



Uncovering a Novel Role for *Duoxa2* in NK Cell Development

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Statement of Originality

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Bí dílis don Chreideamh

Abstract

The DUOX2/DUOX2A system is involved in the generation of hydrogen peroxide. In the thyroid, hydrogen peroxide produced by DUOX2/DUOX2A is essential for thyroid hormone synthesis. Consequently, mutations in *DUOX2* and *DUOX2A* result in congenital hypothyroidism. Recently, *Duoxa2^{-/-}* mice were found to be extremely susceptible to pulmonary colonisation when subjected to an experimental metastasis assay. *Duoxa2^{-/-}* mice were also found to exhibit a perturbation of immune homeostasis, with defects in several immune populations, including NK cells. However, this complex is not known to play a direct role in the immune system. Therefore, this PhD investigated the mechanism by which DUOX2A is involved in immunoregulation, focusing on NK cells. It was found that *Duoxa2^{-/-}* mice exhibit a severe defect in NK cells. Mature NK cells were significantly reduced and an accumulation of refined-NKPs was evident in the bone marrow. Together these results indicate a defect in NK cell maturation in the absence of DUOX2A.

Importantly, *Duoxa2^{-/-}* mice are congenitally hypothyroid. Thyroid hormones have been demonstrated to influence various immune cells, including NK cells. They have been shown to increase NK cell cytotoxicity, but NK cells from hyperthyroid patients also have reduced cytotoxic capacity. However, evidence for the relationship between thyroid hormones and the immune system remains limited. Interestingly, induction of hypothyroidism in mice using a low-iodine 0.15% Propothiouracil diet resulted in a similar NK cell defect to that observed in *Duoxa2^{-/-}* mice. Hypothyroid mice also exhibited a reduction of mature NK cells and a build-up of refined-NKPs, suggesting that hypothyroidism is driving this phenotype. Signalling through thyroid receptors can regulate gene expression and is essential for normal neurodevelopment. Therefore, thyroid hormones may also regulate gene expression during NK cell development. In summary, these results identify a novel and fundamental role for thyroid hormone in the regulation of NK cell development in mice.

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Abbreviations

AF-1 – Activation function 1 domain

AF-2 – Activation function 2 domain

BCR – B cell receptor

cAMP – Cyclic adenosine monophosphate

CAR – CXCL12-abundant reticular cell

cDNA – Complementary DNA

CGD – Chronic granulomatous disease

CILP – Common innate lymphoid progenitor

CCL – CC-motif chemokine ligand

CCR – CC-motif chemokine receptor

CLP – Common lymphoid progenitor

CMP – Common myeloid progenitor

CXCL – CXC-motif chemokine ligand

CXCR – CXC-motif chemokine receptor

CX₃CR1 – CX₃C motif chemokine receptor 1

D2 – Type 2 deiodinase

D3 – Type 3 deiodinase

DBD – DNA binding domain

DC – Dendritic cell

DIT – Diiodotyrosine

DUOX2 – Dual oxidase 2

DUOXA2 – Dual oxidase maturation factor 2

EOMES – Eomesodermin

ER – Endoplasmic reticulum

ERK – Extracellular signal-related kinase

ETS1 – ETS proto-oncogenic 1

FasL – Fas ligand

FMO – Fluorescence minus one

FOXO1 – Forkhead box protein O1

GATA3 – GATA binding protein 3

GFP – Green fluorescent protein

GM-CSF – Granulocyte monocyte colony stimulating factor

HAT – Histone acetyltransferase

HDAC3 – Histone deacetylase 3

HPT – Hypothalamic-pituitary-thyroid

HSC – Haematopoietic stem cell

H₂O₂ – Hydrogen peroxide

IBD – Inflammatory Bowel Disease

ID2 – Inhibitor of DNA binding 2

IFN – Interferon

IL – Interleukin

ILC – Innate lymphoid cells

ITAM – Immunoreceptor tyrosine-based activation motif

ITIM – Immunoreceptor tyrosine-based inhibitory motif

JAK – Janus kinase

kbp – Kilo-base pair

KIR – Killer immunoglobulin-like receptor

LBD – Ligand binding domain

LMPP – Lympho-myeloid primed progenitors

MACS – Magnetic-activated cell sorting

MAPK – Mitogen-activated protein kinase

MHC – Major histocompatibility complex

MIT – Moniodotyrosine

mRNA – Messenger RNA

MTOC – Microtubule organising centre

mTOR – Mammalian target of rapamycin

NADPH – Nicotinamide adenine dinucleotide phosphate

NCoA-1 – Nuclear receptor co-activator 1 NCoA-1

N-CoR1 – Nuclear receptor co-repressor 1

N-CoR2 – Nuclear receptor co-repressor 2

NFIL3 – Nuclear factor interleukin 3 regulated protein

NK – Natural Killer

NKP – Natural Killer Progenitor

NKT – Natural Killer T cell

NOX – NADPH oxidase

PBS – Phosphate buffered saline

PIOD – Partial iodide defect

PI3K – Phosphoinositide 3-kinase

PKA – Protein kinase A

PKC – Protein kinase C

PMA – Phorbol myristate acetate

PolyI:C – Polyinosinic:polycytidylic acid

PTU – Propothiouracil

PVN – Paraventricular nucleus

qRT-PCR – Quantitative real time polymerase chain reaction

rNKP – Refined-NK progenitor

ROS – Reactive oxygen species

ROR γ – Retinoic acid-related orphan receptor gamma

RXR – Retinoid X receptor

STAT – Signal transducer and activator of transcription

T₃ – Triiodothyronine

T₄ – Thyroxine

T-BET – T-box protein expressed in T cells

TCR – T cell receptor

TGF β – Transforming growth factor β

ThOX – Thyroid oxidase

TIOD – Total iodide defect

TLR – Toll-like receptor

TNF – Tumour necrosis factor

TPO – Thyroid peroxidase

TRAIL – Tumour necrosis factor-related apoptosis-inducing ligand

TRE – Thyroid response element

TRH – Thyrotropin-releasing hormone

TR α – Thyroid receptor alpha

TR β – Thyroid receptor beta

TSH – Thyrotropin

TYK – Tyrosine kinase

ZEB2 - Zinc finger E-box binding homeobox 2

γ_c – Common gamma chain

1. Introduction

1.1. Discovery and function of the DUOX2/DUOXA2 system

The generation of reactive oxygen species (ROS) is essential for many physiological processes including immunity and cell signalling, however excess ROS production can also contribute to disease, including carcinogenesis, due to oxidative damage (Bae et al. 2011). Dual oxidase 2 (DUOX2) is a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase flavoprotein. DUOX2 is a member of the NADPH oxidase (NOX) family of proteins, of which there are seven homologs in the mammalian genome. These include NOX 1-5 and the dual oxidases, DUOX1 and DUOX2, all of which play an important role in the generation of ROS (Fig. 1.1) (Brandes et al. 2014). The most well characterised of these enzymes is NOX2, the phagocyte NADPH oxidase. NOX2 is known to play an essential role in innate immunity through the generation of antimicrobial ROS in the respiratory burst in neutrophils and macrophages (Lam et al. 2010). Consequently, mutations in any of the components of NOX2 results in the development of chronic granulomatous disease (CGD) in humans (Segal, 1996). CGD is characterised by an increased susceptibility to certain bacterial and fungal infections and autoinflammation (van den Berg et al. 2009). However, ROS generation is not restricted to phagocytic cells and is essential for many other biological processes. Differential and distinct expression of NOX isoforms in different tissues allows regulation of many different pathways.

The dual oxidases, DUOX1 and DUOX2, are highly expressed in the thyroid gland and play an important role in thyroid hormone synthesis. In particular, the generation of hydrogen peroxide (H_2O_2) by DUOX2 is crucial to this process. Early research into thyroid hormone synthesis identified a key role for H_2O_2 , however, identification of the cellular source of H_2O_2 proved difficult. Early studies demonstrated that generation of H_2O_2 in the thyroid gland was associated with an NADPH oxidase found in thyroid tissue (Virion et al. 1984; Björkman and Ekholm, 1984). In 1999, a single NADPH oxidase was purified and partially cloned for the first time from both pig and human thyroid tissue (Dupuy et al. 1999). At a similar time, another study performed a screen for sequences similar to NOX2 in complementary DNA (cDNA) libraries from primary thyroid cells. This study identified two novel NADPH

oxidase proteins, originally named thyroid oxidase 1 (ThOX1) and thyroid oxidase 2 (ThOX2), now known as the DUOX1 and DUOX2 proteins. The authors identified that these genes had 83% similarity to each other, but also significant similarity to the NOX2 sequence – 53% and 47% respectively. These genes were also found to be similar to a predicted protein in the *Caenorhabditis elegans* genome, with 53% and 61% similarity respectively (De Deken et al. 2000).

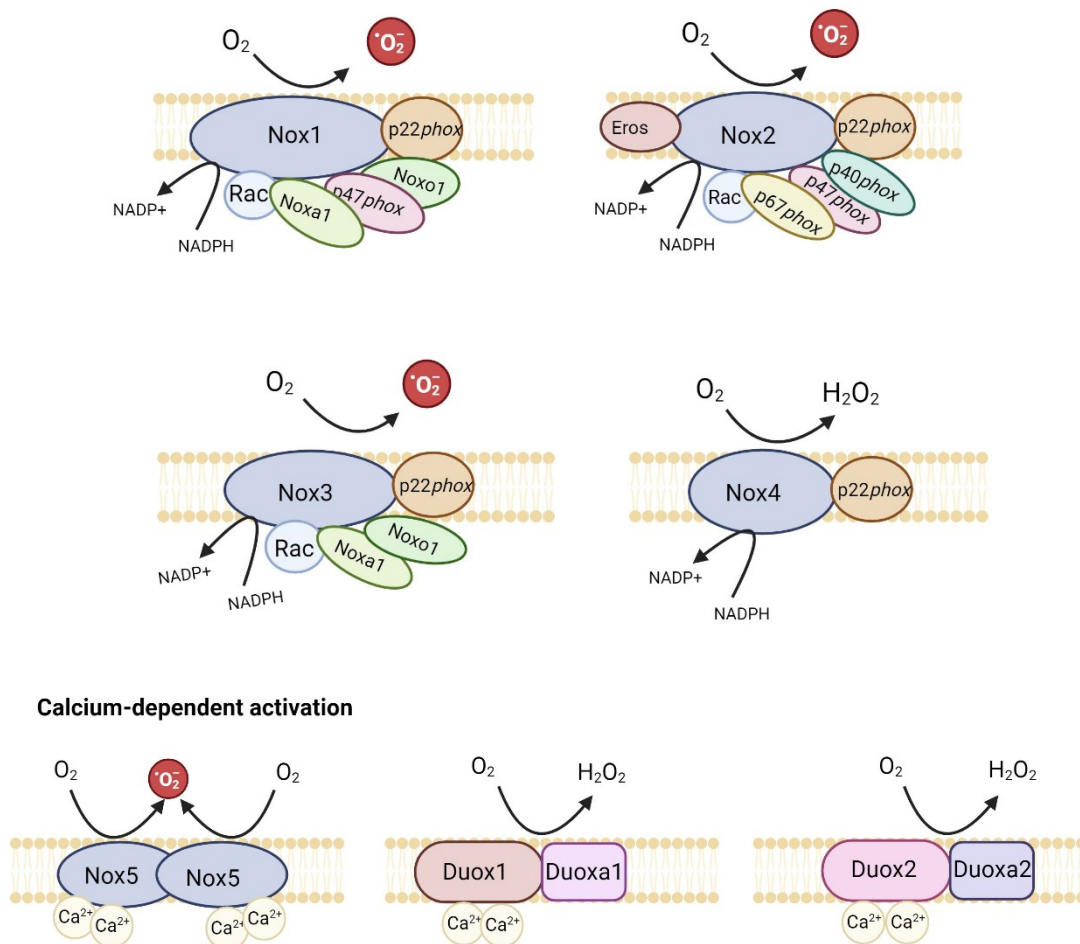


Figure 1.1. Structure of NOX family NADPH oxidase complexes

The NOX family of NADPH oxidases contains seven members – NOX1-5, DUOX1 and DUOX2, each containing transmembrane proteins involved in the generation of reactive oxygen species. NOX1, 2 and 3 are multi-subunit complexes which are dependent on cytosolic proteins for their activation. NOX4 is constitutively active and the NOX5, DUOX1 and DUOX2 complexes exhibit calcium-dependent activation. Most NADPH complexes generate superoxide, but NOX4, DUOX1 and DUOX2 generate hydrogen peroxide.

Both *DUOX1* and *DUOX2* are expressed in the thyroid gland, but *DUOX2* expression is five-fold higher and so is considered to be the dominant oxidase in the thyroid (Ameziane-El-Hassani et al. 2005). The *DUOX1* and *DUOX2* genes are located on Chromosome 15q15.3 in humans, consisting of 35 and 34 exons respectively. The DUOX proteins contain seven transmembrane α -helices. Similar to other NOX family members, the DUOX proteins contain a NOX2 homolog domain. However, they also have an N-terminal extension containing an extracellular peroxidase-like domain, which gives rise to their name as the dual oxidases (Ohye and Sugawara, 2010). These domains are connected by an intracellular loop which contains two EF-hand motifs, resulting in calcium-dependent activation of these oxidases (Ameziane-El-Hassani et al. 2005).

Despite the identification and successful cloning of *DUOX2*, functional DUOX2 protein could not be reconstituted at the cell surface using cellular transfection approaches. This suggested that a necessary component of the NADPH complex was missing. In 2006, a novel gene, dual oxidase maturation factor 2 (*DUOX2*), was identified as the functional maturation factor for DUOX2. Using massively parallel signature sequencing, the authors identified novel transcripts which mapped to the 16 kilo-base pair (kbp) intergenic *DUOX1*/*DUOX2* region (Grasberger and Refetoff, 2006). In the genome, the *DUOX2* gene is oriented head-to-head with *DUOX1*, ensuring co-expression of both genes. *DUOX2* consists of 6 exons encoding 320 amino acids. Similar to DUOX2, *DUOX2* is a transmembrane protein consisting of five transmembrane helices. An extracellular loop connecting the second and third transmembrane helices contains three N-glycosylation sites. A single *DUOX2* paralog, *DUOX1*, was also identified, and was also aligned in a similar orientation with *DUOX1* to promote co-expression. Interestingly, the alignment of the genes in the *DUOX/DUOX1* locus is highly conserved throughout the vertebrate lineage (Grasberger and Refetoff, 2006).

As mentioned previously, functional DUOX2 could not be reconstituted at the cell surface. However, co-transfection of *DUOX2* with *DUOX2* rescued this phenotype, allowing translocation to the cell surface and generation of H_2O_2 . In this system, *DUOX2* was found to remain resident in the endoplasmic reticulum (ER), and so the authors concluded that *DUOX2* was an ER-resident protein essential in facilitating the maturation and ER-exit of

DUOX2 but was not essential for generation of H₂O₂ at the plasma membrane (Grasberger and Refetoff, 2006). A subsequent study published in 2009 demonstrated that DUOX2 and DUOXA2 do in fact form a complex at the plasma membrane using co-expression and immunofluorescence to demonstrate co-localisation (Morand et al. 2009). This demonstrated that DUOXA2 and DUOX2 both undergo ER-to-Golgi transition and translocate to the cell surface. At the plasma membrane, DUOX2 and DUOXA2 together form a catalytic complex which generates H₂O₂. Interestingly, the authors also demonstrated that the maturation factors could cross function and form a complex with either DUOX1 or DUOX2, although H₂O₂ generation was highest when paired with the correct maturation factor, indicating that there is a level of innate specificity. In addition, mismatched pairings such as DUOX2/DUOXA1 appeared to leak superoxide, whereas DUOX2/DUOXA2 resulted in highly efficient and specific H₂O₂ production (Morand et al. 2009).

DUOX1 and DUOX2 undergo ER-to-Golgi transition with their maturation factors and translocate to the plasma membrane where they form a functional complex involved in ROS generation. DUOXA2 is essential to promote the localisation and functional maturation of DUOX2. Unlike other NADPH oxidases including NOX1-4, the activation of both DUOX1 and DUOX2 is regulated in a calcium-dependent manner (Brandes et al. 2014).. Mutation of the glutamate residue to glutamine in the EF-hand motif sequence of DUOX2 abolished the activity of the complex, rendering it unable to generate H₂O₂ (Rigutto et al. 2009). Therefore, the EF-hand motifs are essential in binding calcium which in turn allows the DUOX2/DUOXA2 complex to adopt its active conformation. Although both DUOX1 and DUOX2 exhibit calcium-dependent activation, their activity is also differentially regulated through phosphorylation. DUOX1 is phosphorylated by protein kinase A (PKA) in a cyclic adenosine monophosphate (cAMP)-dependent manner. Addition of phorbol myristate acetate (PMA), an activator of protein kinase C (PKC), to both COS-7 cells and primary thyrocytes resulted in strong activation of DUOX2, even at low concentrations. DUOX1 was also responsive to PMA, but to a much lesser extent. As such, DUOX2 is mainly phosphorylated by PKC and is the predominant NADPH oxidase in the thyroid (Rigutto et al. 2009).

The formation of a stable complex at the cell surface requires disulphide bonds between DUOX2 and DUOXA2 (Carré et al. 2015). Mutation of five key cysteine residues in DUOX2

resulted in either a decrease or complete loss of H₂O₂ production. In this study, the authors also demonstrated that expression of DUOXA2 with mutant DUOX2 resulted in retention of DUOXA2 in the ER, indicating that the structure of DUOX2 is also essential for the translocation of DUOXA2. An intramolecular disulphide bond between cysteine residues 124 and 1162 of DUOX2 in the N terminal region was shown to be essential for the correct folding of DUOX2. Mutation of cysteine 1162 reduced activity of the complex and prevented the trafficking of DUOX2 and DUOXA2 to the cell surface. In addition, mutation of two conserved cysteine residues in DUOXA2, cysteines 167 and 233, reduced the amount of H₂O₂ generated. Both cysteines form part of a disulphide bridge with cysteines 568 and 582 of DUOX2 (Carré et al. 2015). It is evident that both intramolecular and intermolecular disulphide bonds are essential for the development of a functional DUOX2/DUOXA2 complex. Whilst the presence of DUOXA2 as a maturation factor is crucial for the formation of a functional oxidase, DUOX2 also plays a reciprocal role and is necessary for the translocation and stabilisation of DUOXA2 at the plasma membrane.

1.1.1. The role of the DUOX2/DUOXA2 system in thyroid hormone synthesis

Thyroid hormone synthesis occurs at the apical plasma membrane of thyroid follicular cells. DUOX2 and DUOXA2 are highly expressed in the thyroid gland where they act as the primary H₂O₂ generator (De Deken et al. 2000). DUOX2/DUOXA2-derived H₂O₂ is involved in the organification of iodide - a crucial step in thyroid hormone synthesis. Thyroid peroxidase (TPO), a transmembrane protein also found at the plasma membrane apical surface (Godlewska et al. 2014), utilises H₂O₂ to oxidise iodide which is then covalently linked to tyrosine residues of thyroglobulin. TPO then further catalyses the linking of these iodinated tyrosine residues to form the iodothyronines, thyroxine (T₄) and triiodothyronine (T₃). Production of H₂O₂ by the DUOX2/DUOXA2 complex constitutes the rate-limiting step in thyroid hormone synthesis (Grasberger, 2010). Consequently, mutations in either DUOX2 or DUOXA2 cause congenital hypothyroidism in both humans and mice (Grasberger and Refetoff, 2006).

Congenital hypothyroidism is the most common neonatal endocrine disorder which can result in severely impaired neurodevelopment if left untreated. Compulsory newborn screening aids in the detection of congenital hypothyroidism in many countries, including the UK, allowing treatment and prevention of impaired neurodevelopment in children (Peters et al. 2019).

Congenital hypothyroidism can arise from thyroid dysgenesis or defective thyroid hormone synthesis, also known as thyroid dyshormonogenesis. 15-20% of cases of congenital hypothyroidism are due to thyroid dyshormonogenesis (Grasberger, 2010). As the organification of iodide is essential in thyroid hormone synthesis, mutations in this pathway have been identified as potential causes of congenital hypothyroidism. These mutations can result in either a partial iodide defect (PIOD) or a total iodide defect (TIOD), depending on the severity of the mutation. For example, homozygous mutations in the TPO gene can result in TIOD as absence of this enzyme means the organification of iodide cannot occur (de Vijlder, 2003).

Pathogenic mutations in *DUOX2* were first identified in 2002. Heterozygous *DUOX2* mutations result in congenital hypothyroidism, indicating that loss of one allele is enough to result in PIOD in humans (Moreno et al. 2002). Interestingly, heterozygous *DUOX2* mutations only cause transient congenital hypothyroidism. Symptomatic hypothyroidism is often only evident during the neonatal stage in children and resolves itself later in childhood. Similarly, adults identified with heterozygous mutations were found to have normal thyroid hormone levels (Moreno et al. 2002).

A homozygous mutation in *DUOX2* was first reported in 2008 in a child with congenital hypothyroidism (Zamproni et al. 2008). Parents and siblings of the patient were all heterozygous for the mutation and exhibited normal thyroid function and normal serum thyroid hormone levels. Loss of *DUOX2* function does not result in as severe a thyroid defect as loss of *DUOX1*. It has been suggested that *DUOX1* protein may be able to partially compensate for loss of *DUOX2*/*DUOX1* activity, thereby reducing the severity of the defect (Zamproni et al. 2008). A study in 2019 identified that *DUOX2* and *DUOX1* mutations may be more common than previously thought and are often missed in borderline cases of congenital hypothyroidism. Of the 52 patients screened, 26 had *DUOX2* mutations and 6 patients had a *DUOX1* mutation (Peters et al. 2019).

1.1.2. The Role of DUOX2/DUOXA2 in Immunity

The most well-known role of the DUOX2/DUOXA2 system is as a H₂O₂ generator in the thyroid. However, these genes can also be relatively highly expressed at mucosal surfaces, including in the lung and gut epithelia (Geiszt et al. 2003), which suggests it may also function at these sites. Upregulation of these genes has been identified in inflammatory diseases including Crohn's disease and Inflammatory Bowel Disease (IBD). Mutations in *DUOX2* have also been identified in patients with IBD (Hayes et al. 2015). Mutations in *DUOX2* have also been found to be associated with increased interleukin (IL)-17C secretion in the gut which is often associated with increased disease risk (Grasberger et al. 2021).

H₂O₂ generated by the DUOX2/DUOXA2 complex has also been shown to restrict mucosal colonisation by *Helicobacter felis*, highlighting an important role for the complex in mucosal immunity (Grasberger et al. 2013). A further study demonstrated that *Duox2* expression was significantly upregulated in the gastrointestinal tract upon microbial colonisation by segmented filamentous bacterium. It was also associated with preventing translocation of bacteria, demonstrating an active role for DUOX2 in mucosal immunity by maintaining barrier integrity (Grasberger et al. 2015).

DUOX2 is upregulated in IBD and associated with the development of colorectal cancer. Toll-like receptor (TLR) 4 signalling in the gut epithelia was demonstrated to upregulate DUOX2 expression, which resulted in increased ROS production (Burgueño et al. 2021). Using Villin-TLR4 mice which exhibit constitutive TLR4 signalling, the authors found that these mice had an increased risk of developing colorectal cancer compared to wild-type controls using an inflammatory model of colorectal cancer. Interestingly, this effect was microbiota-dependent as it was not recapitulated in germ free mice, and so the microbiota are necessary to promote ROS generation by DUOX2 in a TLR-dependent manner (Burgueño et al. 2021). Although the mechanisms at play require further elucidation, ROS generation by DUOX2/DUOXA2 in the mucosa also appears to play a role in barrier immunity.

More recently, a potential new role for DUOXA2 in immunity has been described. In a large-scale screen for novel regulators of metastasis, *Duoxa2*^{-/-} mice were found to be extremely susceptible to metastatic colonisation following injection with the B16-F10 melanoma cell line. *Duoxa2*^{-/-} mice exhibited the most severe phenotype in the study of 1300 different genes (Van der Weyden et al. 2017; Van der Weyden et al. 2020). A further study described a range of defects in various immune cell subsets, including an increase of neutrophils and eosinophils and a significant decrease in mature KLRG1+ Natural Killer (NK) cells in peripheral blood (Abeler-Dörner et al. 2020). However, DUOXA2 is not highly expressed in immune cells and there is currently no known direct role in the immune system, suggesting a novel immunoregulatory role for DUOXA2.

1.2. NK Cells

NK cells are cytotoxic lymphocytes and are members of the innate lymphoid cell (ILC) family (Spits et al. 2016). NK cells were first described in 1975 and were found to be ‘naturally’ cytotoxic due to their ability to recognise and actively kill cancer cells in athymic mice (Kiessling et al. 1975; Herberman et al. 1975). NK cells are known to play a crucial role in immunity, both in tumour immunosurveillance and in forming the first line of defence against viral infection. The cytotoxic function of NK cells is similar to that of CD8⁺ T lymphocytes. However, unlike T or B lymphocytes, NK cells lack clonotypic receptors such as the T-cell receptor (TCR) and the B-cell receptor (BCR). NK cells do not exert their functions through antigen-specific receptors, and thus are most often characterised as innate immune cells (Abel et al. 2018). Importantly, as NK cells can support or enhance the functions of other lymphocytes, they are a key link between the adaptive and innate immune systems. NK cells were the first innate lymphoid cells to be identified, but recent research has characterised several distinct groups of ILCs. As such, NK cells are members of the ILC family and are most similar to ILC1 cells which also express NK1.1 and can secrete Interferon- γ (IFN- γ). However, ILC1s are tissue resident cells which develop in a variety of tissues and are only weakly cytotoxic.

1.2.1. The Stages of NK Cell Development

NK cells account for 2-5% of lymphocytes in the bone marrow, peripheral blood and spleen of mice (Wang and Zhao, 2021). In humans, they can account for up to 15% of the circulating lymphocyte population with a turnover rate of two weeks (Vivier et al. 2008). The primary site of NK cell development is the bone marrow. NK cells can then egress from the bone marrow and are known to circulate in a wide variety of tissues including peripheral blood, spleen, lung, tonsils, lymph nodes, liver and uterus (Abel et al. 2018).

In the bone marrow in mice, specialised niches maintain haematopoietic stem cells (HSCs) and support the development of the haematopoietic lineage (Schofield, 1978). HSCs are multipotent, self-renewing stem cells from which all blood cells in the body originate. HSCs give rise to the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP), which develop into the lymphoid and myeloid leukocyte lineages respectively. The CLP gives rise to lymphoid progenitors including Pro-B, Pre-T, common innate lymphoid progenitors (CILPs) and NK progenitors (NKPs). As depicted in Figure 1.1, there are two main NKP populations in mice which have been identified. The earliest lineage committed cells are pre-NKPs which were first defined in 2011 as Lineage- CD27⁺ CD244⁺ CD117- CD135- CD122- cells. Acquisition of CD122 expression marks the transition into the refined-NKP (rNKP) stage (Fathman et al. 2011). CD122 expression renders NKPs responsive to IL-15 which is an important regulator of NK cell maturation, and so this represents a crucial step in NK cell development.

Immature NK cells descend from rNKPs in the bone marrow and progress through several developmental stages. Maturation status of NK cells is commonly defined based on CD27 and CD11b expression and involves a four-stage developmental programme. A very small population of CD27⁻ CD11b⁻ NK cells can be detected in many tissues. However, the most common population of immature NK cells detected in tissues are CD27⁺ CD11b⁻. Transition to the next stage of development involves CD11b expression, resulting in double positive CD27⁺ CD11b⁺ NK cells. Terminally mature NK cells are defined as CD11b⁺ CD27⁻ NK cells (Fig. 1.2). Progression through the developmental programme is associated with the acquisition of effector functions including, but not limited to, cytotoxicity and IFN- γ

secretion. Interestingly, CD27⁺ CD11b⁺ NK cells respond better to chemokines, exhibit increased IFN- γ secretion and increased cytotoxicity capacity compared to CD11b⁺ CD27⁻ NK cells, suggesting that they have heightened effector functions compared to terminally mature NK cells (Hayakawa and Smyth, 2006). However, CD11b⁺ CD27⁻ NK cells are the final stage of NK cell development and so important regulatory mechanisms must be acquired during this final step of maturation. Mature cells express more inhibitory Ly49 receptors and a wider range of chemokine receptors and so exhibit increased functional regulation (Chiosonne et al. 2009).

Terminal maturation of NK cells involves the development of functional competency. As mentioned previously, NK cells do not have a large repertoire of antigen specific receptors. Instead, their functionality and activation is regulated through a balance of germline-encoded inhibitory and activating receptors acquired during development. Immature NK cells in mice acquire expression of activating receptors including NK1.1, NKG2C, NKp46 (encoded by *Ncr1*) and NKG2D (Fig. 1.2). Signalling from activating receptors such as NKG2D is mediated through immunoreceptor tyrosine-based activation motifs (ITAMs) in receptor associated adaptor proteins such as DAP10 and DAP12. Fc ϵ RI γ and CD3 ζ also act as adaptor proteins for other activating receptors (Abel et al. 2018).

Terminally mature NK cells express markers such as CD49b and CD51. Acquisition of Ly49 receptors also marks terminal differentiation in NK cells (Fig. 1.2). Ly49 receptors can be activating, such as Ly49D and Ly49H, or they can be inhibitory such as Ly49A or Ly49G (Rahim et al. 2014). The balance of inhibitory and activating signals is essential in regulating NK cell activity. Interaction with a target cell and engagement of activating receptors must generate an activation signal which is large enough to overcome inhibitory signalling in order to shift the balance and initiate the NK cell response.

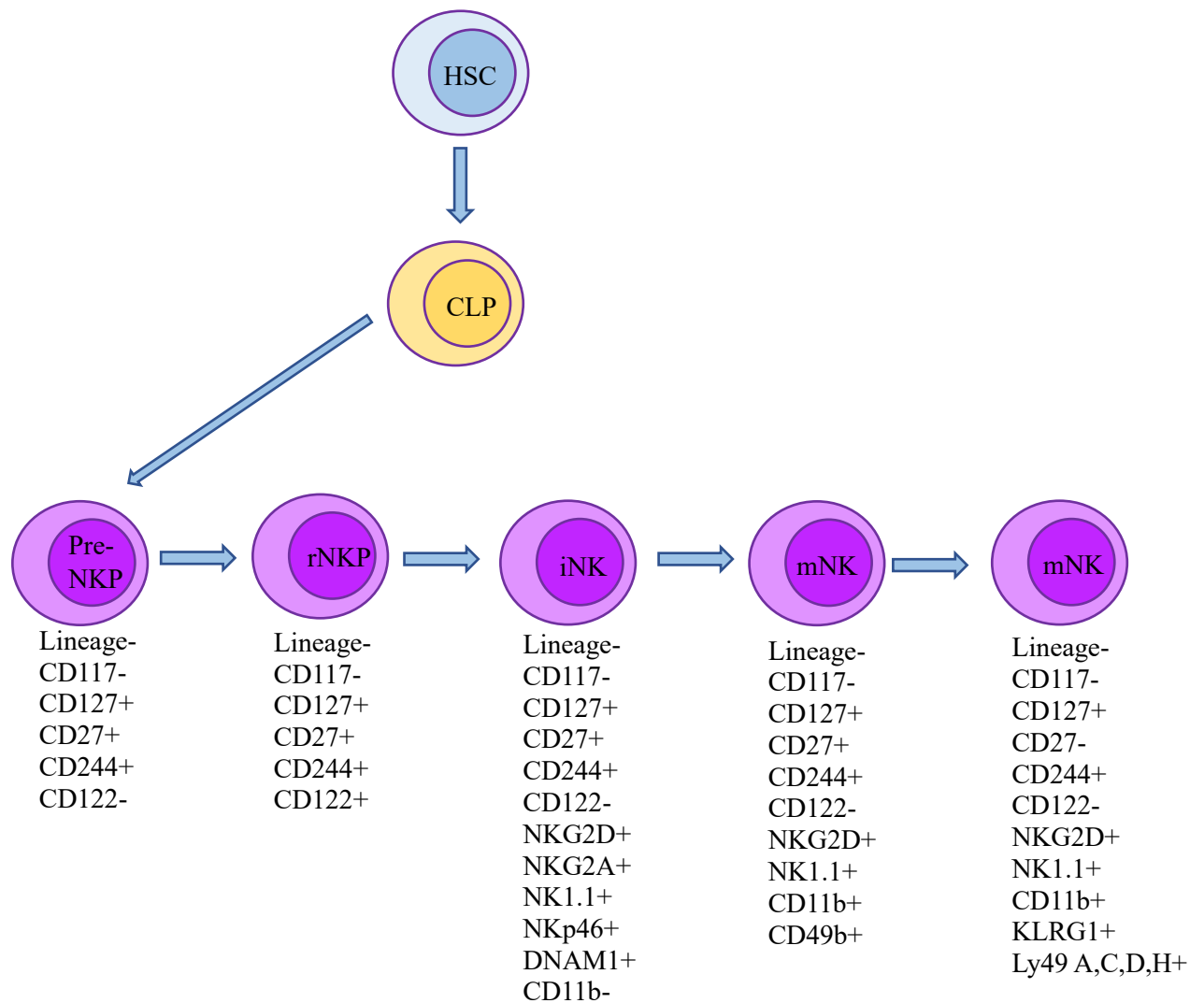


Figure 1.2. The key stages of NK cell development in mice

NK cell development primarily occurs in the bone marrow. The haematopoietic stem cell (HSC) gives rise to the common lymphoid progenitor (CLP) which gives rise to all lymphoid lineages, including NK cells. The NK cell population progresses through several stages of development to produce mature functionally competent NK cells. The developmental stages involve the progressive acquisition and loss of various surface receptors. The first lineage committed cells are the pre-NK progenitors (pre-NKPs) which are defined as Lineage- CD117- CD27+ CD244+ CD127+ CD135- CD122- cells. Acquisition of CD122 expression gives rise to the refined-NKP (rNKP). Expression of CD122 renders the NKP sensitive to IL-15, an essential step in the generation and maturation of NK cells. The rNKP generates immature NK cells which acquire the expression of NKG2D and are CD27+ CD11b-. Immature NK cells also begin to express NK1.1 as they continue to develop. Progression through development is associated with acquisition of CD11b+ expression, generating double positive CD27+ CD11b+ NK cells. At the final stages of NK cell maturation, NK cells acquire expression of the Ly49 family of receptors and KLRG1 and downregulate CD27 expression giving rise to terminally mature CD27- CD11b+ NK cells.

Human NK cells also progress through a multi-step developmental programme, although many of the lineage markers which define human NK cells differ to those in mouse NK cell development. Similar to mouse NK cells, human NK cells also descend from the CLP in the bone marrow. Immature and mature stages can be defined based on expression of CD16 and CD56 (Fig. 1.3). CD16⁺ CD56^{dim} are considered to be the most mature subset as they have increased cytotoxic functions, whilst CD16⁻ CD56^{bright} are more immature (Abel et al. 2018). In humans, the killer immunoglobulin-like receptor (KIR) family bind to major histocompatibility complex class I (MHC-I) to regulate NK cell function and are expressed on NK cells in the later stages of development (Jacobs et al. 2001). It should be noted that this thesis will mainly focus on the pathways driving NK cell development in mice.

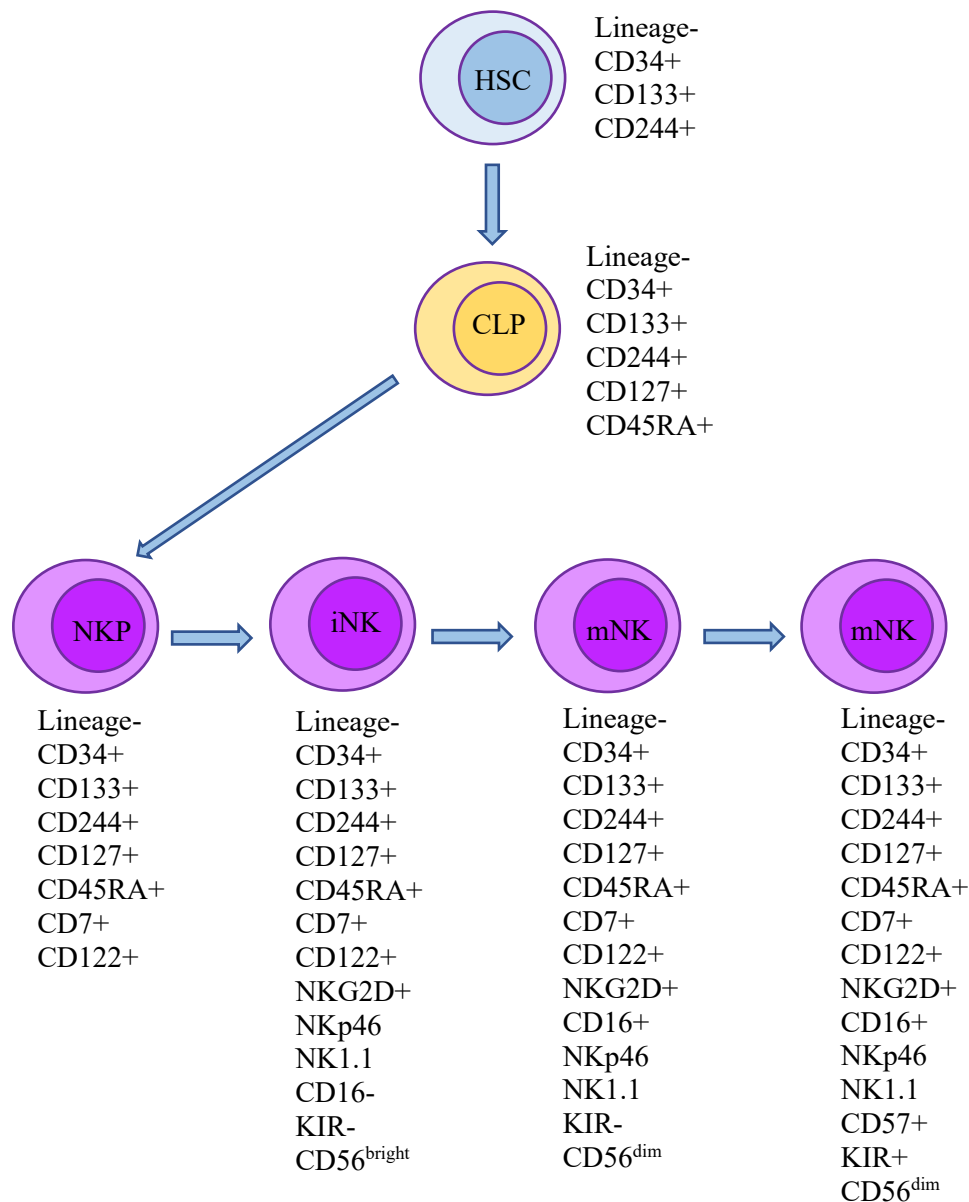


Figure 1.3. The key stages of NK cell development in humans

Human NK cell development primarily occurs in the bone marrow but can also occur in secondary lymphoid organs such as lymph nodes. The haematopoietic stem cell (HSC) gives rise to the common lymphoid progenitor (CLP) which gives rise to all lymphoid lineages, including NK cells. The developmental stages involve the progressive acquisition and loss of various surface receptors. The first lineage committed cells are the NK progenitors (NKPs) which are defined as Lineage- CD34+ CD133+ CD244+ CD122+ cells. Acquisition of CD122 at this stage renders the NKP sensitive to IL-15, constituting an essential step in the generation and maturation of NK cells. Immature NK cells are generated from NKPs which acquire the expression of NKG2D, NK1.1 and NKp46. Immature NK cells are CD16- CD56^{bright} cells. As the developmental programme progresses, CD56 expression decreases, with immature NK cells developing into mature CD16+ CD56^{dim} cells. The final stage of development also involves the acquisition of killer immunoglobulin-like receptors (KIR) which can bind MHC-I molecules and enable functional maturation.

1.2.2. NK Cell Education

In order to exert their effector functions in a precise and targeted manner, NK cells must exhibit self-tolerance and possess an ability to accurately identify and target transformed cells or invading pathogens. Self-tolerance is developed through a process of NK cell education based on MHC-I expression. NK cell receptors, including the Ly49 receptors, can detect MHC-I on healthy cells. The first theory describing NK cell education was termed the ‘missing self’ hypothesis. This model suggests that NK cells are self-tolerant as they can interact with MHC-I which is expressed on healthy host cells, resulting in inhibitory receptor signalling. Conversely, the absence of self MHC-I molecules on a target allows NK cells to selectively kill invading cells or transformed cells which have downregulated MHC-I expression as inhibitory receptors are not engaged (Kärre et al. 1986). Tumours developed in all C57BL/6 mice injected with the RBL-5 lymphoma cell line which expresses MHC-I molecules, as NK cells were not activated due to the presence of MHC-I molecules. In contrast, injection of the RMA subline which does not express MHC-I molecules did not result in tumour growth in mice as NK cells could recognise the absence of MHC-I as foreign (Kärre et al. 1986). It is important to note that activating receptors such as NKG2D can also detect molecules which are upregulated during cellular stress. These stress ligands include Rae1 (Cerwenka et al. 2000), H60 (Diefenbach et al. 2001), and Mult1 (Carayannopoulos et al. 2002) in mice.

In 2005, a new model of NK cell education termed ‘licensing’ was proposed. NK cell functional maturation requires the interaction of NK cell inhibitory receptors with self-MHC-I molecules in order to obtain effector capabilities and become functionally ‘licensed.’ Signalling from the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the tail of inhibitory receptors is essential in this process. Consequently, the small proportion of NK cells which do not express self-MHC-I inhibitory receptors are self-tolerant and remain hyporesponsive as they are not functionally licensed (Kim et al. 2005).

Another model was proposed in 2006, termed the ‘arming’ and ‘disarming’ model. It suggests that NK cells are ‘armed’ through self-MHC and NK cell inhibitory receptor interactions which renders them functionally mature. The ‘disarming’ model suggests that NK cells which

do not express inhibitory receptors are rendered hyporesponsive due to chronic stimulation by activating receptors expressed on NK cells (Raulet and Vance, 2006).

NK cells expressing a wider range of inhibitory receptors were found to have increased effector responses, despite greater inhibitory signals (Höglund and Brodin, 2010). Therefore, it has been suggested that the level of responsiveness can be tuned or adjusted based on these signals to regulate activation. NK cells from MHC-I deficient hosts became responsive and could exert effector functions including IFN- γ secretion when transferred into MHC-I sufficient hosts when they expressed host-specific MHC-I inhibitory receptors (Joncker et al. 2010). In addition, another study demonstrated that mature NK cells from MHC-I sufficient hosts became hyporesponsive when transferred into MHC-I deficient hosts (Elliot et al. 2010). This demonstrates that NK cell education and licensing is not restricted to development in the bone marrow, as previously thought, and modulation of NK responsiveness can occur in different environments (Held et al. 2011).

The exact model and mechanism of NK cell education remains a controversial issue. However, it is clear that MHC-I dependent education of NK cells plays an important role in regulating NK cell effector functions.

1.2.3. The Essential Role of Cytokines in NK Cell Development

1.2.3.1. Common Gamma Chain Cytokines

From the earliest stages of development, NK cells express cytokine receptors as they are important regulators of NK cell development and function. In particular, signalling through the common gamma (γ_c) chain receptor has been shown to play a crucial role in NK cell development and maturation. Mice lacking the γ_c chain show a severe reduction in NK cell numbers in peripheral tissues coupled with significantly reduced cytotoxic function (Cao et al. 1995). Similarly, mutations in the γ_c chain in humans cause X-linked severe combined immunodeficiency (SCID) (Noguchi et al. 1993). In addition to defects in T and B cells, these

patients also exhibit severe developmental defects in their NK cell populations with an absence of mature NK cells and associated functional defects (Rochman et al. 2009).

The γ_c chain acts as a signalling subunit for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, with each receptor complex containing a unique alpha chain to confer cytokine specificity. However, mice which are deficient in IL-2, IL-4, IL-7 or IL-21 possess normal mature NK cell populations with intact effector functions which suggests that they are not essential in NK cell development and maturation (Vosshenrich et al. 2005). In contrast, IL-15 is essential for normal NK cell development (Fig. 1.6). *IL2R β ^{-/-}*, *IL-15^{-/-}* or *IL-15R α ^{-/-}* deficient mice possess a severe defect in their NK cell populations with an absence of functionally mature NK cells (Kennedy et al. 2000), with a similar phenotype observed in humans (Gilmour et al. 2001). Interestingly, γ_c deficient mice have equivalent numbers of NKPs to wild-type controls in the bone marrow (Vosshenrich et al. 2005). This suggests that γ_c cytokines are not required for lineage commitment, but some γ_c cytokines such as IL-15 do play an essential role in NK cell maturation in the later stages of NK cell development. Indeed, acquisition of IL-2R β (CD122) marks the transition from pre-NKP to rNKP and renders the rNKP sensitive to IL-15 (Fathman et al. 2011). As the pre-NKP is not sensitive to IL-15, this suggests that IL-15 does not play a role in NK lineage commitment.

Both IL-2 and IL-15 signal through heterotrimeric complexes consisting of the γ_c subunit (CD132), a shared IL-2R β subunit and unique alpha subunits for each cytokine. In contrast to other cytokines, IL-15 mediates its effects via a trans-presentation mechanism, as shown in Figure 1.4. Expression of IL-15 and IL-15R α by the same cell results in presentation of soluble IL-15 to NK cells expressing CD122/CD132 complexes (Dubois et al. 2002). IL-15 can be trans-presented by haematopoietic cells such as dendritic cells (DCs) or macrophages, but also by stromal cells in the bone marrow microenvironment. In mice deficient in IL-15R α , adoptively transferred wild-type NK cells do not survive in the periphery whereas adoptive transfer of IL-15R α -deficient NK cells into wild type mice results in normal NK cell populations. However, in IL-15R α deficient mice, IL-15R α sufficient NK cells do not survive. These experiments demonstrate that IL-15R α expression by cell types other than NK cells is critical for the maintenance and survival of peripheral NK cells (Koka et al. 2003).

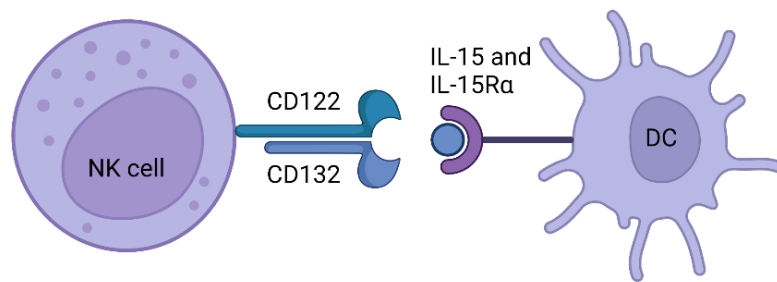


Figure 1.4. Trans-presentation of IL-15 to NK cells

IL-15 is essential for NK cell maturation, but unlike other cytokines it is trans-presented to NK cells by other cells in the microenvironment. IL-15 and IL-15R α are both expressed by the same cell type which then presents IL-15 to NK cells expressing CD122/CD132 complexes. IL-15 can be trans-presented by immune cells including dendritic cells or by stromal cells in the bone marrow microenvironment. Image created using BioRender.com.

1.2.3.2. The important role of JAK/STAT signalling in NK cell development

Signal transduction in response to binding of γ_c cytokines to their cognate receptors is mediated by the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. The JAK/STAT pathway is highly conserved and plays an important role in proliferation, survival and maturation of many different immune cells. Consequently, the JAK/STAT pathway is crucial in the modulation of immune responses. In the mammalian genome there are four members of the JAK family (JAK1-3 and tyrosine kinase 2 (TYK2)) and seven members of the STAT family (STAT1-4, STAT5A, STAT5B and STAT6). Binding of IL-2 and IL-15 results in activation of JAK1 and JAK3, which results in the activation of STAT1 and STAT5, respectively (Gotthardt et al. 2019).

Indicative of its essential role in development, complete loss of JAK1 leads to perinatal lethality in mice. Conditional deletion of JAK1 in immature and mature NK cells using *Jak1^{fl/fl}Ncr1^{Cre}* mice resulted in a significant reduction of mature NK cells in the periphery. In the spleen of wild-type mice, 4.36% of lymphocytes were CD3- NKp46+ compared to only 0.36% in *Jak1^{fl/fl}Ncr1^{Cre}* mice. Loss of a single JAK1 allele also resulted in a reduction in mature NK cells, but to a lesser extent, with NK cells constituting 2.15% of total lymphocytes in the spleen. NK cells were also reduced in number in the bone marrow and exhibited impaired maturation, with a skew towards immature NK cells evident (Witalisz-Siepracka et al. 2018). These results demonstrate that JAK1 plays an essential role in NK cell maturation.

Mutation of JAK3 in humans results in SCID, with patients lacking NK and T cells (Gotthardt et al. 2019). Similarly, *Jak3^{-/-}* mice also exhibit severe defects in T, B and NK cell populations. JAK3-deficient mice exhibit a reduction of NK cells in the periphery and a 50% reduction in both the proportion and number of rNKPs, indicating a block at the pre-NKP stage (Robinette et al. 2018). Similar to NK populations, ILC development is also significantly impaired in these mice, therefore JAK3 is also essential for maturation and survival of both of these lineages.

STAT5 is crucial for NK cell development, maturation and survival in the periphery. STAT5A and STAT5B are highly homologous with 96% sequence similarity, and *Stat5a/b* deficiency also leads to perinatal lethality (Cui et al. 2004). Restriction of *Stat5a/b* deletion to NKp46+ cells also resulted in a severe reduction of NK cells in the periphery, with the spleen containing only 1% CD3- NKp46+ cells amongst total lymphocytes compared to 5% in wild-type mice. In the bone marrow mature NK cells numbers were also reduced (Eckelhart et al. 2011). However, there was a significant increase in NKP numbers, suggesting a developmental block at this stage. As shown in Figure 1.6, this suggests that STAT5 is essential for driving both development and survival of NK cells in mice. Of note, STAT5 is also important in the generation of memory CD8+ T cells (Burchill et al. 2003). Loss of function mutations in STAT5 in humans also result in a significant reduction in NK cell numbers (Scaglia et al. 2012).

It is clear that signalling through the JAK/STAT pathway plays an essential role in driving NK cells. Without intact JAK/STAT signalling, signals from γ_c receptors cannot be transduced and so NK cell development, maturation and survival are severely impacted.

1.2.3.3. The Role of TGF β

As shown in Figure 1.5, there are two main TGF β signalling pathways – the canonical and non-canonical pathways. The canonical pathway involves phosphorylation of SMAD2/SMAD3, which in turn recruits SMAD4 to form a trimer. This complex can then translocate to the nucleus to regulate gene expression. In the non-canonical pathway, the PI3K, Ras and TAK1 signalling pathways, amongst others. Transforming growth factor β (TGF β) has been suggested to be involved in the regulation of NK cell development (Fig. 1.6). TGF β was found to restrict NK cell development as the number of mature NK cells was significantly reduced *in vitro* when TGF β was added to cultured cells. In addition, transgenic mice expressing the dominant negative form of TGF β receptor type II in CD11c⁺ cells had significantly increased numbers of mature NK cells in both the bone marrow and periphery compared to wild-type controls (Marcoe et al. 2012). However, another study which restricted deletion of *Tgfb2* to immature and mature NK cells using *Ncr1^{Cre}Tgfb2^{fl/fl}* mice did not find an increase in peripheral mature NK cells (Viel et al. 2016). The authors demonstrated that mammalian target of rapamycin (mTOR) is a target of TGF β in NK cells. Inhibition of mTOR or constitutive TGF β signalling were found to arrest NK cell proliferation and development in the bone marrow. In addition, deletion of TGF β in NK cells resulted in a better ability to control tumour engraftment as 50% fewer metastases were observed in the B16-F10 melanoma model compared to control mice, suggesting that TGF β signalling also inhibits the effector functions and cytotoxic potential of NK cells (Viel et al. 2016).

More recently, SMAD4 deletion was found to affect the development of the NK cell lineage. SMAD4 plays an important role in signal transduction from TGF β receptors (Shi and Massague, 2003). TGF β imprinting commits cells to the ILC1 lineage. SMAD4 was deleted in immature and mature NK cells and ILC1s using *Smad4^{fl/fl}Ncr1^{iCre}* mice. However, in SMAD4 deficient mice, ILC1 populations were unaffected and NK cells developed an ILC1-like gene signature, such as increased CD49a expression, with reduced expression of mature NK cells markers including KLRG1. Effector functions including granzyme B and IFN- γ secretion were reduced. Granzyme B mRNA expression was reduced and granzyme B expression was also reduced from 74% in wild-type splenic NK cells to 50.2% in *Smad4^{fl/fl}Ncr1^{iCre}* NK cells when measured by flow cytometry. These cells were also unable to control metastases in the B16-F10 melanoma model as *Smad4^{fl/fl}Ncr1^{iCre}* mice had a six-fold increase in the number of lung metastases compared to wild-type controls (Cortez et al. 2017). SMAD4 was found to be a negative regulator of non-canonical TGF β R1 signalling in NK cells, as shown in Figure 1.5. Whilst knockout of SMAD4 disrupts the canonical TGF β signalling pathway, it also prevents the inhibition of the non-canonical pathway, thereby allowing unrestricted TGF β signalling and promoting ILC1 development. Together, these results suggest a role for TGF β in inhibiting NK cell function in the tumour microenvironment and therefore reducing NK cell anti-tumour activity.

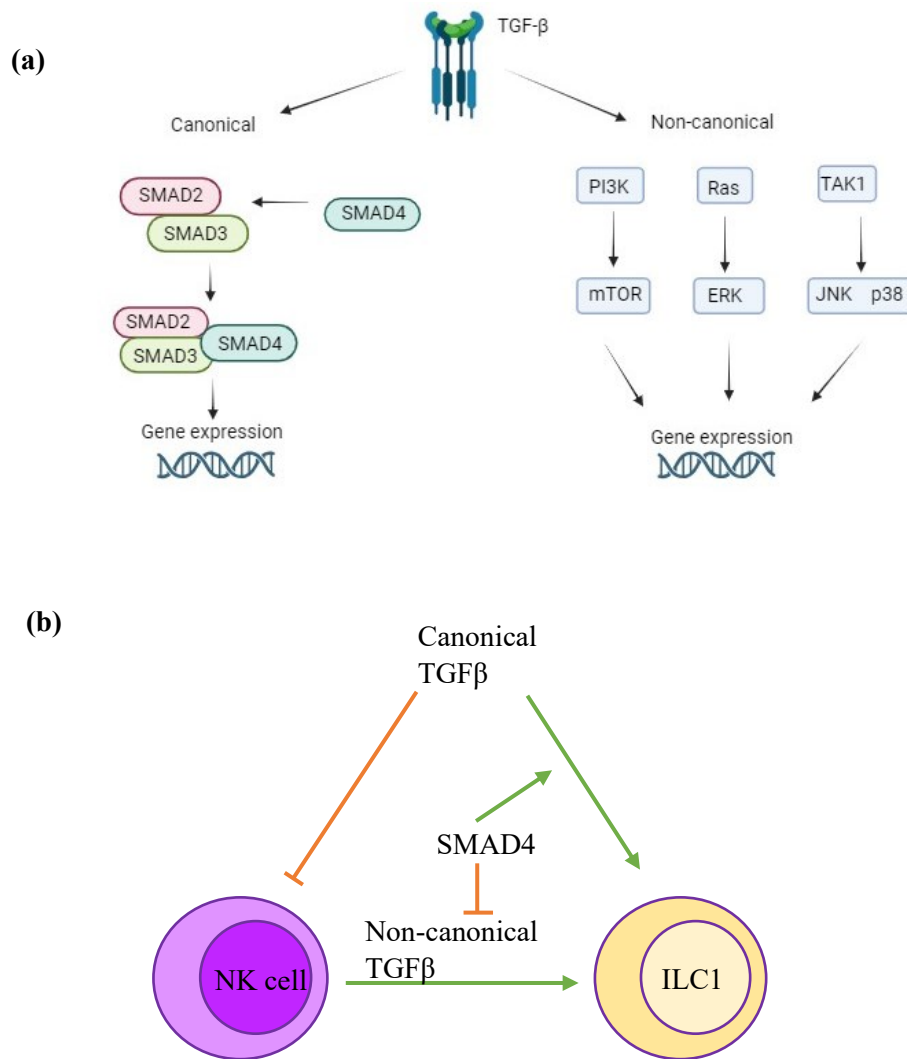


Figure 1.5. TGF β signalling pathway

(a) The TGF- β receptor binds its ligand, TGF β , and can activate both the canonical and non-canonical TGF- β signalling pathways. In the canonical pathway, TGFBR1 recruits and phosphorylates SMAD2 and SMAD3. SMAD4 is then recruited to form a heterotrimer which can translocate to the nucleus and alter gene expression. The non-canonical pathway can activate a variety of signalling pathways upon ligand binding, including PI3K, Ras and TAK1. (b) TGF- β imprinting drives the development of the ILC1 lineage whilst simultaneously negatively regulating NK cell development. SMAD4 is involved in signal transduction in the canonical TGF- β signalling pathway to promote ILC1 development. However, it also acts as a negative regulator of the non-canonical pathway. In the absence of SMAD4, the non-canonical TGF- β pathway is not inhibited and NK cells develop a more ILC1-like phenotype.

1.2.4. Chemokines in the Bone Marrow Microenvironment

Chemokines are a family of chemoattractant molecules which control immune cell migration. Chemokines play a fundamental role in the immune response by attracting immune cells to the site of inflammation or infection. They also play an important role in NK cell development, regulating retention of progenitors and immature NK cells in the bone marrow and promoting trafficking of NK cells from the bone marrow into blood circulation to establish peripheral populations.

1.2.4.1. The Role of CXCL12

CXC-motif chemokine ligand (CXCL) 12 has been demonstrated to play a fundamental role in NK retention in the bone marrow. Administration of AMD-3100 to adult mice, an antagonist of CXC-motif chemokine receptor type 4 (CXCR4), the receptor for CXCL12, resulted in a significant decrease in the number of NK cells in the bone marrow, whilst the number of NK cells in peripheral sites such as the spleen was significantly increased. In addition, CXCR4 expression was highest at the progenitor and immature NK cell stage, with expression decreasing as NK cells mature (Bernardini et al. 2008). This demonstrates that CXCL12/CXCR4 signalling regulates retention of NK cells in the bone marrow.

Cxcr4^{+/-1013} mice contain a mutant form of the receptor resulting in impaired downregulation of CXCR4, leading to constitutive CXCL12/CXCR4 signalling. *Cxcr4*^{+/-1013} mice were found to have an accumulation of NK cells in the bone marrow with a concomitant reduction of NK cells in the bone marrow sinusoids. Evidently, NK cell egress from the bone marrow requires downregulation from the cell surface and internalisation of CXCR4 to reduce CXCL12/CXCR4 signalling in order to permit NK cell egress from the bone marrow (Mayol et al. 2011).

Another study induced conditional deletion of CXCR4 in adult mice using *Mx^{Cre}CXCR4^{f/null}* mice. In this model the Cre recombinase was expressed after the induction of type I IFN by polyinosinic:polycytidylic acid (poly(I:C)), thereby allowing temporal deletion of CXCR4 in

adult mice. The authors observed a reduced number of CLPs, NKPs and both immature and mature NK cells in the bone marrow. Mature NK populations were also reduced in the blood, spleen and lung compared to wild-type mice (Noda et al. 2011). Generation of NK cells from lympho-myeloid primed progenitors (LMPPs) was also increased when cultured with CXCL12. Together these results suggest that CXCL12 plays an important role in NK cell development and promotes the development of NK cells from progenitors, as shown in Figure 1.6. Interestingly, the authors also demonstrated that CXCL12-abundant reticular (CAR) cells are potent producers of IL-15, as IL-15 messenger RNA (mRNA) levels are higher in CAR cells than in DCs or other stromal cells. Using single-cell RT-PCR, the authors demonstrated that all CAR cells analysed expressed IL-15 mRNA and 26% of CAR cells were also found to express IL-15R α . In addition, using immunohistochemistry it was demonstrated that most NKp46+ cells in the bone marrow are in contact with the processes of CAR cells (Noda et al. 2011). As IL-15 and CXCL12 are important in NK cell development, it is likely that CAR cells form a specialised niche for NK cell development within the bone marrow microenvironment.

1.2.4.2. S1P5

Sphingosine 1 phosphate receptor 5 (S1P5) is also involved in the regulation of NK cell trafficking during homeostasis by promoting bone marrow egress. S1P5 is expressed on both human and mouse NK cells and expression levels increase during maturation. In S1P5-deficient mice, NK cell populations were reduced in peripheral tissues such as a 50% reduction of NK cells in the spleen and 70% reduction in the lung but there was a two-fold increase in the bone marrow compared to wild-type controls. In particular, a reduction in mature CD27- NK cells was observed in the periphery (Walzer et al. 2007). Another study demonstrated that S1P5 engagement is required to promote egress of mature NK cells in particular, as mature NK cell numbers were severely reduced in both the bone marrow and lymph node sinusoids of *S1P5*^{-/-} mice (Mayol et al. 2011). However, it is important to note that although both S1P5 and CXCL12 regulate bone marrow egress, S1P5 engagement and CXCR4 internalisation are not dependent on each other. Migration towards CXCL12 was comparable between wild-type and S1P5-deficient NK cells, indicating that S1P5 is not necessary for responsiveness to CXCL12. (Mayol et al. 2011).

1.2.4.3. CX₃CR1

Knockout of CX₃C motif chemokine receptor 1 (CX₃CR1) in mice also led to the accumulation of mature KLRG1⁺ NK cells in the bone marrow, due to impaired NK cell egress from the bone marrow (Ponzetta et al. 2013). It is likely that there are multiple non-redundant compensatory mechanisms regulating NK cell migration, as many chemokines including CXCL12, S1P5 and CX₃CR1 (Fig. 1.6) have been shown to play a role in this process.

1.2.5. Transcriptional Regulation of NK Cell Development

1.2.5.1. The Role of *T-bet* and *Eomes*

The stages of NK cell development are also tightly regulated by transcription factors. T-box transcription factors play a crucial role in immune cells. This was first identified in T cells as T-box protein expressed in T cells (T-BET, encoded by *Tbx21*) expression promotes Th1 lineage commitment and IFN- γ secretion in CD4⁺ cells (Szabo et al. 2000). T-BET and a related T-box transcription factor Eomesodermin (*Eomes*) also play a pivotal role in the generation of memory populations of CD4⁺ and CD8⁺ lymphocytes as well as regulating effector functions of both B and T lymphocytes (Kallies and Jacobson, 2017). T-BET and EOMES are also highly expressed in both human and mouse NK cells (Simonetta et al. 2016). Expression of T-BET and EOMES during different stages of NK cell development is key to promoting the generation of mature, functionally competent NK cells with intact effector functions.

The role of T-BET in NK cell development was first identified based on observations made in T-BET-deficient mice. *Tbx21*^{-/-} mice exhibited a reduction in the number of NK cells in peripheral organs such as the blood, spleen and liver. For example, the proportion of TCR β -NK1.1⁺ cells amongst total lymphocytes was 8.1% in the peripheral blood of wild-type mice, compared to 2.7% in *Tbx21*^{-/-} mice. However, there was an accumulation of NK cells in the bone marrow with NK cells constituting 1.9% of total lymphocytes compared to 1.2% in wild-type controls. NK cells in *Tbx21*^{-/-} mice were also found to be predominantly immature,

with decreased CD49b and CD11b expression (Townsend et al. 2004). These results clearly demonstrate that T-BET expression in NK cells is essential for terminal maturation. The authors suggested that loss of T-BET expression results in a developmental block at the immature NK cell stage, and a possible defect in egress from the bone marrow, thereby resulting in reduced peripheral NK cell populations. T-BET also regulates the expression of S1P5 which is necessary to facilitate bone marrow egress (Simonetta et al. 2016).

More recently, the defective migration of *Tbx21*^{-/-} NK cells was directly demonstrated using sinusoidal labelling of CD45⁺ cells. In *Tbx21*^{-/-} mice, frequencies of NK cells, particularly the mature subset, were not increased in the sinusoids compared to the bone marrow parenchyma (Cuff et al. 2020). This suggests that *Tbx21*^{-/-} NK cells have a reduced ability to migrate into the circulation and therefore T-BET plays a role in regulation of bone marrow egress.

Deduction of the role of *Eomes* in NK cell development initially proved difficult. *Eomes*^{-/-} mice die embryonically, so EOMES was first investigated in a compound *Tbx21*^{-/-} *Eomes*^{+/-} mouse model. Similar to *Tbx21*^{-/-} mice, *Tbx21*^{-/-} *Eomes*^{+/-} mice have a significant reduction in NK cells. However, the defect was more severe in *Tbx21*^{-/-} *Eomes*^{+/-} indicating that EOMES also plays a non-redundant but complementary role in NK cell development (Intlekofer et al. 2005). EOMES was also found to be necessary for the maintenance of CD122 expression, which is essential to render NK cells responsive to IL-15 (Wang and Zhao, 2021).

Restriction of *Eomes* deletion to the haematopoietic lineage in *Eomes*^{fl/fl}- *Vav*^{Cre+} mice resulted in a reduction in NK cells in spleen and blood, but only slight reductions in other organs. In addition, in this model a greater proportion of NK cells were immature and expressed a more limited repertoire of Ly49 receptors which suggests that EOMES is essential for terminal NK maturation. However, T-BET and EOMES were both necessary to maintain maturity in mature NK cells. Deletion of both transcription factors also resulted in a complete absence of NK cells, further demonstrating the essential role of these transcription factors in NK cell development (Gordon et al. 2012).

Interestingly, expression of T-BET and EOMES is inversely correlated in NK cells. *Tbx21*^{-/-} NK cells express very high levels of EOMES, whereas overexpression of T-BET in NK cells results in very low levels of EOMES expression. This suggests that they can regulate each other's expression in NK cells (Daussy et al. 2014). More recently, it was demonstrated that immature CD27⁺CD11b⁻ NK cells expressed high levels of EOMES and low levels of T-BET, whereas mature CD27⁻CD11b⁺ NK cells downregulate EOMES and upregulate expression of T-BET (Fig. 1.6) (Zhang et al. 2021). T-BET expression levels were found to be much lower in NK cells in the bone marrow compared to those in the periphery, but T-BET still plays an important role in NK cell development in the bone marrow (Daussy et al. 2014).

Loss of EOMES in mice results in a decrease in the number of NK cells, and in particular a reduction in the number of immature NK cells, whereas loss of T-BET is associated with loss of mature NK cells. This further supports that EOMES is essential to induce the earliest stages of NK cell development, whilst repression of EOMES by T-BET is necessary to proceed to terminal maturation (Zhang et al. 2021). Indeed, 42% of genes regulated during NK cell maturation were found to be regulated by either T-BET or EOMES. Taken together, these results demonstrate that the delicate balance of T-BET and EOMES expression plays a fundamental role in controlling NK cell development, in concert with cytokines and chemokines in the microenvironment.

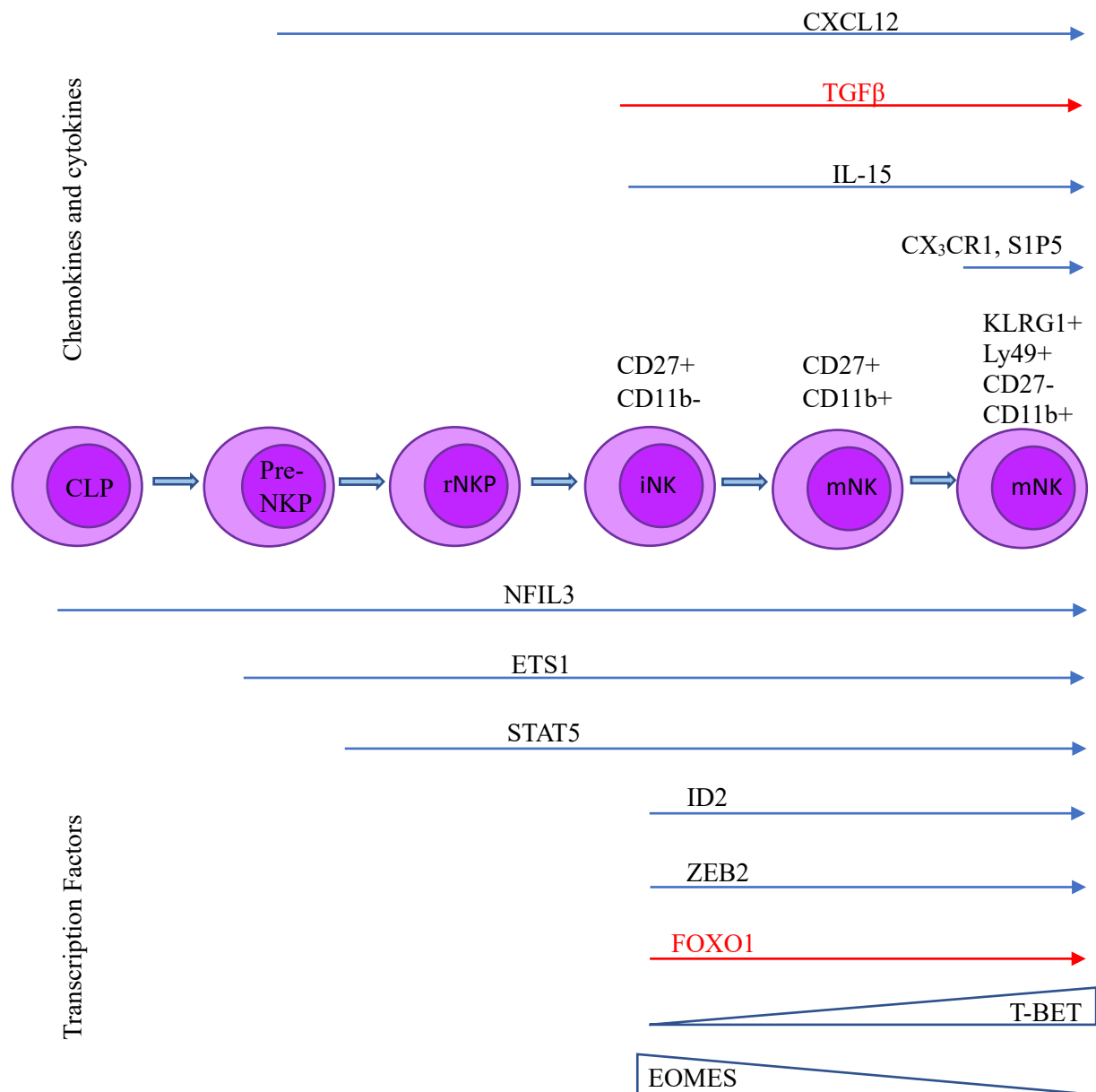


Figure 1.6. Regulation of NK cell development in mice

The stages of NK cell development and egress are tightly regulated by a variety of transcription factors, chemokines and cytokines. Expression of certain transcription factors at specific developmental stages is essential to promote NK lineage commitment, to drive the generation of NK cells from NK progenitors (NKPs) and to promote functional maturation of NK cells. NFIL3 expression in the common lymphoid progenitor (CLP) is essential to promote the generation of lineage committed NKPs. ETS1 and STAT5 expression at the NKP stage is necessary to promote the generation of immature NK (iNK) cells. Various different transcription factors including ID2, ZEB2, T-BET and EOMES are necessary to promote maturation of iNKs into terminally mature NK (mNK) cells. FOXO1 and TGFβ act as negative regulators (red) of this process. IL-15 is also crucial in NK cell development and is required for the generation of mNKs. CXCL12 promotes the generation of iNKs from NKPs, but also plays an important role in NK cell egress from the bone marrow alongside CX₃CR1 and S1P5.

1.2.5.2. *Nfil3*

Whilst T-BET and EOMES are essential to drive NK cell development and maturation, there are several other transcription factors that are important in NK cell development. An important NK lineage commitment transcription factor is *E4bp4*, also known as nuclear factor interleukin 3 regulated protein (*Nfil3*). NK cells are significantly reduced in *Nfil3*^{-/-} mice, again with a significant loss of both immature and mature NK cells (Gascoyne et al. 2009; Kamizono et al. 2009). *Nfil3*^{-/-} mice also exhibit a reduction in the number of both pre-NKPs and rNKPs, with a 7.8-fold reduction in the number of pre-NKPs and a 10.6-fold reduction in the number of rNKPs in the bone marrow (Male et al. 2014). This demonstrates that NFIL3 expression is required to generate lineage-committed NK progenitors and therefore acts upstream of EOMES. Indeed, *Nfil3* is also expressed in CLPs and NK cells cannot arise from *Nfil3*^{-/-} CLPs. Transduction and expression of NFIL3 in *Nfil3*^{-/-} CLPs was sufficient to enable NK cell development in these cells and so NFIL3 expression is crucial to promote the transition from CLP to NKP (Fig. 1.6), delineating the earliest stage of NK lineage commitment (Male et al. 2014). As a master regulator of NK lineage commitment and development, NFIL3 is also involved in the direct regulation of other important transcription factors including EOMES and inhibitor of DNA binding (ID2) (Male et al. 2014).

1.2.5.3. *Ets1*

Ets proto-oncogenic 1 (*Ets1*) also acts at the early stages of NK cell development (Fig. 1.6) to promote the expression of T-BET and ID2. Similar to many of the knockout models discussed above, *Ets1*^{-/-} mice exhibit a significantly underdeveloped NK cell population, with a loss of immature and mature NK cells. rNKPs in particular were also significantly reduced in these mice, with a 50% reduction compared to wild-type cells, again highlighting its importance in early NK cell development at the NKP stage (Ramirez et al. 2012). Loss of ETS1 was also associated with decreased expression of activating receptors such as NKp46 and members of the Ly49 which resulted in decreased cytotoxic capacity in *Ets1*^{-/-} NK cells (Ramirez et al. 2012).

1.2.5.4. *Id2*

Unlike ETS1, ID2 appears to be important later in NK cell development. In a global *Id2*^{-/-} model, NK cell populations are also significantly reduced, with the spleen containing only 10% of the number of NK cells found in control mice (Yokota et al. 1999). Conditional deletion of *Id2* in haematopoietic cells and in NKp46⁺ cells also produced the same phenotype (Delconte et al. 2016). However, progenitor development is unaffected in *Id2*^{-/-} mice and so ID2 is essential for NK maturation but not for lineage commitment in mice (Boos et al. 2007).

1.2.5.5. *Zeb2*

Zinc finger E-box binding homeobox 2 (*Zeb2*) is expressed during the later stages of NK cell development and its expression is induced by T-BET. Knockout of ZEB2 in mice resulted in an accumulation of NK cells in the bone marrow, with a significant reduction in peripheral NK populations. In particular, mature NK cells were significantly lost compared to wild-type controls suggesting it is necessary for the generation and maintenance of mature NK cells (Fig. 1.6). For example, in the spleen of wild-type mice, 60% of NK cells were mature CD27-CD11b⁺ NK cells, whereas in *Zeb2*^{-/-} mice only 4% of splenic NK cells were mature NK cells. In addition, *Zeb2* deletion resulted in decreased expression of S1P5, suggesting that ZEB2 helps promote NK cell egress from the bone marrow (van Helden et al. 2015).

1.2.5.6. *Foxo1*

Forkhead box protein O1 (*Foxo1*) also plays a role in NK cell development. However, unlike the transcription factors discussed so far, it is a negative regulator of NK cell development. Deletion of *Foxo1* in mice resulted in an increase in the proportion of mature NK cells in both the bone marrow and periphery, demonstrating that FOXO1 restrains NK cell maturation. In the bone marrow of FOXO1-deficient mice, mature NK cells accounted for 18.4% of the NK cell population, compared to 13.6% in wild-type mice. T-BET and FOXO1 were also found to have an inverse correlation in their expression patterns, with T-BET

expression higher in CD27- CD11b⁺ cells and FOXO1 expression higher in CD27⁺ CD11b⁻ cells. The differential expression of these genes therefore controls the progression of NK cell development (Deng et al. 2015).

Table 1.1. Summary of factors involved in the regulation of NK cell development

Cytokines
IL-15
TGFβ
Chemokines
CXCL12
S1P5
CX ₃ CR1
Transcription factors
T-BET
EOMES
NFIL3
STAT5
ID2
ZEB2
ETS1
FOXO1

1.2.6. Conventional NK cells and ILC1s in mice

When NK cells were first identified in 1975, they were described as innate cytotoxic lymphoid cells and were thought to exclusively develop in the bone marrow before entering the circulation. However, recent research has led to the identification of a range of ILCs, a tissue resident group of cells, some of which have similarities to NK cells.

The existence of a distinct subset of cells was first observed in the liver. NK cells express EOMES which is required for the early stages of NK cell development. However, in the liver a significant population of NK1.1⁺ EOMES⁻ cells which expressed tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and CD49a but lacked CD49b were detected (Takeda et al. 2005). The majority of these cells were also CD11b⁻, indicating they were immature. Using a green fluorescent protein (GFP) reporter mouse approach, it was identified that EOMES⁻ cells could not give rise to EOMES⁺ cells, indicating that these were not developmental precursors, but rather distinct cell types (Daussy et al. 2014). EOMES⁻ cells arise in the foetal liver and persist in the adult liver (Takeda et al. 2005; Gordon et al. 2012) whereas EOMES⁺ NK cells are only detected 2-3 weeks after birth (Chiossone et al. 2009). As NK cells were initially thought to develop solely in the bone marrow, these results suggested that EOMES⁻ and EOMES⁺ cells represented distinct development pathways.

NK cells enter the circulation from the bone marrow and then circulate throughout peripheral tissues. ILC1s, however, are tissue resident cells which develop and remain resident in the same tissue, such as the liver (Peng et al. 2013). As discussed above, tissue resident ILCs can usually be identified by a lack of EOMES expression and also a CD49a⁺ CD49b⁻ phenotype. However, these markers of tissue-residency have been found to vary between different tissues, complicating the description of these cells. Tissue-resident ILCs in the skin are EOMES⁻ and their development is dependent on T-BET in a similar fashion to liver-resident ILCs (Sojka et al. 2014). However, T-BET and EOMES dependence in ILC1s is reversed in other tissues such as the uterus. Uterine ILC1s express high levels of EOMES and follow a T-BET-independent developmental programme (Montaldo et al. 2016).

Although ILC1s and NK cells can both produce IFN- γ , they differ in terms of their cytotoxic ability. ILCs are considered to be weakly cytotoxic whereas NK cells are potent cytotoxic cells (Guia and Narni-Mancinelli, 2020).

As research into innate lymphoid cells has progressed, characterisation of these cells and the associated nomenclature has evolved. When first identified, they were often described as tissue-resident NK cells. In 2013, three groups of ILCs were proposed according to the cytokines secreted and the transcription factors required for each subset, based on the same principle used for helper T cells. Group 1 ILCs were T-BET-dependent cells which secreted IFN- γ and so was comprised of NK cells and ILC1s. Group 2 ILCs were GATA binding protein 3 (GATA3)-dependent and secreted IL-5 and IL-13. Group 3 ILCs were retinoic acid-related orphan receptor gamma (ROR γ t)-dependent cells, secreted IL-17 and IL-22 and consisted of ILC3s and lymphoid tissue inducer cells (Spits et al. 2013). In 2014, a common innate lymphoid progenitor (CILP) was identified which differentiated from the CLP which gives rise to all innate lymphoid cell subsets (Constantinides et al. 2014). Also in 2014, another novel progenitor was identified which could give rise to all helper-like ILCs, but not NK cells (Klose et al. 2014). This finding provided evidence that ILC1s and NK cells were indeed distinct subsets arising from distinct developmental pathways. In 2018, a new nomenclature was introduced to encapsulate these new findings. This proposed that ILCs could be classified into five distinct subsets – NK cells, ILC1, ILC2, ILC3 and lymphoid tissue inducer cells due to their similar, yet distinct, developmental pathways (Vivier et al. 2018).

Although NK cells and ILC1s are distinct cell types, recent research has demonstrated that NK cells and ILCs can exhibit phenotypic plasticity. TGF β was found to induce the conversion of NK cells into intermediate ILC1s (CD49a⁺ CD49b⁺ cells) and ILC1s in the tumour microenvironment of mice subcutaneously injected with the MCA1956 tumour cell line (Gao et al. 2017). In *R^{CA-FL}* mice which have constitutive TGF β signalling in NKp46⁺ cells, intermediate ILC1s and ILC1s were the predominant cell types found in tumours, with a reduction in the proportion of NK cells to 10% of CD3⁺ NK1.1⁺ NKp46⁺ cells, compared to 50% in wild-type controls. In addition, tumour growth was significantly increased in these mice which suggests that their failure to control cancer growth is due to reduced tumour

immunosurveillance, restricted by TGF β signalling, resulting in fewer NK cells and reduced NK cell anti-tumour activity in the tumour microenvironment.

The characterisation of ILCs remains controversial. Coupled with the difficulty in defining lineage-specific markers which often vary between tissues, great diversity exists within ILCs and so the developmental relationship between each subset remains poorly understood. Further research will help to elucidate the progenitors and mechanisms which govern each subset and will enhance our understanding of the relationship between NK cells and ILC1s.

1.2.7. Functions of NK cells

The activation of the cytotoxic effector functions of NK cells involves the sequential activation of several steps. Upon recognition of a target cell, an immunological synapse forms between the NK cell and the target. Reorganisation of the actin cytoskeleton coupled with polarisation of the microtubule organising centre (MTOC) then allows lytic granules to move along microtubules towards the immunological synapse (Paul and Lal, 2017). The lytic granules then fuse with the target cell membrane, facilitating degranulation and release of cytotoxic enzymes including perforin and Granzyme B into the target cell to initiate lysis and destruction of the cell (Paul and Lal, 2017). This is the primary cytotoxic mechanism by which NK cells can destroy malignant cells.

Another mechanism by which NK cells can initiate target cell death is the initiation of apoptosis through the activation of death receptors on target cells. NK cells express death receptor ligands including Fas ligand (FasL) and TRAIL. These death receptor ligands bind to their cognate receptors on target cells, triggering apoptosis of the target cell (Smyth et al. 2005). Induction of apoptosis by death receptors is particularly important in the destruction of tumour cells (Screpanti et al. 2001). By inhibiting death-receptor mediated apoptosis in the MHC-I deficient lymphoma cell line RMA-S, the authors demonstrated that NK cells failed

to kill these apoptosis-resistant tumour lines. FasL expression was also found to be upregulated in tumours *in vivo* in C57BL/6 mice injected with the RMA-S tumour line. In addition, expression of FasL and TRAIL receptors can be upregulated in response to IFN- γ secretion by NK cells, thereby enhancing the ability of NK cells to target these cells (Screpanti et al. 2001).

In addition to their direct cytotoxic mechanisms, NK cells also act as inflammatory mediators through the secretion of various cytokines and chemokines (Abel et al. 2018). NK cells can secrete IFN- γ , tumour necrosis factor- α (TNF- α) and granulocyte monocyte colony stimulating factor (GM-CSF). They also secrete chemokines such as CC-motif chemokine ligand (CCL) 3, CCL4, CCL5 and CXCL8 (Paul and Lal, 2017) which can attract other immune cells, including T cells and myeloid cells, to amplify the immune response (Walzer et al. 2005; Roda et al. 2006). Their role as inflammatory mediators provides a key link between the innate and adaptive immune responses.

Whilst NK cells require an appropriate balance of activating and inhibitory receptors to initiate activation upon target recognition, they also require additional signals to prime them to promote subsequent activation (Abel et al. 2018). Production of type I IFNs by DCs is essential to promote the priming of NK cells (Paolini et al. 2015). In addition, DCs can secrete cytokines including IL-12, IL-15 and IL-18 which are essential to enhance NK effector functions. In particular, production of IL-15 and its subsequent trans-presentation by DCs is essential for NK cell priming and activation of effector functions. Type I IFN results in the upregulation of IL-15R α and priming of NK cells does not occur in *IL-15*^{-/-} mice, further highlighting the important role of DC-produced IL-15 (Lucas et al. 2007). Together, IL-12, IL-15 and IL-18 act in a synergistic fashion to amplify NK cell activation and effector function (Nielsen et al. 2016). IL-18 can prime NK cells, resulting in significantly increased production of IFN- γ when stimulated with IL-12 (Chaix et al. 2008; Lusty et al. 2017). Consequently, crosstalk between DCs and NK cells plays an important role in regulation and amplification of the immune response in inflammation, infection and anti-tumour immunity

1.3. The Thyroid

1.3.1. Thyroid Hormone Production

Thyroid hormones are essential for normal metabolism, growth and development. Lack of thyroid hormone during development results in severe skeletal and neurodevelopmental defects. Thyroid hormone production and secretion is tightly regulated by the hypothalamic-pituitary-thyroid (HPT) axis. Homeostatic thyroid hormone levels are maintained through a negative feedback loop linked to circulating thyroid hormone levels (Mendoza and Hollenberg, 2017). In response to low levels of circulating T_3 and T_4 , thyrotropin-releasing hormone (TRH) is secreted by TRH neurons in the paraventricular nucleus (PVN) of the hypothalamus. In turn, TRH stimulates the anterior pituitary gland to synthesise and secrete thyrotropin (TSH). TSH secretion then promotes increased production and release of T_3 and T_4 from thyroid follicles in the thyroid gland (Ortiga-Carvalho et al. 2016).

Thyroid hormone synthesis occurs in thyrocytes in the thyroid follicles of the thyroid gland (Fig. 1.6). In the lumen, thyroglobulin acts as a precursor for thyroid hormone synthesis and tyrosine residues of thyroglobulin are linked to iodine. This process involves the organification of iodide using H_2O_2 generated by the DUOX2/DUOXA2 complex, as described earlier. In order to ensure a sufficient supply of iodide, iodide is transported into thyrocytes by the sodium/iodide symporter. Iodide can then be further transported into the lumen through the pendrin transporter. The TPO enzyme catalyses the organification of iodide, generating monoiodotyrosine (MIT) and diiodotyrosine (DIT). TPO then catalyses a coupling reaction, where the iodotyrosines are combined to generate T_3 and T_4 (Szanto et al. 2019). Both T_3 and T_4 are transported across the plasma membrane by the thyroid hormone transporters, monocarboxylate transporter 8 (MCT8) and monocarboxylate transporter 10 (MCT10), which release the hormones into the circulation (Fig. 1.7).

T_4 is an inactive prohormone which is converted into its biologically active form T_3 by type 2 deiodinase (D2). T_3 is the active signalling molecule which can bind to thyroid hormone receptors. T_3 can be converted to reverse T_3 by type 3 deiodinase (D3), rendering it inactive. T_3 can also be converted to T_2 by D2 to prohibit thyroid hormone signalling (Bianco, 2011).

The ability of the deiodinases to generate and inactivate T_3 allows control of thyroid hormone production and consequently permits tight control of hormone levels in circulation. When signals are received via the HPT axis, T_3 and T_4 can be synthesised and then released into the circulation. Importantly, T_3 can act as a signalling molecule or be rendered inactive in any cell expressing thyroid receptors or deiodinase enzymes.

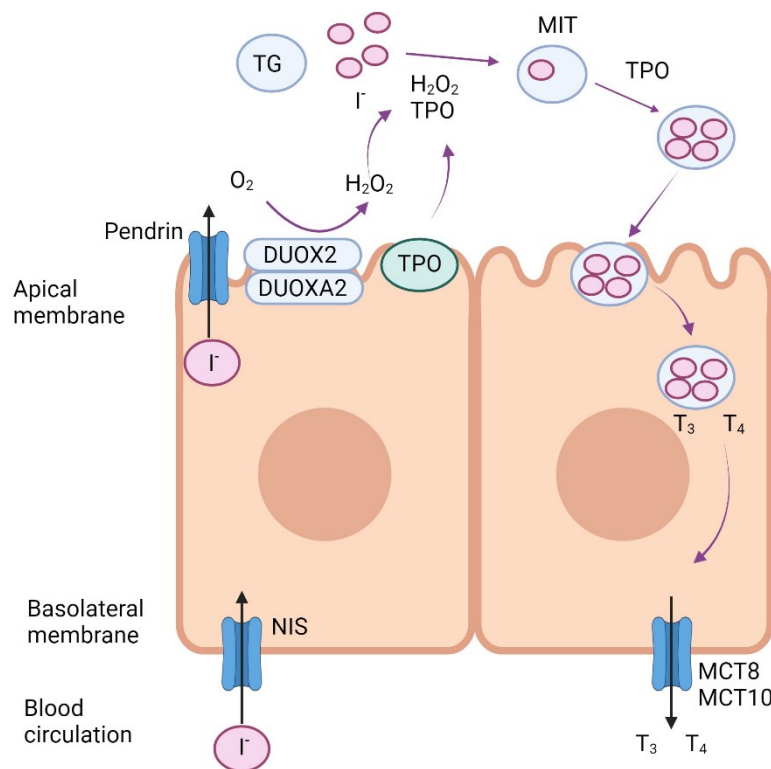


Figure 1.7. Thyroid hormone production in the thyroid gland

Thyroid hormone production occurs at the apical membrane of thyrocytes in the thyroid gland. Iodide is transported into thyrocytes via the sodium/iodide symporter (NIS) and then further transported into the lumen through the pendrin transporter. During thyroid hormone synthesis, thyroglobulin acts as a precursor as tyrosine residues of thyroglobulin are linked to iodine. H_2O_2 generated by the DUOX2/DUOX2A2 system at the cell surface is an essential step in this process as it is used for the organification of iodide. This reaction is catalysed by the TPO enzyme, generating monoiodotyrosines (MIT) and diiodotyrosines (DIT). TPO then further catalyses the coupling reaction, linking the iodotyrosines together to generate T_3 and T_4 . The thyroid hormones can then be released into the blood circulation through the MCT8 and MCT10 transporters when stimulated to do so by the secretion of TSH through the HPT axis.

1.3.2. Thyroid hormone receptors and regulation of gene expression

Thyroid hormone action is mediated through signalling by the thyroid receptors, thyroid receptor α (TR α) and thyroid receptor β (TR β). TR α and TR β are encoded by the *THRA* and *THRB* genes respectively in humans, and the *Thra* and *Thrb* genes in mice. The thyroid receptors are members of the nuclear receptor superfamily which includes receptors such as the estrogen receptor and the retinoid X receptor (RXR) (Oritga-Carvalho et al. 2014). Thyroid receptors are important regulators of gene expression. They are ligand-dependent DNA-binding transcription factors, the ligand for which is T₃ (Wu and Koenig, 2000). Regulation of gene transcription is achieved by binding to thyroid responsive elements (TREs), located in the promoters of genes regulated by thyroid hormone (Oritga-Carvalho et al. 2014).

Thyroid receptors are formed from a single polypeptide chain which consists of several conserved functional domains (Ribeiro et al. 1995; Cheng et al. 2010). The N terminal A/B domain is variable amongst the various thyroid receptors and contains an activation function 1 (AF-1) region. Thyroid receptors also contain a DNA-binding domain (DBD) which is essential for TRE binding to regulate gene expression, and a C-terminal ligand-binding domain (LBD). The LBD contains an activation function 2 (AF-2) region which can bind its cognate ligand T₃, however, the LBD also has the capacity to promote homodimerisation or heterodimerisation with other nuclear receptors. Thyroid receptors can form heterodimers, often with RXRs (Brent, 2012), which has been suggested to enhance DNA binding and increase the number of T₃ target genes (Brent et al. 1992; Zhang and Kahl, 1993).

Several different isoforms are produced by the thyroid receptor genes through alternative splicing mechanisms (Anyetei-Anum et al. 2018). *Thra* produces three isoforms – TR α 1 (Sap et al. 1986), TR α 2 and TR α 3. TR α 2 and TR α 3 are truncated versions of TR α 1 which do not contain the AF-2 region of the LBD and therefore they cannot bind T₃ (Mitsuhashi et al. 1988). As a result, TR α 1 is the main active isoform of TR α . *Thrb* also produces several isoforms – TR β 1 (Weinberger et al. 1986), TR β 2 (Hodin et al. 1989), TR β 3 (Williams, 2000) and the more recently described TR β 4 (Tagami et al. 2010). Expression of thyroid receptor isoforms is tissue-dependent and they also exhibit temporal regulation during development

(Cheng et al. 2010). $Tr\alpha 1$ is highly expressed in the brain, bone and heart. $TR\beta 1$ is predominantly expressed in the kidney and liver whereas $TR\beta 2$ is expressed in the cochlea, retina and the pituitary (Bradley et al. 1992).

Regulation of expression is exerted through the binding of thyroid receptors to TREs, as shown in Figure 1.8, which can activate or repress transcription of thyroid hormone-responsive genes. In the unliganded state, when T_3 is not bound, thyroid hormone receptors are bound to the TRE of a target gene, resulting in the recruitment and association of co-repressors such as nuclear receptor co-repressor 1 (N-CoR1) and nuclear receptor co-repressor 2 (N-CoR2) (Astapova et al. 2008). The presence of co-repressors also activates histone deacetylase 3 (HDAC3), which catalyses deacetylation of histones, thereby promoting chromatin remodelling to a closed conformation (Fig. 1.8a). Consequently, transcription of the target gene is repressed in the closed chromatin state (You et al. 2013).

In the case of a positive TRE, T_3 binding to the LBD of a thyroid receptor results in a conformational change, releasing co-repressors and allowing recruitment and binding of co-activator proteins including nuclear receptor co-activator 1 (NCoA-1) (Fig. 1.8b). Histone acetyltransferase (HAT) is also recruited, resulting in acetylation of histones to promote an open chromatin confirmation, thereby initiating transcription of the target gene (McKenna et al. 1999). Binding of T_3 results in a conformational change in the LBD domain. This conformational change is essential to allow regulation of transcription by T_3 . The AF-2 region of the LBD contains 12 alpha helices and upon ligand binding, helix 12 changes position, which promotes dissociation of co-repressors and binding of co-activators (Souza et al. 2011).

It has been demonstrated that thyroid hormone signalling can both activate and repress transcription of genes. However, the underlying mechanisms regulating target gene repression remain unclear. Thyroid receptors are also involved in negative regulation of the HPT axis, and it has been demonstrated that thyroid receptor binding to a negative TRE is essential in this process (Shibusawa et al. 2003). It has been suggested that an inverse mechanism of that involved in gene activation may mediate gene repression. Therefore, for negatively regulated

genes, T₃ binding would induce repression of gene transcription. However, this has not been directly demonstrated and the necessary co-repressors and activators remain unclear (Ortiga-Carvalho et al. 2014). Indeed, although negative TREs have been identified in some genes, they remain elusive in many negatively regulated genes, suggesting that an indirect mechanism may be involved in negative regulation (Mendoza and Hollenberg, 2017).

Using ChIP-seq analysis, another study demonstrated that increased TRβ1 binding in gene promoters was associated with increased gene transcription, whereas decreased TRβ1 binding in the promotor region was associated with negative regulation or repression of a target gene (Ramadoss et al. 2014). This evidence suggests that negative regulation may in fact be achieved through reduced recruitment of thyroid receptors. Therefore, a combination of indirect and direct mechanisms may be involved for different target genes.

A further study demonstrated that deletion of NCoA-1 was sufficient to induce resistance to thyroid hormone in mice and prevented activation of T₃ target genes. Deletion of the co-repressor N-CoR1 resulted in normal thyroid hormone levels and normal gene transcription. Surprisingly, deletion of N-CoR1 in NCoA-1 deficient mice did not result in any hormone or transcription defects. A different co-activator, NCoA-2, is recruited and can compensate in the absence of NCoA-1 (Vella et al. 2014). Together these results suggest that a balance of co-activator and co-repressor recruitment is essential to regulate the expression of thyroid-responsive genes. In addition, different co-regulators may be required in different tissues, thereby allowing tissue specific T₃ responses.

Thyroid hormones can also translate signals in a rapid manner via non-genomic mechanisms. For example, thyroid hormones can bind to two domains on the integrin αvβ3 which is present on the plasma membrane of many cell types, including immune cells. Integrin αvβ3 can bind both T₃ and T₄, which subsequently results in PKC activation which in turn can activate the mitogen-activated protein kinase (MAPK) pathway (De Vito et al. 2011). Engagement of αvβ3 can regulate processes including angiogenesis and cell proliferation, which can become dysregulated in cancer cells (Davis et al. 2016). However, the role of

integrin $\alpha\beta3$ in thyroid signalling and the regulation of gene expression still remains unclear and requires further investigation.

Whilst the mechanisms involved in thyroid receptor-mediated regulation of gene expression have not yet been fully elucidated, there is clear evidence that thyroid hormone is essential for the positive and negative regulation of many genes.

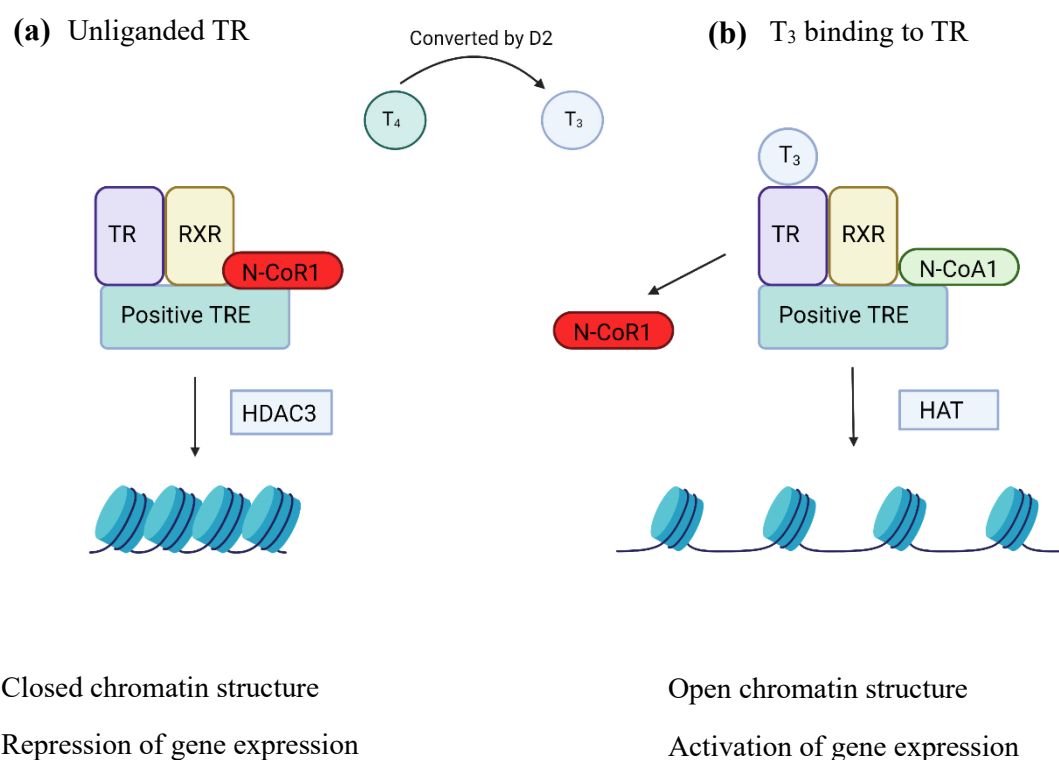


Figure 1.8. Thyroid receptor-mediated regulation of gene expression

Thyroid hormone receptors (TRs) are nuclear receptors which can regulate the expression of a wide variety of genes in a T_3 -dependent manner. They bind to thyroid response elements (TREs) in the promoter region of thyroid responsive genes. In the absence of T_3 , a TR homodimer or a TR-RXR heterodimer are bound to a TRE. The co-repressor N-CoR1 is recruited which activates histone deacetylase 3 (HDAC3) resulting in the deacetylation of histones promoting a closed chromatin structure, thereby repressing gene expression. When T_3 binds to a TR, this results in a conformational change which releases N-CoR1 and promotes recruitment of the co-activator N-CoA1. Histone acetyltransferase (HAT) is also recruited and the acetylation of histones promotes an open chromatin structure, thereby resulting in the activation of gene expression at positive TRE sites.

1.3.3. Thyroid Hormone and the Immune System

Defects or dysregulation of thyroid function have been associated with altered functions of a wide variety of immune cells, including neutrophils, macrophages, B cells, T cells, DCs and NK cells. While crosstalk between thyroid hormones and the immune system has been documented, the relationship remains poorly understood, particularly in terms of the mechanisms at play.

The crosstalk between the endocrine and immune systems is bidirectional. Thyroid hormone levels are influenced by pro-inflammatory mediators, including the cytokines IL-6 and TNF- α , which are secreted by immune cells. Stimulation with IL-6 and TNF- α has been shown to result in decreased T₃, T₄ and TSH serum concentrations and reduced sensitivity to TSH (Pang et al. 1989). Immune cells can also express the thyroid receptors and transporters necessary for thyroid hormone metabolism, which suggests that thyroid hormones can play a role in these cells (Montesinos and Pellizas, 2019). Indeed, T₃ has been detected in various immune cell subsets in humans and mice including monocytes, T lymphocytes and NK cells (De Vito et al. 2011).

1.3.3.1. Thyroid Hormone in Neutrophils and Macrophages

In neutrophils, thyroid hormones have been shown to influence ROS production. Hyperthyroidism is associated with increased ROS generation by neutrophils in both rats (Zamoner et al. 2007) and humans (Sarkar et al. 2006), whereas hypothyroidism is associated with decreased ROS production (Sarkar et al. 2006). Similar results have been demonstrated in macrophages, with ROS production increased under hyperthyroid conditions (Videla et al. 1993). However, a different study observed the opposite effect. Induced hyperthyroidism in rats was associated with decreased ROS production and reduced phagocytic activity. Under hypothyroid conditions, the opposite phenotype was observed with increased ROS production

and enhanced phagocytic activity (Rosa et al. 1995). The study by Rosa et al. used a combination of T₃ and T₄ injection daily for 7 days to render mice hyperthyroid although the route of administration is unclear, whilst daily intraperitoneal injection of T₃ only for 7 days was used in the study by Zamoner and colleagues. The dose of T₃ was also higher so it is possible that the mice used in the study by Rosa and colleagues were more severely hyperthyroid due to higher circulating thyroid hormone levels. However, as these studies find opposite effects of hyperthyroidism, the role of thyroid hormones in ROS production remains unclear.

Stimulation with thyroid hormones *in vitro* has also been demonstrated to result in preferential M1 polarisation in macrophages, suggesting that thyroid hormones drive a pro-inflammatory phenotype (Perrotta et al. 2014; Kwakkel et al. 2014). Stimulation with T₃ during M1 polarisation increased the number of CD14⁺ F4/80⁺ cells and increased CXCL16 and TNF α mRNA expression in these cells by 1.5-fold and 2-fold respectively (Perrotta et al. 2014). Knockout of TR α in mice also results in defective macrophage function, suggesting that thyroid receptors may play a role in mediating the phagocytic activity of macrophages (Billon et al. 2014). However, genomic and non-genomic mechanisms likely act in combination to regulate macrophage function as thyroid hormone binding to α v β 3 on macrophages activates the phosphoinositide 3-kinase (PI3K) and extracellular signal-related kinase 1/2 (ERK1/2) pathways, which in turn increase phagocytotic activity in macrophages (Chen et al. 2012). However, it is important to note that contradictory evidence has been reported in the literature as has been discussed and so the effects of thyroid hormone on the immune system are not yet fully defined or understood.

1.3.3.2. Thyroid Hormones and Dendritic Cells

Thyroid hormones have also been shown to play a role in the maturation of DCs. Thyroid hormones were found to stimulate the differentiation and maturation of DCs from peripheral monocytes (Mooij et al. 1994). Thyroid receptors, particularly TR β 1, are expressed in bone-marrow derived DCs in mice (Mascanfroni et al. 2008). Stimulation of DCs *in vitro* with T₃ resulted in upregulation of the activation markers CD40, CD80 and CD86 (Mascanfroni et al.

2008). DCs also showed enhanced co-stimulatory capacity for T cells, with IFN- γ secretion increased by six-fold in CD4 T cells when cultured with T₃-stimulated DCs, suggesting that T₃ can promote functional maturation of DCs and enhance the adaptive immune response. A further study demonstrated that increased NF- κ B translocation in response to T₃ drove the functional maturation of DCs (Mascanfroni et al. 2010). In addition, it was also demonstrated that DC maturation was regulated by T₃ in a TR β -dependent manner (Mascanfroni et al. 2010). Whilst enhancing DC function, addition of T₃ *in vitro* also enhanced DC viability as apoptosis was reduced from 13% Annexin V⁺ cells in wild-type DCs to 7% Annexin V⁺ cells amongst T₃-stimulated cells. Migration of DCs to the lymph nodes was also increased by four-fold in T₃-stimulated DCs due to an upregulation of CC-chemokine receptor type 7 (CCR7), an important homing signal in DC migration (Alamino et al. 2015). Taken together, these results demonstrate that T₃ can promote the functional maturation of DCs, which in turn leads to enhanced co-stimulation of T cells and increased antigen-presentation.

1.3.3.3. Thyroid Hormone and NK Cells

Thyroid hormones have also been demonstrated to have an impact on NK cell activity. It is important to note that many of the early studies investigating the role of thyroid hormones on NK cells were extremely limited due to the incomplete knowledge of NK cell development and relevant surface markers. Both technical advances in flow cytometry and our increased understanding of NK cell development would now allow a more detailed investigation, but research into this topic remains very limited.

Observations relating to the relationship between thyroid hormone and lymphocyte activity were documented in 1973 in thyroidectomised mice where lymphocyte numbers in peripheral blood were severely reduced (Fabris, 1973). In the 1980s, thyroid hormones were found to increase the cytotoxicity of NK cells by increasing their sensitivity to IFN (Provinciali and Fabris, 1987). Incubation of mouse spleen cells with T₃ or T₄ prior to IFN exposure resulted in increased NK cell activity, with 50% lower levels of IFN required to promote NK cell activity than in unstimulated cells (Provinciali and Fabris, 1990). In addition, stimulation

with thyroid hormones was found to increase NK cell cytotoxicity, particularly in older mice (Provinciali et al. 1991).

Alterations in NK cell function have also been reported in patients with thyroid diseases. Patients with Grave's disease have hyperthyroidism and their NK cells were found to have a reduced ability to lyse K562 tumour cells *in vitro*. NK cells from Grave's disease patients exhibited an average of 1.3% target cell lysis compared to 15.1% with control NK cells at a 25:1 effector/target ratio, suggesting that high T₃ levels may suppress NK cell function (Papic et al. 1987). This was further evidenced in a separate study which found reduced NK cell activity, with 12% lysis with NK cells from hyperthyroid patients compared to 28% lysis for control NK cells (Wang et al. 1988). It was also demonstrated that NK cell function returned to normal when patients returned to the euthyroid state after treatment. However, this study also identified reduced NK cell activity in euthyroid patients who were rendered hypothyroid using antithyroid drugs such as Propothiouracil (PTU) or Methimazole (Wang et al. 1988). Together these results demonstrate that both hypothyroidism and hyperthyroidism can affect NK cell function, suggesting that appropriate physiological T₃ concentrations are necessary for normal NK cell activity. The concept of thyroid hormone-NK cell crosstalk is far from established, however, as another study reported conflicting evidence that T₃ levels were not correlated with NK cell function in patients with Grave's disease (Wenzel et al. 1998).

Further evidence of a positive correlation between T₃ serum concentrations and NK cell activity was identified in a study of NK cell activity in elderly individuals. Stimulation of NK cells with T₃ *in vitro* increased NK cell cytotoxicity. However, this effect was only observed in patients with low T₃ concentrations as there was no effect on NK cell activity in patients who had normal T₃ levels (Kmiec et al. 1991). It is well known that ageing is associated with the decline of immune populations and a reduction in their functionality, as elderly individuals are more prone to infection, illness and the development of cancer (Weyand and Goronzy, 2016). Thyroid hormone levels also decrease with age and so perhaps there may be a link between reduced thyroid hormone serum concentrations and immune cell defects associated with ageing.

Whilst many observations surrounding T₃ and NK cell activity have been published, the underlying mechanisms which facilitate this response remain to be elucidated. It remains entirely unclear whether T₃ can influence NK cell metabolism through thyroid receptors, or whether this occurs in a non-genomic fashion through the $\alpha v \beta 3$ integrin receptor. Further detailed research into the role of thyroid hormones in NK cells is necessary to increase our understanding of this crosstalk. In turn, it is possible that a better understanding of the mechanisms at play may be useful therapeutically in order to boost NK cell activity, for example in order to help prevent tumour growth, or to prevent susceptibility to infections in older or immunocompromised individuals.

Importantly, hormonal regulation of NK cell development has been identified in the uterus. It has been established that progesterone induces transcription and production of IL-15 in stromal cells in the uterus, which in turn promotes development of uterine NK cells (Gordon, 2021). Therefore, although the relationship between thyroid hormone and NK cells remains unclear, it is evident that hormonal regulation of NK cell development occurs in the uterus and therefore may also occur in other organs.

2. Materials and Methods

2.1. Mice

Duoxa2^{tm1b(KOMP)Wtsi} (Van der Weyden et al. 2020) mice (referred to as *Duoxa2^{-/-}* mice in this thesis) were generated at the Wellcome Trust Sanger Institute in Hinxton and were a kind gift of Dr Anneliese Speak. *Duoxa2^{-/-}* mice were rederived into Central Biomedical Services at Imperial College London. Homozygous knockout mice and heterozygous or wild-type littermate controls were generated through breeding of heterozygous mice due to the small stature and moderate phenotype of *Duoxa2^{-/-}* mice. Mice were bred in house at Imperial College London and housed in individually ventilated cages under specific pathogen free conditions. Mice had free access to food and water and were maintained at 21°C and 52% humidity under a 12 hour light:dark cycle. Mice used in experiments were between 8-12 weeks of age unless specified otherwise and all experiments were performed with age-matched controls. All mice were genotyped by PCR to confirm genotype before experiments were conducted. Wild-type C57BL/6 mice used in hypothyroidism experiments were purchased from Charles River. *TRα^{0/0}* mice and mice expressing *Trα1* only (LOF mice) were kindly provided by collaborators Prof. Graham Williams and Prof. Duncan Bassett of Imperial College London. *Slc26a7^{-/-}* mice were kindly provided by Dr Nadia Schoenmakers from the University of Cambridge. All animal experiments were conducted in accordance with local Animal Welfare and Ethical Review Board and UK Home Office guidelines.

2.2. Genotyping

Mice generated from the *Duoxa2* heterozygous breeding programme were genotyped before use in experiments. Ear notches were incubated overnight at 55°C in an orbital shaker in 200µl of lysis buffer (100mM Tris-HCL, 5mM EDTA, 200mM NaCl and 0.2% SDS) and 100µg/ml of Proteinase K (Sigma Aldrich, #P8450) to extract DNA. Samples were then centrifuged at 16000 x g to remove debris. 10µl of supernatant was diluted in 90µl of distilled H₂O and samples were heat-inactivated for 15 minutes at 75°C. Samples were then genotyped using DreamTaq Hot Start Green PCR Master Mix (ThermoFisher, #K9022) using standard PCR reactions in 25µl reactions. Amplification conditions are detailed in Table 2.1. Amplified products were run on a 1.5% agarose gel (ThermoFisher, #16500100) with 1%

ethidium bromide (ThermoFisher, #15585011) at 120V. O'RangeRuler 100bp DNA ladder (ThermoFisher, #SM0623) was loaded in one well of each gel to identify band sizes. Gels were then imaged under UV light. Primers (Sigma Aldrich) used to genotype mice are listed in Table 2.2. Expected band sizes were 489bp for the wild-type *Duoxa2* PCR, 208bp for the mutant *Duoxa2* PCR and 108bp for the LacZ PCR. Wild-type mice contained the wild-type band only. Heterozygous mice contained the wild-type, mutant and LacZ band. *Duoxa2*^{-/-} mice contained the mutant and LacZ band, but no wild-type *Duoxa2* band.

Table 2.1. Amplification Conditions for Genotyping PCR

Cycle	Temperature	Time
1	94°C	5 mins
2	94°C	30 secs
3	58°C	30 secs
4	72°C	45 secs
5	Go to '2' + 34 cycles	-
6	72°C	5 mins
7	12°C	Forever

Table 2.2. Genotyping PCR primers

PCR reaction	Forward Primer	Reverse Primer
Wild-type <i>Duoxa2</i> PCR	TCTCTGAATGCCTTAGCCCC	ATTCTGGGCATGTGTTTCCC
Mutant <i>Duoxa2</i> PCR	TCTCTGAATGCCTTAGCCCC	TCGTGGTATCGTTATGCGCC
LacZ PCR	ATCACGACGCGCTGTATC	ACATCGGGCAAATAATATCG

2.3. Inducing hypothyroidism in mice

Wild-type C57BL/6 mice were given a low-iodine 0.15% PTU diet (Envigo, #TD.95125). Hypothyroidism was induced in both 6 week old and 9.5 week old mice. Mice were maintained on diet for two or six weeks. Wild-type C57BL/6 mice were fed normal chow as a control (SDS Diets, #RM1). Mice were weighed twice a week to monitor for signs of weight

loss and general health. Mice were also observed for signs of lethargy as an indicator of hypothyroidism.

2.4. RNA Extraction and qRT-PCR

Mouse tissues including lung, thyroid, kidney and spleen were homogenised in 1.5ml eppendorfs (StarLabs) in RNA lysis buffer (Qiagen) using the TissueLyserII system (Qiagen). Mouse femurs were cut below the knee and placed cut-down in 0.5ml eppendorfs (StarLabs). Prior to this, holes were inserted into the eppendorfs using 19G sterile needles (Greiner). 0.5ml eppendorfs were placed inside 1.5ml eppendorfs and centrifuged for 30 seconds at 5000 x g to isolate the bone marrow, as described by Amend et al. 2016. Total RNA extraction was then performed using the RNeasy Plus Mini Kit (Qiagen, #74104) according to manufacturer's instructions.

Following RNA extraction, 1µg of total RNA was used to reverse-transcribe cDNA using Superscript IV VILO MasterMix (ThermoFisher, #11756050) according to manufacturer's instructions. Quantitative Real-time Polymerase Chain Reactions (qRT-PCR) were performed using an Applied Biosystems ViiA7 real time PCR system. TaqMan Gene Expression Master Mix (ThermoFisher, #4369016) and Taqman primers for mouse *Duoxa2* (Mm00470560_m1), *Hprt* (Mm03024075_m1), *Il2* (Mm00434256_m1), *Il7* (Mm01295803_m1), *Il15* (Mm00434210_m1) and *Cxcl12* (Mm00445553_m1) were used according to manufacturer's instructions. qRT-PCR reactions were performed in triplicate and relative gene expression was calculated compared to wild-type controls, using *Hprt* as a housekeeping reference gene.

2.5. Single Cell Suspension

For analysis of cell populations by flow cytometry, single cell suspensions were generated from various tissues. Bone marrow was isolated from mouse femurs by centrifugation. Femurs were cut below the knee and placed cut-down in 0.5ml eppendorfs. Prior to this, holes were inserted into the eppendorfs using 19G sterile needles. 0.5ml eppendorfs were

placed inside 1.5ml eppendorfs and centrifuged for 30 seconds at 5000 x g to isolate the bone marrow. Cells were resuspended in 1ml of ice-cold flow cytometry buffer (Phosphate buffered saline (PBS), 2% foetal bovine serum (FBS) and 2mM EDTA) and then passed through a 70 μ M cell strainer (Greiner). Cells were washed with ice-cold PBS and pelleted at 400 x g. Cells were resuspended and incubated in 1ml of Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich, #R7757) for 2 minutes. Cells were washed again in PBS and then stained for flow cytometry as described below.

Spleens were forced through a 70 μ M cell strainer using a syringe plunger and cell strainer was rinsed with 5ml of PBS. Cells were centrifuged at 400 x g for 5 minutes. Red blood cell lysis was performed by incubation in 1ml of Red Blood Cell Lysing Buffer Hybri-Max for 2 minutes. Cells were washed again with PBS, centrifuged at 400 x g and then stained for flow cytometry. The same procedure was used to isolate a single cell suspension from thymic tissue.

For mouse lungs, single cells were released by enzymatic digestion with 0.1mg/ml DNaseI (Sigma Aldrich, #DN25) and 0.1mg/ml Collagenase I (Sigma Aldrich, #SCR103) in RPMI (Gibco). Lungs were minced with scissors in 5ml Bijou tubes (Greiner), transferred to 1.5ml eppendorfs and 1ml of digestion buffer was added. Tissue was incubated at 37°C for 45 minutes in an incubator with continuous agitation. At 15 minute intervals, samples were triturated with a 1ml pipette. After digestion, tissue was passed through a 70 μ M cell strainer. Cells were washed with PBS and centrifuged at 400 x g. Cells were resuspended in 1ml of Red Blood Cell Lysing Buffer Hybri-Max, incubated for 2 mins and washed again with PBS. Cells were stained for flow cytometry as described below.

Single cell suspensions of liver cells were generated by enzymatic tissue digestion with Dulbecco's PBS (DPBS) containing calcium and magnesium (Gibco, #14040117) with 5% FBS, 0.1mg/ml DNase I and 0.5mg/ml Collagenase I. Livers were cut into small pieces using scissors, placed into 15ml falcon tubes and 5ml of digestion buffer was added. Samples were incubated at 37°C for 45 minutes with continuous agitation. 10ml of PBS was added to each sample after incubation. Liver fragments were passed through a 100 μ M cell strainer using a

syringe plunger. Cells were washed in PBS and centrifuged at 35 x g for five minutes to pellet hepatocytes. The supernatant was carefully removed and layered onto 24% Optiprep (Stemcell Technologies, #07820) to purify immune cell populations. Samples were centrifuged at 700 x g for 20 minutes. The interface layer was removed using a pasteur pipette (SLS). Samples were washed again in PBS and centrifuged for 5 minutes at 400 x g. Cells were resuspended in 1ml Red Blood Cell Lysing Buffer Hybri-Max, incubated for 2 mins and washed again with PBS. Cells were stained for flow cytometry as described below.

2.6. NK Progenitor Isolation

To detect progenitors, bone marrow was isolated and combined from both femurs of each mouse. Bone marrow was isolated from mouse femurs by centrifugation as described above. To enrich for NK progenitors, non-NK lineage cells were removed by magnetic depletion using anti-FITC beads (Miltenyi Biotec, #130048701). Cells were incubated with FITC-conjugated anti-CD3, anti-CD8, anti-CD19 and anti-Gr-1 for 45 minutes at 4°C. Cells were then washed and resuspended in magnetic-activated cell sorting (MACS) buffer (PBS, 0.5%BSA (Sigma Aldrich, #A9418), 2mM EDTA). 50µl of anti-FITC beads were added and incubated at 4°C for 15 minutes. Cells were washed again in MACS buffer and resuspended in 2ml of MACS buffer. Magnetic depletion was performed using the MidiMACs separator system (Miltenyi Biotec). LS columns (Miltenyi Biotec, #130042401) were prepared by running 2ml of MACS buffer through. Resuspended cells were then added onto each LS column, with the eluate collected in a 15ml falcon tube. LS columns were further washed three times with 2ml of buffer. The eluate was pelleted by centrifugation at 400 x g for 5 minutes. Cells were then stained for flow cytometry as described below.

2.7. Flow Cytometry Staining

Single cell suspensions were incubated with TruStain FcX Fc block solution (Biolegend, Clone 93) for 15 minutes at 4°C prior to antibody staining in order to block non-specific staining. Surface staining was performed using antibody cocktails in flow cytometry buffer (PBS, 2%FBS, 2mM EDTA). Samples were stained for 45 minutes at 4°C, washed in ice-cold

PBS and resuspended in flow cytometry buffer. Samples were run on a BD Biosciences LSRFortessa X-20 harboring Violet 405nm, Blue 488nm, Yellow-Green 561nm and Red 633nm lasers and data was analysed using FlowJo software. Single stains for compensation calculation were prepared using single cell suspensions or UltraComp eBeads Compensation Beads (Invitrogen, #01222242). Dead cells were excluded using eBioscience Fixable Viability Dye eFluor 780 (Invitrogen, #65086514). The antibodies used are listed in Table 2.3 below.

Table 2.3. List of Antibodies Used in Flow Cytometry

Fluorophore	Antigen	Clone	Dilution	Concentration	Manufacturer
APC	CD8	53-5.8	1:200	1µg/ml	BioLegend
APC	CD11b	M1/70	1:200	1µg/ml	BioLegend
APC	CD49b	HMA2	1:100	2µg/ml	BioLegend
APC	anti-CD135	A2F10.1	1:50	4µg/ml	BD Biosciences
APC-Cy7	CD27	LG.3A10	1:200	1µg/ml	BioLegend
BV421	NK1.1	PK136	1:200	1µg/ml	BioLegend
BV421	CD4	GK1.5	1:200	1µg/ml	BioLegend
Pacific Blue	Sca1	D7	1:100	5µg/ml	BioLegend
Pacific Blue	Annexin V	Annexin V	1:100	2µg/ml	BioLegend
BV605	CD27	LG 3A10	1:100	2µg/ml	BioLegend
BV605	CD44	(IM7	1:100	2µg/ml	BioLegend
BV785	CD8	53-6.7	1:200	1µg/ml	BioLegend
BV785	CD11b	M1/70	1:200	1µg/ml	BioLegend
BV785	CD45	30-F11	1:200	1µg/ml	BioLegend
BV785	CD127	A7R34	1:50	4µg/ml	BioLegend
FITC	CD3	17A2	1:200	2.5µg/ml	BioLegend
FITC	CD8	53-6.7	1:200	2.5µg/ml	BioLegend
FITC	CD19	ID3	1:200	2.5µg/ml	BioLegend
FITC	Gr-1	R86-8C5	1:200	2.5µg/ml	BioLegend
FITC	CD45.2	104	1:200	2.5µg/ml	BioLegend
FITC	TER119	TER119	1:200	2.5µg/ml	BioLegend
PE	CD3	17A	1:200	1µg/ml	BioLegend
PE	CD25	A18264A	1:200	1µg/ml	BioLegend
PE	CD27	LG-3A10	1:200	1µg/ml	BioLegend
PE	CD49a	HMA1	1:100	2µg/ml	BioLegend
PE	CD122	5H4	1:100	2µg/ml	BioLegend
PE	NK1.1	S17016D	1:200	1µg/ml	BioLegend
PE-Cy7	CD117	2B8	1:100	2µg/ml	BioLegend
PerCp-Cy5.5	CD11b	M1/70	1:100	2µg/ml	BioLegend
PerCp-Cy5.5	CD45	30-F11	1:200	1µg/ml	BioLegend
PerCp-Cy5.5	CD244.2	m2B4 (B6) 458.1	1:100	2µg/ml	BioLegend

2.8. Annexin V Staining

Single cell suspensions were generated as described above. Cell suspensions containing 1×10^6 cells were stained with extracellular antibodies and eBioscience Fixable Viability Dye eFluor 780 for 45 minutes at 4°C. 1X Annexin binding buffer was prepared by diluting 5X Annexin binding buffer (Invitrogen, #V13246) with deionised water. Pacific Blue-conjugated anti-Annexin V (BioLegend) was added to 1X Annexin binding buffer at a 1:100 dilution. Cells were washed in ice-cold PBS and then resuspended in 1X Annexin V binding buffer containing anti-Annexin V antibody. Samples were incubated for 15 minutes at room temperature protected from light. 200µl of 1X Annexin Binding Buffer was added, cells were placed on ice and then analysed immediately by flow cytometry using a BD Biosciences Fortessa LSR X-20.

2.9. Statistics

All statistical tests used are identified in figure legends. Data was analysed using Mann-Whitney rank tests or unpaired t-tests. Each data point represents a biological replicate and lines indicate the median. A p value of <0.05 was considered statistically significant and error bars on graphs represent standard error of mean. Statistical analyses were carried out using Prism 9 (GraphPad).

3. Immune Defects in *Duoxa2*^{-/-} mice

3.1. *Duoxa2*^{-/-} mice are extremely susceptible to melanoma metastasis

The global Knockout Mouse Project (KOMP) was established to generate knockout mouse lines for all protein-coding genes, enabling targeted research into gene function. As part of the KOMP project, a large-scale screen was carried out by the Wellcome Trust Sanger Institute in Cambridge to identify novel regulators of metastasis. As part of this project, *Duoxa2*^{-/-} mice were generated and subjected to an experimental metastasis assay. *Duoxa2*^{-/-} mice were injected with B16-F10 melanoma cells and the number of pulmonary metastases were counted macroscopically 10 days later (Van der Weyden et al. 2017; Van der Weyden et al. 2020). As is evident in Figure 3.1, *Duoxa2*^{-/-} mice are extremely susceptible to melanoma metastasis, 700-fold more so than wild-type control mice. *Duoxa2*^{-/-} mice exhibited the most severe phenotype in the study of 1300 different genes.

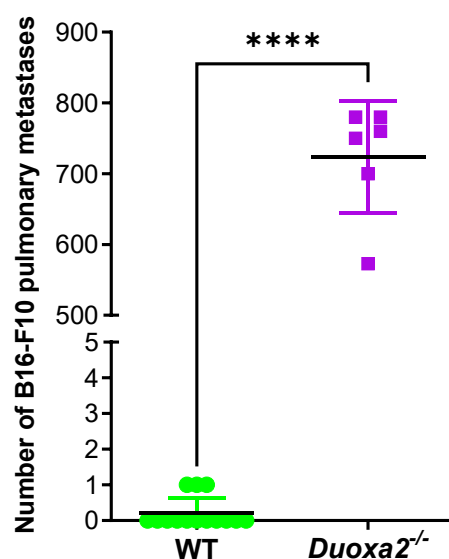


Figure 3.1. Increased B16-F10 metastatic burden of *Duoxa2*^{-/-} mice

C57BL/6 *Duoxa2*^{-/-} mice and wild-type control mice were intravenously injected with 4x10⁴ B16-F10 melanoma cells and the number of pulmonary metastases were counted 10 days later. Statistical significance was determined using a Mann-Whitney rank test. **** p<0.0001. The data presented in this figure was generated at the Wellcome Trust Sanger Institute (Van der Weyden et al. 2017; Van der Weyden et al. 2020).

3.2. Inducible *Duoxa2*^{-/-} mice are also extremely susceptible to melanoma metastasis

Duoxa2^{-/-} mice have congenital hypothyroidism (Zamproni et al. 2008; Grasberger et al. 2012) and as a result are significantly smaller than aged-matched wild-type controls (Van der Weyden et al. 2020). As shown in Figure 3.1, they are extremely susceptible to melanoma metastasis. However, it is possible that their small size contributes to their susceptibility and inability to control metastatic colonisation in the B16-F10 assay.

In order to determine whether DUOXA2 is involved in the regulation of metastasis, inducible Ubiquitin^{+/-CreERT2}: *Duoxa2*^{fl/fl} mice that were comparable in size to wild-type mice were generated. In this model, Cre recombinase was expressed from the ubiquitin promoter under the control of tamoxifen, and so deletion of *Duoxa2* was induced in adulthood upon tamoxifen administration. The mice were allowed to grow until six weeks of age so that they were similar in size to adult wild-type controls before deletion of the gene was induced. As shown in Figure 3.2, inducible Ubiquitin^{+/-CreERT2}: *Duoxa2*^{fl/fl} mice were also significantly more susceptible to melanoma metastasis than their wild-type counterparts. This demonstrates that the susceptibility to metastasis is not solely due to their small size. However, it is important to note that although tamoxifen-induced deletion of *Duoxa2* is effective, the level of knockdown achieved was not as low as that of the *Duoxa2*^{-/-} mice. When measured by qPCR, mRNA expression in *Duoxa2*^{-/-} mice is virtually absent. In Ubiquitin^{+/-CreERT2}: *Duoxa2*^{fl/fl} mice, *Duoxa2* mRNA expression is reduced by approximately 95% compared to wild-type controls (data generated by Dr Anneliese Speak).

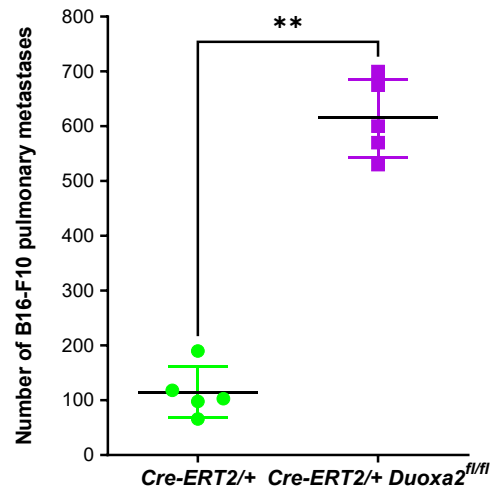


Figure 3.2. Increased B16-F10 metastatic burden of inducible Ubiquitin^{+/CreERT2}; *Duoxa2*^{fl/fl} mice

Deletion of *Duoxa2* was induced by feeding tamoxifen from six weeks of age for a total of six weeks. Ubiquitin^{+/CreERT2}; *Duoxa2*^{fl/fl} mice and Ubiquitin^{+/CreERT2} control mice were injected with B16-F10 melanoma cells and the number of pulmonary metastases were counted 10 days later. Statistical significance was determined using a Mann-Whitney rank test. ** p<0.01. The data shown in Figure 3.2 was generated by Dr Anneliese Speak at the Wellcome Trust Sanger Institute in Cambridge.

3.3. *Duoxa2*^{-/-} mice exhibit immune cell defects

Various immune cell populations are known to play an important role in the tumour microenvironment, including tumour immunosurveillance and the control of metastatic disease. As *Duoxa2*^{-/-} mice are extremely susceptible to melanoma metastasis, it was postulated that they may have defects in their immune system. Immunophenotyping of *Duoxa2*^{-/-} mice and littermate controls was carried out by flow cytometry to characterise several immune cell populations in the bone marrow, spleen and lung.

The T cell populations of *Duoxa2*^{-/-} mice were analysed by flow cytometry to investigate the proportion of CD4⁺ and CD8⁺ T cells. A representative gating strategy is presented in Figure 3.3a. Naïve and memory T cell populations were also investigated on the basis of CD62L and CD44 expression (Fig. 3.3b). T cells recirculate through the bone marrow and it can also act as a reservoir for both effector and memory T cells (Di Rosa and Gebhardt, 2016). In *Duoxa2*^{-/-} mice, there was an expansion of CD4⁺ T cells in the bone marrow (Fig. 3.4a), with a similar trend in the spleen and lung, although this was not significant. There was no significant difference in the overall proportion of CD8⁺ T cells in *Duoxa2*^{-/-} mice (Fig. 3.4b). However, when the naïve and memory T cell populations were investigated, alterations in these subsets were identified, particularly in the CD8 subsets. The proportion of CD8⁺ effector memory T cells (CD44^{hi} CD62L⁻) was significantly reduced in both the bone marrow and spleen of *Duoxa2*^{-/-} mice (Fig. 3.4d). A reduction of CD4⁺ effector memory T cells was also observed but was restricted to the bone marrow only (Fig. 3.4c). CD8⁺ central memory T cells (CD44^{hi} CD62L⁺) were also very significantly reduced in all tissues (Fig. 3.4f), with the most pronounced effect observed in the bone marrow where the proportion of CD4⁺ central memory T cells was also decreased (Fig. 3.4e). The reduction in effector memory subsets was associated with a concomitant increase in the proportion of naïve T cells (CD44^{lo} CD62L⁺). This was observed for the CD8⁺ naïve T cell populations in both the bone marrow and spleen (Fig. 3.4h). A trend for an increase in the proportion of CD4⁺ naïve T cells was also evident in the bone marrow (Fig. 3.4g), but this was not statistically significant. Overall, *Duoxa2*^{-/-} mice demonstrated a loss of CD8⁺ memory T cell populations and a skew towards a naïve T cell phenotype, both of which were particularly evident in the bone marrow. It should be noted that overall proportions were measured in this experiment and absolute cell numbers were not analysed which is a limitation of this experiment.

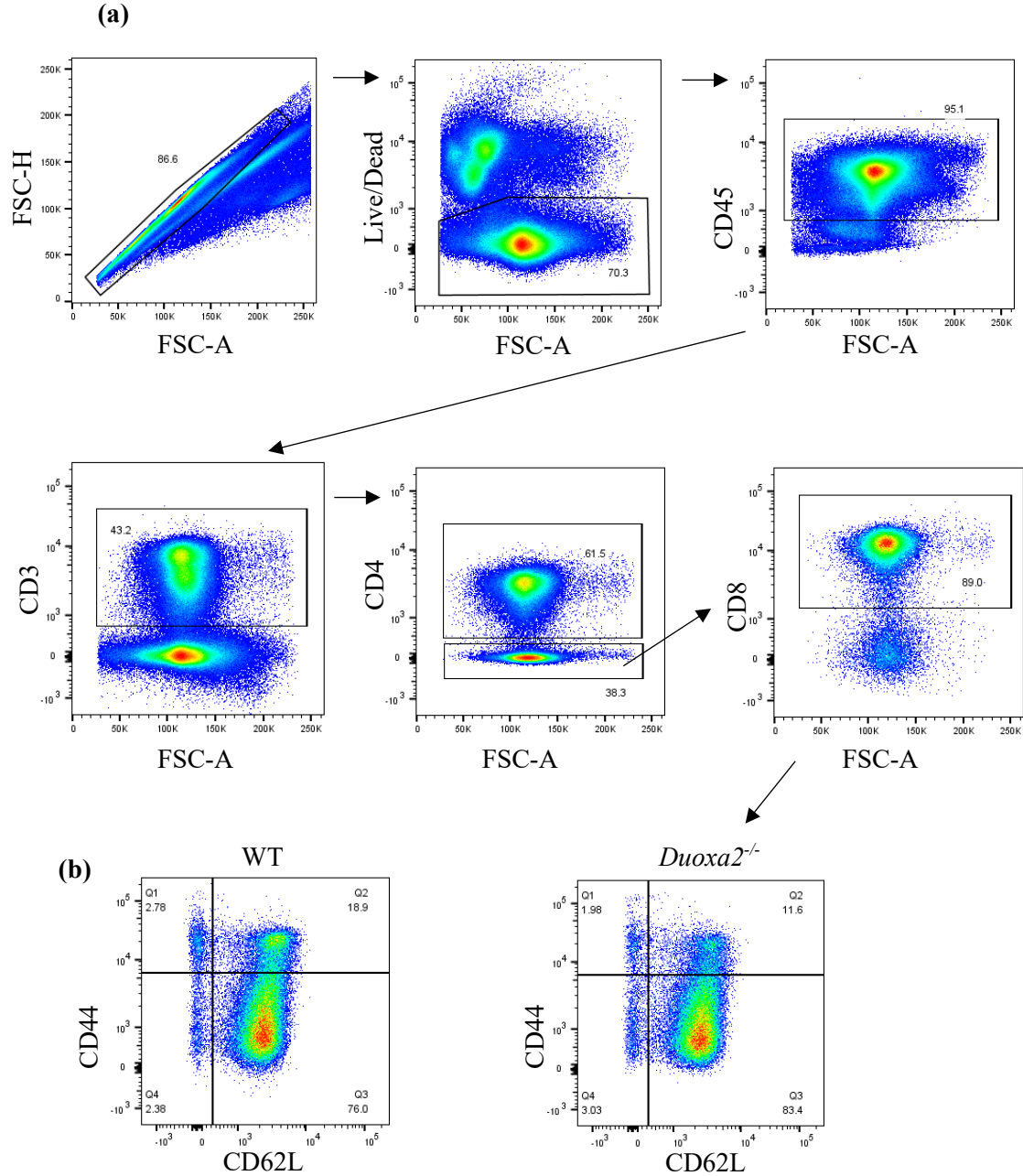


Figure 3.3. Representative gating strategy of the T cell phenotype

The T cell populations of *Duoxa2*^{-/-} mice and age-matched littermate controls were analysed by flow cytometry. (a) Representative gating strategy to define CD8⁺ T cells in the spleen, lung and bone marrow of *Duoxa2*^{-/-} mice and age-matched littermate controls. CD8⁺ T cells were defined as Live CD45⁺ CD3⁺ CD4⁻ CD8⁺ cells. CD4⁺ T cells were defined as Live CD45⁺ CD3⁺ CD8⁻ CD4⁺ cells. (b) Representative gating strategy to define central memory, effector memory and naïve T cells in the bone marrow, lung and spleen of wild-type and *Duoxa2*^{-/-} mice. Central memory T cells were defined as CD44^{hi} CD62L⁺, effector memory cells were defined as CD44^{hi} CD62L⁻ and naïve T cells were defined as CD44⁻ CD62L⁺. Data is representative of three independent experiments.

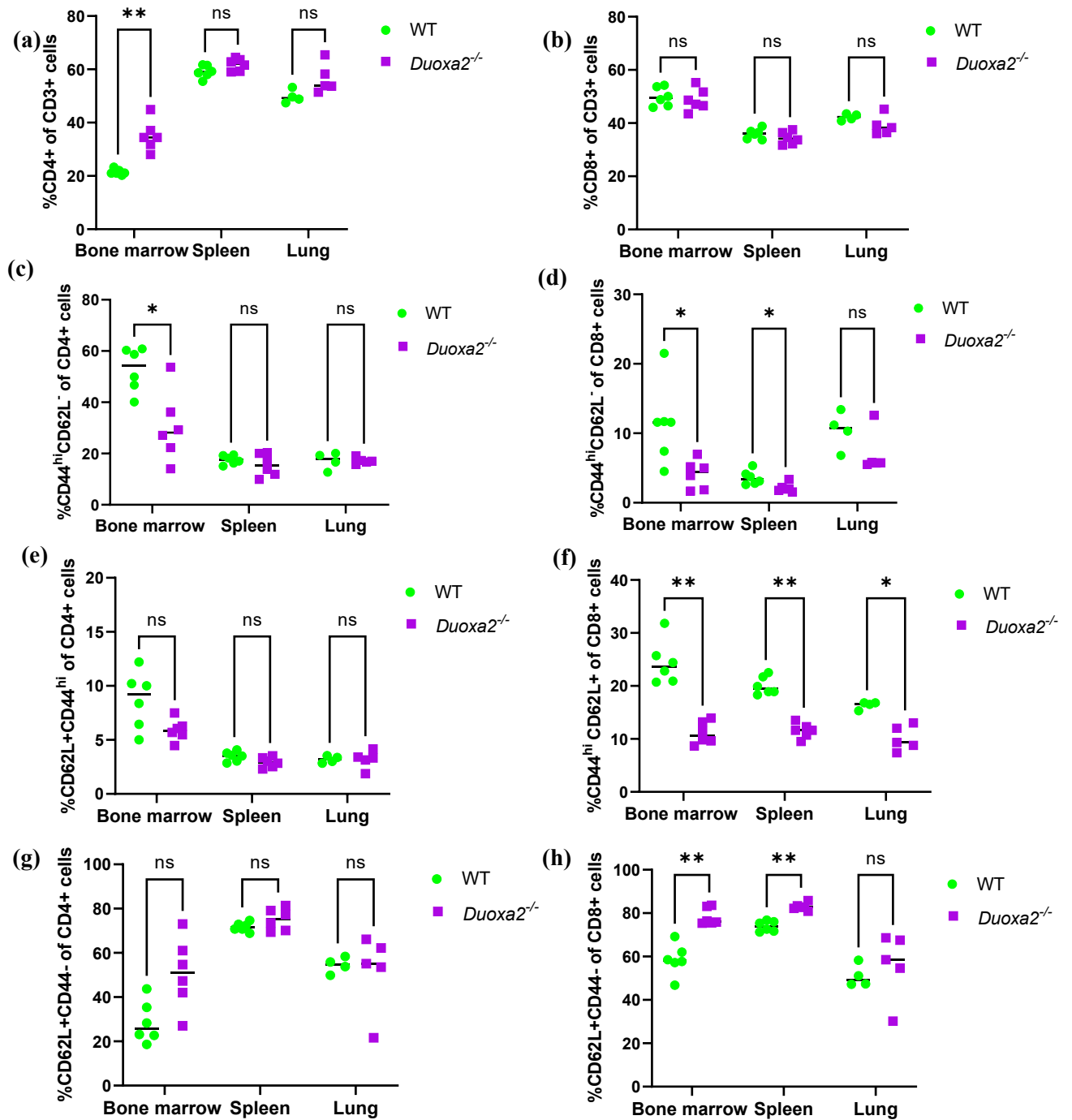


Figure 3.4. Characterisation of the T cell phenotype of *Duoxa2*^{-/-} mice

The T cell populations of *Duoxa2*^{-/-} mice and age-matched littermate controls were analysed by flow cytometry. (a) Percentage of CD4⁺ T cells as a proportion of Live CD45⁺ CD3⁺ cells, (b) percentage of CD8⁺ T cells as a proportion of Live CD45⁺ CD3⁺ cells, (c) percentage of CD44^{hi} CD62L⁻ CD4⁺ effector memory T cells, (d) percentage of CD44^{hi} CD62L⁻ CD8⁺ effector memory T cells, (e) percentage of CD44^{hi} CD62L⁺ CD4⁺ central memory T cells, (f) percentage of CD44^{hi} CD62L⁺ CD8⁺ central memory T cells, (g) percentage of CD44⁻ CD62L⁺ naïve CD4⁺ T cells and (h) percentage of CD44⁻ CD62L⁺ naïve CD8⁺ T cells in the bone marrow, lung and spleen of wild-type and *Duoxa2*^{-/-} mice. Data is representative of three independent experiments. Statistical significance was determined using a Mann-Whitney rank test with Holm-Sidak's multiple testing correction. ns p > 0.05, * p < 0.05 and ** p < 0.01.

As *Duoxa2*^{-/-} mice exhibited alterations in their naïve and memory T cell populations, T cell development in the thymus was also investigated to determine whether defective T cell development was responsible for driving this phenotype. T cell development in the thymus was analysed by flow cytometry to investigate the proportion of single positive CD4⁺ and CD8⁺ cells (Fig. 3.5a), and the earlier stages of development of CD4⁻ CD8⁻ cells (Fig. 3.5b) based on CD44 and CD25 expression (DN1: CD44⁺ CD25⁻, DN2: CD44⁺ CD25⁺, DN3: CD44⁻ CD25⁺, DN4: CD44⁻ CD25⁻).

T cell development in the thymus of *Duoxa2*^{-/-} mice was found to be largely similar to that of littermate controls. As shown in Figure 3.6, the CD4 single positive (SP)/CD8 SP ratio was skewed in *Duoxa2*^{-/-} mice, with an increase in the proportion of CD4⁺ SP cells (Fig. 3.6a) and a decrease in the proportion of CD8⁺ SP cells (Fig. 3.6b). However, there was no significant difference observed in any of the DN1-4 populations. The proportion of DN1 and DN2 populations were comparable between *Duoxa2*^{-/-} mice and controls (Fig. 3.6 c, d). There was a trend for an increase in the proportion of DN3 cells (Fig. 3.6e) and a decrease in the proportion of DN4 cells (Fig. 3.6f), however this was not statistically significant. It should be noted that overall proportions were measured in this experiment and absolute cell numbers were not analysed which is a limitation of this experiment.

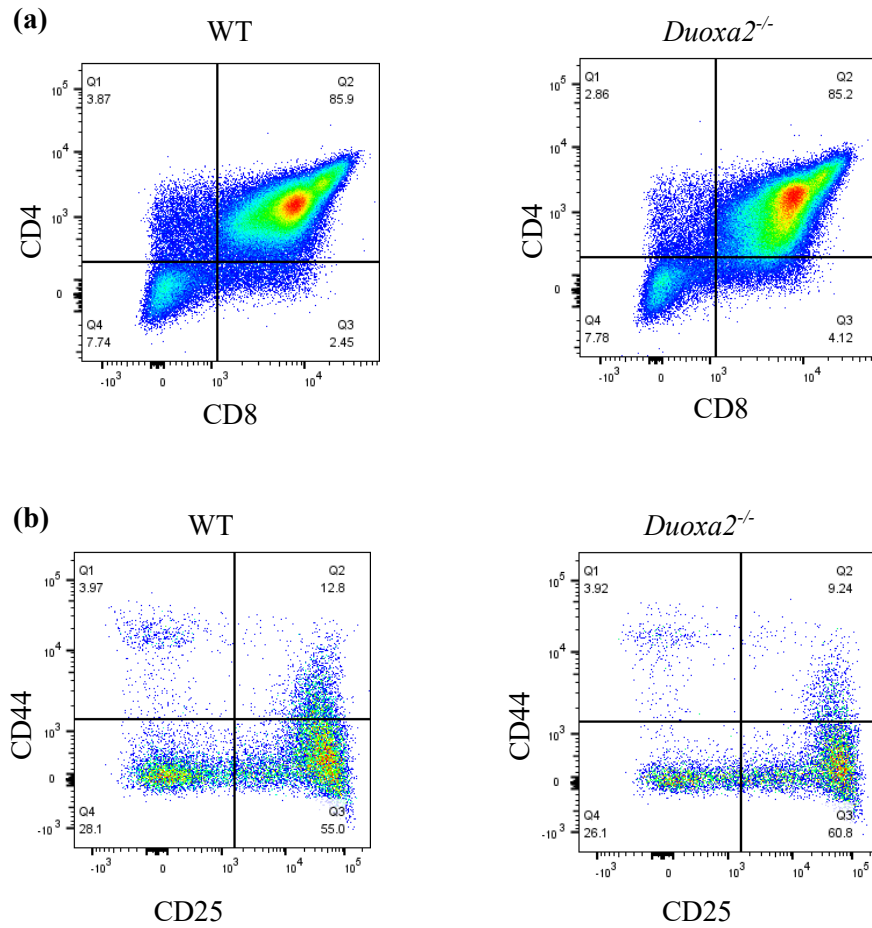


Figure 3.5. Representative gating strategy of the T cell phenotype in the thymus

Cells were isolated from the thymus of *Duoxa2*^{-/-} mice and aged-matched littermate controls and T cell development was analysed by flow cytometry. (a) Representative gating strategy defining CD4 single positive and CD8 single positive T cells in the thymus. (b) Representative gating strategy defining double negative cells in the thymus (DN1: CD44⁺ CD25⁻, DN2: CD44⁺ CD25⁺, DN3: CD44⁻ CD25⁺, DN4: CD44⁻ CD25⁻). Data is representative of two independent experiments.

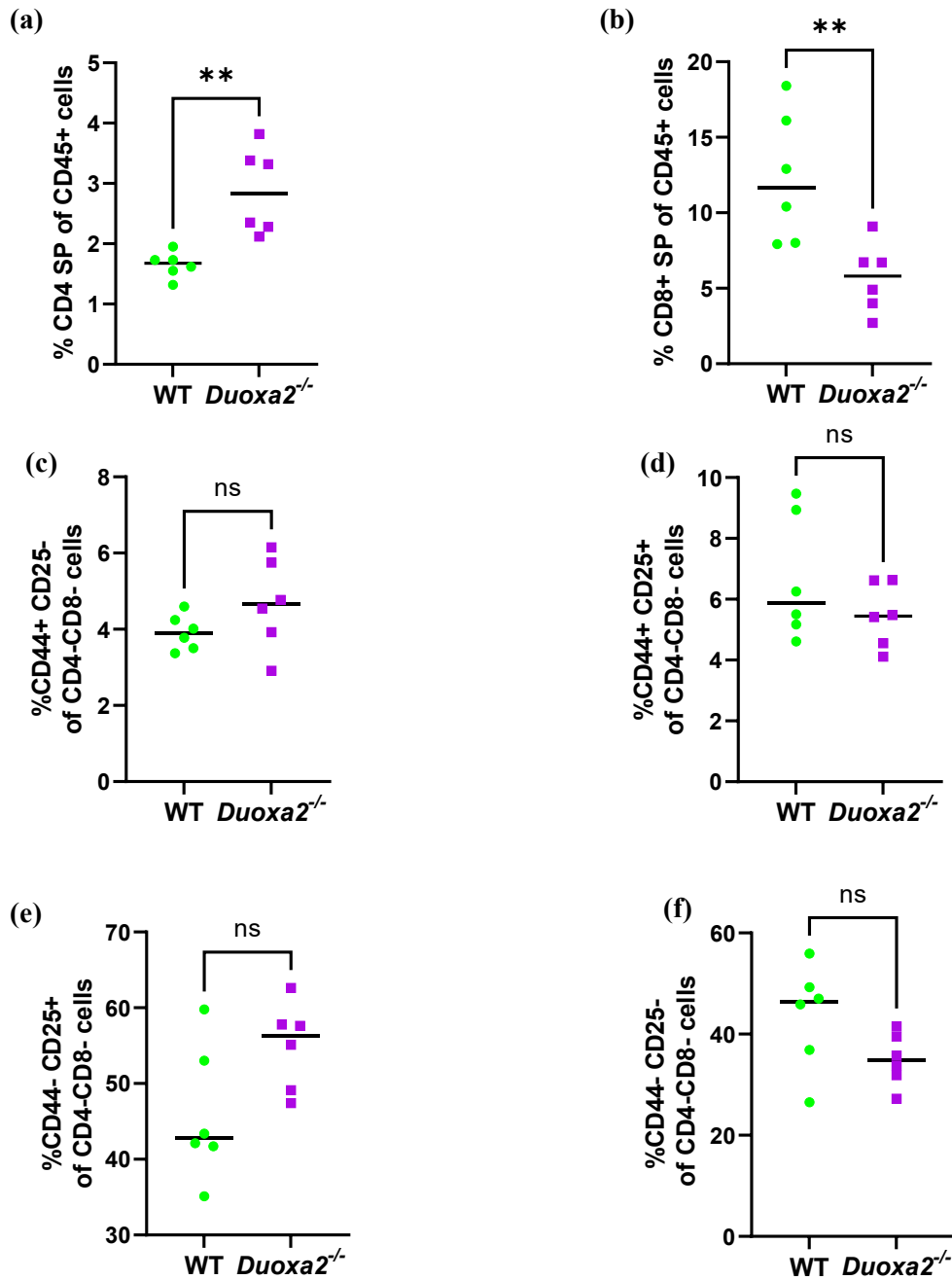


Figure 3.6. Characterisation of the T cell phenotype in the thymus of *Duoxa2*^{-/-} mice

A single cell suspension was isolated from the thymus of *Duoxa2*^{-/-} mice and aged-matched littermate controls and T cell development was analysed by flow cytometry. (a) Percentage of CD4⁺ cells as a proportion of CD45⁺ cells in the thymus, (b) percentage of CD8⁺ cells as a proportion of CD45⁺ cells, (c) percentage of CD44⁺ CD25⁻ (DN1) cells as a proportion of CD4⁺ CD8⁻ cells, (d) percentage of CD44⁺ CD25⁺ (DN2) cells as a proportion of CD4⁺ CD8⁻ cells, (e) percentage of CD44⁻ CD25⁺ (DN3) cells as a proportion of CD4⁺ CD8⁻ cells and (f) percentage of CD44⁻ CD25⁻ (DN4) cells as a proportion of CD4⁺ CD8⁻ cells in the thymus of littermate control and *Duoxa2*^{-/-} mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns p > 0.05 and ** p < 0.01.

Myeloid populations of *Duoxa2*^{-/-} mice were also investigated by flow cytometry. A representative gating strategy is shown in Figure 3.7a. An increase in the proportion of neutrophils (CD11b^{hi} Ly6G^{hi}) as a proportion of live CD45⁺ cells was detected across all tissues (Fig. 3.7b). No significant difference was observed in the proportion of monocytes (CD11b^{hi} Ly6G^{lo}) as a proportion of live CD45⁺ cells, although there was a trend for a slight increase in the bone marrow and a trend for a decrease in the lung of *Duoxa2*^{-/-} mice (Fig. 3.7c).

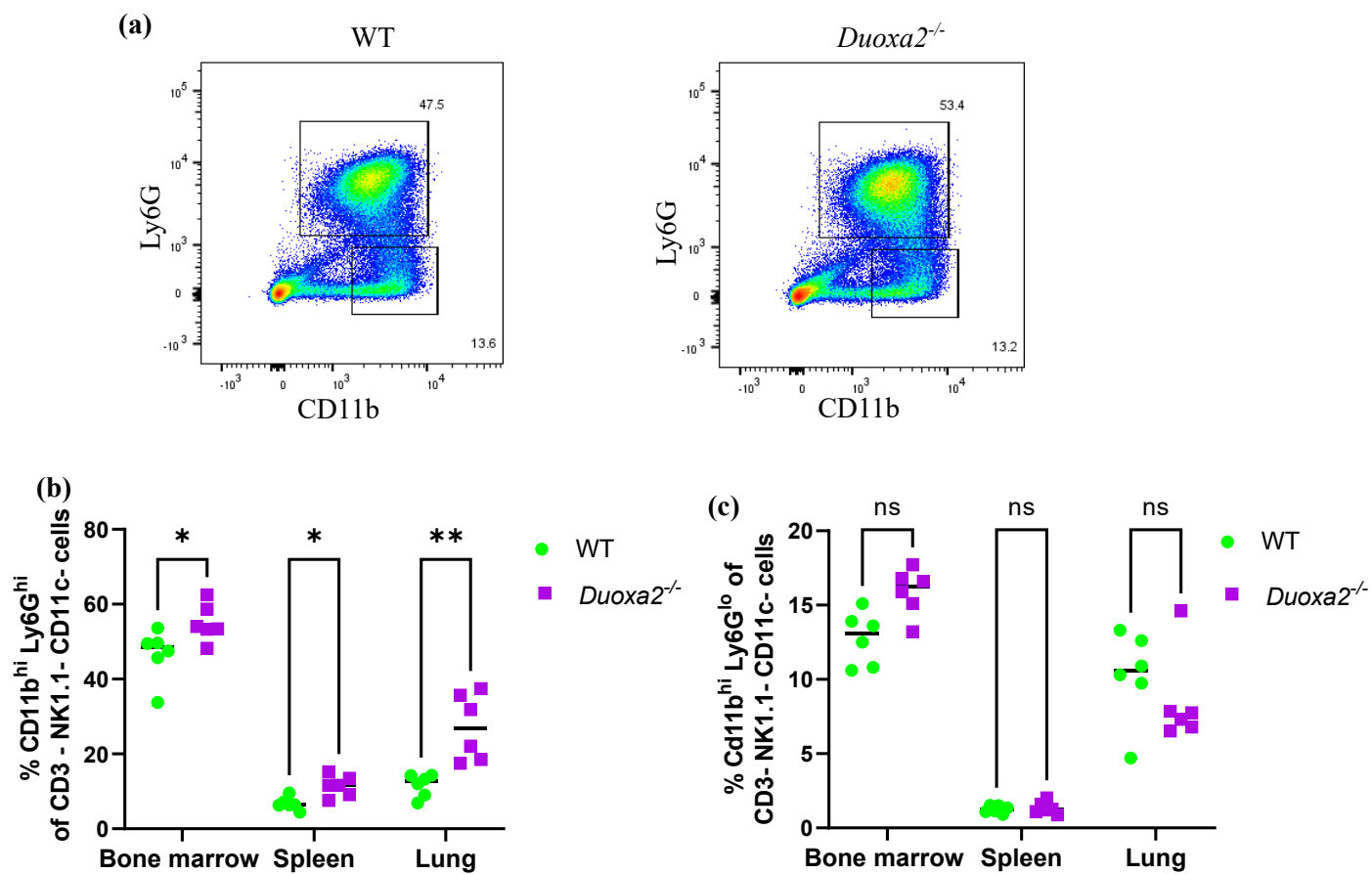


Figure 3.7. Neutrophil and Monocyte populations of *Duoxa2*^{-/-} mice

(a) Representative gating strategy to investigate the neutrophil and monocyte populations in the bone marrow, spleen and lung of *Duoxa2*^{-/-} mice and age-matched littermate controls. (b) Percentage of neutrophils (CD11b^{hi} Ly6G^{hi}) as a proportion of live CD45⁺ CD3⁺ NK1.1⁻ CD11c⁻ cells and (c) percentage of monocytes (CD11b^{hi} Ly6G^{lo}) as a proportion of live CD45⁺ CD3⁺ NK1.1⁻ CD11c⁻ in littermate control and *Duoxa2*^{-/-} mice. Data is representative of three independent experiments. Statistical significance was determined using multiple Mann-Whitney rank tests with Holm-Sidak's multiple testing correction. ns p > 0.05, * p < 0.05 and ** p < 0.01.

NK cells are known to play an important role in anti-tumour immunity by directly killing cancer cells through lytic granule secretion or induction of apoptosis (Paul and Lal, 2017). It has also been demonstrated that NK cells are important in controlling metastasis in the B16-F10 melanoma model (Smyth et al. 2000; Van der Weyden et al. 2017). Due to the inability of *Duoxa2*^{-/-} mice to control engraftment of B16-F10 cells in this assay, the NK cell populations of *Duoxa2*^{-/-} mice were also investigated by flow cytometry. KLRG1⁺ NK populations were severely affected in *Duoxa2*^{-/-} mice. KLRG1 is an inhibitory receptor acquired during maturation and is expressed on terminally mature NK cells (Abel et al. 2018). The proportion of mature KLRG1⁺ NK cells was significantly reduced in the bone marrow, spleen, peripheral blood and lung compared to littermate controls, as demonstrated in Figure 3.8 (Abeler-Dorner et al. 2020). The loss of mature NK cells in peripheral organs was particularly striking. The profound disruption of NK cell development in *Duoxa2*^{-/-} mice was investigated further and will be discussed in more detail in Chapter 4.

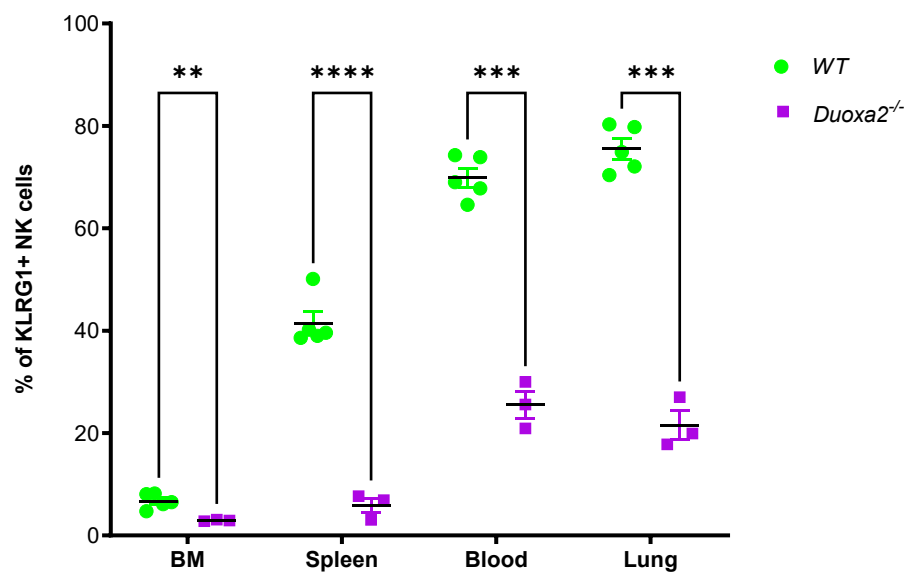


Figure 3.8. Reduction in the proportion of mature KLRG1⁺ NK cell populations in *Duoxa2*^{-/-} mice

The proportion of mature KLRG1⁺ NK cell populations (CD3⁻ NK1.1⁺) in the total NK cell population was determined in the bone marrow (BM), peripheral blood, spleen and lung of *Duoxa2*^{-/-} mice and age-matched littermate controls by flow cytometry. Statistical significance was determined using unpaired t-tests with Holm-Sidak's multiple testing correction. ** p<0.01, *** p<0.001 and ****p<0.0001. The data presented in Figure 3.8 was generated by Dr Anneliese Speak at the Wellcome Trust Sanger Institute in Cambridge.

3.4. *Duoxa2* mRNA is not highly expressed in the bone marrow

Several of the immune cell defects described above were particularly striking in the bone marrow. In particular, NK cells are known to develop in the bone marrow before egressing into the periphery. Therefore, it was hypothesised that DUOXA2 could play a direct role in the bone marrow through paracrine H₂O₂ production by stromal cells, influencing the development of NK cells. To investigate the role of DUOXA2, *Duoxa2* expression was investigated in the bone marrow by qPCR as no reliable antibodies are commercially available.

Duoxa2 is known to be highly expressed in the thyroid gland and at barrier surfaces in the lung and gut. It is generally accepted that any transcripts detected during the latest cycles of qPCR are close to the limit of detection as the cDNA template available is very limited. As is evident in Table 3.1, *Duoxa2* was not highly expressed in the bone marrow of wild-type mice as it was typically detected between 36-38 cycles of the qPCR run. Whilst *Duoxa2* expression was ‘undetermined’ in *Duoxa2*^{-/-} mice, it is clear that *Duoxa2* is not highly expressed in the bone marrow of wild-type mice. As expected, high expression of *Duoxa2* was detected in the thyroid and lung, as has been documented previously, whereas expression in the kidney and spleen was close to or above the limit of detection, similar to the bone marrow as shown in Table 3.2. If *Duoxa2* was directly regulating NK cells in the bone marrow, it would be expected that expression would be similar to that in the lung or thyroid where it is known to function. As such, it was concluded that *Duoxa2* mRNA expression is selective as, of the organs investigated, it is only highly expressed in the thyroid (Grasberger and Refetoff, 2006) and also expressed in the lung to a lesser extent (Geiszt et al. 2003), where it plays an active role in H₂O₂ generation. Publicly available single cell RNA-sequencing databases were also mined to search for *Duoxa2* expression in the bone marrow, but no *Duoxa2* expression was identified.

Table 3.1. Average CT values of *Duoxa2* in the bone marrow of wild-type and *Duoxa2*^{-/-} mice

Sample	Average <i>Duoxa2</i> CT	Average <i>Hprt</i> CT
WT Bone Marrow 1	38.050	23.189
WT Bone Marrow 2	37.363	22.811
WT Bone Marrow 3	36.250	23.153
WT Bone Marrow 4	36.879	23.406
<i>Duoxa2</i> ^{-/-} Bone Marrow 1	Undetermined	22.330
<i>Duoxa2</i> ^{-/-} Bone Marrow 2	Undetermined	21.580

Table 3.2. Average CT values of *Duoxa2* in various organs of wild-type C57BL/6 mice

Sample	Average <i>Duoxa2</i> CT	Average <i>Hprt</i> CT
WT Spleen 1	36.764	21.969
WT Spleen 2	37.772	23.029
WT Kidney 1	37.323	22.605
WT Kidney 2	33.966	21.337
WT Kidney 3	34.153	21.387
WT Lung 1	32.367	23.287
WT Lung 2	30.412	22.223
WT Thyroid 1	21.308	22.993
WT Thyroid 2	23.217	22.750

3.5. Discussion

Duoxa2^{-/-} mice are extremely susceptible to melanoma metastasis when subjected to the B16-F10 melanoma metastasis assay. In fact, they exhibited the most severe phenotype of over 1300 different genes involved in the screen (Van der Weyden et al. 2017; Van der Weyden et al. 2020). However, as *Duoxa2*^{-/-} mice are congenitally hypothyroid, their growth is significantly stunted which likely contributed in part to the metastatic burden observed. An inducible model of *Duoxa2* deletion in adult mice also exhibited a highly significant susceptibility to metastatic colonisation compared to wild-type controls. Taken together, these results suggest a potential role for DUOXA2 in the regulation of metastasis.

Interestingly, *Duoxa2*^{-/-} mice exhibit a severe perturbation of immune homeostasis. *Duoxa2*^{-/-} mice possess immune cell defects in a variety of immune cell subsets. These immune defects likely contribute at least in part to the increased metastatic burden observed in the experimental metastasis assay. Both cytotoxic CD8⁺ T cells and NK cells are known to play an important role in killing cancer cells, particularly in the B16-F10 melanoma assay (Smyth et al. 2000; Van der Weyden et al. 2017). Importantly, previous research has demonstrated that depletion of NK cells in mice using anti-NK1.1 followed by injection of B16-F10 melanoma cells results in approximately a 10 fold increase in lung metastases compared to wild-type control mice (Smyth et al. 2000). As the *Duoxa2*^{-/-} mice are 700-fold more susceptible to engraftment of B16-F10 cells, this phenotype is much more severe than NK-cell deficient mice. Therefore, other immune cells which are disrupted in *Duoxa2*^{-/-} mice, including CD8⁺ T cells, likely also contribute to the increase metastatic burden in this model. Therefore, a reduction in these immune cell types compromises the ability of the immune system to control metastatic colonisation in this model.

However, evidence supporting a role for DUOXA2 in immunity is limited. It has been implicated in barrier immunity as part of its role in ROS generation in the DUOX2/DUOXA2 complex. For example, H₂O₂ generation by DUOX2/DUOXA2 was shown to restrict bacterial colonisation in the mucosa in a mouse model of infection (Grasberger et al. 2015). However, a direct role for DUOXA2 in the regulation of immune cell populations had not previously been identified until immune cell defects were identified in *Duoxa2*^{-/-} mice as part

of a screen at the Wellcome Trust Sanger Institute in Cambridge (Abeler-Dorner et al. 2020). Further in-depth phenotyping would be required to confirm and expand upon the immune defects identified in this screen and to determine how DUOX2 influences immune cells.

Interestingly, several different immune cell populations were found to be altered in *Duoxa2*^{-/-} mice. Whilst the percentage of neutrophils as a proportion of live CD45⁺ cells was increased, NK cell populations were significantly reduced in the periphery and monocytes were unaffected. However, the markers used to define monocytes in this panel were limited and it would be beneficial to repeat this analysis using other markers including CD115 and Ly6C in future to further determine whether any subsets of monocytes are affected. In addition, there was a skew towards a naïve CD8⁺ T cell phenotype, with memory CD8⁺ T cells significantly reduced, particularly in the bone marrow. Importantly, KLRG1 is expressed on both mature NK cells and on cytotoxic effector T cells, particularly in response to T cell activation during inflammation (Joshi et al. 2007). These results indicate that the mechanism of DUOX2 action may be common to many immune cell types. As CD8⁺ T cells and NK cells are functionally related with shared cytotoxic capacity and both are affected in *Duoxa2*^{-/-} mice, it is possible that a transcription or regulatory network common to both cell types may be disrupted in the absence of *Duoxa2*. In particular, the defect in mature NK cells is very severe with a significant reduction in the proportion of mature NK cell populations in *Duoxa2*^{-/-} mice. As NK cell development occurs primarily in the bone marrow, it was hypothesised that DUOX2 expression in the bone marrow may play a direct role in the regulation of NK cell maturation. As DUOX2 is an accessory protein for DUOX1, it is likely that this putative role in the bone marrow would involve H₂O₂ generation by the DUOX1/DUOX2 complex.

Analysis of *Duoxa2* gene expression in the Immgen database (www.immgen.org) demonstrated that *Duoxa2* mRNA is not detected in immune cells. Therefore, it was hypothesised that DUOX2 may regulate immune cell homeostasis in a cell-extrinsic manner. The bone marrow microenvironment consists of a large stromal cell network that forms specialised developmental niches that support specific cell types, such as those that maintain stem cells (Schofield, 1978) and the potential NK cell niche (Noda et al. 2011). As stromal cells are important cellular sources of signalling molecules that direct immune cell trafficking and development, such as chemokines and cytokines, it was hypothesised that a

subset of stromal cells within the bone marrow microenvironment may express *Duoxa2*. However, high levels of *Duoxa2* mRNA expression were not detected by qPCR in the bone marrow, leading to the conclusion that *Duoxa2* is not highly expressed in the bone marrow. Therefore, it is unlikely that DUOXA2 is acting directly on NK cells in the bone marrow. *Duoxa2* expression elsewhere in the body must form part of an overarching mechanism which regulates immune cell maturation and function in an indirect manner.

4. Investigating the NK Cell Defect in *Duoxa2*^{-/-} mice

4.1. Mature NK cell populations are significantly reduced in *Duoxa2*^{-/-} mice

As mature KLRG1⁺ NK cells were found to be significantly reduced in *Duoxa2*^{-/-} mice as part of a large-scale screen (Abeler-Dorner et al. 2020, Chapter 3 Figure 3.8), this NK cell phenotype was further investigated by flow cytometry. NK cells were defined as Live CD45⁺ Lineage⁻ (CD3⁻ CD8⁻ CD19⁻ Gr-1⁻) NK1.1⁺ cells, as demonstrated in a representative gating strategy in Figure 4.1a. Immature and mature NK cell subsets were also investigated on the basis of CD27 and CD11b expression in the bone marrow (Fig. 4.1b), lung (Fig. 4.1c) and spleen (Fig. 4.1d) of *Duoxa2*^{-/-} mice and littermate controls. For the analysis of NK cell populations, as *Duoxa2*^{-/-} mice are significantly smaller than age-matched wild-type controls, absolute cell numbers per milligram of spleen or lung, or per million cells in the bone marrow, were calculated where possible to ensure the observed phenotypes were not driven by a smaller organ size.

The overall proportion of NK cells as a proportion of Live CD45⁺ lineage⁻ cells (Fig. 4.2a) and the absolute number of NK cells (Fig. 4.3a) was significantly increased in the bone marrow of *Duoxa2*^{-/-} mice compared to littermate controls. Conversely, in peripheral tissues such as the lung, the proportion of (Fig. 4.2a) and absolute number (Fig. 4.3a) of NK cells was significantly decreased, with a similar trend observed in the proportion of NK cells in the spleen (Fig. 4.2a) although this was not statistically significant in terms of absolute numbers of splenic NK cells (Fig. 4.3a). To determine whether there was a defect in a particular subpopulation of NK cells, the maturation states of NK cells in *Duoxa2*^{-/-} mice were also analysed by flow cytometry. As shown in Figure 4.2b, the proportion of immature CD27⁺ CD11b⁻ NK cells in the total NK cell population was significantly increased in the spleen and lung of *Duoxa2*^{-/-} mice (Fig. 4.2b). The absolute number of immature NK cells in the total NK cell population was also increased in the bone marrow and spleen (Fig. 4.3b). There was also a drastic reduction in both the proportion (Fig. 4.2c) and absolute number (Fig. 4.3c) of mature CD27⁻ CD11b⁺ NK cells in the total NK cell population in all tissues. This reduction was significant in the bone marrow, lung and spleen but was particularly striking in the spleen and lung as sizable mature NK cell populations are evident in these tissues in littermate controls. This reduction in mature KLRG1⁺ NK cells in the peripheral blood was initially

observed as part of a large-scale screen as described in Chapter 3 (Abeler-Dorner et al. 2020). Importantly, rederivation of the line and subsequent phenotyping at Imperial College London demonstrated that the NK cell phenotype is robust and largely unaffected by housing or environmental conditions, as presented in Figures 4.1- 4.3.

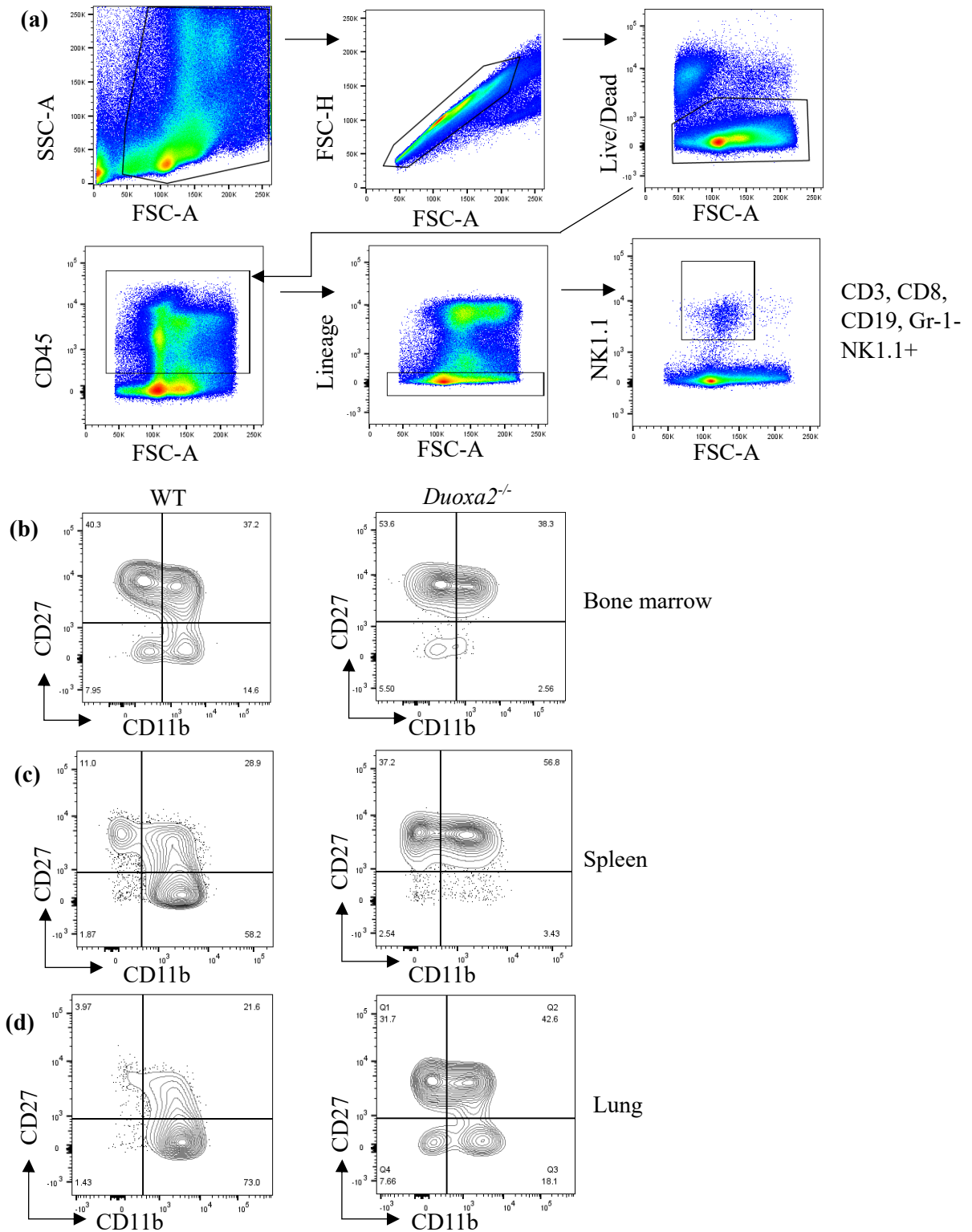


Figure 4.1. Representative gating strategy of NK Cell populations of *Duoxa2*^{-/-} mice

NK cell populations of *Duoxa2*^{-/-} and littermate control mice were analysed by flow cytometry. (a) Representative gating strategy to define NK cells in bone marrow, spleen and lung. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression. (b) Representative flow cytometry plots of NK cell subsets in the bone marrow, (c) NK cell subsets in the spleen and (d) NK cell subsets in the lung of littermate control and *Duoxa2*^{-/-} mice.

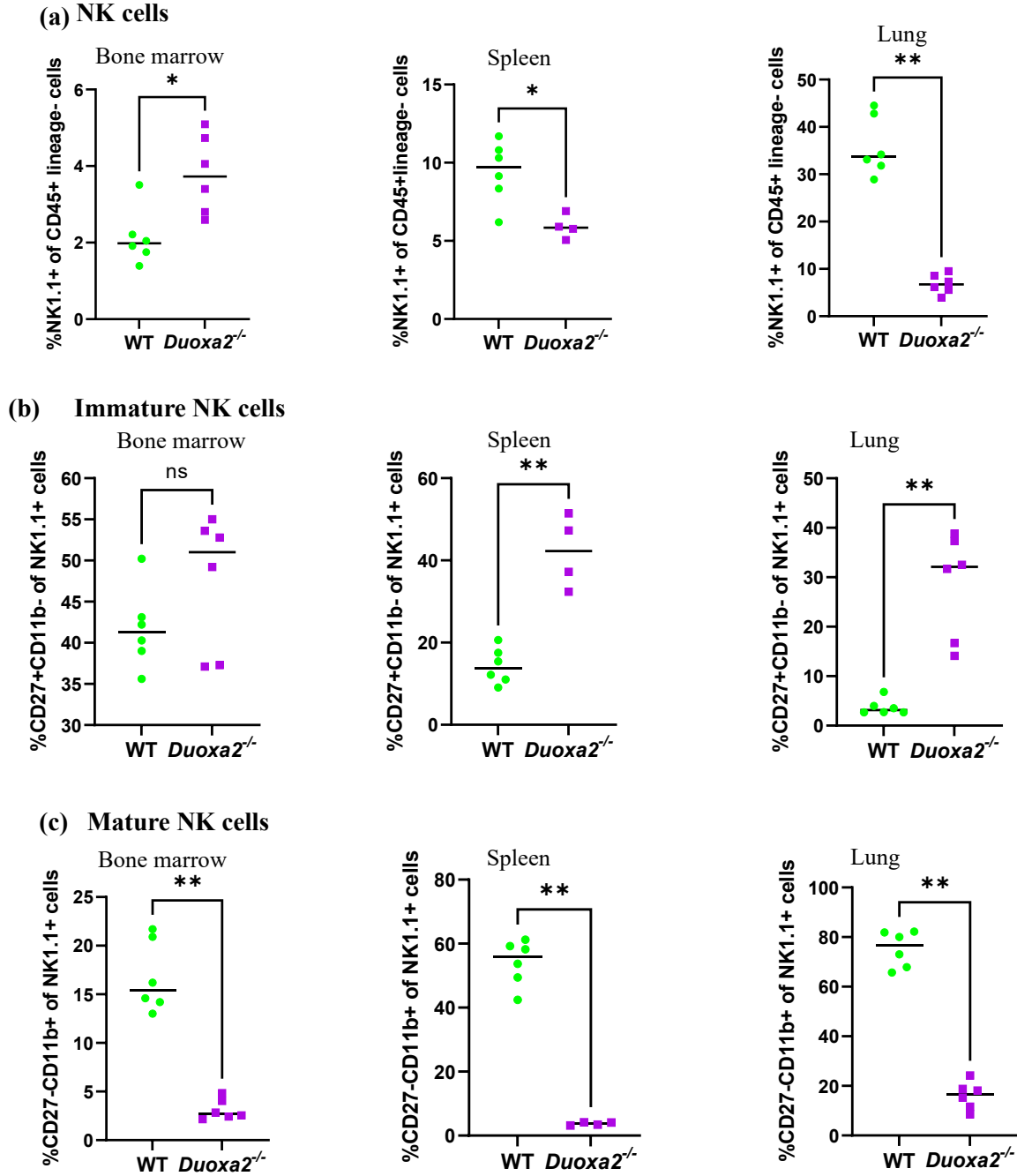


Figure 4.2. Decreased proportion of mature NK cells in *Duoxa2*^{-/-} mice

NK cell populations of *Duoxa2*^{-/-} and littermate control mice were analysed by flow cytometry. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. (a) Percentage of NK cells as a proportion of Live CD45⁺ Lineage⁻ cells, (b) percentage of immature CD27⁺ CD11b⁻ NK cells as a proportion of Live CD45⁺ Lineage⁻ NK cells and (c) Percentage of mature CD27⁻ CD11b⁺ NK cells as a proportion of Live CD45⁺ Lineage⁻ NK cells in the bone marrow, spleen and lung of littermate control and *Duoxa2*^{-/-} mice. Data is representative of three independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

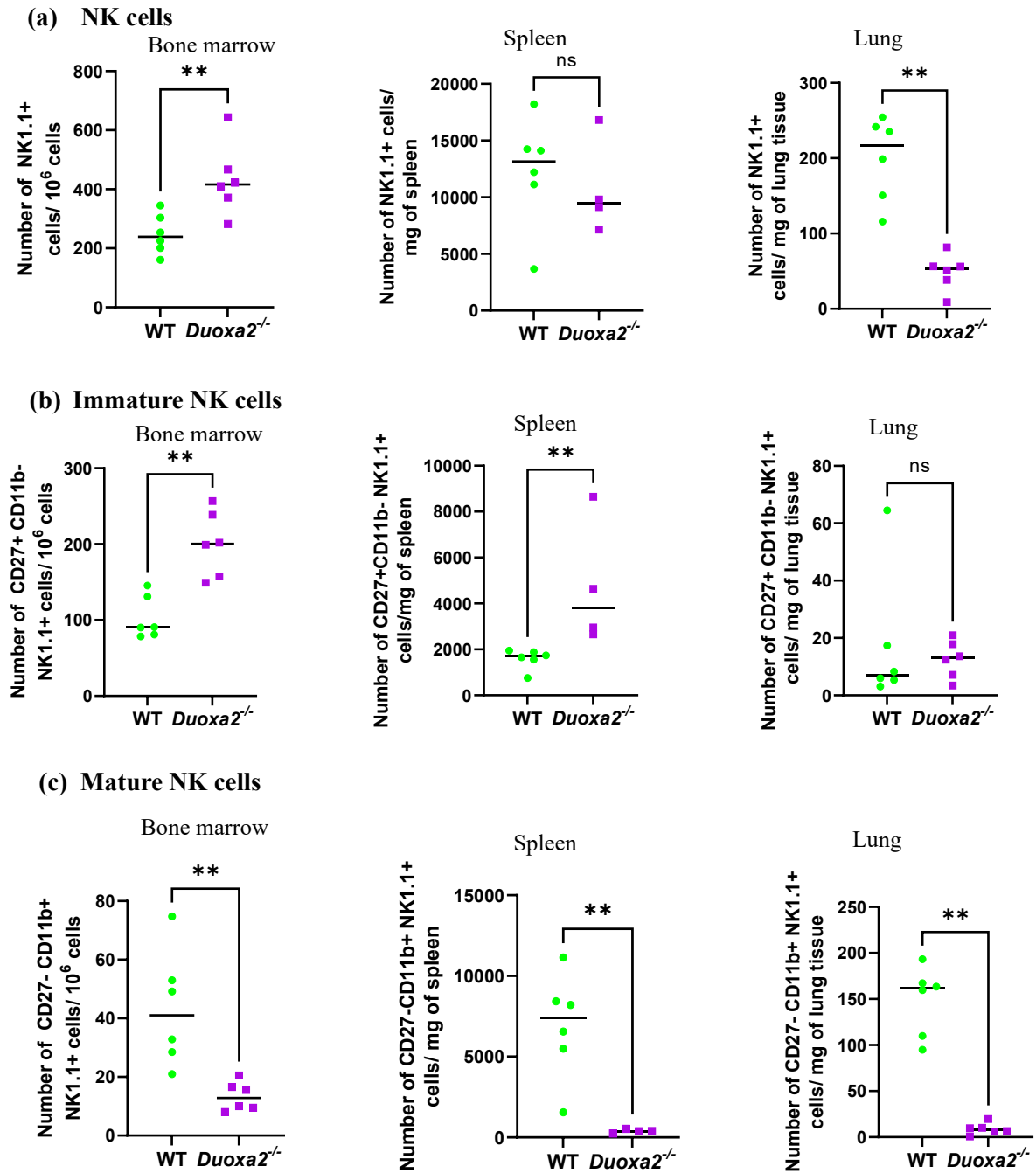


Figure 4.3. Decreased absolute number of mature NK cells in *Duoxa2*^{-/-} mice

NK cell populations of *Duoxa2*^{-/-} and littermate control mice were analysed by flow cytometry. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression. (a) Number of NK cells, (b) number of immature CD27⁺ CD11b⁻ NK cells and (c) number of mature CD27⁻ CD11b⁺ NK cells in the bone marrow, spleen and lung of littermate control and *Duoxa2*^{-/-} mice. Data is representative of three independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns p>0.05, * p<0.01 and ** p<0.001.

4.2. NK cell populations are also reduced in the liver of *Duoxa2*^{-/-} mice

In order to further investigate the NK cell defect in *Duoxa2*^{-/-} mice, the liver NK cell populations (Live CD45⁺ Lineage⁻ NK1.1⁺ cells) and the immature (CD27⁺ CD11b⁻) and mature (CD27⁻ CD11b⁺) NK cell subsets of the liver were analysed by flow cytometry (Fig. 4.4a). Similar to other peripheral tissues, the proportion of total NK cells in Live CD45⁺ Lineage⁻ cells was reduced in *Duoxa2*^{-/-} mice compared to littermate controls, as shown in Figure 4.4b. This was associated with an increase in the proportion of immature CD27⁺ CD11b⁻ NK cells and a decrease in the proportion of mature CD27⁻ CD11b⁺ NK cells in the total NK cell population (Fig. 4.4b). It should be noted that the liver was not perfused in these experiments and so this staining may also have identified cells which were in circulation but not resident in the liver.

The liver also contains a significant population of ILC1 cells as well as conventional NK cells. When first identified, NK cells were characterised as innate cytotoxic lymphoid cells that develop in the bone marrow before trafficking to peripheral sites. More recently, heterogeneous groups of innate lymphoid cells have been identified, now known as the ILC family (Vivier et al. 2018). Most similar to NK cells are the ILC1 cells which also secrete IFN- γ . Unlike NK cells, ILC1s are tissue-resident and are weakly cytotoxic cells (Peng et al. 2013). The liver contains both ILC1s and NK cells, both of which express NK1.1. ILC1 and NK cell populations can be distinguished by analysis of markers of tissue-residency. The resident ILC1 populations can be separated from conventional NK cells as ILC1s are CD49a⁺ CD49b⁻, whereas NK cells are CD49a⁻ CD49b⁺.

In order to determine whether loss of DUOXA2 affects both NK cells and ILC1s, CD49a and CD49b expression was analysed by flow cytometry (Fig. 4.4c). The proportion of ILC1s (CD49a⁺ CD49b⁻) amongst Live CD45⁺ Lineage⁻ NK1.1⁺ cells is significantly increased in *Duoxa2*^{-/-} mice (Fig. 4.4d). As shown in Figure 4.4e, the proportion of NK cells (CD49a⁻ CD49b⁺) amongst Live CD45⁺ Lineage⁻ NK1.1⁺ cells is significantly reduced in the liver in *Duoxa2*^{-/-} mice as expected. This is likely reflective of the reduction in proportion of NK cells in the liver. However, it would be necessary to repeat these experiments to include fluorescence minus one (FMO) controls in order to set the gates as the CD49a stain is not

very strong. In addition, although CD49a is expressed on ILCs, it would have been useful to include CD200R as a marker expressed on ILCs in order to aid in the identification of ILC cells in the liver.

Natural Killer T (NKT) cells are also an important immune cell type in the liver that are similar to NK cells and express NK1.1 (Godfrey et al. 2004). Therefore, NKT cells were investigated in *Duoxa2*^{-/-} mice to determine if NKT populations are also affected by loss of DUOXA2. Analysis of NKT populations determined that the proportion of NKT cells was also significantly decreased in the liver of *Duoxa2*^{-/-} mice (Fig. 4.4f, g).

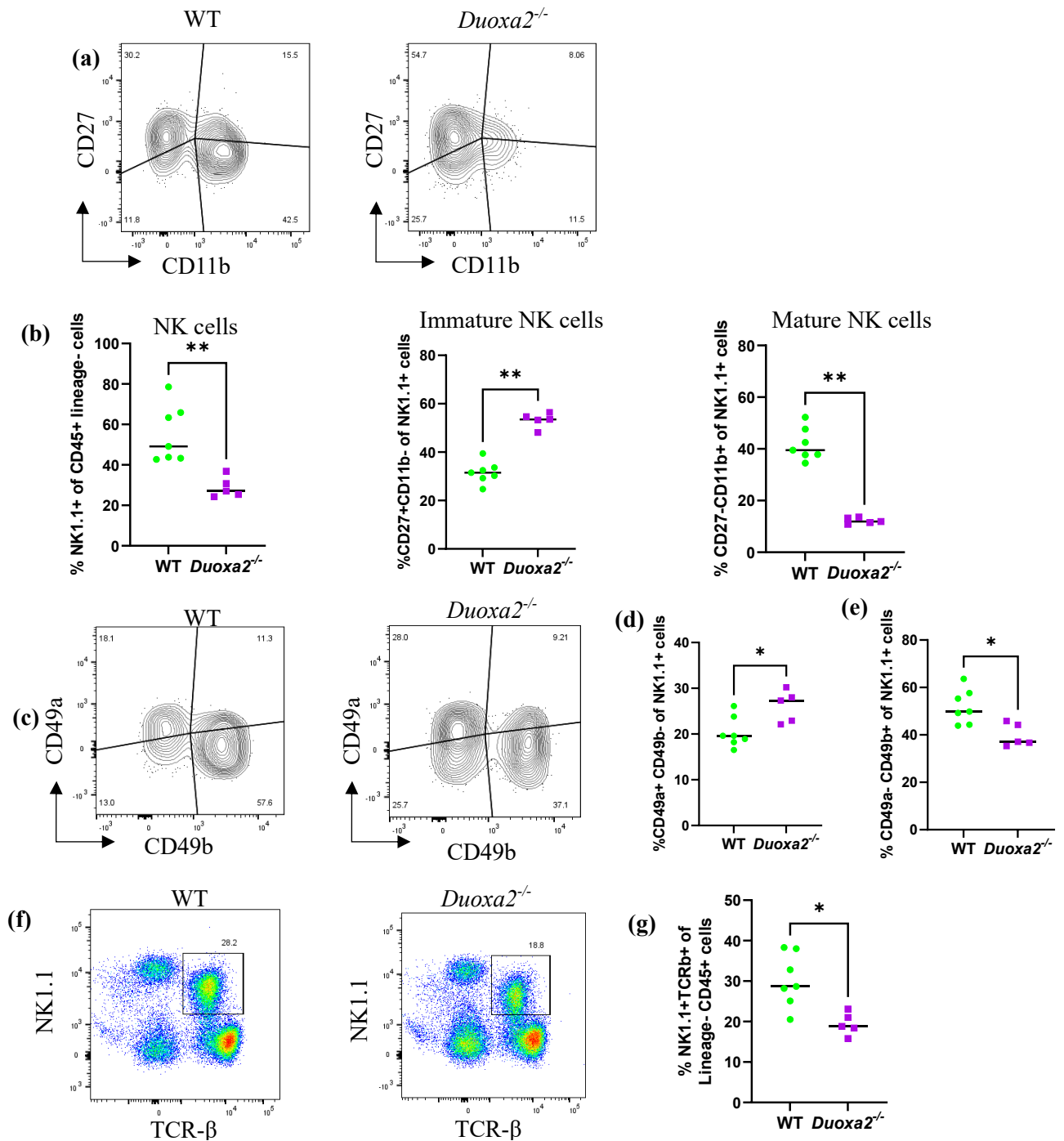


Figure 4.4. NK, ILC1 and NKT phenotyping of the liver of *Duoxa2*^{-/-} mice

NK, ILC1 and NKT cell populations of *Duoxa2*^{-/-} and littermate control mice were analysed by flow cytometry. (a) Representative flow cytometry plots of immature and mature NK cell subsets in the liver. NK cells were defined as Live CD45+ Lineage- NK1.1+ cells. (b) Proportion of total NK, immature and mature NK cells in littermate controls and *Duoxa2*^{-/-} mice. (c) Representative flow cytometry plots to distinguish CD49a+ CD49b- ILC1 cells and CD49a- CD49b+ NK cells. (d) Proportion of ILC1 (CD49a+ CD49b-) cells and (e) proportion of NK cells (CD49a- CD49b+) in littermate controls and *Duoxa2*^{-/-} mice. (f) Representative flow cytometry plots of NKT cell populations in the liver and (g) proportion of NKT cells (NK1.1+ TCRβ+) in the liver of littermate control and *Duoxa2*^{-/-} mice. Data is representative of three independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns>0.05, * p<0.01 and ** p<0.001.

4.3. NK cell defect in *Duoxa2*^{-/-} mice is cell-extrinsic

In order to investigate the mechanism driving alterations in NK cell populations in *Duoxa2*^{-/-} mice, it was important to determine whether the role of DUOXA2 was cell-intrinsic or extrinsic. Using a strain of mice in which Cre recombinase is expressed by NKp46⁺ cells only (*Ncr1*^{Cre}), deletion of *Duoxa2* was restricted to NKp46-expressing NK cells. As shown in Figure 4.5, NK-cell specific deletion of *Duoxa2* had no effect on NK cell populations. The proportion of KLRG1⁺ NK cells in *Duoxa2*^{-/-} mice was equivalent to that found in littermate controls across all tissues analysed. It is important to note that NKp46 is expressed by immature and mature NK cells only, and not on NK progenitors and so deletion of *Duoxa2* was induced in immature and mature NK cells only. The data presented in Figure 4.5 was generated by Dr Anneliese Speak at the Wellcome Trust Sanger Institute in Cambridge. This finding was supported by the generation of bone marrow chimeras where bone marrow from *Duoxa2*^{-/-} mice produced mature NK cells when transplanted into wild-type hosts (Data not shown, data generated by Dr Anneliese Speak).

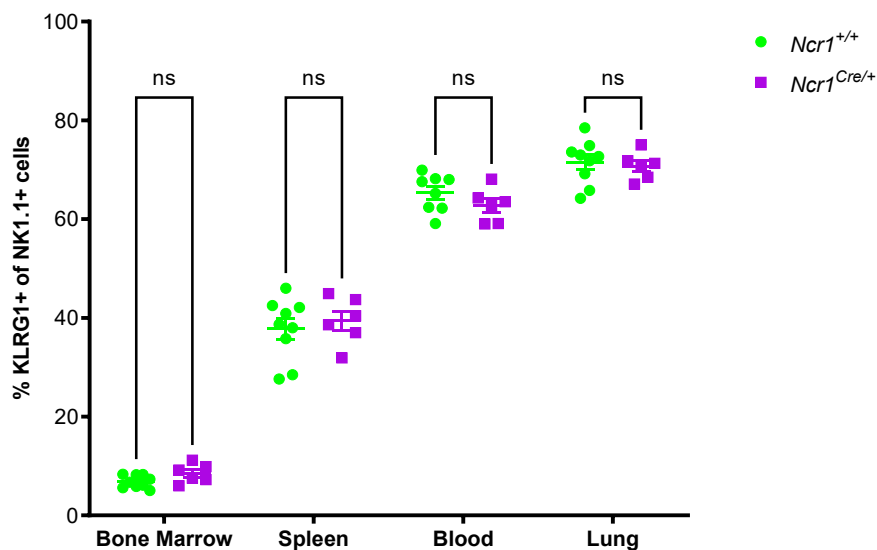


Figure 4.5. Mature KLRG1⁺ NK cell populations are unaffected in mice with an NK-cell specific deletion of *Duoxa2*

Deletion of *Duoxa2* was restricted to NKp46⁺ cells using an *Ncr1*^{Cre/+} line. The percentage of mature KLRG1⁺ NK cells as a proportion of total NK cells in the bone marrow, spleen, blood and lung of *Ncr1*^{Cre/+} mice and control mice. Statistical significance was determined using multiple Mann Whitney rank tests with Holm-Sidak's multiple testing correction. ns p>0.05.

4.4. Heterozygous *Duoxa2*^{+/-} mice do not exhibit NK cell defects

The genetic defect driving the NK cell phenotype in *Duoxa2*^{-/-} mice was further investigated by analysing the NK populations of mice with heterozygous mutations in *Duoxa2* by flow cytometry. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. The proportion of NK cells was similar in the bone marrow, spleen and lung of *Duoxa2*^{+/-} mice compared to wild-type controls (Fig. 4.6a). The proportions of both immature (Fig. 4.6b) and mature NK cells (Fig. 4.6c) were equivalent in *Duoxa2*^{+/-} and wild-type mice.

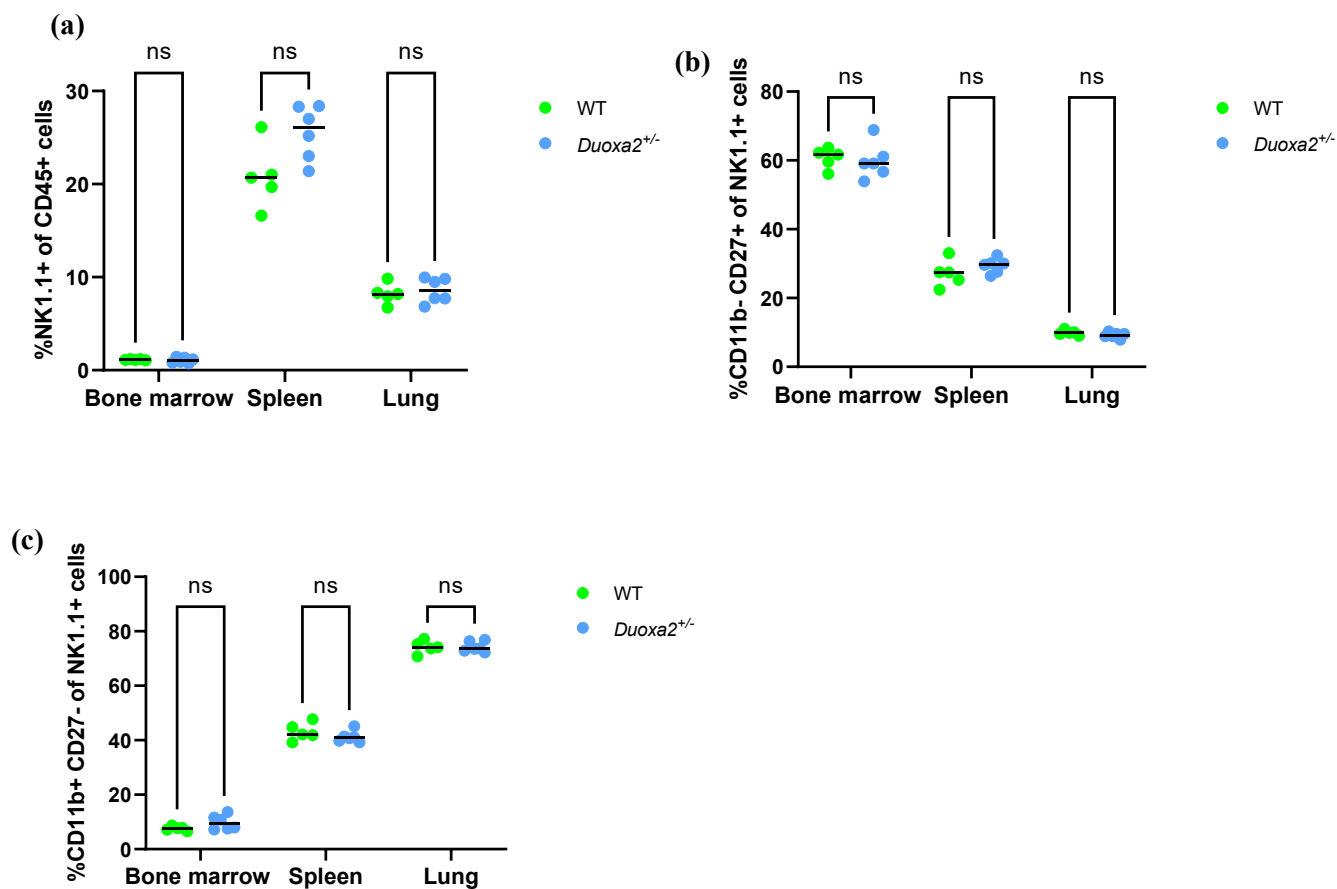


Figure 4.6. NK cell populations are unaffected in *Duoxa2*^{+/-} mice

NK cell populations of *Duoxa2*^{+/-} mice and littermate controls were investigated by flow cytometry. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression. (a) Proportion of NK cells in *Duoxa2*^{+/-} mice. (b) Proportion of immature CD27⁺ CD11b⁻ NK cells in *Duoxa2*^{+/-} mice. (c) Proportion of mature CD27⁻ CD11b⁺ NK cells in *Duoxa2*^{+/-} mice. Data is representative of three independent experiments. Statistical significance was determined using multiple Mann Whitney rank tests with Holm-Sidak's multiple testing correction. ns p>0.05.

4.5. Accumulation of refined-NK progenitors in *Duoxa2*^{-/-} mice

The defect in NK cells observed in *Duoxa2*^{-/-} mice could occur at a number of points in the NK cell developmental pathway. A defect in NK cell development and maturation, a failure to egress from the bone marrow or a failure to survive in the periphery, or a combination of these factors, could result in the loss of mature NK cell populations in *Duoxa2*^{-/-} mice. In order to gain further mechanistic insight into where the NK cell defect may arise, NK progenitor populations were analysed by flow cytometry to investigate the earlier stages of NK cell development. A representative gating strategy is shown in Figure 4.7a, where NK progenitors were gated as lymphocytes based on forward and side scatter and then as single cells to exclude doublets, and then gated on Lineage- (CD3, CD8, CD19 and Gr-1) cells to remove T cells and more abundant lymphocyte populations. NK cell progenitors were gated as Lineage- CD244⁺ CD27⁺ CD127⁺ cells, and then further divided into subpopulations based on CD135 and CD122 expression (Fig. 4.7b). CLPs were defined as Lineage- CD27⁺ CD244⁺ CD127⁺ CD135⁺ CD122⁻ cells. Pre-NKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁻ cells. As the transition from pre-NKP to rNKP is marked by CD122 acquisition, rNKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁺ cells.

Although the proportion of pre-NKPs was also reduced, this was not reflected in terms of absolute numbers of pre-NKPs which were not significantly different in *Duoxa2*^{-/-} mice (Fig. 4.7c). As is evident in Figure 4.7b and d, there is an accumulation of rNKPs in *Duoxa2*^{-/-} mice. Both the proportion and absolute number of rNKPs were significantly increased amongst Lineage- CD244⁺ CD27⁺ CD127⁺ cells in *Duoxa2*^{-/-} mice compared to littermate controls (Fig. 4.7d). Although the proportion of CLPs was decreased amongst Lineage- CD244⁺ CD27⁺ CD127⁺ cells in *Duoxa2*^{-/-} mice, the absolute number of CLPs was actually slightly increased in *Duoxa2*^{-/-} mice compared to littermate controls (Fig. 4.7e). Therefore, the decrease in proportion of CLPs and pre-NKPs is likely driven by the increase in the proportion of rNKPs.

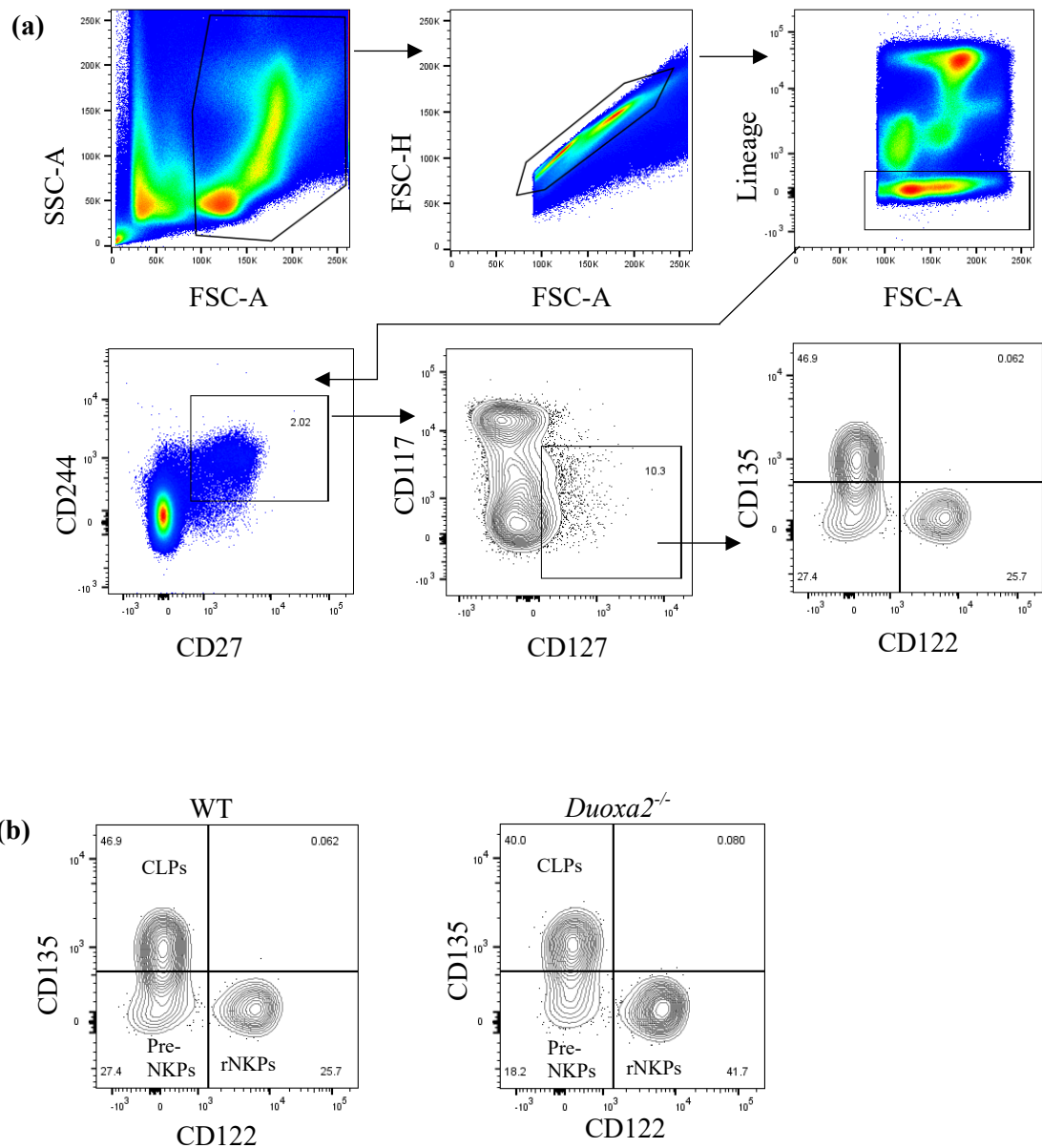


Figure 4.7. Accumulation of refined NK Cell progenitor populations in the bone marrow of *Duoxa2*^{-/-} mice

Bone marrow was isolated from *Duoxa2*^{-/-} and littermate control mice and NK progenitors were identified by flow cytometry. (a) Representative gating strategy used to identify NK progenitor populations. (b) Representative flow cytometry plots of pre-NKP (Lineage- CD27+ CD244+ CD127+ CD135- CD122-), refined NKP cells (Lineage- CD27+ CD244+ CD127+ CD135- CD122+) and CLPs (Lineage- CD27+ CD244+ CD127+ CD135+ CD122-) in littermate controls and *Duoxa2*^{-/-} mice.

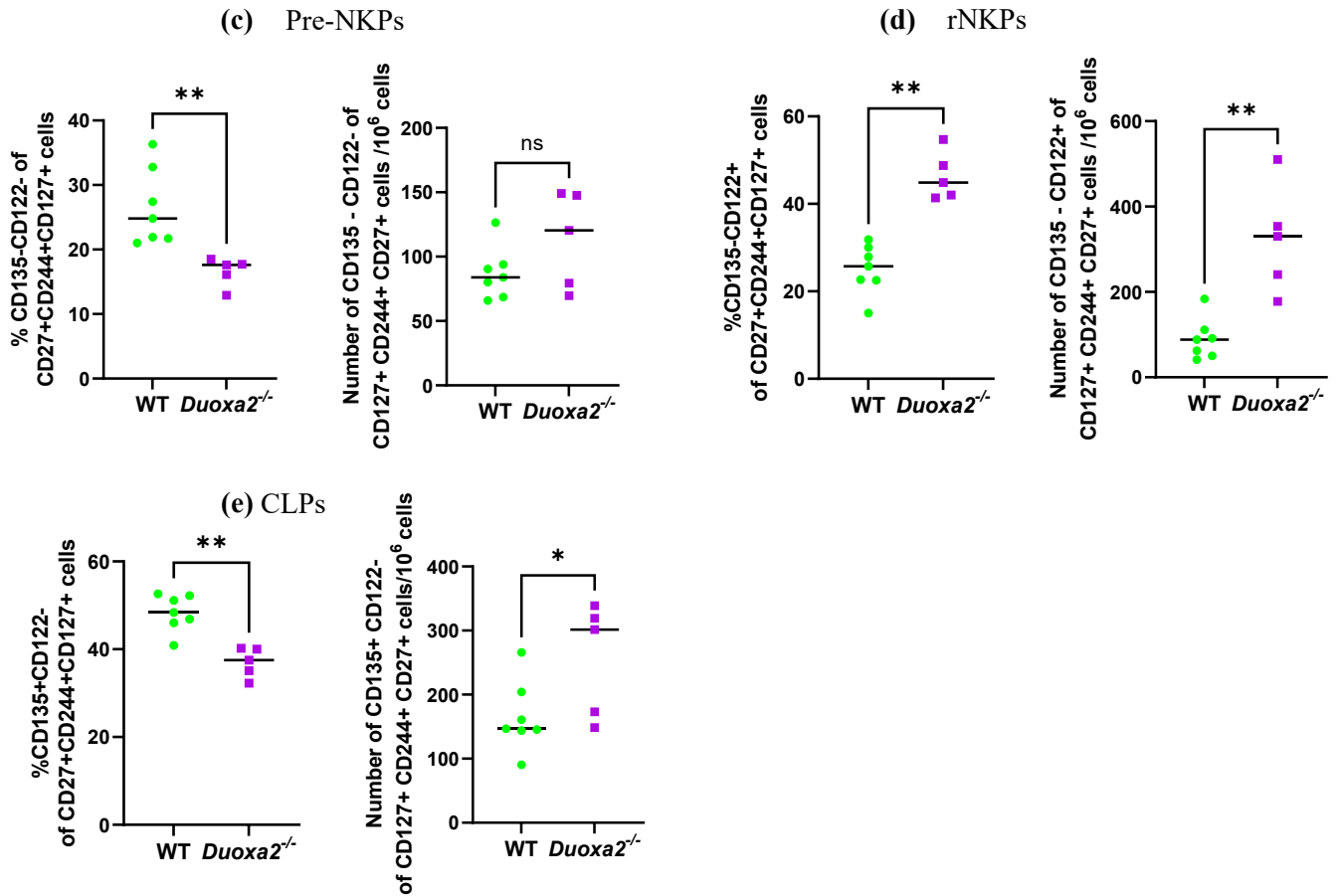


Figure 4.7. Accumulation of refined NK Cell progenitor populations in the bone marrow of *Duoxa2*^{-/-} mice

(c) Proportion and absolute number of pre-NKP cells, (d) proportion and absolute number of rNKP cells and (e) proportion and absolute number of CLPs in littermate controls and *Duoxa2*^{-/-} mice. Data is representative of three independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

4.6. Survival of NK cells is not affected in *Duoxa2*^{-/-} mice

The loss of mature NK cell populations in *Duoxa2*^{-/-} mice could be due to an inability to survive, particularly in the periphery. To further investigate this, apoptosis of NK cells from *Duoxa2*^{-/-} mice at steady state was investigated using Annexin V as a marker of apoptotic cells. As demonstrated in Figure 4.8a, the proportion of Annexin V⁺ cells was not significantly increased in NK cells isolated from the bone marrow (Fig. 4.8b), spleen (Fig. 4.8c) or lung (Fig. 4.8d) of *Duoxa2*^{-/-} mice. In fact, the proportion of Annexin V⁺ cells was significantly decreased in the bone marrow of *Duoxa2*^{-/-} mice compared to littermate controls (Fig. 4.8b). Therefore, no decrease in survival at steady state was detected in NK cells from *Duoxa2*^{-/-} mice compared to littermate controls, and NK cell survival in the bone marrow may in fact be enhanced in *Duoxa2*^{-/-} mice.

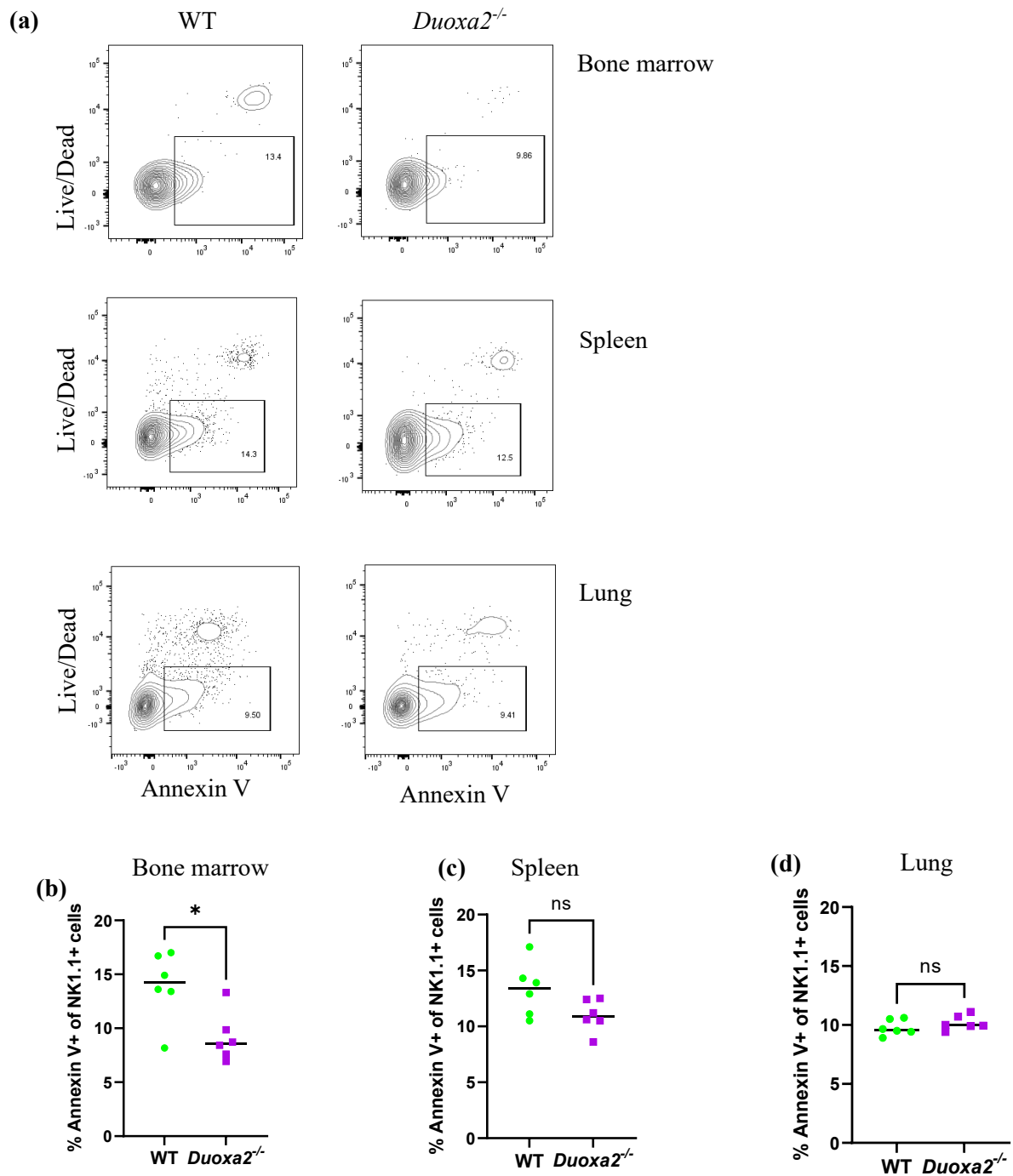


Figure 4.8. Level of apoptosis of NK cells in *Duoxa2*^{-/-} mice at steady state

Annexin V staining was used as a marker of apoptosis to investigate apoptosis occurring at steady state in NK cells from littermate control and *Duoxa2*^{-/-} mice. NK cells were defined as CD45⁺ Lineage⁻ NK1.1⁺ cells. Live/dead stain was used to differentiate dead cells from apoptotic cells. Apoptotic cells were defined as Annexin V⁺ Live/Dead⁻ cells. Dead cells were defined as Annexin V⁺ Live/Dead⁺ cells. (a) Representative flow cytometry plots identifying Annexin V⁺ cells in the bone marrow, spleen and lung of *Duoxa2*^{-/-} mice and littermate controls. Proportion of Annexin V⁺ cells in (b) the bone marrow, (c) spleen and (d) lung of littermate controls and *Duoxa2*^{-/-} mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$ and * $p < 0.01$.

4.7. Cytokine and chemokine transcription is unaffected in the bone marrow of

Duoxa2^{-/-} mice

As there is an accumulation of rNKPs in the bone marrow and a loss of mature NK cell populations in *Duoxa2*^{-/-} mice, it is possible that a defect in the bone marrow microenvironment may be driving defective NK cell maturation. Various chemokines and cytokines secreted in the bone marrow microenvironment are important in NK cell development, maturation and egress. To investigate the conditions of the bone marrow microenvironment in *Duoxa2*^{-/-} mice, the mRNA expression of *Il2*, *Il7*, *Il15* and *Cxcl12* were tested by performing qRT-PCR on RNA extracted from whole bone marrow samples. The expression levels of *Il2*, *Il7*, *Il15* and *Cxcl12* relative to *Hprt* were not significantly different in *Duoxa2*^{-/-} mice compared to littermate controls (Fig. 4.9a-d). There was a trend for a slight decrease in each of the genes, however no statistically significant differences were observed across three independent experiments, indicating that transcription of these genes is not altered in the *Duoxa2*^{-/-} bone marrow microenvironment.

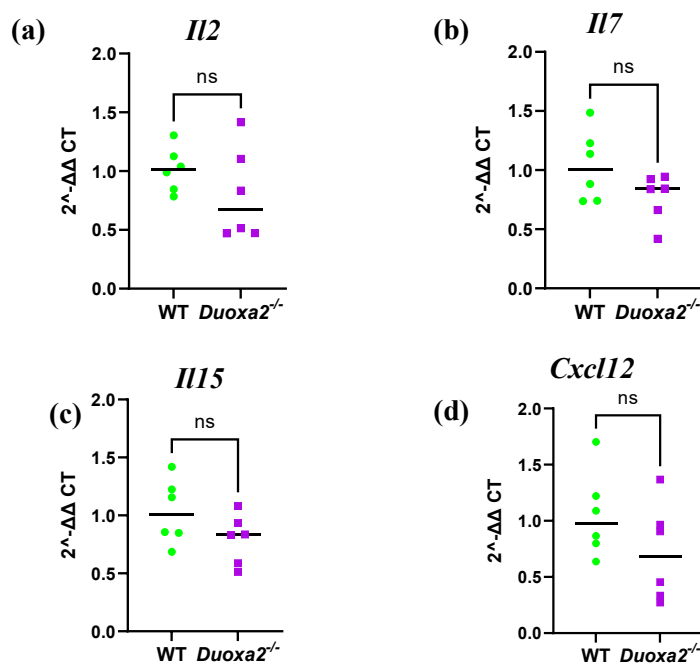


Figure 4.9. *Il2*, *Il7*, *Il15* and *Cxcl12* expression in the bone marrow of *Duoxa2*^{-/-} mice

RNA was extracted from bone marrow isolated from femurs of *Duoxa2*^{-/-} mice and littermate controls. Relative gene expression (2^{-ΔΔCT}) of (a) *Il2*, (b) *Il7*, (c) *Il15* and (d) *Cxcl12* in *Duoxa2*^{-/-} mice and littermate controls. qRT-PCR analysis of gene expression was normalised to the reference gene *Hprt1*. Relative fold change in mRNA expression was compared to littermate controls. Data is representative of three independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns p>0.05.

4.8. Increase in CAR cells in the bone marrow of *Duoxa2*^{-/-} mice

NK cell development primarily occurs in the bone marrow where NKPs give rise to immature NK cells which undergo a maturation process before egressing into the circulation. NK cell development in the bone marrow is supported by the existence of an NK cell niche consisting of a stromal cell network (Noda et al. 2011). Both immune and stromal cell populations are important cellular sources of chemokines and cytokines that are essential for NK cell development and function, including IL-15 and CXCL12. CAR cells are stromal cells located within the bone marrow that exhibit potent secretion of IL-15 and CXCL12, both of which are essential in NK cell development and maturation, with CXCL12 also playing an important role in the regulation of NK cell egress. Most NK cells are found to be in contact with CAR cells in the bone marrow microenvironment, which suggests that they can promote NK cell development by forming an NK cell niche (Noda et al. 2011). NK cells can then egress into bone marrow sinusoids through the sinusoidal epithelium to circulate into the periphery.

To assess whether the NK cell niche was defective in the bone marrow of *Duoxa2*^{-/-} mice, stromal CAR cells were investigated by flow cytometry (Fig. 4.10a). CAR cells were defined as CD45⁻ TER119⁻ CD31⁻ Sca1⁻ PDGFRβ⁺ cells. As depicted in Figure 4.10b, a significant increase in the proportion of PDGFRβ⁺ CAR cells as a proportion of CD45⁻ TER119⁻ CD31⁻ Sca1⁻ cells was detected in the bone marrow stroma of *Duoxa2*^{-/-} mice compared to littermate controls.

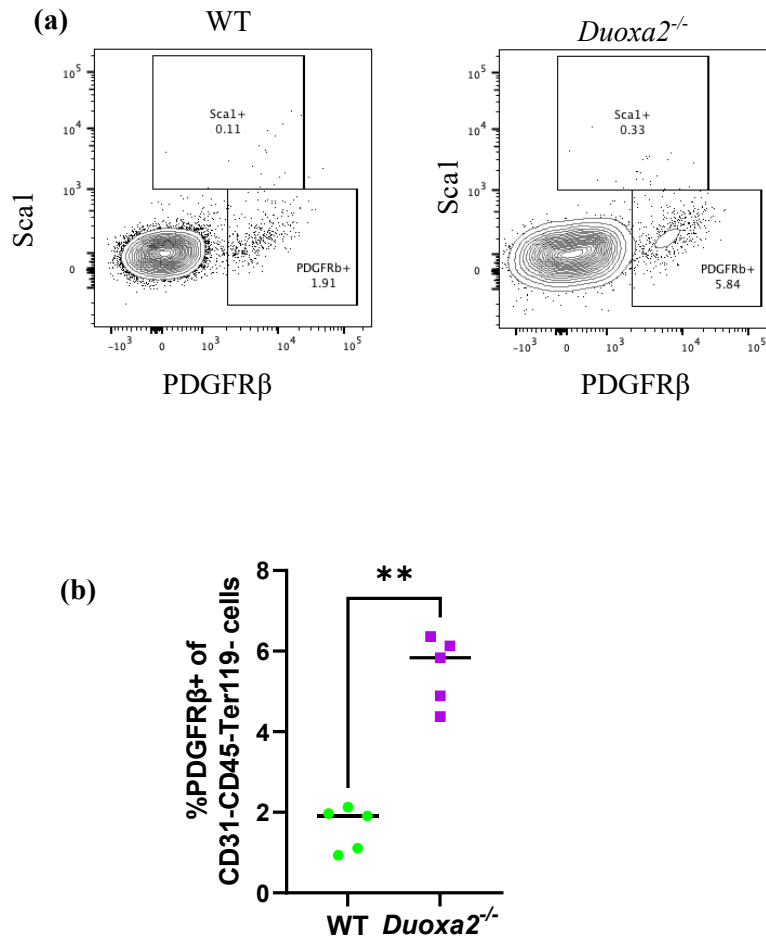


Figure 4.10. Increased proportion of CAR cells in the bone marrow of *Duoxa2*^{-/-} mice

Bone marrow from *Duoxa2*^{-/-} and littermate control mice was enzymatically digested to release stromal cells. (a) Representative flow cytometry plots identifying CAR cells (CD45- TER119- CD31- Scal- PDGFR β +) in the bone marrow of *Duoxa2*^{-/-} and control mice. (b) Proportion of PDGFR β + CAR cells in *Duoxa2*^{-/-} and control mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ** $p < 0.001$.

4.9. Discussion

Mature KLRG1⁺ NK cells were found to be significantly reduced in *Duoxa2*^{-/-} mice (Abeler-Dorner et al. 2020). In this Chapter, NK cell development and maturation were characterised in NKPs, immature and mature NK cells of *Duoxa2*^{-/-} mice to further investigate the mechanism of how loss of DUOXA2 impacts NK cell development in mice. NK cell development is severely disrupted in *Duoxa2*^{-/-} mice, exemplified by a very substantial loss of mature CD27⁻ CD11b⁺ NK cells. This was observed in the bone marrow, spleen, lung and peripheral blood of *Duoxa2*^{-/-} mice. In addition, an accumulation of rNKPs was demonstrated in the bone marrow of *Duoxa2*^{-/-} mice and the proportion and absolute number of immature NK cells amongst total NK cells were also increased, suggesting a developmental block in *Duoxa2*^{-/-} mice. Heterozygous mutations in *Duoxa2* do not result in NK cell defects which suggests that a single copy of *Duoxa2* is sufficient to enable normal NK cell development. In addition, the defect is NK cell-extrinsic as deletion of *Duoxa2* in NK cells specifically did not alter NK cell maturation in this model. Given that *Duoxa2* is not detected in the bone marrow (Chapter 3), this data further supports the hypothesis that DUOXA2 must play a role in a larger global mechanism, and therefore likely indirectly regulates NK cell development and maturation in mice.

In wild-type adult mice, mature NK cells constitute the majority of NK cells found in peripheral tissues, as can be seen in the spleen and lung. The bone marrow also contains mature NK cells, but the majority of NK cells found in this tissue are immature as it is the primary site of NK development (Chiossone et al. 2009), after which NK cells typically egress into the circulation (Abel et al. 2018). Whilst mature NK cell numbers are also reduced in the bone marrow of *Duoxa2*^{-/-} mice, this loss is most striking in the periphery. Although not completely absent, mature NK cells in peripheral sites such as the spleen and lung are drastically reduced both in proportion and absolute number of total NK cells compared to littermate controls. Interestingly, although the overall number of NK cells was decreased in peripheral tissues, an accumulation of total NK cells was observed in the bone marrow, with an increased number compared to littermate controls.

NK cell development in the bone marrow is a complex process involving multiple stages of development to reach terminal functional maturation. Lineage-committed NKPs arise from CLPs, and in turn, they give rise to immature NK cells. Progression through several stages enables immature NK cells to develop into mature NK cells. This process involves the acquisition and loss of various cell surface receptors and requires stage-specific expression of various transcription factors. Mature NK cells then egress into the periphery, although terminal maturation can also continue in the periphery (Chiossone et al. 2009). As *Duoxa2*^{-/-} mice exhibit a build-up of NK cells in the bone marrow and a loss of mature NK cells, the defect could arise at a number of points in this pathway. A block in the developmental programme, an inability to egress into the periphery, or a failure to survive, or indeed a combination of these factors, could underlie the loss of NK cells in *Duoxa2*^{-/-} mice. As *Duoxa2*^{-/-} mice exhibit a paucity of mature NK cells in particular, it is plausible that *Duoxa2*^{-/-} NK cells may possess an innate survival disadvantage and therefore may be unable to survive, especially in the periphery. In addition, as there is also a build up of NK cells in the bone marrow of *Duoxa2*^{-/-} mice, it is also possible that there may be a defect in NK cell egress thereby preventing trafficking of NK cells to peripheral sites resulting in diminished mature NK cell populations. The build-up of NK cells in the bone marrow is also associated with an increase in the proportion and number of immature NK cells when compared to the NK cell populations of littermate controls. This may be indicative of a developmental block at this stage preventing the maturation of immature NK cells.

The results presented in this Chapter demonstrate that the survival of NK cells does not appear to be affected in *Duoxa2*^{-/-} mice. In fact, fewer apoptotic NK cells were detected in the bone marrow of *Duoxa2*^{-/-} mice compared to wild-type controls, suggesting that *Duoxa2* deficiency may in fact enhance NK survival in the bone marrow. As levels of apoptosis were not significantly different in the lung or spleen, it is unlikely that a failure to survive in the periphery is driving the loss of peripheral NK cells in *Duoxa2*^{-/-} mice. However, it would be useful to also investigate NK cell survival by culturing NK cells from *Duoxa2*^{-/-} mice and littermate controls *in vitro* and analysing levels of apoptosis after several days. In addition, incubation of NK cells from *Duoxa2*^{-/-} mice and littermate controls with the OP-9 stromal cell line for 48-72 hours would enable investigation of NK cell survival when *Duoxa2*^{-/-} mice are exposed to wild-type stromal cells.

Investigation of NKP populations identified an accumulation of rNKPs in the bone marrow of *Duoxa2^{-/-}* mice. This is indicative of a defect or block in the NK cell development pathway at the rNKP stage. However, the number of immature NK cells is increased, and not decreased, suggesting that there may in fact be a developmental block preventing the transition and maturation of NK cells from the immature to mature state. As the loss of mature NK cells is so severe, it is tempting to speculate that the accumulation of rNKPs may reflect an effort to upregulate NK cell generation to attempt to release more NK cells into the periphery to bolster the NK cell population. There may also be a developmental block affecting both the rNKP and immature stages of NK cells.

Importantly, egress of NK cells in *Duoxa2^{-/-}* mice has not been addressed in this Chapter. The reduction of NK cells in the periphery and associated accumulation of NK cells in the bone marrow may reflect a defect in egress, rendering NK cells unable to leave the bone marrow. Sinusoidal labelling of CD45⁺ cells by intravenous injection of a fluorescently labelled antibody is a common method used to distinguish NK cells located in the bone marrow parenchyma (CD45⁻) from those which have recently egressed from the bone marrow into the sinusoids (CD45⁺) (Anderson et al. 2014). If the proportion of NK cells in the sinusoids was significantly reduced in *Duoxa2^{-/-}* mice compared to wild-type controls, then this may provide evidence for a defect in egress in *Duoxa2^{-/-}* mice. However, given the small stature of *Duoxa2^{-/-}* mice (Van der Weyden et al. 2020), intravenous injection of these mice is technically difficult and could not be completed during the time frame of this PhD. This could be attempted as part of future experiments with collaborators who have experience with intravenous injection of juvenile mice.

As a defect in NK development was identified in the bone marrow, it is possible that the bone marrow microenvironment may be abnormal in *Duoxa2^{-/-}* mice. Stromal cells and immune cells within the bone marrow are a major source of cytokines and chemokines which are essential in NK cell development. IL-15 is essential in NK cell development and maturation as *Il-15^{-/-}* mice exhibit a severe reduction in peripheral mature NK cell populations (Kennedy et al. 2000). Similarly, CXCL12 plays an important role in promoting both generation of immature NK cells from rNKPs and NK cell maturation as deletion of CXCR4 in adult mice results in diminished numbers of NKPs, immature and mature NK cells (Noda et al. 2011).

Therefore, loss of either of these molecules could result in defective NK cell development. However, the expression levels of *Il15* and *Cxcl12* were unaffected in *Duoxa2^{-/-}* mice. The mRNA expression of *Il2* and *Il7*, which can influence NK cell cytotoxicity, were also unaffected. As transcription levels of all genes investigated were similar to wild-type levels, it is unlikely that alterations in these cytokines and chemokines are driving the NK cell defect. However, analysis of cytokine and chemokine receptor levels by flow cytometry should also be undertaken to confirm these findings. In these experiments, cytokine and chemokine mRNA expression was measured in total RNA extracted from the whole bone marrow. Measuring cytokine protein levels by ELISA could help to confirm if mature cytokine levels are also unaffected in *Duoxa2^{-/-}* mice. It is also important to consider that whilst total gene expression may be unaffected, the spatial localisation of cytokines and therefore localised cytokine concentrations and gradients could potentially be disrupted in the bone marrow microenvironment of *Duoxa2^{-/-}* mice. Temporal and spatial requirement of signalling molecules is essential to promote NK cell development and maturation, and so dysregulation of these signals could potentially result in defective NK cell maturation.

CXCL12/CXCR4 signalling also regulates NK cell egress from the bone marrow as a particular mutation of CXCR4 that prevents downregulation of this receptor results in constitutive CXCR4/CXCL12 signalling, leading to an accumulation of NK cells in the bone marrow and a reduction of NK cells in the bone marrow sinusoids (Mayol et al. 2011). Whilst *Cxcl12* gene transcription was not significantly different, expression of CXCR4 in *Duoxa2^{-/-}* mice must also be investigated in future experiments, as an inability to downregulate CXCR4 could also prevent bone marrow egress. Although CXCR4/CXCL12 signalling is involved in regulation of NK cell egress, it must be acknowledged that other chemokines and proteins are also important in NK cell trafficking, including CX₃CR1 (Ponzetta et al. 2013) and S1P5 (Walzer et al. 2007). Mutation of either of these genes also results in an accumulation of NK cells in the bone marrow and a reduction in the periphery. Loss of S1P5 also results in a reduction in the proportion of mature NK cells in the periphery in particular (Walzer et al. 2007). Consequently, it would be beneficial to also investigate their expression in the bone marrow of *Duoxa2^{-/-}* mice in future.

Interestingly, the proportion of CAR cells in the bone marrow microenvironment was found to be increased in *Duoxa2^{-/-}* mice. CAR cells secrete both CXCL12 and IL-15, and it has been documented that most NK cells in the bone marrow are in contact with CAR cells (Noda et al. 2011). Whilst CXCL12/CXCR4 signalling retains NK cells in the bone marrow, downregulation of CXCR4 and thus the CXCL12/CXCR4 signalling axis permits bone marrow egress. As the proportion of CAR cells was found to be increased, it is possible that the retention signal in the bone marrow is increased in *Duoxa2^{-/-}* mice, thereby preventing bone marrow egress. As CXCL12 can also promote the generation of immature NK cells, it may also be an attempt to increase CXCL12 production to increase NK cell generation. However, mRNA levels of *Cxcl12* were not different to that observed in control mice, despite the conflicting evidence of an increase in the proportion of CAR cells in *Duoxa2^{-/-}* mice identified by flow cytometry. It is possible that although there are more CAR cells in *Duoxa2^{-/-}* mice, individual CAR cells are producing decreased amounts of CXCL12 compared to littermate controls. Further research and characterisation of the bone marrow stroma of *Duoxa2^{-/-}* mice is required to determine whether any stromal cell populations including CAR cells are dysregulated.

Another important factor to consider is the role of transcription factors in NK cell development. Expression of certain transcription factors at discrete stages of NK cell development are essential to ensure the generation of mature, functionally competent NK cells. As the results presented in this Chapter indicate a block in NK cell development, it is possible that transcription of an important transcription factor is not upregulated or downregulated appropriately, thereby resulting in an inability to progress through the subsequent stages of development. As NK cells are still present in *Duoxa2^{-/-}* mice, it is unlikely that transcription factors involved in lineage commitment are affected. NFIL3 is required for lineage commitment as NK cell and NKP numbers are reduced in *Nfil3^{-/-}* mice (Male et al. 2014). ETS1 is also required at the pre-NKP stage as loss of ETS1 resulted in reduced numbers of rNKPs, mature and mature NK cells (Ramirez et al. 2012). Therefore, it is unlikely that *Nfil3* or *Ets1* are dysregulated in *Duoxa2^{-/-}* mice.

T-BET, EOMES, ID2 and ZEB2 are all essential to promote maturation from an immature CD27⁺ CD11b⁻ NK cell into a mature CD27⁻ CD11b⁺ NK cell. EOMES is highly expressed

in immature NK cells where T-BET expression is low. EOMES is subsequently downregulated whilst T-BET is upregulated to promote NK cell maturation (Zhang et al. 2021). ID2 and ZEB2 also play an important role in NK cell maturation and so their expression increases as NK cells mature. Therefore, it is plausible that failure to regulate transcription, or indeed a co-regulator, of one, or perhaps even a combination of these genes, prevents terminal maturation and results in a paucity of mature NK cells as seen in *Duoxa2*^{-/-} mice. Indeed, the phenotype of *Tbx21*^{-/-} mice is similar to that of *Duoxa2*^{-/-} mice, with an increase in NK cells in the bone marrow and a reduction of NK cells in peripheral sites, with a reduction in mature NK cells in particular (Townsend et al. 2004). A similar phenotype is also observed in *Id2*^{-/-} and *Zeb2*^{-/-} mice. NK cell populations are also significantly reduced in *Id2*^{-/-} mice (Yokota et al. 1999), but NKPs are unaffected (Boos et al. 2007). *Zeb2*^{-/-} mice also exhibit a loss of peripheral mature NK cells, with an accumulation of NK cells in the bone marrow (van Helden et al. 2015). STAT5 is also required for normal NK cell development and maturation. Deletion of *Stat5* in NKp46⁺ cells using *Stat5*^{fl/fl}*Ncr1*^{Cre} mice also resulted in reduced peripheral NK cell populations. NKp46 is expressed in NK cells but not NKPs, and so deletion of *Stat5* was induced in immature and mature NK cells only in this model. Interestingly, *Stat5*^{fl/fl}*Ncr1*^{Cre} mice also exhibit an increase in progenitors in the bone marrow (Eckelhart et al. 2011), similar to *Duoxa2*^{-/-} mice which also have an increased number and proportion of rNKPs. As discussed, loss of T-BET, ID2, ZEB2 or STAT5 in mice all result in a loss of mature NK cell populations, particularly in the periphery. This phenotype is very similar to the NK cell defect observed in *Duoxa2*^{-/-} mice and so one, or indeed a combination, of these transcription factors may be dysregulated in *Duoxa2*^{-/-} mice.

The proportion of NKT cells as a proportion of Live CD45⁺ cells was also reduced, but ILC1s were not reduced in the liver of *Duoxa2*^{-/-} mice. Therefore, it may be a transcription factor which is common to both NK cells and NKTs but not ILCs which is dysregulated in the absence of *Duoxa2*. However, many transcription factor networks, including *Tbx21*, *Id2* and *Stat5* are common to both NK and ILC1 development as both cell types are members of the ILC family (Vivier et al. 2018). Therefore, it is possible upstream co-regulators of these genes may differ or may be indirectly regulated by DUOXA2 in specific immune cell types.

It is important to consider that dysregulation of gene expression could in fact affect a combination of genes which are involved in both development and egress. Consequently, it is possible that both NK cell maturation and egress are impaired in *Duoxa2*^{-/-} mice. It is also entirely possible that upstream regulators of the genes involved in NK cell development may be dysregulated, and therefore preventing the appropriate upregulation or downregulation of genes involved in NK cell development. In addition, although NK cell development is defective in *Duoxa2*^{-/-} mice likely due to a transcriptional defect, this may not be entirely intrinsic to NK cells and could also result from an abnormal bone marrow microenvironment if stromal cell populations are also disrupted.

In order to further investigate the mechanism at play, transcription of genes important in NK cell development and trafficking should be investigated in NK cells from *Duoxa2*^{-/-} and wild-type mice. Transcriptomic analysis would provide detailed profiling of all pathways and genes important in the NK cell developmental programme, thereby identifying the exact mechanism which is driving the defect in NK cells in *Duoxa2*^{-/-} mice. Single cell RNA-sequencing of the whole bone marrow could demonstrate which transcription networks are disrupted. However, if this defect is specific to a small group of cells, or NK cells only, whole-bone marrow sequencing may not provide a clearly defined result. RNA sequencing of specific subsets such as rNKPs or immature NK cells would allow focused analysis and therefore would enable identification of the exact stage at which the transcriptional dysregulation occurs.

In summary, *Duoxa2*^{-/-} mice have an accumulation of rNKPs in the bone marrow and reduced mature NK cell populations, particularly in the periphery. This NK cell-extrinsic defect suggests that there is a developmental block in *Duoxa2*^{-/-} mice and that DUOXA2 plays an indirect role in NK cell development and maturation.

5. Investigating the Role of Thyroid Hormone in NK Cell Development

5.1. *Slc26a7*^{-/-} mice do not have a defect in NK cell development

An association between thyroid hormone and immune cell function has been suggested as stimulation with thyroid hormones has been demonstrated to increase the effector functions of various immune cells including macrophages (Videla et al. 1993), DCs (Mascanfroni et al. 2008) and NK cells. To date, the evidence on the role of thyroid hormones in NK cells is conflicting and limited. IFN- γ secretion is increased when NK cells are supplemented with T₃ or T₄ (Provinciali and Fabris, 1987). However, in patients with hyperthyroidism, NK cells exhibit reduced cytotoxic capacity (Papic et al. 1987). As *Duoxa2*^{-/-} mice have congenital hypothyroidism, it was important to investigate the extent to which hypothyroidism may contribute to the NK cell defect observed in this model. Therefore, the NK cell phenotype of a different mouse model of hypothyroidism was investigated. *Slc26a7*^{-/-} mice are a novel model of congenital hypothyroidism that exhibit goitrous congenital hypothyroidism (Cangul et al. 2018). In this study, *SLC26A7* mutations were first identified in humans with congenital hypothyroidism lacking mutations in common causative genes. The mechanism by which *SLC26A7* mutation results in hypothyroidism remains unclear, but it has been suggested to function as an anion transporter in the thyroid (Cangul et al. 2018). Another study suggested that *SLC26A7* may be involved in iodide efflux in the thyroid (Ishii et al. 2019).

Analysis of the NK cell populations of *Slc26a7*^{-/-} mice was undertaken by flow cytometry to determine whether they possessed similar defects to those previously identified in *Duoxa2*^{-/-} mice. No major defects were detected in the NK cell populations in the bone marrow, lung or spleen of *Slc26a7*^{-/-} mice. The overall proportion of NK cells was not significantly altered in either *Slc26a7*^{+/-} or *Slc26a7*^{-/-} mice compared to wild-type controls, as shown in Figure 5.1a. Similarly, no significant differences were detected in the immature CD27⁺ CD11b⁻ (Fig. 5.1b) or mature CD27⁻ CD11b⁺ (Fig. 5.1c) NK cell populations in either *Slc26a7*^{+/-} or *Slc26a7*^{-/-} mice. NK cell populations appeared unaffected in *Slc26a7*^{-/-} hypothyroid mice, suggesting that the defect in NK cell maturation observed in *Duoxa2*^{-/-} mice is independent of thyroid hormone. However, it is important to note that the level of hypothyroidism in *Slc26a7*^{-/-} mice is not as severe as *Duoxa2*^{-/-} mice. As observed during these experiments,

Slc26a7^{-/-} mice do not have a growth defect and so consequently the level of hypothyroidism is not as severe.

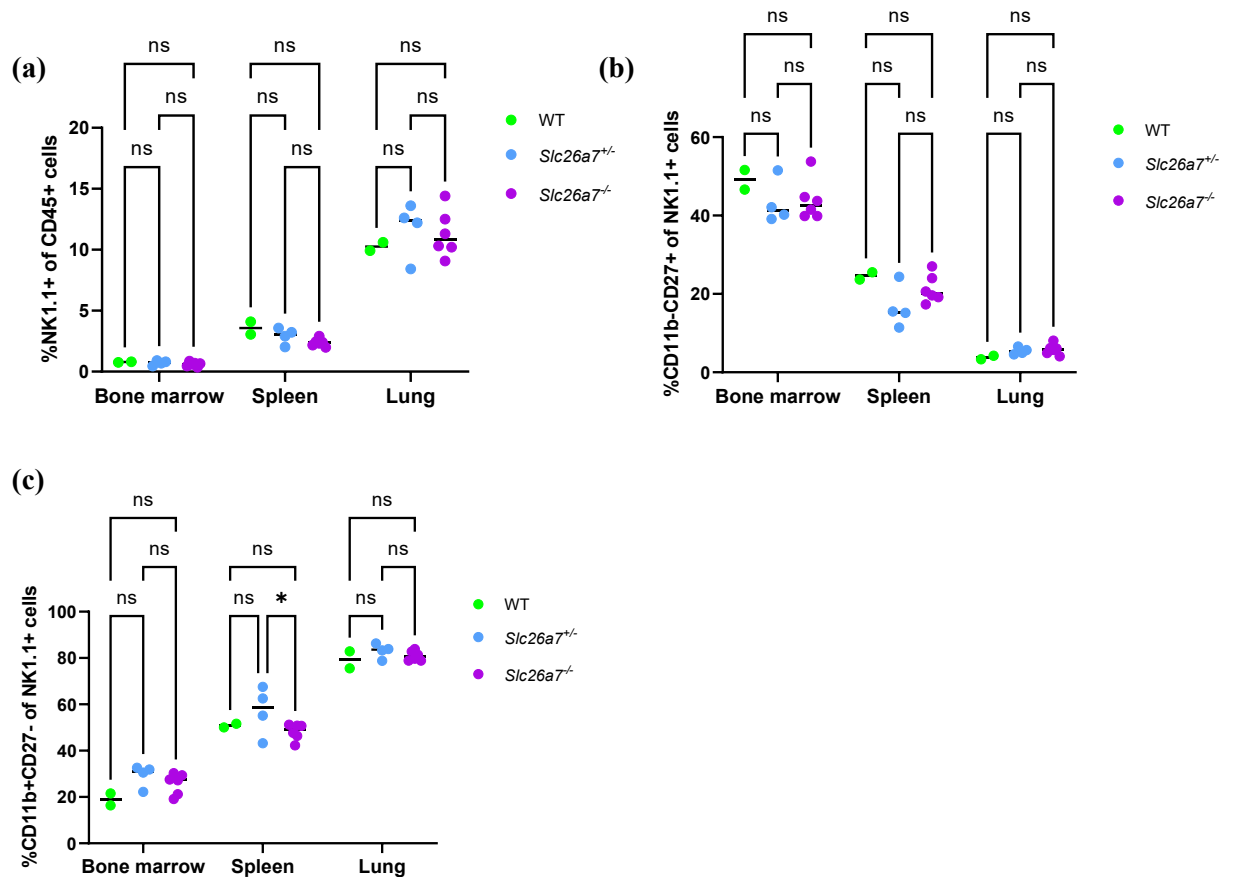


Figure 5.1. NK cell populations are unaffected in *Slc26a7*^{-/-} hypothyroid mice

The NK cell populations of *Slc26a7*^{-/-}, *Slc26a7*^{+/-} and wild-type mice were analysed by flow cytometry. NK cells were defined as Live CD45+ NK1.1+ cells. (a) Proportion of NK cells, (b) proportion of immature CD27+ CD11b- NK cells and (c) proportion of mature CD27- CD11b+ NK cells in the bone marrow, spleen and lung of *Slc26a7*^{-/-}, *Slc26a7*^{+/-} and wild-type mice. Data is representative of two independent experiments. Statistical significance was determined using multiple Mann-Whitney rank tests with Holm Sidak's multiple testing correction. ns p>0.05 and * p < 0.05.

5.2. NK cell populations are defective in PTU-induced hypothyroid mice

In order to further investigate the extent to which hypothyroidism contributes to the NK cell defect, another mouse model of hypothyroidism was tested. PTU-induced hypothyroidism is a common model used to render wild-type mice severely hypothyroid in scientific research. The mechanism of PTU action involves the inhibition of the TPO enzyme which in turn prevents the generation of the thyroid hormones T₃ and T₄ (Vella et al. 2014). Six week old wild-type C57BL/6 mice were fed a low-iodine 0.15% PTU diet for a total of six weeks to render them hypothyroid. After six weeks, their NK cell populations were then investigated by flow cytometry to determine whether any defects similar to those found in *Duoxa2^{-/-}* mice could be identified in the bone marrow, spleen and lung. A representative gating strategy is shown in Figure 5.2a.

As shown in Figure 5.2b, the number of total NK cells and the overall proportion of total NK cells (Fig. 5.3a) in PTU-induced hypothyroid mice is significantly increased in the bone marrow. The absolute number (Fig. 5.2b) and proportion (Fig. 5.3a) of NK cells is significantly reduced in the lung. A trend for a decrease in the number of total NK cells was also observed in the spleen although this was not significant (Fig. 5.2a). The number of immature CD27⁺ CD11b⁻ NK cells amongst total NK cells was also significantly increased in the bone marrow of hypothyroid mice compared to untreated controls (Fig. 5.2c). The proportion of immature NK cells in the spleen and lung was significantly increased (Fig. 5.3b), although no difference in the number of immature NK cells in the spleen or the bone marrow was observed (Fig. 5.2c). Similar to *Duoxa2^{-/-}* mice, PTU-induced hypothyroid mice had a significant loss of both the proportion (Fig. 5.3c) and absolute number of mature CD27⁻ CD11b⁺ NK cells (Fig. 5.2d) amongst total NK cells both in the bone marrow and in peripheral tissues including the spleen and lung.

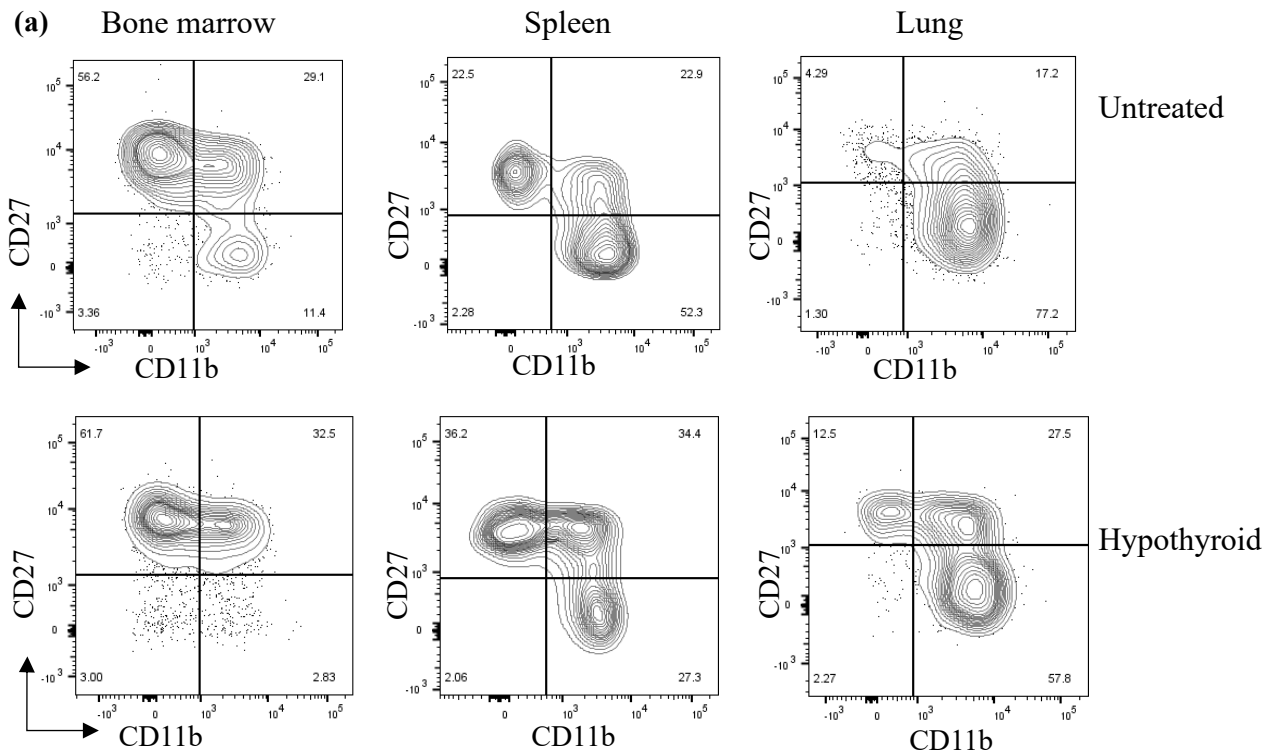


Figure 5.2. Absolute numbers of mature NK cells are decreased in PTU-induced hypothyroid mice

NK cell populations of hypothyroid and untreated C57BL/6 control mice were analysed by flow cytometry. Hypothyroidism was induced in wild-type mice by feeding a low-iodine 0.15% PTU diet for six weeks. Control mice were fed normal chow. (a) Representative gating strategy to define NK cells in bone marrow, spleen and lung. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression.

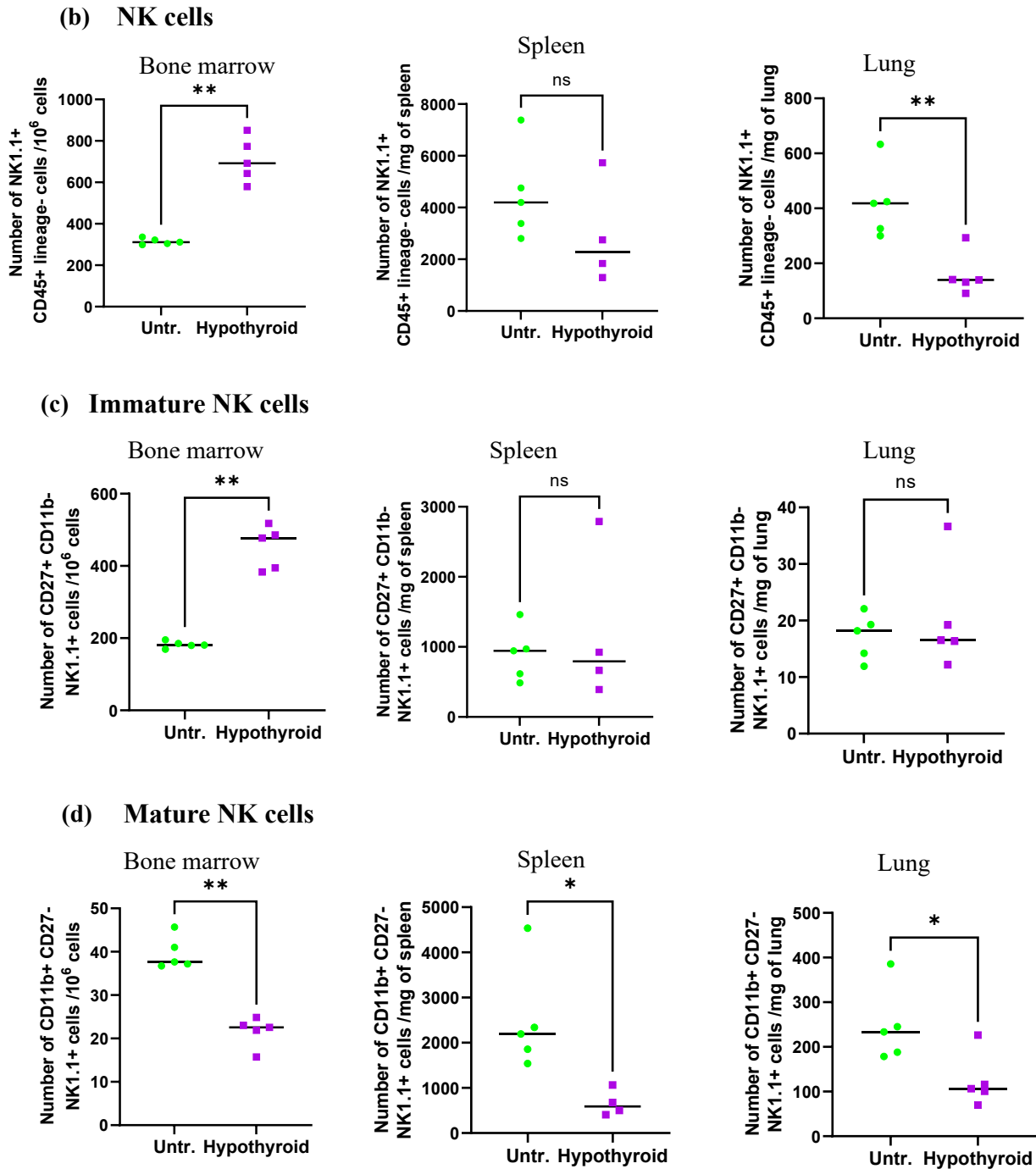
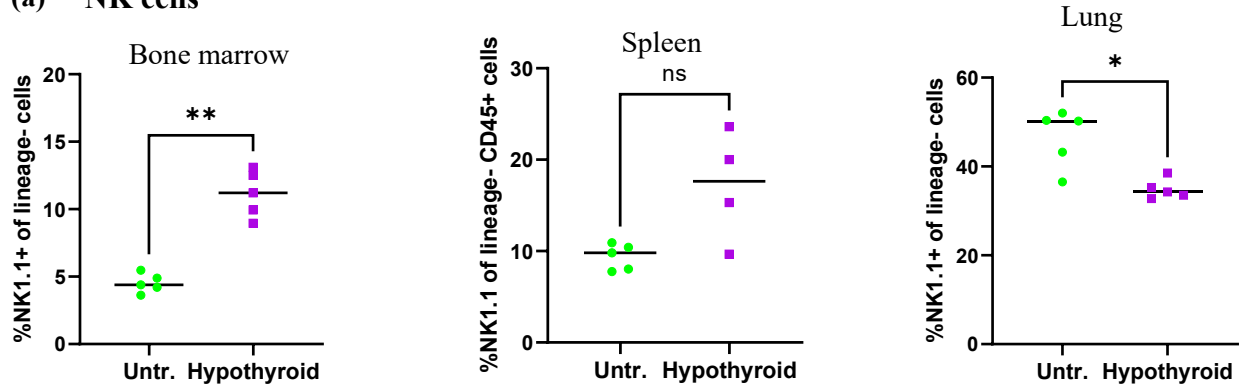


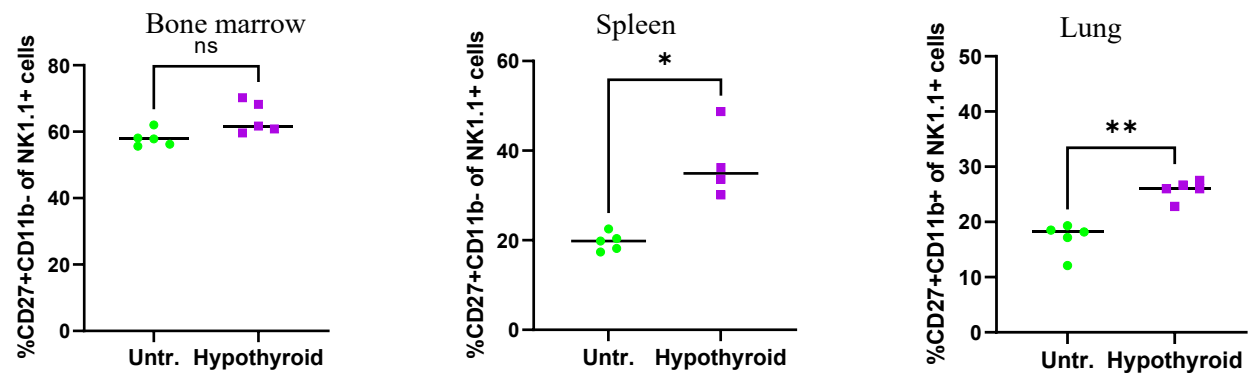
Figure 5.2. Absolute numbers of mature NK cells are decreased in PTU-induced hypothyroid mice

(b) Number of NK cells, (c) number of immature CD27+ CD11b- NK cells and (d) number of mature CD27- CD11b+ NK cells in the bone marrow, lung and spleen of untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

(a) NK cells



(b) Immature NK cells



(c) Mature NK cells

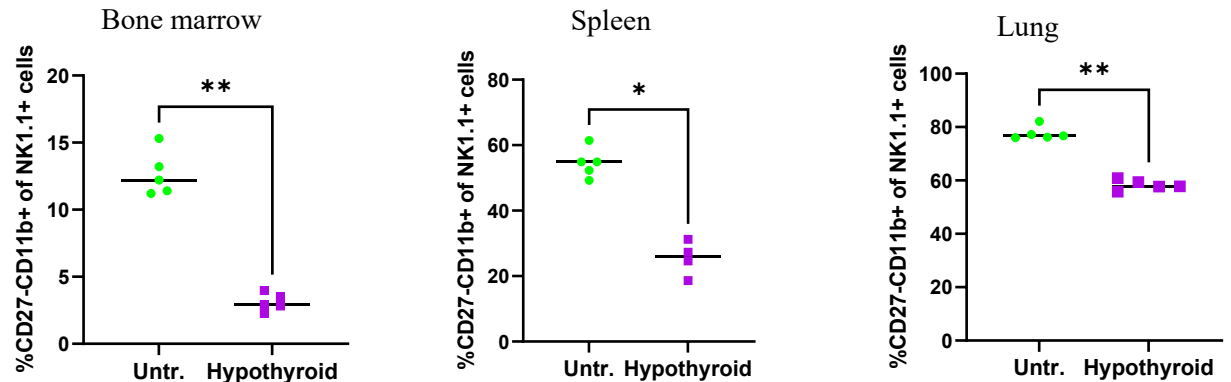


Figure 5.3. Proportion of mature NK cells is reduced in PTU-induced hypothyroid mice

NK cell populations of hypothyroid and untreated C57Bl/6 control mice were analysed by flow cytometry. Hypothyroidism was induced in wild-type mice by feeding a low-iodine 0.15% PTU diet for six weeks. Control mice were fed normal chow. NK cells were defined as Live CD45+ Lineage- NK1.1+ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression. (a) Proportion of NK cells, (b) proportion of immature CD27+ CD11b- NK cells and (c) proportion of mature CD27- CD11b+ NK cells amongst Live CD45+ Lineage- cells in the bone marrow, lung and spleen of untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

5.3. Accumulation of NK progenitors in PTU-induced hypothyroid mice

As discussed previously, a defect in NK cell development and maturation, a failure to egress from the bone marrow or a failure to survive in the periphery could underlie the loss of mature NK cell populations in *Duoxa2^{-/-}* mice. In hypothyroid mice, NK cells were observed to accumulate in the bone marrow and a loss of mature NK cells was also observed (Fig. 5.2). In order to gain further mechanistic insight into the origin of the NK cell defect, NK progenitor populations were investigated by flow cytometry. Representative flow cytometry plots are shown in Figure 5.4a. NK cell progenitors were gated as Lineage- CD244⁺ CD27⁺ CD127⁺ cells, and then further divided into subpopulations based on CD135 and CD122 expression. CLPs were defined as Lineage- CD27⁺ CD244⁺ CD127⁺ CD135⁺ CD122⁻ cells. Pre-NKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁻ cells. As the transition from pre-NKP to rNKP is marked by CD122 acquisition, rNKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁺ cells.

As is evident in Figure 5.4, there is an accumulation of rNKPs in hypothyroid mice. The proportion of CLPs (Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁺ CD122⁻ cells) was increased in hypothyroid mice, but the absolute number of CLPs was decreased (Fig. 5.4b). The absolute number of pre-NKPs (Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁻ cells) was also significantly increased, but the overall proportion of pre-NKPs was not different compared to untreated control mice (Fig. 5.4c). Both the overall proportion and absolute number of rNKPs (Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁺ cells) (Fig. 5.4d) were significantly increased in the bone marrow of PTU-induced hypothyroid mice compared to littermate controls, similar to *Duoxa2^{-/-}* mice.

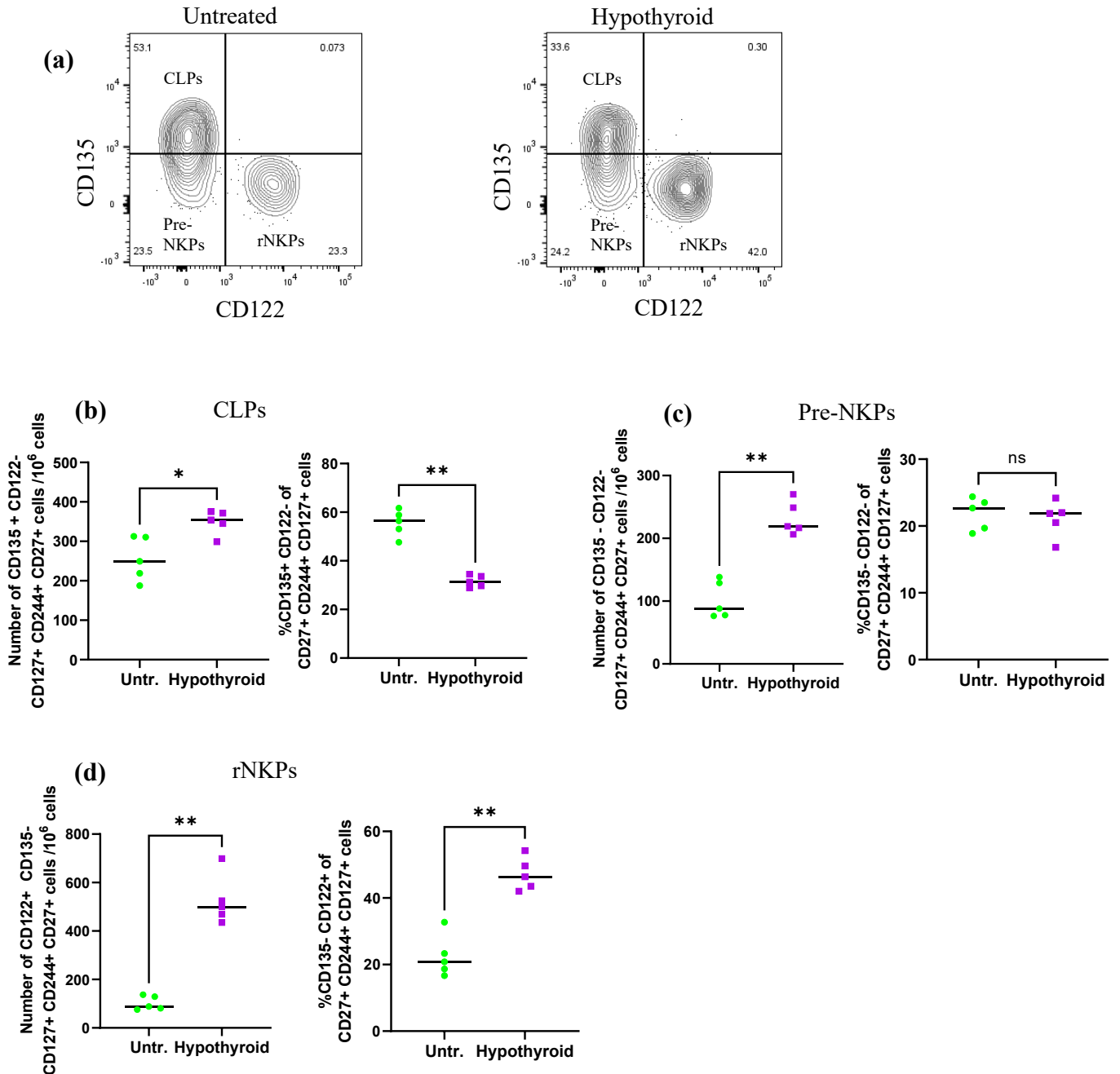


Figure 5.4. Characterisation of NK progenitor populations of hypothyroid mice

Bone marrow was isolated from hypothyroid and untreated C57BL/6 control mice and NK progenitors were identified by flow cytometry. Hypothyroidism was induced in wild-type mice by feeding a low-iodine 0.15% PTU diet for six weeks. Control mice were fed normal chow. (a) Representative flow cytometry plots of CLP (Lineage- CD27+ CD244+ CD127+ CD135+ CD122-), pre-NKP (Lineage- CD27+ CD244+ CD127+ CD135- CD122-) and refined NKP (Lineage- CD27+ CD244+ CD127+ CD135- CD122+) cells in untreated control and hypothyroid mice. (b) Proportion and absolute number of CLPs, (c) pre-NKP cells and (d) rNKP cells in untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

5.4. NK cell populations are defective in hypothyroid mice after two weeks on PTU diet

Ingestion of a low-iodine 0.15% PTU diet for six weeks rendered wild-type mice hypothyroid and resulted in an accumulation of NK progenitors and NK cells in the bone marrow and a loss of mature NK cell populations, similar to the NK cell defect observed in *Duoxa2^{-/-}* mice. To test the dynamics of this effect and whether NK cell maturation may require continuous thyroid hormone under normal conditions, six week old wild-type mice were fed a low-iodine 0.15% PTU diet for a shorter time period of two weeks. After two weeks, their NK cell populations were analysed by flow cytometry. Representative flow cytometry plots are shown in Figure 5.5a.

Both the overall number of NK cells (Fig. 5.5b) and the proportion of total NK cells (Fig. 5.6a) amongst Live CD45⁺ Lineage⁻ cells was significantly increased in the bone marrow of hypothyroid mice compared to untreated controls. The number of NK cells was significantly decreased in the lung of hypothyroid mice with a slight decrease also observed in the spleen (Fig. 5.5b) although the proportion of NK cells in these tissues was not different (Fig. 5.6a). The number of immature CD27⁺ CD11b⁻ NK cells was also significantly increased in the bone marrow and spleen of hypothyroid mice, but no difference was detected in immature NK cells in the lung compared to untreated controls (Fig. 5.5c), although both exhibited an increased overall proportion amongst total NK cells (Fig. 5.6b). There was a loss of mature NK cells in the periphery, as both the number (Fig. 5.5d) and overall proportion (Fig. 5.6c) of CD27⁻ CD11b⁺ NK cells amongst total NK cells were significantly decreased in the spleen and lung of hypothyroid mice. No significant difference was detected in the number of mature NK cells in the bone marrow (Fig. 5.5d), although the overall proportion was decreased compared to wild-type controls (Fig. 5.6c).

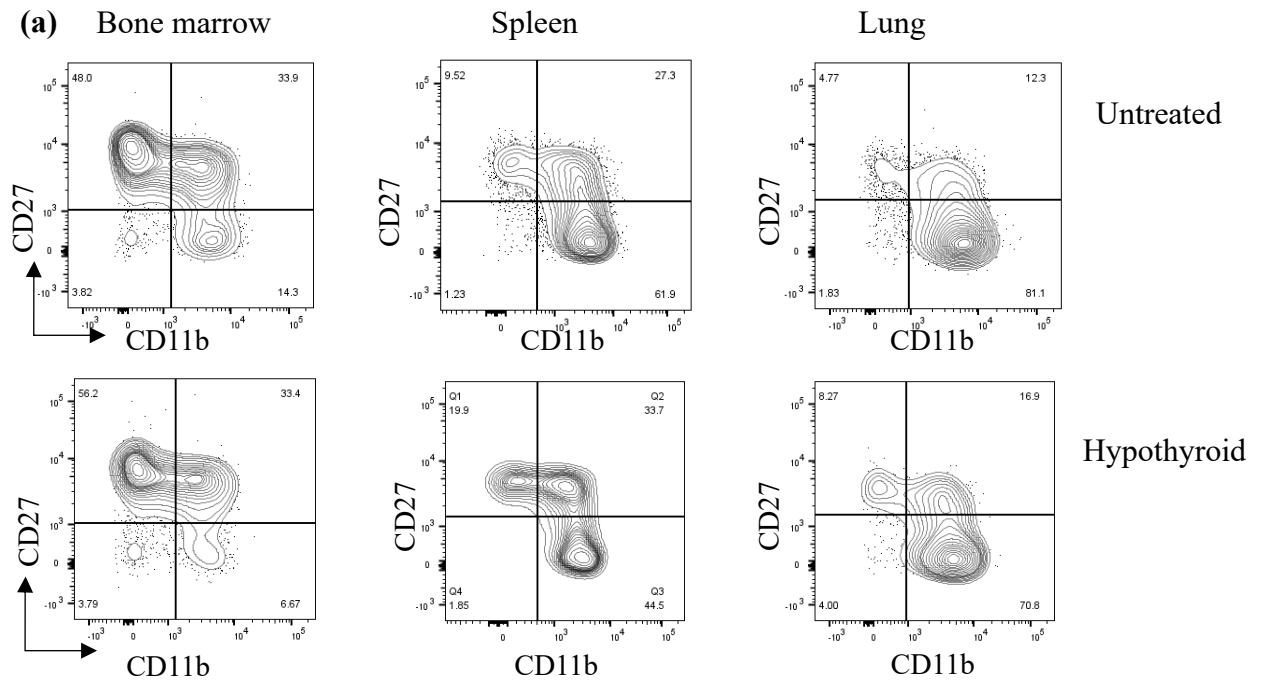
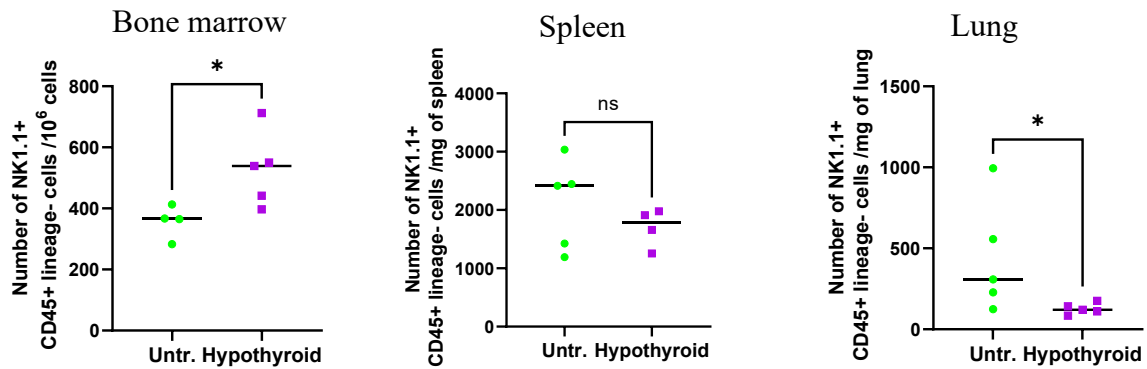


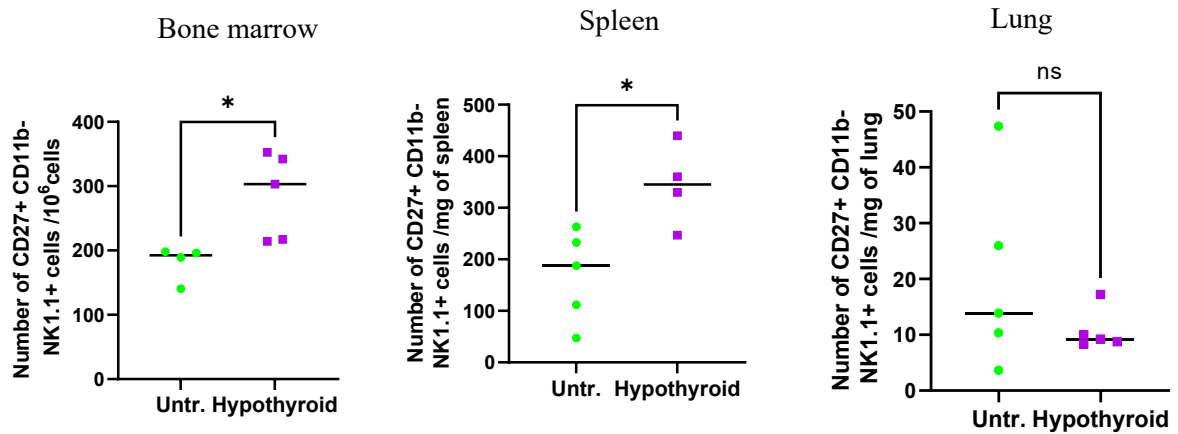
Figure 5.5. NK Cell populations of mice fed a low-iodine 0.15% PTU diet for two weeks

Hypothyroidism was induced in wild-type mice by feeding a low-iodine 0.15% PTU diet for two weeks. Control mice were fed normal chow. NK cell populations of hypothyroid and untreated C57BL/6 control mice were analysed by flow cytometry. (a) Representative flow cytometry plots to define mature and immature NK cells in bone marrow, spleen and lung. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression

(b) NK cells



(c) Immature NK cells



(d) Mature NK cells

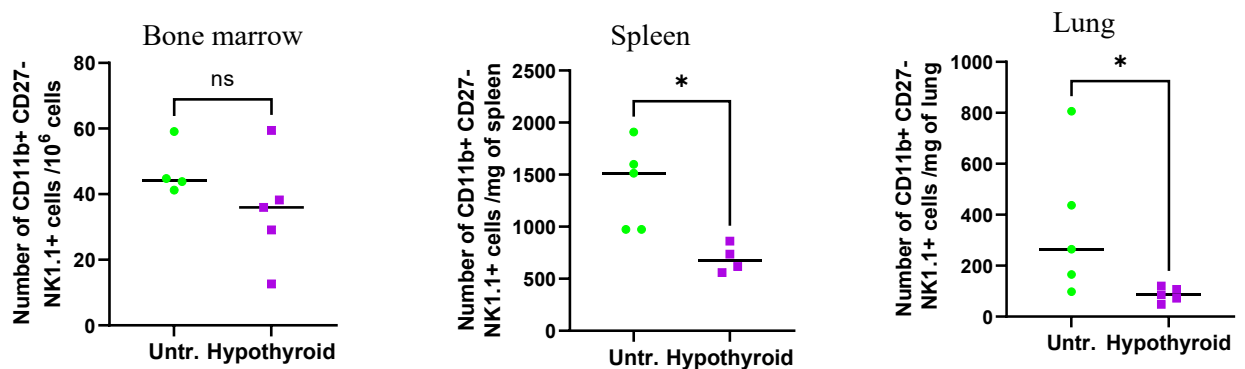


Figure 5.5. NK Cell populations of mice fed a low-iodine 0.15% PTU diet for two weeks

(b) Number of NK cells, (c) number of immature CD27+ CD11b- NK cells and (d) number of mature CD27- CD11b+ NK cells in the bone marrow, lung and spleen of untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

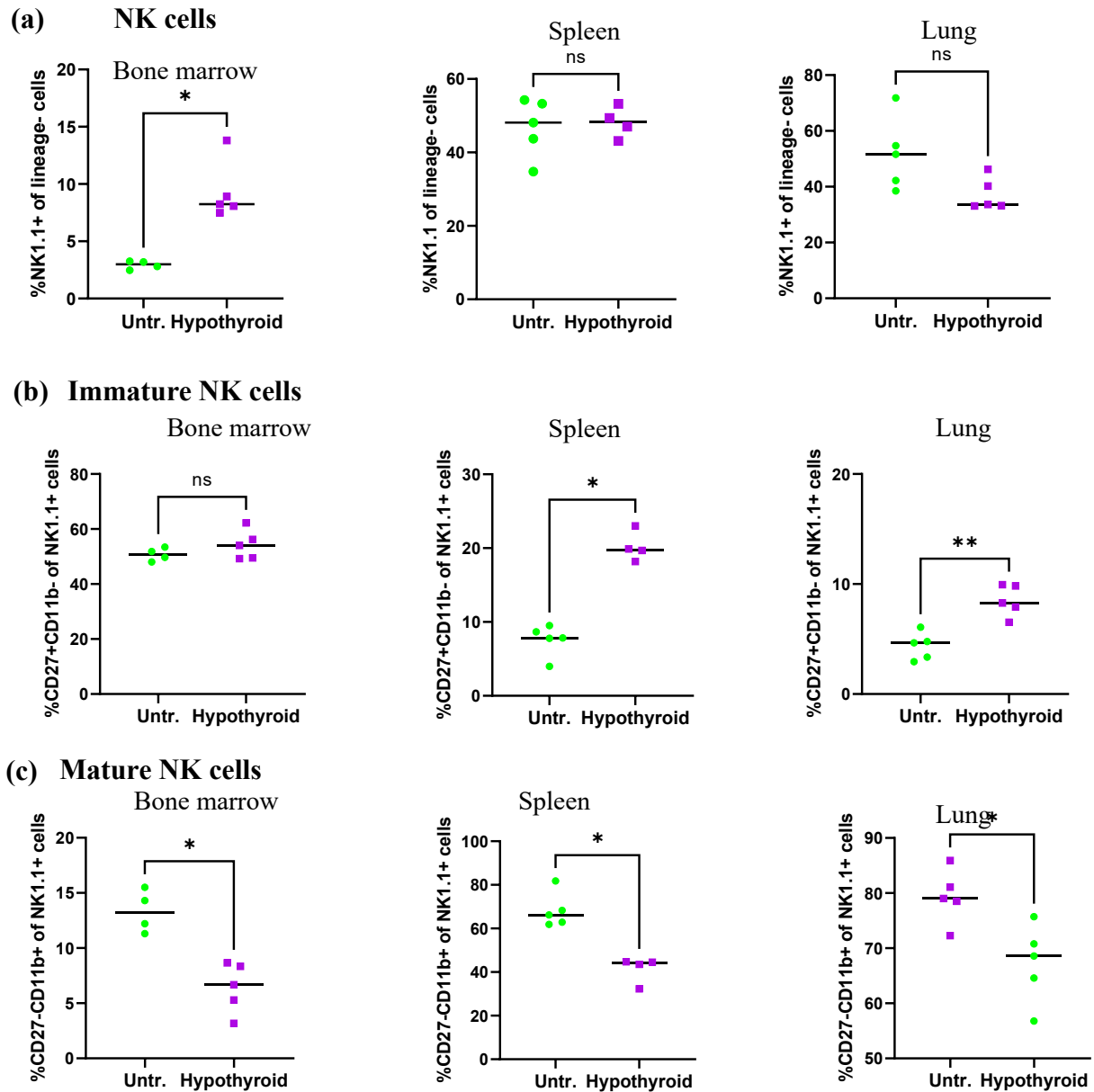


Figure 5.6. Proportion of mature NK cells is reduced in fed a low-iodine 0.15% PTU diet for two weeks

NK cell populations of hypothyroid and untreated C57BL/6 control mice were analysed by flow cytometry. Hypothyroidism was induced in wild-type mice by feeding a low-iodine 0.15% PTU diet for two weeks. Control mice were fed normal chow. NK cells were defined as Live CD45+ Lineage- NK1.1+ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression. (a) Proportion of NK cells, (b) proportion of immature CD27+ CD11b- NK cells and (c) proportion of mature CD27- CD11b+ NK cells amongst Live CD45+ Lineage- cells in the bone marrow, lung and spleen of untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns p>0.05, * p<0.01 and ** p<0.001.

5.5. Accumulation of NK progenitors in hypothyroid mice fed a low-iodine 0.15% PTU diet for two weeks

In hypothyroid mice fed a low-iodine 0.15% PTU diet for two weeks, NK cells accumulated in the bone marrow and a loss of mature NK cells was also observed in the periphery (Fig. 5.5 and 5.6). To assess whether the earlier stages of NK development were affected and gain further mechanistic insight, NK progenitor populations were investigated by flow cytometry. Representative flow cytometry plots are shown in Figure 5.7a. CLPs were defined as Lineage- CD27⁺ CD244⁺ CD127⁺ CD135⁺ CD122⁻ cells. Pre-NKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁻ cells. As the transition from pre-NKP to rNKP is marked by CD122 acquisition, rNKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁺ cells.

The proportion of CLPs amongst Lineage- CD244⁺ CD27⁺ CD127⁺ cells was significantly decreased in hypothyroid mice, although the absolute number of CLPs was similar between hypothyroid mice and untreated controls (Fig. 5.7b). The proportion of pre-NKPs was also significantly decreased in hypothyroid mice, although the absolute number of pre-NKPs was similar between hypothyroid mice and untreated controls (Fig. 5.7c). As is evident in Figure 5.7d, there is an accumulation of rNKPs in hypothyroid mice. Both the proportion and absolute number of rNKPs was significantly increased in the bone marrow of hypothyroid mice after two weeks, similar to *Duoxa2*^{-/-} mice (Fig. 5.7d)

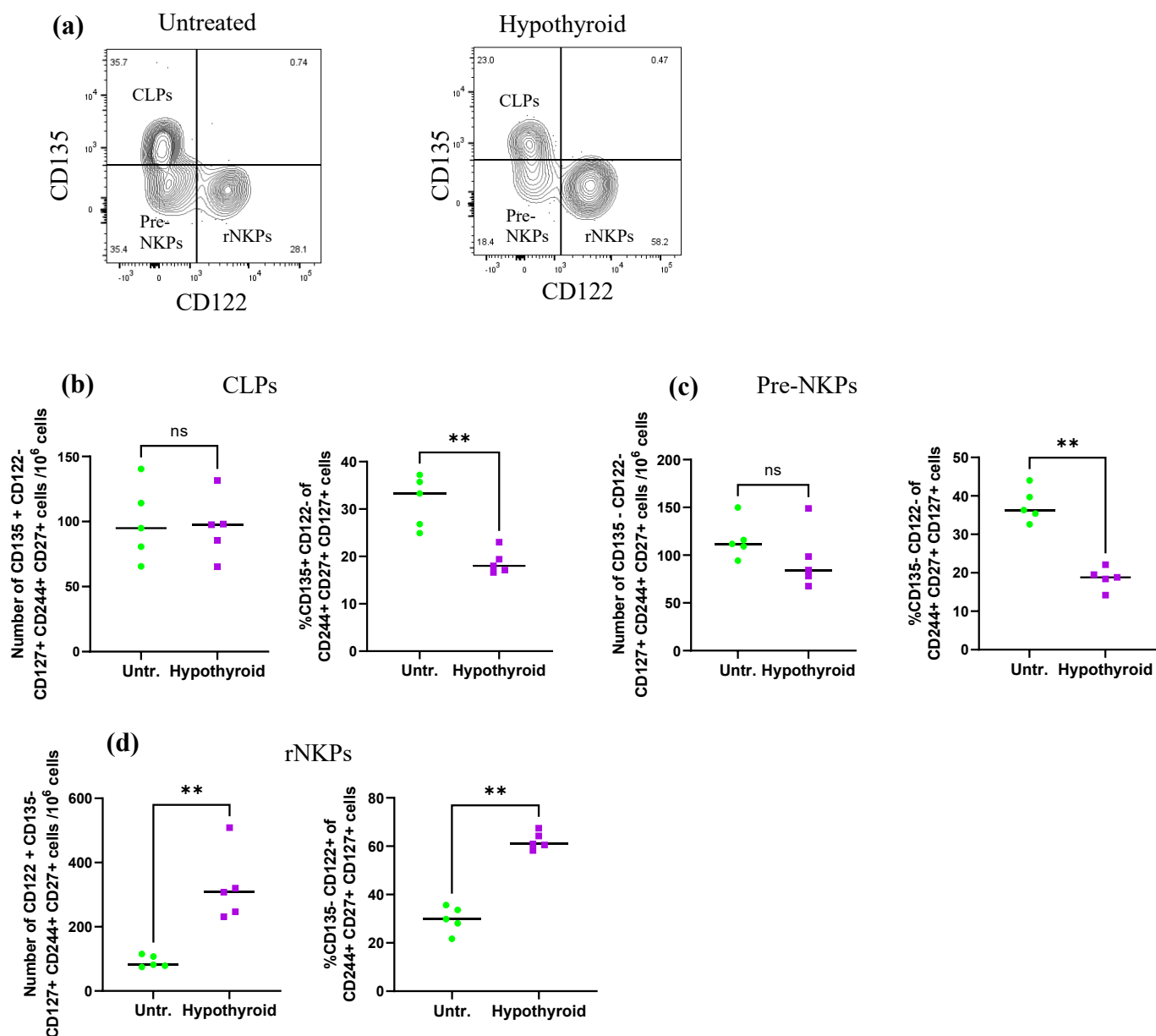


Figure 5.7. Characterisation of NK progenitor populations in mice fed a low-iodine 0.15% PTU diet for two weeks

Hypothyroidism was induced in wild-type mice by feeding a low-iodine 0.15% PTU diet for a short time period of two weeks. Control mice were fed normal chow. Bone marrow was isolated from hypothyroid and untreated control mice and NK progenitors were identified by flow cytometry. (a) Representative flow cytometry plots of CLP (Lineage- CD27+ CD244+ CD127+ CD135+ CD122-), pre-NKP (Lineage- CD27+ CD244+ CD127+ CD135- CD122-) and refined NKP (Lineage- CD27+ CD244+ CD127+ CD135- CD122+) cells in wild-type and hypothyroid mice. (b) Absolute number and proportion of CLPs, (c) pre-NKP cells and (d) rNKP cells in untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

5.6. NK cell populations are also defective in mice rendered hypothyroid from 9.5 weeks of age

In six-week old mice, induction of hypothyroidism led to NK cell defects after both two and six weeks of dietary intervention. To test whether this effect only occurs in younger mice, or whether it can occur at any age, slightly older 9.5 week-old wild-type mice were fed a low-iodine 0.15% PTU diet for six weeks. NK cells in the bone marrow, spleen and lung of untreated and hypothyroid mice were then investigated by flow cytometry (Fig. 5.8a)

An accumulation of NK cells was observed in the bone marrow of mice fed a low-iodine 0.15% PTU diet from 9.5 weeks of age. The absolute number (Fig. 5.8b) and proportion (Fig. 5.9a) of NK cells amongst total NK cells was significantly increased in the bone marrow of hypothyroid mice compared to untreated controls. No significant difference in the number of NK cells was detected in the spleen and lung of hypothyroid mice, although there was a trend for a slight decrease, particularly in the lung (Fig. 5.8b). The number of immature CD27⁺CD11b⁻ NK cells amongst total NK cells was also significantly higher in the bone marrow (Fig. 5.8c) of hypothyroid mice compared to untreated controls, although the overall proportion was unchanged (Fig. 5.9b). This trend was also evident in the spleen and lung and was significant in terms of overall proportion (Fig. 5.9b) but was not statistically significant in terms of absolute number (Fig. 5.8c). A loss of peripheral mature NK cells was also observed in hypothyroid mice as both the overall proportion (Fig. 5.9c) and number of CD27⁻CD11b⁺ NK cells (Fig. 5.8d) was significantly reduced in spleen and lung. The overall proportion of mature NK cells amongst total NK cells was also reduced in the bone marrow (Fig. 5.9c), but no reduction in terms of absolute cell number was detected (Fig. 5.8d).

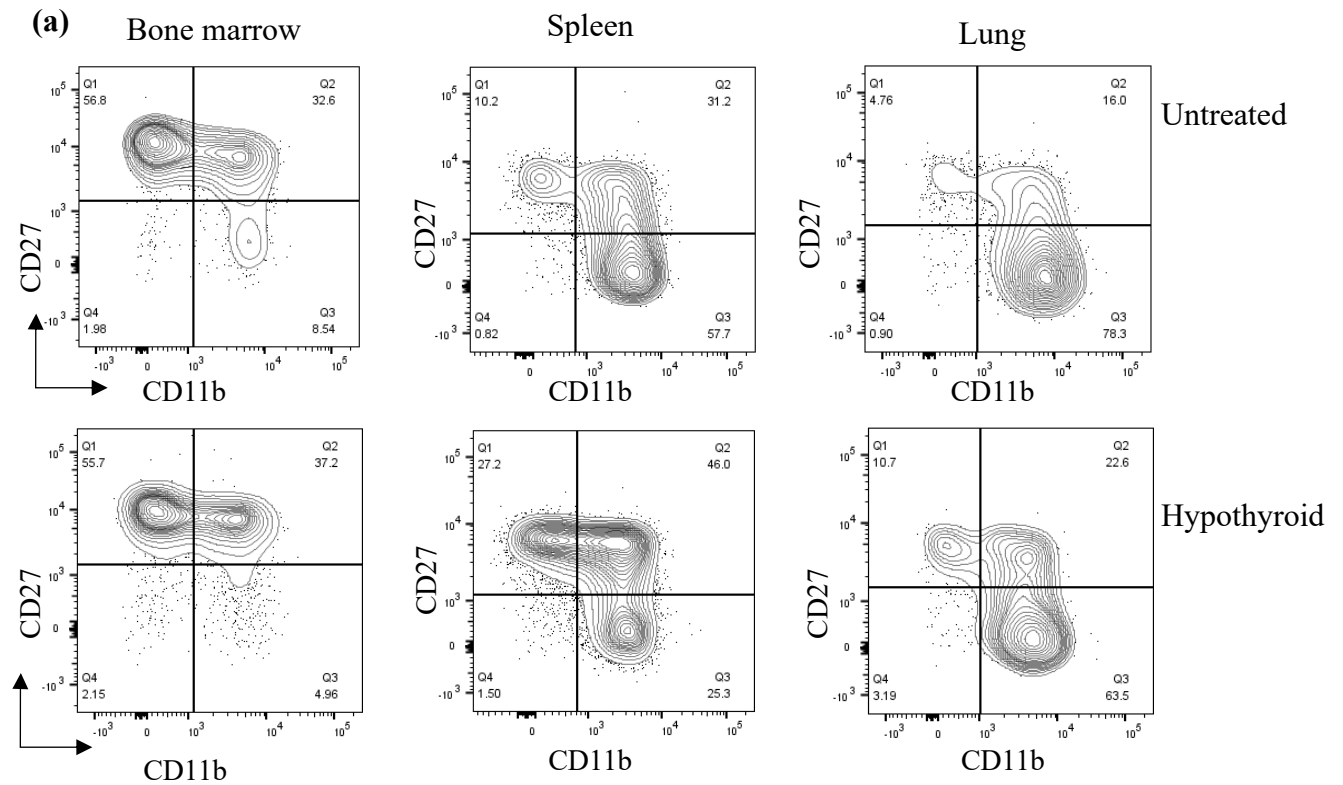
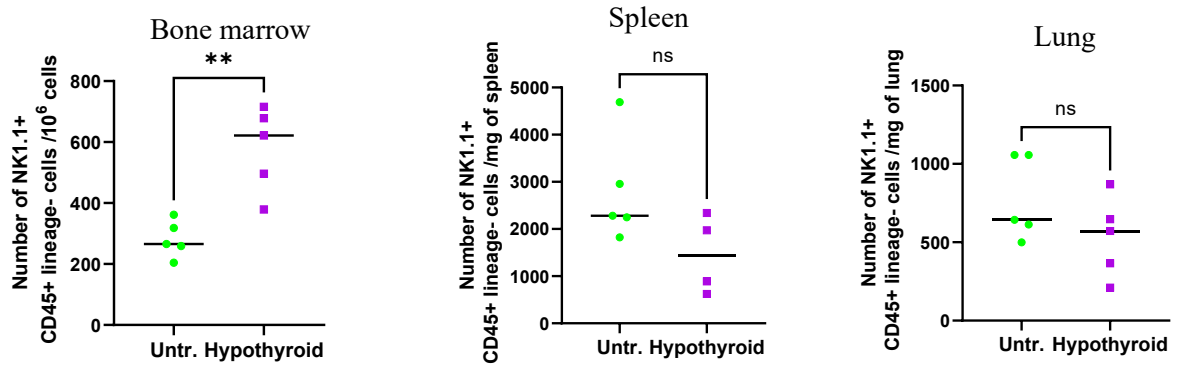


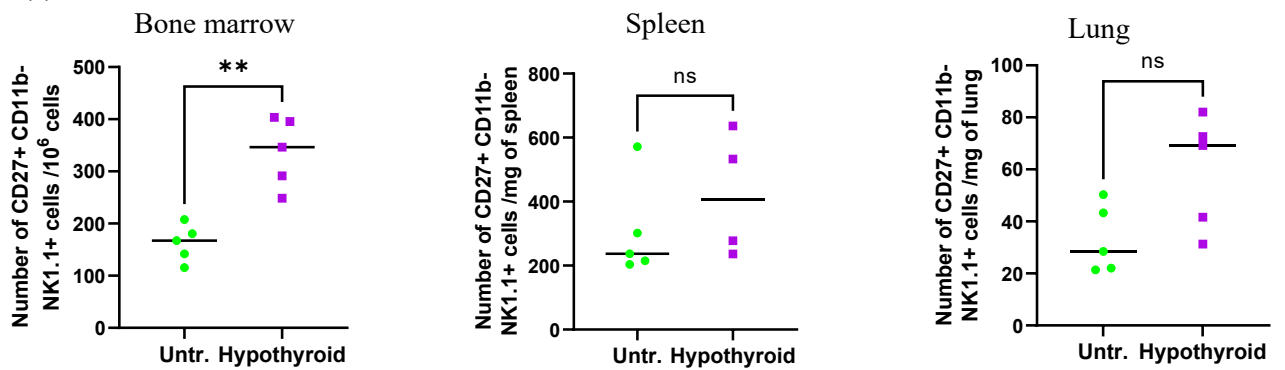
Figure 5.8. Number of mature NK cells is reduced in mice rendered hypothyroid from 9.5 weeks of age

Hypothyroidism was induced in 9.5 week old wild-type mice by feeding a low-iodine 0.15% PTU diet for six weeks. Control mice were fed normal chow. NK cell populations of hypothyroid and untreated C57BL/6 control mice were analysed by flow cytometry. (a) Representative flow cytometry plots to define immature and mature NK cells in bone marrow, spleen and lung. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression.

(b) NK cells



(c) Immature NK cells



(d) Mature NK cells

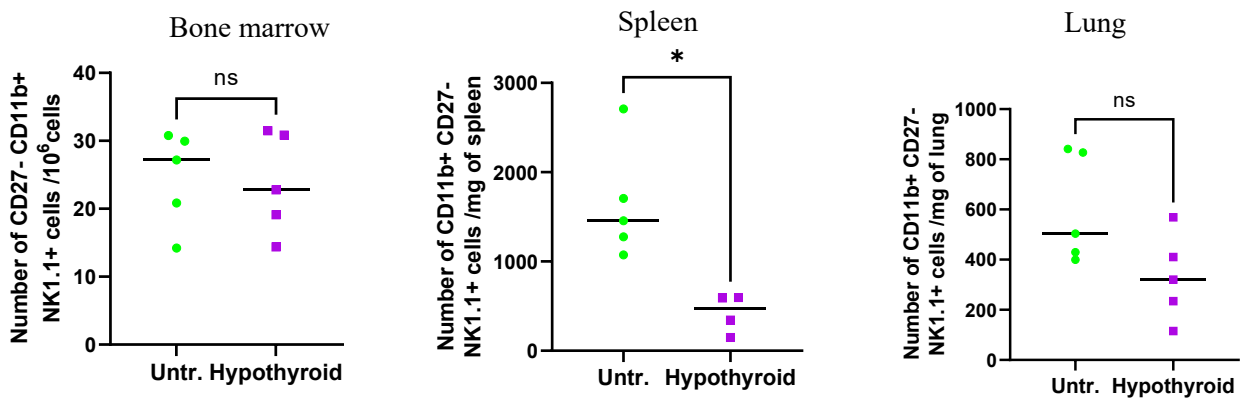


Figure 5.8. Number of mature NK cells is reduced in mice rendered hypothyroid from 9.5 weeks of age

(b) Number of NK cells, (c) number of immature CD27+ CD11b- NK cells and (d) number of mature CD27- CD11b+ NK cells in the bone marrow, lung and spleen of untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns p > 0.05, * p < 0.01 and ** p < 0.001.

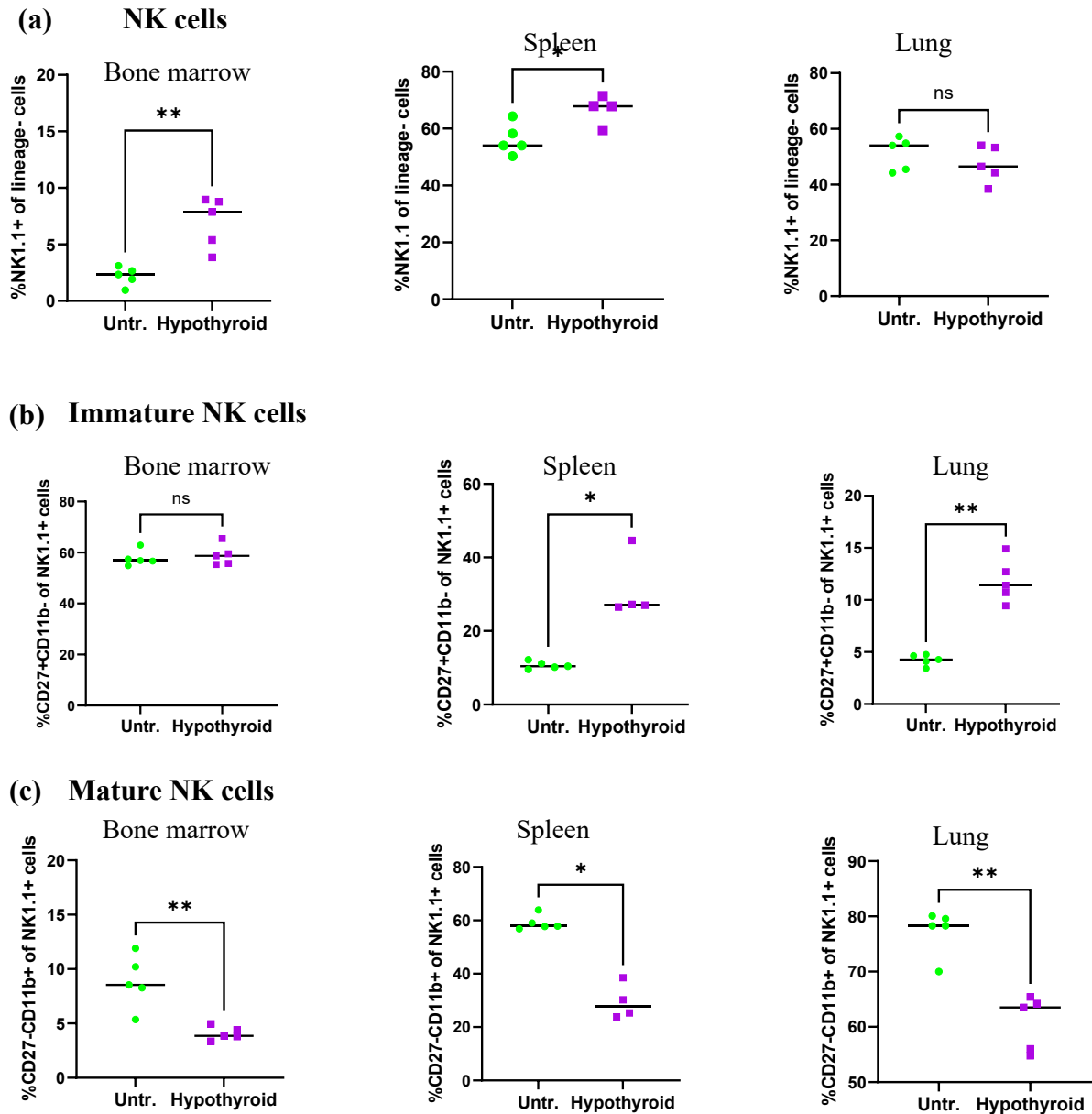


Figure 5.9. Proportion of mature NK cells is reduced in mice rendered hypothyroid from 9.5 weeks of age

NK cell populations of hypothyroid and untreated C57BL/6 control mice were analysed by flow cytometry. Hypothyroidism was induced in wild-type mice by feeding a low-iodine 0.15% PTU diet for six weeks. Control mice were fed normal chow. NK cells were defined as Live CD45⁺ Lineage⁻ NK1.1⁺ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression. (a) Proportion of NK cells, (b) proportion of immature CD27⁺ CD11b⁻ NK cells and (c) proportion of mature CD27⁻ CD11b⁺ NK cells amongst Live CD45⁺ Lineage⁻ cells in the bone marrow, lung and spleen of untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

5.7. Accumulation of refined NKPs in the bone marrow of mice rendered hypothyroid from 9.5 weeks of age

As a build-up of NK cells in the bone marrow and a loss of peripheral mature NK cell populations was observed in mice rendered hypothyroid from 9.5 weeks of age, the earlier stages of NK cell development were also investigated in these mice. NK progenitor populations in the bone marrow of hypothyroid and control mice were analysed by flow cytometry after six weeks of a low-iodine 0.15% PTU diet. A representative gating strategy is shown in Figure 5.10a. NK cell progenitors were gated as Lineage- CD244+ CD27+ CD127+ cells, and then further divided into subpopulations based on CD135 and CD122 expression. CLPs were defined as Lineage- CD27+ CD244+ CD127+ CD135+ CD122- cells. Pre-NKPs were defined as Lineage- CD244+ CD27+ CD127+ CD135- CD122- cells. As rNKPs express CD122, rNKPs were defined as Lineage- CD244+ CD27+ CD127+ CD135- CD122+ cells.

As observed in previous conditions, an accumulation of rNKPs was observed in the bone marrow of hypothyroid mice, as shown in Figure 5.10a. Both the proportion and absolute number of CLPs (Lineage- CD27+ CD244+ CD127+ CD135+ CD122- cells) were not significantly different between hypothyroid and untreated control mice (Fig. 5.10b). Both the proportion and absolute number of pre-NKPs (Lineage- CD27+ CD244+ CD127+ CD135- CD122- cells) were also comparable between hypothyroid and untreated control mice, although there was a trend for an increase in the proportion of these cells (Fig. 5.10c). There was a trend for an increase in the overall proportion of rNKPs although this was not significant, but the absolute number of rNKPs (Lineage- CD244+ CD27+ CD127+ CD135- CD122+ cells) was significantly increased in hypothyroid mice (Fig. 5.10d).

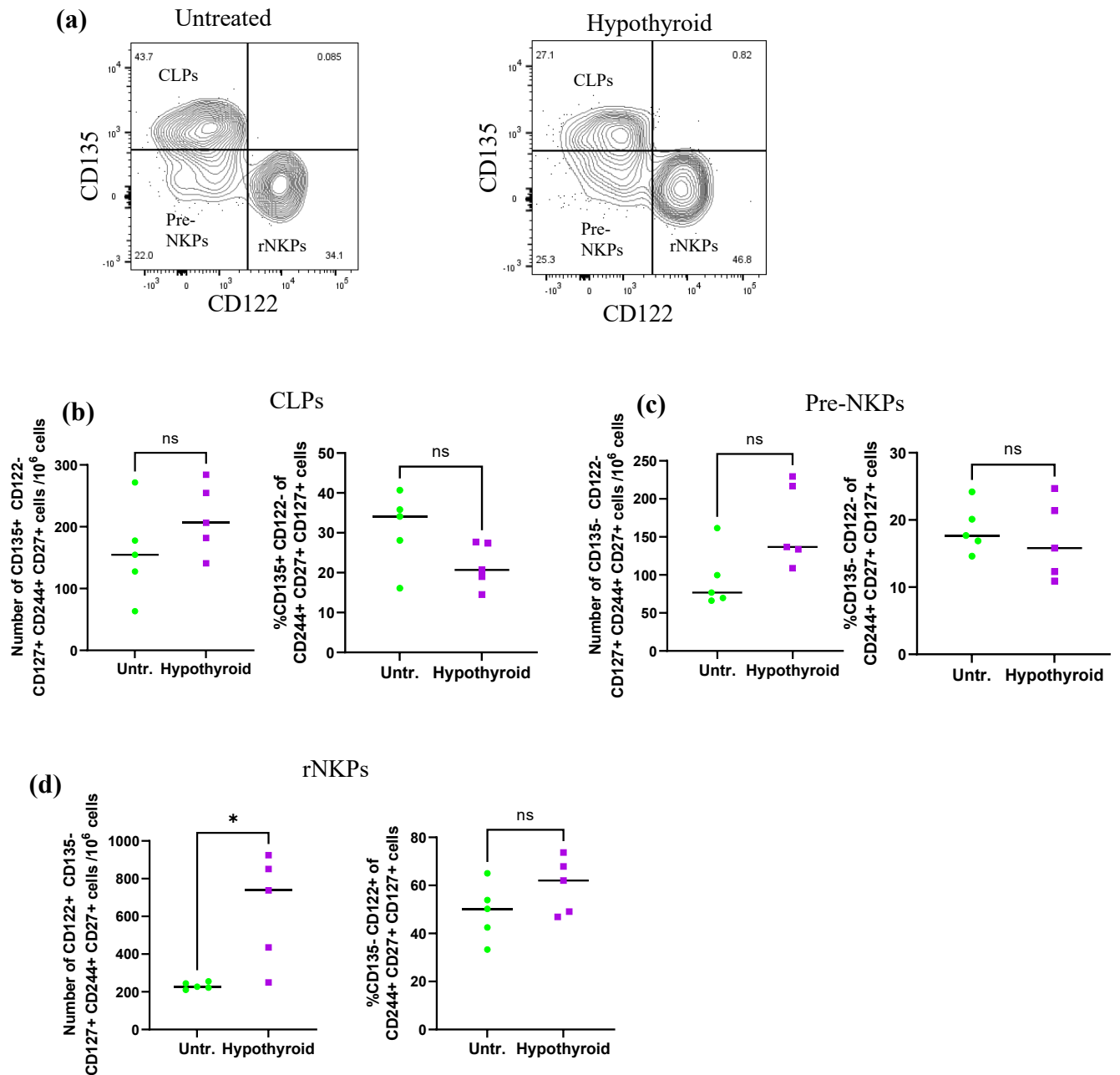


Figure 5.10. Accumulation of rNKPs in mice rendered hypothyroid at 9.5 weeks of age

Hypothyroidism was induced in 9.5-week old wild-type mice by feeding a low-iodine 0.15% PTU diet for six weeks. Control C57BL/6 mice were fed normal chow. Bone marrow was isolated from hypothyroid and untreated control mice and NK progenitors were identified by flow cytometry. (a) Representative flow cytometry plots of CLP (Lineage⁻ CD27⁺ CD244⁺ CD127⁺ CD135⁺ CD122⁻), pre-NKP (Lineage⁻ CD27⁺ CD244⁺ CD127⁺ CD135⁻ CD122⁻) and refined NKP (Lineage⁻ CD27⁺ CD244⁺ CD127⁺ CD135⁻ CD122⁺) cells in wild-type and hypothyroid mice. (b) Absolute number and proportion of CLPs, (c) pre-NKPs and (d) rNKP cells in untreated and hypothyroid mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

5.8. NK cell populations are not altered in $TR\alpha^{0/0}$ mice

As discussed, $Duoxa2^{-/-}$ mice are hypothyroid and both $Duoxa2^{-/-}$ and hypothyroid mice exhibit a similar severe defect in NK cell development, suggesting that thyroid hormone may play a key role in the regulation of NK cell maturation. The effects of thyroid hormone are mediated through the nuclear thyroid hormone receptors, $TR\alpha$ and $TR\beta$. Both $TR\alpha$ and $TR\beta$ act as T_3 -dependent transcription factors involved in the regulation of gene expression by binding to TREs in the promoters of many thyroid responsive genes (Wu and Koenig, 2000). For positively regulated genes, unliganded TRs bind to TREs and repress gene expression. Binding of T_3 releases the co-repressor complexes and recruits co-activators, thereby allowing chromatin remodelling and initiation of gene transcription (Astapova et al. 2008). TRs are also involved in negative gene regulation, although the underlying mechanism is poorly understood.

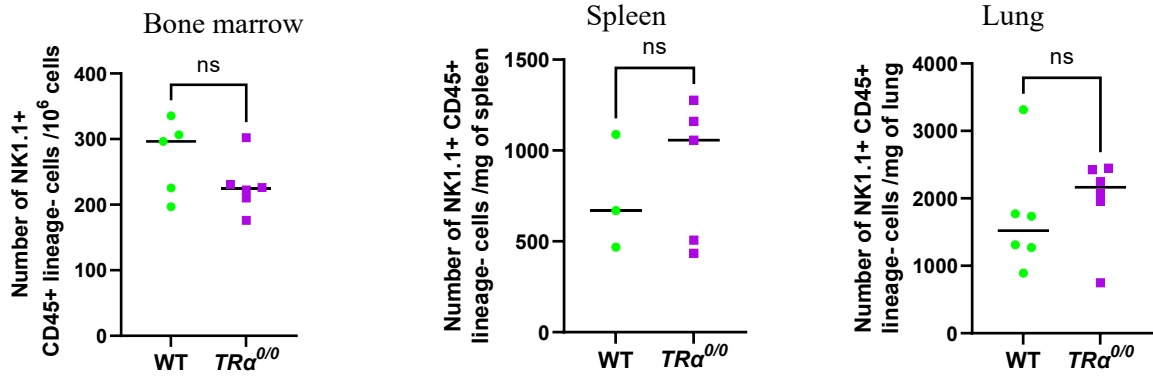
The mouse NK cell developmental programme is tightly regulated by expression or repression of a wide variety of transcription factors at discrete time points during NK cell maturation. Therefore, it was hypothesised that transcription of a gene or genes involved in NK cell development may be regulated by TRs. It is also possible that TRs could regulate other cells, for example stromal cells, in the bone marrow microenvironment which in turn regulate NK cell development. However, given that an accumulation of rNKPs is evident after only two weeks of PTU diet, it is likely that TRs may act directly on NK cell populations. In hypothyroid mice, lack of T_3 may result in disruption of the balance of ligand and receptor as all $TR\alpha$ will remain unoccupied. Therefore, absence of T_3 results in unliganded $TR\alpha$, and consequently unliganded $TR\alpha$ signalling cannot be altered or abrogated and may result in dysregulation of NK cell development in $Duoxa2^{-/-}$ and hypothyroid mice. In summary, it was hypothesised that a combination of severely reduced T_3 levels and the presence of $TR\alpha$ protein is necessary for the dysregulation of transcription.

It is important to note that $TR\alpha^{0/0}$ mice lack normal $TR\alpha$ protein, but the generation of thyroid hormones in these mice is not impaired. If $TR\alpha$ protein is not present, $TR\alpha$ cannot bind to TREs in thyroid-responsive genes and so unliganded T_3 expression or repression of certain genes cannot occur. Therefore, the NK cell populations of $TR\alpha^{0/0}$ mice were expected to be

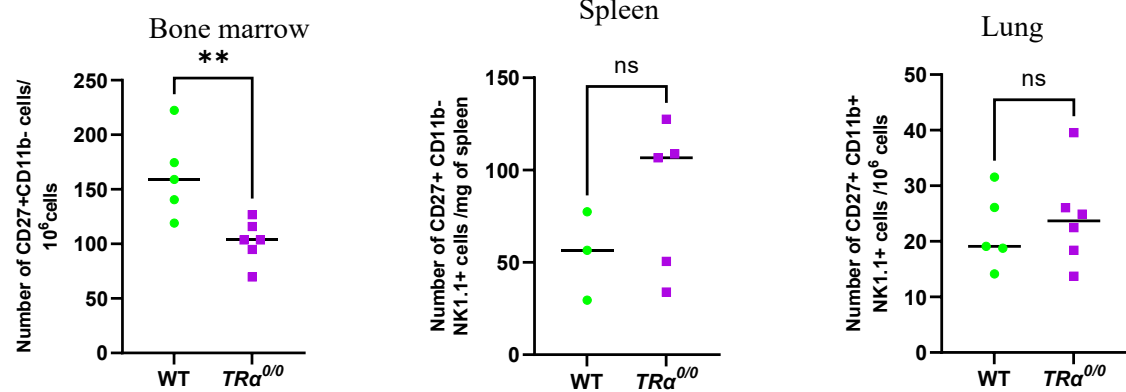
comparable to wild-type controls. In order to investigate this hypothesis, the NK cell populations of $TR\alpha^{0/0}$ mice were analysed by flow cytometry.

In $TR\alpha^{0/0}$ mice, the number of NK cells in the bone marrow, spleen and lung were not significantly different compared to littermate controls (Fig. 5.11a). The stages of NK cell maturation were also analysed to determine whether there were any alterations in individual subsets of NK cells. The number of immature CD27⁺ CD11b⁻ NK cells was significantly decreased in the bone marrow of $TR\alpha^{0/0}$ mice (Fig 5.11b). No significant difference in the number of immature NK cells was detected in spleen or lung, although there was perhaps a slight trend to an increase in the spleen. Mature NK cell populations were also not significantly different between $TR\alpha^{0/0}$ mice and littermate controls in either the bone marrow, spleen or lung (Fig. 5.11c).

(a) NK cells



(b) Immature NK cells



(c) Mature NK cells

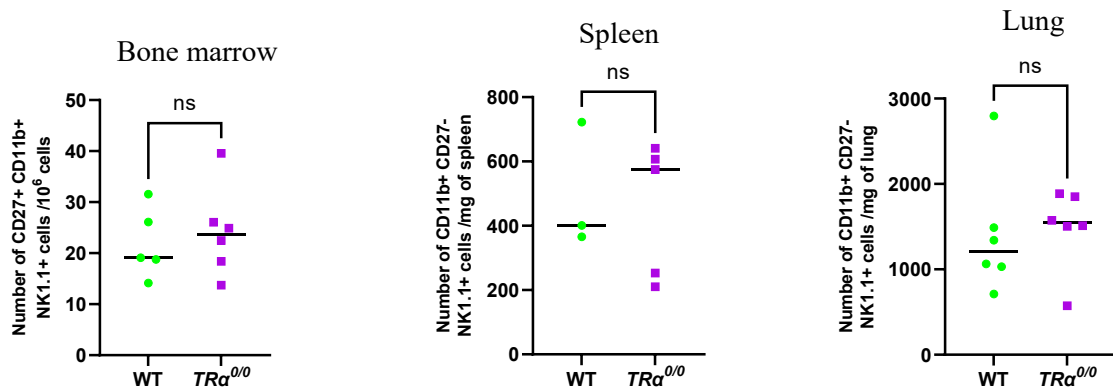


Figure 5.11. NK Cell populations are unaffected in $TR\alpha^{0/0}$ mice

NK cell populations of $TR\alpha^{0/0}$ and littermate control mice were analysed by flow cytometry. NK cells were defined as Live CD45+ Lineage- NK1.1+ cells. Immature and mature NK cells were identified based on CD27 and CD11b expression. (a) Number of NK cells, (b) number of immature CD27+ CD11b- NK cells and (c) number of mature CD27- CD11b+ NK cells in the bone marrow, lung and spleen of littermate control and $TR\alpha^{0/0}$ mice. Data is representative of two independent experiments. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$, * $p < 0.01$ and ** $p < 0.001$.

5.9. NK Progenitor populations were not altered in $TR\alpha^{0/0}$ mice

As discussed above, it was hypothesised that the phenotype of $Duoxa2^{-/-}$ and PTU-induced hypothyroid mice is driven by an absence of T_3 resulting in unliganded $TR\alpha$. The absence of T_3 results in an inability to alter the transcriptional effects of unliganded $TR\alpha$, and so unopposed $TR\alpha$ signalling results in repression or expression of target genes. However, in $TR\alpha^{0/0}$ mice, the lack of $TR\alpha$ means that transcriptional effects mediated by unliganded $TR\alpha$ cannot occur. As a result, progenitor populations were expected to be unaffected in $TR\alpha^{0/0}$ mice. In order to investigate this hypothesis, NK progenitors in the bone marrow of $TR\alpha^{0/0}$ mice were analysed by flow cytometry. NK cell progenitors were gated as Lineage- $CD244^+$ $CD27^+$ $CD127^+$ cells, and then divided into subpopulations based on $CD135$ and $CD122$ expression. CLPs were defined as Lineage- $CD27^+$ $CD244^+$ $CD127^+$ $CD135^+$ $CD122^-$ cells. Pre-NKPs were defined as Lineage- $CD244^+$ $CD27^+$ $CD127^+$ $CD135^-$ $CD122^-$ cells and rNKPs were defined as Lineage- $CD244^+$ $CD27^+$ $CD127^+$ $CD135^-$ $CD122^+$ cells.

As shown in Figure 5.12, the progenitor populations of $TR\alpha^{0/0}$ mice were comparable to those of littermate control mice. No significant difference was detected in either the proportion or absolute number of CLPs (Fig. 5.12a), pre-NKPs (Fig. 5.12b), or rNKPs (Fig. 5.12c) in the bone marrow of $TR\alpha^{0/0}$ mice.

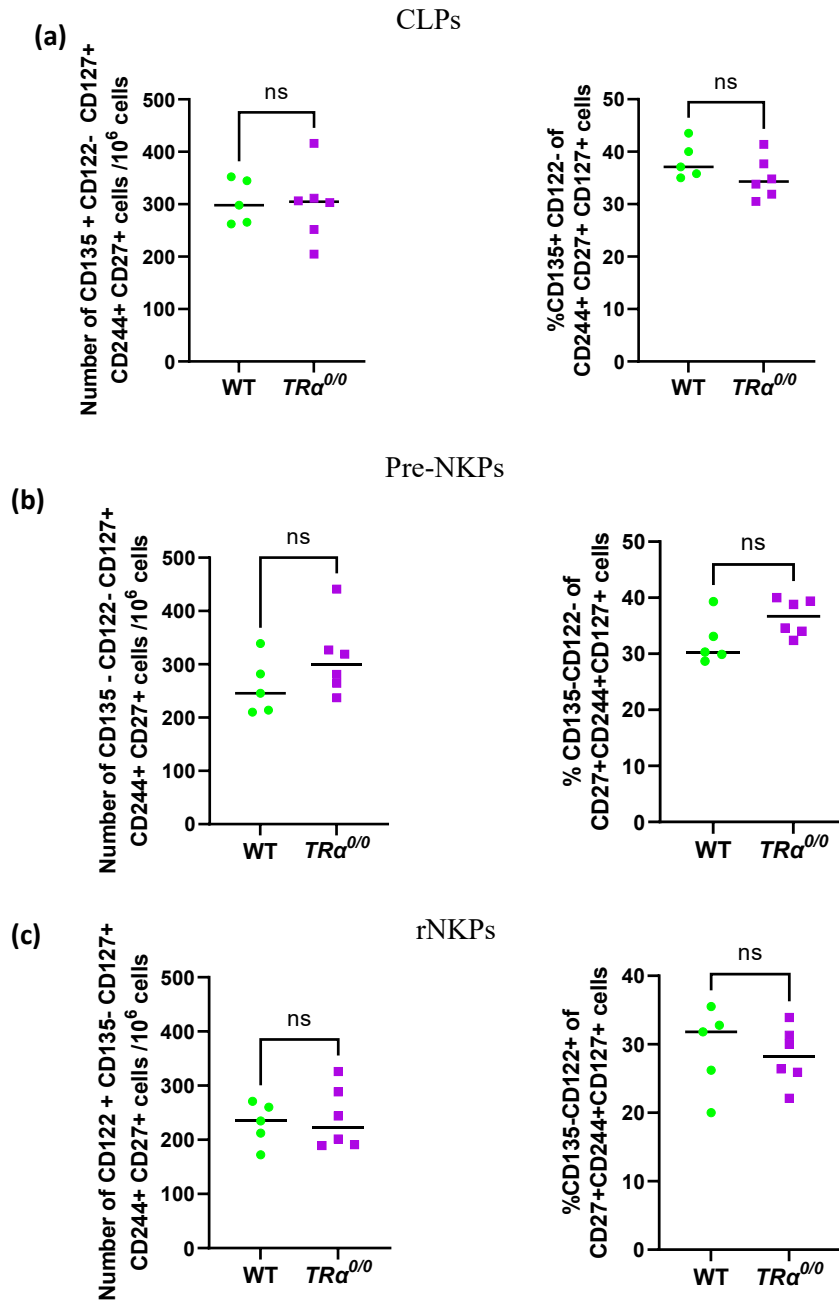


Figure 5.12. Characterisation of NK progenitor populations in $TR\alpha^{0/0}$ mice

Bone marrow was isolated from $TR\alpha^{0/0}$ and littermate control mice and NK progenitors were identified by flow cytometry. (a) Number of CLPs (Lineage- CD27+ CD244+ CD127+ CD135+ CD122-), (b) number of pre-NKPs (Lineage- CD27+ CD244+ CD127+ CD135- CD122-) and (c) number of rNKP cells (Lineage- CD27+ CD244+ CD127+ CD135- CD122+) in $TR\alpha^{0/0}$ mice and littermate controls. Data is representative of one experiment. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$.

5.10. NK cell populations are altered in mice expressing only TR α 1 (LOF mice)

In wild-type mice, alternative splicing occurs for both TR α and TR β , generating multiple different isoforms of each TR (Anyetee-Anum et al. 2018). As TR α 1 is the predominant TR isoform expressed in bone, the role of TR α 1 in NK cell development was further investigated. Alternative splicing mechanisms in wild-type mice generate a mix of both TR α 1 and TR α 2. The TR α 2 isoform is truncated and does not contain the AF-2 region of the LBD and therefore cannot bind T₃ (Mitsuhashi et al. 1988). As a result, TR α 1 is the main active isoform of TR α and is involved in control of gene expression of thyroid-responsive genes. In wild-type mice, these alternative splicing mechanisms regulate TR α 1 levels and in turn the balance of receptor and ligand availability regulates the control of thyroid hormone transcriptional activity. To test the role of TR α 1 in NK cell maturation, a mouse model that constitutively expresses TR α 1 exclusively due to a loss of the alternative splicing mechanism (loss of function, LOF mice) was used. As LOF mice express TR α 1 only, there is increased expression of TR α 1 in this model as alternative splicing in wild-type mice generates a mix of both the TR α 1 and TR α 2 isoforms. Therefore, this model allows for the investigation into the specific role of TR α 1 in regulation in NK cell development and maturation.

During postnatal development, there is a spike in thyroid hormone levels in the serum approximately two weeks after birth, before serum levels begin to fall (van Buul-Offers et al. 1983). As LOF mice express the TR α 1 isoform exclusively, during this spike in thyroid hormone and the days immediately afterwards, T₃ serum levels will be substantially elevated and so more ligand will be available to bind TR α 1 resulting in increased TR α 1 signalling. However, as there is an excess of TR α 1 in LOF mice, not all TR α 1 will be bound by T₃ resulting in some regulation of gene expression by unliganded TR α 1. As a result, unliganded TR α 1 signalling will be enhanced in LOF mice with excess TR α 1 compared to wild-type mice during this time period resulting in increased unliganded gene transcription regulation in LOF mice. However, substantial NK cell populations are only detected 2-3 weeks after birth in mice (Chiossone et al. 2009). Therefore, the NK cell populations of LOF mice were analysed by flow cytometry at 23 days post birth (Fig. 5.13a) in order to maximise T₃ serum levels, whilst allowing NK populations to emerge to enable meaningful analysis. It is important to note that wild-type mice were 27 days old in this experiment.

The number of NK cells was slightly increased in the bone marrow of LOF mice, although this was not statistically significant, as shown in Figure 5.13b. The proportion of NK cells was comparable to wild-type mice. In the spleen of LOF mice, both the overall proportion and absolute number of NK cells was decreased although this was not statistically significant (Fig. 5.13c). No difference in the proportion or number of NK cells was detected in the lung (Fig. 5.13d). It should be noted that NK cells were also absent in one wild-type biological replicate. This is an unusual result and is not consistent with the other wild-type replicates and as a result may be affecting the statistical analysis of this experiment.

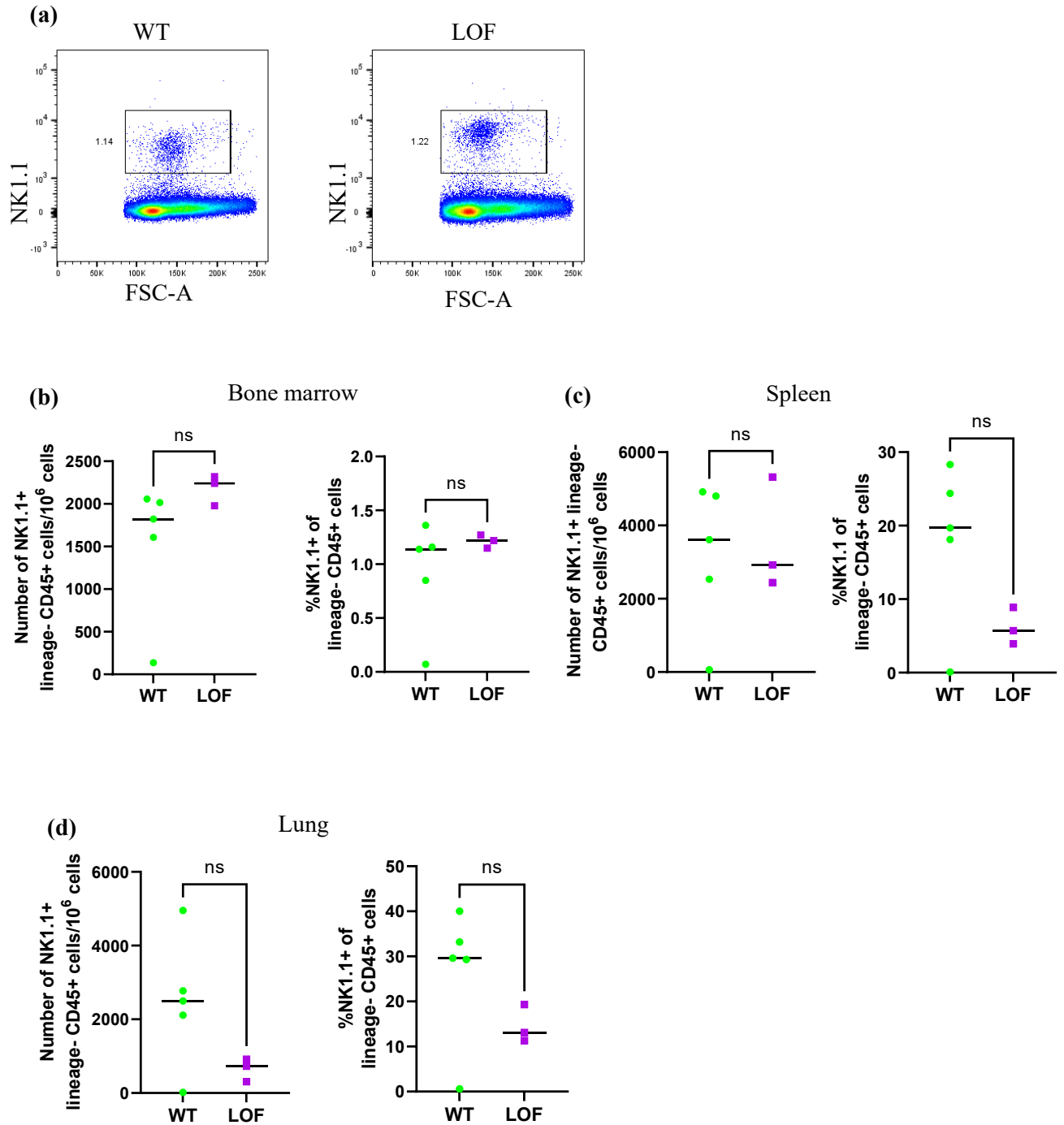


Figure 5.13. Characterisation of NK Cell populations in LOF mice

NK cell populations of LOF and wild-type control mice were analysed by flow cytometry. NK cells were defined as Live CD45+ Lineage- NK1.1+ cells. (a) Representative flow cytometry plots of the NK cell populations in the bone marrow of wild-type and LOF mice. (b) Absolute number and proportion of NK cells in the bone marrow, (c) spleen and (d) lung of wild-type and LOF mice. Data is representative of one experiment. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$.

5.11. NK progenitor populations are altered in mice expressing TRa1 only (LOF mice)

The progenitor populations of LOF mice were also investigated by flow cytometry to determine whether a similar trend to that observed in *Duoxa2*^{-/-} and hypothyroid mice was evident. NK cell progenitors were gated as Lineage- CD244⁺ CD27⁺ CD127⁺ cells, and then further divided into subpopulations based on CD135 and CD122 expression (Fig. 5.14a). CLPs were defined as Lineage- CD27⁺ CD244⁺ CD127⁺ CD135⁺ CD122⁻ cells. Pre-NKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁻ cells and rNKPs were defined as Lineage- CD244⁺ CD27⁺ CD127⁺ CD135⁻ CD122⁺ cells.

The proportion of CLPs was significantly reduced in LOF mice compared to wild-type mice, but the absolute cell numbers of CLPs were not significantly different (Fig. 5.14b). The proportion of pre-NKPs was significantly increased in LOF mice but the absolute number of pre-NKPs was not significantly different, although there was a trend for an increase in number (Fig. 5.14c). Both the proportion and absolute number of rNKPs in LOF mice were significantly increased compared to wild-type control mice (Fig 5.14d), which is similar to the trend observed in *Duoxa2*^{-/-} mice. The proportion of rNKPs in the wild-type mice in this experiment is very low compared to the results observed in the *Duoxa2*^{-/-} and PTU-induced hypothyroid models. This is likely due to the fact that the mice analysed in this experiment are much younger as the wild-type mice are 27 days old, whereas the *Duoxa2*^{-/-} and PTU mice were 10-16 weeks old at the time of the experiment.

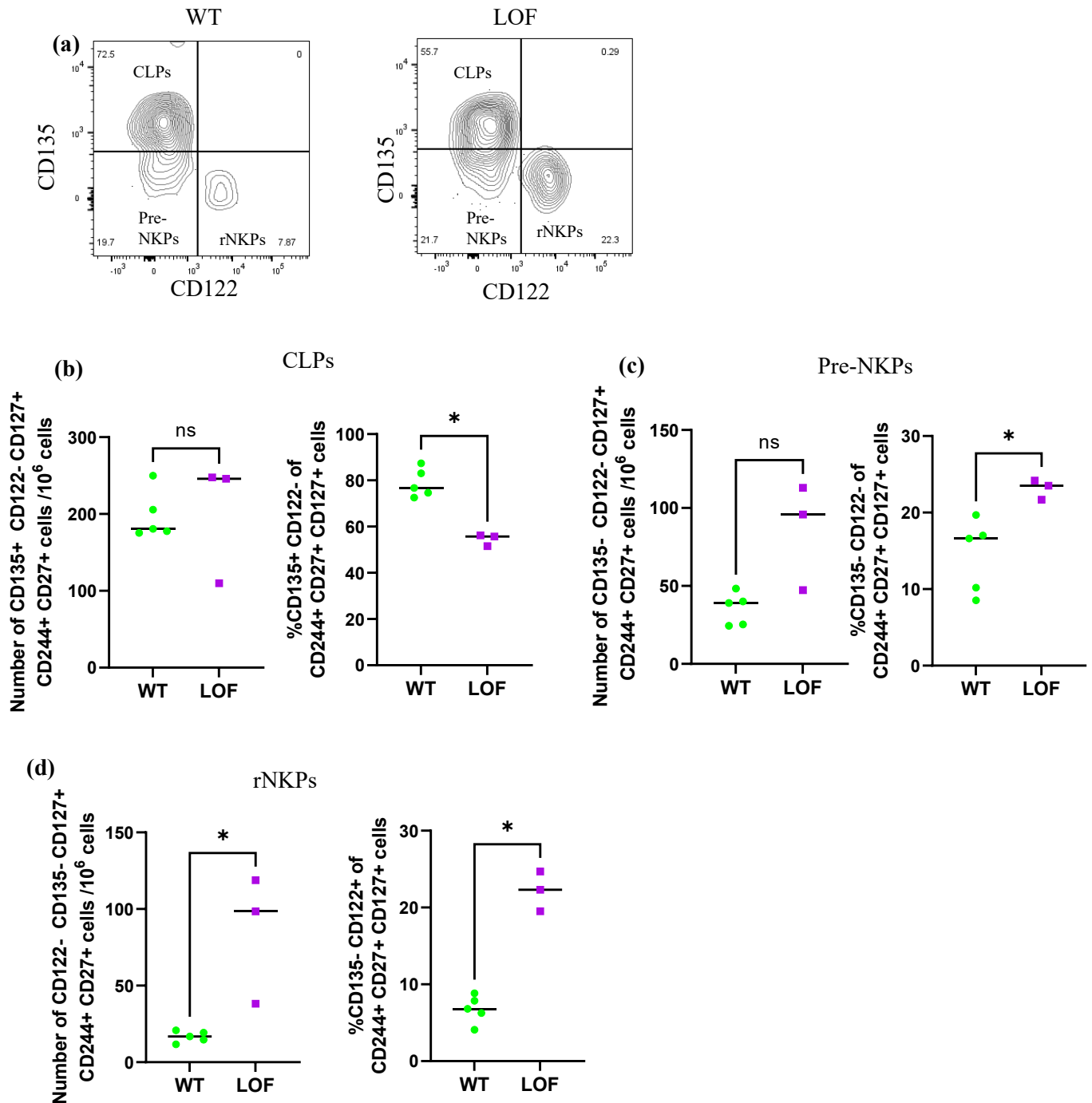


Figure 5.14. Accumulation of rNKPs in the bone marrow of LOF mice

Bone marrow was isolated from LOF and wild-type control mice and NK progenitors were identified by flow cytometry. (a) Representative flow graphs identifying CLPs (Lineage- CD27+ CD244+ CD127+ CD135+ CD122-), pre-NKPs (Lineage- CD27+ CD244+ CD127+ CD135- CD122-) and rNKPs (Lineage- CD27+ CD244+ CD127+ CD135- CD122+) in wild-type and LOF mice. (b) Absolute number and proportion of CLPs, (c) pre-NKP cells and (d) rNKP cells in wild-type and LOF mice. Data is representative of one experiment. Statistical significance was determined using a Mann-Whitney rank test. ns $p > 0.05$ and * $p < 0.01$.

5.12. Discussion

Duoxa2^{-/-} mice exhibit a severe defect in NK cell development and maturation. As *Duoxa2^{-/-}* mice are congenitally hypothyroid (Grasberger and Refetoff, 2006), the results presented in this Chapter sought to investigate the extent to which hypothyroidism may contribute to the NK cell defect observed in *Duoxa2^{-/-}* mice. Surprisingly, mice rendered hypothyroid by feeding a low-iodine 0.15% PTU diet display a very similar NK cell defect to that observed in *Duoxa2^{-/-}* mice. As thyroid hormone has not been previously demonstrated to strongly affect NK cell development in mice, this was an unexpected result. A build-up of NK cells in the bone marrow with a decrease in the number of NK cells in the periphery was observed in both hypothyroid and *Duoxa2^{-/-}* mice. In particular, a paucity of mature NK cells was also clearly evident in both models. Consequently, it is apparent that hypothyroidism must play a role in the dysregulation of NK cell development in mice.

As the NK cell phenotype was observed in both congenitally hypothyroid mice (*Duoxa2^{-/-}*) and in mice where hypothyroidism was induced, this phenotype cannot be solely developmental as it could be induced in mice post-birth. In addition, the defect in NK cells was apparent after both two and six weeks on the diet, and in mice treated from both six and 9.5 weeks of age. This suggests that there must be a continuous need for thyroid hormone during NK cell development, as NK cell defects begin to emerge after only two weeks on the low-iodine 0.15% PTU diet and in mice of various ages. In addition, it is possible that thyroid hormone may also influence NK cell education in the periphery and not solely during development in the bone marrow.

The strongest phenotype was observed in PTU-induced hypothyroid models after six weeks of low-iodine 0.15% PTU diet. As NK cells have a half-life of two weeks (Vivier et al. 2008), it would take around this length of time for population turnover to occur. Therefore, after two weeks of PTU diet, the phenotype is less extreme. If thyroid hormone acts at a specific stage of NK cell development, it would take time for this to become evident in other NK cell subsets. This is because complete population turnover would be just starting to occur, and so it would take time to see this reflected as a loss of mature NK cells which are not being replenished to the same extent. Similarly, in mice fed the diet from 9.5 weeks of age, the

phenotype was also less extreme. It is possible that as they are slightly older, the immune cell populations are more established and therefore it may take slightly longer to see such an extreme reduction. Nonetheless, it is evident that induction of hypothyroidism results in a defect in NK cell maturation in mice.

As thyroid hormone signalling is mostly mediated through the thyroid hormone receptors, it was hypothesised that thyroid hormone receptors may regulate the expression of a gene or genes involved in NK cell development. Both TR α and TR β are T₃-dependent DNA-binding transcription factors (Wu and Koenig, 2000), but TR α is the predominant receptor expressed in bone (Bradley et al. 1992). Regulation of gene expression is controlled by binding of thyroid receptors to TREs in thyroid responsive genes, which can result in activation or repression of target genes (Ortiga-Carvalho et al. 2014). The absence of T₃ means that TR α remains unoccupied and therefore maintains consistent repression or activation of its target genes. Under homeostatic conditions, the balance of TR α and T₃ levels is coordinated to alter gene transcription at distinct developmental stages to activate or repress target genes when necessary. When TR α is absent, as in TR $\alpha^{0/0}$ mice, the unliganded effects of TR α cannot be exerted. Transcriptional dysregulation cannot occur in the absence of TR α as it is dependent on TR α binding to TREs in target genes. As is evident in TR $\alpha^{0/0}$ mice, a defect similar to *Duoxa2*^{-/-} or PTU-induced hypothyroid mice was not observed as NK cells were comparable to littermate controls. Therefore, the absence of T₃ and the presence of TR α is required to drive the defect identified in *Duoxa2*^{-/-} and hypothyroid mice. It should be noted that although two independent TR $\alpha^{0/0}$ experiments were performed, the first was a small preliminary experiment of three littermate controls and three TR $\alpha^{0/0}$ mice provided by collaborators and NK progenitors were not analysed. The data from the second larger experiment is presented in this Chapter. Both experiments yielded similar results, demonstrating normal NK cell populations in TR $\alpha^{0/0}$ mice. However, to further strengthen these results, it would be beneficial to repeat this experiment again to increase the number of mice analysed and confirm that NK cell development is not affected.

As has been demonstrated, wild-type mice rendered hypothyroid using a low-iodine 0.15% PTU diet also exhibit a severe NK cell defect. In this model, T₃ serum levels would be significantly reduced and so unliganded TR α will maintain either expression or repression of

target genes. This mechanism should be equivalent to that occurring in *Duoxa2*^{-/-} mice where T₃ is absent. The results presented in Chapter 5 suggest that this is likely to be a mechanism common to all severely hypothyroid mouse models. To confirm the role of TRα in NK cell development and maturation, crossing *TRα*^{0/0} mice to *Duoxa2*^{-/-} mice should rescue the NK cell phenotype of *Duoxa2*^{-/-} mice. T₃ levels are very low in *Duoxa2*^{-/-} mice, however removing TRα would abrogate the effects of unliganded TRα by preventing TRα dysregulating transcription of target genes. Similarly, it would be assumed that switching *TRα*^{0/0} mice onto the PTU diet would not result in an NK cell defect, as the presence of TRα is essential to mediate unliganded TRα effects.

The experimental data investigating the role of TRα1 was obtained by a collaboration with Professor Graham Williams and Professor Duncan Bassett, Imperial College London, who kindly provided the LOF mice for these experiments. It should be noted that this is preliminary data as this experiment was only performed once, and the number of biological LOF replicates was low. In terms of the NK cell phenotype, the trend observed in LOF mice was similar to that seen in both *Duoxa2*^{-/-} and hypothyroid mice. Although no statistical significance was observed, there was a trend for a slight increase in NK cell numbers in the bone marrow and a decrease was also observed in the spleen. However, as only three LOF mice were analysed, repetition of this experiment with a greater number of biological replicates is necessary to obtain more definitive results.

The number of rNKPs was also significantly increased in hypothyroid mice. This result is quite striking as the accumulation of rNKPs is consistent in all models - *Duoxa2*^{-/-}, hypothyroid mice and in LOF mice. Both *Duoxa2*^{-/-} and hypothyroid mice have constitutive unliganded TRα1 signalling due to the absence of T₃, whereas LOF mice have an excess of TRα1 as these mice do not express TRα2. This will result in a mismatch in the balance of hormone and receptor resulting in increased unliganded TRα1 signalling compared to wild-type control mice. Although it is clear that maturation of NK cells is impaired, this is also associated with an obvious and consistent increase in the number of rNKPs in the bone marrow. It is possible that there may be a partial block at both the progenitor and immature stages of NK cell development and so T₃ may be important at both the progenitor and immature stages of NK cell development. Therefore, careful dissection of the role of stage-

specific TR α 1 signalling is essential to fully understand the thyroid hormone-NK cell regulatory axis.

Together, these common results are highly suggestive of a role of TR α 1 in the regulation of NK cell development and maturation. However, exactly where and how TR α 1 functions in this process remains to be elucidated. TR α can be involved in either positive or negative regulation of genes, depending on the target gene. It could be speculated that T₃ binding to TR α is necessary to activate expression of genes essential for NK cell development. Conversely, it could also be suggested that TR α may be involved in repressing genes which allow NK cell development to proceed. From the experiments conducted in this PhD, it cannot yet be determined whether TR α 1 is involved in positive or negative gene regulation in NK cells under homeostatic conditions. This could be investigated by adding T₃ to NK cell cultures and subsequently extracting RNA and using qPCR to assess whether T₃ alters the expression of key genes involved in NK cell development.

It is plausible that under normal conditions TR α could directly repress a key gene in NK cell development. Both FOXO1 and TGF β are known to be involved in negative regulation of NK cell development. A failure to repress these genes could result in impaired NK cell maturation. It is possible that in hypothyroid mice, the absence of T₃ would result in an inability to switch off the expression of these genes, thereby preventing repression. Constitutive expression would therefore limit NK cell maturation. Expression of EOMES is also vital to promote the development of immature NK cells. However, EOMES expression is downregulated as T-BET is upregulated to allow NK cell maturation. It could also be speculated that TR α 1 may be involved in repression of EOMES at the later stages of development, and a failure to repress this gene would prevent T-BET upregulation, consequently preventing NK cell maturation.

Furthermore, it must also be considered that TR α 1 may be involved in activation of a key gene in NK cell development and maturation under normal conditions. Activation of *Tbx21*, *Zeb2*, *Id2* and *Stat5* expression is essential for the generation of mature NK cells. Failure to activate these genes would also result in defective NK cell maturation. If TR α was involved

in activation of these genes, the absence of T₃ in hypothyroid mice would result in an inability to activate gene transcription, as unliganded TR α would maintain strong repression of these genes. However, research on this topic is very limited and thyroid hormone may positively or negatively regulate a wide variety of genes. One study identified an interaction between TR β and STAT5 and the authors found that STAT5 was negatively regulated by TR β (Favre-Young et al. 2000). T-BET was also found to be upregulated in patients with Hashimoto's thyroiditis (Tokić et al. 2016). It would also be useful to search for TREs in all candidate genes as this would enable identification of potential thyroid-responsive genes in the NK cell development pathway.

Both TR α and TR β act as T₃-dependent transcription factors involved in the regulation of gene expression. As the effects of T₃ are mostly mediated by TR α and TR β , it is likely that the defect observed in *Duoxa2*^{-/-} and hypothyroid mice is driven by thyroid receptor-mediated dysregulation of gene expression. As TR α is the predominant isoform expressed in bone, it is mostly likely that TR α is involved in gene regulation in NK cells. However, TR β is also a T₃-dependent transcription factor, and so it is also possible that it could play a role in the regulation of NK cells. In addition, the role of integrin $\alpha\text{v}\beta 3$ in NK cells should also be investigated as it can act as an alternative receptor for thyroid hormones (De Vito et al. 2011). However, in order to elucidate the exact mechanism at play, investigation of the transcriptome of *Duoxa2*^{-/-} mice is necessary. RNA sequencing of specific NK cell subsets sorted from *Duoxa2*^{-/-}, hypothyroid and wild-type mice would potentially identify any genes or pathways which are dysregulated in NK cell development in *Duoxa2*^{-/-} mice.

One important factor to consider is that the NK cell populations of *Slc26a7*^{-/-} mice appeared normal. As a hypothyroid mouse model, it would be expected that they would display a similar phenotype to *Duoxa2*^{-/-} mice and mice with induced hypothyroidism. However, it is important to consider that *Slc26a7*^{-/-} mice are not as severely hypothyroid as *Duoxa2*^{-/-} mice, which is evident as their growth is not severely stunted. Therefore, it is possible that mice may need to be severely hypothyroid before defects in NK cell populations would be observed. This is further supported by the evidence presented in Chapter 4, which demonstrated that mice with heterozygous mutations in *Duoxa2* do not exhibit NK cell abnormalities. Therefore, T₃ levels in *Duoxa2*^{+/-} mice must be sufficient to permit and drive a

normal NK cell developmental programme. In *Duoxa2*^{-/-} mice, T₃ levels are essentially undetectable, suggesting that the phenotype is dose-dependent. The balance of T₃ and TRα1 levels is essential to regulate gene transcription. Therefore, there may be a threshold of T₃ levels, below which normal gene transcription regulation cannot occur as the amount of unliganded TRα1 is too high, essentially resulting in constitutive activation or repression of target genes. Investigation of the thyroid hormone levels in the serum and correlation with the severity of the NK cell phenotype in *Slc26a7*^{-/-}, *Duoxa2*^{-/-} and hypothyroid mice would provide further evidence on this matter.

To date, the evidence highlighting a role for thyroid hormones in NK cells is limited. Reduced NK cell activity has been reported in hyperthyroid patients (Papic et al. 1987) and hypothyroid patients (Wang et al. 1988). Stimulation with T₃ also increased NK cell activity in patients with low serum T₃ levels (Kmiec et al. 1991). However, most of the evidence published relates to early clinical observations and no investigations into the various subsets of NK cells or the stages of NK cell development have yet been undertaken. In conclusion, whilst the exact mechanism requires further investigation, it is clear that thyroid hormones play a previously undescribed, yet crucial role in NK cell development in mice. Mouse NK cell development appears to be fundamentally dependent on thyroid hormone.

6. Discussion

Whilst the DUOX2/DUOX2A system has been demonstrated to play a role in retaining barrier integrity in mucosal tissues (Grasberger et al. 2015), previous evidence of a role for DUOX2A in immune cells is limited. However, the role of the DUOX2/DUOX2A system in the thyroid is well established as H_2O_2 generation by this complex is essential to enable thyroid hormone synthesis (Grasberger, 2010). Throughout the 1970s to the 1990s, there were several early observations identifying a potential role for thyroid hormones in immune cell physiology. Thyroid hormones have been found to alter the function of various subsets such as neutrophils (Zamoner et al. 2007), DCs (Mascanfroni et al. 2008) and NK cells (Provinciali and Fabris, 1990). However, despite significant advances in our understanding of the development and function of immune cell subsets, including NK cells, research into the relationship between thyroid hormones and immune cells remains very limited. Stimulation of NK cells from patients with low serum thyroid hormone levels with T_3 has been demonstrated to increase NK cell cytotoxicity (Kmiec et al. 1991). Similarly, stimulation with thyroid hormones increased sensitivity to IFN (Provinciali and Fabris, 1987) and also increased NK cell cytotoxicity in mice (Provinciali et al. 1991). Patients with Grave's disease are hyperthyroid and were found to have reduced NK cell activity (Papic et al. 1987). Another study also identified reduced NK cell cytotoxicity in both hyperthyroid and hypothyroid patients (Wang et al. 1988). Although T_3 and T_4 have been shown to alter NK cell function, there has been no investigation into the role of thyroid hormone in specific NK cell subsets. As such, a role for thyroid hormones in regulating NK cell development and maturation has not yet been reported in the literature. However, thyroid hormones are known to play an important role in the regulation of growth and development, including both brain and skeletal development. Therefore, it is entirely possible that they may also be involved in the development and maturation of the immune system.

Duoxa2^{-/-} mice exhibit a severe defect in NK cell development and maturation, with a striking reduction of mature NK cells in particular. An accumulation of rNKPs and NK cells is also evident in the bone marrow of *Duoxa2*^{-/-} mice. Unexpectedly, rendering wild-type mice hypothyroid by feeding a low-iodine 0.15% PTU diet, resulted in a very similar NK cell defect. Together, the results of this PhD provide evidence of a role for thyroid hormones in the regulation of NK cell development and maturation. It is likely that insufficient T_3 levels

abrogate thyroid receptor signalling and consequently disrupt regulation of T₃ target genes through unliganded TR α signalling. The precise mechanism underlying this relationship requires further investigation to determine which exact transcription factors are disrupted in *Duoxa2*^{-/-} and severely hypothyroid mice. Further investigation of this mechanism will enhance our understanding of the regulation and maintenance of NK cell development in mice. In turn, our increased understanding of this relationship could prove useful when trying to increase NK cell production and responses, for example to infiltrate the tumour microenvironment. NK cells are cytotoxic lymphocytes which play an important role in tumour immunosurveillance and so have become an important therapeutic target for cancer treatment (Morvan and Lanier, 2016). Increased infiltration of tumours by NK cells correlates with a decreased risk of death in patients with solid tumours (Nersesian et al. 2021). As such, it is tempting to hypothesise whether administration of T₃ to cancer patients could help to bolster NK cell populations. However, serum T₃ and T₄ levels are tightly controlled under homeostatic conditions and so this would need to be carefully monitored as NK cells from hyperthyroid patients were found to have a reduced cytotoxic activity (Papic et al. 1987; Wang et al. 1988) and so raising T₃ levels too much could result in the opposite of the desired effect. However, it would be interesting to investigate whether T₃ may have therapeutic benefit in cancer patients, particularly those with low NK cell counts. It is possible that for certain patients T₃ may be able to increase NK cell production to aid in anti-tumour immunity. This hypothesis could first be tested in mouse models of cancer to determine whether T₃ or T₄ administration could help to boost the NK cell response to tumours.

6.1. Future work

In terms of future experiments, to help confirm the role of thyroid hormones in mouse NK cells, supplementing *Duoxa2*^{-/-} mice with thyroid hormones to establish normal physiological serum concentrations is necessary. If supplementation with T₄ in drinking water for several weeks could rescue the NK cell defect, this would provide further evidence that thyroid hormones are essential in the regulation of NK cell development and maturation. Similarly, it would also be interesting to perform adoptive transfer experiments of NK cells from *Duoxa2*^{-/-} mice into wild-type mice. If the peripheral NK cell defect corrected when transferred into wild-type mice, this would further support the conclusion that it is driven by a cell-extrinsic microenvironmental defect. It would be hypothesised that normal T₃ levels in a

wild-type host would be sufficient to promote maturation of *Duoxa2*^{-/-} NK cells, which would also provide further evidence that T₃ regulates NK cell maturation.

In addition, if thyroid hormones are directly regulating gene expression in NK cells, preventing T₃ action in NK cells specifically should also result in defective NK cell development. D3 is responsible for the inactivation of thyroid hormone by converting it from T₃ to inactive reverse T₃ (Bianco, 2011). Using a mouse model provided by Professor Graham Williams and Professor Duncan Bassett, it would be possible to cross mice which conditionally overexpress D3 with *Ncr1*^{Cre} mice in order to specifically overexpress the D3 enzyme in NK cells. This would remove T₃ in NK cells, rendering them hypothyroid, thereby preventing T₃-dependent regulation of gene expression. If thyroid hormone acts directly on NK cells, it would be expected that this would produce a similar phenotype to that observed in *Duoxa2*^{-/-} mice. However, as NKp46 is expressed by immature and mature NK cells and not by progenitors, this effect will only be observed if thyroid hormone is in fact necessary during the immature and mature NK cell stages and not solely at the NKP stage. It would also be possible to cross mice which conditionally overexpress D3 with *Vav1*^{Cre} mice to enable overexpression of D3 in all haematopoietic cells, including NKPs. Whilst this would render NKPs hypothyroid and enable analysis of the effects of loss of thyroid hormone signalling in NKPs, it is important to consider that all other haematopoietic cells would also be rendered hypothyroid which could in turn affect NKPs.

It will also be important to investigate the role of thyroid hormones in the immune system in humans. Whilst it has been identified that thyroid hormones regulate NK cell development in mice, it remains unknown as to whether a similar relationship also translates to humans. Several early observations identified reduced NK cell activity in patients with Grave's disease (Papic et al. 1987), but also in both hyperthyroid and hypothyroid patients (Wang et al. 1988). However, in depth analysis of the relationship between thyroid hormones and immune cell defects remains limited and requires further investigation. It could be useful to complete immunophenotyping of peripheral blood from patients with *DUOX2* and *DUOXA2* mutations. However, the effects of mutations in these genes may not be as severe in humans as they are in mice, as human patients with mutations in these genes often exhibit only transient hypothyroidism. It is possible that humans may have more compensatory

mechanisms available, such as DUOX1/DUOXA1, and so perhaps loss of DUOX2/DUOXA2 in humans may not result in as severe a phenotype. As the defects identified in mice are associated with severe hypothyroidism, it would be beneficial to carry out immunophenotyping of peripheral blood of severely hypothyroid patients. However, to date, no definitive link between alterations in thyroid hormone and severe immune defects have been identified. Indeed, the vast majority of hypothyroid patients undergo treatment with T₄ supplementation and so this could also explain why severe immune defects related to hypothyroidism are not common.

Finally, it is crucial to perform transcriptomic analysis on NK cells from *Duoxa2*^{-/-} mice in order to provide further mechanistic insight into which transcription factors or networks are dysregulated in the absence of DUOXA2. As discussed, loss of several genes important in NK cell development and egress result in a similar phenotype of a loss of NK cells in the periphery, especially mature NK cells. Consequently, it is likely that one or more of these genes is dysregulated in *Duoxa2*^{-/-} mice. As NK cell egress has not yet been investigated in *Duoxa2*^{-/-} mice, it is possible that a defect in both NK cell development and egress is responsible for the loss of peripheral NK cells. Indeed, loss of T-BET, ZEB2, ID2, STAT5, S1P5 and CX₃CR1 all result in a reduction of NK cells in the periphery. As T-BET is known to regulate S1P5 expression, it is plausible that a loss of a combination of genes involved in development and egress may be driving the NK cell defect. Focused transcriptional analysis of sorted NK cell subsets, including both rNKPs and immature NK cells will enable the identification of which transcription factor networks are disrupted in *Duoxa2*^{-/-} and hypothyroid mice. RT-PCR could also be used to analyse mRNA expression levels of candidate genes in NK cells from *Duoxa2*^{-/-} and littermate controls. As the *Duoxa2* defect is NK cell-extrinsic, transcriptomics of the CD45⁻ fraction of the bone marrow could also potentially identify whether defects in stromal cells are driving the defect in NK cell development in these mouse models.

As discussed in the Introduction, there is also evidence for a role of thyroid hormones in other immune cell subsets including neutrophils, macrophages and DCs, in particular through the $\alpha\beta3$ integrin receptor. Although the magnitude of its effects may differ in distinct immune cell types, thyroid hormone may be involved in the regulation of many immune cell

populations. Therefore, whilst the results of this PhD have identified an important role for thyroid hormone in NK cell development, it is important to acknowledge that the immunoregulatory role of thyroid hormone likely extends beyond its role in NK cell maturation.

6.2. Concluding Remarks

In conclusion, the evidence presented in this thesis establishes an indirect yet fundamental role for the DUOX2/DUOXA2 system in the regulation of NK cell development and maturation. In mice, H₂O₂ generation by the DUOX2/DUOXA2 complex plays a key role in thyroid hormone synthesis, upon which regulation of T₃-responsive target gene transcription is dependent. It is likely that thyroid hormone-dependent regulation of key genes in NK cell development is essential to permit a normal programme of NK cell development under homeostatic conditions. In conclusion, the results of this PhD suggest a previously undescribed but crucial role for thyroid hormones in the regulation of NK cell development and maturation in mice.

7. References

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