Exploring the role of Apolipoprotein E in normal and malignant Haematopoiesis

Badi Abdulhadi Alotaibi

Thesis submitted in fulfilment of the requirement for

The Degree of Doctor of Philosophy

Cardiff University

March/2021

Acknowledgment

This acknowledgment has been written for the debt I owe to many people for their care, support and encouragement during the course of my PhD. First of all, I sincerely thank Allah the Almighty for his boundless blessing, mercy and for enabling me to complete this thesis successfully. I am truthfully pleased to express my thank, sincere gratitude and deepest appreciation to my academic supervisor Dr Neil Rodrigues for giving me an opportunity to do my PhD in his lab, for his thoughtful support throughout the course of my PhD, especially during COVID- 19 pandemic period, his research constructive directions and his encouragement to accomplish and meet the goals and aims of this project. I would like also to express my deepest and sincere gratitude to Dr Timothy Hughes for his assistance, guidance and research valuable, constructive suggestions and directions throughout my PhD. Thanks to Professor Dipak Ramji for his valuable research suggestions.

I wish to express my thank and respect to all staff and members of School of Biosciences European Cancer stem cells research institute (ESCRI) staff, at Cardiff University for their kind support and welcoming atmosphere. I also wish to extend my thanks and respect to my sponsor, the government of Saudi Arabia represented by Ministry of Education - King Saud Bin Abdulaziz University for Health Sciences and Ministry of National Guard for giving me a chance to pursue my post graduate studies abroad as well as for their generous support financially. Further, Special thanks, sincere gratitude and deepest appreciation are extended to my parents, who have progressively supported me, whose prayers affluence my hardship, brothers, sisters, nephews and nieces for their continuous encouragements, inspiration, and lovely care throughout my research degree.

Abstract

Apolipoprotein E (ApoE) is a gene known for its role in lipid metabolism and has an anti-atherogenic role. Consequently, the deficiency of ApoE in mice results in the development of atherosclerosis and, conversely, re-expression of the gene reduces the development of the disease. Atherosclerosis is known to involve haematopoietic cell subsets and deregulated haematopoiesis drives both clonal haematopoiesis (a pre-leukaemia syndrome) and atherosclerosis. In this thesis, I investigated the broad requirement of ApoE signalling in normal haematopoiesis and the role of ApoE signalling in haematopoiesis and the development of leukaemia in the context of a high fat diet (HFD) that drives atherosclerosis. ApoE-/- mice fed a normal chow diet (NCD) displayed normal abundance of most haematopoietic stem/progenitor (HSPC) subsets and lineage specific myeloid and lymphoid progenitors, with the exception of a marginal reduction in B cells in the bone marrow and spleen, and a decrease in Mac-1+ cells and platelets in the peripheral blood. Functionally, as assessed by colony forming cell (CFC) assays, haematopoietic progenitor formation from ApoE-/- mice was unperturbed.

When ApoE-/- mice were fed an HFD, a select expansion of immunophenotypic HSPCs was observed, including expansion of the LSK compartment reflecting ApoE mediated increases in haematopoietic stem cells (HSCs), multipotent progenitors (MPPs) and committed myeloid progenitors. ApoE-/- mice fed an HFD have a HPSC late survival advantage. Unexpectedly, ApoE signalling in the context an HFD appeared, with some exceptions, to be largely unneeded for HSC functioning, as assessed by transplantation, and for HSC self-renewal, judged by secondary transplantation. To test the impact of an HFD on ApoE signalling in leukaemia, an MLL-AF9 driven model of acute myeloid leukaemia (AML) was employed. MLL-AF9 transformed HSPCs from ApoE-/- mice fed an HFD showed no differences in pre-LSC formation *in vitro* and when pre-LSCs were transplanted allowing the development of AML *in vivo*, no impact was seen on the development of leukaemia, indicating that HFD has no influence on ApoE signalling in the initiation and development of leukaemia.

In conclusion, these data suggest an association between the requirement for ApoE and the regulation of steady state haematopoiesis in select mature blood cell lineages, which requires further investigation in functional *in vivo* experiments. ApoE signalling in HFD expands select HSPC subsets, but functionally these cells behave normally. Further experimentation will be needed to resolve the ApoE mediated transcriptional signature underlying the immunophenotypic changes in HSPCs observed in an HFD. However, ApoE signalling in HFD has no impact on leukaemogenesis in an MLL-AF9 driven model of acute myeloid leukaemia.

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ABCA1: ATP binding cassette transporter

ABCG1: ATB binding cassette transporter subfamily G member

AGM: Aorta gonad mesonephros

ALL: Acute lymphoblastic leukaemia

AMKL: Acute megakaryoblastic leukaemia

AML: Acute myeloid leukaemia

AMPK: Activated protein kinase

ApoE: Apolipoprotein E

Arf: Alternate reading frame of product of the CDKN2A locu

ASXL1: Additional sex combs protein 1

BAFFR: B cells activating factor receptor

Bcl-2: B-cell lymphoma

BET: Bromodomain and extra-terminal proteins

BM: Bone marrow

Bmi1: Polycomb group RING finger protein

Bp: base pair

C/EBPs: CCAAT/enhancer-binding protein alpha

cAMP: Cyclic adenosine monophosphate

CBC: Complete blood count

CBL: Casitas B-lineage Lymphoma

CD: Cluster of differentiation

CFC- Mix: colony forming cells granulocyte, erythrocyte, macrophage, megakaryocyte

CFC: Colony forming assay

CFU-M: Colony forming cells Monocyte

cGM: Cyclic Guanosine Monophosphate

CH: Clonal haematopoiesis

CHIP: Clonal haematopoiesis of intermediate potential

CLPs: Common lymphoid progenitors

CML: Chronic myeloid leukaemia

CMPs: Common myeloid progenitors

CO2: Carbon dioxide

CR: Caloric restriction

CSF: Colony stimulating factors

CVD: Cardiovascular diseases

CXCL 12: C-X-C motif chemokine 12

CXCR - 4: Chemokine receptor type 4

DNA: Deoxyribonucleic acid

DNMTA3: DNA methyltransferase

DOT1L: Disruptor of telomeric silencing-1 L

Dpc: days after conception.

EDTA: Ethylenediaminetetraacetic acid

E-MK cells: Megakaryocyte/erythrocyte cells

ERK: The extracellular-signal-regulated kinase

ETP: Earliest thymic progenitors

ETS: Erythroblast Transformation Specific

ETV2: Variant Transcription Factor 2

FAB: French American British

FACs: Fluorescent activating cell sorting

5-FU: Fluorouracil

Flk1: Foetal liver kinase 1

FLT3: Fms-like tyrosine kinase 3

G-CSF: Granulocyte colony-stimulating factor

GFP+: Green fluorescent protein +

GM-CSF: Granulocyte macrophage colony, stimulating factor receptors

GMPs: Granulocyte and monocyte progenitors

GNAS: Guanine nucleotide binding protein, alpha Stimulating activity polypeptide

HDAC: Histone deacetylase inhibitors

HDL: High-density lipoprotein

HFD: High fat diet

HGB: Haemoglobin

HPCs: Haematopoietic progenitor cells

HSCs: Haematopoietic stem cells

HSPCs: Haematopoietic stem and progenitor cells

ICAM1: Intracellular adhesion molecule 1

ICAM1: Intracellular adhesion molecule 1

IDL: Intermediate density lipoprotein

IGF-1: Insulin/insulin-like growth factor

IHD 1: Isocitrate dehydrogenases 1

IHD 2: Isocitrate dehydrogenases 2

IL-17: Interleukin 17

IL-23: Interleukin-23

IL-3Rβ: Interleukin-3RB

IL-3β: Interleukin 3β

IL-6: Interleukin – 6

IL-7: Interleukin - 7

Ink4: Inhibitors of CDK4

IRAK4: Interleukin-1 receptor-associated kinase 4

ISCs: Intestinal stem cells

JAK2: Janus kinase 2

Ko: Knockout

KSL: C-kit+ sca-1 + lineage

LDL: Low density lipoprotein

LDLR: Low-density lipoprotein receptor

Lin- : lineage negative

LK: Lineage - C-kit+

LMPP: Lymphoid primed multipotent progenitor

LRP: Low density lipoprotein receptor-related protein

LSCs: Leukaemia stem cells

LSK: Lineage - sca-1 + C-kit+

LT: long term

Ly6: Lymphocyte antigen 6

MCP-1: Monocyte chemoattractant protein-1

MDS: Myelodysplastic syndrome

MEPs: Megakaryocyte/erythroid progenitors

MFI: Mean fluorescence intensity

Mll-AF9: Mix lineage leukaemia -AF9

MPN: Myeloproliferative neoplasm

MPPs: Multi-potent progenitor cells

MLP: Multi lymphoid progenitors

mTOR: Mechanistic target of rapamycin

MyD88: Myeloid differentiation primary response 88

NCD: Normal chow diet

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NGS: Next generation sequencing

NK: Nature killer

NO: Nitric oxide

NOD/SCID: Non-obese diabetic/severe combined immunodeficiency

NOD: Non-obese diabetic

NSCLC: Non-small cell lung carcinoma

O2: Oxygen

OCT: optimum temperature formulation

ORO: Oil -Red-O

Ox-LDL: Oxidized low-density lipoprotein

P 53: Protein 53

PA: Polyadenylation signal,

PB: Peripheral blood

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PLT: Platelets

PML-RARα: Promyelocytic leukaemia - retinoic acid receptor

PPM1D: Protein phosphatase 1D magnesium-dependent delta isoform

RBCs: Red blood cells

ROS: Reactive oxygen species

RUNX1: Runt-related transcription factor 1

Sca-1: Stem cells antigen

SCID: Severe combined immunodeficient

SDF-1: Stromal cell derived factor-1

SEM: Standard error of mean

SF3B1: Splicing factor 3b, subunit 1

SFK: Src family kinases

Shh: Sonic hedgehog

SLAM: Single lymphocyte activating molecule

SMCs: smooth muscle cells

Sp: Spleen

SR-BI: Scavenger receptor reporter type BI expression

SRSF2: Serine/arginine-rich splicing factor 2

ST: Short term

STAT: Signal transducer and activator of transcription

TAM: Tumour associated macrophage

TCGA: The Cancer Genome Atlas

TET: Ten-eleven translocation

Thy: Thymus

Thy1.1: Thymus cell antigen

TIIC: Tumour infiltrating immune cells

TLR: Toll like receptor

Tsp: Transcription start point

TX: Transplantation

TF: Transcription factors

U2AF1: U2 small nuclear RNA auxiliary factor 1

VAF: variant allele fraction/frequency

VCAM1: Vascular cell adhesion protein 1

VLDL: Very low-density lipoprotein

WBCs: White blood cells

Wpc: Weeks after conception

Chapter 1: Introduction

1.1 Haematopoiesis

Haematopoiesis is a continuous and regulated process of blood cell production that involves self-renewal, differentiation, proliferation, and maturation (Seita and Weissman 2010). These processes culminate in the formation, development, and specialisation of all blood cells that are released from the bone marrow (BM) to circulation. Blood contains four components, namely, plasma, red blood cells (RBCs) (erythrocytes), white blood cells (WBCs) (leukocytes), and platelets (thrombocytes), with each having various functions (Rodak et al. 2007). Plasma protects the blood vessels and maintains circulation by transporting coagulation enzymes (Rodak et al. 2007). Leukocytes involve several specialised cells that are essential in forming the immune system (Rodak et al. 2007). Erythrocytes are responsible for the transportation of oxygen (O2) and carbon dioxide (CO2) to the tissues, whereas thrombocytes are involved in wound healing and blood coagulation (Rodak et al. 2007). All blood cell types arise from a common cell type - haematopoietic stem cells (HSCs) - that can self-renew and differentiate into any mature haematopoietic effector cells (Rieger and Schroeder 2012).

1.1.1 Ontogeny of haematopoietic system

The ontogeny of the haematopoietic system in vertebrates can be characterised into two waves: embryonic and definitive haematopoiesis (Medvinsky et al. 2011). The embryonic wave produces “temporary” haematopoietic cells, such as some myeloid cells and primitive erythrocytes. This population emerges in the extra-embryonic yolk sac and placenta and potentially in the intra-embryonic location prior to the first production of HSCs. These types of haematopoietic cells are not found in adults; instead, they are soon replaced by their definitive haematopoietic counterparts in the embryo, whose function differs (e.g., primitive RBCs maintain their nuclei and express the foetal form of haemoglobin) (Rieger and Schroeder 2012). Definitive haematopoiesis generates all the mature blood cells that are eventually utilised in the adult. The de novo generation, maintenance, expansion, and locations of haematopoietic stem and progenitor cells dynamically fluctuate during embryogenesis. Anatomically, definitive haematopoietic cells are produced in the extra embryonic yolk sac and later in the allantois and placenta. Eventually, definitive haematopoietic cells are produced de novo in the intra embryonic aorta gonad mesonephros (AGM) region. HSCs are produced in the intra-embryonic AGM region (Müller et al. 1994; Medvinsky and Dzierzak 1996) and the placenta (Gekas, Dieterlen-Lièvre et al. 2005; Ottersbach and Dzierzak 2005). Following their

generation in the AGM region, they migrate to the placenta and the foetal liver, which are the main sites for proliferation and expansion. Thereafter, they migrate to the spleen and, prior to birth, to the BM, which becomes the predominant site for HSCs and haematopoiesis after birth (Rieger and Schroeder 2012) (Figure 1.1).

It has been hypothesized that the first HSCs are derived from the endothelium (Medvinsky et al. 2011). These haemogenic endothelium cells, as they are termed, would be integrated into the vessel wall and be able to generate haematopoietic blood cells under the influence of unknown molecular signals. However, proving the existence of the haemogenic endothelium has been contentious, with the weight of evidence suggesting the existence of haemogenic endothelium. Static analysis of both pre- and post-haemogenic endothelium transition generated data that could indicate that the haematopoietic cells could be produced elsewhere and then migrate to the endothelium. In addition, single cell analysis of the endothelium to haematopoietic transition by time lapse microscopy demonstrated the generation of haematopoietic cells from the haemogenic endothelium (Eilken et al. 2009).

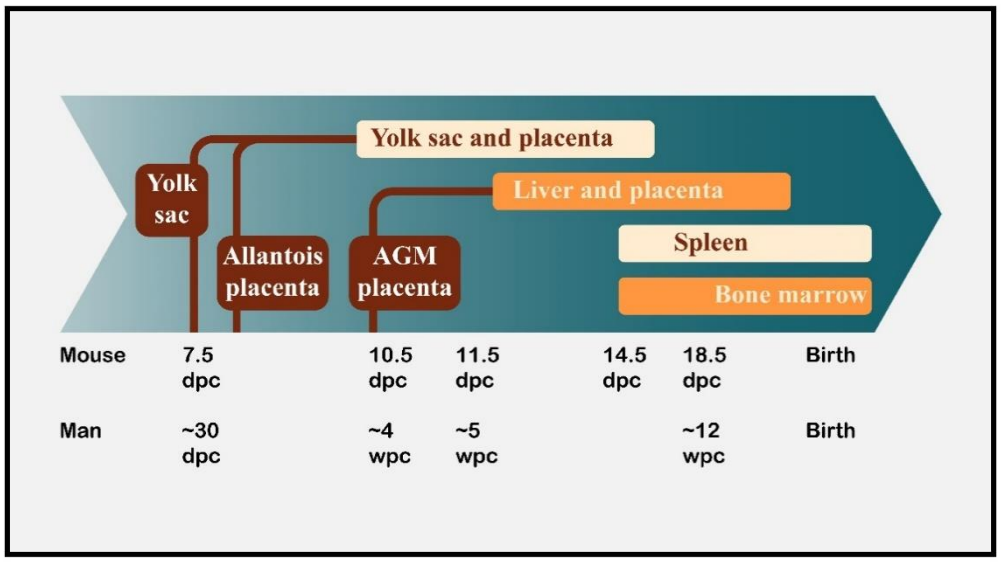


Figure 1.1 Ontogeny of haematopoietic system Dpc, days after conception. Wpc, weeks after conception. Adapted from (Rieger and Schroeder 2012).

These molecular mechanisms regulating haemogenic endothelium are incompletely understood but include complicated signalling from the various cell types surrounding proximal to the haemogenic endothelium at different sites and times in the developing embryo. Mesodermal cells expressing Flk1 and ETV2 were recognized as a common precursor for endothelium and haematopoietic cells (Lee et al. 2008; Kataoka et al. 2011). Furthermore, molecule-like membrane-bound notch ligands, vascular endothelial growth factor, sonic hedgehog bone morphogenetic protein, shear stress, and nitric oxide signalling have been

involved in the induction of the haemogenic program in endothelial cells (Medvinsky et al. 2011). Moreover, several transcription factors within haemogenic endothelial cells, such as RUNX1 and CBFβ, appear to be vital in the induction of the haemogenic program (Chen et al. 2011).

1.1.2 Haematopoietic hierarchy and lineage commitment of HSCs

The functional properties of HSCs have been traditionally defined by the transplantation assay. The first in vivo evidence for the presence of HSCs was noted by rescuing lethally irradiated recipient mice by a BM transplantation assay followed by detection of multi-potential haematopoietic colonies in the recipient's spleen (Till and McCulloch 1961). Since then, scientists have been aiming to develop methods to purify HSCs from BM to fully understand the functional and molecular regulatory networks. The utilization of antibodies and fluorescence-activated cell sorting (FACS) has allowed for the purification of HSCs. Firstly, Weissman and his colleagues used a combination of several surface markers, including Sca-1 and c-kit, to describe enriched HSCs cells (Spangrude et al. 1988). Thereafter, various research groups have dedicated a significant amount of effort to identifying more surface markers to further separate the functional heterogeneity of HSCs.

To date, single lymphocyte activating molecule (SLAM), CD34, Sca-1, and c-kit are commonly used to separate HSCs in different laboratories (Kiel et al. 2005; Oguro et al. 2013; Ikuta and Weissman 1992; Osawa et al. 1996; Okada et al. 1992). In addition, multi- and uni-potent progenitor populations can be isolated according to the surface marker (Pietras et al. 2015; Wilson et al. 2008; Kondo et al. 1997). HSCs have been defined according to two essential properties: self-renewal and multipotent differentiation (Reya 2003; Morrison et al. 1995). On the other hand, progenitor populations have been defined by the lack of self-renewal and restricted differentiation capacity. The isolation of HSCs and committed progenitors using FACS allowed for the formulation of the classical model of haematopoiesis (Akashi et al. 2000; Reya et al. 2001).

All haematopoietic cells arise from the HSCs in the BM. Long-term HSCs are present at a low frequency in the BM and show a lifelong capacity for self-renewal and differentiation potential among all haematopoietic cells. Short-term HSCs have a reduced self-renewal capability, but they still exhibit a multi-lineage differentiation potential. The differentiation of HSCs to the lineage-committed effector cells of the haematopoietic system requires the loss of the ability for self-renewal and the concurrent activation of genes that enhance differentiation (Akashi et al. 2000 Reya et al. 2001).

Initially, HSCs differentiate to multi-potent progenitor cells (MPPs), which no longer have any self-renewal ability but keep the lineage differentiation potential. MPPs differentiate into oligo progenitors with a strict segregation into either lymphoid or myeloid pathways —common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). Collectively, these oligo progenitors differentiate to all the committed lineage effector cells of the haematopoietic system. CMPs give rise to megakaryocyte/erythroid progenitors (MEPs) and granulocyte and monocyte progenitors (GMPs), which in turn, give rise to granulocyte and monocyte fate. MEPs differentiate into megakaryocyte/erythrocyte cells (E-MK) cells. As their name suggests, CLPs give rise to all the cells of lymphoid lineages, but they lack myeloid potential, whereas CMPs form the myeloid lineage cells, but they lack lymphoid potential. CLPs give rise to B cells, the earliest thymic progenitors (ETP), which are committed to T and NK lineages (Doulatov et al. 2012).

In contrast, other models of lineage commitment have been proposed where lymphoid and myeloid fates are shared until the later stages of differentiation. For example, Kawamoto and colleagues in 1999 observed that B, T, and erythroid fates were coupled with myeloid potentials whereas a progenitor with restricted B and cells was not found. This has led to a proposed myeloid based model in which myeloid and lymphoid fates stay coupled (Kawamoto et al. 2010). This model predicts the existence of myelo-lymphoid, which is confirmed by the separation of the lymphoid primed multipotent progenitor (LMPP) from the mouse BM (Adolfsson et al. 2005). LMPP repopulate a transient lympho-myeloid that displays lymphoid bias but has a low potential of E-MK (Månsson et al. 2007) (see Figure 1.2). Multi lymphoid progenitors (MLP) have been proposed as the human counterparts of LMPPs (Doulatov et al. 2012).

Transplantation assays and colony assays have defined HSCs' essential properties as self-renewal and multipotent differentiation (Cheng et al. 2019; Reya 2003; Dick 2003). On the other hand, progenitors are defined by the absence of self-renewal ability and the capacity for restricted lineage differentiation (Cheng et al. 2019). Although the classical model has been very beneficial for understanding the process of differentiation of HSCs, it has some flaws, as it has oversimplified the complexity of lineage commitment in haematopoietic stem and progenitor cells (HSPCs) (Cheng et al. 2019). The analysis of the bulk cells, as opposed to single cells, presumes that each population of cells that have same phenotype will have an identical function. With the advancement of single cell technology and genetic mouse models, the classical models of haematopoiesis have been challenged over the past years. Using single cell transplantation and limiting dilution analysis, Muller-Sieburg et al. (2004) and Dykstra et al. (2007) have defined myeloid biased (My-Bi), balanced (Ba) and lymphoid biased (Ly-Bi) HSCs according to the output of myeloid and lymphoid cells (Figure 1.3, A and B).

Additionally, platelet-biased HSCs have been described as a My–Bi subset that exists at the top of the haematopoietic hierarchy (Cheng et al. 2019) (Figure 1.3, C). Furthermore, with single cell transplantation, Yamamoto et al. (2013) noticed that the self-renewing lineage-restricted progenitors reside in phenotypically defined HSCs comprising megakaryocyte-erythrocyte progenitors (MkRPs), common myeloid repopulating progenitors (CMRPs) and megakaryocyte-erythrocyte repopulating progenitors (MERPs) (Figure 1.3, D).

Pietras et al. (2015) and Wilson et al. (2008) have further divided MPP populations into MPP1, MPP2, MPP3, and MPP4 based on lineage bias, cell cycle status, immunophenotype, BM abundance, and drug treatment resistance. MPP1 is analogous to ST-HSCs, which have the ability to multilineage reconstitute up to 4 months post transplantation, while MPP2/3/4 lack the potential for self-renewal and show short-term myeloid ability to reconstitute. Additionally, MPP2 and MPP3 give rise to low-level T and B cells, and, in vivo, MPP4 populations produce a low level of myeloid cells. MPP 4 populations generate an elevated level of platelets in comparison to MPP3 and MPP4. Collectively, MPP3 is an My-Bi MPP subset, and MPP2 is a megakaryocyte-biased MPP subset (Figure 1.3, E). Post transplantation, HSCs give rise to MPPs (MPP1/2) to determine myeloid yield, followed by lymphoid-primed MPP4 to rebuild lymphoid compartments. Thus, MPPs are a heterogenous population with a differential potential of lineage for bias (Cheng et al. 2019).

To understand blood formation dynamics, various approaches to lineage tracing have been utilized to evaluate the contribution of HSC and HPC subsets to lineage out. Sun et al. (2014), using the approach of doxycycline–induced sleeping beauty transposon tagging in HSPCs revealed that rather than HSCs, MPPs are the main driver of haematopoiesis in adulthood. Busch et al. (2015) allowed for inducible genetic labelling for the most primitive $Tie2^{+}$ HSCs in BM and used limiting dilution analysis and mathematical modelling to quantity label progression along with the development of haematopoiesis. The results revealed that adult haematopoiesis is largely maintained by ST-HSCs and, on other hand, showed that LT-HSCs are used to establish blood cells in the foetal and early life of postnatal. Sawai et al. (2016) demonstrated that Pdzk1ip1 is expressed in HSPCs in murine, and thus, they developed a Pdzk1ip1 – GFP and Pdzk1ip1 – Cre ER murine model and revealed that LT-HSCs make a significant contribution to all mature blood cells. Pei et al. (2017) revealed a basic split between the development of common myeloid and erythroid cells and the development of common lymphoid cells using Rodewald's polylox tracking approach (Pei et al. 2017). However, the megakaryocyte fate was not assessed in this study. The Camargo group carried out a pulse chase experiment in mice for a long-term (30 weeks) evaluation with the sleeping beauty barcode system and reported that the megakaryocyte lineage evolves independently of other

fates of haematopoiesis, and LT-HSCs predominately contribute to the output of megakaryocytes (Rodriguez-Fraticelli et al. 2018).

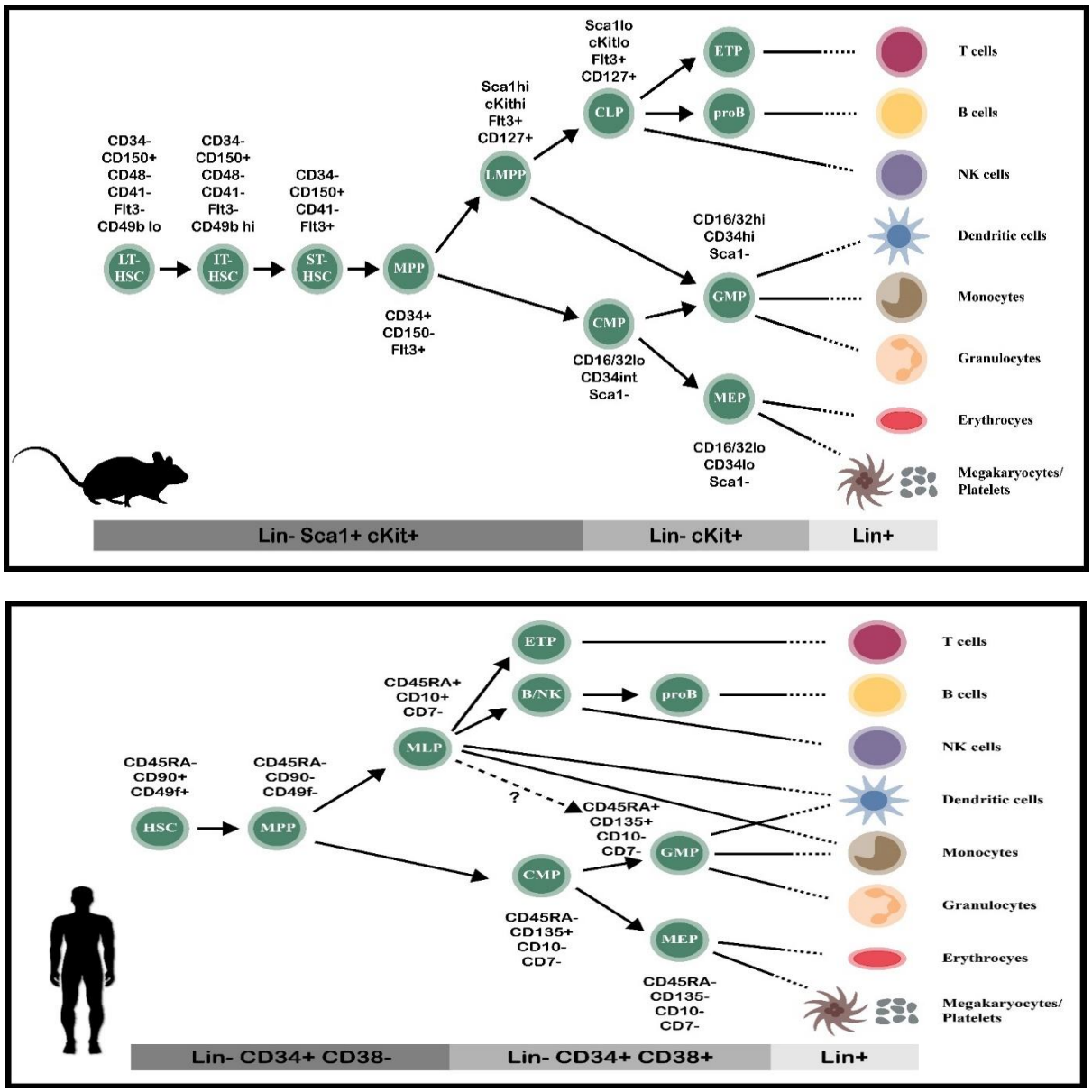


Figure 1.2 Lineage determination of haematopoietic hierarchy for adult mouse and human**.** HSPCs are defined by their surface phenotype, which are listed next to each population. LT: long term, IT: Intermediate term and ST: short term Adapted from (Doulatov et al. 2012).

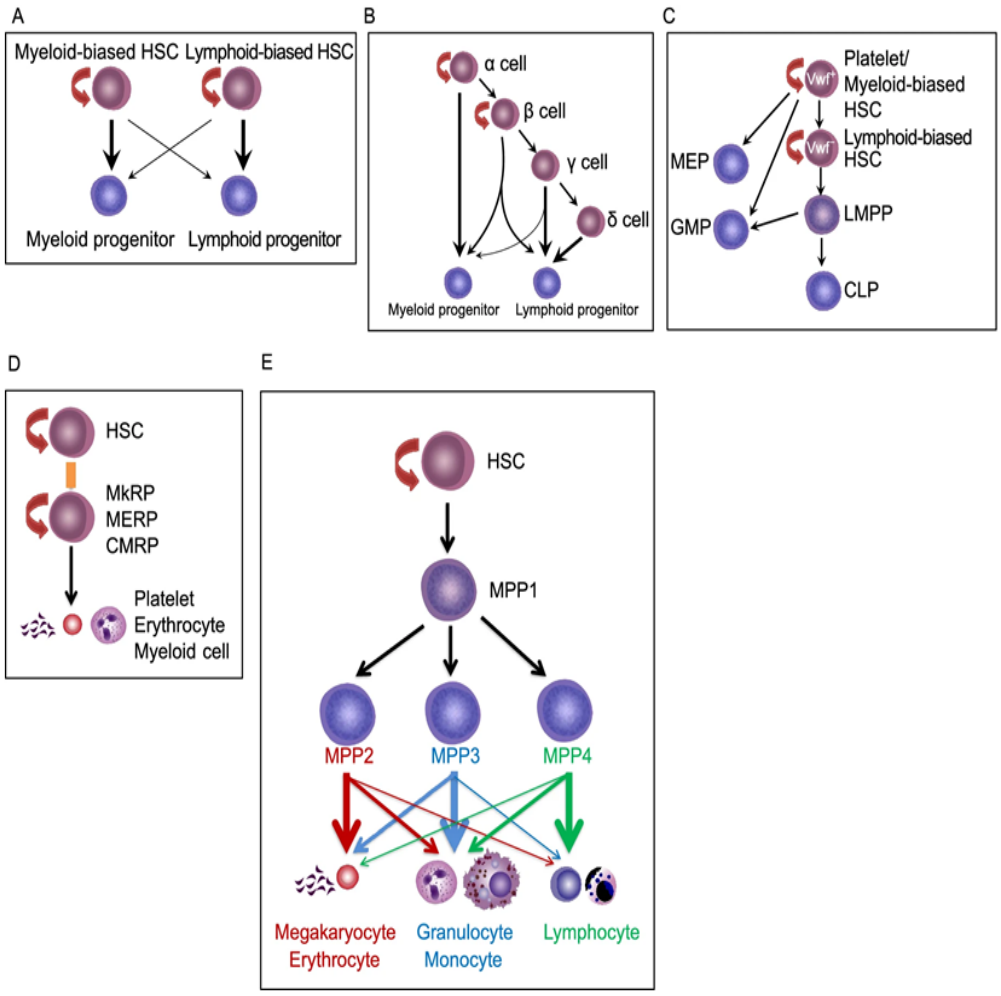


Figure 1.3 The revised models for haematopoietic stem cell differentiation. (A) the HSC models My-Bi and Ly-Bi. Ly-Bi HSCs are less likely to reconstitute the myeloid lineage than the lymphoid lineage, and vice versa. (B) The cells of Eaves' lab have been identified as alpha, β, μ and та to the percentage of the myeloid chemical (M/L ratio). (C) vWF+ platelet-based HSCs rest on the hierarchical apex and can be distinguished into both progenitors and mature cells. VWF− HSCs residing at the bottom of vWF+ HSCs are lymphoid-biased. The megakaryocyte/erythrocyte lineage cannot be produced by the LMPPs. MEPs come from HSCs directly. (D) The LT-HSC population includes CMRPs, MERPs, and MkRPs in the myeloid bypass model. These MyRPs are manufactured directly from HSCs. (E) subtypes of MPP will be divided into MPP1–4. Both lines can be uploaded by MPP1. MPP2/3 was biased with myeloid and MPP4 is biased with lymphoid. MPP2 is also platelet oriented. (Cheng et al. 2019).

1.1.3 Immunophenotypic characterisation of haematopoietic stem cells

HSCs reside primarily in the BM with an estimated frequency of 0.01% of the total nucleated cells in the BM. Numerous efforts have been made to isolate and characterise the HSCS from differentiated cells, which has resulted in identification of the broad antigenic phenotype c-kit+ Sca-1 + lineage - (KSL) to identify HSCs (Rossi et al. 2011). The expression and lack of expression of specific cells' surface markers are one of the main criteria used for characterizing and identifying HSCs. The expression of tyrosine kinase receptor c-kit (CD117) and membrane glycoprotein Sca-1 alongside the lack of expression of terminal differentiation markers (Ter119, Gr-1, MAC-1, B220, CD8, CD4), together known as lineage markers, is used to isolate populations of HSCs. The resultant c-kit+ Sca-1+ lineage, universally known as KSL cells, comprise cells able to reconstitute haematopoiesis. This method depends on either the combination with other cell surface markers, such as Thy1.1 (KSL Thy low), CD34 (KSL CD34 neg / low) and Flk2 (KSL CD34- Flk2-) (see Figure 1.2) or the ability to efficiently efflux Hoechest 33342. In addition, an alternative strategy based on the SLAM family (CD224- CD150+ CD48-) is utilized to identify the highly enriched HSC population.

Human HSCs have been isolated using similar methodologies to those used for murine HSCs. The first cell surface marker utilized for enriching human HSCs was CD34+, which is a ligand for L-selectin expressed by only 0.5-5% of blood cells in adult BM, human cord blood, and the human foetal liver (Civin et al. 1984; DiGiusto et al. 1994; Krause et al. 1996). The first isolation of human HSCs showed the phenotype of CD34+CD90+lin-, where these cells generate lymphoid and myeloid progeny in both in vivo and in vitro assays. Further enrichment of human HSCs within CD34+ populations was recognized by a differential expression of the surface marker CD38+. Although the majority of CD34+ cells co-express CD38+, cells that can give rise to all multi-lineage colonies reside in the negative to low fraction of CD38+ and CD90+ fraction (Civin et al. 1984; Hao et al. 1996). Moreover, CD34+ CD38-contains cells able to reconstitute the absence of the lymphoid compartment of severe combined immunodeficient (SCID) mice (Civin et al. 1984).

1.1.4 Regulation of haematopoiesis

Intrinsic factors maintain HSC characteristics of quiescence, self-renewal, and suppression of differentiation. The Bmi1-p53 axis of cell cycle regulators and the PI3K signalling pathway constitute the major intrinsic factors. Bmi1 controls cell proliferation by repressing the Ink4/Arf locus. Bmi1 supports cell renewal by suppressing the transcription of the cell cycle inhibitors p19ARF and p16Ink4a encoded in the Ink4/Arf locus. Furthermore, the tumour suppressor

p53 helps in the regulation of HSC quiescence through inhibition of the cell cycle (Pietras et al. 2011). On the other hand, the PI3K signalling pathway controls cell growth, proliferation, and survival by integrating numerous upstream signals, such as nutrients, oxygen, and growth factors. Besides intrinsic factors, some extrinsic factors also play a role in maintaining HSC characteristics. Extrinsic factors, such as cytokines (fms-related tyrosine kinase 3-ligand), chemokines (CXCL12), and growth factors (granulocyte-macrophage-CSF), have been linked to HSC stemness. The micro-environment provides soluble factors that support both haematopoietic and non-haematopoietic cells.

To maintain a high level of differentiation and lineage-specific genes, transcription factors (TFs) play a vital role in the regulation of HSCs as well as in the haematopoietic lineage decision. These TFs, in addition to the discovery of the master TFs, were demonstrated to have important clinical applications using a mechanism of activation and repression of specific genes (Fiedler and Brunner 2012). Core determinants of tissue identity are combinatorial interactions of TFs. This was revealed early on by overexpression studies in, for example, non-muscular (Davis et al. 1987), muscle phenotype, or combinations of haematopoietic TFs, which reprogram non-thermogenic mesoderm into their blood during development. The specific power of combinatorial TF interaction has recently been highlighted by the derivation of multipotency-associated TFs into differentiated cells (Takahashi and Yamanaka 2006), by trans-differentiation of pancreatic exocrine cells into insular secreting b-cells (Zhou et al. 2008), and by a direct transformation of fibroblasts. When combined with regulatory elements of the target gene, the requirement for particular TF combinations is likely to reflect the combinatorial interactions of these TFs.

For normal growth of erythroid cells, megakaryocytes, mast cells, and eosinophil granulocytes, GATA-1 is an integral founding member of GATA's family (Nishiyama et al. 2005). For normal haematopoietic cell maturation, the correct combination of GATA-1 activation and repression functions is important, and their disturbance contributes to diseases with underlying GATA-1 mutations such as acute megakaryoblastic leukaemia (AMKL) (Wechsler et al. 2002). SCL is expressed in haematopoietic growth in both early and late stages and is crucial for the normal formation of HSCs and then the complete differentiation of erythroid, megakaryocyte, and mast cell formation (Mikkola et al. 2003). SCL can operate as an activator or repressor like most transcription regulators. At least part of the above mechanism is dominated by interaction with the transcriptional ETO-2 corepressor (Schuh et al. 2005). At many of the regulatory sites, for which GATA-1 is a trigger for or a repressor of the general functioning of SCL complexes

in the GATA 1-dependent transcription activation, SCL, LMO-2, E2A, and Ldb1 have been identified. In contrast, GATA-1 cannot employ the SCL facility in places where it is a repressor. The complex SCL is also used in connection with the GATA-2 before GATA-1 gene repression.

1.1.5 Metabolic regulation of stem cells

According to many studies, diet intake appears to be an essential regulator of organismal physiology and health, especially for stem cell function. Caloric restriction (CR) and fasting increase HSCs' quiescence and protects patients from injury, respectively, while a high-fat diet (HFD) impairs haematopoiesis (Mana et al. 2017). CR is defined as 30-60% of ad libitum feeding without causing malnutrition (Mercken et al. 2012) and is linked to lifespan expansion, reduced incidence of cancers, and delayed onset of age-related diseases (Fontana et al. 2010; Mercken et al. 2012).

The impact of CR on lifespan is believed to be due to mediation by the downregulation of essential nutrient-sensing pathways, including the insulin/insulin-like growth factor (IGF-1) and the mechanistic target of rapamycin (mTOR) signalling (Berryman et al. 2008; Fontana et al. 2010). Many reports on mammalian intestines have shown that CR affects stem cell functions by modulating the microenvironment of stem cells. Indeed, CR increases the frequency of LgR5+ and Paneth (niche) cells with a reduction in the mature enterocyte number of mice, suggesting that CR causes a shift toward self-renewal (Yilmaz et al. 2012). LgR5+ is expressed by most intestinal stem cells (ISCs) through the intestinal tract (Barker et al. 2007). LgRs+ ISCs could self-renew and differentiate throughout the organism's life and inhabit the crypt base sandwiched between Paneth cells (Barker et al. 2007; Yilmaz et al. 2012). In vitro, the addition of the Paneth cells resulted in a marked increase in the potential of LgR5+ ISCs to produce self-renewing organoid bodies reminiscent of the mini-intestine (Sato et al. 2010).

Therefore, Paneth cells comprise a key component of the niche of stem cells both in vivo and in vitro (Barker et al. 2007: Sato et al. 2010: Yilmaz et al. 2012). Yilmaz et al. (2012) revealed how CR affected the frequency and function of the niche of Paneth cells and ISCs. Yilmaz et al. (2012) conducted a CR experiment using Lgr5 – EGFP – IRES – Cre ERT2 knock-in model in which the isolation of Lgr5 – EGFP ^{hi} ISCs, their progenitors, and more differentiated cells EGFP^{low} could be performed. The findings revealed that CR increased the frequency of Lgr5 – EGFP ^{hi} ISCs and Paneth cells. In addition, the frequency of EGFP ^{low} differentiated

progenitors was, however, lower in CR mice compared to ad libitum fed mice. These data are consistent with the phenotypic expansion of ISCs, which suggests that although the CR leads to the expansion of the pool of ISCs, it results in the reduction of more differentiated progenitors. Therefore, CR has an opposing impact on the number of stem cells and their progeny, thus changing the equilibrium in favour of the self-renewal of the stem cells.

Furthermore, the induction of the BM stromal cell antigen (BST1) in the niche of the Paneth cells drives the enhanced activity of ISCs in CR. Igarashi and Guarente (2016) revealed that CR increases the proliferation and self-renewal of ISCS. Moreover, this study shows that the reciprocal mTORC1-S6K1 signalling axis in ISCs is essential for enhancing the functionality of ISCS; this is detected when ISCs are cultured with CR Paneth cells. Together, these results revealed that CR affects intestinal homeostasis by enhancing the function of ISC non-cells autonomously via the downregulation of mTORC1 signalling in the niche of Paneth cells.

CR has both beneficial and adverse effects on haematopoiesis (Tang et al. 2016). During the ageing of C57BL/6 mice, HSCs increase in number but have reduced functionality, such as a lower self-renewal ability and a skewing differentiation toward myeloid lineages due to active HSCs' cycling-related stress (Flach et al. 2014). In addition, CR reduces the proliferation-related stress, as 4-9 months of CR caused increased HSC quiescence and conferred protection against age-related increases in HSCs (Tang et al. 2016). Furthermore, the condition of CR prevented the skewing of HSCs toward a myeloid lineage, as the number of lymphoid- and myeloid-based HSCs is comparable; however, CR induced a reduction in CLP and in the pro-B cell number, whereas it promoted the number of myeloid progenitors, indicating that CR impairs the differentiation of lymphoid-based HSCs. The reduction in the number of common CLPs in aged CR mice might lower the infection response (Gardner 2005). Moreover, treating aged mice with IGF-1 reduced the number of quiescent HSCs and further elevated the number of myeloid cells compared to CR mice.

Treatment with IL-6, IL-7, and IGF-1 restored the differentiation of the lymphoid progenitor, suggesting that the suppression of IL-6, IL-7, and IGF-1 is essential for the HSC phenotype under a CR condition. Collectively, these data demonstrate that the CR condition benefits maintenance of the quiescence of HSCs, but it may adversely affect lymphopoiesis. In other stem cell settings, CR has an advantageous effect on skeletal muscle stem cells by preserving the functionality of muscle stem cells during ageing. Adult stem cells or satellite cells are quiescent under normal circumstances but are able to restore tissue integrity upon exposure to external cues (Yin et al. 2013). Short-term CR in both young and aged mice results in an increased myogenic function of satellite cells, which is probably due to the metabolic shift to more mitochondrial oxidative phosphorylation.

In a similar vein, fasting has been reported to affect stem cells. Fasting is defined as no or less consumption of calories for 12 to multiple days (Longo and Mattson 2014). There are different forms of fasting including intermittent fasting (IF), which involves alternate day fasting or fasting for 2 days or fasting or a week, and periodic fasting (PF), which lasts for 3 days or longer (Longo and Mattson 2014). Fasting is known to cause ketogenesis, to confer resistance against DNA damage, and to reduce the side effects associated with chemotherapy (Safdie et al. 2009; Tinkum et al. 2015). Collectively, these findings indicate that fasting intervention may augment the function of stem cells in ageing and injury. Forty-eight hours of fasting did not alter the number of LgR5+ stem cells per crypt compared to fasted mice (Richmond et al. 2015). Cycling LgR5+ stem cells in a fasting state reduces the number and repopulated less towards the intestinal epithelium.

In the haematopoietic system, PF for 48 hours induces changes in the number of HSCs and differentiation. PF increases the proliferation of MPP and the myeloid progenitor while maintaining the total number of stem and progenitor cells compared to non-fasted animals (Cheng et al. 2014). In addition, stromal niche cells from fasted animals stimulate the expansion of MPPs, which are derived from ad libitum HSCs. The population of HSCs in aged mice is skewed towards myeloid lineage in comparison to a young control, and prolonged fasting restores the balance between aged blood cells as observed in the condition of the young control. Mechanistically, IGF-1/PK9 mediates many of the impacts of PF. Furthermore, IF in an animal model decreases the aged clinical symptoms related to neuronal maladies, such as Alzheimer's disease (Longo and Mattson 2014).

Conversely, increased caloric intake culminates in metabolic, cytokine signalling, such as IL-6, IL -1β and TNF-α, and hormonal imbalances (Hursting 2014; Rodriguez et al. 2015). Overnutrition leads to obesity and multiple diseases, such as diabetes, cardiovascular diseases (CVD), and non-alcoholic fatty liver disease (Jung and Choi 2014; Van Gaal et al. 2006). Long-term HFD feeding (more than 6 months) expands the number of ISCs and decreases the number of niche Paneth cells (Beyaz et al. 2016; DeClercq et al. 2015; Mah et al. 2014). Mechanistically, HFD activates the PPAR-delta programme within ISCs and progenitors. Mice treatment with a PPAR-delta agonist recapitulates many of the ISCs and progenitors induced by an HFD. In addition, under an HFD condition, ISCs and progenitors acquire stemness features involving the ability to form tumours. Effectively, an HFD elevates the number of target cells, such as ISCs, that can initiate tumours by undergoing oncogenic transformation (Beyaz et al. 2016). An HFD also induces alterations to the haematopoietic cells. Feeding an HFD for a few weeks results in a decrease in HSCs (Luo et al. 2015; Berg et al. 2016), while long-term HFD feeding boosts the number of HSCs and functions (Singer et al. 2014).

Interestingly, this phenotype is partially reversible: the number of HSCs in HFD-fed mice returns to normal after the removal of the HFD. HFD feeding in both the short term and the long term skews the differentiation toward myelopoiesis (Luo et al. 2015; Berg et al. 2016). In other stem cell settings, the activation of IKKβ/NFB through an HFD in hypothalamic neuron stem cells reduces the number of stem cells, partially due to apoptosis and impairment in notch-mediated differentiation (Li et al. 2012; Li et al. 2014). An HFD affects the skeletal muscle stem cells. Rodent models of diabetes revealed impaired muscle generation (Vignaud et al. 2007). These effects in satellite cells in response to an HFD rely on AMP-activated protein kinase (AMPK) signalling. For example, AMPK agonist treatment enhances the function of satellite stem cells and muscle regeneration in HFD-fed mice, increasing the possibility that the activation of AMPK might be a therapeutic target for promoting muscle function in obese patients.

Obesity has been linked to several major human cancers such as postmenopausal breast, colon, endometrium, oesophagus, liver, and kidney cancers (Lichtman 2010). Epidemiological studies indicate a significantly elevated risk for haematological malignancies in obese people, such as leukaemia (Larsson and Wolk 2007), non-Hodgkin's lymphoma (Larsson and Wolk 2007; Willett and Roman 2006) and multiple myeloma (Larsson and Wolk 2007) In addition, hypercholesterolaemia has frequently been observed in AML patients (Tatidis et al. 2001; Vitols et al. 1985; Vitols et al. 1990). Leukaemia cells from AML patients have an increased receptor-mediated uptake of low-density lipoprotein (LDL), which is a major carrying lipoprotein cholesterol (Brown and Goldstein 1986). The level of plasma cholesterol returns to normal when the leukaemic cells disappear during chemotherapy, indicating that the hypercholesterolemia is induced by leukaemic cells (Vitols et al. 1990).

1.2 Clonal haematopoiesis

Detecting the premalignant state, such as colonic tubular adenoma, and using initial interventions to prevent malignancies are key accomplishments in public health and cancer biology (Jan et al. 2017). Transformation of the normal state into the malignant state involves consecutive acquisitions of several genetic alterations through the division of many cell types (Knudson 1971; Vogelstein et al. 1988; 2013). Several body tissues harbour populations of pre-cancerous cells that have acquired the alterations necessary for transformation to the malignant state, and identifying these pre-cancerous cells is an essential step in preventative care (Gibson and Steensma 2018). However, the premalignant state for some haematologic malignancies has yet to be elucidated. Studies of blood clonality in healthy women have suggested that clonal haematopoiesis (CH) is an initial step in the pathogenesis of haematologic malignancy (Busque and Gilliland 1998), and a large number of studies investigating the common mutations of haematologic malignancies have determined the

cellular and molecular mechanisms of pre-cancerous lesions in haematopoiesis. Furthermore, several genetic technologies recently utilized by researchers have found that CH is a common, age-related premalignant condition.

HSCs produce approximately 10^10 –10^12 new blood cells every day (Beerman et al. 2010), which means that an estimated 50,000–200,000 HSCs exist in the human body (Lee-Six et al. 2018) and that each HSC may acquire one exon mutation per decade (Welch et al. 2012). These mutations provide a competitive advantage for HSPCs; this culminates in a state of CH, which is defined as any expansion of haematopoietic cell clones with somatic mutation in the WBC (Silver and Jaiswal 2019; Gibson and Steensma 2018; Calvillo-Argüelles et al. 2019). These somatic mutations can lead to diseases, such as acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS), and myeloproliferative neoplasm (MPN). Over time, with each cell division, human HSPCs acquire random somatic mutations. Thus, greater replicative history, such as old age, is associated with the accumulation of these mutations. Mutated genes can be classified according to their functions as follows: epigenetic regulators (e.g., DNMTA3, TET2, and ASXL1) (Heyde et al. 2021), splicing factors (e.g., U2AF1, SRSF2, and SF3B1), tumour suppressor (e.g., PPm1D and TB53), and signal transducers (e.g., JAK2, GNAS, and CBL; Sperling et al. 2016). Many of these mutations will have little to no impact on fitness; however, some will confer a competitive advantage for the expansion of clonal mutant cells in the peripheral blood (PB) (Heyde et al. 2021).

The first evidence of CH was obtained from reports on healthy women that stated that haematopoiesis could be oligoclonal with increasing age (Jan et al. 2017). From a non-random X allele, clonal skewing was noted in leukocytes in the PB in 20–25% of healthy women above the age of 60 years (Busque et al. 1996; Champion et al. 1997). CH can also be detected using next-generation sequencing (NGS) of isolated DNA from leukocytes in the PB (Shumilov et al. 2018; Calvillo-Argüelles et al. 2019). The variant allele fraction/frequency (VAF) is the frequency of mutant clones within the cell population. CH prevalence differs according to the VAF threshold and sequencing depth used. Additionally, according to Genovese et al. (2014), VAF varies with age, ranging from less than 1% in individuals younger than 40 years to more than 20% in individuals older than 90 years (Shlush 2018; Jaiswal et al. 2014; Genovese et al. 2014; Xie et al. 2014; Steensma et al. 2015).

CH can result from various causative mechanisms, such as exposure to radiotherapy (Coombs et al. 2017), post aplastic anaemia (Yoshizato et al. 2015), ageing (Jaiswal et al. 2014), and genetic drift (Zink et al. 2017). As some CH cases will culminate in haematologic malignancies, another term for CH, namely, CH of intermediate potential (CHIP), is used to

differentiate between this condition and other pre-leukaemia states. A VAF greater than 2% has been suggested for defining CHIP (Steensma et al. 2015). Additionally, since CH is observed in ageing and is linked to increased mortality, another term used to describe the condition is age-related CH, which refers to CH recurrent mutations in individuals with no history of aplastic anaemia, radiotherapy exposure, or chemotherapy.

1.2.1 Clonal haematopoiesis, ageing, inflammation, and cardiovascular disease

DNA damage is a well-known characteristic of cellular ageing (Welch et al. 2012). As HSPCs age, the expression of all subunits of the mini-chromosome maintenance helicase decreases (Flach et al. 2014). Changes in DNA repair are related to age and the decreased regenerative ability that comes with ageing (Wagner et al. 2009); this provides the opportunity for clonal selection and expansion (Natarajan et al. 2018). Previous studies have estimated that an average of five coding mutations could accumulate within each HSPC in an individual by the age of 50 (Fuster and Walsh 2018). Ageing has been found to alter haematopoiesis (de Haan et al. 2018), which includes altered lineage composition, increased myeloid malignancies, and reduced regenerative ability (Luis et al. 2019). Collectively, age-associated phenotypes might be a significant factor in clinical outcomes (Figure 1.4).

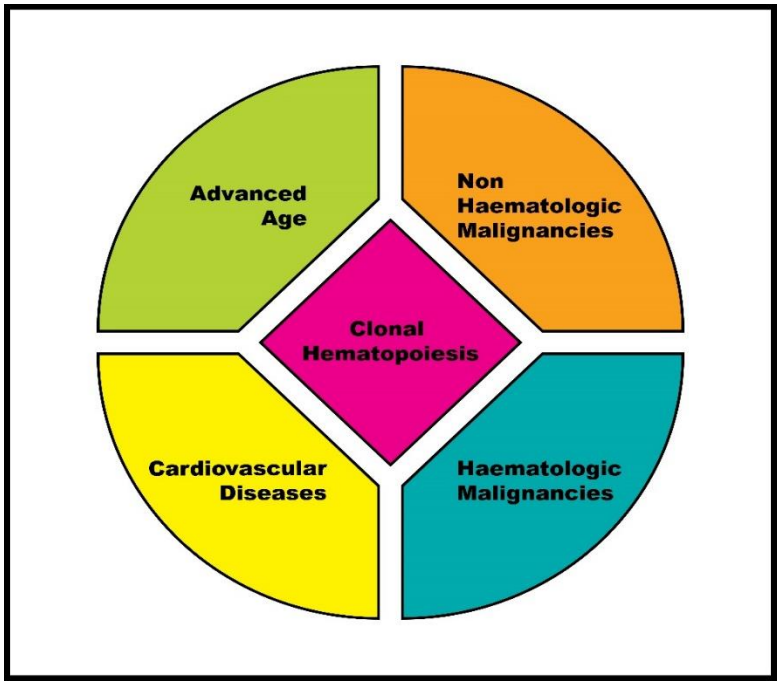


Figure 1.4 Advanced age with the presence of clonal haematopoiesis leads to CVD, haematologic malignancies, and non-haematologic malignancies. Adapted from Páramo Fernández (2018).

CH carriers have double the risk of coronary heart disease and four times the risk of myocardial infarction (Jaiswal et al. 2017). The three most commonly mutated genes in CH (DNMTA3, TET2, and ASXL1) show a comparable risk of developing coronary heart disease. The mechanism by which CH carriers have a greater risk of developing CVD was demonstrated by two seminal studies exploring the impact of ten-eleven translocation 2 (TET2) mutation on atherosclerosis (Fuster et al. 2017; Jaiswal et al. 2017; Luis et al. 2019; Heyde et al. 2021). In these studies, TET2-deficient BM was transplanted into LDL receptor-deficient mice (LDLR-/-), who were fed an HFD. Large atherosclerotic lesions developed in the transplanted mice with TET2 compared to the mice receiving wild-type BM, and gene expression analysis showed increased expression of genes associated with inflammation, including the pro-inflammatory cytokine genes IL-6 and IL-16 and other cytokine genes, such as Cxc13 and Cxc12. Thus, TET2-deficient cells accelerate atherosclerosis by increasing inflammatory immune responses and retention into the arterial wall with increased proinflammatory cytokine production and pro-atherosclerotic activity (Heyde et al. 2021) (see Figure 1.5).

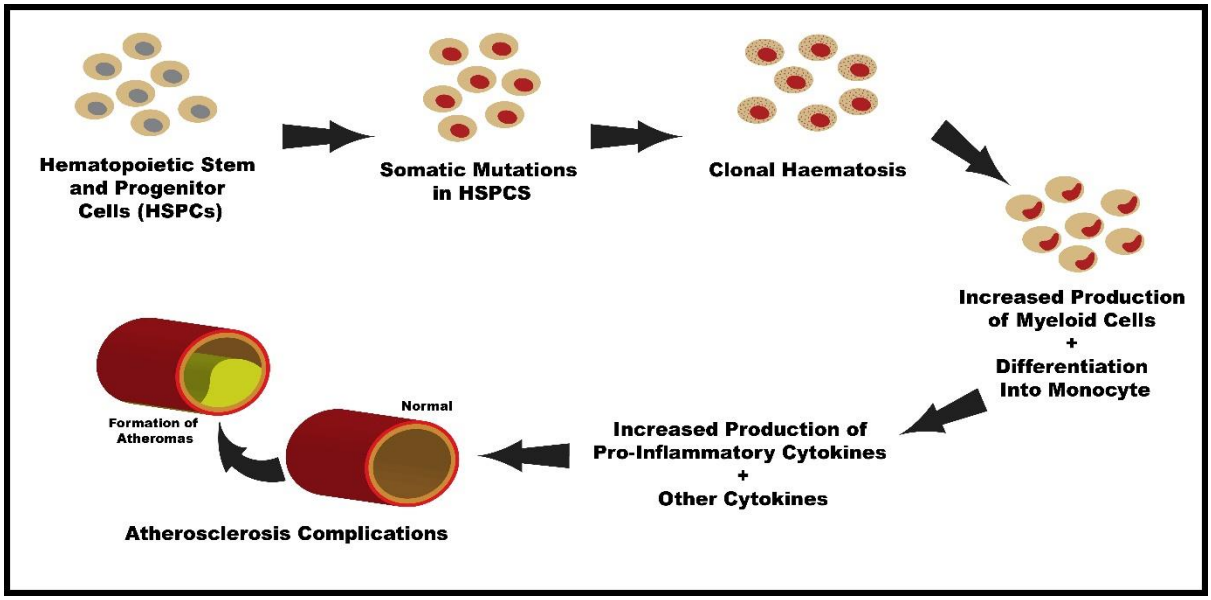


Figure 1.5 Proposed mechanism of CH in atherosclerosis Natarajan et al. (2018).

1.2.2 Clonal haematopoiesis and haematological malignancies

CH is regarded as a future risk factor of haematologic malignancies, such as AML (Genovese et al. 2014; Xie et al. 2014; Heyde et al. 2021). Thus, it is often regarded as a pre-leukaemic state. Clonal studies have identified a subset of HSCs that harbour one or a subset of genetic mutations in a leukaemic blast as pre-leukaemic HSCs (Jan et al. 2017; Shlush et al. 2014). CH tends to harbour a single detectable mutation in other pre-leukaemic states, such as MDS, while multiple genetic mutations are noticed in the PB in MDS patients (Steensma et al. 2015; Jan et al. 2017). Around 10% of CH cases develop AML partially due to CH presentation in the elderly, for whom other diseases cause mortality before the progression of CH to haematologic malignancies. The presence of certain mutations is believed to be a strong predictor of CH progression to AML. These include TP53, U2AF1, and SRSF2 mutations. Other mutations show a similar predictivity of AML and have shown longer latency, including IDH2, IDH1, and RUNX1 mutations. However, other mutations, such as TET2 and DNMTA3, have revealed surprisingly weak predictivity of AML (Abelson et al. 2018).

Enhanced self-renewal or a large pool of HSCs have been suggested as a mechanism for the mutation of TET2, DNMTA3, and ASXL1 (Moran-Crusi et al. 2011; Challen et al. 2012; Yang et al. 2018). However, other mechanisms of the recurrent mutations are less clear. Within functional HSCs, early mutations are believed to enhance multilineage haematopoiesis with a clonal advantage. Shlush et al. (2014) revealed that DNMT3A mutations come from pre-leukaemic cells within HSCs in AML patients. Conversely, later mutations, which are seen only in myeloid leukaemic blasts (such as FLT3 and CEBPA), play a role in driving the pre-

leukaemic differentiation of HSCs to the downstream myeloid cell type (Shlush et al. 2014; Corces-Zimmerman et al. 2014).

1.2.3 The interconnection between clonal haematopoiesis, cardiovascular disease, cancer

The pathogenesis of CVD and of cancers share similar underlying mechanisms (de Boer et al. 2019; Totzeck et al. 2019). The prevalence of CVD and cancer increases with age (de Boer et al. 2019; Koene et al. 2016). Additionally, chronic inflammation plays a significant role in CVDs (Peter Libby 2012) and could enhance malignant transformation and cancer progression (Kuper et al. 2000; Crusz and Balkwill 2015). Inflammation is one of the features of cancer that both causes and stimulates tumour growth (Hagemann 2007). Tumour cells, as well as tumour-associated macrophages, which are potent generators of the pro-inflammatory cytokine, mediate inflammation. The development of pro-inflammatory cytokines by stress-related factors is established (Kiecolt-Glaser et al. 2003). Inflammation, or inflammation from the tumour, also has a known effect on the central nervous system. In cancer patients, it may have severe psychosocial sequelae (Lutgendorf and Andersen 2015). Depression has been linked with higher IL-6 in cancer patients and severe depressed illness (IL-6), while some reports indicate that IL-6 is closer to plant and non-factory depression symptoms (Howren et al. 2009; Jehn et al. 2006; Musselman et al. 2001). Inflammatory cytokines like IL-6 are known to contribute to neurovegetative effects in the central nervous system (Dantzer et al. 2008) and thus to centrally influence IL-6 originating from the tumour.

Inflammatory processes in epithelial tumours are normal, and inflammation is the cause and promoter of the tumour (Balkwill and Mantovani 2001, Hagemann et al. 2007). Tumour cells and TAMs generate significant cytokine inflammatory levels, particularly IL-6 (Hagemann et al. 2007). After treatment for cancer, including radiation, inflammatory cytokines are also made. These pro-inflammatory cytokines, extracted from tumours and medication, could theoretically trigger the CNS pathways, evoking a ‘health actions’ syndrome consisting of behavioural and affective reactions to flu-like symptoms (Maier and Watkins 1998; Raison et al. 2006). These results show a pathway between tumours, which may contribute to chronic inflammation, eventually contributing to infection, through the tumour and treatment-based proinflammatory cytokines. Chronic inflammation also contributes to increased production of cortisol for inflammatory control and hence to dysregulation of the HPA axis (Jehn et al. 2006).

As alluded to above, patients with CH have an increased risk of developing haematologic malignancies (Sperling et al. 2017). Furthermore, patients with haematologic malignancies, such as leukaemia, MDS, and Hodgkin’s lymphoma, are at a higher risk of developing CVDs (Brunner et al. 2017; van Leeuwen and Ng 2016; Van Haelst et al. 2006). Moreover, other

CVD complications are associated with colorectal cancer patients (Wang et al. 2019). Also, CH is associated with cardiovascular diseases (Libby et al. 2019) and non-haematologic cancer (Bolton et al. 2019) (Figure 1.6). Indeed, the CH somatic mutations were found in bladder (Paterson et al. 2003) and stroma of breast cancer patients (Kurose et al. 2002; Patocs et al. 2007).

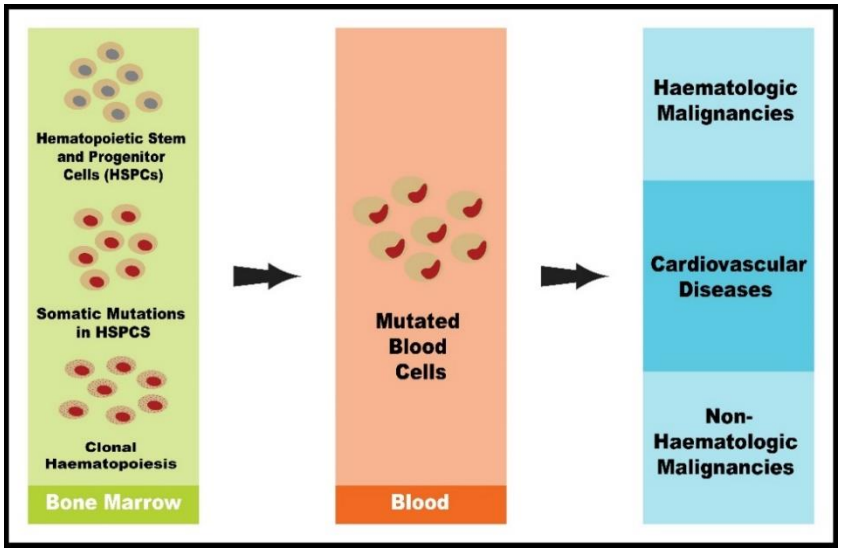


Figure 1.6 Acquires somatic mutation in HSPCs results in a state of clonal mutated blood cells that is associated with presence of CVDs, haematologic malignancies, and non-haematologic malignancies. Adapted from Libby et al. (2019)

1.3 Atherosclerosis

Statistically, one in three deaths in Western countries are caused by CVD. By 2030, approximately 23.6 million individuals globally are expected to die from CVD and related complications, such as myocardial infraction (MI) and stroke, which were estimated to have cost a total of £315.4 billion in 2014 (Go et al. 2014; Buckley and Ramji 2015). Several risk factors have been recognised, and these are generally classified into non-modifiable and modifiable risk factors. The latter include sedentary lifestyle and cigarette smoking (McLaren 2011). The modifiable risk factors involve gender, age, and a genetic predisposition to hypertension, hypercholesterolemia, systemic inflammation, and diabetes (McLaren 2011). CVD is mainly driven by atherosclerotic vascular diseases. Atherogenesis is a complicated process that involves both lipid and immune cells' entrance into and retention in the arterial wall. The entry of these inflammatory leukocytes enhances the progression of the disease and impairs plaque resolution (Bobryshev 2006; Woollard and Geissmann 2010).

Animal models have provided a potential explanation for the cardiovascular outcomes in CH; 10% of TET2 null cells transplanted into the atherosclerotic murine model have displayed clonal expansion, which results in an increased atherosclerotic plaque size, a nucleotide binding oligomerization domain, and a leucine rich repeat and pyrin domain containing inflammasome mediated interleukin 1β (NLRP3) induced by TET2 -/- macrophage (Fuster et al. 2017) . The effect of atherogenesis was reduced by the infusion of NLRP3 inhibitor. Jaiswal et al. (2017) transplanted cells from TET2 heterozygous (TET2+/-) or homozygous (TET2-/-) or control mice into the atherosclerotic murine model and observed that mice that received transplanted TET2-/- cells promoted a larger aortic root than control mice. In addition, the macrophage from TET2-/- mice expressed an increased transcript level of chemokine ligand 1 (CXCCL1), chemokine ligand 2 (CXCL2), chemokine ligand 3 (CXCCL3), IL-Iβ, IL-6, and platelet factor 4 suggesting an increased level of inflammation. Taken together, these results indicate a causal relationship between CH and CVD with inflammation as a possible mechanism.

1.3.1 Pathophysiology of atherosclerosis and link to the haematopoietic system

Atherosclerosis is initiated by an injured endothelium, which permits circulating apolipoprotein B containing lipoprotein to enter and to accumulate in the sub-endothelium and to undergo a chemical modification (Michael et al. 2013; Moss and Ramji 2016). Modified lipoprotein, especially oxidized low-density lipoprotein (Ox-LDL), which induces the increased expression of vascular cell adhesion protein 1 (VCAM1), intracellular adhesion molecule 1 (ICAM1), and proinflammatory cytokine production, causes leukocytes to migrate to the lesion site (Deng et al. 2015; Estruch et al. 2013; Milstone et al. 2015; Zhao et al. 2015).

Subsequently, after infiltration into the lesion site, T lymphocytes, monocytes, and dendritic cells uptake lipids and become foam cells that worsen the atherosclerosis cascade (Cochain and Zernecke 2015; Haka et al. 2015; Oliveira et al. 2013). Concomitantly, smooth muscle cells (SMCs) proliferate and migrate to take part in the progression and formation of plaque (Locher et al. 2002). Consequently, the formation of atherosclerotic plaque with cellular components, such as SMCs, leukocytes, and a cellular component, such as cholesterol and collagen, leads to blood flow restriction. Accumulating evidence has explained the provocation of atherosclerosis and inflammation as detailed in Figure 1.7.

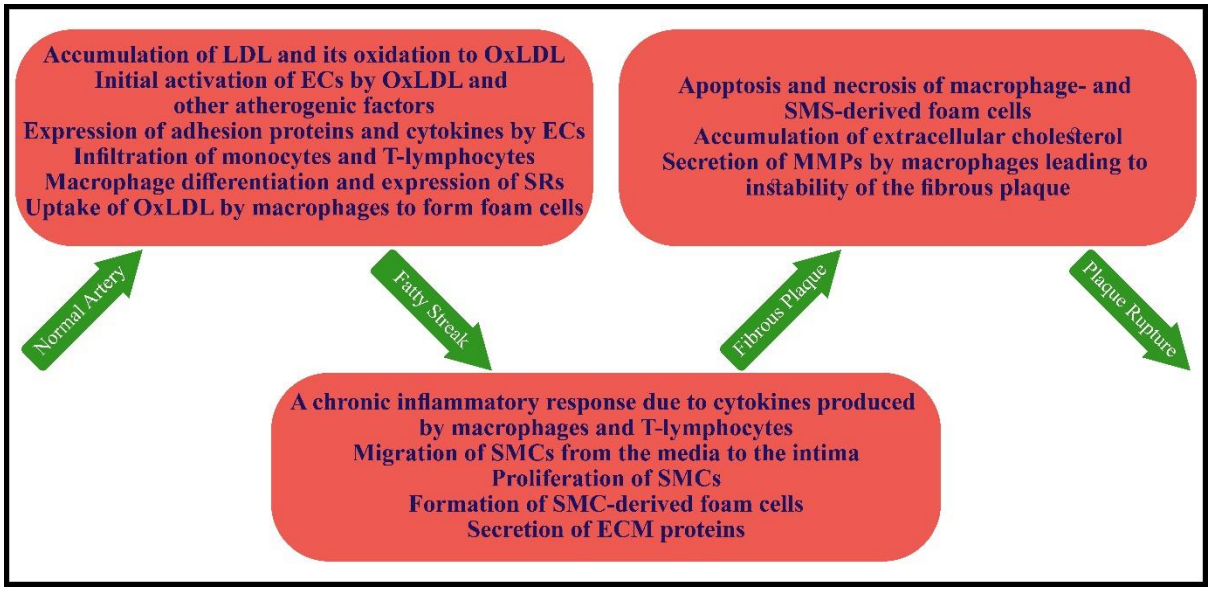


Figure 1.7 Overview of stages of atherosclerosis. Adapted from (Buckley and Ramji 2015).

Numerous in vivo and in vitro studies have demonstrated the accumulation of intracellular cholesterol and Ox-LDL in promoting endothelial dysfunction and apoptosis (Veas et al. 2016; Westerterp et al. 2016; Yu et al. 2014). Increased production of reactive oxygen species (ROS) with a decreased level of nitric oxide and bioavailability is involved in hypercholesterolaemia-induced smooth endothelial apoptosis (Wang et al. 2015b; Westerterp et al. 2016; Yu et al. 2014). Subsequently, a series of proinflammatory cytokines involving c-reactive oxygen, monocyte chemoattractant protein-1 (MCP-1), VCAM1, ICAM1, and tumour necrosis – α are triggered, which induce the recruitment of leukocytes to activate endothelial cells (Capers et al. 1997; Dje N'Guessan et al. 2009; Sanz-Rosa et al. 2005; Verma et al. 2003; Yamagata et al. 2010).

Neutrophils, platelets, and T-lymphocytes regulate the activation of macrophages. Physically, neutrophils are the initial cells that serve as home to the site of infection where they secrete granules to clear microorganisms (Zhang et al. 2009). Neutrophil depletion in apolipoprotein mice (ApoE-/-) fed an HFD diet culminated in a reduced plaque size and a reduction in the number of monocytes/macrophages and dendritic cells in aortic lysates, implicating neutrophils in rolling monocytes/macrophages and dendritic cells to the lesions during atherosclerotic development (Drechsler et al. 2010). Th1 regulates the activity of macrophages by releasing inflammatory cytokines and controls the production of IgE by mast cells (Conti and Shaik-daSthagiRiSaeb 2015; Kounis and Hahalis 2016). Furthermore, platelets interact with leukocytes through receptors and ligands (Etulain et al. 2015; Weber and Springer 1997). When platelets adhere to injured endothelium cells, the adherence facilitates leukocyte extravasation and plaque infiltration.

Platelets could regulate the activity of leukocytes for phagocytosis (Kockx et al. 2003) and the differentiation of monocytes to macrophages (Pervushina et al. 2004) and reactive oxygen species (Badrnya et al. 2014). The contribution of circulating monocytes to CVD has been studied extensively. Johnsen et al. (2005) reported that the monocyte count could be used as an independent risk factor for CVD (Johnsen et al. 2005) as monocytosis was shown to be present in CVD patients compared to those with no heart disease (Olivares et al. 1993). In humans, monocytes can be classified into three subpopulations based on the expression of cells' surface markers CD14 and CD16 (Passlick et al. 1989). The most abundant monocyte subset of all monocytes is the classical monocyte CD14+CD16-. The non-classical monocyte CD14-CD16+ and intermediate CD14+CD16+ subsets are linked to CVD (Auffray et al. 2009; Kashiwagi et al. 2010).

A causal relationship between atherosclerosis development and monocytosis has been established based on evidence accumulated using animal models. For instance, monocyte deletion and a murine model of defective monocytes show a blunted atherogenesis and plaque reduction (Rajavashisth et al. 1998; Stoneman et al. 2007). Murine monocytes are heterogeneous populations classified into two subtypes, namely, Ly6-Chi and Ly6-Clo monocytes, based on the expression of the cell surface Ly6-C (Woollard and Geissmann 2010). Both monocyte subsets enter atherosclerotic lesions but retain phonotypical differences in atherosclerotic plaque (Dragoljevic et al. 2018; Tacke et al. 2007). Ly6-C^{hi} monocytes accumulate in lesions, leading to exacerbated atherosclerosis (Robbins et al. 2012; Tacke et al. 2007).

Conversely, Ly6-C^{lo} seems to develop into $CD11C^{+}$ cells upon entry into the atherosclerotic lesion, which is usually regarded as a marker for dendritic cells or inflammatory macrophages (Tacke et al. 2007). Neutrophils have been reported to enhance atherogenesis and can develop lesions (Drechsler et al. 2010). Platelets are essential drivers of atherosclerosis because their activation and overproduction enhance the growth of plaques (Huo et al. 2003; Murphy et al. 2013). Collectively, the abundance and activation of leukocytes are critical to atherosclerotic development. Hypercholesterolaemia induces HSPC proliferation and differentiation into leukocytes, culminating in leucocytosis and the progression of plaque in ApoE-/- mice (Feng et al. 2012; Murphy et al. 2011), low-density lipoprotein receptor (LDLR-/-) mice (Feng et al. 2012; Seijkens et al. 2014), and scavenger receptor reporter type BI expression (SR-BI-/-) mice (Gao et al. 2014). Similar to mature cells, the haemostasis of intracellular cholesterol depends on the ATP-binding cassette transporter (ABCA1), ATB-binding cassette transporter subfamily G member (ABCG1), and ApoE on the cell surface of these cells (Murphy et al. 2011). Due to the deficiency of these lipoprotein receptors, diet-induced hypercholesterolaemia results in the proliferation and differentiation of HSPCs in the

BM niche, contributing to leucocytosis in PB (Gao et al. 2014; Murphy et al. 2011; Yvan-Charvet et al. 2010).

Mechanistically, Yvan-Charvet et al. (2010) revealed that the absence of ABCG1 and ABCA1 enhances the accumulation of cholesterol in HSPC membranes, particularly in the lipid raft, which increases the expression of the IL-3β subunit granulocyte-macrophage colony-stimulating factor receptors (GM-CSF), and elevates proliferative responses to GM-CSF and IL-3Rβ. Similarly, Murphy et al. (2011) showed that impaired cholesterol efflux did not activate E3-ubiquitin ligase, leading to an increased macrophage colony-stimulating factor (M-CSF) and IL-3Rβ. Eventually, increased IL3/ IL-3R axis, GM-CSF and M-CSF signalling skews the differentiation of HSPCs to the production of the myeloid lineage. In agreement with these findings, Gao et al. (2014) showed an expansion of HSPCs, CMP, and GMP in the BM in SR-BI-/- mice. Nevertheless, the infusion of reconstituted high-density lipoprotein (HDL) or purified ApoA-1 decreases the proliferation of HPCs in these models, implicating the role of cholesterol haemostasis in controlling the biology of HSPCs (Gao et al. 2014; Murphy et al. 2011; Yvan-Charvet et al. 2010).

Hypercholesterolaemia also enhances HSPC mobilization from the BM. HSPCs communicate with various cell types in the environment of the BM via chemokine receptor type 4 (CXCR-4)/stromal cell derived factor-1 (SDF-1). Histological studies have shown that megakaryocytes create clusters near the BM sinusoidal vessel (Gomes et al. 2010), suggesting modified conditions between haematopoietic equilibrium and thrombolysis. Furthermore, hypercholesterolaemia causes a distorted SDF-1 gradient between the PB and the BM, resulting in HSPC mobilisation. Another mechanism was reported by Westerterp et al. (2012), who revealed that a defect in cholesterol efflux induced extramedullary haematopoiesis with an increased production of IL-17/IL-23 and G-CSF in dendritic cells and splenic macrophages (Westerterp et al. 2012). In addition, hypercholesterolaemia modified the structure of the BM microenvironment by decreasing the number of osteoblasts, particularly N-Cadherin+ osteoblasts and Nestin+ mesenchymal stem cells, leading to a reduced production of SDF in the BM and an increased production of IL-17/IL-23 and G-CSF in the PB, which encourages the mobilisation of HSPCs. Collectively, these data indicate that hypercholesterolaemia-mediated changes in multiple aspects of haematopoiesis may drive atherosclerosis.

Successive work based on the link between CVD and CH has revealed that mutant myeloid cells aggravate atherosclerosis via increased activity of proinflammation (Fuster et al. 2017; Sano et al. 2018; Heyde et al. 2021). CH, which is a condition in which the clones of HSCs in an individual give rise to a disproportionate fraction of leukocytes (Heyde et al. 2021), is associated with an increased risk of CVDs. The mechanism of this association is poorly understood. Heyde et al. (2021) showed that the division rates of HSCs are elevated in

humans and mice with atherosclerosis. Indeed, mathematical analysis showed that the increased proliferation of stem cells accelerates clone expansion with driver mutations and somatic evolution. The increased rate of division in patients with atherosclerosis is enough to generate a 3.5-fold increased risk of CH by the age of 70. Heyde et al. (2021) also showed the expansion and acceleration of competitively transplanted Tet2-/- cells in the context of chronically increased activity of haematopoiesis. Thus, increased proliferation of HSCs is an essential factor supporting the link between CH and CVDs.

1.4 Apolipoprotein E

Apolipoproteins are amphipathic molecules that can interact with both the lipoprotein core lipids and the aqueous plasma environment. They act as biochemical keys, which offer lipoprotein particles access to particular locations where lipids are given, recognized, or modified (Semenkovich 2012). The table below displays large apolipoproteins, their chromosome positions, and their characteristics. Apolipoproteins may have therapeutic usefulness for serum measurements. For instance, increased apo B concentrations and reduced apo AI levels are linked to vascular disease.

Apolipoproteins have key roles in lipoprotein particles as structural elements, receptor ligands, and enzyme co-factors. Lipoprotein particles are needed for the transport of lipid energy, and for the synthesis of hormones, vitamins, and bile acids (Liao et al. 2016). In the transport of dietary and endogenous lipids to the peripheral tissues, where these lipids are being used as an energy supply, the apolipoproteins of ApoB and ApoE are important for restoring excess cholesterol from the peripheral tissues to the liver (Chen et al. 2011). The role of apolipoproteins in neurological processes is also important, for instance, for ApoE and ApoJ in the transport of lipids to the brain. No more research has been carried out into the roles of other apolipoproteins, such as ApoH, ApoD, and ApoM.

Table 1.1 Table displays large apolipoproteins, their chromosome positions, and their characteristics

Apolipoprotein	Functions
Apo B100	Atherogenic Lipoprotein Structural Compound (VLDL, IDL, LDL), VLDL secretion, LDL ligand, high vascular-associated levels
Apo B48	Secretion of chylomicron from the intestine
Apo E	Binding of LDL and LRP particles high in triglyceride, possible functions of Alzheimer's disease and neuronal damage Ligand
Apo AI	Structural HDL part, LCAT-activates, high levels of vascular disease prevention
Apo AII	Familial mixed hyperlipidaemia genetically and biochemically related
Apo AIV	A possible function for food intake regulation
Apo AV	Needed for natural lipolysis of lipoproteins rich in triglycerides
Apo CII	LPL activator
Apo CIII	LPL inhibitor
Apo (a)	Lp(a) forms a covalent connection with apo B100 which makes it immune to LDL receptors.

Apolipoprotein E (ApoE) was first described in 1970 as a minor apolipoprotein in very low density lipoprotein (VLDL), which serves to transport triglyceride from the liver to adipose and other tissues (Tsai, Rainey and Bollag, 2017), and, subsequently, was recognised as a main apolipoprotein in cholesterol-fed animals (β VLDL). In addition, it was found to be part of the subclass of a high-density lipoprotein (HDL, HDL1). ApoE is a 34 KDa glycoprotein known for its role in lipid metabolism (Mahley 2016; Tudorache et al. 2017; Zhao et al. 2018; Zhong et al. 2016). It is mainly synthesised in the liver, but other organs and tissues also produce ApoE, including the brain (astrocyte), kidneys, spleen, adrenal glands, gonads, SMCs, and macrophages (Greenow et al. 2005; Meir and Leitersdorf 2004; Yousuf and Iqbal 2015). Furthermore, ApoE is involved in other functions, such as inflammation, antimicrobial defence, oxidative stress, neuroprotection (Tudorache et al. 2017), and immunoregulation (Mahley et al. 2009).

The structure, polymorphisms, and functions of ApoE have been studied extensively. Because ApoE has a major role in lipid metabolism, it was found to be associated with atherosclerosis (Mahley et al. 2009), type 2 diabetes, and CVD (El-Lebedy et al. 2016). In addition, ApoE has been correlated with Alzheimer's disease (Genin et al. 2011), renal diseases (Hsu et al. 2005; Liberopoulos et al. 2004), Parkinson's disease (Jasinska-Myga et al. 2007), and cancer (Davidson 1996; Ito et al. 2006; Liu et al. 2016). In addition, ApoE has been correlated with Alzheimer's disease (Genin et al. 2011), renal diseases (Hsu et al. 2005; Liberopoulos et al. 2004), Parkinson's disease (Jasinska-Myga et al. 2007), and cancer (Davidson 1996; Ito et al. 2006; Liu et al. 2016).

1.4.1 Structure

The ApoE gene is a protein of 299 amino acids localised on the long arm of chromosome 19 at position q 13.2 in humans (El-Lebedy et al. 2016; Zhong et al. 2016). Murine ApoE is located on chromosome 7 band A2 (Maloney et al. 2007). Genes of both humans and mice consist of three introns and four exons. ApoE is a polymorphic gene with a single nucleotide polymorphism (rs42358 and rs7412), resulting in three various alleles, *ε4*, *ε3* and *ε2*, coding for three isoforms, ApoE 2, ApoE 3, and ApoE, with three homozygous phenotypes *(ApoE ε2/ ε2, ApoE ε3/ ε3* and *ApoE ε4/ ε4)* and four heterozygous phenotypes *(ApoE ε3/ ε2, ApoE ε4/ ε2* and *ApoE ε4/ ε3)*. The three proteins vary by a single amino acid change at position 112 and 158, where arginine and cysteine exist (ApoE2: Cys-112, ApoE4: Arg-112, Arg-158; Cys-158; ApoE3: Cys-112, Arg-158) (Greenow et al. 2005).

The differences in the single amino acid affect the protein structure and affect the ability to bind to the lipid and receptor (Depypere et al. 2016; Zhong et al. 2016), and thus ApoE regulates lipid metabolism in an isoform-dependent manner. It has two functional and structural domains, namely, the N-terminal domain (amino acid 1- 191) and the C-terminal domain (amino acid 206-299), separated by a hinge region (Figure 1.8). The N-terminal domain is composed of four anti-parallel helix bundles containing a low-density lipoprotein receptor binding region (residue 136 – 150) and a heparin sulphate proteoglycan binding (HSPG) region with a weak capability of lipid binding. The C-terminal domain consists of an amphipathic α – helix with a high affinity for lipid binding in the region of 244-272.

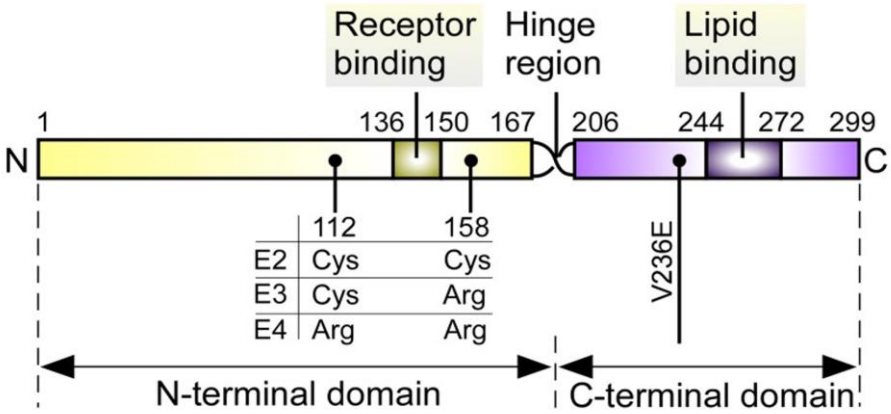


Figure 1.8 Graph showing human ApoE. Adapted from Zhao et al. (2018)

1.4.2 The role of ApoE in atherosclerosis

ApoE is a part of the structural components of all lipoproteins except LDL. ApoE functions as a ligand for all lipoproteins on the cell surface to clear VLDL remnants and chylomicron (Curtiss and Boisvert 2000). Several reports have implied a strong anti-atherogenic role of ApoE. Moreover, the ApoE murine model is severely hypercholesterolemic compared to the wild-type murine model, and the elevation in cholesterol level is mainly distributed in the lower density lipoprotein due to impaired clearance (Plump et al. 1992; Zhang, Reddick et al. 1994). In the ApoE murine model, a complex atherosclerotic lesion is developed even on a low-fat diet due to the accumulation of remnant lipoprotein. Furthermore, in the ApoE murine model, atherosclerosis is prevented by the increasing level of circulating ApoE via either ApoE transgene expression (Shimano et al. 1992), by the infusion of synthetic ApoE peptide mimics (Nikoulin and Curtiss 1998), or by the transfer of the recombinant adenovirus mediated gene to the liver (Desurmont et al. 2000).

BM transplantation techniques have been largely used to explore the role of monocyte/macrophage-derived ApoE on atherosclerosis. Several studies have revealed that macrophage derived ApoE has anti-atherogenic properties separately from its impact on the level of plasma lipids (Van Eck et al. 2000). In addition, methods other than BM transplantation have proved the anti-atherogenic role of the expression of ApoE in the vessel wall (Shimano et al. 1995). Therefore, transgenic mice expressing ApoE in the vessel wall revealed a decrease in the number of atherosclerotic lesions. Additionally, the low-level expression of ApoE in the artery wall of the ApoE murine model utilizing a retroviral transduction system resulted in the decreased formation of early foam cells (Hasty et al. 1999). Collectively,

macrophage-derived ApoE has an anti-atherogenic role through alterations in plasma lipids and its actions on or within the artery wall.

1.4.3 The role of apolipoprotein E in haematopoiesis

Previously, the ApoE gene has been reported to be abundantly expressed in haematopoietic stem and progenitor cells (HSPCs) and has been found to regulate the proliferation of HSPCs, monocytes and monocyte accumulation in atherosclerotic lesions (Murphy et al. 2011). Murphy et al. (2011) revealed that ApoE binds to proteoglycans to regulate the proliferation of HSPCs. LXR-inducible pools of ApoE on the surface of HSPCs revealed that ApoE binds to proteoglycans to regulate the proliferation of HSPCs, the expansion of myeloid cells, monocytosis, and the accumulation of monocytes in atherosclerotic lesions. Furthermore, heparin treatment showed that proteoglycan bound ApoE on the cell surface suppresses the proliferation of HSPCs. The ApoE gene interacts with ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1), both of which are expressed in HSPCs, to promote the efflux of cholesterol and to decrease the downstream signalling of IL3 receptors (Murphy et al. 2011).

The expression of ABCA1 and ABCG1 points to the regulatory role of the ApoE gene in haematopoiesis. In addition, heparin sulphate proteoglycans (HSPGs) help facilitate the interactions of ABCA1, ABCG1 and ApoE, resulting in the promotion of cholesterol efflux and phospholipid efflux (Murphy et al. 2011). Moreover, ApoE, ABCA1, and ABCG1 deficiencies have downstream effects, including cholesterol accumulation, increased cell surface levels, and signalling of the common β subunit of the IL-3/GM-CSF (CBS) through the STAT and ERK signalling pathways. In line with these findings, competitive BM transplantation studies have revealed an increased number of donor HSPCs, monocytes, and CD45.2 monocytes in atherosclerotic plaque.

In addition, a competitive BM transplantation study revealed that the ApoE cells acted autonomously to control the proliferation of HSPCs, monocytes, and neutrophils and the accumulation of monocytosis in atherosclerotic plaque. These data revealed that leucocytosis contributes to the development of atherosclerosis and provides substantial evidence of the link between leucocytosis and hypercholesterolemia. In addition, Tie et al. (2014) revealed that ApoE and WT mice fed an HFD had accumulated oxidized LDL and increased ROS. In their study, they found that hypercholesterolaemia causes oxidant stress in HSCs, which modifies the gene expression and the regulation of the cell cycle. This results in HSC ageing and is demonstrated by the decrease in the long-term HSC compartment, telomere erosion, quiescent loss, and reduced reconstitution capacity of HSCs.

1.4.4 ApoE and LDLR knockout murine models

The use of animal models of atherosclerosis is a key approach to studying and to developing the understanding of the mechanism underlying atherosclerosis development and progression. Generally, animal models of atherosclerosis depend upon the acceleration of plaque formation due to diet manipulation, the genetic manipulation of lipid metabolism, and the induction of additional risk factors of atherosclerosis (Emini Veseli et al. 2017). In the last decade, the murine model became the most predominant species to study atherosclerosis due to its fast reproduction, the simplicity of genetic manipulation, and the monitoring of atherogenesis in a reasonable amount of time (Bond and Jackson 2011; Getz and Reardon 2012; Schwartz et al. 2007). However, mice have a low level of atherogenic LDL and VLDL, as cholesterol is transported in an HDL-like particle. In addition, mice are moderately resilient to atherosclerosis development due to differences in their lipid profiles compared to humans. Thus, the manipulation of the genes involved in lipid metabolism is required (Getz and Reardon 2012; Meir and Leitersdorf 2004).

In 1992, two laboratories developed the first line of ApoE (Piedrahita et al. 1992; Plump et al. 1992) using homologous recombination to delete the ApoE gene in embryonic stem cells. The ApoE-/- mice were normal, had a comparable body weight, and were born at the expected frequency (Jawien, 2012; Plump et al. 1992); however, the profile of lipoproteins showed a significant difference compared to the wild type. Indeed, ApoE-/- mice were unable to clear lipoprotein, resulting in an increased plasma level of cholesterol (400-600 mg/dl) compared to the wild type (75-110 mg/dl) when fed an NCD (Nakashima et al. 1994). This drastic difference was attributed to an elevated level of VLDL particles. Therefore, the deficiency of ApoE is sufficient to induce significant variations in lipoprotein metabolism, resulting in significant hypercholesterolaemia.

Moreover, the absence of ApoE increases sensitivity to a diet high in cholesterol and fat. Indeed, feeding a Western-type diet (composed of 21% fat, 0.15% cholesterol) for several weeks doubled the plasma level of cholesterol in wild-type mice, while it increased fourfold in ApoE-/- mice (Plump 1992). Extensive atherosclerosis was observed in both types of mice by the age of 2-3 months (Reddick et al. 1994). Conversely, the heterozygous ApoE mice (ApoE+/-) did not exhibit differences in the plasma levels of cholesterol, which suggests that a 50% reduction in ApoE was not sufficient to alter plasma lipids.

Indeed, Plump et al. (1992) revealed that the heterozygous ApoE deficient mice fed a Western-type diet showed a similar cholesterol level to control mice, but postprandially, showed a significant elevation in the levels of lipoprotein. Therefore, it seems that the deficiency of ApoE of greater than 50% is essential to show an impact on the fasting level of lipoprotein. In addition, Shimano et al. (1992) overexpressed rat ApoE into transgenic mice reaching a 3 to 4-fold elevation in the plasma level. In comparison to control animals, the transgenic mice showed a 43% lower fasting level of cholesterol, and when transgenic mice were fed a Western-type diet containing 1% of cholesterol, the cholesterol level rose significantly. Therefore, the response to a high cholesterol diet in the over-expressed ApoE mice reduced the magnitude but not the percentage elevation of plasma cholesterol. Therefore, it seems that the overproduction and underproduction of ApoE could affect the responsiveness of ApoE to diet (Plump et al. 1992).

In the setting of atherosclerosis, the attachment of monocytes to the endothelial cells was observed from 6 weeks of age, and after 8 weeks of age, the development of foam cells was visible (Emini Veseli et al. 2017).

An intermediate lesion containing mostly SMCs and fibrous plaque comprising SMCs, an extracellular matrix, and a necrotic core covered with a fibrous cap was noted after 15 – 20 weeks. The time course for lesion formation is accelerated upon feeding a Western-type diet, implying a diet-dependent mechanism. Furthermore, ApoE-/- mice developed an atherosclerotic plaque at vascular branch points, especially at the aortic root, the main branches of the aorta, and the carotid and pulmometry arteries. Although ApoE is regarded as a preferable atherosclerotic model due to the spontaneous development of atherosclerosis even upon feeding an NCD (Meir and Leitersdorf 2004), some limitations have been reported. For instance, ApoE has an impact on inflammation, oxidation, and the reversal transport of cholesterol by macrophages and the proliferation and migration of SMCs. These functions may affect the progression and development of atherosclerotic plaque independent of the plasma level of lipids (Getz and Reardon 2009). In addition, VLDL is the most abundant lipoprotein in ApoE-/- mice, and LDL is the most abundant for human atherosclerosis (Plump et al. 1992).

Low density lipoprotein receptor (LDLR) is a membrane receptor, which has a molecular weight of 160 KDa (Emini Veseli et al. 2017: Marais, 2004). It mediates cholesterol-rich LDL endocytosis and therefore maintains the LDL plasma level (Emini Veseli et al. 2017; Marais

2004). The murine model with target inactivation of LDLR was created in 1993 (Ishibashi et al. 1993). LDLR deficiency (LDLR-/-) in addition to mutations in genes encoding for LDLR leads to similar phenotypic outcomes as described in familial hypercholesterolaemia (Defesche 2004; Marais 2004). Low density lipoprotein (LDL) deficient mice showed a moderate increase in the plasma level of cholesterol, and they developed no or moderate atherosclerosis when fed a normal diet (Ishibashi et al. 1994). In the LDLR-/- murine model, LDL is elevated among lipoprotein particles, while HDL and triglycerides remain unaltered (Ishibashi et al. 1993; Ishibashi et al. 1994). Plaque development is generally like that seen in ApoE-/- mice (Knowles and Maeda 2000).

The development of plaque occurs in a time-dependent manner starting in the proximal aorta and continuing to the distal aorta (Knowles and Maeda 2000). In addition, the locations where blood flow is distributed are more vulnerable to atherosclerosis lesion (Knowles and Maeda 2000). The LDLR-/- murine model has advantages in comparison to the ApoE model. Firstly, it generates a human-like lipid profile, as plasma cholesterol is carried by LDL particles (Emini Veseli et al. 2017). Then, the deficiency of LDLR does not affect inflammation compared to ApoE. Therefore, in the LDLR-/- model, the development of atherosclerosis plaque is dependent on the increased level of plasma lipids (Getz and Reardon 2012). Thirdly, it shares similar features with familial hypercholesterolaemia as seen in humans, which is caused by the deficiency of functional LDLR (Lee et al. 2017). Shortly after the creation of the ApoE and LDLR murine models, the ApoE/LDL receptor double knockout murine model was introduced. It develops severe atherosclerosis and hyperlipidaemia (Bonthu et al. 1997). The development of atherosclerosis is more profound in the ApoE/LDL receptor double knockout than in ApoE deficiency alone (Witting et al. 1999). In addition, there are no significant differences in the lipoprotein profile compared to the ApoE model (Caligiuri et al. 1999) except an observed increase in the B48 and B100 apolipoproteins (Ishibashi et al. 1994). The ApoE/LDL receptor double knockout murine model is a suitable model to explore the anti-atherosclerotic impact of potential treatments without the use of an atherogenic diet (Jawień et al. 2004).

1.5 Leukaemias

Leukaemias are a group of diseases that are characterised by the accumulation of malignant WBC in the blood or BM. These malignant cells result in symptoms that are caused by the failure of BM, such as anaemia and neutropenia, and organ infiltration, such as the spleen and the liver (Hoffbrand 2011). Leukaemias are classified into four main types – first, as either acute or chronic, and then these are further divided into myeloid and lymphoid. Acute

leukaemias, in which genetic alterations occur in HSPCs, are generally aggressive. These genetic alterations lead to an increased proliferation rate, decreased apoptosis, and a blockage in cellular differentiation (Siveen et al. 2017; Zjablovskaja and Florian 2019). Together, these events lead to the accumulation of blast cells in the BM (Hoffbrand 2011).

1.5.1 Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a clonal disorder of HSCs characterised by differentiation pathway blockage and the accumulation of immature cells in the PB and the BM (Ferrara and Schiffer 2013). The incidence of AML increases in older populations (Khaled et al. 2016), with a prevalence of 3.8 cases per 100,000 in the general population compared to 17.9 per 100,000 in adults aged 65 and older (Estey and Döhner 2006). As AML is a heterogeneous disease, several systems have been used to classify AML into subgroups based on immunological and cytogenetic features, cell morphology, and cytochemical stains (Konoplev and Bueso-Ramos 2006). The French American-British (FAB) system classifies AML based on cytomorphology and cytochemistry stains (Kansal 2016) (Table 1.2). The World Health Organisation retains the criteria of FAB classification and incorporates molecular, genetic, and recurrent cytogenetic abnormalities (Arber et al., 2016) (Table 1.3).

AML is diagnosed when the BM contains more than 30% blast cells. Recognition of those patients with AML who are more likely to respond to the current treatments and those who are less likely to do so is of clinical importance. Both patient-specific factors and disease-specific factors influence the prognosis of AML (Liersch et al. 2014). The most important patient-specific factor is the patient's age, while the strongest disease risk factor is chromosome abnormalities (Liersch et al. 2014). Cytogenetic and molecular abnormalities are the most significant factors used to place AML patients into prognostic categories (Mrózek et al. 2012) (Table 1.4).

Table 1.2 Classification of AML using FAB system (Bain and Estcourt, 2013)

Classification	Description
M0	AML with minimal evidence of myeloid differentiation
M1	AML without maturation
M2	AML with maturation
M3	AML with specific cytological features of acute promyelocytic leukaemia
M4	AML showing both granulocytic and monocytic differentiation
M5	AML with monocytic differentiation
M6	Erythroleukaemia, erythroid cells at least 50% of nucleated cells and blast cells at least 30% of non-erythroid cells
M7	AML with megakaryocytic differentiation

Table 1.3 Acute myeloid leukaemia and related neoplasm and acute leukaemia of ambiguous lineage classification by WHO.(2008) (Döhner et al., 2010).

Acute myeloid leukaemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
Acute myeloid leukaemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukaemia not otherwise specified (NOS)
Acute myeloid leukaemia with minimal differentiation
Acute myeloid v with maturation
Acute myelomonocytic leukaemia
Acute monoblastic/monocytic leukaemia
Acute erythroid leukaemia
Pure erythroid leukaemia
Erythroleukaemia, erythroid/myeloid
Acute megakaryoblastic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis (acute myelofibrosis; acute myelosclerosis)
Myeloid sarcoma (extramedullary myeloid tumour; granulocytic sarcoma; chloroma)
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (transient myeloproliferative disorder)
Myeloid leukaemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute undifferentiated leukaemia
Mixed phenotype acute leukaemia with t(9;22)(q34;q11.2); BCR-ABL1
Mixed phenotype acute leukaemia with t(v;11q23); MLL rearranged
Mixed phenotype acute leukaemia, B/myeloid, NOS
Mixed phenotype acute leukaemia, T/myeloid, NOS
Provisional entity: Natural killer (NK)–cell lymphoblastic leukaemia /lymphoma

Table 1.4 European LeukaemiaNet's stratifications for cytogenetic and molecular lesions as prognostic factors (Mrózek et al. 2012).

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD (normal karyotype)
	Mutated CEBPA (normal karyotype)
Intermediate-I	Mutated NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLL-AF9
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
	t(6;9)(p23;q34); DEK-NUP214
	t(v;11)(v;q23); MLL rearranged
	-5 or del(5q); -7; abnl(17p); complex karyotype

The treatment of AML consists of two phases, namely, induction and consolidation, which may include stem cell transplantation. The induction phase is achieved when complete remission is reached. Complete remission is characterised by the presence of less than 5% blast cells in the BM. The standard induction regimen involves an anthracycline-daunorubicin combination given for 3 days with the progressive infusion of cytarabine for 7 days ('3+7'). The aim of the consolidation phase is to eradicate the residual leukaemia cells that might be present after the induction phase. The discovery of the molecular and genomic landscape of AML, the recognition of mutations linked to AML pathogenesis, and understanding of the mechanism of resistance to treatment from translational research helped to increase the treatment options for AML, which led to a remarkable advance and the approval of the Food and Drug Administration (FDA) for new therapeutic treatment for AML patients. Targeted therapies and combinations of therapeutic agents of different classes to overcome treatment resistance expanded further the options of the treatment and improved the survival rate (Liu 2021), for instance, BCL-2 inhibitor, which is a member of anti– and pro–apoptotic proteins of the BCL-2 family. BCL–2 prevents apoptosis, and the expression of BCL-2 in AML has been linked to reduced sensitivity to cytotoxic chemotherapy and an increased rate of relapse (Campos et al. 1993). Isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) inhibitors result in a promising response for the corresponding mutations (DiNardo et al. 2018).

Indeed, IDH1 and IDH2 are essential enzymes for the oxidative isocitrate carboxylation. IDH1 and IDH2 mutations lead to elevated 2-hydroxyglutarate (2-HG). 2-HG induces DNA and histone methylation resulting in a cellular differentiation blockage and tumorigenesis (Liu 2021). IDH1 (ivosidenib) and IDH2 (enasidenib) as an oral small molecule inhibitor have been developed and have shown a promising response for the corresponding mutations with a complete remission of 21.6% (DiNardo et al. 2018). The development of targeted therapies, such as targeting fms-like tyrosine kinase3 (FLT3) mutations, has significantly changed the treatment of AML. The mutation of FLT3 occurs in nearly 30% of patients, that is, 20% to 25% with FLT3-ITD and 5% to 10% with FLT3-TKD) and has been linked to elevated relapse risk and more proliferative disease (Liu 2021). In addition, the study of randomized phase III ADMIRAL with single-agent gilteritinib recognized gilteritinib for the treatment of relapsed refractory FLT3-mutated AML (Perl et al. 2019). Furthermore, immunotherapy including the inhibition of immune negative regulators, antibody-based treatment, and potentially CART cells, may increase the therapeutic options for AML. For example, targeting CD47 has led to the engulfment of leukaemia cells and therapeutic elimination (Chao et al. 2020). Indeed, magrolimab in combination with azacytidine showed a promising result in both MDS and AML patients (Sallman et al. 2019). Moreover, blocking Tim-3 pathways, which has been recognised as an antigen on AML stem cells and leukaemic blast, has demonstrated efficacy in blocking AML engraftment in the mouse xenotransplantation model (Kikushige et al. 2010). Indeed, Sabatolimab which is used to block humanized anti-Tim-3 IgG4 via blocking phosphatidylserine to Tim-3 binding, has shown no treatment-related death (Borate et al. 2019). Furthermore, fewer antigens, such as CD133, CD123, and chimeric antigen target cells (CART), are being evaluated in pre-clinical studies (Liu 2021).

1.5.2 Leukaemia stem cells

The development of adult normal blood cells relies on the balanced self-renewal and differentiation of HSPCs, whereas the abnormal activation of self-renewal or blockage of differentiation pathways could lead to malignant cell proliferation and ontogenesis. Leukaemia stem cells (LSCs), as a rare cell population present in leukaemia, confer chemotherapy resistance and are responsible for the maintenance and relapse of the disease. Therefore, the treatment of leukaemia requires the eradication of LSCs for long-term remission. The treatment outcome of myeloid leukaemia remains poor due to disease relapse. Fialkow et al. who used x-inactivation patterns in different haematopoietic cell lineages, suggested that the early stem and progenitor cells are involved in the development of leukaemogenesis. Dick et al. were pioneers in improving the SCID and the non-obese diabetic (NOD) mice xenotransplantation stem cell assay systems that were described by Weissman et al. (Huntly

and Gilliland 2005). LSCs give rise to identical daughter cells and differentiated cells, thus sustaining and perpetuating AML and CML. LSCs have distinct features compared to the bulk of AML and CML cells and normal HSCs, which makes it very difficult to eliminate them with conventional chemotherapy. It has been shown that LSCs are responsible for the continuous proliferation and growth of chronic myeloid leukaemia (CML) by using separated populations of cells from CML patients or mice with a CML-like disease transduced by BCR-ABL1 (Hu et al. 2016), where LSCs reside in the HSC compartment (Eisterer et al. 2005; Bhatia et al. 1997; Hu et al. 2016).

The AML population is composed of poorly differentiated blasts and is replenished by LSCs. However, they are biologically different, making it difficult to eliminate LSCs with conventional therapy. In AML, seminal studies have resulted in the hypothesis of cancer stem cells (CSCs), which assumes that cancers are formed in a cellular hierarchy similar to normal tissues (Dick 2008). At the hierarchal apex are multipotent, long-lived CSCs, mostly quiescent with a marked capacity for self-renewal, which maintain the disease and differentiate into a bulk of cancer cells (Goardon et al. 2010). Early studies suggested that AML LSCs resemble normal HSCs sharing limited immunophenotype (CD34+CD38-) (Bonnet and Dick 1997; Ishikawa et al. 2007). However, the idea that LSCs exist in the compartment of CD34+CD38- has been challenged (Goardon et al. 2010). In mice, modelling of AML has revealed that mature GMP generates transplantable LSCs (Cozzio 2003; Krivtsov et al. 2006; Goardon et al. 2010). Overall, this indicates that, depending on the leukaemia, LSCs can occur in either HSCs or the more committed progenitor compartment (e.g., GMP). As minimal residual disease is a prognostic factor for disease relapse and poor outcomes, it is possible that the minimal residual disease contains the (rare) LSC population. Therefore, treatment that focuses on the eradication of LSCs will ultimately reduce the minimal residual disease and improve outcomes. The understanding of myeloid leukaemia pathogenesis has led to the identification of many actionable pathways that can be translated into relevant therapeutics. Potential targets with the characteristic features of LSC populations include NF-Kappa B activation, bcl-2 expression, oxidative and metabolic states, and epigenetic modifiers and cell cycle signalling inhibitors.

Many pathways are believed to regulate the development of both stem cells and cancer cells. For instance, the overexpression of oncogene bcl-2 to prevent apoptosis leads to an increased number of HSCs, indicating that cell death has a role in regulating HSC homeostasis (Domen et al. 1998). In addition, signalling pathways, such as Notch, Shh, and Wnt, are involved in

oncogenesis and stem cell self-renewal regulation. These signalling pathways result in tumourigenesis when dysregulated, suggesting that the stem cells are targets for transforming mutations in cancer. Therefore, fewer mutations are required to sustain self-renewal, and stem cells can exist for a long period of time in highly proliferative tissues rather than dying quickly like mature cells. FDA-approved treatment of AML patients in recent years includes DNA alkylates, topoisomerase inhibitors, antibiotics, and steroids (Mussai et al. 2015). However, particularly for elderly patients, AML has a very poor prognosis (Ding et al. 2017). The detection of LSC in AML in the course of the diagnosis, prognosis, surveillance, and drug screening of AML is particularly important. Therapies that target LSCs through aberrant phenotypes, such as antigens expressed by LSCs (e.g., CD123, CLL-1) and kinase families (e.g., c-kit) could be very effective. In addition, targeting NF-Kappa B activation, which is responsible for the regulation of cell survival and proliferation, can also be very effective. Moreover, targeting LSCs through their mitochondrial protein and biogenesis has demonstrated an ability to ablate AML cells and LSCs (Škrtić et al. 2011).

1.6 Thesis aim and objectives

Atherosclerosis is a chronic inflammatory disease in which the vessel wall accumulates lipoproteins and leukocytes infiltrate, inducing the build-up of atherosclerotic plaque, which upon rupture, results in fatal complications, such as stroke and myocardial infraction. In particular, haematopoietic cells are known to be a critical contributor to the initiation and development of atherosclerotic lesion (Poller, Nahrendorf and Swirski, 2020). As HSCs supply and produce inflammatory immune cells, which are vital to the development of CVDs, CVDs in turn significantly influence haematopoiesis. Moreover, the common risk factors of CVDs, such as hyperlipoproteinemia, have a considerable impact on the process of haematopoiesis; similarly, atherosclerosis and its complications strongly affect haematopoiesis. Former reports have identified a causative association between leucocytosis and hypercholesterolaemia, which indicates that atherosclerosis might be enhanced by leucocytosis (Coller 2005). Studies have been conducted to investigate various aspects of the impact of an HFD on monocytes and atherosclerosis in an environment lacking ApoE (Murphy et al. 2011; Tie et al. 2014); however, the broad requirement for ApoE in haematopoiesis and in the initiation and progression of leukaemia in the context of an HFD and a normal chow diet (NCD) remains to be elucidated. Thus, the underlying hypothesis of this thesis is that ApoE plays an essential role in the initiation and development of leukaemia. Here, new studies were conducted in AML to study the ApoE driven effect of an HFD and an NCD on the development of haematopoiesis and leukemogenesis. Therefore, the overall aim of this project was to provide insights into the

requirement of the ApoE gene in haematopoiesis and the development of leukaemia in the context of an HFD and an NCD with the following objectives:

1. To investigate the requirement of the ApoE gene in haematopoiesis using a germline knockout ApoE murine model

2. To assess the impact of an HFD and an NCD on the development of haematopoiesis using a germline knockout ApoE murine model

3. To explore the impact of an HFD and an NCD on the development of leukemogenesis using an MLL-AF9 murine leukaemia model

Chapter 2: Method and Materials

2.1 ApoE knockout mice

2.1.1 Animals, breeding and diets

C57BL/6 male ApoE$^{-/-}$ knockout (KO) mice and wild-type (WT) ApoE$^{+/+}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) (Murphy et al. 2011). The deletion of ApoE was achieved using homologous recombination (Plump et al. 1992). Briefly, positive selection and polyadenylation capture was used to target the ApoE gene. Embryonic stem cells' genomic DNA and mice were processed with EcoRI *(Escherichia Coli)*, and a 3' ApoE flanking probe was utilized to search for southern blots. Site specific integration at the ApoE locus was indicated by the addition of 2.9 kb signals to the endogenous 8.3 kb signal. To ensure the integrity of homologous recombination was achieved, two additional probes (probes B and C) and an additional digestion with NcoI were used. Then, targeted cells were injected into host blastocysts of C57BL/6J and BALB/cJ mice. In total, 12 males and 4 females were generated; 3 of the resulting males transferred the disrupted ApoE allele via germ line. The heterozygote mice were crossed and the target alleles were segregated in a Mendelian fashion. The homozygous mice had viable progeny. The strategy used to disrupt the ApoE gene in a mouse is seen in Figure 2.1.

ApoE$^{-/-}$ (KO) mice were bred with ApoE$^{-/-}$ (KO) to obtain a cohort of germ line mutated ApoE-/- (KO) mice. In addition, (WT) Apo$^{+/+}$ mice were bred with Apo$^{+/+}$ mice to obtain a cohort of control mice. The breeding and the maintenance of mice were conducted at the animal unit of Cardiff University (Heath Hospital) under Home Office-approved legislature (project license 30/3380 – 30/3365 and personnel license I052809AA) and an institutional ethics review. All mice were housed in a pathogen-free environment and remained under suitable 12-hour day/night cycles and fed a normal diet unless otherwise stated. For HFD experiments, male mice aged 4-8 weeks were fed an HFD containing 21% (WT/WT) pork lard supplemented with 0.15% cholesterol (special diet service, Witham, UK) for 12 weeks.

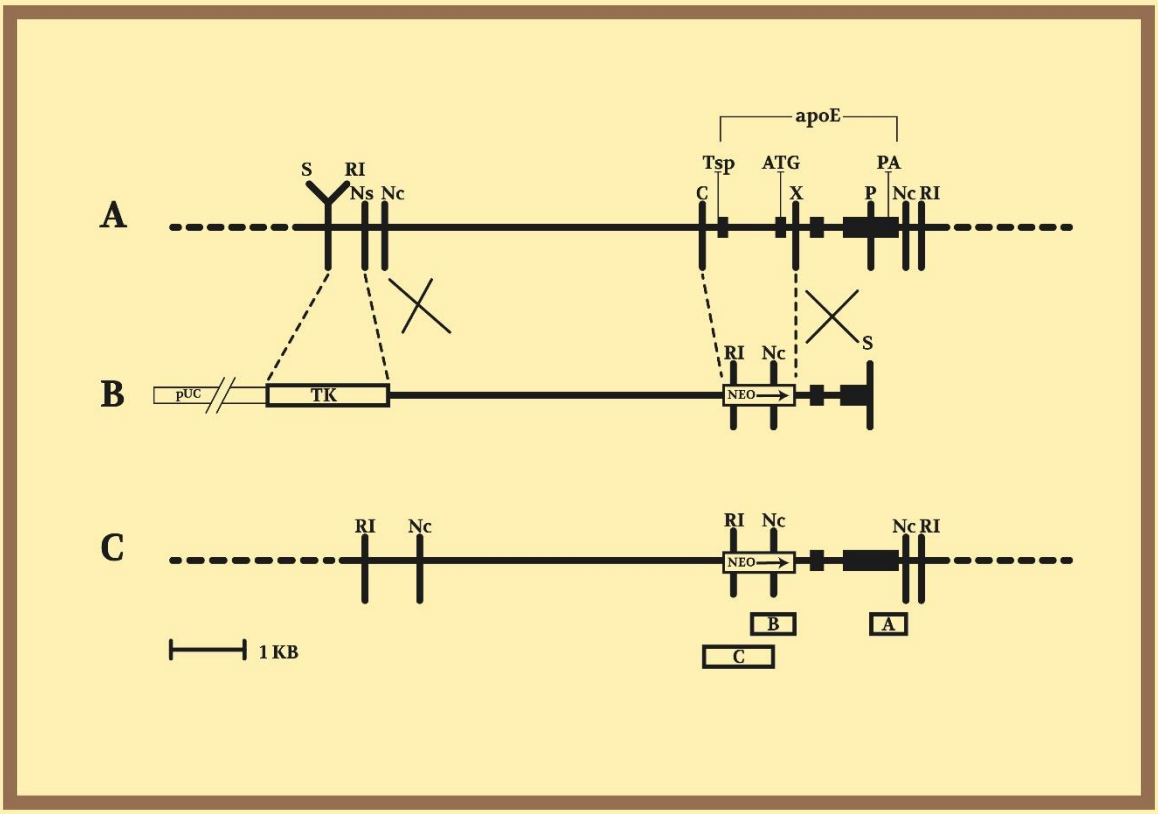


Figure 2.1 Strategy used to disrupt ApoE gene in a mouse. A. shows the map of endogenous ApoE gene in murine as well as its flacking sequences. Solid boxes indicate exon sequence, and solid line indicates flanking and flanking and intron. In addition, endonuclease restriction sites that were used for screening and cloning are shown (RI: EcoRI; Ns, Nsil; P, Pvul X, Xbal; C, Sacll; Nc, Ncol; S, Sail). Essential landmarks are shown (PA, polyadenylation signal, ATG, translation start site; Tsp, transcription start point). B. shows vector used for targeting ApoE locus (NEO, neomycin resistance gene TK, herpes simplex virus thymidine kinase gene, pUC, and pUC18). C. indicates the organization of locus after homologous recombination. The box under line C represents the probe used for screening homologous integrants. B and C are the two probes that confirm specific integrity confirmation (Plump et al. 1992).

2.1.2 Identification method

Eight-week-old mice were ear notched for the purpose of identification (Figure 2.2). Ear notched samples were used for mice genotyping.

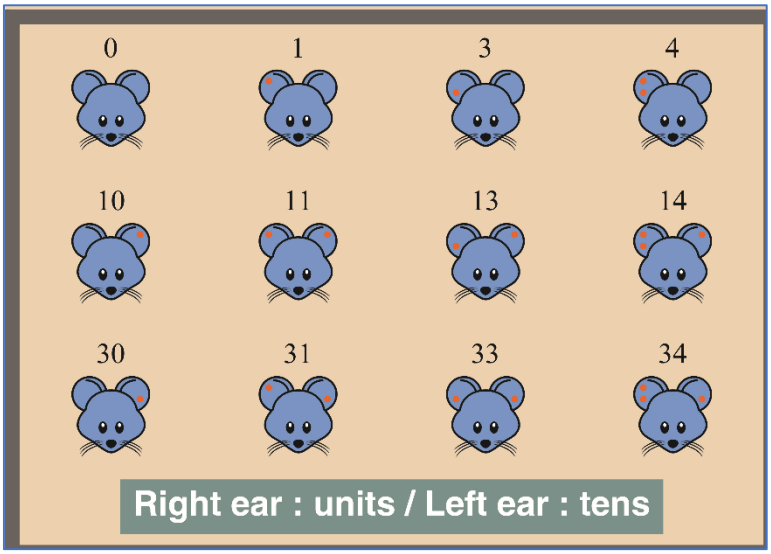


Figure 2.2 Mouse identification method

2.1.3 DNA extraction and PCR

DNA extraction was performed according to the manufacturer's protocol (ISOLATE II GENOMIC DNA KIT, BIOLINE). Ear punch specimens were incubated in Proteinase K and lysis buffer GL for 2-3 hours at 56 °C. Following this, lysis buffer G3 was added to the samples and incubated for 10 min at 70 °C. Then, to adjust the DNA binding, ethanol (97%) was added to the samples and loaded to the ISOLATE II GENOMIC DNA spin column. Next, samples were centrifuged and washed with washed buffers GW1 and GW2 and eluted in 70 °C preheated nuclease-free water. The PCR was performed in agreement with the manufacturer's protocols (Mangomix ™, BIOLINE) and was conducted using T100™ Thermal Cycler (Bio-Rad) to magnify genomic DNA. Then, the mix of PCR reaction involved 12.5 µl of mango mix (Bioline), 0.10 µl of each primer (see Table 2.1), 4 µl of each specimen, and 8.30 µl of nuclease-free water to reach 25 µl as a final volume. The PCR settings and DND primers (Sigma) are listed in Table 2.1. The PCR reaction amplified products were run using 2% gel agarose (Molecular probe) which contains 1:30000 dilution of Saferview (Biolegend). Bio-Rad Gel Doc XR and ImageLab software were used to detect, view, and annotate the gel brands (Figure 2.3).

Table 2.1. Genotyping primers and PCR conditions

Primer name	Final concentration	Sequences 5' --> 3'	PCR conditions
Common	0.50 μM	GCC TAG CCG AGG GAG AGC CG	1. 94◦ C for 3 min 2. 94◦ C for 30 seconds 3. 68◦ C for 40 seconds 4. 72◦ C for 1 minute (repeat step for 35 cycles) 5. 72◦ C for 2 minutes 10◦ C for 2 minutes + hold
Wild-type reverse	0.50 μM	TGT GAC TTG GGA GCT CTG CAG C	
Mutant reverse	0.50 μM	GCC GCC CCG ACT GCA TCT	

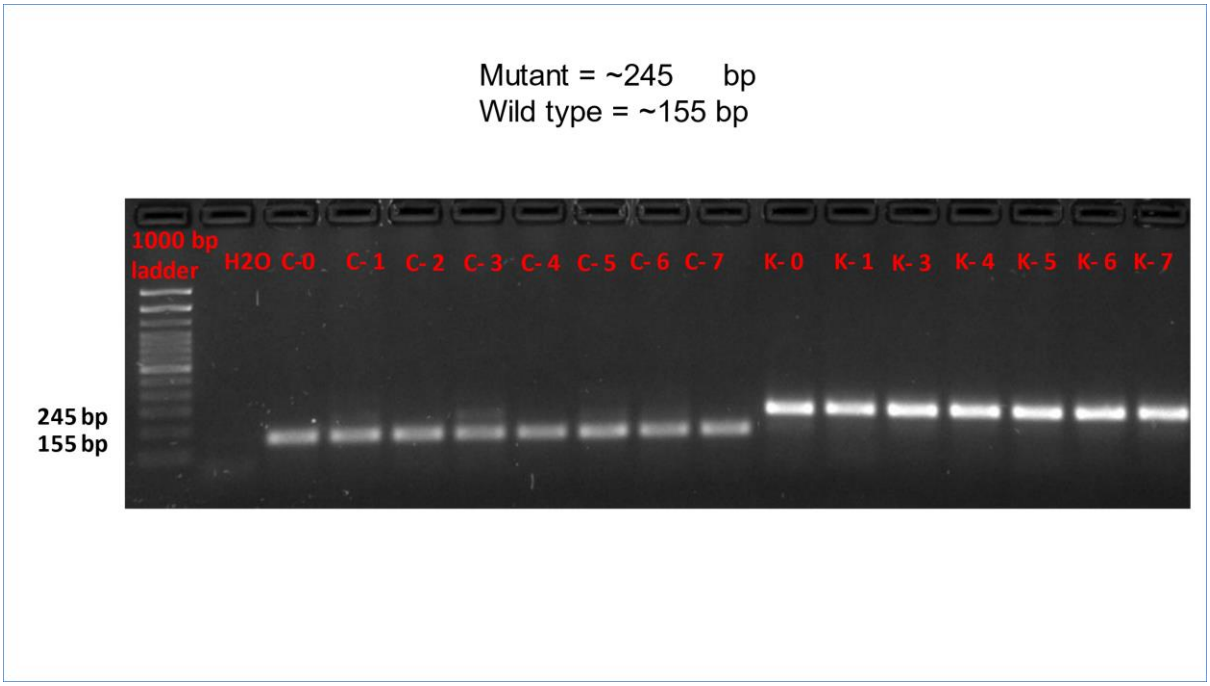


Figure 2.3 Genomic DNA PCR showing ApoE deletion. +/+: control, C: Control, K: Knockout, bp: base pair

2.1.4 Tissue collection

2.1.4.1 Heart

Mice were euthanized by increasing the CO_2 level. Then, after cessation of breathing, palpitation was used to confirm death. Next, 1 ml of whole blood was taken from the heart by cardiac puncture. Following this, the heart was flushed with phosphate buffer saline (PBS)(Gibco™), stored in a Shandon base model, covered in optimum temperature formulation (OCT) (Thermo Fisher Scientific), and snap frozen in -80 °C.

2.1.4.2 Extraction of bone marrow, spleen, and thymus

BM was harvested from the femur and tibia from the hind legs. The BM was crushed with a pestle and mortar and collected in a PBS (Gibco™) supplemented with 2% heat inactivated foetal bovine serum (Gibco™) (2% FBS/PBS) and filtered in a 70 µm strainer (Miltenyi Biotec). This step was repeated three times until a 30 ml cell suspension was obtained. The spleen (Sp) and thymus (Thy) were weighed, and photographs was taken. The Sp and Thy were placed in a 70 µm strainer inside 6-cm petri dish in 2% FBS/PBS and then homogenised using a 5 ml syringe plunger for the Thy and a 10 ml syringe plunger for the Sp. This step was repeated three times until a 7 ml cell suspension for the Sp and a 4 ml cell suspension for the Thy were obtained.

2.1.4.3 Blood extraction, red blood cell lysis and complete blood count analysis

Blood was taken via tail bleeding and collected using a collection tube with Ethylenediaminetetraacetic acid (EDTA) tubes (Mirovette CB 300, Cat 16.444, sarstedt). RBCs were lysed with ammonia chloride (NH4CL) (stem cells technology). Then, 12 µl of blood was added to 600 ml NH4CL and incubated for 6 min. Next, it was mixed completely by inverting it 4-6 times and incubated for 6 min. Then, the sample was centrifuged at 370 g for 10 min at room temperature. The supernatant containing lysed RBCs was then discarded to obtain a WBC pellet. For complete blood count (CBC), the same blood extraction procedures were followed except RBCs were not lysed. CBC test was conducted using Celltac Alpha MEK-6500K, Nihon Kohden.

2.1.5 Heart sectioning

Microtome cryostat sectioning at –20 ^{o}C was used to obtain cryosections of OCT-embedded roots. Next, 10 µM sections from the aortic roots were serially cut to observe clearly all three leaflets of the aortic valves. Finally, polylysin-coated slides were used to collect sections, and these were air dried for 1 hour prior storage at –80 ^{o}C.

2.1.6 Oil Red O staining

Sections were thawed and fixed in 4% paraformaldehyde (PFA) for 15 minutes. Next, slides were washed three times in distilled water. Then, the slides were counterstained for 3 minutes in Gill's haematoxylin. The slides were next rinsed below running tap water. Subsequently, the slides were put in a propylene glycol prior staining with oil red O (ORO) stain at 60^{o}C for 15 minutes. After, the slides were placed in an 85% propylene glycol for 5 minutes and then washed three times in distilled water and mounted via DPX mountant. Leica DMRB brightfield microscope with ProgRes® CapturePro 2.8.8 software was used to capture images at X40 magnification (X4 objective). ImageJ software was used to analyse lipid content and plaque area of Oil red O-stained sections.

Table 2.2 Reagents used for flow cytometry analysis

Population	Antigen	Clone	Fluorochrome	Dilution	Company
Lineage Markers Mac1, GR.1, Ter119, CD3, CD4, CD8a,	Mac1, GR.1, Ter119, CD3, CD4, CD8a,	Shown individually	Biotin	1/1000	Biolegend eBiosciences
HSPCs and progenitor	C-kit (CD117)	2B8	APC	1/100	Biolegend
	Sca-1	D7	PE, PE-CY7	1/25	Biolegend
	CD48	HM48-1	FITC	1/50	Biolegend
	CD150	TC15 - 12F12.2	PE-CY7	1/100	Biolegend
	CD16/32	93	PE-CY7	1/25	Biolegend
	CD34	RAM34	FITC	1/25	eBiosciences
	CD127 (IL-7rα)	A7R34	BV650, PE	1/50	Biolegend
	CD135 (Flt3)	A2F10	PE	1/25	Biolegend
Myeloid cells	Mac-1 (CD11b)	M1/70	APC	1/1000	Biolegend
	Gr.1	RB6-8C5	FITC, PE-CY7	1/1000	Biolegend
Erythroid lineage	CD71	TER-119	APC-CY7	1/1000	Biolegend
	Ter119	RI7217	PE	1/1000	Biolegend
Megakaryocyte	CD41	MWreg30	FITC	1/1000	Biolegend
Lymphoid cells	CD3	17A2	FITC, APC	1/1000	Biolegend
	CD4	GK1.5	PerCP, PE	1/1000	Biolegend
	CD8a	53-6.7	APC-CY7	1/1000	Biolegend
	B cells – (CD45R/B220)	RA3-6B2	FITC, APC	1/1000	Biolegend
Recipient and donor cells	CD45.2	104	PE, APC-CY7, BV510	1/500	Biolegend
	CD45.1	A20	BV510, BV650, APC	1/500	Biolegend
Apoptotic cells staining	Annexin V	NA	APC, PE	1/100	Biolegend
Cell cycle assay staining	Ki-67	16A8	APC, PE	1/100	Biolegend
Other reagents	Streptavidin	NA	Pacific blue, PerCP	1/100	Biolegend eBiosciences
	FC Block	NA	NA	1/50	Biolegend

2.1.7 Immunophenotypic characterisation of HSPCs

For the staining of HSCs (marker), 10 million BM cells were incubated in a CD16/32 antibody (FC block) in 50 µl of 2% FBSPBS. Then, 50 µl of the antibody mix containing a biotin conjugated cocktail of antibodies CD3e, CD4, CD8a, B220, MAC-1, Gr.1 and Ter119. Next, c-kit, Sca-1, CD150, CD48, and CD135 (Flt-3) were added to study populations of HSCs, HPC1, HPC2, MPP, and LMPP. Cells were washed with 2% FBS/PBS following 30 min of incubation at 4 ^{o}C and centrifuged for 5 min at 500G. Then, the pellet was resuspended in 100 µl Streptavidin-Percp. Following 10 min of incubation, cells were washed and resuspended in 2% FBS/PBS (Figure 2.4).

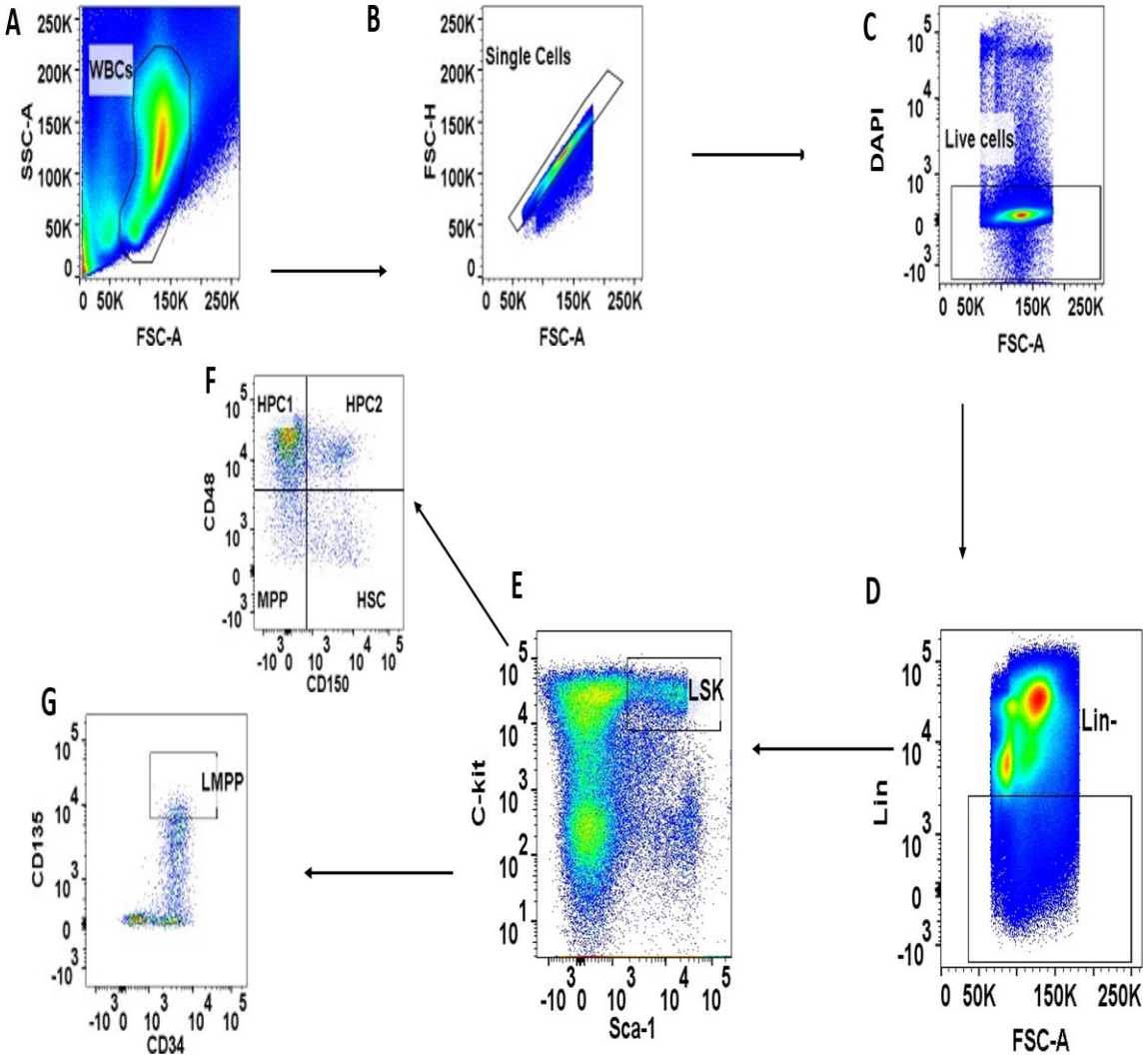


Figure 2.4 Flow cytometry analysis illustrating the gating strategy used for the identification of HSPCs in BM. (A) debris and dead cells were excluded to gate for WBCs. (B) Single cells were gated from WBCs to remove clumps and doublets. (C) Live cells were then gated for an analysis of the lin- population (D), which then is gated for the

analysis of the LSK population (E), which contains the most primitive and progenitor cells HPC1, HPC2, HSC, MPP (F), and LMPP (G). Percentages of each cell type from the BM cells were taken for the calculation of the frequency and the cell count of HSPCs.

For the staining of committed progenitor cells, 10 million BM cells were incubated in 100 µl of an antibody mix containing a biotin conjugated cocktail of antibodies CD3, CD4, CD8, B220, MAC-1, Gr.1 and Ter119. Next, c-kit, Sca-1, CD127, CD34, CD16/32 were added to study the populations of CMP, GMP, and CLP. Cells were washed with 2% FBS/PBS following 30 min of incubation at 4 ^{o}C and centrifuged for 5 min at 500G. Then, the pellet was resuspended in 100 µl Streptavidin-Percp. Following 10 min of incubation, the cells were washed and resuspended in 2% FBS/PBS (Figure 2.5).

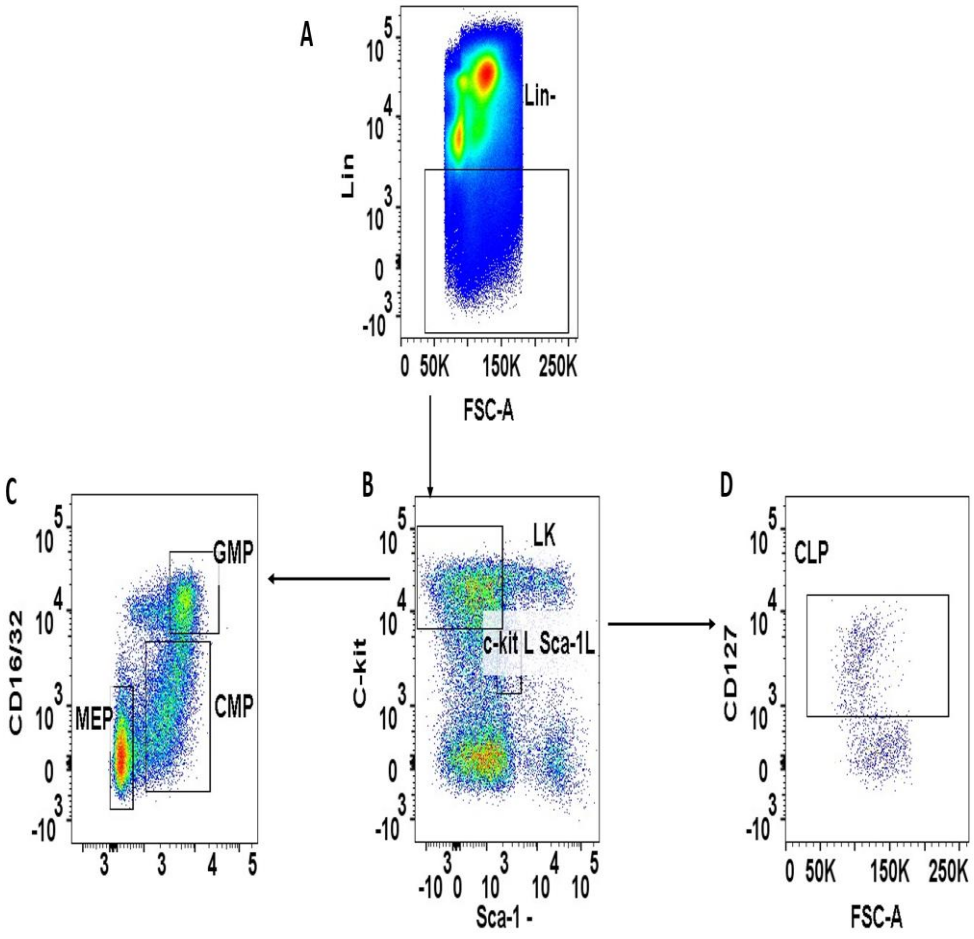


Figure 2.5 Flow cytometry analysis illustrating the gating strategy used for the identification of HSPCs in BM. Lin- populations were gated for the analysis of LK population (B), which contains lineage-restricted progenitors GMP, CMP, MEP (C). CLP (D) population were gated

from c-kit^{low}Sca-1^{low} populations. Percentages of each cell type from the BM cells were taken to calculate the frequency and the cell count of HSPCs.

2.1.8 Immunophenotypic characterisation for lineage positive cells

For the staining of lineage positive cells, 200,000 cells of the BM, PB, Sp, and Thy were incubated with a mix of antibodies containing a myeloid marker (CD11b, Gr-1), Erythroid (Ter119, CD71), a T lymphoid cell marker (CD4, CD8a) and a B lymphoid cell marker (B220) for 30 min at 4 ^{o}C. Next, cells were washed in 2% FBS/PBS and centrifuged for 5 min at 500G. The pellet was resuspended in 2% FBS/PBS. For all experiments, all FAC analyses were carried out using four laser BD LSRFortessa™ (BD Biosciences). Data were acquired and analysed using FlowJo software (Tree star INC, USA) (Figure 2.6).

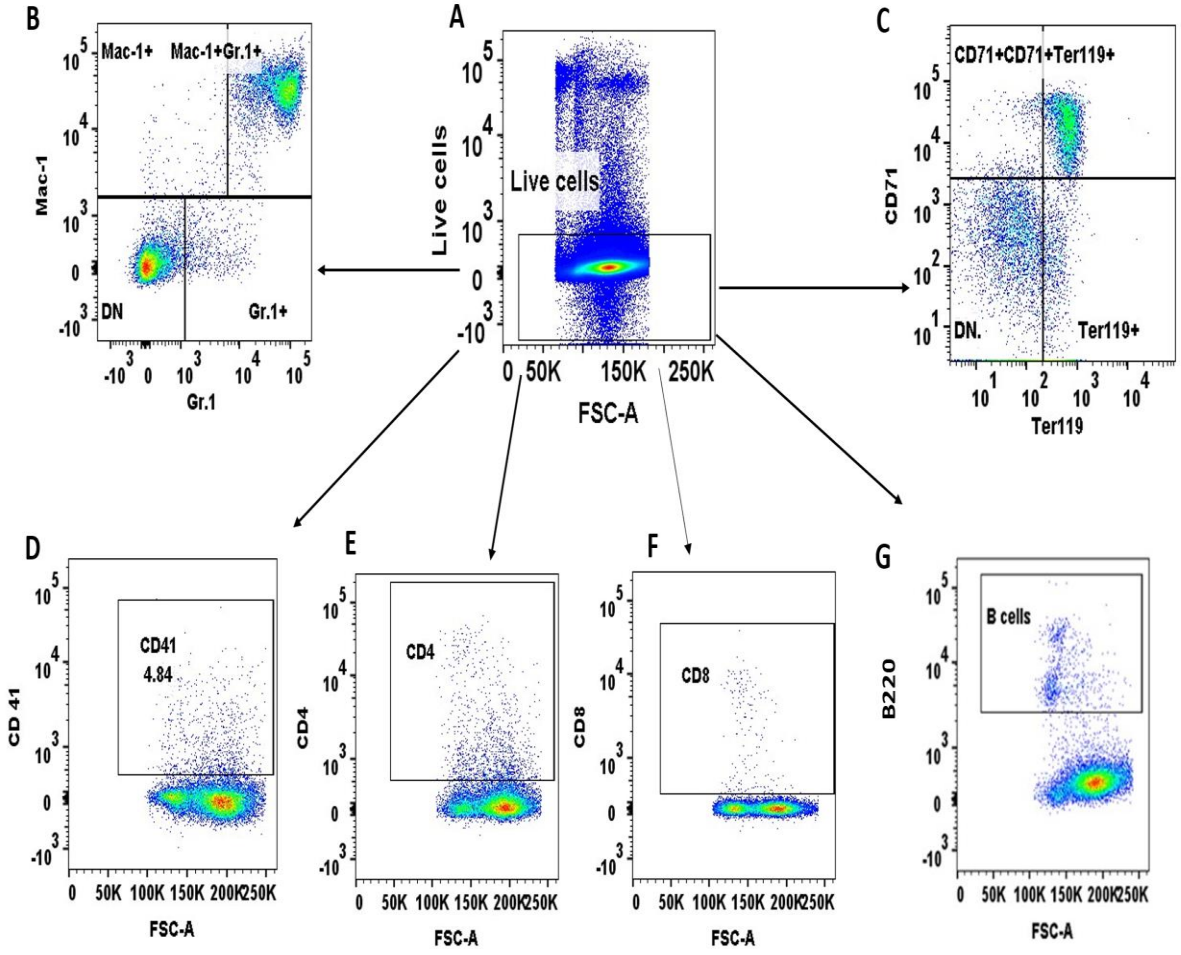


Figure 2.6 Flow cytometry analysis illustrating the gating strategy for the lineage positive cells. (A) live cells were gated for analysis of differentiated cells (B) MAC-1^{+}Gr.1^{+}, (C) CD71 and Ter119, (D) CD41, (E) CD4, (F) CD8 and (G) B cells.

2.1.9 Annexin V assay

BM cells were stained for SLAM and progenitor markers as described previously **(section 2.1.7)**. Then, cells were washed with cold 2% FBS/PBS. An annexin-binding buffer containing annexin V – PE fluorochrome conjugate was added to the BM cells and incubated for 30 min in the dark at room temperature. Next, 2 µg/ml of DAPI was added to the samples and kept on ice before analysis using flow cytometry (Figure 2.7).

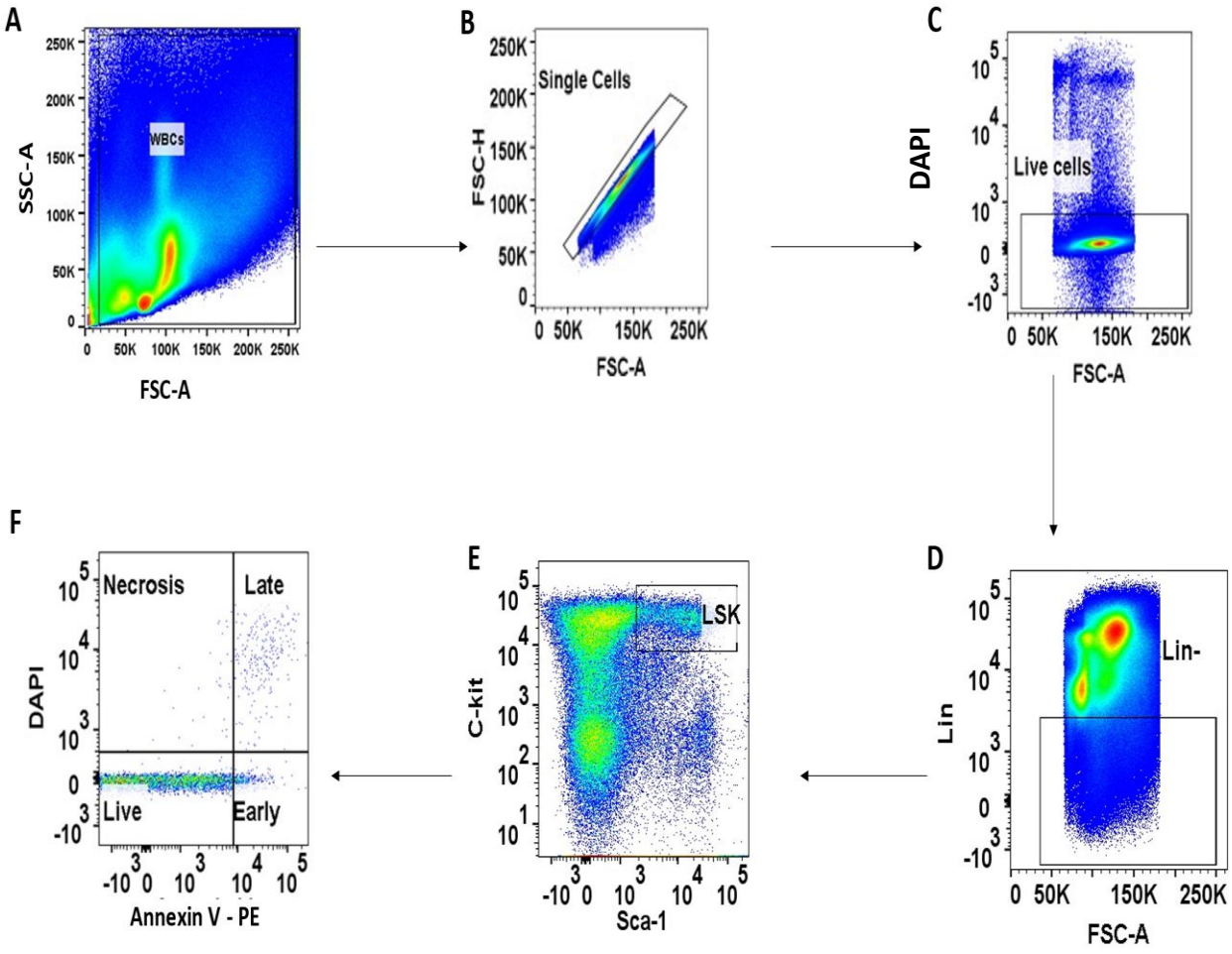


Figure 2.7 Flow cytometry analysis illustrating the gating strategy used for the identification of apoptotic stages. A) debris and dead cells were excluded to gate for WBCs. (B) Single cells were gated from WBCs to remove clumps and doublets. (C) Live cells were then gated for an analysis of the lin- population (D), which then was gated for the analysis of the LSK population (E). (F) The stages of apoptosis were determined by annexin V – PE fluorochrome conjugate/ DAPI: live cells (Annexin V- DAPI-), early apoptosis (Annexin V+ DAPI-), and late early apoptosis (Annexin V+ DAPI+) (F). The percentage of each gate was taken to calculate the frequencies of various stages of apoptosis.

2.1.10 Ki-67 assays

BM cells were stained for SLAM and progenitor markers as described previously (section 2.1.7). Next, cells were washed with cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. Cells were then washed with PBS, and 0.1% saponin containing Ki-67 PE fluorochrome conjugate (10 ul/10 million cells) was added to the samples, which were incubated for 24 h at 4 ^{o}C. Next, the DAPI of a 500 ug/ml final concentration was added to the samples before analysis using flow cytometry (Figure 2.8).

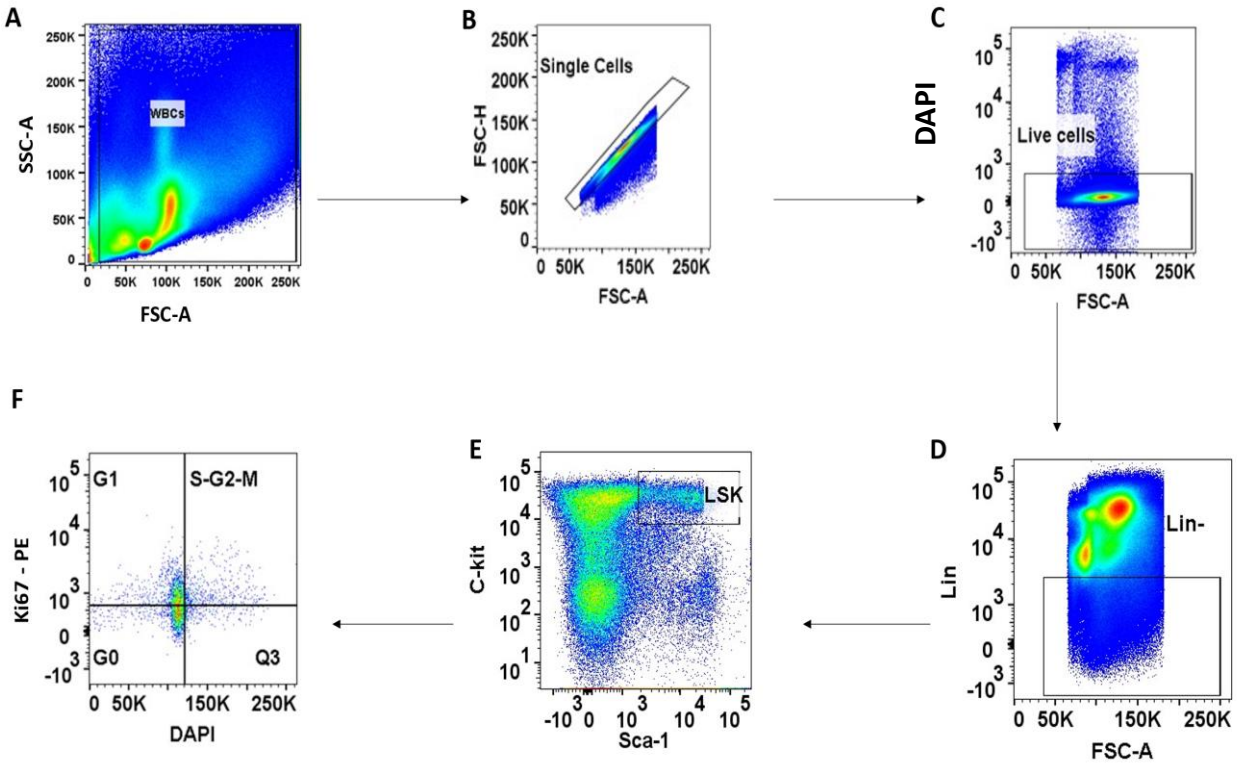


Figure 2.8 Flow cytometry analysis illustrating the gating strategy used for the identification of cell cycle stages. **(**A) debris and dead cells were excluded to gate for WBCs. (B) Single cells were gated from WBCs to remove clumps and doublets. (C) Live cells were then gated for an analysis of the lin- population (D), which then is gate for the analysis for LSK population (E). (F)The stages of the cell cycle were determined by KI67 PE fluorochrome conjugated /DAPI. G0 (KI67- DAPI-), G1 (KI67+ DAPI-) and S-G2-M (KI67+ DAPI+). The percentage of each gate was taken to calculate the frequencies of various stages of the cell cycle.

2.1.11 Colony forming cell assay

Media and cells up to 250 µL were added to methylcellulose (STEMCELL technologies), vortexed and incubated for 5 min and then plated using a 2 ml syringe attached to a 19G blunted needle. For the leukaemia assay, Methocult ™ 3232 media supplemented with 20 ml IMDM, 10 ng/mL GM-CSF (Peprotech), 10 ng/mL IL-6 (Peprotech), 1 ml Pen/Strep (Gibco™), 20 ng/mL SCF (Peprotech), and 10 ng/mL IL-3 (Peprotech) was used to perform the colony forming cell (CFC) in leukaemia assay. Methocult ™ 3434 media was used to perform the normal CFC assay. The colonies were counted and scored using an inverted microscope (Leica) at day 10 for normal CFC and day 6 for leukaemia assay.

2.2 ApoE in leukaemogenesis

2.2.1 C-kit enrichment using AutoMACS magnetic cell separation

After RBC lysis, BM cells pellets were re-suspended in a mix containing a 300 µl 2% FBS/PBS and 20 µl of c-kit (CD117) microbeads (Milteny Biotec) and then incubated while rotating for 12 min at 4 ^{o}C. Next, cells were washed and filtered using a 30 µl strainer (Cells Trics) and transferred into AutoMACS (Milteny Biotec). Then, the Possleds programme was used to separate the c-kit cells. C-kit cells were used for the downstream experiments of HSC transplantation and for performing the leukaemia assay.

2.2.2 Cryopreservation media

Some of the murine BM cells (2 ml) were frozen to perform the leukaemia assay. They were frozen in a 10% DMSO and 90% FBS solution. Cells were spun down at 500G for 10 min at 4 ^{o}C. Then, the pellet was resuspended on cold freezing media and moved to a 1 ml cryovial (Camlab limited) and stored at -80 ^{o}C for short-term storage (1 month) and in liquid nitrogen (-175 ^{o}C) for long-term storage (more than 1 month).

2.2.3 Generation of retroviruses

2.2.3.1 DNA preparation and plasmids

Dr Daniela Krause (Goethe University Frankfurt) kindly gifted MSCV-MLLAF9-GFP and MSCV -GFP. Prof. Kamil Kranc (University of Edinburgh) kindly gifted psPAX2 and pCMV-VSV-G. StbI3 bacteria (Sigma) were used to transform all plasmids. LB broth medium (Sigma) was used to culture all plasmids with 100 µg/mL of ampicillin (Gibco). Bacteria were taken from glycerol stock and cultured in 250 mL of the medium in a shaker overnight. The following day, the culture was harvested, and DNA was extracted according to the instruction of the manufacturer of the EndoFree plasmid Maxi kit (Qiagen). Then, DNAase free water (molecular probe) was used to elute the DNA. The DNA was aliquoted at >1 µg/mL concentration and stored at – 20 ˚C.

2.2.3.2 Culture conditions used for packaging cell lines

Platinum E cells (Cells biolabs) were derived from the HEK293T cell line by introducing packaging plasmid env, gag, and pol under the expression of the g promoter EF1α. To select the packaging plasmid with blasticydin (gag-pol) and puromycin (env), the internal ribosome entry site was added. Platinum E cells were preserved in DMEM (Gibco, UK) supplemented with 2 mM L-glutamine (Gibco) and 10% heat-activated FBS. As soon as the cells reached 70-90% confluency, HEK293T cells were passaged every 3 days.

2.2.3.3 Generation of retroviruses

To generate retroviruses, a calcium phosphate method was used. First, 62.5 µL of calcium chloride (Sigma) was mixed with 437.5 µL of H_2O. Then, 10 µg of retroviral vector containing Mll-af9 (or empty vector) were diluted in the mixture. Next, this mix was added to 500 µL of 2X hebs buffer saline (HBS; sigma) in a drop wise manner and incubated for 15 minutes. Following the incubation period, the mix was placed in a 10 cm dish containing platinum E cells media at confluency 70%. Prior to transfection, 25 µM of chloroquine (Sigma) was added to the media. Following transfection, the supernatant-containing retrovirus was collected 24 hours and 48 hours, filtered through 0.45 µm filter (Sigma), snap freeze in a dry ice and stored at –80ºC.

2.2.4 Murine HSPCs retroviral transduction

On the day prior to transduction, 1 million c-kit positive cells were pre-stimulated with 40 ng/mL SCF (Peprotech), 20 ng/mL IL-3 (Peprotech), and 20 ng/mL IL-6 (Peprotech). Cells were then transduced with a retrovirus containing the oncogene MLL-AF9. Six hours after the first transduction, the cells were transduced again in a new retronectin-coated plate containing retroviruses. MLL-AF9-transduced cells were kept for 72 hours prior to the sorting of live cells according to GFP fluorescence into the colony forming cells assay CFC1 (method described in section 2.1.6). Colonies were replated every 6 days with up to 3 rounds of CFCs. After each round, the colonies were counted and scored using an inverted microscope (Leica) and were immunophenotyped by flow cytometry. The cells from CFC3 were enriched for the c-kit before transplantation into lethally irradiated primary recipients for pre-LSCs generation. LSCs from leukaemic animals were transplanted into lethally irradiated secondary recipients.

2.2.5 Immunophenotypic characterisation of colonies

For the staining of colonies, 200,000 colonies of each round of CFCs were incubated with a mix of antibodies containing a myeloid marker CD11b, Gr.1 and c-kit for 30 min at 4 ºC. Next, cells were washed in 2% FBS/PBS and centrifuged for 5 min at 500G. The pellet was resuspended in 2% FBS/PBS (see Figures 1.1, 1.2, 1.3, and 1.4). For all experiments, all

FACS analyses were carried out using four laser BD LSRFortessa™ (BD Biosciences). Data were acquired and analysed using FlowJo software (Tree star INC, USA).

2.3 Transplantation assay

2.3.1 Normal haematopoiesis transplantation assay

2.3.1.1 HSCs sorting

For sorting HSCs, a BM suspension was obtained as described in section 2.1.4.B. Then, RBCs were lysed by NH4CL and enriched for c-kit as described in section 2.2.1. C-kit positive cells were stained with antibodies as described in section 2.1.8, and HSCs were sorted using a BD FACSAria™ fusion (BD Biosciences).

2.3.1.2 HSCs primary and secondary transplantation

Multilineage reconstitution for long-term haematopoiesis is the gold standard to detect the function of HSCs. For the primary transplantation, 150 HSCs were sorted from donor mice BM cells C57 BL/6 (CD45.2) and transplanted together with 200,000 WT CD45.1 BM cells (support cells) into lethally irradiated CD45.1 recipient mice. The irradiated mice received a dose of 9 Gy (a split dose separated by 4 hours). For the secondary transplantation, 300 HSCs were sorted and transplanted together with 200,000 WT CD45.1 BM cells (support cells) into lethally irradiated CD45.1 recipient mice. Recipient mice were injected with cells in PBS with up to 200 µL per mouse. To test multilineage haematopoiesis reconstitution, the mice were bled every 4 weeks until 16 weeks for both primary and secondary transplantation. After 16 weeks, the mice were dissected and BM, SP, and Thy were harvested to test the engraftment capacity (Figure 2.9).

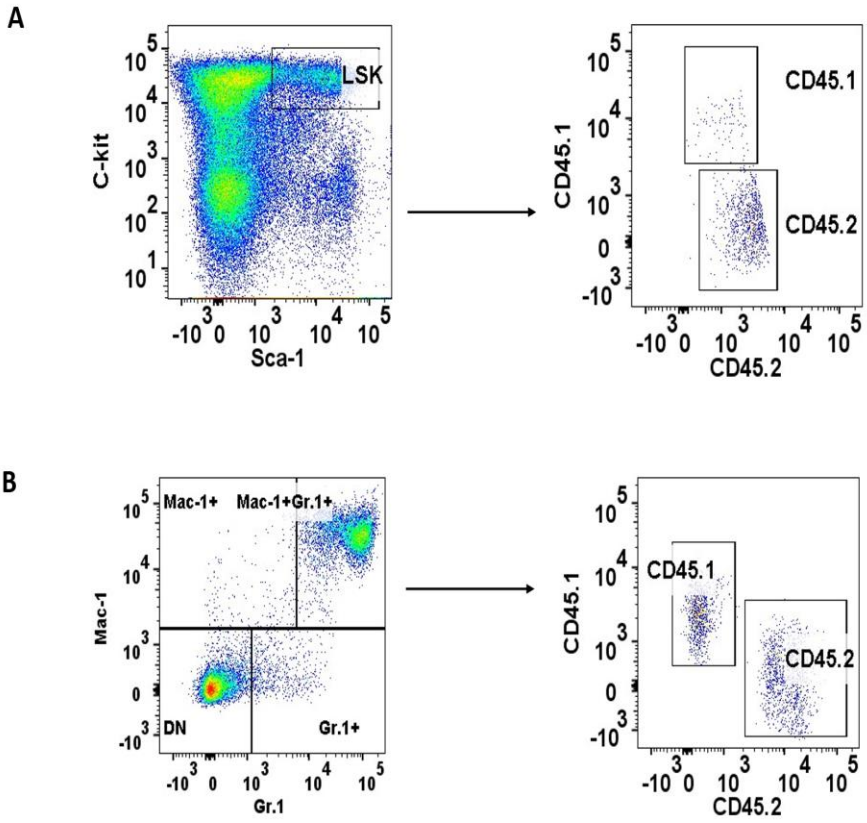


Figure 2.9 Flow cytometry analysis illustrating the gating strategy used for the identification for HSPCs and lineage positive cells in the transplantation assay. A. Representative flow cytometry analysis panel for the identification of the populations of recipient cells (CD45.1) and donor cells (CD45.2) gated from LSK. B. Representative flow cytometry analysis panel for the identification of the populations of recipient cells (CD45.1) and donor cells (CD45.2) gated from Mac-1+Gr-1+.

2.3.2 Leukaemia transplantation assay

For primary Pre-LSC transplantation, 100,000 c-kit positive cells (GFP^+ cells) together with 200,000 WT CD45.1 BM cells (support cells) were injected into lethally irradiated CD45.1 recipient mice. Recipient mice were injected with cells in PBS with up to 200 µL per mouse.

2.4 Statistical analysis

The data of the flow cytometry was analysed using FlowJo software (Tree Star, Inc). Statistical analysis was performed using a Mann Whitney test and one-way ANOVA test (4-group comparison) (GraphPad prism). Figures were arranged using prism (GraphPad Software, Inc). P-values less than 0.05 were considered significant. Data were represented by ± Standard error of mean (SEM).

Chapter 3: Exploring the requirement of Apolipoprotein E in steady-state haematopoiesis

3.1 Introduction

CVD is the number one cause of death globally (Poller et al. 2020). Despite advances in interventional treatments, health education, and drug discoveries, an estimated 17.9 million individuals died due to CVDs worldwide in 2016, accounting for around 31% of universal death that year (Poller et al. 2020). The underpinning of CVDs in most cases is atherosclerosis, a chronic inflammatory disease in which the wall of the vessels accumulates lipoproteins, and leukocytes (immune cell) infiltrate, causing a build-up of atherosclerotic plaque. When the plaque ruptures, it results in fatal complications, such as stroke and myocardial infraction, and is responsible for 85% of CVDs deaths (Poller et al. 2020, Cardiovascular diseases (CVDs), 2020). In particular, haematopoietic cells have been recognized as essential contributors to the initiation, development, and maintenance of atherosclerotic lesions (Randolph 2009; Moore and Tabas 2011; Swirski and Nahrendorf 2013; Poller et al. 2020).

HSCs give rise to all blood lineage cells including immune cells. A healthy mature body produces 4 – 5 x 10^{11} blood cells to maintain the level of haemostasis (Kaushansky 2006). HSCs reside in a specialised environment or niche in the BM (Pinho and Frenette 2019). As consequence of a regulated proliferation and differentiation process, mature blood and immune cells egress the BM in response to different stimuli, circulating before arriving at their target tissues via diapedesis and adhesion (Poller et al. 2020). While HSCs supply and give rise to inflammatory immune cells, which are critical to the development of CVDs, CVDs in turn strongly affect haematopoiesis. Furthermore, the common risk factors for CVDs, such as diabetes mellitus and hyperlipoproteinemia, substantially alter the process of haematopoiesis, and similarly, atherosclerosis and its complications have a significant effect on haematopoiesis.

Previous reports have recognised a causal relationship between hypercholesterolemia, leucocytosis, and CVDs, demonstrating that leucocytosis might exacerbate atherosclerosis (Coller, 2005). A case in point is monocytes, which have specifically been linked to atherosclerosis (Stewart et al. 2005; Afiune Neto et al. 2006; Chapman et al. 2004). Furthermore, an increase in haematopoietic progenitor (CFCs) in BM in hypercholesterolemic rabbit and swine models has been illustrated (Feldman et al. 1991; Averill et al. 1989).

Genetically modified murine models of cholesterol disrupted metabolism, such as ApoE and low-density lipoprotein receptor (LDLR), develop atherosclerosis lesions when mice are fed an HFD (Knowles and Maeda 2000). ApoE is a multifunctional protein that has an essential

role in lipid metabolism (Greenow et al. 2005). Thus, ApoE deficiency leads to differences in lipoprotein metabolism and, as a ramification, significant hypercholesterolemia (Curtiss and Boisvert 2000; Greenow et al. 2005). Recent studies using mice genetically engineered to be deficient in ApoE and other murine models deficient in cholesterol efflux pathways, such as adenosine triphosphate (ATP) binding cassette transport 1 (ABCA1) and ATB binding cassette subfamily G member 1 (ABCG1), have revealed a mechanistic connection between lipid biology and leucocytosis (Swirski and Nahrendorf 2013). Furthermore, it has been reported that ApoE-mediated control of leucocytosis occurs via ABCG1 and ABCA1 respectively to regulate cellular cholesterol efflux (Yvan-Charvet et al. 2007; Matsuura 2006: Remaley et al. 2001). However, while ApoE has been linked to lipid biology, previous studies have not described the role of endogenous ApoE in controlling the HSPC compartment in steady-state haematopoiesis. Thus, this chapter aims to explore the role of ApoE in the regulation of haematopoiesis.

3.1.1 Aims of the chapter

Previous studies have identified a causal relationship between leucocytosis, hypercholesterolemia, and CVDs, which shows that leucocytosis may promote atherosclerosis (Coller 2005), for instance, monocytes, which have explicitly been associated with atherosclerosis (Stewart et al. 2005; Afiune Neto et al. 2006; Chapman et al. 2004). Genetically modified murine models of atherosclerosis, such as ApoE and low-density lipoprotein receptors develop atherosclerosis upon being fed an HFD (Knowles and Maeda, 2000). ApoE is a multifunctional protein that has a fundamental role in lipid metabolism (Greenow et al. 2005). Therefore, a deficiency in ApoE results in differences in the metabolism of lipoprotein and, as a result, significant hypercholesterolaemia (Curtiss and Boisvert 2000; Greenow et al. 2005). Previously, the ApoE gene has been found to be abundantly expressed in HSPCs and has been observed to control the proliferation of HSPCs and monocytes, and the accumulation of monocytes in the lesion of atherosclerosis in the context of an HFD (Murphy et al. 2011). However, how ApoE affects steady-state haematopoiesis in the context of an NCD and in the absence of atherosclerosis remains to be elucidated. Thus, the underlying hypothesis of this chapter is that ApoE plays a fundamental role in regulating steady-state haematopoiesis in the context of an NCD. The overall aim of this chapter is to explore the role of ApoE in steady-state haematopoiesis with the following objectives:

1. To characterise haematopoietic stem and progenitor cells (HSPCs) and lineage committed cells in the bone marrow by flow cytometry
2. To characterise lineage committed cells in peripheral blood, spleen, and thymus by flow cytometry

3. To assess the functional capacity of haematopoietic progenitors by performing colony forming cell (CFC) assay
4. To evaluate peripheral blood by complete blood count (CBC) analysis

To achieve this, a germline deficient ApoE murine model (ApoE-/-) was used, and the haematopoietic cell compartments of ApoE-/- mice, aged 8 – 12 weeks, were comprehensively analysed. Immunophenotypic analysis of haematopoietic cell compartments in BM, PB, Sp, and Thy was conducted on these mice. In addition, at the functional level, CFCs using total BM cells were performed to evaluate the differentiation and growth capacity of haematopoietic progenitor cells (HPCs) from ApoE-/- mice (Figure 3.1).

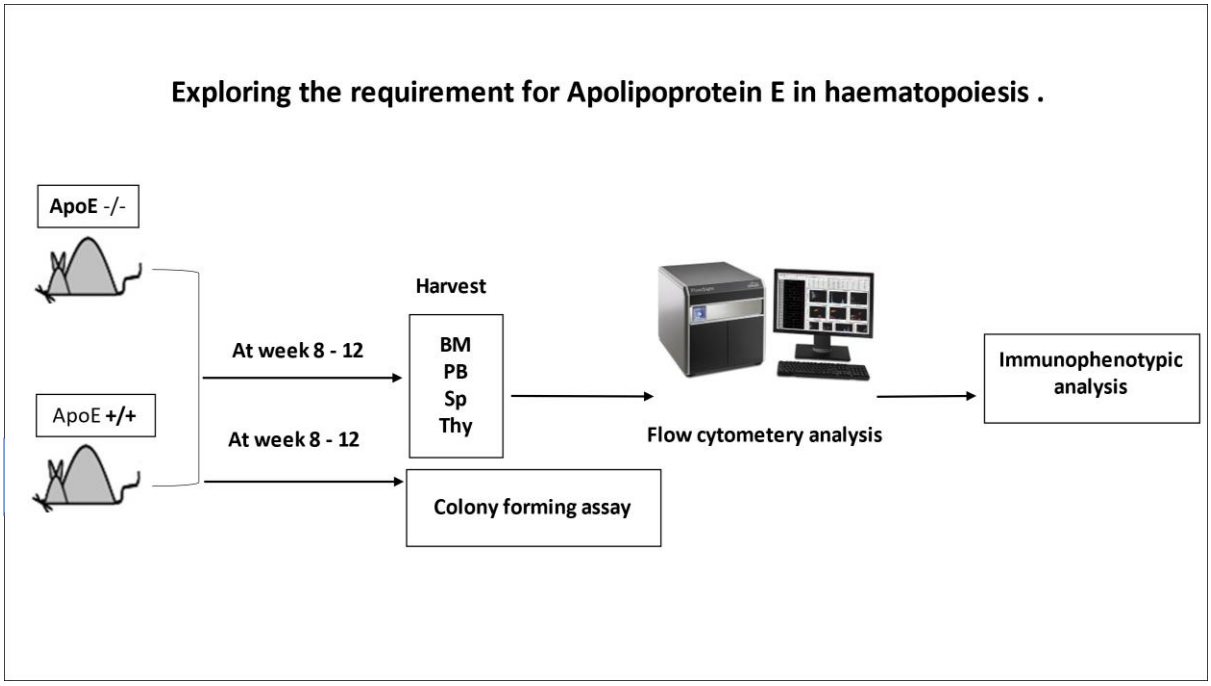


Figure 3.1 Experimental design for exploring the role of ApoE in haematopoiesis. ApoE -/-: ApoE knockout mice, ApoE +/+: wild-type mice, BM: bone marrow, PB: peripheral blood, Sp: spleen and Thy: thymus.

3.1.2 Result

3.1.2.1 Expression of ApoE in normal haematopoiesis

Previously, the ApoE gene has been reported to have abundant expression in HSPCs and was found to regulate the proliferation of HSPCs, monocytes, and monocyte accumulation in atherosclerotic lesions (Murphy et al. 2011) as well as accelerating the ageing of HSCs (Tie et al. 2014). This prompted me to examine the role of ApoE in normal, steady-state haematopoiesis. For this purpose, germline deficient ApoE-/- (KO) or wild-type (Thy1.1) mice were analysed at 8-12 weeks of age (Piedrahita et al. 1992; Plump et al. 1992). PB, BM, Sp and Thy samples were harvested to assess the function of HSPCs in the haematopoietic system. I confirmed complete deletion of the ApoE gene in ApoE-/- mice by genomic PCR of ear notches obtained from each genotype (Figure 3.2).

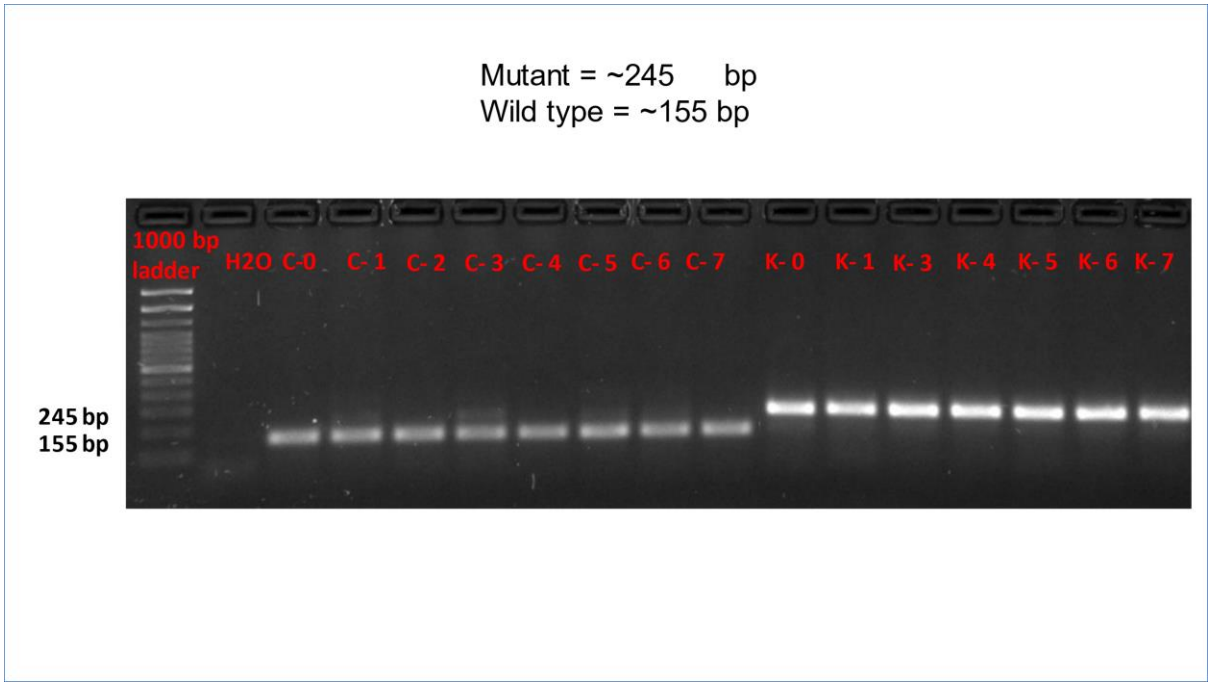


Figure 3.2 Genomic PCR showing ApoE deletion in experimental mice. Gel band showing C: Control, K: knock out bp: base pair.

3.1.2.2 ApoE deficiency has no impact on bone marrow cellularity. First, BM cellularity was compared between control and ApoE-/- mice and no significant difference was observed between the two genotypes (Figure 3.3).

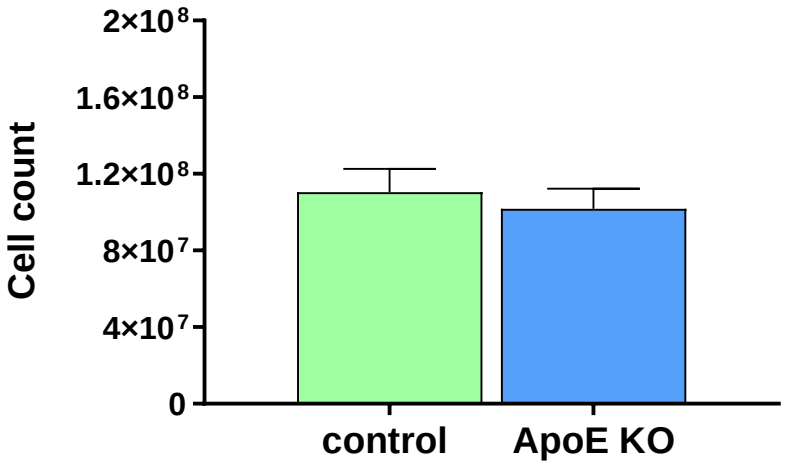


Figure 3.3 ApoE deficiency has no impact on BM cellularity. Data shown here represent total cell number in the BM harvested from 2 tibias and femur. Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using C: 7 mice KO: 9 mice for each genotype.

3.1.2.3 ApoE deficiency has no impact on the abundance of immunophenotypic HSPCs

Next, using flow cytometry, different HSPC sub-populations were immunophenotyped from the BM of each genotype. This analysis revealed that the abundance of the HSPC-enriched LSK compartment, which is a heterogeneous population that is enriched for HSCs (Oguro et al. 2013), was similar in both the control and ApoE-/- groups. By sub-fractionating the LSK compartment into specific HSC and MPP compartments (Oguro et al. 2013), no difference in the frequency and absolute number of HSC and MPP compartments (MPP, HPC1, and HPC2) was observed between genotypes (Figure 3.4). Similarly, an enriched committed progenitor compartment (LK) and lineage-specific progenitors (CMP, GMP, MEP, and CLPs) were assessed, and parity was observed in the frequency and absolute number of each of the populations in each genotype (Figure 3.4). Overall, it can therefore be considered that loss of ApoE has no impact on the abundance of HSPCs in steady-state haematopoiesis (Figure 3.4).

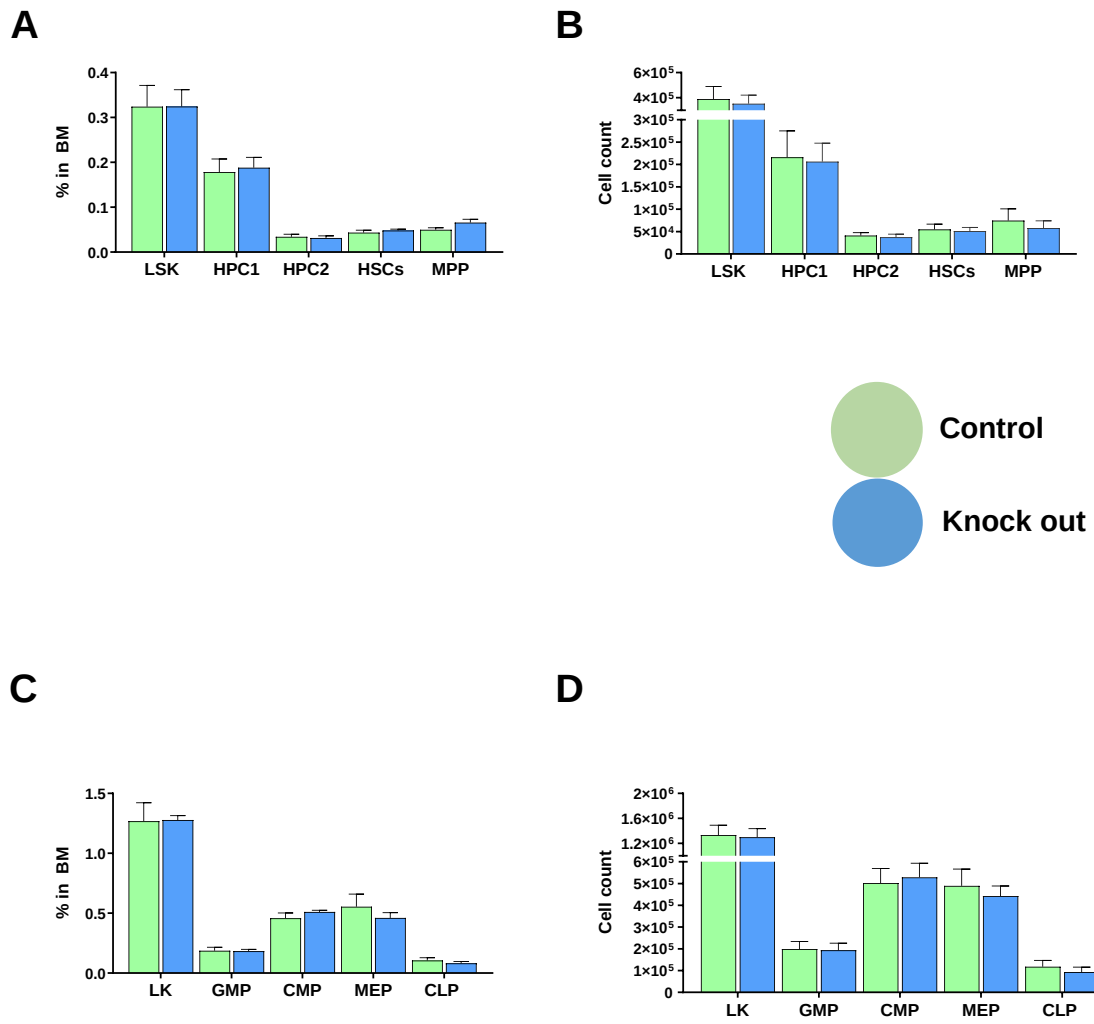


Figure 3.4 No impact of loss of ApoE on immunophenotypic HSPCs. Bone marrow cells were harvested from two tibias and the femur and stained for LSK and LK compartments using flow cytometry analysis. The most primitive HSPCs were analysed. Absolute counts of each type of cell were calculated by multiplying the percentages (frequencies) of each cell type by the total number of cells from populations arising from LSK and LK. Data shown here represent the frequency and total cell number of specific compartments from the total BM. Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using C: 7 mice KO: 9 mice for each genotype. LSK: lin$^-$ Sca-1$^+$ C-kit$^+$, LK: lin$^-$ C-kit$^+$, HPC1: haematopoietic progenitor 1, HPC2: haematopoietic progenitor 2, HSCs: haematopoietic stem cells, MPP: multiple progenitor, GMP: granulocyte monocyte progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor.

3.1.2.2 ApoE deficient bone marrow cells display no functional defect in progenitor formation.

The CFC assay was carried out on BM cells from each genotype to assess the whether the immunophenotypic analysis of HSPCs reflects the functional behaviour of haematopoietic progenitors that are deficient in ApoE. By plating total BM cells, it was found that the total CFC number and specific CFC lineages were not significantly different between the control and ApoE-/- groups. The results demonstrate that ApoE deficient cells do not affect the functional generation of lineage-restricted haematopoietic progenitors (Figure 3.5).

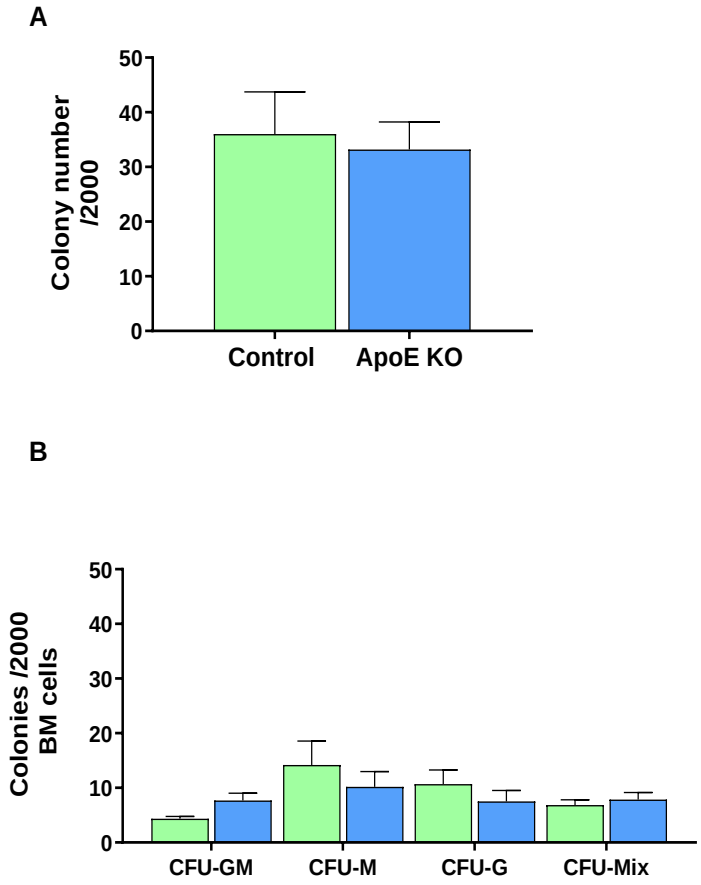
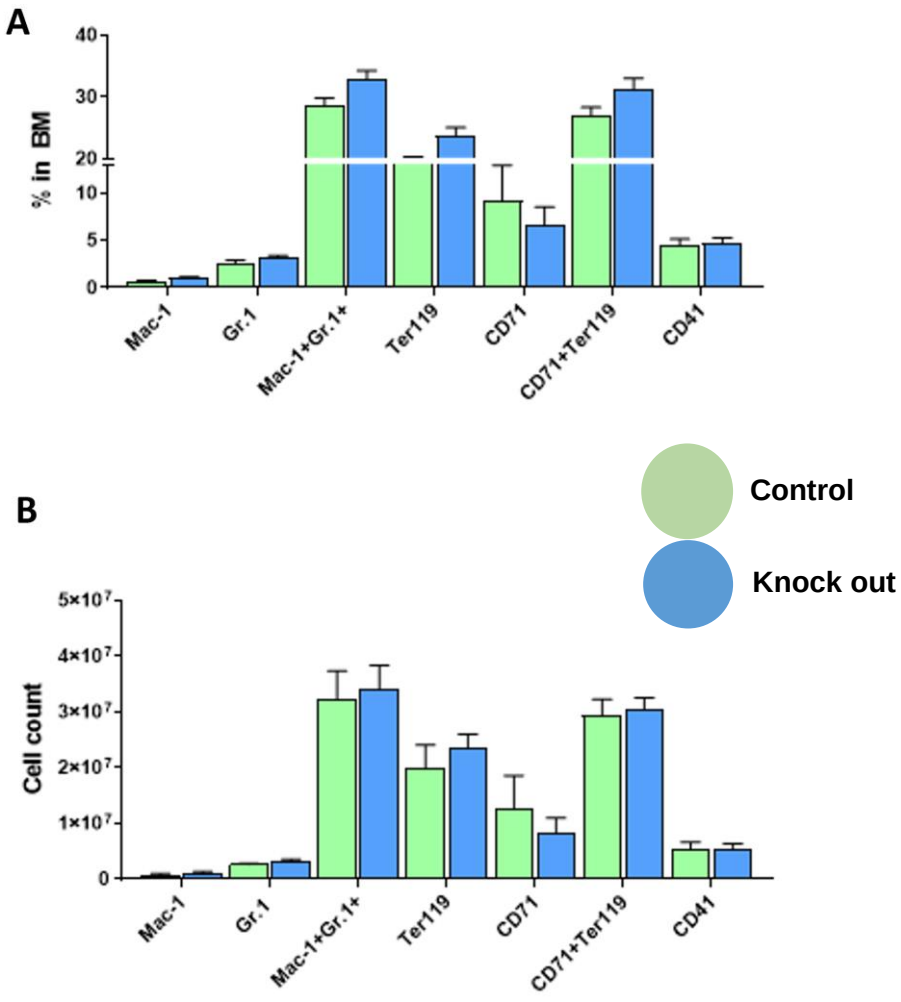


Figure 3.5 Loss of ApoE does not affect the lineage restricted progenitor. A. Bar graph shows the total number of colonies observed / 2000 cells B. The type of colonies: CFU- GM (macrophage granulocyte), CFU-M (monocyte), and CFC-Mix (granulocyte, erythrocyte, macrophage, megakaryocyte). Data shown here represent the frequency and total cell number of said compartments from the total BM. Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using 6 mice for each genotype.

3.1.2.3 Deficiency of ApoE leads to a reduction of B lymphocytes in BM

To analyse the impact of ApoE on BM haematopoietic differentiation, lineage-specific mature blood cells from the BM of both genotypes were immunophenotypically assessed using flow cytometry. Each genotype displayed a normal frequency and absolute number of T-lymphocytes (CD4, 8), myeloid cells (Gr-1, Mac-1), erythroid cells (TER119, CD71), and megakaryocytic cells (CD41) in the BM. However, a significant reduction in the frequency and absolute cell number of B-lymphoid cells was observed in ApoE-/- mice (Figure 3.6).



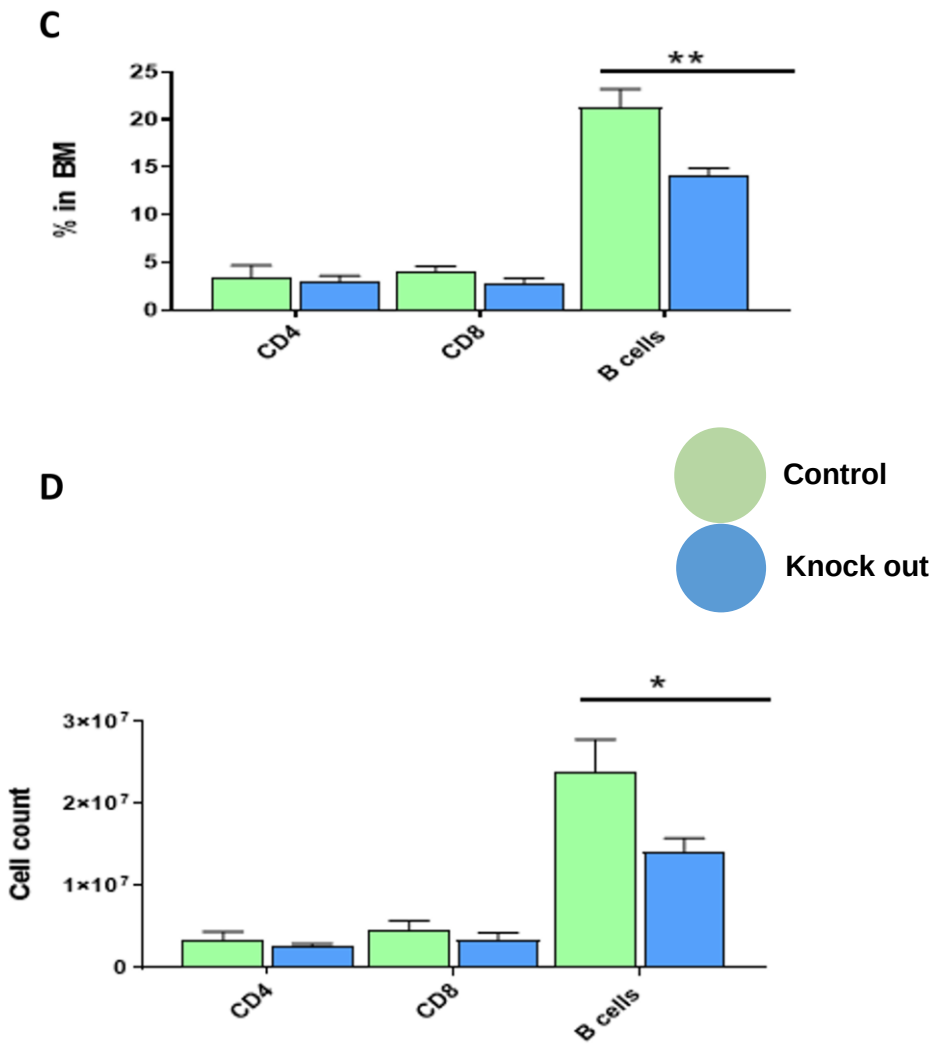


Figure 3.6 Impact of loss of ApoE on multi-lineage haematopoiesis in steady-state BM**.** Loss of ApoE affects the frequency and the total cell count of B cells in BM as assessed using flow cytometry. The bar graphs show the frequency and absolute cell count of myeloid (A, B) and lymphoid cells (C, D). Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control: 7 mice and KO 11 mice. *P <0.05 ** P<0.01.

3.1.2.4 Deficiency of ApoE leads to enlargement of the spleen

ApoE-/- mice also exhibited a significant increase in the weight of the spleen in comparison to their control counterparts (Figures 3.7 A, B) but without a significant increase in cellularity

(Figure 3.7 C). Furthermore, no changes were observed in the frequency and total cell count of myeloid and lymphoid populations in the spleen of ApoE-/- mice (Figure 3.8). From the comparison of myeloid and lymphoid populations, it is observed that the frequency and absolute number of TER119 and B cells in the spleen were insignificantly reduced in ApoE-/- mice. Otherwise, however, a loss of ApoE has no substantial effect on the frequency and the total cell count of the myeloid and lymphoid population in the spleen.

A

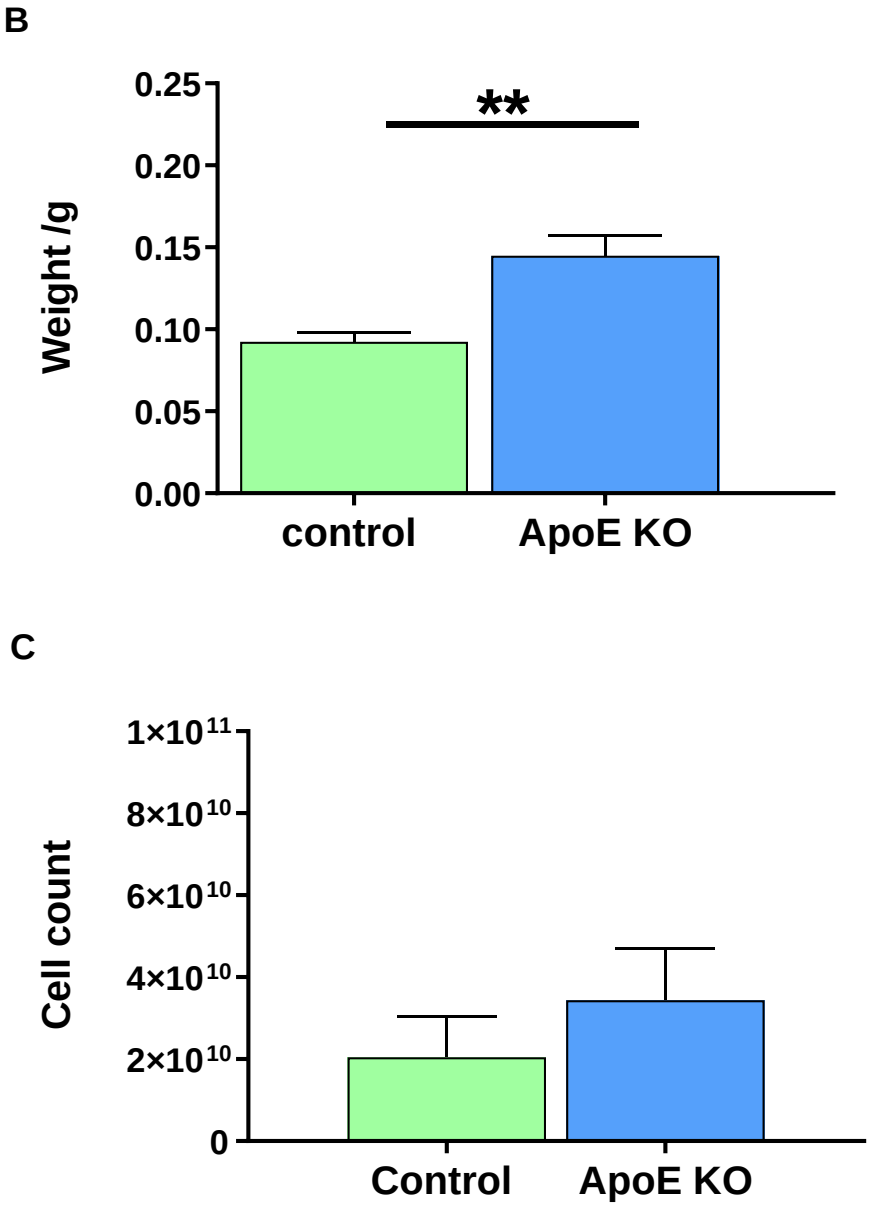
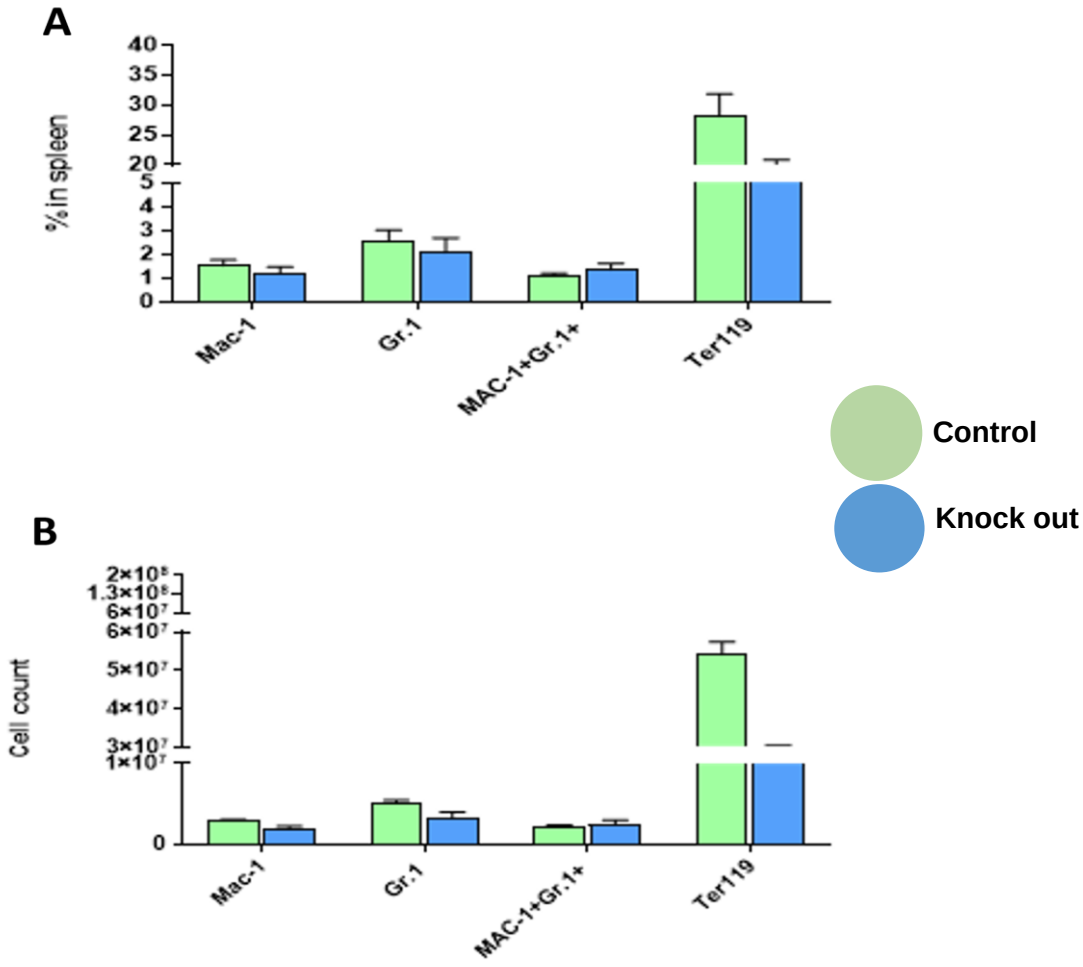


Figure 3.7 Loss of ApoE causes enlargement of the spleen. A representative photo of the spleen from both genotypes; C: control, K: ApoE -/- (Ko) (A). The bar graphs show the weight (B) and the cellularity of the spleen (C). Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using control: 6 and knock out :7 mice for each genotype. ** P<0.01.


A
% In spleen
40
35
30
25
20
5
4
3
2
1
0
Mac-1
Gr.1
MAC-1+Gr.1+
Ter119
Control
Knock out
B
Cell count
2×10⁸
1.3×10⁸
6×10⁷
6×10⁷
5×10⁷
4×10⁷
3×10⁷
1×10⁷
0
Mac-1
Gr.1
MAC-1+Gr.1+
Ter119

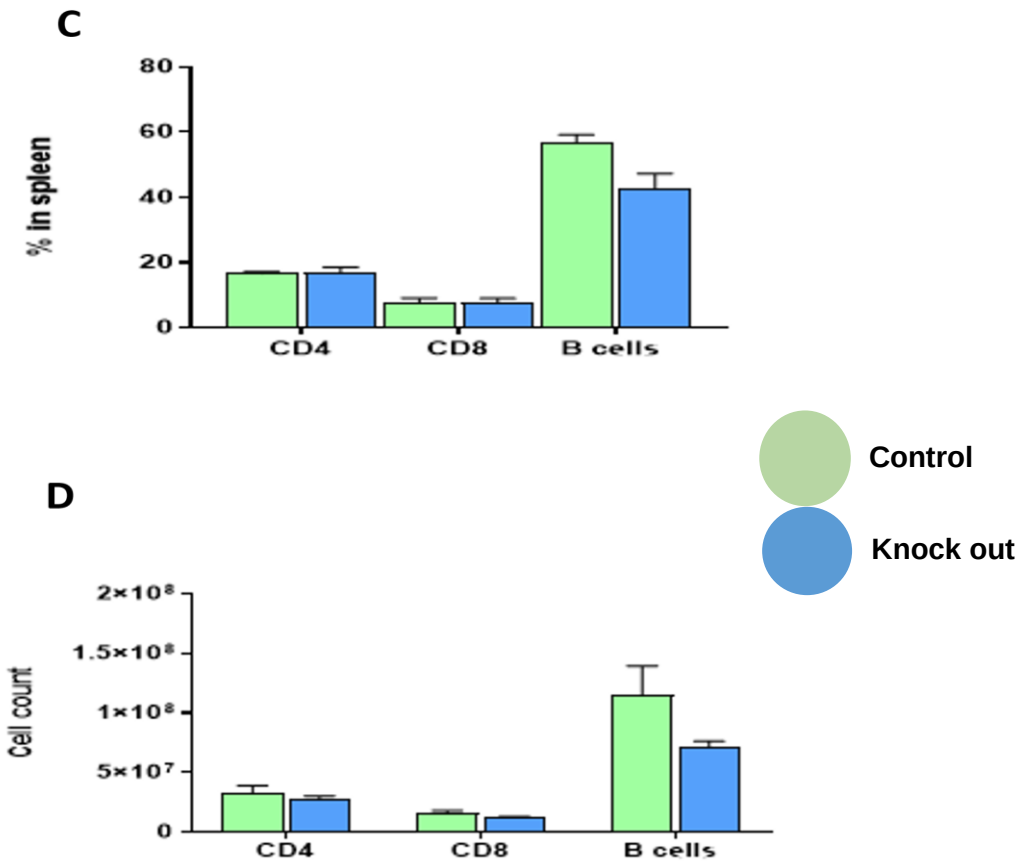
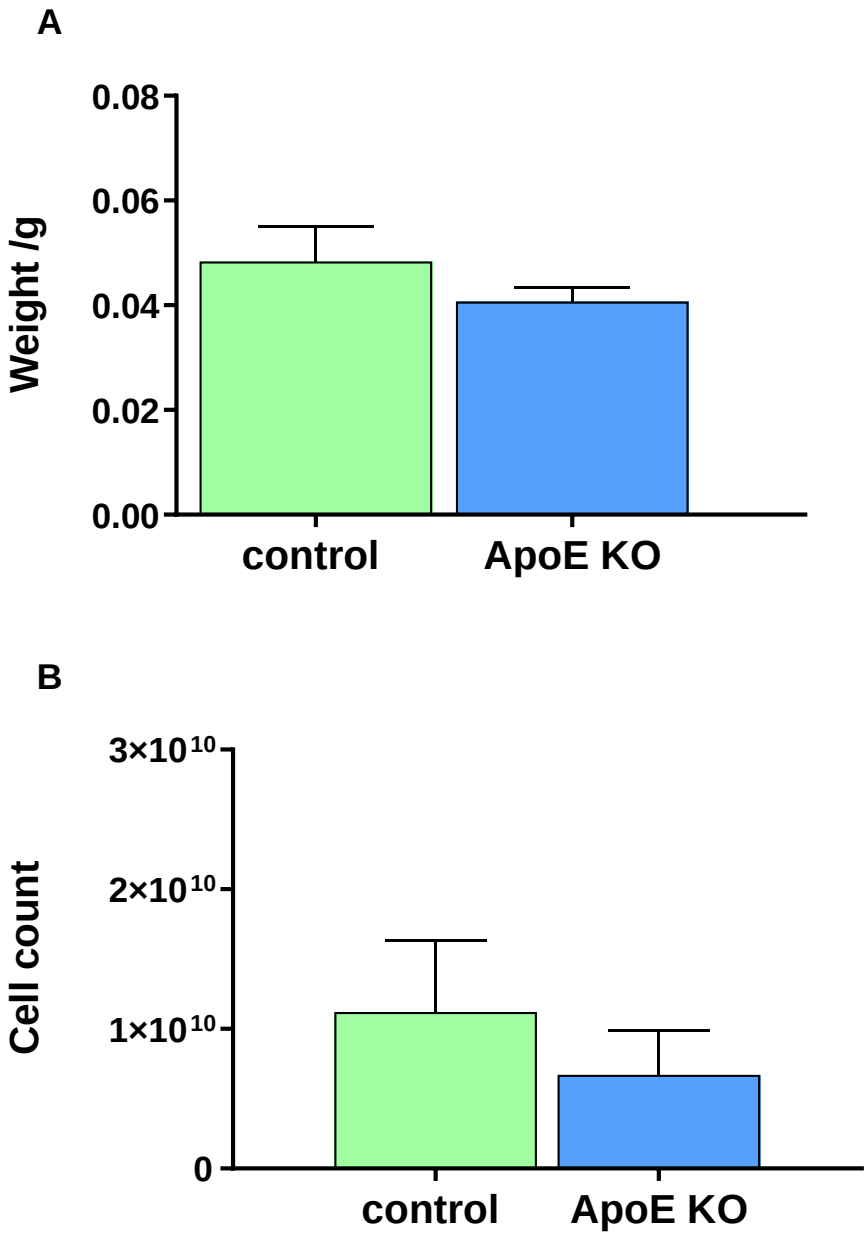


Figure 3.8 Frequency and the total cell count of the myeloid and lymphoid population on the spleen from ApoE-/- spleen. Loss of ApoE has no effect on the frequency and the total cell count of the myeloid and lymphoid population on the spleen. Bar graph shows the frequency of the myeloid (A) and lymphoid (C) and total cell count myeloid (B) and lymphoid (D) populations. Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using 3 mice per genotype.

3.1.2.5 The deficiency of ApoE has no impact on development of T cells in thymus

In Thy, there were no observed changes in the weight and total cell count of T cells and their precursors and mature T cells in ApoE-/- mice (Figure 3.9). A trend decrease was observed

in the number of B cells in the Thy of ApoE-/- mice, consistent with alterations in B-cell abundance in other haematopoietic organs of ApoE-/- mice (Figure 3.9 C, D).



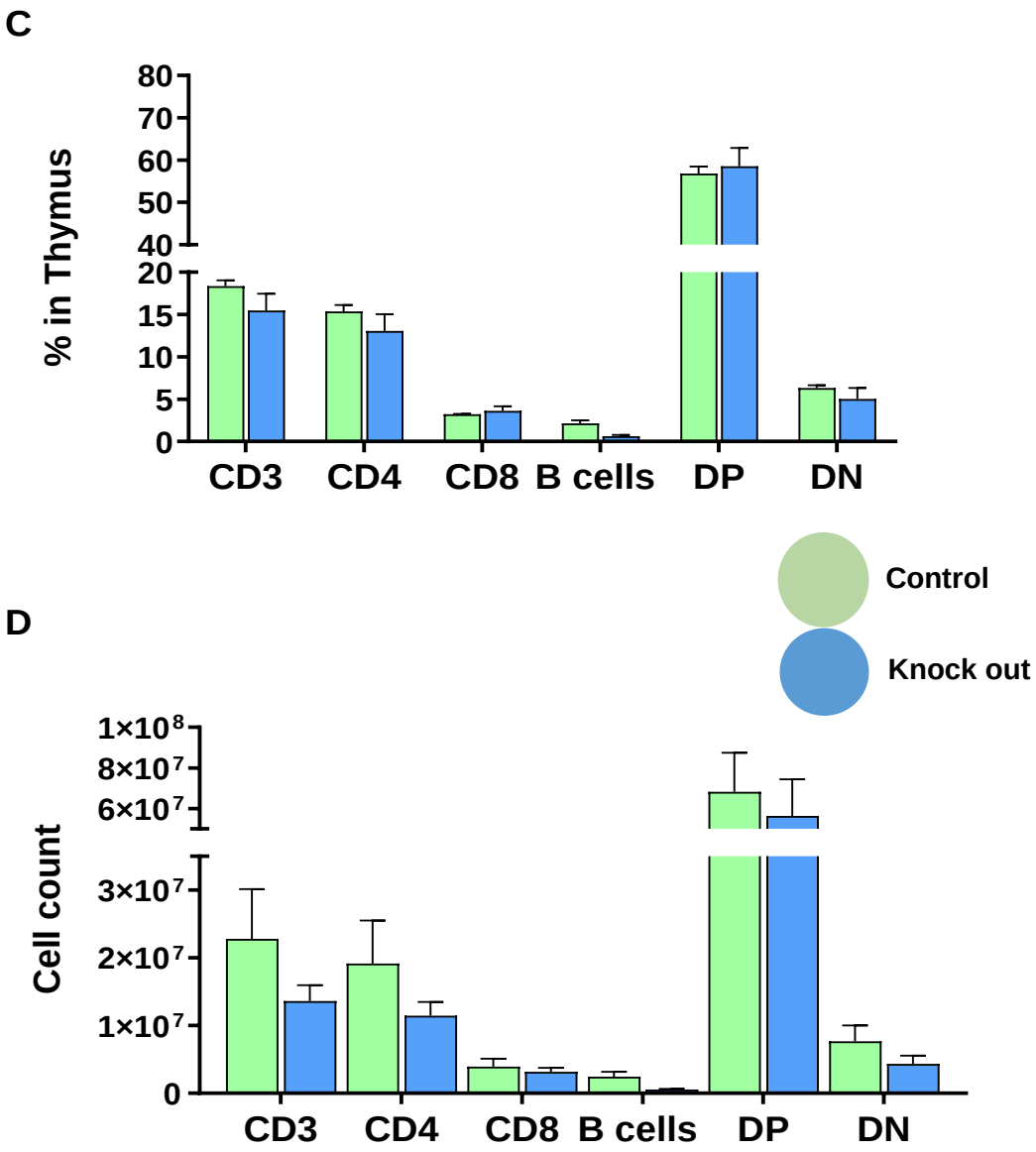


Figure 3.9 Loss of ApoE does not affect T cell precursors or T cell development. Loss of ApoE does not affect the weight (A), or total cell count (B). Loss of ApoE does not affect the frequency (C) and total cell count (D) of the lymphoid population in Thy. Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using 3 mice per genotype.

3.1.2.6 Deficiency of ApoE has impact Mac-1 cells and platelets in peripheral blood

To further analyse the impact of ApoE deficiency in mature blood cells, CBC analysis was performed on PB. A mild, significant reduction in the platelet population was observed, while no changes were observed in the white and red blood cell counts from ApoE-/- mice (Figure 3.10). To dissect lineage-specific changes in mature blood cells from ApoE-/- mice, flow cytometric analysis was performed, and no significant changes were observed in the myeloid

(Mac-1+Gr-1+) and lymphoid population (CD4, CD8, B cells). However, a significant decrease in the Mac-1+ population was noted (Figure 3.11).

Overall, mice lacking ApoE revealed no changes in the frequency and total cell number of HSPCs, which suggests that ApoE has a minimal role in steady-state haematopoiesis. However, our data suggest ApoE may have a role in the maintenance of B–lymphoid cells in the BM and Mac-1+ cells, and platelets from the PB (Table 3.1).

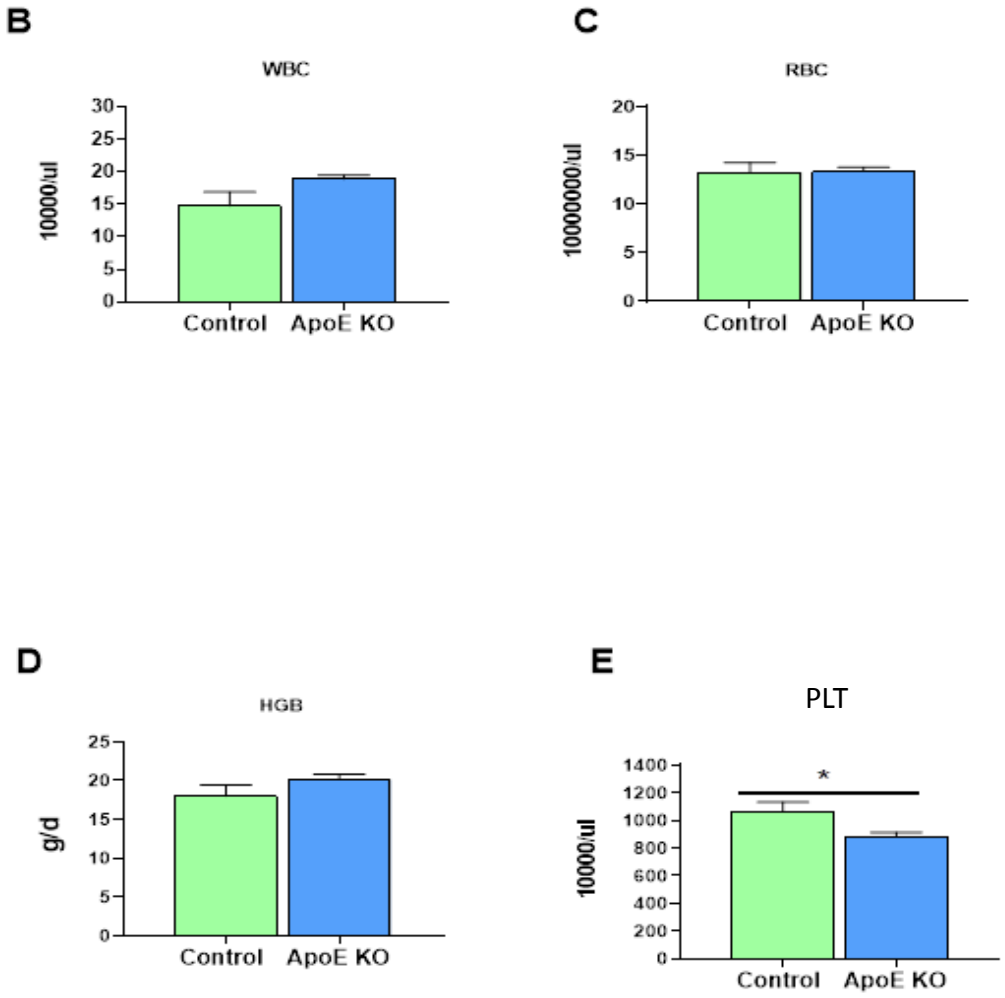


Figure 3.10 Complete blood count (CBC) analysis reveals a mild platelet reduction in ApoE-/- mice. Bar graph shows CBC results. A. total white blood cells (WBC). B. red blood cells (RBC). C. haemoglobin (HGB). D platelet (PLT). Statistical analysis was performed using a Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using control: 3 and KO: 5 mice per genotype. *P <0.05.

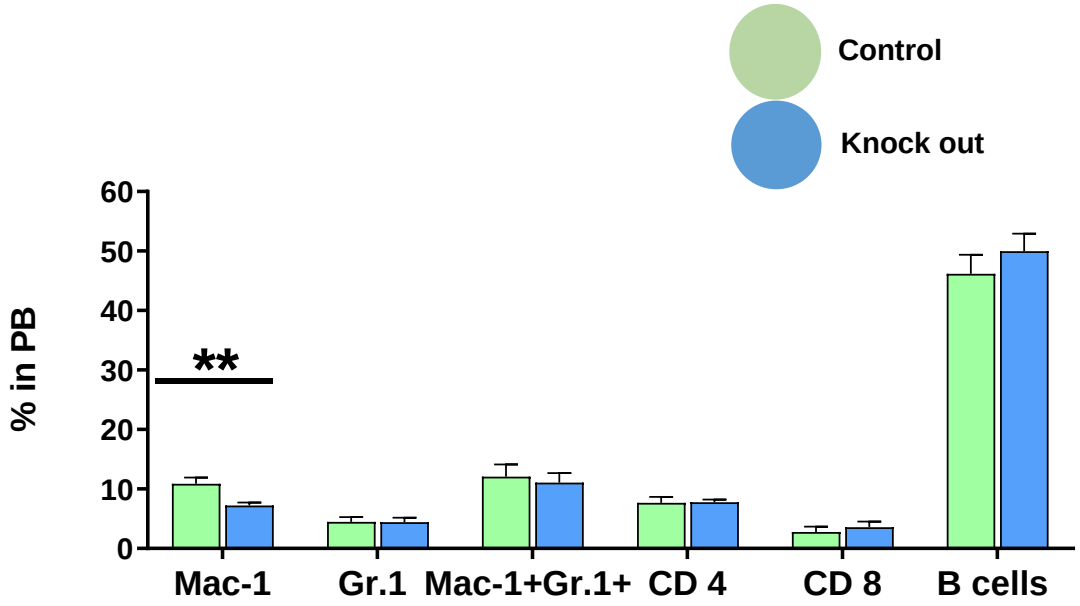


Figure 3.11 Loss of ApoE affects the frequency of Mac-1 cells in PB.

Bar graph shows the frequency myeloid and lymphoid cells in PB. Statistical analysis was performed using Mann Whitney test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using control: 5 and KO: 9 mice ** P<0.01.

Table 3.1 Summary of the observed phenotype in steady-state ApoE-/- mice.

Immunophenotype analysis	ApoE-/- phenotype
HSPCs in BM	No changes
Lin+ in BM	Reduction in B cells
Lin+ in SP	Increase in spleen weight, non-significant reduction in B cells and TER119+ cells
T cells in Thy	No changes, insignificant reduction in B-cells
Lin+ in PB	Decrease in Mac-1+ population
CBCs	Reduction in Platelets

3.1.3 Discussion

Haematopoiesis is the process of the production of cellular components and blood plasma. Within the haematopoietic system, the principal organ contributing to blood cell production is the BM, with the Thy and Sp acting as sites of extramedullary haematopoiesis (Jagannathan-Bogdan and Zon 2013). The ApoE gene is the provider of instructions for making the protein ApoE, which when combined with lipids and fats in the body, produces molecules called lipoproteins. The main responsibility of these lipoproteins is to package cholesterol and other forms of fats, and then those are carried through the blood stream. In this chapter, the effect of ApoE on haematopoiesis was evaluated by immunophenotypic analysis of BM, Sp, Thy, and PB and functional analysis of BM progenitors in vitro.

Formerly, ApoE gene expression in HSPCs has been reported to be abundant and has been associated with regulation of the proliferation of HSPCs, monocytes, and monocyte accumulation in atherosclerotic lesions (Murphy et al. 2011) and to accelerate the ageing of HSCs Tie et al. (2014) in the context of an HFD. This encouraged me to hypothesise that ApoE has a fundamental role in the regulation of haematopoietic cells in a normal steady-state haematopoiesis in the context of an NCD. Therefore, a germline deficient ApoE murine model (ApoE-/-), aged 8–12 weeks and fed an NCD, was used to comprehensively analyse haematopoietic cell compartments in BM, PB, SP, and Thy. In addition, CFCs using total BM cells were performed to assess the differentiation and growth capacity of haematopoietic progenitor cells from ApoE-/- mice at the functional level.

HSPCs and lineage-specific myeloid and lymphoid progenitors from both groups in steady state were unperturbed. In summary, the impact of ApoE in immunophenotypically defined HSPC compartments was not significantly different. Consistent with this, I found no change functionally in the number of CFCs, including CFC-mix activity, which provides a crude surrogate of multi-potential haematopoietic progenitor activity. Yet, immunophenotype may not reflect the functional attributes of HSPCs, CFC assays performed in vitro do not entirely reflect HSPC function in vivo, and I did not perform competitive repopulation and serial transplantation experiments, which are the gold standards for measuring in vivo HSPC function and HSC self-renewal, respectively (Harrison et al. 1997: Micklem et al. 1972). Indeed, other reports suggest a function for ApoE in HSPCs. For example, in the context of atherosclerotic lesions, it has been observed that ApoE acted on cells autonomously to control the proliferation of HSPCs along with monocytosis, neutrophilia, and monocyte accumulation in atherosclerotic lesions (Murphy et al. 2011).

Using an ApoE murine model fed an HFD, Murphy et al. (2011) showed that ApoE binds to proteoglycans to control the proliferation of HSPCs. The LXR-inducible pools of ApoE on the surface of HSPCs revealed that ApoE binds to proteoglycans to regulate the proliferation of

HSPCs, expansion of myeloid cells, and the accumulation of monocytosis and monocytes in atherosclerotic lesions. Furthermore, ApoE interacts with ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1), both of which are expressed in HSPCs to regulate cholesterol efflux and reduce the downstream signalling of IL3 receptors (Murphy et al. 2011). ABCA1 and ABCG1 indicate the regulatory role of the ApoE gene in haematopoiesis.

The deficiencies of ApoE, ABCA1, and ABCG1 have downstream effects including the accumulation of cholesterol, increased cell surface levels, and signalling of the common β subunit of the IL-3/GM-CSF (CBS) through the STAT and ERK signalling pathways. Furthermore, a competitive BM transplantation assay revealed that ApoE acted autonomously to regulate the proliferation of HSPCs, monocytes, and neutrophils, and monocytosis accumulation in atherosclerotic plaque. Additionally, Tie et al. (2014) showed that ApoE murine model fed an HFD LDL and had elevated ROS. Tie et al. (2014) revealed that hypercholesterolaemia induces oxidant stress in HSCs, which modifies the gene expression and the regulation of the cell cycle. This culminates in the aging of HSCs and is exhibited by the reduction in the long-term HSC compartment, telomere erosion, quiescent loss, and reduced reconstitution capacity of HSCs. These data point to the leukocyte contribution to atherosclerosis development and provide considerable evidence of the connection between leucocytosis and hypercholesterolaemia.

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The available literature also suggests that ApoE may have a significant impact on lineage-specific mature cells including a sub-type of NK cell from ApoE-/- mice, which demonstrated much more cytotoxicity than conventional NK cells. These cytotoxic effects of NK cells derived

from ApoE KO mice were associated with a higher expression of Granzyme B, Fas Ligand, IFN-γ, TNF-α, NKG2D, NKp46, and DNAM-1 (Lee et al. 2019). In this chapter, further evaluation of lineage-specific progenitors in the BM showed no sign of any impact of ApoE, with the notable exception of B cells, which were reduced in ApoE-/- mice. This result was also mirrored by a non-significant reduction in B cells from the Sp and Thy of ApoE-/- mice. ApoE-mediated control of B cells appears to be independent of both HSC/HSPC and CLP compartments, which were largely unchanged. Therefore, it is reasonable to hypothesize that ApoE-mediated regulation of B cell abundance occurs during post-CLP B-cell selection. Further immunophenotyping experiments and functional experiments (transplantation and CFC-B assays) will be needed to dissect the precise role of ApoE in pre/pro-B cell development. A role for ApoE in B cell biology has been suggested by other studies. B cells express the activation of LDL-R, and the activation of NKT cells by B cells is entirely dependent on LDL-R, as shown by blocking experiments and the complete absence of antigen presentation while using ApoE2, an isoform of ApoE that is incapable of binding LDL-R. Furthermore, B cells use an apolipoprotein-mediated lipid antigen presentation pathway, which is a type of innate NKT cell support for B cells (Allan et al. 2009).

In the pathological setting, major studies exploring both the protective and pro-atherogenic roles of B cells in atherosclerosis have been conducted by Caligiuri et al. (2002) and Major et al. (2002) utilizing different atherosclerotic models, such as ApoE. Caligiuri et al. (2002) examined spleen-related immune activities in ApoE -/- mice, which revealed that splenectomy significantly exacerbated atherosclerosis in hypercholesterolaemic ApoE-/- mice. Furthermore, the transplantation of spleen cells of atherosclerotic ApoE-/- mice dramatically reduces atherosclerosis development in recipient young ApoE-/- mice. To investigate which subset of lymphocytes was immunoprotective, researchers carried out immunomagnetic isolation prior to cell transfer to isolate T and B cells; they found that B cells give protection to atherosclerosis and alleviate disease in splenectomised mice. The protective impact was related to an elevation in antibody titre to oxLDL (Caligiuri et al. 2002). Similarly, Major et al. (2002) used B cell-deficient mice (μMT) as a BM donor for LDLR-/- mice. Subsequently, recipients were shown to have <1% of normal B cell populations. Moreover, LDLR-/- deficient B cells on an HFD revealed a marked reduction in the levels of total serum antibodies and in the level of antibodies against oxLDL. Additionally, the deficiency of B cells demonstrated 30 – 40% elevation in the lesion area. The results suggest a protective role of B cells in atherosclerosis (Major et al. 2002).

In contrast, these studies were challenged by Ait-Oufella et al. (2010), who studied lesional development in mice with or without the depletion of B cells using CD20 monoclonal antibody. ApoE-/- mice were treated with CD20 monoclonal antibody, which resulted in a sustained and

dramatic reduction in the number of mature B cells in the BM, peritoneum, Sp, and blood. The results showed a significant decrease in lesion development. In addition, to rule out the possibility that this result was due to the specific murine model and diet, the researchers conducted the same experiment in both ApoE-/- and LDLR-/- mice. The findings showed that CD20 monoclonal antibody treatment culminated in a marked depletion of B cells and a significant reduction in atherosclerosis (Ait-Oufella et al. 2010). The depletion of B cells corresponded to a reduction in the proliferation of activated splenic T cells, T cell content in lesions content, and CD4+ T cells. Overall, these findings suggest a proatherogenic role of B cells.

Alongside a trend decrease in B-cells and Ter119+ cells in the spleen, my experiments showed that spleen weight in ApoE-/- mice was relatively higher, which perhaps reflects the known role of the ApoE gene in lipid biosynthesis (Greenow et al. 2005). Cholesterol and lipids which remain in the bloodstream pass through the spleen and, as the spleen acts as a filter, those cholesterol and lipid molecules may be retained in the spleen of ApoE deficient background (Zhou et al. 2015). Further analysis of splenic lipid content together with histological analysis of splenic architecture will be required to dissect the possible role of ApoE in lipid biosynthesis in this setting.

In the PB, alterations were noted in Mac-1+ cells (monocytes) and platelets in ApoE-/- mice, with a decreased abundance of monocytes and platelets. ApoE expression in macrophages has long been known to prevent atherosclerosis by suppressing the formation of foam cells in the vessel wall (Fazio et al. 1997). More recently, ApoE was shown to suppress myelopoiesis (Murphy et al. 2011) and monocyte activation and expansion in the circulation in hyperlipidaemic mice (Gaudreault et al. 2012). My results showing a ApoE knockout mediated decrease in monocytes in steady state haematopoiesis have several possible mechanistic causes. Firstly, cellular cholesterol content influences the synthesis and secretion of ApoE by the macrophage/monocyte lineage (Zhang et al. 1996; Van Eck et al. 1997), which is consistent with the idea that ApoE acts as a cholesterol responsive gene (Dory 1991; Mazzone et al. 1989; Mazzone et al. 1987). Thus, ApoE secretion regulation might be essential in sustaining the balance of cholesterol efflux and influx (Mazzone et al. 1989; Mazzone et al. 1987; Van Eck et al. 1997). For example, ApoE produced by the macrophage/monocyte lineage might facilitate reverse cholesterol transport (Basu et al. 1982; Dory 1991). Indeed, Mazzone and Reardon (1994) and Granot and Eisenberg (1995) confirmed the role of ApoE in reverse cholesterol transport in vitro by showing the transfection of J774 macrophages that do not express endogenous ApoE promoted cholesterol efflux, while the addition of exogenous ApoE did not enhance the efflux of cholesterol. In addition, ApoE not only acts as a lipid transporter but also regulates essential immunoregulatory molecules with an impact on

immune cells, such as the macrophage/monocyte lineage (Grainger et al. 2004; Bellosta et al. 1995: Braesch-Andersen et al. 2013: Vitek et al. 2009; Kelly et al. 1994).

Conversely, monocytosis and neutrophilia have been noted in animal model of atherosclerosis (Averill et al. 1989; Feldman et al. 1991; Wang et al. 2014). Recent reports indicate that the expansion of HSPCs underlie the monocytosis and neutrophilia seen in animal models of atherosclerosis (Tall et al. 2012; Soehnlein and Swirski 2013; Wang et al. 2014). The deficiency of ATP-binding receptor (ABC) transporters (ABCA1 and ABCG1), which enhance myeloid cell cholesterol efflux, develop monocytosis and neutrophilia and HSPC expansion in the BM (Yvan-Charvet et al. 2010). A similar finding was seen in the ApoE murine model using a Western-type diet, where the expansion of HSPCs was associated with monocytosis and neutrophilia (Murphy et al. 2011). The transplantation of chimeric BM showed that ApoE $^{-/-}$ HSPCs outcompeted wild-type HSPCs in producing monocytes and neutrophils when transplanted into LDLr $^{-/-}$ mice and that ABCA1$^{-/-}$ and ABCG1$^{-/-}$ HSPCs outcompeted WT HSPCs when transplanted into wild-type mice, which suggests an advantage of cell intrinsic proliferation of ApoE $^{-/-}$ ABCA1$^{-/-}$, and ABCG1$^{-/-}$ HSPCs compared to WT HSPCs (Murphy et al. 2011). In these studies, the proliferative advantage of a cell intrinsic was associated with an increased level of the cell surface of common β subunit granulocyte macrophage colony stimulating factor (GM-CSF) / interleukin -3 receptor (IL-3) (CBC). Robbins et al. (2012) used a ApoE murine model and revealed an increased number of the cells were producing GM-CSF in the spleen, and they highlighted the importance of the extramedullary expansion of HSPCs in the spleen as an enhancer of monocytosis and atherogenesis. Wang et al. (2014) also revealed that the CBS plays an essential role in regulating and mediating monocytosis that developed in the hypercholesterolaemia ApoE murine model.

Consistent with my results, ApoE has also been shown to regulate platelet formation. In the steady state, Riddell et al. (1997) reported that ApoE regulates anti-platelet activity by promoting the production of endogenous nitric oxide (NO), elevating the activity of platelet NO synthase and the level of intraplatelet of cyclic guanosine monophosphate (cGMP), while inhibitors of NO synthase limited its inhibitory action. Even though ApoE causes an elevation in both cyclic adenosine monophosphate (cAMP) and cGMP, additional experiments implicated that guanylate cyclase activity specific stimulation as well as increased cGMP are required for ApoE antiplatelet action. The anti-aggregatory action of ApoE was also mediated by platelet NO synthase through NO production. Therefore, the inhibitors of NO synthase, such as amino acid of L-arginine, preserve ApoE antiplatelet action.

My observations of the decreased platelet count in ApoE-/- mice are consistent with that of Biswas et al. (2016), who reported that carboxyalkylpyrrole-phosphatidylethanolamine derivatives (CAP-PEs) in the plasma of the ApoE murine model bind to Toll-like receptor 2

(TLR2) and cause the activation of platelet integrin $\alpha_{IIb}\beta_3$ and the expression of P-selectin in a TLR2 dependent manner. The activation of platelets via CAP-Pes including the TLR2/TLR1 receptor complex, the induction of downstream signalling via myeloid differentiation primary response 88 / MyD88 adapter-like (Mal)/TIRAP (MyD88/TIRAP), and interleukin-1 receptor-associated kinase 4 (IRAK4) phosphorylation, and the subsequent activation of necrosis factor receptor–associated factor 6 results in the activation of spleen tyrosine kinase, platelet integrins, and Src family kinases (SFK).

Platelets also play an essential role in atherosclerosis in which their overproduction and activation enhances the growth of plaque (Huo et al. 2002; Murphy et al. 2013). Platelets are implicated in the multiple steps resulting in atherothrombosis, both in enhancing the growth of atherosclerotic plaque and thrombi rupture on eroded plaque (Koenen et al. 2009; Huo et al. 2002). To this end, Van Geffen et al. (2020) studied prothrombotic propensity using hyperlipidaemia murine strain ApoE$^{-/-}$ and LDLr$^{-/-}$. The findings revealed that a moderate increase in plasma cholesterol is sufficient to promote the activation of platelets and fibrin formation. In this setting, Van Geffen et al. (2020) suggested that megakaryocytes adapt to a changing lipid environment and therefore, affect platelet formation (Murphy et al. 2013; Van Geffen et al. 2020). In addition, limited enhancement of coagulation activity in plasma was accompanied by a more enhanced formation of fibrin thrombus. Furthermore, this study suggests that the prothrombotic phenotype of platelets in the atherosclerotic murine model is enhanced by lipid surrounding, leading to moderate alterations in the level of lipid abundance. Altogether, these findings indicate a role of ApoE in regulating platelet cells in steady-state and hyperlipidaemic settings.

This project has explored the role of ApoE in normal haematopoiesis in a young ApoE murine model. The study utilized a young ApoE murine model from whom BM, Thy, and PB were harvested to study blood cells. Even though this project attempted to increase the knowledge and understanding of ApoE in the field of haematopoiesis with regard to the risk of atherosclerosis, the scale of gabs continues to be both extensive and multifaceted. Thus, considering the following for future research may assist in the accomplishment of the mentioned objectives. The results showed a reduction in B cells, but more studies are needed to section B cells to evaluate which B-cell subsets are affected in a young ApoE murine model to provide a precise and accurate view of the involvement of B cells in atherosclerosis and haematopoiesis (Caligiuri et al. 2002; Major et al. 2002; Ait-Oufella et al. 2010). Using immunophenotypic assay by flow cytometry technique to characterise B cells would be essential to determine the involvement of the fundamental role of B cells in haematopoiesis and atherosclerosis. Indeed, like all leukocytes, B cells are derived from HSCs in the BM. CLP has been recognized to produce B and T cells (Baumgarth 2004). In conjunction with an in

vitro culture system, the flow cytometry technique was instrumental in recapitulating the development of B cells from the earliest committed precursor to the immature B cells stage as well as in identifying the B cells phenotype. Several combinations of markers have been utilised to categorise the stages of development of B cells in mice and humans. The expression of CD19 and c-kit cells recognise B cells in the BM of adult mice (Rolink et al. 1996). In addition, CD19 is expressed on the cell surface after the expression of B220 (Allman et al. 1999). The identification of c-kit and CD19 as the earliest committed B cells precursor by Melchers et al. (1995) seems to reconstitute the later stage of B cells' development (Baumgarth 2004). Taken together, the data point to the definition of B-cell lineage commitment rather than the identification of the B-cell phenotype.

Furthermore, the gene expression of RAG1, RAG2, IgM positive, and IgD negative B cells, CD24, represent immature B cells (Baumgarth 2004). The immature B cells, which migrate from the BM to the Sp, are referred to as a transitional B cells. Three populations could be separated in the SP: B-1 cells, marginal zone B cells, and follicular cells. The recognition of B cells is based on cell surface markers B220, CD19, CD5, CD23, and CD21 (Baumgarth 2004). Follicular B cells could be identified based on the cell surface expression of $CD5^{-}$ $CD21^{int}$ and $CD23^{+}$. Marginal zone B cells can be characterised by an increased expression of CD1, CD21, and IgM. B-1 cells can be characterised according to the expression of cell surface $CD11b^{+}$ $CD23^{-}$,IgM^{hi}, and IgD^{lo}. In addition, a mouse pre-B colony forming cells assay was performed, which is dependent upon the ability of the haematopoietic progenitor cells to proliferate and differentiate in response to cytokines stimulation in a semisolid media (Mouse Pre-B Colony Forming Cell (CFC) Assay Using Methylcellulose-based Media 2021). Furthermore, transplantation assays provide a precise role of the B-cell populations in an ApoE murine model. For instance, Major et al. (2002) utilised B-cell deficient mice (μMT) as a BM donor for low density lipoprotein receptor (LDLR-/-) mice. Next, the recipients exhibited <1% of normal B-cell populations. Besides, LDLR-/- deficient B cells on an HFD show a noticeable decrease in the total serum antibodies level and antibodies level against oxLDL. Furthermore, competitive transplantation assays are needed to determine the function and the multilineages reconstitution capacity of HSCs in a young ApoE murine model (Micklem et al. 1972). In addition, a serial transplantation assay is required to evaluate the repopulation and the differentiation capacity of ApoE deficient cells.

As the ApoE murine model is the preferred model for atherosclerosis (Meir and Leitersdorf 2004), more atherosclerotic studies are required to confirm and to study plasma lipid profile in a young ApoE mice in the context of an NCD (Raffai et al. 2005; Choy, Siow, Mymin and O 2004) and aortic arch sectioning to check the lipid content, plaque size, and lesion area (Murphy et al. 2011). Moreover, the finding revealed that there is a decrease in a platelet count

in the PB. However, due to the low sample size, conclusion cannot be drawn. Therefore, more samples size is needed to confirm the result. Additionally, immunohistochemistry technique to stain either B cells or platelets in an atherosclerotic lesion will be useful in determining and confirming the involvement of these cells in haematopoiesis in a less hypercholesterolaemic environment (Murphy et al. 2011; Tie et al. 2014). Furthermore, a quantitative reverse transcription polymerase chain reaction (qRT- PCR) will be needed to identify genes differentially expressed between young mutant ApoE and control mice (Tie et al. 2014). In addition, from the above, a germline mutate ApoE murine model was used; it is worth mentioning that it is very difficult to distinguish between both the intrinsic and extrinsic mechanisms behind the observed immunophenotype. Therefore, exploring the effect of the acute deletion of ApoE in the haematopoietic cells in the young murine ApoE model using an Mx1 – Cre system to delete ApoE conditionally in the haematopoietic cells should be explored. Indeed, this system uses an inducible promoter of the mouse Mx1 gene to regulate Cre recombinase transgene expression (Kuhn et al. 1995). Mx1 promoter could be activated via an increased amount of transcription in many tissues of interferon α or interferon β or of the synthetic double stranded RNA (polyinosinic - polycytidylic acid) (Kuhn et al. 1995). Besides, exploring the requirement of ApoE in HSCs functions via transplantation assay 14 days post ApoE deletion is crucial.

To conclude, evidence is presented in this chapter to suggest an association between ApoE and the regulation of steady-state haematopoiesis in select mature cell lineages, namely, B cells, monocytes, and platelets, which may be understood further in functional transplantation assays and in the transcriptomic analysis of those lineage-specific haematopoietic progenitor populations.

Chapter 4: Exploring the impact of a high fat diet on haematopoiesis in an apolipoprotein murine deficient model

4.1 Introduction

Several studies have concluded that there are intrinsic and extrinsic impacts of diet on stem cell generation (Zon 2008). In general, calorie restriction caused by an NCD causes a boost in the regenerative and self-renewal capacity of the stem cell (Cho and Park 2020). In this case, the calorie or dietary restriction is defined as the reduction of 20-40% of the total calorie in nutrition intake without malnutrition (Johnson et al. 2013). On the contrary, in an HFD, it is found that HSCs are less efficient at mobilizing from BM to the blood (Mihaylova et al. 2014). An HFD induces myelopoiesis in HSCs (Singer et al. 2014). A recent study of 2019 has concluded that an HFD interrupts the maintenance of HSCs in mouse BM by disturbing lipid raft/TGF-β signalling (Hermetet et al. 2019). Furthermore, it has also been found that excess cholesterol results in hypercholesterolaemia, which promotes haematopoietic stem and progenitor cell proliferation (Vanhie et al. 2020).

Deposition of cholesterol-rich lipoproteins in the artery wall causes atherosclerosis, contributing to monocyte-macrophage recruitment and persistent inflammation. Although inflammation associated with atherosclerosis could lead to this relationship, there is also substantial evidence that atherosclerosis and thrombosis are specifically enhanced by leukocytosis (Coller 2005). Numerous risk factors for CVD are all linked with leukocytosis, including obesity, smoking, sedentary habits, and metabolic syndrome (including individual elements of metabolic syndrome, such as elevated triglycerides and low HDL) (Tani et al. 2009). Both prospective and cross-sectional studies have also directly correlated monocytosis with CVD and the atherosclerotic plaque burden (Bovill et al. 1996).

In animal models, monocytosis has also been associated with atherosclerosis: Gerrity first noted the association between dietary hypercholesterolaemia, monocytosis, and atherosclerosis in models of pigs and rabbits and identified an increase in the development of BM-derived CFCs in hypercholesterolaemic pigs (Feldman et al. 1991; Wierenga et al. 1998). Dietary hypercholesterolaemia is characterised by the progressive monocytosis and a rise in the Ly-6Chi (CCR2+) subset of monocytes that reach lesions more readily than Ly-6Clo monocytes in the Apoe-/- mouse model of atherosclerosis (Swirski et al. 2007). ApoE-/-mice monocytosis has previously been shown to entail both increased development and diminished cell clearance, but the detailed mechanisms underlying monocytosis and neutrophilia remain poorly characterised (Swirski et al. 2007).

In conjunction with hypercholesterolaemia, the lack of ABCG1 and ABCA1 in BM-derived cells results in dramatically accelerated atherosclerosis (Yvan-Charvet et al. 2007). These mice exhibit dramatic myeloid cell proliferation, monocytosis, and neutrophilia, with HSPCs underlying proliferative defects. Increased plasma membrane lipid rafts and increased plasma membrane levels of the IL-3/GM-CSF receptor typical β subunit (CBS, also referred to as IL-3Rβ or CD131) were correlated with HSPC hyperproliferation. However, the role of endogenous apolipoproteins in regulating the proliferation of HSPCs was not established by these studies. ApoE has a prominent role in the regulation of leukocytosis, as noted above, and ApoE and ApoE-containing HDL interact with ABCA1 and ABCG1, respectively, to facilitate cellular cholesterol efflux (Matsuura 2006; Remaley et al. 2001).

4.1.1 Aims of the chapter

According to many studies, diet intake appears to be an essential regulator of HSCs, and thus of organismal physiology and health, especially stem cell function. In many studies, caloric restriction and fasting increased HSCs' quiescence and protected them from injury, respectively, while an HFD impaired haematopoiesis (Mana et al. 2017). Research clearly shows that an HFD affects the number and differentiation of HSPCs, but the mechanism governing these effects has not been identified (Mana et al. 2017). For instance, an HFD causes changes to the haematopoietic cells. Feeding an HFD diet for a few weeks results in a decrease in HSCs (Luo et al. 2015; Berg et al. 2016), whereas long-term HFD feeding enhances the number of HSCs and functions (Singer et al. 2014). Since HDF affects HSCs, which supply and produce the inflammatory immune cells that are crucial in the development of atherosclerosis and CVD, HFD in turn strongly affects haematopoiesis, atherosclerosis, and CVD.

Previous researchers have identified the causal relationship between hypercholesterolaemia, leucocytosis, and CVDs, which indicates that leucocytosis may promote atherosclerosis (Coller 2005). Murine models of atherosclerosis, such as ApoE and low-density lipoprotein receptors develop atherosclerosis upon feeding an HFD (Knowles and Maeda 2000). ApoE is a multifunctional protein that has a fundamental role in lipid metabolism (Greenow et al. 2005). Thus, ApoE deficiency leads to differences in lipoprotein metabolism and therefore significant hypercholesterolaemia (Curtiss and Boisvert 2000: Greenow et al. 2005). The ApoE gene has been found to be expressed abundantly in HSPCs and has been observed to control the proliferation of HSPCs and monocytes, and the accumulation of monocyte in the lesion of atherosclerosis in the context of an HFD (Murphy et al. 2011).

Although Murphy et al. (2011) concluded that ApoE regulates the proliferation of HSPCs and monocytes as well as monocytosis accumulation in atherosclerosis lesion in the context of an HFD and an NCD, the broader impact of ApoE on other haematopoietic lineages and HSC self-renewal in the setting of an HFD remains unclear. Thus, the underlying hypothesis of this chapter is that ApoE is an essential regulator for haematopoiesis and the functionality of HSPCs in the context of an HFD and an NCD.

The aim of this chapter is to evaluate the effect of an HFD on haematopoiesis using an ApoE-/- murine model with the following objectives:

1. To immunophenotypically characterise haematopoietic stem and progenitor cells (HSPCs) and lineage positive cells in the bone marrow
2. To characterise lineage positive cells in peripheral blood, spleen, and thymus
3. To evaluate peripheral blood by complete blood count test
4. To test the functionality and self-renewal of haematopoietic stem cells using HSCs transplantation assay

ApoE-/- murine model (8 – 12 weeks) along with WT mice were fed either an HFD or NCD for 12 weeks. After this period, immunophenotypic analysis of HSPCs compartment in BM, lineage differentiated cells in BM, PB, Sp, and Thy was performed. In addition, HSC primary and secondary transplantation was conducted to assess the functionality of HSCs to produce multilineage haematopoiesis and self-renewal respectively. (Figure 4.1-4.3).

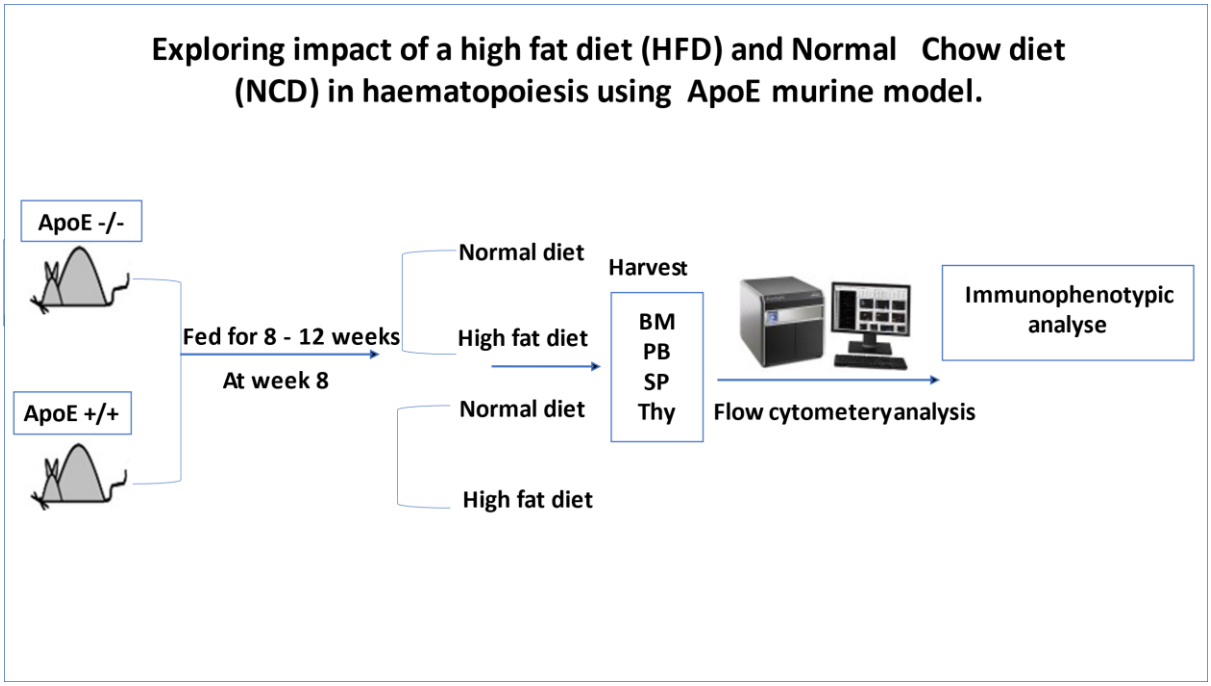


Figure 4.1 The experimental design for exploring the impact of an HFD and NCD on haematopoiesis. One group of ApoE knockout mice was fed an HFD and the other fed an NCD. One group of a ApoE +/+: wild-type mice was fed an HFD and the other was fed an NCD at 8 weeks of age for 12 weeks. Then, BM: bone marrow, PB: peripheral blood, Sp: spleen, and Thy: thymus were harvested for immunophenotypic analysis using flow cytometry.

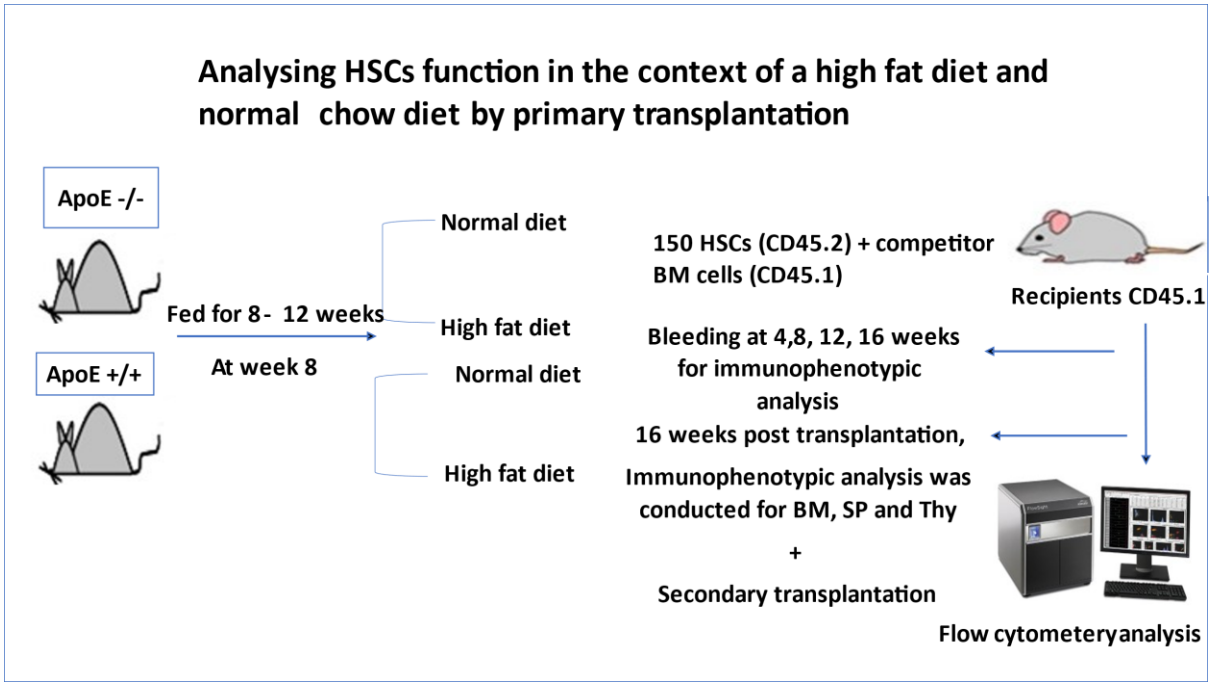


Figure 4.2 The experimental design for assessing HSC function in the context of an HFD and NCD by primary transplantation. 150 HSCs were sorted from group conditions, ApoE knockout and control mice who were fed either an HFD or NCD for 12 weeks and transplanted with competitor BM cells into recipient mice. All recipient mice were bled 4-, 8-, 12-, and 16-weeks post transplantation for immunophenotypic analysis. Then, 16 weeks post transplantation, immunophenotypic analysis was conducted to characterise haematopoietic cells in BM, Sp, and Thy using flow cytometry. In addition, 300 HSCs were sorted from all group conditions for secondary transplantation.

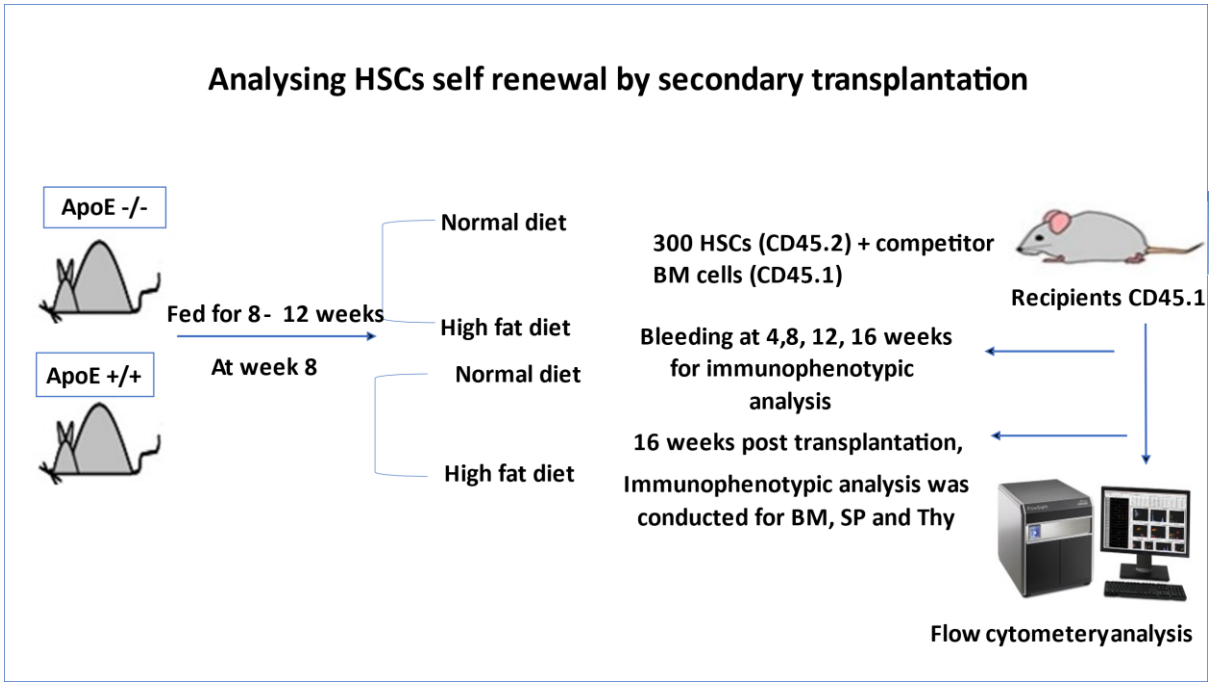


Figure 4.3 The experimental design for assessing HSCs self-renewal function in the context of an HFD and NCD by secondary transplantation. 300 HSCs were sorted from all group conditions and transplanted with competitor BM cells into recipient mice. All recipient mice were bled at 4, 8, 12, and 16 weeks post-secondary transplantation for immunophenotypic analysis. Immunophenotypic analysis was conducted to characterise haematopoietic cells in BM, Sp, and Thy by flow cytometry.

4.1.1 Results

4.1.1.1 Evaluating the effect of a high fat diet on haematopoiesis in ApoE-/- mice

Studies have reported that the ApoE gene is abundantly expressed in HSPCs and is found to regulate the proliferation of HSPCs and monocytes and the accumulation of monocytes in atherosclerotic lesions (Murphy et al. 2011), in addition to accelerating the ageing of HSCs (Tie et al. 2014). This prompted me to evaluate the impact of an HFD more broadly in haematopoiesis and the HSC function in the ApoE murine model. For this, ApoE-/- mice (8 – 12 weeks) along with WT mice were fed an HFD and an NCD for 12 weeks. PB, BM, Sp, and Thy samples were harvested for assessing the effect of an HFD and NCD on haematopoiesis.

4.1.1.2 The impact of a high fat diet impact on bone marrow cellularity in ApoE-/- mice

First, BM from ApoE-/- mice on an HFD showed a non-significant reduction in cellularity compared to ApoE-/- mice on an NCD. In control mice, an HFD does not have any impact on cellularity when compared to control mice fed an NCD (Figure 4.4).

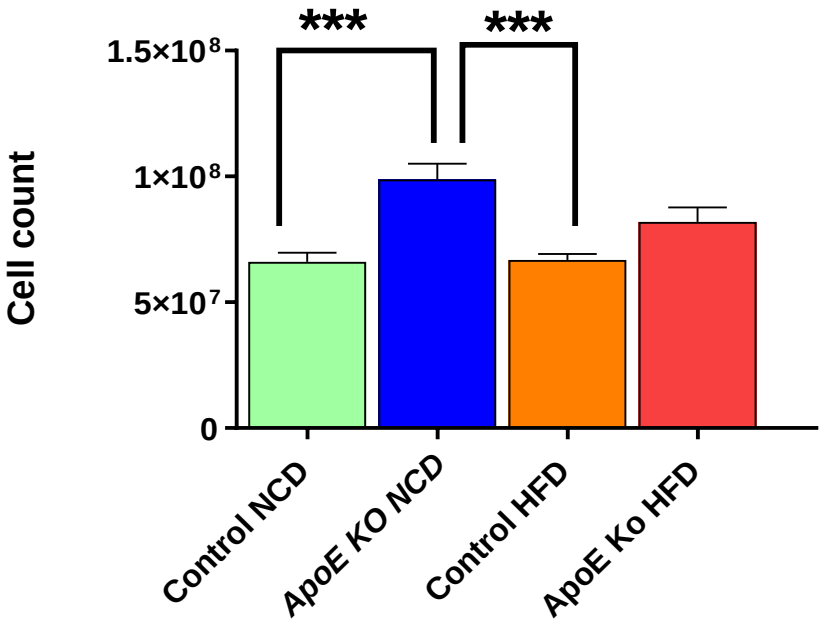
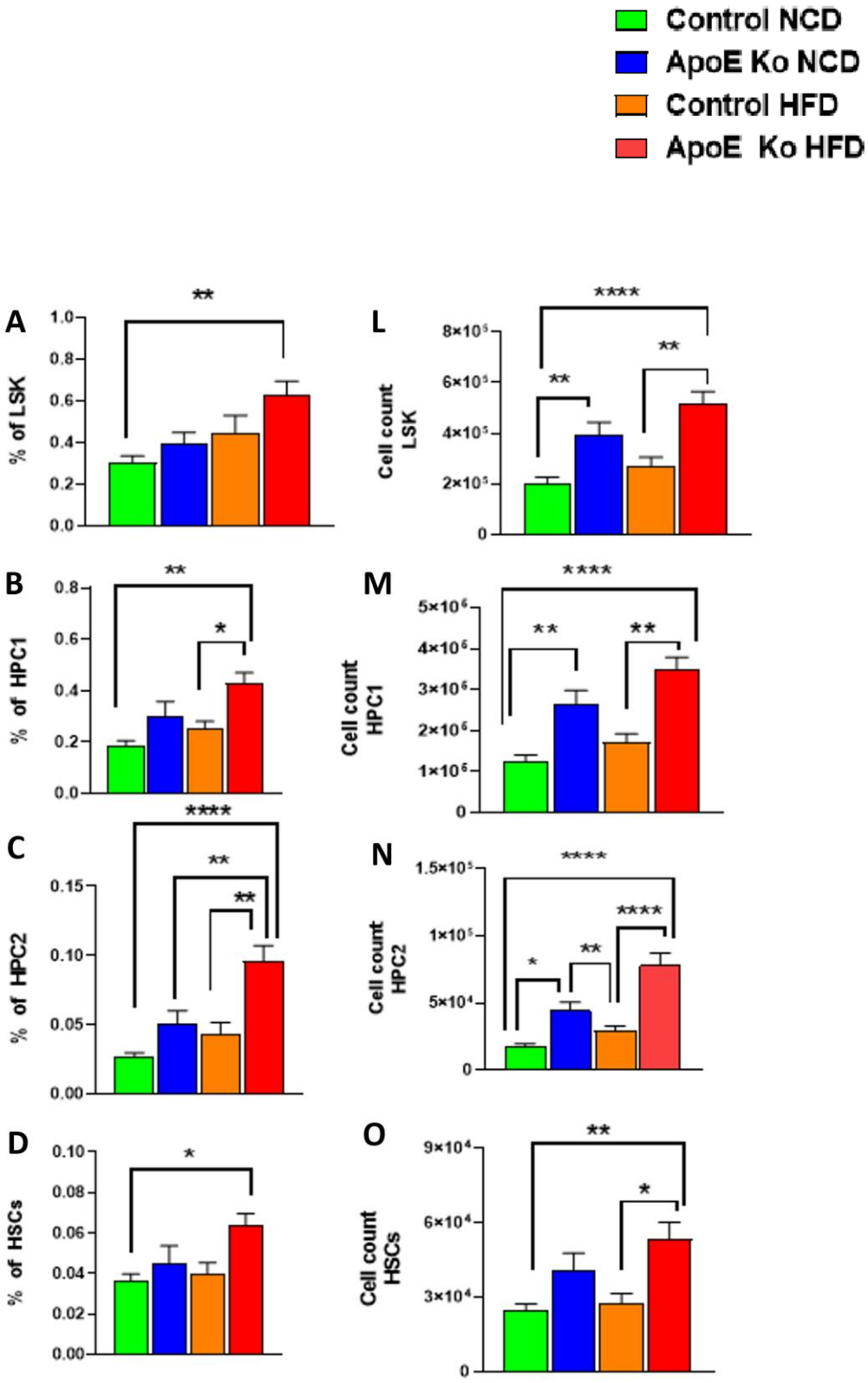


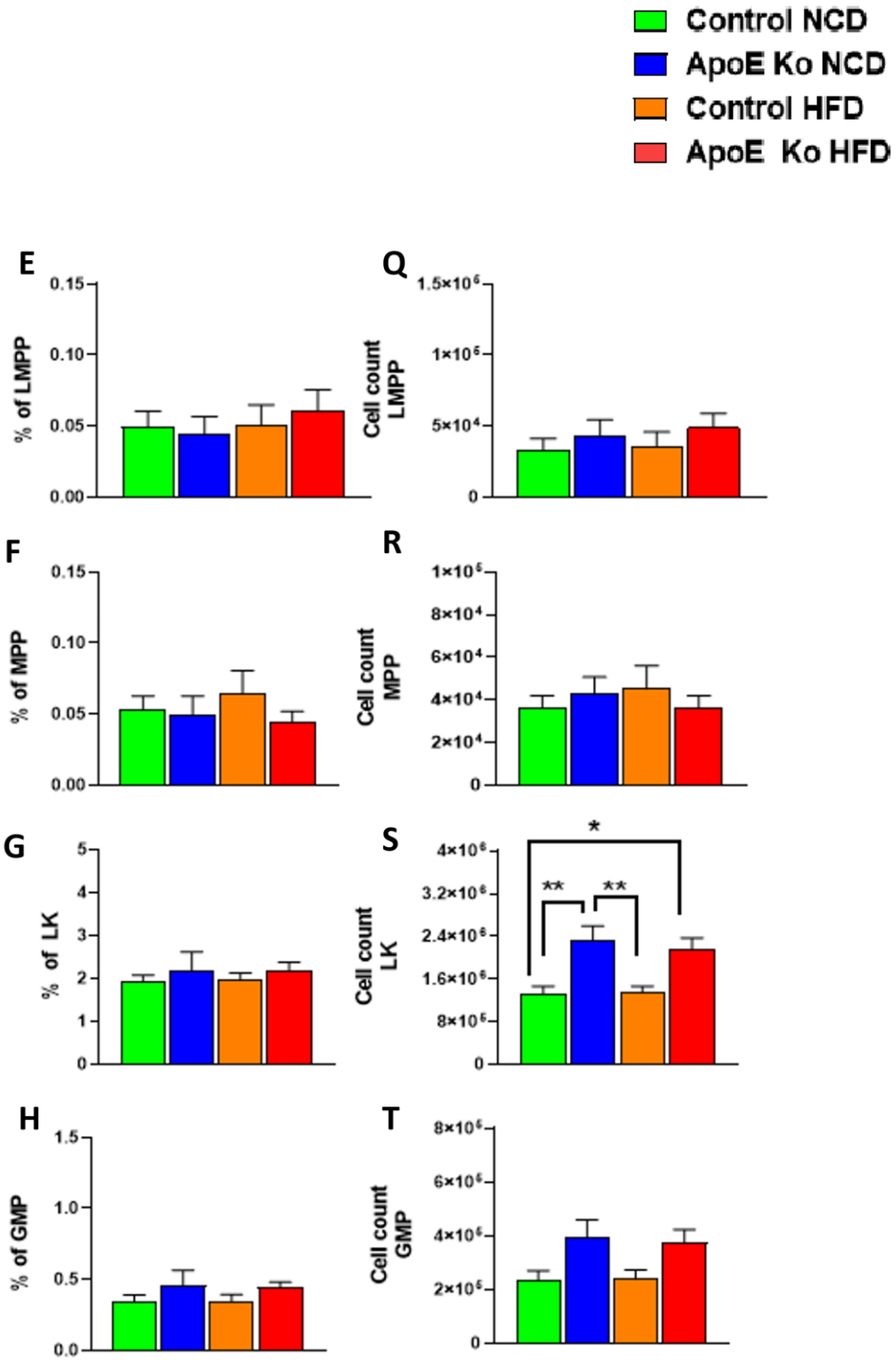
Figure 4.4 Impact of HFD on bone marrow cellularity in ApoE-/- mice. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD: 9, Ko NCD: 9, Control HFD:7, Ko HFD: 8 mice. *** P ≤ 0.001. Control: wild type mice: ApoE KO: ApoE knockout mice, NCD: Normal chow diet, HFD: high fat diet.

4.1.2.3 The impact of a high fat diet on haematopoietic stem and progenitor cells from ApoE-/- mice

Next, I analysed the HSC-containing the LSK population and LSK subpopulations (such as HPC1, HPC2, HSC, and MPP) based on the expression of the SLAMs CD150 and CD48 in the BM in ApoE-/- or control mice fed an HFD or NCD. When the cell count of the immunophenotypically-defined HPCs and highly enriched HSC population were analysed, the most primitive HSPCs (HSC, HPC1, HPC2, and MPP) revealed differential sensitivities to HFD in the presence and absence of ApoE. With an HFD, an insignificant increase in LSK, HPC1, and HPC2 frequency in ApoE-/- mice was noted in comparison to their ApoE-/- counterparts fed an NCD, which translated to significant increase in absolute number in HPC2 in ApoE-/- mice fed HFD (Figure 4.5 (A,B,C and L,M,N). HSCs from ApoE-/- mice fed an HFD were significantly expanded in comparison to control mice fed an NCD but were only insignificantly expanded against ApoE-/- mice fed an NCD (Figure 4.5 D, O).

In contrast, no significant difference in numbers of lymphoid-primed multipotent progenitors (LMPP) or MPPs across the experimental conditions (Figure 4.5 A and C)**.** Within the LK populations that mark myeloid progenitors, I observed no substantial change in the frequency of GMP or MEP in either the ApoE-/- or control mice being fed an HFD or NCD (Figure 4.5 B), and CLPs were similarly unaltered. A significant elevation in the absolute number of the LK population was noted in ApoE-/- mice in both the HFD and NCD settings (Figure 4.5 D). A slight but insignificant increase was observed in the frequency and absolute number of the CMP in ApoE-/- mice fed an HFD compared to ApoE-/- mice fed an NCD (Figure 4.5 B and D). Altogether, these results reveal that combined HFD and loss of ApoE perturbs cell distribution within select subsets of HSPCs.


Control NCD
ApoE Ko NCD
Control HFD
ApoE Ko HFD
A
% of LSK
B
% of HPC1
C
% of HPC2
D
% of HSCs
L
Cell count LSK
M
Cell count HPC1
N
Cell count HPC2
O
Cell count HSCs


Control NCD
ApoE Ko NCD
Control HFD
ApoE Ko HFD
E
% of LMPP
0.15
0.10
0.05
0.00
Q
Cell count LMPP
1.5×10⁵
1×10⁵
5×10⁴
0
F
% of MPP
0.15
0.10
0.05
0.00
R
Cell count MPP
1×10⁵
8×10⁴
6×10⁴
4×10⁴
2×10⁴
0
G
% of LK
5
4
3
2
1
0
S
Cell count LK
4×10⁶
3.2×10⁶
2.4×10⁶
1.6×10⁶
8×10⁵
0
*
**
**
H
% of GMP
1.5
1.0
0.5
0.0
T
Cell count GMP
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0

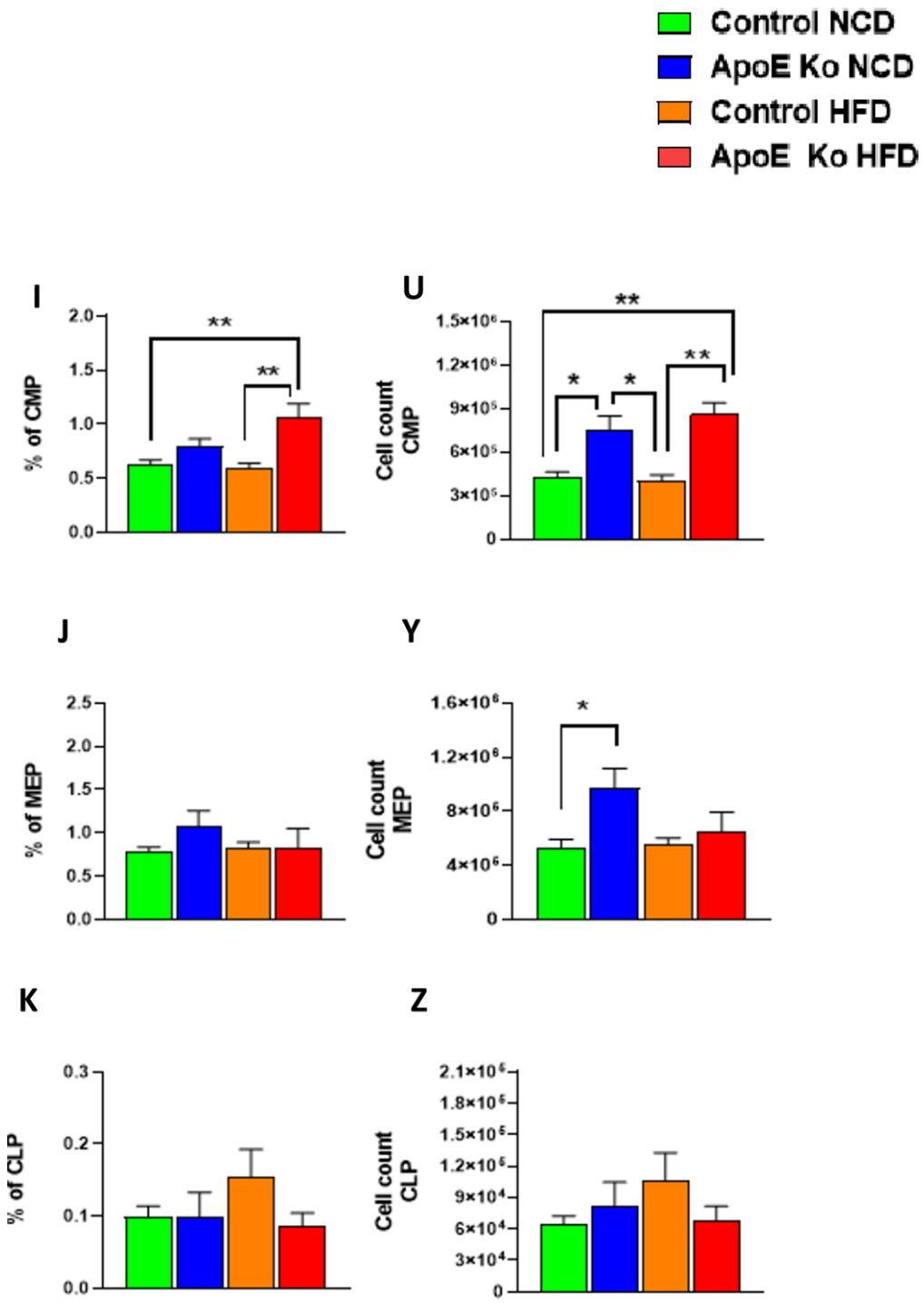
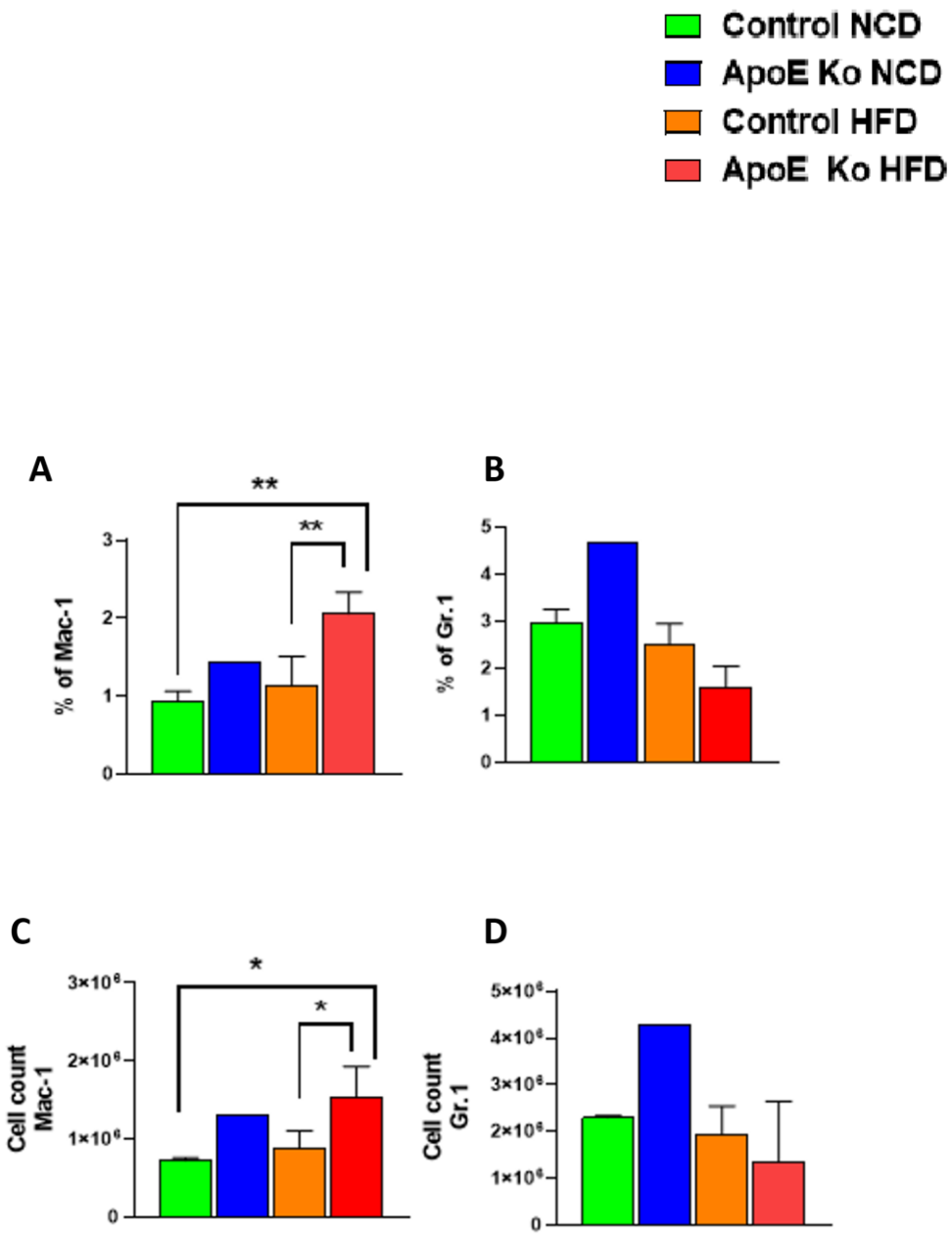


Figure 4.5 High fat diet selectively affects HSPC populations in an environment lacking ApoE. Bar graphs show the frequency of LSK and its subpopulation (A – F), LK and its subpopulation (G – K), the absolute number of LSK and it subpopulation (L – R), and LK and its subpopulation (S – Z) of each population (C, D). Absolute counts of each cell type were calculated by

multiplying the percentages (frequencies) of each cell type by the total number of cells from populations arising from LSK and LK. Data shown here represent the frequency and total cell count of each population in the BM. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD: 8, Ko NCD: 7, Control HFD:6, Ko HFD: 6 mice. *P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 N: 6-8 mice. Control: wild type mice: ApoE KO: ApoE knockout mice Normal chow diet, HFD: high fat diet. LSK: lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$, LK: lin^- $c\text{-}kit^+$, HPC1: haematopoietic progenitor 1, HPC2: haematopoietic progenitor 2, HSCs: haematopoietic stem cells, MPP: multiple progenitors, GMP: granulocyte monocyte progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor.

4.1.2.4 High fat diet impacts select mature myeloid and lymphoid cells in ApoE-/- bone marrow

Turning to analysis of mature blood cells in the BM, it was observed that the frequency and absolute number of Mac-1+ cells in the BM was increased in the ApoE-/- HFD group compared to the ApoE-/- NCD group. While trend differences were observed in other myeloid cells, no significant differences were observed (Fig 4.6 A – E, G). For lymphoid cells, an insignificant reduction in B cells and significant reduction in CD8+ T cells was noted in BM of ApoE-/- from HFD group compared to their NCD counterparts. (Figure 4.6 F and H).


Control NCD
ApoE Ko NCD
Control HFD
ApoE Ko HFD
A
B
C
D
% of Mac-1
% of Gr.1
Cell count Mac-1
Cell count Gr.1
**
**
*
*
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3
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5
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3×10⁶
4×10⁶
5×10⁶

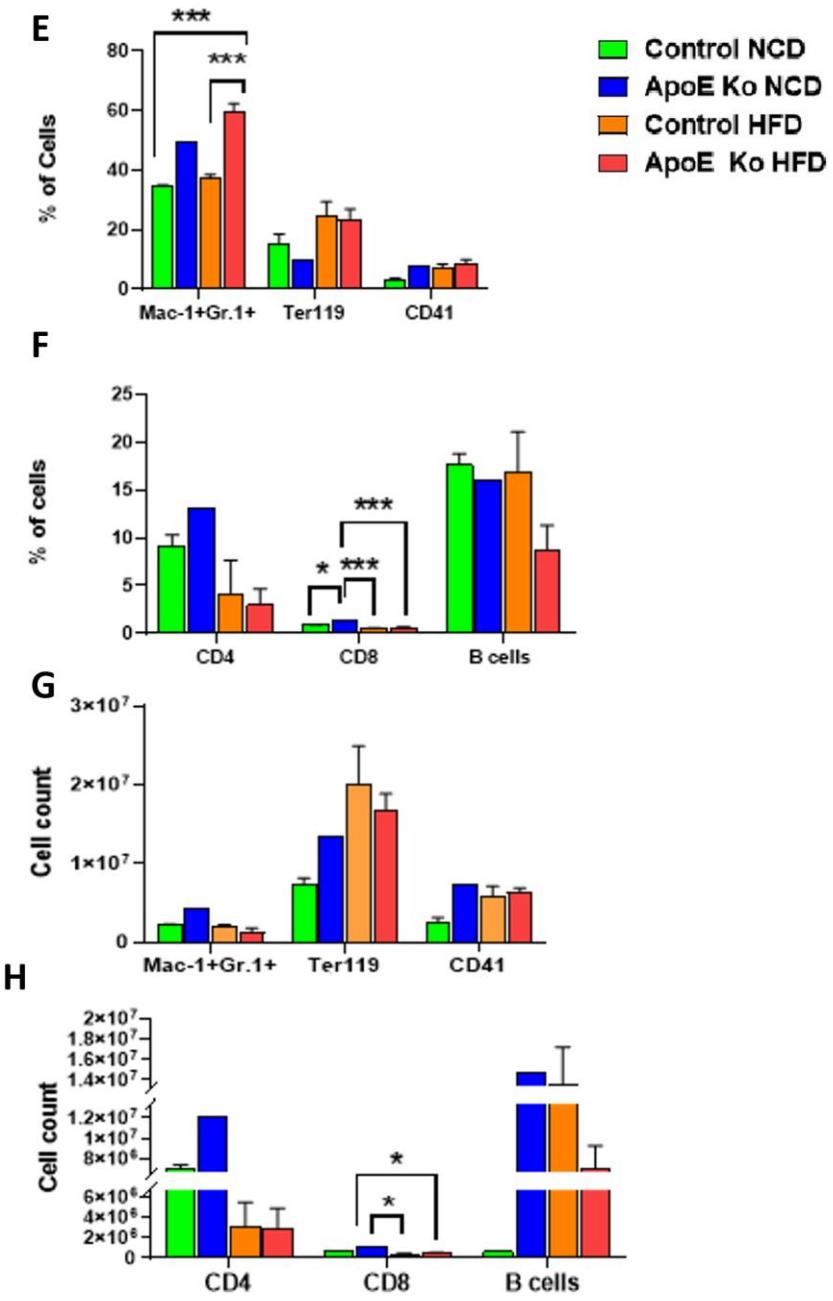


Figure 4.6 Impact of high fat diet and normal diet on mature lineage cells from ApoE-/- bone marrow. Impact of HFD and NCD on multilineage haematopoiesis on BM. HFD and NCD affect the frequency and the total cell count of cells in BM. Bar graphs show the frequency (myeloid (A), (B) and (E) and lymphoid (F) and absolute cell count of myeloid (C), (D) and (G) and lymphoid cells (H). Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:8, Ko NCD: 1, Control HFD:6, Ko HFD: 6 mice. *P <0.05 ** P<0.01 and *** P < 0.001. Control: wild type mice: ApoE KO: ApoE knockout mice, NCD: normal chow diet, HFD: high fat diet.

4.1.2.5 The impact of high fat diet on spleen size and cellularity in ApoE-/- mice

Splenic hypertrophy was noted in ApoE-/- mice on both the HFD and NCD compared to their control counterparts, consistent with a significant elevation in spleen cellularity, but no significant differences were noted between ApoE-/- HFD versus ApoE-/- ND (Figure 4.7 and Figure 4.8). Thus, ApoE-/- induced splenomegaly regardless of their dietary status.

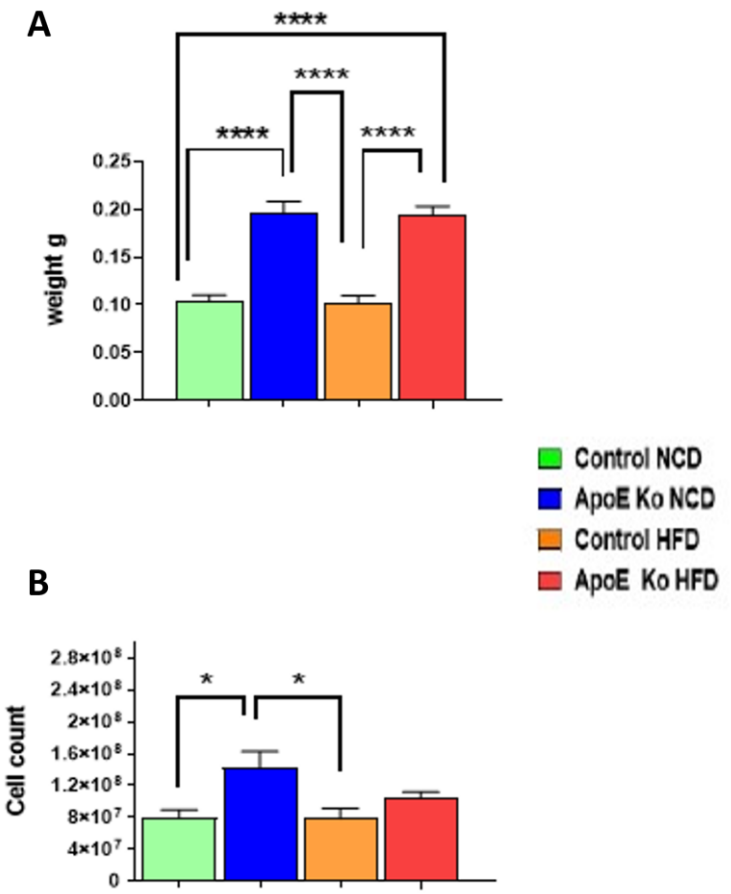


Figure 4.7 Impact of high fat diet on spleen size in ApoE-/- mice (A) and (B) cellularity. HFDs and NDs induce spleen hypertrophy. A. Bar graph represents the weight (A) and cellularity (B) of the spleen and. WT stand for ApoE+/+ and KO stand for ApoE +/+. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD: 9, Ko NCD: 8, Control HFD:7, Ko HFD: 8 mice. *P <0.05, *** P < 0.001. N: 6-9 mice. Control: wild type mice: ApoE KO: ApoE knockout mice, NCD: normal chow diet, HFD: high fat diet.

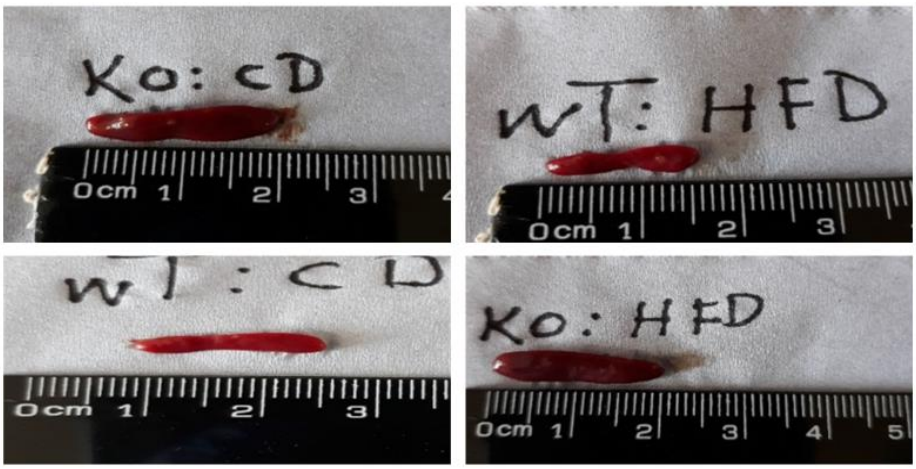


Figure 4.8 Splenomegaly induced in ApoE mice regardless of HFD. Representative photos of spleens in all condition. Ko: CD – ApoE knock out mice on a normal chow diet: WT: HFD – wild type mice on a high fat diet: WT: CD – wild-type mice on an NCD: K:HFD – knock out mice on an HFD.

4.1.2.6 The impact of high fat diet on myeloid and lymphoid cells in the spleen of ApoE-/- mice

There was no significant difference in the numbers of mature myeloid and lymphoid lineage cells from the Sp across the groups (Figure 4.9) except for Gr-1+ which displayed a non-significant reduction in ApoE-/- mice fed an HFD compared to their NCD counterparts and significantly reduced against control mice fed an ND.

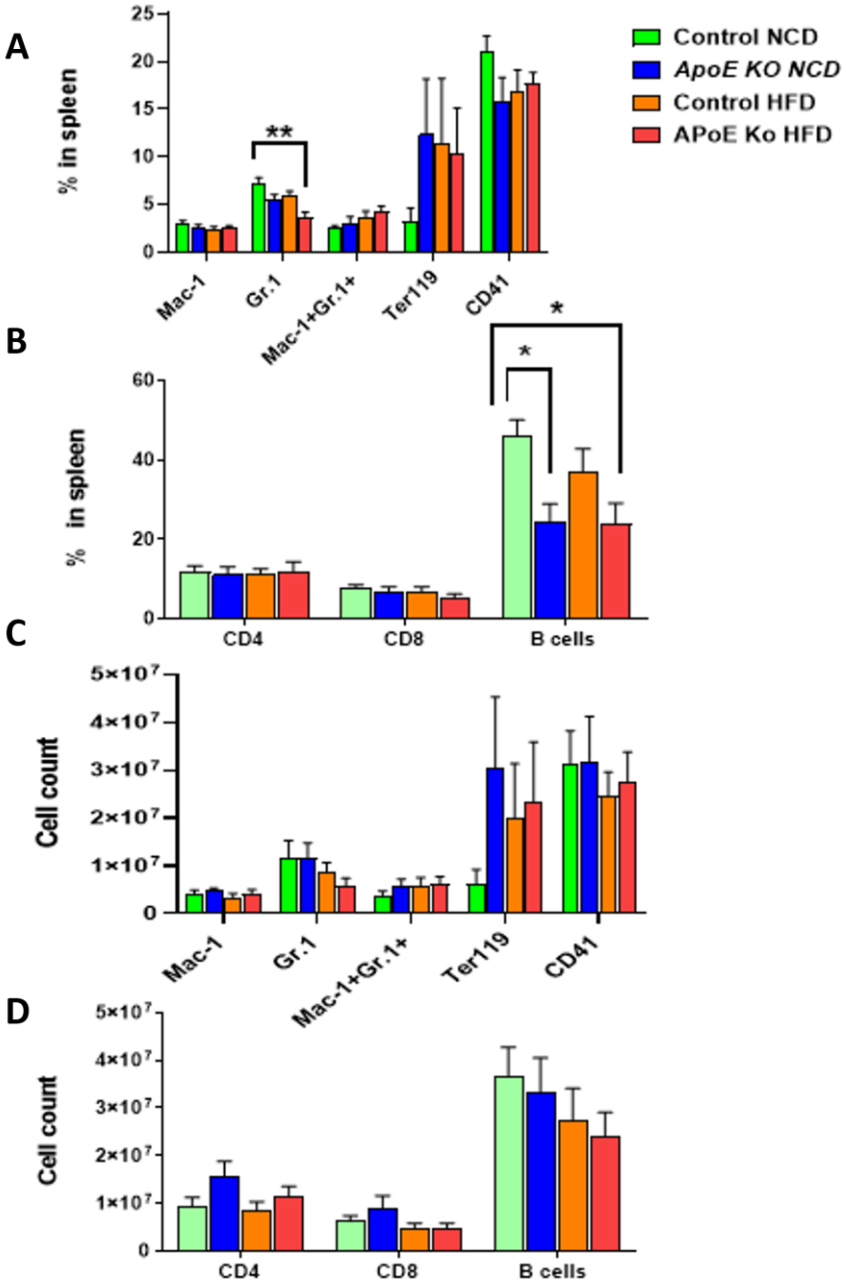
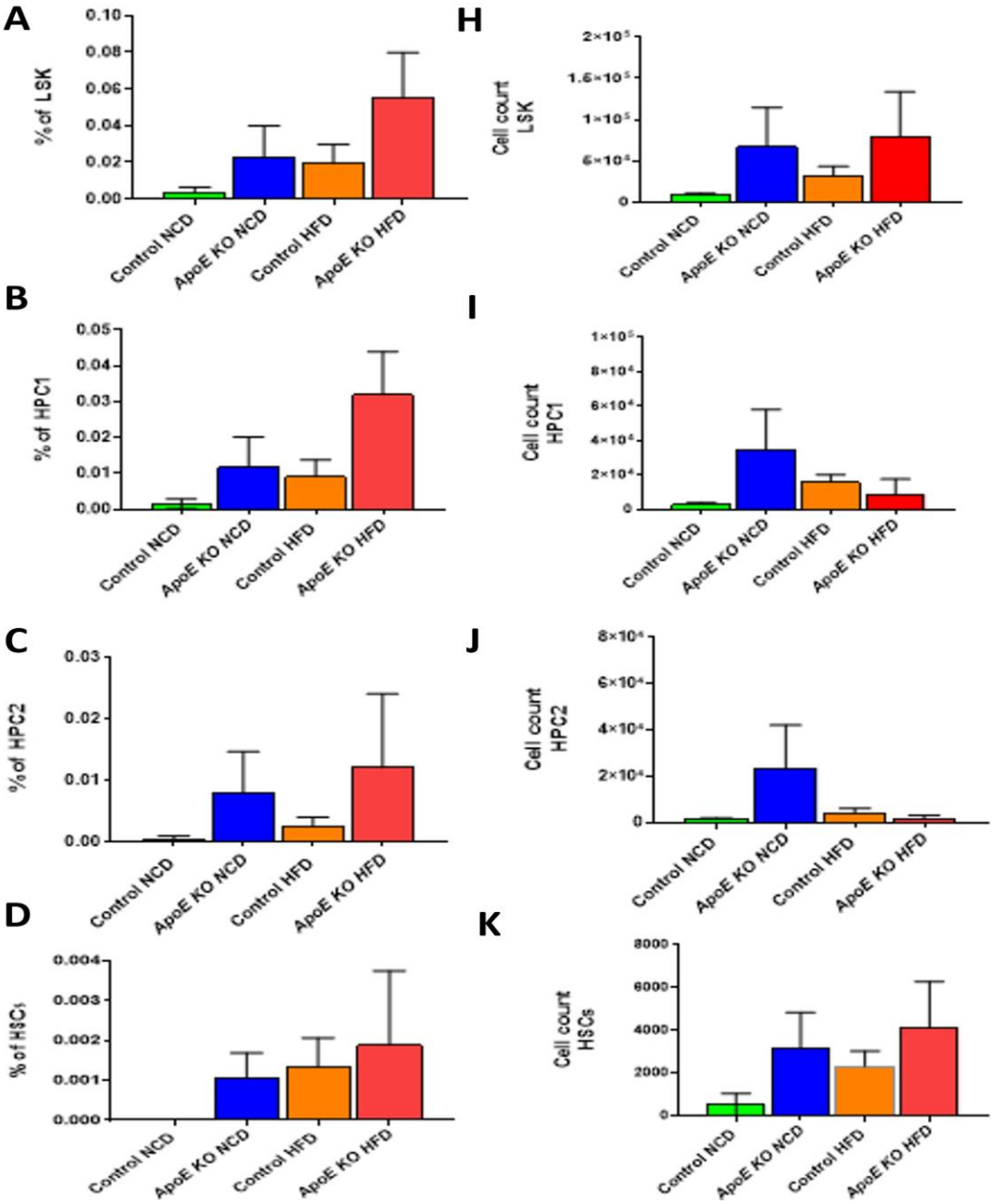
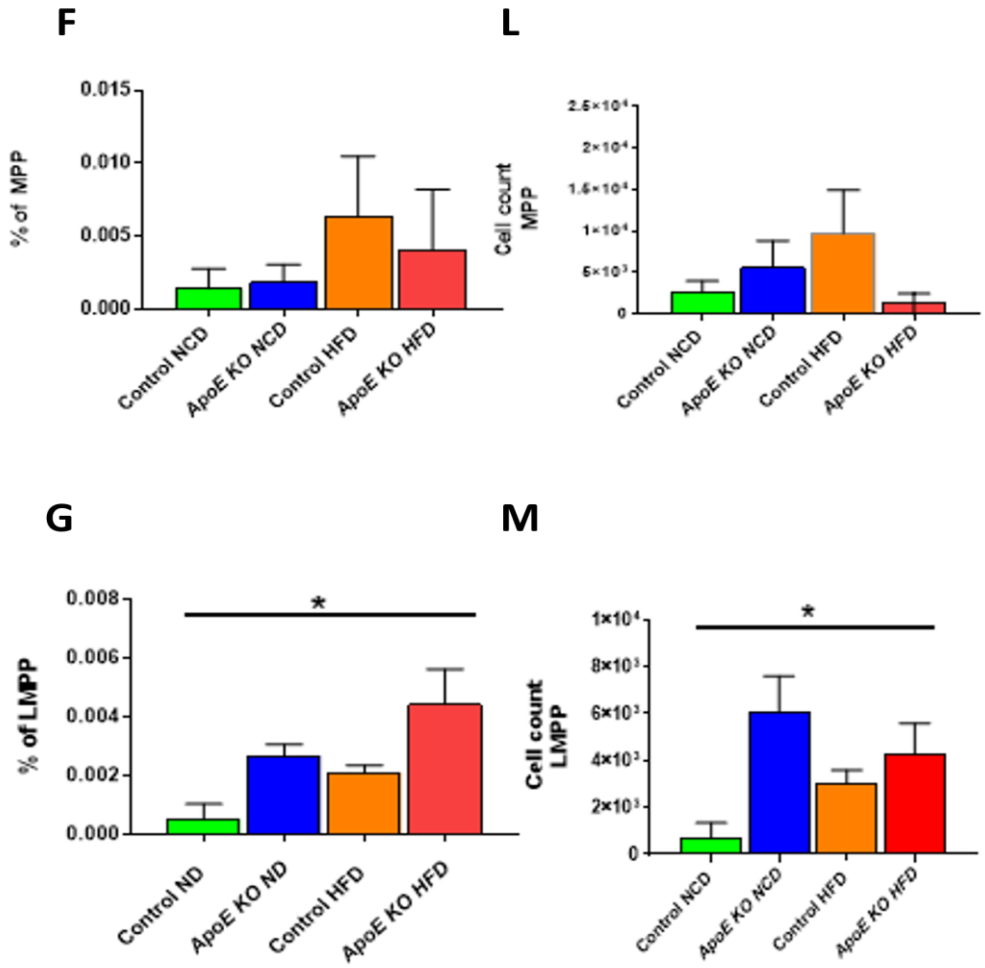


Figure 4.9 Impact of high fat diet on lineage positive cells in spleen in ApoE/- mice. Impact of HFD and NCD on multilineage haematopoiesis on BM. HFD and NCD affect the frequency and the total cell count of cells in BM. Bar graphs show the frequency (myeloid (A) and lymphoid (B) and absolute cell count of myeloid (C) and lymphoid cells (D). Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD: 5, Ko NCD: 4, Control HFD:5, Ko HFD: 5 mice. *P <0.05 ** P<0.01 and 4-5 mice. Control: wild-type mice: ApoE KO: ApoE knockout mice NCD: normal chow diet, HFD: high fat diet

4.1.2.7 The impact of high fat diet on HSPCs in the spleen

There was no significant difference in numbers of HSPC populations was found across groups fed an HFD or NCD (Figure 4.10) with the exception of the LMPP**.** Frequency of LMPP were found to be expanded in ApoE-/- fed an HFD compared to all groups, with significance only being reached against control mice being fed an NCD. (Figure 4.10 G). However, when normalized for cell count no differences were observed in total LMPP number (Figure 4.10 M).




F
% of MPP
0.015
0.010
0.005
0.000
Control NCD
ApoE KO NCD
Control HFD
ApoE KO HFD
L
Cell count MPP
2.5×10⁴
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
Control NCD
ApoE KO NCD
Control HFD
ApoE KO HFD
G
% of LMPP
0.008
0.006
0.004
0.002
0.000
*
Control ND
ApoE KO ND
Control HFD
ApoE KO HFD
M
Cell count LMPP
1×10⁴
8×10³
6×10³
4×10³
2×10³
0
*
Control NCD
ApoE KO NCD
Control HFD
ApoE KO HFD

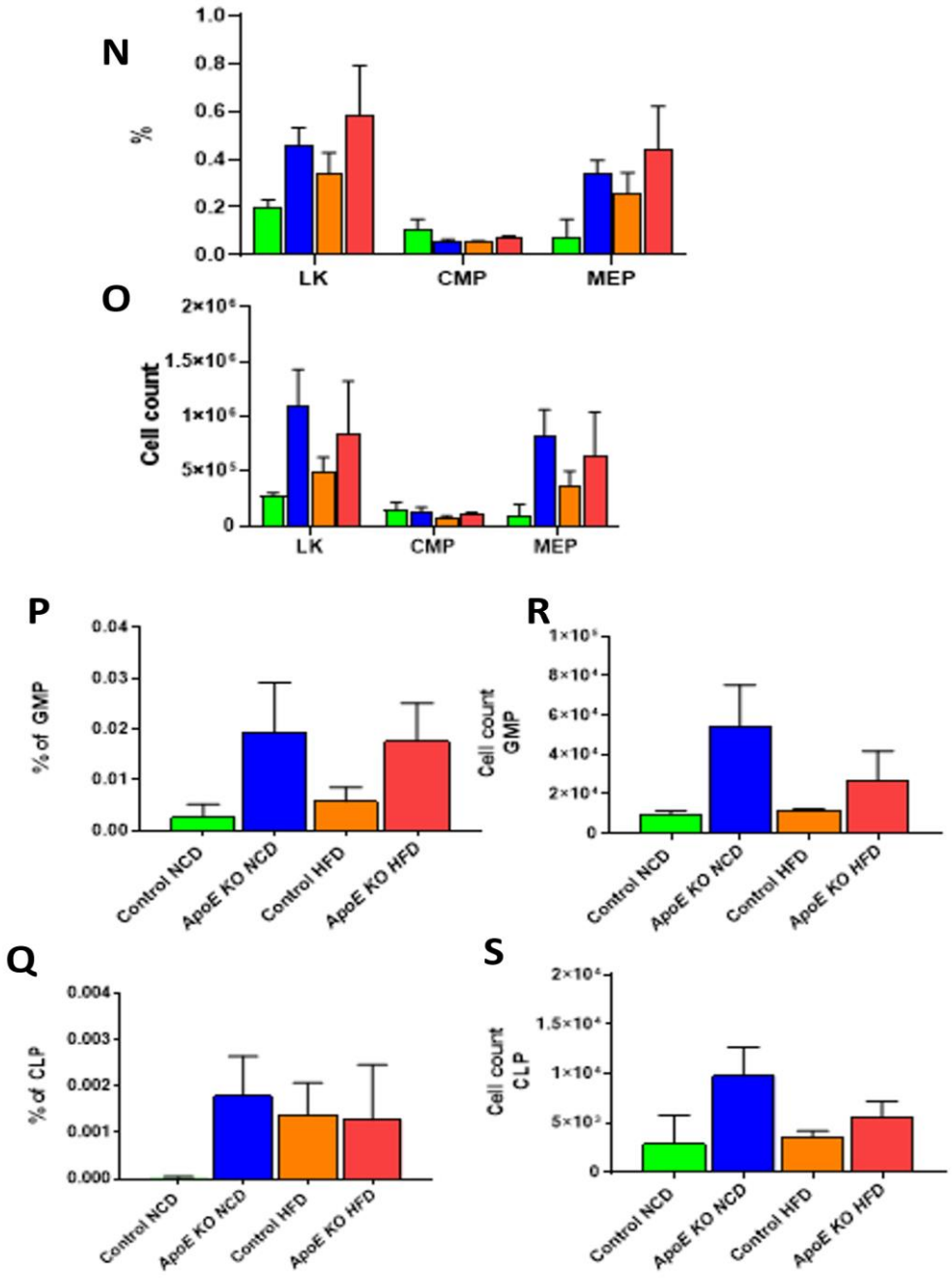
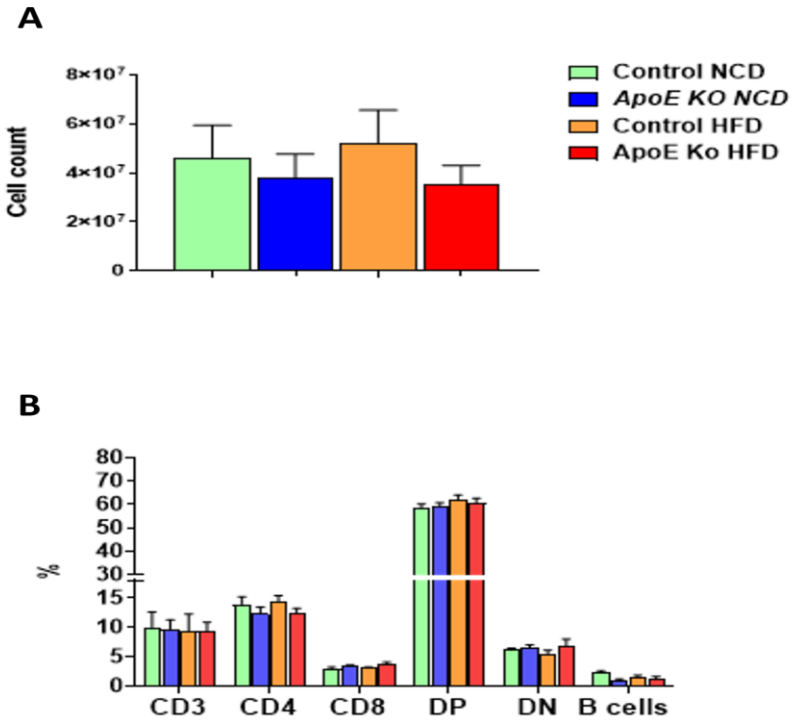


Figure 4.10 Impact of high fat diet on HSPCs from the spleen. Bar graph shows the frequency of LSK and its subpopulation (A) - (G) and LK and its subpopulations (N) – (Q) and total cell count of the LSK and its subpopulation (H) – (C) and LK and it subpopulation (O) – (S). Absolute counts of each cells were calculated by multiplying the percentages (frequencies) of each cell type by the total number of cells from populations arising from LSK and LK. Data shown here represent the frequency and total cell count of each population in the BM. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:2, Ko NCD: 3,

Control HFD:3, Ko HFD: 2 mice *P < 0.05, Control: wild-type mice, ApoE KO: ApoE knockout mice, NCD: normal chow diet, HFD: high fat diet, LSK: lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$, LK: lin^- $C\text{-}kit^+$, HPC1: haematopoietic progenitor 1, HPC2: haematopoietic progenitor 2, HSCs: haematopoietic stem cells, MPP: multiple progenitors, GMP: granulocyte monocyte progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor.

4.1.2.8 High fat diet did not affect lymphoid primed progenitor cells in the thymus

Similarly, no significant impact of diet or ApoE was found from detailed immunophenotypic analysis of Thy during T-cell development and in mature T-cell populations (Figure 4.11).



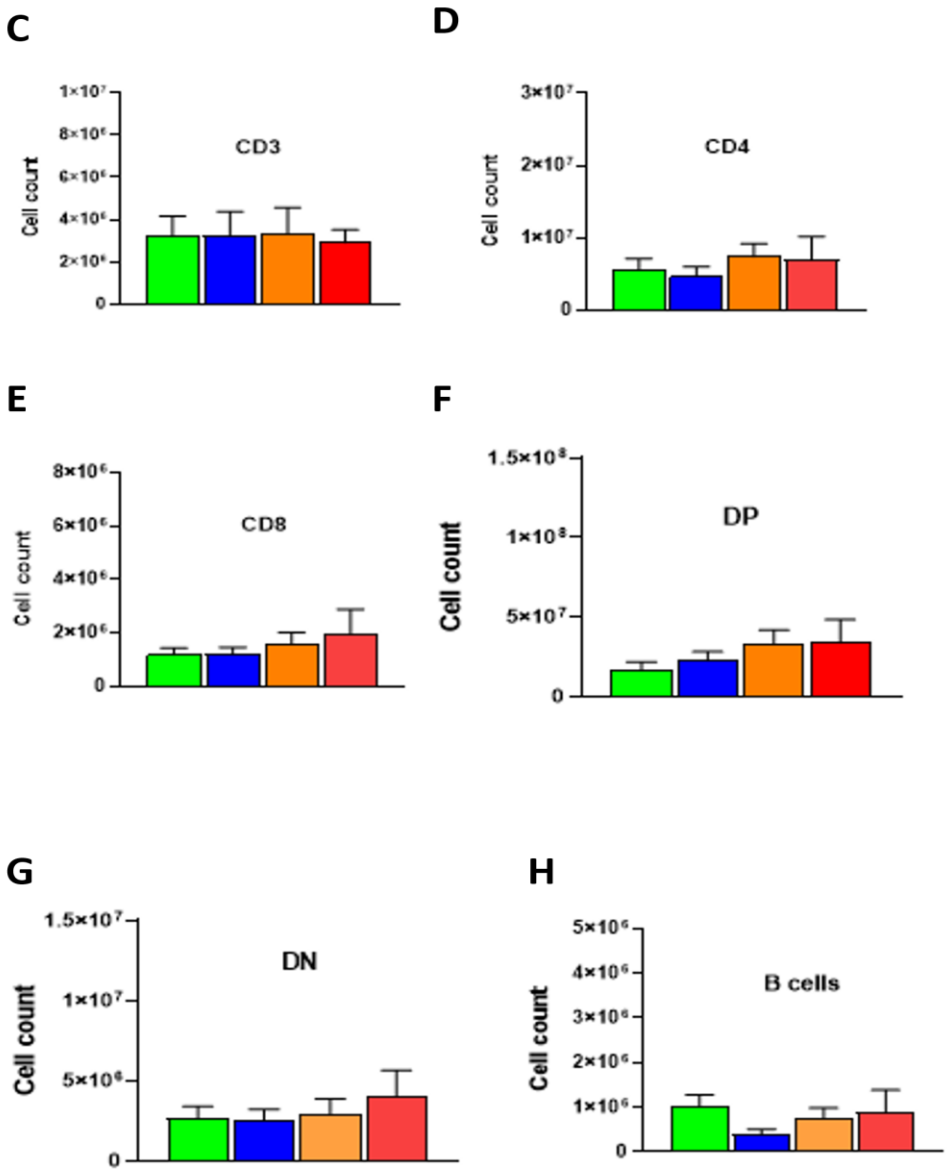


Figure 4.11 Impact of ApoE and HFD on lineage positive cells from the thymus**.** An HFD and NCD has no impact on T-cell development. Bar graphs show the total cell count (A) frequency of T cell development and (C - H) total cell count in Thy. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:8, Ko NCD:7, Control HFD:6, KO HFD: 7 mice. Control: wild type mice: ApoE KO: ApoE knockout mice Normal chow diet, HFD: high fat diet.

4.1.2.9 High fat diet has an impact Gr-1+Mac-1+ myeloid cells in peripheral blood of ApoE-/- mice

Analysis of PB revealed Mac-1+Gr-1+ increases in ApoE-/- HFD in comparison to ApoE-/- NCD, whereas other myeloid and lymphoid cells were largely unaffected by diet or loss of ApoE (Figure 4.12). Furthermore, CBC analysis revealed no significant impact of ApoE and diet where WBCs, RBCs, HGB and, platelets were similar across all conditions examined (Figure 4.13).

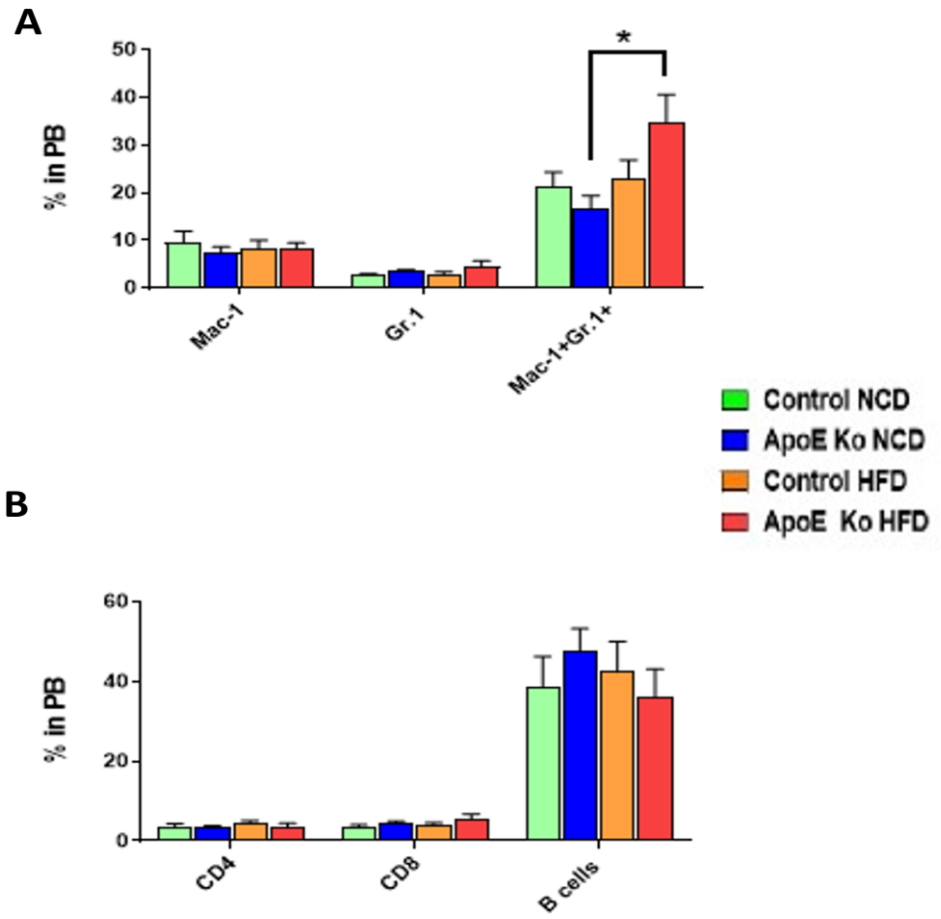


Figure 4.12 Impact of ApoE and diet on lineage positive cells in PB. Bar graphs show the frequency (A) myeloid and (B) lymphoid cells in PB. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple

comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD: KO NCD: 7, Control HFD:7, KO HFD: 8 mice. *P < 0.05. Control: wild-type mice: ApoE KO: ApoE knockout mice, NCD: normal chow diet, HFD: high fat diet.

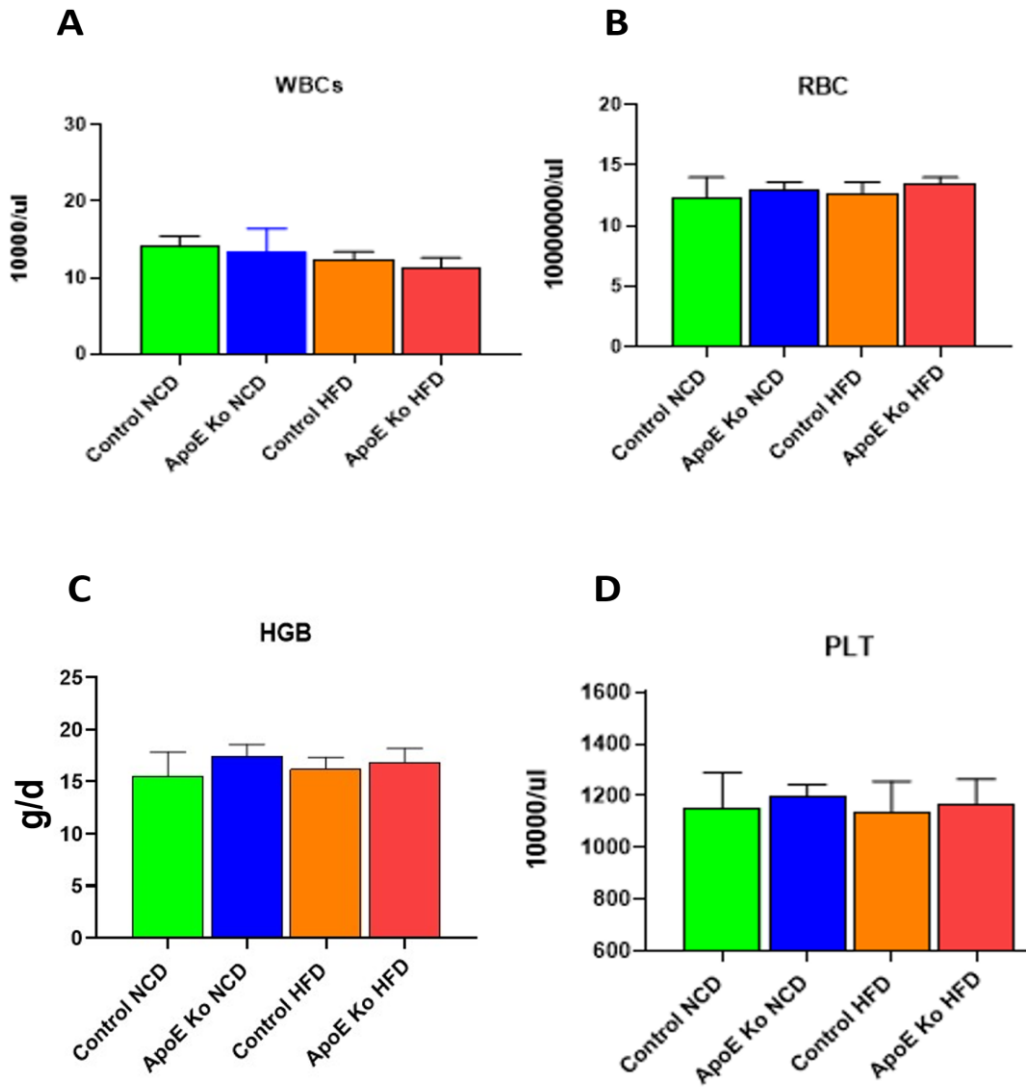


Figure 4.13 Impact of ApoE and high-fat diet as analysed by complete blood counts. An HFD and NCD has no impact on CBC test. A. total white blood cells (WBC). B. red blood cells (RBC). C. haemoglobin (HGB). D platelet (PLT). Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual

experiments using Control NCD:4, KO NCD: 6, Control HFD:5, KO HFD: 5 mice. Control: wild-type mice: ApoE KO: ApoE knockout mice, NCD: normal chow diet, HFD: high fat diet.

Table 4.1 A summary of the immunophenotyping results of HSPCs in BM and Lin + cells in BM

Immunophenotype analysis	ApoE-/- on NCD phenotype	ApoE-/- on HFD phenotype
Cell count	Increased total cell count compared to C: NCD and HFD	No change
% HSPCs in BM	NO change	Increased LSK compared to C: NCD Increased HPC1 compared to C: NCD and HFD Increased HPC2 compare all conditions Increased HSCs compare to C: NCD Increased CMP compared to C: HFD and NCD
Cell count HSPCs in BM	Increased LSK compared to C: NCD Increased HPC1 compared to C: NCD Increased HPC2 compared to C: NCD Increased LK compared to C: NCD and HFD Increased CMP compared to C: NCD and HFD Increased MEP compared to C: NCD	Increased LSK compared to C: NCD and C: HFD Increased HPC1 compared to C: NCD and C: HFD Increased HPC2 compared to all condition Increased HSCs compare to C: NCD and HFD Increased LK compared to C: NCD Increased CMP compared to C: NCD and C: HFD
% Lin+ in BM	Increased CD8 compared to all conditions	Increased Mac-1 compared to C: NCD and C: HFD Increased Mac-1+Gr.1+ compared to C: NCD and C: HFD
Cell count Lin+ in BM	Increased CD8 compared to C:HFD and ApoE :HFD	Increased Mac-1 compared to C: NCD and C: HFD

Table 4.2 A summary of the immunophenotyping results of HSPCs and Lin + cells in spleen

Spleen observation	Increased weight compared to C: NCD and HFD Increased total cell count compared to C: NCD and HFD	Increased weight compared to all conditions
% HSPCs in spleen	No change	Increased LMPP compared to C: NCD
Cell count HSPCs in spleen	No change	Increased LMPP compared to C: NCD
% Lin+ in Spleen	Decreased B cells compare C: NCD	Decreased Gr.1 compared to Ko: HFD Decreased B cells compare to C: NCD
Cell count Lin+ in spleen	No change	No change
Lin + in PB	No change	Increased Mac-1+Gr.1+ compared to Ko: NCD
CBC	No change	No change

4.1.2.10 High fat diet has an impact on apoptosis in haematopoietic stem/progenitor cells from ApoE-/- mice

To investigate whether the changes in the cellularity and the frequencies of HSPCs were due to variations in apoptosis and in proliferation, Annexin V and KI67 assays were carried out, respectively. For the apoptosis assay, it was observed that in early apoptosis, there was no significant impact of ApoE or HFD for most of HSPCs/progenitors, except MEP, which demonstrated a decreased frequency of early apoptosis in the ApoE-/- HFD group, significantly against the control HFD group. Similarly, it was observed that in late apoptosis there was no significant impact of ApoE and diet for most of HSPCs/progenitors except for LSKs where late apoptosis was reduced from ApoE-/- mice fed an HFD, which mapped to specific, statistically insignificant decreases in HPC2 and MPP populations, suggesting a trend

toward late-stage survival advantage for these HSPC populations for ApoE-/- mice fed an HFD (Figure 4.14).

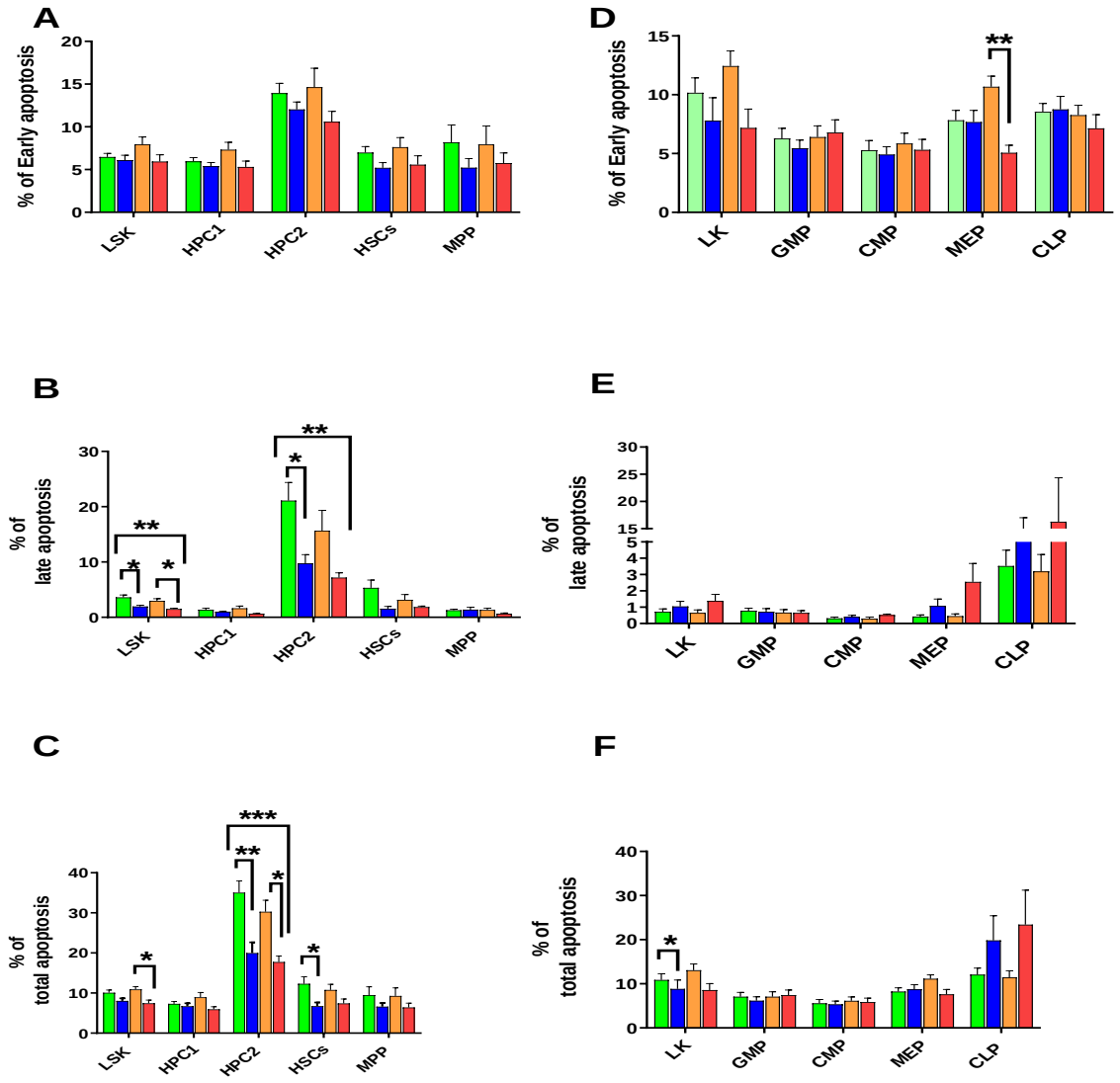


Figure 4.14 High fat diet affected early and late apoptotic stages in select HSPCs from ApoE-/- mice**.** HFD and ND induced changes in apoptosis. Bar graphs show the frequency of early, late, and total apoptotic stages in HSCs containing LSK population, LK population, and LK subpopulation. Data shown here represent the frequency and total cell count of each population in BM. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:8, KO NCD: 6, Control HFD:6, KO HFD: 6 mice. *P < 0.05, ** P<0.01. Control: wild-type mice: ApoE KO: ApoE knockout mice, NCD: normal chow diet, HFD: high fat diet. LSK: lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$, LK: lin^- $C\text{-}kit^+$, HPC1: haematopoietic progenitor 1, HPC2: haematopoietic progenitor 2, HSCs:

HSCs, MPP: multiple progenitor, GMP: granulocyte monocyte progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor.

4.1.2.11 High fat diet has no impact on cell cycling of highly enriched haematopoietic stem cells in ApoE-/- mice

In assessing whether cell cycle status was affected in HSPCs from ApoE-/- mice fed an HFD, the results demonstrated equivalent fractions of HPSCs residing in G0, G1 and S-G2-M phases across all experimental conditions. These data indicate no significant effect of diet and ApoE on the cell cycle status of HSPCs (Figure 4.15).

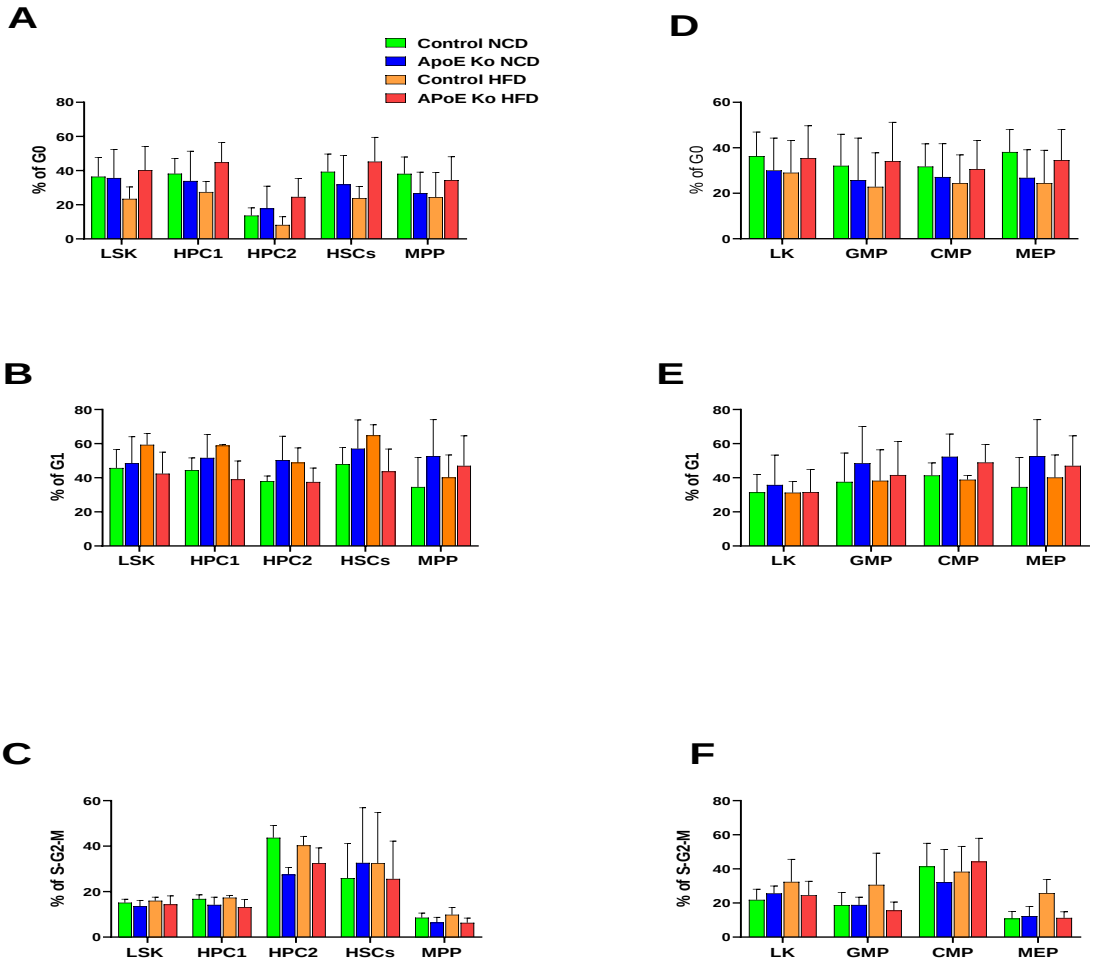


Figure 4.15 High fat diet has no effect on cell cycle status of HSPCs from ApoE-/- mice. Bar graphs show the frequency of G0, G1, and S-G2-M phases of the cell cycle in HSCs containing LSK population and LK and its subpopulation. Data shown here represent the frequency and total cell count of each population in BM. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO NCD: 3, Control HFD:3, KO HFD: 4 mice. P<0.01. Control: wild-type mice:

ApoE KO: ApoE knockout mice, NCD: normal chow diet, HFD: high fat diet. LSK: lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$, LK: lin^- $c\text{-}kit^+$, HPC1: haematopoietic progenitor 1, HPC2: haematopoietic progenitor 2, HSCs: haematopoietic stem cells, MPP: multiple progenitor, GMP: granulocyte monocyte progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor.

Table 4.3 A summary of the apoptosis and cell cycle analysis

Immunophenotype analysis	**ApoE-/- on NCD phenotype**	**ApoE-/- on HFD phenotype**
% of early apoptosis (Annexin v)	No change	Decreased MEP compared to C: HFD
% of late apoptosis (Annexin v)	Decreased LSK compared to NCD Decreased HPC2 compared to NCD	Decreased LSK compared to C: HFD
% of total apoptosis (Annexin v)	Decreased HPC2 compared to C: NCD Decreased HSCs compared to C: NCD Decreased LK compared to C: NCD	Decreased LSK compared to C: HFD Decreased HPC2 compared to C: HFD and C: NCD
Cell cycle analysis (Ki67 assay)	No change	No change

4.1.2 Assessing HSC functionality in the context of a high fat diet using ApoE murine model

4.1.2.1 Impact of HFD and loss of ApoE on HSC engraftment to the peripheral blood

To functionally test the impact of an HFD on ApoE-/- HSPC cell functionality, I performed competitive transplantation experiments where long-term multilineage reconstitution is considered to be the gold standard to detect the function of HSCs (Harrison et al. 1997; Micklem et al. 1972). 150 Lin- c-kit+ Sca-1+ (LSK) CD48-CD150+ HSCs were sorted from donor mice BM cells from each genotype (CD45.2), which were fed either an HFD or an NCD, and transplanted together with 200,000 WT CD45.1 BM cells, into lethally irradiated CD45.1 recipient mice. PB was collected 4 weeks post transplantation and every 4 weeks thereafter to assess the engraftment capacity of ApoE under the effect of an HFD.

The results showed significant differences in overall chimerism 4 weeks post transplantation in ApoE-deficient cells under the effect of both an HFD and an ND when compared to their control counterparts (Figure 4.16). After this time, donor-derived cell contributions to individual myeloid and lymphoid lineages were found similar in almost all cases, with the exception of B-cells, which were decreased in ApoE-/- regardless of dietary intake (Figure 4.16 C). Similar myeloid and lymphoid engraftment patterns were noted up to 16 weeks (Figure 4.17 - 4.19), with a significant decrease in B cells from transplant recipients of ApoE-/- HSPCs fed an HFD compared to their control HFD counterparts noted at 16 weeks.

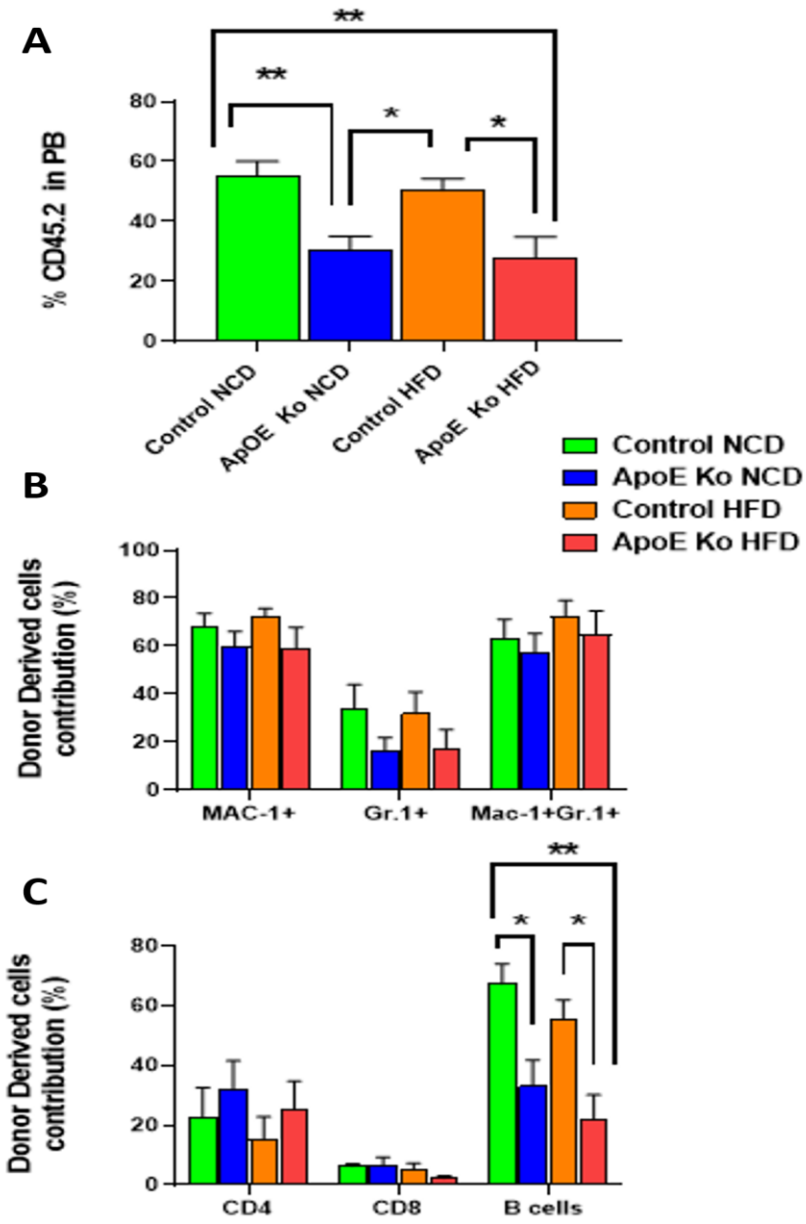


Figure 4.16 The deficiency of ApoE resulted in multilineage haematopoietic defect 4 weeks post HSC transplantation in the context of an HFD. ApoE-deficient cells under the dietary effect contributed more significantly to myeloid lineages. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid lineages. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:9, KO NCD: 7, Control HFD:9, KO HFD: 7 mice. *P <0.05 ** P<0.01 and *** P < 0.001. Control: wild-type mice: ApoE KO: ApoE knockout mice.

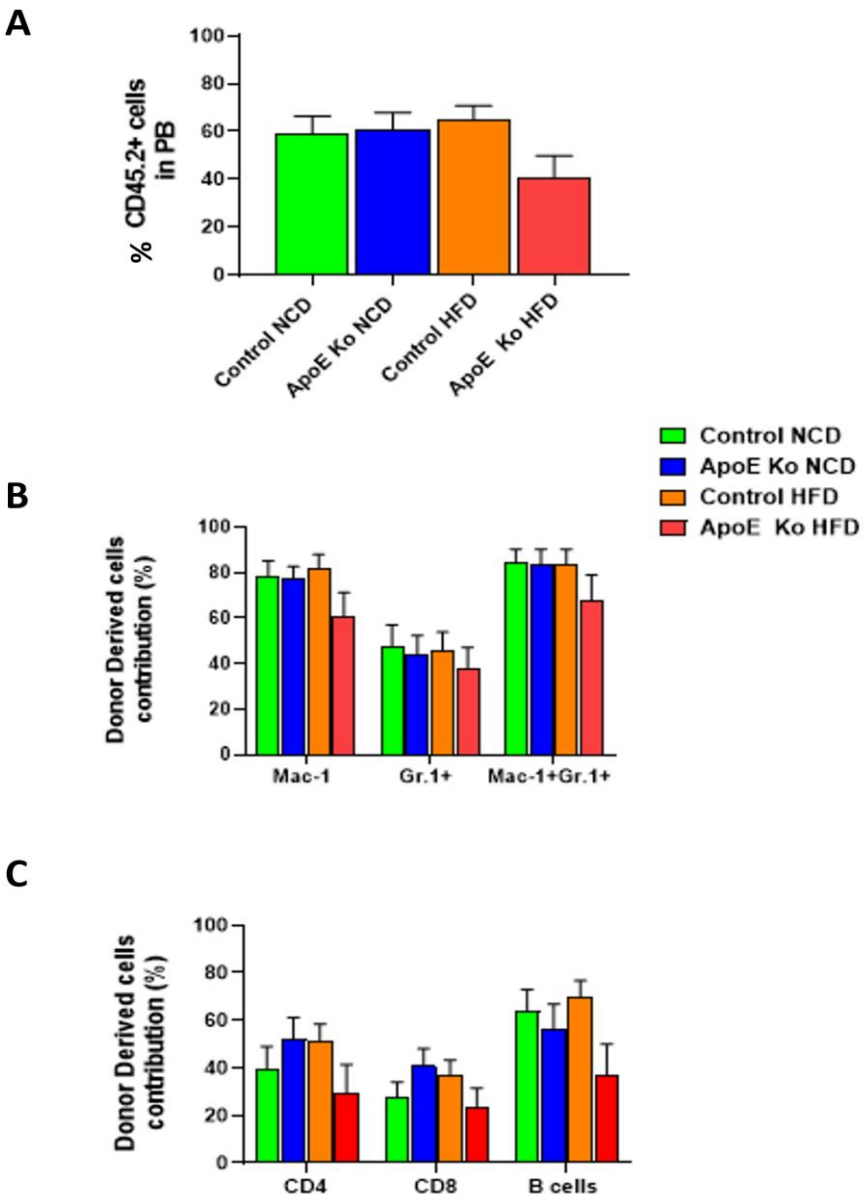


Figure 4.17 The deficiency of ApoE did not impact multilineage haematopoietic reconstitution at 8 weeks post HSCs transplantation in the context of an HFD. ApoE-deficient cells under the dietary effect has no effect on myeloid and lymphoid lineages. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid lineages. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:8, KO NCD: 7, Control HFD:9, KO HFD: 7 mice. Control: wild-type mice: ApoE KO: ApoE knockout mice.

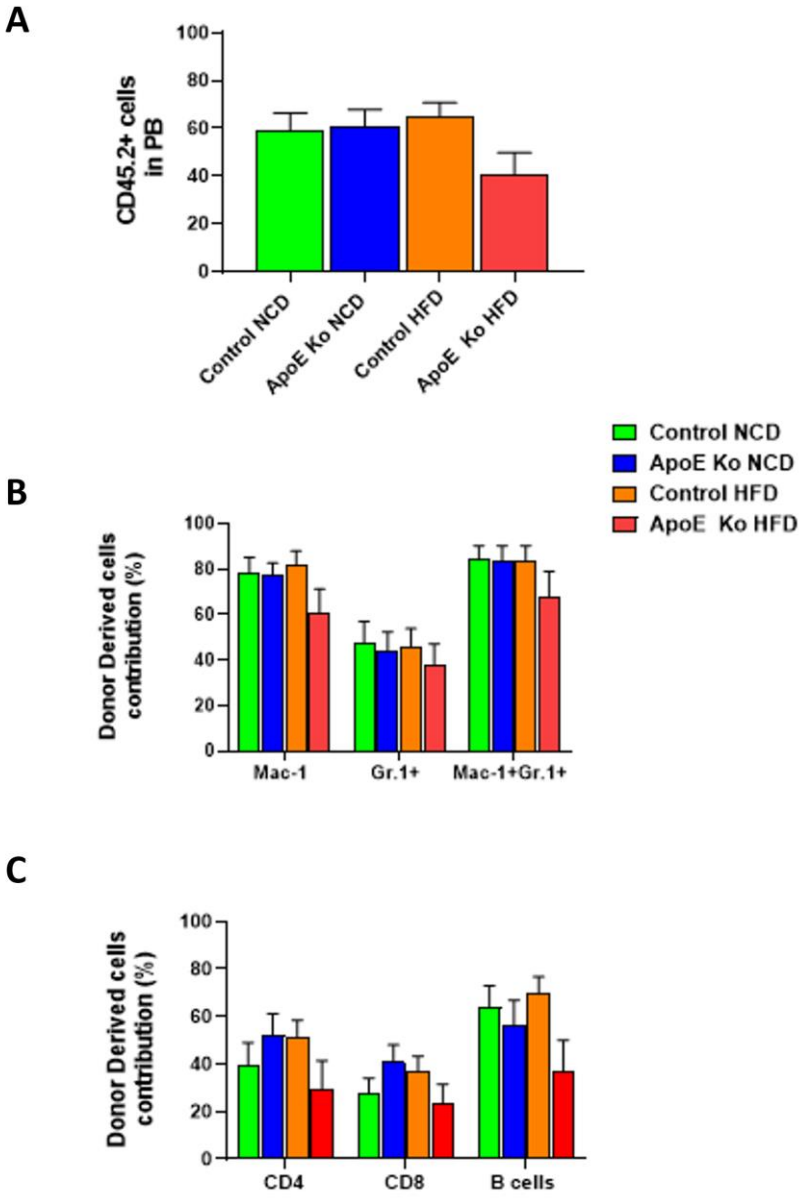


Figure 4.18 The deficiency of ApoE did not impact the multilineage haematopoietic reconstitution at 12 weeks post-HSC transplantation in the context of an HFD. ApoE-deficient cells under the dietary effect h*ave* no effect on myeloid and lymphoid lineages. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid lineages. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD :8, KO NCD: 7, Control HFD:9, Ko HFD: 6 mice. *P <0.05 ** P<0.01 and *** P < 0.001. Control: wild-type mice: ApoE KO: ApoE knockout mice.

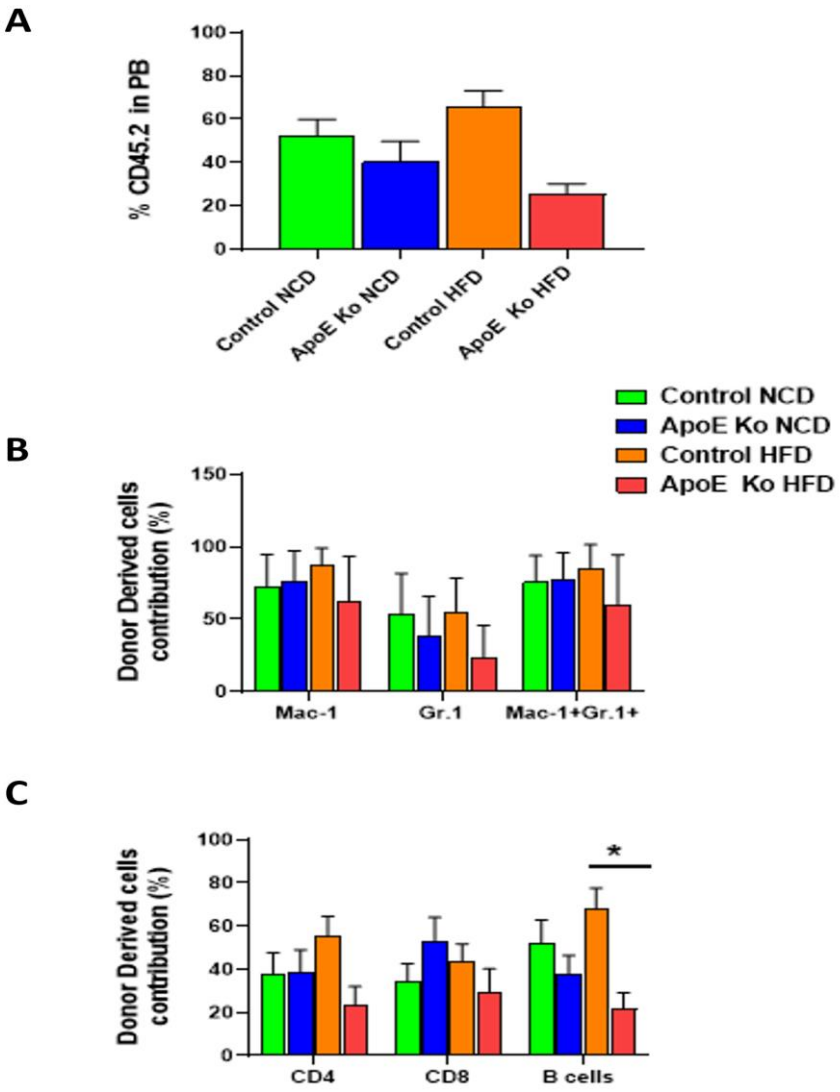


Figure 4.19 The deficiency of ApoE impacted the B lymphopoiesis at 16 weeks post HSC transplantation in the context of an HFD. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid lineage. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:8, Ko NCD: 7, Control HFD:8, KO HFD: 6 mice. *P <0.05 ** P<0.01. Control: wild-type mice: ApoE KO: ApoE knockout mice.

4.1.2.2 High fat diet and chronic loss of ApoE has no impact on the highly enriched hematopoietic stem and progenitor cells and lineage positive cells in BM, Sp, and Thy after transplantation

Next, I analysed the BM of recipients of ApoE-/- HSPCs exposed to an HFD. No significant changes in HSPC engraftment were observed in any condition after primary transplantation, indicating that an HFD has no impact on the engraftment of ApoE-/- HSPCs (Figure 4.20). Similarly, lineage positive cell engraftment was not affected by diet or ApoE (Figure 4.21). In the case of CD3, CD4, CD8 and B-cells, the ApoE Ko group contributed less to donor cell. Engraftment of lineage specific progenitors was highly variable, but overall, a trend of decreased engraftment was observed across all myeloid and lymphoid lineages was observed in the ApoE-/- HFD group. (Figure 4.22). Similarly, CD3, CD4, CD8 and DP engraftment was markedly reduced in the Thy of recipients of ApoE-/- cells fed an HFD (Figure 4.23).

Overall, these data show that an HFD fed to ApoE-/- animals has minimal impact on the function of their HSPCs and PB engraftment of lineage-specific cells in transplantation (Table 4.3).

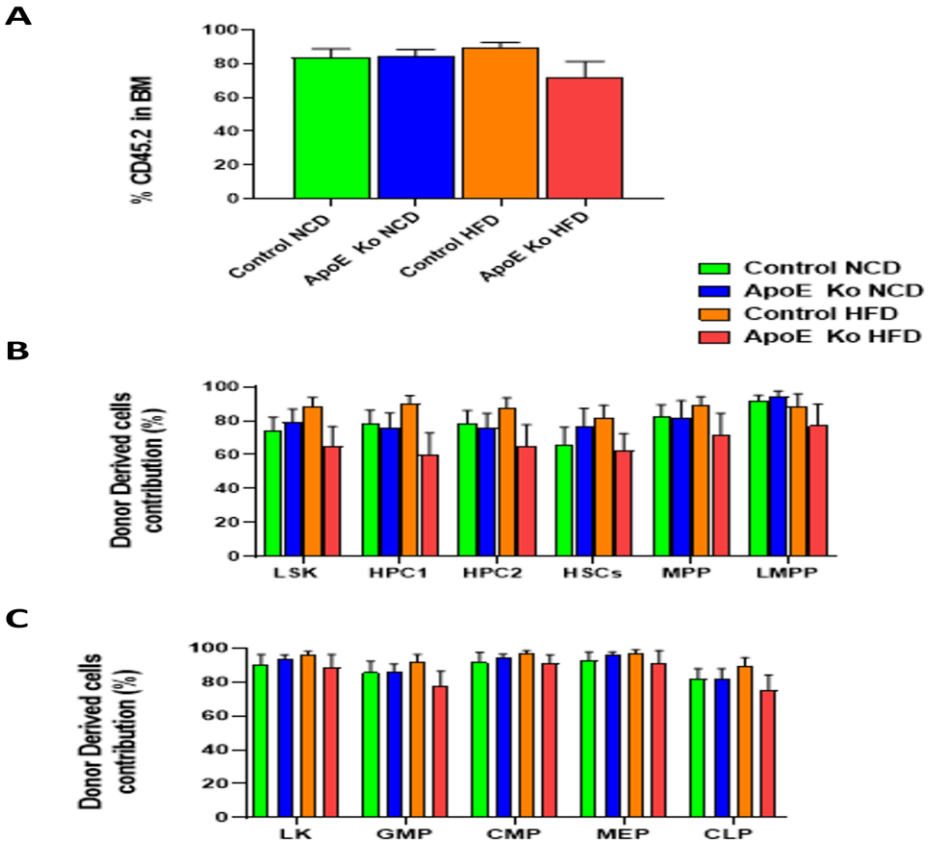


Figure 4.20 The deficiency of ApoE did not impact HSPCs in Bone Marrow after primary HSCs transplantation in the context of an HFD. HFD, NCD, and chronic loss of ApoE have no impact on the total contribution of HSPCs (A), LSK and its subpopulation (B), and LK and its subpopulation (C). Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:7, KO NCD: 8, Control HFD:9, KO HFD: 6 mice. NCD: normal chow diet, HFD: high fat diet. LSK: lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$, LK: lin^- $c\text{-}kit^+$, HPC1: haematopoietic progenitor 1, HPC2: haematopoietic progenitor 2, HSCs: haematopoietic stem cells, MPP: multiple progenitors, GMP: granulocyte monocyte progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor.

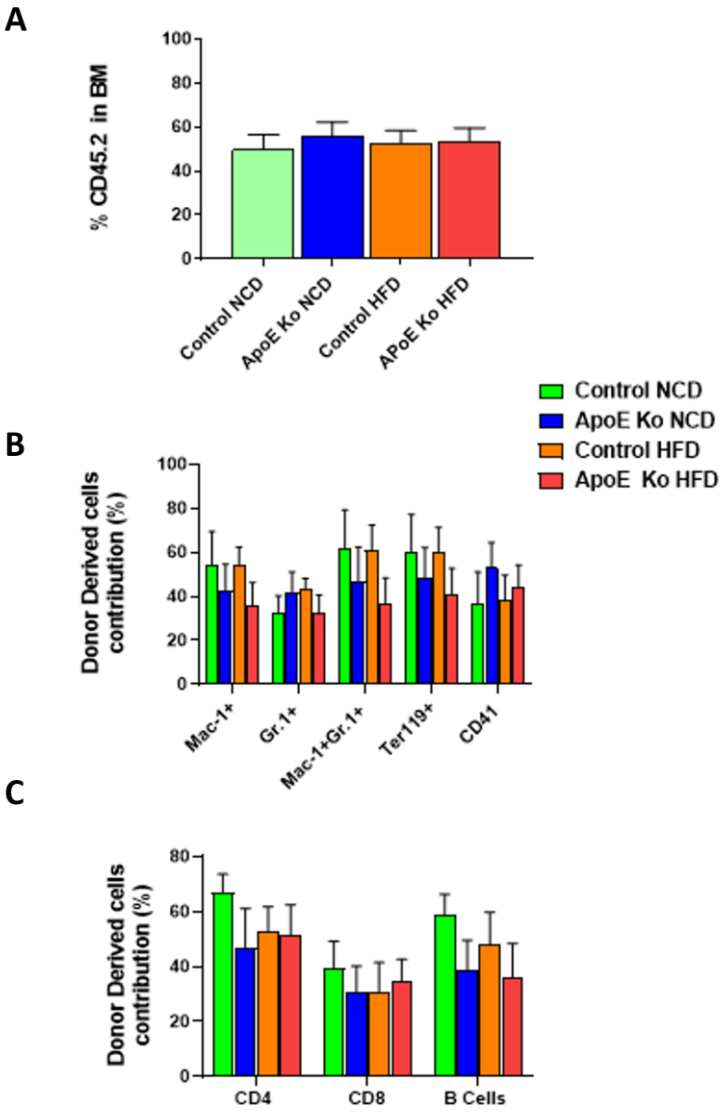


Figure 4.21 The deficiency of ApoE did not affect lineage positive cells in BM after primary HSCs transplantation in the context of an HFD. HFD, NCD, and chronic loss of ApoE have no impact on the total contribution to myeloid and lymphoid lineage (A), myeloid (B) and lymphoid. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:8, KO NCD: 7, Control HFD:7, KO HFD: 6 mice. NCD: normal chow diet, HFD: high fat diet.

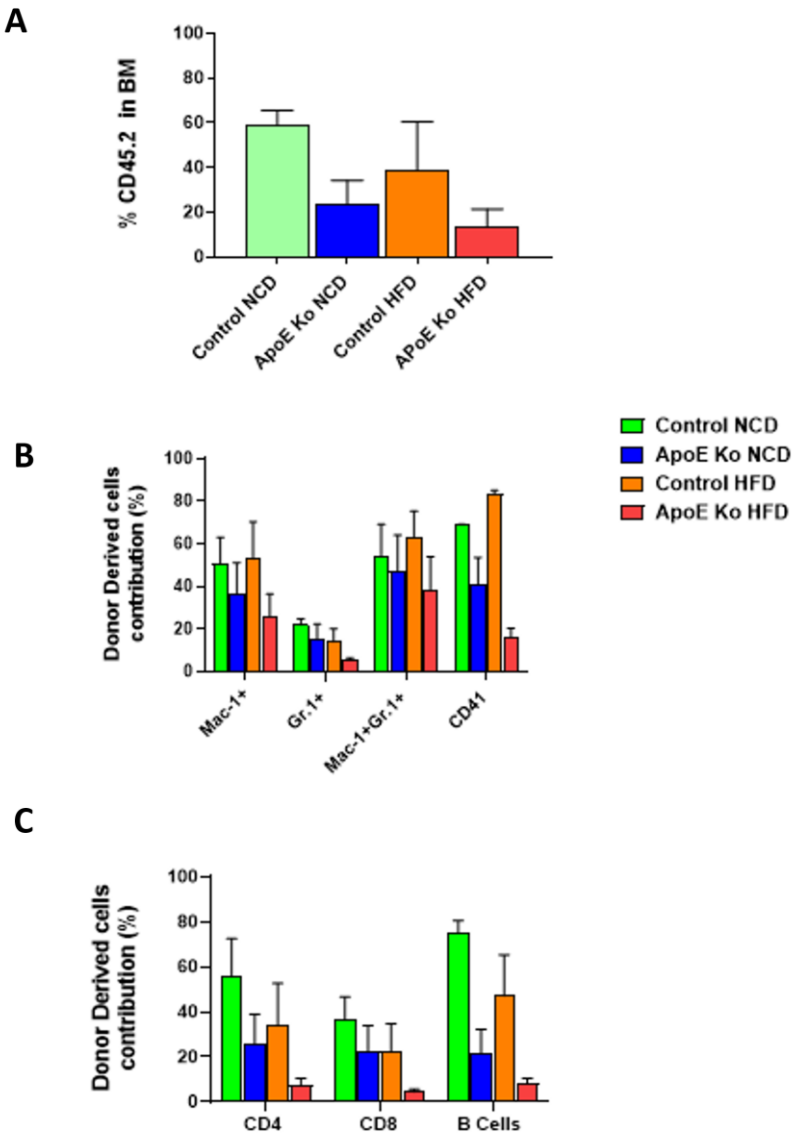


Figure 4.22 The deficiency of ApoE did not affect lineage positive cells in spleen after primary HSC*s* transplantation in the context of an HFD. HFD, NCD, and chronic loss of ApoE has no impact on the total contribution to myeloid and lymphoid lineage (A), myeloid (B) and lymphoid. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:2, KO NCD: 5, Control HFD:4, KO HFD: 5 mice. Control: wild-type mice: ApoE KO: ApoE knockout mice.

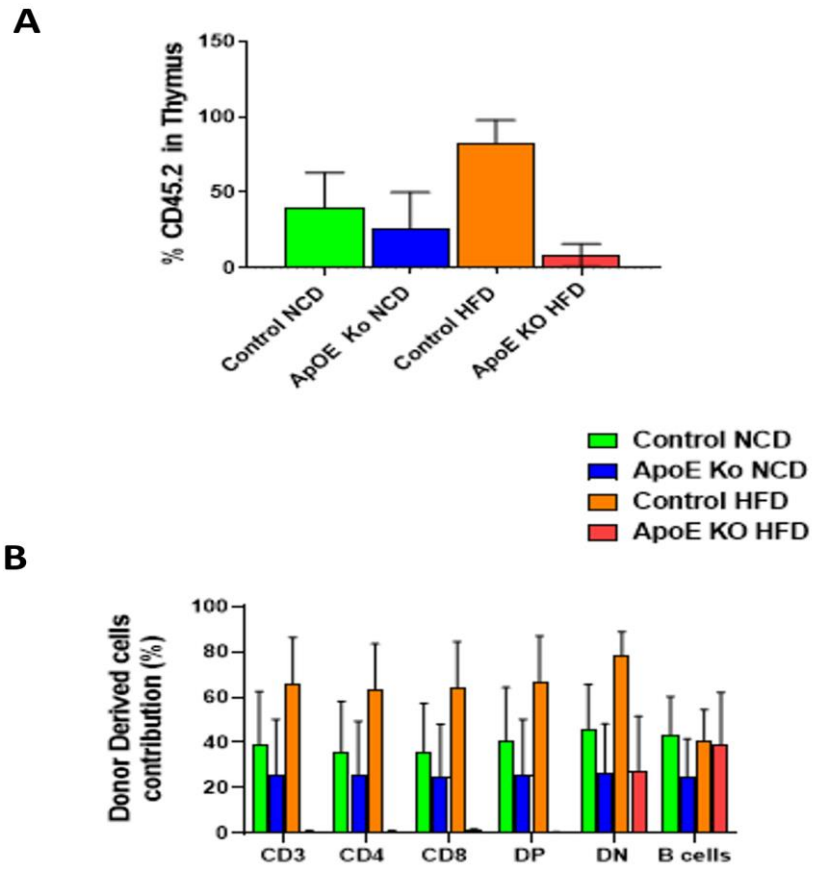


Figure 4.23 The deficiency of ApoE did not affect lineage positive cells in thymus after primary HSC transplantation in the context of an HFD. HFD, NCD, and chronic loss of ApoE has no impact on the total contribution to T cells development (A), and T cells (B). Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD :6, KO NCD: 4, Control HFD:4, KO HFD: 4 mice. Control: wild-type mice: ApoE KO: ApoE knockout mice.

Table 4.4 Summary of the observed phenotype in primary HSCs transplantation in ApoE mice in the context of a high fat diet and normal chow diet.

Immunophenotype analysis	**ApoE-/- on NCD phenotype**	**ApoE-/- on HFD phenotype**
4-week post transplantation	Decreased CD45.2 compared to C: NCD and C: HFD Decreased B cells compare to NCD	Decreased CD45.2 compared to all conditions Decreased B cells compare to C: NCD and C: HFD
8-week post transplantation	No change	No change
12 -week post transplantation	No change	No change
16 -week post transplantation	No change	Decreased B cells compare to C: HFD
HSPCs in BM	No change	No change
Lin+ in BM	No change	No change
Lin+ in spleen	No change	No change
T cells in Thy	No change	No change

4.1.3 Assessing the HSCs self-renewal function in the context of a high fat diet and normal chow diet using the ApoE murine model

To test the self-renewal capacity of HSCs from ApoE-/- mice that had been fed an HFD, donor (CD45.2+) cells from primary transplanted recipients were isolated and transplanted into secondary recipients; 300 lin- c-kit+ SCa-1+ (LSK) CD48-CD150+ HSCs were sorted from donor mice BM cells (CD45.2) and transplanted together with 200,000 WT CD45.1 BM cells, into lethally irradiated CD45.1 recipient mice. There was no significant impact of HFD on ApoE mediated on HSC self-renewal as evidenced by the lack of impact of PB engraftment from 4-16 weeks, with the exception of gradual B cell reduction (Figure. 4.24 - 4.27)

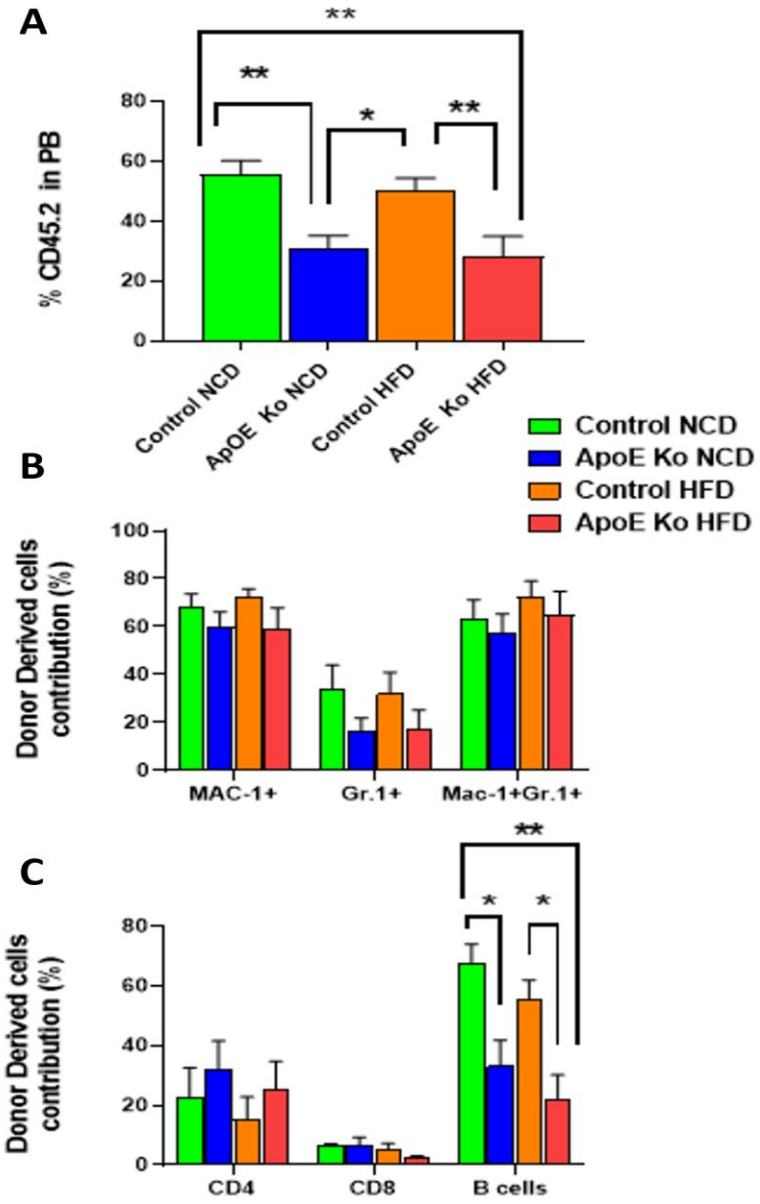


Figure 4.24 The deficiency of ApoE affected B lymphopoiesis 4 weeks post-secondary HSCs transplantation in the context of an HFD. ApoE-deficient cells under the dietary effect contributed more significantly to myeloid lineages. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid lineages. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO NCD: 6, Control HFD:5, KO HFD: 6 mice. *P <0.05 ** P<0.01 and *** P < 0. 001. Control: wild-type mice: ApoE KO: ApoE knockout mice.

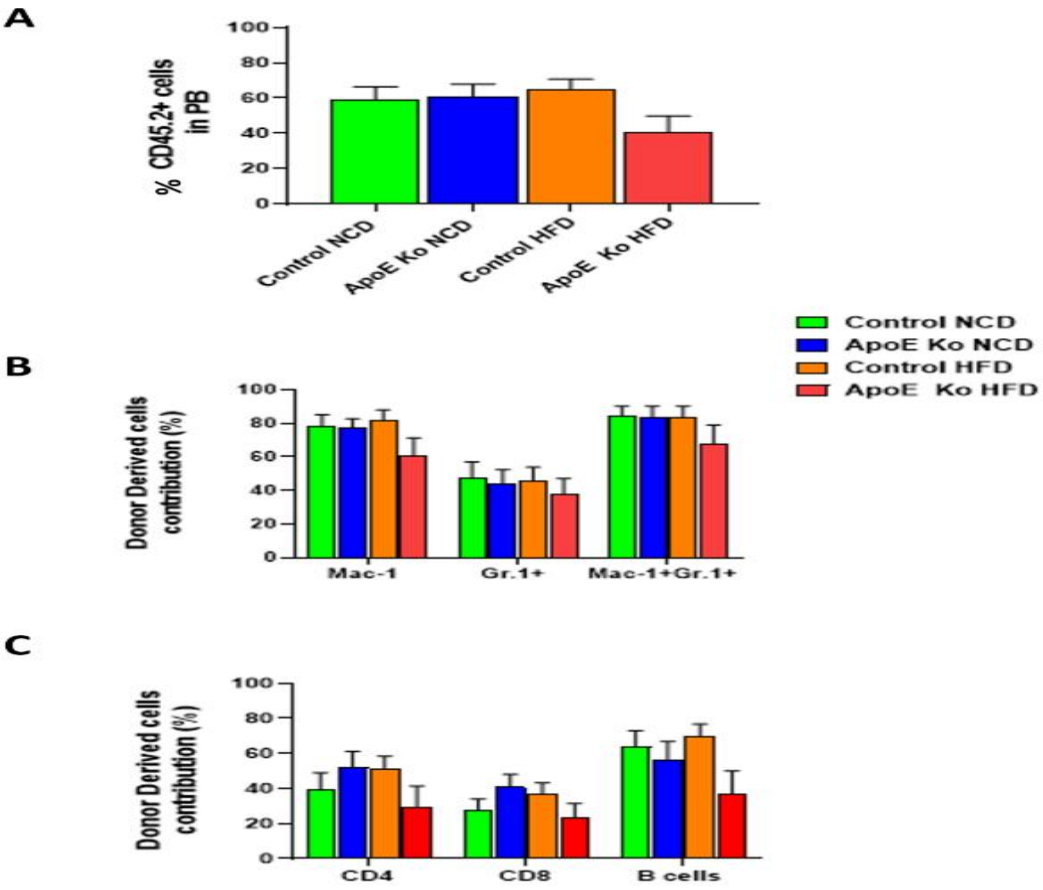


Figure 4.25 The deficiency of ApoE did not affect multilineage haematopoiesis 8 weeks post-secondary HSCs transplantation in the context of an HFD. ApoE-deficient cells under the dietary effect ha*ve* no effect on myeloid and lymphoid lineages. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid lineages. Error bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO NCD: 6, Control HFD:5, KO HFD: 6 mice. *P <0.05 ** P<0.01 and *** P < 0. 001. Control: wild-type mice: ApoE KO: ApoE knockout mice.

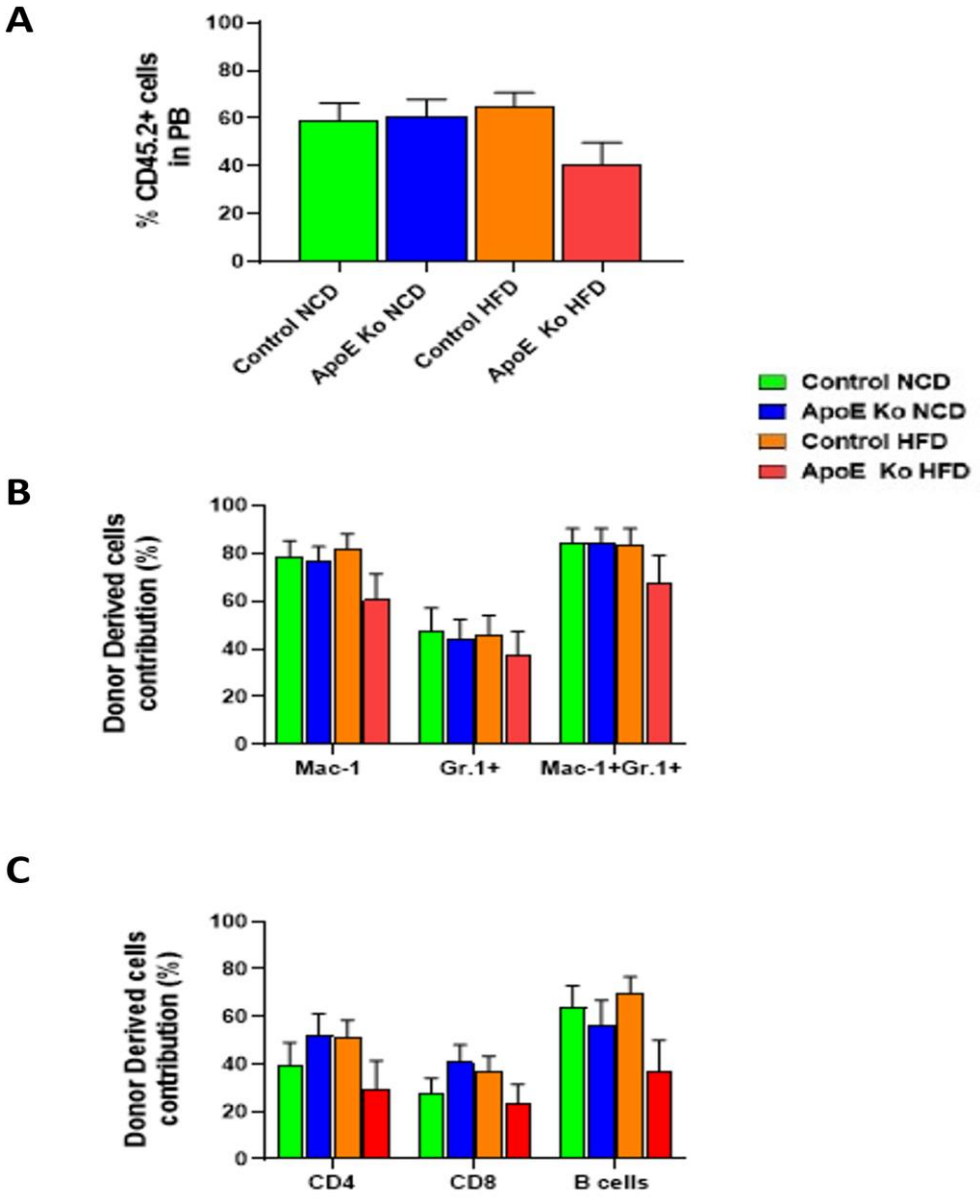


Figure 4.26 The deficiency of ApoE did not affect multilineage haematopoiesis 12 weeks post-secondary HSCs transplantation in the context of an HFD. ApoE-deficient cells under the dietary effect have no effect on myeloid and lymphoid lineages. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO NCD: 6, Control HFD:5, KO HFD: 6 mice. *P <0.05 ** P<0.01 and *** P < 0. 001. Control: wild-type mice: ApoE KO: ApoE knockout mice.

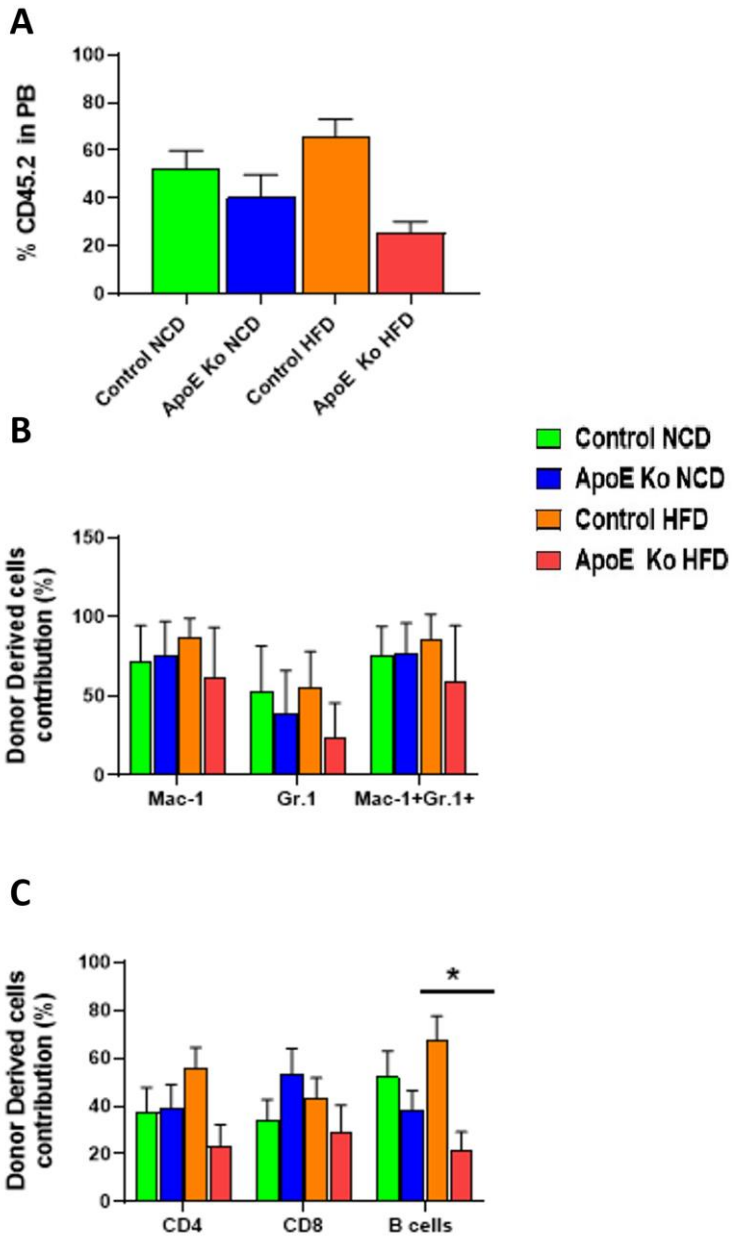


Figure 4.27 The deficiency of ApoE did not affect multilineage haematopoiesis at 16 weeks post-secondary HSCs transplantation in the context of an HFD. ApoE-deficient cells under the dietary effect have no effect on myeloid and lymphoid lineages. A. Percentage of CD45.2 donor-derived cells in PB. B. Contribution of donor-derived cells to (B) myeloid and (C) lymphoid lineages. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error

bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO NCD: 5, Control HFD:5, KO HFD: 6 mice. *P <0.05 ** P<0.01 and *** P < 0. 001. Control: wild-type mice: ApoE KO: ApoE knockout mice.

4.1.3.1 An HFD and chronic loss of ApoE has no impact on the highly enriched hematopoietic stem and progenitor cell engraftment in bone marrow after secondary transplantation

Assessing engraftment in HSPCs in secondary recipients showed no significant effects of an HFD or of ApoE deficiency, further indicating that an HFD has no impact on ApoE-mediated HSC self-renewal (Figure 4.28). There are some minor impacts on myeloid and/or lymphoid lineages in BM and Thys but not Sp (Figure 4.29 – 4.31). However, overall, an HFD has a minimal impact on ApoE-mediated self-renewal in terms of HSPC and lineage engraftment in the BM following secondary transplantation.

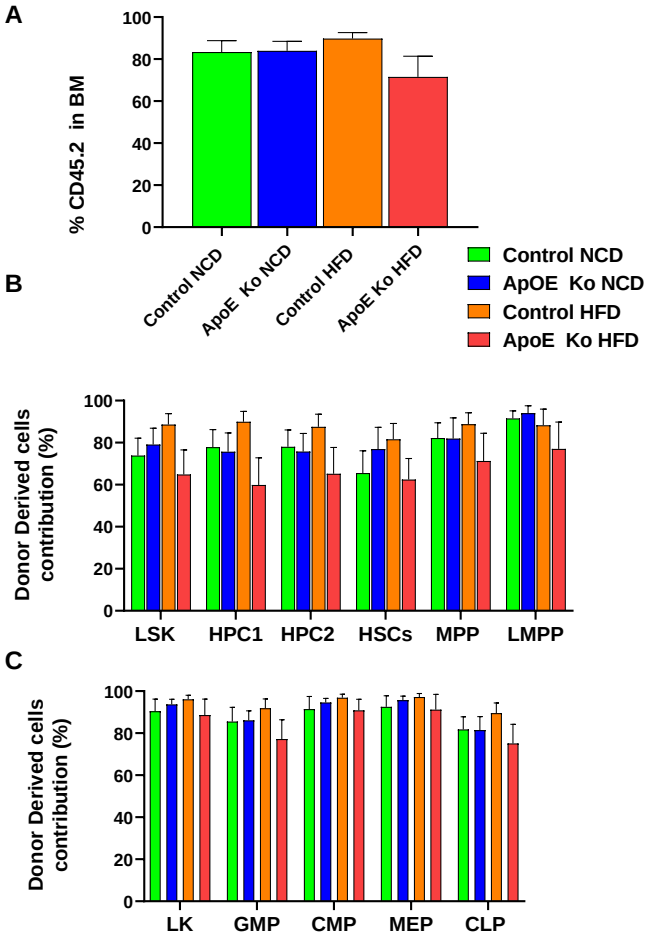


Figure 4.28 The deficiency of ApoE did not affect HSPC engraftment in BM after secondary HSC transplantation in the context of an HFD**.** An HFD, NCD, and chronic loss of ApoE have no impact on the total contribution of HSPCs (A), LSK and it subpopulation (B), and LK and its subpopulation (C). Statistical analysis was performed using one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD :4, KO NCD: 4, Control HFD:5, KO HFD: 6 mice. *P <0.05 ** P<0.01 and *** P < 0. 001. Control: wild-type mice: ApoE KO: ApoE knockout mice.

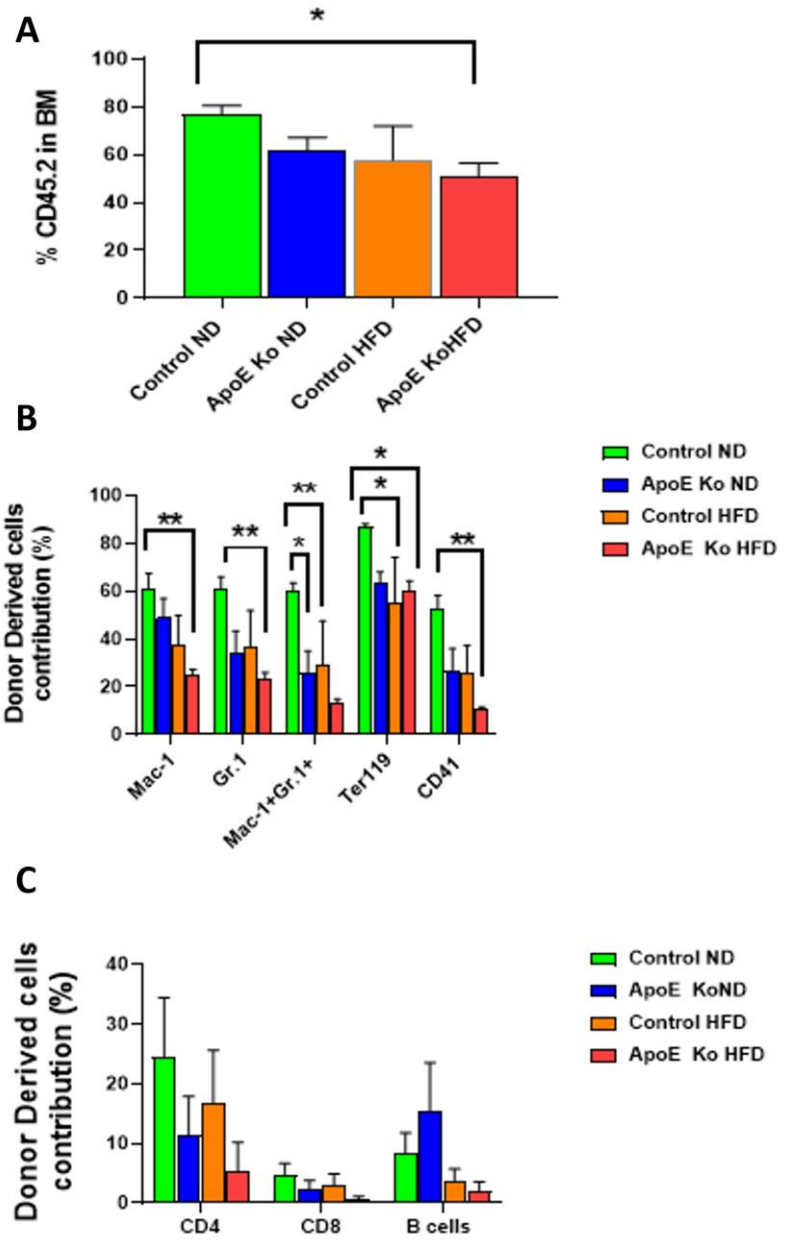


Figure 4.29 The deficiency of ApoE affects the contribution of myeloid and lymphoid cells in BM after secondary HSC transplantation in the context of an HFD. An HFD, NCD, and chronic loss of ApoE have an effect on the total contribution to myeloid and lymphoid lineage (A), and impacted myeloid (B), and show no effect on lymphoid cells. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO: NCD: 4, Control HFD:3, KO HFD: 6 mice. *P <0.05 ** P<0.01. Control: wild-type mice: ApoE KO: ApoE knockout mice.

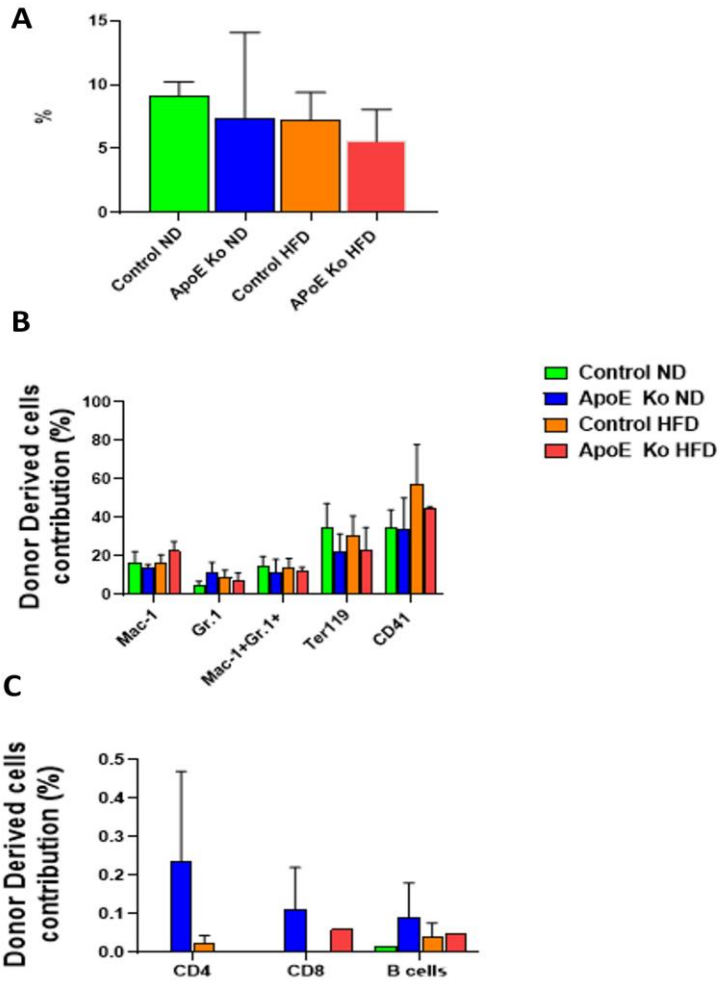


Figure 4.30 The deficiency of ApoE has no impact on lineage positive cells in the spleen after secondary HSC transplantation in the context of an HFD**.** An HFD, NCD, and chronic loss of ApoE has no impact on the total contribution to myeloid and lymphoid lineage (A), myeloid (B), and lymphoid. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO NCD: 4, Control HFD:3, KO HFD: 6 mice. Control: wild-type mice: ApoE KO: ApoE knockout mice.

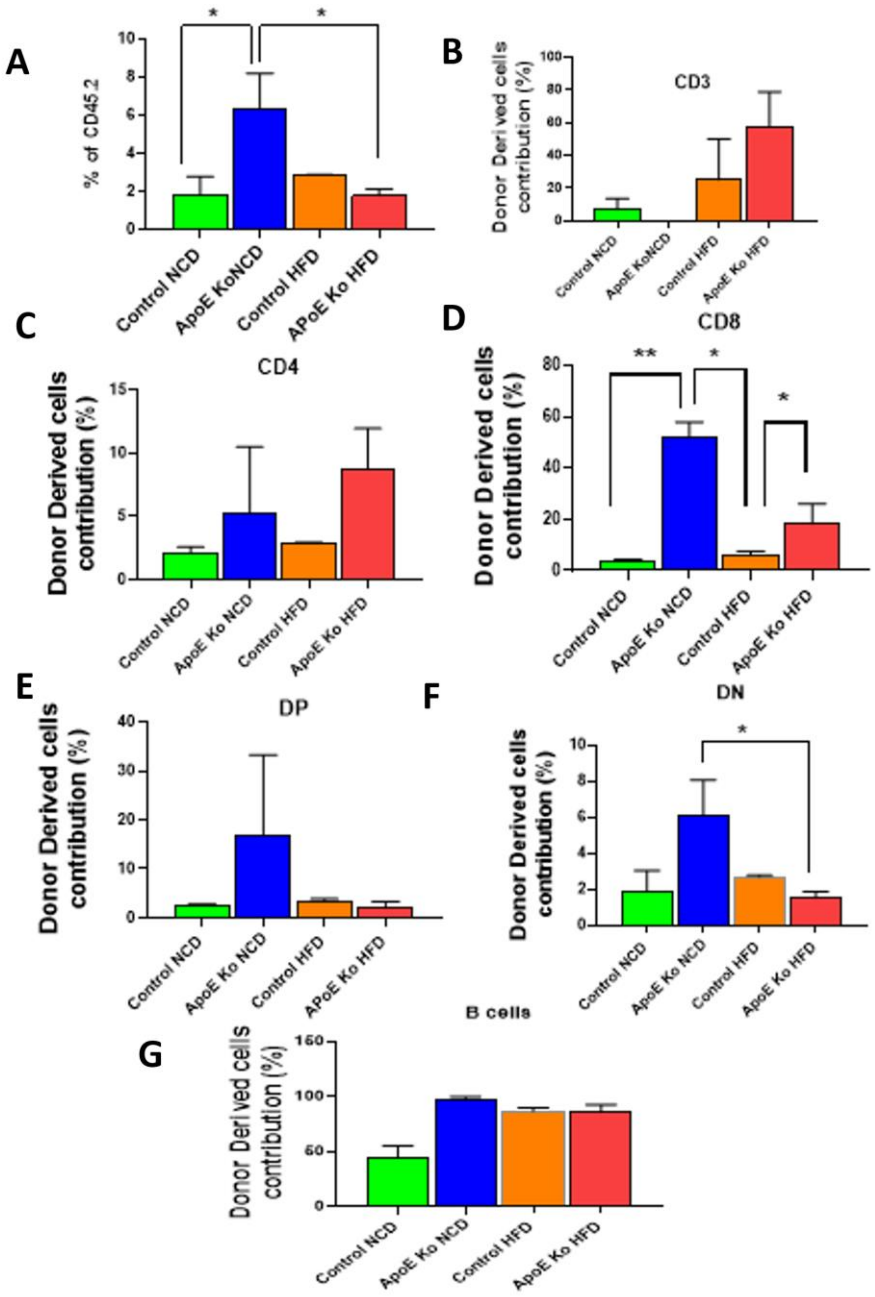


Figure 4.31 The deficiency of ApoE has an impact on T cells and B cells in Thy after secondary HSC transplantation in the context of an HFD. An HFD, NCD, and chronic loss of ApoE has no impact on the total contribution to lymphoid lineage (A), (B) CD3, and (C) CD4, (D) CD8, (E) DP, (F) and (G) B cells. Statistical analysis was performed using a one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD:4, KO NCD:4, Control HFD:2, KO HFD: 3 mice. *P <0.05 ** P<0.01. Control: wild-type mice: ApoE KO: ApoE knockout mice.

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Table 4.5 Summary of the observed phenotype of PB results and HSPCs in BM post-HSCs secondary transplantation in ApoE mice in the impact of an HFD and an NCD

Immunophenotype analysis	**ApoE-/- on NCD phenotype**	**ApoE-/- on HFD phenotype**
4-week post-secondary transplantation	Decreased CD45.2 compared to C: NCD and HFD Decreased B cells compared to C: NCD and C: HFD	Decreased CD45.2 compared to C: NCD and HFD Decreased B cells compared to C: NCD and C: HFD
8-week post-secondary transplantation	No change	No change
12 -week post-secondary transplantation	No change	No change
16 -week post -secondary transplantation	No change	Decreased B cells compared to C: HFD
HSPCs in BM	No change	No change

Table 4.6 Summary of the observed phenotype of lineage positive in BM, Sp and T cells development in Thy post-HSCs secondary transplantation in ApoE mice in the impact of an HFD and an NCD.

Immunophenotype analysis	ApoE-/- on NCD phenotype	ApoE-/- on HFD phenotype
Lin + in BM	Decreased Mac-1+Gr.1+ compared to C: NCD	Decreased CD45.2 compared to C: NCD Decreased Mac-1 compared to C: NCD Decreased Gr.1 compared to C: NCD Decreased Mac-1+Gr.1+ compared to C: NCD Decreased Ter119 compared to C: NCD Decreased CD41 compared to C: NCD
Lin + in spleen	No change	No change
Lin + in Thy	Increased CD45.2 compared to C: NCD and Ko: HFD Increased CD8 compared to C: NCD and C: HFD Increased DN compared to C: NCD Increased B cells compare to C: NCD	Decreased CD45.2 compared to Ko: NCD Increased CD8 compared to C: HFD Increased B cells compare to C: NCD

4.1.3.2 ApoE deficiency leads to atherosclerosis

Diet dependent changes in haematopoiesis were observed in the ApoE-/- mice, which are known to lead to heightened inflammation and atherosclerosis in an HFD regimen (Greenow et al. 2005). To confirm the role of ApoE in atherosclerosis, at week 8 of age, one group of ApoE-/- mice was fed an HFD, and the other was fed an NCD for 12 weeks after which, lipid content was assessed in atherosclerotic plaque from the arteries. ORO staining of frozen sections allows the easy assessment of the lipid content and distribution in tissues utilizing basic equipment in the laboratory (Mehlem et al. 2013). ORO is a lipophilic, fat-soluble dye that dissolves in lipids and stains them to a red colour (Bumbrah et al. 2019). Here, ORO staining was used to confirm the presence of atherosclerosis characterised by the accumulation of lipid droplet and increased atherosclerosis plaque. Unfortunately, ORO staining for the slides of ApoE mice on an NCD could not be performed due to technical issues. Additional, statistical analysis of the ORO experiment was not performed due to the small sample size. However, ORO staining confirmed an increase in atherosclerotic plaque as well as lipid droplet in atherosclerotic plaque in ApoE-/- mice on an HFD (Figure 4.32).

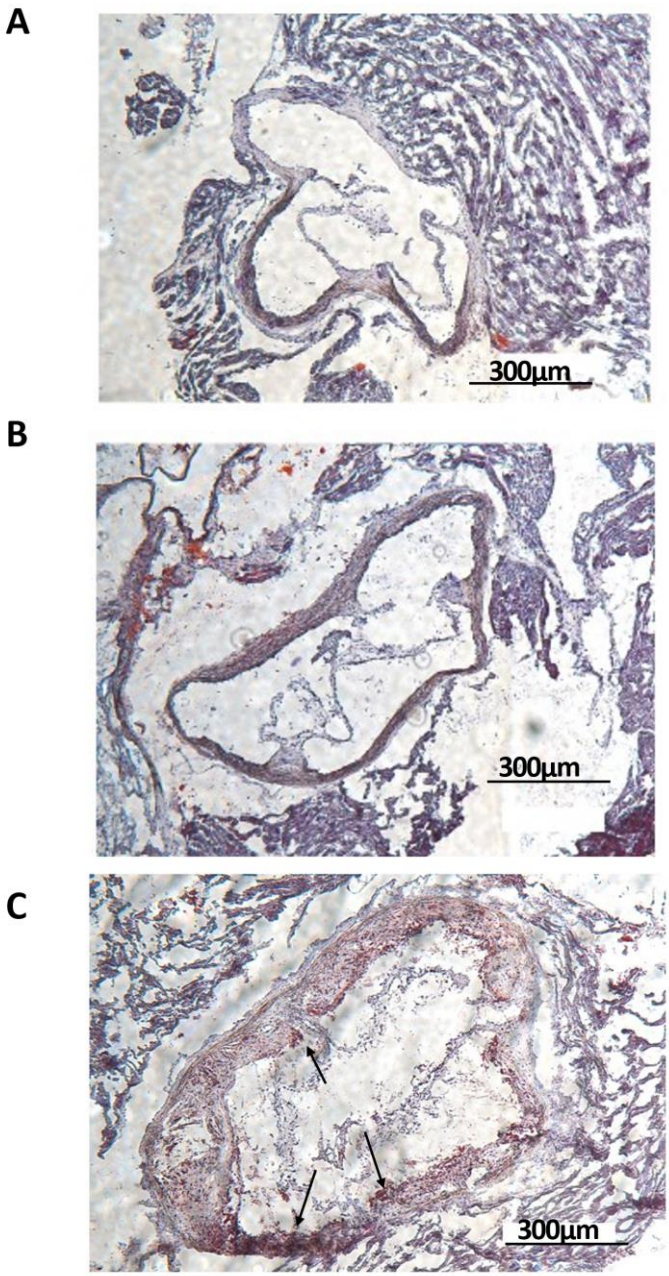

Figure 4.32 Quantification of atherosclerotic lesions in ApoE-/- mice fed an HFD. Aortic root cross section at 300 µm from aortic sinus in mice fed NCD or HFD for 12 weeks. The arrows show lipids, which are stained with red colour by ORO staining (Maganto et al. 2012) A: Control mice on an NCD. B: Control mice on an HFD. C: ApoE KO mice on an HFD. Image's magnifications are taken at x40.

4.1.4 Discussion

Numerous studies have been conducted to study the association between leukocyte count in PB and CVD occurrence (Johnsen et al. 2005; Passlick et al. 1989). The mechanism by which leukocyte count is linked to atherosclerosis development is poorly understood (Arbel et al. 2012). Hypercholesterolaemia is regarded as a major risk for atherosclerosis and might affect HSPCs homeostasis to induce leucocytosis (Murphy et al. 2011; Tie et al. 2014; Yvan-Charvet et al. 2010). Previously, ApoE has been reported to be abundantly expressed in HSPCs, regulating the proliferation of HSPCs, monocytes, and monocyte accumulation in atherosclerotic lesions (Murphy et al. 2011) and accelerates the ageing of HSCs (Tie et al. 2014). This prompted me to examine the broad impact of an HFD on haematopoiesis in ApoE-/- mice. I used germline ApoE -/- (KO) or wild-type (Thy1.1) mice, which were fed an HFD or an NCD at 8 to 12 weeks old, on an NCD. PB, BM, Sp and Thy samples were harvested to assess the function and characteristics of HSPCs in a haematopoietic system.

HSCs exist in at the top of the haematopoietic hierarchy and are capable of self-renewing and differentiating into multilineage haematopoiesis, both of which characteristics are vital for the lifelong sustenance of the pool of stem cells (Hermetet et al. 2019). These features are regulated by cells' extrinsic and intrinsic mechanisms involving transcription factors, cytokines, and cell–cell interaction (Zon 2008; Hermetet et al. 2019). Moreover, many metabolic pathways have been documented as regulatory components for the self-renewal of HSCs and lineage commitment and specification (Ito and Suda 2014). However, lipid dependent regulation of HSCs remains undefined (Hermetet et al. 2019). Although haematopoietic tissues are known to respond to dietary signals, little is known about the influence of an HFD, known also as pro-obesity or Western diets, on the regulation of HSPCs and their functions (Hermetet et al. 2019).

Recent reports on wild type rodent models have revealed that HFD induced obesity induces significant alterations to HSCs and haematopoietic system homeostasis (Berg et al. 2016: Adler et al. 2014: (Singer et al. 2014; Hermetet et al. 2019). However, it is challenging to establish whether these changes are consequences of direct impact such as alterations in HSCs lipid metabolism or linked to obesity pathophysiology, diabetes or inflammation. Hermetet et al. (2019), reported that feeing a wild-type mice a HFD as little as 4 weeks exhibited a half loss of the primitive HSCs in BM, lower reconstitution potential of haematopoiesis post transplantation. BM shows induced monocytes and neutrophils expansion in the context of obesity. This quantitative expansion includes more primitive hematopoietic cells (Nagareddy et al. 2014; Singer et al. 2014; Berg et al. 2016).

Several studies showed obesity-induced diet results in an increase in multiple early myeloid progenitor populations, pre-granulocyte and macrophage progenitors (Pre-GMs), common

myeloid progenitors (CMPs) and multipotent progenitors (MPPs) (Nagareddy et al. 2014: Singer et al. 2014: Berg et al. 2016). Besides, in obese patients, an elevation in the circulating $CD34^{+}$ hematopoietic progenitors have been documented (Bellows et al. 2011). However, by looking at these reports, variability in experimental designs and methodology attributes to these differences. These variabilities comprise the use of genetic modified models of obesity, versus diet-induced obesity, which may vary in calories percent from fat, diet exposure length, and the experimental designs of BM transplantation (Bowers and Singer 2021).

In these experiments, I found select expansion of HSPCs, including expansion of the LSK compartment reflecting ApoE-mediated increases in HSCs, MPP (HPC 1, HPC2), and committed progenitors (CMP) in ApoE-/- mice fed an HFD. HPC1 populations are heterogenous cells of restricted progenitors that contain early lymphoid progenitors and some cells that produce a few level of transient myelo-erythroid reconstitution whereas HPC2 seems to involve a heterogeneous population of restricted progenitors with restricted potential of reconstitution in vivo (Oguro, Ding and Morrison 2013). This was mirrored by decreases in late apoptosis in select HSPC populations but no alteration in cell cycle status, demonstrating that ApoE-/- mice fed an HFD have a survival advantage. Surprisingly, ApoE signalling in the context of an HFD appeared, with exceptions, to be largely expendable for HSC function as assessed by transplantation and HSC self-renewal judged by secondary transplantation. The functional assay report after the primary transplantation demonstrates some interesting results. The impact of ApoE KO was observed in the % CD45.2 in PB, and in both the diet cases, the ApoE KO group demonstrated a lower percentage. Even after the primary transplantation donor-derived B cell contribution was found to be lower in the ApoE KO gene. As it is evident that B cells are responsible for the immune system of the body, thus the ApoE KO group become more vulnerable after the primary transplantation, as the contribution of the donor-derived B cells decreased. The difference was more evident in the 4^{th} week and 16^{th} week. However, in BM, Thy, and Sp lineage positive cells, no difference was observed. From the result of the functional assay after the second transplantation, it has been observed that % CD45.2+ cells in PB is lower in the ApoE KO group. The donor-derived cell contribution was lower in the case of CD4, CD8, and B cells in the ApoE KO group. The impact of ApoE KO in B cells was more evident in 4 weeks and 16 weeks and in 8 and 12 weeks, and the difference was more evident in the case of live cells, mac-1. In the KO group, the frequency was significantly less. In lineage positive cells, the difference was observed after post-secondary transplantation. In each case, the ApoE KO group demonstrated less cellular frequency.

In addition, ApoE appears to be expendable for extramedullary haematopoiesis in the context of an HFD, which was also unexpected given the findings of Tie et al. (2014), who reported that an HFD induces systemic inflammation that results in enhanced extramedullary haematopoiesis. Mechanistically, sympathetic nervous system signalling has been reported to liberate HSPCs from BM niches where they seed in the spleen leading to increased monocyte production (Dutta et al. 2012). Multiple mechanisms have been recognized with regards to inducing mobilization of HSPCs and extramedullary haematopoiesis in a hypercholesterolaemia environment (Soehnlein and Smirks 2013), but my data supports the idea that ApoE signalling is not required in this setting. Besides, by looking at the HSPCs compartment in the SP, a reduction of LMPP populations was observed in ApoE mice in the context of an HFD. However, conclusions cannot be drawn due to the low sample size, and thus, more samples are needed to confirm the impact of an HFD on the SP in the ApoE murine model. This is regarded as a pilot study, where the experiment could be conducted on a smaller scale rather than a full-scale study, which is essential for the improvement in the study's quality and main efficacy compared to 2017. Therefore, it could be concluded that an HFD, an NCD, and ageing have an extramural impact on haematopoiesis, but larger sample sizes are needed to confirm this hypothesis.

The data in this chapter suggest that ApoE-/- mice fed an HFD cause BM HSPCs to expand, proliferate, and enhance the production of myeloid cells, as noted by the increase in Gr1+Mac-1+ cells. Leucocytosis induced by obesity is driven by the upregulation of S1008A8/A9 along with other TLR3 ligands in the visceral adipose tissue; macrophage signals induced by MyD88 pathways cause IL-1ß proceeded by the NLRP3 inflammasome to form mature IL-1ß. Subsequently, IL-1ß migrates to the BM to cause proliferation of HSPCs through IL-1R, eventually culminating in monocytosis and neutrophilia (Nagareddy et al. 2014; Nagareddy et al. 2013; Tie et al. 2014).

My findings show that the ApoE gene plays a regulatory role in the biology of HSPCs in normal haematopoiesis, which might be attributed to its interactions with ABCA1 and ABCG1 and the resulting downstream effect on signalling pathways (Murphy et al. 2011). The ApoE gene interacts with ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1), both of which are expressed in HSPCs to promote the efflux of cholesterol and decrease the downstream signalling of IL3 receptors (Murphy et al. 2011). The expression of ABCA1 and ABCG1 points to the regulatory role of the ApoE gene in haematopoiesis. In addition, heparinise sulphate proteoglycans help facilitate the interactions of ABCA1, ABCG1, and ApoE, resulting in the promotion of cholesterol efflux and phospholipid efflux (Murphy et al. 2011). Moreover, ApoE, ABCA1, and ABCG1 deficiencies have downstream effects, including cholesterol accumulation, increased cell surface levels, and signalling of the common β subunit of the IL-

3/GM-CSF (CBS) through the STAT and ERK signalling pathways. Indeed, ERK could regulate integrin ß2 expression on HSPCs, which is also regulated by hypercholesterolaemia in a dependent and independent manner resulting in increased homing and localization of HSPCs to the injured arteries (Wang et al. 2015a).

My results indicate that ApoE-deficient cells under the effect of an HFD have an impact on lymphoid progenitors and differentiated lymphoid cells in transplantation. For example, there were impacts on T and B cell subsets in transplantation (Oguro et al. 2013). This could be reflective of the myeloid bias caused by an HFD in the context of ApoE-/-. Hypercholesterinaemia induced skewed differentiation has been linked to aging of HSCs ageing (Coller 2005; Desforges et al. 1989). Indeed, Tie et al. (2014) reported that hypercholesterolaemia may cause oxidant stress in HSCs, which might change the expression of their gene and the regulation of the cell cycling resulting in HSC ageing as demonstrated by telomere erosion, quiescence loss, decreased long-term HSC compartment, and impaired capacity of HSCs to reconstitute multi-lineage haematopoiesis. Taken together with my data, this suggests that an HFD may prematurely age HSPCs via ApoE signalling.

My results are broadly in agreement with Murphy et al. (2011), who concluded that the action of ApoE is in a cell-intrinsic fashion to promote the proliferation of HSPCs and monocytosis, though I did not see any impacts on neutrophilia or cell cycle. I also identified some ApoE HFD-mediated impacts on lymphoid cells, which may be a consequence of the myeloid bias observed. In addition to Murphy et al.'s (2011) study, in this project, the role of ApoE in steady-state haematopoiesis using a young ApoE murine model and the role of ApoE in the development of haematopoiesis and leukaemia in the context of an HFD and NCD using an ApoE murine model were used. In comparison to Murphy et al. (2011), the findings of this project covered all aspects of haematopoiesis in more depth as represented by analysing HSPC compartments in BM; multilineage haematopoiesis in BM, Sp, and PB; and T development in Thy. In closing, haematopoiesis is solely related with atherosclerosis, and atherosclerosis is associated with diet and ApoE. HFD and deficiency in ApoE is considered an increasing factor for atherosclerosis (Lu et al. 2017).

It is worth mentioning that this chapter differs from the previous chapter (exploring the role of ApoE in the steady-state haematopoiesis) in the context of the experimental design used. The previous chapter explored the role of ApoE in steady-state haematopoiesis in a young murine model in the context of an NCD. A germline deficient ApoE murine model (ApoE-/-), aged 8–12 weeks and fed an NCD, was performed to comprehensively analyse haematopoietic cell compartments in BM, PB, SP, and Thy. Furthermore, CFCs using total BM cells were performed to assess the differentiation and growth capacity of haematopoietic progenitor cells from ApoE-/- mice at the functional level. The findings revealed that HSPCs and lineage-

specific myeloid and lymphoid progenitors from both groups in steady state were unperturbed. Consistent with this, no change was found functionally in the number of CFCs. However, immunophenotype might not mirror the functional properties of HSPCs, and CFC assays performed in vitro do not entirely reflect HSPC function in vivo. Competitive repopulation and serial transplantation experiments, which are the gold standard for measuring in vivo HSPC function and HSC self-renewal, respectively (Harrison et al. 1997; Micklem et al. 1972) were not performed. Indeed, other studies show a function for ApoE in HSPCs. For example, in the context of atherosclerotic lesions, it has been observed that ApoE acted on cells autonomously to control the proliferation of HSPCs along with monocytosis, neutrophilia, and monocyte accumulation in atherosclerotic lesions (Murphy et al. 2011).

The current chapter explores the effect of an HFD and NCD on haematopoiesis in an ApoE murine model. ApoE and a wild-type murine model were placed on either an HFD or NCD for 12 weeks at 8 weeks of age to induce extensive atherosclerosis (Liu et al. 2018). From this experimental design, ageing, including an HFD and NCD, has been assessed which has been shown to affect haematopoiesis (Geiger et al. 2013; Lazare et al. 2017). The results revealed ageing, HFD, and NCD affect the highly enriched HSPCs, multilineage haematopoiesis, and survival advantage of the HSPCs. In addition, in the context an HFD, ApoE signalling seemed to be mostly unessential for HSC function as evaluated by transplantation and HSC self-renewal judged by secondary transplantation. Furthermore, ApoE appears to be expendable for extramedullary haematopoiesis in the context of an HFD.

According to Hermetet et al. (2019), HSC impaired maintenance is attributed to decreased dormancy post a HFD feeding. In addition, HFD disturb the receptor of TGF-β within lipid raft, linked to alter signalling of Smad2/3-dependent TGF-β as the major molecular mechanism of action. Trottier et al. (2012) showed that diet induced obesity in a rodent model causes significant changes in the function haematopoiesis and lymphopoiesis in BM and thymus. The results showed a sustained elevations in cells number exist in BM and thymus of HFD fed mice. Furthermore, increased thymus size and thymocyte number indicating that the homeostasis was not maintained. The observed results are believed to be due to large increases in leptin in BM and blood (Trottier et al. 2012). Adler et al. (2014) revealed that a HFD reduces B lymphopoiesis by changing the supportive capacity of BM niche. Indeed, feeding wild type mice for 1 and 6 weeks decreased B cells populations and this was caused by in reduced expression of B cells development markers Il-7 , Pax-5 and Ebf-1.

Lee et al. (2017) reported that obesity impair HSCs compartment in response to haematopoietic stress. Mechanistically, obesity causes oxidative stress deregulates GFi1 transcription factor expression. The deficiency of TLR4 signalling modifies the phenotype of HSPC in the BM of obese mice. $Tlr4^{-/-}$, $Myd88^{-/-}$, and $Ticam1^{-/-}$ mice fed an HFD for 16

weeks had a reduction in GMPs and declined production of myeloid colonies via CFU assay (Griffin et al. 2018). Other laboratories have examined the involvement of the NLRP3 inflammasome and IL-1β in the proliferation of myelopoiesis in obesity (Nagareddy et al. 2014: Pietras 2017). $Il1r^{-/-}$ mice fed a HFD for 6 months have reduced expansion of HSCs, GMPs and CMPs in comparison to $Il1r^{+/+}$ HFD controls and reduced production of inflammatory cytokines from BM-derived macrophages (Nagareddy et al. 2014). Recipient mice receiving $Nlrp3^{-/-}$ BM showed reduced expansion of GMPs, circulating neutrophils and monocytes, compared with WT BM recipients (Nagareddy et al. 2014). Ki67 staining has displayed augmented cellular proliferation of the HSC, GMP and MPP, CMP populations of obese mice (Nagareddy et al. 2014: Liu et al. 2018: Hermetet et al. 2019).

HSPCs in the context of obesity revealed consistent elevations in genes expression that are linked to myeloid differentiation such as Runx1, Csfr1 and Spi1 and activity of myeloid such as Stat6 and Stat3 and activator of cell cycle (Cdk1 and Ccna2) (Liu et al. 2018: Bowers and Singer 2021). Furthermore, elevated expression of cMyc , a cell cycle promoter , and reduced expression of cell cycke inhibitor such as p21 and p57 have been documented in purified HSCs in the context of obesity. The expression of the gene profile is attributed to be as a result of the loss of the lipid raft on cellular membrane of HSCs, culminating in the inhibitatin TGF-β signalling (Hermetet et al. 2019). Taken together, these reports revealed the involvement of obesity, inflammation, and the alterations that may happen within the haematopoietic system in response diet induced obesity (Bowers and Singer 2021).

In order to observe that at the time of analysis the mice had developed the expected phenotype in the ApoE model, more atherosclerotic studies are necessary to confirm and to study plasma lipid profile in aged ApoE mice (Raffai, R.L et al., 2005) and aortic arch sectioning to examine the lipid content, plaque size and lesion area(Murphy et al., 2011). In addition, cytokines assay will be needed to confirm the participation of B cells in atherosclerosis and atherosclerosis (DiLillo, Matsushita and Tedder, 2010: DiLillo, Horikawa and Tedder 2010; Lindemann et al. 2001; McEver 2001; Henn et al. 1998). Furthermore, Quantitative reverse transcription polymerase chain reaction (qRT- PCR) is required to identify genes differentially expressed between young mutant ApoE and control mice in the context of a HFD and NCD (Tie et al. 2014).

Different murine atherosclerotic models have essential differences in lipoprotein and cholesterol metabolism, reverse cholesterol pathways and inflammatory process (Oppi, Lüscher and Stein 2019). Endothelial cell activation, impaired efferocytosis macrophage activation VSMCs proliferation, Lipoprotein oxidation, and platelet aggregation are features that lead to the inflammation of the arterial wall, expansion of the lesion and thrombosis. ApoE is a multifunctional protein that influence the processes of inflammation, inhibits the oxidation

of lipoproteins (Oppi et al. 2019). ApoE murine models display raised levels of endothelial cell adhesion molecules, therefore activating monocytes and thymocytes recruitment into the subintimal space (Nakashima et al. 1994: Tian et al. 2005). Furthermore, ApoE prevents the proliferation and migration of VSMC and accordingly ApoE murine models rises VSMCs the proliferation and migration (Hui and Basford 2005). Similarly, ApoE prevents the aggregation of platelet, hence displaying an additional anti-atherogenic function (Riddell et al. 1999). Efferocytosis, the phagocytic clearance of cellular debris and apoptotic cells have essential functions in inflammation resolution and is primarily mediated by macrophages and other immune phagocytes (Kasikara et al. 2018). Indeed, ApoE stimulates apoptotic cells ingestion in macrophages, and hence ApoE murine models show impaired efferocytosis and apoptotic cells and cellular fragments accumulation in the vessel wall and further enhancing the development of lesion (Grainger et al. 2004). These anti-inflammatory properties along with its effect on lipoprotein metabolism of ApoE, elucidate why ApoE murine models revealed a very robust atherosclerosis development in comparison to other mouse models (Oppi, Lüscher and Stein, 2019).

Atherosclerosis can be an indicative parameter of clonal haematopoietic disease (Natarajan et al. 2018). A high serum lipid level, more specifically, low-density lipoprotein, is found to have strong association with atherosclerosis. The lipid profile can be a vital indicator of the condition of the atherosclerosis (Choy et al. 2004). Due to the Covid-19 pandemic, lipid profiling could not be done in this study to confirm atherosclerosis. Also, gene expression and RNA sequencing could not be conducted due to the Covid-19 pandemic; however, this would be helpful to understand some of the ApoE-mediated immunophenotypes affected (e.g., HSPC compartment) and their impact (Nagaoka et al. 2020).

Chapter 5: Exploring the impact of a high fat diet on AML development from ApoE-deficient haematopoietic stem/progenitor cells

5.1 Introduction

Cancer is predicted to be the leading cause of death in every country by the end of this century (Bray et al. 2018). Research has established that there are associations between cancer and lifestyle and, as diet is a part of lifestyle, by extension, this implies diet plays an essential role in cancer. For example, nasopharyngeal cancer is common in the Cantonese population and this was found to be associated with salt-preserved food (Adami et al. 2008). Conversely, fruit, vegetables, and food rich in Vitamin C and antioxidants are found to be reducing factors for oral and pharyngeal cancer (The Effect of Vitamin E and Beta Carotene on the Incidence of Lung Cancer and Other Cancers in Male Smokers 1994).

Acute myeloid leukaemia (AML) is recognized as a group of haematological diseases that affect HSPCs (Stone et al. 2004). Haematopoietic stem cell development is an ordered multi-level procedure in which microenvironmental cues of BM regulate a complex intrinsic network (Boulais and Frenette 2015). Blocked differentiation of myeloid precursors causes the development of AML (Enciso et al. 2015). The median age for this disease is 67 years (Ma et al. 2009), and it accounts for 30% of all leukaemias, which makes it the most frequent leukaemia (Jemal et al. 2008). Lifestyle, including diet, advancing age, and gender are all risk factors for AML (Ma et al. 2009). For example, a study has suggested that there are positive associations between AML and the consumption of meat and alcohol, while in the same study, it was reported that it is negatively associated with the consumption of milk (Li et al. 2006). Consumption of green tea is also found to be negatively associated with the diagnosis of AML (Zhang et al. 2007). Cholesterol metabolism is found to be dysregulated in haematological malignancies, which suggests that there could be an association between ApoE and malignant haematopoietic cells (Oguro 2019).

Cholesterol-derived metabolites also play a number of roles in cancer development and immune suppression. Preclinical and clinical studies have shown that controlling cholesterol metabolism inhibits tumour development, reshapes the immunological landscape, and reinvigorates anti-tumour immunity (Huang et al. 2020). The tumour microenvironment involves different immune effector and immunosuppressive cells together known as tumour infiltrating immune cells (TIIs), which have various anti- and pro-tumour functions based on cancer type and stage (Mantovani et al. 2008). TIIs involve T and B lymphocyte cells, dendritic cells, natural killer cells, neutrophils, and tumour-associated macrophages (TAMs) (Turley et al. 2015). For instance, TAMs could be reprogrammed as a consequence of changes in the

metabolism of cholesterol. Cancer cells produce hyaluronic acid oligomers, which increase the efflux of cholesterol in TAMs, which direct TAMs toward an M2-like phenotype and, as a result, accelerate the progression of tumours (Goossens et al. 2018). Furthermore, activated T cells undergo rapid proliferation and depend upon a high cholesterol metabolism to provide enough cholesterol to be utilized as a building block (Turley et al. 2015). Generally, the metabolism of cholesterol contributes to the progression of cancer including the proliferation, invasion, and migration of cells (Chimento et al. 2019: Liu et al. 2018). The biosynthesis of cholesterol is essential sustaining CSCs by activating signalling pathways downstream of Notch, tyrosine kinase receptors and sonic hedgehogs (Kim 2018). Moreover, high cholesterol biosynthesis has been noted in CSCs derived from patients under mammosphere culture conditions (Ehmsen et al. 2019). These studies suggest that ensuring sufficient cholesterol supply for CSCs might be vital for cancer progression and therefore, highlight an essential role of cholesterol in cancer (Turley, Cremasco and Astarita 2015). A well-defined type of CS namely, LSCs, are killed by the cholesterol lowering drugs Lovastatin, Fluvastatin, and Simvastatin in AML mouse models and patient samples through a mechanism that inhibits HMG-CoA reductase (HMGRC) (Yusenko et al. 2018: Hartwell et al. 2013), an enzyme that catalyses cholesterol biosynthesis rate limiting step (Gazzerro et al. 2011: Yusenko et al. 2018), and mevalonate, a downstream regulator of HMGCR. Together, these data indicate the cholesterol metabolism is central to the biology of CSCs.

ApoE is responsible for the metabolism of cholesterol (Greenow et al. 2005). Thus, the lack of ApoE alters ability to consume cholesterol, reducing it and thus extra cholesterol remains unmetabolized in the blood serum and the cholesterol. This causes a restriction in cell apoptosis and proliferation (Lin and Gustafsson 2015; Russo 2011). Indeed, the agonist of liver X receptor (LXR), which is a nuclear receptor target of metabolites of cholesterol and oxysterol (Calkin and Tontonoz 2010), showed a promising finding for the treatment of different cancers mainly due to the inhibition of the proliferation of cancer cells and cells causing apoptosis (Huang et al. 2020). However, the modulation of LXR signalling affects not only cancer cells but also immune cells (Huang et al. 2020). The RGX–104 LXR agonist reduces myeloid-derived suppressor cells via the upregulation of the LXR transcriptional target of ApoE and consequently elevated activates T cells.

ABCG1 and ApoE are responsible for the metabolism of cholesterol (Murphy et al. 2011; Greenow et al. 2005). Single deletion of the macrophage Abcg1 or Apo causes a mild but non-significant increase in the development of atherosclerotic lesions, while combined deletion of Abcg1 and Apo causes a more severe and significant increase in atherosclerosis (Lammers et al. 2009). Extramedullary haematopoiesis occurs as the spleen and liver regain their foetal haematopoietic roles in haematologic malignancies such as leukaemias and

myeloproliferative neoplasms, resulting in organomegaly. Symptomatic splenomegaly is observed in patients with leukaemia, and this induces severe morbidity. Pressure, early satiety, pancytopenia, portal hypertension, and hypercatabolic modifications are all symptoms of an enlarged Sp. Extramedullary haematopoiesis is thought to be caused by factors that damage the BM microenvironment, allowing stem/progenitor cells to ingress more easily (Westerterp et al. 2012). In other leukaemias, the ApoE genotype is regarded as determinant of the chronic lymphocytic leukaemia survival (Weinberg et al. 2008). The beneficial impact in CLL was due to the regulation of apoptosis of leukaemia cells by the ApoE 4 allele. In other cancers, ApoE mRNA and protein levels were both increased in non-small cell lung carcinoma (NSCLC) (Trost et al. 2008) as well as elevated ApoE serum in NSCLC patients. Elevated ApoE level was associated with lymph node metastasis and poor prognosis (Luo et al. 2016). ApoE was also found to be upregulated in gastric cancer, and there was a robust connection between the level of ApoE and the risk of muscular invasion, which makes it a promising marker for detecting gastric tumours invasion (Tanaka 1994; Oue et al. 2004).

ApoE has been found to be overexpressed in different ovarian cell lines and tissues and is fundamental for the growth and survival for ovarian cancer cells (Chen et al. 2005). Additionally, ApoE expression in PC-3 human prostate cancer cell line is linked to distant and local metastasis and hormone independence (Venanzoni et al. 2003). ApoE expression in the urine of patients with bladder cancer is linked to progression (Lindén et al. 2013). Therefore, the testing of ApoE in urine could offer a possible marker for non-invasive bladder cancer (Urquidi et al. 2012). Increased levels of ApoE have been found in tissue and serum in patients with pancreatic cancer, and this may be used as an early screening tool of the disease (Chen et al. 2013). High levels of ApoE in serum have been linked to the development of breast cancer and poor prognosis (Xu et al. 2016). The protein of ApoE has been shown to be increased in hepatocellular carcinoma (HCC) and might be used as potential marker for HCC (Yokoyama et al. 2006). Finally, multi-miRNAs targeting ApoE drive LDLR-related protein 1 and 8 (LRP1, LRP8) **–** dependent melanoma angiogenesis and metastasis (Pencheva et al. 2012). Overall, these data show the oncogenic association between ApoE and development of a variety of cancers, including AML.

5.2 Aims of the chapter

Several studies have been conducted to establish the link between cancer and lifestyle. As diet is a part of lifestyle, this by extension, implies diet has a crucial role in cancer (Adami, Hunter and Trichopoulos 2008). The dysregulation of cholesterol metabolism has been observed in haematological malignancies, which proposes that there might be a connection between and malignant haematopoietic cells and abnormal cholesterol metabolism (Oguro, 2019). Since ApoE is a multifunctional protein that is essential in regulating lipid metabolism (Greenow et al., 2005) as well as that the ApoE deficiency leads to the differences in the lipoprotein metabolism and, as consequence, significant hypercholesterolemia (Curtiss and Boisvert, 2000: Greenow et al., 2005), ApoE might play a fundamental role in the development of haematopoietic malignancies. Formerly, ApoE gene has been stated abundantly to be expressed in haematopoietic stem and progenitor cells (HSPCs) (Murphy et al. 2011) and many studies have been conducted to explore several aspects of the influence of a HFD on haematopoiesis and atherosclerosis development in an environment lacking ApoE. (Murphy et al., 2011; Tie et al., 2014). ApoE has been linked to the development of malignant hematopoietic cells (Oguro, 2019). However, the requirement for ApoE in leukaemia initiation and progression remains to be elucidated. Thus, the undying hypothesis of this chapter is that ApoE plays an essential role on the initiation and progression of leukaemia in the context of a HFD and NCD. The overall aim of this chapter was to explore the role of ApoE in leukaemogenesis with the following objectives:

1. To evaluate colony forming cell (CFC) capacity of HSPCs (c-kit+ cells) from ApoE-/- mice fed an HFD and transduced with the MLL-AF9 oncogene to generate pre-LSCs.
2. To characterise the immunophenotype of pre-LSCs generated from ApoE-/- mice fed an HFD.
3. To assess the *in vivo* role of ApoE deficient HSPCs fed an HFD in the maintenance and propagation of LSCs using the MLL-AF9 leukaemia murine model.

5.2.1 Results

5.2.1.1 Evaluation of CFC capacity of MLL-AF9 transduced BM c-kit+ cells from ApoE-/- mice fed an HFD

AML is a clonal disorder characterised by differentiation pathway blockage and the accumulation of immature cells in PB and BM (Ferrara and Schiffer 2013). Only a subset of AML cells, referred to as LSCs, are able to expansively proliferate and self-renew with similar properties to normal HSCs, making them essential therapeutic targets for leukaemia treatment (Wang et al. 2010). To examine the role of ApoE in leukaemogenesis, a well-characterised murine model of AML was used in which the progression and development of LSCs is driven by mixed-lineage leukaemia MLL-AF9 oncogene (Somervaille and Cleary 2006).

BM c-kit$^+$ HSPCs from ApoE and control mice that were fed either an HFD or an NCD were isolated and were transduced by a retrovirus containing MLL-AF9 oncogenes (Vukovic et al. 2015). The oncogenes were replated in methylcellulose three times to assess their ability to maintain continual self-renewal as represented by induced colony formation in the methylcellulose through three serial rounds of plating (Pattabiraman et al. 2014). Subsequently, after the three rounds of replating, a population of pre-leukaemic cells (pre-LSC) was generated. When transplanted into primary recipients, these cells produce LSCs, which induce AML; on transplantation into a secondary recipient, they self-renew to further generate LSCs (Figure 5.1).

Mixed-lineage leukaemia is characterised by chromosomal translocation affecting the MLL gene at 11q23 (Slany 2009), which results in chimeric fusion oncogenes and may include genes that are essential in the development of normal haematopoiesis. Examples of such translocations are t(9;11), t(15;17) and t(8;21), which encode MLL-AF9, PML-RARα, and AML1-ETO respectively. Fusion oncogenes produce abnormal gene proteins leading to aberrant haematopoietic signalling, which is a critical initial step in leukaemogenesis (Stubbs et al. 2008).

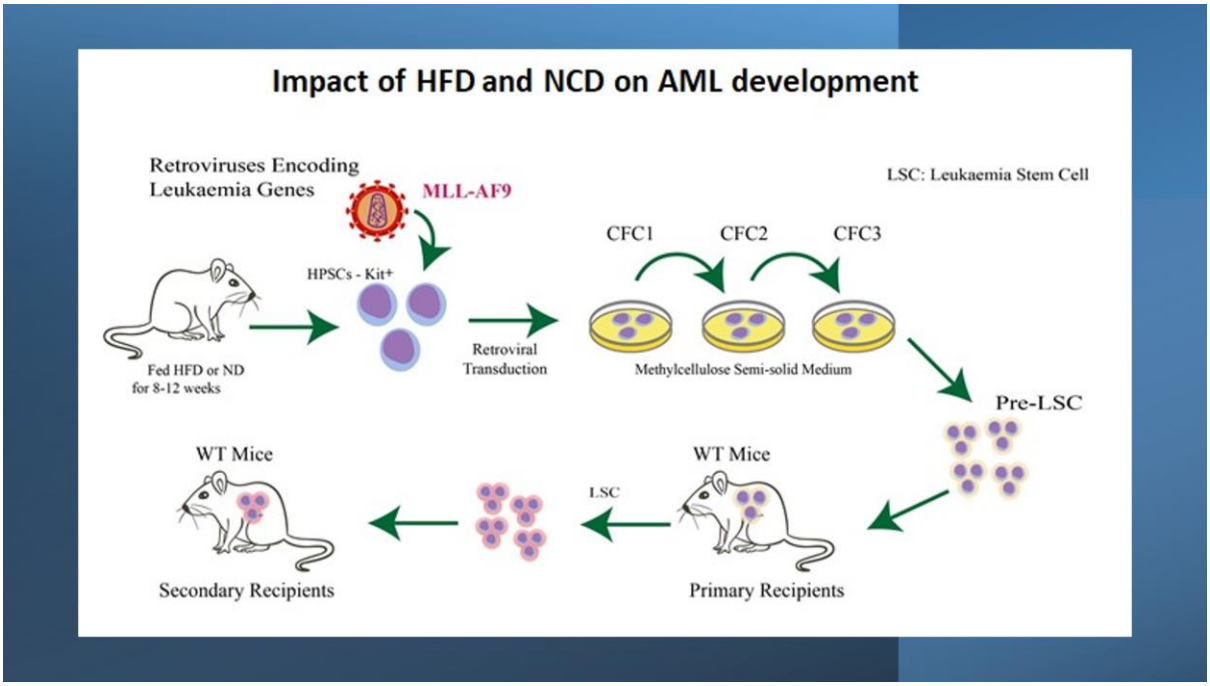

Figure 5.1 Schematic showing leukaemia transformation and transplantation assay. BM c-kit^{+} HSPCs were transduced by a retrovirus containing MLL-AF9 oncogenes. The oncogenes were replated in methylcellulose three times. After the three rounds of replating, a population of pre-leukaemic cells were generated; when transplanted into primary recipients, these cells produce LSCs, which induce AML with long latency. HFD: high fat diet, NCD: normal chow diet.

First, qualitative analysis of the CFC transformation assay was conducted. In this assay, three types of CFC are formed - A: compact colony. B. compact colonies with a corona of migrating cells, and C. small and diffuse colonies of large corona of migrating cells. A and B consist of immature cells, with leukaemic potential, with A representing the strongest leukaemic potential, while C contains more differentiated cells with less leukaemic potential (Mi et al. 2010). Microscopic analysis of the colonies of both genotypes revealed comparable size in all experimental conditions (Figure 5.2). Quantitatively, MLL-AF9 transduced cells derived from ApoE-deficient or control cells from mice that were previously fed either an HFD or an NCD had similar replating capacities (Figure 5.3), even though in CFC 1 ApoE-/- HFD and ND groups exhibited higher total colony numbers.

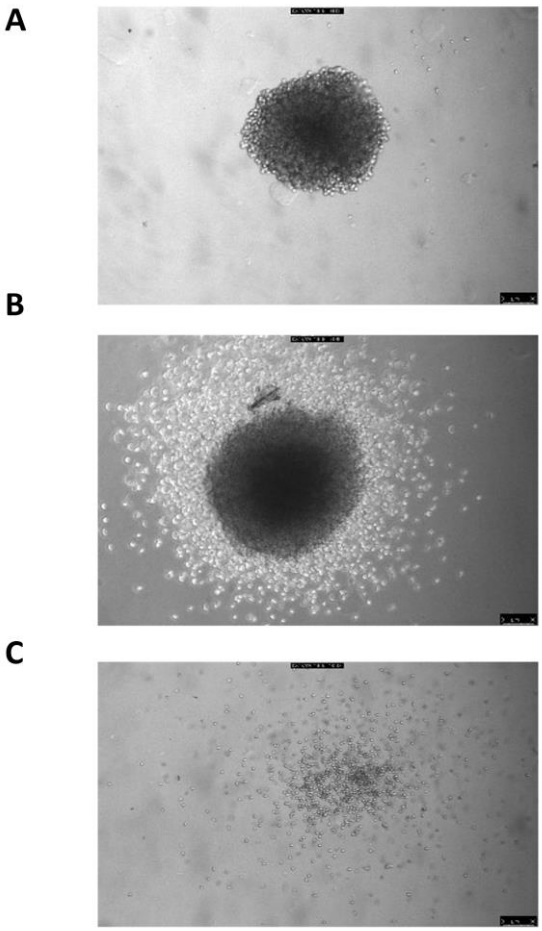

Figure 5.2 Pre-LSCs derived from ApoE-/- HSPCs fed an HFD display normal CFC colony appearance. Representative colonies at CFC3 shown at 10x magnification. A: compact colony. B. compact colonies with corona of migrating cells. C. small and diffuse colonies of large corona of migrating cells. A and B colonies consisted of immature cells, while C contained more differentiated cells (Mi et al. 2010).

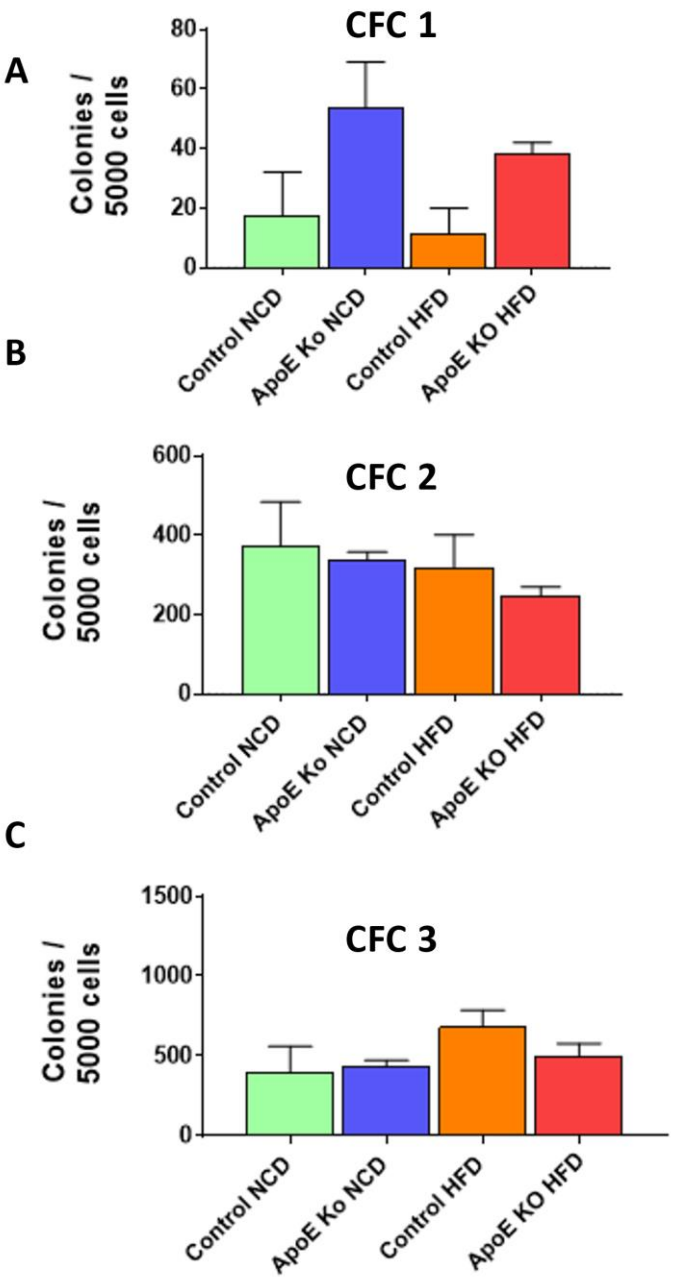

Figure 5.3 Pre-LSCs derived from ApoE-/- HSPCs fed a HFD display normal CFC replating capacity. Number of colonies at each passages (A) CFC 1, (B) CFC 2 and (C) CFC3. Statistical analysis was performed using one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD :2, Ko NCD: 2, Control HFD:5, Ko HFD: 5 mice. NCD: Normal chow diet, HFD: high fat diet.

5.2.1.2 Evaluation of immunophenotypic characteristics of MLL-AF9 transduced BM c-kit+ cells from ApoE-/- mice fed an HFD

Despite the lack of qualitative and quantitative differences in the CFC assay, flow cytometric analysis of the colonies from both genotypes fed either an HFD or an NCD was performed utilizing c-kit$^+$, Gr-1 and MAC-1 markers, which are markers for myeloid-specific antigens consistent with a late-stage myeloid phenotype and indicative of maturational arrest (Somervaille and Cleary 2006). These results showed no significant difference in the percentage of the immunophenotypic composition between the two genotypes of all experimental conditions (Figure 5.4). By looking at the mean fluorescent intensity (MFI), which measures the relative expression of each myeloid marker on a per cell basis, changes in the MFI of each immunophenotypic marker was unchanged in each of the experimental condition during CFC1 and CFC2. In CFC3, a significant increase in the MFI of Mac-1+ cells from control NCD were noted in comparison to other conditions (Figure 5.5).

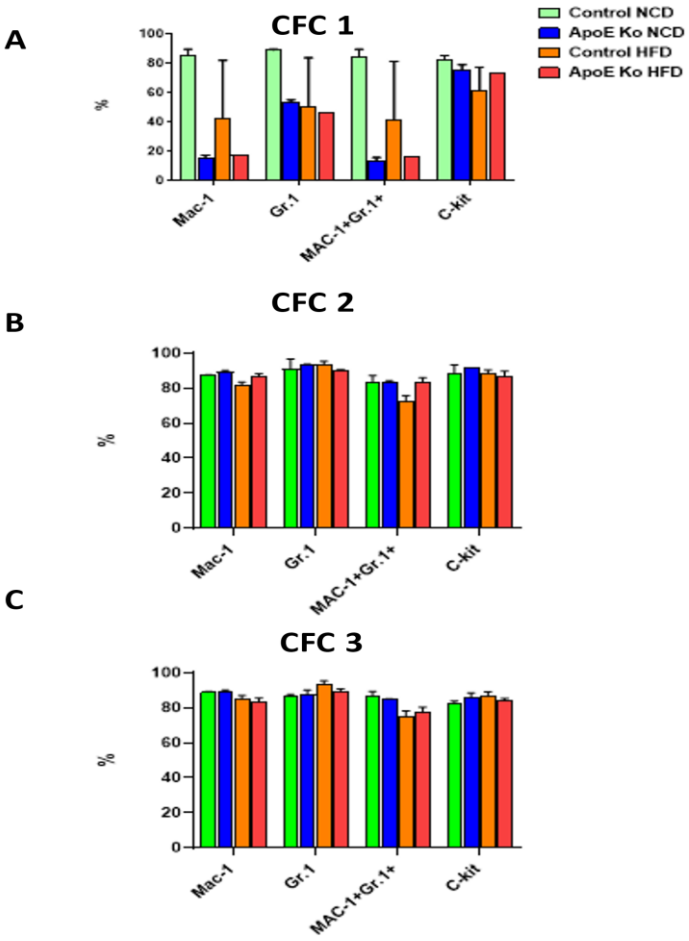

Figure 5.4 Pre-LSCs derived from ApoE-/- HSPCs fed a HFD display normal Immunophenotypic characteristics in CFC assays. % of myeloid cells (Mac-1, Gr.1, Mac-1+Gr.1+ and C-kit+ of CFC 1 (A), CFC2 (B) and CFC 3 (D). Statistical analysis was performed using one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple

comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD :2, Ko NCD: 2, Control HFD:2, Ko HFD: 2 mice. NCD: Normal chow diet, HFD: high fat diet.

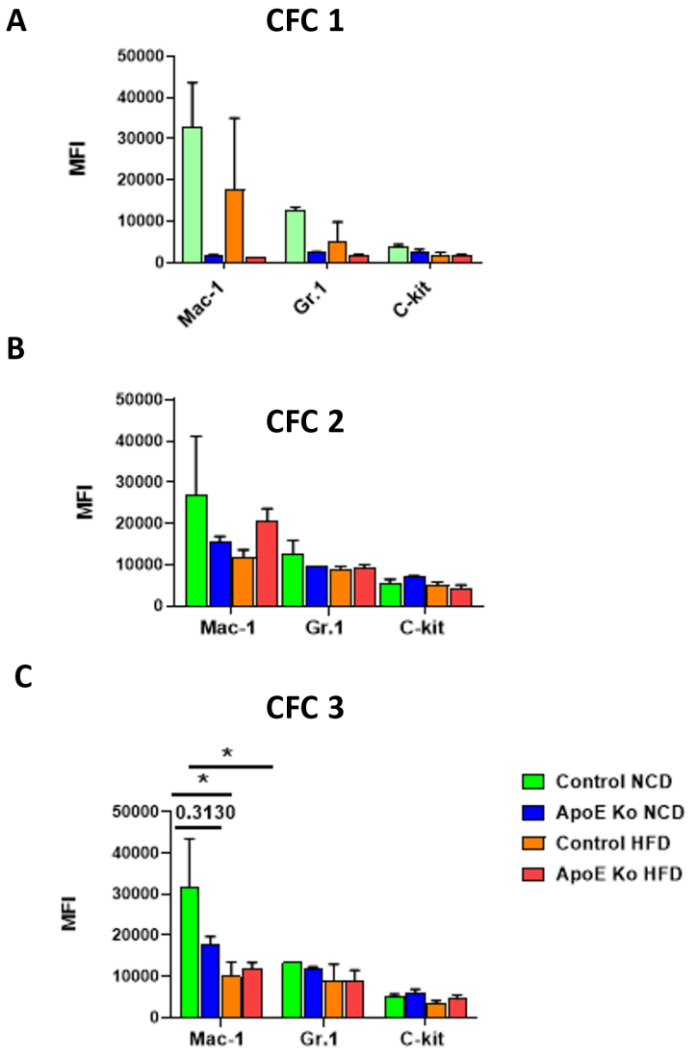

Figure 5.5 Pre-LSCs derived from ApoE-/- HSPCs fed a HFD display unaffected mean fluorescent intensity of myeloid markers in CFC assays**.** MFI for CFC (A), CFC 2 (B), CFC 3 (C). Statistical analysis was performed using one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using Control NCD :2, Ko NCD: 2, Control HFD:2, Ko HFD: 2 mice. *P <0.05. NCD: Normal chow diet, HFD: high fat diet.

5.2.1.3 Evaluating the effect of a high fat diet on development of LSCs and leukaemia in vivo

To explore the impact of an HFD on the development of leukaemia in the ApoE murine model in vivo, after the three rounds of CFC replating, a population of pre-leukaemic cells was generated that when transplanted into primary recipients produced LSCs, which induce AML. A primary transplant was conducted to assess the impact of an HFD on the initiation of MLL-AF9-driven AML from ApoE-/- mice. Blood was sampled from the tail vein to observe the engraftment. The findings show significant changes in leukaemic cell engraftment at the 4th, 7^{th,} and 11th weeks post-transplant for the ApoE KO HFD group compared to the other groups (Figure 5.6). Overall, from the engraftment results, engraftment of leukaemic cells appeared to be higher at weeks 4, 7 and 11 in LSCs derived from ApoE-/- mice fed an HFD.

To analyse the long-term survival of transplanted mice, the Kaplein-Mier survival analysis was conducted. From the survival curve, it can be concluded that survival percentage dropped initially in both NCD groups but was preserved in both HFD groups (Figure 5.7). However, while the ApoE-/- HFD group finally succumbed with the longest latency of AML, ultimately, no statistically significant difference was found in survival to support the hypothesis of an impact of an HFD on the development of MLL-AF9-driven AML from ApoE-/- HSPCs (Figure 5.7).

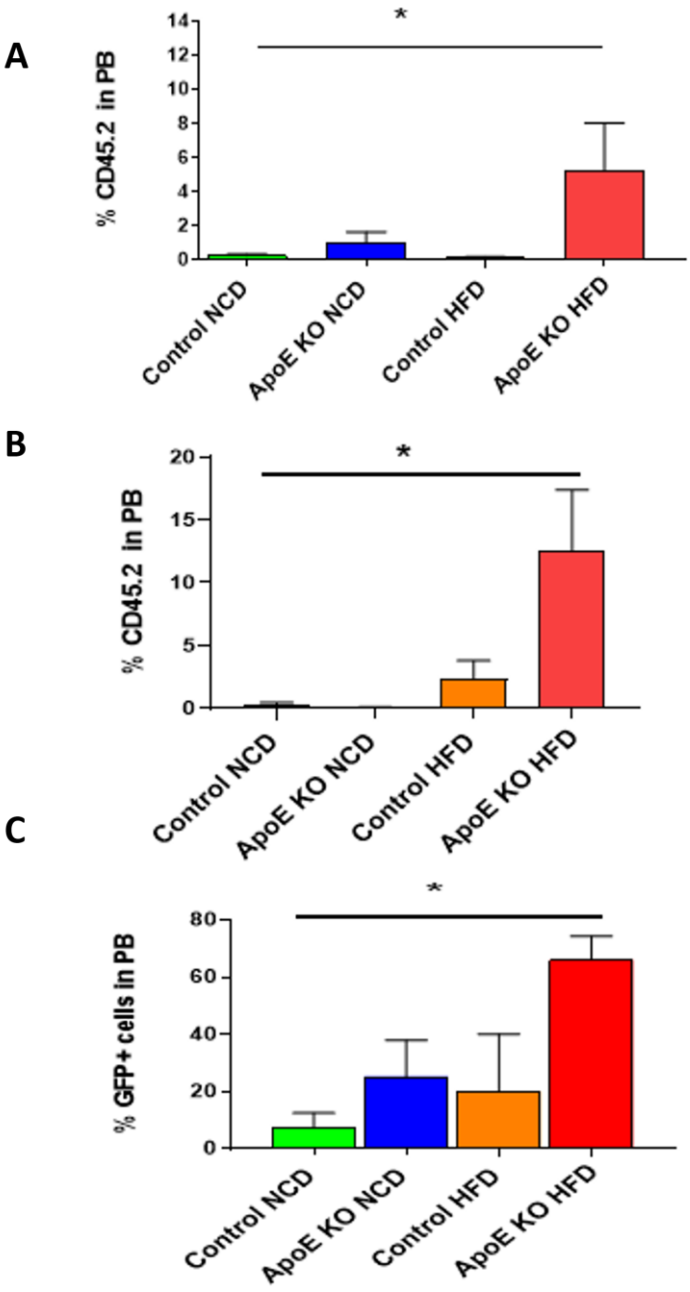

Figure 5.6 Peripheral blood engraftment after transplantation of LSCs from ApoE-/- mice fed an HFD. Bar graph shows a significant increase in the frequency of CD45.2 expressing MLL-AF9 in ApoE-deficient cells compare to the control cells in the context of a HFD and NCD in week 4 (A), week 7 (B) and week 11 (C). Statistical analysis was performed using one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using in 4 weeks Control NCD :6, Ko NCD: 6, Control HFD:4, Ko HFD: 4 mice. 7 weeks Control NCD :6, Ko NCD: 6, Control HFD:4, Ko HFD: 4 mice. 11 weeks Control NCD :5, Ko NCD: 3, Control HFD:3, Ko HFD: 2 mice. *P <0.05. NCD: Normal chow diet, HFD: high fat diet. *P < 0.05. NCD: Normal chow diet, HFD: high fat diet.

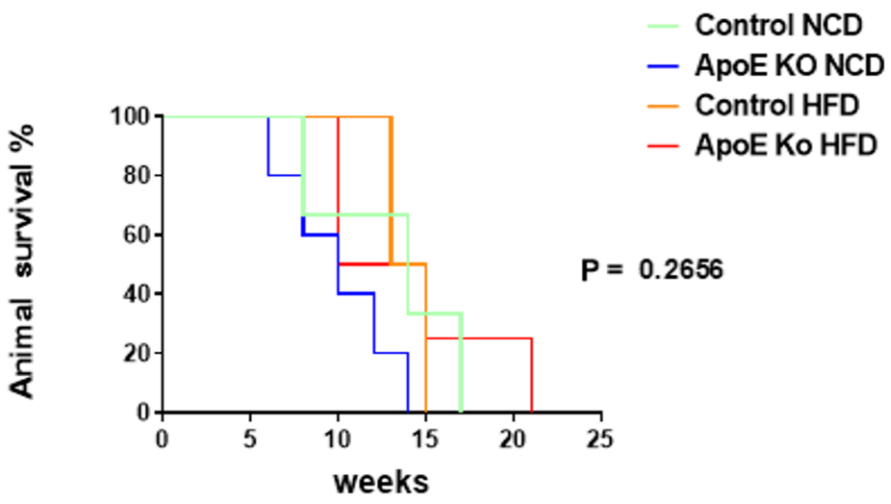

Figure 5.7 HFD does not accelerate leukaemia development in LSCs derived from ApoE-/- mice fed an HFD.

Graph showing the survival animal between control mice on a HFD and ApoE Ko on a HFD and including other conditions. Median survival was (A), control NCD: 14 weeks: ApoE Ko NCD: 10 weeks: Control HFD: 14 weeks: ApoE Ko HFD: 12.5. Statistical analysis was performed using one-way ANOVA nonparametric (4-group comparison) test with Turkey's multiple comparison test (GraphPad prism). Error bars represent the mean ± SEM of the individual experiments using in Control NCD :3, Ko NCD: 5, Control HFD:2, Ko HFD: 4 mice. Normal chow diet, HFD: high fat diet.

To explore the requirement for ApoE expression in human haematopoiesis and clinical cases of AML, I interrogated the www.bloodspot.eu. database. Initial analysis revealed that ApoE is expressed in different haematopoietic cells, consistent with previous reports in mice (Murphy et al 2011). APOE mRNA was expressed at an increased level in MPPs, MEPs, and monocytes, relative to HSCs and other populations of lineage-committed progenitors. In differing AML subtypes, including complex AML karyotypes, ApoE was expressed at higher levels than in normal HSCs, suggesting that ApoE expression may be required for leukaemogenesis in these settings (Figure 5.8.) The steady-state requirement for ApoE expression in AML in normal diet settings requires further exploration to test this hypothesis.

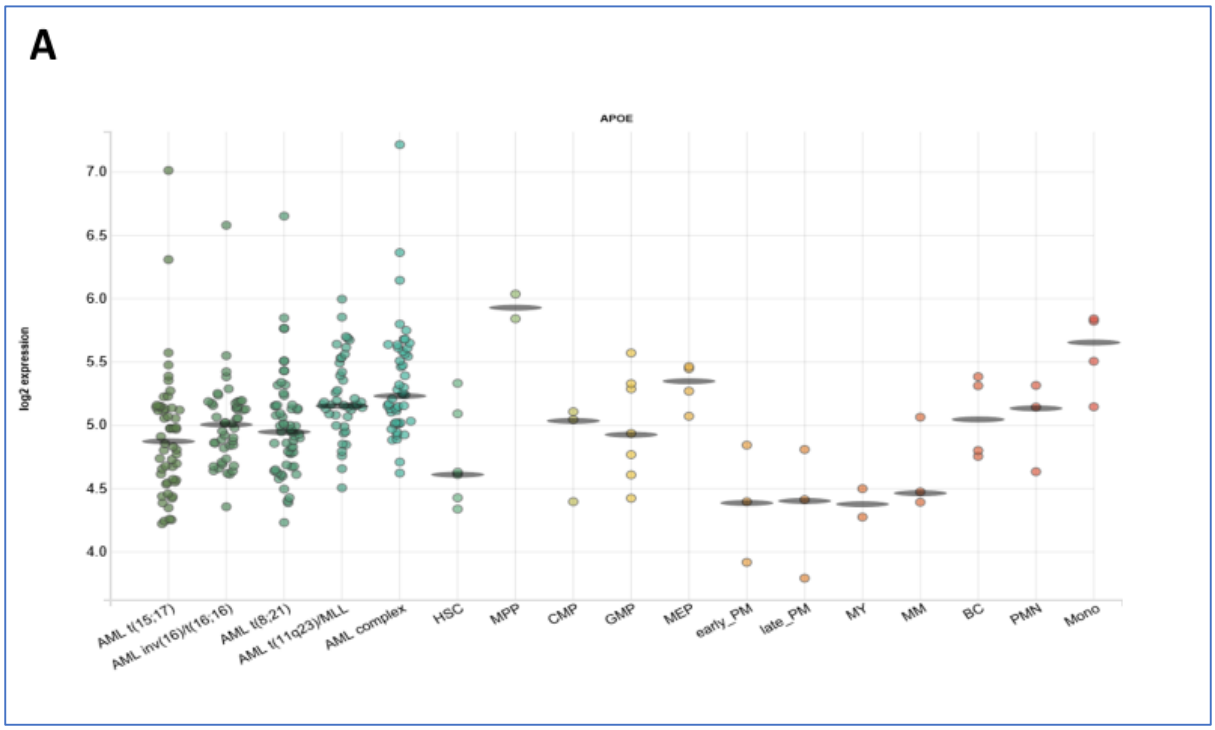

Figure 5.8 Graph showing the expression of ApoE gene for different haematopoietic cells and AML subtypes. Each circle represents one specimen and that the median is seen via grey line in each column. AMLt(15:17): AML with translocation(15:17), AML inv(16)t(16): AML with inversion (16) and translocation with (16), AML t(8:21): AML with translocation (8:21), AML t(11q23)mll: AML with translocation (11q23)/MLL, AML complex: AML with complex aberrant karyotype, HSCs: haematopoietic stem cells, MPP: multipotent progenitor , CMP: common myeloid progenitor, GMP: granulocyte monocyteprogenitor, MEP: megakaryocyte erythroid progenitor, early PM: early promyelocyte, late PM : late promyelocyte, MY: myelocyte, BC: Band cells, PMN: polymorphonuclear cells, Mono: monocyte adapted from (BloodSpot 2021).

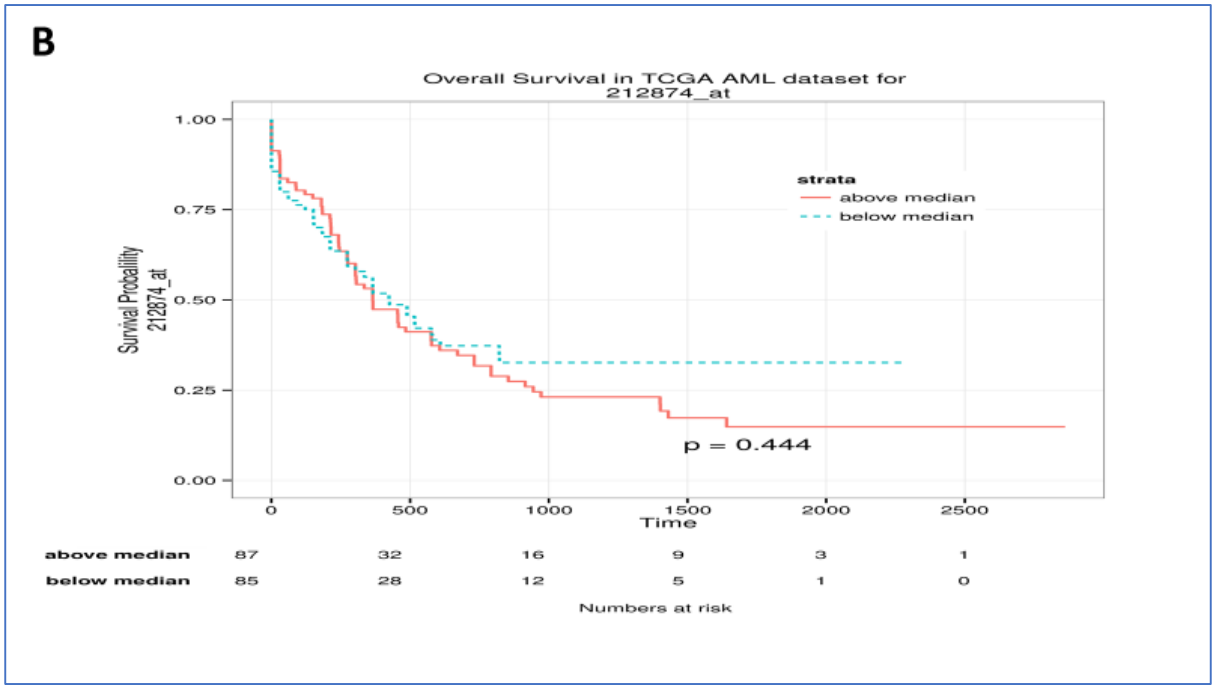

Figure 5.9 Graph showing the predication of the survival probability according to high and low level of ApoE gene expression. The survival plot is based upon AML dataset from The Cancer Genome Atlas (TCGA). The graph visually shows full Kaplan-Meier analysis of survival based on ApoE expression below or above median (BloodSpot 2021). cbioportal.org database

5.2.2 Discussion

Evidence based studies have concluded that diet and nutrition have a direct association with several types of cancer. For example, comprehensive studies have concluded that there is no association between fruit and vegetable intake and AML, but AML is found to be positively associated with smoking and with meat intake (Deschler and Lübbert 2006). However, fat intake and cholesterol are also found to be strongly associated with cancer (Yang et al. 2020). ApoE is related to cholesterol metabolism, and an elevated cholesterol level is found to be associated with cancer diagnosis (Westerterp et al. 2012). In this study, I therefore used ApoE-/- mice to examine the impact of an HFD on the development of leukaemia, which is evaluated by using MLL-AF9 driven AML murine model.

In our murine model, an explicit effect of ApoE in the context of an HFD was not found in the CFC assay, which generates pre-LSCs. This was assessed qualitatively by colony size and quantitatively colony count and immunophenotyping of the myeloid markers associated with the AML differentiation blockade. Significantly, however, the subtypes of leukaemic colonies were not classified, which would have given an additional indication of the leukaemic capacity of ApoE HSPCs fed an HFD. Notwithstanding this, however, it appears an HFD does not affect the transformation of ApoE-/- HSPCs into pre-LSCs in vitro.

When pre-LSCs were transplanted allowing the development of AML, while the ApoE-/- HFD group developed AML with the longest latency, there was no statistically significant difference amongst all conditions, which indicates that an HFD has no influence on the initiation of leukaemia in ApoE murine model. The former finding mirrors the finding in the TCGA dataset, which indicates that the survival was enhanced, albeit insignificantly, in ApoE low populations. Given this TCGA data, the steady-state requirement for ApoE expression in AML in normal diet settings requires further experimentation. Overall, however, these findings indicate that an HFD does not drive ApoE signalling to alter the pathogenesis of MLL-AF9 AML. In the context of HFD other signalling pathways appear to be more important than ApoE in initiating and maintaining AML. For example, the mechanism by which a HFD accelerates AML progression has been ascribed to epigenetic alteration which leads to the activation of cell cycling (Yan et al., 2016). Indeed, the upregulation of fatty acid-binding protein 4 (FABP4) stimulates the aggressiveness AML via the enhancement of DNMT1-dependent DNA methylation (Yan et al. 2017). The upregulation of FABP4 in AML cells happens by vascular endothelial growth factor (VEGF) signalling. Therefore, explaining a key regulatory feedback loop of FABP4-DNMT1 in the biology of AML. Yan et al. (2017) revealed that the dysfunction of FABP4 via its selective inhibitor BMS309403 results in DNMT1 downregulation, reduction of global DNA methylation and $p15^{INK4B}$ tumour suppressor gene re-expression by promoting DNA hypomethylation. Functionally, BMS309403 inhibits the formation of cell colony, causes

cell differentiation, and, notably, impairs the progression of leukaemic disease. In addition, the mechanism by which a HFD accelerates AML progression has been attributed to alteration in the metabolism of leukaemic cells (Tsunekawa-Imai et al., 2013: Yun et al., 2010).

Indeed, cancer cells depend on cholesterol as a cellular building block for the formation of membrane as well as for signalling molecules production (Tsunekawa-Imai et al., 2013). For instance, LSCs are eradiated by the cholesterol lowering drugs Lovastatin, Fluvastatin and Simvastatin in AML mouse models and patient samples via a mechanism that inhibits HMG-CoA reductase (HMGRC) (Yusenko, Jakobs and Klempnauer, 2018: Hartwell et al., 2013), an enzyme that catalyses cholesterol biosynthesis rate limiting step (Gazzerro et al., 2011: Yusenko, Jakobs and Klempnauer, 2018). Alteration in the microenvironment in which cancer cells are proliferating is regarded as a mechanism that accelerates AML progression in the context of a HFD ((Li et al. 2020). For example, the importance of the alterations in the metabolism might not only diverge in accordance with the intrinsic signalling pathways within the cancer cell, but similarly depend upon the interaction between the surrounding tumour microenvironment (TME), including extracellular matrix (ECM) components and soluble factors, stromal cells and immune cells (Schwörer et al. 2019). In other leukaemias, the genotype of ApoE is considered as determinant of survival in chronic lymphocytic leukaemia (Weinberg et al., 2008). The advantageous influence in CLL was attributed to the regulation of apoptosis of leukaemia cells by the ApoE 4 allele.

Hermetet et al. (2019) showed that HFD increases the clustering of the FLT3 receptor within the lipid raft on the surface of HSPCs, which activates the phosphorylation FLT3 receptor and, in turn, enhances the stimulation of the JAK3/STAT3 pathways in these cells. Inhibition of the cholesterol level via statins appears to be effective in targeting LSCs in AML (Hartwell et al. 2013), but why ApoE signalling appears to be dispensable for AML LSCs in this study is unclear. Part of the reason may be that ApoE is a multi-faceted protein influencing, amongst other pathways, inflammation, cholesterol metabolism, and oxidation by macrophages and the migration and proliferation of smooth muscle cells (Getz and Reardon 2009). Thus, compensatory mechanisms may influence ApoE functions in AML independent of the plasma level and the impact of atherosclerosis, inflammation, or cholesterol metabolism (Getz and Reardon 2009). For example, low expression of ApoE in adrenal cells can decrease the severity of atherosclerosis without influencing the plasma lipid level in the ApoE murine model (Klotz et al. 2006).

The strategies of therapy are urgently necessary AML patients. Leukocyte immunoglobulin-like receptor B4/Apolipoprotein E (LILRB4), which suppresses the activation of T-cell and supports AML cells tissue infiltration, is regarded a drug target for anti-AML therapeutics (Gui et al. 2019). The development and identification of an LILRB4-specific humanized mAb (h128-

3,) that blocks the activation of LILRB4 has shown a powerful activity in blocking monocytic AML development in several models such as syngeneic immunocompetent AML mice and patient-derived xenograft mice (Gui et al. 2019). MAb h128-3 promotes the efficacy of chemotherapy treatment of anti-AML by enhancing leukaemia cells mobilization. The activity of anti-AML of h128 has been ascribed to suppression of tissue infiltration of monocytic AML cell, inhibition of reversal of T-cell, cellular phagocytosis of antibody-dependent and cellular cytotoxicity of antibody-dependent. Thus, antibody targeting of LILRB4 represents an active therapeutic strategy for treating monocytic AML (Gui et al. 2019).

Different murine atherosclerotic models have essential differences in lipoprotein and cholesterol metabolism, reverse cholesterol pathways, and inflammatory process (Oppi et al. 2019). It will therefore be important to replicate these experiments in the LDLR model, for example, as an independent model of cholesterol transportation and atherosclerosis, as AML may grow and progress differently. For example, the LDLR model more faithfully recapitulates what is observed clinically in hypercholesterolemia compared to the ApoE model (Oppi et al. 2019; Defesche 2004). Other reasons that LDLR is a favourable to model to study the metabolism of cholesterol over their ApoE counterpart is that cholesterol is carried out via LDL particles in the LDLR model (Defesche 2004; Oppi et al. 2019), like the human setting; in the ApoE model, cholesterol is transported via very low-density lipoprotein (VLDL). Finally, more than 600 mutations in the LDLr genes have been reported, many of which cause familial hypercholesterolaemia, a genetic disorder that leads to an increased level of cholesterol LDL and the development of atherosclerosis (Goldstein 2001).

It is also possible that an effect of an HFD on AML in the context of the ApoE model was not revealed due to the choice of AML model – in our case, MLL-AF9. AML is an extremely heterogeneous disease with at least eight distinct subtypes according to the French, American, and British leukaemia classifications, which depend on the cell from which the leukaemia originates/propagates. It may be that an HFD fed to ApoE-/- HPSCs cells affects their leukaemic capacity in various subtypes of AML but with limited relevance to MLL-AF9. Overall, it is hypothesised that an HFD increases the likelihood of contracting AML. For example, from several studies, it has been found that the subtypes of AML known as M2, M3, and M4 are correlated with fat intake and lifestyle (Ma et al. 2009).

In further studies, the specific impact of am HFD can be evaluated on various subtypes of AML based on a range of mice models. Similarly, an HFD and obesity may have an impact on the development of other types of leukaemia. A 2010 study has concluded that diet-induced obesity accelerates acute lymphoblastic leukaemia progression (Yun et al. 2010). Yun et al.

(2010) developed an animal model of obesity and leukaemia to explore whether obesity can accelerate acute lymphoblastic leukaemia (ALL) using BCR/ABL transgenic mice on a C57BL background, which results in pro-B ALL (Heisterkamp et al. 1990) and AKR/J mice, which leads to T-cell ALL (Cloyd et al. 1980). Aged obese mice showed an accelerated onset of ALL, indicating a time-dependent impact of obesity on ALL. In addition, obese AKR/J mice developed ALL earlier than did control mice. Obese AKR/J mice showed elevated leptin, interleukin-6 level, insulin in comparison to control and these obesity related hormones have potential roles in the pathogenesis of leukaemia (Yun et al. 2010).

A cardinal property of LSCs is their ability to self-renewal and perpetuate leukaemogenesis. While ApoE signalling is dispensable for AML LSC maintenance in the context of an HFD, as revealed by primary transplantation, it is possible that it may be required to drive self-renewal of LSCs in this setting, which would be revealed in secondary transplantation. This experiment was substantially delayed due to the coronavirus pandemic, and when it was eventually performed, it failed due to technical issues with irradiation toxicity. In support of this hypothesis, the accumulated evidence suggests that there is an association between HFD intake and leukaemic stem cell self-renewal in the CML setting (Ye et al. 2016). Future work will be needed to determine the impact of ApoE signalling on an HFD in AML LSC self-renewal. It will also be important to explore the role of atherosclerosis-driven inflammation in the development of AML self-renewal in both ApoE and LDLR models. Several reports have highlighted the important response of HSCs toward inflammation and possible mechanisms of transformation to haematologic malignancies (Sezaki et al. 2020).

HSCs sense inflammation via extracellular and intracellular receptors resulting in the loss of their quiescence and proliferation to the external environment of the infectious agents and inflammatory factors. The common inflammatory signals that have been reported to affect haematopoiesis include interferon INF-α (Essers et al. 2009), IFNγ (Baldridge et al. 2010), macrophage colony stimulating factor (M-CSF) (Mossadegh-Keller et al. 2013), transforming growth factor (TGF)-β (Yamazaki et al. 2011), interleukin (IL)-1 (Pietras et al. 2016) and IL-6 (Reynaud et al. 2011). In addition, infectious agents involve pathogen associated molecular pattern (PAMPs) derived from danger associated molecular pattern (DAMPs) and microbes: both of them are referred to as pattern recognition receptors (PRRs). The activation of the downstream signalling in HSCs causes their differentiation to enhance the immune cell production, proliferation, and mobilization (Pietras 2017).

Indeed, chronic stimulation of immune cells induces DNA damage, stress of cells, and haematopoietic dysfunction as noted in sickle cell disease and developing myeloid malignancies because of the cytokine milieu, which can induce somatic gene mutations (Li et al. 2019). For example, persistent stimulation of cytokines or the stimulation of PAMP via IL-

6, TNF, and IFN signalling, and the dysfunction of HSCs have been well established and may possibly affect the progression and initiation of haematologic malignancies and the failure of BM (Trompouki et al. 201 Kristinsson et al. 2011; Kristinsson et al. 2010; Sezaki et al. 2020). Collectively, these papers explain a causative connection between the dysfunction of HSCs induced by the chronic stimulation of immune cells and the development toward haematologic malignancies (Sezaki et al. 2020).

Chapter 6: General discussion

6.1 Role of ApoE in Steady-State Haematopoiesis

CVD is regarded as the leading cause of death globally (Poller et al. 2020)**.** In most cases, the underlying cause of CVDs is atherosclerosis, an inflammatory disease in which the lipoproteins and leukocytes accumulate infiltrate, inducing the formation of atherosclerotic plaques, which then rupture culminating in potentially fatal complications such as myocardial infraction and stroke (Poller et al. 2020). HSCs give rise to all blood lineage cells including immune cells, and **a** healthy individual generates 4 – 5 x10^{11} blood cells to maintain the level of haemostasis (Kaushansky 2006). HSCs inhabit a specialised environment or niche in the BM (Pinho and Frenette 2019). CVDs strongly affect haematopoiesis, as HSCs supply and give rise to inflammatory immune cells, which are essential to the development of CVDs. Additionally, the common risk factors for CVDs, such as hyperlipoproteinemia and diabetes mellitus, markedly change the haematopoiesis process, and likewise, atherosclerosis and its complications affect haematopoiesis.

Studies have reported a causal relationship between leucocytosis, hypercholesterolemia, and CVDs, which shows that leucocytosis might augment atherosclerosis **(**Coller 2005). ApoE regulates the cholesterol metabolism of the body and hypercholesterolaemia is induced in ApoE knockout mice (Rashidi et al. 2017). Hypercholesterolaemia is found to be associated with acute monocytosis and neutrophilia (Soehnlein and Swirski 2013). Several studies conducted on ApoE knockout mice have suggested that monocytosis and neutrophilia develop in association with the proliferation and expansion of HSPCs in the BM (Chapman et al. 2004). The reason behind this association is described in a recent study, which has found evidence that cholesterol-sensing pathways are the regulators of the proliferation of HSCs (Murphy et al 2011). Another study has suggested that in the ApoE KO mice model of atherosclerosis, an increase was observed in the BM colony forming unit (Feldman et al. 1991). However, as ApoE has been connected to the biology of lipids, previous research has not described the role of endogenous ApoE in regulating HSPC compartments in steady-state haematopoiesis. In this project, the function of ApoE in steady-state haematopoiesis was firstly explored by immunophenotypic analysis of BM, Sp, Thy, and PB and functional analysis of BM progenitors in vitro.

The results revealed that HSPCs and myeloid and lymphoid progenitors' lineage, specific from both groups in steady state, were unperturbed. In summary, the influence of ApoE in immunophenotypically defined HSPC compartments was not significantly altered. Consistent

with this, there is no change functionally in the number of CFCs. Although the immunophenotype may not mirror the functional attributes of HSPCs, CFC assays performed *in vitro* do not entirely mirror the function of HSPCs *in vivo*, and competitive repopulation and serial transplantation experiments were not conducted, which are the gold standards for measuring in vivo HSPC function and HSC self-renewal, respectively (Harrison et al. 1997; Micklem et al. 1972). In addition, the results showed a significant reduction of B cells in BM in ApoE-/- mice, which seems to be independent both of HSC/HSPC and CLP compartments, which were largely unaffected. Thus, it is hypothesised that ApoE-mediated regulation of B-cell abundance takes place during post-CLP B-cell selection. Additionally, more immunophenotyping experiments and functional experiments (transplantation and CFC-B assays) will be required to dissect the precise role of ApoE in pre/pro-B-cell development.

Spleen weight in ApoE-/- mice was relatively increased, which possibly indicates the known role of ApoE gene in lipid biosynthesis (Greenow et al. 2005). Further analysis of lipid content in the Sp alongside histological analysis of splenic architecture will be needed to dissect the potential role of ApoE in lipid biosynthesis in this setting. In the PB, changes were seen in Mac-1+ cells (monocytes) and platelets in ApoE-/- mice, with a decreased abundance of monocytes and platelets. Several possible mechanisms have been suggested. Initially, the content of cellular cholesterol affects the secretion and synthesis of ApoE by the macrophage/monocyte lineage (Zhang et al. 1996; Van Eck et al. 1997), which is consistent with the notion that ApoE acts as a cholesterol responsive gene (Dory 1991; Mazzone et al. 1989; Mazzone et al. 1987). Therefore, the regulation of ApoE secretion may be vital in maintaining the balance of cholesterol efflux and influx (Mazzone et al. 1989; Mazzone et al. 1987; Van Eck et al. 1997). Furthermore, ApoE not only acts as a lipid transporter but also regulates essential immunoregulatory molecules with an impact on immune cells, such as the macrophage/monocyte lineage (Grainger et al. 2004; Bellosta et al. 1995; Braesch-Andersen et al. 2013; Vitek et al. 2009; Kelly et al. 1994).

A reduction in the platelet count was also noted in the PB of ApoE-/- mice. This might be attributed to anti-platelet activity regulation by enhancing the production of endogenous NO (Graham and Owen 1997). In addition, carboxyalkylpyrrole-phosphatidylethanolamine derivatives (CAP-PEs) in the plasma of ApoE murine model binds to TLR2 and induces platelet integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression in a TLR2-dependent manner. The induction of downstream signalling via myeloid differentiation primary response 88 / MyD88 adapter-like (Mal)/TIRAP (MyD88/TIRAP), interleukin-1 receptor-associated kinase 4 (IRAK4) phosphorylation, and subsequent activation of necrosis factor receptor–associated factor 6 results in the activation of Sp tyrosine kinase, platelet integrins, and SFK. It can be concluded that there is an association between ApoE and the regulation of steady state haematopoiesis

in select mature cell lineages, namely, B cells, monocytes, and platelets, which might further be understood in a transcriptomic analysis of those lineage-specific haematopoietic progenitors and functional transplantation assays.

6.2 Impact of a high fat diet on ApoE signalling in haematopoiesis

Several studies have been performed to evaluate the dietary effect on haematopoiesis. Calorie restriction provides a boost in the regenerative and self-renewal capacity of stem cells (Cho and Park 2020). In contrast, in a high fat-based diet, inhibits HSC mobilization (Mihaylova et al. 2014). An HFD induces myelopoiesis from differentiating HSCs (Singer et al. 2014). Finally, hypercholesterolaemia enhances haematopoietic stem and progenitor cell proliferation (Vanhie et al. 2020), and a link has been made to ApoE in this respect. Thus, as ApoE has been linked to the biology of lipids, a previous study was carried out to assess the role of endogenous ApoE in the control of HSPC proliferation and monocytosis in the context of an HFD (Murphy et al. 2011). However, the broader impact of ApoE on other haematopoietic lineages and HSC self-renewal in the setting of an HFD remains incompletely defined.

Immunophenotypic analysis showed the HSC-containing LSK population and LK subpopulation were significantly perturbed, which suggests that an HFD and chronic loss of ApoE changes cellular distribution within the HSPC compartment. This might be attributed to an increased expression of inflammatory molecules, growth factors, and cytokines, which are essential in modulating haematopoiesis (Trottier et al. 2012). Furthermore, it can be concluded that in the context of an HFD, ApoE acts as a key regulator of HSPCs' immunophenotypic status. Mechanistically, the impact observed in HSPCs was due to changes in the late stages of apoptosis rather than the proliferative capacity of these populations (Murphy et al. 2011).

Given the impacts of an HFD on HSPC subsets in ApoE-/- mice, in further experiments, it will be of interest to examine the impact of an HFD on ApoE-mediated signalling in the context of HSPC mobilization from the BM to the PB as well as a further detailed histological examination of extramedullary haematopoiesis. Mechanistically, sympathetic nervous system signalling has been suggested to release HSPCs from BM niches where they could seed the Sp (Dutta et al. 2012). Other mechanisms have been suggested including an increased level of granulocyte-colony stimulating factors (G-CSF) skewing the myeloid lineage commitment in favour of neutrophils rather than monocytes, which reduces the number of macrophages in BM and therefore supports the abundance and network of osteoblasts nestin^{+} mesenchymal stem cells, which express CXCL12, an essential retention signal for HSPCs (Sugiyama et al. 2006). Furthermore, a change in the BM stem cell niche induced by an HFD in ApoE-/- mice may cause an increase in HSPC mobilization due to, for example, cleavage of CXCR4 on

HSPC induced by proteolytic environment caused by increased production of neurophils, leading to an impairment of HSPC retention in the BM (Soehnlein and Swirski 2013). Finally, ERK could control the expression of integrin ß2 expression on HSPCs, which is also regulated by hypercholesterolaemia in a dependent and independent manner culminating in an elevated HSPC mobilization and localization to injured arteries (Wang et al. 2015a). These potential mechanisms should be explored.

ApoE cooperates with ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1), which are expressed in HSPCs, to enhance cholesterol efflux and decrease downstream signalling of IL3 receptors. Moreover, heparinise sulphate proteoglycans aid in facilitating the interactions of ABCA1, ABCG1, and ApoE, leading to the enhancement of cholesterol efflux and phospholipid efflux (Murphy et al. 2011). Additionally, the deficiency of ApoE, ABCA1, and ABCG1 leads to a downstream impact involving the accumulation of cholesterol and the elevated level of cell surface signalling of the common β subunit of the IL-3/GM-CSF (CBS) through the STAT and ERK signalling pathways.

The results of Murphy et al. (2011) and of this study similarly suggest that there is an association between atherosclerosis, haematopoiesis, and ApoE. For example, Murphy et al. (2011) indicated that ApoE functions to cause an expansion of HSPCs, though the exact mechanisms for HSPC expansion differ between this study and theirs. Due to the outbreak of coronavirus, some experiments were not performed. Lipid profile can be an essential marker of atherosclerosis condition (Choy et al. 2004). In addition, gene expression and RNA sequencing could have been valuable to understand the observed phenotypes and mechanisms (Nagaoka et al. 2020). Covering these aspects will give an insight into the basis for the immunophenotypic changes in HSPCs observed in this study.

6.3 Impact of HFD on ApoE signalling in leukaemogenesis.

Leukaemogenesis has a direct association with dietary intake (Tucci et al. 2018). It is a well-established fact that BM function is indeed affected by dietary intake and that an HFD perturbs normal function (Spurny et al. 2020). Lifestyle, involving diet, gender, and advancing age, are all risk factors for AML (Ma et al. 2009). The metabolism of cholesterol is found to be dysregulated in haematological malignancies, which implies that there could be an association between ApoE and malignant haematopoietic cells (Oguro 2019). Given this, the initiation of leukaemia and the progression of HSPCs from ApoE-/- mice fed an HFD was investigated in this chapter. However, no impact of an HFD on ApoE signalling in AML was found. Why signalling of ApoE seems to be dispensable for AML is unclear, but the specific role of atherosclerosis-driven inflammation in AML development in both ApoE and other LDLR models should be further explored as inflammation may provide a positive trigger for

leukaemogenesis (Pietras 2017). In this respect, ApoE-/- mice provide a tractable model to directly assess whether HFD promotes AML in association with the inflammatory status of atherosclerosis development. To test this, wild-type HSCs could undergo the leukaemogenic assay described in this thesis, and pre-leukaemic CFCs could be transplanted into either ApoE-/- mice or control mice and fed an HFD (or a normal diet) for the duration of the experiment; this model will simultaneously promote atherosclerosis in ApoE-/- mice that will proceed to develop AML. To assess the association between atherosclerosis and AML development, the survival of mice and the disease progression could be monitored as described in this thesis. Experiments could also be performed where leukaemic ApoE-/- recipients are fed an HFD for half of the experiment, followed by a switch to a normal diet to allow for the regression of atherosclerotic plaque; the impact on AML development could similarly be monitored in this setting. Collectively, these experiments could establish the relationship between HFD, atherosclerosis-driven inflammatory cues, and the development of AML.

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