Charles University in Prague

First Faculty of Medicine

PhD Thesis

Hematopoietic Stem Cell Properties and Transplantation Preconditioning Studied by Competitive Repopulation of the Hematopoietic Tissue

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DECLARATION

The thesis was worked out at the institute of Pathological Physiology, First Medical Faculty, Charles University in Prague from 2004-2009.

I hereby declare that I have worked out the thesis independently while noting all the resources employed as well as co-authors. I consent to the publication of the thesis under Act No. 111/1998, Coll., on universities, as amended by subsequent regulations.

In Prague 2.6.2009 MUDr. Tereza Hlobeňová

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Mým drahým rodičům

ABBREVIATIONS (In alphabetical order):

AGM	Aorto-gonado-mesonephros
BM	Bone marrow
Bu	Busulphan
CFU-S	Colony forming units-spleen
CLP	Common lymphoid precursor
CMP	Common myeloid precursor
CY	Cyclophosphamide
ER	Estrogen receptor
F1	Filial 1
FL	Fetal liver
G-CSF	Granulocyte colony-stimulating factor
GMP	Granulocyte/macrophage progenitor
HSCs	Hemopoietic stem cells
$Lin^{+/-}$	Lineage positive/negative
LTRCs	Long term repopulating cells
MEP	Megacaryocyte/erythrocyte progenitor
MMP-9	Matrix metalloproteinases 9
MPP	Multipotent progenitors
P0.5	Newborn liver
SCF	Stem cell factor
SDF-1	Stromal derived factor-1
STRCs	Short term repopulation cells

1. INTRODUCTION

1.1 Hematopoietic stem cells, hematopoiesis and its hierarchy

Hemopoietic stem cells (HSCs) are primitive cells capable of replacing terminally differentiated cells throughout life (Mazurier F et al., 2003). HSCs are defined as pluripotent cells able to give rise to at least ten different functional cell types and they posses a huge self-renewal capability (Dexter TM et al., 1984). Two types of stem cells have been identified – long term repopulating cells (LTRCs) and short term repopulating cells (STRCs). LTRCs are potent of producing all blood cell types for the whole life span of an individual organism (Morrison SJ et al., 1994). STRCs are able to reconstitute the myeloid/lymphoid cell pool only for limited period of life of the organism (Morrison SJ et al., 1994 and 1997). The process during which stem cells give rise to terminally differentiated cells occurs through a number of committed progenitor cells in the bone marrow (BM) (Golde DW, 1991). During this process stem cells undergo proliferation and sequential differentiation, which is accompanied by gradual loss in self-renewal capacity and capability to produce mature cells.

It has been estimated that stem cells are very low in number and that they divide relatively rarely. Therefore the transit population of committed progenitors is responsible for most of the cell amplification that takes place to keep blood cell production (Abkowitz JL et al., 2002).

The hierarchy in hemopoietic tissue is as follows. The first progeny of HSCs in adult bone marrow are multipotent progenitors (MPP), which have full pluripotent differentiation potential, but lack in contrast to HSCs the self-renewal ability (Adolfsson J et al., 2001). Two common precursors descend from the MPP, the common myeloid precursor (CMP) and the common lymphoid precursor (CLP) (Akashi K et al., 2000). CMP give rise to megakaryocyte/erythrocyte progenitor (MEP) and

granulocyte/macrophage progenitor (GMP). It has been believed that during commitment, the fate of multilineage progenitors becomes restricted in a stepwise and irreversible manner. Recently alternative lineage choices have been reported, especially in B-cell/macrophage and T-cell progenitor and CMP (Allman D et al., 2003). These surprising findings could be explained by a stochastic model during initial phase of commitment. According to this model, there is a great deal of heterogeneity among progenitors with a continuum of overlapping potentials. Some combinations are less likely to occur (B-cell/macrophages) (Allman D et al., 2003), whereas others are much more advantageous and are more likely to occur (granulocyte/macrophage) (Fig. 1).

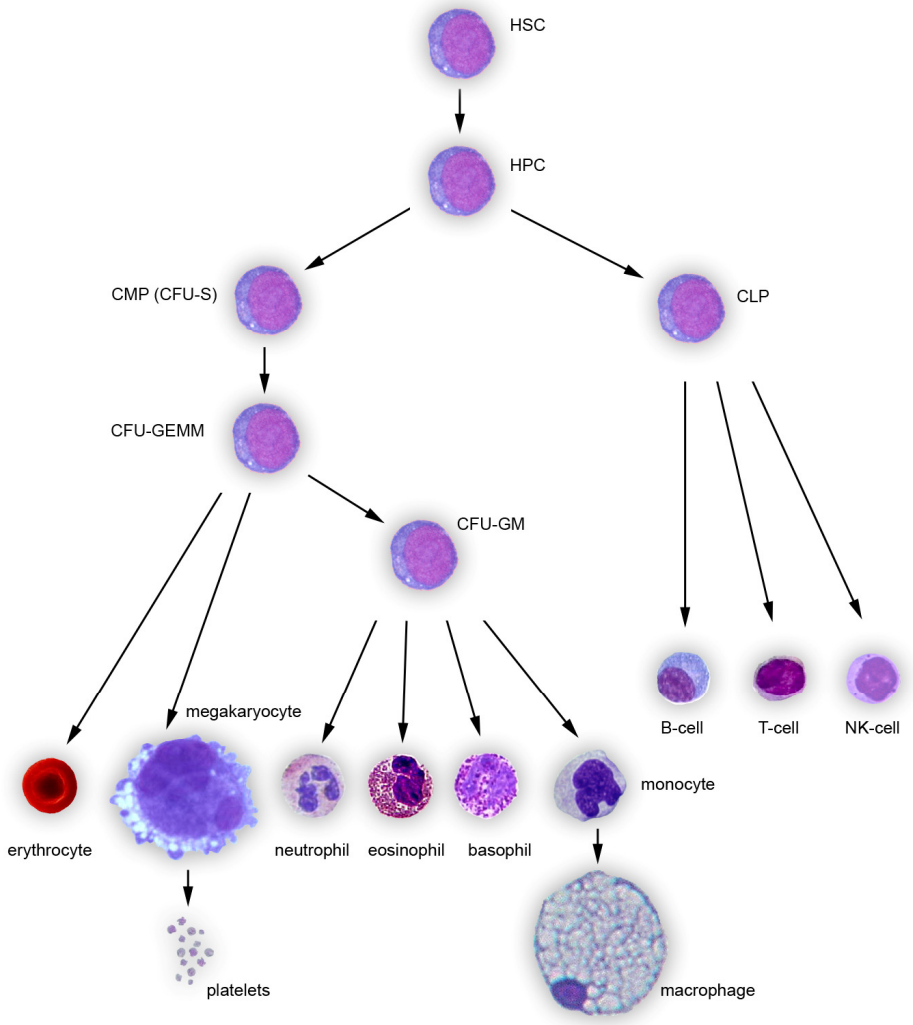


Fig.1: Schematic view of hematopoiesis

1.2 Identification of hemopoietic stem cells and some functional markers

Cell surface markers are used for the identification of stem cells. Most commonly, the HSCs are identified in the absence of all lineage markers (Lin^-). There are some inter-species differences, a panel of cell surface antigens used to identify human HSCs is different from the one used for murine HSCs.

In the experimental setting, the murine HSCs were first described to be in c-Kit^+ $Thy1^{lo}$ Lin^- Sca-1^{hi} population (Spangrude GJ et al., 1988). LTRC, STRC as well as committed progenitors can be distinguished according to the presence of a cocktail of surface antigens. Now, SLAM family markers are used for identification of HSCs. HSCs were shown to be CD150+, CD48- (Yilmaz OH et al. 2006). The most interesting fraction of bone marrow cells capable of long-term repopulation, the LTRC, is present within this $Thy1^{lo}$ fraction (Christensen JL and Weisman IL, 2001).

In humans CD34 has been used to distinguish stem cells in bone marrow for a long time, only recently was found that unstimulated LTRC in BM are CD34 negative (Bhatia M et al, 1998). CD34 is expressed on only 0.5-5% of human bone marrow cells. CD34 is still used when identifying cells mobilized into the peripheral blood. However the most commonly used stem cell marker for human BM is CD133 antigen.

Additionally, the most concentrated population of HSCs resides in the so-called SP fraction. This population is characterized according to the extrusion of Hoechst 3342 from the cells and is largely enriched for HSCs. A number of other markers could be used to identify HSCs, such as CD10, CD7, IL-3R-α-low (Ziegler BL et al., 1999).

However, even with the use of a large antigen identifying cocktail, the HSCs population remains heterogeneous. The markers are not essential for stem cell function and therefore the expression of these markers might not directly correlate with stem cell potential (Matsuoka S et al., 2001). Stem cells thus appear as a population of cells with

random gene transcription that is likely to generate phenotypically heterogeneous cells with identical functional capability. Therefore, nowadays the only conclusive assay for HSCs is to access their ability to give raise to a long-term engraftment in the lymphoid and myeloerythroid lineages in a lethally irradiated host following transplantation. Many years the enumeration of spleen colony forming unit (CFU-S) was considered to fulfill the requirements of HSCs. CFU-S have the ability to generate colonies in the spleen of a mouse eight to twelve days after transplantation, which contained cells of different lineages. A small fraction of these colonies was able to repopulate a secondary irradiated host (Till JE and McCulloch EA, 1961). Currently the long-term repopulating ability of CFU-S is under debate and current opinion regarding Day-12 CFU-S is that they are derived from primitive MPP and Day-8 CFU-S are derived from late myeloerythroid committed progenitors (MEP) (Mazurier F et al., 2003).

The bone marrow microenvironment may selectively support the survival, proliferation and differentiation of HSCs (Na Nakorn T et al., 2002). In experimental settings using a murine model, HSCs are assessed by in vivo competitive repopulation assays after transplantation into irradiated congenic host and long-term engraftment of HSCs can be demonstrated by secondary and tertiary transplantations. For the detection of human HSCs, transplantation of human stem cells into xenogenic hosts is used, particularly into immunodeficient NOD/SCID or RAG-/RAG-mice and presence of human cells in blood and hematopoietic tissue is detected (Cashman J et al., 1997). Terminally differentiated human cells of all hemopoietic lineages should be detected. A weak point of all transplantation assays is that they relate to the future potential of stem cells. The nature of HSCs can be therefore deduced only retrospectively. Any conclusions derived from these assays do not state anything about the behaviour of a single cell, but about the probabilistic behaviour of a population of cells. Additionally it underestimates

the frequencies of HCSc, because it detects cells that have sufficient self-renewal and multipotent differentiation potential, but also express the molecules necessary for homing to the bone marrow of the hosts. The application route of cells seems to play a role as well. Recently the homing interference has been excluded by direct cell delivery of HSCs into the bone marrow cavity by intra-bone injection (Mazurier F et al., 2003, Wang J et al., 2003).

1.3 Ontogeny of HSCs

Hemopoiesis site changes several times during vertebrate embryogenesis according to the demands of the developing organism (Fig. 2). The first site is the yolk sac that contains predominantly erythrocytes, macrophages and megakaryocytes (Naito M et al., 1996), which however differ from their adult counterparts. These differences ensure the developing embryo a necessary quick supply of oxygen, protection from bleeding and rapid clearance of dead cells (Yoder MC et al., 1997). Interestingly, cells from this stage do exert repopulating ability when transplanted into lethally irradiated newborn recipients, but not into adult mice, suggesting special microenvironmental requirement.

The second site of hemopoiesis takes place in the aorto-gonado-mesonephros (AGM) region containing aorta and urogenital system (Müller AM et al., 1994). Hemopoiesis is present here only transiently (Godin I et al., 1999). Cells from this developmental stage have already characteristic of definitive HSCs, which is demonstrated by the capability of multilineage engraftment into adult recipients as well as differentiation into erythroid, myeloid and lymphoid lineages (Medvinsky A and Dzierzak E, 1996). Very soon after presence in the AGM region definitive HSCs can be detected in the liver (Medvinsky A and Dzierzak E, 1996, Müller AM et al., 1994). It seems that AGM is the most potent intraembryonic HSCs site. Fetal liver becomes the main source of

hemopoiesis during fetal life. Around birth, the HSCs migrate from the fetal liver to the site responsible for adult hemopoiesis, the bone marrow and also to the spleen in case of rodents (Delassus S and Cumano A, 1996). Very close juxtaposition and temporally parallel onset of the endothelial and hemopoietic cells is seen in the yolk sac and AGM region. The idea of a common precursor, "the hemangioblast" for the hemopoietic and endothelial lineage was first published many years ago and has been extensively studied recently (Bellantuono I, 2004). Data that support this theory include evidence that genes involved in primitive hemopoiesis are directly responsible for angiogenesis and endothelial cell proliferation (de Bruijn MF et al., 2002). Sorted cells from yolk sac and AGM with endothelial markers from blood cells in culture in presence of stromal cells (Nishikawa SI et al., 1998). This was also proven by humans, where vascular endothelial calls isolated from fetal liver and fetal bone marrow were capable of multilineage hemopoiesis (Oberlin E et al., 2002). These data suggest that liver and bone marrow stroma are inevitable compartments in hemopoietic development. Additionally, HSCs population could be viewed as a heterogeneous one, with changing HSCs pool properties during development. The demand for rapidly increasing numbers of hemopoietic cells in fetal life differs from their steady state maintenance in adult life. Previous studies have shown that the potential of fetal liver HSCs to expand in culture and to repopulate lethally irradiated recipients is greater than that of adult bone marrow HSCs (Chang KT et al., 2005).

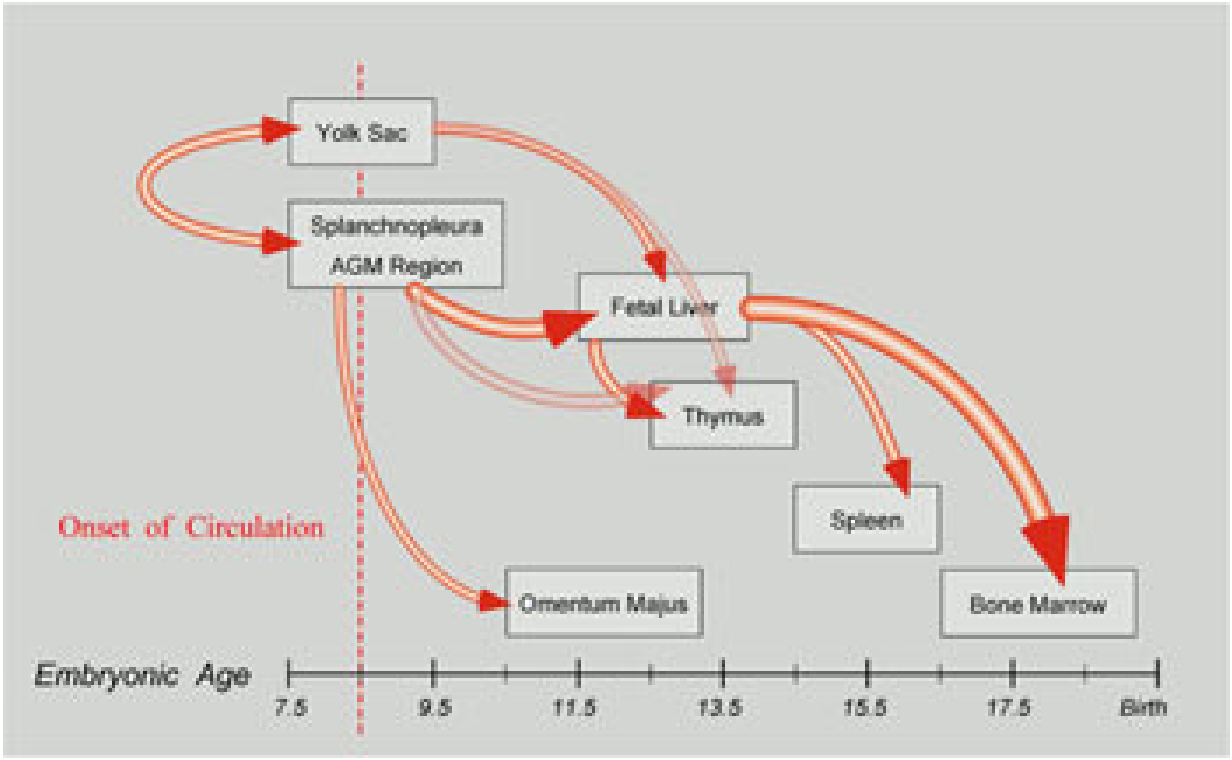


Fig. 2: Hematopoiesis and migration during ontogeny

1.4 Properties of stem-cells – Self-renewal

It is necessary to ensure the maintenance of hemopoiesis over the entire lifespan of an individual. Two models have been proposed. The classical model counts with a fixed number of stem cells set during embryogenesis. These cells then randomly proliferate and differentiate into mature cells (Brecher G et al., 1986, Hellman S et al., 1978). In the second model a small number of stem cells is developed during embryogenesis and these cells can later undergo self-renewal and reproduce. This self-renewal produces daughter cells with the same proliferative and developmental potential as the parental cells. Only a small fraction of HSCs will undergo differentiation and maturation. Definitive evidence proving one of the models is lacking so far. The first model is supported by classical experiments done in the 1960ś where serial transplantations of bone marrow in mice has led to a progressive loss of regenerative potential of the CFU-S (Siminovitch L et al,

1964). Data considering also stem cell aging have been lately published (Kamminga LM et al., 2006).

The second model is supported by data from murine models showing that one mouse has enough stem cells to repopulate a thousand mice (Harrison DE and Zhong RK, 1992). This would implicate that stem cells are able to produce more stem cells.

A compromise between these two models is proposed. It views the hemopoeitic system as a heterogeneous population of stem cells in continuum (Quessenberry PJ et al, 2007). The HSCs pool is again a heterogeneous one containing cells of different levels, ranging from the most immature to more mature ones. Any of these cells has sufficient proliferative potential to repopulate an irradiated host. It can be postulated, that-self renewal is not obligatory for HSCs, but can be realized under certain circumstances. This hypothesis is supported by several facts. First, the expansion of HSCs pool is seen during fetal development and early postnatally (Rebel V.I. et al., 1996, Szilvassy SJ et al., 2001). Second, the progenitor number increases with age (Sudo K et al., 2000). Finally, the telomere length of HSCs decreases during life, suggesting that self-renewal might not be an inexplicit phenomenon (Vaziri H et al., 1994). This has been proven not only in experimental models, but in human studies as well. Telomere length shortening has been shown in patients after transplantation (Wynn RF et al., 1998). It might be explained by proliferative stress exerted after transplantation
(Neben S et al., 1993).

1.5 Properties of stem cells – Commitment

The second main characteristic of stem cells is their commitment to differentiation into hemopoietic lineages. Again, more explanations and predictions exist regarding this feature. It remains unsolved whether the commitment is a pure stochastic event or if it is the result of environmental changes transmitted by soluble factors and cell-cell or cell-matrix interactions. It is probable that the maintenance of HSCs depends mainly on the microenvironment, which provides signals modulating the stem cell pool. However, stochastic gene expression in HSCs takes place in early stages of commitment. Lineage decision would then be probabilistic depending from random formation of transcription complex able to initiate specific transcription or to repress it (Shalaby F et al., 1997). Some combinations of expressed or repressed genes would form an autocrine loop. These combinations would increase the probability of transcription of other genes, which in turn would edit the stem cell response to the signals from the microenvironment (Shalaby F et al., 1997) and further intensify the commitment.

1.6 Properties of stem cells – Plasticity

Traditionally, commitment of progenitor cells has been considered irreversible. In other words, once the progenitor cell looses its multipotency, it can never gain it back again. It has lost a substantial part of the developmental potential and these cells are expected to express lineage specific genes and antigens. Recent studies demonstrating lineage switching have doubted this theory (Graf T et al., 1992, Graf T, 2002). Examples come from B-lineage switching (Nutt SL et al., 1999, Rolink A et al., 1999), neutrophils switching (Heyworth C et al., 2002) and CLP reprogramming (Kondo M et al., 2000).

Additionally, HSCs plasticity was demonstrated not only between hemopoietic cell lineages, but between hemopoietic and non-hemopoietic cells as well, particularly in

regeneration of damaged myocytes, neurons, hepatocytes, myocardiocytes, epithelial and endothelial cells (French SW et al., 2002, Quessenberry PJ et al, 2005). Clinical studies demonstrating benefit of hematopoietic HSCs are now emerging (Syková E and Jendelová P, 2005). It has to be stated that this conversion was demonstrated to be a very rare event (Wagers AJ et al., 2002). However, data obtained from studies in this highly interesting field of medicine have to be interpreted and put into everyday clinical practice with caution due to the following. To trans-differentiate, the HSCs have to undergo genetic reprogramming. Commitment of cells is associated with a relatively small number of genes being transcribed at high levels and a large number of genes, which become transcriptionally inactive. This is mainly determined by the availability of transcription factors, their combination and by the chromatin status. Chromatin could be permissive or refractory to transcription. This specifies a pattern of gene expression that is inherited through subsequent cell division, creating a form of molecular memory. This might provide possible explanations for cell plasticity, because according to such a model it might be relatively easy to reprogram cells where only a few genes have been shut down. In hemopoietic tissue the reprogramming is relatively easy, because the switching between lineages occurs between cells with similar genetic profile and the like-hood of switching these genes is relatively high. Studies demonstrating reprogramming emerge. But although in principle the cell ability to reprogram exists, the final outcome depends on a combination of a series of random events (Boiani M et al., 2002).

1.7 IPS cells

Recently it has been demonstrated that it is possible to reprogramme adult murine and human cells to pluripotent state. These cells are called induced pluripotent stem cells (iPS cells). They are genetically modified by the integration of up to four viral vectors

bearing DNA-transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) into adult cell genome. IPS cells can be induced from a variety of adult cells. However, since they are modified with viruses, there is a potential activation of oncogenes at the moment (Takahashi K et al., 2006).

1.8 Stem cell niche

Bone marrow provides an environment where HSCs can interact with each other, stromal cells, fat containing cells, sinusoidal blood vessels and other components of the extracellular matrix. The blood sinusoids in the bone marrow support differentiation of progenitors and mobilization of mature cells. The niches are specific microenvironments in which stem cell reside in situ. The concept of stem cell niche was first proposed for human hemopoietic system in the 1970ś (Schofield R, 1978). The niche tightly regulates the fate of the stem cells, it controls the balance between commitment and self-renewal. It is conceptually divided into two parts: osteoblastic zone, vascular zone.

The osteoblasts are a critical component of adult BM niche. They are bone-forming cells derived from mesenchymal stem cells (Yasuda H et al., 1998). They are a heterogeneous population including immature cells as well as mature cells, which contribute to the bone formation (Calvi LM et al., 2003). It has been proved that the number of HSCs increases or decreases as the number of osteoblasts changes (Zhang J et al., 2003, Visnjic D et al., 2004). The cells of the niche produce factors that inhibit differentiation of HSCs and maintain them within the microenvironmental compartment. Adhesion molecules, mainly N-cadherins and β1-integrin, play a crucial role in the interaction between HSCs and osteoblasts (Arai F et al., 2004). HSCs express mainly α4β1 and α5β1 integrins which bind to fibronectin and promote adhesion to BM stromal cells (Potocnik AJ et al., 2000). Other group of proteins involved for localization of HSCs

to hemopoietic organs are the chemokines. They are involved in stem cell homing (Nagasawa T et al., 1996, Tokoyoda K et al., 2004, Peled A et al., 1999). The most important chemokine for localization of HSCs in BM is the stromal derived factor-1 (SDF-1) and its receptor CXCR4. Many other molecules expressed on osteoblasts play a role in bone marrow microenvironment interactions, such as leukocyte cell adhesion molecule (Arai F et al., 2002), osteopontin, bone morphogenetic protein, the Notch ligand-δ and angiopoietins (Calvi LM et al., 2003, Arai F et al., 2004).

The vascular niche differs between embryonic and adult life. Whereas during embryonic life well studied tight interactions between HSCs and the developing vascular system take place, interactions during adult life are not as extensively known. During embryogenesis, as stated above, hemopoietic and endothelial cells are derived from common progenitors, the hemangioblasts (Choi K et al., 1998). There exists ligand-receptor signaling between HSCs and endothelial cells. For example, HSCs secrete angiopoietin-1, which induces angiogenesis (Takakura N et al., 1998). Vascular endothelial cells provide inductive signals for organ development. During adult life, however, the situation is not as clear. In adult BM, vascular niche is defined as a place for stem cell mobilization or proliferation and differentiation of progenitors (Heissig B et al., 2002). To supply oxygen and nutrients necessary for dividing cells, the blood supply is required for proliferating progenitor cells rather than for stem cell maintenance. A model for vascular niche has been proposed, in which the activity of matrix mettaloproteinase-9 expressed within the osteoblastic zone results in cleavage of the membrane Kit ligand from BM stromal cells. Soluble Kit ligand then promotes cell cycle entry and motility of stem cells (Avecilla ST et al., 2004). As in the osteoblastic niche, also in the vascular niche are important stem cell regulators. Other important regulator is the VEGF, which regulates the blood vessel formation and hemopoiesis. The difference between

osteoblastic versus vascular niche is that in the osteoblastic niche stem cells are quiescent, whereas in the vascular niche stem cells divide (Heissig B et al., 2002).

Gerber et al (2002) hypothesized that progenitor cells surrounding the stem cells can be considered a third type niche cells. Progenitor cells suppress proliferation of stem cells, similarly to lateral inhibition seen in neural cells. When the progenitor number is decreased, HSCs are released and divide (Gerber HP et al., 2002).

1.9. HSCs homing, seeding and engraftment

Many complex processes are involved in successful transplantation of HSCs. They include homing, seeding and engraftment.

Homing of HSCs by extravasation into the microenvironment of bone marrow and stem cell niche is thought to be a pivotal process, because niche is the only site that normally hosts durable and multilineage differentiation of HCSc (Stein J et al., 2005, Berrios VM et al., 2001, Kucia M 2005 et al., Lapidot T and Kollet O, 2002, Quesenberry PJ and Becker PS, 1998, Voermans C et al., 2001). After transplantation, HSCs injected into systemic circulation distribute in accordance with hydrostatic and chemotactic determinants (Quesenberry PJ and Becker PS, 1998). This process is controlled by multiple interactions between HSCs and endothelial cells. HSCs express adhesion molecules including integrins, sialomucins and CD44 isoforms (Chute JP, 2006). Endothelium expresses selectins, namely E- and P-selectin (Lapidot T et al., 2005). Once the cell is attached to the endothelial wall, chemoattractants such as stromal derived factor-1 (SDF-1) induce an intracellular signaling cascade leading to activation of integrins (Chute JP, 2006). Integrins create firm adhesion by binding to counter-receptors on endothelial cell surface (Chute JP, 2006). A dominant role plays interaction between

α4β1 integrin, very late antigen (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1) (Lapidot T et al., 2005).

After HSCs adhesion and rolling, HSCs transmigrate through the bone marrow vessels. This process is prominently controlled by SDF-1 gradient. SDF-1 is expressed on endothelial cells and bone marrow cells (Orschell-Traycoff CM et al., 2000). HSCs express CXCR4, a receptor for SDF-1. Their mutual interaction is suggested to be a primary axis governing stem cell homing in the bone marrow following transplantation (Chute JP, 2006).

Seeding of HSCs is determined by adhesive interaction with the stroma (Askenasy N and Farkas DL, 2002). Rather than selective seeding due to a single molecular pair, interaction with bone marrow stroma is joined by rapid phenotypic changes in expression of cell molecules during the first days after transplantation (Stein J et al., 2005). Stein et al. hypothesized that cells make use of the pre-existing repertoire of adhesion molecules only for the first steps of homing and early seeding. Soon thereafter HSCs express additional molecules, a process that promotes engraftment (Askenasy N et al., 2003).

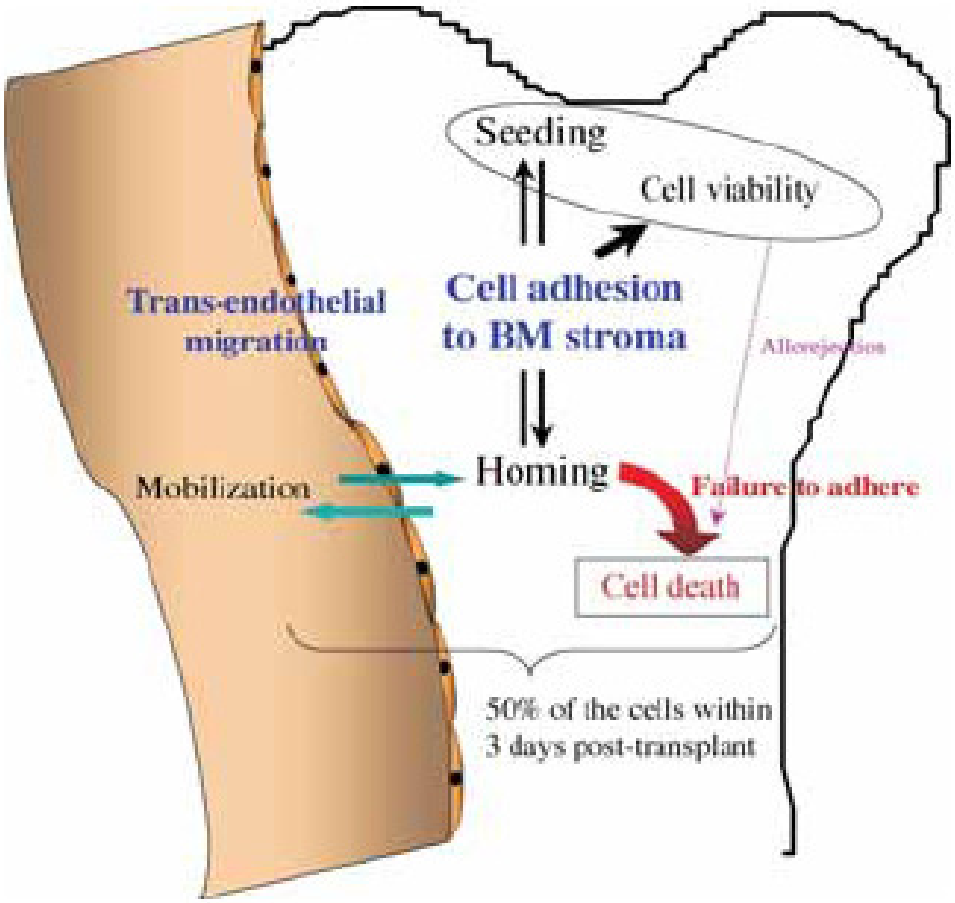


Fig. 3: Mobilization, homing, seeding

Engraftment means proliferation and differentiation of donor HSCs. Two types of niches are involved; niches that maintain functional quiescence of cells for long duration and niches that promote early dividing of cells (Askenasy N et al., 2003). The site of activity shifts with time towards the more central regions of the bone marrow.

The precise organization of seeding and engraftment in space and time illustrates the cooperativity of stromal regulation and both, adaptive and intrinsic HSCs responsiveness in the dynamic of early engraftment.

1.10 Mobilization of HSCs

As described above, retention of HSCs in hematopoietic organs is thought to be controlled by the interactive network of adhesive interactions (Gumbiner BM et al., 1996, Verfaillie CM, 1998). Under normal conditions only very low numbers of HSCs circulate in the peripheral blood (PB), which is the result of a dynamic equilibrium between efflux from the BM into the PB and recapture or rehoming of HSCs into the BM (Wright DE et al., 2001). Compared to homing, mechanisms leading to HSCs mobilization are less well understood. Similarly to homing and retention, important roles of the adhesion molecules in the mobilization has emerged (Lévesque JP et al., 2002).

Few distinct mechanisms may lead to stem cell mobilization (van Os R et al., 2000). Induction of stem cell expansion may lead to egress of stem cells to the peripheral blood and spleen (Bodine DM et al., 1996). This mechanism is dependent on hematopoietic growth factors that affect primitive stem cell growth such as stem cell factor (SCF) and FlT-3 ligand (FL). Another possibility for mobilization is the administration of IL-8 (Laterveer L et al., 1996), with similar overlapping pathways with G-CSF-induced mobilization, implicating the role of metalloproteinases (Laterveer L et al., 1996). Clinically as well as experimentally, mobilization is induced by administration of cytokines, such as granulocyte colony-stimulating factor (G-CSF or GM-CSF) (Sato N et al., 1994). Mobilization induced by G-CSF is dependent in time and dose, with rapid neutrophilia followed by a delayed increase of HSCs numbers in PB with maximum between 3-6 days after G-CSF administration (Sato N et al., 1994, Molineux G et al., 1990). In contrast, mobilization induced by chemotherapeutic agents is delayed, occurring in mice around day 6 during the recovery phase that follows the leucopenia induced by chemotherapy. G-CSF can be added to myelosuppresive drugs, leading to augmentation in HSCs mobilization (Sato N et al., 1994). Number of data now available demonstrates that

mobilization of HSCs is associated with increased production of proteases within the bone marrow space. These enzymes are able to degrade the extracellular matrix (Lataillade JJ et al., 2004). Mobilization induced by G-CSF is associated with increased SDF-1 degradation by elastase (Petit I et al., 2002) and matrix metalloproteinases 9 (MMP-9) (Heissig B et al., 2002). Additionally, it has also been demonstrated that after G-CSF administration occurs proteolysis of the extracellular domain of CXCR4 by working of elastase, parallely to the cleavage of VCAM-1 (Valenzuela-Fernandez A et al., 2002, Lévesque JP et al., 2003 and 2001). Degradation of CXCR4 leads to its internalization, which is a crucial event in HSCs mobilization (Shen H et al., 2001, Gazzit Y and Liu F, 2001). Therefore, directly blocking the interaction between SDF-1/CXCR4 alone can trigger HSCs mobilization from BM to the PB (Liles WC et al., 2003).

Further, administration of monoclonal antibodies against VLA-4 or VCAM-1 may induce mobilization of HSCs requiring signaling through the c-kit/c-kit ligand pathway (Papayannopoulou T et al., 1993; Craddock CF et al., 1997).

Recent studies suggest an important role for neutrophils in HSCs mobilization induced by G-CSF, cytotoxic agents and by chemokines (Lévesque JP et al., 2001). During G-CSF induced mobilization, neutrophils play a key role in this process with their massive degranulation (Hoglund M et al., 1997). It has been demonstrated by Lévesque et al. (2001) that the important step in HSCs mobilization caused by G-CSF is the disruption of the adhesive interaction between VCAM-1 and VLA-4 as a consequence of VCAM-1 cleavage by neutrophil proteases such as neutrophil elastase (NE) and catepsin G (CG), released by neutrophils activation in the extravascular compartment of the BM following G-CSF administration. Further evidence supporting the role of neutrophils in mobilization is that neutrophil depletion by infusion of neutrophil antibody prevents HSCs mobilization and reinfusion of mature neutrophils restores mobilization in response to G-CSF or

chemokines (Aiuti A et al., 1999). It therefore appears that activation of neutrophils within the BM leading to the transformation of the BM extravascular compartment into a highly proteolytic environment is a common feature of mobilization induced by G-CSF or chemotherapy (Lévesque JP et al., 2004, Winkler IG and Lévesque JP, 2006).

1.11 Estrogen and its influence on the hematopoietic tissue

Sex steroids and especially estrogen have a considerable influence over numbers of B-lymphocytes that are produced within bone marrow (Kincade PW et al., 2000). Beyond, data indicate that this might be a situation in humans as well (Kincade PW et al., 2000). It is generally appreciated that newly formed B cells and pre-B cells are normally frequent in bone marrow and markedly depressed in pregnant mice or in mice treated with estrogen (Medina KL et al., 1993, Medina KL et al., 1994). Which cells are the target of estrogen working and which stages of B-cell development are hormone sensitive are subjects of intensive research.

Estrogen influence corresponds to the suppression in IL-7 responsive precursors (Medina KL et al., 2000). Medina et al. provided data that have demonstrated that sex estrogen alter production of B cells by influencing the differentiation, proliferation and survival of early B-cell precursors (Medina KL et al., 2000).

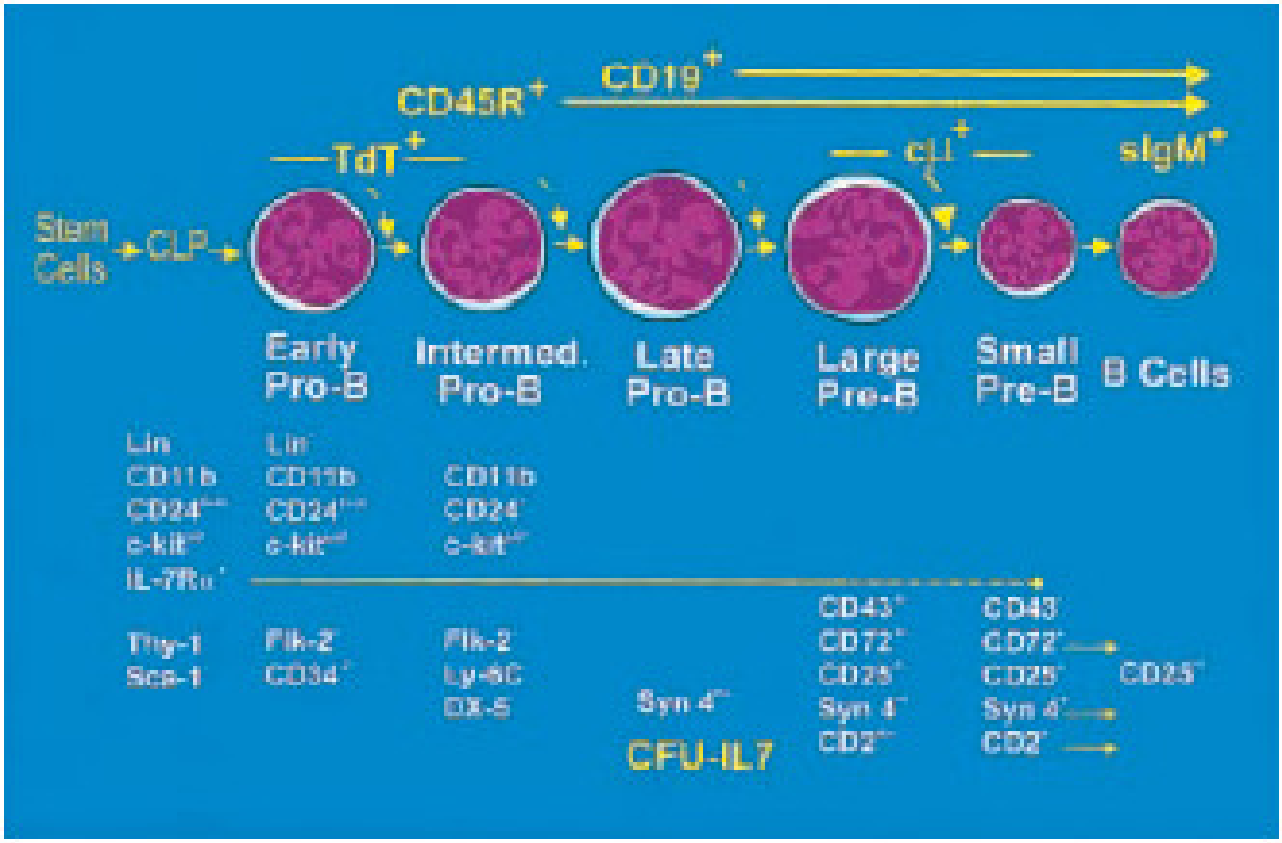


Fig.4: B-cell differentiation in mice

The data suggest that the $CD43^{-}CD45R^{+}CD19^{+}$ cells in estrogen treated mice are derived from very early precursors that exit cell cycle and subsequently retain some pro-B characteristics (Kincade PW et al., 2000) (Fig. 4). The $CD43^{-}$ cells differ from normal small pre-B cells in some aspects, for instance they lack μ heavy chains of Ig and CD25 (Fig.1). More accurate identification is presented by Rolink et al, who named estrogen sensitive population as pre-B II (Rolink A et al., 1994). They noted that these cells are $CD25^{+}$ $CD45R^{+}$. However, cells identified as $CD45R^{+}CD25^{-}$, pre-B I, were also depressed by estrogen (Medina KL et al., 2000). These data indicate that estrogen influence relatively early cells in the lineage. Further, it has been shown that estrogen influence events at an even earlier Lin^{-} stage (Payne KJ et al., 1999). The population of $Lin^{-}TdT^{+}$ cells is reduced by 66% in estrogen treated mice (Payne KJ et al., 1999). Furthermore, it

has been demonstrated that estrogen influences cells upwards of the common lymphoid progenitor stage (Medina KL et al., 2000). These findings are consistent with the fact that the production of B cells decreases during pregnancy or during estrogen treatment (Kincade PW et al., 2000). Numbers and activity of early pro-B cells probably assign the size of subsequent precursor populations. Therefore it can be postulated that a very early stage in lymphoid development may represent a key, estrogen sensitive control point.

Before, it was presumed that estrogen only regulate B-population indirectly through influencing stromal cells (Smithson G et al., 1995 and 1998). The assumption was made on *in vitro* findings that normal IL-7 sensitive B-cells and lymphocyte clones were resistant to estrogen when put in stroma cell-free cultures. These observations were concurrent with preceding data describing functional estrogen receptors in stromal cells (Bellido T et al., 1993). Along, estrogen influence on density and other features of marrow metabolism and physiology could be mediated by estrogen-responsible genes expressed by stromal cells. Therefore it may be necessary to have stromal cells present in bone marrow cultures to trace all estrogen induced effects (Smithson G et al., 1995). However, later studies have found significant decrease in B-cell numbers and differentiation even in stromal cell-free cultures. These studies have used early pro-B cells and multipotential progenitors (Medina KL et al., 2000). It might then seem that early pro-B cells may be particularly estrogen responsive.

Analogous situation is in humans. Estrogen and their antagonists are largely used in contraception, hormone replacement therapy and in the treatment of tumors (Kincade PW et al., 2000). It was demonstrated in murine model that number of newly formed B-cells decreases in peripheral blood and spleen after estrogen injection. Based on animal models, an assay was introduced to examine whether normal lymphocyte production is under

hormone control as is the situation in rodents. First data indicate that there is a fall in B-cell numbers of 70% during normal pregnancy (Medina KL et al., 1999).

Interesting aspect of estrogen influence is the fact, that steroid hormones are normal constitutients of bone marrow. Some tissues, like thymus and brain, have the potential to synthesize and degrade hormones locally (Mellon SH and Deshepper CF, 1993, Vacchio MS et al., 1994). If the same is true for bone marrow, systemic concentration may be less important than the one directly available for lymphocyte precursors. Therefore, production of steroidogenic and degrading enzymes by bone marrow stromal components could represent next control level.

It has successively become evident that the process of blood cell formation is very different between fetal and adult life (Kincade PW et al., 2002). This is particularly the case for lymphopoiesis, where the properties of progenitors and the differentiation pathways change dramatically in a developmental age-dependent manner. Furthermore the importance of steroid hormones as gatekeepers is appreciated, controlling entry into and progression within lymphoid lineages (Kincade PW et al., 2002). There are indices that fetal stem cells are intrinsically unique and do not efficiently acquire adult characteristics when transplanted to adult bone marrow (Igarashi H et al., 2001).

1.12 Adult versus fetal differences

Number of studies suggests that steroid hormones regulate normal steady-state lymphopoiesis (Hirose J et al., 2002). In contrast, the marrow of castrated male or ovariectomized female mice contains abnormally elevated numbers of lymphocyte precursors (Smithson G et al., 1994, 1998). However, estrogen selectively affects early B-cells (Rolink A et al., 1993) with myeloid progenitors being unaffected (Kincade P.W. et al., 2002). Therefore it is of great interest that the fetal immune system develops in an

estrogen-rich environment. Although placental enzymes can protect the fetus from maternal glucocorticoids, there is no such mechanism for estrogen. Igarashi et al demonstrated that estrogen receptors in B-cells are acquired only after birth (Igarashi H et al., 2001). In reality, the adult pattern of receptor expression did not develop until more than 3 weeks of age (Igarashi H et al., 2001). Noticeably, fetal HSCs did not efficiently acquire hormone receptors after transplantation into adult mice. This implies that fetal HSCs can have unique intrinsic properties (Kincade P.W. et al., 2002).

Other examples of fetal versus adult differences in B-cell lineage were presented already more then two decades ago (Landreth KS et al., 1983). Further research in this area brought information regarding expression of Qa-2 antigen (Kincade PW et al., 1980), major histocompatibility complex (MHC) class II expression on pre-B cell (Hayakawa K et al., 1994, Lam KP and Stall Am, 1994) and myosin light chain gene expression (Oltz EM et al., 1992). Kincade and colleagues concluded that the expression of genes is related more to developmental age than to differentiation stage (Kincade PW et al., 1980, 2002). It is also noteworthy that developmental age-related changes continue many weeks after birth (Kikuchi K and Kondo M, 2006). For example, adult mouse B-lymphopoiesis in contrast to its fetal counterparts, is completely dependent on IL-7 presence (Carvalho TL et al., 2001), fetal cells lack TdT (Gilfillan S et al., 1993), there is a different influence of CD40 delivered signals in fetal and adult mice (Martinez-Barnetche J et al., 2002).

Furthermore, expression of recognition molecules made during fetal life is substantially different from adult life (Kincade PW et al., 2002). It was shown, that specific subset of lymphocytes that are prevalent during fetal life, maintain their properties after transplantation into adult microenvironment (Hao Z and Rajewsky K, 2001). A comparable situation exists for the $V\gamma_3$ receptor bearing γ/δ T cells (Ikuta K et al., 1990).

It has been postulated that a developmental clock might influence the intrinsic differentiation potential of hematopoietic stem cells (Kincade PW et al., 2002).

Questions arise whether the patterns of gene expression and dependence on transcription factors are intrinsic or dictated by environmental cues. Extensively, whether stem cells derive directly from well-studied fetal counterparts and if so, residence in adult tissues must confer unique properties on those stem cells and specifically, on the lymphoid progenitors that derive from them (Kincade PW et al., 2002).

Gene or feature	**Finding**
Interleukin 7	Only required for adult lymphopoiesis
ERα, ERβ	Estrogen receptor acquired after birth
MHC II	Adult pattern of expression
TdT	Adult pattern of expression
CD144 (VE-cadherin)	Specific fetal pattern of expression
*Sox*17	Specific fetal pattern expression

Table 1. Examples of differences between fetal and adult HSCs

2. AIMS OF THE STUDY

1. To examine in detail murine congenic transplantation model Ly5.1/Ly5.2. To evaluate dependence of engraftment rate on cell dose transplanted to both lethally and sublethaly irradiated recipients. To study influence of mouse strain and sex on postransplantation chimerism. To establish and test a new F1 transplantation model allowing direct follow up the two competing cell populations in indifferent host microenvironment.

2. To study microenvironment/hematopoietic niche succeptibility to transplantation after preconditioning regimens consisting of cyclophosphamide and total body irradiation or busulphan. In CY-TBI host regimen the aim was to study influence of different time intervals between CY and TBI, different irradiation doses, effect of two CY doses and on the level of post-transplantation chimerism. To study the effect of replacing total body irradiation with busulphan on both the levels of myeloablation and post-transplantation chimerism.

3. To study the succeptibility to transplantation of hematopoietic stem cell niche after drugs affecting its cellular components (biphosphonates), and to investigate different types of hematopoietic microenvironment (fetal, neonatal and adult) in terms of aquisition of HSCs succeptibility to estrogen.

3. MATERIALS AND METHODS

3.1 Mice

C57BL/6 mice (B6-Ly 5.2) and their congenic strain (B6-Ly5.1) were used in the experiments. This mouse strains differ only in surface antigen Ly5 (CD45). They bear either Ly5.1 (CD45.1) or Ly5.2 (CD45.2) isoform. This allows detection of donor-derived blood cells present in recipient organism (chimerism). The animals were maintained in a clean conventional facility with a light-dark cycle of 12 hours and fed ad libitum. Mice taken to the experiments were 8- to 12-weeks-old.

F1 (*Filial 1*) generation mice are obtained from cross mating of parental inbred Ly5.1 and Ly5.2 strains. They express both CD45.1 and CD45.2 molecules.

3.2 Mating

To set up mating, females were examined in the afternoon, and those in estrus phase were placed in cages with males (two females with one male). The morning after mating males were removed and the females were checked for the presence of a copulating plug in the vagina, and this day was designated as embryonic day 0.5.

3.3 Cells

Adult BM was collected from both femurs of normal adult mice. Fetal livers were obtained from embryos at day E14.5 of gestation. Neonatal livers were obtained from newborn mice, day P0.5. A single-cell suspension was prepared in a phosphate-buffer saline (PBS) solution containing 0.5% albumin by repeated flushing the tissues through needles of 18 and 27 Gauges. Finally, cells were passed through a nylon mesh with a pore size of 70 um (Falcon 2350, Becton Dickinson Labware, Franklin Lake, NJ), counted and appropriately diluted.

3.4 Irradiation

Irradiation was delivered from a ^{60}Co source 0.8Gy/min. Mice were irradiated with different doses as described in concrete transplantation settings bellow in results section. Lethal irradiation was 10 Gy (5+5, 30´ interval).

3.5 Drugs/chemicals (in alphabetical order):

3.5.1 Bisphosphonates treatment

Bisphosphonate (natrii risedronas, Actonel, Sanofi-Aventis, s.r.o., Praha, Czech Republic) was incorporated into the animal diet. Each animal received 5.0 g of food per day corresponding to 70 ug/kg/day, which is considered to be comparable to a dose of risendronate used for treatment of osteoporosis in women.

3.5.2 Busulphan treatment

Busulphan (Busulphan, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in DMSO:oil solution (0.35:0.65) and injected s.c. in a dose 25mg or 50mg.

3.5.3 Cyclophosphamide treatment

Cyclophosphamide (cyclophosphamidum, Cyclophosphamide Orion, Orion Corporation, Espoo, Finland) was dissolved in PBS and injected i.p. in dose of 135 mg/kg of cyclophosphamide.

3.5.4 Estrogen treatment

Male mice were used in the experiments and treated with estrogen to avoid possible hormone background in females. Estrogen was delivered in two administration regimens:

A) Estrogen (estradiolum hemihydricum, Estrofem, Novo Nordisk A/S, Bagsvaerd, Denmark) was added to food starting one day before transplantation in an amount corresponding to delivery of approximately 100 ug of 17β estradiol/mouse/day. Additionally, estrogen-treated mice received 100 ug of estradioli dipropionas (Agofollin, Biotika, Slovak Republic) i.m. one day before transplantation to substitute a decreased food intake following preconditioning with irradiation.

B) Estrogen was delivered only as i.m. injections of 250 ug estradioli dipropionas (Agofollin) in 2-weeks intervals starting one day before transplantation.

In both administration protocols the estrogen treatment resulted in diminished weight of the testes and seminal vesicles and in partial obliteration of the cavity of long bones harbouring BM. These are well-known effects of estrogen administration (Samuels 1999, Onoe 2000, Perry 2000, Erlandsson 2003) and these examinations were used to confirm estrogen exposure.

3.6 Analysis of recipients

3.6.1 Flow cytometry

Peripheral blood was obtained from the retro-bulbar plexus in regular intervals 2, 4, 8, 12, 16 and 20 weeks after transplantation. Aliquots (~50 ul) from each blood sample were added to separate tubes filled with 3 ml of lysis buffer (0.15 M NH_4Cl, 0.035 M NaCl and 0.1 mM EDTA) and red blood cells were lysed at room temperature for 10 minutes. Cells were washed twice, resuspended in PBS and stained with phycoerythrin (PE)-conjugated anti-Ly5.1 and fluorescence isothiocyanate (FITC)- conjugated anti-Ly5.2 antibodies. They were simultaneously stained with biotinylated anti-B220 or with biotinylated anti-Gr-1 and anti-Mac-1 or with biotinylated anti-CD3 antibodies. This was followed by addition of streptavidin-PE-Cy-5. All antibodies and reagents were purchased

from Pharmingen (San Diego, Ca, USA). Multicolor analysis was performed on FACS Calibur (Becton Dickinson, San Jose, Ca, USA). Gating for $Ly5.1^+$ and $Ly5.2^+$ cells was performed and when $Ly5.1^+Ly5.2^+$ artificial doublets were present, they were omitted from the analysis.

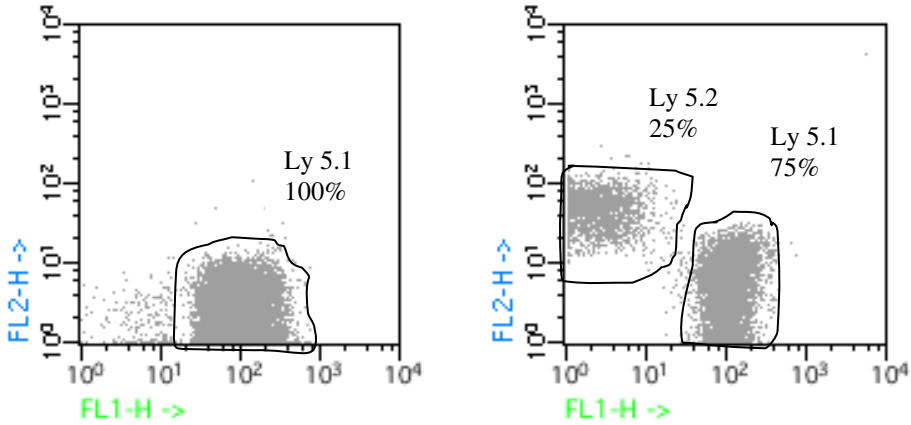


Fig. 5: Example of FACS figure of Ly5.1 mouse and chimeric Ly5.1 mouse.

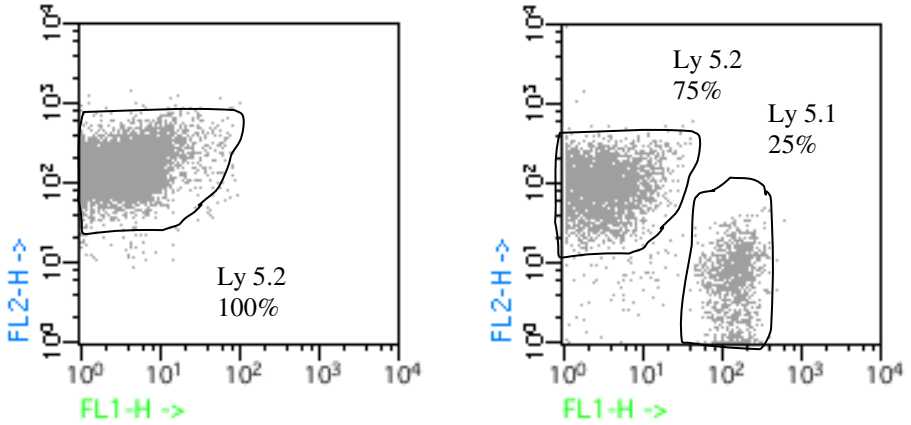


Fig. 6: Example of FACS figure of Ly5.2 mouse and chimeric Ly5.2 mouse.

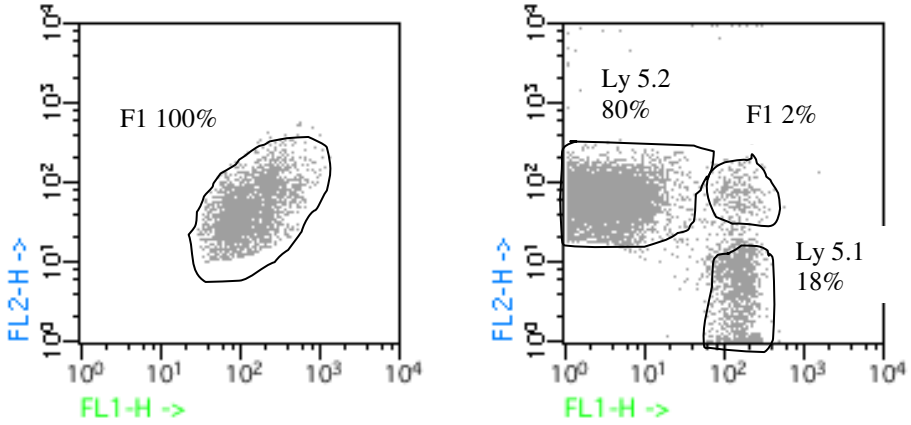


Fig. 7: Example of FACS figure of F1 mouse and chimerism in F1 mouse.

3.6.2 Real-Time PCR, RT-PCR

Tissue samples were stored in RNALater (Sigma Aldrich, USA). Total mRNA was isolated with Qiagen RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). Reverse transcritption was performed using Bio-Rad iScript (Bio-Rad Laboratories, Hercules, CA, USA). Target mRNA levels were determined by real-time PCR using SYBR Green protocol. Target mRNA content was calculated relatively to GAPDH mRNA content, assuming exact doubling of amplified DNA in each PCR cycle (Vokurka at el 2006). ESRα specific primers were:

- F: 5´AAGAACGTTGTCCCCCTCTAT 3´
- R: 5´GGGTAAAATGTTGCAGGGATT 3´

The amplicon length was 249 bp.

3.7 Statistical analysis

Group of eight recipient mice given the same treatment were used to calculate the mean values and standard deviations of donor-origin and recipient-origin cells, both in total chimerism as well as in chimerism in specific hematopoietic cell lineage populations. All experiments were repeated at least 3 times. These values were used to calculate P values using the two-tailed Studentś *t*-test. ANOVA was used for comparison of multiple groups. Linear regression was used for analyses of experiments with expected linear increase in chimerism.

4. RESULTS

4.1 Part I - Verification of murine congenic transplantation models

Congenic Ly5.1/Ly5.2 mice model is widely used in experimental hematology. Ly5.1 and Ly5.2 are two inbred mice strains that differ between each other in the surface antigen CD45. They express isoforms CD45.1 or CD45.2. This murine model is used in functional studies of HSCs behavior.

Van Os et al. have demonstrated, that minor immunogenic reactions can occur in this transplantation model. They can possibly influence HSCs behavior and interfere with gained results.

F1 (*Filial 1*) generation mice are obtained from cross mating of parental inbred Ly5.1 or Ly5.2 strains. They express both CD45.1 and CD45.2 molecules. We have created this mouse model to be able to track competition of two grafts in new parental environment.

Detailed knowledge of the properties of Ly5.1/Ly5.2 model and F1 model is of outmost importance.

In classical Ly5.1/Ly5.2 mice model, following transplantation combinations and their mutual interference have been studied (Part A):

1. To test if post-transplantation chimerism is proportional to ratio of transplanted Ly5.1/5.2 cells into lethally irradiated recipients
2. To test if post-transplantation chimerism is proportional to transplanted congenic cells into sublethally irradiated recipients
3. To test gender influence on the engraftment
4. To test if the same WBCs subpopulation distribution is affected by the percentage of donor cell chimerism

In F1 mice model, the following was studied (Part B):

1. To test if post-transplantation chimerism is proportional to ratio of transplanted Ly5.1/5.2 cells into lethally irradiated recipients. The influence of different parental combinations of F1 mice on the level of engraftment of HSCs originating from individual parental lineages

Part A

4.1.1. Influence of different mutual ratios of donor Ly5.1/Ly5.2 cells on the level of engraftment after transplantation into lethally irradiated recipients

Lethally irradiated Ly5.1 or Ly5.2 mice were used as recipients. Two hours after irradiation they were transplanted with a mixture of bone marrow cells from Ly 5.1 or Ly 5.2 donors. The total number of transplanted cells was 4 x 10^6. Mutual ratio of donor cells is presented in Table 2.

Increasing percentage of donor cells leads to increasing number of donor hematopoiesis in Ly5.1 as well as Ly5.2 recipients (Fig.8 and 9). There was no significant difference between the two strains in the level of donor cells engraftment. Tables 3 and 4 show statistical parameters.

Ly 5.1 (%)	Ly 5.2 (%)
0	100
25	75
50	50
75	25
100	0

Table 2: The mutual ratio of Ly 5.1 and Ly 5.2 donor bone marrow cells transplanted into lethally irradiated recipients.

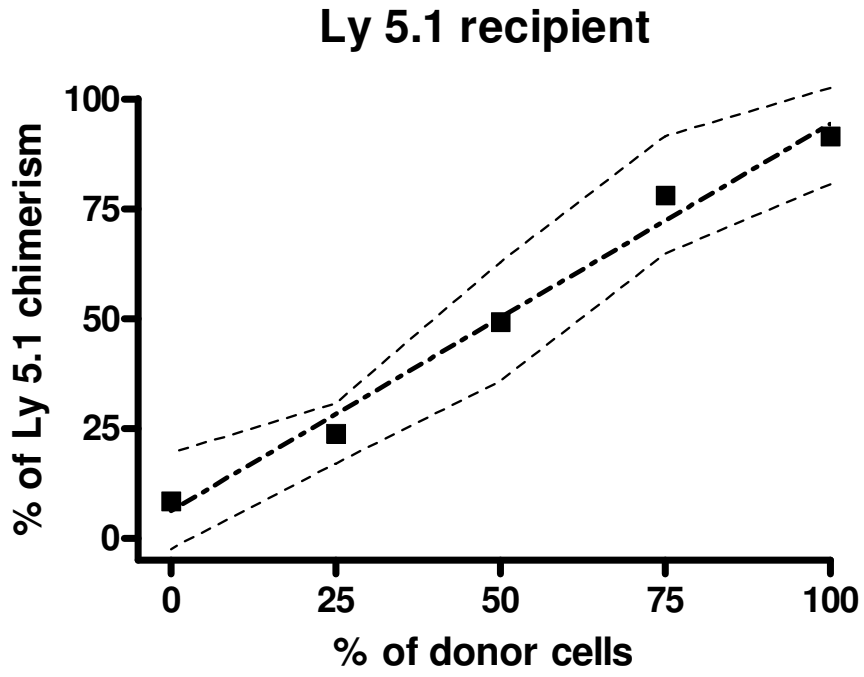


Fig. 8: Percentage of engraftment of Ly5.1 donor cells into lethally irradiated Ly5.1 mice. % of Ly5.2 cells is 100% - Ly5.1%. Regression analysis: chain-dotted line is the regression curve, 95% confidential interval is represented by dashed line.

Regression analysis – linear model	
a	0.8810 $\pm$ 0.05919
y	6.232 $\pm$ 3.640
x	-7.074
r^2	0.8569

Table 3: Parameters of linear regression for Ly 5.1 recipients.

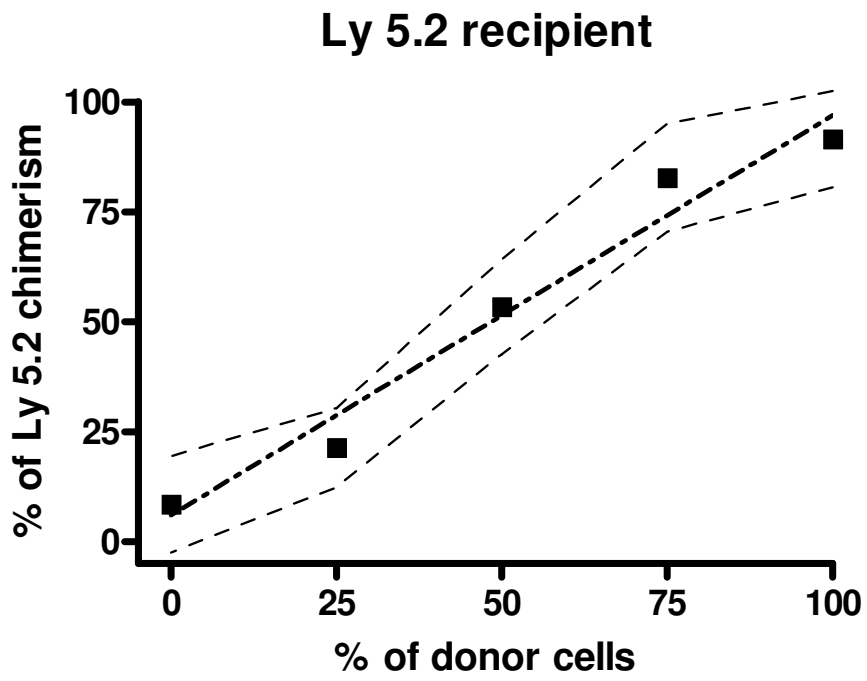


Fig. 9: Percentage of engraftment of donor cells into lethally irradiated Ly5.2 mice. % of Ly5.1 cells is 100% - Ly5.2%. Regression analysis: chain-dotted line is the regression curve, 95% confidential interval is represented by dashed line.

Regression analysis – linear model	
a	0.9095 $\pm$ 0.06157
y	6.025 $\pm$ 3.770
x	-6.625
r^2	0.8517

Table 4: Parameters of linear regression for Ly5.2 recipients.

4.1.2. Influence of the number of transplanted congenic cells on the level of engraftment after transplantation into sublethally irradiated recipients

Ly 5.1/Ly 5.2 mice were used. Recipient mice were sublethally irradiated with 4 Gy and transplanted 2 hours later with 5, 10, 20 or 30 million congenic bone marrow cells. Ly5.1/Ly 5.2 mice were reciprocally used.

Transplantation of 5 million donor cells lead to approximately 30% of donor hematopoiesis. Twofold dose of transplanted cells increased the percentage of donor hematopoiesis to 60%. Transplantation of 20 and 30 million cells ensured near total donor-derived chimerism (Fig.10).

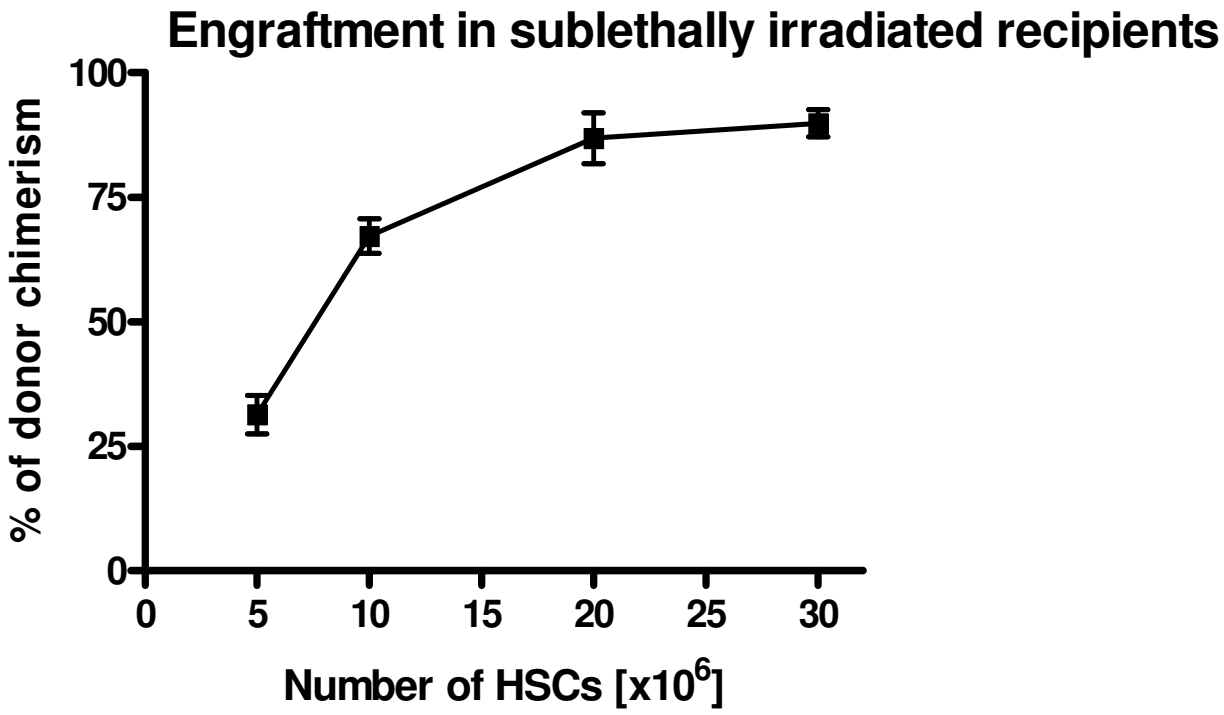


Fig. 10: Engraftment of donor cells into sublethally irradiated recipients.

4.1.3 Gender influence on the engraftment

Lethally irradiated Ly5.1 or Ly5.2 male and female mice were used as recipient. They were lethally irradiated and 2 hours later transplanted with a 5 x 10^6 bone marrow cells in equal amount of Ly5.1 an Ly5.2 cells.

Transplantation of Ly5.1 and Ly5.2 BM cells into male and female recipients lead to comparable level of donor hematopoiesis. There was no difference in the level of engraftment between the sexes. There was also no preference in engrafting of congenic or syngenic cells (Fig. 11 and 12).

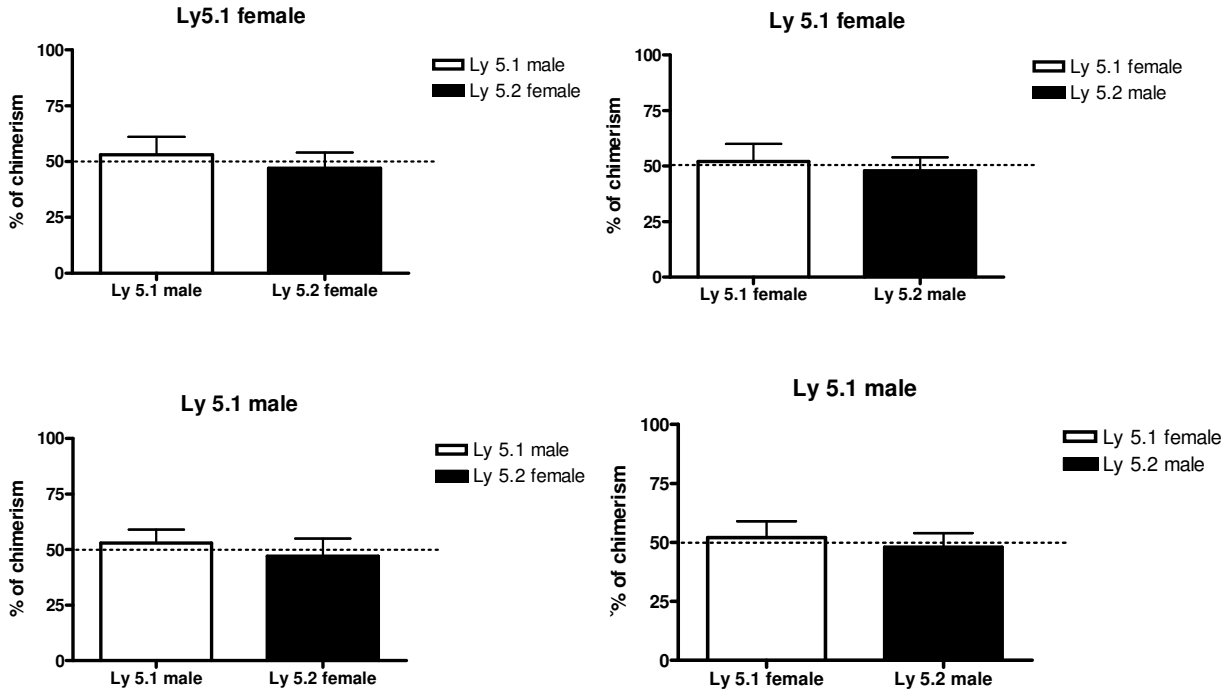


Fig. 11: Engraftment of bone marrow cells into lethally irradiated Ly 5.1 recipients.

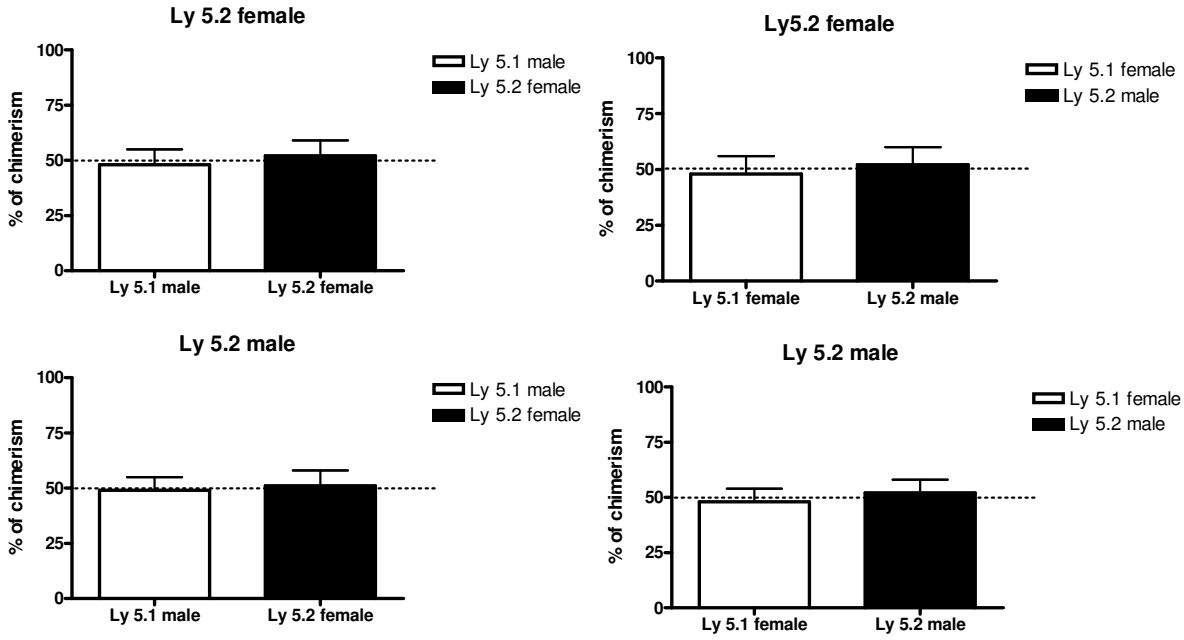


Fig. 12: Engraftment of bone marrow cells into lethally irradiated Ly 5.2 recipients.

4.1.4 The influence of chimerism on WBCs subpopulation distribution

The differential leukocyte count is mainly consisting of B-lymphocytes, followed by CD3+ cells and Gr/Mac+ cells in mice. B-lymphocytes (B220+ cells) encompass about two thirds of the WBC population. There is no significant difference in the distribution of the subpopulations between Ly5.1 and Ly5.2 mice (Fig. 13).

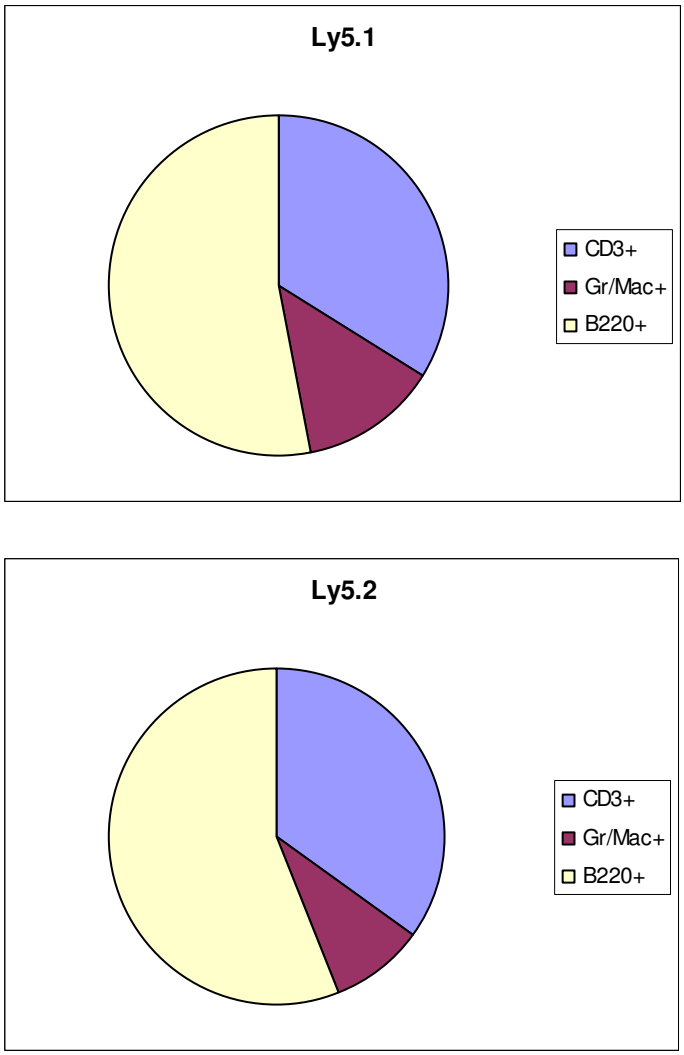
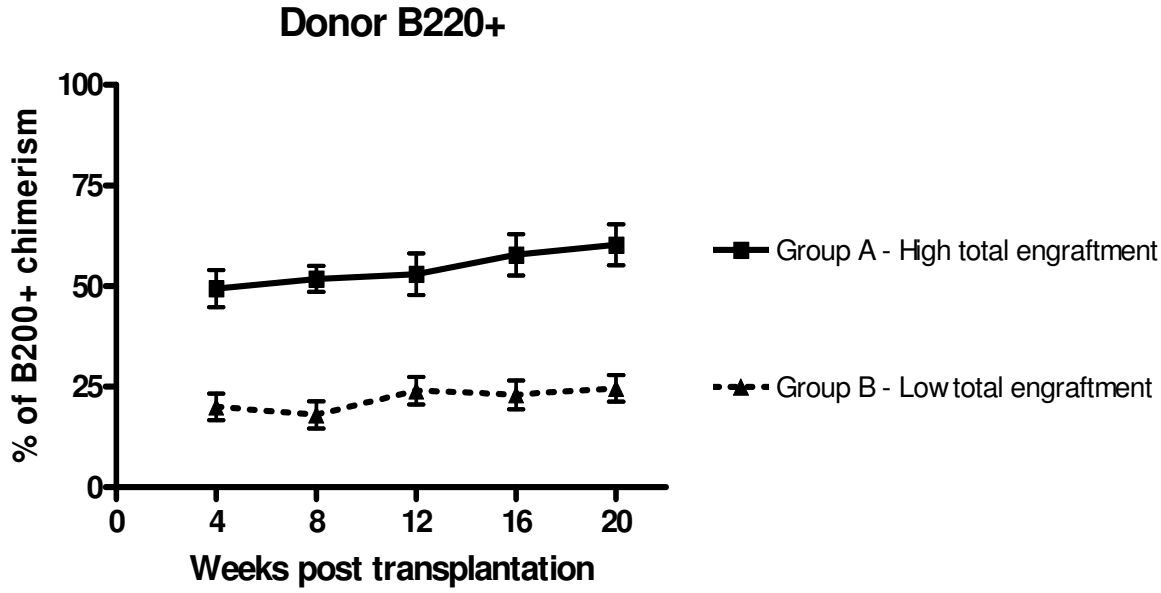


Fig. 13: Distribution of white blood cell subpopulations in Ly5.1 and Ly5.2 mice.

It has been studied whether different levels of HSCs engraftment are related to different distribution of WBC subpopulations using Ly5.1/Ly 5.2 model.

Recipient mice were sublethally irradiated with 4 Gy and transplanted 2 hours later with 5 (Group A) or 20 million (Group B) congenic bone marrow cells. Ly 5.1/Ly 5.2 mice were reciprocally used. Transplantation of 5 million of donor cells lead to approximately 30% of donor hematopoiesis. Transplantation of 20 million cells lead to almost total donor chimerism. In mice with high level of donor engraftment (Group B), the main of donor derived WBCs are B220+ cells. Gr/Mac+ cells are relatively suppressed (Fig. 14). Low levels of donor cell engraftment (Group A) are associated with B220+ suppression and relatively higher contribution of Gr/Mac+ in donor derived part of WBC. Total distribution of WBC remained unchanged from normal mouse, only relative contribution of donor cells to B- and Gr/Mac-subpopulation depends on the level of engraftment.



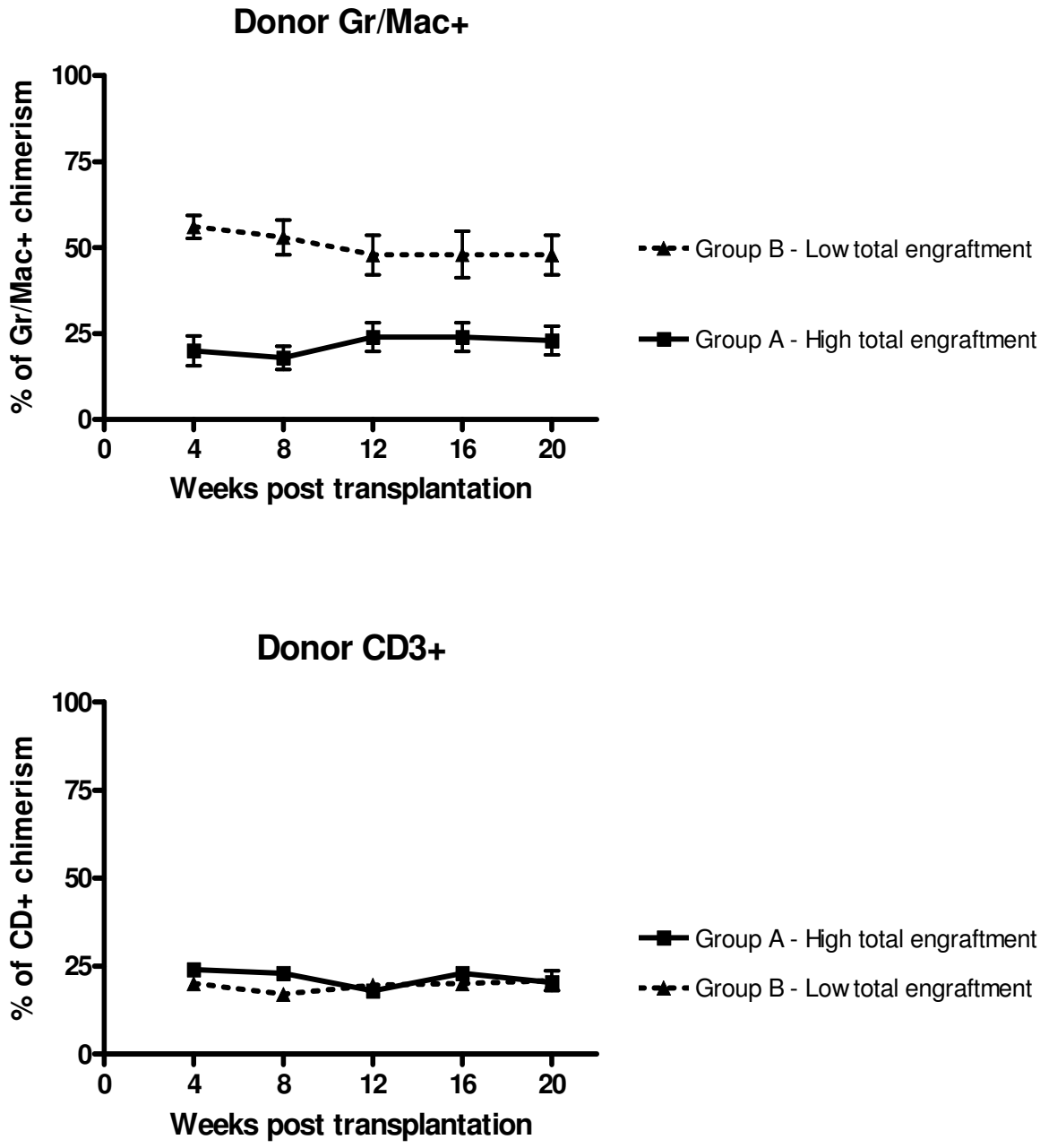


Fig. 14: WBCs subpopulation (B220+, Gr/Mac+, CD3+) distribution in mice with high and low total chimerism.

Part B

4.1.5 The influence of different parental combinations of F1 mice on the level of engraftment of donor cells originating in individual parental lineages

F1 mice were used as recipients. Mice were from both parental combinations (Recipient A and B) (Table 5).

The recipient mice were lethally irradiated. 2 hours after irradiation mice were transplanted with equal number of Ly 5.1 and Ly 5.2 cells (1:1) in total amount of 8 million cells. Chimerism was followed until 20 weeks after transplantation. The percentage of original F1 mice was negligible (<1%). Hematopoiesis from Ly 5.1 and Ly 5.2 origin participated with equal contribution. Small, but non.signififcant preference of Ly5.2 cells was noted. (Fig. 15 and 16).

Recipient A		Recipient B	
Mother Ly 5.2	Father Ly 5.1	Mother Ly 5.1	Father Ly 5.2

Table 5: Origin of recipient F1 mice. Mice obtained from both parental combinations (Mother Ly 5.2, Father Ly 5.1 and vice versa).

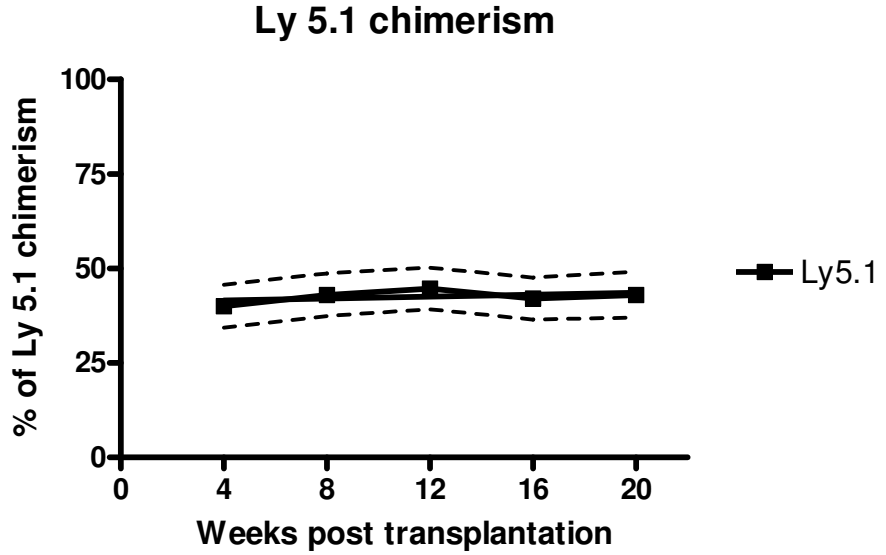


Fig. 15: Engraftment of Ly 5.1 and Ly 5.2 cells into F1 mice. F1 mice – recipient B.

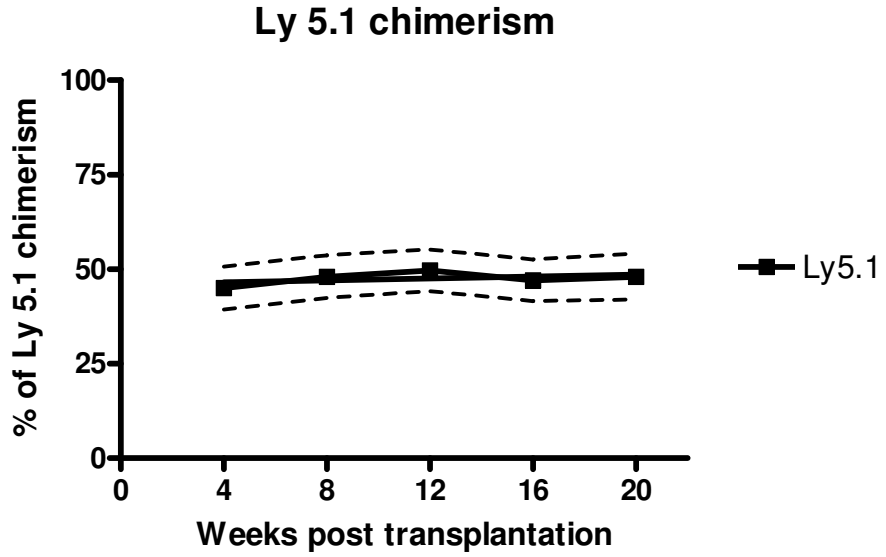


Fig. 16: Engraftment of Ly 5.1 and Ly 5.2 cells into F1 mice. F1 mice – recipient A.

4.2 Evaluation and comparison of different conditioning regimens on the level of engraftment of transplanted HSCs

Cyclophosphamide (CY) is used in management of a wide variety of pathological conditions including treatment of solid tumors, hematological malignancies and some autoimmune diseases such as lupus erythematosus and rheumatoid arthritis (8-11). It has immunosuppressive and antiangiogenic effects (2-4). It is also used for the mobilization of HSCs from the bone marrow into peripheral blood (5-7).

However, the most frequent use of CY is as part of preconditioning regimens before bone marrow transplantation combined either with total body irradiation (TBI) or other myelosuppresive agents.

It has been previously demonstrated that CY administration induces a unique pattern on hematopoietic regeneration in mice (Sefc et al. 2003). Single dose of CY alters the bone marrow microenvironment in such a way that it either suppresses or induces regeneration of CFU-S, in different phases of regeneration (Šefc et.al.). Changes in the microenvironment made the CFU-S susceptible or resistant to irradiation damage according to the time interval between CY and TBI.

We have studied the effectiveness of this CY-induced conditioning *in vivo* on the level of engraftment of transplanted HSCs.

The following was studied:

1. The importance of the time interval between administered CY dose and total body irradiation (TBI) delivery to hosts
2. Effect of different radiation doses on the level of engraftment in CY pretreated mice
3. The effect of CY on donor hematopoiesis
4. Engraftment of donor HSCs into recipients conditioned with single or two separate CY doses and irradiation

5. Influence of replacing myeloablation of TBI with Busulphan - engraftment of donor HSCs in cyclophosphamide –busulphan (CY-Bu) conditioning regimen

4.2.1 Importance of the time interval between CY dose and total body irradiation delivery of hosts

Congenic Ly5.1/5.2 mice model was used. Mice were treated with the same CY dose in different time intervals before TBI. Control mice were irradiated only. 2 hours after irradiation mice were transplanted with bone marrow from congenic mice strain. Detailed conditioning and transplantation scheme is depicted below (Fig. 17).

CY given to recipients two days prior to TBI (CY-2) significantly decreased both short- and long-term engraftment of donor HSCs compared to control mice that were only irradiated. On the other hand, CY administered five (CY-5) or seven (CY-7) days before TBI significantly increased donor-derived chimerism. Administration of CY three (CY-3), ten (CY-10) or fourteen (CY-14) days before TBI lead to intermediate engraftment, achieving higher percentage than in control mice, but not reaching the level of CY-7/5 group (Fig. 18).

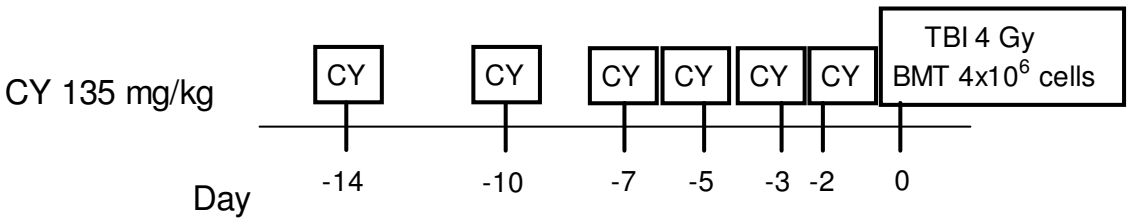


Fig. 17: Conditioning and transplantation scheme.

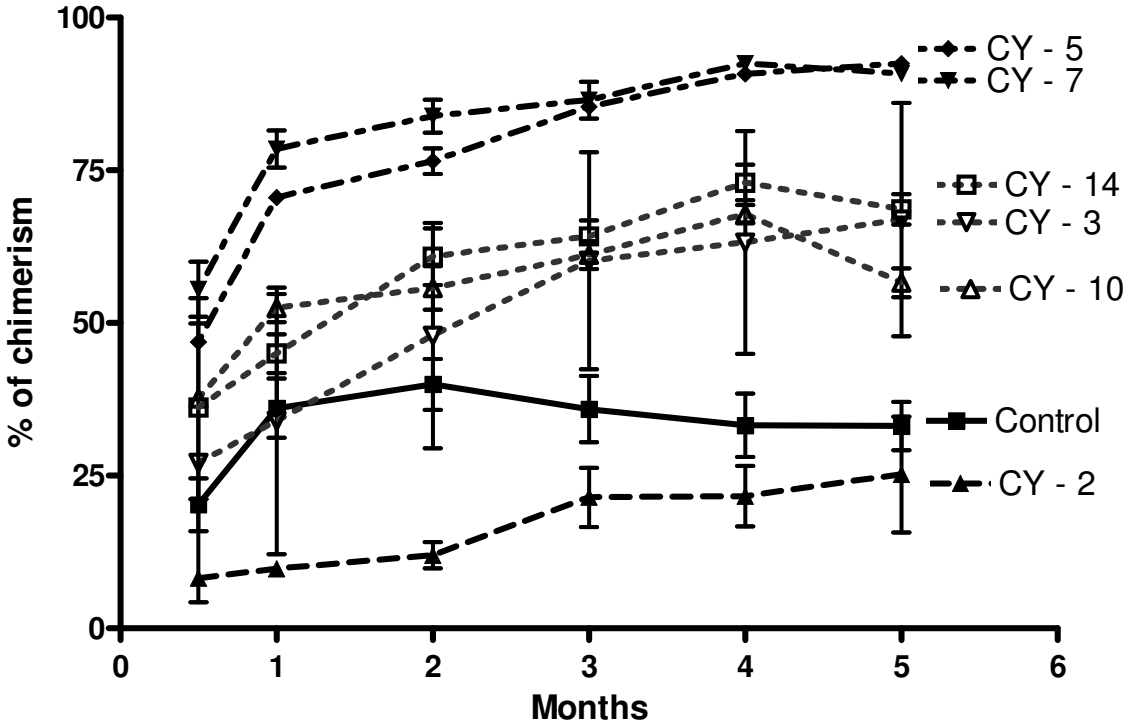


Fig. 18: Donor-derived engraftment (% of chimerism) achieved in recipient mice in particular preconditioned groups.

Month	0.5	1	2	3	4	5
CTRL	20 ± 4.3	36 ± 4.8	40 ± 4.2	36 ± 5.4	33 ± 5.2	33 ± 4.0
CY-2	8.2 ± 1.0 **	9.8 ± 1.2 **	12 ± 2.2 **	21 ± 4.8 **	22 ± 4.9 *	25 ± 9.5 **
CY-3	27 ± 12.8 ns	34 ± 12.2 ns	48 ± 5.8 *	60 ± 10.8 **	63 ± 11.8 *	67 ± 12.0 *
CY-5	47 ± 1.0 **	71 ± 0.8 ***	77 ± 2.1 ***	85 ± 1.3 ***	91 ± 1.1 ***	92 ± 0.4 ***
CY-7	56 ± 4.5 ***	78 ± 3.0 ***	84 ± 2.7 ***	86 ± 3.0 ***	92 ± 1.2 ***	91 ± 1.2 ***
CY-10	38 ± 1.6 **	52 ± 2.3 *	56 ± 3.6 *	61 ± 2.3 **	68 ± 1.5 **	57 ± 2.3 **
CY-14	36 ± 1.0 **	45 ± 3.2 *	61 ± 4.6 **	64 ± 2.6 **	73 ± 2.9 **	69 ± 2.5 **

Table 6: Statistical analysis. Values are expressed as mean ± SEM. Significant difference from controls: (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

4.2.2 Effect of different radiation doses on the level of engraftment in CY pretreated mice

According to the results presented in previous section, the time interval between CY administration and TBI lead to great differences in donor-derived engraftment with the same irradiation dose used. Therefore it was tested whether it is possible to increase the level of chimerism with increasing the irradiation dose. The same murine model was used. Detailed transplantation scheme is depicted in Fig. 19. Mice were divided into three groups (CY-5, CY-2, control mice), representing high, low and medium engraftment levels after the dose of 4 Gy of TBI. In the present experiment, they were irradiated with increasing radiation doses in a range from 0 Gy to 8 Gy and transplanted with $4x10^6$ donor cells. A minimum irradiation dose is needed for successful engraftment in all groups, but its level greatly differs. CY delivered 5 days before TBI, the similar level of engraftment was obtained after smaller TBI dose compared to control. When CY is administered 2 days before TBI the minimum irradiation dose needed for engraftment is higher compared to CY-5 group (1 Gy for CY-5, 2 Gy for CTRL and CY-2). CY-2 gave inferior engraftment compared to CTRL and CY-5 and increasing the radiation over 4 Gy does not lead to the increase in engraftment

(Fig. 20).

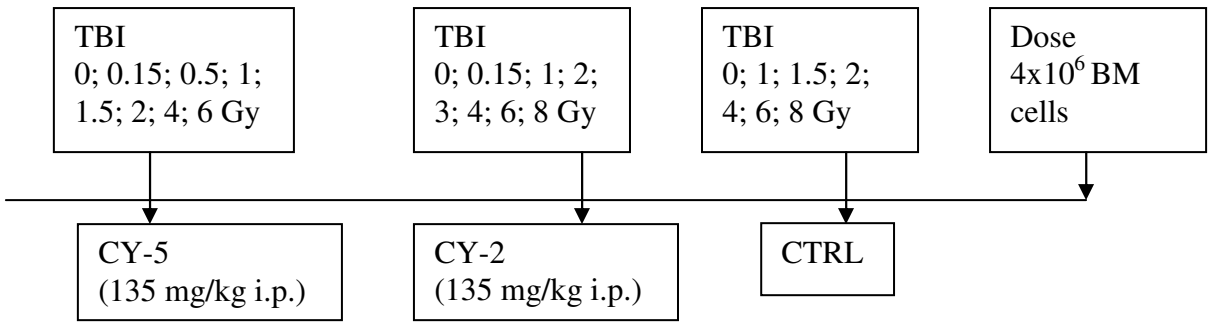


Fig. 19: Preconditioning and transplantation scheme.

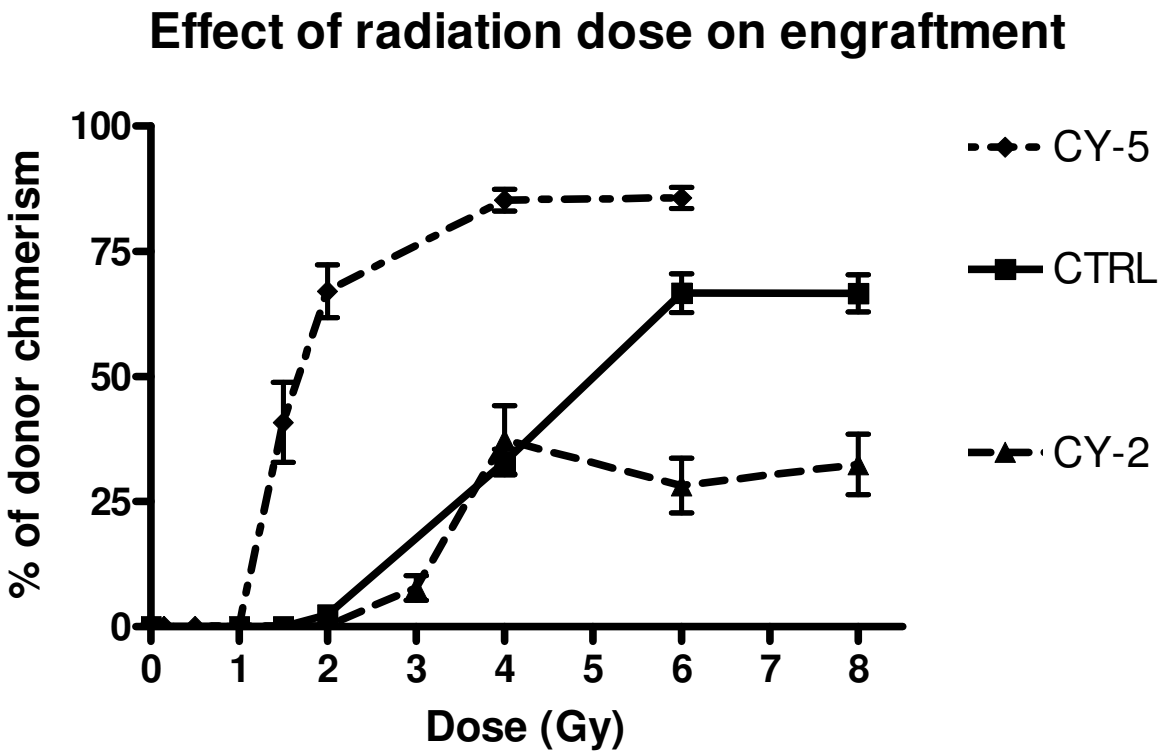


Fig. 20: Donor-derived hematopoiesis achieved with increasing dose of delivered TBI determined 16 weeks after transplantation.

Dose (Gy)	0	0.15	0.5	1	1.5	2	3	4	6	8
CTRL	0	-	-	0	0	2.5 ± 1.4	-	33 ± 2.5	67 ± 3.9	66 ± 3.7
CY-2	0 ns	0 -	-	0 ns	-	0.1 ± 0.05 **	7.7 + 2.4 -	30 ± 6.8 ns	28 ± 5.5 **	32 ± 6.1 **
CY-5	0 ns	0 -	0 -	0.7 ± 0.1 ns	41 ± 8.0 ***	67 ± 5.3 ***	-	85 ± 2.2 ***	86 ± 2.1 *	-

Table 7: Statistical analysis. Values are expressed as mean ± SEM. Significant difference from controls: (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

4.2.3 The effect of CY on donor hematopoiesis

We have studied the repopulation potential of HSCs of BM collected from mice different time after CY treatment. Ly5.1 and Ly5.2 mice were treated with CY. Bone marrow (equal to 1/3 of femur) was collected 2 and 5 days after CY and transplanted into congenic recipients irradiated with 4 Gy (Fig. 21). Control mice received an equal amount of normal saline. BM 2 days after CY (CY-2) gave significantly higher repopulation compared to BM 5 days after CY (CY-5) and control BM (CTRL). (Fig. 22).

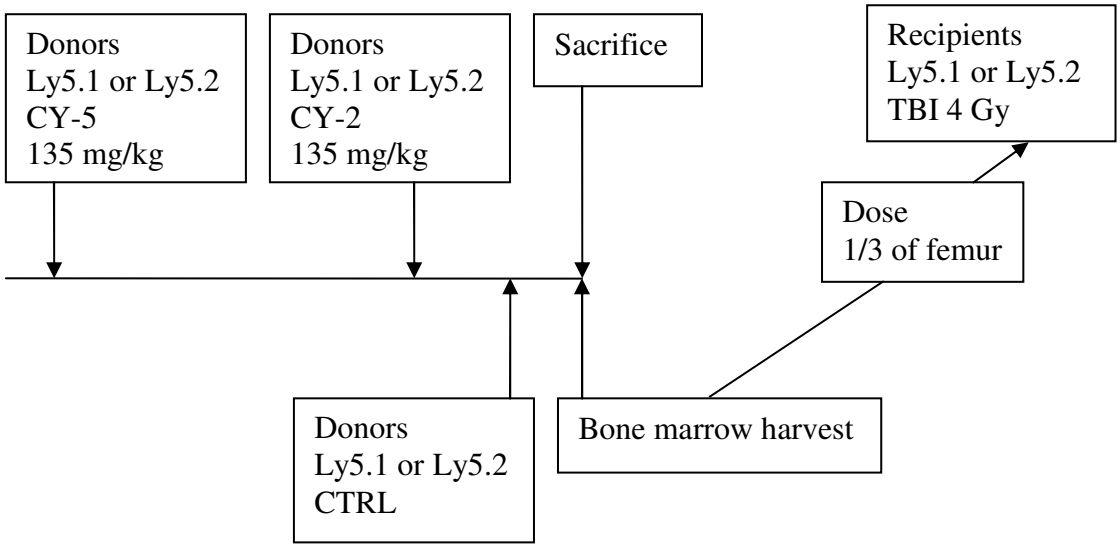


Fig. 21: Preconditioning and transplantation scheme.

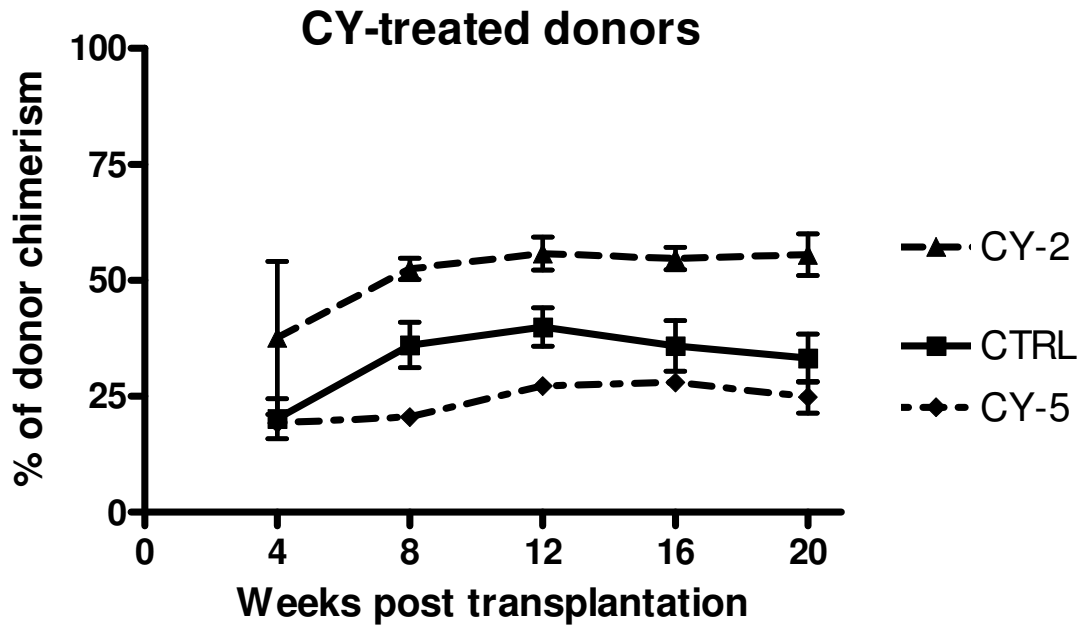


Fig. 22: Hematopoiesis derived from CY treated mice into recipients irradiated with 4 Gy.

Weeks	4	8	12	16	20
CTRL	20 ± 4.3	36 ± 4.9	40 ± 4.2	36 ± 5.4	33 ± 5.2
CY-2	38 ± 16.5 **	52 ± 2.3 **	56 ± 3.6 **	55 ± 2.4 **	56 ± 4.5 **
CY-5	19 ± 1.1 ns	21 ± 1.6 *	27 ± 1.8 *	28 ± 1.7 *	25 ± 3.4 *

Table 8: Statistical analysis. Values are expressed as mean ± SEM. Significant difference from controls: (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

4.2.4 Engraftment of donor HSCs into recipients conditioned with a single or two separate CY doses

Based on previously obtained results demonstrating significant difference in short-term as well as long-term engraftment of HCSc depending on different time between CY administration and TBI, the influence of delivering second CY dose was investigated in Ly5.1/5.2 congenic model. Conditioning scheme is in detail in Fig. 23. Mice were divided into five groups, receiving 135 mg/kg i.p. of CY 7, 2 or (7 and 2) days or (3 and 2) days before irradiation. Control mice were only irradiated. Mice preconditioned with CY 7 days before TBI had a significantly higher level of donor derived engraftment compared to mice that had received CY 2 days before TBI. Interestingly, administration of the second dose of CY 2 days before TBI had decreased just short-term engraftment (up to 2 months). The level of long term engraftment (above 3 moths) was determined by the first dominant dose of CY administered 7 days before TBI, when the engraftment was comparable to a single administered CY dose (Fig. 24). Two doses given to hosts 2 and 3 days before TBI (CY-3 + CY-2) did not increase engraftment of transplanted BM.

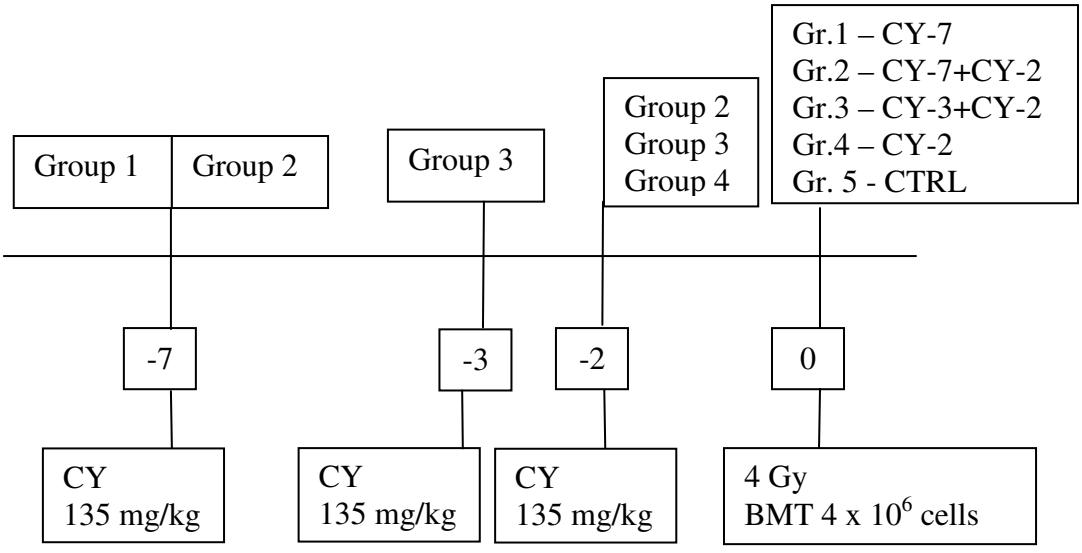


Fig. 23: Preconditioning and transplantation scheme.

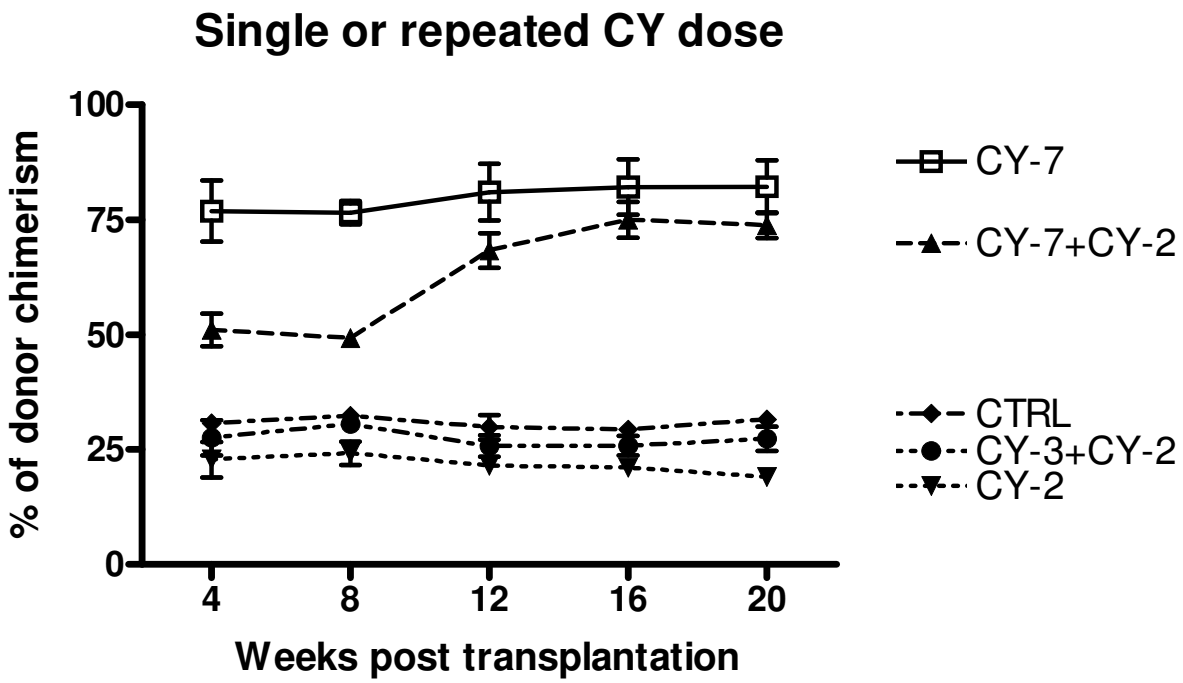


Fig. 24: Engraftment of donor HSCs into recipients conditioned with different numbers of CY doses.

Weeks	4	8	12	16	20
CTRL	31 ± 1.8	32 ± 1.8	30 ± 2.6	29 ± 0.2	32 ± 0.9
CY-2	23 ± 3.9 *	24 ± 2.6 *	22 ± 1.2 *	21 ± 1.5 **	19 ± 1.2 **
CY-7	77 ± 6.6 ***	76 ± 2.5 ***	81 ± 6.2 ***	82 ± 6.1 ***	82 ± 5.8 ***
CY-3+CY-2	26 ± 2.7 ns.	26 ± 3.0 ns.	28 ± 2.9 ns.	26 ± 2.2 ns.	26 ± 3.6 ns.
CY-7+CY-2	51 ± 3.5 **	49 ± 1.5 **	68 ± 3.7 ***	75 ± 3.9 ***	74 ± 2.8 ***

Table 9: Statistical analysis. Values are expressed as mean ± SEM. Significant difference from controls: (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$

4.2.5 Influence of replacing myeloablation of TBI with Busulphan - engraftment of donor HSCs in cyclophosphamide –busulphan (CY-Bu) conditioning regimen

Busulphan is widely used in clinical settings as a part of chemotherapeutic regimens. Therefore its effect on the level of HSCs engraftment compared to irradiation is of great interest. Ly5.1/Ly5.2 murine model was used in the experiments. Mice were pretreated with CY in usual dose (135mg/kg i.p.) and 5 or 2 days later busulphan was delivered. Two doses of busulphan were used, 25 or 50 mg/kg i.p. Control mice received busulphan only. Mice were then transplanted with $4x10^6$ cells i.v. (Fig. 25).

The low dose of busulphan lead to a low level of engraftment comparable between the three groups. The higher dose of busulphan resulted in a significantly higher engraftment of donor cells in all experimental groups. Control mice and mice pretreated with CY 2 days before busulphan administration achieved comparable levels of long-term engraftment. The level of donor hematopoiesis in CY-5 group 20 weeks after transplantation was significantly higher compared to control and CY-2 mice (Fig. 26).

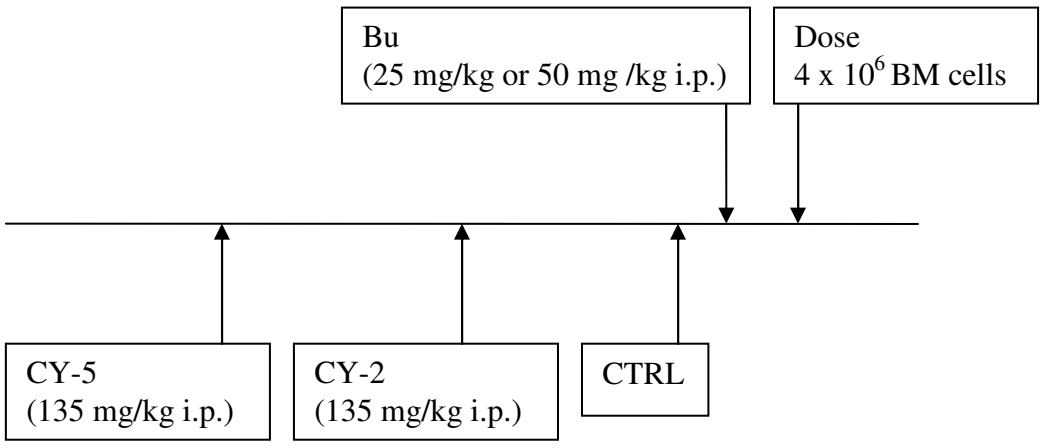


Fig. 25: Transplantation scheme

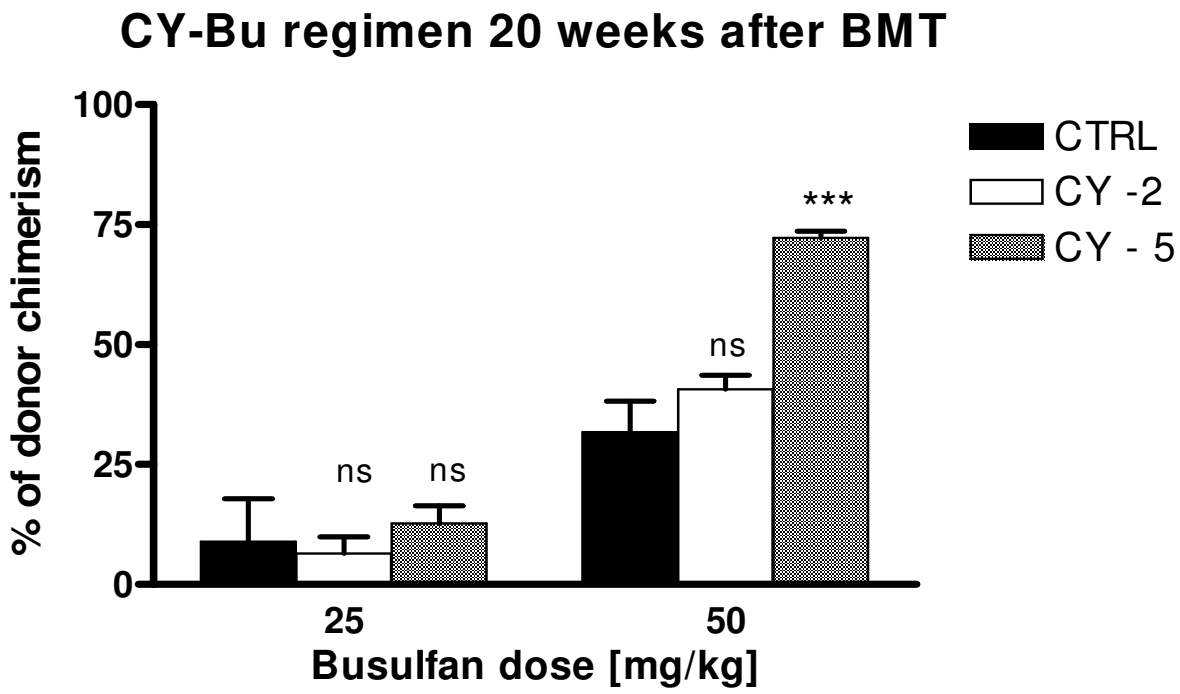


Fig. 26: Engraftment of donor HSCs in CY and busulphan conditioning regime. Significant difference from controls: (***) p<0.001.

	CY-2	**CY-2**	**CTRL**
Bu 25mg/kg	13 ± 3.7 ns.	6.5 ± 3.5 ns.	9 ± 3.5
Bu 50mg/kg	72 ± 1.5 ***	41 ± 3.0 ns.	32 ± 6.5

Table 10: Statistical analysis. Values are expressed as mean ± SEM. Significant difference from controls: (*) p<0.05, (**) p<0.01, (***) p<0.001.

4.3 Difference of fetal and adult HSCs with special emphasis on the development of sensitivity to estrogen

The process of blood cell formation is very different between fetal and adult life (Kincade PW 2002). This is particularly the case for lymphopoiesis, where the properties of progenitors and the differentiation pathways change dramatically in a developmental age-dependent manner. There are indices that fetal stem cells are intrinsically unique and do not rapidly acquire adult characteristics when transplanted to adult recipients (Igarashi H 2001).

Since one of the main features distinguishing fetal from adult HSCs is the responsiveness of B-cell lineage to estrogen, this characteristic can be followed in investigating fetal HSCs conversion to adult ones.

The behavior of HSCs was studied using murine congenic transplantation model with particular attention to the B220+ cells representing the B-lineage.

The following was investigated:

1. Sensitivity of B-lymphopoiesis of fetal liver origin and adult bone marrow origin to estrogen after transplantation into lethally irradiated adult recipients
2. Sensitivity of B-lymphopoiesis of fetal liver origin and adult bone marrow origin to estrogens after secondary transplantation into lethally irradiated adult recipients
3. Sensitivity of B-lymphopoiesis to estrogen of fetal liver origin or newborn liver origin as compared to those of adult BM after transplantation into sublethally irradiated adult recipients
4. Sensitivity of B-lymphopoiesis to estrogen of fetal liver origin and newborn liver origin cells after co-transplantation into lethally irradiated mice and after secondary transplantation

5. Sensitivity of B-lymphopoiesis of newborn liver origin and newborn spleen origin to estrogens as compared to adult bone marrow origin cells after transplantation into lethally irradiated adult recipients
6. Real-time PCR of estrogen receptor-α from fetal liver and adult bone marrow

4.3.1 Sensitivity of B-lymphopoiesis of fetal liver origin and adult bone marrow origin to estrogens after transplantation into lethally irradiated recipients

Lethally irradiated male Ly5.1 or Ly5.2 mice were transplanted with a mixture of $5x10^6$ Ly5.1 FL (E14.5) cells and $5x10^6$ Ly5.2 adult BM cells. Half of the recipients were treated with estrogen (Fig. 27). Estrogen was delivered as described (Regimen A). The percentage of all FL-origin white blood cells (WBCs), as well as the percentage of FL-origin B220+ cells until day 90 after transplantation are presented in Fig. 28. After transplantation of the same amount of FL and adult cells, FL cells were more efficient in restoring hematopoiesis in lethally irradiated recipient mice as compared to BM cells (95% vs 75%). Estrogen affected only B-lymphopoiesis. CD3+ cells and Gr/Mac+ cells were not affected by estrogen administration (Fig. 29).

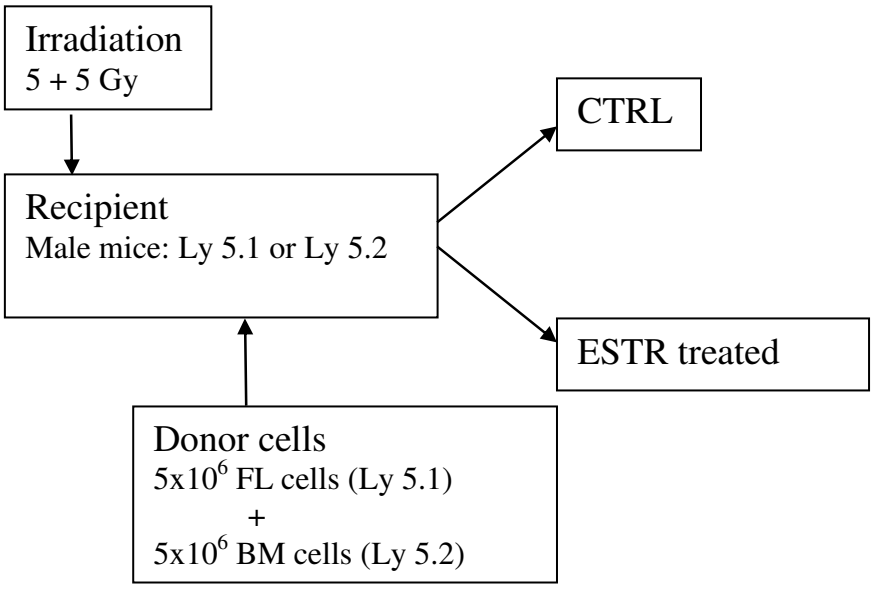


Fig. 27: Transplantation scheme

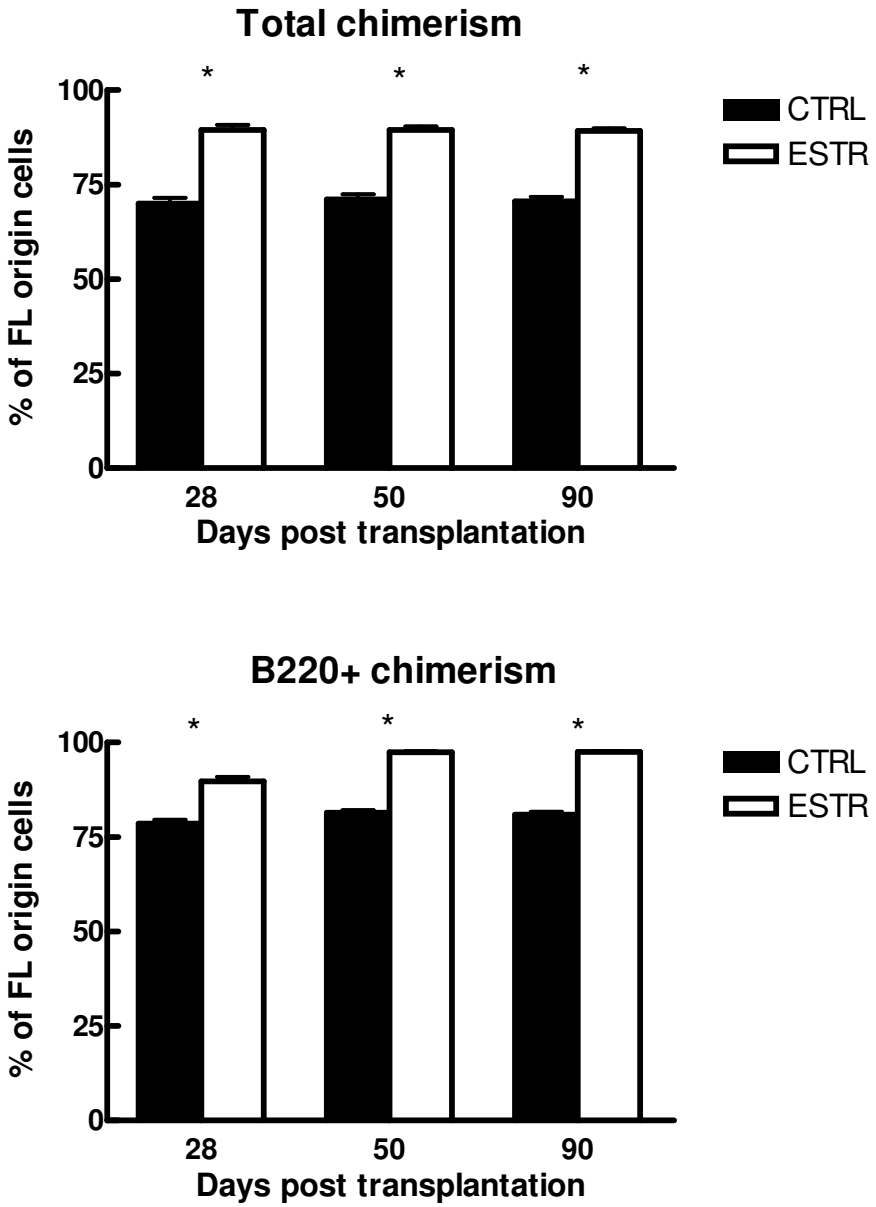


Fig. 28: Cells of FL (Ly5.1) origin in peripheral blood, total and B220+ chimerism after transplantation. (*) $p < 0.05$.

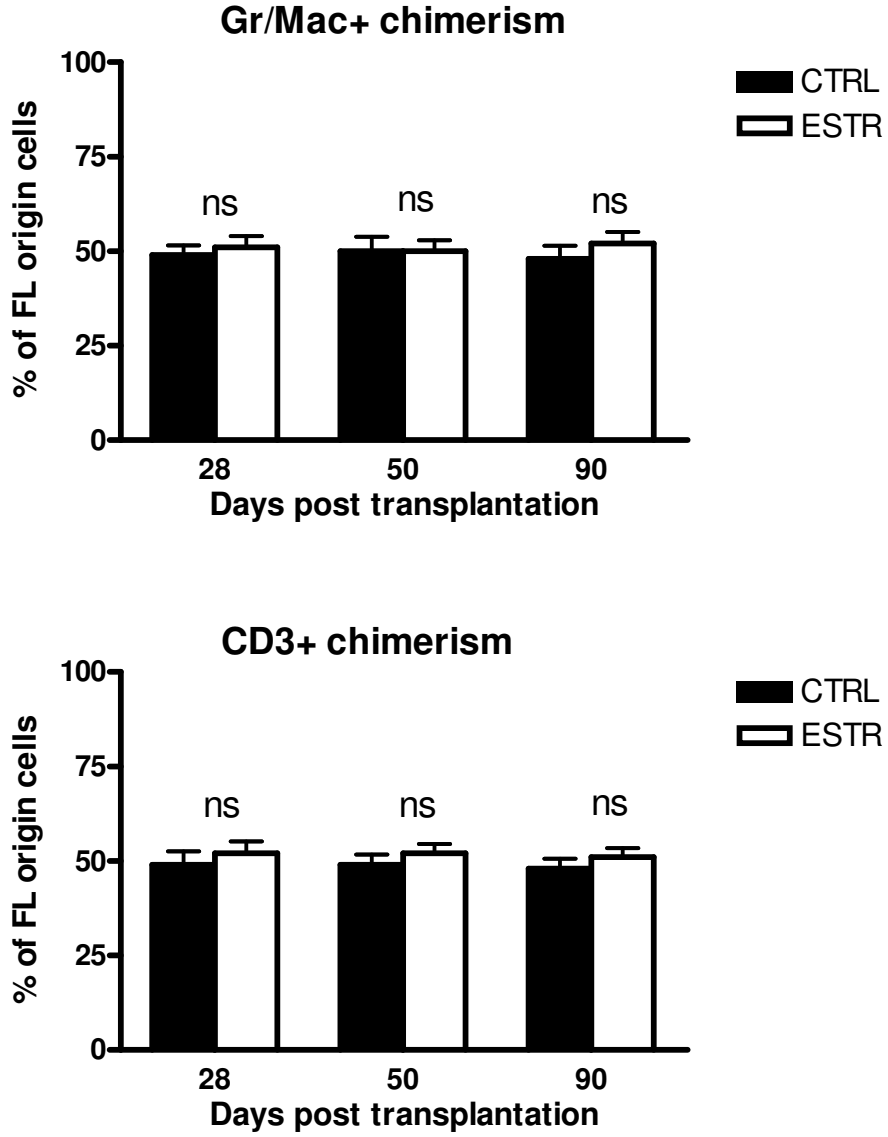


Fig. 29: Cells of FL (Ly5.1) origin in peripheral blood, Gr/Mac+ and CD3+ chimerism after transplantation.

4.3.2 Sensitivity of B-lymphopoiesis of fetal liver origin and adult bone marrow origin to estrogens after secondary transplantation into lethally irradiated recipients

To investigate the behavior of FL HSCs transplanted for a significantly long time into adult environment two consecutive series of experiments were performed (Fig. 30). First, Ly 5.2 male mice were sublethally irradiated with 4 Gy and transplanted with $5x10^6$ Ly5.1 FL cells (E14.5). FL-origin chimerism is illustrated in Fig. 31, estrogen regimen A. The chimerism was significantly higher in the group treated with estrogens. There was 22% chimerism in control mice and 28% in estrogen-treated mice in blood at the time of sacrifice and BM transplantation (1/3 of femur) into lethally irradiated secondary recipients (140 days post transplantation).

After secondary transplantation, the proportion of FL-origin cells remained increased in 28 and 50 days in estrogen treated recipients. Later after secondary transplantation, there was no significant effect of estrogen treatment between FL and adult BM origin cells in total chimerism (Fig. 32).

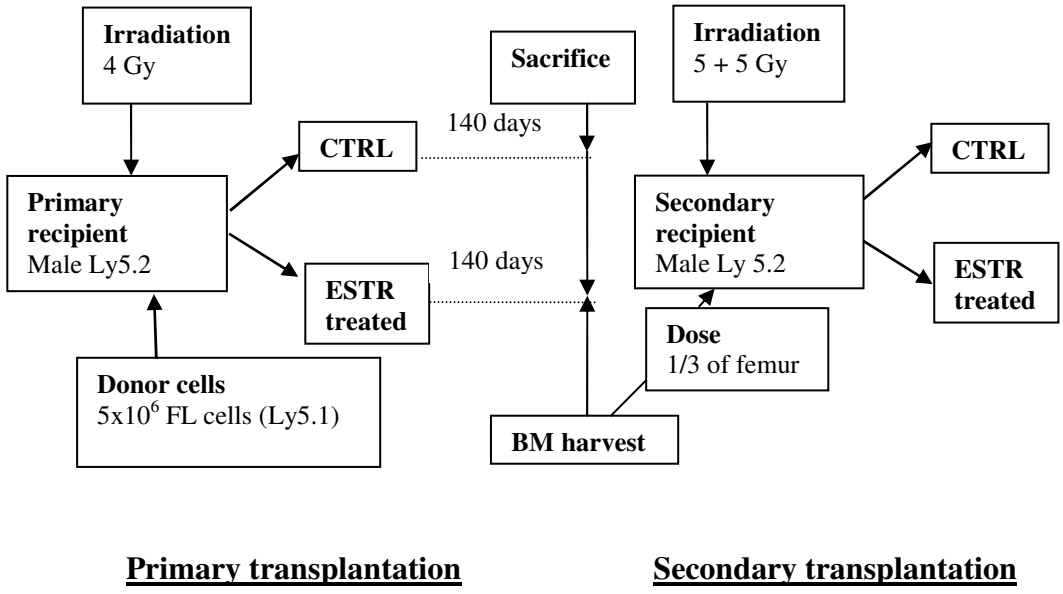


Fig. 30: Scheme of primary transplantation and secondary transplantation

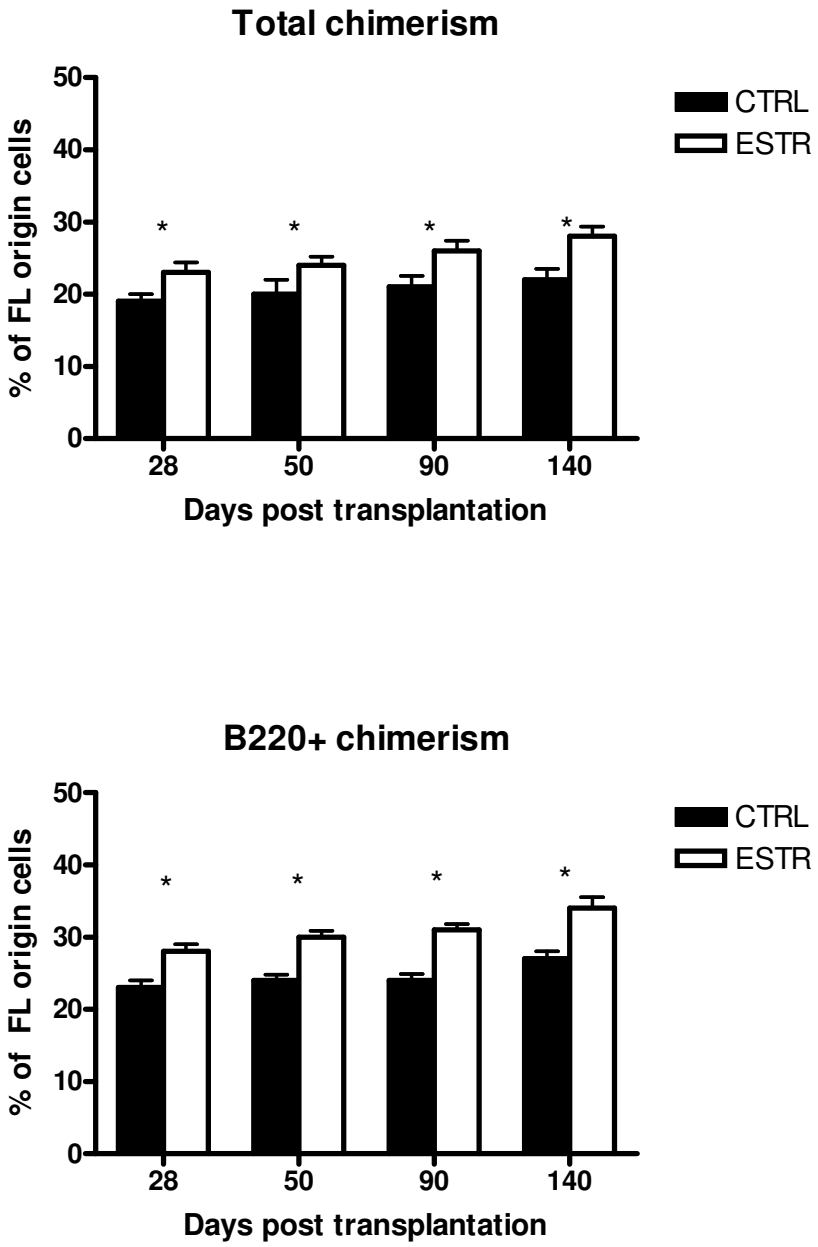


Fig. 31: Cells of FL (Ly5.1) origin in peripheral blood after primary transplantation, total and B220+ chimerism. (*) $p < 0.05$.

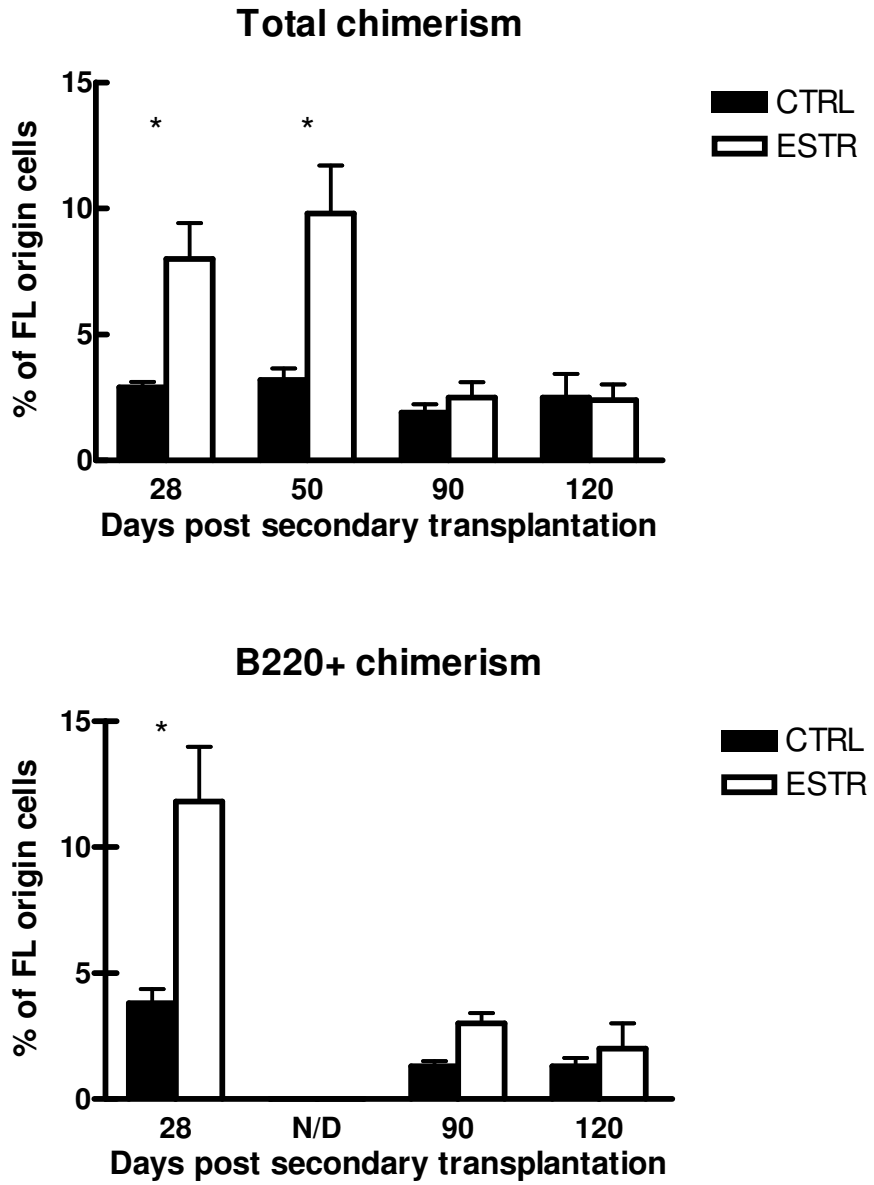


Fig. 32: Cells of FL (Ly5.1) origin in peripheral blood after secondary transplantation, total and B220+ chimerism. (*) $p < 0.05$.

4.3.3 Sensitivity of B-lymphopoiesis to estrogen of fetal liver origin or newborn liver origin as compared to those of adult BM after transplantation into lethally irradiated mice

Lethally irradiated male Ly5.1 and Ly5.2 mice were transplanted with a mixture of either $5x10^6$ FL E14.5 cells or newborn liver P0.5 cells, both of Ly5.2 phenotype, with $5x10^6$ Ly5.1 adult BM cells. Half of the recipients received estrogen, regimen B (Fig. 33). Estrogen administration increased the fraction of B220+ cells of FL origin over that of adult BM origin cells in mice transplanted with a mixture of FL E14.5 cells/BM cells during the whole observation period (90 days) after transplantation (Fig. 34). In contrast to this, in the mice transplanted with a mixture of newborn liver P0.5 cells/BM cells, fraction of B220+ cells of P0.5 liver origin was increased by estrogen treatment only up to six weeks after transplantation. Later on the ratio between B220+ liver (P0.5) origin cells and those of BM origin were no longer affected by estrogen administration (Fig. 35). Because the majority of nucleated cells in blood of mice are B-lymphocytes (~60%) (see Fig. 13), the effect of estrogen was similarly reflected in total chimerism. (Fig. 34 and 35).

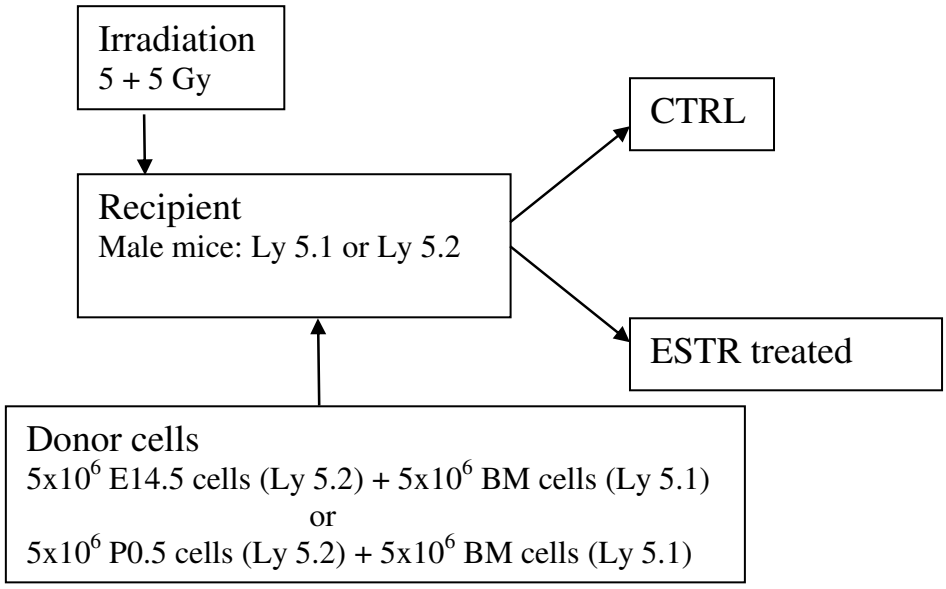


Fig. 33: Transplantation scheme

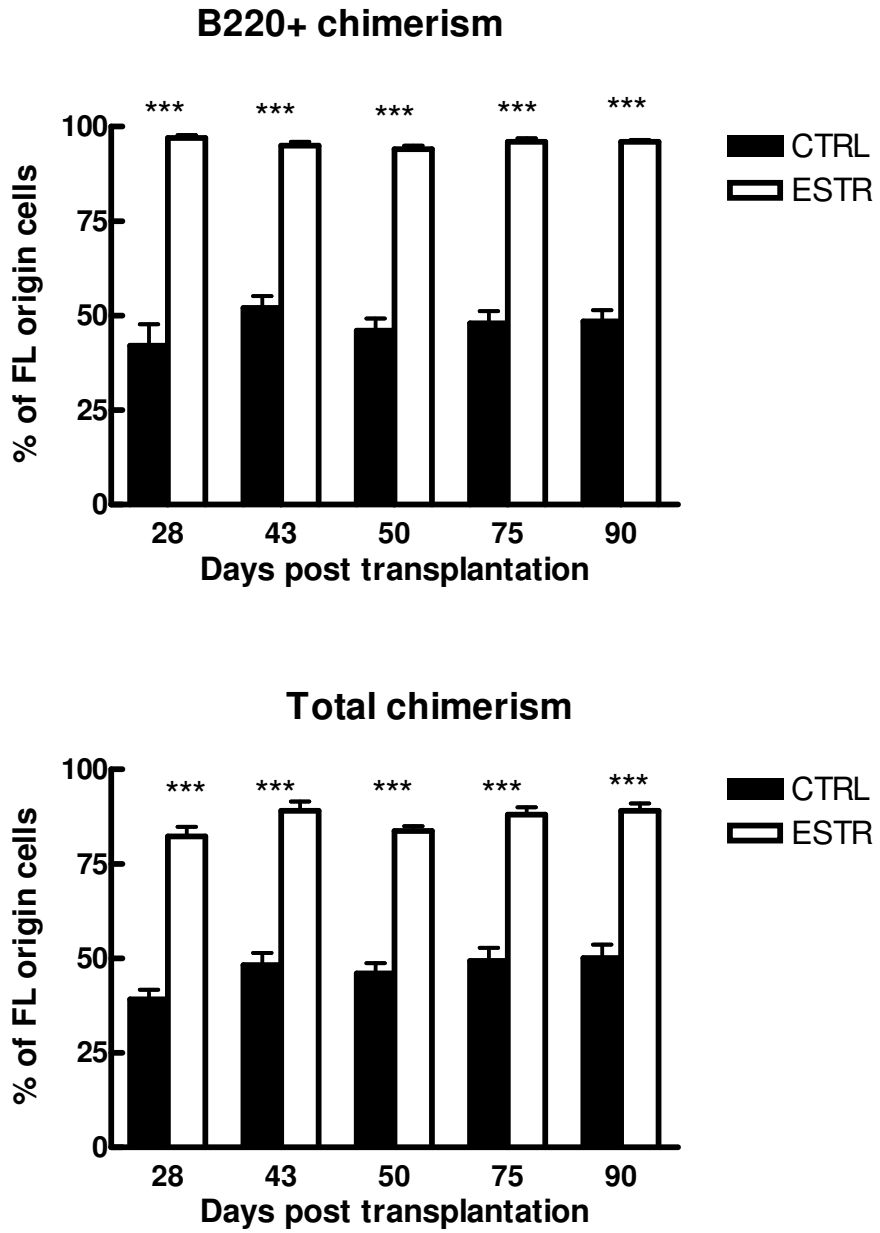


Fig. 34: B220+ chimerism and total chimerism of FL origin cells in peripheral blood up to 90 days after transplantation. (***) $p < 0.001$.

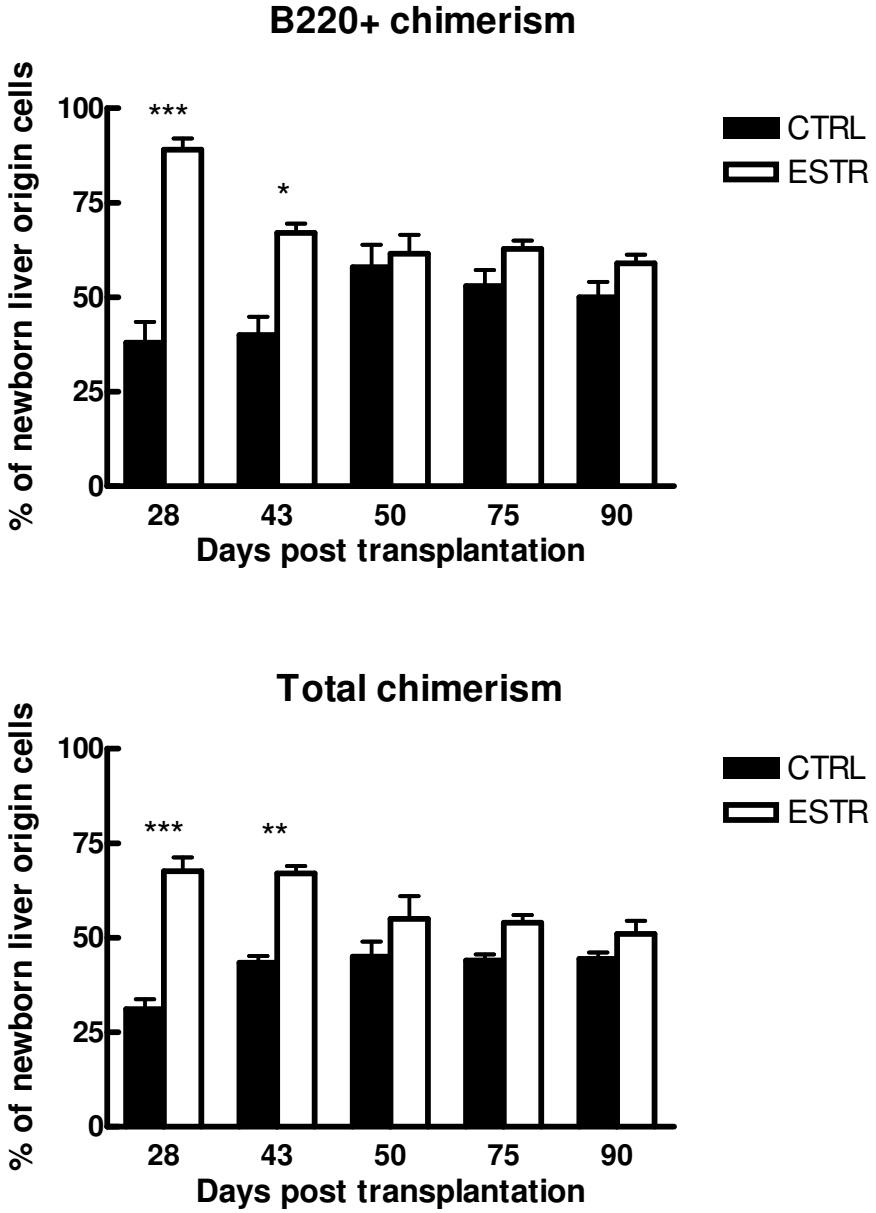


Fig. 35: B220+ chimerism and total chimerism of newborn liver origin cells in peripheral blood up to 90 days after transplantation. (***) $p < 0.001$, (**) $p < 0.01$ (*) $p < 0.05$.

4.3.4 Sensitivity of B-lymphopoiesis to estrogen of fetal liver origin and newborn liver origin cells after co-transplantation into lethally irradiated mice and after secondary transplantation

Lethally irradiated male Ly5.2 mice were transplanted with a mixture of five million of Ly5.1 FL E14.5 cells and Ly5.2 P0.5 cells or Ly5.2 FL E14.5 cells and Ly5.1 P0.5 cells. Half of the recipients were treated with estrogen, regimen B (Fig. 36). There was a stable chimerism in peripheral blood for the whole observed period after transplantation. Chimerism in the BM corresponded to the chimerism in peripheral blood at the time of sacrifice. 20 weeks after the primary transplantation there were approximately 75% FL E14.5 cells in estrogen treated mice and 55% in control mice (Fig. 37 and 38), when the mice were sacrificed and the bone marrow was transplanted into secondary lethally irradiated male recipients. The transplant dose was one third of a femur (Fig. 36). A half of the secondary recipients were again treated with estrogen. Estrogen administration increased the proportion of FL 14.5 derived B220+ cells until 140 days after the secondary transplantation. P0.5 derived cells remained sensitive to suppression by estrogen (Fig. 39 and 40). Estrogen suppressed P0.5 derived cells more than E14.5 derived cells, which then dominated.

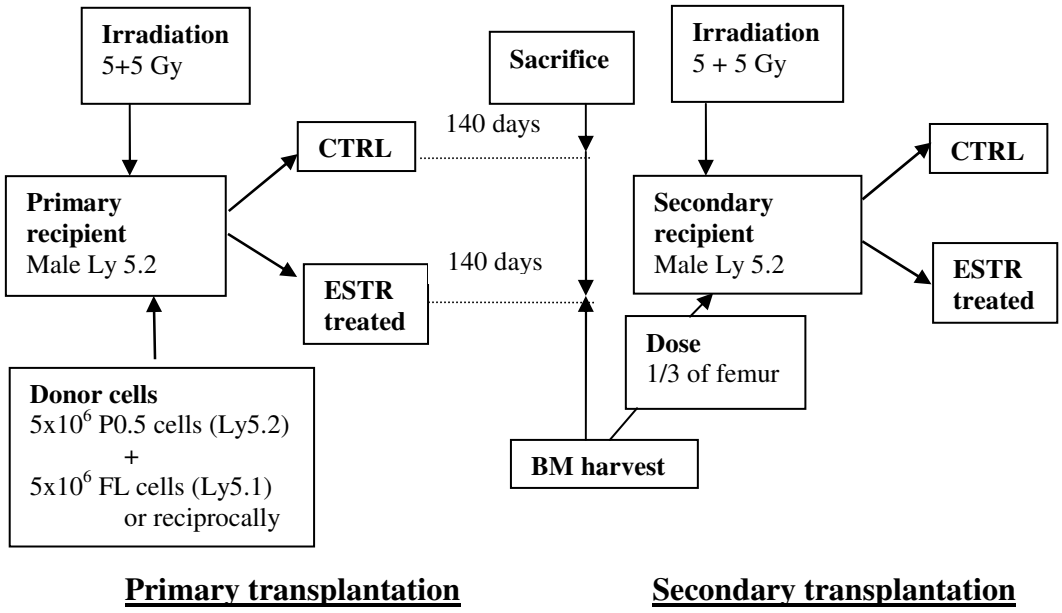


Fig. 36: Scheme of transplantation and secondary transplantation

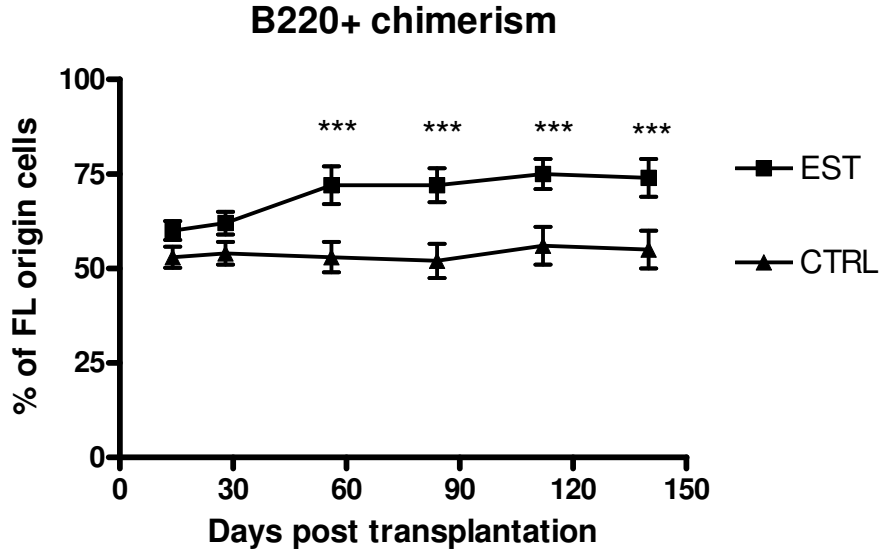


Fig. 37: Chimerism in PB of FL B-cells co-transplanted with P0.5 liver cells into primary recipients. B220+ chimerism; FL Ly5.1 (***) $p < 0.001$.

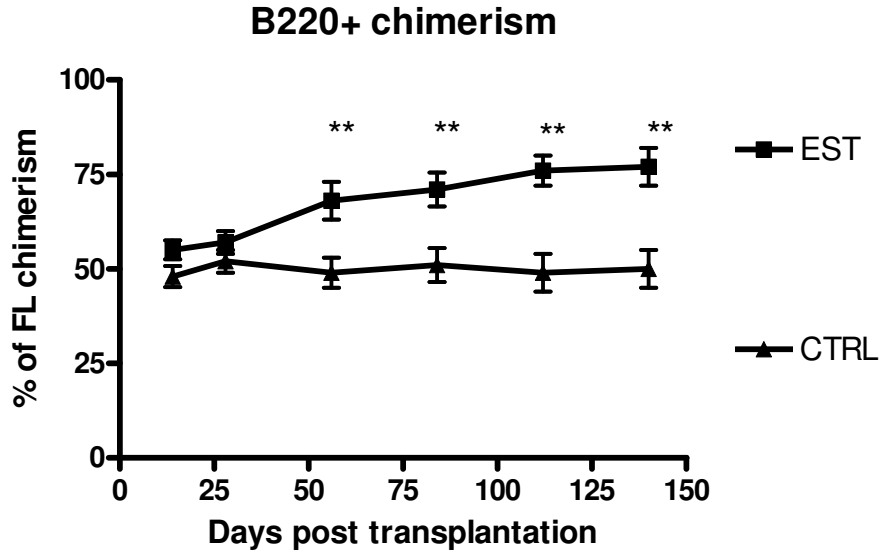


Fig. 38. Chimerism in PB of FL B-cells co-transplanted with P0.5 liver cells into primary recipients. B220+ chimerism; FL Ly5.2 (**) $p < 0.01$.

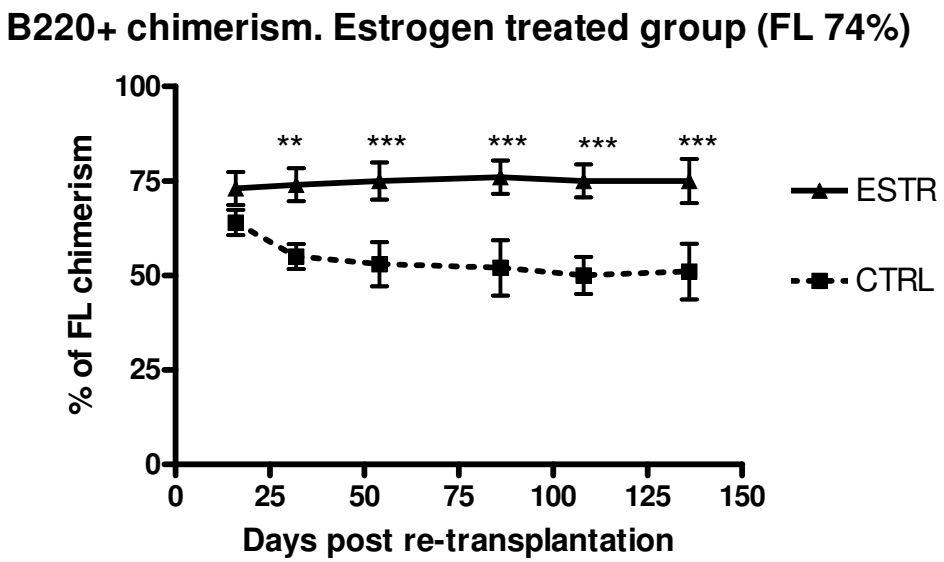


Fig. 39: Chimerism in PB of FL-origin B-cells co-transplanted with P0.5 liver cells (from Fig. 37) after transplantation into secondary recipients. Estrogen treated group. (***) $p < 0.001$, (**) $p < 0.01$

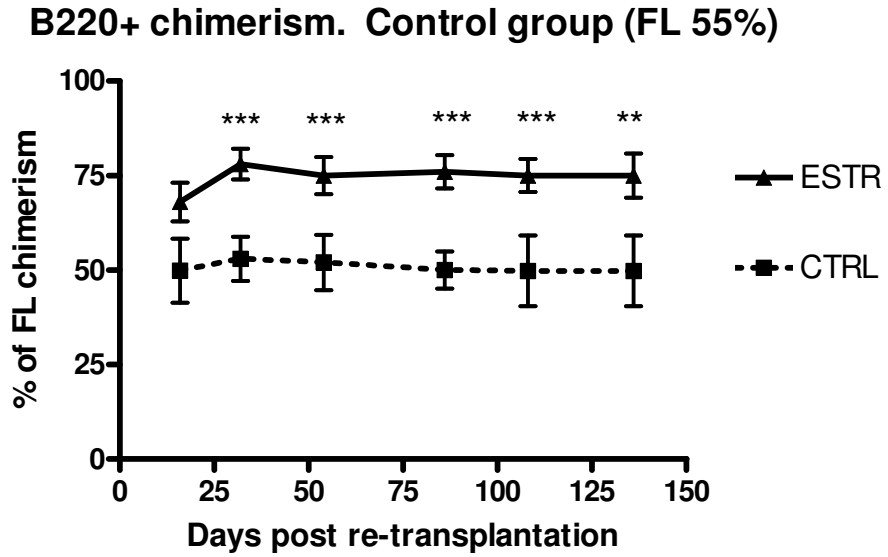


Fig. 40: Chimerism in PB of FL-origin B-cells co-transplanted with P0.5 liver cells (from Fig. 37) after transplantation into secondary recipients. Control group. (***) $p < 0.001$, (**) $p < 0.01$

4.3.5 Sensitivity of B-lymphopoiesis of newborn liver origin and newborn spleen origin to estrogens as compared to adult bone marrow origin cells after transplantation into lethally irradiated adult recipients

In rodents, spleen remains important site of hematopoiesis in adult life. To investigate the sensitivity to estrogen of HSCs obtained from this hematopoietic site, a mixture of adult BM (Ly5.2) cells and cells from newborn liver (P0.5) or newborn spleen (SP0.5) (Ly5.1) was transplanted to lethally irradiated adult male Ly5.2 or Ly5.1. The amount of transplanted cells was (2 x five million). Half of the recipients received estrogen, regimen B (Fig. 41).

The fraction of B220+ cells of P0.5 origin as well as SP0.5 origin was increased by estrogen treatment only up to six weeks after transplantation (Fig. 42). Later on, starting approximately seven weeks after transplantation, the sensitivity to estrogen of P0.5 origin cells and SP0.5 origin cells was no longer different from BM origin cells (Fig. 42 and 43).

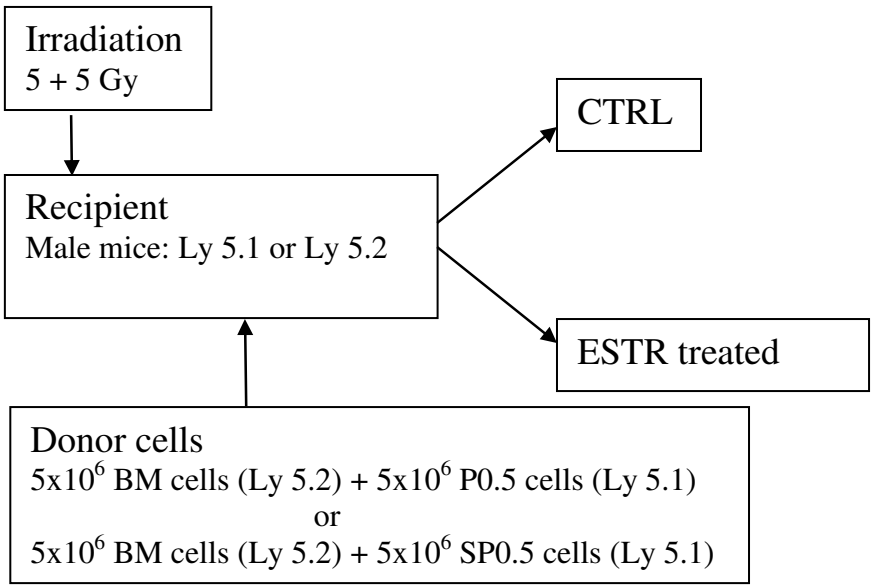


Fig. 41: Transplantation scheme

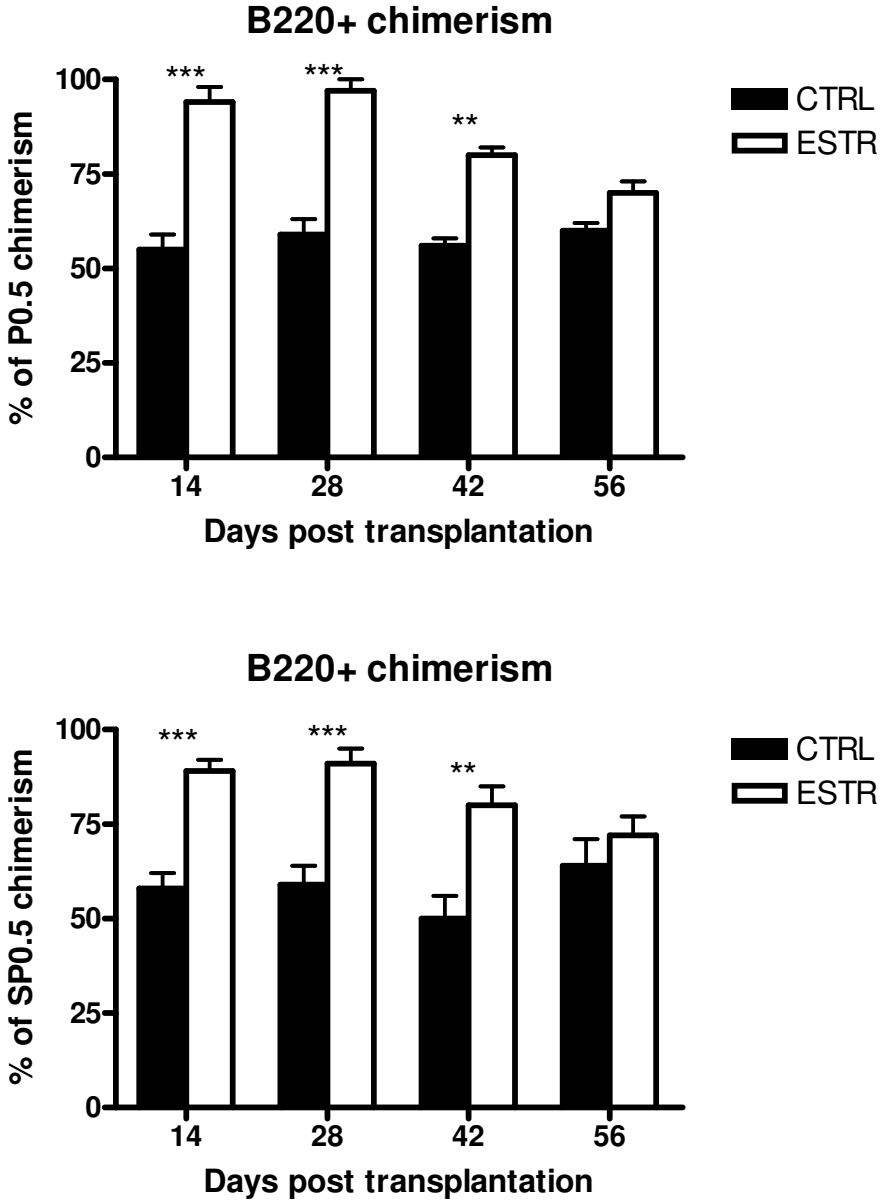


Fig. 42: B220+ chimerism of newborn liver (P0.5) and newborn spleen (SP0.5) cells in peripheral blood after transplantation into adult recipients. (***) $p < 0.001$, (**) $p < 0.01$.

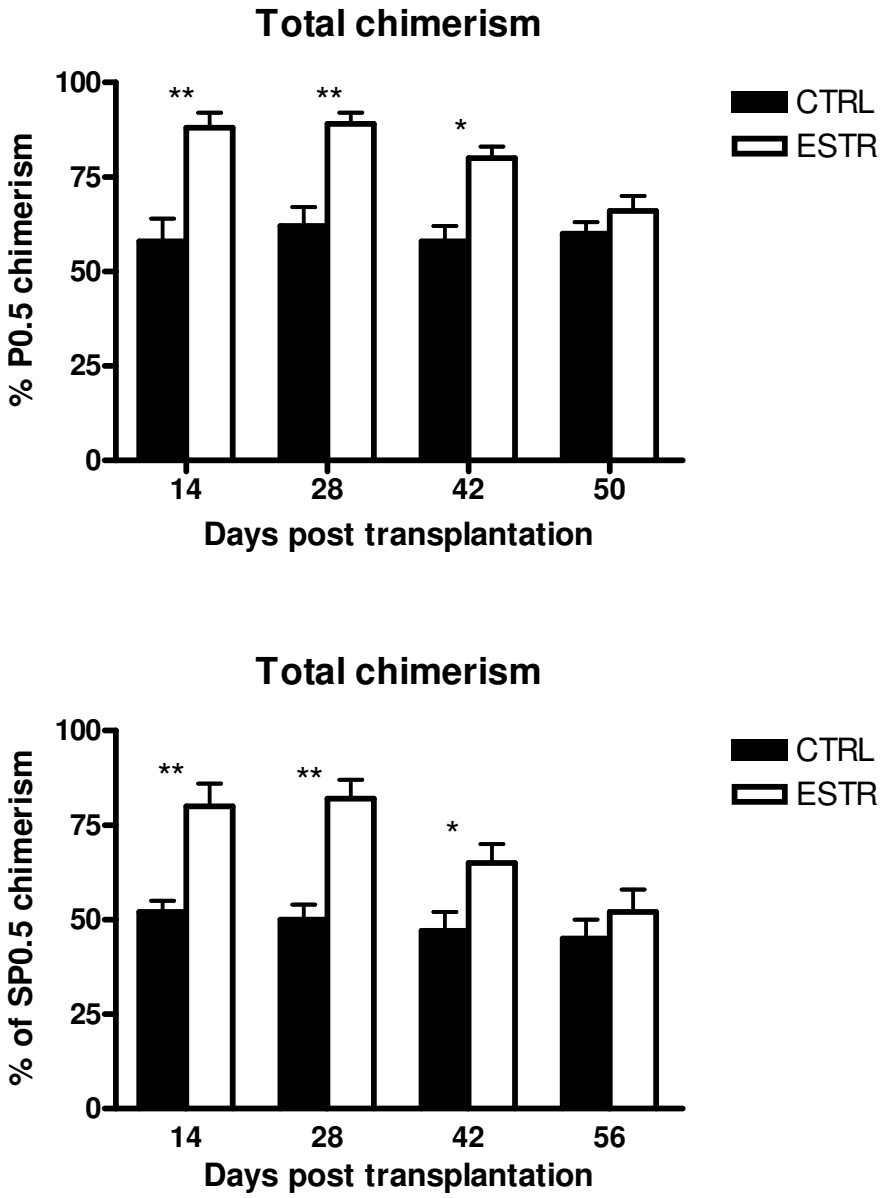


Fig. 43: Total chimerism of newborn liver (P0.5) and newborn spleen (SP0.5) cells in peripheral blood after transplantation into adult recipients. (**) $p < 0.01$, (*) $p < 0.05$.

4.3.6 RT-PCR of estrogen receptor-α mRNA from fetal liver and adult bone marrow

Conversion to adult phenotype of some lymphocytic surface markers takes place in early weeks after birth. We have studied the expression of estrogen receptor-α (ESRα) in fetal liver E14.5 and in newborn liver P0.5. Total mRNA was isolated from fetal and newborn liver of Ly5.1 mice. Similar percentage of B220+ cells was present in the two sources. No significant difference was found between the amount of total mRNA obtained from fetal liver or from newborn liver (Fig. 44).

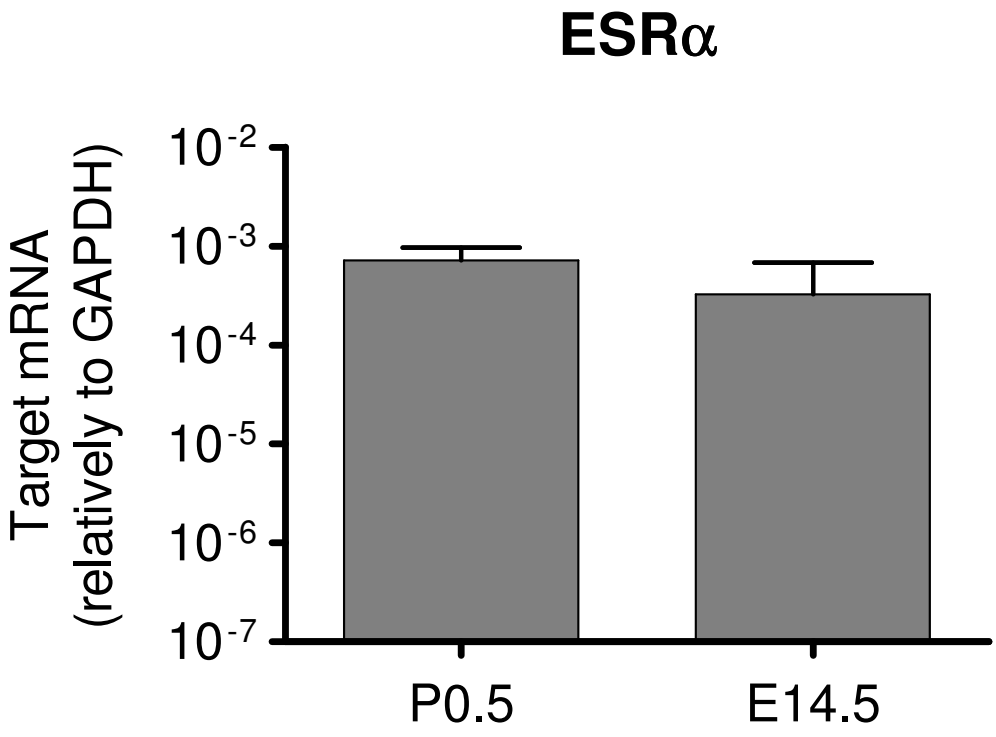


Fig. 44: ESRα mRNA content in fetal liver (E14.5) and newborn liver (P0.5).

4.4 Influence of systemically administered drugs affecting bone marrow microenvironment on hematopoietic tissue

There is a tight dynamic relationship between hematopoietic tissue and bone marrow microenvironment. Systemically delivered drugs affecting the bone tissue and its components could have potential effect on hematopoietic tissue as well.

In clinical settings, biphosphonates are generally used drugs for the treatment of osteoporosis. They inhibit osteoclast activity and in such way bone resorption. It has been shown that they also influence endothelial cells in vitro. Since endothelial cells are integral part of bone marrow microenvironment, biphosphonates could influence bone marrow hematopoiesis and hematopoietic stem cells.

Effect of systemically administered biphosphonates on hematopoietic stem cells was studied in murine transplantation model:

1. Influence of biphosphonates on the ability of HSCs to repopulate the host microenvironment
2. Effect of biphosphonates on host bone metabolism and ability to engraft donor cells

4.4.1 Influence of biphosphonates on the ability of HSCs to repopulate the host microenvironment

Adult male Ly5.1 mice were used as donors. Half of the mice were treated with biphosphonates in diet for two weeks. The administered dose was 70ug/kg/day. Bone marrow was then collected and $4x10^6$ bone marrow cells were transplanted into sublethally irradiated (6 Gy) male Ly5.2 mice (Fig. 45).

There was no significant difference in bone marrow cellularity in biphosphonate-treated and in control mice (Tab.11). Biphosphonates did not effect transplantability of hematopoietic stem cells. There was also no difference in hematopoietic stem cell engraftment between the two groups (Fig. 46).

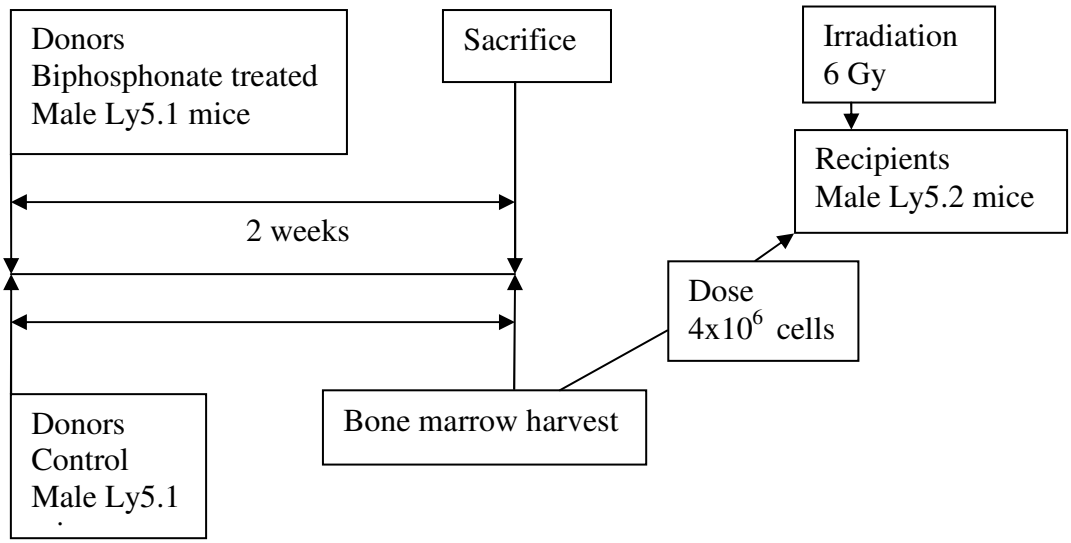


Fig. 45: Scheme of mice treatment and transplantation.

	Weight [g]	Number of cells [million/femur]
Biphosphonate-treated	23.85 (±1.24)	34.82 (±3.58)
Control	24.98 (±1.25)	34.80 (±1.79)

Table11: Weight and bone marrow cellularity of biphosphonate-treated and control mice.

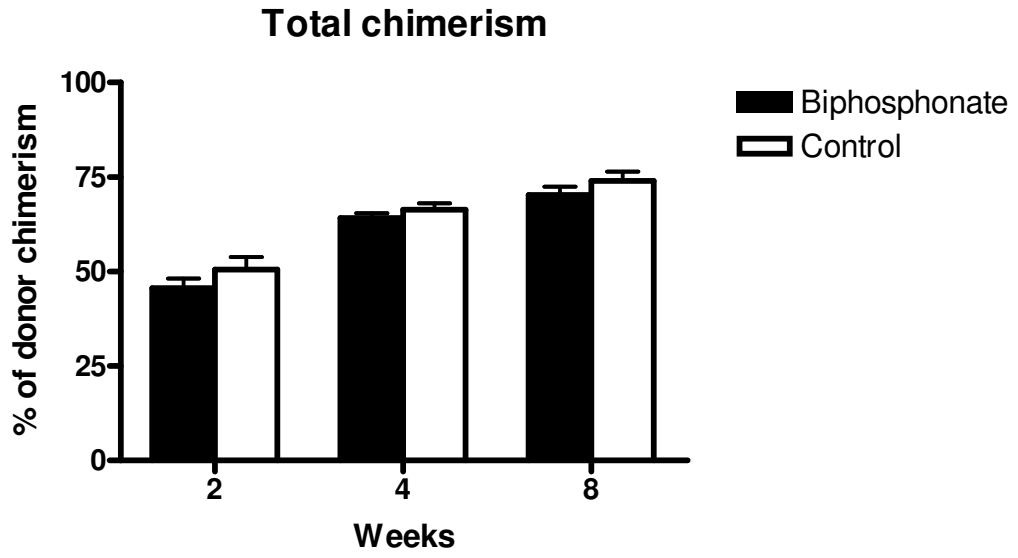


Fig. 46: Donor chimerism in recipient mice up to 8 weeks after transplantation. Donors bisphophonates treated.

4.4.2 Effect of biphosphonates on host bone metabolism and ability to engraft donor cells

Recipient Ly5.2 mice were sublethally irradiated and transplanted with 4 x 10^6 bone marrow Ly5.1 cells (Fig. 47). Half of the recipients was treated with diet bisphosphonate in dose of 70ug/kg/day. Chimerism was followed for 12 weeks.

Administration of bisphosphonate had no influence on the level of donor hematopoietic engraftment. There was no significant difference between biphosphonate-treated and control group (Fig. 48).

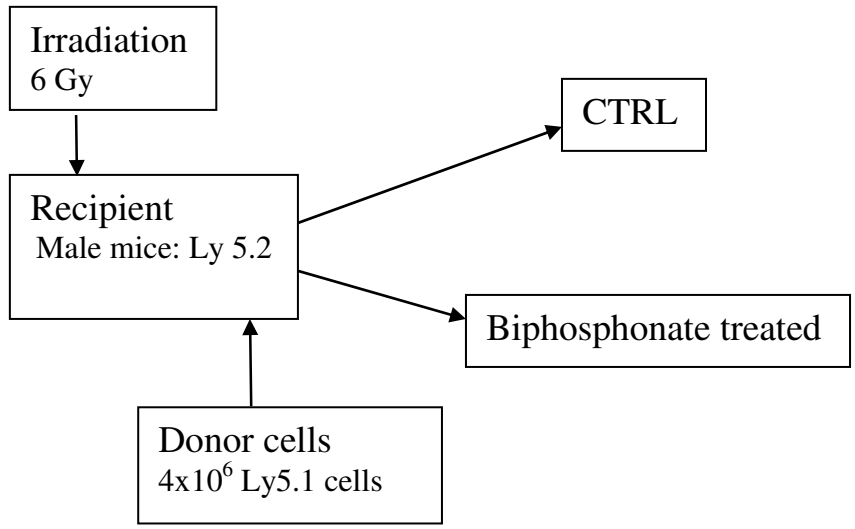


Fig. 47: Transplantation scheme

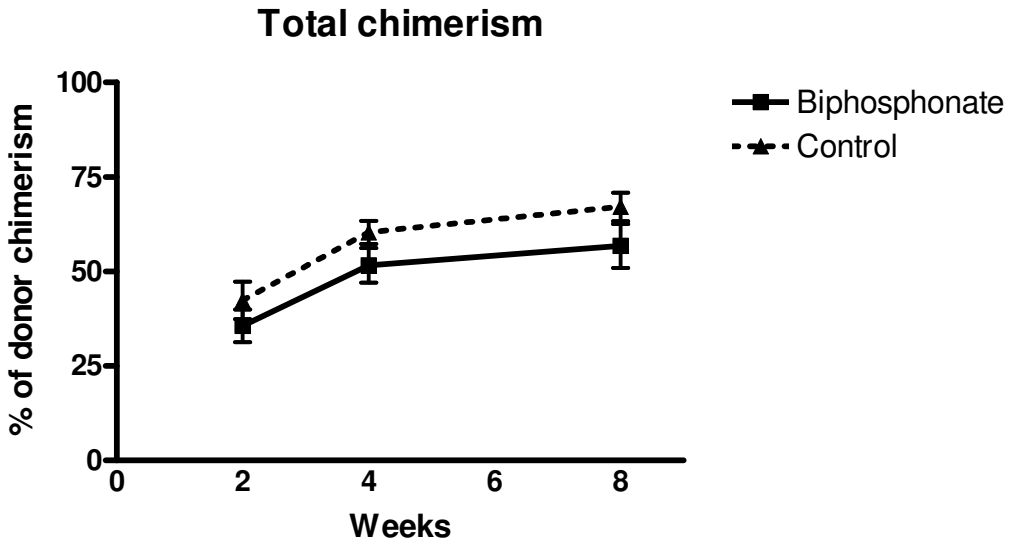


Fig. 48: Chimerism in biphosphonate-treated and in control mice.

5. DISCUSSION

5.1 Verification of murine congenic transplantation models

Congenic Ly5.1/Ly5.2 murine model is widely used in experimental hematology in functional studies of HSCs behavior (Soderling CC et al., 1985, van Os. R et al., 2001). Despite frequent use of this system, no methodological data were published so far describing reciprocity of Ly5.1/Ly5.2 strains, sex and dose dependency of chimerism. Van Os et al. (2001) found that engraftment following experimental BMT relies on the donor and host combination chosen; especially when minimal conditioning is applied. They ascribed it to T-cell-mediated immunity. As most our experimental data were based on chimerism measurement, we have done methodology experiments first to study in detail this transplantation model and to prove its validity for gaining reliable data.

We have studied the influence of sex and dose of BM cells transplanted into lethaly and sublethally irradiated recipients on chimerism. We have analyzed distribution of WBCs subpopulations in relation to the level of achieved chimerism.

We have proved that results obtained in this transplantation model are reliable and reproducible. There are no restrictions for recipients regarding sex of the donors. The post-transplantation chimerism reflected well the number of transplanted cells in both lethally irradiated (10 Gy) and sublethally irradiated (4 Gy) hosts. There is a strong correlation between total engraftment and WBCs subpopulation distribution. In case of a high total engraftment, the subpopulation distribution in the graft remains unchanged from normal mice.

To test relative competition of two grafts in indifferent environment, F1 mouse recipient model was prepared and evaluated. There is no influence of any parental lineage on the level of engraftment. This model has shown to be acceptable and useful for transplantation studies as well.

The main WBCs subpopulation are B-lymphocytes , as in normal mouse, in mice with a high total engraftment. In mice with a low total engraftment, the main subpopulation of engrafted cells is made up by Gr/Mac$^+$ cells. Nevertheless, this could be a general phenomenon after engraftment and cannot be attributed only to Ly5.1/Ly5.2 system.

5.2 Evaluation and comparison of different conditioning regimens on the level of engraftment of transplanted HSCs

The most frequent use of cyclophosphamide in clinical practice is as part of preconditioning regimen before BMT (Zhang J et al., 2006). CY is commonly used in combination with total body irradiation (Toubai T et al., 2004). We have studied the CY-TBI regimen from several perspectives and their influence on the level of engraftment of transplanted HSCs. Recipient preconditioning has been modified in respect of different time intervals between CY administration and TBI delivery, different radiation doses and application of two separate CY doses. Effect of CY administration on donor HSCs has been studied.

Using the same doses of CY and TBI in preconditioning and the same number of BM cells transplanted, just changing the time interval between CY application and TBI delivery, leads to pronounced differences in chimerism. When CY is administered 2 days prior to TBI, the chimerism after transplantation is significantly lower compared to only irradiated hosts. When CY is delivered 5 or 7 days before TBI, the chimerism is significantly increased. Regeneration of BM after CY administration follows a unique pattern (Šefc L. et al., 2003). It has been demonstrated by us that hematopoietic progenitors intensively proliferate one to three days after CY administration while they are quiescent five to seven days after CY administration (Šefc L. et al., 2003). Expression

of SCF and SDF-1 is high in CY-2 group and low in CY-5/7. CY-2 induces regeneration of BM whereas CY-5/7 induces mobilization (Lévesque JP et al., 2002, Pšenák O et al., 2003).

Delivering the second dose of CY 2 days before TBI does not lead to changes in achieved chimerism despite doubling the dose of chemotherapy. In CY-7+CY-2 group, the chimerism is slightly lower compared to CY-7 group, but remains significantly higher compared to controls and to CY-3+CY-2 group. Hence, the time interval between the first CY administration and irradiation seems to be determining for the level of achieved donor engraftment.

Corresponding results are obtained with varying radiation dose applied after the same dose of CY and transplantation of the same number of cells. In controls the chimerism increase is linear proportional to radiation dose. In CY-7 group, a high chimerism is presented already with a low radiation dose used. Markedly, increasing irradiation dose over 4 Gy in CY-2 group does not lead to an increase in engraftment at all.

It has been shown, that CY administration relatively spares HSCs compared to more differentiated hematopoietic cells (Gardner RV et al., 2001). Transplantation of BM from mice treated with CY 2 days before harvest lead to significantly higher donor engraftment compared to BM from mice treated CY 7 days before harvest. Our data show that HSCs (LTRC) follow similar regeneration kinetics as progenitor cells with secondary decrease 5/7 days after CY (Šefc L et al., 2003).

In a clinical setting TBI is replaced with busulphan for several indications and the preconditioning regimen consists only from administration of chemotherapeutic agents (Escalón MP et al., 2009, Bredeson CM et al., 2008). Since busulphan and cyclophosphamide both exert potential liver toxicities (Hassan Z et al., 2002), application

of a minimal possible dose to ensure maximal effect is of great importance. Changing the administration order of CY and Bu has no pharmacodynamical effect (Nilsson C et al., 2005, Sadeghi B et al., 2008). Myelosuppresion with Bu (50 mg/kg) was comparable to the myelosuppresion achieved with TBI (4 Gy) after CY administration as reflected by the level of donor chimerism. Because Bu is delivered as a powder with a very limited solubility its dissolution and later application is a complicated process. Applying irradiation with similar results is thus technically simpler and more feasible at least in mice.

We suppose that transplantation of hematopoietic cells into microenvironment supporting their proliferation stimulates donor stem cells into cycle and decreases their ability for long-term repopulation. Transplantation into non-inductive microenvironment allows donor stem cells to seed and ensures a stable long-term engraftment.

5.3 Difference of fetal and adult HSCs with special emphasis on the development of sensitivity to estrogen

It has been demonstrated previously that HSCs/progenitors from different stages of FL development were ready to home and function in the environment of adult hematopoiesis (Chang KT et al., 2005). However, the process of blood formation is very different between fetal and adult life (Kincade PW et al., 2002). This is particularly the case for lymphopoiesis, where the properties of progenitors and the differentiation pathways change dramatically in a developmental age-dependent manner (Pelayo R et al., 2006, Kikuchi K and Kondo M, 2006). An excellent example is pregnancy, during which adult B-lymphocytes are suppressed by high levels of circulating estrogen, but the B-lymphopoiesis from fetus is unaffected (Medina KL et al., 1993). Igarashi et al have demonstrated that FL-derived HSCs still lacked the receptors for sex steroid hormones

four weeks after transplantation of FL into irradiated RAG-1-deficient mice and that the B-lymphopoiesis derived from these cells was less sensitive to inhibition by estrogens compared to that derived from adult BM (Igarashi H et al., 2001). The different phenotype of FL and adult BM B-cell progenitors reported by Igarashi et al (2001) and Medina et al (2001) thus presented a challenge that we approached by examining the timing of conversion of the estrogen-resistant phenotype of FL B-lymphopoiesis into the sensitive phenotype of the adult BM B-lymphopoiesis. Hematopoietic cells from fetal, neonatal and adult origin were co-transplanted into lethally or sublethally irradiated adult recipients. Our results provide the first *in vivo* functional confirmation of a differential responsiveness of FL- and adult BM-derived B-lymphopoiesis to suppression by estrogen (Pelichovská T et al., 2008). The B-lymphopoiesis derived from FL remained significantly less sensitive to inhibition by estrogen compared to that of adult BM origin. It remained its permanent feature as demonstrated in re-transplantation experiments. In the case of B-lymphopoiesis derived from neonatal liver its non-sensitivity to suppression by estrogen lasted only for about 50 days after transplantation to adult recipients. Later on, there was no difference from adult B-lymphopoiesis. Hence, although HSCs from different stages of FL development can home and engraft to damaged BM microenvironment successfully (Chang KT et al., 2005), an exposure of HSCs to the late-stage FL microenvironment seems to be critical and mandatory for the proper timing and a full conversion of the FL phenotype of the B-lymphopoiesis into that of the adult BM phenotype.

5.4 Influence of systemically administered drugs affecting bone marrow microenvironment on hematopoietic tissue

Bisphosphonates are commonly used in clinical settings for the treatment of osteoporosis (Mahakala A et al., 2003). They are potent inhibitors of osteoclast-mediated

bone resorption (Papapoulos SE et al., 2008, Drake MT et al., 2008). It has been shown that new generation of bisphononates inhibits proliferation of endothelial cells *in vitro* (Yuasa T el at., 2007). Since endothelial cells are an important part of the BM microenvironment, knowledge of the effect of prolonged administration of bisphosphonates on HSCs and BM hematopoiesis is of outmost significance. Using murine transplantation model, we have tested the *in vivo* effect of bisphosphonates administration on HSCs, on their ability to repopulate the host microenvironment and their effect on host metabolism and ability to engraft donor cells. Mice were fed with bisphosphonates in a dose that is comparable with the dose used for osteoporosis treatment in women. No difference between bisphosphonates-treated and control mice was observed in bone marrow cellularity or in the content of functional HSCs (Šefc L et al., 2007). There was no difference in hematopoiesis and in short-term as well as long-term engraftment between treated and control mice.

6. CONCLUSION

Part I – Congenic Ly5.1/Ly5.2 murine transplantation model is reliable. F1 mouse model is also acceptable and usable in transplantation studies and allow to study direct competition of two grafts. Results obtained from both systems are well reproducible and do not rely on strain and sex combinations. In mice with high total chimerism, the main WBC subpopulation of the graft are B-lymphocytes, as in normal mice. In mice with low total chimerism, the main contribution of graft to WBC is made up by Gr/Mac^{+} cells.

Part II – In cyclophosphamide-total body irradiation preconditioning regimen, donor cells engraftment depends on the time interval between the first cyclophophamide administration and irradiation. Prolongation of the interval from 2 days to 5 or 7 days leads to significant increase in donor chimerism. Cyclophosphamide-busulphan preconditioning ensures myelosuppression comparable with CY-TBI regimen. Increasing irradiation in CY-2 group does not lead to an increase in donor cells engraftment.

Part III – The B-lymphopoiesis derived from fetal liver remained significantly less sensitive to suppression by estrogen compared to that of adult and neonatal origin. It remained its permanent feature. Exposure of HSCs to the late-stage FL microenvironment seems to be critical and mandatory for gaining later sensitivity to estrogen.

Part IV – Bisphophonates do not affect hematopoiesis in the mouse. They seem to be safe in regard to the effect on hematopoietic tissue in mice.

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8. APPENDIX:

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