strikingly Ifitm proteins inhibit already the entry of viruses into the cytoplasm, at least in part by preventing cytosolic entry of the virus from endosomes [Feeley et al., 2011]. Furthermore they also interfere with viral replication, although the mechanism is still unknown [Lu et al., 2011]. Antiviral activity of Ifitm proteins is not only regulated on the expression level, but at least in part by post-translational modifications. While S-palmitoylation enhances the membrane affinity and anti-viral activity of ifitm3, lysine ubiquitination decreases antiviral activity [Yount et al., 2012; Yount et al., 2010].

Particularly interesting in regards to protection of stem cells is the fact that Ifitm proteins are the first viral restriction factor described to inhibit already the entry of the virus into the cell and therefore efficiently prevent infection. Unlike that in most other immune defense processes against viral pathogens the infected cell is eliminated either by killing through activated immune cells or by different triggers of apoptosis within the infected cell [Roulston et al., 1999]. Notably replacing infected HSCs during a viral infection would be much more risky for the whole organism compared to replacing more committed or differentiated cells. As expression of Ifitm1 and 3 is already strongly enriched in HSCs and granulocytes and furthermore highly induced by both IFNα as well as LPS in these cells, it is tempting to speculate that in the hematopoietic system this protection mechanism is restricted mainly to these cell types. An interesting question is why such an efficient protection mechanism would not be present in all hematopoietic cells. While there is no definitive answer to this, one possible explanation could be
argued with evolutionary pressure. If all cells would express Ifitm family members, evolutionary pressure on viruses would be much higher to overcome this, compared to just few cells expressing it. More differentiated cells which are easier to replace would be sacrificed to safe HSCs, which are crucial for the survival of the organism (Fig 4.2).

In summary we propose that in the course of viral infections Ifitm family members are crucial for protecting hematopoietic stem and progenitor cells from infection, while more differentiated cells are infected easier and consecutively replaced by cells, which are newly produced from HSCs. Using the IfitmDEL mouse model, we are currently investigating whether the presence of Ifitm family members on HSCs protect them from viral pathogens in vitro and in vivo, similar to other cell types described.

![Diagram of Ifitm protein family as protection against viral pathogens](image)

**Figure 4.2 Proposed model of the Ifitm protein family as protection against viral pathogens**

During homeostasis wild type HSCs and some differentiated cell populations express Ifitm1 and Ifitm3 at moderate levels, while the majority of cells lack expression. This expression is further induced in the course of viral infections. Expression of Ifitm family members renders these cells resistant to infection, and subsequently allows the re-establishment of the hematopoietic system from uninfected HSCs. Conversely, without protection by Ifitm proteins, also HSCs are efficiently infected and the viral pathogens cannot be efficiently cleared.
4.3 Activation of human HSCs in a xenotransplantation mouse model

As previously described hematopoietic stem cells in the mouse are efficiently activated by IFNα [Essers et al., 2009]. Notably interferon is used in human patients to treat a variety of different diseases, ranging from viral infection (e.g. Hepatitis B and C viral infections) to multiple sclerosis and hematologic malignancies including chronic myeloid leukemia (CML) [Cooksley, 2004; Goldstein and Laszlo, 1988; Paolicelli et al., 2009; Shepherd et al., 2000]. Therefore it is crucial to get a better understanding on the influence of IFNα on human HSCs, particularly in regards to possible combination therapies with other drugs (for a detailed discussion for CML see chapter 4.4). As the investigation of the effects of IFNα on human HSCs cannot be performed in vitro, as IFNα only activates HSCs in vivo, we established a xenotransplantation model where human cord blood CD34 enriched cells are transplanted intra-femurally into immune compromised NSG mice to establish a human hematopoietic system within a mouse bone marrow niche (Fig 3.17).

4.3.1 Human HSCs do not achieve quiescence in NSG mice

Human HSCs showed efficient long-term engraftment in NSG mice and furthermore gave rise to human CD19+ B-cells and CD11b+ monocytes (Fig 3.17 and data not shown). Interestingly however cell cycle analysis showed that human CD34+CD38- HSCs were less quiescent compared to mouse HSCs with only 20-30% of human HSCs in the G0 phase of the cell cycle compared to about 75% in mouse LSK SLAM HSCs (Fig 3.19 and 3.21). Furthermore while a subpopulation of mouse HSCs are long-term dormant and can be identified by label retaining assays 100 days after initial labeling [Wilson et al., 2008], we could not detect any label retaining human HSCs as described in chapter 3.5. One possible explanation for the lower quiescence of human HSCs could be the
less strict definition of human HSCs compared to their mouse counterparts, and therefore a higher proportion of highly cycling progenitor cells within the HSC subset. Interestingly however a recent report showed, that single human CD34⁺CD38⁻CD90⁺Rhô⁻CD49f⁺ positive HSCs are capable to long-term multi-lineage engraft mice [Notta et al., 2011]. Notably we did not observe further enrichment of cells in the G₀ phase in CD34⁺CD38⁻CD90⁺CD49f⁺ HSCs compared to CD34⁺CD38⁻ HSCs (data not shown). Therefore whereas human HSCs cannot be defined as precisely as mouse HSCs this discrepancy is unlikely to account for the big difference in quiescence.

Another possible explanation why human HSCs are less quiescent than mouse HSCs is the foreign mouse niche environment human HSCs face in our xenotransplantation model. Beside cell intrinsic regulator of HSC quiescence like p21⁰CIP, p53 or p57⁰KIP2 also extrinsic factors and the interaction of HSCs with their niche is crucial for the maintenance of quiescence [Wilson and Trumpp, 2006]. Amongst the most important niche cells for the maintenance of HSC quiescence are bone lining osteoblasts and Nestin⁺ mesenchymal stem cells (MSCs), which both supply HSCs with factors crucial for their quiescence, including Ang-1, TPO or SCF [Ehninger and Trumpp, 2011]. Further crucial extrinsic factors include N-cadherins, integrins and osteopontin. It is well possible that one or several of these important interactions is functionally impaired between mouse niche cells and human HSCs, leading to impaired quiescence in human HSCs. Approaches how to solve this problem are described below in chapter 4.3.3.

4.3.2 Human HSCs are not activated by interferon but are activated by LPS

To elucidate whether IFNα activates human HSCs similar to mouse HSCs, we treated xenotransplanted mice with different sub-types of type I interferon, including IFNα 1b, IFNα 2a and IFNα 2b. Surprisingly we did not observe any evidence for cell cycle activation, neither an increase in human HSCs incorporating BrdU nor a decrease in human HSCs in the G₀ phase of the cell cycle (Fig 3.18, 3.19 and data not shown). Notably treatment of mice with human
IFNα 1b as well as with poly-(I)-(C) efficiently activates mouse HSCs (Fig 3.19). Similarly to IFNα also the type II interferon, IFNγ, as well as the type III interferon, IFNλ, did not lead to activation of human HSCs (Fig 3.21). In striking contrast to IFNα, treatment of mice with LPS efficiently activated human HSCs. This was evident by a change in cell surface protein expression, most notably up-regulation of CD38, as well as decrease of human HSCs in the G₀ phase of the cell cycle (Fig 3.20 and 3.21).

There are several possible explanations why IFNα is not able to activate human HSCs in our xenotransplantation mouse model. As mouse IFNα efficiently activates mouse HSCs in vivo but fails to do so in vitro [Essers et al., 2009], it is evident that some HSC extrinsic factors are crucial for the activation of HSCs, similar to the factors important for the acquisition and maintenance of quiescence of HSCs described above. It is possible that these extrinsic factors could be missing in our xenotransplantation setting, or alternatively not able to activate human HSCs. Furthermore as described above human HSCs are actively cycling in our model, with no cells being long-term quiescent and only 20-30% of human HSCs in the G₀ phase at homeostasis compared to 75% in mouse HSCs (Fig 3.21). It is possible that in such an actively cycling population no further activation can be detected. However activation of mouse HSCs can be detected also in less stringently defined populations e.g. LSK cells, which also show a similarly low level of quiescent cells during homeostasis (data not shown). Moreover activation of human HSCs could be detected after treatment with LPS.

While the activation processes of HSCs by IFNα and LPS in the mouse share some similar mechanisms (Tab 3.1 and 3.2), some crucial steps are different. Similar to IFNα also activation of HSCs by LPS is dependent on the niche environment [Marieke Essers, personal communication]. However whether the same components from the niche are crucial in both cases is still under investigation. It is therefore possible that crucial factors for the activation by IFNα are missing in the xenotransplantation setting, while the ones crucial for activation by LPS are present. Another explanation could be the effect of LPS on
bone marrow cellularity. Treatment of mice with LPS leads to a pronounced decrease in bone marrow cellularity (data not shown), and this decreased bone marrow cellularity could in turn leads to activation of human HSCs, independent of the initial LPS stimulus.

Microarray analysis of human HSCs after treatment with IFNα + poly-(l)-r(C) or LPS showed that human HSCs at least partly respond to IFNα (Tab 3.3). While no changes in cell cycle regulators could be detected in human HSCs of IFNα treated mice, a strong IFNα response cluster was apparent (Tab 3.3 and Fig 3.22). Strikingly LPS treated mice similarly showed a strong induction of interferon response genes, but additionally also increased expression of cell cycle related genes, correlating with the cell cycle induction in HSCs in these mice (Tab 3.4 and Fig 3.22). In particular human HSCs of xenotransplanted mice treated with LPS showed increased expression of the cyclin-dependent kinase inhibitors p19 and p21Cip1 as well as Cyclin D1, which are all key regulators of the G1/S transition.

Comparison of the gene expression in human HSCs after treatment with IFNα or LPS might provide novel insights into pathways that require interaction with the HSC niche environment, as activation of such pathways should be detectable after LPS treatment but not after treatment with IFNα. Furthermore similarly comparing the expression profile after IFNα treatment in mouse and human HSCs might provide novel insights. To address this we are currently comparing these microarray expression profiles, and moreover also compare the expression of differentially regulated genes that are possibly involved in HSC activation, between mouse and human HSCs within the same mouse by qPCR.

4.3.3 Outlook and possible improvements for the xenotransplantation mouse model

As discussed above one major problem in our xenotransplantation mouse model is the foreign mouse niche environment, which could be an explanation for both, the low quiescence of human HSCs observed in our model, as well as
the lack of activation of human HSCs by IFNα (Fig 4.3). To overcome this lack of compatibility between human HSCs and the mouse niche cells one possibility is to humanize the niche environment similar to the hematopoietic system. This could be achieved by transplantation of human stromal cells, e.g. osteoblasts and mesenchymal stem cells. Notably previous reports showed that the co-transplantation of human mesenchymal stem cells improved the engraftment of human CD34+ HSPCs in NOD/SCID mice [Noort et al., 2002]. Alternatively or additionally NSG mice that produce human cytokines could be used as host mice. Interestingly a recent report showed improved engraftment of human AML samples in NSG mice expressing the three human cytokines IL3, SCF and GM-CSF [Wunderlich et al., 2010].

Another approach to elucidate crucial components of the bone marrow niche for IFN induced HSC activation could be reconstructing the hematopoietic stem cell niche in vitro. This offers the advantage that different stromal compartments of the niche like endothelial cells, mesenchymal stem cells or osteoblasts as well as soluble factors can be added either separately or simultaneously and therefore their role in HSC maintenance and activation unraveled. Notably the three dimensional structure of the bone marrow niche might have to be taken into account, which might require sophisticated 3D culture models. Indeed such 3D co-culture models recently showed that co-culture of human cord blood HSCs with stromal cells like osteoblasts and MSCs increases the proportion of quiescent HSCs in culture [de Barros et al., 2010; Di Maggio et al., 2011]. Beside components of the niche also culture conditions might be very important for such an approach. HSCs in the bone marrow reside at the lowest end of an oxygen gradient under hypoxic conditions [Jang and Sharkis, 2007; Parmar et al., 2007]. Notably low oxygen culture conditions also increase the survival and self-renewal of HSCs also in vitro [Danet et al., 2003]. It would be very interesting to investigate whether HSCs cultured under such advanced culture conditions can be efficiently activated by IFNα also in vitro, contrarily to the single culture of HSCs. Notably the defined setup of these cultures allows the removal of single components which might allow to
identify which of the stromal components are crucial for HSCs activation and which are dispensable.

In conclusion, the stromal cells of the bone marrow HSC niche are very critical components, not only for HSC maintenance but also for quiescence and activation of HSCs. Hence improving the xenotransplantation model by modeling the niche environment as close as possible to a human niche might be crucial to investigate the mechanism of activation of human HSCs in vivo. Similarly, reconstruction of a niche environment similar to the in vivo situation is crucial to study the activating effects of IFNα on HSCs in-vitro.

![Figure 4.3 Incompatibility between mouse stromal cells and human HSCs](image)

Mouse HSCs are quiescent during homeostasis, but can be efficiently activated by IFNα. Both direct interactions with stromal cells from the niche as well as secreted factors are crucial for maintenance of quiescence and activation of HSCs. In our xenotransplantation model human HSCs do not become quiescent and cannot be activated by IFNα. This can be explained by incompatibility in the crosstalk between the murine bone marrow niche with human HSCs. It is currently unknown whether HSCs in humans are dormant during homeostasis and can be activated by IFNα similar to the mouse. To elucidate this advanced xenotransplantation models will be needed, which also include a humanization of the stromal niche compartment, and hence allow efficient cross-talk between human HSCs and their niche.
4.4 Activation of leukemic stem cells in a mouse model for CML

As described in chapter 1.4 chronic myeloid leukemia (CML) is a chronic myeloproliferative disease, characterized by a chromosomal translocation, leading to the formation of the Philadelphia chromosome and the BCR-ABL fusion kinase [Daley et al., 1990]. While inhibition of the BCR-ABL kinase by tyrosine kinase inhibitors (TKI) like Imatinib specifically targets BCR-ABL expressing cells and leads to rapid remission of the disease [O'Brien et al., 2003], leukemic stem cells (LSCs) are not efficiently eliminated by TKI treatment. These residual LSCs can lead to rapid relapse of the disease upon cessation or interruption of TKI treatment [Goldman, 2009]. Therefore treatment approaches that can lead to the elimination of residual LSCs are of high clinical interest. One such approach is the activation of LSCs by IFNα, followed by elimination of activated LSCs by Imatinib [Essers and Trumpp, 2010]. However unlike for HSCs the effects of IFNα on HSCs is currently unknown. To elucidate the effects of IFNα on leukemic stem cells and the feasibility of such approaches we employed a mouse model for chronic myeloid leukemia and investigated the effect of IFNα on LSCs in this model in vivo.

4.4.1 Establishment of a mouse model to elucidate effects of IFNα on CML LSCs

To elucidate the effects of IFNα on leukemic stem cells we obtained a mouse model for chronic myeloid leukemia, which allows the expression of the BCR-ABL transgene specifically in hematopoietic stem and progenitor cells in a Dox inducible manner [Koschmieder et al., 2005]. These mice express the transactivator protein tTA under the control of the murine stem cell leukemia (SCL) gene 3′ enhancer, and the P210 BCR-ABL transgene under control of a tet-responsive element (TRE) (Fig 3.23).

As expected mice expressing the both transgenes developed a CML like disease within few weeks after the cessation of Doxycycline treatment. These
symptoms included increase in spleen size, increase of LSK cells and increase in differentiated und undifferentiated granulocytes in the bone marrow and spleen (Fig 3.24). Notably a major caveat of this model is that unlike in human patients all HSPCs express the BCR-ABL transgene and hence the model is not suitable to study effects of TKI treatment and combinations of TKIs with IFNα on LSCs.

To address this we generated bone marrow chimeras, which harbor both leukemic as well as normal hematopoietic cells. Within few weeks after cessation of Dox treatment SCLtTA/BCR-ABL bone marrow chimeras showed signs of leukemia (Fig 3.25). Notably in the model we employed it is not possible to distinguish normal HSCs from LSCs. This model could be markedly improved by using wild type FVB/N CD45.2 mice, which differ in CD45 antigen expression from the SCLtTA/BCR-ABL strain, which express the CD45.1 antigen (Fig 4.4).

Figure 4.4 Transplantation mouse model to investigate the effects of IFNα and TKIs on LSCs
Model for the generation of mixed bone marrow chimeras of FVB/N CD45.2 wild type mice and SCLtTA/BCR-ABL double transgenic mice. This model allows detection of single leukemic cells by the CD45.2 antigen, and therefore allows monitoring of treatment success and minimal residual disease.
This allows to detect even very low numbers of leukemic stem cells within a majority of normal cells and hence monitor whether residual LSCs can be efficiently removed by treatment with IFNα in combination with TKIs. We are currently employing such a model and investigating the effects of IFNα and TKIs as single agents as well as in combination.

In conclusion SCLtTA/BCR-ABL bone marrow chimeras develop a CML like disease within weeks after transplantation and are suitable for investigating the effects of different treatment regimens on leukemic stem cells. They resemble the situation in a human patient, where leukemic cells are present next to healthy hematopoietic cells, more closely than the original SCLtTA/BCR-ABL model. Furthermore the use of strains expressing different isotypes of CD45 offers the possibility to track individual leukemic cells and is therefore superior for the analysis of minimal residual disease and the effects of combined therapies on LSCs.

4.4.2 Activation of leukemic stem cells by IFNα

The most crucial question for a potential combination therapy with IFNα and TKIs is whether IFNα activates leukemic stem cells similar to normal HSCs. To address this we investigated the effects of IFNα on HSCs of SCLtTA/BCR-ABL single and double transgenic mice. As expected we observed up-regulation of Sca-1 expression and induction of proliferation in HSCs from single transgenic mice (Fig 3.26). Surprisingly only a part of double transgenic mice showed a similar reaction while the majority did not show any activation of LSCs by IFNα (Fig 3.26).

Interestingly a recent report suggested that expression of BCR-ABL in leukemic cells leads to down-regulation of the IFNAR and hence to decreased responsiveness of these cells to IFNα in vitro [Bhattacharya et al., 2011]. This down-regulation is mediated by protein kinase D2, which is activated by BCR-ABL and in turn promotes phosphorylation-dependent degradation of the IFNAR-1-chain of the IFNAR, leading to an attenuation of IFNα signaling.
Interestingly inhibition of the BCR-ABL kinase by the TKI Imatinib increased the anti-proliferative effects of IFNα on CML cells *in vitro*, suggesting that the down-regulation of the IFNAR is reversible by inhibition of BCR-ABL [Bhattacharya et al., 2011]. This is important as this finding further highlights the crucial aspect of timing the two components IFNα and TKI right (Fig 4.5 A). A crucial next step will therefore be to investigate whether pre-treatment with TKIs also *in vivo* restores the responsiveness of CML LSCs to IFNα.

**Figure 4.5 Model of a possible combination therapy of IFNα and TKIs to eliminate leukemic stem cells**

**(A)** LSCs are less responsive to IFNα compared to HSCs, due to BCR-ABL dependent degradation of the IFNAR. By inhibiting the BCR-ABL kinase with TKIs the sensitivity of LSCs to IFNα can be restored. **(B)** While treatment with TKIs alone efficiently targets the bulk of leukemic cells, quiescent LSCs remain unharmed and can lead to relapse of leukemia. Combination therapy with TKIs and IFNα might be a possibility to eliminate also these residual clones. After pre-treatment with TKIs, LSCs can be activated by IFNα and eliminated by another cycle of TKI treatment.
In conclusion the bone marrow transplantation model described above will be a crucial tool to determine the optimal targeting strategy in this combination therapy. Judging from our data an optimal approach might include pre-treatment with Imatinib or other TKIs, to restore full responsiveness of LSCs to IFNα, followed by activation of LSCs by IFNα, followed again by treatment with TKIs to eradicate activated LSCs (Fig 4.5 B). Importantly it might be crucial to interrupt treatment with TKIs briefly at the time of treatment with IFNα, as TKI treatment itself drives LSCs into quiescence and might therefore counteract the effects of IFNα [Graham et al., 2002].
5 MATERIALS & METHODS

5.1 Mouse strains

All animal experiments were conducted in accordance with the German Law for Protection of Animals and the National Institute of Health Guidelines for Care and Use of Laboratory Animals. Mice were maintained in the animal facility of the German Cancer research Center under specific pathogen-free conditions (SPF) and housed in individually ventilated cages (IVC). If not specified differently all mice used for experiments were between 6 and 12 weeks old at the beginning of the respective experiment. NSG mice used for intra femoral transplantations were between 8 and 14 weeks at the time of transplantation.

Wild-type mice
Wild-type and immune compromised mouse strains were ordered from the following companies:

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<th>Common Name</th>
<th>Strain Name</th>
<th>Company</th>
</tr>
</thead>
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<td>C57BL/6 JOlHaHsd</td>
<td>Harlan Laboratories</td>
</tr>
<tr>
<td>Ly5.1</td>
<td>C57/BL6 - Ly 5.1</td>
<td>Charley River, Italy</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD.Cg-Prkdc&lt;scid&gt;Il2rgytm1Wjl&gt;/SzJ</td>
<td>Jackson Laboratory</td>
</tr>
<tr>
<td>FVB/N</td>
<td>FVB/N Crl</td>
<td>Charles River, Sulzfeld</td>
</tr>
</tbody>
</table>

Table 5.1 Commercially obtained mouse strains
NSG mice
As host mice for the xenotransplantation the NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) strain was chosen. NSG mice combine the phenotype of severe combined immune deficiency mutation (scid) with deficiency of the IL2-receptor γ chain. As a result they lack mature B- and T-cells, functional NK cells and are deficient in cytokine signaling [Ishikawa et al., 2005; Shultz et al., 2005]. They therefore accept human cells without graft rejection. NSG mice were obtained from Jackson Laboratory.

Ifitm3EGFP and IfitmDEL
Ifitm3EGFP and IfitmDEL mice were obtained from the laboratory of Prof. Azim Surani [Lange et al., 2008]. Ifitm3EGFP mice were generated by targeted insertion of EGFP 30 nucleotides downstream from and in frame with the Ifitm3 start codon (Fig 5.1), and the mutant ES cells were used to transmit the allele onto the C57BL/6J background. Ifitm3EGFP homozygous mice lack Ifitm3 expression but express EGFP instead, while heterozygous mice have one intact copy of Ifitm3 and simultaneously express EGFP. Ifitm3 null mice had no observed phenotype and developed normally [Lange et al., 2008].

Figure 5.1 Schematic of the targeting strategy used to generate the Ifitm3EGFP allele (adapted from [Lange et al., 2008])
IfitmDEL mice were engineered by deletion of a 120-kb region on chromosome 7 (Fig 5.2), harboring the entire Ifitm family cluster but no other known genes [Lange et al., 2008]. IfitmDEL mice were backcrossed on a C57BL/6J background. The number of homozygous animals born from intercrosses of IfitmDEL heterozygous mice was reported to be below the expected Mendelian ratio.

![Figure 5.2 Schematic of the targeting strategy used to generate the IfitmDEL allele](adapted from [Lange et al., 2008])

**Sca-1 KO and IFNAR KO**
Mice deficient for Sca-1 or the IFNα receptor were maintained on a C57Bl/6 background as described before [Ito et al., 2003; Muller et al., 1994].

**SCLtTA-BCR Abl**
SCLtTA-BCR Abl mice, which allow the targeted expression of the BCR-ABL kinase in hematopoietic stem and progenitor cells using a tet-off system, were obtained from the laboratory of Dr. Steffen Koschmieder [Koschmieder et al., 2005]. In these mice the transactivator protein tTA was placed under the control of the murine stem cell leukemia (SCL) gene 3’ enhancer, and the P210 BCR-ABL transgene under control of a tet-responsive element (TRE). This allows to induction of BCR-ABL expression in hematopoietic stem cells by withdrawal of Doxycycline (Fig 3.23). Within few weeks after induction of BCR-ABL kinase
expression mice develop a disease closely resembling the chronic phase of chronic myeloid leukemia [Koschmieder et al., 2005]. The BCR-ABL mouse line was kept on a FVB/N genetic background. Mice were supplied with 2g/l Doxycycline in the drinking water, containing 5 cubes of sugar (Dox water). Mating cages were kept on Dox water permanently. Disease development was induced in 6-10 week old mice by exchange of Doxycycline water with pure water and blood counts of mice were monitored routinely starting at six weeks after induction. For transplantation of the disease bone marrow cells were isolated from single or double transgenic mice, which were still on Dox water, and transplanted in wildtype FVB/N mice. Doxycycline treatment was stopped at the timepoint of transplantation.

5.2 Genotyping

To determine the genotype of mice DNA was extracted from tail biopsies (PCR Tail Lysis reagent, Peqlab) and genotyping PCR were performed. All PCR protocols used Taq Polymerase (Invitrogen), 10x PCR buffer (670mM TrisHCL pH8.8, 67mM MgCl$_2$, 1,7mg/ml BSA; 166mM (NH$_4$)$_2$SO$_4$) with final concentrations of 10% DMSO or 0,12% Triton X100 and 2µM primers. Amplification was performed for 35 cycles with an annealing temperature of 60°C, with the exception of genotyping for the BCR-ABL transgene (40 cycles, 58°C annealing temperature), SCLtTA transgene (40 cycles, 59°C annealing temperature) and IfitmDEL locus (45 cycles, 62°C annealing temperature). The primers used for genotyping are listed in Table 5.2.
5.3 Isolation of cells from bone marrow, spleen and blood

Bone marrow was isolated from hind legs (femur and tibia) or hind legs, fore legs (humerus) and hips (ilium) depending on the amount of cells required for the experiment. Muscles and connective tissue was removed and bones were crushed in RPMI medium with 2% FCS (Gibco or PAN Biotech). Similarly spleen was minced in RPMI with 2% FCS and cells suspended by pipetting up and down. The cell suspension was filtered through a 40µm mesh and cell numbers were determined with a ViCell automated cell counter (Beckman Coulter) or manually using a Neubauer chamber. If necessary lineage depletion was performed by incubating the cell suspension with a mixture of monoclonal antibodies against CD11b (M1/70), Gr-1 (RB6.8C5), CD4 (GK1.5), CD8a (53.6.7), Ter119 (Ter119) and B220 (RA3-6B2) for 30 minutes on ice. Subsequently the cells were washed...
and incubated 15 min on ice with anti-rat IgG-coated Dynabeads (4.5µm supermagnetic polystyrene beads, Invitrogen), 1ml of beads per 250x10^6 cells. The labeled cells were depleted using a magnet and the remaining lineage depleted cells were washed and again counted. For all washing steps cells were washed with PBS 2% FCS followed by centrifugation at 1600rpm for 5 min at 4°C (Eppendorf 5810r centrifuge).

For analysis of blood chimerism and monitoring of leukemia development 6 drops of blood were collected in tubes covered with EDTA. Red blood cells were lysed with ACK lysis buffer (Life Technologies, #A10492-01) according to manufacturers protocol. The remaining cells were washed as described above and subsequently used for determining blood counts with a Hemavet cell counter (Drew Scientific) or for FACS analysis as described below.

5.4 Isolation of stem cells from the skin

Mice were shaved with depilatory cream; skin was harvested and cut into pieces. The skin was placed in a petri dish and incubated floating in trypsin 0.25% (Gibco) for 16 to 24 hours at 4°C. The epidermal layer was peeled off with a razor blade, the epidermis was chopped with scissors in suspended in HBSS (Gibco). The suspension was pressed through a 40µm cell strainer and washed with HBSS. The suspension was centrifuged at 1200rpm for 5 min at 4°C (Eppendorf 5810r centrifuge) and filtered again through a new 40µm cell strainer. Finally the cells were counted and used for FACS analysis as described below.

5.5 Fluorescence activated cell sorting (FACS)

FACS analysis was performed on BD LSR II (Becton Dickinson), BD Fortessa (Becton Dickinson) or CyAn ADP (Beckman Coulter) flow cytometers. Cell sorting was performed with BD FACSVantage, BD Aria, BD AriaII or BD AriaIII (all
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(Becton Dickinson) cell sorters. Analysis of FACS data was done with FlowJo software (Tree Star Inc.).

Cell surface staining was performed in a mixture of 50% PBS (+2% FCS), and 50% supernatant from the 24G2 hybridoma cell line, which produces rat blocking antibodies directed against the FcγR (CD16/32) antigen. For FACS stainings, which required analysis of CD16/32 expression, the stainings were performed in PBS 2% FCS without 24G2. Cells were incubated with antibodies for 30 minutes on ice. For biotin coupled antibodies cells were subsequently incubated with streptavidin conjugates for 20 minutes on ice.

The following anti-mouse antibodies were used for FACS analysis:
Gr-1 (Ly-6G, RB6-8C5)-FITC and -biotin, Ter119-FITC and -biotin, B220 (RA3-6B2)-FITC and -biotin, CD11b-FITC and -biotin, CD4 (clone GK1.5)-FITC and -biotin and CD8a (53.6.7)-FITC and -biotin were purified and conjugated in the laboratory following standard protocols. CD34 (RAM34)-FITC, cKit (2B8) -PE, -PE-Cy5, -PE-Cy7, -APC, and -APC-Cy7, Sca-1 (D7) -APC, -APC-e780, -Alexa700 and (2B8)-biotin, CD48 (HM48-1)-PE, -PB, CD4 (GK1.5)-PE-Cy7, CD8 (53-6.7)-PE-Cy7, CD11b (M1/70)-PE-Cy7, Gr1 (RB6-8C5)-PE-Cy7, B220 (RA3-6B2)-PE-Cy7, Ter119-PE-Cy7, CD45 (30-F11) -FITC, CD45.1 (A20)-FITC and PE-Cy7 and CD45.2 (104)-Pacific Blue and Alexa647 were all purchased from eBioscience. CD150 (TC15-12F12.2)-PE and PE-Cy5 as well as CD48-Pacific Blue were purchased from Biolegend.

The following anti-human antibodies were used for FACS analysis:
CD4 (RPA-T4)-biotin, CD8 (RPA-T8)-biotin, CD11b (ILRF44)-biotin, CD34 (581/CD34)-PE and -APC, CD38 (HIT2)-PE and -PE-Cy5, CD49f (GoH3)-PE-Cy5, CD90 (5E10)-PE-Cy5 and CD117 (104D2)-PE-Cy7 were all purchased from Becton Dickinson. CD16 (eBio CB16)-biotin, CD19 (HIB19)-biotin and -APC, CD11b (ILRF44)-PE-Cy7, CD235a (HIR2)-biotin, CD49f (eBioGoH3)-PE and CD45 (HI30)-PE were all purchased from eBioscience.

The following Streptavidin conjugates were used for FACS analysis:
APC-e780 (eBioscience) or PE-TexasRed (BD Biosciences)
5.6 BrdU incorporation assays and Ki67/Hoechst cell cycle analysis

Cell cycle profile and proliferation of HSCs was analyzed by combining cell surface stainings as described above with Ki67/Hoechst analysis or BrdU incorporation assays.

For BrdU analysis mice were injected i.p. with 360µg/mouse BrdU (Sigma) 12 hours prior to analysis unless indicated differently. BM cells were harvested and cell surface stainings performed as described above. Cells were fixed and permeabilized with a BrdU staining kit (BD Biosciences) with Cytofix/Cytoperm and Cytoperm plus according to manufacturers protocol. Samples were incubated with DNAse I for one hour at 37°C, followed by intra-cellular staining with anti-BrdU-FITC or –APC antibodies in Permwash solution (BD Biosciences).

For Ki67/Hoechst analysis cells were fixed with Cytofix/Cytoperm (BD Biosciences), and incubated with anti-Ki67-FITC or -Alexa647 antibodies (BD Biosciences) in Permwash solution over night. Before analysis cells were incubated for 10 min with 1:200 diluted Hoechst33342 (Invitrogen) on ice.

5.7 Immunofluorescence

Unfractionated bone marrow cells or FACS sorted hematopoietic populations were fixed with Cytofix/Cytoperm (BD Biosciences) for 15min at 4°C. Cells were spun down on SuperFrost Ultra Plus slides (Menzel) with a Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific). Samples were blocked with 15% goat serum (DAKO) in PBS for one hour at 4°C to reduce unspecific binding of antibodies. Slides were incubated with a rabbit polyclonal primary Antibody against Ifitm3 (Abcam #ab15592) 1:100 diluted in blocking solution over night at 4°C in a humidified chamber. Slides were washed in PBS and incubated for one hour at 4°C with anti-rabbit IgG F(AB)2 fragment conjugated with DyLight 549 or Dylight 649 diluted 1:100 in PBS (Jackson ImmunoResearch). Slides were washed again and mounted with ProLong Gold mounting medium containing DAPI.
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Finally slides were analyzed on a Zeiss Cell Observer and documented with an AxioCam HR (Zeiss).

5.8 RNA isolation and Reverse Transcription

For microarrays of mouse samples, cells were sorted directly in RLT lysis buffer and RNA was isolated with the RNeasy micro kit (Qiagen) according to manufacturers instructions, including an RNAase free DNAse digestion step to digest DNA.

For all qPCR samples as well as human microarrays, cells were FACS sorted directly in extraction buffer (Arcturus PicoPure Kit) and RNA was isolated with the PicoPure RNA isolation kit (Arcturus) according to manufacturers instructions, including an RNAse free DNAse step to digest DNA.

RNA was reverse transcribed with the SuperScript VILO cDNA synthesis kit (Invitrogen) according to manufacturers instructions. Resulting cDNA was diluted 1:4 or 1:10 before use for downstream reactions.

5.9 Microarray analysis

RNA was isolated with RNeasy micro kit (Qiagen) or Picopure RNA isolation kit (Arcturus) as described, including digestion of DNA by RNAse-free DNAse. After isolation RNA quality was checked and quantity determined with an Agilent Bioanalyzer 2100 (Agilent). Samples of insufficient quality (RIN score below 7) were excluded from analysis; samples of insufficient quantity (below 2.5ng) were pooled with other samples or excluded as well. Samples of sufficient quality and quantity were amplified with the Ovation PicoSL WTA system and labeled with the BiotinII Module (Nugene). After amplification and labeling samples were hybridized to the respective microarray, MouseRef-6 V2.0 (Illumina) for mouse samples and HumanHT-12 (Illumina) for human samples.
5.10 Quantitative Real-time PCR analysis

Quantification of mRNA expression by quantitative Real-time PCR (qPCR) was performed using the ABI Power SYBR Green Master Mix (Applied Biosystems) on a Light Cycler 480 (Roche) according to the manufacturers instructions. The following amplification protocol was used: 95°C for 10min; 50 cycles of 95°C for 15s, 60°C for 45s, followed by acquisition of fluorescence; 95°C for 10s; 65°C for 1 min and increase of temperature by 0.11°C/min up to 95°C continuous acquisition for melting curve; 40°C 5min. Results were analyzed using the supplied LightCycler software (Roche).

Primers for qPCR reactions were designed with the Assay Design Center of the Universal Probe Library (Roche¹) or Primer3² [Rozen and Skaletsky, 2000]. Sequences of genes were obtained from ENSEMBL³ or GenBank⁴. Sdha, B2m and Oaz1 were used as housekeeping genes for normalization of gene expression, with the exception of quantification after treatment with IFNα or LPS, where only Sdha and Oaz1 were used as B2m is induced by both treatments.

¹https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000
²http://frodo.wi.mit.edu/
³http://www.ensembl.org
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Table 5.3 Primers used for Real-time PCR analysis
5.11 Statistical analysis

Statistics were calculated and Graphs designed with Microsoft Excel (Microsoft) or GraphPad Prism (GraphPad Software). If not specified differently graphs show the mean expression and error bars indicate standard error of the mean (SEM). Significance of differences between samples was determined using unpaired two-tailed t-tests.

Indication of significance:  
*  p<0.05  
** p<0.01  
*** p<0.005  
**** p<0.001


continuous imatinib vs pulsed imatinib with or without G-CSF in CML patients who have achieved a complete cytogenetic response. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK 23, 1199-1201.


# Appendix

## 7.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AGM</td>
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</tr>
<tr>
<td>Akt</td>
<td>PKB, Protein kinase B</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Angiopoietin-1</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<tr>
<td>CAR</td>
<td>CXC-12 abundant reticular cell</td>
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<tr>
<td>c-Kit</td>
<td>v-kit Hardy-Zuckerman 4 feline sarcoma cellular homologue</td>
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<td>Common lymphoid progenitor</td>
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<td>CMP</td>
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<td>Granulocyte-colony stimulating factor</td>
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<td>GMP</td>
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<td>i.f.</td>
<td>intrafemoral</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>IVC</td>
<td>Individually ventilated cage</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LRC</td>
<td>Label retaining cell</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte erythroid progenitor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
</tr>
<tr>
<td>MPP</td>
<td>Multi-potent progenitor cell</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>mTor</td>
<td>Mammalian target of Rapamycin</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B, Akt</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>pIC</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen 1</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor, c-Kit ligand</td>
</tr>
<tr>
<td>SLAM</td>
<td>Signaling lymphocyte activation molecule</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal-transducer and activator of transcription 1</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
</tbody>
</table>

**Table 7.1 Abbreviations**
### 7.2 Cell surface markers used to define hematopoietic populations

#### Mouse hematopoietic cells:

<table>
<thead>
<tr>
<th>Population</th>
<th>Cell surface phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;</td>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; Gr-1&lt;sup&gt;+&lt;/sup&gt; B220&lt;sup&gt;+&lt;/sup&gt; CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;+&lt;/sup&gt; Ter119&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>LSK</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>LSK SLAM</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;CD150&lt;sup&gt;-&lt;/sup&gt;CD48&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>LK SLAM</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;CD150&lt;sup&gt;-&lt;/sup&gt;CD48&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>LSK SLAM CD34&lt;sup&gt;-&lt;/sup&gt;</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;CD150&lt;sup&gt;-&lt;/sup&gt;CD48&lt;sup&gt;-&lt;/sup&gt;CD34&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPP1/2</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;CD150&lt;sup&gt;-&lt;/sup&gt;CD48&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPP3/4</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;CD150&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>CMP</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;IL7R CD34&lt;sup&gt;-&lt;/sup&gt;FcyR&lt;sub&gt;lo&lt;/sub&gt;</td>
</tr>
<tr>
<td>GMP</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;IL7R CD34&lt;sup&gt;-&lt;/sup&gt;FcyR&lt;sub&gt;hi&lt;/sub&gt;</td>
</tr>
<tr>
<td>MEP</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;IL7R CD34 FcyR&lt;sub&gt;lo&lt;/sub&gt;</td>
</tr>
<tr>
<td>CLP</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt;Sca-1&lt;sup&gt;-&lt;/sup&gt;IL7R CD135&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>T-cells</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; or CD8&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>B-cells</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Ter119&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt;Gr-1&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>CD41&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
</tbody>
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**Table 7.2 Markers used to identify mouse HSCs**

#### Human hematopoietic cells:

<table>
<thead>
<tr>
<th>Population</th>
<th>Cell surface phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;</td>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; CD16&lt;sup&gt;+&lt;/sup&gt; CD19&lt;sup&gt;+&lt;/sup&gt; CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;+&lt;/sup&gt; CD235a&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;-&lt;/sup&gt;</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD34&lt;sup&gt;-&lt;/sup&gt;CD38&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;CD117&lt;sup&gt;+&lt;/sup&gt; HSCs</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD34&lt;sup&gt;-&lt;/sup&gt;CD117&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Progenitor cells</td>
<td>lin&lt;sup&gt;neg&lt;/sup&gt;CD34&lt;sup&gt;-&lt;/sup&gt;CD38&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>T-cells</td>
<td>CD4&lt;sup&gt;-&lt;/sup&gt; or CD8&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>B-cells</td>
<td>CD19&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>CD235a&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD11b&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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