Characterising the Role of *Zeb1* in the Adult Haematopoietic System

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ABSTRACT

Zeb1, a zinc finger E-box binding EMT transcription factor, acts as a critical regulator of cell plasticity and confers properties of 'stemness', such as self-renewal, in cancer. Yet little is known about the function of *Zeb1* in somatic stem cells. We have used the haematopoietic system, as a well-established paradigm of stem cell biology, to explore *Zeb1* mediated regulation of somatic stem cells.

Here, we employed a conditional genetic approach using the Mx1-Cre system to specifically knockout (KO) *Zeb1* in adult haematopoietic stem cells (HSCs) and their downstream progeny. Long-term loss of *Zeb1* resulted in an expansion of HSCs and MPPs that impacted the differentiation to downstream progenitors, while acute deletion of *Zeb1* resulted in a reduction of lymphoid progenitors in BM and no change was observed in HSCs. Transplantation of HSCs after acute and chronic loss of *Zeb1* resulted in a profound self-renewal defect and multi-lineage differentiation block.

Acute loss of *Zeb1* in HSCs activates a transcriptional program of deregulated HSC maintenance and multi-lineage differentiation genes, and of cell polarity, consisting of cytoskeleton, lipid metabolism and cell adhesion related genes. Notably, Epithelial cell adhesion molecule (EpCAM) expression was prodigiously upregulated in *Zeb1* KO HSCs.

Furthermore, acute deletion of *Zeb1* led to a rapid onset thymic atrophy and cell autonomous loss of thymocytes and T cells. This defect in thymocytes was associated with increased cell death and changes in cell cycle kinetics as well as perturbations of memory $CD8^+$ T cell homeostasis. Thus, *Zeb1* acts as a crucial transcriptional repressor in haematopoiesis, co-ordinating HSC self-renewal and multi-lineage differentiation fates.

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ABBREVIATIONS

ALP	all-lymphoid progenitor
AML	acute myeloid leukaemia
ASXL1	ASXL transcriptional regulator 1
BM	bone marrow
bp	base pair
CAR cells	CXCL12-abundant reticular cells
CD	cluster of differentiation
CDC42	cell division control protein 42
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CFC	colony forming cell
CKIs	cyclin-dependent kinase inhibitors
CLP	common lymphoid progenitor
CM	central memory
CMP	common myeloid progenitor
CNS	central nervous system
Crb3	crumbs3
Cre	Cre recombinase
CSC	cancer stem cell
CtBP	carboxyl-terminal binding protein
CTC	circulating tumour cell
CTLA-4	cytotoxic T-Lymphocyte Associated Protein 4
CXCR	CXC chemokine receptors
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
DEG	differentially expressed genes
DN	double negative
DNA	deoxyribonucleic Acid
DNMT3A	DNA Methyltransferase 3A
Dox	doxycycline
DP	double positive
E.	embryonic day
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
EGR1	early growth response 1
EM	effector memory
EMT	epithelial to mesenchymal transition
EnET	endothelial to epithelial transition
EpCAM	epithelial cell adhesion molecule
EPCR	endothelial protein C receptor
ESC	embryonic stem cell
ETP	early thymic/T cell progenitor
Evi1	ecotropic viral integration site 1

FACS	fluorescence activated cell sorting
FBS	foetal bovine serum
fl	floxed, flanked
Flt3	FMS-like tyrosine kinase 3
FOXC2	forkhead box protein C2
GATA	GATA binding protein
Gfi1	growth factor independence 1
GFP	green fluorescent protein
GMP	granulocyte-macrophage progenitor
GO ORA	gene ontology over-representation analysis
Gy	gray
HD	homeodomain
HDAC	histone deacetylation
Hif1α	hypoxia-inducible transcription factor 1
HPC	haematopoietic Progenitor Cell
HRE	hypoxia response element
HSC	haematopoietic stem cell
HSPC	haematopoietic stem and progenitor cell
ID1	inhibitor of DNA binding
IL	Interleukin
ILC2	innate lymphoid cell group2
INK4	inhibitor of CDK4
IP	Intraperitoneal
Jam3	junctional adhesion molecule 3
KEGG	Kyoto Encyclopaedia of Genes and Genomes database
KO	Knockout
LCMV	lymphocytic choriomeningitis virus
$LEPR^+$ cells	leptin receptor-expressing cells
Lin^-	Lineage negative
LK	lineage-, Sca-1-, c-Kit+
LMPP	lymphoid-myeloid multipotent progenitor
LSK	lineage-, Sca-1+, c-Kit+
LT-HSC	long-term haematopoietic stem cell
MCL	mantle cell lymphoma
MDS	myelodysplastic syndrome
MEC	mammary epithelial cells
MEP	megakaryocytic-erythroid progenitor
MET	mesenchymal to epithelial transition
miR	microRNA
MLP	multi-lymphoid progenitor
MPP	multi-potential progenitor
mRNA	messenger Ribonucleic Acid
MSC	mesenchymal stromal cell
Mx1	Myxovirus resistance-1
NK	natural killer
NSG	NOD scid gamma mouse
OB	osteoblast
PB	peripheral blood

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-L1	programmed death-ligand 1
PHF6	plant homeodomain 6 gene
Pimo	pimonidazole
PKC	protein kinase C
Poly I:C	polyinosinic: polycytidylic acid
PPCD	posterior polymorphous corneal dystrophy
qRT-PCR	real-time quantitative reverse transcription PCR
RB	retinoblastoma
RBC	red blood cell
RIN	RNA integrity number
RNA	ribonucleic Acid
RNA-Seq	RNA sequencing
ROS	reactive oxygen species
SBD	smad protein binding
Sca-1	stem cell antigen 1
SCF	stem cell factor
Scl	stem cell leukaemia
SLAM	signalling lymphocytic activation molecule
SP	side population or single positive
ST-HSC	short-term haematopoietic stem cell
T-ALL	T-cell acute lymphoblastic leukaemia
TCR	T cell receptor
TFs	transcription factors
TGF-β	transforming growth factor-β
Thy-1.1	thymus cell antigen 1.1
TPO	thrombopoietin
VLA-4	very late antigen-4
WBC	white blood cell
Wnt	wingless
YFP	yellow fluorescent protein
ZEB	zinc finger E-Box binding
ZFP90	zinc finger protein 90
ΔC-fin	lack of zinc finger sequence proximal to C-terminal in *Zeb1*
δEF-1	δ-crystallin enhancer factor 1

CHAPTER 1 : Introduction

1.1 General overview of haematopoiesis

The haematopoietic system is a dynamic and hierarchical tissue system that produces blood and immune cells in the body (Weissman, 2000, Orkin and Zon, 2008, Sun et al., 2014, Dykstra et al., 2007, Eaves, 2015). In the adult bone marrow (BM), there is a rare subset of primitive long-term haematopoietic stem cells (LT-HSCs) that give rise to all blood and immune cells in the body. These LT-HSCs give rise to short-term HSCs (ST-HSCs) which subsequently generate multi-potential progenitors (MPPs). MPPs undergo further differentiation to oligopotent and lineage-restricted progenitors including common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs), and megakaryocytic-erythroid progenitors (MEPs). Finally, these lineage-restricted progenitors produce terminally differentiated mature blood cells including erythrocytes, platelets, granulocytes, dendritic cells (DCs), mast cells, natural killer cells (NKs), monocytes/macrophages, B lymphocytes (B cells), and T lymphocytes (T cells) (Eaves, 2015, Doulatov et al., 2012, Orkin and Zon, 2008).

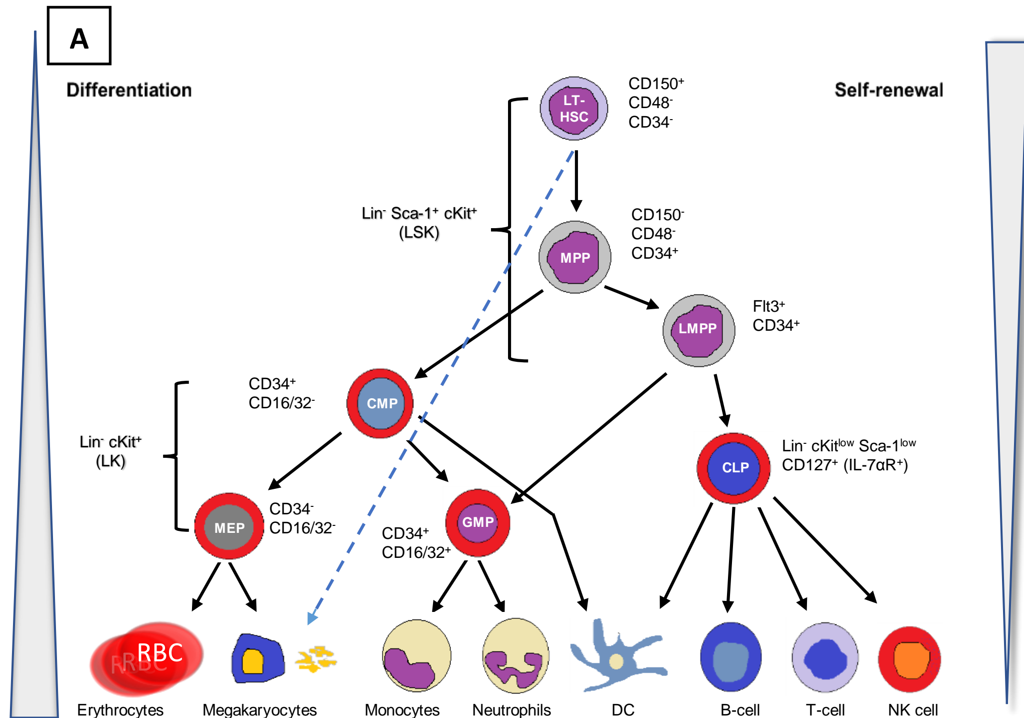
Functional experiments that have been performed on animal models in the last decades suggested a common parental cell at the top of the haematopoietic hierarchy. In 1951, Jacobson and his colleagues rescued a lethally irradiated mouse by transplanting normal adult BM cells that showed both myeloid and lymphoid engraftment (Jacobson et al., 1951). This was further supported by seminal studies of Till and McCulloch and their colleagues when they found colonies on the spleen of irradiated mice after BM transplantation. They examined the colonies using spleen colony forming unit assays and found more than one type of differentiated cells in the colony originating from a cell capable of self-renewal and differentiation (Becker et al., 1963, Siminovitch et al., 1963, Till and Mc, 1961, Wu et al., 1967).

The advent of fluorescence-activated cell sorting (FACS) technology has facilitated a better understanding of the haematopoietic system. It allows for a prospective isolation of HSCs to do single cell transplantation to measure the clonal functionality of HSCs as well as immunophenotypic characterization of HSCs and their progeny after staining the cells with fluorescent labelled antibodies binding to specific cell surface markers (Spangrude et al., 1988, Morrison and Weissman, 1994, Okada et al., 1992, Osawa et al., 1996, Kiel et al., 2005, Morita et al., 2010, Challen et al., 2010, Beerman et al., 2010, Lin and Goodell, 2011, Oguro et al., 2013, Yamamoto et al., 2013). Furthermore, viral genetic barcoding to track HSCs *in vivo* have helped to

better understand HSCs differentiation kinetics and their lineage fates and also to redefine the haematopoietic hierarchy by solving the heterogeneity existing within haematopoietic populations (Lu et al., 2011, Cheung et al., 2013, Gerrits et al., 2010, Rodriguez-Fraticelli et al., 2018, Lu et al., 2019, Pei et al., 2017, Sun et al., 2014, Carrelha et al., 2018, Busch et al., 2015, Azevedo Portilho et al., 2019). Additionally, single cell technologies have improved our understanding of HSC development and function as well as redefining knowledge of HSC differentiation kinetics during steady state and stress haematopoiesis at the genome, transcriptome, proteome, and epigenome level (Jacobsen and Nerlov, 2019, Ye et al., 2017).

1.2 Immunophenotype of haematopoietic cells

Different haematopoietic populations can be studied immunophenotypically using flow cytometry according to their cell surface marker expression. This allows determination of HSC status, lineage commitment, differentiation stage, and terminal maturation. It also allows for prospective isolation of different populations of haematopoietic cells for functional studies including cell transplantation, *in vitro* assays and molecular analysis. Cell surface markers are differentially expressed among distinct populations, so they distinguish stem cells from progenitors, and differentiated cells. Although, the haematopoietic hierarchy exists in mice and human models, there is a considerable difference in the expression of the cell surface markers among the populations in the two species (Figure 1.1 A and B). The differences will be reviewed below in (1.2.3).



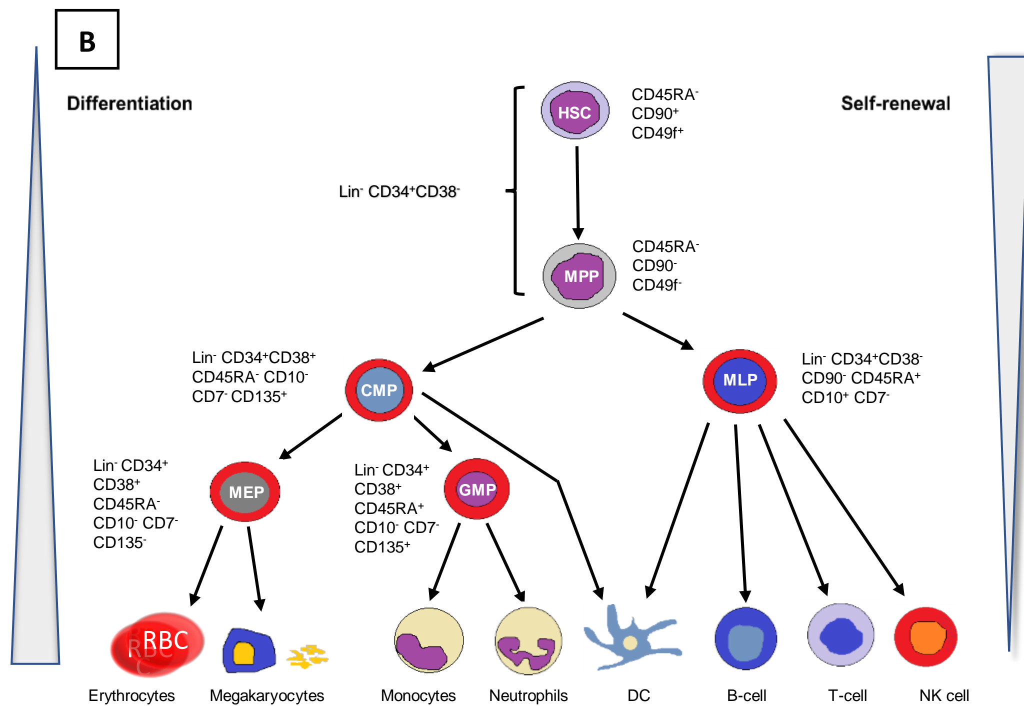


Figure 1.1. The haematopoietic hierarchy. The haematopoietic hierarchy in adult mouse (A) and human (B). Cell surface markers at different developmental stages are shown. LT-HSC: long-term haematopoietic stem cell, MPP: multi-potential progenitor, LMPP: lymphoid-myeloid multipotent progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, GMP: granulocyte-macrophage progenitor, MEP: megakaryocytic-erythroid progenitor, MLP: multi-lymphoid progenitor, DC: dendritic cell, NK: natural killer. The dotted blue line is a newly suggested pathway of direct generation of megakaryocytes from HSCs.

1.2.1 Characterization of adult mouse haematopoietic cells in BM

In the adult mouse BM, HSCs with long-term repopulation capacity were initially identified and enriched within the lineage negative population (cells that do not express mature blood cell markers (known as Lin^-)) combined with expression of stem cell antigen 1 (Sca-1) and low levels of Thy-1.1 (CD90) (Spangrude et al., 1988). Thy1.1 is a cell surface protein that is highly expressed in thymocytes and brain and regulates cell-cell interaction, particularly, in thymus it mediates thymus cells adhesion to thymus epithelia (Haeryfar and Hoskin, 2004). Sca-1 also known as Ly-6A is a cell surface marker that regulates cell signalling and adhesion via modulating receptor tyrosine kinases and Src family kinases (Holmes and Stanford, 2007). Since then more cell surface markers have been used to purify more homogenous HSC populations. In addition to Lin^- $Sca\text{-}1^+$, more purified HSCs have been achieved according to the expression of c-Kit (Okada et al., 1992). C-Kit (CD117) is a cell surface receptor that regulates various aspects of cellular processes upon activation by its ligand stem cell factor (SCF) including apoptosis, differentiation, proliferation, and others (Miettinen and Lasota, 2005). The term LSK will be used from now on to denote to $Lineage^-$, $Sca\text{-}1^+$, $c\text{-}Kit^+$. Work has continued to dissect the LSK population. HSCs and MPPs can be further purified according to the expression of Mac1 and CD4. HSCs are LSK $Thy1.1^{lo}$, $CD4^-$, and $Mac1^-$, while MPPs are LSK $Thy1.1^{lo}$, $CD4^{lo}$, and $Mac\text{-}1^{lo}$ (Morrison et al., 1997, Morrison and Weissman, 1994). LSK has further been divided according to the expression of CD34. CD34 is a cell surface protein that is implicated in the proliferation of stem and progenitor cells (Nielsen and McNagny, 2008). LSK $CD34^-$ mark long-term repopulating HSCs, while LSK $CD34^+$ mark multipotent progenitors (Osawa et al., 1996). LSK $CD34^-$ cells efflux the DNA-binding dye Hoechst 33342 so they are called LSK $CD34^-$ SP (SP: side population) (Goodell et al., 1997) and LSK $CD34^-$ $Flt3^-$ (Adolfsson et al., 2001) have been utilised to purify HSCs. Flt3 denotes for Fms-like tyrosine kinase 3 also known as CD135 is a cell surface receptor that is implicated in proliferation of early BM progenitors specifically in lymphoid specification (Parcells et al., 2006). Mouse ST-HSCs and MPPs are LSK cells and express CD34 and Flt3 but not Thy1.1 with transient reconstitution ability (Adolfsson et al., 2001). Signalling lymphocytic activation molecule (SLAM) family markers: CD150 and CD48 have been used to highly purify HSCs and MPPs. SLAM family is a group of members that control different aspects of lymphocytes such as activation, polarisation, cytotoxicity, and others (Engel et al.,

2003). LT-HSCs are LSK $CD150^+$ $CD48^-$ and MPPs are LSK $CD150^-$ $CD48^-$ (Kiel et al., 2005).

Despite all these strategies to isolate pure HSCs, there is still functional heterogeneity within HSC population with respect to quiescence, cell cycle activity, self-renewal capability, and the ratio of myeloid and lymphoid cells they produce upon transplantation (Wilson et al., 2008, Foudi et al., 2009, Benveniste et al., 2010, Morita et al., 2010, Dykstra et al., 2007, Beerman et al., 2010, Challen et al., 2010). Therefore, in addition to CD150 and CD48, more SLAM markers, CD244, and CD229, have been used to increase HSC purity. These markers divide adult mouse BM LSK cells into seven functionally distinct populations with respect to cell cycle kinetics, self-renewal ability, and repopulation potential upon transplantation (Oguro et al., 2013). HSC1: LSK $CD150^+$ $CD48^{-/low}$ $CD229^{-/low}$ $CD244^-$, HSC2: LSK $CD150^+$ $CD48^{-/low}$ $CD229^+$ $CD244^-$, MPP1: LSK $CD150^-$ $CD48^{-/low}$ $CD229^{-/low}$ $CD244^-$, MPP2: LSK $CD150^-$ $CD48^{-/low}$ $CD229^+$ $CD244^-$, MPP3: LSK $CD150^-$ $CD48^{-/low}$ $CD229^+$ $CD244^+$, HPC1 (haematopoietic progenitor cell 1): LSK $CD150^-$ $CD48^+$, and HPC2 (haematopoietic progenitor cell 2): $CD150^+$ $CD48^+$ (Oguro et al., 2013). Another study to increase the purity of the isolated HSCs using a combination of single-cell transplantation assays and single-cell RNA sequencing linked together with flow cytometric index sorting, found that endothelial protein C receptor (EPCR) is essential to isolate highly pure LT-HSCs, with 67% of LT-HSCs residing within the LSK $CD150^+$ $CD48^-$ $EPCR^{high}$ compartment (Wilson et al., 2015). EPCR is a cell surface receptor that is involved in protein C activation which is required for tissue maintenance against inflammation and coagulation (Esmon, 2004).

Distinct BM progenitors with different lineage choices and kinetics have also been identified. Lympho-myeloid multipotent progenitors (LMPPs) are defined as LSK $CD135^{high}$ ($Flt3^{high}$) and possess myeloid and lymphoid potential but no erythroid or megakaryocytic choices (Adolfsson et al., 2005). Originally, common lymphoid progenitors (CLP) are enriched as Lin^- $c\text{-}Kit^{low}$ $Sca\text{-}1^{low}$ $IL\text{-}7r\alpha^+$ ($CD127^+$) and are found to give rise to B cells ($B220^+$), T cells ($CD4^+$ and $CD8^+$), and NK cells (Kondo et al., 1997b, Coffman and Weissman, 1981, De Rosa et al., 2001). CLP has since been refined according to the expression of CD135 as Lin^- $c\text{-}Kit^{low}$ $Sca\text{-}1^{low}$ $IL\text{-}7r\alpha^+$ $CD135^+$ and have an ability to produce T, B, NK and dendritic cells without myeloid potential (Karsunky et al., 2008). Ly6d has been used to subdivide CLP population into two subsets: $Ly6d^-$ CLP (termed all-lymphoid progenitor ALP) and $Ly6d^+$ CLP (termed B-cell-biased lymphoid progenitor BLP) (Inlay et al., 2009). $Ly6d^-$ CLP cells have a

capacity to produce all lymphoid cells including T and B cells, whereas, $Ly6d^+$ CLP cells are fully committed to B cell lineage and confirmed to be a progeny of $Ly6d^-$ CLP cells (Inlay et al., 2009).

While the pathway of B cell production in the BM via CLPs is well characterised (Kondo et al., 1997b, Inlay et al., 2009, Karsunky et al., 2008), T cell production via BM progenitors are more heterogeneous in terms of the parental progenitor-cell origin/type. As documented previously, CLPs give rise to T cell lineage (Kondo et al., 1997b, Karsunky et al., 2008), though an earlier pathway has been suggested to generate T cells independent of CLPs (Allman et al., 2003, Kondo et al., 1997b). LMPPs expressing CD127 (LSK $CD135^+$ $CD127^+$) have been found to efficiently generate T cells faster than CLPs (Ghaedi et al., 2016).

Common myeloid progenitors (CMP) can be isolated as Lin^- IL-$7r\alpha^-$ c-Kit^+ Sca-1^- $CD34^+$ $CD16/32^-$ to produce all myeloid progenitors including GMP (Lin^- IL-$7r\alpha^-$ c-Kit^+ Sca-1^- $CD34^+$ $CD16/32^+$) and they give rise to monocytes ($Mac1^+$), granulocytes ($Mac1^+$ $Gr1^+$). MEPs are defined as Lin^- IL-$7r\alpha^-$ c-Kit^+ Sca-1^- $CD34^-$ $CD16/32^-$ and produce erythroid cells ($Ter119^+$) and megakaryocytes ($CD41^+$) (Akashi et al., 2000, Koulnis et al., 2011, Chen et al., 2007, Lagasse and Weissman, 1996, Sunderkotter et al., 2004). Recently, *in situ* lineage tracing studies found that megakaryocyte lineage is generated from HSCs independent from MPPs and MEPs (Rodriguez-Fraticelli et al., 2018, Chapple et al., 2018, Carrelha et al., 2018).

1.2.2 Characterization of adult human haematopoietic cells

Human haematopoietic surface markers differ significantly from their mouse counterparts particularly in the HSPC population (Figure 1.1 A and B). The most critical surface marker is CD34 that is expressed in human LT-HSCs, ST-HSCs, and some lineage-committed progenitors (Krause et al., 1996, Andrews et al., 1989). However, there is a subset of human HSCs that do not express CD34 and showed low clonogenicity unveiled in *in vitro* studies (Bhatia et al., 1998). Human LT-HSCs in cord blood and BM are enriched as Lin^- $CD34^+$ $CD38^-$, however, their enrichment based on $CD34^+$ $CD150^+$ $CD48^-$ contributed equally or less to long-term repopulation *in vivo* and colony formation *in vitro* (Larochelle et al., 2011). Because $CD34^+$ cells

are heterogeneous, containing both stem and progenitor cells, researchers have further purified this population to isolate LT-HSCs and have succeeded in purifying HSCs from human cord blood as Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90/Thy1^+$ $Rhodamine123^{Low}$ $CD49f^+$ with long-term multi-lineage reconstitution ability in NOD-scid-$IL2Rgc^{-/-}$ (NSG) mice (Notta et al., 2011). Also, expression of Flt3 is found to mark human HSCs within $CD34^+$ (Sitnicka et al., 2003). Further, c-Kit expression has been used to separate Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^+$ into two subpopulations: intermediate $c\text{-}Kit^{int}$ and high $c\text{-}Kit^{high}$ where Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^+$ $cKit^{int}$ HSCs have a higher engraftment capacity than Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^+$ $cKit^{high}$ HSCs (Grinenko et al., 2014, Cosgun et al., 2014). Upon differentiation, MPPs are defined as Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^-$ $CD49f^-$ (Notta et al., 2011). More committed progenitors including CMPs are enriched as: $CD34^+$ $CD38^+$ $CD10^-$ $FLT3^+$ $CD45RA^-$ (Notta et al., 2016), CLPs: Lin^- $CD34^+$ $CD10^+$ (Galy et al., 1995) and $CD24^-$ (Six et al., 2007), GMPs: $CD34^+$ $CD38^+$ $Thy\text{-}1^-$ $CD45RA^+$ $Flt3^+$ $CD7^-$ $CD10^-$ (Doulatov et al., 2010), and MEPs: $CD34^+$ $CD38^+$ $Thy\text{-}1^-$ $CD45RA^-$ $Flt3^-$ $CD7^-$ $CD10^-$ (Doulatov et al., 2010, Notta et al., 2016).

1.2.3 Immunophenotypic differences between murine and human HSC

Even though human and mouse present a similar pattern of hierarchical haematopoietic differentiation where a stem cell resides at the apex of the hierarchy, differences are present in terms of heterogeneity of HSC pool that needs to be resolved as well as the selection of markers used to study different haematopoietic populations. Such immunophenotypic differences include using CD150 to purify mouse LT-HSCs but it is not used to isolate human HSC (Larochelle et al., 2011). Also, Flt3 expression is found in human HSCs but not murine LT-HSCs (Sitnicka et al., 2003, Adolfsson et al., 2001). Further, high expression of c-Kit marks mouse LT-HSCs while intermediate expression marks human HSCs (Grinenko et al., 2014). More importantly, human HSPCs express CD34 while mouse LT-HSC are negative for CD34 (Andrews et al., 1989, Osawa et al., 1996).

1.3 Clonal dynamics of adult steady-state haematopoiesis

Even though the haematopoietic system is considered one of the most organised systems in the body and HSC differentiation kinetics has been extensively studied in the past several years (Dykstra et al., 2007, Gerrits et al., 2010, Oguro et al., 2013), still more work to be done to solve the functional heterogeneity within the HSPC population and to understand the nature of clonal dynamics of haematopoiesis. HSC functionality and contribution to long-term multilineage haematopoiesis has been clarified by single cell transplantation (Oguro et al., 2013, Yamamoto et al., 2013, Dykstra et al., 2007). Notably, transplanted HSCs show qualitative and quantitative differences in terms of self-renewal status and proliferation kinetics as well as the effect of the injured BM niche due to irradiation on the behaviour of the transplanted HSCs; hence, data from *in vivo* HSC transplantation does not reflect the nature of haematopoiesis during homeostasis (Busch and Rodewald, 2016, Mendelson and Frenette, 2014). The extent of long-term contribution of HSCs to adult steady state haematopoiesis is still controversial and efforts are on-going to solve the puzzle (McRae et al., 2019). Recent emerging studies using *in situ* non-invasive lineage-tracing models have been carried out to unravel the dynamics of long-term unperturbed haematopoiesis and appreciate the molecular mechanism governing adult steady-state haematopoiesis (McRae et al., 2019, Azevedo Portilho et al., 2019, Zhang et al., 2018b, Cheng et al., 2019).

Some groups have suggested that adult steady state haematopoiesis is mainly driven by multipotent progenitors (MPPs), with little contribution from HSCs (Sun et al., 2014, Busch et al., 2015). Using a doxycycline (Dox)-inducible transposon labelling system, Sun et al reported labelling efficiency of about 30% of HSCs, MPPs, and myeloid progenitors all together and their data showed that LT-HSCs contributed less than 5% to PB lineages and MPPs, whereas MPPs contributed to about 50% of mature blood cells at 40 weeks post Dox removal, suggesting that MPPs actively contribute to adult unperturbed haematopoiesis (Sun et al., 2014). However, this study lacks specificity for HSC labelling. Their labelled cells include LT-HSC, MPPs, and myeloid progenitors; hence, because HSC number is very low, their labelling system may underestimate HSCs contribution. However, a subsequent study supported the idea that native haematopoiesis depends on MPPs (Busch et al., 2015). Busch et al used Tie2-Cre system to specifically knock in fluorescent YFP into HSCs. They achieved 1% labelling of HSCs and tracked the lineage output of the tagged HSCs but they did not observe balanced tags between HSC and mature cells throughout the life of mice

suggesting that native haematopoiesis is driven by intermediate progenitors rather than LT-HSC (Busch et al., 2015). The limitation of this study is that the tagged HSCs only 1% may not be representative of all LT-HSC.

In contrast, recent studies have shown that HSCs are the major contributor to the unperturbed adult haematopoiesis (Chapple et al., 2018, Sawen et al., 2018, Sawai et al., 2016). Using Pdzk1ip1-CreER mice, Sawai et al reported an average of 32.6% and 3% labelling of HSCs and MPPs, respectively, after tamoxifen injection and analysis at 36 weeks after HSC tagging showed about 60% labelling in all lineage restricted progenitors (Sawai et al., 2016). This observation was further supported when Krt18-CreERT2 and Fgd5-CreER transgenic system were used (Chapple et al., 2018). About 2% and 32% HSC labelling efficiency was achieved using Krt18-CreERT2 and Fgd5-CreER, respectively. Analysis one year post tagging showed 50-60% labelling in lymphoid and myeloid lineages with striking labelling of platelets about 100% from Krt18-CreERT2 (Chapple et al., 2018). Using Fgd5-CreER, Sawen et al supported the observation that HSCs are major contributor to native haematopoiesis (Sawen et al., 2018). This difference in conclusions between groups is caused by different labelling techniques they used and more importantly the specificity of the technique to label HSCs or multipotent progenitors as all Cre-based labelling techniques labelled some downstream progenitors (McRae et al., 2019). Clearly more work is needed to clarify these conflicting results and elucidate the cellular and molecular mechanisms governing adult steady-state haematopoiesis and the extent of HSC or MPP contribution to adult haematopoiesis under physiologic conditions.

1.4 HSC ageing

Like other somatic cell types, in accordance with the Hayflick limit (Shay and Wright, 2000), HSCs can be negatively affected by ageing (de Haan and Lazare, 2018). A lineage tracing study revealed a decrease in clone numbers in PB and BM in aged mice and showed more functional heterogeneity of the HSC pool associated with slow cell division (Ganuza et al., 2019). This is consistent with previous reports that showed perturbed functional properties of HSCs during ageing (Dykstra et al., 2011, Florian et al., 2012, Chambers et al., 2007). Aged mice show expansion of HSC number with defect in their repopulation capacity, skewing to myeloid lineage, reduction in lymphoid outputs, increased mobilisation from the BM to PB, and a decrease in

homing capacity to the BM after transplantation (Florian et al., 2012, Dykstra et al., 2011, Sawen et al., 2018, Xing et al., 2006, Liang et al., 2005). Also, aged HSCs show DNA damage, senescence, activation of polarity, and impairment of mitochondrial activity (de Haan and Lazare, 2018). Ageing HSCs express high levels of CDC42, a polarity marker of HSCs, and show a dysregulated polarity signature such as CDC42, tubulin, and Per-2 resulting in apolar and non-functional HSCs in terms of repopulation toward BM progenitors and PB (Florian et al., 2012). Pharmacological inhibition of CDC42 reversed ageing HSC defects, allowing them to behave like their young HSC counterparts (Florian et al, 2012). Aged HSCs with changes in cell polarity preferentially self-renew via symmetric divisions that cause expansion of HSC numbers at the expense of differentiation activity (Florian et al., 2018). Further studies are needed to clarify the molecular drivers of ageing in HSCs and to solve the heterogeneity of HSCs in aged mice.

1.5 HSC characteristics and regulation

Adult HSCs in the BM are multipotent and unspecialized cells that have the ability to self-renew to maintain sufficient quantity of HSCs via symmetrical divisions and to differentiate into all functionally and morphologically different blood cells including red blood cells (RBCs), platelets, neutrophils, basophils, eosinophils, mast cells, monocytes, macrophages, NKs, DCs, B cells, and T cells via asymmetrical divisions. Most of the HSCs are in a quiescent state (G0) in the BM niches, and they rarely divide to maintain the HSCs pool and to compensate the loss of cells that results from apoptosis and differentiation (Cheshier et al., 1999, Boulais and Frenette, 2015). These properties of being quiescent, self-renewing, and differentiating are highly regulated by transcription regulators that control gene expression under the influence of signalling pathways, and microenvironments (stem cell niches) to prevent the exhaustion of HSCs and to ensure the lifetime supply of blood.

1.5.1 HSC maintenance

Maintaining the balance between HSCs self-renewal and differentiation is one of the most critical aspects of HSC regulation in the adult bone marrow to sustain blood production throughout life. Transcription factors (TFs) are critical to gene activation and are intrinsic master regulators of HSC behaviour in the BM (Teitell and Mikkola, 2006). Gene knockout (KO) studies have revealed few TFs that are absolutely required for steady-state adult HSC maintenance including *Gata2*, *Cited2*, *Tel/Etv6*, and *Nkap*. Haploinsufficiency of *Gata2* causes a small primitive stem cell with an increase in cell quiescence, tendency to undergo apoptosis and a reduced reconstituting capacity after transplantation (Rodrigues et al., 2005). Conditional deletion using the Mx1-Cre system in adult murine haematopoietic system of *Gata2*, *Cited2*, *Tel/Etv6*, or *Nkap* resulted in complete loss of HSCs (Menendez-Gonzalez et al., 2019, Kranc et al., 2009, Hock et al., 2004b, Pajerowski et al., 2010). However, these TFs apparently regulate HSC function in different manners. *Cited2* was found to regulate HSC behaviour via cell cycle regulators Ink4a/Arf and Trp53, while *Gata2* and *Tel/Etv6* were shown to modulate survival of HSCs and *Gata2* also was shown to regulate quiescence of HSCs (Hock et al., 2004b, de Pater et al., 2013, Rodrigues et al., 2005). *Nkap* was found to regulate HSCs via modulating their survival and proliferation via p21Cip1/Waf1 and p19Ink4d (Pajerowski et al., 2010).

Other transcriptional regulators have been found to regulate HSC function and maintenance. The Growth factor independence 1 (*Gfi 1*) has been found to have an essential role in maintaining HSC number by regulating the self-renewal ability and suppressing proliferation and controlling the long-term repopulation capacity (Zeng et al., 2004). The proto-oncogene *c-Myc* has been implicated in the regulation of adult HSC self-renewal and differentiation via the interaction between HSCs and their niches. *c-Myc*$^{-/-}$ mice showed severe cytopenia, retention of HSCs in the bone marrow, and upregulation of N-cadherin and other stromal adhesion receptors, whereas, overexpression of *c-Myc* led to a loss of self-renewal and increased differentiation of HSCs (Wilson et al., 2004). *c-Myb* is another regulator of self-renewal and lineage differentiation of HSCs. Its deletion in bone marrow HSCs resulted in a reduction of HSC pool via loss of self-renewal and increased differentiation (Lieu and Reddy, 2009). Several Ets TFs have been implicated in HSC haemostasis. For example, *Pu.1* is shown to be necessary for the maintenance of HSC pool in the bone marrow as well as the differentiation into common myeloid and lymphoid progenitors and further maturation of more restricted myeloid progenitors

but not the lymphoid cells (Iwasaki et al., 2005). Other Ets TFs are *Erg* and *Gabp* have been found to regulate adult HSCs self-renewal and differentiation (Ng et al., 2011, Yu et al., 2011). The TF *Evi1*, ecotropic viral integration site 1, had been demonstrated to regulate the balance between HSCs self-renewal and differentiation. *Evi1*$^{+/-}$ mice exhibited a loss of self-renewal activity, while, *Evi1* overexpression led to a suppression of lineage commitment and enhanced self-renewal capacity (Kataoka et al., 2011). *Twist*‐*1* is a TF that belongs to the basic helix-loop-helix (bHLH) family is found to be a critical regulator of HSC self-renewal and specification to myeloid lineage (Dong et al., 2014). *Slug* is a TF belongs to Slug/Snail family of zinc-finger TFs. *Slug*$^{-/-}$ mice showed enhanced HSC self-renewal and repopulation capacity (Sun et al., 2010). Additionally, zinc finger protein 90 (*Zfp90*) is shown to regulate HSC function and its loss in adult HSCs impaired their number and repopulation capacity after transplantation (Liu et al., 2018). Further, loss of the plant homeodomain 6 gene (*Phf6*) and Inhibitor of DNA binding (*Id1*) in adult HSC has been shown to enhance HSC self-renewal and long-term reconstitution via promoting quiescence and protection against DNA damage (Wendorff et al., 2019, Singh et al., 2018).

1.5.2 Cell Cycle Regulation of HSCs

The cell cycle activity in an organism is controlled throughout life in a dynamic manner in response to developmental needs. In the adult BM, most HSCs are in a quiescent state (G0). *In vivo* studies using retention of 5-bromodeoxyuridine (BrdU) and histone 2B (H2B)-green fluorescent protein (GFP) fusion protein showed that mouse adult dormant HSCs (Lin$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^+$ CD48$^-$) are heterogeneous in their proliferation rate and 20% of them divide at a very low rate < or = 0.8 - 1.8% /day (Foudi et al., 2009). Also, dormant HSCs (Lin$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^+$ CD48$^-$ CD34$^-$) were found to divide once every 145 days with a high potential for long-term repopulation (Wilson et al., 2008).

Quiescence is essential for HSC survival and maintenance as they show kinetics different than cycling HSCs that help to protect their genome status and reduce their entry into cell cycle (Szade et al., 2018). Such characteristics that are unique for quiescent HSCs to maintain their integrity and ability to sustain blood production include their localisation in a hypoxic niche in the BM, using glycolysis for their energy instead of oxidative phosphorylation, and having low mitochondrial activities such as

low levels of reactive oxygen species (ROS) and low baseline and maximal respiration (Snoeck, 2017, Ito, 2018, Szade et al., 2018). This mitochondrial activity in HSCs is highly regulated by autophagy, a maintenance process that use lysosomes to degrade damaged mitochondrial components (Ito, 2018). Thus, perturbations in the mitochondrial activities enhance HSC entry into cell cycle and differentiation and eventually exhaustion (Ito, 2018).

Quiescence of HSCs in the bone marrow is regulated by intrinsic (TFs) and extrinsic (cytokines and cell-cell signalling) elements. *Scl* is highly expressed in quiescent LT-HSCs and is found to be an inhibitor of the transition from G0 to G1 as well as its role in preserving the LT-HSCs repopulation potential (Lacombe et al., 2010). *Gata2* also is an important regulator of HSC quiescence. Enforced expression of *GATA2* in human HSCs is found to increase the quiescence and block haematopoiesis *in vivo* (Tipping et al., 2009). In contrast, as mentioned above, haploinsufficiency of *Gata2* causes a small primitive stem cell pool characterised by an increase in cell quiescence (Rodrigues et al., 2005). These data indicate that *Gata2* is an essential regulator of haematopoiesis and its highly balanced levels play an important role in different stem cell functions. Retinoblastoma family members *R*b, *p107*, and *p130* are found to regulate adult HSCs quiescence in which the deletion of the three members results in a loss of quiescence, increased expansion of HSCs, and impaired the repopulation capacity (Viatour et al., 2008). *Egr1*, is a member of immediate response TF family, regulates HSCs quiescence. *Egr1* knockout increases the number of cycling HSCs and enhances HSCs migration into peripheral blood (PB) without impairment of LT-HSCs repopulation ability in primary recipients (Min et al., 2008).

Progression through the cell cycle is regulated including cyclins and a family of serine/threonine protein kinases, cyclin-dependent kinases (CDKs) as well as Cdk inhibitors (CKIs) (Hao et al., 2016). The entry into the cell cycle requires activation of CDK4/6/cyclin D by inhibition of the retinoblastoma proteins by phosphorylation that modulates the G1-S transition (Malumbres et al., 2004, Cooper and Shayman, 2001). LT-HSCs lack CDK6 whereas ST-HSCs express high levels of CDK6 that enhances a quick entry into the cell cycle upon mitogenic stress (Laurenti et al., 2015). The cyclin D family includes cyclin D1, cyclin D2, and cyclin D3, all of them are expressed in HSCs (Passegue et al., 2005). The transition from G1 to S phase is initiated by CDK2/cyclin E binding and it is maintained through S phase by CDK2/cyclin A (Aleem et al., 2005). CDK1 is the only essential CDK that can drive mammalian cell division.

It initiates the transition to G2 and M phase via binding to cyclin A and cyclin B, respectively (Santamaria et al., 2007).

CKIs are proteins that block the activity of the kinase in cyclin/CDKs complexes to arrest the cell cycle in G1 phase. There are two families including INK4 (Inhibitor of CDK4) and CIP/KIP (CDK2 interacting protein/kinase inhibition protein). INK4 family includes p16, p15, p18, and p19, which negatively regulate cyclin D/CDK4/6 complex by inhibiting phosphorylation of Rb proteins and subsequently blocking the entry into S phase. *p19* is a critical regulator of HSC in G0 and inhibits G0\G1 transition. *p19*$^{-/-}$ bone marrow showed a decreased number of quiescent HSCs, increased apoptosis after genotoxic stress due to exhaustion of highly cycling HSCs (Hilpert et al., 2014). CIP/KIP family consists of p21, p27, and p57, which represses cyclin E/CDK2 complex, hence, inhibits entry into S phase and preserve stem cell quiescence (Hao et al., 2016). *p21* is an important regulator of HSC homeostasis and maintenance of stem cell pool. *p21*$^{-/-}$ mice showed increased HSC proliferation and impaired self-renewal capacity in transplanted mice due to HSC exhaustion which resulted in reduced survival of recipient mice to one month after transplantation (Cheng et al., 2000b). Unlike *p21*$^{-/-}$, *p27*$^{-/-}$ mice demonstrated increased active progenitor cell number but not stem cells which had ability to regenerate upon serial transplantation and improved survival rates when compared to *p21*$^{-/-}$ mice (Cheng et al., 2000a). Another regulator of HSC quiescence is *p57*. It is the most abundant cell cycle inhibitor expressed in quiescent HSCs (Matsumoto et al., 2011). HSCs that lack *p57* exhibited a reduction of G0 HSCs, impairment of self-renewal ability, and repopulation defect in lethally irradiated mice after week 16 (Matsumoto et al., 2011).

1.5.3 BM niche

During adult steady state haematopoiesis, HSCs in the BM niche reside in the vascular niche and are regulated directly or indirectly by a variety of cells such as mesenchymal stromal cells (MSC), endothelial cells (ECs), osteoblasts (OBs), megakaryocytes, monocytes, macrophages, Schwann cells, CXCL12-abundant reticular cells (CAR cells), leptin receptor-expressing cells (LEPR^{+} cells) and others (Figure 1.2) (Crane et al., 2017). BM niche factors are essential for HSC maintenance such as SCF (Asada et al., 2017), CXCL12 (Sugiyama et al., 2006, Asada et al., 2017), and thrombopoietin (Yoshihara et al., 2007). Deep imaging analysis of BM

niche found more than 80% of dormant and cycling HSCs expressing c-Kit and α-catulin reside near sinusoids, while about 15% near arterioles and few dividing HSCs in endosteal niche (Crane et al., 2017, Acar et al., 2015). Thus, the interaction of HSCs and stromal cells in perivascular niches (sinusoidal and arteriolar) is essential for their functionality and regulates the balance between self-renewal and differentiation.

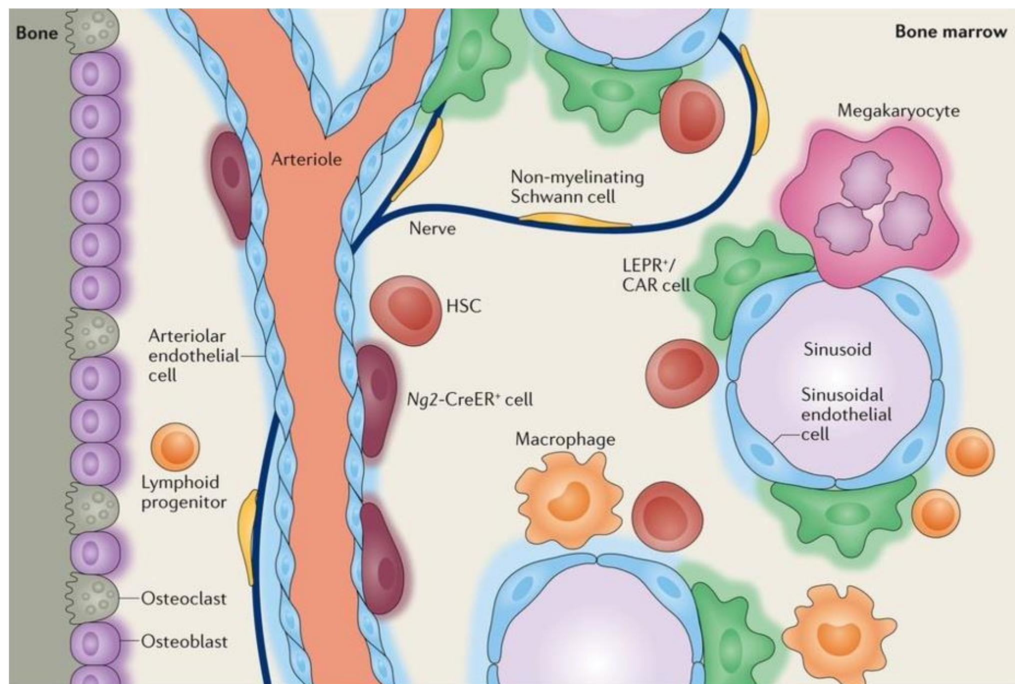


Figure 1.2. Adult BM niche. HSCs resides in the perivascular niche (sinusoidal and arteriolar) and interact with various niche cells: mesenchymal stromal cells (MSC), endothelial cells (ECs), osteoblasts (OBs), megakaryocytes, monocytes, macrophages, Schwann cells, CXCL12-abundant reticular cells (CAR cells), leptin receptor-expressing cells (LEPR$^+$ cells). The figure adapted from Crane et al., 2017.

HSPCs in BM niche are located in hypoxic environment in which HSPCs retain pimonidazole (Pimo), a standard hypoxic marker, and express hypoxia-inducible factor 1 alpha (Hif1α) (Nombela-Arrieta et al., 2013). Hif1α is found to control the generation of embryonic HSCs in hypoxic sites (Imanirad et al., 2014). However, its role in adult HSCs remains to be confirmed as two studies that use the same mouse model and same system for conditional deletion of *Hif1α* (*Hif1α$^{fl/fl}$ Mx1-Cre*) found conflicting results and conclusions (Takubo et al., 2010, Vukovic et al., 2016). According to Takubo et al, HSCs lacking *Hif1α* show differentiation defects after transplantation and a reduction in their number due to increased cell cycle (Takubo et al., 2010). Conversely, the data from Vukovic et al shows that the function of *Hif1α*-deficient HSCs is normal (Vukovic et al., 2016). This discrepancy in conclusion may relate to the experimental design used between the two studies (Sykes, 2016). However, even though there are different conclusions about the role of *HIF1α* in HSCs after loss of function studies, overexpression and stabilising *HIF1α* protein in HSCs enhances their homing and migration and repopulation capacity via upregulation of the chemokine CXCR4 (Speth et al., 2014).

The complex environment of the niche is crucial for HSC quiescence, maintenance, and function as well as physical and chemical support (Suda et al., 2011). Osteoblasts are thought to regulate HSCs quiescence by producing osteopontin, a negative regulator of HSC expansion (Stier et al., 2005), as well as angiopoietin-1 and thrombopoietin that bind the tyrosine kinase receptor Tie2 and thrombopoietin receptor MPL expressed in HSCs, respectively (Arai et al., 2004, Yoshihara et al., 2007). However, studies using conditional deletion of *Cxcl12* or *Scf*, critical factors for HSC regulation, in mature osteoblasts showed normal function and frequency of HSCs and no effect on HSCs mobilisation (Greenbaum et al., 2013, Ding and Morrison, 2013, Ding et al., 2012). This suggests that osteoblasts are not an essential source of CXCL12 and SCF for HSC regulation. Perivascular cells expressing CXCL12, known as CXCL12-abundant reticular (CAR) cells in vascular and endosteal niches have also been found to regulate HSC quiescence by binding to the CXCR4 receptor in HSCs (Sugiyama et al., 2006). Deletion of *Cxcl12* from arteriolar $NG2^+$ cells resulted in a decrease in HSC number (Asada et al., 2017). SCF is an essential component for HSC regulation produced from variety of BM niche cells. Inactivation of Scf in $LepR^+$ cells led to a reduction in HSC number (Asada et al., 2017). However, deletion of *Cxcl12* or Scf in perivascular cells resulted in a decline in HSC number (Asada et al., 2017). Sympathetic nerves in the BM niche control HSC mobilisation from the BM niche in response to granulocyte colony-stimulating factor (G-CSF)-mediated mobilisation and this is dependent on norepinephrine (NE) signalling (Katayama et al., 2006). Furthermore, non-myelinating Schwann cells regulate HSC maintenance in the BM via TGF-β/Smad signalling (Yamazaki et al., 2011). Macrophages are also shown to maintain the number of HSCs in their niches and depletion of macrophages results in HSC mobilisation to PB (Winkler et al., 2010). This tight regulation of HSCs in the BM niche is essential for controlling HSC function and survival.

1.6 *Zeb1*

1.6.1 *Zeb1* structure

The Zinc Finger E-Box Binding homeobox (ZEB) family consists of two structurally highly similar TFs : ZEB1 (also known as δEF-1, TCF8, BZP, ZEB, AREB6, NIL-2-A, Zfhep, and Zfhx1a) and ZEB2 (also known as SIP1, KIAA0569 and Zfhx1b) (Figure 1.3) (Fortini et al., 1991)(Gheldof et al., 2012). Both genes are structurally similar containing two zinc finger DNA binding domains at C-and N-terminals as well as functional central domains including homeodomain (HD), Smad protein binding (SBD) and CtBP interaction domains (CID) encoding relatively large protein size 1117 and 1214 amino acids for ZEB1 and ZEB2 , respectively (Fortini et al., 1991, Vandewalle et al., 2009); however, ZEB2 possesses NuRD interaction domain at N-terminal domain which differentiates it from ZEB1 (Verstappen et al., 2008).

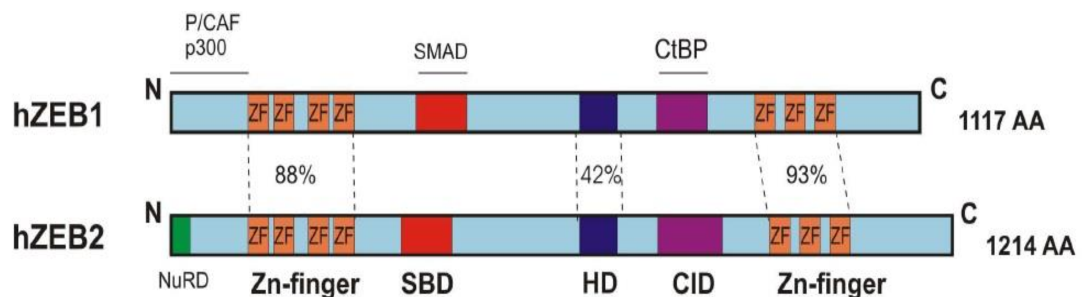


Figure 1.3. ZEB1 and ZEB2 structure. They consist of functional domains Zinc-finger (Zn-finger), homeodomain (HD), Smad protein binding (SBD) and CtBP interaction (CID) domains. Figure adapted from (Goossens and Haigh J, 2012).

ZEB1 and ZEB2 were first discovered in *Drosophila melanogaster* named as zfh1 and zfh2, respectively. ZEB1 gene is located on chromosome 10 (10p 11.2) in humans (Williams et al., 1992) and is found to have a product of nine C2H2 zinc fingers and one homeodomain, while zfh-2 is shown to produce a product with three homeodomains and sixteen H2C2 zinc fingers (Fortini et al., 1991). ZEB1 and ZEB2share a high degree of sequence similarity within zinc finger domains at N-terminal (four zinc fingers, 88%) and C-terminal clusters (three zinc fingers, 93%) which suggests that they can independently bind 5'-CACCT (G)-3' sequences in different promoter regions (Verschueren et al., 1999, Remacle et al., 1999). *Zeb1* (δEF1) consists of nine exons. Exons 5-7 encode for N-terminal zinc finger domain while 8 and 9 exons encode for C-terminal zinc finger domains (Sekido et al., 1996). The zinc finger domains at N-terminal and C-terminal of ZEB1 and ZEB2 proteins are responsible for DNA binding to target genes at the 5'-CACCT (G)-3' sequences, while the homeodomain is responsible for protein-protein interaction (Vandewalle et al., 2009). SBD is another domain that is located near N-terminal zinc finger domain of

ZEB1 and ZEB2. Unlike the zinc finger domains, homeodomain and SBD are structurally different between ZEB1 and ZEB2 (Gheldof et al., 2012). Interactions of ZEB1 and ZEB2 with Smad proteins which are signal transducers downstream of TGF-β receptors, is mainly occur through SBD domain (Miyazono, 2000). However, other domains seem less conserved and different between ZEB1 and ZEB2 and they function as recruiting domains for co-repressors such as CtBP (carboxyl-terminal binding protein) (Postigo and Dean, 1999b, van Grunsven et al., 2007) or co-activators such as p300 or P/CAF (van Grunsven et al., 2006, Postigo et al., 2003). Preferential recruitment of co-repressors (CtBP) and co-activators (p300 or P/CAF) between ZEB1 and ZEB2 results in opposing functions of these proteins. ZEB1 binds TGF-β-activating Smad proteins and p300 to activate TGFβ signalling, while ZEB2 recruits CtBP to inhibit TGFβ signalling (Postigo et al., 2003). In ZEB2 but not ZEB1, there is a recruiting domain for nucleosome remodelling and deacetylase complex (NuRD) which mainly serve as a co-repressor factor (Verstappen et al., 2008). Although there is a similarity in the zinc finger sequences between ZEB1 and ZEB2 which suggests a similar function of ZEB1 and ZEB2, the differences in homeodomain, SBD, and co-factors-recruiting domains may explain possible functional disparities.

1.6.2 ZEB1 expression in normal physiology

Expression of ZEB1 in mouse embryos has been investigated by *in situ* hybridization (Takagi et al., 1998). During early development at E8.5, high ZEB1 expression is found in the head fold with less expression in presomitic mesoderm (Takagi et al., 1998). ZEB1 expression also is found in neural crest derivatives and limb buds at E9.5 and the expression is found at subsequent developmental stages at E11.5 in neural tube, myotomes, limbs, and cartilages (Takagi et al., 1998). Conventional germline *Zeb1* knockout mice show defects in skeletal system (craniofacial, limbs, sternum, ribs), chondrocyte and T cell development (Takagi et al., 1998). They also display oedema, curled tail, and a propensity to haemorrhage. More importantly, *Zeb1* mutants develop until birth but they die as a result of inability to breathe. *Zeb1* heterozygous mice are healthy and do not develop any abnormalities (Takagi et al., 1998). However, null and heterozygous *Zeb1* mutant mice show same corneal defect patterns that resemble posterior polymorphous corneal dystrophy (PPCD) in humans (Liu et al., 2008).

Zfh-1, the Drosophila homologue of *Zeb1*, functions as an essential regulator for myogenesis (Postigo et al., 1999) and neuron development and differentiation of *Caenorhabditis elegans* (Clark and Chiu, 2003). In vertebrates, *Zeb1* has been implicated in post-gastrulation embryogenesis of chicken embryos by binding and repressing δ1-crystallin enhancer and its expression is documented in mesodermal tissues, nervous system, and the lens (Funahashi et al., 1993). However, lack of ZEB1 expression was observed in lens cells during mouse embryogenesis (Takagi et al., 1998). Moreover, *Zeb1* negatively regulates muscle differentiation by binding and repressing muscle genes such as *Mef-2*, *α4-integrin*, and *α7-integrin* (Postigo and Dean, 1997, Postigo and Dean, 1999a, Jethanandani and Kramer, 2005).

ZEB1 expression in adult and foetal human tissues has been reported (Hurt et al., 2008). RNA Master Tissue Blot has been used to test mRNA ZEB1 expression in adult and foetal human tissues and revealed high ZEB1 expression in adult bladder, uterus, thymus, aorta, and skeletal muscles, while in foetal tissues high ZEB1 expression was reported in lung, heart, and thymus (Hurt et al., 2008).

1.6.3 *Zeb1* in EMT and MET

Epithelial to mesenchymal transition (EMT) is a biological process that is implicated in the development of tissues and organs during embryogenesis including mesoderm formation and neural crest delamination (Kalluri and Weinberg, 2009). MET (mesenchymal to epithelial transition) is a reverse process of EMT characterised by downregulation of mesenchymal genes and upregulation of epithelial genes and at the end the cells acquire an epithelial phenotype (Samavarchi-Tehrani et al., 2010). *Zeb1* binds through its zinc finger sequence to the E-box clusters of epithelial genes repressing them and initiating the mesenchymal state (Postigo et al., 1999). E-cadherin, a major cell-cell adhesion molecule and a tumour cell invasion and metastasis suppressor, is an essential gene for EMT (Eger et al., 2005). After *Zeb1* and other EMT inducers repression of epithelial genes, epithelial cells undergo series of changes including alterations in apical-basal cell polarity, losing the adhesion property and becoming motile, acquiring the ability to migrate (Greenburg and Hay, 1982). These changes result from molecular changes including upregulation of mesenchymal markers (N-Cadherin, vimentin, fibronectin, ZEB1, and others),

downregulation of epithelial genes, and changes in cytoskeleton signature (Kalluri and Weinberg, 2009).

EMT is classified into three types with distinct functional outputs. Type1 EMT is responsible for embryogenesis, organ formation, and production of mesenchymal cells (Kalluri and Weinberg, 2009). Type 2 EMT is a process that is implicated in tissue maintenance and regeneration, for example, in wound healing and inflammation; however, this may lead to tissue fibrosis if the inflammation persists becomes protracted (Kalluri and Weinberg, 2009). Type 3 EMT is mainly associated with cancer stem cells (CSC) acquiring mesenchymal state and enhancing cancer invasiveness and metastasis (Kalluri and Weinberg, 2009). EMT is driven by signalling pathways, such as Wnt, TGF-β, Notch pathway (Liu et al., 1999, Thiery and Sleeman, 2006), TFs such as *Zeb1*, *Snail*, *Eomes*, *Mesps*, and others that induce EMT via regulation of cell polarity, cytoskeleton, and cell adhesion (Lindsley et al., 2008, Arnold et al., 2008, Nieto, 2002, Eger et al., 2005).

In addition to EMT and MET processes, a partial (intermediate or hybrid) EMT process can occur where the cells simultaneously acquire both epithelial and mesenchymal phenotypes where intermediate levels of ZEB1 is required to maintain partial EMT (Jolly et al., 2015, Lu et al., 2013, Jia et al., 2017). Partial EMT is found in the context of cancer and characterised by a collective cell migration to form circulating tumour cell clusters (CTC clusters) (Lecharpentier et al., 2011). CTC clusters are resistant to apoptosis and have enhanced metastatic capacity compared to single migrating cells and showing stemness features (Aceto et al., 2014, Grosse-Wilde et al., 2015).

EMT is also implicated in tumour progression and metastasis. Aberrant activation of EMT in epithelial cells and high ZEB1 expression are found in solid tumours and associated with more stem, aggressive, invasive, poor outcomes, and drug resistant cancer stem cells (CSCs) via repressing the tumour suppressor E-cadherin, polarity genes such as Crumbs3, HUGL2 and PATJ, and microRNAs such as miR-203 and the miR-200 family (Zhang et al., 2015a). High expression of ZEB1 is reported in different cancer types that are characterised by a poor prognosis including breast (Eger et al., 2005, Chaffer et al., 2013, Zhou et al., 2017), colorectal (Spaderna et al., 2008, Zhang et al., 2013a, Yuan et al., 2019), pancreatic (Bronsert et al., 2014), uterine (Spoelstra et al., 2006), osteosarcoma (Shen et al., 2012, Yu et al., 2019), lung (Zhang et al., 2013b, Matsubara et al., 2014, Ma et al., 2019), liver (Zhou et al., 2012, Qin et al., 2019), gastric (Okugawa et al., 2012, Jia et al., 2012, Xue et al.,

2019), glioblastoma (Siebzehnrubl et al., 2013), prostate (El Bezawy et al., 2019), oesophageal squamous cell carcinoma (Gu et al., 2019).

1.6.4 Regulation of *Zeb1*

Zeb1 is regulated by various transcription regulators, either as activators or repressors. Such activators include EMT inducers that are found to activate *Zeb1* such as *Slug* that binds E-box sequence in *Zeb1* promoter in melanoma cell lines and *Snail1* in mouse breast epithelial NMuMG cells and human hepatoma cell HepG2 (Dave et al., 2011, Wels et al., 2011, Wu et al., 2017). Forkhead box protein C2 (*FOXC2*) is also found to regulate *Zeb1* via p38 phosphorylation in Immortalized human mammary epithelial cells (HMLE) (Werden et al., 2016). Signalling pathways are also found to positively regulate *Zeb1* such as transforming growth factor (TGF)-beta (TGF-β) (Shirakihara et al., 2007), Wnt canonical pathway and Hypoxia-inducible factor 1 alpha (HIF-1α) in colorectal cancer cells via binding hypoxia response element (HRE) in *Zeb1* promoter (Zhang et al., 2015b).

Transcriptional repressors of *Zeb1* that have been shown to bind and repress *Zeb1* promoter include the retinoblastoma-associated protein 1 (*Rb1*) in mouse embryonic fibroblasts (Liu et al., 2007), Grainyhead-like-2 (*GRHL2*) in the mesenchymal subpopulation cells derived from HMLE (Cieply et al., 2012), and Ovo-like 2 (*Ovol2*) in normal and malignant mammary epithelial cells (MECs) (Watanabe et al., 2014). LIM only 2 (LMO2) is shown to repress the activity of *Zeb1* by histone deacetylation via recruiting HDAC1 and SAP18 in T-cell acute lymphoblastic leukaemia (T-ALL) cells (Wu et al., 2018). *Zeb1* also regulates *Ovol2* and *Zeb1*-*Ovol2* repression circuit to maintain partial EMT in normal human mammary epithelial cells (MCF10 A) (Hong et al., 2015). The bidirectional inhibition of *Zeb1* and other regulators is seen as well with microRNA 200 family (miR-200b~200a~429) as microRNA 200-*Zeb1* mutual inhibition is found in human breast cancer cells (Bracken et al., 2008).

Post-transcriptionally, phosphorylation is found to regulate the activity of *Zeb1* (Xu et al., 2019). *Zeb1* binding to target genes is attenuated upon increased phosphorylation mediated by protein kinase C (PKC) in Jurkat or COS-7 cells (Llorens et al., 2016). In contrast, *Zeb1* can be phosphorylated by ATM kinase in radioresistant

breast cancer stem cells, hence, stabilizing and upregulating *Zeb1* levels (Zhang et al., 2014).

1.6.5 The role of *Zeb1* in haematopoiesis

1.6.5.1 *Zeb1* role in T cells

Complete deletion of *Zeb1* (*Null–LacZ*) in mice embryos showed perinatal deaths and a dramatic reduction in thymus mass and cellularity at E18.5 as well as skeletal and chondrocyte defects (Takagi et al., 1998). However, 80% of mutant mice that only lack the zinc finger sequence proximal to C-terminal portion (*ΔC-fin*) died two days after birth and few survived and showed the same reduction in total cell number and mass of thymus as the *Null–LacZ* (Higashi et al., 1997, Takagi et al., 1998). Further analysis of the thymus and T cell development in neonates and the survived adults in *ΔC-fin* mice showed a depletion of the early stage intrathymic T-cell precursors (c-kit$^+$, CD4$^-$ CD8$^-$ DN population) (Higashi et al., 1997). A small population of intrathymic T-cell precursors <1% escapes the early block and differentiate into CD4$^+$ CD8$^+$ DP cells with high proportion of CD4$^+$ CD8$^-$ SP cells (Higashi et al., 1997). This indicates that different domains in ZEB1protein play different roles in different organs and systems and, in this case, C-terminal cluster is essential for T-cell development but not for skeletal system (Higashi et al., 1997, Takagi et al., 1998, Postigo and Dean, 1999a). However, the N-terminal domain inhibits more haematopoietic genes such as *c-myb*, members of the ets family, and *TFE-III* (Postigo and Dean, 1999a).

CD4 is documented as a target gene of *Zeb1* in T-cell development (Brabletz et al., 1999). *Zeb1* negatively regulates *CD4* via binding to the '5 E-box in the proximal enhancer of the *CD4* promoter rendering it a silencer after a competition with the activators E12 and HEB (Brabletz et al., 1999). Overexpression of *Zeb1* in Jurkat cell line (CD4$^+$ CD8$^-$) and A.301 cell line (CD4$^+$ CD8$^+$) results in a reduction of the activity of the *CD4* proximal enhancer to 48% and 76%, respectively. Overexpression of *Zeb1* also reduces CD4 protein expression in CD4$^+$ SP Jurkat cells but not for CD4$^+$ CD8$^+$ DP A.301 cells (Brabletz et al., 1999), which may explain the predominance of CD4$^+$ CD8$^-$ SP mature T cells seen in *Zeb1 ΔC-fin* mutants (Brabletz et al., 1999, Higashi et al., 1997).

Furthermore, *Zeb1* has been shown to bind and repress α4-integrin, a subunit component of VLA-4, a critical regulator of haematopoiesis that is expressed in HSPCs, T cells, B cells, monocytes, NK cells, eosinophils, and neutrophils (Postigo and Dean, 1999a, Imai et al., 2010). *Zeb1* mutant T cells show high expression of α4-integrin (Higashi et al., 1997). α4-integrin is regulated by the haematopoietic TF *c-Myb* as well as Ets proteins which both synergise to overcome repression by *Zeb1*, whereas, *Zeb1* can repress them individually (Postigo et al., 1997). Moreover, *Zeb1* has been found to bind and block the activity of some genes implicated in T-cell development such as *GATA3* (Gregoire and Romeo, 1999) and interleukin-2 (*IL-2*) (Williams et al., 1991, Yasui et al., 1998, Wang et al., 2009). Recently, *Zeb1* is shown to regulate the function and maintenance of $CD8^+$ T cells (Guan et al., 2018). After exposure to lymphocytic choriomeningitis virus (LCMV), memory $CD8^+$ T cells lacking *Zeb1* showed transient expansion, but this diminished overt time as a result of apoptosis (Guan et al., 2018).

1.6.5.2 *Zeb1* role in B cells

Several lines of evidence suggest a function of *Zeb1* in B cells. *Zeb1* has been shown to bind and repress immunoglobulin heavy chain enhancer that has been implicated in B cell specificity (Genetta et al., 1994). Furthermore, *Zeb1* with the corepressor CtBP have been shown to form a complex suppressing the distal promoter element of the *BCL6* locus, a highly expressed gene in germinal centre B-cells and is required for normal antibody responses (Papadopoulou et al., 2010). Additionally, *Zeb1* has been shown to be required for B cell proliferation upon stimulation with F(ab′)2αIgM during humoral immune responses (Arnold et al., 2012).

1.6.6 Biological differences and similarities in *Zeb1* and *Zeb2* function

Although *Zeb1* and *Zeb2* share a similar gene structure and bind to the same DNA sequence CACCT to repress transcription via recruiting CtBP co-repressors, they present differences in terms of the expression pattern and biological function (Miyoshi et al., 2006, Postigo, 2003, Postigo and Dean, 2000). Using northern blot, both *Zeb1*

and *Zeb2* have overlapping expression in central nervous system (CNS), skeletal muscles, BM, and liver cells, however this redundancy has not been functionally proven yet (Postigo and Dean, 2000). *Zeb1* and *Zeb2* show differential expression within lymphoid cells as *Zeb1* is highly expressed in T cells while *Zeb2* is absent (Postigo and Dean, 2000). Additionally, high *Zeb1* expression is found in foetal lung and placenta, while *Zeb2*is expressed at low levels (Postigo and Dean, 2000). Furthermore, both *Zeb1*and *Zeb2* are expressed in the heart, brain, and spleen with more expression levels for *Zeb2* in these tissues (Postigo and Dean, 2000). ZEB2 protein is expressed during embryogenesis in neuroepithelium of headfolds, neural crest cells, and the branchial arch (Van de Putte et al., 2003). ZEB2 expression during embryogenesis occurs at E7.75 earlier than ZEB1that its expression starts at E8.5 and despite their broad expression after gastrulation the expression pattern differs depending on the tissues (Takagi et al., 1998, Miyoshi et al., 2006). ZEB2at E8.5 is highly expressed in neural tube and paraxial mesodermal tissues while ZEB1 expression is low (Miyoshi et al., 2006). However, ZEB1expression at E8.5 is found to be high in notochord, node and endoderm while ZEB2expression is low (Miyoshi et al., 2006). One clear expression difference between ZEB1 and ZEB2 is during neuron development where ZEB2 is highly expressed early from E9.5-11.5, while ZEB1 is expressed at high levels during differentiation after E11.5 (Miyoshi et al., 2006). However, it also appears that ZEB1 and ZEB2 are capable of compensating for each other in certain circumstance. For example, ZEB1expression increased in E8.5 and E11.5 in *Zeb2*$^{-/-}$ embryos while there was no change in ZEB2expression in *Zeb1*$^{-/-}$ embryos (Miyoshi et al., 2006). *Zeb2*$^{-/-}$ Ly-6C^{+} monocytes display high ZEB1expression (Wu et al., 2016). Also, *Zeb2*$^{-/-}$ effector CD8^{+} T cells show increased ZEB1 expression and *Zeb1*$^{-/-}$ memory CD8^{+} T cells show increased ZEB2expression (Guan et al., 2018). *Zeb1* binds to the *Zeb2* promoter in naive CD8^{+} T cells (Guan et al., 2018). These data suggest that *Zeb1* and *Zeb2* can also negatively regulate each other contrasting with other EMT inducers *Slug* and *Snail1* that positively regulate *Zeb1* (Dave et al., 2011, Wels et al., 2011).

Functionally, *Zeb2* KO embryos show developmental defects such as an inability of neural tube to close and lose the branchial arch and die around E8.5 (Van de Putte et al., 2003) while *Zeb1* KO embryos die perinatally, showing skeletal and T cells development defects and an inability to breathe (Takagi et al., 1998). Compound *Zeb1*$^{-/-}$ *Zeb2*$^{+/-}$ embryos die about E13.5 presenting major defects in maxillary tissues and neural tube including cerebrum and lower region of spinal cord (Miyoshi et al., 2006). Thus, *Zeb1* and *Zeb2* show different and overlapping expression patterns and

functionality suggesting both genetic interaction and distinct function in a temporal and tissue-dependent manner.

1.6.7 The role of *Zeb2* in haematopoiesis

Zeb2 is found to be required for embryonic haematopoiesis and mobilisation of HSCs to the BM during development (Goossens et al., 2011) and for adult murine HSC differentiation (Li et al., 2016b). Using Mx1-Cre system, adult *Zeb2*$^{-/-}$ mice presented a decrease in erythrocytes, leukocytes, and thrombocytes in PB and loss of B cells and monocytes in PB and BM with increased granulocytes, LSK, HSC, and MEP and a reduction in GMP in BM (Li et al., 2016b). Primary, secondary, and tertiary transplantations revealed the same pattern of differentiation defects with preserved number of HSCs (Li et al., 2016b). *Zeb2* loss resulted in a terminal maturation defect of B cells from pre-pro-B cells to pro-B cells (Li et al., 2016b). *Zeb2* is shown to be a critical regulator of the development of plasmacytoid DC and monocytes (Wu et al., 2016). Additionally, *Zeb2* is found to regulate terminal differentiation of effector CD8^{+} T cells and maturation and survival of NK cells (Guan et al., 2018, van Helden et al., 2015).

1.7 Aims of thesis

Under normal physiologic conditions, *Zeb1* is widely expressed and functions in different tissues including nervous system, lung, kidney, skeletal system, muscles, thymus, BM, spleen, and others (Postigo and Dean, 2000, Hurt et al., 2008, Higashi et al., 1997, Sekido et al., 1994, Guan et al., 2018, Arnold et al., 2012). Extensive research has been conducted on the role of *Zeb1* in cancer settings, including haematologic malignancies of T and B cell lineages (Zhang et al., 2015a, Zhang et al., 2019, Sanchez-Tillo et al., 2014, Hidaka et al., 2008). *Zeb1* has also been identified as a regulator of stem cell traits in the context of solid tumours (Zhang et al., 2015a). Furthermore, *Zeb1* has been documented as a critical regulator of T cells during embryogenesis but not adulthood (Higashi et al., 1997, Takagi et al., 1998). However, there is a lack of knowledge on the role of *Zeb1* in normal stem cell biology. This led us to investigate its role in the adult haematopoietic system, where HSC function is well defined and serves as a paradigm for stem cell behaviour in other tissues. Exploring the function of *Zeb1* in normal stem cell biology will thus allow a better understanding of its role in haematopoiesis, as well as providing possible mechanistic insights into how it regulates other adult, tissue-specific stem cells. Here, we utilised a conditional genetic approach using the Mx1-Cre system to specifically knockout (KO) *Zeb1* in adult HSCs, progenitors and differentiated haematopoietic cells. The aims of this thesis are to:

1. Understand the function of *Zeb1* in adult long-term haematopoiesis. In this chapter, *Zeb1* was deleted *in vivo* during adulthood and the haematopoietic compartments including HSCs were analysed at week 32 after deletion. HSCs were functionally tested by primary and secondary transplantations.

2. Assess the impact of acute deletion of *Zeb1* in the adult haematopoietic system. In this chapter, *Zeb1* was deleted *in vivo* during adulthood and the haematopoietic compartments including HSCs were analysed at day 14 after deletion. HSCs were functionally tested by primary and secondary transplantations.

3. Explore the requirement of *Zeb1* in adult T cell development. In this chapter, *Zeb1* role in T cell differentiation, maturation, survival and proliferation was tested in the adult thymus and peripheral T cells.

CHAPTER 2 : Materials and Methods

2.1 *Zeb1* knockout model

2.1.1 Mice breeding and maintenance

All mice used in this thesis were on C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME USA). To study the role of *Zeb1 in vivo*, *Zeb1*$^{fl/fl}$ mice (Brabletz et al., 2017), a kind gift from Dr Florian Siebzehnrubl from the European Cancer Stem Cell Research Institute (ECSCRI), Cardiff University, were bred with *Zeb1*$^{+/+}$ *Mx1-Cre*$^{+/-}$ *mice* (Kuhn et al., 1995) to generate an experimental cohort of *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{-/-}$ (control) and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{+/-}$ (*Zeb1*$^{-\backslash-}$). In this model, exon 6 (contains coding sequences for the majority of *Zeb1* protein) is flanked with loxP sites and under the control of a tissue specific promoter (*Mx1*) that activates Cre recombinase to delete the floxed exon 6, *Zeb1* can be conditionally inactivated at any developmental stage (Figure 2.1). Deletion of exon 6 results in splicing of exon 5—7 and eventually premature stop in translation (Figure 2.1). All experiments were performed under the regulations of the UK Home Office (project number 30/3380).

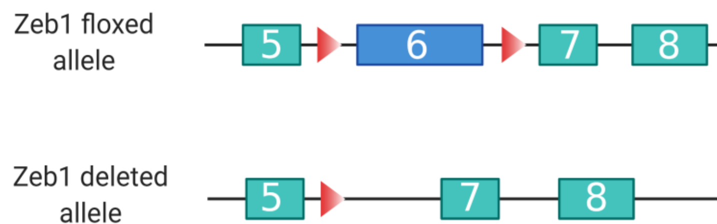


Figure 2.1. Generation of *Zeb1* conditional knockout model. Upon activation of Cre recombinase, floxed exon 6 will be deleted leading to splicing of exon 5-7. Red triangles refer to loxP sites. Rectangles refer to exons.

2.1.2 Genotyping

To genotype mice, genomic DNA was isolated from ear notches, PB, BM and spleen cells using Isolate II Genomic DNA Kit (Bioline) according to the manufacture instructions. The PCR was performed on T100^{TM} Thermal Cycler (Bio-Rad) to amplify the genomic DNA. For PCR reaction mix, 12.5 µL of the Mango Mix (Bioline), 0.10 µL of each primer either *Zeb1* or Cre (the stock concentration was prepared at 100 uM) (Table 2.1), 8.30 µL of nuclease free water and 4 µL of each DNA sample (final DNA concentration ranges from 20-80 ng/µL)to make final volume of 25 µL. The

T100™ Thermal Cycler was set at the following conditions: for *Zeb1*: volume 25 μL, 95°C for 5 min, 95°C for 30 sec, 64°C for 45 sec, 72°C for 1 min 39x, 72°C for 5 min, and 10°C for ∞; for Cre: volume 25 μL, 95°C for 3 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min 30x, 72°C for 5 min, and 10°C for ∞. The amplified products of the PCR reaction were run on a 2% agarose gel containing 1:30000 dilution of SafeView (BioLegend). Gel bands were detected by a Bio-Rad Gel Doc XR and viewed and annotated using ImageLab Software (Figure 2.2).

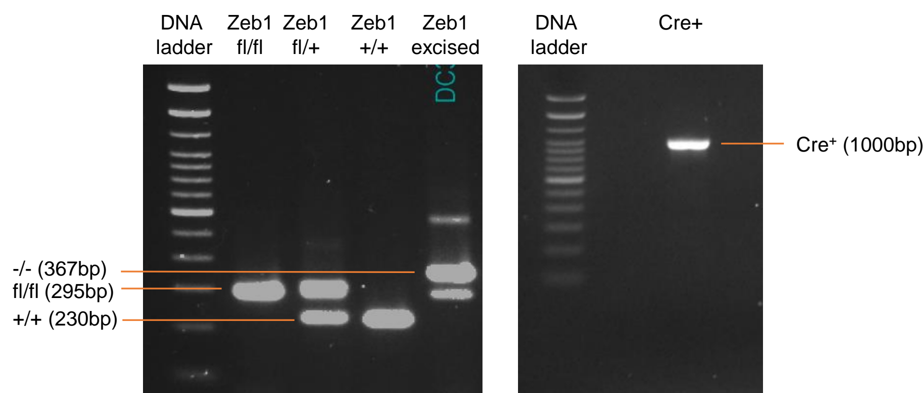


Figure 2.2. Confirmation of *Zeb1* genotyping in the experimental mice. Gel bands of *Zeb1 fl/fl*, *Zeb1 fl/+*, *Zeb1+/+*, and *Zeb1 Mx-1 Cre+/-* mice.

Table 2.1. Information of *Zeb1* and Cre primers used for genotyping.

Gene	Sequence
***Zeb1*fl forward**	5′-CGTGATGGAGCCAGAATCTGACCCC-3′
***Zeb1*fl reverse**	5′-GCCCTGTCTTTCTCAGCAGTGTGG-3′
***Zeb1* excised reverse**	5′-GCCATCTCACCAGCCCTTACTGTGC-3′
Generic Cre forward	5′-TGACCGTACACCAAAATTTG-3′
Generic Cre reverse	5′-ATTGGCCCTGTTTCACTATC-3′

2.1.3 *Zeb1* deletion

8-12 weeks old *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{-/-}$ (control) and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{+/-}$ (*Zeb1*$^{-\backslash-}$) mice were used in all experiments to test the function of *Zeb1 in vivo*. *Zeb1* was deleted after intraperitoneal (IP) administration of polyinosinic: polycytidylic acid (Poly I:C) (6 doses every alternate day, 0.3 mg per dose, GE Healthcare) into *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{-/-}$ (control) and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{+/-}$ (*Zeb1*$^{-\backslash-}$) mice. Poly I:C is a double stranded synthetic RNA that induces type 1 α and β interferons which then drives the expression of *Mx1* promoter (inactive in healthy mice) to activate Cre recombinase to cut the floxed allele (Kuhn et al., 1995). In our model, administration of Poly I:C activated *Mx1* promoter which stimulated Cre recombinase to excise the floxed exon 6 of *Zeb1* (Figure 2.1). Mice were analysed at different time points after the last dose of Poly I:C as it is shown in each respective result chapter. All experiments were performed under the regulations of the UK Home Office.

2.2 Sample harvesting and processing

2.2.1 Peripheral blood (PB)

PB was obtained in EDTA treated tubes (Starstedt) by nicking the tail vein using a scalpel blade. RBCs were lysed by ammonium chloride solution (NH_4Cl) (StemCellTechnologies). Simply, 12 µL of blood was lysed in 600 µL NH_4Cl for 12 minutes. Then, samples were centrifuged for 10 minutes at 370 g at room temperature (RT). The pellet containing white blood cells (WBCs) were used for analysis of PB lineages.

2.2.2 Bone marrow

Mice were culled by cervical dislocation. The mice were sprayed with 70% ethanol. Tibias, femurs, and iliac bones were harvested. After cleaning the surrounding tissues and muscles, the bones were crushed using a pestle and mortar in phosphate-buffered saline (PBS) (Gibco) supplemented with 2% FBS (foetal bovine serum) and the 30 mL BM cell suspension was obtained and filtered through 70 µm cell strainer (Miltenyi Biotec).

2.2.3 Spleen and thymus

After dissection of mice, spleens and thymi were harvested. Weight and pictures were taken. Then, they were minced through 70 µm cell strainer using PBS 2% FBS. 7 mL and 4 mL cell suspension were obtained for spleen and thymus, respectively.

2.3 Flow cytometric analysis

Information about all antibodies used in the thesis for flow cytometry analysis are presented in Table 2.2. Stock concentration of all antibodies is 0.2 mg/mL except B220-FITC, CD48-FITC, CD34-FITC is 0.5 mg/mL and Annexin V-PE is 4 µg/mL. An appropriate staining dilution of each antibody is stated in Table 2.2. Details on staining process of PB, BM, spleen, and thymus cells are described below in the respective section. Analysis of samples was performed on BD LSRFortessa™ (BD Biosciences). Cell sorting was done using BD FACSAria™ Fusion (BD Biosciences). To solve the spectral overlap, compensation was done after performing single staining for each fluorochrome. Each tube containing a single stain was acquired and using algorithms on the software the spectral overlap was solved. Population gates were set using unstained samples and fluorescent minus one control (FMO) as negative controls. In FMO, all the fluorochromes were added in a tube except the fluorochrome that was measured. Details on the staining process and number of cells stained and acquired are stated below in the respective sections. After running the samples and applying the compensation, the experiment files were exported and analysed using FlowJo software (Tree Star, Inc).

Table 2.2. Information about antibodies used for flow cytometry analysis.

	Antigen	Clone	Reactivity	Fluorochrome	Dilution	Manufacturer
HSPCs and committed progenitors	Pool of lineage+ markers: CD3, CD4, CD8a, B220, Mac1, Gr1, Ter119	Shown below individually	Shown below individually	Biotin	CD3 and CD8:1/40, B220 and Gr1:1/20, CD4:1/80, Mac1:1/10, Ter119:1/5	Biolegend, eBioscience
	Sca-1	D7	mouse	APC-Cy7, PE	1/25	Biolegend
	cKit	2B8	mouse	APC, PE	1/100	Biolegend
	CD150	TC15-12F12.2	mouse	PE-Cy7	1/100	Biolegend
	CD48	HM48-1	mouse	FITC	1/50	Biolegend
	CD135 (Flt3)	A2F10	mouse	PE	1/50	Biolegend
	CD34	RAM34	mouse	FITC	1/25	ebioscience
	CD16/32	93	mouse	PE-Cy7	1/25	Biolegend
	CD127 (IL-7rα)	A7R34	mouse	BV650, PE	1/100	Biolegend
Erythroid lineage	Ter119	TER-119	mouse	APC-Cy7	1/1000	Biolegend
	CD71	RI7217	mouse	PE	1/1000	Biolegend
T cell panel	CD3	17A2	mouse	APC, FITC	1/1000	Biolegend
	CD4	GK1.5	mouse	PE, PerCP	1/1000	Biolegend
	CD8a	53-6.7	mouse	APC-Cy7	1/1000	Biolegend
	CD44	IM7	Mouse and human	APC	1/1000	Biolegend
	CD25	PC61	mouse	PerCP	1/1000	Biolegend
	CD62L	MEL-14	mouse	PE-Cy7	1/1000	Biolegend
	CD24	M1/69	mouse	FITC	1/1000	Biolegend
Myeloid cells	CD11b (Mac1)	M1/70	Mouse and human	APC	1/1000	Biolegend
	Gr-1	RB6-8C5	mouse	FITC, PE-Cy7	1/1000	Biolegend
B cells	B220/CD45R	RA3-6B2	Mouse and human	FITC, APC	1/1000	Biolegend
Megakaryocytes	CD41	MWreg30	Mouse	FITC	1/1000	Biolegend
donor and recipient cell staining	CD45.1	A20	mouse	BV510, BV650, APC	1/500	Biolegend
	CD45.2	104	mouse	BV510, PE, APC-Cy7	1/500	Biolegend
	EpCAM (CD326)	G8.8	mouse	BV711	1/50	Biolegend
	Annexin V	NA	All mammalian	APC, PE	1/25	Biolegend
	Ki-67	16A8	mouse	APC, PE	1/25	Biolegend
	Fc Block	93	mouse	NA	1/100	Biolegend
	Streptavidin	NA	Mouse and human	Pacific blue, PerCP	1/100	Biolegend, ebioscience

2.3.1 Immunophenotypic analysis of HSPCs and committed progenitors in BM and spleen

$10X10^6$ BM and spleen cells were stained as follows: for HSPCs (LSK SLAM): Lin^- cocktail was prepared from a pool of biotinylated antibodies of differentiated cell markers in PBS 2% FBS (Mac1 and Gr1 for myeloid cells, Ter119 for erythroid lineage, B220 for B cells, CD3e, CD4, CD8a for T cells) as well as Sca-1, c-Kit, CD150, and CD48, CD127, CD135 (Flt3) to study HSC, MPP, LMPP, HPC1, and HPC2. For committed progenitors (LK) in addition to Lin^- cocktail, Sca-1, c-Kit, CD34, CD16/32, CD127, and CD135 (Flt3) were used to study CMP, GMP, MEP and CLP. Before staining with the antibody mix, the cells were incubated with Fc block (anti CD16/CD32) in 50 µL PBS 2% FBS for 5 minutes. Then, the cells were incubated with antibody mixture in PBS 2% FBS at final volume 100 µL for 30 minutes at 4ºC in dark. Cells were washed with 1 mL PBS 2% FBS and incubated with streptavidin in PBS 2% FBS at final volume 100 µL for 20 minutes at 4ºC in dark. Streptavidin was added as a secondary antibody to detect the biotinylated antibodies in the Lin^- cocktail. Next, the cells were washed with 1 mL PBS 2% FBS and resuspended in 500-800 µL PBS 2% FBS. Before running the samples on the BD LSRFortessa™ (BD Biosciences), DAPI (4′,6-diamidino-2-phenylindole) (Molecular probes) at final concentration of 0.5-1 ug/mL was added to exclude dead cells. For samples harvested from transplantation experiments, CD45.1 and CD45.2 were added to the antibody panel to allow us to differentiate between donor and competitor cells. For EpCAM expression in HSPCs, EpCAM antibody was added to the panel of HSPCs and its expression was quantified in HSC and HPC1. Minimum events that were acquired for HSPCs analysis in BM and spleen was $2.5X10^6$ and $4X10^6$, respectively. For committed progenitor analysis in BM and spleen, $1.5X10^6$ event were acquired. Sample acquisition was set on FSC-A vs SCC-A gate that contain all mononuclear cells excluding dead cells and debris.

2.3.2 Immunophenotypic analysis of mature lineages in PB, BM, spleen, and thymus

200,000 PB, BM and spleen cells were stained for Ter119 (erythroid lineage) (not for PB), Gr-1 and Mac-1 (myeloid cells), CD3, CD4, CD8 (T-cells), CD41 (megakaryocytes) and B220 (B-cells). For thymocytes, 500,000 cells were stained for CD4, CD8, CD44 and CD25, c-Kit. CD62L and CD44 in combination with CD3, CD4, and CD8 were used to study naïve, effector memory, and central memory T cells in different T cell subsets in PB, BM, and spleen. For samples harvested from transplantation experiments, CD45.1 and CD45.2 were added to the antibody panel to allow us to differentiate between donor and competitor cells. DAPI at concentration of 0.5-1 ug/mL was added before running the samples to exclude dead cells.

2.3.3 Apoptosis analysis

For apoptosis assay, after staining the cells for the cell surface markers as described previously, they were stained in 100 µL Annexin V buffer (Biolegend) containing Annexin V antibody (Biolegend) for 30 minutes in the dark at RT. Then, 600 µL Annexin V buffer was added to stop the reaction. DAPI at concentration of 0.5-1 ug/mL was added before running the samples.

2.3.4 Cell cycle analysis

Cell cycle profile was assessed in stem and progenitor cells in BM as well as in thymocytes using Ki-67 antibody. After staining the cells for the cell surface markers as described previously, they were fixed with 1% paraformaldehyde (PFA) (ThermoFisher) for 20 minutes at 4°C, permeabilised using 0.1% saponin (Sigma) prepared in PBS for 30 minutes at 4°C and stained with Ki-67 antibody for 30 minutes at 4°C in dark. Next, they were resuspended in 500-700 µL FACS buffer and they were incubated with DAPI at final concentration of 5 ug/mL in dark for 5 minutes before running the samples.

2.3.5 HSC sorting

For HSC sorting, BM cell suspension was obtained as in 2.2.2, and RBCs were lysed by ammonium chloride solution (StemCellTechnologies) for 2 minutes. Then, the cells were enriched for c-Kit by magnetic-activated cell sorting (MACS) (MACS®, Miltenyi Biotec) after incubation with anti-c-Kit magnetic beads (Miltenyi Biotec) for 20 minutes at 4°C in dark. c-Kit$^+$ cells were stained as described earlier and HSCs were sorted using BD FACSAria™ Fusion (BD Biosciences).

2.4 Colony forming cell assay

For the colony forming unit assay (CFC), 20,000 whole BM and purified 150 HSCs were plated in methylcellulose medium M3434 supplemented with recombinant mouse SCF, recombinant mouse IL-3, recombinant human IL-6, FBS, Bovine serum albumin (BSA), recombinant human insulin, human transferrin (iron-saturated), 2-Mercaptoethanol, and recombinant human erythropoietin (EPO) (Stem Cell Technologies). Cells were incubated for 10 days at 37°C then the colonies were scored under the microscope.

2.5 Transplantation experiments

C57BL/6 SJL mice (CD45.1) were used as recipients for all the transplantations except for the niche transplantation, C57BL/6 (CD45.2) mice were used as recipients. The mice were lethally irradiated at 9 Gy (split dose with 4 hours between the doses). For primary transplantation, 150 HSCs from *Zeb1*$^{-/-}$ and control mice mixed with 2X10^5 whole BM (CD45.1) (supporting cells) were transplanted into lethally irradiated mice (CD45.1). To monitor the engraftment, tail vein bleeding was performed at different time points post-transplant. To further assess the capacity of *Zeb1*-deficient HSCs to repopulate secondary recipients, 300 HSCs from *Zeb1*$^{-/-}$ and control primary recipients were sorted and mixed with 3X10^5 whole BM (CD45.1) (supporting cells). The engraftment ability was monitored via tail vein bleeding until week 12.

For cell autonomous transplantation, *Zeb1* was deleted specifically in haematopoietic cells (but not in BM niche cells) after transplanting 5X10^5 whole BM (CD45.2) from *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^+$ and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^-$ along with 5X10^5 whole BM (CD45.1)

(supporting cells) into lethally irradiated recipients (CD45.1). Six weeks later, 6 doses of Poly I:C (every alternate day, 0.3 mg per dose) were intraperitoneally (IP) injected to delete *Zeb1*. Mice were dissected at day 14 after the last dose of Poly I:C and analysed. 500,000 CD45.2 donor BM cells were then sorted, mixed with 500,000 competitor cells (CD45.1) and re-transplanted into lethally irradiated recipients and monitored via tail vein bleeding until week 16.

For niche transplantation, *Zeb1* was deleted specifically in BM niche cells to test the effect of *Zeb1*-deficient BM niche on HSC function. $1X10^6$ total BM cells from wild type (WT) $CD45.1^+$ mice were transplanted into lethally irradiated *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^+$ and *Zeb1*$^{fl/fl}$ *Mx1-Cr*$^-$ mice. Six weeks later, 6 doses of Poly I:C (every alternate day, 0.3 mg per dose) were IP injected to delete *Zeb1*. Mice were dissected at week 16 after the last dose of pIpC and analysed.

2.6 Homing assay

7,000,000 unfractionated BM cells from *Zeb1*$^{-/-}$ and control mice were transplanted into lethally irradiated mice (CD45.1). 18 hours later, mice were dissected, and the BM cells were stained for CD45.1 and CD45.2. The frequency of CD45.2 was analysed using flow cytometry.

2.7 *Zeb1* expression analysis in haematopoietic compartments

2.7.1 RNA extraction

For *Zeb1* expression analysis, different haematopoietic populations (HSC, MPP, HPC1, HPC2, CMP, GMP, MEP, CLP, $Mac1^+$ $Gr1^+$, $Ter119^+$, B cells, and T cells) from the bone marrow of 8-12-week-old WT C57BL/6 mice were sorted on BD FACSAria™ Fusion (BD Biosciences) and stored in RLT buffer to lyse the cells before RNA extraction. RNA extraction was done using RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. The concentration of RNA was assessed by NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.7.2 cDNA synthesis

Synthesis of cDNA was performed using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) on T100^{TM} Thermal Cycler (Bio-Rad) using random hexamer primers according to the following program: volume: 20 µl, 25°C for 10 minutes, 50°C for 50 minutes, 85°C for 5 minutes, and 4°C for ∞.

2.7.3 qRT-PCR

To analyse the expression of *Zeb1*, Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) was done using Taqman® Universal Master Mix II (Applied Biosystems), and analysis was performed using QuantStudio® 7 Flex Real-Time PCR System (Applied Biosystems) at 95°C for 15 seconds, then 60°C for 1 minute for 40 cycles. cDNA samples were diluted in RNAase free water to ensure having final 10ng cDNA in each well of a 96-well plate used for qRT-PCR. Gene expression was normalised using the housekeeping gene HPRT. Three biological replicates were performed on each cell population for both the reference (HPRT) and target (*Zeb1*) genes. Primers information (ThermoFisher Scientific) are shown in Table 2.3.

Table 2.3. Information of the primers used in qRT-PCR.

Gene	Assay ID
Mouse *Hprt1*	Mm00446968_m1
Mouse *Zeb1*	Mm00495564_m1

2.8 RNA-sequencing

2.8.1 Sample preparation and sequencing

RNA from HSCs (LSK CD150$^+$ CD48$^-$) from 4 control and 4 *Zeb1*$^{-/-}$ mice 14 days after the last dose of Poly I:C injection was extracted using RNAeasy micro kit (Qiagen). Total RNA quality and quantity was assessed using Agilent 2100 Bioanalyzer and RNA Nano 6000 kit (Agilent Technologies). 0.5-6 ng of total RNA with RNA integrity number (RIN) value >8 was used as the input. Sequencing libraries were prepared via the generation of cDNA libraries from total RNA using the NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina® (NEB). The steps for cDNA library preparation included reverse transcription, template switching, cDNA amplification (11-17 cycles), and cleanup of the amplified cDNA with Agencourt Ampure® XP beads (Beckman Coulter®) using 80% ethanol. Sequencing libraries were prepared from the cDNA libraries using the steps of fragmentation, end repair, 5' phosphorylation and dA-tailing, adaptor ligation and excision, clean up and PCR enrichment (7 cycles) of the adaptor-ligated DNA that used the NEBNext® Mulitplex Oligos for Illumina® (Dual Index primers set 2: NEB E7780) followed by a final clean-up of the PCR product with Agencourt AMPure® XP beads (Beckman Coulter®).

The libraries were validated using the Agilent 2100 Bioanalyzer and high-sensitivity kit (Agilent Technologies) to ascertain the insert size, and the Qubit® (Life Technologies) was used to perform fluorometric quantitation. Following validation, the libraries were normalized to 4 nM, pooled together then sequenced using a 75-base paired-end (2x75bp PE) dual index read format on the Illumina® HiSeq4000 according to the manufacturer's instructions.

2.8.2 Raw data processing and analysis

2.8.2.1 Trimming

To remove adapter sequencer and poor-quality ends of reads, trimming was performed using Trim Galore, a wrapper tool which uses cutadapt and FastQC to trim and perform QC on the trimmed. Trimming was performed in paired-end mode.

2.8.2.2 Mapping

Trimmed reads were mapped against the GRCm38.p6 mouse genome using STAR (Alex Dobin, Git Hub). STAR was run with the MultimapNMax=1 flag, meaning reads mapping to more than 1 location were considered unmapped.

2.8.2.3 Expression summarisation

Expression counts for both exons and transcripts were calculated, using Subread featureCounts Version 1.6.2 (Liao et al. 2014), a program for assigning sequence reads to genomic features. To define the exon and transcript locations (raw reads calculation), the GENCODE M18 gene model was used. Read summarization (counting) was generated for paired end read fragments with data summarised at gene level. To provide stringent and robust data, reads overlapping more than one feature were excluded from the count summary (according to authors recommendations Liao et al. 2014).

2.8.2.4 Differential gene expression

Differentially expressed genes were identified using a DEseq2 Bioconductor package (Love et al. 2014) analysis within the R environment for statistical computing software on normalised count data. For multiple testing and false discovery issues, the generated p-values were corrected using the FDR method (Benjamini & Hochberg, 1995).

2.8.2.5 Gene ontology over-representation analysis

The Gene ontology over-representation analysis (GO ORA) analysis was undertaken using the GOstats Bioconductor library. The resultant data was corrected for multiple testing and false discovery using the FDR method. Genes were classified in three categories, its biological processes (BP), molecular functions (MF) and cellular components (CC). To overcome the issue of short gene lists and the limitation of associated terms, analysis was performed on a list of the top 200 ranked genes ranked by p-value.

2.8.2.6 Data visualisation

The heatmap was created using https://software.broadinstitute.org/morpheus/. DEGs according to FDR <0.05 were used. The biological pathway analysis was performed using Kyoto Encyclopaedia of Genes and Genomes database (KEGG), an online database offering data on the genome and genetic information, cellular processes, biological pathway and others (Kanehisa and Goto, 2000). Top enriched pathways were selected, and a graph was generated using Prism (GraphPad Software, Inc.).

2.9 Statistical Analysis

Flow cytometry data was analysed by FlowJo software (Tree Star, Inc). Figures were prepared using Prism (GraphPad Software, Inc.). N in the figure legends represents the total biological replicates (total number mice) that were used for the analysis. The number of independent experiments has been separately indicated in the figure legends. For example, if N=8 and the results were derived from 2 independent experiments, this means the total mice used in two independent experiments is 8. Statistical analyses were done using Mann–Whitney U test to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

CHAPTER 3 :

The effect of *Zeb1* on long-term haematopoiesis

3.1 Introduction

The haematopoietic system is a dynamic tissue system that is responsible for blood and immune cell production. This process is sustained through life by a rare subset of LT-HSCs in adult BM. While most HSCs are quiescent in the adult BM niche, few enter the cell cycle to differentiate and self-renew to meet the body needs and to maintain the stem cell pool (Cheshier et al., 1999, Boulais and Frenette, 2015). These properties of quiescence, self-renewal, and differentiation are highly regulated by TFs that control gene expression of signalling pathways, and microenvironments to prevent premature exhaustion of HSCs and to ensure the lifetime supply of blood.

Although HSC functionality and contribution to long-term multilineage haematopoiesis has been proven by single cell transplantation (Oguro et al., 2013, Yamamoto et al., 2013), the extent of long-term contribution of HSCs to adult steady state haematopoiesis is still controversial (McRae et al., 2019). Lineage tracing studies suggested that adult steady state haematopoiesis is mainly driven by MPPs with little contribution from HSCs (Sun et al., 2014, Busch et al., 2015). However, recent studies have shown that the HSCs are the major contributor to the unperturbed adult haematopoiesis (Chapple et al., 2018, Sawen et al., 2018). This difference in conclusions between groups is caused by different labelling techniques they used and more importantly the specificity of the technique to label HSCs or multipotent progenitors as no HSC specific labelling technique has been discovered yet (McRae et al., 2019).

Steady-state haematopoiesis is negatively affected with time. The functional properties are perturbed of aged HSCs (Dykstra et al., 2011, Florian et al., 2012). *In vivo* tracking of haematopoiesis in aged mice showed heterogeneity in HSC pool associated with perturbed cell division and function resulting in a reduction of clone numbers in PB and BM (Ganuza et al., 2019). Furthermore, during ageing mice present an expansion of HSCs associated with a defect in their functionality, bias to myeloid lineage, and a reduction in lymphoid cells (Florian et al., 2012, Dykstra et al., 2011, Sawen et al., 2018). Yet, further investigation is needed to identify the molecular mechanism of ageing HSCs.

Zeb1 contains two zinc finger DNA binding domains at C-and N-terminals as well as functional central domains including homeodomain (HD), SBD and CtBP interaction (CID) domains encoding 1117 amino acids protein (Fortini et al., 1991, Vandewalle et al., 2009). Some domains function as recruiting domains for co-repressors such as CtBP ((carboxyl-terminal binding protein) (Postigo and Dean, 1999b, van Grunsven

et al., 2007) or co-activators such as p300 or P/CAF (van Grunsven et al., 2006, Postigo et al., 2003).

Zeb1 is a potent inducer of EMT and is implicated in myogenesis (Postigo and Dean, 1997, Postigo and Dean, 1999a, Postigo et al., 1999, Jethanandani and Kramer, 2005), neurones development and differentiation (Clark and Chiu, 2003), post gastrulation embryogenesis (Funahashi et al., 1993), chondrocyte development (Bellon et al., 2009), skeletal, and respiratory systems (Bellon et al., 2009, Takagi et al., 1998). Further, *Zeb1* is found as an essential driver of EMT in cancer stem cells (CSCs) in solid tumours facilitating their metastasis, invasion, and drug resistance (Aigner et al., 2007, Eger et al., 2005, Spaderna et al., 2008).

In the haematopoietic system, *Zeb1* is found to be required for T cell development after germline deletion of *Zeb1* (Higashi et al., 1997) and for B cell proliferation during humoral immune responses (Arnold et al., 2012). A recent study found that *Zeb1* is required for memory $CD8^+$ T cells survival and maintenance (Guan et al., 2018). Still, the role of *Zeb1* in adult haematopoiesis has not been discovered yet.

Conventional *Zeb1* knockout mice presented defects in skeletal system, chondrocyte and T cell development (Takagi et al., 1998). Also, they showed oedema, curled tail, and haemorrhage. More importantly, *Zeb1* mutants developed until birth but they died because of inability to breathe (Takagi et al., 1998). Recently, a *Zeb1* conditional KO mouse model ($Zeb1^{fl/fl}$) has been generated and characterised (Brabletz et al., 2017). In this model, exon 6 was flanked with loxP sites and under the control of a tissue specific promoter that activates Cre recombinase to delete the floxed exon 6, *Zeb1* can be conditionally inactivated at any developmental stage. Deletion of exon 6 resulted in splicing of exon 5—7 and eventually premature stop in translation (Brabletz et al., 2017). $Zeb1^{-/-}$ embryos from this model presented a consistent phenotype as previously described from the conventional *Zeb1* knockout (Takagi et al., 1998).

3.2 Aims

The physiological significance of *Zeb1* during immune cell development led us to investigate its broader role in the adult haematopoietic system specifically on the long-term haematopoiesis. Exploring the function of *Zeb1* in normal biology will allow a better understanding of its role in blood malignancies that will facilitate treatment invention. Thus, we utilized a conditional *Zeb1* knockout mouse model to investigate the function of *Zeb1* in the adult HSCs and other haematopoietic lineages. The aims of this chapter are:

1. Assessing the expression of *Zeb1* in FACS purified haematopoietic compartments including primitive, committed progenitors, and differentiated cells using qPCR.

2. Studying the function of *Zeb1* in adult long-term haematopoiesis using Mx1-Cre interferon inducible system to conditionally delete *Zeb1* in the haematopoietic system at steady state.

3. Studying the role of persistent *Zeb1* loss on HSCs function using transplantation experiments. Sorted HSCs at week 32 after Zeb1 deletion was transplanted and engraftment in PB was monitored for 16 weeks.

4. Studying the cell autonomous effect of prolonged loss of *Zeb1* in adult haematopoiesis.

3.3 Result

3.3.1 *Zeb1* is differentially expressed in haematopoietic stem, progenitor and differentiated cell compartments

Zeb1 mRNA expression has been observed in thymus, BM, spleen, and other haematopoietic tissues (Postigo and Dean, 2000, Higashi et al., 1997). However, *Zeb1* expression in different subsets of haematopoietic cells, including haematopoietic stem and progenitor cells (HSPCs), remains unclear. Therefore, Q-PCR for *Zeb1* mRNA expression analysis was performed on haematopoietic compartments prospectively isolated by FACS. *Zeb1* was expressed at relatively high levels in stem and progenitor cells (HSCs, MPP, HPC1, and HPC2) and differentiated cells (myeloid, erythroid, B, and T lineages) whereas it was lower in committed myeloid and lymphoid progenitors (CMP, GMP, MEP, CLP) (Figure 3.1).

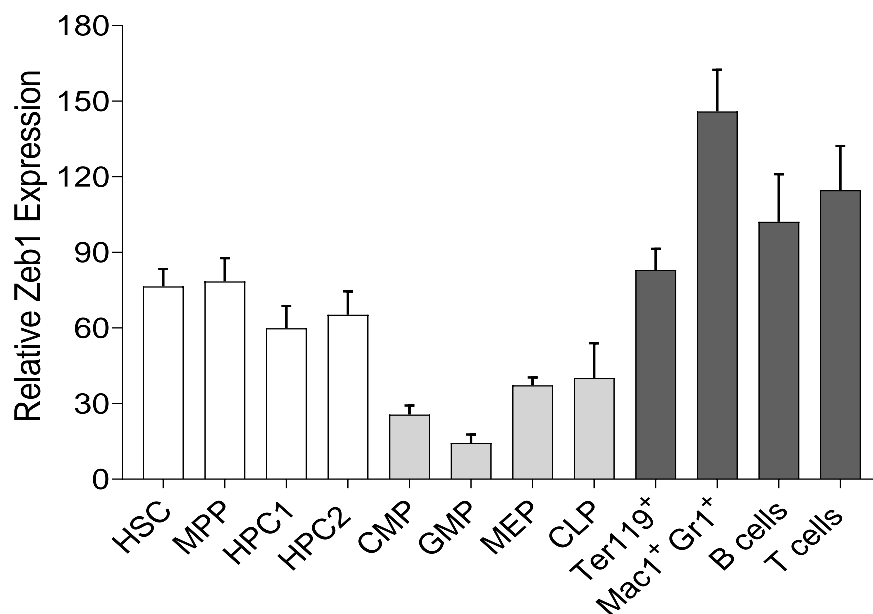


Figure 3.1. *Zeb1* is differentially expressed in haematopoietic cells. mRNA expression analysis using qPCR of *Zeb1* in different mouse haematopoietic populations in the BM. HSCs give rise to primitive progenitors: MPP, HPC1, and HPC2. These primitive progenitors differentiate into myeloid progenitors: CMP, GMP, and MEP, which finally produce mature cells erythrocytes (Ter119) and granulocytes and macrophages ($Gr1^+$ $Mac1^+$) while the lymphoid progenitor CLP gives rise to B cells and T cells. HPRT was used as a reference gene. Data represents mean± SEM (n= 6-7 biological replicates for each population except 3 biological replicates for CLP).

3.3.2 *Zeb1* is required for long-term multilineage haematopoietic differentiation

To study the requirement of *Zeb1* in long-term haematopoiesis, we utilised a conditional inducible *Zeb1* KO mouse model, crossing *Zeb1$^{fl/fl}$* mice (Brabletz et al., 2017) with mice expressing the interferon-inducible Mx1-Cre recombinase (Kuhn et al., 1995). This generated offspring that were either *Zeb1$^{fl/fl}$ Mx1-Cre$^{-/-}$* (control) or *Zeb1$^{fl/fl}$ Mx1-Cre$^{+/-}$* mice. *Zeb1$^{fl/fl}$ Mx1-Cre$^{+/-}$* mice or control mice were injected intraperitoneally with Poly I:C, which activates the *Mx1* promoter to stimulate Cre recombinase expression that excises the floxed *Zeb1* alleles (Kuhn et al., 1995) in haematopoietic tissues to generate *Zeb1$^{-/-}$* mice (Figure 3.2A). After Poly I:C treatment, control or *Zeb1$^{-/-}$* mice were left for 32 weeks and were monitored by bleeding every 4 weeks for changes in PB (PB) after *Zeb1* loss (Figure 3.2A). PB was analysed for the expression of Mac1 and Gr1 in myeloid cells, B220 in B cells, and CD4/CD8 in T cells (Figure 3.2B). Our data showed a significant reduction in the proportion of Mac1$^+$cells (Mac1$^+$ or Mac1$^+$ Gr1$^-$ will be used interchangeably throughout the thesis) that comprise the monocytes (Lagasse and Weissman, 1996, Sunderkotter et al., 2004) after *Zeb1* KO (Figure 3.2C). This reduction started early, at week 4 after *Zeb1* KO and was maintained until week 32 post *Zeb1* deletion (Figure 3.2C). No significant change was observed in Mac1$^+$ Gr1$^+$ cells that contain granulocytes (Sunderkotter et al., 2004, Lagasse and Weissman, 1996) nor in B cells (Figure 3.2D and E). T cell frequency after *Zeb1* deletion was unchanged at week 4 but trended towards a reduction from week 8 onwards, was significantly reduced by week 16 after *Zeb1* deletion and was restored by week 32 post *Zeb1* deletion (Figure 3.2F). *Zeb1* deletion was assessed in PB at week 6 and T cells at week 2 after *Zeb1* deletion and we found incomplete deletion of *Zeb1* in PB and T cells, indicating that some T cells escaped *Zeb1* inactivation (Figure 3.2G).

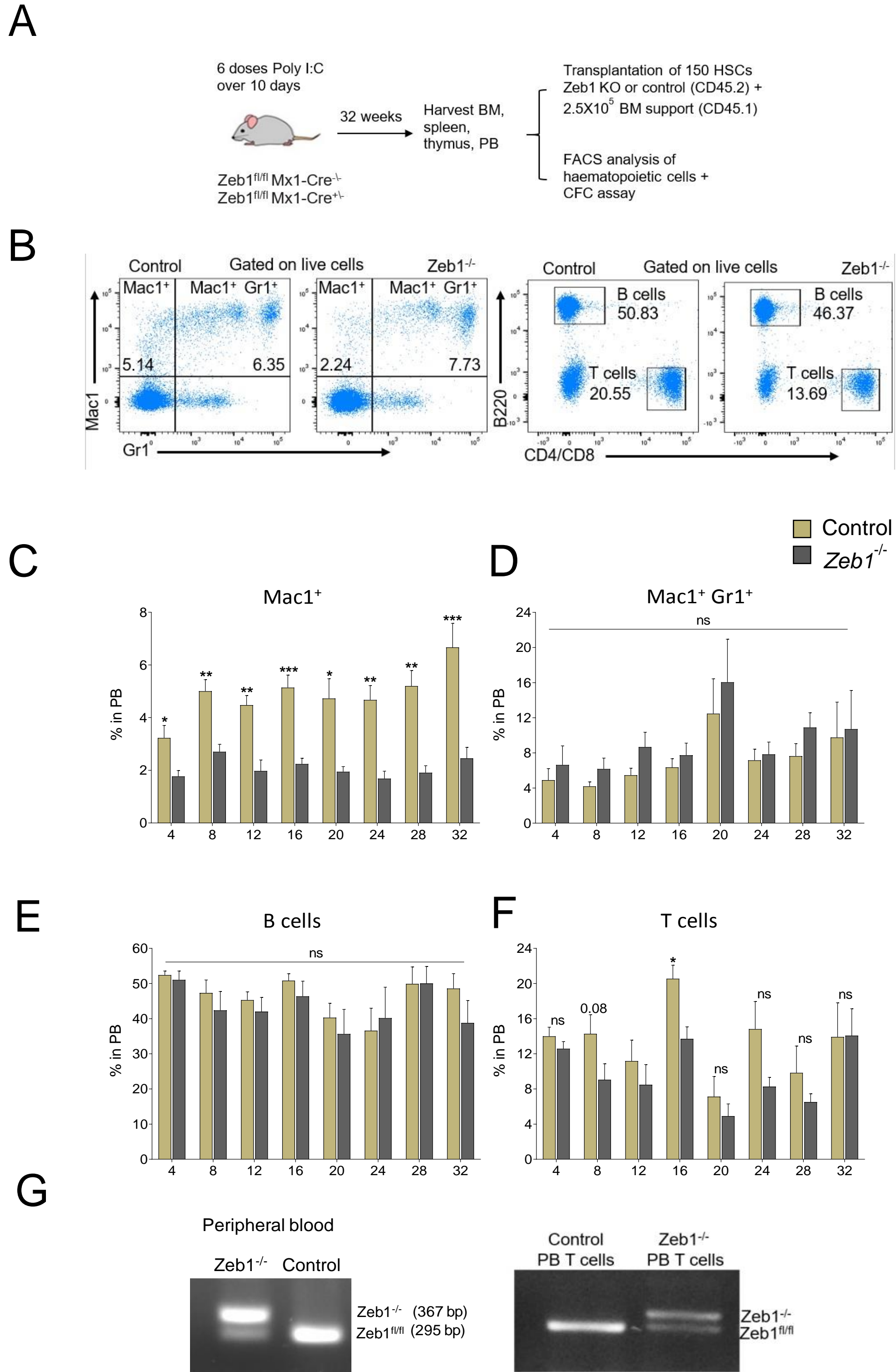
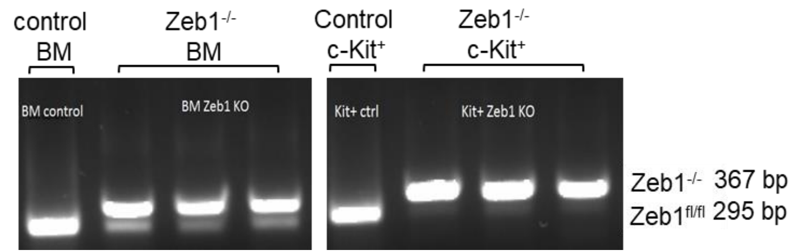


Figure 3.2. Long-term *Zeb1* loss resulted in a reduction of Mac1⁺ Gr1⁻ myeloid cells and a transient reduction of T cells in PB. (A) A scheme of *Zeb1* deletion. 6 doses of Poly I:C were intraperitoneally administered into *Zeb1$^{fl/fl}$ Mx1-Cre$^-$* and *Zeb1$^{fl/fl}$ Mx1-Cre$^+$* and mice were monitored for 32 weeks. (B) Representative FACS plots of PB analysis at week 16 post *Zeb1* deletion of myeloid, B, and T cells gated from DAPI negative cells (live cells). The same gating strategy were followed for PB analysis of the other time points. (C) Analysis of Mac1$^+$ myeloid cells, (D) Mac1$^+$ Gr1$^+$ myeloid cells, (E) B220$^+$ B cells, and (F) CD4$^+$ CD8$^+$ T cells, every 4 weeks after *Zeb1* deletion until W32. Data from 3-4 independent experiments (N=5 at week 4, N= 8 at weeks 8-20, N= 5-6 at weeks 24-28, N=8-9 at week 32). (G) *Zeb1* deletion was assessed using genomic DNA from total PB cells and PB T cells. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

To assess the impact of *Zeb1* on HSPCs and other haematopoietic cell compartments, at 32 weeks after *Zeb1* deletion control and *Zeb1*$^{-/-}$ mice were culled. *Zeb1*$^{-/-}$ mice did not show any gross abnormalities in comparison to their control counterparts. Next, we assessed *Zeb1* deletion in the BM at the DNA level. DNA was extracted from total BM and c-kit^{+} BM cells (a population that contains HSPCs and committed myeloid and lymphoid progenitors). We observed incomplete deletion of *Zeb1* in total BM although most cells showed *Zeb1* deletion. Yet, full deletion of *Zeb1* was achieved in HSPC containing ckit^{+} cells (Figure 3.3A), suggesting that some differentiated haematopoietic cells escaped *Zeb1* inactivation in BM, as was also observed in T cells from PB (Figure 3.2G).

We assessed the total cellularity of the BM and spleen in *Zeb1*$^{-/-}$ and control mice. A trend towards a reduction in *Zeb1*$^{-/-}$ BM cellularity was observed and a near significant reduction in *Zeb1*$^{-/-}$ spleen cellularity (Figure 3.3B). Surprisingly, this was associated with a significant increase in *Zeb1*$^{-/-}$ spleen weight (Figure 3.3B). Thus, *Zeb1* regulates long-term maintenance of cellularity in haematopoietic organs.

A



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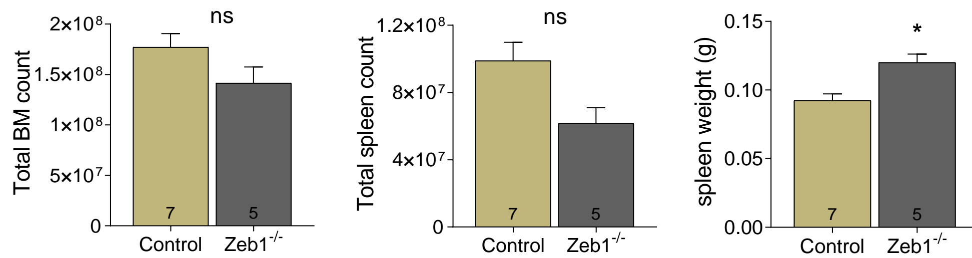
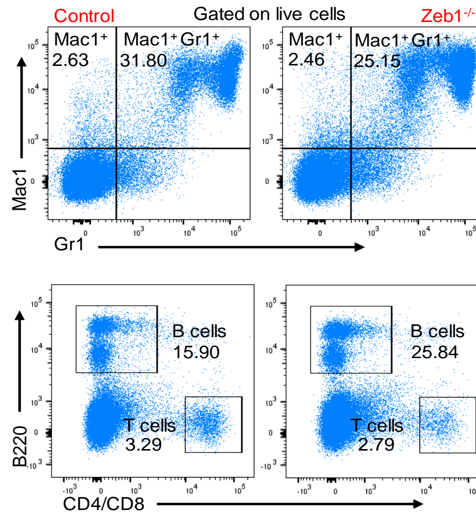


Figure 3.3. Long-term *Zeb1* loss resulted in a slight reduction in BM and spleen cellularity. (A) *Zeb1* deletion was assessed from total BM cells and $cKit^+$ population. A small subset from total BM cells (a faint band) was observed as few cells still have the *Zeb1* floxed alleles un-excised, while $ckit^+$ cells have the full excised band. (B) analysis of total cells of BM and spleen as well as spleen weight from control and $Zeb1^{-/-}$ mice (number of biological replicates are written on the bottom of the bars). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

Next, using flow cytometry we immunophenotyped differentiated haematopoietic cells from BM and spleen (Figure 3.4A). In BM, the frequency of B cells significantly increased in *Zeb1*$^{-/-}$ and no change in myeloid, erythroid, and T cells (Figure 3.4B). However, when we quantified the absolute number of these populations to account for the observed changes in cellularity after *Zeb1* deletion, we found a comparable number of B cells in the BM and a reduction in T cell number in *Zeb1*$^{-/-}$ mice (Figure 3.4B). Also, a statistically insignificant reduction was observed in Mac1^{+} Gr1^{+} myeloid cells from *Zeb1*$^{-/-}$ mice and no change was found in Mac1^{+} cells (Figure 3.4B). In the spleen, we found a reduction in the frequency of Mac1^{+} myeloid cells and T cells (Figure 3.4C) in *Zeb1*$^{-/-}$ mice. Similar to the BM, the frequency of B cells significantly increased but no significant change was found in the absolute count (Figure 3.4C). Also, we found a decrease in the absolute numbers of Mac1^{+}, Mac1^{+} Gr1^{+}, and T cell number after *Zeb1* deletion (Figure 3.4C). These data together indicate the requirement of *Zeb1* in the differentiation of multiple haematopoietic lineages during long-term haematopoiesis.

A



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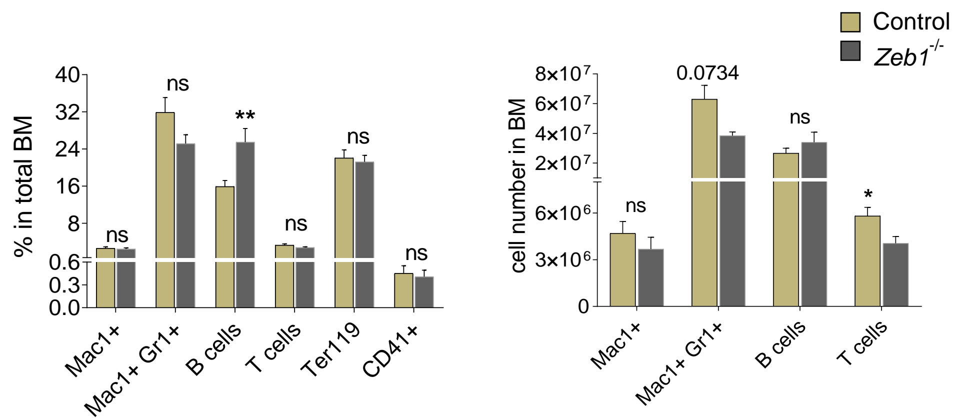

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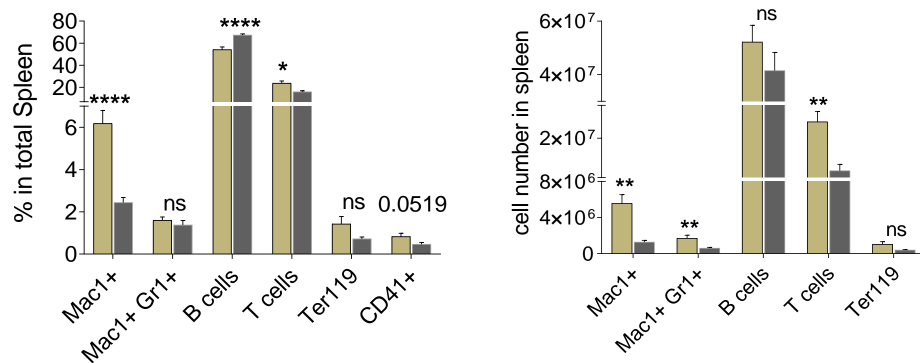


Figure 3.4. Long-term *Zeb1* loss resulted in a multilineage haematopoietic differentiation defect in the BM and spleen. (A) Representative FACS plots of BM analysis at week 32 (W32) after *Zeb1* deletion of myeloid, B, and T cells gated from DAPI negative cells (live cells). The same gating strategy was followed for spleen analysis. (B) Analysis of the frequency and cell count of the differentiated cells in the BM at W32 after *Zeb1* deletion. (C) Analysis of the frequency and cell count of the differentiated cells in spleen at W32 after *Zeb1* deletion. Data from 3-4 independent experiments (N= 9-8 for BM and spleen frequency analysis except for Ter119$^+$ and CD41$^+$ N= 5-7, N= 6-7 for BM cell count and N=5-7 for spleen count). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.3 Persistent loss of *Zeb1* expression resulted in a dramatic reduction of thymocytes and T cells in thymus

Given the transient *Zeb1* dependent reduction in T cells in the PB observed after long-term *Zeb1* deletion, we analysed the thymi of *Zeb1*$^{-/-}$ mice at week 32, which were very small and dramatically reduced in total cellularity (Figure 3.5A). Using CD4 and CD8 expression to assess developing T cell subsets in the thymus of these mice (Figure 3.5B) increased frequencies of immature double negative $CD4^{-}$ $CD8^{-}$ (DN) cells and mature single positive SP CD4 ($CD4^{+}$) and SP CD8 ($CD8^{+}$) T cells were observed, in stark contrast to a significant reduction in double positive $CD4^{+}$ $C8^{+}$ (DP) cells (Figure 3.5C). These data indicate a differentiation defect at select stages of T cell development in the thymus in the context of long-term KO of *Zeb1*. When absolute number of these populations was quantified, we found a significant reduction in total cell count of all populations: DN, DP, $CD4^{+}$ and $CD8^{+}$ in *Zeb1*$^{-/-}$ thymus (Figure 3.5D). Together, these data demonstrate the long-term requirement for *Zeb1* during thymocyte maturation.

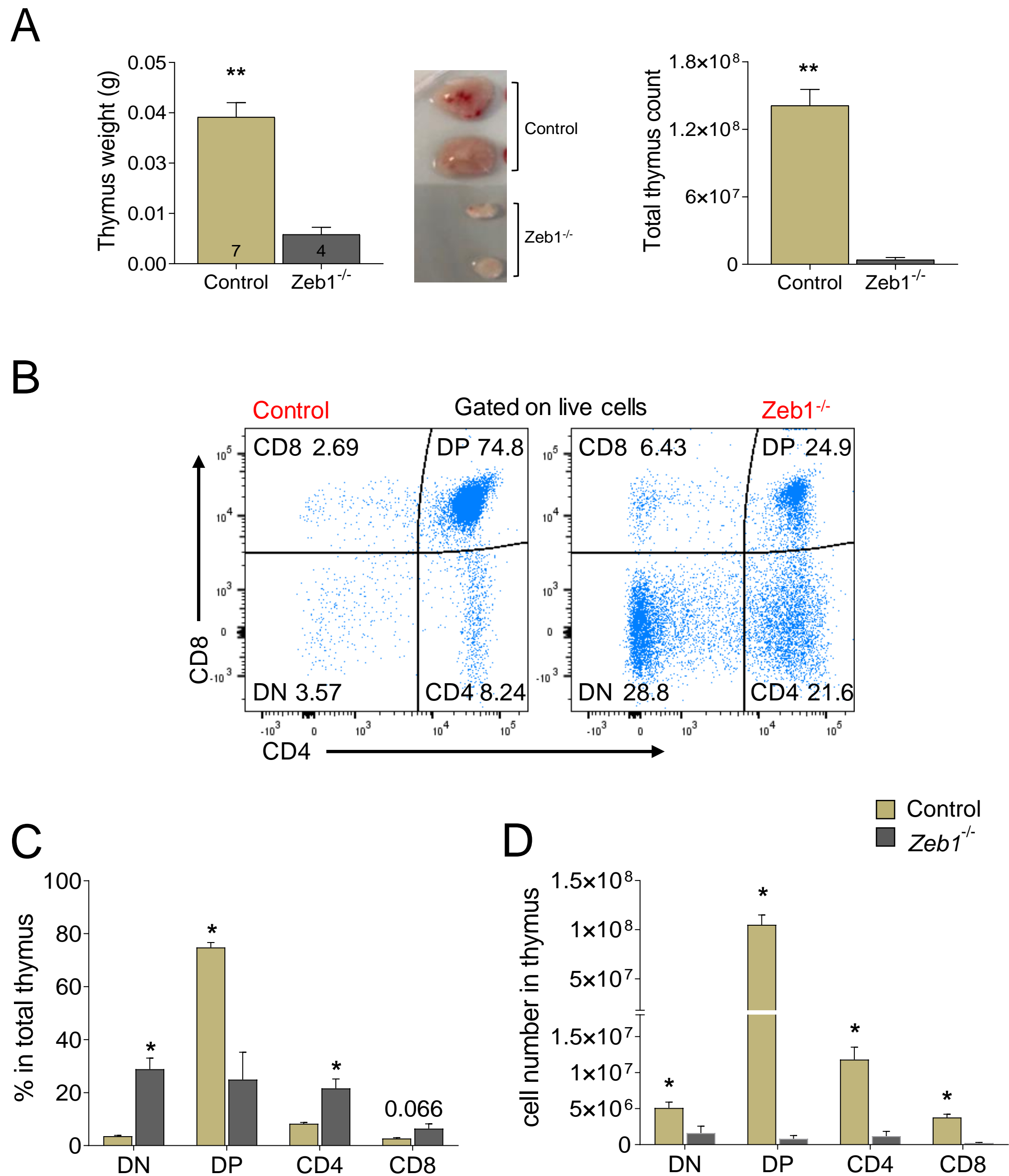
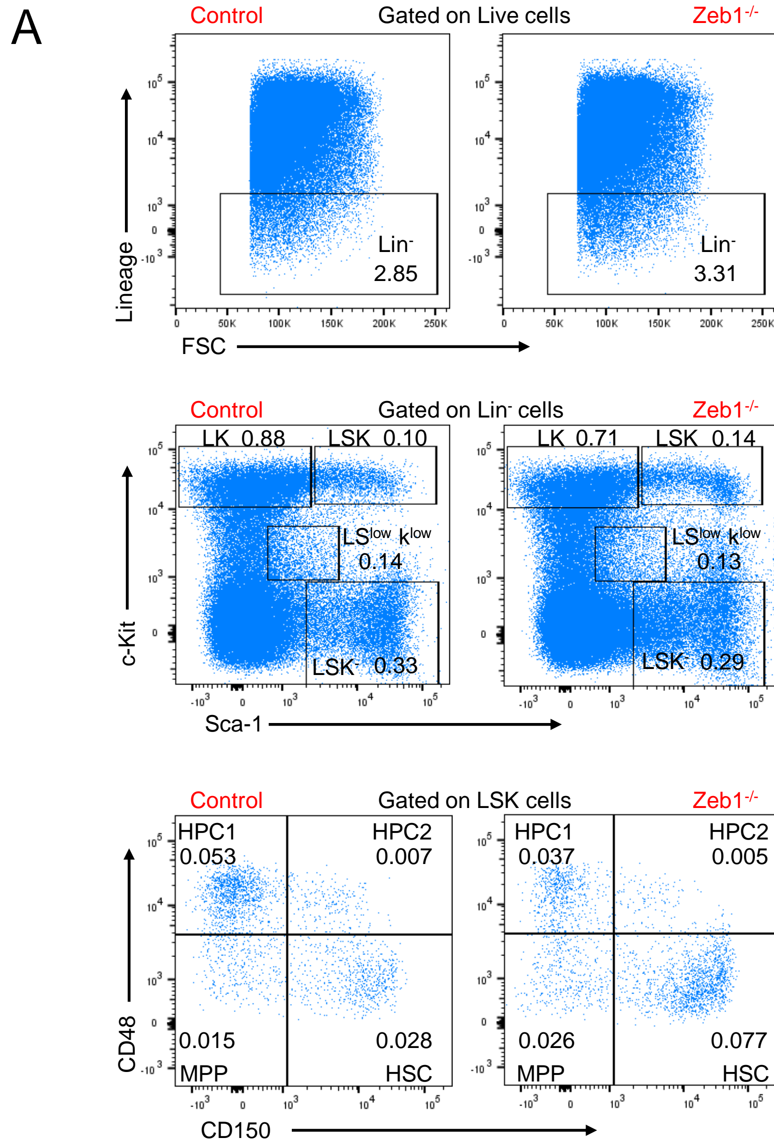
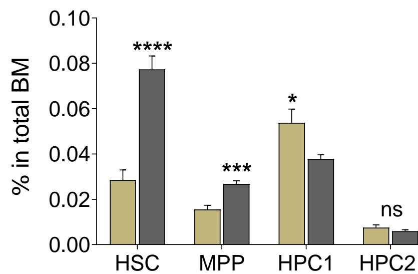


Figure 3.5. Long-term *Zeb1* loss resulted in a dramatic reduction of thymocytes and T cells in thymus. (A) analysis of thymus total cells and weight with a representative picture from control and *Zeb1*$^{-/-}$ mice (number of biological replicates are written on the bottom of the bars, N= 7 control and 4 *Zeb1*$^{-/-}$). (B) Representative FACS plots of thymus analysis at week 32 after *Zeb1* deletion based on the expression of CD4 and CD8 gated from DAPI negative cells (live cells). (C) Analysis of the frequency of T cells in thymus at W32 after *Zeb1* deletion. (D) Analysis of cell count of T cells in thymus at W32 after *Zeb1* deletion. Data from 3 independent experiments (N= 7 control and 3 *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.4 Long-term loss of *Zeb1* resulted in an expansion of HSCs and reduction of HPC1 cells

To examine the requirement of *Zeb1* for long-term maintenance of HSPCs, we documented stem and progenitor cell numbers in the BM at 32 weeks after *Zeb1* deletion (Figure 3.6A). First, we analysed the Lin^- population that contains all the HSPCs and lineage-restricted progenitors and did not find a significant change in Lin^- proportion between control and *Zeb1*$^{-/-}$ (Figure 3.6A). Next, within the Lin^- cells, c-kit and Sca-1 cell surface markers were used to sub-divide the Lin^- population into LSK (Lin^- Sca-1^+ c-Kit^+) HSPCs, LK (Lin^- Sca-1^- c-kit^+) myeloid, erythroid, and megakaryocytic progenitors, Lin^- sca-1^{low} c-Kit^{low} for common lymphoid progenitors, LSK^- (Lin^- Sca-1^+ c-Kit^-) early lymphoid precursors (Kumar et al., 2008) that contain innate lymphoid cell progenitors group 2 (ILC2) (Ghaedi et al., 2016) (Figure 3.6A). Using the SLAM markers CD150 and CD48 we analysed four populations within LSK population: LSK $CD150^+$ $CD48^-$ (HSC), LSK $CD150^-$ $CD48^-$ (MPP), LSK $CD150^-$ $CD48^+$ (HPC1), and LSK $CD150^+$ $CD48^+$ (HPC2) (Figure 3.6A). *Zeb1*$^{-/-}$ mice showed an expansion in the frequency of HSCs (2.7-fold) and MPPs (Figure 3.6B). However, HPC1 frequency was reduced in *Zeb1*$^{-/-}$ mice while there was no change in the frequency of HPC2 (Figure 3.6B). When we checked the number of cells, we found a significant increase of HSCs and near significant increase of MPPs (Figure 3.6C) after *Zeb1* deletion. Furthermore, the absolute number of HPC1 and HPC2 was significantly reduced in *Zeb1*$^{-/-}$ mice compared to control mice (Figure 3.6C). These data indicate that *Zeb1* is required to maintain the abundance of HSPCs during long-term, steady-state adult haematopoiesis.





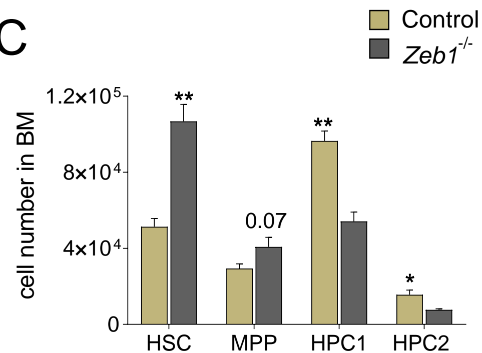


Figure 3.6. Long-term *Zeb1* loss resulted in an expansion of HSCs and reduction of HPC1 cells. (A) Representative FACS plots of BM HSPCs analysis at week 32 after *Zeb1* deletion gated from DAPI negative cells (live cells). Analysis of the frequency (B) and total count (C) of HSPCs at W32 after *Zeb1* deletion. Data from 3-4 independent experiments (N= 8-9 for the analysis of frequency of cells and 5-7 for cell count analysis). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.5 Long-term loss of *Zeb1* did not affect BM lineage-restricted progenitors in steady state haematopoiesis

We next asked if long-term loss of *Zeb1* expression affect committed myeloid and lymphoid progenitors downstream of HSCs and primitive progenitors. Within LK population using CD34 and CD16/32 we analysed three populations: LK $CD34^+$ $CD16/32^-$ (CMP), LK $CD34^+$ $CD16/32^+$ (GMP), and LK $CD34^-$ $CD16/32^-$ (MEP) (Figure 3.7A). These populations give rise to myeloid, megakaryocytic, and erythroid lineages. By analysing the frequency and absolute numbers of these cells, we did not find significant differences between the two genotypes, although GMP and MEP showed a trend toward reduction after *Zeb1* deletion (Figure 3.7B and C). Looking at the common lymphoid progenitor (CLP), defined as Lin^- $c\text{-}kit^{low}$ $Sca\text{-}1^{low}$ $CD127^+$ (IL7rα), and ILC2, defined as Lin^- $c\text{-}Kit^-$ $Sca\text{-}1^+$ (LSK^-) $CD127^+$ (Figure 3.7A), no difference was observed in the frequency and number between control and *Zeb1*$^{-/-}$ mice at 32 weeks after the last dose of Poly I:C (figure 3.7B and C). These data indicate that *Zeb1* is dispensable for maintenance of BM committed progenitors during long-term, steady-state adult haematopoiesis.

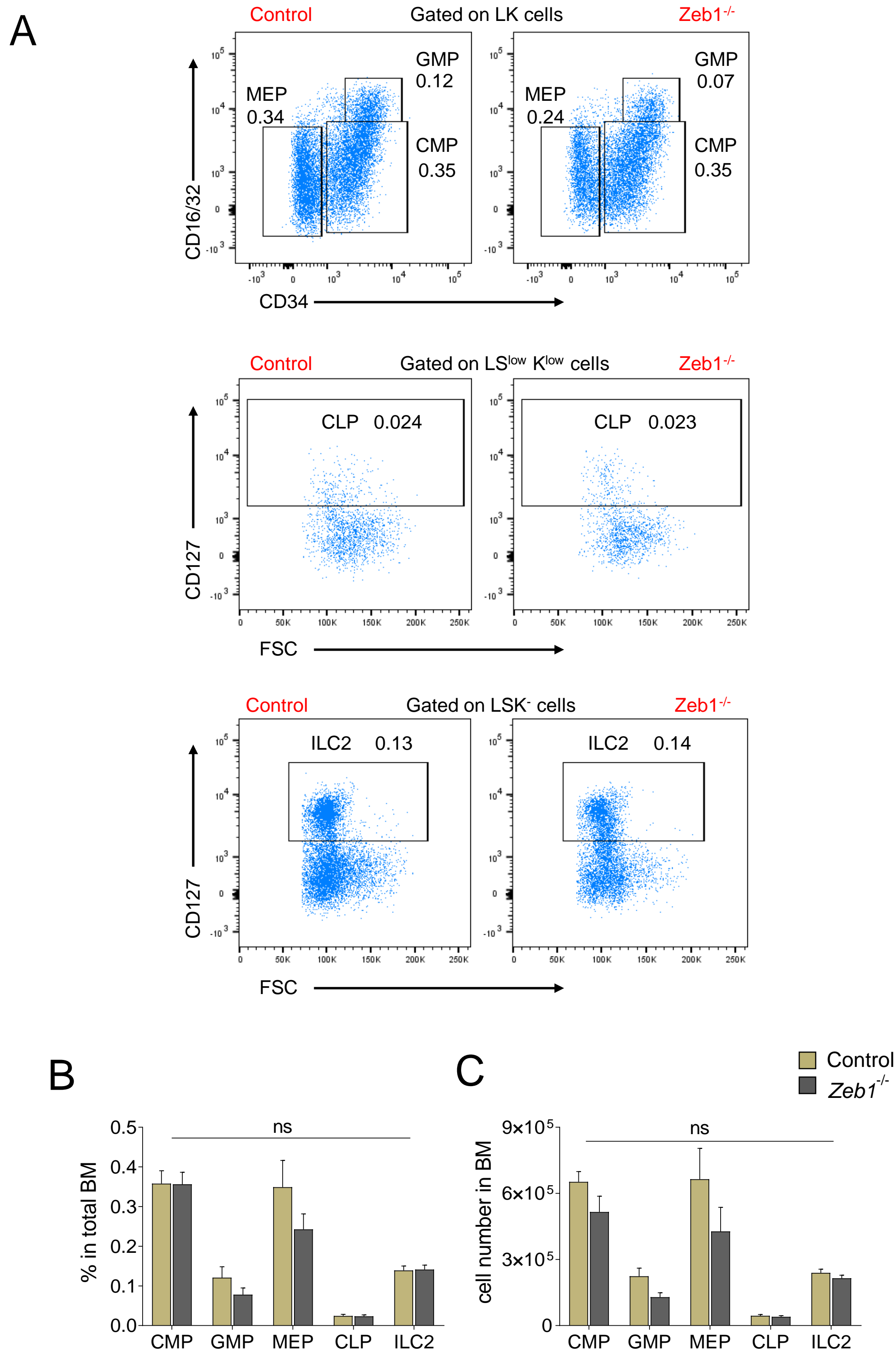


Figure 3.7. Long-term *Zeb1* loss did not affect lineage-restricted progenitors in the BM during steady state haematopoiesis. (A) Representative FACS plots of BM committed progenitors: GMP, CMP, MEP, CLP, and ILC2 analysis at week 32 after *Zeb1* deletion. Analysis of the frequency (B) and total count (C) of CMP, GMP, MEP, CLP, and ILC2 at W32 after *Zeb1* deletion. Data from 3-4 independent experiments (N= 8-9 for the analysis of frequency of cells and 5-7 for cell count analysis. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.6 Long-term *Zeb1* loss conferred cell survival advantage on HSPCs

Next, we asked if the changes within HSPCs in LSK population were caused by differential cell survival. We used a common flow cytometry-based assay for the measurement of cell apoptosis using Annexin V binding to the cell surface phosphatidylserine (PS) in conjunction with DAPI (Vermes et al., 1995). Immunophenotypic expansion of HSCs and MPPs (Figure 3.6) was associated with a reduction in apoptotic levels, while the reduction in HPC1 and HPC2 numbers was not associated with deregulated apoptosis, as judged by comparable apoptotic levels between control and *Zeb1*$^{-/-}$ mice (Figure 3.8A and B). Even though we did not see a significant difference in numbers of CMP and GMP between control and *Zeb1*$^{-/-}$, curiously, apoptosis was lower in these populations in *Zeb1*$^{-/-}$ compared to control (Figure 3.8C). Together, these data imply that *Zeb1* acts as an important regulator of long-term cell survival and homeostasis of HSPCs.

3.3.7 Long-term *Zeb1* loss causes HSC expansion independently of cell cycle status

Next, we asked whether the expansion of HSCs in *Zeb1*$^{-/-}$ mice was associated with changes in cell cycle status. Intracellular staining of Ki-67, a nuclear proliferation antigen (Gerdes et al., 1984), and DAPI allows analysis of quiescent cells in G0 (cells do not express Ki-67) and different cell cycle phases G1, S, and G2-M phases of cell cycle (Gerdes et al., 1984, Kim and Sederstrom, 2015, Schwarting et al., 1986). No difference in G0, G1, or S-G2-M was observed between control and *Zeb1*$^{-/-}$ HSPCs (Figure 3.9A and B). Thus, *Zeb1* modulates the long-term homeostasis of HSCs independently of cell cycle regulation.

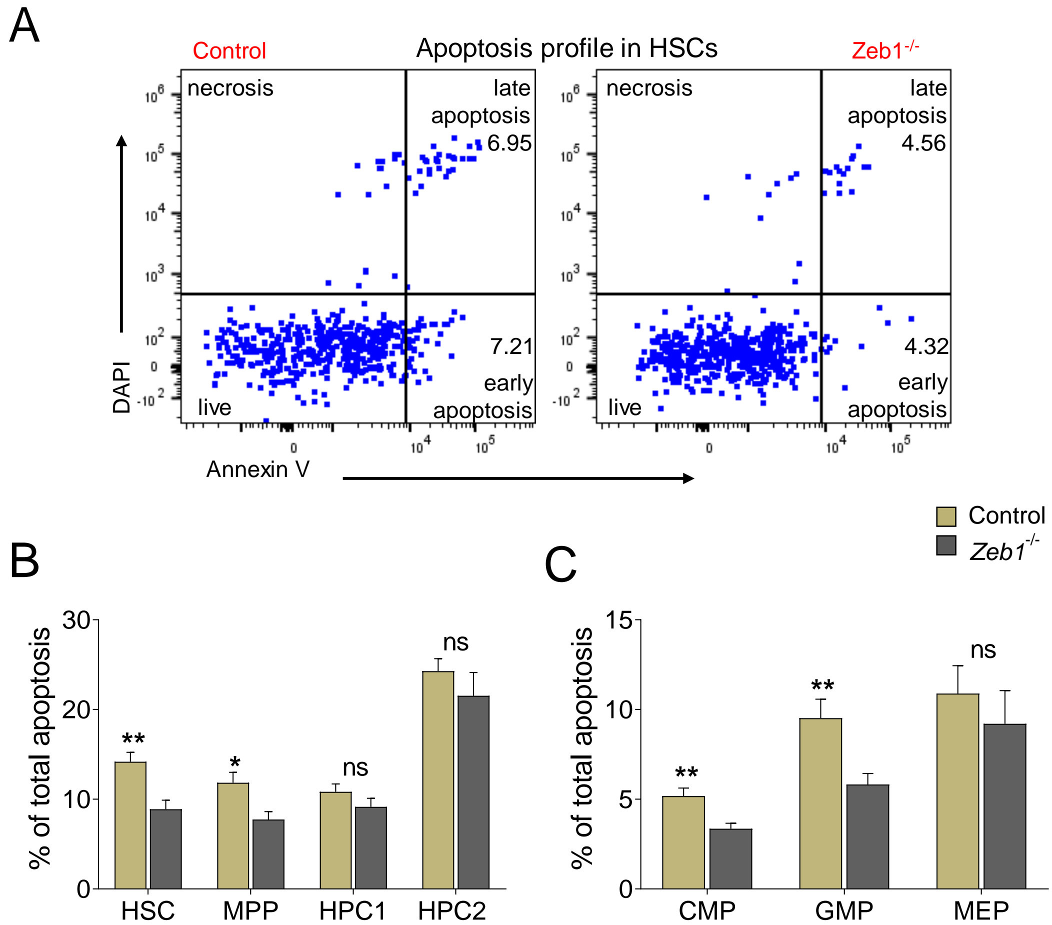
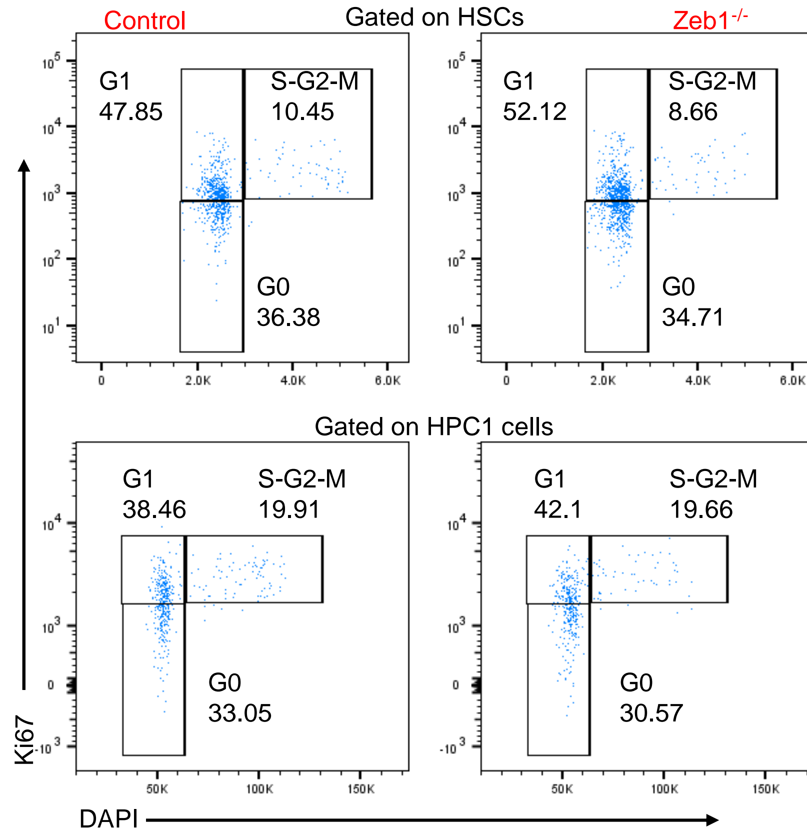


Figure 3.8. Long-term *Zeb1* loss conferred survival advantage of HSPCs. (A) Representative FACS plots of apoptosis analysis of HSCs at week 32 after *Zeb1* deletion. Analysis of total apoptosis in BM HSPCs (B) and committed progenitors (C) at W32 after *Zeb1* deletion. Data from 4 independent experiments (N= 7 for control and *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.



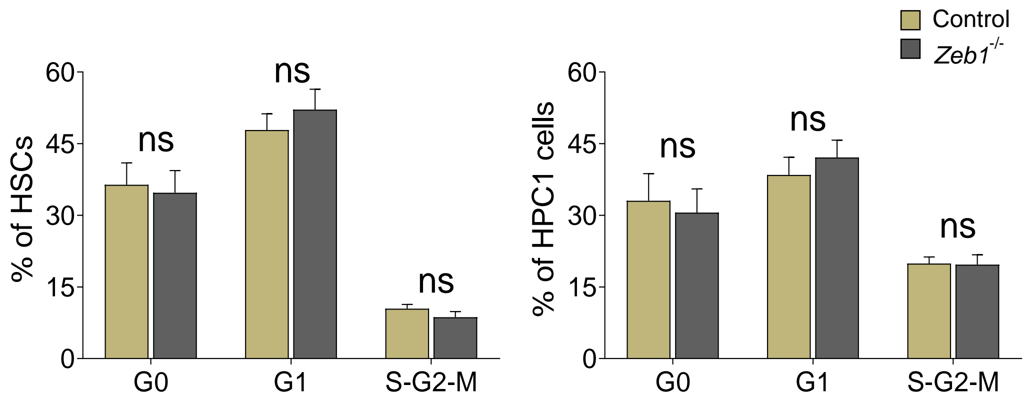


Figure 3.9. Long-term *Zeb1* loss did not affect cell cycle status of HSPCs. (A) Representative FACS plots of cell cycle analysis of HSCs and HPC1 at week 32 after *Zeb1* deletion. (B) Analysis of G0 and cell cycle phases of BM HSCs and HPC1 cells at W32 after *Zeb1* deletion. Data from 4 independent experiments (N= 8 for control and *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.8 Long-term loss of *Zeb1* expression resulted in a reduction of HSCs and myeloid and lymphoid progenitors in spleen

Because we observed perturbed haematopoiesis in the BM characterised by accumulation of HSCs, we hypothesised that prolonged loss of *Zeb1* would affect extramedullary haematopoiesis in extramedullary organs. Thus, we analysed haematopoietic compartments in spleen. First, we quantified the number of Lin^- cells in spleen and found a significant reduction after *Zeb1* deletion (Figure 3.10A). This contrasts with the findings in BM that showed similar Lin^- cell frequency between control and *Zeb1*$^{-/-}$ genotypes (Figure 3.6A). Also, we found no difference in LSK number, however the number of LK cells showed a trend toward reduction in *Zeb1*$^{-/-}$ (Figure 3.10B). Interestingly, while there was an expansion of HSCs in the BM, we found a dramatic reduction of splenic HSC numbers in *Zeb1*$^{-/-}$ mice compared to control, with no significant changes in MPP, HPC1, and HPC2 (Figure 3.10C). Within the LK compartment, the number of CMPs and GMPs significantly decreased with trend toward reduction in MEPs after *Zeb1* loss (Figure 3.10D). A more profound decrease was found in lymphoid progenitors CLPs and ILC2 after *Zeb1* deletion (Figure 3.10D). Altogether, these data show that long-term *Zeb1* loss resulted in a defect in extramedullary haematopoiesis in the spleen.

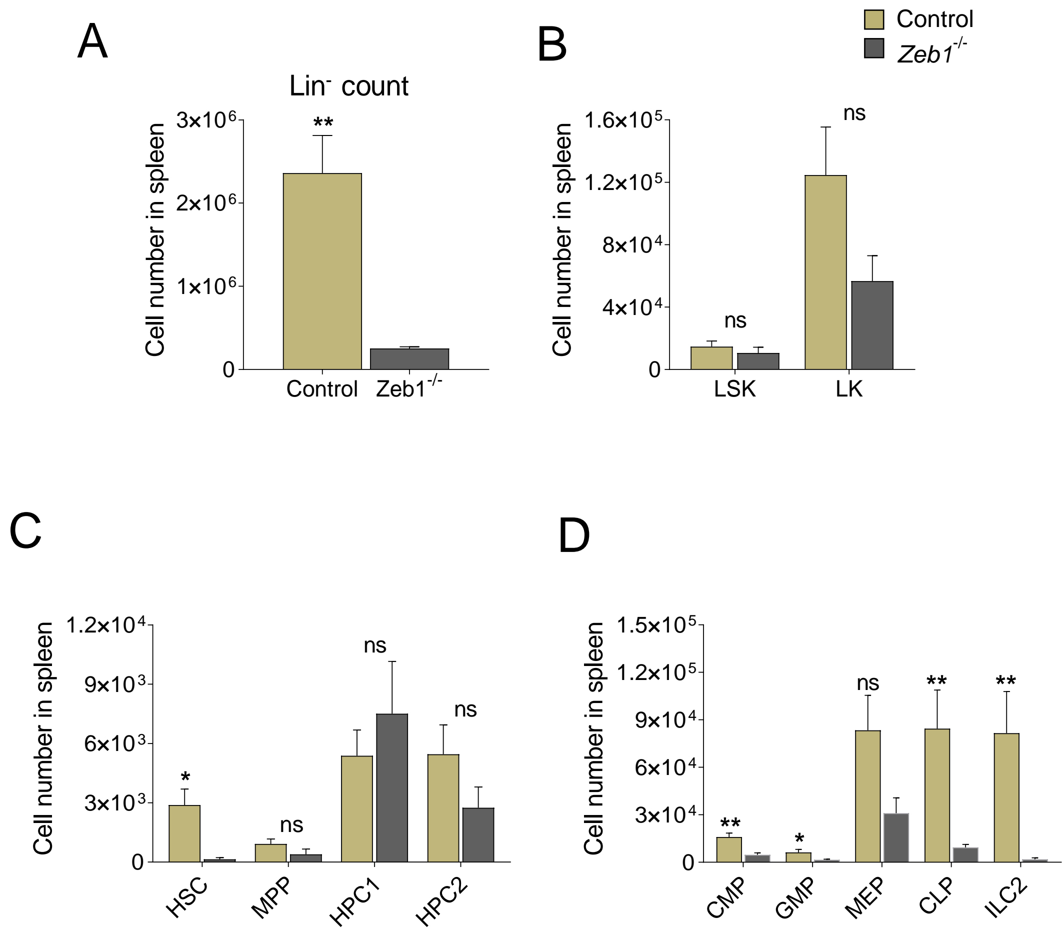


Figure 3.10. Long-term *Zeb1* loss resulted in a reduction of HSCs and myeloid and lymphoid progenitors in spleen. Analysis of the total count of Lin⁻ cells (A), LK and LSK (B), HSPCs (C), and committed progenitors (D) in spleen at W32 after *Zeb1* deletion. Data from 3 independent experiments (N= 7 control and 5 *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.9 HSCs lacking *Zeb1* long-term expression showed a multilineage haematopoietic differentiation defect after transplantation

To test the functionality of HSCs from *Zeb1*$^{-/-}$ mice, we cell sorted 150 HSCs (CD45.2) from control or *Zeb1*$^{-/-}$ mice at 32 weeks following deletion, mixed them with $2X10^5$ BM competitor cells (CD45.1) and these were transplanted into lethally irradiated recipients (CD45.1) (Figure 3.11A). We monitored the engraftment capacity in PB until week 16 (Figure 3.11A). A trend towards a reduction in the total engraftment of *Zeb1*-deficient HSCs in PB compared to control at week 4 was observed (Figure 3.11B). Significant engraftment failure was observed at week 8 and continued to decrease progressively until week 16 (Figure 3.11B). To test the donor contribution to PB haematopoietic lineages, we analysed PB for CD45.2 (donor) and CD45.1 (competitor) in conjunction with Mac1$^+$ myeloid, Mac1$^+$ Gr1$^+$ myeloid, B220$^+$ B cells, and CD4$^+$/CD8$^+$ T cells. Consistent with steady-state data regarding the dramatic reduction of thymic T cells, no engrafted T cells were derived from recipients transplanted with *Zeb1*$^{-/-}$ HSCs (Figure 3.11C). Also, a profound reduction in donor contribution to B cells (Figure 3.11D) and Mac1$^+$ (Figure 3.11E) was observed in recipients of *Zeb1*$^{-/-}$ HSCs. Furthermore, we noticed a reduction in donor contribution to Mac1$^+$ Gr1$^+$ myeloid cells (Figure 3.11F). Thus, long-term *Zeb1* expression is required for the multi-lineage differentiation function of HSCs.

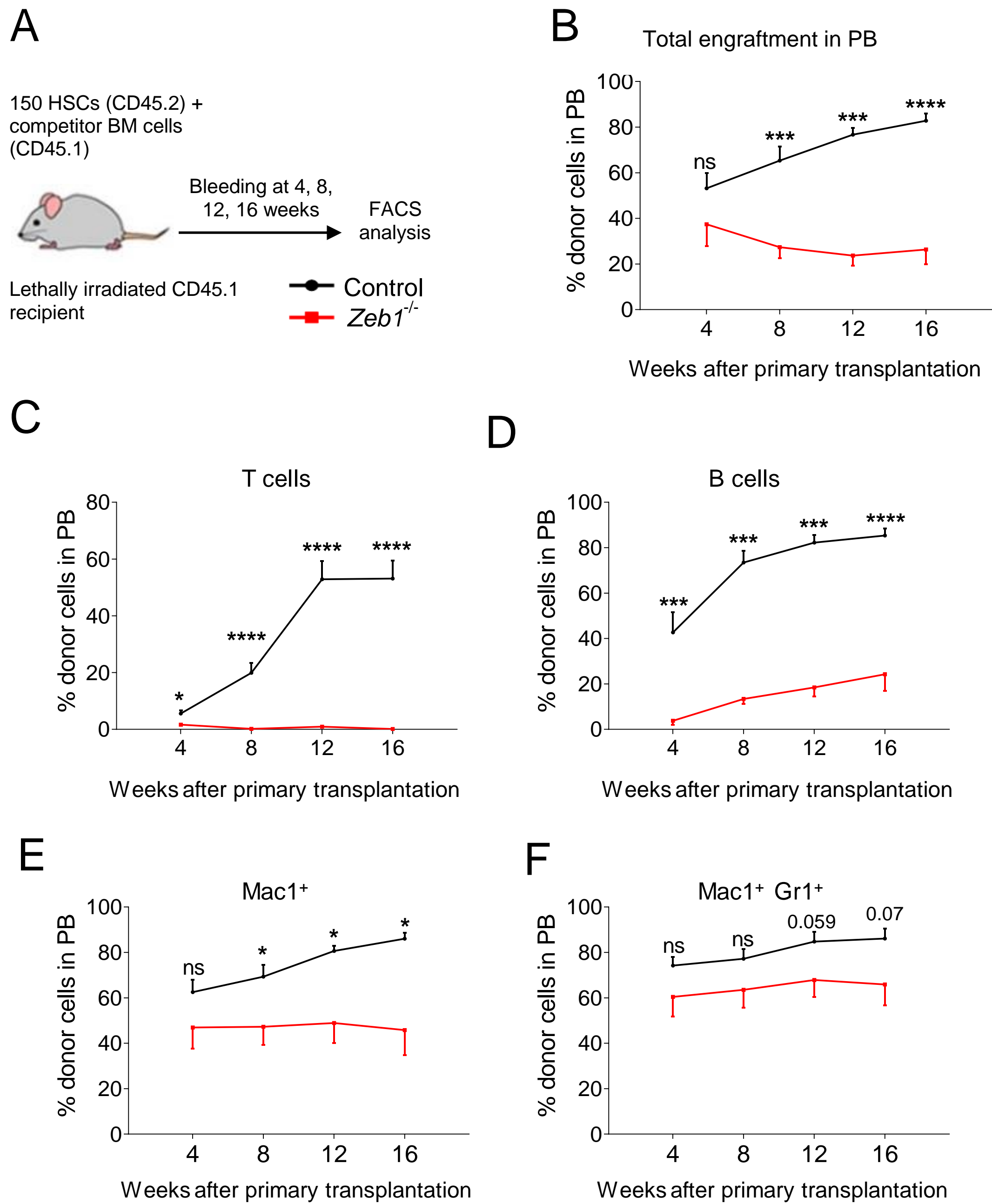


Figure 3.11. HSCs lacking long-term *Zeb1* expression displayed a multilineage haematopoietic differentiation defect after transplantation. (A) A scheme of competitive HSC transplantation experiment. 150 HSCs from control or *Zeb1*$^{-/-}$ mice (donor CD45.2) mixed with 2X10^5 BM competitor cells (CD45.1) were transplanted into lethally irradiated recipients (CD45.1) and mice were monitored by bleeding the tail vein at different time points until week 16. (B) The percentage of donor cells in PB at different time points post-transplant from control and *Zeb1*$^{-/-}$ mice. Donor contribution to PB T cells (C), B cells (D), Mac1$^+$ (E), and Mac1$^+$ Gr1$^+$ myeloid cells (F) from control and *Zeb1*$^{-/-}$ mice. Data from 2 independent experiments (N= 9-10 control and 8-9 *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

At 16 weeks after primary HSC transplantation, we evaluated donor engraftment in the BM, spleen, and thymus. Consistent with PB data, we found a reduced donor contribution to total BM (Figure 3.12A). We found no donor T cell engraftment from *Zeb1*$^{-/-}$ HSCs (Figure 3.12A) and donor contribution to B cells was dramatically reduced as well as Mac1^{+} and Mac1^{+} Gr1^{+} myeloid cells from recipients receiving *Zeb1*$^{-/-}$ HSCs (Figure 3.12A). A similar pattern of engraftment defect was observed in the spleen (Figure 3.12B). A profound effect was found in thymus and different T cell subsets as well as DN B220^{+} thymic B cells (Figure 3.12C). Overall, these data demonstrate that while long-term *Zeb1* loss expands the immunophenotypically defined HSC compartment, those HSCs are functionally compromised, as evidenced by the multilineage haematopoietic differentiation defects observed after transplantation of *Zeb1*$^{-/-}$ HSCs.

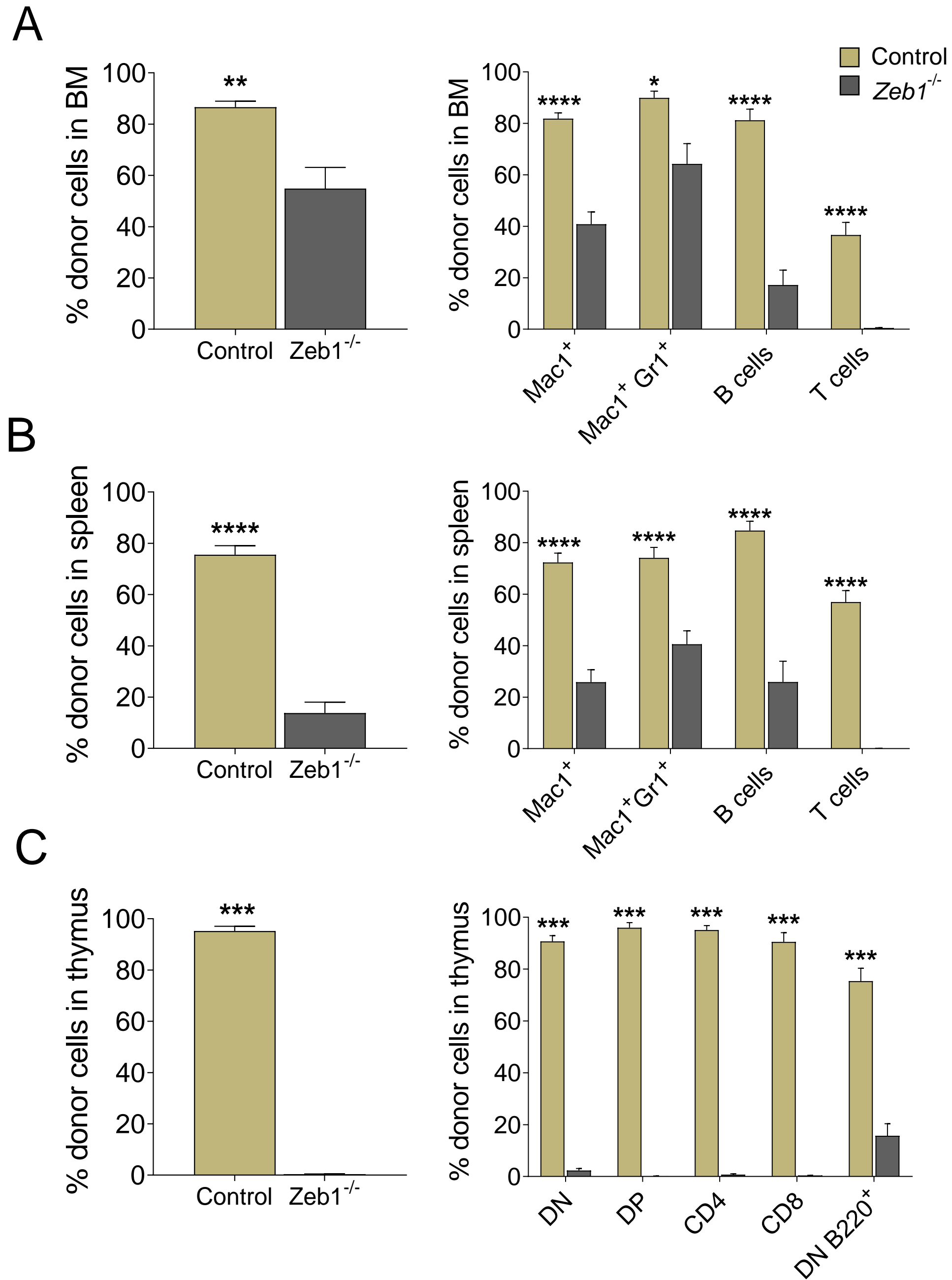


Figure 3.12. HSCs lacking *Zeb1* long-term displayed a multilineage haematopoietic differentiation defect after transplantation. (A) The percentage of total donor cells and differentiated cells in the BM 16 weeks after transplantation from control and *Zeb1*$^{-/-}$ mice. (B) The percentage of total donor cells and differentiated cells in spleen 16 weeks after transplantation from control and *Zeb1*$^{-/-}$ mice. (C) The percentage of total donor cells and T cells in thymus 16 weeks after transplantation from control and *Zeb1*$^{-/-}$ mice. Data from 2 independent experiments (N= 9-10 control and 8-9 *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.10 *Zeb1*$^{-/-}$ HSCs showed a reduced donor contribution to BM HPC1 and myeloid and lymphoid progenitors

We asked whether the multi-lineage differentiation defects observed in *Zeb1*$^{-/-}$ HSCs originates from engraftment defects in HSCs or committed progenitors. Within LSK compartments, the donor contribution to HSC, MPP, and HPC2 was equal between recipients of control or *Zeb1*$^{-/-}$ HSCs (Figure 3.13A). However, there was a significant reduction in the donor contribution to HPC1 in *Zeb1*$^{-/-}$ compared to control (Figure 3.13A). We also analysed committed progenitors downstream of HSPCs and found a dramatic reduction in donor contribution to GMP, CLP and ILC2 populations and near significant reductions in CMP and MEP populations in recipients of *Zeb1*$^{-/-}$ HSCs (Figure 3.13B).

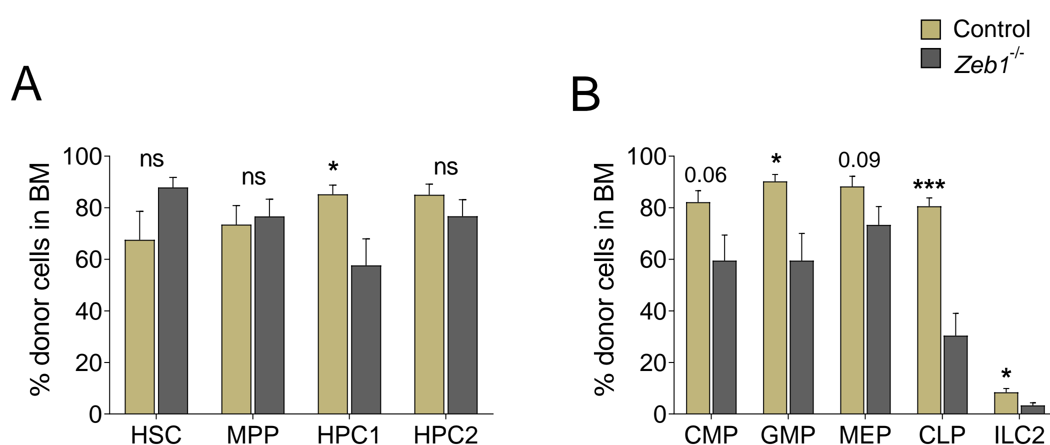


Figure 3.13. *Zeb1*$^{-/-}$ HSCs showed a reduced donor contribution to BM HPC1 and lineage-restricted progenitors. (A) Analysis of donor contribution to HSPCs in the BM 16 weeks after transplantation from control and *Zeb1*$^{-/-}$ mice. (B) Analysis of donor contribution to the committed progenitors in the BM 16 weeks after transplantation from control and *Zeb1*$^{-/-}$ mice. Data from 2 independent experiments (N= 9-10 control and 9 *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.11 Long-term *Zeb1* expression was required for cell-autonomous HSC functionality

Since the Mx1-Cre system can delete genes in non-haematopoietic tissues such as BM niche cells (Kuhn et al., 1995, Zhang et al., 2003), we asked whether the requirement for *Zeb1* in the maintenance of long-term HSC function was cell autonomous. To this end, we performed a competitive BM transplantation by transplanting $5X10^5$ BM cells from *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^-$ and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^+$ (CD45.2) admixed with an equal number of competitor cells (CD45.1) into lethally irradiated recipients (CD45.1). 8 weeks later *Zeb1* deletion was induced by administering recipients with Poly I:C (Figure 3.14A). Mice were monitored by PB bleeding for 32 weeks following *Zeb1* deletion (Figure 3.14B). Prior to treating mice with Poly I:C, donor cells were comparable between both genotypes (Figure 3.14C). However, we observed a gradual reduction in *Zeb1*$^{-/-}$ donor cells in PB and Mac1$^+$ and Mac1$^+$ Gr1$^+$ myeloid cells from 4 weeks after *Zeb1* deletion and more marked changes in B cell, and T cell engraftment (Figure 3.14C).

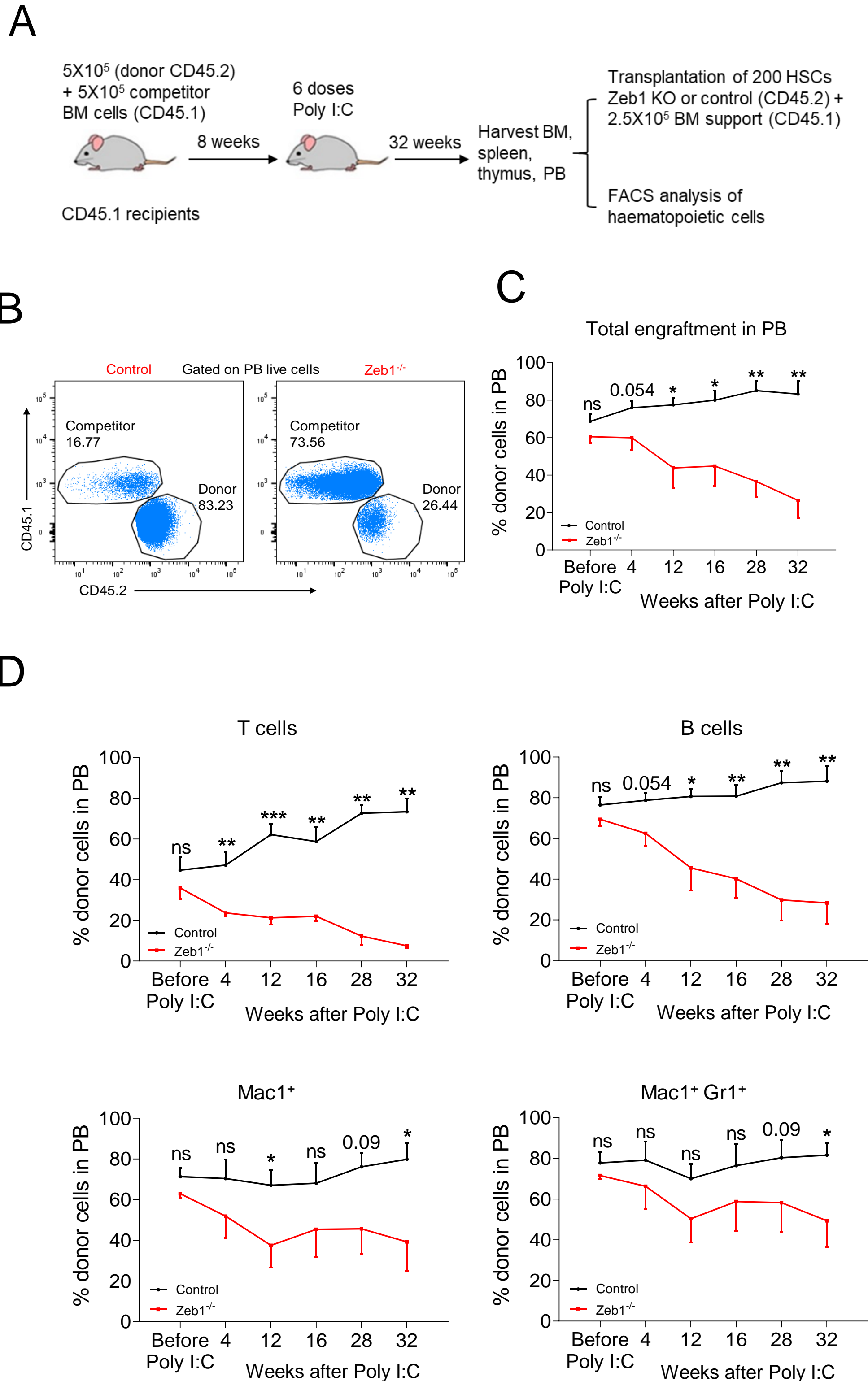


Figure 3.14. Long-term *Zeb1* expression is required for multilineage haematopoietic differentiation in PB via cell autonomous manner. (A) A scheme of cell autonomous experiment. (B) Representative FACS plots for the analysis of engraftment in PB at week 32 after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ gated from DAPI negative cells (live cells). (C) The percentage of donor cells in PB at different time points after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. (D) Donor contribution to PB T cells, B cells, Mac1^{+}, and Mac1^{+} Gr1^{+} myeloid cells from control and *Zeb1*$^{-/-}$ mice. Data from 2 independent experiments (N= 5-7 control and *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

Consistent with PB data, reduced donor cells were found at all haematopoietic sites: BM, spleen, and thymus (Figure 3.15A-C). When differentiated cells were analysed, a robust reduction in donor contribution to T cells in both the BM and spleen (Figure 3.15A and B) and T cells from the thymus was observed (Figure 3.15C). Although we found a reduced donor contribution to myeloid and lymphoid lineages in the BM and spleen after *Zeb1* deletion, donor contribution to $Ter119^+$ erythroid cells in the BM and spleen was comparable between control and $Zeb1^{-/-}$ (Figure 3.145A and B). These data demonstrate that long-term *Zeb1* expression modulates HSC multi-lineage differentiation in a cell autonomous manner.

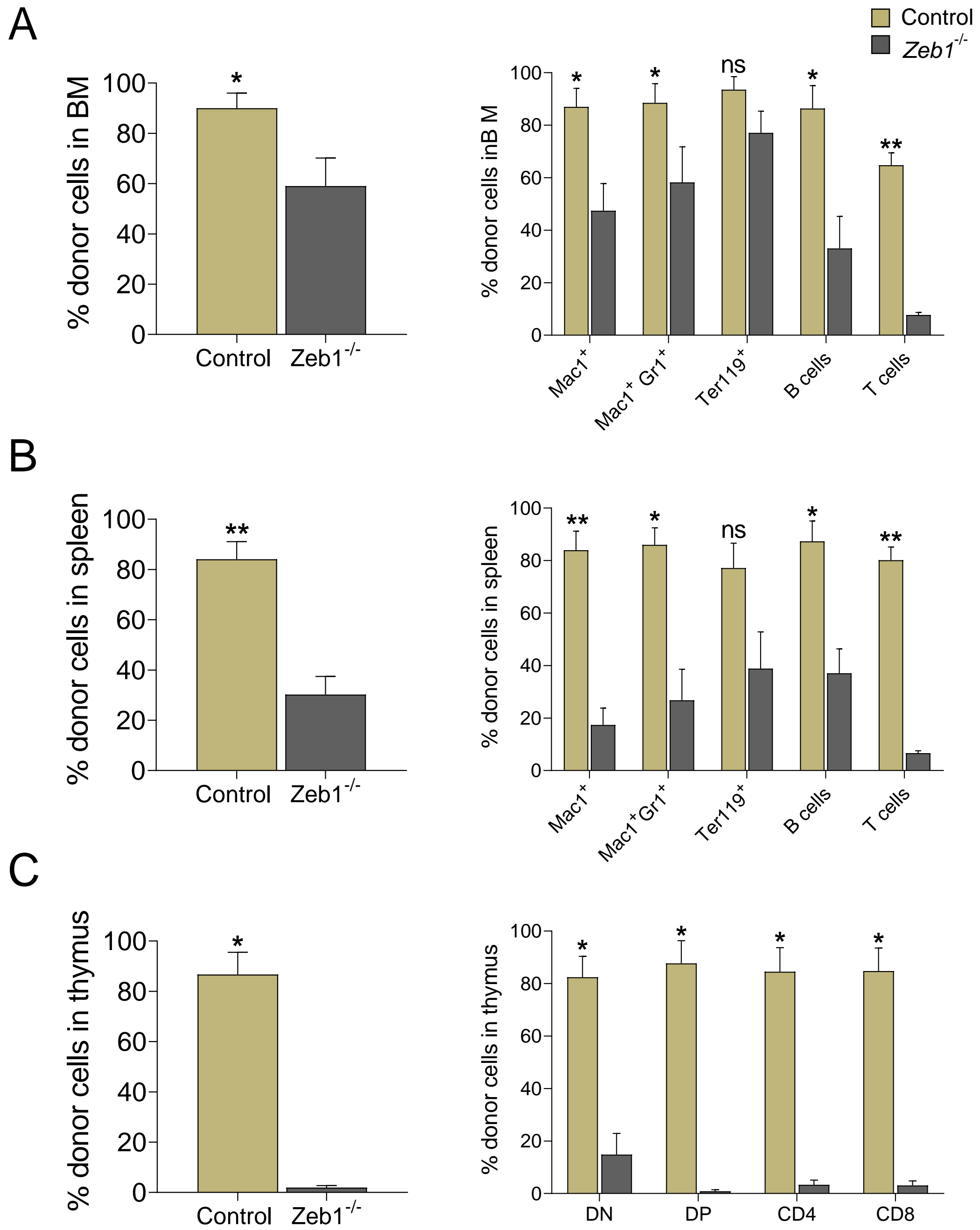


Figure 3.15. Long-term *Zeb1* expression is required for multilineage haematopoietic differentiation in BM and spleen in a cell autonomous. (A) The percentage of total donor cells and differentiated cells in the BM 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. (B) The percentage of total donor cells and differentiated cells in spleen 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. (C) The percentage of total donor cells and T cells in thymus 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. Data from 2 independent experiments (N= 5 control and *Zeb1*$^{-/-}$ except for thymus N= 5 control and 3 *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.12 *Zeb1* was required for long-term BM myeloid and lymphoid progenitor differentiation in a cell autonomous manner

At 32 weeks after the last dose of Poly I:C, we evaluated whether donor contribution to HSPC and progenitor compartments in the BM was perturbed in the context of long-term *Zeb1* deletion. No significant change was noted in donor contribution of *Zeb1*$^{-/-}$ HSCs (Figure 3.16A). However, during the differentiation toward MPP, HPC1, and HPC2, a statistically insignificant reduction in donor contribution to MPP and HPC2 was found and a significant reduction in HPC1 was observed (Figure 3.16A). There was also a reduction in GMP (P= 0.055) and CLP (P=0.055) (Figure 3.16B) and a general trend of reduction in CMP and MEP populations from *Zeb1*$^{-/-}$ mice (Figure 3.16B). Finally, we found a significant reduction in ILC2 from the *Zeb1*$^{-/-}$ genotype (Figure 3.16B). In concert, these data indicate *Zeb1* is required for differentiation of HSCs toward multipotent and committed progenitors in a cell autonomous manner.

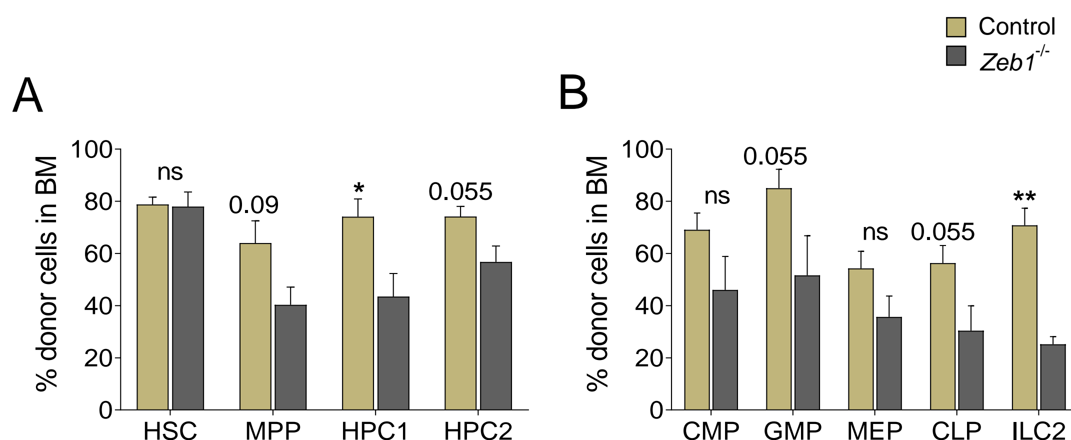


Figure 3.16. Long-term *Zeb1* expression is required for HSC differentiation to BM progenitors in a cell autonomous manner. (A) Analysis of donor contribution to HSPCs in the BM 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. (B) Analysis of donor contribution to the committed progenitors in the BM 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. Data from 2 independent experiments (N= 5 control and *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.13 *Zeb1* mediated defect observed in myeloid and lymphoid progenitors in the BM was not associated with apoptosis

To evaluate whether the reduction in HPC1 and myeloid and lymphoid progenitors was associated with changes in apoptosis, 32 weeks after the last dose of Poly I:C BM progenitors were assessed using the Annexin V assay, but no changes were observed between control and *Zeb1*$^{-/-}$ (Figure 3.17A and B). Thus, long-term deficiency of *Zeb1* modulates myeloid and lymphoid progenitors independently of cell survival mechanisms, suggesting that *Zeb1* predominantly regulates initial lineage commitment of HSCs in this context.

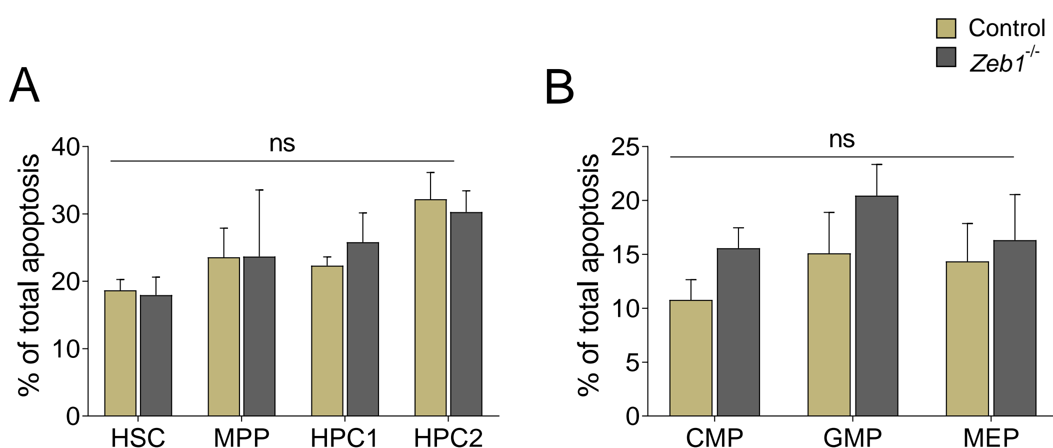


Figure 3.17. The defect observed in myeloid and lymphoid progenitors in the BM is not associated with apoptosis. Analysis of total apoptosis in BM HSPCs (A) and committed progenitors (B) 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$. Data from one independent experiment (N= 4 control and *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.14 Persistent *Zeb1* loss resulted in a reduced cell autonomous donor contribution to LSK CD34$^-$ cells HSCs in spleen

Because we found a severe impairment of haematopoiesis in spleen at steady state, we wanted to know if these changes are cell intrinsic or spleen niche dependent. We analysed the donor contribution to HSPCs in spleen via cell autonomous manner. Although there was a trend toward reduction in donor contribution to Lin$^-$ and LK populations, we found a significant reduction in LSK population (Figure 3.18A). Further, we studied LSK cells according to the expression of CD34. LSK CD34$^-$ that contains HSCs (Osawa et al., 1996) showed a significant reduction whereas the donor contribution to LSK CD34$^+$ cells that contain multipotent progenitors was comparable between control and *Zeb1*$^{-/-}$ (Figure 3.18B). Further, we analysed committed myeloid and lymphoid progenitors in the spleen. No significant change was observed in CMP, GMP, MEP, and CLP between control and *Zeb1*$^{-/-}$ (Figure 3.18C). However, ILC2 cells derived from donor cells were attenuated in *Zeb1*$^{-/-}$ (Figure 3.18C). Thus, *Zeb1* regulates long-term extra-medullary HSC abundance in a cell-autonomous manner.

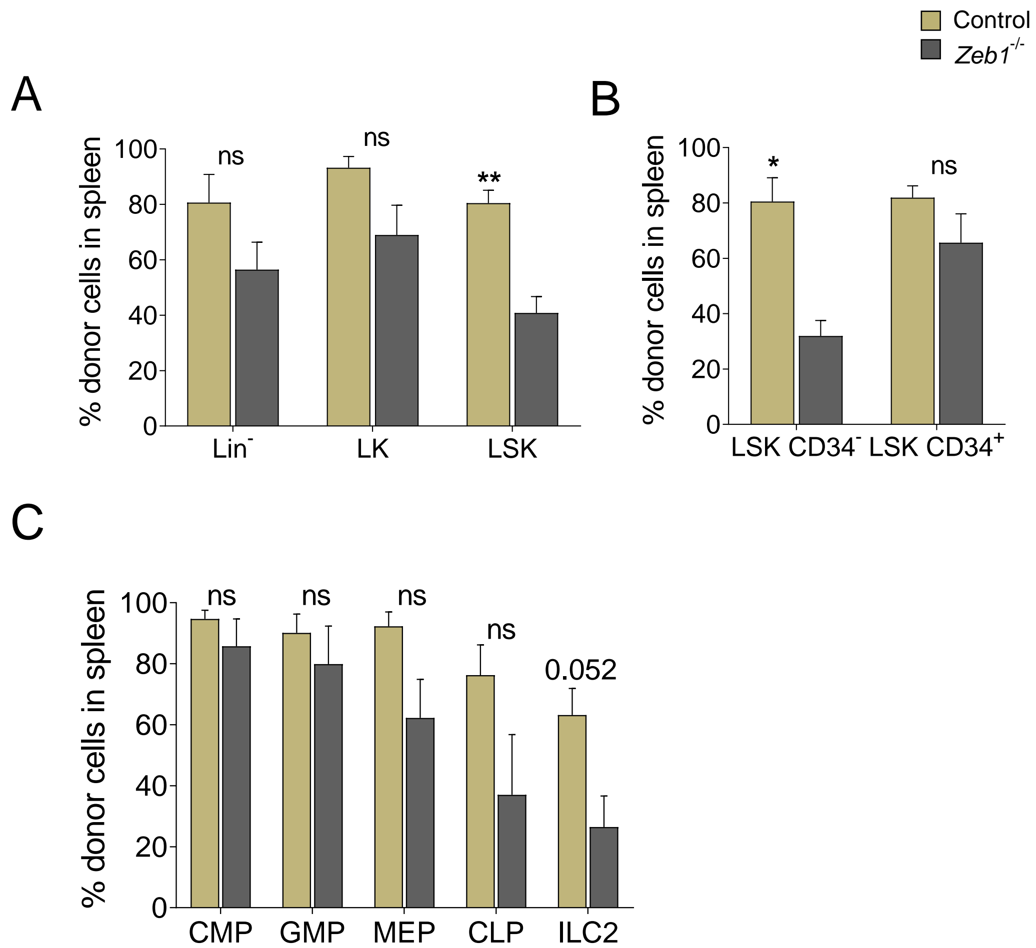


Figure 3.18. *Zeb1* loss resulted in a reduced donor contribution to LSK CD34⁻ cells in spleen via cell autonomous manner. (A) Analysis of donor contribution to Lin⁻, LK, and LSK in spleen 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. (B) Analysis of donor contribution to LSK CD34^{+} and LSK CD34^{-} in spleen 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice. (C) Analysis of donor contribution to committed progenitors in spleen 32 weeks after the last dose of Poly I:C from control and *Zeb1*$^{-/-}$ mice Data from one independent experiment (N= 3 control and *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.15 Extended *Zeb1* loss resulted in a cell-autonomous multilineage haematopoietic differentiation defect after transplantation

To test the cell autonomous functionality of HSCs after long-term deletion of *Zeb1* we sorted 200 HSCs from control and *Zeb1*$^{-/-}$ primary recipients 32 weeks after *Zeb1* deletion and transplanted them with BM competitor cells into lethally irradiated recipients (Figure 3.19A). Analysis of engraftment in PB of transplant recipients revealed a rapid engraftment defect at week 4 (out of 5 *Zeb1*$^{-/-}$ recipients, 4 showed an engraftment of less than 6% compared to average of 18.21% engraftment in control group, however one *Zeb1*$^{-/-}$ recipient had 46.4 %) (Figure 3.19B). After that, PB was analysed at weeks 12 and 17 post transplantation and, interestingly, a near complete loss of donor cells in PB at weeks 12 and 17 (Figure 3.19B). This was associated with a loss of donor derived T, B, and Mac1^{+} cells in PB and a substantial reduction in Mac1^{+} Gr1^{+} myeloid donor cells from *Zeb1*$^{-/-}$ recipients (Figure 3.19C). Consistent with PB data, a near complete loss of donor cells in BM and spleen and a complete loss of donor cells in thymus was observed in transplant recipients receiving *Zeb1*$^{-/-}$ HSCs (Figure 3.19D). Thus, persistent loss *Zeb1* severely perturbs the differentiation capacity of HSCs after transplantation in a cell autonomous fashion.

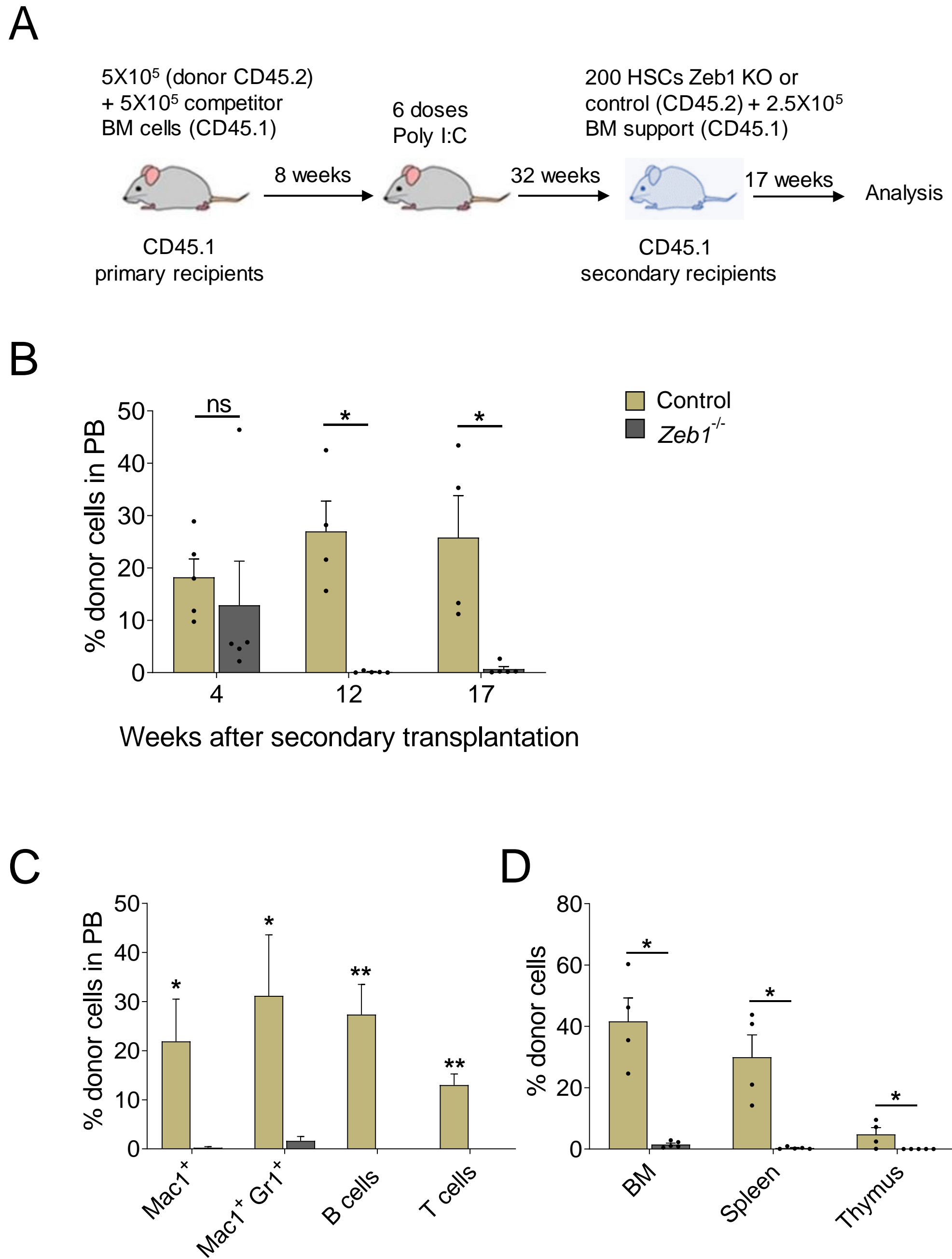
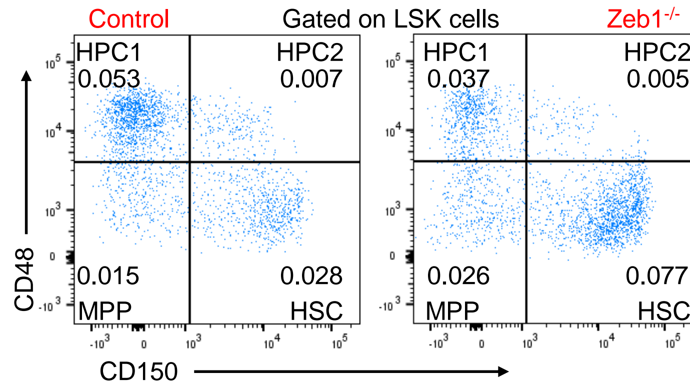


Figure 3.19. Persistent *Zeb1* loss resulted in cell autonomous multilineage haematopoietic differentiation failure after transplantation into secondary. (A) A scheme of secondary HSC transplantation via cell autonomous manner. (B) The percentage of donor cells in PB at different time points after HSC transplantation from control and *Zeb1*$^{-/-}$ mice. (C) Analysis of donor contribution to PB myeloid and lymphoid cells at week 16 after secondary HSC transplantation from control and *Zeb1*$^{-/-}$ mice. (C) Analysis of donor cells in BM, spleen, and thymus at week 16 after secondary HSC transplantation from control and *Zeb1*$^{-/-}$ mice Data from one independent experiment (N= 4 control and 5 *Zeb1*$^{-/-}$). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

3.3.16 Persistent *Zeb1* loss resulted in increased EpCAM expression in HSCs

RNA-sequencing (RNA-Seq) data from control and *Zeb1* KO HSCs 14 days after *Zeb1* deletion showed a striking upregulation of EpCAM (RNA-Seq data will be discussed in Chapter 4, Figure 4.10). Thus, we analysed the expression of EpCAM protein in HSC and HPC1 32 weeks after *Zeb1* deletion using flow cytometry (Figure 3.20B). Strikingly, EpCAM expression was dramatically increased in *Zeb1*$^{-/-}$ HSC and HPC1 populations (Figure 3.20B). EpCAM has been shown to be expressed in stem cells and enhance survival in other tissues (Zheng et al., 2017, Gao et al., 2014, Gonzalez et al., 2009). This may indicate that the survival advantage seen in *Zeb1*$^{-/-}$ HSC at week 32 after *Zeb1* deletion (Figure 3.8), is mediated, in part, by the increase in EpCAM.

A



B

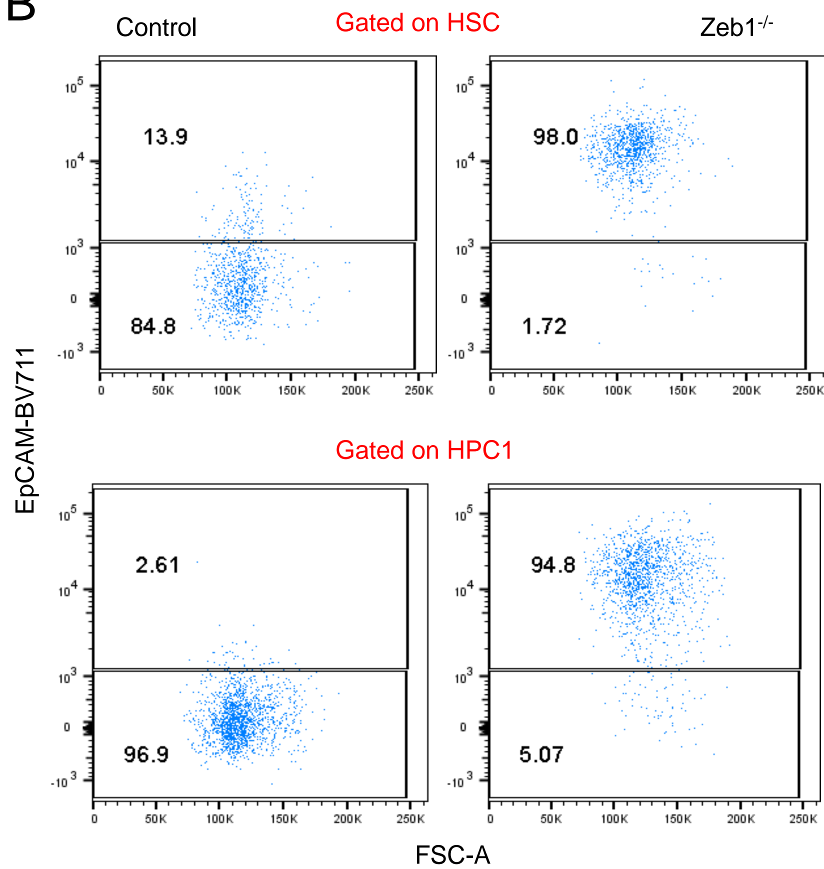


Figure 3.20. Persistent *Zeb1* loss resulted in increased EpCAM expression in HSCs. (A) FACS plots showing the analysis of HSPCs at week 32 after *Zeb1* deletion. (B) FACS plots showing protein expression of EpCAM in HSC and HPC1 populations at week 32 after *Zeb1* deletion.

3.4 Discussion

Zeb1 is widely expressed in different tissues including nervous system, lung, kidney, skeletal system, muscles, thymus, BM, spleen, and others (Postigo and Dean, 2000, Hurt et al., 2008, Higashi et al., 1997, Sekido et al., 1994). Although the oncogenic role of *Zeb1* in driving cancer stem cell metastasis, invasion, and chemoresistance cancer has been gaining much interest in the last few years (Preca et al., 2015, Zhang et al., 2013a, Zhou et al., 2012, Kahlert et al., 2015, Siebzehnrubl et al., 2013, Zhang et al., 2018a, Caramel et al., 2018), knowledge about the specific role for *Zeb1* in the long-term maintenance of normal adult stem cell biology has been lacking. The haematopoietic system has been well defined in terms of their stem cell and progenitor biology. Initial analysis of *Zeb1* expression in purified populations of HSPCs and their progeny revealed high *Zeb1* levels in HSPCs (HSC, MPP, HPC1, HPC2) that declines during differentiation to lineage-restricted progenitors (CMP, GMP, MEP, CLP), followed by up-regulated *Zeb1* expression during the terminal maturation of the cells (myeloid, erythroid, B, and T lymphoid cells). This pattern of expression in haematopoietic cell subsets suggests a pivotal role for *Zeb1* in haematopoiesis. Thus, here we characterised the role of *Zeb1* in the long-term functioning of the adult haematopoietic system.

Given that germline KO of *Zeb1* in mice is associated with perinatal deaths (Takagi et al., 1998, Brabletz et al., 2017), we exploited a conditional *Zeb1* knockout mouse model (*Zeb1*$^{fl/fl}$) that, under the control of an inducible haematopoietic-specific *Mx1* promoter, drives Cre expression to spatially and temporally inactivate *Zeb1* expression in HSPCs and their downstream progeny (Brabletz et al., 2017). At the HSPC level, we showed that long-term deletion of *Zeb1* (for 8 months) resulted in an accumulation of HSCs and MPPs and a reduction in HPC1 and HPC2 in the absence of alterations in committed myeloid and lymphoid progenitors. This expansion of HSCs and MPPs was associated with diminished apoptosis, but not cell cycle alterations. Interestingly, reduction in HPC1 and HPC2 populations was not caused by changes in apoptosis, suggesting that there may be a *Zeb1* mediated block in differentiation of HSCs and MPPs towards HPC1 and HPC2. Altogether, this data suggests that *Zeb1* controls the differentiation fates of HSPCs in the context of long-term, steady-state haematopoiesis. In support of this conclusion, multilineage haematopoietic defects resulted from the chronic loss of *Zeb1* and *Zeb1*$^{-/-}$ HSCs were functionally compromised in their capacity to differentiate to myeloid and lymphoid lineages after transplantation.

Given that long-term absence of *Zeb1* resulted in HSC expansion in the BM, the main site of haematopoiesis in adult, and as *Zeb1* regulates migration in other tissue settings (Caramel et al., 2018, Xue et al., 2019), we posited that expanded HSCs in the BM may result in alterations in HSCs and other progenitors in the spleen, a site of extramedullary haematopoiesis. Indeed, a dramatic reduction in the number of Lin^- cells, HSCs, CMP, GMP, CLP, and other lymphoid progenitors and a trend towards a reduction of MPPs was observed in spleens of *Zeb1*$^{-/-}$ mice at 32 weeks after induction of deletion. Notably, this data contrasts with that observed in BM, particularly with respect to HSCs frequency. *Zeb1* is a known EMT inducer that involves *Zeb1*-mediated repression of adhesion genes such as CDH1 that encodes the protein E-cadherin and polarity genes such as Crumbs3 and HUGL2 which in turns convert the epithelial cells to lose their polarity and acquire a motile state (Aigner et al., 2007, Sanchez-Tillo et al., 2010, Vandewalle et al., 2009). Thus, loss of *Zeb1* in HSCs in the BM may lead to increased expression of cell adhesion molecules and polarity genes as evidenced by a striking expression of EpCAM in *Zeb1*$^{-/-}$ HSCs. Consistent with this, our observation of a decreased splenic HSC pool in *Zeb1*$^{-/-}$ mice and increased cell adhesion molecules such as EpCAM suggests that long-term expression of *Zeb1* is required to restrain the motility of HSCs to egress from the BM to the spleen. Together, these data suggest that *Zeb1* acts as an essential regulator of HSC homeostasis in the BM in terms of their localisation in the BM niche.

An earlier report showed that *Zeb1* was essential for T cell development during embryogenesis (Higashi et al., 1997) and another more recent report found that *Zeb1* is required for the function and survival of CD8 memory T cells (Guan et al., 2018). We employed a conditional mouse model of chronic *Zeb1* loss that differs from that of Higashi et al, where we conditionally deleted *Zeb1* in adult haematopoietic tissues while their phenotype related an embryonic stage that resulted from a germline mutation of *Zeb1* that translates protein lacking c-terminus (Higashi et al., 1997). In this chapter, we provide evidence and define for the first time that persistent *Zeb1* expression in the adult haematopoietic system is required for (i) thymocyte maturation in the thymus, (ii) T cell differentiation in homeostasis and (iii) the ability of HSCs to functionally differentiate to T cells.

Expansion of HSCs in the BM and a defect in the repopulation capacity are features during ageing of HSCs (Chambers et al., 2007, Ergen and Goodell, 2010). Ageing HSCs express high levels of CDC42, a polarity marker of HSCs, and show a dysregulated polarity signature such as CDC42, tubulin, and Per-2 resulting in apolar and unfunctional HSCs in terms of repopulation toward BM progenitors and PB

(Florian et al., 2012). Interestingly, dysregulation of actin cytoskeleton and activation of CDC42 in LSKs that were exposed to Wnt5a haploinsufficient BM niche resulted in a repopulation defect in secondary recipients and showed differentially expressed genes enriched in *Zeb1* DNA binding motif (Schreck et al., 2017). These pathways alter HSC polarity during ageing by preferentially favouring self-renewal symmetric divisions that expand HSCs and diminish differentiation activity (Florian et al., 2018). This phenotype is similar to our observations, where long-term loss of *Zeb1* expression over 8 months resulted in an expanded HSC compartment in BM, reduced HSCs located in the spleen, and impairment of repopulation capacity after transplantation.

Ageing-associated changes in haematopoiesis, particularly, the impairment of HSC function and expansion of HSPC number have been linked to the development of haematopoietic malignancies (McKerrell and Vassiliou, 2015, Shlush, 2018). Age-related clonal haematopoiesis (ARCH) is an emerging term to describe the condition characterised by expanded HSPCs that have specific gene mutations (ASXL1, TET2, DNMT3A) without observed symptoms of haematopoietic neoplasms which can lead to development of several types of leukaemia in elderly by acquiring further genetic mutations (Konieczny and Arranz, 2018, Genovese et al., 2014, Shlush, 2018). Our *Zeb1* KO mice presented with expansion of HSPCs and attenuated differentiation ability at eight months after *Zeb1* deletion, which equates to middle age. Thus, because of the strong relationship between ageing and development of haematopoietic neoplasms it would be interesting to investigate whether long-term *Zeb1* deficiency into old age (approx.18 - 24 months) would result in development of haematopoietic-related malignancies.

CHAPTER 4 :

Evaluating the impact of acute deletion of *Zeb1* in the adult haematopoietic system

4.1 Introduction

Zeb1 regulates key cellular processes such as differentiation, adhesion and migration (Wang et al., 2019, Haensel et al., 2019). A key role of *Zeb1* is induction of EMT during development (Zhang et al., 2015a). Thus, *Zeb1* controls other processes through EMT (when it is expressed) or MET (when it is repressed). One major feature of EMT is repression of cell-cell adhesion and polarity molecules and vice versa for MET (Thiery et al., 2009). Hence, both expression and repression of ZEB1 are involved in regulation of stem cell differentiation. *Zeb1* is found to positively control neuron differentiation and migration via regulation of polarity molecules (Singh et al., 2016, Wang et al., 2019, Yan et al., 2017). Further, expression of ZEB1 is essential for late stage neuron differentiation from human embryonic stem cells, while its loss results in death of the differentiated cells (Jiang et al., 2018). Also, ZEB1expression is required for smooth muscle cells (SMCs) differentiation (Nishimura et al., 2006). However, while ZEB1 expression maintains muscle satellite cells, its reduction promotes differentiation into myoblasts (Siles et al., 2019). Also, ZEB1 expression is repressed during the differentiation of human embryonic stem cells (hESCs) to endothelial cells (Luo et al., 2013) and hepatic cells, while it is increased when differentiating into mesodermal cells expressing CD34 (Kim et al., 2017). Supporting the notion that ZEB1 loss induces MET, ZEB1is downregulated and E-cadherin is upregulated during conversion of fibroblasts into multipotent cells while other EMT inducers Snai1, Snai2, and ZEB2 remain unchanged (Liu et al., 2013). Also, after *Zeb1* ablation, corneal endothelial cells undergo a reverse process named endothelial to epithelial transition (EnET) (Frausto et al., 2019). Thus, ZEB1 expression is differentially required for differentiation in different cellular contexts at distinct developmental stages by adopting either EMT or MET.

Although a role of *Zeb1* in stem cell maintenance has not been fully elucidated in normal physiology compared to its role in stem cell differentiation, this idea is supported by the role of ZEB1 in epithelial cancer stem cells (CSCs). Aberrant activation of EMT in epithelial cells and high ZEB1 expression are found in solid tumours and associated with more stem, aggressive, invasive, and drug resistant CSCs. Upregulation of ZEB1 is reported in different cancer types including breast (Eger et al., 2005, Chaffer et al., 2013, Zhou et al., 2017), colorectal (Spaderna et al., 2008, Zhang et al., 2013a, Yuan et al., 2019), pancreatic (Graham et al., 2008), uterine (Spoelstra et al., 2006), osteosarcoma (Shen et al., 2012, Yu et al., 2019), lung (Zhang et al., 2013b, Matsubara et al., 2014, Ma et al., 2019), liver (Zhou et al., 2012, Qin et al., 2019), gastric (Okugawa et al., 2012, Jia et al., 2012, Xue et al.,

2019), glioblastoma (Siebzehnrubl et al., 2013), prostate (El Bezawy et al., 2019), and others. These findings enforce the oncogenic role of ZEB1 in cancers in terms of inducing stemness.

As discussed earlier, ZEB1 can be upregulated or downregulated during cell differentiation, the same pattern occurs in cancer where it can act as an oncogene or tumour suppressor. These opposing functions are best modelled in haematological malignancies. ZEB1 is found to be a tumour suppressor in T cell-associated leukaemias/lymphomas as in Sézary syndrome, an aggressive form of cutaneous T-cell lymphoma, and in adult T-cell leukaemia/lymphoma (ATLL) (Vermeer et al., 2008, Hidaka et al., 2008). On the other hand, ZEB1 is documented to be an oncogene in B cell-associated leukaemias as in mantle cell lymphoma (MCL), a B-cell malignancy characterised by a poor prognosis (Sanchez-Tillo et al., 2014). However, its role in myeloid leukaemias may involve both as an oncogene or tumour suppressor. It is reported to be an oncogene in AML as in MLL-AF9 LT-HSCs AML (Stavropoulou et al., 2016). However, the expression of ZEB1 is downregulated in patients with myelodysplastic syndrome (MDS) (Nilsson et al., 2007, Pellagatti et al., 2006). This line of evidence from normal and malignant settings indicate that ZEB1 is essential regulator of cell processes and its function is tissue-specific and dependent on the development stage.

4.2 Aims

In Chapter 3, we evaluated the role of *Zeb1* in the long-term maintenance of haematopoiesis in homeostasis and after HSC transplantation using the Mx1-Cre system to conditionally delete *Zeb1* in the haematopoietic system. We found that 32 weeks (8 months) after *Zeb1* deletion, LT-HSCs accumulated in the BM and this was associated with enhanced cell survival and declining numbers of splenic LT-HSCs. We found a reduction in *Zeb1* KO HSPCs, specifically HPC1 and HPC2 in the BM, and no alterations in lineage-restricted progenitors in the BM. Long-term loss of *Zeb1* also reduced the numbers of splenic myeloid and lymphoid progenitors, T-cell progenitors in the thymus, BM, and spleen and Mac1^{+} myeloid cells and T cells in PB. Functionally, long-term *Zeb1* loss in HSCs perturbed their functional capacity, as judged by the pan-haematopoietic engraftment failure observed after primary and secondary transplantation.

As these immunophenotypic and functional changes occurred at 32 weeks after *Zeb1* deletion, in middle-aged mice, we cannot exclude the possibility that ageing of HSCs had an impact on the observed phenotype. To address this confounding factor and to provide a complementary analysis into *Zeb1* function in adult haematopoiesis, we sought to examine the function of *Zeb1* after acute deletion, 14 days after induction of *Zeb1* deletion using the Mx1-Cre system. The aims of this chapter are to:

1. Study the effect of acute deletion of *Zeb1* in the adult murine haematopoietic system using the Mx1-Cre system to conditionally delete *Zeb1* in the haematopoietic system at steady state.

2. Study the requirement of *Zeb1* for HSC function by using transplantation assays 14 days after acute *Zeb1* deletion.

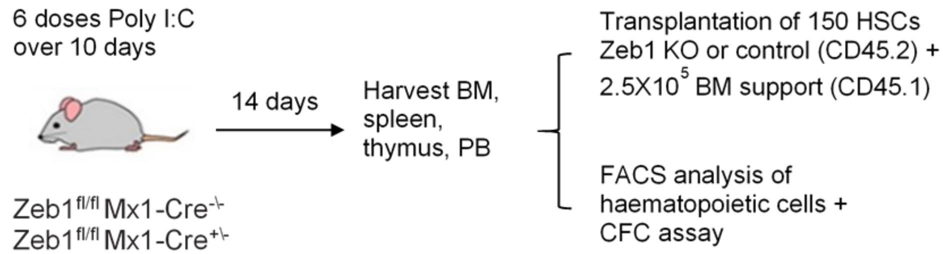
3. Elucidating the transcriptional programme of HSCs after acute deletion of *Zeb1* using RNA-sequencing

4.3 Result

4.3.1 *Zeb1* was successfully deleted in HSPCs in the BM

We reported in Chapter 3 that chronic loss of *Zeb1* in the adult haematopoietic system results in a cell autonomous defect in the differentiation of HSCs to committed progenitors and differentiated cells at steady state and after transplantation. Herein, we have assessed the impact of acute *Zeb1* deletion in the adult haematopoietic system. To do this, we utilised the same mouse model described in Chapter 3, but instead of leaving mice for 32 weeks after *Zeb1* deletion, we analysed mice 14 days after the last dose of Poly I:C into $Zeb1^{fl/fl}$ $Mx1\text{-}Cre^{-\backslash-}$ (control) and $Zeb1^{fl/fl}$ $Mx1\text{-} Cre^{+/-}$ ($Zeb1^{-/-}$) mice (Figure 4.1A). We initially checked deletion of *Zeb1* in different haematopoietic cell compartments 14 days after the last dose of Poly I:C and found that *Zeb1* was largely deleted in total BM cells; however, few cells escaped *Zeb1* deletion (Figure 4.1B). To specifically confirm that *Zeb1* was absent in HSPCs, we prospectively isolated LSK populations that contain HSPCs and, by genotyping PCR, observed complete deletion of *Zeb1* (Figure 4.1B). We also isolated $CD3^+$ T cells and $B220^+$ B cells from spleen and HSPC containing c-Kit^+ cells from the BM and checked *Zeb1* deletion in these populations. While HSPC containing c-Kit^+ cells had a full deletion of *Zeb1*, only partial deletion was observed in T and B cells (Figure 4.1C). These data imply that residual cells from the BM that escaped *Zeb1* deletion are those circulating, long-lived differentiated T and B cells, while HSCs and progenitors in the BM contained entirely deleted *Zeb1* alleles.

A



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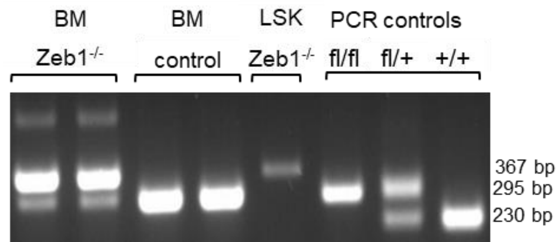

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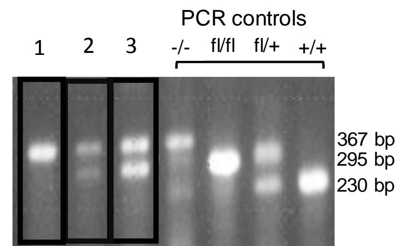


Figure 4.1. Efficient Cre-mediated recombination of *Zeb1* in BM HSPCs but not in T and B lineages. (A) A scheme of *Zeb1* deletion. 6 doses of Poly I:C were intraperitoneally administered into *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{-}$ and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{+}$. The mice were dissected at D14 after the last dose of Poly I:C. (B) *Zeb1* deletion was assessed in total BM cells and LSK population 14 days after the last dose of Poly I:C. A small subset from total BM cells (a faint band) was observed as few cells still have *Zeb1* floxed alleles not deleted, while LSK cells have a fully excised band. (C) *Zeb1* deletion was also assessed in HSPC containing cKit^{+} BM cells, T cells and B cells from spleen 14 days after the last dose of Poly I:C. A complete ablation of *Zeb1* was found in c-Kit^{+} cells, however a partial deletion was observed in T and B cells.

4.3.2 Acute deletion of *Zeb1* resulted in a reduction in the frequency of Mac1$^+$ myeloid cells in PB

14 days after *Zeb1* ablation, we did not find a significant change in BM and spleen cellularity nor spleen size (Figure 4.2A). PB was analysed for expression of Mac1 and Gr1 for myeloid cells, B220 for B cells, and CD4/CD8 for T cells. A significant reduction in the proportion of Mac1$^+$ Gr1$^-$ population that contain monocytes was observed after *Zeb1* knockout, while no significant changes were observed in Mac1$^+$ Gr1$^+$ population that contains granulocytes or T cells, and B cells (Figure 4.2B). We also analysed the differentiated cells in the BM and spleen, which were comparable in all haematopoietic lineages between the two genotypes (Figure 4.2C and D). Taking into consideration the incomplete deletion of *Zeb1* in T cells and B cells, we cannot confirm the role for acute loss of *Zeb1* in these populations at steady state. However, in Sections 4.3.6 and 4.3.11, we will test the requirement of *Zeb1* in circulating B and T cell lineages by transplanting *Zeb1*-deficient HSCs in cell autonomous and non-cell autonomous settings and assessing differentiation into T and B lymphoid lineages.

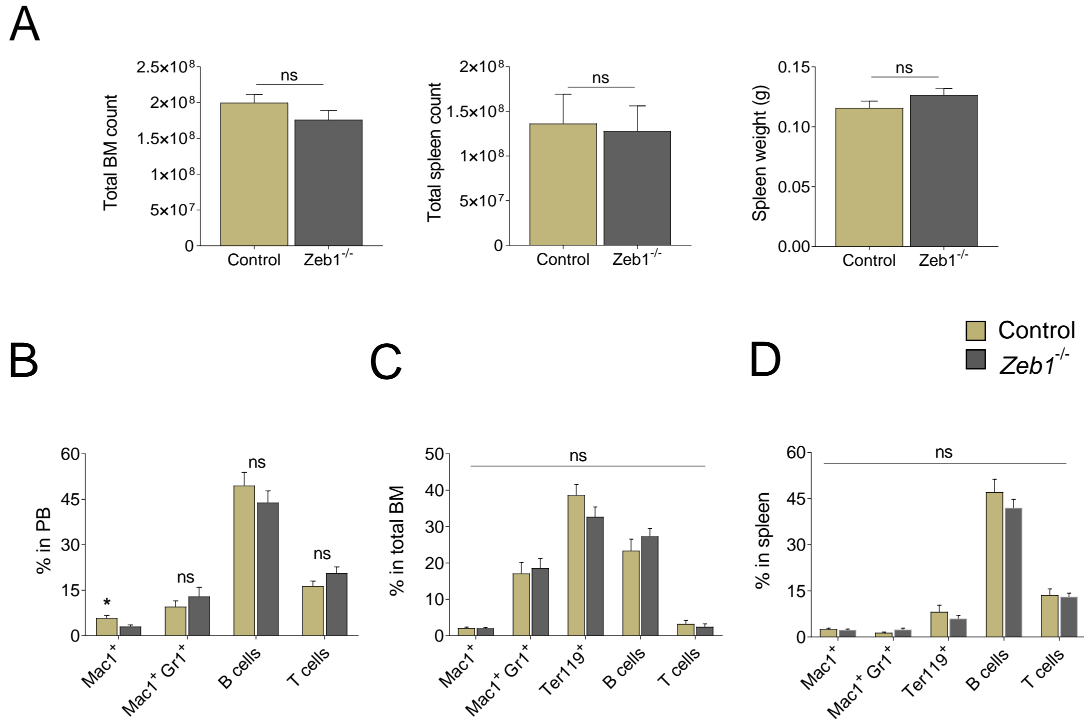


Figure 4.2. Acute *Zeb1* loss resulted in a reduction of Mac1⁺ myeloid cells in PB. (A) Analysis of total cells of BM (N= 12 control and 11 *Zeb1*$^{-/-}$) and spleen (N= 6 control and *Zeb1*$^{-/-}$) as well as spleen weight (N= 8 control and *Zeb1*$^{-/-}$) from control and *Zeb1*$^{-/-}$ mice 14 days after the last dose of Ploy I:C. (B) Analysis of the frequency of the differentiated cells in PB at D14 after *Zeb1* deletion (N= 8-11 in each genotype). Analysis of the frequency of the differentiated cells in the BM (C) and spleen (D) at D14 after *Zeb1* deletion (N=10 except spleen N= 6 each genotype). Data from 3-4 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.3 Acute deletion of *Zeb1* resulted in a reduction of LMPP and CLP in BM

Given that *Zeb1* appears to be an important regulator of HSPC functionality (Chapter 3), the effect of acute deletion of *Zeb1* on immunophenotypically defined HSCs and multipotent progenitor population in BM was assessed. Within LSK populations, we found increased frequency of *Zeb1*$^{-/-}$ HSCs (LSK CD150^{+} CD48^{-}) but no significant changes in the frequency of MPPs (LSK CD150^{-} CD48^{-}), HPC1 (LSK CD150^{-} CD48^{+}), and HPC2 (LSK CD150^{+} CD48^{+}) between control and *Zeb1*$^{-/-}$ genotypes (Figure 4.3A). However, despite the increased frequency of HSCs in LSK cells in *Zeb1*$^{-/-}$, their frequency in total BM as well as the frequency of MPP were comparable (Figure 4.3B). HPC1 frequency showed a near significant reduction and HPC2 frequency significantly reduced after *Zeb1* deletion (Figure 4.3B). We also assessed HSPC frequency by using another immunophenotypic definition of HSPCs according to CD34 and CD135 (Flt3) expression (Adolfsson et al., 2005, Yang et al., 2005, Huang et al., 2008, Osawa et al., 1996). Consistent with SLAM marker (CD150/CD48) analysis, no significant difference was observed in the frequency of LT-HSC (LSK CD34^{-} CD135^{-}), ST-HSC (LSK CD34^{+} CD135^{-}), and MPP (LSK CD34^{+} CD135^{+}) between control and *Zeb1*$^{-/-}$ mice (Figure 4.3C). Then, we analysed the LMPP population (Lympho-myeloid multipotent progenitors) marked by LSK CD135^{high} CD127^{high} expression (Figure 4.3D). This population has a rapid and efficient capacity to generate T and innate lymphoid cells (Ghaedi et al., 2016) compared with conventional LMPP (LSK CD34^{+} CD135^{high}) (Adolfsson et al., 2005) or HPC1 (LSK CD150^{-} CD48^{+}) that overlap functionally with LMPP by 80% (Pietras et al., 2015, Oguro et al., 2013, Mooney et al., 2017). Interestingly, we found a significant reduction in the proportion of LMPP CD127^{+} but not LMPP (LSK CD34^{+} CD135^{high}) that showed a statistically insignificant trend towards reduction after acute *Zeb1* ablation (Figure 4.3E). Given the defect in early lymphoid committed progenitors in BM after *Zeb1* ablation, we assessed other BM lymphoid progenitor compartments including CLP: Lin^{-} Sca1^{low} c-Kit^{low} CD127^{high} CD135^{high} (Sitnicka et al., 2002, Ghaedi et al., 2016) and Lin^{-} c-Kit^{-} Sca1^{+} CD127^{+} CD135^{+} (Kumar et al., 2008) and found a significant reduction after acute *Zeb1* knockout in both populations (Figure 4.4B and C). Together, these data suggest that *Zeb1* acts as a critical modulator of incipient lymphoid progenitor commitment from HSCs.

Having observed a reduction of Mac1^{+} myeloid cells in PB following acute deletion of *Zeb1* in the haematopoietic system, we asked whether this was due to defects in committed myeloid progenitors from BM at D14 after *Zeb1* deletion. No significant

difference in CMP (LK $CD34^+$ $CD16/32^-$), GMP (LK $CD34^+$ $CD16/32^+$) and MEP (LK $CD34^-$ $CD16/32^-$) populations was noted between control and *Zeb1*$^{-/-}$ mice (Figure 4.4A). Thus, *Zeb1* mediated regulation of terminal Mac-1 myeloid cell maturation appears to be independent of committed myeloid progenitor generation from BM.

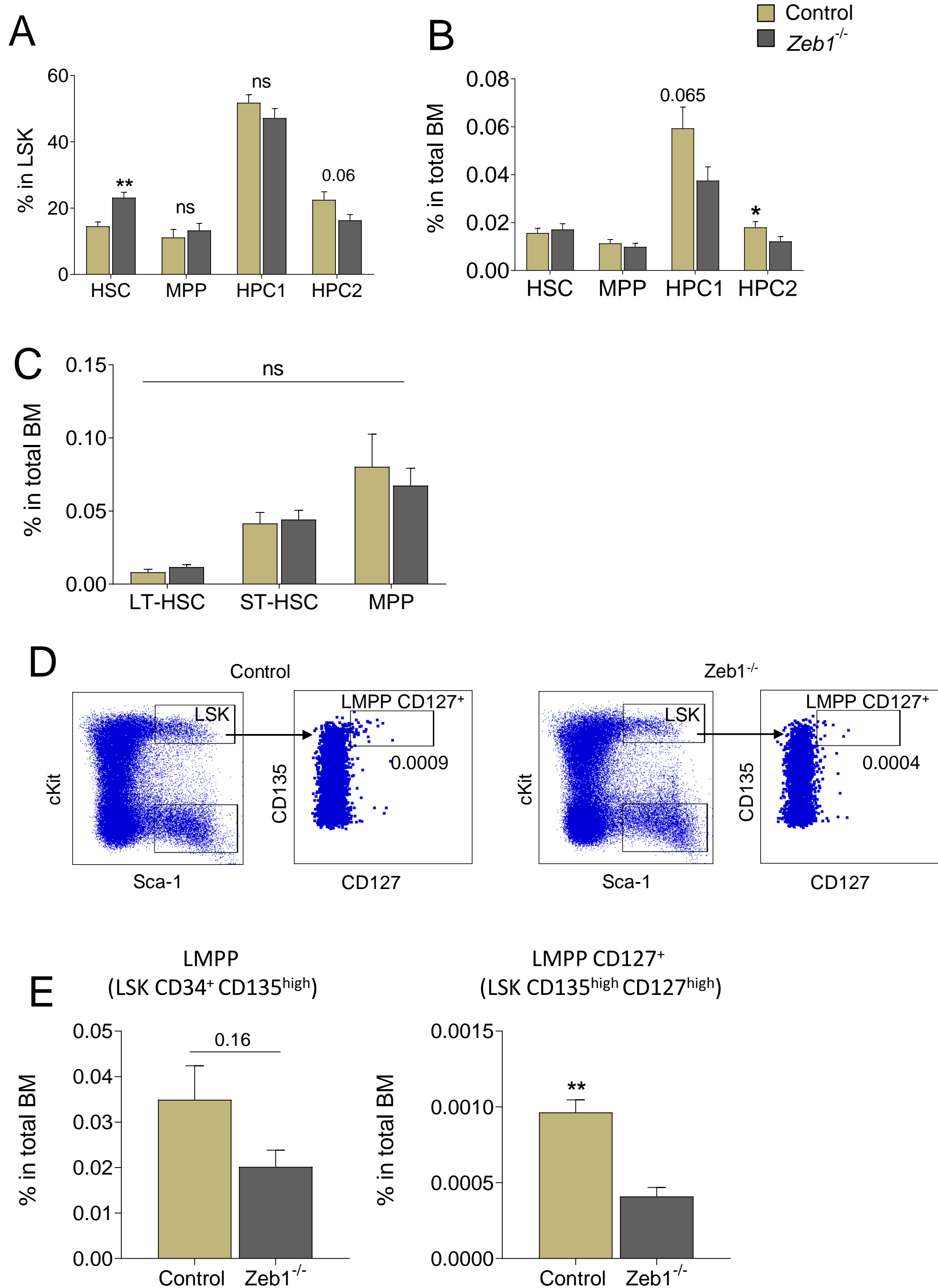


Figure 4.3. Acute deletion of *Zeb1* resulted in a reduction of LMPP in BM. (A) Analysis of the frequency of HSPCs in LSK population at D14 after *Zeb1* deletion (N= 6 in each genotype from 3 independent experiments). (B) Analysis of the frequency of HSPCs in total BM at D14 after *Zeb1* deletion (N= 15-17 in each genotype from 7 independent experiments). (C) Analysis of the frequency of LT-HSC (LSK CD34$^-$ CD135$^-$), ST-HSC (LSK CD34$^+$ CD135$^-$), and MPP (LSK CD34$^+$ CD135$^+$) in total BM at D14 after *Zeb1* deletion (N= 4 control and 5 *Zeb1*$^{-/-}$ from 2 independent experiments). (D) Representative FACS plots of BM LMPP (LSK CD135^{high} CD127^{high}) analysis at D14 after *Zeb1* deletion. (E) Analysis of the frequency of LMPP in total BM at D14 after *Zeb1* deletion (N= 6 control and 7 *Zeb1*$^{-/-}$ from 3 independent experiments for LMPP CD127$^+$ analysis; N=8 control and 10 *Zeb1*$^{-/-}$from 4 independent experiments). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

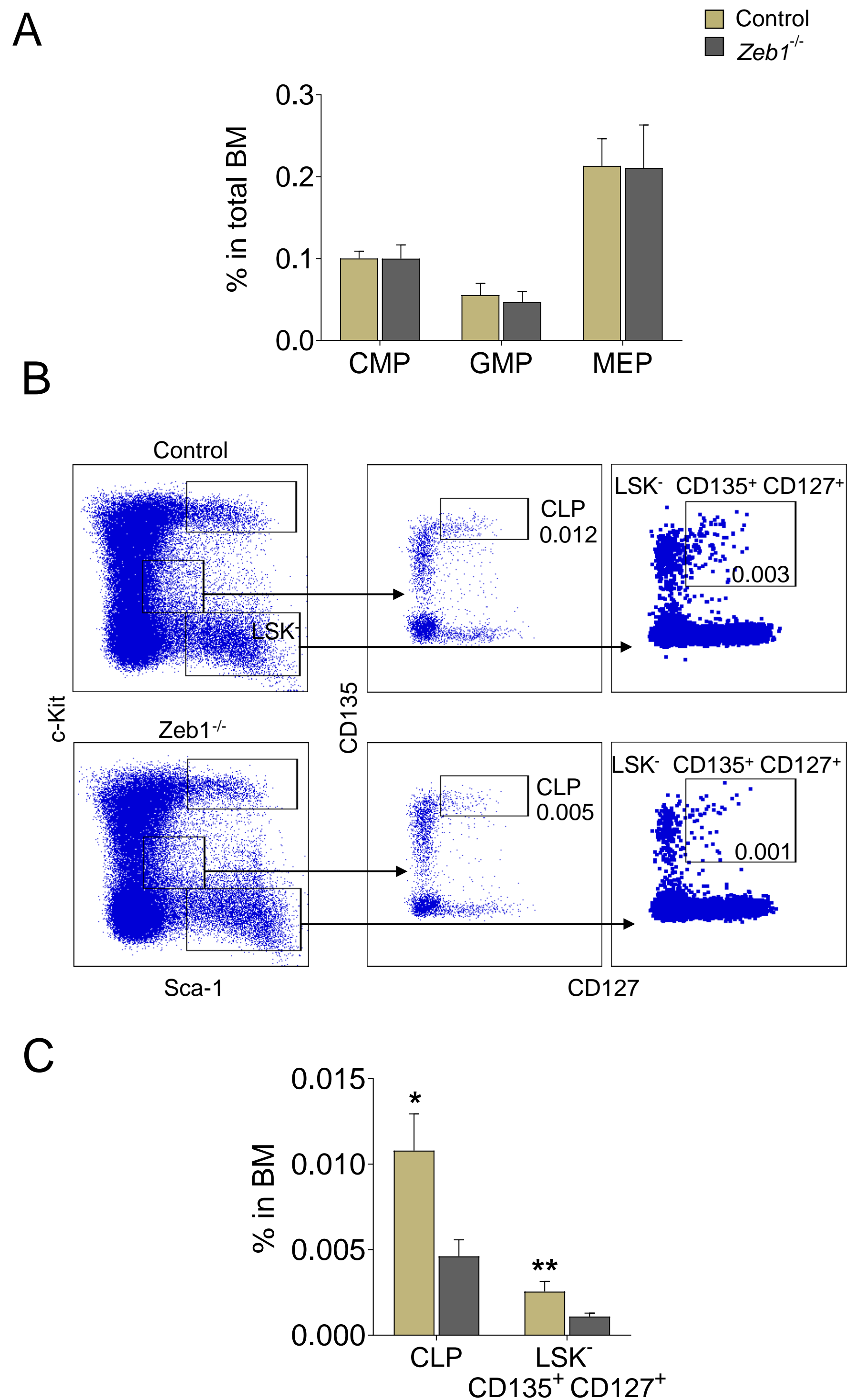


Figure 4.4. Acute deletion of *Zeb1* resulted in a reduction of CLP from BM. (A) Analysis of the frequency of committed progenitors in LK population at D14 after *Zeb1* deletion (N= 6 in each genotype from 3 independent experiments). (B) Representative FACS plots of BM CLP and lymphoid progenitor LSK$^-$ CD135$^+$ CD127$^+$ analysis at D14 after *Zeb1* deletion. (C) Analysis of the frequency of CLP and lymphoid progenitor LSK$^-$ CD135$^+$ CD127$^+$ in total BM at D14 after *Zeb1* deletion (N= 8 control and 10 *Zeb1*$^{-/-}$ from 3 independent experiments). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.4 Acute deletion of *Zeb1* resulted in a reduction of LMPP in spleen

Given that persistent loss of *Zeb1* led to a reduction in splenic HSPCs (Chapter 3), we asked if acute *Zeb1* inactivation would similarly impact splenic HSPC and committed progenitors. In the spleen, we analysed the frequency of HSC, MPP, HPC1, and HPC2 and found no difference in the abundance of these populations (Figure 4.5A) or lineage-restricted myeloid progenitors (CMP, GMP, and MEP) in the spleen (Figure 4.5B) or lymphoid committed progenitors (Lin^{-} $Sca1^{low}$ $c\text{-}Kit^{low}$ $CD127^{high}$ $CD135^{high}$ CLP) (Figure 4.5D). Strikingly, however, a reduction in the number of LMPP residing in the spleen was observed after *Zeb1* deletion (Figure 4.5C and D).

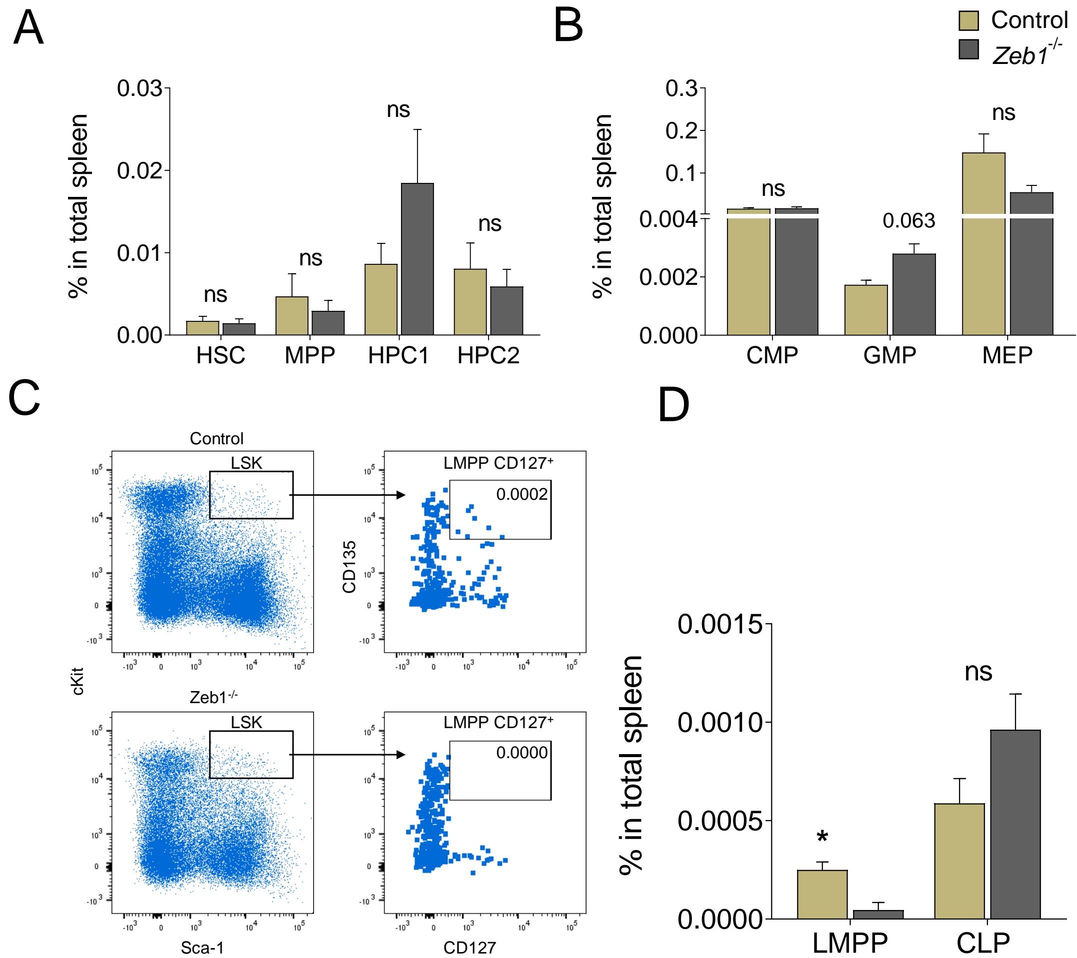


Figure 4.5. Acute deletion of *Zeb1* resulted in a reduction of LMPP in spleen. (A) Analysis of the frequency of HSPCs in spleen at D14 after *Zeb1* deletion (N= 6 control and 7 *Zeb1*$^{-/-}$ from 3 independent experiments). (B) Analysis of the frequency of committed progenitors in spleen at D14 after *Zeb1* deletion (N= 4 control and 5 *Zeb1*$^{-/-}$ from 2 independent experiments). (C) Representative FACS plots of spleen LMPP CD127^{+} at D14 after *Zeb1* deletion. (D) Analysis of the frequency of LMPP and CLP in spleen at D14 after *Zeb1* deletion (N= 5 for each genotype from 3 independent experiments). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.5 Acute *Zeb1* loss did not affect HSC colony formation *in vitro*

While the preceding data suggest a differentiation defect of *Zeb1* KO HSCs, as judged by immunophenotyping, it remains unclear the extent to which this reflects the functional defects in *Zeb1* HSC or progenitor differentiation. To assess this directly, we first assessed the capacity of *Zeb1* HSCs to differentiate *in vitro* using a colony forming unit assay (CFC). 14 days after *Zeb1* deletion, we plated 20,000 BM cells from control and *Zeb1*$^{-/-}$ in methylcellulose, and after incubation for 10 days at 37°C, we scored myeloid-specific colony formation. Similarly, we prospectively isolated 150 HSCs from control and *Zeb1*$^{-/-}$ mice by FACS and plated them in CFC assays, scoring colonies at Day 10 after incubation. Our data showed no significant difference in CFC capacity from either total BM or purified HSC in either genotype (Figure 4.6).

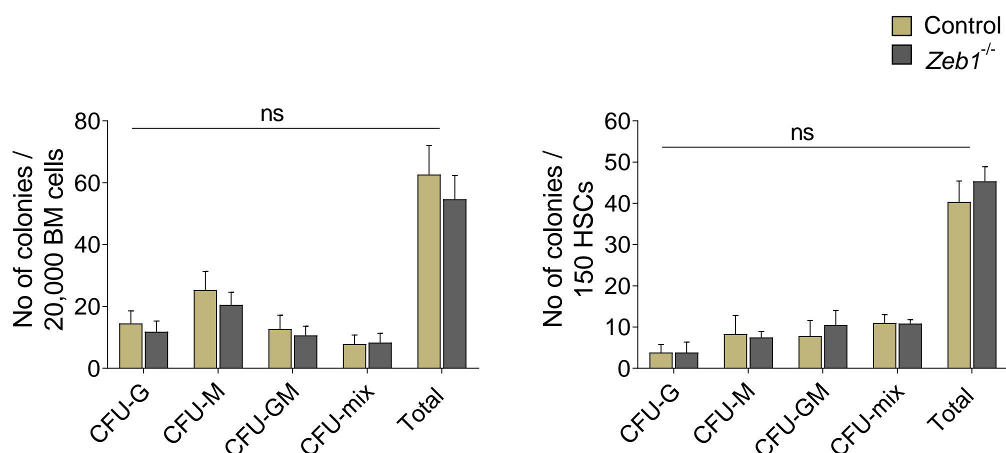


Figure 4.6. Acute *Zeb1* loss did not affect the *in vitro* differentiation activity of HSCs and BM progenitors after primary CFC plating. The number of colonies scored at D10 of incubation after plating 150 HSC and 20,000 total BM cells from control and *Zeb1*$^{-/-}$ 14 days after *Zeb1* deletion (N= 6 for each genotype). Data from 3 independent experiments). Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.6 Acute *Zeb1* loss resulted in a multilineage haematopoietic differentiation defect after HSC transplantation

To more stringently test the functionality and differentiation capacity of HSCs from *Zeb1*$^{-/-}$ mice, we prospectively isolated 150 HSCs (CD45.2) from control or *Zeb1*$^{-/-}$ mice at 14 days following deletion, mixed them with $2X10^5$ BM competitor cells (CD45.1) and transplanted this cell preparation into lethally irradiated recipients (CD45.1) (Figure 4.7A). The engraftment capacity of transplant recipients in PB was monitored until week 16. Significant engraftment failure was observed by week 6 and continued to decrease progressively until week 16 (Figure 4.7B). To test the donor contribution to PB of specific haematopoietic lineages, we analysed PB for CD45.2 (donor) and CD45.1 (competitor) in conjunction with Mac1$^+$ myeloid, Mac1$^+$ Gr1$^+$ myeloid, B220$^+$ B cells, and CD4$^+$/CD8$^+$ T cells. A profound reduction in donor contribution to B cells (Figure 4.7C), Mac1$^+$ Gr1$^-$ myeloid cells (Figure 4.7E), and Mac1$^+$ Gr1$^+$ myeloid cells (Figure 4.7F) was observed in recipients of *Zeb1*$^{-/-}$ HSCs. No engrafted T cells were derived from recipients transplanted with *Zeb1*$^{-/-}$ HSCs (Figure 4.7D). This data on the function of HSCs lacking *Zeb1* for short term (14 days) mirrors the data of primary HSC transplantation of HSCs lacking *Zeb1* for long term (32 weeks) and re-enforces the notion that *Zeb1* is an essential requirement for HSC multilineage differentiation.

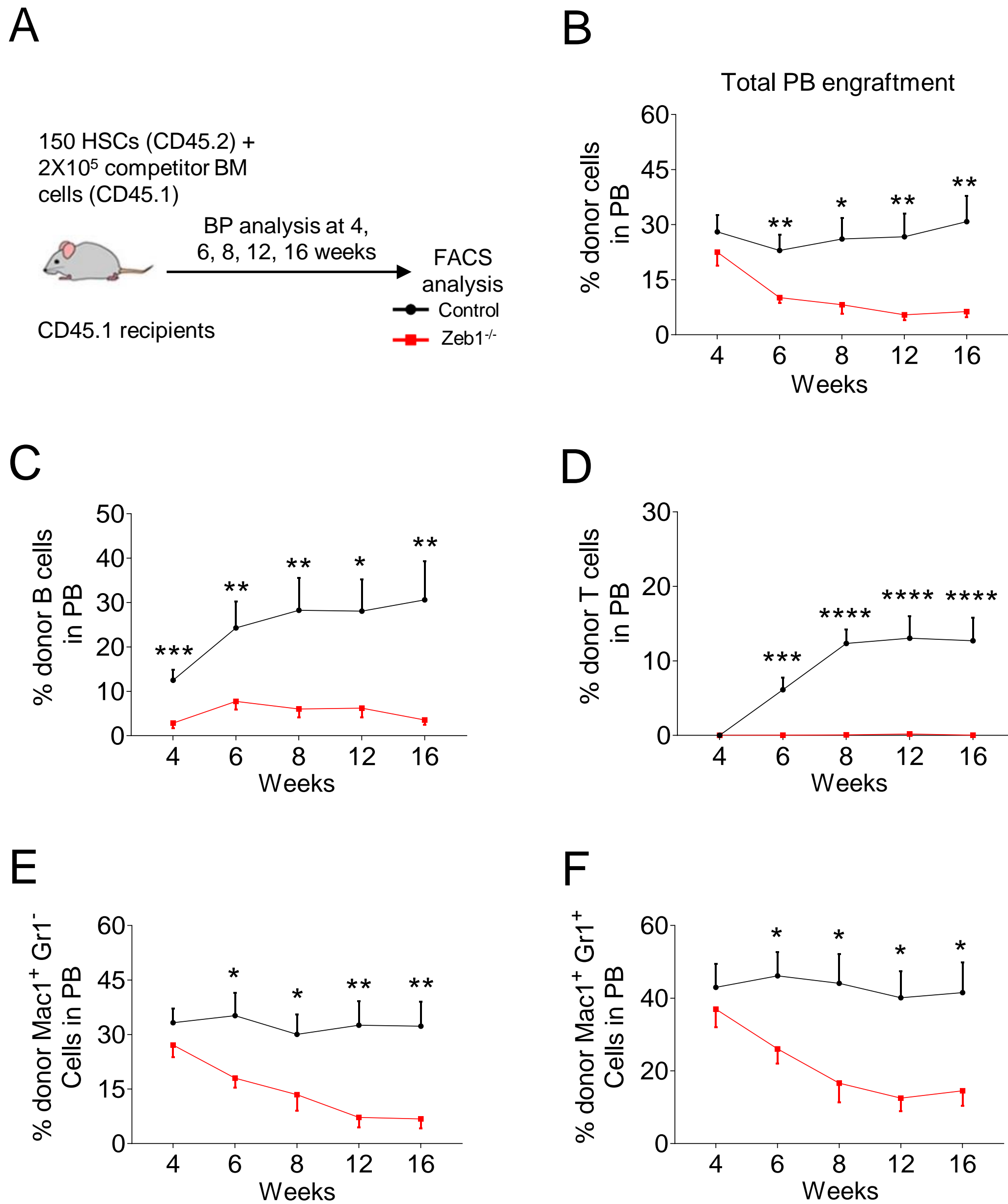


Figure 4.7. Acute *Zeb1* loss resulted in a multilineage haematopoietic defect in PB after HSC transplantation. (A) A scheme of the competitive HSC transplantation. 150 HSCs from control or *Zeb1*$^{-/-}$ mice (donor CD45.2) mixed with 2X10^5 BM competitor cells (CD45.1) were transplanted into lethally irradiated recipients (CD45.1) and the mice were monitored by bleeding the tail vein at different time points until week 16. (B) The percentage of donor cells in PB at different time points post-transplant from control (n=10) and *Zeb1*$^{-/-}$ (n= 10) mice from 2 independent experiments. PB donor contribution to B cells (B220$^+$) (C), T cells (CD4$^+$ CD8$^+$) (D), Mac1$^+$Gr1$^-$ (E), and Mac1$^+$ Gr1$^+$ myeloid cells (F) from control (n=10) and *Zeb1*$^{-/-}$ (n=10) mice from 3 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

At 16 weeks after HSC transplantation, we evaluated donor engraftment in the BM and spleen. Consistent with PB data, a reduced donor contribution to total BM was observed (Figure 4.8A). Donor T cell engraftment from *Zeb1*$^{-/-}$ HSCs was extinguished (Figure 4.8B) and donor contribution to Mac1^{+} Gr1^{+} myeloid cells and B cells was dramatically reduced in recipients receiving *Zeb1*$^{-/-}$ HSCs (Figure 4.8B). A similar defective pattern of engraftment was observed in the spleen of recipients receiving *Zeb1*$^{-/-}$ HSCs (Figure 4.8C and D).

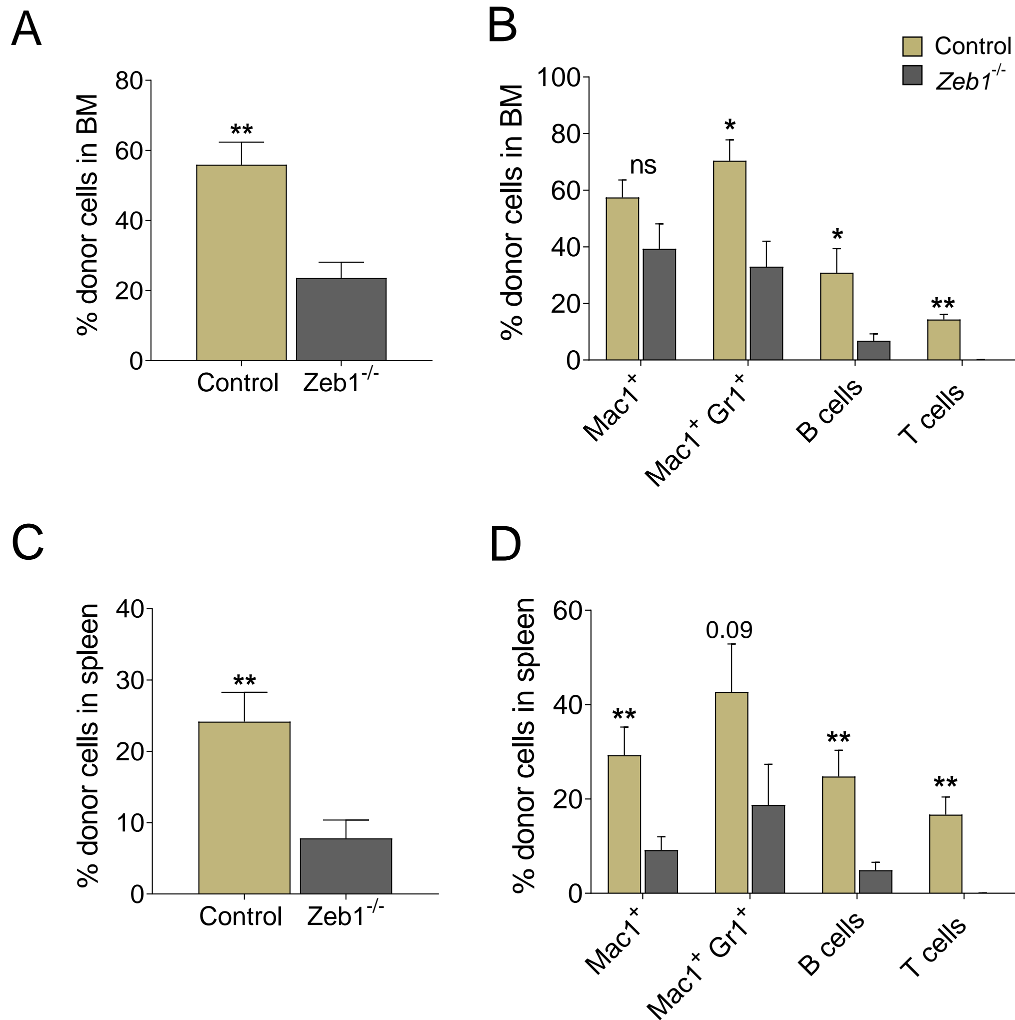


Figure 4.8. Acute *Zeb1* loss resulted in a multilineage haematopoietic defect in BM and spleen after HSC transplantation. The percentage of donor cells in BM (A) and differentiated cells (B) at week 16 after HSC transplantation from control (n=6) and *Zeb1*$^{-/-}$ (n= 7) mice from 2 independent experiments. The percentage of donor cells in spleen (c) and differentiated cells (d) at week 16 after HSC transplantation from control (n=6) and *Zeb1*$^{-/-}$ (n= 6) mice from 2 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.7 *Zeb1*$^{-/-}$ HSCs showed a reduced donor contribution to BM primitive and committed progenitors after transplantation

Having observed multi-lineage defects in terminally differentiated blood cells from the BM and spleen of recipients of *Zeb1*$^{-/-}$ HSCs, we asked whether these defects originated in parental HSPCs or lineage-committed progenitors. Within LSK compartments, the donor contribution to HSC was equal between recipients of control or *Zeb1*$^{-/-}$ HSCs (Figure 4.9A). However, there was a significant reduction in the donor contribution to MPP, HPC1, and HPC2 in *Zeb1*$^{-/-}$ compared to control (Figure 4.9A). We also analysed committed progenitors downstream of HSPCs and found a dramatic reduction in donor contribution to CMP, GMP, MEP, and ILC2 but no change observed in donor contribution to CLP (Figure 4.9B). These data directly link the functional defects observed after transplantation of *Zeb1* deficient HSCs to alterations in specific HSPC and lineage committed progenitor compartments.

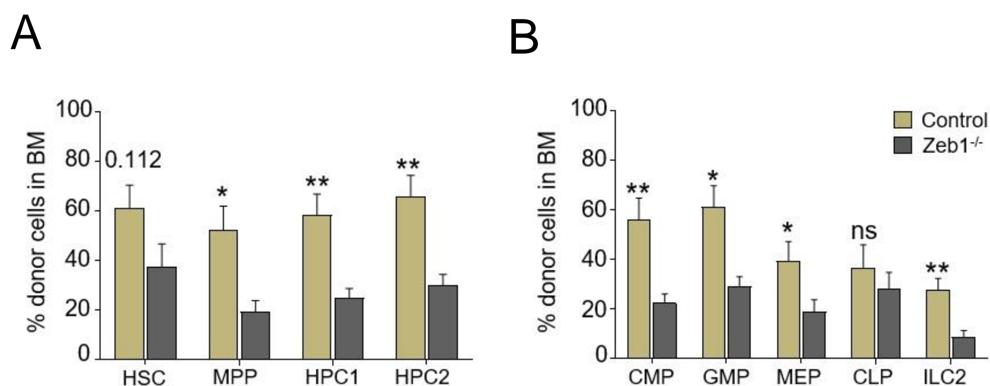


Figure 4.9. *Zeb1*$^{-/-}$ HSCs showed a reduced donor contribution to BM primitive and committed progenitors. Donor contribution to BM HSPCs (A) and committed progenitors (B) at week 16 after primary HSC transplantation from control (n=6) and *Zeb1*$^{-/-}$ (n=7) from 2 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.8 *Zeb1*$^{-/-}$ HSCs displayed deregulated haematopoietic differentiation and cell polarity transcriptional signatures

In order to understand the transcriptional signature underpinning *Zeb1* mediated regulation of HSC function, we performed RNA Sequencing on purified HSCs (LSK CD150^{+} CD48^{-}) 14 days after *Zeb1* deletion from 4 control and 4 *Zeb1*$^{-/-}$ mice. Our data showed 221 differentially expressed genes (DEG) (46 downregulated / 175 upregulated) (FDR < 0.05). Out of all DEGs, 79.18% were upregulated after *Zeb1* loss which was consistent with the notion that *Zeb1* largely acts as a transcriptional repressor (Zhang et al., 2015a). Biological pathway analysis using Kyoto Encyclopaedia of Genes and Genomes database (KEGG), an online database offering data on the genome and genetic information, cellular processes, biological pathway and others (Kanehisa and Goto, 2000), revealed that all enriched pathways were upregulated and included tight junction, cell adhesion, cell junction organisation, immune system, endocytosis, and others (Figure 4.10A). We also created a heatmap for the DEGs after classifying them according to their function in the literature. Reflecting multi-lineage differentiation defects of *Zeb1*$^{-/-}$ HSCs, we found a robust pattern of deregulated HSC maintenance and haematopoietic lineage-affiliated genes of myeloid and lymphoid genes (Figure 4.10C). Interestingly, *Zeb1* appears to regulate a transcriptional signature related to cell polarity consisting of genes related to cytoskeleton (e.g. *Crb3*, *Prkcz*, *Pard6b*, *Cxadr*), cell adhesion (e.g. *Epcam*, *Cdh1*, *Jam3*), and lipid metabolism (e.g. *Pld3*, *Spns3*, *Klf11*) (Figure 4.10B). It appeared that genes responsible for cell adhesion and cytoskeleton mainly were upregulated, consistent with the role of *Zeb1* as a potent inducer of EMT process that involves *Zeb1*-mediated repression of epithelial adhesion and polarity genes (Aigner et al., 2007, Sanchez-Tillo et al., 2010, Vannier et al., 2013). We found the most upregulated gene in the list was epithelial cell adhesion molecule *Epcam* that was found to maintain stemness in mouse embryonic stem cells (ESCs) (Gonzalez et al., 2009) and regulate various cellular processes including proliferation, differentiation, cell cycle, and adhesion as well as in epithelial cancer can work as an oncogene or suppressor (Schnell et al., 2013). *Zeb1* also was shown to regulate another adhesion molecule CDH1 during normal development and cancer (Aigner et al., 2007, Sanchez-Tillo et al., 2010, Vannier et al., 2013, Wong et al., 2014), Thus, our RNA-Seq results are consistent with the other's showing *Zeb1* as a critical regulator of cell adhesion and polarity genes and show a novel gene signature in *Zeb1*-deficient HSCs that might acquire epithelial cell like phenotype.

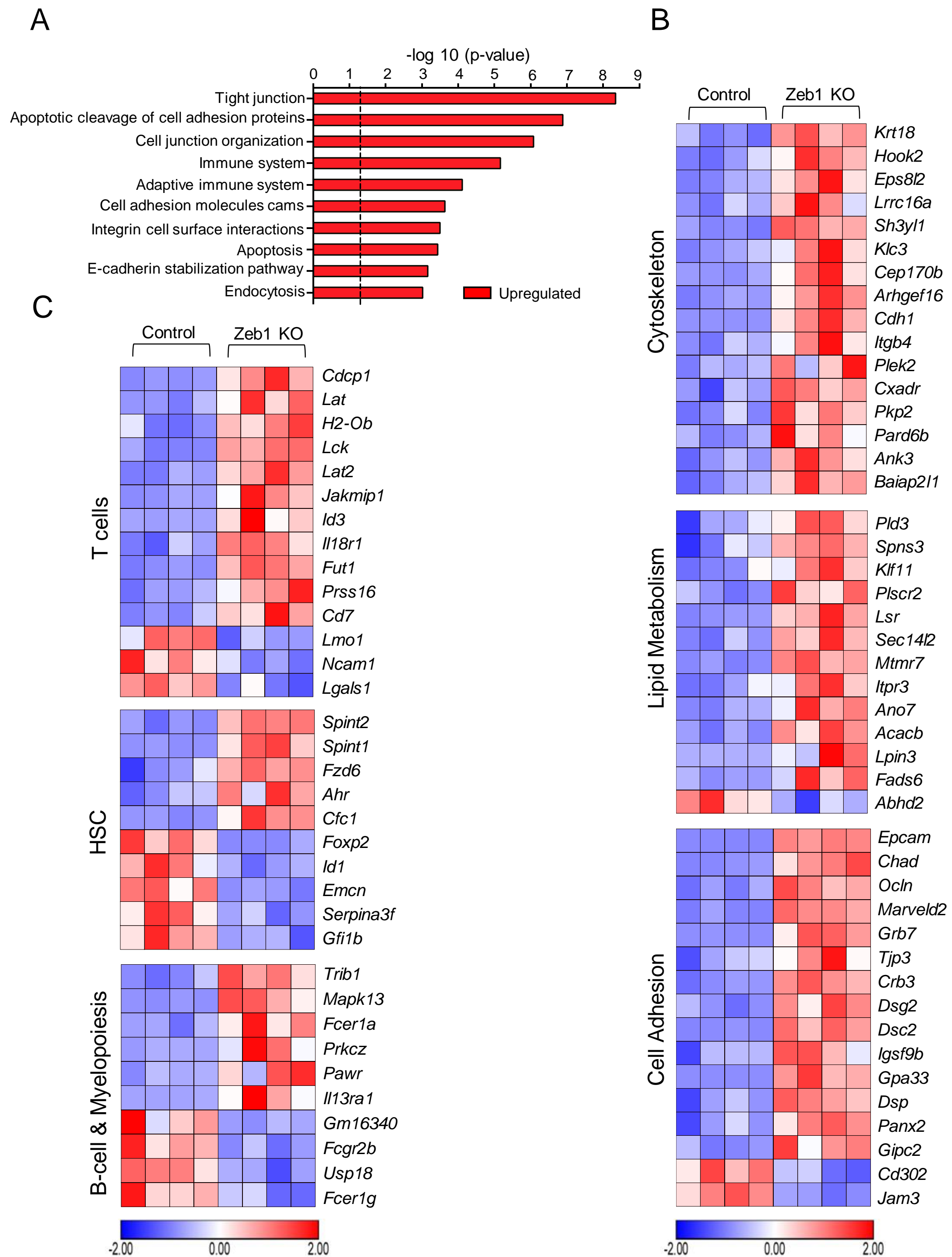


Figure 4.10. *Zeb1*$^{-/-}$ HSCs display deregulation of haematopoietic function and cell polarity transcriptional programming. RNA sequencing was performed in sorted control and *Zeb1*$^{-/-}$ HSCs (LSK CD150^{+} CD48^{-}) 14 days after the last Poly IC dose (n=4). (A) Analysis of most enriched pathways using KEGG database in *Zeb1*$^{-/-}$ HSCs. Data is shown as –log10 (p-value), and the dotted black line indicates p-value = 0.05. (B) and (C) Heat maps of the differentially expressed genes after *Zeb1* deletion where in (B) genes related to haematopoiesis and in (C) genes related to cytoskeleton, lipid metabolism, and cell adhesion. The scale represents Z-score.

4.3.9 *Zeb1*$^{-/-}$ HSCs display a profound self-renewal defect

RNA-sequencing demonstrated that *Zeb1* drives a transcriptional programme of cell polarity, which has been hypothesized to be a critical biological mechanism that asserts symmetric versus asymmetric cell divisions in stem cells – namely, striking a strict balance between self-renewal and differentiation fates (Florian et al., 2018). Therefore, to directly test the impact of *Zeb1* on the self-renewal capacity of *Zeb1*-deficient HSCs we performed secondary transplantation of *Zeb1*$^{-/-}$ HSCs. We sorted 300 HSCs (CD45.2) from control or *Zeb1*$^{-/-}$ primary recipients and admixed them with 3X10^{5} competitor BM cells before transplanting them into lethally irradiated recipients. We observed a strong defect in PB engraftment associated with multilineage haematopoietic impairment in secondary transplant recipients by week 12 (Figure 4.11B), suggestive of a self-renewal defect in HSCs where *Zeb1* has been deleted acutely (Figure 4.11C and D).

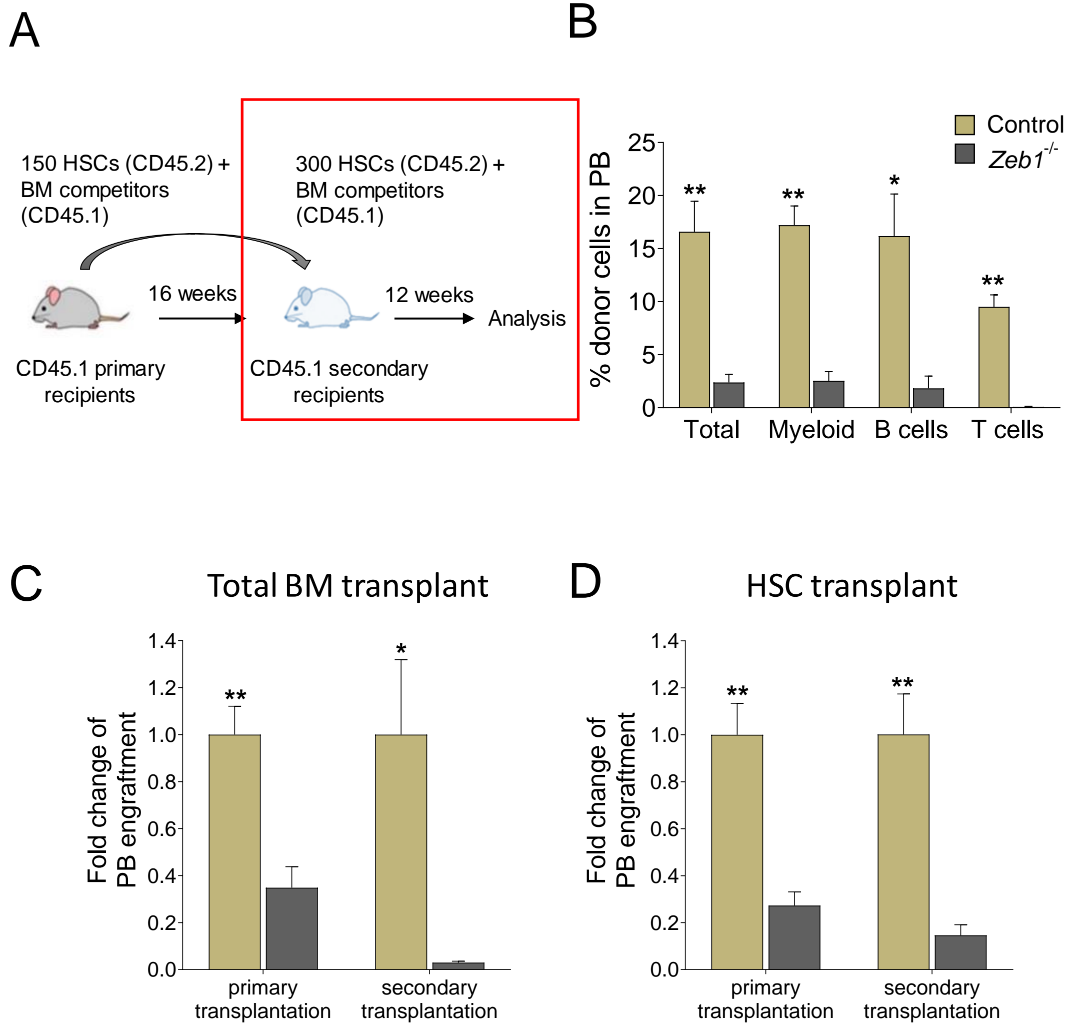


Figure 4.11. *Zeb1$^{-/-}$* HSCs have a profound differentiation defect after secondary transplantation. (A) A scheme of the competitive secondary HSC transplantation. 300 HSCs from control or *Zeb1$^{-/-}$* primary recipients (donor CD45.2) mixed with $3X10^5$ BM competitor cells (CD45.1) were transplanted into lethally irradiated recipients (CD45.1) and the mice were analysed at week 12. (B) The percentage of donor cells in PB and the donor contribution to myeloid, B, and T cells at week 12 after HSC secondary transplantation from control (n=7) and *Zeb1$^{-/-}$* (n= 5) mice from 2 independent experiments. Fold change of PB engraftment from total BM transplantation from primary (n=9 control, n= 7 *Zeb1$^{-/-}$*) from 3 independent experiments and secondary recipients (n=7 control, n= 3 *Zeb1$^{-/-}$*) from 2 independent experiments (C) and HSC transplantation from primary (n=6 control, n= 7 *Zeb1$^{-/-}$*) and secondary recipients (n=7 control, n= 5 *Zeb1$^{-/-}$*) (D) from 2 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.10 *Zeb1* deficient BM niche did not affect haematopoiesis and HSC function

Cell adhesion molecules and cytoskeleton pathways are critical for the maintenance of HSC function through interaction of HSCs with the BM niche. Given that RNA-sequencing of HSCs lacking *Zeb1* revealed deregulation of cell adhesion and cytoskeleton genes (Figure 4.10B), we investigated the effect of *Zeb1*-deficient BM niche on haematopoiesis. We transplanted $1X10^6$ wild type (WT) BM cells (CD45.1) into lethally irradiated $Zeb1^{fl/fl}$ $Mx1\text{-}Cre^{-}$ or $Zeb1^{fl/fl}$ $Mx1\text{-}Cre^{+}$ (CD45.2) (non-competitive transplantation), then six weeks later, we injected the recipients with Poly I:C to delete *Zeb1* and analysed the mice at week 16 after the last dose of Poly I:C (Figure 4.12A). PB analysis of myeloid cells ($Mac1^{+}$, $Mac1^{+}$ $Gr1^{+}$), B cells ($B220^{+}$), and T cells ($CD4^{+}$/$CD8^{+}$) showed no significant difference between control and $Zeb1^{-/-}$ (Figure 4.12B). Similarly, we analysed myeloid cells ($Mac1^{+}$, $Mac1^{+}$ $Gr1^{+}$), erythroid cells ($Ter119^{+}$), B cells ($B220^{+}$), and T cells ($CD4^{+}$/$CD8^{+}$) in the BM, which were comparable between control and $Zeb1^{-/-}$ recipients (Figure 4.12C). Next, we asked whether the altered BM niche would impact the composition of HSPC and committed progenitors. The data showed that the frequency of these populations was comparable between control and $Zeb1^{-/-}$ (Figure 4.12D).

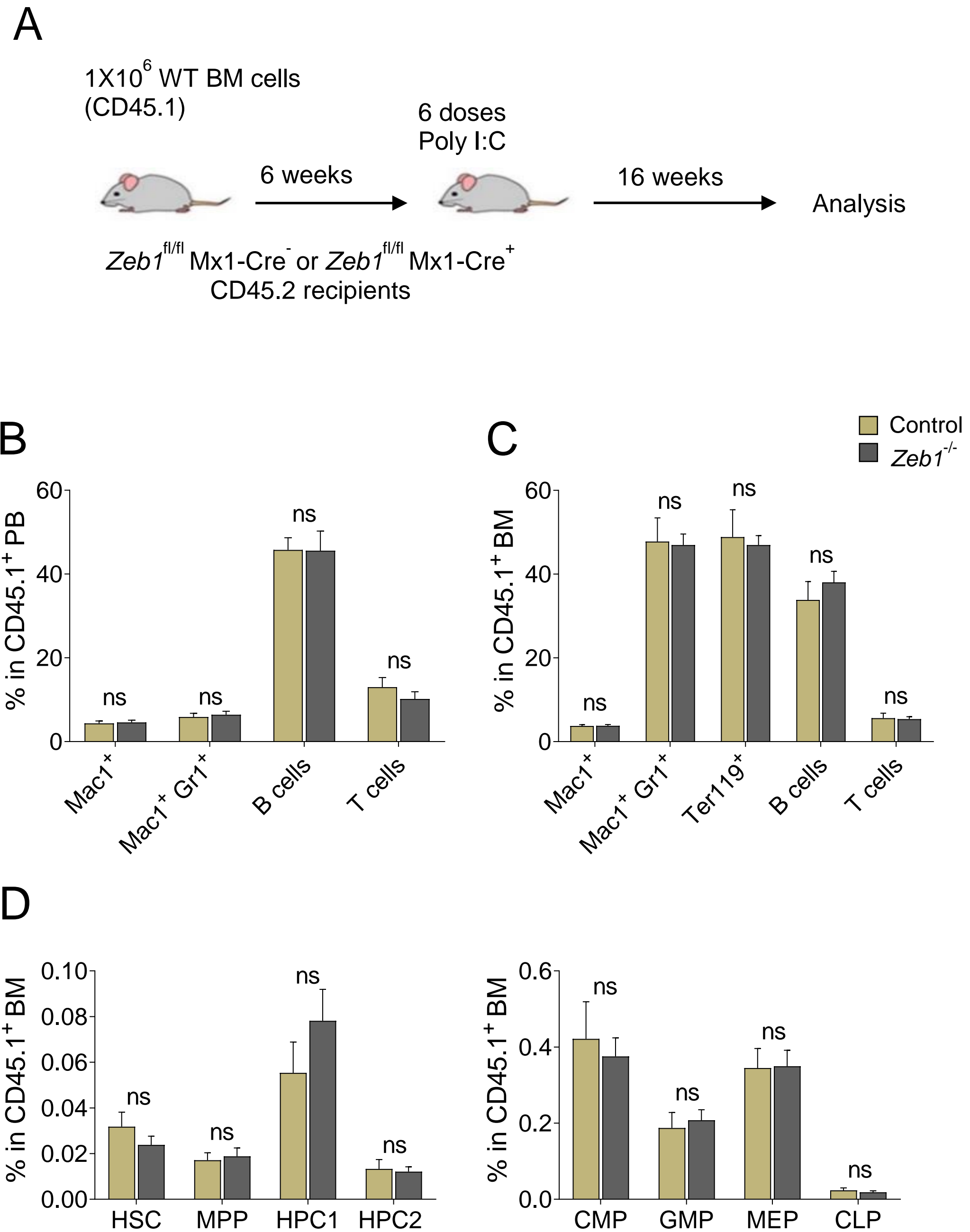


Figure 4.12. *Zeb1*-deficient BM niche did not alter haematopoiesis. (A) A scheme of niche experiment. Analysis of engraftment of differentiated cells in PB (B) and BM (C) at 16 weeks after primary BM transplantation to *Zeb1*-deficient BM niche from control (n=10 PB, 6 BM) and *Zeb1*$^{-/-}$ (n= 10 PB, 9 BM) mice from 2-3 independent experiments. (D) Analysis of engraftment of HSPCs and committed progenitors in BM at 16 weeks after primary BM transplantation to *Zeb1*-deficient BM niche from control (n= 6) and *Zeb1*$^{-/-}$ (n= 7-9) mice from 2-3 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

Although the frequency of HSCs that were exposed to *Zeb1*-deficient BM niche for 16 weeks was unchanged, it remains possible that their functional capacity could be altered. To evaluate this, at week 16, we prospectively isolated 200 HSCs from mice transplanted into control or *Zeb1*$^{-/-}$ (CD45.1) primary recipients, mixed with competitor BM cells (CD45.2) and transplanted into lethally irradiated wild-type recipients (CD45.2) (Figure 4.13A). HSCs that were exposed to the *Zeb1*$^{-/-}$ BM niche for 16 weeks showed a similar PB engraftment as control HSCs at 9 weeks after transplantation (Figure 4.13B) and also, equally contributed to myeloid, B, and T cells as control (Figure 4.13B). These data indicate that *Zeb1* mediates HSC function independently of the niche.

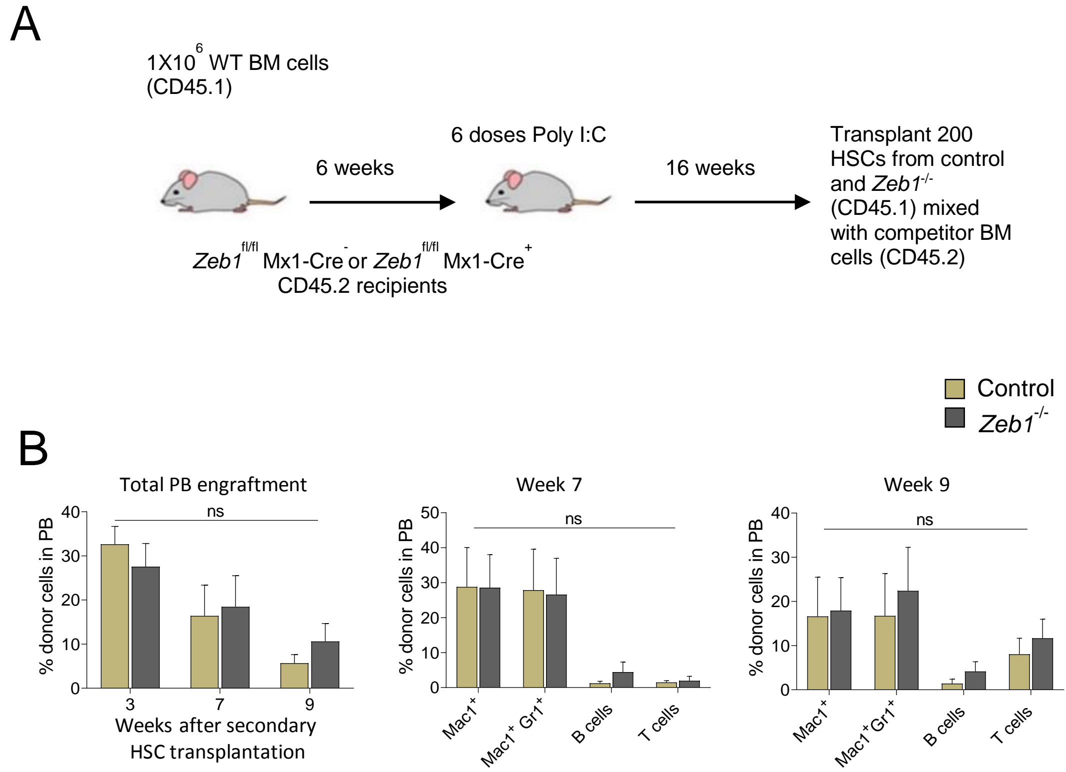
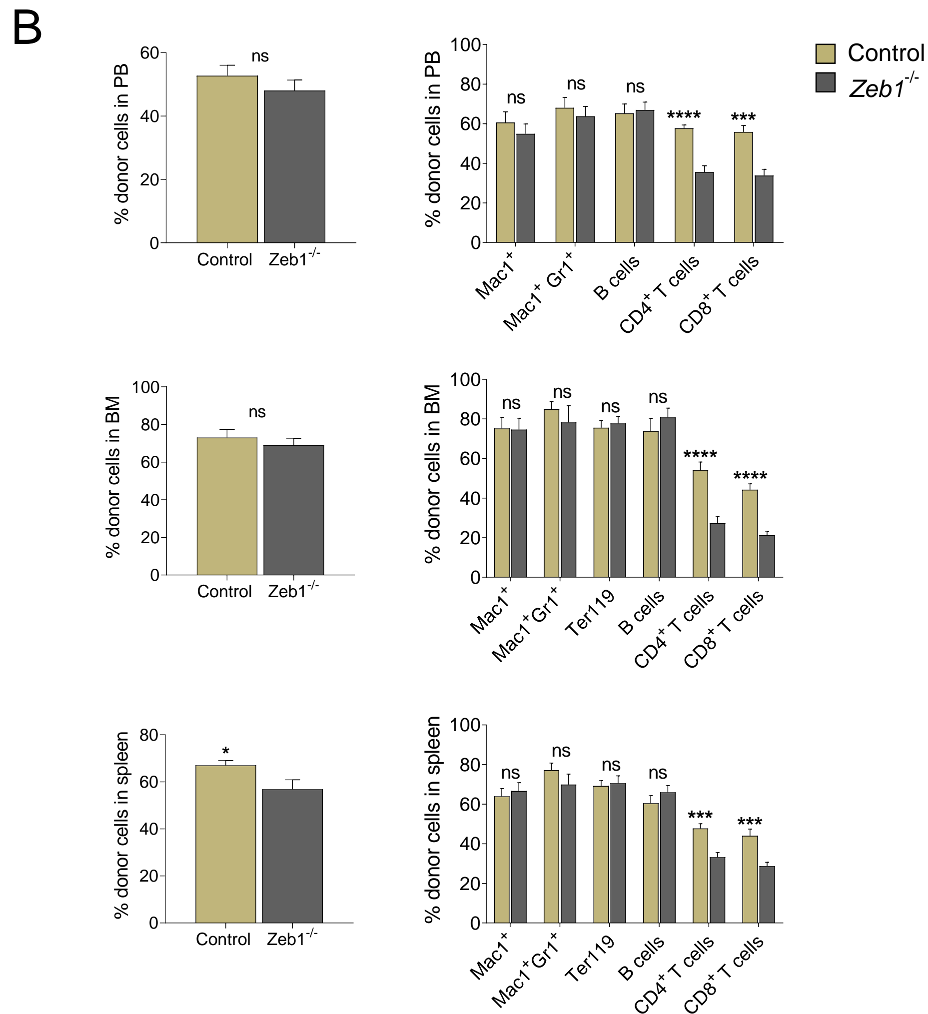


Figure 4.13. *Zeb1*-deficient BM niche did not alter HSC function. (A) A scheme of secondary HSC transplantation from niche experiment. (B) Analysis of engraftment in PB at weeks 3, 7, and 9 and differentiated cells at weeks 7 and 9 after secondary HSC transplantation from control (n=7) and *Zeb1*$^{-/-}$ (n= 7) mice from one independent experiment. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.11 Acute deletion of *Zeb1* resulted in a cell autonomous T cell reduction

To assess whether the acute requirement for *Zeb1* in maintaining HSC function was cell autonomous, we performed a competitive BM transplantation by transplanting $5X10^5$ BM cells from *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^-$ and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^+$ (CD45.2) admixed with equal number of competitor cells (CD45.1) into lethally irradiated recipients (CD45.1). 8 weeks later *Zeb1* deletion was induced by administering recipients with Poly I:C and 14 days after the last dose of Poly I:C, the mice were sacrificed for analysis of haematopoietic chimerism (Figure 4.14A). Total donor cells in PB and BM remained unchanged after *Zeb1* deletion compared with control (Figure 4.14B). However, donor cell contribution to spleen was lower in *Zeb1*$^{-/-}$ compared to control (Figure 4.14B). Among haematopoietic lineages, we found a reduced donor contribution to T cells in PB, BM, and spleen while there was no change in donor contribution to myeloid, B lymphoid, and erythroid lineages (Figure 4.14B). The reduction in T cells accounted for the reduction in donor cell engraftment in spleen as the spleen mainly consists of T and B cells. Full *Zeb1* deletion was confirmed in total BM cells 14 days after the last Poly I:C dose (Figure 14.4C). This data shows that cell autonomous acute loss of *Zeb1* is dispensable for $Mac1^+$ myeloid cell function in PB which contrasts the D14 steady state data on acute loss of *Zeb1* that showed a reduction in $Mac1^+$ myeloid cells in PB (Figure 4.2), suggesting that the effect in the D14 setting was due to an extrinsic effect of *Zeb1* loss in other tissues. Overall, these data show that *Zeb1* is essential for cell autonomous regulation of T cells, which will be further investigated in Chapter 5.



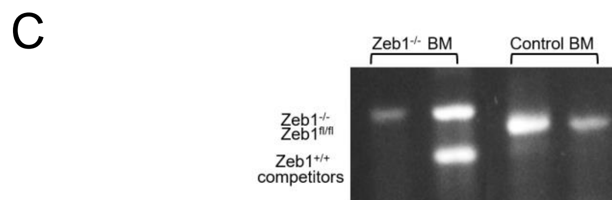


Figure 4.14. Acute deletion of *Zeb1* resulted in a cell autonomous T cell defect. (A) A scheme of cell autonomous experiment. (B) The percentage of donor cells in total PB, BM, and spleen and the donor contribution to myeloid, B, and T cells at 14 days after the last dose of Poly I:C from control (n= 11-16 for PB and spleen, 7-11 for BM) and *Zeb1*$^{-/-}$ (n= 11-19 for PB and spleen, 6-12 for BM) mice from 3-4 independent experiments. (C) full *Zeb1* deletion in BM 14 days after the last dose of Poly I:C from cell autonomous experiment. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.12 Acute deletion of *Zeb1* did not affect BM and spleen HSPCs and committed progenitors via cell autonomous manner

14 days after the last dose of Poly I:C in primary recipients from the cell autonomous experiment (Figure 4.14A), we analysed HSPCs and lineage restricted progenitors in the BM. Consistent with steady state data on HSPC immunophenotyping (Figure 4.3 and 4.4), we found no significant difference in the donor contribution to HSC, HPC1, MPP, CMP, GMP, MEP, CLP, or ILC2 populations between control and *Zeb1* KO (Figure 4.15A). Although the donor contribution to HPC2 was not affected (Figure 4.15A), HPC2 frequency 14 days after *Zeb1* deletion at steady state was reduced (Figure 4.3B) suggestive of an extrinsic effect from the BM niche affected HPC2 proportion. Additionally, we evaluated donor contribution to HSPCs and committed progenitors in spleen, which was comparable between control and *Zeb1*$^{-/-}$ (Figure 4.15B).

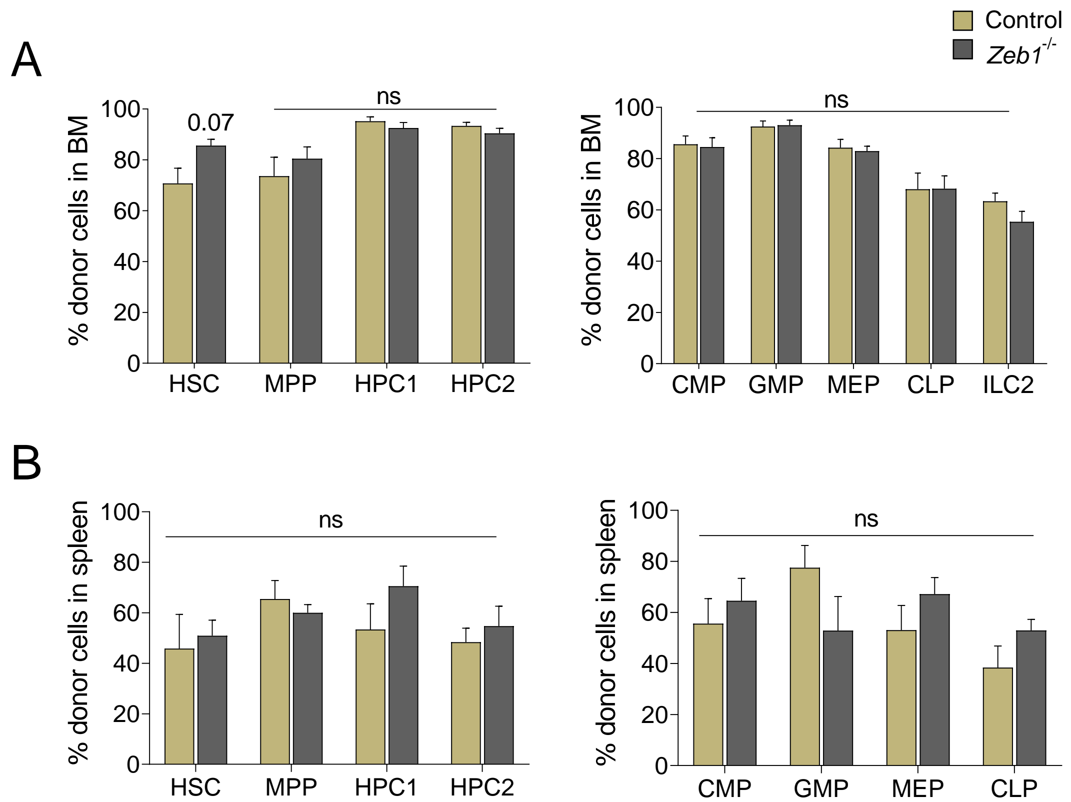
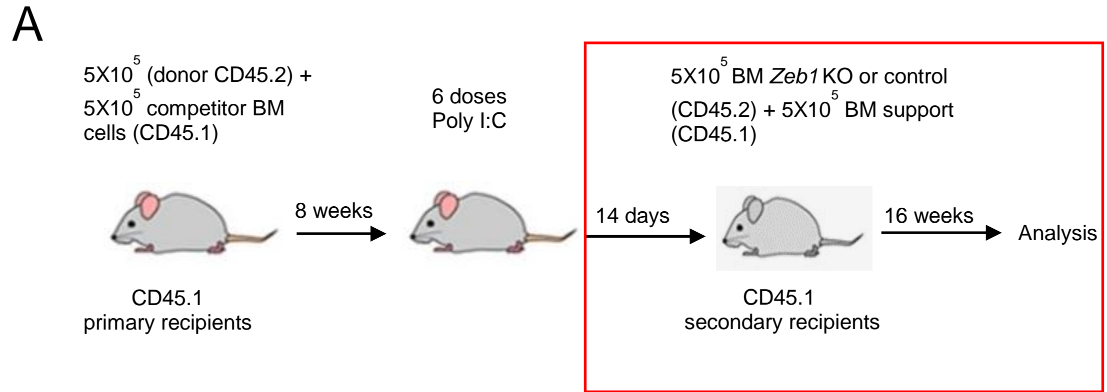


Figure 4.15. Acute deletion of *Zeb1* did not affect BM and spleen HSPCs and committed progenitors via cell autonomous manner. Analysis of donor contribution to BM HSPCs and committed progenitors (A) and spleen HSPCs and committed progenitors (B) at 14 days after the last dose of Poly I:C from control (n=9 for BM, 4 for spleen HSPCs, 5 spleen progenitors) and *Zeb1*$^{-/-}$ (n= 8 for BM, 6 spleen HSPCs, 7 spleen progenitors) mice from 2-3 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.13 *Zeb1* is required for cell autonomous HSC function after transplantation

Next to test the cell autonomous repopulation capacity of HSCs and BM progenitors after acute *Zeb1* KO, we isolated 500,000 donor BM cells (CD45.2) from primary recipients (14 days after *Zeb1* ablation) mixed with 500,000 competitor BM cells (CD45.1) and transplanted them into lethally irradiated recipients (Figure 4.16A). Consistent with HSC secondary transplantation data that were originally transplanted from steady state HSC (Figure 4.11), a dramatic reduction in donor engraftment in PB and BM was observed (Figure 4.16B). Further analysis of donor contribution to PB lineages revealed a marked reduction in myeloid cells, near loss of B cells, and a complete loss of T cells (Figure 4.16C). Thus, *Zeb1* is required for cell autonomous HSC function after transplantation.



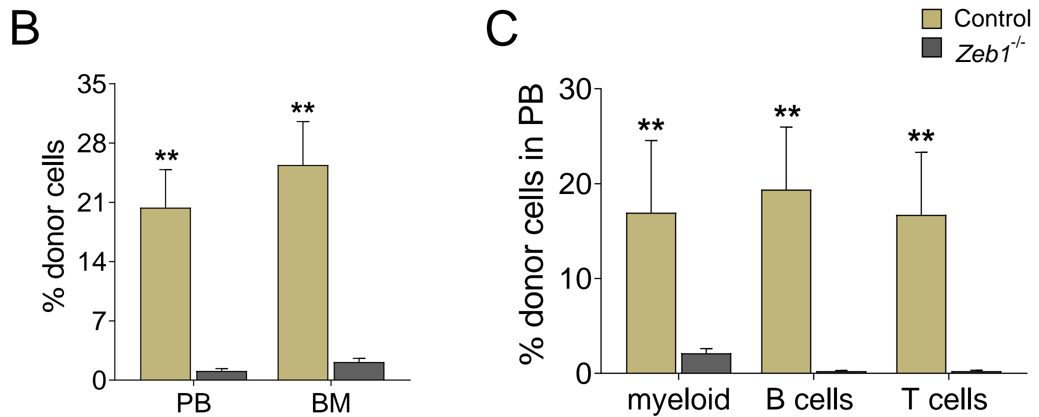


Figure 4.16. *Zeb1* is required for HSC function after secondary transplantation via cell autonomous manner. (A) A scheme of the competitive secondary BM transplantation via cell autonomous manner. (B) Analysis of engraftment in PB and BM at 16 weeks after secondary BM transplantation from control (n=5 PB, 6 BM) and *Zeb1*$^{-/-}$ (n= 8 PB, 6 BM) mice from one independent experiment. (C) Analysis of donor contribution to PB myeloid, B, and T cells at 16 weeks after secondary BM transplantation from control (n=5) and *Zeb1*$^{-/-}$ (n= 7-8) mice from one independent experiment. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.3.14 Acute *Zeb1* loss does not affect homing capacity of BM and LSK cells

An integral part of successful engraftment after BM transplantation is homing of intravenously infused HSPCs to the BM niche, the main site of adult haematopoiesis. Primary and secondary transplantations of HSCs from *Zeb1*$^{-/-}$ mice showed an early defect in engraftment. To assess whether this was due to abnormal homing of *Zeb1*$^{-/-}$ HSPCs, we transplanted $7X10^6$ total BM cells (CD45.2) from control and *Zeb1*$^{-/-}$ 14 days after the last dose of Poly I:C into lethally irradiated recipients (CD45.1) and analysed the donor cells 16 hours after transplantation (Figure 4.17A). Our data showed an equal homing capacity of total BM cells or LSK population between the two genotypes, as judged by the presence of donor cells CD45.2 (Figure 4.17B). Similarly, homing of donor cells to the spleen and thymus was comparable between control and *Zeb1*$^{-/-}$ genotypes (Figure 4.17C). Thus, acute deletion of *Zeb1* does not impact the homing ability of haematopoietic cells *in vivo*.

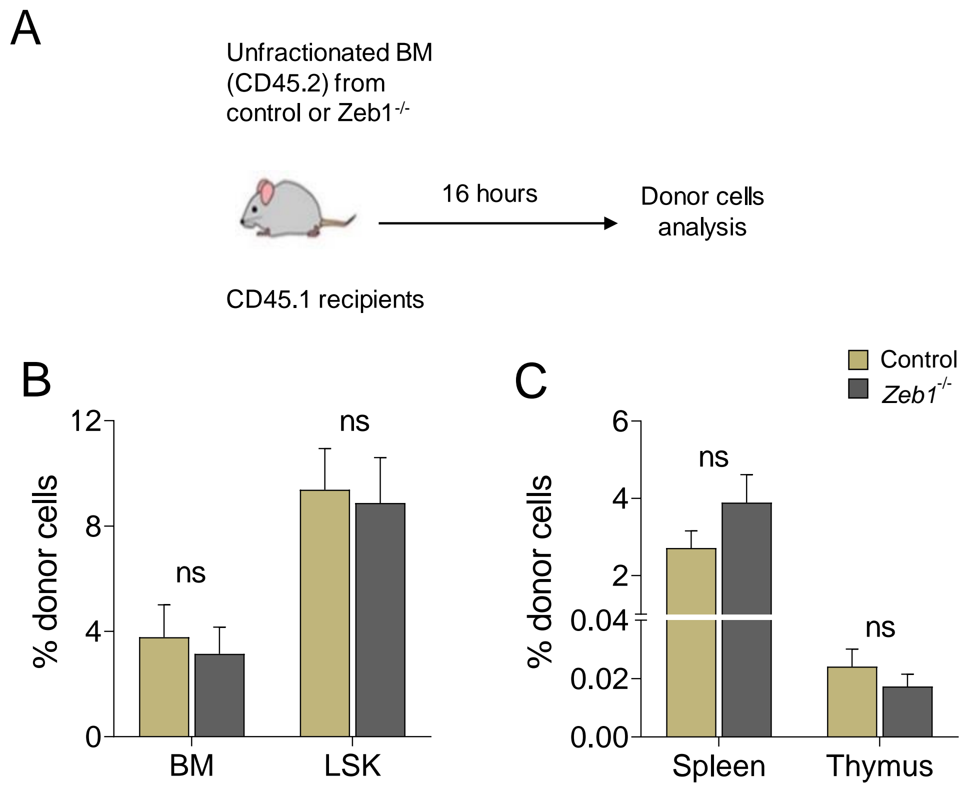


Figure 4.17. *Zeb1* loss did not affect homing capacity of BM cells and LSK. (A) A scheme of homing experiment. (B) Analysis of BM and LSK donor cells (B), spleen and thymus (C) 16 hours after total BM transplantation from control (n= 3 LSK, 8 BM and spleen, 7 thymus) and *Zeb1*$^{-/-}$ (n= 2 LSK, 6 BM, 5 spleen and thymus) mice from 1-2 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

4.4 Discussion

In this Chapter, we investigated the role of *Zeb1* in adult haematopoiesis by acutely deleting *Zeb1* using the Mx1-Cre system, where we analysed HSPC function by immunophenotyping and a series of cell autonomous and non-cell autonomous transplantation experiments 14 days after induction of gene deletion. These data reveal an essential, acute requirement for *Zeb1* in HSPC function, in particular to balance self-renewal and differentiation fates.

Our data on *Zeb1* mediated regulation of HSPC function presented here, for the most part, are consistent with that generated in Chapter 3, with some notable exceptions. Acute deletion of *Zeb1* did not lead to immunophenotypic alterations in HSCs, MPP, and HPC1 compartments. This contrasts with the expansion of HSC and MPP populations and a reduction in HPC1 and HPC2 observed in mice where *Zeb1* was persistently absent. Also, the specific HSPC and committed progenitor populations contributing to the differentiation defect of HSPC after transplantation were different under conditions of acute versus long-term deletion of *Zeb1*. The reasons for these differences are unclear but could be due to the length of *Zeb1* deletion in HSCs in the acute versus long-term setting. Thus, for example, expanded HSCs after prolonged loss of *Zeb1* may reflect distinct cellular and molecular changes occurring over time compared to HSCs where *Zeb1* was deleted acutely. This may also underlie why, HSC differentiation kinetics after transplantation are slightly different in the two distinct settings of *Zeb1* deletion.

Given the defects in the lymphoid compartment on persistent loss of *Zeb1*, in this chapter we analysed, in detail, the impact of acute deletion of *Zeb1* on initial commitment of lymphoid progenitors in the BM. We showed that CLP (Lin^{-} $Sca1^{low}$ $cKit^{low}$ $CD127^{high}$ $CD135^{high}$) and LSK^{-} $CD135^{+}$ $CD127^{+}$ lymphoid progenitors were reduced in $Zeb1^{-/-}$ mice. At an earlier stage of lymphoid commitment in the BM or spleen, LMPPs (LSK $CD127^{high}$ $CD135^{high}$) were reduced in $Zeb1^{-/-}$ mice. Thus, our data shows that LMPP, CLP, and LSK^{-} $CD135^{+}$ $CD127^{+}$ lymphoid progenitors are heavily dependent on *Zeb1* during steady-state adult haematopoiesis. Taken together, with the cell-autonomous role for *Zeb1* in T cells (Figure 4.14), these data also suggest a pivotal role for *Zeb1* mediating T cell commitment and development in adults, mirroring the developmental requirement for *Zeb1* during T-cell genesis in the developing embryo (Higashi et al., 1997, Takagi et al., 1998).

Acute deletion of *Zeb1* resulted in multi-lineage differentiation failure of HSPCs reflecting predominant deregulation of T, B, and myeloid related genes and deregulation of HSC maintenance genes, as evidenced by RNA-seq. Interestingly, the RNA-Seq data also unveiled a dysregulated gene signature of cell adhesion, polarity and cytoskeleton. *Zeb1* is known as a potent repressor of epithelial genes to induce EMT. This process involves *Zeb1*-mediated repression of adhesion genes such as *CDH1* that encodes the protein E-cadherin and polarity genes such as Crumbs3 (*Crb3*), HUGL2 (*Llgl2*), and Pals1-associated tight junction protein (*PATJ*) (*Inadl*) which in turns convert the epithelial cells to lose their polarity and acquire a motile state (Aigner et al., 2007, Sanchez-Tillo et al., 2010, Vandewalle et al., 2009). Our data showed increased expression of several genes responsible for cell adhesion (*Epcam, Cdh1, Jam3* and others) and cytoskeleton and polarity (*Crb3, Prkcz, Pard6b, Cxadr*, and others). We found the most upregulated gene in the list was epithelial cell adhesion molecule EpCAM. EpCAM maintains stemness in mouse embryonic stem cells (ESCs) (Gonzalez et al., 2009) and regulate various cellular processes including proliferation, differentiation, cell cycle, and adhesion as well as in epithelial cancer can work as an oncogene or suppressor (Schnell et al., 2013). *Zeb1* was shown to bind and repress EpCAM in Zebrafish during development and human breast and pancreatic cancer cell lines as well as in mouse during liver fibrosis (Vannier et al., 2013, Song et al., 2017). However, its role in HSC and normal haematopoiesis is unclear. Furthermore, *Zeb1* was documented to regulate polarity and cytoskeleton genes *Pard6b* and *Cxadr* during neuron differentiation (Wang et al., 2019, Singh et al., 2016) and *Crb3* and *Inadl* in colorectal cancer (Aigner et al., 2007, Spaderna et al., 2008). Thus, our RNA-Seq results are consistent with *Zeb1* acting as a critical regulator of cell adhesion and polarity genes and show a novel gene signature in *Zeb1*-deficient HSCs that might acquire epithelial cell like phenotype.

Regulation of polarity components has been found to control HSC function and balance between self-renewal and differentiation. Increased expression of Crumbs3, CDC42, and others was found in aged HSCs that were characterised by an expansion in number and defect in their repopulation capacity and these changes were reversed by pharmacological inhibition of CDC42 (Florian et al, 2012). Furthermore, aged HSCs with changes in polarity preferred self-renewal symmetric divisions that ended up with expansion of HSCs and diminished differentiation activity (Florian et al., 2018). This suggests that *Zeb1* KO HSCs after transplantation had more self-renewal versus differentiation divisions, hence they retained their numbers and resulted in differentiation defects. Interestingly, dysregulation of actin cytoskeleton and

activation of CDC42 in LSKs that were exposed to Wnt5a haploinsufficient BM niche were causative of repopulation defect in secondary recipients and showed differentially expressed genes enriched in *Zeb1* DNA binding motif (Schreck et al., 2017). *Zeb1* was shown to regulate actin cytoskeleton during the migration of CSCs via repressing miR-34a and regulation of CDC42 levels (Ahn et al., 2012). miR34a is required for HSC survival after irradiation via DNA damage repair process (Zeng et al., 2019). This line of evidence about the role of *Zeb1* in driving cell migration through actin cytoskeleton and polarity regulation supports our phenotype that HSCs that lack *Zeb1* with upregulation of cytoskeleton and polarity genes may restrict their motility and alter their interaction with BM niche components and eventually impair their function.

The role of other polarity proteins in HSC function has been reported. Scribble (Scrib) was found to regulate HSC maintenance and loss of Scrib resulted in HSC self-renewal defect (Mohr et al., 2018). Another member of Scribble complex, Llgl (lethal giant larvae homolog 1) was found to regulate HSC function. *Llgl*$^{-/-}$ HSCs showed enhanced self-renewal ability sustained through tertiary transplantation (Heidel et al., 2013). Therefore, we speculate that the upregulation of polarity genes after *Zeb1* KO confers on HSCs a cell-autonomous epithelial-like feature, which facilitates interaction with components of the BM niche components such as osteoblasts, MSCs, endothelial cells, CAR cells, and others. This eventually restrains the motility of HSCs outside of the BM leading to a reduction in HSCs in extramedullary sites of haematopoiesis, such as the spleen (data discussed in Chapter 3) and impacts between self-renewal and differentiation fates as observed in HSC transplantation in the setting of both acute and chronic loss of *Zeb1*.

Zeb2, the other member of Zeb family, was found to be essential for embryonic haematopoiesis and mobilisation of HSCs to the BM during development (Goossens et al., 2011) and for adult murine HSC differentiation (Li et al., 2016b). *Zeb1* and *Zeb2* KO HSC both resulted in a multilineage haematopoietic differentiation defect during steady state and post transplantation with no significant reduction in HSC number although *Zeb1* KO HSCs showed a trend towards reduction; however, the defect was more profound in *Zeb1*-deficient HSCs. Some differences exist which suggest different roles of *Zeb1* and *Zeb2* during haematopoiesis. *Zeb2*$^{-/-}$ mice at 8 weeks after *Zeb2* deletion presented a decrease in erythrocytes, leukocytes, and thrombocytes in PB and loss of B cells and monocytes in PB and BM with increased granulocytes, LSK, HSC, and MEP and a reduction in GMP in BM (Li et al., 2016b). Primary, secondary, and tertiary transplantation revealed the same pattern of

differentiation defects with preserved number of HSCs (Li et al., 2016b). In contrast, *Zeb1* KO mice at 2 weeks after *Zeb1* deletion showed a dramatic reduction of T cells in thymus and a decrease in lymphoid progenitors in BM and reduction of donor contribution to monocytic, granulocytic, B cell lineages as well as HSPCs and committed progenitors after HSC transplantation. One major difference between our findings and *Zeb2* phenotype is that inactivation of *Zeb2* had a huge impact on B lymphocyte lineage while our data showed a prominent effect on T cell lineage and thymus. Another difference is that we found a multilineage differentiation defect including monocytic, granulocytic, B, and T lymphoid lineages while they reported the same pattern apart from granulocytic lineage that was not affected after transplantation in *Zeb2* KO.

ZEB1 and ZEB2 expression has been reported to overlap in some tissues including nervous and skeletal tissues which suggests a compensatory mechanism for each other (Postigo and Dean, 2000). This applies to T cells where ZEB1 is highly expressed in thymus whereas ZEB2 is almost absent in thymus. Thus, loss of *Zeb1* resulted in severe thymus and T cell defects as we observed and others (Higashi et al., 1997, Takagi et al., 1998) while in *Zeb2* KO no change was observed in T cells during steady state (Li et al., 2016b). A compensatory mechanism also may explain why the HSC number in *Zeb1* KO and *Zeb2* KO was not significantly altered as both *Zeb1* and *Zeb2* are highly expressed in HSC and absence of one of them might be compensated by the other. This might apply as well in B cell phenotype in *Zeb2* KO. Although our qPCR analysis showed high levels of *Zeb1* in terminally differentiated $B220^+$ B cells, expression data from ImmGen database (http://www.immgen.org/) revealed increased expression of *Zeb2* in early B cell precursors in BM while *Zeb1* expression in these precursors was lower than *Zeb2*, hence potentially accounting for the early defect of B cells in *Zeb2* KO and the inability of *Zeb1* to compensate for loss of *Zeb2*. Similarly, GMP was reduced in *Zeb2* KO but was preserved in *Zeb1* KO during steady state. This might be explained by the fact that *Zeb2* is highly expressed in GMP while *Zeb1* had a lower expression in GMP and as a result could not compensate for the loss of *Zeb2*. Thus, differential and overlapping expression and functions in different haematopoietic cell subsets may explain the different phenotypes observed between *Zeb1* and *Zeb2* KO.

The role of other EMT inducers in haematopoiesis and HSC function has similarly been shown. Slug is a TF belongs to Slug/Snail family of zinc-finger TFs. It is found to directly bind and activate *Zeb1* by binding to *Zeb1* E-box sequence in *Zeb1* promoter in melanoma cell lines (Wels et al., 2011). *Slug*$^{-/-}$ mice showed enhanced

HSC self-renewal and repopulation capacity (Sun et al., 2010). This phenotype contrasts with *Zeb1* KO phenotype that showed HSC differentiation defects and suggests that Slug regulates haematopoiesis independent of *Zeb1*. Twist-1 is a TF that belongs to the basic helix-loop-helix (bHLH) family was found to be a critical regulator of HSC self-renewal and specification to myeloid lineage (Dong et al., 2014). Interestingly, this line of evidence shows different EMT inducers have distinct roles in haematopoiesis.

In closing, we report, using the Mx-1 Cre *Zeb1*$^{fl/fl}$ mouse model, the intrinsic role of *Zeb1* in regulating the self-renewal and differentiation processes of adult HSC and its requirement for different haematopoietic lineages.

Table 4.1. Comparison between the phenotypes of acute and long-term deletion of *Zeb1* in the haematopoietic system during steady state haematopoiesis and after transplantation.

		During steady state		After transplantation	
Cell type	**site**	**Acute deletion (14 days)**	**Long-term deletion (32 weeks)**	**Acute deletion**	**Long-term deletion**
HSPCs	BM	LMPP CD127$^+$, HPC2 ↓	HSC, MPP ↑ HPC1, HPC2 ↓	MPP, HPC1, HPC2 ↓	HPC1 ↓
	Spleen	LMPP CD127$^+$ ↓	HSC ↓	It was not analysed	It was not analysed
Committed Progenitors	BM	CLP Flt3$^+$, LS CD127$^+$ Flt3$^+$ ↓	No difference	CMP. GMP, MEP, ILC2 ↓	CMP. GMP, CLP, ILC2 ↓
	Spleen	No difference	CMP. GMP, CLP, ILC2 ↓	It was not analysed	It was not analysed
Mature cells	BM	EM CD8$^+$ ↓	T cells ↓	myeloid, B, T ↓	myeloid, B, T ↓
	PB	Mac1$^+$ cells EM CD8$^+$ ↓	Mac1$^+$ cells ↓	myeloid, B, T ↓	myeloid, B, T ↓
	Spleen	CM CD8$^+$ ↓	Mac1$^+$, Mac1$^+$ Gr1$^+$, T cells ↓	myeloid, B, T ↓	myeloid, B, T ↓

CHAPTER 5 :

The role of *Zeb1* in adult T cell development

5.1 Introduction

T lymphocytes develop and mature in thymus (Miller and Osoba, 1967). The majority of T cells express either CD4 or CD8 cell surface markers. They are essential components in adaptive immunity to fight foreign antigens (Luckheeram et al., 2012). For sustained T cell production and development throughout life, the thymus is continually seeded by BM progenitors (Donskoy and Goldschneider, 1992, Wu et al., 1991, Antica et al., 1994, Scollay et al., 1986, Goldschneider et al., 1986). These BM progenitors are called early thymic progenitors / early T cell progenitors (ETPs) (Ceredig and Rolink, 2002). ETPs in the thymus are identified as Lin^{-} $CD44^{+}$ $CD25^{-}$ c-Kit^{+} which can differentiate into B cells, myeloid cells, DCs, and NKs in addition to T cells (Luc et al., 2012, Ceredig et al., 2007, Bell and Bhandoola, 2008, Bhandoola and Sambandam, 2006).

Immature thymic progenitors can be first characterised as double negative $CD4^{-}$ $CD8^{-}$ (DN) (Ceredig et al., 1983). The DN population can be subdivided into four temporally restricted cellular compartments according to the expression of CD44 and CD25: DN1 ($CD44^{+}$ $CD25^{-}$), DN2 ($CD44^{+}$ $CD25^{+}$), DN3 ($CD44^{-}$ $CD25^{+}$), and DN4 ($CD44^{-}$ $CD25^{-}$) (Figure 5.1) (Godfrey et al., 1993). Differentiation to the T cell lineage starts when DN1 cells that contain ETPs progress into DN2 cell when the cells lose B cell potential but retain T and DC lineages (Wu et al., 1996). Next, upon transition to DN3 stage, the cells are fully committed to the T cell lineage and contain rearranged T cell receptor (TCR) β (Capone et al., 1998). Successful TCR-β chain rearrangement is essential for thymocyte differentiation and progression through DN4 (Krueger et al., 2017, Bhandoola and Sambandam, 2006, Dudley et al., 1994, Michie and Zuniga-Pflucker, 2002). Subsequently, the cells begin to express both CD4 and CD8, DP (double positive cells), and rearrange TCR-αβ and undergo positive and negative selection to finally mature to SP CD4 or SP CD8 and exit the thymus to the periphery (Bhandoola and Sambandam, 2006, Krueger et al., 2017, Moore and Zlotnik, 1995, Huesmann et al., 1991).

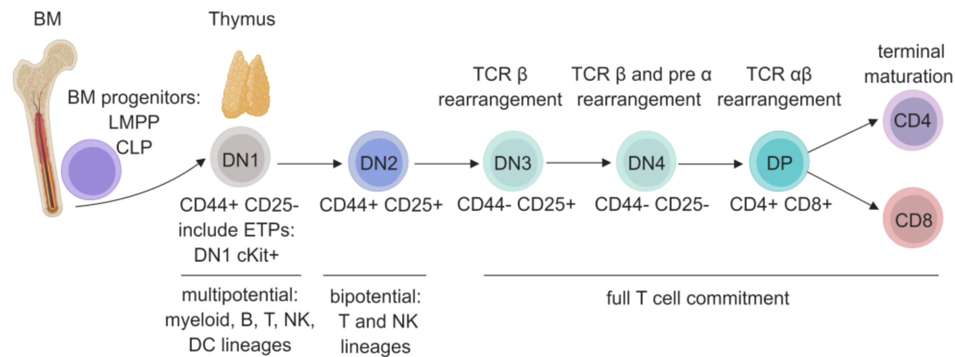


Figure 5.1. T cell development in thymus. Lymphoid progenitors such as LMPPs and CLPs migrate to the thymus via blood stream. In the thymus, ETPs are considered canonical T cell progenitors, that differentiate into the subsequent double negative (DN) CD4 and CD8 populations DN2, DN3, and DN4. Cells in DN4 upon a successful rearrangement of TCR-β and pre α develop to double positive (DP) CD4 and CD8 cells where the cells that are negatively selected die and cells that are positively selected differentiate into mature single positive (SP) CD4 or CD8. Mature T cells exit the thymus and migrate to lymphoid tissues, PB, BM, and others.

After development in the thymus, $CD4^+$ and $CD8^+$ T cells emigrate and circulate in defined areas including PB, spleen, lymph node, and others to provide immunological protection to the host (Weinreich and Hogquist, 2008). $CD8^+$ T cells are cytotoxic and have the ability to directly kill the infected cells after being induced by interaction with antigen presenting cells and release of cytokines and cytotoxic granules (Harty et al., 2000). $CD4^+$ T cells mainly are helper cells that produce cytokines to facilitate the function of other immune cells including B cells, $CD8^+$ T cells, granulocytes, macrophages and others (Zhu and Paul, 2008). Most T cells in the body are naïve, that is they have not encountered an antigen to activate them, and they are characterised by high expression of CD62L and low expression of CD44, though some memory T cells ($CD44^{high}$) normally exist during homeostasis (Takada and Jameson, 2009). T cell homeostasis is mainly regulated by interleukin 7 (IL7) and contact with self-peptide MHC via TCR (Fry and Mackall, 2005, Sprent and Surh, 2011). After encountering antigen, a small subset of naïve T cells become activated and proliferate and differentiate into effector memory ($CD44^{high}$ $CD62^{low}$) and central memory ($CD44^{high}$ $CD62^{high}$) T cells (Sprent and Surh, 2011, Carrette and Surh, 2012). After antigen clearance, effector cells exhaust while the memory cells remain, forming immunological memory to quickly respond to the second infection (Harty et al., 2000).

Few studies have shown that *Zeb1* is essential for normal T-cell development. Complete deletion of *Zeb1* (*Null–LacZ*) in mice embryos results in a sharp decrease

of thymocytes at E18.5, postnatal death and skeletal and respiratory defects (Takagi et al., 1998). In contrast to *Null–LacZ*, 80% of homozygous mice that lack the zinc finger sequence proximal to C-terminal portion (*ΔC-fin*) die perinatally but few survive (Higashi et al., 1997). Homozygous *ΔC-fin* mutant embryos and survived adults at 6 weeks old show a decline of total cell number in thymus and a reduction in c-kit$^+$ DN population, DN2, and DN3 as well as a decline in T cells in spleen and lymph nodes (Higashi et al., 1997). This indicates that different domains in ZEB1 protein play different roles in different cellular contexts and, in this case, the C-terminal cluster is essential for T-cell development but not for skeletal system (Higashi et al., 1997, Takagi et al., 1998, Postigo and Dean, 1999a).

CD4 has been documented as a target gene of *ZEB1* in T-cell development. *ZEB1*represses *CD4* via binding to the '5 E-box in the proximal enhancer of the *CD4* promoter rendering it a silencer after a competition with the activators E12 and HEB (Brabletz et al., 1999). Overexpression of ZEB1 in Jurkat cell line (CD4$^+$ CD8$^-$) and A.301 cell line (CD4$^+$CD8$^+$) results in a reduction of the activity of the CD4 proximal enhancer to 48% and 76%, respectively (Brabletz et al., 1999). Overexpression of ZEB1 also reduces CD4 protein expression in CD4$^+$ SP Jurkat cells but not CD4$^+$CD8$^+$ DP A.301 cells (Brabletz et al., 1999). Moreover, *ZEB1* binds and blocks the activity of some genes implicated in T-cell development such as *GATA3* (Gregoire and Romeo, 1999) and interleukin-2 (*IL-2*) (Williams et al., 1991, Yasui et al., 1998, Wang et al., 2009). Recently, *Zeb1* was found to regulate the function and maintenance of memory CD8$^+$ T cells (Guan et al., 2018). After exposure to lymphocytic choriomeningitis virus (LCMV), memory CD8$^+$ T cells lacking *Zeb1* showed transient expansion, but this diminished over time as a result of apoptosis (Guan et al., 2018).

5.2 Aims

Germline deletion of *Zeb1* in mice revealed impairment in T cell development (Higashi et al., 1997, Takagi et al., 1998). However, there is a lack evidence of how this T cell defect occurred and whether this phenotype is intrinsic to *Zeb1* KO in T cells or whether it was a systemic effect. Also, as this defect occurred because of germline deletion of *Zeb1* during embryogenesis, the requirement of *Zeb1* in adult T cell development is unknown. The aims of this chapter are:

1- Studying the effect of acute deletion of *Zeb1* in adult T cell development in thymus using Mx1-Cre system. T cell subsets were analysed using CD4 and CD8 markers from control and *Zeb1*$^{-/-}$ 14 days after Zeb1 deletion.

2- Studying the impact of *Zeb1* in early commitment to the T cell lineage in adults. This included studying the early populations in thymus within CD4 and CD8 double negative compartment from control and *Zeb1*$^{-/-}$ 14 days after Zeb1 deletion.

3- Assessing *Zeb1* mediated regulation of BM progenitor seeding to the thymus. To fulfil this aim, ETPs, the early population entering the thymus from the BM, was analysed from control and *Zeb1*$^{-/-}$ 14 days after Zeb1 deletion.

4- Elucidating the cellular mechanism of how *Zeb1* regulates T cell development in thymus in adults. Apoptosis and cell cycle analyses were performed in immature and mature T cell subsets in thymus from control and *Zeb1*$^{-/-}$ 14 days after Zeb1 deletion.

5- Assessing the requirement for *Zeb1* in adult T cell subsets in the periphery. CD4^{+} and CD8^{+} T cells in PB, BM, and spleen from control and *Zeb1*$^{-/-}$ 14 days after Zeb1 deletion were analysed using CD44 and CD62L markers to study their homeostasis and activation status.

5.3 Result

5.3.1 Acute deletion of *Zeb1* resulted in a defect in T cell development in thymus

Germline KO of *Zeb1* resulted in a developmental defect of T cell lineage (Higashi et al., 1997, Takagi et al., 1998). However, the requirement of *Zeb1* during T cell development in adulthood is unknown. We used the Mx1-Cre conditional mouse model to fully understand the role of *Zeb1* in adult T cell development (Figure 5.2A). Fourteen days after the last Poly I:C dose, *Zeb1*$^{-/-}$ mice showed very small thymi associated with a dramatic reduction in total cellularity (Fig. 5.2B-D).

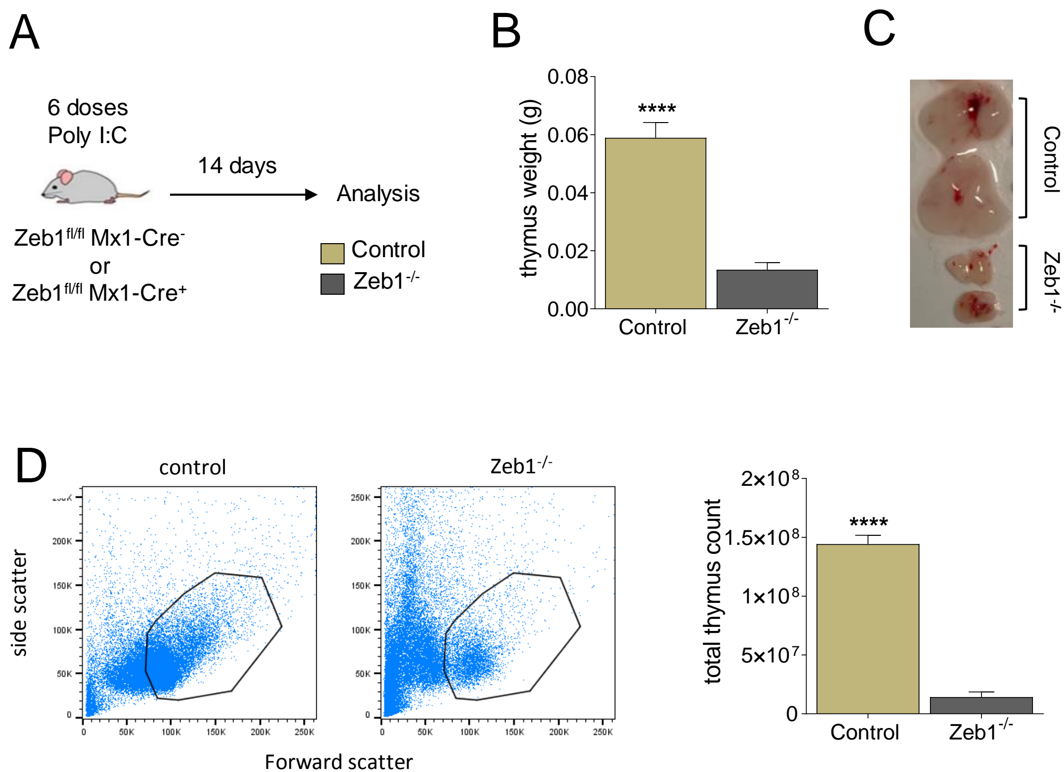


Figure 5.2. Acute deletion of *Zeb1* resulted in impairment of thymus mass and cellularity. (A) A scheme of *Zeb1* deletion. 6 doses of Poly I:C were intraperitoneally administered into *Zeb1*$^{fl/fl}$ Mx1-Cre$^-$ and *Zeb1*$^{fl/fl}$ Mx1-Cre$^+$. The mice were dissected at D14 after the last dose of Poly I:C. Thymus weight (B), a representative picture (C) and total thymus cellularity (D) from control (n=9) and *Zeb1*$^{-/-}$ (n=8) mice from 5 independent experiments at D14 after the last Poly I:C dose. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

Analysis of T cell subsets in the thymus, using CD4 and CD8 markers, showed an increased frequency of immature CD4 CD8 DN (DN) cells and mature SP CD4 ($CD4^+$) and SP CD8 ($CD8^+$) T cells and a significant reduction in the frequency of CD4 C8 DP (DP) cells in *Zeb1*$^{-/-}$ (Figure 5.3A and B). We also quantified the abundance of these populations in total thymocytes and, because of the profoundly reduced thymic cellularity, found a significant reduction in absolute numbers of DN, DP, $CD4^+$ and $CD8^+$ after *Zeb1* KO (Figure 5.3C). This data demonstrates the requirement of *Zeb1* in T cell maturation in the thymus.

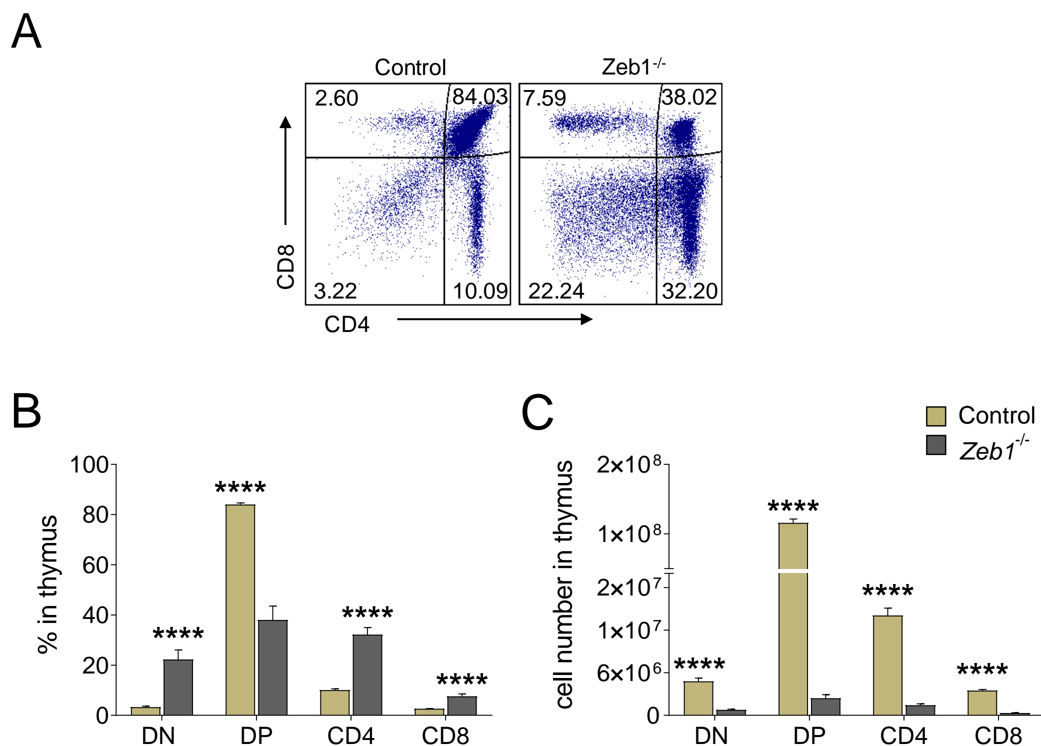
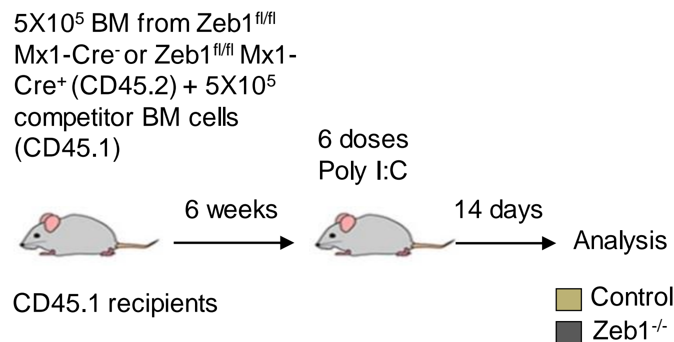


Figure 5.3. Acute deletion of *Zeb1* resulted in a dramatic reduction of T cell subsets in thymus. (A) Representative FACS plots of T cell analysis in thymus based on CD4 and CD8 cell surface markers (DN: $CD4^-$ $CD8^-$, DP: $CD4^+$ $CD8^+$, $CD4^+$, $CD8^+$) gated from DAPI negative cells (live cells). (B) Frequency of T cell subsets in thymus from control (n=13) and *Zeb1*$^{-/-}$ (n=12) mice from 6 independent experiments at D14 after the last Poly I:C dose. (C) Total cell count of T cell subsets in thymus from control (n=14-15) and *Zeb1*$^{-/-}$ (n=14-15) mice from 6 independent experiments at D14 after the last Poly I:C dose. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

Since Mx1-Cre system can delete genes in non-haematopoietic tissues such as thymus and BM niche (Kuhn et al., 1995, Zhang et al., 2003), we tested the cell autonomous role of *Zeb1* in T cell development (Figure 5.4A). Fourteen days after the last injection of Poly I:C, we analysed donor cells in thymus. Strikingly, donor engraftment in the thymus was dramatically reduced compared to control (Figure 5.4B). Next, we checked the donor contribution to T cell populations in thymus. Consistent with lack of thymic donor engraftment, we found a massive reduction in donor contribution to DN, DP, CD4⁺, and CD8⁺ cells (Figure 5.4C). This data demonstrates the cell-autonomous role of *Zeb1* in T cell development and maintenance in adults.

A



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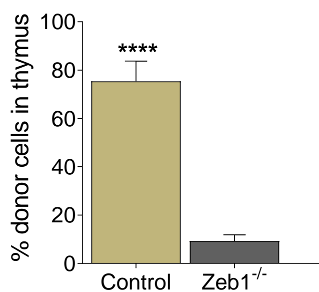

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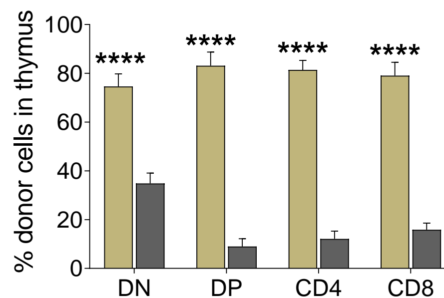
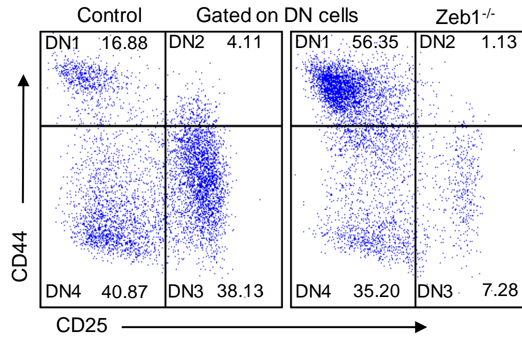


Figure 5.4. Acute deletion of *Zeb1* resulted in a dramatic reduction of T cell subsets in thymus via cell autonomous. (A) A scheme of cell autonomous transplant to assess the effect of *Zeb1* loss in haematopoietic cells but not in niche cells. 5X10^5 BM cells from *Zeb1$^{fl/fl}$ Mx1-Cre$^-$* or *Zeb1$^{fl/fl}$ Mx1-Cre$^+$* (CD45.2) + 5X10^5 competitor BM cells (CD45.1) were transplanted into lethally irradiated recipients (CD45.1), then six weeks later mice were injected with Poly I:C to delete *Zeb1* and analysed at 14 days after the last dose of Poly I:C. (B) The percentage of donor cells in thymus from control (n=11) and *Zeb1$^{-/-}$* (n=14) mice from 3 independent experiments via cell autonomous manner. (C) Analysis of donor contribution to T cell populations in thymus from control (n=10) and *Zeb1$^{-/-}$* (n=14) mice from 3 independent experiments via cell autonomous manner. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

5.3.2 Acute loss of *Zeb1* resulted in a partial differentiation block of T cells in thymus

To understand the observed defect occurring in the DN fraction in the thymus after *Zeb1* ablation, we further studied DN cells (Figure 5.5A). Using CD44 and CD25 markers, the DN population can be subdivided into 4 populations: DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^+$), DN3 ($CD44^-$ $CD25^+$), and DN4 ($CD44^-$ $CD25^-$) (Godfrey et al., 1993). DN1 cells differentiate into DN2 and the differentiation process proceeds through DN3 to DN4 reaching DP and finally single $CD4^+$ and $CD8^+$ mature cells (Godfrey et al., 1993). Our analysis of the frequency of DN1, DN2, DN3, and DN4 showed increased DN1 cells and a reduction in DN2, DN3 while no change was observed in the frequency of DN4 after *Zeb1* KO (Figure 5.5B). By quantifying the absolute number of these populations, we found a significant decrease in DN1, DN2, DN3, and DN4 absolute numbers after *Zeb1* deletion (Figure 5.5C). In cell-autonomous experiments (Figure 5.4A), we found a significant reduction in donor contribution to DN1, DN3 and DN4 and a trend towards reduction in DN2 after *Zeb1* deletion (Figure 5.5D). Thus, *Zeb1* is required for cell-autonomous regulation of the earliest stages of thymocyte commitment to the T cell lineage in adults.

A



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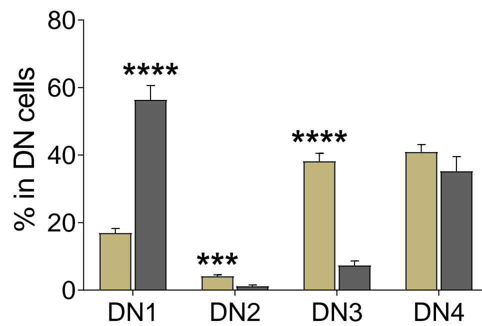

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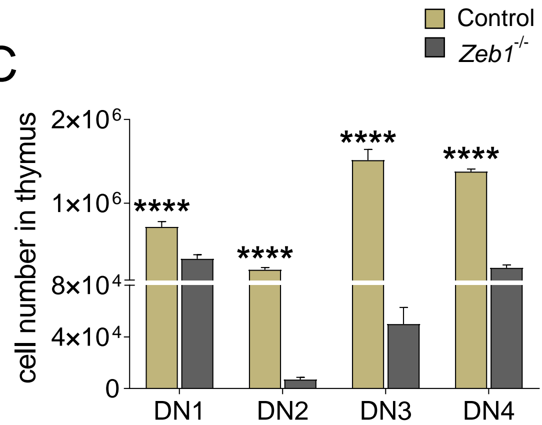

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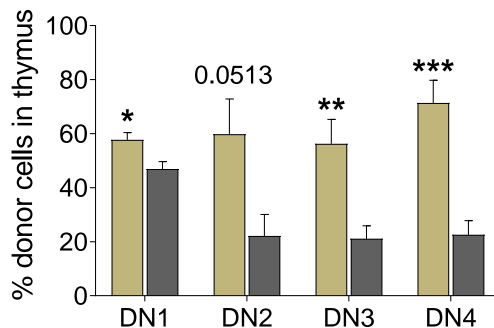

Figure 5.5. Acute deletion of *Zeb1* resulted in differentiation block of T cells in thymus. (A) Representative FACS plots showing the gating strategy of early stages within CD4 CD8 DN population using CD25 and CD44 (DN1: $CD44^+$ $CD25^-$, DN2: $CD44^+$ $CD25^+$, DN3: $CD44^-$ $CD25^+$, DN4: $CD44^-$ $CD25^-$) between control and *Zeb1*$^{-/-}$ at D14 after the last Poly I:C dose. (B) Frequency of DN populations in DN cells from control (n=12) and *Zeb1*$^{-/-}$ (n=12) mice from 5 independent experiments at D14 after the last Poly I:C dose. (C) Total cell count of DN populations in thymus from control (n=12-13) and *Zeb1*$^{-/-}$ (n=12-13) mice from 5 independent experiments at D14 after the last Poly I:C dose. (D) Donor contribution to DN populations (DN1, DN2, DN3, DN4) in thymus from control (n= 7-8) and *Zeb1*$^{-/-}$ (n=10-12) mice from 2 independent experiments via cell autonomous manner. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

5.3.3 Acute loss of *Zeb1* does not impact cell-autonomous BM seeding to the thymus

Next, due to the observed impact on DN1 population after *Zeb1* loss, we examined the effect of *Zeb1* deletion on the subpopulations within DN1 including ETPs, the earliest population entering the thymus from the BM. DN1 population is a heterogenous population and can be subdivided using CD24 and c-Kit markers to 5 different subpopulations according to their proliferation capacity and lineage choice: 1a (c-Kit$^+$ CD24$^-$), 1b (c-Kit$^+$ CD24^{low}), 1c (c-Kit$^+$ CD24$^+$), 1d (c-Kit$^-$ CD24$^+$), and 1e (c-Kit$^-$ CD24$^-$) (Porritt et al., 2004). ETPs express high levels of c-Kit, so 1a and 1b together are ETPs. Fourteen days after the last dose of Poly I:C, we found no significant difference in the frequency of DN1c, d, and e populations (Figure 5.6A and B). We then analysed total cell count and found a significant reduction in 1d and 1e and no change in 1c after *Zeb1* deletion (Figure 5.6C). Further, we analysed ETPs (CD4$^-$ CD8$^-$ CD44$^+$ CD25$^-$ c-Kit^{high}) (Matsuzaki et al., 1993, Ceredig and Rolink, 2002, Allman et al., 2003, Bhandoola et al., 2003). We found a comparable frequency between control and *Zeb1*$^{-/-}$ (Figure 5.6D and E). However, due to a reduction in the cellularity of the thymus, the absolute count of ETPs in *Zeb1*$^{-/-}$ thymus showed a near significant reduction compared to control (Figure 5.6F). Next, in cell-autonomous experiments (Figure 5.4A), we found a reduced donor contribution to 1e population after *Zeb1* deletion, while there was no change in donor contribution to the ETPs, 1c, and 1d populations (Figure 5.6G). Thus, these data suggest that BM seeding to the thymus remains unperturbed in the context of *Zeb1* deficiency and the observed *Zeb1* mediated defect is intrinsic to the differentiation process during T cell maturation in adults.

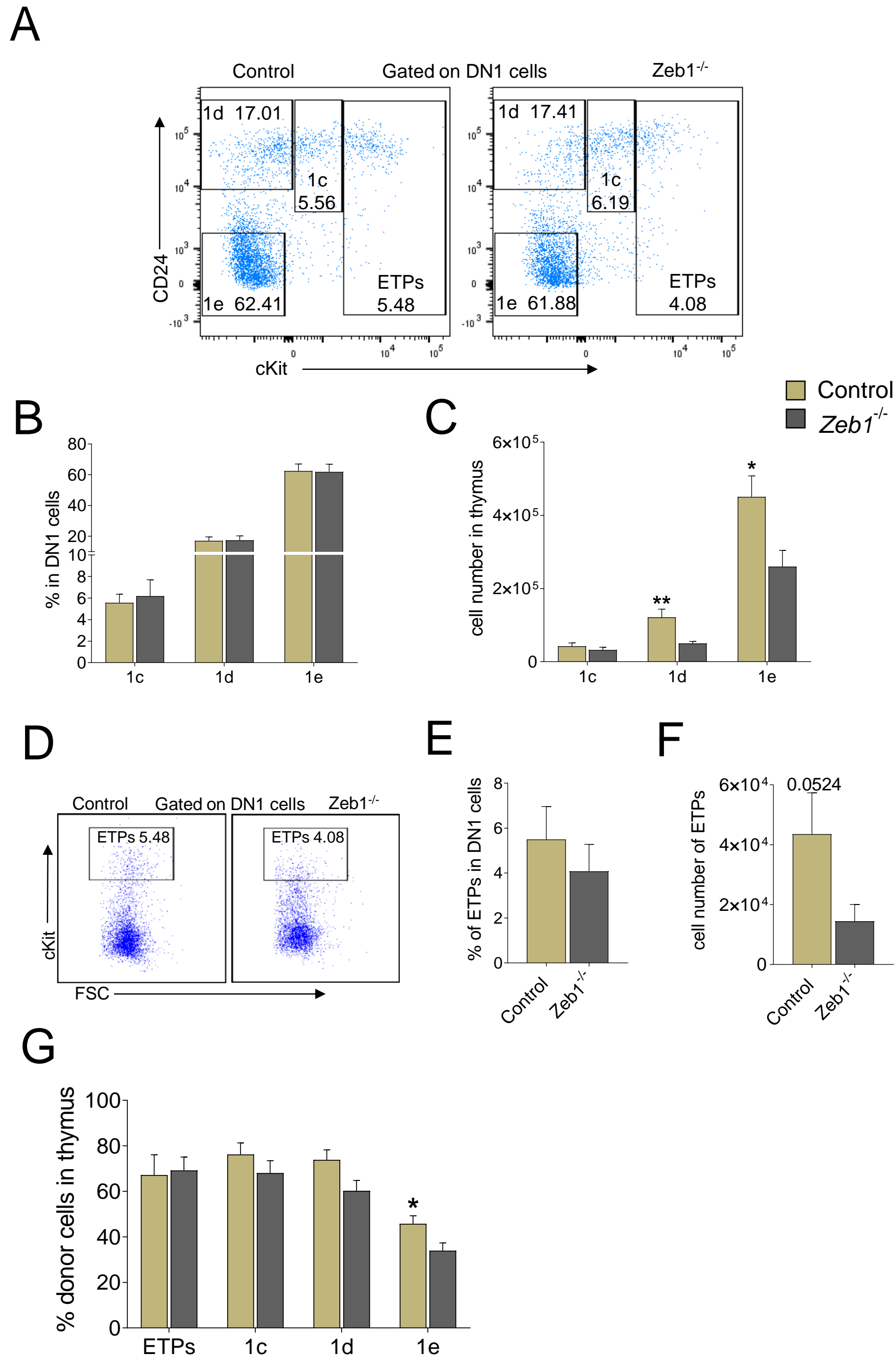

Figure 5.6. Acute loss of *Zeb1* did not impact BM seeding to the thymus. (A) Representative FACS plots showing the gating strategy of DN1 populations at D14 after the last Poly I:C dose. (B) Frequency of DN1 populations in DN1 from control (n=9) and *Zeb1*$^{-/-}$ (n=9) mice and (C) total cell count of the cells in thymus from control (n=9) and *Zeb1*$^{-/-}$ (n=9) mice from 3 independent experiments at D14 after the last Poly I:C dose. (D) Representative FACS plots showing the gating strategy of ETPs (DN1 c-Kit$^{high)}$ at D14 after the last Poly I:C dose. (E) Frequency of ETPs in DN1 cells from control (n=10) and *Zeb1*$^{-/-}$ (n=11) mice and (F) total cell count of ETPs in thymus from control (n=10) and *Zeb1*$^{-/-}$ (n=10) mice from 4 independent experiments at D14 after the last Poly I:C dose. (G) Donor contribution to DN1 populations and ETPs in thymus from control (n= 8) and *Zeb1*$^{-/-}$ (n=12) mice from 2 independent experiments via cell autonomous manner. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

5.3.4 Acute loss of *Zeb1* resulted in increased apoptosis in thymic T cells

We further asked if the differentiation defect in thymus is associated with changes in cell survival after *Zeb1* KO. We analysed Annexin V in T cell subsets in thymus. Fourteen days after the last dose of Poly I:C, Annexin V frequency was significantly high in *Zeb1* deficient DP, $CD4^+$, and $CD8^+$ cells whereas the level in DN population was comparable to the control (Figure 5.7A and B). However, further analysis of apoptosis in DN subsets revealed increased apoptotic levels in DN2 and DN3 after *Zeb1* deletion whereas DN1 and DN4 were comparable to control (Figure 5.7C and D). This data indicates that *Zeb1* is essential for cell survival during T cell development in adults.

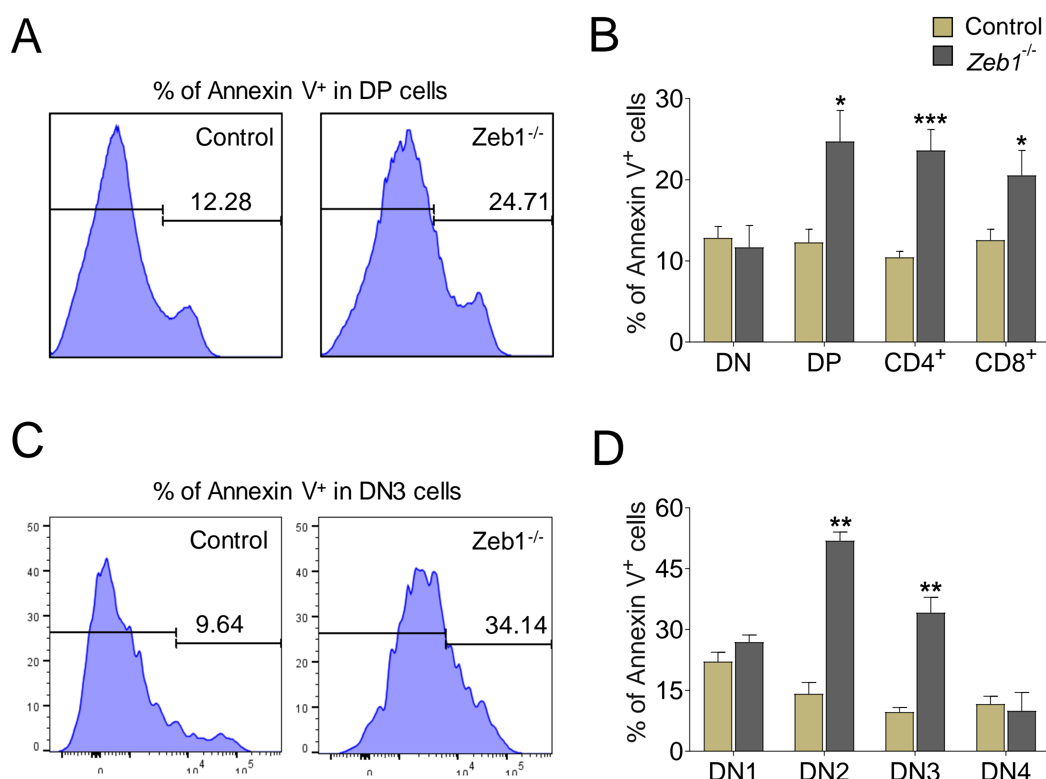

Figure 5.7. Acute loss of *Zeb1* resulted in increased apoptosis in thymus cells. (A) Representative FACS plots showing histograms of Annexin V analysis in DP cells at D14 after the last Poly I:C dose. (B) Percentage of Annexin V^+ in DN, DP, $CD4^+$, and $CD8^+$ from control (n=8) and *Zeb1*$^{-/-}$ (n=8) mice from 4 independent experiments at D14 after the last Poly I:C dose. (C) Representative FACS plots showing histograms of Annexin V analysis in DN populations (DN1, DN2, DN3, and DN4) at D14 after the last Poly I:C dose. (D) Percentage of Annexin V^+ in DN1, DN2, DN3, and DN4 from control (n=7) and *Zeb1*$^{-/-}$ (n=6) mice from 3 independent experiments at D14 after the last Poly I:C dose. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

5.3.5 Acute loss of *Zeb1* resulted in changes in cell cycle in thymus T cells

Next, we asked if the observed defect in DN populations was associated with changes in cell cycle. Using Ki-67 and DAPI we assessed G0, G1, and S-G2-M in all thymocytes (Figure 5.8A). Fourteen days after *Zeb1* KO, we found no change in cell cycle profile of DN1 cells (Figure 5.8B). Cells in DN2 stage showed a trend towards reduction in G0 after *Zeb1* deletion but no change was observed in G1 and S-G2-M (Figure 5.8B). However, *Zeb1*-deficient DN3 cells showed a strong reduction in G0 and increase in G1 and no changes in S-G2-M (Figure 5.8A and B). Cells in DN4 stage showed a near significant increase in G0, no change in G1, and a significant reduction in S-G2-M after *Zeb1* KO (Figure 5.8B). Next, we analysed cell cycle phases in DP, $CD4^+$, and $CD8^+$ (Figure 5.8C). DP cells lacking *Zeb1* showed comparable proportions in G0 and G1 to the control, however they significantly decreased in S-G2-M (Figure 5.8D). Mature $CD4^+$ T cells showed a trend towards reduction in G0 and a significant increase in G1 while there was no change in S-G2-M after *Zeb1* deletion (Figure 5.8D). Mature $CD8^+$ T cells after *Zeb1* deletion showed an increased proportion in G0 and a significant reduction in G1 and S-G2-M (Figure 5.8C and D). Therefore, *Zeb1* acts as a critical regulator of T cells in adults by modulating cell cycle kinetics during their maturation.

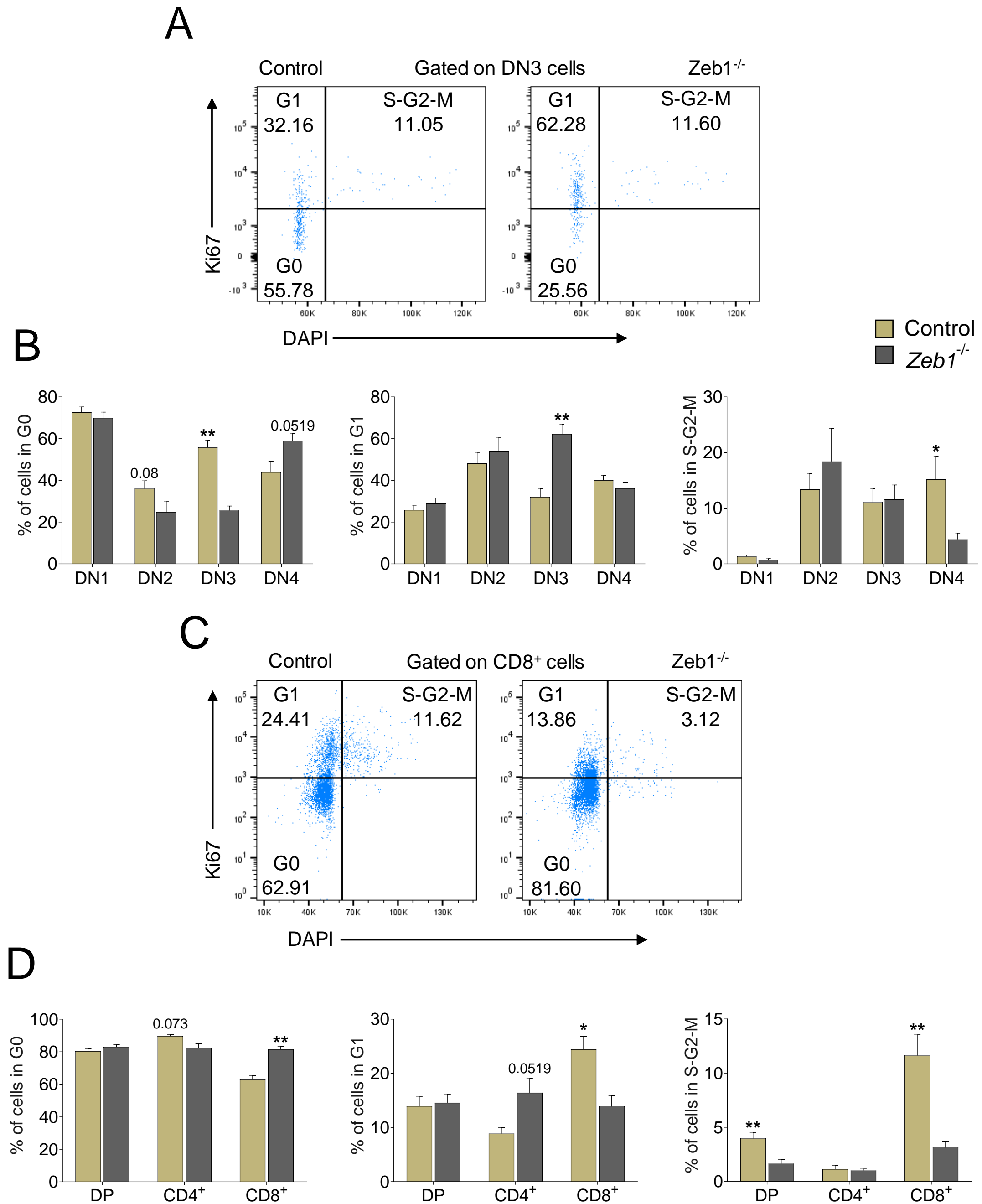

Figure 5.8. Acute loss of *Zeb1* resulted in changes in cell cycle in thymic T cells. (A) Representative FACS plots showing analysis of G0, G1, and S-G2-M in DN3 cells at D14 after the last Poly I:C dose. (B) Frequency of DN1, DN2, DN3, and DN4 in G0, G1, and S-G2-M from control (n=6) and *Zeb1*$^{-/-}$ (n=5) mice from 2 independent experiments at D14 after the last Poly I:C dose. (C) Representative FACS plots showing analysis of G0, G1, and S-G2-M in CD8^{+} cells at D14 after the last Poly I:C dose. (D) Frequency of DP, CD4^{+}, and CD8^{+} in G0, G1, and S-G2-M from control (n=6) and *Zeb1*$^{-/-}$ (n=5) mice from 2 independent experiments at D14 after the last Poly I:C dose. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

5.3.6 Acute loss of *Zeb1* resulted in a reduction of $CD8^+$ memory T cells

While we observed a striking loss of T cells in the thymus of *Zeb1* KO mice, the frequency and number of mature T cells in BM, PB, and spleen was unchanged (Chapter 4), perhaps due to the observed inefficient excision of *Zeb1* alleles using the Mx1-Cre system (Chapter 4). Nonetheless, we also assessed T cell activation markers CD44 and CD62L in combination with T cells markers CD3, CD4, and CD8 to study naïve ($CD62L^+$ $CD44^{-/med}$), effector memory (EM) ($CD44^{high}$ $CD62L^-$) and central memory (CM) ($CD44^+$ $CD62L^+$) respectively at D14 after *Zeb1* deletion (Figure 5.9A). We found a reduction of $CD8^+$ EM T cells in PB and BM as well as in splenic $CD8^+$ CM T cells after *Zeb1* KO while no change was noted in splenic $CD8^+$ EM T cells and CD8+ naïve in any lymphoid tissue (Figure 5.9C). Proportions of CD4+ naïve, EM, or CM in PB, BM, and spleen were unperturbed in the context of *Zeb1* KO (Figure 5.9B). This reduction in CD8+ EM T cells in PB was confirmed in cell autonomous based experiments (Figure 5.9D). This data reveals an important role of *Zeb1* in memory $CD8^+$ T cell homeostasis.

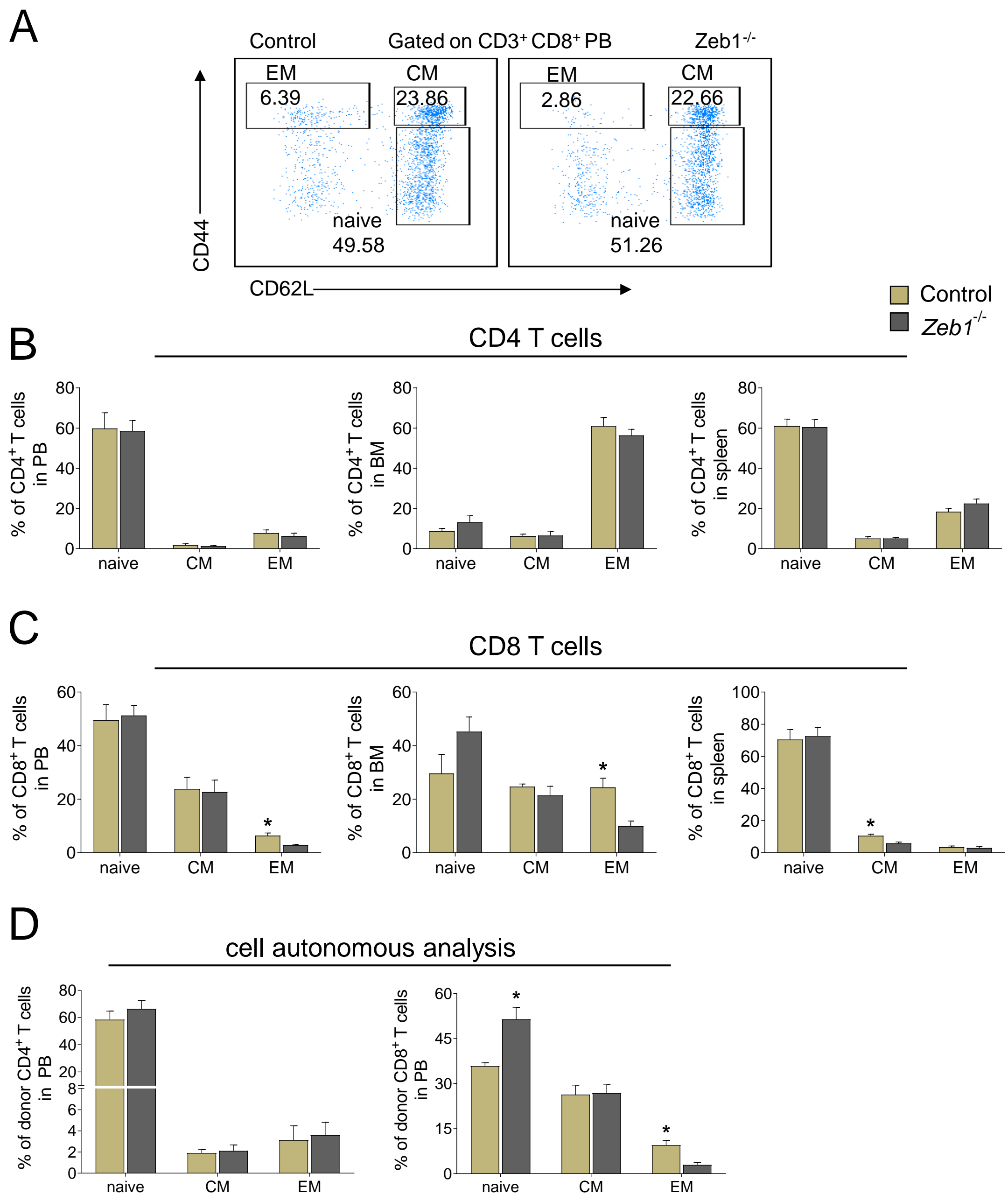

Figure 5.9. Acute loss of *Zeb1* resulted in a reduction of CD8⁺ memory T cells. (A) Representative FACS plots showing gating strategy of analysing CD44 and CD62L in CD8 T cells in PB at D14 after the last Poly I:C dose. (B) Analysis of naïve, CM, and EM in CD4 T cells in PB, BM, and spleen from control (n=5 for PB and BM, 6 for spleen) and *Zeb1*$^{-/-}$ (n= 5 for PB and BM, 7 for spleen) mice from 3 independent experiments at D14 after the last Poly I:C dose. (C) Analysis of naïve, CM, and EM in CD8 T cells in PB, BM, and spleen from control (n=5 for PB and BM, 6 for spleen) and *Zeb1*$^{-/-}$ (n= 5 for PB and BM, 7 for spleen) mice from 3 independent experiments at D14 after the last Poly I:C dose (D) Percentage of naïve, CM, and EM in CD3 CD4 or CD8 donor cells from control (n=5) and *Zeb1*$^{-/-}$ (n=5) mice at D14 after the last Poly I:C dose via cell autonomous manner. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01, ***P < .001, ****P <0.0001.

5.4 Discussion

Earlier reports have shown that *Zeb1* is essential for T cell development after embryonic deletion of *Zeb1* (Higashi et al., 1997, Postigo and Dean, 1999a, Takagi et al., 1998). A recent report found that *Zeb1* is required for the regulation of $CD8^+$ memory T cells (Guan et al., 2018). Here, we employed a conditional mouse model of acute *Zeb1* loss that differs from that of previously published reports where we conditionally deleted *Zeb1* in adult haematopoietic tissues. In this chapter, we provide evidence and define that *Zeb1* expression in the adult haematopoietic system is required for (i) thymocyte maturation, (ii) T cell differentiation and homeostasis and (iii) T cell survival and cell cycle regulation.

14 days after *Zeb1* deletion, we found a strong reduction in total cellularity of thymus associated with a dramatic decrease in thymus mass. Our data revealed a T cell defect in thymus at early and late developmental stages represented by an increased frequency of DN1 population and a reduction in DN2 and DN3 populations which marks the onset of T cell lineage commitment suggestive of an early partial block in T cell differentiation between DN1-DN2/DN3. This stage of development was reported to be mainly dependent on Notch and pre-TCR signalling (Hosokawa and Rothenberg, 2018) suggestive of *Zeb1*-mediated defect in these signalling pathways. We also found at the same time a reduced proportion of DP cells and an increase in SP $CD4^+$ and $CD8^+$ cells. As our system simultaneously deleted *Zeb1* in all thymus populations, this late defect might be independent of the early block observed in DN populations or possibly combined with an intrinsic role of *Zeb1* in DP and mature $CD4^+$ and $CD8^+$ cells and these possibilities will be tested in future experiments

Our data present *Zeb1* as a novel regulator of T cell development and maturation in adult and *Zeb1* joins a few TFs and signalling molecules that are essential for T cell development including *Notch1*, *Gata3, E2a, Tcf1* and *Bcl11b* (Hosokawa and Rothenberg, 2018). Among these, *Zeb1* was reported to bind and repress *GATA3* and *IL-2* (Gregoire and Romeo, 1999, Wang et al., 2009). *Gata3, Tcf1, and E2a* loss resulted in a reduced number of ETPs and DN2 which impacted subsequent T cell differentiation (Hosoya et al., 2009, Germar et al., 2011, Xu et al., 2013), while deletion of *IL-2* did not impact thymus development (Schorle et al., 1991). Defective DN2 from *$E2A^{-/-}$* mice showed overexpression of *Gata3* and knockdown of *Gata3* rescued T cell phenotype (Xu et al., 2013). Notch signalling enhances the specification to T cell lineage via regulation of *Gata3* and *Tcf1* (Hosokawa and Rothenberg, 2018). *Notch1* loss autonomously resulted in a block at DN1-DN2

(Radtke et al., 1999). Further, *Bcl11b*$^{-/-}$ mice showed accumulation of DN3 and loss of TCRβ which failed to develop to DN4 and DP cells (Inoue et al., 2006). Other TFs regulating T cell development includes *Gfi1* (Yucel et al., 2003), *Runx1* and *Runx3* (Egawa et al., 2007). The broader impact of the *Zeb1* phenotype in thymus suggests a complex interaction between *Zeb1* and these T cell regulators, making *Zeb1* one of the essential regulators of T cell development in thymus.

Mechanistically, *Zeb1* mediated control of T cell differentiation was associated with alterations in cell cycle and apoptosis. These changes commenced early from DN2 stage, which represent the early events towards T cell lineage commitment (Wu et al., 1996), through maturation to SP CD4$^+$ and CD8$^+$ T cells. Cell cycle and apoptosis analysis showed differential requirement of *Zeb1* in different thymus populations. This was evident in DN3 cells after *Zeb1* loss that significantly entered the cell cycle as shown by a reduction in G0 and increase in G1, while DN4 cells showed a decrease in S-G2-M phase and no change in G0 or G1. The increase in the frequency of DN3 cells in G1 phase might be an expected response to compensate for their loss by increased apoptosis. The reduction in cell division during S-G2-M phase of DN4 cells possibly impacted the transition from DN4-DP, hence resulting in a reduction of DP cells that was associated with increased apoptosis and a reduction in S-G2-M phase. Survival of DN thymocytes is regulated by IL-7 signalling (IL7 and its receptor CD127) and BCL-2 (Peschon et al., 1994, Kondo et al., 1997a). Overexpression of the antiapoptotic gene *Bcl-2* reversed T cell defects that was observed in CD127-deficient mice which showed a block at DN2 (Kondo et al., 1997a, Peschon et al., 1994). Interestingly, we found that lymphoid progenitors expressing CD127 in *Zeb1*$^{-/-}$ BM such as LMPP CD127$^+$ and CLP CD127$^+$ CD135$^+$ were reduced and these populations are known to seed the thymus (Donskoy and Goldschneider, 1992) which indicates that the attenuation of the expression of CD127 after *Zeb1* deletion maybe responsible for cell death in DN2 and DN3 thymocytes. In support of this postulate, BCL-2levels were found reduced in defective *Zeb1*$^{-/-}$ memory CD8$^+$ T cells (Guan et al., 2018). Also, in DN3 cells successful rearrangement of TCR β and pre-α TCR are critical for cell survival as well as *Bcl2-A1, Akt, and Cxcr4* (Hernandez et al., 2010). Overall, this line of evidence suggests that *Zeb1* modulates thymocyte survival via regulation of CD127 and BCL-2.

Cell cycle analysis also revealed that *Zeb1* differentially regulates mature CD4$^+$ and CD8$^+$ T cells in the thymus. CD4$^+$ T cells showed a near significant reduction in G0 and increased proportions of cells in G1 phase and no change in S-G2-M, while CD8$^+$ T cells showed an increased proportion of G0 cells and reduced proportions in G1

and S-G2-M and both $CD4^+$ and $CD8^+$ T cells in thymus showed increased cell death. Clearly, we found a selective and strong effect of *Zeb1* loss on the proliferation of $CD8^+$ T cells suggestive of a critical requirement of *Zeb1* in their function and survival. *Zeb1* binds and represses CD4 (Brabletz et al., 1999). Thus, loss of *Zeb1* may lead to increased expression of CD4 but whether this increase in CD4 was intrinsically responsible for $CD4^+$ T cell death and changes in cell cycle is unknown. This may explain the mild changes we observed on proliferation of $CD4^+$ T cells, which predicts a strong involvement of *Zeb1* in $CD8^+$ T cell function more than $CD4^+$ T cells. At this stage mature $CD4^+$ and $CD8^+$ T cells normally proliferate before exiting the thymus to increase the T cell pool and live for several weeks or months and are maintained by interaction with major histocompatibility complex (MHC) ligands (Sprent and Surh, 2011, Le Campion et al., 2002). Thus, the reduction in mature $CD4^+$ and $CD8^+$ T cells in thymus after *Zeb1* deletion and the changes in their cell cycle profile predict changes in their homeostasis in periphery. Although the frequency and number of mature T cells in BM, PB, and spleen were not changed during steady state (chapter 4) because of inefficient Cre-mediated *Zeb1* deletion in T cells, our cell autonomous data, where full *Zeb1* deletion was achieved, showed a significant reduction in donor contribution to $CD4^+$ and $CD8^+$ T cells in PB, BM, and spleen. We also analysed T cells in naïve, EM and CM states at 14 days after *Zeb1* deletion. Our data showed a reduced frequency of EM $CD8^+$ T cells in PB and BM as well as CM $CD8^+$ T cells in spleen and no change in $CD4^+$ T cell state. We also confirmed this observation via cell autonomous experiments in PB, where we found a reduced EM $CD8^+$ and increased naïve $CD8^+$. This preferential regulation of *Zeb1* of mature memory $CD8^+$ T cells but not $CD4^+$ T cells in normal setting (without immunological challenge) suggests that *Zeb1* is required for regulation of T cell subsets at different developmental stages. This notion is supported by our cell cycle data of $CD4^+$ and $CD8^+$ in thymus which showed differential kinetics after *Zeb1* deletion. Furthermore, it is consistent with a recent report that found *Zeb1* as an essential regulator for the function and survival of memory $CD8^+$ T cells that was positively regulated by TGF-β (Guan et al., 2018). The difference was that Guan et al challenged $CD8^+$ T cells with LCMV infection while our data was carried out under normal physiologic conditions. Thus, our data together with Guan et al, demonstrate the importance of *Zeb1* in the maintenance of memory $CD8^+$ T cells during normal and activated conditions and predicts a critical role of *Zeb1* in mediating the immune response of $CD8^+$ T cells.

In terms of BM seeding to the thymus, our analysis of ETPs revealed a comparable level of ETPs within DN1 population while the total cell count was slightly decreased

(p= 0.0524) after *Zeb1* deletion. This reduction maybe ascribed to the strong reduction in the thymus cellularity. However, our cell autonomous data showed that donor contribution to ETPs was comparable to control when analysed 14 days after *Zeb1* deletion. The reason for the variation between steady state and cell autonomous data is unknown but suggests that *Zeb1* might be deleted in thymus epithelial tissues, hence altered some cell populations. Given that we observed a reduction in early lymphoid progenitors that seed the thymus which might be responsible for the near significant reduction in ETPs in the thymus, analysing ETPs beyond 14 days after *Zeb1* ablation via cell autonomous manner may reveal a reduction. However, the quick profound thymus defect observed at 14 days after *Zeb1* deletion confirms that *Zeb1* autonomously regulate T cell development independent of BM seeding to the thymus.

In closing, *Zeb1* KO mice presented with small thymi associated with hypocellularity. We also found a dramatic reduction in all T cell populations in thymus from DN1 until final maturation stages $CD4^+$ and $CD8^+$ T cells in association with increased cell cycle and increased apoptosis (Figure 5.10). These alterations in T cells are intrinsic to T cells and not a failure of BM to seed the thymus. Furthermore, *Zeb1* regulates memory $CD8^+$ T cells. Thus, we report for the first time that *Zeb1* is indispensable regulator of adult T cell development.

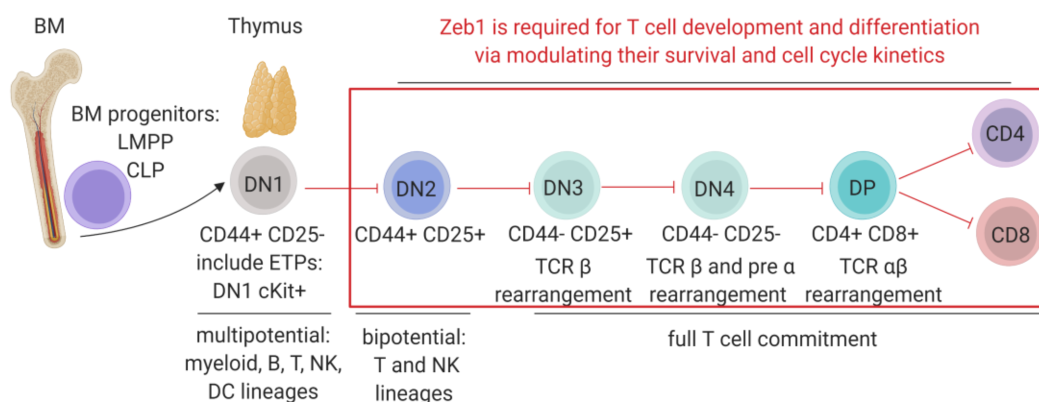

Figure 5.10. Summary of the role of *Zeb1* in T cell development in thymus. At steady state, ETPs lacking *Zeb1* showed a comparable proportion to the control, however, there was a dramatic reduction in DN2 and DN3 which impacted the subsequent T cell differentiation. This was associated with defect in survival and cell cycle status.

CHAPTER 6 : General Discussion

Zeb1 is widely expressed in different tissues including nervous system, lung, kidney, skeletal system, muscles, thymus, BM, spleen, and others (Postigo and Dean, 2000, Hurt et al., 2008, Higashi et al., 1997, Sekido et al., 1994). This pattern of expression of *Zeb1* reflects its critical role in various tissues. Although the oncogenic role of *Zeb1* in driving cancer stem cell metastasis, invasion, and chemoresistance has been gaining much interest in the last few years (Preca et al., 2015, Zhang et al., 2013a, Zhou et al., 2012, Kahlert et al., 2015, Siebzehnrubl et al., 2013, Zhang et al., 2018a, Caramel et al., 2018), knowledge about the specific role for *Zeb1* in normal adult stem cell biology has been lacking.

In the haematopoietic system, analysis of *Zeb1* expression in purified populations of HSPCs and their progeny revealed high *Zeb1* levels in HSPCs (HSC, MPP, HPC1, HPC2) that declines during differentiation to lineage-restricted progenitors (CMP, GMP, MEP, CLP), followed by up-regulated *Zeb1* expression during the terminal maturation of the cells (myeloid, erythroid, B, and T lymphoid cells). This pattern of expression suggests a pivotal role for *Zeb1* in haematopoiesis, including the HSPC compartment. Thus, to gain insight into the specific role of *Zeb1* in normal stem cells, this thesis focused on the characterisation of *Zeb1* in the adult murine haematopoietic system. We have utilised Mx1-Cre system (Kuhn et al., 1995) to conditionally inactivate *Zeb1* in the adult haematopoietic system using *Zeb1*$^{fl/fl}$ mice (Brabletz et al., 2017). Our data showed that *Zeb1* is required for the cell-autonomous long-term maintenance of haematopoiesis as evidenced by perturbations in stem and progenitor compartments and more differentiated, mature cell types. Additionally, acute deletion of *Zeb1* showed that *Zeb1* is an essential regulator of BM lymphoid progenitors and is required for multi-lineage haematopoietic differentiation as evidenced by differentiation defects observed after transplantation. Furthermore, *Zeb1* acts as a critical regulator for adult T cell development and maintenance as evidenced by a dramatic decrease in thymus mass and cellularity and a differentiation defect in T cells at multiple developmental stages in the thymus and periphery.

6.1 *Zeb1* as a transcriptional repressor involved in stem cell differentiation

Zeb1 mediated repression of gene expression to regulate differentiation has been reported (Siles et al., 2013, Jiang et al., 2018). *Zeb1* is shown to bind and repress tissue specific genes to regulate stem cell differentiation in neurons and muscles (Jiang et al., 2018, Wang et al., 2019, Singh et al., 2016, Siles et al., 2013, Siles et al., 2019). Consistent with transcriptional repression executed by *Zeb1*, our data revealed a pivotal role for *Zeb1* in HSC differentiation exemplified by upregulation of 79% of genes in *Zeb1*-deficient HSCs. Few transcriptional repressors have been shown to regulate HSC function including *Gfi1, Gfi1b, Bmi1*, and *Tel/Etv6* (Teitell and Mikkola, 2006). Interestingly, *Gfi1b* appeared to be downregulated in *Zeb1*$^{-/-}$ HSCs, suggesting a positive regulation by *Zeb1* (Figure 4.10). *Gfi1b*$^{-/-}$ mice showed an accumulation of HSCs in BM, spleen, and PB without HSC self-renewal perturbation (Khandanpour et al., 2010). This suggests that *Gfi1b* downregulation in *Zeb1* KO HSCs, possibly results in the expansion of *Zeb1*$^{-/-}$ HSCs in the BM. However, *Zeb1*$^{-/-}$ HSCs showed a defect in their differentiation contrasting with *Gfi1b*$^{-/-}$ HSCs that showed comparable reconstitution capacity to their normal counterparts after transplantation (Khandanpour et al., 2010), suggesting another molecular mechanism apart from *Gfi1b* is responsible for the differentiation defect seen in *Zeb1*$^{-/-}$ HSC. *Gfi1* also appeared to be downregulated in *Zeb1*$^{-/-}$ HSCs (P value: < 0.002; FDR value: <0.11). *Gfi1*$^{-/-}$ HSCs showed multi-lineage differentiation defects after transplantation (Zeng et al., 2004, Hock et al., 2004a). Thus, we report that *Zeb1* as a novel transcriptional repressor that regulates HSC self-renewal and global differentiation through a transcriptional repressor network that likely includes both Gfi1 and Gfi1b (Khandanpour et al., 2010, Zeng et al., 2004).

6.2 *Zeb1* modulates cell adhesion and cytoskeleton in HSCs

Acute deletion of *Zeb1* unveiled a dysregulated gene signature of cell adhesion, polarity and cytoskeleton. As mentioned above, *Zeb1* is shown to regulate stem cell differentiation in neurons and muscles (Jiang et al., 2018, Wang et al., 2019, Singh et al., 2016, Siles et al., 2013, Siles et al., 2019). Interestingly, one of the mechanisms that *Zeb1* utilises to regulate the neuron differentiation is through control of polarity and cell adhesion genes, such as *Pard6b* and *Cxadr* (Wang et al., 2019). This is consistent with our data as *Zeb1* loss resulted in HSC differentiation defects

associated with dysregulation of polarity and cell adhesion genes including *Pard6b* and *Cxadr*.

Zeb1 is known as an EMT inducer, particularly, in cancer where CSC acquire EMT to promote cancer progression and CSC migration and invasion via repression of E-cadherin, EpCAM and other epithelial molecules (Aigner et al., 2007, Eger et al., 2005). *Zeb1*-deficient HSCs with upregulation of epithelial proteins showed expansion in their number, differentiation defect, and resistance to apoptosis. We found an approximately 6-fold increase in EpCAM protein in HSCs lacking *Zeb1* compared to control. EpCAM is expressed in undifferentiated ESC and in CSCs and its down regulation is seen during differentiation (Gonzalez et al., 2009). It regulates various cellular processes including proliferation, differentiation, cell cycle, and adhesion and, in epithelial cancer, can work as either an oncogene or suppressor (Schnell et al., 2013). This is consistent with our data as *Zeb1* KO HSCs showed upregulation of EpCAM associated with expansion of HSC number that showed cell survival advantage compared to control, and a defect in differentiation. This data is consistent with the role of EpCAM in modulating cell survival in which upregulation of EpCAM in cancer cells conferred survival and silencing EpCAM induced apoptosis in cancer cells (Gao et al., 2014). Similarly, preliminary data from our laboratory (Neil Rodrigues laboratory) showed that *Zeb1* KO *EpCAM*$^{-}$ HSCs were more apoptotic compared to control and *Zeb1* KO *EpCAM*$^{+}$ HSCs. Together, these data indicate that the *Zeb1*-Epcam axis is essential for HSC survival and differentiation. Future work should be focused on examining the requirement for EpCAM in *Zeb1* KO HSPCs and transplanting them after knockdown of EpCAM to test their functionality. Considering the role of EpCAM in regulating cell differentiation and its high expression in maintaining stemness (Gonzalez et al., 2009), possibly, lowering EpCAM levels in *Zeb1*$^{-/-}$ HSCs will force them to normally differentiate as control HSCs.

6.3 *Zeb1* regulates HSC function in BM niche via EMT/MET?

Regulation of HSCs in the BM niche has gained much interest in the last few years (Crane et al., 2017). HSCs interact with various cells in the niche via cell-cell (receptors and ligands), cell-extracellular matrix (integrins) routes and by factors released by niche cells such as CXCL12, thrombopoietin, and SCF (Crane et al., 2017). Such an interaction between HSCs and niche cells suggests an involvement of an intermediate EMT/MET process (Hamidi and Sheng, 2018).

Downregulation of mesenchymal genes and upregulation of epithelial genes are a reminiscent of MET process (Samavarchi-Tehrani et al., 2010). During MET, the cells transition from mesenchymal to epithelial state, they might develop full epithelial or a hybrid (partial/intermediate) state expressing both mesenchymal and epithelial genes and may acquire functional properties of both processes (Jolly et al., 2016) . This is seen in circulating tumour cells (CTCs) where they migrate in clusters keeping cell adhesion property as a feature of MET and migration property as a feature of EMT (Jolly et al., 2016).

Zeb1 is known to repress epithelial genes to induce mesenchymal state which results in motility and migration of cells (Aigner et al., 2007). Such properties are seen in HSC as they are non-adherent cells and individually move as seen in their homing and migration to the BM after transplantation or mobilisation and egression from the BM due to physiological or pathological stress (Gazitt, 2004, Suarez-Alvarez et al., 2012). The acquisition of *Zeb1* KO HSC to an epithelial signature suggests that *Zeb1*-deficient HSCs may have undergone MET where *Zeb1*, a mesenchymal marker, was abolished and epithelial proteins such as EpCAM, E-cadherin, and others were increased.

Although these *Zeb1* KO HSCs efficiently home to the BM after tail vein injections, we speculate that this epithelial signature in *Zeb1* KO HSCs is critical for their interaction within the BM niche but not outside of the BM. Thus, once *Zeb1* KO HSCs safely home to the BM niche after transplantation or during steady state where they normally reside in the niche, they undergo abnormal interaction with components of the BM niche. Therefore, we postulate that the upregulation of polarity and cell adhesion genes after *Zeb1* KO confers on HSCs a cell-autonomous epithelial-like feature, which facilitates interaction with components of the BM niche such as osteoblasts, MSCs, endothelial cells, CAR cells, and others. This eventually restrains the motility of HSCs outside of the BM leading to a reduction in HSCs in extramedullary sites of haematopoiesis, such as the spleen, and impacts the balance between self-renewal and differentiation fates as observed in HSC transplantation in the setting of both acute and chronic loss of *Zeb1*. To directly test this hypothesis, deep *in vivo* imaging should be performed to visualise *Zeb1* KO HSCs in the BM niche and to define their localisation and interaction within various niche locations.

6.4 *Zeb1* as a regulator of stem cell polarity and HSC division

The expanded number of HSCs and the differentiation defect after *Zeb1* KO possibly is caused by increased symmetric divisions at the expense of asymmetric divisions (Wu et al., 2007). Cell polarity genes play an important role in maintaining the balance between self-renewal and differentiation (symmetric vs asymmetric) (Wu et al., 2007, Florian et al., 2018). For example, *Crb3* (significantly upregulated in *Zeb1*$^{-/-}$ HSC), *Numb* (P value: <0.001; FDR: <0.07 in *Zeb1*$^{-/-}$ HSC), Cdc42 are polarity markers that their distribution is critical for cell division in HSCs (Wu et al., 2007, Florian et al., 2018, Florian et al., 2012). Increased expression of Crumbs3 and CDC42 resulted in an expansion of aged HSCs that favoured self-renewal symmetric divisions and defect in their repopulation capacity and these changes were reversed by pharmacological inhibition of CDC42 (Florian et al, 2012). This suggests that the upregulation of polarity markers in *Zeb1* KO HSCs is responsible for the differentiation defects which may result from increased symmetric divisions favouring HSC differentiation rather than self-renewal, hence, differentiation defects. *Zeb1* was found to regulate polarity and cytoskeleton genes that appeared significantly upregulated in our list such as *Pard6b* in neuron differentiation (Wang et al., 2019, Singh et al., 2016) and *Crb3* and *Inadl* in colorectal cancer (Aigner et al., 2007, Spaderna et al., 2008). Interestingly, *Zeb1* was also shown to regulate symmetrical division via Numb-MiR31 axis in lung carcinoma (Wang et al., 2018). Thus, these findings are strongly correlated with molecular and cellular phenotypes observed in other tissues and pathological settings and suggest deregulation in cell polarity/cytoskeleton as a critical cause of the defect in *Zeb1*-deficient HSCs. We have identified *Zeb1* target genes critical to cell polarity in HSCs. Further experiments should focus on loss of function experiments to decipher the function of these genes, including *Pard6b, Numb*, and *Crb3*. These experiments should show which of these genes are critical to rescue the *Zeb1* KO phenotype, as was reported when CDC42 was pharmacologically inhibited in aged HSCs and rejuvenated their functionality (Florian et al., 2012).

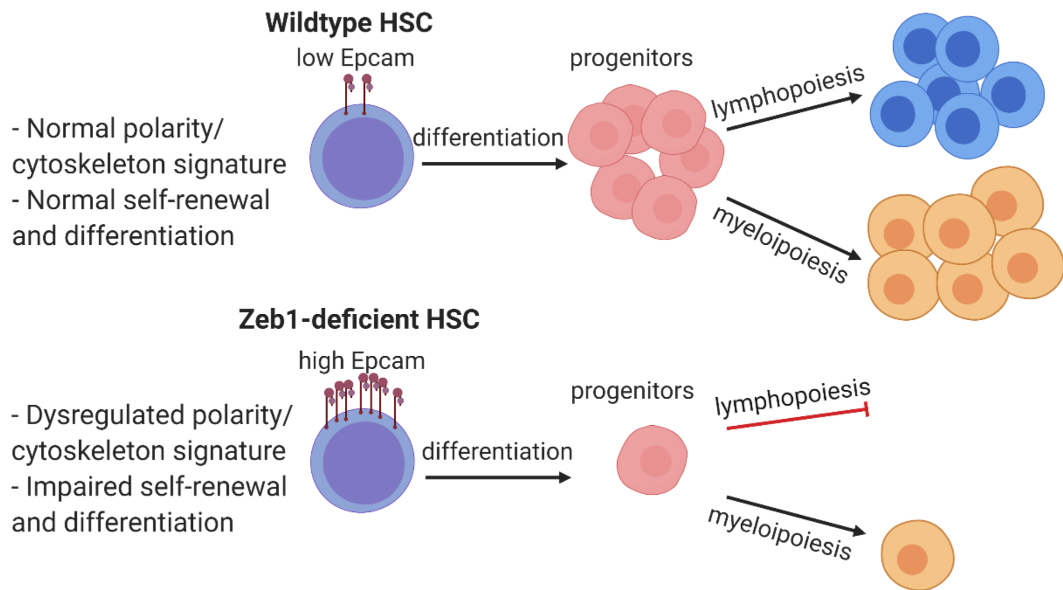

Figure 6.1. A summary of the role of *Zeb1* in HSC. Deletion of *Zeb1* in HSCs resulted in upregulation of EpCAM. Primary and secondary transplantation of *Zeb1*-deficient HSCs resulted in a profound defect in their self-renewal and differentiation capacity to downstream progenitors and mature cells in particular to lymphoid lineage (T and B cells).

6.5 *Zeb1* in cancer immunotherapy

Given the requirement of *Zeb1* in T cell maintenance, function, and survival that our data revealed together with other laboratories (Guan et al., 2018) and the role of T cells in fighting cancer, we posit that *Zeb1* is playing an essential role in modulating the immune response against tumour cells, specifically $CD8^{+}$ T mediated immunosurveillance. Cancer cells escape killing mediated by T cells via upregulating immune checkpoints that suppress the T cell activity such as PD-L1 and CTLA-4 (Zappasodi et al., 2018). PD-L1 binds PD-1 on T cells and CTLA-4 binds B7 and this inhibitory mechanism between cancer and T cells reduces the antitumor activity of T cells (Okazaki et al., 2013). Immune checkpoint blockades are blocking antibodies that are used in clinics to treat different cancers including melanoma, liver, gastric, and Hodgkin lymphoma (Zappasodi et al., 2018) and patients show a higher response when they receive combined Immune checkpoint blockades rather than a monotherapy (Zappasodi et al., 2018). CD47 is another molecule has been utilised in cancer immunotherapy (Matlung et al., 2017). Targeting CD47- SIRPα enhances innate and adaptive immunity against tumour cells via increasing macrophage-mediated phagocytosis of cancer cells and also modulating the balance between effector and regulatory T cells (Folkes et al., 2018, Kauder et al., 2018). Interestingly, cancer stem cells with high ZEB1 expression also show high levels of PD-L1 suggestive of ZEB1 positive regulation of the inhibitory molecule PD-L1 (Dong et al., 2018). Also, in breast cancer cells *ZEB1* is found to positively regulate CD47 and they both show high expression (Noman et al., 2018). Thus, combined immunotherapy against ZEB1, PD-L1-PD1, and/or CD47 might prove benefits and increase the response rates in several cancers.

6.6 *Zeb1* and haematological malignancies

The role of ZEB1 in haematological malignancies has been reported, with both tumour suppressor and oncogene dependent functions being suggested. ZEB1 was found to be a tumour suppressor in Sézary syndrome, an aggressive form of cutaneous T-cell lymphoma, and in adult T-cell leukaemia/lymphoma (ATLL) (Vermeer et al., 2008, Hidaka et al., 2008). In Sezary syndrome, loss of ZEB1 is found in 45% of patients (9 of 20) (Vermeer et al., 2008). Moreover, in ATLL, *Zeb1* mutant mice frequently demonstrate an invasive $CD4^{+}$ T-cell lymphoma as well *Zeb1* knockdown is associated with resistance to TGF-β1-mediated growth suppression which results

from upregulation of SMAD7, an inhibitory factor of TGF-β1 (Hidaka et al., 2008, Nakahata et al., 2010).

Conversely, ZEB1 has been documented as an oncogene in mantle cell lymphoma (MCL), a B-cell malignancy characterised by a poor prognosis. ZEB1 expression is detected in primary MCL cells, and its knockdown resulted in a reduction of MCL cells viability and proliferation *in vitro* as well as tumour growth suppression in mouse models (Sanchez-Tillo et al., 2014). Furthermore, *Zeb1* was found to control invasion and migration in AML. The researchers documented a high *Zeb1* expression in MLL-AF9 LT-HSCs while its expression in MLL-AF9 GMP-derived cells was lower. *Zeb1* knockdown reduced the invasiveness of LT-HSC-early-AML cells on MS-5 stroma and through Matrigel and reduced their growth in the methylcellulose (MC) with growth factors as well as it impaired the limited migration ability of GMP-derived AML cells. *In vivo*, deletion of *Zeb1* significantly compromised the infiltration of LT-HSC-early-AML cells in the bone marrow and other organs (Stavropoulou et al., 2016). However, the requirement for *Zeb1* in AML maintenance and propagation remains unclear and requires further experimentation.

In our experiments, mice lacking *Zeb1* for 8 months showed an expansion of HSCs and MPPs, differentiation defects, and resistance to apoptosis as well as splenomegaly, reminiscent of some pre-leukaemic syndromes (Koeffler and Leong, 2017). *Zeb2* has also been implicated in the development of myeloproliferative disorders and found to be highly expressed in different types of leukaemia and associated with poor prognosis (Goossens et al., 2017, Goossens et al., 2015, Li et al., 2016a). *Zeb2* KO mice at 16 months showed splenomegaly and increased extramedullary haematopoiesis which are signs of myeloproliferative disorders (Li et al., 2016b). In contrast to *Zeb2*, *Zeb1* KO mice at 8 months showed a slightly larger spleen associated with hypocellularity. Considering our observations: increased BM HSCs and MPPs, differentiation defect, resistance of BM cells to apoptosis as well as reduced extramedullary haematopoiesis in spleen, we speculate that these are the early signs of a myelodysplastic syndrome (MDS) related phenotype. Therefore, ageing *Zeb1* mice for longer timeframes (18 months) might alter the genetics of BM HSCs and MPPs and may eventually lead to the development of leukaemia.

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