THE ROLE OF RESISTIN-LIKE GAMMA EXPRESSED BY HAEMATOPOIETIC STEM CELLS IN CONTROLLING THEIR MAINTENANCE AND COMMITMENT TO DIFFERENTIATION

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Abstract

Haematopoietic stem cells (HSC) are at the top of a hierarchy of cell commitment, proliferation, and differentiation that leads to the life-long production of the functional cell types that constitute the blood. The maintenance of HSC is tightly controlled by an interplay between cell intrinsic and extrinsic mechanisms, the latter including the bone marrow niche environment and systemic factors. One intrinsic factor, the transcriptional regulator MYB, is important in normal HSC maintenance and in the initiation of haematological disease and ageing. From genetic manipulation and RNA-seq experiments, the Resistin-like gamma (*RETNLγ*) gene has emerged as a predominant target of the action of MYB. This project involves the investigation of the expression and function of the *RETNLγ* gene and protein in HSC.

RETNLγ is a member of a small family of Resistin-related proteins that appear to act as soluble mediators of a number of biological processes, working by binding to cognate receptors. Very little is known about RETNLγ apart from a demonstrated expression in several tissues, including bone marrow. This project seeks to determine the nature of RETNLγ expression in the HSC compartment at the RNA and protein level. In order to investigate the function of RETNLγ in HSC in the bone marrow, a mouse knock out (KO) of the *RETNLγ* gene was used in a combination of *in vitro* and *in vivo* assays to measure the consequences of absence of the protein.

The *RETNLγ* gene was found to be expressed throughout the haematopoietic hierarchy, the level of expression varying widely with mature myelomonocytic cells and HSC expressing the highest and lowest levels, respectively. Interestingly, comparing males and females, significantly lower expression of *RETNLγ* RNA was noted in the most immature HSC and in

megakaryocytes. RETNLγ protein levels correlated with the RNA expression data. The gender-specific differences in RETNLγ RNA expression were reflected in corresponding differences in HSC numbers in the bone marrow. Transcriptome analysis of female HSC showed that the absence of RETNLγ results in significant gene expression differences, particularly in terms of increased expression of immune- and stress-related genes in the knockout, which were not affected in males. Attempts to rescue the effects of the absence of RETNLγ in knockout female HSC were unsuccessful in that the normal level of affected genes could not be restored, most likely because of a failure to achieve physiological levels of RETNLγ activity.

Overall, the results point to a female-specific function of RETNLγ in HSC, which opens up a whole new area of research into the mechanisms at play, both in terms of the role of RETNLγ and the way in which this is influenced by the gender background.

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AGM	Aorta-Gonad-Mesonephros region
BM	Bone marrow
c-Kit	Receptor tyrosine kinase
CAFC	Cobblestone-Area Forming Cell assay
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMRP	Common myeloid repopulating progenitor
CSF	Colony-stimulating factor
E	Embryonic day
ECM	Extracellular matrix
Eryth	Erythrocyte
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
Flt3	FMS-like tyrosine kinase 3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte macrophage progenitor
Gran	Granulocyte
HSC	Haematopoietic stem cell
IL-3	Interleukin-3
iPSC	Induced pluripotent stem cell

LDA	Limiting dilution assay
Lin	Lineage
LT-HSC	Long-term haematopoietic stem cell
M-CSF	Macrophage colony-stimulating factor
Meg	Megakaryocyte
MEP	Megakaryocyte-erythrocyte progenitor
MERP	Megakaryocyte-erythrocyte repopulating progenitor
MkRP	Megakaryocyte repopulating progenitor
Mono	Monocyte
MPP	Multipotent progenitor
MSC	Mesenchymal stem cell
PSC	Pluripotent stem cell
RBC	Red blood cell
RELM	Resistin-like molecule
RETN	Resistin
RETNLα	Resistin-like alpha
RETNLβ	Resistin-like beta
RETNLγ	Resistin-like gamma
Sca-1	Stem cell antigen Sca-1
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SL-MkP	Stem-like megakaryocyte committed progenitor
ST-HSC	Short-term haematopoietic stem cell
TF	Transcription factor

TGFβ1	Transforming growth factor beta 1
Ub	Ubiquitin
vWF	von Willebrand factor
YS	Yolk sack

CHAPTER 1 INTRODUCTION

Haematopoiesis is the process by which the body produces blood cells. This system is essential for vertebrate life and is composed of several specialised cell types. Haematopoietic stem cells (HSC) are responsible for maintaining haematopoiesis and include a group of cells that have the potential for both self-renewal (generating of daughter HSC by cell division), and multipotent differentiation (generating mature adult blood cell types) (Eaves, 2015; Weissman and Shizuru, 2008; Seita and Weissman, 2010). Having these two functional properties enables HSC to persist throughout life (Wilkinson et al., 2020). These fundamental properties of HSC enable them to reconstitute the haematopoietic system of irradiated mice, which are ablated for their bone marrow (BM) stem cells, following transplantation (Copelan, 2006; Chabannon et al., 2018). The ability of HSC to re-establish the haematopoietic system following transplantation has been much utilised as a curative therapy for several haematological malignancy diseases, such as leukaemia and lymphoma, and non-malignant blood disorders, including immunodeficiency diseases, autoimmune diseases, hereditary blood disorders, and anaemia (Copelan, 2006; Chabannon et al., 2018). Collectively, the research into and therapeutic application of HSC make them a paradigm for stem cell biology as a whole and has allowed the development of technologies and experimental approaches that are widely used in studying stem cells.

1.1 The haematopoietic hierarchy

1.1.1 The haematopoietic hierarchy includes stem cells, progenitors, and fully differentiated cells

Like all differentiation systems, haematopoiesis can be regarded as a hierarchical organisation of cells progressing from an immature state towards more differentiated, functional cell types that supply peripheral blood and other haematopoietic organs. The haematopoietic hierarchy can be divided into three compartments. At the top of the hierarchy are the HSC, which possess self-renewal capacity that allows these cells to be maintained throughout life. These HSC have the ability fully to reconstitute the haematopoietic system in lethally-irradiated mice for the long term (Spangrude et al., 1988). The capability of HSC to self-renew gradually declines as they mature towards haematopoietic progenitors. HSC are not committed to a specific cell lineage but give rise to the second compartment of cells in the hierarchy called multipotent progenitors (MPP) (Morrison and Weissman, 1994; Christensen and Weissman, 2001). These MPP are heterogenous (Osawa et al., 1996; Christensen and Weissman, 2001; Kiel et al., 2005b) and can differentiate into common myeloid progenitors (CMP) (Kondo et al., 1997), and common lymphoid progenitors (CLP) (Akashi et al., 2000). These progenitors are immature cells with a limited self-renewal capacity, but with the capability for mass proliferation in order to produce sufficient differentiated cells when required. The third compartment consists of fully mature cells that can circulate in the peripheral blood and other haematopoietic tissues (Figure 1.1). Mature cells have no self-renewal or proliferation capacity, and they are divided into (1) myeloid lineage cells, which are produced from CMP and include red blood cells (RBC), megakaryocytes, monocyte/macrophages, and granulocytes; and (2) lymphoid lineage cells, which are cells that are produced from CLP and are responsible for acquired immunity (T- and B-lymphocytes, and NK cells) (Figure 1.1).

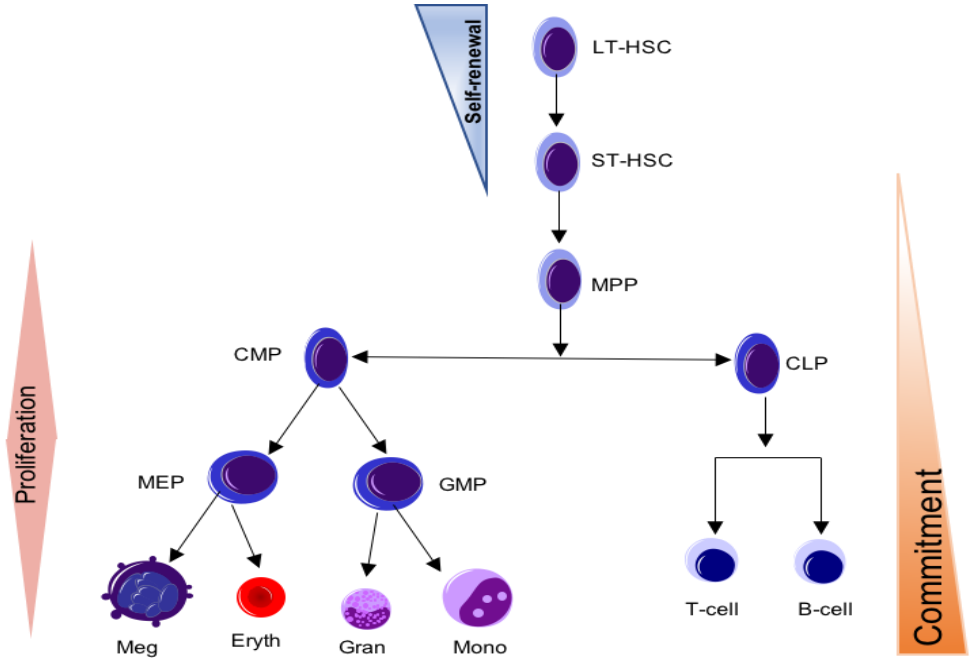


Figure 1.1 The haematopoietic hierarchy. Schematic diagram of the haematopoietic hierarchy, which at the top is initiated by the multipotent, long-term haematopoietic stem cell (LT-HSC) that gives rise to the short-term haematopoietic stem cell (ST-HSC), which in turn differentiates into multipotential progenitors (MPP) (Morrison and Weissman, 1994; Christensen and Weissman, 2001). The blue triangle illustrates the reduction of self-renewal capacity during maturation in the stem cell compartment. Cells in the middle are progenitor cells (CMP, common myeloid progenitor (Kondo et al., 1997); CLP, common lymphoid progenitor (Akashi et al., 2000); MEP, megakaryocyte-erythrocyte progenitor; and GMP, granulocyte macrophage progenitor) that have no self-renewal capacity. These progenitor cells are committed towards a specific lineage and have a high proliferation rate. The cells at the bottom of the hierarchy (Meg, megakaryocyte; Eryth, erythrocyte; Gran, granulocyte; and Mono, monocyte) are terminally differentiated and can be found in peripheral blood. Microsoft PowerPoint has been used to obtain this figure.

1.1.2 Mammalian blood cells serve a wide range of functions

Peripheral blood contains more than ten distinct mature cell types with specific functions, which are ultimately derived from the HSC. RBC are the most abundant cells in the bloodstream, being responsible for the delivery of oxygen to body tissues and organs and removal of carbon

dioxide. Megakaryocytes are responsible for producing platelets, which are essential for haemostasis. Monocytes, macrophage, and granulocytes together with NK cells contribute to innate immunity and cell-mediated immunity. Finally, lymphoid cells T- and B-cells constitute the adaptive immune system.

Under normal physiological conditions, HSC are responsible for maintaining homeostasis in the haematopoietic system but must increase their activity to the maximum in response to any haematological stress (eg bleeding), and then return the normal steady-state (Ogawa, 1993; Morrison and Weissman, 1994; Kondo et al., 1997; Akashi et al., 2000; Chao et al., 2008).

1.2 Development of the haematopoietic system

1.2.1 Haematopoiesis develops in distinct phases

The development of the adult haematopoietic system in vertebrates is a complex process that has been under investigation over several decades. The fact that dysregulation or mutation in critical regulators of embryonic blood development can lead to haematological malignancy in later life is one reason why understanding the ontogeny of haematopoiesis has been a major research direction in the field. Detailed knowledge of both the anatomical sites and specific temporal sequence of HSC evolution is necessary in order fully to understand haematopoiesis, and the mouse has served as the principal model in which to conduct such studies.

During mouse embryogenesis, haematopoiesis occurs sequentially in a number of distinct locations (Figure 1.2), which correlate with changes in the anatomy of the embryo during organogenesis. Broadly, two phases of haematopoietic development can be defined, namely ‘primitive’ and ‘definitive’, the latter giving rise to adult haematopoiesis.

During the early embryonic stage starting at E7.5, primitive nucleated erythrocytes are produced in the blood islands of the extra-embryonic yolk sac (Dzierzak and Medvinsky, 1995; Palis and Yoder, 2001). The haematopoietic and endothelial cells of the blood island are produced from a common mesodermal precursor known as the haemangioblast (Ferkowicz and Yoder, 2005; Shalaby et al., 1995; Choi et al., 1998). The primary function of the primitive wave is to produce erythroid progenitors that can transport oxygen as the embryo undergoes rapid growth (Orkin and Zon, 2008). These erythroid progenitors are unipotent and do not have self-renewal capacity.

Under experimental conditions, erythroid-myeloid progenitors start to appear in the YS (Moore and Metcalf, 1970) and in the early stage of the placenta and the umbilical cord, known as the chorion and allantois (Palis et al., 1999; McGrath et al., 2011; Alvarez-Silva et al., 2003; Corbel et al., 2007; Zeigler et al., 2006; Boisset and Robin, 2012). From E8.5, erythroid, myeloid, and lymphoid progenitors are found in the YS in addition to the Para-aortic Splanchnopleura (P-Sp region formed by the dorsal aorta, omphalomesenteric, artery, gut, and splanchnopleure) (Godin et al., 1995; Bertrand et al., 2005). Multipotent stem cells are also detected in the YS and the intra-embryonic Aorta-Gonad-Mesonephros region (AGM, the region corresponding to the earlier P-Sp) at E10 (Medvinsky et al., 1993).

For a period of time, it was believed that the YS produced adult HSC, and that these migrated and colonised the foetal liver followed by the adult BM (Moore and Metcalf, 1970). However, it was found that embryonic HSC failed to support erythropoiesis and haematopoietic precursors found throughout life, which are actually derived from a different site in the embryo (Figure 1.2) (Dieterlen-Lievre, 1975). Despite the ultimate origin of the cells, the main site of adult HSC differentiation and expansion in the second, definitive wave of haematopoiesis is the foetal liver. However, HSC are not produced *de novo* by the foetal liver, but are believed to be

seeded by circulating haematopoietic cells that arise in the AGM region of the developing embryo (Houssaint, 1981; Johnson and Moore, 1975; Mikkola and Orkin, 2006; Cumano and Godin, 2007). Foetal liver seeding starts at E9.5 to E10.5 when the liver rudiment becomes colonised by myeloid and erythroid progenitors that generate definitive erythroid cells (Mikkola and Orkin, 2006; Jagannathan-Bogdan and Zon, 2013).

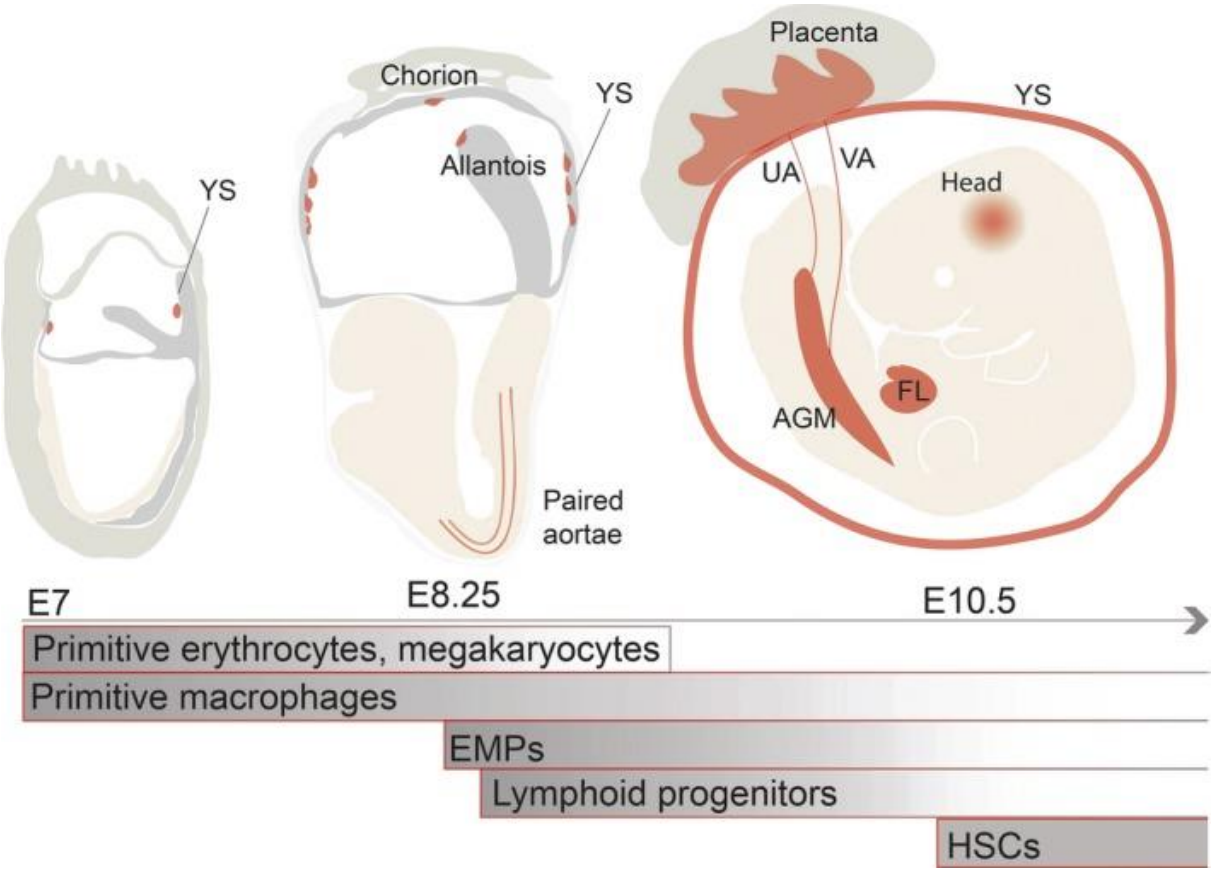


Figure 1.2 Establishment of primitive and definitive haematopoietic stem cell in mouse. The production site of primitive HSC can be found in the blood island during embryonic stage (Dzierzak and Bigas, 2018). Definitive HSC are produced from foetal liver after it has been seeded by circulating cells. Also, a time line and duration of the production of both primitive and definitive HSC and the sites of each of each period of time (Orkin and Zon, 2008). Image taken from (Kauts et al., 2016).

1.2.2 Adult HSC arise in the AGM before progressing to the foetal liver and then the bone marrow

At E11.5, the majority of the HSC of the second phase that colonising the foetal liver derive from endothelium with haemogenic properties known as haemogenic endothelium at the level of AGM (Jagannathan-Bogdan and Zon, 2013). HSC and progenitors travel through the AGM and the placenta via the umbilical vessels, which is the second major vascular circuit that connects to the foetal liver (Ivanovs et al., 2011; Ivanovs et al., 2014). At E12.5 the foetal liver becomes the main site where HSC undergo expansion and differentiation, reaching a plateau (around 1000 HSC) by E15.5 to E16.5 before declining (Ema and Nakauchi, 2000; Gekas et al., 2005; Morrison et al., 1995).

In order for HSC to be maintained into and throughout adult life, a unique microenvironment becomes established within skeletal system development. At E12.5, skeletal development starts when mesenchymal stem cells (MSC) give rise to chondrocytes that create a cartilaginous skeletal framework (mesenchymal condensation). The chondrocytes are then replaced by osteoblasts, which in turn generates calcified bone through endochondral ossification. The seeding of HSC and progenitors into the developing bones is facilitated by vascular invasion (Olsen et al., 2000). Clonogenic progenitor activity in the long bone can be found at E15.5, and the functional HSC are found from E17.5 onwards (Christensen et al., 2004; Gekas et al., 2005), although functional HSC are found in the circulation several days before, which means that the early foetal BM microenvironment is unable to attract HSC and support their engraftment and self-renewal (Mikkola and Orkin, 2006).

1.3 The haematopoietic stem cell

1.3.1 The history of the discovery and investigation of HSC spans more than 70 years

For several decades, starting around the middle of the 20th century, HSC have been the subject of intense investigation, particularly in relation to their ability to protect an organism against the consequences of lethal irradiation (Ng and Alexander, 2017). In 1945, Owen observed that fraternal twin cattle shared for life the blood cell types of both calves (Owen, 1945). Owen wrote, "Since many of the twins in this study were adults when they were tested, and since the interchange of formed erythrocytes alone between embryos could be expected to result in only a transient modification of the variety of circulating cells, it is further indicated that the critical interchange is of embryonal cells ancestral to the erythrocytes of the adult animal. These cells are apparently capable of becoming established in the haematopoietic tissues of their co-twin hosts and continuing to provide a source of blood cells distinct from those of the host, presumably throughout his life." In 1952, cattle twins were used again by Medawar and Billingham for skin transplantation experiments between heterozygotic (mixed sex) and monozygotic (identical) twins. An unexpected outcome was the observation that grafts were invariably accepted in all circumstances (Billingham and Reynolds, 1952). Their further studies in mice demonstrated that tolerance could be induced by haematopoietic cell infusion in foetal and neonatal mice, research that ultimately led to the Nobel Prize in Medicine being awarded to Medawar in 1960 (Billingham et al., 1953).

The occurrence of haematological failure, and often death, in people exposed to radiation from the atomic bomb explosions in Hiroshima and Nagasaki during the Second World War, led to much of the research focussed on the capacity of HSC to reconstitute the haematopoietic system. It was found that mice could be protected from radiation syndrome by shielding their spleen with lead, then by injecting spleen or marrow cells (Jacobson et al., 1950; Lorenz et al.,

1951; Jacobson et al., 1951). In 1955, the first successful transplantation experiment was performed in mice when allogenic marrow was transplanted into lethally-irradiated mice without immune suppression, implying that this experiment is similar to Owen's experiment (Main and Prehn, 1955). A massive breakthrough in the field of HSC research came in 1961 when Till and McCulloch published their findings indicating that: (1) haematopoiesis could be studied as a quantitative science; (2) clonal HSC in the marrow can give rise to all blood cell types; (3) some of these cells made more of themselves (self-renewal potential); and (4) lymphocytes are produced from cells that existed in the spleen, which initially come from the BM (Till and Mc, 1961; Becker et al., 1963; Siminovitch et al., 1963; Wu et al., 1967; Wu et al., 1968).

Between the 1970s and 1980s, the focus of research became the ability of HSC to be transplanted and survive for a long time, involving different autologous and allogeneic haematopoietic cell transplants of different cell types, including progenitors and stem cells. The HSC has been defined as a single cell that has the ability to undergo self-renewal, produce all blood cell types (multipotency), and produce lifelong reconstitution of the BM (Becker et al., 1963; Lemischka et al., 1986). Subsequently, different aspects of HSC have been studied, predominantly using the mouse. Key features of HSC studied, with the goal to improve how HSC can be used to treat leukaemia and other haematological malignancies, include their surface antigen characteristics and how best to isolate them, the degree of heterogeneity, and mechanisms of regulation of HSC by both intrinsic and extrinsic factors.

1.3.2 Stem cells are defined by a number of fundamental characteristics

1.3.2.1 Potency

Stem cells are defined by two main characteristics, that is, their ability to self-renew to secure their existence throughout life, and the ability to differentiate into one or more cell types.

The zygote can be considered as a totipotent stem cell that can divide and differentiate into cells of the whole organism. A totipotent stem cell has the greatest degree of differentiation potential, being able to give rise to both embryonic and extraembryonic (placenta) lineages (Zakrzewski et al., 2019).

Pluripotent stem cells (PSC) are similar to totipotent stem cells; however, they are unable to produce extraembryonic cell types. Embryonic stem cells (ESC) are an example of PSC (Sukoyan et al., 1993). Induced pluripotent stem cells (iPSC) are another form of PSC that are artificially generated from somatic cells and in many respects are similar to ESC. Research in the iPSC field has been intense since 2006, largely because of the promise they offer for regenerative medicine (Larijani et al., 2012; Zakrzewski et al., 2019). A multipotent stem cell has self-renewal capacity similar to that of totipotent and pluripotent stem cells but has a more restricted spectrum of differentiation. The most studied example of this type of stem cell is the HSC (Zakrzewski et al., 2019). Oligopotent stem cells can differentiate into different cell types of a small number of lineages, while unipotent stem cells can give rise to just one cell type.

1.3.2.2 Quiescence

For decades, there was debate about the nature of the cells that are non-cycling but have the ability to proliferate in response to extrinsic stimuli. Some researchers considered these cells to be in a prolonged G1 phase of the cell cycle, while others regarded it as a non-proliferative phase G0, which is also referred to the quiescence state (Cheung and Rando, 2013).

Quiescence is a state of the cell that does not divide but that retains the ability to re-enter the cell cycle. In the haematopoietic system, all HSC are capable of entering the cell cycle; however, only 1-3% are cycling under normal conditions. It is clear that this property is important to maintain lifelong stem cell activity (Challen et al., 2009), and also as a protective mechanism to guard against malignant transformation and malfunction and to avoid depletion (Wang and Dick, 2005). Different intrinsic and extrinsic factors contribute to the decision whether or not HSC exit quiescence, including in response of inflammation, blood loss, etc. (Morrison and Weissman, 1994; Figure 1.3). Quiescent and cycling HSC can be distinguished in a number of ways based on DNA and RNA content and by staining for proliferation markers. Quiescent HSC contain less RNA compared to active HSC (Darzynkiewicz et al., 1980), while dual staining with Hoechst 33342 together with anti-Ki67 antibody, can be used specifically to label proliferating cells (Wilson et al., 2004). Staining with Pyronin Y, which is an RNA binding dye, was used to show that approximately two thirds of LT-HSC are quiescent (Eddaoudi et al., 2018; Nakamura-Ishizu et al., 2014; Bigas and Espinosa, 2012) (Figure 1.3).

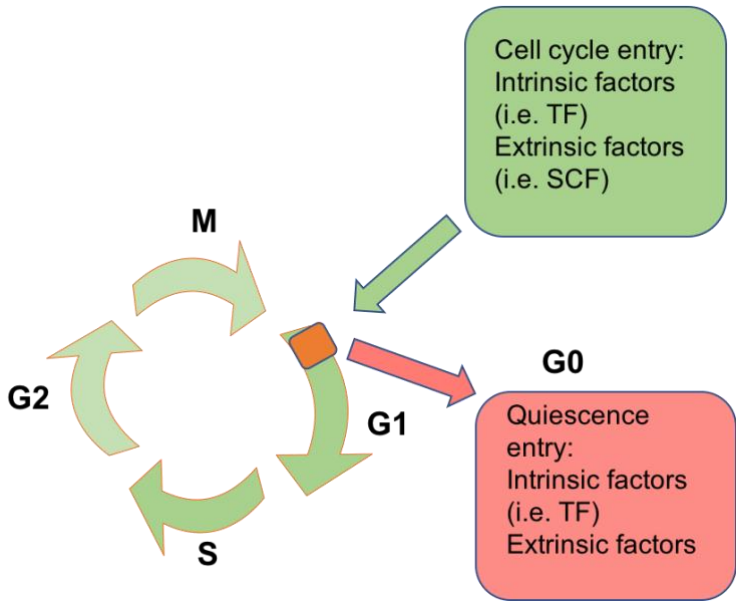


Figure 1.3 HSC quiescence. Most HSC are in a quiescent state in the G0 phase of the cell cycle in normal haematopoiesis. Normally, only 1-3% of the HSC are activated by intrinsic and extrinsic factors to pass the

restriction point in G1 phase and progress through the cell cycle phases (G1 phase, S phase, G2 phase, and M phase) and divide. TF, transcription factor; SCF, stem cell factor; G1, cell increase in size phase; S, cell copies its DNA; G2, cell prepare to divide; M, mitosis phase. Microsoft PowerPoint has been used to obtain this figure.

1.3.2.3 Self-renewal

One stem cell can divide into either two identical cells with the same developmental potential as the original cell (symmetrical division) or one identical cell and one committed progenitor (asymmetrical division), the production of an identical cell generally being referred to as 'self-renewal' (Figure 1.4). Two models have been proposed to describe the HSC choice to commit to differentiation. In the 'stochastic' model, HSC randomly commit without the influence of cytokines, which instead allow the survival of the cells as they start to differentiate. In the other, 'instructive', model specific cytokines direct the HSC and determine the outcome of each mitosis. Several studies support the instructive model; however, the initial division of HSC are likely to involve stochastic fate choices (Wagers et al., 2002). So, HSC self-renewal is controlled by intrinsic factors, mainly transcription factors, and by the environment (BM niche). Self-renewal is a critical property of HSC enabling the maintenance of homeostasis, keeping a fairly constant number of cells in the stem cell pool throughout life while allowing the continuous production of blood cells. Rigorous quantitative transplantation assays can measure the extent of HSC self-renewal. (Zon, 2008; Szilvassy, 2003).

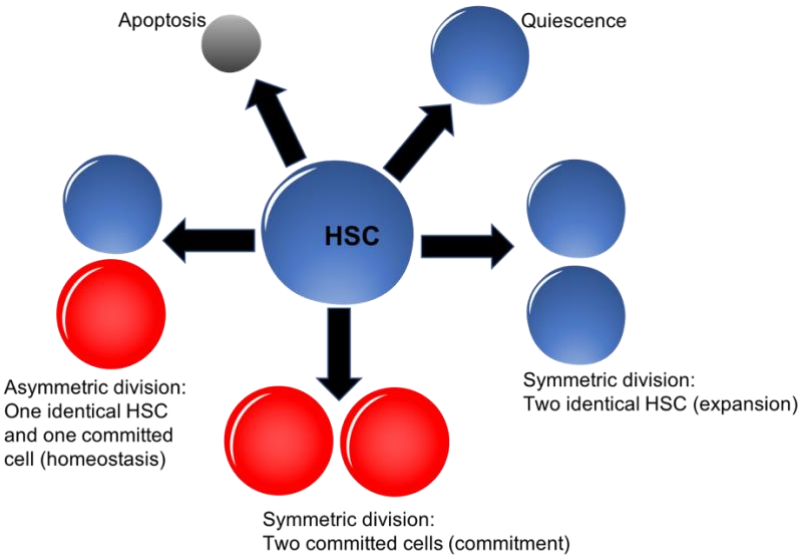


Figure 1.4 HSC fate choices. This schematic diagram represents the HSC fate choices in the BM. The process of homeostasis, commitment, and expansion results in generation of one identical HSC and one committed cell, two committed cells, and two identical HSC, respectively. HSC and quiescence cells are represented in blue, committed cells are represented in red, and apoptotic cell in black. Microsoft PowerPoint has been used to obtain this figure.

1.3.2.4 Apoptosis

Programmed cell death, also known as apoptosis, influences the maintenance of normal HSC numbers (Nebel et al., 2006). Any defect in HSC apoptosis could lead to failure in the production of sufficient mature cells or, conversely, to increase in the production of one or several types of cells that might lead to haematological malignancy (Oguro and Iwama, 2007). Some genes (intrinsic factors) have been identified in response to DNA-damage, i.e. RUNX1 (Bellissimo and Speck, 2017). Also, longevity-related signalling (extrinsic factors) contribute to the HSC to apoptosis (Alenzi et al., 2009).

1.3.2.5 Differentiation

When HSC undergo differentiation, along either the myeloid or lymphoid lineages, this is usually associated with asymmetric stem cell division (Figure 1.4). Several growth factors affect the type of division that the HSC adopts (Gunsilius et al., 2001; Rossi et al., 2007).

1.3.3 The methods for the identification, isolation, and analysis of HSC are highly developed and underpin much of stem cell science

1.3.3.1 Identification and isolation of HSC using surface marker expression

The top part of the haematopoietic hierarchy contains a heterogenous group of HSC, which are different in their properties from each other. In 1988, HSC were described after using a combination of several surface markers, including enzymes, glycoproteins, chemokines, amongst others. These markers are generally known as cluster of differentiation (CD) antigens. Different CD antigens can be used to distinguish different cell populations, often using combinations of multiple markers. Fluorescently-labelled monoclonal antibodies against surface antigens combined with fluorescence-activated cell sorting (FACS) was utilised by Weissman and his colleagues to describe the first HSC-enriched cells (Spangrude et al., 1988; Cheng et al., 2020). To the present day, this method remains the most powerful strategy that can be applied to define even very small subsets of HSC within the overall pool (Eaves, 2015; Kawahara and Shiozawa, 2015; Ng and Alexander, 2017).

One of the first, and still much used, definition of HSC in mice involved detection of surface-expressed tyrosine kinase receptor for stem cell factor (c-Kit) and the stem cell antigen Sca-1 (Ly6A/E) on cells that do not express lineage (Lin^-) markers of B cells, T cells, granulocytes, monocytes/macrophages, erythrocytes, and megakaryocytes (Huang et al., 2007; Ikuta and Weissman, 1992). Mouse HSC defined through use of these markers are often referred to as

'KSL' or 'LSK' cells. Subsequently, ever more markers have been discovered better to define homogenous sub populations of HSC, including FMS-like tyrosine kinase 3 (Flt3) and CD34 (Adolfsson et al., 2001; Kiel et al., 2005a; Osawa et al., 1996).

Cells sorted from a heterogenous population of HSC can be functionally defined on the basis of their ability to reconstitute haematopoiesis in a lethally-irradiated mouse. Three types are usually described, all of which are included in the KSL pool; namely, long-term (LT) HSC, short-term (ST) HSC, and multipotent progenitors (MPP). LT-HSC are the most immature, have the greatest self-renewal potential, are able to reconstitute lethally-irradiated mice for more than six months, and can be serially transplanted into secondary recipients. LT-HSC are defined by a number of markers, including expression of the SLAM marker (signalling lymphocyte activation molecule) CD150, and lack of expression of the SLAM marker CD48, CD34, and Flt3 (Morrison and Weissman, 1994; Adolfsson et al., 2001; Oguro et al., 2013). Like LT-HSC, ST-HSC can repopulate irradiated mice, but for a shorter time (three months), and are distinguished from LT-HSC by being positive for CD34 expression (Adolfsson et al., 2001). The most mature component of the KSL pool are the MPP, which can be further subdivided into three cell types: MPP1, which has a low level of CD34; MPP2, which is $CD34^+$ and has low level Flt3 expression; and MPP3, which is $CD34^+$ and is $Flt3^-$. Beyond the MPP, differentiation can proceed along a myeloid or lymphoid path via committed progenitors CMP and CLP, respectively. CMP, which are $CD34^+CD16/32^-$, progress to GMP, which express CD16/32, or MEP, which does not express CD16/32 or CD34 (Figure 1.5).

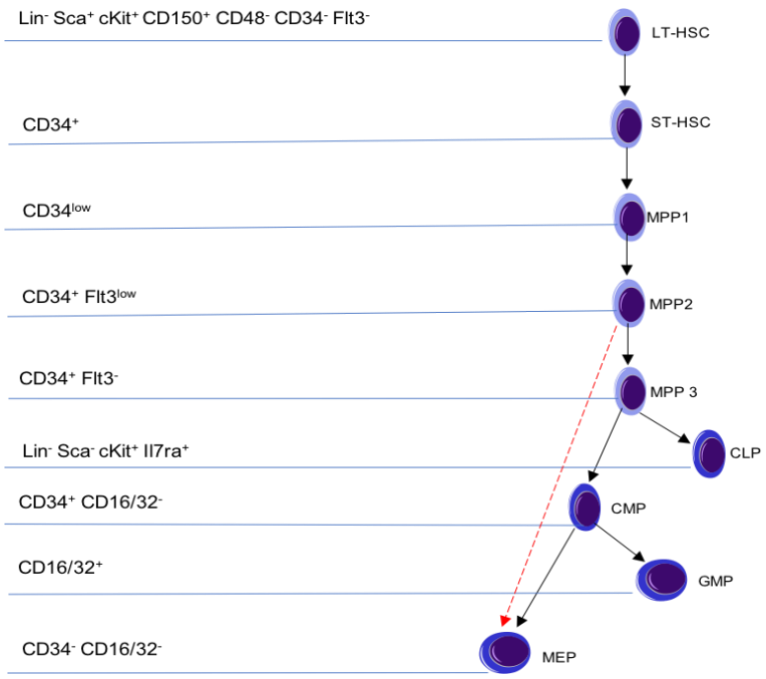


Figure 1.5 Cell surface markers of haematopoietic stem and progenitor cells (HSPC). This schematic diagram represents the cell surface markers of murine HSPC. The pool of MPP has a heterogenous cells that can be differentiated according to their specific cell surface markers, and functional differences have been found in this group of cells. For example, MPP2 can give rise directly to MEP without passing through CMP (red dashed arrow) (Chao et al., 2008; Mayle et al., 2013; Goodell et al., 1996; Yang et al., 2005; Kondo et al., 1997; Akashi et al., 2000). LT-HSC, long-term haematopoietic stem cell; ST-HSC, short-term haematopoietic stem cell; MPP, multipotent progenitors; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; GMP, granulocyte-macrophage progenitors; MEP, megakaryocyte-erythrocyte progenitors; Lin, lineage markers. Microsoft PowerPoint has been used to obtain this figure.

Surface immunofluorescent staining of HSC combined with fluorescence-activated cell sorting (FACS) is the preferred method for the isolation of HSC, providing high degrees of purity and viability, even for the very small numbers of cells that can be recovered for subtypes such as the LT-HSC.

1.3.3.2 In vitro assays of HSC

In vitro, the colony-forming unit (CFU) assay can be used to characterise sorted cell populations. This method is considered the second, and most rapid, method of identifying HSC and progenitors. It is based on cell culture in a semi-solid medium that incorporates different cytokines and growth factors, including so-called colony-stimulating factors (CSF) (Metcalf, 2013). There are four distinct CSF, which are named after the major types of colony formation stimulated by their action; granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and multipotential colony-stimulating factor, known as interleukin-3 (IL-3). Purification and cloning of CSF were achieved during the 1970s and 1980s (Burgess et al., 1977; Stanley and Heard, 1977; Nicola et al., 1983; Ihle et al., 1982). This CFU assay is considered as the second most rapid method of identifying HSC and progenitors, providing a limited functional assessment by showing the ability of HSC to differentiate as well as some limited self-renewal (Frisch and Calvi, 2014).

The Cobblestone-Area Forming Cell (CAFC) assay is another form of *in vitro* test that can be used to analyse HSC. It is a long-term co-culture assay in which HSC are plated on a pre-cultured MSC (de Haan and Ploemacher, 2002). The CAFC assay relates to *in vivo* studies of the BM because it allows the growth of HSC in close association with supporting MSC (Dexter et al., 1977a; de Haan and Ploemacher, 2002). This method can measure the activity of HSC and the frequencies of different progenitor cells (Ploemacher et al., 1989; Ploemacher et al., 1991; Ploemacher et al., 1993). Despite the time saving of such *in vitro* assays, they are considered as indicative assays for HSC activity. In vivo assay (HSC transplantation) is the only assay, which known as a definitive assay.

1.3.3.3 In vivo assays of HSC

Stem cell transplantation into irradiated recipients is the preferred method for the characterisation and assessment of HSC. First developed in the 1950s, the *in vivo* method evolved out of attempts to identify BM cells that could be transplanted to overcome the effects of haematopoietic cell ablation in lethally-irradiated mice, reconstituting all blood cell types (Jacobson et al., 1951; Ford et al., 1956). From these early experiments, the methods were refined for the purposes of identifying, characterising, and quantifying BM cells with repopulating activity (Dick et al., 1985; Keller et al., 1985; Uchida et al., 2003). Further refinements included the limiting dilution assay (LDA), which defines the minimal number of cells required in a BM sample that are sufficient to elicit reconstitution. Earlier experiments showed how the clonal behaviour of HSC could be visualised by examination of a recipient spleen for the presence of visible nodules (colonies) on the surface 9 to 14 days post-transplantation, each nodule being derived from a single donor cell, which was therefore designated as a colony forming unit spleen (CFU-S, (McCulloch and Till, 1960; Till and Mc, 1961). The spleen colonies in such assays were found to consist of a mixture of mature myeloid cells and to be formed from cells that can also produce lymphoid progeny (Becker et al., 1963; Wu et al., 1967). Moreover, some colonies were shown to contain daughter cells that produce similar multilineage colonies in the spleen of a secondary irradiated host, which was therefore the birth of the concept of self-renewal (Siminovitch et al., 1963).

Most experiments using the mouse transplantation assay do so at present using a competitive approach, that is, the host animal is transplanted with a mixture of wild type and test (usually genetically modified) donor cells so that their relative behaviour can be determined. The reference and test donor cells are genetically identical with the exception of (i) any genetic modification that is being explored in the test cells, and (ii) a difference in the allele of the

surface marker CD45 that enables discrimination by flow cytometry or during cell sorting. Hence, the two alleles CD45.1 and CD45.2 are employed, the hosts being CD45.1 homozygotes, the test donor cells being CD45.2 homozygotes, and the reference donor wild type cells being CD45.1/CD45.2 heterozygotes. Such a competitive assay can be refined very precisely to define the number of fully reconstituting HSC by performing a limiting dilution of the test donor against a fixed number of the reference donor cells (Eaves, 2015).

1.3.4 The precise nature of the HSC has become progressively more refined, revealing a complex phenotypic and functional heterogeneity in the stem cell pool

The understanding of the HSC hierarchy developed through over 50 years of experimentation has served the field well, although there is an appreciation that some of the strategies adopted, including a reliance on the use of surface antigens to identify and sort cells, have limitations (Cheng et al., 2020). In the past few years, with the advances in various single cell technologies, new HSC hierarchy models have been proposed. Myeloid-biased HSC (My-Bi) and lymphoid-biased HSC (Ly-Bi) have been identified after using single-cell transplantation and LDA. My-Bi can reconstitute the lymphoid lineage to a lesser extent than the myeloid lineage, and vice versa (Muller-Sieburg et al., 2002; Muller-Sieburg et al., 2004; Dykstra et al., 2007; Benz et al., 2012) (Figure 1.6A). In addition, platelet/myeloid-biased HSC have been recognised at the top of the haematopoietic hierarchy. These cells were found to be LT-HSC that express the surface marker von Willebrand factor (vWF). Roughly 25% of LT-HSC express the vWF and can give rise to all blood cell types, including vWF^- HSC, which is a lymphoid-biased HSC (Sanjuan-Pla et al., 2013). MEP arise directly from platelet/myeloid-biased HSC (Sanjuan-Pla et al., 2013; Yamamoto et al., 2013) (Figure 1.6B). In 2015, stem-like megakaryocyte committed progenitors (SL-MkP) have also been recognised within the HSC compartment. This population shares many features with HSC and becomes activated upon stress to replenish

platelets (Haas et al., 2015). Moreover, using single-cell transplantation, a distinct class of HSC found to replenish megakaryocyte/platelets without producing any other blood cell types (Carrelha et al., 2018).

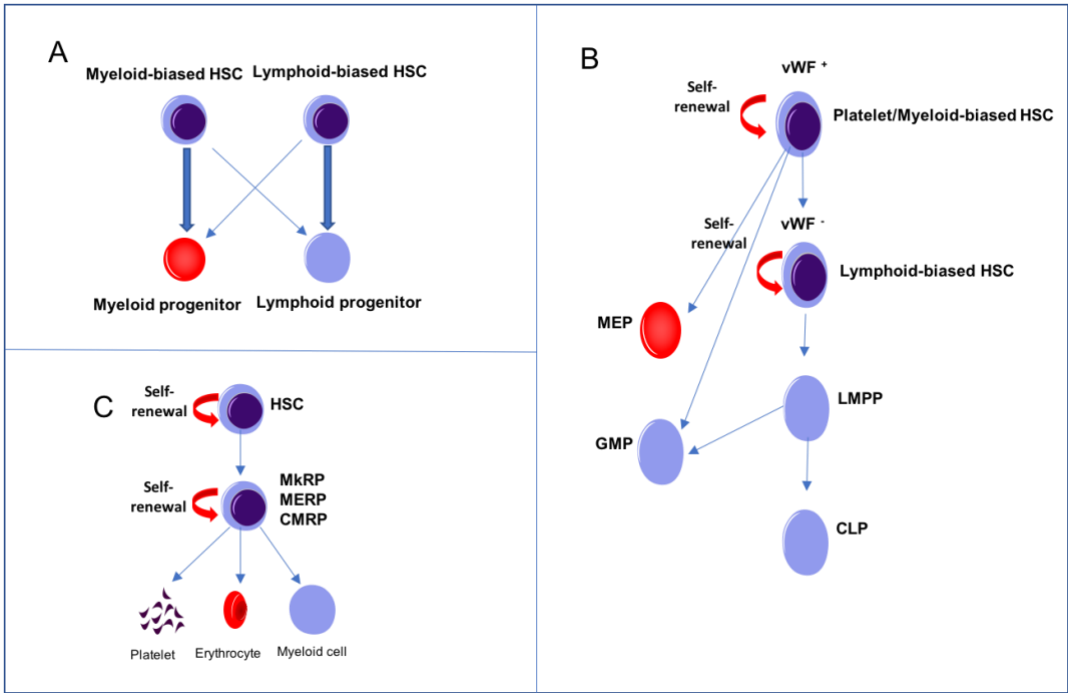


Figure 1.6 The refined models of HSC differentiation. (A) Myeloid-biased HSC and lymphoid-biased HSC model. Myeloid-biased HSC has the ability to reconstitute the lymphoid lineage to a lesser extent than the lymphoid-biased HSC, and vice versa. (B) vWF^{+} platelet/myeloid-biased HSC sit on the top of the hierarchy and has the ability to differentiated into all blood cell types. vWF^{-} lymphoid-biased HSC is derived from vWF^{+} platelet/myeloid-biased HSC. And can differentiate into LMPP. vWF^{-} lymphoid-biased HSC and LMPP cannot give rise to megakaryocyte. (C) MkRP, MERP, and CMRP are heterogenous group that can be found in the HSC pool and they possess the ability of self-renewal. MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; MkRP, megakaryocyte repopulating progenitor; MERP, megakaryocyte-erythrocyte repopulating progenitor; and CMRP, common myeloid repopulating progenitor. Microsoft PowerPoint has been used to obtain this figure.

In recent single-cell transplantation assays, it has also been found that lineage-restricted progenitors exist which have self-renew capacity. These include megakaryocyte repopulating progenitors (MkRP), megakaryocyte-erythrocyte repopulating progenitors (MERP), and common myeloid repopulating progenitors (CMRP). These findings suggest that the HSC pool contains oligo-, bi-, and uni-potent stem cells (Yamamoto et al., 2013) (Figure 1.6C).

Differences in HSC reconstitution ability have also been found with respect to their expression of the SLAM family markers CD150 and CD229. $CD150^{med}$ HSC, $CD150^{hi}$ HSC, and $CD229^{-}$ HSC, exhibit higher self-renewal potential with myeloid-biased differentiation (Morita et al., 2010; Oguro et al., 2013), whereas $CD229^{+}$ HSC have less self-renewal potential with lymphoid bias (Oguro et al., 2013).

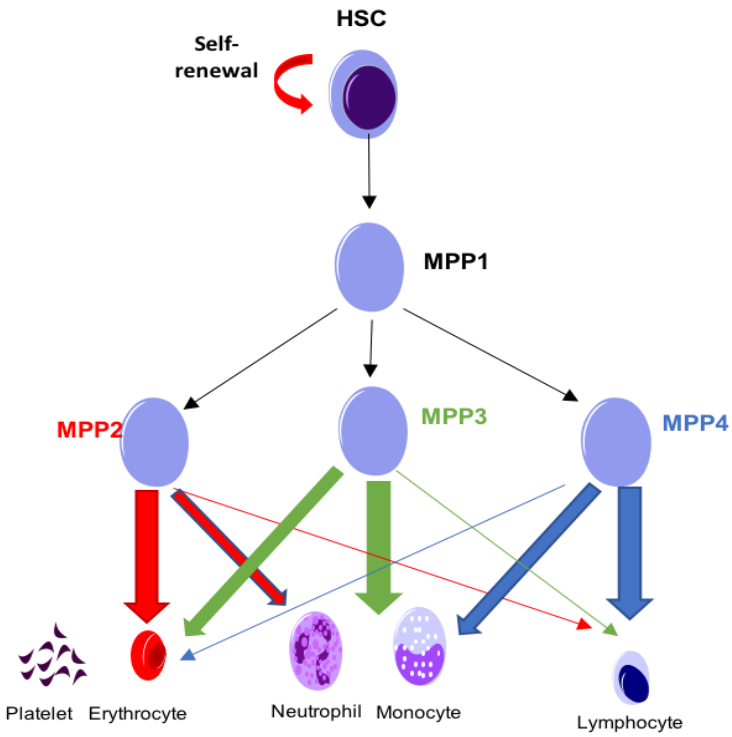


Figure 1.7 An alternative model of HSC differentiation. MPP can be separated into four different populations (MPP1-4). MPP1 can give rise to all other MPP populations. MPP2 is a platelet-erythrocyte biased progenitor. In addition, MPP2 and MPP3 are myeloid-biased progenitors. MPP4 is lymphocyte-biased progenitors. MPP2-4 can

each give rise to all blood cell types to a limited extent. MPP, multipotent progenitors. Microsoft PowerPoint has been used to obtain this figure.

Heterogeneity has also been defined within MPP populations. Hence, based on immunophenotype, cell cycle status, lineage bias, resistance to drug treatment, and BM abundance, MPP populations can be segregated into four categories, MPP1-4. MPP1 have multi-lineage reconstitution ability (up to 4 months) in the first transplantation, whereas, MPP2/3/4 have no self-renewal potential and only show short-term myeloid reconstitution ability (<1 month). MPP2 is considered to be a platelet-biased progenitor because it produces higher numbers of platelets. MPP3 seems to be a myeloid-biased progenitor because it produces higher levels of myeloid cells. Both MPP2 and MPP3 produce a low level of lymphocytes. On the other hand, MPP4 produces a high level of lymphocytes and only a low number of myeloid cells. It is important to mention that MPP1 can produce all MPP populations; however, none of MPP2/3/4 can produce each other. *In vivo*, MPP1/2/3 are produced first after transplantation enabling the myeloid compartment to be established rapidly, followed by MPP4 which is capable of rebuilding the lymphoid compartment (Wilson and Kotton, 2008; Pietras et al., 2015) (Figure 1.7).

1.4 The haematopoietic stem cell niche

A stem cell 'niche' refers to a specific region within a tissue that provides a microenvironment in which stem cells can reside and regulate their functions. Both cellular and non-cellular elements are involved in this microenvironment, which provides a complex and dynamic molecular crosstalk between the stem cell and its surroundings (Figure 1.8) (Lo Celso and Scadden, 2011).

The concept of the BM stem cell niche was first proposed in 1978 (Schofield, 1978). This hypothesis has been supported by a variety of *in vitro* coculture experiments and by BM transplantation, in which the niche is the first target to be 'emptied' through irradiation (Dexter et al., 1977b; Moore et al., 1997; Rios and Williams, 1990). It has been shown that HSC move toward the BM niche in the embryo and after transplantation. The migration and localisation of HSC into a specific microenvironment implies that there are mechanisms of communication between the stem cells and the rest of the bone marrow. The BM niche plays a role in determining stem cell fate (Li and Xie, 2005; Spradling et al., 2001) and in protecting HSC from excessive proliferation, which in turn reduces the number of genetic mutations that might lead to disease (Schofield, 1978).

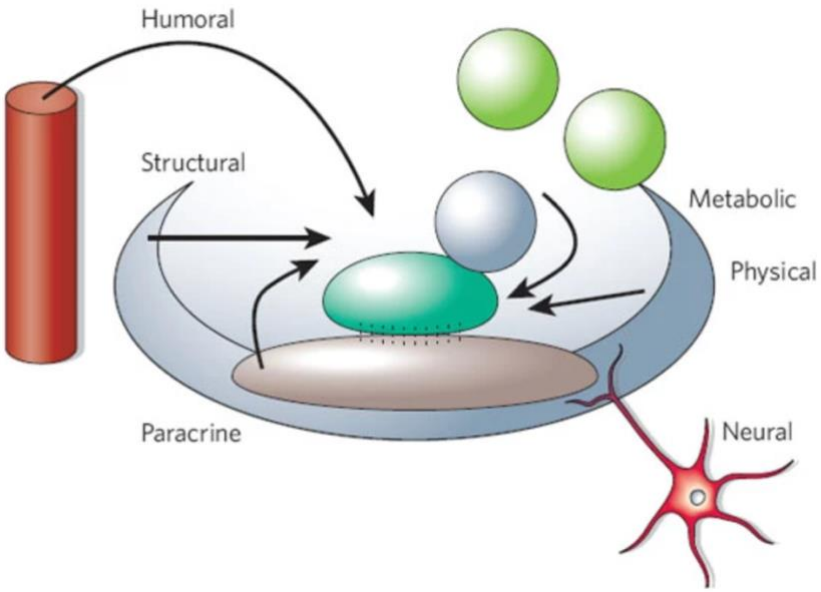


Figure 1.8 Potential components of a stem cell niche. Both cellular and non-cellular components are involved in any stem cell niche. Cellular components include all signals that are received from neighbouring cells. Non-cellular components include the structures that surround the stem cells. Figure taken from (Scadden, 2006).

1.4.1 HSC are located in at least two distinct niches in the bone marrow

The BM microenvironment contains at least two distinct niches for HSC; namely, the endosteal and vascular (Mosaad, 2014). These two niches are comprised of a broad range of cells, including osteoblasts, MSC, immune cells, and several others that play different roles in the regulation of the HSC (Oh and Kwon, 2010). The endosteal niche lies at the inner surface of the BM cavity, which is lined by osteoblasts and osteoclasts, and is an ideal space for HSC to be maintained long-term and to be mobilised for tissue turnover (Gong, 1978; Islam et al., 1990; Taichman, 2005). The vascular niche (also known as a sinusoidal niche) involves thin-walled vessels that acts as a connection between the marrow cavity and blood circulation (Nwajei and Konopleva, 2013) and incorporates perivascular stromal cells, MSC, and neurons, amongst other cell types (Li and Li, 2006; Oh and Kwon, 2010; Nwajei and Konopleva, 2013) (Figure 1.9).

The osteoblasts localised on the inner surface of the endosteal niche are cells derived from mesenchymal stem cells (MSC) (Calvi et al., 2003). Osteoblasts exhibit HSC supportive properties in co-culture experiments *in vitro* (Taichman, 2005; Taichman et al., 1996). The primary role of osteoblasts is to act as a reservoir for essential minerals, protect vital organs from trauma, and support for locomotion (Neiva et al., 2005). Mature osteoblasts share several phenotypic characteristics with the haematopoietic-supportive stromal cell lines (Dorheim et al., 1993; Nelissen et al., 2000) and are responsible for the expression of factors in the niche that are involved in HSC regulation. For example, stromal-cell derived factor-1 (SDF-1) and N-cadherin on so-called spindle-shaped N-Cadherin osteoblasts (SNO) (Zhang et al., 2003; Kanji et al., 2011; Nwajei and Konopleva, 2013; Morrison and Spradling, 2008). SNO act as a facilitator of binding of the HSC to the niche due to the asymmetrical expression of N-Cadherin

on the side adjacent to the HSC (Zhang et al., 2003; Wilson and Trumpp, 2006; Kiel et al., 2007; Li and Xie, 2005).

HSC themselves can affect the regulation of secretion of factors by osteoblasts, some even not being detectable in the absence of HSC, implying a reciprocal relationship between osteoblast and HSC (Taichman et al., 2002). Cytokines secreted by osteoblasts that regulate HSC and progenitors include IL-1, IL-6, granulocyte-macrophage-stimulating factor (GM-CSF), granulocyte-stimulating factor (G-CSF), and macrophage-stimulating factor (M-CSF) (Taichman and Emerson, 1998).

Adipocytes in the BM niche have been shown to be negative regulators of HSC. Hence, a reduction in HSC frequency was found in the adipocyte-rich vertebrae of the mouse (Naveiras et al., 2009). Moreover, osteoclasts, a specialised population of endosteal cells, play a role in HSC migration from the BM (Kollet et al., 2006).

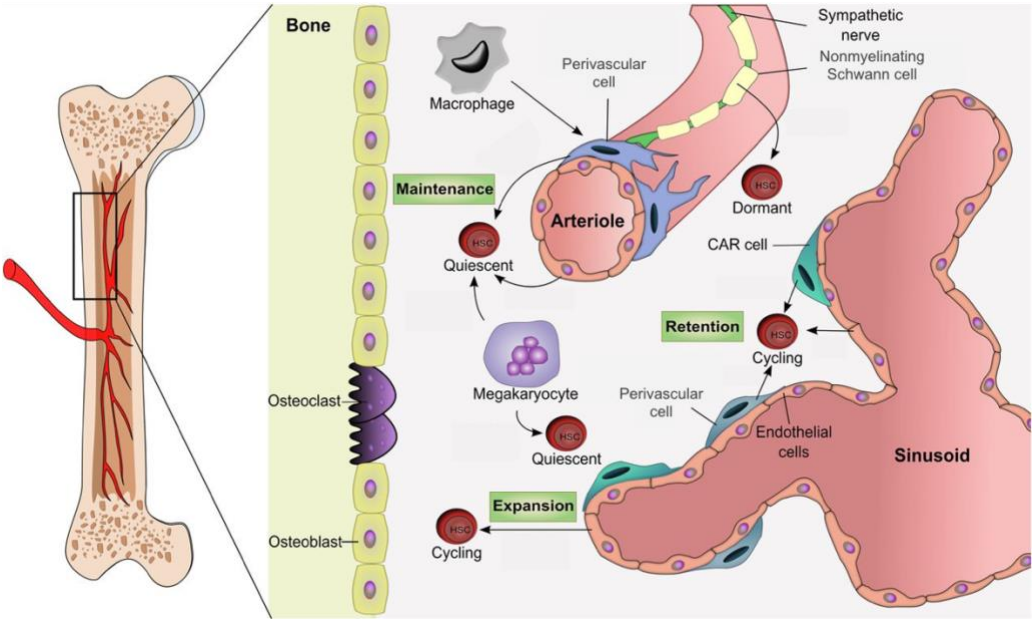


Figure 1.9 The HSC niche. Quiescent HSC are found around perivascular, endothelial, and neuronal cells, where CXCL12, TGFβ1, and SCF are secreted. HSC enter the cell cycle and start to expand when they associate with the sinusoidal niche and are exposed to different signals, eg SCF and Notch. Figure taken from (Boulais and Frenette, 2015).

Several studies have been made about the signals that are involved in the maintenance of HSC in the BM niche. It has been demonstrated that complex interactions take place between HSC and its microenvironment components. The research revealed that there are a complex array of cell-surface and adhesion molecules, growth factors, and chemokines are working in networks to maintain the BM niche and its function toward HSC (Figure 1.10).

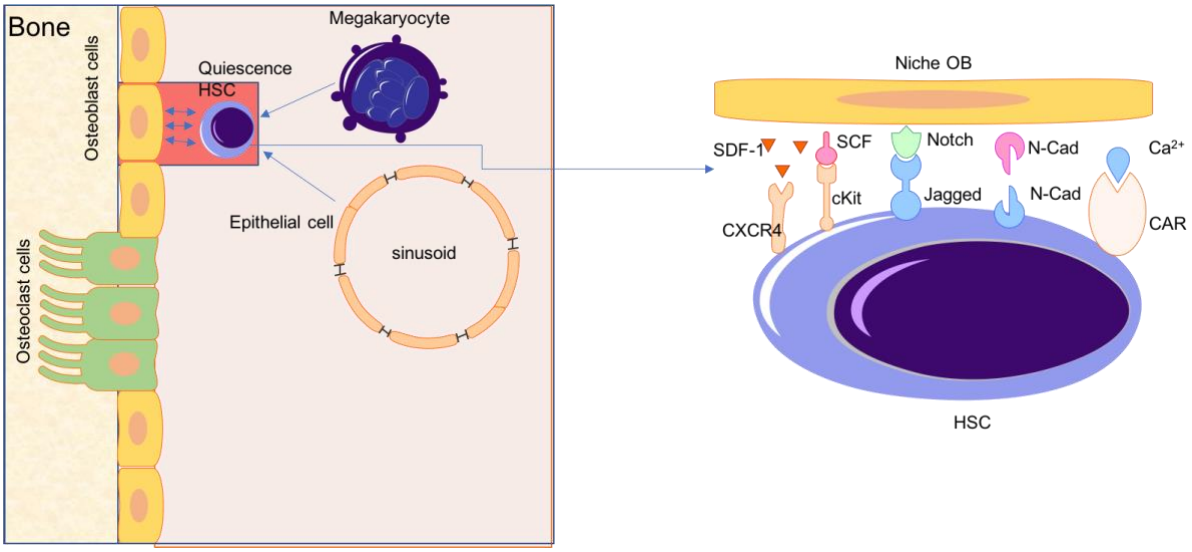


Figure 1.10 HSC are regulated by several signalling molecules in the BM niche. These factors include interactions between HSC and their environment (the niche), including cells, extracellular matrix, and growth factors Microsoft PowerPoint has been used to obtain this figure.

1.4.2 Cells and the extracellular matrix form the basis for the HSC niche

In addition to the contribution of cells directly to the interactions of HSC with their BM microenvironment, an important component is the extracellular matrix (ECM) that is produced by some of the cells in the niche. Both niche cells and ECM provide a supportive role to HSC and can have an influence on their fate (Connelly et al., 2010). ECM is composed largely of glycoproteins, which provide support, cell interactions, and a repository for the accumulation and presentation of various soluble molecules, including growth factors (Li and Li, 2006; Scadden, 2006; Taichman, 2005). Secreted phosphoprotein 1 (SPP1), a protein derived from osteoblasts, has been shown to accumulate in the ECM where it is able to affect HSC number and function; a decrease in SPP1 leading to a stroma-dependent increase of HSC. SPP1-deficient mice acquire haematological malignancy because HSC expansion is not accompanied by their exhaustion (Nilsson et al., 2005; Stier et al., 2005).

1.5 Regulation of haematopoietic stem cells

HSC have an enormous capacity for proliferation and differentiation in order to meet the demand for the production of blood cells during homeostasis and stress. HSC are tightly regulated by both intrinsic and extrinsic factors, and it is important to understand how the various components of the BM niche affect their function.

1.5.1 Signalling from the niche

1.5.1.1 Stem cell factor (SCF)

SCF is a growth factor that is expressed perivascularly around sinusoids throughout the BM (Ding et al., 2012). SCF binds to its receptor, the tyrosine kinase KIT, which is expressed by HSC at all stages of development (Kent et al., 2008). SCF is required for HSC proliferation and survival *in vivo*. Knockout of SCF is embryonically lethal, death *in utero* resulting from severe anaemia (Broudy, 1997). HSC number and function were not affected when SCF was conditionally deleted from osteoblast and NESTIN-expressing cells, although the number of HSC was dramatically reduced when SCF was deleted from endothelial cells or Leptin receptor (LEPR) expressing perivascular stromal cells (Ding et al., 2012; Zhao and Li, 2015b). This implies that endothelial and LEPR-expressing perivascular cells express the majority of the SCF that contributes to the regulation of HSC in the BM.

1.5.1.2 CXCR4/CXCL12

The chemokine CXCL12, also known as stromal cell-derived factor 1 (SDF-1), plays an important role in the regulation of leukocyte and haematopoietic precursor migration, and HSC homing and engraftment (Rafii et al., 1997; Saftig et al., 1998). CXCR4, which is a receptor for CXCL12, is involved in HSC maintenance. Deletion of the genes encoding CXCR4 or CXCL12 impaired HSC homing and engraftment in the BM (Nagasawa et al., 1996; Kopp et

al., 2005). CXCL12 is expressed by osteoblasts and other stromal cells in the BM. Conditional deletion of CXCL12 from haematopoietic cells or NESTIN-expressing cells had little or no effect on HSC and restricted progenitors; however, its conditional deletion from osteoblasts led to depletion of early lymphoid progenitors (Ding and Morrison, 2013; Greenbaum et al., 2013). This implies that CXCL12 secreted from different cellular niches in BM might have a different function.

1.5.1.3 Transforming growth factor beta 1 (TGFβ1)

Another important extrinsic factor is Transforming Growth Factor beta 1 (TGFβ1), which is involved in the control of cell growth, proliferation, differentiation, and apoptosis (Massague, 2012). TGFβ1 signalling is critical in maintaining the quiescent state of the HSC by inhibiting cell division (Yamazaki et al., 2009). The main source of TGFβ1 in the niche is megakaryocytes (Zhao et al., 2014). In addition, the protein is expressed in a latent form by different elements in the niche, including osteoblasts and other stromal cells (Yamazaki et al., 2009). The activation of the protein occurs through non-myelinating Schwann cells (glial cells that surround autonomic nerves), which have been identified as a critical TGFβ signal regulator (Zhao et al., 2014; Zhao and Li, 2015a). TGFβ1 has two receptors (TGFβR1 and TGFβR2), which when knocked out result in increased HSC proliferation and impaired long-term self-renewal potential (Larsson et al., 2003; Yamazaki et al., 2011; Zhao and Li, 2015b).

1.5.1.4 Notch signalling

NOTCH is a family of four transmembrane proteins (NOTCH 1, 2, 3, and 4) found on the cell surface (Canalis, 2008) that play a critical role in cell fate decision. Notch receptors are activated by direct contact with the NOTCH ligands DELTA-LIKE 1, 3, and 4 and JAGGED 1 and 2 (Mumm and Kopan, 2000; Pursglove and Mackay, 2005). NOTCH signalling triggers the

expression of various target genes (Iso et al., 2003) and cross-talk between the HSC niche and osteoblasts through NOTCH signalling has been described (Engin and Lee, 2010). Signalling through NOTCH promotes HSC self-renewal and inhibits differentiation (Varnum-Finney et al., 2003; Calvi et al., 2003; Lampreia et al., 2017) so that when NOTCH is inhibited *in vitro* differentiation of HSC is accelerated (Duncan et al., 2005).

1.5.2 Transcriptional regulation of HSC

The intrinsic regulation of HSC is largely the result of the action of specific transcriptional regulators, many of which act together in complex networks that allow for maximum responsiveness to changing environmental conditions and the demand for blood cell production. Many of the transcriptional factors critical for the normal function of HSC and down-stream progenitors have been identified through the analysis of genes that are aberrantly expressed in haematological malignancies, often being first identified as chromosomal aberrations that characterise individual diseases (Orkin, 1995; Zhang, 2008). Changes in the transcriptome associated with altered conditions, for example HSC undergoing the stress of an acute haemorrhage or the effects of ageing as compared to cells under homeostatic conditions in young adulthood, illustrate the sort of role played by the transcriptional regulator network (Lionberger and Stirewalt, 2009).

The use of genetically altered mouse lines (Capecchi, 1989) has contributed to the identification of the specific roles played by individual transcriptional regulators in the function of HSC and the ways in which their aberrant expression can lead to disease. Some examples of key transcription factors involved in maintaining the HSC phenotype are illustrated in Table 1.1.

Table 1.1 Examples of transcription factors involved in the regulation of HSPC

Transcription factor	Haematopoietic phenotype in mice	References
RUNX1	Knockout: lack of all definitive haematopoiesis. Conditional Knockout: impaired megakaryocytic maturation, defective T-cell and B-cell development, myeloid proliferation	(Okuda et al., 1996; Ichikawa et al., 2004; Growney et al., 2005)
SCL	Knockout: complete absence of yolk sac haematopoiesis, lack of angiogenesis. Conditional Knockout: decreased erythrocytes and megakaryocytes, impaired SH-HSC, normal LT-HSC	(Mikkola et al., 2003; Robb et al., 1995; Curtis et al., 2004)
PU.1	Knockout: lack of mature myeloid cells and b cells. Conditional Knockout: block prior to CMP and CLP stages, increased granulopoiesis, defective HSC.	(Iwasaki et al., 2005; Dakic et al., 2005; Scott et al., 1994; McKercher et al., 1996)
C/EBPα	Knockout: lack of GMPs and granulocytes, impaired monocytes, increased immature myeloid cells. Conditional Knockout: same as knockout mice, plus increased HSC self-renewal.	(Zhang et al., 1997; Zhang et al., 2004)
GFI1	Knockout: reduction in earliest lymphoid progenitors, complete block in late neutrophil maturation, defective HSC.	(Hock et al., 2003; Karsunky et al., 2002; Zeng et al., 2004; Hock et al., 2004)

1.5.3 Epigenetic regulation of HSC

Covalent histone modifications are significant for all cellular processes that modulate the access to genomic DNA, like transcription. In order to activate or inhibit gene expression in the haematopoietic system, cofactors are recruited by transcription factors to their binding sites to

regulate the accessibility of regulatory regions (Kosan and Godmann, 2016). Epigenetic regulation involves those processes that can affect gene expression, and thereby phenotype, without alteration to the genomic DNA sequence (Sharma and Gurudutta, 2016). In the haematopoietic system, changes to the epigenetic landscape over time may be responsible for differences in HSC phenotypes, especially age-related dysfunction (Kramer and Challen, 2017). Epigenetic modifications can be inherited through cell proliferation and have been demonstrated to play key roles in the long-term fate of HSC (Geiger et al., 2013; Kamminga and de Haan, 2006).

The major covalent modifications that occur at histone tails are acetylation/deacetylation elicited by histone acetyltransferases / histone deacetylases, and methylation / demethylation, which require specific histone methyltransferases / histone demethylases (Sharma and Gurudutta, 2016).

HSC self-renewal, proliferation, and differentiation are regulated by protein acetylation. Histone acetyltransferases (HAT) transfer the acetyl group from acetyl coenzyme A (acetyl-CoA) (Sharma and Gurudutta, 2016) and several examples are involved in the regulation of normal and malignant haematopoiesis, including p300/CBP, MYST, and GNAT, in addition to multiple chromatin complexes such as NuA4.P300/CBP/HBO1 (Santini et al., 2013; Sun et al., 2015). Histone deacetylase inhibitors lead to chromatin remodifying to modulate re-expression of silenced tumour suppressor genes in leukaemia stem and progenitor cells, which in turn lead to increase cellular differentiation, inhibition of their proliferation and self-renewal properties (Munoz et al., 2012). Furthermore, transcription factors recruit histone deacetylases to the promoter region of target genes to down regulate expression (Montoya-Durango et al., 2008; Heyd et al., 2011).

DNA methylation occurs by transferring a methyl group to the C5 position of the cytosine ring of DNA and generally has the effect of inhibiting gene expression. Different methylation enzymes are involved in HSC regulation, for example, DNA methyltransferases DMNT3a and DMNT3b (involved in *de novo* DNA methylation), and DNMT1 (involved in maintaining DNA methylation patterns) are regulate HSC self-renewal (Tadokoro et al., 2007; Challen et al., 2011). In addition, methylcytosine dioxygenase (TET2) is required for HSC homeostasis (Ko et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011). At the histone level, methylation affected by two groups of enzymes, namely histone methyltransferases and histone demethylating enzymes. Histones have several methylation sites, each site being responsible for a specific function, for example the three lysine methylation sites that are implicated in activation of transcription: H3K4, H3K36, and H3k79. Methylation at two of these lysines giving rise to H3K4me and H3K36me, have been implicated in transcriptional elongation. Three lysines are connected to transcription repression: H3K9, H3K27, and H4K20 (Sharma and Gurudutta, 2016).

1.5.4 Post-transcriptional and post-translational regulation of HSC

In addition to the regulation of gene expression through the combined actions of transcription factors and epigenetic modulators, there are several post-transcriptional mechanisms that influence the level and activity of individual gene products that are crucial to HSC function. A variety of conserved micro-RNAs (miRNA) and other non-coding RNAs have been identified that regulate crucial genes through post-transcriptional mechanisms (Lim et al., 2003). A given miRNA binds to the 3' untranslated region (3'UTR) of target mRNA, resulting in their cleavage or translational repression (Bartel, 2004). Such post-transcriptional regulation has considered as an important aspect in the control of HSC fate, especially quiescence (Lechman et al., 2012). For example, miR-126 attenuates multiple components in the PI3K-AKT signalling pathway

that control HSC quiescence, a reduction in miR-126 activity allowing HSC proliferation without inducing exhaustion (Lechman et al., 2012; Cheung and Rando, 2013).

In addition to post-transcriptional regulation, the modification of amino acid side chains, that is post-translational modification (PTM), contributes significantly to the regulation of the functional activity of proteins (Wilkinson and Gottgens, 2013). PTMs include ubiquitination, phosphorylation, acetylation, glycosylation, and hydroxylation, which individually or in combination can affect enzyme activity, protein turnover and localisation, and protein-protein interactions, and are involved in modulating several signalling cascades, DNA repair processes, and cell division (Karve and Cheema, 2011).

Ubiquitylation, which entails the covalent attachment of a small regulatory protein called ubiquitin (Ub, either as a monomer or a polymer) to a target protein through a multistep enzymatic process (Wilkinson, 1987; Rechsteiner, 1987), is a key regulatory mechanism that has a role in modulating cellular functions such as cell proliferation and differentiation, apoptosis, DNA repair, and stress response (Shi and Kehrl, 2010; Jin et al., 2011; Bosanac et al., 2010). Three enzyme classes are involved in ubiquitinylation, which act in a strict order: Ub activating enzymes (E1), Ub conjugating enzymes (E2), and Ub ligases (E3) (Pickart, 1997).

Phosphorylation is another important PTM affecting protein function in stem cells, influencing cellular metabolism, protein-protein interaction, enzyme reactions, and protein degradation, particularly in the context of intracellular signalling cascades (Hunter, 1995; Ghosh and Adams, 2011). Protein phosphorylation involves the addition of a phosphate group to individual amino acids through the action of specific protein kinases. The most commonly observed phosphorylated amino acid residues are serine/threonine (Ser/Thr) and tyrosine, and they have been frequently implicated in the progression of cancers (Karve and Cheema, 2011). For example, the transcription factor RUNX1, which plays an important part in the development

and function of HSC, is phosphorylated by cyclin-dependent kinases (CDKs) in a cell cycle-specific manner, leading to effects on its activity, protein-protein interaction, stability, and degradation (Guo and Friedman, 2011; Biggs et al., 2006; Wilkinson and Gottgens, 2013). On the other hand, dephosphorylation, that is, the removal of a phosphate group from a phosphorylated amino acid, is catalysed by different protein phosphatases (Hunter, 1995). A balance between the activity of specific protein kinases and protein phosphatases is important for the maintenance of homeostasis.

1.6 The role of MYB in HSC regulation

1.6.1 MYB is a highly conserved transcriptional regulator

The transcription factor MYB (also known as c-MYB) was originally identified as a truncated oncogenic version transduced by avian retroviruses that induce leukaemia. Such a truncated MYB (v-MYB) was identified in viruses, namely avian myeloblastosis virus (AMV) and E26 (Lipsick and Wang, 1999). MYB is one of three related MYB family proteins in vertebrates, the others being A-MYB and B-MYB (now called MYBL1 and MYBL2, respectively) (Nomura et al., 1988). After its identification in the chicken, *Myb* was subsequently cloned in mice and humans (Gonda et al., 1985; Rosson and Reddy, 1986; Bender et al., 2004).

MYB proteins consist of three domains: (1) the N-terminal domain; (2) the C-terminal domain; and (3) a transactivation domain. The N-terminal domain contains three repeats of 50-52 amino acids (R1, R2, and R3) each of which has a structure made up of three alpha helices. This highly conserved structure is seen in related proteins across vertebrates, invertebrates, plants and yeasts. The C-terminal domain of MYB is considered as a negative regulatory domain (Anton and Frampton, 1988; Biedenkapp et al., 1988; Frampton et al., 1991; Oh and Reddy, 1999).

Alternative splicing of the *MYB* gene has been detected in different cell types (Stenman et al., 2010) and results in a variety of different mature proteins, the two predominant forms being 75 and 89 kDa, the latter incorporating an additional exon between exon 9 and exon 10 (Shen-Ong, 1987; Weston and Bishop, 1989). MYB can partner with a number of proteins that serve as regulators of its function, the most significant of which are the protein acetylases p300/CBP (Sandberg et al., 2005; Alm-Kristiansen et al., 2008).

Following on from the discovery of MYB through its leukaemogenic activity as part of the avian retroviruses, studies showed that the insertion of mutated *MYB* by retroviruses in mice can cause myeloid and lymphoid malignancies. Linking with such leukaemogenic potential, it was also found that somatic duplication of *MYB* occurs as a causal event in human T-cell leukaemia (Lipsick, 2010). Combining these observations it is clear that MYB gain-of-function can be a cause of haematopoietic malignancies in vertebrates (Lipsick, 2010).

MYB is considered to be one of the key regulators of vertebrate haematopoiesis (Davidson et al., 2005). It is part of a complex genetic network which plays a crucial role in the maintaining of HSPC and regulating their self-renewal, proliferation, and differentiation (Burns et al., 2009; Soza-Ried et al., 2010). Appropriate levels of MYB expression are required at specific differentiation stages of each cell lineage in haematopoietic progenitor cells (Emambokus et al., 2003; Sakamoto et al., 2006). Beyond haematopoiesis, MYB is now known to be expressed in many different tissues. For example, colorectal cancer cells (CRC) are tightly regulated by MYB, the Cyclin *E1* gene being an important cell cycle related target gene. Down regulation of MYB expression leads to CRC (Cheasley et al., 2015; Mitra, 2018).

1.6.2 MYB is a critical transcriptional regulator in HSC

Within the haematopoietic system, MYB expression is generally highest in the immature progenitors, and is less highly expressed (or absent) in most of the mature differentiated blood cells (Westin et al., 1982; Gonda and Metcalf, 1984). MYB found to be important in adult HSC (Garcia et al., 2009; Sandberg et al., 2005; Mucenski et al., 1991). Knockout of *MYB* results in embryonic lethality due to impaired of definitive intraembryonic haematopoiesis, with death occurring at day 15 of gestation (Mucenski et al., 1991). Conditional deletion of *MYB* in adult mice affects HSC by reducing self-renewal capacity due to accelerated differentiation and impaired proliferation (Lieu and Reddy, 2009). Moreover, the level of MYB is important to maintain multilineage long-term engraftment of HSC, modulating the balance between asymmetric and symmetric cell division (Sakamoto et al., 2015).

The Frampton group has been studying aspects of the role of MYB in HSC over a number of years. A central approach to these studies has been the use of mouse lines that have either constitutively reduced levels of MYB or in which gene ablation can be achieved conditionally (in time or space). A reduction of the level of MYB in the *MYB* knockdown (KD) line (Emambokus et al., 2003) leads to a myeloproliferative phenotype characterised by a high number of platelets and monocytes in the peripheral blood (Garcia et al., 2009). Further investigation of the KD revealed that HSC lose their potential while at the same time more committed progenitors gain stem cell capacity, seemingly as the result of accentuated cytokine signalling responses (Clarke et al., 2017). In addition, the group has found that haploinsufficiency of MYB in $MYB^{+/-}$ (Mucenski et al., 1991) animals leads to a loss of HSC self-renewal capacity and the ability to repopulate after transplantation, and furthermore results in a high frequency of age-related development of MPN and MDS (Clarke et al., 2017).

1.6.3 MYB regulates genes expression in haematopoietic and progenitor stem cells

In order better to understand how MYB regulates the behaviour of HSC and downstream progenitors, the Frampton group has performed a number of transcriptome analyses using RNA-seq. Comparing myeloid progenitors from wild type and *MYB* KD mice it was found that a number of genes involved in cytokine signalling are affected by the level of MYB (Clarke et al., 2017). For example, the *SPROUTY 2* gene, which encodes a protein that inhibits the Ras/MAP kinase pathway, was found to be down regulated in *MYB* KD cells, perhaps contributing to the myeloproliferative phenotype exhibited by these cells. The group has also investigated how a reduction of MYB levels affects gene expression in a number of other contexts related to HSC or myeloid leukaemia. In both model myeloid leukaemia cells (HOXA9/MEIS1 transformed) and HSC, conditional KO or haploinsufficiency of *MYB* reveals that the expression of the resistin-like gamma gene (*RETNLγ*) is significantly reduced, suggesting that it is a target gene of MYB (Clarke et al, unpublished) (Table 1.2; Figure 1.11). The degree of down regulation upon loss of MYB activity makes this a highly interesting MYB target gene for further investigation, especially in respect to its importance in HSC function.

Table 1.2 Microarray analysis of gene expression differences comparing KSL HSC from WT and conditional knockout (MybF/F:Cre) mice

Genes expressed at lower levels	log2(fold_change)	P-value
Ngp	9.27719	5.00E-05
Ltf	8.71257	5.00E-05
Camp	8.56818	5.00E-05
Retnlg	8.31404	0.0001
Prg2	8.29462	0.0003
Cd177	8.22059	0.0004
Fcnb	7.0801	5.00E-05
Wfdc21	6.80673	5.00E-05
Mmp8	6.73089	5.00E-05
Lcn2	6.55764	5.00E-05
Col1a2	5.56688	5.00E-05
Slfn4	5.54406	5.00E-05
C1qb	5.363	5.00E-05
Mcemp1	5.35111	5.00E-05
Adam8	5.21706	5.00E-05
Lgals3	4.94644	5.00E-05
Clec4a2	4.28791	5.00E-05
Hmox1	4.14844	0.0001
Slc40a1	4.01701	5.00E-05
Fn1	3.99016	5.00E-05
Ccl6	3.90373	5.00E-05
Slc11a1	3.8555	0.0002
Pglyrp1	3.82797	5.00E-05
Hsd11b1	3.80493	5.00E-05
C3	3.632	5.00E-05
Ppbp	3.59102	5.00E-05
Fgr	3.3843	0.0001
Ifitm6	3.36026	5.00E-05
Lyz1	3.13581	5.00E-05
Cd79a	2.94796	0.00035
Fcgr3	2.82895	5.00E-05
Lilrb4a	2.82494	0.00025
Hp	2.63802	5.00E-05
Itgam	2.55068	5.00E-05
Gda	2.53784	0.00025
Pirb	2.52299	0.0001
Cybb	2.33798	5.00E-05
Anxa1	2.27477	5.00E-05
Fam101b	2.24971	0.0003
Ltb4r1	2.22007	5.00E-05
Trem3	2.18417	5.00E-05
Elane	2.17325	5.00E-05
Hdc	2.16717	5.00E-05
Gca	2.1536	0.0004
Pde2a	2.08328	0.00015
Mxd1	2.0588	0.0001
Plaur	2.00691	5.00E-05
Dok3	1.99235	5.00E-05
Ms4a3	1.74146	5.00E-05
Mgst1	1.73071	0.00015
S100a6	1.69236	5.00E-05
Rgcc	1.6486	5.00E-05
Adpgk	1.53284	0.0001
Dstn	1.39378	0.0002
Gstm1	1.37341	0.0001

The table lists those genes exhibiting the highest degree of decreased expression in KSL HSC following conditional deletion of *MYB* (achieved through Cre-mediated deletion of floxed alleles). The arrow shows that

RETNLγ is one of the most affected genes. Similar findings were also derived from microarray analysis of leukaemia model cells and using RNA-seq analysis. Result was supplied by Mary Clarke but not published.

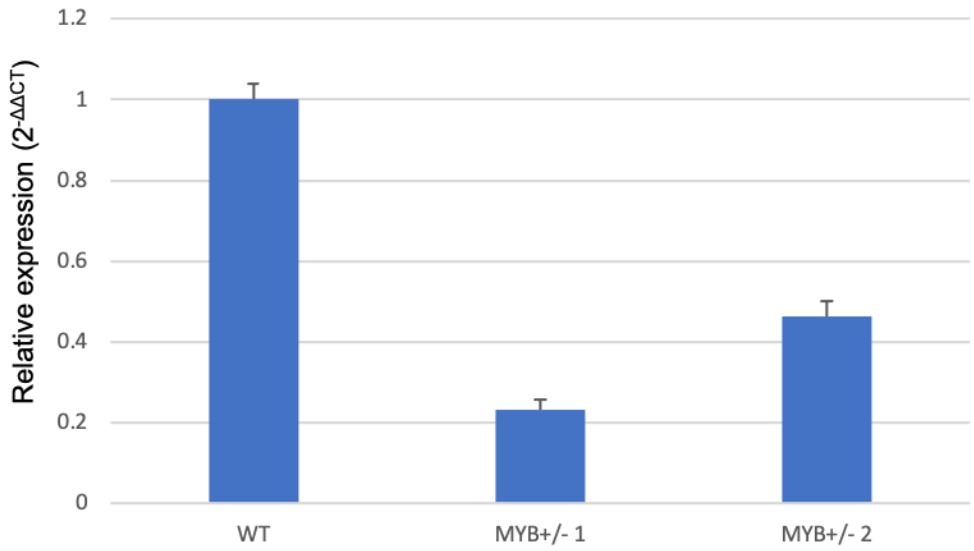


Figure 1.11 *Retnlγ* RNA expression in WT versus *MYB* haploinsufficient (*Myb*+/-) KSL HSC. qRT-PCR analysis of *Retnlγ* RNA levels, performed in triplicate, is represented. Result was supplied by Mary Clarke but not published.

1.7 Resistin family proteins

1.7.1 The resistin family of proteins is large with a wide range of activities in different tissues

Resistin-like molecules (RELM) represent a family of small hormone-like proteins, with diverse, and in some cases unknown functions. The RELM family is represented by four genes in rodents and two genes in human. *RELM* genes are expressed differentially in a wide range of tissues (Table 1.3). The first gene identified was mouse Resistin (*RETN*) (Steppan et al., 2001a). RETN has no homology to other hormones, cytokines, or intracellular signalling molecules and was named after its ability to antagonize insulin action. Studies showed that the

level of *RETN* is increased in mouse models of obesity and insulin resistance. A major site of expression in mice is adipose tissue (Steppan et al., 2001a).

In addition to *RETN* gene, three other genes have been described in rodents: Resistin-like alpha (*RETNLα*), Resistin-like beta (*RETNLβ*), and Resistin-like gamma (*RETNLγ*). All of the mouse genes are located on chromosome 16 with the exception of *RETN*, which is located on chromosome 8 (Table 1.2). In humans, the two *RELM* genes identified are Resistin (*RETN*), located on chromosome 19, and Resistin-like beta (*RETNLβ*), located on chromosome 3.

All of the *RELM* genes encode secreted proteins of 105-114 amino acids that are constituted of three domains: a N-terminal signal sequence, a variable middle section, and a conserved C-terminal portion. The conserved C-terminal sequences share a unique cysteine-rich region (Steppan et al., 2001b; Gerstmayer et al., 2003; Holcomb et al., 2000; Yang et al., 2003; Banerjee and Lazar, 2003).

RELM genes have been known to have distinct expression patterns between different species (Park and Ahima, 2013; Codoner-Franch and Alonso-Iglesias, 2015; Schwartz and Lazar, 2011). The occurrence of four genes in mice (*RETN*, *RETNLα*, *RETNLβ*, and ***RETNLγ***) and only two genes in humans (*RETN* and *RETNLβ*) implies that different roles have arisen during evolution.

RETNLα is expressed at a high level in the white adipose tissue, and at lower levels in the heart, lung, and tongue. The similarity of the RETNLα and RETN proteins is approximately 63% in the C-terminus. One study showed that RETNLα is involved in a complex regulatory function in adipocyte differentiation. Blagoev et al found that RETNLα can form heterodimers or multimers with RETN (Blagoev et al., 2002). RETNLβ and RETN are 55% similar in the C-terminus. Compared to RETNLα, RETN and RETNLβ have an additional cysteine residue at

position 26, which is responsible for dimer formation. In both genes, mutation of this extra cysteine residue to alanine prevents dimerization yielding a monomeric protein similar to RETNLα (Banerjee and Lazar, 2001). The mRNA of *RETNLβ* is specifically expressed in the colon where its levels are high in non-proliferative epithelial cells compared to proliferative epithelial cells, suggesting that it has a role in cell proliferation. In addition, high expression of *RETNLβ* has been demonstrated in a mouse colon cancer model, but not in a normal colon tissue, which suggests that it has a role in cancer malignancies (Steppan et al., 2001b). Human RETNLβ and mouse RETNLβ are highly similar, especially in the C-terminus. In addition, a similarity has been found in the N-terminus, in a presumed signal sequence (Steppan et al., 2001b).

Table 1.3 Different resistin-like molecule genes

Official symbol	Species	Other designations	Chromosomal location	Expression
RETN	Mouse	xcp4/Fizz3	8	White adipose tissue
RETNLα	Mouse	xcp2/Fizz1	16	Pancreas, tongue, skin, peritoneal exudates
RETNLβ	Mouse	xcp3/Fizz2	16	Colon, small intestine
RETNLγ	Mouse	xcp1/Fizz3	16	Bone marrow, spleen, peritoneal exudates, embryonic liver, neonatal pancreas, gut, lung, and heart
RETN	Human	XCP1/FIZZ3	19	Adipose tissue
RETNLβ	Human	XCP2/FIZZ1 or 2	3	GI tract, colon

RELM family genes are expressed in several tissues. A number of different names have been attached to each. Genes with all capital letters represent human genes. FIZZ or Fizz; found in inflammatory zone (Holcomb et al., 2000; Steppan et al., 2001a; Steppan et al., 2001b; Chumakov et al., 2004; Kubota et al., 2000; Ort et al., 2005).

1.7.2 *RETNLγ* is expressed predominantly in myelomonocytic cells although its function is unclear

RETNLγ was the last *RELM* gene to be identified. It is most closely related to *RETNLα*, being 86% homologous at the nucleotide level. As with other RELM proteins, RETNLγ consists of an N-terminal domain and cystine-rich C-terminal domain (CX_{11}-C-X_8-C-X-C-X_3-C-X_{10}-C-X-C-X-C-X_9-CC). Similar to RETN and RETNLβ, RETNLγ contains an extra cysteine residue, but in this case located at position 45.

The expression of *RETNLγ* is highly tissue-specific. The highest level of *RETNLγ* RNA is seen in the cells and organs of the haematopoietic system like BM, spleen, and thymus, being particularly high in myeloid cells. This expression pattern has led to the suggestion that RETNLγ has a cytokine-like signalling function, similar to other RELM family members (Gerstmayer et al., 2003). A functional overlap between *RETNLγ* and *RETNLα* was proposed by Gerstmayer because of the high degree of similarity in their gene sequence, but this idea has not been investigated. In addition, this question was addressed after a formation of heterodimers and multidimers between RETN and RETNLα was observed. The similar formation is possible between RETNLγ with RETN, RETNLβ, or RETNLα. However, the multimers formation between RETNLγ and other genes has not been investigated (Gerstmayer et al., 2003; Blagoev et al., 2002). Despite the expression of *RETNLγ* in both mouse and rat, the ortholog of this gene has not been defined yet in humans. Moreover, the role of *RETNLγ* in HSC and the haematopoietic system is still unknown.

1.7.3 Mouse *RETNLγ* is possibly the functional equivalent of human *RETN*

RETN expression is high in human primary acute leukaemia cells, macrophages, and myeloid cell lines such as U937 and HL60. In addition, *RETN* mRNA is only detected at very low levels in human adipose tissue, about 1/250 of that in the mouse (Patel et al., 2003; Yang et al., 2003; Hu et al., 2015; Jang et al., 2015). In addition, *RETN* is linked with inflammation, which means that it has an association with granulocytes/leukocyte that are produced originally in the BM, while a link between *RETN* and myeloid cell chemotaxis has been reported (Chumakov et al., 2004). This expression pattern best matches with *Retnlγ* amongst the mouse RELM family members.

A further link between *RETN* in humans and *RETNLγ* in mice is that there appear to be parallels in the mechanism of their transcriptional regulation, in particular by *C/EBPε*, which is a member of C/EBP family of transcriptional factors. *C/EBPε* is highly expressed in myeloid cells and its genetic knockout leads to the production of dysfunctional neutrophils and eosinophils. Moreover, *C/EBPε* KO mice die of opportunistic infections, implying that the transcription factor is crucial for myeloid differentiation (Kubota et al., 2000; Antonson et al., 1996; Chumakov et al., 1997; Morosetti et al., 1997). Expression of both *RETN* and *RETNLγ*, in humans and mice respectively, is dependent on *C/EBPε*. Taking these observations together it is likely that *RETN* is the biological analogue of *RETNLγ* and as such that these two factors share a similar function in human haematopoietic system.

1.8 Hypothesis and aims of the research programme

The working hypothesis is that mouse *RETNLγ* is an important target gene of MYB that contributes to MYB-dependent mechanisms regulating the normal function of HSC. Further, the expression of *RETNLγ* in HSC suggests that it has a cell autonomous role in HSC behaviour.

The programme of investigation described in this thesis is divided into three major objectives, which are explored *in vitro* and *in vivo* using cells derived from wild type and *RETNLγ* KO mice:

1. To determine the level of *RETNLγ* gene and protein expression in HSC and its downstream progenitors and differentiated cells.

2. To assess the consequences of the absence of *RETNLγ* by comparing male and female WT and *RENTLγ* KO mice, assessing the haematopoietic profile in the blood and BM and determining the properties of HSC using *in vitro* and *in vivo* functional assays.

3. To define the differences caused by the absence of *RETNLγ* in terms of the HSC phenotype at the level of the transcriptome and to explore possible mechanisms by which endogenous or exogenous *RETNLγ* is able to influence the pattern of genes expressed.

CHAPTER 2 MATERIALS AND METHODS

2.1 Mouse work

2.1.1 Mice

Wild type and *RETNLγ* KO mice were obtained from The Jackson Laboratory and maintained on a C57/6BL background. Mice were kept for breeding in a pathogen-free facility (Biomedical Service Unit BMSU, College of Medical and Dental Sciences, University of Birmingham). In addition, C57/BL6 mice congenic for the *PTPRC*^{a} allele (B6.SJL-*PTPRCa*, also known as BoyJ) of the pan leukocyte marker commonly known as CD45.1 or Ly5.1 were obtained from BMSU for use as hosts in transplantation studies in order to distinguish them from donor cells derived from C57BL/6 inbred mice expressing the *PTPRC*^{b} (CD45.2 or Ly5.2) allele. All animal experiments were carried out according to Animals (Scientific Procedures) Act 1986, monitored by the Home Office in the United Kingdom.

2.1.2 Genomic DNA extraction

Genotyping of wild type and *RETNLγ* KO mice arising from crosses of animals heterozygous for the KO allele was carried out on using an ear clip sample as a source of genomic DNA. DNA was prepared by placing the tissue in 50 µl of an extraction mix (250 mM NaOH and 2 mM EDTA) in an Eppendorf tube and heated to 90°C for 20 minutes in order to dissolve the cell membrane to extract the DNA. Then, 50 µl of Tris-HCL (pH7.5, 40 mM) was added to the tube prior to amplification by PCR.

2.1.3 DNA amplification and detection

In order to prepare for the PCR amplification reaction, 12.5 µl of DreamTaq green PCR master mix (2x; K1081, ThermoFisher) was added to 10.5 µl of nuclease-free water. 0.5 µl of forward and reverse primers (Table 2.1) at 12 mM was added to the mix to a final volume of 24 µl. From the DNA template, 1 µl was added to a final volume of 25 µl. PCR reactions were performed using a MJ Research PTC-200 machine. Each cycle of the PCR reaction consisted of 94 °C for 60 second, 62°C for 60 second, and 72°C for 60 second. This cycle was repeated 35 times.

Amplified DNA was detected by running on a 1.2% (w/v) agarose gel in TAE buffer plus 0.5 µg/ml ethidium bromide (E1510-10ml, Sigma-Aldrich). The wild type DNA band size is 582 bp and the mutant DNA band size is 339 bp.

Table 2.1 Forward and reverse primer sequences for *RETNLγ* KO mouse genotyping

***RETNLγ* primer**	**Sequence**
Forward	gtcctttcccttacagcca
Reverse	agctcttattgaagaggtctaaag

2.2 Blood counting and analysis

Blood counts were used on a regular basis to assess the haematological phenotype of genetically altered mice (*RETNLγ* KO) and the status of transplantation hosts following engraftment. Blood was withdrawn through tail puncture and collected into acid-citrate-dextrose ACD solution (6.8 mM citric acid, 11.2 mM trisodium citrate, 24 mM glucose). Full blood counts were performed using an ABX Pentra 60 (ABX Diagnostic) automatic blood counter. Absolute numbers were

calculated by multiplying the blood counter values by the dilution factor of the sample. GraphPad Prism software was used to calculate the mean and standard error of the mean (SEM).

2.3 Bone marrow cell preparation

Most, if not all, of the experiments in this project were based on the cells that obtained from mouse bone marrow (BM). Haematopoietic stem and progenitor cells were obtained by flushing the BM from tibias and femurs of adult mice (6-10 weeks old) using a 25-gauge needle and phosphate buffer saline (PBS) supplemented with 10% (v/v) foetal bovine serum (FBS, F7524, Sigma).

Ammonium-Chloride-Potassium buffer (ACK, 0.15 M ammonium chloride, 1 mM potassium bicarbonate, pH7.3 disodium EDTA) was used to lyse all red blood cells for all immunostainings except those for Ter119/CD71. After flushing the BM, the cell suspension was centrifuged at 300 rcf for 5 minutes at 4°C and resuspended in ACK lysis buffer for 5 minutes at room temperature. The cell suspension was centrifuged at 300 rcf for 5 minutes at 4°C and resuspended in 100 µl of PBS supplemented with 10% (v/v) FBS prior to immunostaining with specific antibodies.

2.4 Haematopoietic cell analysis using flow cytometry and specific cell sorting

All cells that required antibody (Ab) immunostaining were kept in 100 µl of 10% FBS/PBS per mouse and washed in 200 µl of 10% (v/v) FBS/PBS in 1.5 ml Eppendorf tube or in a 96 V-shaped well plate for cell culture, BM transplantation experiments, colony assays, RNA expression analysis, and intracellular protein production. All washing steps were performed at 300 rcf for 5 minutes at 4°C. Non-specific binding was blocked as necessary by adding purified

anti-CD16/32 (FcγRI II/III) Ab to cells and incubated for 15 minutes at 4°C. Cells were washed before performing specific Ab staining.

Ab staining was performed by adding each Ab to the 100 µl of cell suspension at 1:100 (except for anti-CD34, which was added at 1:50). Cell suspensions were incubated for 30-60 minutes on ice in the dark. After incubation, cells were washed in 100 µl of 10% (v/v) FBS/PBS and centrifuged at 300 rcf for 5 minutes. Cell pellets were re-suspended in 500 µl of 10% (v/v) FBS/PBS. The suspensions were filtered using a 50 µm CellTrics filter (04-0042-2317, Sysmex) and placed in a tube compatible with the flow cytometry machine. The conjugated fluorescent antibodies that were used are listed in (Table 2.2). For multicolour immunofluorescent staining, single colour stained controls were used in order to set up the fluorescence emission compensation. Stained cells were analysed on CyAn flow cytometry using Summit 4.3 software. Forward scatter and side scatter were used to gate live cells.

Table 2.2 Flow cytometry antibodies

Surface antigen	Monoclonal Ab clone	Conjugated fluor	Supplier
CD8a	53-6.7	FITC, APC	eBioscience
Ly-6g/Ly-6c	RB6-8C5	FITC, APC	eBioscience
Ter119	Ter-119	FITC, APC	eBioscience
CD45R (B220)	RA3-6B2	FITC, APC	eBioscience
CD11b	M1/70	FITC, APC	eBioscience
CD5	53-7.3	FITC, APC	eBioscience
CD34	RAM34	FITC	eBioscience
CD134 (Flt)	A2F10.1	PE-CF594	BD Horizon
CD41	eBioMWReg30	PE	eBioscience
Ly-6a/E (Sca-1)	D7	PE	eBioscience
CD71	R17217	eFluor450, PE	eBioscience
CD45.1	A20	eFlour450, PE Cy5	eBioscience
CD45.2	104	APC, eFlour780	eBioscience
CD48	HM48-1	BV510	BD Horizon
CD117 (cKit)	2B8	PE Cy5	eBioscience
CD16/32	93	PE	eBioscience
CD150	Mshad150	PE Cy7	eBioscience
CD43	eBioR2/60	PE	eBioscience
CD4	GK1.5	APC	eBioscience

The gating strategy employed in order to delineate HSC and downstream progenitors is illustrated in Appendix 1.

For cell sorting, BM cells were centrifuged at 300 rcf for 5 minutes and re-suspended in 1 ml ACK buffer. Cells were incubated in the ACK buffer for 5 minutes at room temperature. Then, cells were centrifuged at 300 rcf for 5 minutes and resuspended in 100 µl of 10% (v/v) FBS/PBS. Fluorescence Ab staining was performed as described previously. Cells of interest were sorted in sterile tubes by MoFlo XDP cell sorted (Beckman Coulter).

2.5 Transplantation using HSC and competitive BM cells to reconstitute lethally-irradiated recipients

BM transplantation is used to examine the ability of specific HSC populations to reconstitute haematopoiesis following lethal irradiation. B6.SJL (BoyJ, CD45.1) mice were used as recipients, and received two doses of 450 Gy radiation to clear the BM of resident haematopoietic cells.

For transplantation, $5x10^5$ ACK-treated WT reference BM cells, (B6 x B6.SJL F1s, CD45.1/CD45.2 heterozygotes) were tail vein injected together with 100 test donor cells (B6, CD45.2) from WT or *RETNLγ* KO mice (Table 2.3). Three or more recipients were used per experimental group. Recipient mice were observed daily over the course of two weeks for any weight loss or abnormal phenotype resulting from the procedure.

In order to assess the extent of engraftment in each recipient, monthly sampling was performed by withdrawing peripheral blood from the tail vein into ACD solution. The sample was centrifuged and cells were resuspended into 500 µl ACK buffer and incubated for 10 minutes at room temperature. Cells were centrifuged at 300 rcf for 5 minutes, resuspended in 100 µl of 10% (v/v) FBS/PBS and stained with fluorescent conjugated antibodies. Antibodies specific for CD45.1 and CD45.2 were used to distinguish reference (CD45.1/CD45.2) and test (CD45.2/CD45.2) donor cells and to identify any residual host cells (CD45.1/CD45.1). Depending on the extent and progression of engraftment, animals were culled at specific time points and their BM analysed by immunofluorescence / flow cytometry for the profile of donor-derived cells.

Table 2.3 Number of donor and competitor cells used in transplantation experiments

HSC population	Test donor cell number	Reference donor cell number

LT-HSC	100	$5x10^5$
$Kit^+Sca^+Lineage^-$ (KSL)	500	$5x10^5$
$Kit^+Sca^+Lineage^-$ (reciprocal)	500	None

2.6 Cell culture

2.6.1 Liquid culture of KSL treated with recombinant RETNLγ protein

In order to examine the effect of RETNLγ protein on HSC, KSL cells were sorted as described in Section 2.4. The sorted cells were plated in Iscove's Modified Dulbecco's Medium (IMDM, Gico 13390-500ML) supplemented with 10% (v/v) FBS (F7524, Sigma), 100 U/ml penicillin/streptomycin (15070-063, Gibco), 50 µg/ml gentamicin (15710-064, Gibco), 20 ng/ml SCF (250-03, PeproTech), 10 ng/ml Flt3 (250-31L, PeproTech), and 10 ng/ml TPO (315-14, PeproTech). The cells were split into two wells of a 96 U-shape well plate. One well was left without RETNLγ protein, while the other well was supplemented with RETNLγ protein at 100 ng/ml (450-26G, PeproTech). Cells were incubated for 18 hours at 37°C, 5% CO_2 in a fully humidified atmosphere.

2.6.2 Semisolid methylcellulose culture

Semisolid methylcellulose media (Methocult M3434, Stem Cell Technologies) was used to examine the ability of HSC to form haematopoietic cell colonies (numbers and phenotype). 600 BM KSL cells from WT and *RETNLγ* KO mice were placed in 3 ml of M3434 supplemented with cytokines that promote the growth of myeloid lineage colonies. Penicillin/streptomycin (15070-063, Gibco) was added at 50 U/ml, and gentamicin (15710-64, Gibco) at 50 µg/ml. The cell suspensions were vortexed and left for 15 minutes at room temperature. An 18-gauge blunt needle was used to dispense 1.3 ml of cell suspension in a 35 mm Petri dish. Duplicates were

plated in each experiment. Cultures were incubated for 10 days at 37°C, 5% CO_2 in a fully humidified incubator. Colony numbers and phenotypes were assessed, and in some cases the total cell content of the dish was recovered for immunofluorescence staining.

2.7 *RETNLγ* gene over expression

2.7.1 *RETNLγ* expressing lentiviral vector and virus production

A *RETNLγ* over expression lentiviral vector was purchased from VectorBuilder (vector ID VB900025-4807acy). The plasmid was supplied in 60% (v/v) glycerol stock. The bacteria were expanded on LB agar supplemented with 100 µg/ml ampicillin antibiotic. Lentivirus packaging was performed in the Lentiviral Production Facility in the Weatherall Institute of Molecular Medicine, University of Oxford.

2.7.2 Transduction of *RETNLγ* KO HSC using the *RETNLγ* expressing lentivirus

Lin^-Kit^+ cells were sorted from *RETNLγ* KO mouse BM. Cells were split into two wells of a round bottom 96-well plate containing IMDM (13390-500ML) supplemented by 10% (v/v) FBS (F7524, Sigma), 100 U/ml penicillin/streptomycin (15070-063, Gibco), 50 µg/ml gentamicin (15710-064, Gibco), 20 ng/ml SCF (250-03, PeproTech), 10 ng/ml Flt3 (250-31L, PeproTech), and 10 ng/ml TPO (315-14, PeproTech). 2 µg/ml of polybrene was added to each well. Test cells were infected with 1 µl of *RETNLγ* lentivirus and incubated overnight.

2.7.3 Over expression of *RETNLγ* in 293T cells

SF Cell Line 4D-Nucleofector X Kit S (Lonza, V4XP-2032) was used and the nucleofector solution SF cell line with supplement was prepared in the proportions described on the manufacturer's instructions (Lonza). To electroporate the 293T cells, $1x10^6$ cells of 293T cells were suspended in nucleofector reagent (solution SF cell line + supplement) 1 µg pmaxGFP +

10 µg RETNLγ over expression plasmid and 1 µg of pmaxGFP alone as control reaction. The mixture was transferred into a 16-well nucleocuvette strip and placed into 4D nucleofection system (Lonza) with CM-150 electroporation programme. Next day, the transfection efficiency was assessed according to the green fluorescence expression (pmaxGFP). The *RETNLγ* over expression plasmid was received from the group of Dr Tomoishiro Asano at Hiroshima University.

2.8 Gene expression analysis

2.8.1 RNA extraction

Sorted cells were centrifuged at 300 rcf for 5 minutes at 4°C and the cell pellets were re-suspended in 250 µl Trizol reagent (15596026, Invitrogen) and incubated for 15 minutes at room temperature. 0.2 volumes of RNA-free chloroform (BP1145-1, Fisher Scientific) per sample was added and shaken vigorously for 15 seconds. Samples were incubated at room temperature for 10 minutes, then centrifuged at 14,000 rcf for 15 minutes at 4°C. The aqueous phase was collected into RNA-free Eppendorf tubes. 0.5 volumes of RNA-free isopropanol (P17490/15, Fisher Scientific) was added together with 1 µl of RNA-free glycogen (10901393001, Roche). Samples were centrifuged at 14,000 rcf for 10 minutes at 4°C, the supernatant was removed and pellets were washed with 70% RNA-free ethanol. Samples were centrifuged at 14,000 rcf for 5 minutes at 4°C and the ethanol was removed. Pellets were air-dried and re-suspended in 14 µl of RNA-free water (Figure 2.1).

2.8.2 cDNA synthesis by reverse transcription

One microliter of 500 µg/ml of oligo dT 15 primer (C1101, Promega) was added to each RNA sample and heated at 70°C for 5 minutes. Samples were cooled in ice for 5 minutes before

adding 1.25 µl of 10 mM dNTPs (10297018, Invitrogen), 5 µl of M-MLV reverse transcriptase 5X reaction buffer, 1 µl of 200 U/µl M-MLV (M1701, Promega), and 1 µl of RNase-out (10777019, Invitrogen) (Figure 2.1). Samples were incubated at 40°C for 1 hour and stored at -20°C.

2.8.3 Quantitative polymerase chain reaction (q-PCR)

The quantitative (q) PCR was performed in a MicroAmp optical 96-well reaction plate (430673, Life Technology). For each sample, 10 µl of Taqman Universal Master Mix II (4426710, Thermo Fisher Scientific) was added together with 8 µl of RNA-free water. One microliter of Taqman oligo for the gene of interest was added. One microliter of sample was added to the mix in the plate. Each sample was performed in triplicate. Then, the plate was sealed with a film and centrifuged at 3,000 rcf for 5 minutes at 4°C. The qPCR was carried out in a Stratagene Mx3005P (Agilent Technologies). The PCR conditions were 50°C for 2 minutes, 90°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The Ct values generated were used to calculate relative gene expression compared to β_2 microglobulin (Figure 2.1).

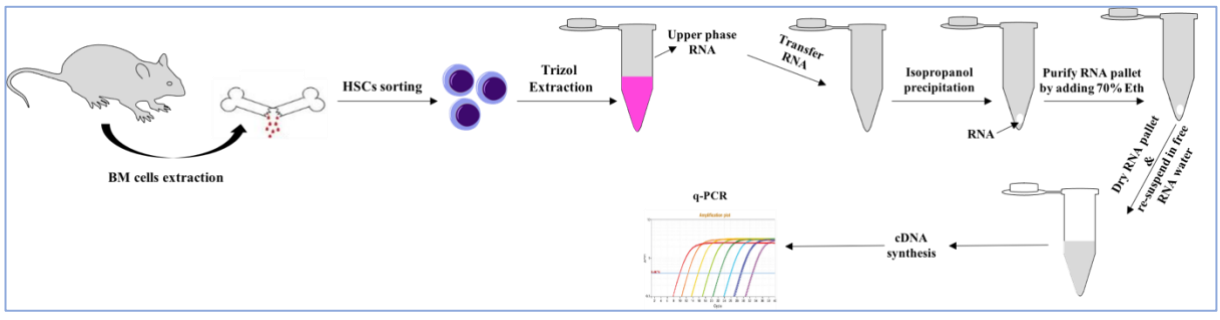


Figure 2.1 A schematic diagram of RNA extraction, cDNA synthesis, and q-PCR. Microsoft PowerPoint has been used to obtain this figure.

2.8.4 RNA-seq

RNA was extracted from sorted KSL cells using Trizol extraction described in section 2.8.1. The quality of the RNA was checked through the University of Birmingham genomics service using a Qubit High Sensitivity RNA assay and an RNA tape on the Agilent TapeStation. The concentrations from the Tapestation were used to normalise the RNA that fell below the detection limit of the Qubit (<5ng/ml), as the kit input minimum is 2pg/ml. RNA-seq was performed using the NEBNext® Single Low Input RNA Library Prep and sequencing. Libraries were generated using a NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina®_24 kit. The protocol for the kit was followed. RNA was normalised to 1.4ng/ml, and 10ng of RNA input was used for the prep, which involved 13 cycles of amplification. cDNA generated in the first step of the prep was quality checked to ensure sufficient cDNA had been generated to continue. Libraries were indexed using NEBNext® Multiplex Oligos for Illumina set 1 and set 2. Libraries were quality checked using a high sensitivity DNA Qubit assay and sized using a D1000 screentape. Libraries were normalised and pooled in equal volumes and made to 4nM for sequencing. The sequencing platform was NextSeq 500, using a v2.5 150 cycles Mid output (75 paired end) Flowcell.

2.8.5 Bioinformatic analysis

Bioinformatic analysis of the raw output data and generation of plots was performed by Dr. Boris Noyvert and Dr. Mary Clarke. Sequencing reads were mapped to mm10 mouse genome with STAR aligner (v2.5.2b) (Dobin et al., 2013). Reads mapping to genes were counted by the same software. Normalisation of read counts and differential expression analysis were performed with DESeq2 (v.1.26.0) R Bioconductor package (Love et al., 2014). Gene set enrichment analysis was done using GAGE (v.2.36.0) R Bioconductor package with Gene

Ontology databases (Luo et al., 2009). The computations were performed on the CaStLeS infrastructure [4] at the University of Birmingham.

2.9 *In situ* protein detection in HSC

Cells sorted from BM were centrifuged and re-suspended in 100 µl PBS. Cell suspensions were placed into a cytofunnel attached to a slide and centrifuged in a cytospin centrifuge at 800 rpm for 8 minutes. The slides were left to air-dry for 10 minutes and then fixed with 4% (w/v) PFA for 20 min at room temperature, washed 3x with PBS, and aldehyde groups quenched for 10 minutes with 50 mM NH_4Cl (254134, Sigma) in PBS at room temperature, followed by 3x washes in PBS. Cells were then permeabilized with 0.3% (v/v) Triton X-100 (11332481001, Sigma) in PBS for 15 minutes at room temperature and blocked with PBS containing 3% (w/v) of bovine serum albumin (BSA, A1933, Sigma), 0.3% (v/v) of Triton X-100, 10% (v/v) FBS for 1 hour in a humidified environment at room temperature. The cells were then incubated with diluted primary rabbit Ab in blocking buffer for 1 hour at room temperature or 4°C overnight (Table 2.5).

Table 2.4 Primary and secondary antibodies in that were used in *in situ* protein detection in HSC

Antibody	Immunoglobulin	Stock concentration (µg/µl)	Dilution Used	Supplier	Cat. number	Comment
RETNLγ	Rabbit IgG	1	1:100	2B Scientific	V4XC-2032	Primary Ab
Goat anti-rabbit Alexa Flour 488	Goat IgG	2	1:200	Invitrogen	A11034	Secondary Ab

After incubating with the primary antibody, the cells were washed for 30 minutes with PBS containing 0.1% (v/v) Tween-20 (P9416, Sigma), and goat anti-rabbit secondary antibody (Table 2.5) was added to the cells for 1 hour at room temperature. Finally, the cells were washed with PBS, mounted, and observed and imaged using a fluorescence microscope (Leica DM6000, Leica Microsystems).

2.10 Intracellular protein detection using flow cytometry

Cells were fixed in 1.6% (w/v) PFA and incubated at room temperature for 15 minutes, then centrifuged at 300 rcf for 5 minutes. The pellets were washed by FBS/PBS/BSA solution and centrifuged at 300 rcf for 5 minutes. Cells were permeabilised by re-suspending in ice cold acetone, incubated for 10 minutes at 4°C, then centrifuged at 300 rcf for 5 minutes followed by a final washing step in FBS/PBS/BSA solution and re-suspension in 100 µl FBS/PBS/BSA. F_C regions were blocked by incubating the cells with 1:20 anti-CD16/32 and incubated for 15 minutes at 4°C. Primary rabbit Ab was added (Table 2.5), and cells were incubated for 30 minutes at 4°C, washed 3x with FBS/PBS/BSA, centrifuged at 300 rcf for 5 minutes at 4°C, and re-suspended in 100 µl. Secondary Ab was added at the concentration indicated in Table 2.5 and incubated for 30 minutes in the dark at 4°C. Another 3x washing steps were performed and cells were resuspended in 400 µl of FBS/PBS/BSA. Samples were run through the CyAn ADP analyser (Beckman Coulter) and Summit 4.3 software was used to analyse the results.

2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Prism version 8.0 for Mac, GraphPad Software, San Diego California USA). All data are expressed as Mean ± Standard Error of the Mean (SEM). P values less than 0.05 were considered statistically significant and a star (*) was annotated in the figure. The following statistical analyses were used: Two-way ANOVA to analyse data describes the two factors across multiple parametric groups. One-way ANOVA or Student T-tests to analyse data describes one factor across multiple parametric.

CHAPTER 3 *RETNLγ* EXPRESSION IN THE HAEMATOPOIETIC STEM CELL HIERARCHY

3.1 Introduction

Previously, RETNL*γ* has been described to be expressed in the BM of both mice and rats (Gerstmayer et al., 2003; Hu et al., 2015). In this chapter, experiments are described that sought to provide a comprehensive picture of the extent of *RETNLγ* RNA and protein expression in the haematopoietic hierarchy of wild type mice. In addition, since RELM proteins are known to be secreted and to be present in the blood, the ability of haematopoietic cells to secrete RETNLγ was investigated. The expression information obtained was expected to serve as a precursor to functional studies on RETNLγ and examination of the consequences of its absence.

3.2 *RETNLγ* RNA expression profile in the haematopoietic hierarchy and HSC subsets

As a first step in trying to understand how the expression of RETNLγ might impact upon the haematopoietic hierarchy and potentially relate to the function of individual cell types, a comprehensive quantitative RT-PCR analysis was performed using cells purified from the bone marrow of 2-month-old wild type C57BL6 mice. High-speed cell sorting was used to isolate cell populations following surface staining with combinations of fluorescently conjugated antibodies (Table 3.1; Appendix 1).

Table 3.1 Surface antigen characteristics used to identify and isolate specific bone marrow HSC subsets and progenitors

Haematopoietic cell type	Surface antigen characteristics used for cell sorting
Long term haematopoietic stem cell (LT-HSC)	Lin- Sca1+ Kit+ CD150+ CD48-
Short term haematopoietic stem cell (ST-HSC)	Lin- Sca1+ Kit+ CD150+ CD48+
Multipotent progenitor (MPP)	Lin- Sca1+ Kit+ CD150- CD48- CD135-
Granulocyte-macrophage progenitor (GMP)	Lin- Sca1- Kit+ CD16/32+ CD34+
Common myeloid progenitor (CMP)	Lin- Sca1- Kit+ CD16/32- CD34+
Megakaryocyte-erythroid progenitor (MEP)	Lin- Sca1- Kit+ CD16/32- CD34-
Immature erythrocyte (Ter119-)	Ter119- CD71+
Immature erythrocyte (Ter119+)	Ter119+ CD71+
Mature erythrocyte	Ter119+ CD71-
Megakaryocyte	Kit- CD41+
Myelomonocytic progenitor	Kit+ CD11b+ Gr1+
Monocytic progenitor	Kit+ CD11b+
Mature myeloid cell	CD11b+ Gr1+

Following RNA extraction and synthesis of cDNA, PCR amplification was performed using primers specific for *RETNLγ* and *β2M* as a house-keeping gene reference. Each cell population

was isolated from three female and three male individuals, and each qPCR determination was repeated in triplicate. The results represented as the relative abundance ($2^{-\Delta Ct}$) of *RETNLγ* and *β2m* RNA are shown in Figures 3.1 to 3.5.

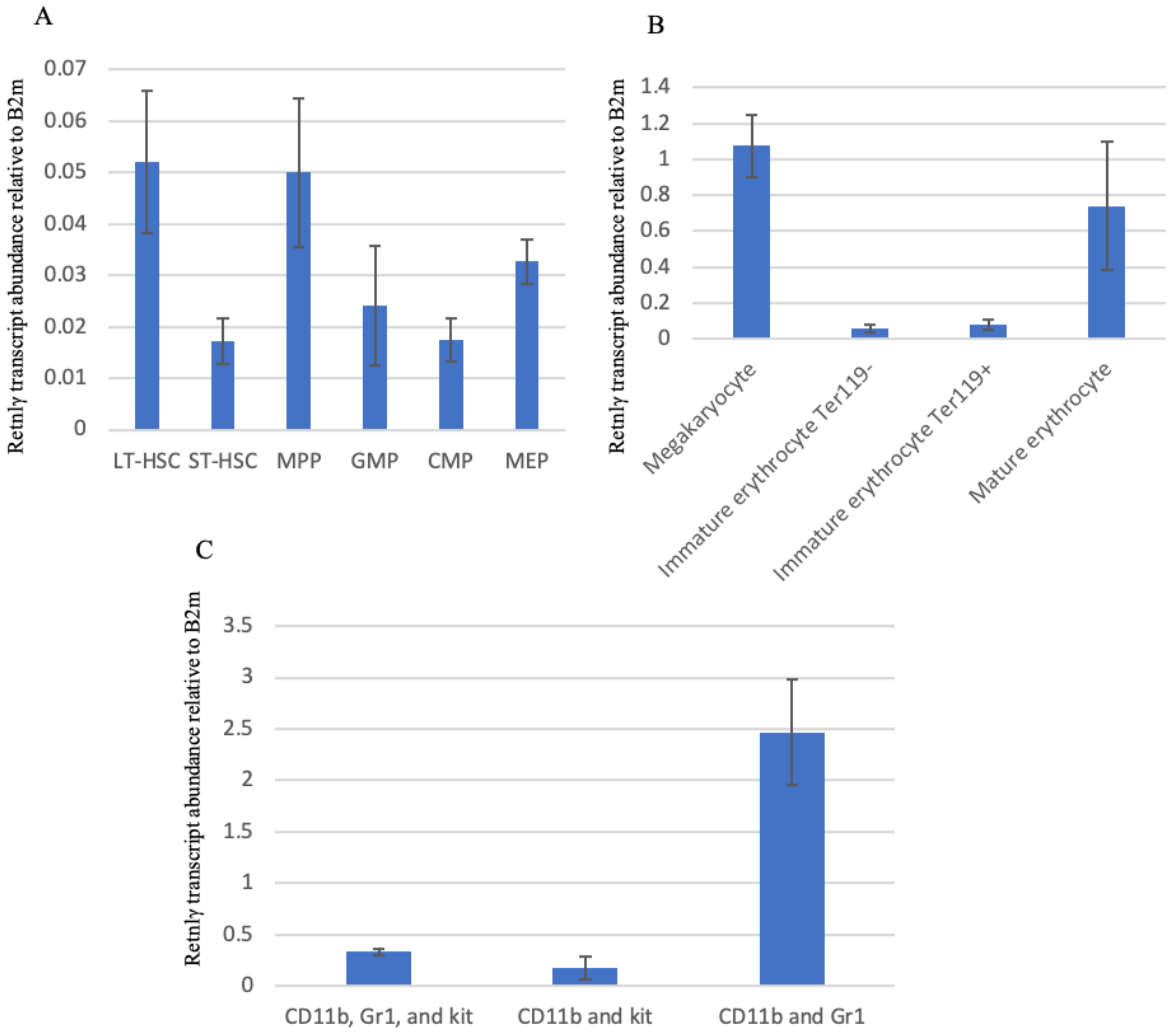


Figure 3.1 *RETNLγ* RNA expression in the haematopoietic hierarchy. BM from WT mice was stained and sorted (Appendix 1) using the surface antigen characteristics defined in Table 3.1. qRT-PCR was performed using Taqman primers for both *β2* microglobulin and *RETNLγ*. The histograms represent the relative expression of *RETNLγ* compared to *β2* microglobulin ($2^{\Delta Ct}$). (A) HSC and committed progenitors. (B) Megakaryocyte compared to erythrocytes and erythroid progenitors. (C) Mature myelomonocytic cells compared to progenitors in the same lineage. Each histogram represents the mean of samples derived from three mice, and qRT-PCR reactions that were repeated in triplicate. SEM is indicated. Student T-test was performed.

3.2.1 The highest levels of *RETNLγ* expression occur in mature haematopoietic lineages

Figure 3.1 illustrates the range of *RETNLγ* RNA expression detected in adult BM HSC, progenitors, and mature cells. The highest levels of *RETNLγ* RNA expression were detected in mature erythrocytes, megakaryocytes, and myelomonocytic cells ($2^{-\Delta Ct}$ of ~0.07-2.4). Immature erythrocytes, myeloid progenitors, and monocytic progenitors showed a lower level of *RETNLγ* expression ($2^{-\Delta Ct} < 0.05$), while the lowest levels were observed in HSC and progenitor cell populations, including the LT-HSC, ST-HSC, MPP, GMP, CMP, and MEP ($2^{-\Delta Ct}$ of 0.01-0.05).

3.2.2 Sex-specific differences in relative *RETNLγ* RNA expression in some haematopoietic cell types

A few studies have suggested that there can be sex-specific differences in the activity of members of the resistin family of proteins (Morash et al., 2004; Le Lay et al., 2001; Gui et al., 2004). In light of these observations, further analyses and additional qRT-PCR determinations were carried out to determine if there are any significant differences between male and female mice in terms of *RETNLγ* expression in BM haematopoietic cells (Figures 3.2-5, summarised in Figure 3.5).

Comparing cells of the erythroid and megakaryocytic lineages, there was some indication of differences in the relative expression of *RETNLγ* in mature erythrocytes and megakaryocytes, females exhibiting higher levels in the former and males having proportionately greater expression in megakaryocytes (Figure 3.2). Similar to the mature erythroid and megakaryocytic cells, female mature $CD11^{+}Gr1^{+}$ myelomonocytic cells were found to express a higher level of *RETNLγ*, especially when comparing to more immature cells in this lineage (Figure 3.3).

The overall levels of *RETNL*γ RNA expression in the most immature stem cells and progenitors of the haematopoietic hierarchy are much lower than in mature cells (approximately 10-100-fold). Although there seem to be no significant differences in *RETNL*γ RNA comparing committed progenitors between males and females, there does appear to be a much lower level of expression in female LT-HSC and a corresponding relatively higher level in MPP (Figure 3.4).

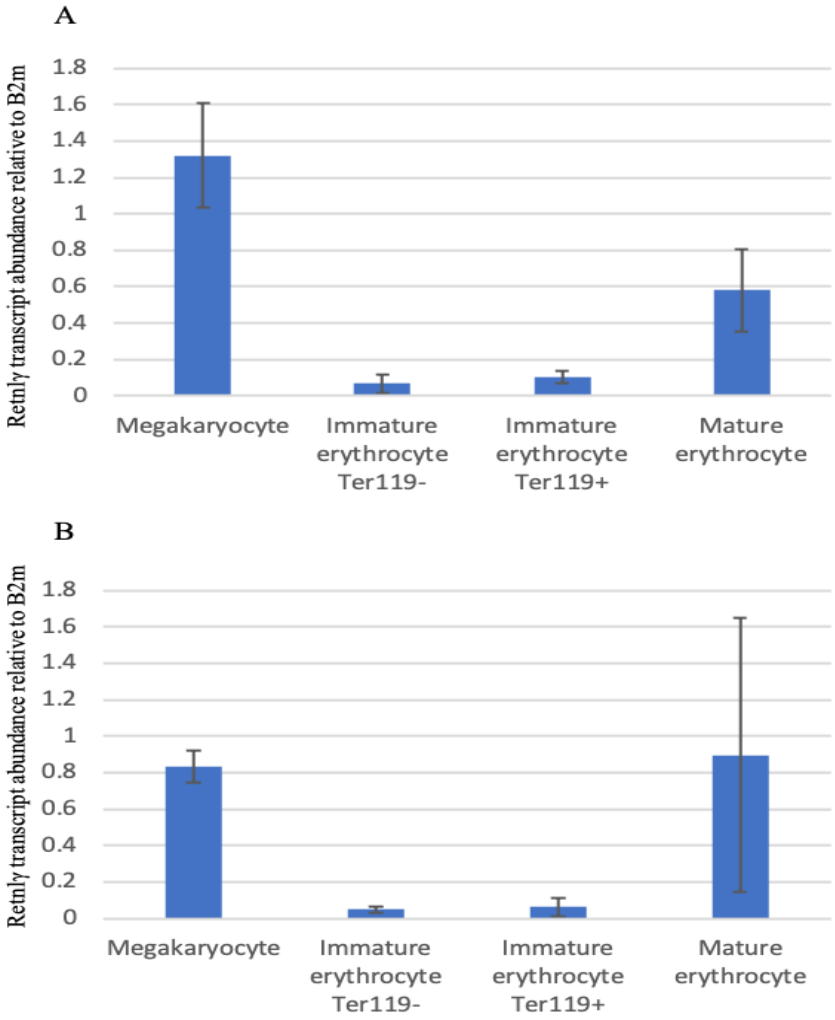


Figure 3.2 *RETNLγ* RNA expression in the haematopoietic hierarchy of male and female mice. BM from WT male and female mice was stained and sorted using the surface antigen characteristics defined in Table 3.1. qRT-PCR was performed using Taqman primers for both *β2* microglobulin and *RETNLγ*. The histograms represent the relative expression of *RETNLγ* compared to *β2 microglobulin* ($2^{-\Delta Ct}$). (A) Megakaryocyte compared to erythrocytes and erythroid progenitors in male mice. (B) Megakaryocyte compared to erythrocytes and erythroid progenitors in female mice. Each histogram represents the average of samples derived from three mice, and qRT-PCR reactions that were repeated in triplicate. SEM is indicated. Student T-test was performed.

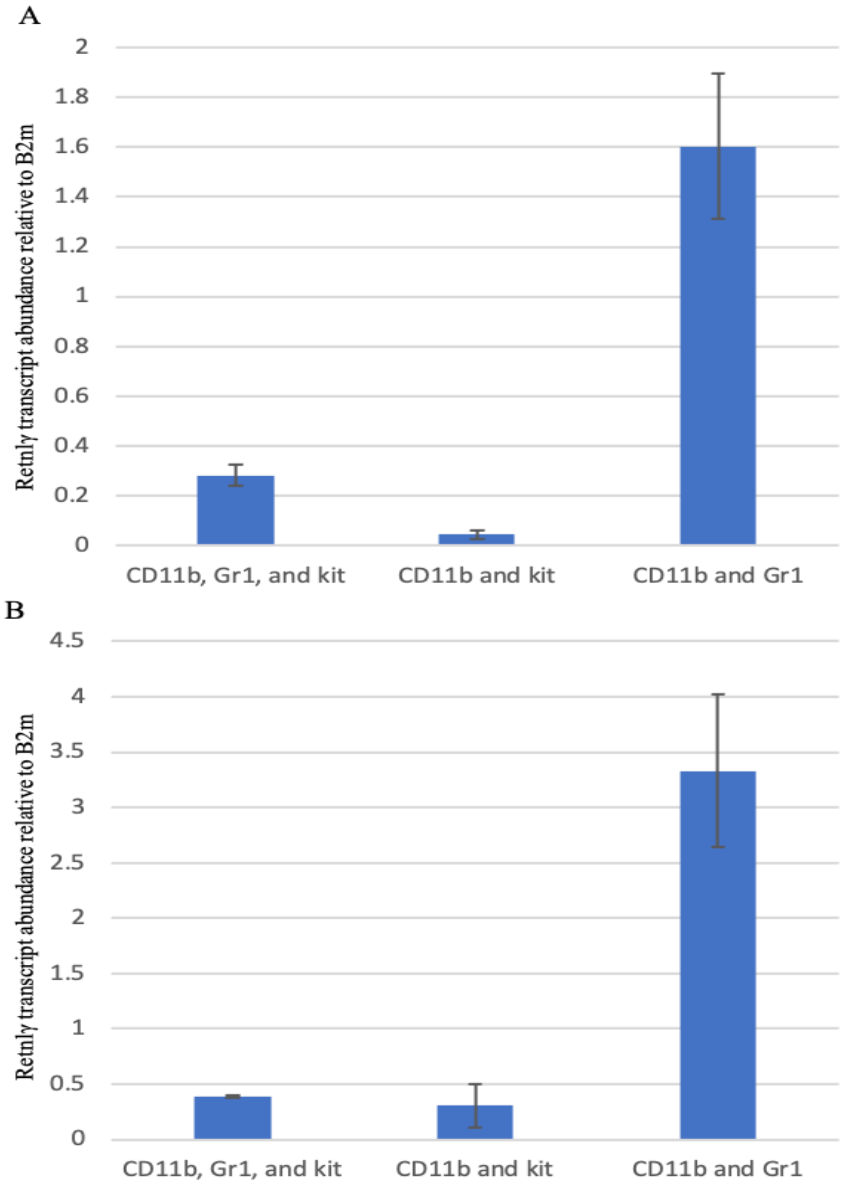


Figure 3.3 *RETNLγ* RNA expression in HSC progenitors in both male and female mice. BM from WT male and female mice was stained and sorted using the surface antigen characteristics defined in Table 3.1. qRT-PCR was performed using Taqman primers for both *β2 microglobulin* and *Retnlγ*. The histograms represent the relative expression of *RETNLγ* compared to *β2 microglobulin* ($2^{-\Delta Ct}$). (A) A comparison between the Retnlγ expression in myelomonocytic progenitors, monocytic progenitors, and mature myeloid cells in male mice. (B) A comparison between the *RETNLγ* expression in myelomonocytic progenitors, monocytic progenitors, and mature myeloid cells in female mice. Each histogram represents the average of samples derived from three mice, and qRT-PCR reactions that were repeated in triplicate. SEM is indicated. Student T-test was performed.

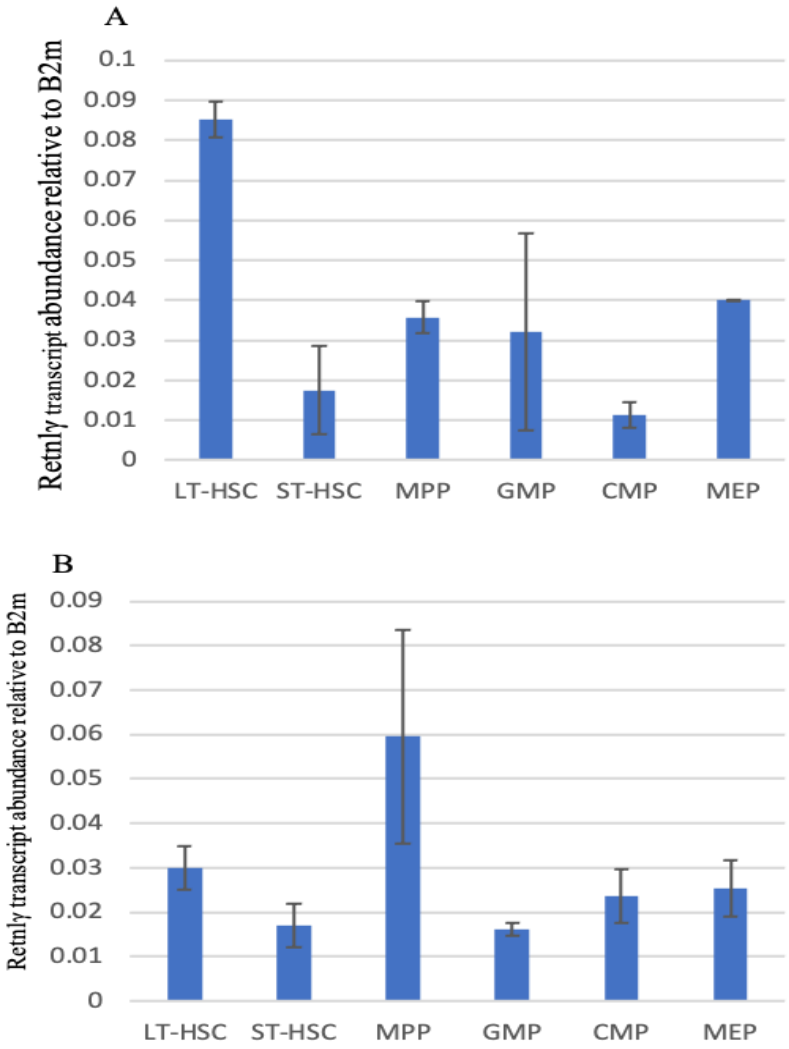


Figure 3.4 *RETNLγ* RNA expression in HSC and progenitors in both male and female mice. BM from WT male and female mice was stained and sorted using the surface antigen characteristics defined in Table 3.1. qRT-PCR was performed using Taqman primers for both *β2 microglobulin* and *RETNLγ.* The histograms represent the relative expression of *RETNLγ* compared to *β2 microglobulin* ($2^{-\Delta Ct}$). (A) HSC and committed progenitors in male mice. (B) HSC and committed progenitors in female mice. Each histogram represents the average of samples derived from three mice, and qRT-PCR reactions that were repeated in triplicate. SEM is indicated. Student T-test was performed.

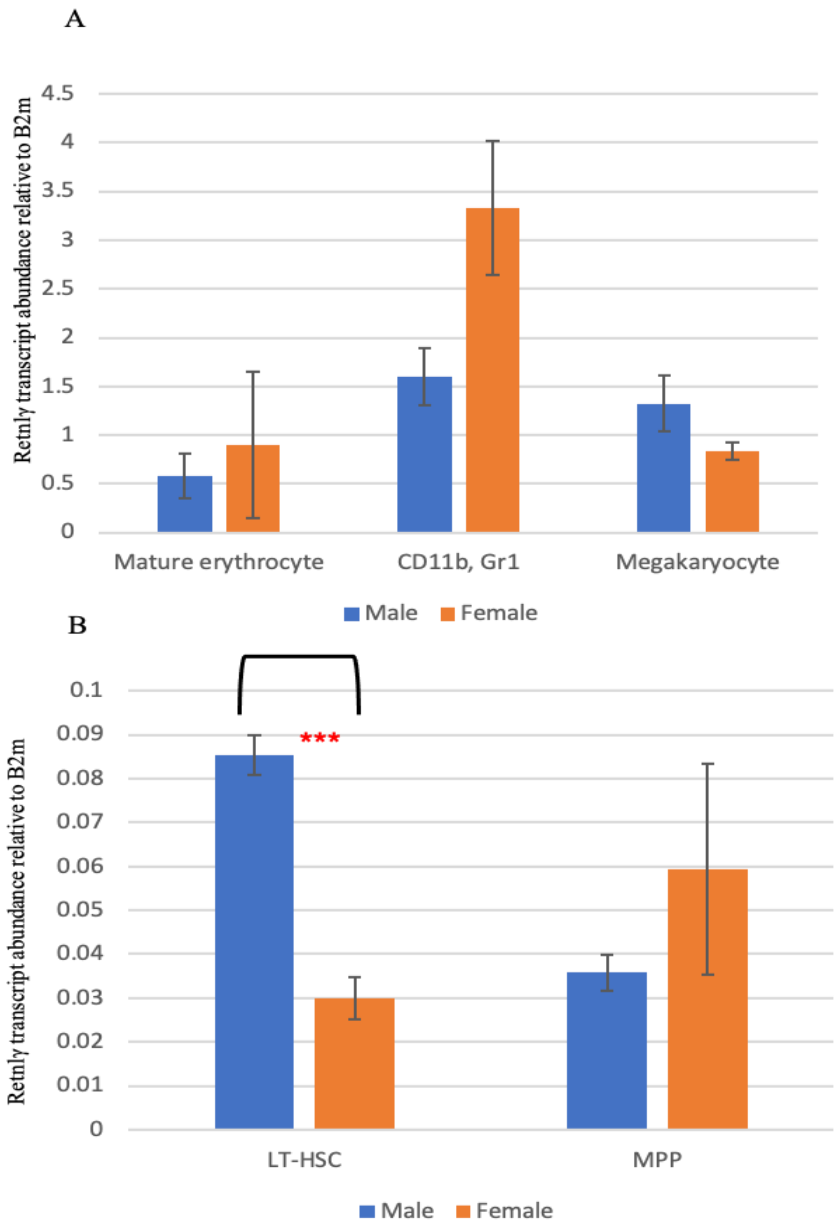


Figure 3.5 *RETNLγ* RNA expression in HSC and progenitors in both male and female mice. BM from WT male and female mice was stained and sorted using the surface antigen characteristics defined in Table 3.1. qRT-PCR was performed using Taqman primers for both *β2 microglobulin* and *RETNLγ*. The histograms represent the relative expression of *RETNLγ* compared to *β2* microglobulin ($2^{-\Delta Ct}$). A) A comparison of *RETNLγ* expression between male and female mice in three different cell populations, mature erythrocyte, mature myeloid cells, and megakaryocyte. B) A comparison of *RETNLγ* expression between male and female mice in LT-HSC and MPP. Each histogram represents the average of samples derived from three mice, and qRT-PCR reactions that were repeated in triplicate. SEM is indicated. Student T-test was performed.

3.3 RETNLγ protein expression in HSC

The variation in *RETNLγ* RNA expression within the haematopoietic hierarchy described in the previous section shows a considerable difference between immature stages, including stem cells, and the most mature lineages, with the highest levels being seen in mature myelomoncytic cells. Two important points arise from these findings and reports in the literature about the secreted nature of resistin proteins (Gerstmayer et al., 2003; Holcomb et al., 2000; Fasshauer et al., 2001). First, is the level of *RETNLγ* RNA reflected in the expression of RETNLγ protein? Second, is RETNLγ protein secreted?

3.3.1 High-level expression of RETNLγ protein can be detected in haematopoietic cells using *in situ* immunofluorescence assay

Since $CD11b^+$ myelomonocytic cells were shown to express the highest amounts of *RETNLγ* at the RNA level, these cells were used as a starting point for analysis of RETNLγ protein expression. Cells were derived from both WT and *RETNLγ* KO mouse BM, the latter providing a control for the specificity of Ab staining. In addition, a positive control was employed involving 293T cells (HEK cells) that had been transiently transfected with a *RETNLγ* over expression plasmid, as described in Section 2.7.3.

Sorted WT or *RETNLγ* KO $CD11b^+$ myelomonocytic cells or 293T/*RETNLγ* cells were fixed on slides, permeabilised, and stained with rabbit anti-mouse RETNLγ protein Ab or control IgG Ab. Goat anti-rabbit fluorescent Ab (Alexa Fluor 488, green) was used to detect binding of the primary Ab. Stained cells were observed using a Leica DM6000 fluorescent microscope (Figure 3.6).

Clear specific staining of RETNLγ was seen comparing 293T with and without over expression of the protein (Figure 3.6A). A somewhat weaker signal was seen in WT $CD11b^+$ cells, and

specific since no fluorescence was detected when using *RETNLγ* KO cells (Figure 3.6B). Amongst the population of WT $CD11b^+$ cells some variation in the intensity of specific RETNLγ staining was apparent, indicating that there is probably a degree of heterogeneity. Such heterogeneity in the population is expected, as it is possible to define subpopulations with additional surface antigen markers, such as Gr1.

3.3.2 Detection of low-level expression of RETNLγ protein in HSC requires flow cytometric analysis of immunofluorescent staining

The degree of sensitivity achieved in the *in situ* staining experiments and the number of cells required suggested that it would not be possible to apply the same strategy in order to detect expression of RETNLγ protein in HSC, which was expected to be 10 to 100 times lower than that in the mature myelomonocytic cells. Therefore. in order to study RETNLγ protein levels in HSC, cells were sorted from WT and *RETNLγ* KO BM and analysed for RETNLγ protein expression using immunofluorescence and flow cytometry. Isolated BM cells were stained for surface antigens to allow discrimination of HSC and progenitor populations, then fixed and permeabilised to enable intracellular staining with anti-RETNLγ antibody and secondary goat anti-rabbit Ig conjugated to Alexa Fluor 488. Using this approach, it was possible to detect RETNLγ protein expression in all populations tested, including LT-HSC, even though only approximately 100-200 cells could be obtained per mouse (Figure 2.7).

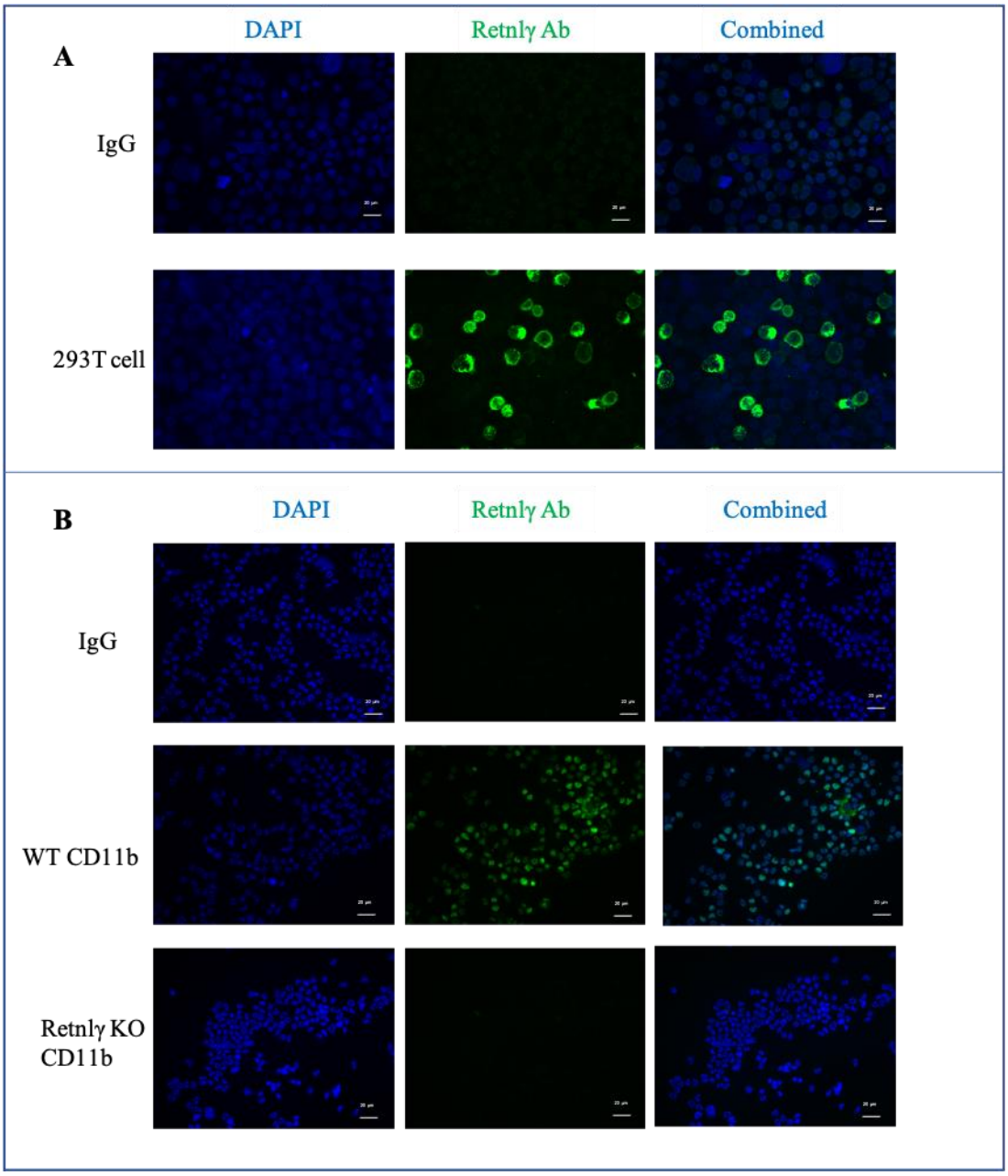


Figure 3.6 Intracellular RETNLγ protein expression by in situ immunofluorescence assay. (A) Transfected 293T cells by RETNLγ over expression plasmid were fixed on slides, permeabilised, stained by non-fluorescent rabbit anti-mouse RETNLγ protein Ab and IgG Ab separately. Secondary Ab, goat anti-rabbit fluorescent Ab (Alexa fluor 488, green), was used to detect any binding between specific Ab and RETNLγ protein. Non-transfected 293T cells were used as a control. (B) Mature monocytic cells were sorted from wild type and RETNLγ KO mice using a high-speed cell sorting machine, permeabilised, and stained by non-fluorescent rabbit anti-mouse RETNLγ protein Ab and IgG Ab separately. Secondary Ab, goat anti-rabbit fluorescent Ab (Alexa fluor 488,

green), was used to detect any binding between specific Ab and RETNLγ protein. *RETNLγ* KO mouse was used as a negative control. Cells were observed under 40X lens (n=5 independent experiments).

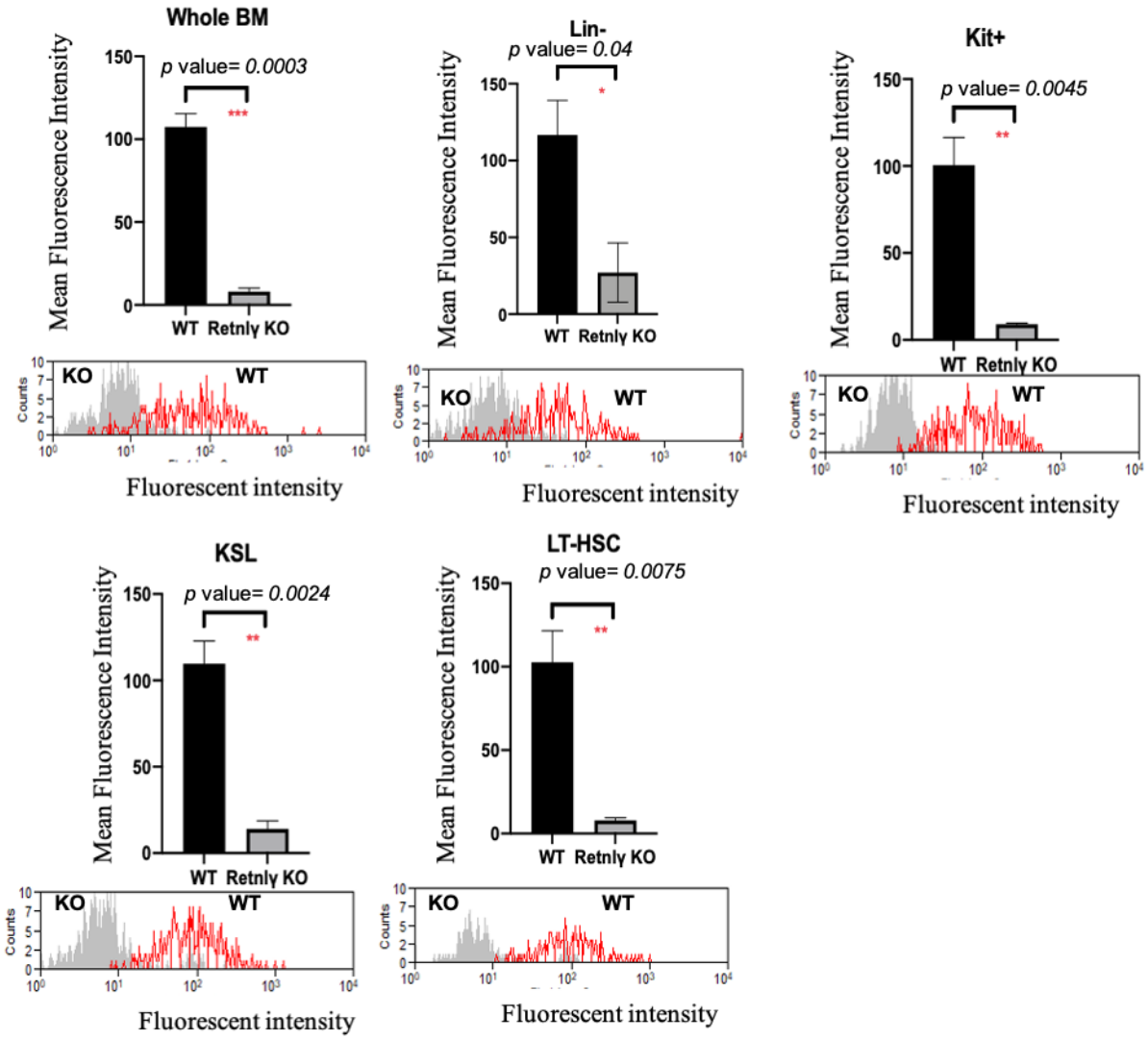


Figure 3.7 Intracellular RETNLγ protein expression in HSC by flow cytometry. BM from WT and *RETNLγ* KO mice was extracted and stained for surface antigen characteristics in Table 3.1. Cells were permeabilised and stained using specific non-fluorescence Ab for RETNLγ protein followed by secondary Ab to detect the binding between the primary Ab and RETNLγ protein. RETNLγ protein was detected intracellularly in all HSC populations. GraphPad Prism software was used to calculate the mean and standard error of the mean (SEM). Unpaired T-test was performed to calculate statistical differences.

3.4 Summary

The results in this chapter have been demonstrating that *RETNLγ* mRNA is expressed throughout the haematopoietic hierarchy in different levels. The highest level of *RETNLγ* can be found in mature erythroid cells, megakaryocytes, and myelomonocytic cells. It is noticeable that the level of *RETNLγ* in myelomonocytic cells is about 100-fold greater than that in HSC. Intracellular RETNLγ protein expression was similarly detected in cells representing high RNA expression (myelomonocytes) and low expression (HSC), and it is anticipated that the protein could be secreted, although technical issues prevented this from being thoroughly investigated here. In addition, differences were observed in the expression of the *RETNLγ* mRNA between males and females, and these were statically significant in LT-HSC. Higher levels have been noticed in female mice in mature erythrocytes, megakaryocytes, and MPPs; and lower levels in female LT-HSC comparing to male LT-HSC. How these gender-specific differences relate to the function of HSC is considered in Chapters 4 and 5.

CHAPTER 4 FUNCTIONAL CONSEQUENCES OF LOSS OF RETNLγ EXPRESSION IN HAEMATOPOIETIC STEM CELLS

4.1 Introduction

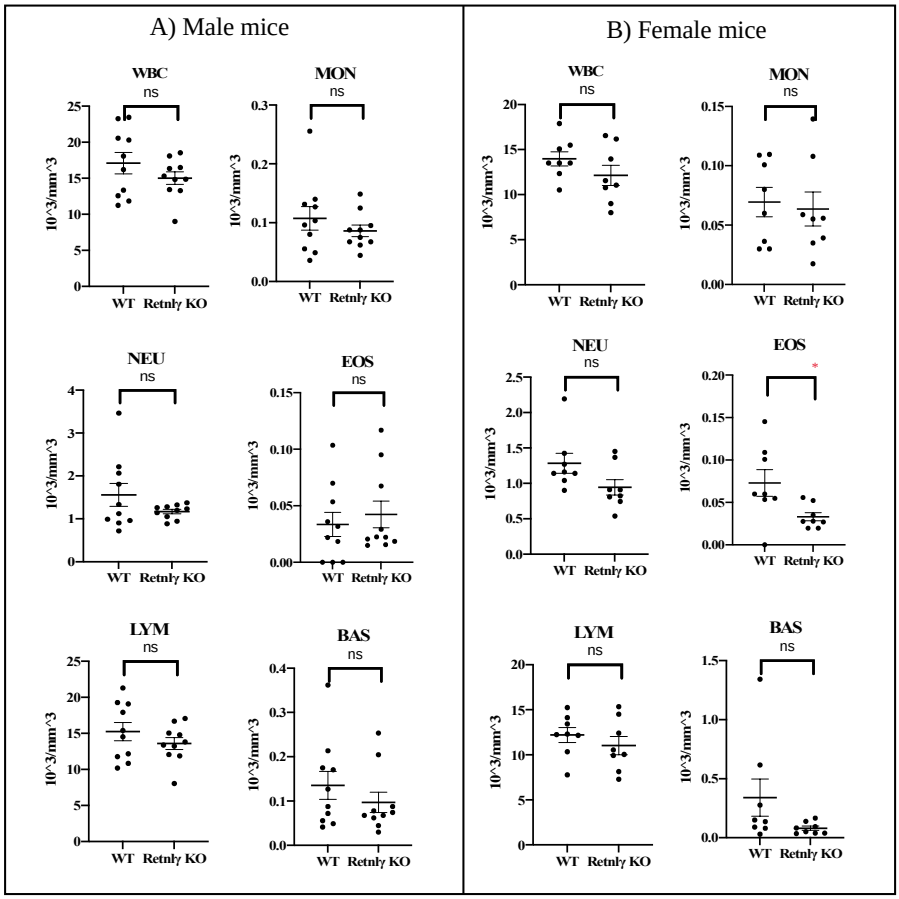
In Chapter 3 a comprehensive analysis of *RETNLγ* RNA in the haematopoietic hierarchy showed that it is expressed throughout, including in the various stem cell stages. There are wide variations in the expression seen, ranging from the highest levels in mature erythroid and myelomonocytic cells and megakaryocytes to roughly 1-10% of these levels in immature stem cells and progenitors.

Given the previously unrecognised expression of RETNLγ in HSC, the next objective was to determine its functional relevance in these cells. In this chapter, results are described for the comparative analysis of the cell composition of the peripheral blood and the BM from wild type and *RETNLγ* KO mice. Furthermore, some preliminary studies are performed to determine if any functional effect caused by the absence of RETNLγ is compounded during ageing. In addition, both *in vitro* and *in vivo* assays of HSC differentiation and self-renewal potential are employed to compare cells derived from wild type and *RETNLγ* KO mouse BM.

4.2 Comparison of peripheral blood cell numbers in wild type and *RETNLγ* knockout mice

In order to examine the effect of *RETNLγ* KO in the haematopoietic system, twenty littermate pairs of mice were collected to examine their peripheral blood. Equal numbers of male and

female pairs were studied. Peripheral blood was collected from the tail vein into a tube containing anticoagulant, and a full blood count was obtained using an automated machine. The results are represented in Figure 4.1, showing white blood cells, monocytes, neutrophils, eosinophils, basophils, and lymphoid cells, and in Figure 4.2, showing red blood cells (including number, haemoglobin content, and haematocrit) and platelets.



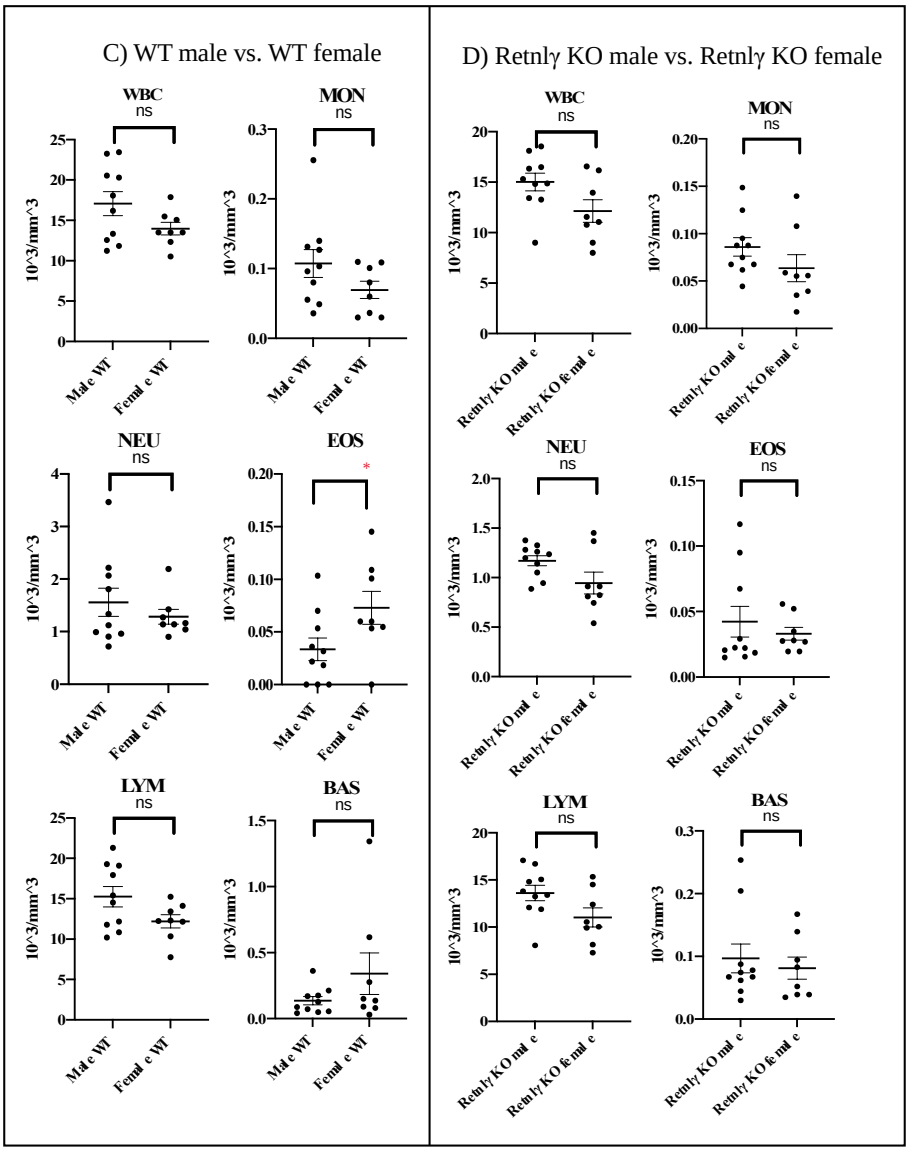


Figure 4.1 Complete blood cell counts comparing male and female wild type and *RETNLγ* KO mice: myeloid and lymphoid cells. Peripheral blood was sampled through the tail vein from 10 WT and 10 KO males and the same number of females. Counting was carried out using an automated machine. The absolute cell numbers for individual mice are represented as scatter plots, showing the mean and SEM. Each panel shows values for white blood cells (WBC), monocytes (Mono), neutrophils (Neu), eosinophils (Eos), basophils (Bas) and lymphocytes (Lym). Each panel shows a different comparison between males and females and WT and KO: (A) WT vs KO in males; (B) WT vs KO in females; (C) WT male vs female; and (D) KO male vs female. Unpaired t-test was used to detect any significant result. ns, not significant. GraphPad Prism software was used to calculate the mean and

standard error of the mean (SEM). Unpaired T-test was performed to calculate statistical differences. *Gating strategies can be found in* Appendix 1.

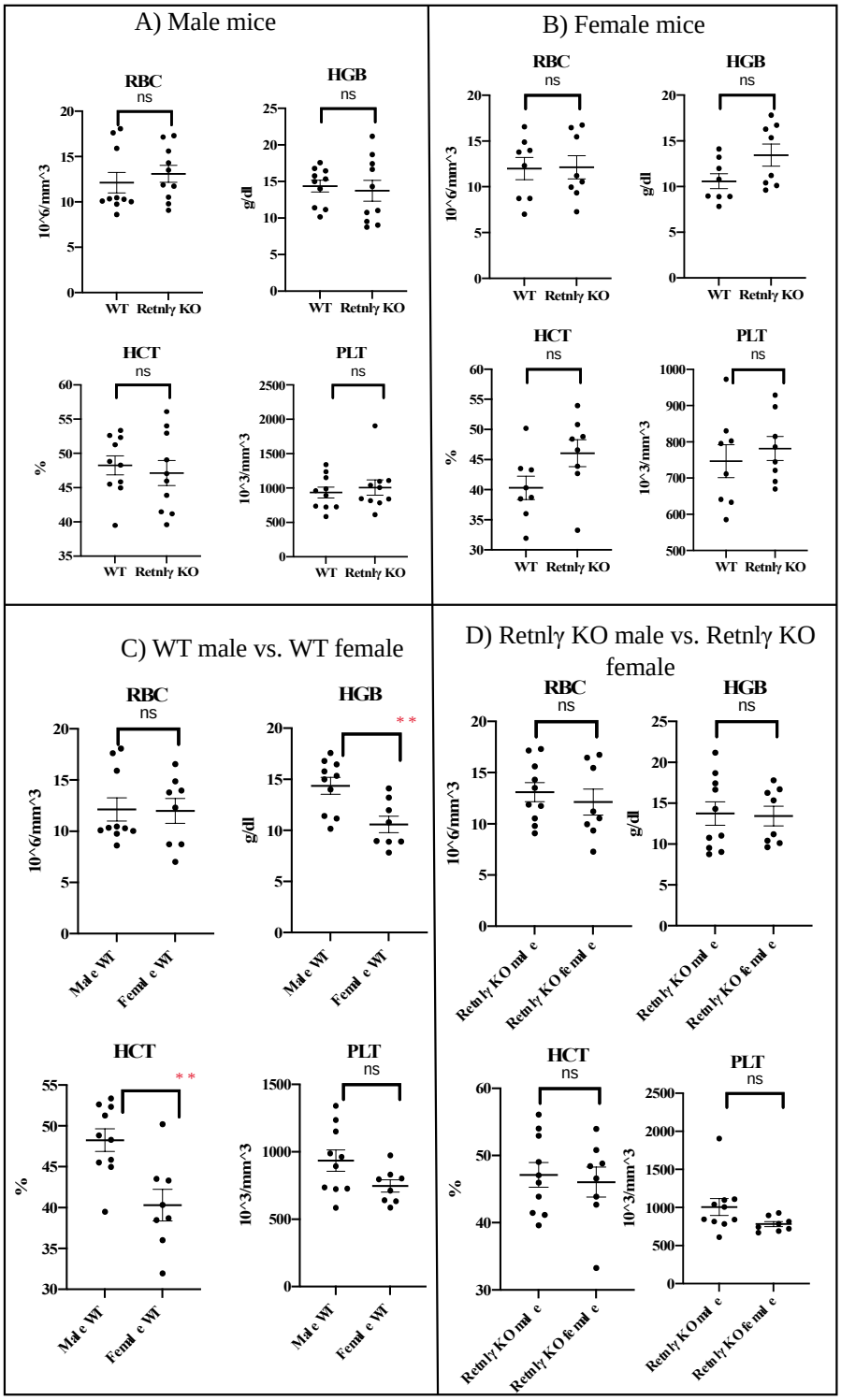


Figure 4.2 : Complete blood cell counts comparing male and female wild type and RETNLγ mice: red blood cells and platelets. Peripheral blood was sampled through the tail vein from 10 WT and 10 KO males and the same number of females. Counting was carried out using an automated machine. The absolute cell numbers for individual mice are represented as scatter plots, showing the mean and SEM. Each panel shows values for red

blood cells (RBC), the red cell parameters of haemoglobin content (HGB) and haematocrit (HCT), and platelets (Plt). Each panel shows a different comparison between males and females and WT and KO: (A) WT vs KO in males; (B) WT vs KO in females; (C) WT male vs female; and (D) KO male vs female. Unpaired t-test was used to detect any significant result (* $p< 0.05$ ** $p<0.01$), ns, not significant. GraphPad Prism software was used to calculate the mean and standard error of the mean (SEM). Unpaired T-test was performed to calculate statistical differences. Gating strategies can be found in Appendix 1.

In both figures, the results are presented as WT versus KO for males and females separately (parts A and B, respectively), and as males versus females for WT and KO separately (parts C and D). With one exception, statistically significant differences were only detected in respect to male versus female comparisons of WT mice, which showed lower haemoglobin and haematocrit values in females, and higher eosinophils in females. One difference between WT and KO was observed in females in that the levels of eosinophils were significantly lower in the KO ($p = 0.029$).

4.3 Assessment of any compounding effect of age on the peripheral blood phenotype caused by the absence of RETNLγ

Ageing is known to have effects on HSC in terms of their proliferation and lineage bias and can reveal underlying effects of gene deficiencies, as has been seen for example in the studies by the Frampton group (García et al, 2009; Clarke et al, submitted). In order to assess if the absence of RETNLγ could have an effect that becomes more apparent with age, cohorts of female WT and KO mice were monitored over a period of a year and a half. Peripheral blood samples were collected from the tail vein starting from 2 month, and repeated on a monthly basis. The results are presented in Figure 4.3 (numbers for white blood cells, monocytes, neutrophils, eosinophils,

basophils, and lymphoid cells) and Figure 4.4 (red blood cell number, haemoglobin content, haematocrit, and platelet number).

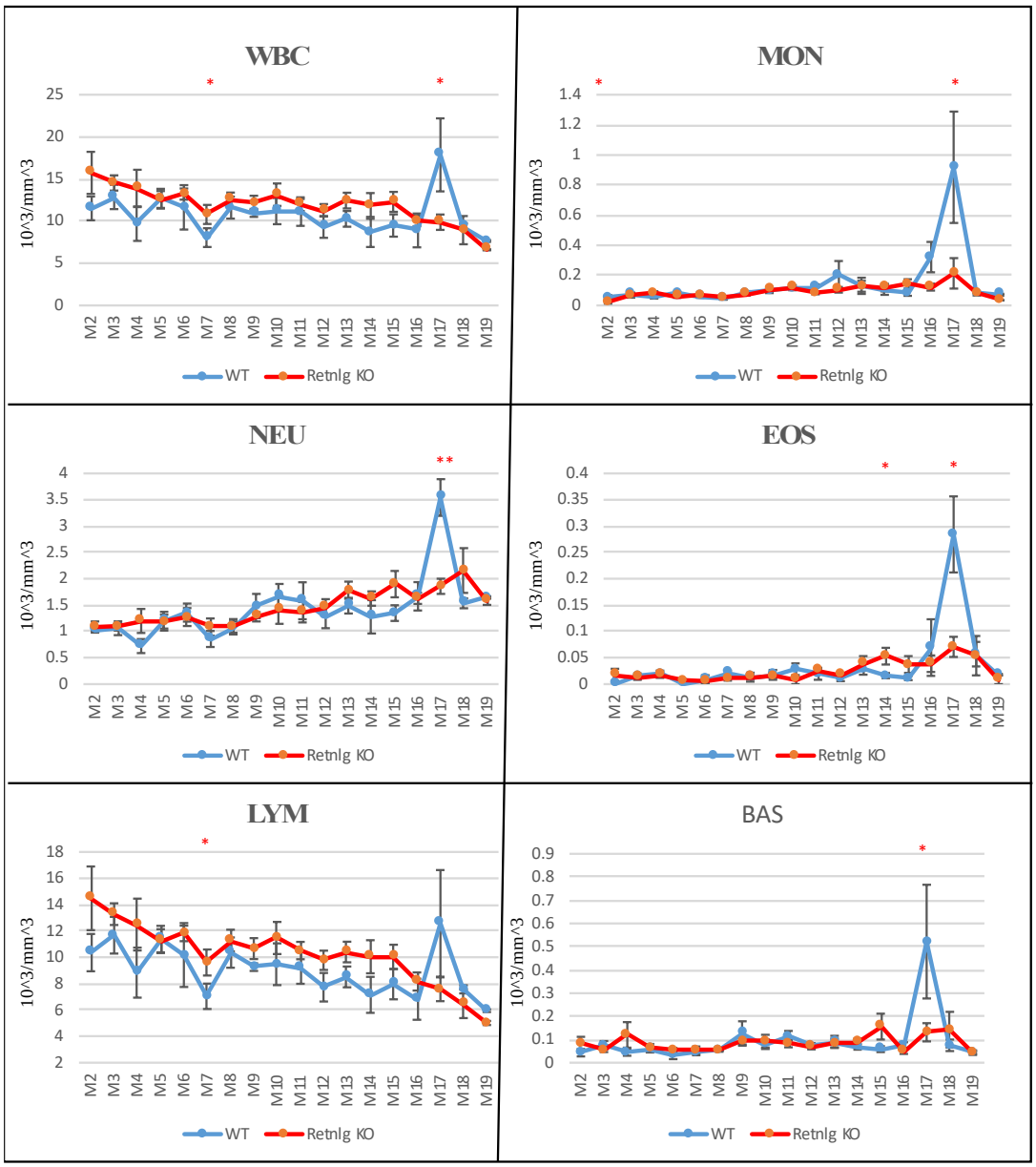


Figure 4.3 Complete blood cell counts comparing female wild type and *RETNLγ* KO mice during ageing: myeloid and lymphoid cells. Peripheral blood was sampled every month through the tail vein from 5 WT and 8 KO females. Counting was carried out using an automated machine. The average cell numbers are represented by the blue (WT) and red (KO) lines. White blood cells (WBC), monocytes (Mono), neutrophils (Neu), eosinophils

(Eos), basophils (Bas) and lymphocytes (Lym). Vertical bars indicate the SEM. Two-way ANOVA test (multiple comparisons) was used to detect any significant result (* $p < 0.05$) (** $p < 0.01$).

Although some consistent and expected changes in individual blood cell types were observed (eg decreases in lymphoid cells and red blood cells, and increase in platelets), these were not statistically different when comparing WT and KO mice.

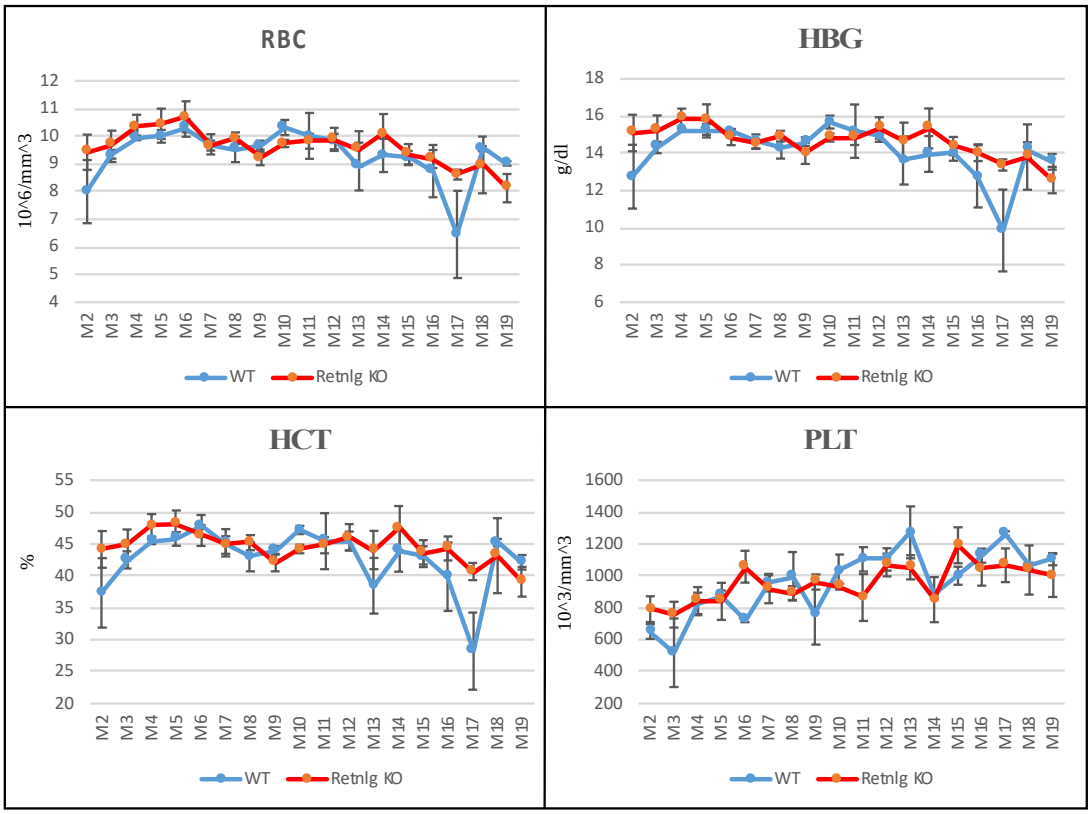
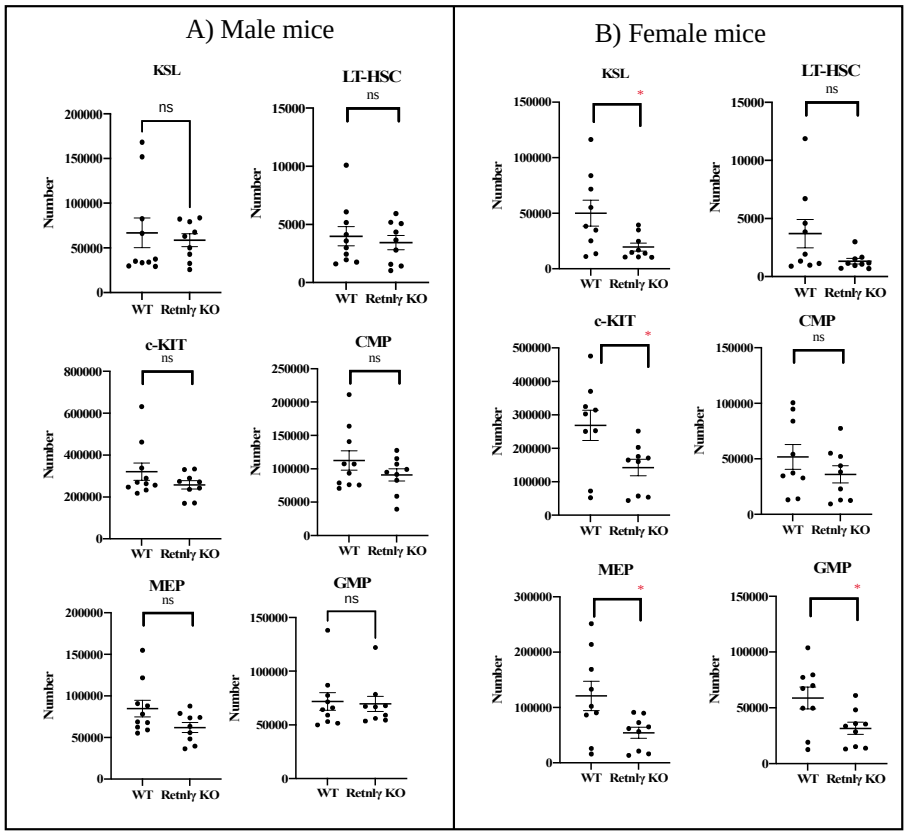


Figure 4.4 Complete blood cell counts comparing female wild type and *RETNLγ* KO mice during ageing: red blood cells and platelets. Peripheral blood was sampled every month through the tail vein from 5 WT and 8 KO females. Counting was carried out using an automated machine. The average values are represented by the blue (WT) and red (KO) lines. Red blood cells (RBC), the red cell parameters of haemoglobin content (HGB) and haematocrit (HCT), and platelets (Plt). Vertical bars indicate the SEM. Two-way ANOVA test (multiple comparisons) was used to detect any significant result.

4.4 Comparison of bone marrow haematopoietic cell numbers in wild type and *RETNLγ* knockout mice

Although little or no difference was seen when comparing peripheral blood values between WT and *RETNLγ* KO mice, this may mask an underlying defect within the bone marrow haematopoietic stem and progenitor cell compartment. The same cohorts of animals that were analysed for their peripheral blood values at 3-months of age (Figures 4.1 and 4.2) were also examined for their bone marrow cell content. BM cells were immune-stained for specific cell surface markers that define the majority of haematopoietic stem and progenitor cell populations (Table 3.1). Flow cytometry was used to identify and quantify the individual populations (Appendix 1). The results are represented in Figure 4.5, showing long-term HSC (LT-HSC), KSL cells (combined LT-HSC, ST-HSC, and MPP), Kit+ cells (mainly bulk progenitors), common myeloid progenitors (CMP), granulocyte myeloid progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP). The results are presented as WT versus KO for males and females separately (parts A and B, respectively), and as males versus females for WT and KO separately (parts C and D).


A) Male mice
B) Female mice
KSL
LT-HSC
c-KIT
CMP
MEP
GMP
Number
ns
*
WT
Retnlγ KO

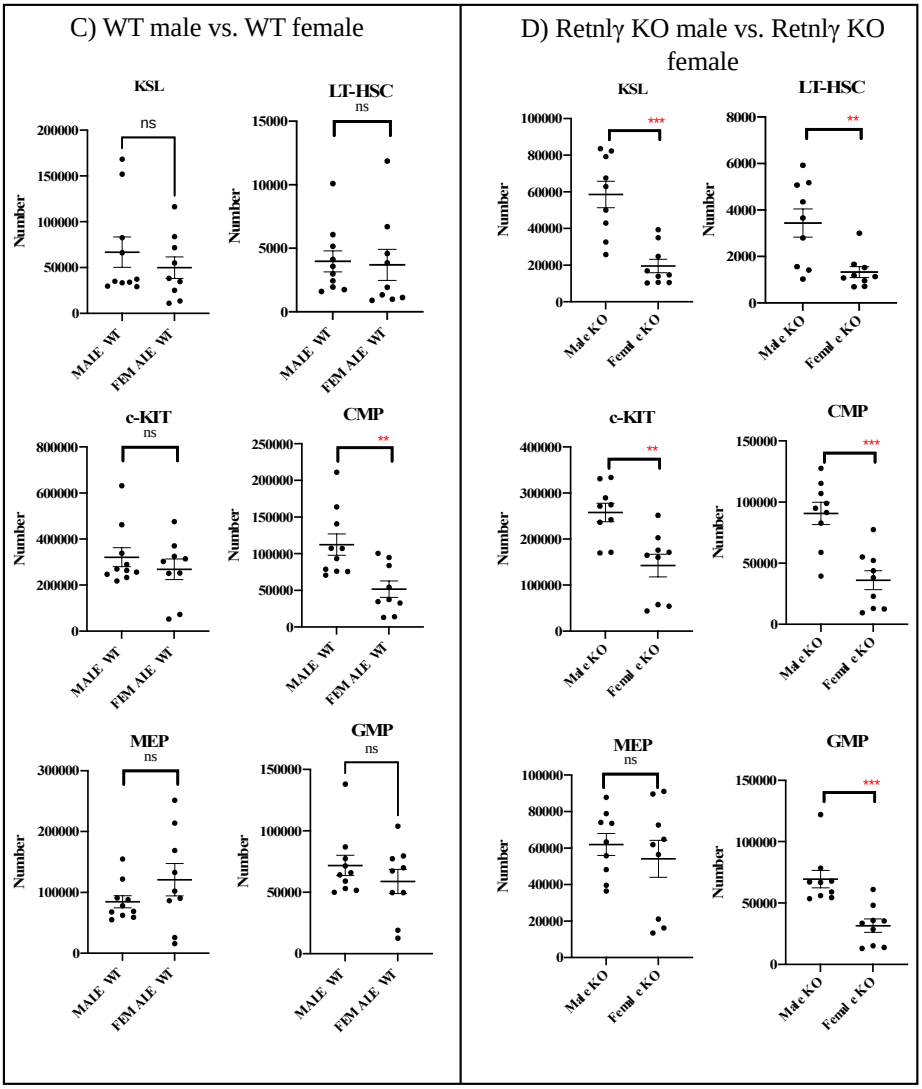
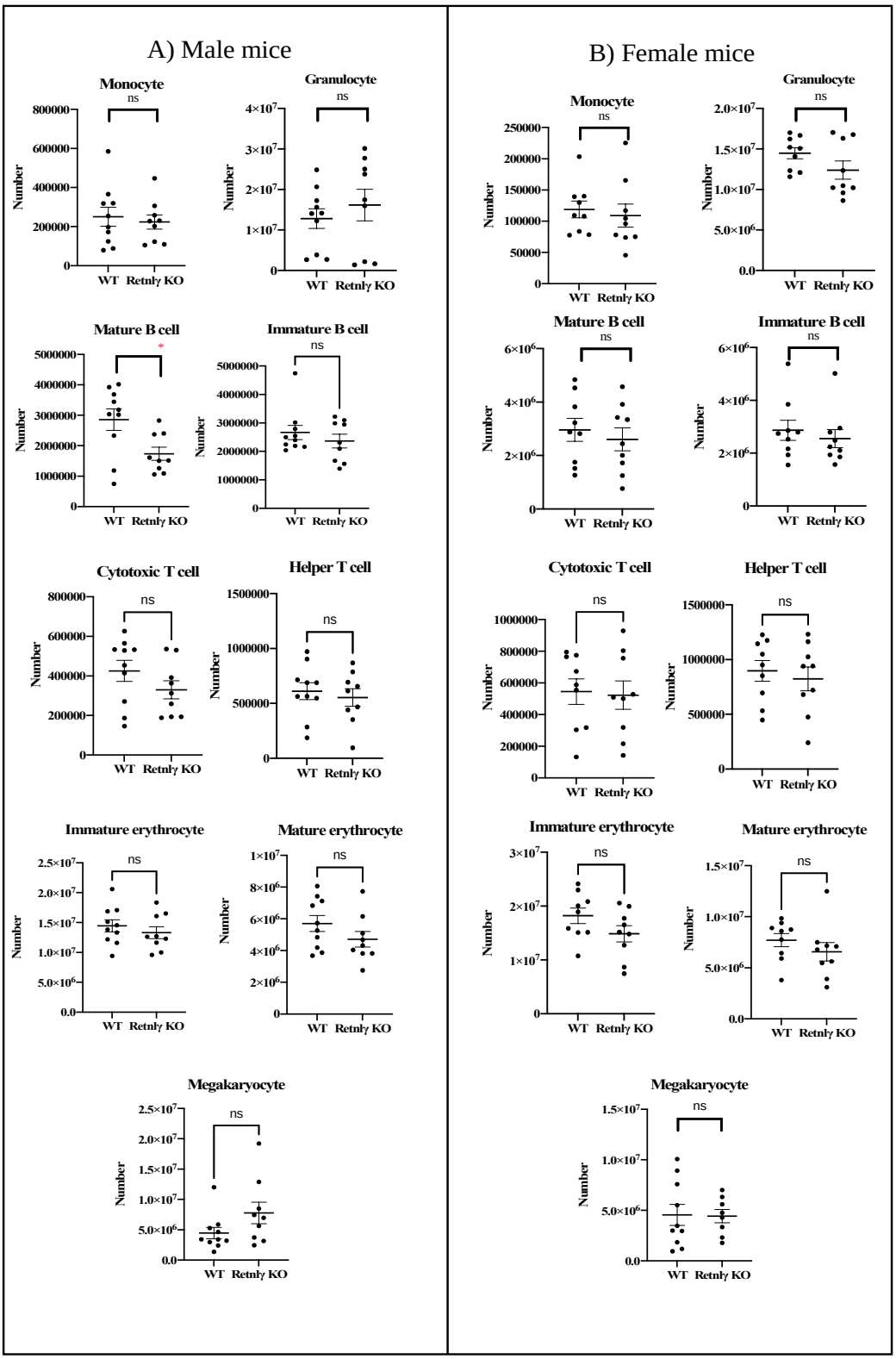


Figure 4.5 Analysis of BM cell content by immunofluorescence: stem cells and progenitors. Bone marrow was collected from the femurs of 10 WT and 10 *RETNLγ* KO 3-month old males and the same number of females. Cells were immuno-stained with antibodies to enable discrimination of distinct stem and progenitor population by flow cytometry (Table 3.1). The absolute cell numbers are represented in the scatter plots. Each panel shows values for long-term HSC (LT-HSC), KSL cells, Kit+ progenitors, common myeloid progenitors (CMP), granulocyte myeloid progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP). Each panel shows a different comparison between males and females and WT and *RETNLγ* KO: (A) WT vs KO in males; (B) WT vs KO in females; (C) WT male vs female; and (D) KO male vs female. The mean is indicated by the horizontal lines and the SEM is shown by T-bars. Unpaired t-test was used to detect any significant result (* $p<0.05$, ** $p<0.01$, *** $p<0.005$), ns, not significant. Gating strategies can be found in Appendix 1.

A number of statistically significant differences were identified and, intriguingly, some strong, female-specific effects in the KO. No significant differences were seen in males comparing WT and KO mice, although MEP showed a trend towards lower levels in the KO (p=0.075). In contrast, in females statistically significant lower levels were seen for KSL (p=0.025), Kit+ cells (p=0.025), GMP (p=0.027), and MEP (p=0.032). Approaching the comparisons from a different direction, that is, male versus female separately for the WT and KO, revealed one significant difference in WT mice, CMP being significantly lower in females (p=0.0047). The male versus female comparison of KO BM cells further strengthened the conclusion that female KO mice have a profound sex-specific haematopoietic stem and progenitor cell deficiency. This latter comparison showed highly significant decreases in all cell types examined with the exception of MEP, the respective p-values for LT-HSC, KSL cells, Kit+ cells, CMP, and GMP being 0.0051, 0.0002, 0.0021, 0.0003, and 0.0006.

BM cells were also immune-stained for specific cell surface markers that define the mature lineage cells. The results are represented in Figure 4.6, showing monocytes, granulocytes, mature and immature B-cells, cytotoxic (CD8+) and helper (CD4+) T-cells, immature and mature erythroid cells, and megakaryocytes. The results are presented as WT versus KO for males and females separately (parts A and B, respectively), and as males versus females for WT and KO separately (parts C and D). Only one significant difference was seen when comparing WT versus KO either specifically in males or in females, namely mature B-cells were lower in male KO animals (p=0.017). However, when comparing male versus females specifically in WT or in KO mice, a few differences were noted. Hence, in WT mice monocytes were significantly lower in females (p=0.024), while CD4+ T-cells, and immature and mature erythroid cells were higher in females (p=0.030, 0.044, and 0.025 respectively). Likewise,

comparing KO mice, females exhibited a highly significant lower level of monocytes (p=0.013).



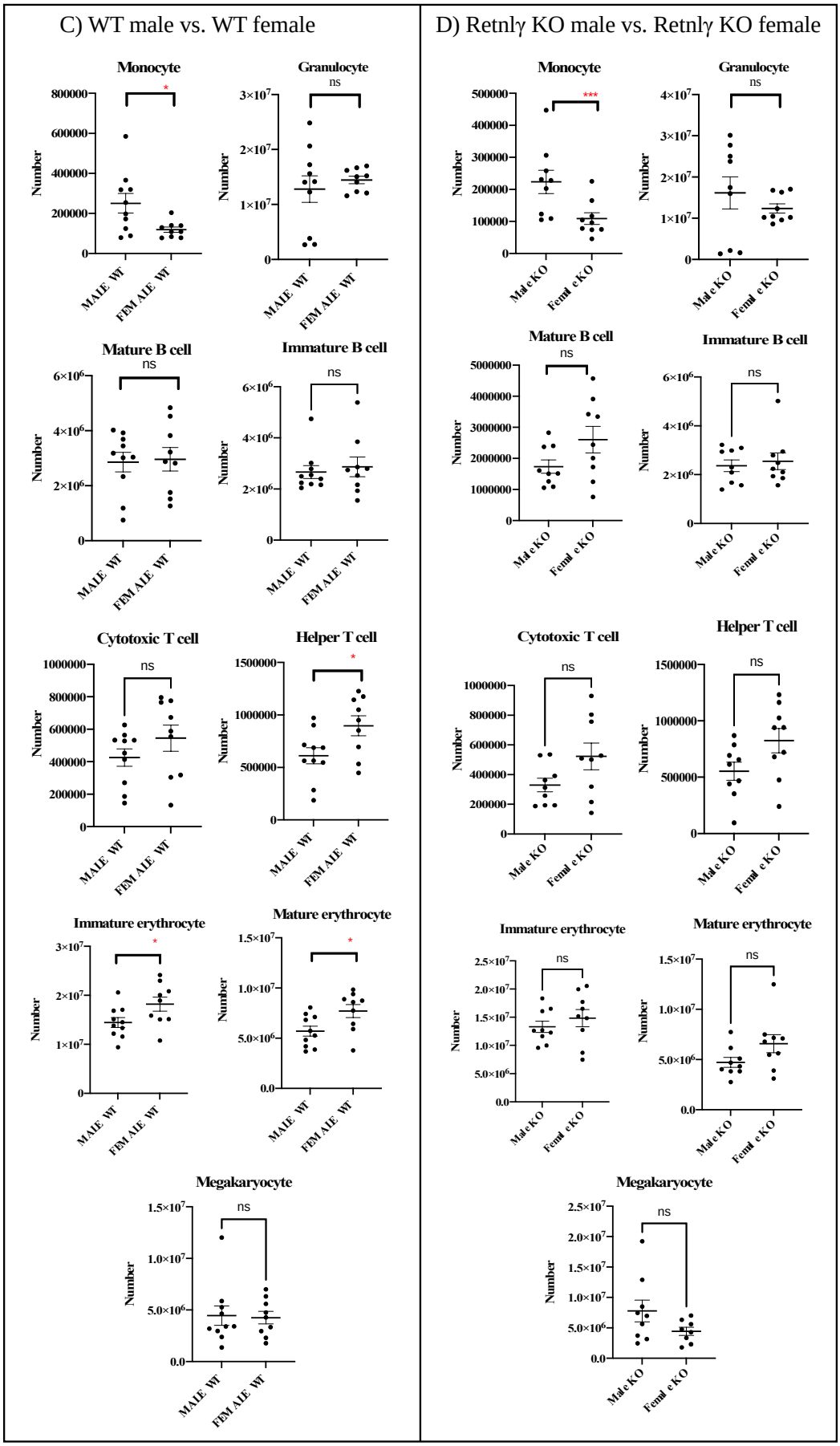

C) WT male vs. WT female
D) Retnlγ KO male vs. Retnlγ KO female
Monocyte
Granulocyte
Mature B cell
Immature B cell
Cytotoxic T cell
Helper T cell
Immature erythrocyte
Mature erythrocyte
Megakaryocyte
Number
MALE WT
FEMALE WT
Male cKO
Femal e cKO
ns
*
***

Figure 4.6 Analysis of BM cell content by immunofluorescence: differentiated cells. Bone marrow was collected and immune-stained as described in Figure 4.5. The absolute cell numbers are represented in the scatter plots. The scatter plots show values for the differentiated cell types indicated. Each panel shows a different comparison between males and females and WT and KO: (A) WT vs KO in males; (B) WT vs KO in females; (C) WT male vs female; and (D) KO male vs female. The mean is indicated by the horizontal lines and the SEM is shown by T-bars. Unpaired t-test was used to detect any significant result (* $p<0.05$, ** $p<0.01$, *** $p<0.005$), ns, not significant. Gating strategies can be found in Appendix 1.

4.5 *In vitro* assay of the differentiation and proliferative capacity of wild type compared to *RETNLγ* knockout haematopoietic stem and progenitor cells

The capacity of haematopoietic stem and progenitor cells to proliferate and differentiate can be assessed through in vitro culture in semi-solid medium (methylcellulose) supplemented with growth factors that are permissive for the various stages of myeloid cell lineage commitment and differentiation. BM cells from male or female 2-month old WT and *RETNLγ* KO mice (as littermate pairs, with three animals per group) were sorted and 300 KSL cells plated in M3434 semi-solid medium supplemented with 25ng/ml thrombopoietin (TPO). Plates were incubated at 37°C for 10 days, during which colony phenotypes were assessed. At the final 10-day count, the total plate cell content was recovered, washed, counted, and immune-stained (as per Table 3.1). The colony number and morphology results are presented in Figure 4.7, the progenitors giving rise to individual colonies being categorised as CFU-GEMM, CFU-GM, CFU-M, CFU-G, and CFU-MK. Immuno-staining of the pooled cells is shown in Figure 4.8. The comparisons between WT and KO are shown separately for males and females, and for each comparison, regardless of sex, no significant differences in colony number, morphology, or cell content were observed.

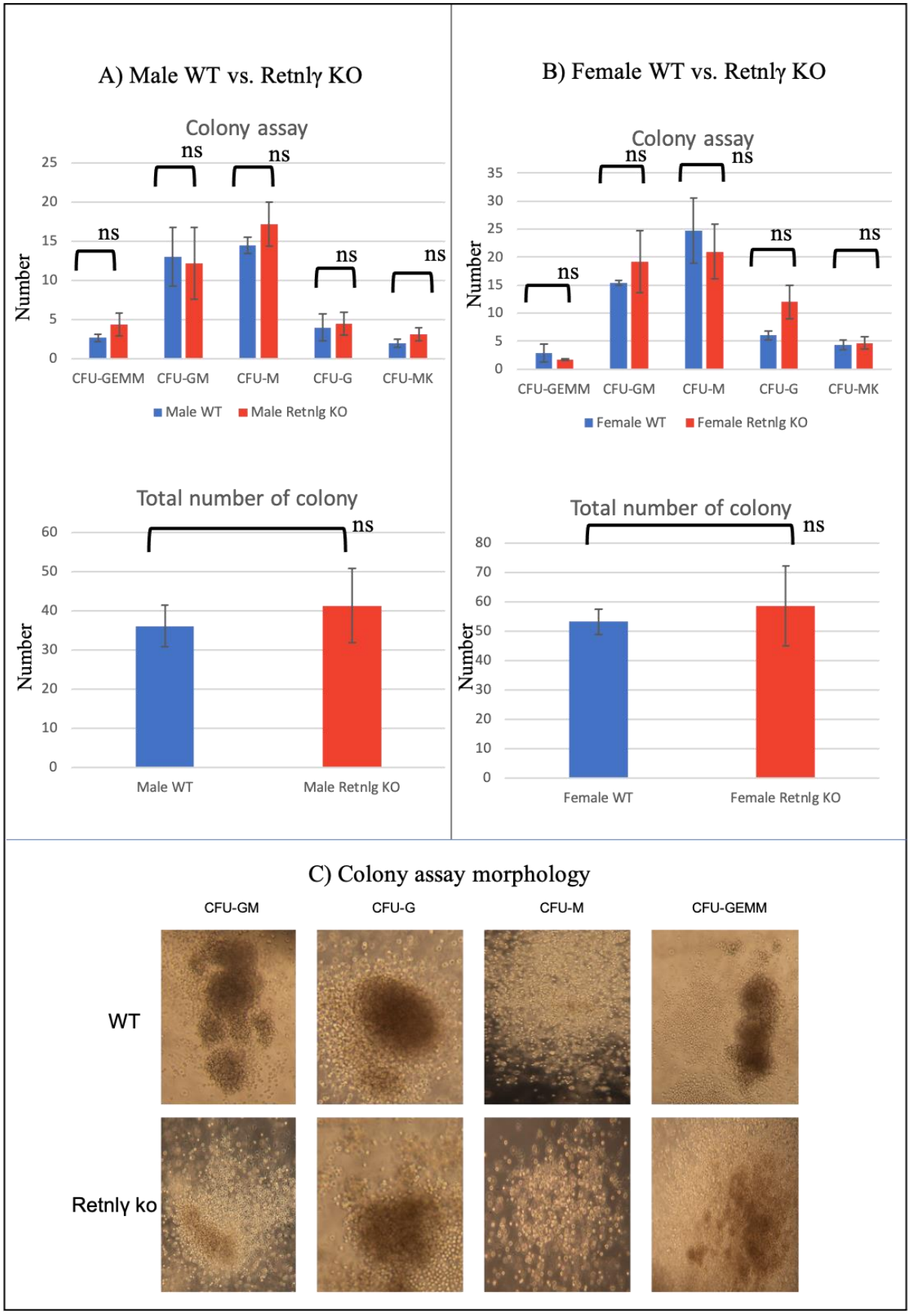
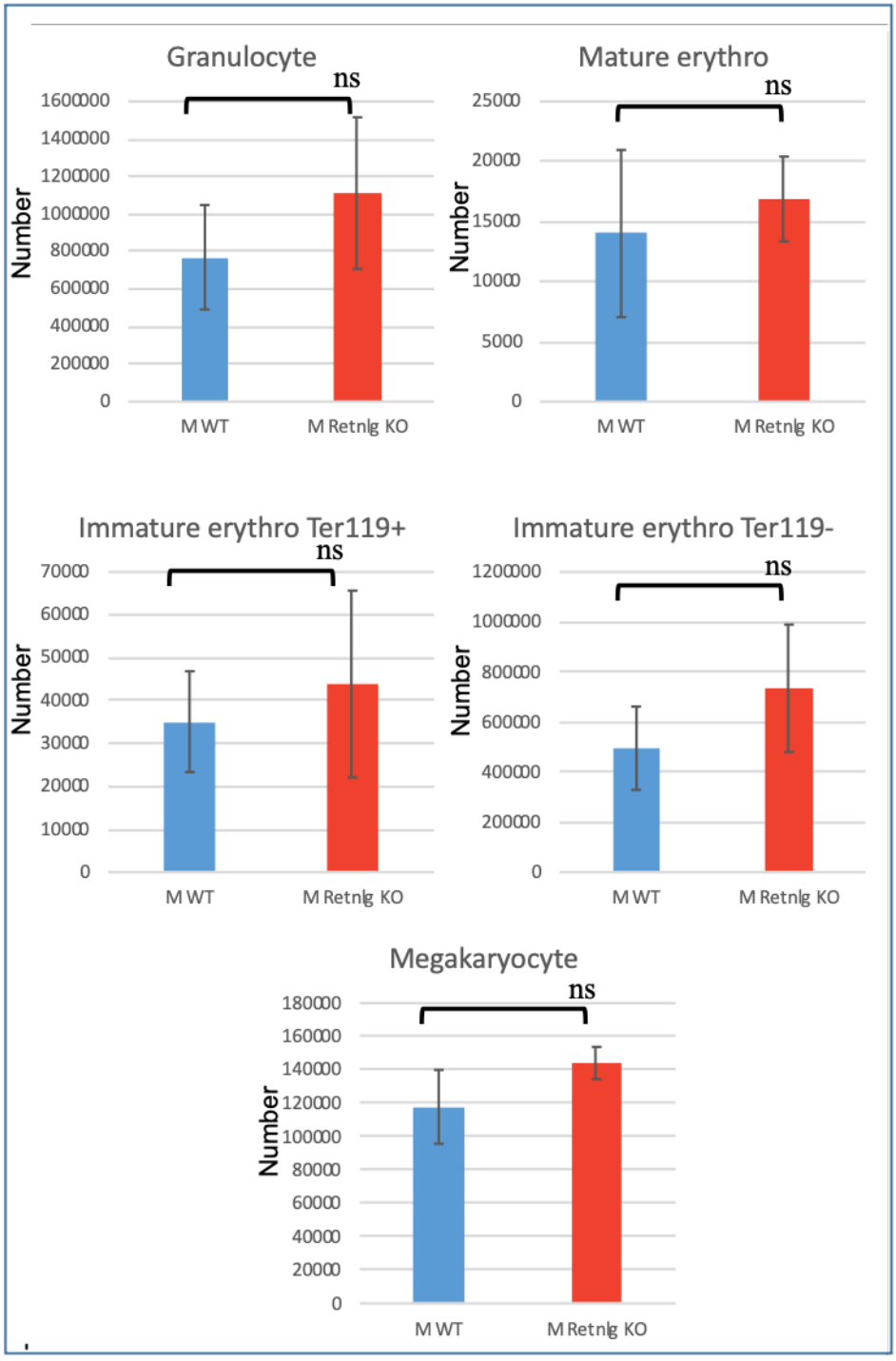

A) Male WT vs. Retnlγ KO
Colony assay
Number
ns
CFU-GEMM
CFU-GM
CFU-M
CFU-G
CFU-MK
Male WT
Male Retnlg KO
B) Female WT vs. Retnlγ KO
Colony assay
Female WT
Female Retnlg KO
Total number of colony
Male WT
Male Retnlg KO
Female WT
Female Retnlg KO
C) Colony assay morphology
CFU-GM
CFU-G
CFU-M
CFU-GEMM
WT
Retnlγ ko

Figure 4.7 *In vitro* colony assays of BM progenitors from WT and *RETNLγ* KO mice. 300 sorted KSL cells were plated in methylcellulose supplemented with growth factors. Colonies were assessed after 10 days incubation at 37°C. The histograms represent the average values from three independent experiments, each of which was performed in duplicate. The top panels show the total colony numbers in each category, while the lower panels show the overall total colony numbers. (A) BM from male mice. (B) BM from female mice. (C) Colony morphology of WT and *RETNLγ* KO (no gender-specific differences were noted). T-bars show the SEM. Unpaired t-test was used to detect any significant result (* $p<0.05$, ** $p<0.01$, *** $p<0.005$), ns, not significant.


Granulocyte
ns
Number
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
M WT
M Retnlg KO
Mature erythro
ns
Number
25000
20000
15000
10000
5000
0
M WT
M Retnlg KO
Immature erythro Ter119+
ns
Number
70000
60000
50000
40000
30000
20000
10000
0
M WT
M Retnlg KO
Immature erythro Ter119-
ns
Number
1200000
1000000
800000
600000
400000
200000
0
M WT
M Retnlg KO
Megakaryocyte
ns
Number
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
M WT
M Retnlg KO

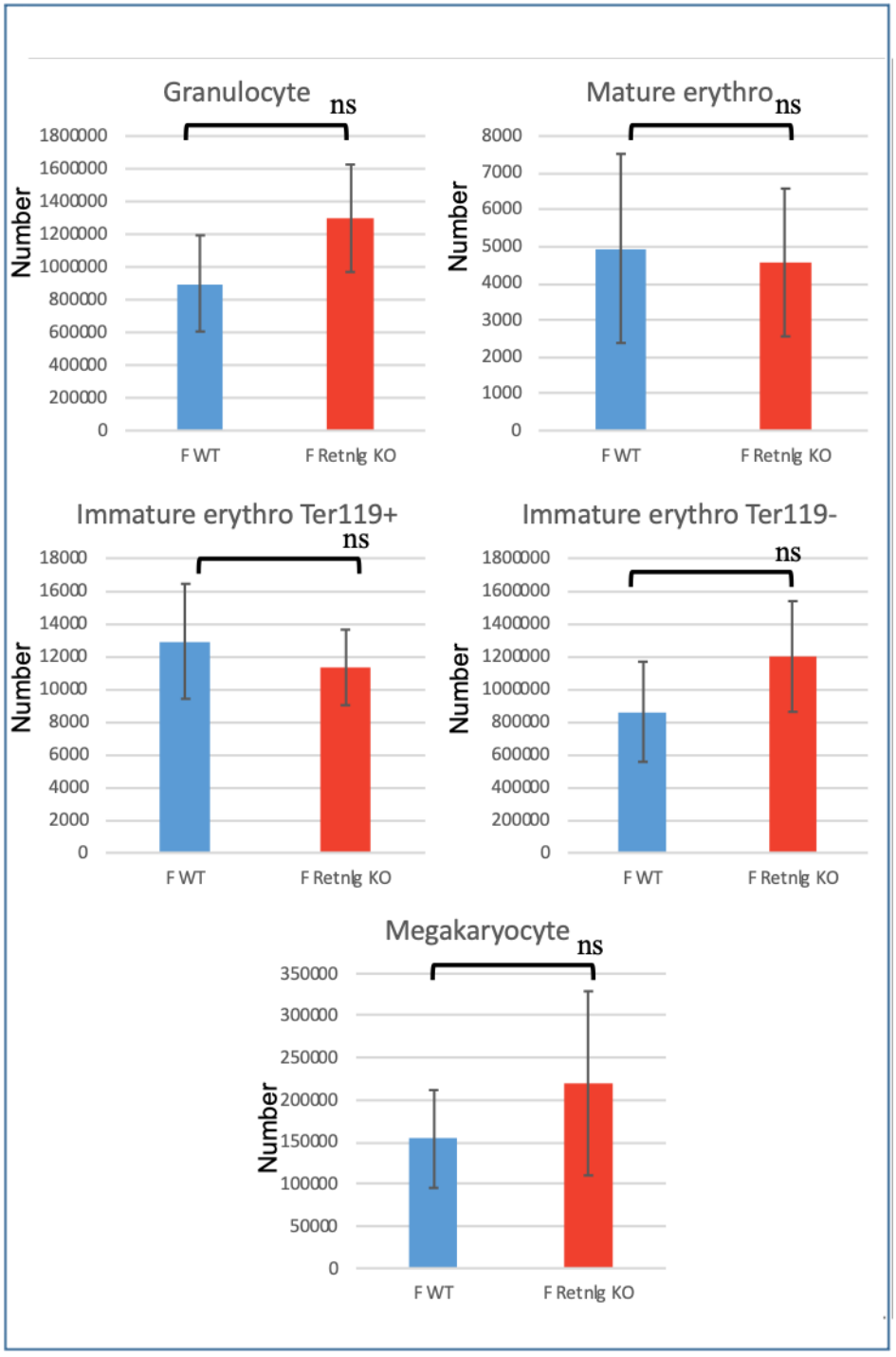


Figure 4.8 *In vitro* colony assays of BM progenitors from WT and *RETNLγ* KO mice: flow cytometric analysis of total colonies. The colonies depicted in Figure 4.7 were recovered from the tissue culture dishes, washed, and immune-stained to discriminate individual mature cell types present. The histograms represent the average values from the three independent experiments performed in duplicate. The top panel shows the values

for males, while the lower panel shows the equivalent results from the female samples. T-bars show the SEM. Unpaired t-test was used to detect any significant result. ns, not significant.

4.6 *In vivo* assay of the differentiation and self-renewal capacity of wild type compared to *RETNLγ* knockout haematopoietic stem cells

Transplantation of haematopoietic stem and progenitor cells into lethally-irradiated recipients is the gold standard for the assessment of stem cell potential, the reconstitution of haematopoiesis achieved being a direct reflection of both the self-renewal and differentiation capacity of the test cells. A number of transplantation assays were undertaken to assess how the loss of RETNLγ affects the fundamental properties of HSC. Given the observations of a sex dependency in the phenotype of BM haematopoietic stem and progenitor cells defined in the previous sections, transplantations were performed specifically to compare the engraftment potential of cells derived from female animals. Female LT-HSC from WT or KO were transplanted into WT male hosts (Figures 4.9, 4.10, and 4.11). In parallel, male LT-HSC from WT or KO mice were transplanted into WT male hosts (Figures 4.12 and 4.13). Finally, although the principal objective was to use transplantation to assess the role played by RETNLγ intrinsically in HSC, one set of experiments was also performed to examine if WT HSC (female, in this case KSL cells) are differently influenced in their engraftment potential by being placed in either a WT or KO host (female) background (Figure 4.15). For each transplantation experiment, the extent of engraftment was determined monthly over a 4-month period by sampling peripheral blood and immuno-staining for the presence of test donor-derived cells (CD45.2) compared to reference donor-derived cells (CD45.1/CD45.2) (Figures 4.9, 4.12, and 4.15). At the end of each experiment, the transplantations host were sacrificed and their BM

and spleen analysed for test and reference donor-derived cells, including both haematopoietic stem and progenitor cells and more mature cell types.

The transplantations involving female WT and KO LT-HSC donor cells utilised two donor littermate pairs, the cells from each donor being transplanted into three hosts. The extent of engraftment showed a trend to being more effective for the KO cells, although the difference between WT and KO engraftment did not reach statistical significance, with p values in the range of 0.09 to 0.32 (Figure 4.9).

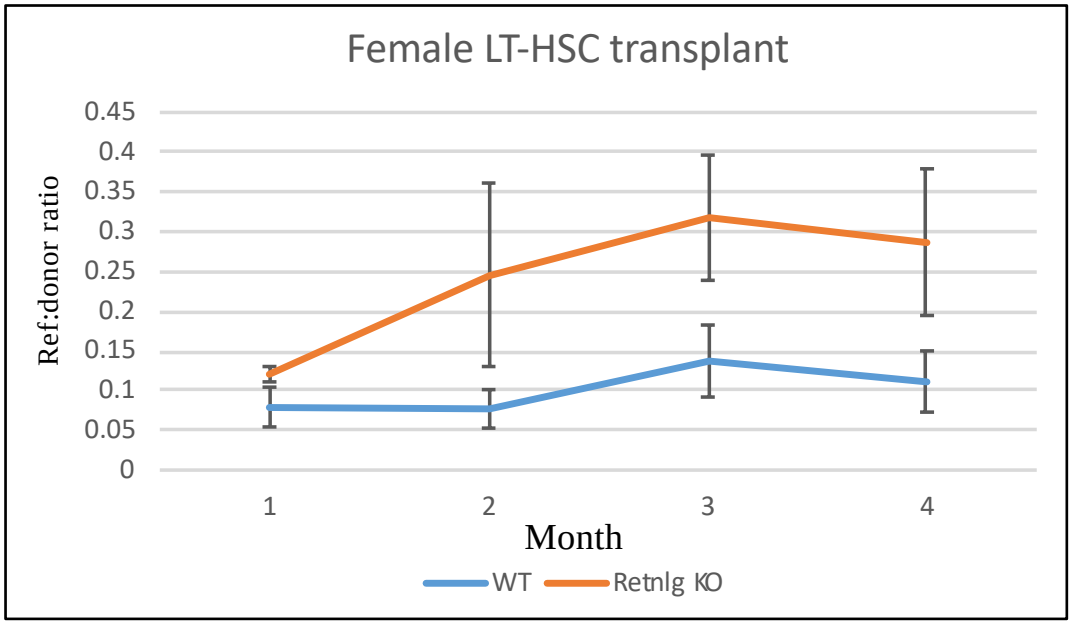


Figure 4.9 Transplantation assay of female LT-HSC. 100 LT-HSC sorted from the BM of 2-month old female WT or *RETNLγ* KO mice were injected into lethally-irradiated Boy/J hosts (3 pairs of recipients). Peripheral blood was sampled every month for 4 months, and analysed by immuno-staining and flow cytometry to determine the relative engraftment of test and reference donor cells. Two sets of WT and KO littermate donor pairs were used to isolate LT-HSC, which was injected into triplicate hosts. Unpaired t-test was used to detect any significant result.

Examination of the BM of the engrafted animals from these experiments 4 months after transplantation revealed a trend towards higher absolute numbers of test donor-derived KO

compared to WT cells, paralleling the peripheral blood values, but these too were generally not statistically significant (Figure 4.10). However, three exceptions were the values for mature B-cells, monocytes, and megakaryocytes (p values of 0.036, 0.056, and 0.029 respectively). Examination of the spleens of the engrafted animals (Figure 4.11) revealed one statistically significant difference between the WT and KO donor-engrafted animals in that immature erythroid cells were relatively lower when the donor cells were KO (p=0.037).

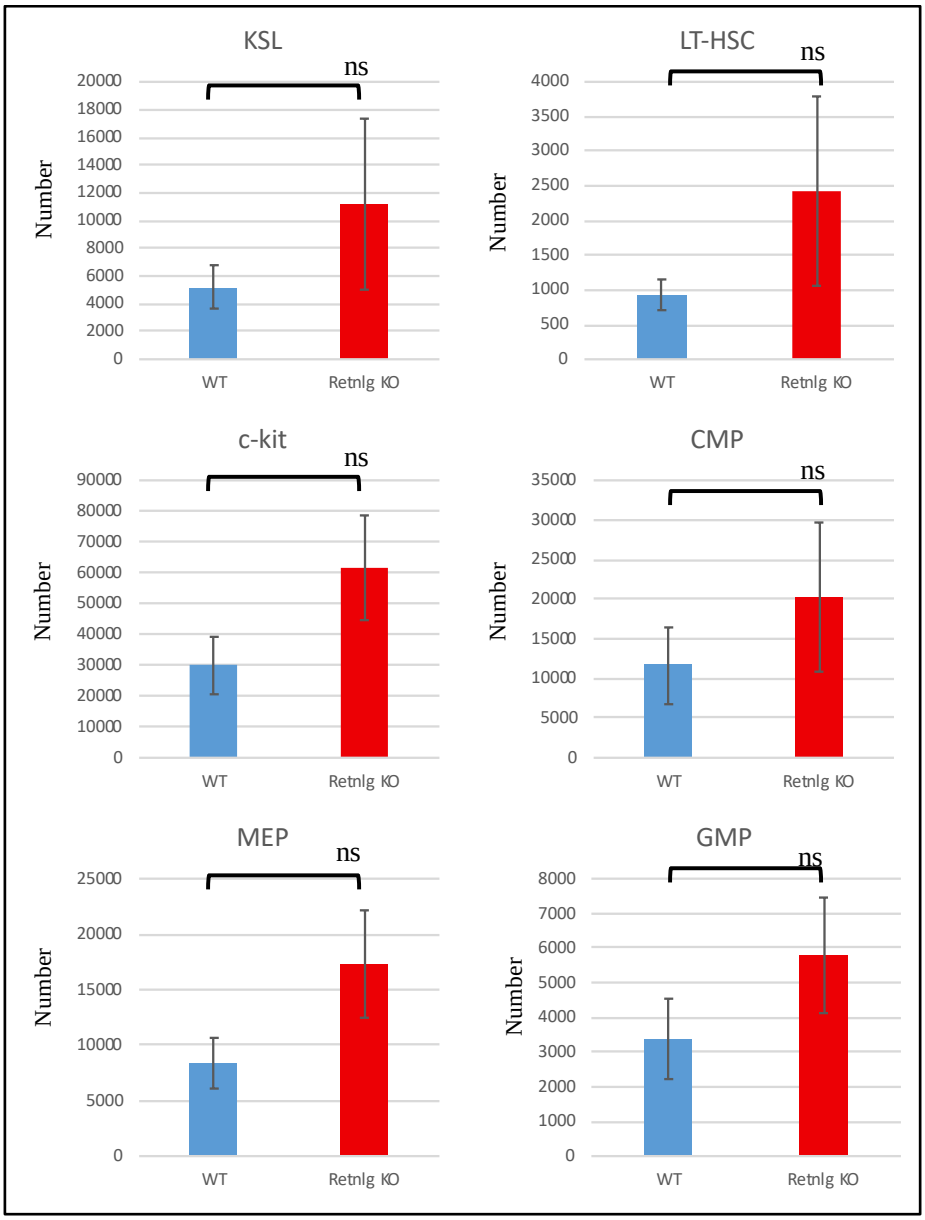
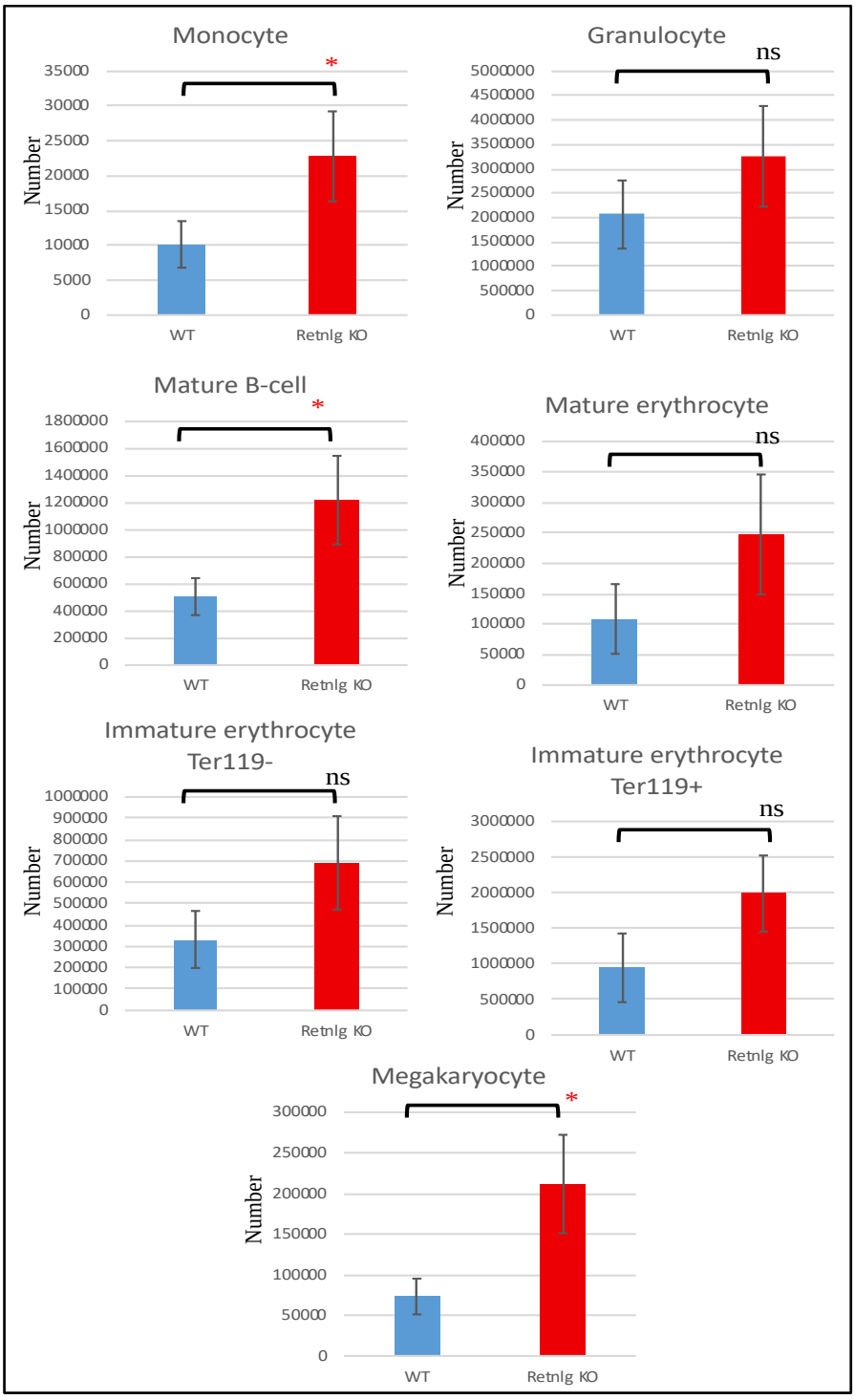


Figure 4.10 Analysis of BM cell content by immunofluorescence. Bone marrow was collected from the femurs of transplanted animals at the end of the experiment described in Figure 4.9. Cells were immuno-stained with antibodies to enable discrimination of distinct stem and progenitor population by flow cytometry (Table 3.1). The average absolute cell numbers, represented in the histograms, are shown in the panel above for long-term HSC (LT-HSC), KSL cells, Kit+ progenitors, common myeloid progenitors (CMP), granulocyte myeloid progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP). Unpaired T-test was performed to calculate statistical differences. Gating strategies can be found in Appendix 1.



The average absolute cell numbers, represented in the histograms, are shown in the panel above for the indicated mature cell types. The SEM is shown by T-bars. Unpaired t-test was used to detect any significant result (* $p<0.05$, ** $p<0.01$, *** $p<0.005$), ns, not significant. Gating strategies can be found in Appendix 1.

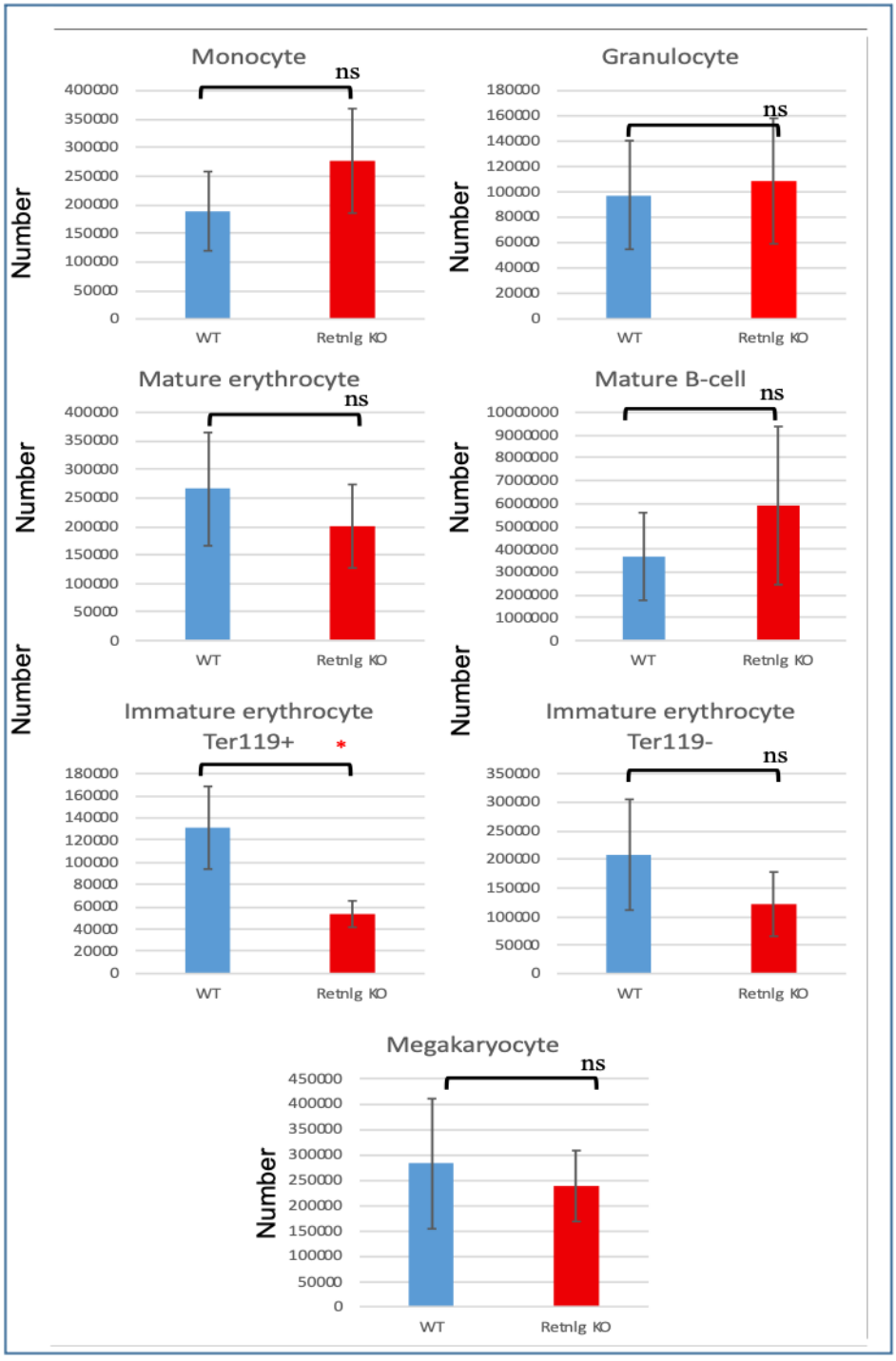


Figure 4.11 Analysis of spleen cell content by immunofluorescence. Spleen was collected from transplanted animals at the end of the experiment described in Figure 4.9. Cells were immuno-stained with antibodies to enable discrimination of mature haematopoietic cell types. The average absolute cell numbers are shown. The SEM is shown by T-bars. Unpaired t-test was used to detect any significant result (* $p<0.05$, ** $p<0.01$, *** $p<0.005$), ns, not significant. Gating strategies can be found in Appendix 1.

The transplantations involving male WT and KO LT-HSC donor cells utilised one donor littermate pair, the cells from each donor being transplanted into three hosts. The extent of engraftment again showed a trend to being more effective for the KO cells, although as for the previous experiment, the difference between WT and KO engraftment did not reach statistical significance, with p values in the range of 0.15 to 0.21 (Figure 4.12).

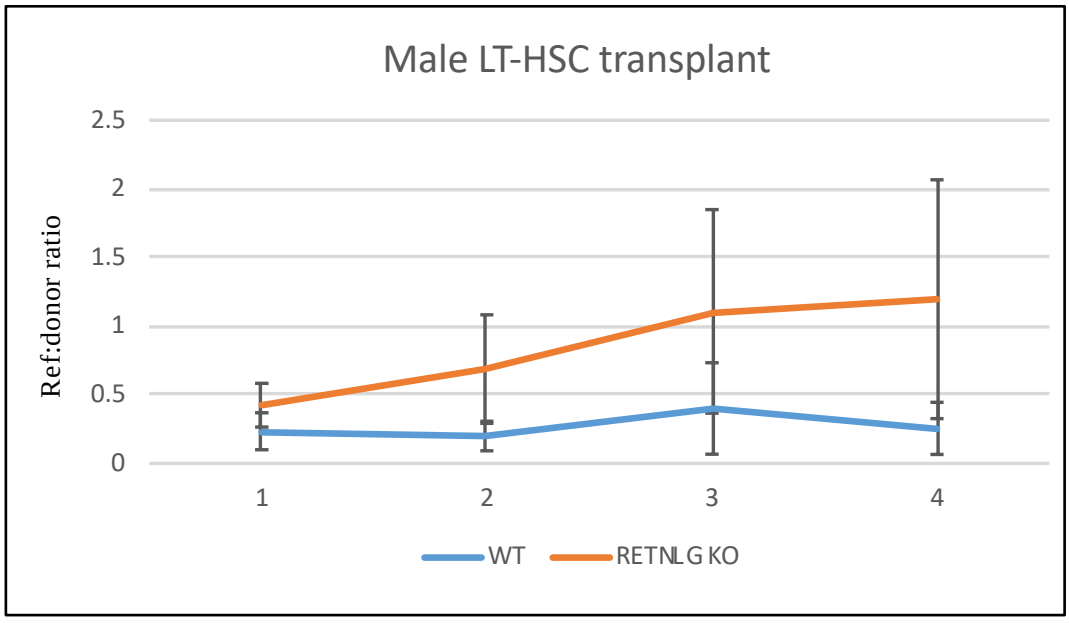
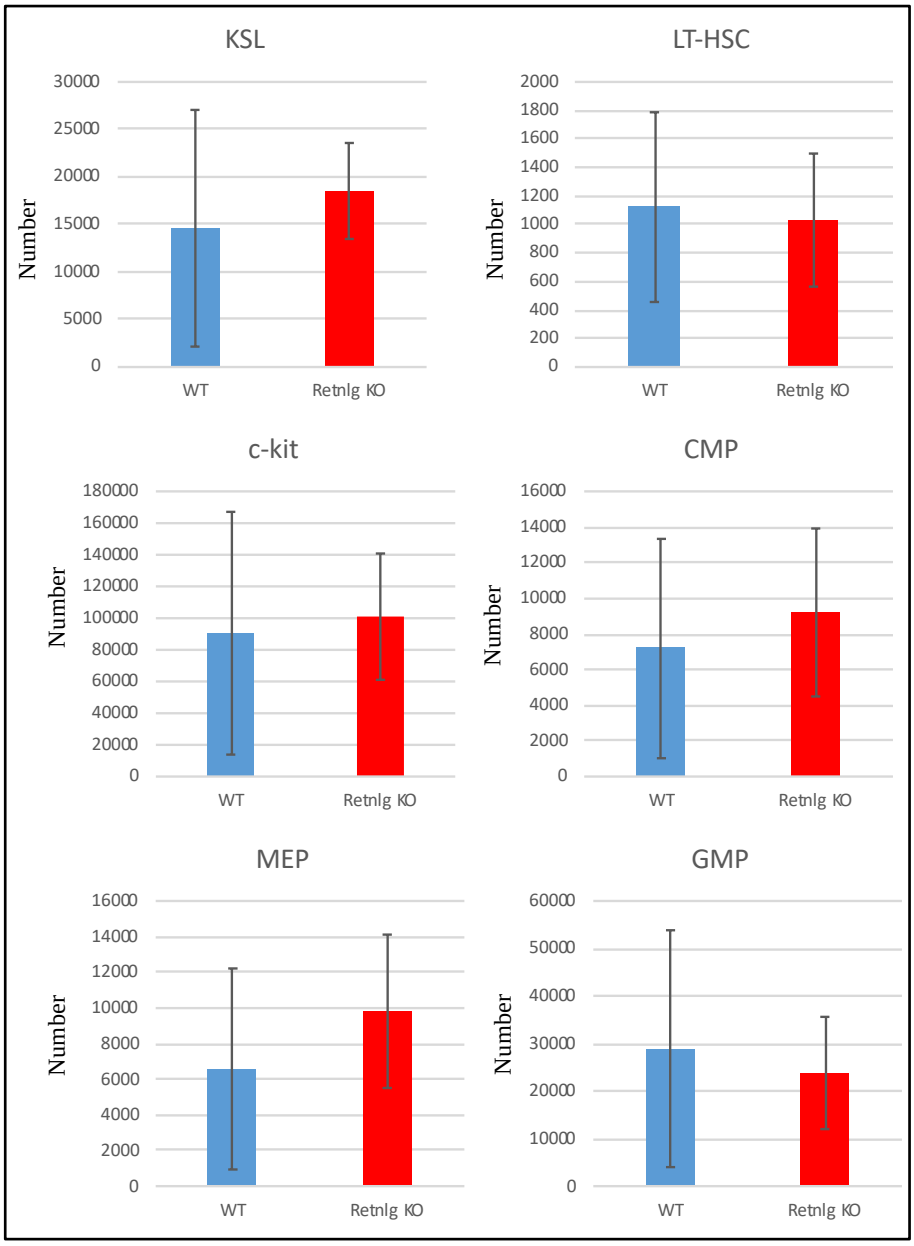


Figure 4.12 Transplantation assay of male LT-HSC. 100 LT-HSC sorted from the BM of 2-month old male WT or *RETNLγ* KO mice were injected into lethally-irradiated Boy/J hosts. Peripheral blood was sampled every month for 4 months and analysed by immuno-staining and flow cytometry to determine the relative engraftment of test and reference donor cells. One set of WT and KO littermate donor pairs was used to isolate LT-HSC, which was injected into triplicate hosts. Unpaired t-test was used to detect any significant result.

Examination of the BM of the engrafted animals from these experiments 4 months after transplantation revealed no significant trend in pattern of WT versus KO test donor-derived cells, although one significant difference was seen in immature B-cells, which were higher in KO donor transplanted animals (p=0.025) (Figure 4.13). Examination of the spleens of the engrafted animals (Figure 4.14) revealed a number of statistically significant difference

between the WT and KO donor-engrafted animals, the relative level of donor-derived mature B-cells, monocytes, and megakaryocytes all being higher in those recipients transplanted with KO cells (p=0.042, 0.014, and 0.049 respectively).



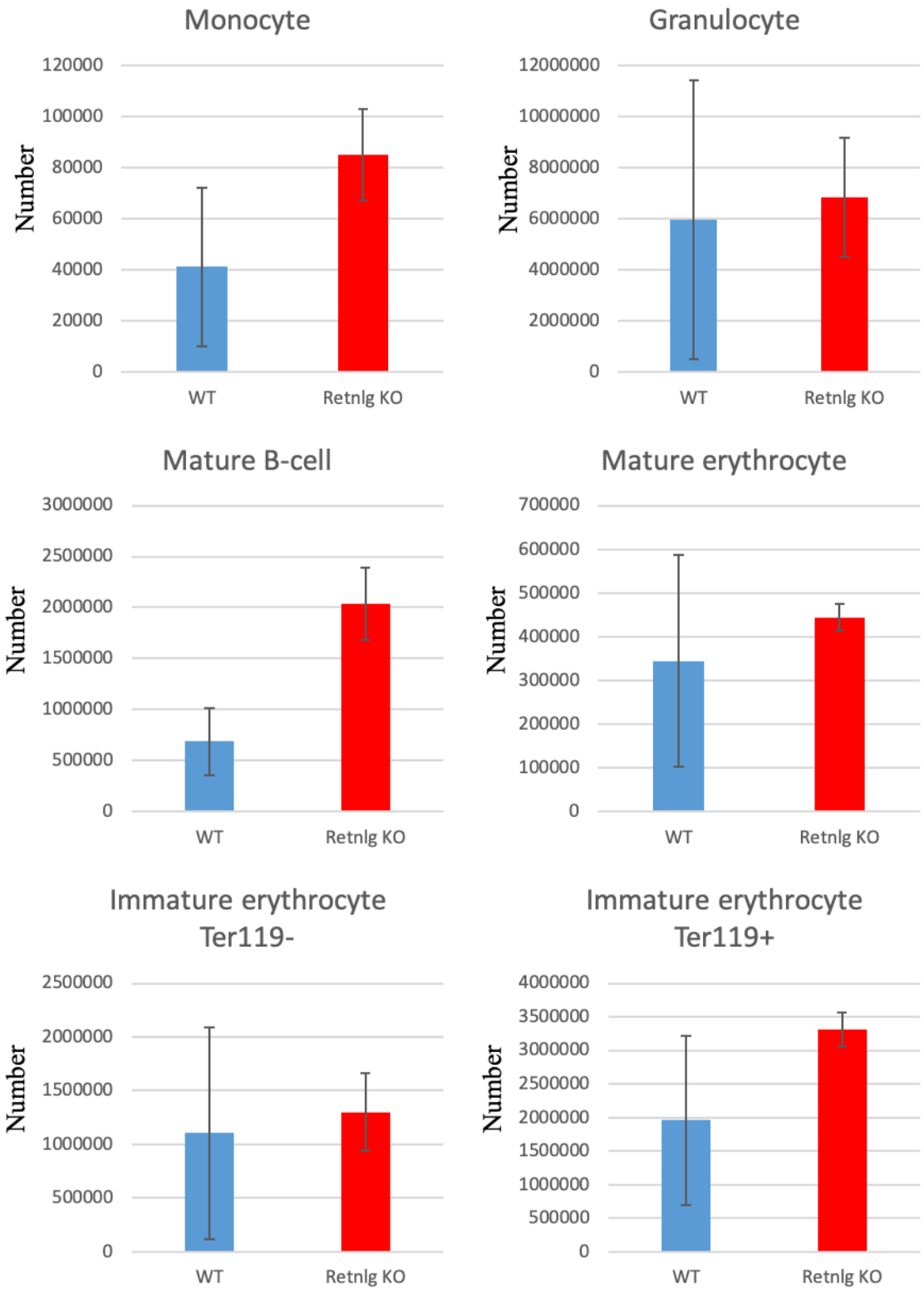


Figure 4.13 Analysis of BM cell content by immunofluorescence. Bone marrow was collected from the femurs of transplanted animals at the end of the experiment described in Figure 4.12. Cells were immuno-stained with

antibodies to enable discrimination of distinct stem and progenitor populations (Table 3.1) and mature cells by flow cytometry. The average absolute cell numbers, represented in the histograms, are shown in the upper panel for long-term HSC (LT-HSC), KSL cells, Kit+ progenitors, common myeloid progenitors (CMP), granulocyte myeloid progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP), and in the lower panel for mature cell types. The SEM is shown by T-bars. Unpaired t-test was used to detect any significant result (* $p<0.05$, ** $p<0.01$, *** $p<0.005$), ns, not significant. Gating strategies can be found in Appendix 1.

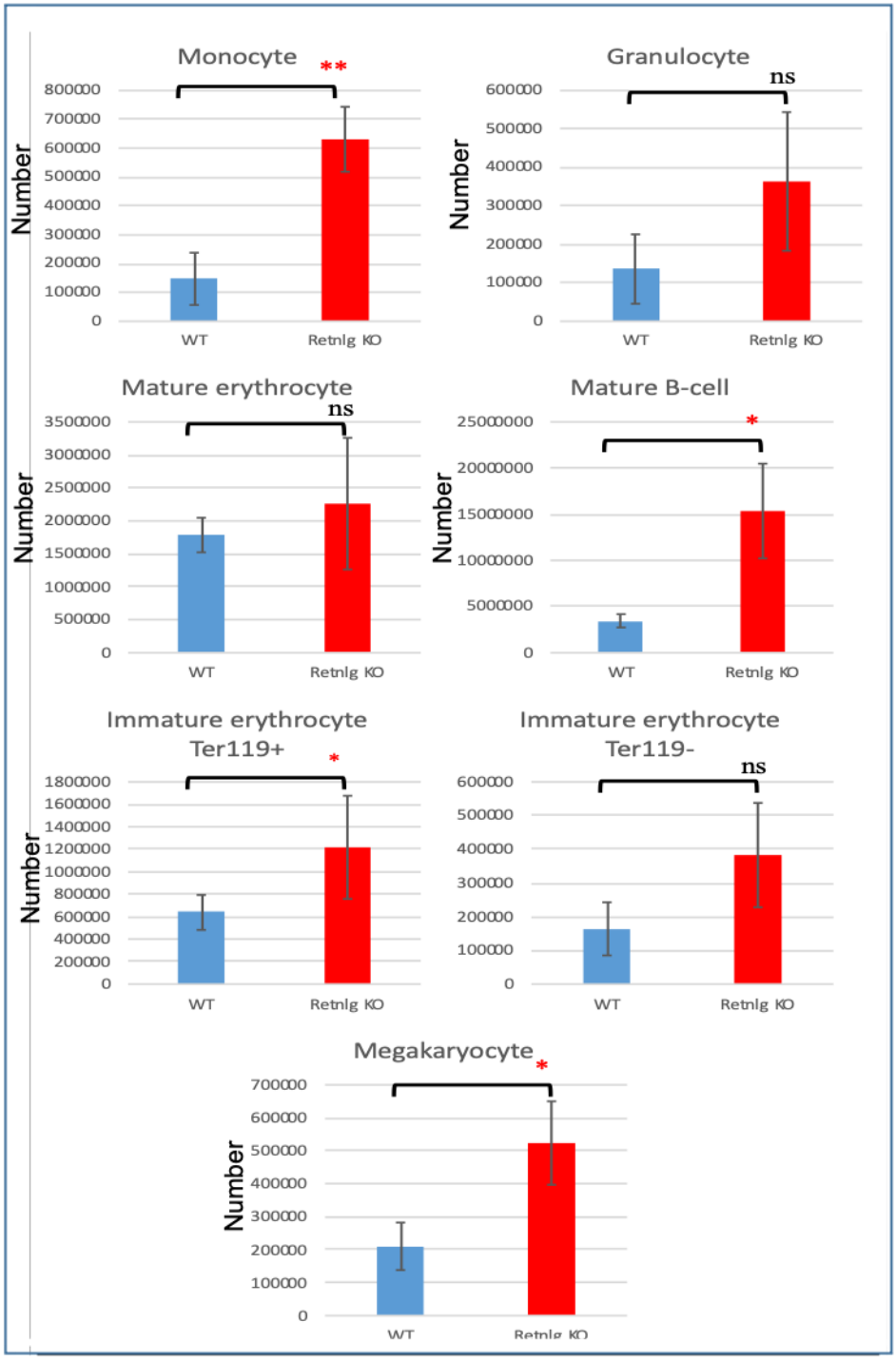


Figure 4.14 Analysis of spleen cell content by immunofluorescence. Spleen was collected from transplanted animals at the end of the experiment described in Figure 4.12. Cells were immuno-stained with antibodies to enable discrimination of mature haematopoietic cell types. The average absolute cell numbers are shown. The SEM is shown by T-bars. Unpaired t-test was used to detect any significant result (* $p<0.05$, ** $p<0.01$, *** $p<0.005$), ns, not significant. Gating strategies can be found in Appendix 1.

Finally, the reciprocal transplantations of WT female HSC into either WT or *RETNLγ* KO hosts showed no significant or trending differences in the degree of engraftment (Figure 4.15).

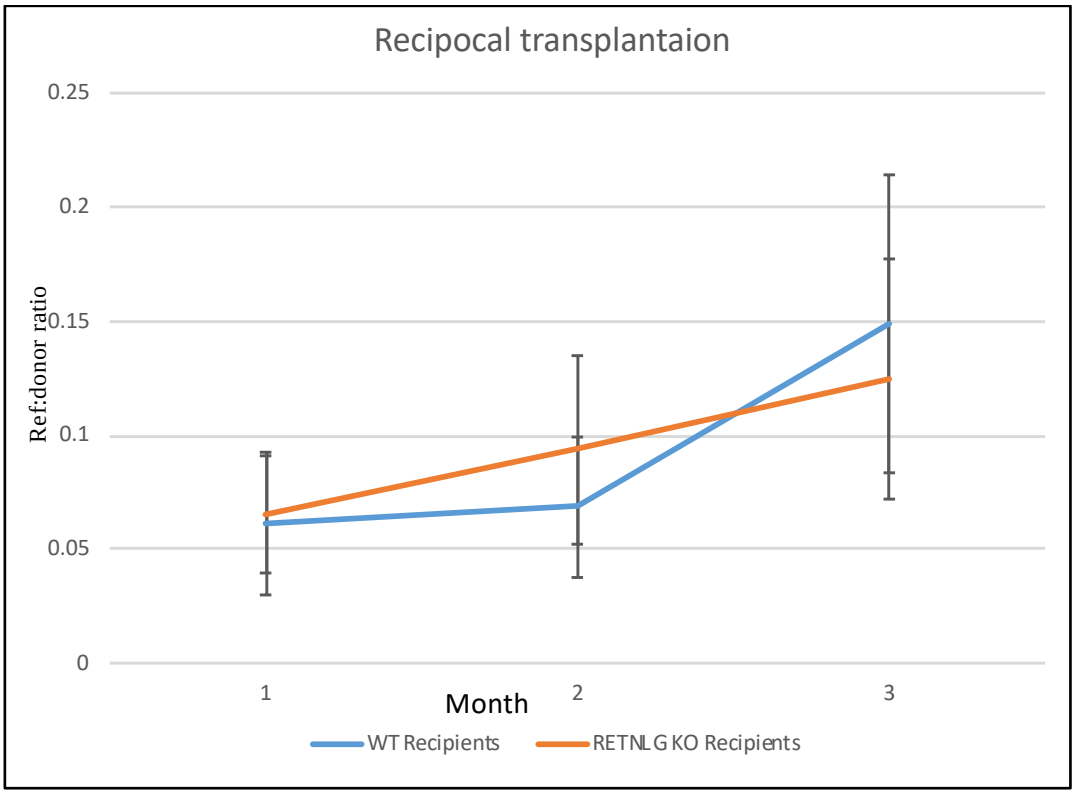


Figure 4.15 Transplantation assay of WT female HSC into WT or KO female hosts. 1000 KSL HSC sorted from the BM of a 2-month old female WT mouse were injected into 3 lethally-irradiated female WT or KO Boy/J hosts. Peripheral blood was sampled every month for 3 months, and analysed by immuno-staining and flow cytometry to determine the relative engraftment of test and reference donor cells. Unpaired t-test was used to detect any significant result.

4.7 Summary

The experiments described in this chapter have shed light on the differences in the haematopoietic profile and HSC characteristics resulting from the constitutive absence of RETNLγ in the KO mouse line. Although many parameters seem little different between WT and KO, a strikingly sex-specific effect was seen in female KO BM in which the numbers of stem cells and committed progenitors were found to be significantly reduced compared to both WT females and KO males. In terms of isolated stem cell functional assays, the *in vitro* assays were unable to demonstrate any differences between WT and KO. *In vivo* assay of HSC by transplantation showed some weak evidence for enhanced engraftment by both male and female KO HSC, but these findings were not statistically significant. The female-specific effect of *RETNLγ* KO on HSC is explored further in Chapter 5 using transcriptome analysis as a way to provide overall phenotypic information about differences between WT and KO cells and the extent to which any differences are gender-specific.

CHAPTER 5 MECHANISM OF ACTION OF RETNLγ IN HAEMATOPOIETIC STEM CELLS

5.1 Introduction

The results and observations described in Chapter 3 and 4 indicate that the expression of RETNLγ in HSC has a phenotypic consequence, at least in females, which is most apparent in the reduced numbers of stem and progenitor cells present in adult bone marrow. *In vitro* and *in vivo* assay of WT versus *RETNLγ* KO HSC revealed only weak evidence of a functional impact to the absence of RETNLγ although, at least in the case of the *in vivo* assays, it can be argued that RETNLγ is present in the environment into which the KO cells were transplanted.

In order to gain further insight into the role played by RETNLγ in HSC, the experiments in this chapter sought to identify transcriptomic differences between WT and KO HSC. With this knowledge of the differences in RNA expression resulting from the absence of RETNLγ, the question was addressed as to whether restoration of RETNLγ, either by addition to cultured KO HSC or by lentiviral over expression in KO HSC, could revert gene expression back to a WT profile.

5.2 Transcriptome analysis

In order to assess the extent to which the absence of RETNLγ in HSC might affect the profile of RNA expression, RNA-seq was performed on both WT and KO KSL HSC (see Appendix 4 for validation of the individual mouse genotypes). Given that the significant differences in

haematopoietic stem and progenitor cell numbers were seen in females, HSC were sorted from 3-month old female bone marrow, using 3 littermate pairs of WT and KO animals. Sequencing reads were mapped to the mouse genome, counted, normalised, and differential expression determined.

5.2.1 Significant gene expression differences between WT and KO KSL HSC

A total of 49 genes exhibited a significant difference in expression using an adjusted p value threshold of $p < 0.05$ (Table 5.1).

Table 5.1 Gene ontogeny categorisation of gene expression differences between WT and *RETNLγ* KO HSC: Cellular processes

Gene Name	Adjusted p value	WT mean counts	Retnlγ KO mean counts
LY6C2	9.1e-07	915	2159.1
LTF	3.98e-06	5686.8	12033.9
ELANE	1.13e-05	2838.8	7723.8
NGP	4.61e-05	20088.9	47785.8
SLC4A1	0.000116	28.2	206.1
CHIL3	0.000123	3556.7	9188.4
CLEC4A2	0.000123	121.8	369.9
FCNB	0.000188	615.9	1584.2
TNFSF13B	0.000266	21.1	102.2
PRG3	0.000661	3.7	111.5
MMP9	0.000701	435.2	923
WFDC21	0.00131	550.1	2165
MS4A3	0.00142	399	977.6
LYZ2	0.00145	9458.5	19189.8
MMP8	0.00148	1254	2438.6
CAMP	0.00154	9660.1	34466.8
S100A8	0.00157	27588.3	95485.6
IGKV8-21	0.0016	0.3	1432
LCN2	0.00177	1065.2	3684
CEBPE	0.00179	46.3	207.2
LY6A2	0.00179	285.3	916.2
S100A9	0.00179	9863.5	32957.9
PROM1	0.00326	44.1	152.5
NCAM1	0.00604	85.5	205.2
HP	0.00604	959.7	2062.5

C3	0.00655	124.5	342.4
HBA-A1	0.00901	1571.5	4804.7
CYBB	0.0125	1261.7	2458.1
TMEM30A	0.0125	641.7	1341.3
HBB-BS	0.0134	3957.9	10512.4
TREM3	0.014	296.5	688.7
PRG2	0.0154	59.9	2191.4
PGLYRP1	0.0186	930.5	2274.5
LY6G	0.0193	80.9	237.8
FGR	0.0227	196	410.3
CAMK1	0.023	132.6	331.6
FER	0.0267	137.4	46.9
REDRUM	0.0278	1.6	26
6720464F23RIK	0.0318	10.2	82.5
FCGR3	0.0327	527.8	961.2
DGAT2	0.0357	42.2	121.2
HBB-BT	0.0417	894.5	2491.4
HBA-A2	0.0418	1022.6	2602.4
CHIL1	0.0455	408.6	724.8
TYROBP	0.046	1314.6	2527.1
ASPRV1	0.0467	24.3	90.9
PI16	0.0494	102.5	263.6
ACPP	0.05	21.1	81.2
CLDN15	0.05	81.7	205.6

The table shows the top 49 genes that exhibit a significant difference in expression between WT and *RETNLγ* KO HSC. Genes were selected with an adjusted p value of less than 0.05.

Unsupervised clustering of the sequence results for the triplicate WT and KO samples was performed (Figure 5.1), and the top 50 statistically significant gene expression differences are represented in the heatmap presented in Figure 5.1.

Two immediate conclusions can be drawn from the significant genes listed in Table 5.1. First, there are many significant differences between WT and *RETNLγ* KO HSC. Second, the vast majority of the differences are genes that are expressed more highly in the KO.

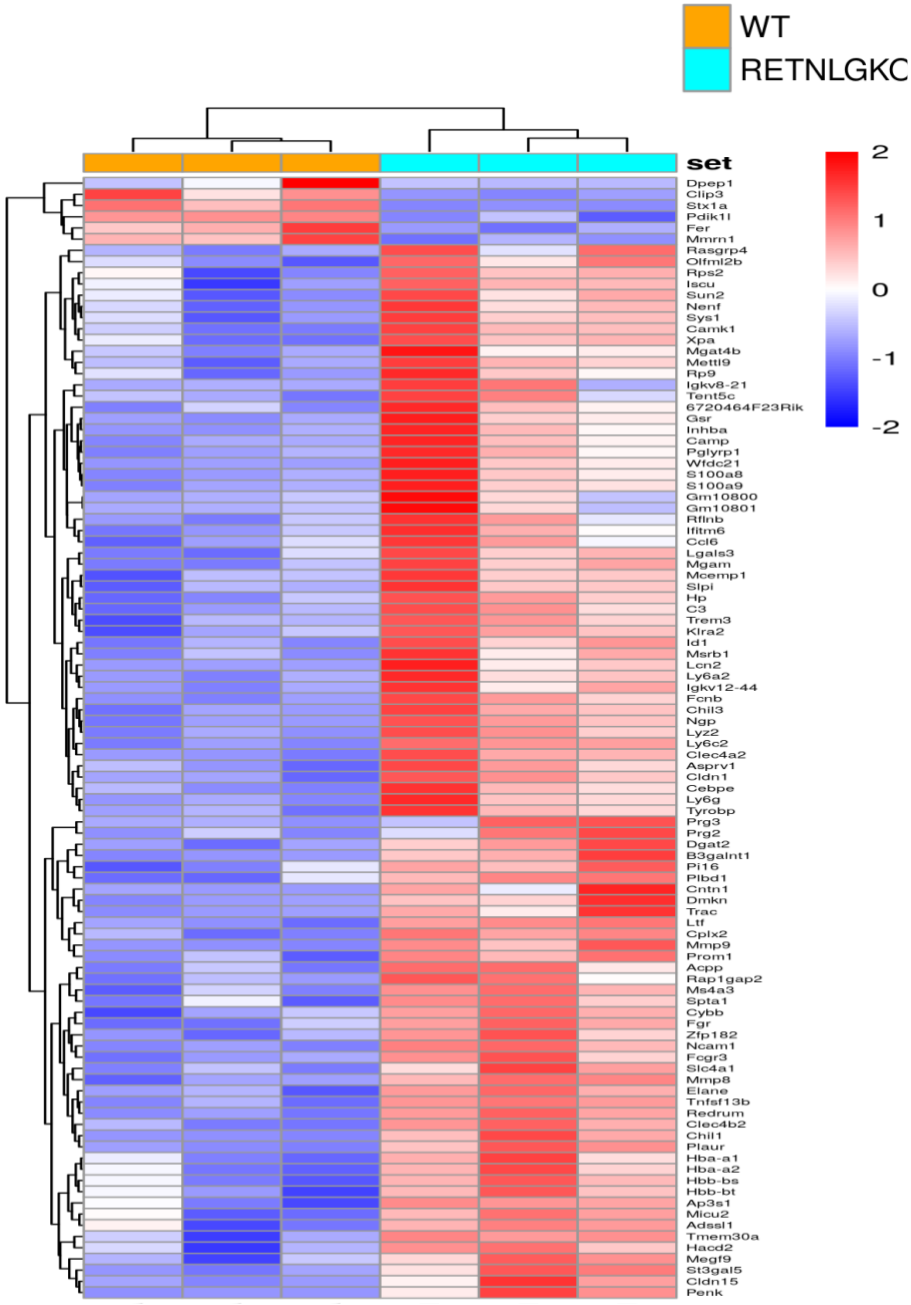


Figure 5.1 Gene expression differences comparing WT and *RETNLγ* KO KSL HSC from adult female mice. BM from 3 female WT mice and 3 *RETNLγ* KO mice was stained and KSL sorted using the surface antigen characteristics defined in Table 3.1. Trizol was used to extract the RNA. RNA-seq was performed using the NEBNext® Single Low Input RNA Library Prep and sequencing.

Next, a selection of genes exhibiting significant differences in expression between WT and KO were selected for qRT-PCR analysis on independent RNA samples as a means to validate the observations derived from the RNA-seq data. Although the RNA-seq had been performed on the heterogenous KSL HSC population, it was decided to conduct the validation experiments on the more immature LT-HSC subpopulation because these cells were both most affected by the absence of RETNLγ and were those tested in the *in vivo* stem cell assays. The analysis was performed on both male and female LT-HSC samples to determine if the female-specific effects of the absence of RETNLγ in respect to stem cell and progenitor numbers is similarly restricted in terms of gene expression differences between WT and KO.

Genes for validation were selected on the combined basis of the degree of their differential expression and their level of expression as represented by the RNA-seq read counts, aiming for genes that are expressed at levels sufficiently high so that even with small numbers of sorted cells (of the order of 300 LT-HSC). The genes chosen were *HBA* and *HBB* (haemoglobin alpha and beta), *CAMP* (cathelicidin antimicrobial peptide), and *S100A8* (S100 Calcium Binding Protein A8). The results, shown in Figure 5.2 and Appendix 5, do not achieve statistical significance (2 male and female independent LT-HSC samples were tested), but the trends are consistent with the RNA-seq data, particularly for the samples derived from female LT-HSC, for which each of the four genes tested exhibited higher relative levels in the *RETNLγ* KO. Overall, differences between WT and KO in the male LT-HSC samples were less than seen in the case of the female samples.

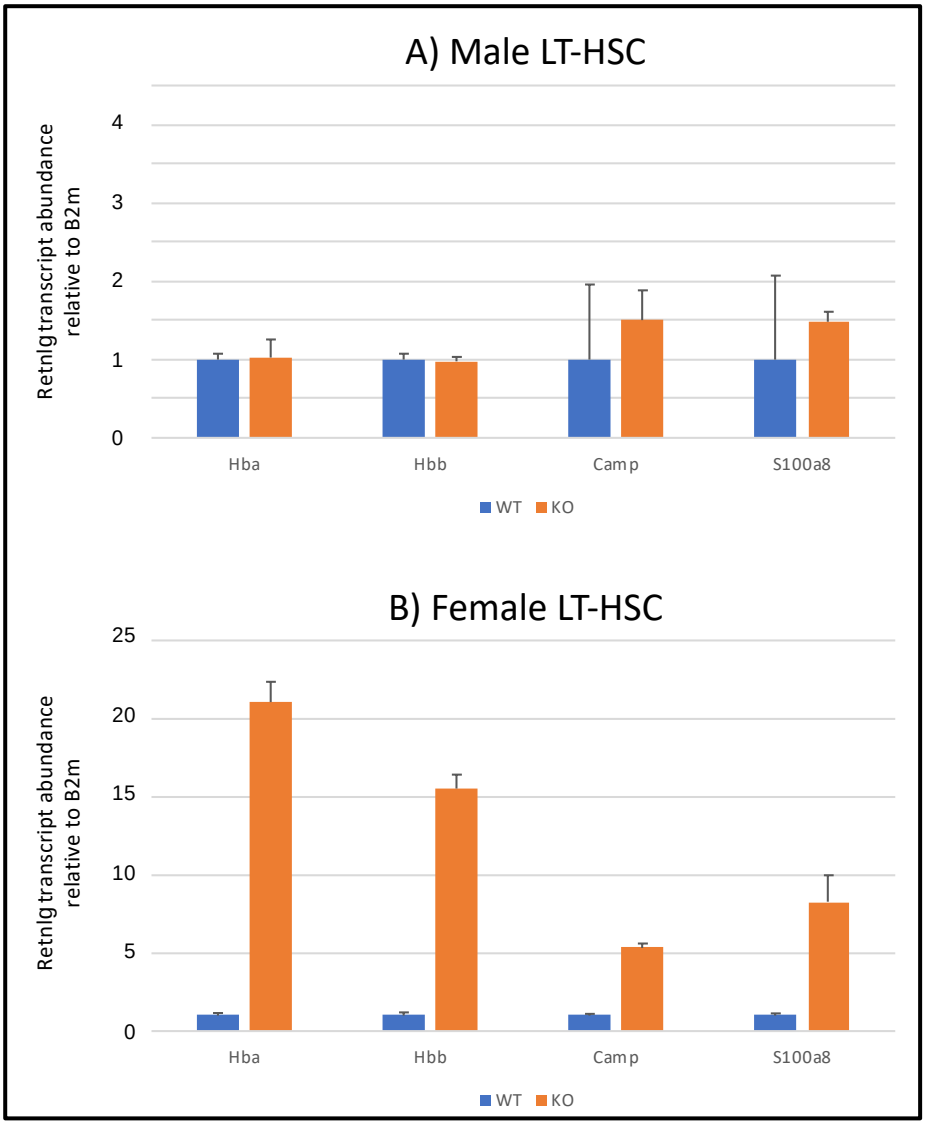


Figure 5.2 Validation of RNA-seq results. BM from WT and *RETNLγ* KO mice was stained and LT-HSC was sorted using the surface antigen characteristics defined in Table 3.1. qRT-PCR was performed using Taqman primers for *HBA*, *HBB*, *CAMP*, and *S100A8* genes. The histograms represent the relative expression of the mentioned genes compared to *β2* microglobulin ($2^{-\Delta\Delta Ct}$). (A) The result of LT-HSC from 2 WT and 2 *RETNLγ* KO male mice. (B) The result of LT-HSC from 3 WT and 3 *RETNLγ* KO female mice.

5.2.2 Gene ontology analysis shows that the gene expression differences related to RETNLγ in HSC are restricted to specific cell and molecular processes

Further examination of the RNA expression differences between the WT and KO HSC was performed by conducting a gene ontology (GO) analysis on the 49 genes that exhibited a statistically significant difference in Table 5.1. Using the GO group "biological processes" the 49 significant genes were put into functional categories, the most significant of which in terms of enrichment are illustrated in Table 5.2.

Table 5.2 Gene ontogeny categorisation of gene expression differences between WT and KO HSC: Cellular processes

Enrichment FDR	Genes in list	Total genes	Functional Category	Genes
2.28E-11	22	1439	Defence response	CYBB ELANE PGLYRP1 HP CAMP LYZ2 TREM3 C3 LCN2 FCNB PRG2 FGR CLEC4A2 NGP LTF CHIL3 S100A8 S100A9 CHIL1 MMP8 TYROBP CEBPE
1.47E-10	16	693	Response to bacterium	PGLYRP1 CAMP LYZ2 WFDC21 ELANE LCN2 PRG2 HP LTF CEBPE C3 HBA-A2 HBA-A1 FGR TREM3 FER
1.47E-10	25	2299	Immune system process	FER ELANE PGLYRP1 CAMP C3 FGR TYROBP TNFSF13B TREM3 CYBB MMP9 LCN2 FCNB PRG3 PRG2 CLEC4A2 HP LTF CEBPE S100A8 S100A9 HBA-A2 HBA-A1 MMP8 SLC4A1
1.28E-09	15	702	Cytokine production	PGLYRP1 FGR CHIL1 CYBB ELANE C3 FCNB PRG3 LTF TREM3 MMP8 PRG2 TYROBP CEBPE CLEC4A2
1.28E-09	11	273	Defence response to bacterium	PGLYRP1 CAMP LYZ2 ELANE LCN2 PRG2 HP LTF FGR TREM3 CEBPE
1.28E-09	17	987	Response to external biotic stimulus	PGLYRP1 CAMP LYZ2 LCN2 WFDC21 ELANE PRG2 HP LTF CEBPE C3 HBA-A2 HBA-A1 FGR TREM3 FER S100A9
1.28E-09	17	984	Response to other organism	PGLYRP1 CAMP LYZ2 LCN2 WFDC21 ELANE PRG2 HP LTF CEBPE C3 HBA-A2 HBA-A1 FGR TREM3 FER S100A9
1.57E-09	17	1020	Response to biotic stimulus	PGLYRP1 CAMP LYZ2 LCN2 WFDC21 ELANE PRG2 HP LTF CEBPE C3 HBA-A2 HBA-A1 FGR TREM3 FER S100A9
1.43E-08	18	1367	Immune response	FER ELANE PGLYRP1 CAMP C3 FGR TREM3 CYBB LCN2 FCNB PRG3 PRG2 CLEC4A2 TNFSF13B LTF S100A8 S100A9 TYROBP
4.94E-08	22	2378	Response to external stimulus	FER PGLYRP1 CAMP LYZ2 LCN2 WFDC21 CYBB ELANE PRG2 HP LTF CEBPE S100A8 S100A9 CHIL1 C3 HBA-A2 HBA-A1 FGR TREM3 MMP8 MMP9
1.09E-07	7	97	Cellular oxidant detoxification	HP HBB-BS S100A8 S100A9 HBA-A2 HBA-A1 HBB-BT
1.64E-07	9	247	Cellular response to toxic substance	CYBB LCN2 HP HBB-BS S100A8 S100A9 HBA-A2 HBA-A1 HBB-BT
1.72E-07	7	106	Cellular detoxification	HP HBB-BS S100A8 S100A9 HBA-A2 HBA-A1 HBB-BT
3.12E-07	7	117	Detoxification	HP HBB-BS S100A8 S100A9 HBA-A2 HBA-A1 HBB-BT
3.12E-07	25	3522	Response to stress	CYBB ELANE PGLYRP1 HP CAMP LYZ2 TREM3 MMP9 C3 LCN2 FCNB PRG2 FGR CLEC4A2 NGP LTF CHIL3 S100A8 S100A9 CHIL1 FER MMP8 TYROBP CEBPE SLC4A1
3.30E-07	12	633	Regulation of cytokine production	PGLYRP1 FGR CYBB ELANE C3 FCNB PRG3 LTF MMP8 PRG2 TYROBP CLEC4A2
5.37E-07	11	526	Defence response to other organism	PGLYRP1 CAMP LYZ2 ELANE LCN2 PRG2 HP LTF FGR TREM3 CEBPE
5.44E-07	23	3062	Regulation of multicellular organismal process	PGLYRP1 NGP MMP9 PI16 FGR TMEM30A CYBB ELANE C3 FCNB PRG3 PROM1 CAMK1 LTF ACPP CAMP CHIL1 TYROBP MMP8 LCN2 PRG2 S100A9 CLEC4A2
5.62E-07	4	13	Oxygen transport	HBB-BS HBA-A2 HBA-A1 HBB-BT
6.58E-07	18	1827	Positive regulation of multicellular organismal process	FGR TMEM30A CYBB MMP9 ELANE C3 FCNB PRG3 PROM1 CAMK1 LTF CAMP CHIL1 MMP8 LCN2 PRG2 TYROBP S100A9
8.05E-07	12	703	Innate immune response	CAMP TREM3 CYBB C3 LCN2 FCNB FGR CLEC4A2 PGLYRP1 LTF S100A8 S100A9
8.06E-07	5	40	Killing of cells of other organism	PGLYRP1 ELANE LTF TREM3 CAMP
8.06E-07	5	40	Disruption of cells of other organism	PGLYRP1 ELANE LTF TREM3 CAMP
8.68E-07	7	146	Modification of morphology or physiology of other organism	PGLYRP1 ELANE LTF TREM3 CAMP MMP9 S100A9
1.14E-06	13	901	Positive regulation of immune system process	FER C3 FGR TYROBP TNFSF13B TREM3 ELANE FCNB PGLYRP1 LTF MMP8 SLC4A1 MMP9
1.51E-06	7	160	Cell killing	PGLYRP1 ELANE LTF TREM3 C3 TYROBP CAMP

2.12E-06	4	19	Gas transport	HBB-BS HBA-A2 HBA-A1 HBB-BT
3.71E-06	11	668	Inflammatory response	ELANE HP C3 CHIL3 S100A8 S100A9 CHIL1 MMP8 CYBB PGLYRP1 TYROBP
3.72E-06	19	2348	Multi-organism process	PGLYRP1 CAMP LYZ2 MMP9 LCN2 WFDC21 ELANE FCNB PRG2 HP LTF CEBPE C3 HBA-A2 HBA-A1 FGR TREM3 FER S100A9
4.74E-06	6	116	Cytokine metabolic process	CYBB ELANE PRG3 TREM3 TYROBP CEBPE

Strikingly, the significant genes fall predominantly into functional categories reflecting immune/stress responses that normally involves either neutrophils granulocytes and monocyte / macrophage or erythroid cells. Examples of proteins encoded by genes falling into several of the significant functional categories include: (i) ELANE (neutrophil elastase), which is the most abundant protein in neutrophils, achieving millimolar concentration, and has been associated with endoplasmic reticulum stress if it acquires a mutation; (ii) PGLYRP1 (peptidoglycan recognition protein 1), which is an antibacterial and pro-inflammatory innate immunity protein that plays a role in maintaining anti- and pro-inflammatory homeostasis; (iii) CAMP (cathelicidin antimicrobial peptide), which in addition to its actions against bacteria, fungi, and viruses has a role in cell chemotaxis, immune mediator induction, and inflammatory response regulation, and has been shown to prime the responsiveness of hematopoietic stem and progenitor cells to a stromal-derived factor-1 (SDF-1) chemokine gradient; and (iv) S100A8 (S100 calcium binding protein A8), which is involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation, and in neutrophils and monocytes acts as a Ca2+ sensor, participating in cytoskeleton rearrangement and arachidonic acid metabolism, and during inflammation it is released actively and exerts a critical role in modulating the inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion.

5.3 Attempt to restore a WT pattern of gene expression in *RETNLγ* KO HSC through exposure to exogenous RETNLγ protein or by ectopic expression of the *RETNLγ* gene

The transcriptome analysis shows that KO HSC exhibit significant changes in gene expression, particularly in females, and that these changes are most apparent in genes encoding proteins associated with differentiated cell function, particularly in relation to immune / stress responses. Since the KO HSC used for the RNA-seq analysis were from constitutive KO mice, then it is possible that either or both endogenous RETNLγ or RETNLγ in the niche environment could be influencing gene expression. To investigate if restoration of exogenous or endogenous RETNLγ can revert the gene expression difference, KO HSC were either treated in vitro with RETNLγ protein or transduced with a lentivirus encoding RETNLγ.

5.3.1 The influence of exogenous RETNLγ protein on the expression of genes that are differentially expressed in KO versus WT HSC

To explore if exogenously applied RETNLγ protein can influence gene expression in KO HSC, KSL cells were sorted from KO BM and then cultured *in vitro* with or without the addition of RETNLγ protein. After 24 hours in culture, RNA was prepared and qRT-PCR performed for genes characteristic of the changes seen in HSC from female KO animals. Both male (n=3) and female (n=2) HSC were used. The results obtained for *HBA*, *CAMP*, and *S100A8* are depicted in Figure 5.3 and Appendix 6.

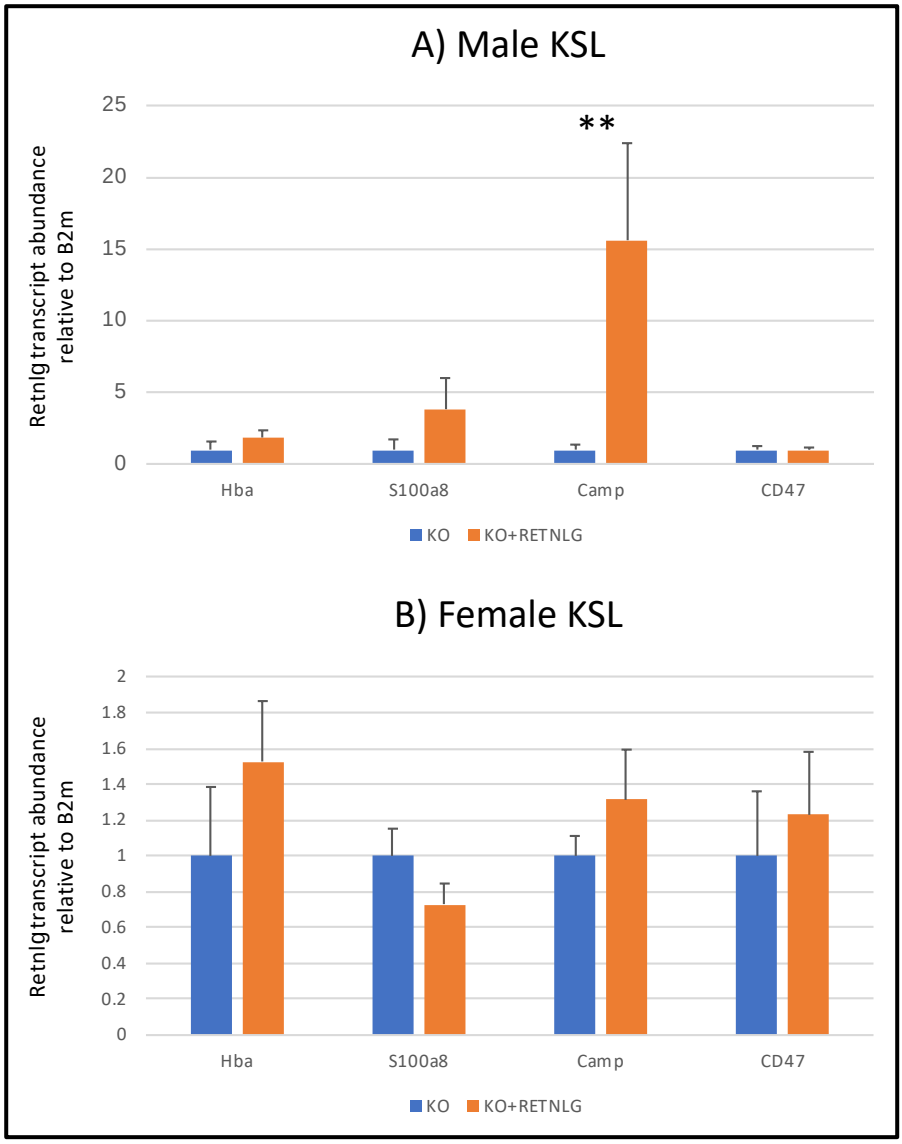


Figure 5.3 qRT-PCR analysis of gene expression changes in *Retnlγ* KO HSC treated with RETNLγ protein. Sorted *RETNLγ* KO KSL cells were divided into two parts and placed in culture media supplemented with growth factors. RETNLγ protein was added, and cells were incubated for an overnight. qRT-PCR was performed using Taqman primers for *HBA*, *S100A8*, *CAMP*, and *CD47* genes. The histograms represent the relative expression of the mentioned genes compared to *β2* microglobulin ($2^{-\Delta\Delta Ct}$). (A) The result of KSL from 3 *Retnlγ* KO male mice. (B) The result of KSL from 3 *RETNLγ* KO female mice.

Unexpectedly, RETNLγ protein applied to KO HSC had significant effects in male cells but not those derived from female mice. The effect on *CAMP* expression was most notable, and reached significance ($p = 0.004$). Also, contrary to expectation, the effects observed in male

cells involved increased expression of the genes that had been chosen because they exhibited increased expression in female KO HSC. The positive effects of exogenously applied RETNLγ could be opposite to expectation for a number of reasons, including that as an external agent a distinct signalling pathway(s) is elicited, or perhaps the amounts applied were not equivalent to what would normally be experienced by an HSC, most likely being much higher than the level expressed internally.

5.3.2 Endogenous re-expression of RETNLγ protein does not influence the expression of genes that are differentially expressed in KO versus WT HSC

Next, the consequences of the restoration of endogenous *RETNLγ* expression in KO HSC was explored by transduction of cells with lentiviral vectors.

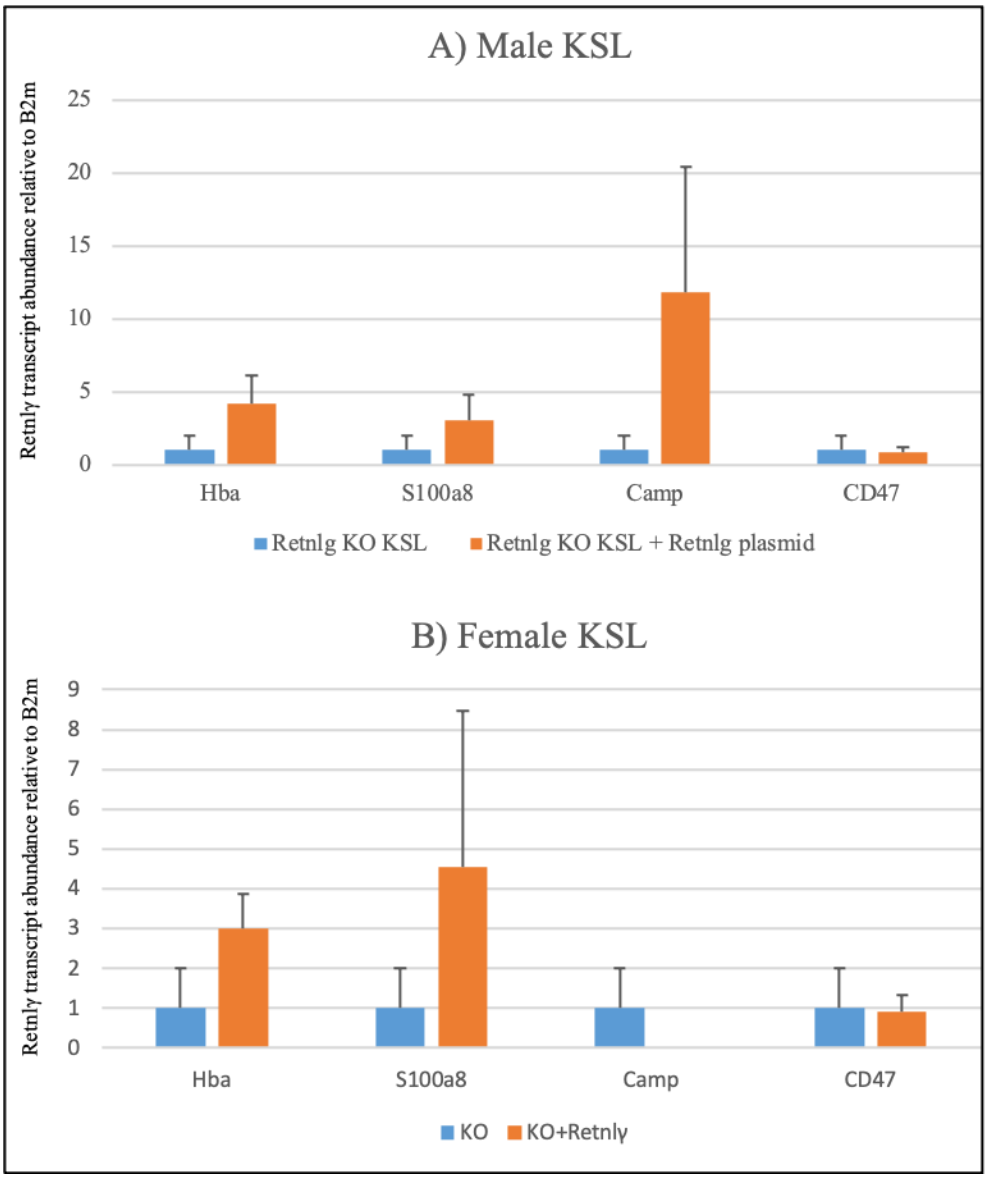


Figure 5.4 qRT-PCR analysis of gene expression changes in KO HSC transduced with lentivirus expressing RETNLγ. Sorted *RETNLγ* KO $Lin^{-}Kit^{+}$ cells were transduced by an over expression *RETNLγ* lentivirus. The cells were placed in culture media supplemented with growth factors. After 24 hours of incubation, KSL which has GFP^{+}(transduced)was sorted. The level of selected genes was examined by qPCR. (A) The result of KSL from 3 *RETNLγ* KO male mice. (B) The result of KSL from 3 *RETNLγ* KO female mice. The level of *RETNLγ* RNA after transduction can be found in Appendix 8.

KSL cells were sorted from KO BM and then cultured *in vitro* after infection with either a control or a *RETNLγ*-expressing lentivirus. After 24 hours in culture, RNA was prepared and qRT-PCR performed for the same genes examined in the previous experiment involving addition of RETNLγ protein. Both male and female HSC were used. The results obtained are depicted in Figure 5.4 and Appendix 7.

Although significance was not reached for any of the gene expression changes elicited as a result of the lentiviral transductions, a similar trend appeared to that seen as a result of addition of RETNLγ protein, that is, there was a male-specific increase in the three test genes (the increase in Camp gene expression achieved a p value of 0.083). As for the explanation proposed for the opposite response to re-expression compared to the absence of RETNLγ expression, it is possible that this was not physiological in the context of the HSC (see level of over expression in Appendix 8). Examination of qRT-PCR data for *RETNLγ* RNA over expression in male KO cells shows no differences to the results of exogenous protein effect on these genes. In female KO cells a difference in genes level after transduction was observed. However, these result shows no significance when compared to the non-transduced cells.

5.4 Summary

The experiments described in this chapter represent a preliminary approach to understand what molecular changes result when the expression of RETNLγ is constitutively ablated in KO mice. Clearly, a number of significant differences are seen in the profile of gene expression comparing female WT and KO HSC, and these appear to involve increased levels RNA for genes related to immune / stress responses that normally involve myelomonocytic cells and to a lesser extent erythroid cells.

Attempts to revert these differences in the KO HSC, either using exogenous protein or lentiviral transduction, produced unexpected results in that (i) the effects were restricted to male HSC, and (ii) over expression of RETNLγ in the KO context actually had the reverse effect to that anticipated. So, although these results are at face value conflicting, they do demonstrate that RETNLγ is able to influence the expression of specific genes in HSC, and in a manner that has a sex-specific aspect. Future rescue studies will require a more controlled approach to re-expression of RETNLγ in the KO context, as clearly the levels achieved in the experiments reported were way above normal physiological expression.

Future work should be directed at understanding how the observed gene expression differences might influence the function of HSC. The transplantation experiments in Chapter 4 failed to demonstrate a convincing difference in HSC engraftment potential, but more could be revealed through a round of secondary transplantations, which are a more stringent test of self-renewal potential. Given the nature of the gene expression differences, it would also be interesting to explore how WT and KO HSC respond to conditions of induced stress, such as haematopoietic cell deficit or to infection.

CHAPTER 6 GENERAL DISCUSSION

6.1 Introduction

Resistin-like gamma (RETNLγ) is a member of a family of proteins, the resistin-like molecules (RELM), which are present throughout the vertebrates, although gene duplication and diversification have led to the evolution of a diversity of roles, only some of which are fully understood. The RELM proteins are small, hormone-like molecules that can be secreted and have the potential to multimerise. Previous studies on a range of tissues in the mouse showed that *RETNLγ* is most highly expressed in the haematopoietic tissue (spleen, thymus, and BM), suggesting that the protein might have a cytokine-like role in haematopoiesis (Gerstmayer et al., 2003).

The overall aim of the research described in this thesis has been to understand the functional significance of the expression of RETNLγ in haematopoietic stem cells (HSC). Interest in RETNLγ in this context arose because its expression was found to be dependent on the activity of the transcription factor MYB, which itself is an essential regulator of gene expression throughout the haematopoietic hierarchy, including in the stem cell compartment. A role for MYB in the regulation of *RETNLγ* is also suggested by the finding that it cooperated with C/EBPε in the stimulation of *RETN* gene expression in a non-haematopoietic cell line, both *RETNLγ* and *RETN* being co-expressed with C/EBPε (Chumakov et al., 2004).

The level of expression of the *RETNLγ* gene in HSC, and the extent to which this decreased upon loss of MYB activity suggested that the RETNLγ protein might be responsible for at least some of the phenotypic consequences of the transcription factor. No role has yet been described

for RETNLγ or any other RELM protein in mouse HSC, and therefore a major objective of the research carried out in this thesis has been to determine how its expression varies across the haematopoietic hierarchy, what effect it has on HSC function, and by what mechanisms it might act.

6.2 *RETNLγ* expression across the haematopoietic hierarchy

Quantitative analysis of *RETNLγ* RNA in sorted cell populations representing the haematopoietic hierarchy from the stem cells at the top to committed progenitors and differentiated lineage cells revealed expression throughout, although at widely different levels. Highest expression was seen in committed myelomonocytic, erythroid, and megakaryocytic lineage cells, about 5- to 10-fold lower levels in lineage-committed progenitors, and a further 5- to 10-fold lower still in multilineage progenitors and stem cells. Intriguingly, the expression of *RETNLγ* RNA in some haematopoietic cell types showed a degree of gender specificity. Most notably, the most immature stem cells tested, the LT-HSC, showed lower expression in females compared to males.

In parallel with the analysis of *RETNLγ* RNA, protein levels were assessed using immunofluorescence to confirm both the variation across the haematopoietic hierarchy and the actual presence of the protein in HSC. The high expression of RETNLγ protein in mature cells were sufficient to enable *in situ* detection, while much lower levels in HSC, reflecting the RNA expression results, necessitated the more sensitive approach using flow cytometry. Attempts to demonstrate secretion of RETNLγ from haematopoietic cells proved inconclusive, although some evidence was obtained (data not shown) that high RETNLγ expressers like

myelomonocytic cells do shed the protein into the culture supernatant. Consideration needs to be given to the sensitivity of the method of detection, which will be particularly important if it is to be possible to show that HSC can secrete RETNLγ.

The relative expression of RETNLγ in HSC and progenitors compared to mature BM cells, such as monocytes and megakaryocytes, combined with the fact that RETNLγ is secreted poses an interesting question about the possible contribution of intrinsic and extrinsic RETNLγ protein in respect to its potential role in HSC. Stem cells and progenitors in the bone marrow are likely to be exposed to higher levels of RETNLγ produced from mature cells than they are capable of producing themselves, begging the question about a possible distinct role for intrinsic RETNLγ in the HSC, that is, could the intracellular protein be acting through different mechanisms than that in the extracellular environment? How RELM proteins act on their target cells remains a largely unresolved issue, and nothing is specifically known regarding RETNLγ. Receptors and downstream signalling pathways have been suggested for some of the cellular scenarios in which these hormones are implicated (Pine et al., 2018; Acquarone et al., 2019). Five potential receptors have been proposed, namely adenylyl cyclase-associated protein 1 (CAP1), Toll-like receptor 4 (TLR4), insulin-like growth factor receptor 1 (IGF1R), tyrosine kinase-like orphan receptor (ROR1), and Decorin (DCN). RNA-seq data obtained by the Frampton group confirms that both CAP1, TLR4, and IGF1R are expressed in LT-HSC, while ROR1 and DCN are not. If HSC-intrinsic RETNLγ has a role that is distinct to the extracellular protein then an intracellular mechanism would be consistent with CAP1 being the receptor since, although described to be associated with the cell membrane (Lee et al., 2014), it can also operate as an intracellular receptor.

6.3 RETNLγ affects haematopoietic cell numbers in a sex-specific manner and has some limited influence on stem cell function *in vivo*

In an attempt to understand what role RETNLγ might play in HSC a number of analyses were performed comparing WT and *RETNLγ* KO mice. A comprehensive measurement of peripheral blood and bone marrow cell numbers revealed that although most measurements showed little difference between WT and KO, a striking sex-specific effect was seen in female KO BM in which the numbers of stem cells and committed progenitors, and possibly monocytes, were significantly lower compared to both WT females and KO males. However, these cell number differences were less obviously reflected in the behaviour of isolated HSC when tested in either *in vitro* colony or *in vivo* transplantation assays. *In vivo* assay of HSC by transplantation showed some weak evidence for enhanced engraftment by both male and female KO HSC, but these findings were not statistically significant. Examination of the bone marrow and spleens of transplanted animals did, however, show some signs that RETNLγ might affect the fate of HSC and that some aspects are sex-specific. Animals transplanted with KO female LT-HSC exhibited generally higher numbers of most lineages in the bone marrow, in particular monocytes, megakaryocytes, and B-cells, while their spleens had relatively low levels of donor-derived erythroid cells compared to WT donor transplants. In contrast, animals transplanted with KO male LT-HSC showed no signs of higher levels of donor-derived bone marrow cells while their spleens had increased relative numbers of most lineages.

The lower numbers of HSC and progenitors in KO versus WT female BM combined with the observations of seemingly enhanced engraftment of KO versus WT female LT-HSC (based on peripheral blood values and BM cell numbers) might suggest that the absence of RETNLγ leads to a greater tendency towards differentiation and a consequent depletion of the immature stem

cells. As discussed below, the comparative transcriptome analysis of KO versus WT female HSC lends further support to this idea.

At this point, it can only be speculated what might underlie the largely female-specific differences observed. However, a likely explanation lies in the effect that female sex hormones have on HSC (Nakada et al., 2014) in that it was found that mouse HSC exhibit sex differences in cell-cycle regulation by oestrogen, cells in female mice dividing significantly more frequently than in male mice. This difference was shown to depend on the ovaries but not the testes, while administration of oestradiol increased HSC division in both males and females. HSC express high levels of oestrogen receptor α, which when conditionally deleted reduced HSC division in female, but not male, mice. Perhaps oestrogen signalling integrates with the effect of RETNLγ in some way.

6.4 The effect of RETNLγ on HSC gene expression

The comparison of transcriptomes between female WT and KO HSC revealed a strong effect of the absence of RETNLγ on the profile of gene expression. Validation using selected genes, comparing both male and female HSC, again showed a strong female-specific effect in that the genes were only seen to be affected in the female cells, which as discussed above might suggest that the female hormone environment in some way cooperates with the absence of RETNLγ in bringing about a phenotypic change in HSC.

The differences in gene expression in female HSC resulting from the absence of RETNLγ are very interesting in terms of what function the protein performs in stem cells. Most of the significant changes appear to involve increased levels RNA for genes related to immune / stress responses that normally involve myelomonocytic cells and to a lesser extent erythroid cells.

Whether these gene expression differences reflect altered properties of the HSC *per se* or reflect an increased tendency of the cells to differentiate towards myelomoncytic and erythroid lineage cells cannot be concluded, but certainly form the basis for further investigations.

The elevation in gene expression in the absence of RETNLγ but only in the female context could suggest that the protein normally represses genes that have a link to immune / stress responses, but when absent then oestrogen receptor signalling leads to transcriptional activation. It would be interesting to explore if a similar pattern of activated gene expression could be observed in male mice treated with oestradiol, or if knockout of the oestrogen receptor in female mice negates the effect of *RETNLγ* KO. It would also be interesting to determine if there is any change in the binding of oestrogen receptor to the regulatory regions of genes in KO compared to WT female HSC, and if this correlates with the profile of over expressed immune / stress related genes.

How RETNLγ might normally repress gene expression, either directly or indirectly, is another issue that warrants further investigation. There is no evidence in the literature that RELM proteins can bind to DNA, but findings mainly derived from studies on RETN show that it can initiate signalling that could therefore impact indirectly on transcriptional regulation. The experiments performed in this thesis regarding the restoration of RETNLγ expression in KO HSC could form the basis for such further studies, although at this point the results are difficult to interpret. If restoration of expression of RETNLγ by either addition of protein or through lentiviral transduction could elicit a reversal in the gene expression profile from the KO to the WT pattern, then such a system could be used to perform comparative genome-wide chromatin experiments such as ATAC-seq or ChIP-seq to determine what features are influenced.

6.5 The relevance of RETNLγ function in the mouse in the context of human HSC

As pointed out in Chapter 1, the nomenclature of the RELM family genes can be confusing, especially in trying to make comparisons between mouse and humans. The evidence available, especially the cell types expressing the protein and the regulatory mechanism controlling expression, seems strongly to suggest that RETNLγ is equivalent to RETN in humans, the so called RETN protein in the mouse being distinct in its expression domain (Chumakov et al., 2004; Patel et al., 2003; Yang et al., 2003). If this is indeed correct, then it would be an obvious next step to start to investigate the role of human RETN, which could have translational implications in the context of managing clinical situations in which haemostasis is perturbed.

6.6 Limitations and future studies

This study of the expression and function of RETNLγ was completely dependent on the availability of appropriate genetically altered mice carrying the KO allele for the gene. Given the importance of using littermate pairs of WT and KO animals, combined with the need to look separately at males and females, meant that it was always a challenge to accumulate sufficient experimental animals. However, experiments conducted in this way ultimately generate more biologically relevant and statistically significant results. Another practical limiting challenge given the cell type of interest and its derivation from mice is that fact that most experiments required highly purified sorted HSC, which represent a very small proportion of the bone marrow cell population.

Although assays for HSC function are well-established, they are also resource- and time-consuming, especially when transplantation into donor animals is required. The latter approach is the ‘gold standard’ in the field, and in the case of the RETNLγ study was deemed essential

as no definitive conclusions could be drawn from *in vitro* colony assays of stem cell potential. Given the limited time and resource available, it was not possible to pursue further *in vivo* experiments, although the next steps should involve secondary BM transplantations fully to explore the self-renewal capacity of WT versus KO HSC, as well as *in vivo* stress or challenge experiments, especially taking account of the immune / stress response profile of genes that appear to be influenced by RETNLγ, at least in females. Such *in vivo* challenges should include models of haematopoietic cell depletion, such as induced anaemia or bone marrow ablation using 5-fluorouracil.

Preliminary attempts to rescue the RETNLγ KO phenotype at the level of gene expression were inconclusive, although lentivirally-mediated transduction did prove to be a feasible way to restore expression in primary HSC. An obvious problem that needs to be resolved is how to control re-expression so that the levels of RETNLγ are close to normal physiological levels seen in freshly isolated cells. As a first next step, some form of controllable over expression lentiviral system should be explored, probably utilising doxycycline-mediated transcriptional activation of RETNLγ expression.

Probably the most interesting aspect of this project that should be pursued is how the gender background of the mice influences the effects of RETNLγ on gene expression. As mentioned already, it would be good in the first instance to explore the relationship between RETNLγ and oestrogen: it could be that RETNLγ affects the way in which oestrogen and its receptor affect HSC, presumably through effects on gene expression, or alternatively oestrogen / oestrogen receptor might impinge on a mechanism of gene regulation requiring RETNLγ. Although there are indications that male HSC do not respond in the same way as female cells to the absence of RETNLγ in gene expression terms, this should be examined more comprehensively through RNA-seq. In addition, it would be very informative if the chromatin status of WT versus KO

HSC for both males and females could be compared, preferably using ATAC-seq, as this would provide insight into the genes and gene regulatory features that are affected by RETNLγ and how this might tie in with the actions of the oestrogen receptor.

Lastly, and most importantly from a medical translation perspective, it would be interesting to try to perform experiments that support the arguments presented in this thesis that mouse RETNLγ is the functional equivalent of human RETN. Although, as already discussed, further development of the *in vivo* biological function assays in the mouse is necessary, the gene expression consequences of RETNLγ ablation and the female-specific aspects of this and parallel HSC deficiencies in the BM at least provide a workable platform. This being the case, the ideal model would be to generate a knock-in mouse line in which mouse RETNLγ gene is replaced by the human RETN coding sequences. This would be a big undertaking, but if the theory is correct about the functional homology of these genes, then the knock-in line should demonstrate all of the features of normal animals expressing RETNLγ.

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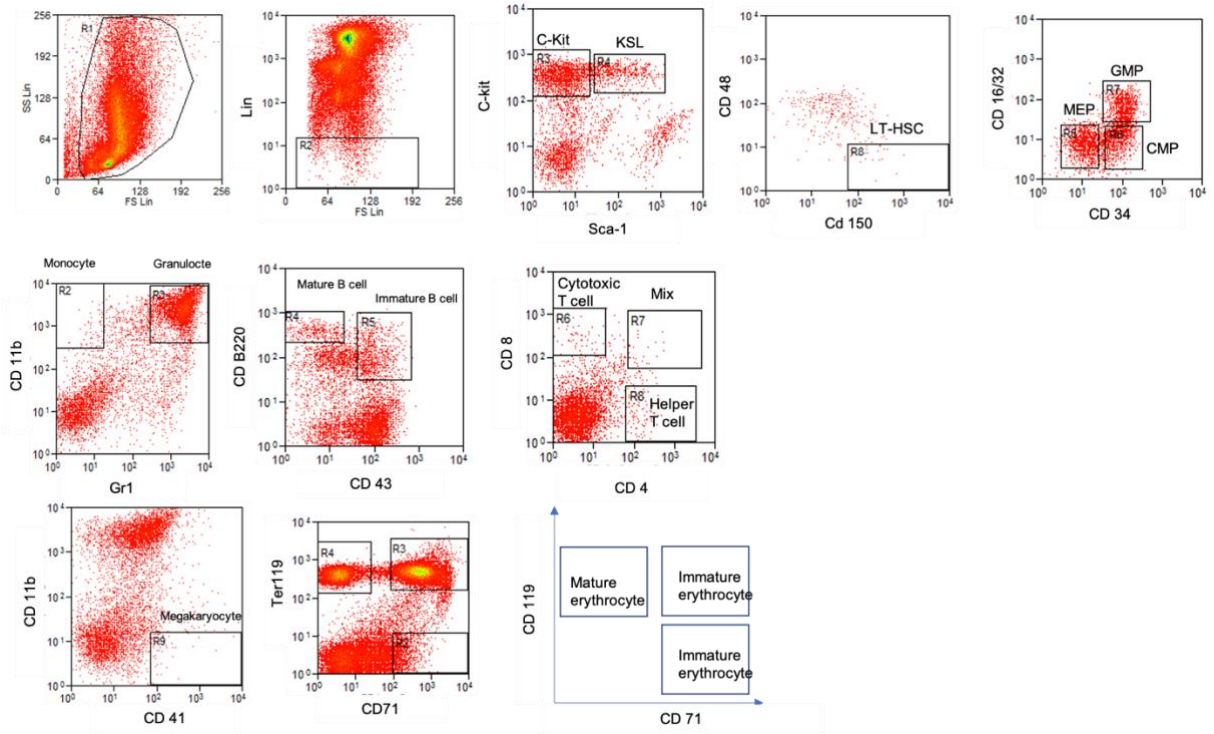
Appendix 1: Gating strategies used for BM HSC characterisation and isolation



Bone marrow was collected from the femurs of 3-onth old mice. Cells were immuno-stained with antibodies to enable discrimination of distinct stem and progenitor population by flow cytometry (Table 3.1).

Appendix 2: Ct values for qRT-PCR analysis of RNA from HSC and progenitor populations from WT male mice

	B2M ct value			Retnlg ct value		
LT-HSC	31.95	32.14	32.25	35.8	35.54	35.89
ST-HSC	29.49	29.18	29.41	36.95	36.62	36.37
MPP	30.33	30.38	30.44	34.94	34.99	35.17
GMP	29.44	29.42	29.55	35.93	36.19	36.06
CMP	29.27	29.42	29.48	35.85	35.47	36.07
MEP	33.65	33.79	34.08	38.56	38.56	38.34
Megakaryocyte	29.86	29.95	29.95	29.1	28.97	29.52
immature Ter119-	31.23	31.91	31.86	37.41	37.26	37.18
immature Ter119+	29.74	29.76	29.82	33	33.43	34.08
mature erythro	35.25	35.52	35.14	37.64	37.63	37.81

	B2M ct value			Retnlg ct value		
LT-HSC	35.2	35.59	35.5	39.03	38.81	38.88
ST-HSC	33.76	33.68	33.7	38.32	38.71	39.49
MPP	32.53	32.79	32.44	37.62	37.11	37.97
GMP	30.56	30.46	30.65	38.14	38.23	38.41
CMP	32.3	32.52	32.55	37.77	38.49	38.89
MEP	34.04	34.28	33.88	38.6	38.69	38.82
Megakaryocyte	30.48	30.49	30.58	29.78	29.87	29.97
immature Ter119-	31.63	31.74	31.82	37.54	37.64	37.62
immature Ter119+	31.64	31.5	31.79	35.28	35.37	35.66
mmature erythroature	35.71	35.69	35.7	36.64	36.21	36.58

Male 1	B2M			Retnlg		
KSL	30.15	30.34	30.23	38.75	39.09	38.8
Cd11b, Gr1, ckit	24.02	24.04	24.02	26.25	26.11	26.32
CdD11b, c-kit	26.79	26.87	26.83	30.57	31	30.72
CD11b, Gr1	23.01	23.04	22.97	21.81	21.88	22.06

Male 2	B2M			Retnlg		
KSL	27.86	27.26	27.88	34.94	35.77	35.45
Cd11b, Gr1, ckit	22.1	22.07	22.25	23.55	23.58	23.67
CdD11b, c-kit	26.23	26.43	26.62	30.6	30.67	30.71
CD11b, Gr1	22.88	22.71	22.84	21.94	22.47	22.09

Male 3	B2M			Retnlg		
KSL	29.25	29.25	29.25	35.92	35.75	36.65
Cd11b, Gr1, ckit	28.43	28.47	28.41	30.28	30.22	30.53
CdD11b, c-kit	28.67	28.8	28.93	34.85	34.96	35.22
CD11b, Gr1	30.05	29.79	30.01	29.7	29.78	29.89

Appendix 3: Ct values for qRT-PCR analysis of RNA from HSC and progenitor populations from WT female mice

Female 1	B2M ct value			Retnlg ct value		
LT-HSC	31.33	31.31	31.45	37.04	36.36	36.74
ST-HSC	31.96	31.99	32.05	37.44	37.63	36.71
MPP	34.42	34.67	34.43	38.44	38.48	38.53
GMP	30.62	30.36	30.43	36.04	36.81	36.39
CMP	29.61	29.81	29.91	35.23	34.84	34.92
MEP	28.55	28.53	28.58	32.98	33.46	33.48
Megakaryocyte	29.31	29.31	29.56	29.84	29.82	30.2
immature Ter119-	29.91	30.05	30.32	35.18	35.33	35.13
immature Ter119+	31.3	31.48	31.7	37.53	38.48	38.74
mature erythro	36.76	36.55	37.23	38.8	39.39	39.69

Female 2	B2M ct value			Retnlg ct value		
LT-HSC	34.69	34.51	34.78	39.44	39.75	38.77
ST-HSC	29.81	29.99	30.05	35.92	35.9	36.15
MPP	32.81	32.9	32.95	38.93	38.98	38.64
GMP	27.24	27.24	27.13	33.67	33.25	33.37
CMP	29.55	29.55	29.69	35.98	35.86	36.16
MEP	29.3	29.43	29.35	35.51	35.31	35.37
Megakaryocyte	30.63	30.75	30.72	30.55	30.65	30.96
immature Ter119-	30.87	31.34	30.9	35.97	35.61	35.66
immature Ter119+	32.69	32.79	32.8	38.94	39.46	38.69
mature erythro	33.42	33.89	33.95	36.85	36.6	37.29

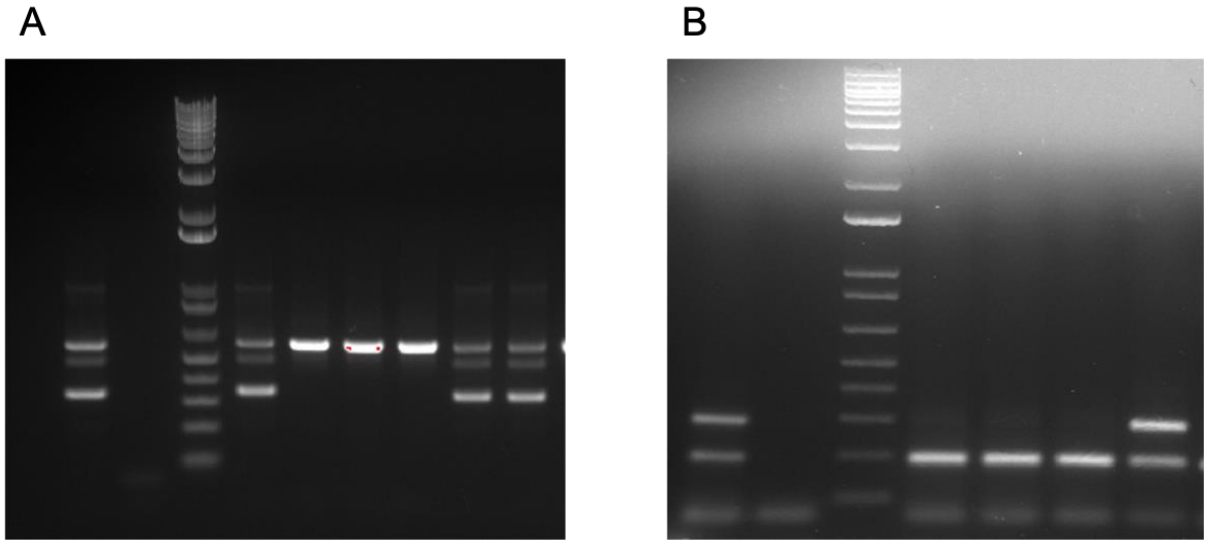
Female 3	B2M ct value			Retnlg ct value		
LT-HSC	33.96	34.1	34.17	39.27	39.18	39.57
ST-HSC	28.7	28.75	28.93	35.77	35.39	35.26
MPP	29.32	29.27	29.35	32.86	32.64	32.45
GMP	33.31	32.97	32.92	36.94	37.38	37.52
CMP	26.96	26.97	27.22	33.96	33.57	34.45
MEP	30.44	30.2	30.24	35.21	35.54	35.33
Megakaryocyte	34.42	34.03	34.23	34.61	34.5	34.35
immature Ter119-	27.04	27.05	26.96	30.62	31.29	30.36
immature Ter119+	29.42	29.46	29.42	32	31.91	32.21
mature erythro	38.82	38.21	39.32	37.93	37.93	36.71

Female 1	B2M			Retnlg		
Cd11b, Gr1, ckit	31.07	31.23	30.74	32.37	32.38	32.51
CdD11b, c-kit	33.71	33.94	33.84	36.47	36.77	36.31
CD11b, Gr1	23.9	24.1	24.07	22.54	23	22.91

Female 2	B2M			Retnlg		
Cd11b, Gr1, ckit	25.19	25.53	25.37	26.58	26.86	26.71
CdD11b, c-kit	37.66	37.56	37.08	38.69	37.3	37.84
CD11b, Gr1	22.86	23.29	23.28	21.45	21.62	21.54

Female 3	B2M			Retnlg		
Cd11b, Gr1, ckit	25.35	24.98	25.23	26.4	26.4	26.76
CdD11b, c-kit	33.2	33.09	33.16	37.49	37.11	36.78
CD11b, Gr1	23.74	24.44	24.52	21.82	22.09	22.15

Appendix 4: PCR-based genotyping of WT and *RETNLγ* KO mice that were used in RNA-seq analysis of purified KSL HSC



Genomic DNA was isolated from ear-clip samples taken from mice at weaning. PCR was performed using primers listed in Table 2.1 and the PCR product was run on a 1.5% w/v agarose gel. The three WT (A, 582bp) and three KO (B, 339bp) PCR products are indicated.

Appendix 5: Ct values for qRT-PCR validation of genes identified by RNA-seq

A

Male 1		WT 1			Retnlg KO 1		
B2M	31.73	31.62	31.58	B2M	33.12	32.95	33.25
Hba	29.88	29.99	29.84	Hba	31.66	31.59	31.38
Hbb	28.51	28.46	28.77	Hbb	30.15	30.24	30.41
Camp	32.19	31.87	32.28	Camp	31.39	31.2	31.13
S100a8	33.53	33.6	33.75	S100a8	32.75	32.8	33

Male 2		WT2			Retnlg KO 2		
B2M	31.63	31.47	31.61	B2M	32.01	31.95	31.93
Hba	29.61	29.6	29.63	Hba	29.72	29.79	29.75
Hbb	28.75	28.68	28.74	Hbb	28.91	28.88	29.08
Camp	29.29	29.33	29.2	Camp	30.7	30.87	30.91
S100a8	30.37	30.44	30.57	S100a8	31.82	32.04	32

B

Female 1		WT			Retnlg KO		
B2M	30.08	30.13	29.95	B2M	30.91	30.82	30.84
Hba	35.39	35.84	35.58	Hba	31.96	32.1	31.97
Hbb	34.22	34.17	34.74	Hbb	31.23	31.15	31.29
Camp	30.1	30.31	30.34	Camp	28.68	28.57	28.67
S100a8	31.84	32.09	32.2	S100a8	29.41	29.83	30.15

Female 2		WT2			Retnlg KO 2		
B2M	34.19	34.19	34.4	B2M	34.37	34.16	34.48
Hba	34.24	34.54	34.55	Hba	36.13	35.98	35.97
Hbb	33.8	33.71	33.95	Hbb	35.36	35.35	35.45
Camp	32.03	31.93	31.92	Camp	34.73	35.14	34.98
S100a8	34.51	34.96	35.38	S100a8	38.69	38.71	38.77

Female 3		WT			Retnlg KO		
B2M	34.81	34.52	34.42	B2M	35.6	35.36	35.42
Hba	34.97	35.01	34.77	Hba	35.27	35.62	35.27
Hbb	33.9	33.66	33.82	Hbb	34.73	34.44	34.52
Camp	32.92	33.22	33.57	Camp	31.59	31.5	31.32
S100a8	37.19	36.97	37.24	S100a8	34.51	34.63	34.73

A) male mice, B) female mice.

Appendix 6: Ct values for qRT-PCR assessment of selected Retnlγ influenced genes in RETNLγ KO KSL HSC following addition of exogenous Retnlγ protein

A

Male	B2M			Hba			S100a8			Camp			CD47		
M1 Retnlg KO KSL	29.46	29.62	29.57	37.44	37.45	37.68	32.02	31.85	32.01	34.18	34.57	34.31	32.96	33.27	33.57
M1 Retnlg KO KSL + Retnlg protein	29.78	29.91	29.9	35.52	35.28	35.87	30.46	30.77	30.88	30.65	30.68	30.53	33.76	33.64	33.13
M2Retnlg KO KSL	31.32	31.45	31.19	36.53	37.05	36.7	35.85	35.75	35.78	37.69	38.33	37.36	36.31	35.69	36.28
M2 Retnlg KO KSL + Retnlg protein	30.75	30.76	30.86	37.79	37.56	37	33.36	33.28	33.39	33.78	33.9	33.47	35.09	34.93	34.72
M3 Retnlg KO KSL	26.63	26.61	26.37	34.38	34.56	33.98	32.41	32.29	32.83	32.75	32.44	32.83	30.01	30.34	30.2
M3 Retnlg KO KSL + Retnlg protein	34.68	35.3	34.71	No Ct	No Ct	No Ct	38.21	39	38.47	36.82	36.53	36.86	39.5	39.07	

B

Female	B2M			Hba			S100a8			Camp			CD47		
F1 Retnlg KO KSL	30.9	30.92	31	38.94	38.76	38.8	30.76	30.7	30.73	31.58	31.69	31.59	35.35	35.39	35.99
F1 Retnlg KO KSL + Retnlg protein	30.2	30.15	30.22	38.89	38.86	38.78	31	30.96	31.03	31.18	31.2	31.06	33.34	33.46	33.34
F2 Retnlg KO KSL	31.22	31.29	31.07	37.98	38.02	37.83	35.37	35.26	35.25	36.39	36.7	36.19	36.09	35.99	36.14
F2 Retnlg KO KSL + Retnlg protein	31.53	31.69	31.49	37.62	37.93	37.84	35.55	35.7	35.78	36.89	36.15	36.64	36.14	36.02	35.98
F3 Retnlg KO KSL	27.31	27.19	27.27	35.42	34.8	35.13	30.93	31.22	30.61	32.21	32.13	32.17	31.06	31.33	30.9
F3 Retnlg KO KSL + Retnlg protein	27.39	27.42	27.65	34.63	34.76	34.11	32.18	31.69	32.28	31.89	31.65	32.04	31.27	31.16	30.98

A) male mice, B) female mice.

Appendix 7: Ct values for qRT-PCR assessment of selected RETNLγ-influenced genes in *RETNLγ* KO KSL HSC following transduction with *RETNLγ*-expressing lentivirus

A

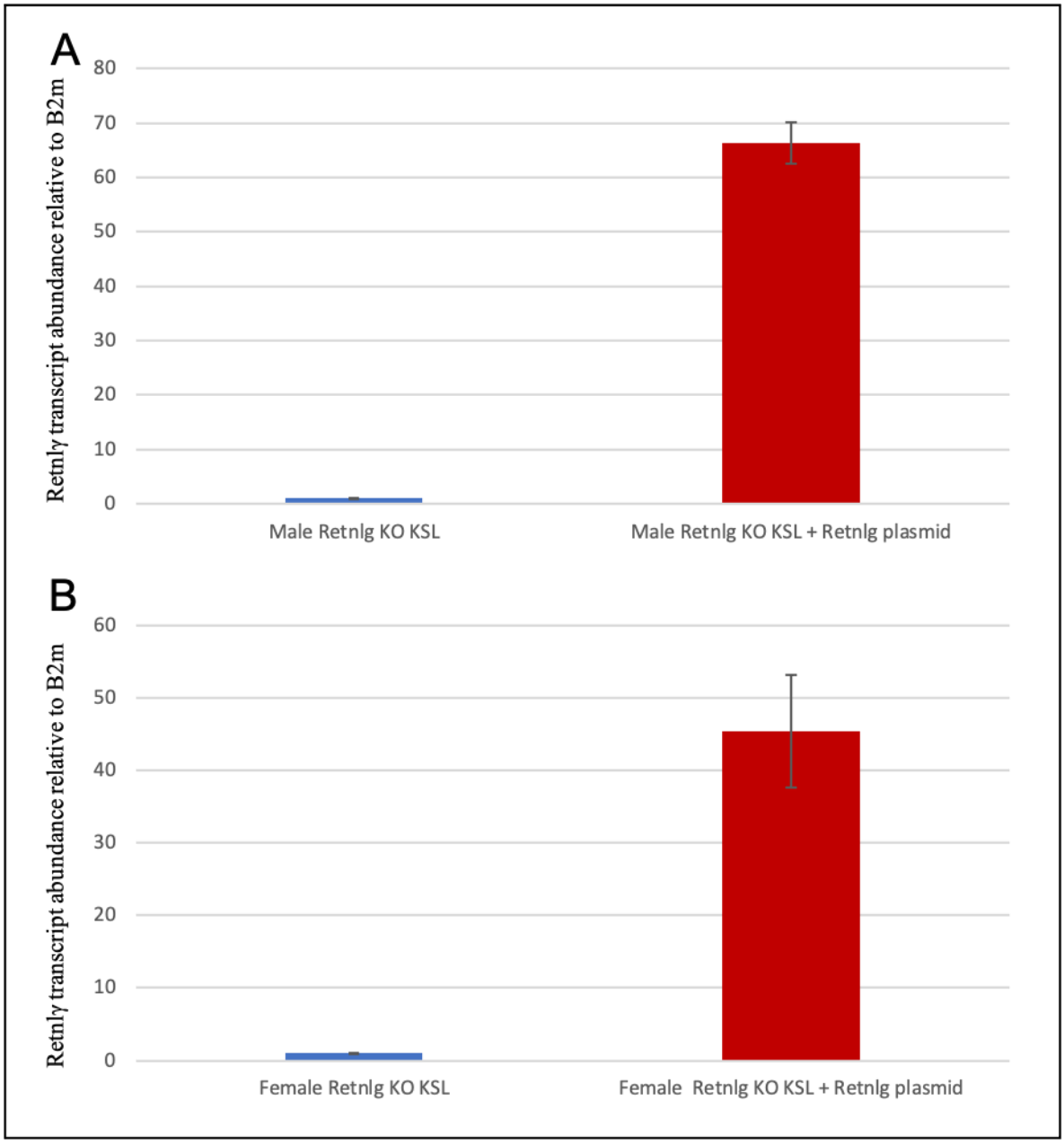
Male	B2M			Hba			S100a8			Camp			CD47		
M1 Retnlg KO KSL	28.76	28.61	28.65	37.3	36.93	37.59	30.79	30.84	30.98	32.98	33	33.12	31.64	31.56	31.49
M1 Retnlg KO KSL + Retnlg plasmid	31.08	31.29	31.48	37.55	37.88	37.05	32.56	32.51	32.3	33.12	32.93	32.96	35.85	35.58	35.9
M3 Retnlg KO KSL	28.67	28.64	28.71	33.06	33.32	33.03	29.49	29.37	29.52	31.01	31.25	31.17	32.04	32.26	31.9
M3 Retnlg KO KSL + Retnlg plasmid	30.77	30.95	31.37	36.45	37.18	37.08	32.5	32.43	32.28	34.8	34.35	34.77	34.55	34.94	34.55
M2 Retnlg KO KSL	30.94	30.82	30.69	36.85	36.54	36.22	34.78	34.38	34.463	36.7	36.94	36.69	35.55	35.25	35.21
M2 Retnlg KO KSL + Retnlg plasmid	34.64	34.47	34.46	37.48	37.33	37.62	35.5	35.5	35.65	33.46	33.72	33.75	38.58	38.3	38.64

B

Female	B2M			Hba			S100a8			Camp			CD47		
F1 Retnlg KO KSL	31.62	31.6	31.82	38.08	38.24	38.61	34.62	34.36	34.7	35.48	35.61	35.48	36.57	36.25	36.76
F1 Retnlg KO KSL + Retnlg plasmid	31.72	32	32.19	37.48	37.78	37.2	35.17	35.56	35.77	No Ct	No Ct	No Ct	36.38	36.1	36.77
F1 KO KSL	28.33	28.18	28.23	33.79	33.78	33.36	38.77	39.3	38.93				31.37	31.26	31.07
F1 KO KSL + Retnlg plasmid	29.23	29.86	29.78	33.14	33.1	32.96	37.19	37.66	37.03	37.84			33.39	33.66	33.69
F4 KO KSL	29.88	29.61	2955	33.6	33.85	33.65	38.64	38.07	37.6	37.37	39.24	37.61	32.89	33	33.67
F4 KO KSL + Retnlg plasmid	30.6	30.6	30.53	33.62	33.76	33.43	38.41	38.46	38.14	39.7	36.08	37.77	34.99	34.76	34.07

A) male mice, B) female mice.

Appendix 8: qRT-PCR assessment of the level of *RETNLγ* RNA in *RETNLγ* KO KSL HSC after transduction with *RETNLγ*-expressing lentivirus



Sorted *RETNLγ* KO $Lin^{-}Kit^{+}$ cells were transduced by an over expression *RETNLγ* lentivirus. The cells were placed in culture media supplemented with growth factors. After 24 hours of incubation, *GFP*+ KSL *HSC were* sorted. The level of selected genes was examined by q*RT*-PCR. (A) The result of KSL *HSC* from 3 *RETNLγ* KO male mice. (B) The result of KSL from 3 *RETNLγ* KO female mice.