NOVEL PATHWAY OF THYMUS-DEPENDENT NK CELL DEVELOPMENT

by

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B.Sc.H, Acadia University, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

December 2006

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ABSTRACT

Natural killer (NK) cells are the major lymphocytes of the innate immune system, but their developmental pathway is not fully defined. It is commonly assumed that all NK cells develop in the bone marrow. In this thesis, I describe a novel thymus-dependent pathway of NK cell development that is specific to those in the thymus and lymph nodes. Microarray analysis revealed TCRγ mRNA expression in NK cells. Genomic and RT-PCR showed that some NK cells have rearranged TCRγ genes while TCRβ and TCRδ genes are in germline order. NK cells with rearranged TCRγ (TcrγNK cells) were absent in nude mice indicating that they are thymus-dependent. Approximately half of thymus NK cells have rearranged TCRγ genes and in vitro cultures of immature thymocytes (double negative (DN)1 and DN2 progenitors) produced TcrγNK cells, strongly suggesting that these are the thymus-dependent NK cell-progenitors in vivo. Thymus NK cells are CD94/NKG2$^\text{hi}$ Ly49$^\text{lo}$ Mac-1$^\text{lo}$ IL-7Ra$^\text{hi}$ and they have normal cytotoxicity levels but reduced IFNγ production. By using TCRγ gene rearrangement as a marker of thymus-dependent NK cells, we showed that they are also present in lymph nodes (LNs) but in no other tissues tested. NK progenitors similar to immature thymocytes were found in LNs and LN TcrγNK cells and LN progenitors were also absent in nude mice. In vitro cultures and preliminary in vivo studies suggest that the NK progenitors in the LN give rise to mature NK cells. The results suggest that immature thymocytes migrate to LNs and differentiate into NK cells. It is likely that the thymus-dependent NK cells play a special role in the immune response since their phenotype is unique.

Finally, this study suggests that multiple pathways of NK cell commitment exist in multiple tissues. The differences in tissue environment may influence the phenotype and function of NK cells, resulting in multiple subsets of NK cells throughout the body.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DEC</td>
<td>dendritic epidermal T cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>ELP</td>
<td>early lymphoid precursor</td>
</tr>
<tr>
<td>ETP</td>
<td>early thymus progenitor</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FL</td>
<td>fetal liver</td>
</tr>
<tr>
<td>Flt3L</td>
<td>Flt-3 ligand</td>
</tr>
<tr>
<td>FTOC</td>
<td>fetal thymic organ culture</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venules</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenously</td>
</tr>
<tr>
<td>Id</td>
<td>inhibitors of DNA binding</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon γ</td>
</tr>
<tr>
<td>KIR</td>
<td>killer inhibitory receptor</td>
</tr>
<tr>
<td>LAK</td>
<td>lymphokine activated killer cell</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LTβR</td>
<td>lymphotxin beta receptor</td>
</tr>
<tr>
<td>MCMV</td>
<td>mouse cytomegalovirus</td>
</tr>
<tr>
<td>2ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium eagle</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mNK</td>
<td>mature NK cell</td>
</tr>
<tr>
<td>MPP</td>
<td>multipotent progenitor</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NKP</td>
<td>NK precursor</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin streptomycin</td>
</tr>
<tr>
<td>RAET1</td>
<td>retinoic acid early transcripts</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequence</td>
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</tbody>
</table>
SIP₁: sphingosine 1-phosphate type 1 receptor
SAGE: serial analysis of gene expression
SCF: stem cell factor
SP: single positive
TCR: T cell receptor

Tcρ NK: NK cell with TCRγ gene rearrangement
TdT: terminal deoxyribonucleotide transferase
TEC: thymic epithelial cell
T-IEL: T intraepithelial lymphocyte
TNF: tumour necrosis factor
TNKP: bipotent T/NK cell precursor
TRAF6: tumor necrosis factor receptor associated factor 6

TRAIL: TNF-related apoptosis-inducing ligand
VDUP1: vitamin D3 upregulated protein 1

γε: common γ chain
ACKNOWLEDGEMENTS

Firstly, thank you to my supervisor Dr. Fumio Takei. My studies were greatly enhanced by having you as a supervisor. I feel very lucky to have had you as my supervisor. You have been extremely helpful and always interested and involved in my research. You have put so much time into working with me on various projects and have helped so much with writing the thesis. You have always been enthusiastic and encouraging and I have learned so much from you. Thank you!

Thank you to my graduate committee members, Drs. Dixie Mager, Rob Kay, and Kelly McNagny, for their advice and guidance throughout my studies.

Thank you to all of my lab members, past and present, for their friendship and help. Special thanks to Motoi Maeda for teaching me many laboratory techniques when I first started working in the lab. To Nooshin Tabatabaei, we have gone through classes, research, travelling to conferences together, etc. at the same time. It was nice to have you around to talk with.

Thanks to Evette Haddad, Emily Mace, Erica Wilson, Eva Backstrom, Valeria Alcon, Lisa Dreolini, Matt MacLeod, Reza Marwali, Carmine Carpenito, for teaching me how to do certain techniques, for answering questions, helping me through problems, and for making the lab such a great place to be! You are what made my grad studies so much fun!

Thank you to Nastaran Mohammadi and Christine Parachoniak for doing the cloning and sequencing of the Vy-Cγ RT-PCR products. Thanks to Chelsea Greenwood for helping me work on single cell RT-PCR. And finally thanks to Tim Halim for working on the LN DN progenitor in vitro cultures and for working together with me on the transplantation studies, especially for getting all of the peripheral LNs and learning how to do intravenous injections.

A very big thank you to the FACS sorting staff, Lindsey, Gayle, Jaime, Cam, and Rick. You were always a big help. Thank you to Kai Lucke for help with i.v. injections and supplying mice. Thank you to the Marcel Bally lab for nude mice and to the JAF and ARC staff.

I would like to acknowledge my funding from the Michael Smith Foundation for Health Research for both junior and senior trainee awards and from UBC UGF awards.

Thank you to my family, especially my parents, for their support and encouragement. And finally, thank you most of all to my husband, Matt, to whom I owe very much. Thank you for giving up so much to come to Vancouver for me to do my PhD. Thank you for your constant support and understanding throughout the five years. I really needed your encouragement to keep working hard and to strive to always do my best. You always make me want to reach higher. You mean the world to me. Thank you!
1 INTRODUCTION

1.1. Introduction to NK cells

Natural killer (NK) cells are lymphocytes that belong to the innate immune system. They have a large granular morphology and can mediate cellular cytotoxicity as well as release chemokines and inflammatory cytokines. NK cells become activated by cytokines or upon encountering target cells that express ligands for NK cell receptors. NK cells also play a role in activating adaptive immunity and participate in cross talk with dendritic cells (DCs). Upon activation, NK cells can directly lyse target cells by exocytosis of perforin and granzymes. They also secrete cytokines such as interferon γ (IFNγ) and tumor necrosis factor (TNF) and chemokines. Elimination of cells via FasL and TNF-related apoptosis-inducing ligand (TRAIL) pathways also occurs in NK cells. These ligands are mediators of caspase-dependent apoptosis in target cells expressing the corresponding receptors Fas and TRAIL-R. NK cells also express CD16 (FcγRIII) which binds IgG antibody coated cells and mediates antibody-dependent cell-mediated cytotoxicity (ADCC).

NK cells have a recognition system that is encoded by non-rearranged genes and it involves multiple types of receptors rather than one dominant receptor. These receptors can trigger NK cells individually or in combination, depending on each target cell it encounters. NK cells use various strategies to recognize target cells including recognition of pathogen-encoded molecules, induced self recognition, and the most classically described missing-self recognition (Fig. 1.1).
As part of the well characterized missing self recognition strategy, NK cells express receptors that are specific for major histocompatibility complex (MHC) class I molecules (reviewed in 8). MHC class I molecules are expressed on the surface of all healthy nucleated cells but are often downregulated as a consequence of infection, mutation, or transformation. Most of the MHC class I-specific receptors transduce an inhibitory signal. Ligation of these receptors delivers a dominant-negative signal to NK cells that prevents natural killing of self cells. It is
the balance of inhibitory and stimulatory signaling that determines the outcome of NK-target cell interactions such that if a NK cell encounters a healthy cell, its inhibitory signals received will override activating signals. If, on the other hand, an NK cell encounters an unhealthy cell (MHC class I′), an inhibitory signal is not received and the activating signals will result in cytolytic function. Adult mice express two families of MHC class I-specific receptors, Ly49 and CD94/NKG2, whereas fetal and neonatal NK cells express only the latter. Human NK cells express CD94/NKG2 receptors and the killer inhibitory receptor (KIR) family.

Another recognition strategy is detection of pathogen-encoded molecules. NK cells express the Ly49H receptor which is stimulatory and binds to an MHC class I-like protein, m157, which is a mouse cytomegalovirus (MCMV) encoded protein. Ly49H enables NK cells to undergo considerable proliferation and to limit early stage MCMV infection. Other NK cell receptors that are specific for pathogens are NKp46 and NKp44 which bind the influenza virus hemagglutinin.

NK cell receptors can also recognize induced self signals, (stress signals) that are upregulated during infection or on tumor cells. NKp46, NKp44, and NKp30 receptors likely recognize ligands on tumor cells but the ligand identities are not yet known. The best characterized receptor is NKG2D, which is expressed by almost all NK cells and plays a key role in immune responses, especially as an activating receptor that triggers NK cells to respond against tumors (reviewed by ). NKG2D ligands are stress signals encoded by the host genome. These include a diverse family of ligands, retinoic acid early transcripts (RAET1) that are shared between mice and humans. Family members include Rae-1, H-60, Mult1 and ULBP. Although the expression patterns of NKG2D ligands are diverse and complex, the general
pattern is that they are expressed poorly on healthy cells but are upregulated on infected or tumor cells.

NK cells express multiple activating and inhibitory receptors that recognize MHC class I as well as many non-MHC class I ligands such as pathogen encoded molecules and self-induced ligands. It is ultimately the balance of signals that determines the functional outcome of the NK cell:target cell interaction. NK cells exhibit complex repertoires of receptors because there is random coexpression of many possible combinations of NK cell receptors. This process of variable receptor gene expression results in a repertoire of NK cells that can detect multiple changes in cells. This somewhat resembles T cell and B cell lymphocytes but NK cells differ from T and B cells since they can kill target cells without prior sensitization, their NK cell receptor loci do not require gene rearrangement and their repertoire is much smaller.

Although many of the activating/inhibitory receptors and their ligands have been discovered, the biological relevance of these molecules in host defense and the interactions between NK cells and other immune system cells is less well characterized. Through multiple pathways (cytokine secretion, direct killing, interaction with other cells), NK cells have been shown to be involved in combating viral infections (cytomegalovirus, sendai virus, influenza virus, HIV, and ebola virus), parasites (P. falciparum which causes malaria, P. berghei, and T. cruzi), and bacteria (Shigella flexneri, M. tuberculosis). These roles are reviewed by Lodoen, et al. NK cells also interact with DCs. They can reciprocally activate one-another by unknown receptor-ligand pairs and by cytokines (reviewed in). DCs secrete IL-2, IL-12, IL-18, and IFNα/β which induces cytotoxicity, proliferation, CD69 expression and IFNγ production in NK cells. NK cells, on the other hand, activate or induce maturation of DCs through cell-
contact and TNFα. One example of NK cell-DC cross talk is during MCMV infection. DCs induce NK cell expansion by release of IL-12 and IL-18 and through Ly49H. Also, the presence of Ly49H+ NK cells results in the maintenance of DCs in the spleen during acute MCMV infection.

Since NK cells play such an important role in the immune system, it is very important to understand all aspects of NK cell biology, including their lineage commitment and maturation pathways as well as factors that affect their final phenotype and function. In this study, we have identified a new pathway of NK cell lineage commitment that is thymus-dependent and gives rise to NK cells that reside in the thymus and LNs. This thymus-dependent pathway influences the NK cell phenotype and function. Therefore, not all NK cells develop via the BM pathway that is currently accepted in the literature as the sole location of NK cell development. We will therefore review the current understanding of NK cell precursors and their development in the BM as well as their maturation steps and factors involved in this process. T cell development and V(D)J recombination of T cell receptors (TCRs) will also be reviewed since this step of development occurs in the thymus-dependent pathway of NK cell development.

1.2. Hematopoietic lineage commitment

All blood cells develop from hematopoietic stem cells (HSCs). These cells are multipotent and are capable of self renewal. Fetal hematopoiesis occurs in the liver and it switches to the BM in the adult. The first step in hematopoiesis is typically described as the division into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). This
model of hematopoiesis assumes that cells consistently reach branch points in the same order and that the timing of the developmental choices is fixed such that when a cell reaches a branching point, it cannot progress further without choosing one branch or the other. It also assumes that the choice is binary so that if a cell makes a positive choice for the one branch, it automatically makes a negative choice for the other branch. If developmental choices are considered in terms of gene regulatory mechanisms though there is no longer a need for cells to encounter binary choices but rather cells pass through overlapping ‘windows of opportunity’ to give rise to certain cell types\textsuperscript{20}. Lineage opportunities remain open as long as the cells express essential enabling factors. By turning on or off other subsets of transcription factors, a precursor cell can switch its cell type to more than one other choice. The exact timing and order of the choices may not always be the same and one cell fate can be blocked without commitment to another. Since there is diversity in gene expression of progenitors within the same stage, the lineage decision of each cell can occur somewhat randomly due to the fluctuations in gene expression. The end point could be reached via more than one pathway\textsuperscript{20}. Oligonucleotide array analysis of HSCs, MPPs (multipotent progenitors), CLPs, and CMPs revealed that HSCs express non-hematopoietic genes as well as many hematopoietic genes in a lineage promiscuous manner\textsuperscript{21}. MPPs express both myeloid and lymphoid genes and CLPs express genes for T, B, and NK cells before they’re committed to a lineage. Interestingly, CLPs express the germline transcripts of many TCR and BCRs, including germline TCR\textgreek{y} and TCR\textgreek{b}. CLPs also express RAG-1\textsuperscript{21}. Lineage commitment is a result of inactivation of non-lineage specific genes and the retention or activation of enabling factors for the lineage. Therefore cells can express genes specific for a certain lineage before they are committed to that lineage and cells can express genes of other lineages even though they will differentiate.
into another lineage. This stresses the separation of the terms “specification” and “commitment”\textsuperscript{20}.

1.3. Commitment to the NK cell lineage

It is the common assumption that all NK cells develop in the BM of adult mice. NK cells and B cells are both dependent on the BM, because BM ablation by \textsuperscript{89}Sr\textsuperscript{22} or oestradiol\textsuperscript{23} results in NK cell deficiencies. This defect is also seen in osteopetrotic animals which have a defective and much smaller BM compartment due to an osteoclast deficiency\textsuperscript{24}. Commitment to the NK cell lineage requires progressive restriction of lineage potential from multipotent HSCs to oligopotent precursors to unipotent committed NK cell precursors which no longer have the ability to become any other cell type. Several oligopotent precursors have been described that retain NK cell potential (Fig.1.2). The two main populations in the adult BM are lymphocyte precursors which posses B cell, T cell, and NK cell potential. Early lymphoid precursors (ELPs) are Lin\textsuperscript{c-kit}\textsuperscript{−}Flt3L\textsuperscript{+} and common lymphoid progenitors (CLPs) are Lin\textsuperscript{c-kit}\textsuperscript{−}IL-7Ra\textsuperscript{+}. It is unclear whether these are obligate intermediates for NK cells to arise. In c-kit-deficient mice, that lack all CLPs, NKP and mature NK cell numbers are normal\textsuperscript{25,26}.

On the other hand, several studies suggest that NK cells do arise from CLPs or ELPs. Two studies using reporter genes for V(D)J recombinase\textsuperscript{27} and RAG1\textsuperscript{28} show that NK cells arise from progenitors which express RAG1 or V(D)J recombinase. One study showed that V(D)J recombinase is active at the CLP stage and its activity or evidence of its activity is detectable in B, T, NK, and DC lineages\textsuperscript{27}. This does not necessarily mean that NK cells or others arise only from CLPs because V(D)J recombinase could be active in other progenitors as well. The
recombinase expression at the CLP stage was shown to be controlled by the B cell specific enhancer, \textit{Erag} and approximately 5\% of NK cells do have IgH rearrangements\textsuperscript{27}. Therefore, perhaps this IgH\(^+\) portion does arise from CLPs in which the B cell specific \textit{Erag} enhancer controls recombination. In the other study, RAG1 expression was detectable in a progenitor upstream of the CLP stage, the ELP progenitors\textsuperscript{28}. RAG expression (or evidence of previous expression) was also detectable in mature NK cells in this study. This earlier activity in ELPs suggests that NK cells can arise from earlier progenitors as well as CLPs. Kouro, \textit{et al.}\textsuperscript{29} examined potentials in BM precursors. NK cells can develop from early Lin\(^\text{c-kit}\^{hi}\) cells (like ELPs) but also from more mature Lin\(^\text{c-kit}\^{lo}(\textit{Flt3L})\) cells (which include CLPs) (Fig.1.2). It seems that a significant proportion of BM-derived NK cells do arise from a Lin\(^\text{c-kit}\^{lo}\) subset, because when mice are treated with estrogen, the precursors that produce NK and B cells are diminished from the Lin\(^\text{c-kit}\^{lo}\) fraction. When the remaining Lin\(^\text{c-kit}\^{lo}\) cells are cultured, the B and NK cell numbers are reduced. Most of the progenitors for B and NK cells within the Lin\(^\text{c-kit}\^{lo}\) subset are in separate populations though, because upon single cell assays most wells produced either B cells (1 of 6-10) or NK cells (1 of 19-25) but a bipotent B/NK cell precursor was rarer (1 of 64-96). The Lin\(^\text{c-kit}\^{lo}\) population is heterogeneous with NK cell precursors and B cell precursors having substantial differences. For example, all B cell precursors are IL-7R\(\alpha\)\(^+\) while only half of NK cell precursors are. Also, NK cell precursors are more resistant to \(\beta\)-estradiol hormone treatment than B cells\textsuperscript{29}. A committed NK cell precursor (NKP) was isolated in the adult BM\textsuperscript{30}. These cells are Lin\(^\text{CD122}^{+}\text{NK1.1}^{+}\text{DX5}^{+}\). CD122 is IL-2/IL-15R\(\beta\) and this is used by two cytokines, IL-2 and IL-15\textsuperscript{31}, both dependent on signaling through a third subunit, known as common \(\gamma\) chain (\(\gamma c\))\textsuperscript{32}. These cells lack mature NK cell markers and are not functionally developed. NKPs produce mature NK cells via an in vitro culture system and they lack all other cell potential.
Figure 1.2. Progenitors with NK cell lineage potential. Multiple progenitors in the bone marrow and thymus have been shown to possess NK cell lineage potential.

NK cells may share a closer developmental relationship with T cells in fetal mice and B cells in adult mice. Numerous studies have shown that fetal T cell precursors possess NK lineage potential (Fig.1.3). A bipotent T/NK cell precursor (TNKP) is present in the fetal liver, spleen, and blood. The earliest TNKP is B220lo c-kit+ CD19- and is found in the fetal liver (FL)33. They reconstitute the T and NK cell compartments upon transplantation into mice lacking T, B and NK cells. These cells have been shown to be truly bipotent, because a single cell gives rise to both T and NK cells in a fetal thymic organ culture (FTOC). This bipotent population represents 70% of the cells that seed the thymus33. The earliest prethymic T cell progenitors in the fetal blood34 and the first cells to colonize the fetal thymus35 both have T, NK, and dendritic cell potential. Of 40 cells examined from the fetal thymic anlage, 7 cells gave rise to
T cells and all of these 7 cells also gave rise to NK cells. Four of these cells produced dendritic cells as well. Subpopulations of immature CD4⁺CD8⁻ (DN) thymocytes in adult mice also possess NK cell potential. NK cell potential of DN1 thymocytes was also shown by Balciunaite et al. Adult DN1 c-kit⁺ cells, as well as DN2 cells, upon culturing on OP9 cells developed into functional NK cells. Limiting dilution analysis showed that 1 in 15 DN1 cells and 1 in 7 DN2 cells developed into NK cells. Taken together, these studies suggest a link between T cells and NK cells. This notion has further been supported by a transgenic mouse model of human CD3γ which displays a block in development of NK and T cells but not B cells. Mice transgenic for FceRIγ exhibit a similar phenotype.

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<th>Fetal liver</th>
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<th>Fetal blood</th>
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<td>TNKP</td>
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<td>B220⁺</td>
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<td>CD19⁻</td>
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<td>IL-7R⁺</td>
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**Figure 1.3. Bipotent T/NK cell progenitors.** TNKP cells are present in the fetal liver, blood, and thymus. Figure is revised from that designed by Tabatabaei.

### 1.4. NK cell development

Much about the NK cell commitment pathway was first determined from in vitro cultures. Nearly a decade ago, an in vitro culture system was used to demonstrate that there are two major stages in NK cell development. The first stage involves acquisition of CD122, which marks commitment to the NK cell lineage and allows the cells to become responsive to IL-15 which is crucial for the second stage of NK cell maturation. When Williams et al. cultured multipotent progenitors with IL-15 alone there was no cell expansion whereas when the cells
were cultured in IL-6, IL-7, SCF, and flt3L substantial expansion occurred but little to no NK cells were produced. But if these cells were first cultured in the cytokine cocktail (where they acquired CD122 and became IL-15-responsive) and then switched to a culture with IL-15 alone, cells expanded well and almost all became NK1.1+ and had lytic capabilities. There is also a critical requirement for stromal cells in NK cell development. Cells cannot express Ly49 receptors without stroma cells in the culture\textsuperscript{41}. 

1.4.1. Stages of NK cell Development: from NKP to the mature NK cell

Recently Kim \textit{et al.}\textsuperscript{10} defined a developmental model of NK cell maturation that divides their in vivo development in the BM into five stages (Fig. 1.4). These stages are defined by the cells' functional characteristics and expression of molecules which may not provide specific functional significance but work for defining specific stages of maturity in NK cells. Developmental stage I represent the NKPs which are defined as CD122\textsuperscript{−}NK1.1\textsuperscript{−}DX5\textsuperscript{−}.

Vossenrich \textit{et al.}\textsuperscript{42} have further characterized this population and have shown that a subset of NKPs express CD127 (IL-7Rα), IL-21R, CD117(c-Kit), and CD135 (Flk2), but are CD25 (IL-2Rα) negative. These markers are characteristic of early hematopoietic precursors but CD122 is not. In addition, they found that NKPs express NKG2D, while earlier studies have seen this at stage II. Stage II of development involves acquisition of the pan-NK cell markers; high NK1.1 expression and low DX5 expression. The cells also express their first functional receptors, CD94/NKG2 and, as reported by Kim \textit{et al.}\textsuperscript{10}, NKG2D. This stage of development resembles mature fetal/neonatal NK cells which do not express Ly49 receptors. Stage II cells also express the integrin α\textgamma\textsuperscript{+} and are Mac-1\textsuperscript{−}CD43\textsuperscript{−}c-kit\textsuperscript{−}. The profile of Stage III NK cells is similar to that of the previous stage except that they are now c-kit\textsuperscript{+} and they begin to acquire
the Ly49 molecules. DX5 is upregulated in stage IV and the NK cells undergo a substantial proliferation in the bone marrow. At stage V, NK cells complete their maturation by highly expressing Mac-1 and CD43, and proliferation is slowed until NK cells are stimulated by pathogens\textsuperscript{10}. These mature NK cells have full functional capabilities as well. The NK cell maturation status varies in the peripheral tissues. Mature NK cells in the periphery express DX5 and CD11b and lack CD51 and CD117. Expression of KLRG1, a killer cell lectin-like receptor, is restricted to these most mature cells. Therefore, DX5\textsuperscript{+}CD11b\textsuperscript{+}KLRG1\textsuperscript{+}CD51\textsuperscript{−}CD117\textsuperscript{−}NK cell population is the most mature phenotype in the periphery\textsuperscript{43}.

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**Figure 1.4. Stages of NK cell maturation.** NK cells proceed through multiple stages following commitment to the NK cell lineage until they are fully mature. Each stage is marked by changes in receptor expression, proliferation and functional capabilities. Figure is from Veinotte et al\textsuperscript{8}. 

<table>
<thead>
<tr>
<th>Stage I</th>
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<tr>
<td>NKP</td>
<td>MHC class I receptor acquisition</td>
<td>Expansion</td>
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<td>CD122\textsuperscript{+} NK1.1\textsuperscript{+} DX5\textsuperscript{hi} NKG2 &amp; NKG2 &amp; Mac-1\textsuperscript{lo} &amp; α\textsubscript{v}\textsuperscript{lo} &amp; CD43\textsuperscript{lo} &amp; c-kit\textsuperscript{+}</td>
<td>CD122\textsuperscript{+} NK1.1\textsuperscript{+} DX5\textsuperscript{hi} NKG2 &amp; NKG2 &amp; Mac-1\textsuperscript{hi} &amp; CD43\textsuperscript{hi} &amp; c-kit\textsuperscript{+} &amp; Ly49\textsuperscript{+}</td>
<td>CD122\textsuperscript{+} NK1.1\textsuperscript{+} DX5\textsuperscript{hi} NKG2 &amp; NKG2 &amp; Mac-1\textsuperscript{hi} &amp; CD43\textsuperscript{hi} &amp; c-kit\textsuperscript{+} &amp; Ly49\textsuperscript{+} &amp; Cytotoxicity\textsuperscript{lo} &amp; IFNγ\textsuperscript{lo}</td>
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Mature NK cells express a full repertoire of receptors and have the functional capacity to kill target cells and produce cytokines. NK cells are functionally mature when they can distinguish between self-MHC class I+ and self class I- (expressing allogeneic class I or no class I) cells, rather than just when the cells acquire cytotoxicity potential. Mature NK cells are relatively static in terms of self renewal and proliferative capacity, except after pathogen infection.

1.4.2. Factors involved in NKP production: Cytokines

To delineate the crucial cytokines for NK cell commitment, Williams et al.\textsuperscript{41} cultured multipotent progenitors with various combinations of IL-7, SCF, and Flt3L. IL-7 and Flt3L combination yielded the highest proportion of NKP. Although addition of SCF did not change NKP numbers, it did increase NK cell yield. Therefore, IL-7 and Flt3L expand or induce NKP, and SCF has an additive effect to the overall NK cell numbers. The IL-7Ra chain is expressed by some NKP\textsuperscript{30} and splenic NK cells are reduced 3-fold in IL-7-/- mice\textsuperscript{44}, whereas normal numbers of NK cells are produced in IL-7Ra +/- mice\textsuperscript{45}. Since IL-7 only minimally affects NK cell development, Williams et al.\textsuperscript{41} further concentrated on Flt3L and its role in NK cell development. Flt3L+ progenitors produce more NK cells than Flt3L- progenitors. It seems that Flt3L either induces NKP or more likely expands the NKP population, because NKP cell numbers are only slightly reduced in Flt3L-/- mice\textsuperscript{46}. Flt3L-/- mice have a 5.3-fold reduction of mature NK cells and a loss of cytotoxicity against YAC-1\textsuperscript{47}. As for a role for SCF, when c-kit-/- (receptor for SCF) progenitors were transplanted into RAG2/-/- mice, they produced NK cells at lower levels; absolute cell numbers were 40% of that of wild type progenitors. Their lytic capacity was greatly reduced\textsuperscript{48}. NKP cell numbers are not affected in c-kit-/- mice though so it appears that it is important for survival and
proliferation of committed NK cells\textsuperscript{25, 26}. Individually, these pathways are not essential for the first step of NK cell lineage commitment but there may be redundancy in the system and analysis of the double-mutant mice would be informative.

Even though early studies suggested that signaling via the common \(\gamma\) chain (\(\gamma c\)) is essential for NK cell commitment since defects in the gene coding for \(\gamma c\) (\(il2rg\)) cause an X-linked severe combined immune deficiency characterized by several immune defects, including an absence of mature NK cells and IL15\textsuperscript{−/−} mice have reduced NK cell numbers\textsuperscript{49–52}, further investigations showed that the commitment to the NK lineage does not occur through \(\gamma c\)-dependent cytokine stimulation\textsuperscript{42}, indicating that factors other than early-acting cytokines or \(\gamma c\)-cytokines lead to NK cell lineage commitment. Recent studies indicates the expression NKG2D at the NKP cell stage\textsuperscript{42} but it has yet to be clarified whether it plays a role in restriction of the progenitors to the NK cell lineage.

1.4.3. Factors involved in NKP production: Transcription factors

There are some factors that indirectly affect the production of NKP cells probably by affecting earlier upstream progenitors. One example is Ikaros, a zinc-finger DNA binding protein that is present in HSCs and is up-regulated during lymphocyte differentiation. An Ikaros null mutation in mice causes a severe defect in the lymphoid compartment including NK cells\textsuperscript{53}. Analyses of BM hematopoietic progenitors from Ikaros mutant mice revealed very low expression of Flt3 and c-Kit receptors in these progenitors\textsuperscript{54}, which suggested that Ikaros might regulate ELP/CLP homeostasis through growth factor receptors, thereby affecting the development of all lymphoid lineages including NKPs. Another factor is PU.1, an Ets family
transcription factor, which is highly expressed in most hematopoietic cells and regulates the development of myeloid and lymphoid lineages. PU.1 and GATA1 have a mutually antagonistic relationship because they bind each other in the cytoplasm. This causes them to lose their translocation and binding ability respectively. The levels of these two transcription factors mediate the decision between lymphoid and myeloid lineages. High PU.1 induces myeloid genes and high GATA-1 promotes erythroid and megakaryocyte genes. At low PU.1 levels, lymphocyte lineages are permitted. This is likely because only at low levels can PU.1 induce IL-7Rα expression which promotes survival and proliferation of T and B cells. PU.1 knockouts do show defects in NK cells, but they are less severe than T and B cell defects, and this is likely due to NK cells' reduced dependency on IL-7. When transferred to Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice, PU.1-deficient fetal liver cells generate normal numbers of HSCs, but produce reduced numbers of NKPs and NK cells. Although the NKP cell numbers were affected, the phenotype of mature NK cells was not substantially different but the deficiency does affect cell expansion and homeostasis.

Ets-1, a winged helix-turn-helix transcription factor is essential for development of NK cells. In Ets-1<sup>−/−</sup> mice, T and B cells develop normally but the number of DX5<sup>+</sup>CD3<sup>−</sup> splenic NK cells and their cytolytic activity is remarkably reduced. The defects in Ets-1<sup>−/−</sup> NK cells are intrinsic to NK precursors. One mechanism by which Ets-1 controls NK cell development might be through survival, as the increased apoptosis has been reported in B- and T-cell populations from Ets-1<sup>−/−</sup> mice.

A class of bHLH proteins known as inhibitors of DNA binding (Id) proteins are key molecules for NK cell development. They negatively regulate E proteins that are crucial for B and T cell
lineages. Overexpression of Id3 in human CD34+ HSCs has been shown to block T cell
development and promotes NK cell development in fetal thymic organ cultures. Development
of NK cells is also accelerated when both Id2 and Id3 are constitutively expressed in CD34+
HSCs, whereas the generation of T, B, and lymphoid-DCs is completely abrogated. These
observations were further complemented by the finding that targeted disruption of Id2 gene
results in a selective block in NK cell development, accompanied by a lack of NKPs. These
data present a model in which Id proteins promote commitment of common lymphoid
precursors to NKPs while inhibiting their option to develop to committed T- and B-
progenitors.

1.5. NK cell maturation

1.5.1. MHC Class I Receptor acquisition

The MHC-class I receptor acquisition on NK cells is complex. Although the numbers of Ly49
and CD94/NKG2 receptors is quite low in comparison to T and B cell receptor diversity, the
repertoire of the NK cell population, as a whole, is very diverse. Each Ly49 or CD94/NKG2
receptor family member is expressed on a subset of NK cells. Therefore, their expression
partially overlaps with the expression of the other receptors. NK cells express at least one
MHC I-specific receptor and individual NK cells can coexpress up to five different Ly49
receptors in addition to CD94/NKG2 receptors. Expression of the receptors is not co-
regulated and it appears that they are expressed with a great degree of independence from each
other.
The receptor expression on developing NK cells differs between the fetal and neonatal mouse and the adult mouse. Most fetal and neonatal NK cells express CD94/NKG2 but not Ly49 receptors, with the exception of Ly49E, which is expressed in the fetal cells but not the adult cells\textsuperscript{65}. As the mouse matures, NK cells begin to acquire Ly49 receptors, while CD94/NKG2\textsuperscript{+} NK cells decline. The frequency of NK cells expressing high levels of CD94/NKG2 decreases from \textasciitilde90\% on neonatal NK cells to \textasciitilde50\% on adult NK cells\textsuperscript{66}. This decrease in CD94/NKG2 expression coincides with the increase in the frequencies of NK cells expressing Ly49. Ly49 receptor expression begins very gradually, starting at approximately 1 week after birth and reaching adult levels at 6 to 8 weeks of life\textsuperscript{67}. The decrease of CD94/NKG2 is not due to a downregulation in cells but rather due to a generation of CD94/NKG2\textsuperscript{+}Ly49\textsuperscript{+} NK cells in young mice\textsuperscript{67}.

NK cells appear to undergo a process of ensuring self tolerance during development. Since many normal self cells express ligands for NK cells, it is important that NK cells express self MHC class I inhibitory receptors to ensure that autoreactivity does not occur. Evidence shows that NK cells that lack inhibitory receptors for self MHC class I are hyporesponsive. Two main theories exist on how this self tolerance is acquired by NK cells during development (reviewed in \textsuperscript{7}). The first is the arming model. In this model, NK cells receive positive signals if their Ly49 receptors bind self MHC class I. This positive signal ‘arms’ the NK cells and allows them to mature into functional NK cells while those that do not express a self Ly49 remain unarmed and unresponsive. The second model is the disarming model. In this model NK cells express a variety of stimulatory and inhibitory signals and interact with self cells. If the NK cell binds both activating ligands and inhibitory ligands (the self MHC class I) no net stimulation occurs and the responsiveness of the NK cell is maintained. Conversely, if an NK
cell without a self Ly49 binds a self cell, it will only receive activating signals and hyporesponsiveness will be induced in this cell. In other words, the cell is ‘disarmed’.

1.5.2. Factors involved in NK cell maturation

NK cell maturation requires cell-to-cell interactions and soluble factors derived from BM stromal cells. In vitro culture systems that are designed to give rise to mature NK cells, as described earlier, are not complete without stromal cells in the culture. Multipotent HSCs can be cultured with certain cytokines to produce CD122+ NKPs but without addition of IL-15 and stromal cells in a second stage of NK cell culture, the cells will not fully mature. If IL-15 is added without stromal cells, the cells become functionally mature and express CD94/NKG2 but not Ly-49. This shows the importance of cell-cell interactions between developing NK cells and stromal cells.

IL-15 is the critical cytokine, or the “essential fuel” for NK cell development. IL-15 works by binding the IL-15R complex, which is made up of IL-2/IL-15Rβ (CD122), IL-15Rα, and the γc but it appears that there may be other methods in which IL-15 signaling occurs. There is an NK cell deficiency, as well as deficiencies in NKT cells, intestinal epithelial cells, and memory CD8+ T cells, in IL-15 and IL-15Rα-deficient mice. Disrupting γc, the third subunit of IL-15R, generates a similar defect in NK cell production, suggesting an essential role for IL-15, overall, in NK cell development. In an effort to identify the developmental stage at which IL-15 and other γc cytokines are important for NK cell development, Vossehenrich et al. produced various γc deficient mice (IL-2, IL-4, IL-7, IL-15) including multiple γc deficiencies per mouse (i.e. IL-2γcIL-7γc, IL-2γcIL-4γcIL-7γc, and IL-4γcIL-7γcIL-15γc) on a Rag2-deficient
background. They were able to show that NKPs do not require γc cytokines because they are present in normal numbers and IL-2, IL-4, and IL-7 have no role in the generation of mature NK cells. Conversely, there is an essential singular role for IL-15 in the generation of immature and mature NK cells in the BM and mature NK cells in the spleen. The NK cells that do remain in IL-15-Rag2 deficient mice have an immature phenotype of Mac-1loCD43+. The cells were also CD94, NKG2A/C/E and Ly49D+ but Ly49G2 and Ly49C/I NK cell numbers were reduced. The cells could kill YAC-1 and produce IFNγ upon stimulation but at reduced levels42. One clue to IL-15 signaling function is the antiapoptotic factor Bcl-2. Transgenic overexpression of Bcl-2 in IL-2Rβ-deficient mice leads to normal NK cell numbers71. It also allows peripheral NK cells to persist in IL-15 deficient hosts after adoptive transfer2,72. A recent study has isolated a downstream target of IL-15 in human NK cells and therefore provides another new clue to its function73. Because IL-2 and IL-15 signal through common receptor subunits, it is likely that they regulate a shared set of downstream target genes. They showed that IL-2 and IL-15 stimulation results in the post-transcriptional increase in Ets-1 protein. The exact role for Ets-1 in NK cell function and the particular target genes controlled by Ets-1 are still unknown73. These studies still have not revealed the exact role of IL-15 in NK cell development and many possibilities exist. To summarize, IL-15 may promote NK cell development by indirectly controlling the responsiveness of maturing NK cells to other growth and survival factors. It may also act as a survival factor for NK cells at certain developmental stages or it may act to support proliferation of developing NK cells. Also, IL-15 may have an indirect effect on NK cells by regulating their susceptibility to the IL-21 maturation factor42.

Factors that regulate the IL-15 signaling pathway and its effects on NK cells have been identified. These include lymphotoxin and IRF-1. The lymphotoxin LTα1β2 is expressed on NK
cells and it binds the LTβ receptor on non-lymphoid cells. A reduction of NK cell numbers is seen in mice lacking the LTα on NK cells or LTβR on stromal cells\textsuperscript{74, 75}. The impaired NK cell development is due to the absence of LTβR triggered signals in the stromal cells. FL cells from wild type mice cannot develop into NK cells when grown in vitro on LTβR deficient stromal cells. If the reverse culture is performed, LTα-deficient FL cells can differentiate into NK cells when grown on wild type stroma\textsuperscript{76}. Lian \textit{et al.}\textsuperscript{77} recently suggested that this impaired NK cell development is a result of impaired IL-15 production in the stromal cells. The signals derived from LT and LTβR interaction result in the up-regulation of IL-15 production by the BM stroma\textsuperscript{77}. IRF-1 deficient mice also have severely reduced numbers of NK cells due to a defect in the production of IL-15 by the bone marrow microenvironment\textsuperscript{78, 79}.

A very recent study has made a breakthrough in determining factors required for Ly49 receptor expression and NK cell maturation\textsuperscript{80}. Tyro3 receptors' (Axl, Tyro3, Mertk) expression on committed NK cell precursors is crucial for both the acquisition of nearly all inhibitory and activating receptors on NK precursors in the BM and for the functional maturation of these cells in the spleen. NK cells express all three receptors and BM stroma expresses their two ligands (Gas6 and protein S)\textsuperscript{80}. They seem to be required for progression from the immature NK cell stage because NK cells in Tyro3-deficient mice have high NKG2 and CD94 expression and low Ly49, DX5, NKG2D, Mac-1, CD43 expression. Their cytotoxicity is substantially decreased and the cells cannot produce IFNγ either. The Tyro3 receptors seem to act through an uncharacterized pathway because they do not alter expression of known factors that play roles in NK cell development\textsuperscript{80}. 

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Other factors, besides those that play a role in the BM stroma signals, are important for NK cell maturation and/or effector function. For example, VDUP1 (Vitamin D3 upregulated protein 1)" mice show a profound reduction in the number of mature NK cells in the spleen and BM. Expression of CD122 (the marker of NKPs) was reduced and VDUP1 expression is known to be induced from the NKP stage. Therefore VDUP1 may be important for the differentiation of NKPs or for their maturation because upregulated VDUP1 may induce cell cycle arrest, which is necessary for the onset of differentiation. The GATA-3 transcription factor may also play an important role in the transition from the immature Mac-1<sup>lo</sup>CD43<sup>lo</sup> stage to the Mac-1<sup>hi</sup>CD43<sup>hi</sup> stage. Peripheral NK cells that develop in GATA-3<sup>−/−</sup> mice resemble immature Mac-1<sup>lo</sup>CD43<sup>lo</sup> NK cells. A third factor that controls NK cell maturation is NF-κB. The NF-κB family members normally are kept in an inactive state by inhibitors but if these inhibitors are degraded the NF-κB transcription factors become active. When the NF-κB transcription factor family members are hyperactivated, NK cell maturation is arrested and the residual NK cells in the BM, spleen, and liver are immature (Mac-1<sup>lo</sup>CD43<sup>lo</sup>). NK cell cytotoxicity is not affected but there is reduced capacity for IFN-γ production. The defect in maturation is likely a result of NK cells being unable to provide adequate proliferative signals. The cells express normal levels of IL-2Rβ and γ<sub>c</sub> but that they could not proliferate in the presence of IL-2 or IL-15. The molecular mechanism for this defect is unclear.

T-bet is a transcription factor that regulates NK cell maturation and effector functions (cytokine production and to a lesser extent, cytotoxicity). In T-bet deficient mice there is a reduction in NK cell number, specifically in cells with late maturation markers. The perforin and granzyme B genes are T-bet target genes even though T-bet only plays a minor role in controlling cytotoxicity. There may be a compensatory relationship with T-bet. EOMES,
which is also a T-box family transcription factor, is highly expressed in NK cells (even in T-bet^−/− mice) and it regulates IFNγ, perforin, and granzyme expression. It will be informative to study these transcription factors’ roles in EOMES^−/− T-bet^−/− mice. GATA3 participates with T-bet in NK cell function as well. There is an intrinsic defect in IFN-γ production in GATA-3^−/− NK cells. In these mice, T-bet expression was 4-fold reduced while Hlx (another transcription factor that controls IFNγ production) expression was 10-fold reduced. T-bet establishes an active chromatin configuration of the IFN-γ locus and it cooperates with Hlx, which is broadly expressed in multiple hematopoietic cell lineages, including NK cells, to upregulate IFN-γ expression. The combined deficiency in T-bet and Hlx expression may explain the poor inducible IFN-γ production in GATA3^−/− mice.

A few other factors that are critical for NK cell effector function rather than maturation are MEF-1, C/EBPγ, MITF and NEMO. A member of the ETS-family, MEF-1, is required for NK and NKT cell development as a profound reduction of these cells is observed in MEF-deficient mice. The few NK cells found in these mice are functionally impaired because MEF regulates transcription of the perforin gene. MITF-deficient mice have a cytotoxicity defect due to decreased perforin expression while NEMO-deficient NK cells have a defect in their NF-κB signaling pathway which is important in the regulation of perforin and other cytotoxic factors. The basic leucine zipper transcription factor, C/EBPγ, is also important for proper NK cell cytotoxicity and IFNγ production but not for earlier NK cell development. C/EBPγ expression is ubiquitous and constitutive and while it does not have a transcription activating domain, it can interact with other transcription factors and augment their DNA binding. In C/EBPγ^−/− mice, NK cell cytotoxic activity and IFNγ production are impaired.
A cytokine that plays a late role in NK maturation is IL-21. IL-21 induces further differentiation of activated, mature NK cells and is an initiator of IFN-γ production as well. The terminal differentiation of mouse NK cells leads to an increase in cell size and granularity, loss of NK1.1 expression, and upregulation of the NKG2-CD94 complex. It also leads to a significant increase in cytotoxicity and a massive induction of cytokine secretion. This late function of IL-21 is important for activated NK cells because it enhances IFN-γ production more than IL-15 alone.

In conclusion, the precise pathway of NK cell lineage commitment and subsequent maturation is still unclear. The stages of NK cell development are not yet fully characterized but we are reaching a point now where an accepted model of NK cell development is present and being built upon. Future studies will contribute to this model by characterizing more cell surface markers and molecular pathways which contribute to either the commitment or the maturation of NK cells.

Table 1.1. Genetic mutations affecting the maturation of NK cells. Revised from DiSanto.

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<thead>
<tr>
<th>Transcription factor</th>
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<th>Repertoire</th>
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<th>Cytotoxicity</th>
<th>Cytokines</th>
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<td>NKP</td>
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<td>Normal</td>
<td>Late mature NK</td>
<td>Reduced</td>
<td>Normal</td>
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1.6. T cells and development

The T cell population is able to mount a response to virtually any foreign antigen because each T cell expresses a unique variant of heterodimeric receptors (αβ or γδ)\textsuperscript{95}. These T cell receptors (TCRs) are formed from rearrangement and co-expression of either α and β genes or γ and δ genes. αβ T cells are part of the adaptive immune system. They localize primarily in secondary lymphoid organs and respond to infection by facilitating the production of antibodies and by lysing target cells. αβ T cells recognize peptide ligands presented by class I and II MHC molecules. γδ T cells participate in the early immune response, similar to the innate immune system\textsuperscript{96}. They comprise only a minor population in the blood (1-5%) but in epithelial tissues, they represent 50% of the T cells. They recognize a much wider variety of antigens such as nonclassical MHC molecules, heat shock proteins, and lipids\textsuperscript{96}. The structure and signaling potential of the γδTCR complex differs slightly from the αβTCR complex. Both complexes include invariant accessory chains such as the CD3 proteins. While in the αβTCR complex there is one heterodimer of CD3ε and δ and another heterodimer of CD3ε and γ, the γδTCR complex lacks CD3δ and has two heterodimers of CD3ε and γ\textsuperscript{97}. The signal transduction by the γδTCR complex is superior to that of the αβTCR complex\textsuperscript{97}.

T cell development occurs in the thymus and T cell precursors migrate from the fetal liver and the adult BM to continuously seed the thymus. The first half of T cell development is independent of the TCR while the second half is TCR dependent (Fig. 1.5). T cell development can be defined by a series of stages (reviewed by Janeway\textsuperscript{95}). The initial stages are traditionally termed double negative since they do not express CD4 or CD8 but they are also referred to as triple negative to include CD3 as well. The initial precursor stage is termed
double negative 1 (DN1) and the cells are CD44+CD25-. The DN2 stage is marked by gain of CD25+ and the initiation of TCR gene rearrangement. This rearrangement continues in CD44+CD25+ cells at the DN3 stage. T cell development then becomes TCR dependent and cells die if they fail to generate a productive in-frame TCRβ chain or a pair of TCRγ and TCRδ chains. Because the joining events of V(D)J recombination are imprecise, two out of three attempts are non-productive and fail to maintain the translational reading frame of the TCR subunit. Cells that do successfully rearrange and express a productive TCR chain(s) on their surface will then continue differentiation to either the γδ lineage or the αβ lineage. How the T cell progenitors decide to follow the γδ T cell lineage versus the αβ T cell lineage is not yet known. One model suggests that it depends on the strength of the signal. If a weak signal is received from either a pre-TCR (the TCRβ chain is expressed on the surface at this stage with an invariant p1α chain) or a γδ TCR that has not encountered ligand, the cells will become αβ T cells. Conversely, if a stronger signal is received from a γδ TCR that has encountered a ligand of intermediate affinity, cells will become γδ T cells. Finally, if a very strong signal is received from a γδ TCR and ligand, the cells will die. αβ T cells continue their development by undergoing proliferation and differentiate into CD4+CD8+ double positive (DP) αβ T cells. These αβ T cells then rearrange their TCRα genes and undergo positive and negative selection for self/nonself discrimination. TCRs must be able to recognize MHC class I or II on cortical thymic epithelial cells to pass positive selection and become CD8 SP cells or CD4 SP cell respectively. If SP cells recognize a self antigen presented by BM DCs or macrophages in the thymus, they are killed by negative selection to ensure that self-reactive cells do not leave the thymus. After selection and maturation, T cells finally exit the thymus to take up their roles in the immune system.
Natural killer T (NKT) cells are a conserved T cell sublineage with unique properties (reviewed by Kronenberg\textsuperscript{99}). The main subpopulation of NKT cells are invariant NKT cells, which likely arise from DP T cells during development in the thymus (Fig. 1.5). These cells express invariant TCRs and they recognize a synthetic glycolipid presented by an MHC class I-like molecule, CD1d. Upon stimulation, NKT cells rapidly produce many cytokines and can influence diverse immune responses. Another subset of NKT cells is CD1d independent and their developmental pathway is not defined.

Figure 1.5. Stages of T cell development. The initial stages are TCR independent and involve V(D)J recombination of TCR\(\gamma\), \(\delta\), and \(\beta\) genes as well as loss of alternate cell potential and final commitment to the T cell lineage at the DN3 stage. The remainder of the stages are TCR dependent and result in mature single positive CD4 and CD8 T cells.

Two groups characterized the kinetics and timing of TCR recombination during T cell development\textsuperscript{100,101}. TCR\(\gamma\), \(\delta\), and \(\beta\) recombination occurs before TCR\(\alpha\). TCR rearrangements
at the DN1 stage are negligible and rearrangements that were detected are likely contamination. At the DN2 stage, both TCRγ and TCRδ rearrangements are present while TCRβ rearrangements are almost absent. Specifically for TCRγ, Vγ2-Jγ1 rearrangements were more abundant than Vγ5-Jγ1 and by the DN3 stage, the level of Vγ2-Jγ1 rearrangements were within range of mature γδ T cells while Vγ5-Jγ1 levels were still lower. For TCRδ, Capone et al.\textsuperscript{100} detected Vδ5-Jδ1 and Vδ4-Jδ1 rearrangements at DN2 stage but Vδ5 rearrangements are more prominent at this stage. Livak et al.\textsuperscript{101} saw that DN2 cells have substantial amounts of partial Dδ1 and Dδ2 to Jδ1 rearrangements but not Vδ-DJδ and that by DN3 75-100% have completed V-DJδ rearrangements where only a minority of cells have full V-DJβ rearrangements. All major γ and δ genes are rearranged at DN3 or DN4 at levels similar to total thymocytes. Therefore, the recombination of the majority of TCRγ and TCRδ loci is completed by DN3 but completion of V-DJβ rearrangement to maximal levels is not seen until more advanced stages. Also, rearrangement of the TCRγ and TCRδ loci occurs in most αβ T cells\textsuperscript{102-107} and productive TCRβ rearrangements can be found in γδ T cells\textsuperscript{104,108-111}.

1.7. TCR rearrangement: V(D)J recombination

The ability of T cells to respond to a vast array of antigens is dependent on the generation of unique surface receptors with diverse binding specificities. The TCRs are assembled during lymphocyte development from germline variable (V), diversity (D), and joining (J) gene segments by the process of V(D)J recombination (Fig. 1. 6) (reviewed by \textsuperscript{112}). The segments are flanked by recombination signal sequences (RSSs). These conserved noncoding sequences guide the DNA rearrangements. The RSS includes a heptamer sequence which is always
contiguous with the coding sequence. Next is a nonconserved spacer region that is either 12 or 23 bases long, which is followed by a second conserved nonamer sequence. Therefore the heptamer-spacer-nonamer sequence motif makes up the RSS and it is always directly adjacent to the coding sequence of the V, D, or J gene segment. Normally, a 12-RSS is joined with a 23-RSS. The complex of enzymes that carries out recombination is collectively called V(D)J recombinase. This contains lymphocyte specific enzymes, RAG-1 and RAG-2, and ubiquitously expressed DNA-modifying proteins. Two RAG complexes recognize and bind two RSS sequences. The RAG complexes then bind each other and therefore bring together the two gene segments to be joined. The RAG complexes each introduce a single strand nick precisely between the RSS and the coding sequence. This leaves a free 3’ OH group which attacks the phosphodiester bond on the other strand, creating a hairpin at the end of the gene coding segment. This process simultaneously creates a double-stranded break at the ends of the heptamer sequences. The two non-coding RSSs are joined in a precise head to head linkage to form a signal joint. The coding joint formation involves extra steps. The RAG complex remains bound to the hairpin structures and proteins in the complex cleave the hairpins at random. The DNA repair enzymes in the complex may remove some nucleotides while the terminal deoxynucleotidyl transferase (TdT) enzyme adds nucleotides randomly. Finally, DNA ligase IV joins the ends together and repair enzymes trim off non-matching bases and synthesize complementary bases to fill in the remaining single stranded DNA. Since the number of nucleotides added by TdT is random, the added nucleotides often disrupt the reading frame of the coding sequence. This produces a nonproductive rearrangement, which occurs in 2/3 of rearrangements, as mentioned above.
Figure 1.6. The process of V(D)J recombination. 1. Two RAG complexes recognize and bind two RSS sequences, one on the V segment and one on the J segment. 2. The RAG complexes then bind each other and therefore bring together the two gene segments to be joined. 3. The RAG complexes cleave the DNA to create hairpin ends on the V and J segments. Other proteins bind the hairpins and the cleaved RSS ends of the gene coding segment. 4. The DNA hairpins are cleaved at random. Additional bases are added (TdT) or subtracted (exonuclease) to create imprecise ends. 5. DNA ligase IV joins the ends of the segments to form the coding joint. It also joins the RSSs to form a signal joint.

The process of V(D)J recombination is regulated by the availability of the recombination machinery and also by accessibility of the target gene segments. Multiple epigenetic mechanisms are involved in regulating rearrangements including: histone acetylation, DNA methylation, allelic exclusion, nuclear location, and cis- and trans- acting factors.

Specifically for TCRγ recombination, IL-7 regulates chromatin accessibility for germline expression and RAG enzymes via histone acetylation which opens the chromatin structure.

1.8. TCRγ locus and rearrangement patterns

The TCRγ locus in mice features four clusters of Vγ, Jγ, and Cγ regions containing 7 Vγ segments, 4 Jγ segments and Cγ regions (Fig. 2.1). Each cluster contains one C region,
one J segment and one to four V segments. The V segments rearrange to the J segment in the same cluster. Cluster 3 has been deleted in most TCRγ haplotypes and is believed to be non-functional in some strains$^{102,118}$. The Cγ1 cluster contains four closely linked but distantly related V region genes.

The utilization of Vγ genes is developmentally regulated such that Vγ3 and Vγ4 gene rearrangements are prominent in the fetal thymus but are very rare in the adult while Vγ2 and Vγ5 rearrangements have the opposite pattern$^{103,119,120}$. This rearrangement pattern is reflected in the γδ T cells present in the mouse. Vγ3$^+$ γδ T cells appear first at approximately embryonic day 13 and disappear from the thymus by embryonic day 18$^{121}$. Vγ4$^+$ γδ T cells are also seen in the fetal mouse$^{122}$. Interestingly, the cells expressing different Vγ rearrangements have specific functions as well. Vγ3$^+$ γδ T cells home to epidermal epithelial tissues and are called dendritic epidermal T cells (DECs)$^{123}$. These cells are unique in that they secrete keratinocyte growth factor$^{124}$. Vγ4$^+$ γδ T cells, on the other hand, concentrate in the female reproductive tract and tongue. These cells also have a fixed TCRδ chain and therefore, the population only has one TCR specificity. These cells likely recognize common stress-induced self antigens and eliminate damaged cells as well as promote epithelial growth and differentiation$^{123}$. In the adult environment, γδ T cells rearrange Vγ2 and Vγ5 chains but they are not as limited with TCR specificity since they can pair to different TCRδ chains, to some extent, and they also have V(D)J junctional diversity, which is lacking in the fetal cells.

Therefore, these cells likely recognize foreign antigens$^{125}$. 
It appears that developmentally regulated Vγ gene recombination is an intrinsic genetically programmed process. In models where the TCRγ locus contains frame shift mutations that prevent functional expression, the Vγ genes are still rearranged at the appropriate times even though they cannot influence the fate of the cells. For the fetal TCR genes (Vγ3 and Vγ4), the location of the V segment influences the rearrangement pattern. In a transgenic model where Vγ2 and Vγ3 are switched, fetal cells had higher Vγ2 rearrangement levels than Vγ3, the opposite of what normally occurs. V segment location does not affect the rearrangement pattern in adult thymocytes though and they are mostly influenced by promoters. The sequences upstream of Vγ2, 3, and 4 are quite divergent, suggesting that each promoter could be regulated by distinct trans-acting factors. Baker et al.126 swapped the promoter regions of Vγ2 and Vγ3 and the pattern was reversed in adult with Vγ3 being rearranged. Since germline transcription is present for both Vγ2 and Vγ3 in the fetal stage, it seems that both genes are accessible in the early stages, with an advantage to the more proximal gene, followed by a stage when Vγ3 is repressed and/or Vγ2 is activated, leading to a strong preference for Vγ2 rearrangement. Therefore, the "developmental switch" (i.e. Vγ3,4 to Vγ2,5) in Vγ gene usage is imposed by two mechanisms, one sensitive to gene location and the other dependent on differential V-promoter activity.

During development, each precursor can try multiple rearrangements at the TCRγ locus. For example when γδ T cells were sorted for Vγ2, Vγ5 or Vγ1.1+ γδ TCRs on their cell surface (these three represent 90% of the γδ thymocytes in adult B6 mice), all clones had multiple rearrangements at the γ locus. Most cells had 2 to 6 rearrangements and Vγ2+ cells normally
had a maximum of 4 rearrangements. Rearrangements involving Jγ1 were found in all cells and about half had Jγ1 rearrangements in both chromosomes.

Since multiple V-J rearrangements occur in a single cell there are several factors that ensure that γδ T cells will not express more than one TCR specificity on the cell surface. These include: 1. the frequencies at which each Vγ and Vδ gene segments participate in recombination, 2. the frequencies at which the rearranged products produce a functional chain, and 3. whether the functionally rearranged TCRγ and TCRδ chains can pair to form a γδTCR\textsuperscript{127}. The end result is the formation of a pool of γδ T cells expressing a diverse repertoire of Vγ and Vδ chains in which the majority of the cells bear a single TCR specificity at the cell surface. This works in these cells without specific checkpoints to test and control for the functionality of each of the rearranged chains, unlike αβ T cells. This is only possible because the three mechanisms work differently on each V gene segment. For example, Vγ2 and Vγ1.2 rearrange at similar frequencies but they are approximately ~10-12 times more frequent than Vγ1.1 rearrangements and 16-20 times more common than Vγ5 rearrangements. Vδ segments also rearrange at different frequencies\textsuperscript{127}.

The frequencies at which different Vγ and Vδ segments participate in recombination are inversely correlated with the apparent ability of the resulting TCRγ or TCRδ chain to participate in the formation of a functional γδTCR. For example, Vγ1.2 is one of the most frequent rearrangements but it shows the highest level of restriction for pairing with a TCRδ chain. In contrast, Vγ1.1 and Vγ5, which are rearranged at low frequencies, lack restriction in pairing with Vδ chains.
Also, there is a stop codon at the 3'end of the very frequently rearranged germline Vγ2 gene segment\textsuperscript{100, 128}. This substantially lowers the frequency at which rearrangement will produce a functional chain. This therefore lowers the frequency of a functional Vγ2 being expressed on the cell surface and therefore increases the chances that γδ T cells can express TCRγ chains on their surface that are from low frequency rearrangements (i.e. Vγ5).

1.9. Thesis objectives and hypotheses

The **original objective of my thesis** was to determine if adult and neonatal NK cells follow separate pathways of lineage commitment. Since adult and neonatal NK cells differ in their phenotype and function, I hypothesized that the differences are due to their developmental pathways. In Chapter 3, this hypothesis was tested by examining differences in gene expression patterns between the two types of NK cells. This study resulted in the detection of TCRγ gene rearrangement in a subset of NK cells. Based on this interesting and unexpected finding, my subsequent thesis research was redirected to further characterize NK cells that have rearranged TCRγ genes. The **objectives of my subsequent study** were to determine the developmental pathway responsible for the generation of NK cells with TCRγ gene rearrangement and to further characterize these NK cells. My **hypotheses were**: 1) there are at least two separate developmental pathways for NK cells, one in the bone marrow and the other in the thymus, 2) a subset of NK cells arises from immature T cell progenitors that have begun TCRγ gene rearrangement but still retain NK cell potential, and 3) NK cells with rearranged TCRγ genes are different from bone marrow-derived conventional NK cells in phenotype and function. **To test these hypotheses, the following objectives were set**: 1. to determine if the TCRγ gene-rearranged NK cells are thymus-dependent, 2. to identify the progenitors that give
rise to the NK cells with TCRγ gene rearrangement, 3. to determine if NK cells generated by the developmental pathway involving TCRγ gene rearrangement differ from conventional BM-derived NK cells in phenotype, function, and tissue distribution.
2 MATERIALS AND METHODS

2.1. Mice. C57BL/6 (B6) mice were bred in our animal facility. Adult mice used in this study were 6 to 10 weeks old and neonatal mice were 1 to 3 days old. RAG2<sup>−/−</sup>HY TCR transgenic mice were also bred from breeders purchased from Taconic Farms (Germantown, NY). Adult TCRβ/TCRδ-double knockout (B6.129P2-Tcrβ<sup>tm1Mom</sup>Tcrδ<sup>tm1Mom</sup>/J) mice were purchased from The Jackson Laboratories (Bar Harbour, ME). Heterozygous nude (B6.Cg-Foxn1nu) mice were purchased from The Jackson Laboratories and were mated, and athymic nude neonatal mice (3 days old) were used. IL-15<sup>−/−</sup> mice (C57BL/6NTac-IL15<sup>tm1Iwx</sup>) were purchased from Taconic Farms. NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ mice were from The Jackson Laboratories.

2.2. Antibodies. Antibodies used in this thesis are listed in Table 2.1.
Table 2.1. List of antibodies:

<table>
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2.3. Microarray Sample Preparation and Analysis. Total RNA was isolated by using the RNeasy Mini Kit (QIAGEN Inc., Mississauga, ON). Double stranded cDNA was synthesized from total RNA with the Superscript double stranded cDNA kit (Invitrogen, Carlsbad, CA). The Enzo BioArray high yield RNA transcript labeling kit (Affymetrix Inc., Santa Clara, CA) produced biotin labeled cRNA which was fragmented and hybridized to Affymetrix GeneChip Mouse Genome U74Av2 arrays. The first two microarray experiments were performed at the DNA Array Laboratory, Wine Research Centre, University of British Columbia and the third experiment was performed at the Affymetrix GeneChip Facility at the Michael Smith Genome Sciences Centre, British Columbia Cancer Agency. All data analysis was performed with Genespring version 7 (Silicon Genetics, Redwood City, CA). Expression values were background corrected, normalized, and summarized by using the default settings of the program package.

2.4. Measuring DNA. For determining the percentage of NK cells that had TCRγ gene rearrangements, DNA templates were first quantitated using Pico Green® dsDNA quantitation kit (Molecular Probe, Invitrogen, Carlsbad, CA), and the fluorescence was measured with a CytoFluor™ 2300 Fluorescence Measurement System (Millipore, Billerica, MA).

2.5. Genomic PCR. To isolate genomic DNA, cells were lysed with 50 µl of dH2O and vigorous pipetting, placed at 98°C for 10 minutes, and then 5 µl of 1 mg/ml proteinase K was added and incubated at 55°C for 2 hours followed by incubation at 98°C for 10 minutes. DNA thus isolated was used as template for PCR. Primers for Vγ-Jγ and NKG2A genomic PCR are listed in Table 2.2. The genomic PCR scheme is shown in Fig. 2.1. The reaction volume for these PCRs was 50 µl, containing 5 µl of 10× PCR buffer, 1.5 µl of 50 mM MgCl2, 1 µl of 10
mM dNTPs, 1.25 μl each of 10 μM primers, and 0.5 μl of 5 U/μl Taq DNA polymerase.

Thermocycling conditions were as follows: 3 minutes at 94°C followed by 30 cycles of 45 seconds at 94°C, 2 minutes at 55°C, 1 minute at 72°C and finally 7 minutes at 72°C. 10 μl of PCR products mixed with 1 μl 10× loading buffer were analyzed on a 1% agarose gel. TCRβ and TCRδ PCR primers are listed in Table 2.2. PCR for TCRβ gene rearrangement was as described by Ikawa, et al.63. The reaction volume was 20 μl, containing 1.5 μl of 10× PCR buffer (with MgCl₂), 0.16 μl of 25 mM dNTPs, 0.4 μl each of 10 μM primers, and 0.2 μl of 5U/μl Taq DNA polymerase. Thermocycling conditions were as follows: 5 minutes at 94°C followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, 2 minutes at 72°C and finally 10 minutes at 72°C. Primers for TCRδ PCR were described by Capone et al.100. Thermocycling conditions were as follows: 5 minutes at 94°C followed by 32 cycles of 1 minute at 94°C, 30 seconds at 55°C, 2 minutes at 72°C and finally 7 minutes at 72°C.
Figure 2.1. **A. The murine TCRγ locus.** There are 4 clusters in the TCRγ locus. Each cluster contains one J segment and one C segment and variable numbers of V segments. The nomenclature is that of Garman et al.\(^{103}\)  **B. Schematic of genomic PCR design.** The genomic PCR design was based on that by Itohara et al.\(^ {129}\). If the locus is in germline order, the primers specific to the V and J segment will be too far apart to produce a PCR product. On the other hand, if the V and J segment have been rearranged, the primers will be close enough together and a PCR product will result.

### 2.6. Southern blot

10 μl of PCR products mixed with 1 μl 10× loading buffer were analyzed on a 1% agarose gel. The gel was alkaline blotted to BioRad’s (Hercules, CA) Zeta-Probe\(^ {\circledR}\) membrane. The southern blot was probed with a biotin-labeled oligonucleotide and visualized by Pierce’s (Rockford, IL) North2South\(^ {\circledR}\) Chemiluminescent Nucleic Acid Hybridization and Detection kit. The oligonucleotide probes were labeled with 3’ end labeling DNA with biotin-14-dATP Invitrogen protocol.
2.7. **RT-PCR.** RNA was isolated from cells with QIAGEN’s RNeasy® Mini Kit and reversed transcribed into cDNA with QIAGEN’s Omniscript® Reverse Transcription kit. The cDNA samples for RT-PCR templates were equal to 100ng of RNA. Forward primers for Vγ2, 3, 4, and 5 were paired with a reverse primer for a constant region sequence that is shared by all TCRγ gene clusters (Cγ1, 2, and 4). The primer sequences are listed in Table 2.2. The reaction volume was 50 µl, containing 5µl of 10× PCR buffer, 1.5 µl of 50 mM MgCl₂, 1 µl of 10 mM dNTPs, 1.25 µl each of 10 µM forward and reverse primers, and 0.5 µl of 5U/µl Taq DNA polymerase. Thermocycling conditions were as follows: 5 minutes at 96°C followed by 32 cycles of 15 seconds at 96°C, 40 seconds at 50°C, 1 minutes at 72°C and finally 10 minutes at 72°C. 1 µl of PCR product was analyzed on a 1% agarose gel.
Table 2.2. List of primers

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<td>C-region: CTTATGGAGATTGTTCAGC</td>
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2.8. **Sequencing of PCR products.** RT-PCR products from thymocytes, IL-2-activated adult NK and newborn NK cells were purified using Wizard PCR preps DNA purification from Promega (Madison, WI). The PCR products were ligated into the pGEM-T easy vector (Promega). The plasmid clones were sequenced at the NAPS Sequencing Service (University of British Columbia, Vancouver, Canada).

2.9. **Tissue culture:**

2.9.1. **L cells.** The murine fibroblast L-cells were cultured in Dulbecco’s modified eagle’s medium (with 4500 mg D-glucose/L) (DMEM) plus 10% FBS, L-glutamine, penicillin, streptomycin, and $5 \times 10^{-5}$ M 2-mercaptoethanol.

2.9.2. **OP9 cells.** OP9 stroma cells were cultured in Minimum essential medium eagle, alpha modification with nucleosides (MEM) with 10% FBS, penicillin, and streptomycin.

2.9.3. **LAK cells.** Single cell suspensions of bulk cells from spleen, lymph node, BM, thymus, liver or lung were cultured with 1000 U/ml IL-2 (PeproTech, Rocky Hill, NJ) to expand the population. Cells were incubated in tissue culture dishes for 3 hours at 37°C and the non-adherent cells were cultured for 7-10 days in RPMI1640 media containing 10% FBS, L-glutamine, penicillin, streptomycin, and $5 \times 10^{-5}$ M 2-mercaptoethanol. Media was changed during the middle of the culture.

2.9.4. **Thymus or LN DN progenitor culture and BM NKP progenitor culture.** Thymocytes, LN cells, or BM cells were blocked with 2.4G2 hybridoma culture supernantant and then stained with lineage marker mAbs (CD3, CD8, TCRβ, TCRγδ, CD19, B220, Mac-1, GR-1, NK1.1 (and possibly NKG2A/C/E, Ly49G, and Ly49D), and Ter119). Lineage marker positive cells were removed from the sample with EasySep FITC Positive Selection kit (StemCell Technologies). Thymus and LN cells were then stained with CD44 and CD25.
mAbs and DN1 (Lin\(^{CD44^{+}CD25^{+}}\)) and DN2 (Lin\(^{CD44^{+}CD25^{+}}\)) or pre-DN2 (Lin\(^{CD44^{+}CD25^{lo}}\)) cells were sorted. For BM NKP cells, cells were stained with NK1.1 and CD122 and NKP cells were sorted (Lin\(^{NK1.1^{+}CD122^{+}}\)). Cells were then seeded onto OP9 stroma at 20,000-40,000 cells per 500 µl well of a 24-well plate. If less cells were sorted, all cells were seeded into one well. OP9 stroma was grown 2 days in advance in Minimum essential medium eagle, alpha modification with nucleosides (MEM) with 10% FBS and P/S to ensure that the stroma was confluent before DN or NKP progenitors were added. The OP9 media was removed and the progenitor cells were grown in MEM with 10% FBS and P/S as well as 150 µM monothioglycerol, 30 ng/ml stem cell factor (SCF), 100 ng/ml recombinant human Flt-3 ligand (Flt3L), 1 ng/ml IL-7, and 25 ng/ml IL-15. Half of the media was replaced on day 4 and if necessary, cells were transferred to new OP9 with new media at a later stage. Cultures were grown for 10-12 days.

2.10. Cell preparation. Cell suspensions were prepared from spleen, thymus, or LN tissue and passed through a 70 µm filter. Cells were washed, red blood cells were lysed with ammonium chloride and washed again. To prepare single cell suspensions of BM, muscle was removed from femur and tibia and BM was plunged from the bone with a syringe and 28 gauge needle. BM was then made to single cell suspension by passing through a 21 gauge needle repeatedly. Cells were washed, red blood cells were lysed and cells were washed again. For lung and liver, the tissues were perfused with 2% Phosphate buffered saline (PBS). The tissue was cut into small sections and digested with DNase and collagenase. Liver was digested in RPMI with 5% FBS, P/S, 2ME. Lung was digested in DMEM with 5% FBS, P/S. Liver tissue was digested with 25 U/ml Dnase I and 250 U/ml collagenase IV while rotating at room temperature for 45 minutes. Lung tissue was digested with 50 U/ml Dnase and 250U/ml
collagenase IV while rotating at 37°C for 1 hour. Tissues were then passed through a 70 µm filter and washed. The liver pellet was resuspended in 40% percoll (dilutions made with PBS) and layered on top of 70% percoll. For the lung, the percoll was first diluted to 90% percoll with 10X PBS. The lung pellet was resuspended in 44% percoll and layered on 67% percoll (dilutions made with DMEM). Gradients were spun at 2100 rpm for 20 minutes. The interface was collected, cells were washed, and red blood cells were lysed.

To isolate B cells, bulk splenocytes from adult and newborn mice were stained with anti-CD19-biotin plus streptavidin-PE and anti-CD3-FITC, and CD19⁺CD3⁻ cells were purified by cell sorting.

2.11. Staining and FACS sorting or analysis of cells. Cells were washed in 2% PBS, counted and pellets were incubated on ice for 15 minutes in 50 µl 2.4G2 hybridoma supernantant per 4x10⁶ cells to block Fc receptors. mAb was then added at appropriate concentration for 30 minutes at 4°C. Finally, propidium iodide was added to 5 µg/ml. Cells were purified by cell sorting by a FACS Caliber® (BD, Mountain View, CA). Sorted cells were checked for purity. For analysis of FACS data CellQuestPro and Win MDI were used.

2.12. IFNγ production assay. Bulk cells (2 x 10⁶) from LN, thymus or spleen were resuspended in 2 ml of RPMI media with 10% FBS, P/S, and 2-ME with 1 ng of IL-12 and 0.5 ng of IL-18. These cells were incubated at 37°C for 24 hours. Approximately 6 hours before the end of the culture, 1 µl of Golgi Plug (BD Biosciences) was added to each well. This inhibits the secretion of the IFNγ produced and makes it accumulate in the cells. Intracellular
staining of IFNγ was performed with the BD Biosciences BD Cytofix/Cytoperm™ Plus kit. Cells were then analyzed by FACS.

2.13. **Cytotoxicity assay.** RMA-S cells were used as target cells. These cells were fluorescence labeled using Invitrogen Vybrant CFDA SE Cell Tracer kit. A stock CFDA SE solution was made at 100 μM and a working dilution of 1 μM was used to stain the RMA-S cells. The effector cells were either splenic or thymus NK cells that were cultured with IL-2 in a LAK culture. Thymus NK cells were prepared from B6 thymocytes depleted of CD3+ cells or from TCRβ−δ− mice. 10,000 CFDA SE labeled RMA-S cells were mixed with either thymus NK or splenic NK cells at ratios of 1:1, 1:2, 1:5, 1:10, and 1:20 in 500 μl of RPMI in 24-well plates. Following a four-hour incubation, the cells were collected, washed and resuspended in PI buffer. The cells were then analyzed by FACS and the percentage of CFDA SE+ cells that were positive for PI buffer was recorded.

2.14. **LN DN cell transplantation.**

2.14.1. **Intraperitoneal injection.** DN1 and pre-DN2 cells were sorted from IL-15−/− mice as above. 20,000 cells were injected intraperitoneally in 500 μl PBS into three Nod Scid IL-2Rγ−/− mice. Three weeks later, spleens, thymuses, BM, and LN were removed and examined by FACS analysis for NK cells.

2.14.2. **Intravenous injection.** DN1 and pre-DN2 cells were sorted from Pep3b mice. 20,000 cells were injected intravenously in 500 μl PBS into two Nod Scid IL-2Rγ−/− mice. Four weeks later, spleens and BM were removed and examined by FACS analysis for NK cells.
2.15. **Statistics.** Data was analyzed statistically using the Student’s T-test (Microsoft Excel). Differences of $p<.05$ were considered statistically significant.
3 IDENTIFICATION OF A NOVEL PATHWAY OF NK CELL DEVELOPMENT THAT IS THYMUS-DEPENDENT AND INCLUDES TCR GENE REARRANGEMENT

3.1. Introduction

Currently, the relationship between BM-derived NK cells and the NK cell potential demonstrated by T cell progenitors is unclear. Although adult DN thymocytes have NK cell potential, it is believed that this pathway is not realized in steady state NK cell development in normal mice since NK cell numbers are normal in nude mice, which lack a thymus. While it is well demonstrated that NK cells arise from bipotent T/NK cell progenitors found in the fetal liver, blood, spleen and thymus, it is currently thought that this pathway is restricted to the fetal environment. As hematopoiesis switches from fetal liver to adult BM, it is assumed that the NK cell developmental pathway switches exclusively to the BM as well. In this regard, it is of interest that NK cells in fetal and neonatal mice are different from those in adult mice. The former express CD94/NKG2, which recognizes non-classical MHC class I Qa-1b, and Ly49E, but not other Ly49 receptors that recognize classical MHC class I, whereas the latter express a full repertoire of NK cell receptors. What causes the differences between fetal/neonatal and adult NK cells is still unknown, especially the control of Ly49 expression.

We began this study by comparing gene expression patterns between adult and neonatal NK cells to look for differences that may suggest different pathways of NK lineage commitment in neonatal and adult mice. Unexpectedly, we found that a subpopulation of NK cells expresses

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TCRγ genes and that expression is higher in neonatal NK cells than adult NK cells. These studies suggest that a subset of NK cells develop in the thymus from T cell precursors that have rearranged TCRγ genes. Therefore, since both adult and neonatal NK cells express TCRγ genes, the bipotent T/NK cell pathway still contributes in the adult environment.

3.2. Results

3.2.1. Microarray analysis reveals expression of TCRγ gene in NK cells

To examine whether adult and neonatal NK cells follow different pathways of lineage commitment, global gene expression of IL-2 activated NK cells, which were purified by two rounds of cell sorting (over 99% NK1.1⁺ CD3⁺), were compared by performing triplicate microarray experiments on Affymetrix MG-U74Av2 chips. The raw expression values of the microarray experiments were submitted to an online microarray database and they are available at EBI ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession # E-MEXP-354. The genes were normalized and then filtered on expression, confidence, and fold change. A parametric student's t-test with a p-value cut off of 0.05 and a Benjamini and Hochberg false discovery rate multiple testing correction was applied. Out of 12,488 genes on the chip, 12 had statistically significant differences in expression between the two samples (Fig. 3.1). As expected, 7 of the 12 differences in gene expression were from Ly49 genes with high expression in adult NK cells and low or absent in neonatal NK cells.
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Figure 3.1. Microarray data analysis of differentially expressed genes between adult NK and neonatal NK cell samples.

Genes with statistically significant differences when grouped by ‘Sample Source’ (adult vs. neonatal samples); parametric test, variances assumed equal (Student's t-test). p-value cutoff 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate. Genes from adult and neonatal were normalized and were filtered on expression, confidence, and fold change. These remaining genes were then tested by 1-way ANOVA. (a) List of the genes with statistically significant differences after 1-way ANOVA test. (b) Graph representation of the same group of genes as in (a).
Figure 3.1. Microarray data analysis of differentially expressed genes between adult NK and neonatal NK cell samples.

(c) Graph representation of the same group of genes as in (a).
The most striking and unexpected result was the expression of TCRγ genes in both adult and neonatal NK cells. An analysis of various TCR gene expression in NK cells showed that only TCRγ genes were consistently detected in both neonatal and adult NK cells (Fig. 3.2a). TCRδ gene expression was detectable at a lower level and the probe identification describes it as germline TCRδ expression. TCRβ gene expression was undetectable. Only one of three TCRα gene probes detected positive expression. The other TCRα and the pre-TCRα probes were negative and TCRα rearrangement only begins after full T cell lineage commitment and therefore, it is likely that this probe detected non-specific gene expression. Not only was TCRγ gene mRNA expression in NK cells detected, its expression was also shown to be significantly higher in neonatal NK cells than in adult NK cells with a student’s t-test p-value of 0.0475 (Fig. 3.2b). To confirm that TCRγ is not expressed on the NK cell surface, T cells and NK cells were stained with TCRγδ mAb and FACS analysis showed no expression on NK cells (Fig. 3.2c).
Figure 3.2. Detection of TCRγ gene expression in NK cells by microarray analysis.

(a) Gene expression patterns of purified IL-2 activated adult and neonatal NK cells were analyzed in triplicate using Affymetrix GeneChip Mouse Genome U74Av2 arrays. Expression of α, β, γ, and δ TCR genes ± s.d. are shown. The black bars represent neonatal NK cells and the white bars represent adult NK cells. The expression values (0-700 on the graph) are based on raw values after default normalization of the 6 chips, with the 3 adult samples grouped together and the 3 neonatal samples grouped together. For Affymetrix gene chips, each gene is represented by a probe set of 10-25 oligonucleotide pairs, each pair consisting of a perfectly matching probe and a probe with one nucleotide mismatch in the middle of the sequence. The detection call of whether a gene is present (expressed) or absent (not expressed) is based on binding to the perfect match and mismatch pairs. Therefore, an expression value can be assigned but it does not necessarily mean the gene will be called present. Those with a * show that they are present and the expression values are valid. The affymetrix probe numbers for these genes are: β1: 101311, β2: 94202, β3: 99798, α1: 101823, α2: 97944, α3: 97945, pα: (preTa): 98354, δ: 92328, γ1: 102745, γ2: 102685, γ3: 102744.

(b) Significant difference in expression levels of TCRγ gene (probe 102744) in IL-2 activated adult NK cells (white bar) and neonatal NK cells (black bar) determined by a 1-way ANOVA (p-value: 0.0475). The values represent the log ratio which is the intensity ratio (adult NK cell sample gene divided by the neonatal NK cell sample gene) log transformed (log2). (c) Surface staining for TCRδγ on neonatal NK cells and T cells from a splenic LAK culture show that NK cells do not express TCR on their cell surface.
3.2.2. TCRγ genes are rearranged and expressed in NK cells

The microarray data revealed TCRγ gene expression in NK cells. However, the probes for the TCRγ genes on the microarrays were specific for the 3’ end of the transcripts and the results did not reveal whether the microarray data were detecting germline expression or expression of rearranged TCRγ gene segments that resulted from VJ recombination. In addition, if it was detecting rearrangement, the microarray data did not show the extent of possible rearrangement combinations that were expressed. There are four clusters in the murine TCRγ locus, each containing variable (V), joining (J), and constant (C) regions. Cluster 1, which is the most commonly studied, consists of four V segments (Vγ2, 3, 4, and 5), one J segment (Jγ1) and one constant region (Cγ1). To further characterize the TCRγ gene expression in NK cells, genomic PCR was performed to determine whether TCRγ genes are rearranged in NK cells. Forward primers specific to the V segments in the locus (Vγ2, 3, 4, 5) and the reverse primers specific to their respective J segment (Jγ1) were used. The genomic PCR was designed so that if the locus was in germline configuration, the V and J primers would bind to segments too far apart from each other to produce a PCR product. On the other hand if the V and J segments were rearranged, the primers would be close enough to each other to produce a PCR product of about 350 bp. IL-2-activated NK cells from adult and neonatal mouse spleen were purified as above and analyzed by genomic PCR. Southern blot analysis of the PCR products hybridized to Jγ-specific oligonucleotide probes determined that neonatal NK cells exhibited rearrangement of all possible V-J combinations that were examined while adult NK cells had some of the possible rearrangements (Fig. 3.3a, left panel). The identity of the larger band seen for the adult NK cell Vγ3-Jγ1 PCR is unknown. These results showed that TCRγ gene rearrangement with multiple V-J combinations does occur in NK cells. To determine whether the rearranged TCRγ genes are expressed in NK cells, RT-PCR was performed using the
primers specific to the V-segments and their corresponding C-region. Consistent with the
genomic PCR results, neonatal NK cells expressed all of the possible combinations while adult
NK cells expressed only some (Fig. 3.3a, right panel). The genomic and RT-PCR experiments
were also performed with freshly isolated NK cells without culturing with IL-2, and the results
were the same to those with IL-2 activated NK cells (data not shown).

Figure 3.3. TCRγ gene rearrangement and expression in NK cells.
(a) Southern hybridization with Jγ1-specific oligonucleotide probe of genomic PCR (left
panel) or RT-PCR (right panel) of IL-2 activated adult and neonatal NK cells to test for
rearrangement of TCRγ locus and expression of rearranged TCRγ genes. Thymocytes are the
positive control and fibroblasts (L cells) are the negative control. NKG2A PCR and
Glyeraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR were used as control for
genomic and RT-PCR, respectively. (b) NK cell DNA from adult Rag2−/− was tested by
genomic PCR for Vγ2-Jγ1 rearrangement as in (a).

3.2.3. Specificity of rearrangement

To confirm that the detected TCRγ gene rearrangement occurs as a result of normal V(D)J
recombination that requires RAG enzymes, NK cells from adult RAG2−/− mice were sorted and
tested by genomic PCR. As expected, no TCRγ gene rearrangement was detected in these NK
cells (Fig. 3.3b). The specificity of the RT-PCR was also confirmed by cloning and
sequencing the PCR products from thymocytes and from purified adult and neonatal NK cells. The sequences showed that PCR cross-amplified non-specific TCRγ genes. However, Southern hybridization to Jγ-specific oligonucleotide probes detected only the specific sequences (data not shown).

3.2.4. TCRγ gene rearrangements in NK cells represent unselected, random recombination

Since Vγ2-Jγ1 recombination was most prominent among NK cells, RT-PCR sequencing results for this rearrangement were further analyzed. Vγ2 has an in-frame stop codon at the 3’ end, which can be removed during the VJ gene recombination process. Out of the Vγ2-Jγ1 rearrangements that were sequenced, 4 out of 9 (44%) were in-frame, productive rearrangements in adult NK cells and 3 out of 8 (44%) were in-frame, productive rearrangements in neonatal NK cells (Fig. 3.4). These are similar to the expected frequencies (33%) of random unselected rearrangements.
Figure 3.4. Sequences of productive and non-productive TCRγ gene rearrangements. Sequences of neonatal NK cell RT-PCR products for Vγ2-Cγ1 transcripts. Only the sequences at the junction of Vγ2-Jγ1 are shown. The in-frame stop codon in Vγ2 is underlined.

3.2.5. Tcry⁺ NK cells represent a small population of total splenic NK cells

To determine the percentage of splenic NK cells with TCRγ gene rearrangement (termed Tcry⁺ NK cells hereafter), fresh and IL-2 activated NK cells from adult and neonatal mice were purified by two rounds of cell sorting. The levels of purity of the NK cell samples used in this experiment were always over 99% with 0-0.05% CD3⁺ or TCRγδ⁺ (Fig. 3.5a). DNA was isolated from the purified NK cells and subjected to genomic PCR analysis for TCR Vγ2-Jγ1 gene rearrangement. To determine the frequency of Vγ2-Jγ1 rearrangements among NK cells, DNA was also isolated from purified γδT cells and mixed with fibroblast (L-cell) DNA at various ratios, and genomic PCR was performed in the same way. The intensity of the bands for adult and neonatal NK cells was compared to the various control percentages. To ensure that the starting amount of DNA was identical for each sample, the DNA was first measured with PICO green staining. Results consistently showed that about 5% of neonatal splenic NK
cells and about 1% of adult splenic NK cells had Vγ2-Jγ1 gene rearrangements (Fig. 3.5b, c). The frequency was the same with freshly isolated NK cells and IL-2-activated NK cells, as the PCR bands were of similar intensity (Fig. 3.5b, d). Since these percentages are low, it was important to rule out the possibility of T cell contamination in the NK cell samples. The same experiment was also performed with NK cells from TCRβ−/−δ−/− mice which have no T cells in the spleen. TCRγ gene rearrangement was still observed in these NK cell samples, thus ruling out the possibility of T cell contamination (Fig. 3.6).
Figure 3.5. Low frequency of NK cells with rearranged TCRγ genes.
(a) Purity of IL-2 activated NK cell samples from adult and neonatal mice after two rounds of cell sorting. The numbers show the percentages of NK cells (NK1.1+CD3ε). (b) Genomic PCR (Vγ2-Jγ1) performed with δγT cell DNA and fibroblast DNA mixed at various ratios and with fresh and IL-2 activated adult and neonatal NK cells. The PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. (c) Southern hybridization to Jγ1 probe of the genomic PCR products generated from IL-2-activated NK cells from adult and neonatal mice in (b). The top panel shows a short exposure of the southern blot whereas the bottom panel shows a long exposure to visualize rearranged Vγ2- Jγ1 in adult NK cells. (d) Southern blot of genomic PCR, as in (c), but with freshly isolated adult and neonatal cell DNA. Gels divided by lines are groupings of images from different parts of the same gel.
Figure 3.6. Tcργ NK cells are not due to contamination of T cells.
The frequency of IL-2 activated NK cells from TCRβ⁻/⁻TCRδ⁻/⁻ mice with Vγ2-Jγ1 rearrangement was estimated by genomic PCR and ethidium bromide staining of agarose gel. Genomic PCR (Vγ2-Jγ1) was performed with δγT cell DNA and fibroblast DNA mixed at various ratios and with NK cell DNA.

3.2.6. NK cells have a germline TCRβ locus and may have initiated TCRδ rearrangement

Rearrangement of TCRβ and TCRδ genes in NK cells was also tested by genomic PCR.

Rearrangement of TCRβ genes was not detectable in neonatal and adult NK cells with the genomic PCR scheme used (Fig. 3.7a). On the other hand, a very small fraction (less than 1%) of neonatal NK cells had Vδ4-Jδ1 rearrangement (Fig. 3.7b).
Figure 3.7. TCRβ and TCRδ gene rearrangements in NK cells.
DNA from purified IL-2 activated adult and neonatal NK cells was tested by genomic PCR for TCRβ (a) or TCRδ (b) rearrangement. (a) Genomic PCR using primers specific to Dβ2 and Jβ2.6 genes was analyzed by agarose gel electrophoresis and stained with ethidium bromide. The largest band represents non-rearranged germline TCRβ locus whereas multiple smaller bands represent TCRβ gene rearrangements. Thymocyte DNA was used as positive control and fibroblast DNA was used as negative control. (b) Genomic PCR (Vδ4-Jδ1 or Vδ5-Jδ1) performed with γδT cell DNA and fibroblast DNA mixed at various ratios and with fresh and IL-2 activated adult and neonatal NK cells. The PCR products were analyzed by agarose gel electrophoresis, blotted and hybridized to Jδ1-specific oligonucleotide probe. Gels divided by lines are groupings of images from different parts of the same gel.

3.2.7 TCRγ gene rearrangement found in NK cells does not occur in CLPs which generate mature B cells

RAG genes have been shown to be activated in CLPs in the BM²⁷,²⁸, and about 5% of adult NK cells have been shown to have rearranged immunoglobulin heavy chain gene²⁷. Therefore, whether TCRγ genes are also rearranged in B cells in neonatal and adult mice was tested. Genomic PCR analysis of purified B cells detected no Vγ2-Jγ1 rearrangement in B cells (Fig. 3.8). These results suggest that a subpopulation of NK cells develop from thymic T/NK bipotential progenitors that have rearranged TCRγ genes and lost B cell potential.
Figure 3.8. Lack of TCRγ gene rearrangement in B6 mouse B cells. Southern blot with JY1 probe of genomic PCR (Vγ2-JY1) of B6 adult and neonatal splenic B (CD19⁺CD3⁻). Genomic PCR for a part of NKG2A gene confirms the presence of B cell DNA.

3.2.8. The thymus is required for the development of Tcry⁺ NK cells

The TCR gene rearrangement in NK cell subsets suggested that they develop in the thymus, since this is the location where the majority of T cells undergo TCR V(D)J recombination. To examine whether the Tcry⁺ NK cells develop in the thymus, NK cells were isolated from nude mice, which lack a proper thymic environment and lack conventional T cells. Only extrathymic T cells accumulate in the spleen of older nude mice (up to 5.4% of splenocytes) (Fig. 3.9a). It was known that NK cells were present at normal or elevated levels in adult nude mice, but the NK cell status of neonatal nude mice was not known. Normal numbers of NK cells were found in the spleen of neonatal (3 day old) nude mice. Genomic PCR analysis of freshly isolated NK cells and IL-2-activated NK cells from nude mice showed no Vγ2-JY1 rearrangement in NK cells from nude mice (Fig. 3.9b). It should be noted that extrathymic T cells, which accumulate in the spleen of old nude mice, had rearranged TCRγ genes, but NK cells isolated from the same mice did not (Fig. 3.9b) demonstrating that the TCR rearrangement can still occur in these mice and the absence of it in NK cells is due to the lack of the thymus.
Furthermore, genomic PCR analysis of highly purified fresh and IL-2 activated NK cells from the BM and thymus showed that TCRγ gene rearrangement is present in at least half of thymic NK cells while very low (~5% or less) rearrangement was detected in BM NK cells (Fig. 3.10). Therefore, the thymus is required for the development of T<sub>crγ</sub><sup>+</sup> NK cells.

![Figure 3.9. Lack of TCRγ gene rearrangement in nude mouse NK cells and high TCRγ gene rearrangement in thymus NK cells.](image)

(a) Percentages of NK (NK1.1<sup>+</sup>CD3<sup>-</sup>) cells and T (NK1.1<sup>-</sup>CD3<sup>+</sup>) cells in the spleen of 1 year old (left) and three day old (right) nude mice. (b) Agarose gel electrophoresis and ethidium bromide staining of genomic PCR (Vγ2-Jγ1) of IL-2 activated and fresh NK cells from adult and neonatal nude mice. T cells that accumulate in aged nude mice were also isolated from spleen of the same adult mouse by cell sorting. Thymocytes were used as positive control and fibroblasts (L cells) were used as negative control. Genomic PCR for a part of NKG2A gene confirms that comparative amounts of template DNA was used for all the genomic PCR.
Figure 3.10. Thymus NK cells have high levels of TCRγ gene rearrangement.
Southern blot with Jyl probe of genomic PCR products generated from IL-2 activated NK cells from adult thymuses and BM and δγ T cell and fibroblast DNA mixed at various ratios. The middle panel is a longer exposure of the same membrane. The bottom panel shows control NKG2A PCR confirming that comparable amounts of DNA were used for the analysis.

3.3. Discussion

The microarray analysis suggested that adult and neonatal NK cells have very similar gene expression profiles. This argues against our initial hypothesis that neonatal and adult NK cells follow different pathways of development. Although the microarray data was not further analyzed with respect to this original objective, it was extremely useful because it suggested that NK cells may follow more than one pathway of development within the body. The discovery of TCRγ mRNA in NK cells led to the discovery and characterization of a thymus-dependent NK cell developmental pathway.
The microarray analysis revealed that adult and neonatal NK cells do not differ much in gene expression other than the Ly49 receptors, which has been well characterized. Fetal and neonatal NK cells do not express Ly49 receptors, except Ly49E^{66}. Cells begin to acquire Ly49 receptors after birth and only reach full expression levels at 6 weeks of age. This is what we observe in the microarray expression results as well. The other four receptors that were significantly higher in adult NK cells than neonatal NK cells were NK cell functional factors: CCL3, CCL4, CCL5 and gp49B.

The most striking result was that NK cells express a T cell specific gene, TCRγ. This is unexpected because it is commonly assumed that TCR rearrangement marks final commitment to the T cell lineage. Both adult and neonatal NK cells express the TCRγ and the difference in expression between the two is significant as well. More recently, another study has also detected TCRγ expression (but not other TCR expression) in NK cells. Kang et al.^{130} performed serial analysis of gene expression (SAGE) of HSCs, NK precursors (NKPs), and mature NK cells (mNKs) and they detected TCRγ expression in mNK cells but did not expand upon this discovery.

It is important to note that the TCRγ mRNA expression detected in the NK cells does not indicate this protein is expressed on the cell surface. Firstly, the NK cells were sorted as NK1.1^{+}CD3^{−} cells and DN thymocytes cannot assemble a functional TCR complex on the cell surface in the absence of CD3ε^{131,132}. TCRγ cannot be expressed on the cell surface without TCRδ either^{129}. This was confirmed by flow cytometry and no NK cells express TCRγ on their surface. It is only known that TCRγ mRNA is expressed in NK cells since a western or intracellular staining for the TCRγ chain was not performed to check if the protein was
expressed. Since the TCR is not expressed on the cell surface, it likely has no functional role in the NK cells. Rather, it serves as a marker of NK cells that have followed this developmental pathway.

There are several examples of cells that are not T cells or B cells, respectively, with TCR or Ig gene rearrangements. For example, 5% of NK cells have IgH gene rearrangements, 30-50% of peripheral T cells have IgH gene rearrangement, and 16% of adult B cells had V-D TCRδ rearrangements. There are multiple cases of gene expression promiscuity in hematopoietic progenitors, as elaborated upon in the Introduction.

This novel pathway of NK cell lineage commitment includes TCR rearrangement and the thymus. The higher expression of TCRγ mRNA in neonatal NK cells suggests that the pathway is more common in the fetal/neonatal mouse as would be expected due to the lack of a BM environment during fetal development and the multiple studies that reveal bipotent T/NK progenitors in multiple tissues. The adult pathway is important for the production of NK cells for most of the lifetime and it is of more interest that the thymus dependent pathway is maintained in the adult. While adult NK cells do develop in the BM, we now see that this is not the case for all adult NK cells. This is significant because it shows that NK cells follow at least two separate pathways of lineage commitment and it is important to know that the BM pathway is not representative of all NK cells in steady-state NK cell development.

The presence of TCRγ gene rearrangement in NK cells does not necessarily prove that a subset of NK cells develop in the thymus since RAG enzymes are also expressed in CLPs in the BM. However, B cells, which arise from CLPs, do not have TCRγ gene rearrangement. This
suggests the TCR rearrangement in NK cells does not occur due to RAG expression in the BM progenitors. We have also directly shown that Tcry+ NK cells are thymus dependent since they are absent in nude mice. It also rules out the possibility of the Tcry+ NK cells arising from an extrathymic pathway that undergoes V(D)J recombination outside of the thymus because extrathymic T cells that accumulate in old nude mice have TCRγ gene rearrangement, while any NK cells still present do not. In addition, to confirm that the absence of NK cells in nude mice is not due to the defective FoxN1 gene instead of the lack of a thymus, we show that half of thymus NK cells have TCRγ gene rearrangement while BM NK cells do not. It is likely that the small percent of BM NK cells that are Tcry+ are circulating NK cells. The thymus NK cells that do not have Vy2-Jγ1 rearrangement may have other Vγ rearrangements or they may be NK cells that arise from DN1 progenitors before V(D)J recombination begins.

Recently a second functional thymus that is the size of a small lymph node in the neck has been described135. It is unlikely that Tcry+ NK cells arise exclusively from this second thymus, since they are absent in nude mice, which lack the classic thymus. The only way that they may arise from the second thymus is if this thymus is also defective in nude mice, which is not yet known or the number of NK cells produced from this thymus is so small that they are undetectable in nude mice.

It is most likely that Tcry+ NK cells arise from DN2 T cell precursors since this is the stage that TCRγ gene rearrangement begins100. TCRγ and TCRδ gene rearrangements occur at least one full stage ahead of TCRβ gene rearrangement. Specifically, Vγ2-Jγ1 rearrangements are clearly seen at the DN2 stage and maximal rearrangement is reached by the DN3 and DN4 stages. This is similar for TCRδ. While a few complete TCRγ and TCRδ gene rearrangements
can be detected at the DN2 stage, the TCRβ locus is mainly in germline form\textsuperscript{100}. The possibility that \textit{Tcry}\textsuperscript{+} NK cells arise from DN2 thymocytes is consistent with what has been described about TNKP development in the fetal thymus as well\textsuperscript{63}. Fetal DN1 cells include both TNKPs and NK progenitors while DN2 cells on the other hand, have a large number of T progenitors as well as TNKP and NK progenitors. By the DN3 stage, there is little progenitor activity left except for a limited number of T progenitors\textsuperscript{63}. In our study, TCRβ gene rearrangements were undetectable in NK cells, suggesting that there may be a narrow window of opportunity for TNKPs in the thymus to rearrange TCRγ genes and still retain NK potential. It is likely that once thymocytes rearrange both TCRγ and TCRδ genes or TCRβ genes, they become committed to the T lineage and lose NK potential. Our results suggest that TCRγ gene rearrangements may initiate earlier than TCRδ since multiple Vγ-Jγ recombinations were detected whereas TCRδ gene rearrangement is very rare (although microarray results do report germline TCRδ expression). Although only Vδ4-Jδ1 and Vδ5-Jδ1 recombinations were examined in our study, the adult TCRδ repertoire is dominated by Vδ4, Vδ5, and Vδ7 representing 80-90% of the cells\textsuperscript{136}. Studies on the kinetics of TCR rearrangement state that TCRγ and TCRδ gene rearrangements begin at the same stage. So, another possibility is that NK cells diverge from the T cell lineage at the beginning of the DN2 stage with germline TCRγ expression. The NK cells may continue TCRγ gene rearrangement following NK cell lineage commitment if they retain the enabling factors for TCRγ gene rearrangement but lack those for TCRδ. Some NKPs express IL-7Rα and this signal does induce TCRγ gene rearrangement specifically\textsuperscript{114-116}. Our results also dispute the common assumption that TCR gene rearrangement marks the final irreversible commitment of cells to the T cell lineage as NK cells can still arise after TCR gene rearrangement has begun. It would be of interest to test
TCRγ gene rearrangement in DCs since their potential is seen at the DN1 and DN2 stage as well.

Our studies concentrate on cluster 1 rearrangement. Primers were originally used for Vγ1.1 and Vγ1.2 but following sequence analysis to test RT-PCR specificity, it was determined that the PCR was not specific and we therefore ended the analysis of these clusters. Another study experienced technical problems with Vγ1.1 as well and their PCR was not specific and amplified other Vγ genes. Once it was determined that rearrangements involving all Vγ segments of cluster 1 were seen in NK cells, we primarily focused on Vγ2-Jγ1 recombination as it was most prominent among several possible Vγ-Jγ recombinations. Vγ2 and Vγ1.2 rearrangements are about ~10-12 times more frequent than Vγ1.1 and 16-20 times more than Vγ5 rearrangements. Although Vγ2-Jγ1 is one of the most common rearrangements in adult γδ T cells, the percentage of NK cells with TCRγ gene rearrangements is likely an underestimate since we are basing the value on only Vγ2-Jγ1 rearrangements and other cells may have rearrangements other than this combination. Also, additional NK cells likely arise from DN1 cells but since they are not expected to have rearranged TCRγ genes, we cannot make any conclusions about whether a larger proportion of NK cells develop in the thymus before the initiation of TCRγ gene rearrangement. Ikawa et al. examined TCR gene rearrangement in NK cells generated from fetal thymic progenitors by FTOC and did not detect rearrangement of TCRβ genes or TCR Vγ3-Jγ1 or Vγ4-Jγ1 gene rearrangement. This is inconsistent with our current results, but the discrepancy may be explained by the fact that Vγ2-Jγ1 is the most prevalent rearrangement detected in our study whereas Ikawa et al. did not examine this rearrangement. It is also possible that their genomic PCR may not have been
sensitive enough to detect the small percentage of rearrangements. Older studies examined TCRβ, δ, and γ gene rearrangement in NK cells via Northern blot and did not detect rearrangement. These studies used splenic NK cells, which have low levels of rearrangement so it was likely that their method was not sensitive enough.\textsuperscript{137-139}

The method used to calculate the percentage of cells with TCRγ gene rearrangement can only serve as a rough estimate. Originally, single cell RT-PCR was attempted but the method was unsuccessful. Multiple attempts to optimize the sensitivity of the PCR to detect gene expression of housekeeping genes at low cell numbers did not work. Therefore, the alternative approach was to use γδ T cell DNA mixed with fibroblast DNA at various percentages. The majority, but not all, of adult γδ T cells have rearranged Vγ2-Jγ1 and therefore 100% of γδ T cells does not completely equal 100% Vγ2-Jγ1 rearranged loci for the genomic PCR. When comparing the intensity of the γδ T cell/fibroblast percentage bands with the NK cell bands, it is not a quantitative comparison of intensity, rather it is just by visual comparison that conclusions are made. Southern blot analysis of the PCR is useful because at different exposures, only certain bands appear. Therefore if two bands appear at the same time, they are similar in intensity. This method serves only as a rough measurement of TCRγ gene rearrangement and provides a general idea of the percentage of rearrangements but is not an accurate quantitative tool.

To conclude, our study provides compelling evidence that a population of NK cells does, in fact, develop in the thymus during steady state NK cell production. The NK cell potential demonstrated by others in the bipotent T/NK progenitors in the fetal environment and in the
adult DN1 and DN2 thymocytes does represent a normal pathway of NK cell development and reveals a close developmental relationship between T cells and NK cells.
4 THE THYMUS-DEPENDENT DEVELOPMENTAL PATHWAY GENERATES
UNIQUE SUBSETS OF NK CELLS IN THYMUS AND LYMPH NODE

4.1. Introduction

In the previous chapter we determined that Tcryn NK cells are produced via a thymus-dependent pathway. Now that the pathway has been identified, it is important to identify the precursors that give rise to the thymus-dependent NK cells. We hypothesize that the thymus progenitors that produce Tcryn NK cells are the DN2 progenitors. DN3 cells have irreversibly committed to the T cell lineage while DN1 and DN2 cells retain NK cell potential and DN2 cells undergo TCR rearrangement. It has been shown that 1 in 9 DN1 cells and 1 in 14 DN2 cells become NK cells in vitro. Although NK cell potential has previously been demonstrated in these early T cell progenitors, the NK cells produced in culture have not been further characterized beyond NK1.1 expression. Also, it was assumed that this pathway is not realized in steady state NK cell development in vivo. The knowledge of TCRγ gene rearrangement in thymus-dependent NK cells can be used as a marker to test whether DN progenitors are the precursors to thymus NK cells. It is also important to determine if the thymus-dependent pathway plays a unique role in the generation of NK cells. For example, do the NK cells have different functional capabilities or a phenotype that would suggest a unique role for the cells? Does this pathway produce cells that localize in other tissues? While we have determined that the thymus-dependent pathway does not significantly contribute to NK cell populations in the spleen or BM since their percentage of NK cells with TCRγ gene rearrangement is very low, it is unknown if this pathway is important in other tissues besides the thymus. The significance of the thymus-dependent pathway is addressed in this chapter.
4.2. Results

4.2.1. DN1 and DN2 thymocytes differentiate into NK cells

An NK cell differentiation culture system was used to generate NK cells from DN1 and DN2 thymocytes (Figure 4.1).

Figure 4.1. Scheme of thymus (or LN) DN progenitor culture. DN progenitors are sorted and then cultured with a cytokine cocktail to induce NK cell development. The NK cells produced are then analyzed by FACS or sorted and DNA is isolated for genomic PCR analysis.

In previous studies, NK cell potential was reported in T cell differentiation conditions using OP9-DL1 stroma, which promotes T cell development. Bulk thymocytes were first depleted of lineage marker+ (CD3, CD8, TCRβ, TCRγδ, CD19, B220, Mac-1, GR-1, NK1.1, and Ter119)
cells and then DN1 (Lin'CD44+CD25\(^+\)) and DN2 (Lin'CD44+CD25\(^+\)) cells were sorted. To compare the NK cell differentiation of these cells with those of defined NK cell progenitors, we also sorted and cultured BM NKP (Lin'NK1.1'CD122\(^+\)) cells. Cells were grown on OP9 stroma with a cytokine cocktail of IL-15, IL-7, SCF, and flt3L. After 10-12 days cells were analyzed by FACS. At the end of all cultures, the majority (over 90% in all cases) of cells were NK1.1\(^+\) (Fig. 4.2). NKG2A and CD94 expression was very high while Ly49 expression was variable but was always lower than normal in vivo NK cell levels (Fig. 4.2b). This does not appear to be a result of the progenitor tissue origin as the same was evident in both the thymus DN cultures and the BM NKP cultures. Rather, it appears to be a result of the culture conditions. Although the Ly49 positive population was small, it was always a clearly visible, separate population, as seen in Fig.4.2a. Cells also expressed low levels of IL-7R\(\alpha\), Mac-1 and NKG2D. 2B4 expression was only examined in one culture of DN1 and DN2 cells but not NKP cells and its expression was very high (Fig. 4.2b). All other values are taken from 5 to 9 replicate cultures.
Figure 4.2. Thymus DN1 and DN2 progenitors have the potential to give rise to NK cells during in vitro cultures. BM NKP and DN1 and DN2 progenitors were cultured on OP9 in NK cell differentiation conditions. (a, b) Profiles of cells at the end of cultures. (a) Values are representative of one culture set. (b) Averages of receptor expression each from at least 4 cultures (except 2B4).
4.2.2. DN2-derived NK cells are $T_{cry}^+$

The DN1- and DN2-derived NK cells were sorted and TCRγ gene rearrangements were examined. Semi-quantitative genomic PCR showed that 25-50% of DN2-derived NK cells had $\gamma_2\gamma_1$ rearrangements while DN1-derived NK cells had low TCRγ gene rearrangement with about 5% of cells being positive (Fig. 4.3a,b). This is to be expected since TCR gene rearrangement only begins at the DN2 cell stage. Therefore, $T_{cry}^+$ NK cells likely arise from DN2 cells in vivo while other thymus NK cells likely arise from the earlier DN1 progenitors which have not begun TCRγ gene rearrangement yet.

4.2.3. Thymus NK cells are phenotypically different from NK cells in other tissues

In the previous chapter, it was shown that almost 50% of thymus NK cells have rearranged TCRγ genes, suggesting that the majority of thymus NK cells develop in the thymus. To determine if the thymus-dependent pathway of NK cell development produces NK cells that...
differ from those in other tissues, we performed FACS analysis of NK cells from the thymus and compared them to NK cells from the spleen, liver, lung, BM, and mesenteric lymph nodes. The NK cell profile is difficult to visualize in the wild type thymus with such a large percentage of CD3$^+$ cells, and depletion of T cells seems to skew the NK cell profile as well. Therefore, thymuses from TCRβ$^{-/-}$ mice were used. No T cells or NKT cells are present in these mice and the NK cell population is easily visualized. As expected, NK cell receptor expression on splenic NK cells from these mice is the same as B6 splenic NK cells (Table 4.1b). There are many significant differences in receptor expression on thymus NK cells compared to those of other tissues. Thymus NK cells exhibit both extremes of expression when compared to other tissues (Table 4.1a).
Table 4.1. Global FACS analysis of NK cells from the thymus, LN, BM, spleen, liver and lung.

(a) Averages of percentages of receptor expression on NK cells from various tissues determined by FACS analysis. Values are from triplicate (or more) experiments. Values marked with * are significantly different than spleen NK cells with a p-value <0.05. (b) Percentages of receptor expression on NK cells from the spleen of TCRp^+δ^- mice.

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Cells expressing NKG2A, CD94, 2B4, and IL-7Rα are most abundant among thymus NK cells while those expressing Mac-1, Ly49A, C/I, G, and D are very rare (Fig. 4.4, 4.5, 4.6). More thymus NK cells express Ly49H than lymph node and BM NK cells but less than splenic NK cells. For the Ly49H values, double staining with 5E6 (Ly49C/I) and 1F8 (Ly49C/I/H) mAbs was performed. The Ly49H value represents Ly49H single positive cells, so the value is lower.
than reported values since double positive (Ly49H\textsuperscript{+}Ly49C/I\textsuperscript{+}) cells were excluded. Interestingly, the percentage of IL-7Rα\textsuperscript{+} cells is very high in thymus NK cells and LN NK cells are the only other population with a moderate percentage of IL-7Rα\textsuperscript{+} cells (Fig. 4.4). Also, thymus NK cells have the lowest Mac-1 expression, with LN NK cells a distant second. All thymus NK cell receptors have significantly different expression pattern from splenic NK cells except Ly49H and 2B4. The intensity of receptor staining did not differ between NK cell populations. These results suggest an immature NK cell phenotype (CD94/NKG2\textsuperscript{hi}Ly49\textsuperscript{lo}Mac-1\textsuperscript{lo}) of thymus NK cells and also a most closely shared phenotype with LN NK cells.

Figure 4.4. Thymus NK cells appear 'immature' (Mac-1\textsuperscript{lo}IL-7Rα\textsuperscript{hi}) compared to other tissue NK cells. FACS analysis of Mac-1 and IL7Rα expression on NK cells from various tissues. The percentages of IL-7Rα and Mac-1 expression in the thymus NK cells are statistically significant from spleen NK cells with a p-value >0.05.
Figure 4.5. Thymus NK cells have the highest percentages of NKG2A/C/E and CD94 expression and average 2B4 expression. FACS analysis of NKG2A/C/E, CD94, and 2B4 expression on NK cells from various tissues. The percentages of NKG2A/C/E and CD94 on thymus NK cells are statistically significant from spleen NK cells with a p-value >0.05.

Figure 4.6. Thymus NK cells have the lowest percentages of Ly49A, G, D, and C/I expression. FACS analysis of Ly49 expression on NK cells from various tissues. The expression of Ly49A, Ly49G, Ly49C/I and Ly49D are all significantly different between thymus and spleen NK cells.
4.2.4. TCRγ gene rearrangement in LN NK cells suggests a link with DN-derived thymus NK cells

To determine whether the NK cells produced via the thymus-dependent developmental pathway migrate to other tissues, we purified NK cells from lung, liver, and both mesenteric and peripheral LNs and checked for presence of TCRγ gene rearrangement by genomic PCR since this is a marker of NK cells that originated from the thymus pathway rather than the BM pathway. Tissues with a significant Tcrrγ+ NK cell population have NK cells that originate via the thymus-dependent pathway and selectively migrate to the tissue. As shown in chapter 3, 50% of thymus NK cells have TCRγ gene rearrangement while less than 5% of splenic and BM NK cells do (Fig. 4.7c). Lung NK cells have negligible levels of TCRγ gene rearrangement as do liver NK cells, except on one occasion where a visible band was detected. Both peripheral and mesenteric LN NK cells, on the other hand, consistently had TCRγ gene rearrangement (Fig. 4.7a). Semi-quantitative genomic PCR showed that approximately 20-25% of LN NK cells are positive for TCRγ gene rearrangement (Fig. 4.7b).
Figure 4.7. LN NK cells have the highest percentage of Tcrγ+ NK cells other than thymus NK cells.

(a) Equivalent amounts of DNA from NK cells from each tissue were examined by genomic PCR for the presence of TCRγ gene rearrangement. Agarose gel electrophoresis and ethidium bromide staining of genomic PCR is shown. (b) Southern blots of genomic PCR with Jγ1 probe was carried out as in Figure 3.5 to estimate the percentage of TCRγ gene rearrangement in mesenteric (M-LN) and peripheral (P-LN) LN NK cells. Genomic PCR (Vγ2-Jγ1) was performed with δγT cell DNA and fibroblast DNA mixed at various ratios and with NK cell DNA. (c) A comparison of the percentage of TCRγ gene rearrangement in NK cells of multiple tissues.

4.2.5. DN1 and pre-DN2 cells in LNs give rise to NK cells in culture

The above results suggested thymic origin of LN NK cells. However, it was unknown whether they develop in the thymus and migrate to the LN or whether immature thymocytes migrate to the LN and differentiate into NK cells within the LN. Terra et al. previously showed that LNs have DN1 and pre-DN2 (CD44+CD25lo) progenitors present. The DN1 cells differ from the ETP profile of the thymus, and no CD25hiDN2 or DN3 cells are present in the LN.

Furthermore, LN DN cells in normal mice are unable to differentiate into T cells whereas their
Figure 4.8. **DN1 and pre-DN2 progenitors are present in the lymph node and they possess NK cell potential in vitro.**

(a) Lin− cells in the LN were gated and DN1 and pre-DN2 profiles are shown. (b) DN1 and pre-DN2 cells were sorted and cultured in NK cell differentiation conditions as in Figure 4.1. FACS profile of cells at the end of one representative culture is shown. (c) Southern blot of genomic PCR with Jyl probe of NK cells that differentiate during the culture was used to estimate the percentage of NK cells with TCRγ gene rearrangement. Genomic PCR (γ2-Jγ1) was performed with γT cell DNA and fibroblast DNA mixed at various ratios and with NK cell DNA.

NK cell potential has not been addressed. To test the possibility of thymus-derived progenitors migrating to the LN and producing thymus-dependent NK cells, the NK cell potential of the LN DN1 and pre-DN2 cells was tested. The DN cells were purified and cultured for NK cell differentiation as described for DN thymocytes. The majority of cells recovered from the
cultures were NK cells very similar to those from DN thymocyte cultures. These NK cells expressed a detectable level of Ly49 receptors as well (Fig. 4.8a,b). LN DN-derived NK cells were purified by cell sorting and TCRγ gene rearrangement was checked by genomic PCR. As seen in the thymus cultures, DN1-derived NK cells had negligible levels of rearrangement while pre-DN2-derived NK cells had significant levels of rearrangement (Fig. 4.8c).

To confirm that the NK cells were not from contaminating NK cells that expanded during the culture, the same in vitro culture was performed with DN1 and pre-DN2 cells from IL-15+/− mice which have greatly reduced NK cell numbers. The end result of these cultures was the same as the wild type cultures. Once again the majority of cells were NK cells and they expressed detectable levels of Ly49 receptors (Fig. 4.9).

![Image](image.png)

**Figure 4.9.** DN progenitors from IL-15+/− mouse LNs still show NK cell potential in vitro. DN progenitors from IL-15+/− mice, which lack NK cells, were cultured in NK cell differentiation conditions as in Figure 4.1. FACS profiles of cells recovered from a representative culture.
4.2.6. The DN cells and Tcrγ+ NK cells in the LN are thymus-dependent

To confirm that the Tcrγ+ NK cells in the LN were thymus-dependent rather than being derived through an alternate BM developmental pathway, we checked for Tcrγ+ NK cells in the LNs of nude mice, which lack a thymus. The NK cells in nude LNs lack TCRγ gene rearrangement. Therefore, the population from the thymus-dependent pathway is absent in these mice (Fig. 4.10b). In addition, we found that DN1 and pre-DN2 cells are greatly reduced in nude mouse LN. In wild type mice, approximately 50% of Lin− cells are DN1 whereas in the nude mouse, only 3-9% of Lin− cells are DN1 progenitors. Pre-DN2 cells were 7 fold fewer in nude LNs (Fig. 4.10a). These results indicate that LN DN cells derive from the thymus.

Figure 4.10. Nude mouse LNs have lower levels of DN1 and pre-DN2 progenitors than wild type LNs and nude LN NK cells do not have TCRγ gene rearrangement. (a) Lin− cells were gated and then DN1 and pre-DN2 profiles from nude mouse mesenteric LNs were examined. (b) Agarose gel electrophoresis and ethidium bromide staining of genomic PCR to test for TCRγ gene rearrangement in nude mouse LN NK cells and NKG2A genomic PCR control.

4.2.7. Preliminary results suggest that LN DN progenitors give rise to NK cells in vivo

LN DN1 and pre-DN2 progenitors were sorted from IL-15−/− donors and were injected intraperitoneally (i.p.) into three Nod Scid IL-2 receptor gamma−/− mice, which are deficient for all lymphocytes including NK cells. After three weeks, the spleens, thymuses, BM, and LNs
were examined for the presence of CD3\(^+\)NK1.1\(^+\) cells (Table 4.2a). The host strain does not express NK1.1 and therefore all NK1.1\(^+\) cells are donor derived. Lymph nodes of untreated hosts were invisible. Donor-derived NK cells were undetectable in the BM but detectable in the LN, thymus, and spleen. Mouse 1 and 3 had small numbers of CD3\(^+\)NK1.1\(^+\) cells while mouse 2 did not. Mouse 3 had the highest percentage of NK cells in all tissues. Interestingly, when CD3\(^+\)DX5\(^+\) cells were examined, the population was much higher (Table 4.2b). The host mouse NK cells express DX5 but not NK1.1. However, NK cells are deficient in the host mice and undetectable in the spleen. Therefore, the CD3\(^+\)DX5\(^+\) cells in the transplanted mice were likely donor derived, but it remains to be confirmed. The reason for the difference in NK1.1 versus DX5 expression is unknown. It is possible that the LN DN progenitors selectively give rise to a DX5\(^+\)NK1.1\(^-\) cells. Although such cells are readily detected in normal B6 mouse LN (data not shown), their identity is unknown. To conclude, LN DN progenitors do appear to give rise to NK cells in vivo although the number of LN DN-derived NK cells is low.

A second study was performed with Pep3b mice as DN progenitor donors and Nod Scid IL-2 receptor gamma\(^-\) mice were once again recipients. Two mice were intravenously (i.v.) injected with LN DN cells. Four weeks later, the spleen and BM were examined for donor-derived NK1.1\(^+\) NK cells (Table 4.2a) as well as DX5\(^+\) NK cells (Table 4.2b). Once again, small numbers of NK cells were observed.
Table 4.2. LN DN1/pre-DN2 cells produce small numbers of NK cells following one i.p. and one i.v. transplantation.

(a) LN DN cells (Lin\(^{-}\)CD44\(^{+}\)CD25\(^{-}\)) from B6 mice were purified by FACS sorting and 2\(\times\)10\(^{4}\) cells were injected i.p. or i.v. into each NOD Scid gc\(^{-}\) mouse. Tissues were examined 3 weeks (i.p.) or 4 weeks (i.v.) after transplantation for donor-derived NK cells by NK1.1 expression. (b) The spleens (and BM for i.v.) of each mouse were examined for DX5\(^{+}\) NK cells. All values are minus the background staining in control mice.

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4.2.8. Thymus NK and LN NK cells produce lower levels of IFN\(\gamma\) upon stimulation

NK cells from thymus, LN, and spleen were stimulated with IL-12 and IL-18 for 24 hours and their level of intracellular IFN\(\gamma\) was determined by FACS analysis. About 18% of thymus NK cells and 33% of LN NK cells produced IFN\(\gamma\) whereas 45-48% of splenic NK cells produced IFN\(\gamma\) (Fig. 4.11). Although the fractions of IFN\(\gamma\)-producing NK cells in the thymus and LN were significantly smaller than those in the spleen, the amount of IFN\(\gamma\), as determined by the fluorescence intensity, in the IFN\(\gamma\)-positive NK cells from the thymus and LN was higher than that of spleen NK cells.
Figure 4.11. LN NK cells and thymus NK cells produce lower levels of IFNy after IL-12 and IL-18 stimulation than spleen NK cells.
LN cells, thymocytes and spleen cells were stimulated with IL-12 and IL-18 for 24 hours, stained for CD3, NK1.1 and intracellular IFNy, and the percentage of NK (CD3⁻NK1.1⁺) cells that were positive for intracellular IFNy over isotype matched control antibody staining was determined. Values are representative of triplicate experiments. Both LN (p-value= 2.40947E⁻¹³) and thymus (p-value= 1.03E⁻⁷) NK cell IFNy levels are significantly lower than spleen NK cells.

4.2.9. Thymus NK cells have normal levels of cytotoxicity
Spleen cells and thymocytes were cultured with IL-2 to generate LAK cells and their cytotoxicity was tested. A cytotoxicity assay measures NK cells' ability to kill MHC class I deficient target cells or in other words, their natural cytotoxicity level. NK cells are the effector cells and they are mixed with target cells at various ratios and the number of target cells killed is observed. LAK cells were mixed with CFSE-labeled RMA-S cells at various effector:target (E:T) ratios (1:1, 1:2, 1:5, 1:10 and 1:20) for four hours and their cytotoxicity was measured by FACS analysis. The level of cytotoxicity was determined as the percentage of CFSE⁺ target cells that were PI⁺ (minus the background level). Although thymus NK cells have slightly lower cytotoxicity than spleen NK cells, the difference is not significant. The p-value from the student's t-test was lower as the E:T ratio increased but at 1:20 E:T ratio the p-value=0.08 and was therefore still not significantly different (Fig. 4.12).
Figure 4.12. Spleen and thymus NK cells have similar levels of cytotoxicity.
Spleen cells and thymocytes were cultured with IL-2 to stimulate and amplify NK cells. IL-2-stimulated NK cells recovered from the cultures were mixed with CFSE labeled RMA-S cells at ratios of 1:1, 1:2, 1:5, 1:10 and 1:20. Cytotoxicity was determined by the percentage of PI+ cells among CFSE+ (RMA-S) cells by FACS analysis. Values are representative of quadruplicate experiments.

4.3. Discussion

The results presented in this chapter have shown that significant fractions of NK cells in the thymus and lymph nodes of normal mice have rearranged TCRγ genes. The lack of these NK cells in nude mice indicates that they are thymus-dependent. Therefore, the thymus-dependent developmental pathway proposed in chapter 3 is not restricted to thymus NK cells but a subset of LN NK cells also develop through this pathway. The thymus NK cells most probably derive from DN1 and DN2 thymocytes that readily differentiate into NK cells in vitro while those derived from DN2, but not DN1 are Tcrγ+.

DN progenitors are also present in LNs\textsuperscript{141}. We have demonstrated that they have NK cell potential and can specifically give rise to Tcrγ+ NK cells in vitro. Out of the Lin− cells in the LN, 56% of cells are CD44+CD25− (DN1) and 6.7% are CD44+CD25\textsuperscript{lo} (pre-DN2)\textsuperscript{141}. It has
been reported that the DN1 progenitors in the LN differ from those in the thymus. In the thymus there are two progenitors that can generate T cells, the ETPs, which are Lin"Sca-1"c-Kit"IL-7Rα" and then also, Lin"Sca-1"c-Kit"IL-7Rα" cells. Only the second population is located in the LNs. These DN1 cells in the LN progress to a pre-DN2 stage with CD44"CD25" expression but then become cell cycle arrested in the G1 phase. This is because they lack expression of Wnt4 which is needed to upregulate certain genes and downregulate others for the cells to progress in T cell development.  

Although we can conclude that a subset of LN NK cells is thymus-dependent, it is still unclear whether NK cells that develop in the thymus migrate to the LN or DN thymocytes migrate to the LN and differentiate into NK cells. Since thymus NK cells and LN NK cells significantly differ in their phenotypes, it is unlikely that thymus NK cells significantly contribute to the thymus-dependent LN NK cell population. It is more likely that thymus-derived DN progenitors migrate to LN and differentiate into TCRγ+ NK cells. This is supported by the fact that LN DN1 and pre-DN2 cells efficiently differentiate into NK cells during in vitro cultures. They also give rise to NK cells, albeit in small numbers, in vivo as revealed by our preliminary transplantation studies. The LN environment also seems to be supportive for NK cell development. The gut (mesenteric LNs) has high IL-7 expression which would likely allow extrathymic TCRγ gene rearrangement to occur. Also, human LN DCs produce IL-15 upon stimulation, which is crucial for NK cell maturation. Finally, early T cell progenitors do have the capacity to migrate out of the thymus without losing their differentiation potential or their capacity to colonize primary lymphoid organs. Until very recently it was believed that only mature T cells could exit the thymus. However, a new study has shown that early T cell progenitors, specifically DN2 and DN3 progenitors, migrate to the gut and produce CD8αα+T-
IELs in the gut cryptopatches\textsuperscript{142}. The thymus is only important for the establishment of the CD8\alpha\alpha\^+ pool during development because if a thymectomy is performed after the neonatal period, this cell population still developed normally. It therefore seems that the DN2 and DN3 cells that migrate and settle in the cryptopatches have a longer lifespan and can self renew.

One contradiction to the hypothesis that LN DN progenitors produce NK cells is that the LN DN1 phenotype resembles CLPs, rather than the thymic T cell precursors, which share NK cell potential. It would be expected that since the cells both share a thymus-dependent pathway via common precursors, that the DN1 phenotype in the LN would be the same as that in the thymus. One possibility may be that the phenotype of DN1 changes as the cells migrate from the thymus and settle in the LN.

It is still unknown what fractions of LN NK cells develop through this pathway. Although about 20\% of normal NK cells are Tcrγ\^+, it is very likely that more LN NK cells are thymus-dependent, because not all thymus-dependent NK cells are Tcrγ\^+. Both DN1 and DN2 thymocytes show NK potential in NK differentiation cultures in vitro while only DN2-derived, but not DN1-derived, NK cells are Tcrγ\^+. Similarly, NK cells generated from pre-DN2, but not DN1, LN cells in vitro are Tcrγ\^+. Since no marker is available to identify DN1-derived NK cells, the extent of DN1-derived NK cell development cannot be determined.

The thymus-dependent pathway that produces thymus and LN NK cells very likely plays a unique role in the generation of NK cells that differs from the BM pathway of development. It produces NK cells with unique phenotypes and functional abilities. FACS analysis of NK cells from the spleen, BM, liver, lung, LN and thymus revealed that each tissue exhibits specific
differences in NK cell phenotype. Strikingly, thymus NK cells are the most different with receptor expression values on both extremes: the highest levels of certain receptors and the lowest levels of others. The thymus NK cell phenotype resembles that of an immature NK cell at an intermediate stage of development. They have high expression of NKG2A/C/E and CD94 and low expression of most Ly49 receptors. Also, immature NK cells are normally classified as Mac-1$^{lo}$CD43$^{lo}$ and cells at this stage can also express IL-7Rα. Thus, most thymus NK cells have the immature phenotype. Whether the thymus NK cell are 'immature' or whether this phenotype does not represent an immature developmental stage in the thymus-dependent pathway remains to be further studied.

While LN NK cells are not as different from other tissue NK cells (and presumably BM-derived NK cells) in receptor expression, they are the population that is the most similar to thymus NK cells based on the expression of IL-7Rα and Mac-1. Their CD94/NKG2 and Ly49 receptor expression patterns are more similar to those of other tissue NK cells but other similarities exist between the LN and thymus NK cells as well. They are both poor IFNγ producers but have regular cytotoxicity compared to splenic NK cells. Since a thymus-dependent pathway is shared between thymus NK cells and at least a subset of LN NK cells, one may expect the phenotype to be the same for the two populations. This need not be the case as the difference in thymus and LN profiles could either be due to further maturing of thymus NK cells after they migrate to the LN or most likely due to DN progenitors giving rise to NK cells in the LN. The difference in the LN developmental environment (and perhaps DN1 progenitor identity) likely creates the differences in NK cell phenotypes.
Chen, et al.\(^{144}\) previously showed that LN NK cells produced lower IFN\(\gamma\) levels than splenic NK cells but they tested this following poly I:C stimulation in vivo, which stimulates NK cell effector functions via TLR3 ligation\(^{145}\). The LN NK cell cytotoxicity was not tested in our study because it has already been shown to be the same as spleen NK cells\(^{144}\). Although we tested thymus NK cell cytotoxicity following LAK culture (to increase the cell number for the assay), Carlyle et al. previously examined the cytotoxicity of fresh fetal and adult thymus NK cells without stimulation and showed that they were functionally mature and lysed Yac-1 target cells in a cytotoxicity assay\(^{146}\). Therefore, the IL-2 stimulation did not induce functional maturation in the cells and they are cytotoxic as fresh cells as well.

In this study, we defined thymus DN1 cells for our in vitro cultures as CD4\(^{+}\)CD8\(^{-}\)CD3\(^{-}\)CD44\(^{+}\)CD25\(^{-}\), which is very heterogenous and includes committed B cell progenitors, myeloid cells, and DCs in addition to T cell progenitors. Balciunaite et al.\(^{36}\) compared c-kit\(^{+}\) and c-kit\(^{-}\) DN1 and DN2 cells and the lineage marker expression difference between CD117\(^{+}\) and CD117\(^{-}\) DN cells was striking. The CD117\(^{-}\) cells expressed a broad array of markers (CD19, NK1.1, CD11c, and CD11b) but the CD117\(^{+}\) cells did not express any of these. They further characterized CD117\(^{+}\) DN1 and DN2 cells to show that they have T cell and NK cell potential but lack B cell potential. Also, early thymus progenitors (ETPs), which are the earliest and most efficient T cell progenitors, fall within the CD117\(^{+}\) DN1 classification. More than half of the cells express CD4 and therefore CD4 was not included in our lineage marker cocktail for lineage negative selection, to ensure that the ETP/DN1 cells were not removed. The novel classification of ETP/DN1 is Lin\(^{10}\)CD25\(^{-}\)CD117\(^{+}\). These cells are uniformly CD44\(^{+}\) so this marker does not need to be included. Although we used the older classification of DN1 cells, which includes the committed cells in the heterogenous mix, it is not likely that the NK cells in
our culture arose from these other cell types. Firstly, committed NK cells were removed since NK1.1 was included in the lineage marker cocktail. Secondly, Porritt, et al. further divided the CD117+DN1 population into DN1a-e by CD24 expression. Porritt demonstrated that the true ETP is DN1a (CD117+CD24+CD127). These cells have the slowest kinetics of differentiation, the highest proliferative burst potential, and the ability to home to the thymus following i.v. transplantation. Most importantly for our study, only the true T cell progenitor populations (DN1a and its progeny, DN1b) have NK cell potential. Therefore, by excluding mature NK cells from the sort and gating on DN1 cells only as LinCD44+CD25+, only the true T cell precursor within the broader DN1 classification possesses NK cell potential.

Therefore, when we demonstrate that Tcrγδ+ NK cells arise from thymus DN1 (and DN2) cells, they are truly arising from the same progenitors that produce T cells.

To conclude, this chapter revealed that the thymus-dependent developmental pathway produces NK cells in both the thymus and LNs. It is very likely that thymus-dependent DN progenitors, in both the thymus and LN, give rise to Tcrγδ (and Tcrγδ cells, which cannot be measured) NK cells. These NK cells are distinct and likely have unique roles that differ from BM-derived NK cells.
5 GENERAL DISCUSSION

The most significant outcome of this thesis research is the discovery of novel pathways of NK cell development. We have demonstrated that TCRγ genes are rearranged in subsets of NK cells in the thymus and LN and that they develop via thymus-dependent pathways. Furthermore, the phenotypes and functions of NK cells in the thymus are different from those of spleen NK cells. Most studies on mouse NK cell development have focused on NK cells in the spleen, liver, and BM, and it has commonly been assumed that all NK cells developed via the same pathway in the BM. Differences in receptor expression on NK cells in various tissues have been thought to be due to different developmental stages/maturation levels or due to migration of certain subpopulations of BM-derived NK cells. Our results now suggest that various developmental pathways and locations may produce different types of NK cells. Our updated NK cell developmental pathway scheme is shown in Figure 5.1.
Figure 5.1. Revised model of NK cell development in the mouse. The BM and thymus-dependent pathway of NK cell development are illustrated. Participation of the LN in the thymus-dependent pathway is shown as well as potential locations of other unidentified NK cell developmental pathways.

In a very recent review, Di Santo$^{46}$ addresses the topic of NK cell development in other tissues as well. Two important points from the review are that NKPs have been identified in LNs and spleen as well as the previously described fetal thymus and adult BM locations. Presence of NKPs in LNs strengthens the hypothesis that NK cells develop from thymus-derived DN progenitors in the LN. Other developmental intermediates need to be looked for in the LN. Our laboratory is also currently examining the possibility of NKPs in lungs. Secondly, Di
Santo addresses the fact that BM ablation greatly affects NK cell numbers in the periphery. Although this is often cited as evidence that the BM is required for NK cell development perhaps these treatments may affect the capacity of NK precursors to respond to maturation signals that they would encounter elsewhere in the body. The BM may also only be involved in the initial steps of NK cell differentiation, with other sites required for NK cell maturation.

5.1. Thymus-dependent NK developmental pathway

Our studies with in vitro differentiation of DN1/DN2 thymocytes and DN1/pre-DN2 LN cells into NK cells clearly demonstrated that they have NK cell potentials. Furthermore, TCRγ gene rearrangement in large fractions of thymic and LN NK cells in normal mice and the lack of such cells in nude mice indicate that NK cell potentials of these cells are realized in normal mice. Regardless of the origin of the LN Tcργ+ NK cells, whether it is thymus DN progenitors that migrate to the LN and give rise to LN NK cells or immature thymus NK cells that migrate to the LNs and further mature, both LN Tcργ+ NK cells and thymus NK cells are thymus dependent and develop from a shared pathway. This pathway includes T cell precursors that retain NK cell potential up until a stage after V(D)J recombination has begun and this pathway differs from the BM pathway in location, precursors, and output.

As discussed in the previous chapter, not all thymus-dependent NK cells are Tcργ+, and no specific marker is available to identify all thymus-dependent NK cells. Therefore, the extent of the contribution of this developmental pathway in the overall NK population is difficult to assess. However, it should be noted that neonatal as well as adult nude mice have normal number of NK cells in the spleen. Therefore, it seems likely that the contribution of thymus-dependent NK cell development is tissue-specific. We have found that Tcργ+ NK cells are
abundant in the thymus and LNs and rare in spleen, liver, lung and BM, but we have not
examined other tissues. It is possible that Tcrγ+ NK cells may localize in other tissues. Gut-
associate lymphoid tissues are of particular interest, because DN2 (and DN3) thymocytes have
been shown to migrate and become a unique T cell population in gut cryptopatches. Perhaps
NK cell potential is realized in this location as well.

It is of great interest to determine what the significance of this pathway is. This pathway could
simply be an evolutionary remnant since studies have suggested that the NK cell fate may be
the default fate of thymocytes\textsuperscript{35}. Why does the thymus retain NK cell potential? Perhaps the
thymus is a main supplier of NK cells to the peripheral lymphoid tissues during the
fetal/neonatal stages since NK cells are generated first in the FT during ontogeny. This is
supported by the bipotent T/NK progenitors in the fetal development and by our studies which
show higher TCRγ mRNA expression in neonatal NK cells than in adult NK cells. The
pathway may remain in the adult while most hematopoiesis switches to the BM. Another
option is that there may just be no necessity in cutting off NK cell potential before entering the
thymus since it does not seem to disturb T cell generation. Since the NK cell potential is
retained in DN progenitors and few signals are likely required to switch the cell’s fate from a T
cell to an NK cell and vice versa, it may be that the NK cell potential is occasionally realized in
vivo. As discussed in more detail later, it is possible that there are certain areas in the thymic
environment that are more permissive of the NK cell lineage decision. Also, the NK cell fate
may be adopted when a crucial signal for the T cell lineage is not received or perhaps when
TCR gene rearrangements are not successful. Two findings argue against this hypothesis. First,
the NK cell fate is lost after the DN2 stage, when only complete TCRγ and TCRδ gene
rearrangements are seen but rarely TCRβ gene rearrangements. Secondly, one would expect to
see more incomplete TCRδ gene rearrangements as well as TCRγ gene rearrangements in NK cells. Regardless of how the pathway arose, it does not seem to be redundant, because the thymus-dependent pathway produces distinct NK cells.

5.2. Mouse NK cell subsets

We hypothesize that the developmental pathway and location influence the NK cell phenotype and function resulting in distinct NK cell subsets. Our FACS analysis of NK cells in various tissues indeed showed that surface phenotypes of NK cells in the thymus are significantly different from those in spleen, liver, lung and BM. However, the interpretation of this finding is complicated, because NK cells in a given tissue are heterogeneous with respect to their maturity. Kim et al. identified distinct steps in NK cell development in the BM based on the expression of various receptors. According to their scheme, immature NK cells are Mac-1lo whereas mature NK cells are Mac-1hi. Hayakawa et al. have recently defined two mature NK cell subpopulations, namely a Mac-1hiCD27hi and a Mac-1hiCD27lo population. They differ in receptor expression, tissue distribution, proliferation, function, and chemokine sensitivity. CD27lo cells have higher Ly49C/I levels, which are the self-MHC I receptors in B6 mice, and higher KLRG1 expression. They are also long-lived, senescent cell and they localize in peripheral tissues such as spleen, liver, lung and blood. CD27hi cells show greater responsiveness to activatory ligand on tumor cells and are cytotoxic to tumor cells even in the presence of MHC class I expression. These cells also respond more strongly to IL-12 and IL-18 stimulation as well as to stimulation directly from DCs. Finally, CD27hi cells express high levels of CXCR3 which is crucial for LN migration and they localize in the LNs and BM. Importantly, the cells in each tissue retain the same receptor profiles that accompany the Mac-1hiCD27hi/lo phenotypes. Immature NK cells and two subsets of mature NK cells defined by
Hayakawa et al. coexist in individual tissues. These NK subsets differ from each other in the pattern of Ly49 and other cell surface marker expression, resulting in high degree of heterogeneity in the overall NK population in individual tissues. Since the majority of thymus NK cells are Mac-1\(^{-10}\) and IL-7R\(\alpha^+\) and only small fractions express Ly49, they can be considered immature. On the other hand, the immature phenotype is less obvious with LN NK cells. Almost 50% of them are Mac-1\(^{hi}\), and their Ly49 expression pattern is similar to that of spleen NK cells. Therefore, it still remains to be determined whether thymus-dependent developmental pathway generates unique population of NK cells and whether the location of NK development influences the phenotype and functions of the products.

5.3. Human NK cell subsets and LN pathway of development

There are two main subsets of NK cells in the human (reviewed in \(^{149}\)). One subset, defined by CD56\(^{dim}\), has high cytotoxicity but low cytokine production. The other subset, CD56\(^{bright}\), has poor natural cytotoxicity but plays an immunoregulatory role and are potent cytokine producers. Interestingly, CD56\(^{bright}\) NK cells are ten times more frequent in the LNs than in the blood. It is likely that the CD56\(^{bright}\) subset develops in the LNs since CD34\(^+\) NK precursors, which differentiate in vitro into CD56\(^{bright}\) NK cells, are found in the T cell rich region of LNs\(^{143}\). A follow up study showed that LNs contain all developmental intermediates spanning from CD34\(^+\) progenitors to CD56\(^{bright}\) NK cells\(^{150}\). Each population is capable of downstream NK cell differentiation ex vivo. T cell and DC potential are lost during progression through the developmental stages and NK cell cytotoxicity, cytokine production and NK cell receptor repertoire is acquired during development\(^{150}\). These studies demonstrate that a distinct subset of NK cells develops in human LN. The correlation between this developmental pathway in humans and the thymus-dependent pathway we found in mice is unclear. NK progenitors in
human LN likely derive from BM whereas Tcrγ NK cells in mouse LN seem to develop from DN2 thymocytes or pre-DN2 cells in the LN. No TCR rearrangement in human LN NK cells has been reported. Nevertheless, it is of interest that NK cells generated from adult CD34+ progenitor cells in BM stroma-free cultures with IL-15 resemble the CD56bright NK cells with high CD94/NKG2 expression but little or no CD16 or KIR expression. This phenotype is similar to mouse thymus NK cells, which are mostly CD94/NKG2hi and Ly49- (mouse counterpart of KIR). However, the functions of mouse thymus NK cells are different from human LN-derived NK cells as the former are poor producers of IFNγ upon stimulation with IL-12 and IL-18. Further analyses are needed to determine how these subsets relate to each other.

5.4. Thymus NK cells

While we have identified their precursors and characterized their phenotype, we have not defined the role of thymus NK cells. It does not appear that thymus NK cells are hyporesponsive because their cytotoxic abilities are at normal levels even though IFNγ production is low. Although Ly49C/I expression is very low, NKG2A and CD94 expression are very high. The three self-MHC class I specific receptors in C57BL/6 are Ly49C, Ly49I, and NKG2A/CD94. Therefore, it is likely that these cells passed through the stage of self tolerance induction without becoming (disarming model) or remaining (arming model) hyporesponsive. Perhaps since there are fewer cells (i.e. DCs) to influence with IFNγ in the thymus, only a lower level is required to be effective.

Thymus derived NK cells may be locally involved in the regulation of thymopoiesis. LAK cells generated by IL-2 stimulation of DN progenitors selectively killed DP progenitors but
not any other progenitors. Therefore, NK cells may aid in the elimination of the majority of DP cells that do not proceed through positive selection. DP thymic autoreactive clones are expected to be “activated” after interaction with their substrate and thus their cell membrane may look ‘foreign’ due to expression of activation antigens. Ballas et al.\textsuperscript{151} sought indirect evidence in support of this hypothesis. Thymocytes were activated by PHA stimulation and tested for their LAK susceptibility. Such activated thymocytes were readily lysed by DN-derived LAK, suggesting that LAK may indeed play a role in thymic selection and elimination of DP thymocytes. The DP cells that are marked to die may be lysed by NK cells. Small cortical thymocytes (DN progenitors) have low or no MHC class I expression while subcapsular thymocytes (DP progenitors) and thymus migrant cells (mature SP thymocytes) all express MHC class I receptors\textsuperscript{152}. However, Ballas et al.\textsuperscript{151} did not detect killing of the DN progenitors by the thymus LAK cells. It should be noted that IL-2-stimulated thymic NK cells were used in these studies to demonstrate their cytotoxicity. Whether thymic NK cells in vivo display any cytotoxicity is currently unknown. Moreover, intrathymic T cell development appears to be normal in IL-15-deficient mice and Id2-deficient mice, both of which have greatly reduced number of NK cells. Therefore, it seems unlikely that NK cells play a major role in intrathymic T cell development.

Another very likely role of thymus NK cells is to monitor the thymus for infectious agents and tumor cells. Di Santo suggested that NK cells may be important for detection of thymocyte transformation\textsuperscript{46}. NK cells could lyse early thymocytes and could be involved in immunosurveillance of rapidly dividing thymic precursors. The thymus is a target of certain infectious agents. Despite the blood-thymus barrier, viruses (HIV, SIV, lymphocoriomeningitis), parasites (\textit{T. cruzi}), and fungi (\textit{Paracoccidioides brasiliensis}) have
been detected within the thymus\textsuperscript{153}. It is well documented that NK cells play a role in the combat against HIV and \textit{T. cruzi}\textsuperscript{13} which have both been detected in the thymus. One way to test the role of NK cells in the thymus is to infect IL15\textsuperscript{−/−} mice or thymuses directly with \textit{T. cruzi}, for example, and determine if the outcome is different in the absence of NK cells.

5.5. LN NK cells

Human CD56\textsuperscript{bright} NK cells are located in the paracortex where circulating lymphocytes enter the LNs via high endothelial venules (HEVs) and where T cells and DCs interact. Approximately 0.5\% of total lymphocytes are NK cells in resting mouse LNs. Many NK cells migrate to LNs following stimulation. While only 20\% of resting LN NK cells have rearranged TCR\textgamma, this may be due to the lower number of DN2 cells in the LN. It is possible that the remainder of resting LN NK cells derive from DN1 progenitors. We hypothesize that the NK cells in resting LNs are thymus-dependent NK cells, whereas the massive migration of NK cells following stimulation is from BM-derived NK cells. Therefore, to study NK cells that are thymus-dependent, one would have to use resting LNs. In L-selectin, E-selectin, and P-selectin triple deficient mice there are very low numbers of NK cells in the LNs. NK cells in these mice cannot migrate to the LNs so the cells remaining are resident NK cells. These mice may be useful to study thymus-dependent NK cell function. Another option would be to examine the LNs of nude mice which would lack thymus-dependent NK cells and compare functional abilities and phenotype to wild type mice. If a function or phenotype were changed, one could deduce that the function was normally controlled by the thymus-dependent NK cells that are now absent.
Most studies that have examined the role of LN NK cells focused on those that migrate to the LN, rather than those that are resident in the LN. These NK cells play a role in controlling tumor formation. When NK cell migration to the LNs is blocked in L-selectin−/− mice, there is aggressive tumor formation in LNs following B16 melanoma injection. Another study focused on an NK cell role in priming T\(_{H1}\) cells. Recruitment of NK cells correlates with induction of T\(_{H1}\) responses as they provide the early source of IFN\(\gamma\) which is necessary for the T\(_{H1}\) polarization.

Confocal microscopy of LN sections shows that NK cells reside in two areas of the LN: the medulla and the T cell rich paracortex. In resting LNs 44% of NK cells are in the paracortex and 56% are in the medulla. By intravital imaging it was shown that NK cells are relatively immotile. After stimulation, NK cells accumulate in the paracortex whereas the NK cell numbers in the medulla remain the same. NK cells were also shown to make long steady contacts with DCs. One possible resident (we hypothesize thymus-dependent) NK cell function may be to monitor the LN for pathogens. One clue to this is that some NK cells reside in the paracortex and can interact with blood borne cells and molecules entering the LNs. From here they interact with DCs as has already been shown. Likely, lymph borne pathogens include \textit{Yersinia pestis} (the bubonic plague), HIV, mycobacteria and anthrax.

5.6. T cell vs. NK cell lineage commitment

The discovery of DN2-derived NK cells poses an interesting question of what determines whether DN cells differentiate into T cells or NK cells. Obviously the T cell pathway is dominant over the NK pathway in the thymus whereas the T cell pathway of DN cells appears
to be blocked in the LN possibly leaving only the NK pathway open. There are two candidates that may play critical role in the T vs. NK commitment, namely Notch and Id2.

5.6.1. Notch signals

The Notch pathway is crucial for commitment to the T cell lineage. Notch receptors interact with two kinds of ligands, Delta and Jagged, and although they both activate the same downstream target, their developmental responses differ. NK cells can develop in the presence of Notch ligands. When DN1 and DN2 progenitors are cultured on OP9 or OP9-Delta1 stroma, both conditions produce NK cells. The NK cell potential is decreased in the presence of Notch signaling but it is not fully removed, especially for DN1 progenitors. When Notch inhibitor is added to OP9-DL1 cultures, there is a striking increase in NK cells generated as the inhibitor concentration increases. It appears that the Notch ligand and therefore signal strength are determinants of the switch between T and NK cell fate. Delta1 signals are stronger than Jagged1 signals. Both Jagged1 and Delta1 can inhibit B cell potential but only Delta1 signals are strong enough to induce T cell potential. NK cells are the favored fate of intermediate Notch signals. DN1 cells grown on OP9-Jag1 do not produce T cells or B cells but preferentially develop into NK cells (20-65%). Jagged1 signals may only promote NK cell development by default since they inhibit B cell potential and fail to promote T cell potential. Or, they may induce commitment to the NK cell lineage. While 5% of multipotent progenitors become NK cells after culture on OP9 stroma, 30% of cells become NK cells following OP9-Jagged2 culture. The absolute NK cell numbers were 7.5 fold higher in the OP9-Jagged2 cultures than the OP9 cultures. Perhaps the NK cell fate is revealed during normal development in areas of the thymus where Notch ligands are sparser or in areas of the thymus that are more concentrated with Jagged rather than Delta ligand. Jagged1 is expressed...
in the correct anatomic location to influence this decision in vivo. Precursors enter the thymus through blood vessels near the corticomedullary junction and Jagged 1 is expressed at high levels on endothelial cells lining the thymus blood vessels\textsuperscript{156}.

5.6.2. E proteins vs. Id2

Class I HLH transcription factors play key roles in T cell development (reviewed in \textsuperscript{158}). These transcription factors, also known as E proteins, bind to a palindromic DNA sequence called the E box site. E boxes are found in promoter and enhancer regions of many T cell specific genes including CD4\textsuperscript{159} and pre-T\alpha\textsuperscript{160}. The E protein family includes four members in vertebrates: E12, E47, HEB, and E2-2. The Id (inhibitors of DNA binding) proteins act as dominant-negative HLH proteins and can regulate the level of E proteins. Members of the Id group lack DNA binding domains but they heterodimerize very well with E proteins and block their DNA binding\textsuperscript{161}. E protein expression begins to rise at the DN2 stage of T cell development and Id expression is also high in DN progenitors\textsuperscript{158}. HLH proteins play a direct role in the T vs. NK cell lineage decision. Ikawa \textit{et al.}\textsuperscript{162} demonstrated that Id2 directly controls the production of NKPs from fetal DN1 T cell precursors. This group previously used a clonal culture system that supports both fetal T and NK cell development to show that DN1 and DN2 cells contain bipotent T/NK progenitors as well as pT and NKPs and that DN1 progenitors can be divided into CD122\textsuperscript{−} and CD122\textsuperscript{+} populations, with the CD122\textsuperscript{+} cells representing NKPs\textsuperscript{63}. Ikawa \textit{et al.}\textsuperscript{162} showed that in Id2\textsuperscript{−/−} mice, the NK cell percentage in the fetal thymus was 0.5% compared to 2.5% of a wild type thymus. Also, the CD122\textsuperscript{+} population of DN1 cells is absent in Id2\textsuperscript{−/−} mice. To analyze the commitment status of the DN1 CD122\textsuperscript{−} cells, they cultured them in the progenitor culture for a single cell assay. As previously reported, the WT DN1 CD122\textsuperscript{−} progenitors contained all three progenitors (pT, NKPs, and TNKPs) but the Id2\textsuperscript{−/−} DN1 CD122\textsuperscript{−} progenitors contained only pT cells. Interestingly, the number of pT in the DN1 CD122\textsuperscript{−}
progenitors of Id2<sup>−/−</sup> mice corresponded to the total number of pT, NKP, and TNKPs cells of WT mice. Loss of Id2<sup>−/−</sup> failed to support commitment of bipotent precursors to NKP<sub>s</sub> and therefore they all committed to the T cell lineage<sup>162</sup>. E proteins promote the T cell lineage and their functional inactivation by Id promotes the NK cell lineage. While lack of E proteins inhibits T cell commitment, too high levels of E proteins can cause G1 arrest at stages when this is not required (i.e. proliferation stages). Therefore, a balance of E proteins and Id proteins is important. Interestingly, rearrangement of specific fetal types of TCRγ and TCRδ chains are facilitated by lower levels of activity of E2A and are disfavored by higher levels<sup>163</sup>.

Both Notch signaling and E protein activity are crucial for the DN stages of T cell development but at a later stage, the γδ T cell lineage does not require as high levels. It seems that there is a continuum in levels required between αβ T cells, γδ T cells, and NK cells. Therefore, in DN1 and DN2 precursors, a weak Notch signal and low E protein/high Id levels appear to be crucial in producing thymus-dependent NK cells.

### 5.7. Medical relevance

A pathway that produces TCRγ<sup>+</sup> NK cells in humans is likely. A rare blastic NK-cell like leukemia/lymphoma with TCRγ gene rearrangement has been described in several patients<sup>164</sup>-<sup>166</sup>. These TCRγ<sup>+</sup> NK cells may be equivalent to the population we have described in mice. It has been recently reported that 3.2-36% of human peripheral NK cells have incomplete TCRδ rearrangements<sup>167</sup>. TCRγ rearrangements on the other hand were much rarer. These Tcrδ<sup>+</sup> cells likely occur through a thymus-dependent pathway shared with early T cell progenitors. In humans, the earliest T cell progenitor in the thymus (CD34<sup>+</sup>CD1a<sup>+</sup>) has begun V(D)J recombination with immature TCRδ gene rearrangements although they still retain NK
cell and DC potential. In humans, TCRγ rearrangements do not begin until the next stage (CD34+CD1a+) when NK cell potential is much lower. Therefore, this appears to be very similar to what we have described in mice, with NK cells splitting from the T cell lineage at the very early stages of V(D)J recombination, except with TCRδ rearrangement first. It seems likely that some NK cells still branch at the later stage since the TCRγ+ NK cell leukemia/lymphoma have been described.

5.8. Final conclusions

At least two pathways of lineage commitment produce NK cells in the adult mouse. We discovered a thymus-dependent pathway that has shared progenitors with T cells and TCR rearrangement. This thymus and LN-specific pathway gives rise to NK cells with unique phenotype and functional capabilities. It is likely that thymus-derived T cell precursors migrate to the LN where they give rise to NK cells although it is still possible that thymus NK cells migrate to the LN and further mature. This study refutes the idea that all NK cells develop in the BM and it also brings to light the possibility that multiple pathways of NK cell development may occur in multiple locations. The division of NK cells into distinct subsets is relatively new and it is possible that the subsets of NK cells are the result of multiple developmental environments. We hypothesize that differences in tissue environment influence the final NK cell phenotype and function, resulting in multiple subsets of NK cells throughout the body. This may be how the body produces distinct NK cell subsets that may each play specific roles in the immune response. For example, the thymus-dependent NK cells may fill a specific niche which requires them to have low levels of MHC-class I inhibitory receptors and IFNγ production. The BM pathway may be unable to fill this niche since its environment produces NK cells with a different phenotype (i.e. BM stroma stimulates higher Ly49 receptor
expression levels). Future characterization of NK cell subsets and the environments that produce them may reveal developmental triggers specific to each subset. If different subsets play distinct roles in the immune response, the specific NK cell subset that is best suited to combat the threat at hand (i.e. tumors, pathogens, viruses) may be used in treatments by stimulating their development or proliferation.
BIBLIOGRAPHY


40. Williams, N. S. et al. Generation of lytic natural killer 1.1+, Ly-49- cells from multipotential murine bone marrow progenitors in a stroma-free culture: definition of


65. Fraser, K. P. et al. NK cells developing in vitro from fetal mouse progenitors express at least one member of the Ly49 family that is acquired in a time-dependent and


111. Vicari, A. P., Mocci, S., Openshaw, P., O'Garra, A. & Zlotnik, A. Mouse gamma delta TCR+NK1.1+ thymocytes specifically produce interleukin-4, are major histocompatibility complex class I independent, and are developmentally related to alpha beta TCR+NK1.1+ thymocytes. Eur J Immunol 26, 1424-9 (1996).


