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TRANSPLANTACE KOSTNÍ DŘENĚ PŘÍJEMCŮM S REGENERUJÍCÍ KRVETVORBOU: ÚČINNOST TRANSPLANTACE A STAV REGENERUJÍCÍ KOSTNÍ DŘENĚ

BONE MARROW TRANSPLANTATION TO HOSTS WITH REGENERATING HEMATOPOIESIS: EFFICIENCY OF TRANSPLANTATION AND CONDITION OF REGENERATING BONE MARROW

Disertační práce

Ph.D. Thesis

Školitel/Supervisor: Prof. MUDr. Emanuel Nečas, DrSc

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Abstract

Hematopoietic stem cells (HSCs) have the ability of both self-renewal and differentiation. After bone marrow damage, surviving host HSCs or transplanted donor HSCs are able to restore hematopoiesis and maintain it for a long time due to the self-renewal potential. HSCs reside in a specific microenvironment in the bone marrow, in stem cell niche, which supports their survival and controls their functioning.

In this study, we investigated the impact of bone marrow damage induced by increasing doses of irradiation on engraftment efficiency of transplanted donor repopulating cells. Using the CD45.1/CD45.2 congenic mouse model, we developed a new approach enabling estimation of surviving HSCs in damaged hematopoietic tissue. Its principle is in measuring of the donor chimerism resulting from transplantation of a defined dose of normal congenic bone marrow cells. The transplanted donor cells contain repopulating cells, progenitors (STRCs) and HSCs (LTRCs) that give rise to blood cell production which proceeds in parallel with that present in the host hematopoietic tissue. We applied this approach to monitor spontaneous regeneration of repopulating cells, including HSCs, in mice irradiated with a sublethal dose of 6 Gy or by a lethal dose of 9 Gy and rescued by syngenic bone marrow cells. This was accompanied by functional assays testing the transplantability of regenerating bone marrow cells, recovery of productive hematopoiesis, and analysis of the Lin^{low}c-$kit^{+}$$Sca\text{-}1^{+}$ (LSK) population of the bone marrow which is highly enriched in progenitors and HSCs. LSK cells were further analyzed according to CD150 and CD48 markers.

Our results demonstrate that the damage caused by sublethal irradiation does not interfere with the engraftment of intravenously administered progenitors and HSCs; what is more, they are engrafted with very high efficiency. We experimentally demonstrated existence of different types of niches for progenitors and HSCs in the bone marrow. While niches for HSCs were available for transplanted repopulating cells for a relatively long time after both sublethal and lethal irradiation, niches for transplanted progenitors “closed” more rapidly. Although the cellularity of regenerating bone marrow normalized in approximately 20 days after irradiation, and it produced high numbers of blood cells, its repopulating capability was very low still after 30 days. Subfractions of the LSK population classified according to expression of CD150 and CD48 markers were significantly altered during the entire 30 day regeneration period following sublethal irradiation.

Abstrakt (Czech)

Kmenové buňky krvetvorné tkáně (dále kmenové buňky) mají schopnost sebeobnovy i schopnost diferenciace. Kmenové buňky, které přežijí poškození krvetvorné tkáně, nebo které jsou transplantovány jedinci s poškozenou nebo zcela zničenou krvetvornou tkání, mohou krvetvorbu obnovit a udržovat ji po dlouhou dobu.

V této práci jsme studovali důsledek poškození krvetvorné tkáně různými dávkami ionizujícího záření na účinnost přihojení intravenózně transplantovaných krvetvorbu obnovujících dárcových buněk, buněk progenitorových (STRCs) a buněk kmenových (LTRCs). Použili jsme kongenní linie pokusných myší, lišící se antigeny CD45.1 a CD45.2 přítomnými na krevních buňkách, k vypracování citlivé metody, která umožňuje určit množství kmenových buněk v poškozené krvetvorné tkáni. Spočívá ve stanovení úrovně chimerismu v krvi a v kostní dřeni, který je výsledkem transplantace známého množství kostní dřeně od kongenního dárce pokusným myším s poškozenou krvetvornou tkání. Metodu jsme použili ke sledování regenerace populací buněk obnovujících poškozenou krvetvorbu, včetně buněk kmenových, po subletálním ozáření myší dávkou 6 Gy nebo po jejich ozáření dávkou letální (9 Gy) spojenou s transplantací syngenní kostní dřeně. Současně jsme, v případě subletálního ozáření, vyšetřili schopnost regenerující kostní dřeně obnovit krvetvorbu po její transplantaci, obnovení tvorby krevních buněk a v kostní dřeni jsme analyzovali populaci buněk LSK ($Lin^{low}c\text{-}kit^{+}Sca\text{-}1^{+}$), složenou z progenitorových a kmenových buněk, z hlediska zastoupení buněk lišících se přítomností antigenních znaků CD150 a CD48.

Výsledky ukazují, že poškození krvetvorné tkáně ionizujícím zářením nesnižuje významně přihojení intravenózně transplantovaných progenitorových a kmenových buněk, které jsou z velké části využity pro obnovu poškozené krvetvorby. Experimentálně prokazují existenci odlišných nik pro buňky kmenové (LTRCs) a buňky progenitorové (STRCs). Niky kmenových buněk přijímají (přihojují) transplantované kmenové buňky po dlouhou dobu po poškození krvetvorné tkáně, zatímco niky pro buňky progenitorové se progresivně uzavírají dříve. Kostní dřeň poškozená subletální dávkou záření 6 Gy dosáhla normálního počtu buněk za přibližně 20 dnů a zcela obnovila tvorbu myeloidních krevních buněk. Její transplantovatelnost však byla velmi nízká ještě i za 30 dnů. Složení populace buněk LSK, z hlediska zastoupení buněk s různou expresí antigenních znaků CD150 a CD48, bylo významně změněné během celého 30 denního období regenerace.

Content

LIST OF ABBREVIATONS

7-AAD	7-aminoactinomycin D
ANOVA	analysis of variance
APC	allophycocyanin
APC/Cy7	allophycocyanin cyanin 7
APC/H7	allophycocyanin-Hilite® 7-BD
Bas	basophil
B-cells	B lymphocytes; B220 positive cells
BFU-E	burst-forming unit-erythroid
BM	bone marrow
BMCs	bone marrow cells
BrdU	5-bromo-2´-deoxyuridine
BV	brilliant violet
CAR cells	CXCL12 abundant reticular cells
CFC-Meg	colony forming cells - megakaryocyte
CFU-E	colony-forming unit - erythrocyte
CFU-S	colony forming unit - spleen
c-kit	CD117, stem cell factor receptor
CLP	common lymphoid progenitor
CTRL	control
CXCR4	receptor for stromal-derived factor-1 (CXCL12)
CY	cyclophosphamide
ECM	extracellular matrix
EDTA	ethylendiaminetetraacetic acid
Eos	eosinophil

f	femur
FACS	fluorescence-activated cell sorting
FITC	fluoresceine isothiocyanate
G	gauge; unit of needle thickness
G-CFC	granulocyte colony-forming cell
Gy	Gray; unit of ionizing radiation
GM	granulo-macrophage cells
GM-CFC	granulocyte-macrophage colony-forming cell
HA	hyaluronic acid
Hb	hemoglobin
HPCs	hematopoietic progenitor cells
HSCs	hematopoietic stem cells
LFA-1	lymphocyte function-associated antigen 1
Lin	lineage
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$
LTRCs	long-term repopulating cells
Ly5.1	CD45.1; Leukocyte common antigen 1
Ly5.2	CD45.2; Leukocyte common antigen 2
Lym	lymphocyte
M-CFC	macrophage colony-forming cell
Mon	monocyte
MPP	multipotent progenitor cells
Neu	neutrophil
NK–cells	natural killer cells
p	probability

P	P-value
PB	peripheral blood
PBS-BSA	phosphate buffer saline supplemented with 0.5% bovine serum albumin
PE	phycoerytrin
PerCP	peridinin chlorophyll protein complex
PLT	platelets
RBC	red blood cells
RIC	reduced intensity conditioning
ROS	reactive oxygen species
SCF	stem cell factor
SDF-1	stromal-derived factor-1, CXCL12 chemokine
SEM	standard error of the mean
SLAM	signaling lymphocyte activation molecule; surface molecules CD150 and CD48
SP	side population; Hoechst 33342 negative cells
STRCs	short-term repopulating cells
TBI	total body irradiation
T-cells	T lymphocytes; CD3 positive cells
VLA-4	very late antigen 4
WBC	white blood cells

DEFINITIONS

Asymmetric cell division – cell division, which results in two daughter cells with different fates

Bone marrow transplantation – intravenous application of bone marrow cells in order to reestablish hematopoiesis after its damage or to induce chimeric hematopoiesis

Chimerism – coexistence of hematopoiesis of both donor- and recipient-type, typically after pre-transplantation conditioning with sublethal doses of radiation or cytostatics

Congenic mice – mice that are genetically identical except for a small genetic region, typically a single genetic locus (i.e. a single gene)

Differentiation – development of cells connected with obtaining of specific functions

Engraftment – outcome of successful bone marrow transplantation, when transplanted bone marrow cells found their niches, repopulate them and subsequently produce blood cells

Homing – coordinated, multistep process by which the transplanted hematopoietic cells are directed into the bone marrow niches

Lethal (myeloablative) irradiation – total destruction of the hematopoiesis which without rescue transplantation has fatal outcome for the organism

Long-Term Repopulating Cells – stem cells, which have the ability to restore damaged hematopoiesis and maintain it for a long time

Niche availability – amount of "empty" niches for transplanted repopulating cells in damaged hematopoiesis

Proliferation – cell divisions generating new cells which may increase cell numbers but not necessarily if proliferation is balanced by cell losses

Self-renewal – ability of cell to divide and generate at least one daughter cell with identical characteristics of the parent cell

Short-Term Repopulating Cells – progenitor cells, which maintain hematopoiesis only transiently for a few weeks and maximally to three months

Stem cell niche – specific microenvironment within the bone marrow, where hematopoietic stem cells reside and their fates are controlled

Sublethal (submyeloablative) irradiation – not complete destruction of the hematopoietic tissue with possibility of its spontaneous regeneration

Symmetric cell division – both of the daughter cells are identical

Syngenic mice – mice that are genetically identical, particularly with respect to antigens or immunological reactions

1 INTRODUCTION

1.1 Hematopoietic stem cells

Hematopoietic stem cells (HSCs) are by far the best characterized adult stem cells. A very specific functional feature of HSCs is their ability to reproduce themselves (self-renewal) and to recover their number in case it was reduced. Due to this unique property, a single HSC can reestablish and maintain the whole hematopoiesis for a long time [1-3]. Therefore they are called the **Long-Term Repopulating Cells (LTRCs)** and pluripotent stem cells.

Figure 1 provides an overview of the hierarchical organization of murine hematopoietic tissue with the central role of HSCs and indicates the numbers of blood cells produced daily. The hematopoietic tissue of adult mice must replace approximately $2.4x10^8$ red blood cells and $4x10^6$ non-lymphoid nucleated blood cells each day and compensate for hematological stresses such as blood loss, infection, and exposure of cytotoxic chemicals or to irradiation. HSCs are source of both the myeloid and the lymphoid cells.

HSCs are only a very tiny fraction of all bone marrow cells (BMCs). The specific phenotype of HSCs and progenitor cells is characterized by antigenic cell surface markers which are detectable by flow cytometry. Murine HSCs lack the lineage markers (Lin) of blood precursor cells (B220, CD4, CD8, Gr-1, Mac1-, Ter-119) and are positive for Sca-1 (an adhesion molecule) and c-kit (receptor for Stem Cell Factor) surface antigens. Although cells with the $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) phenotype greatly contribute to hematopoietic reconstituting activity, this bone marrow is only enriched for HSCs (approximately 1 out of 10 of LSK cells has repopulation capacity) [4]. For further subdivision of this population several additional markers are used. Pluripotent

(multilineage) reconstituting HSCs are considered as LSK Flt3^{-} [5], LSK CD150^{+}CD48^{-} [6], LSK Thy1.1^{lo}Flk-2^{-} [7] or as the Hoechst-effluxing side population (SP) [8] cells of the murine bone marrow.

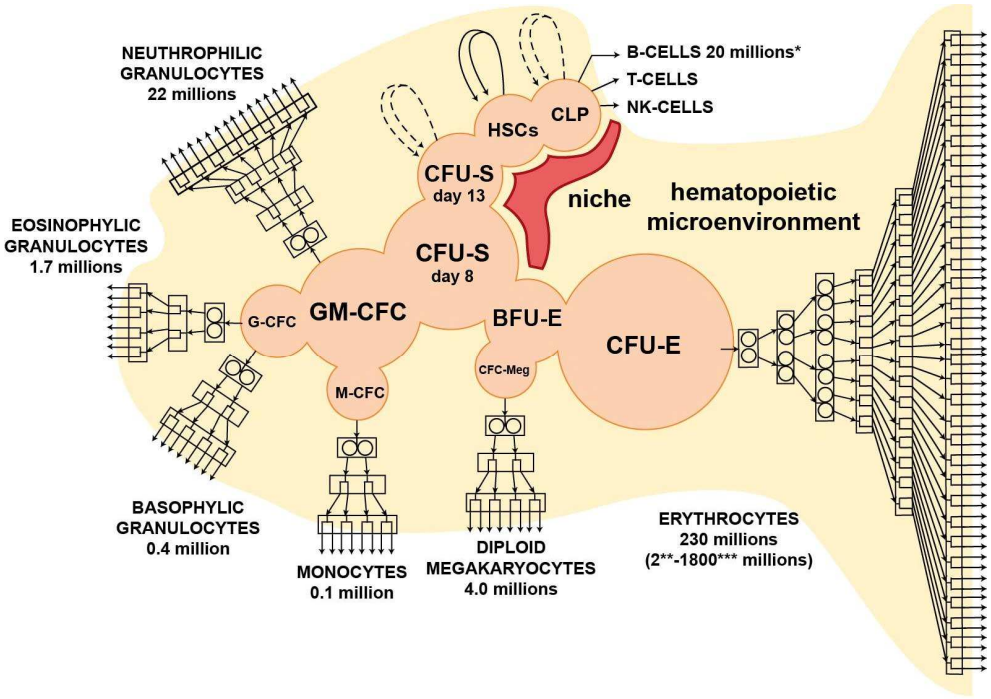

Figure 1 – Cellular hierarchy of murine hematopoiesis and the rates of blood cell production. HSCs – hematopoietic stem cells, CLP – common lymphoid progenitor, CFU-S, BFU-E, CFU-E, CFC-Meg, GM-CFC, G-CFC, M-CFC progenitors with variable capacity for repeated cell divisions and limited or absent capacity for self-renewal. B-cells, T-cells and NK-cells represent the lymphopoiesis. Dendritic cell and mast cell lineages are not represented. Precursors of blood cells are depicted as binomically multiplicating and maturing cells. The estimates of the daily production of mature blood cells and megakaryocytes in the mouse are from the paper by Novak and Necas [9]. The large range on the possible production of erythrocytes corresponds to conditions of suppressed or enhanced erythropoietin stimulation. Only HSCs can replace themselves by renewing asymmetric or symmetric cell divisions next to their ability to differentiate ("commit themselves") into more intensively proliferating progenitors which are the ultimate source of blood precursor cells belonging to different developmental lineages of blood cells. Proliferation of progenitors leads to a loss of the ability to self-renew and makes them thus dependent on supply of new cells from the compartment of HSCs [10].

1.1.1 Symmetric and asymmetric cell division of stem cells

As mentioned above, HSCs are defined as pluripotent cells with immense self-renewal capacity. Despite this high proliferative potential, they rarely divide under physiological conditions. Several studies [8,11,12] have shown that most of the HSCs are in the G_0 state. They may divide only every 145 days or five times within the lifespan of the mouse [13]. Therefore, this rarity of divisions and very low total

numbers makes them technically difficult to image. Although the cell cycle time of HSCs is relatively slow under steady-state conditions, it is highly responsive to changes in the hematopoietic environment [13].

The mechanisms underlying the proliferation and differentiation of HSCs are incompletely understood. The proliferation of HSCs apparently takes place “on demand” in vivo, when the body needs more blood cells and, particularly, when the number of HSCs has been reduced after a damage to the hematopoietic tissue.

Ability of stem cells to produce populations of differentiated blood cells and concurrently reproduce themselves to avoid their exhaustion requires their **asymmetric divisions**. Therefore, asymmetric division is a mechanism by which cells can give rise to daughter cells with different fates, in other words one daughter cell retains its “stemness” while the other becomes a differentiated progenitor cell [14-16]. Both extrinsic (growth factors, HSC-niche interactions) and intrinsic factors (transcription factors) are involved in the regulation of asymmetric division. Unequal distribution of a parental factor (e.g. transcription factors) leads to generation of two different daughter cells at the time of division. On the other hand, similar daughter cells at the time of division could become different due to subsequent different signals from the microenvironment (Figure 2) [16,17].

Asymmetric division is a property of polarized cells. Polarity can be ensured by their location and interaction with surrounding cells, or by unequal partitioning of cell fate determinant as a result of orientation of the mitotic spindle, which ensures the unequal distribution of cellular constituents between the daughter cells. Hematopoietic stem and progenitor cells are detected as round, non-polarized cells. The polarization probably takes place after the redistribution of cell surface molecules [18].

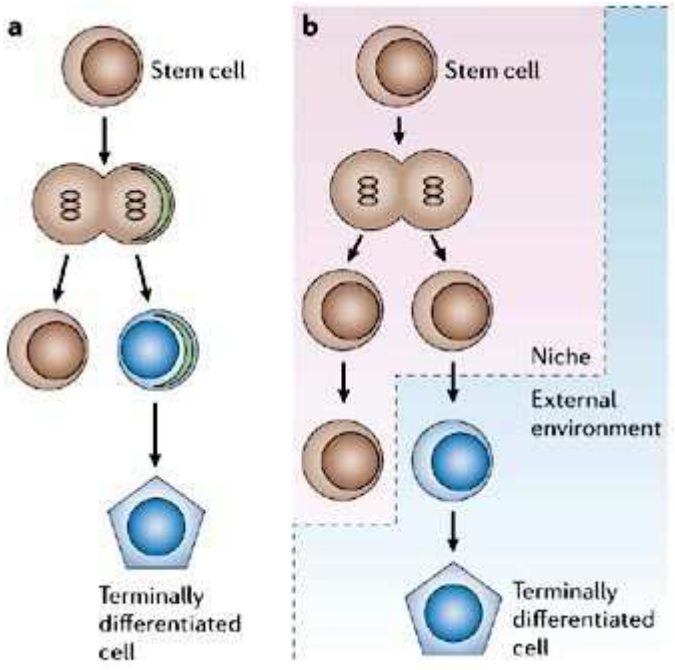

Figure 2 – Asymmetric division of stem cells intrinsically (a) or extrinsically (b) [17].

Stem cells may also undergo **symmetric divisions**. During symmetric division, a stem cell divides to become two stem cells (symmetric renewal) or two committed cells (symmetric commitment) [15,16].

Alterations in the balance between asymmetric and symmetric division can result in increased renewal which in extreme case can erupt in cancer growth or, otherwise, in decreased renewal which can lead to exhaustion of HSCs pool. Hence, switching between the two types of the self-renewing divisions of HSCs must be tightly controlled. Mechanistically, the control is assumed to be provided by stem cell niches which availability, the "niche space", determines the likelihood with which the HSC maintains its specific stem cell phenotype, covering the theoretically unlimited self-renewal potential as relate to the life time of the whole organism, or converts into a progenitor cell which though capable to generate millions of blood cells, can do this only for a limited time that is much shorter compared to the life time of whole organism.

1.2 Hematopoietic progenitor cells and multipotent progenitors

Beside hematopoietic stem cells, there are also other primitive subpopulations in the bone marrow, namely the hematopoietic progenitor cells (HPCs) and multipotent progenitor cells (MPPs). Similarly to HSCs, these cells are also able to differentiate into a spectrum of mature blood cells, but differ in their self-renewal and proliferative capacity. As HSCs differentiate into HPCs and then MPPs, the self-renewal capability progressively declines. Therefore, cells derived from transplanted HPCs and MPPs are detectable only for about ten weeks post-transplant [7].

HPCs and MPPs differ from HSCs also in their cell cycle status. In contrast with HSCs, both HPCs and MPPs are actively proliferating cells.

Hematopoietic progenitor cells

Hematopoietic progenitors are actively proliferating cells [19,20] with a large potential for cell production [21]. They provide a large functional reserve, enabling a relatively rapid increase in the production of a specific type of blood cells [22]. In contrast to HSCs, they have a limited potential for self-renewal; after a damage to the bone marrow they restore hematopoiesis only transiently. Therefore they are also called **Short-Term Repopulating Cells (STRCs)**. The probability of self-renewal compared to their losses due to differentiation/apoptosis is biased towards the latter. They are thus destined to clonal extinction after various periods of functioning and the production of blood cells [23,24]. They have a larger volume cell than stem cells [25]. Using flow cytometry analysis, they can be distinguished from HSCs by the expression of CD34, Flt3 and Thy1.1 [5,7] and the SLAM pattern of $CD150^-$, $CD48^+$ [6]. They encompass a large spectrum of cells with a highly variable predestination for further differentiation

and a limited self-renewal and proliferation potential. Their differences from HSCs and among themselves are determined by a specific gene expression profile projected into the presence, amount and mutual ratio of specific transcription factors [26-29].

Multipotent progenitors

Multipotent progenitors lack the ability of self-renewal. They have multilineage potential but in comparison with HPCs shorter reconstitution capability. They can be distinguished from HSCs and HPCs by flow cytometry by the divergent expression of characteristic surface markers. MPPs are characterized as LSK Thy1.1$^-$Flk-2$^+$ [7] or LSK CD150$^-$CD48$^-$ [6] population of the bone marrow.

1.3 Hematopoietic stem cell niche

Although during homeostasis a small number of HSCs is present in the circulation [30], the majority of HSCs reside in specific microenvironment in the bone marrow called “stem cell niche” (see Figure 3). The hematopoietic stem cell niche is a specialized stroma supporting and controlling HSCs. Together they form a principal functional unit of hematopoietic tissue. HSCs can be physically separated from their niches, handled in vitro, and transplanted to conditioned recipients either directly into the hematopoietic tissue or via the circulation. They engraft with a very high efficiency [2,31] which assumes guidance and strong attraction of transplanted HSCs and specific recognition of the niche structures.

1.3.1 Structural components of niche

Structurally, hematopoietic stem cell - niche unit can be divided into two main parts: hematopoietic stem or progenitor cells and stromal components. Stromal components contain blood vessels and include endothelial cells, adipocytes and other stromal cells, as well as specialized osteoblasts and macrophages. These supporting cells express specific membrane-bound molecules and secrete paracrine factors, as well as various extracellular matrix components which contribute to the chemical signature of the niche that include also the long-range signals that reach the niche and HSCs through the circulation. The nervous system is represented by sympathetic nerve endings; reviewed e.g. in [32,33].

In the bone marrow, two types of niches have been identified [6,34,35]. These two types of niches differ in their localization in bone marrow, cellular composition, and function. The **endosteal niche** is situated in a close proximity of inner bone surface (in endosteum) of trabecular bone and comprise specialized osteoblasts, CAR (CXCL12

abundant reticular) cells, osteoclasts and stromal fibroblasts. The **vascular niche** is located more centrally in the bone cavity, next to the sinusoids. It consists of endothelial cells and CAR cells and contains dividing multipotent progenitors (see Figure 3, [4]).

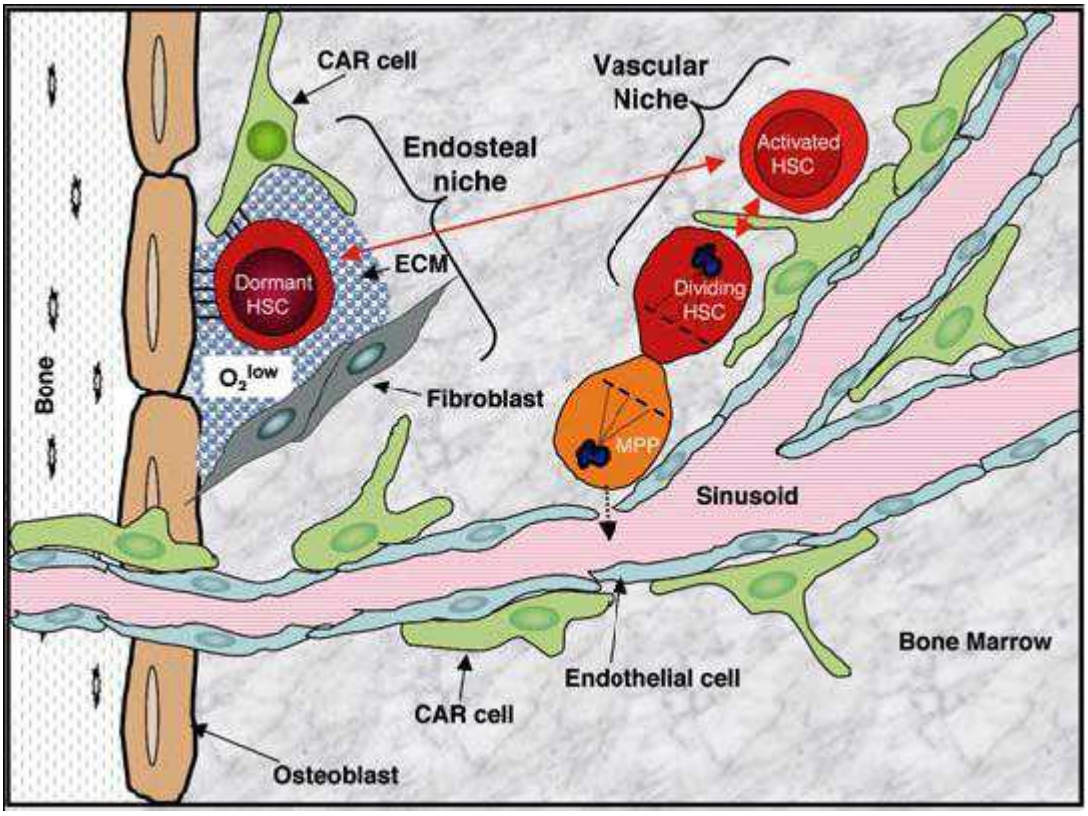

Figure 3 – Dormant and activated HSCs and their niches [4].

Although the very first concept of the niche was established more than 30 years ago [36], this microenvironment was inaccessible for direct observations for a long time. In 2003, Askenasy et al. [37] developed a technique that enabled the optical tracking of cells labeled with fluorescent markers in recipient bone marrow in vivo. They defined the hematopoietic niche as a three-dimensional functional unit composed of several stromal cells, extracellular matrix, and bone surface, which hosts a cluster of transplanted cells. Lo Celso et al. [38] visualized hematopoietic stem cells in vivo using advanced light microscopy techniques. The transplantation of bone marrow cells, or cells highly enriched in HSCs into the circulation of non-irradiated or irradiated recipients resulted in the migration of donor cells to the vicinity of the endosteal bone of

the calvaria or epiphyseal trabecular bone of the tibia [38-40]. However, transplanted cells started to proliferate only in irradiated recipients [38]. Yoshimoto et al. [41], using transplanted cells isolated from transgenic GFP mice, demonstrated that HSCs preferentially engraft at the epiphysis of the femurs or short flat bones.

1.3.2 The role of the stem cell niche

Crucial role of the niche is maintenance of HSCs and control of their self-renewal, differentiation and eventually apoptosis and migration.

The niche is assumed to exert control over the fate of a HSCs and progenitors within limits given by the gene expression status of the particular cell. The options are therefore slightly different for stem cells and progenitors. Apart from their inner differences in the activity of critical genes, stem cells and progenitors also use different niches. This is understandable, since while progenitors should actively proliferate and differentiate into more mature cells, stem cells should self-renew and, in the case of hematopoietic stem cells, remain quiescent and metabolically inactive. The various possible fates of a hematopoietic stem cell or a progenitor are depicted in Figure 4. Under normal circumstances, HSCs remain predominantly in the G_0-state and can repeatedly self-renew. Their long-term probability (p) to self-renew is 0.5 and can be transiently > 0.5 after transplantation or after a partial loss of HSCs. In contrast, progenitors are actively proliferating cells biased to differentiation into hematopoietic precursors and consequently their long-term probability to self-renew is always < 0.5. This makes them dependent on supply of cells from the stem cell compartment.

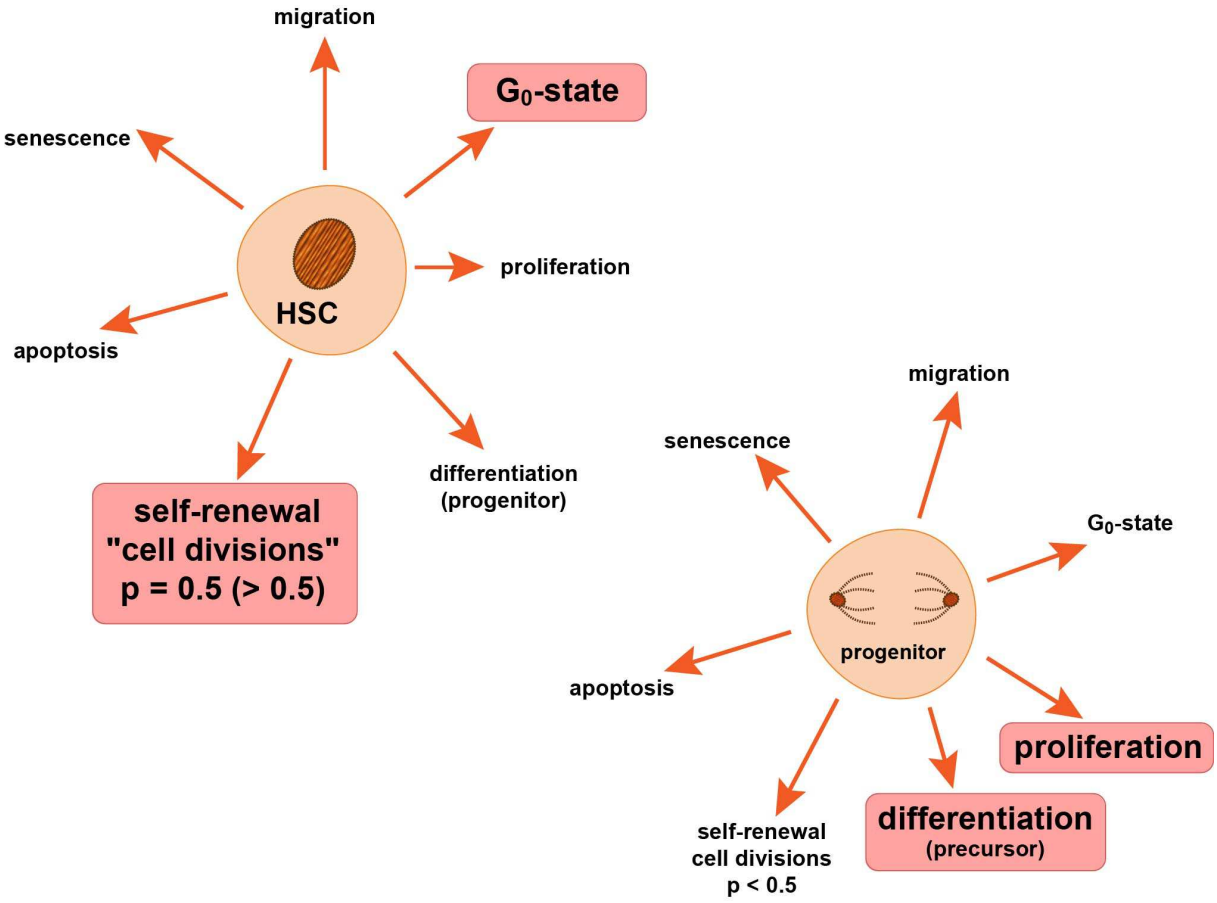

Figure 4 – Possible fates of hematopoietic stem (HSC) and progenitor cells. The cells, when affected by various stimuli, may undergo either asymmetric or symmetric self-renewal division, differentiation (commitment), remain in the G0-state, migrate into the blood as well as succumb to apoptosis or proliferative senescence [10].

1.3.3 Established interactions between niche and HSCs

As it is indicated above, the essential role of the niche is to keep HSCs in a quiescent state, to control their self-renewal cell divisions so they would precisely replace the differentiation of HSCs into progenitor cells and lead to the recovery of their number after loss due to damage to the hematopoietic tissue. These functions are ensured by specific interactions between the niche and HSCs. Specialized osteoblasts have been shown to be principal cellular components of the HSC niche in the bone marrow. Together with other stroma cells (reviewed in [33]), these osteoblasts produce factors maintaining stem cells quiescent (angiopoietin 1, thrombopoietin), inducing their proliferation in instances of tissue damage or stress (Wnt, interferon γ), control their

migration and localization in the bone marrow (CXCL12) and maintain their vitality (SCF, thrombopoietin, interleukins). The increase of osteoblasts correlates with an increased number of HSCs [42] and the elimination of osteoblasts causes HSCs to exit the bone marrow and induces extramedullary hematopoiesis [43,44]. Also, high concentrations of extracellular calcium seem to take part in the stem cell – niche interactions. Mice with deficient calcium-sensing receptor in HSCs failed to engraft [45]. The "osteoblastic niche" harbors multipotent HSCs with unrestricted self-renewal capability and is responsible for the lifelong maintenance of hematopoiesis. When transplanted and accepted by the niches, HSCs can reproduce themselves, colonize available niches and support and maintain hematopoiesis for the rest of the life of the organism, hence they are designated as Long-Term Repopulating Cells (LTRCs) in a rigorous syngenic mouse based transplantation assay.

1.3.4 A niche for a single HSC or for several HSCs? An inducible niche?

The number of HSCs is constant in adult bone marrow, which is thought to be due to a limited and fixed number of niches. At steady state, the majority of HSCs are probably single cells [13,38] located near the bone surface, the endosteum, which is lined with osteoblasts. The niches for HSCs are rare inside hematopoietic tissue, as is indicated by the low numbers of HSCs. The number of HSC niches should approach the total number of HSCs, i.e. 9,000 in the whole of the hematopoietic tissue or approximately 600 in the femoral bone marrow, often employed in analyses as a representative unit of total bone marrow. The numbers of niches should be even lower if there was only approximately 1 HSC among 100,000 marrow cells [46] or if a niche could harbor more than one HSC. Maloney et al. estimated that the whole bone marrow of W/W^v mice is compartmentalized into approximately 2,600 stem cell regulatory units [47]. The low and limited number of HSC niches likely determines the maximum

number of HSCs. This is also an important function of the niches with respect to the intrinsically unrestricted capacity of HSCs to self-renew [4,48-50].

The niche concept includes several assumptions which are supported by mostly indirect evidence. One of the assumptions is that niches determine the maximum number of HSCs. The evidence for this consists in a relatively constant number of HSCs throughout the life of the organism, and in no significant overshoot during regeneration from damage based on enhanced symmetric self-renewing cell divisions. By inducing large ectopic bones in otherwise normal mice, it was possible to increase the total number of progenitor cells CFU-S two- to four-fold [51]. This result can also be regarded as a piece of supporting evidence that the niches determine the number of early progenitor CFUs. The stimulation of bone formation through the action of the parathyroid-hormone related peptide also increased number of HSCs twofold and this was attributed to an increased number of niches [43]. The most slowly proliferating hematopoietic cells, probably hematopoietic stem cells, were shown to exist as single cells [13]. A niche which could accommodate only a single cell could be predetermined for the asymmetric divisions which provide progenitor cells for blood cell production and keep the pool of stem cells constant (Figure 5A).

Mice deficient in the Lnk signaling adaptor protein had approximately ten-fold increased numbers of HSCs due to their increased self-renewal and compromised apoptotic machinery [52-54]. This raises the questions whether self-renewing hematopoietic stem cells could accumulate in the niche (Figure 5B) or whether self-renewing stem cells could induce new niches. Recently, also mice overexpressing the miR-125 regulatory microribonucleic acid have been shown to have up to 8-fold increase number of HSCs [55-57]. Lnk-deficient mice and miR-125 overexpressing mice thus represent a significant challenge to current thinking on niche - HSCs

relationships and, in future may lead to new and deeper understanding of the niche – HSC interactions.

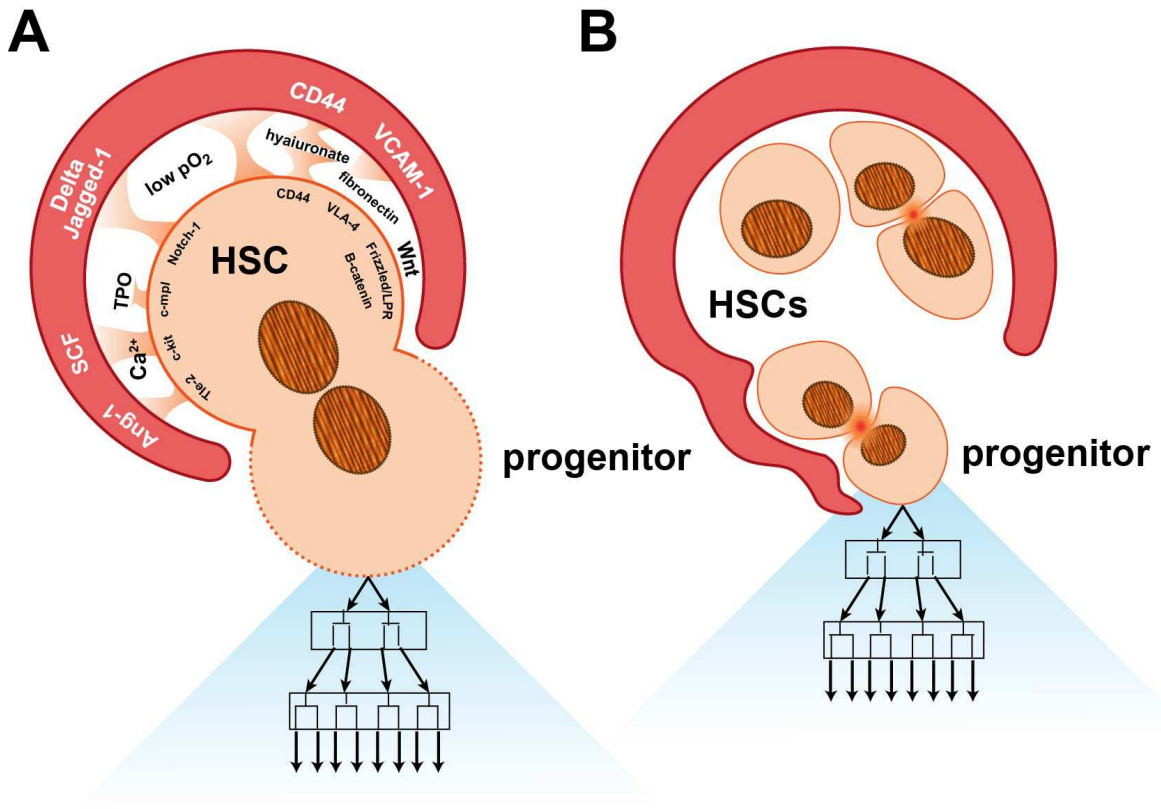

Figure 5 – Hematopoietic stem cell – niche unit. A – Niche harboring a single stem cell favoring asymmetric division due to extrinsic and eventually intrinsic (unequal distribution of transcription factors and other cellular components) factors. **B** – Niche containing a group of HSCs and eventually progenitors [10].

1.4 Availability of hematopoietic stem cell niches for transplanted stem cells

Hematopoietic stem cell transplantation is a procedure when intravenously administered HSCs restore hematopoiesis after its entire or partial destruction by radio- or chemotherapy-based conditioning. All interactions between HSCs, progenitor cells, and their niches are temporarily interrupted to be reestablished in the host hematopoietic tissue. Without conditioning, transplantation may result in a low level of chimerism [58]. The ratio can be ameliorated by conditioning the recipients with treatments which result in the cytoreduction of the host hematopoiesis. Ideally, the conditioning treatment should deprive stem cell niches of HSCs while preserving the niches functionally intact. Preferably, progenitor and blood precursor cells should also be spared to avoid bone marrow aplasia and transient pancytopenia in the blood. No such ideal conditioning treatment has been developed to these date. Also, relatively little is known regarding damage done to stem and progenitor cells and their niches by different conditioning treatments.

1.4.1 Homing and engraftment of donor HSCs introduced into circulation

After intravenous application of hematopoietic stem cells into the circulation they locate and identify the bone marrow and overcome the endothelial barrier of bone marrow sinusoids. This procedure requires active navigation, a process termed **homing**. Homing is the first and essential step in clinical or experimental stem cell transplantation. It is a rapid process, transplanted cells home to femoral bone marrow within minutes after intravenous injection [37]. Homing is thought to be a coordinated, multistep process, which involves signaling chemoattraction by stromal-derived factor 1 (SDF-1; CXCL12 chemokine) and stem cell factor (SCF), activation of the lymphocyte

function-associated antigen 1 (LFA-1), very late antigen 4 (VLA-4) and CD44 cytoskeleton rearrangement, membrane type 1 (MT1)-matrix metalloproteinase (MMP) activation and secretion of MMP2/9. SCF is the product of the murine Sl locus. It can be produced in both membrane-bound and soluble forms and is a ligand for the receptor encoded by the c-kit protooncogene, a member of the type III receptor tyrosine kinase family. The membrane-bound form of SCF stimulates the adherence of stem cells to the stroma.

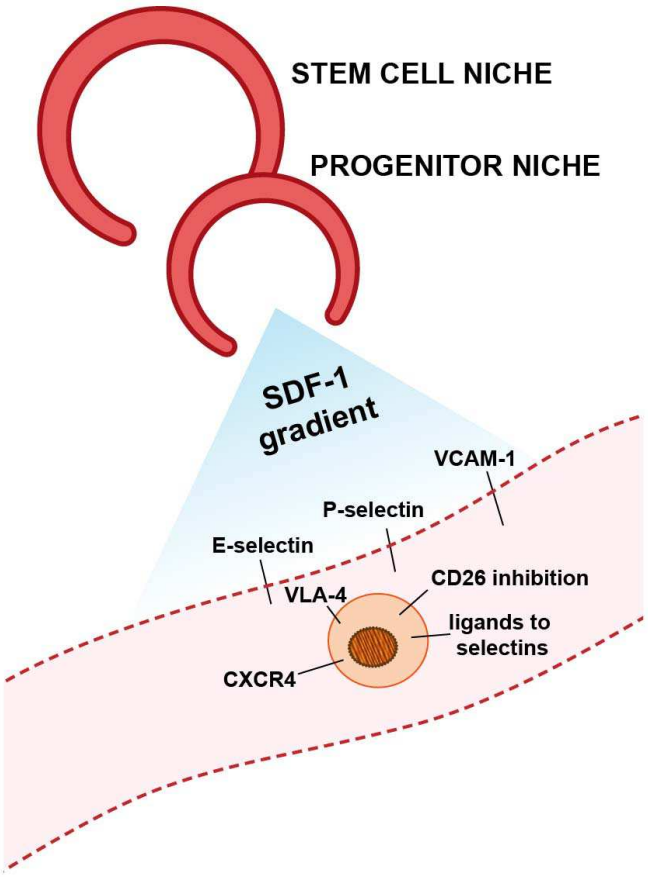

Figure 6 – Schematic view of the homing of donor HSCs into niches (see text) [10].

Rolling and firm adhesion of progenitors to endothelial cells in small marrow sinusoids under blood flow is followed by trans-endothelial migration across the physical endothelium/extracellular matrix (ECM) barrier. HSCs synthesize and express the glycosaminoglycan hyaluronic acid (HA), whose presence on HSCs is critical for

their transmarrow migration to the endosteal region after transplantation [59]. HA is the first molecule identified to have a significant impact on the lodgment of engrafting HSCs. Stem cells finalize their homing by selective access and anchorage to their specialized niches in the extravascular space of the endosteum region and in periarterial sites [60]. Askenasy et al., using in vivo tracking of HSCs, observed that successful homing and lodgment occur when several cells adhere to form a primary cluster in the subendosteal areas of the femoral epiphysis, close to the endosteal surface [61]. The clustering pattern of donor cells and formation of early clusters was observed not only in recipients conditioned by radio or chemotherapy (busulfan), but also in non-conditioned recipients. The location of the clusters in the epiphysis and the size of the early clusters are independent of the number of donor cells.

Many cell types, including long-term repopulating HSCs, short-term repopulating progenitors as well as some specialized mature cells can home to the bone marrow, but only HSCs initiate long-term repopulation.

1.4.2 Pre-transplantation conditioning regimes

As mentioned above, without conditioning, engraftment of transplanted cells is limited. The aim of the pre-transplantation condition treatment is to eradicate the disease and concurrently to create “space” for transplanted cells. Myeloablative pre-transplantation conditioning regime, which includes high doses of cytostatics combined with total body irradiation (TBI), suppress recipient’s hematopoiesis as well as his immune response against transplanted cells. On the other hand it is very toxic for several tissues (GIT, liver, heart). This led to implementation of submyeloablative (sublethal) pre-transplantation conditioning regimes (RIC – Reduced Intensity Conditioning). The aim of RIC is establishment of donor hematopoiesis under reduced exposure of recipient to potentially toxic doses of radiation or chemotherapy. Result of

RIC is a chimera, which is characterized by coexistence of donor and recipient's hematopoiesis.

Conventional way of pre-transplantation conditioning includes use of cytostatic drugs or TBI or combination of both. There are some new, less toxic regimens, which utilizes targeting of surface structures characteristic for HSCs. For example blocking of c-kit by specific c-kit blocking antibody [62,63] or sunitinib [64] enhance the engraftment of transplanted HSCs. The CXCR4 receptor, which is present on the surface of HSCs and is crucial for their maintenance and homing to their niches, could be also targeted. AMD3100 (Plerixafor), a CXCR4 receptor antagonist, increases niche availability through the mobilization of the recipient's residual stem cells and enhances donor engraftment [65].

Cytostatic drugs target proliferating cells and may induce significant hypoplasia of the hematopoietic tissue. As most of the HSCs are under normal circumstances quiescent, usually repeated administration of cytostatic drug is required.

Ionizing irradiation appears to be the most effective in "emptying" niches for transplanted HSCs. High doses of irradiation, which are lethal without the transplantation of donor HSCs, result in conversion of the hematopoiesis to purely of donor type due to absolute elimination of the host's HSCs. Lower doses of irradiation result in a partial chimerism, the degree of which is quantitatively related to the radiation dose [31,66]. The efficiency with which ionizing radiation increases engraftment of donor HSCs is the major argument for the necessity of creating space for donor HSCs by emptying niches.

1.4.2.1 Effect of irradiation on HSCs and their niche

Irradiation damages cells by inducing double strand DNA breaks and causing other damage mediated predominantly by reactive oxygen species (ROS) which also damage

other cellular components by causing lipid peroxidations and protein alkylation. Generally, proliferating cells are more sensitive to radiation-induced damage compared to differentiated, functionally specialized and non-proliferating cells. Consequently the small intestinal epithelium, hematopoietic tissue and the epidermis of the skin are among the most sensitive tissues. Exceptions to this rule are non-proliferating (G_0) lymphocytes which are highly sensitive [67]. Depending on the extent of damage, the outcome for an affected cell can be full recovery, cellular senescence or death. All hematopoietic cells, including proliferatively quiescent (G_0) stem cells, are highly sensitive to radiation damage.

The irradiation of mouse hematopoietic tissue with doses from 1 to 10 Gy causes a transient or permanent bone marrow hypoplasia, depending on the dose. The minimum bone marrow cellularity is reached approximately12 hours after submyeloablative doses [68]. The hematopoietic stroma, including its cellular part, is more resistant to irradiation. However, it is also subject to damage and the bone marrow undergoes extensive remodeling following irradiation [40]. Several authors demonstrated significant damage to sinusoidal endothelial cells and to the circulation [38,69]. Dominici et al. demonstrated damage to the osteoblasts in myeloablatively irradiated mice followed by their rapid regeneration [70]. Also the trabecular bone, a predominant location to which transplanted HSCs home and engraft is damaged even by submyeloablative irradiation [71]. Surprisingly, this does not seem to compromise the homing and engraftment of transplanted HSCs, which is highly efficient [31,72].

There is persuasive evidence that the intracellular signaling initiated by up-regulation of the p53 protein has a dominant role in the death of HSCs in irradiated hematopoietic tissue. p53 is up-regulated by DNA damage shortly after irradiation. Of the several p53 target genes, the one for the BH3-only protein Puma (p53 up-regulated

modifier of apoptosis) is critical. Similarly to mice lacking functional *Trp-53* genes coding the p53 protein, mice lacking functional genes for Puma are increasingly resistant to the irradiation-induced hematological tissue failure [73,74]. On the other hand, the hematopoietic tissue of mice lacking the gene for *Mdm2*, coding an inhibitor of p53 and thus increasing p53 signaling, as well as of the mice lacking the gene *Slug* coding inhibitor of Puma, are more sensitive to irradiation. Marusyk et al. observed no significant loss of phenotypic HSCs within 48 h of irradiation, but a functional assay indicated significantly reduced numbers of them [75]. Due to a low proliferation rate, HSCs can physically remain in their niches after irradiation before they succumb to apoptosis. Submyeloablative doses of irradiation induced apoptosis in bone marrow cells after several hours and apoptotic cells were present up to four days after irradiation [76].

2 AIM OF THE STUDY

- to examine whether the damage to the hematopoietic tissue resulting from progressive doses of irradiation negatively influences engraftment of intravenously transplanted stem cells

- to study engraftment of transplanted hematopoietic stem cells relative to regeneration of the hematopoietic tissue derived from surviving or transplanted HSCs

3 MATERIALS AND METHODS

3.1 Materials

Chemicals

7-AAD (7-aminoactomycin D) (*BD Pharmingen, USA*)

Ammonium chloride (NH_4Cl) (*IPL, Czech Republic*)

Bovine serum albumin – Fraction V, bovine serum albumin – Fraction V, biotin free (*Carl Roth GmbH*)

BrdU (5-bromo-2´-deoxyuridine) (*BD Pharmingen, San Jose, CA*)

BrdU flow kit (*BD Pharmingen, San Jose, CA*)

Disodium phosphate ($Na_2HPO_4.12H_2O$) (*IPL, Czech Republic*)

EDTA (ethylendiaminetetraacetic acid, disodium salt: dihydrate; $C_{10}H_{14}N_2O_8Na_2.2H_2O$) (*Sigma-Aldrich, USA*)

Halothane (*Hospira INC., USA*)

Heparin (*Zentiva, Czech Republic*)

Hoechst 33342 (*Honeywell Riedel-de Haën, Germany*)

Iscove's Modified Dulbecco's Medium with HEPES and L-glutamine (*Lonza, Switzerland*)

Monosodium phosphate ($NaH_2PO_4.2H_2O$) (*IPL, Czech Republic*)

Sodium chloride (NaCl) (*IPL, Czech Rebublic*)

Türck solution (*Penta, Czech Republic*)

Cystostatic and immunosuppressive drugs

Endoxan (Cyclophosphamidum monohydricum, *Baxter Oncology GmbH, Germany*)

Dexamed (Dexamethasoni phosphas, *Medochemie, Cyprus*)

Antibodies

Chimerism analysis:

FITC-conjugated anti-mouse CD45.1 (*BioLegend, USA*)

PE-conjugated anti-mouse CD45.2 (*BioLegend, USA*)

"LSK SLAM" panel:

APC conjugated anti-mouse Ly-6A/E (Sca-1) (*BioLegend, USA*)

APC/H7-conjugated anti-mouse CD117 (c-kit) (*BioLegend, USA*)

FITC-conjugated anti-mouse CD48 (*BioLegend, USA*)

Lineage biotin-antibody cocktail (*Miltenyi Biotec GmbH, Germany*)

PE-conjugated anti-mouse CD150 (*BioLegend, USA*)

PE/Cy7-conjugated streptavidin (*BioLegend, USA*)

"Cell cycle" panel:

7-AAD (DNA labeling) (*BD Pharmingen, USA*)

Alexa Fluor 700-conjugated anti-mouse CD48 (*BioLegend, USA*)

APC-conjugated anti-BrdU (*BD Pharmingen, USA*)

Brilliant Violet 421 (BV421)-conjugated anti-mouse CD117 (c-kit) (*BioLegend, USA*)

FITC-conjugated lineage antibody cocktail (*BioLegend, USA*)

PE-conjugated anti-mouse CD150 (*BioLegend, USA*)

PE/Cy7-conjugated anti-mouse Ly-6A/E (Sca-1) (*BioLegend, USA*)

"Subpopulations" panel:

Alexa Fluor 700-conjugated anti-mouse CD45R/B220 (*BioLegend, USA*)

APC-conjugated anti-mouse CD11b (Mac-1) (*BioLegend, USA*)

APC-conjugated anti-mouse Ly-6G/Ly6C (Gr-1) (*BioLegend, USA*)

PerCP-conjugated anti-mouse TER119/erythroid cells (*BioLegend, USA*)

Buffers

Phosphate buffered saline (PBS): 16 mmol/dm^3 $Na_2HPO_4.12H_2O$, 4 mmol/dm^3 $NaH_2PO_4.2H_2O$, 0.15 mol/dm^3 NaCl, pH 7.4

Ammonium chloride lysis solution: 0.15 mol/dm3 NH4Cl, 0.1 mmol/dm^3 EDTA, 0.032 mol/dm^3 NaCl

3.2 Instruments

Analytical balance AB 104, Mettler Toledo, Czech Republic

Auto Hematology Analyzer BC-5300 Vet, Mindray, China

Automatic micropipettes, Eppendorf, Germany

Cellometer AUTO T4, Nexcelom Bioscience, USA

Centrifuge 5804R, Eppendorf, Germany

CO2 incubator, IGO 150 Cell life, Jouan GmbH, Germany

Flow box, Holten LaminAir, Model 1.2, Thermo-Scientific Inc., USA

Flow cytometers: BD FACS Aria Ilu equipped with UV-laser, BD FACS Canto II, Becton Dickinson, USA

Source of ionizing radiation: 60Co Chisobalt, Chirana, Czech Republic

Orbital incubator SI50, Stuart Scientific, UK

Vortex mixer, Velp Scientifica, Italy

Water bath, Heto lab equipment, Denmark

3.3 Animals

C57BL/6NCrl mice (CD45.2) and congenic B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) were bred in the specific pathogen-free facility of the Center of Experimental Biomodels, First Faculty of Medicine, Charles University in Prague, maintained in a clean conventional part of the facility during the experiments with a light-dark cycle of 12 hours, and fed ad libitum. Three- to six-month-old mice, 20 – 25 g of body weight were used in the experiments. All experiments were approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University in Prague and were performed in accordance with national and international guidelines for laboratory animal care.

3.4 Total body irradiation (TBI)

Before bone marrow transplantation, recipient mice were irradiated with various doses of ionizing radiation. Total body irradiation of ~0.58 Gy/min from a ^{60}Co source from a distance of 123.5 cm was used. Doses of 1 –7 Gy and 9 Gy were used for the sublethal and the lethal irradiation, respectively.

3.5 Peripheral blood collection for complete blood count analysis with differential of white blood cells

Blood samples for complete blood count were collected from retroorbital venous plexus of anesthetized mice using microhematocrit capillaries (Keraglass, Czech

Republic) containing10 μL of EDTA. The samples were analyzed on Auto Hematology Analyzer BC-5300 Vet (Mindray, China).

3.6 Harvesting of bone marrow cells

Bone marrow cells (BMCs) were collected from the femurs of mice sacrificed by cervical dislocation. Femurs were removed from the body, carefully cleaned of muscles, and repeatedly flushed with 1-milliliter insulin syringe (21G needle) into 1 ml PBS supplemented with 0.5% BSA (PBS-BSA). A single cell suspension was created with 25G needle. The cells were counted with Cellometer AUTO T4, using Türck solution for white blood cell count. The average cellularity of the bone marrow obtained from one femur was 29.2 ± 4.6 (n=51) in normal male mice and 29.8 ± 4.4 (n=20) in normal female mice.

3.7 Bone marrow transplantation (BMT)

Ly5.1/Ly5.2 congenic experimental model was used in transplantation experiments. C57BL/6 Ly5.1 and C57BL/6 Ly5.2 are two inbred mouse strains which differ in the surface antigen CD45 (Leukocyte Common Antigen). Isoforms CD45.1 (Ly5.1) and CD45.2 (Ly5.2) are functionally identical but distinguishable by flow cytometry using monoclonal antibodies. Because CD45 is expressed by all nucleated cells, Ly5.1/Ly5.2 ratio in peripheral blood reflects the percentage of transplanted HSCs which have engrafted to recipient's bone marrow (chimerism of donor cells).

BMCs were administered intravenously into the retroorbital venous plexus (see Figure 7) in a volume of 0.5 ml PBS-BSA. At least three mice were used per group.

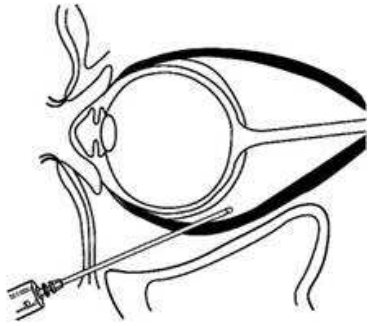

Figure 7 – Transplantation into the retroorbital venous plexus in mouse

(image from http://www.iacuc.ucsf.edu/policies/awspretroorbitalinjection.asp).

After sublethal irradiation (1-7 Gy), a standard dose of BMCs corresponding to a half of the femur (approximately$1x10^7$ BMCs; ~3.5% of total bone marrow of the mouse) was transplanted in most of the experiments.

After lethal irradiation (9 Gy) the mice received immediately a syngenic rescue transplant defined as a fraction of the femur. Applied fractions of the femur and corresponding amount of BMCs in each fraction are shown in Table 1.

fraction of the femur	*Approximate number of BMCs*	*Approximate % of total BMCs*
1/200 of femur	10^5 BMCs	0.035
1/100 of femur	2.10^5 BMCs	0.07
1/10 of femur	2.10^6 BMCs	0.7
½ of femur	10.10^6 BMCs	3.5
1 femur	20.10^6 BMCs	7
2 femurs	40.10^6 BMCs	14

Table 1 –Transplanted fractions of the femoral bone marrow.

The following transplantation experiments were performed:

→ **congenic transplantation into sublethally irradiated mice:**

- normal non-irradiated BMCs were transplanted into congenic recipients irradiated with progressive sublethal doses of irradiation (0 – 7 Gy)
- normal non-irradiated BMCs, as well as BMCs from donors irradiated with progressive sublethal doses of irradiation (1 – 7 Gy) 24 hours before bone marrow collection were transplanted into sublethally (5 Gy) irradiated congenic recipients

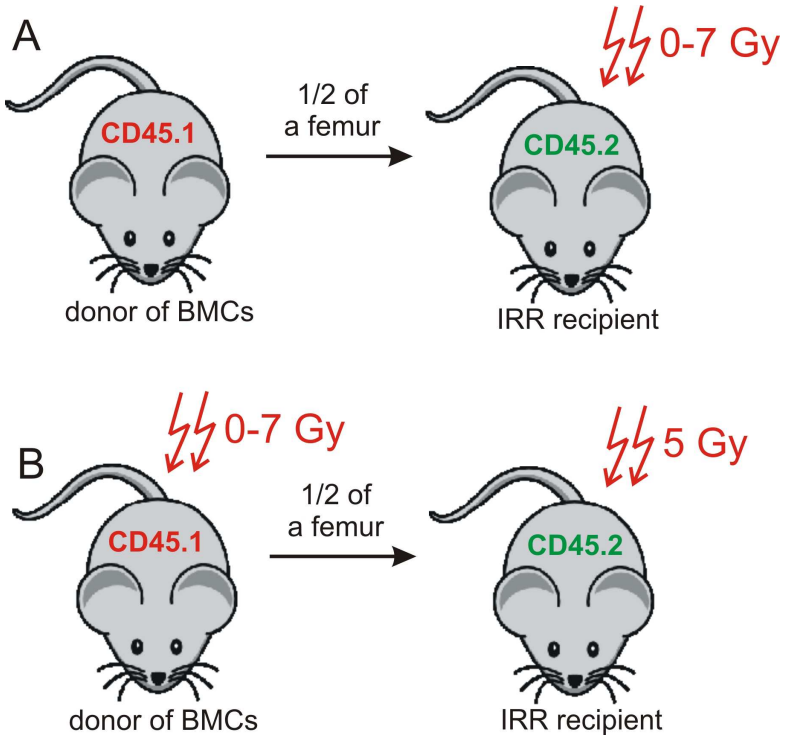

Figure 8 – Congenic transplantation into sublethally irradiated mice to determine a proportion of surviving hematopoietic stem (LTRCs) and progenitor (STRCs) cells. BMCs from one-half of the congenic femur from normal non-irradiated donors was transplanted into non-irradiated or sublethally irradiated (1-7 Gy) recipients (**A**). BMCs from one-half of the congenic femur from non-irradiated or sublethally irradiated (1-7 Gy) donors was transplanted into sublethally (5 Gy) irradiated recipients (**B**).

→ **delayed transplantation after sublethal irradiation**

- normal non-irradiated BMCs were transplanted into 6 Gy irradiated congenic recipients with a delay from 0 to 30 days

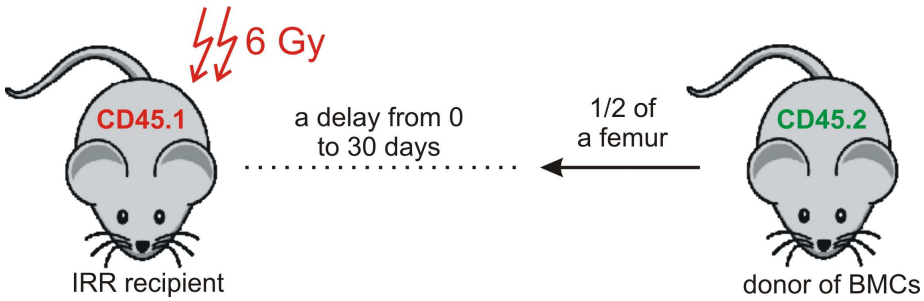

Figure 9 – Delayed transplantation after sublethal irradiation to determine "space" available for transplanted stem (LTRCs) and progenitor (STRCs) cells in the recipient's hematopoietic tissue. Sublethally irradiated (6 Gy) recipients were transplanted with a delay from 0 to 30 days with congenic BMCs from one-half of the femur.

→ **delayed transplantation after lethal irradiation and rescue syngenic transplantation**

- normal non-irradiated BMCs were transplanted with a delay from 0 to 180 days into 9 Gy irradiated congenic recipients, which received a syngenic rescue transplant of various size
- normal non-irradiated congenic BMCs were transplanted with a delay 20 or 30 days into lethally irradiated recipients, which received a rescue transplant from syngenic donors regenerating 30 or 60 days from TBI by 6 Gy

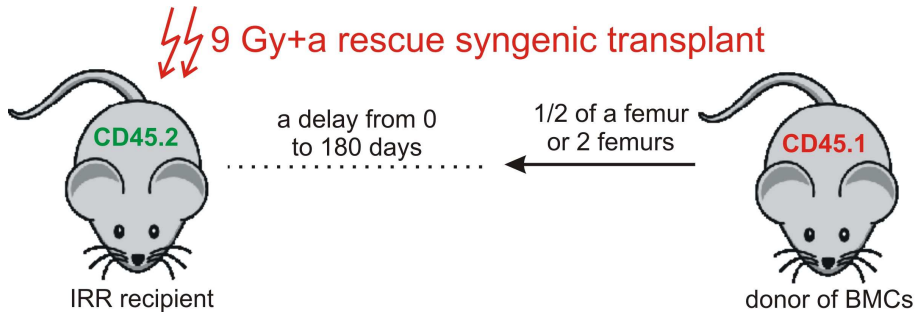

Figure 10 – Delayed transplantation after lethal irradiation to determine "space" available for transplanted stem (LTRCs) and progenitor (STRCs) cells in the recipient's hematopoietic tissue. Lethally (9 Gy) irradiated recipients were immediately transplanted with a rescue syngenic transplant according to Table 1 and subsequently transplanted with a delay from 0 to 180 days with congenic BMCs from a half of the femur or two femurs.

→ **co-transplantation (competitive transplantation) of regenerating BMCs with normal congenic BMCs into lethally irradiated recipients**

- BMCs regenerating from TBI by 6 Gy were co-transplanted with normal non-irradiated BMCs into lethally irradiated recipients in 1:1 or 10:1 ratio

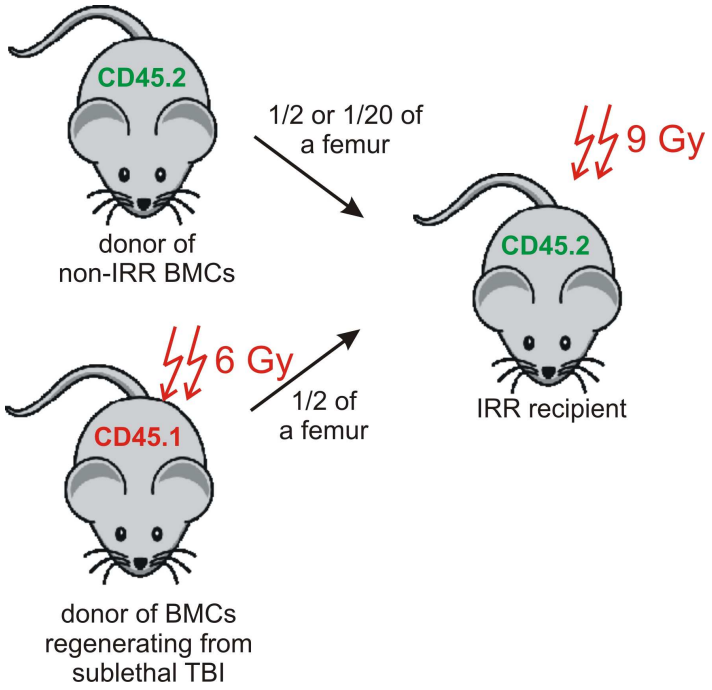

Figure 11 – Co-transplantation of regenerating BMCs with normal congenic BMCs into lethally irradiated recipients. BMCs from congenic donors regenerating from sublethal irradiation was co-transplanted with BMCs from normal non-irradiated donor in 1:1 or 10:1 ratio into lethally irradiated recipients.

The concrete transplantation setting of each experiment is described in detail in the appropriate results section. As the CD45.1/CD45.2 system is interchangeable, the donors/recipients were chosen according to the actual status in the animal facility, i.e. do not strictly correspond with illustrations above in every single experiment.

3.8 Flow cytometry

3.8.1 Determination of chimerism in peripheral blood and bone marrow

A sample of peripheral blood was taken from the retroorbital venous plexus using heparinized capillary tubes from one to six months after transplantation to distinguish the chimerism arising from the short-term repopulating cells (STRCs – chimerism level determined in blood after 1 month) and long-term repopulating cells (LTRCs – chimerism level determined in blood or bone marrow after 3 and more months). Briefly, 50 µL blood samples were added to 3 mL ammonium chloride lysing buffer (0.15 M NH_4Cl, 0.035 M NaCl, and 0.1 mM EDTA) and agitated at 37°C for 15 minutes to lyse the red blood cells, then washed twice with PBS-BSA, resuspended in 100 µL PBS-BSA, and stained with PE–conjugated anti-CD45.1 and FITC-conjugated anti-CD45.2 antibodies - 0.5 µL of each antibody per sample - for 30 minutes on ice in the dark. Bone marrow cells were collected from the femurs of mice sacrificed by cervical dislocation. Twenty µL of the cell suspension was added to 3 mL of PBS-BSA and centrifuged (4 °C, 400g, 6 min). After removing the supernatant, the cells were stained as described above. After washing with PBS-BSA, samples were analyzed by flow cytometry. A gating for $CD45.1^+$ and $CD45.2^+$ cells was performed. $CD45.1^+/CD45.2^+$ artificial doublets, if present, were omitted from the analysis.

3.8.2 Analysis of primitive cell populations of bone marrow

Staining for $Lin^{low}Sca\text{-}1^+c\text{-}kit^+$ and SLAM markers (CD150 and CD48) according to Kiel et al. [6] together with detection of LSK side population (LSK SP) according to Goodell et al. [8] were used to identify the most primitive cells within the bone marrow.

BMCs isolated from the femurs of irradiated or control mice were counted and stained with Hoechst 33342 and fluorescein-labeled monoclonal antibodies. Briefly,

BMCs in an amount corresponding to one femur were labeled in a preheated IMDM medium (10^6 cells per 1 mL of the medium) with 5μg/mL Hoechst 33342 dye at 37°C for 90 min. Cells were then washed with PBS-BSA and stained with a mouse lineage biotin-antibody cocktail (3 μL per sample), APC-conjugated anti-mouse Ly-6A/E (Sca-1) antibody, APC/H7-conjugated anti-mouse CD117 (c-kit) antibody, PE-conjugated anti-mouse CD150 antibody, FITC-conjugated anti-mouse CD48 antibody (1 μL of each antibody per sample) for 30 min on ice in the dark. Cells were washed with PBS-BSA and secondary staining with 3 μL streptavidin PE/Cy7 per sample was performed. After washing with PBS-BSA, cells were resuspended in 300 μL of PBS-BSA and analyzed by flow cytometry. Doublets and debris were discriminated and the LSK/SP and LSK/SLAM populations were gated (Supplementary Figure 1).

3.8.3 Analysis of precursors of blood cells in bone marrow

For detecting and quantifying precursors of blood cells in the marrow, 300 μL of a bone marrow cell suspension, containing approximately six million cells, was incubated with Alexa Fluor 700-conjugated anti-mouse CD45R/B220 antibody, APC-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) antibody, APC-conjugated anti-mouse CD11b (Mac-1) antibody, PerCP-conjugated anti-mouse TER-119/erythroid cells antibody (1 μL of each antibody per sample) on ice for 30 min in the dark. After washing with PBS-BSA, the samples were resuspended in 300 μL of PBS-BSA and analyzed for particular subpopulations.

3.8.4 Cell cycle analysis

BrdU staining for cell cycle analysis was performed according to the BrdU flow kit staining protocol. Briefly, mice recovering various times from sublethal irradiation by 6 Gy as well as non-irradiated controls were injected i.p. with 1.5 mg/mouse of BrdU.

One hour later, BMCs were isolated from the femur of donors sacrificed by cervical dislocation. 5.10^6 of cells were incubated with FITC-conjugated lineage antibody cocktail (2.5 μL/sample), PE/Cy7-conjugated anti-mouse Ly-6A/E (Sca-1), Brilliant Violet 421 (BV421)-conjugated anti-mouse CD117 (c-kit), Alexa Fluor 700-conjugated anti-mouse CD48, PE-conjugated anti-mouse CD150 (1 μL of each antibody per sample) for 15 min on ice in the dark. Cells were then washed, fixed, permeabilized and subsequently treated with DNase. Then, the samples were incubated with 1 μL/sample APC-conjugated anti-BrdU for 20 min at room temperature in the dark. Finally, 20 μL/sample 7-AAD was added, samples were filtered though 70 μm filter and analyzed by flow cytometry (FACS Canto, BD Biosciences, San Jose, CA). Gating strategy used for identifying of populations of our interest is shown in Supplementary Figure 2.

3.9 Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, USA, www.graphpad.com). Data are presented as the mean ± standard error of the mean (SEM). One-way ANOVA using Dunnett's post test was used to compare each group to the control. P values < 0.05 were considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$). The log (inhibitor) vs. response curve - variable slope (four parameters) calculation using a least squares fit was used for the nonlinear regression of radiation dose-dependence data. A least square fit was also used to compare the conformity of the experimental to the calculated data.

4 RESULTS

4.1 Efficiency of engraftment of transplanted donor cells

To establish engraftment efficiency of transplanted donor progenitors (STRCs) and HSCs (LTRCs), we first determined the fraction of the cells which survived after different doses of irradiation in host mice. The surviving host progenitors and HSCs compete with the transplanted donor progenitors and HSCs which is reflected in the level of blood cell chimerism in peripheral blood and bone marrow. Knowledge of the ratio of competing donor and host cells enables calculation of the expected chimerism for different levels of the efficiency of engraftment of transplanted donor cells.

4.1.1 Survival of STRCs and LTRCs in sublethally irradiated mice

To determine the fraction of host repopulating cells surviving different doses of irradiation, groups of three mice CD45.1 were irradiated with 0.5, 1, 2, 3, 4, 5, 6, and 7 Gy. The control group was not irradiated. BMCs were collected 22 hours after irradiation and transplanted in an amount corresponding to half of a femur into sublethally (5 Gy) irradiated congenic CD45.2 recipients. Donor chimerism was determined in peripheral blood (PB) 1 (STRCs), 3 and 6 months (LTRCs) after the transplantation; after 6 months the chimerism was also determined in the bone marrow (BM) (Figure 12A; the results obtained after 3 months are not shown, as they are similar to those after six months). To estimate the surviving fractions of STRCs and LTRCs after irradiation with various doses, engraftment of the marrow from non-irradiated donors (0 group) was taken to be 100% (donor chimerism 54.7% for STRCs and 73.3% for LTRCs). Hence, the chimerism values presented in Figure 12A were corrected by a factor of 1.828 (100 %/54.7 %) for STRCs and 1.362 (100 %/73.4 %) for LTRCs. A

least square fit was used for the nonlinear regression of the radiation dose – chimerism dependence data. The obtained curves were then used to estimate the proportional survival of STRCs and LTRCs after various doses of irradiation (Figure 12B). Survival of STRCs and LTRCs was negatively proportional to progressive doses of irradiation and confirmed higher radiosensitivity of STRCs, especially after lower doses.

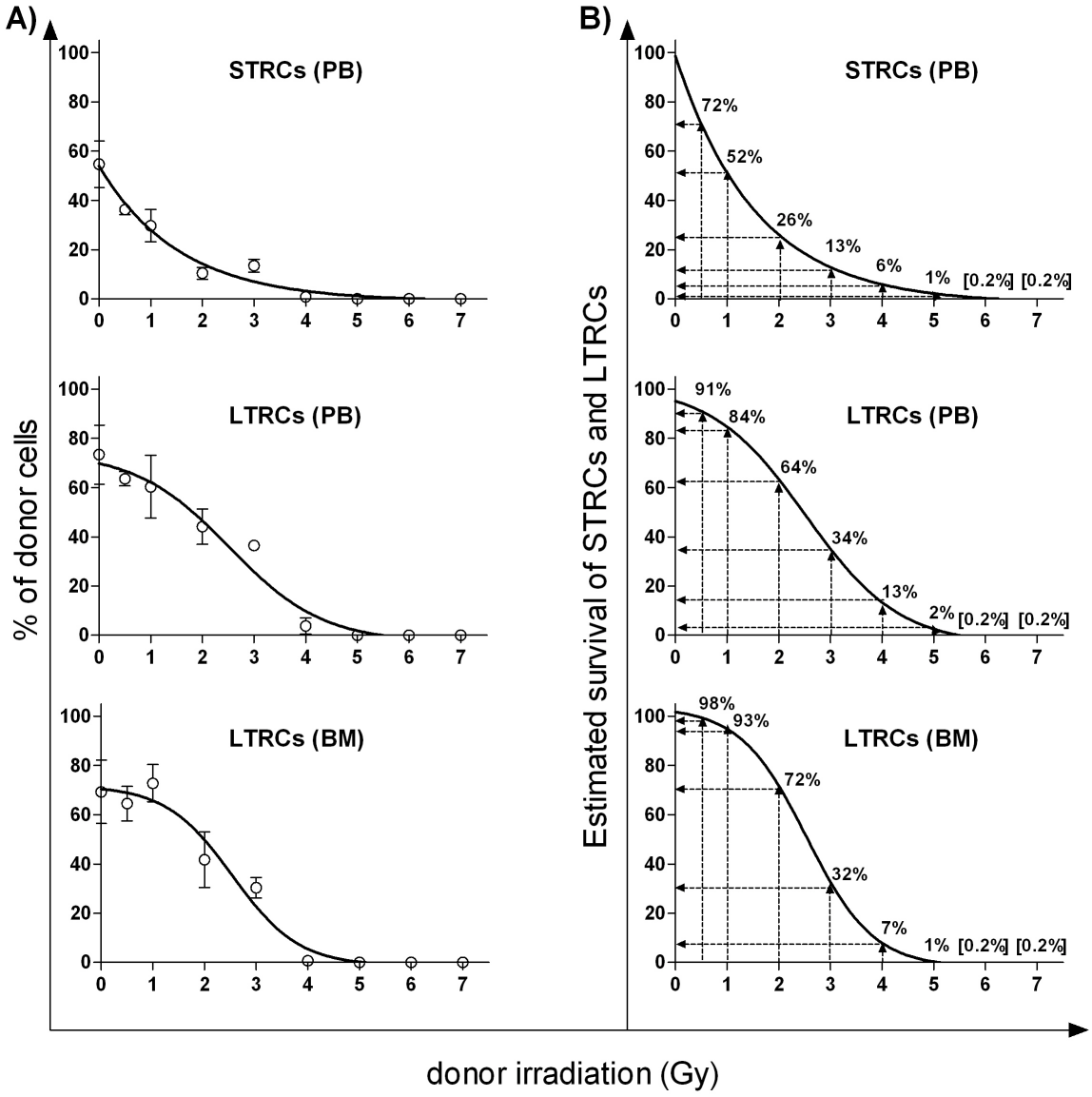

Figure 12 - Survival of STRCs and LTRCs after irradiation with various doses. (A) experimental data, (B) derivation of the proportional survival estimates. Data are presented as mean ± SEM (n=3). Regression analysis: nonlinear regression using the log (inhibitor) vs. response curve - variable slope (four parameters) calculation.

4.1.2 Calculation of expected chimerism after a standard dose of donor cells

The expected (theoretical) engraftment of transplanted STRCs and LTRCs administered in a standard dose corresponding to 3.5% of their total pool in normal non-irradiated bone marrow (one half of the femur) and competing with a surviving fraction of the host's cells was calculated from Figure 12B for different doses of irradiation using the formula:

$$\%D_{CH} = \frac{\%D_C}{\%D_C + \%H_C} \times 100$$

where $\%D_{CH}$ is the theoretical engraftment of transplanted donor cells, $\%D_C$ is the percentage of the donor repopulating cells administered and $\%H_C$ is the percentage of the host repopulating cells which survive irradiation.

Since two independent measurements of donor chimerism derived from LTRCs were available in each experiment, one in the peripheral blood and the second in the bone marrow, a mean value from both survival estimates was used in the calculations (Figure 13). The calculations assumed efficiencies of donor STRCs and LTRCs engraftment ranging from 100% to 20% (see Supplementary Table 1).

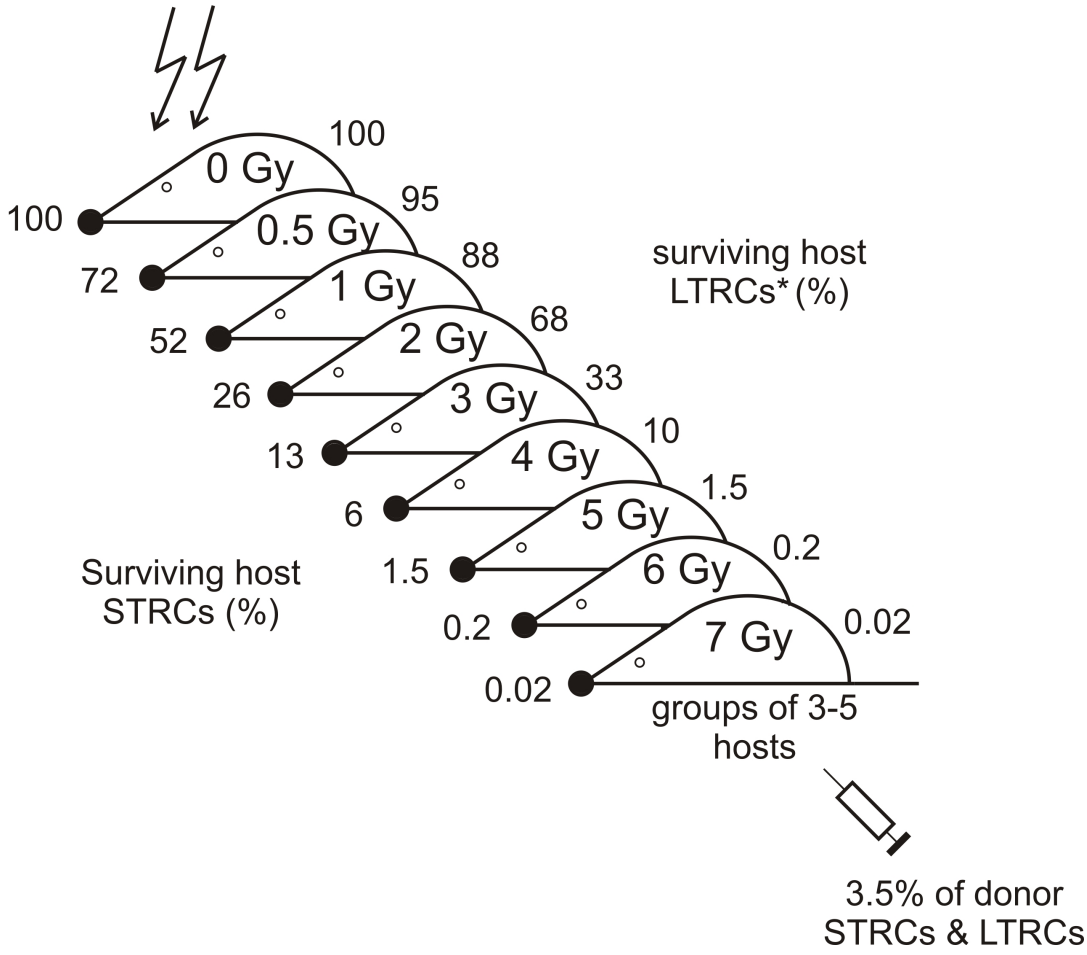

Figure 13 - Schematic representation of experiments for determining dependence of donor chimerism on submyeloablative conditioning irradiation of host congenic mice.
* Average from estimates derived from chimerism determined in blood and bone marrow (see Figure 1B)

4.1.3 Experimental chimerism after a standard dose of donor cells

Four separate experiments in which marrow cells from one-half of the femur of normal donors were intravenously injected into congenic progressively irradiated (0 – 7 Gy) mice were performed (Figure 14). The donor chimerism was determined after 1 month (STRCs) and after 4 or 6 months (LTRCs). At the end of the experiments, either after 4 months or after 6 months, donor chimerism was determined both in the peripheral blood and in the bone marrow. There was an S-shape relationship between the dose of irradiation and the engraftment of donor cells.

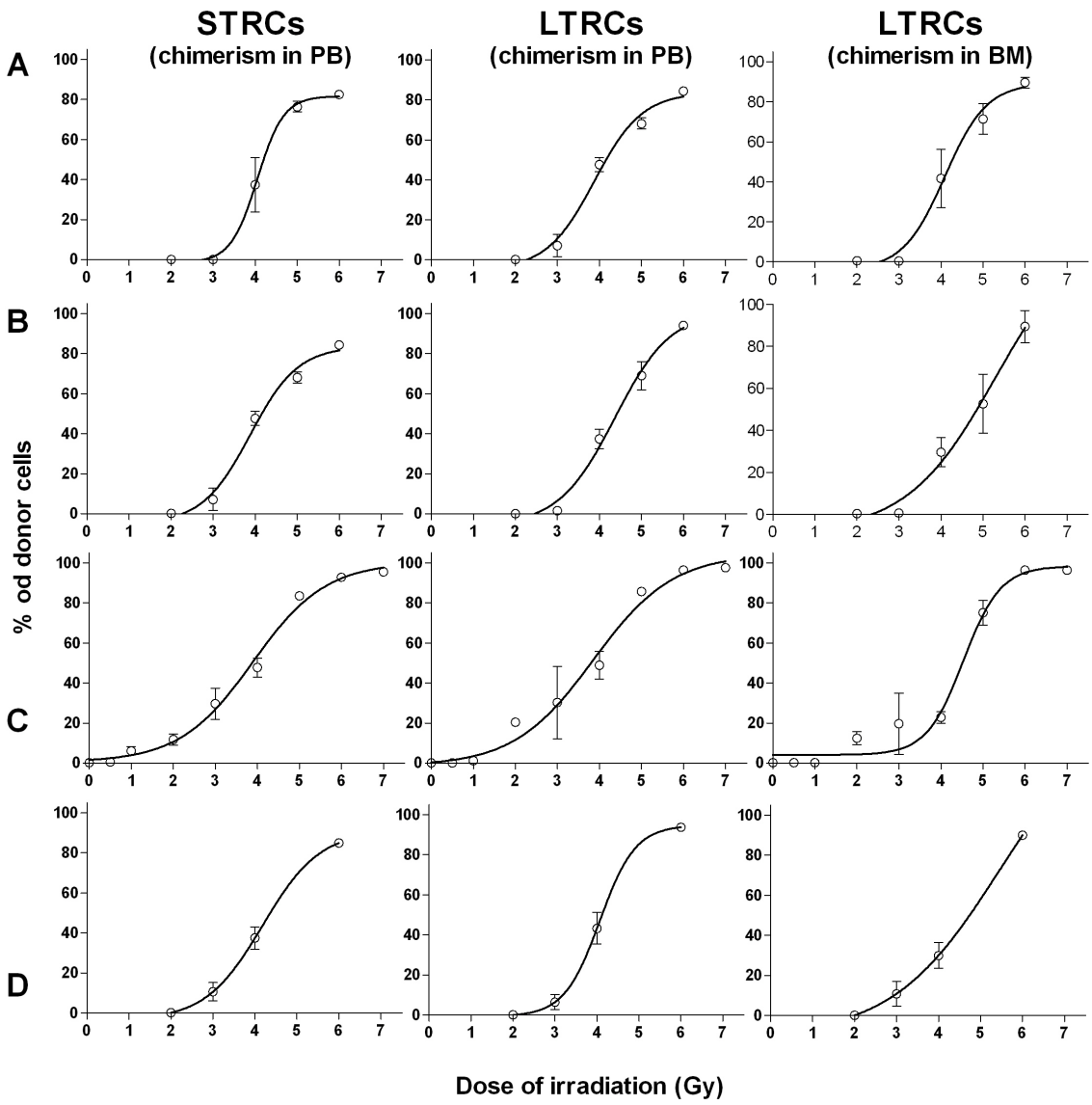

Figure 14 - Donor chimerism determined after 1 month (STRCs) in peripheral blood (PB) and after either 4 (A,B) or 6 (C,D) months (LTRCs) in peripheral blood and bone marrow (BM) in progressively irradiated recipients transplanted with a standard dose of normal bone marrow cells. Data are presented as mean ± SEM (n=3). Regression analysis: nonlinear regression using the log (inhibitor) vs. response curve - variable slope (four parameters) calculation.

4.1.4 Comparison of experimental and theoretically calculated engraftment results

The results of the four separate experiments presented in Figure 14 were pooled, nonlinear regression curves were plotted through the pooled data and they were compared to the curves similarly derived from the calculated estimates presented in

Supplementary Table 1 (Figure 15A). The consensus between the experimental chimerism values and the theoretical calculated values was also evaluated by summing of the squared differences between the two values for various assumed efficiency engraftment levels. The best fit between the experimental and theoretical data was for the calculations assuming 100% engraftment efficiency (Supplementary Table 1).

We further established how the fit between the experimental and the calculated data assuming 100% efficiency would be affected by the variation in the fraction of bone marrow cells contained in one-half of the femur. This was assumed to be 3.5% of the total marrow in all previous calculations. We calculated a theoretical donor chimerism assuming a 100% engraftment efficiency of transplanted donor STRCs and LTRCs, but with their content in one-half of the femur being in the range from 2.5 to 4.5% of their total marrow number (Supplementary Table 2). The obtained data were plotted against the curves and these were presented in Figure 15B, together with the chimerism data from real experiments.

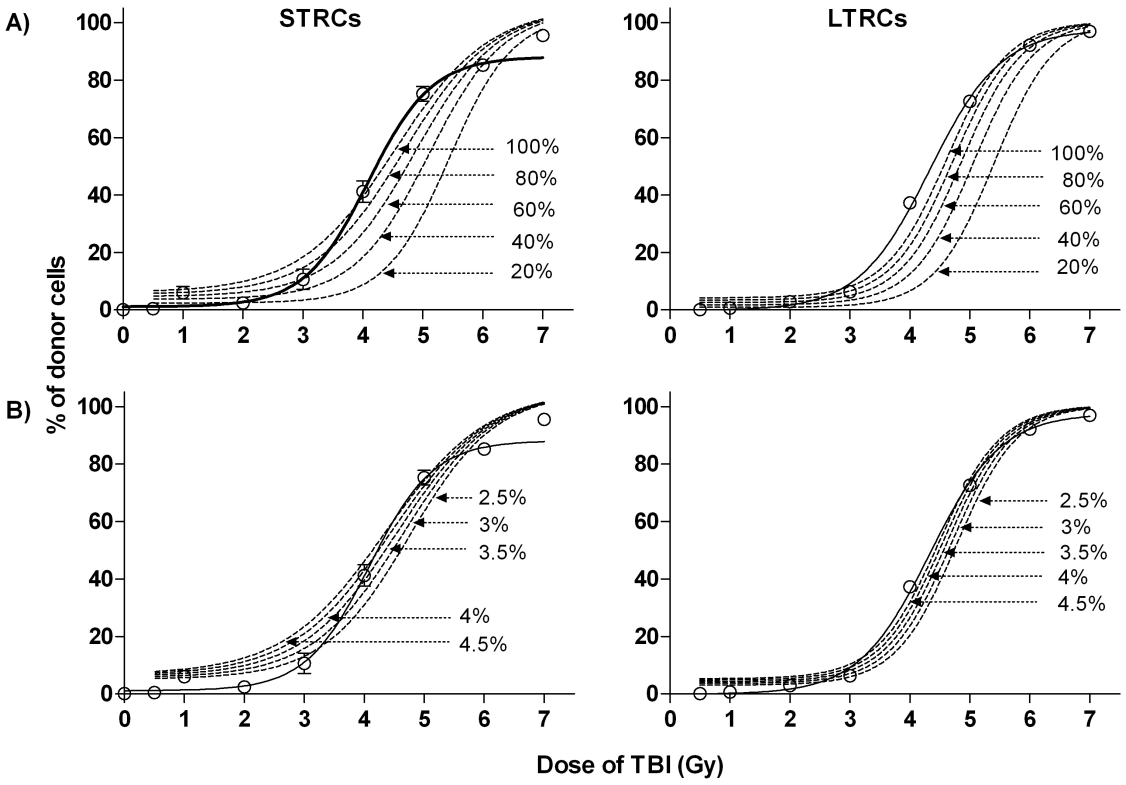

Figure 15 - Pooled results from the four separate experiments presented in Figure 14 (empty circles and solid curve) were plotted against (A) curves showing the theoretically expected chimerism corresponding to 100–80–60–40-20% engraftment efficiency derived from calculated data presented in Supplementary Table 1 (dashed curves); (B) curves showing the theoretically expected chimerism corresponding to 100% engraftment efficiency and the bone marrow from a half femur representing from 2.5 to 4.5% of total marrow cells (dashed curved).

4.1.5 Calculated (theoretical) and experimental chimerism after different doses of donor cells

Additional experiments used progressively irradiated recipients as in previous experiments but transplanted with a different fraction of normal congenic marrow ranging from 14.0% to 0.9% of its total murine content. The results of the two experiments were pooled and are presented in Table 2. There was a significant correlation between experimental and calculated donor chimerism levels which assumed a 100% engraftment efficiency.

		Recipient irradiation (Gy)					
		Estimated fraction of STRCs survived (%)					
		1 Gy	**2 Gy**	**3 Gy**	**4 Gy**	**5 Gy**	**6 Gy**
		52%	*26%*	*13%*	*6%*	*1.5%*	*0.20%*
Donor BMCs transplanted *Estimated fraction of STRCs transplanted (%)*	**2f ~** *14.00%*	*(21)* **9**	*(35)* **28**	*(52)* **44**	*(70)* **77**	-	-
	1f ~ *7.00%*	-	*(21)* **21**	*(35)* **26***	*(54)* **58***	*(82)* **88**	-
	0.5f ~ *3.5%*	-	*(12)* **16**	*(21)* **19***	*(37)* **44***	*(70)* **78***	*(95)* **92***
	0.25f ~ *1.75%*	-	-	-	*(23)* **49**	*(54)* **64***	*(90)* **84***
	0.125f ~ *0.90%*	-	-	-	-	*(38)* **64**	*(82)* **80***

Table 2 – Calculated and experimentally determined donor chimerism after transplantation of various fractions of marrow from normal donors to progressively irradiated recipients. Groups of five mice, either CD45.2 or CD45.1, were irradiated with doses of 1 to 6 Gy. Two hours later, the mice were injected with congenic marrow in an amount ranging from the equivalent of 0.125 to 2 femurs. The calculated (theoretical) donor chimerism level *(numbers in italics and parentheses)* was calculated assuming a 100% engraftment efficiency of donor STRCs. The experimental chimerism (**numbers in bold**) was determined 5 weeks after transplantation in peripheral blood. The correlation coefficient between the calculated and the experimental values was $r = 0.9227$ ($p<0.0001$).
*indicates a mean from two experiments that used opposing combinations of CD45.1 and CD45.2 mice as donors and recipients, the remaining data are from a single experiment.

4.1.6 Immunosuppressive treatment to reduce immune disparity in the CD45.1/CD45.2 experimental system

Because the expected and calculated chimerism values deviated in recipient mice irradiated with radiation doses lower then 3 Gy, which suggested only a partial utilization of donor repopulating cells, the possibility of partial immune intolerance in the congenic mice (CD45.1, CD45.2) which had been suggested by van Os et al. [77] and by results of Tomita et al. [78] was tested. We applied an immunosuppressive treatment to normal (non-irradiated) mice, recipients of a standard dose of intravenously transplanted donor congenic bone marrow cells.

Groups of 3 CD45.2 non-irradiated recipients were transplanted with congenic donor cells in an amount corresponding to either half of a femur or two femurs. Mice were then i.p. injected with 350 mg/kg cyclophosphamide immediately or 24 hours after transplantation. Other groups of transplanted recipients were given 1 mg/100 ml or 2 mg/100 ml Dexamed in drinking water. Non-treated transplanted mice were used as controls. Donor engraftment determined 14 days and 1 month after transplantation is shown in Table 3.

		transplanted cells	donor engraftment		
			experimental		expected
			14d	30d	
A	**without suppression**	0.5f	-	0.13 ± 0.03	3.4
		2f	0.71 ± 0.21	0.49 ± 0.18	12.3
B	**CY (immediately)**	0.5f	1.71 ± 0.24	1.21 ± 0.23	3.4
		2f	5.46 ± 0.92	4.28 ± 0.71	12.3
	CY (24h)	0.5f	1.24 ± 0.42	1.23 ± 0.18	3.4
		2f	5.31 ± 0.57	4.47 ± 0.42	12.3
C	**Dexamed (1mg/100ml)**	0.5f	-	-	3.4
		2f	8.20 ± 0.97	5.80 ± 2.13	12.3
	Dexamed (2mg/100ml)	0.5f	2.05 ± 0.11	1.53 ± 0.38	3.4
		2f	7.10 ± 0.81	6.46 ± 2.15	12.3

Table 3 – Effect of immunosuppressive treatment on normal recipients of congenic bone marrow. Non-irradiated recipients were transplanted with congenic donor cells in an amount corresponding to either one-half of the femur or two femurs (**A**). The immunosuppressed mice were then treated with (**B**) 350 mg/kg cyclophosphamide immediately or 24 hours after transplantation; (**C**) Dexamed in dose of 1 mg/100 ml or 2 mg/100 ml of drinking water, respectively. Data are presented as mean ± SEM. (n=3) The theoretical donor chimerism levels for 100% engraftment efficiency are also indicated.

4.1.7 Stability of established chimeric hematopoiesis

Quantitative comparison of donor and host hematopoiesis in chimeric mice assumes that the donor and the host repopulating cells (STRCs and LTRCs) contribute equally to productive hematopoiesis, i.e. that one does not overcompete (overgrow) the other.

Values from these and other experiments (in total 21 independent experiments) in which a partial chimerism was induced in submyeloablatively irradiated mice by injecting congenic donor bone marrow cells from normal donors were used to evaluate the stability of engraftment during a three-month period. The results presented in Table 4 indicate an equal fitness of the competing donor and the host LTRCs in generating nucleated blood cells during the three-month period.

TBI	***Donor chimerism 3M***	***Donor chimerism 6M***	***Ratio 3M/6M***	***n***
4 Gy	58.2 ± 10.281	52.70 ± 9.780	1.13 ± 0.113	n = 5
5 Gy	87.20 ± 6.872	85.53 ± 8.583	1.02 ± 0.030	n = 3
6 Gy	89.48 ± 1.488	88.35 ± 1.964	1.02 ± 0.014	n = 13

Table 4 - Chimerism stability between 3 (3M) and 6 (6M) months after transplantation. Recipient mice, either CD45.2 or CD45.1, were irradiated with a sublethal dose of ionizing radiation (4 to 6 Gy) and were transplanted with congenic donor cells (mostly with an aliquot containing BMCs from a half femur). Donor chimerism was determined in peripheral blood after 3 and 6 months.

4.2 Restoration of pools of hematopoietic stem cells after bone marrow damage induced by sublethal irradiation

The hematopoietic tissue is one of the tissues which are very sensitive to damage by ionizing radiation. HSCs and progenitors of blood cells are increasingly destroyed by progressive doses of irradiation. The damage is lethal if none of the HSCs survive since only HSCs possess the capacity to restore whole hematopoiesis and maintain it permanently. HSCs restore damaged hematopoiesis by switching from proliferative quiescence to active proliferation and by switching between self-renewing asymmetric and self-renewing symmetric cell divisions. These fundamental processes in the stem cell biology and functioning are difficult to study because HSCs are very rare cells after the irradiation induced damage; only a very tiny fraction (single cells or only a few dozens of them) may survive in the whole body.

We developed a sensitive method for quantification of HSCs surviving irradiation by means of competition with a known number of transplanted donor HSCs (see 4.1.1, p.46) and utilized this method to study early phases of the restoration of the pool of HSCs from a very low number of HSCs surviving in mice irradiated with 6 Gy (^{60}Co). This was complemented by examination of progenitor pools and HSCs typified by particular cell surface epitope profiles and by examination of the general bone marrow condition and the recovery of blood cell production.

4.2.1 Recovery of STRCs and LTRCs after sublethal irradiation by 6 Gy determined by three functional assays

At various times after sublethal irradiation mice were injected with a defined amount of congenic BMCs corresponding to one-half of the femur (approximately 3.5% of total BMCs). Clonogenic repopulating cells (STRCs and LTRCs) were estimated

from resulting donor chimerism level. This was interpreted as reflecting the magnitude of empty niches available to the incoming donor cells. 100% donor hematopoiesis would indicate that all niches in the irradiated recipients were available to donor repopulating cells and 0% donor chimerism would indicate a full occupancy of the niches by the host STRCs and LTRCs. A chimerism level in between these extreme values indicates the fraction of niches lacking the endogenous host STRCs and LTRCs which are available to transplanted donor STRCs and LTRCs present in the standard dose corresponding to approximately 3.5% of their total normal value.

The second assay based on co-transplantation of regenerating BMCs, collected at different times after 6 Gy irradiation, with normal congenic BMCs, mixed in determined ratios to lethally irradiated mice.

Finally, BMCs regenerating 30 or 60 days after 6 Gy irradiation or normal BMCs were transplanted to lethally irradiated syngenic mice. The ability of the regenerating and normal BMCs to "close" hematopoietic tissue against a defined dose of congenic normal BMCs was tested after 20 and 30 days.

4.2.1.1 STRCs and LTRCs determined according to decreased availability of niches to transplanted congenic BMCs

Groups of 5 CD45.1 mice were exposed to irradiation by 6 Gy and with a delay ranging from 2 hours to 30 days they were transplanted with congenic CD45.2 BMCs from one-half of the femur of normal donors. Resulting chimerism which was composed of donor and host cells was determined after 1 month in blood and again after 6 months in blood and bone marrow. Results from six experiments consisting in total of 25 groups and presenting both the donor and the host chimerism levels are showed in Figure 16. Nonlinear regression curves were fitted to the experimental data and the time when the host and the donor cells are equally represented was indicated (13.9 days for

the early repopulation determined one month (STRCs) and 18.0/18.5 days in case of the long-term repopulation (LTRCs)). The equal donor and host chimerism indicates that the number of the host STRCs and LTRCs were the same as in the transplanted BMCs (3.5% of total).

Lower chimerism in peripheral blood after 1 month compared to that after 6 months (Figure 16 and confirmed in other 8 experiments which are not presented) was unexpected regarding a higher sensitivity of STRCs to irradiation than of LTRCs. To reveal this difference, a group of 8 CD45.2 recipient mice were irradiated by a sublethal dose 6 Gy and transplanted with congenic CD45.1 BMCs corresponding to one-half of the femur. One month after transplantation, the total chimerism was determined in the peripheral blood as well as in lineages of nucleated blood cells ($B220^+$ B-cells, $Gr\text{-}1^+$ granulocytes, $Mac\text{-}1^+$ macrophages and $CD3^+$ T-cells). Four of the mice were sacrificed and chimerism was also determined in the bone marrow (Figure 17A). Lower donor chimerism in peripheral blood compared to bone marrow can be explained by dominance of the host-derived $CD3^+$ cells (more than 70% of all $CD3^+$ cells) in blood 1 month after transplantation. Two months after transplantation, the ratio of donor- and host-derived $CD3^+$ cells was already reversed; most of the $CD3^+$ cells were of donor-origin (78%) similar to chimerism level in other blood cell lineages (Figure 17B). Bone marrow does not contain any significant number of T-cells and consequently is not affected by the delayed onset of donor T-cell production.

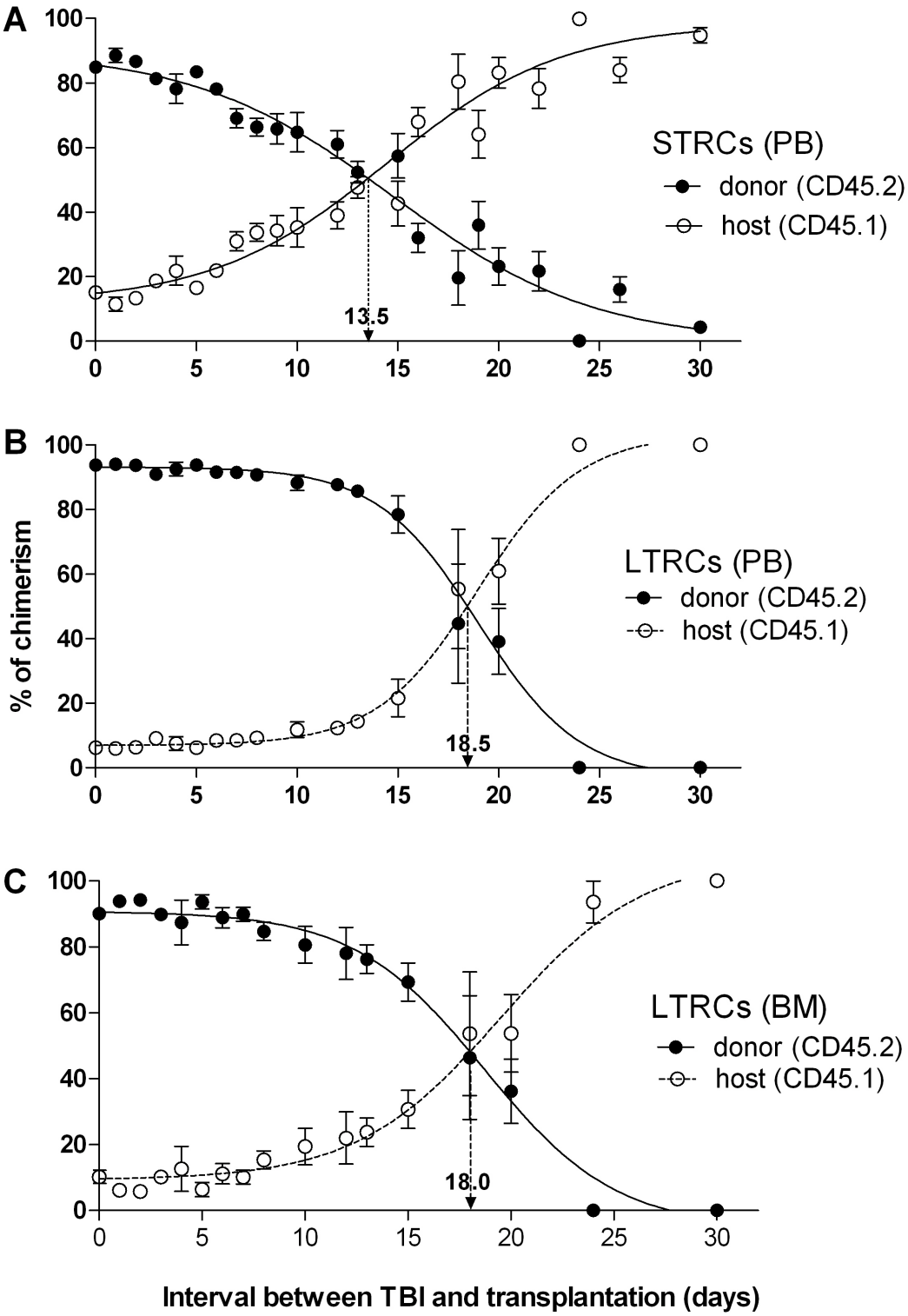

Figure 16 – Chimerism of donor cells transplanted with a delay from 2 hours to 30 days and appropriate host chimerism. Chimerism was determined in peripheral blood 1 month (**A**) and 6 months (**B**) after transplantation; after 6 months the chimerism was determined also in bone marrow (**C**). Data are presented as mean ± SEM (n=5-34). Error bars represent ± 1 SEM for 5 recipients from one to six separate experiments. The time of equally representation of the donor and host cells was estimated using nonlinear regression curves. Regression analysis: nonlinear regression using the log (inhibitor) vs. response curve - variable slope (four parameters) calculation.

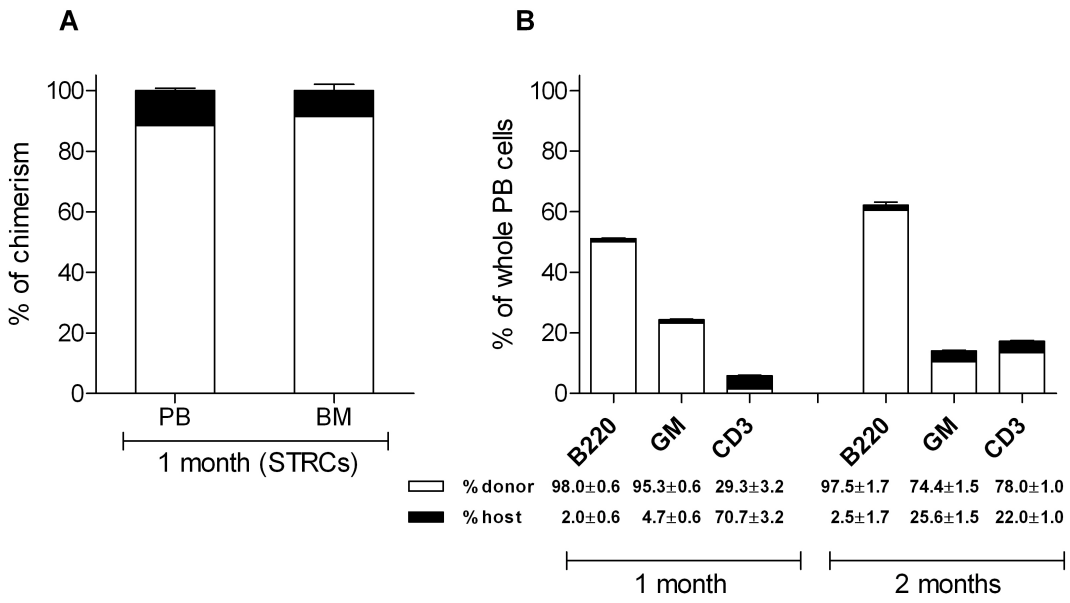

Figure 17 – Difference between the short-term chimerism in peripheral blood and bone marrow. Chimerism of STRCs in peripheral blood and bone marrow (**A**). Chimerism in peripheral blood subpopulations (B220, GM, CD3) one and two months after transplantation (**B**). Data are presented as mean ± SEM (n=4-8).

4.2.1.2 STRCs and LTRCs estimated according to repopulating ability in competitive transplantation test

The presence of repopulating cells, progenitors (STRCs) and HSCs (LTRCs) in the bone marrow regenerating after sublethal irradiation with 6 Gy was tested by their co-transplantation with normal congenic BMCs in defined ratios. CD45.1 BMCs were collected at various times after exposition of mice to irradiation and co-transplanted with normal CD45.2 BMCs from congenic donors in either 1:1 or 10:1 ratio into lethally irradiated (9 Gy) CD45.2 recipients. Co-transplantation of normal BMCs from CD45.1 and CD45.2 donors mixed in 1:1 ratio provided expected chimerism of approximately 50%. The regenerating BMCs competed poorly with that of normal BMCs during the entire follow-up period extending from 12 to 30 days after irradiation. Despite that the cellularity of femoral bone marrow was already normal after 20, 25 and 30 days (see Figure 24A), and most of the niches were no longer available to transplanted cells after 20 and 30 days (Figure 16), the regenerating BMCs were

significantly inferior to normal BMCs even when administered in a 10:1 ratio (Figure 18). Repopulating activity of the regenerating BMCs collected after 30 days was approximately only 4 – 6%[1] of normal BMCs for STRCs. A similar estimate could not be done for LTRCs since the chimerism levels were too low even when the regenerating BMCs were given in a 10 fold excess.

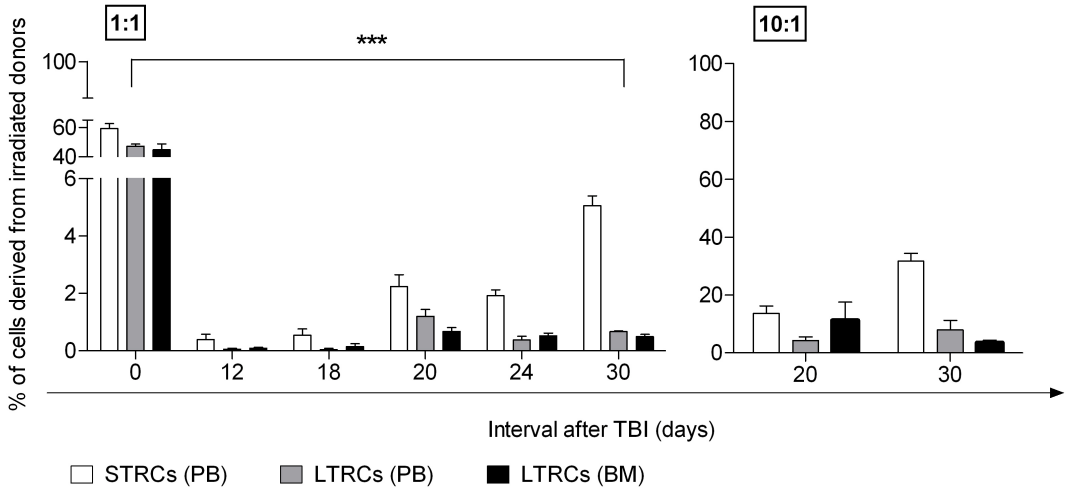

Figure 18 – Engraftment of donor cells collected at various time points after irradiation by 6 Gy and co-transplanted with normal non-irradiated congenic cells into lethally irradiated recipients in a 1:1 or 10:1 ratio. Engraftment was determined in peripheral blood 1 month (white bars) and six months (grey bars) after transplantation; after six months the chimerism was determined also in bone marrow (black bars). Data are presented as mean ± SEM (n = 5). Significance of difference from the controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

4.2.1.3 STRCs and LTRCs estimated according to ability to close hematopoiesis of lethally irradiated mice to transplanted cells

Repopulating potential of regenerating and normal BMCs was compared in lethally irradiated recipients. The potential was evaluated according to inhibition of the engraftment of congenic BMCs administered after 20 or 30 days. Groups of 3 CD45.1 mice were irradiated by a sublethal dose of 6 Gy. BMCs were collected either 30 or 60 days after the irradiation and also from normal mice of the same sex and age, and transplanted in an amount corresponding to one-half of the femur into groups of lethally

[1] $\%D_{ch}/(100-\%D_{ch})$ x 100 in the case of the 1:1 ratio and multiplied by 10 in the case of 10:1 ratio, respectively; D_{ch} is donor chimerism

(9 Gy) irradiated syngenic mice. Twenty or 30 days later, recipients were transplanted with BMCs from one-half of the femur of normal congenic CD45.2 donors (Figure 19A). Engraftment of the congenic BMCs was assessed after 1 and 6 months in blood samples (Figure 19B, C) and after 6 months also in whole bone marrow and in LSK subpopulation (Figure 20). Transplantation of normal BMCs to lethally irradiated syngenic mice inhibited engraftment of congenic STRCs below 10% already after 20 days (Figure 19B). Engraftment of congenic LTRCs was inhibited by normal BMCs to 29% in blood and 37% in bone marrow after 20 days (Figure 19C, Figure 20A). After 30 days the engraftment of congenic LTRCs was inhibited to 20% in blood and 29% in bone marrow (Figure 19C, Figure 20A). Transplantation of the regenerating BMCs, collected 30 days after irradiation by 6 Gy, and to a lesser degree also the regenerating BMCs collected after 60 days, inhibited engraftment of congenic BMCs significantly less (Figure 19B, Figure 20A).

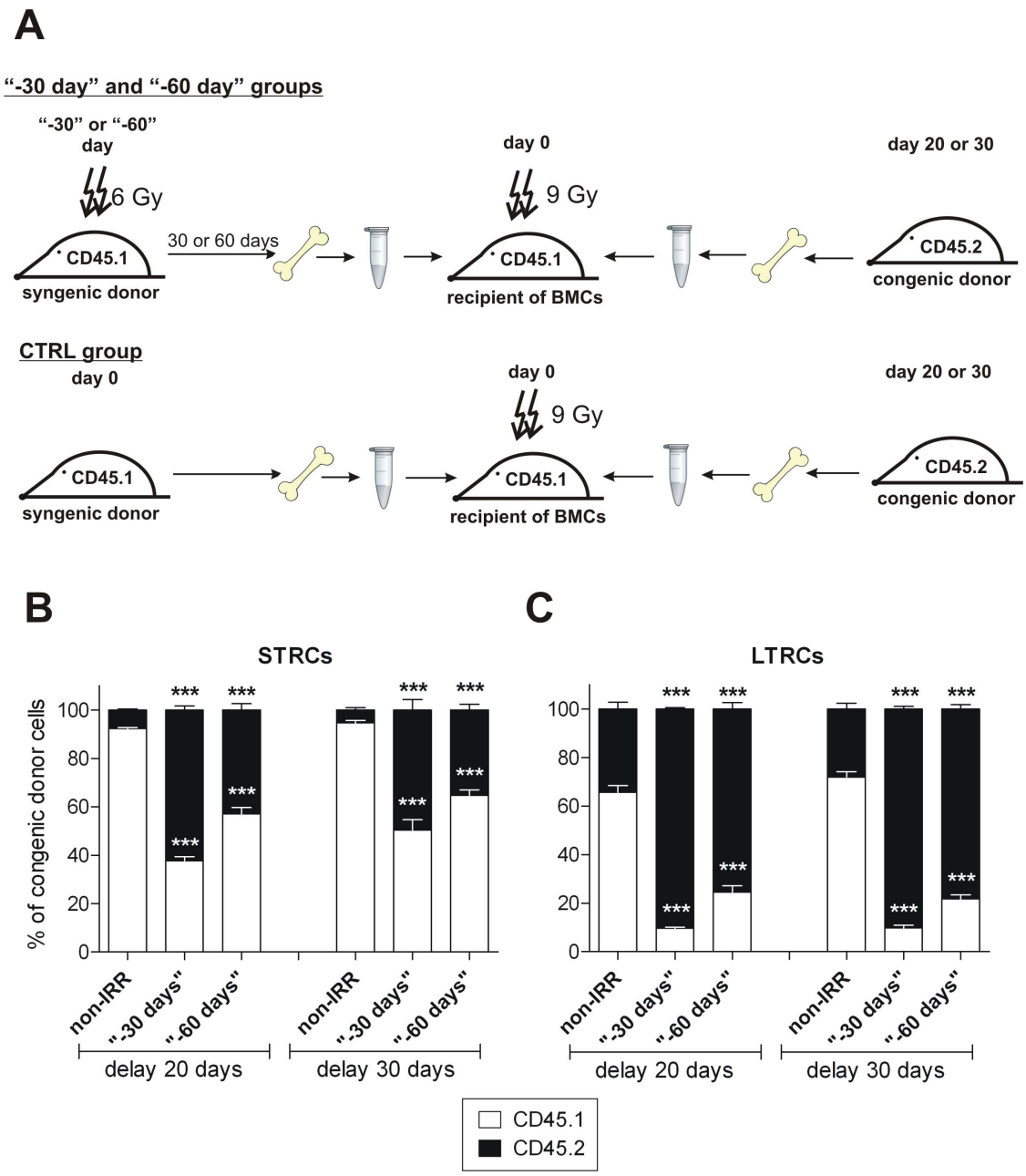

Figure 19 – Chimerism of syngenic (empty parts of the columns) and congenic (black parts of the columns) cells in blood determined 1 month (STRCs) or 6 months (LTRCs) after the congenic transplantation. Congenic BMCs, testing the availability of niches to donor BMCs, were transplanted 20 or 30 days after transplantation of the syngenic BMCs. Syngenic BMCs was from normal non-irradiated (non-IRR) mice or from mice regenerating from a 6 Gy irradiation either 30 days ("-30 days") or 60 days ("-60 days"). Recipients of the syngenic BMCs, followed by congenic BMCs after 20 or 30 days, were lethally irradiated (**A**). The chimerism between syngenic and congenic cells was determined in blood collected either 1 month (**B**) or 6 months (**C**) after the congenic transplantation. Data are presented as mean ± SEM (n=5). Significance of difference from the non-irradiated controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

Six months after the transplantation of congenic BMCs the chimerism between syngenic and congenic cells was also determined in the LSK population, which is a subfraction of BMCs enriched in progenitors and HSCs. In the mice transplanted with

normal syngenic BMCs, congenic LSK cells represented 36% and 20% when the congenic transplant was given after 20 days or 30 days, respectively. The regenerating BMCs, collected 30 days after 6 Gy irradiation, turned out to be very weak in repopulating of the hematopoietic tissue of lethally irradiated syngenic mice since more than 95% of LSK cells was of the congenic phenotype. However, the regenerating BMCs collected 60 days after 6 Gy irradiation repopulated the LSK population of lethally irradiated syngenic mice to the same degree as the BMCs (Figure 20B).

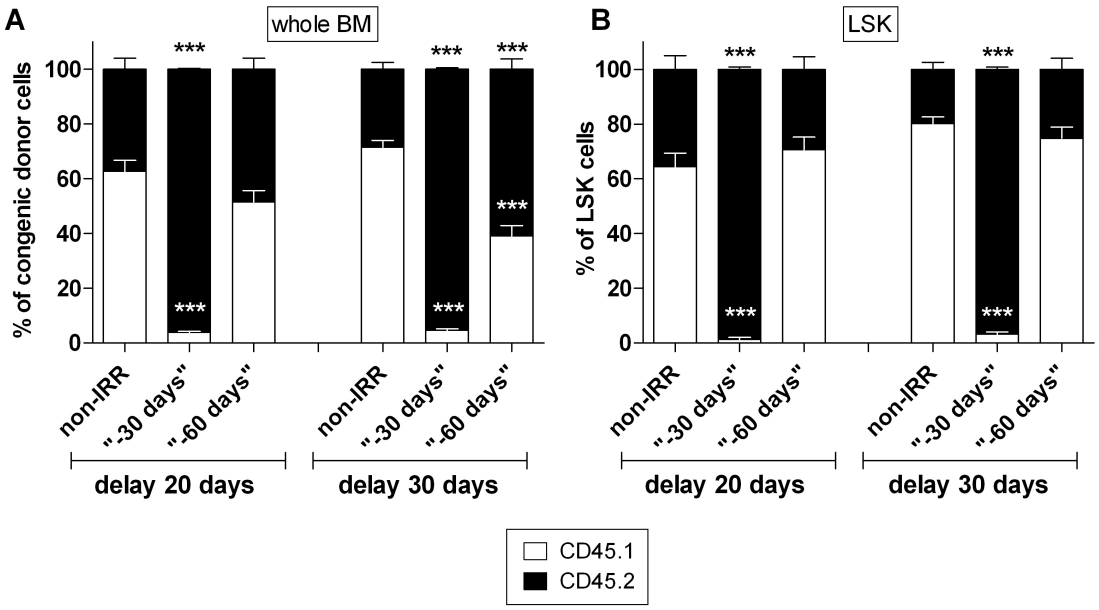

Figure 20 – Chimerism of syngenic (empty parts of the columns) and congenic (black parts of the columns) cells in bone marrow determined six months after the congenic transplantation. Congenic BMCs, testing availability of niches to donor cells, were transplanted 20 or 30 days after transplantation of the syngenic BMCs. Syngenic BMCs were from normal non-irradiated mice (non-IRR) or from mice regenerating from a 6 Gy irradiation 30 days ("-30 days") or 60 days ("-60 days"). Recipients of the syngenic BMCs, followed after 20 or 30 days by congenic BMCs, were lethally irradiated. The chimerism between syngenic and congenic cells was determined in whole bone marrow (**A**) and in the LSK population (**B**). Data are presented as mean ± SEM (n=5). Significance of difference from the non-irradiated controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

4.2.2 State of the host hematopoiesis during its regeneration from damage by sublethal 6 Gy irradiation

General state of hematopoiesis during its regeneration from sublethal irradiation by 6 Gy was examined between days 10 and 30. Samples of blood, bone marrow and spleens were collected and subsequently analyzed. Complete blood count with differential of white blood cells, cellularity of bone marrow, percentages of differentiated bone marrow subpopulations, frequencies of progenitors and HSCs characterized by the LSK and CD150/CD48 phenotypes and their proliferation activity were determined.

4.2.2.1 Restoration of blood cell production

Complete blood count with differential of white blood cells was determined in control mice and at various time points after sublethal irradiation by 6 Gy. Numbers of total white blood cells (WBC), red blood cells (RBC), platelets (PLT) and the hemoglobin concentration (Hb) are shown in Figure 21. Percentages of lymphocytes (Lym), neutrophils (Neu), eosinophils (Eos), basophils (Bas) and monocytes (Mon) are presented in Figure 22. On day 15 after irradiation the WBC and platelet counts started to increase. The red blood cell count and hemoglobin concentration reached their nadirs on day 15 and started to increase on day 20. These results thus demonstrate significant blood cell production, derived from the progenitors (STRCs) and HSCs (LTRCs) which survived irradiation, after day 15.

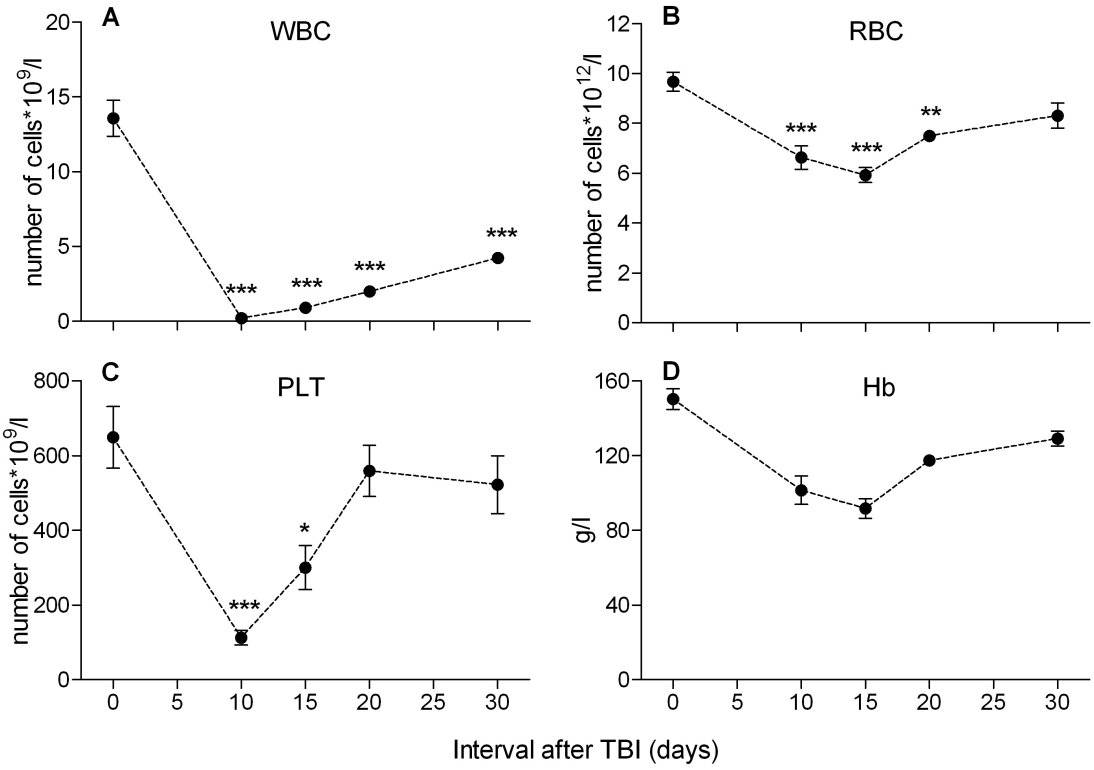

Figure 21 – Complete blood cell count at various time points after irradiation by 6 Gy. Levels of white blood cells (WBC; **A**), red blood cells (RBC; **B**), platelets (PLT; **C**), and hemoglobin concentrations (Hb, **D**) are presented as mean ± SEM (n = 3-6). Significance of difference from the controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

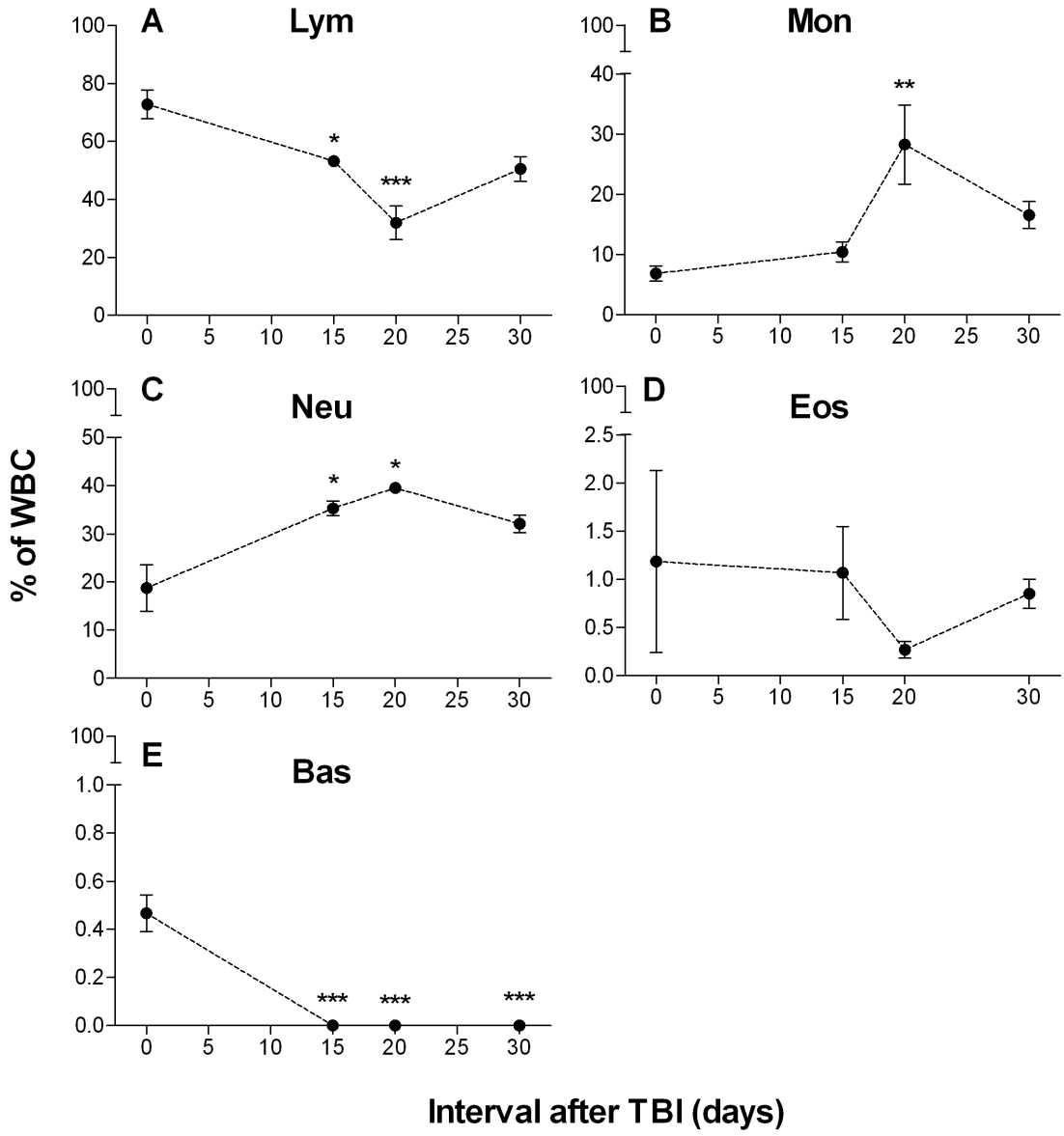

Figure 22 – Differential of white blood cells. Percentages of lymphocytes (Lym; **A**), monocytes (Mon; **B**), neutrophils (Neu; **C**), eosinophils (Eos; **D**), and basophils (Bas; **E**) are calculated from the total white blood cell count. Data are presented as mean ± SEM (n = 3-6). Significance of difference from the controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

4.2.2.2 Spleen weight

The splenic hematopoiesis, which occurs naturally in mice, becomes increasingly active in conditions connected with accelerated blood cell production. This activation was monitored by determining spleen weight at different time points after irradiation with 6 Gy. The size and weight of spleens was reduced 10 days after irradiation, started to increase on day 12 and peaked on day 15 with a weight exceeding that of non-irradiated control mice approximately twice (Figure 23). The spleen weight changes did not reach statistically significant levels when tested by analysis of variance.

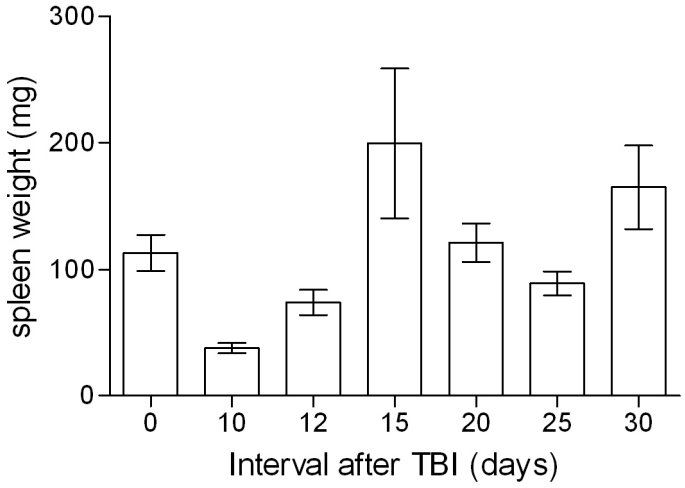

Figure 23 – Spleen weights at various time points after 6 Gy. Data are presented as mean ± SEM (n = 3-6). Difference from the controls at individual time points was not statistically significant.

4.2.2.3 Bone marrow cellularity and differentiated precursors of blood cells

Bone marrow cellularity and numbers of bone marrow subpopulations defined by specific antigenic markers (anti-Gr-1/Mac-1 for granulocytes and macrophages (GM cells), anti-B220 for B-cells and anti-Ter119 against erythroid cells) were determined at different times after sublethal irradiation by 6 Gy. BMCs from CD45.2, 5-6-months old male mice were collected at various time points after irradiation by 6 Gy. Non-irradiated mice were used as control. Total bone marrow cellularity started to increase on day 12 and reached the values of control on day 20. The myeloid precursors (Ter119

and GM cells) achieved normal values between day 15 (Ter119 significantly elevated, $P<0.05$) and 20 (GM). B cells (B220) peaked on day 25 but did not recover completely. On day 30, Ter119 cells and B220 cells were significantly decreased compared to control values and also the total bone marrow cellularity decreased (Figure 24).

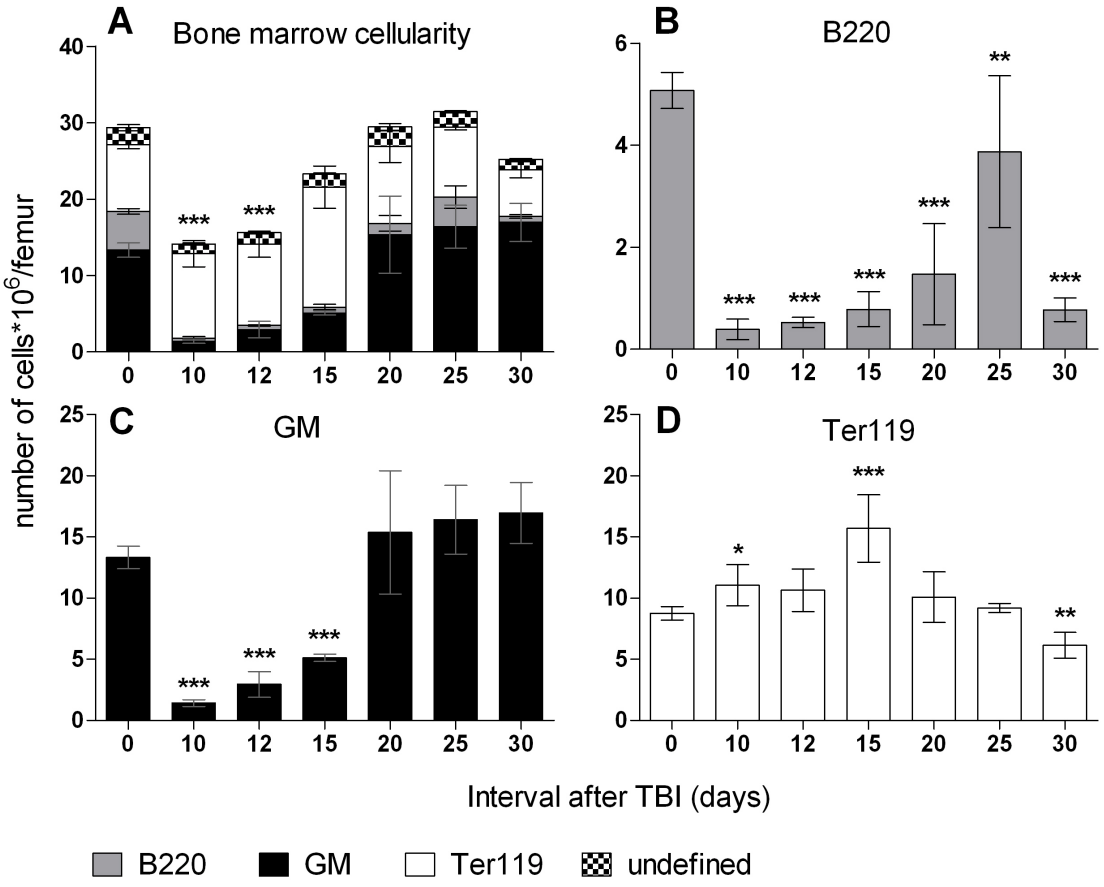

Figure 24 – Bone marrow cellularity and representation of subpopulations. Number of total BMCs (**A**), B200 (**B**), GM (**C**), and Ter119 (**D**) cells in 1 femur was determined. Data are presented as mean ± SEM (n = 3). Significance of difference from the controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

4.2.2.4 Subpopulations of lineage negative (low) c-kit positive Sca-1 positive (LSK) cells

Bone marrow from irradiated and control mice was further analyzed for subpopulations known to be highly enriched for progenitors and HSCs. Numbers of LSK cells determined in non-irradiated control and at various times after 6 Gy are shown in Figure 25. The LSK population was further subdivided into 4 populations according to surface markers CD150 and CD48 (Figure 26).

Approximately 0.23% of the BMCs belonged to the $Lin^{low}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) population in control mice (~65 000 cells per femur). Irradiation significantly reduced their numbers; however, the population was fully recovered by day 30.

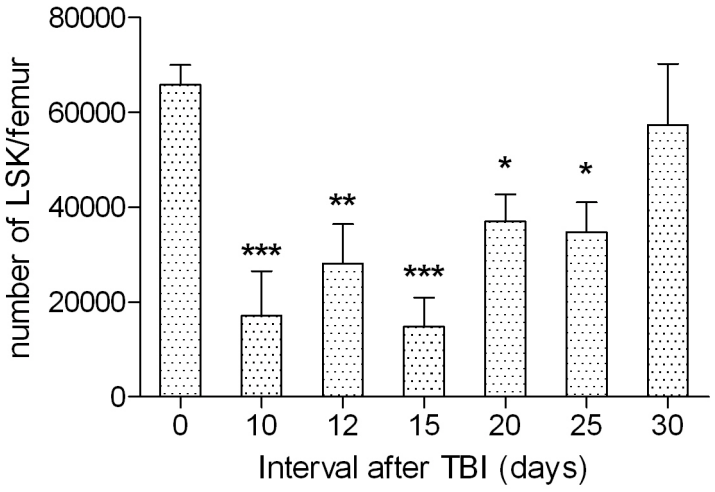

Figure 25 - Numbers of LSK cells per femur. Data are presented as mean ± SEM (n = 3-6). Significance of difference from the controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

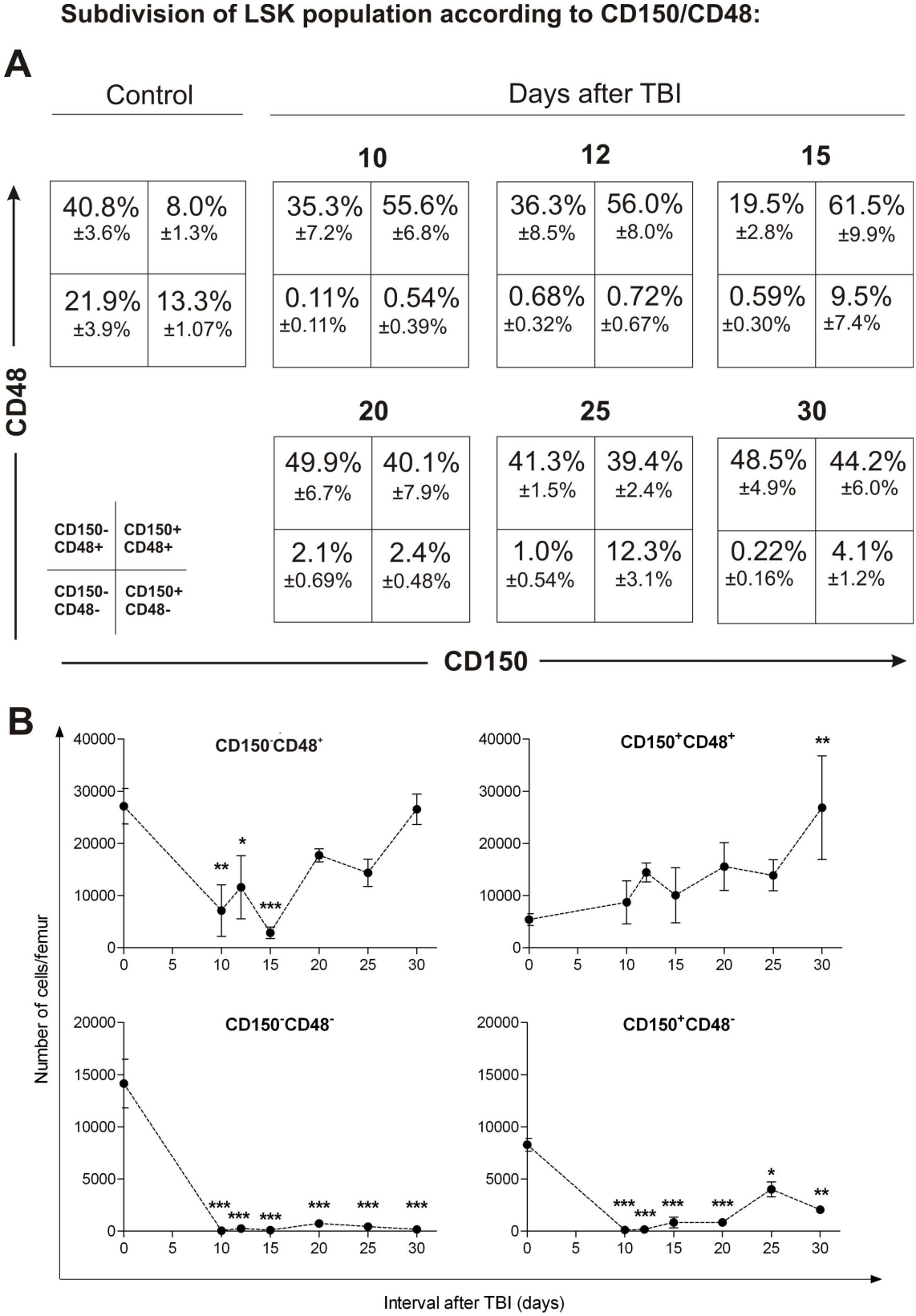

Figure 26 – Subdivision of LSK population according to surface markers CD150 and CD48 in control non-irradiated mice and at various time intervals after sublethal irradiation by 6 Gy. Representation of all 4 populations as percentages of LSK (**A**); Numbers of the cells in femoral bone marrow (**B**). Data are presented as mean ± SEM (n = 3-6). Significance of difference from the controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

Approximately 41% of LSK cells (0.093% of BMCs) carried the $CD150^{-}CD48^{+}$ phenotype characteristic for progenitors [6]. On days 10 to 15 after irradiation their numbers were decreased to 10 – 30%. This was followed by recovery of their numbers which reached normal levels on day 30 (Figure 26B).

The $CD150^{-}CD48^{-}$ phenotype characteristic for multipotent hematopoietic progenitors (MPP) [6] represented approximately 22% of LSK cells (0.05% of BMCs). After irradiation, this population almost completely disappeared (<1% of LSK) and their numbers remained very low still 30 days after sublethal irradiation (Figure 26B).

The $CD150^{+}CD48^{-}$ phenotype characteristic for HSCs [6] represented 13.3% of LSK cells (0.04% of bone marrow cells). Ten days after irradiation their numbers were reduced to approximately 1% of normal values. Their numbers steadily slowly increased between days 10 and 30 but remained subnormal for the whole follow-up period.

The $CD150^{+}CD48^{+}$ cells represented only approximately 8% of LSK cells (0.02% of BMCs) in control mice. After irradiation, their relative representation increased significantly throughout the whole follow-up period (Figure 26A). They absolute numbers were normal and more than doubled on day 30 (Figure 26B).

The LSK SP population, which normally represented 0.12 % of BMCs, is shown in Figure 27. Ten days after irradiation their numbers were reduced to 4.6% of normal values and remained low till day 25, however were ~73% of the control values on day 30.

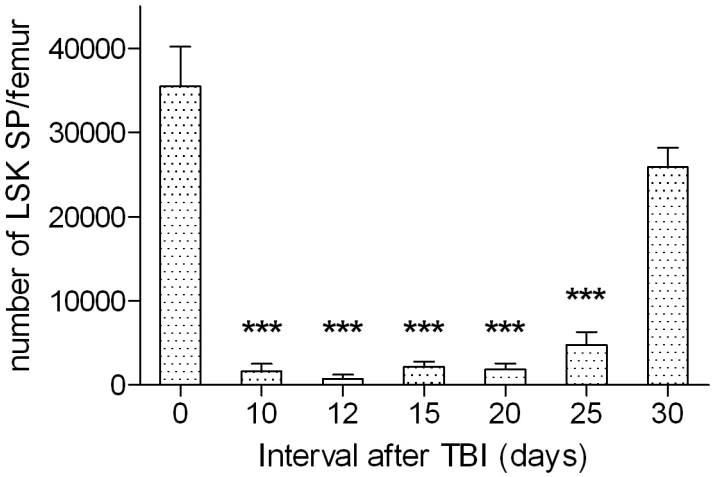

Figure 27 – Numbers of LSK SP cells/femur in bone marrow regenerating from sublethal irradiation by 6 Gy. Data are presented as mean ± SEM (n = 3-6). Significance of difference from the controls at individual time points: $^{***}P < 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$.

To determine the cell cycle status of LSK cells and its subpopulations according to CD150/CD48, groups of four CD45.1 female mice (2-3 months old) were irradiated with a sublethal dose of 6 Gy. Non-irradiated mice were used as control. Various times after irradiation the mice were sacrificed and BMCs collected from femurs were stained for cell cycle analysis as described in 3.8.4. Cell cycle status of LSK, LSK $CD150^{-}CD48^{+}$, LSK $CD150^{+}CD48^{-}$ and LSK $CD150^{+}CD48^{-}$ populations is shown in Figure 28.

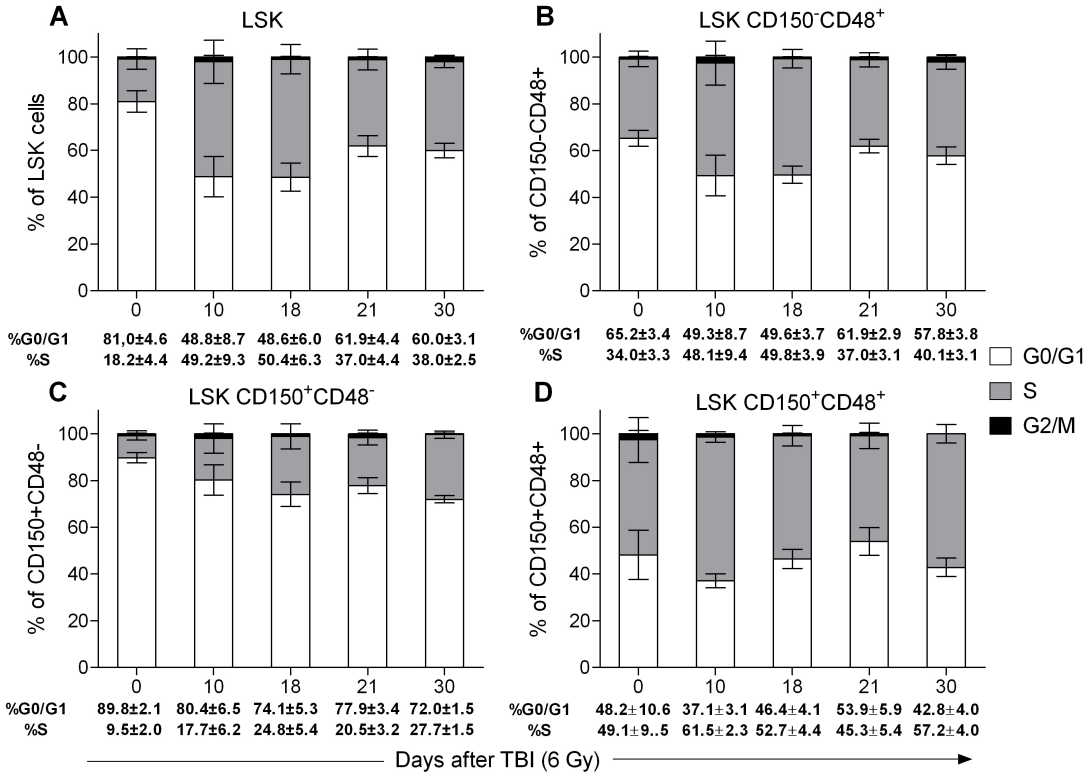

Figure 28 – Cell cycle analysis after 6 Gy in LSK (A), LSK $CD150^{-}CD48^{+}$ (B), LSK $CD150^{+}CD48^{-}$ (C) and LSK $CD150^{+}CD48^{+}$ populations. Data are presented as mean ± SEM (n = 3-6).

4.3 Restoration of pools of hematopoietic stem cells after lethal irradiation and rescue transplantation of syngenic BMCs

Availability of niches for STRCs and LTRCs after lethal irradiation and immediate application of syngenic rescue transplant was tested by delayed transplantation of congenic BMCs. Moreover, the possibility of niche saturation was investigated after repeated large transplantations of syngenic BMCs administered during the first four days after lethal irradiation. Available niche space was determined by transplantation of a defined dose of congenic BMCs and subsequent analysis of resulting chimeric hematopoiesis.

4.3.1 Engraftment of congenic BMCs transplanted with a delay from 2 hours to 180 days to lethally irradiated mice given a rescue syngenic transplant of different size immediately after irradiation

Similarly to experiments using sublethal irradiation, the process of restoration of the STRCs and LTRCs pools was examined after lethal irradiation and subsequent syngenic rescue transplantation. At various times after lethal irradiation with 9 Gy and immediate rescue transplantation of syngenic BMCs, recipients received a defined dose of congenic BMCs. The chimerism level derived from congenic BMCs, determined in blood after 1 and 6 months and in bone marrow after 6 months, was interpreted as reflecting the availability of empty niches to congenic donor STRCs and LTRCs in course of the regeneration induced by previous syngenic transplant. Table 5 shows the expected chimerism if both transplants were performed simultaneously, i.e. when STRCs and LTRCs contained in transplanted syngenic BMCs could not yet multiplicate

and expand their pools and hereby inhibit engraftment of the congenic repopulating cells (STRCs and LTRCs).

syngenic transplant	*congenic transplant*	*expected congenic chimerism if administered simultaneously*
1/200f	1/2f	99%
1/100f	1/2f	98%
1/10f	1/2f	83%
0.5f	2f	80%
2f	2f	50%

Table 5 – Combination of utilized doses of transplanted syngenic and congenic BMCs and expected congenic chimerism in the case of their simultaneous transplantation

4.3.1.1 Rescue syngenic transplant corresponding to 1/200 - 1/10 of the femoral bone marrow

Engraftment of congenic BMCs from one-half of the femur and transplanted at different times after lethal irradiation and immediate rescue transplantation of syngenic BMCs corresponding to 1/200 or 1/10 of the femur is presented in Figure 29.

Groups of 5 CD45.2 mice were exposed to lethal irradiation (9 Gy) and were immediately injected with a syngenic rescue transplant in an amount of 1/200 or 1/10 of the femur. They were subsequently transplanted with congenic CD45.1 BMCs from one-half of the femur with a delay from 2 hours to 30 days. Resulting chimerism of donor cells, which was determined after 1 month in peripheral blood (STRCs) and again after 6 months in peripheral blood and bone marrow (LTRCs), is shown in Figure 29.

In the experiments using 1/10 of syngenic femoral BMCs, transplantation of congenic BMCs was delayed also by 40 days. Resulting chimerism was almost undetectable (chimerism was below 0.1%; data not shown)

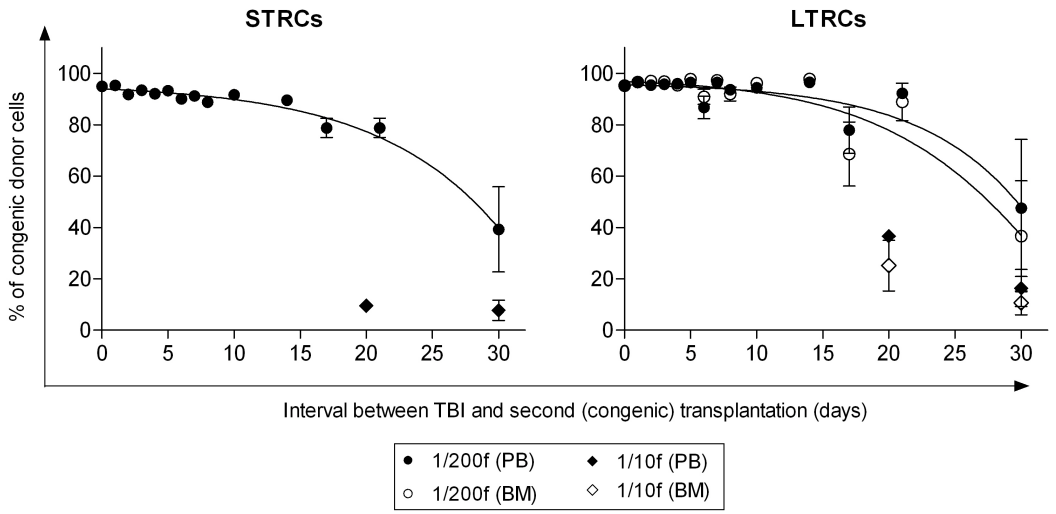

Figure 29 – Chimerism of congenic donor cells transplanted with a delay from 2 hours to 30 days after 9 Gy irradiation and rescue syngenic transplantation. Chimerism was determined in peripheral blood 1 and 6 months after transplantation of congenic BMCs; after 6 months the chimerism was determined also in bone marrow. As rescue syngenic transplant were administered BMCs corresponding to 1/200 of the femur (black dots – STRCs and LTRCs in peripheral blood, empty dots – LTRCs in bone marrow) or to 1/10 of the femur (black squares – STRCs and LTRCs in peripheral blood, empty squares – LTRCs in bone marrow). Data are presented as mean ± SEM (n=5). Regression analysis: nonlinear regression using the log (inhibitor) vs. response curve - variable slope (four parameters) calculation.

We next tested whether a higher dose of congenic BMCs collected from 2 femurs will demonstrate available niches 60 or 180 days after irradiation and syngenic transplantation. Groups of 6 CD45.2 mice were lethally irradiated (9 Gy) and transplanted with syngenic BMCs from one-half of the femur. After 60 or 180 days they received congenic CD45.1 BMCs from two femurs. One and six months after this second congenic transplantation, blood and bone marrow were examined for presence of cells derived from the congenic transplant. Chimerism resulting from the transplantation of congenic BMCs from two femurs was not significantly different between normal non-irradiated and lethally irradiated and rescue-transplanted mice (Figure 30).

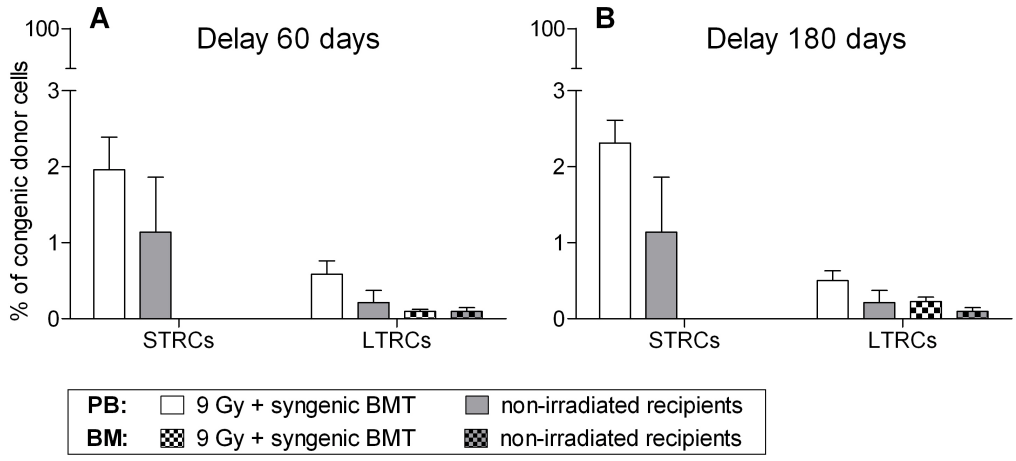

Figure 30 – Chimerism of donor cells transplanted with a delay 60 (A) and 180 days (B) after lethal irradiation and immediate syngenic transplantation. Groups of 6 CD45.2 mice were lethally irradiated (9 Gy) and transplanted with syngenic BMCs from one-half of the femur. After 60 or 180 days they received congenic CD45.1 BMCs from two femurs. Chimerism was determined in peripheral blood 1 month (STRCs) and 6 months (LTRCs). After 6 months, the chimerism was determined also in bone marrow. Data are presented as mean ± SEM (n=6).

4.3.1.2 Rescue syngenic transplant consisting of BMCs from 2 femurs

Groups of 5 CD45.1 mice were irradiated with a lethal dose of 9 Gy and immediately injected with syngenic BMCs in an amount corresponding to two femurs (approximately ~40.10^6 BMCs). With a delay from 2 hours to 17 days they received congenic CD45.2 BMCs from two femurs per mouse. Chimerism resulting from the second, congenic transplant was determined after 1 and 6 months. The chimerism data obtained after 1 month reflected predominantly the engraftment of STRCs. Analogously, chimerism determined after 6 months was from LTRCs. Due to the experimental setup, a 50% chimerism was expected in the case when competing syngenic and congenic repopulating cells (STRCs and LTRCs) had the same chance to engraft into supportive niches. This was likely to be the case at the time point "0", when the congenic transplant was given within two hours after the syngenic one. Unexpectedly, syngenic transplant corresponding to approximately 14% of total murine BMCs (two femurs) did not measurably inhibit engraftment of LTRCs from the same amount of congenic BMCs for more than two weeks (Figure 31B). On the other hand,

engraftment of STRCs progressively decreased (Figure 31A). After 10 days the engraftment of STRCs was approximately 10%. Since recipient mice irradiated with a lethal dose would be still highly immunosuppressed, the value of 10% could indicate a complete restoration of STRCs pool from the rescue syngenic transplant.

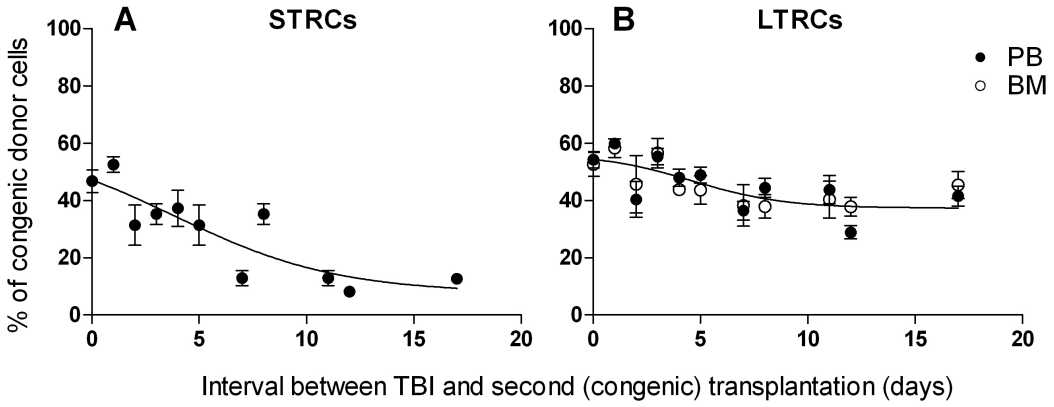

Figure 31 – Chimerism of congenic donor cells transplanted with a delay from 2 hours to 17 days after lethal irradiation and immediate syngenic transplantation. Chimerism was determined in peripheral blood 1 month (STRCs, **A**) and 6 months (LTRCs, **B**, black dots) after second congenic transplantation and after 6 months also in bone marrow (**B**, empty dots). Data are presented as mean ± SEM (n=5). Regression analysis: nonlinear regression using the log (inhibitor) vs. response curve - variable slope (four parameters) calculation.

4.3.2 Saturation of hematopoietic stem cell niches with repeated syngenic transplantation of BMCs from 2 femurs

The high availability of niches for LTRCs persisting still on day 17 after transplantation of a large dose of BMCs (from two femurs corresponding to ~14% of total BMCs) prompted us to test whether repeated administration of such doses of normal BMCs will limit availability of niches to transplanted congenic BMCs.

Groups of 4 CD45.1 recipients were irradiated with lethal dose of TBI (9 Gy) and were transplanted as shown in Table 6. Chimerism of donor cells from the second, congenic transplantation, which was determined one and 6 months later in blood and after 6 months also in bone marrow, is shown in Figure 32. It is compared to an

expected (theoretical) chimerism calculated from the ratio between total amounts of the syngenic and congenic BMCs administered

	day 0	*day 1*	*day 2*	*day 3*	*day 4*
group 1 0%*	-	-	-	-	2f CD45.2
group 2 14%*	2f CD45.1	-	-	-	2f CD45.2
group 3 28%*	2f CD45.1	2f CD45.1	-	-	2f CD45.2
group 4 42%*	2f CD45.1	2f CD45.1	2f CD45.1	-	2f CD45.2
group 5 56%*	2f CD45.1	2f CD45.1	2f CD45.1	2f CD45.1	2f CD45.2

Table 6 – Transplantation schema of niche saturation. Groups of 4 recipient mice were irradiated by 9 Gy on day 0. Individual groups were transplanted by one to four doses of syngenic BMCs corresponding to two femurs per dose (approximately 14% of total BMCs). On day 4 they were all transplanted with congenic BMCs from two femurs.
* Percentage of the whole syngenic BMCs administered.

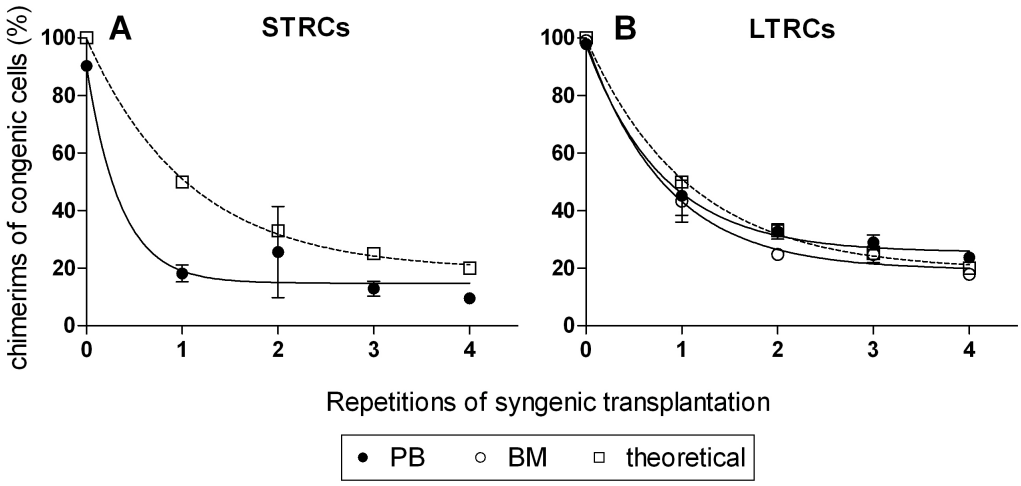

Figure 32 - Chimerism of congenic cells transplanted on day 4 after lethal irradiation followed by a single or repeated transplantations of syngenic BMCs according to Table 6. Chimerism was determined in peripheral blood 1 (**A**, STRCs, black dots) and 6 months (**B**, LTRCs, black dots) after TBI followed by repeated syngenic transplantation and after 6 months also in bone marrow (**B**, empty dots). Empty squares are estimates of theoretical chimerism of congenic donor cells. Data are presented as mean ± SEM (n=4). Regression analysis: nonlinear regression using the log (inhibitor) vs. response curve - variable slope (four parameters) calculation.

Niche availability for the congenic STRCs was significantly suppressed already by one dose of syngenic BMCs and results deviated from the theoretical ones (Figure 32A). In contrast, availability of niches for the congenic LTRCs was not significantly limited by one dose of syngenic BMCs (chimerism close to 50%), but became progressively limited after their repeated doses, in a close agreement with the theoretical values. It was ~20% after 4 doses of syngenic BMCs; expected (theoretical) chimerism, was calculated under assumption that the syngenic and congenic cells were administered together, i.e. in ratios 1:1 (expected chimerism 50%) up to 4:1 (expected chimerism 20%).

5 DISCUSSION

Hematopoietic stem cells (HSCs), although they have been widely studied for more than a half century, are still the subject of intensive investigation. Hematopoietic stem cells are very rare inside the bone marrow and this makes their research difficult. Although at this time they are phenotypically well characterized, the most reliable and rigorous method of their determination is a functional test, when after their transplantation into a damaged hematopoietic microenvironment they reestablish and maintain hematopoiesis for a long time [79].

In this study we monitored the effect of ionizing radiation on HSCs and their microenvironment with focus on ensuing regeneration. Deciphering of processes underlying regeneration of tissues from their stem cells is of the utmost importance since they are basic not only for generation of new tissues during embryogenesis but also for their regeneration after damage and, particularly, for tumor growth and relapsing of tumors after a therapy. We have chosen the regeneration of hematopoietic tissue as it is the most affordable for a quantitative evaluation of the regeneration process. The ultimate goal of the study was contribution to existing knowledge of the stem cell biology in general and, specifically, an expansion of understanding of the processes driving the regeneration of hematopoietic tissue derived from hematopoietic stem cells.

5.1 Efficiency of HSCs transplantation

Irradiation is assumed to generate space in the recipient's hematopoietic tissue, enabling engraftment of transplanted HSCs (reviewed in [10]). Although conditioning of mice with irradiation negatively influences the homing of transplanted BMCs from the circulation to the bone marrow [80-82], the HSCs present in transplanted BMCs engraft with a high efficiency [2,66,72,78]. However, not all studies confirmed this high engraftment efficiency of transplanted HSCs [83] and several studies unanimously demonstrated significant structural damage of different parts of the bone marrow stroma elicited by irradiation [38,40,69-71]. Interestingly, in normal non-irradiated syngenic mice all intravenously transplanted donor stem cells, present in the bone marrow delivered intravenously, compete equally with those of the host and replace a respective portion of them [58,84,85]. Hence, a highly efficient mechanism for selective trapping of HSCs from the blood and for their engraftment into niches operates in normal mice. The mechanism might serve for physiological recirculation of HSCs among different parts of the hematopoietic tissue which is distributed throughout the entire body. Whether the mechanism is compromised by the bone marrow structural damage caused by irradiation, and how much this could negatively influence engraftment of intravenously administered HSCs, was unclear. We have tackled the problem in sublethally irradiated mice using essentially the approach developed and applied by the Dr. Quesenberry's group [58,86] for studies in normal mice. In difference from the studies using normal mice as recipients of transplanted bone marrow, a proportion (number) of HSCs surviving in mice exposed to different sublethal doses of ionizing radiation had to be established at first.

The proportional survival of hematopoietic STRCs (progenitors) and LTRCs (HSCs) in progressively irradiated mice confirmed a higher radiosensitivity of progenitors as compared to HSCs, especially after low doses (Figure 12, p.47), as reported previously in several studies [87,88]. Ionizing radiation induces double-strand DNA breaks and LTRCs and STRCs differ in their capacity for non-homologous end-joining repair mechanism. This enables the survival of HSCs (LTRCs) after doses of ionizing radiation which already result in apoptosis of progenitors (STRCs) [89,90].

The donor HSCs and the HSCs surviving irradiation in recipient mice should be equal in their contribution to blood cell production to enable quantitative assessment of their ratio after transplantation of a testing dose of donor cells. We have tested this assumption by examining the stability of the partial donor chimerism in submyeloablatively irradiated mice between 3 and 6 months after irradiation and transplantation. Any difference in the fitness of the both competing HSCs should result in a progressive overgrowth of the "stronger" branch of the chimeric hematopoiesis. This was not the case (Table 4, p.55).

We used congenic CD45.1/CD45.2 mice enabling the discrimination of donor and host hematopoietic cells in chimeric mice by the two isoforms of the CD45 cell membrane phosphatase. In most experiments a standard dose of normal BMCs, corresponding to approximately 3.5% of their total content in the mouse was transplanted to progressively irradiated congenic recipient mice. The resulting donor chimerism was determined after 1 month and after 4 or 6 months. The results were compared to theoretical chimerism levels calculated with the assumption that from 100% to 20% of the transplanted STRCs and LTRCs engrafted and contributed just as much to the production of blood cells as those surviving irradiation in the hosts (Figure 15, p.52). The highest agreement between experimental and theoretical data was

constantly for calculations assuming 100% utilization of donor STRCs and LTRCs in recipients irradiated with 3 Gy and higher doses. The consensus of the theoretical and experimental data was also analyzed by calculating squares of the differences between the two sets of data. This result was further verified in two experiments in which different doses of donor BMCs cells were administered to progressively irradiated hosts (Table 2, p.53).

The efficiency seemed to be lower in recipients irradiated with doses lower than 3 Gy. Using also CD45.1/CD45.2 congenic mice, Tomita et al. [78] demonstrated that stable chimerism was uniformly established in 3 Gy-irradiated CD45.2 recipients of CD45.1 bone marrow cells but not in 1.5 Gy-irradiated recipients. van Os et al. [77] demonstrated immunogenicity of Ly5 (CD45)-antigens in transplantation experiments. We hypothesized that the lower than expected engraftment of donor STRCs and LTRCs in mice irradiated by doses below 3 Gy, and particularly in normal non-irradiated mice might be caused by a partial immune disparity between the congenic CD45.1/CD45.2 mice. Hence, we applied immunosuppressive treatment to normal recipients of congenic BMCs which should had increase resulting chimerism to theoretically expected values. The immunosuppression was partially effective (Table 3, p.54) and approximated our experimental results to those expected from evidence provided by previous experiments in normal mice [58,84,85]. Hence, the lower than expected engraftment of transplanted marrow in recipients irradiated by doses lower than 3 Gy was likely due to the minor immune disparity of CD45.1/CD45.2 congenic mice.

Results of the experiments presented in the first part of the dissertation thesis and published [31] provided us a possibility for estimating very low numbers of STRCs and LTRCs in a damaged hematopoiesis by competition with a known number of

transplanted donor STRCs and LTRCs. This was utilized in ensuing experiments aimed at elucidation of the process effective in regenerating hematopoiesis.

5.2 Regeneration of hematopoiesis from surviving or transplanted HSCs

In adulthood, HSCs are slowly proliferating cells [13,91] residing in a specific cellular and extracellular microenvironment known as the osteoblastic niche [33,43]. After transplantation or a damage affecting the hematopoietic tissue, HSCs can repopulate the whole hematopoiesis and maintain it throughout the organism's lifetime.

A current view of the hematopoietic tissue regeneration assumes that:

- → the ultimate regeneration is possible only from HSCs
- → HSCs can arise only from surviving or transplanted HSCs by their self-renewal
- → self-renewal of HSCs results from renewing cell divisions of HSCs which can be either asymmetric, replacing the HSCs that had divided by its single identical copy and providing one progenitor cell utilizable for generation of blood cells, or symmetric, replacing the HSC that had divided by their two identical copies
- → the only way how the number of HSCs can increase are their self-renewing symmetric divisions

First, we indirectly estimated pools of STRCs and LTRCs in the hematopoiesis of mice recovering from a sublethal irradiation by 6 Gy by transplanting them with a standard dose of congenic BMCs. Long-term donor chimerism, determined 6 months after transplantation in the bone marrow, was 90% ± 2% when congenic donor cells were administered within 2 hours after irradiation (Figure 16, p.59). According to the relationship between donor chimerism and ratio of the host and donor LTRCs (see

equation in 4.1.2, p.48), the surviving fraction of the host LTRCs (HSCs) was 0.39%. If the total number of HSCs in normal murine hematopoiesis were 9000 (see Introduction part 1.3.4, p.24), approximately 35 HSCs would survive irradiation with 6 Gy.

The long-term donor chimerism (determined after 6 months) was constantly high when congenic BMCs were administered with a delay of up to 15 days. Hence, during these 15 days the majority of stem cell niches made available to transplanted donor LTRCs by sublethal irradiation with 6 Gy remained unoccupied by the host LTRCs. By assuming that niches are "closed" by presence of LTRCs, we interpret these experimental findings as an evidence for LTRCs numbers remaining low during the first two weeks of spontaneous regeneration of the hematopoietic tissue damaged by sublethal irradiation. Niches for STRCs (progenitors) closed more rapidly and with a different kinetics (Figure 16, p.59). When transplantation of congenic BMCs was delayed by 18.0 to 18.5 days, contribution of the host and the donor LTRCs to blood cell production was equal, which was demonstrated by a 50% chimerism level. Using the quantitative relationships established in the first part of this thesis and published [31], we estimated, that during a period of approximately 3 – 3.5 days, between days 15 and 18-18.5, the pool of LTRCs increased from ~35 to ~320. Thereafter, the pool of the host LTRCs continued to grow which was reflected by progressively decreasing chimerism derived from donor congenic BMCs. The immeasurable donor chimerism in mice which were injected with congenic BMCs 24 or 30 days after irradiation then indicates lack of available niches for transplanted donor LTRCs suggesting that numbers of the host LTRCs reached or approached their normal numbers.

We used two transplantation-based assays to estimate HSCs in regenerating bone marrow. One was a competitive transplantation assay comparing hematopoietic chimerism after transplantation of tested cells together with normal non-irradiated cells

in a defined ratio and administered to lethally irradiated recipient mice. This assay demonstrated presence of only approximately 5% of STRCs in the hematopoietic tissue 30 days after irradiation. The number of LTRCs was immeasurable even after 30 days (Figure 18, p.61). In this assay, the regenerating BMCs were not able to fully compete with normal BMCs even when transplanted in a 10-fold preponderance. The second transplantation-based assay compared capacity of normal and regenerating bone marrow to reconstitute hematopoiesis in lethally irradiated mice. Syngenic BMCs collected 30 days after irradiation of mice by 6 Gy were significantly inferior in their capacity to close hematopoiesis against congenic transplant, given 20 or 30 days after the syngenic transplantation, when compared to either normal BMCs or BMCs collected 60 days after irradiation (Figure 19, p.63 and Figure 20, p.64).

Since the three assays examining the recovery of STRCs and LTRCs pools in sublethally irradiated mice provided different results, we further analyzed the hematopoiesis spontaneously regenerating in mice irradiated by 6 Gy by determining recovery of the blood cell production and by measuring BMCs highly enriched in progenitors and HSCs. A quantitatively significant myelopoiesis was present from day 15 after irradiation with lymphopoiesis lagging behind. Cellularity of the bone marrow reached normal values about day 20 after irradiation and was characterized by stimulated myelopoiesis at the expense of lymphopoiesis (Figure 21, p.66 and Figure 22, p.67). Cellularity of the spleen, indirectly determined according to the development of spleen weights, recovered earlier on day 15.

Interesting data provided analysis of the LSK cells, a subpopulation of BMCs enriched in progenitors and HSCs and accounting to approximately 0.2% of total BMCs. Total number of LSK cells fully recovered by day 30. However, representation of their subtypes characterized by expression of CD150 and CD48 markers was

markedly skewed (Figure 26, p.71). Cells bearing the phenotype of multipotent progenitors, LSK $CD150^{-}CD48^{-}$ cells [6] essentially disappeared. The LSK $CD150^{+}CD48^{-}$ cells [6], which are most close to HSCs, recovered very slowly and achieved less than 20% of their normal values by day 30. Progenitors, the LSK $CD150^{-}$ $CD48^{+}$ cells [6] recovered fully by day 30. However, the most abundant became the LSK $CD150^{+}CD48^{+}$ cells which exceed their normal numbers already 10 days after irradiation and remained high till day 30.

As it is generally assumed that HSCs can multiply only by self-renewing symmetric cell divisions, we examined the cell cycle status in the studied subcategories of LSK cells using a pulse labeling with bromodeoxyuridine (BdrU) administered in vivo. The most quiescent were the LSK $CD150^{+}CD48^{-}$ cells (HSCs) with the percentage of S-phase cells about 10% in normal bone marrow and about 20% in regenerating bone marrow. On the other hand, the LSK CD150+ cells expressing also the CD48 marker (LSK $CD150^{+}CD48^{+}$ cells) incorporated BrdU in normal bone marrow as well as in regenerating bone marrow. By assuming that S-phase of the cell cycle lasts 6 hours, these cells would duplicated twice a day. CD48 molecule is expressed throughout all short-term progenitors, but it is excluded from long-term HSCs [92,93]. Venezia et al. also described a transient up-regulation of CD48 on phenotypically defined HSCs in bone marrow damaged by 5-fluorouracil [94].

Proliferation rate of the LSK $CD150^{-}CD48^{+}$ cells was in between of the two above discussed cells.

5.3 Closing of the hematopoiesis of lethally irradiated mice transplanted with syngenic bone marrow to delayed transplant of congenic BMCs

Further we studied regeneration of hematopoiesis fully damaged by a lethal dose of irradiation and rescued by transplantation of syngenic BMCs. A measure of the regeneration, aimed at estimation of the pools of STRCs and LTRCs, was the chimerism resulting from transplantation of a defined fraction of normal congenic BMCs administered from 2 hours to 180 days after irradiation and rescue transplantation. Initiation of the regeneration of hematopoietic tissue was from transplanted syngenic STRCs and LTRCs.

Rescue transplantation of approximately ~10^5 syngenic BMCs (~1/200 of the femoral bone marrow) did not result in complete inhibition of engraftment of congenic BMCs till day 30.

After enlarging the rescue transplant to approximately $2x10^6$ of syngenic BMCs (~1/10 of the femoral bone marrow), the engraftment of congenic cells became more suppressed after 20 and 30 days of regeneration and was immeasurable after 40 days. Surprisingly, a much larger syngenic transplant, corresponding to two femurs representing approximately 14% of whole BMCs, did not inhibit engraftment of congenic LTRCs up to 17 days of regeneration. Engraftment of STRCs present in the congenic transplant was much more affected by previous transplantation and regeneration of syngenic BMCs. Similarly as after the sublethal irradiation with a dose of 6 Gy, when hematopoiesis regenerated spontaneously from surviving STRCs and LTRCs, closing of regenerating hematopoiesis against transplanted STRCs started earlier and followed a different kinetics from that of LTRCs.

Finally, we attempted to inhibit engraftment of congenic BMCs by repeated injections of large syngenic transplants, each comprising approximately 14% of total murine BMCs. In contrast to a single dose of syngenic BMCs, two doses representing together approximately 28% of whole BMCs, as well as three and four doses representing 42 and 56% of BMCs, respectively, progressively inhibited engraftment of congenic LTRCs. Moreover, when the experimental values were compared with the theoretical ones calculated from ratios in which syngenic (increasing number) and congenic (constant number) BMCs were administered (Table 6, p.80), the values agreed very well (Figure 32, p.80). The experimental values for STRCs deviated from the theoretical ones. Closing of the niches for congenic STRCs was more rapid than it was predicted due to their saturation by increasing numbers of transplanted syngenic BMCs. This suggests early multiplication of STRCs in regenerating bone marrow.

These results, together with those presented in Figure 31, indicate that niches occupied by transplanted STRCs are different from those for LTRCs; they close more rapidly during regeneration of the hematopoietic tissue compared to those for LTRCs. The results also show that there is a limited number of niches for both STRCs and LTRCs and they engraft transplanted repopulating cells only until they are saturated with appropriate type of cells. Moreover, there is no evidence for a significant reduction of niches for LTRCs (HSCs) in the hematopoietic tissue of mice after lethal irradiation mice, since their saturation by successive doses of BMCs equivalent to 14% of their normal numbers followed the predicted development assuming undisturbed transplantation space.

By assuming that STRCs and LTRCs inhibit engraftment of transplanted repopulating cells by occupying (closing) their niches, our results suggest that while the

pool of STRCs expands rapidly during regeneration of damaged hematopoietic tissue, the pool of LTRCs remains low for and extended time.

6 CONCLUSIONS

The present study provides experimental evidence that

- a very high proportion of intravenously administered hematopoietic stem cells is captured from circulation and engraft in the hematopoietic tissue damaged by irradiation
- in the bone marrow there are two different types of niches for hematopoietic repopulating cells which are differently engrafted and repopulated by transplanted progenitors (STRCs) and HSCs (LTRCs)
- niches remain available for transplanted LTRCs (HSCs) for a relatively long time after both sublethal and lethal irradiation, while those for STRCs (progenitors) close more rapidly
- regenerated bone marrow has low repopulating capacity although it resumed the blood cell production and the engraftment of transplanted cells is markedly inhibited; thus HSCs generated in regenerating hematopoietic tissue might be transiently deficient in capacity to be transplanted
- damaged hematopoiesis restored preferentially myelopoiesis while the restoration of lymphopoiesis and of pools of the transplantable repopulating cells lagged behind
- representation of the subtypes of LSK cells characterized by presence/absence of antigens CD150 and CD48 is significantly changed during bone marrow regeneration
- the stem cell phenotype and some of their functional properties may transiently dissociate in newly produced HSCs

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Forgacova K, Savvulidi F, Sefc L, Linhartova J, Necas E. All hematopoietic stem cells engraft in submyeloablatively irradiated mice. Biol Blood Marrow Transplant. 2013 [Epub ahead of print]. **IF = 3.873**

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Manuscripts in preparation

Forgacova K, Savvulidi F, Paral P, Sefc L, Necas E. Asymmetric versus symmetric divisions in regenerating hematopoiesis

Savvulidi F, **Forgacova K**, Michalova J, Paral P, Necas E, Sefc L. Omitting lineage markers in determination of hematopoietic stem and progenitor cells

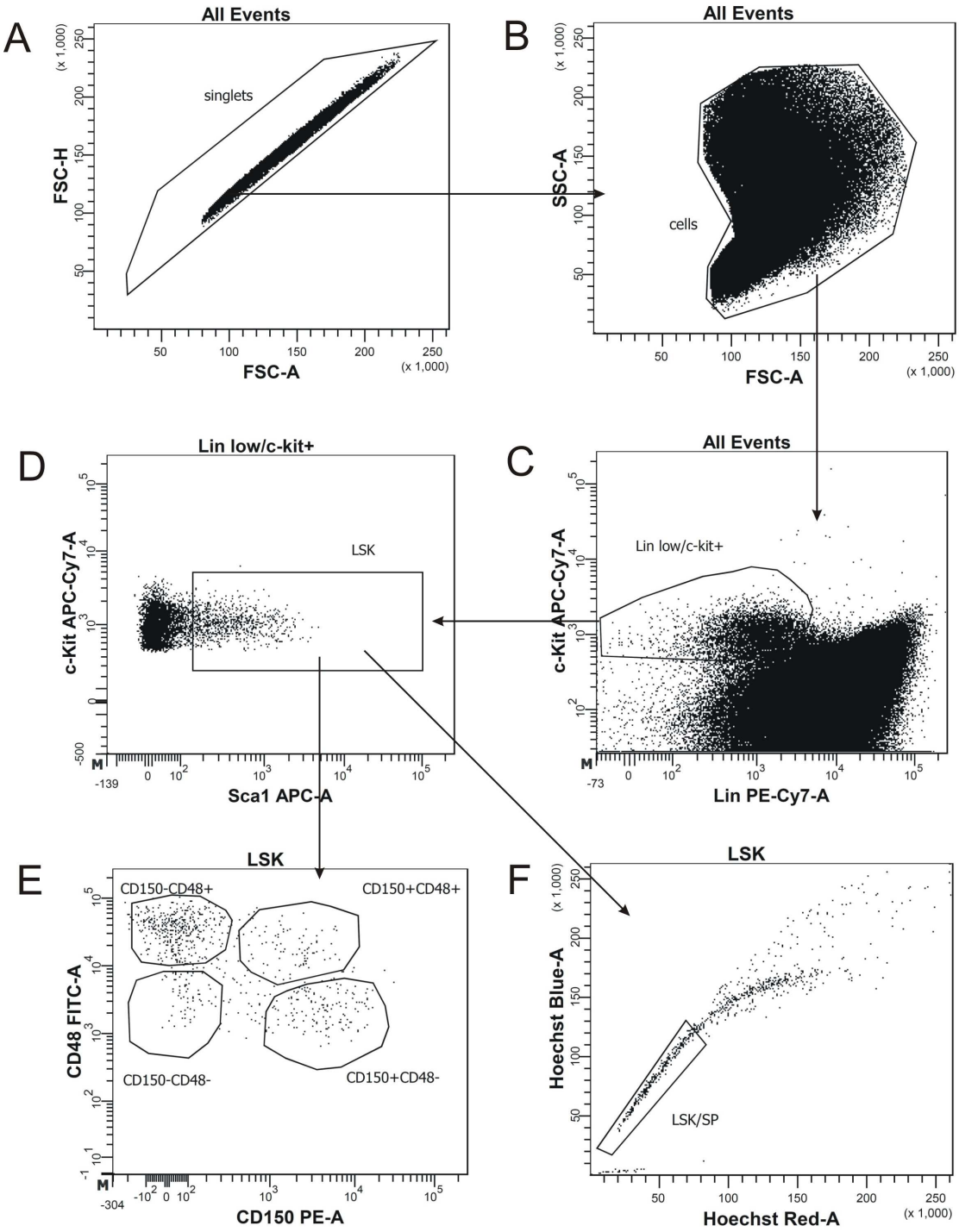
9 APPENDICES

Appendix 1

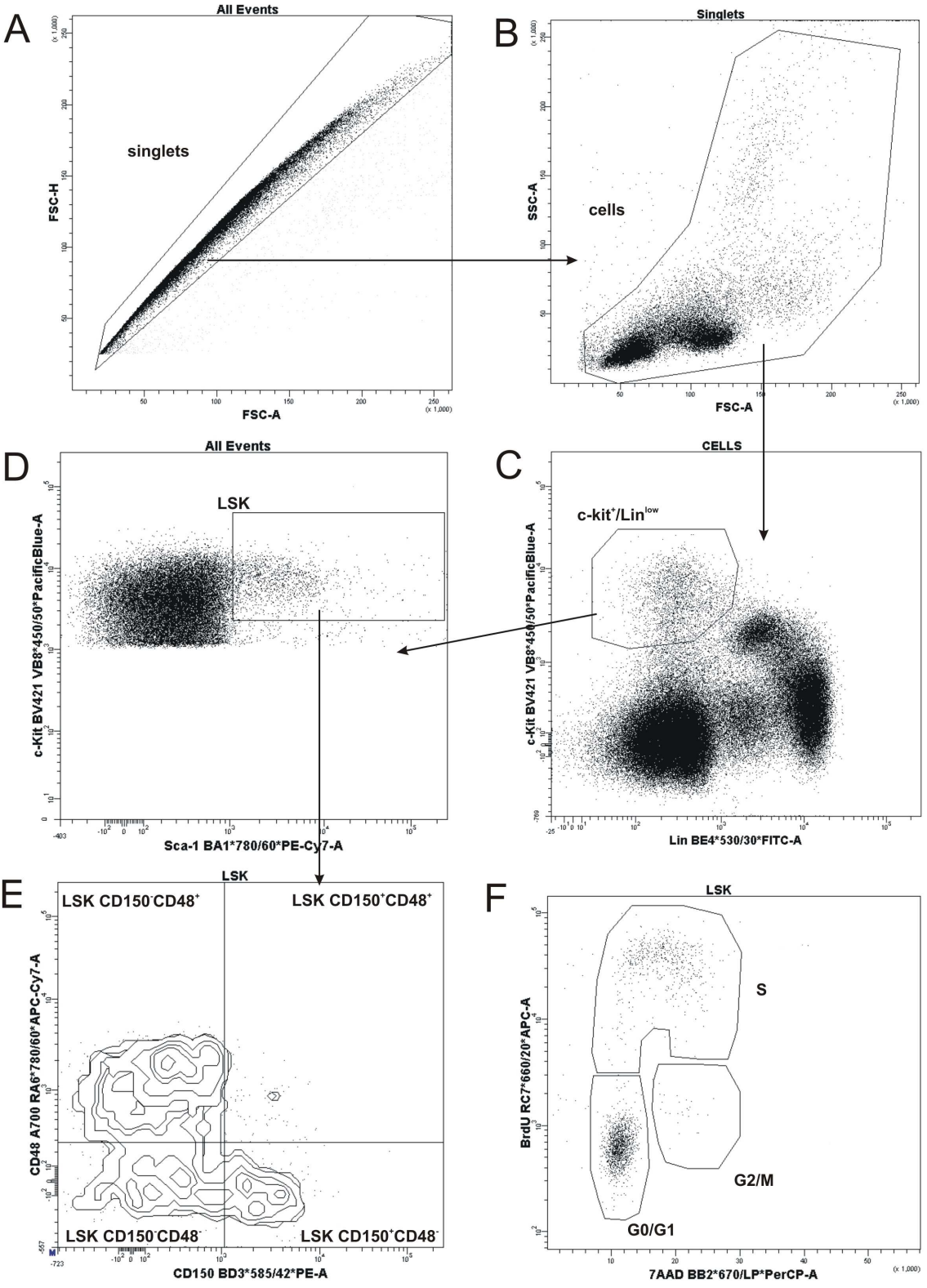
Supplementary figures

Appendix 2

Supplementary tables



Supplementary Figure 1 – Gating of the most primitive populations of the bone marrow. (**A**) exlusion od derbis by gating of cell singlets, (**B**) gating of cells, (**C**) gating of Linlow/c-kit+ population, (**D**) gating of Linlowc-kit+Sca-1+ (LSK) population, (**E**) gating of subpopulations of LSK according to CD150 and CD48 surface markers, (**F**) gating of Hoechst 33342 negative cells (side population, SP).



Supplementary Figure 2 – Cell cycle analysis gating. (**A**) exlusion od derbis by gating of cell singlets, (**B**) gating of cells, (**C**) gating of Linlow/c-kit+ population, (**D**) gating of Linlowc-kit+Sca-1+ (LSK) population, (**E**) gating of subpopulations of LSK according to CD150 and CD48 surface markers, (**F**) gating of cells in G0/G1 – S – G2/M phases of the cell cycle according to BrdU and 7-AAD uptake.

	IRR (Gy)	% of surviving cells	experimental chimerism mean ± SEM	efficiency (%) *corresponding % of whole BM content*				
				100	**80**	**60**	**40**	**20**
				3.5	***2.8***	***2.1***	***1.4***	***0.7***
A) STRCs	0.5	72	0.053 ± 0.43	4.64	3.74	2.83	1.91	0.96
	1	52	6.03 ± 2.12	6.31	5.11	3.88	2.62	1.33
	2	26	2.42 ± 1.34	11.86	9.72	7.47	5.11	2.62
	3	13	10.64 ± 3.50	21.21	17.72	13.91	9.72	5.11
	4	6	41.19 ± 3.74	36.84	31.82	25.93	18.92	10.45
	5	1.5	75.22 ± 2.52	70.00	65.12	58.33	48.28	31.82
	6	0.2	85.19 ± 1.12	94.59	93.33	91.30	87.50	77.78
	7	0.02	95.57 ± 1.25	99.43	99.29	99.06	98.59	97.22
	$\Sigma (x_1 - x_2)^2$			**371.56**	388.01	616.25	1259.58	2940.04
B) LTRCs (PB)	0.5	95	0.03 ± 0.03	3.55	2.86	2.16	1.45	0.73
	1	88	1.07 ± 0.50	3.83	3.08	2.33	1.57	0.79
	2	68	3.14 ± 2.12	4.90	3.95	3.00	2.02	1.02
	3	33	6.56 ± 3.51	9.59	7.82	5.98	4.07	2.08
	4	10	43.29 ± 4.59	25.93	21.88	17.36	12.28	6.54
	5	1.5	80.25 ± 4.10	70.00	65.12	58.33	48.28	31.82
	6	0.2	93.98 ± 0.64	94.59	93.33	91.30	87.50	77.78
	7	0.02	97.62 ± 0.69	99.43	99.29	99.06	98.59	97.22
	$\Sigma (x_1 - x_2)^2$			**442.49**	705.18	1168.66	2036.59	3983.89
C) LTRCs (BM)	0.5	95	0.15 ± 0.08	3.55	2.86	2.16	1.45	0.73
	1	88	0.23 ± 0.10	3.83	3.08	2.33	1.57	0.79
	2	68	2.58 ± 1.53	4.90	3.95	3.00	2.02	1.02
	3	33	6.18 ± 2.99	9.59	7.82	5.98	4.07	2.08
	4	10	31.32 ± 4.32	25.93	21.88	17.36	12.28	6.54
	5	1.5	64.98 ± 6.63	70.00	65.12	58.33	48.28	31.82
	6	0.2	90.37 ± 1.64	94.59	93.33	91.30	87.50	77.78
	7	0.02	96.31 ± 0.50	99.43	99.29	99.06	98.59	97.22
	$\Sigma (x_1 - x_2)^2$			**123.38**	126.98	256.28	663.22	1892.97
D) LTRCs	0.5	95	0.09	3.55	2.86	2.16	1.45	0.73
	1	88	0.65	3.83	3.08	2.33	1.57	0.79
	2	68	2.86	4.90	3.95	3.00	2.02	1.02
	3	33	6.37	9.59	7.82	5.98	4.07	2.08
	4	10	37.3	25.93	21.88	17.36	12.28	6.54
	5	1.5	72.61	70.00	65.12	58.33	48.28	31.82
	6	0.2	92.18	94.59	93.33	91.30	87.50	77.78
	7	0.02	96.96	99.43	99.29	99.06	98.59	97.22
	$\Sigma (x_1 - x_2)^2$			**184.70**	317.77	614.06	1251.38	2839.77

Supplementary Table 1 - Comparison of experimental and theoretical donor chimerism levels assuming different efficiencies of the engraftment of intravenously administered donor BMCs.
Estimates of surviving fractions of STRCs and LTRCs for different submyeloablative doses of irradiation (IRR) derived from Figure 1B. The experimental chimerism was determined by pooling the results from the four experiments presented in Fig. 3. The theoretical chimerism was calculated using the assumptions that half of the

femoral bone marrow contains 3.5% of the total STRCs and LTRCs, 100 – 80 – 60 – 40 or 20% of them engraft, and those of the donor and the host contribute equally to blood cell production. The square roots of the differences between the experimental and the calculated values were determined (x1 – x2)^2 and their summation is given below the columns. The best fit between the experimental results and calculated values was for 100% engraftment efficiency which is indicated by the least sum of squared differences (bold numbers below columns). Results are given for STRCs derived from donor chimerism determined in blood one month after transplantation (**A**), LTRCs derived from donor chimerism determined either in blood (**B**) or bone marrow (**C**) four or six months after transplantation and for LTRCs derived from the mean donor chimerism calculated from that measured in the blood and that measured in the marrow (**D**).

	IRR (Gy)	**% of surviving cells**	**experimental chimerism mean ± SEM**	***corresponding % of whole BM content***				
				2.5	***3***	***3.5***	***4***	***4.5***
A) STRCs	0.5	72	0.053 ± 0.43	3.36	4.00	4.64	5.26	5.88
	1	52	6.03 ± 2.12	4.59	5.45	6.31	7.14	7.96
	2	26	2.42 ± 1.34	8.77	10.34	11.86	13.33	14.75
	3	13	10.64 ± 3.50	16.13	18.75	21.21	23.53	25.71
	4	6	41.19 ± 3.74	29.41	33.33	36.84	40.00	42.86
	5	1.5	75.22 ± 2.52	62.50	66.67	70.00	72.73	75.00
	6	0.2	85.19 ± 1.12	92.59	93.75	94.59	95.24	95.74
	7	0.02	95.57 ± 1.25	99.21	99.34	99.43	99.50	99.56
	$\Sigma (x_1 - x_2)\text{^}2$			452.01	**366.84**	371.56	437.68	547.22
B) LTRCs	0.5	95	0.09	2.56	3.06	3.55	4.04	4.52
	1	88	0.65	2.76	3.30	3.83	4.35	4.86
	2	68	2.86	3.55	4.23	4.90	5.56	6.21
	3	33	6.37	7.04	8.33	9.59	10.81	12.00
	4	10	37.3	20.00	23.08	25.93	28.57	31.03
	5	1.5	72.61	62.50	66.67	70.00	72.73	75.00
	6	0.2	92.18	92.59	93.75	94.59	95.24	95.74
	7	0.02	96.96	99.21	99.34	99.34	99.50	99.56
	$\Sigma (x_1 - x_2)\text{^}2$			418.22	267.29	184.70	148.28	**144.73**

Supplementary Table 2 - Effect of varying the fraction of STRCs and LTRCs in BMCs contained in bone marrow collected from a half of the femur.

Calculation of theoretically expected donor chimerism in progressively irradiated hosts assuming 100% engraftment efficiency of STRCs and LTRCs but their representation in the bone marrow from half of a femur varying from 2.5 to 4.5%.The proportional survival of the host STRCs (**A**) and LTRCs (**B**) are those from Figure 1B, the latter being the mean from estimates derived from the chimerism level determined either in the blood or in the marrow.