SMALL MOLECULE MEDIATED TARGETING OF HAEMATOPOIETIC STEM/PROGENITOR CELL AND LEUKAEMIC STEM CELL FUNCTION

A thesis submitted in fulfilment of the requirements for the degree of

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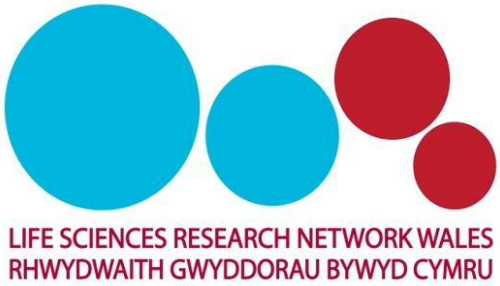

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Abstract

Haematopoietic stem cells (HSC) are a rare population of cells that have the ability to self-renew and differentiate giving rise to various blood lineages, thereby reconstituting the whole haematopoietic system. This is an essential characteristic, exploited in bone marrow transplantation therapy in response to myeloablative treatment. Due to their rarity, the lack of sufficient HSC numbers for transplantation has proved to be a major clinical issue. Separately, in the development of leukaemia, acquired mutations in HSCs give rise to malignant cells. These cells, like HSCs, have the ability to self-renew and differentiate forming immature blasts and are termed cancer (leukaemic) stem cells. They are thought to remain in a quiescent state and are therefore not targeted by standard chemotherapy, inducing relapse in haematopoietic malignancies. In this study, a cross species stem cell based screen was conducted on a 12,000 small molecule library across a range of adult and embryonic tissue types with a view to identifying compounds that would (i) expand HSCs ex vivo and *in vivo* for transplantation and (ii) eradicate cancer stem cells in leukaemia. A number of small molecules were identified as lead compounds and were assessed in our investigation. We found that Yohimbine, an alpha-2 adrenergic receptor (adra-2) antagonist, and Oxa-22, cis-2-Methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide (M3 Muscarinic acetylcholine receptor agonist) elicited a 2- and 1.5- fold increase in HSC frequency (respectively) *in vivo*. Further competitive transplantation studies showed that Yohimbine and Oxa-22 treated cells also enhanced the reconstitution of B cells and T cells respectively. In parallel, we also assessed Oxa-22 and a third compound, Phthalylsulfathiazole- an antibacterial sulphonamide, in the leukaemic setting to ascertain whether compounds could target leukaemic stem cells (LSCs). We found that these compounds promoted proliferation in acute myeloid leukaemia (AML) cell lines. Furthermore, when Oxa-22 and Phthalylsulfathiazole were administered *in vivo* models of AML, they accelerated disease progression by increasing the number of LSCs.

Collectively, these results show that using small molecules we can target neuronal related pathways to enhance HSC number and function. Further investigation is required to elucidate the exact mechanisms of the compounds however, these data may prove to be

influential in directing new methods of stem cell expansion for transplantation therapies. Small molecules targeting neuronal or antibacterial related pathways were also found to target malignant LSCs and alter their behaviour. By driving LSCs out of their dormant state, these small molecules may pave the way for potential targeting of LSCs in conjuction with standard current chemotherapies that incorporate and kill proliferating cancer cells.

Abbreviations and Signs

Symbols

°C= Degrees Celsius

µg = Micrograms

µl = Microlitre

µm = Micrometre

µM = Micromolar

nM = Nanomolar

mM = Millimolar

A

Ab = monoclonal Antibody

Adra-A2 = α2 Adrenergic receptor

ALL = Acute Lymphocytic Leukeamia

AML = Acute Myeloid Leukeamia

APL = Acute Promyelocytic Leukeamia

Ara-c = Cytarabine

B

BFU-E = Burst Forming Unit-Erythrocyte

BM = Bone marrow

BMP = Bone Morphogenic Protein

BMT = Bone marrow transplant

bp = Base Pair

BrdU = 5'-bromo-2-deoxyuridine

C

CB = Cord blood

CFC = Colony Forming Cell

CFU = Colony Forming Unit

CFU-E = Colony Forming Unit- Erythrocyte

CFU-G = Colony Forming Unit- Granulocyte

CFU-GEMM = Colony Forming Unit-Granulocyte/Erythrocyte/Macrophage/Mega-aryocyte

CFU-GM = Colony Forming Unit-Granulocyte/Macrophage

CFU-M = Colony Forming Unit- Macrophage

CFU-Meg/Mk = Colony Forming Unit-Megakaryocyte

CFU-s = Colony Forming Unit- Spleen

cDNA = Complementary DNA

CLP = Common Lymphoid Prgogenitor

CMP = Common myeloid Progenitor

Cre = Cre recombinase

CSC = Cancer Stem Cell

CT = Cycle threshold

D

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic Acid

DNase = Deoxyribonuclease

DNMT = DNA Methyltransferase

dNTP = Deoxynucleotide triphosphate

E

EDTA = Ethylenediaminetetraacetic acid

EGF = Epidermal Growth Factor

EGFR = Epidermal Growth Factor Receptor

F

FACs = Fluorescence Activated Cell Sorting

FBS = Fetal Bovine Serum

G

g = Gram

G-CSF = Granulocyte- colony Stimulating Factor

GFP = Green Fluorescent Protein

GMP = Granulocyte Macrophage Progenitor

GVHD = Graft versus Host Disease

H

HLA = Human Leukocyte Antigen

HPC = Haematopoietic Progenitor Cell

HSC = Haematopoietic Stem Cell

HSPC = HaematopoieticStem/Progenitor Cell

I

Il- = Interlukin-

K

kg = Kilogram

L

L = Litre

Lin = Lineage

LoxP = Locus of crossover of Bacteriophage P1

LSC = Leukaemic Stem Cell

LT-HSC = Long Term- HSC

LTC-IC = Long term culture initiating cell

M

mAChR = Muscarinic acetyle choline receptor

MEP = Megakaryocyte Erythrocyte Progenitor

mg = Milligram

Min = Multiple Intestinal Neoplasia

mins = Minutes

ml = Millilitre

MLL- = Mixed Lineage Leukemia

mM = Millimolar

MPP = Multipotent Progenitor

mRNA = Messenger Ribonucleic Acid

MSC = Mesenchymal Stem Cell

N

n = Number

NOD/SCID = Non-obese diabetic/severe combined immune deficiency

O

OB = Osteoblasts

P

PIpC = polyinosinic:polycytidylic acid

PBS = Phosphate Buffered Saline

PCR = Polymerase Chain Reaction

Q

qRT-PCR = Quantitative Reverse Transcription Polymerase Chain Reaction

R

RNA = Ribonucleic Acid

RNase = Ribonuclease

rpm = Revolutions per minute

RT = Room Temperature

S

SA = Streptavidin

SEM = Standard error of mean

ST-HSC = Short term-HSCs

T

TBI = Total Body Irradiation

TGF-β = Transforming Growth Factor-β

TPO = Thrombopoietin

U

UV = Ultra Violet

V

VEGF = Vascular Endothelial Growth Factor

VEGFR = Vascular Endothelial Growth Factor Receptor

W

WBM = Whole Bone Marrow

Wnt = Wingless

WT = Wild Type

Y

YFP = Yellow Fluorescent Protein

123

5-FU = 5'-fluorouracil

6-OHDA = 6-Hydroxydopamine

Chapter 1: Introduction

1.1 Haematopoietic Stem Cells

The haematopoietic system is primarily comprised of a variety of differentiated cells such as erythrocytes, platelets and immune cells. However, many of these mature blood cells are short lived, and so their replenishment is regulated by a small, rare, population of haematopoietic stem cells (HSC) resident in the bone marrow. HSCs are one of the best understood examples of somatic stem cells. They are capable of self-renewal, whereby cells undergo symmetrical division, and therefore maintain a population of long-term progenies. HSCs also possess the ability to differentiate into precursors which are fated to uni-lineage differentiation, eventually giving rise to mature blood cells (red blood cells, megakaryocytes, myeloid cells and lymphocytes) (Ogawa 1993).

The existence and functional role of HSCs was first demonstrated in the early 1950's. Animals that underwent total body irradiation (TBI) succumbed due to bone marrow (BM) failure. However, irradiated recipients could be rescued following transplantation of spleen or BM cells from unirradiated donors (Lorenz et al. 1951). This initial study showed that BM cells have the ability to regenerate the haematopoietic system upon bone marrow transplantation (BMT). However, the question of whether this was due to a clonally derived HSC population or multiple stem cells that are fated to each particular blood lineage remained unanswered. Seminal work from Till and McCulloch showed that bone marrow cells transferred to lethally irradiated mice could proliferate and form macroscopic colonies in the spleen (colony unit forming-spleen (CFU-S)) (Till and McCulloch 1961; Becker et al. 1963). These colonies are clusters of haematopoietic cells undergoing maturation towards the erythrocytic, granulocytic and megakaryocytic lineages and not lymphoid lineages (Till and Mculloch 1961). This assay therefore provided evidence for the existence of multipotent precursors or progenitor cells rather than HSCs in transplanted cells. *In vivo* repopulation assays and *in vitro* colony forming studies in combination with fluorescence activated cell sorting (FACS) have since been used to comprehensively characterise a complete hierarchy of progenitor cell types that are derived clonally from a common parental cell type, the HSC.

Long-term HSCs (LT-HSCs) are fundamental for hematopoiesis. Adult HSCs are a quiescent population of stem cells and it is their slow progression into the cell cycle that maintains their regulation of the homeostatic and continual turnover of the haematopoietic system throughout life (Cheshier et al. 1999; Arai et al. 2004). In addition to self-renewal, LT-HSCs give rise to short-term HSCs (ST-HSCs) that possess extensive proliferation and differentiation properties which contribute to the generation of multipotent progenitors, MPPs. Advances in fluorescent activated cell sorting and analysis has allowed the development of new techniques to identify and characterise different cells of the haematopoietic hierarchy based on the expression of cell surface markers (Figure 1.1).

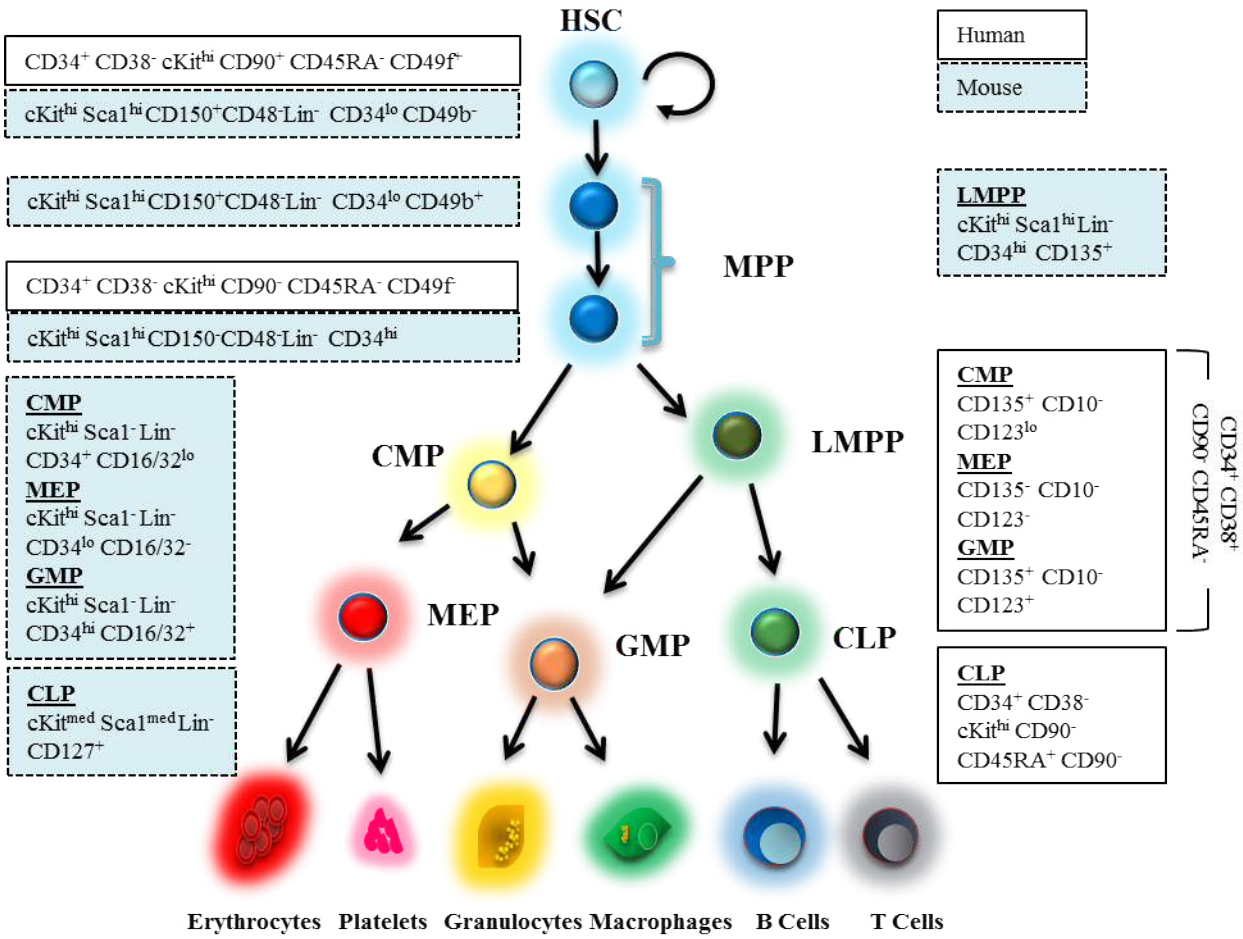

Figure 1. 1 Schematic of the hematopoietic hierarchy.

In this model, self-renewing HSCs give rise to multipotent progenitors. These lack self-renewal potential but differentiate into mature blood cells (Adapted from Rieger and Schroeder 2012).

1.2 Human HSCs

In humans, HSCs and primitive progenitors lack lineage (Lin) markers (Wognum et al. 2003) which are associated with terminally differentiated cell types. Isolation of Lin^- cells can give rise to 20- to 500-fold enrichment of HSCs (Terstappen et al. 1991). However, one of the first recognized markers of primitive human haematopoietic cells used to isolate human HSCs and progenitors was CD34 (Civin et al. 1984). $CD34^+$ cells make up 1-4% of the nucleated cells in human BM samples (Wognum et al. 2003) which possess *in vivo* repopulation potential (i.e. stem cell identity). In addition to HSCs, CD34 is also expressed on mature progenitors such as long-term culture initiating cells (LTC-IC), burst forming units (BFU-E) and colony forming units (CFU-GEMM) (Civin et al 1984).

It was also discovered that the CD38 antigen was expressed in over 90% of $CD34^+$ cells (as well as most sources of HSCs) (Terstappen et a. 1991) and around 60% of LTC-IC (Vogel et al. 2000). However, later studies identified CD38 as marker of differentiated progenitors and not HSCs (Bhatia et al. 1997). On the other hand, $CD34^+CD38^-$ were shown to reconstitute to the multilineage haematopoietic cells after transplantation in immunodeficient mice (Mazurier et al. 2003). The $CD34^+CD38^+$ population of cells are much more abundant and can also cause a transient repopulation of immunodeficient mice. However, this is enhanced in further immunodeficient NOD/SCID/IL-2Ry mice (mice with depleted NK activity) (Verstegen et al. 1998; Hogan et al. 2002) or in mice bypassing the immune system through intrafemoral injection (Mazurier et al. 2003). In 1992, CD90 (Thy1) was identified by Baum et al as a stem cell marker using the Scid-hu model (Baum et al. 1992). This model consists of the transplantation of human fetal thymus or bone grafts into SCID mice hosts. It was shown that $CD34^+Thy^-1^+Lin^-$ cells could engraft and undergo multilineage differentiation at 8 weeks in the Scid-hu bone assay (Murray et al. 1995). In addition to this, Scid-hu thymus assays showed the generation of donor T-cell engraftment in response to both $Thy\text{-}1^+$ and $Thy\text{-}1^-$ subpopulations suggesting that $CD34^+Thy^-1^+Lin^-$ possess long term *in vivo* haematopoietic activity and giving rise to B-, T- and myeloid cells (Murray et al. 1995). Other cell markers that are highly expressed on more lineage-committed cells but not, or weakly, on HSCs include CD33, CD71 CD45RA and HLA-DR (Andrews et al. 1989; Sutherland et al. 1989; Lansdorp et al. 1990; Mayani et

al. 1993) and therefore, since the 1990's human HSCs were classified as $CD34^{+}CD38^{-}Thy1^{+}$ $CD45RA^{-}$.

From the described transplantation experiments, these studies agree that self-renewing $Lin^{-}CD34^{+}CD38^{-}Thy1^{+}$ HSCs reside at the top of the haematopoietic hierarchy. However, the existence of downstream short-term HSCs (ST-HSCs)/multipotent progenitors still remained unclear. These multipotent intermediates were thought to drive the process of differentiation and maturation, each of which possess differing self-renewal capabilities (found by earlier mouse experiments described below) residing between HSCs and committed progenitors. The first suggestion of the existence of such multipotent intermediates was explored in 2003 where human $Lin^{-}CD34^{+}CD38^{lo}$ cells derived from umbilical cord blood were transplanted into NOD-Scid mice (Mazurier et al. 2003). Here, it was discovered that the $Lin^{-}CD34^{+}CD38^{lo}$ population was able to rapidly give rise to myelo-erythroid cells within 2 weeks after intrafemoral bone marrow transplant. They saw similar results when $Lin^{-}CD34^{+}CD38^{+}$ cells were transplanted suggesting that both subsets were able to contribute to rapid reconstitution, this was not the case for the $Lin^{-}CD34^{+}CD38^{-}$ subset. Instead, $Lin^{-}CD34^{+}CD38^{-}$ and $Lin^{-}CD34^{+}CD38^{lo}$ were observed to engraft at 6 weeks whereas the $Lin^{-}CD34^{+}CD38^{+}$ populations did not, suggesting a heterogeneous population with the $Lin^{-}CD34^{+}CD38^{lo}$ subpopulation. This was confirmed by analysing clonal integration sites in transplanted lentiviral-transduced human cells. Sothern blot analysis showed the presence of the DNA of transduced cells in the transplanted femur as well as other bones demonstrating the mobilisation and migration abilities of the $Lin^{-}CD34^{+}CD38^{lo}$ cells. In addition to this, the intensity of the bands correlates to cell number per clone. In this experiment it was found that there was variation in band intensities from different bones. This illustrates the existence of repopulating cells with heterogeneic proliferative capacities (Mazurier et al. 2003). A later study then claimed to have identified a candidate multipotent progenitor characterised as $Lin^{-}CD34^{+}CD38^{-}CD90^{-}CD45RA^{-}$ (Majeti et al. 2007). They found that transplanted $CD90^{+}CD45RA^{-}$ (HSCs) and $CD90^{-}CD45RA^{-}$ gave rise to mature blood cells 12 weeks after transplantation. Of course a larger number of $CD90^{-}CD45RA^{-}$ cells were transplanted to see this effect so when engraftment per 100 transplanted cells was analysed, it was found that

$CD90^{+}CD45RA^{-}$ engrafted cells contributed to a 7-fold greater (human) chimerism than $CD90^{-}$ $CD45RA^{-}$ cells. Although this demonstrates some characteristics of multipotent progenitors, long term chimerism suggests that there may be a small population of long-term repopulating cells within the $CD90^{-}CD45RA^{-}$ compartment. More recently, another marker was identified to further distinguish between HSCs and primitive progenitors. An investigation conducted by the John Dick lab identified CD49f as a positive marker of long-term HSCs (Notta et al. 2011). Building on the CD90 (Thy1) findings, it was discovered that CD49f was expressed on both $Thy1^{+}$ (50%) and $Thy1^{-}$ (25%) cells. In addition to this, both $CD49f^{+}$ fractions were able to contribute to long term engraftment. Conversely, $Thy1^{-}CD49f^{-}$ cells only resulted in multilineage repopulation within 4 weeks, this effect diminished after 16 weeks suggestive of MPP activity (Notta et al. 2011). These findings show the demarcation of human HSCs and MPPs and identify the loss of the critical self-renewal properties which can ultimately be used in therapy.

1.3 Mouse HSCs

Mouse models have proven to be a valuable resource for our understanding of the development of haematopoiesis. Mouse HSCs and progenitor cells can be distinguished by cell surface markers (Spangrude et al. 1988). Before deciphering the identity of murine HSCs, a large number of studies were undertaken between the late 1980's throughout until the early 2000's, to identify mature blood cells utilising monoclonal antibodies (Ab). From these experiments, a number of cell surface markers were identified and used to characterise differentiated lineages. For example it was found that the Ter-119 antibody targets glycophorin A-associated molecules and was used to specifically identify late erythroid cells (Kina et al. 2000). Prior to this, monoclonal Abs were also used to characterise B-cells, T-cells (including their subsets) as well as granulocytic lineages (Coffman and Weissman 1981). An interesting discovery arose when B lineage engrafting cells, which give rise to myeloid and B clones, were found to lack the expression of B220, a B lineage marker (Muller-Sieburg et al. 1986). Since early B cell lineages do not express B-cell markers, this finding led to the belief that earlier progenitors would also lack the expression of committed blood lineage markers. Indeed this was the

case as 7-10 lineage Abs were combined to form the lineage (Lin) combination to negatively select populations of bone marrow (reviewed by (Weissman and Shizuru 2008)). It was also proven that Lin^- cells consisted of lineage progenitors and reconstituting cells (Muller-Sieburg et al. 1986). These progenitors and reconstituting cells, like humans, expressed the murine Thy1 marker (Muller-Sieburg et al. 1989). Additional Abs were explored to further enrich Thy1 expressing HSCs. From a library of monoclonal Abs produced against $Thy1^+$ cells of the marrow (Aihara et al. 1986), Spangrude et al identified Sca1 as a potential marker of stem cells as they were able to show that $Thy1^{lo}$ Lin^- $Sca1^+$ cells were able to rescue lethally irradiated mice and contribute to the generation of myelomonocytic cells as well as B- and T- cells (Spangrude et al. 1988). However it was later shown by Morrison et al that the $Thy1^{lo}$ Lin^- $Sca1^+$ populations could be subdivided into three compartments consisting of long-term HSCs (with self-renewal capacities), short-term HSCs (which have a limited self-renewal capacity but still contribute to the reconstitution of the haematopoietic system) and MPPs (Morrison and Weissman 1994). In parallel to this finding, the expression of CD117 (cKit) on $Thy1^{lo}$ Lin^- $Sca1^+$ was discovered (Ikuta and Weissman 1992). A few years later the mouse homologue of CD34 was investigated and found that in contrast to humans, mouse HSCs did not express CD34 (Osawa et al. 1996). Here it was demonstrated that a single $CD34^-$ $Lin^-Sca1^+cKit^-$ cell could give rise to the long-term reconstitution of the haematopoietic system. Termed as LSK, $Thy1^{lo}$ $CD34^-$ Lin^- $Sca1^+cKit^+$ cells were scrutinised as only 10% of LSK cells were 'true' long-term HSC (Challen et al. 2009). A study in 2001 identified a cytokine tyrosine receptor, flt3, as a regulator of HSCs (Adolfsson et al. 2001). Expression of both ckit and flt3 are observed during the early stages of haematopoiesis (Lyman and Jacobsen 1998) however flt3 is only expressed on 60% of LSK cells (Adolfsson et al. 2001). In addition to this, it was shown that single cell clones of LSK $flt3^+$ and LSK $flt3^-$ cells demonstrate proliferative capabilities and differentiation potentials (give rise to myeloid and lymphoid cells) but it is only the LSK $flt3^-$ cells that maintained life-long reconstitution (Adolfsson et al. 2001). In a later attempt to enrich a more purified subset of HSCs, the signalling lymphocytic activation molecule (SLAM) family receptors were studied (Kiel et al. 2005). The SLAM family of receptors have multiple roles in varying immune cell types (Veillette 2010). Mutations in SLAM are also found in human immunodeficiency and X-linked lymphoproliferative disease (Engel et al. 2003). It was found

that the SLAM receptors CD150, CD244 and CD48 were expressed throughout HSC and functionally differing progenitor populations (Kiel et al. 2005). They characterised the most purified subset of HSCs as $CD150^{+}$ $CD244^{-}CD48^{-}$ and defined MPPs as $CD150^{-}CD244^{+}CD48^{-}$ (Kiel et al. 2005). These findings were confirmed more recently when $CD34^{-}$ $CD150^{+}$ LSK were shown to reconstitute the haematopoietic system in secondary recipient mice (Morita et al. 2010). Extensive analysis by Oguro et al led to the expansion of the SLAM family markers which subdivided murine LSK cells into seven fractions, two within the HSC (HSC-1 and HSC-2), three in the MPP (MPP-1, MPP-2 and MPP-3) and 2 that were characterised as restricted haematopoietic progenitors (HPC-1 and HPC-2) (Oguro et al. 2013). In addition to HSC and MPP immunophenotypical characterisation, HSC1 and HSC2 were characterised as LSK $CD150^{-}CD48^{+}$ and LSK $CD150^{-}CD48^{-}$ respectively (Oguro et al. 2013). Furthermore, these fractions within LSK were found to be functionally distinct. For example, 90% of HSCs and MPPs resided in G0 of the cell cycle where as HPC-1 and HPC-2 cells were actively cycling (Oguro et al. 2013). In addition to this, transplantation of these fractions gave rise to varying reconstitution capabilities demonstrating their functional differences. In this study they showed that transplanted HSC-1 cells gave rise to all downstream populations (MPPs, HPC-1 HPC-2, CMP, MEP, GMP and CLP) including HSC-2, whereas HSC-2 transplanted cells only gave rise to HSC-2 and downstream populations (Oguro et al. 2013). Serial transplantation of HSC-1alsodemonstrated greater self-renewal potential compared to HSC-2 cells. Similarly to HSC-2, MPP-1 cells have very little reconstituting and self-renewing potential although a small number of MPP-1 cells are able to contribute to long-term multilineage reconstitution (Oguro et al. 2013). MPP-2 and MPP-3 sub-populations were characterised as transiently reconstituting multipotent progenitors as MPP-2 cells gave rise to predominantly erythrocytes and platelets, on the other hand, MPP-3 reconstituted multiple lineages though both were short-term (Oguro et al. 2013). Upon transplantation of the HPC population, it was shown that HPC-1 is a heterogeneous population that favours lymphoid reconstitution whereas HPC-2 transiently reconstitutederythrocytes and platelets.

The above studies have demonstrated the complexity of the haematopoietic hierarchy and it is only a small subset within the HSCs that are responsible for sustaining life-long haematopoiesis. Using a

similar approach, lineage specific progenitor populations have been identified. For example, it was reported that a subset of cells characterised by Lin^- $Thy1^-$ $Sca1^+$ $cKit^-$ $IL7\text{-}R^+$ would give rise to lymphoid cells (B-, T- and NK cells) (Kondo et al. 1997). These IL-7R expressing cells lack the potential to give rise to cells of the erythromyeloid lineages and therefore are known as common lymphoid progenitors (CLP) (Karsunky et al. 2008). On the other hand, cells that did give rise to erythromyeloid cells lacked the expression of IL-7R. Instead, the expression profiles of CD34 and the Fcγ receptor-II/III (CD16/32) were used to isolate cells that gave rise to megakaryocytes, erythrocytes, granulocytes and macrophages (Akashi et al. 2000) and thus termed common myeloid progenitors (CMP). It was found that these Lin^- $Thy1^-$ $Sca1^+$ $cKit^-$ $IL7\text{-}R^-$ cells can be divided into three subsets: $FcγRloCD34^+$, $FcγRloCD34^-$, and FcγR $hiCD34^+$. To distinctly differentiate between each subset, CFU assays were conducted. It was shown that cells $FcγRloCD34^+$ formed a wide range of colonies, similar to that of HSC colonies. FcγR $hiCD34^+$ cells only gave rise to colonies rich in macrophages and granulocytes (CFU-M, CFU-G and CFU-GM) and so were called granulocyte/macrophage lineage restricted progenitors (GMP). $FcγRloCD34^-$ cells were given the name megakaryocyte/erythrocyte lineage-restricted progenitors (MEP) as they gave rise to colonies consisting of megakaryocytes and erythroid cells (BFU-E and CFU-Meg) (Akashi et al. 2000). Each of these subtypes was also assayed *in vivo* and the resulting reconstitution mimicked what was observed *in vitro*. Transplantation of CMPs resulted in the detection of donor derived myeloid cells ($Mac1^+Gr1^+$) cells and erythroid ($Ter119^+$) cells in the spleen and bone marrow (Akashi et al. 2000). As expected, transplantation of GMPs only gave rise to $Mac1^+Gr1^+$ cells; conversely transplanted MEPs gave rise exclusively to $Ter119^+$ cells (Akashi et al. 2000). All progenitor subtypes diminished after 4 weeks demonstrating the lack of self-renewal properties. This study suggests that the CLP and CMP are the earliest branching of lineage fated progenitors (Figure 1.2), however more recent studies have sought to challenge this theory (discussed below).

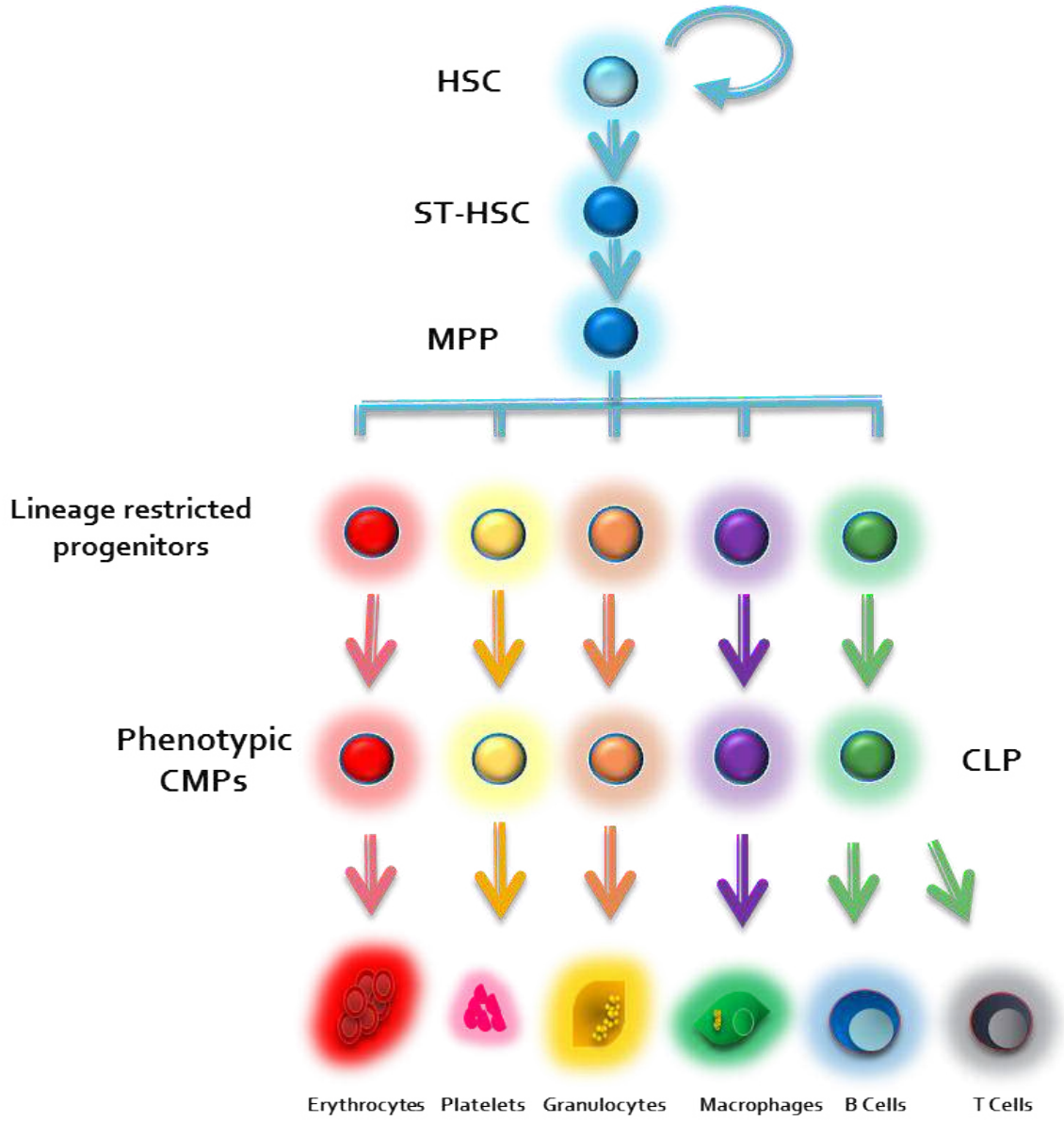

Figure 1. 2 Schematic of the revised haematopoietic hierarchy.

Here, the common myeloid progenitors (CMPs) are a very heterogenous population that have been shown to give rise to either myeloid cells or erythrocytes after transplantation providing evidence of early parallel lineage branching (adapted from Mercier and Scadden 2015).

1.4 A refined model of haematopoiesis

As described above, the classic model describes haematopoiesis as a linear process whereby the HSC sits at the peak of a hierarchical tree losing its self-renewing capacity as it descends through the differentiation stages. The various differentiated states within the hierarchy have been identified through advances in immunophenotypic analysis and transplant in order to assess their differentiation potential (Chao et al. 2008). From this, it is known that these states are defined by progenitors and mature cell types bound within specific differentiation pathways. This model proposes that terminally mature cell types can be traced back through the hierarchy in a theoretical progression of progenitors originating from a HSC. Historically, the proof-of-principle assay to demonstrate these properties of HSCs is through bone marrow transplant where they are able to give rise to the long term reconstitution of the haematopoietic system. However, very recently it has been argued that HSCs may not be the sole contributors to the production of blood under homeostasis. It was hypothesised that this may be due to the differences in haematopoiesis within the physiological environments of normal and irradiated recipients (Sun et al. 2014). In this study, they utilised in situ labelling and tracking of HSCs which would help determine cell origins and lineage development. Here a hyper active transposase, Sleeping Beauty (HSB), was used to mediate the mobilisation of Tn (marked by DsRed) which is a DNA transposon (Mates et al. 2009). The expression of HSB was under the control of M2, a Doxycyclin (Dox)-dependent transcriptional activator, were inserted into mice carrying these three alleles (M2/HSB/Tn mice). Administration of Dox results in the expression of HSB and thus the mobilisation of Tn. The random integration of Tn meant that any cell which transposition occurs, will possess a unique insertion site. This insertion site, with the cessation of Dox exposure, will act as a 'genetic tag' within a cell and thus its progeny (Sun et al. 2014). Using this model, they investigated the ability of long term HSC to contribute to the haematopoietic system. The first approach they took was comparing the clonal repertoire of bone marrow granulocytes of M2/HSB/Tn mice and granulocytes after transplantation. It was discovered that less than 10% of donor-derived granulocyte tags were detected in recipient granulocytes. Moreover, 73 weeks after transplant, two clones were detected in granulocytes within the bone marrow, however these did not arise from long term HSCs

(Sun et al. 2014). This suggests that granulopoiesis in situ is driven by progenitors within the first 12 months of transplant. Looking at the steady state bone marrow, it was shown that half of the clones found in myeloid- and multipotent progenitors were detected in differentiated populations (Sun et al. 2014). In contrast, it was observed that only a small percentage (5%) of long-term HSC tags were detected in the differentiated populations. In addition to this observation, it was also surprising to find that less than 5% of long term HSCs tags were common with MPPs. Therefore, it was hypothesised that the progenitors were responsible for myelopoiesis and lymphopoiesis whereas long-term HSCs are limited in their output under homeostatic conditions.

Another study reports that short term HSCs are actually responsible for sustaining haematopoiesis (Busch et al. 2015). Similarly to the above investigation, they developed a way of genetically labelling HSCs using yellow fluorescent protein (YFP). Here, the Tie2 locus was targeted, as it is expressed throughout adult HSCs (Yano et al. 1997), and used as a driver for Cre recombinase ($Tie2^{MCM/+)}$. These mice were then crossed with YFP expressing mice ($Rosa^{YFP}$) which would generate $Tie2^{MCM/+}$ $Rosa^{YFP}$ mice (Busch et al. 2015). As a result of tamoxifen treatment, cells expressing Cre will be detected as YFP-positive as well as their non-Cre expressing progeny. This was demonstrated when adult $Tie2^{MCM/+}$ $Rosa^{YFP}$ mice treated with tamoxifen resulted in YFP^{+} HSCs and transplantation of single YFP^{+} HSCs would lead to long term engraftment and reconstitution (Busch et al. 2015). An interesting observation was noted in this study when no label was detected in short term HSCs and MPP; instead, HSCs had retained the label up to 3 weeks after tamoxifen treatment. It was only after 4 weeks when the first labelled cell emerged and after 16 weeks labelling was detected in all progenitors and differentiated cells. When compared to HSCs during development (E12.5), it was shown that only HSCs were labelled in the foetal liver at this stage. However, it was described that 1 week after birth, the labelling of the peripheral system equalled that of HSCs. This demonstrates that quiescent HSCs are responsible for maintaining the haematopoietic system but it is the ST-HSCs that give rise to rapid haematopoiesis during development. On the contrary, under perturbed conditions (transplantation), it was found that the level of labelling of donor HSCs was inconsistent with their labelling prior to transplant. For example, it was discovered in one mouse that YFP^{+} HSCs represent

0.3% of HSCs prior to transplant but 17% after transplant suggesting a higher rate of proliferation. Also, it was seen that donor HSC labelling had been lost in 14 out of 32 recipients suggesting a heterogeneous repopulating ability of individual HSCs (Busch et al. 2015). The most striking result observed arose from quantification of haematopoietic flux. Flux was defined as the differentiation and progression of a compartment into the downstream progeny whilst being replaced by influx from the above compartment (e.g. ST-HSCs moving towards MPPs while being replaced by either self-renewal or LT-HSC maturation). Using this model they showed that self-renewal capacities can be calculated by the amount of time spent in one compartment at one given time. As expected, the HSC compartment demonstrated constant labelling regardless of efflux consistent with complete self-renewal capacities. More interestingly, ST-HSCs proliferation contributed exclusively to the efflux into MPPs however it was observed that influx from the HSC compartment was marginal suggesting that self-renewal maintained their maintenance. In addition to this, these ST-HSCs also demonstrated a considerably prolonged life within its compartment and further labelling studies showed that SH-HSC were also able to retain labelling throughout the duration of the mouse life (Busch et al. 2015). Although it was agreed that that HSCs give rise to short term HSCs and in turn give rise to MPPs, it was postulated that since HSCs emanated such a low flux, it is in fact the short term HSC that is responsible, primarily, for maintaining haematopoiesis (Busch et al. 2015). These studies support the theory that under homestatic conditions, ST-HSC (and even MPPs) is responsible for haematopoiesis. However when stressed, for example in transplant or chemical injury, both LT- and ST-HSCs are responsible for the recovery of the haematopoietic system. These challenge the prevailing dogma of LT-HSCs and the model where they are solely responsible for the long-term regeneration of the haematopoietic system.

1.5 Regulation of haematopoiesis

1.5.1 Cell cycle

As discussed, HSCs are characterised by their abilities to differentiate, self-renew, undergo apoptosis or remain in quiescence. These are hallmarks of stem cells which are regulated by intrinsic and extrinsic factors. It is understood that a complex network of regulatory factors exist that determine the quiescent-differentiation balance of HSCs. One of these is cell cycle regulatory factors. These are largely controlled by cyclin-dependent kinases (Cdk), which drive the cell through the cell cycle, and Cdk inhibitors (CKI) which conversely prevent progression of the cell cycle (Morgan 1997). One family of such inhibitors are the CIP/KIP family which include $p21^{Cip}$, $p27^{Kip}$ and $p57^{Kip2}$ (CKIs). These prevent entry into the S phase by inhibiting the cyclin E–Cdk2 complex activity. The role of p21 was investigated in mice constitutively lacking p21 ($p21^{-/-}$). It was shown that $p21^{-/-}$ mice did indeed develop normally however in response to irradiation, it was not possible for the cells to arrest the cell cycle (Deng et al. 1995). This suggested that p21 may have a role in maintaining quiescence. This was tested in the HSC setting by Cheng et al. In their study, they found that $p21^{-/-}$ mice exhibited an increase in HSC numbers compared to $p21^{+/+}$ mice suggesting a lack of cell cycle inhibition (Cheng et al. 2000b). They also showed under stress conditions, survival of $p21^{-/-}$ mice was dramatically hindered due to increased cell cycling and thus leading to the exhaustion of stem cells (Cheng et al. 2000b). A similar investigation was conducted, however they were unable to reproduce the results observed above (van Os et al. 2007). No difference in HSC numbers were observed compared to wild type mice, this was also the case in competitive repopulating ability in serial transplant assays. They did however see a reduced competitive repopulating ability of irradiated HSCs in $p21^{-/-}$ mice. It must be mentioned that this investigation was conducted on a different strain of mice (C57BL6) compared to the previous study signifying the differences of gene modifications and functions in different strains give rise to different outcomes. Importantly, this study suggests that p21 may not be essential in regulating HSC cell cycle under homeostatic conditions but instead under stress. More recently, further rigorous assessment in the 129sv strain (used in Cheng et al 200b) demonstrated that indeed a

reduced competitive repopulating capability was observed in $p21^{-/-}$ mice (Rodrigues et al, unpublished observation).

In addition to p21, investigations have been undertaken to study the roles of p27 and p57. By knocking out p27, it was found that $p27^{-/-}$ animals showed no changes in HSC numbers, self-renewal or cell cycling (Cheng et al. 2000a). Instead, committed progenitor cells exhibited a larger pool size marked by increased CFC numbers. It was also shown that an increased proportion of progenitors in $p27^{-/-}$ mice were actively progressing through the cell cycle. In the transplantation setting, progenitors from $p27^{-/-}$ mice demonstrated expansion and reconstitution capabilities in contrast to wild type animals in which this effect was diminished (Cheng et al. 2000a). This demonstrates the widespread expression and roles of cell cycle regulators which act in specific stem/progenitor compartments. More recently, an investigation studying the role of p57 showed its essential function in maintaining HSC quiescence (Matsumoto et al. 2011). HSCs that lacked p57 demonstrated perturbed self-renewal capabilities as a few cells remained in the G_0 phase of the cell cycle shown by Hoechst 33342 and pyronin y staining. Again, HSCs lacking p57 failed to contribute to long term reconstitution of the haematopoietic system in primary and secondary competitive transplants (Matsumoto et al. 2011). An interesting observation was discovered when p21 was deleted in HSCs lacking p57. Ablation of p57 did not cause a change in colony formation; however combined deletion did elicit a decrease in colony number. In addition to this it was found that there were no differences between HSCs in the G_0 phase within p57 and p57/p21 double knockout suggesting that deletion of the CKIs may not be directly affecting HSC cell cycles but also progenitors (Matsumoto et al. 2011). Further investigation revealed that p27 knock-in was able to recover the phenotypes observed in cells lacking p57 (Matsumoto et al. 2011; Zou et al. 2011). This demonstrates the intricate pathways involved in the regulation of maintaining quiescence in HSCs ensuring their long-term survival for life-long reconstitution of the haematopoietic system.

1.5.2 Transcriptional Regulation

HSCs and haematopoiesis are regulated by a vast complex network of transcriptional factors that regulate gene expression within cells. Initial studies looked at expression of particular genes during

the development of HSCs which would further translate into adult HSCs. One of the earliest studies looked at a basic-helix-loop-helix transcription factor known as SCL/tal1. It was discovered that deleting SCL/tal1 in embryonic stem cells, prevented the differentiation and maturation of erythromyeloid and lymphoid cells (Porcher et al. 1996). Moreover, SCL/tal1 acts upstream of the transcription factor GATA1 (Tripic et al. 2009). During haematopoiesis, HSCs are able to make a choice to either progress down a myeloid or lymphoid lineage differentiation pathway. The decision that the HSC makes on whether it differentiates into a myeloid progenitor or a lymphoid progenitor is tightly regulated by gene expression programmes under the control of lineage-determining transcription factors. One of these factors, GATA1, was initially identified as a regulator of lineage determining genes (Zon et al. 1991) and later shown to be expressed in eosinophils, erythroid cells and megakaryocytes (Martin et al. 1990; Zon et al. 1993; Kulessa et al. 1995; Fujiwara et al. 1996). Erythromyeloid determination of GATA1 was investigated in embryonic stem cells deficient in GATA1, these cells were able to give rise to all cell types in chimeric mice apart from differentiated erythroid cells (Pevny et al. 1991). This was confirmed by *in vitro* analysis of GATA1 deficient embryonic stem cells where erythropoiesis ceased at primitive and late stages (Weiss et al. 1994). *In vivo*, it was found that GATA1 null mice have perturbed red blood cell development and die from anaemia between E10.5 and E11.5 (Fujiwara et al. 1996). Another member of the GATA family heavily involved in haematopoiesis is GATA2. The role of GATA2 was identified as a major regulator of HSC development (Tsai et al. 1994). This was determined when it was observed that GATA2 knockout mice died at E9.5-E10.5 due to deficient haematopoiesis (Ezoe et al. 2002). GATA2 knockout in multipotent progenitors underwent apoptosis however those which did survive proliferated very poorly (Tsai and Orkin 1997). Further *in vitro* studies have also shown the reduced ability of $GATA2^{+/-}$ progenitors to form CFCs (Rodrigues et al. 2005). In the same study, it was shown that $GATA2^{+/-}$ animals possessed a lower number of $CD34^{-}$ LSK cells. $GATA2^{+/-}$ mice also demonstrated decreased stem cell activity measured by the reduced number of mature blood cells ($Mac1^{+}$, $B220^{+}$ and $CD3^{+}$) within the peripheral blood of competitively transplanted animals (Rodrigues et al. 2005).

A large number of transcriptional factors, in addition to the above, have been characterised as major regulators of haematopoiesis, particularly during the primitive (or developmental) stages of haematopoiesis. Genes such as Pu.1, Cebp-α, Runx1 and ikaros have been implicated not only in the development of HSCs but also in lineage specification. Pu.1 is a transcription factor that is expressed in myeloid and B cells in which deletion of Pu.1 results in the failure of the differentiation and maturation of these cell types (DeKoter et al. 1998; Glimcher and Singh 1999). Interestingly it was shown that Pu.1 was able to direct the differentiation of MEPs into myeloblasts by antagonising GATA1 (Nerlov and Graf 1998). Cebp-α is also a transcription factor and has a role in granulopoiesis (Radomska et al. 1998) and, like Pu.1, is also expressed in myeloid cells (Iwama et al. 1998). A study using non-conditionally knockout Cebp-α showed a block in granulocyte differentiation (from CMP to GMP) in fetal liver. This led to the accumulation of immature myeloid cells in the adult bone marrow, mimicking the effects of human AML (Zhang et al. 1997). Moreover, the role of Cebp-α has also been implicated in self-renewal. It was shown that HSCs lacking Cebp-α exhibited increased levels of the polycomb gene Bmi-1 (Zhang et al. 2004), which has been identified as a promoter of HSC self-renewal (Iwama et al. 2004). When Cebp-$\alpha^{-/-}$ HSCs were transplanted competitively with Cebp-$\alpha^{+/+}$ HSCs, it was discovered that Cebp-$\alpha^{-/-}$ HSCs out-competed Cebp-$\alpha^{+/+}$ HSCs by giving rise to a higher level of chimerism and reconstituting the haematopoietic system in secondary transplants (Zhang et al. 2004). A major regulator of HSCs and haematopoiesis in development is Runx1 (North et al. 1999). Runx1 is associated with, and required for, the emergence of HSCs (Cai et al. 2000) Deletion of Runx1 resulted in embryo lethality due to a block in haematopoiesis (Wang et al. 1996; Ichikawa et al. 2004). During adulthood, it was observed that Runx1 expression was maintained in HSCs as well as myeloid and lymphoid cells although this was not the case in late erythroid cells (North et al. 2004). Deletion of Runx1 in adult HSCs did not hinder HSC number (in fact an increase in HSCs was observed) however the lack of expression of Runx1 did decrease the repopulating abilities in competitive transplantation suggesting that Runx1 is not essential for adulthood haematopoiesis (Growney et al. 2005). The effects of excising Runx1 were also seen in CLPs where the inhibition of B- and T-cell lineages occurred (Growney et al. 2005). On the other hand, an expansion of GMPs was observed in these mice. Moreover, in the same animals, marrows exhibited

hypercellularity due to an expansion of $Mac1^{+}$ cells suggestive of a myeloproliferative dysfunction (Growney et al. 2005). This demonstrates the differing roles of such transcriptional regulators between haematopoiesis in development and adulthood.

1.5.3 Cytokines regulate haematopoiesis

As well as cell intrinsic regulators, extrinsic factors also regulate HSC activity. For example, the chemokine CXCL12/SDF-1 and its receptor CXCR4 have been shown to have an important role during development as mutant mice died at E18.5 (Nagasawa et al. 1998). Furthermore, it was shown that SDF-1 mutant embryos were deficient in B-cell progenitors within fetal liver and bone marrow whereas myeloid progenitors were decreased only in the bone marrow (Nagasawa et al. 1996). Not only is SDF-1 and CXCR4 required for primitive lympho-myelopoiesis, the SDF-1-CXCR4 pathway has also been reported to be important in the maintenance of HSC quiescence. Deletion of CXCR4 in LSK cells using a tamoxifen system resulted in an expansion of these cells due to increased rates of proliferation detected by BrDU labelling (Nie et al. 2008). Moreover, RNA and DNA analysis revealed that wild type HSCs *in vitro* exposed to SDF-1 remained in the G0 phase of the cell cycle whereas $CXCR4^{-/-}$ HSCs were unaffected by SDF-1 (Nie et al. 2008). Interestingly, it was reported that cyclin D1 expression was significantly higher in $CXCR4^{-/-}$ HSCs (Nie et al. 2008). As mentioned above, the CKI p57 is required to maintain G0 status of HSCs, it was discovered that $CXCR4^{-/-}$ HSCs expressed lower p57 than normal HSCs (Nie et al. 2008). The cytokine Thrombopoietin (TPO) has also been extensively studied in the haematopoietic setting; more specifically it has been characterised as a regulator of megakaryocyte and platelet differentiation (Broudy et al. 1995). It was also suggested that TPO induced HSC proliferation in combination with SCF and/or IL-3 (Sitnicka et al. 1996). TPO was also shown to prolong HSC viability *in vitro* (Borge et al. 1996). The effects of TPO on HSCs may be explained by the high expression of its receptor, mpl, on HSCs in contrast to the low detection levels of mpl on short term HSCs and MPPs (Buza-Vidas et al. 2006). Deletion of mpl resulted in a decrease in progenitor numbers (Kimura et al. 1998). In addition to this, it was shown that transplanted bone marrow cells from $mpl^{-/-}$ mice lost the ability to compete against wild type cells for long term reconstitution (Kimura et al. 1998). Self-renewal capabilities of stem cells were also

perturbed when $mpl^{-/-}$ transplanted cells failed to repopulate in secondary recipients (Kimura et al. 1998). A more recent study looked at the effects of the loss of TPO, it was shown that there was a significant decrease in stem cell numbers (Qian et al. 2007). They also showed that $TPO^{-/-}$ cells also failed to contribute to the reconstitution of the haematopoietic system after competitive transplant. They further investigated the underlying cell cycle mechanisms of $TPO^{-/-}$ HSCs. Using quantitative real-time PCR, the downregulation of p57 and p19 were observed (Qian et al. 2007). In addition to this, a reduction in the expression of the Hox genes Hoxb4, Hoxa5 and Hoxa9 was observed, all of which are involved in supporting HSC self-renewal (Antonchuk et al. 2002; Lawrence et al. 2005). BrDU staining also proved a higher rate of cell division as well as Ki67/Hoechst analysis showing a larger proportion of $TPO^{-/-}$ HSCs in G1 and SG2M phases of the cell cycle (Qian et al. 2007).

An alternative extrinsic factor important in the regulation of HSC quiescence is Stem Cell Factor, SCF (also known as kit), which is a tyrosine kinase receptor (Williams et al. 1990). The role of kit in the maintenance of HSCs was explored by Thoren et al, they found that a partial loss of kit led to a decrease in long-term HSC frequency (Thoren et al. 2008). Studying the cycle kinetics of HSCs of these mice, it was observed that long term HSCs exhibited increased BrDU uptake while residing in the G1 and SG2M phases of the cell cycle observed by Ki67 and DAPI staining (Thoren et al. 2008). These studies show the importance of such extrinsic factors in maintaining stem cell quiescence, highlighting the intricate pathways that relay extracellular signals through to intracellular transcriptional regulators.

1.6 The Stem Cell Niche

In the adult, HSCs reside in the BM which serves as a complex microenvironment comprising of a number of cell types and extracellular components. Structures and molecular components within the niche regulate the maintenance of HSC by controlling their self-renewal, differentiation and egression (Fuchs et al. 2004; Morrison and Spradling 2008). The BM, according to current hypotheses, can be divided into two categories of niche: the osteoblastic niche (Calvi et al. 2003) and the BM vascular niche (Kiel et al. 2005). Within the endosteal niche, HSCs are positioned in close proximity to

osteoblasts (OBs) of trabecular bone (Frassoni et al. 1982). However, HSCs in the vascular niche reside nearer to the sinusoid endothelium (Zhang et al. 2003).

1.6.1 The endosteal niche

It is hypothesised that HSC quiescence is maintained by the hypoxic environment of the endosteal niche (Parmar et al. 2007; Winkler et al. 2010; Suda et al. 2011). Conversely, HSCs residing within the vascular niche are under oxygenated conditions that encourage proliferation and differentiation. This was demonstrated in E-selectin knockout mice where the inhibition of E-selectin (expressed exclusively in vascular endothelial cells within the bone marrow) increased HSC quiescence and self-renewal suggesting the importance of the vascular niche in HSC proliferation and differentiation (Winkler et al. 2012). Although hypoxic, the endosteal environment is highly vascularised by arterioles (Draenert and Draenert 1980). A study conducted in 2011 utilised 3D bone marrow imaging and $Nestin^{+}$ MSCs with differential GFP expression levels revealed the spatial difference between arterial ($NesGFP^{bright}$) and sinusoidal ($NesGFP^{dim}$) environments (Kunisaki et al. 2013). In addition to this, it was also shown that quiescent HSCs were associated with $NesGFP^{bright}$ arterioles but more interestingly proliferative HSCs migrated away from these arterioles (Kunisaki et al. 2013).

Moreover, it is also important to acknowledge that the metabolic status of HSCs is considerably different to that of its more differentiated downstream progeny, thus allowing them to survive such hypoxic conditions. HSCs survival and quiescence under hypoxia is owed to the high expression of the transcription factor hypoxia-inducible factor 1α (HIF-1α). In fact long-term HSCs highly express HIF-1α mRNA and protein, and is essential for the survival and resistance of HSCs to hypoxia (Simsek et al. 2010; Takubo et al. 2010). This was demonstrated in HSCs of HIF-1α knockout mice that lacked the ability to reconstitute the haematopoietic system after bone marrow transplant (Takubo et al. 2010). In this study, they also showed that HIF-1α knockout HSCs underwent higher rates of proliferation as they exited the G0 phase of the cell cycle. More recently however, it was shown that HIF-1α was in fact not required for the maintenance and survival of HSCs under hypoxic conditions (Vukovic et al. 2016). Here, it was shown that deletion of HIF-1α after bone marrow transplant did not affect chimerism and reconstitution of haematopoietic lineages (Vukovic et al. 2016). In addition

to this they also demonstrated that deletion of HIF-1α did not affect self-renewal of HSCs when serially transplanted into secondary recipients suggesting that HIF-1α is not essential for HSC quiescence within the hypoxic bone marrow niche. The varying results between the studies can be explained by differences in experimental setups. For example, the LSK Hif-1α mediated deletion induced by pIpC, may also induce its deletion from the surrounding BM mesenchymal progenitors as it is recognised that Mx1-Cre recombines with the niche cells of the bone marrow (Walkley et al. 2007b). It has also been shown that Hif-1α is essential for the normal function of bone marrow niche cells (Guarnerio et al. 2014) which may explain the subsequent perturbed HSC function observed in the Takubo et al investigation (Takubo et al. 2010). These studies demonstrate the intricate pathways that exist between HSCs and the niche and that disruption of these pathways result in the exacerbation of HSC function.

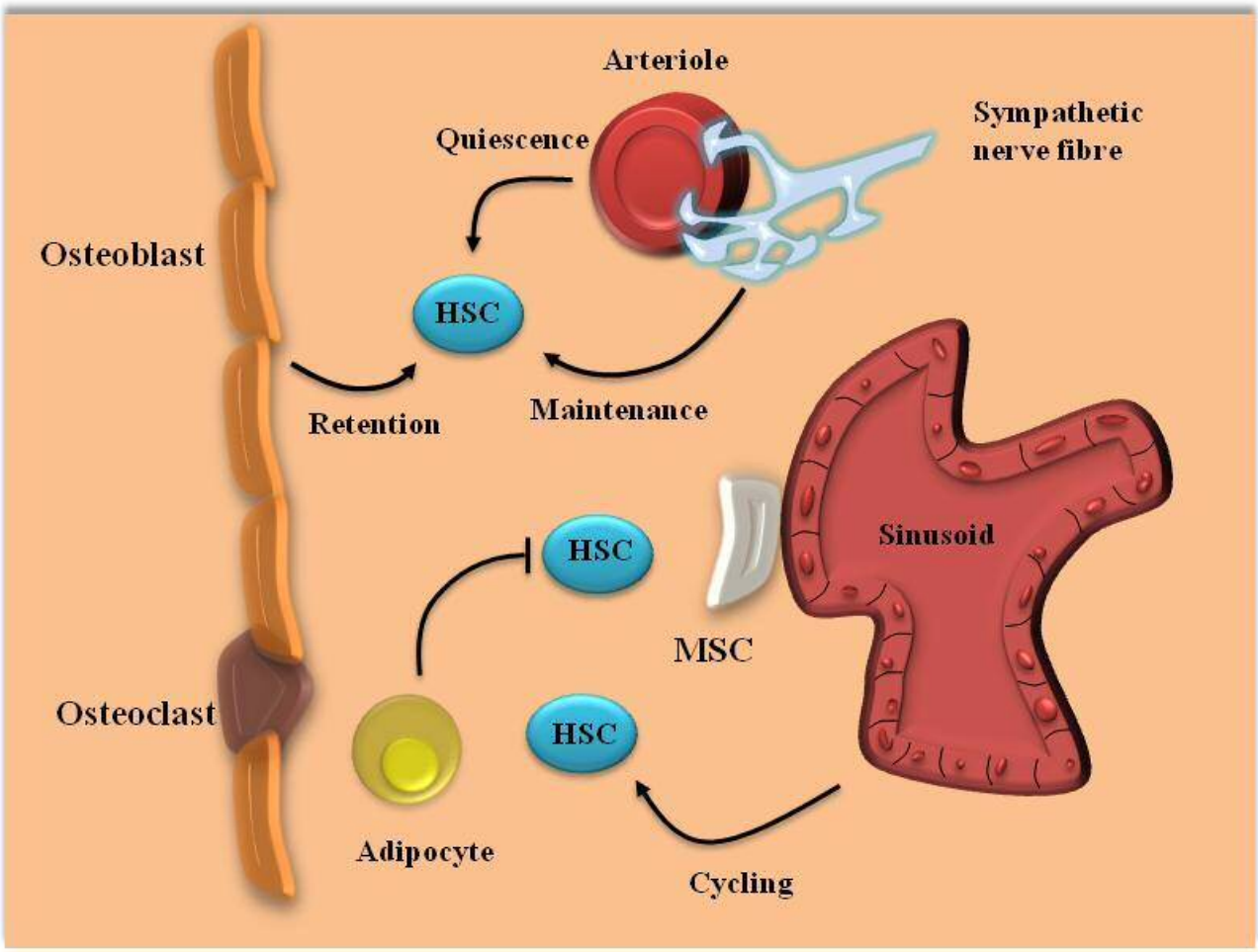

Figure 1. 3 Niche cells regulate HSC function

HSC quiescence is known to be maintained by factors such as CXCL12 and SCF which are secreted by structures such as perivascular cells surrounding arterioles and sympathetic neuronal cells. HSCs that are less dormant tend to reside within the vascular (sinusoidal) niche which promotes self-renewal, proliferation and differentiation. Osteoblasts also regulate HSCs promoting their survival (adapted from Mendelson and Frenette 2014)

1.6.2 Osteoblasts within the endosteal niche regulate HSCs

The endosteal niche is also known as the osteoblastic niche and it is here where the role of osteoblasts (OBs) was shown to be of significance on HSC function (Taichman and Emerson 1998; Levesque et al. 2010). The first experiments to demonstrate this were conducted in the early 2000's. When the BMP receptor BMPR1A (*BMPR1a* gene which is expressed by osteoblasts) was conditionally inactivated in adult mice, there was a significant increase in the number of HSCs which correlated with the increase of N-cadherin^{+} OB numbers (Zhang et al. 2003). In parallel to this study, Calvi et al studied the effects of parathyroid hormone 1 receptor (PTHR1) in osteoblasts (Calvi et al. 2003). Here, they showed that constitutive activation of PTHR1 in osteoblasts resulted in increased numbers of HSCs. In addition to this, to support the notion of osteoblastic effects on HSCs, they demonstrated that administration of PTH (ligand to PTHR1) also resulted in an increase in numbers of HSCs within the bone marrow (Calvi et al. 2003).

Signalling pathways between OBs and HSCs have also proven to be a critical component to the maintenance of HSCs within the niche. OBs produce the chemokine stroma-derived factor 1 (SDF1; also known as CXCL2) which attract both human and mouse HSC (Peled et al. 1999). SDF1 also has an important role in survival, cell cycle and retention of HSCs (Lapid et al. 2008). In addition to SDF1, other ligand-receptor interactions and signalling molecules have been shown to have important roles in maintaining the regulation of HSCs by OBs. Ligand-receptor pairs such as thrombopoietin (TPO) and myeloproliferative leukaemia virus oncogene (MPL), Stem cell factor (SCF) and its receptor KIT, and angiopoietin1 and TIE2 tyrosine kinase not only have roles in maintaining HSC function, but also induce the mobilisation of HSCs from BM into the peripheral blood (Arai et al. 2004; Nakamura et al. 2004; Yoshihara et al. 2007). This is a significant aspect in HSC transplantation therapies where BM cells are forced to exit the BM environment and mobilise to the peripheral blood (discussed below). It was also shown that signalling molecules secreted by OBs, such as bone morphogenetic protein (BMP), have regulatory functions on HSCs. BMP has an important role during development. It is also essential for the induction of haematopoietic tissue

(Maeno et al. 1996; Davidson and Zon 2000). These results highlight the importance of OBs to the maintenance and survival of HSCs within the BM.

1.6.3 The vascular niche

In addition to the osteoblastic niche, the vascular niche also plays an important role in supporting the proliferation, differentiation and migration of HSCs. Endothelial cells of the vascular niches originate from the same progenitor cells (hemangioblasts) as HSCs during embryogenesis (Lacaud et al. 2001) and have similar ontogeny of haematopoiesis in a number of foetal organs as well as adult BM (Li 2005). A study showed that HSCs expressing SLAM markers resided on both OBs of the trabecular bona and were also located adjacently to sinousoidal endothelial cells (Kiel et al. 2005). In addition to this, endothelial cells were also shown to have the ability to maintain HSCs in culture (Li et al. 2004). These findings may support the model that the vascular niche may support the survival and maintenance of the HSC population under different conditions (Wagers 2005), however it was proposed that the vascular niche may be involved in promoting the proliferation and differentiation of HSC whereas the osteoblastic niche would provide a quiescent microenvironment (Kopp et al. 2005). To show this, studies using thrombopoietin mutant ($TPO^{-/-}$) mice showed that under stress conditions, the recruitment of HSCs to the sinusoid endothelial surface was observed to reconstitute thrombopoiesis (Avecilla et al. 2004). HSCs translocated from the osteoblastic niche to the vascular niche where they were seen to differentiate into megakaryocyte progenitors and undergo further maturation into megakaryocytes and platelet release (Avecilla et al. 2004; Kopp et al. 2005). More recently however, it has been argued that the perivascular niche is in fact responsible for maintaining HSC quiescence. This was demonstrated by Ding and Morrison in 2013 where it was discovered that Cxcl12 was expressed by stromal cells within the perivascular microenvironment, and to a lesser extent, in endothelial cells of the endosteal niche (Ding and Morrison 2013). In addition to this finding, the authors also showed that deletion of osteoblast-derived Cxcl12 did not deplete HSCs however Cxcl12 of perivascular stromal cells resulted in HSC depletion and the mobilization of restricted progenitors into the peripheral blood (Ding and Morrison 2013). In support of this, using deep confocal imaging, it was possible to visualise GFP-labelled HSCs within the bone marrow (Acar

et al. 2015). The majority of GFP^+ HSCs were found located in the central marrow rather than bone surface (endosteal) regions. In addition to this, they observed that 85% of these HSCs resided within 10μm of a sinusoidal blood vessel (Acar et al. 2015). In this study, it was also shown that both dividing and non-dividing HSCs reside mainly in the perivascular regions of the bone marrow (Acar et al. 2015). This suggests that cycling and non-cycling HSCs do not reside in spatially distinct regions. In parallel, a separate group sought to question the hypoxic status of the bone marrow (Spencer et al. 2014). In this study, live *in vivo* measurements of oxygen tension (*P*O2) within the bone marrow using two-photon phosphorescence lifetime microscopy. They discovered that the lowest *P*O2 readings were found in the deeper regions of the perivascular niche compared to that of the endosteal microenvironment. The vascular niche was also thought to have an important role for the homing and mobilisation of HSCs (Lapidot et al. 2005; Cancelas and Williams 2006). As mentioned previously, chemokines such as SDF-1 are also expressed on endothelial cells (Peled et al. 1999). SDF-1^+ endothelial cells cause HSCs to undergo transendothelial migration, this process is mediated by E- and P-selectins (Katayama et al. 2003) as well as adhesion molecules (e.g. VLA-4) and the leukocyte function antigen-1 (LFA-1) (Kopp et al. 2005). From these studies, it is clear that both osteoblastic and vascular niches play important roles in the regulation of the mobilisation, homing and quiescence of HSCs and it is clear that the differences in each niche impacts on HSC function (Figure 1.3).

1.6.4 Other niche regulators of HSCs

There are a number of other cellular components within the niches that contribute to the survival, mobilisation and egression of HSCs. For example, the autonomous nervous system has been shown to cause HSCs to egress from the BM (Katayama et al. 2006). Although it is established that neuromodulation of the bone marrow (and bone) is existent, it is still controversial whether neural activity influences stem cell function directly (Benestad et al. 1998). However, more recent studies have shown that the sympathetic nervous system does in fact have an effect on bone cells which in turn modulate haematopoiesis and HSC mobilisation (Artico et al. 2002; Bajayo et al. 2012; Eimar et al. 2013). On the other hand, adipocytes that reside in the BM negatively regulate HSCs (Naveiras et al. 2009). Naveiras et al studied the different regions of the mouse skeleton that differ in adiposity. They found that there was a decreased frequency of HSC and short term progenitors in the vertebrae of the tail (adipocyte-rich) compared to the vertebrae of the thorax (adipocyte-free) (Naveiras et al. 2009). It was also demonstrated that, upon transplantation, BM engraftment was accelerated in lipoatrophic (fatless) mice compared to wild type and untreated mice (Naveiras et al. 2009). These findings may help decipher the mechanisms and interactions of the cells of the BM microenvironment that could enhance haematopoietic recovery in BM transplantation therapy.

1.7 HSC Niche and Disease

The bone marrow niche provides support for the function of HSCs. However this can be greatly disrupted in the case of disease. Specifically looking at malignancies where maturation and differentiation of haematopoietic cells are impeded such as in the case of acute myeloid leukaemia (AML). It is understood that changes within the bone marrow niche occur to support AML blasts at the expense of normal HSCs. Bone marrow failure is one of the main characteristics of AML, not only is this due to the inhibition of myeloid cell maturation, but also due to HSC dysfunction (Risitano et al. 2007). In addition to HSCs, it is understood that AML cells influence the surrounding bone marrow niche in order to support their survival and proliferation. This has been shown to be the case in a number of studies (Ryningen et al. 2005; Pezeshkian et al. 2013; Krevvata et al. 2014).

Mesenchymal stem cells also reside in the bone cavity (Yin and Li 2006). These give rise to the stromal cell lineages (such as osteoblasts, endothelial cells adipocytes and chondrocytes (Short et al. 2003; Wang et al. 2006)) that contribute to the stroma of the bone marrow and are found to be an important component of the HSC niche (Mendez-Ferrer et al. 2010). However, aberrant alterations of the bone marrow stroma can lead to a loss of function of HSCs and result in the development of malignancy in the form of myelodyplastic syndromes and AML (Walkley et al. 2007a; Raaijmakers et al. 2010). Raaijmakers et al show that deletion of Dicer1, a microRNA processing enzyme, in mesenchymal osteoprogenitors causes a loss of function within the HSC population resulting in perturbed haematopoiesis (Raaijmakers et al. 2010). This gave rise to a myelodysplastic syndrome phenotype which further led to AML (Raaijmakers et al. 2010). To support the idea that osteoblastic dysfunction results in AML generation and progression, a recent study showed FoxO1 interaction with β-catenin resulted in the expression of Jagged-1 (notch ligand). This activation of osteoblastic notch signalling with HSCs resulted in their leukemogenic transformation and the progression of AML (Kode et al. 2016). This shows how the misregulation of niche cells gives rise to disease; however studies have sought to decipher how the interaction between the niche and AML cells promote leukaemic cell survival. Signalling molecules that regulate HSCs have also been described to have important roles in leukaemic cell maintenance. This idea was demonstrated when leukaemic cells were co-cultured with mouse stromal cells (Konopleva et al. 2002). In this study they found that apoptosis induced by ara-C was significantly lower in AML cells co-cultured with stromal cells (Konopleva et al. 2002). Furthermore, the release of the signalling molecule SDF-1α from osteoblasts not only regulates HSCs but also AML cells. Using CXCR4 inhibitors, it is possible to test the hypothesis that SDF-1α/CXCR4 interactions promote leukaemic cell survival (Zeng et al. 2006; Zeng et al. 2009; Cho et al. 2015). For example, it was shown that the CXCR4 anatagonist LY2510924 efficiently inhibited SDF-1α prosurvival signals to AML cells shown by the abolishment of leukaemic cell migration and inhibited ERK and AKT phosphorylation (Cho et al. 2015). Moreover in the *in vivo* leukaemia setting, it was shown that leukaemic mice treated with the antagonist exhibited lower tumour burden through the increase in mobilised of leukaemic cells from the bone marrow which correlated with prolonged survival. In addition to this, coupled with chemotherapy, the anti-leukaemic

effects of LY2510924 were enhanced suggesting that not only does the niche under disease protect leukaemic cells, but the SDF-1α/CXCR4 interaction between the niche and leukaemic cells can be targeted for therapy.

As well as dysregulation of BM stroma, normal HSC mobilisation also seems to be impaired in the disease setting (Colmone et al. 2008). In this study, HSCs were transplanted in SCID mouse models of acute lymphoblastic leukemia (ALL) to assess HSC mobilisation. They found that transplanted $CD34^+$ HSPCs localised to SDF-1^+ vascular niches in normal animals (Colmone et al. 2008). However in leukaemic mice, $CD34^+$ cells were observed to migrate to SDF-1^- tumour beds suggesting that the disease can generate a new malignant niche specifically for the recruitment of $CD34^+$ cells (Colmone et al. 2008). In addition to this, they looked to inhibit leukaemic cell and HSC interaction by neutralising stem cell factor (SCF) which mobilises HSC to endosteal niches (Driessen et al. 2003). They found that $CD34^+$ HSC mobilisation was restored in leukaemic mice as well as normalised HSC numbers (Colmone et al. 2008). The notion of HSC displacement however was tested by the Taussiq laboratory. In their study, they showed that by transplanting AML cells into mice, residual HSCs were unchanged in number however their downstream progenitors were significantly reduced suggesting that AML cells block the differentiation of HSCs rather than inducing migration (Miraki-Moud et al. 2013). More interestingly, when these normal HSCs were removed from the leukaemic environment, they were able to contribute to normal haematopoietic reconstitution (Miraki-Moud et al. 2013). This shows that in the AML setting, the bone marrow niche is altered to support AML cells.

Findings from the above studies support the model of BM stroma having a role in the development of disease, either by favouring conditions to maintain leukaemic cells, or by exerting disadvantageous conditions for normal HSCs (Lo Celso and Scadden 2011). These findings also raise the issue of whether it would be possible to pharmacologically manipulate HSC-leukaemic cell interactions to selectively support HSCs or confer a disadvantage for the leukaemic cell population.

1.8 HSCs in therapy

Experimental BM transplantation (BMT) models show not only the differentiation potential of transplanted HSCs, they also demonstrate how HSCs can be used in therapy to reconstitute the haematopoietic system. This property of HSCs is now routinely exploited in clinical BMT to treat a range of blood and solid tumours in combination with chemotherapy and/or radiotherapy. Recipients may receive HSC derived from their own BM (autologous), or from another individual (allogeneic) either related or HLA matched donors. BMT, first conducted in the late 1960's, involves harvesting HSCs from within the BM, typically from the iliac crest of the hip bone (Figure 1.4). Recent advances however have allowed for the retrieval of HSCs through other sources.

1.8.1 Autologous and Allogeneic Transplantation

Autologous transplantation involves the reinfusion of a patient's own HSC population. HSCs are harvested either from BM or peripheral blood, prior to myeloblative treatments, and are cryopreserved. The cells can then be re-infused into the patient to reconstitute the haematopoietic system. Since the HSCs are derived from the same individual, immunosuppression is not required. However, a drawback of autologous transplantation, in the example of blood cancers (or bone marrow diseases), is that malignant cells remaining in the harvested sample may lead to relapse following transplantation.

Allogeneic HSC transplantation can be a potentially curative option for a number of haematological malignancies. Survival rates were improved in response to allogeneic transplantation in patients that suffered from acute and chronic leukaemias (Hishizawa et al. 2010; Sarina et al. 2010) as well as lymphoid neoplasms (Sarina et al. 2010; Schlaak et al. 2012). In allogeneic BMT, HSCs are harvested from a donor and infused into a recipient following myeloblative treatment. Donors must be HLA-matched to prevent host rejection. In addition to this, the recipient must undergo heavy immunosuppression which increases the risk of infection and other medical complications. Although immune reconstitution may be slower than that of autologous transplantation, transplanted blood is free of malignant cells eliminating the risk of relapse. Allogeneic transplants may also confer an

immune mediated graft-vs-malignancy (GVM) effect where transplanted donor-derived lymphoid T-cells recognise and target residual cancer cells, thereby eliminating malignant cells that remain post high dose cytotoxic therapy. However the graft may also lead to GVHD whereby the donor immunocompetent lymphocytes become sensitised and target host cellular antigens (Hardy and Ikpeazu 1989).

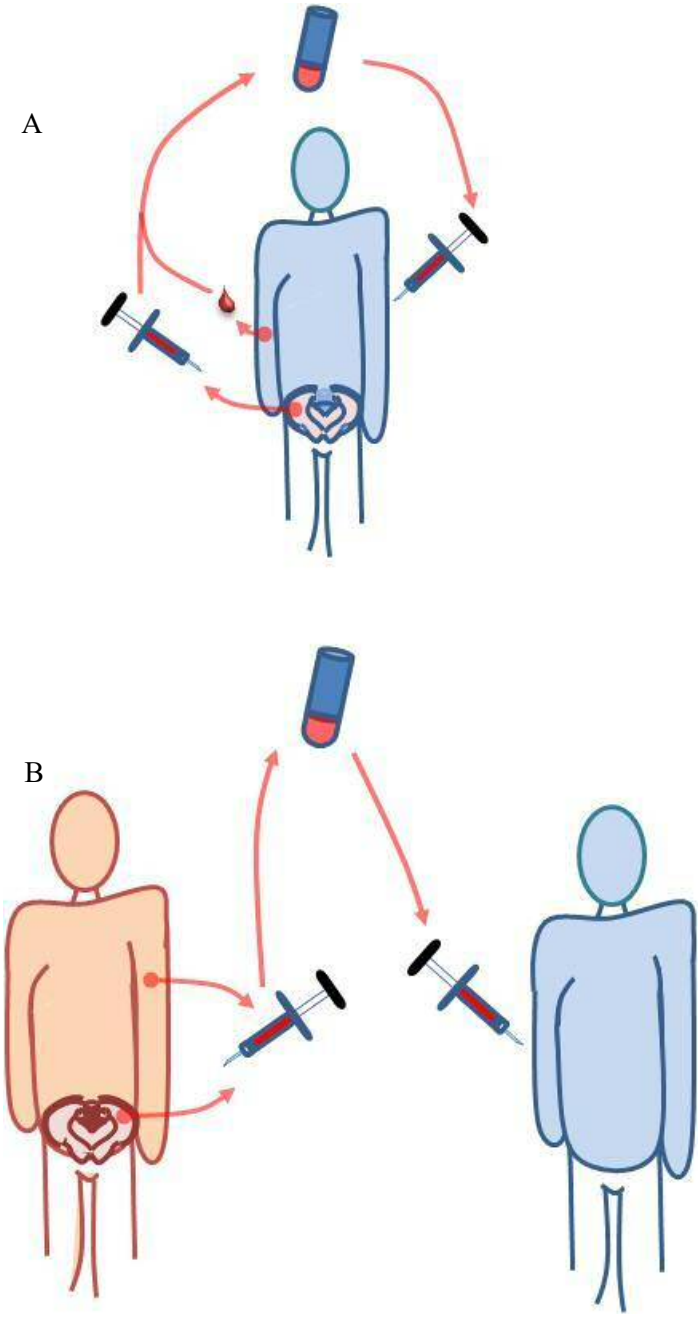

Figure 1. 4 The principles of bone marrow transplant

Autologous transplant consists of harvesting the patients' bone marrow prior to myeloablative treatment (A). Cells are processed in which stem cells are isolated and re-infused back into the patient. Provided there is a HLA-matched donor, stem cells can be harvested from healthy donors either by obtaining mobilised stem cells from the blood, or directly from the bone marrow. Stem cells are then transplanted into the patient (B).

1.8.2 HSC expansion: Umbilical Cord Blood - a source of HSCs/HPCs for allogeneic transplantation

Umbilical cord has been discovered to be a rich source of HSCs. In 1988, a young child suffering from Fanconi anemia received the first successful umbilical cord transplant which was derived from an HLA-matched sibling (Gluckman et al. 1989). It was then recognised that cord blood (CB) contained a source of HSCs and HPCs (Broxmeyer et al. 1989) and could theoretically be used in adult transplantation therapy. CB transplantation provides many advantages over BMT and mobilised peripheral blood. One outstanding advantage of CB is that it can be matched to four of six HLA class I and II molecules (Rao et al. 2012), i.e. it allows for partial HLA-mismatching. In addition to this, there is a lower incidence of GVHD. This can be explained by the low number of T-cells and the naive status of the lymphocytes within the CB (Garderet et al. 1998; Grewal et al. 2003). CB transplant therapy so far has mainly focused on paediatric patients as there are enough $CD34^+$ HSCs within a single unit of CB to engraft these patients, but not adults. It was initially found that 40% of adults were dying before 100 days (Laughlin et al. 2001). Considerable effort has therefore been invested in methods to expand this rich source of HSC to make CB a suitable alternative for adults (Broxmeyer et al. 1992; Piacibello et al. 1999; Robinson et al. 2006). One approach is to infuse two units of CB into a single recipient. Patients are given two units of CB that are at least four of six HLA-matched to the patient and each other. This has been shown to have successful clinical results with improved time-to-engraftment for adults compared to single CB unit transplantation (Brunstein and Wagner 2006)(Wagner et al. 2002). An alternative approach in overcoming the limited number of CB HSCs is to define conditions for ex vivo expansion of HSCs so that long-term multi-lineage engraftment can be obtained. Studies attempting such expansion methods have only been able to demonstrate the production of haematopoietic progenitors that provide short term engraftment of myeloid cell lineages (Robinson et al. 2005; Haylock and Nilsson 2007). In addition to this, there is a delayed time do donor engraftment (usually more than 3 weeks), to achieve adequate haematopoietic cell numbers to contribute to the reconstitution of the host haematopoietic system. This leaves the patient susceptible to infections resulting in morbidity and mortality (Wagner et al. 2002; Laughlin et al. 2004; Rocha et al. 2004). To overcome this engraftment delay, a number of strategies have been

considered to improve CB HSC expansion. Early studies utilised cytokine-mediated expansion protocols to increase the number of blood cells showing that this method is safe and feasible (Shpall et al. 2002; Jaroscak et al. 2003; de Lima et al. 2008). One investigation showed that they were able to expand umbilical CB, in a device developed by Aastrom Biosciences, within 12 days by observing the appearance of CFU-GM (Jaroscak et al. 2003). The expanded cells were then transplanted into patients and after 47 months were still showing engraftment. They also demonstrated the safety of the procedure as they found that from 22 patients that were evaluable for acute GVHD, 8 developed moderate-to- high grade GVHD (Jaroscak et al. 2003). This was also confirmed by de Lima et al (2008) where they showed a low incidence of relapse (1 from 9 patients). However, the small cohort used does not give significant clinical outcomes. In addition to this the cells expanded by Jaroscak et al (2003) gave a large number of CFU-GM, but expansion of $CD34^{+}$Lin- cells was poor, suggesting that further investigations are required to improve the expansion and transplantation efficacy of umbilical CB.

Another group used a similar approach but using the signalling pathway molecule, Notch, that may promote HSC expansion (Delaney et al. 2010). During development, Notch is necessary for the emergence of HSCs (Hadland et al. 2004). In early studies, it was possible to detect the human Notch1 gene in $CD34^{+}Lin^{-}$ HPSCs (Milner et al. 1994) and upon retrovirus-mediated expression of the constitutively active form of Notch1, they saw an increase in self-renewal abilities of HSCs (Varnum-Finney et al. 2000). Notch has been previously identified as a regulator of HSC maintenance within the stem cell niche; this was demonstrated by a number of studies when exposure of notch ligands resulted in increased self-renewal of HPSCs at the expense of differentiation (Karanu et al. 2001; Varnum-Finney et al. 2003; Suzuki et al. 2006). It was also shown that Notch-mediated expansion of CB resulted in a significant increase in absolute $CD34^{+}$ stem and progenitor cell numbers (Delaney et al. 2010). In addition to this, expanded $CD34^{+}$ cells also generated rapid multi-lineage reconstitution in NOD/SCID mice (Delaney et al. 2010). More significantly, their phase 1 preliminary results show that myeloid engraftment was enhanced as neutrophil recovery could be seen within 17 days. However, retention of the long-term repopulating HPSC population was only seen in

two out of ten patients. This may be explained by the loss of stem cell self-renewal capacity during expansion in culture or immune rejection.

Prostaglandin E2 (PGE2) was also discovered to be a strong candidate for expanding HSCs (North et al. 2007; Frisch et al. 2009; Porter et al. 2013). PGE2 and other prostaglandin metabolites are synthesised by a number of cells of the stem cell niche and exert their effects through EP2 and EP4 receptors (Tetradis et al. 1997; Tintut et al. 2002; North et al. 2007). It was first shown that PGE2 synthesis increased HSCs in zebrafish screens, and an inhibition of PGE2 synthesis would in turn lead to a decrease in HSCs (Nort et al. 2007). In addition to this, ex vivo assays showed that exposure to PGE2 correlated with an increased frequency of long-term repopulating HSCs (North et al. 2007). Further studies were able to demonstrate the expansion-inducing properties of PGE2 *in vivo* (Frisch et al. 2009). Here, mice treated with PGE2 experienced increased numbers of $Lin^{-}Sca\text{-}1^{+}cKit^{+}$ bone marrow cells (Frisch et al. 2009). Interestingly, it was also found that PGE2 could also inhibit HSC apoptosis under stress conditions (Porter et al. 2013). Cultures pretreated with PGE2 were discovered to have fewer apoptotic LSK cells compared to vehicle-treated cells in response to cytarabine (Ara-C) treatment (Porter et al. 2013). This anti-apototic affect was also seen to improve haematopoietic recovery in response to total body irradtiation indicated by the increased number of 'highly proliferative progenitors' in PGE2 pretreated irradiated mice (Porter et al. 2013). Moreover, another study was able to demonstrate the effects of PGE2 on human CB (Goessling et al. 2011). In this investigation, they first showed that colony formation, derived from $CD34^{+}$ cells, was enhanced in response to PGE2 exposure. In addition to this, human CB treated with PGE2 ex vivo were transplanted into sublethally irradiated NOD/SCID mice. These mice showed significantly increased percentages of CB-derived stem/progenitor cells as well as myeloid and T-Cell lineages compared to matched controls (Goessling et al. 2011). These studies are a 'proof of priciple' of CB expansion via manipulation of the PGE2 pathway and illustrate the potential of CB expansion for transplantation therapy. However, it still remains imperative that alternative areas of CB expansion are explored to improve both engraftment time and long-term survival of HSPCs.

More recent studies have attempted CB expansion using alternative approaches. One approach is by using compounds to target receptors involved in maintaining HSCs self renewal. StemRegenin-1 (SR-1) had been discovered in a compound screen (Boitano et al. 2010). HSCs exposed to SR-1 resulted in a 50-fold increase in $CD34^+$ cells ex vivo and it was these treated cells that contributed to rapid human engraftment in recipient mice (Boitano et al. 2010). It was also discovered that SR-1 exerted its effects through the antagonism of the aryl hydrocarbon receptor (Boitano et al. 2010). A recent clinical trial has documented the engraftment rates of SR-1 expanded $CD34^+$ CB cells (Wagner et al. 2016). They demonstrated a huge expansion of $CD34^+$ cells (over 300 fold) in response to SR-1 exposure. These were also able to engraft in all patients (17) in which neutrophil and platelet reconsitsution were seen at 15 and 49 days respectively, much quicker than untreated CB cells. As well as screening for compounds, it is also possible to comercially obtain supplementary factors that may aid expansion of $CD34^+$ cells. For example, StemCell2Max have formulated a supplementary mix of neurotrophic factors (GDNF). The GDNF ligand targets the RET tyrosine kinase receptor. RET has been implicated in the survival and expansion of HSCs (Fonseca-Pereira et al. 2014). The same study was also able to show the improved CB $CD34^+$ expansion and engraftment *in vivo* in response to RET activation. These studies demonstrate the latest advances in CB expansion.

1.9 HSCs in Disease: Leukaemic (cancer) stem cells

The connection between stem cells and cancer was first observed in the nineteenth century by Askanazy et al where they described similarities between embryonic tissue and tumours. This gave rise to the hypothesis that cancers may be caused by cells that share similar properties to those of the early embryo (Askanaze et al. 1907). Later, it was found that malignant structures that consisted of differentiated cells that are derived from all the germ layers (teratocarcinoma) originate from tumourogenic stem cells (Jackson 1941). In the late 1960's and 70's, a series of studies were conducted to test the theory of cancer stem cells. It was first described in solid tumours and leukaemia that only small subsets of cancer cells were able to extensively proliferate and form colonies (Southam and Brunschwig 1961; Fialkow et al. 1967; Hamburger and Salmon 1977).

Separate from normal HSCs, only 1 in 10,000 to 1 in 100 cancer cells could form colonies (Park et al. 1971). Leukaemic cells transplanted *in vivo* also showed a weak ability to form spleen colonies (Bruce and Van Der Gaag 1963). Clonogenic leukaemia cells were described as leukaemic stem cells since the differences in clonogenicity within leukaemia cells resembled the clonogenic differences among normal HSCs (Park et al. 1971). From these studies, two possibilities arose regarding leukaemic cells: i) all leukaemic cells showed low proliferating potential in the assays and thereby have the ability to behave as leukaemic stem cells (LSCs) or ii) most leukaemic cells showed low proliferating potential but only a small subset population of cells were clonogenic (Reya et al. 2001).

Dick and colleagues set out to test the latter possibility by separating different classes of leukaemia using flow cytometry and showing that one subset is highly enriched in clonogenic potential *in vivo*, whereas the other subset lack clonogenicity (Bonnet and Dick 1997). Here they showed that human acute myeloid leukaemia (AML) cells obtained from patient samples could be obtained and purified based on $CD34^{+}CD38^{-}$ expression. These cells only represent 0.2% of the AML cells in the patient sample; however they were the only cells that could transfer AML into NOD/SCID mice from patients. These cells were then transplanted into secondary recipients giving rise to AML showing the self-renewal abilities of the LSCs. At the same time, this eliminated the first possibility that all AML cells have the same clonogenic potentials, but instead small subsets of cells retain the capability to proliferate and transfer disease into recipients (the LSCs). A number of studies have utilised different mouse strains and transplantation methods to further support the idea of LSCs residing in the $CD34^{+}CD38^{-}$ compartment (Ishikawa et al. 2007; Eppert et al. 2011; Goardon et al. 2011; Sarry et al. 2011). However, it has also been described that LSCs (or here termed leukaemia initiating cells (LICs)) also reside in the $CD34^{+}CD38^{+}$ fraction (Kreso and Dick 2014). A study looking at these two distinct populations within the $CD34^{+}$ compartment discovered that the $CD34^{+}CD38^{-}$ subpopulation resembled LMPPs where as the $CD34^{+}CD38^{+}$ LICs resembled the GMPs (Goardon et al. 2011). However it has been shown that $CD34^{+}CD38^{-}$ AML contained a higher frequency of LSCs compared to $CD34^{+}CD38^{+}$ populations suggesting that $CD34^{+}CD38^{-}$ LSCs possess higher self-renewal potentials (Eppert et al. 2011; Sarry et al. 2011). It has also been discovered that a small number of

AML cases (approximately 25%) lack CD34 expression (Thomas and Majeti 2017). Unlike $CD34^+$ AML where a 'semihierarchichal' structure exists (i.e. the higher frequency $CD34^+CD38^-$ LSCs give rise to the fewer $CD34^+CD38^+$ LSC population (and vice versa in many cases)), $CD34^-$ AML patients lack the CD34 marker give rise to a non-LSC cell of a myeloid immunophenotype (Quek et al. 2016). This demonstrates that CD34 is not a suitable fixed marker of LSCs, particularly in $CD34^-$ AML. As in normal haematopoiesis, the cancer stem cell model in AML is arranged in a hierarchy where the LSCs are a population of self-renewing cells that give rise to more mature leukaemic blasts (Horton and Huntly 2012). More recent studies have elucidated further markers specific to LSCs, these include CLL-1, CD96, CD47, CD25, CD32 and TIM3 (reviewed by Horton and Huntly 2012). Though these markers have been identified to be LSC-specific, definitive LSC markers have yet to be elucidated in AML. This emphasises the requirement for patient specific targeting of LSCs in therapy.

Leukaemic stem cells are capable of long-term self-renewal, evade apoptosis and differentiate giving rise to a phenotypically diverse population (Lapidot et al. 1994). Their ability to maintain their stem cell properties rely on a number of signalling pathways such as the Wnt/β-catenin and Hedgehog signalling (Dierks et al. 2008; Zhao et al. 2009; Griffiths et al. 2010; Gandillet et al. 2011; Siapati et al. 2011). Interestingly, these same signalling pathways are essential for HSC development and regulation (Reya et al. 2001). It has been shown that Wnt signalling is essential for the self-renewal capabilities of LSCs that are derived from HSCs and GMPs (Wang et al. 2010). Interestingly, this data suggests that reactivation of β-catenin signalling is essential for progenitor cell leukaemogenic transformation as Wnt/β-catenin signalling only occurs in HSCs and not in downstream progeny. Other signalling pathways have also been extensively studied such JAK/STAT and PI3K/AKT which have been strongly linked with LSC survival as well as other cancer formation (Stahl et al. 2016; Wang et al. 2017). Current cancer therapies fail to target cancer stem cells due to their quiescent status which is important for their ability to retain their tumorigenic repopulation potential (Costea et al. 2006). Understanding the signalling pathways will allow for the generation of therapeutics to interfere with LSC maintenance.

Recent studies have shown that inhibitors of these pathways have been to impede LSC growth and induce apoptosis in AML (Konopleva et al. 2014; Sandhofer et al. 2015; Stahl et al. 2016). These therapeutics are becoming the new approach in targeting LSCs. One therapeutic intervention aims to target LSC-specific cell surface antigens. A recent study showed that LSCs, along with their progenies, expressed CD33 (Hauswirth et al. 2007), and using Gemtuzumab ozogamicin (Myelotarg- an antibody against CD33) conjugated to calicheamicin (toxin) could induce remission in some patients during clinical trials (Sievers et al. 2001). However, these patients were susceptible to relapse due to the LSCs being resistant to the toxin (Linenberger et al. 2001). Although this approach was able to induce remission, 97% of the patients in the trial developed thrombocytopenia (Sievers et al. 2001), this was explained by the fact that normal HSCs also expressed the CD33 cell surface antigen (Taussig et al. 2005). Further studies to target cell surface antigens, more specific to LSCs, have been conducted in attempt to selectively target LSCs (Jin et al. 2006; Jin et al. 2009; Majeti et al. 2009). In these studies, AML xenografts (in NOD/SCID mice) failed to engraft as well as causing a decrease in leukaemic cell burden in diseased animals in response to treatment with neutralising antibodies to CD123 (Jin et al. 2009), CD44 (Jin et al. 2006) and CD47 (Majeti et al. 2009). The promising result of these findings is that the normal HSC population were less affected by the treatment compared to the LSCs. Many challenges still exist in LSC-targeted therapy, this is mainly due to the lack of understanding of how extracellular and intracellular mechanisms regulate LSCs. Therefore, for successful eradication of LSCs, a combination of different approaches is required which need to target surface molecules, signalling pathways and microenvironment interaction.

1.10 LSCs in Acute Myeloid Leukaemia

AML is a heterogeneously defined disease at the phenotypic and molecular level. It is characterised by a variety of genetic alterations in either HSCs or haematopoietic progenitor cells that cause a misregulation in self-renewal, proliferation and differentiation (Frohling et al. 2005). Approximately 55% of AML patients present with clonal chromosomal aberrations (e.g. t(15;17), t(8;21), inv (16), t(9;21), t(9;11), del5, del7, etc.) (Welch et al. 2012). These chromosomal translocations generate

fusion proteins that affect proliferation, apoptosis and differentiation. This 'leukaemic stem cell' (LSC) therefore possesses dominance and confers a proliferative advantage over the normal HSC population, and it is this LSC population that maintains AML (Warner et al. 2004). However, AML is phenotypically heterogenic between AML patients which raises the question: which cell in the normal haematopoietic hierarchy acquires the genetic changes that are required to become an LSC? It was firstly hypothesised a number of cell types, including committed progenitors, could be subjected to genetic alterations (Griffin and Lowenberg 1986). This suggests that cell differentiation is inhibited at that particular stage of maturation, giving rise to immature leukaemic blasts that are reflective of the original transformed cell resulting in the heterogeneity between AML patients. For example, oncogenes such as MLL-AF9 and MLL-ENL are able to induce LSCs in restricted progenitor populations (Cozzio et al. 2003; Krivtsov et al. 2006). Another theory proposes that genetic alterations occur in only primitive cells and result in leukaemic transformation (Lapidot et al. 1994; Bonnet and Dick 1997). In this case, the specific genetic changes determine the leukaemic blast phenotype, and so the heterogeneity that is observed is due to the genetic alterations of the transformed cell. An intermediate model was also hypothesised to describe the 'preleukaemic' stem cell (Reya et al. 2001). The preleukaemic stem cell arises from an initial event that occurs in a stem cell, but retains the ability to differentiate into multiple lineages. LSCs then are generated when downstream progenitors undergo additional oncogenic mutations (Reya et al. 2001). Intriguingly, the LSC population is heterogenic within the same patient (Stahl et al. 2016). Lentiviral gene marking showed that LSCs varied in their ability to repopulate secondary and tertiary recipients giving rise to potential long term- and short term- LSCs (Hope et al. 2004). Furthermore, evidence has also shown that LSC phenotype differs between patients depending on their AML subtype. For example, patients suffering from M4 and M5 AML are characterized by $CD34^{+}CD38^{-}$ LSCs, whereas $CD34^{+}CD38^{+}$ LSCs are found in MLL-ENL patients (Lapidot et al. 1994; McKenzie et al. 2006; Taussig et al. 2008).

Rather than LSC immunophenotype, AML subtypes are classified by a combination of morphology, morphology and genetics. Traditionally, AML classification was proposed by the French-American-British Cooperative Group (FAB) which used a 30% bone marrow cell blast limit (Varela et al. 1985).

Here, AML is divided into eight subtypes (table 1.1) based on morphology and immuno/cytochemical phenotype (Bennett et al. 1976). More recently, the World Health Organisation (WHO) have provided an updated classification of AML subtypes. These are termed as (1) AML with recurrent genetic abnormalities and with gene mutations (2) AML with myelodysplasia-related changes (3) Therapy related myeloid neoplasms (4) AML not otherwise specified (NOS) (5) Myeloid sarcoma (6) Myeloid proliferations related to Down syndrome and (7) Blastic plasmocytoid dendritic cell neoplasm (Vardiman et al. 2009). In addition to these, AML classifications have been further detailed by genetic mutations and gene expression alterations which have become powerful tools in predicting outcome and potential therapeutics for AML patients (Saultz and Garzon 2016). For example, mutation of Nucleophosmin 1 (NPM1) occurs in 25-30% of AML patients and thus is the most frequent mutation in AML (Schnittger et al. 2005; Falini et al. 2007). Studies have shown that the *NPM1* protein is aberrantly expressed in the cytoplasm rather than the nucleus resulting in myeloid proliferation and leukaemia (Falini et al. 2006; Falini et al. 2007; Cheng et al. 2010). NPM1 mutations are also linked with other genetic abnormalities such as DNMT3A and FLT3-ITD mutations (Marcucci et al. 2011). DNMT3A mutations account for 18-22% of AML cases (Ley et al. 2010). Defective haematopoiesis and methylation is largely caused by a missense mutation which affects the arginine codon 882 (R882-DNMT3A) however, less frequently, other codons are also affected (non-R882-DNMT3A) (Marcucci et al. 2012). A number of mutations and genetic aberrations have been described (outlined in figure 1.6) however treatment has remained unchanged for AML patients. The standard treatment consists of an intensive anthracycline and cytarabine regimen with the goal of reaching complete remission (Saultz and Garzon 2016). 65-73% of young AML patients will achieve remission using this regimen whereas this is lower in patients >60 years old (38-62%) (Fernandez et al. 2009; Lowenberg et al. 2009). Studies have shown that for older patients that are not fit for intensive therapy (particularly those that carry the complex karyotype without NPM1 mutations), hypomethylating agents such as decitabine and azacitidine have proven to be beneficial (Blum et al. 2010; Quintas-Cardama et al. 2012; Klepin 2014). This was also the case of patients possessing the FLT3 mutation. It was shown that these patients could also be treated with FLT3 inhibitors such as sorafenib, in combination with chemotherapy, vastly improved remission rates (Ravandi et al. 2013). The above

findings demonstrate the complexity of AML as a disease with a diverse genetic background. As further studies are being undertaken to decipher molecular mechanisms, it is becoming evident that single drug therapy may become less successful compared to a multiple drug approach.

FAB Subtype	Description
M0	Minimally differentiated AML
M1	AML without maturation
M2	AML with maturation
M3	Acute promyelocytic leukaemia (APL)
M4	Acute myelomonocytic leukaemia (AMML)
M5	Acute monocytic leukaemia
M6	Acute erythroid leukaemia
M7	Acute megakaryoblastic leukaemia

Table 1.1 FAB classification of AML

The French-American-British (FAB) system characterizes different leukaemia subtypes based on morphological features and cytochemical phenotypes. Further characteristics were added to the classification using immunophenotyping and electron microscopic studies. In addition to AML subtypes, ALL can also be divided into 3 subtypes (L1-L3).

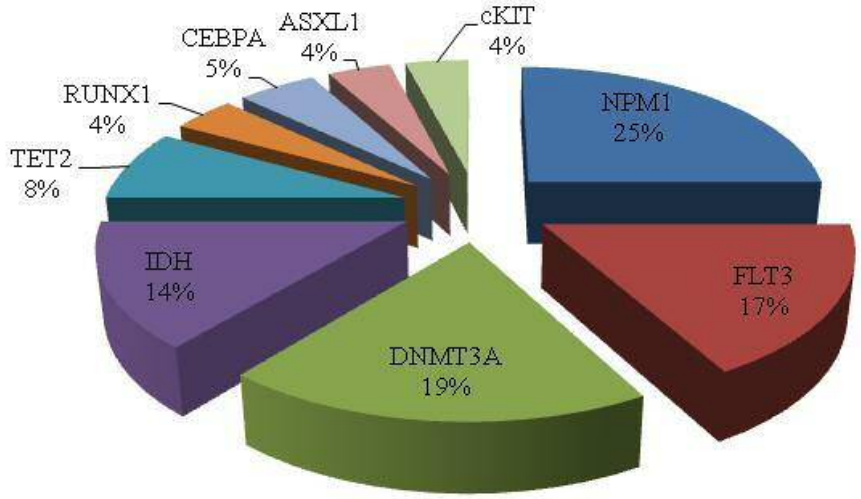

Figure 1.5 Pie chart describing incidences of AML subtypes

Mutations in NPM1, DNMT3A and FLT3 are the most common mutations found within AML patients occurring in approximately 25-30%, 18-22% and 20% in AML cases respectively. NPM1 (Nucleophosmin 1), FLT3 (Fms-Like Tyrosine Kinase 3), DNMT3A (DNA Methyltansferase 3A), IDH (isocitrate dehydrogenase), TET2 (Ten–Eleven Translocation 2), Runx1 (Runt-Related Transcription Factor), CEBPA (CCAAT Enhancer Binding Protein α), ASXL1 (Additional Sex Comb-Like 1)

1.11 Project rationale and preliminary data

From the preceding discussion, it is evident that there is a need to find potential agents that may promote HSC expansion and improve reconstitution of the haematopoietic system after transplant. It is also important to discover cancer stem cell-targeting therapies in an attempt to specifically target quiescent, self-renewing cancer stem cells in disease.

In a collaborative effort with Vastox (now Summit), a screen of 12,000 small molecules (low molecular weight drugs that are able to bind to receptors and target signalling pathways) was conducted to identify stem cell active compounds. Working on the premise that the cellular machinery between HSCs and cancer stem cells/LSCs is often conserved (Shackleton 2010). It has been described that, like normal HSCs, LSCs are able to self-renew, proliferate and give rise to immature leukaemic blasts (Clarke et al. 2006). It was hypothesised that stem cell active compounds identified may target HSCs and/or cancer stem cells. The library consisted of compounds expected to disrupt signalling pathways, protein-protein interactions and transcriptional control mechanisms. We first screened test compounds on wild type adult and embryonic fruit flies (Drosophila). Test compounds were directly fed to tester strain flies to screen the impact on the gut stem cell population; DAPI staining of the gut can differentially identify the morphology of somatic cells versus stem cells; large nuclei indicate the presence of somatic cells, whereas smaller nuclei represent stem cells (Figure 1.6A). Separately, fruit fly larvae were also used to screen compounds that influenced asymmetric division, a key hallmark of stem cell behaviour. Brains of the larvae were dissected and bathed in a solution of compound. Asymmetric division was monitored using green fluorescent protein (GFP) and by immunostaining with Miranda protein and drug hits were defined by modification of division patterns.

Small molecules that were identified as positive hits in either or both of these fruit fly screens were prioritised for further screening in zebrafish embryos which provide a high-throughput vertebrate model in which HSC behaviour can be assessed. Embryos were immersed in media containing the test compounds and assessing the formation, expansion or survival of HSC. The emergence of HSCs was

detected using an in situ hybridisation probe for *Runx-1* (Figure 1.6B), a transcriptional factor that is important in the regulation of HSC differentiation into mature blood cells. HSCs emerge from the ventral wall of the dorsal aorta and these HSCs migrate to the posterior region in the tail called the caudal haematopoietic tissue. Test compounds causing either an increase or decrease of Runx-1 positive cells in the dorsal aorta were defined as a positive hit and prioritised for further study in mouse HSC screens (Table 1.2).

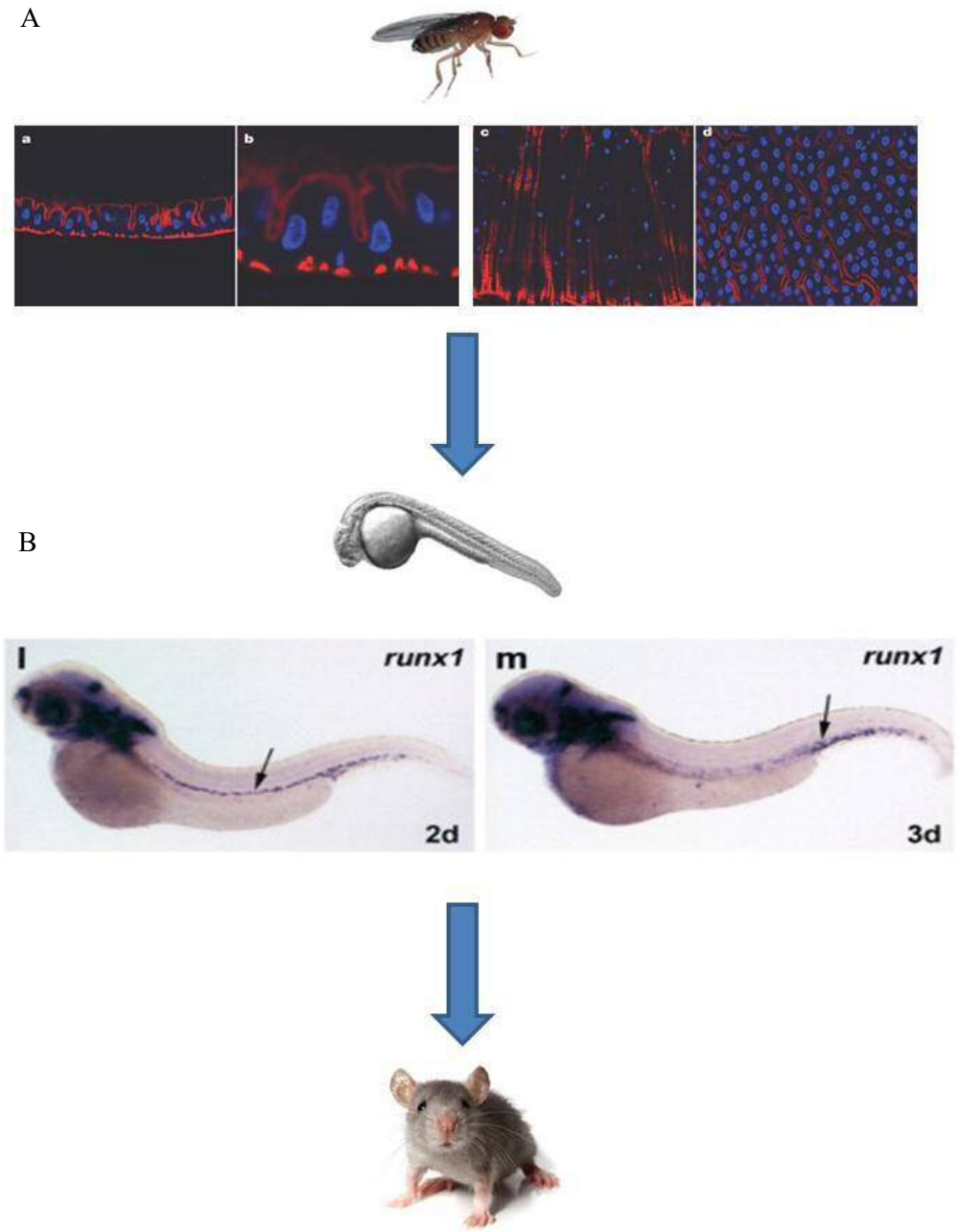

Figure 1. 6 Lower organism library screen identifies compounds with stem cell activity.

(A) DAPI staining of the gut reveals cell nuclei. Large nuclei indicate presence of a somatic cell and small nuclei indicate the presence of gut stem cell. (B) Emergence of HSCs in zebra fish is readily detected by a probe for the expression of the transcription factor Runx-1from the ventral wall of the dorsal aorta.

Screen	Hit (+) or No Hit (-)						
Fruitfly Brain/ Asymmetry	+	+	+	-	-	+	-
Fruitfly Gut	+	-	-	+	-	+	+
Zebrafish Blood	+	+	-	+	+	-	-
Priority for mouse screen	1	2	3	4	5	6	7

Table 1.2 Prioritisation to identify lead small molecules for study.

Compounds with the most positive hits are prioritised for screening in mouse HSCs.

Although rigorous *in vivo* studies are required to assess HSC function of the compounds, preliminary *in vitro* experiments were conducted by exposing mouse HSC/HPCs to the lead small molecules ex vivo and performing *in vitro* colony forming cell (CFC) assays as an assessment of stem cell/multi-potential cell function *in vitro*. From this, we identified the following small molecules as potential targets of HSCs, and perhaps leukaemia (cancer) stem cells:

1.11.1 Fluvastatin - HMG CoA reductase inhibitor

Statins are potent regulators of cholesterol biosynthesis via the inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase. In addition to this, it was shown that statins also possessed anti-proliferative effects on cancer cell lines. Early studies showed that simvastatin was able to decrease cell numbers from normal bone marrow cells, AML cell populations and HL60 (APL) (luekaemic cell line) cells (Newman et al. 1994). As well as inhibiting proliferation, statins have also shown to have a role in inducing differentiation. Dimmeler et al (2001) showed that $CD34^+$ (HSCs) isolated from blood, were able to differentiate into epithelial progenitor cells (EPCs) when treated with atorvastatin. Mice fed simvastatin exhibited a 2-fold increase in EPC numbers in the periphery showing further differentiating potential *in vivo* (Dimmeler et al. 2001). Previous studies showed that statins are involved in the PI3-kinase/Akt pathway (Kureishi et al. 2000). Using PI3-K inhibitors, the statin-stimulated increase of EPCs was abolished. This was also seen when there was an overexpression of a dominant negative Akt where the increase of EPCs was inhibited when stimulated with statins (Dimmeler et al 2001). Statins activate Akt through the inhibition of mevalonate formation (Kureishi et al 2000) however; it is still unclear how statins activate the PI3-k pathway.

Looking specifically at the effects of statins on AML cells, Sassano et al (2007) showed that both atorvastatin and fluvastatin induced cell differentiation and apoptosis. In this study, they showed that fluvastatin (in the NB4 acute promyelocytic leukemia (APL) cell line) was involved in the activation of Rac1/cdc42 and further downstream pathways (c-Jun NH2-terminal kinase kinase pathway) which is essential for the induction of apoptosis (Sassano et al. 2007). Earlier studies showed it was possible to induce apoptosis using lovastatin (Wu et al. 2004). Acute myelogenous leukemia-derived cells were exposed to lovastatin which resulted in disruption of ERK1/2 phosphorylation, ultimately

leading to apoptosis. Constitutive activation of the Raf/MEK/ERK pathway represses (but does not completely block) lovastatin-induced apoptosis. This may suggest that statins may interfere with other signalling pathways that lead to differentiation/apoptosis. In a more recent study, it was shown that lovastatin could inhibit LSC cobblestone formation in human and mouse AML cell lines (Hartwell et al. 2013). This was also the case in primary patient derived AML samples but normal HSPCs were unaffected. This was further proven in co-culture of $DsRed^+$ leukaemic cells and GFP^+ HSPCs where lovastatin selectively inhibited the growth of $DsRed^+$ leukaemic cells while sparing HSPCs (Hartwell et al. 2013). To further demonstrate the anti-leukaemic effects of lovastatin, treated LSCs were transplanted into irradiated recipients, these animals survived considerably longer than animals transplanted with DMSO treated cells (which developed leukemia within 3 weeks) (Hartwell et al. 2013). From these studies, fluvastatin may prove to be an interesting compound for targeting cancer cells in particular.

1.11.2 Fluphenazine - Dopamine receptor Antagonist

Flow cytometry was used to show the presence of the dopamine receptors DR3 and DR5 on the surfaces of human $CD34^+$ cells in cord blood and mobilised peripheral blood $CD34^+$(Spiegel et al. 2007). Also, cord blood and bone marrow $CD34^+$ cells incubated with granulocyte colony-stimulating factor (GCSF) resulted in upregulation of dopamine receptor expression. Dopamine receptor agonists doubled the percentage of human cord blood $CD34^+$ cells with polarised morphology and cellular elongation compared to control untreated cells. Agonists also stimulated cord blood $CD34^+$ colony formation, however, only in the presence of the myeloid cytokines GM-CSF and G-CSF (*in vitro*). $CD34^+$ were stimulated with GCSF ex vivo and transplanted into NOD-SCID mice. A 2-fold increase in engraftment in the bone marrow was observed compared to untreated control cells, suggesting a role for dopaminergic agonists in augmenting the *in vivo* population.

A recent study looked at the effects of phenothiazine compounds on neoplastic human pluripotent cells (hPSC) and AML-blasts (Sachlos et al. 2012) Treatment of Thioridazine (anti-psychotic dopamine antagonist) to neoplastic hPSC inhibited self-renewal and induced differentiation. Using blood from AML patients, Thioridazine caused a decrease in AML proliferation/clonogenic capacity,

while at the same time maintaining HSPC multilineage differentiation (Sachlos et al 2012). They also assessed the effects of other phenothiasine, one of which was fluphenazine. Treatment with fluphenazine caused morphological changes in neoplastic HSPCs. Further work may be required to test the possible anti-proliferative effects of fluphenazine in particular, although it was mentioned that Thioridazine possessed the lowest EC50 making it the most potent agent for targeting AML cancer stem cells (Sachlos et al 2012).

1.11.3 Yohimbine- Alpha-2 Adrenoreceptor Antagonist

Alpha2 adrenoreceptor (A2-AR) mRNA expression was studied in the mouse embryo (Wang & Limbird 1997). They detected A2-AR (α_{2B} subtype) mRNA expression in the liver, a 'blood-forming' organ in embryogenesis, between 11.5-14.5 days post-conception. This may suggest that A2-adrenoreceptors may have a role in embryonic haematopoiesis. However, it was shown that expression of the α_{2B} subtype A2-AR diminished in embryonic development by 5 weeks (Cussac et al. 2001).

A study on the function of adrenergic-receptor antagonists, for the reconstitution of haematopoietic and immune function, was conducted by Maestroni et al. Mice (C57BL/6) underwent syngeneic bone marrow transplantation after lethal irradiation. Mice were treated with prazosin (A1-AR antagonist) after BMT, increased numbers of peripheral blood leukocytes, platelets and spleen cells were observed. It was also shown that prazosin enhanced granulocyte and macrophage reconstitution (Maestroni et al. 1992). Further tests showed that prazosin also enhanced myelopoiesis and platelet formation in normal mice (Maestroni and Conti 1994). A1-AR agonists (noradrenaline) were shown to inhibit the *in vitro* granulocyte/macrophage- colony-forming units. Interestingly, other adrenergic antagonists were compared to prazosin, one of which was Yohimbine, an A2-AR antagonist. Like prazosin, Yohimbine counteracted the inhibitory effects of noradrenaline (Maestroni et al 1994). However, the effects of these compounds on HSCs are still unknown, particularly the role of A2-ARs and their respective antagonists in the regulation of HSC self-renewal, proliferation and differentiation.

1.11.4 Phthalylsulfathiazole - Folic acid synthesis inhibitor

Sulphonamides are anti-bacterial drugs that have recently been shown to possess anti-cancer properties. Chloroquinoxaline sulphonamide (CQS) has been demonstrated to inhibit colony formation against solid tumours such as breast, lung, melanoma and ovarian carcinomas (Fisherman et al. 1993). The mechanisms of CQS are still not understood, however murine B16 melanoma cells or human peripheral blood mononuclear cells incubated with CQS underwent cell cycle arrest in the G0/G1 phase (Scozzafava et al. 2003). Further tests have been conducted to elucidate the potential mechanisms of sulphonamides in the inhibition of cell proliferation. At low concentrations, indole sulphonamides depolymerised the spindle microtubules and disorganised the chromosomes without affecting mechanisms of the cell cycle (Mohan et al. 2006). However at higher concentrations, in addition to the mentioned effects, interphase microtubules were also depolymerised. It was also shown that apoptosis-induced bcl-2 phosphorylation correlated with the treatment of sulphonamides (Mohan et al. 2006). This study has shown that sulphonamides may exert their anticancer effects through the disruption of molecular mechanisms that are involved in cell division, as well as signalling pathways that lead to apoptosis.

1.11.5 Oxa-22 (cis-2-Methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide)- Muscarinic Acetylcholine receptor (mAChR) agonist

Studies have shown that activation of mAChRs can lead to cell proliferation through a number of different pathways (reviewed by(Resende and Adhikari 2009)). Interestingly, M2, M3 and M4 receptors were found to be present in K562 erythroleukemia cells. Using K562 cells, Aydin et al (2013) targeted the downstream signalling molecules of the mAChRs to observe the effects of mAChr-mediated cell proliferation (Aydin et al. 2013). They were able to demonstrate inhibited proliferation of K562 cells with treatment of a mAChR, carbachol (CCh) in the presence of serum. They described an opposite effect in the absence of serum. The CCh-mediated inhibition was due to the activation of the M3 mAChR, they used a number of inhibitors that target downstream pathways of the GPCR. U73122 is a PLC inhibitor, K562 cells treated with U73122 partly antagonised the inhibitory effect of CCh suggesting a role of PLC in the mAChR-mediated inhibition. They also

showed that $Ca2^+$ is an important molecule for proliferation in K562 cell. 2APB, a $Ca2^+$ mobilisation blocker, lifted the inhibitory effect on proliferation of CCh implicating a role for $Ca2^+$ in the muscarinic control of cell proliferation (Aydin et al 2013). This study suggests that mAChR agonist may inhibit the proliferation of leukaemic cell lines, but it is uncertain for this mAChR agonist.

1.12 Project Aims

Since the screen was initially conducted, fluvastatin and fluphenazine, which were identified in our screen, have been comprehensively shown to possess potent anti-leukaemic stem cell properties by other laboratories (Adams et al. 2007; Sassano et al. 2007; Sachlos et al. 2012). This demonstrates the robustness of our approach to identify stem cell specific compounds. However, since fluvastatin and fluphenazine have now been well characterised in that setting (without an impact on normal HSCs), we directed our efforts towards the remaining, less well characterised compounds. Yohimbine (adrenergic antagonist) and Oxa-22 (muscarinic agonist) are targets of the nervous system. Of interest, the nervous system has been implicated in the regulation of HSCs and haematopoiesis through the bone marrow niche (Mendelson and Frenette 2014). We therefore hypothesised that these compounds could alter HSC behaviour. In addition to this, as previously discussed, individuals undergoing myeloablative therapy and consequent bone marrow transplant are at risk of infection during the haematopoietic recovery period. We therefore explored whether these small molecules can influence HSPC function in the transplant setting in order to aid haematopoietic recovery.

Separately in the disease setting, recent findings have shown that a number of antibacterial drugs possess anticancer properties (Felício et al. 2017; Ramirez-Prada et al. 2017; Rubino et al. 2017). Moreover, it has been shown that sulphonamides in particular also possess anti-cancer properties (Scozzafava et al. 2003; Alaoui et al. 2017), we therefore sought to test the impact of sulphonamide in the leukaemic setting as well as exploring its impact on normal haematopoiesis. Finally, we explored the role of Oxa-22 in the leukaemia setting. It is understood that cholinergic signalling through the M3 muscarinic receptor is involved in tumorigenesis (Alea et al. 2011; Zhao et al. 2014). Thus we sought to investigate the effects of Oxa-22 and cholinergic signalling in leukaemia.

1.12.1 Summary of aims

Using small molecules that were identified from a library screen, we are attempting to target HSCs within the healthy and disease setting, ultimately allowing us to better understand HSC function for transplant therapy and understanding mechanisms of disease in AML. We specifically asked whether these compounds can:

- Expand or alter the behaviour of HSCs ex vivo and *in vivo* under steady state conditions
    - If so, what are the functional effects in a competitive transplant setting?
- Improve haematopoietic reconstitution after bone marrow transplant

In addition to normal HSC function, we also seek to investigate the effects of these compounds on leukaemic cells and stem cells. Since there are a number of conserved properties and mechanisms between LSCs and HSCs, we hypothesise that we can target LSCs by driving them into the cell cycle. Initially we will investigate leukaemic cell growth *in vitro*, then leukaemic progression *in vivo*.

Chapter 2: Methods and Materials

2.1 Mice

C57BL/6 mice aged 8 to 12 weeks old (male and female) were used in all experiments. Experiments undertaken on animals were performed under UK Home Office authorization. Project licence (PPL) 30/3381, Personal licence (PIL) IA4825DA6

2.2 Ex vivo analysis of HSCs and Progenitors

Tibias and femurs were dissected from 8-12 week old C57BL6 mice. The bones were crushed using a pestle and mortar and washed with PBS (2% FBS). Collected cells underwent red blood lysis (using NH_4Cl) for counting using the Trypan Blue (Sigma) exclusion test of viability. $1x10^6$ mononucleated cells from whole (unlysed) bone marrow were plated in a 24 well plate in 1ml of StemSpan media (STEMCell). Cells were then exposed to a 1nM-1mM concentration range of Yohimbine (Sigma) or Oxa-22 (Sigma) increasing in 10-fold (Figure 2.1). Cells were plated in triplicate. Cells were exposed to the above compounds for 2, 6, 12 and 24 hours at which point they were removed by pipette and underwent antibody staining for LSK analysis by flow cytometry.

2.2.1 Colony Forming Cell Assay (CFC)

Tibias and femurs were dissected from 8-12 week old C57BL6 mice. The bones were crushed using a pestle and mortar and washed with PBS (2% FBS). Collected cells underwent red blood lysis (using NH_4Cl) for counting using the Trypan Blue (Sigma) exclusion test of viability. $1x10^6$ mononucleated cells from whole (unlysed) bone marrow were plated in a 24 well plate in 1ml of StemSpan media (STEMCell). Cells were then exposed to 100nM, 1uM or 10uM of Yohimbine or Oxa-22 for 2 and 6 hours. Cells were washed with PBS (2% FBS) and counted. 10,000 mononucleated cells were added to 1ml of Methylcellulose (MethoCult M3434, Stemcell Technologies) and plated into a 6 well plate using a 20g needle. Cells were plated in duplicates in the centre wells while the surrounding wells were filled with sterile water. Colonies were scored after 10 days of incubation at 37°C and 5% CO_2.

2.3 Flow cytometry and cell sorting

Flow cytometry analysis was conducted using the BD Fortessa II. Fluorescent activated cell sorting was conducted on the BD Aria Fusion. Data analysis was conducted on BD diva and FlowJo sofwares.

2.3.1 Analysis of ex vivo LSKs

For the staining of HSCs and progenitors (LK and LSK markers) the cells were incubated in a cocktail of biotinylated Lineage differentiated marker antibodies (Lin cocktail): CD3, CD4, CD8, Ter119, B220, CD11b (Mac-1), Gr-1. In addition to the Lin cocktail, cells were simultaneously stained with cKit-APC, Sca-1-PE. A further stain with SA-PerCp was applied after washing. All antibodies were purchased from Biolegend.

2.3.2 Analysis of *in vivo* HSPC populations

For the staining of HSCs (SLAM markers) BM/spleen tissue was incubated in a cocktail of biotinylated Lineage differentiated marker antibodies (Lin cocktail): CD3, CD4, CD8, Ter119, B220, CD11b (Mac-1), Gr-1. In addition to the Lin cocktail, cells were simultaneously stained with cKit-APC, Sca-1-PE, CD48-FITC, CD150-PECy7. A further stain with SA-PerCp was applied after washing. This allows the identification of HSC (Lin^- $Sca\text{-}1^+$ $cKit^+$ $CD150^+$ $CD48^-$), MPP (Lin^- $Sca\text{-}1^+$ $cKit^+$ $CD150^-$ $CD48^-$), HPC1 (Lin^- $Sca\text{-}1^+$ $cKit^+$ $CD150^-$ $CD48^+$) and HPC2 (Lin^- $Sca\text{-}1^+$ $cKit^+$ $CD150^+$ $CD48^+$) populations. Fc block (1:50) was also used to allow for specific SLAM marker binding. To determine progenitor populations, cells underwent similar staining protocols however with the following antibodies: Lin cocktail, cKit-APC, Sca-1-PE, CD34-FITC, CD16/32-PECy7 followed by SA-PerCp. This characterised GMPs (Lin- $cKit^+$ $CD16/32^+$ $CD34^+$), CMPs (Lin^- $cKit^+$ $CD16/32^-$ $CD34^+$) and MEPs (Lin^- $cKit^+$ $CD16/32^+$ $CD34^-$). Lineage differentiated cells were stained with CD4-PE, CD8-PE, Ter119-APC-Cy7, Gr1-FITC and Mac1-APC, B220 was a biotinylated antibody which was followed by SA-PeCy7. All antibodies were purchased from Biolegend.

2.3.3 Analysis of engraftment of transplanted cells

Engraftment was assessed by bleeding mice via tail venepuncture. 10-20ul of blood was collected per animal and lysed with NH_4Cl and stained for CD45.1-Pacific Blue and CD45.2-FITC and lineage differentiated cells were stained with CD4-PE, CD8-PE, Ter119-APC-Cy7, Gr1-PeCy7 and Mac1-APC, B220-APC (Myeloid cells were stained separately to lymphoid cells due to fluorochrome clash) for flow cytometry analysis. All antibodies were purchased from Biolegend.

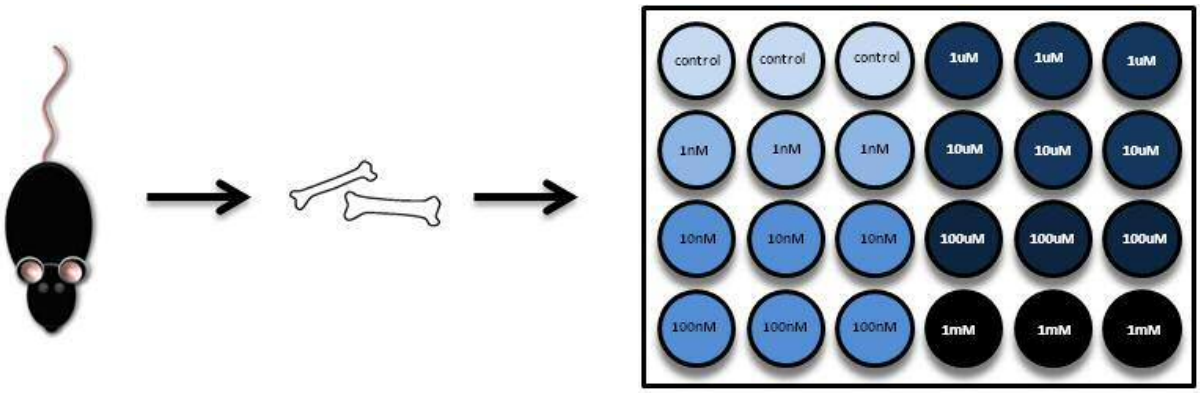

Figure 2. 1 Schematic of bone marrow plating procedure for ex vivo analysis of the effects of compounds

Bone marrow harvested from the tibia and femur of the mouse is plated at a density of $1x10^6$ cells per well. Triplicate wells were used for each respective concentration. PBS was used as the vehicle (termed control)

2.4 RNA Extraction and RT-qPCR.

Whole bone marrow was harvested from C57BL/6 mice (8-12 weeks old male and female). From these cells cKit$^+$ cells were enriched with the use of cKit magnetic beads (Miltenyi Biotec). WBM was incubated with beads for 20mins (at 4°C) and filtered before running the sample in the magnetic-activated cell sorter (MACS®, Miltenyi Biotec). For specific cell populations, FACS was used to sort stem and progenitor cells as well as more mature differentiated cell types (e.g. myeloid cells lymphoid cells). RNA was extracted from WBM and cKit$^+$ cells using Trizol® reagent with phase lock gel heavy. Cells were pelleted and resuspended in the Trizol® reagent for homogenization before being placed into tubes containing phase lock gel heavy. After a brief incubation, chloroform is added, vigorously shaken and centrifuged. Phasing occurs where the cloudy phase gel lock forms as the interphase separating a clear aqueous phase (above the gel) from the pink/purple phenolchloroform phase (below the gel) (Figure 2.2). RNA content is contained within the upper-most aqueous phase and this was decanted into a fresh tube. Isopropyl alcohol was added to the aqueous phase to precipitate the RNA in which a pellet is observed after centrifugation. The supernatant was decanted and the RNA pellet re-suspended in molecular biology grade water. Nanodrop was then used to measure RNA concentration. In the situation where cell numbers were low (for sorted populations), RNA was extracted using the RNAeasy Plus Micro/Mini Kit following manufacturer's instructions (Qiagen). cDNA first strand was synthesised using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). Quantitative PCR (qPCR) was performed employing SybrGreen probe primers (Sigma).

Primers were designed to target the genes in question (Table 2.1) and run in a QuantStudio® 7 Flex Real-Time PCR System (Applied biosystems) at 95°C for 2 minutes then at 60-65°C for 20-30 seconds for 40 cycles. (PCRBiosystems).

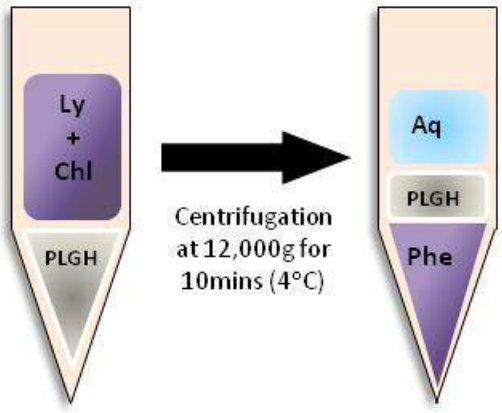

Figure 2. 2 Phase Lock Gel Heavy phasing

After homogenization of the cells, the lysate (Ly) is placed in the phase lock gel heavy tube (along with Chloroform (Chl)) and centrifuged. The resulting phases form in three layers, the RNA-containing aqueous phase (Aq), the Phase Lock Gel Heavy phase in the middle (PLGH) and the phenolchloroform phase (Phe).

Gene	Forward '	Reverse '
ADRA2a	AGCTGCAAGATCAACGACCA	ACGCTTGGCGATCTGGTAAA
CHRM3	CGGGTCATAGCACCATCCTC	TTGGTGTCAGAGGTCTTGGC

Table 2.1 SybrGreen Primers.

Primers were designed based on the sequences of target genes: Adrenergic Receptor a2 (ADRA2a) and Muscarinic Receptor M3 (CHRM3).

2.5 *In vivo* administration of small molecules

Mice were treated with Yohimbine (Sigma) fell into three groups 1) PBS 2) 1mg/kg and 3) 10mg/kg. Oxa-22 (Sigma), the following dosages were used: PBS (control), 10ng/kg, 100ng/kg and 1ug/kg. Phthalylsulfathiazole (Sigma): 1mg/kg, 10mg/kg and 100mg/kg dosages were used. Since Phthalylsulfathiazole is soluble in DMSO, the control group received PBS (10% DMSO). Yohimbine treated animals underwent treatment twice daily for 10 days. Phthalylsulfathiazole was administered once daily for a period of 10 days. However, for Oxa-22, animals were treated only once daily for 5 days. At each respective time points, BM was harvested from femurs and tibias of both legs of each mouse. Bones were crushed with pestle and mortar and washed with PBS (Gibco) 2% FBS to obtain single-cell suspension. Spleens were dissected after culling and smashed with syringe plungers in cell strainers. These too were washed with PBS (Gibco) 2% FBS to obtain single-cell suspension. Blood was collected via venepuncture of the lateral tail vein and into EDTA treated tubes (Starstedt). For HSPC analysis, 100ul of blood was lysed in 5ml of NH_4Cl. As for analysis of lineage differentiated populations, 12ul of blood was lysed in 600ul of NH_4Cl. Cells were then stained and analysed by flow cytometry.

2.6 Competitive Transplantation

Donor mice underwent compound pulsing as mentioned above. Recipient mice received two doses of total body irradiation (TBI) at 400cGy separated by 4-6hours. 24 hours later, Recipients were transplanted with a total of $7.5x10^5$ cells composed of donor cells as well as competitive cells (of the same recipient strain). The ratios of donor: competitor cells were dependent on the changes in cellularity in response to the compound (Table 2.2).

2.6.1 Serial transplantation

After 16 weeks, primary recipients were culled and femurs and tibias were harvested. Bones were crushed and stained for donor LSKs and underwent cell sorting. 2000 donor LSKs were sorted per secondary recipient. Donor cells were transplanted with $5x10^5$ supporting bone marrow cells via tail

vein injection. Secondary recipient mice received two doses of total body irradiation (TBI) at 400cGy separated by 4-6hours and underwent transplant 24 hours later.

2.6.2 Colony forming cell assay

After 16 weeks, recipients were culled and femurs and tibias were harvested. Bones were crushed and stained for donor cells. Cell sorting was used to sort 10,000 donor cells for CFC plating (as described above).

2.6.3 Annexin V assay

To measure apoptosis, Annexin V staining was utilised. This is because Annexin V, a calcium-dependent phospholipid-binding protein, binds to phosphatidylserine (PS) of the inner surface of the plasma membrane. Under apoptosis, PS is translocated to the extracellular surface of the plasma membrane thereby making it available for detection by fluorescently labelled Annexin V. In our study, 16 weeks after transplant, recipient mice were culled and femurs and tibias were harvested. Bones were crushed and stained for LSK cells (As described above). After this, cells are resuspended in 100ul of 1x Annexin V buffer (BD Bioscience 10x) and 2.5ul of Annexin V. Importantly, Annexin buffer contains calcium which is essential for the binding of Annexin V to PS. The calcium molecules, when bound to Annexin V, cause a conformational change which allows the binding of Annexin V to PS. Cells are then incubated in the dark at room temperature for 30 minutes. After this, an additional 300ul of 1x Annexin V buffer is added to the cells along with DAPI (0.1ug/ml final concentration) which binds to the exposed DNA of apoptotic cells. These are then kept on ice and are immediately analysed by flow cytometry.

2.6.4 Ki67 assay

Ki-67 is a protein that is expressed in the nuclei of proliferating cells. Its function is to control heterochromatin organisation during cell proliferation. Cells are therefore permeabilised so that the Ki67 antibody my reach the nucleus of the cell. DAPI is also used in this assay to measure DNA content. This is because DNA replication occurs during cell division and so DAPI is used. With this, recipient mice were culled 16 weeks after transplant and femurs and tibias were harvested. Bones

were crushed and stained for LSK-SLAM cells (As described above). After this, cells are washed with PBS and centrifuged for 5 minutes (500g at 4°C). The supernatant is discarded and cells are resuspended in 200 µl fix buffer. Cells are incubated in fix buffer for 15 minutes on ice. Cells are the centrifuged for 5 minutes (500g at 4°C). After removing the supernatant, cells are resuspended in 200 µl of the permeabilise buffer and incubated for 15 minutes on ice. These are then centrifuged for 10 minutes (300g 4°C) and the supernatant is removed. The cells are then washed with 1 ml wash buffer and again centrifuged for 5 minutes (500g 4°C). The supernatant is removed and cells are then resuspended in 100 µl staining buffer and incubated for 30 minutes on ice in the dark. Cells are washed with 1 ml FACS buffer and centrifuged for 5 minutes (500g at 4°C). The supernatant is discarded and cells are resuspended in 400 µl FACS buffer with DAPI (5 µM final concentration). Cells are then analysed by flow cytometry.

2.6.4.1 Buffers

Fix Buffer: PBS 1%PFA. To prepare 1 mL: 250 µl PFA 4% $^{+}$ 750 µl PBS.

Permeabilise Buffer: PBS 0.1% Saponin 2%BSA. To prepare 1 mL: 267 µl BSA 7.5% $^{+}$ 734 µl PBS $^{+}$ 1 µl X-100 Triton.

Wash Buffer: PBS 0.1% Saponin. To prepare 1 mL: 1000 µl PBS $^{+}$ 1 µl Saponin.

FACS Buffer: PBS 2% BSA. To prepare 1 mL: 267 µl BSA 7.5% $^{+}$ 734 µl PBS.

Staining Buffer: Permeabilise buffer $^{+}$ Ki-67 antibody (dilution 1:100)

	Donor pulse concentration	Number of cells/recipient		Ratio
		Donor	Competitor	
Yohimbine	PBS	$3.75x10^5$	$3.75x10^5$	1:1
	100mg/kg	$2.5x10^5$	$5x10^5$	1:2
	100mg/kg	$3x10^5$	$4.5x10^5$	1:1.5
Oxa-22	PBS	$3.75x10^5$	$3.75x10^5$	1:1
	10ng/kg	$3.75x10^5$	$3.75x10^5$	1:1
	100ng/kg	$3x10^5$	$4.5x10^5$	1:1.5

Table 2.2 Donor: Competitor ratios for competitive transplant.

Ratios were calculated based on the effects of the compounds on HSC numbers *in vivo*. Where a significant increase in HSC was observed after *in vivo* administration of the compound, the competitor was increased proportionally so that ultimately the same ratio of donor: competitor HSCs was transplanted.

2.7 Administration of small molecules following bone marrow transplantation

Recipient mice were transplanted with a limiting dose of cells ($5x10^5$ per recipient). They were then treated with Yohimbine every two days for a duration which equalled 10 treatments with the mentioned doses (PBS, 1mg/kg, 10mg/kg and 100mg/kg). This was also the case for Phthalylsulfathiazole (DMSO, 1mg/kg, 10mg/kg and 100mg/kg for 10 treatments) and Oxa-22 (PBS, 10ng/kg, 100ng/kg and 1ug/kg for 5 treatments). Again, engraftment was assessed by weekly bleeds and analysed by flow cytometry as mentioned above. Secondary transplant, analysis of apoptosis (Annexin V assay) was conducted as described above.

2.8 Leukaemic cell viability assay

For the viability analysis of leukaemic cells, 6 human leukaemic cell lines were used (Table 2.3). Cells were seeded in 96-well plates at 3000 cells per well. They were then treated 1 hour after being plated and incubated for 5 days (Figure 2.3). All cell lines were treated with the compounds at a concentration range of 1nM-1mM increasing in 10 fold. At the end points, cell viability was measured using The CellTiter-Blue® Cell Viability Assay (Promega). This assay is dependent on the fluorescence emitted by resorufin. Viable cells reduce resazurin to resorufin, this then emits a detectable fluorescence, and thus the fluorescence is proportional to the number of viable cells. Fluorescence was measured using the Clariostar Reader (BMG Labtech Inc).

Cell line	Leukaemic Type	Origin	Gene
K562	CML in blast crisis	53-year-old woman with chronic myeloid leukemia (CML) in blast crisis in 1970	cells carry the BCR-ABL1 e14-a2 (b3-a2) fusion gene
KG-1	CML in blast crisis	59-year-old man with erythroleukemia that developed into acute myeloid leukemia (AML)	8p11 FGFR1OP2-FGFR1 fusion
NOMO1	AML	Bone marrow of 31-year-old woman with acute myeloid leukemia (AML FAB M5a)	cells carry the t(9;11)(q23;p22) MLL-MLLT3 (MLL-AF9) alteration
NB-4	APL	bone marrow of a 23-year-old woman with acute promyelocytic leukemia (APL = AML FAB M3)	cells carry the t(15;17) PML-RARA fusion gene
HL-60	APL	35-year-old woman with acute promyelocytic leukemia (AML FAB M2)	8q24 amplification harbouring MYC

Table 2.3 Human leukaemic cell lines.

The mentioned cell lines used in the viability assays with the test compounds. (CML: Chronic myeloid leukemia, AML: Acute Myeloid leukaemia, APL: Acute Promyelocytic leukemia)

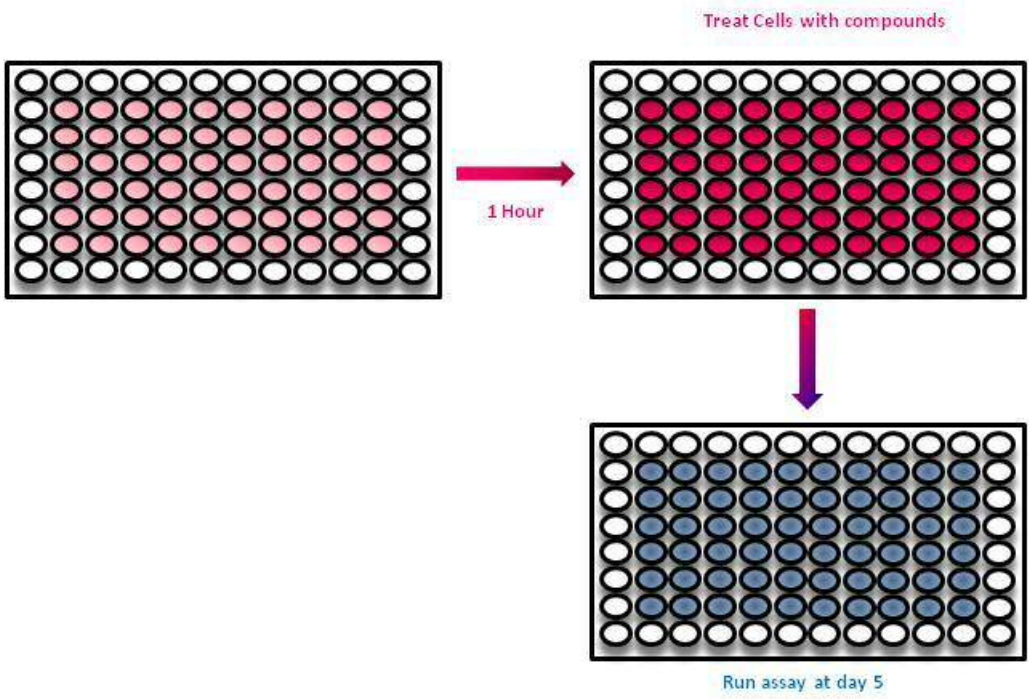

Figure 2. 3 Schematic of experimental set up of the *in vitro* AML proliferation assay

Cells were plated in the first instance and treated with a range of drug concentrations. The treated cells were incubated for 1, 3 and 5 days. At each time point treated cells are exposed to the cell viability reagent where the fluorescent product is detected in a plate reader. The fluorescence produced corresponds to the number of viable cells in the sample.

2.9 Generation of *in vivo* leukaemic mouse models

Bone marrow was harvested from wildtype C57BL6 mice and underwent cKit enrichment using cKit$^-$ positive magnetic beads. The AutoMACS was used to enrich for purified cKit$^+$ cells. cKit$^+$ cells were then transduced with a retrovirus containing the MLL-AF9 translocation and a GFP marker. The construct is a MSCV- based plasmid containing the MLL-AF9 translocation, IRES and GFP (MSCV-MLL-AF9-IRES-GFP) kindly donated by Daniella Krause (Krause et al. 2013). MLL-AF9 GFP$^+$ transduced cells were isolated by FACS and plated in colony forming cell assay (CFC). Colonies are positively selected and serially plated another two times. Purified MLL-AF9 GFP$^+$ cells are harvested and enriched further for cKit, these are termed as pre-leukaemic stem cells (Pre-LSCs). These are then transplanted into irradiated recipients where they propagate. When mice become moribund or succumb to disease, bone marrow is harvested and stained for leukaemic stem cells (LSCs), LSCs are characterised by cKit$^+$ GFP$^+$ CD16/32 $^+$ CD34$^+$.

2.10 Administration of small molecules on leukaemic mice

Upon the generation and transplantation of MLL-AF9 GFP$^+$ LSCs into lethally irradiated mice (900cGy), treatment with Oxa-22 (PBS, 10ng/kg and 100ng/kg) and Phthalylsulfathiazole (DMSO, 1mg/kg, 10mg/kg and 100mg/kg) commenced and continued every other day for the duration of the life of the mouse. When mice became moribund, bone marrow was harvested, crushed and stained for LSCs (cKit-PERCP, CD16/32-PeCy7 and CD34-APC). In addition to this spleen sizes were measured and weighed.

Chapter 3: The impact of Yohimbine, an adrenergic receptor antagonist, on haematopoietic stem and progenitor cell function

3.1 Introduction

It is recognised that complex signalling occurs within the bone marrow niche (Morrison and Scadden 2014). In addition to this, it is also understood that bone and the bone marrow niche may be influenced by external signals, particularly through neural innervation (Cosentino et al. 2015). The majority of blood vessels around the body are innervated by the sympathetic (adrenergic) system via the release of norepinephrine (noradrenaline) (Engel et al. 1992). The sympathetic nervous system is a branch of the autonomic nervous system which regulates the body's "fight-or-flight" responses such as increased heart rates and the dilation of blood vessels and the bronchioles (Gordan et al. 2015). Some of these innervated blood vessels convene with bones and cartilage and continue into the bone marrow in parallel with adrenergic sympathetic fibres (Nance and Sanders 2007). The role of the sympathetic innervation within the bone marrow has been extensively studied to elucidate its effects on HSCs and other bone marrow cells. The modulation of HSCs by the sympathetic nervous system was first demonstrated by studies conducted on circadian rhythms. It was shown that the release of noradrenaline displayed a circadian rhythm within the bone marrow in which a peak was observed at night and resulted in an increase in cells entering the cell cycle (Maestroni et al. 1998). It was also shown that treatment with noradrenaline resulted in increased proliferation of bone marrow cells and recovered mice that underwent chemotherapy (Maestroni et al. 1997; Lucas et al. 2013). More importantly, noradrenaline was discovered to induce HSC proliferation and migration (Spiegel et al. 2007). In support of this, it was found that sympathetic innervation aids HSC mobilisation from the bone marrow (Katayama et al. 2006). In this study, they looked at the role of UDP-galactose:ceramide galactosyltransferase (Cgt) which produces galactocerebrosides (GCs). These GCs are the main components of the myelin sheath that encompasses neural fibres. They found that $Cgt^{-/-}$ mice are unable to undergo HSC in response to G-CSF stimulation (Katayama et al. 2006). Interestingly however, it was discovered that this effect was caused due to deficient signalling and innervation of the surrounding osteoblasts and in turn gave rise to perturbed mobilisation of HSCs (Katayama et al. 2006). This suggested that cells of the bone marrow niche were also sensitive to adrenergic signalling; this was shown indeed to be the case. It was demonstrated that adrenergic signalling within the bone

marrow negatively impact mesenchymal stem cells (MSC) expressing HSC-maintaining genes (Mendez-Ferrer et al. 2010). Adrenergic denervation resulted in the proliferation and expansion of otherwise quiescent MSCs whereas adrenergic stimulation decreased the differentiation of osteoblasts and thus a decrease in HSC-maintaining genes (Mendez-Ferrer et al. 2010; Lucas et al. 2013). It still remains unclear how adrenergic ligands exert their effects on adrenergic receptors; however these receptors have been extensively studied and characterised physiologically. For example, it is known that Yohimbine, which is the focus of this chapter, is an antagonist of the α-2 subtype of the adrenergic receptors (Verwaerde et al. 1997). This subtype of receptors belongs to the Gi G-protein coupled receptor family which is involved in the inhibition of intracellular cAMP production and further neuro-signalling pathways (Philipp et al. 2002). This demonstrates the existence of a complex network of pathways within the bone marrow niche and its regulation on HSCs through the sympathetic nervous system.

It has previously been shown that adrenergic receptor expression is widespread within haematopoietic populations (Muthu et al. 2007). Studies have also shown targets of adrenergic receptors have been implemented in stem cell function (Maestroni and Conti 1994). These early studies have shown that administration of adrenergic receptor antagonists, including Yohimbine, elicited increased neutrophil and platelet production in normal mice and after syngeneic bone marrow transplant. However, the direct impact of Yohimbine on HSC/HPCs on ex vivo expansion and, *in vivo*, in normal and transplant settings remains unclear.

3.2 Adrenergic receptor expression in haematopoietic cells

Yohimbine is a known to target of adrenergic receptors, more specifically the α2-adrenergic receptor (Adra-A2) (Inoue et al. 1991). These receptors have been shown to be present in the bone marrow and the surrounding niche (Muthu et al. 2007). Here, we examined the expression of this receptor in highly specific FACS isolated populations. HPSCs from bone marrow were isolated and RT q-PCR was conducted to confirm the expression of Adra-A2 receptors (Figure 3.1). We observed expression of the Adra-A2 receptor throughout the HSPC populations as well as in mature myeloid ($Mac1^{+}Gr1^{+}$), B- and T- cells (Figure 3.1A). We also explored the expression of Adra-A2 in bone marrow derived MSCs that populate bone marrow niche elements (e.g. osteoblasts) and found expression in MSCs (Figure 3.1A). These data collectively show that Adra-A2 is expressed on prospectively isolated HSC, HPC and mature bone marrow cell populations and bone marrow niche precursors.

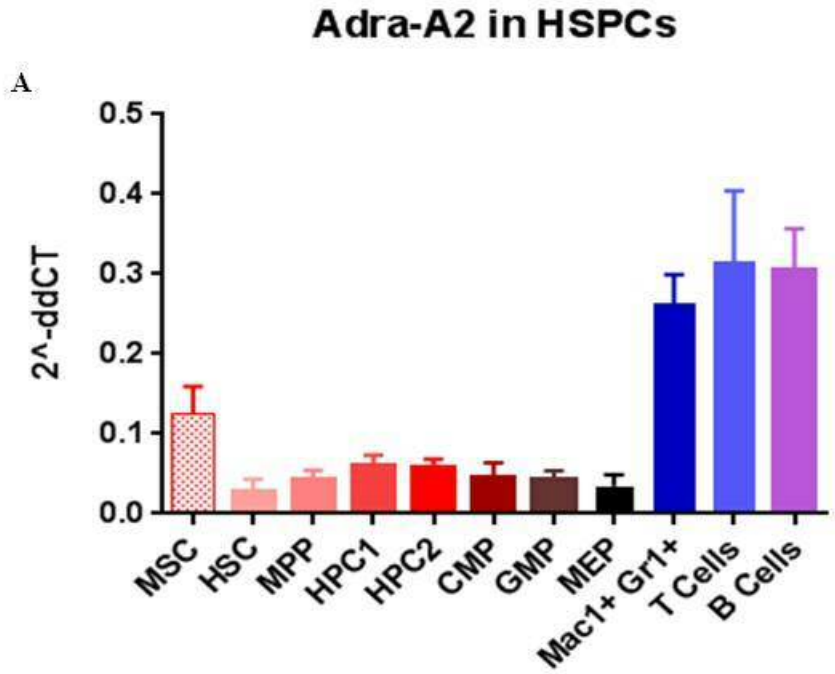

Figure 3. 1 Expression of α2- adrenergic (Adra-A2) receptor in MSCs, HSPCs and lineage differentiated populations.

(A) Adra-a2 RNA levels in MSCs, primitive stem cells and lineage restricted progenitors. Adra-a2 RNA levels also shown in Myeloid ($Mac1^{+}Gr1^{+}$) and lymphoid compartments (B- ($B220^{+}$) and T- ($CD4^{+}/CD8^{+}$) cells). Data represents mean $^{+}$ SEM from *n*=4. MSCs-Mesenchymal stem cells ($CD45^{-}Ter119^{-}Sca1^{+}CD140^{+}$)

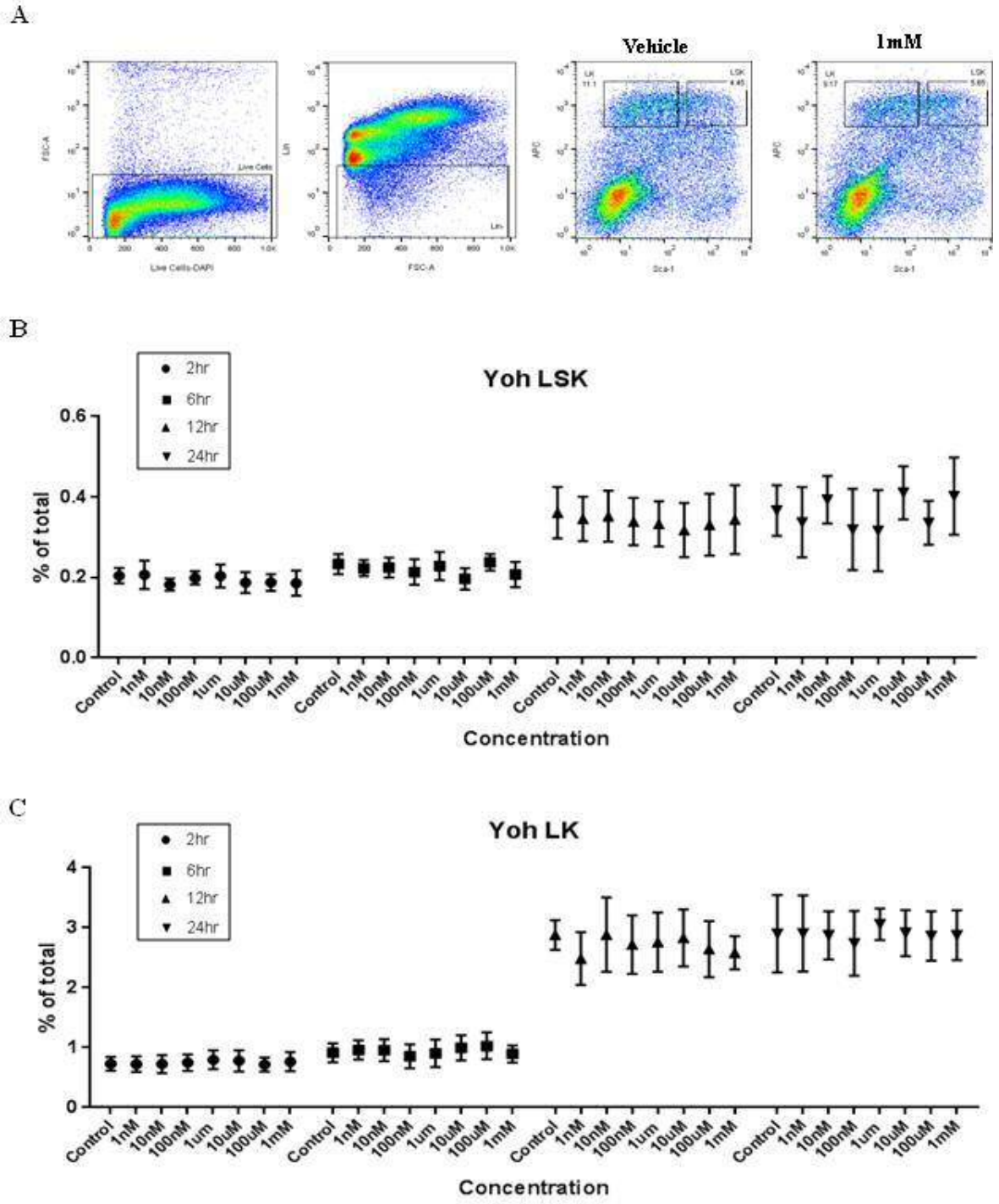

Figure 3. 2 Yohimbine does not affect immunophenotypic expansion of HSPCs ex vivo

Whole bone marrow (WBM) was harvested and plated at a density of $1x10^6$ cells per well. Cells were treated for 2, 6, 12 and 24 hours ex vivo using a range of concentrations (1nM-1mM). (A) Representative flow cytometric plots identifying LK (Lin- Sca-1^+) and LSK (Lin- Sca-1^+ $Ckit^+$) populations between vehicle treated cells compared to 1mM treated cells at 24hrs. The prevalence of (B) HSC enriched LSK and (C) LK cell populations were initially assessed after ex vivo exposure to Yohimbine. *N=3*, cells plated in triplicates, three separate experiments

3.3 Impact of Yohimbine on HSCs/HPCs ex vivo

To test the ability of Yohimbine on the expansion of HSCs ex vivo, we exposed whole bone marrow cells (suspended in StemSpan™ media without cytokines) with a range of Yohimbine concentrations (1nM-1mM) for a period of up to 24 hours. We found that Yohimbine did not elicit any changes in the frequencies of either LK or LSK cells during the measured timed periods (2, 6, 12 and 24 hours) compared to control cells exposed to vehicle (PBS) (Figures 3.2B and 3.2C). Although no differences were observed in the immunophenotypic analysis of HSCs/HPCs, this does not reflect their function in response to drug exposure. To assess this, analysis of colony formation was conducted through the colony forming unit (CFU) assay on WBM cells exposed to Yohimbine. We observed that exposure to Yohimbine for 2 hours caused a concentration dependent increase in total colony number; however this was not statistically significant (Figure 3.3E). The general rise in the total number of colonies may be explained by the increased occurrence of CFU-M (Figure 3.3E). 6 hour exposure to Yohimbine did not elicit any effects on progenitor cell function or lineage distribution of colonies (Figure 3.3E). Importantly, no change in multi-potent CFU-GEMM colony formation was noted after 2 and 6 hours of Yohimbine exposure (Figure 3.3E). This data shows that Yohimbine has little effect on HPSC number and function in the ex vivo setting.

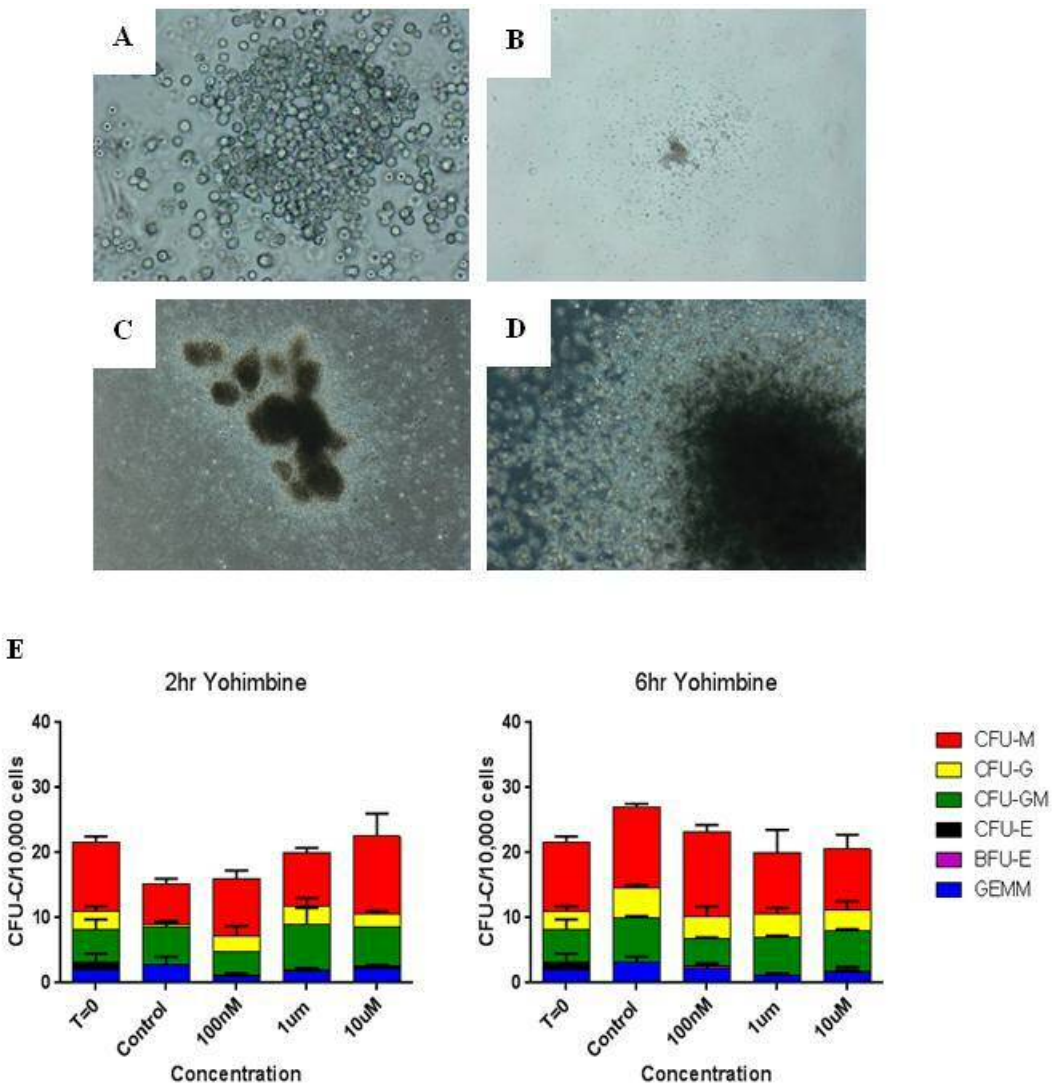

Figure 3. 3 Bone marrow cells cultured with Yohimbine possess normal colony forming potential

WBM cells were exposed to Yohimbine for 2 and 6 hours at concentrations of 100nM, 1uM and 10uM before washing and re-plating in Methylcellulose. After 10 days of maturation in both control and treated settings, multi-potential and lineage-restricted progenitors of the CFU-M, macrophage (A), CFU-G, granulocytic (B), CFU-GEMM, mixed and CFU-GM, granulocyte/monocyte (C and D) formed. Colonies are scored for each respective concentration (E) through duplicate wells in 3 separate experiments.

3.4 The impact of *in vivo* administration of Yohimbine treatment on HSC and HPC populations under homeostatic conditions

3.4.1 The impact of Yohimbine on HSCs

Yohimbine does not have an effect on HSC/HPC frequency or function ex vivo. This may be partly due to the *in vitro* conditions that the cells were incubated in, which is not a true reflection of the *in vivo* setting. For example, ex vivo expansion of HSCs was conducted in the absence of important bone marrow niche cells. It was therefore imperative to assess the impact of Yohimbine *in vivo*. (Figure 3.4A). Mice were treated with either Yohimbine (1 mg/kg or 10 mg/kg) or PBS (controls) twice daily for a period of 10 days. Bone marrow, spleen and blood were harvested at the end points and analysed by flow cytometry for HSC (SLAM markers (LSK $CD150^{+}CD48^{-}$)) and HPC (LK CD34/CD16/32) populations (Figure 3.4B). Lineage-positive fully differentiated cells were also investigated in the respective tissues.

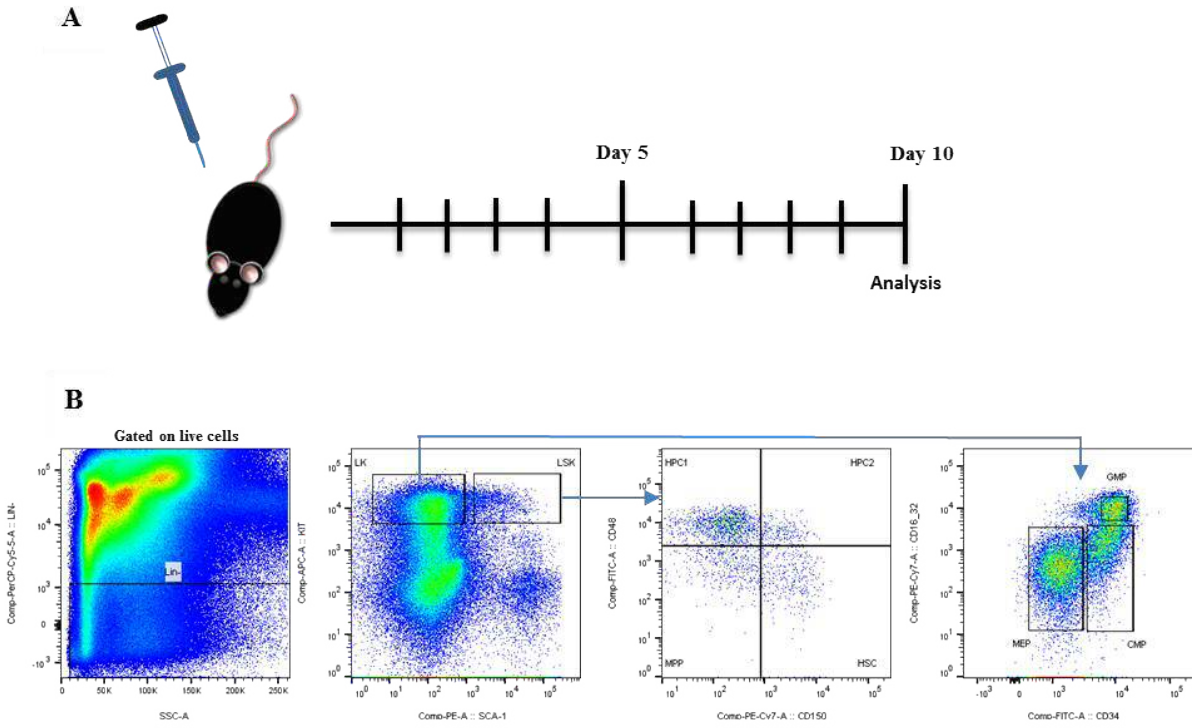

Figure 3. 4 Assessing the impact of Yohimbine on HSPCs *in vivo*

(A) Schematic of Yohimbine treatment. 8-10 week old mice treated twice daily over a period of 10 days. (B) Gating strategy of HSCs and progenitors: SLAM markers, derived from the LSK compartment giving rise to HSCs and primitive progenitors. Lineage restricted progenitors arise from the LK compartment giving rise to the GMP, CMP and MEP populations.

Treatment of Yohimbine caused a small, statistically insignificant increase in the frequency of LSK cells (Figure 3.5C). However, by assessing specific cell types within the LK and LSK compartments, we found that the specific HSC and HPC populations show differential sensitivity to Yohimbine treatment (Figure 3.5D-G). HSC, MPP and HPC1 frequencies were significantly elevated after 10 days of treatment of Yohimbine at 1 mg/kg and 10 mg/kg. A moderate effect was seen in the HPC2 subpopulation, though this was statistically insignificant (Figure 3.5G). However, the frequencies of myeloid lineage restricted progenitor populations (CMP, GMP, MEP) were not significantly affected (Figure 3.6). In addition to myeloid progenitors, we also looked at lymphoid restricted progenitors. The common lymphoid progenitor (CLP) was characterised as $Lin^{-}Sca^{med}cKit^{med}CD127^{+}$ (Kondo et al. 1997; Mayle et al. 2013). In addition to the CLP population, we also looked to study the lymphocyte-primed multipotent progenitors (LMPP). These were identified as LSK $CD34^{+}Flt3^{+}$ as shown in Figure 3.7 (Adolfsson et al. 2005). Treatment with Yohimbine (10 mg/kg) elicited a significant increase in CLPs however no effects were seen on the LMPP subpopulation (Figure 3.7B). These data show that Yohimbine acts to expand primitive HSPC populations and lymphoid restricted progenitors, as judged by immunophenotypic analysis.

Total cell counts from bone marrow were also measured in response to Yohimbine treatment (Figure 3.8). A significant increase in total cellularity was observed within treated animals compared to PBS controls (Figure 3.8A). Normalising for the increase in cellularity after Yohimbine treatment, the effect on the absolute HSC/MPP populations was statistically significant within the 1mg/kg treated animals (Figure 3.8B-F). These data overall show that *in vivo* administration of Yohimbine causes an absolute expansion in immunophenotypically defined bone marrow HSC and primitive progenitor populations under homeostatic conditions.

Haematopoiesis also occurs in the spleen (O'Neill et al. 2011). We therefore assessed HSC/HPC frequency at this site following *in vivo* administration of Yohimbine. Yohimbine did not elicit the same effects on HSPCs in the spleen as seen in the bone marrow (Figure 3.9) and no significant changes in myeloid populations (Figure 3.10) suggesting that the effects of Yohimbine are bone marrow specific.

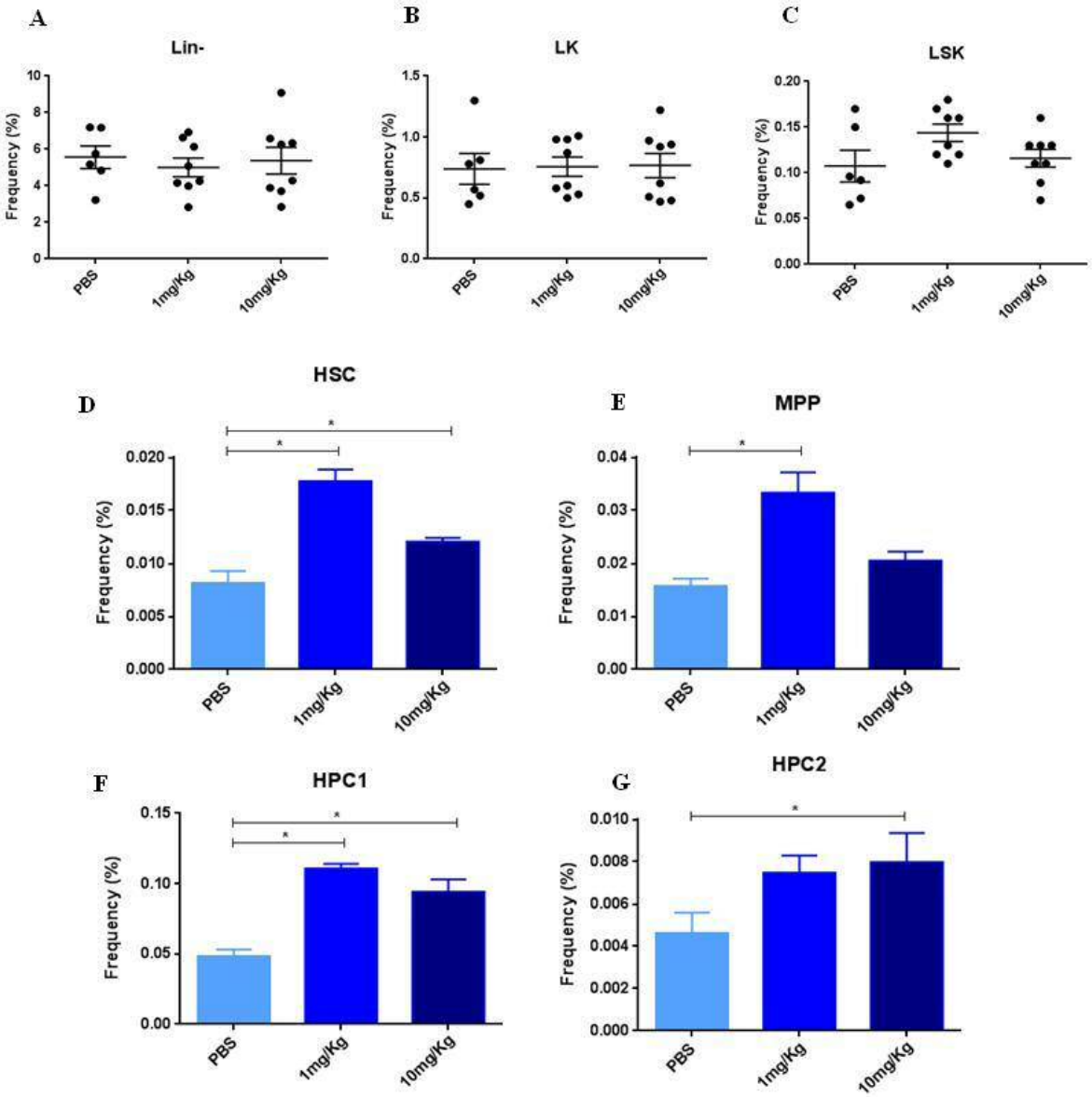

Figure 3. 5 Yohimbine expands immunophenotypically defined HSPC populations *in vivo*

Mice were treated with 1 mg/kg or 10 mg/kg of Yohimbine, or PBS twice daily for 10 days. 1 hour after the final administration, bone marrow was harvested and stained for Lin- (A), LK (B) and LSK (C) markers for flow cytometry analysis. Specific HSC and primitive progenitor populations from within the LSK compartment were also analysed (D-G). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *p<0.05.

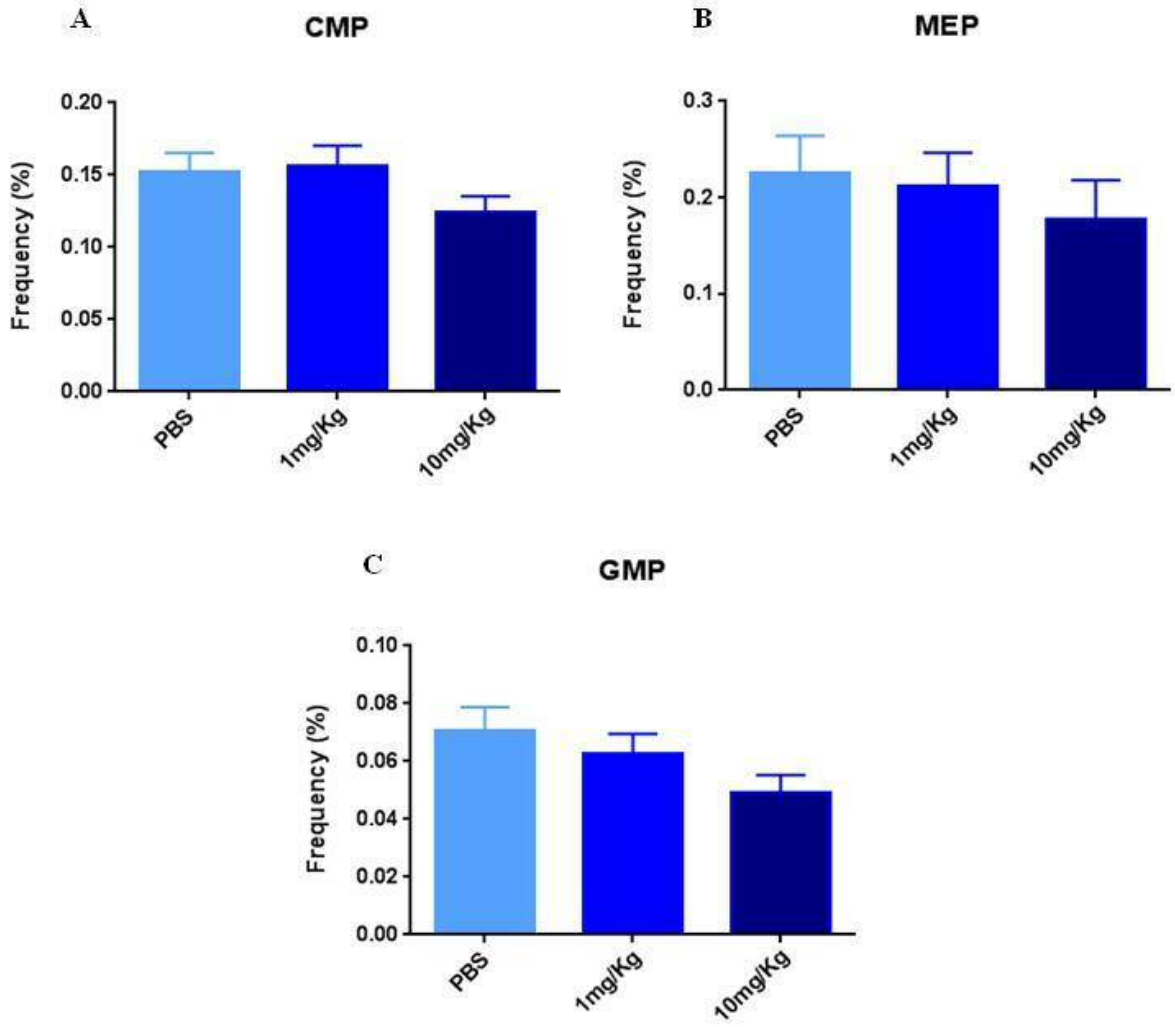

Figure 3. 6 Yohimbine treatment does not affect lineage restricted progenitors *in vivo*

Mice were treated with 1 mg/kg or 10 mg/kg of Yohimbine, or PBS twice daily for 10 days. 1 hour after the final administration, bone marrow was harvested and stained for progenitor populations derived from the LK compartment were analysed (A) CMP (common myeloid progenitor), (B) MEP (megakaryocyte-erythroid progenitor) and (C) GMP (granulocyte-macrophage progenitor). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS)

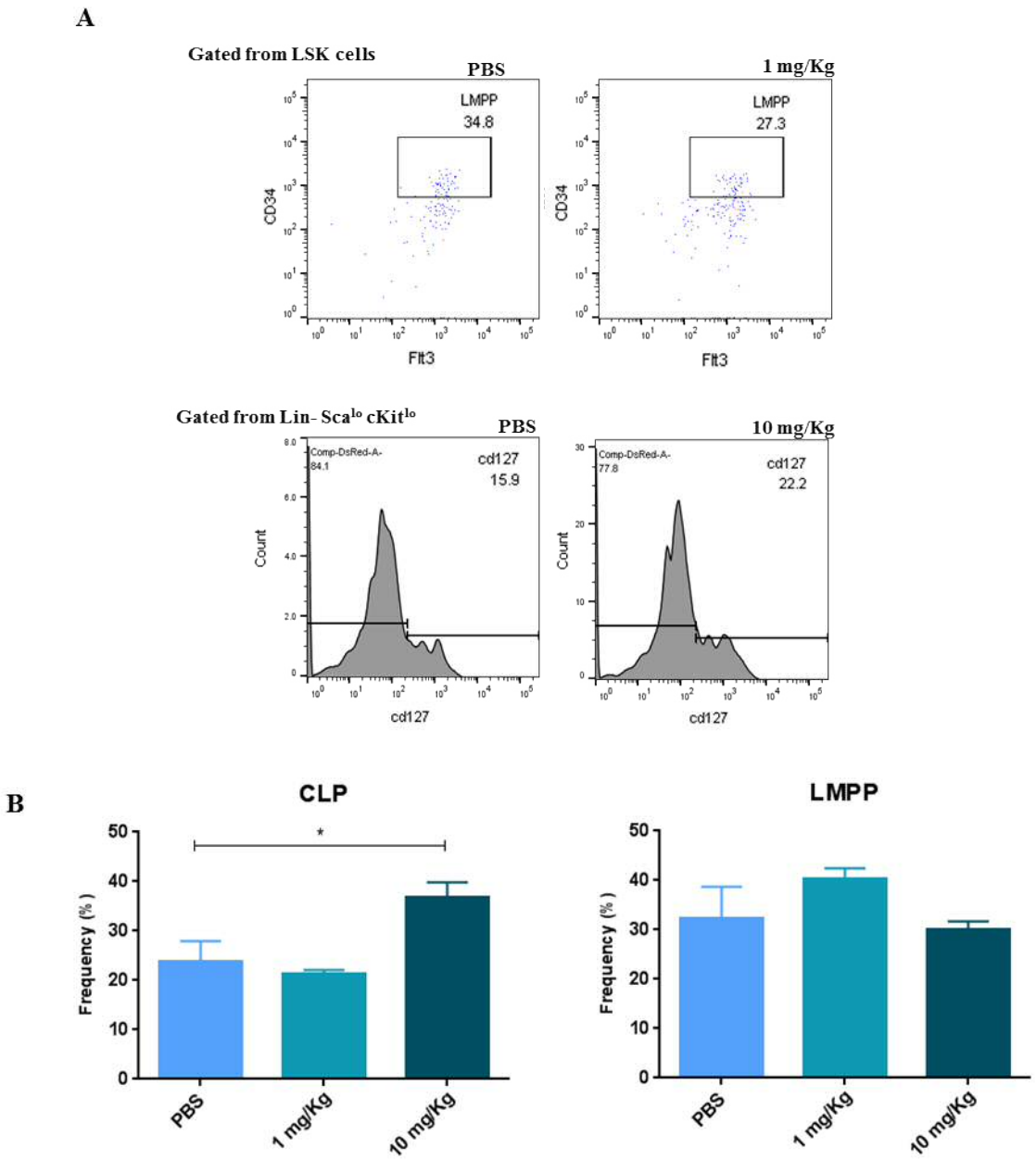

Figure 3. 7 *In vivo* treatment of Yohimbine increases lymphoid restricted progenitor frequencies

Mice were treated with 1 mg/kg or 10 mg/kg of Yohimbine, or PBS twice daily for 10 days. 1 hour after the final administration, bone marrow was harvested (A) The common lymphoid progenitors (CLP) are derived from the Lin-Sca^{med}cKit^{med}CD127^{+} compartment. Lymphoid-primed multipotent progenitors (LMPP) are derived from the LSK compartment and are characterized by CD34^{+}Flt3^{+}. (B) The frequency of CLP and LMPP populations in response to Yohimbine treatment. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$.

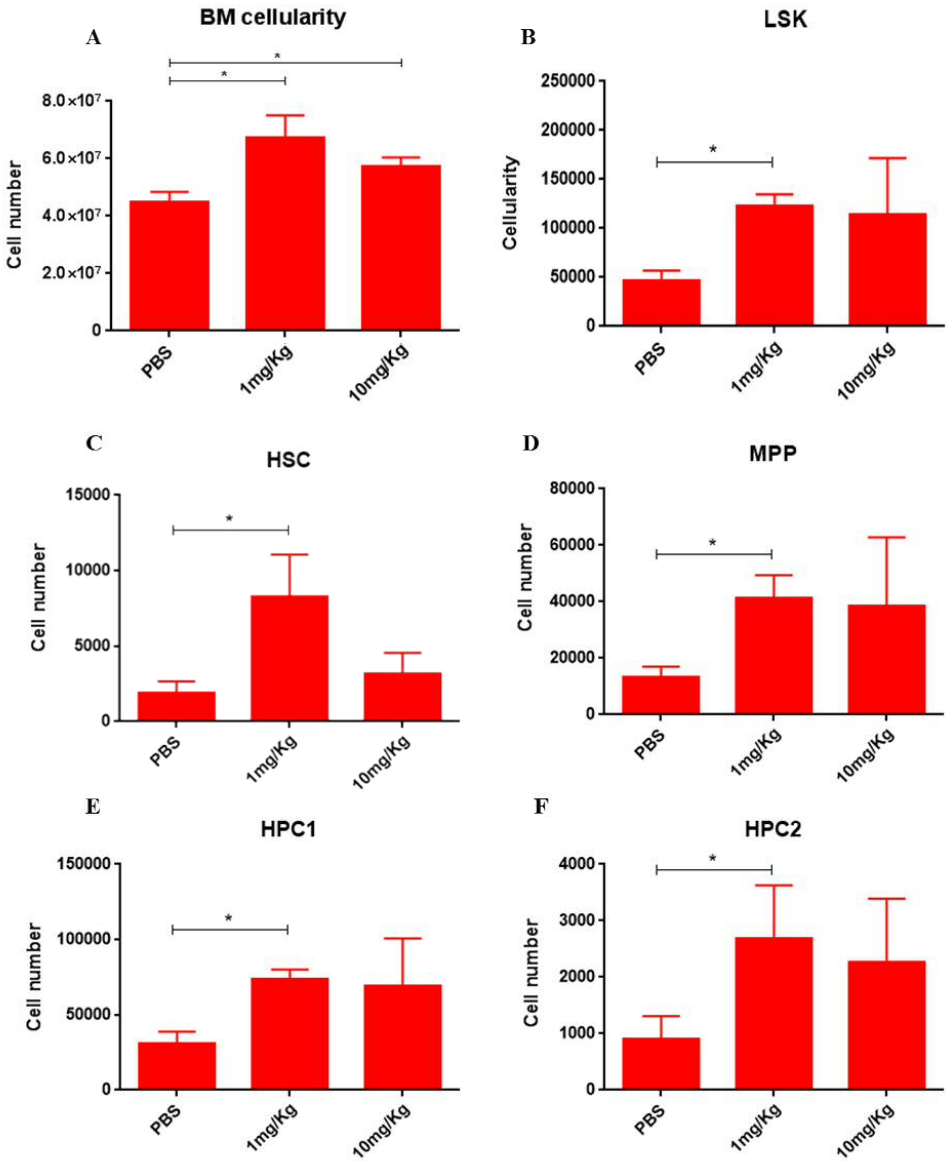

Figure 3. 8 Increased cellularity and expansion of immunophenotypic HSPCs after *in vivo* administration of Yohimbine

Mice were treated with Yohimbine (1 mg/kg or 10 mg/kg of) or PBS twice daily for 10 days. 1 hour after the final administration, bone marrow was harvested and counted (A). Cells harvested from one tibia and one femur. Cell numbers were calculated using the frequencies (percentages) of each cell type against the total number of live cells from populations arising from within the LSK compartment (B-F). Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$.

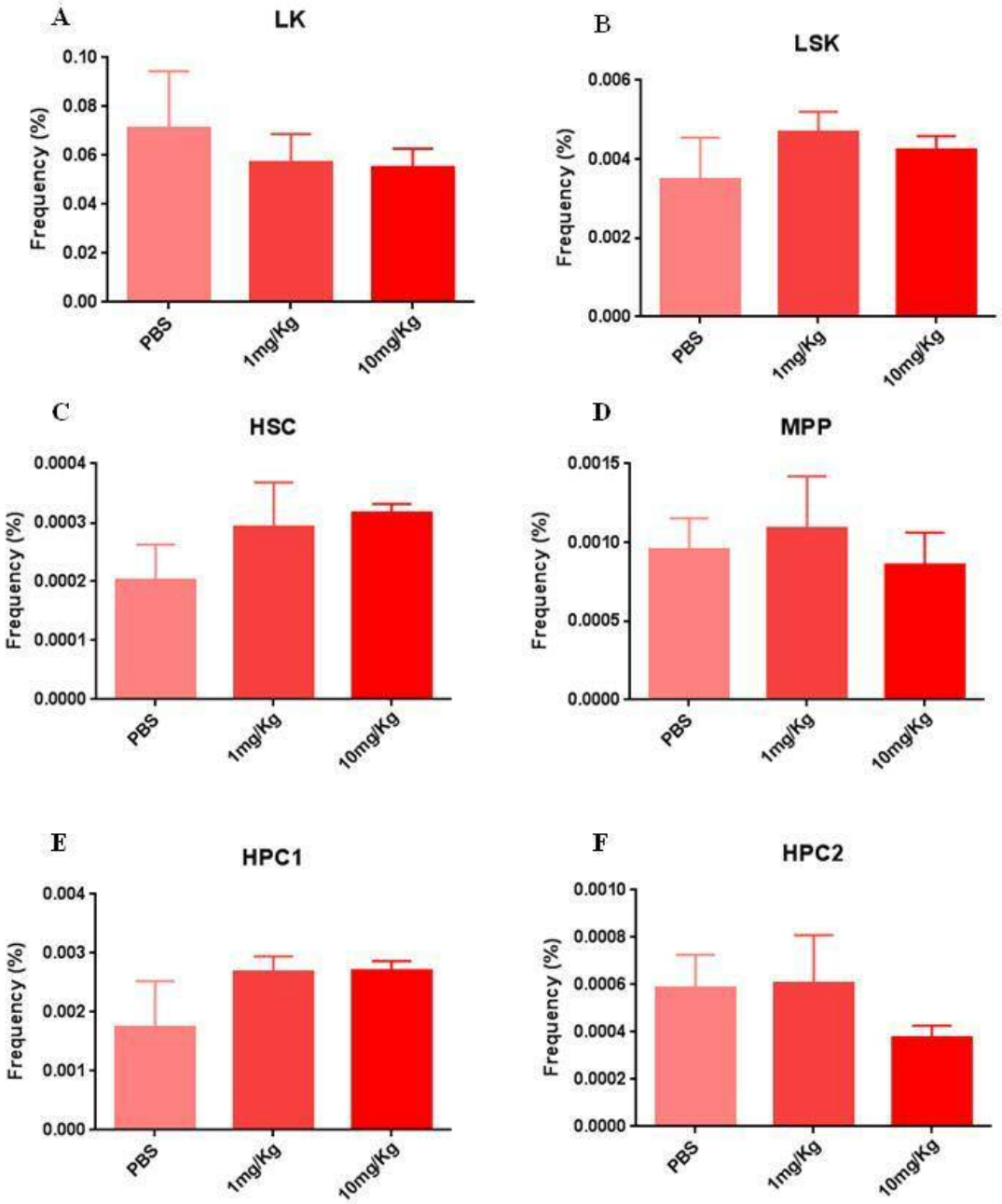

Figure 3. 9 Administration of Yohimbine does not affect splenic HSPC population *in vivo*

Mice were treated with Yohimbine (1 mg/kg or 10 mg/kg of) or PBS twice daily for 10 days. 1 hour after the final administration, the spleens of the animals were obtained and processed into suspension. LK (A), LSK (B) and specific HSPC populations from within the LSK compartment were also analysed by flow cytometry (C-F). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS)

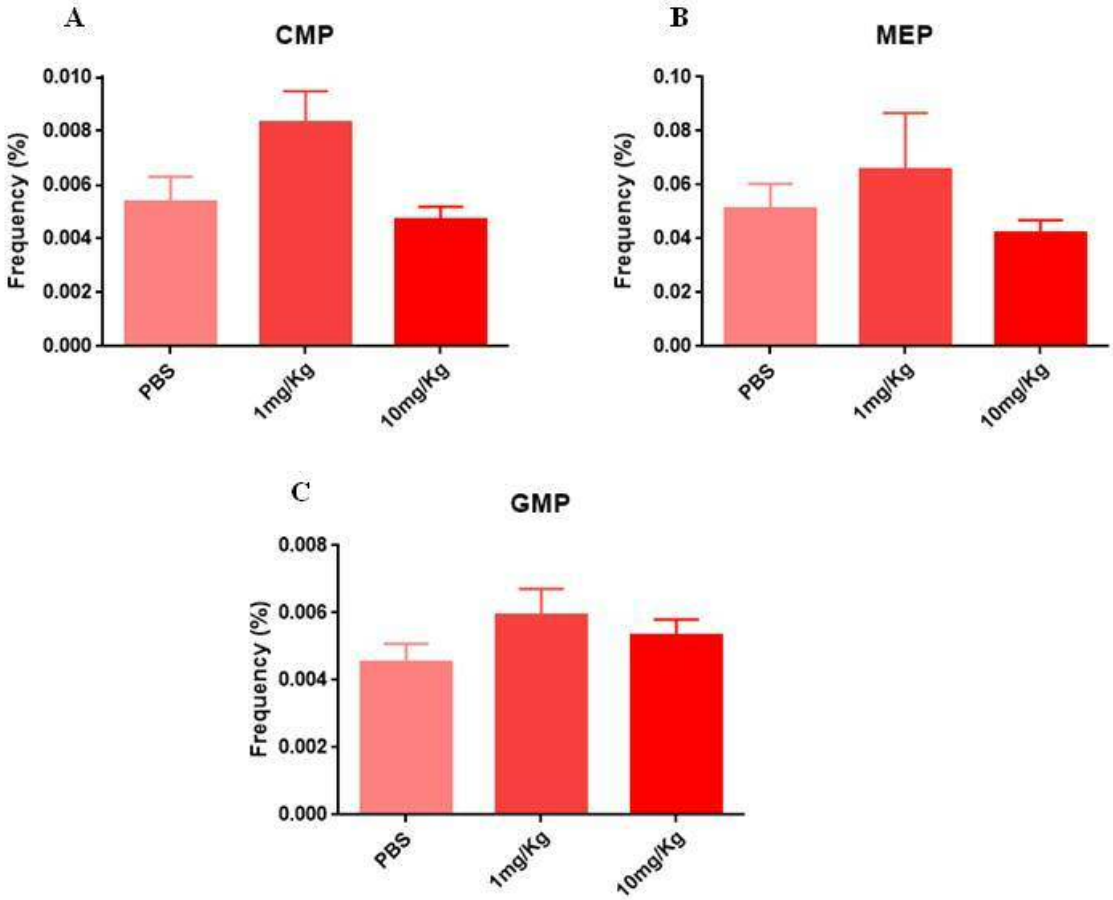

Figure 3. 10 *In vivo* Yohimbine treatment does not affect progenitor cells of spleen tissue

Mice were treated with Yohimbine (1 mg/kg or 10 mg/kg of) or PBS twice daily for 10 days. 1 hour after the final administration, the spleens of the animals were obtained and processed into suspension. Progenitor populations derived from the LK compartment were analysed (A) CMP (common myeloid progenitor), (B) MEP (megakaryocyte-erythroid progenitor) and (C) GMP (granulocyte-macrophage progenitor). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS)

Since it has been shown that adrenergic innervation is also involved in the egress of HSCs from the bone marrow (Katayama et al. 2006), we hypothesised that Yohimbine in addition to HSC/MPP expansion, could induce HSC mobilization. Peripheral blood was obtained from all experimental mice prior to culling for analysis (Figure 3.11). We saw that from all blood samples, no LK or LSK cells were present within the peripheral blood (Figure 3.11E). Thus, Yohimbine administration alone does not elicit HSC mobilisation from the bone marrow to the peripheral blood.

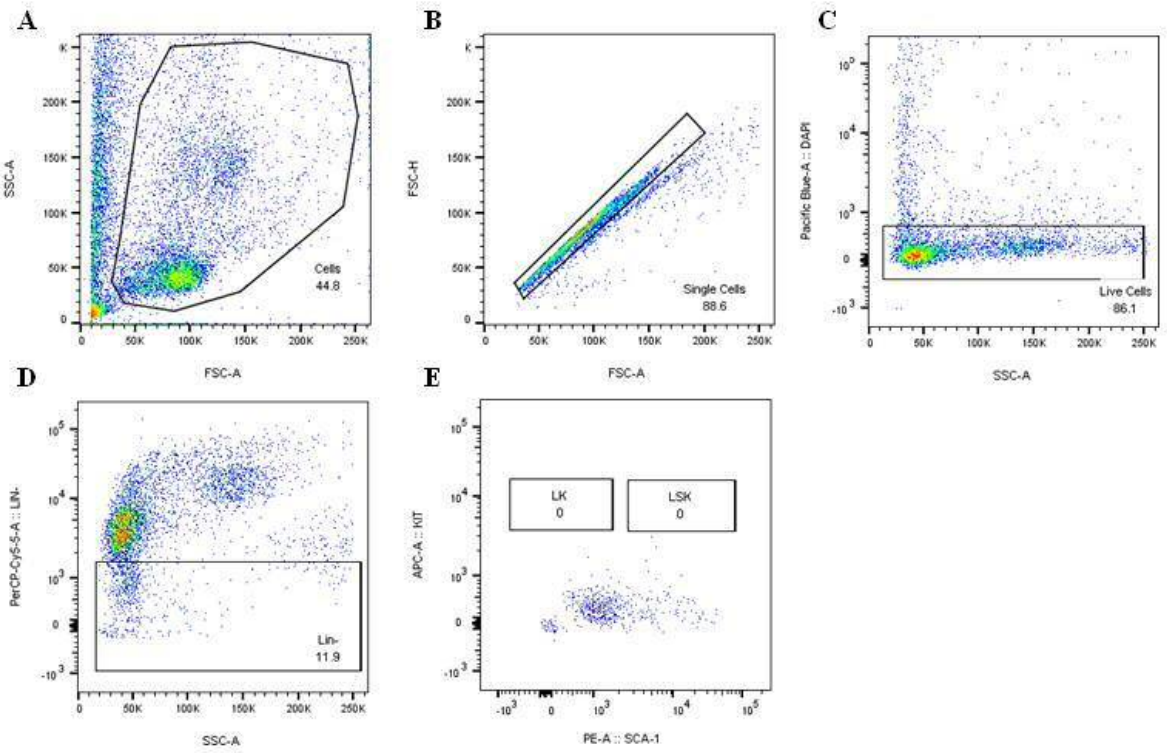

Figure 3. 11 Yohimbine does not mobilize bone marrow HSPCs to the peripheral blood in treated mice

Mice were treated with either Yohimbine or PBS twice daily for 10 days. 1 hour after the final administration, blood was taken via an incision of the lateral tail vein and stained for LK (Lin-cKit^{+}) and LSK (Lin-Sca1^{+}cKit^{+}) populations. (A-E) The gating strategy applied to determine HSPC populations of blood samples taken from treated mice

3.4.2 The impact of Yohimbine on committed progenitor formation and mature differentiated cells

To assess the impact of Yohimbine on committed progenitor cell formation, we performed the CFU assay and found that there was a profound increase in the total number of colonies from mice treated with 1 mg/kg compared to PBS controls. These differences were largely due to the significant increases in CFU-M and CFU-GM colonies. This effect however was not observed in response to 10 mg/kg. (Figure 3.12). These findings strongly support the idea that Yohimbine promotes myelopoiesis under steady state conditions

Next, by flow cytometry, we analysed the differentiation potential of lineage-committed progenitors and lineage differentiated cell populations in response to drug treatment *in vivo*. Lymphoid (T-cells ($CD4/8^+$) and B-cells ($CD19^+$) (Figure 3.13A)), Myeloid ($Mac1^+Gr1^+$ (figure3.13B)) and erythroid-like ($Ter119^+$ (Figure 3.13C) cells were studied in the bone marrow, spleens and blood of treated animals. Figure 3.14 shows the effects of Yohimbine on lineage differentiated cells in the bone marrow after 10 days of treatment. We observed that Yohimbine did not impact the frequency of lymphoid cells (Figure 3.14A and 3.14B). Surprisingly, 10 mg/kg of Yohimbine treatment caused a significant decrease in myeloid cells ($Mac1^+Gr1^+$) (Figure 3.14C). The same analysis was conducted from spleen tissue (Figure 3.15) and blood (Figure 3.16). B-cell frequency was significantly reduced in response to 10 mg/kg in the spleen (Figure 3.15A) however other cell types were not significantly affected. Within peripheral blood lineage differentiated cells, Yohimbine, at both doses, caused a detrimental decrease in the frequency of B-cells (Figure 3.16A) contrasting with an increase in the frequency of T-cells (Figure 3.16B) in response to 10 mg/kg. Animals treated with 10mg/kg Yohimbine also exhibited elevated frequencies of $Mac1^+Gr1^+$ and Gr1 cells (Figure 3.16C and 3.16E respectively). These data collectively show that Yohimbine does not affect lymphoid populations within the bone marrow but it enhances T cells in the peripheral blood and mildly depletes B-cells in the spleen and peripheral blood. Although a decrease in bone marrow $Mac1^+Gr1^+$ cells is observed, the opposite effect is seen in the peripheral blood suggesting that Yohimbine may either promote the survival or migration of myeloid cells to the peripheral blood.

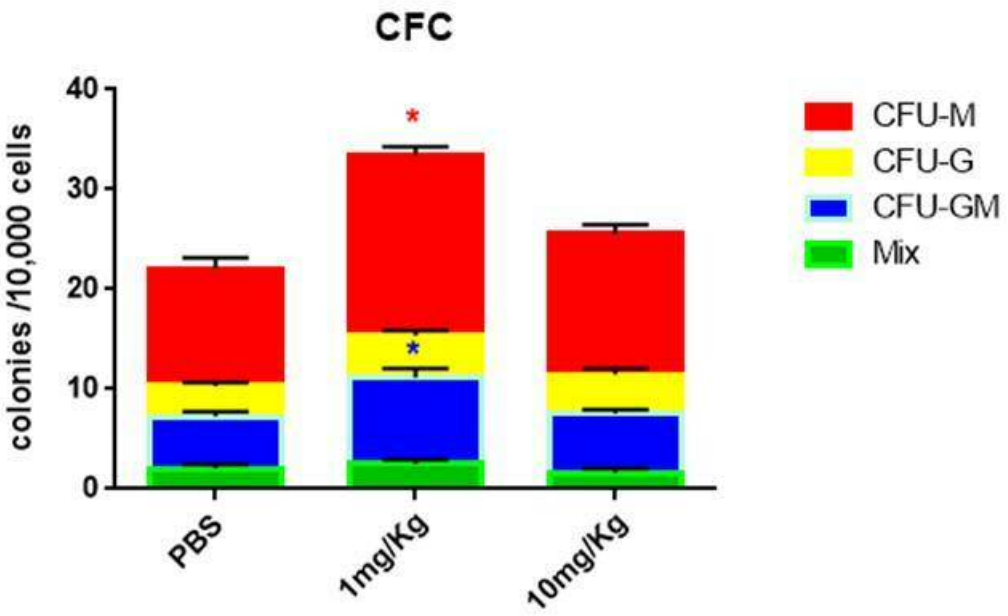

Figure 3. 12 *In vivo* treatment with Yohimbine impacts bone marrow colony forming potential by increasing CFU-M and CFU-GM

Animals were treated with Yohimbine twice daily for 10 consecutive days. After the final injection, bone marrow from the respective treated animals were harvested and plated in methylcellulose at a density of 10,000 cells. Colonies are scored after 10 days from plating in duplicate wells in two separate experiments, $n=3$ in each experiment. *$p<0.05$

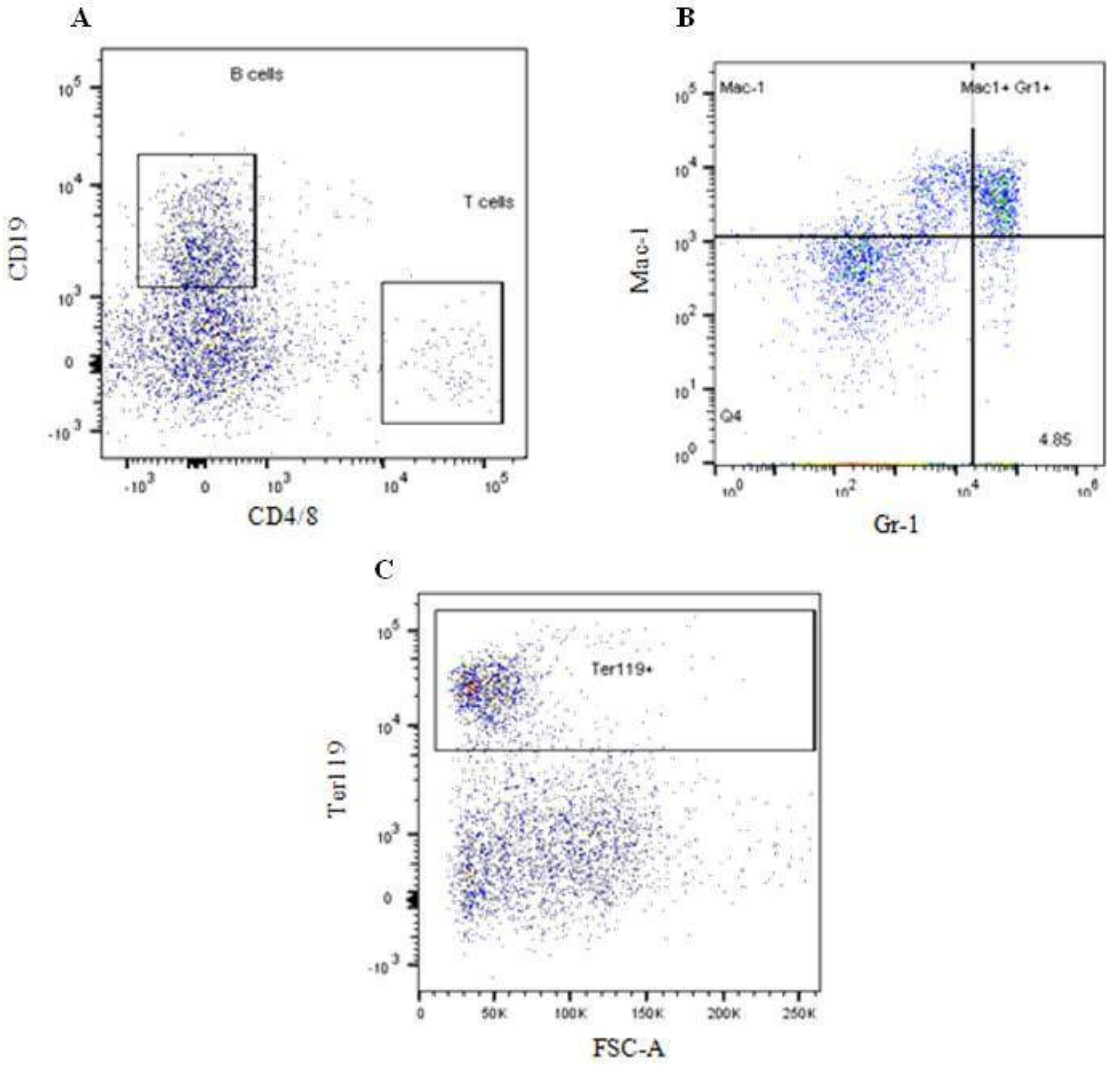

Figure 3. 13 The impact of Yohimbine treatment *in vivo* on lineage committed cells in the peripheral blood

Mice were treated with either Yohimbine or PBS twice daily for 10 days. 1 hour after the final administration, blood was taken via an incision of the lateral tail vein and stained for mature differentiated populations. Representative examples of flow cytometry analysis to assess the effects of Yohimbine on B- (B220) and T-cells ($CD4^+CD8^+$) (A), myeloid cells (Mac1 and Gr1) (B) and erythroid-like cells ($Ter119^+$) (C) .

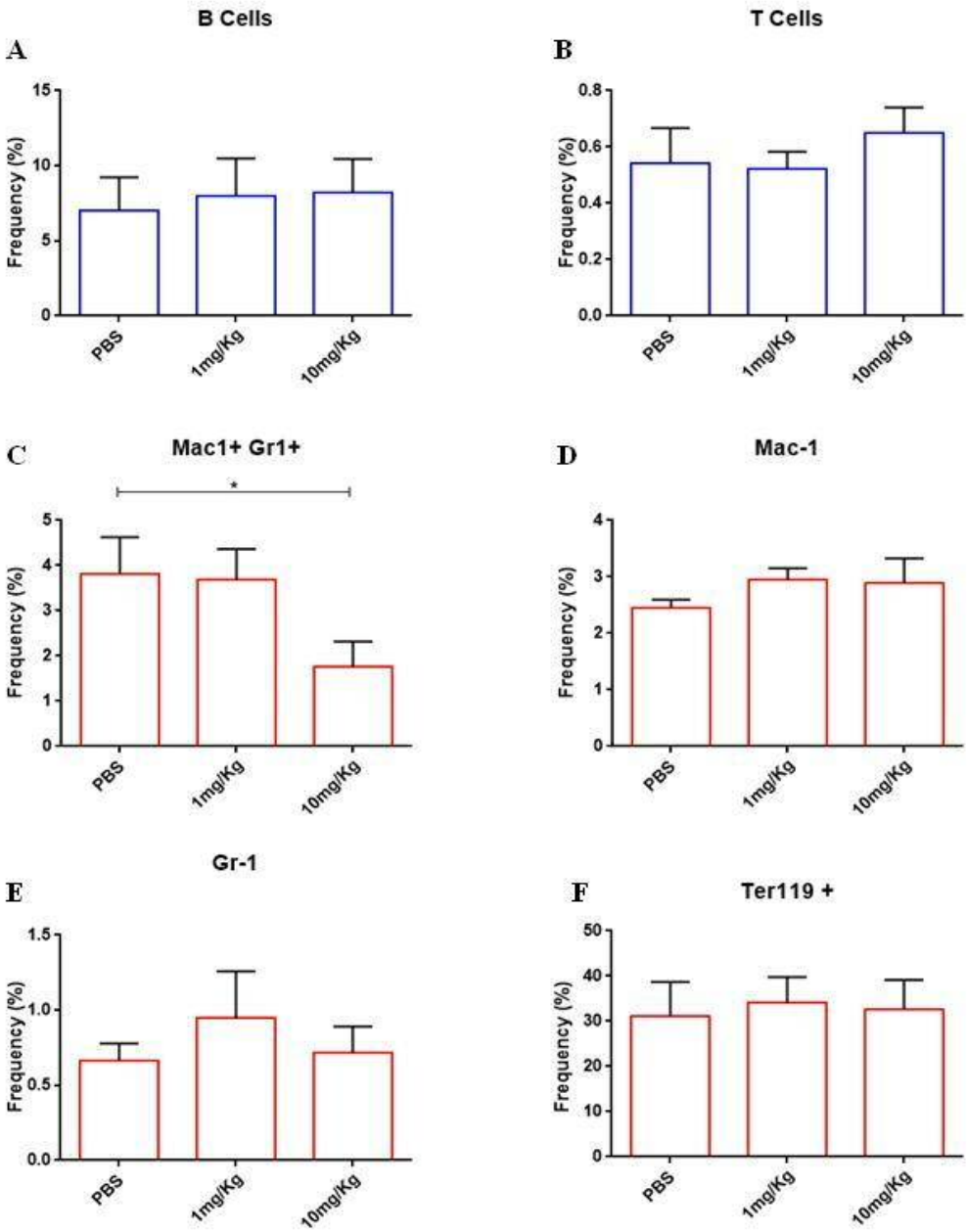

Figure 3. 14 *In vivo* treatment of Yohimbine does not affect lineage committed cells within the bone marrow

Yohimbine was administered twice daily for 10 days, after which bone marrow was harvested. Lymphoid cells include B- (A) and T- cells (B). Cells of the myeloid compartment were defined as myeloid cells ($Mac1^{+}Gr1^{+}$) (C), monocyte/macrophage ($Mac1^{+}$) (D), Granulocyte ($GR1^{+}$) (E) and erythrocyte ($Ter119^{+}$) (F) Data shown as the frequency of populations of live cells. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) * $p<0.05$

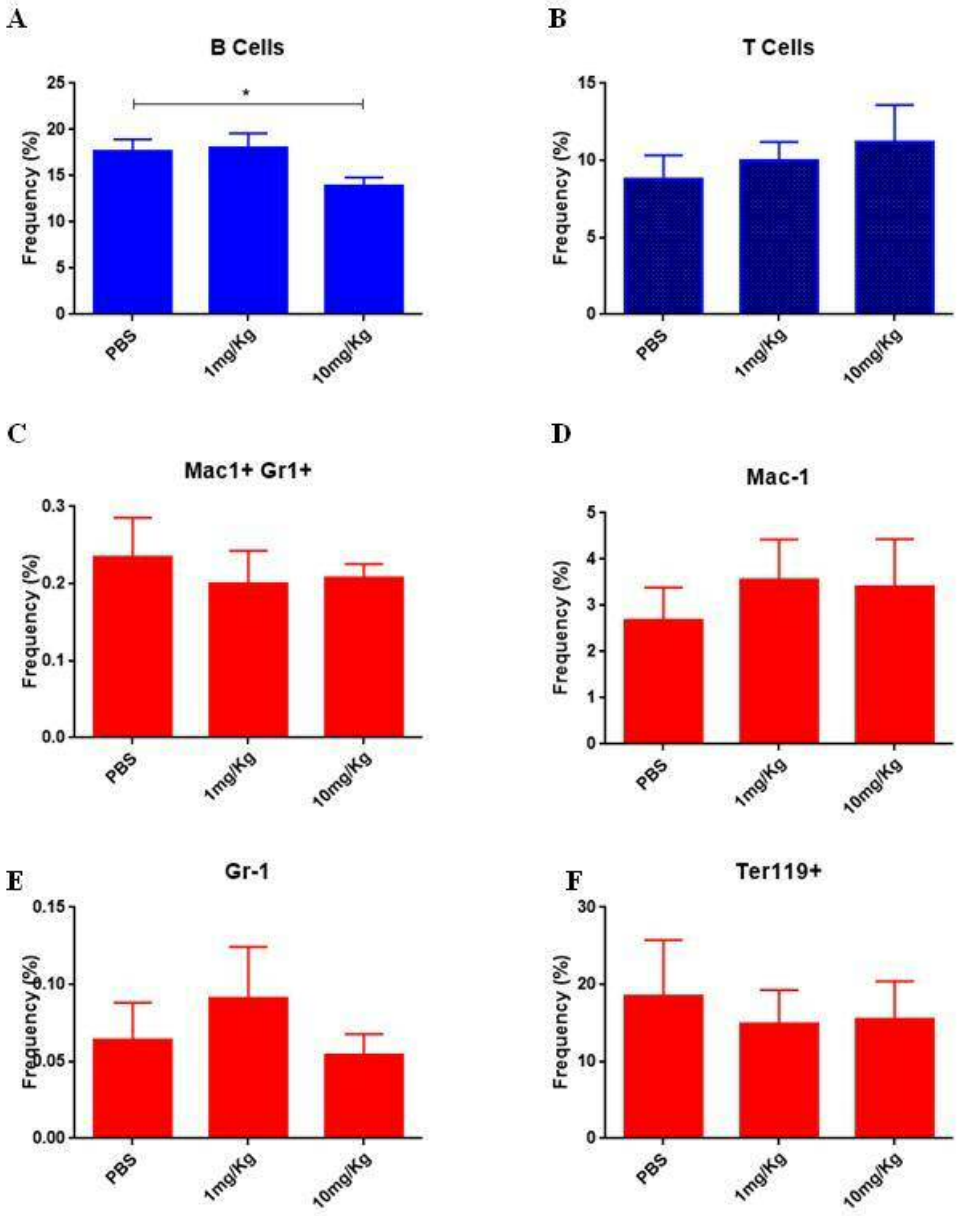

Figure 3. 15 Mild B-cell depletion in the spleen in response to Yohimbine treatment

Yohimbine was administered twice daily for 10 days, after which spleens were harvested and processed into suspension Lymphoid cells include B- (A) and T- cells (B). Cells of the myeloid compartment were defined as myeloid cells ($Mac1^{+}Gr1^{+}$) (C), monocyte/macrophage ($Mac1^{+}$) (D), Granulocyte ($GR1^{+}$) (E) and erythrocyte ($Ter119^{+}$) (F) Data shown as the frequency of populations of live cells. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$

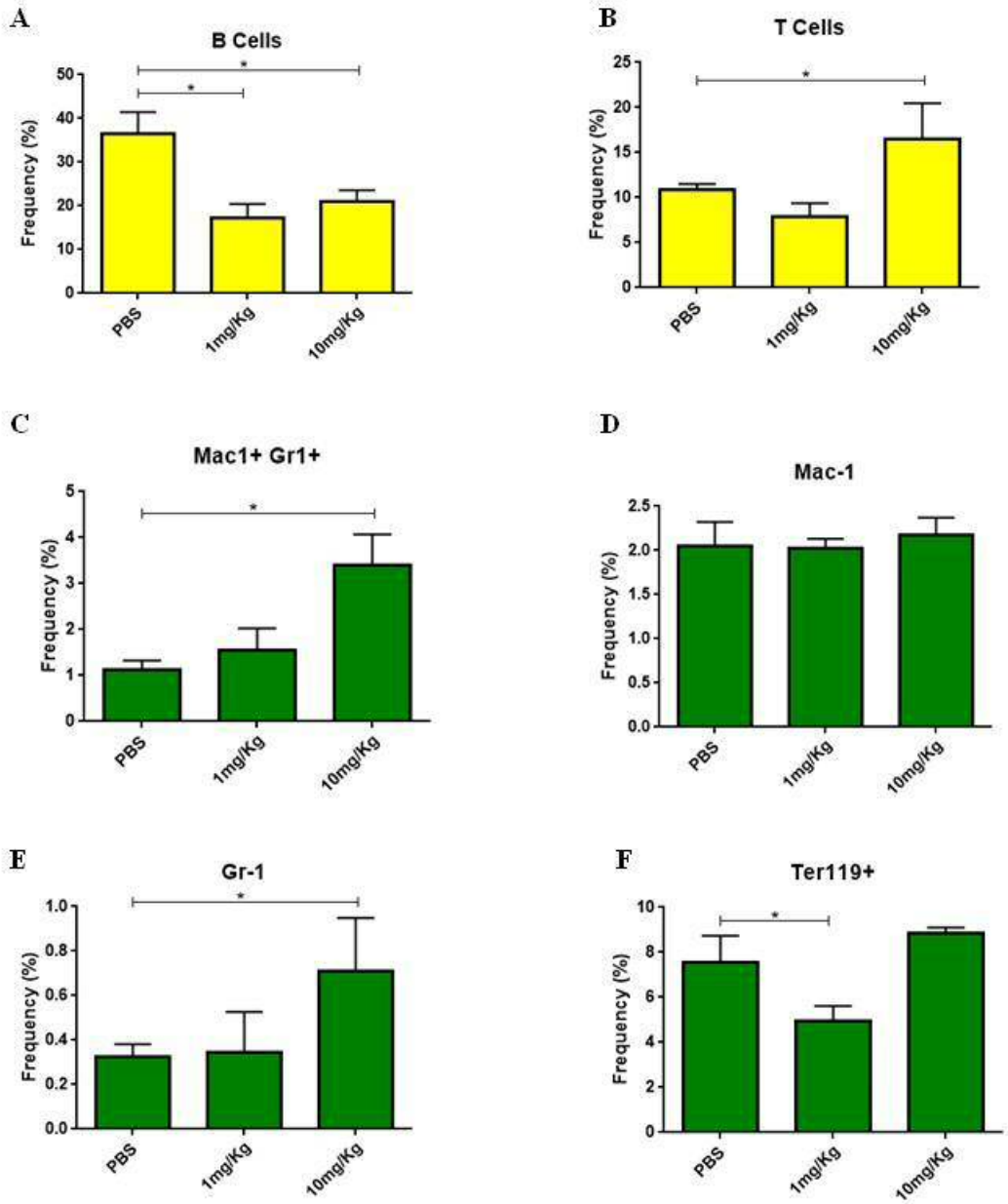

Figure 3. 16 Reduction in TER119 and B cells but increase in circulating myeloid cells and T cells after *in vivo* Yohimbine

Mice were treated with either Yohimbine or PBS twice daily for 10 days. 1 hour after the final administration, blood was taken via an incision of the lateral tail vein and stained for mature differentiated populations. Blood collected from treated animals and analysed for lymphoid (A and B), myeloid (C-E) and erythroid (F) cells. Data shown as the frequency of populations of live cells. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) * $p<0.05$

3.4.3 The effects of Yohimbine on HSC apoptosis *in vivo*

The preceding results show that Yohimbine elicits an increase in HSC/HPC number. To explore the mechanism for increased HSC/HPC frequency, we asked whether Yohimbine influences proliferative versus cell survival capabilities. Animals were treated twice a day with Yohimbine for 10 days. Bone marrow was harvested and stained for LSK SLAM populations. We also sought to investigate the impact of Yohimbine on cell survival *in vivo*. From bone marrow derived LSK SLAM populations, we used the Annexin V assay to identify the different stages of apoptosis in each population. Our results showed that there were no significant differences in cell survival between Yohimbine treated animals and PBS controls (Figure 3.17) suggesting that the increased number of HSPCs are due to proliferation without an impact on cell survival.

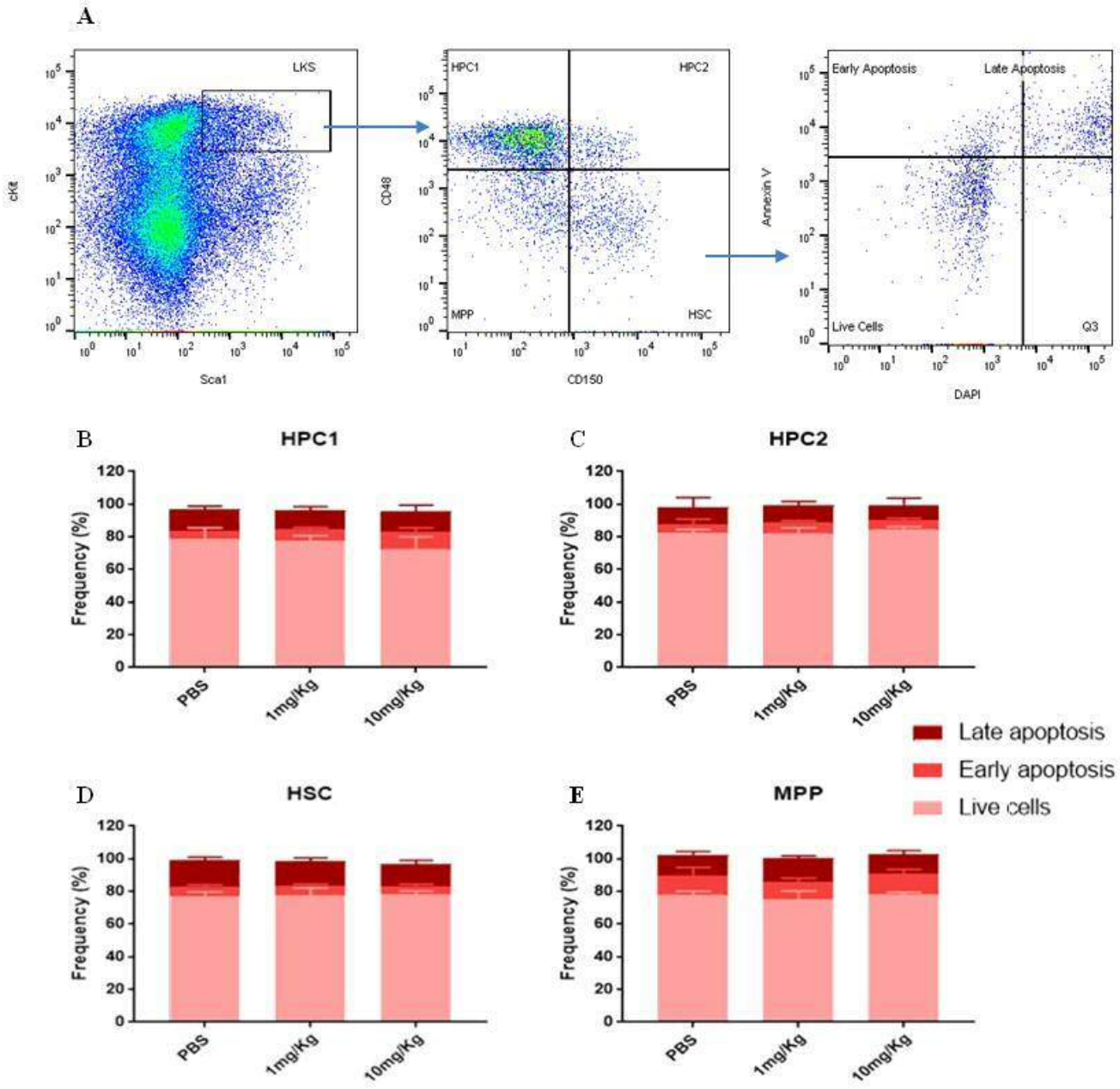

Figure 3. 17 The impact of *in vivo* Yohimbine administration on HSP viability

Animals were treated with Yohimbine twice daily for 10 consecutive days. After the final injection, bone marrow from the respective treated animals were harvested and stained for Lin-Sca1^{+}cKit^{+} SLAM populations. Further staining was conducted with Annexin V conjugated antibody and the DNA dye, DAPI/

3.5 Assessing the Functionality of Yohimbine treated HSCs/HPCs

While HSCs/MPP numbers appear to increase in response to Yohimbine treatment, whether Yohimbine treatment impacts the functionality of HSCs/HPCs remains unclear (Maestroni et al. 1992). To assess this, a competitive transplant experiment was conducted with Yohimbine treated bone marrow. Since an increase in HSC number was observed in Yohimbine treated animals, differing ratios of competitor to donor were transplanted so that equal frequencies of donor and competitor HSCs were used in experiments. The donor: competitor ratios were determined by the 2- and 1.5- fold increase in HSC frequencies in response to 1 mg/kg and 10 mg/kg doses respectively. Hence the ratios 1:2 (1mg/kg) and 1:1.5 (10mg/kg) donor: competitor were used in this experiment. Engraftment and reconstitution of donor HSCs was measured by assessing mature lineage differentiated cells within the peripheral blood on a weekly basis (Figure 3.18A). Treated donor cells in the blood did not show any differences in overall engraftment capacity compared to PBS treated controls over 16 weeks (Figure 3.18B and 3.18C). However, when 10 mg/kg treated cells were transplanted, a rapid reconstitution of B cells, but not T cells, was observed (Figure 3.19). In addition, a rapid increase in $Ter119^{+}$ cells (erythroid cells) was also observed in response to competitive transplantation of 10 mg/kg Yohimbine treated cells (Figure 3.20A-C). This effect was maintained throughout the transplant period. Cells of the myeloid compartment were unchanged between PBS and treated groups (Figure 3.20D-F) suggesting that Yohimbine promotes the generation of B-cells within the lymphoid compartment and erythrocytes after transplantation.

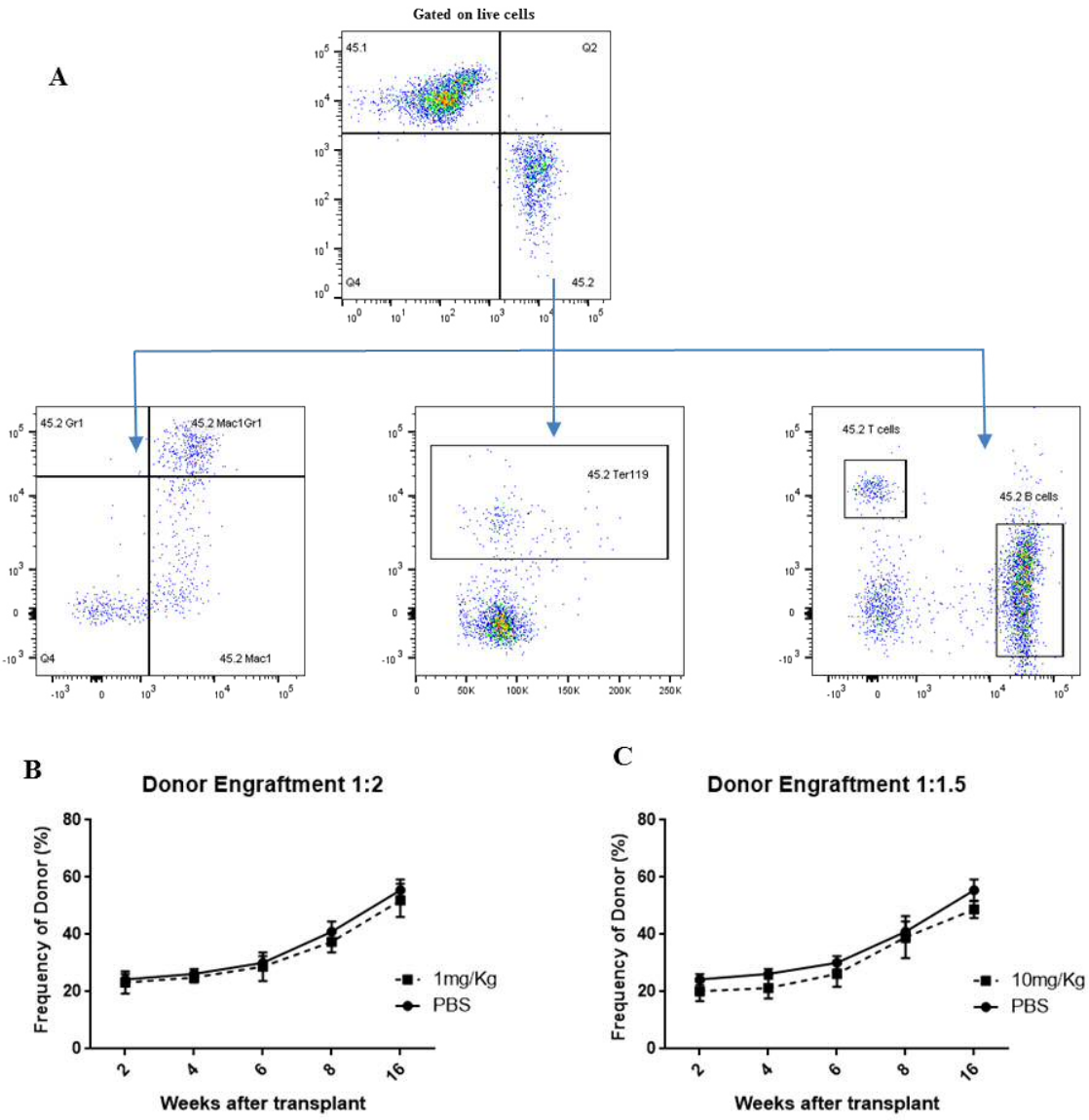

Figure 3. 18 Yohimbine treated HSPCs do not affect engraftment after competitive bone marrow transplant

Donor mice (CD45.2) were treated with Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated recipients (CD45.1). Samples of blood were taken weekly to assess reconstitution and engraftment. Recipient and donor cells were identified using CD45.1 and CD45.2 antibodies respectively (A). From within the donor compartment, lineage differentiated populations were identified as myeloid (Mac1, Gr1), erythrocitic (Ter119) and lymphoid (B220, CD4/CD8) cells. The frequency of donor cells was determined by CD45.2 expression in transplanted mice (B)

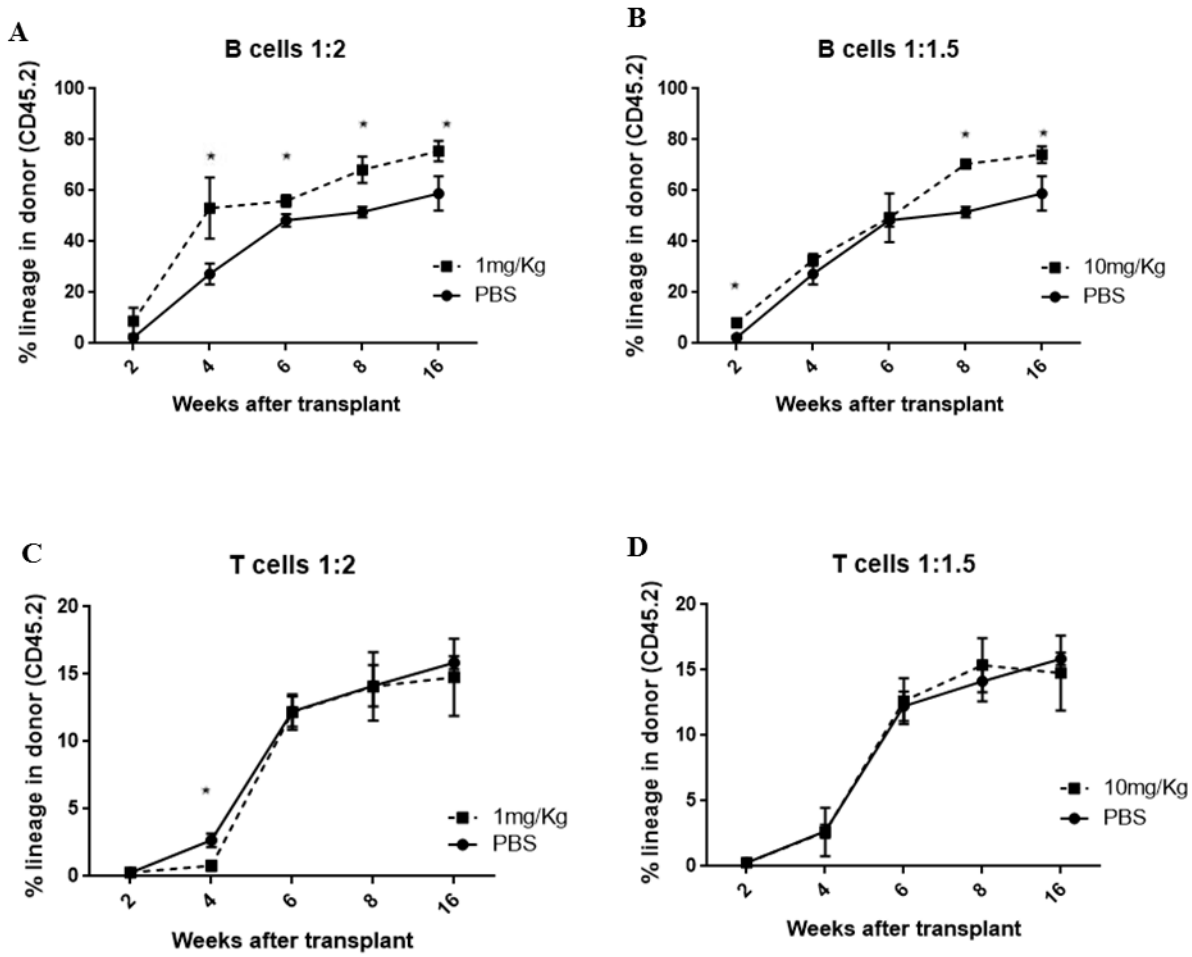

Figure 3. 19 Yohimbine treated cells enhance donor B-cell reconstitution in competitive transplantation

Donor mice were treated with 1 mg/kg and 10 mg/kg Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively irradiated recipients. Samples of blood were taken weekly to assess reconstitution and engraftment. From within the donor compartment, lymphoid populations were identified as (A and B) B-cells (B220) and (C and D) T-cells (CD4/CD8). The frequency of donor cells was determined by CD45.2 expression in transplanted mice (B) Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS).

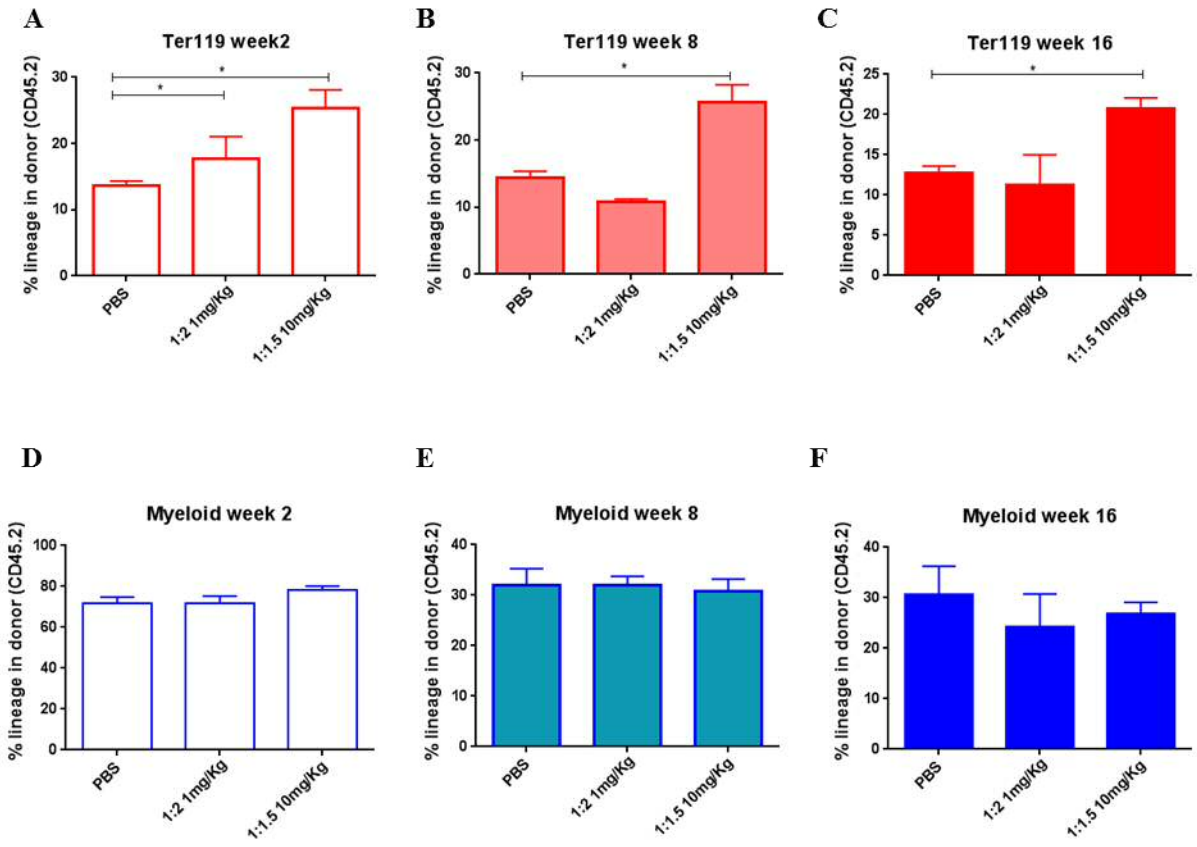

Figure 3. 20 Yohimbine treated cells demonstrate enhanced donor erythrocytic reconstitution.

Donor mice were treated with 1 mg/kg and 10 mg/kg Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively irradiated recipients. Samples of blood were taken weekly to assess reconstitution and engraftment from within the donor compartment, erythrocyte populations were identified as (A) $Ter119^{+}$. (D) Myeloid cells were identified as $Mac1^{+}$, $Gr1^{+}$ and $Mac1^{+}Gr1^{+}$. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$.

We also assessed the donor contribution to lineage differentiated cells as shown in the example in Figure 3.21A. Donor contribution to B cell, T cell and TER119 lineages were dramatically improved in the highest dose of Yohimbine (Figure 3.21B and Figure 3.23). Conversely, the myeloid compartment was unaffected (Figure 3.23). The data shows augmentation of specific mature blood cell lineage distribution in engrafting Yohimbine treated cells.

After 16 weeks, bone marrow of transplanted animals was harvested and analysed. Examining HSPCs derived from treated donor cells, we saw that there were no significant differences in HSCs, MPPs and HPC1 donor cell engraftment between the groups (Figure 3.24A, 3.24C and 3.24D respectively). A higher frequency of HPC2 cells was evident in response to 1 mg/kg (Figure. 3.24B). In parallel to immunophenotypical analysis of HSPCs, a CFC assay was conducted to test the colony forming potential of the donor cells. We observed no differences in the number of colonies between the groups (Figure 3.25). We further assessed the function of long term HSCs by secondary transplantation. Primary recipients were sacrificed and from the harvested bone marrow, donor derived LSKs were sorted by FACS and transplanted at a density of 2000 LSK cells per recipient. From this study we saw that there was no difference in engraftment from treated donor cells compared to PBS controls (Figure 3.26A). We also did not observe any differences in lympho-myeloid reconstitution throughout the secondary transplant (Figure 3.26B-C). These data show that the effects of Yohimbine are specific to HSCs involved with the reconstitution of the haematopoietic system in primary recipients but not on the self-renewal of long term HSCs.

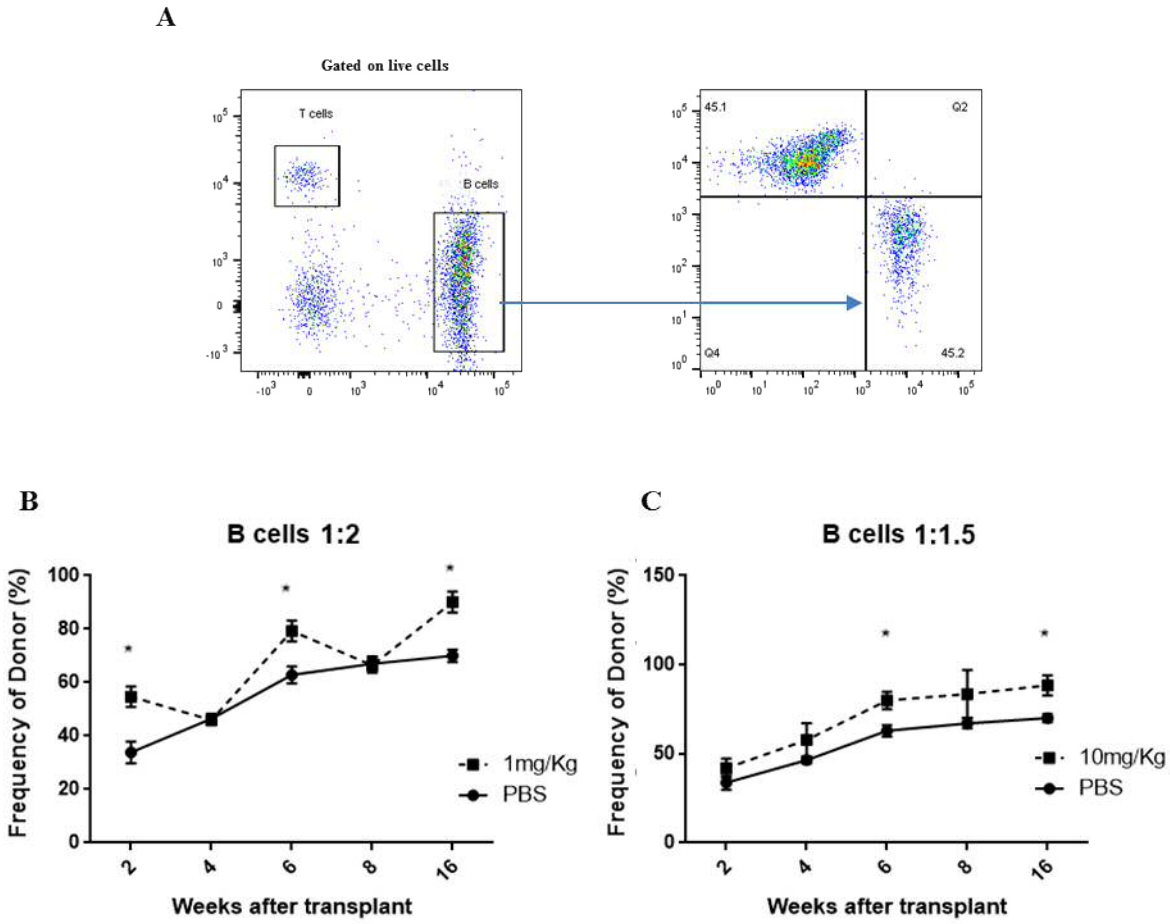

Figure 3. 21 Yohimbine treated cells result in enhanced donor distribution in the B-cell compartment

Donor mice (CD45.2) were treated with Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated recipients (CD45.1). Samples of blood were taken weekly to assess reconstitution and engraftment. Recipient and donor cells were identified using CD45.1 and CD45.2 antibodies respectively. (A) From within the lineage differentiated populations, the distribution of donor cells were identified (B) CD45.2 distribution was measured from B-cells.

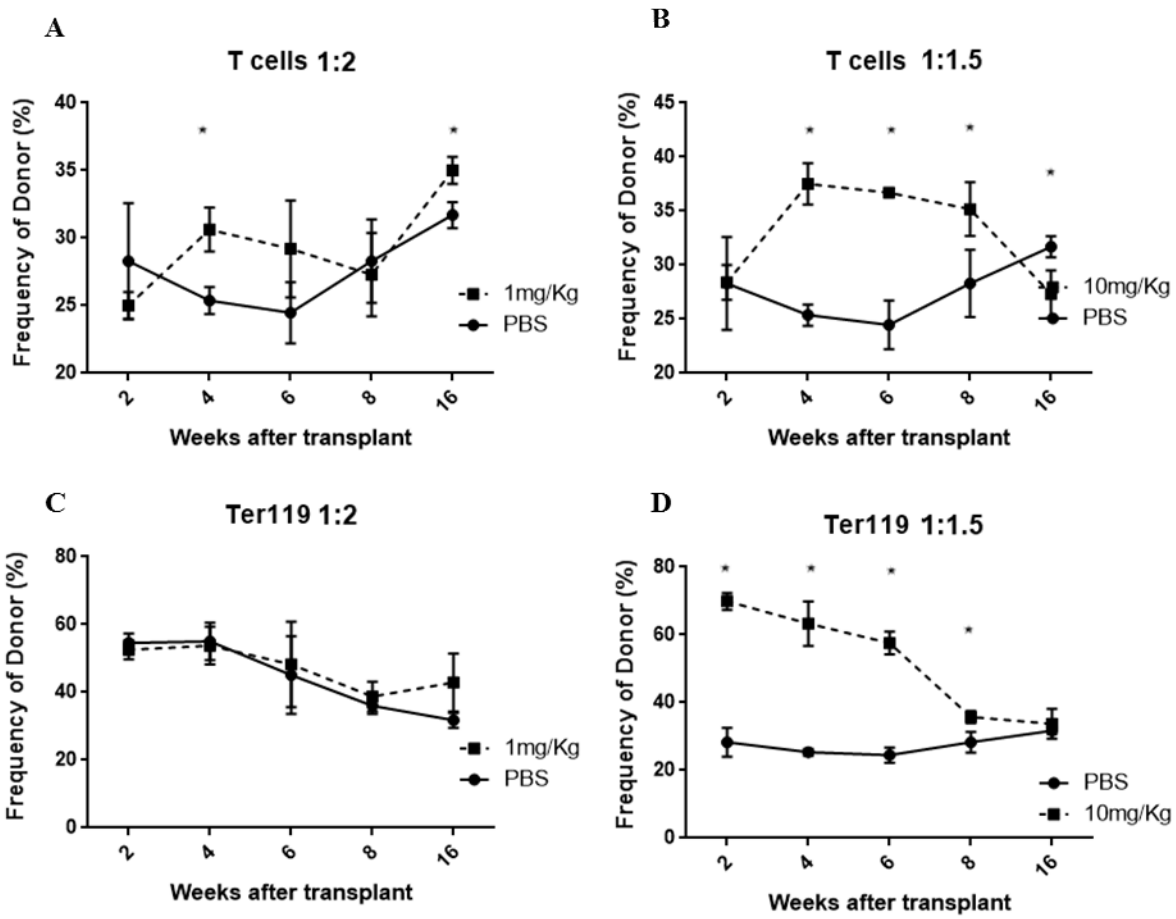

Figure 3. 22 Yohimbine treated cells results in an improved competitive donor distribution in the T-cell and erythrocyte compartment

Donor mice (CD45.2) were treated with Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated recipients (CD45.1). Samples of blood were taken weekly to assess reconstitution and engraftment. Recipient and donor cells were identified using CD45.1 and CD45.2 antibodies respectively. From within the lineage differentiated populations, (A) T-cells ($CD4^+/CD8^+$) and (C) erythrocytic cells (Ter119), the distribution of donor cells were identified.

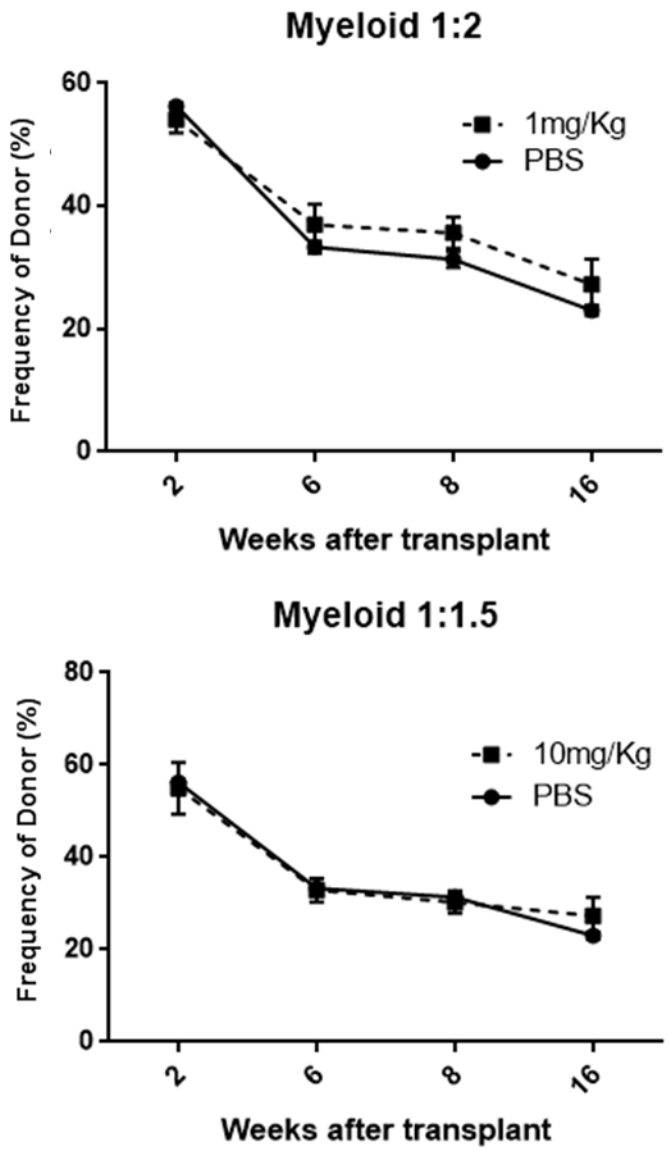

Figure 3. 23 Yohimbine treated cells do not affect the competitive reconstitution of donor cells within the myeloid cell compartment

Donor mice (CD45.2) were treated with Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated recipients (CD45.1). Samples of blood were taken weekly to assess reconstitution and engraftment. Recipient and donor cells were identified using CD45.1 and CD45.2 antibodies respectively. From within the myeloid (Mac1, Gr1 and $Mac1^{+}Gr1^{+}$) populations, the distribution of donor cells were identified.

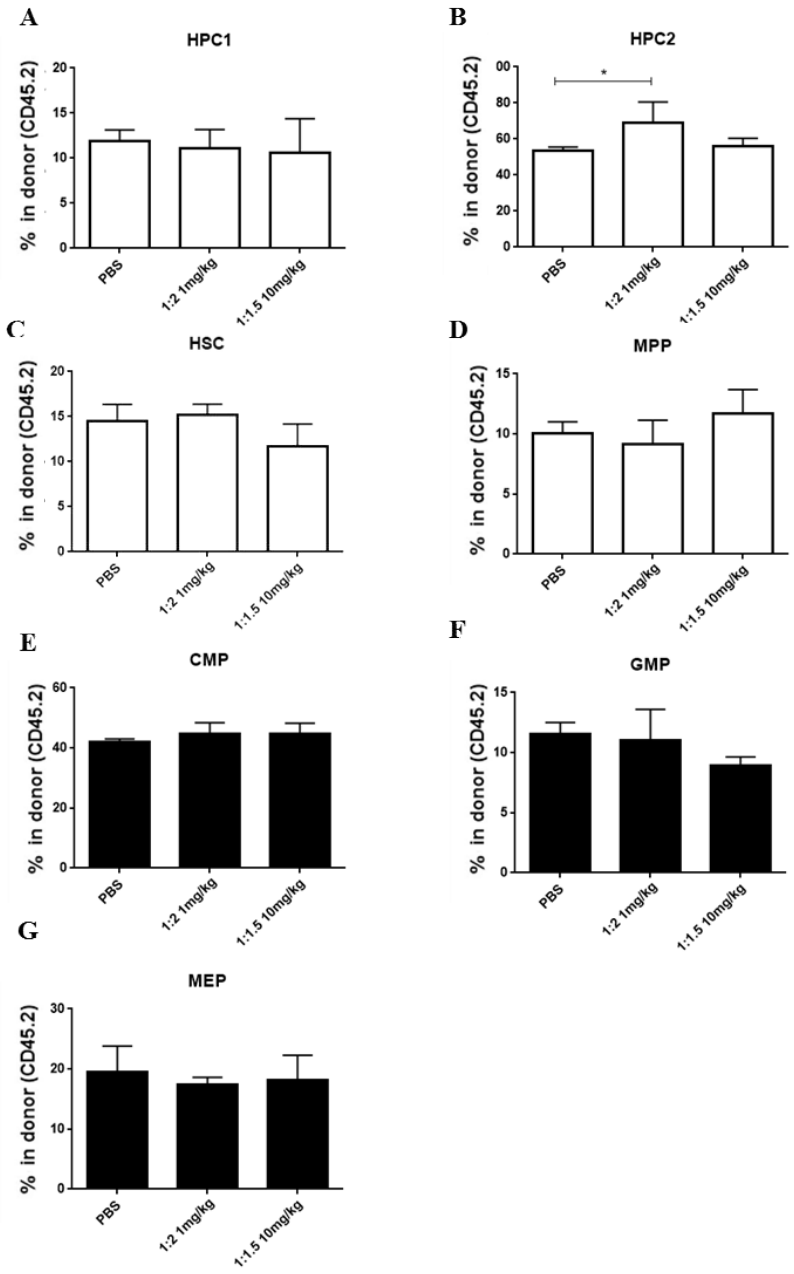

Figure 3. 24 Increased HSPCs in the competitive transplant of Yohimbine treated cells

Donor mice (CD45.2) were treated with Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively into irradiated recipients (CD45.1). 16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Specific HSPC and lineage restricted progenitor populations from within the LSK (A-D) and LK (E-G) compartment were analysed. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$.

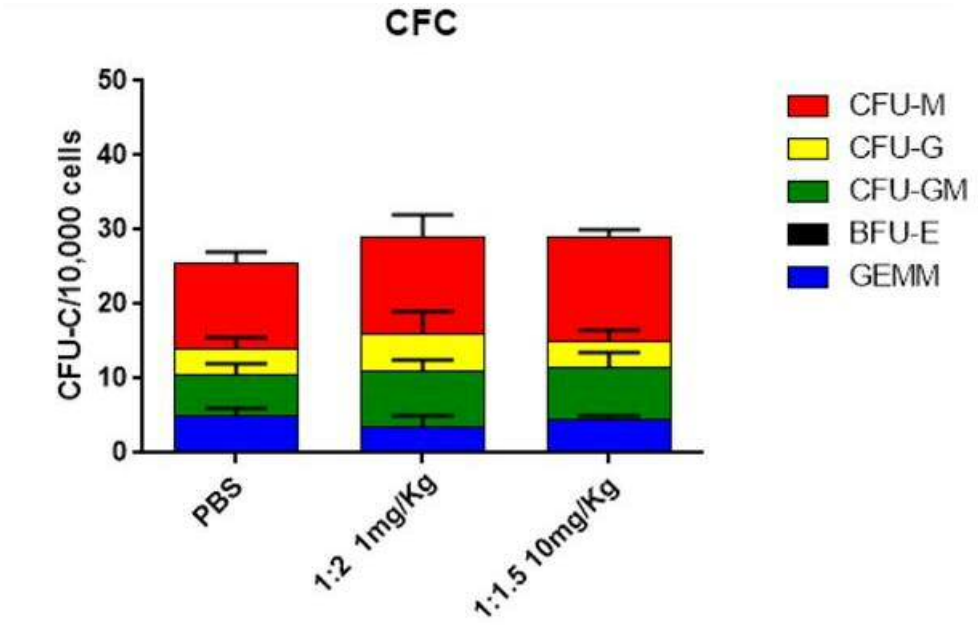

Figure 3. 25 Colony forming potential of transplanted Yohimbine treated HSPCs is unaffected

Donor mice (CD45.2) were treated with Yohimbine twice daily for 10 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively into irradiated recipients (CD45.1). 16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Donor cells were FACS sorted and plated in Methylcellulose at a density of 10,000 cells

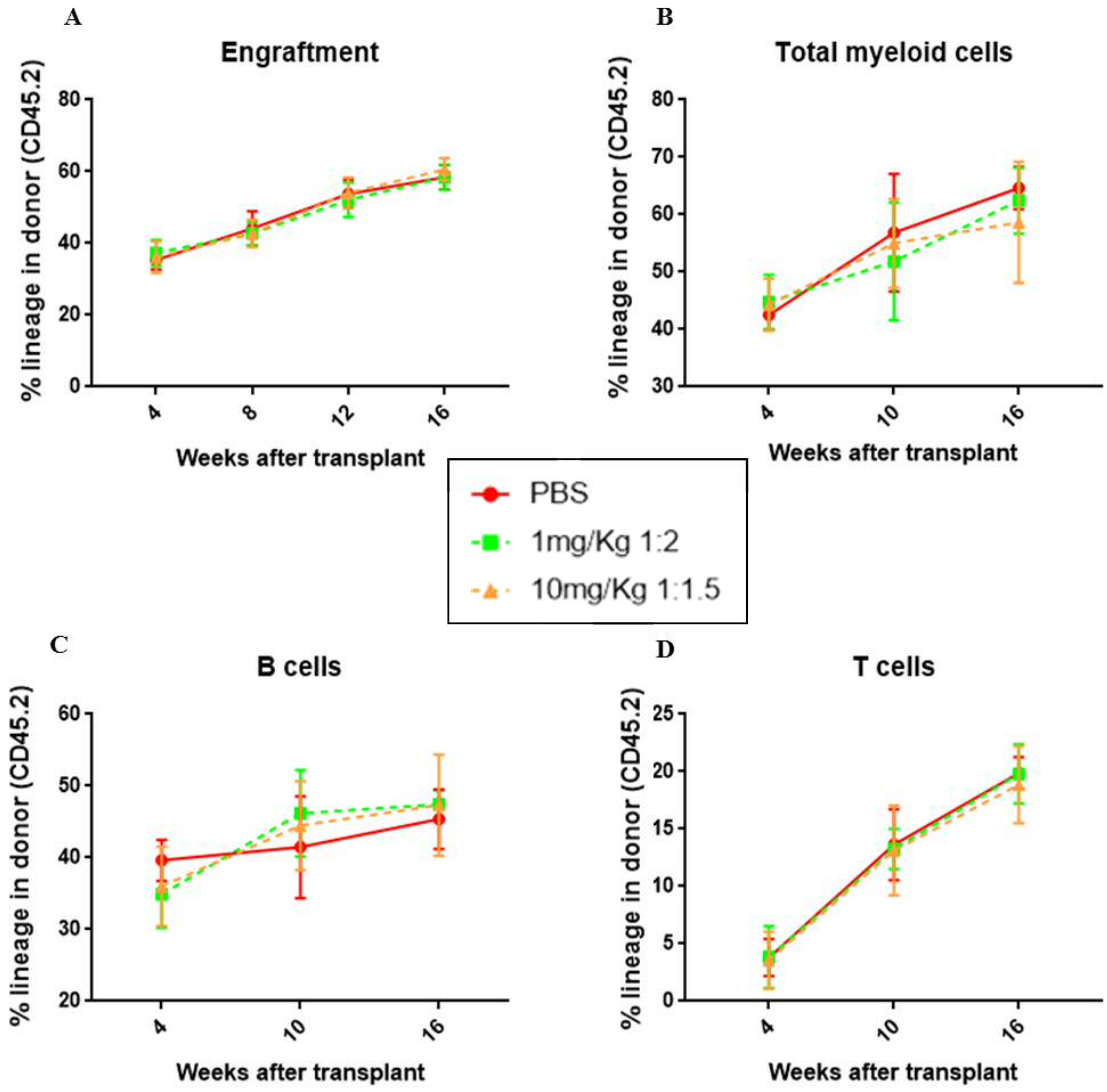

Figure 3. 26 Yohimbine treatment does not affect long-term HSC function in secondary recipients

Donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin\text{-}Sca1^{+}cKit^{+}$ cells per recipient). Blood samples were taken to measure engraftment and reconstitution of lymphoid (B and C) and myeloid (D) using T- ($CD4^{+}/8^{+}$) and B cell (B220), and myeloid (Mac1/Gr1) markers.

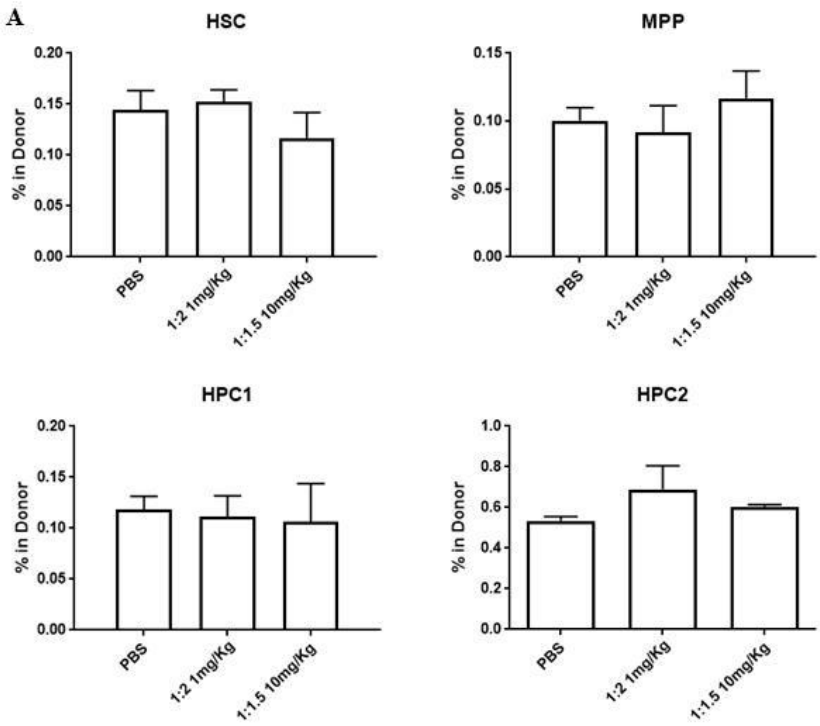

Figure 3. 27 Yohimbine treated HSCs have normal function and self-renewal

Donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin^{-}Sca1^{+}cKit^{+}$ cells per recipient). 16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Specific HSPC and lineage restricted progenitor populations from within the LSK compartment we analysed (A).

3.6 The impact of Yohimbine on haematopoietic reconstitution after BM transplant

BM transplant follows irradiation and/or high-dose chemotherapy which results in pancytopenia. During this period, patients are at risk of infection and therefore it is imperative to seek methods to improve haematopoietic reconstitution. Early studies have shown that adrenergic antagonists promote myelopoiesis after BM transplant (Maestroni et al. 1992). We therefore sought to assess whether Yohimbine would influence post-transplant reconstitution at the level of HSC/HPC expansion. Here, animals treated with PBS, 1 mg/kg and 10 mg/kg of Yohimbine after BM. Figure 3. 28 Yohimbine treatment does not affect engraftment after transplant (Figure 3. A). Treatment with Yohimbine did not show to affect the levels of engraftment of donor cells at any of the doses (Figure 3. B). Interestingly, we observe a small but significant rapid increase in T-cell repopulation 2 weeks after transplant in response to 1 mg/kg Figure 3.). No effect was observed in either the B-cell or the myeloid populations (Figure 3. B). It was also interesting to observe that in the treatment after transplant setting, Yohimbine demonstrated improved erythrocytic reconstitution compared to PBS controls (Figure 3. C and Figure 3. D). The myeloid compartment ($Mac1^{+}Gr1^{+}$) (Figure 3. E and Figure 3. F) was enhanced after Yohimbine administration where 1 mg/kg and 10 mg/kg of Yohimbine elicited a 2.5 fold increase after 4 weeks of transplant. Analysing lineage distribution of donor engrafting cells after Yohimbine treatment showed similar trends as described above (Figure 3.). We found that the distribution of donor cells within B-cells were considerably higher in treated animals compared to the control group up to 4 weeks after transplantation (Figure 3. A). This was also observed in the Ter119 cells (Figure 3. D) but not in any of the other compartments. These data show augmentation of myeloid, erythrocyte and B-cell production is in response to Yohimbine treatment in the early recovery stages after bone marrow transplant.

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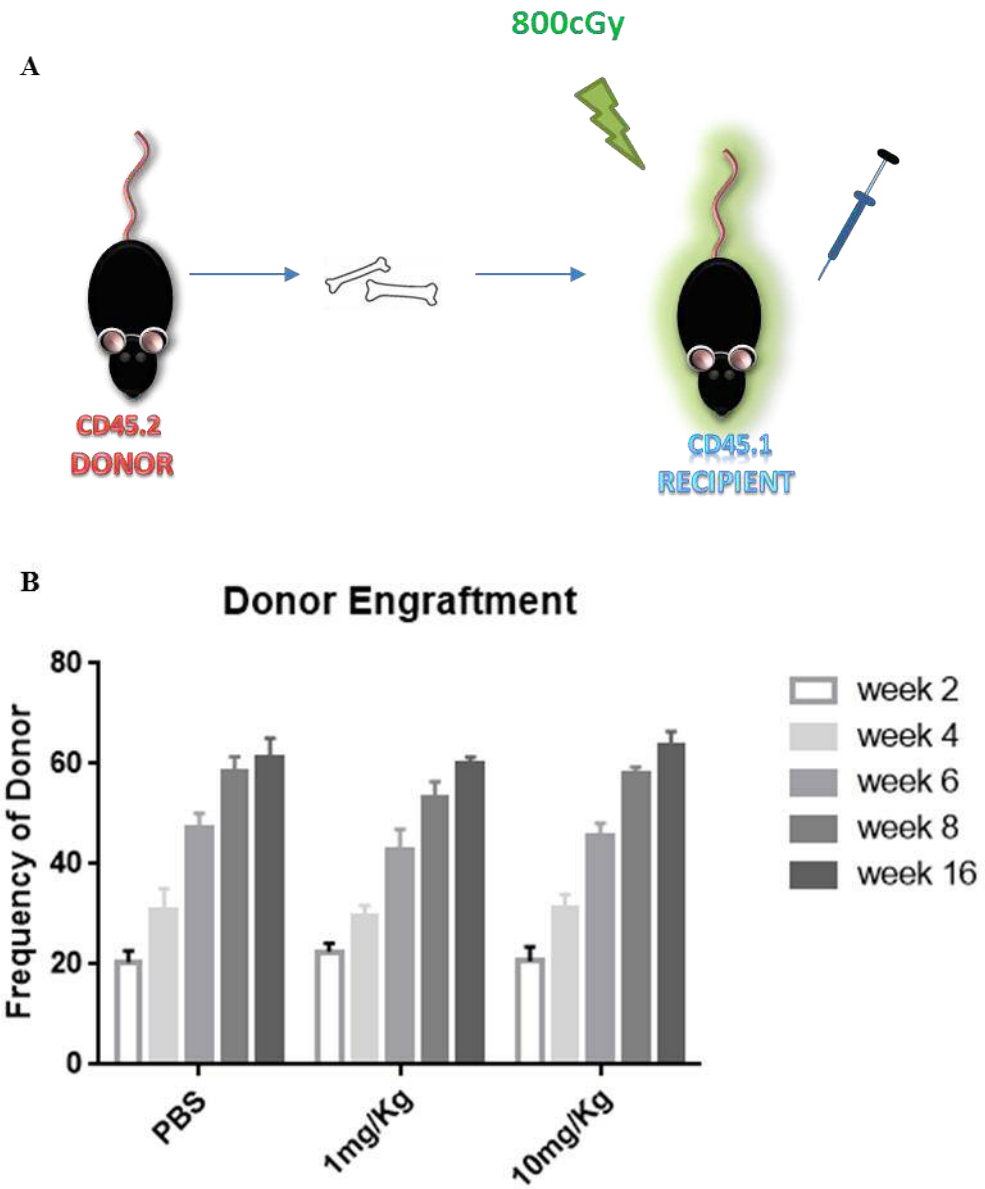


Figure 3. 28 Yohimbine treatment does not affect engraftment after transplant

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Yohimbine (or vehicle) was then administered once every two days for a total of 10 treatments (A). Weekly bleedings were conducted to assess engraftment of donor cells (CD45.2)(B)

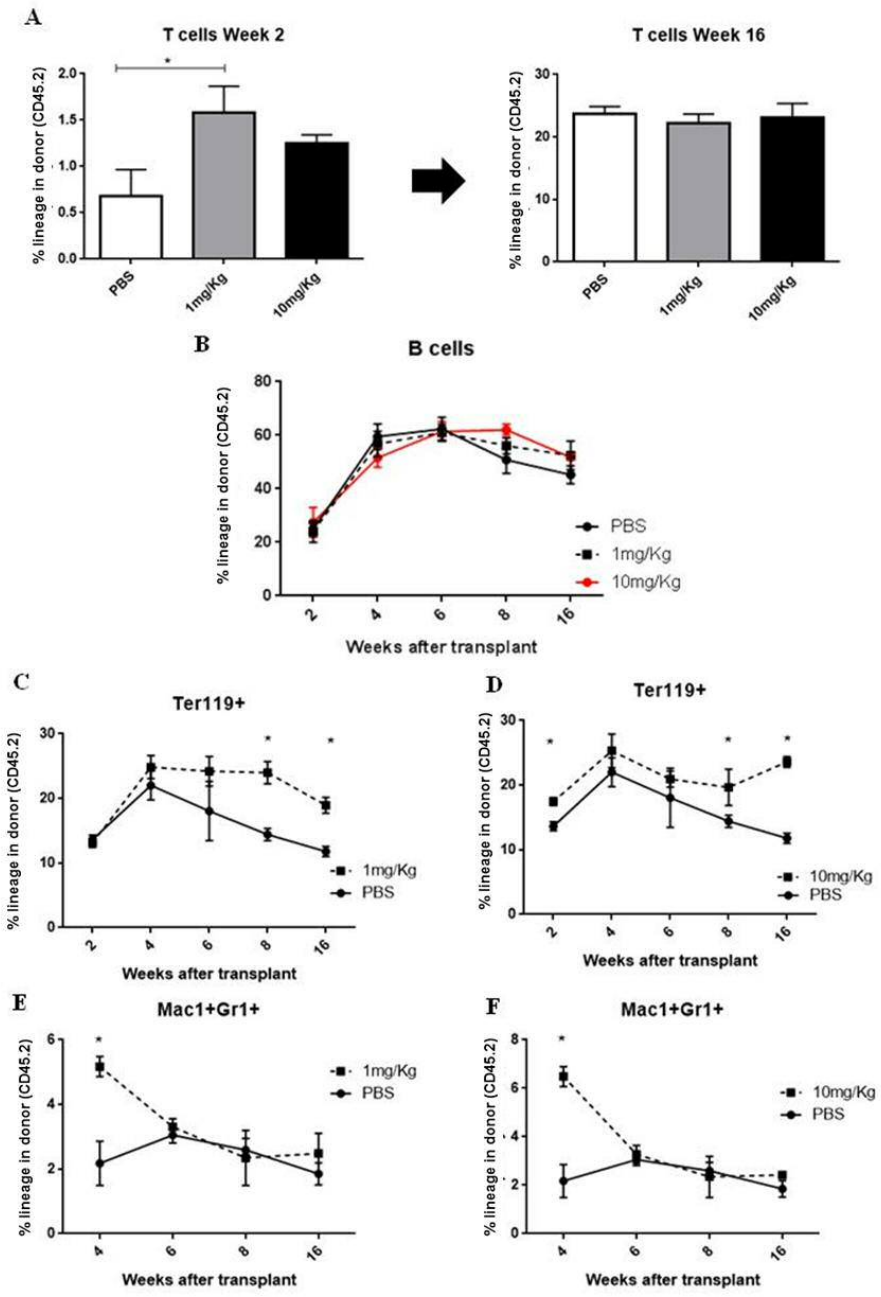


Figure 3. 29 Yohimbine treatment after transplantation elicits an increase in early myeloid and T-cell reconstitution

CD45.1 recipient mice transplanted with $5x10^5$ CD45.2 donor cells. Yohimbine (or vehicle) was then administered once every two days for a total of 10 treatments. Weekly bleedings were conducted to assess reconstitution of lymphoid (A and B), erythrocytes (C and D) and myeloid cells (E and F).

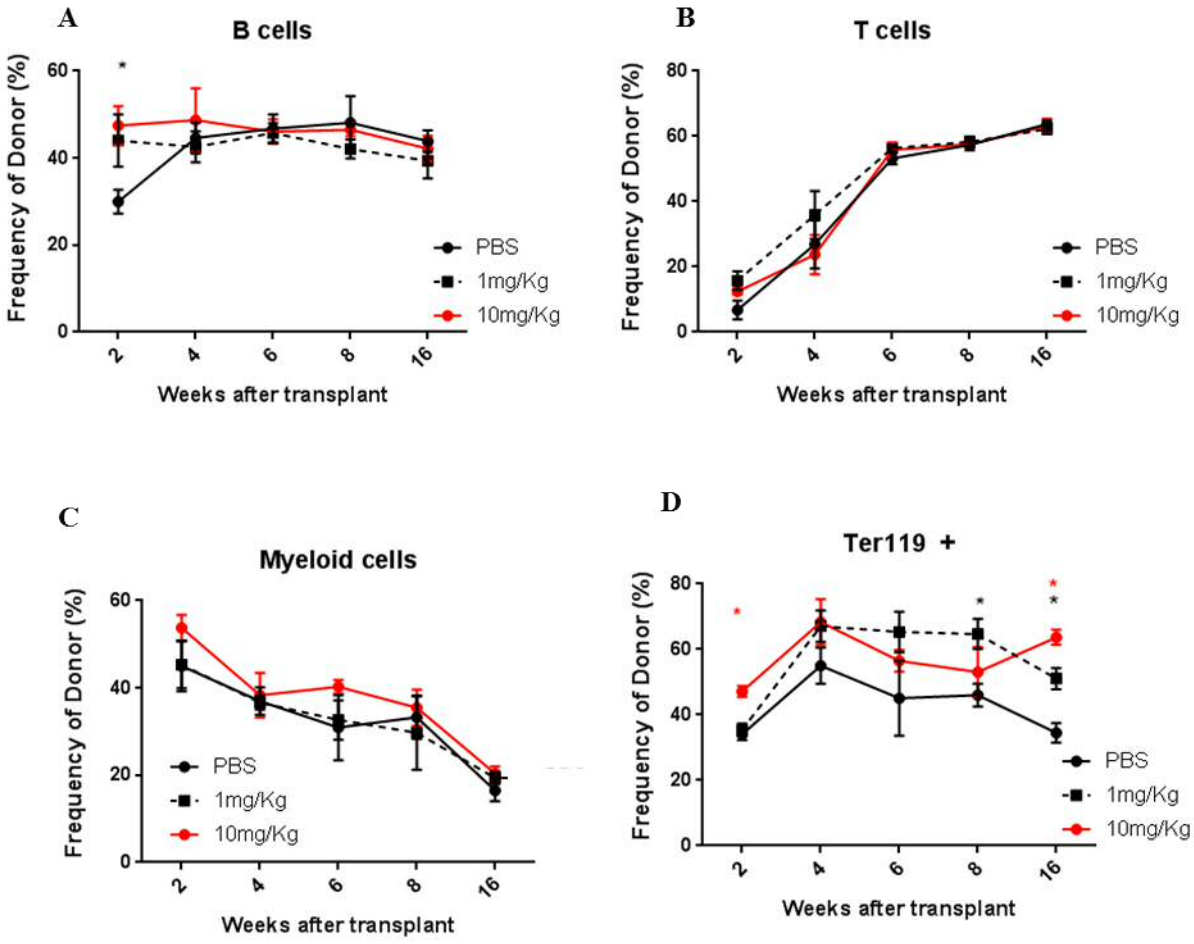


Figure 3. 30 Post-transplant treatment of Yohimbine elicits a donor advantage in the erythrocytic compartment

CD45.1 recipient mice transplanted with $5x10^5$ CD45.2 donor cells. Yohimbine (or vehicle) was then administered once every two days for a total of 10 treatments. Weekly bleedings were conducted to assess reconstitution of (A) B- and (B) T- cells, myeloid cells (C) erythrocytes (D). Data shown as the frequency of donor cells within lineage specific populations.

After 16 weeks, bone marrow of these animals was harvested and studied immunophenotypically. We found that there was an increase in the frequency of donor HPC1 and HPC2 (not significant) in response to 10 mg/kg Yohimbine (Figure 3. A and Figure 3. B). It was also interesting to see a significant increase in the most primitive progenitor populations (MPP) but this was not the case for HSCs (Figure 3. C and Figure 3. D). Treatment of Yohimbine also did not elicit any effects of lineage restricted progenitors after 16 weeks post-transplant (Figure 3. E-G). CFC assays of donor derived cells also showed an increase the total number of colonies compared to PBS however these were not statistically significant (Figure 3.). Annexin V assay was used to determine cell survival of donor-derived LSK cells (Figure 3.). Interestingly, we observed a significantly lower number of LSK cells that were undergoing early apoptosis compared to PBS controls (Figure 3.). This suggests that Yohimbine treatment after transplantation is preserving cell survival whilst promoting differentiation of select mature blood cell lineages.

From the treated recipients, we isolated donor LSK cells after 16 weeks and conducted a serial transplant into secondary recipients to assess the impact of Yohimbine on true long term HSCs. Analysing blood of secondary recipients showed that that engraftment was not affected in Yohimbine treated donors compared to controls (Figure 3. A). Yohimbine also did not elicit any changes in the reconstitution of myeloid or lymphoid cells of secondary recipients (Figure 3. B-D). After 16 weeks of the secondary transplant, bone marrow tissue was harvested and analysed for HSPC populations. We observed no changes in the frequencies of HSPCs in treated animals (Figure 3.). These data suggest that Yohimbine exerts its effects in primary recipients that have been directly exposed to Yohimbine treatment.

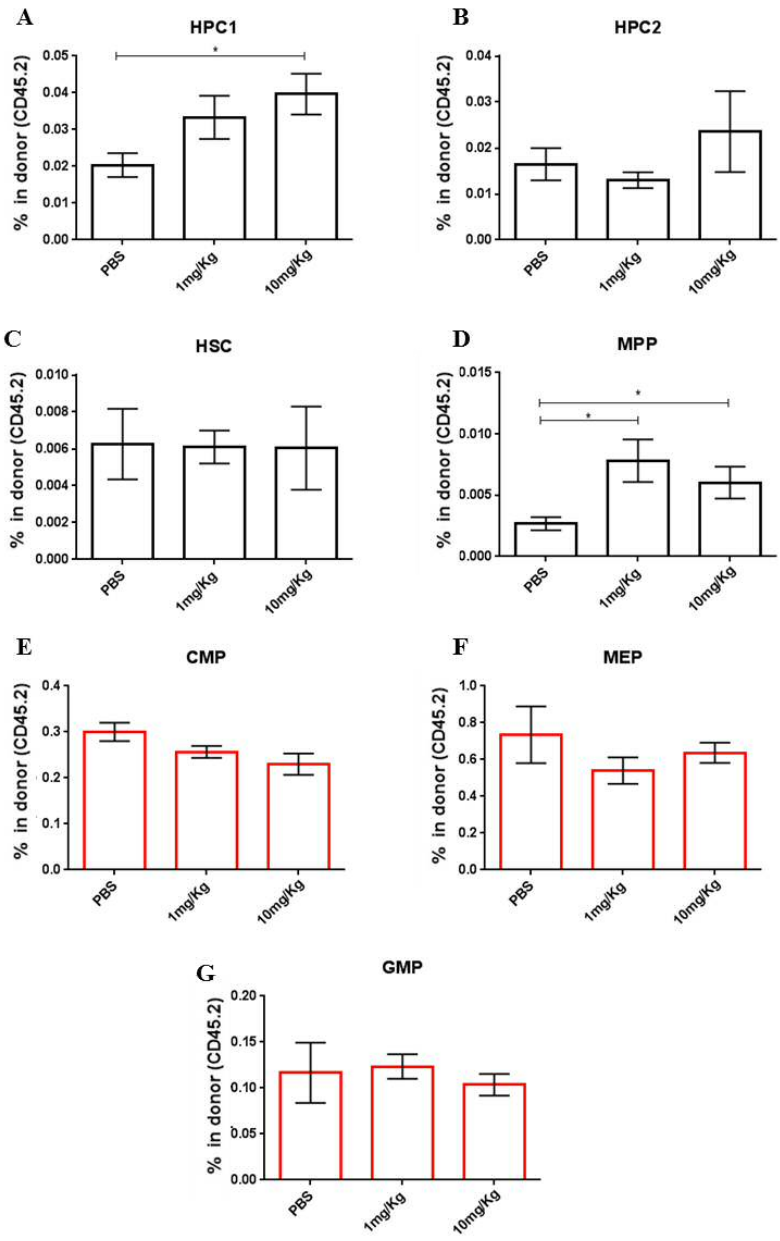


Figure 3. 31 *In vivo* treatment of Yohimbine after transplant increases the primitive HPC1 and MPP populations

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Yohimbine (or vehicle) was then administered once every two days for a total of 10 treatments.16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Specific HSPC and lineage restricted progenitor populations from within the LSK (A-D) and LK (E-G) compartment were analysed. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$.

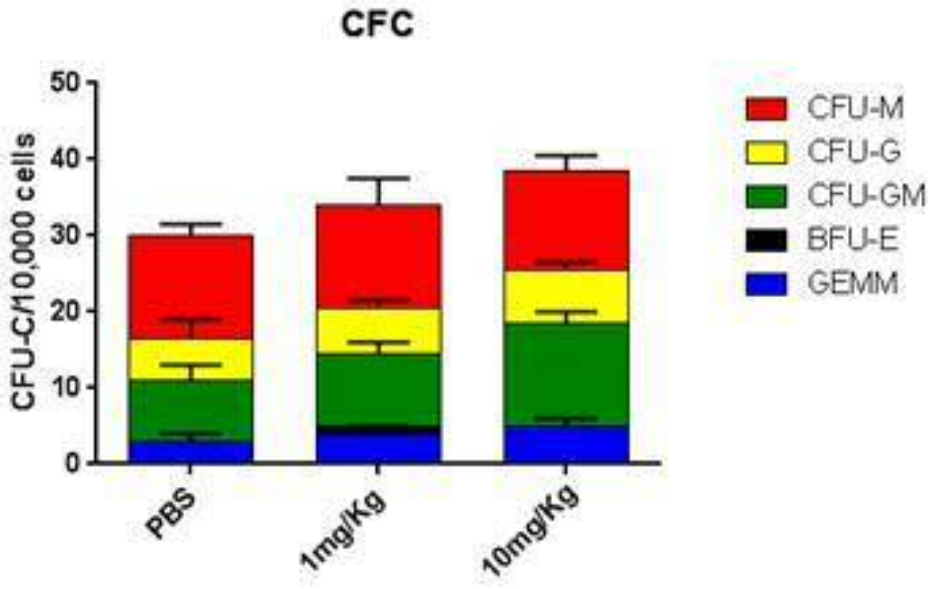


Figure 3. 32 Yohimbine treated cells after transplant do not differ in progenitor function

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Yohimbine (or vehicle) was then administered once every two days for a total of 10 treatments.16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. (A) LSK staining was conducted on bone marrow gated from donor cells. Donor cells were FACS sorted and plated in Methylcellulose at a density of 10,000 cells. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$

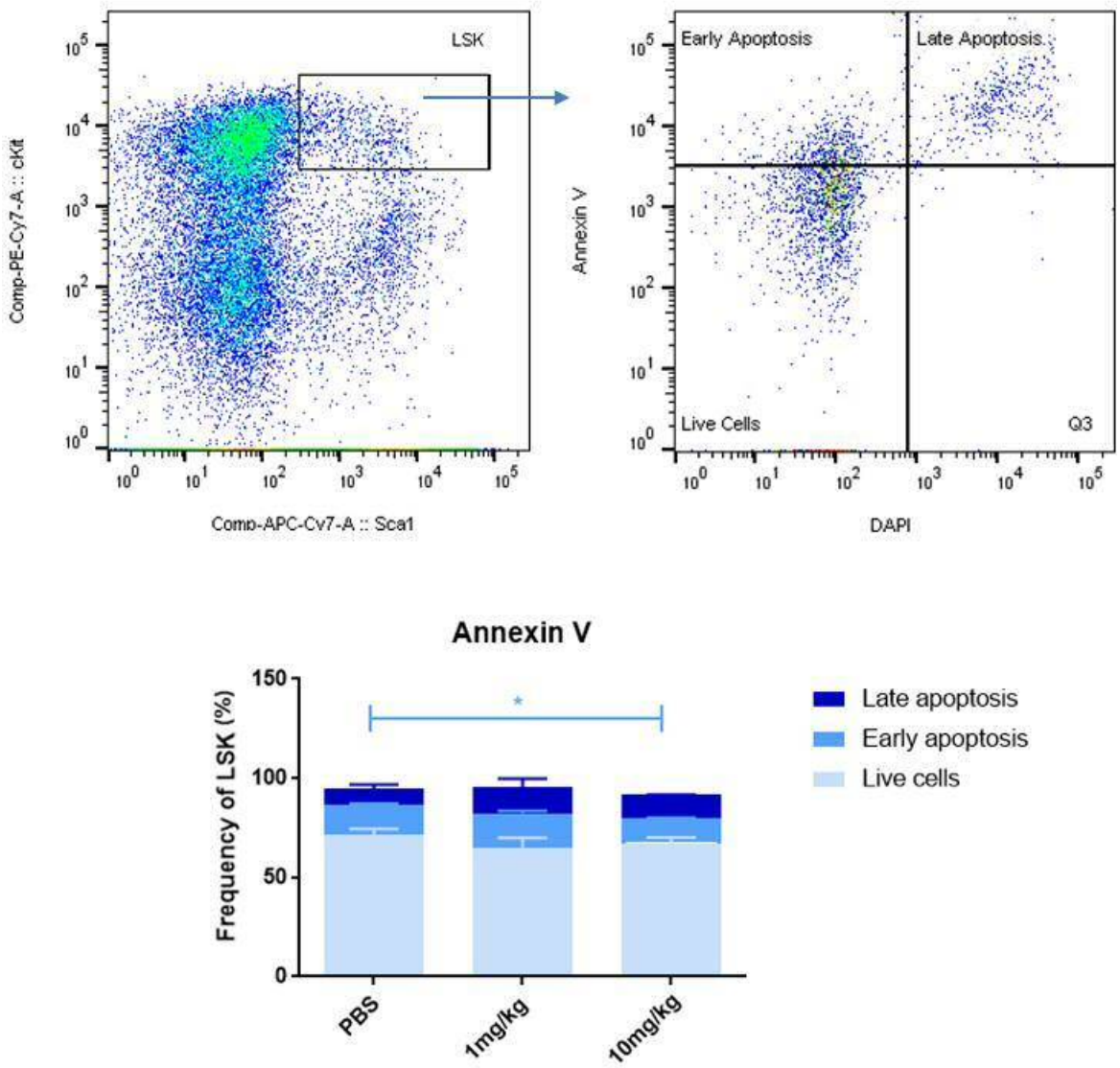


Figure 3. 33Yohimbine treatment after transplant does not affect the survival and apoptotic status of HSCs

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Yohimbine (or vehicle) was then administered once every two days for a total of 10 treatments.16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. (A) LSK staining was conducted on bone marrow gated from donor cells. Further staining was conducted with Annexin V conjugated antibody and the DNA dye, DAPI. Different stages of apoptosis were determined by AnnexinV/DAPI: Live cells (AnnV-DAPI-), Early apoptosis ($AnnV^+DAPI^-$), Late apoptosis ($AnnV^+DAPI^+$). . Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$.

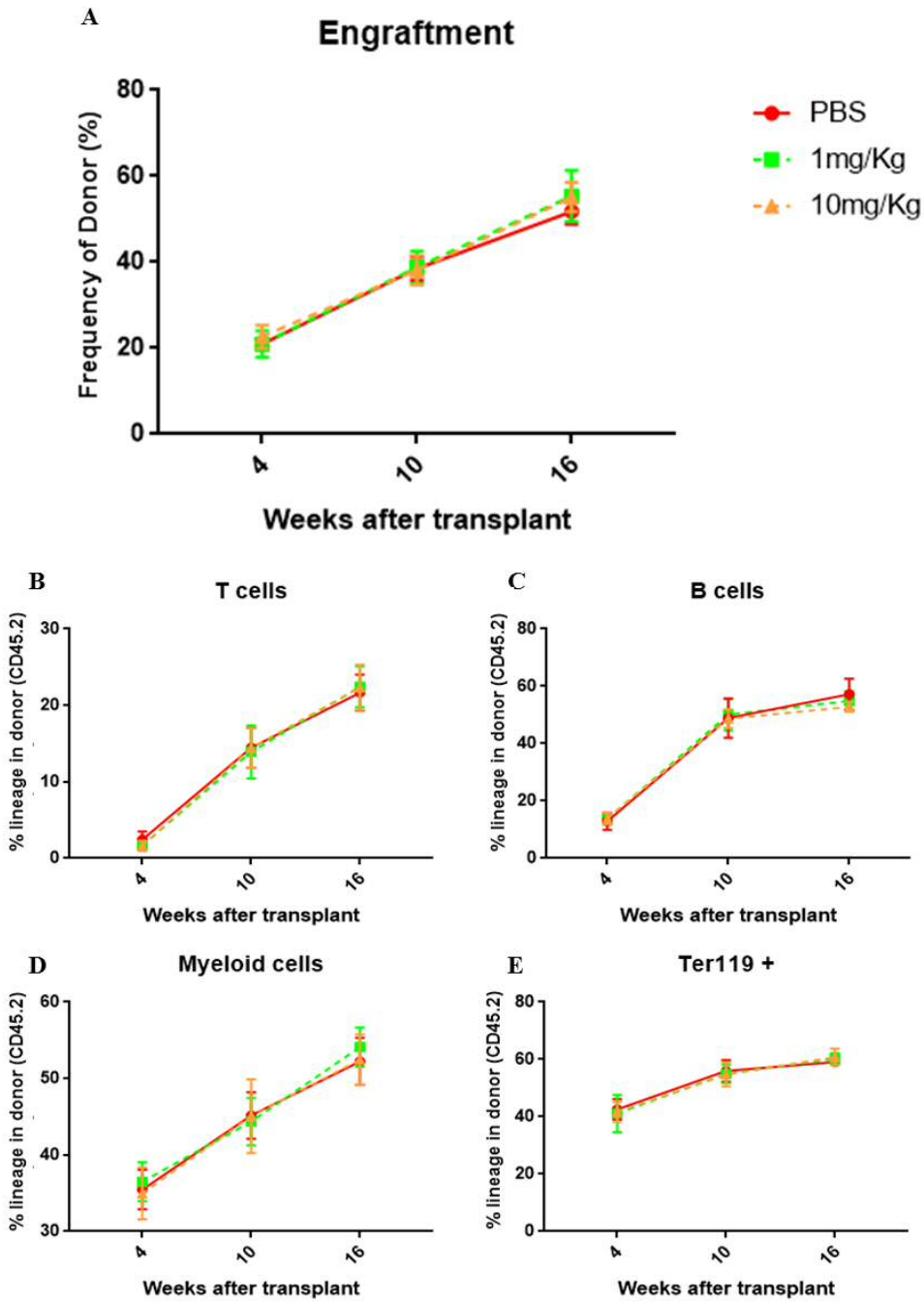


Figure 3. 34 *In vivo* treatment of Yohimbine after transplant does not affect HSC self-renewal in secondary recipients

Primary recipients were treated with Yohimbine or PBS. After 16 weeks, donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}$Lin-$Sca1^{+}cKit^{+}$ cells per recipient). Blood samples were taken to measure engraftment (A) and reconstitution of lymphoid (B and C), myeloid (D) and erythrocyte (E) cells using T- ($CD4^{+}/8^{+}$) and B cell (B220), myeloid (Mac1/Gr1) and erythroid (Ter119) markers.

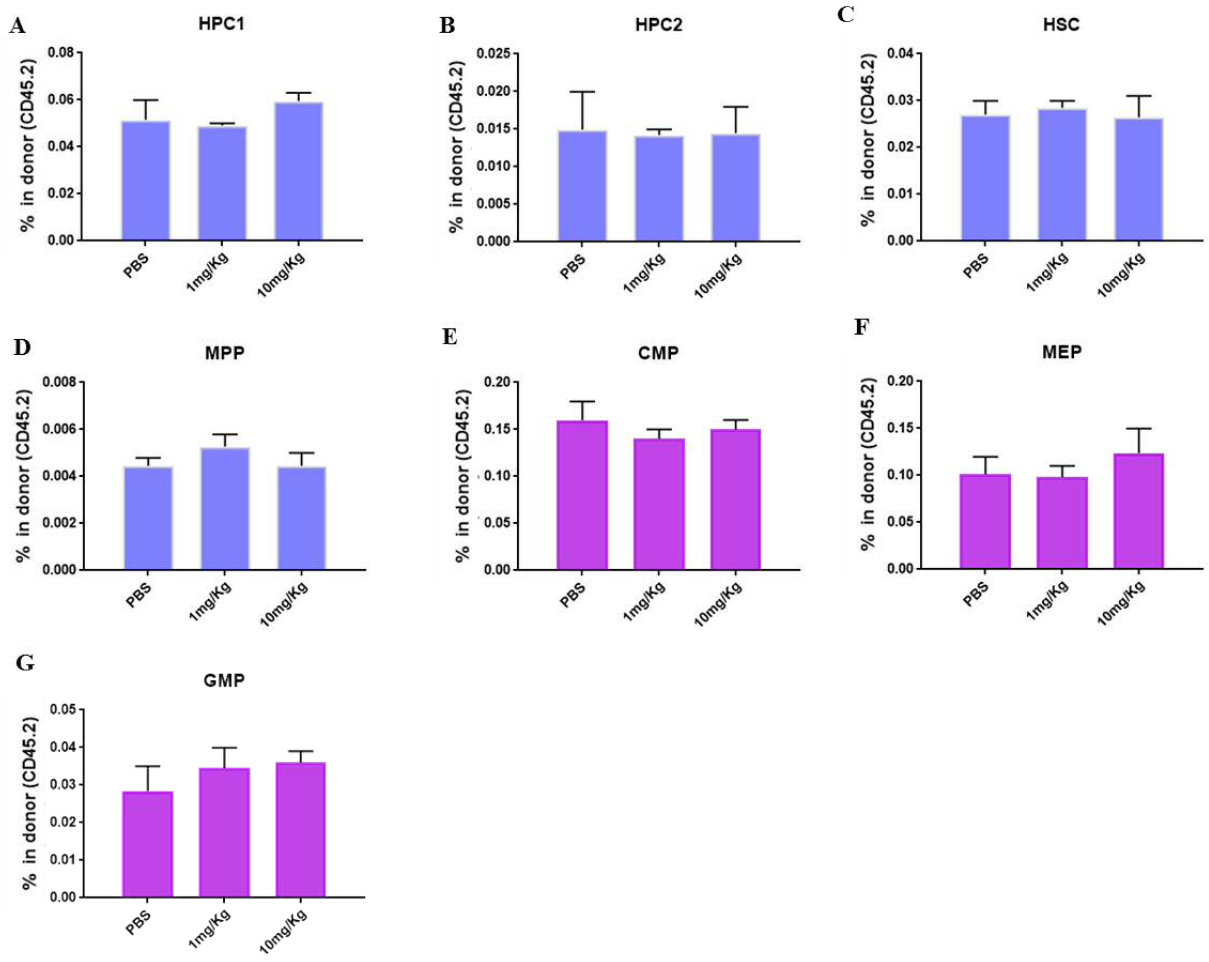


Figure 3. 35 No impact of Yohimbine administration after transplant on long term HSCs or HPCs

Primary recipients were treated with Yohimbine (or vehicle) was then administered once every two days for a total of 10 treatments. After 16 weeks, donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin\text{-}Sca1^{+}cKit^{+}$ cells per recipient). Analysis of SLAM (A-D) and progenitor (E-F) bone marrow populations was conducted 16 weeks after secondary transplant. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group

3.7 Discussion

Growing evidence has shown that bone marrow is innervated by the sympathetic nervous system. Moreover, agents targeting adrenergic signalling have also been shown to modulate HSC function in bone marrow transplant. We therefore sought to investigate the impact of adrenergic signalling on HSPC function. From our compound library screen, we found that Yohimbine, an α2-adrenergic receptor antagonist, targeted stem cell activity. Thus, we assessed whether Yohimbine can be utilised therapeutically. Firstly we investigated the impact of Yohimbine on stem cell expansion ex vivo. We also studied the effects of Yohimbine *in vivo* under homeostasis. Lastly, as it is understood that patients undergoing bone marrow transplant are at an increased risk of infection, we investigated the ability of Yohimbine to rescue animals after bone marrow transplant (as summarised in table 3.1).

We initially investigated HSC function ex vivo when treated with Yohimbine. Little effect on stem cell frequency was observed in bone marrow cells in response to the compound when they were exposed at 2, 6, 12 and 24 hours. However, the bone marrow cells were kept in cytokine-free media, this was to eliminate factors that may affect stem cell function. This may have caused an unfavourable environment for survival of the cells, particularly for extended periods of time. As an alternative approach to this study, more purified HSC and HPC populations could have been used to study the effects of Yohimbine *in vitro* with and/or without supporting media conditions. In previous studies, the use of serum-free media has been described as suitable conditions to maintain HSCs maintenance. For example, StemSpan medium was used with cytokines (Tpo, Flt3L and stem cell factor) to maintain HSCs ex vivo for transplant (Liu et al. 2015). Another study investigated the regulation of the Wnt and mTor pathways in HSC maintenance. Interestingly, they used the X VIVO 15 medium in serum- and cytokine-free conditions (Huang et al. 2012). These demonstrate that HSC maintenance is affected by culture conditions and this raises the idea that these conditions can be utilised in conjunction with our compound.

Upon treatment with Yohimbine, cells re-plated in semi-solid medium showed to have increased macrophage colony-forming units (CFU-M) and total colony number suggesting that the compound

may have altered lineage differentiation of HSPCs. Of course CFC assay is a vigorous method of testing multi-potential progenitor function; however a more stringent test would have been through transplant to assess the true function of HSPC populations.

Although WBM was used in ex vivo studies (meaning that all bone marrow cell types are present), it is not a representation of the natural stem cell niche and so therefore it was essential that these compounds were investigated *in vivo*. Prior to this, it was important to recognise the presence of the receptors that these compounds could potentially target. It has already been shown that adrenergic receptors reside in the bone marrow (Pereira et al. 2003). In fact in an earlier study, it was shown that adrenergic antagonists enhanced granulopoiesis and platelet production in mice under homeostatic conditions and after transplant (Maestroni et al. 1992; Maestroni and Conti 1994). However it was still unclear where the target cell of these antagonists resided. More interestingly, a recent study was able to show the expression of alpha1- and alpha-2 adrenergic receptors on murine HSPCs (Muthu et al. 2007). In our receptor expression analysis conducted, we also observed the expression of alpha2-adrenergic receptor in whole bone marrow, as well as HSPCs. However protein validation was not conducted to prove the existence of this receptor at the protein level. In addition to this, online RNAseq analysis has shown that in fact haematopoietic cells lack the expression of adrenergic receptors. This may suggest that Yohimbine exerts its effects through the central nervous system that indirectly influence haematopoiesis..

On the basis of the expression of the above receptors, it was possible to administer the compound in mice under homeostasis to assess the impact of the drug on HSCs/HPCs *in vivo*. We saw elevated frequencies of HSCs and primitive progenitors in Yohimbine treated animals. This was only the case after 10 days of treatment (instead of 5 days); suggesting prolonged treatments are required to elicit an expansion of HSCs *in vivo*. In addition to this, we did not observe greater effects at higher concentrations suggesting off target effects result from higher dosages. We also only observed this effect in bone marrow tissue which is the primary site of adult haematopoiesis. This may suggest that the effect of Yohimbine is exclusively through neuromodulation of the bone marrow. We also hypothesised that Yohimbine could elicit egression of HSCs to the peripheral blood however this was

not the case. In fact we observed a depleted neutrophil frequency in the blood which may be caused to harsh lysis. It will be intriguing to test the possibility that when coupled with a mobilizing agent (for example G-CSF) expanded HSCs can be driven from the bone marrow niche into the peripheral blood and could pose a therapeutic mobilizing agent. This would lead to a more efficient mobilisation regimen as currently G-CSF mobilisation strategies demonstrate a 5-30% failure rate in healthy donors (To et al. 2011). In our study, myeloid cells were significantly elevated in the peripheral blood. This raises the possibility of mobilisation or expansion of myeloid cells in the peripheral circulation as a result of Yohimbine administration. It may prove to be useful in patients that have undergone bone marrow transplant and require circulating immune cells to decrease the risk of infection in such a critically vulnerable stage of recovery.

We functionally tested Yohimbine treated HSPCs in transplantation assays. We found that in primary transplants, Yohimbine treated donor cells gave rise to rapid repopulation of B-cells. This was also observed when Yohimbine was administered after transplant in a separate experiment, suggesting that Yohimbine may influence the progression of differentiation towards the lymphocytic lineages after bone marrow transplant. It is not yet possible to determine how Yohimbine elicits the generation of B-cells. However Yohimbine could be acting on CLP and LMPP populations which have not been assessed in this study after primary transplant. We also observed an increased production of donor-derived erythrocytes throughout the 16 week transplant period in 10 mg/kg treated donor cells. Interestingly, it has been previously discovered that lymphocytes and erythrocytes express α2-adrenergic receptors (Hoffman et al. 1982; Titinchi and Clark 1984). Moreover, it was also shown that the majority of lymphoid organs are primarily innervated by sympathetic (adrenergic) nerve fibres (Felten et al. 1985). Yohimbine may thus be acting on direct signalling to lymphoid organs eliciting changes in B-cell production of transplanted cells.

It is understood that delays in reconstitution of immune cells after bone marrow transplant is associated with increased risk of infection and disease relapse (Auletta and Lazarus 2005). We therefore hypothesised that Yohimbine may prove to be a critical therapeutic in improving immune reconstitution. We therefore assessed the effects of Yohimbine treatment after syngeneic bone marrow

transplant. We found that there was an enhanced donor T cell reconstitution 2 weeks after transplant. In contrast to our competitive transplant study, we did not observe significant changes in B cell reconstitution. This difference in response may be caused by the direct interaction of Yohimbine on the adrenergic innervation of immune cells after transplant. Under steady state conditions, Yohimbine treatment elicited similar responses suggesting that adrenergic signalling plays a role in immune regulation. In addition to T cells, erythrocytic recovery was also improved as a result of Yohimbine treatment after transplant. Although this was previously observed (Maestroni et al. 1992), it was not described that specific α2-antagonists could elicit such responses. These results provide an encouraging prospect for combating the development of infections and anaemia during the recovery period after bone marrow transplant.

It is still unclear how Yohimbine may be exerting its effects, however it is already known, and as previously described, Yohimbine is an antagonist of the α2-adrenergic receptor (Millan et al. 2000). It is also understood that the α2-adrenergic receptor is a g-protein coupled receptor (GPCR), more specifically of the Gi subfamily. In the haematopoietic setting, a major regulator of HSC function is the CXCR4 receptor which in fact is a Gi GPCR (Busillo and Benovic 2007). CXCR4 has been implemented in maintaining quiescence of HSCs (Nie et al. 2008). Although the specific mechanism of action of Yohimbine is yet to be determined, it is hypothesised that the antagonistic effects of Yohimbine may promote the inhibition of quiescence in HSCs.. It is therefore imperative to investigate the cell cycle status of HSCs after Yohimbine treatment.

In this investigation, we have demonstrated that Yohimbine causes *in vivo* expansion of HSCs and primitive HPC within the bone marrow. Moreover, increased circulating myeloid cells were observed in the peripheral blood of animals treated with Yohimbine. We further assessed the function of *in vivo*-treated HSCs in a competitor transplant experiment. Here, observed a marked increase in circulating erythrocytes and B-cells. In parallel, we also studied the effects of Yohimbine on haematopoietic recovery after transplant. We showed that Yohimbine treatment elicited an enhancement in early T cell reconstitution. Interestingly, improved erythrocytic recovery was also evident in this setting. This may be an indication of the differing influences of neuromodulation

during homeostasis and in transplant setting. It also shows that neuronal signalling can be influenced by active compounds to elicit altered HSC activity which may prove to be critical in bone marrow transplant therapies.

		HSC (LSK)	Progenitors (LK)	Lin$^+$			
				B-cells	T-Cells	Erythroid	Myeloid
In vitro		-	-				
Bone Marrow		↑	↑CLP	-	-	-	-
Blood		-	-	↓	↑	-	↑
Competitor Transplant	Primary	-	-	↑	-	↑	-
	Secondary	-	-	-	-	-	-
Treatment following BMT	Primary	↑ HPC1 ↑MPP	-	-	↑	↑	-
	Secondary	-	-	-	-	-	-

Table 3.1 Summary of the impact of Yohimbine *in vitro* and *in vivo*

Yohimbine was initially studied on whole bone marrow *in vitro*. Since the *in vitro* conditions did not mimic the normal physiological bone marrow environment, *in vivo* assessment of Yohimbine was conducted to assess its impact on HSPCs. Further functional assessment of HSPCs in response to Yohimbine treatment was undertaken in transplant studies.

Chapter 4: The impact of the muscarinic receptor agonist, Oxa-22 (cis-2-Methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide), on haematopoietic stem and progenitor cell function and leukaemia

4.1 Introduction

Cis-2-Methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide, Oxa-22, is an agonist of the M3 muscarinic receptor. Typically M3 receptors are found in regions of the brain, lung and pancreas (Buels and Fryer 2012; de Azua et al. 2012; Weston-Green et al. 2012). However it has been shown that the M3 receptor is also expressed in the bone marrow (Pereira et al. 2003). On the otherhand, ProteinAtlas data shows that this receptor is not expressed in human marrow. A physiological agonist of the M3 receptor is acetylcholine (Ach) which is traditionally recognised as a signalling neurotransmitter. In addition to the nervous system, Ach has also been found to have important functional roles in the autocrine, paracrine and immune systems. It has been shown that T-lymphocytes are an abundant source of Ach in the blood and express choline acetyltransferase (ChAT, an enzyme involved in Ach synthesis) in addition to muscarinic and nicotininc Ach receptors (Kawashima et al. 2012). The role of T-cell mediated Ach signalling has been elucidated in the anti-inflammatory pathway also known as the inflammatory reflex (Martelli et al. 2014). It is yet unknown of the exact roles of this receptor, or Ach signalling, in haematopoiesis.

It wasn't until the early 1900's where the presence of Ach in the spleen was identified in the ox and horse giving rise to the theory of cholinergic innervation of the spleen (Dale and Dudley 1929). This was further supported by experiments using retrograde tracing techniques (techniques that follows neurons from their synapses to their origins) where rodent spleens were thought to possess cholinergic input (Buijs et al. 2008). On the other hand, Bellinger et al discounted the idea of splenic innervation (Bellinger et al. 1993) and more recent studies have described the origin of Ach is actually from epithelial cells and splenic lymphocytes (Wessler et al. 1998; Rosas-Ballina et al. 2011). The debate of cholinergic innervation of the spleen is ongoing as thorough investigations *in vivo* have yet to be conducted.

Many studies have shown the importance of cholinergic regulation of bone modelling and the surrounding bone marrow niche. Muscarinic receptor expression was found to be evident in haematopoietic niche cells such as osteoblasts, osteoclasts and mesenchymal stem cells (En-Nosse et

al. 2009; Hoogduijn et al. 2009; Sato et al. 2010; Liu et al. 2011; Tang et al. 2012). *In vitro* investigations have shown that osteoblast proliferation was increased in response to muscarinic stimulation (Sato et al. 2010; Liu et al. 2011). In addition to this, the M3 subtype has been found to be essential in bone physiology (Shi et al. 2010; Bajayo et al. 2012; Kliemann et al. 2012). Loss of the M3 muscarinic receptor results in mice exhibiting osteoporotic bones due to a decrease in osteoblasts (Shi et al. 2010), a critical component of a complex bone marrow micro-environmental network involved in the maintenance of HSCs (Calvi et al. 2003). It can therefore be hypothesised that cholinergic innervation may indirectly impact the function and behaviour of HSCs and other haematopoietic cells within the bone marrow. Moreover, the agonistic effects of Oxa-22 on the M3 muscarinic receptor may provide an insight of how cholinergic innervation impacts haematopoiesis under steady state- and transplant conditions.

In addition to healthy steady state conditions, cholinergic innervation has also been described in the disease setting. A study looking into gastric tumorigenesis demonstrated the importance of cholinergic signalling in maintaining the tumour (Zhao et al. 2014). Here they showed that surgical and pharmacological vagal denervation reduced tumour progression and prolonged survival in mice models (Zhao et al. 2014). Additionally, they discovered that denervation also inhibited Wnt signalling and stem cell expansion. It was also shown that gastric organoid growth was promoted when co-cultured with neurons. More interestingly, genetic knockdown or inhibition of the M3 receptor supressed the neuronal stimulatory effects (Zhao et al. 2014). The M3 receptor has also been described in other cancers such as melanoma and even involved in metastatic processes (Lammerding-Koppel et al. 1997; Boss et al. 2005; Oppitz et al. 2008). More closely in the leukaemic setting, as it is known that T-cells synthesise Ach, it was found that Ach levels were significantly higher in T-cell leukaemic cell lines compared to healthy T-cells (Fujii et al. 1996). Further analysis showed that the M3 receptor was strongly expressed in Jurkat cells (Alea et al. 2011). This supported the finding of an earlier study where they also showed that an M3 agonist elicited elevated intracellular Ca^{2+} levels in response to PLC and IP3 signalling within Jurkat cells (Kaneda et al.

1993).This suggests that the M3 receptor and cholinergic signalling has a role in tumorigenesis. We therefore sought to study the agonistic effects of Oxa-22 within the leukaemic setting.

4.2 Muscarinic receptor expression in haematopoietic cells

The expression of muscarinic receptors has previously been reported in human neutrophils, mononuclear cells and stromal cells (Pereira et al. 2003). We therefore sought to identify the presence of these receptors in murine HSPCs. Specific HSPC populations were isolated by FACS and further q-RT PCR analysis was conducted to assess expression levels of the M3 receptor, the target receptor for Oxa-22. Gene expression analysis revealed that the M3 receptor is expressed throughout HSPC populations (Figure 4.1). Moreover, we also observed the expression of the M3 receptor in MSCs (Figure 4.1).

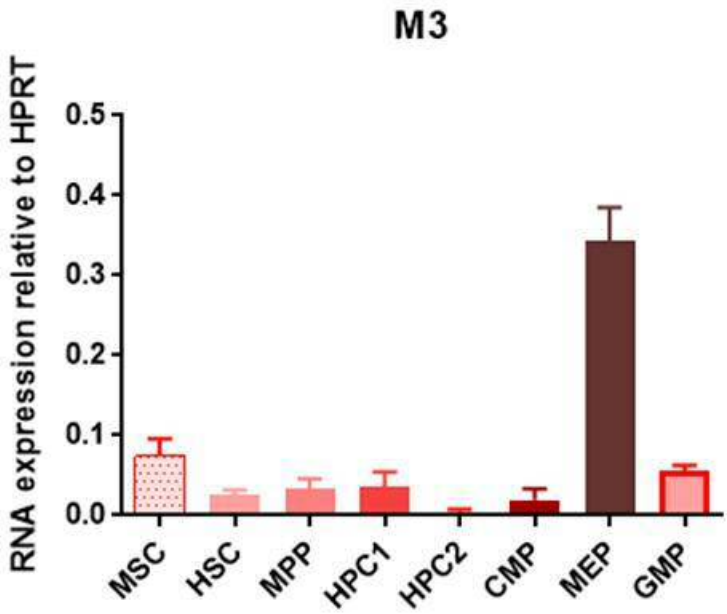


Figure 4. 1 MSCs and HSPCs express the M3 muscarinic ACh receptor

(A) M3 muscarinic receptor RNA levels in MSCs, primitive stem cells and lineage restricted progenitors. Data represents mean $^{+}$ SEM from *n*=4. (B) Electrophoresis with q-PCR products of MSCs and haematopoietic populations.

4.3 The ex vivo effects of Oxa-22 on HSCs and HPCs

We first assessed whether Oxa-22 could impact HSPC function ex vivo. We treated whole bone marrow (suspended in StemSpan™ media without cytokines) with a range of concentrations (1nM-1mM). Exposure of Oxa-22 on whole bone marrow did not affect the frequencies of LSK or LK cells *in vitro* (Figure 4.2). In addition to immunophenotypical analysis, functional analysis also revealed that ex vivo treatment with Oxa-22 did not elicit any changes in the colony forming capacity of stem and progenitor cells in a CFC assay (Figure 4.3). This data shows that ex vivo exposure of HSCs/HPCs to Oxa-22 has no immunophenotypic or functional effect.

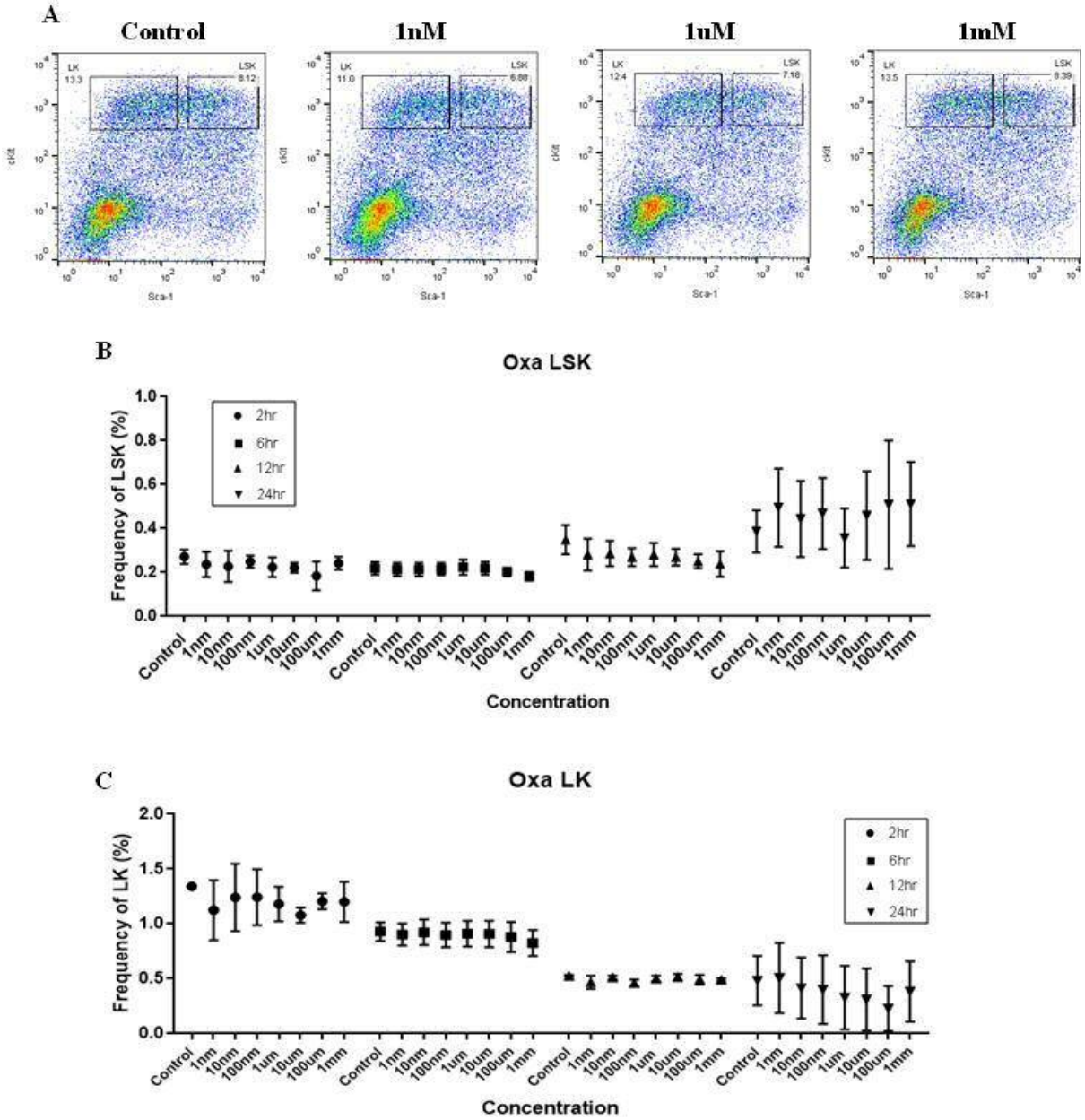


Figure 4. 2 Bone marrow cultured with Oxa-22 does not affect HSPCs ex vivo

Whole bone marrow (WBM) was harvested and plated at a density of $1x10^6$ cells per well. Cells were treated for 2, 6, 12 and 24 hours ex vivo using a range of concentrations (1nM-1mM). (A) Representative flow cytometric plots identifying LK (Lin- Sca-1^+) and LSK (Lin- Sca-1^+ Ckit$^+$) populations between vehicle treated cells compared to 1mM treated cells at 24hrs. The prevalence of (B) HSC enriched LSK and (C) LK cell populations were initially assessed after ex vivo exposure to Yohimbine. *N=3*, cells plated in triplicates, three separate experiments

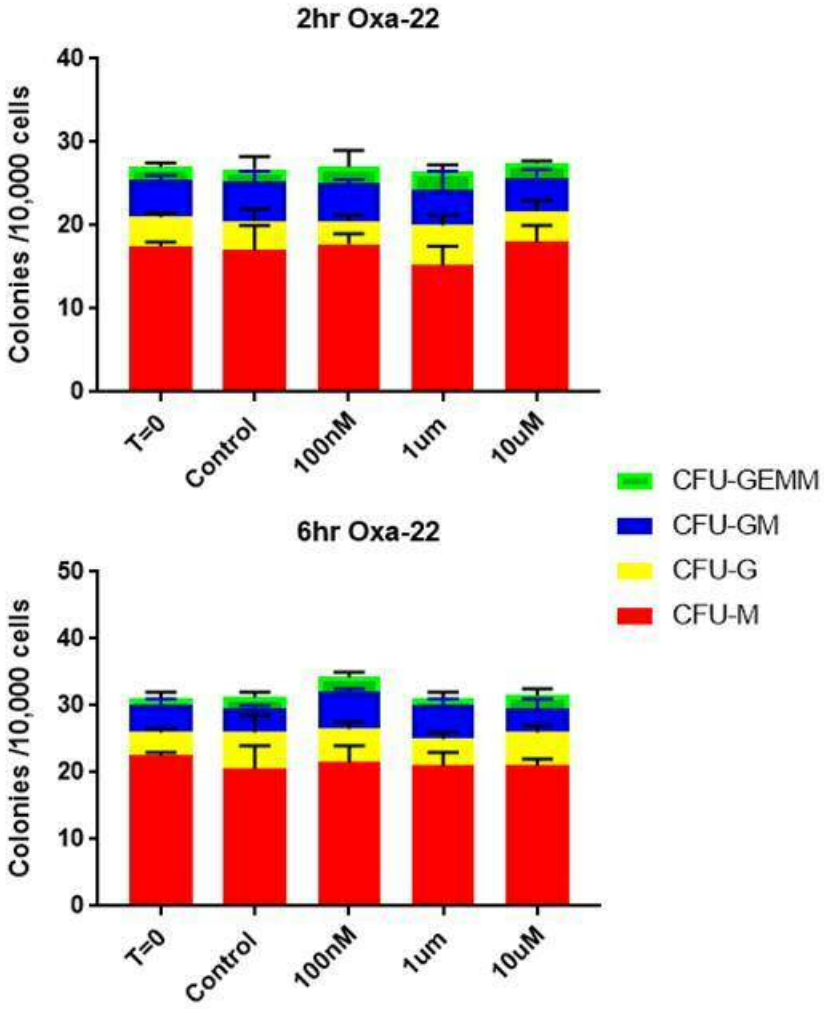


Figure 4. 3 Bone marrow cells exposed to Oxa-22 have unaltered colony forming potential

WBM cells were exposed to Yohimbine for 2 and 6 hours at concentrations of 100nM, 1uM and 10uM before washing and re-plating in Methylcellulose. After 10 days of maturation in both control and treated settings, multi-potential and lineage-restricted progenitors of the CFU-M, macrophage, CFU-G, granulocytic, CFU-GEMM, mixed and CFU-GM, granulocyte/monocyte formed. Colonies are scored for each respective concentration through duplicate wells in 3 separate experiments.

4.4 The impact of *in vivo* administration of Oxa-22 on HSPCs

4.4.1 *In vivo* Oxa-22 administration enhances bone marrow HSC numbers

Next we sought to study the impact of Oxa-22 *in vivo*. Here wild-type animals were treated with Oxa-22 once daily for 5 days. After the final administration of Oxa-22, bone marrow, spleen and blood were harvested and analysed by flow cytometry for HSC (SLAM markers (LSK $CD150^{+}CD48^{-}$)) and HPC (LK CD34/CD16/32) populations (Figure 4.4A). We found that the frequency of HSCs were significantly higher in 100 ng/kg treated animals compared to PBS controls (Figure 4.4B). Interestingly, we also observed a similar effect in the MPP populations in both 10 and 100 ng/kg of the compound (Figure 4.4C). Animals treated with 1 ug/kg, however, demonstrated a decrease in stem cells and primitive progenitors (Figure 4.4B-E). Unlike the LSK compartment, committed myeloid progenitors of the LK compartment were unaffected by the treatment of Oxa-22 (Figure 4.5). In addition to this, total cell numbers of whole bone marrow after treatment were documented and no differences in the cellularity of treated and control animals were found (Figure 4.6A). However, increased numbers of HSC and MPP populations was observed correlating with the observed frequencies above (Figure 4.6B and 4.6C). We also examined HSC and HPC populations within the spleen; we found that treatment with Oxa-22 did not affect the HSPC population (Figure 4.7). These data show that Oxa-22 specifically targets primitive stem and progenitor cell populations of the bone marrow, however at high doses; Oxa-22 depletes HSCs and primitive progenitor populations within the bone marrow.

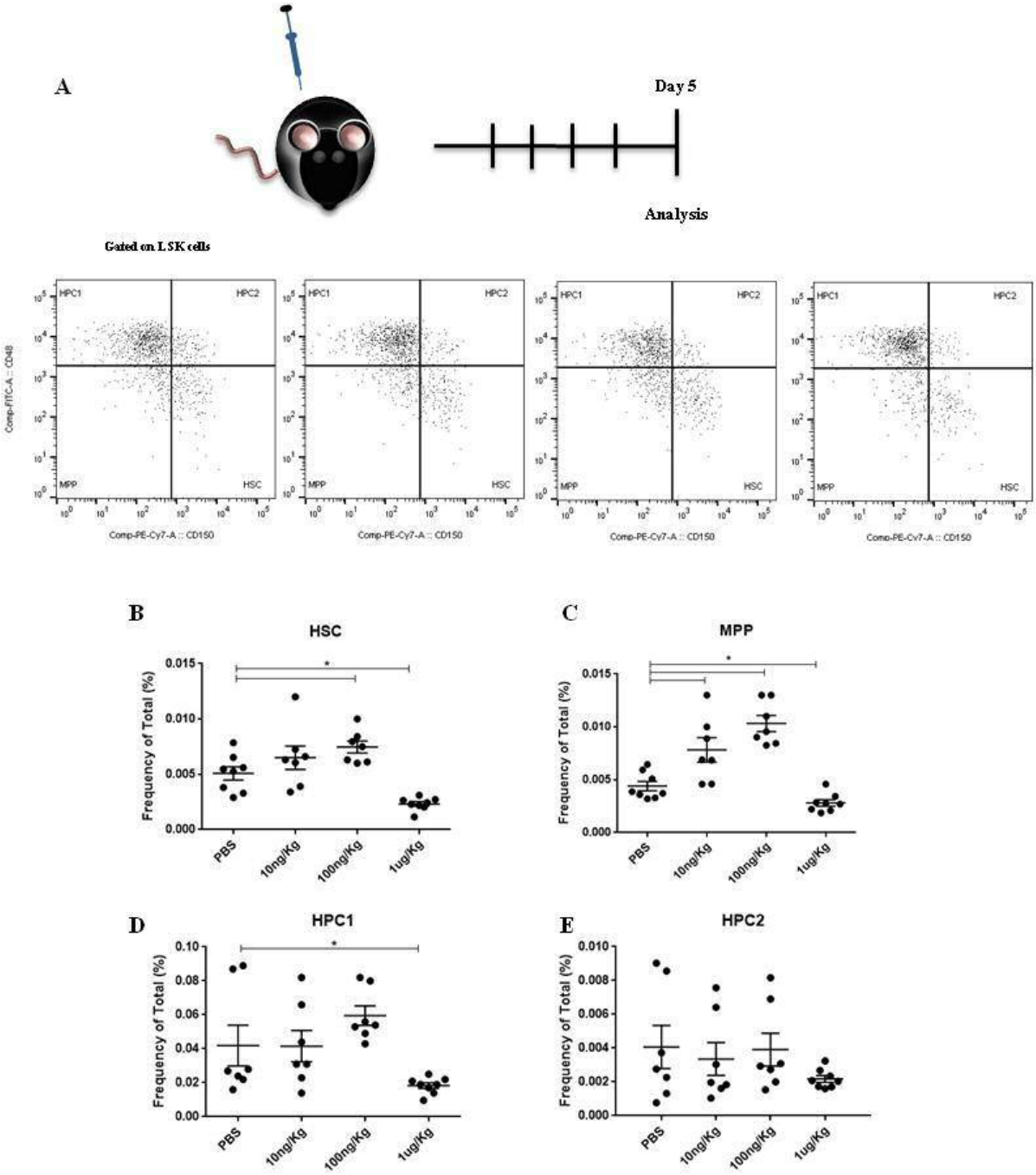


Figure 4. 4 *In vivo* Oxa-22 treatment expands bone marrow HSC and primitive HPC populations

Mice were treated with 10 ng/Kg or 100 ng/Kg of Oxa-22, or PBS daily for 5 days. 1 hour after the final administration, bone marrow was harvested and stained for SLAM markers for flow cytometry analysis. Specific HSC and primitive progenitor populations from within the LSK compartment were analysed (B-E). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, n=7 per group) *$p<0.05$.

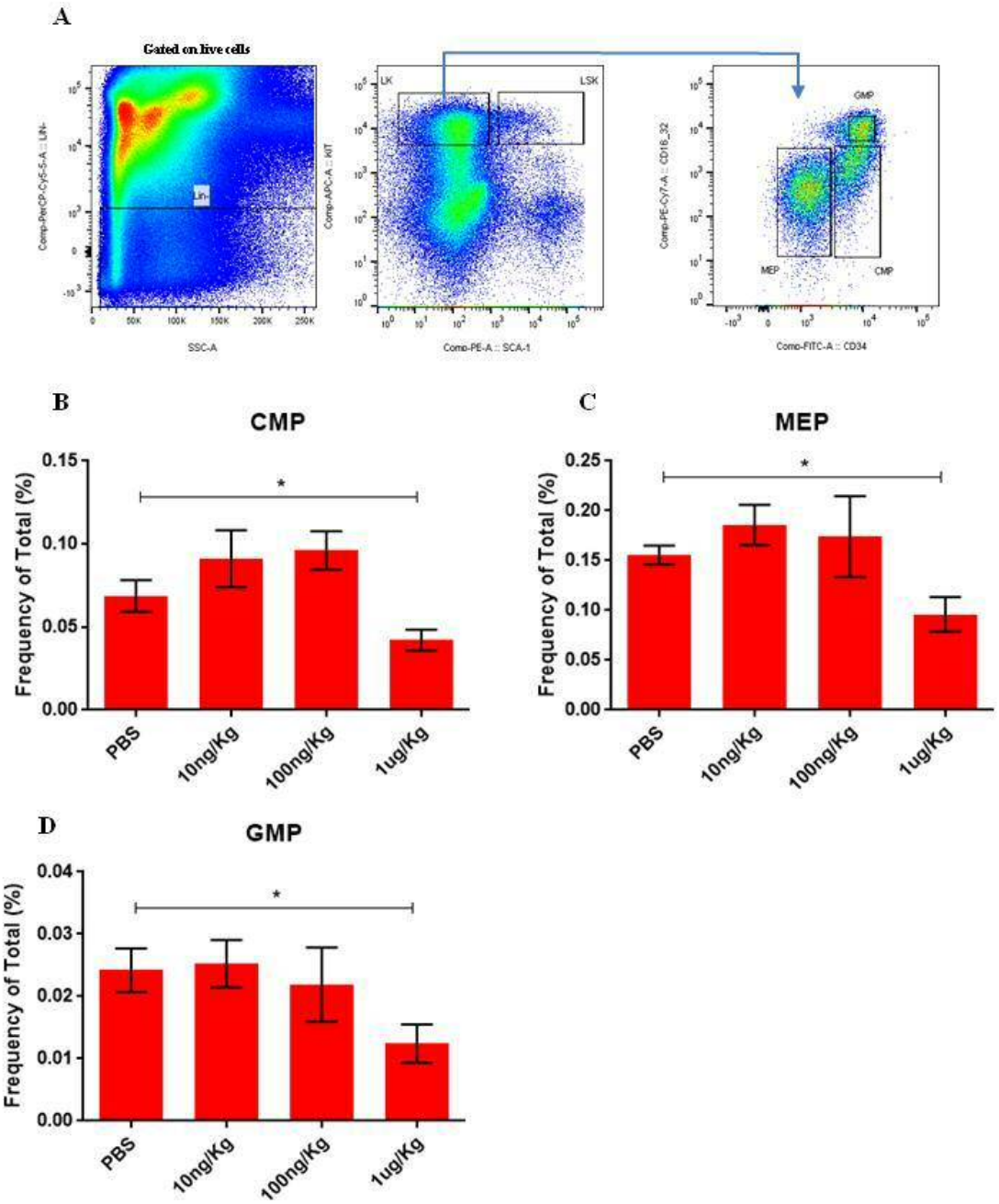


Figure 4. 5 Oxa-22 *in vivo* treatment decreases bone marrow lineage restricted progenitor cells at higher doses

Mice were treated with 10 ng/Kg, 100 ng/Kg or 1 ug/Kg of Oxa-22, or PBS daily for 5 days. 1 hour after the final administration, bone marrow was harvested and stained for progenitor populations derived from the LK compartment were analysed (A). (B) CMP (common myeloid progenitor), (C) MEP (megakaryocyte-erythroid progenitor) and (D) GMP (granulocyte-macrophage progenitor). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments n=7 per group *$p<0.05$.

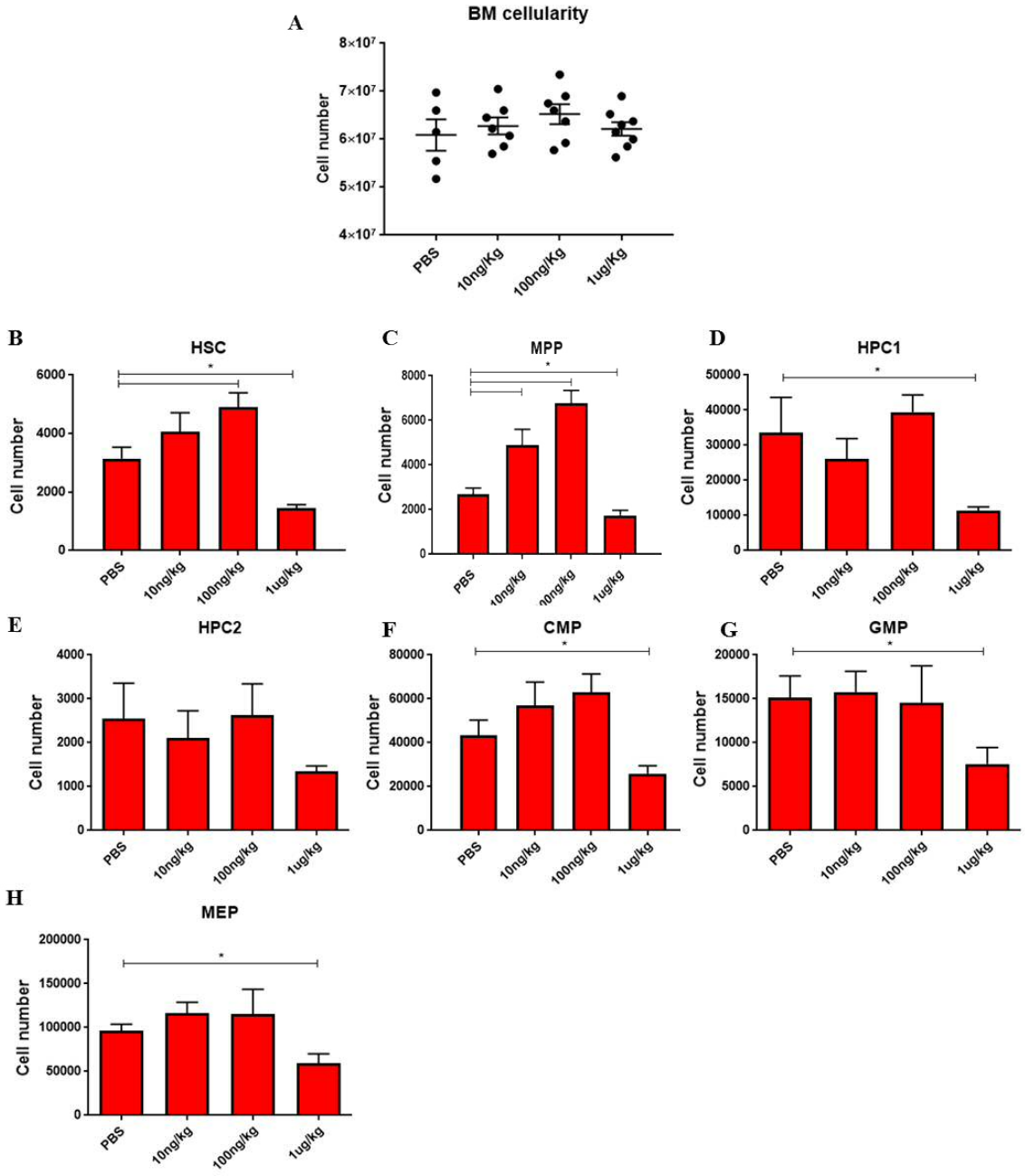


Figure 4. 6 Administration of Oxa-22 does not affect absolute bone marrow cell numbers

Mice were treated with 10 ng/Kg, 100 ng/Kg or 1ug/Kg of Oxa-22, or PBS daily for 5 days. 1 hour after the final administration, bone marrow was harvested and counted (A). Cells harvested from one tibia and one femur. Cell numbers were calculated using the frequencies (percentages) of each cell type against the total number of live cells from populations arising from within the LSK (B-E) and LK compartments (F-H). Error bars represent mean ± SEM of 2 independent experiments, PBS n=5, 10 ng/Kg n=7, 100 ng/Kg n=6, 1 ug/Kg n=8*$p<0.05$.

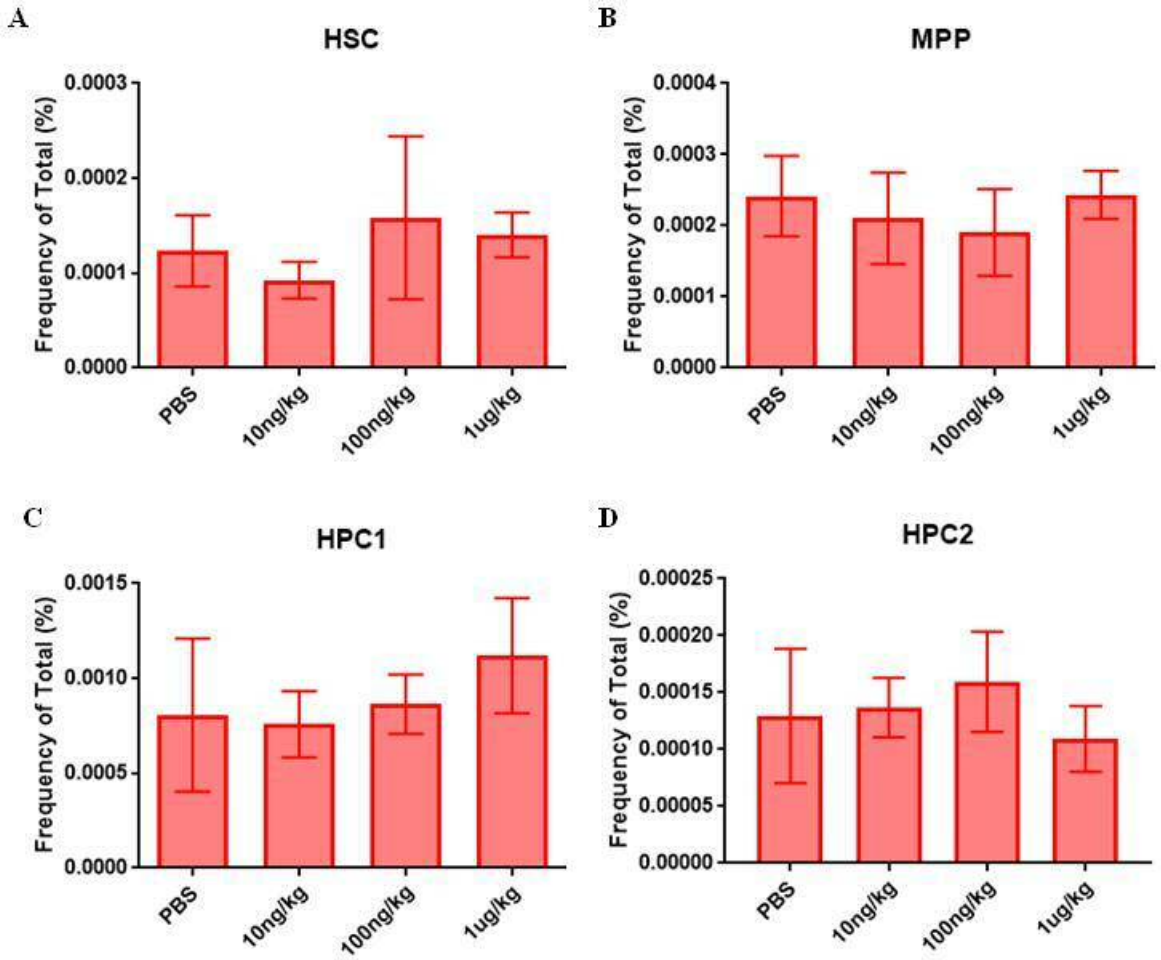


Figure 4. 7 *In vivo* treatment of Oxa-22 does not affect splenic HSPC populations

Mice were treated with 10 ng/Kg, 100 ng/Kg or 1ug/Kg of Oxa-22, or PBS daily for 5 days. 1 hour after the final administration, spleens were harvested and processed into suspension. Specific HSPC populations from within the LSK compartment were analysed by flow cytometry (A-D). Error bars represent mean ± SEM of 2 independent experiments, Error bars represent mean ± SEM of 2 independent experiments n=7 per group *$p<0.05$.

4.4.2 The *in vivo* impact of Oxa-22 on lineage differentiated cells

In addition to HSPC populations, we also analysed the effects of Oxa-22 on mature differentiated cell populations. Assessing, specifically, the bone marrow cells, we see that Oxa-22 treated animals have a no changes in B-cell and T-cell populations (Figure 4.8A and 4.8B). Conversely, cells within the myeloid compartment ($Gr1^+$, $Mac1^+$ and $Mac1^+Gr1^+$) are significantly higher in the 100ng/kg than that of the PBS control group (Figure 4.8D-F). This result was not recapitulated in the spleen; in fact we saw that the higher doses of Oxa-22 (100 ug/kg and 1 ug/kg) elicited a significant decrease in $Mac1^+Gr1^+$ cells (Figure 4.9D). This was also observed in both B- and T-cells (Figure 4.9A and 4.9B). Treatment with Oxa-22 did not result in any significant changes in blood lineage differentiated populations (Figure 4.10). Bone marrow cells from treated mice were also harvested for functional analysis in CFC. We did not see differences in colonies derived from treated cells compared to PBS controls (Figure 4.11). This data shows that Oxa-22 promotes the differentiation of myeloid cells within the bone morrow without an impact on peripheral blood.

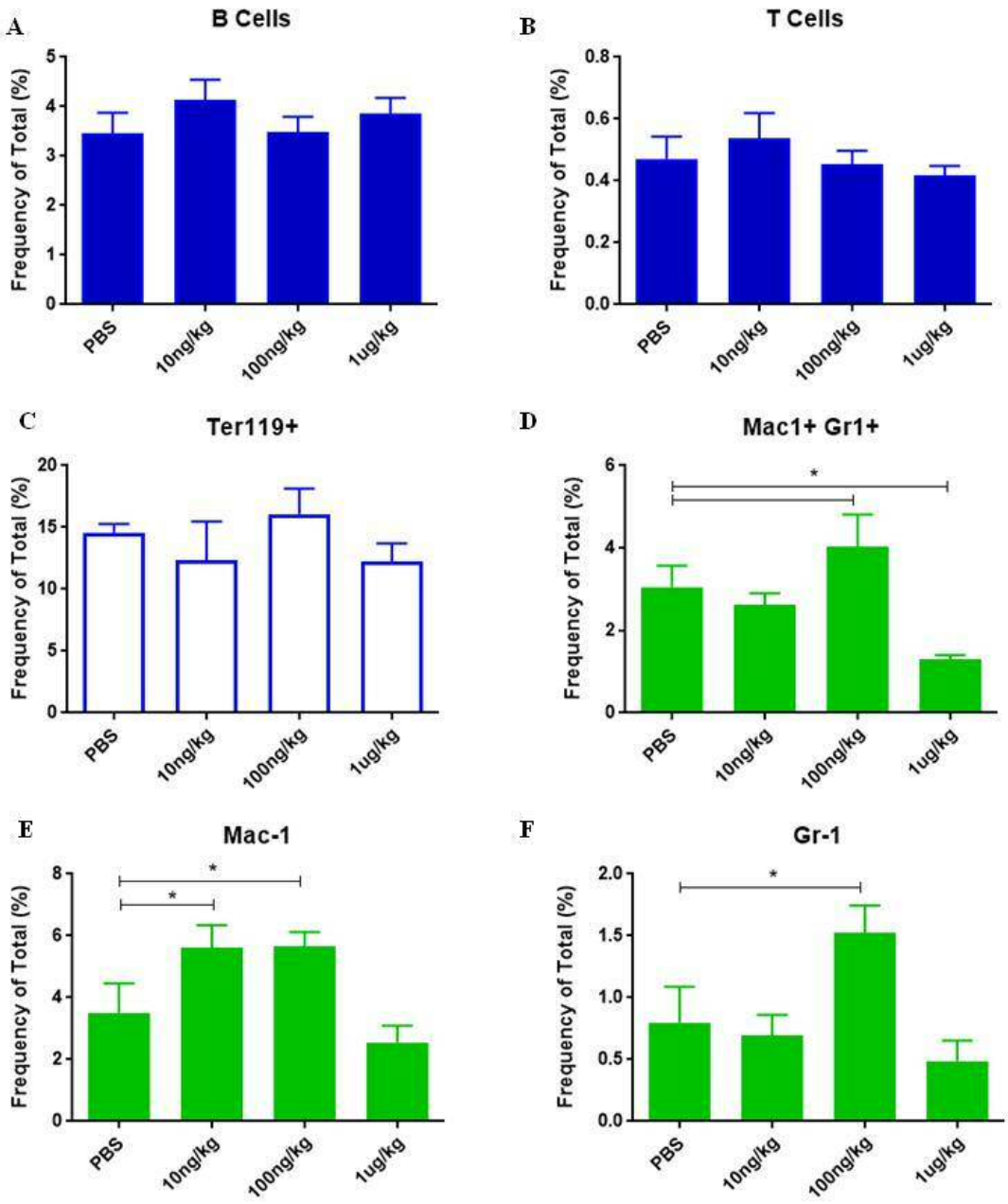


Figure 4. 8 *In vivo* Oxa-22 treatment increases bone marrow myeloid cells

Mice were treated with 10 ng/Kg, 100 ng/Kg or 1 ug/Kg of Oxa-22, or PBS daily for 5 days. 1 hour after the final administration, bone marrow was harvested and stained for lineage differentiated cells. Lymphoid cells include B- (A) and T- cells (B). Erythrocytes were identified as $Ter119^{+}$ (C) and cells of the myeloid compartment were defined as myeloid cells ($Mac1^{+}Gr1^{+}$) (D), monocyte/macrophage ($Mac1^{+}$) (E), Granulocyte ($GR1^{+}$) (F)**.** Error bars represent mean ± SEM of 2 independent experiments, Error bars represent mean ± SEM of 2 independent experiments n=7 per group *$p<0.05$.

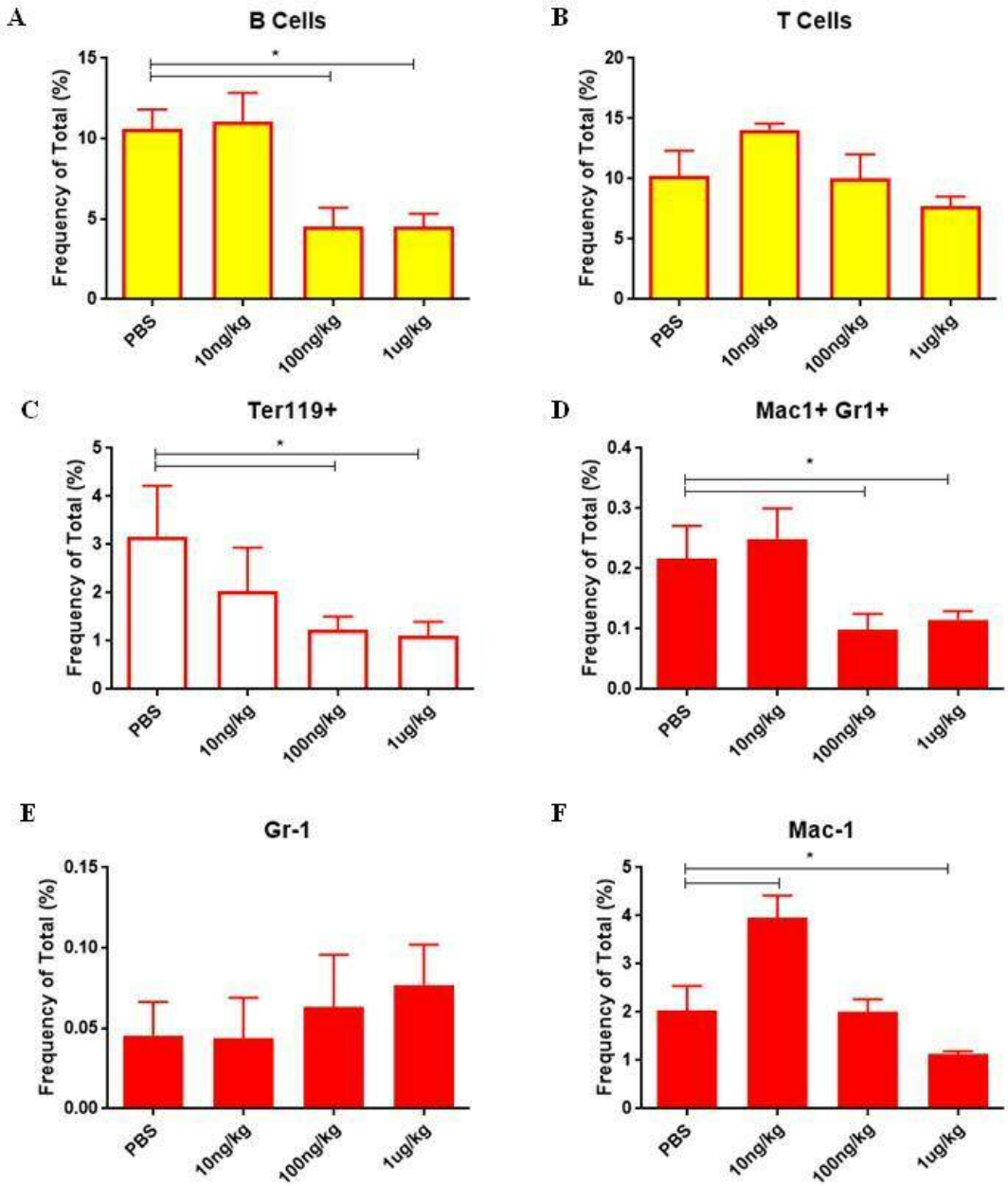


Figure 4. 9 Oxa-22 treatment *in vivo* depletes B cells, erythrocytes and myeloid cells within the spleen

Mice were treated with 10 ng/Kg, 100 ng/Kg or 1 ug/Kg of Oxa-22, or PBS daily for 5 days. 1 hour after the final administration, spleens were harvested, processed into suspension and stained for lineage differentiated cells. Lymphoid cells include B- (A) and T- cells (B). Erythrocytes were identified as $Ter119^{+}$ (C) and cells of the myeloid compartment were defined as myeloid cells ($Mac1^{+}Gr1^{+}$) (D), monocyte/macrophage ($Mac1^{+}$) (E), Granulocyte ($GR1^{+}$) (F)**.** Error bars represent mean ± SEM of 2 independent experiments, Error bars represent mean ± SEM of 2 independent experiments n=7 per group *$p<0.05$.

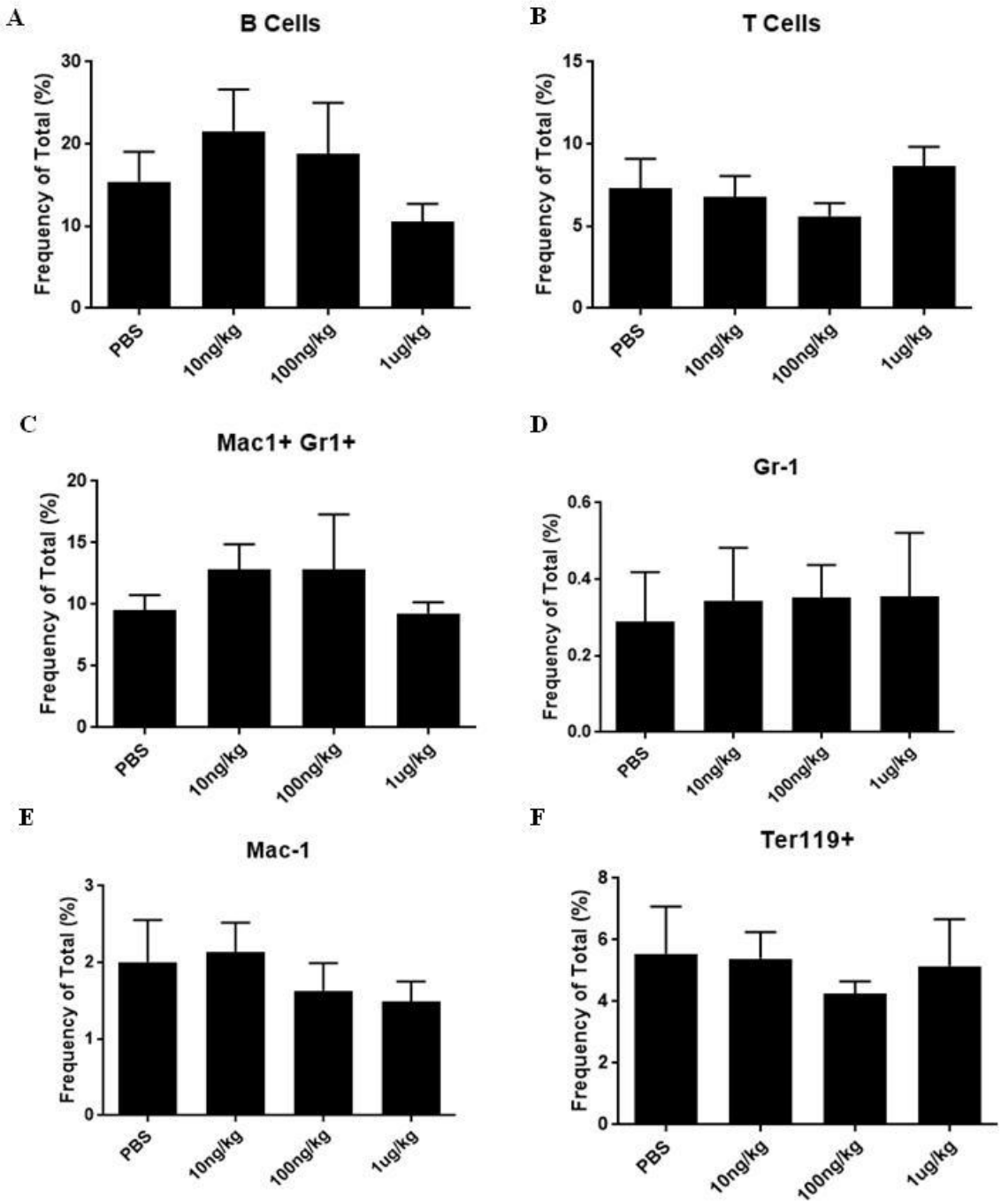
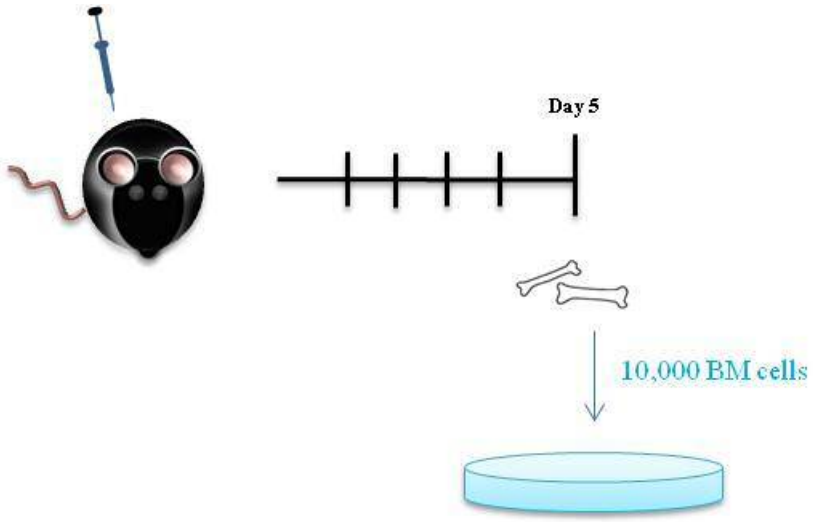


Figure 4. 10 Administration of Oxa-22 *in vivo* does not affect lineage differentiated cells of the peripheral blood

Mice were treated with 10 ng/Kg, 100 ng/Kg or 1 ug/Kg of Oxa-22, or PBS daily for 5 days, 1 hour after the final administration, blood was taken via an incision of the lateral tail vein and stained for mature differentiated populations. Blood collected from treated animals and analysed for lymphoid (A and B), myeloid (C-E) and erythroid (F) cells. Error bars represent mean ± SEM of 2 independent experiments, Error bars represent mean ± SEM of 2 independent experiments n=7 per group *$p<0.05$.



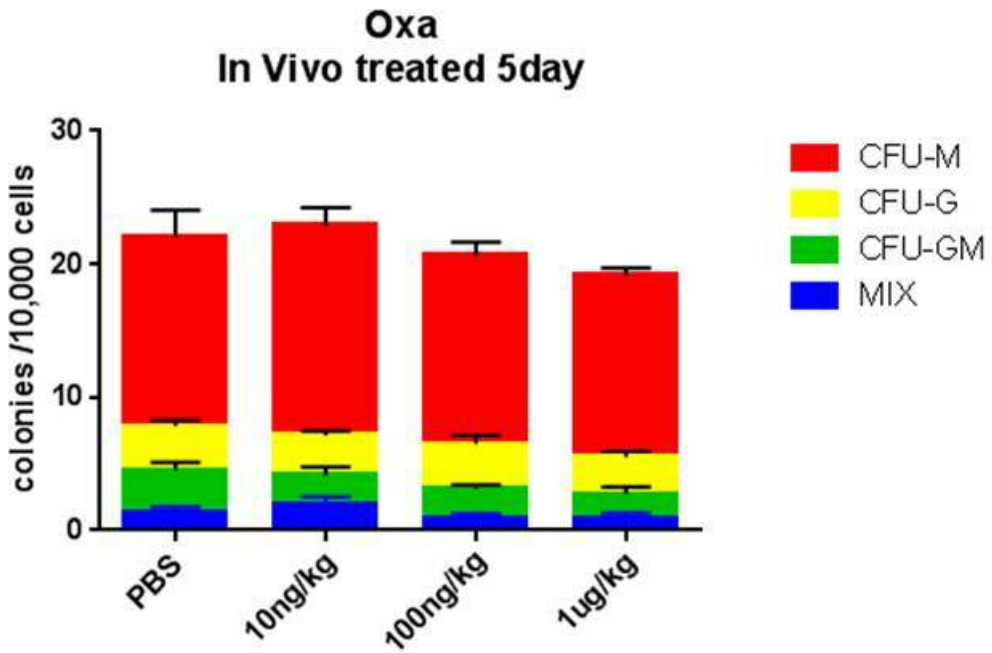


Figure 4. 11 *In vivo* Oxa-22 treated cells show no difference in colony forming potential

Mice were treated with 10 ng/kg, 100 ng/kg or 1 ug/kg of Oxa-22, or PBS daily for 5 days. After the final injection, bone marrow from the respective treated animals were harvested and plated in methylcellulose at a density of 10,000 cells. Colonies are scored after 10 days from plating in duplicate wells in two separate experiments, $n=3$ in each experiment. *$p<0.05$

4.4.3 The impact of Oxa-22 on HSC apoptosis

We observed enhanced HSC and MPP frequencies in Oxa-22 treated animals. To explore the mechanism for augmentation of the HSPC compartment of Oxa-22 treated animals, we assessed the impact of Oxa-22 on the apoptotic stateof HSCs and primitive progenitors. Here we used the same treatment regimen (5 days of treatment) followed by bone marrow harvest. Bone marrow cells were stained for SLAM markers and Annexin V to assess the frequency of apoptotic cells. We found that the frequency of live, and apoptotic, HSCs (and MPPs) were not altered in response to Oxa-22 treatment (Figure 4.12C and 4.12D). This data suggests that Oxa-22 may have little effect on HSC survival.

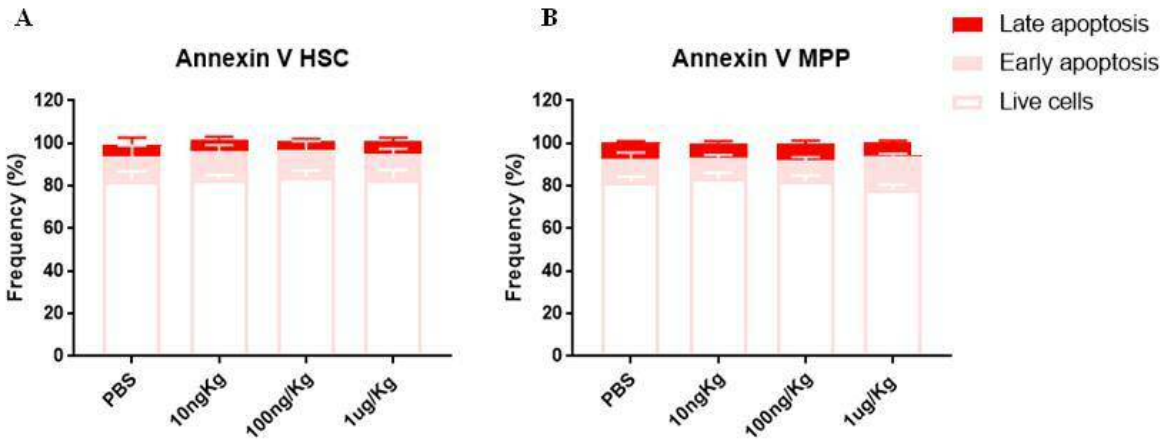


Figure 4. 12 *In vivo* treatment of Oxa-22 does not affect the apoptoic status of HSPCs

Mice were treated with 10 ng/Kg, 100 ng/kg or 1 ug/kg of Oxa-22, or PBS daily for 5 days. After the final injection, bone marrow from the respective treated animals were harvested and stained for Lin-Sca1^{+}cKit^{+} SLAM populations. Further intracellular staining was conducted with Annexin V (A and B) conjugated antibody and DAPI. Data shown as the frequency of the respective SLAM population. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group (3 for PBS) *$p<0.05$.

4.5 The impact of Oxa-22 treated cells on haematopoietic reconstitution

Under homeostatic conditions, Oxa-22 treatment *in vivo* caused an increase in HSCs; we next sought to investigate the functional ability of these *in vivo* treated HSCs/HPCs. Cells from treated animals (5 day treatment regimen) were harvested and transplanted competitively with untreated bone marrow (Figure 4.13A). As an increase in HSC frequency was observed in animals treated with 100ng/kg (1.5 fold), we normalised bone marrow number used so that a 1:1 ratio of HSC donor: competitor was transplanted. In the case of the 10ng/kg dose, we competitively transplanted equal numbers of cells as HSC frequency was not affected. In transplanted recipients, we discovered that Oxa-22 treated cells exhibited normal donor engraftment as seen in PBS treated cells (Figure 4.13B). We also measured donor reconstitution of myeloid and lymphoid lineages in the transplanted animals (Figure 4.13 and 4.14). Within the donor compartment, we found that Oxa-22 treated cells resulted in enhanced B-cell reconstitution after 8 weeks after transplant (Figure 4.13C). This was also seen in the T-cell population however this was not prolonged after 6 weeks (Figure 4.13D). We also assessed donor-derived myeloid reconstitution and found that there were no differences in $Mac1^{+}Gr1^{+}$ reconstitution in Oxa-22 treated donor cells compared to PBS treated controls (Figure 4.14A). However, donor cells treated with compound (100ng/kg) were able to contribute to an increased $Ter119^{+}$ reconstitution throughout the transplant period (Figure 4.14B) After 16 weeks, we analysed HSPC populations of the bone marrow; we did not observe any changes in donor derived HSCs or in the primitive progenitor populations (Figure 4.15A-D). A significant increase in the frequency of donor MEPs was observed but not in the CMP or GMP cells (Figure 4.15 F-G). This data suggests that when transplanted, Oxa-22 treated cells favoured lymphoid and red cell reconstitution without an obstruction of myeloid reconstitution.

In addition to the above, we analysed reconstitution by looking at the donor distribution within lineage differentiated cells. We found that there was a significantly increased frequency in donor cells from within the B-cell compartment from after 8 weeks in treated donor cells (Figure 4.16A). We also observed an increased reconstitution of donor cells within the T-cell population in 100 ng/kg treated donor cells (Figure 4.16B). We observed a deficiency in the early reconstitution of $Mac1^{+}Gr1^{+}$ cells in

10 ng/kg treated donor cells (Figure 4.16C). Conversely, donor frequency of erythrocytic cells was significantly higher in cells treated with 10 ng/kg after 2 weeks but this effect is short lasting and no differences are observed after this (Figure 4.16D). This further suggests that Oxa-22 treated donor HSPC may promote lymphoid reconstitution in the competitive transplant setting.

We further looked at the distribution of donor HSPC populations of primary recipient bone marrow. We found that there were no differences in the frequencies of HSCs in control groups compared to treated donor cells (Figure 4.17A). This was also the case in the MPP populations however a small but significant decrease in HPC1 and HPC2 cells were observed in Oxa-22 treated donors (Figure 4.17C-D). Donor LSK cells from primary recipients were isolated using FACS and transplanted into secondary recipients to assess long term HSC self-renewal. By conducting analysis of the peripheral blood at time points throughout the transplant period, we found that there were no differences in engraftment in treated donor cells compared to controls (Figure 4.18A). We also found that there were no differences in myeloid and lymphoid reconstitution (Figure 4.18A). At 16 weeks after the secondary transplant, we also studied donor LSK populations (Figure 4.18B). We did not observe a change in HSCs function in treated donor cells compared to PBS controls (Figure 4.18B). In addition to the LSK cells, we also assessed their apoptosis status' (Figure 4.18C). We observed no significant differences in cycling and apoptotic LSK cells. This data suggests that Oxa-22 treatment does not affect the long term function of transplanted HSCs.

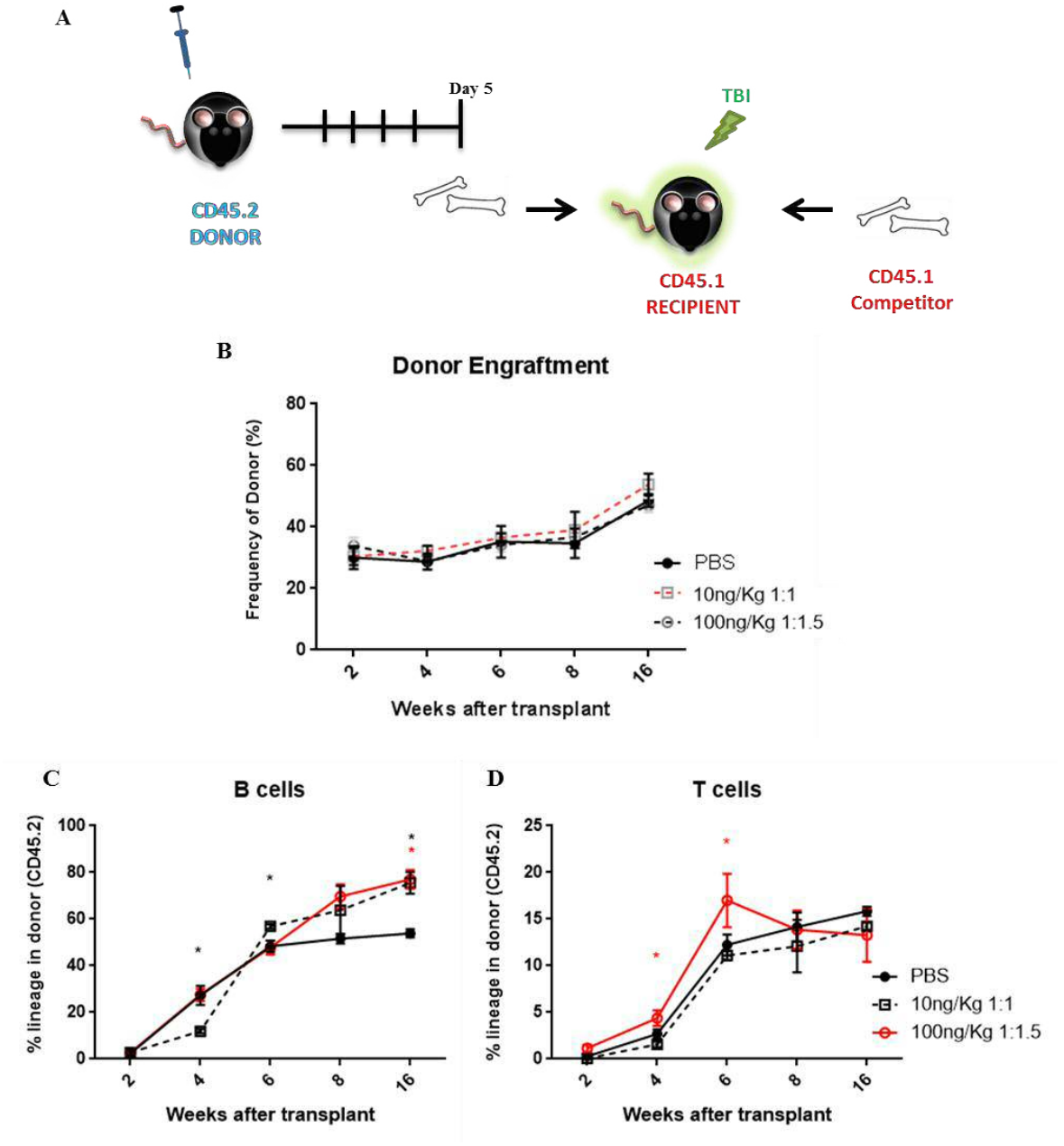


Figure 4. 13 Oxa-22 treated donor cells enhance B cell and T cell reconstitution

Donor mice (CD45.2) were treated with Oxa-22 daily for 5 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated recipients (CD45.1) (A). Samples of blood were taken weekly to assess reconstitution and engraftment (B). Recipient and donor cells were identified using CD45.1 and CD45.2 antibodies respectively. From within the donor compartment, lymphoid cells were identified as B-cells (B220) and T-cells ($CD4^+/CD8^+$) (C and D).

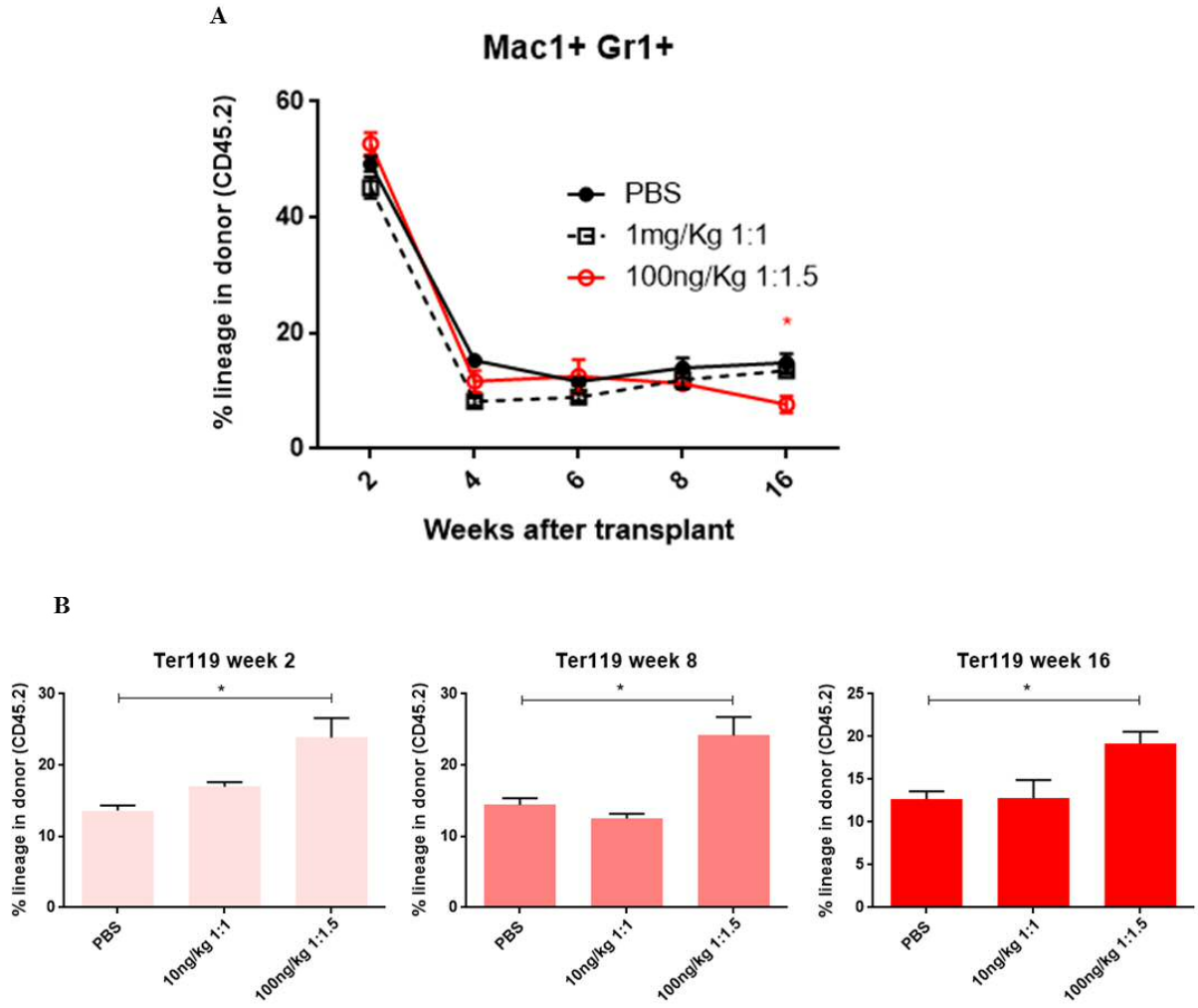


Figure 4. 14 Oxa-22 treated donor cells support enhanced erythrocytic reconstitution

CD45.2 donor mice were treated with Oxa-22 daily for 5 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated CD45.1 recipients. Donor (A) $Mac1^{+}Gr1^{+}$ cell and (B) erythrocytic reconstitution was assessed throughout the transplant period.

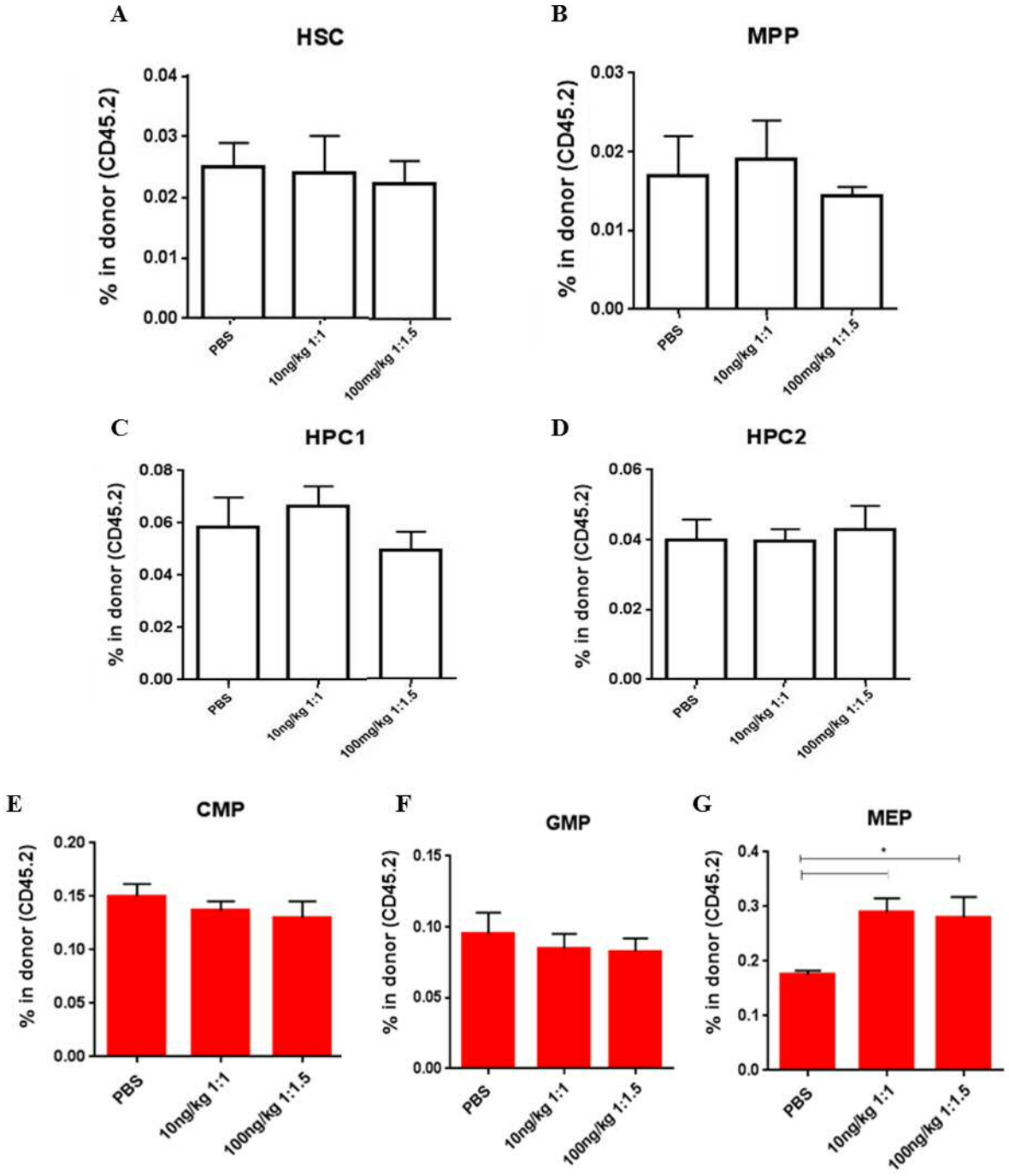


Figure 4. 15 Oxa-22 treated HSCs perform normally in competitive transplant

16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Specific donor HSPC and lineage restricted progenitor populations from within the LSK (A-D) and LK (E-G) compartment were analysed. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

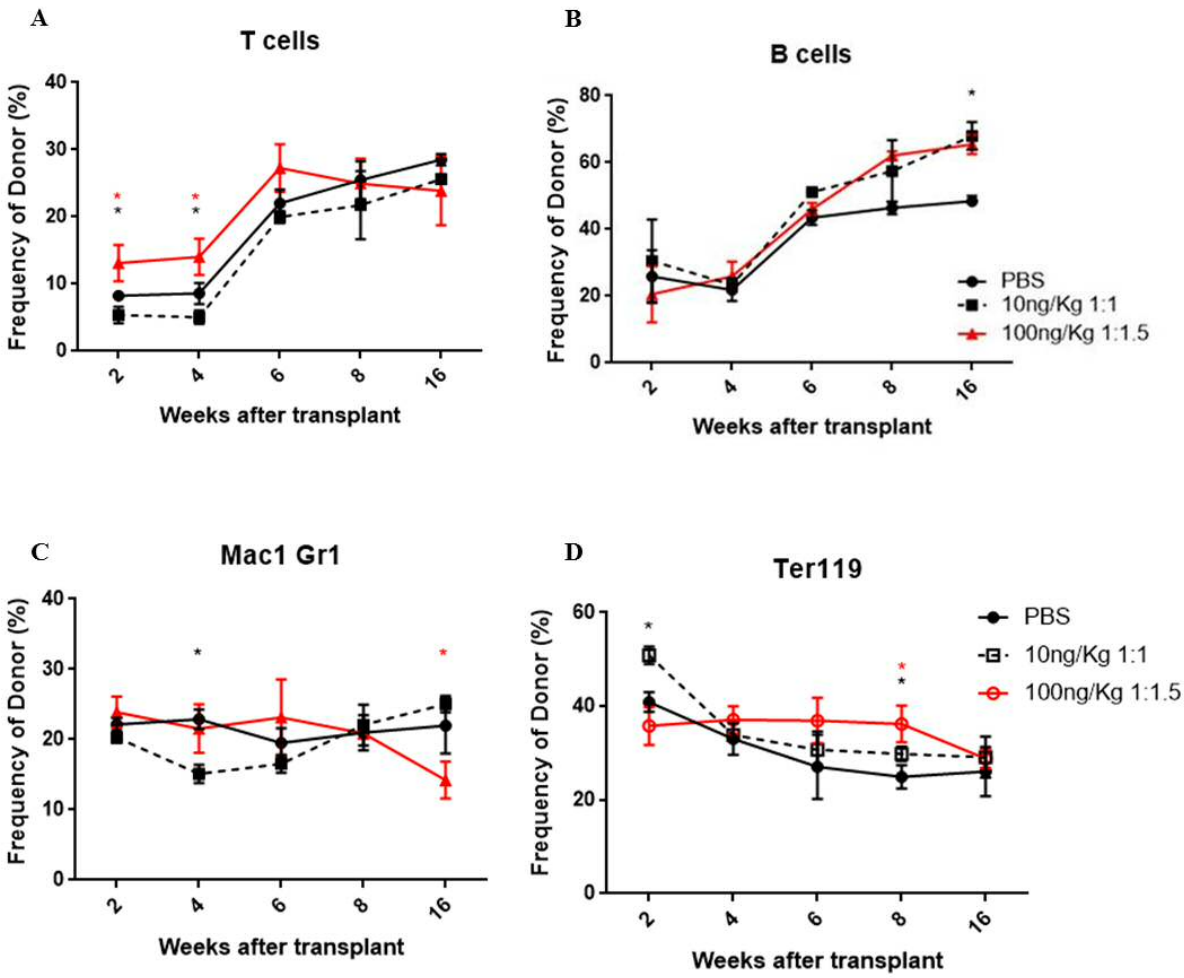


Figure 4. 16 Transplanted Oxa-22 treated cells demonstrate enhanced lymphoid and erythrocyte engraftment

Donor mice (CD45.2) were treated with Oxa-22 daily for 5 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated recipients (CD45.1). Recipient and donor cells were identified using CD45.1 and CD45.2 antibodies respectively. From within the lineage differentiated populations, (A and B) lymphoid (T - and B cells), $Mac1^{+}/Gr1^{+}$ myeloid (C) and eryhthrocytic cells (D), the distribution of CD45.2 donor cells were identified.

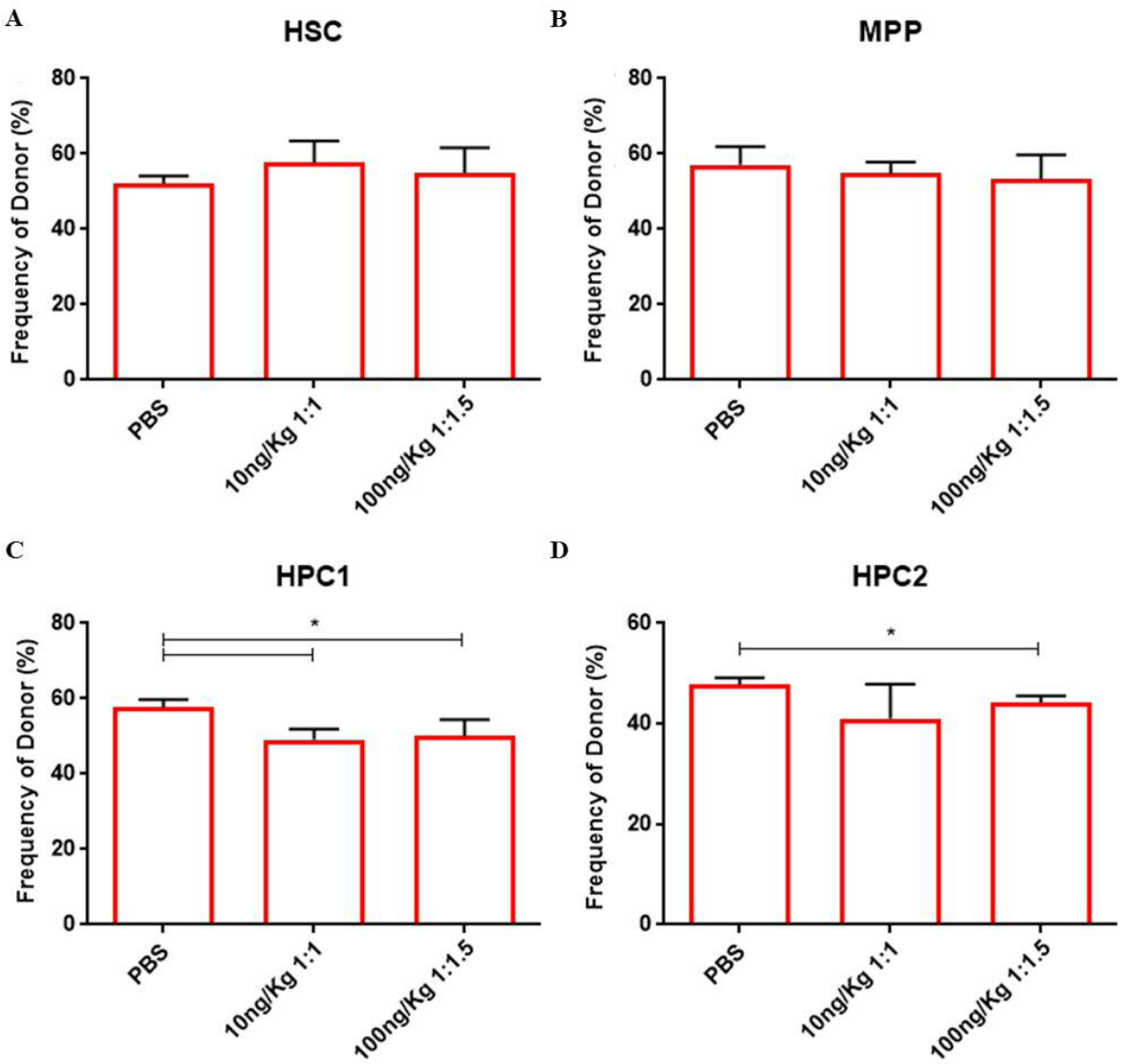


Figure 4. 17Donor competitiveness of Oxa-22 treated HSCs is unaffected

Donor mice (CD45.2) were treated with Oxa-22 daily for 5 consecutive days. Bone marrow of treated animals was harvested and transplanted competitively with CD45.1 bone marrow cells into irradiated recipients (CD45.1). Recipient and donor cells were identified using CD45.1 and CD45.2 antibodies respectively. From within the SLAM marker population, HSC (A), MPP (B) HPC1 (C) and HPC2 (D), the distribution of CD45.2 donor cells were identified. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

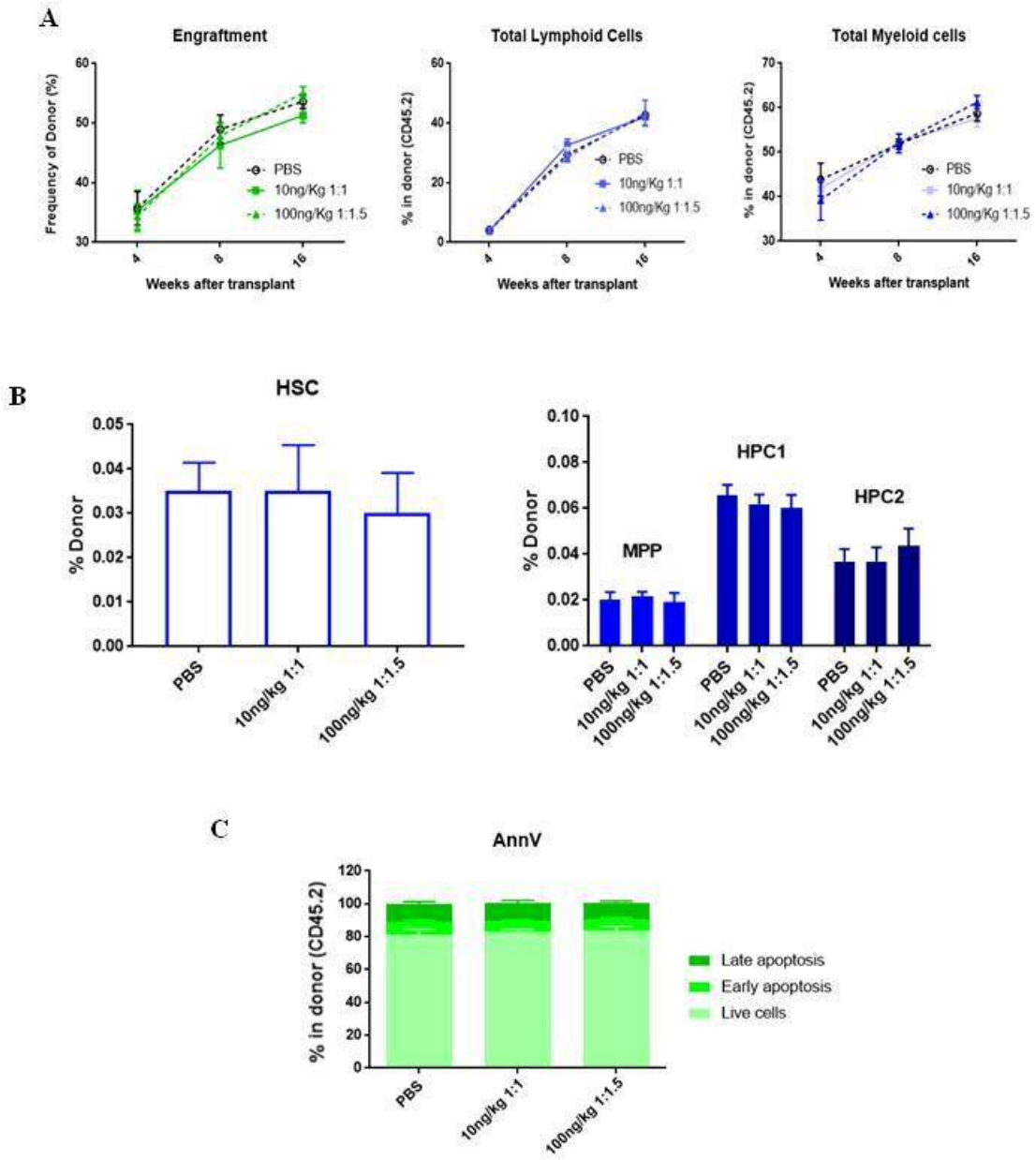


Figure 4. 18 Oxa-22 treated cells does not affect self-renewal of long-term HSCs in secondary recipients

Donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin\text{-}Sca1^{+}cKit^{+}$ cells per recipient). Blood samples were taken to measure engraftment (A) and reconstitution of myeloid (Mac1, Gr1 and Mac1Gr1) (B) and lymphoid (B- and T cells) (C). At 16 weeks, Bone marrow was harvested and donor LSK populations were analysed for apoptotic cell frequency (E) using Ki67 and Annexin V staining respectively.

4.6 The impact of Oxa-22 on haematopoietic reconstitution after BM transplant

As previously described, bone marrow transplant is essential for individuals that have undergone chemotherapy which has led to a depletion in haematopoiesis. After bone marrow transplant, these individuals are at a risk of contracting infections due to the lack of immunity; thus, we next directed our efforts in determining whether Oxa-22 was able to improve haematopoietic reconstitution following bone marrow transplant. In this investigation, we transplanted a limiting dose of $2x10^5$ bone marrow cells into irradiated recipients. These mice were then treated with PBS or Oxa-22 (10 ng/kg or 100 ng/kg) every other day for a total of 5 treatments. Weekly analysis of engraftment did not show any differences in total donor engraftment between PBS controls compared to treated animals (Figure 4.19). Assessing the lympho-myeloid reconstitution, we saw that there were no effects on $Mac1^+/Gr1^+$ reconstitution in treated animals (Figure 4.20A). On the other hand, we observed no significant differences in T-cell and B-cell reconstitution after transplant in response to Oxa-22 treatment (Figure 4.20B and 4.20C). It was however interesting to see a small but significant increase in early erythrocytic reconstitution in Oxa-22 treated recipients (Figure 4.21). This shows that Oxa-22 may have mild suppressive activity in myeloid reconstitution; however it has little overall effect on haematopoietic reconstitution when administered after bone marrow transplant.

At 16 weeks after transplant, bone marrow was harvested from primary recipients that were treated with Oxa-22. HSPC populations were analysed by flow cytometry and we found that treatment of Oxa-22 did not elicit any changes in donor HSCs, primitive progenitors or lineage restricted progenitors (Figure 4.22 and 4.23). In addition to immunophenotypical analysis, functional *in vitro* CFC assays revealed no differences in colony forming capacities of cells deriving from treated animals (Figure 4.24). We also assessed the effects of Oxa-22 on self-renewal capacities of HSCs from treated animals by conducting secondary transplants. 2000 donor derived LSK of treated and PBS control animals were transplanted into respective irradiated secondary recipients. We observed no differences in donor engraftment of secondary recipients of treated animals compared to the control group (Figure 4.25A). We also did not see any changes in lympho-myeloid reconstitution in Oxa-22 treated donors throughout the transplant period (Figure 4.25B-D). Again at 16 weeks after the

secondary transplant, we analysed the HSPC populations of the bone marrow of the secondary recipients and did not find any differences in the frequencies of HSPCs (Figure 4.26). Overall these data suggest that Oxa-22 administration after BM transplantation does not impact long-term self-renewal of HSC.

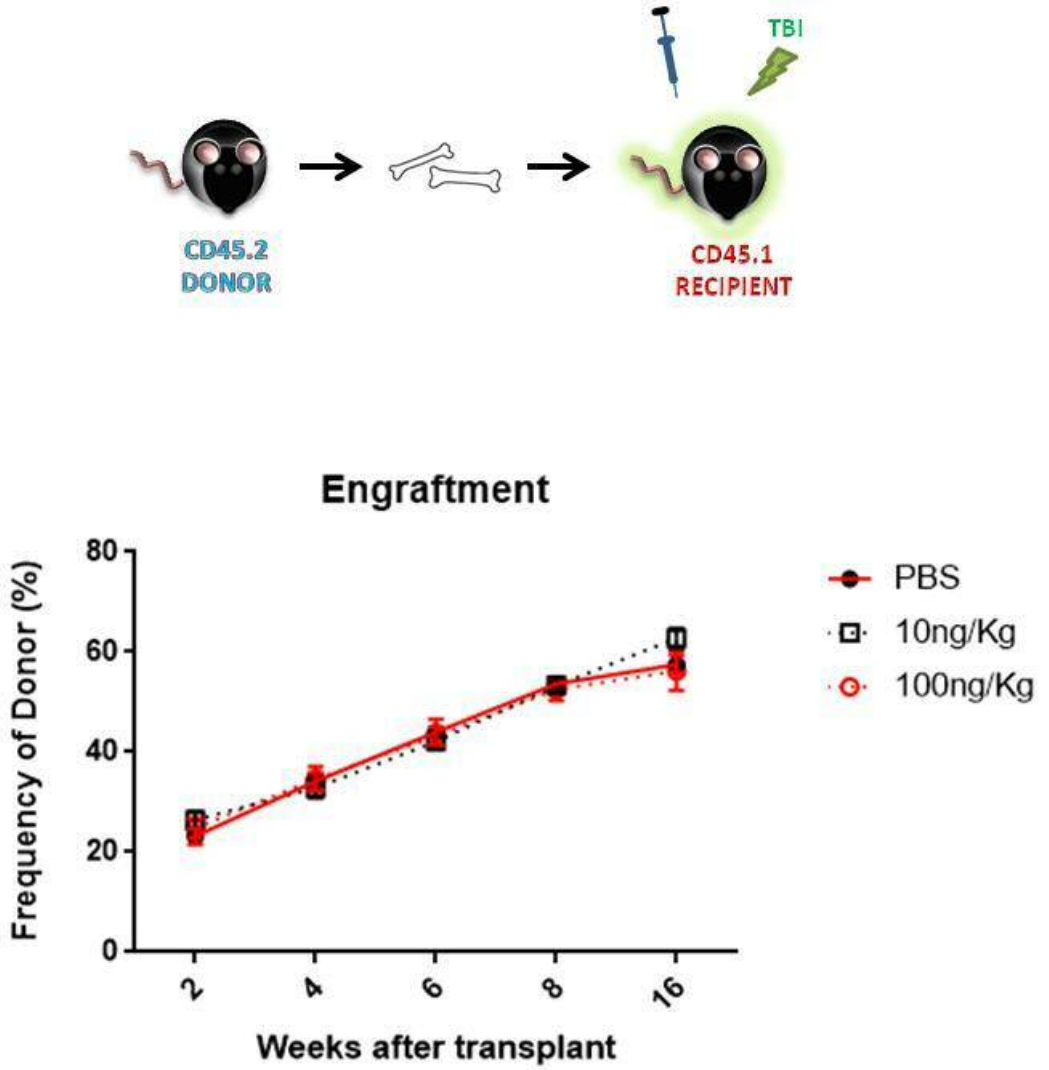


Figure 4. 19 Oxa-22 treatment after transplant does not affect haematopoietic reconstitution

(A) CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments.(B) Weekly bleedings were conducted to assess engraftment of donor cells (CD45.2)

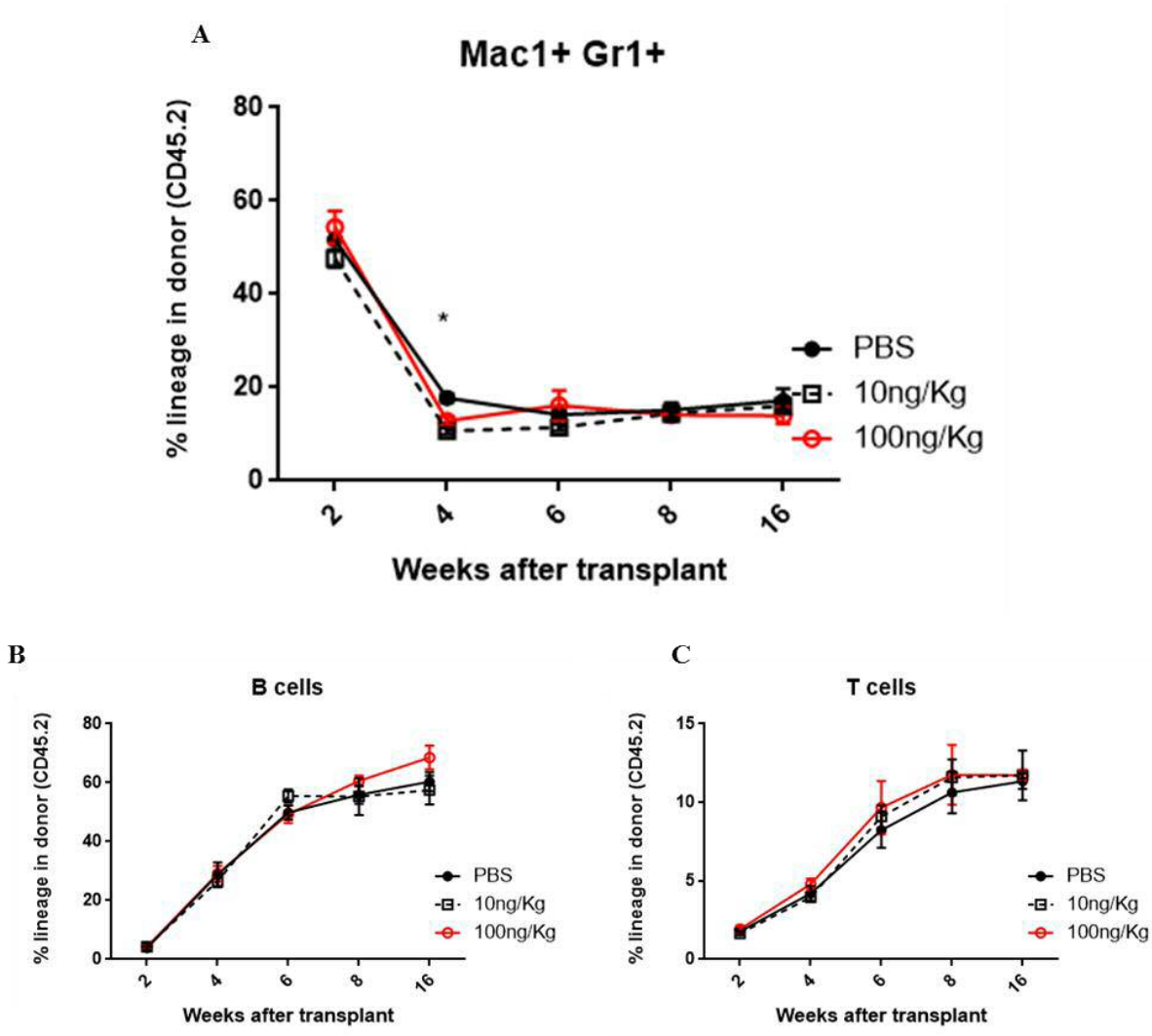


Figure 4. 20 Oxa-22 treatment after transplant does not impact on myeloid and lymphoid recovery

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments. Weekly bleedings were conducted to assess reconstitution of myeloid and lymphoid cells (A and B-C respectively) cells

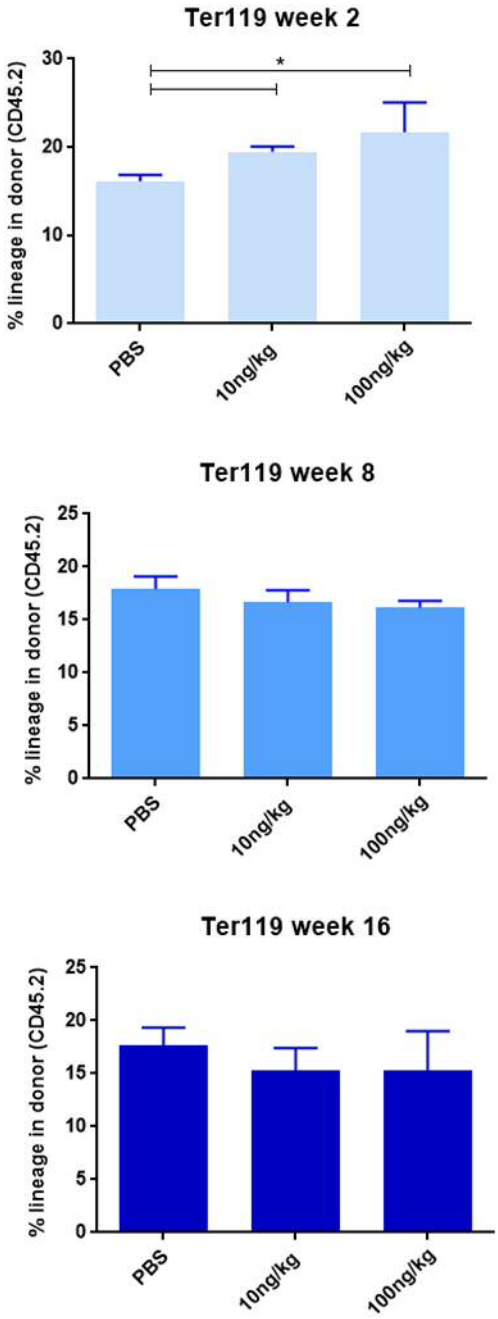


Figure 4. 21 Oxa-22 treatment after transplant enhances early erythrocytic reconstitution

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments. Weekly bleedings were conducted to assess reconstitution of $Ter119^+$ erythrocytes.

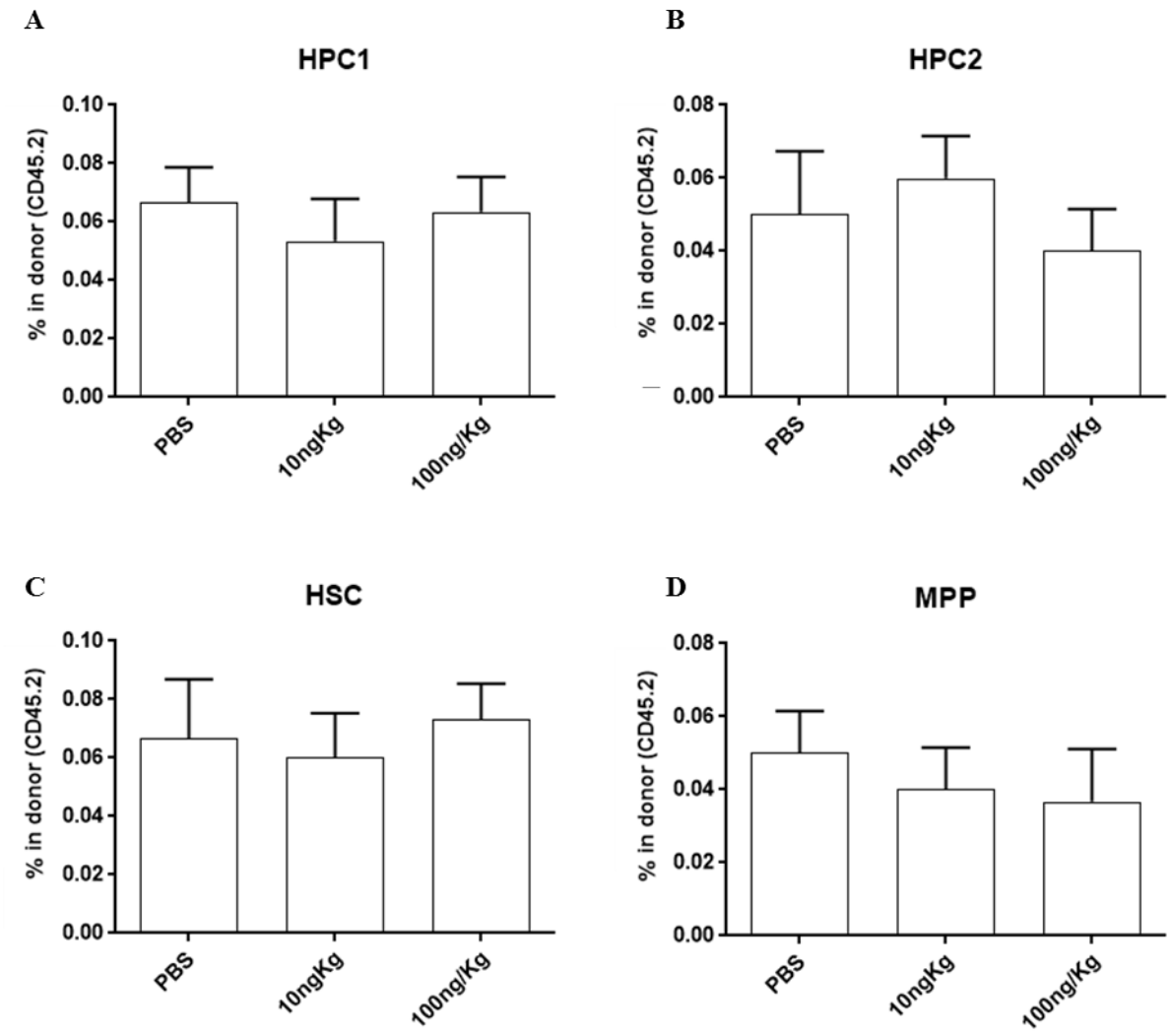


Figure 4. 22 Treatment of Oxa-22 after transplant does not affect HSC and primitive progenitor function

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments. 16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Donor SLAM populations were analysed (A-D) by flow cytometry based on Lin- $Sca1^+$ $ckit^+$ markers. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

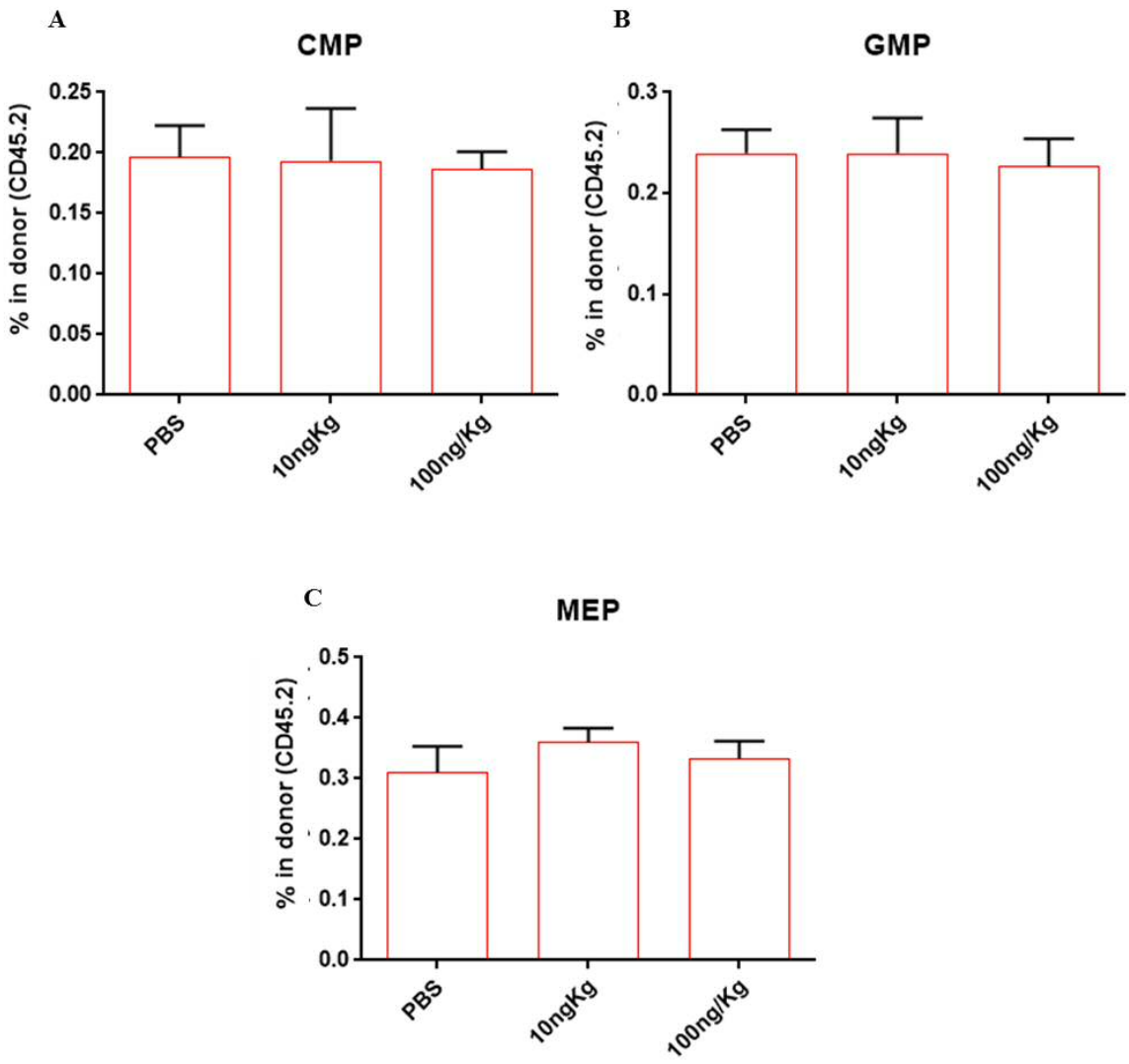


Figure 4. 23 Oxa-22 treatment post-transplant does not affect lineage committed progenitor frequencies

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments. 16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Donor progenitor populations were analysed (A-C) by flow cytometry based on Lin- $ckit^+$ markers. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

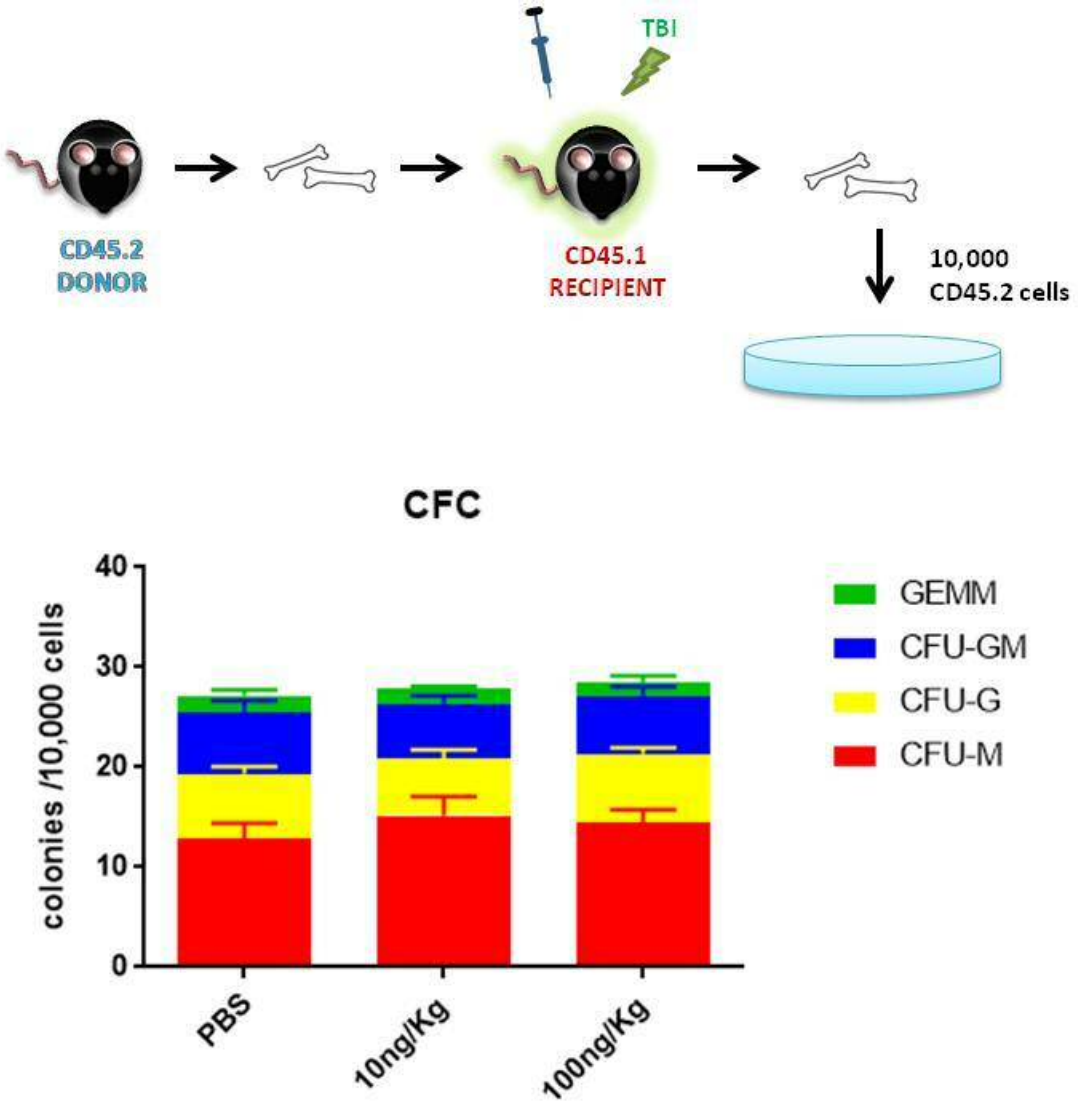


Figure 4. 16 Oxa-22 treatment after transplant does not affect donor HSPC colony forming potential

CD45.1 recipient mice were transplanted with $5x10^5$ donor cells (CD45.2). Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments. 16 weeks after transplant, recipient bone marrow was harvested and processed. FACS was used to sort and isolate CD45.2 donor cells which were then plated into methylcellulose

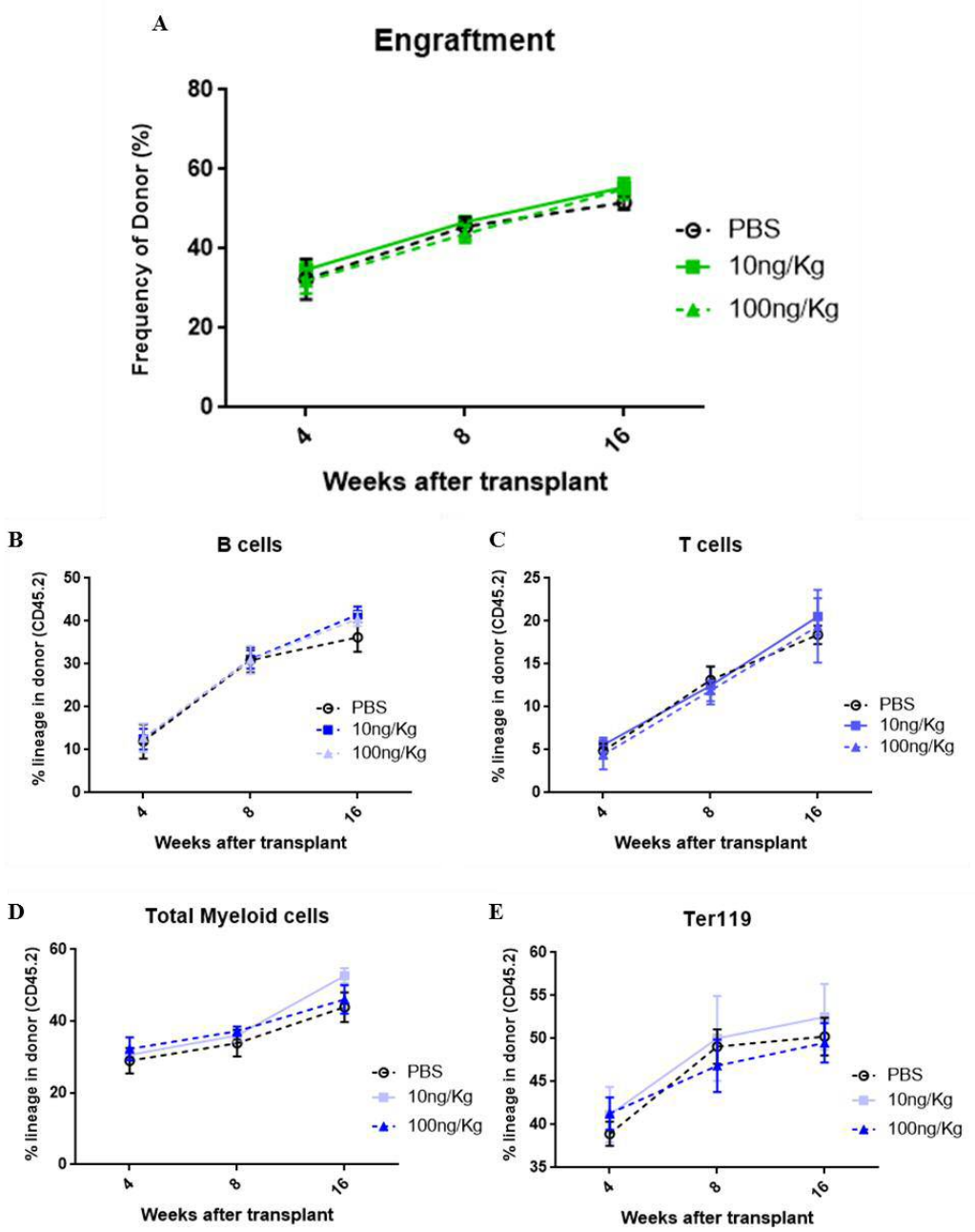


Figure 4. 17 Oxa-22 treatment after transplant does not affect self-renewal capacity of long term HSCs in secondary recipients

Primary recipients were treated with Oxa-22 or PBS. After 16 weeks, donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin\text{-}Sca1^{+}cKit^{+}$ cells per recipient). Blood samples were taken to measure engraftment (A) and reconstitution of lymphoid (B and C), myeloid (D) and erythrocyte (E) cells using T- ($CD4^{+}/8^{+}$) and B cell (B220), myeloid (Mac1/Gr1) and erythroid (Ter119) markers.

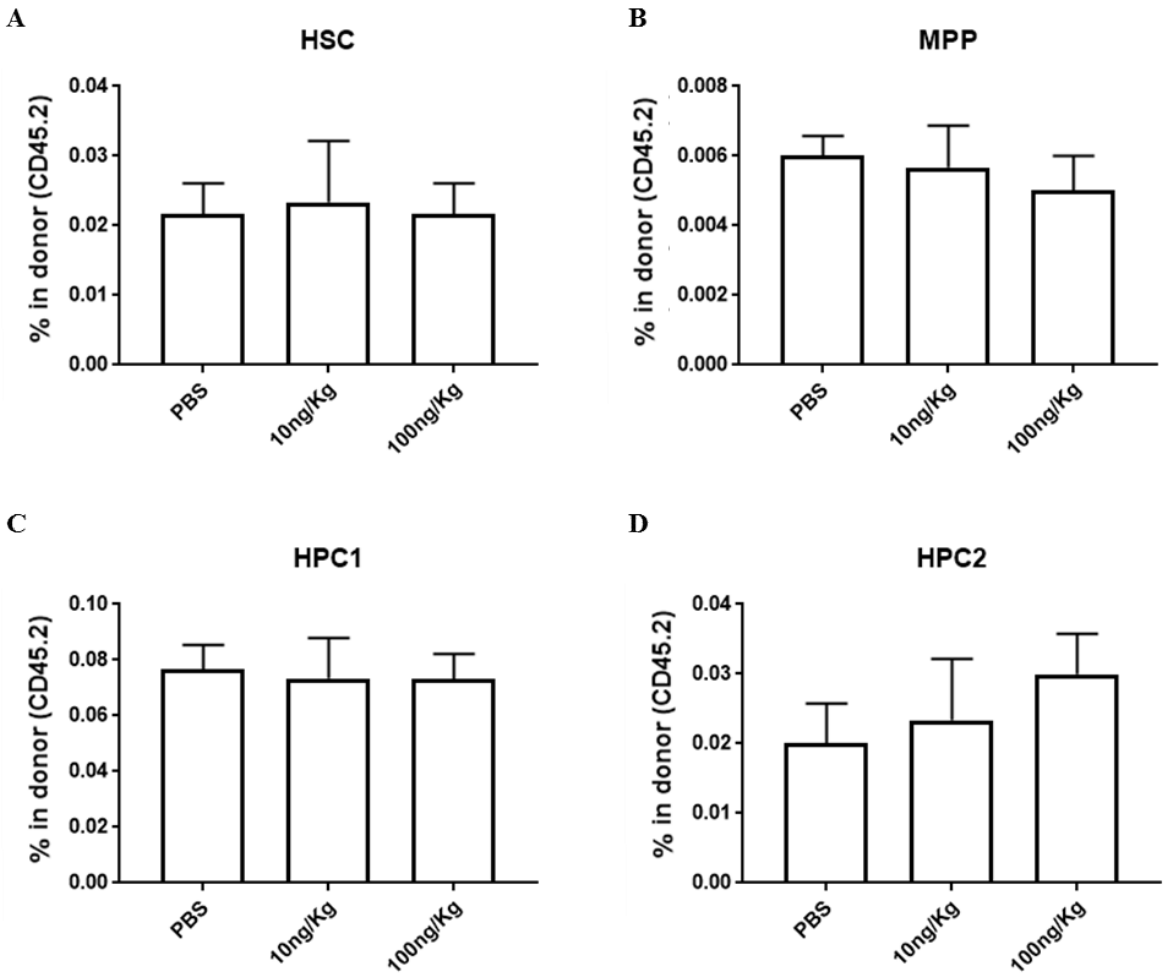


Figure 4. 18 Treatment of Oxa-22 after transplant has no impact on long-term HSPCs

Primary recipients were treated with Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments. After 16 weeks, donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin\text{-}Sca1^{+}cKit^{+}$ cells per recipient). Analysis of bone marrow LSK populations (A-D) was conducted 16 weeks after secondary transplant. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group

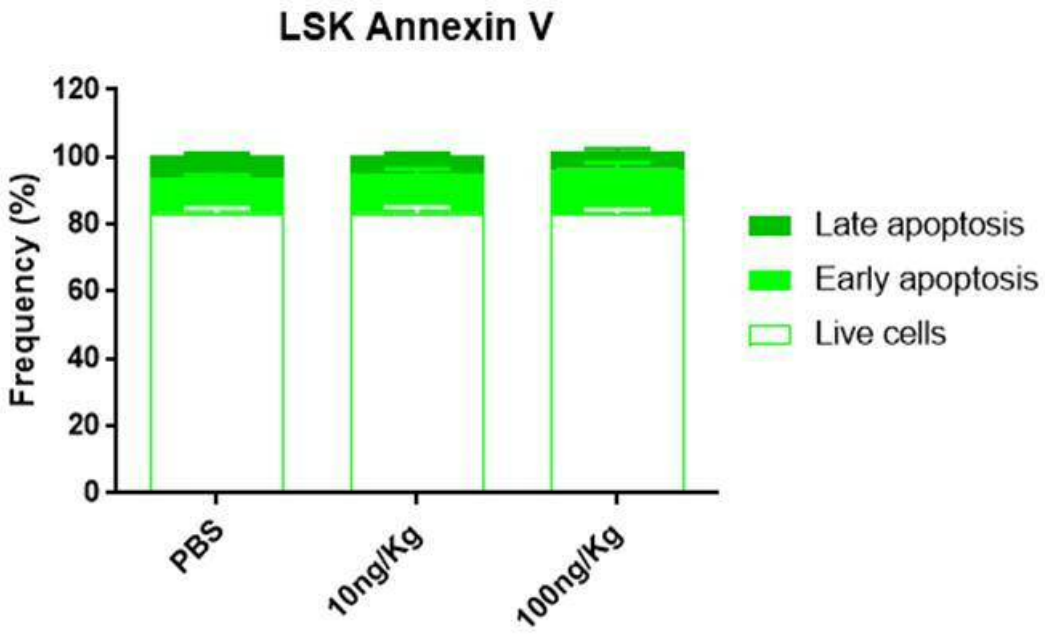


Figure 4. 19 Oxa-22 treatment after transplant does not affect the apoptotic status of long-term HSCs

Primary recipients were treated with Oxa-22 (or PBS) was then administered once every two days for a total of 5 treatments. After 16 weeks, donor CD45.2 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin\text{-}Sca1^{+}cKit^{+}$ cells per recipient). 16 weeks post-secondary transplant, bone marrow was harvested. LSK staining was conducted on bone marrow gated from donor cells. Further staining was conducted with Annexin V conjugated antibody and the DNA dye, DAPI. Different stages of apoptosis were determined by AnnexinV/DAPI: Live cells (AnnV-DAPI-), Early apoptosis ($AnnV^{+}DAPI$-) Late apoptosis ($AnnV^{+}DAPI^{+}$) . Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group.

4.7 The impact of Oxa-22 on Leukaemic Stem Cells

Since it is understood that stem cells and cancer stem cells may share common cellular mechanisms (Shackleton 2010), we explored whether Oxa-22 could exert its effects on leukaemic stem cells (LSC). We firstly conducted a cell proliferation assay on human AML cell lines, which act as a crude surrogate to model LSC behaviour, in response to Oxa-22 exposure (described in materials and methods). From this initial investigation we found that Oxa-22 broadly increased the number of leukaemic cells after 5 days of exposure (Figure 4.28). We observed that concentrations of 100uM and above elicited significant increases in cell numbers of the NB4 and HL-60 cell lines. To assess whether this effect can be recapitulated *in vivo*, we utilised the MLL-AF9 murine model (Krivtsov et al. 2006). Here, we transplanted 10,000 LSCs generated through $cKit^+$ enriched bone marrow cells virally transduced with MLL-AF9 containing a GFP reporter (Figure 4.29). Weekly bleeding analysis revealed the progression of leukaemia. We initiated drug (10ng/kg and 100 ng/kg or vehicle (PBS) only) treatment immediately after transplant every other day for the duration of the study (when an animal becomes moribund or succumbs to disease) (Figure 4.30A). We saw that the progression of leukaemia did not differ between treated animals compared to the control group within the 5 weeks after transplant (Figure 4.30B). However after this, we observe a significantly higher level of GFP^+ cells in the treated animals (Figure 4.30B). We also discovered that mice treated with the highest dose of Oxa-22 (100ng/kg) succumbed to disease quicker and survival rates were significantly lower compared to control animals (Figure 4.30C). When animals succumbed to disease, they were sacrificed and bone marrow and spleens were harvested. In this model, LSCs were identified by $cKit^+$ GFP^+ $CD16/32^+/CD34^+$ (Krivtsov et al. 2006) (Figure 4.31A). Although not statistically significant, we observed an increased frequency in LSCs in 100ng/kg treated mice (Figure 4.31B). We also assessed the spleens of these animals and found that although there were no significant differences in spleen sizes of Oxa-22 treated animals and PBS controls, there was a small but significant increase in the weight of spleens of 100 ng/kg treated animals (Figure 4.31). This data collectively suggests that Oxa-22 may promote the progression of disease through the increase of LSC numbers.

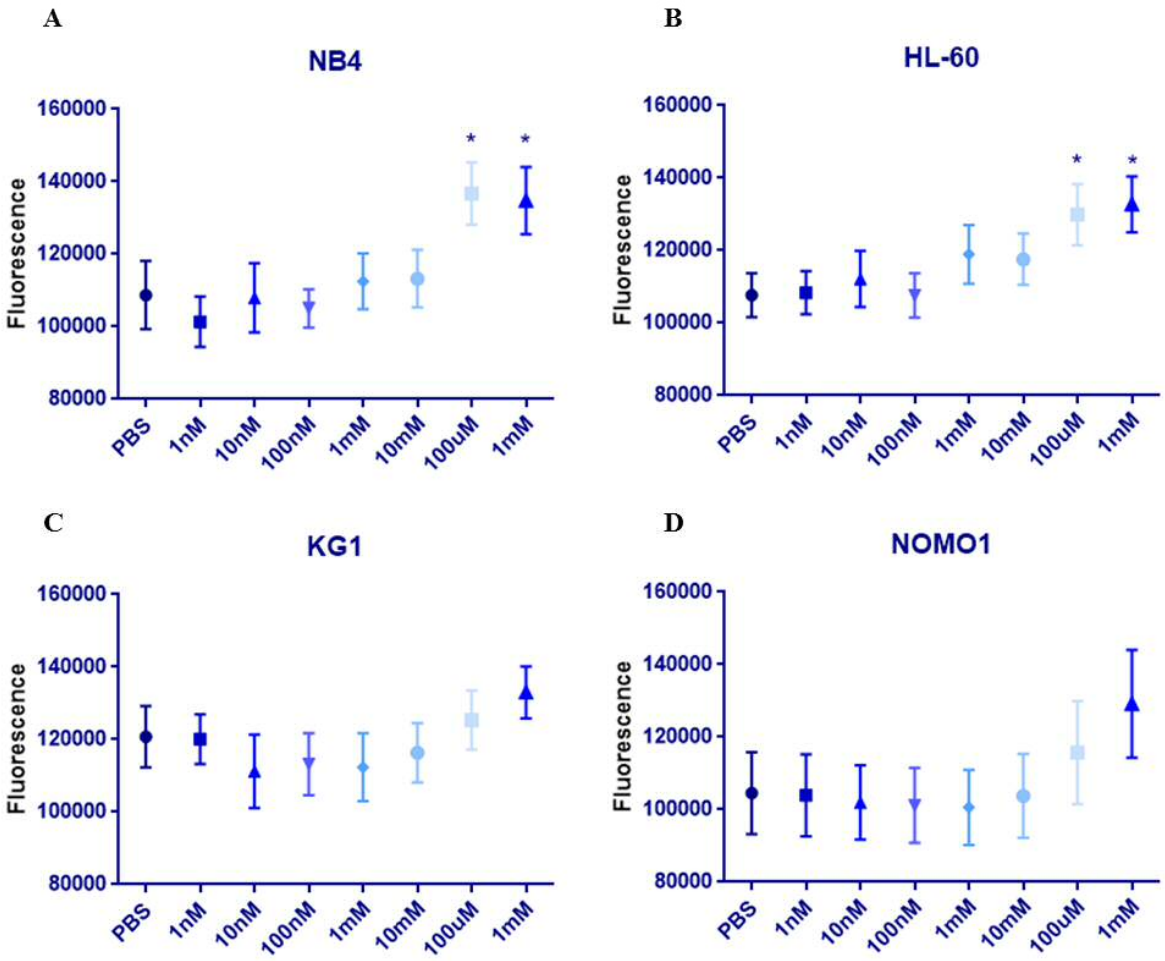


Figure 4. 28 *In vitro* exposure to Oxa-22 increases proliferation in AML cell lines

Human AML cell lines were incubated for 5 days with a range of concentrations of Oxa-22 (1nM-1mM). Cells were exposed to Cell Titre Blue which emits a fluorescence in response to live cells, therefore fluorescence is indicative of number of cells. (A) NB4 and HL-60 (B) cell lines are derived from APL patients whereas (C) KG-1 and (D) NOMO1 are AML patient derived cell lines. Error bars represent mean ± SEM of 3 independent experiments, each with triplicate groups *$p<0.05$.

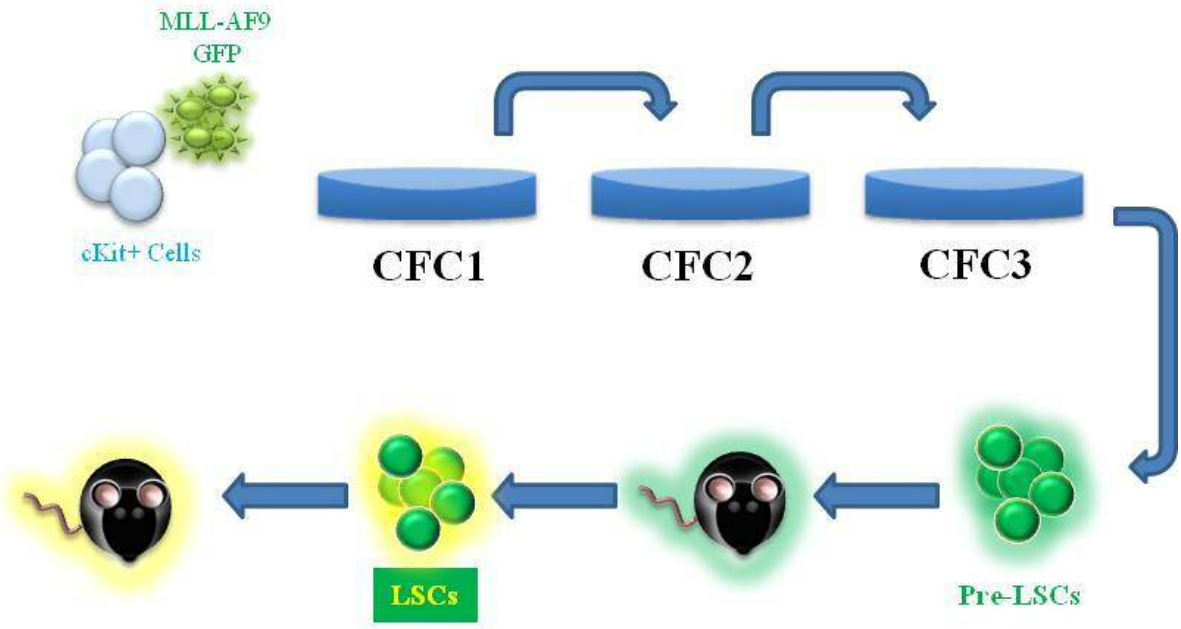


Figure 4. 29 Retroviral transduction of cKit$^+$ cells to generate the MLL-AF9 leukaemic mouse model

Bone marrow was harvested from wildtype C57BL6 mice and underwent cKit enrichment using cKit-positive magnetic beads. The AutoMACS was used to enrich for purified cKit$^+$ cells. cKit$^+$ cells were then transduced with a retrovirus containing the MLL-AF9 translocation and a GFP marker. MLL-AF9 GFP$^+$ transduced cells were isolated by FACS and plated in colony forming cell assay (CFC). Colonies are positively selected and serially plated another two times. Purified MLL-AF9 GFP$^+$ cells are harvested and enriched further for cKit, these are termed as pre-leukaemic stem cells (Pre-LSCs). These are then transplanted into irradiated recipients where they propagate. When mice become moribund or succumb to disease, bone marrow is harvested and stained for leukaemic stem cells (LSCs), LSCs are characterised by cKit$^+$ GFP$^+$ CD16/32 $^+$ CD34$^+$.

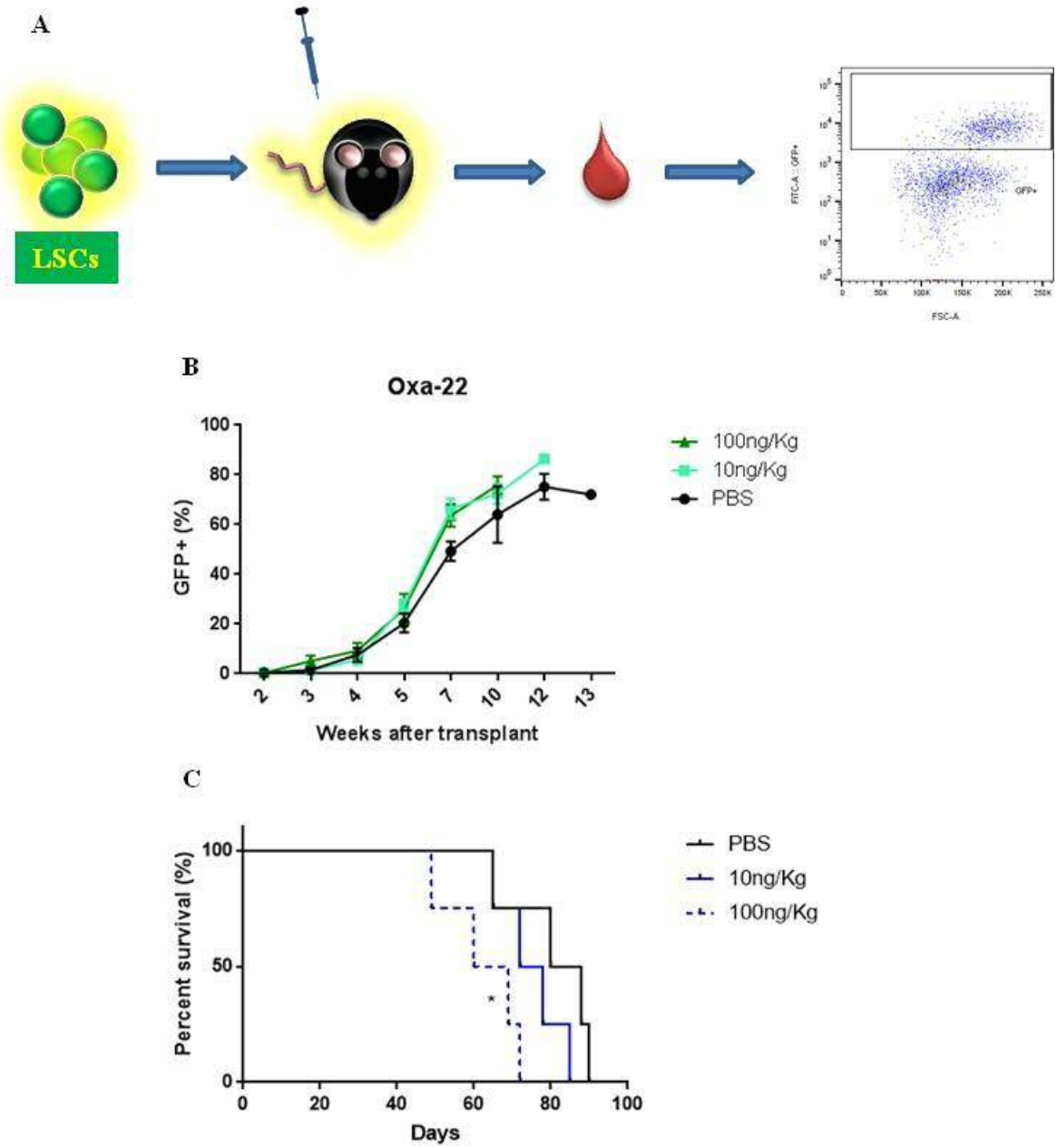


Figure 4. 30 Oxa-22 treatment accelerates disease progression in leukaemic mice

(A) Mice were transplanted with 10,000 LSCs (MLL-AF9/GFP$^+$ cKit$^+$ cells). Immediately after transplantation, mice were treated with PBS or Oxa-22 (10 or 100 ng/kg). (B) Weekly bleeding analysis was conducted to measure disease progression. Flow cytometry was used to analyse GFP$^+$ leukaemic cells. (C) Survival was also measured when mice became moribund or succumbed to disease. Error bars represent mean ± SEM of one experiment, with 4 mice per group *$p<0.05$.

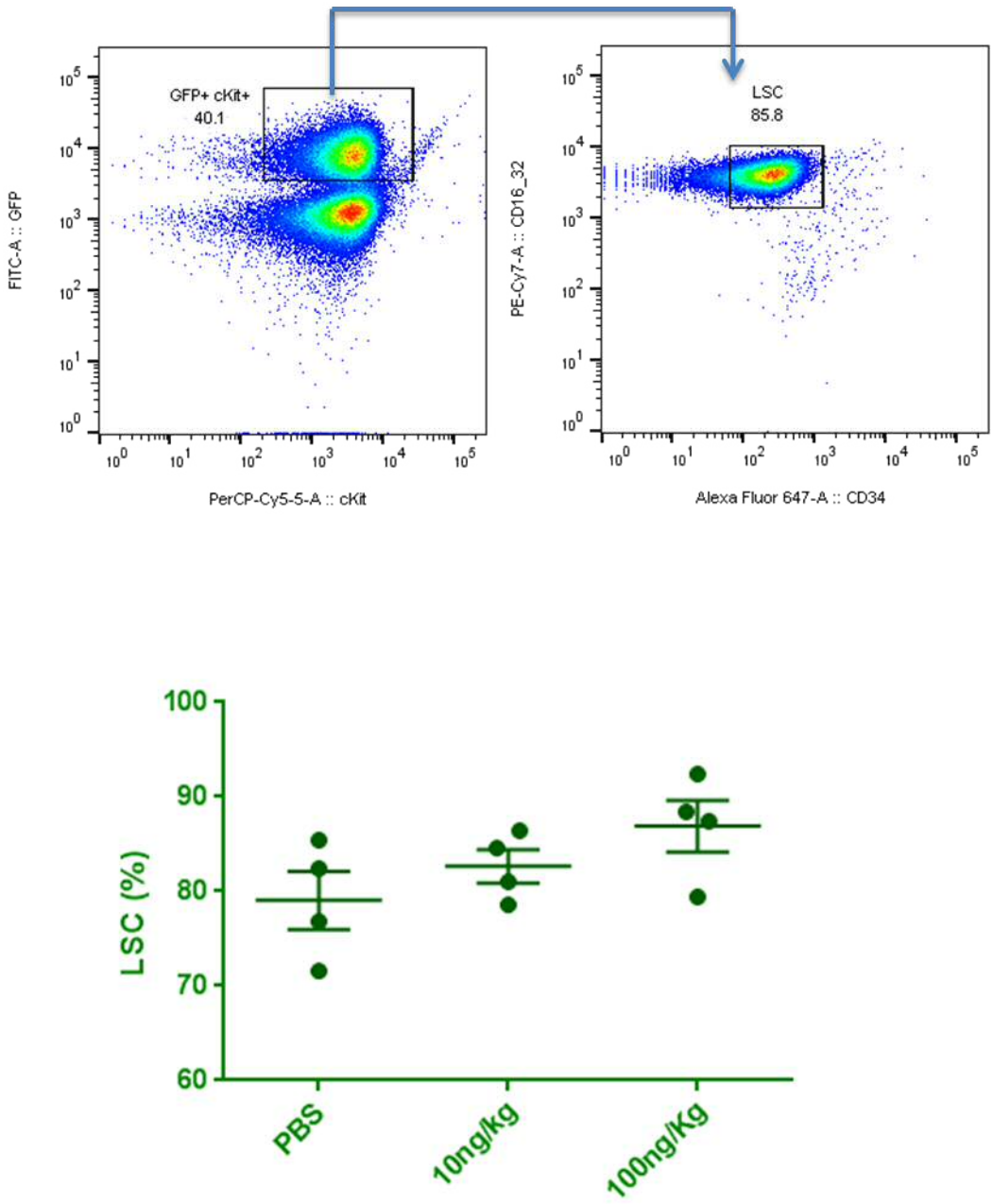


Figure 4. 31 LSC frequency after Oxa-22 treatment

Mice were transplanted with MLL-AF9/GFP cKit^{+} cells. When mice became moribund or succumb to disease, bone marrow was harvested and analysed for LSC populations. LSCs were identified as cKit^{+} GFP^{+} CD16/32^{+}CD34^{+}. Error bars represent mean ± SEM of one experiment, with 4 mice per group.

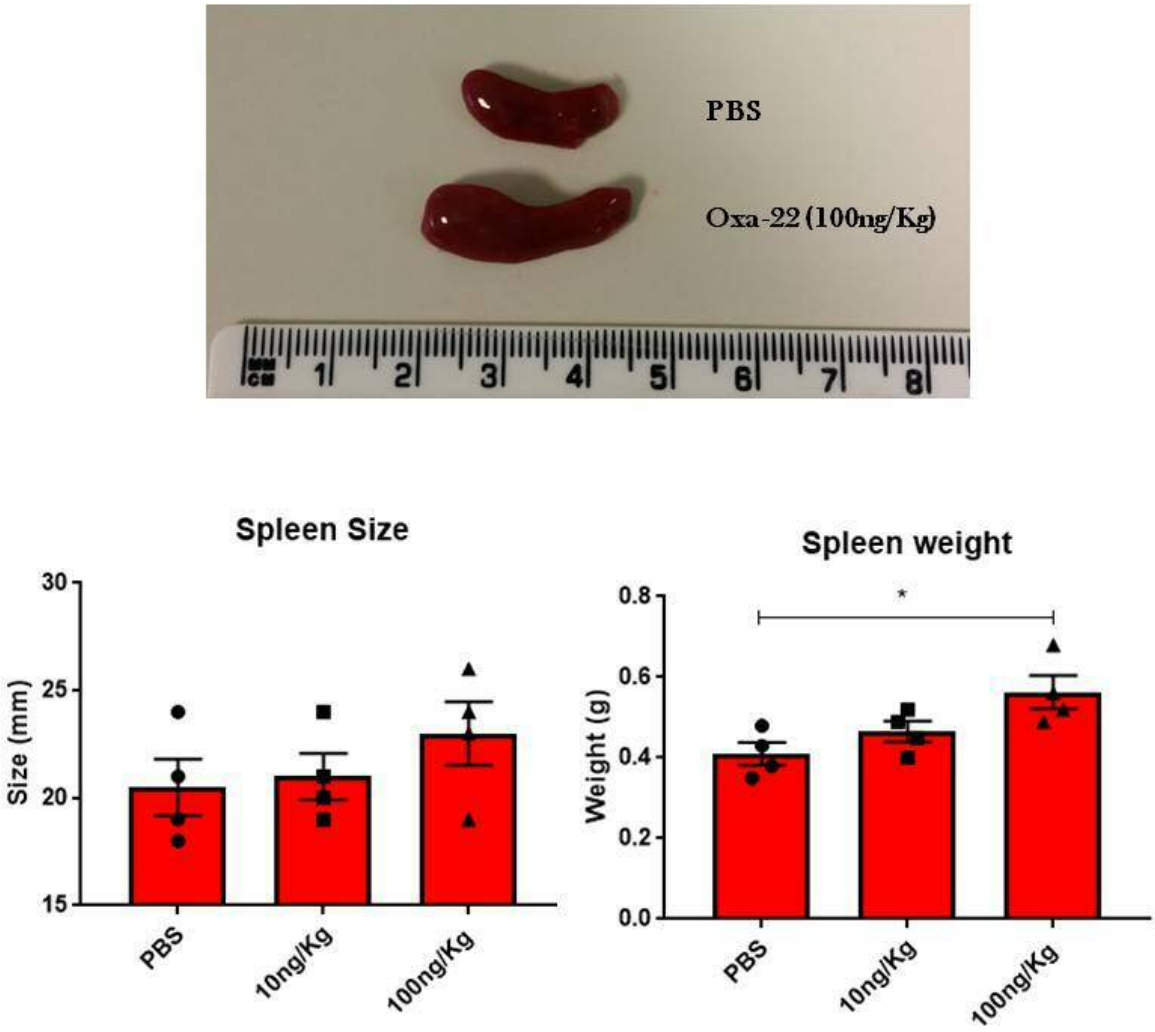


Figure 4. 32 Enhanced splenomegaly is observed in Oxa-22 treated MLL-AF9 mice

Mice were transplanted with MLL-AF9/GFP cKit^{+} cells. When mice became moribund or succumb to disease, spleens were harvested and measured to assess for splenomegaly. Error bars represent mean ± SEM of one experiment, with 4 mice per group *$p<0.05$.

4.8 Discussion

Although it still remains unclear whether cholinergic innervation occurs in the bone marrow, evidence strongly suggests that the surrounding bone and bone marrow niche can also be innervated by the cholinergic nervous system. Oxa-22, an agonist of the M3 muscarinic acetylcholine receptor, was identified from our library screen as a potential target of stem cell activity. We therefore sought to assess the impact of Oxa-22 on cholinergic signalling on HSPC function. We initially investigated the effects of Oxa-22 on bone marrow cells ex vivo then advanced our investigation into *in vivo* treatments under homeostatic conditions. We then turned our attention towards the impact of Oxa-22 on HSC function under homeostatic conditions. In parallel we also assessed the effects of Oxa-22 treatment in mice that had underwent bone marrow transplant in order to explore the effects of the compound on haematopoietic reconstitution. Finally we sought to investigate the effects of this compound in the leukaemic setting in order to understand the role of cholinergic signalling in disease (Alea et al. 2011).

Primarily, we questioned whether Oxa-22 could influence the activity of HSPCs, so we exposed whole bone marrow cells to the compound ex vivo and discovered the Oxa-22 did not elicit any changes in LK and LSK populations immunophenotypically or functionally. As previously mentioned, bone marrow cells grown in cytokine- and serum-free conditions may prove to be an unfavourable condition. In addition to this, $1x10^6$ nucleated cells were plated in this experiment, as the LSK population is relatively small, the lack of effect of the drug may be due to its uptake by the large number of differentiated cells masking it effects on LSK cells. This can be overcome by isolating LKS and/or specific HSPC populations and exposing these cells to the compound. Although we did not observe any effects ex vivo, it may be possible that this compound may impact on the very rare long-term HSC population. To test for this, transplantation of ex vivo-treated HSCs would clarify this question and determine if Oxa-22 impacts HSCs.

In our gene expression analysis study, we found expression of the Oxa-22 receptor, the M3 muscarinic acetylcholine receptor, on HSPC populations. This finding was not followed by protein

expression which would have been an ideal method for validation. Also, online ProteinAtlas data does not support the expression of the muscarinic receptors in haematopoietic populations. this, along with the importance of cholinergic innervation of the surrounding bone marrow niche and the nervous system, we explored the effects of Oxa-22 on HSPCs *in vivo*. No prior haematological investigations were conducted with Oxa-22 so we initially tested a range of doses. Although we found that Oxa-22 resulted in an increase in the frequencies of HSCs at 100ng/kg, we also observed a detrimental decrease in HSPCs at 1ug/kg. It is unclear how the highest dose of Oxa-22 elicited this depletion; however it may be speculated that overstimulation induced by cholinergic innervation results in the adverse depletion of HSCs. Further investigation is required to assess whether apoptosis, quiescence or excess proliferation is the reason for the depletion in these HSCs. On the other hand however, 10ng/kg and 100ng/kg proved to be safe dose. It still remains unclear how Oxa-22 elicits an increased HSC frequency however we can hypothesis that Oxa-22 may alter the behaviour of niche cells such as MSCs and osteoblasts. Sato et al demonstrated the expression of a range of muscarinic receptors on osteoblasts but did not assess cholinergic effects on proliferation and differentiation which has yet to be elucidated (Sato et al. 2010). Assuming that osteoblast behaviour is indeed altered in response to Oxa-22 treatment, we can hypothesise that these in turn impact on HSCs. Osteoblasts have been shown to increase HSC number via Notch signalling (Calvi et al. 2003). Further work could therefore be conducted to assess Notch signalling in response to Oxa-22 treatment. Furthermore, it is also known that cholinergic signalling occurs in the hypothalamus which in turn can impact on bone function and remodelling (Harada and Rodan 2003; Zaidi 2007). Very recently, it was demonstrated that signalling via the M1 muscarinic receptor within the hypothalamus promoted G-CSF-induced HSC mobilisation and mice lacking this receptor demonstrated impaired mobilisation (Pierce et al. 2017). It still remains unclear whether M3 receptor signalling in the central nervous system has an effect on HSC function however further investigation of downstream signalling pathways must be explored.

We also sought to assess the functional effects of Oxa-22 cells that were treated *in vivo*. We did this by treating donor animals with Oxa-22 before competitively transplanting them into irradiated

recipients. Here we found that lymphoid reconstitution was significantly higher 6 weeks after transplant in donor cells treated with 100ng/kg. Within these animals, myeloid reconstitution was not affected however increased donor-derived erythrocytes were detected throughout the transplant period. This may suggest that *in vivo* exposure of donor HSPC cells to Oxa-22 as encourages the generation of B- and T-cells. An investigation that may support this notion was conducted in 2005 where it was shown that the deletion of the M1 muscarinic resulted in the failure of T lymphocyte differentiation (Zimring et al. 2005). Although not the M3 receptor subtype, Oxa-22 activation of these receptors on donor cells may have elicited the increased number of donor derived T cells yet it still remains unclear whether this is the case as M3 deletion has not been investigated. It has also been described that activation of the M3 muscarinic receptor elicits increased levels of intracellular Ca^{2+} (Fujii and Kawashima 2000). This also leads to the activation of the AKT and MAPK pathway which has been found to be essential in T-cell proliferation (Gutkind 1998; Gudermann et al. 2000). An alternative explanation to increased lymphoid cells is by assessing thymic cells and progenitors of the lymphoid compartment (common lymphoid progenitor, CLP) within these primary recipients. This was not explored however it is imperative to understand how Oxa-22 treated donor cells are affected outside the bone marrow niche. We did not observe any changes in engraftment and haematopoietic reconstitution of secondary recipients suggested that the function of long term HSCs were unaffected by Oxa-22.

In parallel to the above study, we also looked at the impact on haematopoietic reconstitution by Oxa-22 treatment after bone marrow transplant. In contrast to the previous investigation, we did not see any effects in T cell, or B cell, reconstitution. We also did not observe effects of Oxa-22 treatment on myeloid reconstitution after transplant. Again, in this setting we did not observe any changes in the self-renewal abilities of long-term HSCs in secondary recipients. An alternative approach in testing the compound in this setting could be that we administer Oxa-22 in secondary recipients as a means of continued exposure and assess whether cholinergic signalling has an impact in long-term HSCs. Interestingly, we observed enhanced immune recovery from Oxa-22 treated donor cells though this was not observed when Oxa-22 is administered after transplant. This may be due to the impact of

irradiation on cholinergic signalling within the bone marrow niche. In fact it has been shown that after irradiation, muscarinic receptor agonists were unable to initiate intracellular calcium release (Coppes et al. 2005). They also showed that receptor density and G protein receptor coupling were not affected by irradiation suggesting that receptor function was unaltered. Instead, muscarinic receptor-induced protein kinase Cα (PKCα) activation was markedly impeded and thus inhibiting downstream signalling pathways (Coppes et al. 2005). This may also be the case in our transplant setting where transplant after irradiation may have perturbed muscarinic signalling, hence the diminished effect of Oxa-22. This may have been overcome in the competitor transplant experiment as transplanted cells have had prior exposure to the effects of Oxa-22 (directly or indirectly), thus are able to function in an irradiated environment after transplantation.

As it is understood that molecular mechanisms are often conserved between stem cells and cancer stem cells (Shackleton 2010) we therefore postulated that Oxa-22 can target leukaemia and leukaemic stem cells. Initially we tested this hypothesis by conducting a cell proliferation assay to assess the effects of Oxa-22 on a range of human AML cell lines. We saw a general increase in the number of cells in response to Oxa-22 exposure, more significantly however in the HL-60 and NB4 cell lines. It is unknown whether the cells express the M3 muscarinic receptor however its expression has been implicated in a number of cancers such as lung, skin and colon cancers (Spindel 2012). Leukaemic cell lines however have been found to express the M3 muscarinic receptor (Cabadak et al. 2011; Aydin et al. 2013). In addition to this it was also found that muscarinic signalling results in an increase in cytosolic Ca^{2+} and the upregulation of c-*fos* (Kawashima and Fujii 2000). Interestingly c-*fos* has been found in the regulation of haematopoietic malignancies (Pinto et al. 1987; Ishihara et al. 1993; Hwang et al. 1999). It may, therefore, be that the agonistic effects of Oxa-22 are resulting in the increased proliferation of these cells. In light of this, we hypothesised that coupled with conventional chemotherapy; the effects of Oxa-22 may induce proliferation of LSC population by forcing them out of quiescence making them more chemosensitive. We used the MLL-AF9 leukaemic murine model to test the effects of Oxa-22 *in vivo*. As expected from the primary *in vitro* study, disease progression was accelerated in leukaemic mice treated with the highest dose of Oxa-22. These mice also

succumbed to disease quicker. By definition, serial transplantation of 'true' LSCs in secondary recipients would result in the generation of leukaemia. Therefore it would be interesting to conduct a re-transplant Oxa-22 treated LSCs into secondary recipients to assess the self-renewal capacity of LSCs and their ability to contribute to leukemogenesis. If this was not to occur then we can confirm that these LSCs have lost their cancer initiating potential and therefore their ability to remain in quiescence avoiding impact by current chemotherapies. In addition to targeting LSCs, further analysis can be undertaken with Oxa-22 to further understand the effects of cholinergic signalling and innervation in the development and progression of leukaemia. For example it has been described in T-ALL that T-ALL blasts are able to interact with the microenvironment through ACh (as osteoblasts express muscarinic receptors (Sato et al. 2010; Behringer and Segal 2015)) which results in CXCL12 osteoblast-mediated release. CXCL12 in turn acts through the CXCR4 which are also expressed on leukaemic cells (Pitt et al. 2015; Passaro et al. 2016). In addition to this, it was also shown that activation of the M3 receptor leads to an increase in intracellular Ca^{2+} which further activates the AKT and MAPK pathway and hence enhance cancer progression (Song et al. 2003; Song et al. 2008) This suggests that leukaemic cells are able to contribute to the remodelling of the environment to promote their growth and survival. Therefore, further studies are required to assess whether cholinergic innervation of the niche plays a role in the development of disease. More importantly, it is imperative to investigate how muscarinic receptors and their downstream signalling pathways lead to the activation of genes that promote LSC survival and proliferation providing us with an insight into LSC behaviour.

In this study, we attempted to elucidate whether cholinergic signalling impacted HSPC function. We found that, through the agonistic effects of Oxa-22, HSC expansion was evident *in vivo* under steady state conditions. Although the exact mechanism is not known, Oxa-22 may be acting on HSCs indirectly through the bone marrow niche. In addition to this, we found that donor T cell reconstitution was enhanced in response to Oxa-22 donor-treated cells suggesting that muscarinic activation may enhance lymphoid differentiation. This may prove useful in long term immune reconstitution in patients that have undergone bone marrow transplant. Finally, we assessed the

impact of Oxa-22 on leukaemic cells. We found that leukaemic cells are enhanced both *in vitro* and *in vivo*. *In vivo* studies also showed that LSC proliferation in treated animals did not differ from controls however the progression of disease was markedly accelerated in Oxa-22 treated animals. However, we theorise that coupled with current therapies, we may able to utilise Oxa-22 to chemosensitise otherwise quiescent LSCs.

		HSC (LSK)	Progenitors (LK)	Lin$^+$			
				B-cells	T-Cells	Erythroid	Myeloid
In vitro		-	-				
Bone Marrow		↑	-	-	-	-	↑
Blood		-	-	↓		↓	↓
Competitor Transplant	Primary		↑MEP	↑(mild)	↑(mild)	↑	-
	Secondary	-	-	-	-	-	-
Treatment following BMT	Primary	-	-	-	-	-	-
	Secondary	-	-	-	-	-	-
Leukaemic setting	*In vitro*	↑ AML cell line viability					
	In vivo	↑ GFP$^+$ leukaemic cells No change in LSC frequency No change in survival					

Table 4.1 Summary of the impact of Oxa-22 *in vitro* and *in vivo*

Oxa-22 was initially studied on whole bone marrow *in vitro*. Since the *in vitro* conditions did not mimic the normal physiological bone marrow environment, *in vivo* assessment of Yohimbine was conducted to assess its impact on HSPCs. Further functional assessment of HSPCs in response to Oxa-22 treatment was undertaken in transplant studies. In parallel, the effects of Oxa-22 were also investigated in the disease setting using AML cell lines and mouse models of leukaemia.

Chapter 5: The impact of Phthalylsulfathiazole, a folic acid synthesis inhibitor, on haematopoietic stem/progenitor cells and acute myeloid leukaemia cell function

5.1 Introduction

Phthalylsulfathiazole is part of a family of anti-bacterial drugs known as sulphonamides. Normally, Phthalylsulfathiazole is used to treat patients that are either undergoing or have undergone colon surgery, dysentery and other intestinal dysfunctions (Popkova and Iurkov 1963; Gerritsen and Hendriks 1982; Turner 1983). Sulphonamides exert their effects through a common pathway - the inhibition of folic acid synthesis and in turn DNA synthesis, as shown in figure 5.0, (Woods 1940). Folic acid, a subtype of vitamin B, is typically used as a supplement during pregnancy in an attempt to prevent the development of neural tube defects (Wolff et al. 2009). In the haematopoietic setting, a role for folic acid has been identified in erythropoiesis (Wickramasinghe 1995). The importance of folic acid in erythropoiesis is demonstrated in megaloblastic anemia. Folic acid deficiency has been described as the major clinical manifestation in megaloblastic anemia where disruption of DNA synthesis results in a block in erythrocytic differentiation and apoptosis and thus anemias and ineffective erythropoiesis arise (Rundles 1959; Koury and Ponka 2004). It was found that patients suffering from folic acid deficiency and anemia exhibited a larger proportion of erythroblasts that resided in the S-phase, an indication of dysfunctional cell cycling mechanisms (Menzies et al. 1966; Wickramasinghe et al. 1968; Yoshida et al. 1968). This was also observed in the bone marrow cells of megaloblastic anemia patients (Ingram et al. 1997). Although the exact role of Phthalylsulfathiazole in haematopoiesis in unclear, it has been reported that patients treated with Phthalylsulfathiazole exhibited side effects such as granulocytpoenia (Menten and Graff 1946; Taylor 1946; Fisch and Sacks 1950). To further assess the effects of Phthalylsulfathiazole on haematopoiesis we tested its effects *in vivo* under steady state conditions and in transplantation settings.

Recently, a flurry of studies has been published looking at the impact of antibacterial/antibiotic agents in the targeting of cancer and cancer stem cells. From such studies it seems that disruption of mitochondrial biogenesis may be an interesting target for the treatment of a number of tumours (Lamb et al. 2015; Zhang et al. 2017). Other studies have looked at alternative agents, such as the antibiotics Actinomycin D and Salinomycin, that selectively targets cancer stem cells though the exact mechanisms are yet to be elucidated (Fiorillo et al. 2015; Jangamreddy et al. 2015; Das et al. 2017;

Mai et al. 2017). Specifically, it has also been shown that sulphonamides possess anti-cancer properties (Scozzafava et al. 2003; Mohan et al. 2006). We therefore sought to assess the impact of Phthalylsulfathiazole in AML cells *in vitro* and in the MLL-AF9 AML mouse model. This model serves as an ideal *in vivo* disease setting to test this compound due to its aggressive nature and the presence of leukaemic stem cells (LSCs) (Schoch et al. 2003; Krivtsov et al. 2006).

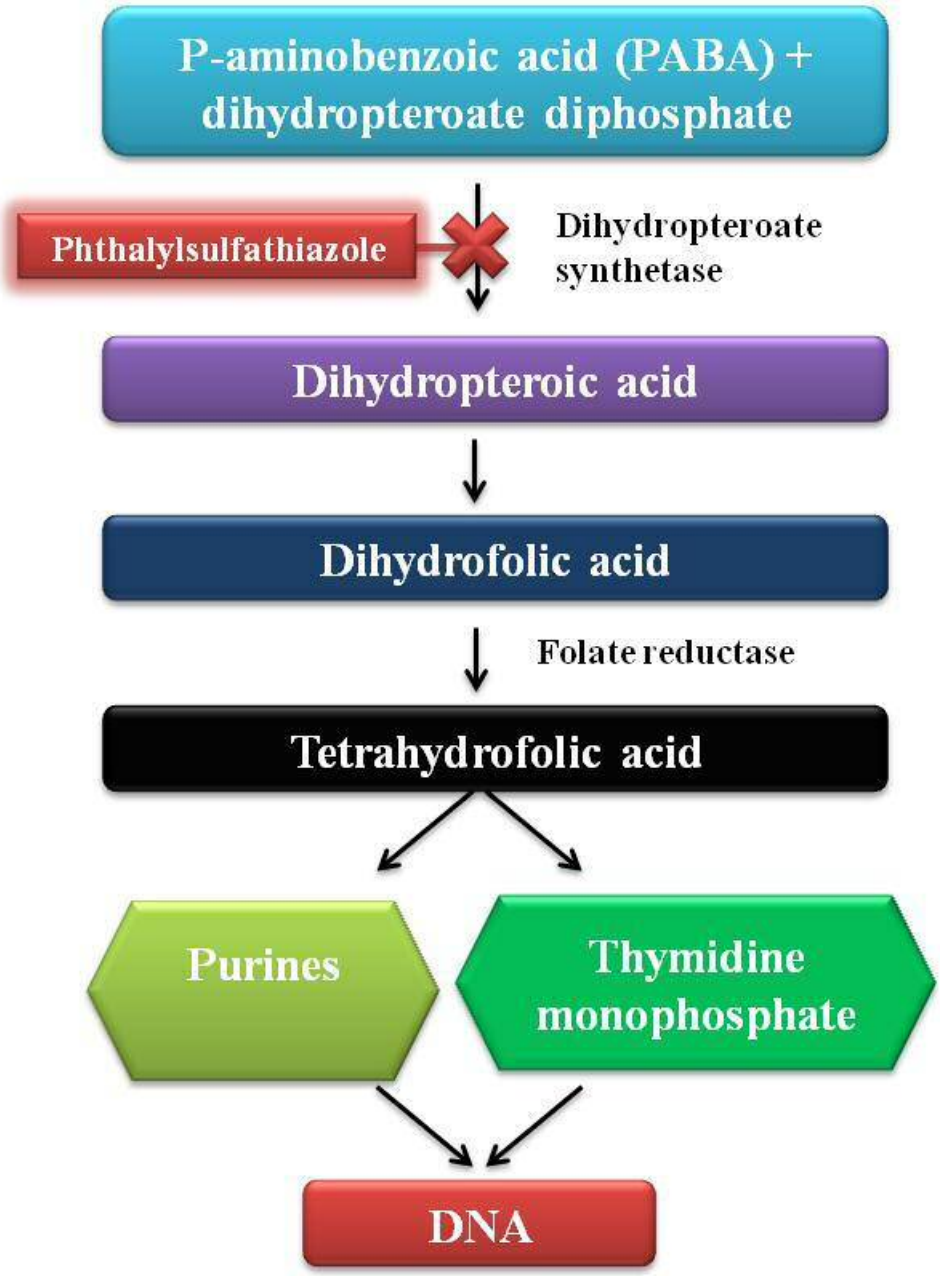


Figure 5.0 Schematic representation of the inhibitory effects of Phthalylsulfathiazole.

Phthalylsulfathiazole, as many other sulphonamides, act to inhibit folic acid synthesis. It does this by inhibiting the action of dihydropteroate synthetase thereby impairing dihydrofolic acid synthesis. This in turn leads to the inhibition of the necessary metabolites required for DNA synthesis (adapted from Williams et al 2000).

5.2 The impact of Phthalylsulfathiazole treatment on HSC and HPC populations under homeostatic conditions *in vivo*

5.2.1 The impact of Phthalylsulfathiazole on HSPCs

Mice were treated with either Phthalylsulfathiazole (1 mg/kg, 10 mg/kg or 100 mg/kg) or DMSO (controls) daily for a period of 10 days. Bone marrow and spleen were harvested at the end points and analysed by flow cytometry for HSC (SLAM markers (LSK $CD150^{+}CD48^{-}$)) and HPC (LK CD34/CD16/32) populations (Figure 5.1). Lineage-positive fully differentiated cells were also investigated in the respective tissues. Phthalylsulfathiazole treatment did not elicit any changes in HSC frequencies within the bone marrow (Figure 5.2A). Within the primitive progenitor populations, we did observe a small but significant increase in HPC1 cells, but not in the MPP and HPC2 in the 100 mg/kg treated group (Figure 5.2B-D). On the other hand, we discovered that upon treatment, Phthalylsulfathiazole resulted in a significant decrease in the CMP and GMP progenitor populations (Figure 5.3A and 5.3B). We also assessed HSPC populations within the spleen and found that there were no differences in LSK and LK populations between vehicle and compound treated animals (Figure 5.4). This suggests that Phthalylsulfathiazole interferes with granulocyte differentiation as previously documented (Fisch and Sacks 1950).

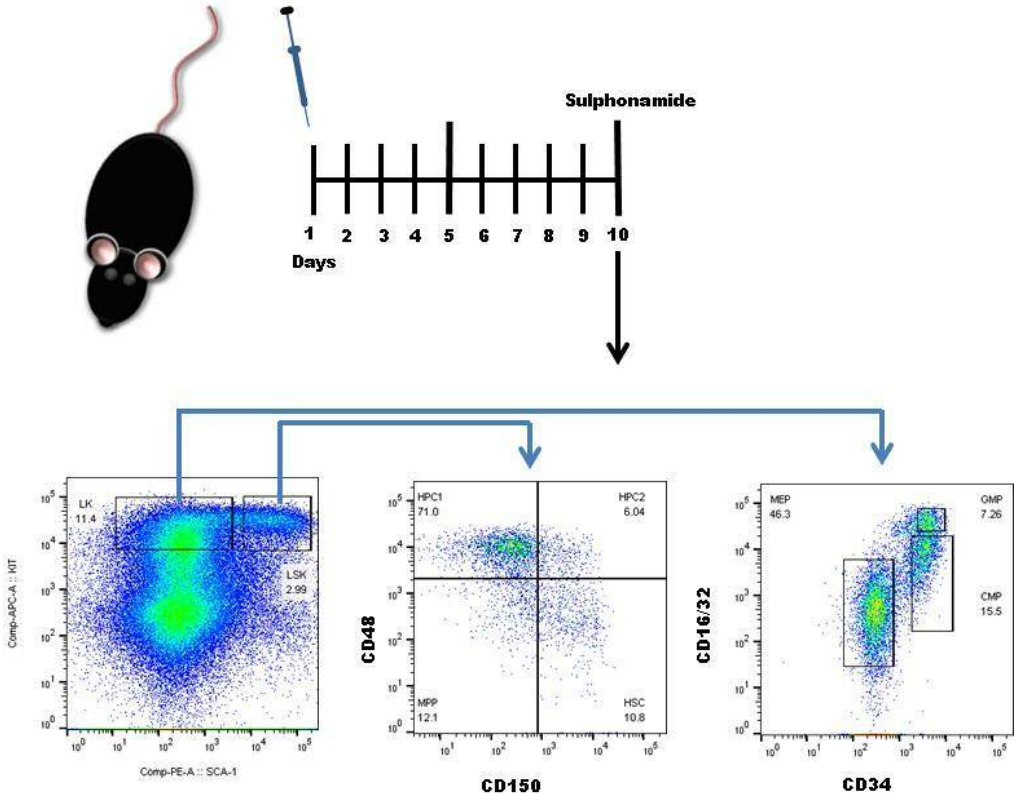


Figure 5. 1 The impact of Phthalylsulfathiazole on HSPC *in vivo*

8-10 week old mice treated daily over a period of 10 days. Gating strategy of HSCs and progenitors: SLAM markers, derived from the LSK compartment giving rise to HSCs and primitive progenitors. Lineage restricted progenitors arise from the LK compartment giving rise to the GMP, CMP and MEP populations.

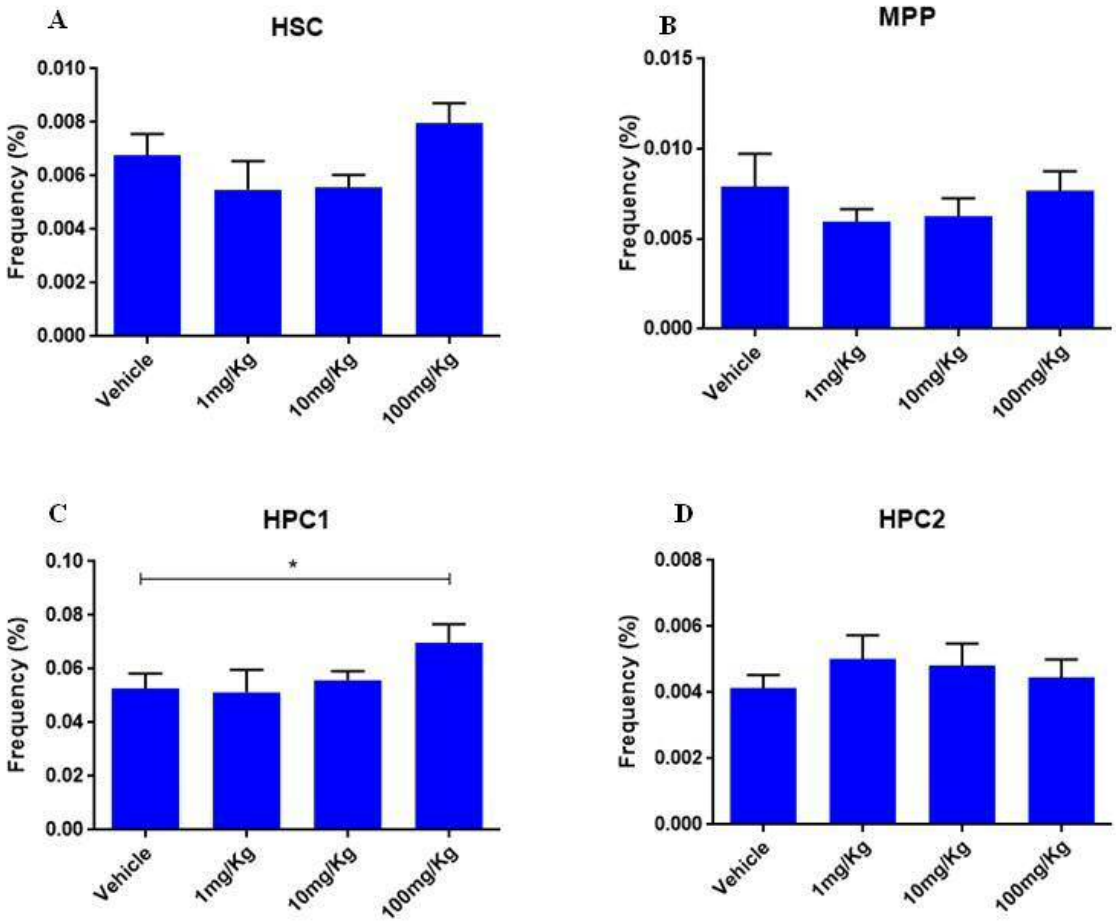


Figure 5. 2 *In vivo* treatment of Phthalylsulfathiazole does not affect bone marrow HSPCs

Mice were treated with 1 mg/kg, 10 mg/kg or 100 mg/kg of Phthalylsulfathiazole, or PBS daily for 10 days. 1 hour after the final administration, bone marrow was harvested and stained for SLAM markers for flow cytometry analysis. Specific HSC and primitive progenitor populations from within the LSK compartment were analysed (A-D). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

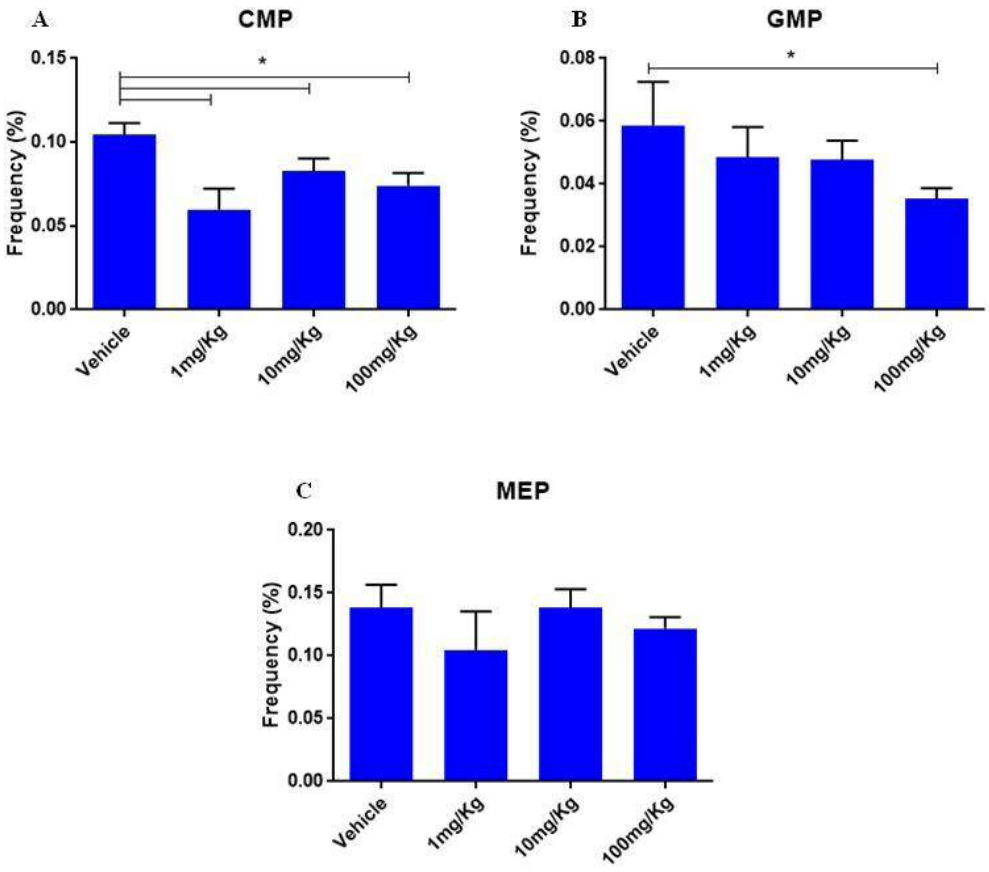


Figure 5. 3 Phthalylsulfathiazole treatment *in vivo* decreases myeloid restricted progenitors.

Mice were treated with 1 mg/kg, 10 mg/kg or 100 mg/kg of Phthalylsulfathiazole, or PBS daily for 10 days. 1 hour after the final administration, bone marrow was harvested and stained for LK markers for flow cytometry analysis (A-C). Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

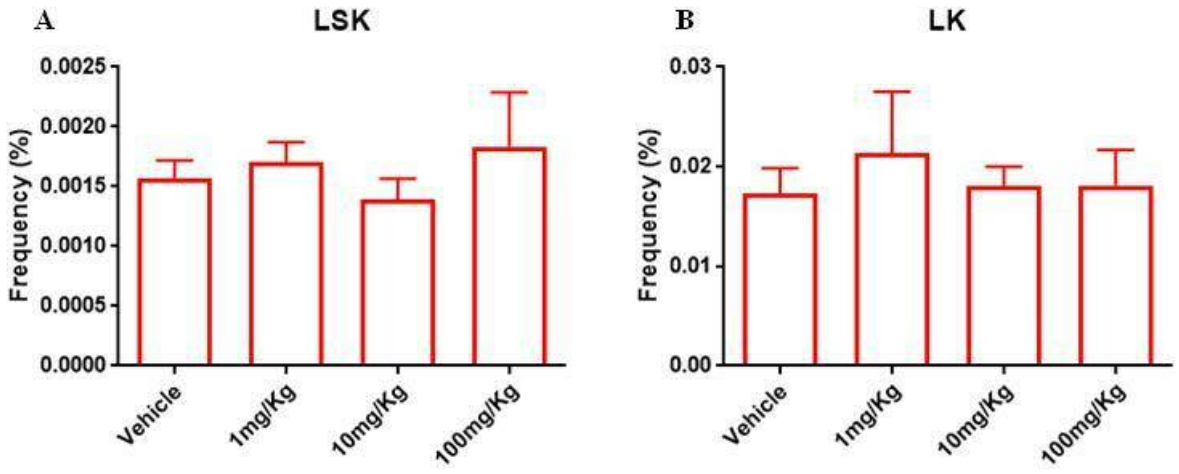


Figure 5. 4 Splenic HSPC populations are unaffected by *in vivo* treatment of Phthalylsulfathiazole.

Spleens of mice were harvested after 10 days. Mice were treated with 1 mg/kg, 10 mg/kg or 100 mg/kg of Phthalylsulfathiazole, or vehicle daily for 10 days. Analysis of (A) LSK (Lin- cKit^{+} Sca1^{+}) and (B) LK (Lin- cKit^{+}) populations were conducted by flow cytometry. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

5.2.2 The impact of Phthalylsulfathiazole on lineage differentiated cells

We also sought to investigate the effects of Phthalylsulfathiazole treatment on mature differentiated blood cell populations. Initially looking at cells of the bone marrow, we found that there were no significant differences within the lymphoid compartment (T- and B cells) between treated and non-treated animals (Figure 5.5A and 5.5B). Interestingly however we saw that Phthalylsulfathiazole treatment caused a significant increase in granulocytes (Gr-1) compared to controls (Figure 5.5C). We did not observe any changes in the remaining cells of the myeloid and erythrocytic compartments (Figure 5.5D-F). We also looked at these populations in the spleen but no changes in the lineage differentiated populations were observed (Figure 5.6). There were also no differences in the lineage differentiated cells of the blood in response to Phthalylsulfathiazole treatment (Figure 5.7). This data suggests that Phthalylsulfathiazole may promote the differentiation or expansion of the granulocyte lineage from GMPs in bone marrow.

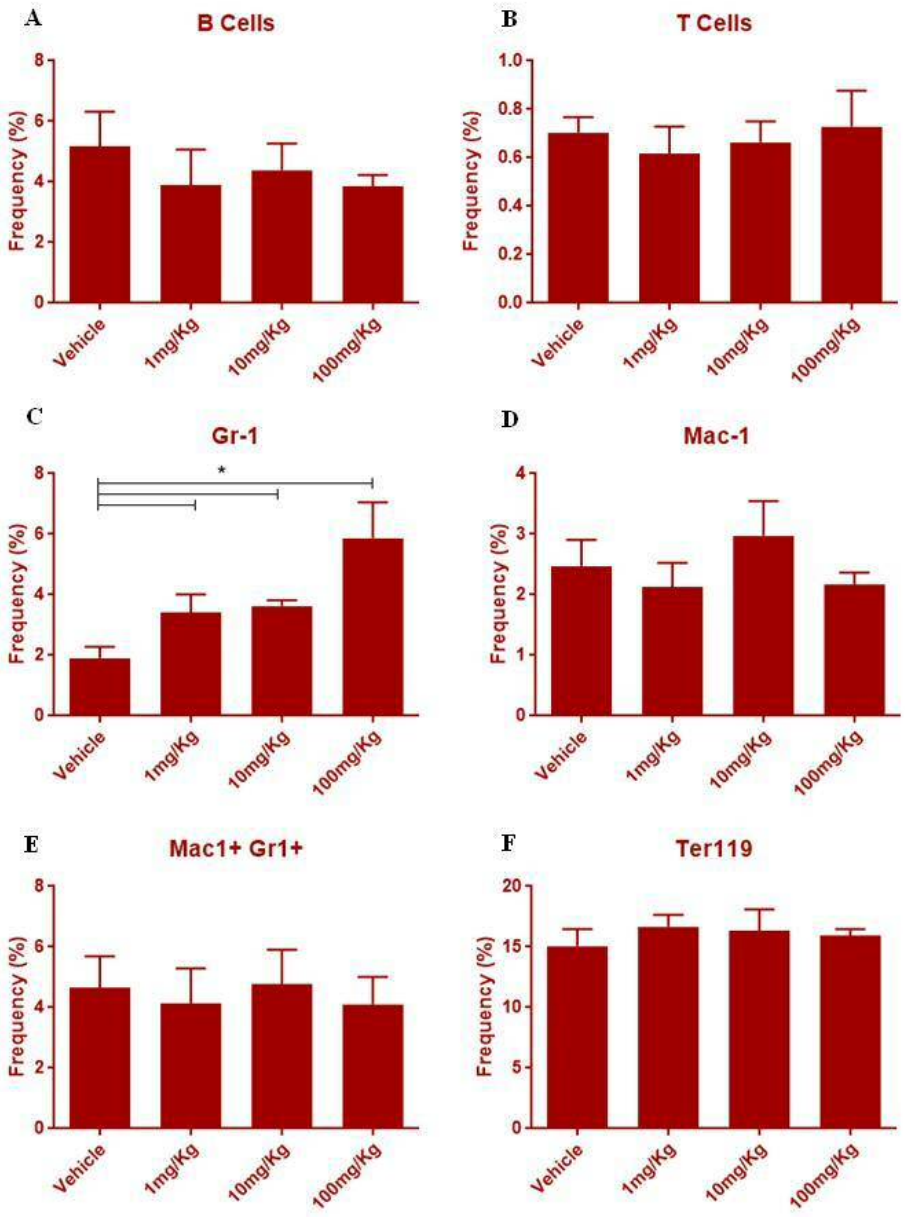


Figure 5. 5 *In vivo* Phthalylsulfathiazole treatment causes an increase in bone marrow granulocytes

Phthalylsulfathiazole was administered twice daily for 10 days, after which bone marrow was harvested. Lymphoid cells include B- (A) and T- cells (B). Cells of the myeloid compartment were defined as myeloid cells Granulocyte ($GR1^+$) (C) , monocyte/macrophage ($Mac1^+$) (D), myeloid cells ($Mac1^+Gr1^+$) (E), and erythrocyte ($Ter119^+$) (F) Data shown as the frequency of populations of live cells. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group * $p<0.05$

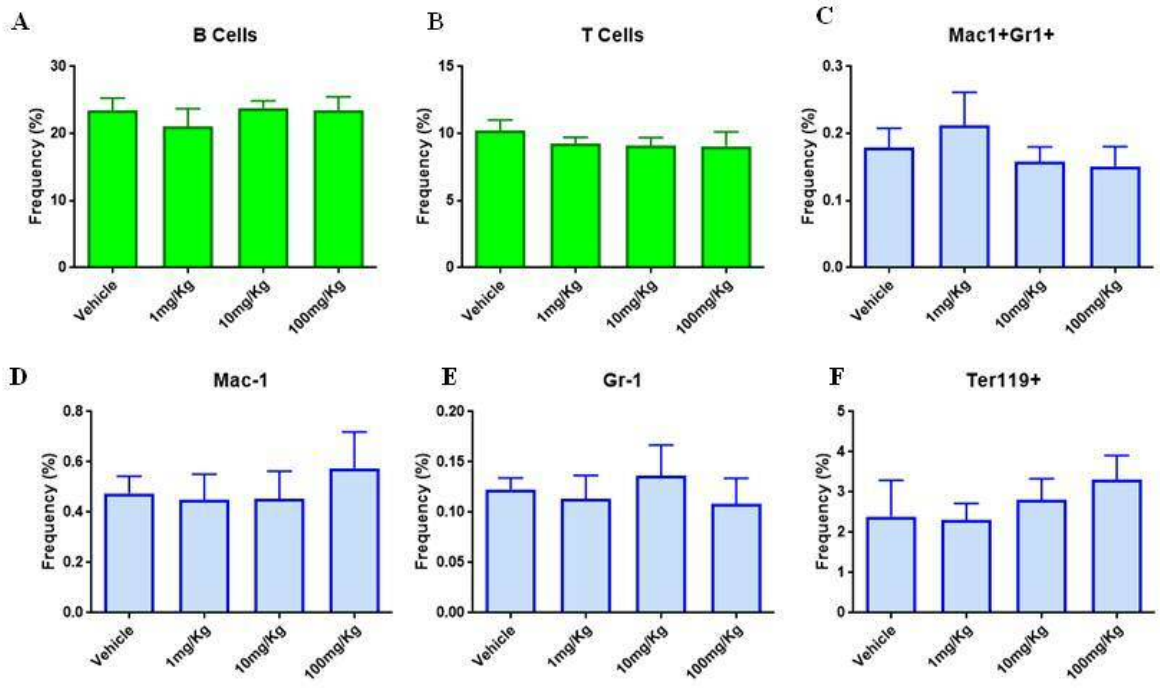


Figure 5. 6 Phthalylsulfathiazole treatment *in vivo* does not affect lineage differentiated cells of the spleen

Phthalylsulfathiazole was administered twice daily for 10 days, after which spleens were harvested. Lymphoid cells include B- (A) and T- cells (B). Cells of the myeloid compartment were defined as myeloid cells ($Mac1^{+}Gr1^{+}$) (C), monocyte/macrophage ($Mac1^{+}$) (D), Granulocyte ($GR1^{+}$) (E) and erythrocyte ($Ter119^{+}$) (F) Data shown as the frequency of populations of live cells. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group * $p<0.05$

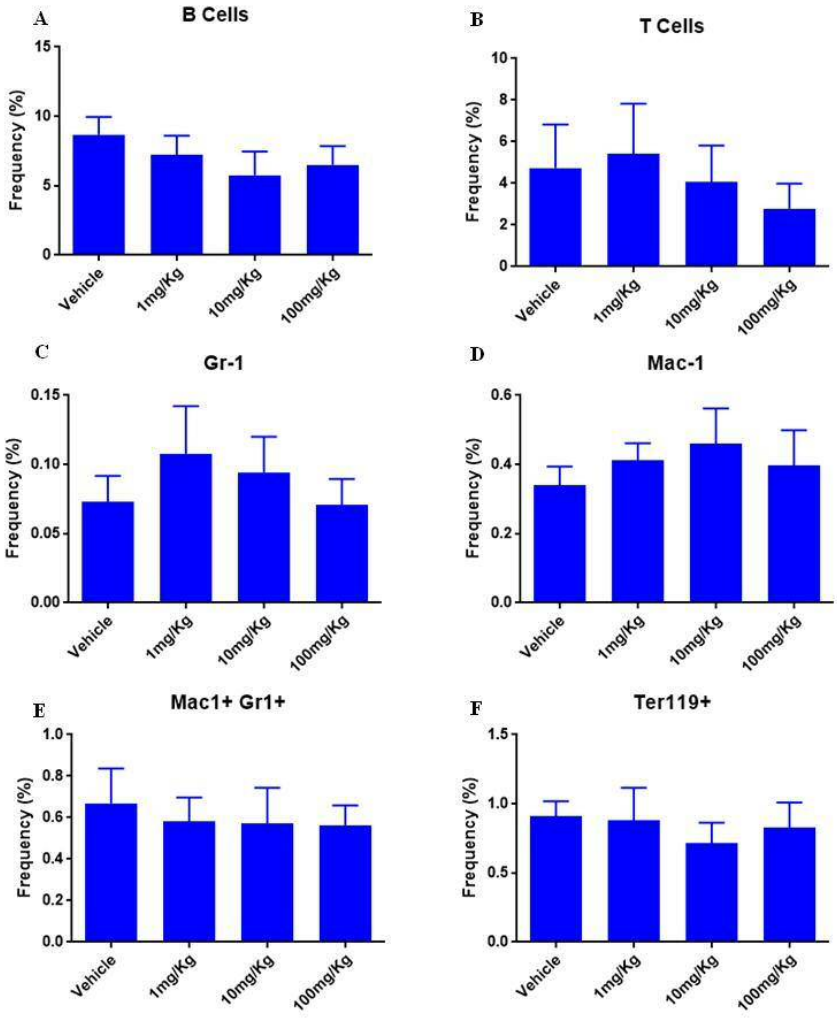


Figure 5. 7 Administration of Phthalylsulfathiazole *in vivo* does not affect circulating lineage differentiated cells

Blood was harvested at the end point of the experiment. Lymphoid cells include B- (A) and T-cells (B). Cells of the myeloid compartment were defined as Granulocyte ($GR1^+$) (C) , monocyte/macrophage ($Mac1^+$) (D), myeloid cells ($Mac1^+Gr1^+$) (E), and erythrocyte ($Ter119^+$) (F) .Data shown as the frequency of populations of live cells. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group * $p<0.05$

5.3 The impact of Phthalylsulfathiazole treatment on the functionality of HSPCs

Although we did not observe any changes in HSPCs immunophentotypically, we assessed whether Phthalylsulfathiazole treated HSPCs cells were functionally altered. We therefore treated animals with Phthalylsulfathiazole for 10 days with 3 doses (1, 10 and 100 mg/kg). After the final administration, bone marrow was harvested and transplanted with bone marrow competitors at a ratio of 1:1 into lethally irradiated recipients (Figure 5.8). We found that there were no differences in donor (CD45.1) engraftment between the control and treated donor groups from 2 to 16 weeks post-transplantation (Figure 5.9A). We also observed no differences in myelo-lymphoid reconstitution in these mice (Figure 5.9B-5.9D). After 16 weeks, we sacrificed the animals and analysed the donor HSPC populations in bone marrow. We found that there were no changes in the frequencies of HSCs of transplanted treated cells compared to vehicle treated cells (Figure 5.10A). This was observed in primitive progenitors (Figure 5.10B-D) and the more lineage restricted progenitors (Figure 5.10E-G). To analyse whether there were alterations in apoptotic characteristics, of HSPCs we performed an Annexin V assay in donor Phthalylsulfathiazole treated HSPCS (Figure 5.11). We observed no differences in the apoptotic (Figure 5.11) status in transplanted Phthalylsulfathiazole treated HSPCs. This suggests that Phthalylsulfathiazole treated HSPCs do not affect haematopoietic reconstitution after transplant. Investigation of the impact of Phthalylsulfathiazole-treated HSCs on their self-renewal capability is currently under investigation in secondary transplanted recipients.

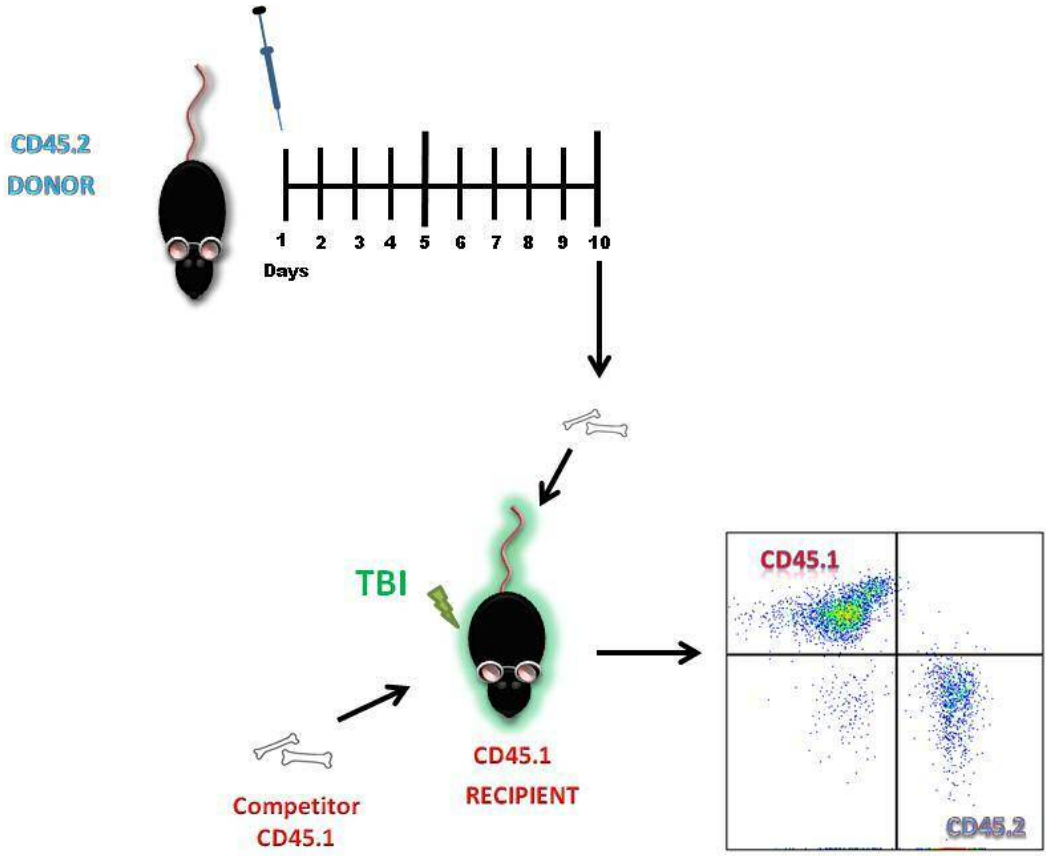


Figure 5. 8 The impact of Phthalylsulfathiazole treated cells in competitive transplantation

Donor CD45.1 mice were treated with Phthalylsulfathiazole (or vehicle) for 10 consecutive days. After this, donor bone marrow was harvested and transplanted competitively with CD45.2 cells into CD45.2 recipients. Engraftment was measured by weekly bleeds post-transplant.

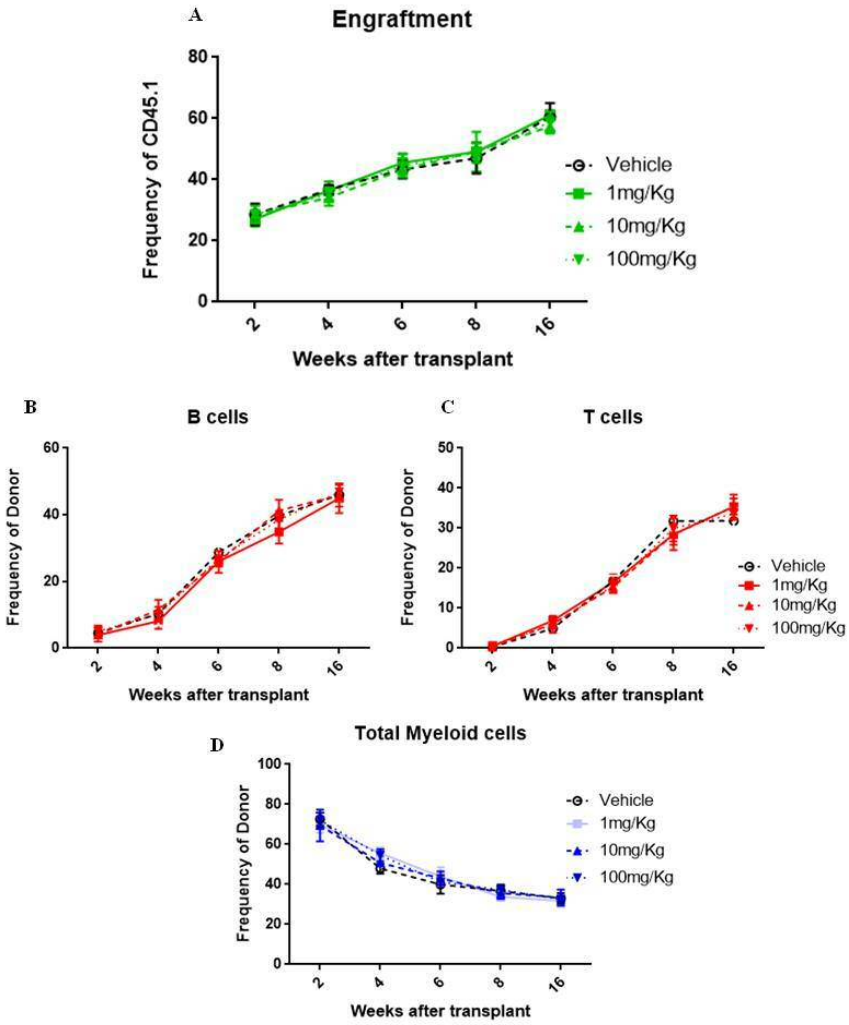


Figure 5. 9 Phthalylsulfathiazole treated cells exhibit normal haematopoietic reconstitution

Donor CD45.1 mice were treated with Phthalylsulfathiazole (or vehicle) for 10 consecutive days. After this, donor bone marrow cells were harvested and transplanted competitively with CD45.2 cells into CD45.2 recipients. Weekly bleeding analysis was conducted to assess haematopoietic reconstitution. Recipient and donor cells were identified using CD45.2 and CD45.1 antibodies respectively (A). From within the donor compartment, lineage differentiated populations were identified as total (B and C) lymphoid (B220, CD4/CD80) and (D) myeloid (Mac1 and Gr1) cells.

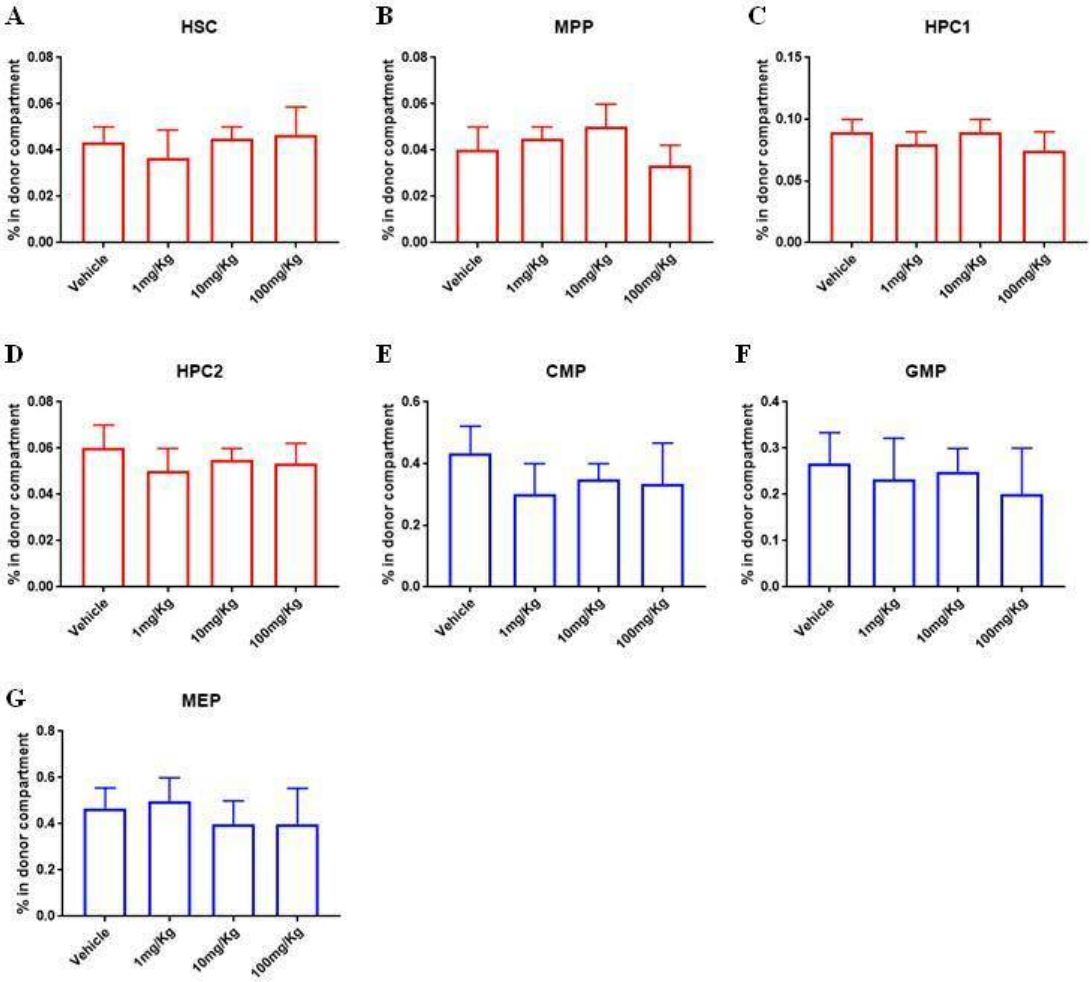


Figure 5. 10 Phthalylsulfathiazole treated HSPCs function normally after primary competitive transplantation.

16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Specific donor HSPC and lineage restricted progenitor populations from within the LSK (A-D) and LK (E-G) compartment were analysed. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

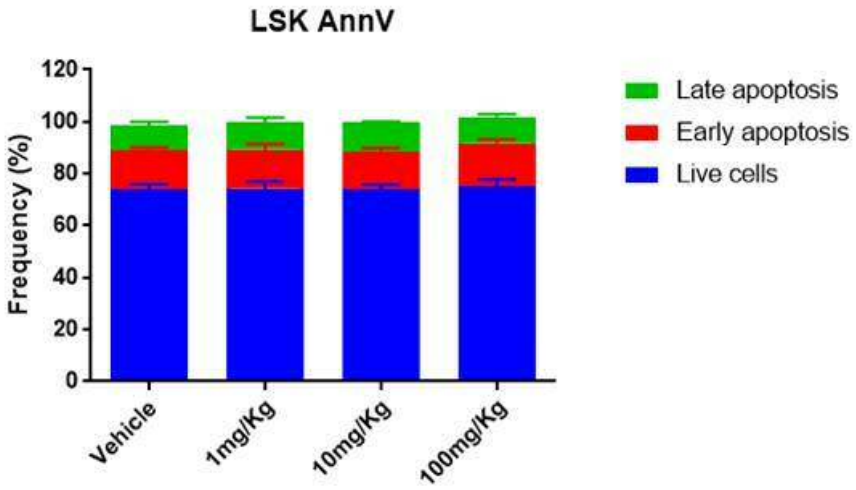


Figure 5. 11 Phthalylsulfathiazole treated HSPCs exhibit normal apoptotic status

16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested and stained for LSK (Lin-Sca1^{+}cKit^{+}) populations. Further staining was conducted with Annexin V staining (B) with DAPI. Data shown as the frequency of the respective SLAM population. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

5.4 The impact of administering Phthalylsulfathiazole following BM transplant

In addition to competitive analysis, we also sought to investigate the effects of this compound on haematopoietic reconstitution following bone marrow transplant. Here we transplanted $5x10^5$ bone marrow cells into irradiated recipients, which were then treated with Phthalylsulfathiazole every other day for a total of 10 treatments. Using this strategy, we did not observe any changes in engraftment capacity over the 16 week transplant period (Figure 5.12A). Again, this was seen in the reconstitution of donor lymphoid and myeloid cells (Figure 5.12B-5.12D). We also assessed the HSPC populations within the bone marrow after 16 weeks and found that donor HSCs and primitive progenitor populations were unaffected by Phthalylsulfathiazole (Figure 5.13A-D). This was also the case in the lineage restricted progenitor (CMP, GMP and MEP) populations (Figure 5.13E-G). Although we did not observe immunophenotypical differences within the above populations, we further looked at the apoptotic status of the LSK compartment. Coinciding with the immunophenotypic analysis, Annexin V staining revealed that there were no differences ofviable LSK cells between control and treated groups (Figure 5.14). These data show that treatment of Phthalylsulfathiazole after bone marrow transplant does not affect haematopoietic recovery.

To test whether the treatment of Pthalylsulfathiazole impacted long term HSC self-renewal, we conducted secondary serial transplants of donor derived LSK cells. We isolated 2000 LSK cells and transplanted them into secondary recipients. We found that engraftment of Phthalylsulfathiazole treated donor LSK cells was unchanged compared to vehicle treated mice (Figure 5.15A). We also did not observe any differences in the reconstitution of lymphoid and myeloid cells within secondary recipients (Figure 5.15B-D). After the 16 week time point, we harvested the bone marrow of secondary recipients and analysed the HSPC populations (Figure 5.16). We found that Phthalylsulfathiazole did not affect HSC function as there were no differences in the frequencies of donor HSCs and primitive progenitors (Figure 5.16A-D). This was also evident in the lineage restricted progenitor populations (Figure 5.16E-G).

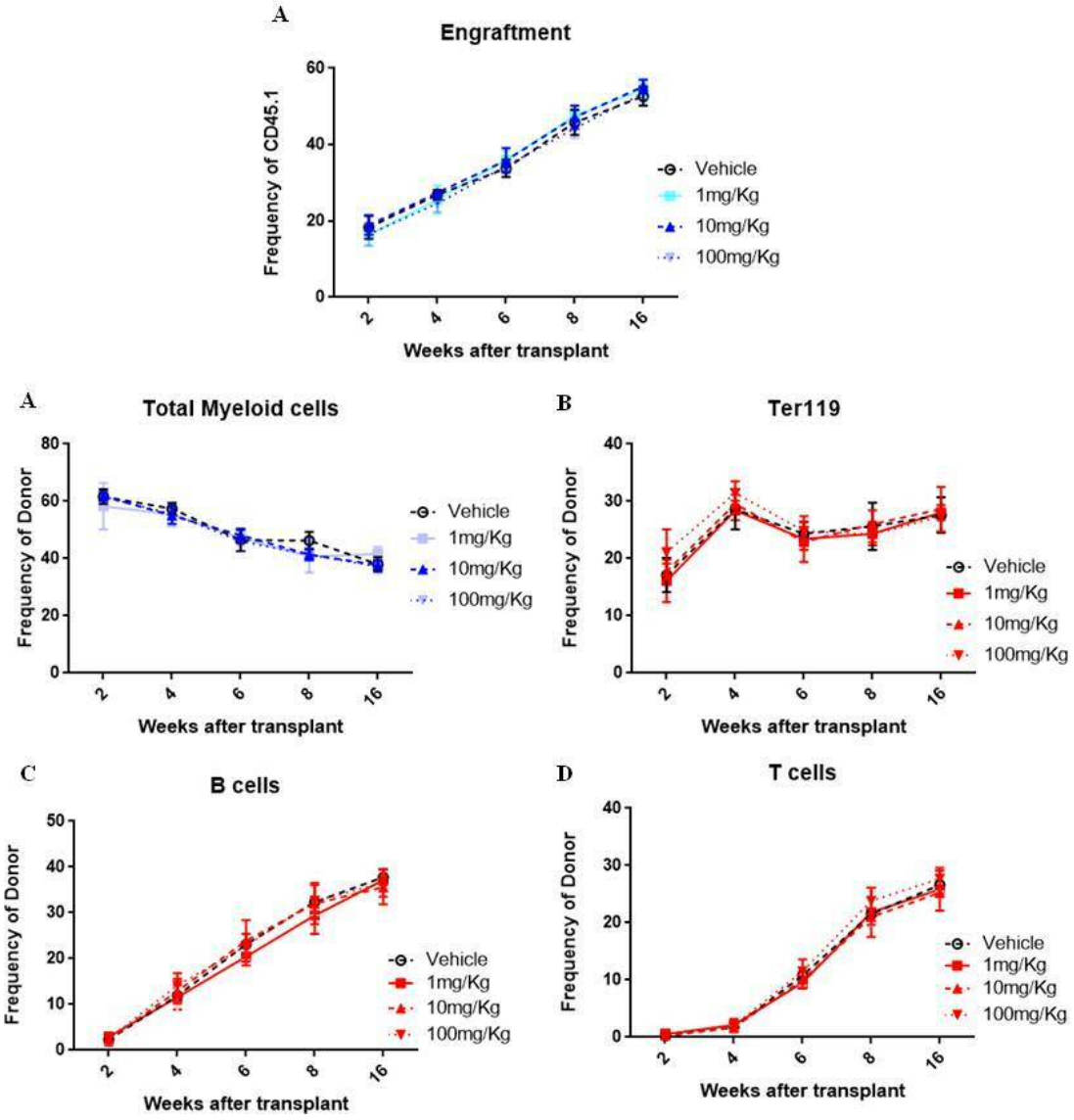


Figure 5. 12 *In vivo* Phthalylsulfathiazole treatment after bone marrow transplant does not affect haematopoietic recovery

CD45.2 recipient mice were transplanted with $5x10^5$ donor cells (CD45.1). Phthalylsulfathiazole (or vehicle (DMSO)) was then administered once every two days for a total of 10 treatments (A). Weekly bleedings were conducted to assess engraftment of donor cells (CD45.1). Recovery of (B) myeloid (Mac1/Gr1, Mac1 and Gr1), (C) erythrocyte (Ter119) and (D and E) lymphoid cells were also assessed.

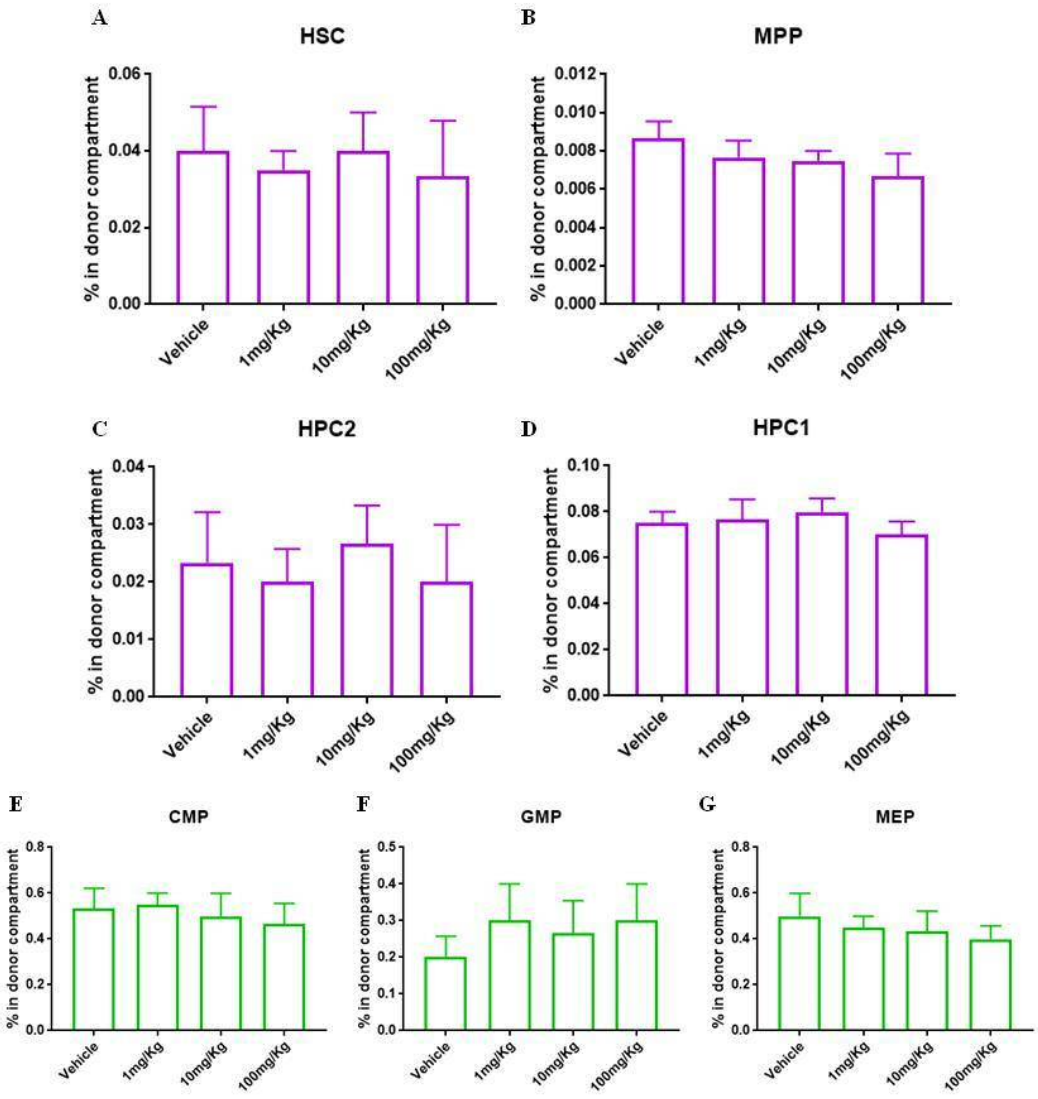


Figure 5. 13 *In vivo* Phthalylsulfathiazole treatment after bone marrow transplant does not affect HSPC function

16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested. Specific donor HSPC and lineage restricted progenitor populations from within the LSK (A-D) and LK (E-G) compartment were analysed. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

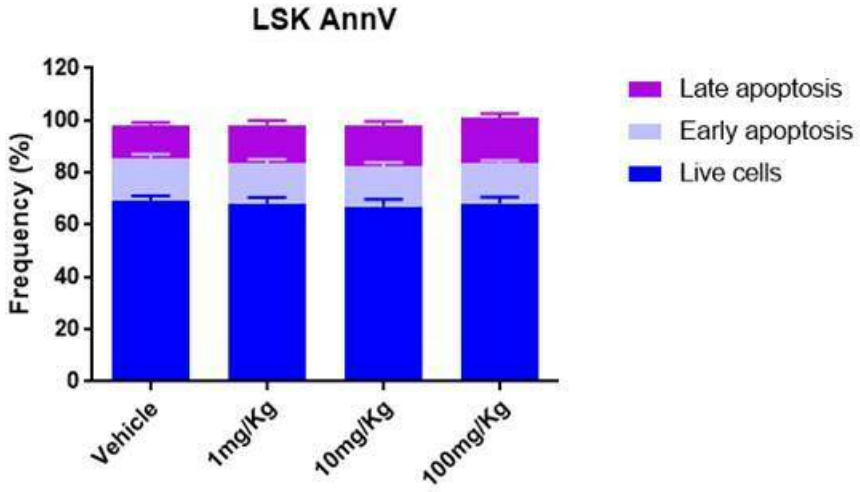


Figure 5. 14 HSPC viability is unaffected by *in vivo* Phthalylsulfathiazole treatment after transplant

16 weeks after transplant, recipient mice were sacrificed and bone marrow was harvested and stained for LSK (Lin-Sca1^{+}cKit^{+}) populations. Further staining was conducted with Annexin V staining and DAPI. Data shown as the frequency of the respective SLAM population. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

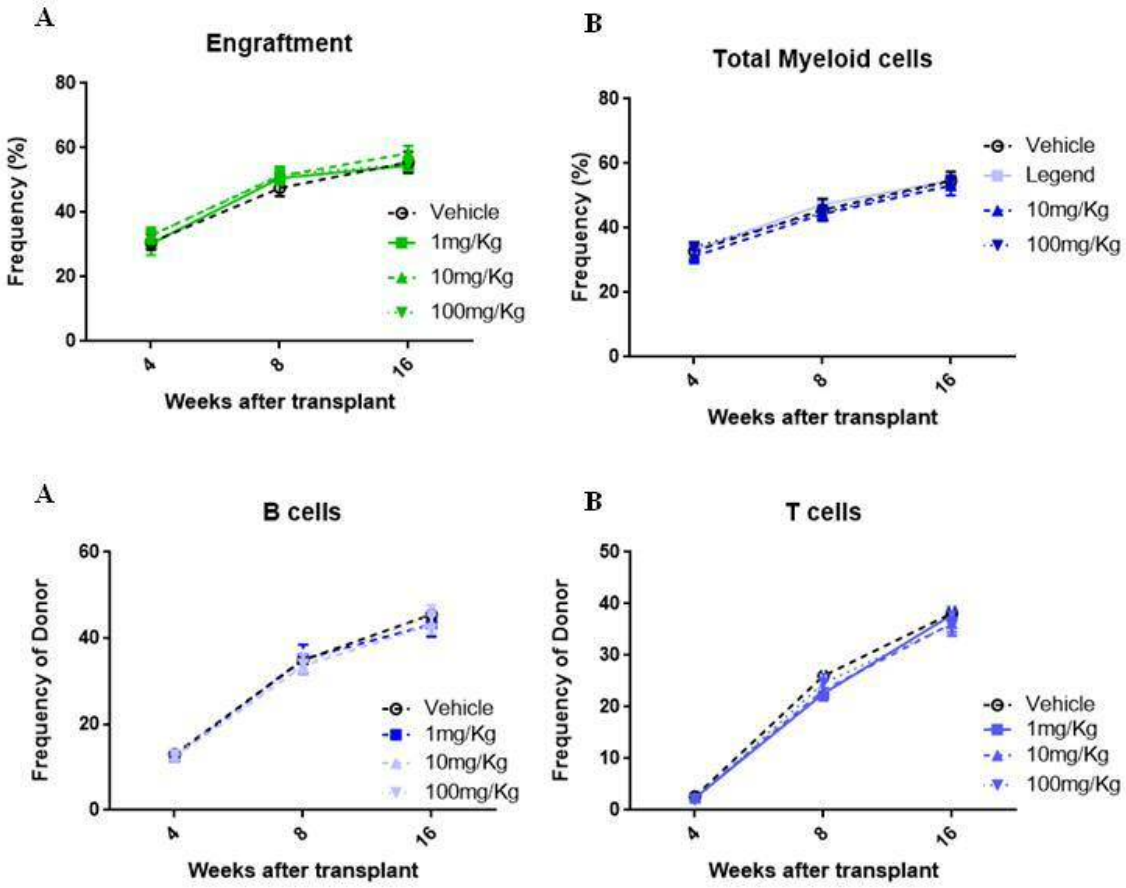


Figure 5. 15 Phthalylsulfathiazole treatment after transplant does not affect self-renewal capacitates of HSCs

Primary recipients were treated with Phthalylsulfathiazole or vehicle. After 16 weeks, donor CD45.1 LSK cells were isolated by FACS sorting and transplanted into secondary recipients (2000 $CD45.2^{+}Lin\text{-}Sca1^{+}cKit^{+}$ cells per recipient). Blood samples were taken to measure engraftment (A) and reconstitution of myeloid (B) and lymphoid (C and D) cells. Myeloid (Mac1/Gr1) and T-($CD4^{+}/8^{+}$) and B cell (B220) markers used respectively.

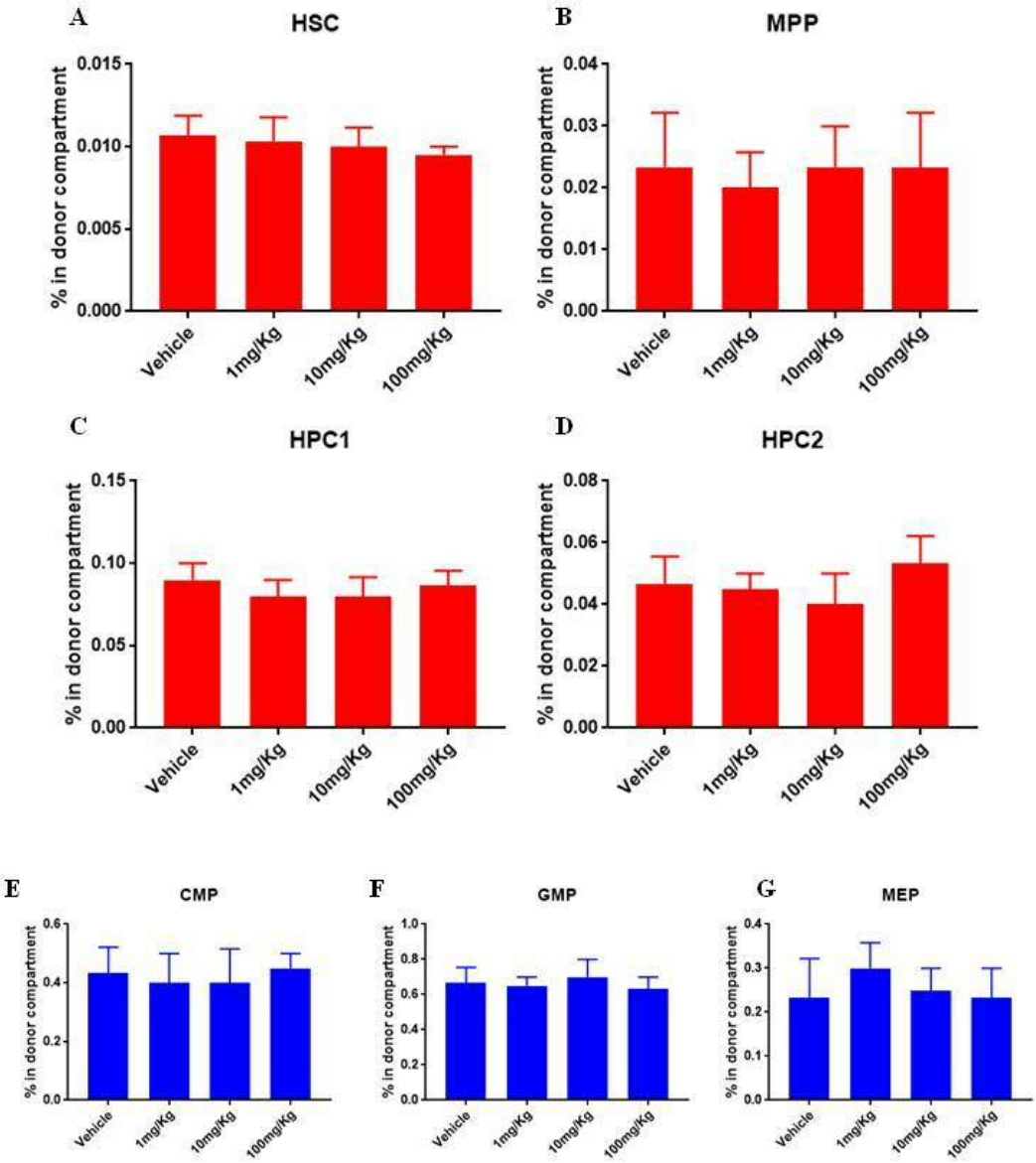


Figure 5. 16 Phthalylsulfathiazole treatment after transplant does not impact HSC function after secondary transplant

16 weeks after secondary transplantation. Mice were sacrificed and bone marrow was harvested from the fibias and tibias. Analysis of bone marrow LSK (A-D) and LK (E-G) was conducted. Data shown as the frequency of populations of total bone marrow. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group

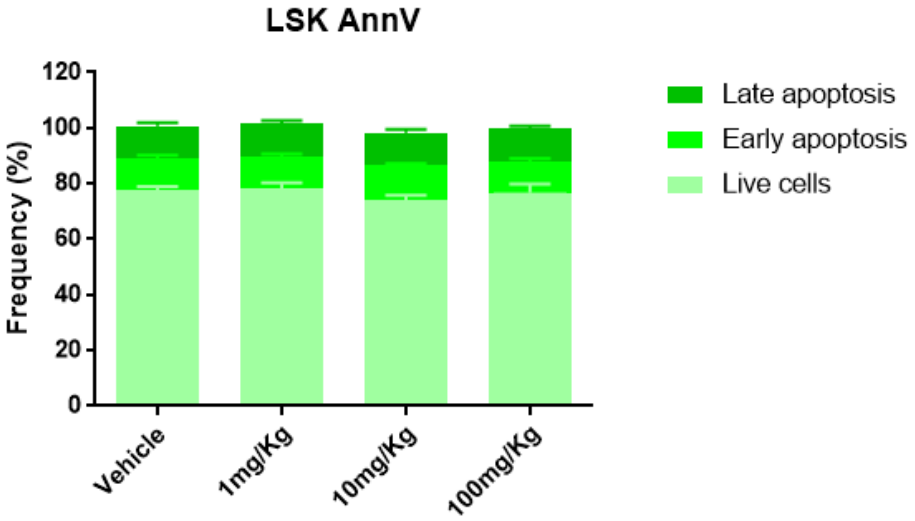


Figure 5. 17 Viability of HSPCs are unaffected by *in vivo* Phthalylsulfathiazole treatment

16 weeks after secondary transplantation. Mice were sacrificed and bone marrow was harvested from the fibias and tibias. Harvested bone marrow was stained for LSK ($Lin^{-}Sca1^{+}cKit^{+}$) populations. Further staining was conducted with Annexin V conjugated antibody and DAPI. Data shown as the frequency of the respective SLAM population. Error bars represent mean ± SEM of 2 independent experiments, each with 4 mice per group *$p<0.05$.

5.5 The impact of Phthalylsulfathiazole in leukaemic cells *in vitro* and *in vivo*

Since it has been reported that some types of sulphonamides have anti-cancer properties (Scozzafava et al. 2003), we assessed whether Phthalylsulfathiazole possessed a similar cancer-targeting ability. We firstly tested this in human AML cell lines *in vitro*. In this study we exposed a number of cell lines (described in materials and methods) to Phthalylsulfathiazole for a period of 5 days, after which we conducted a proliferation assay to determine cell numbers. We found that concentrations above 100nM, Phthalylsulfathiazole elicited in a significant increase in proliferation in the NB4 cell line (Figure 5.18A) and at 10mM in K562 cells (Figure 5.18B). We also investigated the effects of Phthalylsulfathiazole treatment on leukaemic cells *in vivo*. Using the MLL-AF9 mouse model (described in preceding chapter), we transplanted 10,000 leukaemic stem cells (LSCs) into irradiated recipients and commenced treatment (via intraperitoneal injection) 24 hours after transplant. Administration of Phthalylsulfathiazole (or vehicle) continued every other day for the duration of the experiment. Simultaneously, we conducted bleeding analysis at weekly intervals to assess disease progression. We saw that the progression of leukaemia was accelerated in animals treated with 100 mg/kg Phthalylsulfathiazole as there were a significantly higher number of GFP^+ leukaemic cells by 5 weeks compared to vehicle treated controls (Figure 5.19A). The increased rate of disease progression was also mirrored by poor survival rates of Phthalylsulfathiazole treated animals. We observed significantly poorer survival rates in animals treated with 10 and 100 mg/kg of Phthalylsulfathiazole compared to control mice (Figure 5.19B). Moribund mice were culled and bone marrow was harvested. From this, we identified the LSCs populations ($cKit^+$ GFP^+ CD16/32/CD34). We observed that animals treated with 10 and 100 mg/kg Phthalylsulfathiazole possessed a significantly higher frequency of LSCs compared to vehicle treated animals (Figure 5.20). In addition to this, spleens were also harvested and splenomegaly was assessed by measuring the size and weight. We found that the spleens of animals treated with 10 and 100 mg/kg Phthalylsulfathiazole were considerably larger in size and heavier suggesting sever splenomegaly (Figure 5.21). This data suggests that Phthalylsulfathiazole accelerates the progression of disease by increasing the rate of proliferation of leukaemic cells *in vitro* and *in vivo*.

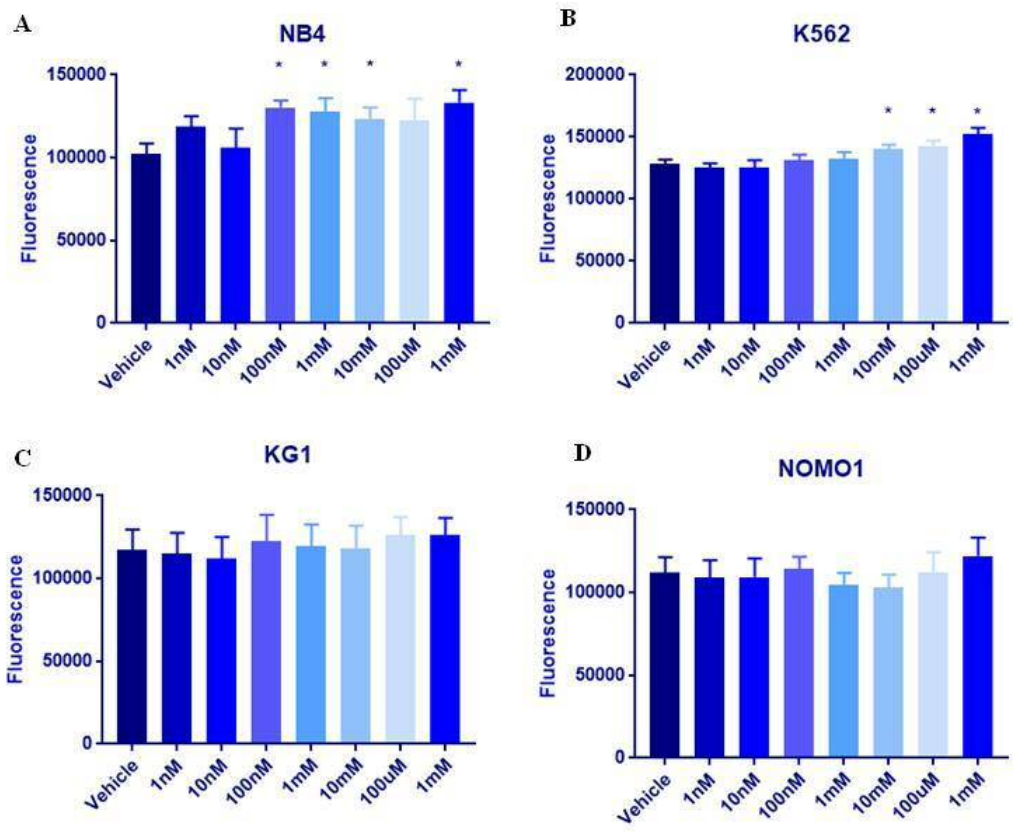


Figure 5. 18 Phthalylsulfathiazole promotes proliferation in AML cell lines

Human AML cell lines were incubated for 5 days with a range of concentrations of Phthalylsulfathiazole (1nM-1mM). Cells were exposed to Cell Titre Blue which emits a fluorescence in response to live cells, therefore fluorescence is indicative of number of cells. (A) NB4 cell line is derived from an APL patient whereas (B) K562 (CML in blast crisis) (C) KG-1 and (D) NOMO1 are AML patient derived cell lines. Error bars represent mean ± SEM of 3 independent experiments, each with triplicate groups *$p<0.05$.

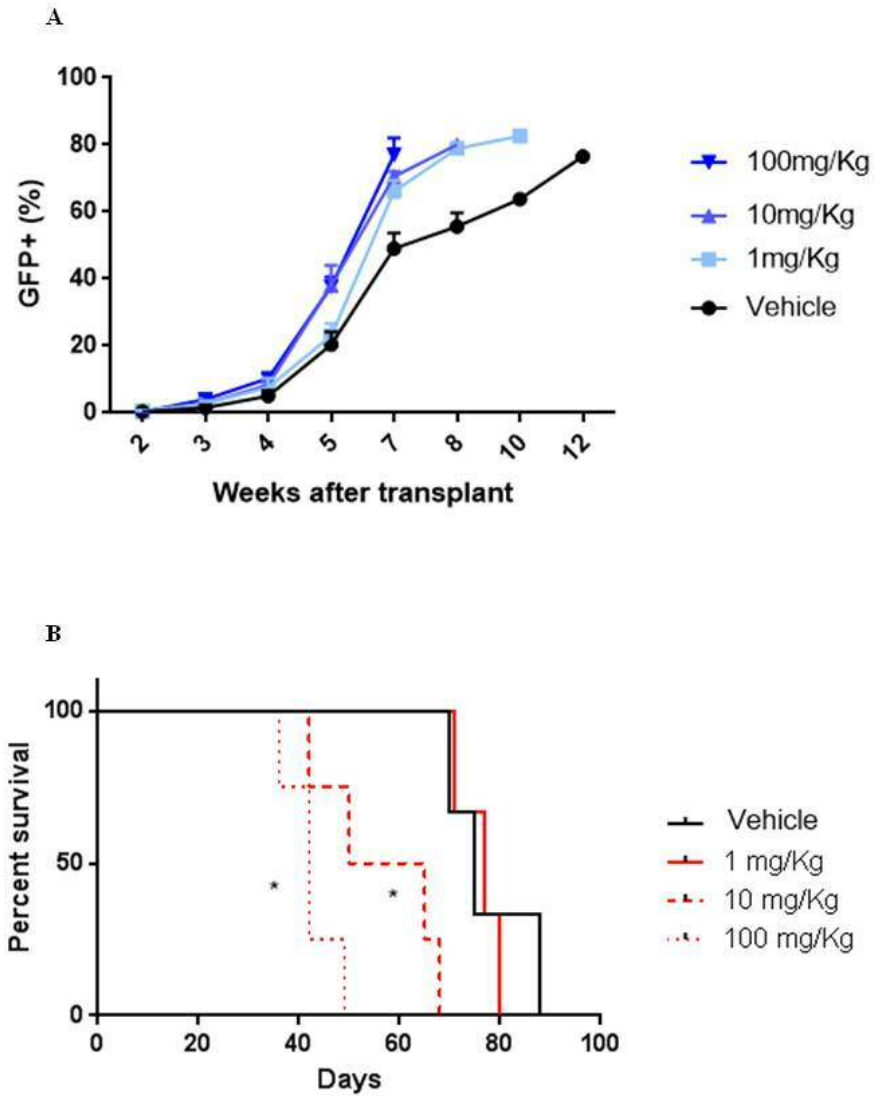


Figure 5. 19 Administration of Phthalylsulfathiazole accelerates disease progression in MLL-AF9 leukaemic mice

Mice were transplanted with MLL-AF9/GFP transduced cKit^{+} cells. Immediately after transplantation, mice were treated with vehicle or Phthalylsulfathiazole (1, 10 or 100 mg/kg). Weekly bleeding analysis was conducted to measure disease progression. Flow cytometry was used to analyse GFP^{+} leukaemic cells (A). Survival was also measured when mice became moribund or succumbed to disease (B). Error bars represent mean ± SEM of one experiment, with 4 mice per group *$p<0.05$.

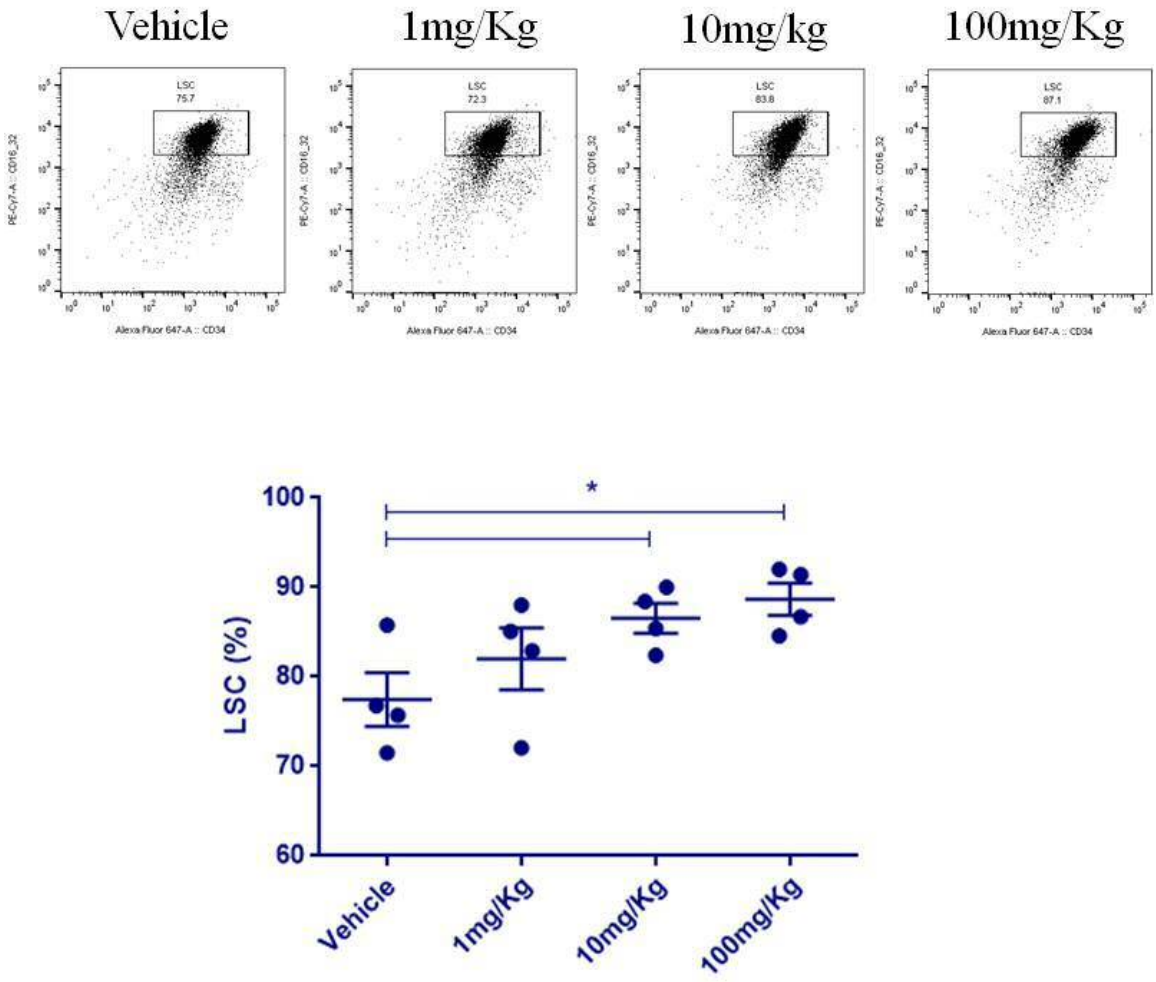


Figure 5. 20 Phthalylsulfathiazole treatment enhances LSC frequencies

Mice were transplanted with MLL-AF9/GFP transduced cKit$^+$ cells. When mice became moribund, bone marrow was harvested and analysed for LSC populations. LSCs were identified as cKit$^+$ GFP$^+$ CD16/32hi CD34hi. Error bars represent mean ± SEM of one experiment, with 4 mice per group *$p<0.05$.

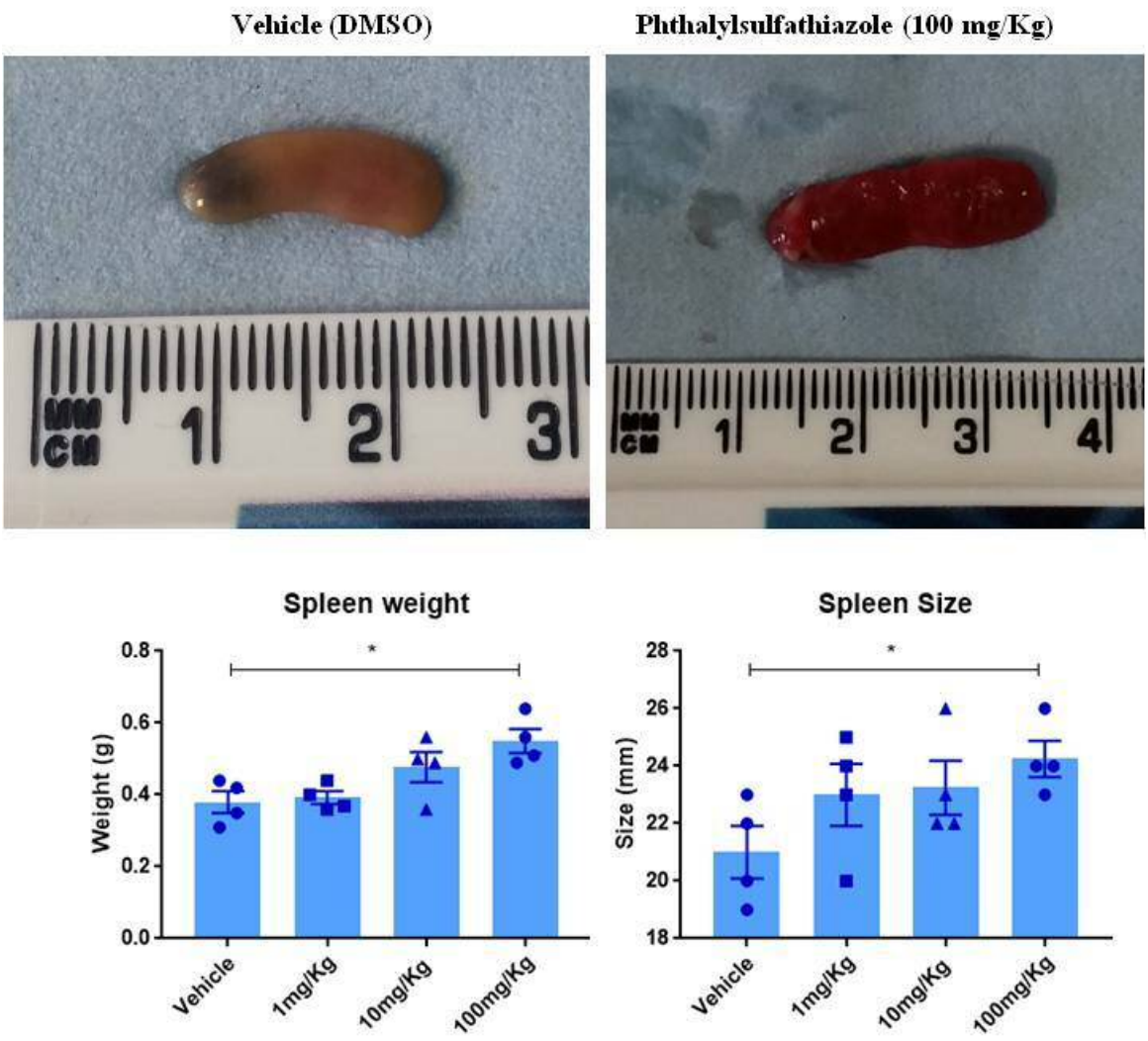


Figure 5. 21 Enhanced splenomegaly in Phthalylsulfathiazole treated MLL-AF9 mice

Mice were transplanted with MLL-AF9/GFP cKit^{+} cells. When mice became moribund, spleens were harvested and measured to assess for splenomegaly. Error bars represent mean ± SEM of one experiment, with 4 mice per group *$p<0.05$.

5.6 Discussion

The sulphonamide Phthalylsulfathiazole was identified as a potential candidate in targeting stem cells from our library screen. We therefore assessed whether Phthalylsulfathiazole exerted its effect on HSCs *in vivo*. This was initially investigated in mice under homeostatic conditions and then taken further into a competitive transplant setting where donor cells were treated *in vivo* and transplanted into irradiated recipients. In addition to this we also sought to study the effects of Phthalylsulfathiazole treatment on haematopoietic reconstitution after bone marrow transplant. Knowing that a number of sulphonamides have been implicated in targeting cancer (Scozzafava et al. 2003; Mohan et al. 2006; Han et al. 2017), we finally investigated the impact of Phthalylsulfathiazole in AML.

We initially sought to study the effects of *in vivo* administration of Phthalylsulfathiazole on HSPC populations. Although we did not see any effects in the HSC and primitive progenitors (with the exception of the HPC1 population), we did observe a significant decrease in the CMP and GMP cells in response to Phthalylsulfathiazole treatment. This was an interesting observation as Phthalylsulfathiazole has already been documented to cause bone marrow failure such as aplastic anaemia and granulocytopenia (Johnson et al. 1948; Fisch and Sacks 1950). However when we looked at differentiated granulocytes within the bone marrow, we found that in fact there was a higher frequency in Gr-1^{+} cells in these animals compared to vehicle treated controls. This may suggest that the decrease in the granulocyte-macrophage progenitors may be due to the increased differentiation of its downstream progenitors in the bone marrow. This of course contradicts the existing literature however these cases were reported between the 1940's and 1960's (Menten and Graff 1946; Taylor 1946; Fisch and Sacks 1950) and may be because if species-specific differences. Apart from these studies, little is known on the effects of Phthalylsulfathiazole on haematopoiesis, partly because more potent and efficient antimicrobial drugs have been developed and are used clinically. In addition to this, in the above cases (and in current Phthalylsulfathiazole treatment regimens), the compound is administered orally as its main role is to target the flora of the gut by disrupting DNA synthesis (Johnson et al. 1948). It has also been reported that Phthalylsulfathiazole is poorly absorbed from the

gut into the blood stream (Florestano and Bahler 1948), therefore it maybe hypothesised that the disruption of the gut flora may impact the haematopoietic system as described by Peled et al (Peled et al. 2016) but the exact mechanisms still remain unclear on how these side effects arise. More importantly, in our studies, as previously mentioned, mice were injected with Phthalylsulfathiazole intraperitoneally and thus bypassing the gut. However the compound is still absorbed into the blood as compounds administered intraperitoneally are absorbed by mesenteric vessels (similarly to that of oral administration) (Turner et al. 2011). Although this may suggest that this provides a direct route to haematopoietic sites, it shows that Phthalylsulfathiazole is not toxic. In competitive transplant experiments, we found that haematopoietic reconstitution in recipients transplanted with Phthalylsulfathiazole-treated cells did not differ from recipients transplanted with vehicle cells. This was also the case in our parallel experiment where we studied the effects of Phthalylsulfathiazole treatment following bone marrow treatment. This demonstrates that Phthalylsulfathiazole does not have an impact on the function of HSPCs. Our data from the secondary transplant experiment also shows that Phthalylsulfathiazole does not impact on the self-renewal abilities of long term HSCs when treated after transplant. Analysis of long term HSCs in secondary recipients is still under investigations in the attempt to observe the impacts of Phthalylsulfathiazole on HSC self-renewal.

Finally we assessed the impact of Phthalylsulfathiazole on cancer and cancer stem cells. Initially we investigated the effects of Phthalylsulfathiazole on leukaemic cells *in vitro*. We found that at higher concentrations, there were a significantly higher number of blast cells of the NB4 and K562 cell lines in response to Phthalylsulfathiazole. We know that the NB4 cell line is derived from an acute promyelocytic leukaemia (APL) and that APL can be treated in a differentiation driven therapy with the use of retinoic acid (Zhou et al. 2007). We therefore investigated whether Phthalylsulfathiazole could exert similar effects in an AML setting *in vivo*. By treating mice induced with MLL-AF9 driven leukaemia with Phthalylsulfathiazole, we in fact observed an accelerated progression of the disease in treated animals followed by an increased rate of death. This was also mirrored by an increased frequency of proliferating LSCs in Phthalylsulfathiazole treated animals. This raises the prospect that if Phthalylsulfathiazole is able to drive otherwise quiescent LSCs into proliferation, which would

render them more sensitive to current chemotherapeutics that target dividing cells. In addition to this, we saw that there was a lack of side effects observed in historical studies in response to Phthalylsulfathiazole suggesting that normal HSCs were unaffected. This raises the idea that Phthalylsulfathiazole can be used to specifically target LSCs without impacting normal HSCs. To further elucidate the mechanism of disease progression in response to Phthalylsulfathiazole, the downstream targets of the compound must be studied. It is understood that Phthalylsulfathiazole inhibits folic acid synthesis which has a role in DNA synthesis and methylation (Crider et al. 2012). We, however, did not observe any effects of Phthalylsulfathiazole on normal HSCs suggesting that the Phthalylsulfathiazole-mediated disruption of folic acid synthesis is not sufficient to elicit a change in behaviour in healthy HSCs. It may also indicate that other mechanisms exist that are able to compensate for the disruption of DNA methylation induced by folic acid inhibition under homeostasis. On the other hand in the leukaemic setting, it has been shown that a number of molecular subtypes of AML demonstrate aberrant DNA methylation (Schoofs et al. 2014). Interestingly this is also seen in MLL- positive AML patients (Alvarez et al. 2010). With this understanding, it may be postulated that Phthalylsulfathiazole may induce further changes to DNA in LSCs thereby altering their behaviour such as promoting their proliferation.

From this investigation of Phthalylsulfathiazole, we found that this compound does not affect the function of HSCs under homeostatic and transplant settings. We also focussed on the action of Phthalylsulfathiazole in the disease setting in AML, where we hypothesised that it could target leukaemic cells. We discovered that in fact it has the opposite effect where disease progression was greatly enhanced due to an increase in LSCs. Further studies are required to determine whether coupling current chemotherapies with Phthalylsulfathiazole treatment could lead to more efficient treatments in AML

		HSC (LSK)	Progenitors (LK)	Lin$^+$			
				B-cells	T-Cells	Erythroid	Myeloid
Bone Marrow		-	↓ CMP	-	-	-	↑ Gr1
Blood		-	-	-	-	-	-
Competitor Transplant	Primary	-	-	-	-	-	-
	Secondary	-	-	-	-	-	-
Treatment following BMT	Primary	-	-	-	-	-	-
	Secondary	-	-	-	-	-	-
Leukaemic setting	*In vitro*	↑ (mild) AML cell line viability					
	In vivo	↑ GFP$^+$ leukaemic cells ↑ LSC frequency ↓ Survival					

Table 5.1 Summary of the impact of Phthalylsulfathiazole *in vitro* and *in vivo*

Phthalylsulfathiazole was initially studied on whole bone marrow *in vitro*. Since the *in vitro* conditions did not mimic the normal physiological bone marrow environment, *in vivo* assessment of Phthalylsulfathiazole was conducted to assess its impact on HSPCs. Further functional assessment of HSPCs in response to Yohimbine treatment was undertaken in transplant studies. In parallel, the effects of Phthalylsulfathiazole were also investigated in the disease setting using AML cell lines and mouse models of leukaemia.

Chapter 6: General Discussion

6.1 General discussion

Using high-throughput assays in Drosophila and zebra fish, we screened a library of small molecules to identify stem cell active compounds. Initially, 6 lead compounds were identified and from these two were discovered by independent laboratories to target leukaemic stem cells (Sassano et al. 2007; Sachlos et al. 2012; Hartwell et al. 2013). These findings validate our overall screening approach to identify novel compounds targeting stem cell activity. In this thesis, we characterised the impact of three lead small molecules (Yohimbine, Oxa-22 and Phthalylsulfathiazole) on mouse haematopoietic stem cell and leukaemic stem cell behaviour.

6.2 The impact of small molecules on HSC function *in vitro* and *in vivo*

Our first approach was to assess the impact of the small molecules on mouse HSCs *in vitro*. We directed our interest to Yohimbine and Oxa-22 and their impact on bone marrow cells ex vivo, which was inspired by the hitherto lack of success in current HSC expansion methods. We firstly asked whether these compounds could affect HSC behaviour. From our investigation of the two compounds, Yohimbine and Oxa-22, we found that HSC activity was unaltered in response to drug treatment as judged by immunophenotyping and also shown by functional colony forming assays after ex vivo treatment. Understanding the importance of the bone marrow niche and its regulation of HSCs *in vivo*, we thus sought to investigate the effects of the compounds *in vivo* under homeostatic conditions. Firstly, we found that HSPCs expressed target receptors for Yohimbine and Oxa-22 (α-2 adrenergic and M3 muscarinic receptors respectively). These receptors were also found to be expressed on the MSC population, a critical player within the bone marrow niche (Frenette et al. 2013). It has already been described that HSCs can be influenced, either directly or indirectly through the niche, by adrenergic and cholinergic innervation (Katayama et al. 2006; Mendez-Ferrer et al. 2008; Eimar et al. 2013). We found that administration of Yohimbine and Oxa-22 in wild-type animals elicited an expansion of HSCs within the bone marrow. In contrast, Phthalylsulfathiazole administration *in vivo* did not elicit changes in HSC frequency. We also sought to investigate whether these treated HSCs were altered in function, as judged by competitive transplant experiments. We found that Yohimbine

treated cells enhanced B cell reconstitution whereas Oxa-22 treated donor cells showed a preferential bias for T cell reconstitution. As seen under normal conditions, Phthalylsulfathiazole treated cells did not impact on haematopoietic reconstitution. In parallel, we investigated the impact of the small molecules on haematopoietic reconstitution after bone marrow transplant to simulate the clinical setting. We pursued these experiments in an attempt to enhance transplantation where HSC/HPCs need to engraft rapidly in order to circumvent infection, a major contributor to morbidity after bone marrow transplant (Pandey et al. 2014). We found that mice treated with Yohimbine after bone marrow transplant exhibited a sharp, but early (after 2 weeks), increase in T cells compared to control mice. Also erythrocytic recovery was enhanced in Yohimbine treated animals. Interestingly, this increase in erythrocytic recovery was also observed in Oxa-22 treated animals following transplant.

6.3 Ex vivo HSC expansion

Although recent advances in allogeneic transplant therapies have been made, such as better supportive care and improved control of complications after transplantation, donor shortage has proved to be a major limitation. For example, HLA-mismatching has prevented over 40% of North American patients from receiving urgent HSC transplant and only 20-45% of African American patients will find an unrelated HLA-matched donor (Park et al. 2015). This of course has led to other sources of HSCs to be explored and the identification of umbilical cord blood (CB) has been a momentous discovery. CB is a rich source of HSCs and has a number of advantages over adult HSC donors such as possessing a lower requirement for HLA matching and reduced incidence of GVHD. However, one unit of CB does not meet the minimum cell dose required for successful transplantation ($2.5x10^7$ nucleated cells/kg body weight (Scaradavou et al. 2013)). In fact, the low cell dose results in the failure of successful engraftment, or delayed engraftment, which ultimately leads to an increased risk of infection. To overcome this issue, a number of studies have utilised the simple approach of combining two CB units for transplant (Barker et al. 2005; Oran and Shpall 2012; Scaradavou et al. 2013). However for this to be a feasible transplant strategy the access to CB has to be readily available and over the past 20 years this has become the case. Currently there are approximately 167

CB banks worldwide, 142 of which are public (McKenna and Sheth 2011). Nonetheless, with the growing number of CB banks comes the need for laws and legislations to regulate the use of CB in therapy. These exist to protect the quality of preserved tissues and also parties involved such as the donor (confidentiality). This also raises a number of socio-ethical issues in regards to obtaining and storing CB. For example within natives of Indonesia, CB is preserved as it is considered "the home of the soul" and that it is "where the soul finds refuge after death" (Petrini 2010). With this belief, Cordlife Indonesia established its first private CB bank in 2007. India, where there is the highest birth rate (per woman) in the world, could potentially position itself as one of the largest collector of CB. However, due to the high costs and the lack of 'functional' public banks (dominated by private banks), CB distribution and treatment has not been made affordable or easily accessed.

To overcome the above issues, but more importantly to enhance CB cell dose, recent studies have looked at expanding HSCs ex vivo. A wide range of approaches have been undertaken to amplify HSCs using a wide array of protocols using co-culture methods, cytokine cocktails and small molecules. Most recent studies have compared the efficiency of expanded and non-expanded HSCs in haematopoietic recovery. In fact, it has been demonstrated that co-transplant of expanded and non-expanded units provide the best solution for overcoming delayed engraftment. This was shown by Delaney et al where they showed that notch-1 mediated expansion of $CD34^+$ HSCs resulted in rapid myeloid reconstitution after transplant (Delaney et al. 2010). Moreover, later myeloid reconstitution was found to be a result of the non-manipulated CB unit. More recently, it was also shown that HSCs expanded in co-culture with mesenchymal stem cells (MSCs) resulted in successful short-term neutrophil and platelet reconstitution however engraftment was improved with un-expanded HSCs (de Lima et al. 2012).

In our studies, we harvested bone marrow derived HSCs from adult (8-10 week old) mice with the caveat that these differ functionally from younger (foetal or post-natal) HSCs (Babovic and Eaves 2014). The Zon laboratory have demonstrated, with the use of small molecules, how it is possible to target developing (embryonic) and adult HSCs (North et al. 2007). Here, they identified a compound (PGE2) that elicited altered HSC behaviour within the zebra fish embryo. Following this step, further

assessment of PGE2 on HSCs in the adult zebra fish was conducted where they demonstrated improved haematopoietic reconstitution after irradiation. In the mammalian setting, they studied the effects of PGE2 on the differentiation of murine embryonic stem cells and found that it resulted in an expansion of MPPs. They also found that ex vivo exposure of PGE2 increased spleen colony-forming units and resulted in an *in vivo* expansion of bone marrow HSCs in competitive transplantation in adult mice (North et al. 2007). This study serves as a gold standard in HSC expansion approaches using small molecules. It also demonstrates the principle of screening compounds in different HSC settings (embryonic and adult). With this, we assessed the impacts of Yohimbine and Oxa-22 on adult bone marrow cells. As previously described, we used cytokine-free conditions, however by observing the preceding studies, the effects of the compounds could have been assessed in established conditions that maintain HSCs so that we can observe the impacts on HSC expansion and/or maintenance. In addition to this, our study looked at whole bone marrow exposure to the compounds rather than specific HSPC populations; the latter approach would minimise the metabolic uptake of the compounds by the surrounding haematopoietic cells which may be masking the effects of the compounds on HSCs. Furthermore, transplantation of these drug-treated HSCs will give the best indication of their functionality. Finally, co-culture of HSPCs with MSCs may have been an alternative approach to study the effects of the small molecules (Robinson et al. 2006; de Lima et al. 2012). Concurring with the literature (Pereira et al. 2003; Muthu et al. 2007), we found that HSPCs and MSCs express target receptors for Yohimbine and Oxa-22

6.4 Neural regulation of HSCs and the marrow niche

We found that treatment of Yohimbine or Oxa-22 in wild-type animals elicited a 2 and 1.5 fold increase in bone marrow HSCs respectively. We also found that cells treated with Yohimbine resulted in enhanced lymphoid and erythrocytic reconstitution when competitively transplanted. In parallel we observed an improved T cell reconstitution from Oxa-22 treated cells *in vivo*. These observations strengthened the hypothesis that HSCs and the microenvironment in which they reside in are modulated by neuronal innervation. It also shows that factors, such as small molecules, can impact

the HSC function directly or indirectly through the niche, though the exact actions of the molecules have yet to be established. As previously discussed, it is widely recognised that the bone marrow receives neural innervation from the sympathetic and the cholinergic nervous system (Artico et al. 2002; Cosentino et al. 2015; Hanoun et al. 2015; Jung et al. 2017; Pierce et al. 2017). Yohimbine (α2-adrenergic receptor (Adra-A2) antagonist) and Oxa-22 (M3 muscarinic acetylcholine receptor (mAChR) agonist) target the above neuronal signalling pathways. Using Yohimbine and Oxa-22 will therefore provide a useful tool to further investigate and dissect the impact of the sympathetic or cholinergic innervation of HSCs in the niche.

6.4.1 Adrenergic signalling modulates HSCs under homeostasis and transplantation

Adrenergic modulation of HSCs and haematopoiesis was first described in the early 90's by Maestroni et al (Maestroni et al. 1992). Here they showed that ablation of sympathetic fibres by 6-hydroxydopamine (6-OHDA)-induced sympathectomy, results in the enhanced production of leukocytes after bone marrow transplant. They also showed that the α1 antagonist prazosin (and Yohimbine to a lesser extent) could elicit the same results (Maestroni et al. 1992). Moreover, these results were reproduced in mice under normal conditions where increased myelopoiesis and platelet formation were observed in response to prazosin and the use of agonists reversed these effects (Maestroni and Conti 1994). Interestingly in this thesis, Yohimbine treated mice (under steady-state conditions), in addition to the HSC expansion, we found that myeloid ($Mac1^+/Gr1^+$) cells were depleted in the bone marrow but were significantly higher in the peripheral blood coinciding with the observations of Maestroni et al. In the transplant setting, we also found that Yohimbine promoted erythrocyte and B cell reconstitution without affecting myeloid reconstitution. The exact mechanisms of how Yohimbine causes these effects are unclear however we can postulate that Yohimbine may be exerting its effects through the common lymphoid progenitor (CLPs) (Figure 6.1). Although we did not investigate this in our primary transplant studies, we did observe a 1.8 fold increase in CLPs in Yohimbine treated wild-type mice. Interestingly under normal conditions, *in vivo* treatment of Yohimbine elicited an increase in circulating T cells as opposed to the increase in circulating B cells within the transplant setting. This may suggest that the role of adrenergic signalling is altered under

stress conditions in order to elicit the relevant response. For example, it has been shown that the release of pro- and anti-inflammatory cytokines, such as IL-1, IL-4, IL-5 and IL-6 occurs after irradiation (Lu-Hesselmann et al. 1997; Han et al. 2006; Schaue et al. 2012; Di Maggio et al. 2015). It has also been suggested that cells of the immune system are regulated by neuronal innervation (Hanoun et al. 2015). Interestingly, neuronal-mediated immune responses have been found to be mediated by alpha (α1) - and beta (β2)-adrenergic receptors (Lomsadze et al. 2013; Pongratz and Straub 2013; Huang et al. 2015). Proimflammatory responses were found to be mediated by the α-adrenergic receptors whereas β-adrenergic receptors mediate anti-inflammatory responses (Pongratz and Straub 2013). The role of the α2 receptor has not been studied extensively in lymphopoiesis, however it is clear that a number of adrenergic receptors are involved in regulating different aspects of the immune system. This may provide an explanation of how Yohimbine elicits different responses in lymphopoiesis under normal and transplant conditions.

The expression of the α2 receptor on CLPs has also previously been reported which may indicate that CLPs may be under adrenergic modulation (Muthu et al. 2007). In addition to this, indirect stimulation of CLPs may also explain the observations in our study. It was shown that osteoblasts can regulate and support the differentiation of CLPs into B-cell precursors (Zhu et al. 2007). In support of this, it was further determined that early CLPs resided in the endosteal niche exemplifying the importance of osteoblast-CLP interactions (Ding and Morrison 2013). Additionally, although the expression of the α2 receptor has been described in HSPCs, very little evidence shows that they are directly stimulated. In fact it has been shown that cytokine responses mediated by activation of adrenergic receptors were only elicited by stimulation of α1- and β2 adrenergic receptors, not the α2, suggesting that the receptor is weak/non-functional (Muthu et al. 2007). Although here they explain the lack of response by activation, Yohimbine is in fact an antagonist of the receptor and so it acts to inhibit the downstream signalling cascade of the α2 receptor-mediated pathway. Therefore further elucidation to the exact inhibitory mechanisms of Yohimbine is required and its effects on direct modulation of HSCs. We thereby hypothesise that adrenergic signalling plays an important role in modulating HSCs, as well as myeloid and lymphoid progenitors, directly and indirectly through the

bone marrow niche. Additionally, with the use of small molecules such as Yohimbine, it may be possible to enhance HSC numbers and improve lineage specific reconstitution. This could prove to be useful in the clinic in aiding haematopoietic recovery and minimising severe risks of infection.

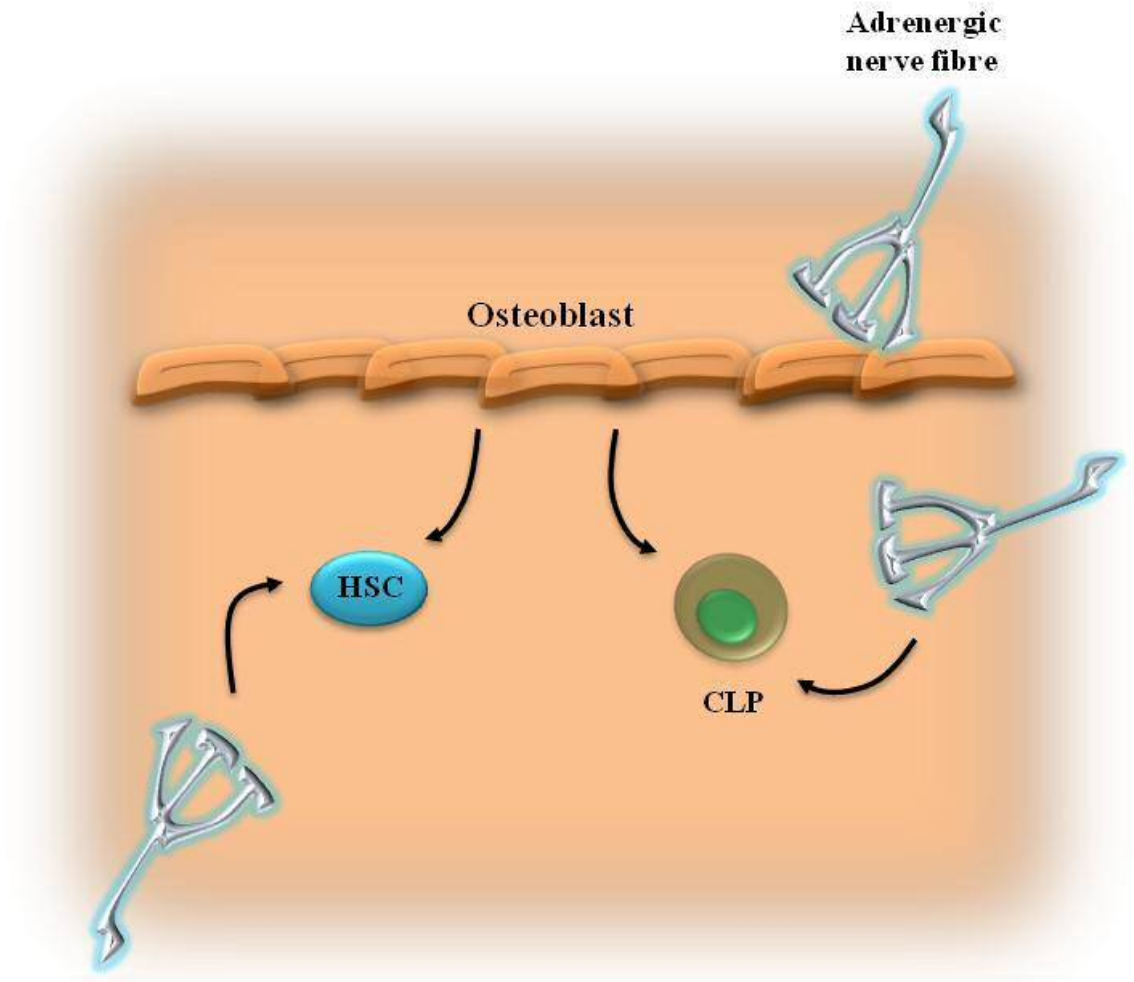


Figure 6. 1 Adrenergic modulation of HSCs and CLPs

Adrenergic innervation of the osteoblastic niche elicits multiple responses. Osteoblast and direct adrenergic signalling impacts on CLP behaviour. It is unclear how adrenergic signalling directly impacts on HSC, however evidence implicates that adrenergic stimulation in turn modulates HSC function.

6.4.2 Cholinergic innervation of the bone marrow niche expands HSCs *in vivo*

In addition to adrenergic innervation, cholinergic innervation has also been described in the modulation of HSCs and the bone marrow niche (Artico et al. 2002; Eimar et al. 2013; Pierce et al. 2017). Our gene expression analysis shows that HSPCs express the M3 mAChR as well as MSCs. Although the expression of the receptor has been shown in bone marrow mononuclear cells (Pereira et al. 2003), there is very little existing evidence for its expression on HSPCs. Conversely, a number of studies have showed that the surrounding cells of the niche derived from MSCs express the M3 receptor and are under the influence of cholinergic signalling (Liu et al. 2011; Tang et al. 2012; Eimar et al. 2013). For example, the loss of the M3 receptor resulted in a reduced number of MSC- derived osteoblasts (Shi et al. 2010), whereas osteoblast proliferation can be induced in response to muscarinic activation (Sato et al. 2010). We therefore speculate that Oxa-22 mediated HSC expansion in wild-type animals results from activation of osteoblasts. The mechanisms for this are unclear however this hypothesis can be strongly supported by the study of Calvi et al where they showed that stimulation of parathyroid hormone receptors (PPRs) on osteoblasts stimulates their proliferation (Calvi et al. 2003). It was also demonstrated that the increased number of osteoblasts produced elevated levels of Jagged 1 (Notch ligand) which in turn promotes the increase in HSC numbers (Calvi et al. 2003). In addition to Notch signalling, other osteoblast-HSC interactions may also be involved in supporting the increase in HSCs. For example, it has been suggested that osteoblasts support HSCs through the interaction of the adhesive molecules N-cadherin (expressed on osteoblastic cells) and β-catenin (Zhang et al. 2003). In this study, they too implicate that the expansion of osteoblasts results in an increase in HSCs within the bone marrow niche. These demonstrate the importance of the osteoblastic environment for the maintenance of HSCs. More importantly, these studies support the notion that the increased number of osteoblasts, potentially induced by cholinergic signalling through the activation of M3 receptors, result in increased HSC numbers (Figure 6.2).

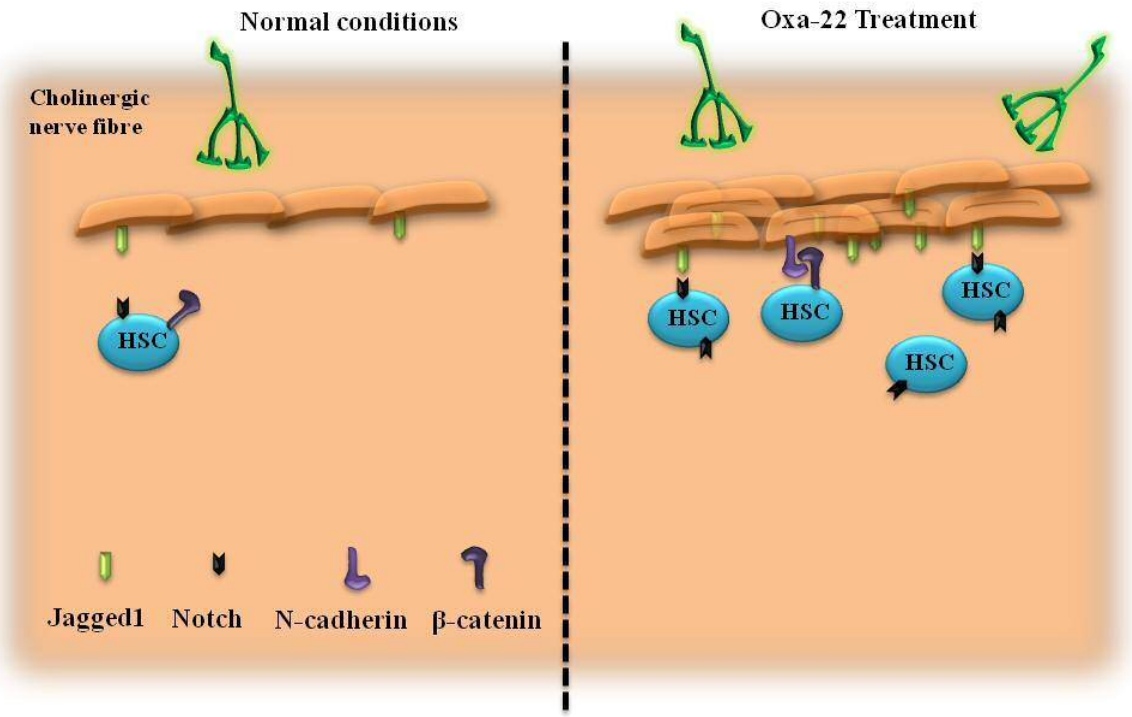


Figure 6. 2 Cholinergic stimulation of the bone marrow niche

We hypothesise that upon treatment of Oxa-22, the activation of M3 muscarinic receptors induces an increase in osteoblast numbers. This in turn increases the number of signalling molecules that stimulates HSC proliferation and maintenance.

6.5 Expanding the use of bone marrow transplant therapy in immunological disorders

In this thesis, we explored using the small molecules to expand HSCs and HPCs *in vivo* and improving haematopoietic reconstitution after bone transplantation therapy. Ironically, bone marrow transplant, a therapy used to save lives, was motivated by the devastation of the atomic bomb in the Second World War which killed hundreds of thousands of people and had lasting adverse effects for decades. This prompted researchers to elucidate ways of restoring bone marrow function after irradiation. Studies in the 50's determined that radiation-induced aplasia can be rescued by bone marrow transplant (Rekers et al. 1950). Moreover, leukaemic mice could be irradiated as therapy then undergo bone marrow transplant in order to overcome bone marrow aplasia and ultimately recover the haematopoietic system (Barnes et al. 1956). A year later, the first allogeneic transplant was conducted by Thomas et al (Thomas et al. 1957). Although 2 out of six patients demonstrated engraftment, all died within 100 days due to HLA-mismatching between donor and recipient. Further characterisation of human leukocyte antigens (HLA) allowed for better recipient-donor matching and the development of successful bone marrow transplants (Thomas et al. 1979).

Bone marrow transplants have been predominantly used in haematopoietic malignancies (such as acute myeloid leukemia (AML)), myelodysplastic syndromes and bone marrow failure syndromes (Henig and Zuckerman 2014). Interestingly, due to the haematopoietic 'restoration' nature of bone marrow transplant, it has also been utilised in autoimmune diseases such as multiple sclerosis and HIV (Krishnan 2009; Radaelli et al. 2014). Bone marrow transplant has also been used in therapies for Crohn's disease and Type 1 Diabetes (Li and Ikehara 2014; Duran and Hommes 2016). Stem cell therapies in these contexts are based on the premise that transplanted HSCs give rise to normal immune cells (e.g. T and B cells) thereby resetting the immune system and restoring immune tolerance (Potter et al. 1999; Ramaswamy et al. 2016). We observed that HSCs treated with Yohimbine or Oxa-22 resulted in enhanced lymphopoiesis after transplant. These small molecules could therefore potentially be exploited in settings where restoring normal immunity is essential for cure of disease.

6.6 Targeting Leukaemic Stem Cells

AML patients have poor outcomes with only 40-50% of long term survival in younger patients, and less than one year for older patients (Pollyea et al. 2014). It is also appreciated that standard remission therapies such as infusional cytarabine and anthracycline have not provided further durable remissions (Stein and Tallman 2012). The main reason for the lack of success in treating AML is hypothesised to be due to the presence of therapy resistant cancer stem cells - LSCs. LSCs share many properties with normal HSCs such as having the ability to self-renew, give rise to immature progeny and more significantly remain in quiescence (Reya et al. 2001; Tan et al. 2006; Dick 2008). These properties allow them to maintain the bulk AML population and also evade standard chemotherapy making them the cause of relapse (Costea et al. 2006). In our investigation into targeting LSCs, we identified Oxa-22 and Phthalylsulfathiazole as interesting potential candidate small molecules. Firstly, cholinergic signalling through the M3 receptor has been observed in solid tumour types as well as T-cell leukaemia (Boss et al. 2005; Alea et al. 2011; Zhao et al. 2014). Secondly, it has come to light that antimicrobial agents also possess anti-cancer/leukaemic properties (Casini et al. 2002; Scozzafava et al. 2003; Han et al. 2017). We therefore investigated the effects of Oxa-22 and Phthalylsulfathiazole in the MLL-AF9 mouse leukaemia model. The MLL-AF9 fusion gene originates from the t(9; 11) translocation (Tkachuk et al. 1992) and it is understood that the arising AML phenotype is of an aggressive nature due to its poor prognosis and chemo-resistive potential in MLL-AF9 patients (Schoch et al. 2003; Zuber et al. 2009). We utilised this model as HPCs, more specifically GMPs, (Lin- $Sca1^{+}cKit^{+}$ $CD34^{+}$ $CD16/32^{+}$) expressing the MLL-AF9/GFP fusion gene possess the ability to undergo limitless expansion, referred to as LSCs (Krivtsov et al. 2006). These LSCs were primarily expanded ex vivo in colony assays to determine blast colony forming potential and allowed for their selection as they have increased blast forming capabilities. As expected, mice transplanted with ex vivo expanded MLL-AF9 LSCs developed leukaemia. Further isolation and transplantation of $cKit^{+}CD34^{+}CD16/32^{+}$ GFP^{+} LSCs from primary leukaemic mice resulted in the development of a phenotypically identical AML in secondary recipients (Krivtsov et al. 2006). Thus, the MLL-AF9

leukaemic mouse provides a suitable model for LSCs *in vivo*, thus allowing for the assessment of the impact of small molecules on cancer and cancer stem cells.

6.6.1 Small molecules combined with chemotherapy to target LSCs

In the cholinergic setting it was understood that M3 receptor signalling is associated with tumorigenesis and indeed we did observe in an accelerated progression of disease in Oxa-22 treated animals. Unexpectedly, we observed the same result in Phthalylsulfathiazole treated leukaemic mice. However in these mice, not only was disease progression significantly accelerated but also higher frequencies of LSCs were detected. Phthalylsulfathiazole treated mice exhibited a higher percentage of proliferating LSCs. This is a significant finding as this demonstrates the transition of LSCs to a more active proliferative state, thus potentially making them easily targeted by standard chemotherapies, which require active proliferation to kill cancer cells. Current approaches are being undertaken (reviewed by (Trumpp et al. 2010)) to test this hypothesis. For example, the use of the mobilising agent G-CSF has been described to stimulate LSCs to enter the cell cycle and further treatment with chemotherapy successfully induced apoptosis and the eradication of LSCs *in vivo* (Saito et al. 2010). Interestingly, when coupled with chemotherapy, G-CSF treated LSCs were unable to propagate disease in secondary recipients demonstrating the loss of self-renewal capabilities (Saito et al. 2010). Arsenic trioxide was also found to drive LSCs out of quiescence and coupled with Ara-C eradicated LSCs (Ito et al. 2008). From this, it is now also possible to successfully treat PML–retinoic acid receptor-α (RARα) induced acute promyelocytic leukaemia (Nasr et al. 2008; Nasr et al. 2009). These are examples of stimulating dormant, and otherwise chemo-resistant, LSCs making them sensitive to chemo- or differentiation therapy. Future studies are therefore warranted to examine whether Phthalylsulfathiazole and Oxa-22 administration, when coupled with standard chemotherapies, could eradicate LSCs (Figure 6.3).

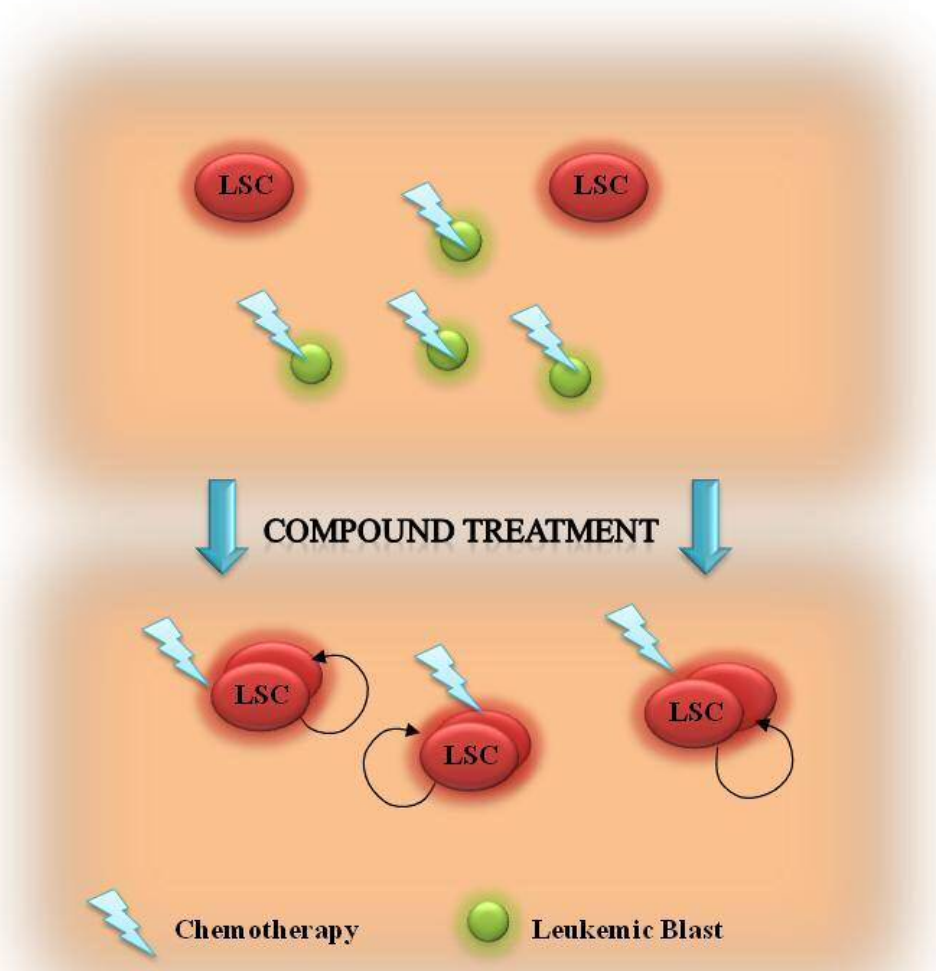


Figure 6. 3 Compound treatment coupled with standard chemotherapy

Dormant LSCs are resistant to standard chemotherapies resulting in disease relapse. Upon compound treatment (Phthalylsulfathiazole), LSCs are pushed into the cell cycle. These dividing cells can then be targeted with standard chemotherapy.

6.6.2 Targeting cancer stem cells with antibacterial agents

As mentioned above, new studies have assessed the potential of antibiotics and antibacterial agents in anti-cancer therapies. Early studies have demonstrated the anti-cancer properties of sulphonamides in a number of tumours (Casini et al. 2002; Scozzafava et al. 2003). These studies show that sulphonamides induce G1 arrest and thus prevent entry to the cell cycle and inhibiting mitosis. Another study also showed that a number of sulphonamide drugs inhibited mitosis by disrupting microtubule assembly (Mohan et al. 2006). They also showed that these drugs induced apoptosis in cancer cells through the phosphorylation of Bcl-2 and thereby impacting on its pathway (Mohan et al. 2006). From these interesting observations, sulphonamides such as Indisulam, Tasisulam and CQS have been taken into phase I and phase II trials in patients with solid tumours (Dittrich et al. 2003; Haddad et al. 2004; Talbot et al. 2007; Han et al. 2017). Alternative antibacterial agents have been established as having anti-cancer properties and more specifically target cancer stem cells (CSCs). Salinomycin, an antibiotic, has been extensively studied in the cancer setting. In fact it has been shown by a number of studies that it is able to target and kill cancer stem cells (Gupta et al. 2009; Germain et al. 2012; Naujokat and Steinhart 2012; Mai et al. 2017). The exact mechanisms of Salinomycin-induced cell death are unclear, however a number of studies have attempted to explain the mode of action of the compound. For example, induction of apoptosis, inhibition of Wnt/β-catenin signalling and oxidative phosphorylation have all been observed in response to Salinomycin treatment (reviewed by (Naujokat and Steinhart 2012)). Furthermore, other antimicrobial agents have been described in the eradication of CSCs by targeting mitochondrial processes and inducing cell death (Lamb et al. 2015). Additionally, antibiotics can also be used to inhibit physical processes such as epithelial-to-mesenchymal transition and molecular pathways such as the inhibition of the stem cell transcription factor SOX2, thus inducing cell death in CSCs (Das et al. 2017; Zhang et al. 2017). These studies demonstrate that in addition to current cancer-targeting therapies, antibiotics and antibacterial agents are an interesting and potentially efficient alternative in cancer therapies.

6.7 Future directions

In this project, we focused on small molecules that target neuronal innervation of HSCs and the surrounding bone marrow niche under the normal steady state and the transplant setting. However, it would have been interesting to directly assess the effects of the compounds on the stem cell niche in these settings. One way of addressing this could be conducted in a niche autonomous manner. For example, wild type mice can be treated with the respective compounds (using the determined treatment regimens) and then undergo total body irradiation. This would ablate treated bone marrow HSCs and leave the remaining treated niche cells. Irradiated mice are then transplanted with untreated bone marrow cells and reconstitution of the haematopoietic system can be assessed under the influence of the treated niche. Alternatively, conditional knockout of the target receptors (α2-adrenergic and M3 muscarinic receptors), specifically in niche cells, with the use of the *Mx1-cre* and pIpC system for example, would eliminate molecule-niche interaction. Thus any results observed would possibly be due to direct molecule-HSC interaction. On the other hand, haematopoietic-specific knockout of these receptors (with the use of a Vav-Cre system) could eliminate direct stimulation of the compounds with HSCs thereby restricting their influence on the niche cells. Haematopoiesis under normal and transplant settings in knockout conditions would allow for the determination of specific HSC/niche-compound stimulation. Previous studies have also shown that molecules that regulate HSC and niche function also possess cytotoxic-protective properties (Porter et al. 2013). We therefore postulate whether Yohimbine or Oxa-22 could offer protection in response to cytotoxicity and radiation. There are two possible approaches to test this hypothesis. The first approach comprises of the animal being treated with the compound followed by administration of a cytotoxic compound (ara-C or 5-fu for example). HSPC populations can be assessed in treated animals compared to controls. The second approach could utilise total body irradiation where animals are treated with the respective compound followed by irradiation. Haematopoietic recovery and HSPC numbers can then be assessed between treated and non-treated animals. Alternatively, compound administration can proceed after cytotoxic treatment (or irradiation) to assess their abilities in aiding haematopoietic recovery.

In order to gain insights into the molecular mechanisms underpinning the small molecules biological function, we would need to perform global gene expression analysis. For example, expanded HSCs from our *in vivo* expansion studies would be isolated and undergo gene expression analysis to assess which genes are responsible for the modulation or enhancement of HSC fate programmes. Similarly, transplanted cells can also be harvested and isolated for gene expression analysis that would provide an insight to what lineage specific genes are activated or suppressed. Importantly, this can also be applied to drug treated LSCs to better understand the genetic and molecular mechanisms underlying quiescence and proliferation. In summary, gene expression analysis will offer insights into the possible mechanisms by which these small molecules may act biologically and therapeutically.

Finally, in our investigations of the effects of the Yohimbine on HSCs, we questioned whether they could elicit HSC mobilisation. G-CSF is commonly used as an agent for promoting the mobilisation of bone marrow HSCs to the peripheral blood (Lapid et al. 2008; Bendall and Bradstock 2014). However, G-CSF induced mobilisation is unsuccessful in 5-30% of healthy donors and 60% in patients with disease suggesting that alternative approaches need to be explored (Demirer et al. 1996; Morgan et al. 2004; Gertz 2010). From our studies, we found that Yohimbine did not elicit HSC mobilisation. However we now ask whether, after administration of Yohimbine, current mobilizing agents can elicit the egression of expanded HSCs from the bone marrow into the peripheral blood.

7. Conclusion

In this thesis, we identified small molecules that have an impact on HSCs under homeostasis and in transplantation. In parallel, due to the properties shared by HSCs and malignant LSCs, we hypothesised that LSCs could be targeted with small molecules. We found that Yohimbine and Oxa-22 elicited *in vivo* expansion of HSCs. This could prove to be a significant observation as HSC expansion has become a major aspect of sourcing HSCs for transplant. It is therefore imperative to assess these compounds comprehensively on human cord blood HSCs in the attempt to expand them for transplantation therapies. We also observed improved B cell and erythrocytic reconstitution in Yohimbine treated cells when competitively transplanted. In addition, we observed improved T cell recovery when Yohimbine was administered after bone marrow transplant. These findings can play a major role in the fight against infection, which is one of the major causes of death, during haematopoietic recovery after transplant. Finally we assessed the impact of Oxa-22 and Phthalylsulfathiazole in the disease setting where we observed a significant enhancement in disease progression in treated animals. This was found to be as a result of increased cycling LSCs in the case of Phthalylsulfathiazole treated animals. We therefore postulate that by stimulating these quiescent LSCs into the cell cycle, they could essentially become more sensitive to standard chemotherapy. In conclusion our results have shown that normal HSCs can be directly, or indirectly, influenced by neuronal innervation with the use of small molecules. In addition to this, we have shown that small molecules can also target disease HSCs in the leukaemic setting. Further characterisation of these small molecules will provide better understanding of the mechanisms underlying HSC innervation and disease progression in AML.

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