MONASH University

The role of NF-κB molecules in natural killer cell development

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Abstract

Natural killer (NK) cells constitute the frontline immune defence against infections and malignant transformation. NK cells develop from committed precursors (NKPs) in the bone marrow and undergo subsequent maturation in peripheral tissues. Murine NK cells are delineated into distinct maturation stages based on differential CD27 and CD11b expression. The maturation pathway of NK cells is a 4 step process that involves the transition of double negative $CD11b^{-}CD27^{-}$ cells to $CD11b^{-}CD27^{+}$ cells, which then mature into $CD27^{+}CD11b^{+}$ cells that finally undergo terminal differentiation into $CD27^{-}CD11b^{+}$ cells. During NK cell development, this pathway is thought to occur in a linear fashion with the generation of each subset arising from its direct precursor. Analysis of the NK cell developmental pathway has shown the importance of transcription factors in this setting and results from these studies have largely supported the linear development pathway. Using genetically manipulated mice, this study sought to investigate the importance of the Nuclear Factor of Kappa B (NF-kB) family of transcription factors in NK cell development and maturation. The development of NK cells was shown to be independent of individual c-Rel and NF-kB1/p50 proteins whereas maturation of peripheral NK cells required *RelA*. The absence of RelA was characterized by a significant reduction in peripheral NK cells expressing CD27 but was also associated with normal numbers of terminally differentiated NK cells. Using confocal microscopy, two distinct populations of NK cells with and without nuclear translocated RelA were identified, suggesting RelA dependent and independent mechanisms may control NK cell development. Collectively, this study demonstrates the importance of RelA during the generation of peripheral NK cells and should prompt further investigation of the role of RelA in NK cell-dependent control of disease

Declarations

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

The isolation of nuclear and cytoplasmic fractions and electrophoretic mobility shift assay were prepared with the assistance of Raelene Grumont (Monash University, Melbourne, Australia) (Chapter 5, Figure 5.1).

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Abbreviations

Bcl11b	B-Cell CLL/Lymphoma 11B
BAFF-R	B-cell-activating factor receptors
bHLH	Basic helix-loop-helix
bZIP	Basic leucine zipper
BM	Bone marrow
DN	$CD11b^{-}CD27^{-}$
SP	$CD11b^{-}CD27^{+}$
TD	$CD11b^{+}CD27^{-}$
DP	$CD11b^{+}CD27^{+}$
ChIP	Chromatin immunoprecipitation
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
CTLs	Cytotoxic T-lymphocytes
DLL	Delta-like ligands
DC	Dendritic cell
ID2	DNA binding protein 2
DNAM-1	DNAX Accessory Molecule-1
PBS	Dulbecco's phosphate buffered saline
EMSA	Electrophoretic mobility shift assay
ELISA	Enzyme-linked Immunosorbent Assay
Eomes	Eomesodermin
FL	flt3 ligand
FOXO1	Forkhead box protein O1
GM-CSF	Granulocyte macrophage colony stimulating factor
HSC	Hematopoietic stem cell
HLA	Human leukocyte antigen
HED-ID	Hypohidrotic ectodermal dysplasia with immunodeficiency
iNK	Immature Natural cells
iNK	Immature natural killer cells
Ig	Immunoglobulin
ILC	Innate lymphoid cell
IFN	Interferon
IL-15Rβ/CD122	Interleukin-15 receptor β chain
IL-7Rα/CD127	Interleukin-17 receptor α chain
IKK	IκB kinase
JAK	Janus kinase
KIR	Killer-cell immunoglobulin-like receptors
KLRG1	Killer-cell lectin-like receptor subfamily G, member 1
LN	Lymph node
LAK	Lymphokine-activated killer
LTβR	lymphotoxin β-receptor
MIP1	Macrophage inflammatory protein-1
MHC	Major histocompatibility complex

MC	Mast cell
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
mAb	Monoclonal antibody
MULT1	Mouse UL16-binding protein-like Transcript 1
NCRs	Natural cytotoxicity receptors
NK cell	Natural killer cell
NKP	Natural killer cell precursor
NKG2D	Natural killer group 2 member D
NEMO	NF-kappa-B essential modulator
NIK	NF-κB-inducing kinase
Nfil3	Nuclear factor IL-3
NF-κB	Nuclear Factor-kB
NLS	Nuclear localization sequence
PALS	Periarteriolar lymphatic sheaths
PBMC	Peripheral blood mononuclear cells
Pre-NKP	Pre-Natural killer cell precursors
RAG	Recombinase activating gene
RBC	Red blood cell
RHR	Rel homology region
RAE-1α-ε	Retinoic acid early inducible 1
RT	Room temperature
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
T-bet	T-box expressed in T cells
TAD	Transactivation domain
TNF	Tumor necrosis factor
ULBP	UL16 binding protein

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Chapter 1

Literature Review

Transcriptional regulation of Natural Killer Cell Development and Maturation

1.1 Introduction

The immune system is comprised of a pool of cells that originate from bone marrow (BM) resident hematopoietic stem cells (HSCs). These hematopoietic cells are separated into two major lineages: the lymphoid lineage, which include the T, B and natural killer (NK) cells, or the myeloid lineage, composed of monocyte/macrophage, erythrocytes, megakaryocytes and granulocytes. Another cell of hematopoietic origin are termed dendritic cells (DCs), however, which lineage they belong to is unclear [2]. In addition to this, a group of innate lymphoid cells (ILCs) derived from common lymphoid progenitor (CLP) have been identified [3]. Based on their phenotype and cytokine secretion profile, ILCs are divided into three subsets: group 1 ILCs, group 2 ILCs and group 3 ILCs [4]. The main function of the immune system is to protect the body from pathogenic microbes, including viruses and bacteria, and to eliminate tumour cells, as well as toxins and other foreign substances that threaten normal homeostasis [5,6]. To do this, the immune system employs an elegant mechanism that relies on the distinction of detecting structural features specific to disease-causing agents. In other words, the immune system has the ability to distinguish "self" from "non-self", avoiding excessive damage to host cells and tissues [5]. While there are many cells that form part of the immune response, they can be classified into two major branches, based on the kinetics with which they respond to insult. These are termed innate and adaptive immunity, which although separated by the time it takes them to become fully active, interweave with each other to ensure the survival of the host [5,6].

1.1.1 The innate immune system

The innate immune system provides a first-line of defense against pathogenic agents, and is characterized by the speed in which it generates a productive immune response, within minute to hours of exposure to the disease-causing agent[6,7]. The cells of the innate immune system are comprised of mast cells (MCs), macrophages, neutrophils, eosinophils and NK

cells [8], and mostly rely on germline encoded receptors for the recognition of molecular patterns that are not present in the host [9]. Moreover, innate immunity is characterized by speed in which it generates a productive immune response, usually within minutes of exposure to the disease-causing agent [6,7]. In addition to the initial response, the innate immune system has a role in activating the adaptive immune response [7]. In cases where the innate immune response is not successful in eliminating the potential threat, the adaptive immune system can be activated to help eliminate the danger. This response is delayed, taking days or weeks to be established, however it is more specific to each unique pathogen and can form memory, meaning, the immune reaction is improved with repeated exposure to the same pathogenic agent [6,9,10].

1.1.2 The adaptive immune system

The adaptive immune system can be divided into humoral and cellular immunity and is carried out by different classes of lymphocytes, called B- and T-cells. Humoral immunity refers to the recognition of foreign antigens by B-cells, which secrete immunoglobulin proteins called antibodies. These circulate in the bloodstream and bind specifically, and with great sensitivity, to the foreign antigen that triggered its production. It is the binding of antibody that inactivates viruses and microbial toxins by blocking their binding to host cells and opsonizing the pathogen for phagocytic cells to ingest [9,11]. On the other hand, cellular immunity is meditated by $CD4^+$ T-cells (T helper cells) and $CD8^+$ T-cells (cytotoxic T-lymphocytes (CTL) [2,9]. Armed CTLs target intracellular pathogens, such as cells infected with viruses or certain bacteria, with specificity dictated by an interaction between the T-cell receptor (TCR) and the class I major histocompatibility complex (MHC) [12]. In contrast to innate immunity, the capacity of $CD8^+$ T-cells to recognize MHC is not germline encoded but involves genetic recombination of the TCR. This process occurs via the recombinase activating gene (RAG), which works to generate an extraordinarily large range of $CD8^+$ T-

cells with differing TCR specificities. From this pool of almost 10^8 unique $CD8^+$ T-cells, it is highly likely that at least one will demonstrate exquisite specificity for the pathogen. During the primary infection, this cell expands to control the disease but, more importantly, generates a pool of long lived cells that provide secondary control. It is this process, termed immunological memory, that prevents re-infection and is the basis for vaccination. In an analogous fashion, although with distinctions, pathogenic antigens derived from ingested extracellular bacteria and toxins, are carried to the cell surface and presented to $CD4^+$ T-cells. These cells provide signals to activate antigen specific B-cells, a process that shares the principal of immunological memory, albeit generated by contrasting mechanisms [13].

1.2 Natural killer cells in the immune system

Natural killer cells are an important member of the innate immune system, and were first discovered in 1974 and described as being "spontaneous killer cells" [14-17]. These cells were identified as "large, granular" lymphocytes with the ability to exert a cytotoxic effect against certain mouse lymphoma cells *in vitro* [14,17,18]. Since their discovery over 40 years ago, much has been learned about their cellular lineage development and function. Functionally, NK cells have been shown to have a significant role in the elimination of infected cells, or those that have undergone malignant transformation [19,20].

As alluded to earlier, the function of NK cells in innate defense is regulated by a series of germ-line encoded receptors. Significantly, these can be activating or inhibitory, providing the balance mechanism by which NK cells recognize "self" and "non-self". Without prior sensitization [21], the outcome of NK cell recognition of "non-self" is the release of chemokines, pro-inflammatory cytokines and contact-dependent killing of target cells through the release of the pore-forming molecule perforin, and apoptosis-inducing proteases [22,23].

1.2.1 Natural killer receptors

The germ-line encoded receptors on the NK cells of mice includes the C-type lectin-like Ly49 NK cell receptor family [24]. In contrast, humans express a structurally distinct but functionally similar receptor family termed the killer-cell immunoglobulin-like receptors (KIR). Both Ly49 and KIR are specific for polymorphic class I MHC, being H-$2^{b,d,l}$ in mice and Human leukocyte antigen (HLA)-A, -B and -C molecules in humans **(see Table 1)** [25]. The activation of NK cells is regulated by a balance between activation and inhibition signals transduced upon the interaction between NK cell receptors and their respective ligands on the target cell surface. In this regard, target cell lysis will take place when the activation signals outweigh the inhibition signals [25-27] **(Figure 1)**. However, to prevent damage to healthy cells, NK activation is tightly regulated through the "missing-self" or the "induced self" theory, which is commonly used as a guiding principle for understanding target cell recognition by NK cells [28].

Table 1. NK cell Ly49 and KIR receptors

Receptor family	Species	Ligands	Activation/Inhibition
Ly49	**Mouse**	**MHC class I**	**Activation / Inhibition**
Ly49A		H-$2D^{d,k,p}$	Inhibition
Ly49C		H-$2K^{b,d,}$ H-$2D^{b,d,k}$	Inhibition
Ly49D		H-$2D^{d}$	Activation
Ly49H		m157	Activation
Ly49I		H-$2K/D^{b,d,s,q,v}$	Inhibition
Ly49P		H-$2D^{d}$	Inhibition
KIR	**Human**	**HLA-A/-B/-C**	**Activation / Inhibition**
KIR2DL1		HLA-C2	Inhibition
KIR2DL2/3		HLA-C1	Inhibition
KIR2DL4		HLA-G	Activation
KIR3DL1		HLA-Bw4	Inhibition
KIR3DL2		HLA-A3, -A11	Inhibition
KIR2DS1		HLA-C2	Activation
KIR2DS2		HLA-C1	Activation
KIR3DS1		HLA-Bw4	Activation

Adapted from Pegram, Andrews et al.2011 [25].

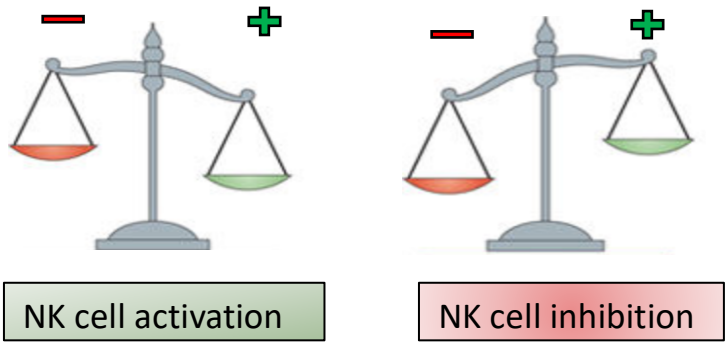


Figure 1. The activation of NK cells is regulated through a balance between activation and inhibition signals. NK cells use their inhibitory receptors to detect the absence of self-molecules on potential target cells, and their activating receptors to detect danger, or stress signals. When activation signals outweigh the inhibition signals, NK cells become activated and endeavour to kill the target cell. Similarly, NK cell activation is inhibited when the inhibition signals outweigh the activation. Figure adapted from Vivier, Ugolini et al [29].

1.3. NK cells acquire self-tolerance during development

In an early study, all mature NK cells were reported to express at least one inhibitory receptor (Ly49 in mice and KIR in humans) that recognizes self MHC class I protein [30,31]. This interaction is required for NK cells to achieve functional maturation and self-tolerance via a process termed "licensing". This process prevents NK cells from attacking healthy self-cells by ensuring that mature NK cells can be inhibited by the presence of self MHC, and therefore allows them to distinguish infected cells or tumor cells from normal self-cells [32,33]. The importance of this interaction is seen during NK cell development, in which the absence of inhibitory receptors specific for self-MHC class I results in the development of NK cells that lack the ability to recognize and kill MHC class I-deficient tumor cells *in vitro*, and MHC class I deficient BM *in vivo* [24,30,34].

Two self-tolerance mechanisms have been proposed to explain the "licensing" of NK cells by inhibitory receptors. The first hypothesis, termed the "arming" model, claims NK cells are initially hyporesponsive during development until the engagement of an inhibitory receptor with its corresponding self-MHC class I protein subsequently "arms" the NK to acquire

functional competence [32]. Alternatively, the "disarming" hypothesis suggests that all NK cells are initially responsive and that NK cells are persistently stimulated *in vivo* by stimulatory ligands expressed on normal self-cells in the body. However, hyporesponsive NK cells arise if the stimulatory signals are not counteracted by inhibitory signals from MHC class I-specific receptors [24,32,35]. Therefore, in either model, expression of inhibitory receptors for self-MHC class I is required for functional maturation of NK cells via an MHC class I-dependent 'licensing' [30,34]. Once licensed, NK cells are equipped with the ability to regulate NK cell activation through the "missing self" or "induced-self" model of NK cell activation.

1.3.1 The "missing-self" and "induced-self" theory of NK cell activation

The "missing self" theory of NK cell activation is dependent on the recognition of MHC-I, MHC-I-related molecules or HLA class I (expressed on healthy nucleated cells) by inhibitory receptors on NK cells, which dampen NK cell activation [28,36]. For instance, a number of Ly49 receptors in mice [37,38], KIR receptors in human [39,40] and the heterodimeric CD94/NKG2A lectin-like inhibitory receptor in both species recognize and attack cells that lack MHC-I expression [28]. Whereas, the presence of MHC I on target cells engage with inhibitory receptors on NK cells and inhibits NK cell activation and thus blocks target cell killing [25,28,41]. In regards to their role in diseases, viral infection and malignancies, pathogens have evolved ways to manipulate the immune system to inhibit cytotoxic T-lymphocytes (CTLs) responses, including down-regulation or complete loss of MHC-I expression, thereby rendering antigen-specific T-cell responses unresponsive, but increasing susceptibility to NK cell-mediated killing [42-44].

In contrast, the “induced-self” model of NK cell activation describes NK cell recognition of ligands that are upregulated upon cellular stress, malignant transformation or viral invasion [45]. For example, the NK cell activating receptor, NKG2D recognizes the “induced-self” ligands expressed upon cellular stress, these include MHC class I chain-related gene MICA/MICB and UL16 binding proteins (ULBP 1–6) in humans, and mouse UL16-binding protein-like Transcript 1 (MULT1), Retinoic acid early inducible 1 (RAE-1α-ε), and the minor histocompatibility (H) antigen (H60a–c) expressed in BALB.B mice [25,45-47]. Thus, NK cell recognition mechanisms are crucial for immediate defense against tumor cells, virally infected cells, stressed cells and T-cell elusive diseases [26,42,44,48].

1.4 Natural killer cell development

The stages of NK cell development have been distinguished based on phenotypic and functional differences **(Figure 1.2A)**. First, the CLP differentiates into pre-NK cell precursors (pre-NKP), which can be distinguished by the increased expression of CD27 and CD244 and decreased expression of c-kit (CD117), however at this stage they still lack common lineage markers ($CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $Ter119^-$, $GR\text{-}1^-$ and $NK1.1^-$). Subsequently, pre-NKP cells differentiate into an NKP population, defined by the expression the IL-15 receptor (IL-15R) beta chain (CD122) and differential expression of IL-7Rα (CD127). Similar to pre-NKP these cells continue to lack NK cell specific markers. From the NKP stage, cells develop into immature NK cells (iNK), which is coupled with the loss of IL-7Rα expression but the gain of the inhibitory receptor CD161, which belongs to the C-type lectin family and is recognized by the NK1.1 antibody in C57BL/6 and C57Bl/10 mouse strains [49,50]. However, the expression of CD161 is not NK cell-specific as it is expressed by 15–25% of peripheral blood T-cells, activated $CD8^+$ T-cells and NKT cells [51]. Therefore, iNK cells express the NK cell marker NKp46, which belongs to a family of natural cytotoxicity receptors (NCRs) that is highly specific for NK cells. These iNK cells express low levels of CD11b (also known as integrin αM) and members of the Ly49 family of receptors [52,53]. In order to be functionally

competent in regards to cytotoxicity and production of interferon (IFN)-γ, the iNK cell population undergoes a linear maturation process in which the expression of Ly49 molecules, CD27 and CD11b are sequentially regulated [54] (**Figure 1.2**). The development of NK cells rely heavily on specific cytokines and transcription factors at each stage of development, which will be explored in Section 1.4.1.1.

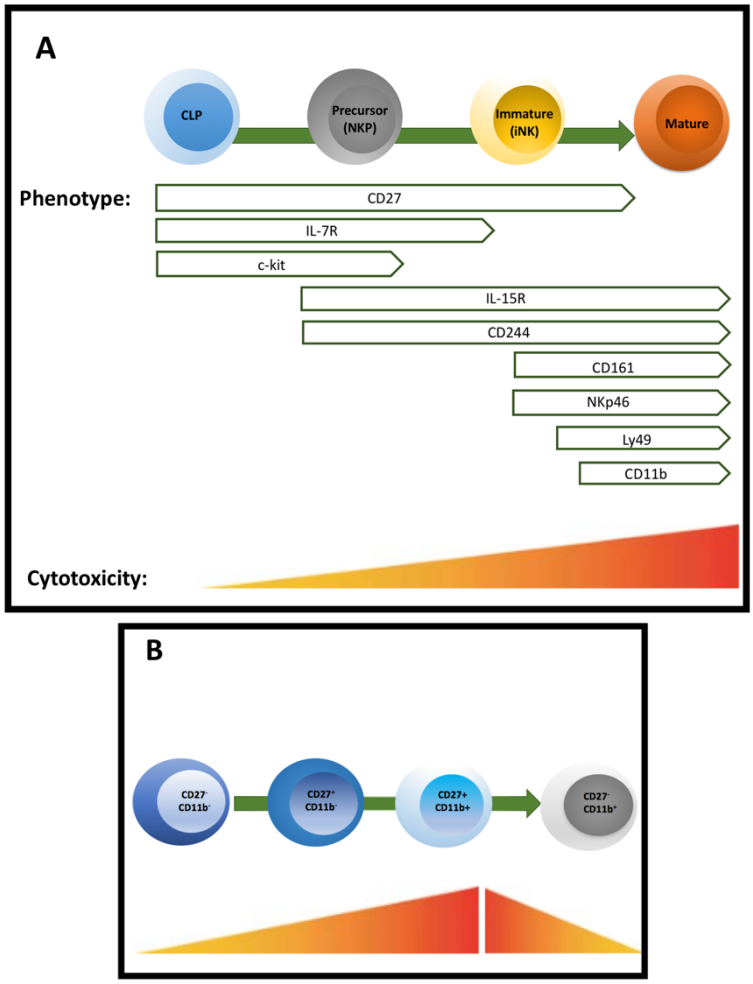


Figure 1.2. Schematic representation of NK cell developmental and maturation in the bone marrow and peripheral organs: A. Mouse NK cell development in the BM follows a linear pathway from the CLP to mature NK cells, which correlates with increased cytotoxicity. The stages of development can be identified by the pattern of surface antigen expression. **B.** Mature NK cells leave the BM and undergo further maturation in peripheral tissues, such as the spleen and liver. NK cell maturation in the periphery follows a linear model of differentiation on the basis of CD27 and CD11b expression. In **A** and **B**, the levels of cytotoxicity within populations is marked by the size of the triangle.

1.4.1. NK cell development into NK cell committed precursors

The acquisition of the IL-15R beta chain (CD122) at the NKP stage marks an important step of NK cell differentiation as IL15 promotes NK cell differentiation, functional maturation and survival in both mouse and human [21,55,56]. The IL-15R complex is composed of the unique IL-15Rα chain, the shared IL-2R (common) γ (γc) and IL-15Rβ chains, which is used by both the IL-2R and IL-15R complex [21,57] **(Figure 1.3)**. Due to the cytokine-receptor subunits being shared between IL-2 and IL-15, signal-transduction pathways are also shared. These include the Janus kinase 1 (JAK1), Janus kinase 3 (JAK3) and Signal transducer and activator of transcription 5 (STAT-5) signaling pathways, ultimately leading to gene transcription and initiation of the NK cell developmental program. Mice lacking a functional IL-15 receptor (IL-15RβR$^{-/-}$) exhibit a significant reduction of NK cells in the BM and spleen, indicating that IL-15 is essential for NK cell development [58]. In further support of this, targeted mutations the IL-15R complex components IL-15Rα and IL-2Rβ, which forms part of the IL-15R complex, results in NK cell deficiency, whereas the absence of IL-2Rα does not affect NK cell development [21,58]. In addition, mouse NK cells have been shown to develop from BM-derived NKPs when cultured with the cytokines, IL-15, IL-7 and the factors, c-kit ligand (CD117) and flt3 ligand (FL); however, IL-15 alone had no effect on the development of mature NK cells [21,59], suggesting that IL-15 promotes the survival and differentiation of the developing NK cell but not lineage commitment.

The IL-15R and IL-7R complex share a structural similarity **(Figure 1.3),** prompting speculation that IL-7 played an important role in NK cell development and survival. However, surprisingly results have shown that the expression of IL-7Rα isn't essential for the development of NK cells in the BM [60,61]. In fact, a decrease in IL-7Rα expression is observed in NKPs in the BM, which is followed by complete loss of expression in circulating mature NK cells in the spleen [60,61] . However, the IL-7Rα subunit is critical for the development of B

and T-cells [62,63], suggesting the down-regulation and subsequent loss in NKPs is required to push the CLP towards an NK cell lineage. Interestingly, however, there is a population of IL-7Rα expressing NK cells that is thought to develop in the thymus, and the genetic deletion of IL-7 results in a significant reduction of these thymic NK cells [63,64]. This suggests that IL-7R plays opposing roles in NK cell homeostasis, dependent on the organ in which they are generated. In support of this, it has been confirmed that IL-7Rα^{+} thymic NK cells are a true NK cell population and not derived from the T-cell precursors [65]. In contract, BM-derived NKPs only mature in peripheral tissues, such as the thymus [64-66], which may explain the difference in IL-7Rα expression between thymic and BM-derived NK cells.

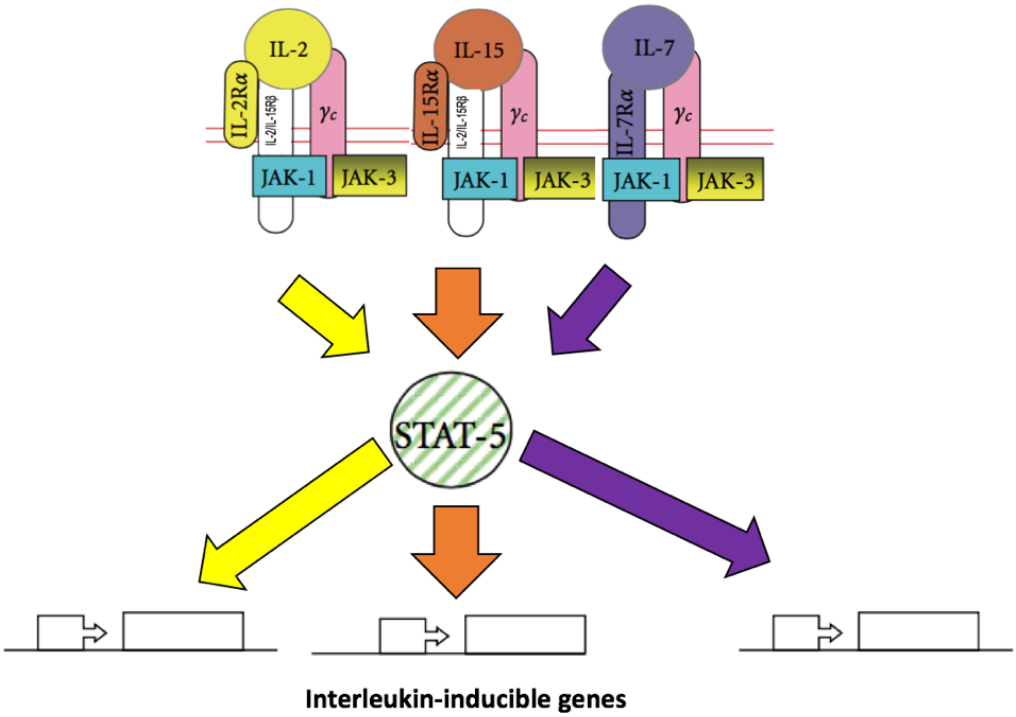


Figure 1.3. Shared signaling pathways of the NK cell cytokine receptors IL-2, IL-15 and IL-7. IL-2, IL-15 and IL-7 share the common cytokine-receptor γc in their receptor complexes, and IL-2 and IL-15 also share the IL-2/IL-15Rβ subunit. Upon binding, cytokines interact with the alpha subunit within the complex. In the case of the IL-7R complex, the IL-7 cytokine forms a dimeric complex, whereas the IL-2 and IL-15 cytokines form a trimeric complex with its corresponding receptor. All three receptor complexes mediate signal transduction via the kinases JAK-1 and JAK-3, which then activate the STAT-5 signaling pathway, resulting in gene transcription which initiates the NK cell developmental program. Figure adapted from Meazza et al [61].

1.4.1.1 Transcriptional regulation of NK cell development

A complex network of transcription factors promote and control lineage commitment of innate and adaptive lymphocytes from the CLP [60]. Although both innate and adaptive lymphocytes share multiple transcription factors, it is the differential kinetics of expression during lineage development that dictate cell fate. In NK cells, several transcription factors play a significant role in development at distinct stages, including Notch1, Nuclear factor IL-3 (Nfil3) and Inhibitor of DNA binding (Id) protein 2, T-box expressed in T cells (T-bet) and the homolog of Blimp1 in T cells (Hobit). Interestingly, despite the similarities between the group 1 ILCs and NK cells, they can be separated on the basis of transcription regulation during development. For instance, the group 1 ILCs require T-bet and Hobbit, but not Eomes and nfil3, which are important for NK cell development [4,67]. However, the precise hierarchy of the transcription factors required for NK cell development is still poorly understood, and more research is needed to comprehensively identify the full repertoire of transcription factors necessary for NK cell development.

1.4.1.1.1 Notch signaling pathway regulates early NK cell development

Notch1 belongs to the Notch family of proteins and plays an important role in NK cell and T-cell lineage commitment by signaling through Jagged1, Jagged2 and delta-like ligands (DLL) [68]. Signaling via Jagged1 and Jagged2 promote NK cell development, whereas signaling through DLL favors T-cell development. The importance of Notch1 signaling in NK cell development was shown via the deletion of the Notch1-dependent transcription factor, B-Cell CLL/Lymphoma 11B (Bcl11b). Bcl11b is poorly expressed in immature NK cells and is completely absent in precursor and mature NK cells, however it is highly specific for T-cell lineage commitment, and its expression is maintained throughout development [69,70]. In the absence of Bcl11b, thymocytes from all stages of development are diverted into an NK cell-like fate, characterized by NKp46 expression and the loss of CD3. In addition, these NK cell-

like lymphocytes express a number of NK-associated transcription factors, including high amounts of Nfil3 transcript [70]. Therefore, Notch1 signaling is thought to be very important for early stages of NK cell development from hematopoietic stem cells (HSC), dictating the transition of precursors to NK or T-cells [71] **(Figure 1.4A)**. This is supported by a complete loss of NK cells when Notch-blocking antibodies are introduced during development [72]. However, whether Notch1 also plays a role in mature NK cell development and survival is yet to be determined.

1.4.1.1.2. Nfil3 signaling required for late stage NK cell commitment

The basic leucine zipper (bZIP) transcription factor, Nfil3, also known as E4bp4, has a variety of roles in the immune system. Nfil3-deficient mice have been used in a number of studies to investigate its function in the development of NK cells. The deletion of the Nfil3 gene in mice revealed a significant reduction of NK cells in the spleen, liver and lungs, however they did not display any deficiencies in lymphoid organ T-cell subsets [73,74], highlighting a critical role for Nfil3 in NK cell lineage commitment through to the NKP stage. Considering that the BM is the main site of NK cell development [75], this suggests that Nfil3 is a key regulator of the development of NK cells. In support of this, Nfil3-deficient mice exhibit a developmental block at the immature and mature stage in BM, however, the NKP cells are unaffected. The mature NK cell impairment is global, with markedly reduced populations in the spleen, lung, and liver of Nfil3-deficient mice [74]. Due to the unaffected NKP cells, this reveals a critical role of Nfil3 in the later stages of NK cell development, more specifically targeting the differentiation of NKP cells into immature and mature NK cells **(Figure 1.4A)**, which ultimately leads to a reduction in NK cell *migration* and localization into peripheral tissue. Moreover, Nfil3 has been seen to regulate both the phenotypic and functional maturation of NK cells in the BM and the spleen. In the spleen, this is characterized by the reduction of activation receptors 2B4, Ly49D, Nkp46 and CD11b,

coinciding with a reduction of IFN-γ production and cytolytic capacity in the Nfil3 deficient mice, compared to WT [74]. However, the function of NFil3 as a regulator of NK cell development is still under investigation, with several groups attempting a targeted deletion in NK cell precursors (Dan Andrews, Personal Communication).

1.4.1.1.3. Id2 protein required for NK cell maturation

The Id protein belongs to a family of basic helix-loop-helix (bHLH) proteins, which can form either a hetero- or homodimer with E-protein transcription factors via the HLH domain. These subsequently bind to target DNA sequences through the basic DNA-binding region. Homo-dimerization of the HLH proteins result in transcriptional activation, whereas hetero-dimerization acts as either an activator or repressor of downstream gene transcription [76]. The Id proteins contain a HLH domain, but unlike E-proteins, they lack a basic DNA-binding region [76]. Id proteins negatively regulate the function of E-protein transcription factors (encoded by the genes E2A (Tcf3), E2-2 (Tcf4), and HEB (Tcf12)) [76], by quenching them through dimer formation and thereby preventing E-protein from binding with its target E-box DNA sequence [77,78]. Interestingly, E-boxes have been identified in genes required for T and B cell development and are thought to be key regulators of both B- and T-cell differentiation [76,79].

Id2, a member of the Id family of proteins, is required for NK cell development in the bone marrow and in the thymus [78]. The role of Id2 in NK cell lineage commitment has been investigated using Id2-deficient mice, which reported an arrest at the iNK stage in Id2-deficient mice and no difference in the NKP population. This suggests that Id2 is not essential for NK cell lineage commitment, but is required for the transition from immature to mature NK cell in adult BM **(Figure 1.4A)** [60,80,81].

1.5. NK cell maturation in the periphery

As previously discussed, the main site of NK cell development is in the BM, where they develop from HSCs into NKPs that give rise to iNK and mature NK cells [82] **(Figure 1.2).** Mature NK cells egress from the BM and take up residence in peripheral sites, mainly in the spleen, liver and lungs with some residing in the lymph node (LN) and BM [49,82,83]. Peripheral NK cell maturation enables the development of full effector function, demonstrated by functional capability and acquisition of certain cell surface markers [82].

Mature murine NK cells express a range of markers, which include CD49b, CD122, CD161, NKG2D, NKp46, Ly49 receptors, as well as high levels of CD11b. Murine and human NK cells can be subdivided by CD27 and CD11b expression [53,84,85]. CD27 belongs to the TNF receptor superfamily and can functionally separate murine and human NK cells into $CD27^{high}$ and $CD27^{low}$ subsets. These two NK cell subsets differ in effector functions, proliferative capacity, tissue organization and interaction with other immune cells [86,87]. Interestingly, CD27 has different functions in human compared to mouse NK cells. In humans, the majority of $CD27^-$ NK cells are more cytotoxic compared to the $CD27^+$ subsets, with an associated increased expression of cytolytic proteins (perforin and granzyme B) and *in-vitro*, show higher toxicity towards the NK cell-sensitive cell line K562 compared with their $CD27^+$ counterparts [87]. In contrast, $CD27^+$ expressing NK cells in mice display enhanced effector function, demonstrated by greater cytotoxic capacity compared to $CD27^-$ cells and greater production of IFN-γ [86-89]. These findings demonstrate the major role of CD27 surface expression in the regulation of NK cell effector function in both humans and mice, however, they emphasize that NK cell populations within the mouse do not always directly reflect that of humans. Moreover, two additional NK cell populations have been identified based on CD11b expression. $CD11b^-$ NK cells are found mostly in bone marrow and LN and display an “immature” phenotype, characterized by a high rate of homeostatic proliferation. Whereas, $CD11b^+$ NK cells are predominant in peripheral sites, such as the spleen and

peripheral blood mononuclear cells (PBMCs), and represent the main "mature" subset in adult mice. $CD11b^+$ NK cells have high levels of Ly49 receptors and potent effector functions compared to the "immature" $CD11b^-$ subset [53,84,90]. Unlike murine NK cells that do not express the marker CD56, humans NK cells are comprised of a heterogeneous population of $CD56^+$ NK cells [91]. In addition, NK cell subpopulations can be defined on the basis of relative CD56 expression. The $CD56^{dim}$ are more cytotoxic and mirror the murine $CD27^+$ NK cells, whereas, the $CD56^{bright}$ NK cells closely resemble the $CD27^-$ NK cells [92]. Interesting, however, brief priming of $CD56^{bright}$ NK cells with IL-15 has been shown to enhanced their anti-tumour response, including degranulation, cytotoxicity, and cytokine production [93].

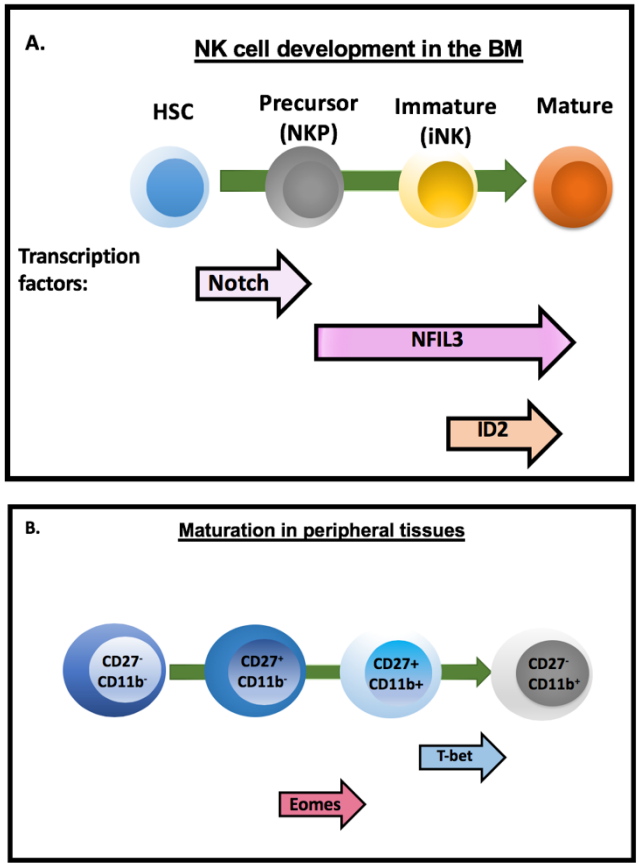


Figure 1.4. Schematic representation of the transcription factors regulating the stages of NK cell development in the BM and the mature subsets in the peripheral tissues. **A.** In the BM, Notch1 is suggested to regulate the differentiation of the CLP to NKPs, while Nfil3 controls the transition of NKPs to mature NK cells and Id2 is required for the final stage of NK cell maturation. **B.** In the periphery, Eomes controls differentiation of $CD27^+CD11b^-$ cells to the $CD27^+CD11b^+$ subset, and T-bet regulates the transition of $CD27^+CD11b^+$ NK cells to the terminally differentiated $CD27^-CD11b^+$ NK cell subset.

1.5.1 NK cell maturation is separated based on CD27 and CD11b surface expression

Maturation of peripheral NK cells follow a linear model of differentiation on the basis of CD27 and CD11b expression. The pool of peripheral NK cells are subdivided into phenotypically and functionally distinct stages of maturation from the most immature $CD11b^{-}CD27^{-}$ (DN) stage through $CD11b^{-}CD27^{+}$ ($CD27^{+}$), which then mature into $CD27^{+}CD11b^{+}$ (DP) cells that finally undergo differentiation into $CD27^{-}CD11b^{+}$ ($CD11b^{+}$) NK cells [49,88,94], (**Figure 1.4B**). This model was proposed based upon results using an adoptive transfer method where the injection of DN cells gave rise to $CD27^{+}$, DP and $CD11b^{+}$ cells in a linear fashion[84]. Interestingly, $CD27^{+}$ cells are mostly localized in the LN and BM, whereas $CD11b^{+}$ cells are predominately in the blood, spleen and lungs [49,84], suggesting organs specific factors can regulate NK cell maturation.

The $CD27^{+}CD11b^{+}$ NK cell subset is considered “mature” and the most cytotoxic and potent cytokine producers. In line with the observation that they are the last stage of NK cell development, NK cells belonging to the $CD27^{-}CD11b^{+}$ subset is classified “terminally differentiated”. This is characterized by increased expression of KLRG1 (killer-cell lectin-like receptor subfamily G, member 1) [95], a marker associated with decreased proliferative capacity, increased apoptosis, and reduced effector function of NK cells [94,96].

1.5.1.1 Transcriptional regulation of NK cell maturation

A number of transcription factors regulate NK cell maturation, including T-bet and Eomesodermin (Eomes), which belong to a phylogenetically related family of T-box transcription factors that share a T-box DNA-binding domain [97,98]. T–bet was first identified in murine $CD4^{+}$ T-cells with a key role in regulating T helper 1 cell lineage commitment by trans-activating the IFN-γ gene and inducing IFN-γ production [98-100]. Eomes expression was

first discovered in Xenopus gastrula embryos and was demonstrated to be involved in embryogenesis of vertebrates [98,101]. Since then, Eomes and T-bet expression have also been identified in major human and murine blood cell subsets, including NK cells, where they play a significant role in NK cell maturation [97]. In the absence of Eomes, NK cells arrest at the $CD27^{+}CD11b^{-}$ stage [102,103], whereas, T-bet drives the terminal differentiation of NK cells to the $CD27^{-}CD11b^{+}$ cells [104,105] **(Figure 1.4**)**,** characterized by a decreased number in the spleen, liver, and peripheral blood [104]. Forkhead box protein O1 (FOXO1) negatively regulates NK cell maturation by acting upstream of the T-bet gene and suppressing T-bet expression, and subsequent to a conditional deletion of FOXO1 from NK cells, there is an observed loss of CD27 expression and an increased proportion of $KLRG1^{+}$ cells [105]. Overall, this demonstrates the importance of the transcriptional regulators Eomes and T-bet in NK cell maturation and thus function.

1.6. The role of Nuclear Factor-κB (NF-κB) in the immune response

A diverse family of transcription factors are modulated upon surface receptor signaling, in turn, reprogramming gene transcription for cytokine and chemokine production [106]. Many of the transcription factors controlling cytokine production in NK cells have been inferred on the basis of similarity to $CD8^{+}$ T-cells. Production of IFN-γ by $CD8^{+}$ T-cells requires T-box factors, STAT proteins and the NF-κB family members. In NK cells, IL-12 is required to activate STAT4 and T-bet and lead to subsequent IFN-γ production [107,108]. However, these pathways are not absolute and T-bet/STAT4 independent pathways to IFN-γ production do exist. In this regard, treatment of NK cells with the Th1 cytokines IL-18 and TNF-α can activate NF-κB, which in conjunction with IL-12 enhances NK cell production of IFN-γ [109]. In addition, NF-κB signaling has been shown to have a profound role in T-cell survival, activation, proliferation and cytokine secretion [109-111]. Given the similarity between T- and

NK cells in regards to transcription factor regulation, an investigation of the contribution of individual NF-κB members in NK cell biology is warranted.

1.6.1 The NF-κB signalling pathway

NF-κB is a transcription factor first identified in 1986 as a nuclear factor associated with an enhancer element of the immunoglobulin (Ig) κ light chain genes in B cells [112]. Since then, a heterogeneous collection of NF-κB dimers has been identified in almost all cell types [113]. The NF-κB family contains five mammalian members, NF-κB1 (encoding the precursor molecule p105 and the processed form p50), NF-κB2 (encoding the precursor p100 and the processed form p52), RelA (p65), RelB and c-Rel, which all recognize a common sequence motif [114]. The three Rel proteins, RelA, RelB and c-Rel contain a nuclear localization sequence (NLS) and share a conserved 300-amino-acid Rel homology region (RHR), made up of two Ig-like domains. The RHR domain is responsible for interaction with IκB proteins, dimerization and DNA binding [113]. In addition, NF-κB proteins differ the ability to activate transcription, for instance, RelA, RelB and c-Rel, have a carboxy-terminal transactivation domain (TAD) that initiates transcription from NF-κB–binding sites in target genes[113,115-117], whereas, NF-kB proteins lack the transcriptional domain and function as transcriptional repressors, such as homodimeric NF-kB1 (p50) and NF-kB2 (p52) [113].

RelA-deficient mice exhibit embryonic lethality at embryonic days 14–15 due to liver degeneration via increased hepatocyte apoptosis, demonstrating the essential role of RelA for survival [118]. The RelA-p50 heterodimer is the most abundant NF-kB complex in most cell types, but other homo- and heterodimeric complexes are possible depending on the cell type and the activation signal [113,119]. Alternatively, RelB is a potent transcriptional activator that dimerizes with only p50 or p52, with expression restricted to specific regions of the lymphoid organs such as the thymic medulla, paracortex of lymph nodes and the

periarteriolar lymphatic sheaths (PALS) of the spleen [120]. Unlike RelA and c-Rel complexes that are involved in an inducible kB-binding activity and gene activation, RelB-p50 and RelB-p52 heterodimers are associated with basal kB-binding in thymus and spleen, indicating that RelB controls the constitutive expression of kB-regulated genes in these tissues [120,121]. For instance, *relb* gene disruption in the spleen results in reduced DC numbers, moreover, these DCs have an impaired antigen presenting function [122,123]. Similar to the other Rel proteins, c-Rel can form homo- or heterodimers with other NF-kB proteins, but unlike RelA and RelB that are activated by immature lymphocytes, c-Rel is activate in mature lymphocytes [124]. The deletion of c-Rel interferes with a number of immune cell-specific functions, including reduced B-cell proliferation, survival and antibody expression [115], as well as reduced IL-2 production by T-cells and impaired T-cell proliferation and differentiation [124].

1.6.1.2 The canonical and non-canonical NF-kB activation pathway

Rel and NF-kB proteins become activated via a well characterized "canonical" or "non-canonical" NF-κB activation signaling pathway [125] **(Figure 1.5)**. The "canonical" pathway regulates the activation of RelA, c-Rel and NF-kB1/p50 hetero or homodimers. As illustrated in figure 1.5, the majority of the NF-kB dimers are retained in a resting state in the cytoplasm, bound to the IkB subunits IkB-α, IkB-β, or IkB-ϵ. This pathway becomes activated by a diverse family of receptors, such as the tumor necrosis factor (TNF) and Toll-like receptors, leading to the phosphorylation of IkB molecules by the IκB kinase (IKK) complex, composed of IKK-α–IKK-β and the regulatory subunit, IKK-γ (termed NEMO). As a result, the IκB molecule undergoes proteasomal degradation and the NF-kB1 dimers are released into the cytoplasm where they subsequently translocate into the nucleus to bind to the kB sequences and initiate gene transcription [116,126]. For example, signaling through the TNF receptor superfamily member CD27 results in activation of the RelA/p50 dimer through an IKKβ- and IKKγ-dependent IκBα degradation [127] [128]. In contrast to the "canonical" pathway, the "non-

canonical" signaling pathway activates the RelB/p52 dimer complex through a mechanism that involves processing of NF-kB2 precursors, p100 to p52, in an IKK-independent manner. This pathway uses NF-κB-inducing kinase (NIK) to phosphorylate and activate an IKKα complex, which then phosphorylates p100, leading to the release of RelB/p52 complex for subsequent entry into the nucleus, where it binds to the kB sequences for gene transcription [116,129,130] . This pathway is activated by specific receptors, such as a subset of TNFR superfamily members, including B-cell-activating factor receptors (BAFF-R) [131], CD40 [132] and lymphotoxin β-receptor (LTβR) [133]. The "non-canonical pathway" regulates a number of immune responses, such as B-cell survival/maturation and DC activation. Additionally, the absence of this pathway is associated with lymphoid malignancies [129]. Therefore, both the "canonical" and "non-canonical" NF-kB signaling pathway has a key role in immune regulation.

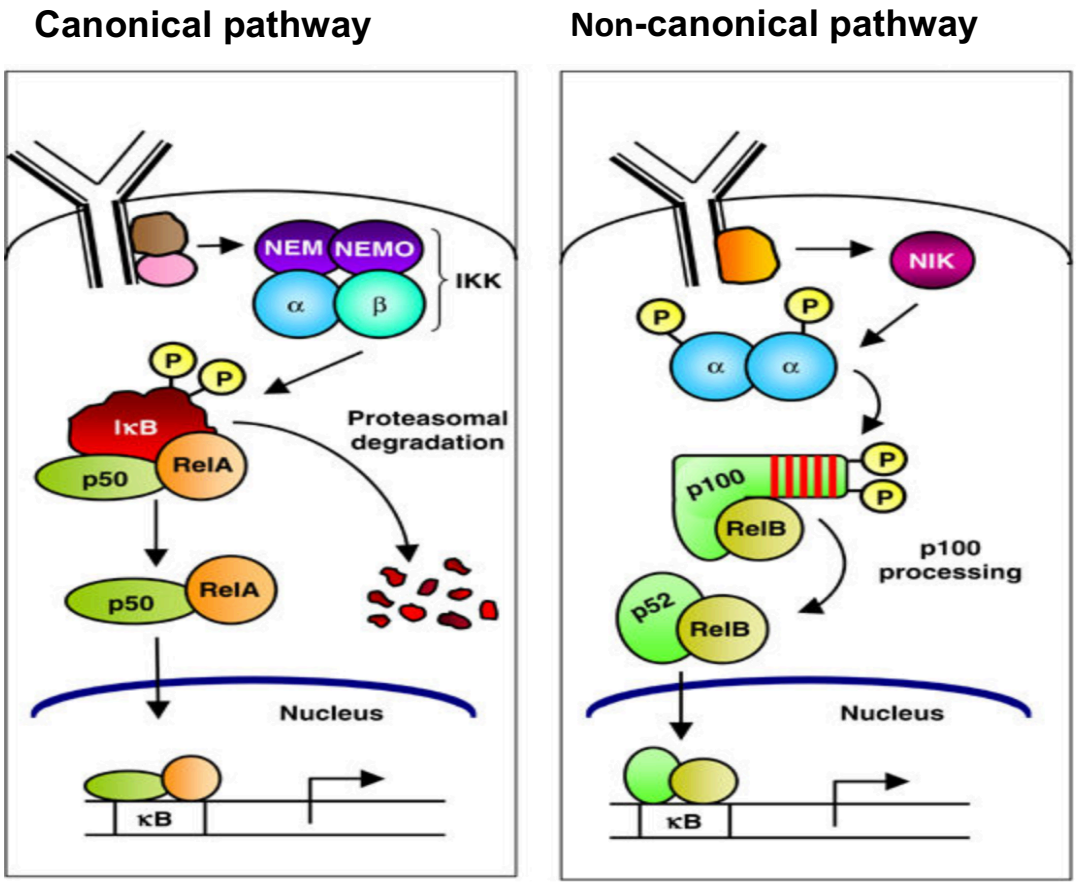


Figure 1.5. NF-κB signal transduction pathways. In the canonical NF-κB pathway (left), the NF-kB dimers are sequestered in the cytoplasm bound to the IκB molecule (often IκBα). Upon stimulation, the IKK complex (containing the α and β catalytic subunits and two molecules of the regulatory IKKγ (NEMO) are recruited to phosphorylate the IκB molecule. This leads to the ubiquitination and subsequent proteasomal degradation of the IκB molecule. The NF-κB dimers are released and enter the nucleus where they bind to the kB element to initiate target gene transcription. In the non-canonical pathway (right), signaling through specific receptor-ligand interactions leads to activation of the NF-κB-inducing kinase NIK, which then phosphorylates and activates the IKKα complex to phosphorylate and process p100 to P52. This pathway leads to the release of the p52/RelB complex into the cytoplasm, before entering the nucleus and binding the kB element to initiate target gene transcription. Figure taken from [130].

1.6.3 The role of NF-κB in natural killer cells

Despite the in-depth characterization of the effect of NK-kB in most other cell types, there has been very little research into the role it plays within NK cells. One study observed a relationship between NK cell activity and NF-κB activation. For example, NF-κB1/p50 has been associated with perforin production via IL-2Rß signaling [134], and NF-κB1/p50 and RelA are required for controlling Ly49 gene expression *in vitro* by binding to the Ly49 promoter, Pro1 [135]. Furthermore, NF-κB/p50 and c-Rel knock-out mice have addressed the role of NF-κB in NK cell proliferation and IFN-γ production. Interestingly, c-Rel behaves as an agonist, whereas NF-κB/p50 plays an opposing role as an antagonist for NK cell proliferation and IFN-γ production [109]. These studies, however, have only scratched the surface of NK cell biology in terms of NF-κB family members. Importantly, the role of individual NF-κB family members in NK cell development was not delineated. Furthermore, the analysis of NK cell activation was restricted only to IFN-γ production without any functional data on NK cell cytotoxicity. Moreover, paired engagement of DNAX Accessory Molecule 1 (DNAM-1) with the coactivation receptor, 2B4 (CD244) was shown to be required for RelA activation [136], however this was briefly explored in NK cells.

1.7 DNAM-1 is important for immune surveillance

DNAM-1 is a 65 kDa transmembrane glycoprotein consisting of two Ig-like domains in the extracellular portion, and a cytoplasmic tail containing three phosphorylation sites for intracellular kinases [137,138]. It has been identified as an adhesion molecule that belongs to the immunoglobulin (Ig) immunoreceptor superfamily and binds to the Nectin and Nectin-like molecules, CD155 (Polivirus Receptor PVR; Necl5) and CD112 (Nectin-2, PRR2) [90,137-139]. DNAM-1 is broadly expressed on human and murine lymphocytes. In humans, DNAM-1 is expressed on all NK cells, T-cells and monocytes, whereas in mice, it is constitutively expressed on all $CD8^+$ T-cells, activated $CD4^+$ T-cells, but only on 25-50% of splenic NK

cells [140-142].

Upon binding to its ligands, DNAM-1 plays a predominant role in inducing cytotoxicity in NK cells, demonstrated in monoclonal antibody (mAb)-dependent blocking experiments using human NK cell lines, including the YT line that lacks CD16, NKp30, NKp46, NKp44, and NKG2D, but expresses DNAM-1[138]. Moreover, DNAM-1 has been implicated as a major trigger for NK cell-mediated killing of tumors over-expressing CD155 and CD112 [140,143], characterized by the inhibition of NK-cell mediated lysis of cell transfectants and tumor cell targets when DNAM-1 binding with either CD155 or CD112 is blocked [138], and increased tumor development in NK cells lacking the DNAM-1 surface receptor [90,144]. Furthermore, CD155 has been shown to be the key ligand for triggering NK-cell mediated tumor suppression, which coincides with CD155 overexpression on tumor cells [142,145,146] and may be explained by different binding affinity seen between DNAM-1 and CD155 or CD112, with CD122 binding less efficiently to DNAM-1 compared to CD115 [139,147]. However, the role of DNAM-1 in cytotoxicity and tumor elimination is redundant in the presence of the NKG2D-ligand pathway [142], thereby revealing a complex hierarchy among NK cell receptors. Therefore, DNAM-1-CD155 interaction by NK cells is important for immune surveillance of tumor development, in particular, tumors that are weakly immunogenic and do not interact with the activating molecule, NKG2D.

1.7.1 DNAM-1 marks an alternative of NK cell maturation in peripheral sites

Recently, a new program of NK cell maturation has been proposed based on DNAM-1 expression [90]. In this model, DNAM-1$^-$ NK cell develop from DNAM-1$^+$ NK cells independent of the CD27 and CD11b maturation scheme. DNAM-1$^+$ NK cells are more active and produce larger amounts of pro-inflammatory cytokines IFN-γ, IL-6 and Granulocyte-macrophage colony-stimulating factor (GM-CSF), whereas DNAM-1$^-$ NK cells produce macrophage

inflammatory protein-1 (MIP1) chemokines with phagocyte-stimulating and pro-inflammatory properties [90]. DNAM-1$^-$ NK cells express genes involved in NK cell receptor signaling as well as certain cytokines and chemokines. In addition, a molecular network analysis revealed up-regulation of nfkb1 in DNAM-1$^-$ NK cells. On the other hand, DNAM-1$^+$ NK cells overexpress cytokine-related genes, consistent with their pro-inflammatory profile and a more activated phenotype [90]. Considering DNAM-1 has a role in NK cell effector function, tumor elimination and more recently represent a new pathway of peripheral NK cell maturation, an investigation in the transcriptional control of this molecule is warranted.

1.8 Summary and thesis aims

NK cells play a pivotal role in providing rapid defense against pathogens and malignant cells. They acquire cytotoxic and cytolytic potential throughout development, primarily in the BM, and during subsequent maturation in peripheral tissues, such as the spleen. The main regulators of NK cell development and function are a pool of transcription factors which influence the stages of NK cell development, cytotoxicity and receptor expression. The NF-κB family members are expressed in almost all cell types and known to regulate the development and function of various lymphocytes, however, only little is known about the role of NF-κB in NK cells. Therefore, in this thesis, I hypothesize that the NF-κB family members regulate the development and maturation of NK cells.

In summary, this thesis aims to investigate the following:

1. Investigate the role of the canonical NF-κB family members in natural killer cell development and maturation *ex vivo* (Chapter 3).
2. Characterising the role of RelA in natural killer cell development and maturation *ex vivo* (Chapter 4).
3. Investigate NF-κB activation during peripheral NK cell maturation (Chapter 5)

Chapter 2

Materials and Methods

2.1 General

All tissue culture experiments were undertaken in a certified PC2 facility following the guidelines set out by the Office of the Gene Technology Regulator (OGTF) at the Monash University. All mammalian cells were cultured at 37°C and 5% CO_2 in a Heracell 150*i* incubator (Thermo Fisher Scientific).

All mouse experiments and procedures were conducted in accordance with the Australian code for the care and use of animals for scientific purposes (National Health and Medical Research Council of Australia, EA28). All experiments were approved by the AMREP Animal Ethics Committee. All mice were house in a specific pathogen-free (SPF) facility in individually ventilated cages (IVS).

2.2 Mice

The NF-kB1/p50 null mice were generated and characterized by Sha *et al.* [148] and c-Rel null mice were derived as described previously by Kontgen *et al.* in 1995 [149]. Green fluorescent protein (GFP)-RelA knock-in mice were generated and characterized by De Lorenzi, R., *et al*. [150]. In these mice, the RelA coding sequence is aligned in frame with a GFP reporter. This strain was provided by Professor Manolis Pasparakis (EMBL, Germany). C57BL/6 mice were used as wild-type healthy controls for all mutant strains and were purchased from AMREP Animal Services (AAS) (AMREP Precinct, Melbourne, Australia). All mouse strains were maintained as a homozygous colony at the Animal Resources Laboratory, Monash University, Clayton.

2.2.1 Generation of Rela-chimeric mice

C57BL/6-CD45.2 Rela-chimeric mice, were generated previously [151]. Fetal liver cells were harvested from ED13.5 embryos and injected intravenously into an 6-week-old irradiated C57BL/6-CD45.1 male host mouse. Cells were harvested from these mice 4-6-weeks post cellular reconstitution.

2.3 Cell Isolation and culture

2.3.1 Isolation of mouse leukocytes from spleen

Spleens were harvested and passed through a 40 µm cell strainer before being centrifuged at 300 *g* for 10 min at 4 °C. To remove red blood cells (RBC), splenocytes were re-suspended in red blood cell lysis buffer (150 mM NH_4CL, 10 mM $KHCO_3$, 0.1mM Na2 Ethylenediamine tetracetic acid (EDTA) for 4 min at room temperature (RT), before the reaction was stopped by the addition of 40 mL phosphate buffered saline (PBS) (Life Technologies) and centrifuged at 300 *g* for 10 min at 4 °C.

2.3.2 Isolation of leukocytes from mouse liver

Harvested mouse livers were passed through a metal sieve and washed with PBS. Cells were transferred to a Falcon tube and centrifuged (300 *g,* 7 minutes, RT). The supernatant was aspirated and resuspended with PBS and centrifuged (300 *g,* 7 minutes, RT). The cells were layered upon 37.5% Percoll (Sigma Aldrich, St. Louis, Missouri, USA) which was first made to 90% and then further diluted to 37.5% in PBS, cells were then centrifuged (690 *g,* 12 minutes, RT), and the supernatant was aspirated. To lyse RBCs, the cell pellet was re-suspended in RBC lysis buffer for 4-minutes at RT. Cells were diluted with PBS and centrifuged (300 g, 10 minutes, 4 °C).

2.3.3 Isolation of leukocytes from mouse BM

Epiphyses of the bones (femur and tibia) were cut off the using clean scissors. A 27-gauge needle and a 12 cc syringe filled with PBS was then used to flush the BM cells from both ends of the bone shafts onto a 50 ml screw top Falcon tube fitted with 40 µm filter followed by centrifugation (1,500 rpm, 5 minutes, 4 °C). To lyse RBCs, BM cells were re-suspended in RBC lysis buffer for 4 min at room temperature. Cells were then diluted with PBS and centrifuged (300 g, 10 minutes, 4 °C).

2.3.4 Flow cytometry

Cells were pre-incubated with sterile PBS containing 5% goat serum (Life Technologies, Carlsbad, CA, USA) for 30 minutes to prevent non-specific Fc binding. Cells were

centrifuged (300 g, 10 minutes, 4 °C) and resuspended with FACS wash buffer containing a combination of fluorescent-labelled mAbs (Table 2.1-2.3), incubated on ice for 30 minutes and washed twice by centrifugation (300 g, 10 minutes, 4 °C). Cells were then resuspended with FACS wash buffer and assayed using BD LSR Fortessa (BD Biosciences, San Jose, CA, USA). Output files were analysed using FlowJo software (Version 10) (LLC, Ashland, OR, USA). Cell populations were determined as follows: splenic NK cells from WT, *crel*$^{-/-}$ and *nfkb1*$^{-/-}$ ($CD3^{-}NK1.1^{+}$ or $CD3^{-}NKp46^{+}$), reconstituted splenic NK cells from the foetal liver RelA chimeric mice ($CD45.2^{+}CD45.1^{-}CD3\text{-}NK1.1^{+}$ or $CD45.2^{+}CD45.1^{-}CD3^{-}NKp46^{+}$), liver NK cells from WT, *crel*$^{-/-}$ and *nfkb1*$^{-/-}$ ($B220^{-}CD5^{-}Ly6G^{-}CD3^{-}NKp46^{+}Ly49a^{+}$), reconstituted liver NK cells from the foetal liver RelA chimeric mice ($B220^{-}CD5^{-}Ly6G^{-}CD3^{-}NKp46^{+}Ly49a^{+}$), BM NK cell precursors ($CD3^{-}B220^{-}Ly6G^{-}CD122^{+}NKp46^{-}$) and mature BM NK cells ($CD3^{-}B220^{-}Ly6G^{-}CD122^{+}NKp46^{+}$). Total numbers of individual cell populations were quantified by multiplying total cell counts by frequencies obtained from flow cytometry. An example of the typical gating strategy of cells using forward- and side-scatter (FSC, SSC) parameters is shown in Figure 2.1.

Table 2.1 *Antibodies used for the detection of spleen NK cell subsets by flow cytometry.*

Antibody	Clone	Company	Dilution
α-mouse CD27-PE	LG.7F9	eBioscience	1:100
α-mouse CD3-BV605	145-2C11	BioLegend	1:100
Anti-mouse CD45.1-BV711	A20	BioLegend	1:100
Anti-mouse CD45.2-BUV395	104	BD Pharmingen	1:100
Anti-mouse CD45.2-BV785	Ly-5.2	BioLegend	1:100
Anti-mouse CD94-PE	18d3	BioLegend	1:100
Anti-mouse CD96-BV421	6A6	BD Biosciences	1:100
Anti-mouse DNAM-1-APC	1OE5	BD Biosciences	1:100
Anti-mouse Ly49A-PB	YE1/48.10.6	BioLegend	1:100
Anti-mouse Ly49C/I/F-FITC	14B11	eBioscience	1:100
Anti-mouse Ly49D-APC	4 E5	BioLegend	1:100
Anti-mouse Ly49E/F-APC	CM4	eBioscience	1:100
Anti-mouse Ly49G2-FITC	4D11	eBioscience	1:100
Anti-mouse Ly49H-APC	3D10	Life Technologies	1:100
Anti-mouse NK1.1-APC	PK136	eBioscience	1:100
Anti-mouse NK1.1-FITC	PK136	eBioscience	1:100
Anti-mouse NKp46-BV421	29A1.4	BioLegend	1:100
Anti-mouse NKp46-PE	29A1.4	BioLegend	1:50

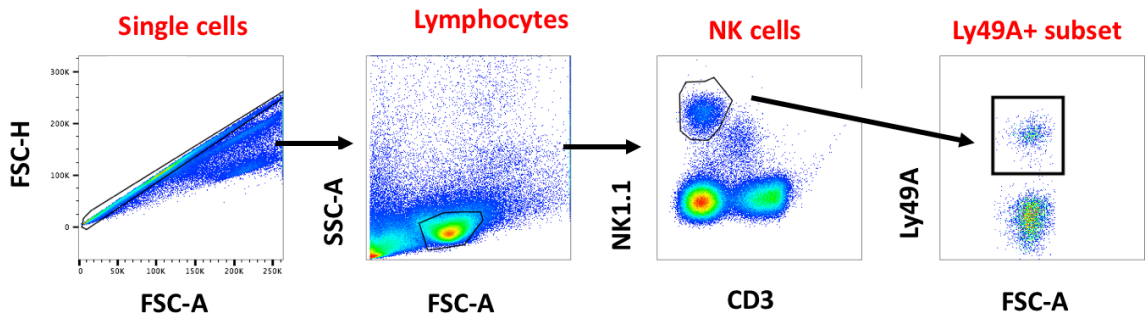
Table 2.2 Antibodies used for the detection of liver NK cell subsets by flow cytometry

Antibody	Clone	Company	Dilution
Anti-mouse B220-BV605	RA3-6B2	BioLegend	1:100
Anti-mouse CD3-BV605	145-2C11	BioLegend	1:100
Anti-mouse CD45.1-BV711	A20	BioLegend	1:100
Anti-mouse CD45.2-BUV395	104	BD Pharmingen	1:100
Anti-mouse CD45.2-BV785	Ly-5.2	BioLegend	1:100
Anti-mouse CD49a-APC	HMa1	BioLegend	1:100
Anti-mouse CD49b-PE	DX5	BioLegend	1:100
Anti-mouse CD5-BV605	RAE-6B2	BioLegend	1:100
Anti-mouse Ly6G-BV605	1A8	BioLegend	1:100
Anti-mouse NKp46-BV421	29A1.4	BioLegend	1:100

Table 2.3 Antibodies used for the detection of BM NK cell subsets by flow cytometry

Antibody	Clone	Company	Dilution
Anti-mouse B220-BV605	RA3-6B2	BioLegend	1:100
Anti-mouse CD122-FITC	TM-B1	BioLegend	1:100
Anti-mouse CD3-BV605	145-2C11	BioLegend	1:100
Anti-mouse CD45.1-BV711	A20	BioLegend	1:100
Anti-mouse CD45.2-BUV395	104	BD Pharmingen	1:100
Anti-mouse CD45.2-BV785	Ly-5.2	BioLegend	1:100
Anti-mouse CD5-BV605	RAE-6B2	BioLegend	1:100
Anti-mouse Ly6G-BV605	1A8	BioLegend	1:100
Anti-mouse NKp46-BV421	29A1.4	BioLegend	1:100

a.



b.

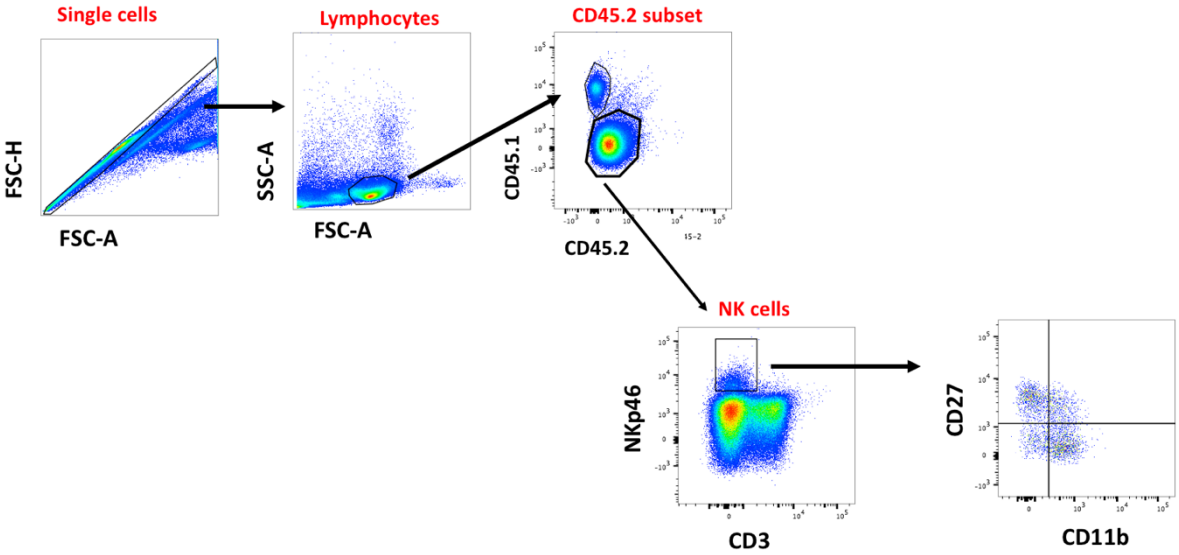


Figure 2.1 Gating strategy for flow cytometry and FACS analysis. **a.** The flow cytometry gating strategy using the expression of Ly49A on NK1.1$^+$ CD3$^-$ splenic NK cells of WT mice. **b.** The flow cytometry gating strategy using CD27 and CD11b expression to classify NKp46$^+$ CD3$^-$ splenic NK cells of WT mice into stages 1–4 of maturity. SSC = Side scatter. FSC = Forward scatter.

2.4 Enrichment of mouse NK cells

To enrich NK cells from splenocytes, isolated mouse leukocytes were subjected to immunomagnetic negative selection using EasySep mouse NK cell isolation kit (StemCell Technologies, Vancouver, Canada). Cells were resuspended in 50μl/1x10^8 cells of the EasySep mouse NK cell isolation and incubated for 10 min at RT. EasySep streptavidin Rapid Spheres were added at 100 μl per 1x10^8 cells for 5 min at RT. Magnetically labelled NK cell were separated using an EasySep™ magnet and enriched NK cells were collected in the negative enrichment unbound fraction. The enriched cell population routinely consisted of ~70% NK cells based on CD3-NK1.1+ staining, and therefore underwent further purification via FACS.

2.4.1 Preparation of cell for cell sorting

Cell pellet was resuspended in sort buffer (1% foetal calf serum (FCS) (Thermo Fisher Scientific), 1Mm EDTA in PBS) at 10^8 cells/ml (counted using Z2 Coulter Counter, Beckman Coulter, Mount Waverly, VIC, Australia). Cells were sorted using a BD Influx or Aria (BD Biosciences, San Jose, CA, USA).

2.5 Stimulation and cytometric bead array assay of splenic NK cells

Enriched and cell sorted splenic NK cells were stimulated with recombinant purified mouse IL-12 (1ng/ml) in combination with either IL-18 (10ng/ml) or IL-15 (50ng/ml) (BioLegend, San Diego, CA, USA) in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) containing 10% FCS and 1% Pen/Step for 6- and 18- hours at 37ºC, 5% CO_2.

At the indicated time, the supernatant from the cytokine stimulated cells, were collected and the concentration of TNF-α, IFN-γ and MIP-1α was determined using a custom BioLegend LEGENDplex, consisting of antibody-immobilized beads for TNF-α, IFN-γ and MIP-1α.

Sample preparation and assay procedures were performed as outlined in the LEGENDplex Mutli-Analyte Flow Assay Kit (BioLegend) protocol. The BD FACSCanto II (BD Biosciences, San Jose, CA, USA) was used to detect the antibody-immobilized beads and the output was analyzed using LEGENDplex (Version 7) and GraphPad Prism (Version 7) (GraphPad Software, CA, USA).

2.6 Electrophoretic mobility shift assays and super-shift assay

The EMSA was carried out as previously described [152] in the following buffer: 10 mM HEPES pH 7.9, 50 mM sodium chloride, 1.5 mM EDTA, 5% glycerol and 0.1% NP-40. Each reaction mixture also contained 3 µg/ml of poly (dI:dC) (Amersham, Little Chalfont, United Kingdom) and phosphorus-32-end-labelled probe. For super-shift experiments, antibodies specific for NF-κB1, RelA or c-Rel (Santa Cruz Biotechnology, CA) were incubated in the buffer at 4°C for 60-minutes before the addition of phosphorus-32-labelled (P^{32}) probe. Reaction mixtures were incubated at room temperature for 30-minutes and the DNA–protein complexes were resolved on a native 4% polyacrylamide gel (Santa Cruz Biotechnology, CA).

2.7 Confocal Microscopy

FACS sorted NK cells ($CD3^{+}NKp46^{+}$) populations from GFP-RelA knock-in mice (section 2.4.1), were fixed in 4% Paraformaldehyde and kept on ice for 1-hour. Cells were then washed twice with PBS, each wash comprised of centrifugation at 1,500 rpm for 5 minutes at RT. To stain the nucleus, cells were incubated with Hoechst (Thermo Fisher Scientific) at a dilution of 1:1000 in PBS followed by incubation on ice for 20-minutes. To remove excess Hoechst, cells were washed twice with PBS, each wash comprised of centrifugation at 1,500 rpm for 5 minutes at RT. To measure the nuclear translocation of GFP, we used a confocal microscopy method using the Nikon A1r plus instrument. The data was analysed and quantified using Nikon NIS AR (version 4.51)(Tokyo, Japan). For quantitative analysis, the

Nikon NIS AR software was pre-set to include cells with a detectable level of both nuclear and GFP fluorescence intensity.

Chapter 3

Investigating the role of the canonical NF-κB family members in natural killer cell development and maturation *ex vivo*

3.1 Introduction

NF-κB is a critical transcription factor involved in a broad range of biological processes, including immune response, cell survival and maturation of various cell types [153]. There are two pathways of NF-κB activation, canonical and non-canonical [154]. Upon activation, the canonical activation pathway regulates the nuclear translocation and activation of NF-κB1/p50, RelA and c-Rel, which can homodimerize and heterodimerize in numerous combinations in response to an array of stimulants, such as cytokines and toll-like receptor (TLR) stimulation [155]. In contrast, the non-classical activation pathway is initiated in response to a specific set of receptors, such a subset of TNFR superfamily members, including the B-cell-activating factor (BAFFR) [131] and CD40 [131,132]. Upon activation, the non-classical pathway regulates the nuclear translocation of the RelB/p52 NF-κB complex using a mechanism that relies on the inducible processing of NF-κB2/p100 into p52 [156].

The NF-κB family members involved in the canonical signaling pathway control the development, maturation and function of some immune cells. Upon activation, the canonical family members RelA and c-Rel either form homodimers or heterodimerise with the transactivating domain lacking NF-KB family member p50. For example, combined *crel/rela* or *nfκb1/crel* deficient mice display an arrest in B cell development [157,158] and profound defects in DC development, survival and IL-12 production [159], whereas, peripheral CD8 and $CD4^+$ T-cell numbers develop normally [158,160]. While the canonical NF-kB family members do not seem to be involved in T-cell generation, the T-cells from these mice display several functional abnormalities, including an impaired production of IL-2, IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF). Considering the production of IL-2 is essential for the maturation and differentiation of CTL effectors, $crel^{-/-}$ T-cells fail to differentiate into CTLs and execute cytotoxicity [161-163].

The innate counterpart to CTLs are NK cells, which are also cytotoxic in response to invading pathogens [164]. NK cells develop in the BM [75] and undergo further maturation in the periphery, in which lineage committed NK cells gain full cytotoxic competence and the ability to produce IFN-γ in response to the engagement of their activating receptors and cytokine stimulation [49,165,166]. Therefore, to complete their developmental process and to be fully functional, NK cells require expression of an array of germ-line encoded receptors. In mice, these include the C-type lectin-like Ly49 receptor family [24], which comprise the activating Ly49D and Ly49H receptors and the inhibitory Ly49A, Ly49C/I/F, Ly49E-F and Ly49G2 molecules [20]. Moreover, CD94 can act as an activating or inhibitory accessory molecule through dimerization with members of the NKG2 family of receptors. For example, CD94/NKG2C functions as an activating receptor, whereas CD94/NKG2A-E is inhibitory [135,167]. Secondary to the expression of receptors, NK cell activation and effector function can also be achieved through cytokine stimulation. For instance, upon stimulation with the cytokines IL-12 and IL-18, mature NK cells in the spleen produce and secrete IFN-γ and TNF for target cell lysis [49], whereas IL-12 and IL-15 mediates the secretion of the chemokine, MIP1-α [168].

There are very few studies into the role of the NF-κB family members in NK cell function. However, patients with Hypohidrotic ectodermal dysplasia with immunodeficiency (HED-ID), a condition resulting from a mutation in the canonical NF-kB protein complex, NEMO, have defective NK cells, characterized by recurrent viral infections with cytomegalovirus (CMV) [12]. Moreover, the cytotoxic activity of the NK cells against tumour-cell targets was completely abolished but was partially reversed by *in vitro* addition of IL-2, which was also able to induce NF-κB activation [161,169]. Another study using *nfkb1* and *crel* knockout mice suggested that c-Rel and NF-κB1/p50 play opposing roles in NK cell proliferation and IFN-γ production, with c-Rel identified as an agonist and NF-κB1/p50 as an antagonist [109]. Further to this, p50 and RelA binding sites have been identified on the bi-directional ly49 promoter, Pro1, which

controls ly49 gene expression [135]. Considering NK cell effector function is regulated by a series of germ-line encoded receptors, such as the ly49 molecules and CD94 (couples to either NKG2A/C/D) [135]. These studies have highlighted the importance of the canonical NF-κB family members in NK cell function, however, the role of individual family members in NK cell development, maturation and cell surface receptor expression has been not been explored. Therefore, using *crel*$^{-/-}$ or *nfkb1*$^{-/-}$ mice, this chapter aims to elucidate the role of c-Rel and NF-κB1/p50 in NK cell development, receptor expression and cytokine production.

3.2 Results

3.2.1 c-Rel is not required for NK cell development

c-Rel has been shown to play a major role in lymphocyte homeostasis, including B-cell development [170], IL-2 production by T-cells [162] and the control of Treg differentiation in the thymus [171]. To investigate the requirement of c-Rel for NK cell development, leukocytes from the peripheral organs of the spleen and the liver of WT and *crel*$^{-/-}$ mice were harvested and NK cells were defined as CD3^{-}, NKp46^{+}. In the liver, CD49a was used to further differentiate between ILCs and NK cells, as CD49a is expressed on ILCs and not on NK cells. Flow cytometric analysis of spleens and livers of *crel*$^{-/-}$ mice indicated that the absolute NK cell numbers were unchanged when compared to WT mice (Figure 3.1a-b). However, the frequency of liver NK cells from *crel*$^{-/-}$ mice were significantly reduced compared to the WT mice ($p<0.05$), whereas no significant change in the frequency of spleen NK were observed (Figure 3.1c-d). This data suggests c-Rel may contribute to the homeostasis of NK cells in the liver but has no role in the spleen.

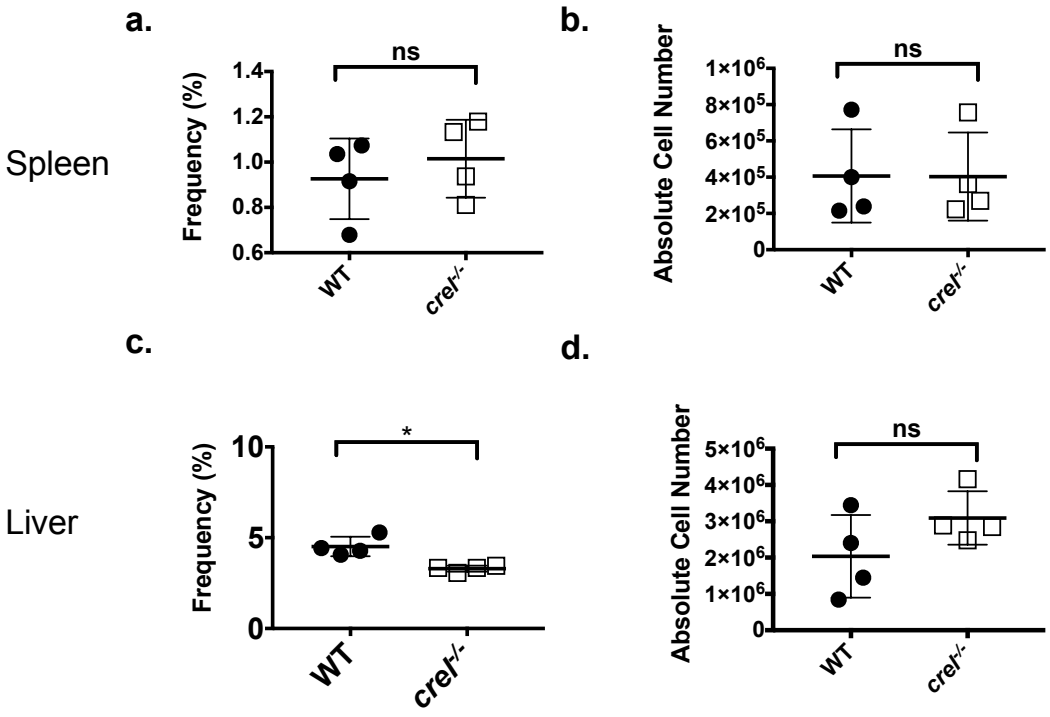


Figure 3.1 c-Rel is not essential for NK cell development in the spleen and liver. The percentage and absolute cell number of splenic (NKp46^{+} CD3^{-}) **(a-b)** and liver (NKp46^{+} CD3^{-} CD49a^{-}) **(c-d)** derived NK cells in WT and c-Rel$^{-/-}$ mice. Data represent the mean ± SD (n = 4). Statistical significance determined by Mann-Whitney test (non-parametric t-test), * $p<0.05$.

3.2.1.1 c-Rel is redundant for NK cell receptor expression

The acquisition of certain NK cell receptors, including the Ly49 family and CD94, characterize the final stage of NK cell development and functional competence [135] . For example, the inhibitory Ly49C receptor is expressed on NK cells that secrete more IFN-γ after stimulation than do Ly49C^{-} NK cells [172] and the activating Ly49H receptor is required for clearance and protection against mouse cytomegalovirus (MCMV) [173]. To further investigate the role of c-Rel in NK cell development and functional competence, the activating and inhibitory Ly49 repertoire and CD94, which is required for the dimerization of the CD94/NKG2 family of receptors [167], was studied in *crel*$^{-/-}$ mice. The percentage of splenic NK cells expressing individual Ly49 proteins and CD94 receptors in *crel*$^{-/-}$ NK mice were compared with that in WT mice (Figure 3.2) In *crel*$^{-/-}$ mice, there was no significant change in proportion of the activating/inhibitory CD94, inhibitory Ly49C/I/F/, G2, E/F and activating Ly49D/H subsets compared with WT mice (Figure 3.2). Therefore, these data demonstrate that c-Rel is not critical for CD94 and Ly49 protein expression on NK cells.

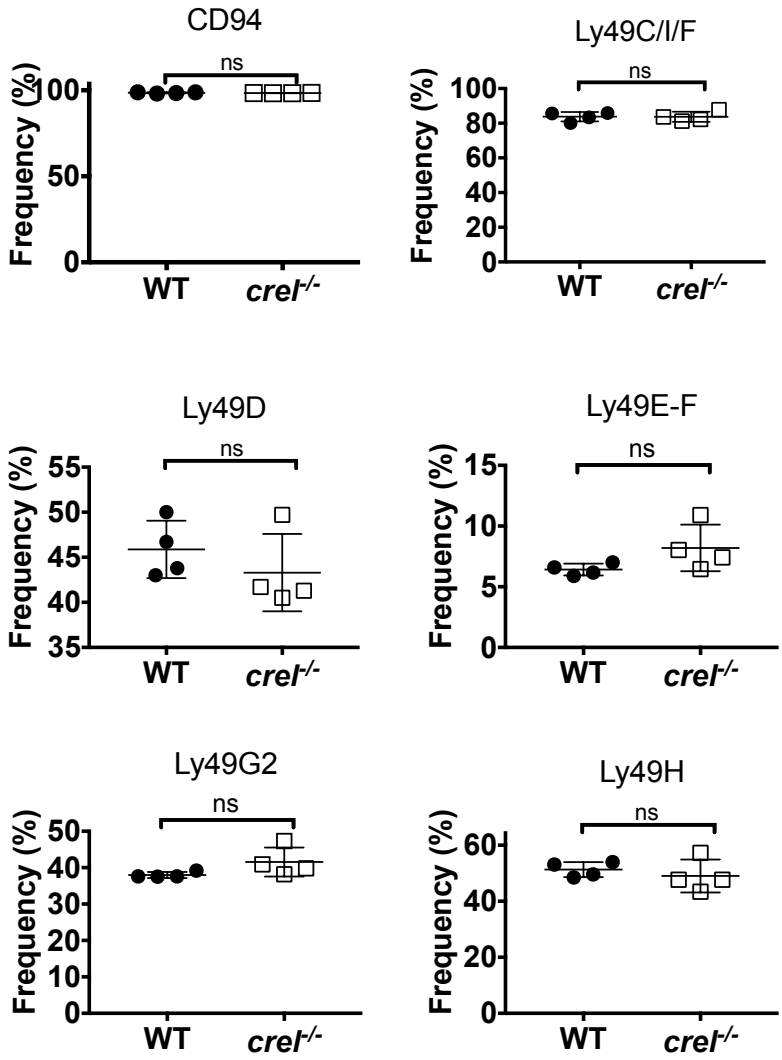


Figure 3.2 CD94 and Ly49 protein expression on individual NK cells was not affected by the absence of c-Rel. The percentage of CD94 and the indicated Ly49 molecules derived from splenic primary mouse NKp46^{+} CD3^{-} NK cells in WT and c-Rel$^{-/-}$ mice. Data represent mean ± SD (n = 4). Statistical significance determined by Mann-Whitney test (non-parametric t-test), * $p<0.05$.

3.2.1.2 The role of c-Rel in cytokine production by NK cells

Having established that the receptor repertoire on NK cells are unchanged between WT and *crel*$^{-/-}$ mice, we next assessed the capacity of these NK cells to respond to cytokine-induced activation. Upon costimulation with either IL-12 & IL-18 or IL-12 & IL-15, human and murine NK cells rapidly produce and secrete IFN-γ and MIP1-α respectively [174,175]. IFN-γ production and secretion by NK cells is critical for the initiation of immune responses against infectious agents and malignancies [176,177], whereas MIP1-α secretion recruits mononuclear cells such as B- and T-cells to the site of infection [168]. To determine the role of the c-Rel subunit in regulating the production of IFN-γ and MIP1-α, we harvested fresh murine splenic NK cells from *crel*$^{-/-}$ and WT mice and stimulated them with either IL-12 and IL-18 (Figure 3.3a) or IL-12 and IL-15 (Figure 3.3b) and assessed for early (6-hours) and late (18- hours) cytokine and chemokine secretion. The concentration of IFN-γ and MIP1-α secretion was quantified by a CBA assay via flow cytometry. Co-stimulation with IL-12 and IL-18 for both 6- and 18-hours did not alter the concentration of IFN-γ secreted by splenic NK cells in *crel*$^{-/-}$ mice when compared with the WT mice (Figure 3.3a). Likewise, co-stimulating NK cells with IL-12 and IL-15 for 6- and 18-hours didn't change the concentration of MIP1-α secretion in crel$^{-/-}$ mice when compared with WT mice (Figure 3.3b). These data suggest that c-Rel is not required for cytokine-induced activation of splenic NK cells.

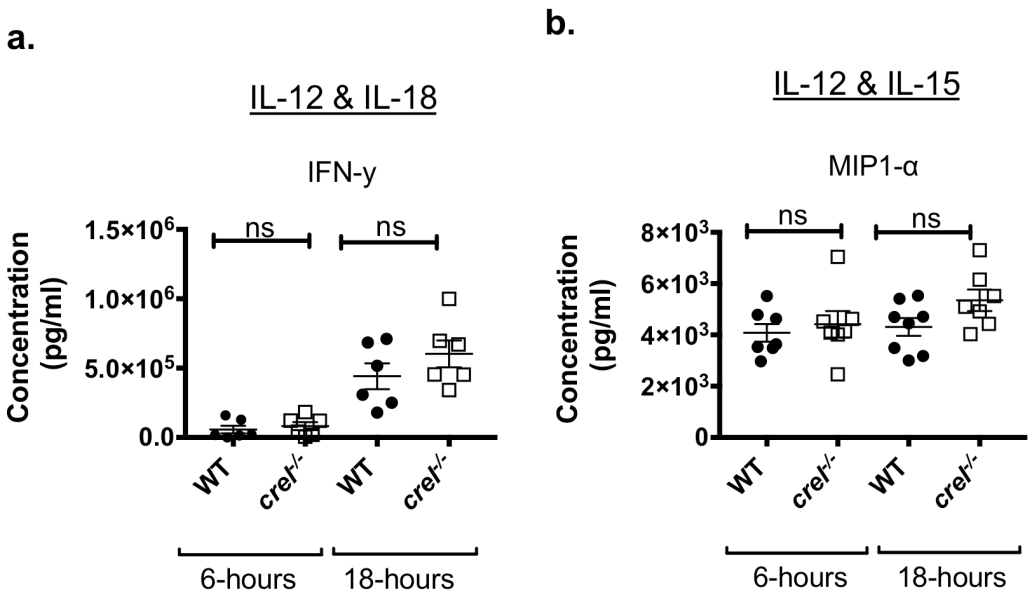


Figure 3.3 c-Rel is not directly regulating IFN-γ and MIP1-α secretion upon cytokine stimulation. Quantitation of IFN-γ and MIP1-α released by splenic NKp46^{+} CD3^{-} NK cells after treatment for 6 or 18 hours with IL-12 (1ng/ml) and IL-18 (10ng/ml) **(a)** or IL-12 (1ng/ml) and IL-15 (50ng/ml) **(b)** from wild-type or *crel*$^{-/-}$ mice. Data pooled from two independent experiments. Bars represent mean ± SEM. P-values were determined by Mann-Whitney test (non-parametric t-test). n = > 6.

3.2.3 NK cell development occurs independent of NFKB1/p50

As my results suggested that c-Rel is not required for NK cell development or effector function, I next investigated the role of the NF-kB1/p50 subunit. In most cell types the NF-kB complex is composed of p50 hetero or homodimers. For example, RelA-p50 heterodimer is the most abundant NF-kB complex [113,119]. However, p50 can also dimerise with RelB and c-Rel to form a potent transcriptional activator [120], whereas p50/p50 homodimers function as transcriptional repressors [113]. Moreover, NF-kB1/p50 has been shown to play a major role in B-cell [157,158] and DC development and survival [159]. Furthermore, studies have associated NF-κB1/p50 with perforin production in NK cells [135]. Therefore, to investigate the requirement of NF-kB1/p50 in NK cell development, leukocytes from the spleen and the liver of WT and *nfkb1*$^{-/-}$ mice were harvested, and NK cells were defined as CD3^{-}, NKp46^{+}. In the liver, CD49a was used to differentiate between ILCs and NK cells as CD49a is expressed on ILCs and not on NK cells. Flow cytometric analysis of spleens and livers of *nfkb1*$^{-/-}$ mice indicated a significant reduction in the frequency of splenic NK cells in *nfkb1*$^{-/-}$ mice in comparison to the WT mice (Figure 3.4a), whereas the absolute NK cell numbers were not changed (Figure 3.4b). However, the frequency of liver NK cells from *nfkb1*$^{-/-}$ mice were significantly higher ($p<0.01$) compared to WT mice (Figure 3.4c) while the absolute NK cell numbers were comparable to WT (Figure 3.4d). This data suggests NF-kB1/p50 may be contributing to NK cell homeostasis in the spleen and in the liver.

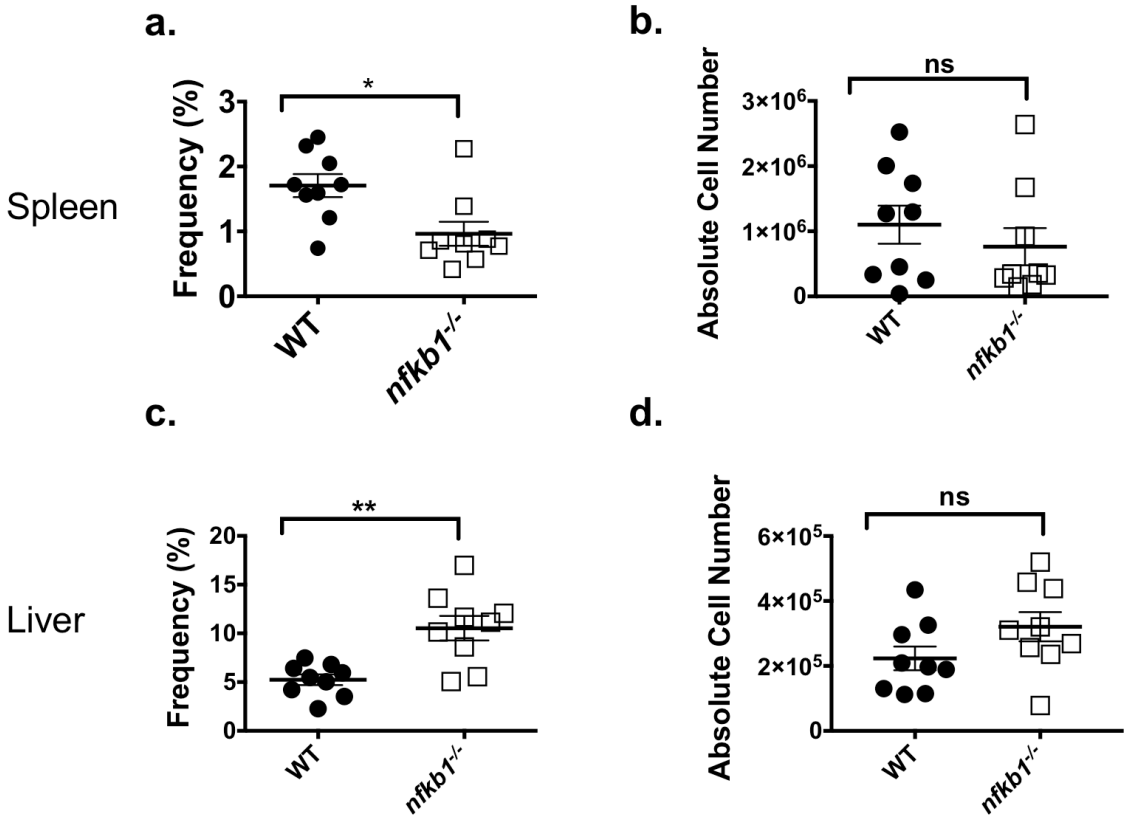


Figure 3.4 NF-kB1/p50 doesn't regulate NK cell development in the spleen and liver. The frequency and absolute cell number of splenic (NKp46^{+} CD3^{-}) **(a-b)** and liver (NKp46^{+} CD3^{-} CD49a^{-}) **(c-d)** derived NK cells from *nfkb1*$^{-/-}$ mice. The absolute number of NK cells was calculated based on the frequency of NK cells of total cells analyzed by flow cytometry The data has been pooled from two independent experiments. Bars represent mean ± SEM. Statistical significance determined by Mann-Whitney test (non-parametric t-test), * $p<0.05$, ** $p<0.01$.

3.2.3.1 NF-KB1/p50 dependent regulation of NK cell receptor expression

Similar to section 3.2.1, to investigate the requirement of NF-kB1/p50 for NK cell development, the expression of the activating and inhibitory Ly49 repertoire and CD94 were studied in *nfkb1*$^{-/-}$ mice (Figure 3.5). Resembling section 3.2.1, the percentage of cells expressing individual Ly49 proteins and CD94 on *nfkb1*$^{-/-}$ NK cells was compared with WT mice (Figure 3.5). In the spleen of the *nfkb1*$^{-/-}$ mice, there was a significant decrease in the proportion of the activating Ly49D NK cell subset. In addition to this, a significant proportion ($p<0.05$) of the activating/inhibitory heterodimeric CD94 and inhibitory Ly49E-F receptors were also reduced in *nfkb1*$^{-/-}$ mice compared to the WT. However, the presence or absence of NF-kB1/p50 does not affect the expression of the inhibitory Ly49A, Ly49G2 and Ly49C/I/F receptor on individual NK cells (Figure 3.5). This data suggests that the expression of the Ly49 repertoire and CD94 receptors on *nfkb1*$^{-/-}$ NK cells is independent of its inhibitory or activating function. Overall, it can be concluded that c-Rel does not appear to have a major regulatory role in NK cell development and activation, yet NF-kB1/p50 may potentially have a role in development and receptor expression.

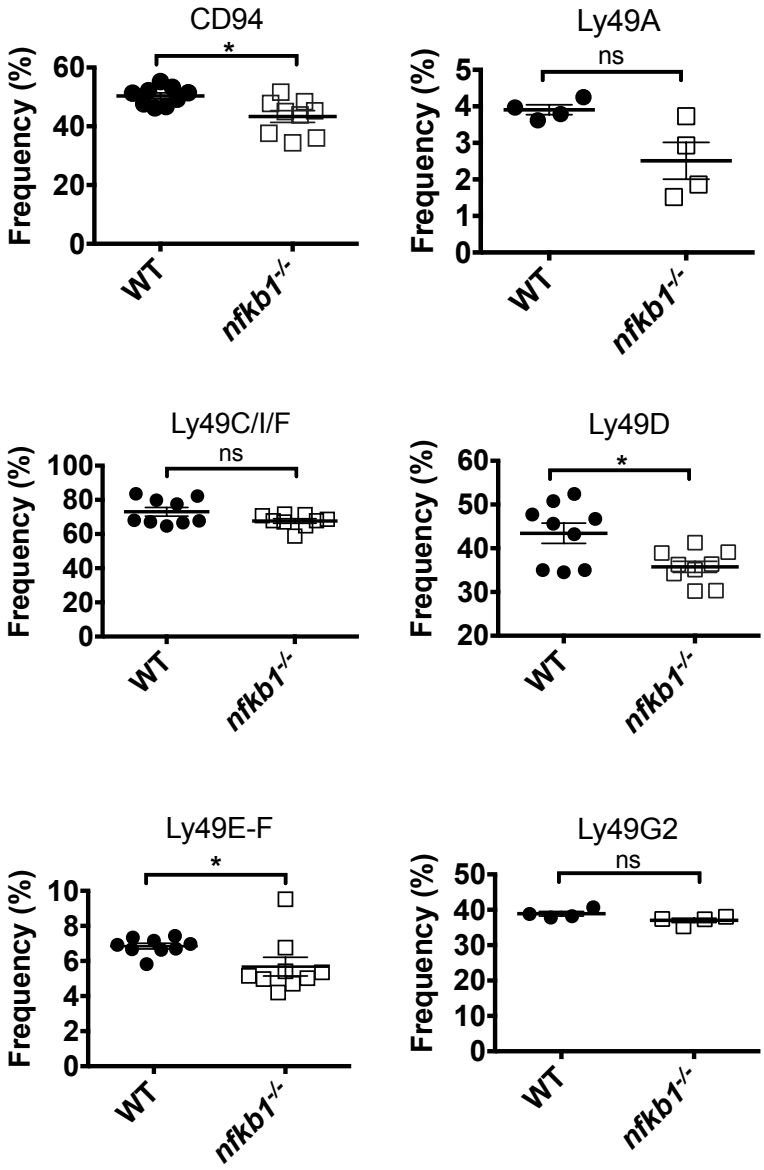


Figure 3.5 Decreased expression of CD94, Ly49D and Ly49E-F in nfkb1$^{-/-}$ NK cells. The expression of the CD94 and the indicated *Ly49 molecules* on spleen NK cells. The Data are expressed as the percentage of CD3−NK1.1+ NK cells in *nfkb1* knockout and WT mice. The data in panel been pooled from two independent experiments. Bars represent mean ± SEM. Statistical significance determined by Mann-Whitney test (non-parametric t-test), * $p<0.05$.

3.3 Discussion

The primary site of NK cell development is in the BM, in which mature NK cells migrate from the BM and take up residence in peripheral sites, mainly in the spleen, liver and lungs with some residing in the LN and BM [49,82,83]. Peripheral NK cell maturation enables lineage committed NK cells to gain full effector function, demonstrated by their ability to produce IFN-γ in response to the engagement of their activating receptors and cytokine stimulation [82]. In addition, the expression of at least one inhibitory receptor Ly49 receptor that recognizes self MHC class I protein is required for NK cells to achieve functional maturation and self-tolerance during development via a process termed "licensing" [30,31]. This process ensures mature NK cells can distinguish infected cells or tumour cells from normal self-cells [32,33]. Therefore, to complete their developmental process and to gain functional competence, NK cells require Ly49 and cytokine receptor expression on their cell surface [135,178]. Both NK cell development and receptor expression require transcriptional activity, however the knowledge of which factors are involved in these processes are poorly understood. Interestingly, most of the findings regarding transcriptional regulation of NK cell activity, such as IFN-γ production, has been extrapolated from research into cytotoxic T-cells [109]. These studies have led to the identification of a number of transcription factors that are shared between NK and T-cells, such as Id2 [76] and T-bet [179]. Interestingly, NK and T-cell function is enhanced through the cytokines IL-1, IL-18 and TNF-α, which the activates the NF-κB pathway, and when used in combination with IL-12 increase the production of IFN-γ by both NK and T-cells. Recently, NF-κB family members have been shown to regulate IFN-γ by T-cells, as well as the production of IL-2, IL-3 and GM-CSF [109,161-163]. Therefore, in this chapter, the role of the transcription factors, c-Rel along with NF-kB1/p50 in NK cell development and homeostasis has been investigated.

The transcription factor, c-Rel is exclusively expressed in hematopoietic cells [180,181], with the highest levels in T- and B-cells [149]. Due to this, T-cells from c-Rel-deficient mice have poor proliferation and display defects in cytokine production, such as IL-2, GM-CSF and IL-3 [149,182]. Furthermore, nuclear translocation of c-Rel has been shown to be required for B-cell development [183]. As NK cells share many critical transcription factors with T-cells, it stands to reason that c-Rel may play an important role in NK cell development or activation, however, research into the role of c-Rel in NK cells is limited. One of the few studies into the role of c-Rel and NK cells is by Tato and colleagues [109], revealed that in the absence of c-Rel, NK cells have a defect in their ability produce IFN-γ. Interestingly, in this chapter, c-Rel was shown not to play a role in the activation and cytokine release of NK cells, nor was it involved in the development of NK cells or the expression of the Ly49 repertoire and CD94 on NK cells. While the current study examined NK cells freshly isolated from WT or c-Rel $^{-/-}$ mice, the study by Tato and colleagues assessed intracellular IFN-γ production using lymphokine-activated killer (LAK) cells, which are purified NK cells activated by IL-2 treatment for 7 days before being cultured *in vitro* with other cytokine combinations However, IL-2 treatment rapidly boosts cytolytic activity and enables responses to weak stimulation by both NK cells and LAK cells [184,185]. Therefore, stimulation with IL-2 is not an ideal physiological activator. Nevertheless, in the current study, c-Rel did not regulate the secretion of IFN-γ and MIP1-α by cytokine-mediated activated NK cells. Taken together, these studies suggest that c-Rel may potentially be regulating intracellular production of IFN-γ but does not regulate cytokine release.

Having established a lack of regulatory function c-Rel in NK cell development, we next assessed the role of NF-kB1/p50 in NK cells. The expression of key cytokine receptors required for NK cells development and cytokine secretion are regulated by a number of transcription factors [78,166]. In addition, *in vivo* studies by Pascal and colleagues have

investigated the role of the transcription factor, NF-kB1/p50, in NK cell development and Ly49 gene expression by using nfkb1$^{-/-}$ mice [135]. In this study, there was no statistically significant difference in the total number of NK cells present in liver, BM, and spleen between WT and *nfkb1*$^{-/-}$ mice, which compliments the findings of this chapter, suggesting that NF-kB1/p50 is expendable for the development of NK cells. Therefore, the decreased frequency of NK cells in the liver and increased frequency in the spleen may be due to an NK cell-extrinsic defect in the lymphocyte population, such as a reduced number of Marginal zone (MZ) B cells in *nfkb1*$^{-/-}$ mice [186]. Additionally, in the study by Pascal and colleagues [135], a statistically significant decrease in the proportion of each Ly49 subset (Ly49A, Ly49D, Ly49C/I, Ly49G and Ly49H) was observed in the spleen and in the BM of *nfkb1*$^{-/-}$ mice [135], which propose that NF-kB1/p50 is involved in the *in vivo* activation of Ly49 expression in NK cells. However, the data presented in this chapter only demonstrate the requirement of NF-kB1/p50 for the expression of activating Ly49D, inhibitory Ly49E-F and the activating/inhibitory CD94 receptors. The inability to reproduce the results obtained by Pascal and colleagues may be attributed to a number of unknown differences, including the antibodies used for flow cytometric analysis, as well as differences in the genetically modified mice caused by environmental variability across laboratories. None the less, taken together, both studies conclude that NF-kB1/p50 doesn't regulate the development of NK cells. Therefore, the decreased Ly49 and CD94 expression on *nfkb1*$^{-/-}$ NK cells in this current study are not due to a reduction of total NK cell numbers, instead, these results may reflect a regulatory role of NF-kB1/p50 in activating the genes encoding Ly49D/E-F and CD94 on NK cells. Finally, the regulation of the cell surface receptors is not dependent on its activating or inhibitory function. To confirm these finding, in the future we aim to use computer-assisted binding site analysis as well as EMSA experiments to identify p50 binding sites in the Ly49D and CD94 promoter region of WT mice. In addition to this, future studies involve investigating the role of NF-kB1/p50 in cytokine and chemokine production, in particular,

IFN-γ and MIP1-α by NK from *nfkb1*$^{-/-}$ mice. Furthermore, considering the primary effector function of NK cells is target cell lysis, we also wish to assess the role of NF-kB1/p50 and c-Rel in regulating NK cell killing by performing a cytotoxicity assay against NK-sensitive YAC-1 target cells from *nfkb1*$^{-/-}$ and *crel*$^{-/-}$ mice.

In summary, the development of NK cells is largely unaffected by global deletion c-Rel and NF-kB1/p50. In addition, our data show that c-Rel-deficiency does not influence the secretion of IFN-γ and the MIP1-α nor the expression of NK cell surface receptors. However, NF-kB1/p50 is required for the expression of Ly49D/E-F and CD94. Next, it is of great interest to study the role of the other classical NF-kB member, RelA, in NK cell development and maturation *in vivo,* which is presented in chapter 4 of this thesis.

Chapter 4

Characterising the role of RelA in natural killer cell development and maturation *ex vivo*

4.1 Introduction

The NF-κB subunit, RelA, is a member of the canonical signalling pathway and is required for survival, with RelA-deficiency in mice being embryonically lethal due to apoptosis of developing hepatocytes [187-189]. Therefore, until recently, the development and function of hematopoietic cells in the absence of RelA had not been examined. To examine the role of RelA using mice, radiation chimeras have been produced, in which lethally irradiated hosts were reconstituted with donor fetal liver cells deficient in RelA [187]. Since then, RelA has been widely studied and identified as an important regulator of HSC differentiation and progenitor cell cycling as well as differentiation and function of lineage-committed hematopoietic cells [190]. In DCs, whilst the deletion of individual NF-κB subunits NF-κB1/p50, c-Rel and RelA did not impair DC development and function, in the *nfkb1* $^{-/-}$ and *rela*$^{-/-}$ mice, DC development was impaired [159]. In addition, mice grafted with *crel*$^{-/-}$ *rela* $^{-/-}$ fetal liver cells displayed a deficit of peripheral $CD4^+$- and $CD8^+$ T-cells and an absence of mature B cells [191,192], highlighting the importance of RelA in T- and B-cell development. Moreover, RelA has been shown to regulate the transition of $NK1.1^-$ to $NK1.1^+$ cells during NKT-cell development, as well as IL-15- and IL-7-induced proliferation of NKT-cell precursors [157,193]. Interestingly, NK and NKT cells are both characterised by the expression of IL-15Rβ/CD122 and differential expression of IL-7Rα during development [49,50]. In addition, immature NK cells are characterised by the expression of NK1.1 [49,50], however, the study of the role of RelA in NK cell development is minimal and requires more investigation. To date, the role of RelA in NK cells has been linked to Ly49 receptor expression [135] and IL-2 induced up-regulation of perforin via nuclear translocation of p50/RelA complexes [194].

The development and subsequent maturation of NK cells is regulated by a number of transcription factors. In the BM of mice, the development of mature NK cells from the CLP is controlled by Notch1 [71], NFIL3 [74]. and ID2 [4,73,74]. Similarly, peripheral maturation of NK cells is highly dependent on transcriptional regulation, for instance, Eomes control the

differentiation of immature SP cells to the mature DP subset [102,103], whereas, T-bet regulates the transition of DP NK cells to the terminally differentiated $CD27^{-}$ $CD11b^{+}$ subset [104,105]. More recently, NK cells have been reported to have an 'alternative' maturation pathway, independent of the previously described CD27 and CD11b, in which cells develop from 'immature' DNAM-1^{+} to 'mature' DNAM-1^{-} cells. The ligands of DNAM-1, are upregulated in cells under stress conditions or associated with malignant transformation or viral infection [195]. Upon, DNAM-1-ligand interactions, NK cells become activated and trigger NK-cell mediated cytotoxicity and tumour elimination [196]. Interestingly, recent research has indicated that full RelA activation requires the engagement of DNAM-1 with its corresponding ligand [136], suggesting RelA may play a role in both NK cell development and effector function through DNAM-1 regulation.

The previous chapter provided a description of the role of the canonical NF-κB family members, NF-κB1/p50 and c-Rel in NK cells, however, the role of RelA was not assessed. Therefore, this chapter aims to shed light on the role of RelA in the development and maturation of NK cells.

4.2 Results

4.2.1 RelA is required for NK cell development

Whilst RelA is required for the development and survival of DC [159], B-, T- [191,192] and NKT [157,193] cells, its involvement in NK cell development and subsequent differentiation into mature NK cell subsets in the periphery is completely unknown. Therefore, to investigate the regulatory role of RelA in NK cell development, RelA chimeras were generated, and leukocytes from the spleen, liver and BM were harvested from WT and *Rela*$^{-/-}$ chimeric mice. Donor NK cells were identified as CD45.2^{+} CD3^{-} NKp46^{+} in the spleen and BM, and CD45.2^{+} CD3^{-} NK1.1^{+} in the liver. In the spleen and liver, CD49a was used to further differentiate between ILC and NK cells. To determine NK cell lineage-committed cells in the BM, NK cells were CD45.2^{+} CD3^{-} NKp46^{+} CD122^{+} B220^{-} GR-1^{-} CD11b^{-}. Flow cytometric analysis of BM, spleens and livers of *Rela*$^{-/-}$ chimeric mice indicated a significant ($p<0.01$) reduction in both the frequency and the absolute number of NK cell reconstitution from *Rela*$^{-/-}$ chimeras compared to the control WT chimeric mice in all three organs (Figure 4.1 a-f). This data suggests RelA has a vital role in NK cell development.

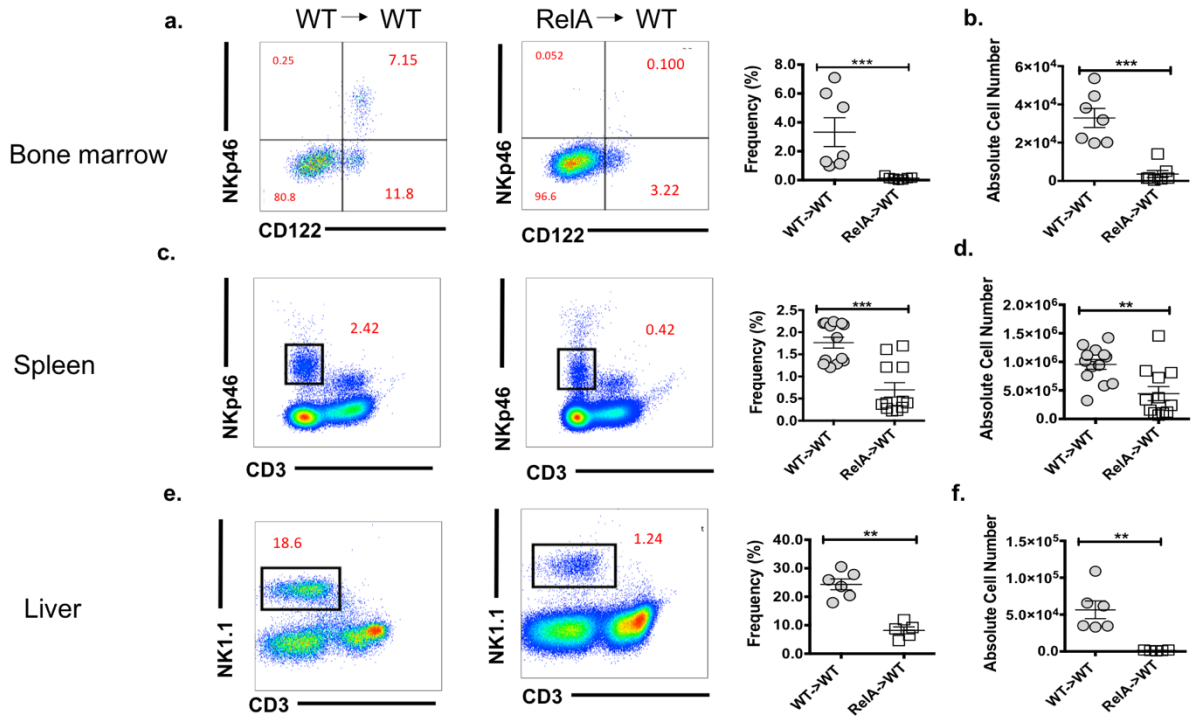


Figure 4.1 .NK cell development is impaired in the absence of RelA in the BM and the periphery. The representative FACS dot plots and quantification of the percentage and the absolute cell number of donor (CD45.2$^+$) derived-bone marrow (CD3$^-$ B220$^-$ GR-1$^-$ CD11b$^-$ NKp46$^+$ CD122$^+$) **(a-b)**, splenic (NKp46$^+$ CD3$^-$) **(c-d)** and liver (NK1.1$^+$ CD3$^-$ CD49a$^-$) **(e-f)** NK cells in WT and *Rela*$^{-/-}$ chimeric mice. Data represent the mean ± SEM (n ≥ 6). Statistical significance determined by Mann-Whitney test (non-parametric t-test), * p<0.05, ** p<0.01, *** p<0.001.

4.2.2 RelA controls Natural Killer Cell development downstream of bone marrow precursors

The development of NK cells in the BM follows a sequential developmental process in which mature NK cells arise from the NK cell committed NKP cells, which then migrate to peripheral organs to undergo further maturation [49,53]. Therefore, to pinpoint the point of development at which NK cells are impaired in RelA deficient mice, we sought to determine the effect of RelA deficiency in the generation of NKP cells. Leukocytes from the BM of WT and RelA-deficient mice were harvested and NK cell lineage-committed donor ($CD45.2^+$) NKP cells were identified as $CD45.2^+$ $CD3^-$ $B220^-$ $GR\text{-}1^-$ $CD11b^-$ $NKp46^-$ $CD122^+$, whereas donor ($CD45.2^+$) mature NK cells were identified as $CD45.2^+$ $CD3^-$ $B220^-$ $GR\text{-}1^-$ $CD11b^-$ $NKp46^+$ $CD122^+$ (Figure 4.2). There was a significant reduction in the proportion of NKP cells between WT and *Rela*$^{-/-}$ chimeric mice (Figure 4.2a), whereas the absolute cell numbers were not changed (Figure 4.2b). In contrast, there was a significant ($p<0.001$) reduction in the proportion (Figure 4.2a) and absolute cell number (Figure 4.2b) of mature NK cells in *Rela*$^{-/-}$ chimeric mice compared to the WT chimeric mice. These results imply RelA controls NK cell development after commitment to the NK cell lineage.

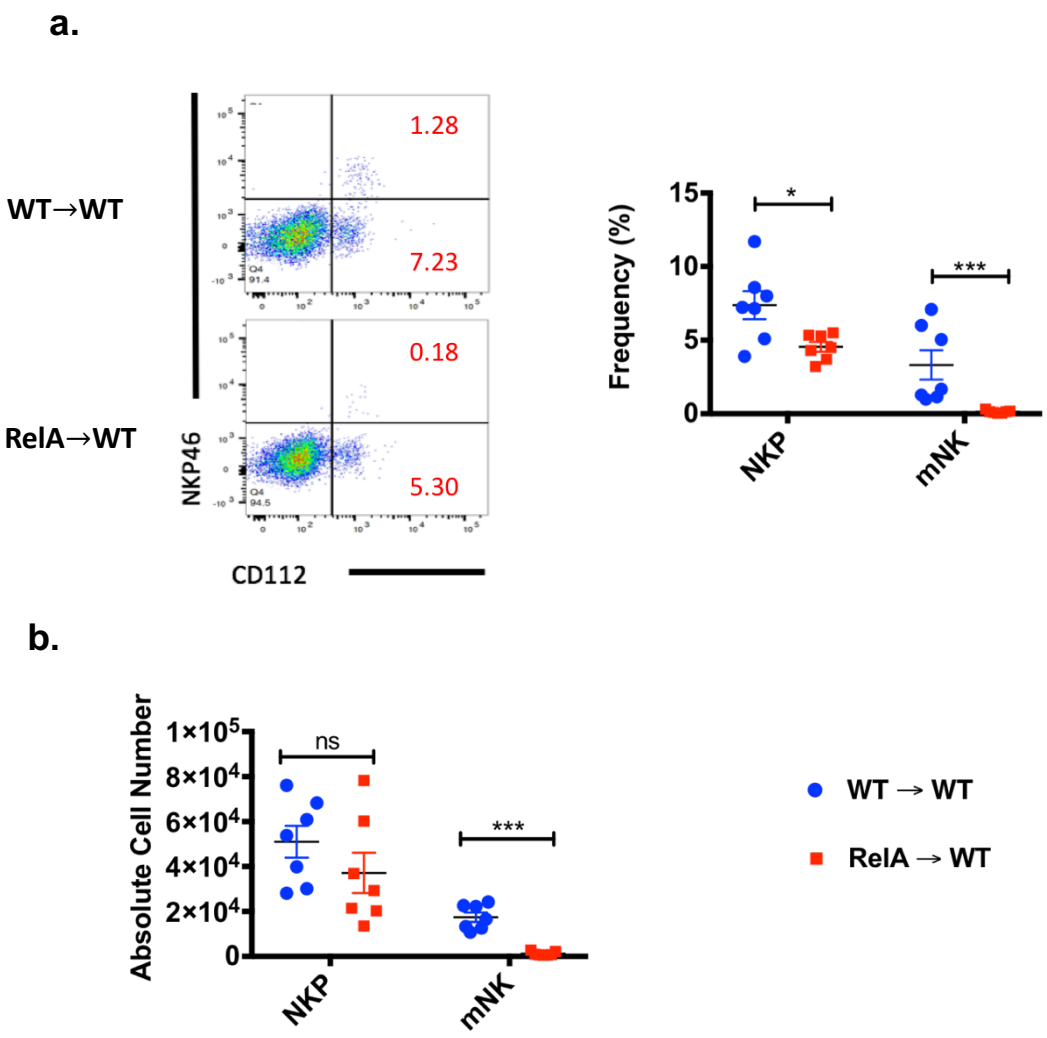


Figure 4.2 Reduction of mature NK cells in the BM of in RelA-deficient mice. The representative FACS dot plots and quantification of frequencies **(a)** and absolute number **(b)** of donor (CD45.2$^+$) derived bone marrow NK cell precursors (CD3$^-$ B220$^-$ GR-1$^-$ CD11b$^-$ NKp46$^+$ CD122$^-$) and mature NK cells (CD3$^-$ B220$^-$ GR-1$^-$ CD11b$^-$ NKp46$^+$ CD122$^+$) in *Rela*$^{-/-}$ and WT chimeric mice. Bars represent mean ± SEM (n=2). Statistical significance determined by Mann-Whitney test (non-parametric t-test), * $p<0.05$, *** $p<0.001$.

4.2.3 RelA regulates a subset of NK cell receptors

Having demonstrated that RelA is required for the development of NK cells, next, the role of RelA in regulating peripheral maturation was assessed. The expression of NK cell receptors characterize the stages of NK cell maturation, for example, the inhibitory Ly49E receptor is expressed on immature NK cells [140,197], whereas, Ly49H expression is abundant on mature NK cells and CD94 expression precedes Ly49 expression in the normal development of NK cells [135]. Therefore, the current study examined the role of RelA in regulating the expression of CD94 and Ly49 receptors. The percentage of splenic NK cells expressing individual Ly49 and CD94 proteins in *Rela*$^{-/-}$ chimeric mice were compared with that of WT chimeric mice (Figure 4.3). In *Rela*$^{-/-}$ chimeric mice, there was a profound increase in the proportion of inhibitory Ly49E/F and a decrease in the activating Ly49H subsets compared with WT chimeric mice (Figure 4.3). This data suggests that RelA may be regulating the expression of Ly49E/F and Ly49H on NK cells.

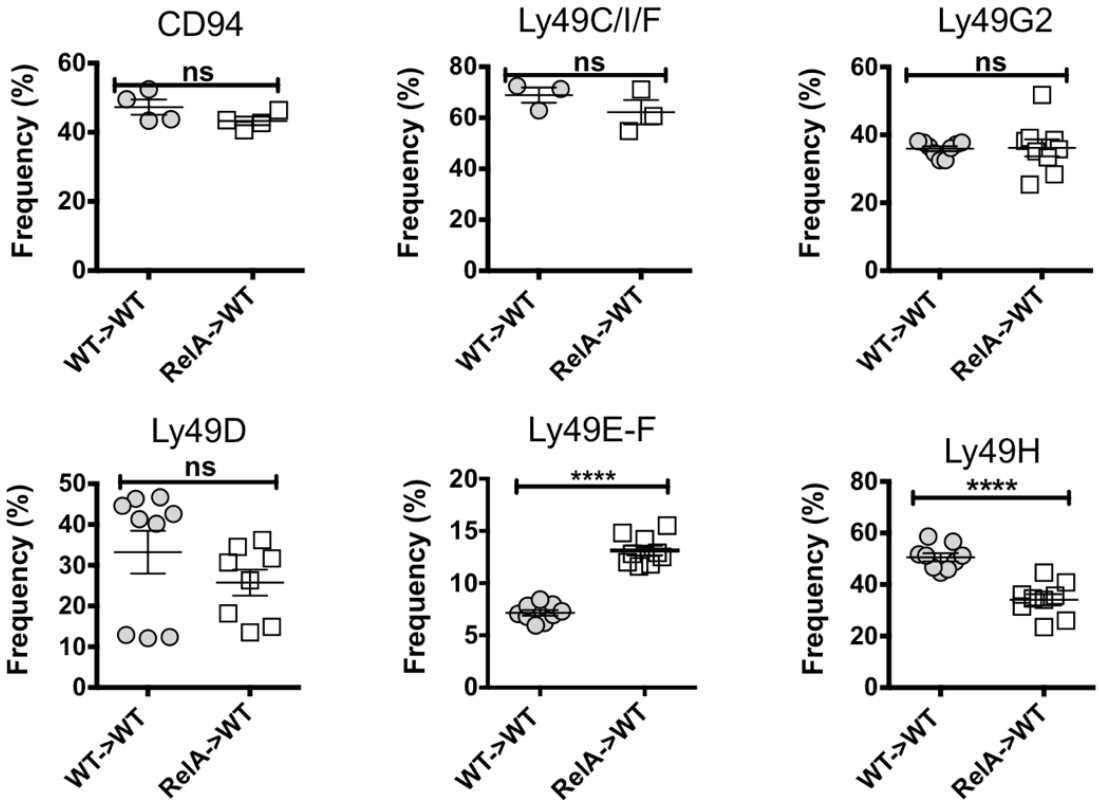


Figure 4.3. RelA deficient NK cells display largely normal activating/inhibitory receptors, apart from a decreased expression of Ly49H and increased expression of Ly49E-F. The expression of the CD94 and the indicated *Ly49 molecules* on spleen NK cells. The data are expressed as the percentage of donor ($CD45.2^+$) derived Splenic $CD3^-NK1.1^+$ NK cells in *Rela*$^{-/-}$ and WT chimeric mice. The data in panel been pooled from two independent experiments. Bars represent mean ± SEM. Statistical significance determined by Mann-Whitney test (non-parametric t-test), * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.3.1. RelA regulates peripheral maturation of NK cells

Having established that RelA is required for NK cell development and the expression of some NK cell receptors, next, the role of RelA in the generation of mature NK cell subsets in the spleen was assessed. In the conventional model of NK cell maturation, peripheral NK cells follow a linear model of differentiation on the basis of CD27 and CD11b expression [84,91]. The percentage and the absolute number of donor NK cells ($CD45.2^+$ $CD3^-$ $NKp46^+$) expressing CD11b and CD27 proteins in the spleen of *Rela*$^{-/-}$ and WT chimeric mice were examined (Figure 4.4a-b). In *Rela*$^{-/-}$ chimeric mice, there was a significant ($p<0.05$) increase in the proportion (Figure 4.4a) and absolute number (Figure 4.4b) of the least mature $CD27^-$ $CD11b^-$ (DN) cells compared to WT chimeric mice. However, the proportion (Figure 4.4a) and absolute number (Figure 4.4b) of the $CD27^+$ $CD11b^-$ (SP) and $CD27^+$ $CD11b^+$ (DP) NK cell subsets in *Rela*$^{-/-}$ chimeric mice were significantly ($p<0.05$) reduced compared to the WT chimeric mice. Interestingly, however, the terminally differentiated $CD27^-CD11b^+$ subset was unchanged. Collectively, this data indicates that only the CD27 expressing NK cell subset relies on RelA for development, whereas the $CD11b^+$ population is unchanged.

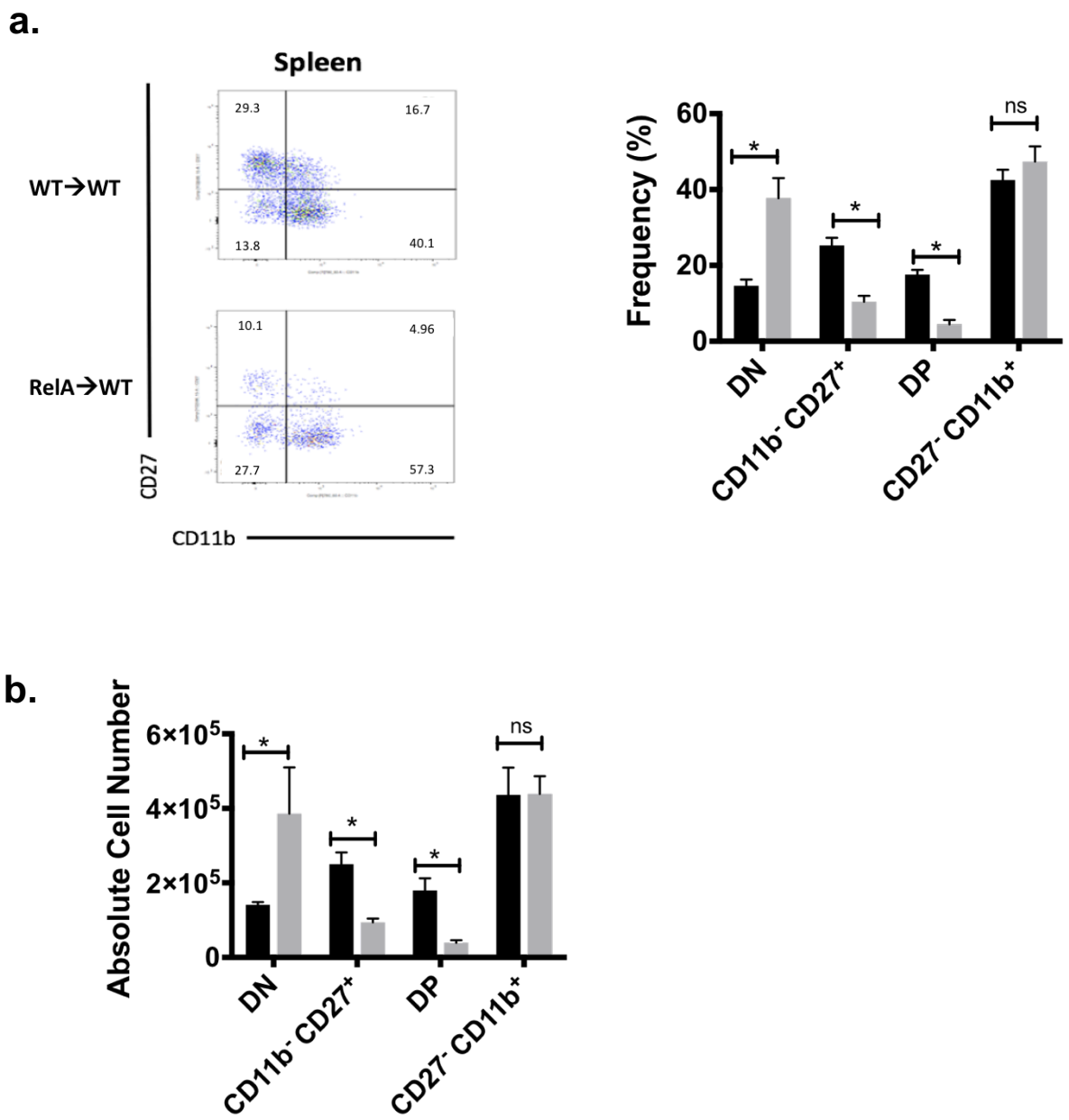


Figure 4.4 Reduced mature NK cells in RelA-deficient mice. The representative FACS dot plots and quantification of frequencies **(a)** and absolute number **(b)** of donor ($CD45.2^+$) derived splenic NK cells ($CD3^-$ $NKp46^+$) gated on DN, $CD11b^-$ $CD27^+$, DP, and $CD11b^+$ $CD27^-$ populations in *Rela*$^{-/-}$ and WT chimeric mice. The data in panel been pooled from two independent experiments. Bars represent mean ± SEM. Statistical significance determined by Mann-Whitney test (non-parametric t-test), * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.4 Decreased DNAM-1 expression on peripheral natural killer cells

The activating receptor DNAM-1 and inhibitory receptor CD96 both belong to the immunoglobulin (Ig) immune-receptor superfamily of Nectins [198,199]. However, DNAM-1 and CD96 have opposing roles in the regulation of NK cell functions [200]. DNAM-1 is crucial for NK-dependent anti-tumor immunity [142] and eradicating infections [199], whereas, CD96 limits NK cell function by direct inhibition [200]. Recently, independent of the CD11b and CD27 maturation scheme, NK cells have been functionally separated into "mature" DNAM-1$^-$ NK cells which differentiate from "immature" DNAM-1$^+$ NK cells [195]. Therefore, to determine if RelA impacts NK cell maturation in the spleen based on DNAM-1 expression patterns, the percentage of splenic donor (CD45.2$^+$) NK cells (CD3$^-$ NKp46$^+$) expressing DNAM-1 or CD96 was assessed (Figure 4.5a). There was a significant reduction in the frequency of DNAM-1 expressing splenic NK cells in *Rela*$^{-/-}$ chimeric mice compared to the WT controls (Figure 4.5a), implying RelA is necessary for DNAM-1 expression on NK cells. To determine whether the effect of RelA is specific for DNAM-1, the percentage of donor (CD45.2$^+$) NK cells (CD3$^-$ NKp46$^+$) expressing individual CD96 proteins was investigated (Figure 4.5b). The expression of CD96 on *Rela*$^{-/-}$ chimeric mice was comparable between the *Rela*$^{-/-}$ and WT chimeric mice (Figure 4.5b), suggesting RelA is specifically regulating DNAM-1 expression on NK cells. Therefore, overall, it can be concluded that RelA has a major regulatory role in the development of NK cells in the BM and subsequent peripheral maturation, particularly the development of functionally mature CD27$^+$ and DNAM-1$^+$ NK cell subsets.

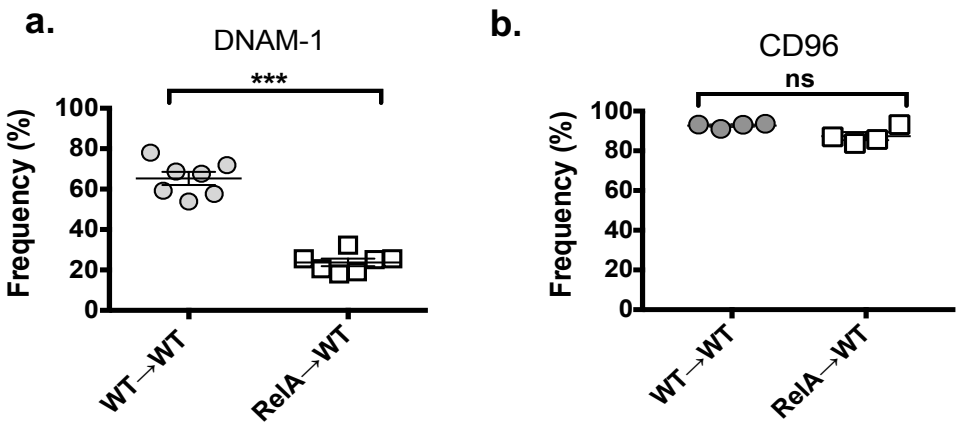


Figure 4.5. RelA specifically regulates DNAM-1 expression in NK cells. The frequency of DNAM-1 (**a**) and CD96 (b) expression among total donor ($CD45.2^+$) derived splenic NK cells ($CD3^-$ $NKp46^+$) from *Rela*$^{-/-}$ and WT chimeric mice. Data represent the mean ± SEM (n > 1). Statistical significance determined by Mann-Whitney test (non-parametric t-test), *** $p<0.001$.

4.3 Discussion

A limited number of studies have explored the role of NF-kB in NK cell development. Early reports identified a critical role of the canonical NF-kB activation pathway in NK cell effector function, seen in patients with NEMO or IKK2 mutations [169], however, the specific NF-kB family members regulating this response was not identified. In contrast, a later study by Zhou and colleagues[194], identified nuclear translocation of p50/RelA complexes to be regulating IL-2- induced up-regulation of perforin in primary mouse NK cells, which was then followed by the Pascal et al. [135], who linked p50/RelA activation with NK cell development based on its regulatory role in NK cell receptor expression. However, to date, no research has investigated the direct role of RelA in NK cell development in the BM and lineage commitment in the periphery. Therefore, in this chapter, the role of the transcription factors, RelA in NK cell development and peripheral maturation has been investigated.

The development of NK cells is tightly regulated by a pool of transcription factors, for example, the loss of the transcription factors Nfil3 [201], ID2 [76], T-bet [108] and Eomes [98] result in a block in NK cell development. With this in mind, in the current study, the loss of RelA was examined in NK cell development, and it was shown to have an *in vivo* role in the development of BM and peripheral NK cells. However, unlike mice deficient in the aforementioned transcription factors, a complete loss of peripheral NK cells was not observed, which suggests of a RelA dependent and independent pathway of NK cell development. Therefore, it was important to further investigate the maturity and functionality of these remaining NK cells.

Having demonstrated that RelA controls NK cell development, the generation of NK cells in the BM was then assessed. This study demonstrated that RelA controls the development of NK cells after commitment to the NK cell lineage. However, the absence of RelA is known

alters the number of CLP, which serves as a precursor for B- T and NK cells [190]. Therefore, the decreased frequency of NKP cells in the BM may be due to an NK cell-extrinsic defect in the lymphocyte population, such as B- and T-cell numbers.

As discussed in chapter 3, the expression of the Ly49 receptors in mice is required for the functional maturation of NK cells and their expression characterizes distinct stages of NK cell development [30,31]. For example, through binding to the viral MCMV protein, m157, the activating Ly49H receptor triggers NK cell activation and the clearance of MCMV-infected cells [202]. However, the role of RelA in regulating the expression of Ly49 receptors in NK cells has not been previously studied. With that being said, the study by Pascal and colleagues [135], showed a significant reduction of Ly49H in NF-κB1/p50 knock-out mice, which is mirrored by the results obtained in this current study. Considering that the most *common* NF-κB complex is the p50/RelA heterodimer [203], it can be hypothesised that both p50 and RelA may be regulating Ly49H expression in NK cell. However, further studies are required to confirm this, including determining Ly49 expression in combined *nfkb1/rela*-deficient mice. Furthermore, considering Ly49H expression is associated with the clearance of the MCMV infection [204], it will be interesting to investigate the functional relevance of RelA in controlling viral infection by challenging *Rela*$^{-/-}$ chimeric mice with MCMV. In contrast to Ly49H, Ly49E/F are inhibitory receptors which recognize MHC class I molecules as ligands [204]. Interestingly, the expression of Ly49E is associated with an immature phenotype on account of being almost exclusively expressed on fetal NK cells but not on resting adult NK cells [205,206]. Therefore, the increased expression of Ly49E in mature NK cells from *Rela*$^{-/-}$ chimeric mice suggest that RelA may have a regulatory role in NK cell development.

In mice, the current paradigm of peripheral NK cell maturation follows a linear pathway based on the co-expression of CD11b and CD27 [84]. However, CD27 expressing NK cells

have an enhanced cytotoxic capacity and are potent producers of pro-inflammatory cytokines, such as IFN-γ [86-89]. Therefore, using the co-expression of CD11b and CD27 as a marker for NK cell maturation, this study found evidence that RelA is required but not essential for the development of the peripheral NK cells. Interestingly, however, the major loss of NK cell subsets are the functionally active $CD27^+$ NK cells. This may be attributed to the finding that a region required for the activation of the p50/RelA complex has been identified on the CD27 molecule [127], and upon ligation by CD70, p50/RelA activation enhances T-cell receptor-mediated proliferative signals [207]. Therefore, considering NK cells share many critical transcription factors with T-cells [109], it stands to reason that the differentiation of NK cells into the $CD27^+$ intermediates during peripheral maturation may be regulated by RelA activation via CD27 signalling. Furthermore, the increase in $CD27^-$ $CD11b^-$ cells but normal numbers of terminally differentiated NK cells in the RelA-deficient mice cannot be explained by the current paradigm of peripheral NK cell maturation, which relies on the differentiation of NK cells through the $CD27^+$ intermediates [84]. Therefore, these results propose a new maturation pathway, in which in the absence of RelA, NK cells have the ability to mature directly from $CD27^-$ $CD11b^-$ to $CD27^+$ $CD11b^+$ populations. Interestingly, however, as there is a small population of $CD27^+$ cells that do exist in these mice, this raises the possibility that there may be two pathways that exist simultaneously, the RelA-dependent and the Rel-A independent pathway (Figure 4.6).

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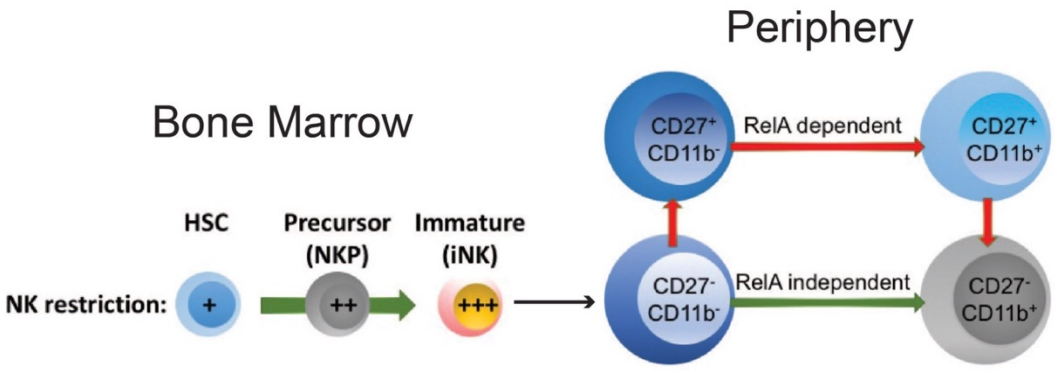

Figure 4.6. The new proposed model of NK cell development. Based on the RelA data generated in this study, the proposed "RelA-dependent" maturation pathway follows the linear paradigm (red arrow), involving CD27 up-regulated, followed by CD11b up-regulation and lastly, CD27 down-regulation on terminally differentiated NK cells. In contrast, the "RelA-independent" pathway of peripheral NK cell maturation (green arrow), does not require the expression of the CD27 intermediates, instead, the DN cells transition directly to the terminally differentiated $CD27^-$ NK cells.

Having established the critical role played by RelA in the peripheral maturation of NK cells on the basis of CD11b and CD27 expression, we next assessed the role of RelA in regulating the "alternative" NK cell maturation based on DNAM-1 expression patterns. The current study demonstrated a critical role of RelA in regulating DNAM-1 expression. According to the maturation scheme proposed by Martinet, L. et al [90], the absence of RelA result in an increased number mature $DNAM\text{-}1^-$ NK cells, suggesting RelA activation may be blocking NK cell maturation. However, these results are conflicting with those obtained on the basis of CD11b and CD27 expression, which showed that RelA is required for the maturation into functionally active $CD27^+$ NK cells. However, the $DNAM\text{-}1^-$ NK cell population most closely resemble the $CD27^+$ subsets, as they produce more pro-inflammatory cytokines, including IFN-y. Therefore, these data indicate that RelA is involved in the maturation of NK cells, but also seems to play a critical role in the development of cytotoxic NK cells. Furthermore, considering, tumour immune surveillance strongly relies on DNAM-1 expression [140,143,144], it will interesting to examine cancer patients for RelA mutations, as this may be causing a reduction in DNAM-1 expression and thus impaired immune surveillance of tumour development. Moreover, a study by Nabekura, T and colleagues [208], have shown that in the

absence of DNAM-1, the expansion of MCMV-specific Ly49H$^+$ NK cells are suppressed, which consequently results in a reduction of host defence against MCMV infection [208]. Interestingly, these results complement those obtained in the current study, which revealed a significant reduction of both DNAM-1 and Ly49H expression in the absence of RelA. Therefore, based on these findings, analysis of RelA mutations in patients with cancer or chronic viral infections may provide significant clues as to the mechanisms underlying their illness.

In summary, RelA was shown to regulate NK cell-lineage commitment in the BM and subsequent maturation into functionally active CD27$^+$ NK cells in the periphery. In addition, RelA was seen to control DNAM-1 and Ly49H expression, which both play a major role in disease clearance. Next, it is of great interest to study RelA activity during NK cell maturation in order to elucidate the link between RelA and CD27 expression in murine NK cells, which is presented in chapter 5 of this thesis.

Chapter 5

Investigating NF-κB activation during peripheral NK cell maturation

5.1 Introduction

The results presented in the preceding chapter demonstrates the role of RelA in regulating DNAM-1 expression and the generation of $CD27^+$ NK cells. CD27 is a member of the TNF receptor superfamily and is expressed on the majority of activated T-cells [209] and cytotoxic NK cells [91]. Furthermore, murine NK cells are separated into distinct maturation stages based on differential CD27 and CD11b expression [84,91]. Starting from "least mature" $CD11b^-$ $CD27^-$ cells, NK cells differentiate into the "immature" $CD11b^-CD27^+$, "mature" $CD11b^+CD27^+$ and finally, the terminally differentiated $CD11b^+CD27^-$ NK cells [84]. This 4-stage process of NK cell maturation follows a linear pathway and no other pathway, apart from the recently proposed alternative pathway based on DNAM-1 expression [90], has been proposed. Interestingly, recent reports have identified RelA activation in response to CD27 [127] and DNAM-1 [136] signals.

The activation of NF-κB is characterised through the ability to detect protein binding to a specific DNA sequence. This interaction is usually detected through the use of an electrophoretic mobility shift assay (EMSA) and the super-shift assay, which is used as a general readout of the activation state of NF-κB [210]. Although newer techniques, such as chromatin immunoprecipitation (ChIP), are often used to assess NF-κB binding to the promoters and enhancers of specific genes *in vitro* and *in vivo* [211], the EMSA remains a powerful experimental tool to quickly test for the presence of NF-κB that is capable of binding to DNA [210]. In contrast to the EMSA and ChIP method which are often used for qualitative purposes, fluorescence microscopy, in particular, confocal microscopy, can quantitate the translocation of NF-κB molecules. The laser-scanning confocal microscopy enables the visualization and quantification of NF-κB activity at the cellular level [212]. Moreover, this technique generates high-resolution images of structures within the cell that could not be detected by conventional means. Therefore, this chapter will utilize a number of different

techniques to measure NF-κB activation during NK cell maturation, which will consequently provide a better insight into the role of NF-κB in NK cell lineage commitment.

5.2 Results

5.2.1 RelA activity is reduced upon peripheral NK cells maturation

Having confirmed that RelA is required for peripheral NK cell maturation in Chapter 4, it became necessary to determine the stage of NK cell development during which RelA has translocated to the nucleus. As such, splenic $CD11b^{-}CD27^{+}$, $CD11b^{+}CD27^{+}$ and terminally differentiated $CD11b^{+}CD27^{-}$ NK cell subsets from WT mice were purified and nuclear extracts were prepared to determine the levels of NF-kB DNA-binding activity through the use of EMSA. This technique is based on the principle that molecules of different molecular weight and charge will differ in motility when running on a non-denaturing gel. Therefore, due to an increase in size, a DNA-Protein complex will run at a much slower rate when compared with free DNA or protein [210,213,214]. Two DNA/NF-kB complexes (Complex 1 (C1) and Complex 2 (C2)) were identified within the $CD11b^{-}CD27^{+}$ and $CD11b^{+}CD27^{+}$ NK cells, but not in the terminally differentiated $CD11b^{+}CD27^{-}$ subset (Figure 5.1a). This data suggest that the early stages of NK cell development are associated with NF-kB translocation and prompted subsequent studies aimed at identifying the specific NF-kB family members that translocated. To identify the specific NF-kB family member bound to the DNA to form "C1 and C2", an electrophoretic "supershift" assay was performed using NF-kB subunit-specific antibodies and control reactions were performed with pre-immune sera (Figure 5.1b). A further shift in the mobility of the protein–DNA complex was observed due to the addition of specific antibodies for NF-κB1 (p50) and RelA (Figure 5.1b). This demonstrated that C1 was comprised of homodimers of NF-κB1 (p50), while C2 was mainly NF-κB1/RelA (p50/p65) heterodimers (Figure 5.1b). Collectively, this data demonstrates a similar DNA/NF-kB complexes formation between the $CD11b^{-}CD27^{+}$ and $CD11b^{+}CD27^{+}$ cell

subsets, which suggest that RelA may be regulating the differentiation of NK cells into $CD27^+$ expressing subsets during peripheral maturation.

Having demonstrated in Chapter 4 that RelA is required for the generation of CD27 expressing NK cells and that DNAM-1 expressing NK cells are compromised in the absence of RelA, we next sought to determine whether RelA activity is dependent on DNAM-1 or CD27 expression using an EMSA assay (Figure 5.1c). Two nuclear NF-kB/DNA complexes (C1 and C2) are present in the immediate "ex-vivo" $CD27^+DNAM\text{-}1^+$ and $CD27^+DNAM\text{-}1^-$ cells, but not in the $CD27^-DNAM\text{-}1^+$ and $CD27^-DNAM\text{-}1^-$ subset (Figure 5.1c). This data demonstrates no association between NF-kB/RelA activity and DNAM-1 expression and, instead, indicate that NF-kB/RelA is linked with CD27 expression on NK cells.

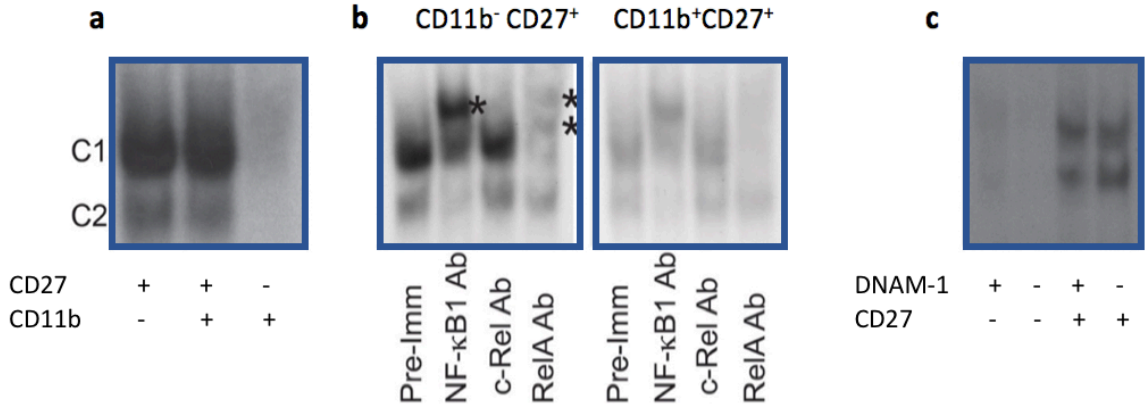

Figure 5.1. NF-κB nuclear translocation in mature NK cell subsets. (a) An EMSA was performed on nuclear extracts isolated from '*ex vivo'* purified splenic NK cell subsets of indicated maturity. Two NF-kB/DNA complexes (C1 and C2) were present in $CD11b^{-}$ $CD27^{+}$ and $CD11b^{+}$ $CD27^{+}$ cells (lanes 1 & 2). **(b)** Antibody shifts demonstrated that in $CD11b^{-}$ /$CD27^{+}$ and $CD11b^{+}$/ $CD27^{+}$ subsets, C1 comprises of homodimers of NF-κB1 (p50), while C2 contains NF-κB1/RelA (p50/p65) heterodimers. **c)** EMSA performed on nuclear extracts isolated from 'ex vivo' purified WT splenic NK cell subsets based on CD27 and DNAM-1 expression. C1 and C2 refers to NF-kB/DNA complexes. Pre-Imm refers to pre-immune sera and *=Antibody super-shifted NF-kB/DNA complex. n=1.

5.2.2 RelA defines a novel pathway of NK cell development

For a quantitative detection of RelA nuclear translocation at a single-cell level, NF-kB translocation was detected using a mouse line in which RelA is constitutively expressing GFP. The $CD11b^{-}$ $CD27^{+}$, $CD11b^{+}$ $CD27^{+}$ and terminally differentiated $CD11b^{+}$ $CD27^{-}$ NK cell subsets from the spleen of these mice were purified and RelA nuclear translocation was enumerated using confocal microscopy. Interestingly, despite the previous data in Chapter 4 indicating that CD27 is completely reliant upon NF-kB, within each of the NK cell subsets, there are three distinct populations of cells based on RelA localization (Figure 5.2a). However, upon quantifying the intensity of RelA in the nucleus across the three subsets, it is clear that the $CD11b^{-}CD27^{+}$ NK cell subset contains the highest intensity of RelA in the nucleus (red arrow), and less cells displaying RelA in the cytoplasm (white arrow) or incomplete RelA translocation (orange arrow) (Figure 5.2a). Indeed, the intensity of nuclear RelA consistently decreased as NK cells matured and terminally differentiated (Figure 5.2b). Collectively, these data corroborate the EMSA results and suggest that RelA may be involved in regulating early stages of NK cell maturation.

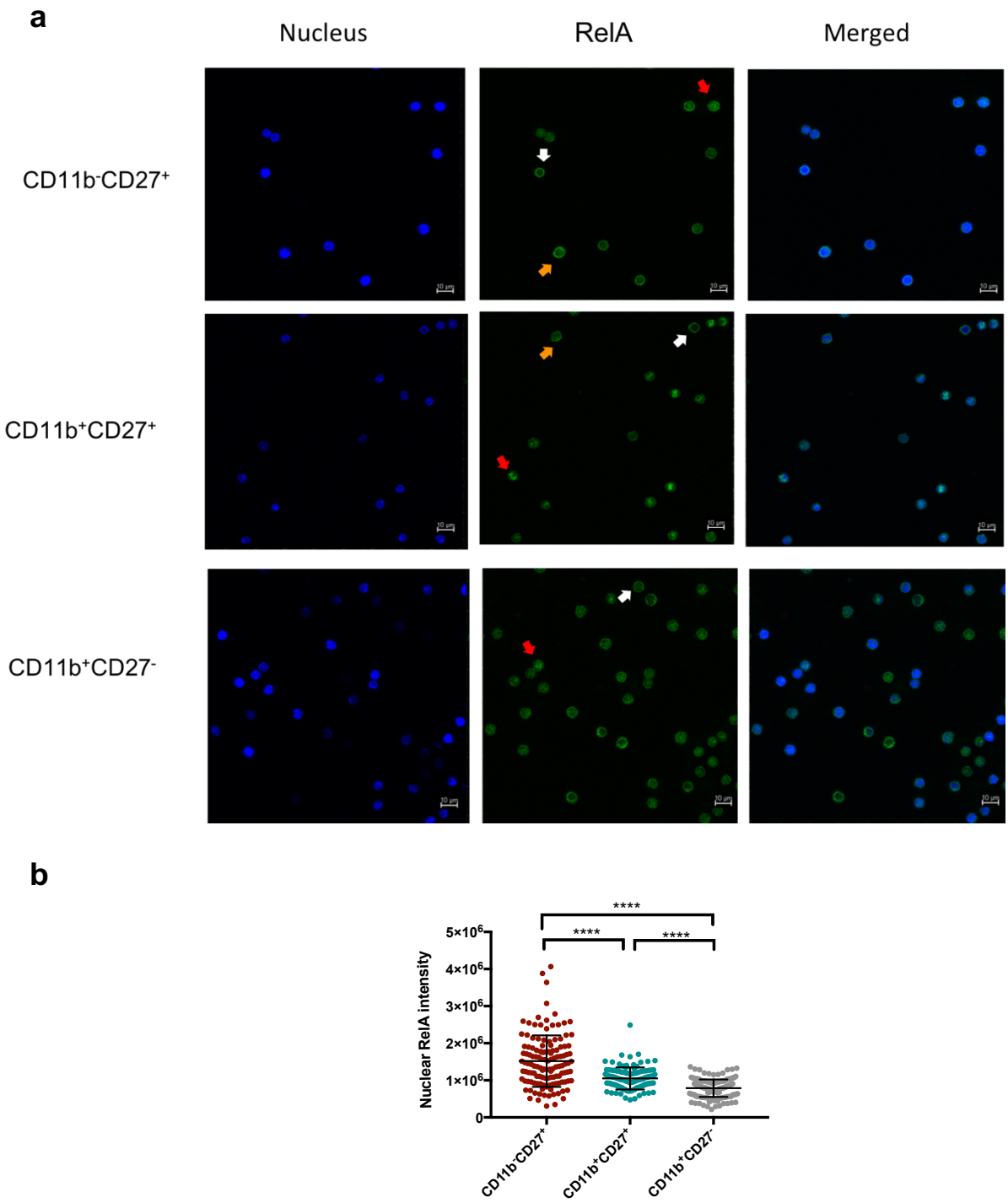

Figure 5.2. Nuclear RelA translocation during lineage commitment. (a) Representative images single confocal optical slices of splenic NK cells isolated from GFP-RelA knock-in mice at different maturation stages. RelA is depicted in green (GFP) and the nucleus in blue (Hoechst). The arrows represent cells with differing RelA activity. **(b)** The sum fluorescence intensity of nuclear GFP from at least 100 cells of each stage of maturation. Data represent the mean ± SD (n = 2). Statistical significance determined by Mann-Whitney test (non-parametric t-test), **** P<0.0001.

5.3 Discussion

There is emerging evidence that supports the requirement of transcription factors for NK cell development [74,215]. However, there are currently no studies focusing on the role of RelA in peripheral NK maturation on the basis of CD27 and CD11b expression. Whereas, there are only a very few studies that have explored their role in DNAM-1 expression [106]. In this chapter, using the EMSA and gel supershift assay, we identified the presence of the classical NF-κB heterodimer of RelA/p50 and p50 homodimer in the nucleus of the CD27 expressing "immature" $CD11b^{-}CD27^{+}$ and "functionally mature" $CD11b^{+}$ $CD27^{+}$ NK cell subsets. This "mature" subset display increased cytotoxicity and cytokine production [216], and interestingly, Pandey et al. [217] have also identified that nuclear translocation of p50 homodimers and RelA/p50 heterodimers occur in *ex-vivo* activated NK cells, in which the cells are more cytotoxic. In addition, due to the lack of the transcription activation domain (TAD), p50 homodimers have been identified to serve an inhibitory function in other cell types [218,219], therefore, the abundant presence of p50 homodimers in the nucleus of $CD11b^{-}CD27^{+}$ and $CD11b^{+}CD27^{+}$ NK cells in this study may be due to both exiting and entering of the nucleus to enable or disable gene transcription, respectively. Furthermore, the presence of the classical NF-κB heterodimer of RelA/p50 and p50 homodimer appear to be restricted to the nucleus of the $CD27^{+}$ NK cells independent of DNAM-1 expression, which ultimately explains the absence of NF-κB/DNA complex in the terminally differentiated $CD11b^{+}CD27^{-}$ NK cell subset by EMSA. Interestingly, an intact DNAM-1 pathway has been shown to be required for optimal NK cell-mediated killing of CD70 expressing tumour cells, which is the ligand for CD27 [142]. Therefore, considering that RelA has been shown in this study to be required for the generation of $CD27^{+}DNAM\text{-}1^{+}$ NK cells, RelA can be hypothesized to play a regulatory role in NK-cell mediated antitumor activity. However, further experiments such as ChIP-Seq analysis is required to be performed on $CD11b^{-}$ $CD27^{+}$ and $CD11b^{+}CD27^{+}$ cells to determine which genes expressed in these subsets are

likely to direct p50 homodimer and RelA/p50 heterodimer transcription, as this may serve as a key to underpin the regulatory role of NF-κB, in particular, the RelA/p50 complex in peripheral NK cell maturation and DNAM-1 expression.

Interestingly, although NF-κB heterodimer of RelA/p50 appeared to translocate to the nucleus of NK cells in only the $CD11b^{-}CD27^{+}$ and $CD11b^{+}$ $CD27^{+}$ NK cell subsets when measured by EMSA, nuclear RelA translocation was also identified in the terminally differentiated $CD11b^{+}CD27^{-}$ cells by confocal microscopy, albeit to a significantly lower level. However, similar to the results obtained by the EMSA, the $CD27^{+}$ NK cells have the highest amount of nuclear RelA translocation, which complements previous research which identified CD27 signalling to activate both the canonical and alternative NF-κB pathway, in particular, nuclear RelA translocation [220]. Although this requires further investigation, the presence of an "intermediate" cell population that contains both cytoplasmic and nuclear RelA may be due to images being captured during nuclear RelA translocation, or during post-induction repression [221,222], in which RelA is returning to the cytoplasm in an inactivated state.

As the microscopy data can identify nuclear translocation of RelA on a single-cell level, this result gives a powerful insight into a possible dual pathway of dependence upon NF-kB, and indeed may be the explanation as to how NK cells are able to mature into terminally differentiated subsets as seen in Chapter 3 in the absence of RelA. This hypothesis can be further explored by using a strain of mice that conditionally target floxed *Rela* in NK cells, which will also determine if RelA plays an intrinsic role in NK cell maturation. Collectively, a more complete picture of the regulatory role of RelA in NK cell maturation is now beginning to form. This thesis has demonstrated that RelA is regulating the maturation of NK cells and DNAM-1 expression. Furthermore, it provides preliminary evidence of a RelA dependent

and independent pathway of NK cell maturation. This is the first documentation for the role of RelA in regulating NK cell lineage commitment in the periphery.

Chapter 6

General Discussion

This thesis provides important data regarding the canonical NF-κB family members in NK cell development. Here, three of the most important findings identify a unique role of RelA in regulating NK cell development. First, RelA regulates NK cell lineage commitment in the BM and subsequent functional maturation in the periphery through CD27 expression. Second, NK cell maturation may not be linear and $CD11b^{-}CD27^{-}$ cells might be able to develop through RelA-dependent or -independent pathways. Lastly, RelA regulates the expression of the critical activating receptors DNAM-1 and Ly49H on NK cells.

6.1 NF-kB in the immune system and in NK cells

This thesis aimed to determine the possible function of the NF-κB family members in the development of NK cells. Therefore, NK cells were analysed from mice specifically lacking each of these proteins. The individual loss of NF-κB1/p50 and c-Rel proteins resulted in no obvious defect in NK cell development. With that being said, the absence of NF-κB1/p50 altered the expression of NK cell receptors, CD94, Ly49D and Ly49E-F **(Chapter 3, Figure 3.5).** These results suggest that p50 may be the major subunit required for the expression of the indicated NK cell receptors, while c-Rel is redundant. However, the canonical NF-κB Rel family members, RelA and c-Rel, form either hetero- or homodimers with each other, or with p50, the precursor of NF-κB1 [223]. Thus, the deletion of c-Rel may have no effect on NK cell development due to compensation and/or redundancy by other family members that may mask their importance. However, considering p50 may be regulating the expression of CD94, Ly49A and Ly49D, in the future, investigations to specifically determine the gene protomer region bound by p50 is required. None the less, these results do not indicate an exclusive role for c-Rel and NF-κB1/p50 in NK cell development.

In contrast to NF-κB1/p50 and c-Rel, RelA was shown to be required for the development of mature NK cells from NKP cells in the BM **(Chapter 4, Figure 4.2)** and their subsequent

maturational stages in the periphery **(Chapter 4, Figure 4.4)**. Unlike the linear paradigm of NK cell maturation, which is based on CD27 and CD11b expression, this study provides data which is suggestive of a new model of NK cell development based on RelA translocation. In this model, the RelA independent pathway involves the direct transition of $CD11b^{-}CD27^{-}$ cells to the terminally differentiated $CD11b^{+}CD27^{-}$ NK cells without acquiring CD27 expression. This model is supported by the presence of two distinct NK cell populations that either require or do not require RelA during peripheral lineage commitment **(Chapter 5, Figure 5.2)**. Considering the development and subsequent peripheral maturation is essential in defining NK cell effector function [82], the impaired expression of NK cell surface receptors that mediate NK cell activation is not surprising. However, with that being said, DNAM-1 regulation by RelA activation is intriguing **(Chapter 4, Figure 4.5 and Chapter 5, Figure 5.1).**

In the current study, RelA was revealed to be a key transcription factor required for the generation of $CD27^{+}$ NK cells **(Chapter 4, Figure 4.4 and Chapter 5, Figure 5.1)** and furthermore, RelA was required for DNAM-1 expression in NK cells **(Chapter 4, Figure 4.5).** Interestingly, Wagner, A. K. *et al*. identified DNAM-1 upregulation to be linked with CD27 and therefore, DNAM-1 expression was diminished in terminally differentiated $CD11b^{+}CD27^{-}$ NK cells [224]. These results complement the findings of the current study and support the proposed RelA-independent pathway **(Chapter 4, Figure 4.6)**, in which NK cells undergo terminal differentiation without prior CD27 expression, combined with a decrease in DNAM-1 expression. Interestingly, the co-engagement of DNAM-1 with 2B4 has been shown to enable RelA activation in NK cells [106], and DNAM-1 along with 2B4 expression is reduced in tumour-associated NK cells [225]. Thus, together with results presented in this thesis, RelA activation has been demonstrated to have a key role in regulating DNAM-1 expression in NK cells. Considering DNAM-1 is an activating receptor

that promotes NK cell-mediated elimination of virally infected cells and various tumours, including ovarian carcinoma [225,226], identifying the transcription factor regulating its expression can be used as a tool for promoting immune surveillance and increasing anti-tumour activity. Overall, the absence of RelA in NK cells may be associated with a decreased responsiveness to cytokines and the production of effector cytokines [165]. This is reinforced by the decreased expression of Ly49H in the absence of RelA **(Chapter 4, Figure 4.3)**, which is required for the clearance of MCMV through the production of perforin and IFN-γ [227].

Moreover, increased knowledge about the transcriptional regulators that guide NK cell development can be used to augment the overall immune response. For instance, the interaction between NK cells and DCs promote DC maturation and therefore their ability to activate naïve T-cells [228]. In addition, during HIV infection, the interaction between NK-DCs are impaired [229]. Therefore, RelA may be indirectly governing this interaction by regulating NK cell generation. For this reason, RelA activation may be an attractive target for defence against viral infections and effective anti-tumour responses where the expression of DNAM-1 is required.

6.2 Future directions

Several questions have arisen from the work presented in this thesis. First, the cytokine and chemokine production as well as, the cytotoxicity of RelA-deficient NK cells at different stages of development, is required. NK cells from the $CD11b^{+}CD27^{+}$ subset produce high levels of IFN-γ and MIP-1α in response to IL-12 and IL-18 [230] or IL-12 and IL-15 [174], respectively. Therefore, in accordance to the results obtained in this study, RelA-deficient NK cells are expected to produce low levels of the aforementioned cytokine and chemokine, which should result in an impaired ability to control infection. Second, to determine the

functional relevance of RelA in controlling viral infection, RelA-deficient mice should be challenged with MCMV and their ability to clear the infection will be assessed. Considering cytotoxicity of NK cells is mostly driven by the activating and inhibiting NK cell receptors, and in the case of MCMV, by Ly49H engagement [231], the viral load is expected to increase in the RelA-deficient mice. Additionally, to determine the ramification of the loss of RelA in tumour clearance, NK cells from RelA-deficient mice will be challenged with the murine B16 melanoma tumour cells and the melanoma burden will be assessed. Finally, as this study suggests of a RelA- dependent and independent pathway of NK cell maturation, a study should be performed using a strain of mice that conditionally target floxed *Rela* in NK cells to determine if, in the absence of RelA, $CD11b^{-}CD27^{-}$ cells differentiate directly into the terminally differentiated NK cells. This will also determine if RelA plays an intrinsic role in NK cell maturation. These results add to knowledge surrounding the transcriptional regulation of NK cell development and DNAM-1 expression, which will make a significant contribution to prospective studies of NK cell effector function and tumour control.

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