Úloha kmenových a progenitorových buněk v regeneraci krvetvorné tkáně

The role of stem and progenitor cells in regeneration of hematopoietic tissue

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Content

1. Abstract

Tissue regeneration is a complex and highly orchestrated process dependent on cells with the potential to restore structures and functions and on controlling factors from the tissue microenvironment. Hematopoietic tissue has a high ability to regenerate, which is attributed to the presence of stem cells, but the regeneration of severely damaged adult tissue is still only partially understood. Hematopoietic tissue provides a unique opportunity to study tissue regeneration due to its well-established steady-state structure and function, easy accessibility, advanced research methods, and well-defined embryonic, fetal, and adult stages of development. Embryonic/fetal liver hematopoiesis and adult hematopoiesis recovering from damage share the need to expand populations of progenitors and stem cells in parallel with increasing production of mature blood cells.

We analyzed adult hematopoiesis in mice subjected to a submyeloablative dose (6 Gy) of gamma radiation, in which only a few cells with reconstituting capacity survived. We targeted the period of regeneration characterized by the renewed massive production of mature blood cells and the ongoing expansion of immature hematopoietic cells. Cells from the top of the hematopoietic hierarchy, hematopoietic stem cells, and multipotent progenitors are almost missing in this period of hematopoiesis regeneration. We uncovered significantly expanded populations of developmentally advanced erythroid and myeloid progenitors with significantly altered immunophenotype and with the ability for intensive proliferation. These immature hematopoietic cells differ from the progenitor cells present in normal bone marrow by the decreased expression level of the c-Kit receptor for stem cell factor, the expression of Sca-1 antigen also in the cells which express transferrin receptor 1 (CD71), by expression of CD16/32 in most of the cells, and by altered expression of CD41. These progenitors activated the erythroid developmental program independently from erythropoietin production. Despite decreased expression of the c-Kit receptor, progenitors require effective stimulation by stem cell factor (SCF) for their expansion.

Hematopoietic stem cells, defined by their ability to reconstitute destroyed hematopoiesis in the host, were reduced to $1 - 2$ % of their normal number in the intensively regenerating hematopoiesis.

It was shown that the early reconstitution of hematopoiesis from transplanted cells that were not exposed to radiation gives rise to populations of altered progenitors, which are similar to those identified in the bone marrow regenerating from endogenous cells surviving exposure to ionizing radiation.

Regenerating hematopoiesis differs significantly from the expanding hematopoiesis in the fetal liver by the virtual lack of stem cells and different immunophenotypes of progenitor cells.

The data presented in this study provide a novel insight into tissue regeneration by suggesting that cells other than stem cells and multipotent progenitors can be of fundamental importance for the rapid recovery of tissue function, and the regenerating adult hematopoiesis shares some features with the embryonic hematopoiesis preceding the development of stem cells.

2. Abstrakt

Regenerace tkáně je komplexní řízený proces závislý na buňkách s potenciálem obnovit buněčnou stavbu tkáně a její funkci, jakož i na řídících faktorech z tkáňového mikroprostředí. Krvetvorná tkáň má velkou schopnost regenerace, která je přičítána přítomnosti kmenových buněk. Regenerace silně poškozené dospělé krvetvorné tkáně je však prozkoumána jen částečně, a důraz na klíčovou úlohu kmenových buněk může zastírat poznání jiných významných mechanismů regenerace. Krvetvorná tkáň přitom poskytuje unikátní možnost poznání tkáňové regenerace díky její známé buněčné hierarchii, existenci pokročilých výzkumných metod včetně možnosti její transplantace, a také dobře definovaným embryonálním, fetálním a dospělým vývojovým fázím. Embryonální/fetální krvetvorba a regenerující dospělá krvetvorba spolu sdílejí potřebu zvyšování populací progenitorových a kmenových buněk při současné potřebě stálého zvyšování tvorby zralých krevních buněk.

Analyzovali jsme dospělou krvetvornou tkáň myší po jejím submyeloablativním poškození ionizujícím zářením (6 Gy). Použitou dávku celotělového záření přežije jen velmi málo buněk s kapacitou rekonstituce (regenerace) hematopoezy. Zaměřili jsme se na období intenzivní regenerace charakterizované obnovenou masivní produkcí zralých krevních buněk a souběžnou expanzí nezralých krvetvorných buněk progenitorových a kmenových. Zjistili jsme, že v této fázi regenerace kmenové buňky a multipotentní progenitorové buňky téměř chybí. Popsali jsme a definovali silně expandovanou populaci vývojové pokročilých erytroidních a myeloidních progenitorových buněk s významně změněným imunofenotypem. Od obdobných buněk přítomných v normální kostní dřeni se lišily sníženou expresí receptoru c-Kit pro cytokin stem cell factor (SCF), expresí Sca-1 antigenu na buňkách, které současně exprimovaly znak CD71 (transferinový receptor 1), zvýšenou expresí CD16/32 antigenu a změněnou expresí znaku CD41. Tyto fenotypové změny nebyly způsobeny stimulací erytropoetinem v důsledku anémie. Přestože buňky měly sníženou expresi c-Kit receptoru, byla jejich stimulace SCF nezbytná pro jejich početní expanzi.

Krvetvorné kmenové buňky definované schopností obnovit poškozenou krvetvorbu hostitele byly v intenzivně regenerující krvetvorbě redukovány na 1 – 2 % svého normálního počtu.

Regenerace krvetvorby se významně lišila od expandující krvetvorby ve fetálních játrech.

Regenerace krvetvorby vycházející z malého počtu z transplantovaných buněk, které nebyly vystaveny ionizujícímu záření, vykazovala shodné znaky s regenerací vycházející z endogenních buněk, které zůstaly v tkáni po působení ionizujícího záření.

Výzkum přináší nový pohled na regeneraci poškozené tkáně především tím, že odhaluje klíčovou úlohu vývojově pokročilých buněk progenitorových, které nahrazují funkci buněk kmenových a multipotentních progenitorů. Poprvé je také upozorněno na podobnost regenerace krvetvorné tkáně se specifickou fází embryonální krvetvorby, která předchází vzniku krvetvorných kmenových buněk.

1. List of Abbreviations

ACK2	c-Kit blocking antibody
AGM	aorta-gonad-mesonephros
ANOVA	analysis of variance
BFU-E	burst-forming unit-erythroid
BM	bone marrow
c-Kit	CD117, stem cell factor receptor
CFU-E	colony-forming unit-erythroid
CFU-GEMM	colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte
CFU-GM	colony-forming unit-granulocyte-macrophage
CFU-M	colony-forming unit-megakaryocyte
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CTRL	control
D	day after irradiation of mice
dHSC	definitive hematpoietic stem cell
E	embyronic day
EHT	endothelial-to-hematopoietic transition
EMP	erythro-myeloid progenitors
EPO	erythropoietin
EryP-CFC	primitive erythroid progenitors
FACS	fluorescence-activated cell sorting
FSC	forward scatter
FL	fetal liver
GFP	green fluorescein protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte-megakaryocyte progenitor
HCT	hematokcrit
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
LK	Lin$^-$c-Kit$^+$
LSK	Lin$^-$c-Kit$^+$Sca-1$^+$
LS-K	Lin$^-$c-Kit$^+$Sca-1$^-$
LT-HSC	long term repopulating hematopoietic stem cell
Mac-CFC	primitive macrofage progenitors
MEP	megakaryocyte-erythrocyte progenitor
MPP	multipotent progenitor
M-CSF	monocyte colony-stimulating factor
PB	peripheral blood
Pre-HSC	precursor of hematopoietic stem cell

RBC	red blood cell
SC	side scatter
Sca-1	stem cell antigen
SCF	stem cell factor
SP	side population
ST-HSC	short term repopulating hematopoietic stem cell
T	transfusion of red cells
TPO	trombopoetin

2. Introduction

Decades of research into the adult murine hematopoiesis have established a hierarchical organization of hematopoiesis in which hematopoietic stem cells (HSCs) give rise to multipotent progenitors (MPPs), and MPPs further develop into lineage-committed and progressively developmentally restricted progenitor cells which finally give rise to differentiated myeloid and lymphoid precursor cells (Weissman, 2000; Adolfsson et al., 2001; Na Nakorn et al., 2002; Kiel et al., 2005; Yang et al., 2005; Pronk et al., 2007; Wilson et al., 2007; Morita et al., 2010; Oguro et al., 2013).

However, several experimental findings have indicated a more complex organization of the immature hematopoietic cells and also challenged the idea that the extensive self-renewal capacity is a unique property of HSCs (Adolfsson et al., 2005; England et al., 2011; Yamamoto et al., 2013; Kim et al., 2015). It was also demonstrated that the undisturbed murine hematopoiesis is maintained by multiple clones acting in parallel (Zavidij et al., 2012; Sun et al., 2014) without any significant contribution from HSCs. Busch et al. (2015) also demonstrated that undisturbed adult hematopoiesis is largely sustained by cells downstream of HSCs, and Schoedel et al. (2016) reported a long-term hematopoiesis occurring in the absence of HSCs while, in contrast, Sawai et al. (2016) and Akinduro et al. (2018) presented the data supporting the continuous contribution of HSCs for steady state hematopoiesis. The controversy in published reports and the question whether transplantable HSCs are required for adult hematopoiesis have been recently discussed by McRae et al. (2019). Further, the megakaryocyte-deficient lympho-erythro-myeloid progenitors and megakaryocyte-restricted progenitors with the properties of long-term HSC were also described in unperturbed adult hematopoiesis (Carrelha et al., 2018; Rodriguez-Fraticelli et al., 2018).

The formation of adult steady state hematopoiesis wherein HSCs and progenitors steadily generate mature blood cells with limited life-span is preceded by its prenatal and early postnatal expansion derived from a small number of founder cells. During the embryonic, fetal and early postnatal periods of life, hematopoietic tissue has to establish its hierarchical organization in parallel with the essential production of functional blood cells. This represents a non-steady state situation when two contradictory processes co-exist, the one requiring self-renewal of produced cells, while the other one requiring their efficient differentiation, both in competition with each other (Figure 1).
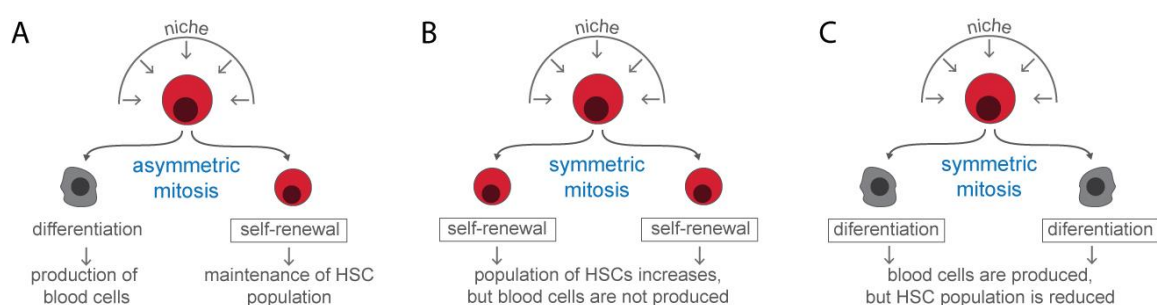
Figure 1. Cell division associated with cell self-renewal or differentiation. A) asymmetric self-renewal and differentiation division, **B)** symmetric self-renewal division, **C)** symmetric differentiation division

In the mouse, the transient primitive hematopoiesis is established in the yolk sac at the embryonic day E7.5 producing mainly primitive red blood cells which undergo the process of maturation in the circulation. These primitive red blood cells are distinguishable from the later fetal and adult definitive red blood cells by their large size and embryonic globin expression (Palis, 2014). This is followed by emergence of the erythro-myeloid progenitors (EMP), also in the yolk sac, which colonize the fetal liver at E10.5 and give rise to definitive

erythrocytes. EMPs also have potential for production of myeloid cells and megakaryocytes but not lymphocytes (Frame et al., 2013; McGrath et al., 2015). These cells lack the capacity to be transplanted and to reconstitute damaged hematopoiesis which is the hallmark of HSCs. The HSCs differentiate later from a specialized hemogenic endothelium in large arteries in the AGM (aorta-gonad-mesonephros) region of the embryo and in the vitelline arteries and also in the placenta [reviewed in Palis (2016); Dzierzak and Bigas (2018)] and are the founder cells for the hierarchically organized adult hematopoiesis producing myeloid and lymphoid blood cells (Figure 2).
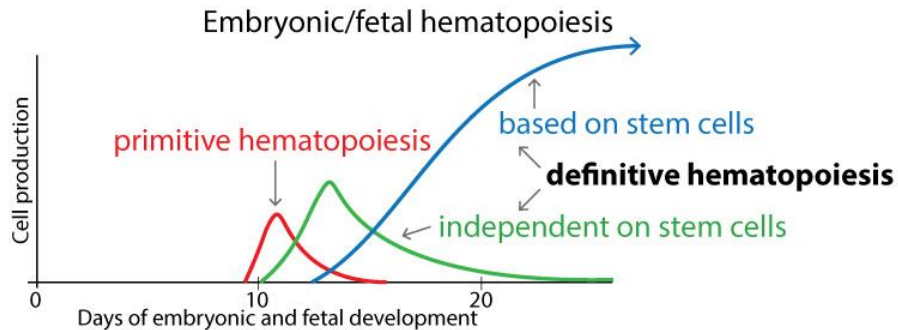

Figure 2. Primitive and definitive hematopoiesis in the yolk sac, embryo, and fetus (delivery of mouse fetuses occurs on days 20-21 after oocyte fertilization).

A similar situation to that in the embryo/fetus arises in the adult hematopoiesis after a severe bone marrow damage. The hierarchy of immature hematopoietic cells has to be reconstituted in parallel with the life-saving recovery of blood cell production. McCarthy (1997) demonstrated that after irradiation of mice with a dose of 6 Gy some mice develop, after several months, monoclonal hematopoiesis derived from a single cell. This demonstrates that in the adult life, after such a significant damage to the bone marrow, hematopoiesis can finally re-establish its HSCs-progenitor cells hierarchy. However, a detailed knowledge about the period of early regeneration of adult bone marrow when it expands the populations of immature cells together with intensive production of blood cells is lacking. In this thesis, we attempt here to fill this significant gap in knowledge by comprehensively examining the immature lineage negative and c-Kit positive hematopoietic cells (Lin⁻c-Kit⁺; LK cells) and their subsets in bone marrow at its vigorous regeneration phase following its major damage induced by total body irradiation of mice with a dose of 6 Gy.

3. Hypothesis

Stem cells are not the exclusive carriers of regeneration of damaged hematopoiesis. Progenitor cells significantly participate in the recovery of blood cell production and early hematopoietic tissue reconstitution. It is assumed that a transient period in the regeneration of damaged hematopoiesis mimics the embryonic stage when production of blood cells is not derived from stem cells.

4. Aims

1) Analysis of progenitor and stem cells in intensively regenerating hematopoiesis.

2) Determination of the role of the SCF/c-Kit receptor signaling and erythropoietin stimulation in early phase of hematopoiesis regeneration.

3) Comparison of the expanding adult hematopoiesis during its regeneration with the expanding hematopoiesis in the fetal liver.

5. Materials and methods

6.1 Mice

C57BL/6J (CD45.2) and B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) mice were used.

6.2 Irradiation

Irradiation was performed in a plastic cage and a ^{60}Co source (Chisobalt 2-B75, MEDIN, formerly Chirana, Czech Republic) from a distance of 123.5 cm with a dose rate of ~ 0.35 Gy per minute.

6.3 Spleen colonies (CFU-S)

Spleen colonies were determined in submyeloablatively irradiated mice (4 Gy or 6 Gy) 8-14 days after irradiation (endogenous spleen colonies). Spleens were fixed in Tellesniczky´s solution (18:1:1 volume parts of 70 % ethanol, glacial acetic acid and formalin), and spleen colonies were observed with the naked eye and counted.

6.4 Bone marrow collection

Bone marrow cells were obtained from the long bones (femurs and tibias, or femurs only) by flushing the bone cavity with PBS supplemented with 1% bovine serum albumin (BSA) through a hole in one end of the bone without clipping off the epiphyses. A single-cell suspension was obtained by repeated passage through the needle (25G) and kept on ice before further handling.

6.5 Flow cytometry and cell sorting

Cells were filtered through a 70μm nylon cell filter (BD Biosciences, San Jose, CA, USA) and stained with fluorochrome-labeled antibodies for 20 minutes at 4° C in the dark with optimal dilutions of commercially prepared antibodies. Stained bone marrow cells were analyzed using a digital FACS Canto II flow cytometer or

sorted with a FACSAria IIu cell sorter (BD Biosciences). LK cells and their subsets were identified by flow cytometry, as is shown in the result figures. LK cells were analyzed in their c-Kit^{high} and c-Kit^{low} fractions.

6.6   Red blood cell transfusion

Mice under deep anesthesia were exsanguinated from the retro-orbital venous sinus by heparinized capillaries and sacrificed by cervical dislocation. Red blood cells were washed with an excess of PBS, and a 75% suspension of red blood cells in PBS was prepared. The suspension was intravenously injected in a volume of 0.5-0.6 ml to mice via the retro-orbital route (29G needle).

6.7   Erythropoietin administration to normal mice

Male mice were injected with recombinant human erythropoietin (NeoRecormon epoetin beta; Roche, Basel, Switzerland) intraperitoneally for four consecutive days, thecumulative dose was 200 IU/mouse. Bone marrow was collected 24 hours after the last EPO injection.

6.8   A state of iron deficiency

Male mice were put on a low-Fe diet (C 1038; Altromin Spezialfutter GmbH & Co. KG, Hamburg, Germany) for 7 days and were bled 0.5-0.6 ml from the retro-orbital venous sinus 5 days and 1 day before the examination of peripheral blood and bone marrow.

6.9   ACK2 c-Kit blocking antibody administration

ACK2 In Vivo Ready^{TM} Anti-Mouse CD117 (c-Kit) was used as the c-Kit blocking antibody. The reagent was from Tonbo Biosciences (U.S.A.). ACK2 antibody was administered to mice intravenously (0.5 mg in 0.25 ml per mouse).

6.10 Peripheral blood analysis

Peripheral blood was collected from the retro-orbital venous sinus of anesthetized mice using capillaries (75mm/60µl; KERAGLASS, Otovice, Czech Republic) containing a small volume of EDTA and was analyzed with a BC-5300Vet Auto Hemato Analyzer (Mindray Bio-Medical Electronics, Shenzhen, China) calibrated for mouse blood samples.

6.11 Bone marrow transplantation

A single-cell suspension of bone marrow cells was transplanted intravenously through the retro-orbital route. Recipient mice were dual CD45.1/CD45.2 (F1) mice irradiated at 8.5 Gy before transplantation. The recipients were transplanted with a mixture of bone marrow cells or sorted LSK CD150$^+$CD48$^-$ cells of regenerating (CD45.2) and untreated (CD45.1) mice (*vice versa* in one experiment). Chimeric CD45.2/CD45.1 bone marrow from transplanted mice was retransplanted to secondary 8.5 Gy-irradiated F1 recipient mice.

6.12 Analysis of chimeric hematopoiesis in peripheral blood and bone marrow

The ratio of donor to host nucleated blood cells was determined in samples of peripheral blood drawn from the retro-orbital venous plexus of transplanted mice using capillaries containing 5µL of 0.5M EDTA. Approximately 50µL blood samples were stained with anti-CD45.1 and anti-CD45.2 antibodies for 30 minutes on ice in the dark and washed after. The samples were also stained for Gr-1/Mac-1, B220, and CD3 or CD4 and CD8 markers.

Femoral bone marrow cells were collected into PBS. Four million bone marrow cells were washed with PBS and centrifuged (4 °C, 400 g, 6 min). After removing the supernatant, the cells were stained for the CD45.2 and CD45.1 allotypes and Gr-1/Mac-1, B220 and CD3 or CD4 and CD8 markers to determine the ratio between CD45.2 cells (originating from regenerating bone marrow) and CD45.1 cells (originating from control bone marrow; *vice versa* in one experiment).

3. Results

7.1 Immature hematopoietic cells expand their population rapidly and in parallel to increasing production of blood cells in regenerating hematopoiesis following significant damage

To define the experimental model of hematopoietic regeneration used in the present study, we estimated the extent of the initial damage inflicted on the hematopoietic tissue by irradiating mice with a dose of 6 Gy by determining the occurrence of endogenous spleen colonies arising from myeloid progenitor cells which survived irradiation. The colony numbers are shown in Table 1, and examples of spleens are shown in Figure 3A. There were only occasional spleen colonies in mice irradiated with a dose of 6 Gy. The rare colonies became large after 11 days, and from the kinetics of spleen colony development (Nečas and Znojil, 1989) it can be deduced that they originated from cells that had survived after irradiation and began their clonal expansion shortly after that. Numerous tiny colonies became visible on the spleens examined 12 – 14 days after irradiation. We hypothesize that they reflect the migration of progenitors from regenerating bone marrow to the spleen, as was reported by Peslak et al. (2012). Based on the spleen colony results and the results from our previous study (Forgacova et al., 2013) and the results reported by McCarthy (1997), we estimate that only very few cells with the hematopoietic tissue reconstituting capacity survived in the 6 Gy-irradiated mice.

Bone marrow cells are severely reduced by the damage caused by irradiation. In untreated mice, the bone marrow of one femur consists of $28.3 \pm 5.3 \times 10^6$ cells. Ten days after irradiation at dose 6 Gy, bone marrow cells were reduced to one-quarter of its number ($7.0 \pm 2.7 \times 10^6$ cells), whereas the population of immature hematopoietic cells lacking lineage markers and expressing the c-Kit receptor (LK cells) was reduced to one-tenth (from 451,300 to only 4,400 LK cells/femur) at this time (not shown). The population of immature LK cells in the bone marrow rapidly expanded between the 14th and the 15th days after irradiation (Figure 3C). Intensive production of blood cells occurred 12 days and later after irradiation, as indicated by the increasing number of red blood cells in peripheral blood (Figure 3B). Altogether, these results suggest the similarity between the vigorously regenerating adult hematopoiesis and the physiologically expanding embryonic/fetal and early postnatal hematopoiesis in the concomitant increase of blood cell production and the expansion of populations of immature hematopoietic cells.

Day	8	11*	12*	12*	14*
4 Gy	12.5±1.9 (4)	n.d.	n.d.	n.d.	n.d.
6 Gy	0.6±0.3 (12)	0.4±0.2 (14)	0.2±0.2 (#) (6)	0.6±0.2 # (24)	0.3±0.2 # (24)

Table 1. The number of spleen colonies in submyeloablatively irradiated mice. Values are means ±SD; the number of mice is indicated in brackets; * - colonies > 2mm; # - a large number of tiny colonies
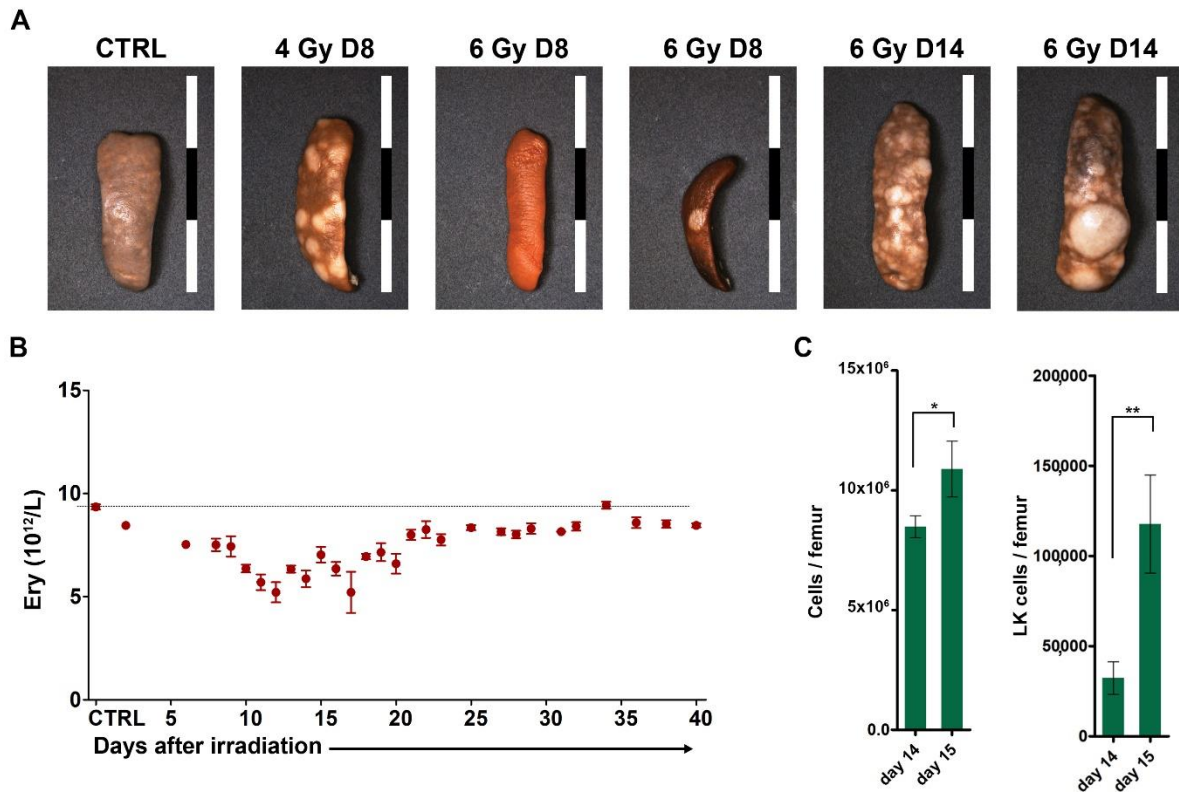
Figure 3. Endogenous spleen colonies, red blood cell numbers, and expansion of hematopoiesis in bone marrow between days 14 and 15 after irradiation of mice with a dose of 6 Gy. **A)** Representative spleens of untreated (CTRL) and submyeloablatively irradiated mice; D – day after irradiation; each segment of the scale bar equals 5 mm. Two spleens are shown after irradiation of mice at 6 Gy for spleen collected either after 8 or 14 days. **B)** Red blood cell (Ery) number achieves its nadir 12 days after irradiation of mice with 6 Gy. Data are from 52 untreated mice and 131 irradiated mice (both males). **C)** Bone marrow cellularity and the number of immature Lin⁻c-Kit⁺ (LK) cells significantly increased between days 14 (16 male mice) and 15 (12 male mice) after irradiation. There were 28.255 ± 5.386 million cells and 451,309 ± 153,982 LK cells in the femur of untreated mice (mean ± SD; 127 male mice).

7.2 The immunophenotype of LK cells is significantly altered in regenerating bone marrow

First, we analyzed the immunophenotype of the strongly reduced population of LK cells in intensively regenerating bone marrow by flow cytometry. We used the CD48 and CD150 markers according to Kiel et al. (2005) to visualize the subtypes of Sca-1 positive (LSK) cells with HSCs, MPP, and myeloid progenitor developmental potential. Figure 4A shows the representative sample wherein the immunophenotype of LK cells is shown in regenerating and normal bone marrow. The c-Kit expression level is significantly decreased, and the proportion of Sca-1 positive (LSK) cells is increased in the LK cells with low c-Kit expression level in regenerating bone marrow. The LSK cells are all CD48 positive, and the fraction of CD150 positive cells is increased.

Figure 4B compares LK cell numbers, and the ratio between Sca-1 negative (LS⁻K) cells and LSK cells in untreated mice (CTRL) and mice examined 15 days after irradiation (6 Gy D15). In the regenerating bone marrow, the proportion of LSK cells was significantly increased, and consequently, the LS⁻K/LSK ratio decreased. Figure 4B also shows the distribution of LSK cells in four CD48/CD150 subtypes in normal (CTRL) and regenerating (6 Gy D15) bone marrow. Because of the significantly altered c-Kit expression level of LK cells in regenerating bone

marrow, we analyzed the CD48 and CD150 expression in LSK cells in the c-Kit^{high} or c-Kit^{low} gates separately so as not to exclude Sca-1 positive cells with decreased c-Kit expression from the analysis.
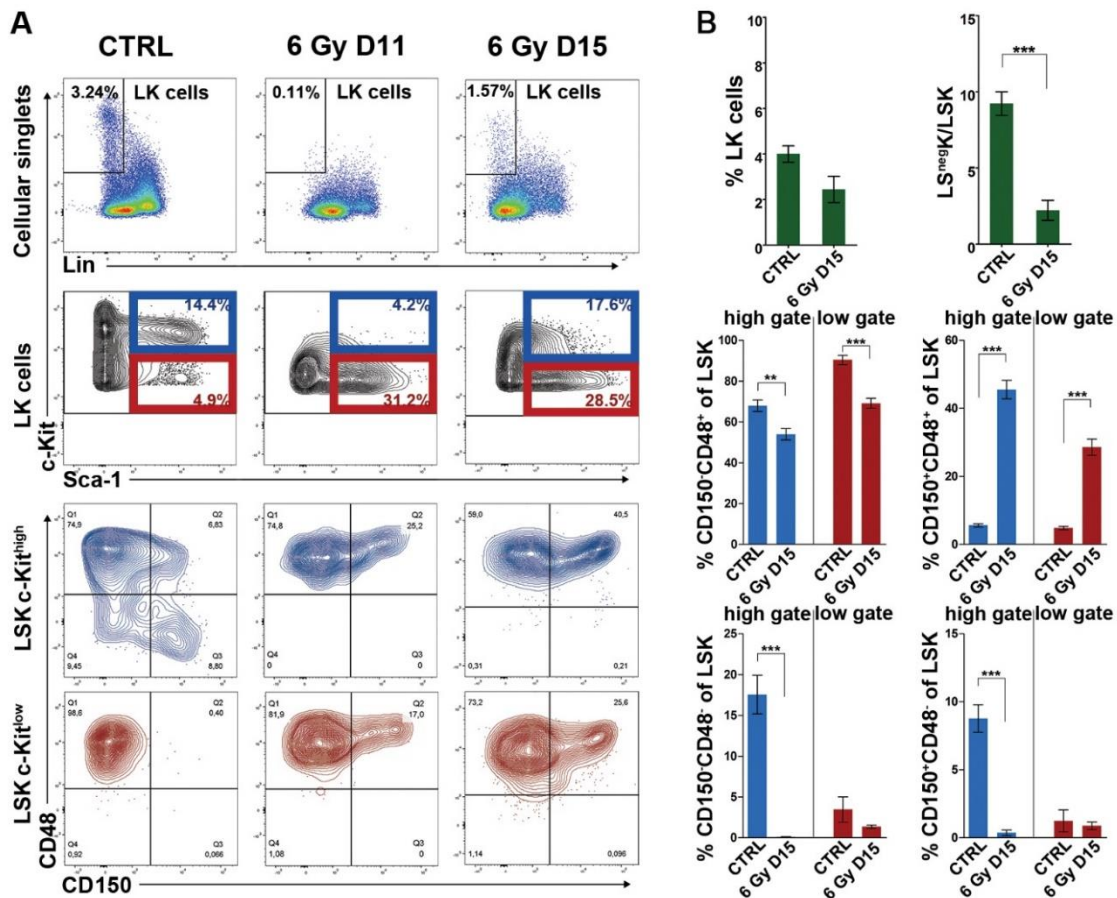

Figure 4. LK cells are c-Kit^{low} and are enriched for Sca-1$^+$ cells; LSK cells are CD48$^+$ and enriched for CD150$^+$ in regenerating bone marrow from irradiated mice. A) Examples of immunophenotypes of LK cells present in normal bone marrow (CTRL) and bone marrow collected 11 (6 Gy D11) or 15 (6 Gy D15) days after irradiation of mice with 6 Gy. The CD150/CD48 expression profile was determined in c-Kit^{high} and c-Kit^{low} subsets of LSK cells. **B)** The percentage of LK cells and the ratio between LS$^-$K and LSK cells in normal (CTRL) and regenerating (6 Gy D15) bone marrow. All LK cells were analyzed as shown in Figure 4A by cell dotplots. However, the subsequent analysis of LSK cells regarding their distribution into the four CD150/CD48 expression profile subtypes was made separately for the LSK cells with "high" and "low" c-Kit expression levels distinguished by blue and dark red colors. Data were pooled from three independent experiments, including 5 untreated mice and 8 mice examined 15 days after irradiation. ** p<0.01, ***p<0. 001.

We added an anti-CD71 antibody to the antibody cocktail used to stain the analyzed bone marrow samples. The CD71/Sca-1 plot of LK cells is highly constant in the normal bone marrow and clearly distinguishes between LSK cells, which are uniformly CD71 negative, and LS$^-$K cells that show variable expression CD71 ranging from CD71$^-$ to CD71^{high} expression (CTRL samples in Figure 5). Unexpectedly, there was a very high frequency of CD71 positive LSK cells in the regenerating bone marrow, which was in striking contrast to LSK cells in the normal bone marrow (Figure 5A). The CD71 marker corresponds to the transferrin receptor 1, which is highly expressed in erythroid cells and induced by a low iron body state. Therefore, we functionally tested the possibility that increased erythropoietin stimulation due to post-irradiation anemia (see Figure 3B) or a relative iron deficiency due to intensive erythropoiesis induced CD71 expression in LK cells in regenerating bone marrow. To differentiate these possibilities, we prevented the development of anemia by repeated transfusions of red blood cells. However, CD71 expression remained high in LSK cells in regenerating bone marrow and was equal in LSK

and LS⁻K cells (Figure 5A). In non-irradiated mice, we tested, with a negative outcome, whether CD71 expression in LSK cells would respond to administration of erythropoietin (EPO) or induction of a state of iron deficiency (Figure 5B).

As the expression of CD71 in LSK cells was quite unusual in regenerating bone marrow, we plotted the CD71 expression level against that of Sca-1 in LK cells from normal and regenerating bone marrow. The Sca-1/CD71 expression profile of LK cells, highly conserved in normal bone marrow, was significantly altered in regenerating bone marrow and presented as irregular cell clusters (Figure 5C). The occurrence of the clusters was not affected by red blood cell transfusions and polycythemia (Figure 5C).

These results confirm the previously reported decreased c-Kit expression, increased Sca-1 expression, and increased CD150 expression in immature cells in post-irradiation bone marrow (Simonnet et al., 2009). They are novel in showing the virtual absence of CD48-negative LK cells, the expression of CD71 in LSK cells, and the previously unrecognized clusters of LK cells with highly variable Sca-1/CD71 expression profiles. They demonstrate that the altered immunophenotype of LK cells is not induced by erythropoietin stimulation.
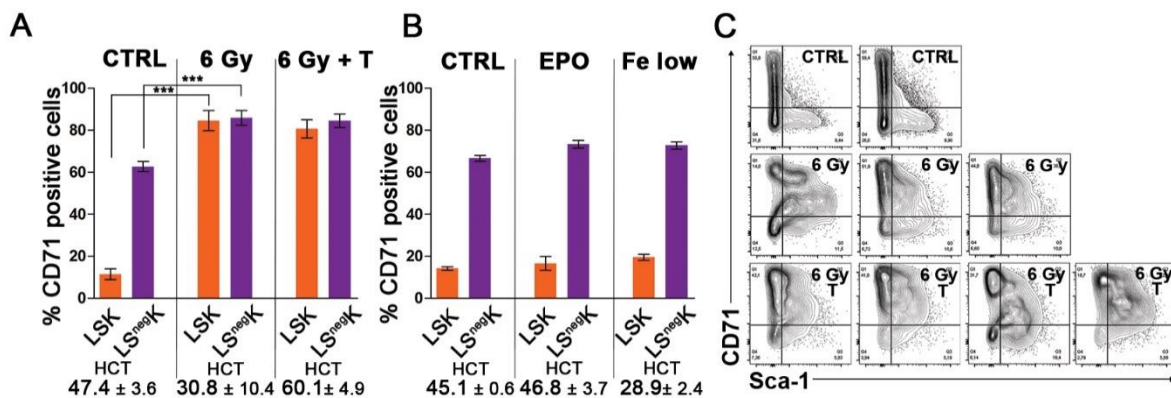

Figure 5. Altered immunophenotype of LK cells is not induced by EPO stimulation. A) Percentage of CD71 (transferrin receptor 1) positive LK cells increased in regenerating bone marrow, particularly in LSK cells. As defined in Figure 4A, all LK cells were divided into LS⁻K and LSK cells and analyzed. In a part of the mice, transfusions of red blood cells were started five days after irradiation and were given in 3-4 day intervals to prevent post-irradiation anemia. Polycythemia induced in irradiated mice by repeated transfusions of red blood cells did not abolish the increase in CD71 expression. Data were pooled from 5 independent experiments, including a total of 6 untreated mice (CTRL), 13 irradiated mice (6 Gy), and 15 irradiated and transfused mice (6 Gy + T). Mice were examined on day 14 in 3 experiments and days 15 and 16 after irradiation in another 2 experiments. **B)** Administration of erythropoietin (EPO) to normal (non-irradiated) mice, or an iron-deficient diet combined with bleeding (Fe low), did not influence the expression level of CD71 in LK cells. **C)** The Sca-1/CD71 expression profile of LK cells, highly conserved in normal bone marrow, became erratic in regenerating bone marrow and transfusions of red blood cells did not abrogate this. Examples of the Sca-1/CD71 immunophenotypes in LK cells from 2 untreated (CTRL), 3 irradiated (6 Gy) and 4 irradiated and transfused (6 Gy T) mice examined 15 days after irradiation are shown. HCT – hematocrit; T – transfusions of red blood cells.

7.3 LSK cells in regenerating bone marrow are similar to granulocyte-macrophage and erythroid progenitors

The unexpected finding of highly CD71 positive LSK cells in regenerating bone marrow prompted us to further explore LSK cells in regenerating bone marrow. We suspected that these cells could be developmentally restricted myeloid progenitors, originally Sca-1 negative, which had started reexpressing Sca-1. The myeloid Sca-

1 negative progenitors can be further differentiated utilizing their CD34 and CD16/32 (FcγRIII/II) expression profiles into the common myeloid progenitors (CMPs; CD34$^+$CD16/32$^-$), granulocyte-macrophage progenitors (GMPs; CD34$^+$CD16/32$^+$), and megakaryocyte-erythroid progenitors (MEPs; CD34$^-$CD16/32$^-$) (Akashi et al., 2000). As we became suspicious that LSK cells in regenerating bone marrow are myeloid progenitor cells that reexpressed Sca-1, we applied the CD34 and CD16/32 marker analysis on all LK cells normal and regenerating bone marrow. We examined LK cells divided into four subsets: c-Kit^{high} - c-Kit^{low} and Sca-1^{negative} - Sca-1^{positive} (see Figure 6A, subgroups 1,2,3,4 of LK cells). Figure 6A shows examples of the CD34/CD16-32 expression profile in the four subsets of LK cells in normal (CTRL) and regenerating (6 Gy D13) bone marrow. Figure 6B shows the distribution of cells with the four CD34/CD16-32 immunophenotypes in female and male mice either normal (CTRL) or examined 13 days (6 Gy D13) or 14 days (6 Gy D14) after irradiation. The most striking difference between the LSK cells (subgroups 3 and 4 in Figure 6A) from normal and regenerating bone marrow is in the expression of CD16/32. LSK cells in the normal bone marrow are uniformly CD16/32 negative, while regenerating bone marrow LSK cells are mostly CD16/32 positive and became GMP-like (Figure 6C).
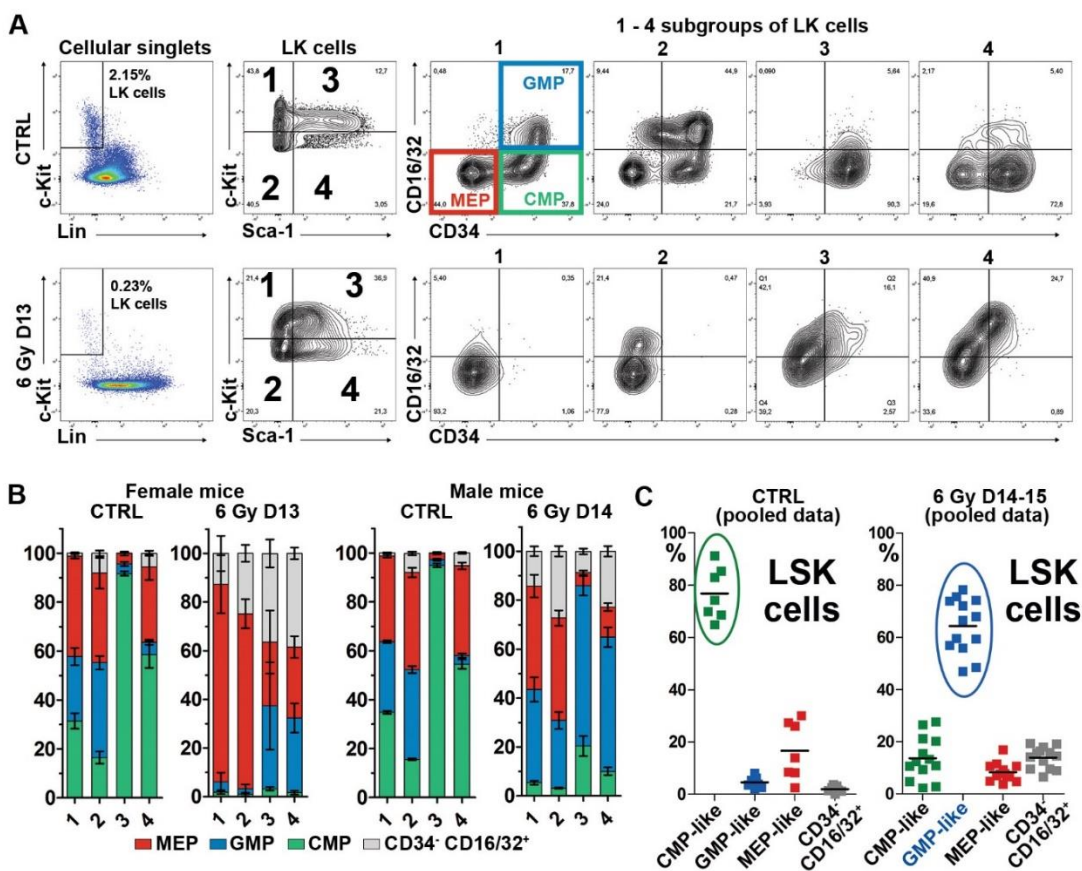

Figure 6. The CD34$^+$CD16/32$^-$ cells are significantly reduced in regenerating bone marrow, and LSK cells express CD16/32 A) Example of the CD34/CD16/32 expression profile in c-Kit^{high} - c-Kit^{low} and Sca-1$^-$ - Sca-1$^+$ (subgroups 1, 2, 3, 4) LK cells. CTRL – and untreated mouse; 6 Gy D13 – a mouse irradiated with 6 Gy before 13 days. The CD34/CD16/32 immunophenotypes characteristic for the common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) shown in subgroup 1 in the CTRL mouse were applied on cells in other subgroups of LK cells (2,3,4) and on the LK cells in the irradiated (6 Gy D13) mouse. **B)** Proportion of cells with the MEP, GMP, CMP, and CD34$^-$/CD16-32$^+$ immunophenotype profiles in 1, 2, 3, and 4 subgroups (see **A**) of LK cells in five untreated female mice (CTRL) and two female mice examined 13 days after irradiation (6 Gy D13), and four untreated male mice (CTRL) and six male mice examined 14 days after irradiation (6 Gy D14). **C)** LSK cells were analyzed for the CD34/CD16-32 immunophenotype in 7 untreated mice (CTRL; from five independent experiments) and 13 irradiated mice examined 14-15 days after irradiation (6 Gy D14-15; in four independent experiments). All mice were males.

Cells immuphenotypically similar to CMP, GMP, or MEP cells have the suffix "-like" because of the Sca-1 positivity. The increased expression of CD16/32 in LSK cells in regenerating bone marrow changed their major immunophenotype from the CMP-like to the GMP-like.

We noticed that the GMPs and MEPs present in the bone marrow of untreated mice significantly differ from common myeloid progenitors (CMPs) and all LSK cells by their increased forward scatter (FSC; the size of cells), and in GMPs, also by their higher side scatter (SSC; the granularity of cells) (Figure 7). Importantly, in the regenerating bone marrow, LSK cells had the FSC and SSC characteristics corresponding to of Sca-1⁻ GMPs and MEPs in the bone marrow of untreated mice (Figure 7).
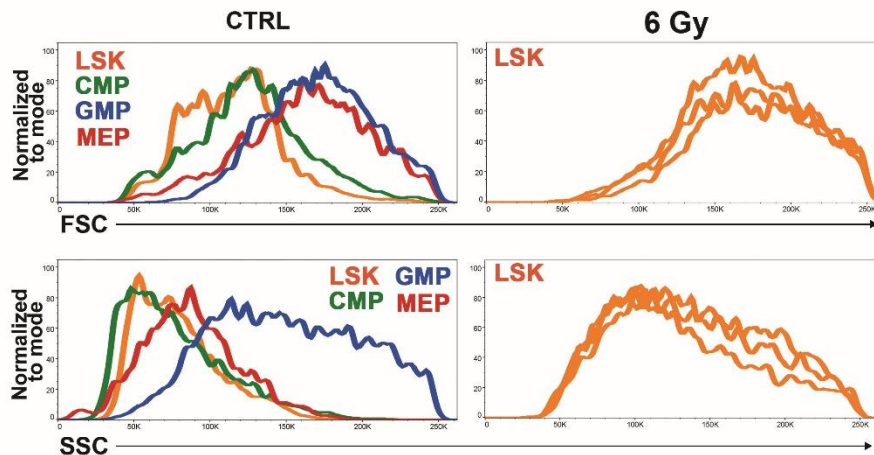

Figure 7 Forward scatter (FSC) and side scatter (SSC) histograms of LSK and CMP, GMP, and MEP cells in the bone marrow of one untreated mouse compared to the histograms of LSK cells of three mice irradiated with 6 Gy examined after 14 days.

We also compared the expression of Flt3 (CD135/Flk2), which marks lymphoid-biased MPPs (Buza-Vidas et al., 2011), in LK cells in normal and regenerating bone marrow. Approximately 40 % of the LSK cells in normal bone marrow expressed Flt3 while the LS⁻K cells were homogenously Flt3^{low} (Figure 8A). In regenerating bone marrow, the Flt3 expression became low in LSK cells and similar to its expression in LS⁻K cells in normal bone marrow (Figure 8A).

The embryonic EMPs are mostly CD41 (integrin αIIb) positive (McGrath et al., 2015). Therefore, we determined CD41 expression in LK cells in normal and regenerating bone marrow. In the normal bone marrow, CD41 is highly expressed in a fraction of LS⁻K cells (corresponding to CMPs; not shown). LK cells in regenerating bone marrow lack CD41^{high} cells, and only a part of LSK cells express CD41 at a medium level (Figure 8B, C).
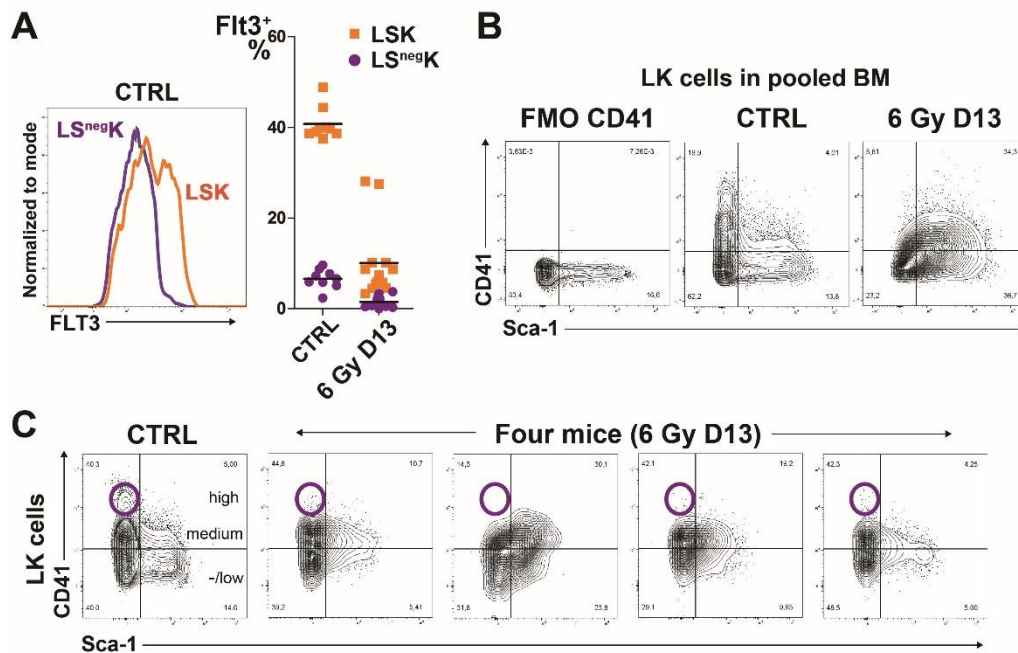
Figure 8. LSK cells in regenerating bone marrow have diminished expression of Flt3 and altered expression of CD41 A) Flt3 expression in LS⁻K and LSK cells in normal bone marrow (CTRL), and the percentage of Flt3 positive cells in LS⁻K and LSK cells in 9 untreated mice (CTRL; data pooled from four independent experiments) and 13 mice irradiated with 6 Gy (6 Gy D13; data pooled from three independent experiments). All mice were males. **B)** CD41 expression in LK cells compared to samples of bone marrow pooled from two normal (CTRL) or three irradiated (6 Gy D13) mice. FMO CD41 is an aliquot of normal bone marrow stained with the omission of anti-CD41 antibody. **C)** CD41 expression in LK cells of one normal (CTRL) mouse and four mice examined 13 days after irradiation (6 Gy D13).

7.4 LK cells in regenerating bone marrow are significantly different from the LK cells in expanding hematopoiesis in the fetal liver and early postnatal bone marrow

Regenerating bone marrow mimics hematopoiesis in the fetal liver and early postnatal bone marrow by rapidly expanding populations of immature cells accompanied by rapidly increasing production of mature blood cells. Therefore, we interrogated whether the vigorous expansion of regenerating bone marrow is similar in some aspects to the hematopoiesis in the fetal liver and early postnatal bone marrow. Immunophenotyping of LK cells from the normal adult bone marrow, fetal liver, and early postnatal bone marrow revealed significant differences from the LK cells in regenerating adult bone marrow (Figure 9B compared with Figure 4A). LK cells in the fetal liver and postnatal bone marrow were more frequent than in the adult and regenerating bone marrow (Figure 9C), exhibited high c-Kit expression (Figure 9B), and elevated LS⁻K/LSK ratio (Figure 9C). The regenerating bone marrow lacked the CD41high LK cells, which occur in the LS⁻K cells in the normal adult and postnatal bone marrow and the fetal liver (Figure 9D). CD41 is highly expressed in CMPs in normal bone marrow (not shown). The cells with the immunophenotype of CMPs are significantly suppressed in regenerating bone marrow (see Figure 6B), and the altered expression of CD41 thus correlates with this change in the composition of LK cells.
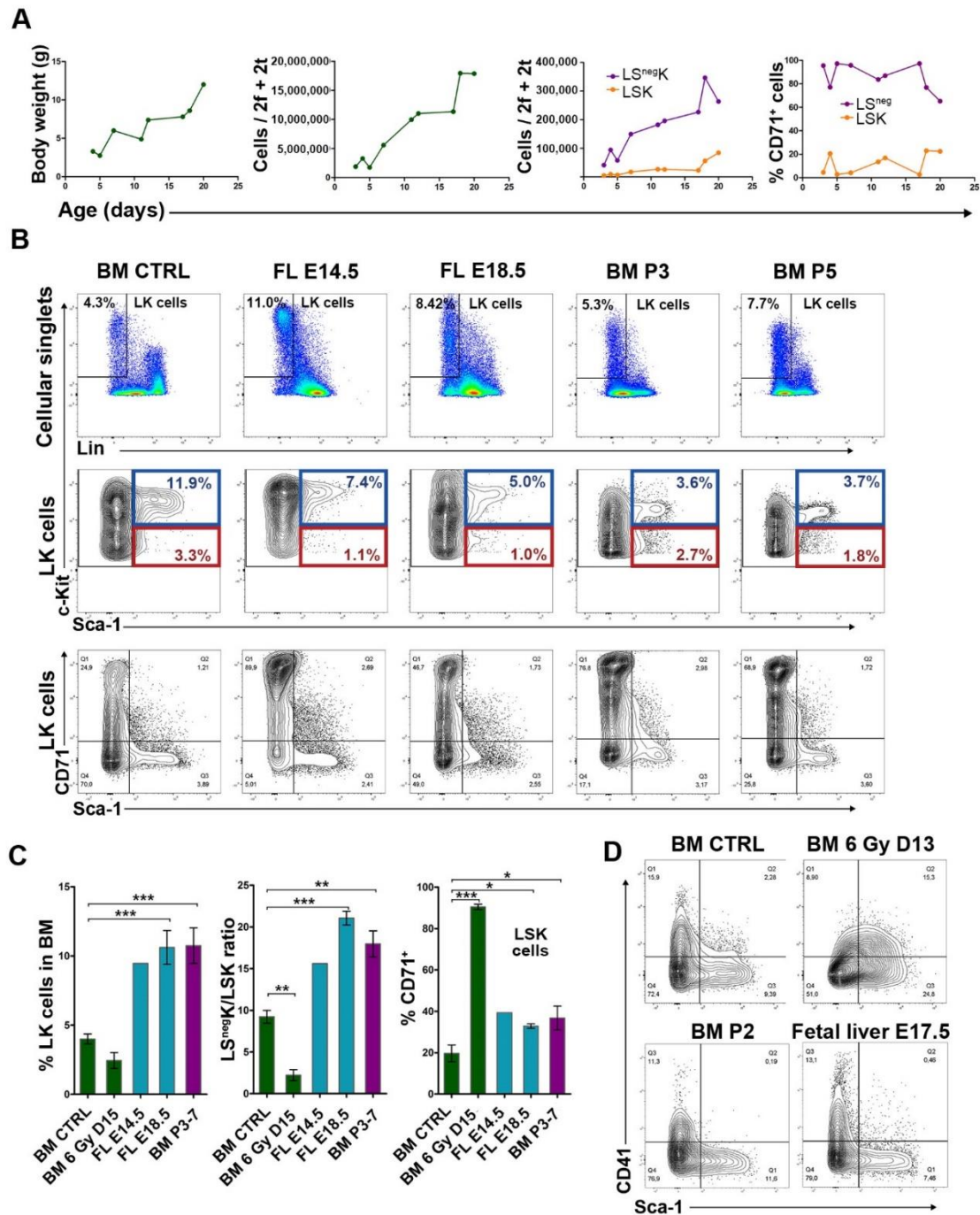
Figure 9. Expanding hematopoiesis in fetal liver and postnatal bone marrow compared to normal adult and regenerating bone marrow. A) Body weight, bone marrow cellularity, LSK, and LS⁻K cells, and CD71 expression in LSK and LS⁻K cells in postnatal mice. f – femur; t – the tibia. **B)** Examples of the immunophenotype of LK cells in adult bone marrow (Adult BM), fetal liver (FL-E14.5 or 18.5), and postnatal bone marrow (BM-P3 or P5). E – embryonic day; P – postnatal day. **C)** The percentage of LK cells, LS⁻K/LSK cell ratio, and percentage of CD71⁺ LSK cells in normal adult BM (BM – CTRL; 5 untreated male mice from three independent experiments); regenerating adult BM (6 Gy BM - D15; 8 adult male mice examined 15 days after the irradiation with 6 Gy from three independent experiments); in FL (FL – E14.5 and E18.5; 3 and 4 mice) and postnatal BM (BM – P3-7; 4 mice examined 3-7 days postpartum). *P<0.05, **P<0.01, ***P<0.001 **D)** Sca-1/CD41 expression profile in adult BM (BM CTRL), regenerating adult BM collected 13 days after irradiation (BM 6 Gy D13), BM collected on postnatal day 2 (BM P2) and fetal liver (FL - E17.5).

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7.5 LK cells in regenerating bone marrow have a meager transplantation potential

Expanding hematopoiesis in the fetal liver is very potent in transplantation, and fetal liver cells outcompete bone marrow cells in co-transplantation assays (Rebel et al., 1996; Bowie et al., 2007; Copley et al., 2013). On the other hand, bone marrow collected 13 days after irradiation of mice with a dose of 5.5 Gy failed to produce blood cells when competitively transplanted with normal bone marrow (Harrison and Astle, 1982). This finding prompted us to perform a series of experiments that compared the transplantation power of the regenerating bone marrow to that of normal bone marrow. Preliminary experiments showed that regenerating bone marrow cells should be transplanted in a significant excess to the competing normal bone marrow cells to obtain similar blood cells in both branches of the resulting chimeric hematopoiesis. We then performed a series of experiments in which the transplantation and developmental potential of regenerating bone marrow were compared to that of normal bone marrow. The experimental design of these experiments is shown in Figure 10A.

In two experiments, we tested the short-term repopulating potential of co-transplanted regenerating and normal bone marrow. Cells from regenerating bone marrow (CD45.2) were given in 30-80 excess to the cells from normal bone marrow (CD45.1). The proportions of CD45.2 and CD45.1 cells were determined in blood and bone marrow 20 days after transplantation (Figure 10B, C). The capacity of regenerating bone marrow to produce bone marrow and mature blood cells was only ~ 2 % of the capacity of normal bone marrow. Regenerating bone marrow also produced fewer B-cells (B220) than granulocytes and macrophages (GM; Figure 10B, C).

We then compared the long-term reconstitution of damaged hematopoiesis in mice transplanted with a mixture of regenerating and normal bone marrow cells and employed three experimental settings in these experiments.

First, we co-transplanted bone marrow collected from mice 14 days after irradiation (CD45.2) with normal bone marrow (CD45.1) at an 80:1 ratio. The peripheral blood of the recipient mice was analyzed for the presence of CD45.2 nucleated blood cells for four months (Figure 10D). The 80-fold excess of transplanted regenerating bone marrow resulted in half of the blood cells of the regenerating bone marrow origin in three out of seven transplanted mice after 3 months (Figure 10D). More granulocytes and macrophages (GM cells) were produced by transplanted regenerating bone marrow than B-cells (B220) and T-cells (CD4+CD8) (Figure 10D). The bone marrow of the three mice was pooled, examined for the frequency of CD45.2 cells, and transplanted to secondary recipient mice to confirm the capacity of regenerating bone marrow to support hematopoiesis in the long term further. The transplanted chimeric bone marrow contained ~ 60 % of CD45.2 cells (Figure 10E, hatched column). The production of CD45.2 (regenerating) blood cells remained steady after the second transplantation for four months and was skewed for myeloid (GM) cells (Figure 10E). Four mice with the chimeric bone marrow containing approximately 25 % of CD45.2 cells (see Figure 10F, the hatched column) were sacrificed four months after co-transplantation of regenerating (CD45.2), and normal (CD45.1) bone marrow cells (see Figure 10D) and their bone marrow were transplanted to secondary recipient mice. The production of CD45.2 blood cells was then followed for 8 months (Figure 10F). The percentage of CD45.2 cells in peripheral blood steadily declined, but CD45.2 cells were still present in the peripheral blood of the secondary transplanted mice after 8 months (Figure 10F). These experiments revealed the occurrence of transplantable cells with the long-term repopulating capacity constituting ~ 1 % of their occurrence in the normal bone marrow. A hundred times more regenerating cells were needed to establish a 50: 50 chimeric hematopoiesis in the co-transplantation experiments.
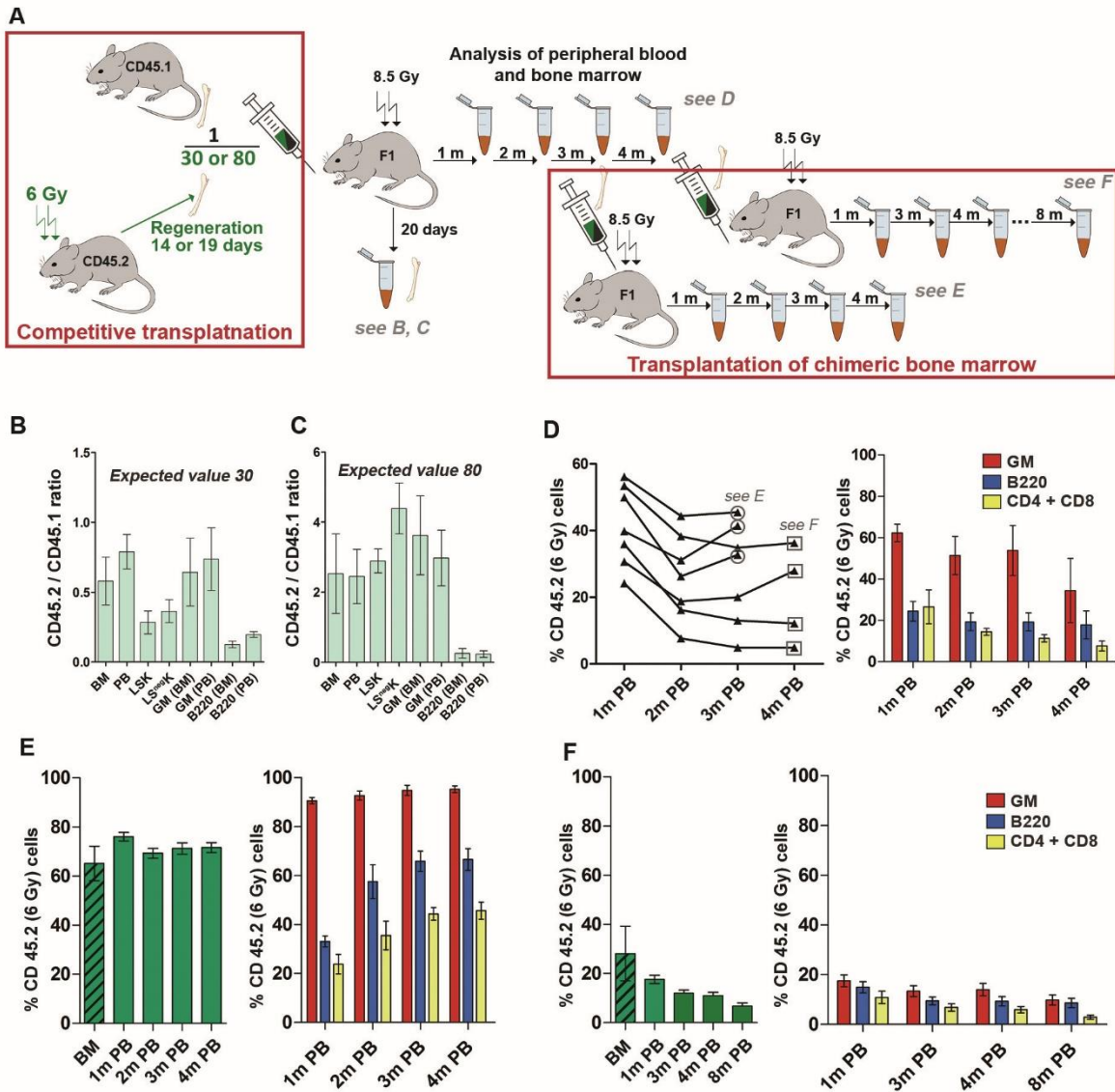
Figure 10. Developmental potential of LK cells in regenerating bone marrow after transplantation. A) Design of experiments studying the transplantability and developmental potential of regenerating bone marrow. **B), C)** Capacity of regenerating (CD45.2) and normal (CD45.1) bone marrow to restore blood cell production 20 days after transplantation (short-term hematopoiesis repopulation) was tested. 30 **(B)** or 80 **(C)** fold more CD45.2 bone marrow cells, collected 19 days in **B** and 14 days in **C** after irradiation, were mixed with CD45.1 normal bone marrow cells and co-transplanted to lethally irradiated dual CD45.1/CD45.2 F1 (F1) recipient mice. CD45.2 and CD45.1 cells were determined in various nucleated blood cells (PB) and bone marrow cells (BM; LSK, LS⁻K) 20 days after transplantation. The CD45.2/CD451 ratio in various types of cells is shown. **D)** Regenerating (CD45.2; collected 14 days after irradiation of mice at 6 Gy) and normal (CD45.1) bone marrow was mixed in an 80: 1 ratio and transplanted (17.5 x 10⁶ per recipient mouse) to lethally (8.5 Gy) irradiated F1 mice. The percentage of CD45.2 nucleated blood cells originating from regenerating bone marrow was determined after 1, 2, and 3 months in seven individual mice and four mice after 4 months. The percentage of CD45.2 Gr-1/Mac-1(GM), B220, and CD4⁺CD8 blood cells is shown in the column diagram. **E)** The CD45.2/CD45.1 chimeric bone marrow pooled from three mice sacrificed 3 months after transplantation (see D) was analyzed for the presence of CD45.2 cells (hatched green column) and re-transplanted to secondary lethally irradiated F1 mice. Peripheral blood of the secondary transplanted mice was examined after 1, 2, 3, and 4 months similar to the first transplantation (see the empty green columns for all nucleated CD45.2 cells and the red, blue, and yellow columns for the GM, B220, and CD4+CD8 cells). **F)** The CD45.2/CD45.1 chimeric bone marrow pooled from four mice sacrificed 4 months after transplantation of the mixture of CD45.2 and CD45.1 bone marrow cells (see D) was similarly treated as

that of the three mice sacrificed one month earlier (see E). Peripheral blood of the secondary transplanted mice was examined for the presence of CD45.2 cells for up to 8 months after transplantation.

Second, we compared the long-term reconstituting potential of LSK cells from regenerating and normal bone marrow. The regenerating bone marrow (CD45.2) was collected either 14 days or 23 days after irradiation. Normal bone marrow was from CD45.1 mice. To reduce pre-transplantation stress on LSK cells to be transplanted, we first determined the number of LSK cells in aliquots of CD45.2 and CD45.1 bone marrow samples kept at 4° C during the flow cytometry analysis of their aliquots. The regenerating and normal bone marrow cells were then mixed in the ratio wherein equal numbers of CD45.2 and CD45.1 LSK cells were present. The cell mixture was transplanted, and the percentage of CD45.2 and CD45.1 nucleated blood cells was determined in the peripheral blood of transplanted mice after 1 month and 6 months. Both these experiments revealed a meager capacity of the LSK cells from regenerating bone marrow to reconstitute and support hematopoiesis after transplantation (Table 2).

Experiment	PB examined after	F1 (recipients)	normal BM (CD 45.1)	regenerating BM (CD 45.2)	45.1/45.2 ratio
6 Gy 14D	1 month	38.63 ± 6.33	60.63 ± 6.34	0.74 ± 0.09	83
	6 months	33.76 ± 6.62	66.1 ± 6.60	0.15 ± 0.05	478
6 Gy 23D	1 month	21.28 ± 3.82	77.55 ± 3.77	1.16 ± 0.29	70
	6 months	12.34 ± 4.39	87.41 ± 4.34	0.25 ± 0.12	516

Table 2. Chimeric hematopoiesis resulting from co-transplantation of bone marrow cells from normal or irradiated mice containing equal number of LSK cells; two independent experiments (6 Gy 14D and 6 Gy 23D). Five dual CD45.1/CD45.2 F1 (F1) hybrid mice were irradiated (5 Gy) and transplanted with a mixture of bone marrow (BM) cells from normal (CD45.1) and (CD45.2) irradiated mice. Mice were irradiated for either 14 days (14D) or 23 (23D) before BM collection. The number of LSK cells was determined in pooled samples of normal and regenerating BM cells by flow cytometry, and the two BM samples were mixed in such a ratio that 10,000 CD45.1 and 10,000 CD45.2 LSK cells were administered to F1 recipient mice. The transplanted cells competed with each other and against the repopulating cells, which survived in submyeloablatively irradiated F1 recipient mice. The contribution of the three types of repopulating cells to blood cell production was examined in the peripheral blood (PB) of F1 transplant recipients after 1 and 6 months, and their proportion is shown as mean ± standard deviation. PB – peripheral blood; BM – bone marrow; D – day after irradiation of mice at 6 Gy

Thus, all these experiments demonstrated that expanding regenerating bone marrow contains a very low number (1 - 2 %) of cells that can reconstitute damaged hematopoiesis after transplantation compared to steady-state adult bone marrow.

7.6 The c-Kit receptor - stem cell factor interaction is essential for the expansion of myeloid progenitors in regenerating hematopoiesis

The low c-Kit expression level in LK cells in regenerating bone marrow was confusing since a high c-Kit expression level is a hallmark of hematopoietic stem and progenitor cells in the mouse (Okada et al., 1991; Osawa et al.,

1996). We hypothesized that c-Kit might have been downregulated on the surface of LK cells by SCF, as we confirm in our laboratory in vitro (Chen et al., 2016). Therefore, we determined the expression of the mRNAs for the membrane-bound and soluble forms of SCF. Both mRNAs were significantly upregulated after irradiation (Figure 11A). To resolve the discrepancy between the downregulation of c-Kit by SCF and the inherently decreased production of c-Kit in LK cells in regenerating bone marrow, we determined the mRNA for c-Kit in normal and regenerating bone marrow. The mRNA for c-Kit was low in regenerating bone marrow still 14 days after irradiation (Figure 11B), suggesting decreased production of c-Kit in LK cells.
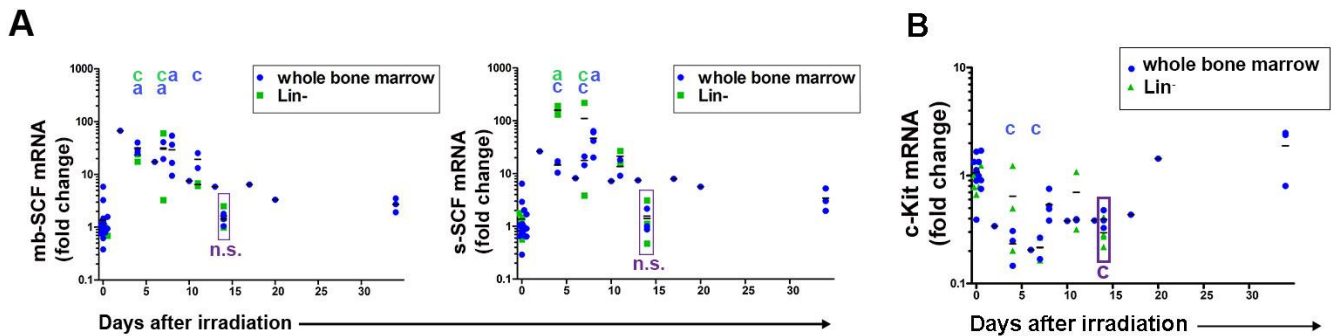

Figure 11. A) Stem cell factor (SCF) mRNA level was significantly increased in total bone marrow cells or magnetically separated Lin⁻ cells of irradiated mice**. mb - membrane-bound; s – soluble **B)** mRNA for c-Kit determined in all bone marrow cells and magnetically separated lineage negative fraction of bone marrow cells (Lin⁻). The statistical significance between results from the bone marrow of irradiated mice and untreated mice is indicated by letters a: p<0.05, c: p<0.005.

Therefore, we functionally tested the role of c-Kit receptor-mediated signaling in developing myeloid progenitors in regenerating hematopoiesis by blocking c-Kit through the administration of a c-Kit-blocking antibody (ACK2) to mice.

In a series of experiments, ACK2 was given to mice following their irradiation and untreated control mice. The mice were then examined 13 days after irradiation. Mice given ACK2 up to four days after irradiation did not survive, except for the one in a moribund condition (Table 3).

Table 3. ACK2 blocking antibody was given to mice following their irradiation and untreated mice. c-Kit blocking ACK2 antibody was lethal if given during the first 4 days after irradiation (●), except in one mouse. Normal mice (○) and mice given ACK2 6, 8, and 10 days after irradiation survived for 13 days. Mouse death is indicated with a Ø.

We then asked how much the reconstitution of damaged hematopoiesis still depended upon SCF/c-Kit signaling 10-13 days after irradiation, when the SCF mRNAs level started to decline (see Figure 11A) steeply, but the mRNA for c-Kit (Figure 11B) and c-Kit expression on LK cells (Table 1) was still low. All nine mice, eight males and one female, given ACK2 10 days after irradiation, survived until day 13 (Table 3). In these mice, ACK2 treatment significantly inhibited the recovery of LK cells (Figure 12D), and particularly their LS⁻K fraction (Figure 12A, B, D). LSK cells were also depressed after ACK2 administration (Figure 12D), but significantly less than LS⁻K cells, as shown by the LS⁻K/LSK cell ratio, which was approximately 50-fold decreased in the ACK2-treated mice (Figure 27D). LK cells surviving ACK2 treatment in irradiated mice were almost exclusively positive for CD16/32 expression (Figure 12A, B). The differentiated precursors of red blood cells (Ter119) and granulocytes (Gr-1) were significantly suppressed in ACK2 treated mice, in contrast to B-cells (B220), which were not affected (Figure 12E).

These results reveal that the SCF/c-Kit signaling is essential for the development and population expansion of erythro-myeloid progenitors in regenerating bone marrow despite the decreased expression of the c-Kit receptor in these cells.
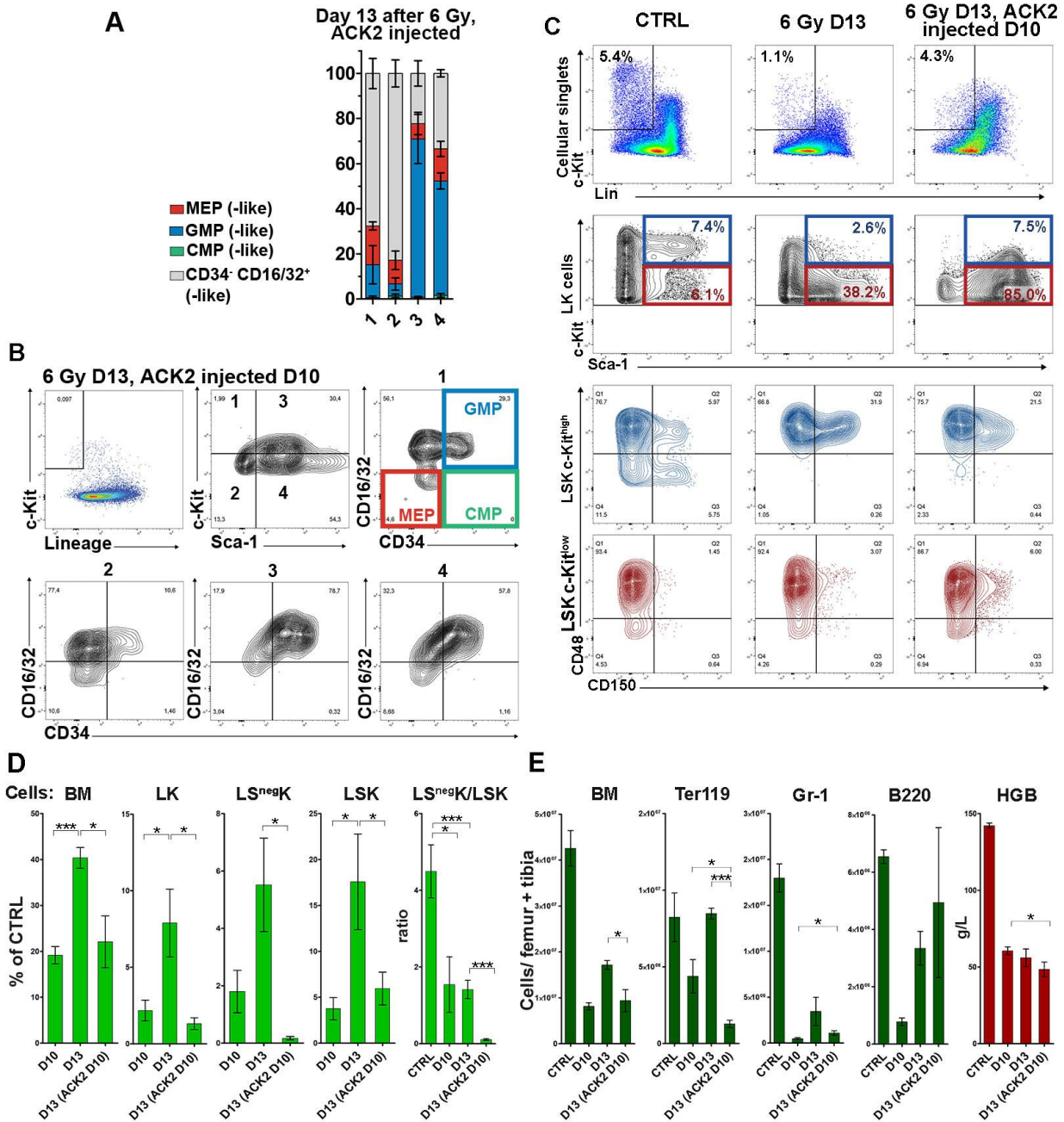
Figure 12. Abrogation of c-Kit receptor signaling reveals its role in bone marrow regeneration. A,D) The proportion of cells with the MEP-, GMP-, CMP-(like) and CD34⁻/CD16-32⁺ immunophenotypes in four subtypes of LK (see **B**) in mice given ACK2 10 days after irradiation and examined 13 days after irradiation. For the corresponding values in the bone marrow of untreated mice (CTRL) and those of only 6 Gy-irradiated mice, see Figure 6B. **B, C)** Representative immunophenotypes of Lin⁻c-Kit⁺ (LK) cells in the bone marrow of untreated (CTRL), irradiated (6 Gy D13) and irradiated and given ACK2 10 days after irradiation (6 Gy D13, ACK2 injected D10) mice examined 13 days after irradiation. **D)** Relative numbers of all bone marrow cells (BM) and LK, LS⁻K, LSK cells in six irradiated mice examined after 10 days or after13 days, and five irradiated mice given ACK2 three days before examination on day 13. All mice were males, and the values from normal eight mice served as the reference 100% values. **E)** The absolute number of total BM cells, Ter119⁺ cells, Gr-1⁺ cells and B220⁺ cells in these mice (**A**). Also, their hemoglobin concentration in the peripheral blood is shown. D10, D13 – days after irradiation with 6 Gy; HGB – hemoglobin concentration in blood. *P<0.05, **P<0.01, ***P<0.001

7.7 Transplanted bone marrow also regenerates by expanded committed myeloid progenitors partly masked by Sca-1 expression

We asked whether transplanted normal bone marrow regenerates similarly as spontaneously regenerating bone marrow, i.e., give rise to expanded populations of megakaryocyte-erythroid and granulocyte-macrophage progenitors. To answer this, we transplanted 1/100 (≈250.000 cells) of femoral bone marrow cells from untreated mice to lethally (myeloablative) irradiated syngeneic recipient mice. Fourteen days after transplantation, the Lin-c-Kit$^+$ (LK) cells in the bone marrow of recipient mice had features highly similar to the features of the LK cells in the spontaneously regenerating bone marrow of submyeloablatively irradiated mice (Figure 13A, B, compared to 13 A, 16 A). The fraction of LSK cells within LK cells was increased, and these cells were uniformly CD48 positive and similar in the c-Kit^{high} and c-Kit^{low} fractions (Figure 13A).
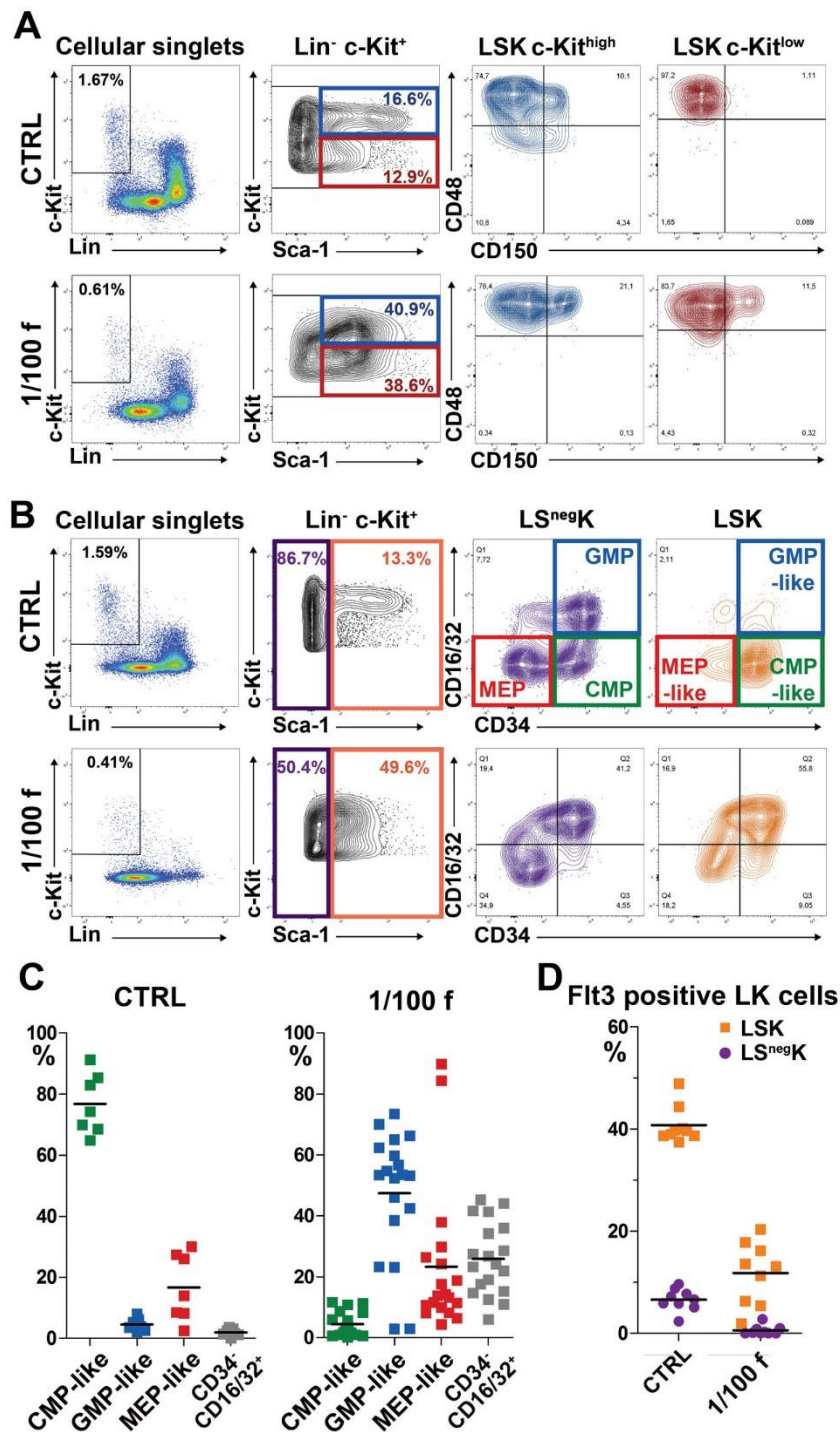
Figure 13. Immature hematopoietic cells in the bone marrow regenerating from transplanted cells have the phenotype of granulocyte-macrophage and megakaryocyte-erythroid myeloid progenitors also when they express Sca-1 antigen. A) Bone marrow pooled from 2 untreated mice (CTRL) and 3 mice irradiated with 9 Gy and transplanted with 1/100 of femoral bone marrow from a syngeneic donor (1/100 f) was analyzed 14 days after transplantation. Representative CD150/CD48 expression pattern in their c-Kit^high (blue) and c-Kit^low (red) portions of LSK cells. **B)** Representative CD34/CD16-32 expression patterns in LS⁻K (violet) and LSK (orange) cells. **C)** LSK cells were analyzed for the CD34/CD16-32 expression pattern (see b) in 7 untreated mice (from five independent experiments; CTRL) and 19 mice irradiated with 8 or 9 Gy and transplanted with 1/100 of syngeneic femoral bone marrow (1/100 f; examined after 14 days in four independent experiments). **D)** Flt3 positive cells in LSK and LS⁻K cells of 9 untreated male mice (from four independent experiments; CTRL) and 9 mice irradiated with 8 Gy and transplanted with 1/100 femoral bone marrow.

To further extend the characterization of LK cells present in bone marrow regenerating from transplanted cells, we compared the CD34/CD16-32 expression profile with the emphasis on LSK cells. Similarly, as in the spontaneously regenerating bone marrow (see Figure 6C), in bone marrow regenerating from transplanted cells, cells with the phenotype of CMPs (CMP-like) were suppressed, and those with the phenotype of GMPs and MEPs were significantly increased (Figure 13C).

Because in spontaneously regenerating bone marrow, the Flt3 (Flk2, marking the lymphoid-primed MPPs) expression became low (see Figure 8A), we explored whether the proportion of Flt3+ cells in LSK cells present in bone marrow regenerating from transplanted cells is also similarly decreased. This analysis demonstrated the significantly diminished proportion of Flt3+ within LSK cells in bone marrow regenerating from transplanted cells (Figure 13D) that corresponded to their low proportion in Sca-1− cells, similarly as in bone marrow regenerating spontaneously without transplantation.

In the regenerating bone marrow, LSK cells had the FSC and SSC characteristics corresponding to Sca-1 negative GMPs and MEPs in the bone marrow of untreated mice (Figure 7). The histograms in Figure 14 confirm that the situation is almost identical in bone marrow regenerating from transplanted cells.
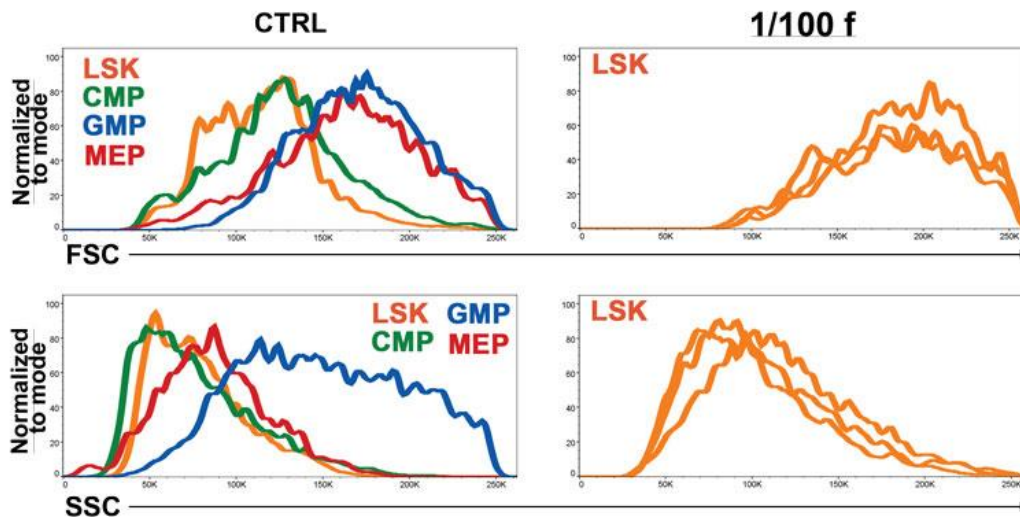
Figure 14. LSK cells in BM regenerating from transplanted cells have the FSC and SSC characteristics corresponding to those of Sca-1 negative GMPs and MEPs in the bone marrow of untreated mice. Forward scatter (FSC) and side scatter (SSC) histograms of LSK and CMP, GMP and MEP cells in the bone marrow of one untreated mouse (similar as in Figure 7) compared to the histograms of LSK cells of three mice irradiated with 8 Gy and transplanted with 1/100 of syngeneic femoral bone marrow examined after 14 days.

Thus, these data collectively confirm the similarity between hematopoiesis regenerating after submyeloablative irradiation and hematopoiesis regenerating after lethal irradiation from transplanted cells. In both, our analyzes revealed significantly expanded populations of MEPs and GMPs, partly presenting as LSK cells due to reexpression of Sca-1 antigen. On the other hand, LK cells with the immunophenotype of CMP, multipotent progenitors LSK CD150−CD48−, and HSCs are significantly suppressed.

7.8 The microenvironment of damaged bone marrow could activate reexpression of Sca-1 antigen in myeloid progenitors

Because LSK cells in regenerating hematopoiesis are similar to granulocyte-macrophage and erythroid progenitors, which are Sca-1 negative, we tested whether the microenvironment of damaged bone marrow would induce reexpression of Sca-1 in myeloid progenitor cells. We isolated LS⁻K cells from the bone marrow of UBC-GFP transgenic mice and transplanted them into congenic wild-type mice, either irradiated beforehand or untreated. Two days later, we analyzed GFP-positive cells in the bone marrow of recipient mice, focusing on their Sca-1 expression profile. In untreated recipient mice, transplanted LS⁻K cells remained predominantly Sca-1^{neg}. However, a significant proportion of LS⁻K cells transplanted into irradiated recipient mice expressed Sca-1 antigen and presented as LSK cells (Figure 15). Thus, these results directly demonstrate the activation of developmentally advanced myeloid progenitors by the stroma of damaged bone marrow.
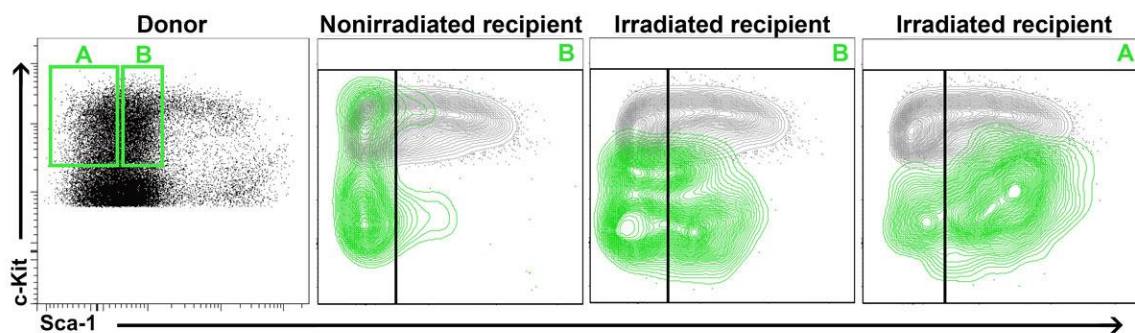

Figure 15. Activation of developmentally advanced Sca-1⁻ myeloid progenitors by the stroma of damaged bone marrow. Lin⁻Sca-1⁻c-Kit+ cells were sorted from the bone marrow of untreated UBC-GFP mice (GFP-LS⁻K cells) by BD FACS Aria IIu cell sorter into two fractions (A and B) according to the gating strategy shown here. 400 000-800 000 GFP-LS⁻K cells were injected intravenously into C57Bl/6 mice, either untreated or irradiated two days prior with 7 Gy. Bone marrow from the femurs and tibias of the recipients was collected 48 hours after transplantation. Lin-c-Kit+ (LK) cells were sorted from the bone marrow of untreated C57Bl/6 mice and were mixed with bone marrow cells isolated from the transplant recipients for analysis. Red blood cells were lysed, and nuclear cells were stained with antibodies against lineage markers, the Sca-1 antigen, and the c-Kit receptor. The Sca-1 and c-Kit expression profiles were determined in lineage-negative cells, and the expression profiles of GFP-positive cells (green) were compared to those of the added untreated LK cells (grey). Representative results from three experiments are shown.

4. Discussion

This thesis provides a deep insight into the intensively regenerating bone marrow during its recovery from severe damage or after transplantation. It compares it to the physiologically expanding embryonic/fetal liver and early postnatal hematopoiesis which concomitantly execute two competing tasks: multiply their immature hematopoietic cells and increase production of mature blood cells.

In the embryo, the transient primitive hematopoiesis starts in the yolk sac and produces mainly large nucleated erythroblasts with fetal hemoglobin. This very early phase of hematopoiesis is followed by another transient hematopoiesis driven by erythro-myeloid progenitor cells, which originate in the yolk sac and migrate the newly

established fetal liver. These two phases of embryonic hematopoiesis precede the appearance of transplantable HSCs (Frame et al., 2013; McGrath et al., 2015; reviewed in Palis, 2016 and Dzierzak and Bigas, 2018). HSCs are then generated separately in the embryo proper by differentiation from the hemogenic endothelium of large arteries in the aorta-gonad-mesonephros region and the vitelline arteries placenta in a stepwise process (Rybtsov et al., 2014, 2016). HSCs migrate into the fetal liver, where they initiate the production of all blood cells, including lymphocytes. The hierarchical structure of hematopoiesis with HSCs – MPPs and developmentally restricted progenitors is first established in the fetal liver. These HSCs are multipotent and have a high self-renewal and transplantation capacity (Copley et al., 2013). HSCs actively proliferate in the fetal liver and in bone marrow during the first three weeks of life in the mouse (Bowie et al., 2006). Afterward, many HSCs enter a dormant state but can still contribute to hematopoiesis (Sawai et al., 2016; Akinduro et al., 2018). The HSCs are induced to proliferate after bone marrow damage, infection, or sustained increased red blood cell production (Baldridge et al., 2010; Trumpp et al., 2010; Singh et al., 2018).

In this study, we targeted the brief period of bone marrow regeneration wherein the hematopoiesis, derived from a tiny number of founder cells, is challenged with similar tasks as in the embryonic hematopoiesis: to concurrently produce blood cells in large amounts, expand the pools of progenitors and to reconstruct the hierarchical structure of hematopoiesis. We focused on all immature cells lacking lineage markers and expressing the c-Kit receptor (LK cells). Thetargeted cell type of immature hematopoietic cells differs from the cells studied by Simonnet et al. (2009). These authors analyzed a subtype of LSK cells characterized by a high efflux of Hoechst 433342 dye ("side population" in flow cytometry; SP) in mice irradiated with either 3 or 6 Gy. They analyzed bone marrow in mice irradiated with 3 Gy after 2-14 days and after 10 weeks in mice irradiated with 3 or 6 Gy. These authors found decreased c-Kit expression and increased Sca-1 expression in SP-LSK cells during the first four days after the dose of 3 Gy and increased expression of CD150 marker up to 10 weeks-post irradiation. A significant deficit in Flt3 (CD135) cells after ten weeks and SP-LSK CD150$^+$ cells showed reduced repopulating potential after transplantation. Our study thus principally differs from the study of Simonnet et al. (2009) in targeting all lineage-negative and c-Kit expressing cells (LK cells) in their c-Kit^{low} and c-Kit^{high} fractions, and also by targeting the transient regenerative phase characterized by the intensive production of mature blood cells and massive concurrent expansion of progenitor cells.

While studying Sca-1 expression in LK cells, we noticed Sca-1 expression in CD71-positive LSK cells in regenerating bone marrow, while the LSK cells in normal bone marrow were uniformly CD71 negative. CD71 is highly expressed in erythroid cells stimulated by erythropoietin, and its expression in LSK cells in regenerating bone marrow might signal their activation towards the erythroid commitment. The functional in vivo tests did not support this hypothesis. Therefore, we hypothesized that the normal Sca-1 negative and CD71 expressing early erythroid progenitors re-expressed the Sca-1 antigen in regenerating bone marrow as part of their activation.

These findings prompted us to focus on LSK cells in regenerating bone marrow and their comparison with LS⁻K cells. Since we became suspected that LSK cells in regenerating bone marrow are, in fact, the myeloid progenitor cells which re-expressed Sca-1, we applied the CD34 and CD16/32 markers, traditionally used only for analysis of LS⁻K cells, also on the analysis of LSK cells. This analysis revealed several similarities between the LSK cells and LS⁻K cells in regenerating bone marrow. LSK cells in regenerating bone marrow had fewer Flt3 (CD135) positive cells: the lymphoid-primed multipotent progenitors with down-regulated megakaryocyte-erythroid potential (Buza-Vidas et al., 2011). LSK cells in regenerating bone marrow were mostly CD16/32 positive and expressed CD71 at a variable level. The expression of CD16/32 characterizes the granulocyte-macrophage progenitors. The CD71 expression is linked to the erythroid developmental lineage.

The flow cytometry analysis of regenerating bone marrow thus uncovered expanded populations of cells with phenotypic markers of the erythroid and myeloid (granulocyte-macrophage) progenitors masked by expression

of Sca-1 in part of them and by the expression of CD16/32 by a majority of the cells. The CD16/32 expression makes these myeloid progenitors similar to the EMPs in the embryo (Frame et al., 2013; McGrath et al., 2015; Palis, 2016). However, there are significant differences between the EMPs and the regenerating myeloid progenitors since EMPs are c-Kit[high], uniformly Sca-1 and CD150 negative, and all express CD41. At the same time, LK cells in regenerating hematopoiesis are c-Kit low, partly Sca-1 and CD150 positive, and express CD41 only in a small fraction of LSK cells.

The gene expression analysis performed in connection with this thesis, revealed a strongly activated erythroid program in LS⁻K and LSK cells in regenerating bone marrow(Faltusová et al., 2020a; the gene expression analyzis was performed by Chia-Ling Chen). Correspondingly, the number of erythrocytes reached its nadir on day 12 after irradiation and then began to increase (Figure 3B). The blockade of the c-Kit receptor suppressed erythropoiesis in regenerating bone marrow significantly (Figure 12E). All these findings demonstrate significant erythroid activity in immature LK cells in intensively regenerating hematopoiesis. This intense erythroid activity was not fully reflected in in vitro clonogenic cultures of cells from regenerating bone marrow. We hypothesize that the culture conditions in GF M3434 medium did not fully substitute for the support and stimulation provided to erythro-myeloid progenitors by the microenvironment in regenerating bone marrow. The culture lacks the membrane-bound SCF and macrophages known to participate in the erythroblast differentiation and maturation. Thus, the conditions for the growth of erythroid cells are often in cultures suboptimal (Monette and Holden, 1982).

Analysis of the gene expression (Faltusová et al., 2020; not included in this thesis) in LSK and LS⁻K cells separated from normal and regenerating bone marrow further supported the erythro-myeloid character of LK cells but revealed differences between their LSK and LS⁻K subtypes. While LSK cells were both erythroid and granulocytic (myeloid) according to the enhanced gene expression linked to both these developmental lineages, the LS⁻K cells had only the erythroid program enhanced (Faltusová et al., 2020). The suppression of erythropoietin stimulation by posttransfusion polycythemia did not inhibit the erythroid program in LK cells in regenerating hematopoiesis, nor did it suppress the enhanced expression of CD71. Peslak et al. (2012) showed that only Day-3 BFU-E and CFU-E erythroid progenitors were responsive to erythropoietin stimulation in regenerating bone marrow. Therefore, most LK cells in regenerating bone marrow have a less advanced erythroid status than Day-3 BFU-E. Contrary to the apparent erythropoietin independence, the expansion of LK cells in regenerating bone marrow, particularly of their LS⁻K fraction, required stimulation by SCF mediated by the c-Kit receptor.

The LK cells in regenerating bone marrow differed markedly from the LK cells in the fetal liver and early postnatal bone marrow phenotypically and in their capacity to be transplanted. The expansion power of the fetal liver hematopoiesis can be transferred to adult mice with damaged hematopoiesis by transplantation and is markedly superior to adult bone marrow (Pawliuk et al., 1996; Harrison et al., 1997; Copley et al., 2013). Therefore, we explored the capacity of regenerating bone marrow to be similarly transplanted. This ultimate functional tests for HSCs revealed the most striking difference between the fetal liver hematopoietic cells and the regenerating bone marrow. The transplantation potential of regenerating bone marrow cells was severely reduced and deficient in producing of the lymphoid cells. In contrast, Harrison et al. (1997) demonstrated that 14-day fetal liver contains several times more long-term repopulating cells (corresponding to stem cells) relative to short-term repopulating cells (corresponding to progenitors) than adult marrow.

Because the population expansion of HSCs in the foetal liver is mainly independent of SCF induced signaling mediated by c-Kit receptor (Ikuta and Weissman, 1992; Thorén et al., 2008), we abrogated the c-Kit receptor signaling with the ACK2 antibody in irradiated mice to simulate the condition under which the fetal hematopoiesis rapidly expands. ACK2 administration to irradiated mice fully inhibited the expansion of Lin-c-Kit⁺ cells and suppressed differentiated bone marrow cells excempt for B-cells. The expansion of MEPs appeared to

be fully, and that of GMPs partly, dependent on uninterrupted SCF/c-Kit signalling. This difference in the SCF dependence further highlights the significant differences between the physiological expansion of hematopoiesis and its expansion during regeneration.

In the present study, we describe significantly expanded populations of altered erythro-myeloid progenitor cells in the Sca-1 positive and Sca-1 negative LK cells in regenerating bone marrow. The most significant feature of these cells, expressing c-Kit at a low level, is their highly restricted capacity for reconstitution of damaged hematopoiesis after transplantation which contrasts with their massive performance in the production of blood cells in situ and their concurrent population expansion. Randall and Weissman (1998) described cells present in murine bone marrow phenotypically similar to HSCs (Sca-1^{high}) lacking the c-Kit expression and the ability to reconstitute hematopoiesis in transplanted mice. Other reports found HSCs in c-Kit^{low} immature cells (Doi et al., 1997; Lian et al., 1999; Yang et al., 2002; Thorén et al., 2008; Grinenko et al., 2014). However, these cells were identified as having a high capacity to reconstitute damaged hematopoiesis after their transplantation which contrasts with the meager capacity to transplant the erythro-myeloid progenitors from regenerating bone marrow we describe here. Our study could not identify the very few cells which were at the beginning of the bone marrow regeneration because of the significant initial damage to bone marrow.

However, and bearing the limitations of our study in mind, we provide compelling evidence that hematopoiesis in regenerating bone marrow contains expanded populations of activated erythro-myeloid progenitors which markedly outweigh the severely reduced populations of the short-term and long-term repopulating cells corresponding to MPPs and HSCs. We also find some previously unknown similarities between regenerating adult bone marrow and definitive embryonic hematopoiesis before the emergence of HSCs.

These novel experimental results shed light on how bone marrow regenerates after submyeloablative damage or after transplantation. They prompted us to develop a mechanistic model, inspired by Waddington´s landscape model of cell differentiation, describing the activation of developmentally very late progenitor cells governed by supporting stroma tissue (Figure 16). After that, we verified the model experimentally by analyzing of Sca-1-negative progenitor cells transplanted into either unconditioned or irradiated mice.

Using a two-dimensional model of the stroma and the hematopoietic stem and progenitor cells interactions, we present the significant differences between the steady-state hematopoiesis and the regenerating hematopoiesis in Figure 16. In regenerating bone marrow, the late myeloid progenitors (GMPs and MEPs) are significantly expanded, while the populations of HSCs, MPPs, and CMPs are diminished. A part of GMPs and MEPs reacquire Sca-1 expression. The Sca-1 expression in GMPs and MEPs may reflect their increased self-renewal capacity exceeding their differentiation and leading to their rapid population expansion. The model assumes that the transient suppression of the differentiation is initially induced in GMPs and MEPs by the external cues provided by the microenvironment of damaged bone marrow. Hypothetically, the highly upregulated gene for SCF in damaged bone marrow (Figure 11A) could provide one of the external cues inducing the early expansion of GMPs and MEPs.
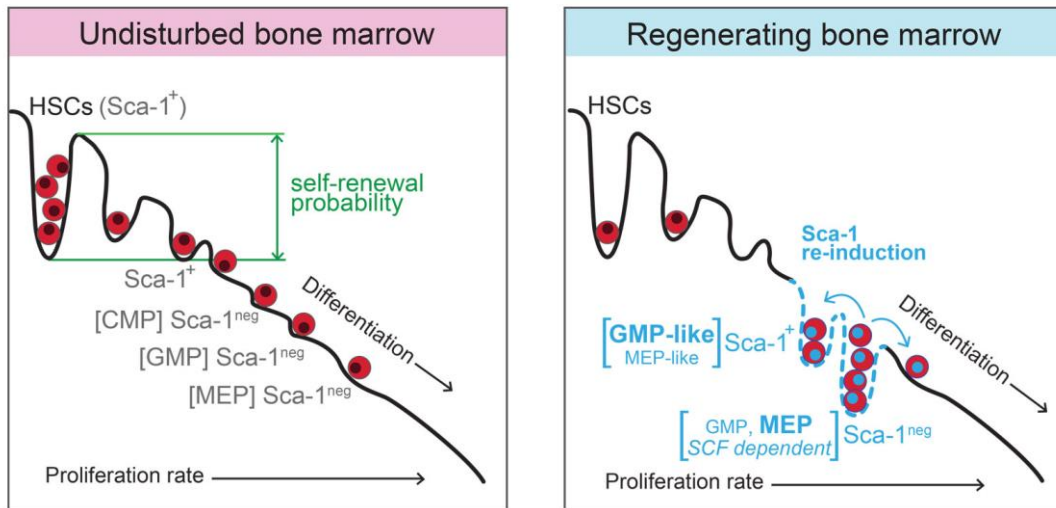
Figure 16. The model of bone marrow regeneration with a robust response of the committed myeloid progenitors. Sca-1 negative cells have most features of megakaryocyte-macrophage progenitors (MEPs) and are highly dependent on stimulation by SCF. Sca-1$^+$ cells are activated granulocyte-macrophage progenitors (GMPs) and MEPs which re-expressed Sca-1. MEPs and GMPs proliferate intensively. The model assumes that external cues emanating from injured bone marrow, e.g., SCF, transiently inhibit the differentiation of these cells, which leads to their early and rapid population expansion. After the initial expansion, a portion of the cells resume differentiation and become the early source of myeloid blood cells.

In conclusion, our research unveils the unexpected extensive regenerative potential of the cells commonly attributed only to amplification function. Conceptually, it draws attention to the latent potential of developmentally advanced and committed progenitor cells to carry out the initial phase of tissue regeneration. Intensive proliferation and the proximity to mature blood cells predisposes these cells for a rapid and vigorous response to tissue damage. The conceptual advance demonstrates that bone marrow regeneration is a biologically markedly distinct process from the expansion of hematopoietic tissues occurring during the fetal and early postnatal development. Our results demonstrate, for the first time, that the function of stem cells and multipotent progenitors in blood cell production can be temporarily highly efficiently substituted by developmentally very distant progenitor cells.

5. Conclusions

The present study provides experimental evidence that stem cells are not the exclusive carriers of regeneration of damaged hematopoiesis. Expanded populations of developmentally advanced erythroid and myeloid progenitors with altered immunophenotype significantly participate in the recovery of blood cell production and early hematopoietic tissue reconstitution. These progenitors activate the erythroid developmental program independently from erythropoietin production but require effective stimulation by stem cell factor (SCF) for their expansion.

It was confirmed that a short period in the regeneration of damaged hematopoiesis mimics the early embryonic definitive stage of production of blood cells before the population of hematopoietic stem cells is established.

The data presnted in this study provide a novel insight into tissue regeneration by suggesting that cells other than stem cells and multipotent progenitors can be of fundamental importance for the rapid recovery of tissue function.

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7. List of publications

Publications with a direct relation to the thesis:

Faltusová, K., Chen, C.L., Heizer, T., Báječný, M., Szikszai, K., Páral,P., Savvulidi, F., Renešová,N., and Necas, E. (2020). Altered Erythro-Myeloid Progenitor Cells Are Highly Expanded in Intensively Regenerating Hematopoiesis. *Frontiers in Cell and Developmental Biology* 8:98. doi: 10.3389/fcell.2020.00098. IF=5.186 (2019).

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Other publications related to the research field:

Faltusová, K., Báječný, M., Heizer, T., Páral, P., and Nečas, E. (2020). T-lymphopoiesis is Severely Compromised in Ubiquitin-Green Fluorescent Protein Transgenic Mice. *Folia Biol. (Praha)* 66:47–59. IF=0.691 (2019)

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