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Effect of the Pretransplantation Conditioning on the Effectiveness of Bone Marrow Transplantation in a Mouse Model

Vliv předtransplantační přípravy na efektivitu transplantace kostní dřeně v myším modelu

Diplomová práce
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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 12. 8. 2013

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Nicol Renešová

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ABSTRAKT

Hematologické malignity patří mezi jedny z nejčastěji se vyskytujících typů rakovinných onemocnění. Při jejich léčbě se často využívá léčebných protokolů zahrnujících předtransplantační přípravu pacienta kombinací cytostatik a ionizujícího záření, po které následuje transplantace zdravých dárcovských krvetvorných kmenových buněk. Jednou z nejobvyklejších používaných kombinací je ošetření alkylačním činidlem cyklofosfamidem a celotělovým ozářením.

V této studii jsme použili kongenní myší kmeny Ly5.1 a Ly5.2, které exprimují různé varianty CD45 antigenu, ke zhodnocení efektu různých časových intervalů mezi cyklofosfamidem a ozářením na přihojování krvetvorných kmenových buněk a jejich schopnost obnovit krvetvorbu. Pro toto stanovení byla použita kompetitivní repopulační studie.

Výsledky ukazují, že míra přihojení a obnovení krvetvorby se mohou významně lišit v závislosti na zvoleném intervalu mezi cyklofosfamidem a ozářením. Shromážděná data navíc ukazují, že pacienti s hematologickými malignitami by mohli vykazovat lepší léčebné výsledky, zejména pokud by interval mezi cyklofosfamidem a ozářením před transplantací byl sedm dní, protože jejich vlastní kmenové buňky by byly nejméně kompetitivní.

Klíčová slova: Cyklofosfamid, hematopoietické kmenové buňky, chimérizmus, ionizující záření, transplantace kostní dřeně

ABSTRACT

Hematologic malignancies are among the most often diagnosed forms of cancers. Treatment regimens often utilise various combination of cytostatic drugs and total body irradiation and subsequent transplantation of hematopoietic stem cells. One of the most common combinations includes ionising radiation with the antineoplastic alkylating agent cyclophosphamide.

In this study we used congenic Ly5.2 and L5.1 mouse strains that express different isoforms of CD45 antigen to evaluate the effects of various time interval between cyclophosphamide and irradiation treatments on the viability of hematopoietic stem cells and their viability. This was done by competitive repopulation assay.

The results revealed that level of engraftment and subsequent reconstitution of hematopoiesis can significantly vary and depend on the time interval between cyclophosphamide and total body irradiation administrations. The results indicate that patients with hematologic malignancies could possibly benefit from the treatment especially if they received transplants after being irradiated five or seven days after cyclophosphamide because at that time point their own stem cells would be least competitive.

Key words: bone marrow transplantation, cyclophosphamide, chimerism, hematopoietic stem cells, ionising radiation

LIST OF CONTENTS

LIST OF ABREVIATIONS

7-AAD	7-aminoactinomycin D
ANOVA	analysis of variance
APC	allophycocyanin
APC/Cy7	allophycocyanin cyanin 7
BSA	bovine serum albumin, Fraction V, biotin free
b.w.	body weight
c-kit	CD117, stem cell factor receptor
CY	cyclophosphamide
CYP	cytochrome P450
CYP reductase	cytochrome P450 reductase (cytochrome c reductase)
CXCR4	C-X-C chemokine receptor 4, receptor for SDF-1
EDTA	ethylendiamintetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
FITC	fluoresceine isothiocyanate
G	gauge, unit of needle thickness
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gy	gray, unit of ionising radiation
HPC	haematopoietic progenitor cell
HSC	haematopoietic stem cell
IMDM	Iscove's Modified Dulbecco's Medium
LET	linear energy transfer,
LT-HSC	long-term haematopoietic stem cells
Ly5.1	expressing CD45.1 antigen
Ly5.2	expressing CD45.2 antigen
MPP	hematopoietic multipotent progenitor cell
NO	nitric oxide
NF-κB	nuclear factor kappaB
NOS	nitric oxide synthase
P	P-value
PBS	phosphate buffered saline

PBS-BSA	phosphate buffered saline supplemented with 1% BSA
PE	phycoerytrin
PerCP	peridin chlorophyll protein complex
PI	propidium iodide
RNS	reactive nitrogen species
ROS	reactive oxygen species
Sca-1	Ly-6A/E
SEM	standard error of the mean
SK SLAM cells	Sca-1^{+}c-kit^{+}CD48^{-}CD150^{+} hematopoietic stem cells
SLAM	singnalling lymfocyte activation molecule, molecules CD150 and CD48
SDF-1	stromal cell-derived factor 1
ST-HSC	short-term haematopoietic stem cells
TBI	total body irradiation
T-cells	T-lymphocytes; CD3ε positive cells
VLA-4	very late antigen-4

1 INTRODUCTION

Hematologic malignancies are widely in occurrence worldwide. They include diseases such as lymphomas (Non-Hodkin lymphoma being the most common), myelomas and several types of leukemias. All of the named malignancies are on the list of twenty most often diagnosed cancers in the United Kingdom (http://www.cancerresearchuk.org/cancer-info/cancerstats/incidence/commoncancers/#Twenty).

The treatment protocols for hematologic malignancies often include hematopoietic stem cell transplantation, with the aim of restoring normal hematopoiesis from donor stem cells. However, before stem cells can be transplanted, the recipient of the transplant needs to be rid of their own aberrant hematopoiesis. For that reason, pretransplantation conditioning regimens destroying the patient's hematopoiesis are employed.

There exist many types of those regimens, but one of the most common subgroups utilises ionising radiation and the cytostatic drug cyclophosphamide (Cant et al, 2007). As the regimens vary in time intervals between the irradiation and cytostatic treatments, it is of great interest to evaluate possible differences between them, so that the best possible outcome of hematopoietic stem cell transplantations can be ensured.

2 REVIEW OF LITERATURE

2.1 HAEMATOPOIESIS AND HAEMATOPOIETIC STEM CELLS

Haematopoiesis is a process during which mature blood cells are created from primitive haematopoietic stem cells (HSCs), which reside in the hematopoietic tissues. Extramedullary (occurring outside the bone marrow compartment) haematopoiesis is rare in humans, and it is always a sign of disease (Singer et al, 2004). In mice, however, spleen is counted as a haematopoietic organ and contributes to normal blood homeostasis (Tan & O'Neill, 2010).

Haematopoietic stem cells are tissue specific stem cells of the body – they have the potential to give rise to a plethora of finally differentiated blood cells, and thus facilitate replenishment of the tissue by new cells (see Figure 1). They are capable of supporting haematopoiesis during the whole lifetime of an organism, which is due to holding on to their "stemness" and the specific ability to self-renew. The cells retain these characteristics by being very slow-cycling in the adult hematopoiesis, and leaving the proliferation and massive production of blood cells to their more differentiated, yet more short-lived progeny.

HSCs are described by several things – **(a)**surface markers – distinct expression profile of a given set of molecules exposed on the surface of the cell. One of the strategies is looking for LSK SLAM cells, which are negative for lineage markers (Lin^-) and CD48 ($CD48^-$ and are positive for c-kit, Sca-1 and CD150 (Kiel et al, 2005), **(b)**their localisation within the bone marrow compartment (Guezguez et al, 2013), and **(c)** their ability to reconstitute haematopoiesis in a myeloablated recipient of a HSC transplant (Osawa et al, 1996).

At any given time there is also a very small fraction of HSCs circulating in the peripheral blood. It seem that HSCs periodically leave a tiny number of their niches vacant and free to be colonised even in unconditioned recipients of HSC transplants (Bhattacharya et al, 2006). It seems that this minor mobility of HSCs is important for generation of innate immune effector cells in extramedullary tissues (Massberg & von Andrian, 2009).

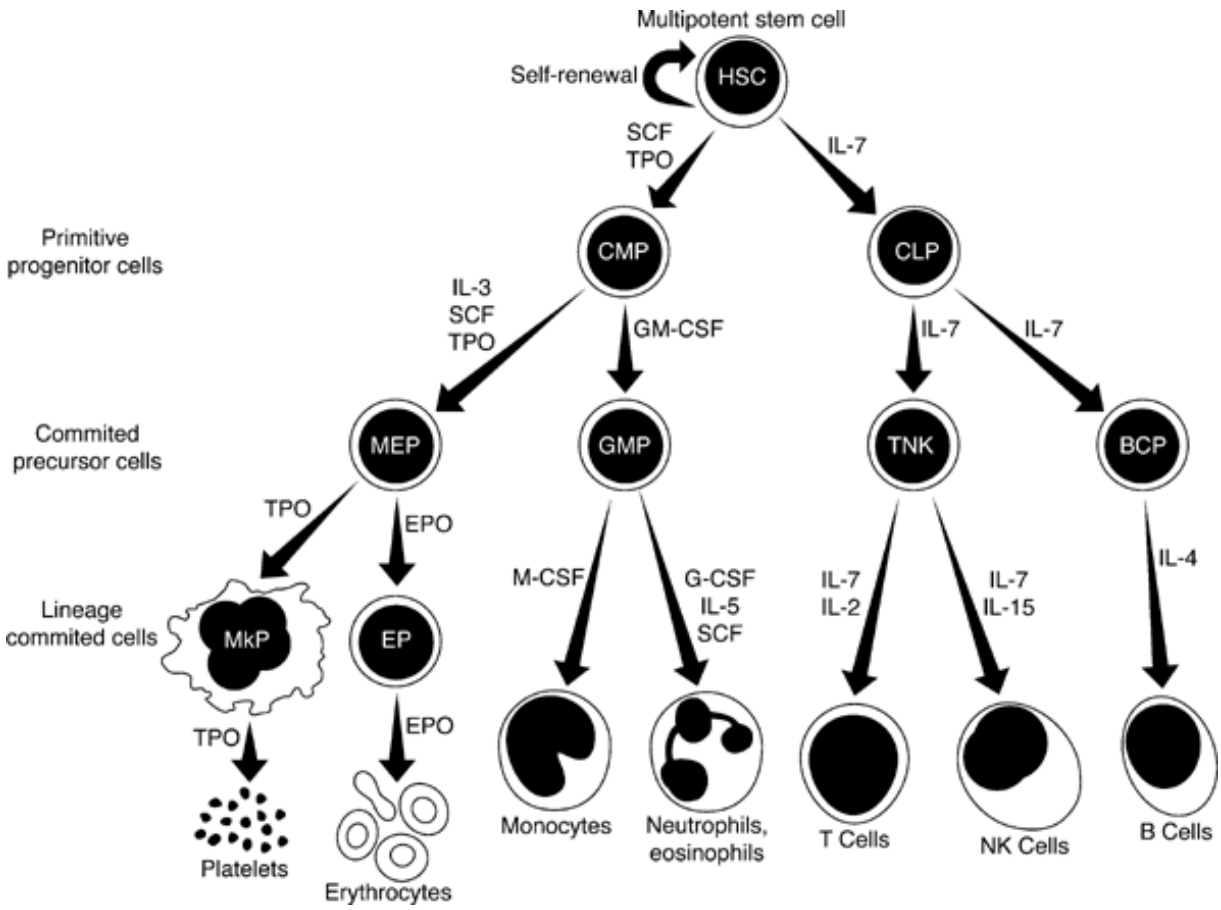

Fig.1 A schematic diagram of haematopoiesis. Self-renewal capacity of HSCs is indicated by an arrow. Gradual loss of potency and increased differentiation is shown in the top to bottom direction (Robb, 2007).

2.1.1 STEM CELL NICHE

A haematopoietic stem cell niche is a place of residence of HSCs that has been postulated several decades ago. Nowadays, there is a consensus that more than one niche exists, and that the niches differ in their composition, localisation and even in the type of HSCs that inhabit them. It is also thought that a niche is not solely a space inhabited by HSCs, but also an important regulator of their function by means of retaining their self-renewal capacity, or by promoting their proliferation and genesis of more mature multipotent progenitor cells (MPPs), which in turn give rise to cells of individual blood lineages. At least three distinct type of HSC niches are thought to exist in the marrow of trabecular bones. Those are: the vascular niche, the reticular niche, and the endosteal niche (see Figure 2).

2.1.1.1 Vascular Niche

The vascular niche is a space directly neighbouring with vascular sinuses of the bone. As the vascular sinuses of the bone marrow are comprised of one layer of epithelial cells with no additional outer layer of connective tissue, they allow for transmigration of differentiated blood cells. In this niche, short-term HSCs and rapidly proliferating lineage-commited progenitors are localised (Grassinger et al, 2010).

Research has validated the existence of the vascular niche – when vascular endothelial growth factor receptor 2 is conditionally deleted (and the regeneration of sinusoidal endothel is hindered) in adult mice, haematopoietic reconstitution in sub-lethally irradiated animals is inhibited (Hooper et al, 2009).

2.1.1.2 Endosteal Niche

As the name suggests, the endosteal niche is located near the endosteum of haematopoietically active bones. This niche has been deemed to harbour the most primitive populations of HSCs (Grassinger et al, 2010; Guezguez et al, 2013; Nilsson et al, 1997). It has also been demonstrated that this is the place where a rare pool of dormant HSCs, which only divide about five times during a lifetime of a mouse (approximately every 145 days) reside. Such stem cells remain in the G_0 phase of the cell cycle, unless a significant injury to the haematopoietic compartment occurs. Remarkably, they are able to re-enter the dormant state after physiological haematopoiesis has been re-established (Wilson et al, 2008).

A special type of osteoblasts has been found in the endosteal niche. These spindle-shaped N-cadherin$^+$CD45$^-$ osteoblastic cells (SNO cells) directly interact with HSCs through homotypic N-cadherin interactions (Zhang et al, 2003). Osteoblast population in the bone marrow seems to be absolutely crucial for the maintenance of HSC numbers. It has been demonstrated that when the numbers of osteoblasts in the bone are lowered, the numbers of HSCs and MPPs plummet (Visnjic et al, 2004).

The endosteal niche is naturally low in oxygen – this hypoxic environment is important, but not sufficient for maintaining quiescence of HSCs (Guitart et al, 2010). Yet another regulator of HSC quiescence is angiopoietin-1 (Ang-1). HSCs that express receptor tyrosine kinase Tie2 which interacts with angiopoietin-1 expressed by the niche osteoblasts are driven to quiescence and adhesion to bone, and exhibit elevated resistence to myelosuppressive stress (Arai et al, 2004).

2.1.1.3 Reticular Niche

The reticular stem cell niche is made up of reticular cells which express high amounts of the chemokine CXCL12, also known as stromal cell-derived factor 1 (SDF-1), and vascular cell adhesion molecule (VCAM)-1. Therefore they are called CXCL12-abundant reticular cells, or "CAR cells" in short (Sugiyama et al, 2006). SDF-1 is a chemotactic molecule which stimulates homing during ontogenesis as well as after transplantation of HSCs (Ara et al, 2003). The importance of SDF-1 signalling in haematopoiesis has been deduced from the fact that *in vivo* studies with knock-out mice deficient in both SDF-1 and its receptor CXCR4 developed severe defects in myelopoiesis and B lymphopoiesis (Nagasawa et al, 1996).

Apart from the SDF-1, CAR cells are also major producers of the stem cell factor (SCF), which through its tyrosine kinase receptor c-kit (CD117) promotes proliferation, and in combination with other growth factor also differentiation of HSCs and MPPs. In the endosteal niche SCF-1 also plays an important role in lodgement and retention of HSCs, if not in their homing (Driessen et al, 2003).

CAR cells span the bone marrow from the vascular sinuses to the endosteal region, and therefore seem to be mediators of HSC migration within the compartment (Sugiyama et al, 2006).

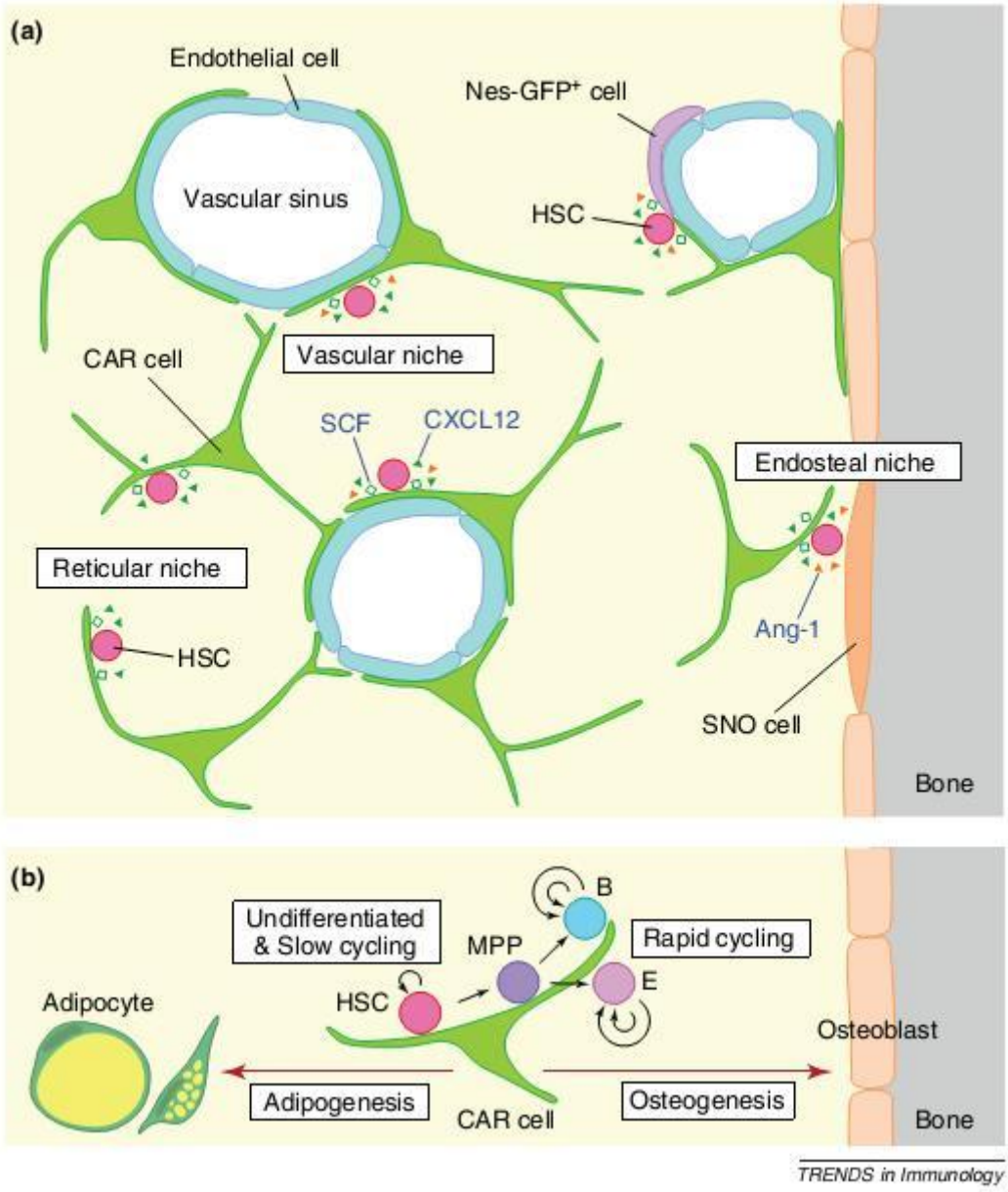

Fig.2 **(a)** A model for the localization of HSCs and their association with cellular niches in the bone marrow compartment. **b)** CAR cells are adipo-osteogenic progenitors and are required for proliferation of lymphoid and erythroid progenitors, as well as HSCs, and maintenance of HSCs in an undifferentiated state. B - B-cell progenitor; E – erythroid progenitor (Nagasawa et al, 2011).

2.2 PRECONDITIONING OF HSC RECIPIENTS

2.2.1 CYCLOPHOSPHAMIDE

2.2.1.1 CYTOCHROME P450

Cyclophosphamide in itself is not a harmful substance. It is a prodrug, which means that it needs to be activated by metabolic processing in the liver before it can have any therapeutic effects (see Figure 5). The enzymes that facilitate the conversion of cyclophosphamide into active metabolites are cytochrome P450s, abbreviated "CYPs" (Pass et al, 2005; Ren et al, 1997).

Cytochrome P450 is a name encompassing a group of enzymes of the oxidoreductase family, which exhibit the highest absorbancy at 450 nm during a spectroscopic analysis. The enzymes occur in both eukaryotic and some of the prokaryotic organisms. In the prokaryotes, it is mostly species utilising hydrocarbons as a source of both energy and carbon that express CYPs.

CYPs are crucial enzymes which are responsible for numerous metabolic processes in a mammalian body. They take part in such diverse processes as are steroidogenesis, eicosanoid metabolism, transformation of xenobiotics, and metabolism of vitamins A and D. Not all of the CYPs are capable of metabolising xenobiotics, but all of those that are capable of that are also capable of processing endogenous substrates. Nevertheless, those are usually processed with a lower metabolic rate, specificity, or both (Guengerich, 2005).

The enzymes include an iron-(III) protoporphyrin-IX linked with a proximal cysteine ligand as a prosthetic group in their molecular structure (see Figure 3). These membrane-bound proteins occur mostly in the endoplasmic reticulum of hepatocytes, but a subpopulation can be found in mitochondria as well (Ghazarian et al, 1974).

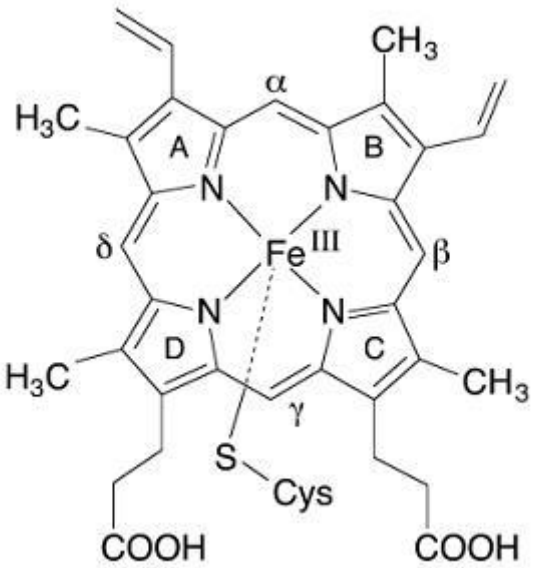

Fig.3 The prosthethic group of CYPs (Meunier et al, 2004)

During the bioactivation of cyclophosphamide by hepatic CYPs a hydroxylation reaction takes place on its C-4 atom. It is a monooxygenase reaction characterised by the equation: $RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H2O$. The two electrons are provided by NAD(P)H via a reductase.

As mentioned above, several isoforms of CYPs, which exhibit different characteristics, exist. A number of them have been found to be particularly effective in bioactiovation of cyclophosphamide. Among these isoforms are: CYP2A6, CYP2B6, CYP3A4, and CYP2C9 (Roy et al, 1999). However, activation is not the only metabolic pathway cyclophosphamide might follow. There are also a few deactivation pathways that produce inactive and/or undesirable metabolites, such as chloracetaldehyde. This neurotoxic substance is produced by a dechloroethylation reaction instead of a 4-hydroxylation of cyclophosphamide, and CYP3A4 seems to be its most active producent (Huang et al, 2000).

As with many other genes, CYPs also exhibit genetic polymorphism. Some patients might therefore not be as responsive to cyclophosphamide therapy as the majority, or can experience unexpected side effects (Griskevicius et al, 2003; Xie et al, 2003).

While some CYPs are expressed constitutively, others are inducible. Cyclophosphamide is even one of the factors regulating its own metabolism – it induces CYPs (Chang et al, 1997). As a direct consequence the 4-hydroxylation reaction increases in rate. On the other hand, the metabolic product acrolein is responsible for the inhibition of both CYPs and the CYP reductase (LeBlanc & Waxman, 1990).

2.2.1.2 CYP REDUCTASE

Cytochrome P450 reductase (alternatively named cytochrome c reductase) is protein of endoplasmic reticulum mostly occurring in liver cells (Phillips & Langdon, 1962). It has two domains localised on the cytosolic side, and a region of about sixty aminacids is anchoring it in the membrane of the ER membrane. The tertiary structure of the enzyme is highly conserved, indicating its importance during evolution. Of the cytosolic domains, one has flavin mononucleotide (FMN) as a cofactor and is responsible for interaction with an isoform of CYP. The other cytosolic domain uses a flavin adenine dinucleotide as a cofactor, and possesses two binding sites for NADPH.

The transport of electrons by the CYP reductase to a CYP molecule is a multi-step process. First, NADPH is bound to the FAD-domain of the reductase, which leads to simultaneous reduction by two hydride ions. Second, two electrons are chanelled to the FMN-domain in sequence; then follows transfer to the CYP (Vermilion et al, 1981).

The enzyme is known to exist in two separate conformational states (see Figure 4). The so-called "open" state allows the electron transfer between FMN and heme, while the "closed" conformation is compatible with transfer of electrons between FAD and FMN (Ellis et al, 2009).

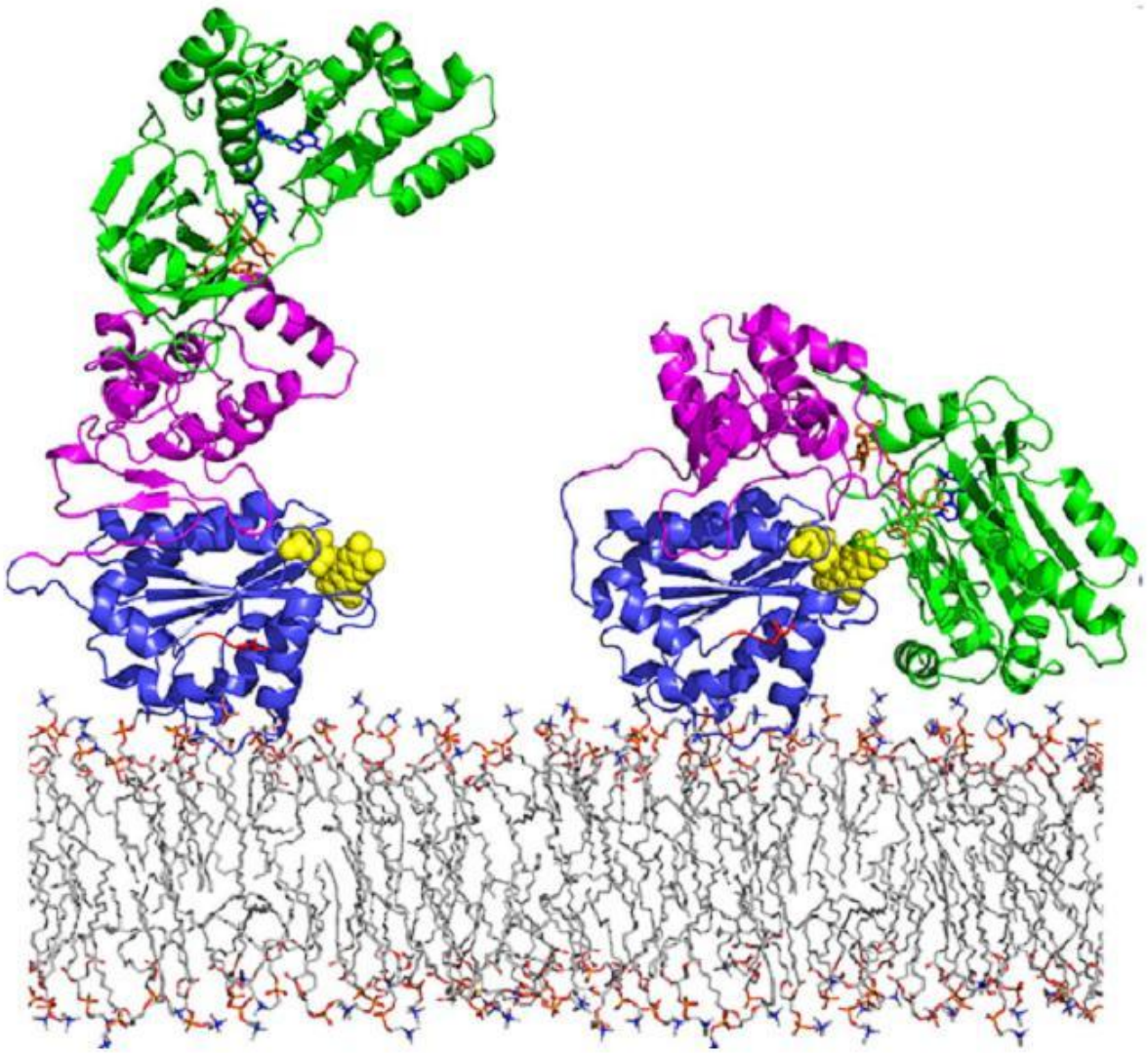

Fig.4 The two conformational states of the CYP reductase – "open" (left) and "closed" (right). Blue – FMN-domain, green – FAD-domain, violet – linker domain. The cofactors are shown: yellow – FMN, red – FAD, blue – NADPH (Ellis et al, 2009).

Only one of the two sites which bind NADPH is catalytic. When two NADPH molecules bind at the same time, it actually attenuates the reduction of FAD. Binding to the second site probably interferes with the release of $NADP^+$ (Paine et al, 2005).

The importance of CYP reductase for the CYP-facilitated drug metabolism has been studied in a knockout mouse strain that exhibits almost no reductase activity. These Hepatic Cytochrome P450 Reductase Null (HRN) mice express less than 1 % of the normal amount of the enzyme found in wild-type mice. The biotransformation of cyclophosphamide in these mice is severely inhibited,

and the little amount of 4-hydroxycyclophosphamide that is generated has its half-life in plasma significantly increased (Pass et al, 2005). Even though the HRN mice exhibit almost no hepatic reductase activity, cyclophosphamide is still metabolised, albeit significantly less effectively. This phenomenon is attributable to extra-hepatic activation, as has been demonstrated on another mouse strain - the CYP reductase-low, liver-CYP reductase-null (CL-LCN) mice (Gu et al, 2007)

2.2.1.3 CYCLOPHOSPHAMIDE BIOACTIVATION

2.2.1.3.1 4-hydroxycyclophosphamide and Aldophosphamide

Hepatic (and in minority also extra-hepatic) CYPs metabolise cyclophosphamide and generate 4-hydroxycyclophosphamide. This product does not exhibit significant cytotoxic effects, but it is an important first step in the activation of CY. In this form, the drug is transported to the target tissues. It has previously been thought that the transport might be facilitated by red blood cells (Highley et al, 1997), but nowadays the general consensus is that it is distributed by plasma.

Once it reaches the target cell, 4-hydrocyclophosphamide undergoes tautomerisation into aldophosphamide. Aldophosphamide then undergoes a non-enzymatic elimination reaction, which produces the final therapeutic agent phosphoramide mustard, and acrolein.

In the deactivation pathway, aldophosphamide might also be metabolised by an aldehyde dehydrogenase, which oxidises it and forms inactive carbophosphamide. For this reason the upregulation of the enzyme cause resistence to cyclophosphamide (Manthey et al, 1990).

2.2.1.3.2 Phosphoramide Mustard

Phosphoramide mustard is the final metabolic and therapeutic product generated from aldophosphamide. Although the generation of phosphoramide mustard does not require a catalyst, it has been established that serum albumin

might make the reaction happen faster (Kwon et al, 1987). This is however of little importance, as phosphoramide mustard is mostly ionised at physiological pH, and therefore cannot pass through cellular membranes.

In the target cells phosphoramide mustard acts as an alkylating agent. It contains an electrophilic aziridinium group that most often reacts with N-7 atom of guanines in nucleic acids, especially if they are flanked by other guanines. Phosphoramide mustard possesses two functional groups, therefore it is capable of producing both intra- and interstrand crosslinks, as well as nucleic acid-protein crosslinks (Kohn et al, 1987).

2.2.1.3.3 Acrolein

Acrolein is a toxic substance, which generation is mostly undesirable, but inevitable because it is produced from aldophosphamide in amount equalling those of phosphoramide mustard.

It contributes to cytotoxicyty of cyclophosphamide by generation of single-strand breaks in the DNA and binding to cellular proteins (Crook et al, 1986), however it is the major cause of occurrence of haemorrhagic cystitis (affects the urinary bladder) in patients treated with cyclophosphamide (Lawrence et al, 1975). For this reason, acrolein is usually depleted by sulphhydryl-containing protective drugs, a practise derived from observation of its depletion of cellular glutathione. This supportive treatment is not counter-productive because the phosphoramide mustard is at least one order in magnitude less effective in depletion of glutathion, therefore its therapeutic capacity remains unhindered (Gurtoo et al, 1981).

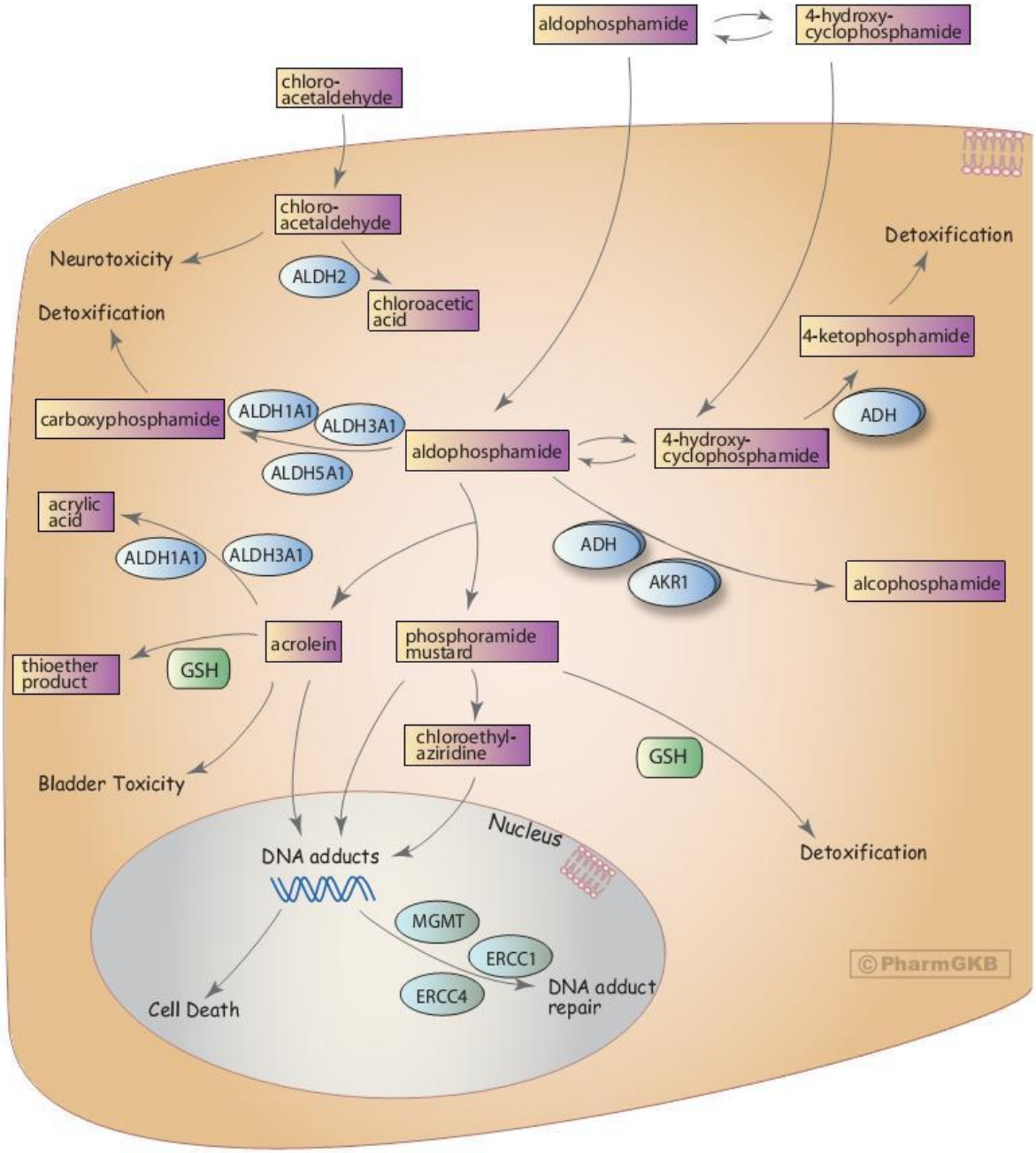

Fig.5 Pharmacodynamics of cyclophosphamide in a target cell.
(source: http://www.pharmgkb.org/views/pathway/PA2034.pdf - reproduced with permission from PharmGKB and Stanford University)

2.2.1.4 Cyclophosphamide Effects on Hematopoiesis

2.2.1.4.1 Myelosuppression and Immunosuppresion

Cyclophosphamide is the result of concentrated scientific effort to develop a potent antineoplastic drug. It was developed in the 1960 and is still widely used in human medicine to this day. As with virtually every single anti-cancer treatment, cyclophosphamide also affects non-cancerous tissues. Haematopoietic system is one of the most severely affected, as the turnover of cells is high (DeWys et al, 1970; Valeriote et al, 1968).

The haematopoietic response to cyclophosphamide regimens is usually characterised by a decline in the numbers of granulocytes, lymphocytes and thrombocytes (Fried & Johnson, 1968; Mosienko et al, 2002). This is not surprising as there is also an underlying decrease of bone marrow cellularity (Sefc et al, 2003). Neutrophils are particularly sensitive to the drug, and patients who experience severe neutropenia need to be given antibiotics as they are almost defenceless to pathogens (Tjan-Heijnen et al, 2001). Neutropenia can be at least partially corrected by administration of G-CSF (Neidhart et al, 1989), nevertheless, to this day myelosuppression is one of the key dose-limiting factors of cyclophosphamide treatment regimens (Presant & Klahr, 1977).

The transient pattern of myelosuppression caused by cyclophosphamide is in accordance with its mode of action. Rapidly proliferating cells are the ones most severely damaged – in the haematopoietic system those would be mostly lineage progenitors. HSCs are in majority slow-cycling and therefore not as affected. However, when they enter the proliferative state in order to compensate for the damage, they are also making themselves more susceptible to it (DeWys et al, 1970).

Notably, cyclophosphamide negatively affects lymphocytes, induces lymphopenia and immunosuppression (Borg et al, 2004). This state might be (a)a by-product of chemotherapy regimen (b)the reason for utilising

cyclophosphamide in the first place – for example in treating autoimmune diseases (Katsifis et al, 2002; Martin-Suarez et al, 1997).

It is currently widely used in the clinic in protocols for conditioning before HSC transplantation due to its combined ability to eliminate host lymphocytes (Litterman et al, 2013), which could otherwise mediate an alloreactive response culminating in rejection of a transplant (Aversa et al, 1999), and to free niches for the incoming transplanted HSCs.

2.2.1.4.2 Mobilisation of Haematopoietic Stem Cells

Cyclophosphamide possesses the ability to mobilise HSCs from their niches into the peripheral blood (Craddock et al, 1992). Mobilised HSCs from can be collected from the blood by apheresis and used for autologous transplantations (Rosen et al, 2000). G-CSF and GM-CSF are also capable of HSC mobilisation (Abkowitz et al, 2003), and are therefore used in combination with cyclophosphamide to maximise the numbers of mobilised cells (Caporali et al, 2001). For ethical reasons cyclophosphamide is not used for HSC mobilisation in healthy donors – it is after all a cancerogenous agent.

Although not yet completely elucidated, some facts are known about the mechanics of HSC mobilisation. The process is basically a direct opposite of the homing process, therefore adhesive interactions and chemokine-receptor interactions are being vigorously studied. Research done with antibodies against very late antigen-4 (VLA-4) lead to the discovery that their administration leads to HSC mobilisation (Kikuta et al, 2000; Papayannopoulou & Nakamoto, 1993). Inhibition of VLA-4 also reduces homing of transplanted HSCs (Zanjani et al, 1999).

As was previously stated, SDF-1 is a chemoattractant for HSCs, and is thought to be mandatory for homing of HSCs both in steady-state hematopoiesis and after transplantation of HSCs (Ara et al, 2003; Sugiyama et al, 2006). Its importance for haematopoiesis is demonstrated *in vivo* in knock-out mice that develop severe defects in B-lymphopoiesis and myelopoiesis (Nagasawa et al, 1996). However, artificially induced over-expression of SDF-1 can cause HSC mobilisation in mice owing to the desensitisation and internalisation of its receptor CXCR4 (Alsayed et al,

2007). This discovery shed some light on findings previously thought to be contradictory - of cyclophosphamide-induced HSC mobilisation and simultaneous increase in expression of SDF-1 in the bone marrow (Gazitt & Liu, 2001). Exposure to chemokines in the blood is thought to drive the expression of new CXCR4 molecules on the surface of transplanted HSCs, as they are capable of homing (Carion et al, 2003).

Exposure to cyclophosphamide turns the bone marrow into highly proteolytic environment (Levesque et al, 2002). Proteolytic enzymes in the bone marrow, namely matrix metalloproteinase 9, leucocyte elastase and cathepsin G are all responsible for cleavage of SDF-1 *in vitro*. Cleaved SDF-1 is not capable of engaging with its receptor and loses the function of a chemoattractant (Carion et al, 2003).

Even though the dynamics of interaction partners are usually studied in isolation, haematopoiesis is a complex system and a crosstalk between several pathways takes place (Peled et al, 2000).

2.2.2 IONISING RADIATION

Ionising radiation can damage cells in two ways. One of these is the direct damage to cellular components by absorption of the radiation; the other way is indirect and facilitated by chemicals occurring in the cell in the aftermath of the radiation. Notable molecules of the indirect route are reactive oxygen species (ROS) and reactive nitrogen species (RNS).

A crucial target for radiation damage is cellular DNA. Depending on the dose and the value of linear energy transfer (LET) of secondary electrons generated by ionising radiation, several scenarios for the damage to a DNA molecule are possible: a) single strand breaks, b) double strand breaks and c) base damage – modification, depurination and depyrimidation (Powsner & Powsner, 2006a).

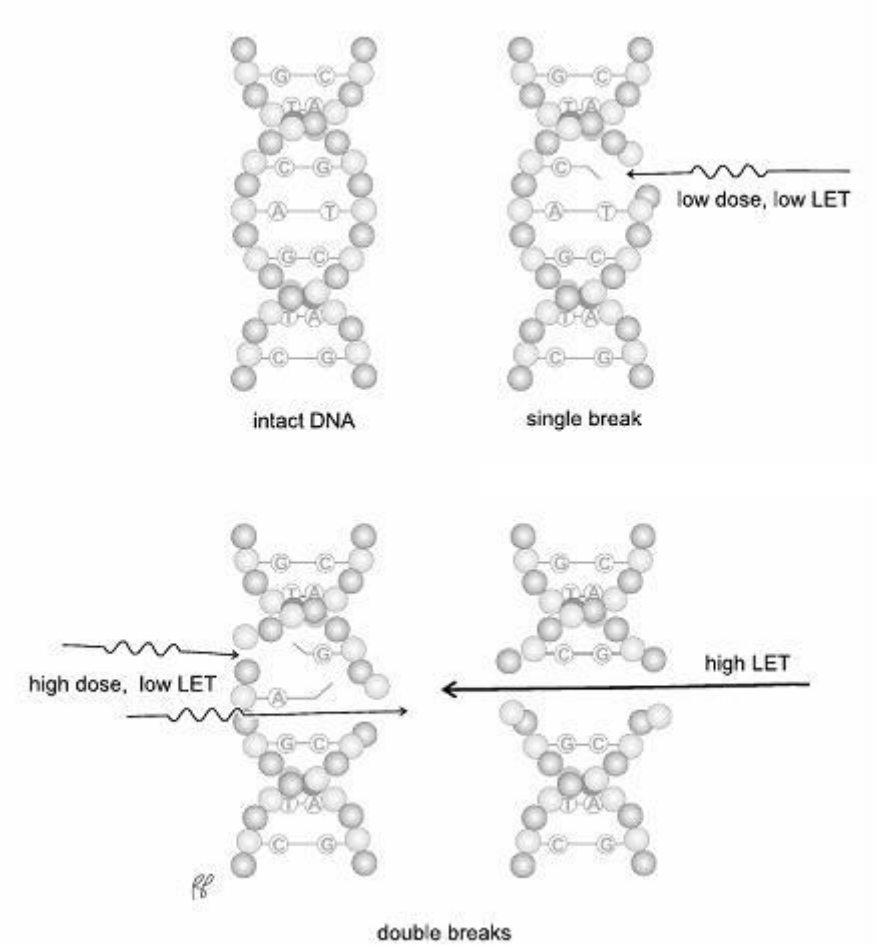

Fig.6 Effects of radiation on a DNA molecule(Powsner & Powsner, 2006b).

In response to insult of its genetic information, a cell recruits repair machinery in order to restore genetic stability and promote survival. Cells which are deficient in the DNA-damage response are also markedly more susceptible to radiation-induced damage and consequent death. This was demonstrated in cells from patients with ataxia telangiectasia, who have a mutation in the gene encoding the ATM kinase, a key component of DNA damage checkpoint in the cell cycle (Houldsworth & Lavin, 1980).

2.2.2.1 ROS/RNS Production

Reactive oxygen species are mostly produced as a result of radiolysis of water molecules. In this multistep process unstable hydroxyl radicals are formed, which in turn can generate formation of hydrogen peroxide and/or organic free radicals.

The generation of organic free radicals in DNA leads to strand breakage and cross-linking (Banjar et al, 1983).

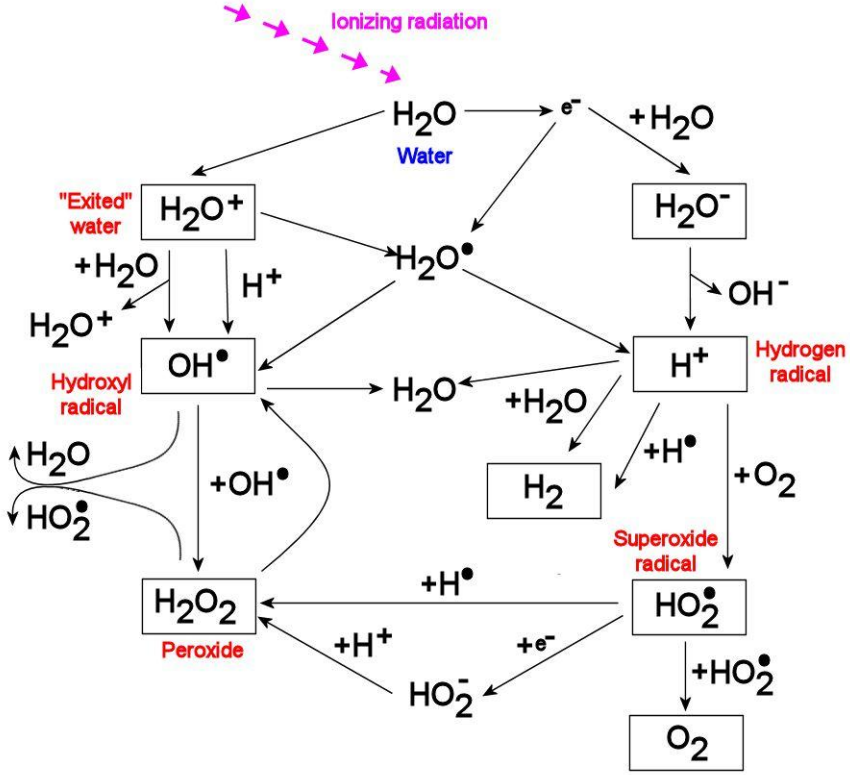

Fig.7 Radiolysis of cellular H_2O (http://www.mun.ca/biology/scarr/Radiolysis_of_Water.html)

Interestingly, hydroxyl radicals formed in mitochondria after exposure to ionising radiation seem to lead to oxidation of the components of electron transport chain. In consequence, mitochondria are dysfunctional, which leads to the occurrence of persistent oxidative stress (Yoshida et al, 2012).

Research performed on a chinese hamster ovary cell line showed that one of the constitutive nitrogen oxide synthases (NOS; namely NOS1) can be activated upon exposition to ionising radiation. Its activation promotes production of NO, which the authors speculate might be used in inter-cellular signalisation of oxidative stress (Leach et al, 2002). Additionally, RNS might be generated from NO, and act further as oxidising agents.

In a murine macrophage cell line, DNA damage induced by ionising radiation leads to the activation of NF-κB. Poly (ADP-ribose) polymerase (PARP), an enzyme

of the DNA damage response machinery, was found to be responsible for the NF-κB activation. This activation causes heightened expression of inducible nitric oxide synthase (iNOS), which also consequently leads to a noticable increase in NO production (Ibuki et al, 2003).

2.2.2.2 Effects of Ionising Radiation on Hematopoiesis

The effects ionising radiation has on haematopoietic compartment have been thoroughly studied since the discovery of the radiation. Much insight has also been gained thanks to the analysis of data collected from victims of nuclear disasters such as were the explosions in Nagasaki and Hiroshima, and in the Chernobyl accident.

The haematopoietic compartment is extremely sensitive to ionising radiation and some injury is caused every time it is subjected to it, even if the dose is small (Jefferies et al, 1998). Peripheral blood cells gradually decrease in number, which reflects damage to differentiated blood precursor cells. Notably, lymphocytes are hugely sensitive and lymphopenia occurs almost immediately after irradiation (Mauch et al, 1995).

During the nuclear bomb testing at the Bikini island, experimental animals (pigs and goats) were subjected to the radiation following the explosions. Significantly decreased white blood cell counts were observed almost immediately afterwards. Before death, the animals gradually developed thrombocytopenia and increased fragility of blood capillaries, which lead to the haemorrhaging syndrome (Cronkite, 1950).

By analysis of past nuclear incidents, classification of the severity of post-irradiation symptoms has been devised. With respect to haematology, the victims are classed in one of four groups (H1-4), where H4 exhibits the most severe manifestations of radiation damage (Fliedner et al, 2001).

Persons in the H4 group experience symptoms so severe that they were deemed to be in accordance with complete and irreversible loss of HSCs. On the other hand, patients in the H3 group exhibit changes in quantity and quality of blood cells, which indicate a few surviving HSCs. Differences between these two

groups lead to summarising the indicators of reversible and irreversible damage to the haematopoietic compartment by ionising radiation (Fliedner et al, 2007).

	INDICATORS	
	reversible injury	**irreversible injury**
granulocytes	moderate granulocytosis; decline: days 4-10; abortive recovery followed by a nadir: days 20-30	initial granulocytosis; progressive decline on days 4-6
lymphocytes	decline to nadir levels within 2 days, thereafter recovery	progressive decline within 24 hours
platelets	decline from day 10; nadir: days 20-30	progressive decline within the first 10 days

Tab.1 Indicators of reversible and irreversible injury to the haematopoietic compartment caused by ionising radiation (after Fliedner et al, 2007).

It has been noted that radiation-induced disruption of blood homeostasis is a problem stemming not only from the damage to differentiated cells and lineage-commited progenitors, but also from damage to HSCs. Stem cells from irradiated mice transitionally change expression of several stem cell markers - the levels of Sca-1 and c-kit are elevated and lowered respectively. A CD150-positive HSCs also manifest higher susceptibility to apoptosis (Meng et al, 2003), and non-negligible engraftment of untreated bone marrow cells takes place even ten weeks after the irradiation (Simonnet et al, 2009).

It has also been found out that irradiated HSCs manifest chronic increase of ROS production. This was attributed to the function of NADPH oxidase 4 (NOX4), which is up-regulated post-irradiation, and its inhibition by diphenylene iodonium significantly reduced the phenomenon. HSCs subjected to such prolonged oxidative stress were found to be prone to senescence (Wang et al, 2010). Moreover,

this damage might even be increased by radiation-induced senescence of stromal cells of the haematopoietic stem cell niche (Carbonneau et al, 2012).

3 PRELIMINARY RESULTS AS BASIS FOR CURRENT EXPERIMENTS

HSCs subjected to various pretransplantation conditioning regimens (differing in the time intervals between CY and TBI treatments) exhibited significantly lower (CY-5 and CY-7) or lower (CY-2) ability to compete with transplanted HSCs. (Šefc,L., unpublished data)

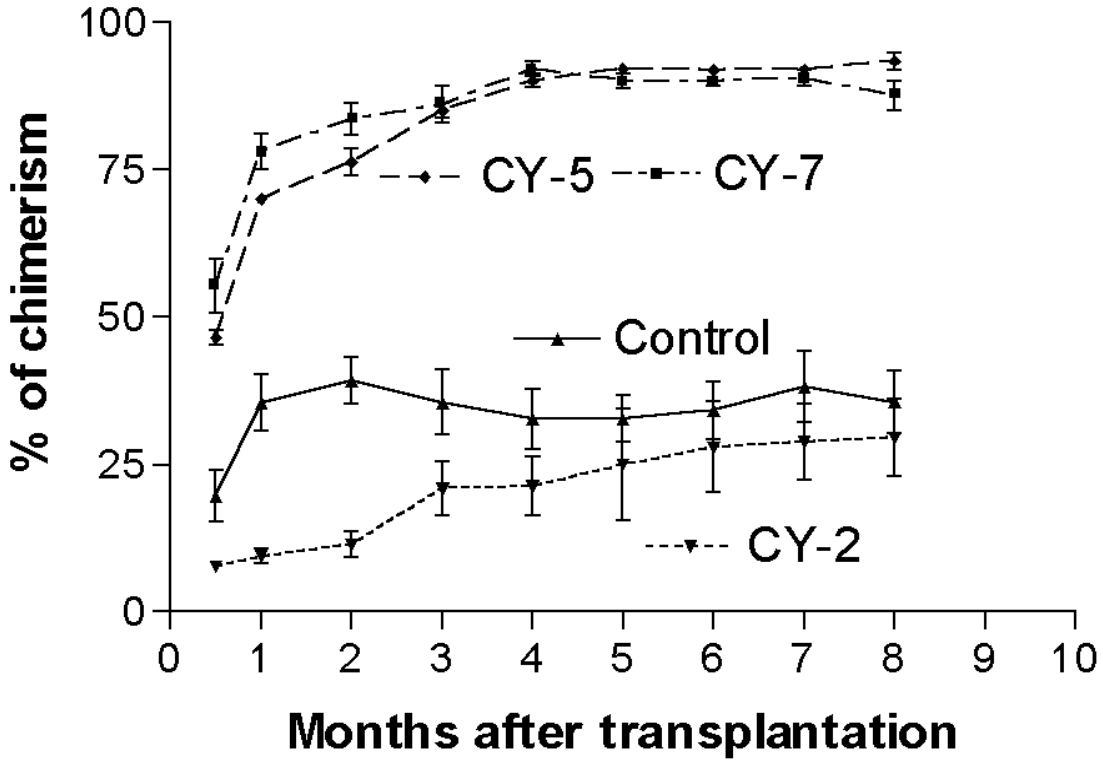

Fig.8 Blood chimerims after transplantation. Mice received cyclophosphamide (CY 135 mg/kg b.w.) 2, 5, 7 days before being subjected to total body irradiation of 4 Gy. After the irradiation mice received a transplant of 4 million untreated bone marrow cells.

4 AIMS OF THE STUDY

- To evaluate various time intervals between cyclophosphamide and total body irradiation treatments with respect to bone marrow cellularity and total numbers and the proliferative state of hematopoietic stem cells.
- To evaluate differences between transplanted sets of hematopoietic stem cells in terms of their engraftment and long term repopulating ability and subsequently discover the optimal pretransplantation conditioning regimen.

5 MATERIALS AND METHODS

5.1 MATERIALS

5.1.1 MICE

- **donors:** C57BL/6NCrl mouse strain (Ly5.2, expresses CD45.2 antigen)
- **recipients:** congenic B6.SJL-Ptprca Pepcb/BoyJ mouse strain (Ly5.1, expresses CD45.1 antigen)

The mice were bred in a specific pathogen free facility of the Center of the Experimental Biomodels, First Faculty of Medicine, Charles University in Prague. During the experiments they were kept in a clean conventional part of the facility with a light-dark cycle of 12 hours. They received food ad libitum. Two- to six-month-old mice with the bodyweight of 20-25 grams were used.

All experiments were approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University in Prague and were performed in accordance with national and international guidelines for laboratory animal care.

There were at least three animals in each experimental group.

5.1.2 CHEMICALS

- Ammonium chloride (NH_4Cl), *IPL, Czech Republic*
- Bovine serum albumin Fraction V, biotin free, *Carl Roth GmbH, Germany*
- Click-iT® EdU Pacific Blue® Flow Cytometry Assay Kit, *Life Technologies Corporation, USA*
- Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit, *Life Technologies Corporation, USA*
- Endoxan (Cyclophosphamidum monohydricum, *Baxter Oncology GmbH, Germany*)
- Deionised water
- Diethyl ether, *Penta, Czech Republic*

- Disodium phosphate (Na2HPO4.12H2O), *IPL, Czech Republic*
- EDTA (ethylendiaminetetraacetic acid, disodium salt: dihydrate; $C_{10}H_{14}N_2O_8Na_2{\cdot}2H_2O$), *Sigma-Aldrich, USA*
- Heparin, *Zentiva, Czech Republic*
- Hoechst 33342, *Honeywell Riedel-de Haën, Germany*
- Iscove's Modified Dulbecco's Medium with sodium bicarbonate and without L-glutamine, *Sigma-Aldrich, USA*
- Monosodium phosphate ($NaH_2PO_4{\cdot}2H_2O$), *IPL, Czech Republic*
- Propidium iodide, *Life Technologies Corporation, USA*
- Sodium chloride (NaCl), *IPL, Czech Rebublic*
- Türck solution, *Penta, Czech Republic*

5.1.3 ANTIBODIES

5.1.3.1 Ly5.1/Ly5.2 Chimerism

- FITC anti-mouse CD45.2, *BioLegend, USA*
- PE anti-mouse CD45.1, *BioLegend, USA*

5.1.3.2 Cell Cycle Analysis

- 7-AAD (DNA labelling), *Life Technologies Corporation, USA*
- APC anti-mouse CD150, *BioLegend, USA*
- APC/Cy7 anti-mouse CD117 (c-kit) , *BioLegend, USA*
- FITC anti-mouse CD48, *BioLegend, USA*
- PE anti-mouse Ly-6A/E (Sca-1) , *BioLegend, USA*
- Pacific Blue (EdU labelling), *Life Technologies Corporation, USA*

OR

- Alexa Fluor® 488 (EdU labelling), *Life Technologies Corporation, USA*
- Alexa Fluor® 700 anti-mouse CD48, *BioLegend, USA*
- APC anti-mouse CD150, *BioLegend, USA*
- biotin anti-mouse CD117 (c-kit) , *BioLegend, USA*
- FxCycle™ Violet Stain (DNA labelling) *Life Technologies Corporation, USA*

- PerCP anti-mouse Ly-6A/E (Sca-1) , *BioLegend, USA*
- Streptavidin-Brilliant Violet™ 570, *BioLegend, USA*

5.1.3.3 Analysis of Apoptosis

- Alexa Fluor® 700 anti-mouse CD48, *BioLegend, USA*
- APC anti-mouse CD150, *BioLegend, USA*
- APC/Cy7 anti-mouse CD117 (c-kit) , *BioLegend, USA*
- FITC anti-mouse Ly-6A/E (Sca-1) , *BioLegend, USA*
- Hoechst 33342, *Honeywell Riedel-de Haën, Germany*
- Propidium iodide, *Life Technologies Corporation, USA*

5.1.4 BUFFERS

- Phosphate buffered saline (PBS): 16 mmol/dm^3 $Na_2HPO_4 \cdot 12H_2O$, 4 mmol/dm^3 $NaH_2PO_4 \cdot 2H_2O$, 0.15 mol/dm^3 NaCl, pH 7.4
- Ammonium chloride lysis solution: 0.15 mol/dm^3 NH4Cl, 0.1 mmol/dm^3 EDTA, 0.032 mol/dm^3 NaCl

5.1.5 INSTRUMENTS

- AccuBlock Digital Dry Bath (D1200-230V), *Labnet International, USA*
- Analytical balance AB 104, *Mettler Toledo, Czech Republic*
- Auto Hematology Analyzer BC-5300 Vet, *Mindray, China*
- Automatic micropipettes, *Eppendorf, Germany*
- Cellometer AUTO T4, *Nexcelom Bioscience, USA*
- Centrifuge 5804R, *Eppendorf, Germany*
- CO_2 incubator, IGO 150 Cell life, *Jouan GmbH, Germany*
- Flow box, Holten LaminAir, Model 1.2, *Thermo-Scientific Inc., USA*
- Flow cytometers: BD FACS Aria Ilu Special Order System - equipped with UV-laser, BD FACS Canto II, *Becton Dickinson, USA*
- Milli-Q RG, *Merck Millipore, USA*

- Source of ionizing radiation: 60Co Chisobalt, *Chirana, Czech Republic*
- Orbital incubator SI50, *Stuart Scientific, UK*
- Vortex mixer, *Velp Scientifica, Italy*

5.2 METHODS

5.2.1 ADMINISTRATION OF CYCLOPHOSPHAMIDE

- Cyclophosphamide powder was weighted and dissolved in PBS to achieve a dose of 135 mg/kg body weight per 0.5 ml of solution.
- Donor Ly5.2 mice received 0.5 ml of the solution by way of intraperitoneal injection.
- Control groups received no cyclophosphamide.

5.2.2 TOTAL BODY IRRADIATION

- Total body irradiation of ~0.58 Gy/min from a ^{60}Co source from a distance of 123.5 cm was used.
- Donor mice received a dose of 2 Gy in total body irradiation.
- Recipient mice received a dose of 4 Gy several hours prior to the bone marrow transplantation. A higher dose (6 Gy) was used in supplementary experiments.

5.2.3 BONE MARROW EXTRACTION

- Donor mice were sacrificed at given time intervals after the administration of cyclophosphamide by cervical dislocation.

		Days after CY									
		Ctrl	1	2	3	5	7	9	12	14	16
Time after IRR	10 min	x	x	x	x	x	x	x	x	x	x
	24 hrs	x	x	x	x	x	x	x	x	x	x
	5 days	x		x		x	x			x	

Tab.2 Overview of the experiment – mice were irradiated a given number of days after CY. The bone marrow was extracted 10 min, 24 hr, 5 days after IRR. Analysed time intervals are marked by "x".

- Bone marrow cells were flushed out of femurs by 1 ml of 1% solution of BSA in PBS. One-milliliter insulin syringe (21G needle) was used in the procedure. A single-cell suspension was achieved by repeated aspiration and expiration.
- The cells were counted with Cellometer AUTO T4, using Türck solution for white blood cell count.

5.2.4 BONE MARROW TRANSPLANTATION

Bone marrow cells from three donors (one femur from each) were pooled into a single tube with 1 ml of PBS-BSA. Cellularity of the transplant was determined by Cellometer AUTO T4, using Türck solution for white blood cell count. The cell suspension was then diluted to the concentration of 1 femur/0.5 ml and stored on ice. The transplant was warmed to room temperature directly before the transplantation.

The transplantation was performed by injection of 0.5 ml of the suspension into the retro-orbital sinus of an anesthetised recipient. One-millilitre insulin syringe was used.

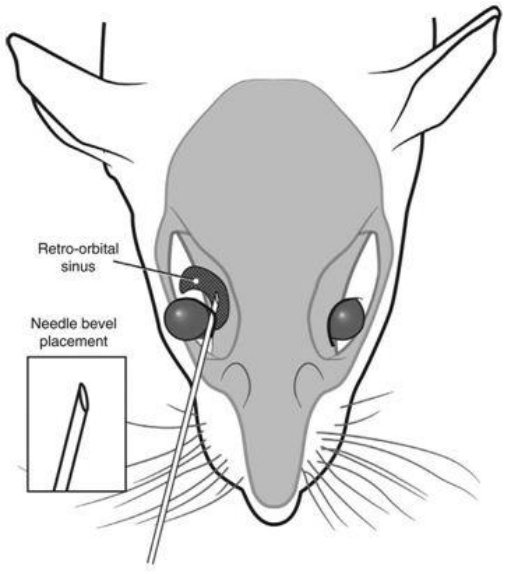

Fig.9 Transplantation into the retroorbital sinus in a mouse (Yardeni et al, 2011)

5.2.5 PERIPHERAL BLOOD COLLECTION FOR COMPLETE BLOOD COUNT WITH DIFFERENTIAL

Approximately 50 µl of blood were collected from the retro-orbital venous plexus of anesthetised mice. Microhematocrit capillaries (Keraglass, Czech Republic) dusted inside with EDTA powder were used. The samples were then analysed by Auto Hematology Analyzer BC-5300 Vet (Mindray, China).

5.2.6 PERIPHERAL BLOOD COLLECTION FOR CHIMERISM ANALYSIS

Approximately 100 µl of blood were collected from the retro-orbital venous plexus of anesthetised mice. Heparinised microhematocrit capillaries (Keraglass, Czech Republic) were used in the process.

50 µl of each blood sample was then stained with 0,2 µl both FITC anti-mouse CD45.2 and PE anti-mouse CD45.1 antibodies. Samples were incubated for 10-15 min on ice. Afterwards 200 µl of PBS-BSA were added and the samples were analysed by flow cytometry.

5.2.7 FLOW CYTOMETRY

5.2.7.1 Analysis of Ly5.1/Ly5.2 Chimerism after Transplantation

CD45 is a surface cellular antigen expressed on cells of the haematopoietic lineage with the exception of mature red blood cells. As the two mouse strains used in this study are congenic and differ only in the expression of the CD45 molecule variant, the ratio between CD45.1 and CD45.2 expressing cells in the peripheral blood reflects the ratio of the recipients' haematopoietic stem cells to engrafted donor stem cells.

The contribution of short-term HSCs (ST-HSCs) and progenitors to haematopoiesis was evaluated at one month after the transplantation; whereas three and six months after the transplantation only long-term HSCs (LT-HSC) were still present and giving rise to progeny of differentiated blood cells.

5.2.7.2 SK SLAM Cell Cycle Analysis

Cell cycle analysis was performed according to manufacturer's instructions using the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit, or Click-iT® EdU Pacific Blue® Flow Cytometry Assay Kit, depending on which kit was in stock at the time. The procedure was always performed on cell suspensions containing $4x10^6$ bone marrow cells.

Briefly, the calculated volume of cell suspension containg 4×10^6 cells were incubated with 2 of EdU and IMDM (volume calculated to the combined value of 2 ml) for 45 min at 37 °C. After the incubation, the samples were centrifuged (400 g, 4 °C, 6 min) and the supernatant was discarded. Samples were washed with 3 ml of PBS-BSA (500 g, 4 °C, 5 min), stained with antibodies (1 µl/sample) and incubated for 15 min on ice in the dark. The samples were then washed with PBS-BSA (500 g, 4 °C, 5 min), the supernatant was discarded, and the samples were incubated with 50 µl parafolmaldehyde for 15 min(room temperature, dark). After fixation the samples were washed again (3 ml of PBS-BSA, 500 g, 4 °C, 5 min), and the pellets were treated with 100 µl of saponin solution (room temperature, dark). After the permeabilisation the samples were washed (3 ml of saponin, 500 g, 4 °C, 5 min), the supernatant was discarded. The pellets were treated with the Click-iT® reaction components (250 µl/sample) and incubated (30 min, room temeperature, dark). At the end of the incubation the samples were washed (3 ml of PBS-BSA, 500 g, 4 °C, 5 min), the supernatant discarded and the 2,5 µl of ribonuclease, 1 µl of 7-AAD and 250 µl of PBS-BSA were added to the pellets and left to incubated (ice, 30 min, dark). Prepared samples were analysed on a flow cytometer (gating strategy in supplements, Fig 22).

5.2.7.3 Analysis of Apoptosis

- Calculated volumes of cell suspension were added to tubes containing 3 ml of PBS-BSA. For analysis of SK SLAM cell apoptosis $5x10^6$ cells were used. Both bone marrow and spleen cells underwent the analysis.

- The samples were washed with 3 ml of PBS-BSA (400g, 4°C, 6 min) and the supernatant was discarded.
    - 1 µl/sample of Alexa Fluor® 700 anti-mouse CD48, APC anti-mouse CD150, APC/Cy7 anti-mouse CD117 (c-kit) and FITC anti-mouse Ly-6A/E (Sca-1) antibodies was added to the samples
- Samples were incubated on ice in the dark for 15 minutes**.**
- Samples were washed with 3 ml of PBS-BSA (400 g, 4 °C, 6 min).
- 500 µl of PBS-BSA and 0.5 µl of Hoechst 33342 were added to each sample, and the samples were incubated in the test tube incubator at 37 °C for 7 minutes. Afterwards the samples were put on ice and stored in the dark.
- 1 µl of PI was added to each sample just before flow cytometry analysis (gating strategy in supplements, Figure 23)

5.2.8 STATISTICS

For the creation of graphs and statistical analysis, GraphPad Prism 5 software (GraphPad Software, USA) was used. Data are presented in the form of mean ± the standard error of the mean (SEM). One-way ANOVA with Dunett's post-test was used to compare the data with corresponding control groups. P values ≤ 0.05 were considered statistically significant. Statistically significant results were marked by asterisks according to the following criteria: * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

6 RESULTS

6.1 Bone Marrow Cellularity

The results show the combined effect of cyclophosphamide (CY, 135 mg/kg b.w.) and total body irradiation (TBI, 2 Gy) on donor Ly5.2 bone marrow cells. Mice were irradiated at different time intervals after the peritoneal administration of cyclophosphamide (exception – control groups did not receive CY). Bone marrow was harvested from femurs and tibias 10 minutes, 24 hours, and 5 days after TBI (see Table 2).

The ten-minute interval represented marrow cellularity after CY treatment alone as cells damaged by TBI had not yet had enough time to finish their apoptotic program induced by irradiation and were included in the cell counts (Collins et al, 1997). Bone marrow cellularity dropped to approximately 10 % of control in the first three days after CY, but a full recovery was achieved already one week after the treatment (Fig 10).

The regeneration of bone marrow cells after 24 hours and 5 days (selected intervals) after TBI respectively in mice pretreated with CY has been determined and obtained results are presented in Figures 11 and 12. Collectively, the results presented in Figures 10-12 are combined in Figure 13.

The main difference between samples collected 10 min and 24 hr after TBI respectively was observable when CY was administered 5 and more days before TBI. In the 10-min interval, cellularity reached normal levels on day 5 after CY and stayed virtually constant up to pretreatment with CY till day 16. In the 24-hour interval after TBI, however, the cellularity of bone marrow in mice pretreated with CY on day 5 reaches double the level of control group (no CY treatment) and then falls back to control levels on days 7 and 9, continues to decrease further (nadir on day 14), and finally normalises at control levels again in mice on day 16.

In mice given CY 7 days before TBI, a decrease in cellularity compared to the 10-min interval is observable 24 hr after TBI and a vast defect in bone marrow regeneration is apparent when bone marrow is examined 5 days after TBI.

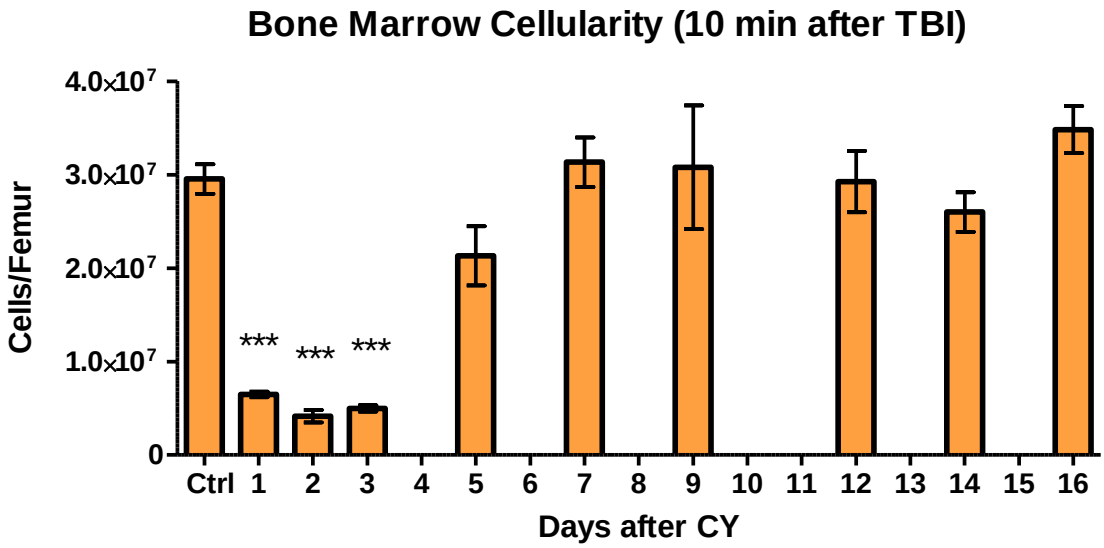

Fig.10 Bone marrow cellularity (number of cells per femur) 10 minutes after total body irradiation (TBI). Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.), and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min after TBI. Significance of difference to the control group (no CY, TBI only): *** ~ $P \leq 0.001$.

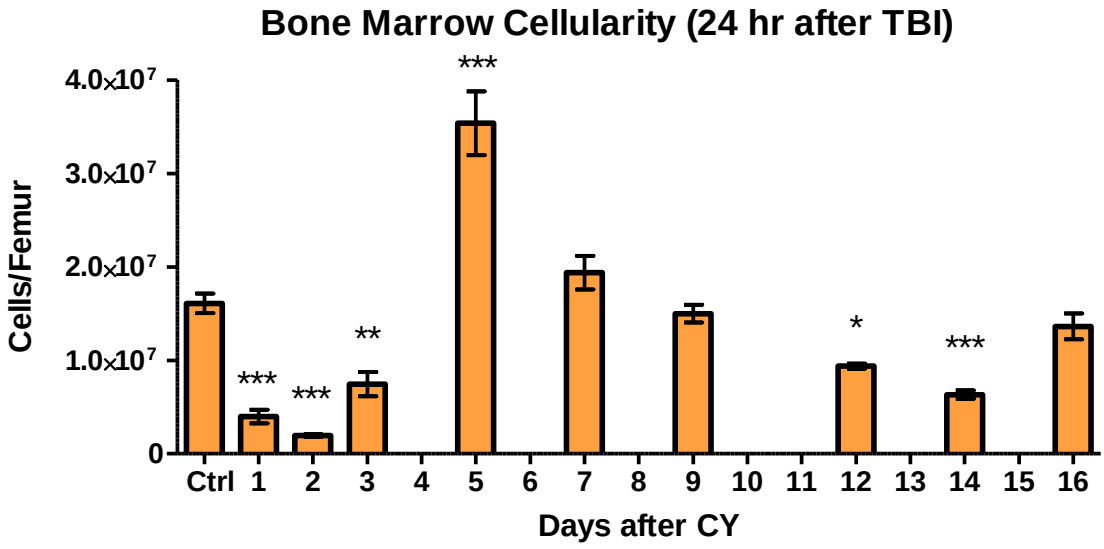

Fig.11 Bone marrow cellularity (number of cells per femur) 24 hours after total body irradiation (TBI). Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.), and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 24 hr after TBI. Significance of difference to the control group (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

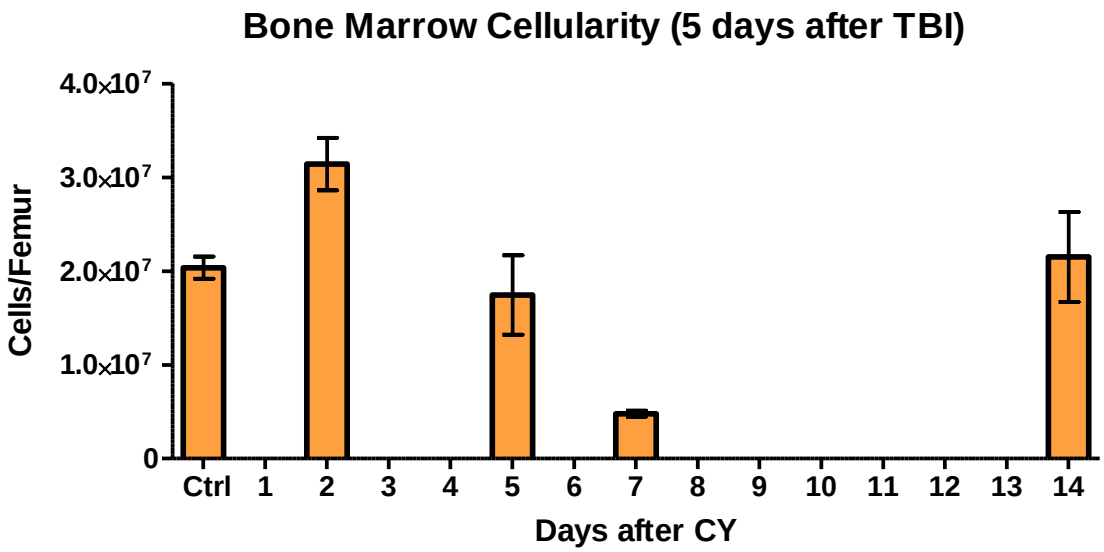

Fig.12 Bone marrow cellularity (number of cells per femur) 5 days after total body irradiation (TBI). Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.), and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 5 days after TBI.

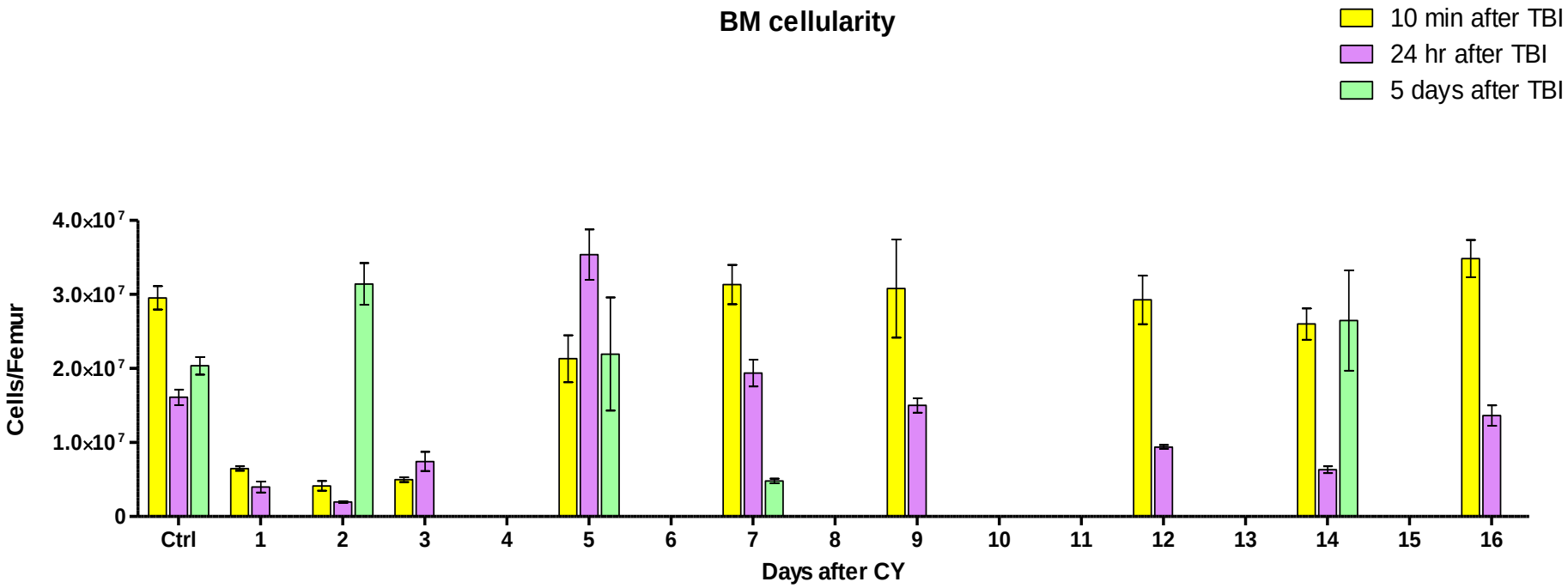

Fig.13 A combined graph of bone marrow cellularity. Asterisks showing significant results were omitted in order not to decrease the clarity.

6.2 Number of Hematopoietic Stem Cells and Their Proliferation

The numbers of hematopoietic stem cells (HSCs) and their proliferation were evaluated in CY-TBI treated mice. The SK SLAM ($Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{+}CD48^{-}$) cells represented stem cells (Kiel et al, 2005). Initial decrease and a subsequent increase in the numbers of HSCs was observed during the first 5 days after CY in all three groups, regardless of the time passed between CY and TBI. 5 days after the administration of CY, the HSC population in the bone marrow significantly exceeded the control levels when examined 10 minutes after TBI and also when examined 5 days after TBI. The only exception was thus the 24-hr interval in which the increase of HSCs on day 5 did not occur, and the numbers of HSCs actually were those of the control group.

As for the proliferation of HSCs, it was elevated 24 hr after irradiation, although no significant difference from the control group was observed on any given day after CY. In the 10-min interval proliferation rapidly increased and peaked on day 2 after CY. On this day, the increase in proliferation was almost 3-fold. Beyond this day, the proliferation decreased, reached the lowest levels on days 5 and 7, and later went back to control levels. In the group irradiated 5 days after a dose of CY proliferation is lowered in all cases, except for the CY-14 group, where it reaches the level of Ctrl(10min.

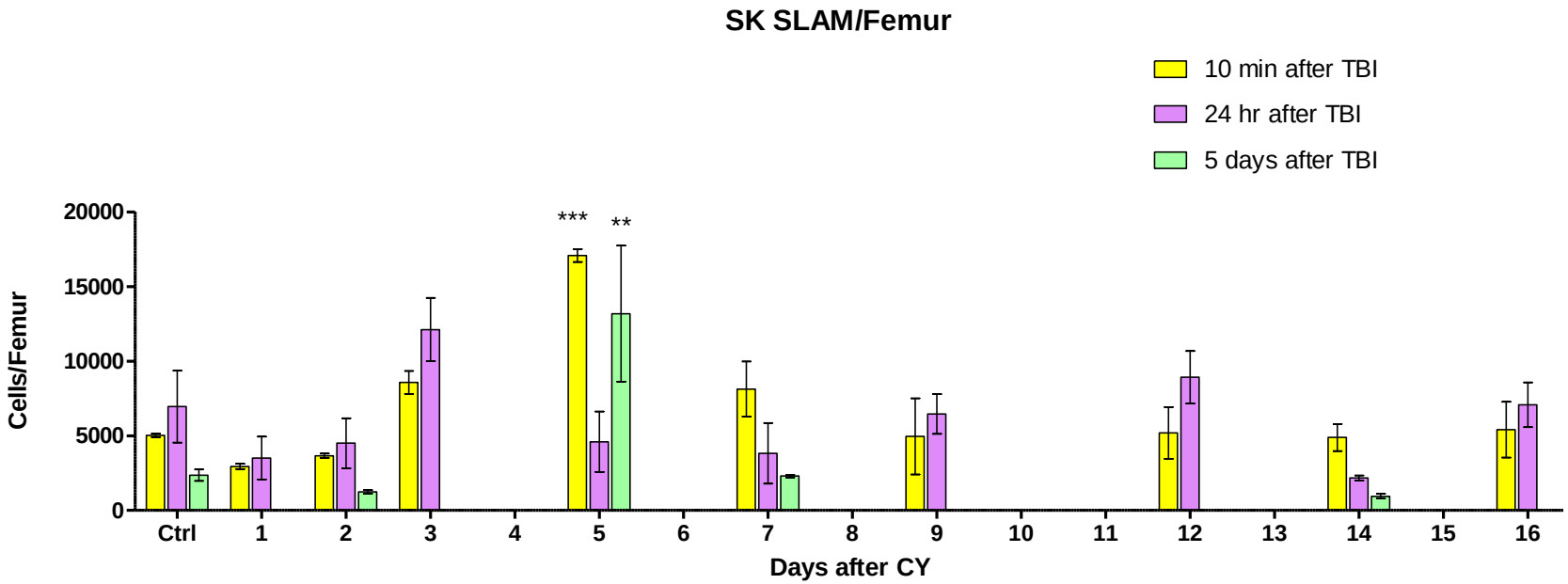

Fig.14 HSC numbers after total body irradiation (TBI). Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Significance of difference to the corresponding control groups (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

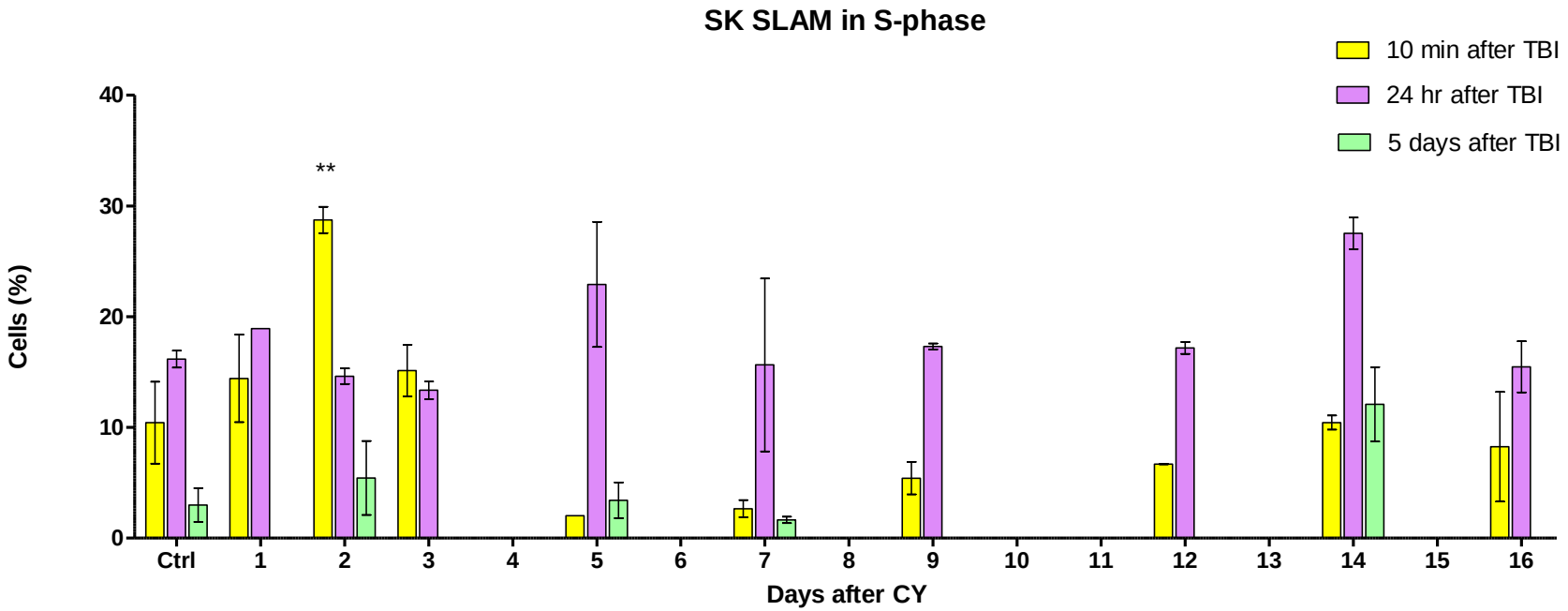

Fig.15 Percentages of actively proliferating haematopoietic stem cells. Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Significance of difference from the corresponding control groups (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

6.3 Ly5.1/Ly5.2 chimerism after transplantation of HSCs

As congenic Ly5.1 and Ly5.2 mouse strains were used in the experiments, progeny of their haematopoietic stem cells were easily distinguishable due to expressing CD45.1 and CD45.2 isoforms on their cell surface. With the use of fluorescently labelled antibodies these distinct populations were evaluated by flow cytometry analysis of collected blood samples one, three (results not shown) and six months after the transplantation.

As the transplant always consist of the suspension of the whole bone marrow (1 femur/0.5 ml PBS-BSA), not only long-term repopulating haematopoietic stem cells, but also short-term stem cells, progenitor cells and more differentiated cell types are transplanted. All of these cell types transiently contribute to haematopoiesis after transplantation, therefore at one month after the transplantation donor progenitors distort the results of the analysis, at three months they do not any longer contribute significantly to blood cell production, and after six months all donor-derived haematopoiesis is assumed to be the product of successfully engrafted long-term HSCs only.

Ly5.1 recipients of transplanted Ly5.2 HSCs were subjected to total body irradiation of 4 Gy a few hours (approximately 3-4) before a transplant was administered by injection of cell suspension into the retro-orbital sinus.

Another control group was added – the recipients received completely untreated donor bone marrow. Even thought all of the CY-TBI groups exhibit significantly lower engraftment capability than the untreated control, differences manifest between the groups. Notably CY-5 and CY-7 groups at all three time interval post-irradiation exhibit chimerism so low, it is on the limit of being virtually undetectable.

Interestingly, the HSCs from group CY-2 isolated 24 hr after TBI engrafted twice as effectively as the corresponding control.

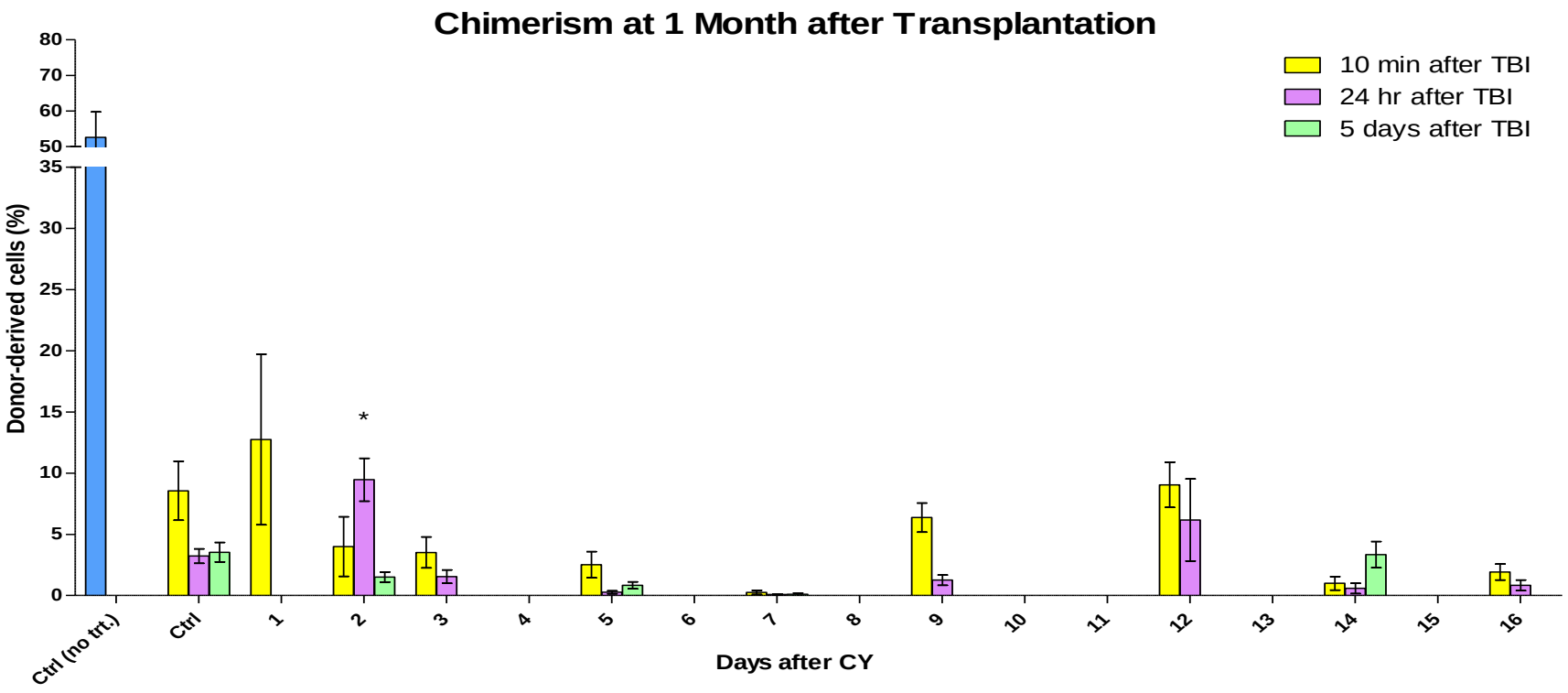

Fig.16 Percentages of donor-derived (Ly5.2) blood cells in the peripheral blood one month after transplantation. Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Ly5.1 recipients underwent TBI (4 Gy) a few hours prior to receiving a transplant (1 femur/0.5 ml PBS-BSA). Significance of difference from the corresponding control groups (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

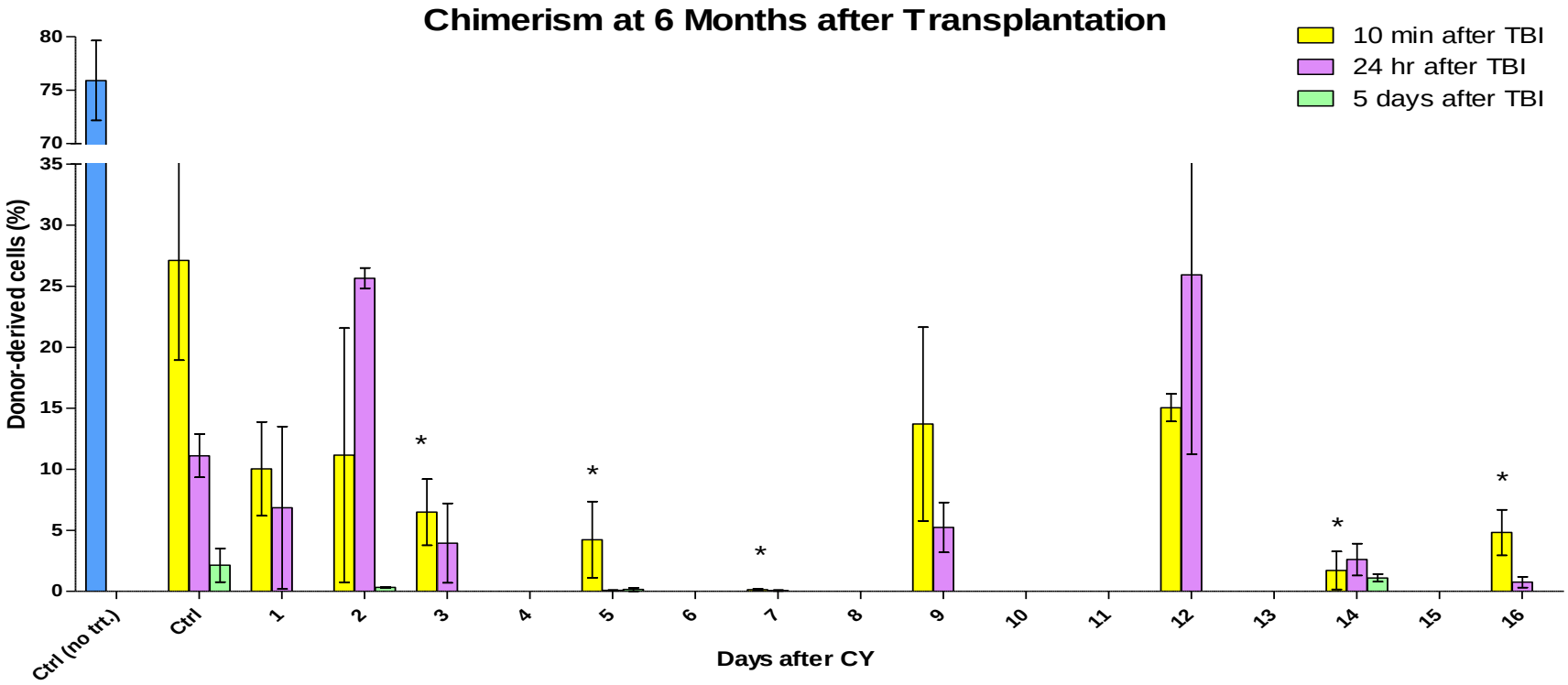

Fig.17 Percentages of donor-derived (Ly5.2) blood cells in the peripheral blood one month after transplantation. Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Ly5.1 recipients underwent TBI (4 Gy) a few hours prior to receiving a transplant (1 femur/0.5 ml PBS-BSA). Significance of difference from the corresponding control groups (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

As was stated above, the blood chimerism in recipients of transplants from groups CY-5 and CY-7 was almost undetectable, which is why we decided to repeat the experiment with the groups that showed the greatest differences (CY-2 and CY-7, together with control). This time we used recipients conditioned by a higher radiation dose (6 Gy) with greater haematologic injury and thus less competing cells present.

This set of experimental data did not reveal as dramatic a difference between the two groups, however, a less effective engraftment of CY-7 HSCs is still observable in the 10-minute and 5-day intervals. Remarkably, CY-7 harvested from the donors 24 hr after TBI exhibit similar engraftment capacity as the CY-2 group of the same post-irradiation interval.

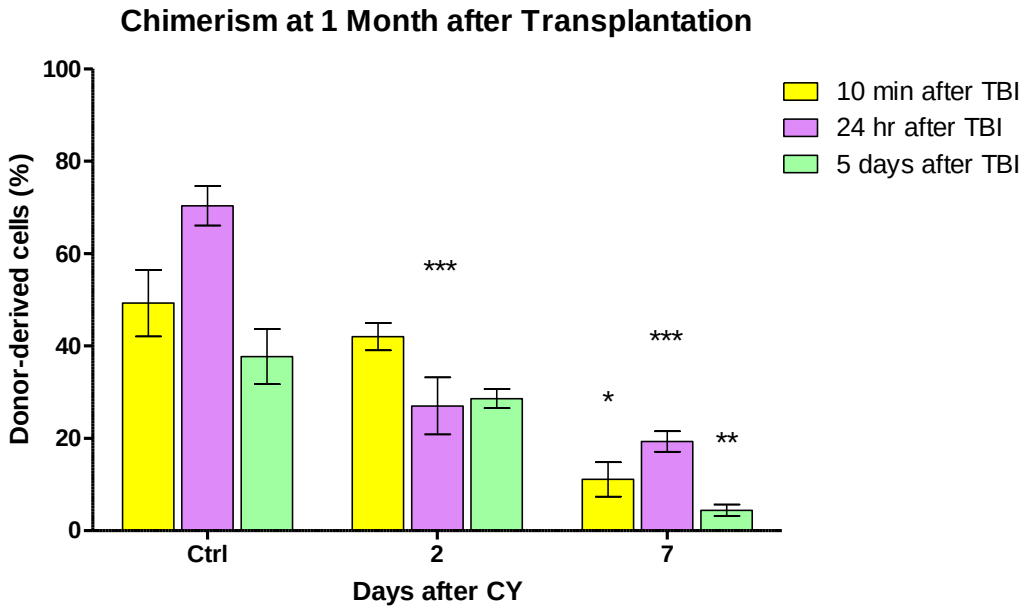

Fig.18 Percentages of donor-derived (Ly5.2) blood cells in the peripheral blood one month after transplantation. Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Ly5.1 recipients underwent TBI (6 Gy) a few hours prior to receiving a transplant (1 femur/0.5 ml PBS-BSA). Significance of difference from the corresponding control groups (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

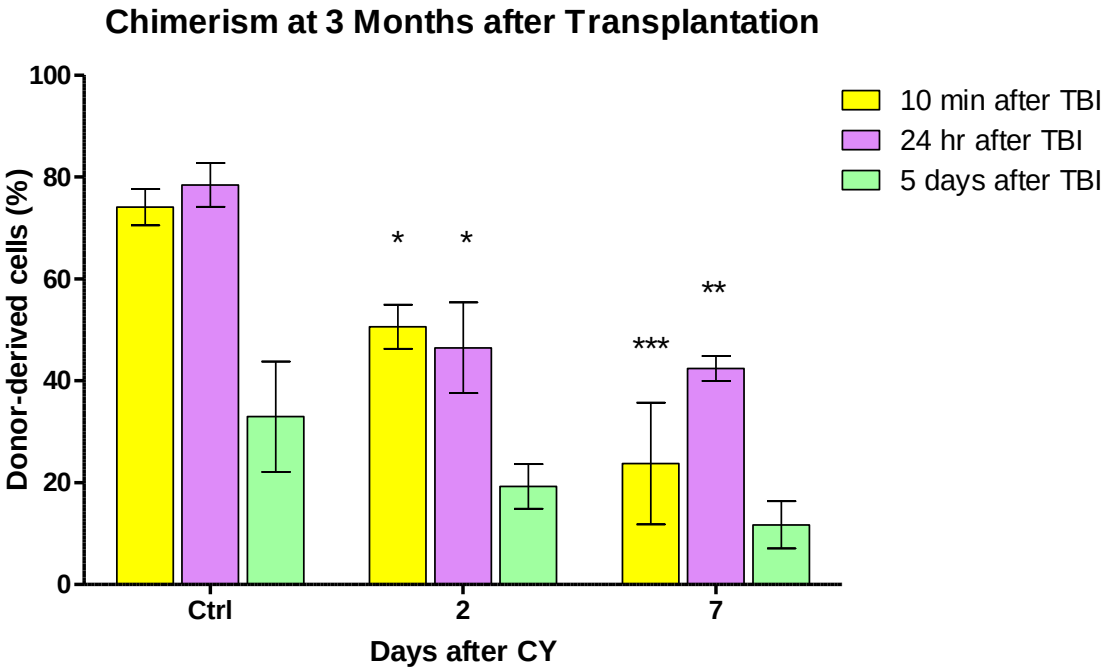

Fig.19 Percentages of donor-derived (Ly5.2) blood cells in the peripheral blood 3 months after transplantation. Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Ly5.1 recipients underwent TBI (6 Gy) a few hours prior to receiving a transplant (1 femur/0.5 ml PBS-BSA). Significance of difference from the corresponding control groups (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

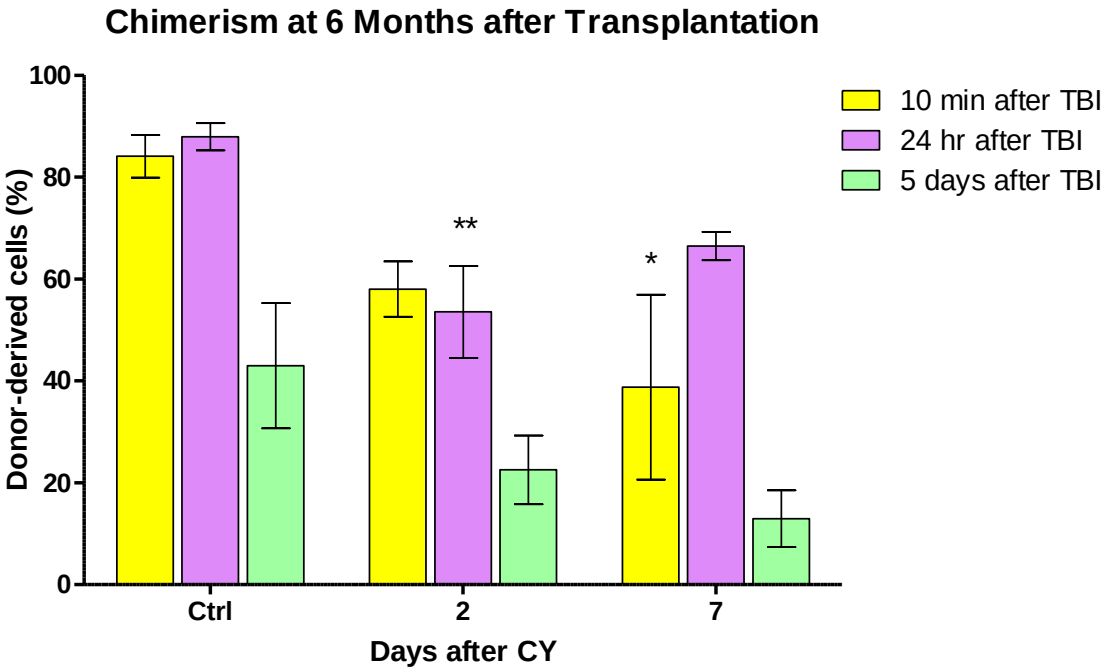

Fig.20 Percentages of donor-derived (Ly5.2) blood cells in the peripheral blood 6 months after transplantation. Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Ly5.1 recipients underwent TBI (6 Gy) a few hours prior to receiving a transplant (1 femur/0.5 ml PBS-BSA). Significance of difference from the corresponding control groups (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

6.4 Apoptosis of Bone Marrow HSCs

At strategic intervals (Ctrl, CY-2, CY-7) an evaluation of percentages of live, apoptotic and dead cells were made by flow cytometry. The mice underwent the same CY-TBI conditioning regimen as described previously. The intervals were chosen on the basis of the huge difference between their engraftment potential (see Figures 7 and 8). Moreover the level of engraftment of CY-7 groups seemed to be in stark contrast with the relatively high numbers of HSCs in the donor bone marrow.

The Hoechst-propidium iodide staining was used for the analysis - the Hoechst dye stains the DNA of all cells, while intercalating agent propidium iodide accumulates only in apoptotic and already dead cells (as it cannot pass the plasma membrane of viable cells). For the statistical analysis apoptotic and dead cells were counted as one group, and the "dead to alive" ratio was evaluated.

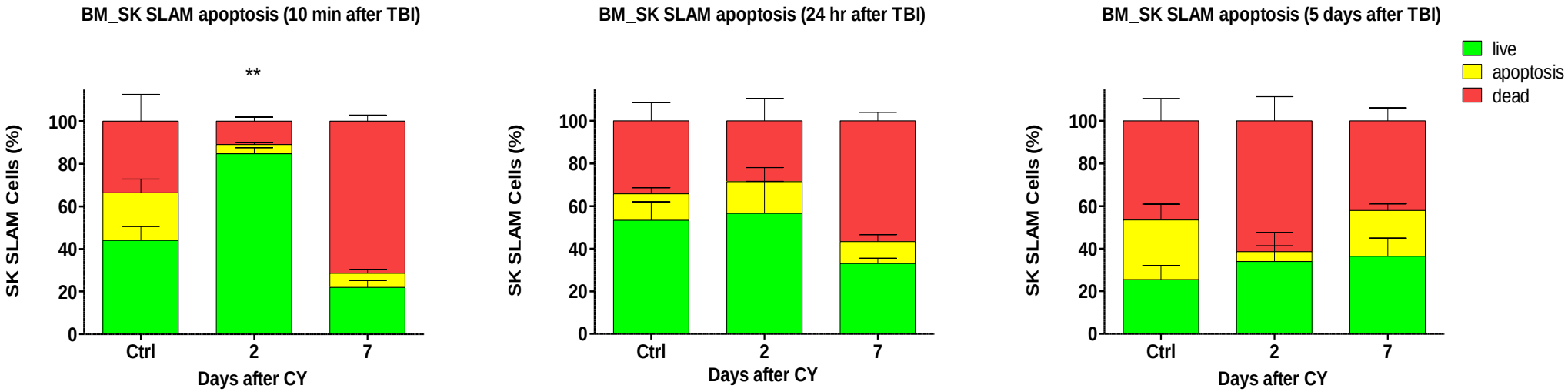

Fig.21 Percentages of live, apoptotic and dead HSCs in the bone marrow. Mice were injected with cyclophosphamide (CY, 135 mg/kg b.w.) and irradiated at different times post-injection with 2 Gy. Bone marrow samples were collected 10 min, 24 hr, 5 days after TBI. Significance of difference from the control group (no CY, TBI only): * ~ $P \leq 0.05$, ** ~ $P \leq 0.01$, *** ~ $P \leq 0.001$.

7 Discussion

Conditioning regimens using the alkylating chemotherapeutic agent cyclophosphamide, which was developed in the 1960s specifically to be used in cancer therapy, are widely used in clinical medicine. This drug is notably used in patients with haematological malignancies who are to receive a transplant of haematopoietic stem cells from a healthy donor. Together with total body irradiation, the drug is used to cause myeloablation and immunosuppression. The vacated stem cell niches are then meant to be occupied by the transplanted donor HSCs. Several treatment protocols exist and are used routinely, dependent on the country and local conventions. Total body irradiation follows a few days (the numbers vary) after administration of cyclophosphamide.

In this thesis project the reaction of bone marrow HSCs to various CY-TBI conditioning regimens was evaluated in terms of their total numbers, proliferation, viability, and ability to repopulate the bone marrow of an irradiated host and facilitate haematopoiesis in the long term.

Previous projects in our laboratory led to the discovery of the kinetics of bone marrow and spleen cellularities and numbers of haematopoietic stem cells after the administration of the cytostatic drug cyclophosphamide. This thesis project aimed to evaluate these characteristic in situations where both cyclophosphamide and total body irradiation were administered with different time intervals passing in between the treatments. It was postulated that bone marrow cellularity values 10 minutes after TBI should not differ from a situation in which only cyclophosphamide has been administered – as the cells have not yet had time to initiate response to radiation damage (Collins et al, 1997). On the other hand, changes were anticipated 24 hrs and five days after irradiation.

7.1 Bone Marrow Cellularity

As predicted, bone marrow extracted mere ten minutes after irradiation did not deviate from the previous findings and exhibited the same cellularity as samples froom CY-only treated bone marrow.

Irradiation of 2 Gy alone (see Ctrl, Figure 13) lowered the cellularity of bone marrow to approximately 60 % one day after the exposure. During the next five days regenerated startered.

Interestingly, no major changes in bone marrow cellularity were distinguishable in CY-1 to CY-5 samples collected 10 min and 24 hr after TBI respectively. Regeneration of bone marrow was immediate and was not influenced by irradiation. However, radiation did cause a decrease of BM cellularity on days 7-16 after CY, whereas the samples collected 10 min after TBI retained the cellularity of the control at those intervals.

In the CY-7 group, irradiation caused a decrease in cellularity 24 hr after TBI. The numbers of cells in the bone marrow continued to fall significantly to the day 5 after irradiation.

7.2 SK SLAM HSCs

The numbers of SK SLAM HSCs decreased after CY alone – here represented by the 10-min post-irradiation interval as their numbers are estimated by flow cytometry analysis of surface markers that do not change, nor the cells disappear so fast after the treatment. Then on days 3-7 occurred an overshot which peaked on day 5 with significantly heightened numbers of HSCs.

TBI alone (see Ctrl, Figure 5) causes a slow gradual fall of HSC numbers during 5 days after the irradiation by 2 Gy.

In mice that had time to develop response to both CY and TBI we saw a slow but pronounced decrease in the numbers of HSCs during the first 5 days after CY treatment. The exception to that was day 5 after CY, in which case the cells were irradiated at the peak of their increase and we already saw them recover 5 days after

TBI; and day 3 in the 24-hr interval, when the HSCs peaked and then fell again on day 5.

Numbers of SK SLAM HSCs in the CY-7 groups were all comparable to their control samples, regardless of the post-irradiation intervals.

7.3 Proliferation of SK SLAM HSCs

SK SLAM HSCs proliferate slowly, and the S-phase fraction represents about 10 % of the population. Cyclophosphamide drives HSCs to proliferation within the first 2 days after administration, and we saw a peak in CY-2 group. Beyond this day, proliferation plummeted (nadir on day 5-7) to 2 %.

In the 24-hour interval between irradiation and collection of bone marrow, number of proliferating HSCs rose slightly, in control mice it reached a 15% rate. Even though all of the CY-treated groups exhibited increased proliferation 24 hr after TBI, none of them differed significantly from the control. Response to radiation damage is initiated very quickly after the injury (Hashiguchi et al, 2007), therefore the increase in proliferation is most probably not attributable to DNA repair synthesis.

7.4 Ly5.1/Ly5.2 Chimerism

The only way to test the viability and functionality of HSCs is via the competitive repopulation assay. In this assay HSCs have to repopulate preconditioned bone marrow of a host. They also have to compete with the remnants of host hematopoietic tissue that survided the conditioning regimen.

In a clinical setting, combination of CY and TBI is used as pre-transplantation conditioning regimen in leukemic recipients of HSC transplants. This treatment aims to destroy patients' aberrant hematopoiesis and clear the space for engraftment of healthy donor HSCs (Cant et al, 2007).

In order to test the effectivity of pre-conditioning, we collected bone marrow cells from pre-conditioned mice (Ly5.1) and measured their repopulation ability by the competitive repopulation assay. Thus, low repopulation ability of HSCs

represents an effective pre-conditioning regimen and low competition of host HSCs to the transplant.

Blood cell Ly5.1/Ly5.2 chimerism 1 month after the transplantation represents short-term repopulating cells (SH-HSCs, MPPs and more mature progenitors; all with limited self-renewing capacity). On the other hand, chimerism 6 months after the transplantation represents long-term repopulation by LT-HSCs, the "real" stem cells (Zhong et al, 1996).

- Hosts irradiated by 4 Gy

  Progenitor cells were more susceptible to damage by the conditioning regimens, than HSC. In all cases, higher chimerism is observed 6 months after the transplantation, when all progenitors are thought to have had differentiated and/or died (Zhong et al, 1996).

  When irradiation was applied very early after CY (groups CY-1 and CY-2) or between days 9 and 12, the damage to donor HSCs is not significantly different from that caused by irradiation alone, and the cells exhibit similar repopulation capacity as the controls.

  On the other hand, prolonging the interval between CY and TBI to 5-7 or 14-16 days lead to significantly pronounced damage to the donor HSC, which manifested as lower ability to repopulate recipient bone marrow. This corresponds with the preliminary results (See Figure 8).

  Moreover, prolonging the 5-day interval between TBI and collection of the HSCs lead to lower chimerim, indicating that bone marrow microenvironment contributes to further decline of HSC viability after irradiation alone and also in combination with CY.

- Hosts irradiated by 6 Gy

  When we increased the dose of ionising radiation for the recipienst in order to reach reliable detection levels of chimerism in CY-7 groups, we did not observe such a pronounced suppression of donor haematopoiesis in the 10-minute and 24-hour post-radiation intervals as in the previous experiments.

Host haematopoiesis seemed to be severely hindered and both CY intervals exhibited about as much engraftment as controls. However, a noticably lowered engraftment is still shown 5 days after TBI.

Seeing that at day 7 after CY we detected relatively high numbers of SK SLAM HSCs in the bone marrow, but the cells were uncompetitive in host irradiated by 4 Gy, we also evaluated apoptosis of bone marrow HSCs that were going to be transplanted into host irradiated by 6 Gy.

The results showed that 7 days after CY approximately 30 % of HSCs are alive in all of the groups. In a contrast to that, controls and CY-2 groups show greater variability, with CY-2 having more than 80 % of HSCs in the live fraction 10 min after TBI, and almost 60 % after another 24 hr. Together, the results of the analysis of apoptosis suggest that in the host irradiated by 4 Gy CY-2 interval was beneficial in terms of engraftment of donor cells simply because the cells did not succumb to apoptosis as often as CY-7 samples.

7.5 SUMMARY

All together the results of this study show that the time interval employed between the cyclophosphamide and total body irradiation administrations is absolutely crucial for the outcome of transplantations – a time interval of 2 days is not better than irradiation alone and HSCs quickly regenerate. In clinical setting that would mean swift and highly undesirable regeneration of leukemic hematopoiesis and its competition with transplanted HSCs in patients. On the other hand, a seven-day interval between the treatments seems to be devastating for the HSCs, which is an ideal characteristic of a pre-transplantation conditioning regimen.

These intervals might be different in humans (as the research was done on mice), but the study indicates that by studying human HSCs transplanted into immunodeficient mice, it might be able to accomplish improvement in the outcome of HSC transplantations in humans.

8 CONCLUSIONS

- The kinetics of bone marrow cellularity and HSC numbers after CY and TBI regimens are comparable to CY-only regimens at short intervals after CY (1-5 days).
- Rapid proliferation of HSCs in short intervals after CY precedes regeneration of their numbers in the bone marrow. Regeneration of the numbers of HSCs precedes regeneration of bone marrow cellularity.
- HSC proliferation is slightly increased 24 hr after TBI, and already subdued 5 days after TBI.
- Survival of HSCs 2 days after CY is increased to 80 %, and is still at 60 % 24 hrs after TBI, whereas 7 days after CY all groups manifest 70 % of apoptotic/dead cells.
- The repopulation ability of HSCs was greatest in cells collected 2 or 12 days after CY, and 10 min or 24 hr after TBI. It was lowest in cells collected 5 or 7 days after CY.
- The time interval between CY and TBI is a key determinant of viability of HSCs, their ability to engraft, and thus the outcome of transplantations.

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