Nuclear Factor κB transcriptional regulator function in haemopoietic stem cells

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ABSTRACT

The NF-κB family of transcription factors are essential for different stages of murine haemopoiesis as supported by studies on single or double knockout mice. The function of NF-κB1 and NF-κB2 in haemopoietic stem cells (HSCs) has never been described before. In mice, the bone marrow c-Kit^{+}Sca1^{+}Lin^{-} (KSL) population represent HSCs that have the ability to repopulate the bone marrow of lethally irradiated animals. Using knockout mice technology we were able to study the function of NF-κB1 and NF-κB2 in HSCs by performing amongst others transplantation assays, which is the most precise way to test HSC potential. *Nfκb1*$^{-/-}$ KSL cells are fully able to reconstitute lethally irradiated recipients indicating that the *in vivo* self-renewal capacity is unaffected. In contrast, colony assays showed NF-κB1 dependency. Upon serial replating, knockout cells were not able to form colonies and lost their *in vitro* self-renewal capacity. Transplantation of the *nfκb2*$^{-/-}$ KSL cells engraft with a proliferative phenotype and a competitive advantage, with changes in the B-cell and myeloid cell lineages. Similar to *nfκb1*$^{-/-}$, *nfκb2*$^{-/-}$ KSL cells lose their function as stem cells upon serial replating. These findings highlight the importance of NF-κB family proteins in HSCs, especially NF-κB2 and should be considered in the development of future cancer treatment that target NF-κB proteins.

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"The best and safest thing is to keep a balance in your life, acknowledge the great powers around us and in us. If you can do that, and live that way, you are really a wise man."

Euripides quotes (Greek playwright, c. 480-406 BC)

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And……

I ask forgiveness from my children for sacrificing my time away from them…..

…….I hope it has been worthwhile and justified.

DECLARATION

I hereby declare to the University of Birmingham that this thesis is my own work and effort and all results were obtained by myself with the exception of the following work:

1. Operation of the cell sorter was carried out partially by Roger Bird and partially by Dr Mary Clarke, Division of Immunity and Infection, The University of Birmingham.
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ABBREVIATIONS

-/-	Knockout
+/-	Heterozygous
ALDH	Aldehyde dehydrogenase
ALL	Acute lymphocytic leukaemia
AML	Acute myelogenous leukaemia
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cytochrome7
BAFF/-R	B-cell activating factor/-receptor
BM	Bone marrow
CAFC	Cobble-stone area forming cell
CaR	Calcium receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU-C	Colony-forming unit in culture assays
CFU-S	Colony-forming-unit spleen
CLL	Chronic lymphocytic leukaemia
CLP	Common lymphoid progenitor
CML	Chronic myelogenous leukaemia
CMP	Common myeloid progenitor
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
DC	Dendritic cells
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetracetic acid
EPO	Erythropoietin
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein-isothiocyanate
Flt3	FMS-like tyrosine
FSC	Forward scatter
GMP	Granulocyte/macrophage progenitor
Gy	Grays
HPRT	Hypoxanthine-guanine phosporibosyl transferase
HSC	Haemopoietic stem cell
Ig	Immunoglobulin
IKK	IκB kinase
IL-1	interleukin-1
IMDM	Isocove's Modified Dulbecco's medium
iPS	Induced pluripotent stem cells
IκB	Inhibitor of NF-κB
Kb	Kilobase pairs
KSL	c-Kit^{+}Sca1^{+}Lin^{-}
LSC	Leukaemic stem cell
LTC-IC	Long-term culture-initiating cell
LT-HSC	Long-term haemopoietic stem cells
LTβ	Lymphotoxin β
MACS	Magnetic cell separation
MM	Multiple myeloma

MP	Major population
MPP	Multipotent progenitor
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa B
NIK	NF-κB- inducing kinase
NK	Natural killer cells
NLS	Nuclear localisation signal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythin
PE-Cy5	Phycoerythin-Cytochrome5
RBC	Red blood cells
RHD	Rel homology domain
RNA	Ribonucleic acid
g	g force
SCF	Stem cell factor
SCF	Stem cell factor
SCID	Severe combined immunodeficient
SEM	Standard error of the mean
SLAM	Signalling lymphocyte activation molecule
SP	Side population
SRC	SCID-repopulating cell
SSC	Side scatter
ST-HSC	Short-term haemopoietic stem cells
TAE	Tris-acetic-EDTA
T-ALL	T-cell acute lymphoblastic leukaemia

Tie-2	angiopoietin-2 receptor
TNFα	Tumor necrosis factor alpha
TPO	Thrombopoietin
WT	Wild type

CHAPTER 1

INTRODUCTION

1.1 Stem cells

In the 19th century the term ‘stem cell’ was used to describe germ line cells that give rise to eggs and sperms. However, more recently stem cells have been defined as unspecialized cells that give rise to differentiated cells. In 1961 Till and McCulloch demonstrated that colonies of myeloid cells developing in the spleen were clonal following transplantation of bone marrow into lethally irradiated mice (Till and McCulloch, 1961).

Stem cells play an important role for the life of multi-cellular organisms as they are required during the development of the embryo and also throughout life for the maintenance of tissues and organs. Stem cells have two defining properties that distinguish them from other cells: the ability to differentiate into one or more cell types and a capacity to self-renew (i.e. to divide and produce another of itself so that it does not become depleted). The stem cell potency depends on the developmental stage, and is operationally defined by the number of distinct cell lineages they are able to give rise to. Totipotent stem cells are cells that after fertilization give rise to both the complete embryo and the extraembryonic tissues such as the placenta. Pluripotency is seen in embryonic stem cells (ESCs), which have an unlimited capacity for growth and can differentiate into all embryonic cell types. Adult stem cells have a more restricted potency and are associated with providing cells for tissues and organs they are part of. These cells are also referred to as tissue or somatic stem cells and may be multipotent (e.g. bone marrow derived stem cells that give rise to all cellular components of

the blood) or monopotent (such as those found in skeletal muscle which give rise to a single lineage).

A different category of stem cells can be created by so called ‘reprogramming’. In this process the gene expression pattern of differentiated cells of the adult body, such as skin fibroblasts, is changed artificially to recapitulate the earliest stage of development. In this way the reprogrammed stem cells acquire a pluripotency property like ESCs. There are different ways to achieve reprogramming, however the two essential approaches are ‘nuclear transfer’ and the generation of ‘induced pluripotent stem’ cells (iPS cells).

Nuclear transfer was achieved 50 years ago by John Gurdon, who inserted the nucleus of a differentiated skin cell into an enucleated egg and generated cloned frogs (Gurdon et al., 1958). This strategy was also used later when Dolly the sheep was cloned from the nucleus of a mammary gland epithelial cell (Campbell et al., 1996). In the cloning process there are proteins and probably other molecules in the cytoplasm of the oocyte that reset the developmental clock and thus revert the gene expression profile.

The second approach of reprogramming was achieved by Takahashi and Yamanaka in 2006, when they showed that expression of four transcription factors in differentiated murine somatic cells induced iPS cells with a pluripotency phenotype that is very similar to that of ESCs (Takahashi and Yamanaka, 2006). One year later iPS cells were also generated from adult human fibroblasts (Takahashi et al., 2007). The use of iPS cells solves many practical and ethical problems that apply to ESCs, as these are patient-specific, do not involve destruction of an embryo and are able to provide an unlimited supply of stem cells. However these cells have still some potential problems as they tend to keep some of the epigenetic

profiles of the adult cells from which they were derived, and this might affect the differentiation properties of the iPS cells with time.

1.2 The haemopoietic hierarchy

Haemopoiesis is defined as the cellular process by which the eight major lineages of blood cells are generated. These cells mature to functionally active cells and are often short-lived. Thus, haemopoiesis continues actively throughout the life of an individual. The haemopoietic system can be sub-divided into three compartments. The first compartment contains haemopoietic stem cells (HSCs), including long-term (LT) HSCs, which are able to self-renew for an unlimited time period, and short-term (ST) HSCs, which self-renew for a limited time period (Passegue et al., 2003). These later cells, known as 'KSL' cells in the mouse, are positive for the stem cell surface markers c-Kit (the receptor for stem cell factor, SCF) and Sca-1 (Okada et al., 1992), and negative for lineage markers which usually include B220, CD5, CD8, Gr1, CD11b (Mac-1) and Ter119. The ST-HSCs differentiate into multipotent progenitors (MPPs), which in turn differentiate into two lineage-restricted progenitors, common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLP give rise to T-lymphocytes, B-lymphocytes and natural killer (NK) cells while CMPs differentiate first into myelomonocytic (granulocyte/macrophage) progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). GMPs differentiate then into monocytes/macrophages and granulocytes whilst MEPs produce megakaryocytes/platelets and erythrocytes. CMPs as well as CLPs produce dendritic cells (Figure 1.1) (Passegue et al., 2003, Bonnet and Dick, 1997).

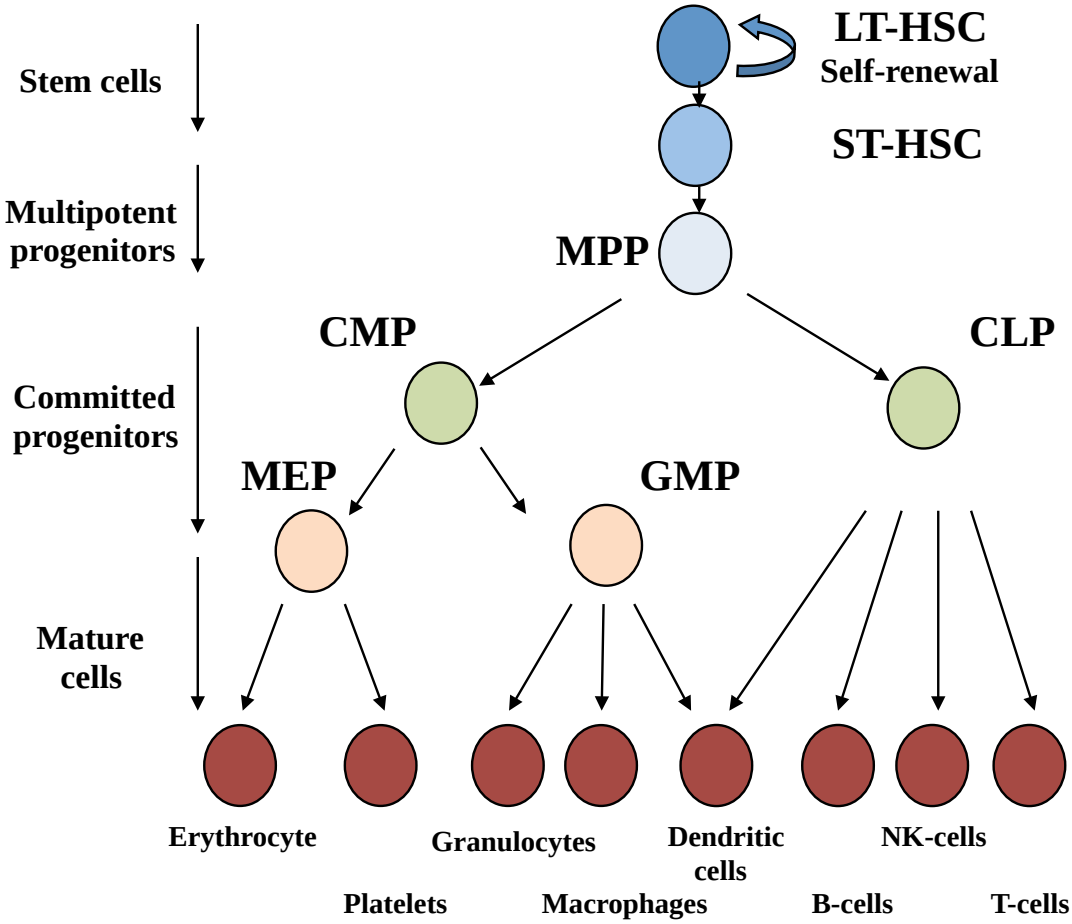

Figure 1.1: Schematic of the haemopoietic hierarchy. Model of the haemopoietic developmental hierarchy consisting of (i) Stem cells and multipotent progenitors: long-term haemopoietic stem cells (LT-HSC), short-term haemopoietic stem cells (ST-HSC), multipotent progenitor (MPP); (ii) Committed proliferating progenitors: common myeloid progenitor (CMP), common lymphoid progenitor (CLP), megakaryocyte / erythroid progenitor (MEP), granulocyte / macrophage progenitor (GMP); and (iii) Mature differentiated cells: erythrocyte, platelets, granulocytes, macrophages, dendritic cells, B- cells, natural killer cells (NK-cells) and T-cells.

1.3 Development of the haemopoietic system

Several anatomical parts of the foetus participate in foetal haemopoiesis. The location of haemopoiesis changes from one site to another as the embryo grows and its anatomy changes during organogenesis. In mice the earliest blood cells arise between embryonic day 7 and 8 (E7-E8) in the yolk sac (Moore and Metcalf, 1970, Haar and Ackerman, 1971). These cells are nucleated primitive erythroblasts that synthesise haemoglobin and enter the embryo when the circulation is established. At around E12 to E13 the foetal liver becomes the predominant haemopoietic organ where definitive haemopoiesis takes place and non-nucleated erythroblasts are formed (Barker, 1968). The liver displays continuous haemopoietic activity throughout development *in utero* and also several weeks postnatally (Wolf et al., 1995, Harrison et al., 1979, Kurata et al., 1998). By E16 to E18 the murine bone marrow compartments are the main and final sites in which haemopoiesis is initiated (Velardi and Cooper, 1984). Both the spleen and the bone marrow function as the primary organs of blood cell production throughout the life of the mouse.

1.4 Haemopoietic stem cell identification, isolation and characterisation

Different techniques have been developed to identify and isolate HSCs, which include enrichment (magnetic cell separation- MACS), single cell sorting (fluorescence-activated cell sorting-FACS) based on cell surface markers and vital dye staining (Challen et al., 2009).

1.4.1 Haemopoietic stem cell isolation using cell surface antigens

There is no single marker that separates HSCs from other haemopoietic cells. However, different antibody combinations have been established to purify these cells using multiparameter flow cytometry. Murine HSCs are a population of cells that phenotypically do not express surface markers characteristic of differentiated haemopoietic cells (Muller-Sieburg et al., 1986), which include CD5, B220, CD4, CD8, Gr1, CD11b (Mac-1) and Ter119, while they display high levels of Sca1 (Spangrude et al., 1988) and receptor tyrosine kinase c-Kit (Okada et al., 1992). Thus, these cells are c-Kit^{+}Sca1^{+}Lin^{-}, known as KSL-cells. However the KSL compartment is heterogeneous, containing a mixture of LT-HSCs, ST-HSCs and MPPs.

With the markers CD34 and Flt3 (also known as Flk-2or CD135) it is possible to further divide the KSL population into more homogeneous populations. LT-HSCs are the true transplantable HSCs and are negative for CD34 (KSL CD34^{-}) (Osawa et al., 1996). Addition of the Flt3 marker makes it possible to not only define the LT-HSC (KSL CD34^{-} Flt3^{-}) but also the ST-HSC population (KSL CD34^{+} Flt3^{-}) and MPP population (KSL CD34^{+} Flt3^{+}) within the KSL cells (Christensen and Weissman, 2001, Adolfsson et al., 2001).

In addition to Flt3 and CD34, angiopoietin-2 receptor (Tie-2) and the cell surface receptors of the signalling lymphocyte activation molecule family (SLAM), including CD150, CD244 and CD48, allow identification of more homogeneous populations within the KSL cells (Arai et al., 2004, Kiel et al., 2005). The HSCs were purified as CD150^{+}CD48^{-}CD244^{-} while MPPs were defined as CD150^{-}CD48^{-}CD244^{+} (Kiel et al., 2005). However it is still debatable if the HSC population isolated by the SLAM markers can be compared to the ones isolated by using the Sca1, c-Kit and lineage markers (Weksberg et al., 2008). Further it has been demonstrated

that HSCs expressing Tie-2 are the subpopulation that are more quiescent and so are enriched in the LT-HSCs. Thus, Tie-2 allows the isolation of the most immature HSCs (Arai et al., 2004).

1.4.2 Haemopoietic stem cell identification using DNA binding dye Hoechst 33342

In addition to surface markers, HSCs can be identified and isolated using the DNA binding dye Hoechst 33342. HSCs have the ability to efflux this fluorescent dye via membrane transport pumps called ABC transporters. These transmembrane proteins are present in many cell types with the function to remove different chemicals in the cytoplasm. ABC-G2 is one of the protein members present in stem cells, and removal of the Hoechst 33342 dye can be observed with the flow cytometer by measuring red and blue fluorescent light emission after stimulating with a UV laser. Stem cells show low red and blue fluorescence and appear as a so-called side population (SP) (Goodell et al., 1996). Bone marrow cells displaying SP characteristic have been shown to have the capability of long term repopulation in lethally irradiated recipients. Cells that lack the ability to expel the dye are called the major population (MP).

1.4.3 Human HSCs

The isolation and characterisation of long-term reconstituting human HSCs is less well-advanced than with the mouse HSCs and only 1 in 10^6 cells in the human bone marrow is a true transplantable HSC (Wang et al., 1997). In humans, there are two main sources for HSCs that are being applied for therapeutic purposes; the bone marrow and the umbilical cord. CD34 is the most convenient cell surface marker for human HSCs and its discovery has helped in the study of human haemopoietic development. This surface marker is expressed on

less than 5% of all blood cells and was the first marker found to enrich for human HSCs and progenitors (Civin et al., 1984). In the nineties, the group of Baum et al and others proved that the human Lin^- $CD34^+$ cells were able to engraft in severe combined immunodeficient (SCID) mice and develop towards multiple lineages (Hao et al., 1998, Miller et al., 1999, Baum et al., 1992). This assay measured the repopulating capacity of human stem cells. However, recent studies have shown that SCID-repopulating cell (SRC) activity resides in $CD34^-$ cells from human cord blood when transplanted using the intra-bone marrow injection method (Kimura et al., 2010). To further define the phenotype of cord-blood derived $CD34^-$ SRCs, Kimura et al suggested that the immunophenotype of very primitive long-term repopulating human HSCs is Lin^- $CD34^-$ $c\text{-}Kit^-$ $Flt3^-$. In addition Goodell et al have shown that $CD34^-$ cells exhibit side population characteristics like the mouse HSCs (Goodell et al., 1997), whereas McKenzie et al have identified long-term human HSCs within the Lin^- $CD34^+$ $CD38^-$ population characterized by low retention of rhodamine 123 dye (McKenzie et al., 2007). Furthermore Baum et al identified CD90 (Thy1) as a further stem cell marker (Baum et al., 1992), while further studies introduced CD45RA and CD38 as markers of more differentiated cells; cells negative for these two markers are enriched in HSCs (Bhatia et al., 1997, Conneally et al., 1997, Lansdorp et al., 1990). Therefore, over the past decade human HSCs have generally been defined as $CD34^+CD38^-Thy1^+CD45RA^-$. Further isolation of very immature human HSCs can be performed using flow cytometry to select for the expression levels of aldehyde dehydrogenase (ALDH), as it has been proven that haemopoietic progenitors exhibit high levels of this enzyme (Kastan et al., 1990). Another important cell surface antigen that is expressed on the surface of long-term repopulating human HSCs is CD133. This antigen has been shown to be expressed on several types of adult stem cells (Yin et al., 1997).

1.4.4 Bioassays of haemopoietic stem cells

Assays have been developed to characterise haemopoietic stem and progenitor cells *in vitro* and *in vivo*.

Short-term in vivo assays - The first *in vivo* assay to be developed for HSCs was the Spleen colony forming unit assay (CFU-S). James Till and Ernest McCulloch demonstrated that colonies of myeloid cells developed in the spleen after injecting bone marrow cells into lethally irradiated mice (Till and McCulloch, 1961). The CFU-S are therefore early engrafting cells, that allow the mouse to survive in the first 2-3 weeks post-transplantation when pancytopenia would otherwise occur following the irradiation. The advantage of this assay is that it is short-term; usually around day 12 colonies can be counted in the spleen.

Long-term in vivo assays - The gold standard assay that truly defines HSCs is the long-term reconstitution assay. Lethally irradiated mice are injected with either the HSCs being tested or whole bone marrow cells, which is a reference non-selected population. There are different types of long-term repopulation assays that measure the functional potential of HSC. The competitive repopulation assay was developed by Harrison et al (1980) and measures the functional potential of test HSCs against whole bone marrow cells from congenic wild type mice (Harrison et al., 1993). This study provides information about the function of the HSCs in their repopulation capacity compared to the competing bone marrow cells, but it does not provide any details about the progeny produced per HSC.

The limiting dilution assay, another type of competitive repopulation assay, determines the frequency of the HSCs through a series of dilutions of the donor test cells that are competed against bone marrow cells (Szilvassy et al., 1990).

The most important assay to test the lifespan and the expansion limits of HSCs is the serial transplantation assay. In this assay, HSCs are transplanted from the primary reconstituted recipient to a secondary irradiated host, thus demonstrating that engrafting cells are undergoing self-renewal and are able to sustain haemopoiesis. *In vivo* assays are important for identifying and quantifying stem cell potential but they have limitations, due to the length of time required to monitor the mice after the transplantation and the significant number of mice that is used for the experiments and therefore the costs.

Long-term in vitro assays - There are also a number of *in vitro* assays for measuring the frequency of progenitors and stem cells. These include long-term culture-initiating cell (LTC-IC), cobble-stone area forming cell (CAFC) and colony-forming unit in culture assays (CFU-C) (Dexter et al., 1977, Whitlock and Witte, 1982, Sutherland et al., 1990, Ploemacher et al., 1989).

In CFU-C, immature cells like HSCs are grown in rich semisolid methylcellulose-based culture media, which allow the cells to differentiate along different pathways to give rise to more mature cell types. The particular differentiation pathway depends on the presence of specific growth factors and nutrients within the culture conditions (Bradley and Metcalf, 1966, Moore et al., 1973). In CAFC and LTC-IC assays, HSCs are seeded and grown on a stromal layer, which mimics the natural HSC microenvironment. The first assay measures the frequency of cells that are able to grow under the stromal layer as CAFCs. The LTC-IC is very similar to the CAF-C assay, but the outcome is the presence of progenitor cells with colony-forming ability (Dexter et al., 1977).

1.5 Haemopoietic stem cell biology

1.5.1 Haemopoietic stem cell fate choices

Since Till and McCulloch proved the existence of HSCs in 1961, these have become the most extensively characterised stem cells. HSCs, like any other stem cells have the ability to self-renew symmetrically or asymmetrically. When a HSC divides asymmetrically one of the daughter cells is able to retain the stem cell fate while the other daughter cell can embark on the pathway of differentiation to give rise to mature blood cell types. In the case of symmetrical division, HSCs produce either two new stem cells or two differentiated cells. Self-renewal is an important property of HSCs, as sustaining these compartments is required for lifelong haemopoiesis. HSC division is under strict physiological control. For example, it is suggested that during embryonic development there is an increase in HSCs through symmetrical division, which expands the stem cell pool, while during adult tissue homeostasis asymmetrical division provides for self-renewal to sustain haemopoiesis and also differentiation to give rise to mature blood cells. In cases of haemopoietic stress such as haemorrhage, it is suggested that HSC undergo symmetric division favouring differentiation to replenish the mature blood cells.

In steady-state conditions, HSCs divide infrequently and usually do not enter the cell cycle. This non-dividing condition is called quiescence. Preserving HSCs in a quiescent state and inhibiting them from differentiation is important for HSC maintenance and survival. As this process suppresses cell division and therefore DNA replication, it also reduces the number of mutations that may occur during replication. However, even if DNA mutations do occur in the quiescent state, HSCs would have enough time to repair the DNA damage. Moreover

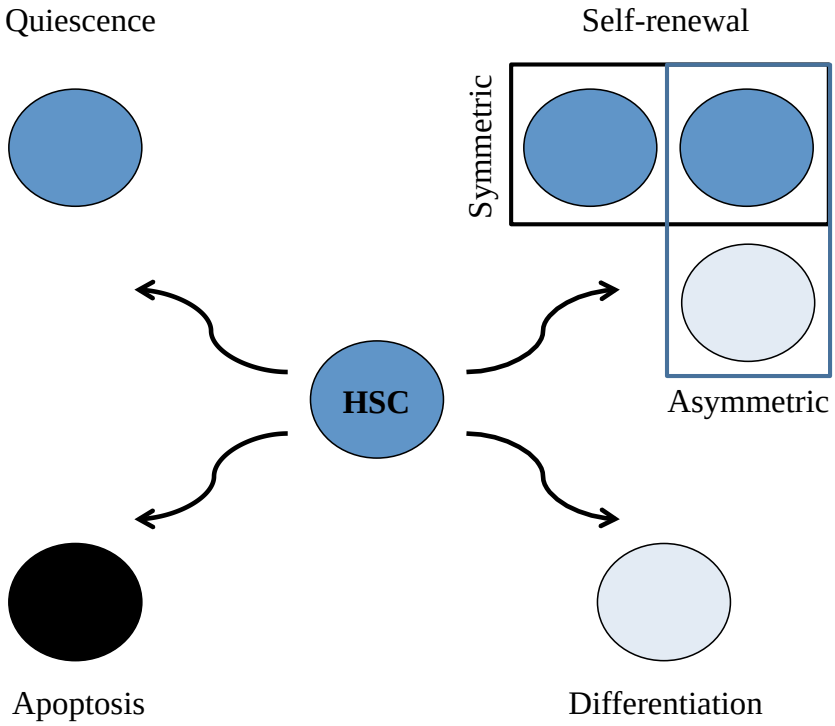

Figure 1.2: Haemopoietic stem cell fate choices. The figure shows the haemopoietic stem cell (HSC) fate decisions in the bone marrow. Self-renewed and quiescent HSCs are shown in blue, apoptotic HSCs are represented in black while committed cells are in light blue.

quiescence protects HSCs from toxic drugs. Quiescence of HSCs depends on the microenvironment within the bone marrow that is called the HSC niche.

Another mechanism that might regulate HSC homeostasis in the body is apoptosis, the process of cell death without inflammation. If there is an excess in HSC number, these cells would not divide but instead would undergo apoptosis to keep the required number in the body (Alenzi et al., 2009) .

1.5.2 The haemopoetic stem cell niche

HSCs are localised in the bone cavities of the bone marrow in a microenvironment that is referred to as the stem cell 'niche', as first proposed in 1978 by Schofield (Schofield, 1978). The bone microenvironment is composed of several potential niches with different specific cell types, the two best described are the osteoblastic (or endosteal) niche and the perivascular niche (Mitsiadis et al., 2007). An essential function of the niches and the signals within them is regulating and maintaining the balance between self-renewal and differentiation, thus maintaining the HSC population and protecting it from excess self-renewal that might lead to cancer (Moore and Lemischka, 2006). In the osteoblastic niche, osteoblasts seem to be the main cell type responsible for the signalling between stroma and HSCs, and this niche is thought to keep HSCs in a quiescent state. A key interaction which has been linked with the maintenance of HSC quiescence is between angiopoietin-1 on osteoblasts and its receptor Tie-2 on HSCs (Arai et al., 2004). In order for these signal transduction exchanges to occur the two cell populations need to be in close contact with each other, and several adhesion

molecules have been reported to be necessary for this interaction, such as N-cadherin and SCF-Kit (Wilson and Trumpp, 2006).

In comparison to the osteoblastic niche, the perivascular niche is thought to contain self-renewing HSCs, which are important for rapid expansion and differentiation of blood cells (Wilson and Trumpp, 2006). In the perivascular niche endothelial and the sinusoidal reticular cells are thought to be in contact with HSCs and to support HSC proliferation and differentiation (Kopp et al., 2005). Also, the interaction between sinusoidal reticular cells expressing CXCL12 and HSCs expressing CXCR4 is reported to be important for HSC mobilization, homing and engraftment (Rafii et al., 1997, Sipkins et al., 2005). Recent studies have been able observe in real time, at a single cell level the precise localisation of haemopoietic cells at different stages of maturity by using a combination of high-resolution confocal microscopy and two-photon video imaging (Lo Celso et al., 2009). These studies could be used to define changes in the homing properties of stem cells that have been genetically modified.

1.6 Leukaemic stem cells

Leukaemia is a malignant disease of myeloid or lymphoid blood cells, characterised by rapid proliferation of abnormal cells, which in turn disturbs the homeostasis of the blood system. Leukaemias are generally divided into four categories: (i) Acute myelogenous leukaemia (AML); (ii) Acute lymphocytic leukaemia (ALL); (iii) Chronic lymphocytic leukaemia (CLL); and (iv) Chronic myelogenous leukaemia (CML). Many haematological diseases have a stem cell component that has the capacity for indefinite self-renewal. Bonnet and Dick

demonstrated that the AML cell population contains a subfraction of cells capable of transferring the disease to irradiated immunocompromised mice. These cells were termed the leukaemic stem cells (LSC) (Bonnet and Dick, 1997). A stem cell component has also been defined in CML (Jamieson et al., 2004), T-cell acute lymphoblastic leukaemia (T-ALL) (Cox et al., 2007), B-cell precursor ALL (Cox et al., 2004), and myeloma (Matsui et al., 2004). In the last few years, various studies have shown that AML LSCs share key features with normal HSCs, which include self-renewal ability and the quiescent cell cycle profile (Hope et al., 2004, Guzman et al., 2002). Although AML LSCs are phenotypically very similar to HSCs, being $CD34^+$ / $CD38^-$ / $HLA\text{-}DR^-$, there are three antigens that distinguish HSCs from AML LSCs, namely CD90, CD117 and CD123 (Blair et al., 1998).

Three possible scenarios can be envisaged for the origin of LSCs within the haemopoietic hierarchy (Figure 1.3). In the first, LSCs could be formed at the level of the HSC as the result of an initial mutation leading to a pre-leukaemic stem cell, followed by a secondary mutation that gives rise to the LSC. In a second scenario, the initial mutation in the HSC leads to a pre-leukaemic myeloid progenitor, in which a second mutation is required to generate the LSC. The third scenario requires normal HSC differentiation into myeloid progenitors, which are then the target for an initial mutation to form a pre-leukaemic myeloid progenitor that gives rise to the LSC after a second mutation (Jordan and Guzman, 2004).

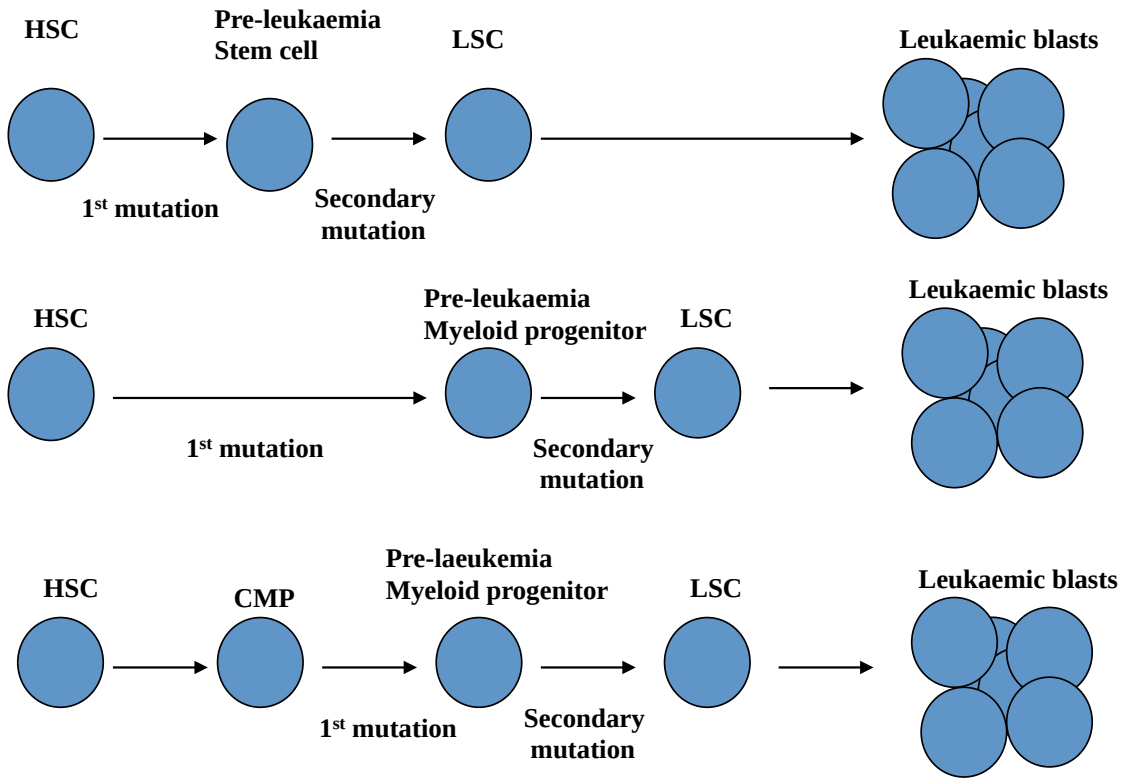

Figure 1.3: Model for the origin of leukaemic stem cells (adapted from (Jordan and Guzman 2004)) In the first, LSCs could be formed at the level of the HSC as the result of an initial mutation leading to a pre-leukaemic stem cell, followed by a secondary mutation that gives rise to the LSC. In a second scenario, the initial mutation in the HSC leads to a pre-leukaemic myeloid progenitor, in which a second mutation is required to generate the LSC. The third scenario requires normal HSC differentiation into myeloid progenitors, which are then the target for an initial mutation to form a pre-leukaemic myeloid progenitor that gives rise to the LSC after a second mutation

1.7 Transcriptional regulation of haemopoiesis

A number of transcription factors e.g SCL/TAL1, RUNX1, Mll, c-Myc, Pu.1, Tel/Etv6, Bmi-1 have been found to be critical in the regulation of HSC establishment and maintenance at various stages of development (summarised in Figure 1.4). Transcription factors not only play a decisive role in HSC development and homeostasis but also in determining the lineage fate from HSCs. Each commitment step requires the activation of lineage-specific genes, whereas unnecessary genes are being repressed (Nerlov et al., 2000). One of the very early regulators is the SCL/TAL1 (stem cell leukemia protein) that is required for blood cell formation. It has been demonstrated that SCL knockout mice and ESC are not able to form haemopoietic cells nor are they able to express blood specific genes (Shivdasani et al., 1995, Robertson et al., 2000). The generation of adult-type HSCs is dependent on the emergence of definitive haemopoietic precursor cell population and this process requires the presence of Runx1 (Runt-related transcription factor 1, also known as Acute Myelogenous Leukaemia factor 1, AML1) (Okuda et al., 1996). Further, for the HSC to mature and to be functional in the foetus the Mll (mixed lineage leukemia) protein crucial. Mll activates the expression of Hox genes which drive haemopoietic progenitor expansion (Ernst et al., 2004). It has been demonstrated that Pu.1 is required for HSC-self-renewal and in addition controls the commitment of HSCs along some individual pathways; disruption of Pu.1 blocks the generation of CLPs and CMPs, demonstrating that Pu.1 is crucial for myeloid and lymphoid cell genesis (Iwasaki et al., 2005). Another transcription factor that has been implicated in regulating the balance between adult HSC self-renewal and differentiation is the proto-oncogene c-Myc. c-Myc deficient mice show an increased number of HSCs due to impaired differentiation in all lineages (Wilson et al., 2004). Moreover, Tel/Etv6 is a transcription factor that is necessary for maintenance of HSC survival (Hock et al., 2004), in this way

indirectly controlling HSC self-renewal and reconstitution capacity. Bmi-1 is a transcriptional repressor that has been implicated in regulating HSC self-renewal ability (Park et al., 2003). Bmi-$1^{-/-}$ mice die soon after birth due to a reduction in HSC number in the bone marrow (Park et al., 2003).

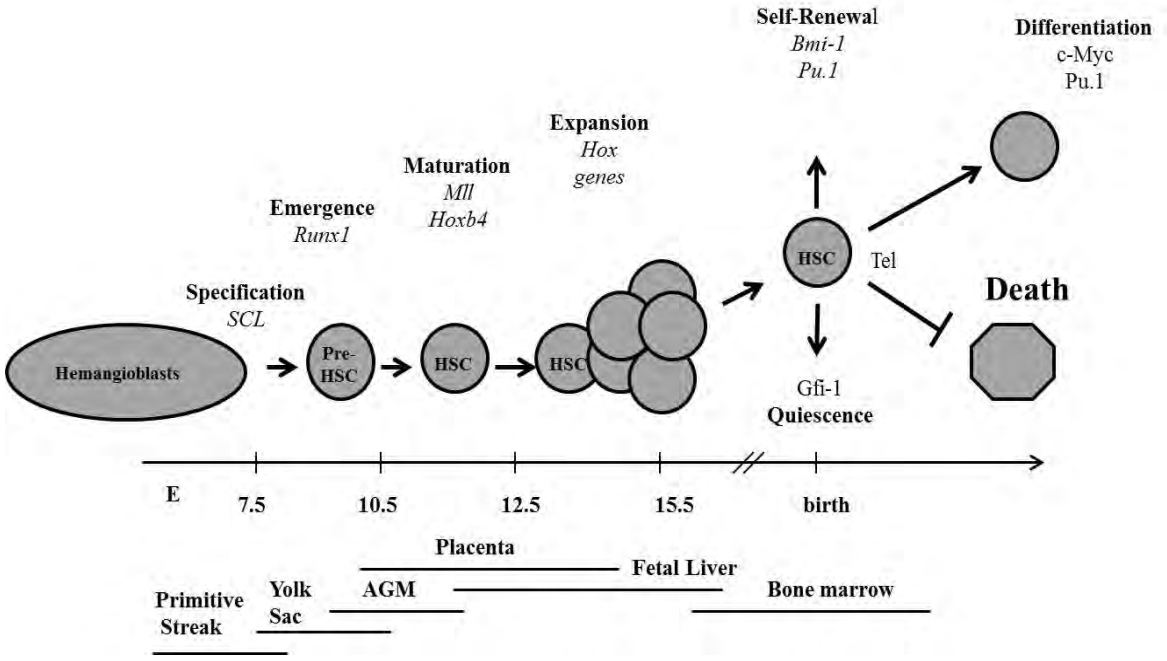

Figure 1.4: Examples of transcription factors that regulate the establishment and maintenance of HSCs at different stages of haemopoietic development (adapted from (Teitell and Mikkola, 2006))

1.8 NF-κB signalling pathways

NF-κB (nuclear factor κB) is another transcription factor that has been implicated in haemopoiesis, but its particular role in HSCs had not been defined. However, the expression patterns of members of the NF-κB family suggest they are likely to play pivotal roles in haemopoietic differentiation. For example, c-Rel expression is restricted to lymphocytes, monocytes, granulocytes and erythroid cells (Grossmann et al., 1999), RelB is expressed in dendritic cells and lymphocytes (Burkly et al., 1995), whilst the importance of NF-κB2 has been shown in expression has been shown in dendritic cells and lymphocytes (Grossmann et al., 1999, Caamano et al., 1998).

NF-κB transcription factors are involved in regulating and activating a wide variety of biological processes including: (i) the immune response; (ii) inflammatory process; (iii) cell stress response; (iv) cell cycle regulation; (v) cell differentiation; and (vi) cell survival (Campbell and Perkins, 2006) that will be further discussed in section 1.8.3 and 1.8.4.

NF-κB can be activated through two main pathways: the Classical and the Alternative pathway

1.8.1 NF-κB family transcriptional regulators

The NF-κB family consists of five distinct members, namely NF-κB1 (the precursor p105 and the active protein p50), NF-κB2 (the precursor p100 and the active protein p52), RelA (p65), RelB and c-Rel. Each protein contains a so-called Rel homology domain (RHD) which is responsible for dimerisation with itself and other family members and also for interactions with the inhibitors of NF-κB (IκBs) (Beg et al., 1995). RelA, c-Rel, NF-κB1 and NF-κB2 can form homo- and heterodimers with each other, whereas RelB can only dimerise with NF-

κB1 and NF-κB2. Each family member has a nuclear localisation signal (NLS) that allows the dimers to enter the nucleus. Furthermore the members RelA, RelB and c-Rel each possess a transactivation domain, hence only dimers containing at least one of these proteins are able to induce gene transcription. In contrast, NF-κB1 (p50) and NF-κB2 (p52) do not carry this domain, and as homodimers act as repressors (Caamano and Hunter, 2002).

1.8.2 IκB and IKK family proteins

In unstimulated conditions, NF-κB is localised in the cytoplasm and is inactive as a consequence of binding with members of the family of IκBs, which includes IκB-α, IκB-β, IκB-ε, and Bcl-3. The IκB family of proteins is characterized by the presence of ankyrin repeats that bind to the RHD to block the NLS. The precursor form of NF-κB1 and NF-κB2 (p105 and p100 respectively) are also considered as members of the IκB family. These proteins have ankyrin repeats in their C-terminal region that are able to fold back onto the RHD in the N-terminus, thereby masking the NLS and thus inhibiting their own nuclear localisation (Ghosh et al., 1998).

Another family of proteins that is associated with NF-κB is the IκB kinase (IKK) family of serine kinases that phosphorylate IκB proteins and target them for degradation in order to transduce signals through NF-κB. There are three members within this family of proteins: IKKα, IKKβ and IKKγ or NEMO. IKKα and IKKβ are catalytic subunits of the complex whilst and IKKγ/NEMO plays a regulatory role.

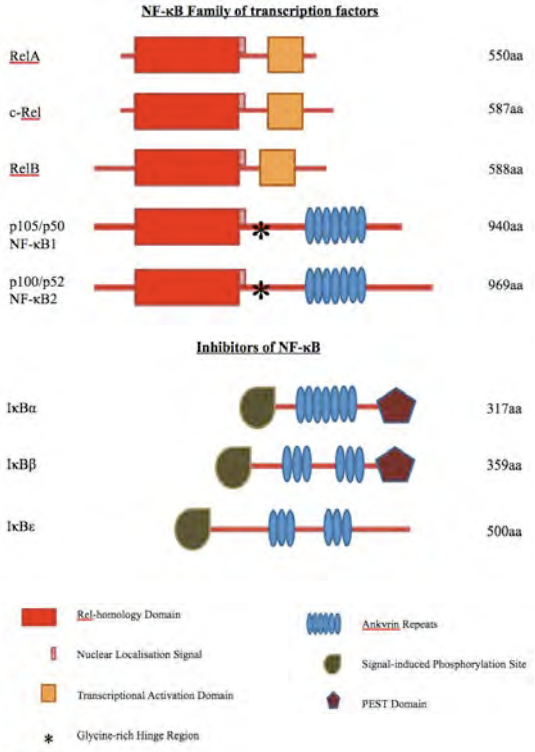

Figure 1.5: Rel/NF-κB family of transcription factors and inhibitors of NF-κB. In mammals there are five members of the Rel/NF-κB family of transcription factors: RelA, c-Rel, RelB, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). All five members share a Rel-homology domain (RHD). RelA, c-Rel and RelB have transcriptional activation domains. NF-κB1 and NF-κB2 are transcribed as the inactive precursors p105 and p100, respectively, which have a series of ankyrin repeats at the C-terminal. These proteins can also act as inhibitiors of NF-κB by dimerising with other members of the Rel/NF-κB family and retaining the complex in the cytoplasm. Phosphorylation and proteosomal degradation cleaves the ankyrin repeats from p105 and p100 to produce the active p50 and p52 proteins, respectively. The inhibitors of NF-κB (IκBs) possess similar ankyrin repeats, and retain Rel/NF-κB dimers in the cytoplasm.

1.8.3 The classical NF-κB pathway

The best studied NF-κB pathway is the classical pathway, which plays the main role in cell proliferation, apoptosis and pro-inflammatory responses (Beinke and Ley, 2004, Ghosh et al., 1998). This pathway is activated by pro-inflammatory cytokines such as TNFα and interleukin-1 (IL-1) or microbial infections. The typical NF-κB heterodimer in this pathway is RelA/p50, but c-Rel/p50 complex can also be involved. In an unstimulated state these heterodimers are retained in the cytoplasm bound to IκB. Upon stimulation, the classical pathway is activated through the IKK-mediated phosphorylation of IκB-α at two serine residues (Ser32 Ser36) near the N-terminus of IκB-α (Chen et al., 1995). This leads to ubiquitination of Lys21 and Lys22 of IκB-α by a specific ubiquitin ligase of the SCF family (Skp1/Cul1/F-box) (Karin and Ben-Neriah, 2000) and proteasomal degradation by the 26S proteasome (Scherer et al., 1995). This results in the release of NF-κB into the nucleus where it binds to DNA and influences the transcription of target genes encoding cytokines, chemokines and adhesion molecules (Pahl, 1999). The kinases responsible for the phosphorylation of IκB are a trimeric complex consisting of the catalytic subunits IKKα and IKKβ together with the regulatory subunit IKKγ.

1.8.4 The alternative NF-κB pathway

The alternative pathway has been implicated in B-cell maturation and lymphoid development and immune processes such as promotion of adaptive immune responses. The pathway is predominantly active in B-cells and occurs downstream of several TNF receptor family members, such as lymphotoxin β (LTβ), B-cell activating factor receptor (BAFF-R) or CD40 (Claudio et al., 2002, Berberich et al., 1994). These stimuli activate NF-κB - inducing kinase

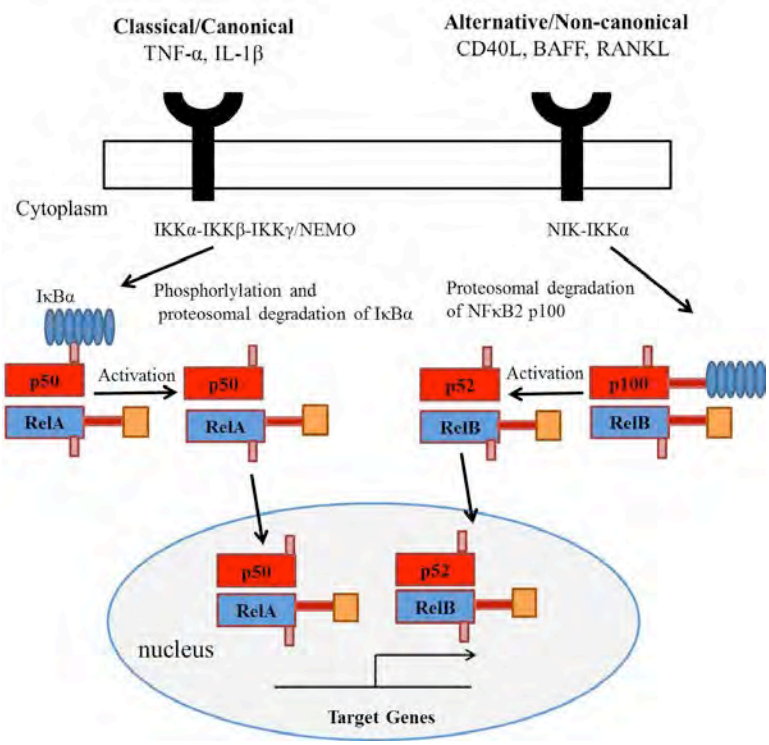

Figure 1.6: Activation of NF-κB. The classical and the alternative pathway. The classical NF-κB pathway is activated by cytokines such as TNFα and interleukin-1 (IL-1) or microbial infections and revolves around nuclear translocation of RelA/p50 and c-Rel/p50 heterodimers, by degradation of IκBs. Activation of the classical NF-κB pathway results in transcription of target genes including cytokines, chemokines, cell adhesion molecules and enzymes. In the alternative NF-κB activation pathway, signals triggered via CD40 or BAFF-R are mediated by NIK and IKKα, which leads to p100 processing and subsequent nuclear translocation of predominantly p52/RelB heterodimers. Activation of this pathway results in transcription of chemokines, cytokines and cell adhesion molecules.

(NIK), which in turn activates IKKα to phosphorylate p100 bound to RelB in an inactive state in the cytoplasm. Ubiquitination and proteolytic removal of the ankyrin-repeat containing domain in the p100 C-terminus at the glycine-rich hinge region (Heusch et al., 1999) allows the resultant p52/RelB dimer to enter the nucleus. The p52/RelB heterodimer then drives transcription of several genes that are involved in developmental programmes in lymphoid cells (Dejardin et al., 1998, Yilmaz et al., 2003, Fusco et al., 2009). The alternative pathway only occurs through NIK and IKKα, and does not depend on either IKKβ or IKKγ (Senftleben et al., 2001a).

1.9 The role of NF-κB family proteins in lymphoid cell regulation

To investigate the physiological roles of the different NF-κB proteins *in vivo*, especially in the regulation of the lymphoid system, several studies have utilised mice carrying deletions of individual or multiple NF-κB genes.

Mice null for the *nfκb1* gene are phenotypically normal and they are able to develop secondary lymphoid organs such as lymph nodes and Peyer's patches, although the Peyer's patches number is decreased (Paxian et al., 2002, Weih and Caamano, 2003). However this subunit seems to plays a role in regulating responses of mature lymphocytes and also nonspecific immune responses to pathogens. In *nfκb1*$^{-/-}$ mice, B cells are not able to proliferate in response to pathogens and are defective in antibody production (Sha et al., 1995). Also *nfκb1*$^{-/-}$ mice show an increase in B-cell apoptosis, as a result of a cell cycle block early in G1 (Grumont et al., 1998).

nfkb2 is mainly expressed in stomach epithelium and immune cells, and mice null for this gene exhibit minor defects in their splenic and lymph node architecture. The spleen of these mice contains irregular follicular B-cell areas and there is a decreased B-cell number in the spleen, bone marrow and lymph nodes (Caamano et al., 1998). In addition, mice lacking *nfκb2* are impaired in their ability to produce antibodies to thymus-dependent antigens (Franzoso et al., 1998). *nfκb2* has been shown to play a role in the regulation of dendritic cells (DC) through its effect on RelB in the Alternative pathway. Usually, RelB is retained in the cytoplasm in association with p100, however in mice lacking *nfκb2* the loss of p100 causes dramatically higher RelB activity that causes modified DC function (Speirs et al., 2004).

It is known that c-Rel is mainly expressed in immune cells and regulated accessory cells and plays a role in lymphocyte function. In its absence B-cells do not proliferate due to a block in the cell cycle at the G1-stage and there is an increase in B cell apoptosis (Grumont et al., 1998).

Mice lacking RelA exhibit the most severe phenotype and die at ~E15 due to hepatocyte apoptosis induced by TNF-α (Beg et al., 1995). In $relA^{-/-}$ mice in which the TNF-α pathway is inhibited, embryos are able to develop and to survive. However, they die after birth due to increased susceptibility to microbial infections (Alcamo et al., 2001). RelA protects several immune cells, and is involved in the regulation of anti-apoptotic genes (Beg and Baltimore, 1996, Senftleben et al., 2001b).

RelB is the only NF-κB component that is not able to bind DNA and forms active heterodimers with p50 or p52. RelB is mainly involved in the formation of secondary lymphoid structures and in the development and function of T-cells (Gerondakis et al., 2006).

Usually RelB is expressed in lymphocytes and dendritic cells and therefore *RelB*$^{-/-}$ mice lack certain populations of dendritic cells (Wu et al., 1998, Burkly et al., 1995). Also, the deletion of RelB causes defects in acquired and innate immune response (Weih et al., 1995).

1.10 Association of NF-κB family proteins with haemopoietic stem cells and the leukemic stem cell phenotype

Several studies have pointed to the significance of the NF-κB proteins in HSCs and LSCs. The study of Pyatt et al has shown that NF-κB is expressed in human CD34^{+} bone marrow cells and it might even be involved in the survival and colony formation of the cells (Pyatt et al., 1999). In turn, its activation can be induced by several cytokines that are involved in the regulation of haemopoiesis. NF-κB is required for the expression of several gene products that are important for haemopoiesis, such as interleukin-1β (IL-1β) (Hiscott et al., 1993), TNF-α (Ziegler-Heitbrock et al., 1993), erythropoietin (EPO) (Lee-Huang et al., 1993), c-myb (Toth et al., 1995) and more. Increased NF-κB signalling is a distinctive feature of primitive umbilical cord blood CD34^{+} HSCs (Panepucci et al., 2007). The same group recently showed that CD133^{+} cells, which define a more primitive subset among CD34^{+} HSCs, have a higher expression of many transcription factors including NF-κB. By analysing promoter occupation of these transcription factors they revealed a higher frequency of NF-κB binding sites in genes such as RUNX1, GATA3 and USF-1, all of which play important roles in the regulation of the early stages of haemopoietic development (Panepucci et al., Panepucci et al., 2010).

Studies have also demonstrated that NF-κB proteins are constitutively active in many cancer cells, whether derived from solid cancers like breast cancer (Nakshatri et al., 1997) or haemopoietic cancers like acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), acute myeloid leukaemia (AML) and multiple myeloma (MM) (Kordes et al., 2000). Also, Guzman et al have observed constitutive activation of NF-κB transcription factor in primary AML LSCs linked to the survival of these cells (Guzman et al., 2001). The anti-apoptotic activity of NF-κB proteins is thought to be critical in their role as survival factors in cancer cells (Wang et al., 1996, Wang et al., 1998).

The reasons why NF-κB proteins are persistently active in tumour cells are not yet fully understood, but there are studies that may explain their involvement. For example, oncogenes such as HRAS or BCR-ABL-1, together with positive regulators of NF-κB such as NIK protein, might undergo gain-of-function mutations that lead to continuous signalling to the IKK complex, thus causing constitutive activation of NF-κB. Alternatively, permanent NF-κB activity can be caused by mutations that occur in negative regulators involved in the pathway, thus preventing the NF-κB proteins to be switched off properly (Kim et al., 2006, Harhaj and Dixit, 2011, Staudt, 2010, Gilmore, 2007). For example, inactivating mutations in IκB-α prevent retention of NF-κB proteins in the cytoplasm and initiate continuous activation (Krappmann et al., 1999)

In contrast, inhibiting NF-κB causes apoptosis of cancer cells and LSCs. Work from the Guzman laboratory has proven that treatment of LSCs with a combination of the proteasome inhibitor MG-132 and the anthracycline idarubicin induces a very rapid apoptosis of LSCs. Proteasome inhibitors are known to prevent NF-κB activation through effects on IκB, supporting the observation that NF-κB is involved in LSC survival (Guzman et al., 2002).

Fortunately, normal HSCs are resistant to these drugs and therefore survive (Gilliland et al., 2004).

1.11 Hypothesis and Aims

Hypothesis:

NF-κB family proteins play a crucial role in maintaining the normal balance of HSC proliferation and survival. Altering the expression of NF-κB proteins can lead to disruption of this balance, resulting in a transformed phenotype or aberrant stem cell function.

Aims of the project:

To assess the role of NF-κB family proteins in bone marrow HSCs

Objectives:

To assess the genetic ablation of NF-κB1 and NF-κB2 and the consequences on HSC number, phenotype and function

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines

2.1.1 HPC7

HPC7 is a HSC-like cell line that has been generated by retroviral transduction of the LIM-homeobox gene Lhx2 into haemopoietic progenitors derived from embryonic stem cells (Pinto do et al., 1998). Lhx2 can immortalise both haemopoietic progenitors derived from embryonic stem cells as well as haemapoietic progenitor / stem cells obtained from adult mouse bone marrow (Pinto do et al., 2002).

HPC7 cells at a density of 1x 10^6 cells/ml were maintained in Stem Pro medium (Invitrogen) supplemented with serum replacement (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco), 12 µM L-glutamine (Invitrogen) and 50 ng/ml stem cell factor (Peprotech) at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 75% N_2.

2.2 Mouse studies

2.2.1 Mice and genotyping

Both *nfκb1*$^{-/-}$ and *nfκb2*$^{-/-}$ mice were maintained on a C57BL6/J background. The *nfκb1*$^{-/-}$ mouse has been generated in the Sha et al laboratory. The *PGK-neo* cassette was inserted into exon 6 of the *NFKB1* gene (Sha et al., 1995). The *nfκb2*$^{-/-}$ mice were generated by Caamano et

al by introduces the PGK-*neo* cassette into exon 4, disrupting the open reading frame of the gene (Caamano et al., 1998). The mice were genotyped by PCR using genomic DNA obtained from ear samples. The primers used for genotyping NF-κB1 mice were 3'WT50 (GCAAACCTGGGAATACTTCATGTGACTAAG), 3'BS-7 (ATAGGCAAGGTCAGAATGCACCAGAAGTCC) and 3'HH-Neo (AAATGTGTCAGTTTCATAGCCTGAAGAACG), which results in products of 120bp for the wild type allele and 200bp for the knockout allele. The primers used for the NF-κB2 mice were 3'Ex4 (GCCTGGATGGCATCCCCG), 3'Ex5 (GTTTGGGCTGTTCCACAA) and 3'PGK-1 (CCAGACTGCCTTGGGAAA), which result in products of 230bp for the wild type allele and 120bp for the knockout allele. The primers were purchased from Eurofins MWG.

To extract the genomic DNA, the ear samples were heated in 100 µl water at 95°C for 5 minutes. 3 µl of 15 mg/ml proteinase K solution (Roche) was added and incubated at 37°C for 20 minutes. The proteinase K was then inactivated by incubating at 95°C for 5 minutes. 1 µl of genomic DNA, 0.5 µl of each primer at the concentration of 0.5 µM was added to 22.5 µl of ReddyMix PCR MasterMix containing 2.5 mM magnesium chloride, deoxynucleotides and Taq polymerase. The PCR products were amplified using the following thermal cycler parameters: denaturation at 95°C for 1 minute, followed by annealing at either 60°C for NF-κB1 or 55°C for NF-κB2 for 1 minute and then extension at 72°C for another 1 minute. This cycle was repeated 35 times. The PCR products were analysed by electrophoresis on a 2.5% agarose gel in TAE and 0.5 µg/ml ethidium bromide (Sigma).

2.2.2 Bone marrow preparation

Whole bone marrow was extracted from femurs and tibias of mice aged between 8 and 12 weeks and flushed using 10 ml per mouse of Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) containing 10% FBS through a 25 gauge needle. Spleen cells were prepared by mincing the tissue in PBS supplemented with 10% FBS. The cells were then filtered through a 70 μM cell strainer and were centrifuged for 5 minutes at 230 x g and the supernatant removed.

The red cells were lysed in all stainings, except for Ter119/CD71/c-Kit, by adding 1 ml of ACK (Red Blood cell lysis buffer containing 0.15 M ammonium chloride, 1 mM potassium bicarbonate and disodium EDTA pH 7.3) per mouse and incubating at room temperature for 5 minutes. An equal volume of PBS was added to wash the cells, then the cells were centrifuged at 230 x g for 5 minutes, before re-suspending in IMDM containing FBS.

For cell counting the samples were first resuspended in 1 ml of PBS supplemented with 10% FBS, then a 1 μl aliquot was further diluted in 100 μl of PBS and 10 μl 0.4% Trypan Blue (Sigma) was added. 10 μl of the dilution was mounted on a Neubauer Haemocytometer and counted under the microscope (Carl Zeiss, Axiovert 25).

2.3 Cell analysis

2.3.1 Immunofluorescent flow cytometry and cell sorting

Stainings were performed in 100 μl IMDM containing 10% FBS. 200 μl of medium was then added prior centrifugation at 230 x g for 2 minutes at 4°C in a 96'V' well plate. To block non-specific binding, 4 μl of 5 μg/ml of anti-CD16/32 Fc-block (FcγRII/III) (eBiosciences) was added to the cells and incubated on ice for 5 minutes. Fc-block was not added to

stainings that required analysis with FcγRII/III. The primary antibodies were then added at a concentration of 10 µg/ml for 30 minutes and incubated on ice in the dark. Single staining controls were done to setup the compensations between fluorescent channels. The antibodies used are listed in Table 2.1. To remove the excess antibodies, double the amount of medium was added prior to centrifugation at 198 x g for 3 minutes. The supernatant was removed and the cells re-suspended in 100 µl medium per mouse. The stained cells were analysed on a Cyan flow cytometer using Summit 4.3 software.

For cell sorting, cells were first extracted from the bone marrow as described previously. The red cells were lysed and the cells stained with the appropriate antibodies. After washing the cells were re-suspended in 300 µl of IMDM containing 10% FBS. The samples were filtered through a sterile 70 µM cell strainer and sorted on a MoFlo machine (Cytomation) into sterile IMDM containing 10% FBS for further usage.

Primary antibody	Conjugate	Isotype	Supplier	Clone
CD4	FITC, APC	Rat IgG2b	eBioscience	RM4 -5
CD5	FITC, APC	Rat IgG2a	eBioscience	53-7.3
CD8a	FITC, APC, PE	Rat IgG2a	eBioscience	53-6.7
CD11b/Mac1	FITC, APC	Rat IgG2b	eBioscience	M1-70
CD16/32	PE	Rat IgG2a	eBioscience	93
CD34	FITC, PE	Rat IgG2a	eBioscience	RAM34
CD41/61	PE	Rat IgG1	eBiosciences	MWReg30
CD43	PE	Rat IgM	eBiosciences	eBioR2/60
CD45.1	PE-Cy5, PE, Pacific Blue	Mouse IgG2a	eBioscience	A20
CD45.2	FITC, PE, APC-Cy7	Mouse IgG2a	eBioscience	104
CD71	PE	Rat IgG2a	eBioscience	R17217
CD177 (c-Kit)	PE-Cy5, APC	Rat IgG2b	eBioscience	ACK2
CD45R (B220)	FITC, APC, Pacific Blue	Rat IgG2b	eBioscience	HIS24
Gr1	FITC, PE, APC	Rat IgG2b	eBioscience	RB6-8C5

CD135 (Flt3)	PE	Rat IgG2a	eBioscience	A2F10.1
Sca1	PE-Cy5, APC, Pe-Cy7, PE	Rat IgG2a	eBioscience	D7. RUO
Ter119	FITC, APC, PE	Rat IgG1	eBioscience	TER-119

Table 2.1: Primary antibodies used for flow cytometric analysis and sorting of haemopoietic cells

2.3.2 Side population staining

Goodell et al described a procedure for staining the side population (Goodell et al., 1997). After counting, the bone marrow cells were re-suspended at $1x10^6$ cells/ml in pre-warmed Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) which contained 2% FBS (Bioclear) and 10 mM HEPES buffer (Invitrogen). Hoechst 33342 (Sigma) dye was added to a final concentration of 5 μg/ml. The cells were mixed and incubated in a 37°C water bath for 90 minutes. The cells were mixed every 15 minutes to ensure that the temperature of the cells was maintained at 37°C. After the incubation the cells were centrifuged at 230 x g at 4°C for 5 minutes. The cells were re-suspended in ice-cold medium and stained with antibodies against lineage associated cell surface markers (CD5, CD8a, CD11b, Gr1, B220 and Ter119) c-Kit and Sca1. Finally the cells were analysed using the MoFlo FACS machine.

2.3.3 Cell cycle analysis of haemopoietic stem cells

The cell cycle status of wild type and *nfκb1*$^{-/-}$ and *nfκb2*$^{-/-}$ mice were determined by BrdU (bromodeoxyuridine) staining. For *in vitro* BrdU incorporation assay, KSL cells were sorted and plated in IMDM containing 10% horse serum (Sigma), L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), murine SCF (20 ng/ml), FL (10 ng/ml) and TPO (5 ng/ml) together with 25 μM BrdU (Sigma-Aldrich). After 12 hours incubation at 37°C, the

cells were collected and centrifuged at 230 x g for 5 minutes. The supernatant was removed and the KSL cells were resuspended in 100 µl PBS, followed by centrifugation onto glass coverslips. The cells were then fixed in ethanol/acetic acid (75%:25%) for 20 minutes. Following fixation the cells were then incubated in PBS for 20 minutes and washed twice with demineralized water. The KSL cells were denatured for 1 hour with 1 N HCL containing 0.5% Tween-20 which is prepared on the day of the experiment. The cells were washed twice with PBS and twice with the blocking buffer, that contains 5% FBS, 0.1%NaN_3, PBS and 0.1% Triton X-100 and treated with RNAaseA (100 µg/ml) in PBS at 37 ºC for 30 minutes. The cells were washed twice with the blocking buffer and incubated with the blocking buffer for 1 hour at room temperature. The cells were finally incubated overnight with the primary monoclonal rat BrdU antibody (1:100 dilution, clone BU1/75, Abcam) at room temperature.

The following day, the cells were washed 3 times with blocking buffer and 3 times with PBS and were then fixed with 4% paraformaldehyde in PBS for 10 minutes, washed twice with PBS and quenched 10 minutes 50 mM NH_4Cl. The cells were then washed again twice with PBS and twice with blocking buffer before being incubated for 2 hours with AlexaFluor anti-rat IgG donkey (1:1000 dilution Invitrogen). After washing 3 times with PBS, the cells were mounted using DAPI-containing Vectashield (Vector Laboratories). At least two coverslips per sample were analysed. The images were acquired by using the Zeiss LSM 510 Meta confocal fluorescence microscope (10 and 25 water immersion objective). For the basic analysis of the confocal images, Zeiss LSM Image Browser v4.2 was used (Carl Zeiss Ltd).

For the in vivo BrdU incorporation study, mice received 1 mg BrdU in 100 µl PBS via intraperitoneal injection. After 24 hours the mice were sacrificed for analysis as described above.

2.4 Cell culture of haemopoietic stem cells

2.4.1 Semisolid methylcellulose cultures of sorted KSL cells

To assess individual cell proliferation and differentiation capacity 350 sorted KSL cells were plated into 1.5 ml methylcellulose Semisolid methylcellulose media supplemented with 25 ng/ml thrombopoietin (TPO) (Methocult M3434, Stem cell Technologies, Vancouver). The sorted KSL cells were added, vortexed gently and finally plated in a 35 mm Petri dish using an 18-gauge needle. At least duplicates were plated in each experiment. Colony numbers and phenotypes were assessed at day 7. For secondary and successive replatings, all cells from previous replating were collected by diluting the methylcellulose with PBS and pippeting it from the plate into a 10 ml falcon tube, cells were counted and 5000 cells replated in fresh methylcellulose. All cultures were incubated at 37ºC in a fully humidified 5% CO_2 in air atmosphere. For identification of colonies, colonies were picked with a pipette tip, and resuspended in 100 µl PBS, which was loaded into cytospin funnels (Shandon) for centrifugation. Cells were centrifuged for 8 minutes at 23 x g onto microscope slides (BDH) and air-dried. Slides were further stained using Diff-Quick set of stains (Dade Behring) for differential staining of cytospins. Cytospins were dipped f15 times for 1 second into the following three solutions: i) 2 µg /ml Fast Green methanol, ii) 1.22 mg/ml Eosin G in phosphate buffer (pH 6.6) and iii) 1.1 mg/ml Thiazine Dye in phosphate buffer (pH 6.6). Using paper towels excess fluid was drained between each solution. Finally the slides were washed gently with distilled water, air dried and covered with neutral mounting medium (BDH) and cover glass.

2.5 Competitive bone marrow reconstitution assay

One week before transplantation, recipient animals were treated with Baytril antibiotic. Following transplantation, the antibiotic was given until the body weight of the mice was stable. For competitive repopulation assays, recipient mice (B6:SJL, CD45.1/CD45.1) were lethally irradiated (900 Gy) and injected via the tail vein with 1 x 10^6 reference wild type ACK-treated whole bone marrow cells (B6 x B6:SJL F1, CD45.1/CD45.2) together with 500 sorted KSL donor cells from either wild type or *nfκb* knockout mice (C57BL6/J, CD45.2/CD45.2) (Figure 2.1). The reference cells were used as an internal reference and also served to keep the mice alive after irradiation. Every four weeks until six months post-transplantation, blood samples were taken from the tail vein to assess reconstitution by the donor cells. Using antibodies against CD45.1 and CD45.2 enabled discrimination of the donor and reference cells. After 6 months the mice were sacrificed and the bone marrow cells analysed by immunofluorescent flow cytometery.

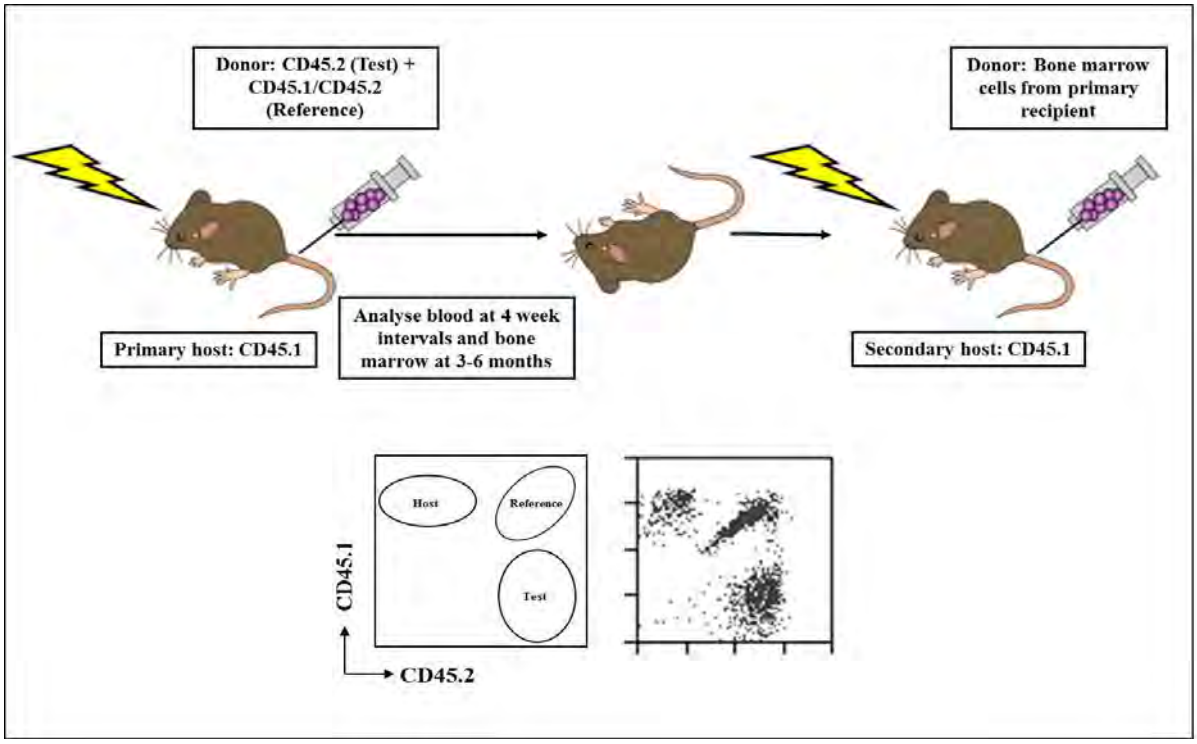

Figure 2.1: Transplantation of wild type and *nfκb* knockout KSL cells into lethally irradiated recipients: recipient mice (B6:SJL, CD45.1/CD45.1) were lethally irradiated (900 Gy) and injected via the tail vein with 1 x 10^6 reference wild type ACK-treated whole bone marrow cells (B6 x B6:SJL F1, CD45.1/CD45.2) together with 500 sorted KSL donor cells from either wild type or *nfκb* knockout mice (C57BL6/J, CD45.2/CD45.2). Every four weeks until three to six months post-transplantation, blood samples were taken from the tail vein to assess reconstitution by the donor cells. Using antibodies against CD45.1 and CD45.2 enabled discrimination of the donor and reference cells. After three to six months the mice were sacrificed and the bone marrow cells analysed by immunofluorescent flow cytometery.

2.6 RNA analysis

2.6.1 RNA extraction

After KSL cells were sorted they were centrifuged at 230 x g for 5 minutes at 4ºC and resuspended in 250 µl TRIZOL Reagent (Invitrogen). The lysates were either stored at -20ºC or processed immediately as indicated by the manufacturer by adding 0.2 volumes of chloroform (Sigma) per sample and shaking vigorously by hand for 15 seconds. The lysates

were incubated for 5 minutes on ice and then micro-centrifuged at 17900 x g for 15 minutes at 4ºC. The upper aqueous phase was collected into a fresh RNase-free tube. To aid RNA precipitation 1 µl of RNase-free glycogen (Roche) was added together with 0.5 volumes of isopropanol (Sigma). The samples were centrifuged at 17900 g for 10 minutes at 4ºC, air-dried and resuspended in 10 µl of RNase-free water.

2.6.2 cDNA synthesis

The single-stranded cDNA was prepared by first adding 1 µl of 500 ng/µl of oligo (dT) primers and 2 µl of 0.5 mM dNTPs (Roche) to 10 µl RNA and incubating at 65ºC for 5 minutes. Samples were then placed on ice for 1 minute, 4 µl of 5x First-strand buffer (Invitrogen), 1 µl dithiothreitol (10nM, Invitrogen), 1 µl RNase-out and 1 µl Superscript reverse transcriptase (Invitrogen) were added to the mixture and reverse transcription was carried out for 1 hour in a 50ºC water bath, followed by the inactivation of the enzymes at 70ºC for 15 minutes.

2.6.3 Semi-quantitative RT-PCR

By measuring the expression level of the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) using real time PCR the cDNA samples were normalised. PCR reactions were performed in a final volume of 50 µl Reddy Mix PCR Master Mix (ABgene) containing appropriate concentrations of gene-specific oligonucleotides (Table 2.3) using the MJ Research PTC100/200 machines. The PCR products were amplified using the following thermal cycler parameters: denaturation at 95°C for 40 secs, followed by annealing at 50-60°C (can vary, depend on the primers) for 40 secs, and extension at 72°C for 40 secs. For each PCR reaction, samples were taken at intervals of 3 cycles to estimate the linear phase

of amplification. The PCR products were analysed by electrophoresis on 2.5% agarose gels. The experiment was performed in duplicate.

Gene	Forward primer	Reverse primer	T_{anneal} (°C)	PCR Product size
HPRT	CCTGCTGGATTACATCAAAGCACTG	GTCAAGGGCATATCCTACAACAAAC	59	620
NF-κB 1	GATAACCTCTTTCTCGAGAAGGCTC	TCCCCATTCTCATCCTGCACGGCGG	60	233
NF-κB 2	GCCTGGATGGCATCCCCG	CTTCTCACTGGAGGCACCT	60	193
RelA	GAGAGAAGCACAGATACCACCAAGAC	GGTTATCAAAAATCGGATGTGAGAGG	60	415
RelB	ATGCCGAGTCGCCGCGCTGCCAGAGATGCTCTGTGATGACCAGGTAC	AAGCGCATGCCACGCTGCTTTGGCGTCCGCGCCCGAGCTAGGG	60	352
c-Rel	TGGCTGACTGACTCACTGACTGACTGACTCGTGCCTTGC	CCAACTAAATCATGAGGATGAGGCTTATATGGATCATTC	60	263
IκBα	CAGGACTGGGCCATGGAGGG	TGGCCGTTGTAGTTGGTGGC	60	526
IκBβ	CCCTTCCTGGATTTCCTCCTGGGCTTTTC	GTGGGGTCAGCACCGGCTTTCAGGAGA	60	589

Table 2.2: Sequences of semiquantitative RT- PCR oligonucleotide primers

2.6.4 Real time PCR

The real time PCR reactions were performed using SYBR Green and the gene-specific primers. Primers were designed using the Primer Express Software (MWG Biotech) to span an intron, generating products between 100-300 bp and no unspecific product, such as primer dimers. Different concentrations of the primers were tested on serial dilutions of cDNA samples to confirm the efficiency of the primers and the validity of the assay. For each reaction 1-2 µl of cDNA dilutions were used in an 18 µl of reaction with Absolute QPCR SYBR Green Mix (ABgene) and 200-400 nM of each primer. The reactions were performed and analysed on the Stratagene Real time PCR machine using the following thermal cycler parameters: an initial denaturation step (95ºC/ 15 minutes), cycling included 95ºC for 15 sec,

57-60ºC for 20 sec and 72ºC for 20-30 sec(40 cycles) (Stratagene Mx3005P). The MxPro software of the Stratagene Real Time PCR machine calculated the amplification efficiency and the Ct-value of each sample.

Gene	Forward Primer	Reverse Primer	T_{anneal} (°C)
HPRT	TGGACTGATTATGGACAGGAC	AGGGCATATCCAACAACAAAC	60
NF-κB1	ACATGGTGGTTGGCTTTGC	CCCTAATACACGCCTCTGTCATC	60
NF-κB2	GGGCCGGAAGACCTATCCTA	ACGAGGTGGGTCACTGTGTGT	60
RelA	AAGCCATTAGCCAGCGAATC	GCCCGGTTATCAAAAATCGG	60
c-Rel	CAGAATTTGGACCAGAACGCA	TGCTGTTCAGGGACATTGAAAG	60

Table 2.3: Sequences of RT- PCR oligonucleotide primers

2.7 Statistical Analysis

Statistical analyses were performed using unpaired two-tailed *t* test. For all tests, a p value lower than 0.05 was considered statistically significant.

CHAPTER 3

ANALYSIS OF THE ROLE OF NF-κB1 IN HAEMOPOIETIC STEM CELLS

3.1 Introduction

As detailed in Chapter 1, NF-kB activity has been implicated in HSC survival and development, however, it was not known which members of this transcription factor family played significant roles in these stem cells nor were their precise functions defined. To study the importance of NF-κB1 function in HSC in greater detail, I have used gene knockout mice of NF-κB1 and performed *in vitro* and *in vivo* assays to assess the HSCs function and compared them to those obtained from the wild type cells. Using PCR we detected NF-κB1 in HSCs. To study the function of NF-κB1 in HSCs *in vivo* we performed serial transplantation assays. For *in vitro* study we performed colony formation assays and proliferation assays were performed.

3.2 The expression of NF-κB family members in HPC7 and KSL cells

In order to gain some understanding of the expression pattern of NF-κB family members in HSCs, semi-quantitative real-time PCR analyses were performed on RNAs derived from the HSC-like cell line, HPC7, and from sorted primary KSL cells. These analyses revealed clearly detectable expression of NF-κB1, NF-κB2, and RelA in HPC7 cells and of NF-κB1, NF-κB2 and RelA in KSL cells (Figure 3.1A and B). RelA appears to be the most abundantly

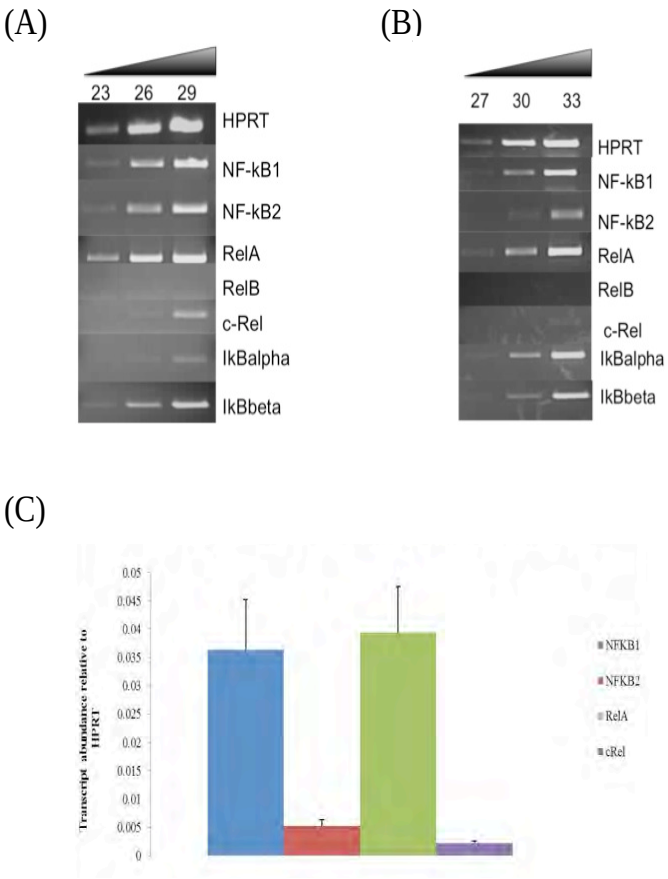

Figure 3.1: Gene expression analysis of the NF-κB family members in HPC7 and KSL cells. (A) The panel shows the expression of different NF-κB family members in HPC7 cells. cDNA was prepared from extracted RNA and semi-quantitative PCR analysis was performed. cDNA was normalised against the HPRT gene as a positive control. The PCR reactions were sampled at cycles 23, 26, 29. (B) The panel shows the expression of the NF-κB family members in sorted KSL cells from the bone marrow of wild type C57BL6 mice. PCR reactions were sampled at cycles 27, 30, 33. (C) Real time PCR was performed on cDNA samples of HPC7 cells as described in (A) using SYBR Green and primers specific for the NF-κB family member genes. Normalisation was performed against HPRT. Reactions were repeated at least 3 times. The normalised average fold-change expression values are shown. Error bars represent the SEM.

expressed NF-κB family gene in HSCs. c-Rel was detected in HPC7, but at a lower level, and was barely detectable in KSL cells. RelB could not be detected in either HPC7 or KSL cells unless the number of amplification cycles was significantly increased (data not shown).

NF-κB1 and NF-κB2 are expressed in HSCs and subsequent work described in this and the next chapter was directed towards understanding their significance in these cells.

3.3 Comparison of haemopoietic cells in the bone marrow of wild type versus *nfκb1*$^{-/-}$ mice

In order to study the effect of the transcription factor NF-κB1 in multiple haemopoietic lineages, an analysis of different haemopoietic cell types in the bone marrow of wild type mice was performed and compared to bone marrow from *nfκb1*$^{-/-}$ mice. The study of different haemopoietic cell types was done using immunofluorescent staining for specific lineage-associated markers. Using lymphoid markers for B-cells (B220/CD43) and T-cells (CD4/CD8) it was clear that *nfκb1*$^{-/-}$ mice exhibit no difference in their lymphoid cell number compared to the wild type mice. Staining for myelomonocytic markers (CD11b/Gr-1) also revealed no difference in the cell number between wild type and *nfκb1*$^{-/-}$ mice. Further immunofluorescent staining of *nfκb1*$^{-/-}$ mice with antibodies against the red cell markers Ter119 and CD71 showed equivalent erythroid cell numbers compared to the wild type mice. Finally, staining with antibodies recognising the progenitor-associated SCF receptor c-Kit and the stem cell/megakaryocyte alpha integrin GPIIb (CD41) showed no change in the progenitor numbers committed towards megakaryocytic differentiation (Table 3.1 and see Appendix Figure A1 and A2).

Cell type	Surface markers	Wild type versus *nfκb1* knockout
Lymphoid	B220/CD43 (B-cells) CD4/CD8 (T-cells)	Equivalent
Myelomonocytic	CD11b/Gr1	Equivalent
Erythroid	TER119/CD71/c-Kit	Equivalent
Megakaryocytic	c-Kit/CD41	Equivalent

Table 3.1: Immunofluorescent analysis of bone marrow cells from wild type versus *nfκb1*$^{-/-}$ mice. Bone marrow was flushed from the femur and tibias of mice aged 8-10 weeks. The table represents different surface markers that were used to study the different haemopoietic lineages. (n=6)

3.4 Analysis of the progenitor and haemopoietic stem cell compartment in wild type and *nfκb1*$^{-/-}$ mice

The progenitor and HSC compartments of wild type and *nfκb1*$^{-/-}$ bone marrow were analysed using a cocktail of antibodies against lineage-associated markers (CD5, CD8a, CD11b, Gr-1, B220 and Ter119) combined with antibodies against progenitor-associated SCF receptor, c-Kit and stem cell antigen Sca-1. The different myeloid progenitor populations can be studied in the Sca1 negative portion of the KSL, which can be further subdivided into (i) GMP ($CD34^{+}CD16/32^{+}$); (ii) CMP ($CD34^{-}CD16/32^{+}$); and (iii) MEP ($CD34^{-}CD16/32^{-}$). The markers CD34 and Flt3 can be used to separate the KSL compartment into; (i) LT-HSCs ($CD34^{-}Flt3^{-}$); (ii) ST-HSCs ($CD34^{+}Flt3^{-}$); and (iii) multipotent progenitor (MPP) cells ($CD34^{+}Flt3^{+}$).

Staining with lineage markers revealed a small increase of lineage negative cells in the *nfκb1*$^{-/-}$ bone marrow mice. However, further analysis of several animals showed that there is a

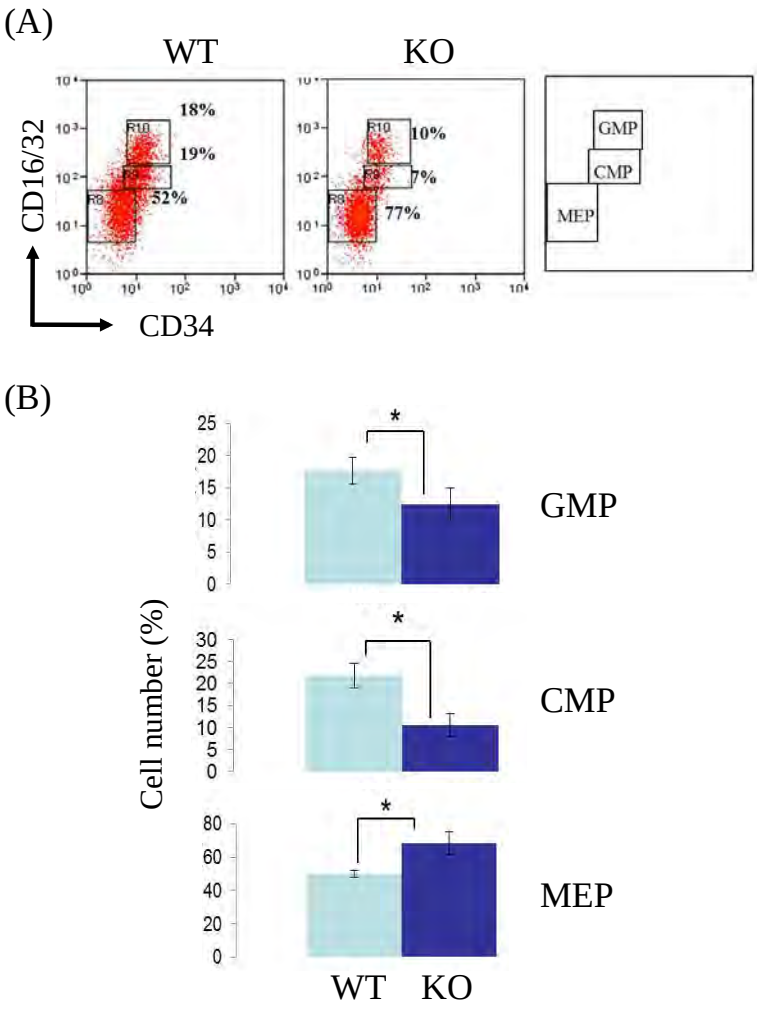

Figure 3.3: Immunofluorescence analysis of progenitor cells of wild type and *nfkb1*$^{-/-}$ mice. To analyse the GMP, CMP and MEP populations, wild type (WT) and *nfkb1*$^{-/-}$ (KO) Lin^{-}c-Kit^{+}Sca-1^{-} cells were co-stained with antibodies against CD16/32 (Fcγ receptor) and CD34. (A) Two-dimensional dot plots of cells gated on FSC against SSC (live cell gate), lineage antigen negativity, and on c-Kit^{+}Sca-1^{-}. The GMP, CMP and MEP cells are indicated by the gates. The schematic indicates the position of the GMP, CMP and MEP populations. (B) The histograms represent the average percentage of GMP, CMP and MEP cells from wild type and *nfkb1*$^{-/-}$ mice. The bars indicate the SEM (n=6). * indicates significance ($P<0.05$)

tendency towards more lineage negative cells in the knockout bone marrow compared to the wild type, but this difference is not statistically significant (Figure 3.2A). No difference was observed in the $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}$ population, which contains GMPs, CMPs, and MEPs (Figure 3.2B). However, analysing these progenitor populations separately by co-staining with antibodies against CD34 and CD16/32 showed that there was a significant decrease in GMP and CMP population in the *nfκb1*$^{-/-}$ mice, while the MEP population was significantly increased (Figure 3.3). This result suggests that there might be a decrease in the proportion of more mature myeloid cells and an increase in the erythroid cell proportion in the *nfκb1*$^{-/-}$ mice. However, analysis of these mature cells using cell surface markers revealed that this is not the case that might be due to homeostatic adjustments.

Further study of the KSL population of the *nfκb1*$^{-/-}$ mice indicated that there is an at least a 7-fold increase in this population compared to the wild type mice. The total number of bone marrow does not change between wild type and knockout mice. However the absolute number of KSL cells is increased mirroring the increased percentage. Since it is known that the murine bone marrow KSL fraction consists of a heterogeneous population of cells with different repopulation capacity when transplanted into lethally irradiated mice, the KSL cells were subdivided into three different compartments based on their expression of CD34 and the fms-like tyrosine kinase receptor, Flt3. Co-staining of wild type and *nfκb1*$^{-/-}$ bone marrow cells with antibodies against KSL markers, CD34 and Flt3 revealed that there are no major differences in the proportion of the KSL sub-populations in the knockout mice compared to the wild type. Knockout mice show a slight increase in the proportion of $CD34^{-}\,Flt3^{-}$ KSL cells, however, this was not statistically significant (Figure 3.4). Although there is no

significant difference within the KSL compartment, corresponding to the increase of the KSL absolute numbers the LT-, ST-HSC and MPP absolute numbers are also increased.

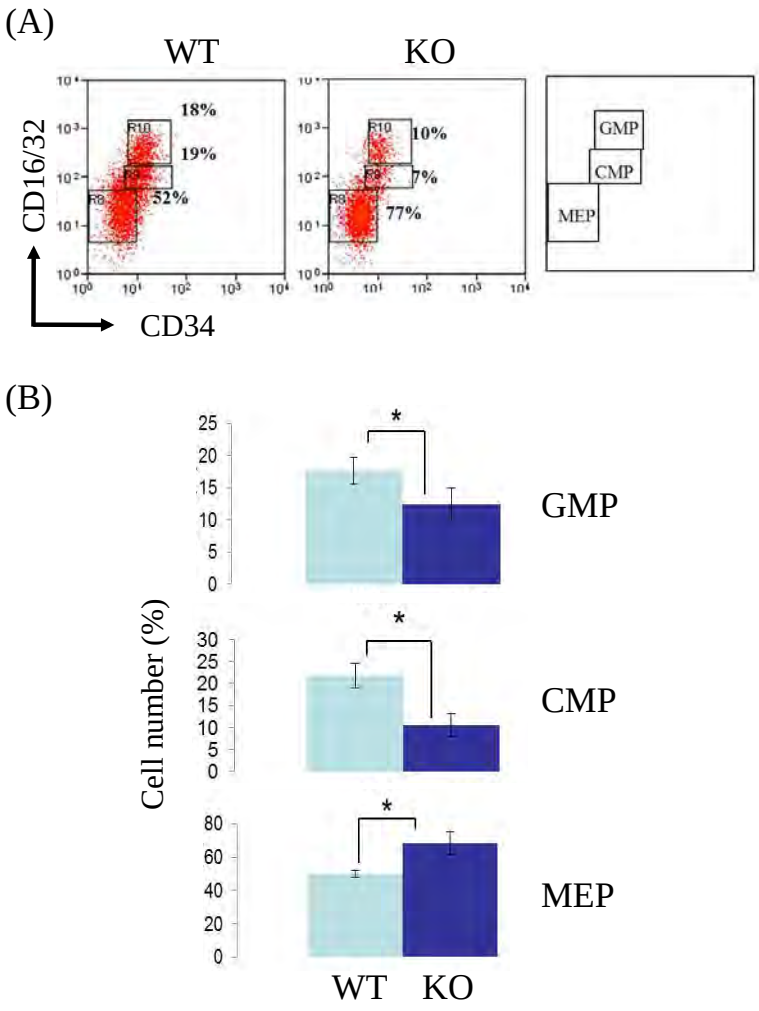

Figure 3.3: Immunofluorescence analysis of progenitor cells of wild type and *nfkb1*$^{-/-}$ mice. To analyse the GMP, CMP and MEP populations, wild type (WT) and *nfkb1*$^{-/-}$ (KO) Lin^{-}c-Kit^{+}Sca-1^{-} cells were co-stained with antibodies against CD16/32 (Fcγ receptor) and CD34. (A) Two-dimensional dot plots of cells gated on FSC against SSC (live cell gate), lineage antigen negativity, and on c-Kit^{+}Sca-1^{-}. The GMP, CMP and MEP cells are indicated by the gates. The schematic indicates the position of the GMP, CMP and MEP populations. (B) The histograms represent the average percentage of GMP, CMP and MEP cells from wild type and *nfkb1*$^{-/-}$ mice. The bars indicate the SEM (n=6). * indicates significance ($P<0.05$)

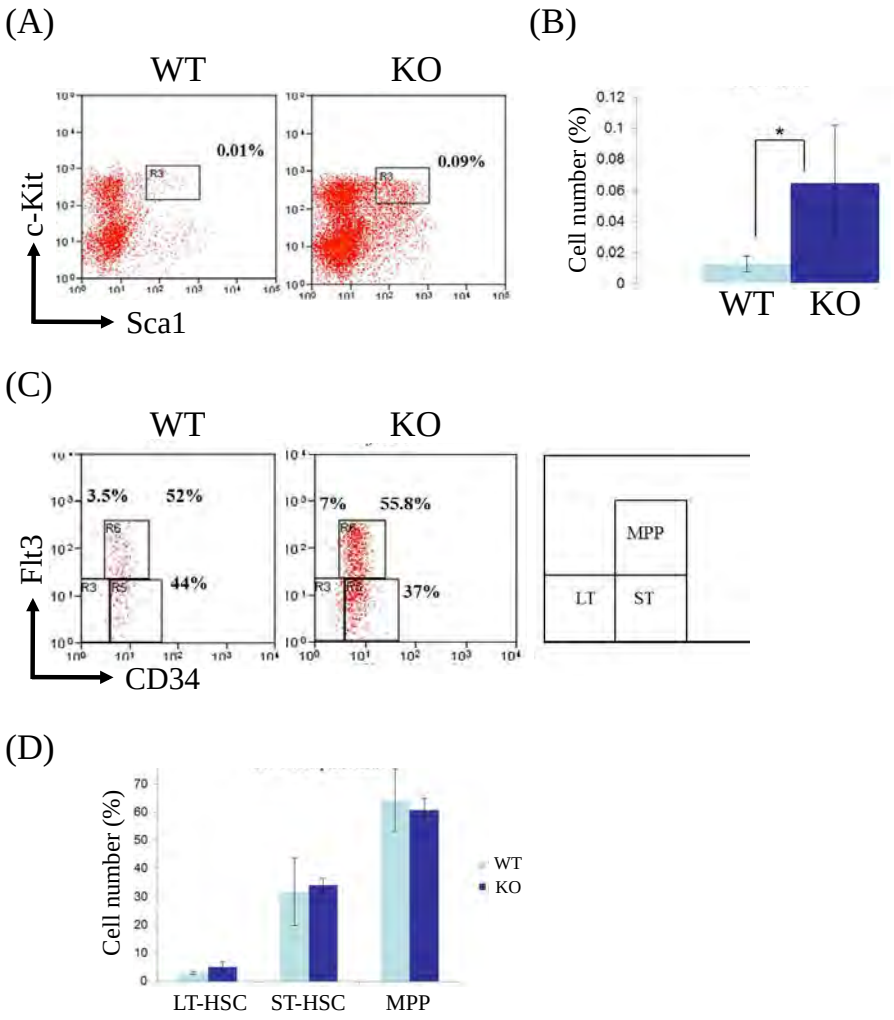

Figure 3.4: Immunofluorescence analysis of stem cell compartments of wild type and *nfkb1*$^{-/-}$ mice. Bone marrow from wild type (WT) and *nfκb1*$^{-/-}$ (KO) mice was extracted and the red cells were lysed. (A) Two-dimensional dot plots showing c-Kit and Sca1 staining of cells gated on FSC against SSC (live cell gate) and lineage antigen negativity. The gate shown represents the KSL fraction. (B) The histogram represents the average percentage of KSL cells from WT and KO mice. The bars indicate the SEM. * indicates significance ($P<0.05$) (C) KSL cells co-stained with Flt3 and CD34. The two dimensional dot plot represents KSL gated cells. The schematic indicates the position of long-term (LT) HSC, short-term (ST) HSC and multipotential progenitors (MPP). (D) The histogram represents the average percentage of cells within each gate. The bars show SEM (n=6).

3.5 Analysis of wild type versus *nfκb1*$^{-/-}$ KSL cells using a phenotypic assay of stem cells

Goodell et al used the Hoechst 33342 DNA binding dye as a means to define the HSC population in murine bone marrow. Stem cells express the ABC-G2 protein, an ABC transporter, which has the ability to export dyes such as Hoechst 33342 that have entered the cytoplasm by passive diffusion. The expression of this transporter protein is thought to correlate with the immaturity of the cell type, the more immature the cells are, the higher the expression of the ABC-G2 transporter protein. Hoechst 33342 dye was used to evaluate the proportion of immature HSCs in wild type compared to *nfκb1*$^{-/-}$ mice. Red cells were lysed and the remaining cells were co-stained with Hoechst 33342 and the antibodies defining the KSL population (Goodell et al., 1997). It is known that the side population (SP) that excludes Hoechst 33342 includes quiescent, immature stem cells. KSL cells in wild type and *nfκb1*$^{-/-}$ bone marrow mice were gated and the proportion of the cells within the SP gate was assessed. This analysis revealed a significant decrease in the percentage of KSL cells that fell within the SP in the *nfκb1*$^{-/-}$ *mice*. However, there was no significant difference in the proportion of KSL cells within the main population (MP) (Figure 3.5A and B). This would suggest that there is a decrease in quiescent HSCs within the bone marrow of *nfκb1*$^{-/-}$ mice.

3.6 Comparative analysis of properties of wild type and *nfκb1*$^{-/-}$ bone marrow HSCs

3.6.1 Cell cycle analysis of wild type and nfκb1$^{-/-}$ *bone marrow haemopoietic stem cells*

Since it is believed that the side population includes quiescent HSC, and the SP analysis showed a decrease of *nfκb1*$^{-/-}$ KSL cells within this fraction, we decided to study the cell cycle

status of wild type and *nfκb1*$^{-/-}$ HSCs in order to gain some understanding about their proliferative capacity. BrdU incorporation assays were performed *in vitro* and *in vivo.*

For the *in vitro* BrdU incorporation assay, KSL cells from the bone marrow of wild type and *nfκb1*$^{-/-}$ mice were sorted and plated in IMDM containing BrdU (section 2.2.3.2) at 37ºC. After labelling with BrdU, the cells were collected, fixed and incubated overnight with primary rat anti-BrdU antibody. The cells were finally stained with secondary Alexa Fluor488 labelled anti-rat IgG and mounted using DAPI-containing Vectashield. Cells that were positive for BrdU, as viewed by confocal microscopy, were counted as proliferating KSL cells.

For the *in vivo* BrdU incorporation assay, BrdU was injected intra-peritoneally into the mice which were then sacrificed after 24 hours. Bone marrow cells were sorted for KSL cells, followed by staining with antibodies as described for the *in vitro* BrdU assay.

The *in vitro* BrdU incorporation assay (Figure 3.6A) showed that there was a small increase in the percentage of BrdU positive KSL cells in *nfκb1*$^{-/-}$ compared to the wild type. However, this increase was not statistically significant as there was a high degree of variation from mouse to mouse. The *in vivo* BrdU incorporation assay showed no differences in the proliferative capacity of the *nfκb1*$^{-/-}$ compared to the wild type KSL cells (Figure 3.6B).

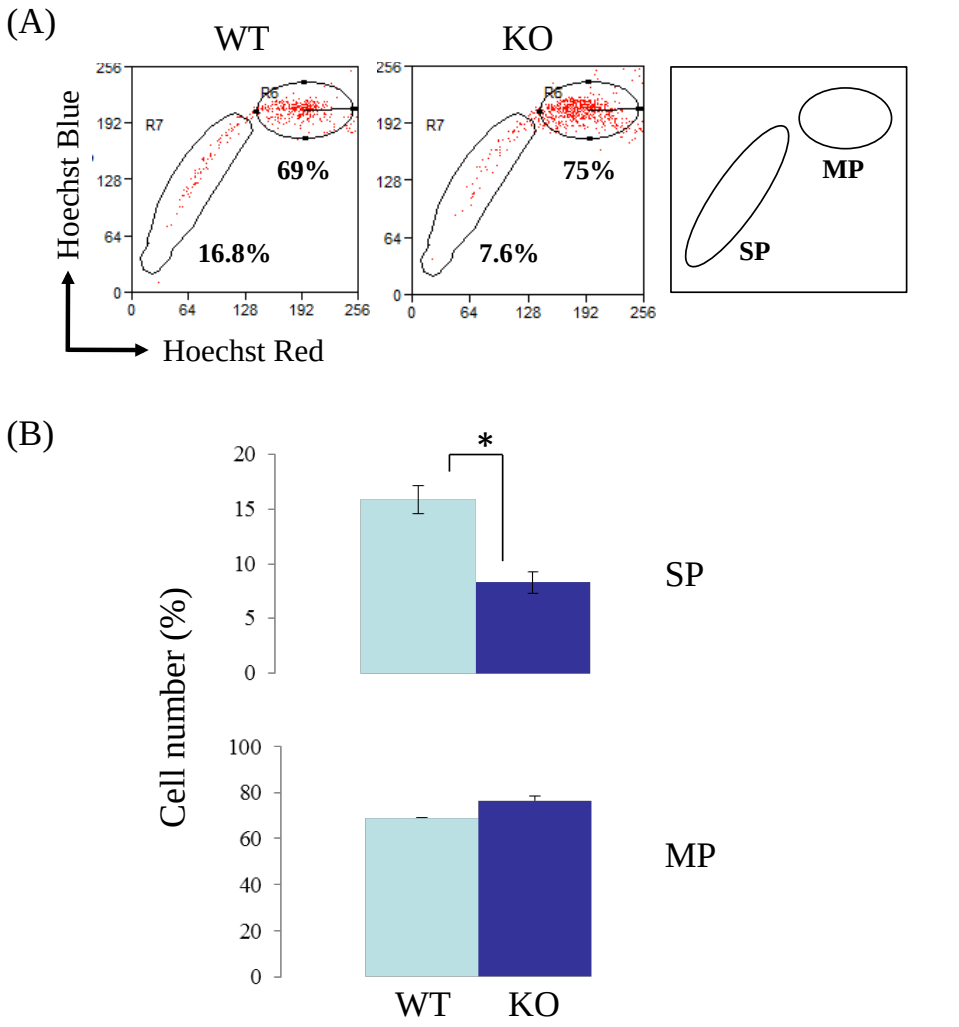

Figure 3.5: Analysis of bone marrow from wild type and *nfκb1*-/- mice using the DNA binding dye Hoechst 33342. (A) The bone marrow of wild type (WT) and *nfκb1*-/- (KO) mice of 8-10 weeks of age was extracted and the red cells lysed. Cells were then stained with Hoechst 33342 as previously described (Goodell et al., 1997) and subsequently co-stained with a cocktail of antibodies against lineage-specific surface markers (CD5, CD8a, CD11b, Gr1, Ter119 and B220) and the stem cell markers c-Kit and Sca-1. The Hoechst 33342 profile analysis was performed following gating for forward scatter (FSC) versus side scatter (SSC), focussing on the lineage negative and KSL fractions. The schematic indicates the position of the side population (SP) and the major population (MP). (B) The histogram represents the average percentage of cells in the SP and the MP gate with bars indicating the SEM (n=4). * indicates significance ($P<0.05$)

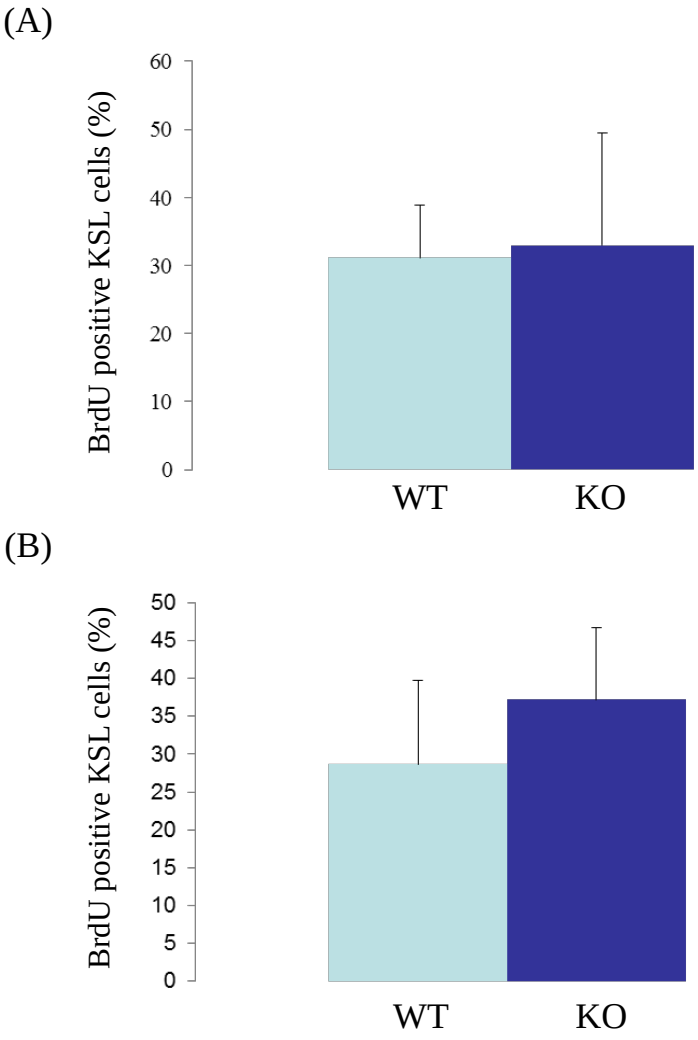

Figure 3.6: Analysis of the proliferation of HSCs from wild type and *nfκb1*$^{-/-}$ mice. (A) Bone marrow cells from wild type (WT) and *nfκb1*$^{-/-}$ (KO) mice at 8-10 weeks of age was extracted and the red cells lysed. KSL cells were sorted and placed in a liquid culture for 12 hours in IMDM containing BrdU. After labelling, the cells were collected, fixed and denatured. After blocking, the cells were incubated overnight with the primary monoclonal rat anti-BrdU antibody. One day later the cells were washed and fixed with paraformaldehyde and quenched with NH_4Cl and finally incubated for 2 hours with AlexaFluor 488 donkey anti-rat. The cells were mounted with DAPI-containing Vectashield and images were acquired using the Zeiss LSM 510 Meta confocal fluorescence microscope. (B) For *in vivo* BrdU incorporation assay, mice were injected intra-peritoneally with BrdU and sacrificed after 24 hours. Bone marrow cells were sorted for KSL cells, and stained as described in (A) (n=4) $P>0.05$

3.6.2 Colony forming potential

In order to compare the proliferation and differentiation potential of wild type and *nfκb1*$^{-/-}$ HSCs *in vitro*, colony assays were performed using sorted KSL cells. On first plating, both wild type and *nfκb1*$^{-/-}$ KSL cells formed approximately the same number of colonies (Figure 3.7A), the small decrease in *nfκb1*$^{-/-}$ colonies mirrors the slight decrease in the total number of cells recovered from the tissue culture plates (Figure 3.7C). After performing several independent experiments, it was concluded that there were no significant changes in the generation of colony types (Figure 3.7 B, D and E). Upon secondary replating, the colony forming capacity of the *nfκb1*$^{-/-}$ cells was significantly impaired compared to the wild type, giving rise to 2.4-fold fewer colonies than the wild type KSL cells (Figure 3.8A). Moreover, the size of colonies was smaller with *nfκb1*$^{-/-}$ cells compared to the wild type (Figure 3.8C), which was also reflected in the total number of cells recovered from the tissue culture plates (Figure 3.8B). On tertiary replating, *nfκb1*$^{-/-}$ cells were not able to form proper colonies and instead spread over the plate. These looked more dispersed and differentiated (Figure 3.9B), and this explains the high number of cells that was collected from the *nfκb1*$^{-/-}$ plates (Figure 3.9A). In contrast, the wild type cells retained their clonogenic potential.

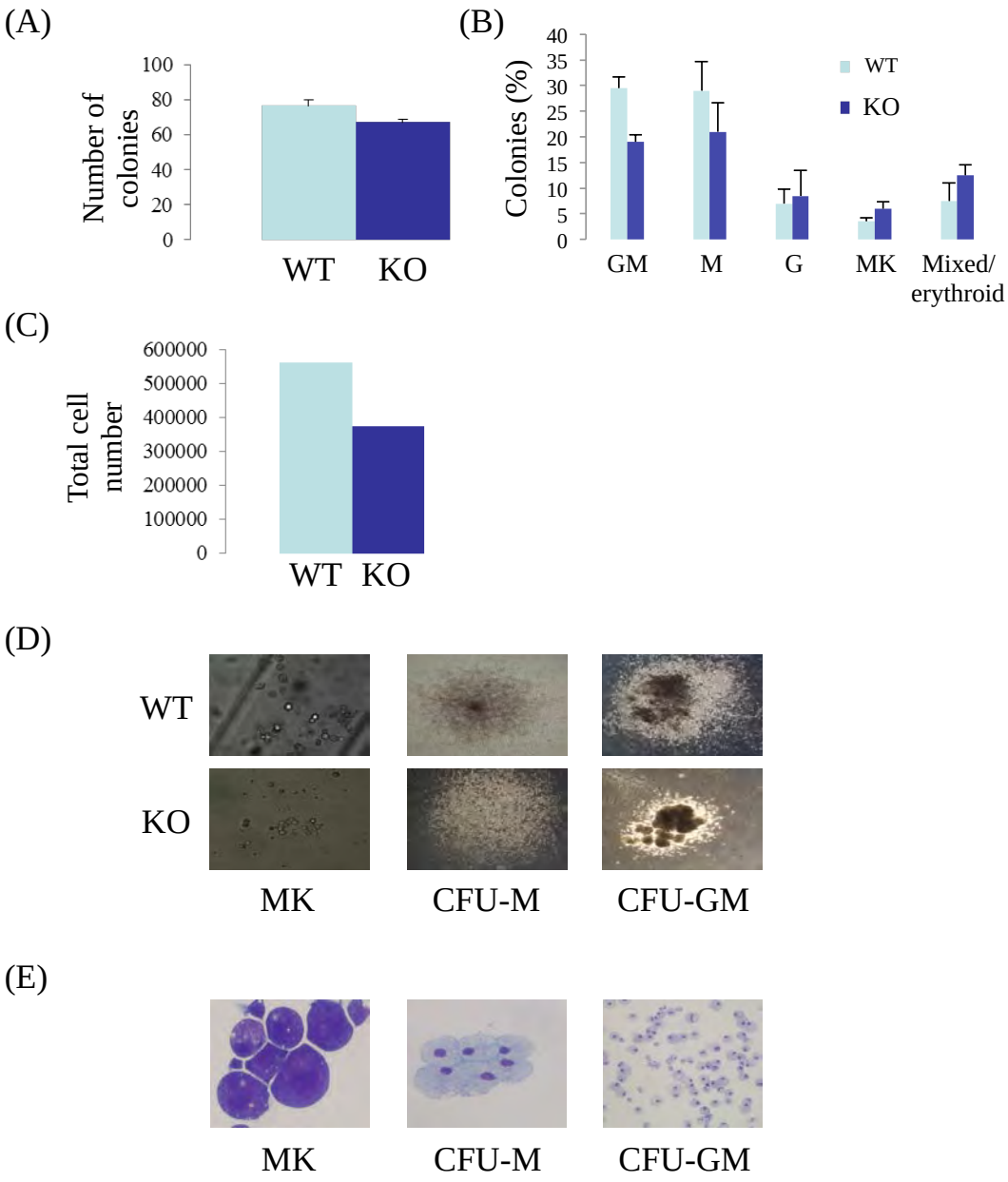

Figure 3.7: Analysis of the primary colony forming potential of wild type and *nfκb1*$^{-/-}$ KSL cells. 350 KSL cells of wild type (WT) and *nfκb1*$^{-/-}$ (KO) mice were plated in semisolid methylcellulose medium (Methocult M3434) supplemented with TPO. (A and B) Histograms show the number and morphology of colonies counted after 7 days. (C) For assessment of the total number of cells within each plate all cells were collected and counted. (D) Pictures of colonies were taken at 5x magnification. (E) Following cytospin, cells were stained with Diff-Quick (n=5) P>0.05.

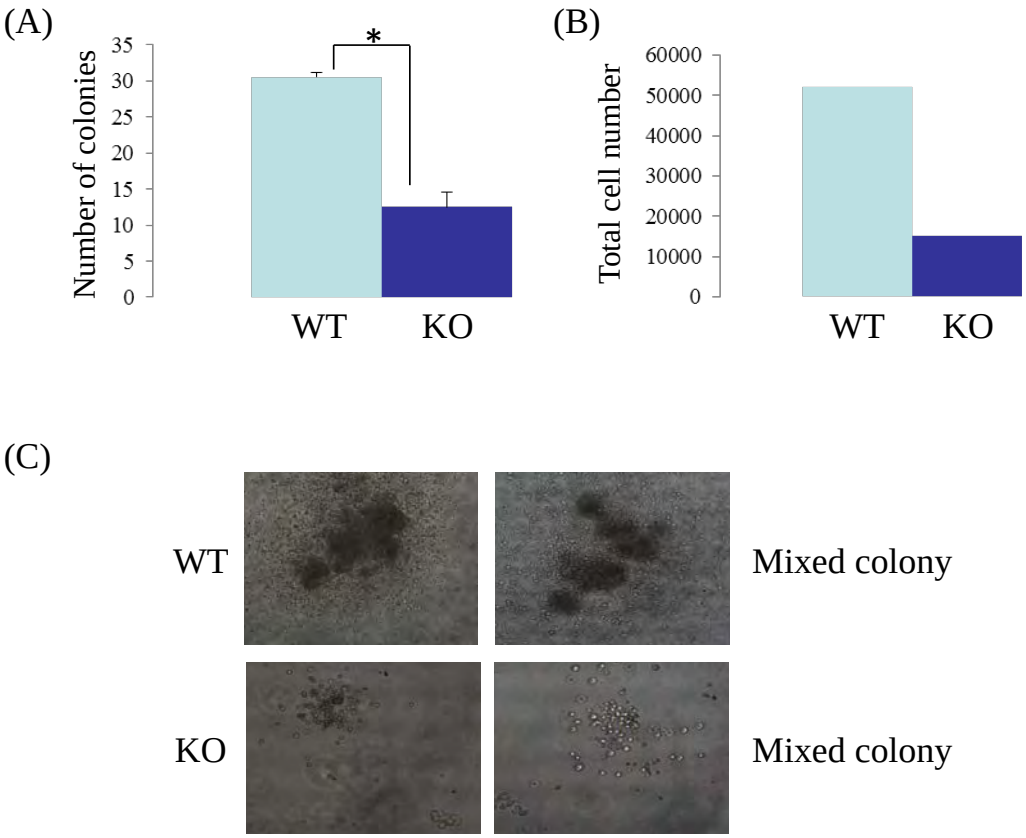

Figure 3.8: Analysis of secondary colony forming potential of wild type and *nfκb1*$^{-/-}$ KSL cells. For secondary replating all cells primary colony assay plates were collected, counted and 5000 cells were replated in fresh methylcellulose, and number of colonies and number of cells was assessed after 7 days. All cultures were incubated at 37°C in a fully humidified 5% CO_2 in air atmosphere. (A) Histograms show the number of colonies counted after 7 days. (B) For assessment of the total number of cells within each plate all cells were collected and counted. (C) Pictures of colonies were taken at 5x magnification. * indicates significance ($p<0.05$)

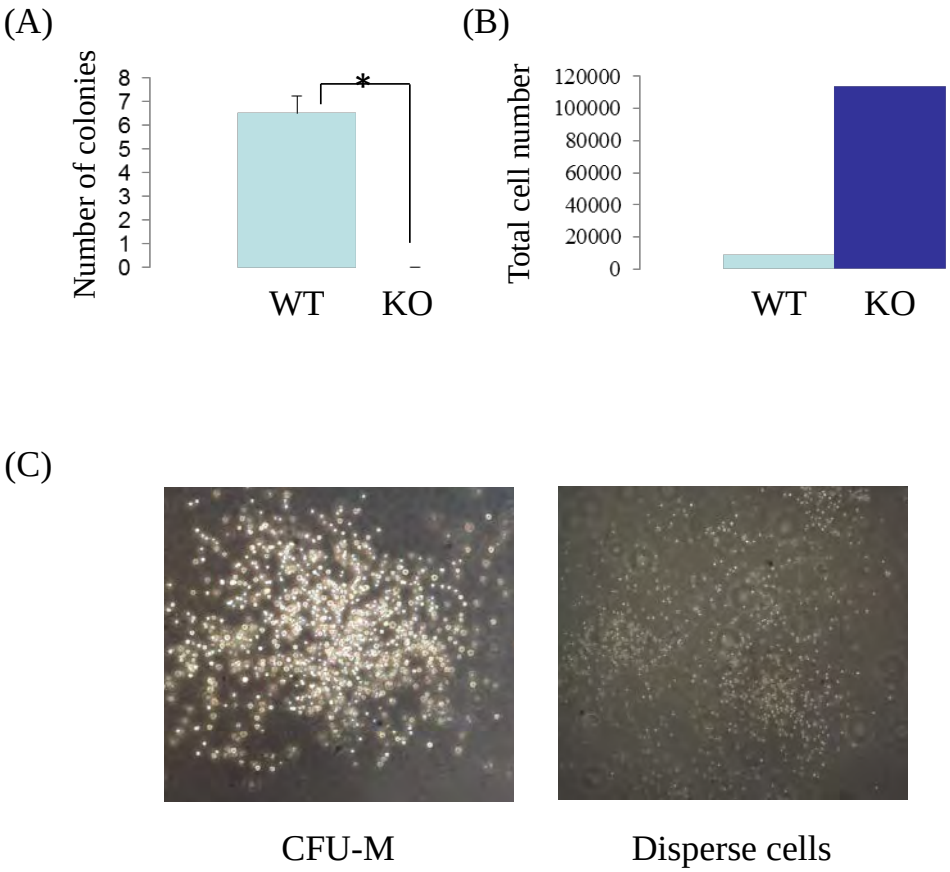

Figure 3.9: Analysis of tertiary colony forming potential of wild type and *nfκb1*$^{-/-}$ KSL cells. For tertiary plating, all cells from secondary colony forming assay plates were collected, counted and 5000 cells replated in fresh methylcellulose and number of colonies and number of cells were assessed after 7 days. All cultures were incubated at 37°C in a fully humidified 5% CO_2 in air atmosphere. (A) The histogram shows the number of colonies within each plate. (B) For assessment of the total number of cells within each plate all cells were collected and counted. (C) Pictures of colonies, taken at 5x magnification. * indicates significance ($p<0.05$)

3.7 RNA analysis

The mild phenotypic differences seen in the *nfκb1*$^{-/-}$ KSL cells suggest that the continued expression of the other NF-κB family members might partially compensate for the absence of NF-κB1. To assess this, expression of the other NF-κB genes was assessed. RT-PCR analysis (Figure 3.10) revealed that *nfκb1* gene deletion increased c-rel expression by up to 6-fold in KSL cells. In contrast, *nfκb2* gene expression was hardly affected, whereas the relA gene appeared to be expressed at lower levels in the *nfκb1*$^{-/-}$ KSL cells compared to the wild type.

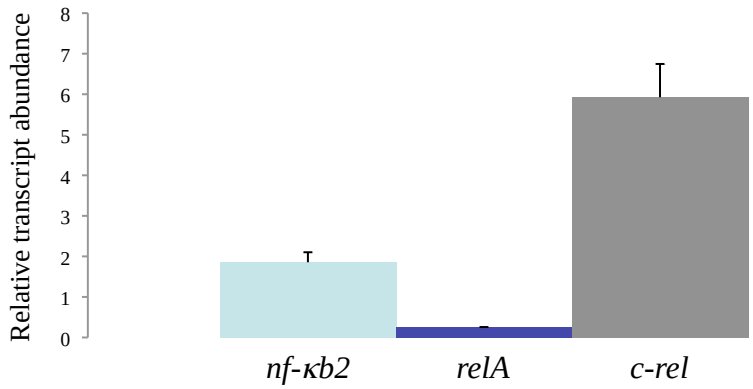

Figure 3.10: Expression of NF-κB family genes in wild type versus *nfκb1*-/- KSL cells. RNA was extracted from KSL cells from the bone marrow of wild type and *nfκb1*$^{-/-}$ mice and converted into cDNA. Real time PCR was then performed on the cDNA using SYBR Green and primers specific for the *nf-κb* family members. The cDNA was normalised using the HPRT gene as a control. The figure represents results obtained from two experiments and shows the abundance in *nf-kb1* knockout cells relative to the wild type. Each PCR was performed in triplicate and the error bars represent the SEM.

3.8 Assessment of *nfκb1*$^{-/-}$ HSC function by transplantation into lethally irradiated recipients

The gold standard test of HSC function is to demonstrate their ability to reconstitute lethally irradiated recipient mice. Furthermore, by performing transplantations using combinations of wild type and mutant KSL cells, it is possible to assess the relative HSC potency.

Recipient mice (BoyJ CD45.1/CD45.1) between 8-10 weeks in age were lethally irradiated (900 Gy) and injected via the tail vein with 10^6 whole bone marrow reference BoyJ:B6F1 CD45.2/CD45.1 cells and 500 sorted KSL donor cells either from wild type or *nfκb1*$^{-/-}$ mice (B6 CD45.2/CD45.2). The reference cells act as both an internal control and to ensure survival of the recipient animals. Host and donor cells were distinguished using different alleles of the CD45 surface antigen, which is expressed on all haemopoietic cells except mature erythrocytes and platelets. Specific antibodies were used against CD45.1 for the recipient, and against CD45.2 on the donor cells. Recipient mice receiving such mixed donor transplantations provide a very sensitive way of comparing the behaviour and potency of wild type and knockout HSC. Cell engraftment was determined by assessment of the proportion of test and reference donor cells in the peripheral blood at various time points following transplantation. At three to six months post transplantation, the recipients were sacrificed. The bone marrow was extracted, analysed and 10^6 total bone marrow cells were transplanted into lethally irradiated BoyJ recipients. Table 3.2 presents a summary of the sets of primary transplantations that were performed, displaying the number of donor wild type and *nfκb1*$^{-/-}$ mice (always littermates) and the number of recipients that were injected with each of the donor KSL populations. The donor to reference (D:REF) ratios in the peripheral blood

samples are presented together with the equivalent ratio in the bone marrow at the end of the experiment when the recipients were sacrificed.

3.8.1 Peripheral blood analysis

Peripheral blood analysis of animals transplanted with *nfκb1*$^{-/-}$ KSL cells revealed two different phenotypes. When peripheral blood was analysed at 4, 8, 12, and 16 weeks, half of the *nfκb1*$^{-/-}$ transplants did not exhibit reconstitution (*nfκb1*$^{-/-}$ non-engrafting), while the others showed good engraftment (*nfκb1*$^{-/-}$ engrafting). Figure 3.11 represents an analysis of transplantations from Set 2. The *nfκb1*$^{-/-}$ non-engrafted recipients all received KSL cells from the same knockout donor while the *nfκb1*$^{-/-}$ engrafted recipients all received KSL cells from another knockout littermate. This result has been confirmed in three independent experiments using different donor and host mice (Table 3.2). Furthermore, analysis of the different lineages revealed that transplanted KSL cells from *nfκb1*$^{-/-}$ mice were able to differentiate towards all B-, T- and myelomonocytic cells without any deficiency. Hence, staining for markers of B-cells (B220/CD43), T-cells (CD4/CD8) and myelomonocytic cells (Gr1/CD11b) showed that there was no difference in the development of these lineages from the transplanted *nfκb1*$^{-/-}$ KSL cells compared to WT KSL cells (Figure 3.12).

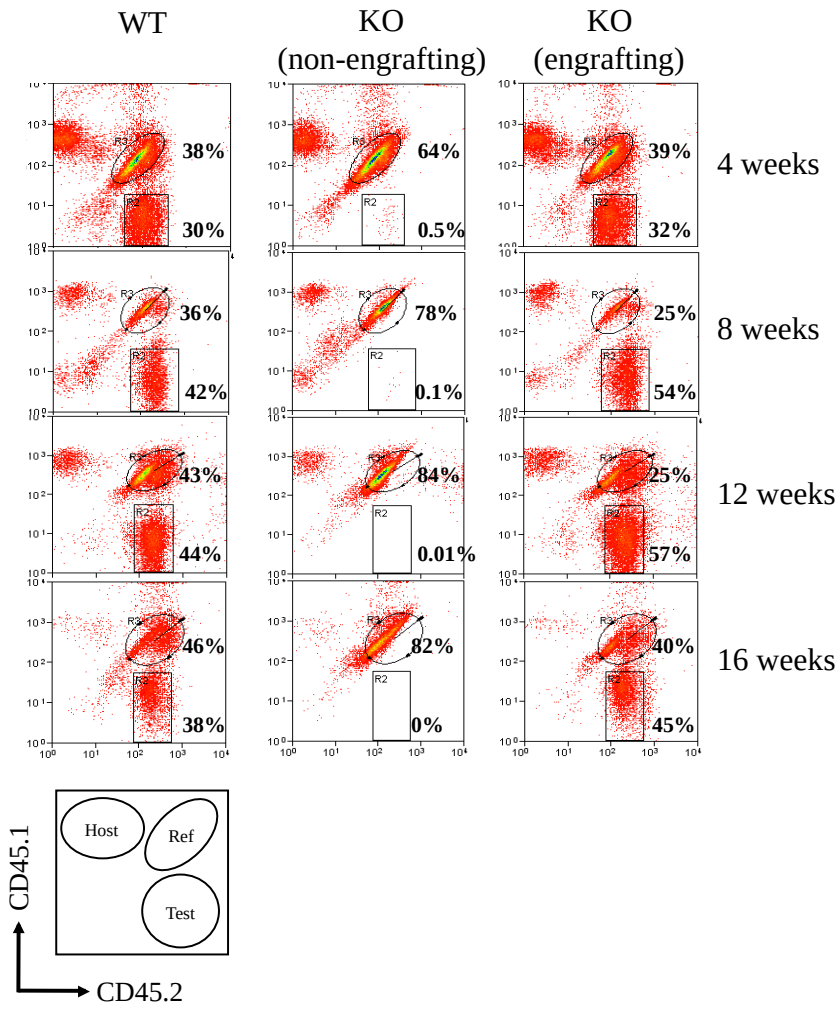

Figure 3.11: Transplantation of wild type and *nfκb1-/-* KSL cells into lethally irradiated recipients. For competitive repopulation assays recipient mice (B6:SJL, CD45.1/CD45.1) were lethally irradiated (900 Gy) and injected via the tail vain with 1x10^6 reference wild type ACK-treated whole bone marrow cells (B6XB6:SJL F1, CD45.1/CD45.2) together with 500 KSL donor cells from either wild type (WT) or nfκb*1-/-* (KO) mice (C57BL6/J, CD45.2/CD45.2). The figures show analysis of peripheral blood samples at 4, 8, 12 and 16 weeks following transplantation using anti-CD45.1 and anti-CD45.2 to distinguish donor and reference cells. The schematic indicates the expected positions of the donor (CD45.2/CD45.2), reference (CD45.1/ CD45.2) and host (CD45.1/CD45.1) populations.

3.8.2 Bone marrow analysis

It is important to analyse the bone marrow of the primary recipients to evaluate the extent of engraftment and to assess the profile of the HSC compartment that develops following transplantation. Thus, at 20 or 27 weeks post-transplantation, the primary recipients were sacrificed and their bone marrow was analysed. Bone marrow cells were also used for transplantation into secondary irradiated recipient mice.

To confirm that recipient mice transplanted with *nfκb1*$^{-/-}$ donor KSL cells revealed two different phenotypes in the bone marrow as seen in the peripheral blood, cells were stained with antibodies against CD45.1 and CD45.2. As expected from the peripheral blood analyses, half of the *nfκb1*$^{-/-}$ transplants did not exhibit reconstitution (*nfκb1*$^{-/-}$ non-engrafting) while the others showed good engraftment (*nfκb1*$^{-/-}$ engrafting) in the bone marrow that was comparable to a wild type engraftment (Figure 3.13). To assess if there was any comparison of the observed phenotypes in the adult *nfκb1*$^{-/-}$ mice to the recipients that were transplanted with *nfκb1*$^{-/-}$ KSL cells, an analysis of progenitor and KSL compartments was performed by immunofluorescent flow cytometry. Analysis of the lineage negative fraction revealed a small increase in the mice receiving knockout cells compared to those transplanted with the wild type cells. However, there were no significant differences in the progenitor populations (GMP, CMP and MEP) in the recipients receiving *nfκb1*$^{-/-}$ KSL cells compared to the wild type (Figure 3.14B), even though the adult bone marrow of the *nfκb1*$^{-/-}$ mice exhibited a significantly lower number of GMPs and CMPs and a significant increase in the MEP population (Figure 3.2). Similar to the knockout donor mice, there was an increase in the KSL population amongst the donor population in the recipient animals, however this was not statistically significant. Similar to the adult bone marrow (Figure 3.3), very small differences

Table 3.2: Primary transplantations of wild type versus *nfκb1*$^{-/-}$ bone marrow HSCs

			Ratio (D:REF)						
Set	**Mouse number**	**CD45.2 (Donor) KSL cells**	**4 weeks**	**8 weeks**	**12 weeks**	**16 weeks**	**20 weeks**	**27 weeks**	**BM Analysis**
1	13	WT1	0.45	1	1.2				1.45
	14	WT2	0.33	0.68	1.04				1.2
	15	*nfκb1*$^{-/-}$ 1	0.02	0.01	0.05				0.1
	16	*nfκb1*$^{-/-}$ 1	0.01	0.01	0.16				0.4
	17	*nfκb1*$^{-/-}$ 2	0.04	0.07	0				0
	19	*nfκb1*$^{-/-}$ 2	0.03	0.06	0.01				0
2	20	WT3	0.7	1.46	1.86	3.1		1.7	6
	21	WT4	0.5	1.86	1.28	0.97		1	1.08
	22	WT4	0.78	1.18	1.0	0. 82		1.1	1.9
	23	*nfκb1*$^{-/-}$ 3	0.01	0	0	0		0	0
	24	*nfκb1*$^{-/-}$ 3	0	0	0	0		0	0
	25	*nfκb1*$^{-/-}$ 4	0.48	0.16	0.3	0.58		0.3	0.5
	26	*nfκb1*$^{-/-}$ 4	3.8	5.4	2.28	1.12		1.3	0.7
	27	*nfκb1*$^{-/-}$ 4	0.79	2.2	2.9	2.25		1.6	1.29
3	30	WT5	0.24	0.29	1.86		0.93	1.7	6
	31	WT6	0.28	0.87	1.28		2.79	1.93	1.08
	32	WT6	0.17	0.42	1.0		0.37	1.1	1.9
	33	*nfκb1*$^{-/-}$ 5	0	0	0		0	0	0
	34	*nfκb1*$^{-/-}$ 5	0	0	0		0	0	0
	35	*nfκb1*$^{-/-}$ 5	0	0	0.3		0	0	0
	36	*nfκb1*$^{-/-}$ 6	0.28	0.12	2.28		0.38	0.3	0.5
	37	*nfκb1*$^{-/-}$ 6	0.3	0.98	2.9		0.9	1.3	0.7
	38	*nfκb1*$^{-/-}$ 6	0.58	0.84	1.52		2.2	1.6	1.29

The table details the sets of transplantations performed. The individual donors and recipients used in each set are indicated, as are the ratios of the donor to reference (D:REF) engrafted cells in the peripheral blood at various time points and when the bone marrow was eventually analysed.

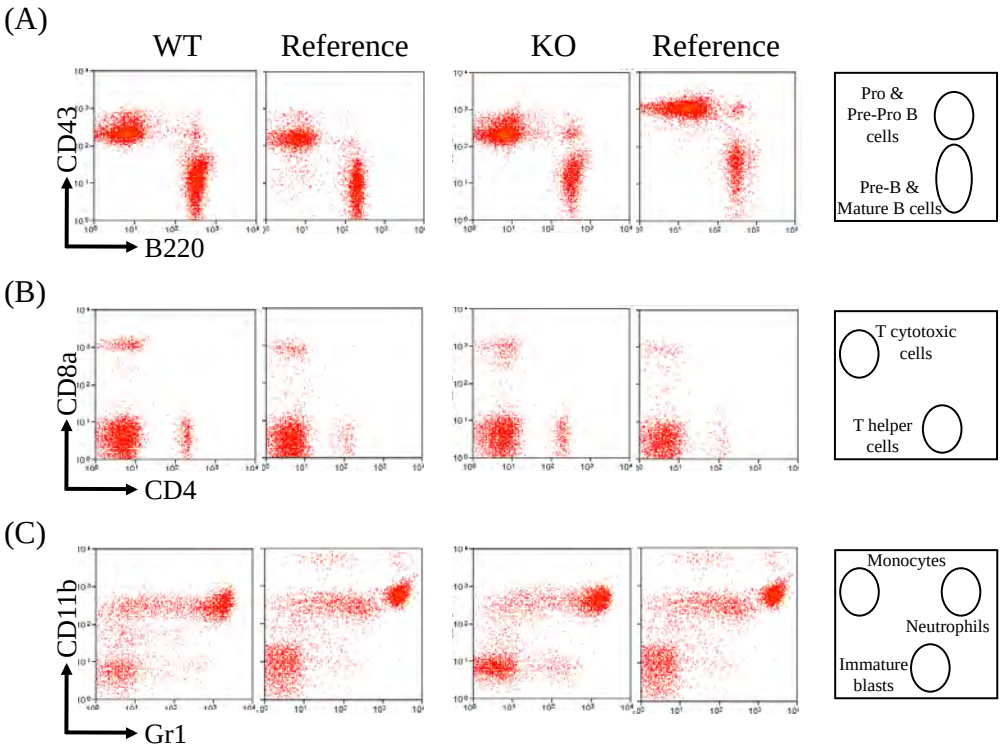

Figure 3.12: Analysis of lymphoid and myeloid lineages in the peripheral blood of engrafted recipients transplanted with wild type and *nfκb1-/-* sorted bone marrow KSL cells. At 12 weeks post transplantation peripheral blood samples were collected from recipient mice numbers 20 and 27. Following red cell lysis the samples were stained with antibodies against lymphoid- and myeloid-associated surface markers. Samples were gated on forward scatter (FSC) and side scatter (SSC) to select live cells and gated for host (CD45.1/ CD45.1), reference (CD45.1/CD45.2) and donor/test (CD45.2/CD45.2) populations. (A) B-cell markers B220 and CD43. The schematic indicates the positions for pro- and pre-pro B cells, pre B cells and mature B cells. (B) T-cell markers CD4 and CD8a. The schematic indicates the expected positions for T-helper (T_H) and T-cytotoxic (T_C) cells. (C) Myelomonocytic cell markers Gr1 and CD11b. The schematic indicates the positions for immature blasts, monocytes and neutrophils.

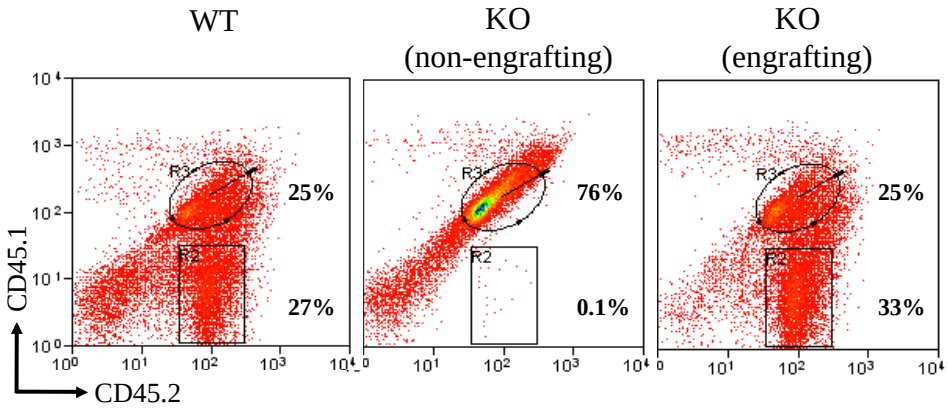

Figure 3.13: Analysis of bone marrow cells from recipients transplanted with wild type and *nfκb1*$^{-/-}$ donor sorted KSL cells. Bone marrow was extracted and red cells lysed from femurs and tibias of primary recipients receiving 500 KLS cells either from wild type (WT) or *nfκb1*$^{-/-}$ (KO) mice at 20 weeks post-transplantation. The dot plots represent the engraftment of recipient mice numbers 21, 24 and 27. Bone marrow cells were analysed using anti-CD45.1 and anti-CD45.2 to distinguish between donor and reference cells.

were observed in the recipient animals in the proportion of MPP and ST-KSL populations in the *nfκb1*$^{-/-}$ compared to the wild type donor populations (Figure 3.15 C and D). Donor *nfκb1*$^{-/-}$ LT-KSL cells were lower compared to the donor wild type equivalent in the engrafted recipients (Figure 3.15A). The side population of bone marrow HSCs was shown to be lower in *nfκb1*$^{-/-}$ compared to wild type mice (Figure 3.4). To assess if this phenotype was maintained following transplantation of KSL cells, the side population was analysed. This revealed that transplantation of *nfκb1*$^{-/-}$ KSL cells into the wild type environment of the recipients did not promote any adoption of the KSL side population phenotype (Figure 3.16); however, the decrease in the donor *nfκb1*$^{-/-}$ KSL side population was not statistically significant in comparison to that seen in the adult *nfκb1*$^{-/-}$ mice.

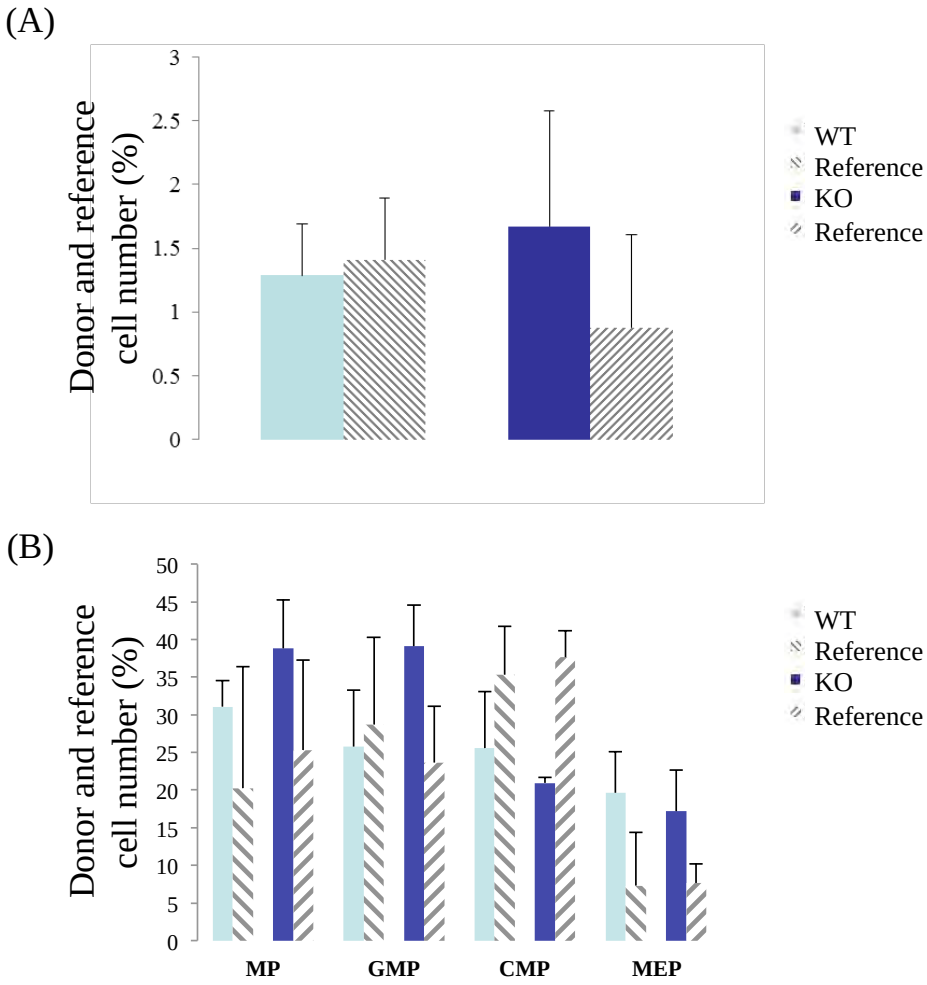

Figure 3.14: Immunofluorescence analysis of engrafted progenitor cells in primary recipient bone marrow. Bone marrow cells from primary transplanted recipients of wild type (WT:REF) and *nfkb1-/-* (KO:REF) mice were stained with a cocktail of antibodies against lineage associated surface markers (CD5, CD8a, CD11b, Gr1, Ter119 and B220). The histogram represents the average percentage of lineage negative cells from at least 4 WT:REF and 4 KO:REF transplanted mice. The bars indicate the SEM. (B) Using the stem cell markers c-Kit and Sca-1 in combination with lineage negativity and CD16/32 and CD34 antibodies the progenitor populations were analysed containing the granulocyte / monocyte progenitor (GMP), common myeloid progenitor (CMP) and megakaryocyte / erythroid progenitor (MEP). The histogram represents the average percentage of the different progenitor cell populations from WT:REF and KO:REF transplanted mice. The bars indicate the SEM. P>0.05

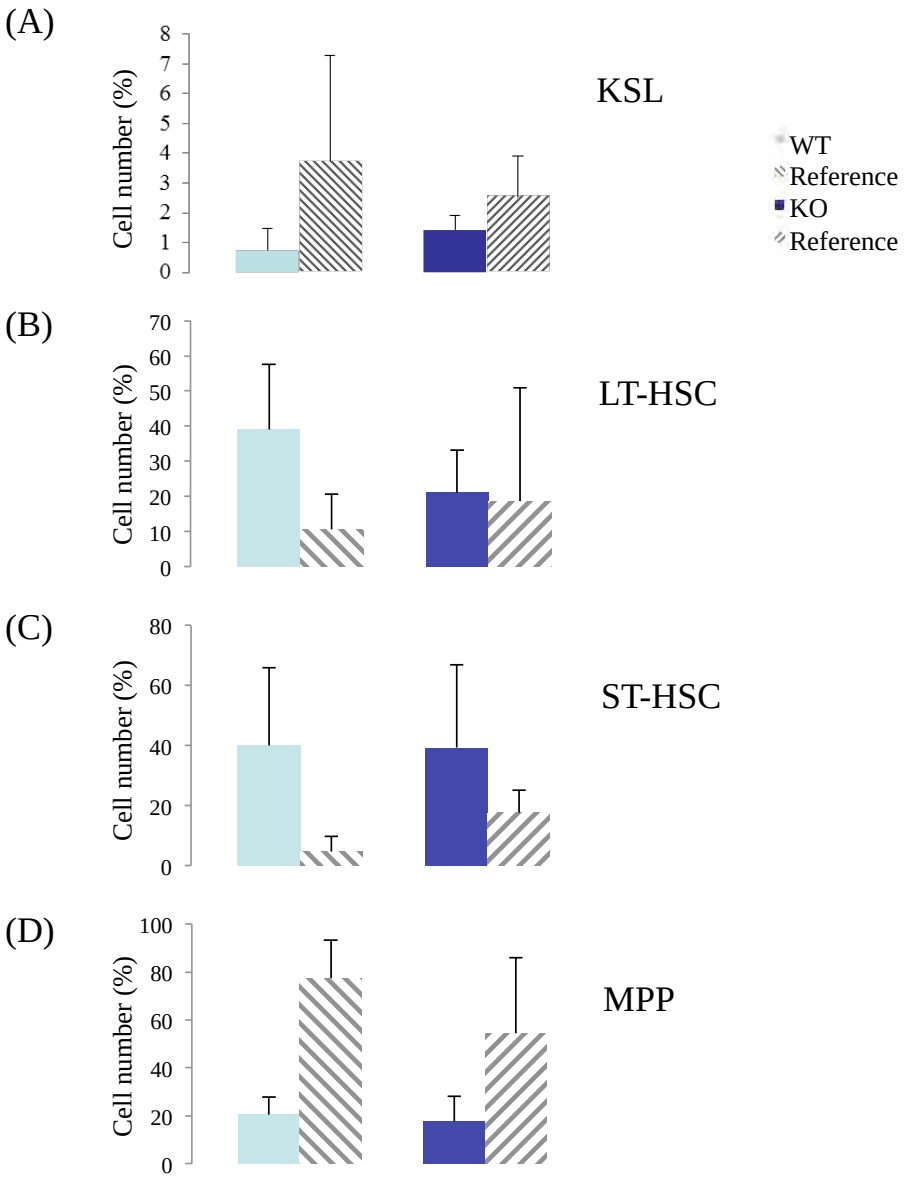

Figure 3.15: Immunofluorescence analysis of engrafted stem cells in primary recipient bone marrow. (A) The bone marrow from recipients of wild type (WT:REF) and *nfκb1*$^{-/-}$ (KO:REF) was stained with a cocktail of antibodies against lineage associated surface markers (CD5, CD8a, CD11b, Gr1, Ter119 and B220) and stem cell markers c-Kit and Sca-1. The histograms represents the average percentage of of donor (WT or KO) and reference (REF) KSL cells in transplanted mice. The bars indicate the SEM. (B) (C) and (D) KSL cells co-stained with Flt3 and CD34. The histograms represents the average percentage of donor (WT or KO) and reference (REF) LT-HSC, ST-HSC and MPP cells in transplanted mice. The bars show SEM (n=4) P>0.05

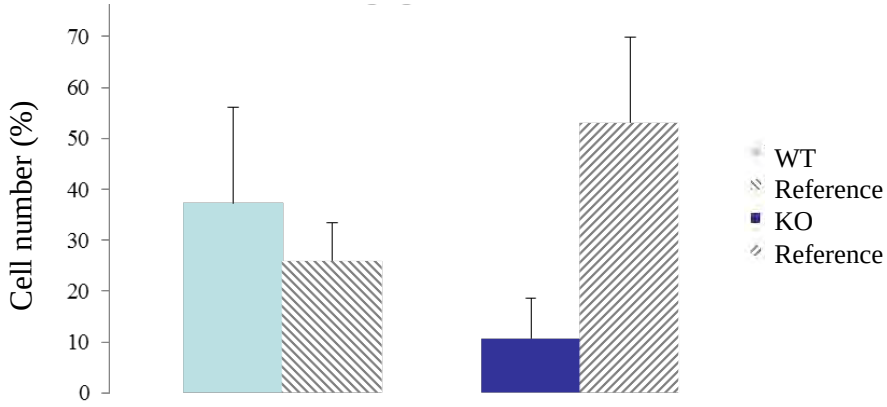

Figure 3.16: Analysis of bone marrow from wild type and *nfκb1*$^{-/-}$ primary recipients using the DNA binding dye Hoechst 33342. The bone marrow from recipients of wild type (WT:REF) and *nfκb1*$^{-/-}$ (KO:REF) was extracted, red cells lysed, then stained with Hoechst 33342 as previously described (Goodell et al, 1996) and subsequently co-stained with a cocktail of antibodies against lineage-specific surface markers (CD5, CD8a, CD11b, Gr1, Ter119 and B220) and stem cell markers c-Kit and Sca-1. The Hoechst 33342 profile analysis was performed following gating for forward scatter (FSC) versus side scatter (SSC), focussing on the lineage negative and KSL fractions. The histogram represents the average percentage of cells in the SP gate with bars indicating the SEM (n=3) P>0.05.

3.9 Secondary transplantation of *nfκb1*$^{-/-}$ bone marrow cells into lethally irradiated recipients

The most precise way to test HSC potential is to assess their ability to be serially transplanted from the primary reconstituted recipients into a secondary lethally irradiated host, demonstrating that the engrafted cells are undergoing self-renewal. Therefore, one million total bone marrow cells from primary recipients were transplanted into BoyJ (CD45.1) host mice. These bone marrow cells were a mixture of either wild type (CD45.2/CD45.2) and reference cells (CD45.1/CD45.2) or *nfκb1*$^{-/-}$ and reference cells. Peripheral blood samples were taken and analysed every 4 weeks to assess secondary engraftment using antibodies against CD45.1 and CD45.2. Analysis of the peripheral blood of the secondary recipients revealed the presence of wild type and *nfκb1*$^{-/-}$ donor cells in the host mice (Figure 3.17). The peripheral analysis at various time points of different recipients is presented in Figures 3.18, 3.19, and 3.20 and a summary of all recipients is presented in Table 3.3.

Analysis of the bone marrow of the secondary recipients proved problematic. Hence, using the surface markers CD45.1 and CD45.2 it was not possible to separate clearly donor or reference populations (See Appendix for further bone marrow analyses (Figure A5)). For that reason, it was decided not to analyse further the bone marrow of the secondary transplanted recipients.

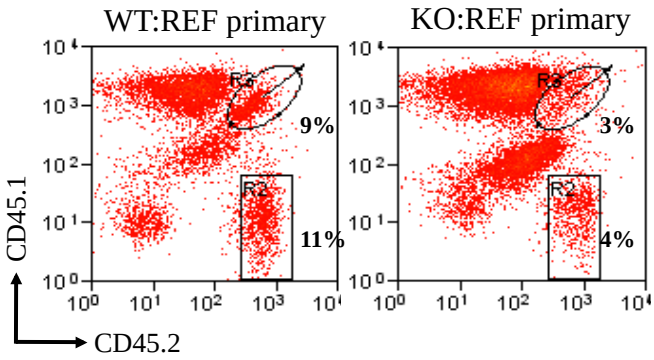

Figure 3.17: Immunofluorescence analysis of peripheral blood of secondary recipients of wild type and *nfκb1*$^{-/-}$ donor bone marrow cells. Peripheral blood samples were collected from secondary recipient mice numbers 51 and 59 that had received 1 x 10^6 wild type (WT:REF primary) or *nfκb1*$^{-/-}$ (KO:REF primary) test cells from primary recipient mice. Following red cell lysis the samples were stained with antibodies against CD45.1 and CD45.2.

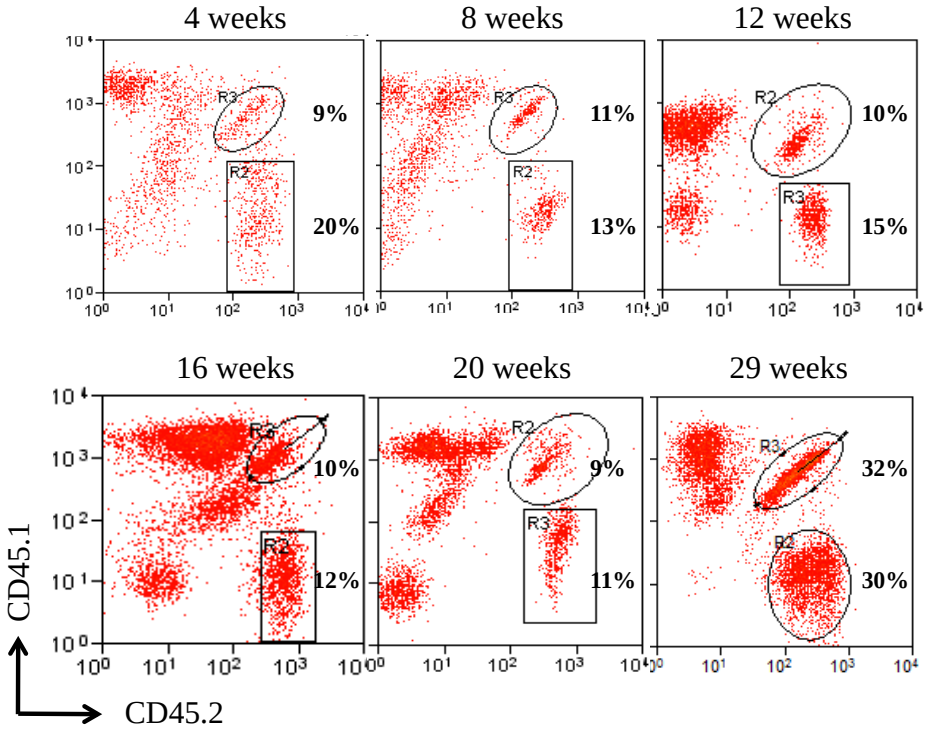

Figure 3.18: Immunofluorescence flow cytometry analysis of peripheral blood from secondary bone marrow transplant of WT:REF primary cells. Peripheral blood samples were collected at different time points from recipient mouse 51 that had received 1 x 10^6 bone marrow cells from primary WT:REF recipient number 30 (see Table 3.3). Following red cell lysis, the samples were stained with antibodies against CD45.1 and CD45.2 to distinguish between host (CD45.1/CD45.1), donor reference (REF) (CD45.1/CD45.2) and donor test (WT) (CD45.2/CD45.2) cells.

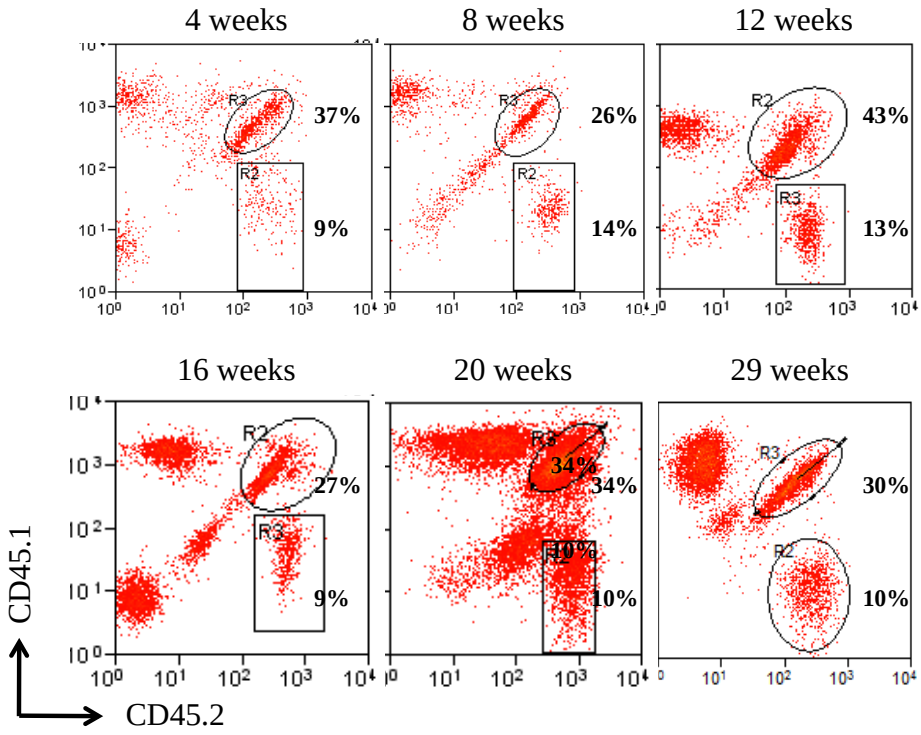

Figure 3.19: Immunofluorescence flow cytometry analysis of peripheral blood from secondary bone marrow transplant of KO:REF primary cells. Peripheral blood samples were collected at different time points from recipient mouse 55 that had received 1 x 10^6 bone marrow cells from primary KO:REF recipient number 36 (see Table 3.3). Following red cell lysis, the samples were stained with antibodies against CD45.1 and CD45.2 to distinguish between host (CD45.1/CD45.1), donor reference (REF) (CD45.1/CD45.2) and donor test (KO) (CD45.2/CD45.2) cells.

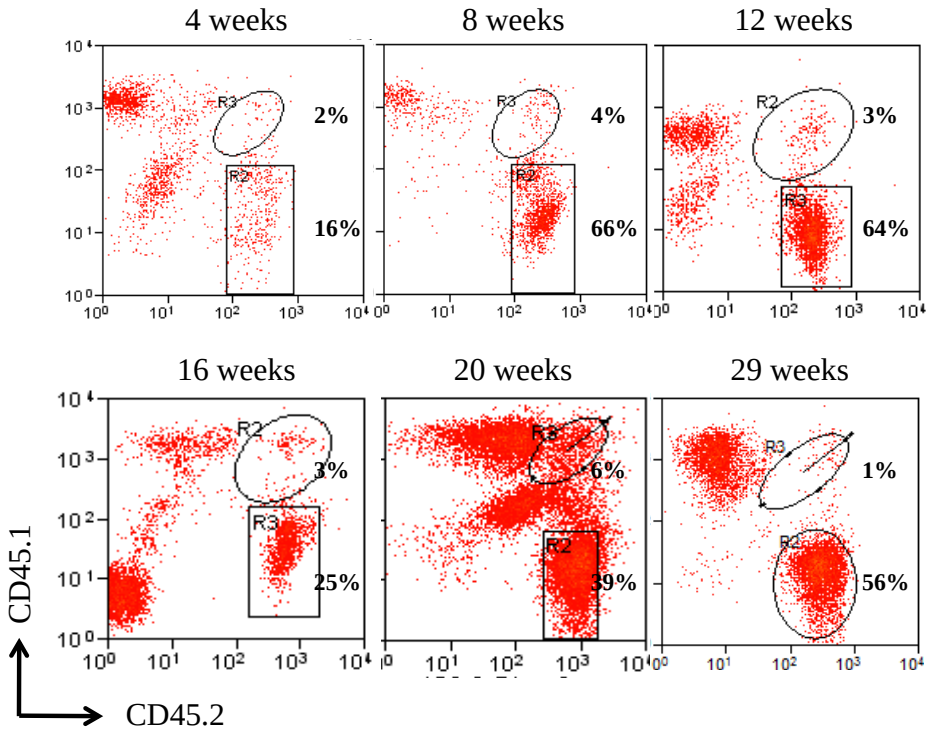

Figure 3.20: Immunofluorescence flow cytometry analysis of peripheral blood from secondary bone marrow transplant KO:REF primary cells. Peripheral blood samples were collected at different time points from recipient mouse 60 that had received 1 x 10^6 bone marrow cells from primary KO:REF recipient number 38 (see Table 3.3). Following red cell lysis, the samples were stained with antibodies against CD45.1 and CD45.2 to distinguish between host (CD45.1/CD45.1), donor reference (REF) (CD45.1/CD45.2) and donor test (KO) (CD45.2/CD45.2) cells.

		Ratio (D:REF)					
Mouse number	**Recipients from primary transplantation 1000000 cells**	**4 week**	**8 week**	**12 week**	**16 week**	**20 week**	**29 week**
49	WT 30 (WT:Ref primary)	0.83	1.7	1.18	1.6	1.8	1.64
51	WT 30 (WT:Ref primary)	2.22	1.1	1.5	1.2	1.2	0.93
52	WT 32 (WT:Ref primary)	2.72	1	0.45	0.5	0.5	0.56
53	WT 32 (WT:Ref primary)	1.31	0.35	0.5	0.24	0.4	0.44
55	*nfκb1*$^{-/-}$ 36 (KO:Ref primary)	0.2	0.5	0.3	0.3	0.3	0.33
59	*nfκb1*$^{-/-}$ 37 (KO:Ref primary)	6.75	12	2.6	1.6	1.3	4.56
60	*nfκb1*$^{-/-}$ 38 (KO:Ref primary)	8	16.5	21	8.3	6.5	77
61	*nfκb1*$^{-/-}$ 38 (KO:Ref primary)	6.2	2.5	2.6	1.5	2.4	1.1

Table 3.3: Secondary transplantation: Donors were taken from primary transplantation Set 3. Number of donors and recipients used in the transplantation, ratio of the donor to reference cell engraftments (D:REF) in the peripheral blood at various time points.

3.10 Discussion

The main aim of this project was to examine the role of the two transcription factors NF-κB1 and NF-κB2 in the maintenance and function of HSCs through analysis of the consequences of their individual ablation in genetically modified mice. The hypothesis of the study was that alteration of the expression of NF-κB proteins can disturb the normal balance of HSC proliferation and survival resulting in aberrant HSC function.

In order to investigate this hypothesis we first looked at the expression pattern of the NF-κB family members in an HSC cell line (HPC7) in comparison to primary murine HSCs (KSL

cells). Interestingly all five members of the family tested were expressed in HSCs, although RelB was expressed at a very low level. Based on the expression pattern, we decided to concentrate on the properties of HSCs in *nfκb1*$^{-/-}$ and *nfκb2*$^{-/-}$ mice, in both cases examining HSC quiescence, proliferation, and differentiation. Most importantly, serial bone marrow transplantation assays were performed in order to assess the true stem cell characteristics of the identifiable HSCs.

As a first step in characterising the knockout animals, haemopoietic cell populations in the bone marrow from *nfκb1*$^{-/-}$ mice were analysed by cell surface immunophenotyping, but this did not reveal any differences compared to wild type mice. Likewise, assessment of the different HSC compartments in the *nfκb1*$^{-/-}$ revealed no obvious alterations in the proportion of the LT-HSC, ST-HSC or MPP populations; although a 7 to 9 fold increase in the total KSL population was seen which might be due to decrese in apoptosis as the BrdU incorporation assay did not show any increase in proliferation. Analysis of the various myeloid progenitor compartments showed a significant decrease in the GMP and CMP populations and a significant increase in the MEP population. It has already been described that p50/p65 complex is essential for lymphopoiesis, whereas normal myeloid cells can develop in presence of either p65 or c-Rel containing complexes, in this case showing a potential of compensation (Liou, 2002). Thus, the significant increase in the *nfκb1*$^{-/-}$ MEP population might have resulted from compensation of NF-κB1 activity by other NF-κB members as we see in KSL cells (Figure 3.10).

The assessment of side population numbers within the KSL HSC compartment showed a significant decrease of *nfκb1*$^{-/-}$ cells within this fraction, and for that reason we decided to study the cell cycle status of wild type and *nfκb1*$^{-/-}$ HSCs in order to gain some understanding

about their proliferation capacity. But as mentioned before labelling of replicating cells by incorporation of BrdU revealed no significant differences between wild type and *nfκb1*$^{-/-}$ HSCs.

Since, bone marrow cells displaying side population characteristics have been shown to have the capability of long term repopulation in lethally irradiated recipients, we performed both *in vitro* serial replating assays and *in vivo* transplantations to study the self-renewing capacity of *nfκb1*$^{-/-}$ HSCs.

The replating assay of *nfκb1*$^{-/-}$ KSL cells showed a significant decrease in the number of colonies and changes in the colony sizes after the second round of replating and the large number of disperse differentiated cells over the plates after the third round. This suggests that the *nfκb1*$^{-/-}$ KSL cells have significantly reduced self renewal capacity compared to the wild type cells.

When tested by transplantation in vivo, the *nfκb1*$^{-/-}$ KSL cells exhibited two different patterns of engraftment. Thus, half of the transplantations showed no repopulation while the others repopulated efficiently, at a similar level to that seen for wild type cells. Given that the engraftments of knockout cells that failed corresponded to specific donor animals and that in these cases no engraftment was seen even at early time points, coupled with the fact that secondary transplantations always succeeded from those animals that had successfully engrafted with knockout cells suggests that the explanation lies in immune rejection. The donor mouse population had been extensively backcrossed to a C57BL6 background (at least 8 generations), so it seems reasonable to conclude that a rejection antigen is closely linked to the *nfκb1* locus. Another reason that might cause that half of the *nfκb1* donor cells are not transplanting is their sensitivity to the FACS machine. With other words cells might die

during the sorting procedure. If any of these explanations are correct, then the fact that half of the engraftments of knockout cells were very similar to those achieved with wild type cells implies that *nfκb1*$^{-/-}$ HSCs are functionally unaffected at this level.

CHAPTER 4

ANALYSIS OF THE ROLE OF NF-κB2 IN HAEMOPOIETIC STEM CELLS

4.1 Introduction

A recent studyng using RelB/NF-κB2 double knockout mice has shown the involvement of the non-canonical NF-κB signalling pathway in the regulation of haemaopoietic stem and progenitor cell (HSPC) self-renewal, expansion and lineage commitment through an influence on the bone marrow microenvironment (Zhao et al., 2012). The aim of the work described in this chapter was to investigate the function of NF-κB2 in HSC using single knockout mice. The analysis of RNA levels in HSCs described in Chapter 3 show that the NF-κB2 gene is expressed. To determine the importance of NF-κB2 activity in HSCs we performed a parallel set of experiments to those described in Chapter 3 for NF- κB1.

4.2 Comparison of haemopoietic cells in the bone marrow of wild type and *nfkb2*$^{-/-}$ mice

To increase our understanding about the importance of the transcription factor NF-κB2 within the haemopoietic cell types constituting the haemopoietic hierarchy, the bone marrow of *nfκb2*$^{-/-}$ mice was extensively characterised in comparison to that from wild type littermates. Flow cytometry using the antibodies against B220/CD43 and CD4/CD8a revealed no differences in the number of lymphoid cells, and similarly analysis of myeloid populations,

identified with antibodies against CD11b/Gr1 and c-Kit/CD41, did not show any change comparing knockout mice to wild type (Table 4.1and see Appendix Figure A3 and A4).

Cell type	Surface markers	Wild type versus *nfκb2* knockout
Lymphoid	B220/CD43 (B-cells) CD4/CD8 (T-cells)	Equivalent
Myelomonocytic	CD11b/Gr1	Equivalent
Erythroid	TER119/CD71/c-Kit	Equivalent
Megakaryocytic	c-Kit/CD41	Equivalent

Table 4.1: Immunofluorescent analysis of bone marrow cells from wild type versus *nfκb2*$^{-/-}$ mice. Bone marrow was flushed from the femur and tibias of mice aged 8-10 weeks. The table represents different surface markers that were used to study the different haemopoietic lineages. (n=6)

4.3 Analysis of the haemopoietic stem cell and progenitor compartments in wild type and *nfkb2*$^{-/-}$ mice

The fact that there is no difference in the number of different mature haemopoietic cells comparing wild type and knockout bone marrow does not necessarily mean that there is no difference in the progenitor and stem cell compartments. Therefore, the HSC compartments within the bone marrow of wild type and *nfκb2*$^{-/-}$ mice were analysed using a cocktail of antibodies against lineage-associated markers combined with antibodies against c-Kit and stem cell antigen Sca-1. The KSL compartment defined by this staining strategy was further subdivided into LT-KSL, ST-KSL and MPP using antibodies against CD34 and Flt3. The GMP, CMP and MEP committed progenitor populations were also analysed using an antibody against CD34 in combination with anti-CD16/32 (FcγRII/III). This latter analysis

revealed no differences in the number of progenitors between wild type and knockout mice (data not shown).

Staining with lineage markers revealed a statistically significant reduction in the proportion of lineage negative cells in the bone marrow of *nfκb2*$^{-/-}$ mice compared to the wild type littermates (Figure 4.1A). Despite this, within lineage negative cells the proportions of Lin^{-}c-Kit^{+}Sca-1^{-} and KSL populations were similar in *nfκb2*$^{-/-}$ and wild type mice (Figure 4.1B). However, sub-fractioning the KSL compartment by co-staining with antibodies against CD34 and Flt3 showed that there was a significant increase in the ST-HSC population and a significant decrease in the MPP population of *nfκb2*$^{-/-}$ mice compared to wild type. The LT-HSC population had very similar proportions between wild type and knockout animals (Figure 4.2).

4.4 Analysis of wild type versus *nfκb2*$^{-/-}$ KSL cells using a phenotypic assay of stem cells

As mentioned above, efflux of the DNA binding dye Hoechst is another way to characterise HSCs in murine bone marrow (Goodell et al., 1997), and this assay was used to evaluate the HSCs of *nfκb2*$^{-/-}$ mice. The bone marrow was extracted and treated to lyse red blood cells, then co-stained with Hoechst 33342 and antibodies to define the KSL population (Goodell et al., 1997). Forward scatter (FSC) and side scatter (SSC) were used to gate live cells. KSL cells from wild type and *nfκb2*$^{-/-}$ were gated and the distribution of the cells within the side population gate was assessed. This analysis showed a lower percentage of side population cells in the knockout KSL population. However, analysing several mice revealed that this

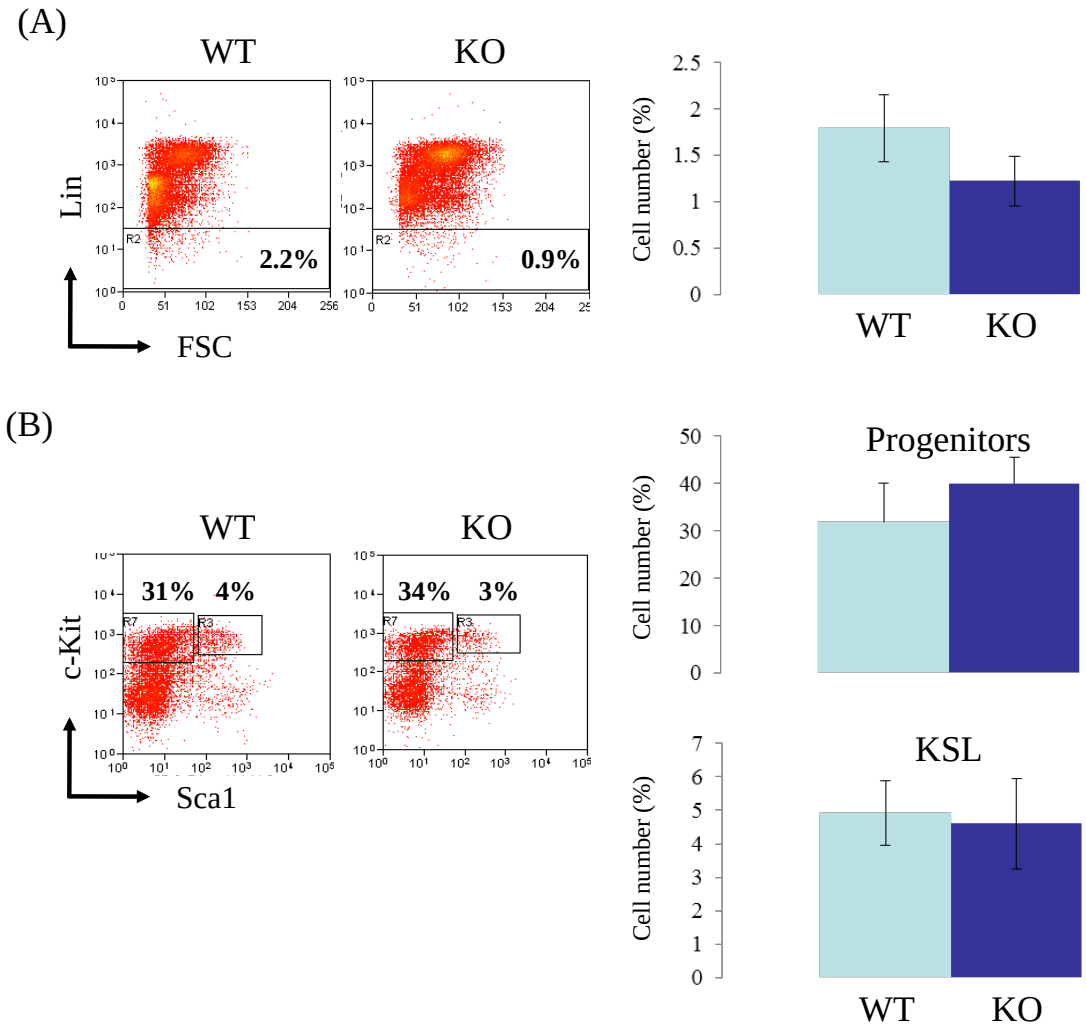

Figure 4.1: Immunofluorescence analysis of stem cell and progenitor populations in wild type and *nfκb2*$^{-/-}$ mice. The bone marrow from wild type (WT) and *nfκb2*$^{-/-}$ (KO) mice was extracted and the red cells lysed. Bone marrow cells were stained with a range of antibodies against lineage-committed markers (CD5, CD8a, CD11b, Gr1, Ter119 and B220). (A) Two-dimensional dot plots following gating of forward scatter (FSC) and side scatter (SSC) for live cells. The indicated gates represent the lineage negative fraction with the percentage of cells indicated. The histogram represents the average percentage of lineage negative cells from assessment of 4 WT and 4 KO mice. The bars indicate the SEM. (B) Two-dimensional dot plots showing the expression of Sca-1 and c-Kit in the gated lineage negative fraction. The R7 gate represents the progenitor population while R3 represents the KSL population. The histograms represent the average percentage of progenitor (upper) and KSL (lower) cells from least 4 WT and 4 KO mice. The bars indicate the SEM (n=5) P>0.05

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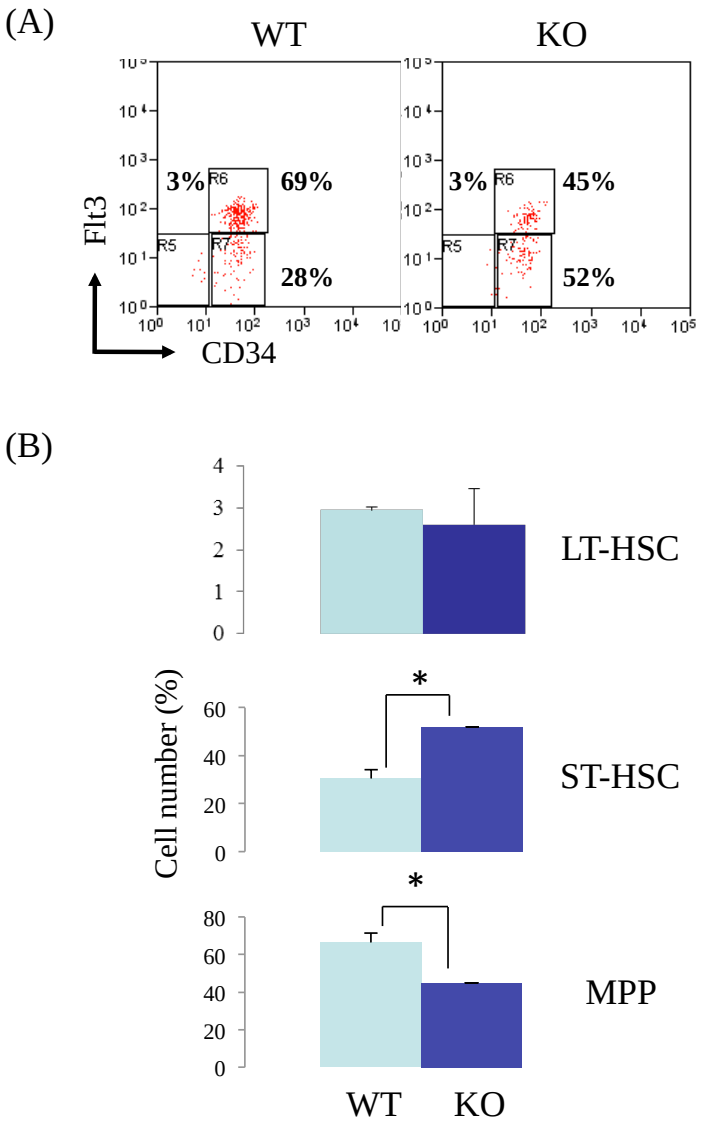


Figure 4.2: Immunofluorescence analysis of stem cell and progenitor populations in wild type and *nfκb2*$^{-/-}$ mice. Bone marrow from wild type (WT) and *nfκb2*$^{-/-}$ (KO) mice was extracted and the red cells were lysed. WT and KO bone marrow cells were stained with antibodies to define the KSL population. (A) Two-dimensional dot plots of gated KSL cells co-stained with antibodies against Flt3 (CD135) and CD34. The gates define the long-term HSC (LT-HSC: Flt3-CD34-), short-term HSC (ST-HSC: Flt3-CD34+) and multipotential progenitor (MPP: Flt3+CD34+) cells. (B) The histograms represents the average percentage of cells within each gate. The bars show SEM (n=4). * indicates significance (P<0.05)

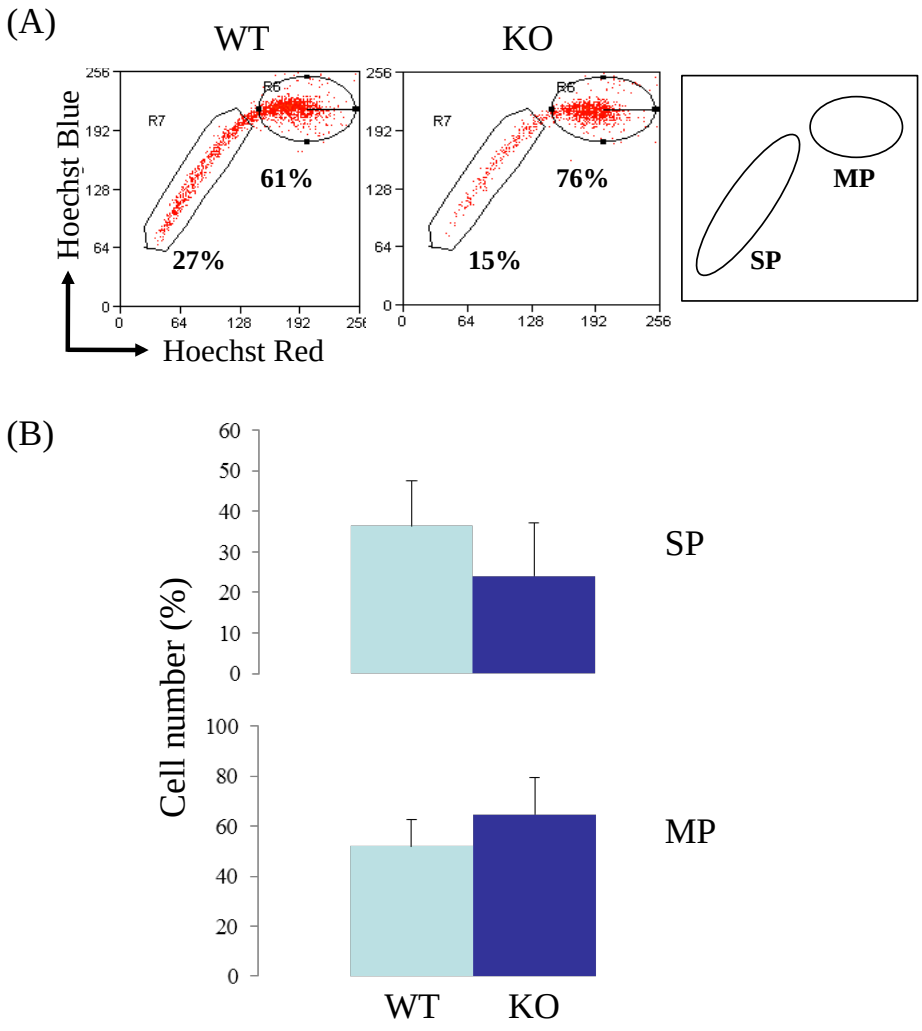


Figure 4.3: Analysis of bone marrow from wild type and *nfκb2*$^{-/-}$ mice using the DNA binding dye Hoechst 33342. (A) The bone marrow of wild type (WT) and *nfκb2*$^{-/-}$ (KO) mice of 8-10 weeks of age was extracted and the red cells lysed. Cells were then stained with Hoechst 33342 as previously described (Goodell et al., 1997) and subsequently co-stained with a cocktail of antibodies against lineage-specific surface markers (CD5, CD8a, CD11b, Gr1, Ter119 and B220) and the stem cell markers c-Kit and Sca-1. The Hoechst 33342 profile analysis was performed following gating for forward scatter (FSC) versus side scatter (SSC), focussing on the lineage negative and KSL fractions. The schematic indicates the position of the side population (SP) and the major population (MP). (B) The histogram represents the average percentage of cells in the SP and the MP gate with bars indicating the SEM (n=4) P>0.05.

change was statistically not significant. The main population showed a very small change between wild type and knockout KSL cells (Figure 4.3).

4.5 Comparative analysis of properties of wild type and *nfκb2*$^{-/-}$ bone marrow HSCs

4.5.1 Cell cycle analysis of wild type and nfκb2$^{-/-}$ *bone marrow haemopoietic stem cells*

To study the cell cycle status and proliferation capacity of the *nfκb2*$^{-/-}$ KSL cells, BrdU incorporation assays were performed *in vitro* and *in vivo* as described above, using confocal microscopy to analyse the data. The *in vitro* BrdU incorporation assay (Figure 4.4A) showed a smaller percentage of BrdU-positive *nfκb2*$^{-/-}$ KSL cells compared to the wild type; however, this decrease is not statistically significant. Similarly, the *in vivo* BrdU incorporation assay demonstrated a decrease in the percentage of BrdU-positive *nfκb2*$^{-/-}$ KSL cells compared to the wild type, which was also not statistically significant (Figure 4.4B).

4.5.2 Colony forming potential

To evaluate the proliferation and differentiation potential of the *nfκb2*$^{-/-}$ HSCs *in vitro*, colony assays were performed using sorted KSL cells. 350 KSL cells from wild type or *nfκb2*$^{-/-}$ mice were plated in methylcellulose. The first plating showed a statistically significant higher number of colonies derived from *nfκb2*$^{-/-}$ KSL cells compared to the wild type. Nevertheless, there were no changes observed in the types of colonies produced (Figure 4.5A). For secondary replating, all cells from previous plates were collected, and 5000 cells were re-seeded in fresh methylcellulose. Upon secondary replating the capacity of the *nfκb2*$^{-/-}$ cells to form colonies was decreased relative to the wild type cells; although this was not significant (Figure 4.5B). There was no difference in the size or morphology of colonies produced by the

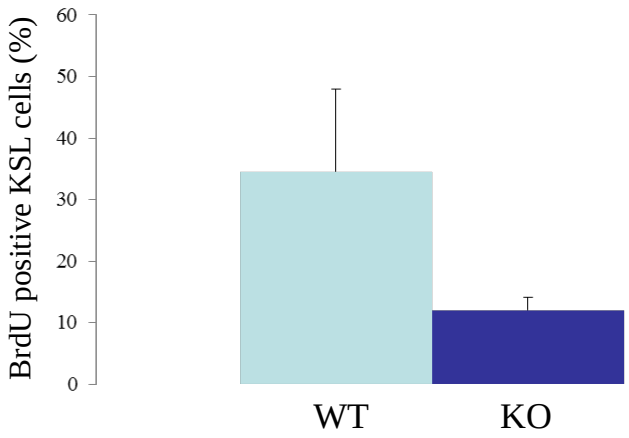
nfκb2$^{-/-}$ cells compared to the wild type. Upon tertiary replating, *nfκb2*$^{-/-}$ cells were hardly able to form colonies while the wild type cells retained their clonogenic potential and formed colonies (Figure 4.5C). This result may suggest that *nfκb2*$^{-/-}$ KSL cells have diminished self-renewal capacity *in vitro*.

Another explanation for that could be that in the *nfκb2*$^{-/-}$ plate were more differentiated cells, which upon replating were not able to self-renew while in the wild type plates there were more immature cells which were able to form colonies.

4.6 RNA analysis

As for *nfκb1*$^{-/-}$ KSL cells (Figure 3.10), a gene expression profiling was also performed on sorted *nfκb2*$^{-/-}$ KSL cells to compare the expression of NF-κB family genes in the knockout with wild type cells. Real-time PCR was performed for the NF-κB family member genes that are expressed in HSCs, using normalisation against HPRT. As deletion of transcription factor NF-κB2 in KSL cells showed only a modest phenotype, it was speculated that this transcription factor might be compensated by other members of the family within KSL cells. In fact the RT-PCR analysis revealed that *nfκb2* gene deletion resulted in increased *nfκb1* expression by up to 4.5-fold and *rel-A* expression by up to 4 fold in KSL cells (Figure 4.6). No difference could be seen in the expression of *c-rel* comparing wild type and *nfκb2*$^{-/-}$.

(A)



(B)

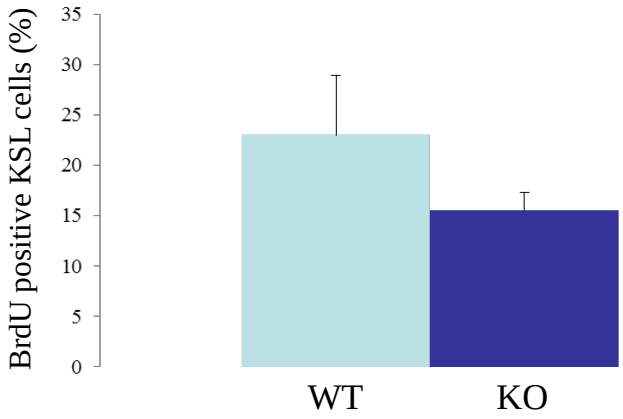


Figure 4.4: Analysis of the proliferation of HSCs from wild type and *nfκb2*$^{-/-}$ mice. (A) Bone marrow cells from wild type (WT) and *nfκb2*$^{-/-}$ (KO) mice at 8-10 weeks of age was extracted and the red cells lysed. KSL cells were sorted and placed in a liquid culture for 12 hours in IMDM containing BrdU. After labelling, the cells were collected, fixed and denatured. After blocking, the cells were incubated overnight with the primary monoclonal rat anti-BrdU antibody. One day later the cells were washed and fixed with paraformaldehyde and quenched with NH_4Cl and finally incubated for 2 hours with AlexaFluor 488 donkey anti-rat. The cells were mounted with DAPI-containing Vectashield and images were acquired using the Zeiss LSM 510 Meta confocal fluorescence microscope. (B) For *in vivo* BrdU incorporation assay, mice were injected intra-peritoneally with BrdU and sacrificed after 24 hours. Bone marrow cells were sorted for KSL cells, and stained as described in (A) (n=4) P>0.05

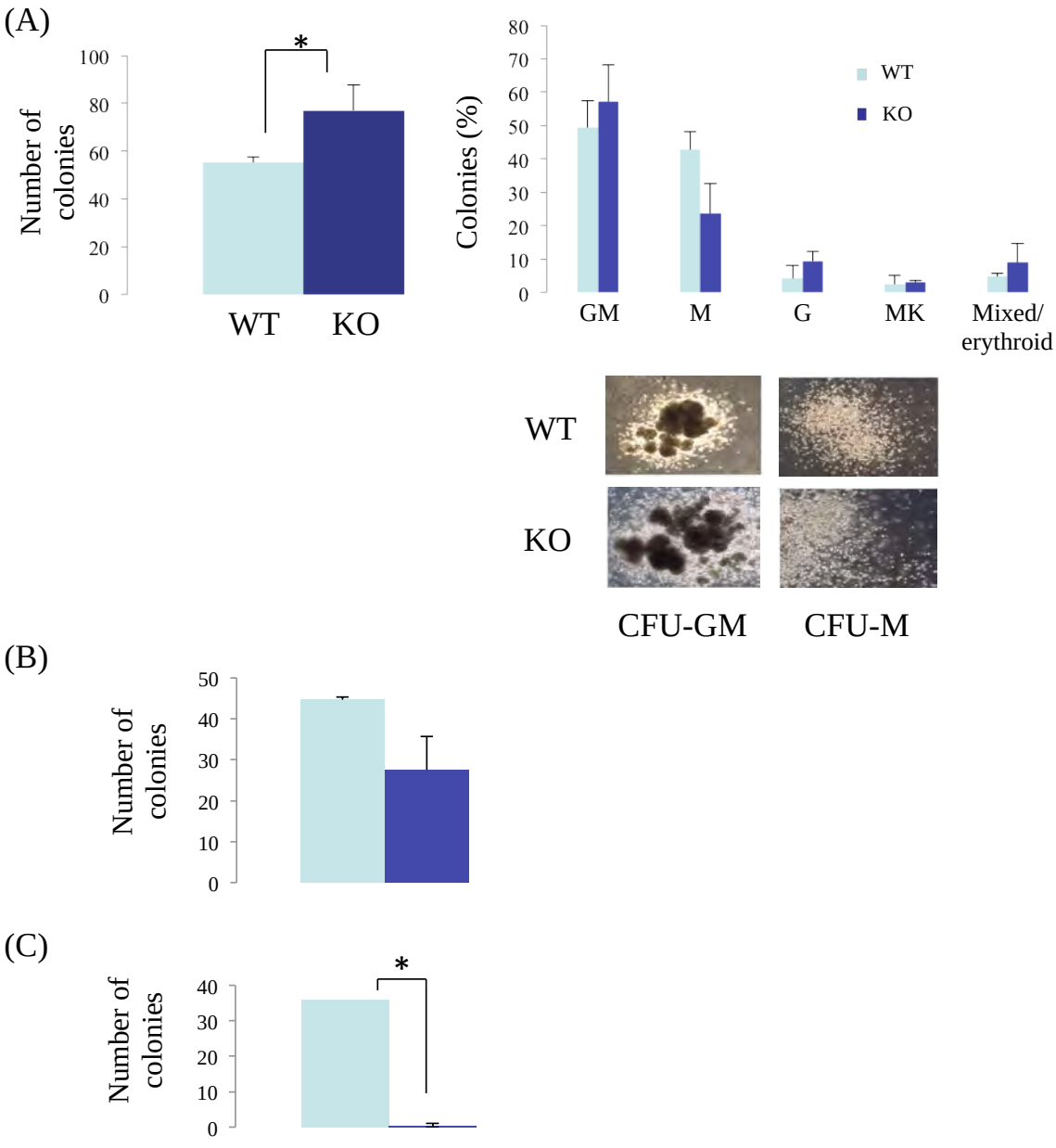


Figure 4.5: Analysis of the colony forming potential of wild type and *nfκb2*$^{-/-}$ KSL cells. 350 sorted KSL cells from wild type (WT) and *nfκb2*$^{-/-}$ (KO) mice were plated in semisolid methylcellulose medium (Methocult M3434) supplemented with TPO. (A) Histograms showing the number and morphology of colonies after 7 days. The pictures illustrate the morphology of CFU-GM and CFU-G colonies. Secondary (B) and tertiary (C) replating of 5000 cells was performed using cells collected from previous plates. The number of colonies and the total number of cells were assessed after 7 days. All cultures were incubated at 37°C in a fully humidified 5% CO_2 in air atmosphere (n=5) * indicates significance (P<0.05)

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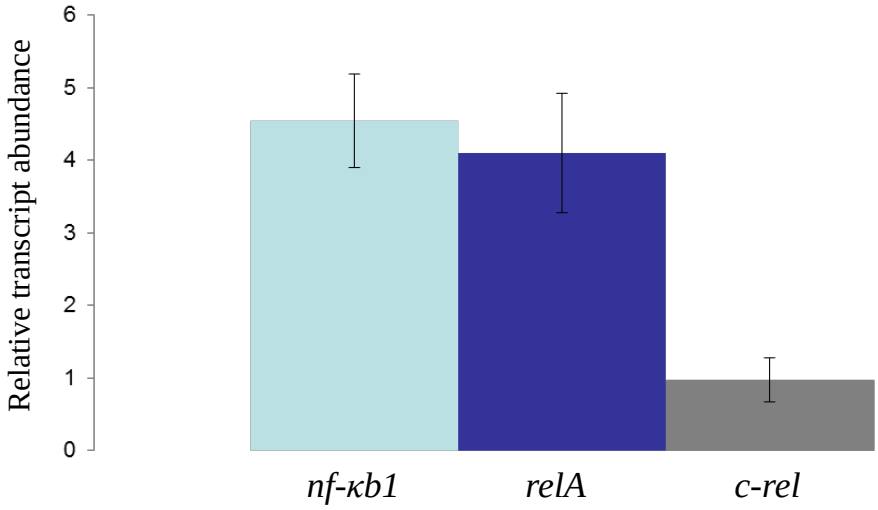

Figure 4.6: Expression of NF-κB family genes in wild type versus *nfκb2*$^{-/-}$ KSL cells. RNA was extracted from KSL cells from the bone marrow of wild type and *nfκb2*$^{-/-}$ mice and converted into cDNA. Real time PCR was then performed on the cDNA using SYBR Green and primers specific for the *nf-κb* family members. The cDNA was normalised using the HPRT gene as a control. The figure represents results obtained from two experiments and shows the abundance in *nf-kb2* knockout cells relative to the wild type. Each PCR was performed in triplicate and the error bars represent the SEM.

4.7 Assessment of *nfκb2*$^{-/-}$ HSC function by transplantation into lethally irradiated recipients

To assess the function and potency of *nfκb2*$^{-/-}$ HSC *in vivo*, serial transplantation assays were performed into lethally irradiated recipient mice as described before in Chapter 3.

After transplantation of 500 *nfκb2*$^{-/-}$ or wild type KSL cells into recipient mice, together with 10^6 whole bone marrow reference cells, peripheral blood samples were taken at different time points to assess the relative engraftment of donor and reference cells. Table 4.2 summarises the number of sets of transplantations that have been done during the project and details the individual donor wild type and *nfκb2*$^{-/-}$ mice (always littermates) and the recipients that have been injected with donor KSL cells. Set 2 transplanations did not show any engraftment, either of wild type or knockout cells, and for this reason these transplantations are not presented. For each transplanted animal, the ratios of donor to reference cells in the peripheral blood and bone marrow of recipients were calculated (Table 4.2) as described in Sections 4.7.1 and 4.7.2.

4.7.1 Peripheral blood analysis

Peripheral blood as taken from recipient animals at several stages following transplantation, and the donor and reference haemopoietic cells were distinguished by flow cytometry after staining with antibodies against CD45.1 and CD45.2. This analysis showed that recipients receiving KSL cells from a given *nfκb2*$^{-/-}$ donor mouse gave rise to two different transplantation phenotypes. Cells either did not engraft (*nfκb2*$^{-/-}$ non-engrafted) or engrafted efficiently, both in the short and long term, and had a competitive advantage relative to the reference cells (*nfκb2*$^{-/-}$ engrafted). Figure 4.7 illustrates an example of engraftments in

recipients 101, 112 and 108 taken from Set 1 transplantations. The efficiency of engraftment with *nfκb2*$^{-/-}$ donor KSL cells was only 25 to 33.3%, despite the *nfκb2*$^{-/-}$ engrafted mice showing an over-proliferation and a competitive advantage of the donor cells. The remaining *nfκb2*$^{-/-}$ recipients showed no engraftment of donor cells, even though KSL cells from the same knockout mouse were able to engraft in other recipients. In complete contrast, KSL donor cells from wild type animals showed consistent engraftment, with virtually all of the recipient mice showing evidence of transplant engraftment (Table 4.2).

			Ratio (D:REF)							
Set	Mouse number	CD45.2 (Donor) KSL cells	4 weeks	8 weeks	12 weeks	16 weeks	17 weeks	19 weeks	20 weeks	BM analysis
1	101	WT1	0.29	0.3	0.47		0.34			1.23
	102	WT1	0.22	0.48	0.51		0.07			0.24
	103	WT2	0.08	0.42	0.38		0. 35			0.39
	104	*nfκb2*$^{-/-}$ 1	0	0.01	0.02		0.01			0.01
	105	*nfκb2*$^{-/-}$ 1	0.08	0.04	0.04		0.01			0.03
	106	*nfκb2*$^{-/-}$ 1	0.3	0.07	0.005		0.01			0.002
	107	*nfκb2*$^{-/-}$ 1	0.47	3.92	11.5		3			14.4
	108	*nfκb2*$^{-/-}$ 2	4.8	5.7	4.6		2.8			18.85
	109	*nfκb2*$^{-/-}$ 2	0.19	0.1	0.09		0.03			0.03
	110	*nfκb2*$^{-/-}$ 2	1.25	0.01	0.02		0.01			0.005
	111	*nfκb2*$^{-/-}$ 2	0.29	0.37	1.96		1.16			7
	112	*nfκb2*$^{-/-}$ 2	0.02	0.005	0.01		0.004			0.001
3	82	WT5	0.07	0.2	0.3	0.4			0.4	1.5
	83	WT5	0.2	0.7	0.5	0.5			0.5	5.2
	84	WT5	0.15	0.36	0.5	0.67			0.61	1.04
	85	WT5	0.06	0.19	0.48	0.7			------	1.25
	86	WT5	0.10	0.32	0.56	0.79			0.68	0.83
	87	WT6	0.01	0.56	1.25	1.93			2.5	2.2
	88	WT6	0.14	0.08	0.05	0			0.06	0.3
	89	*nfκb2*$^{-/-}$ 5	0.006	0	0	0			0	0.3
	90	*nfκb2*$^{-/-}$ 5	0.03	0.01	0.02	0			0	0.4
	91	*nfκb2*$^{-/-}$ 5	0.04	0.19	1.07	1			0.86	10.5
	92	*nfκb2*$^{-/-}$ 5	0.05	0.02	0.12	0.16			0.14	0.3
	93	*nfκb2*$^{-/-}$ 6	0.04	0.27	0.58	0.8			0.69	9.2
	94	*nfκb2*$^{-/-}$ 6	0.08	0	0	0			0	0.09
	95	*nfκb2*$^{-/-}$ 6	0.03	0.01	0.01	0.01			0.01	0.2
	96	*nfκb2*$^{-/-}$ 6	0	0	0	0			0	0.4
	97	*nfκb2*$^{-/-}$ 6	0	0	0.02	0.01			0.01	0.3

Table 4.2 Primary transplantation of wild type versus *nfκb2*$^{-/-}$ bone marrow HSCs. The table details the sets of transplantations performed. The individual donors and recipients used in each set are indicated, as are the ratios of the Donor: Reference (D:REF) engrafted cells in the peripheral blood at various time points and when the bone marrow was eventually analysed.

Immunofluorescence flow cytometry of the peripheral blood of engrafted *nfκb2*$^{-/-}$ engrafted recipients following staining with antibodies specific for the B-cell lineage (CD43/B220) revealed that transplanted *nfκb2*$^{-/-}$ KSL cells had a deficiency in B-cell development, showing up to 50% reduction of mature B cells compared to control wild type transplanted KSL cells or the reference cells (Figure 4.8A). In contrast, staining for markers against T-cells (CD4/CD8) showed no change in the development of this population arising from the *nfκb2*$^{-/-}$ KSL donor cells (Figure 4.8B). However, recipient mice engrafted with *nfκb2*$^{-/-}$ cells exhibited a 4- to 5-fold increase in myeloid populations with CD11b^{+}Gr1^{-} and CD11b^{+}Gr1^{+} phenotypes, when compared to transplanted wild type KSL cells or reference cells (Figure 4.8C). These findings were unexpected, as previous analyses of *nfκb2*$^{-/-}$ mice showed no decrease in mature B-cell development or any increase in myeloid populations (Section 4.2). The changes in the *nfκb2*$^{-/-}$ lineage development therefore revealed only upon transplantation.

After seeing the changes in B-cell and myeloid cell development of *nfκb2*$^{-/-}$ engrafted cells in the peripheral blood, it was necessary to investigate if there were any other phenotypic changes in the blood of the recipient animals. Therefore, on the day of sacrifice, cardiac punctures were performed and blood counts were obtained. On average there were no significant changes in the white blood cell (WBC) and red blood cell (RBC) counts or the haemoglobin (HGB) levels, but there was a statistically significant increase in the platelet value, which is shown in the Appendix (Figure A6).

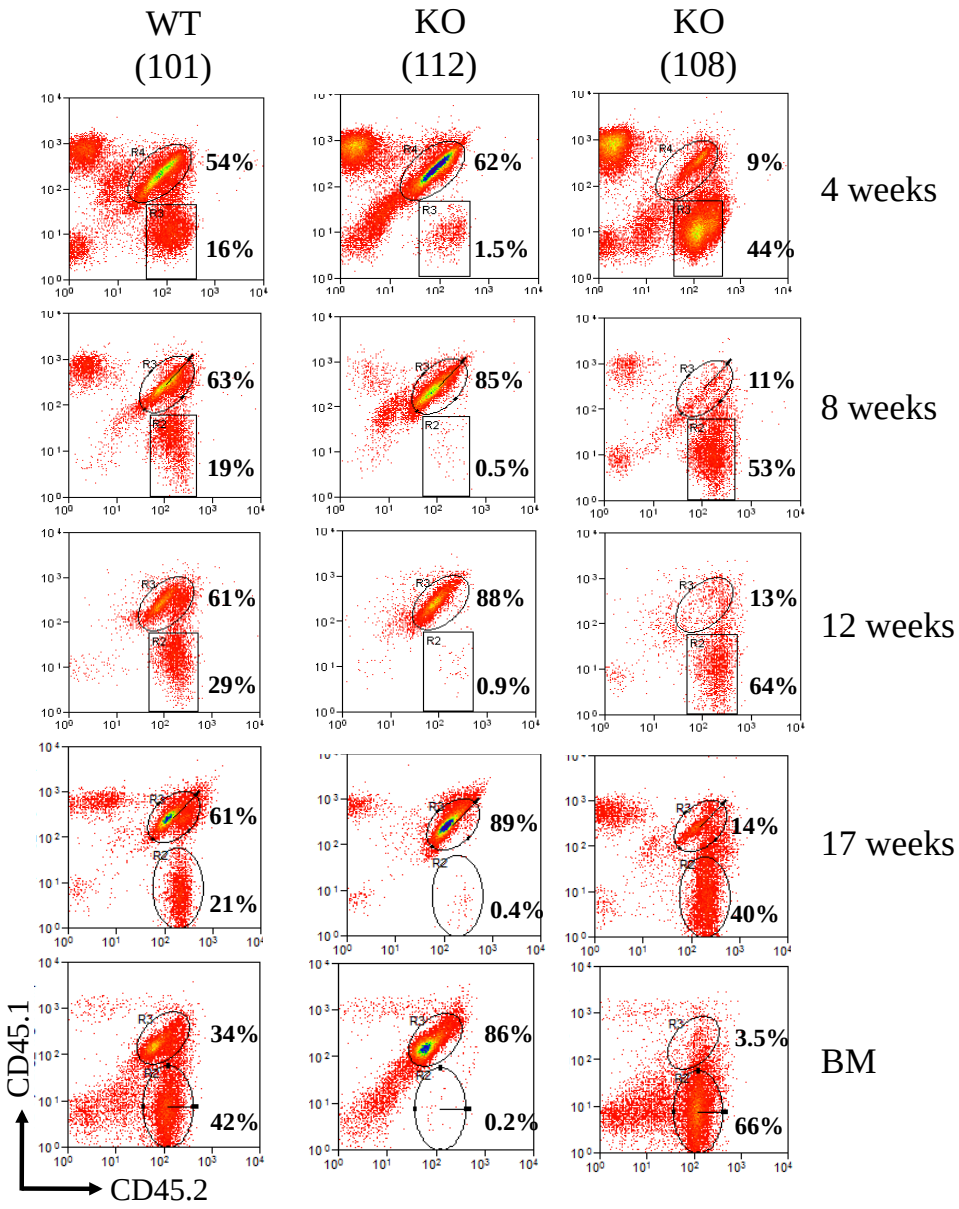

Figure 4.7: Transplantation of wild type and *nfκb2*$^{-/-}$ KSL cells into lethally irradiated recipients. For competitive repopulation assays recipient mice (B6:SJL, CD45.1/CD45.1) were lethally irradiated (900 Gy) and injected via the tail vain with 1x10^6 reference wild type ACK-treated whole bone marrow cells (B6XB6:SJL F1, CD45.1/CD45.2) together with 500 KSL donor cells from either wild type (WT) or *nfκb2*$^{-/-}$ (KO) mice (C57BL6/J, CD45.2/ CD45.2). The figures show analysis of peripheral blood samples at 4, 8, 12 and 17 weeks following transplantation and eventually when the bone marrow (BM) was analysed using anti-CD45.1 and anti-CD45.2 to distinguish donor and reference cells. These dot plots are representing the peripheral blood analysis of three recipient mice (101, 112 and 108) at different time points taken from transplantation Set 1.

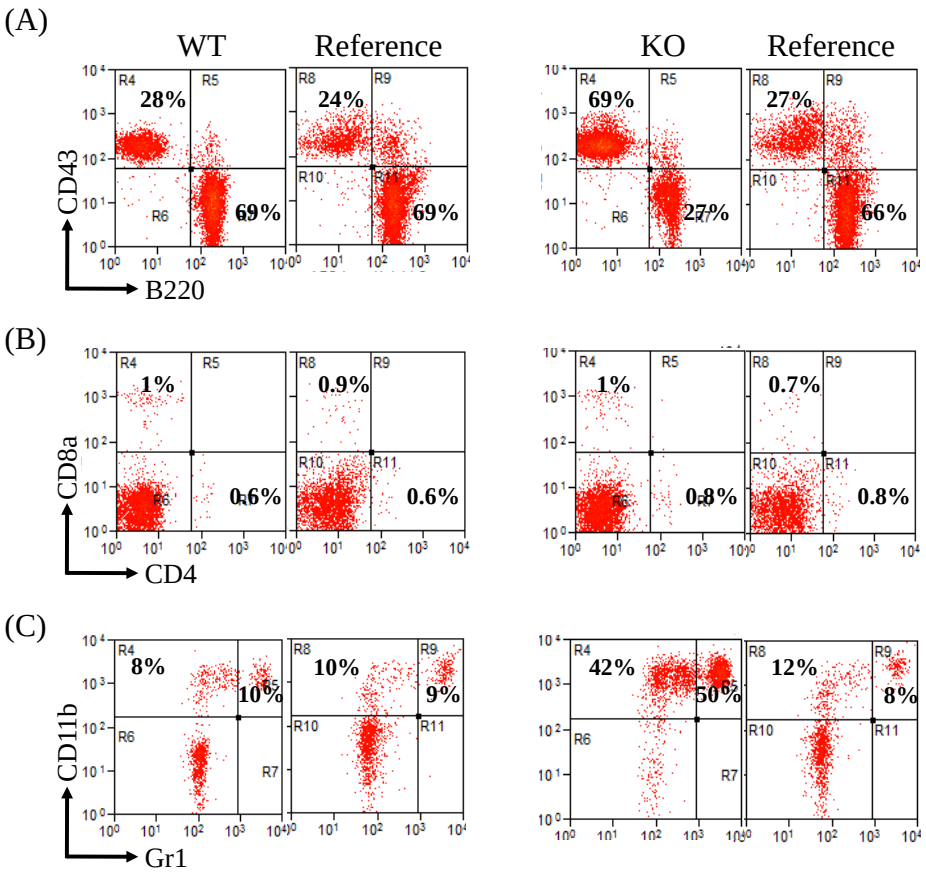

Figure 4.8: Analysis of lymphoid and myeloid lineages in the peripheral blood of engrafted recipients transplanted with wild type and *nfκb2*$^{-/-}$ KSL cells. At 12 weeks post transplantation peripheral blood samples were collected from recipient mice number 101 and 108 that received wild type or *nfκb2*$^{-/-}$ KSL cells. Following red cell lysis the samples were stained with antibodies against lymphoid- and myeloid-associated surface markers. For live cells, samples were gated on forward scatter (FSC) and side scatter (SSC), and for host (CD45.1/CD45.1), reference (CD45.1/CD45.2) and donor (CD45.2/CD45.2) populations. (A) B-cell markers B220 and CD43. (B) T-cell markers CD4 and CD8a. (C) Myelomonocytic cell markers Gr1 and CD11b.

4.7.2 Bone marrow analysis

At approximately six months post-transplantation, the recipients of the primary *nfκb2*$^{-/-}$ and wild type donor KSL cells were sacrificed and their bone marrow was analysed to assess engraftment of the donor and reference cells. Bone marrow cells were also used for secondary transplantation into lethally irradiated mice.

To determine whether recipient mice transplanted with *nfκb2*$^{-/-}$ donor KSL cells exhibited two different phenotypes in the bone marrow, as seen previously in the peripheral blood, cells were stained with antibodies against CD45.1 and CD45.2. In general, individual recipients that showed no engraftment of *nfκb2*$^{-/-}$ KSL cells in the peripheral blood also showed no engraftment of donor cells in the bone marrow (Table 4.2). Similar to the peripheral blood analysis, the 25 to 33% of the *nfκb2*$^{-/-}$ engrafted recipients showed an over-proliferation and a competitive advantage of these donor cells in the bone marrow compared to the reference population.

The previous analysis of the bone marrow HSC in *nfκb2*$^{-/-}$ mice showed a significantly lower number of lineage negative cells compared to the wild type (Section 4.3). In addition, sub-fractionation of the *nfκb2*$^{-/-}$ KSL cells revealed an increase in the ST-HSC and a decrease in the MPP population compared to the wild type, whereas no difference was observed in the LT-HSC sub-fraction (Section 4.3). To evaluate whether these differences in the progenitor/stem cell compartments is also be found in the bone marrow following transplantation, an analysis of progenitor and KSL compartments was performed using appropriate antibodies by immunofluorescence flow cytometry. Analysis of the lineage negative fraction revealed no differences between *nfκb2*$^{-/-}$ or wild type engrafted cells in the

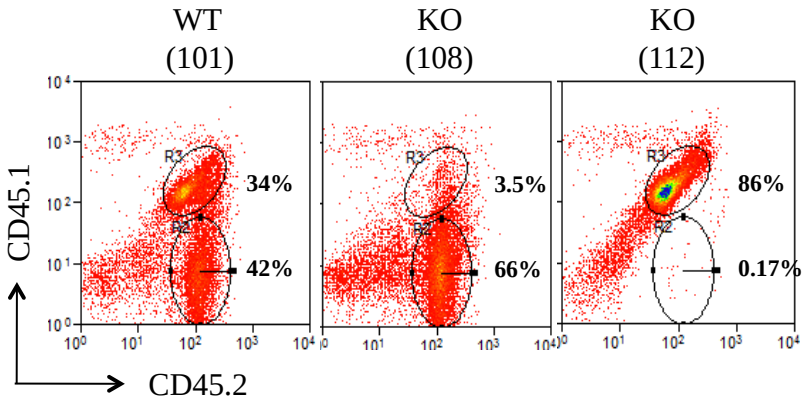

Figure 4.9: Analysis of bone marrow cells from recipients transplanted with wild type and *nfκb2*$^{-/-}$ test donor sorted KSL cells. Bone marrow was extracted from femurs and tibias of primary recipients receiving 500 KSL cells from either wild type (WT) or *nfκb2*$^{-/-}$ (KO*)* mice at approximately 17 weeks post-transplantation. Bone marrow samples were lysed to remove red cells and analysed using anti-CD45.1 and anti-CD45.2 to distinguish between donor and reference cells. The dot plots represent the engraftment of recipient mice 101, 108 and 112.

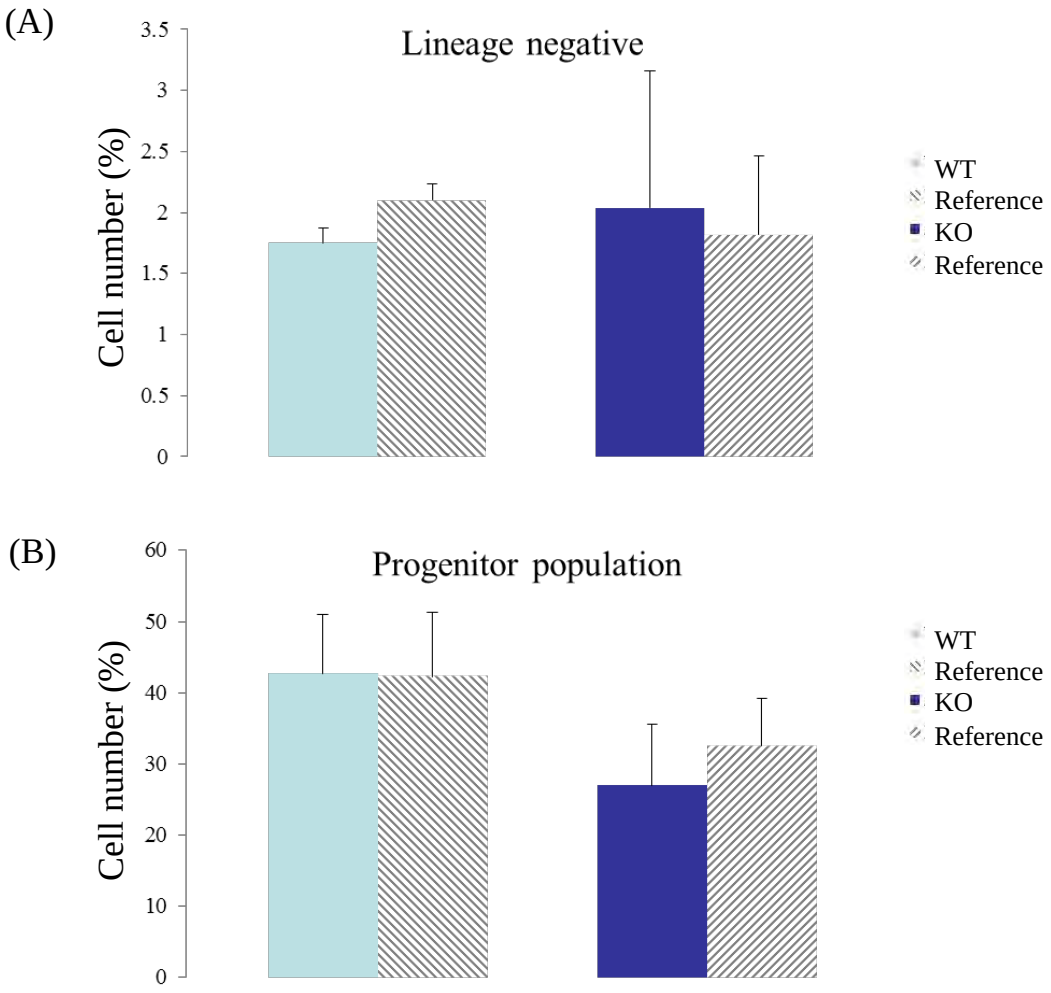

Figure 4.10: Immunofluorescence analysis of engrafted progenitor cells in primary recipient bone marrow. Bone marrow from primary transplanted recipients of wild type (WT:REF) and *nfkb2*$^{-/-}$ (KO:REF) mice were stained with a cocktail of antibodies against lineage associated surface markers which consisted (CD5, CD8a, CD11b, Gr1, Ter119 and B220). (A) The histograms represents the average percentage of lineage negative cells from 4 WT:REF and 5 KO:REF mice. The bars indicate the SEM. (B) Using the stem cell markers c-Kit and Sca-1 in combination with lineage negative surface markers the progenitor population was analysed. The histograms represents the average percentage of the progenitor cell populations from 4 WT:REF and 5 KO:REF mice. The bars indicate the SEM P>0.05

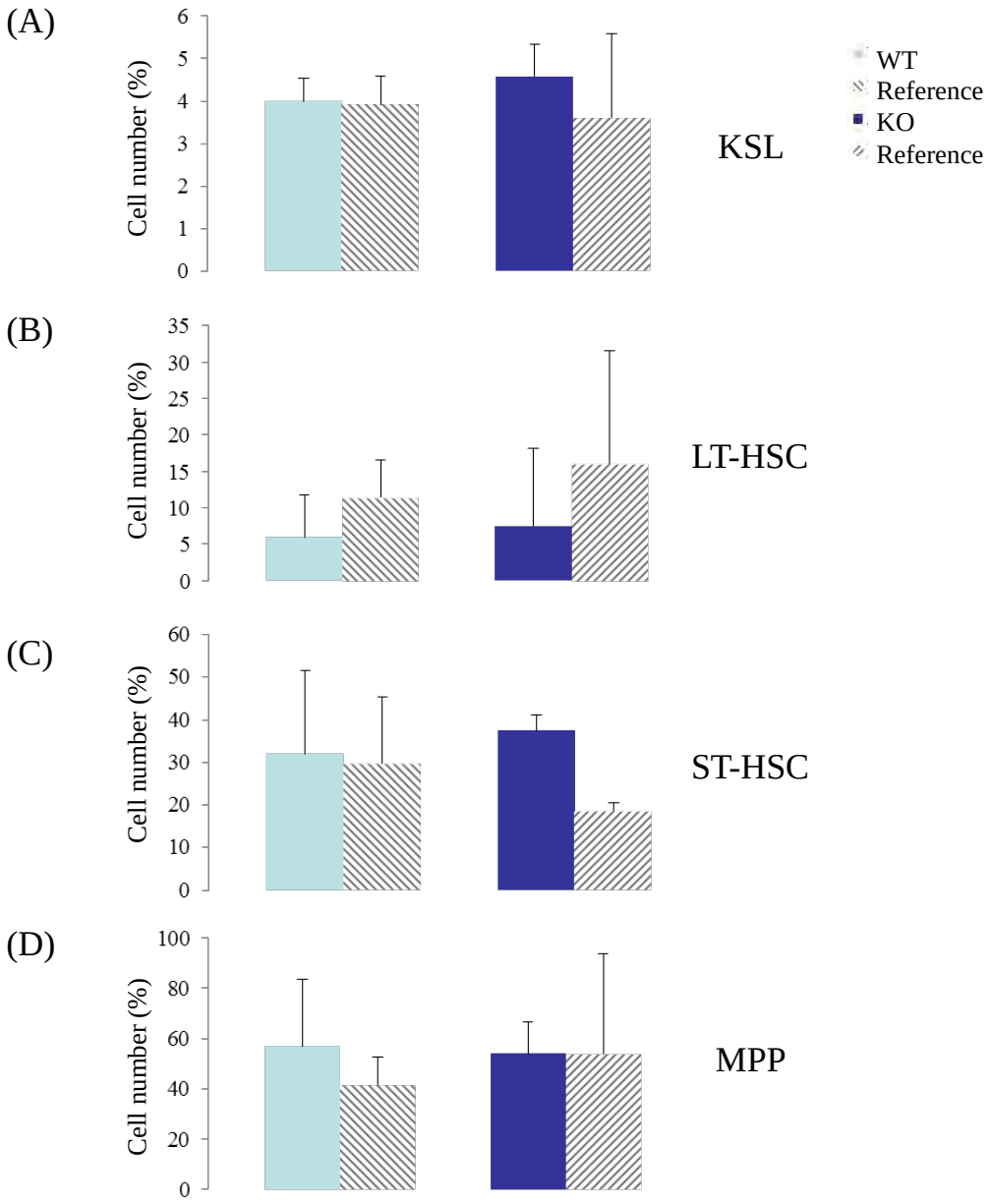

Figure 4.11: Immunofluorescence analysis of stem cell compartments of wild type and *nfκb2*$^{-/-}$ mice. The bone marrow cells from recipients of wild type (WT:REF) and *nfκb2*$^{-/-}$ (KO:REF) mice were were stained with a cocktail of antibodies against lineage associated surface markers (CD5, CD8a, CD11b, Gr1, Ter119 and B220) and stem cell markers c-Kit and Sca-1. (A) The histogram represents the average percentage of KSL cells from WT:REF and KO:REF mice. The bars indicate the SEM. (B) (C) and (D) KSL cells co-stained with Flt3 and CD34. The histograms represents the average percentage of donor (WT or KO) and reference (REF) LT-HSC, ST-HSC and MPP cells in transplanted mice. The bars show SEM P>0.05

bone marrow of recipient mice (Figure 4.10 A), which contrasts with the findings in non-transplanted knockout mice (Figure 4.1A). Whereas non-transplanted *nfκb2*$^{-/-}$ and wild type mice had similar progenitor (Lin^{-}Sca1^{-}c-Kit^{+}) populations, the progenitor cells derived from *nfκb2*$^{-/-}$ grafts were decreased in comparison to the wild type grafts (Figure 4.10B). However, within the progenitor population of *nfκb2*$^{-/-}$ engrafted cells, there were no evident differences compared to wild type in the GMP, CMP and MEP sub-populations (data not shown). As with non-transplanted *nfκb2*$^{-/-}$ mice, *nfκb2*$^{-/-}$ grafts showed hardly any differences compared to wild type in the proportion of KSL cells in the bone marrow of the recipients mice following transplantation (Figure 4.11A). Within this KSL compartment, ST-HSC cells were slightly increased in *nfκb2*$^{-/-}$ grafts (Figure 4.11C). Although this was superficially similar to the observation in non-transplanted *nfκb2*$^{-/-}$ mice (Section 4.3), the increase in the ST-HSC sub-fraction in *nfκb2*$^{-/-}$ grafts was not statistically significant (Figure 4.11C). The LT- HSC sub-fraction was similar in *nfκb2*$^{-/-}$ and wild type grafts (Figure 4.11B), as seen in non-transplanted mice (Section 4.3). Whereas a significant decrease in MPP cells was observed in non-transplanted *nfκb2*$^{-/-}$ mice compared to wild type, this difference was not detected in grafts (Figure 4.11 D).

4.8 Discussion

The NF-κB2 transcription factor is part of the non-canonical pathway that is suggested to regulate important biological functions, such as lymphoid organogenesis and B-cell survival and maturation. The function of this transcription factor in HSCs has not been described before.

As in the *nfκb1*$^{-/-}$ mice, *nfκb2*$^{-/-}$ mice did not demonstrate any differences in the number of different haemopoietic cells when wild type and knockout bone marrow were compared. Interestingly, the *nfκb2*$^{-/-}$ mice exhibited a statistically significant increase in the ST-HSC compartments and a significant decrease in the MPP population (Figure 4.2), reflecting the results obtained from the BrdU incorporation assays (Figure 4.4). The increase in ST-HSC compartment miht be due to the fact that this population is more proliferative. However this need to be further tested by e.g performing BrdU incorporation assay on KSL subpopulations, or by pyroninY staining, or Ki67 staining. Furthermore the decrease in MPP population might be du to the fact that there might be a blockage in the differentaion stage, thus when ST-HSCs differentiate into MPP cells.

The serial replating assay of *nfκb2*$^{-/-}$ KSL cells showed that these cells were not able to form colonies compared to the wild type cells after three cycles of replating, indicating that they have lost their *in vitro* potential for self-renewal.

Transplantation of the *nfκb2*$^{-/-}$ KSL cells into lethally irradiated recipients also gave two distinct outcomes; however, unlike the situation seen for the *nfκb1*$^{-/-}$ transplantations in which a co-segregating transplantation antigen appeared to be responsible for the two transplantation behaviours, in the case of the *nfκb2*$^{-/-}$ donors, KSL cells from the same donor knockout mouse yielded both phenotypes, that is, no engraftment or engraftment with an increased proliferation phenotype and a competitive advantage.

The recipients that showed a proliferation phenotype also exhibited an increase in myelomonocytic cells in the peripheral blood, and a decrease in $CD43^{-}B220^{+}$ cells, indicating a blockage in B-cell development ($B220^{+}$ cells include pre-B and mature B-cells) but normal T-cell development. The increase in $CD11b^{+}Gr1^{+}$ cells in the peripheral blood might be the

result of extramedullary haemopoiesis. NF-κB activity is necessary for the development of B-cells and their survival at various developmental stages (Claudio et al., 2009). Hence, it has already been shown that *nfκb2*$^{-/-}$ mice have abnormalities in the splenic architecture, lymph nodes and Peyer's patches and show a reduction in the B-cell compartments (Caamano et al., 1998). However, another study performed by Claudio and colleagues demonstrated that *nfκb1*/*nfκb2* double knockout mice show a partial reduction in late pre-B and immature B-cells populations ($CD43^{-}B220^{+}$). This encompasses the later B-cell developmental stage in the bone marrow, suggesting that NF-κB1 and NF-κB2 promote B-cell development during transit through the late pre-B and immature stages (Claudio et al., 2009).

In the case of those recipients that did not exhibit engraftment, an immunological mismatch is not a credible explanation as KSL cells from a single knockout donor mouse exhibited the two different phenotypes. These results therefore suggest that the absence of NF-κB2 leads directly to a change in proliferation/self-renewal potential, the precise outcome of which in a transplantation assay depends on yet to be determined factors.

One possibility why the *nfκb2*$^{-/-}$ KSL cells did not repopulate in some animals is that migration or homing processes might be affected. Usually when transplanting cells intravenously into the tail vein the cells home from the peripheral circulation to the vascular niche, undergoing transendothelial migration and finally entering the osteoblastic niche (Lapidot et al., 2005). After homing to the bone marrow and settling in the niche, HSCs have to go through several steps to achieve successful long-term engraftment including proliferation and differentiation into all blood cell types (Adams et al., 2007). By perforning intrafemular injection of the KO cells we would be able to assess if the cells are having defect in their homing upon transplantation. The HSC niche has been shown to play an important

role in the maintenance of stem cell function. Thus there are different gene products involved in the HSC and niche interaction. Several chemokines and signalling and adhesive molecules are involved in HSC mobilisation and homing. NF-κB activity is induced by over 150 different proteins and in turn is involved in the transcription of over 150 target genes (Pahl, 1999), so that it could potentially influence many of the molecules that that are involved in HSC mobilisation and homing. It has already been shown that some genes that are involved in HSC function and niche interaction are NF-κB target genes in other cell types, such as SCF and angiopoietin-1/Tie2 (Da Silva and Frossard, 2005, Scott et al., 2005). Another future experiment that could give us an idea about the bone marrow microenvironment of the KO mice would be transplanting wild type bone marrow KSL cells into KO mice. This would show if the KO KSL cells are having an autonomous or a non-autonomous effect.

The results of the secondary transplantations performed in the case of assessment of the effect of the *nfκb2* knockout were difficult to interpret as these had been obtained from Set 2 primary transplantations that showed very little engraftment of either wild type or knockout KSL cells. This was most likely due to technical issues such as problems with the irradiation of the recipient. Due to time restrictions it was not possible to repeat the secondary transplantations.

CHAPTER 5

DISCUSSION

As detailed in Chapter 1, NF-κB activity has been implicated in HSC and LSC survival and development. Several studies have shown that there is constitutive NF-κB activity in haemopoietic cancers including acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), and multiple myeloma (MM). Guzman and colleagues were the first to observe constitutive activation of NF-κB transcription factor activity in primary AML LSCs, and this is thought to be associated with anti-apoptotic activity of the NF-κB proteins that leads to the survival of these cells (Guzman et al., 2001, Wang et al., 1996, Wang et al., 1998). In addition, the same group has shown that treatment of LSCs with a combination of anthracycline idarubicin and the proteasome inhibitor MG-132, which prevents NF-κB activation, induces a very rapid onset of apoptosis in LSCs, supporting the view that NF-κB is involved in LSC survival (Guzman et al., 2002).

The expression of NF-κB has also been shown in primitive umbilical cord blood $CD34^+$ HSCs (Panepucci et al., 2007), and only recently Zhao and colleagues showed the importance of the NF-κB non-canonical signalling pathway in haemopoietic stem/progenitor populations (Zhao et al., 2012). In the latter study it was shown that *relb/nfkb2* double-knockout (dKO) HSPCs had impaired engraftment and self-renewal activity upon transplantation into lethally irradiated recipient mice. The *relb/nfkb2* dKO mice also showed a significant decrease of bone marrow stromal cell numbers suggesting a decrease in the stromal niche size (Zhao et al., 2012), suggesting that the noncanonical NF-κB signalling pathway can regulate

interactions of HSCs with their microenvironment. However, the detailed role of individual members of this transcription factor family in murine HSCs has not been described before.

The aim of the work described in this thesis was to clarify which NF-κB family genes are significant for HSC function. Expression of NF-κB genes was initially examined using PCR, and revealed a clear expression of NF-κB1, NF-κB2, RelA and c-Rel, whereas RelB could only be detected after a high number of PCR amplification cycles. For that reason we decided to assess the function of NF-κB1 and NF-κB2 in murine HSCs *in vitro* and *in vivo* using single gene knockout mice.

The HSC properties that were tested in cells derived from NF-κB1 and NF-κB2 knockout mice included quiescence, proliferation and differentiation capacity. As part of the study of HSC properties, serial transplantation assays were performed into lethally irradiated mice that are the only precise way to test HSC potential. From our study we concluded that NF-κB1 and NF-κB2 behave differently in term of their *in vivo* self-renewal capacity. The fact that half of the recipients transplanted with *nfκb1*$^{-/-}$ KSL cells were engrafted as effectively as those transplanted with wild type cells, and that secondary transplantations of these engrafted animals were equally successful implies that *nfκb1* knockout cells can self-renew to a similar extent to the wild type cells. In comparison, *nfκb2*$^{-/-}$ KSL transplantations showed a more severe phenotype, KSL cells from the same knockout donor mouse either did not engraft efficiently or engrafted and exhibited an over-proliferation phenotype and competitive advantage with defects in lymphoid and myeloid development. This implies that NF-κB2 has an important function in HSC self-renewal and that its loss affects proliferation control, possibly leading to *in vivo* stem cell exhaustion. However as mentioned already in section 4.8 it need to be further tested if these cells are undergoing a autonomous or a non-autonomous

defect. It is well known that transplation assays are the most precise way to assess stem cell function and also the self-renewal capacity of HSCs. But the disadvantage of these experiments are that they are very long-term, time consuming and also expensive, as you have to keep the mice for more than a year especially if you are performing serial transplantation. A cheaper and short term way to gain some idea about self-renewal capacity of HSCs in vivo, would be to treat the mice with 5-FU (Fluorouracil). However this would not be as precise as transplantation assays.

Similar to this effect of the absence of NF-κB2 activity, the knockout of a number of genes involved in HSC regulation has been shown to have an effect on stem cell self-renewal leading to depletion of the stem cell pool when assayed *in vivo*. For example, constitutive loss of the genes encoding RUNX1, Bmi1, PTEN and Skp2 was demonstrated to cause stem cell exhaustion upon transplantation into lethally irradiated mice (Wang et al., 2011, Jacob et al., 2010, Park et al., 2003, Yilmaz et al., 2006). Hence, *Bmi1*$^{-/-}$ foetal liver and bone marrow cells were only able to contribute transiently to haemopoeisis upon transplantation and had lost their self-renewal capacity (Park et al., 2003). A key transcription factor in the maintenance of normal HSC function that is being studied in our laboratory, namely c-Myb, appears to have a similar controlling effect on self-renewal potential (Garcia et al., 2009). Thus, upon transplantation of total bone marrow cells derived from mice with a genetic knock down in c-Myb levels, the cells exhibit extensive proliferation.

Although HSCs from mice lacking NF-κB1 or NF-κB2 behave differently in terms of their *in vivo* self-renewal capacity, *in vitro* serial replating assays show that the absence of either transcription factor can have an effect on self-renewal potential in certain circumstances.

The RNA analysis of the NF-κB family members showed that both *nfκb1*$^{-/-}$ and *nfκb2*$^{-/-}$ KSL cells exhibited an increased level of expression of other NF-κB family members, suggesting the possibility for compensation for the loss of the specific factor. In the case of *nfκb1*$^{-/-}$ KSL cells, there was increased expression c-Rel, suggesting that the mild phenotype observed in *nfκb1*$^{-/-}$ KSL cells could be due to compensation through c-Rel. In comparison, the *in vivo* and *in vitro* assays performed on *nfκb2*$^{-/-}$ KSL cells showed that this transcription factor is absolutely required, although the RNA analysis showed an increased level of NFκB1 and RelA expression. It has already been demonstrated that there are multiple mechanisms of cross-regulation within the NF-κB family, such as the transcriptional feedback between the RHD that is responsible for DNA binding and dimerisation and the IκB protein family members (Hoffmann et al., 2003). c-Rel, RelB and IκBα are known to be NF-κB target genes (Grumont et al., 1993, Bren et al., 2001, Scott et al., 1993). To study the specific roles of p50 and p65 in NF-κB dependent gene expression, Hoffmann and colleagues generated fibroblast cell lines from *nfκb1*$^{-/-}$, *rela*$^{-/-}$ and *nfκb1*$^{-/-}$*rela*$^{-/-}$ embryos. This showed that *p50/p65* (dKO) cells exhibit decreased protein levels of c-Rel, RelB and IκBα. In addition, *nfκb1*$^{-/-}$ fibroblast cells showed enhanced levels of p52, while c-Rel levels were increased in *rela*$^{-/-}$ cells (Hoffmann et al., 2003). Thus, this study combined with our RNA analysis of *nfκb1*$^{-/-}$ and *nfκb2*$^{-/-}$ KSL cells, shows that the NF-κB family members can regulate each other, and that the loss of one family member can cause changes in the expression of other members.

One way to study the effect of NF-κB family members in KSL cells, would be to generate double knockout mice and to assess the consequences on HSC maintenance and function. *RelA*$^{-/-}$ mice are embryonically lethal due to liver apoptosis, but animals heterozygous for the knockout allele are viable. Our future plan is to study the function of HSCs in *nfκb2*$^{-/-}$*rela*$^{+/-}$, *nfκb*$^{-/-}$*nfκb2*$^{-/-}$ and *nfκb1*$^{-/-}$*c-rel*$^{-/-}$mice. However this would also be time and cost consuming.

For that reason the plans is to knockout different genes within the NF-κB family by sh-RNA and assess the effect on proliferation differentiation e.g by performing conlony asseys and then from there to cross the mice which the specific knockout genes to investigate their importance in in vivo in KSL cells. In addition, for us to get a better insight in these processes a genome-wide gene analysis of changes in gene expression and parallel studies of NF-κB family protein binding to chromatin would be a useful, hoping that NF-κB is biding genes that are involved in self-renewal, cell cycle and proliferation such as e.g cyclin D1, Runx1 or GATA3.. Such analyses will also help in understanding the proliferative phenotype and of *nfκb2*$^{-/-}$ KSL cells upon transplantation, and could give an overview of the expression of niche cytokines, chemokines, adhesion molecules, or cell cycle proteins that might be responsible for the proliferation phenotype.

It is well described that NF-κB activation mediates cell proliferation and that several cell cycle regulatory genes are NF-κB target genes. These include cyclin D1 (Guttridge et al., 1999), E2F (Cheng et al., 2003), Skp2 (Schneider et al., 2006) and p21 (Hinata et al., 2003), each of which is a known key regulator of the cell cycle in HSCs. In particular, NF-κB2-dependent pathways have been implicated in cell proliferation and tumourigenesis and it is known that the p52 subunit is an important regulator of cyclin D1 expression (Guttridge et al., 1999). Furthermore, the RelB/p52 complex has been implicated in the control of transcription of the *skp2* (Schneider et al., 2006) and *c-myc* (Demicco et al., 2005) genes. The fact that NF-κB can regulate the transcription of these cell cycle regulatory genes, confirms its role as a regulator of cell survival, angiogenesis, metastasis and proliferation in cancer cells (Kim et al., 2006).

It is important to note that inhibition of NF-κB in other cell types does not necessarily lead to anti-cancer activity. For example, inhibition of NF-κB in murine skin resulted in an increase in cancer activity (Aggarwal, 2004). Therefore, depending on the environment, NF-κB inhibition or activation can have either a pro- or an anti-cancer outcome. This is important to consider at a clinical level, especially when it is being claimed that an NF-κB inhibiting drug is suitable as a therapeutic cancer treatment, when the side effects could include increased pro-cancer activity. Recent research has demonstrated that the long term inhibition of NF-κB using Bortezomib, which is a drug used to treat multiple myeloma, can cause increased lung tumour growth in mice (Karabela et al., 2012). In the context of the work peformed in this thesis, the fact that loss of NF-κB2 activity can have a proliferative effect, at least within haemopoietic stem cells, needs to be taken into account when considering NF-κB family member inhibition as a possible treatement to target leukaemic stem cells.

APPENDIX

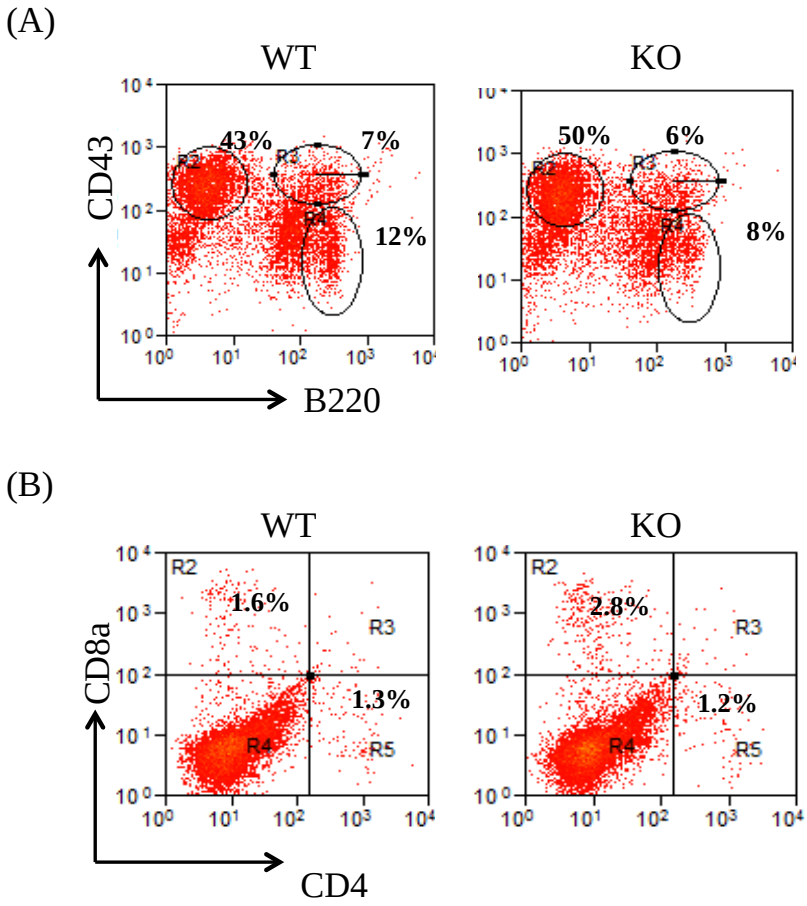

Figure A1: Immunofluorescence analysis of bone marrow cells of wild type and *nfκb1*$^{-/-}$ mice. Bone marrow from wild type (WT) and *nfκb1*$^{-/-}$ (KO) mice was extracted and the red cells were lysed. Two-dimensional dot plots represent cells gated on FSC against SSC (live cell gate). Immunofluorescent staining of bone marrow was performed using antibodies against the indicated lineage associated surface markers. (A) B-cell markers B220 and CD43. (B) T-cell markers CD4 and CD8a. This figure represents typical dot plots from 6 analysed wild type and 6 analysed KO bone marrow mice

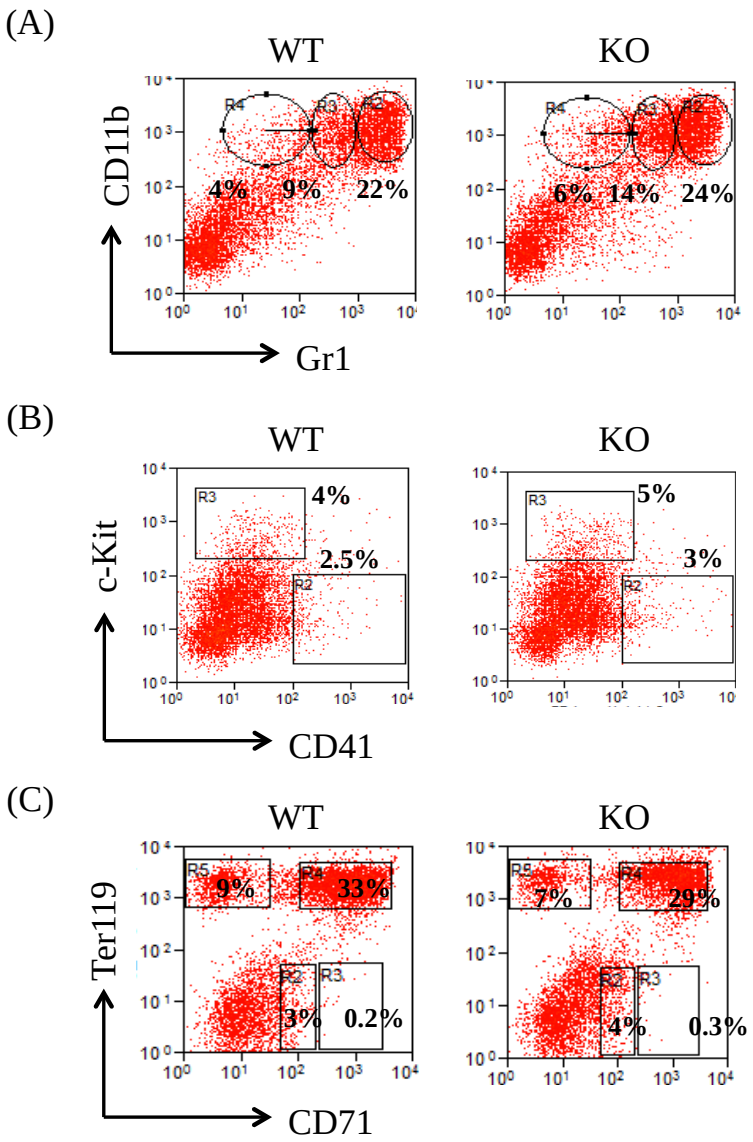

Figure A2: Immunofluorescence analysis of bone marrow cells of wild type and *nfkb1*$^{-/-}$ mice. Bone marrow from wild type (WT) and *nfκb1*$^{-/-}$ (KO) mice was extracted and the red cells were lysed. Two-dimensional dot plots represent cells gated on FSC against SSC (live cell gate). Immunofluorescent staining of bone marrow was performed using antibodies against the indicated lineage associated surface markers. (A) Myelomonocytic markers CD11b and Gr1. (B) Progenitor and megakaryocytic markers c-Kit and CD41. (C) Erythroid markers Ter119 and CD71. This figure represents typical dot plots from 6 analysed wild type and 6 analysed KO bone marrow mice

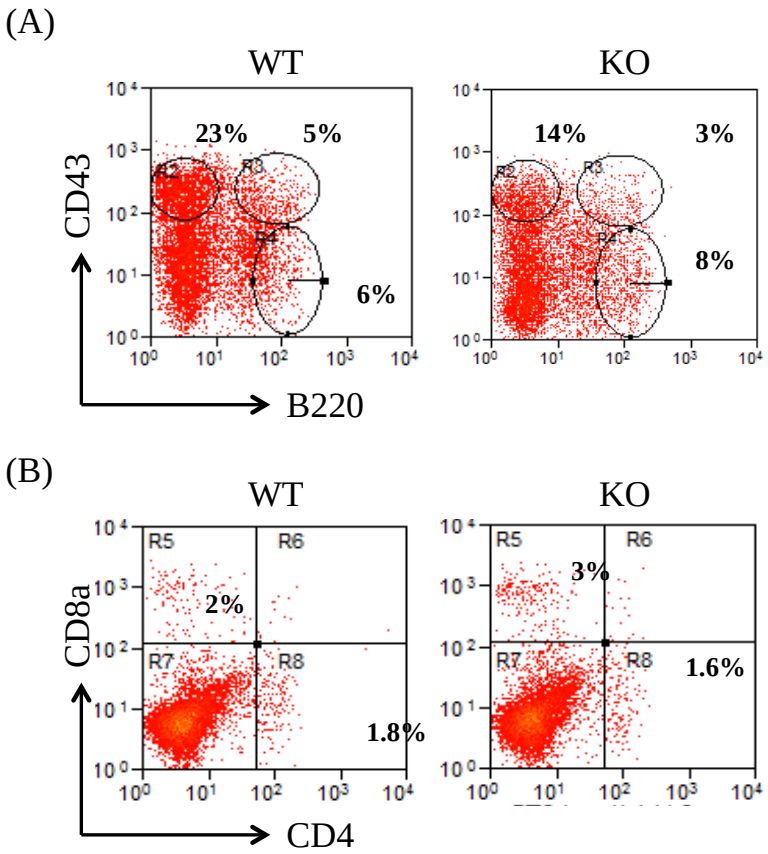

Figure A3: Immunofluorescence analysis of bone marrow cells of wild type and *nfκb2*$^{-/-}$ mice. Bone marrow from wild type (WT) and *nfκb2*$^{-/-}$ (KO) mice was extracted and the red cells were lysed. Two-dimensional dot plots represent cells gated on FSC against SSC (live cell gate). Immunofluorescent staining of bone marrow was performed using antibodies against the indicated lineage associated surface markers. (A) B-cell markers B220 and CD43. (B) T-cell markers CD4 and CD8a. This figure represents typical dot plots from 6 analysed wild type and 6 analysed KO bone marrow mice

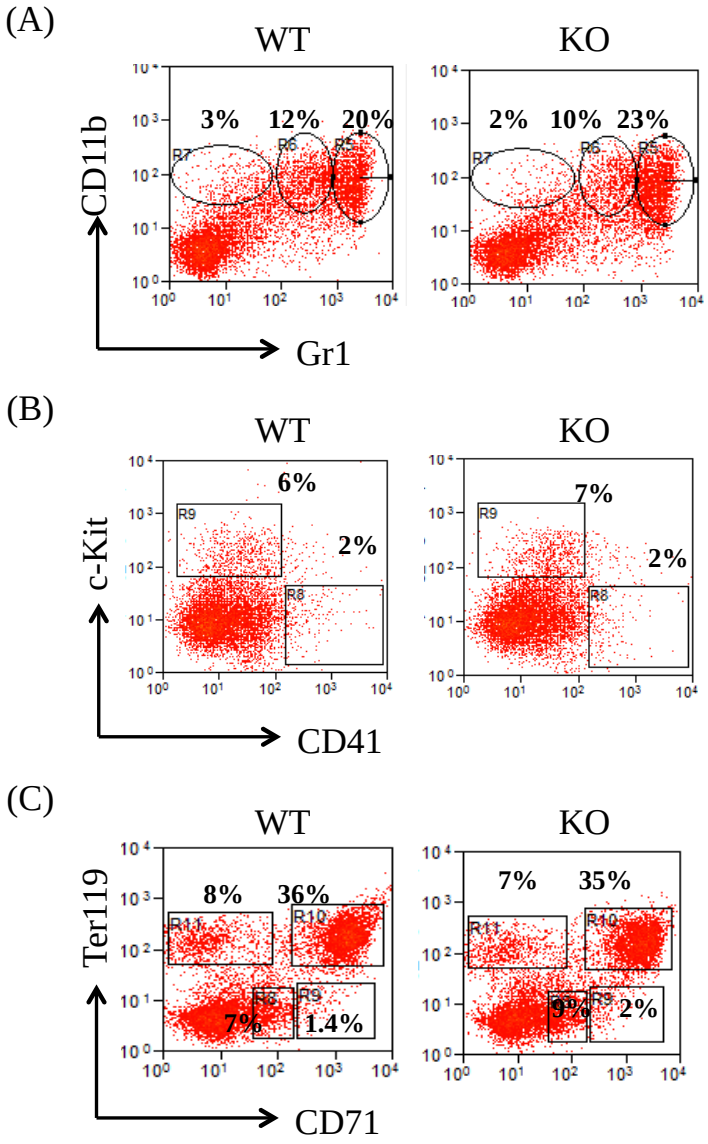

Figure A4: Immunofluorescence analysis of bone marrow cells of wild type and *nfκb2*$^{-/-}$ mice. Bone marrow from wild type (WT) and *nfκb2*$^{-/-}$ (KO) mice was extracted and the red cells were lysed. Two-dimensional dot plots represent cells gated on FSC against SSC (live cell gate). Immunofluorescent staining of bone marrow was performed using antibodies against the indicated lineage associated surface markers. (A) Myelomonocytic markers CD11b and Gr1. (B) Progenitor and megakaryocytic markers c-Kit and CD41. (C) Erythroid markers Ter119 and CD71. This figure represents typical dot plots from 6 analysed wild type and 6 analysed KO bone marrow mice

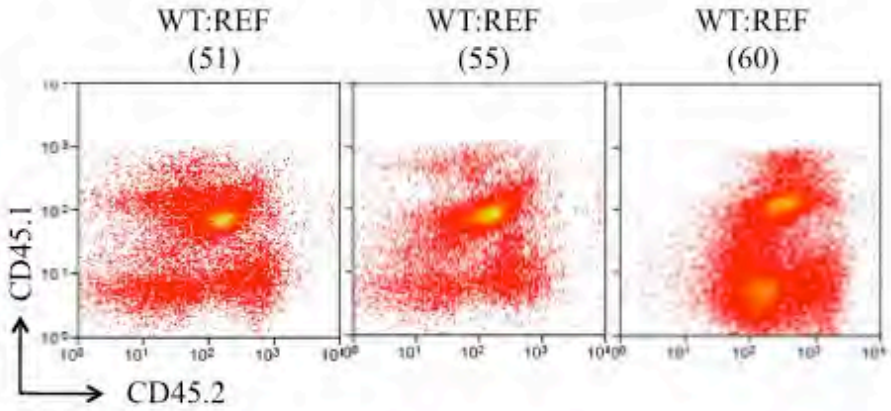

Figure A5: Immunofluorescence flow cytometry analysis of bone marrow from secondary transplant recipients. Bone marrow samples from recipients of wild type (WT:REF) and *nfκb1*$^{-/-}$ (KO:REF) was extracted, red cell lysis and the samples were stained with antibodies against CD45.1 and CD45.2 to distinguish between host (CD45.1/CD45.1), donor reference (REF) (CD45.1/CD45.2) and donor test (WT) (CD45.2/CD45.2) . The dot plot represent the bone marrow staining of mouse 51, 55 and 60. Recipient 51 had received 1 x 10^6 bone marrow cells from primary WT:REF recipient number 30, recipient 55 had received 1 x 10^6 bone marrow cells from primary KO:REF recipient number 36 and recipient 60 had received 1 x 10^6 bone marrow cells from primary KO:REF recipient number 38 (see Table 3.3).

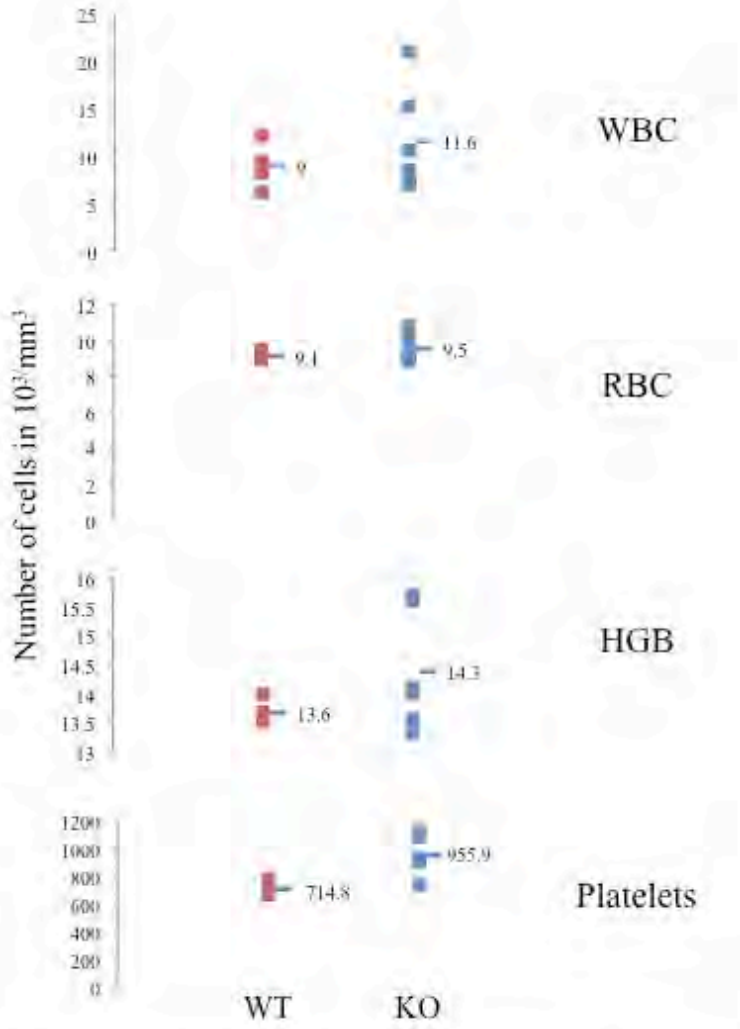

Figure A6: Analysis of peripheral blood cells in wild type and *nfκb2*$^{-/-}$ primary transplanted recipient mice. Cardiac punctures were performed on wild type (WT) and *nfκb2*$^{-/-}$ (KO) primary transplanted recipients and blood was collected into anticoagulant solution. Cell counts were performed using an ABX Pentra 60 automatic blood counter. The plots represent data of 4 WT and 6 KO Set 1 transplanted recipients. The averages are indicated in the plots.

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