1-1-2011

Development Of Thymic Natural Killer Cells From Double Negative 1 Thymocyte Precursors

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DEVELOPMENT OF THYMIC NATURAL KILLER CELLS FROM DOUBLE NEGATIVE 1 THYMOCYTE PRECURSORS

by

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A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the degree
of Doctor of Philosophy

August 2011

Saint Louis, Missouri

ABSTRACT OF DISSERTATION

Development of Thymic NK Cells From Double Negative 1

(DN1) Thymocyte Precursors

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Doctor of Philosophy in Biology and Biomedical Sciences (Immunology)

Washington University in St. Louis, 2011

Professor Wayne M. Yokoyama, Chairperson

While there has been much progress in defining the specificity and function of natural killer (NK) cells, their differentiation has not been fully elucidated. Previous studies of thymocyte development *in vitro* indicate that double negative ($CD4^-CD8^-$, DN) thymocytes can develop into cells with NK cell markers, but these cells have not been well characterized. Moreover, a subpopulation of NK cells which requires an intact thymus, i.e, thymic NK cells, has been described with selective expression of CD127 although their origin and differentiation are also poorly understood. Herein, we generated and characterized NK cells differentiating from thymic DN precursors. We enriched potential progenitors by sorting DN1 ($CD44^+CD25^-$) $CD122^-NK1.1^-$ thymocytes from *Rag1*$^{-/-}$ mice for adoptive transfer into *Rag1*$^{-/-}$Ly5.1 congenic mice. Following intrathymic injection, donor-derived cells phenotypically resembling thymic $CD127^+$ NK cells were found in thymus and spleen. To further characterize these cells, we seeded sorted DN1 $CD122^-NK1.1^-$ thymocytes on a confluent monolayer of irradiated OP9 bone marrow stromal cells in the presence of IL15, IL7, FMS-like tyrosine kinase 3 ligand

(Flt3L) and stem cell factor (SCF). Flow cytometry results showed NK1.1^{+} cells emerged after at least 7 days in culture. By using limiting dilution analysis, we demonstrated a cell frequency of 0.24% (1 out of 414 sorted thymocytes were able to generate an NK1.1^{+} cell population). *In vitro* differentiated NK cells acquired markers associated with the development of conventional bone marrow-derived splenic NK cells, but also expressed CD127, which is typically found on thymic NK cells. In-depth studies using gene chip microarrays further confirmed *in vitro* differentiated NK cells more closely resembled thymic NK cells as both expressed novel markers such as CD25 and CD103, which were not expressed by splenic NK cells. Finally, we found that *in vitro* cells generated from thymic precursors secreted cytokines when stimulated and degranulated upon target exposure, indicating that they were functional. Together, these data indicate that thymic NK cells can develop from a DN1 progenitor cell and may perhaps have a specific role that sets them apart from their splenic counterparts.

ACKNOWLEDGEMENTS

While these past couple of years have been challenging, they have also been some of the most educational. I would like to extend my sincere gratitude to Dr. Wayne Yokoyama for believing in me as a student, giving me the opportunity to work with him and grow as a scientist, and for all his support, scientific guidance, and personal advice. He has taught me how to think, act, question, and doubt like a scientist. For that, I thank him.

I would also like to acknowledge and thank the members of my thesis committee- Dr. Marco Colonna, Dr. Todd A. Fehniger, Dr. Jason C. Mills, Dr. Thaddeus S. Stappenbeck, and Dr. Emil R. Unanue. Along with my mentor, they have provided me with a tremendous graduate education and have encouraged me to be a better scientist with their guidance, enthusiasm, and support. Their high standards for science has challenged me think outside the box and answer difficult questions.

In addition, I would also like to thank past and present Yokoyama lab members for all the emotional support, camaraderie, entertainment, and love they provided. I will truly miss our conversations and lab outings. To Minji Byun, Helena Jonsson, Julie Elliott, Melissa Pak, and Chris Affolter, who have either experienced this grueling time, are experiencing it, or will be soon. Good luck to you all! To Beatrice Plougastel, Samantha Taffner, Stephen Ferris, Melissa Berrien, Diana Beckman, Josh Rivenbark, Megan Cooper, Bijal Parikh, Maria Gainey, Joe Wahle, and Takashi Ebihara-- who have always enlightened me with their scientific knowledge, all while keeping a positive outlook. To Jian Gao and Darryl Higuchi for their technical support, and Lorraine Schwartz and Tammy Derleth for their exceptional administrative support (and fantastic

lunch conversations). I would also like to recognize and thank Jennifer Poursine-Laurent and Liping Yang for all their help and contributions to this project. Their magical hands have taken this project to new and interesting heights.

Finally, I would like to thank the three people whom I owe everything to, my significant other and my parents. To Jason, who has been with me through the highs and lows of this crazy time in my life called graduate school. Always providing the support necessary when my experiments failed, but always there to celebrate when they worked. Thank you. To my parents, whose constant love, support, faith, and confidence in my abilities has allowed me to be the person I am today. To my father, thank you for always guiding me through life, teaching me the value of a great education and hard work, being a great role model for me, and for teaching me how to be self sufficient, curious, and patient. "Para todo hay tiempo." Gracias a ti mamá, por siempre creer en mi, por apoyarme en todas mis locuras, por ser mi mejor amiga, y por estar siempre ahí cuando te necesito. To them, I dedicate this thesis.

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LIST OF ABBREVIATIONS

B6	C57BL/6 mice
BM	Bone marrow
CBA	Cytometric bead array
CCR7	C-C chemokine receptor 7
CEBP-γ	CCAAT/enhancer-binding protein gamma
CLP	Common lymphoid progenitor
DC	Dendritic cells
DL1	Delta like 1
DN	Double negative
DP	Double positive
ELP	Early lymphocyte progenitor
ES	Embryonic stem cells
Flt3L	FMS-like tyrosine kinase 3 ligand
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte macrophage colony stimulating factor
GMG	Granulated metrial gland
HSC	Hematopoietic stem cells
IFNγ	Interferon gamma
IL-	Interleukin-
iNK	Immature natural killer cells
IP	Intraperitoneal

IRF-2	Interferon regulatory factor-2
KIR-	Killer-cell immunoglobulin-like receptors-
KO	Knockout
LD	Low dose
LI	Large intestine
LN	Lymph nodes
LTα	Lymphotoxin alpha
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
MEF	Myeloid ELF1-like factor
MFI	Mean fluorescence intensity
MHC	Major histocompatability complex
MITF	Microphthalmia-associated transcription factor
mNK	Mature natural killer cells
NK	Natural killer cells
NKP	Natural killer cell progenitor
PCA	Principal component analysis
qFDR	q value of false discover rate
RAG	Recombination activating gene
SCF	Stem cell factor
SI	Small intestine
SLT	Secondary lymphoid tissue
TCR	T cells receptor

TF	Transcription factor
TGF-β1	Transforming growth factor-β1
TNFα	Tumor necrosis factor alpha
T/NKP	T/NK bipotential progenitor
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
WT	Wild type

CHAPTER 1

INTRODUCTION

Natural killer (NK) cells are a critical component of the innate immune system that do not require prior sensitization to be activated upon exposure to tumor or infected cells [1]. These cells have been implicated in the priming of T cells, B cell autoimmunity and tissue inflammation [2-4]. Unlike cells from the adaptive immune system, which take days to weeks to exert their effector function, NK cells can respond in a matter of hours [5]. Upon activation, NK cells lyse target cells through the degranulation of granzymes and perforin, and also produce cytokines such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) [6-9]. Furthermore, NK cells do not require somatic rearrangement of genes for expression of their functional receptors, and can be found in normal numbers in mice defective in antigen receptor gene rearrangement, such as *Rag1*$^{-/-}$ mice [10-12]. While there has been much progress in defining the specificity and function of NK cells, their differentiation process has yet to be fully elucidated.

Development of conventional NK cells

NK cells are generally thought to be bone marrow (BM)-derived lymphocytes that differentiate through the sequential acquisition of markers and functional receptors [8, 13]. Current NK differentiation models are based on observations made during *in vitro* or *in vivo* transplantation assays. Studies have shown that NK cell development, which requires an intact BM microenvironment, can be divided into several major steps [14-16]. First, hematopoietic stem cells (HSCs) commit to the NK lineage, becoming natural killer cell precursors (NKPs). Second, the NKPs acquire receptors and molecules involved in target detection, making them immature NK cells (iNKs). Finally, the iNKs are terminally differentiated into mature NK (mNK) cells that have the ability to execute

appropriate effector functions. Thus, NK cells, like other lymphocytes, undergo several developmental steps in order to become phenotypically and functionally mature.

In greater detail, the earliest step of murine NK cell differentiation in the BM involves the commitment of the HSC to the lymphoid linage. Once this commitment is made, HSCs become early lymphoid progenitors (ELP) that can be identified as $Lin^{-}cKit^{high}Sca1^{+}Flt3^{+}$ [14]. At this early developmental stage, the progenitors lose the capacity to produce myeloid or erythroid lineages. Further differentiation of the ELPs generates common lymphoid progenitors (CLP) that are $Lin^{-}cKit^{low}Sca1^{low}IL7R\alpha^{+}$ [15] . Shortly thereafter, the acquisition of CD122 (IL2/IL15Rβ) defines the transition from a CLP to a committed NKP [16]. The expression of CD122 allows the NKPs to be responsive to IL15, which is critical for NK cell development and exerts its effects through the IL15R complex, consisting of IL15Rα, IL2/IL15Rβ, and the IL2R common γ (γc) chains [17]. In addition, signals received from FMS-like tyrosine kinase 3 ligand (Flt3L) and cKit ligand on the BM stromal cells, along with common γ-dependent cytokines influence NK cell commitment since NKPs express CD135 (Flt3) and CD117 (cKit) [18]. Furthermore, studies where reciprocal BM transfers between lymphotoxin-α (LT-α) and wild type mice showed that close interactions between membrane LT-α-expressing NKPs and LT-responsive radioresistant stromal cells are also necessary for NK development [19]. Taken together, the generation of NKPs from HSCs not only defines the first step in NK cell development, but also suggest that initial commitment to the NK lineage is tightly regulated by stromal cells.

The maturation of NKP to iNK and later mNK cells can be further characterized by several putative intermediate stages (Figure 1), based on correlating marker expression

[8, 20]. The generation of CD122-expressing NKPs marks the beginning of intermediate stage I while NK1.1, CD94/NKG2 and NKG2D expression defines stage II. Immature NK cells at this stage are comparable to fetal or neonatal murine NK cells that express CD94/NKG2 receptors without expressing Ly49 receptors [21]. While the stochastic expression of Ly49 receptors follows, CD94/NKG2 expression is not a prerequisite since there are NK cells that are $Ly49^{+}CD94/NKG2^{-}$. During stages III and IV, immature NK cells acquire CD117 and integrin α_2 (DX5). At stage IV, NK cells undergo expansion in the BM. After proliferation, iNK cells become mature NK cells characterized by high levels of CD11b (Mac1) and CD43. Hence, with the acquisition of these markers, NK cells are terminally differentiated with the capacity to kill targets and produce IFNγ. However, it is important to note that these putative developmental stages are based on correlating marker expression *in vivo* and developmental progression has not been directly observed.

In vitro studies with CLP

While the *in vivo* studies have provided some insight into important phenotypic changes that occur during cell development, direct experimental observation of marker acquisition has been challenging. *In vitro* studies showed that NK cells can be generated from bone marrow ELPs in the presence of cytokines including stem cell factor (SCF), IL7, Flt3L, and IL15 [22-24]. However, the stages through which NK cells differentiate *in vitro* have not been described. Moreover, cytokines alone are insufficient at generating mature NK cells; they require direct contact with stromal cells, such as the OP9 stromal cell line, to acquire a mature and functional phenotype [25]. OP9 stromal cells are

adherent BM stromal cells that were established from newborn B6xC3H op/op mouse calvaria [26]. This stromal cell line has an osteopetrotic mutation in the gene encoding macrophage colony stimulating factor (M-CSF). This mutation favors the differentiation of ES cells into hematopoietic cells other than the monocyte-macrophage lineage. With the help of *in vitro* models, significant progress has been made in discerning some cellular and soluble factors provided by stromal cells and progenitors that may be instrumental in NK cell differentiation. All things considered, *in vivo* and *in vitro* studies have provided a framework for considering NK cell commitment, development, and maturation.

Bipotential thymic progenitors

Although CLP have been shown to directly give rise to NKPs *in vivo* and *in vitro*, studies have shown that they also have the ability to become bipotential T/NK progenitors (T/NKPs) and are found in various fetal organs including blood, thymus, spleen, and liver [27-31]. The T/NKPs found in fetal thymus have the ability to generate either TCRαβ T cells or NK cells, but no other lineages, when transferred into a thymic environment [32]. *In vitro* studies have indicated that T/NKPs residing in different tissues may differentiate in a somewhat different manner than NKPs in the BM. For example, fetal thymus and blood T/NKPs express NK1.1 at later stages of development [28, 32], whereas T/NKPs in fetal liver do not up-regulate this specific serological NK marker at any stage of development [30]. Taken together, this may suggest that a minor progenitor population has the potential to emigrate from the BM to the periphery, thereby

differentiating in a different environment which ultimately affects their receptor repertoire and perhaps even their function.

Furthermore, early studies showed that the earliest thymocyte precursors in adult mice also harbor T/NK potential [33]. In these studies, precursors among the double negative 1 (DN1; $CD44^{+}CD25^{-}$) population were sorted based on their expression or absence of CD24, a heat stable antigen, and CD117. Five distinct populations were identified and designated DN1a to DN1e based on the frequency of these two markers. DN1a was identified as $CD117^{+}CD24^{-}$; DN1b was $CD117^{+}CD24^{low}$; DN1c was $CD117^{low}CD24^{+}$; DN1d was $CD117^{-}CD24^{+}$; DN1e was $CD117^{-}CD24^{-}$. Each sorted population was cultured on either OP9 stromal cells transfected with delta-like 1 (OP9-DL1), a Notch receptor ligand, or control non-manipulated OP9 cells. Results showed that DN1c, DN1d and DN1e differentiated into $CD4^{+}$, $CD8^{+}$ or $CD4^{+}CD8^{+}$ T cells only when seeded on OP9-DL1 cells, while DN1a and DN1b progenitors gave rise to $NK1.1^{+}$ cells when cultured with control OP9 cells. The limitation to this study was that the $NK1.1^{+}$ population that differentiated *in vitro* was not further characterized in terms of phenotype or function. Regardless, these studies have demonstrated that a small thymic DN1 population in adult mice also harbor T/NK potential.

NK cells beyond the BM

While a large population of BM derived mature NK cells are found in the spleen, studies have also shown the presence of NKPs and immature NK cells in other extramedullary tissues in both adult humans and adult mice. NKPs in adult humans that reside in the lymph nodes (LN) have been shown to traffic from the BM via the blood

[34]. Similarly, NKPs and immature NK cells have been detected in murine liver and spleen [20, 35]. Although these NKPs and immature NK cells have been shown to differentiate *in vitro*, it is still unknown if they can further differentiate *in vivo*. These studies suggest that perhaps the BM represents the initial site for HSCs to commit to the NK linage, but a small NKP or iNK population can emigrate from the BM and finish their maturation elsewhere.

Interestingly, the so-called "immature" NK cells in the periphery have been shown to be functional, thereby raising the question of whether they truly are immature or whether they are NK subsets that differ in phenotype. For example, fetal hepatic NK cells were shown to be $NK1.1^{+}TRAIL^{+}CD11b^{low}DX5^{-}$ but can eliminate tumor cells in a TRAIL (TNF-related apoptosis inducing ligand)- dependent manner [35, 36]. Meanwhile, two distinct subsets of $CD122^{+}CD3^{-}$ NK cells within a mouse uterus at mid-gestations could be identified. The smaller subset was comparable to splenic NK cells in that they expressed NK1.1, DX5, Ly49s and CD43. However, the larger subset lacked the commonly expressed markers of mature NK cells, including NK1.1 and DX5, yet expressed NKp46, NKG2D and CD16 [37, 38].

Moreover, an interesting NK subset with phenotypically and functionally distinct characteristics was shown to be present in murine thymus by the Di Santo group [39]. These cells had uniform expression of several NK markers, including CD122, NK1.1, DX5, and NKG2D, but were $CD127^{+}CD69^{high}Ly49^{low}CD16^{-}CD11b^{low}$. CD127 is also known at the α subunit of the IL7 receptor and is thought to be a good marker for activated T cells that have the potential to become long-lived memory cells [40]. IL7Rα is also expressed on naive T cells, which is rapidly lost upon activation, and can also be

identified on immature B cells through the early pre-B stage [41, 42] . The $CD127^+$ thymic NK cells failed to lyse YAC-1 target cells as well as their splenic counterpart, yet were more efficient at producing cytokines. Together, these data suggest the functional difference may perhaps be attributed to the unique phenotype such as the low CD11b expression and absence of CD16 (FcγRIII).

Thymic $CD127^+$ NK cells are absent in athymic nude mice, indicating that a functional thymus is required for their development [39]. However, these cells are not only present in *Rag1/2*-deficient thymus, but also resemble wild type counterparts, suggesting that neither more mature thymocytes nor a fully developed thymic medullary stromal compartment is required for thymic NK cell homeostasis [13]. Nevertheless, it is not known if their progenitors seed the thymus as cells already committed to the NK cell lineage or if they differentiate from uncommitted thymic precursors. While the exact progenitor that gives rise to thymic NK cells is not known, recent *in vivo* experiments showed that thymic NK cells do not rearrange the TCRγ locus, suggesting that they are not derived from a committed T cell progenitor [43]. Although these do not arise from failed T cell progenitors, their relationship to previously described T/NKP cells has not been elucidated.

While it is possible that they are generated from T/NKPs, others have suggested that thymic $CD127^+$ NK cells are recirculating BM derived NK cells that have altered their phenotype to serve a precise function within the specific tissue. Adoptive transfer experiments have shown that conventional splenic NK cells can repopulate the spleen, liver, and BM of recipient mice [44-46]. However, studies to determine whether these

NK cells migrate equally to all tissues or whether the recirculation is selective, have not been exhausted.

So what are the biological implications of having NK cell subsets residing in extramedullary tissues such as the thymus? For one, the unique phenotype of each NK subset may provide a plasticity for NK cell effector function that would allow the cells to adapt to different conditions. Recently, a study showed that conventional NK cells have the potential to acquire immunoregulatory function (IL10 secretion) as a consequence of extensive activation in visceral leishmaniasis [47]. Furthermore, thymic NK cells have been implicated in immunosurveillance due to their lysing of rapidly dividing thymic precursors [48]. Whether thymic $CD127^{+}$ NK cells have any other roles such as modulation of thymopoiesis, maintaining thymic architecture, or providing a tolerogenic function, is still not known.

NK cell subsets in humans

The phenotypic and functional differences between thymic $CD127^{+}$ and splenic $CD127^{-}$ NK cells from adult mice bear similarities to the different subsets described in humans. Human NK cells comprise approximately 10-15% of circulating lymphocytes and can be identified phenotypically by their expression of CD56 [49]. Studies have shown that there are two distinct populations of human NK cells: $CD56^{dim}$ (90% of NK cells) and $CD56^{bright}$ (10% of NK cells) [50]. The $CD56^{dim}$ subset express higher levels of CD16 (FcgRIII) and killer cell Ig-like receptors (KIR), which are similar to mouse Ly49 receptors, than their $CD56^{bright}$ counterparts. While the latter subset is dominantly

present in secondary lymphoid tissues (SLT; lymph nodes and tonsils), $CD56^{dim}$ NK cells can be found in the BM, blood, and spleen [51].

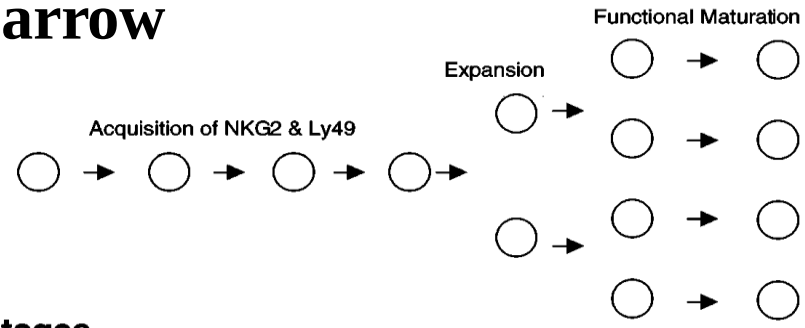
Developmental *ex vivo* studies assessing CD16 versus CD94 expression within the total $CD3^{-}CD56^{+}$ populations of the SLT and blood suggest that human NK cells progress from $CD94^{+}CD16^{-}$ to $CD94^{+/-}CD16^{+}$, marking the final stage of NK cell development *in vivo* [49]. These results suggest $CD56^{bright}$ NK cells, which are $CD94^{+}CD16^{-}$, differentiate into $CD56^{dim}$ NK cells ($CD94^{+/-}CD16^{+}$). In addition, more conclusive studies showed that $CD56^{bright}$ NK cells can further develop into $CD56^{dim}$ by culturing these cells with synovial fibroblast *in vitro* [52]. Although *in vitro* studies show $CD56^{bright}$ NK cells can be further matured, it is not known if this can also occur *in vivo*. Regardless, limitations in human studies have made it difficult to determine the location and differentiation process for the $CD56^{bright}$ population.

Functional analysis showed that $CD56^{dim}$ NK cells have a natural cytotoxicity, while their bright counterpart are more efficient at producing high levels of immunoregulatory cytokines [53]. This includes IFNγ, tumor necrosis factor (TNF), granuloctye-macrophage colony stimulating factor (GM-CSF) and IL10. In addition, $CD56^{bright}$ NK cells express a functional CC-chemokine receptor (CCR7) and CD62L, suggesting that this subset has the ability to traffic to STL [50]. These differences between human NK cells bear resemblance to those found in adult mice. Based on their phenotypic and functional similarities, $CD127^{+}$ NK cells from adult mice may correspond to the $CD56^{bright}CD16^{-}$ NK subset. This perhaps suggests that NK cell heterogeneity may be evolutionary conserved and biologically relevant.

Many questions still remain about NK cell development, including the sequential acquisition of surface markers as well as the development of NKPs or T/NKPs found in the periphery. In this study, we used adoptive transfer to show that the DN1 $CD122^-$ $NK1.1^-$ thymic population harbors cells that have the potential to differentiate into $NK1.1^+$ cells that are phenotypically similar to thymic NK cells. While the number of differentiated cells was low in adoptive transfer experiments, we were successful at further characterizing these NK cells following *in vitro* differentiation. By seeding DN1 $CD122^-NK1.1^-$ thymocytes on a monolayer of irradiated OP9 cells and adding cytokines, we were able to generate $NK1.1^+$ cells within 7 days and sequentially follow the developmental stages. Phenotypic, gene chip, and functional analysis studies show that the DN1 $CD122^-NK1.1^-$ thymic population generate NK cells that very closely resemble freshly isolated thymic NK cells. These studies should improve our understanding of mouse thymic NK cell development, which in turn should aid our knowledge of human NK cells.

Figure 1. Developmental stages of NK cells in the BM. NK cell development can be divided into 5 stages based on phenotypic markers. NKPs expressing CD122 have been shown to give rise to mature NK cells. Expression of NK1.1, CD94/NKG2, Ly49, and cKit define stages II and III. NK cell expansion and increase effector function occur between stages IV and V.

Bone marrow



	Stages				
Marker	**I**	**II**	**III**	**IV**	**V**
IL-2/IL-15Rβ	+	+	+	+	+
NK1.1	-	+	+	+	+
CD94/NKG2	-	+	+	+	+
NKG2D	-	+	+	+	+
Ly49	-	-	+	+	+
c-kit		-	+	+	+
αv		hi	hi	lo	-
α2		lo	lo	hi	hi
αM (Mac-1)		lo	lo	lo	hi
CD43		lo	lo	lo	hi
Function					
Cytotoxicity				lo	hi
IFN-γ production				lo	hi

Yokoyama, W.M., et al. Annu Rev Immunol, 2004. **22**: p. 405-29.

CHAPTER 2

MATERIALS AND METHODS

Mice

C57BL/6J (B6), B6.129S7-*Rag1*^{tm1Mom}/J (*Rag1*$^{-/-}$) Ly5.2, and B6.129S4(C)-Itgae^{tm1Cmp}/J (CD103$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Rag1*$^{-/-}$ Ly5.1 congenic mice on the C57BL/6 genetic background were generated by crossing B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (Ly5.1) mice with *Rag1*$^{-/-}$ mice (both from The Jackson Laboratory). CD25*Rag1*$^{-/-}$ mice were generated by crossing B6.129S4-*Il2ra*^{tm1Dw}/J (CD25$^{-/-}$, The Jackson Laboratory) with *Rag1*$^{-/-}$ mice. All mice were used between 4 and 16 weeks of age. Mice were housed in specific pathogen free conditions and were used in accordance with the animal protocol approved by the Animal Studies committee at Washington University in Saint Louis.

Antibodies, flow cytometry, and cytokines

The following antibodies were obtained from BD Biosciences (San Jose, CA): anti-CD44 (clone IM7); anti-CD25 (PC61); anti-NK1.1 (PK136); anti-CD122 (TM-β1); anti-CD45.1 (Ly5.1, clone A20); anti-CD45.2 (Ly5.2, clone 104); anti-CD43 (Ly48); anti-CD117 (2B8); anti-CD62L (MEL-14); anti-B220 (RA3-62); anti-KLRG1 (2F1); anti-Ly49A (JR9); anti-Ly49F (HBF-719); anti-Ly49G2 (4D11); and streptavidin PerCP-Cy5.5 (SA-PerCP-Cy5.5). The following antibodies were purchased from eBioscience: anti-Ly49A/D (12A8); anti-Ly49C/I/F/H (14B11); anti-CD49 (DX5); anti-CD11b (M1/70); anti-NKG2D (CX5); anti-CD127 (A7R34); anti-CD69 (H1.2F3); anti-CD94 (18d3); anti-CD16/32 (clone 93); anti-CD103 (2E7); CD86 (GL1); anti-CD45.1 (A20); SA-PerCP-Cy5.5; anti-KLRG1 (2F1); anti-IFNγ (XMG1.2); anti-Ly49H (3D10); anti-Ly49I (YLI-90); and anti-CD107a (eBio1D4B). Anti-Ly49C (4LO33) was produced from a

hybridoma kindly provided by Suzanne Lemieux, Institut National de la Recherche Scientifique-Institut Armand- Frappier, Laval, Quebec, Canada). Anti-Ly49D (4E4) was developed in our laboratory as previously described [54]. Surface staining was performed on ice in staining buffer (3% FBS, 0.1% NaN_3 in PBS). Nonspecific antibody binding was blocked with 2.4G2 (anti-FcγRII/III, American Type Culture Collection, Manassas, VA). All samples were collected on a FACSCanto (BD Biosciences) using FACSDiva software (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). The following cytokines were purchased from PeproTech (Rocky Hill, NJ): IL15, IL7, Flt3L, and IL12. In addition, we used stem cell factor (SCF, Fitzgerald Industries, Concord, MA) and IL18 (R&D Systems, Minneapolis, MN).

Cell sorting

DN1 $CD122^-NK1.1^-$ cells were sorted from *Rag1*$^{-/-}$ mice by generation of thymus single cell suspensions and stained with a mixture of antibodies that included fluorescein isothiocyanate (FITC)-conjugated anti-CD44, phycoerythrin (PE)-conjugated anti-CD25, allophycocyanin (APC)-conjugated anti-NK1.1, biotinylated anti-CD122 and PerCP-Cy5.5 conjugated streptavidin. Pilot studies showed that *Rag1*$^{-/-}$ thymus contains only $CD4^-CD8^-$ (DN) cells so routine staining for CD4 or CD8 expression was not required. Cells were stained in MTHc buffer which contained MTH (308 mOsm solution of 10X Hanks Balanced Salt Solution, 1M HEPES and milliQ H_2O), 5% v/v FBS and 0.5% v/v 1mg/ml DNase I stock solution. Cells were sorted in MTHs buffer which contained MTH, 0.5% v/v FBS and 0.5% v/v 1mg/ml DNase I stock solution. Thymic and splenic NK cells were enriched by generation of single cell suspensions from *Rag1*$^{-/-}$ mice and

stained with APC-conjugated anti-NK1.1 in sorting buffer (1% FBS, 0.2% NaN_3 in PBS). All cells were sorted by flow cytometry to >98% purity on a Dako MoFlo (Beckman Coulter) in the Siteman Cancer Center Flow Cytometry Core.

Adoptive transfer

DN1 CD122$^-$NK1.1$^-$ cells were sorted from the thymi of 50-80 *Rag1*$^{-/-}$Ly5.2 mice. Highly purified cells were washed several times with PBS to avoid contaminating recipient mice with remaining antibodies or FBS. Approximately $2x10^5$ cells were transferred into sub-lethally γ-irradiated (700cGy) *Rag1*$^{-/-}$Ly5.1 congenic mice. Cell suspensions were injected into either the lateral tail vein (IV) or intrathymically (IT) of age- and sex-matched recipient mice. IT recipient mice were given buprenorphine hydorchloride for pain every 24 hrs for a period of 72 hrs and were supplied with sulfamethoxazole and trimethoprim antibiotics for the duration of the experiment. All experiments were approved by the Animal Studies Committee at Washington University in Saint Louis.

Co-culture and resting cells

OP9 stromal cells (American Tissue Culture Collection) were resuspended in fresh OP9 media (DMEM, 20% FBS and 1% HEPES) and γ-irradiated at 2,500 rad. Cells were then seeded at $1.5x10^4$ cell per well in 96-well U-bottom plates and incubated for 20-24 hrs at 37°C in 5% CO_2. DN1 CD122$^-$NK1.1$^-$ cells were sorted from the thymi of 10-60 *Rag1*$^{-/-}$ Ly5.2 mice. Highly purified cells were seeded in wells containing the monolayer of OP9 cells that were plated the day before. The co-culture was done in R10 media (RPMI,

10% FBS, L-glutamine, 2-βME, and pen/strep) supplemented with IL7 (5 ng/ml), Flt3L (5 ng/ml), stem cell factor (SCF, 5 ng/ml) and IL15 (20 ng/ml). Cells received fresh media every 3 days. Thymocytes were allowed to differentiate anywhere from 4 to 22 days. To rest *in vitro* generated NK cells, we pooled wells with cells after culture for 19 days, washed them twice with PBS, resuspended them in R10 supplemented with low dose IL15 (10 ng/ml) and seeded them at 1-2x10^6 cells per well in 12 well plates for 36 hrs. To rest freshly isolated and sorted splenic and thymic NK cells, we cultured them in R10 supplemented with low dose IL15 for a period of 16-20 hrs. These cells were not cultured longer due to lower yield at later time points. There was no change in phenotypic markers or activation during this incubation period. All cells were incubated at 37°C in 5% CO_2.

In vitro stimulation assays, intracellular cytokine staining and cytometric bead array

In vitro generated, splenic and thymic NK cells from *Rag1*$^{-/-}$ mice were stimulated in a target cell-free assay. Briefly, 1 X 10^5 cells were cultured in either a 96-well plate that contained R10 media alone, R10 media with IL12 (10 ng/mL) and IL18 (50 ng/mL), or a 24 well plate pre-coated with anti-NK1.1 (clone PK136, 10ug/ml; 1.5 hr at 37°C) and washed 3 times with PBS. Cells were incubated at 37°C and 5% CO_2 for 1 hr and then further incubated in the presence of brefeldin A (GolgiPlug, BD Biosciences) for an additional 7 hr. Cells were fixed and permeabilized (Cytofix/Cytoperm; BD) and IFN-γ was detected by intracellular cytokine staining and flow cytometry as described previously[20]. For the cytometric bead array, cells were stimulated for 8 hrs without the

addition of brefeldin A. At the end of the culture period, we collected the supernatants and followed the manufacturer's protocol for the Mouse Inflammation Kit (BD Biosciences). Cytokine secretion was measured with the FACSCalibur using CellQuest (BD) and analyzed with the BD CBA Software.

Degranulation assay

Sorted *in vitro* generated, splenic and thymic NK cells from *Rag1*$^{-/-}$ mice were co-cultured with YAC-1 target cells at varying ratios in 96-well V-bottom plates. Anti-CD107a antibody and monensin (eBioscience) were added to each well. Plates were incubated for 2 hr at 37°C, after which surface staining for flow cytometry was performed as described above.

Microarray sample preparation and analysis

NK1.1^{+} cells were sorted from *in vitro* generated NK1.1^{+} cells along with splenic and thymic NK1.1^{+} cells from *Rag1*$^{-/-}$ mice. Total RNA was isolated using the QIAGEN RNeasy Mini Kit (Valencia, CA) and target synthesis for hybridization to Affymetrix 430 v2.0 GeneChips was performed with two rounds of linear amplification by the Washington University Siteman Cancer Center Microarray Core Facility. Total RNA from 3 different experiments was pooled for target synthesis and three target samples were pooled and hybridized to each chip, resulting in three chips for each cell type. Data analysis was performed using Partek® Genomics Suite™ (St. Louis, MO). The chips were normalized and the Bonferroni Correction was applied to correct for multiple test samples according to the program instructions.

Isolation of lymphocytes from the small intestine

After euthanizing mice, we performed a midline incision and retracted the skin. The small intestine was cut 0.5 cm below the stomach and 1 cm above the cecum. The intestine was flushed out with 20ml filter sterilized CMF solution (100ml 10X Ca^{2+} and Mg^{2+} HBSS + 100ml 10X HEPES-bicarbonate buffer + 20ml FBS + 1L H_2O) at 4°C. We then cut the intestine longitudinally and laterally into 0.5 cm pieces and rinsed them 3X with CMF solution. Intestinal pieces were then placed in a 50ml conical tube with CMF/ 10% FBS/ 0.1 mM EDTA solution and stirred for 20 minutes at 37°C at medium speed. After vortexing the intestinal pieces and allowing the bigger particles to settle, we transferred the supernatant to another 50 ml conical tube. We added more CMF/ FBS/ EDTA solution to the intestinal pieces and repeated the stirring process. We combined the supernatants, incubated them on ice for 10 minutes, and centrifuged them for 5 minutes at 1500 rpm at 4°C. Supernatant was discarded and cells were resuspended in R10.

Isolation of lymphocytes from the large intestine

After euthanizing mice, we performed a midline incision and retracted the skin. The colon was cut 1 cm below the cecum and near the anus. The intestine was flushed out with RPMI supplemented with Pen/Strep. We cut the colon longitudinally and laterally into 0.5 cm pieces, placed them in a 50ml conical tube with 40 ml prewarmed RPMI/ Pen/Strep/ 3% FBS/ 5mM EDTA/ 154mg/ml DL-Dithiotreitol (Sigma), and incubated them in a orbital shaker for 20 minutes at 37°C at medium speed. The contents were then strained through a sterile kitchen strainer. After washing the intestinal pieces 3X with

RPMI/ Pen/Strep/ 5mM EDTA solution, we minced them and placed them in a new 50ml conical tube with RPMI/ Pen/Step/ 0.155mg/ml liberase (Roche)/ 0.1mg/ml DNase. Once again, we placed them in an orbital shaker for 30 minutes at 37°C at medium speed. At the end of the incubation, we collected the solution in a 50ml conical tube and mashed the intestinal pieces with a syringe plunger. We washed the pieces with PRMI/ 3% FBS 3X. The solution was centrifuged for 10 minutes at 1500 rpm at 4°C, supernatant was discarded and cells were resuspended in R10.

CHAPTER 3

IN VIVO DEVELOPMENT OF THYMIC NK CELLS FROM DOUBLE NEGATIVE 1 (DN1) PRECURSORS

Most studies done on mouse NK cells focus on the conventional splenic population, which are known to develop and mature in the BM. Unlike their splenic counterpart, very little is known about the development of thymic NK cells. Here, we explore the possibility that they develop from progenitors that have seeded the thymus, and therefore are not re-circulating conventional NK cells.

Sorting the progenitor population from the thymus of *Rag1*$^{-/-}$ mice

To initiate our experiments, we enriched potential progenitors by sorting a subset of double negative ($CD4^-CD8^-$) thymocytes, known as DN1 based on their expression of CD44 and low or absent CD25, from *Rag1*$^{-/-}$ mice (Figure 2). Presort analysis showed that only 4% of thymic lymphocytes were in the DN1 ($CD44^+CD25^-$) stage of development. The majority of the thymocytes were arrested in the DN3 ($CD44^+CD25^-$) stage due to the absence of RAG genes that would normally drive the differentiation of thymic progenitors into the T cell lineage. Within the small DN1 population, we identified $CD122^-NK1.1^-$ cells, $CD122^+NK1.1^-$ NK precursors, and $NK1.1^+CD122^+$ NK cells, which constituted 21%, 11%, and 65% of the cells, respectively. To study the NK cell developmental potential of DN1 cells, we further enriched the population by collecting only the $CD122^-NK1.1^-$ cells which represent cells that have yet to commit to the NK cell lineage [16]. Since this is the basis for all future experiments, we did a post-sort analysis which showed that the DN1 $CD122^-NK1.1^-$ thymic population had a 99% purity level. These results suggest there were little to no contaminating mature NK cells that could potentially skew our results.

In vivo differentiation of NK1.1$^+$ cells from thymic DN1 progenitors

To assess whether the DN1 CD122$^-$NK1.1$^-$ population had the potential to generate NK cells, we transferred highly purified cells from *Rag1*$^{-/-}$ Ly5.2 mice into sub-lethally irradiated *Rag1*$^{-/-}$ Ly5.1 congenic recipients. Cells were adoptively transferred either intravenously (IV) or intrathymically (IT). Thirty two days post transfer, we analyzed the spleen and thymus of recipient mice for donor-derived NK1.1$^+$ cells (Figure 3). In mice that received IV transferred cells, a small number of splenic NK cells were donor-derived (Ly5.2$^+$), while the thymus was completely devoid of such cells. On the other hand, a larger pool of donor-derived cells was present in mice receiving the sorted progenitors intrathymically, where approximately 50% of the thymic NK cells were derived from Ly5.2$^+$ donor cells. In addition, NK cells generated from these donor thymocytes were also detected in the spleen of the same mice where they constituted close to 6% of all NK cells. These results suggest that, upon intrathymic adoptive transfer, the DN1 CD122$^-$NK1.1$^-$ thymic subpopulation can differentiate into NK1.1$^+$ cells in the thymus and that a small percent of these NK cells has the potential to emigrate to the spleen.

In vivo differentiation of thymic DN1 cells generates non-conventional NK cells

While the number of donor cells in recipient mice receiving IV transferred DN1 CD122$^-$NK1.1$^-$ thymocytes was too low for analysis, we were able to further characterize the NK cells generated from donor cells transferred intrathymically (Figure 4). By gating on NK1.1$^+$ cells in the thymus, we found analyzed informative markers associated with thymic and splenic NK cells. Although the staining patterns for each marker were

somewhat different, i.e., some markers were expressed on all cells while others were expressed on subsets, we used the percent of positive cells (above isotype control staining) as a convenient (although technically imprecise) means to compare and describe the various staining profiles. With this approach, we found that IT transferred $NK1.1^{+}Ly5.2^{+}$ cells in the thymus expressed the thymic marker CD127, similar to thymic NK cells from unmanipulated mice, albeit at somewhat lower levels for unclear reasons. In addition, we found CD25 to be selectively expressed by thymic NK cells in both control and IT mice. This was a surprising finding since previous studies did not note CD25 expression on thymic NK cells in C57BL/6 mice [39, 55]. This marker may be related to the origin of our NK cells from *Rag1*$^{-/-}$ mice since we also did not find it expressed on thymic NK cells from C57BL/6 mice (data not shown) Regardless, unmanipulated splenic $NK1.1^{+}$ cells do not express either receptor, while thymic $NK1.1^{+}$ cells from both unmanipulated and IT mice had $CD25^{low}$ and $CD25^{high}$ populations, the latter usually falling outside the range of detection. The $CD25^{high}$ staining was unlikely due to non-specific Fc receptor staining because splenic *Rag1*$^{-/-}$ cells which contain an abundant number of FcR-bearing cells did not stain. For Ly49 expression, due to the low return yield of $Ly5.2^{+}$ NK cells in the IT mice, we were unable to assess the expression of the individual Ly49 molecules, thus pan-Ly49 antibodies were used to detect Ly49C/I/F/H and Ly49A/D. Similar to thymic NK cells from unmanipulated mice, a sizable fraction of donor $NK1.1^{+}Ly5.2^{+}$ cells in the thymus were $Ly49^{low}$, whereas a smaller fraction expressed Ly49 receptors at higher levels. However, essentially all splenic NK cells from control mice were $Ly49^{high}$. Finally, we were able to detect DX5 and CD11b on donor-derived cells in the thymus, although their expression level was

lower than on unmanipulated thymic NK cells. Thus, these data suggest that IT transferred DN1 CD122$^-$NK1.1$^-$ cells can differentiate into cells that resemble thymic NK cells.

In the spleen, donor NK1.1$^+$Ly5.2$^+$ cells had a phenotype consistent with that of donor and control NK cells in the thymus (Figure 4). Similar to unmanipulated thymic NK cells, NK1.1$^+$Ly5.2$^+$ cells found in the spleen expressed CD127 and CD25, although at much lower levels. In addition, they expressed both Ly49^{low} and Ly49^{high} and were CD11b^{low}. This phenotype was distinct from control splenic NK cells, suggesting that the adoptive transferred progenitor cells differentiate into thymic NK cells which can then emigrate to the periphery.

Discussion

Conventional NK cells found in the periphery, particularly in the spleen, have been shown to develop from progenitors in the BM [8, 16]. On the contrary, the identification of the specific progenitor and developmental site that generates thymic NK cells has been challenging. To date, there are speculations on whether these unique NK cells begin their life as T/NK bipotential progenitors that initiate their development in the thymus, or whether they are re-circulating conventional NK cells that have altered their phenotype based on their environment. While studies have shown that T/NK bipotential progenitors found within the DN1 population in the thymus are responsible for generating NK cells *in vitro* [33], it has yet to be shown whether they differentiate into thymic NK cells in particular. Moreover, these studies showed that it was the DN1 CD117$^+$ cells that generated NK1.1$^+$ cells upon culturing them with OP9 cells. One major caveat to these

experiments is that they failed to exclude $NK1.1^+$ cells that are within this population. In our hands, approximately 5% of the DN1 $CD117^+$ population in *Rag1*$^{-/-}$ mice were $CD122^+NK1.1^+$ (data not shown). This small pool of NK cells was also found in C57BL/6 mice. Our results suggest that there was a contaminating population of NK cells in early experiments. Since only 1.5% of DN1 cells expressed CD117, we chose to collect all cells within the DN1 population, excluding $CD122^+NK1.1^-$ and $CD122^+NK1.1^+$ cells. This not only increased our recovery yield, it also included cells that were $CD117^+$, approximately 3% of DN1 $CD122^-NK1.1^-$ cells.

To address the question of whether this population differentiated into thymic NK cells, we adoptively transferred $2x10^5$ highly purified DN1 $CD122^-NK1.1^-$ cells from *Rag1*$^{-/-}$Ly5.2 mice into sub-lethally irradiated congenic mice. Pilot studies where we transferred less cells or used non-irradiated hosts yielded negative results as we could not find the $Ly5.2^+$ cells (data not shown). Perhaps this may indicate that these thymocytes needed "space" in a niche created by irradiation. Furthermore, harvesting the cells at earlier time points proved to be difficult since there were too few $Ly5.2^+$ cells to analyze (data not shown). Results from these pilot experiments suggest an appropriate progenitor number, space, and time were essential to our *in vivo* studies.

Using these guidelines, we were able to characterize donor-derived cells that had differentiated *in vivo* for 32 days. Interestingly, we detected a Ly5.2 and Ly5.1 double positive population in the spleen of the IT mice. This may be due to discrepancies in the staining process, but considering the absence of this double positive population in the thymus, it is highly unlikely. A second explanation may be that there are more dead/dying cells in the spleen, thus appearing as double positive. Yet a third explanation

is that the donor cells acquired the Ly5.1 molecule from recipient cells found in the spleen. This could be likely since NK cells have been shown to acquire MHC class I molecules from target cells by stripping them from the cell's membrane [56, 57].

A second interesting finding was the expression level of most phenotypic markers in both thymic and splenic $NK1.1^{+}Ly5.2^{+}$ cells. Similar to unmanipulated thymic cells, the thymic donor-derived IT cells were $CD127^{+}$, $DX5^{+}$, and $CD11b^{low}$, although their expression level (MFI) was much lower. These results suggest that one or more factors, such as stromal cells, that aid in differentiation and may be necessary for marker expression was affected when the mice were irradiated, also explaining why our NK numbers were much lower than those in unmanipulated mice. In addition, DX5 expression in splenic donor-derived IT cells was also detected at much lower levels, once again supporting our idea that the process of irradiating the mice leads to some unforeseen changes that may affect marker repertoire. Furthermore, although CD127 and CD25 were expressed on splenic $NK1.1^{+}Ly5.2^{+}$ cells from IT transferred mice indicating some similarity to unmanipulated thymic NK cells, their expression was not much higher than the isotype control. This could indicate one of two things: 1) DN1 $CD122^{-}NK1.1^{-}$ cells initiate their developmental process in the thymus, allowing for the expression of CD127, CD25, and $Ly49^{low}$ receptors, but emigrate to the periphery to continue their maturation, which includes the down-regulation of CD127 and CD25. 2) DN1 $CD122^{-}$ $NK1.1^{-}$ progenitors fully develop in the thymus, but retain the capacity to alter their receptor repertoire according to their environment, as seen by the emerging $Ly49^{high}$ population. Whereas both possibilities are plausible, it will be difficult to verify these speculations without further knowledge of their *in vivo* differentiation process.

While further *in vivo* studies need to be done, we were able to show that the DN1 population harbors cells with the potential to differentiate into thymic NK cells. This suggest $CD127^+$ NK cells are not recirculating conventional BM-derived NK cells. Though the majority of these cells developed and resided in the thymus, a small pool had the potential to emigrate into the periphery. These results further validate the idea that not all NK cells develop in the BM, more specifically, that $CD127^+$ NK cells develop in the thymus.

Figure 2. Sorting DN1 CD122$^-$NK1.1$^-$ cells from *Rag1*$^{-/-}$ mice yields a highly enriched progenitor population. Thymi from 10 *Rag1*$^{-/-}$ mice were removed and cell suspensions were made. Cells were labeled with anti-CD44, anti-CD25, anti-NK1.1 and anti-CD122 and sorted using the Dako MoFlo. Presort analysis shows cells were gated on lymphocytes based on the forward/side scatter profile. Approximately $2x10^4$ DN1 CD122$^-$NK1.1$^-$ cells were used in a post sort analysis. Data represents more than two experiments.

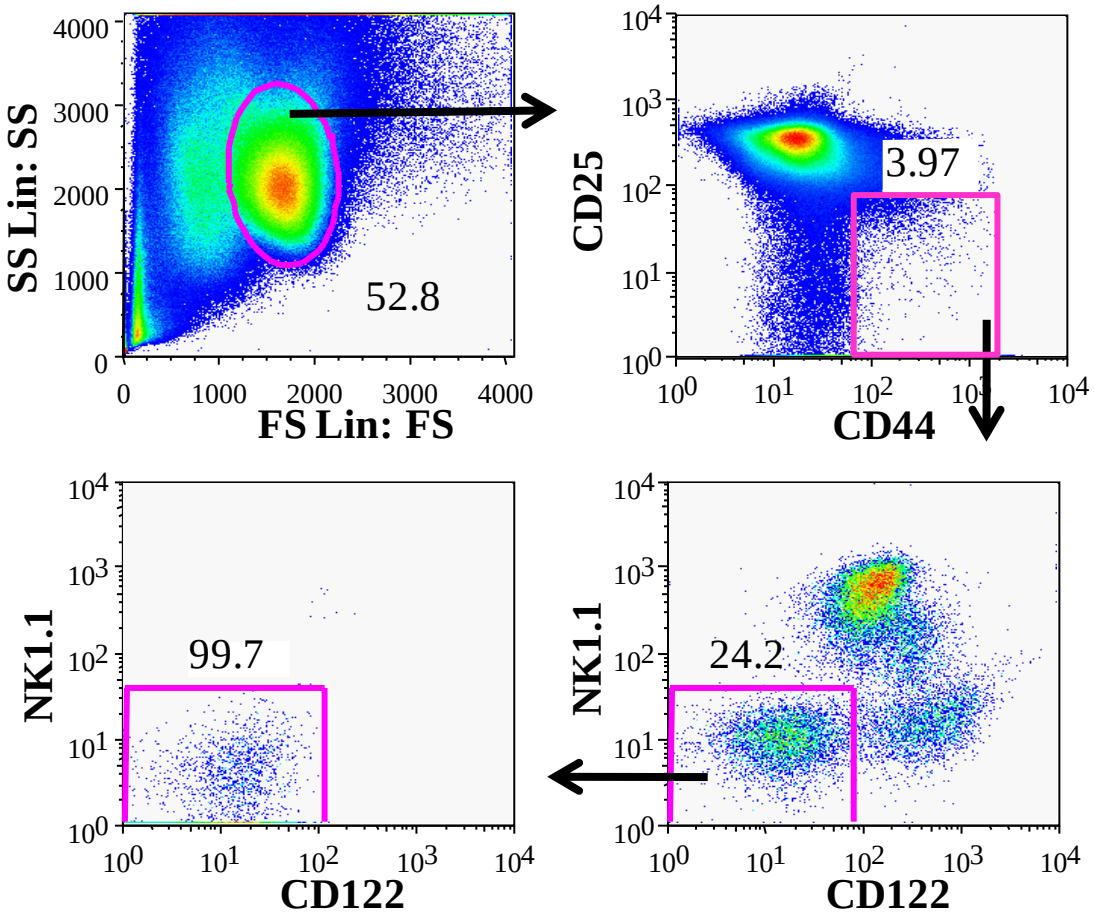

SS Lin: SS
FS Lin: FS
52.8
CD25
CD44
3.97
NK1.1
CD122
99.7
NK1.1
CD122
24.2

Figure 3. Donor derived DN1 CD122$^-$NK1.1$^-$ thymocytes differentiate into NK1.1$^+$ cells in the thymus. Thymi from *Rag1*$^{-/-}$ Ly5.2 mice were harvested and sorted based on DN1 CD122$^-$NK1.1$^-$ phenotype as shown in Figure 2. Cells were transferred into irradiated *Rag1*$^{-/-}$ Ly5.1 mice either intravenous (IV) or intrathymically (IT). Irradiated littermates were used as controls. Cells were harvested from the spleen and thymus 32 days post-transfer. NK1.1$^+$ cells from the lymphocyte population were then examined for host (Ly5.1$^+$) and donor (Ly5.2$^+$) cells. Data are representative of two experiments.

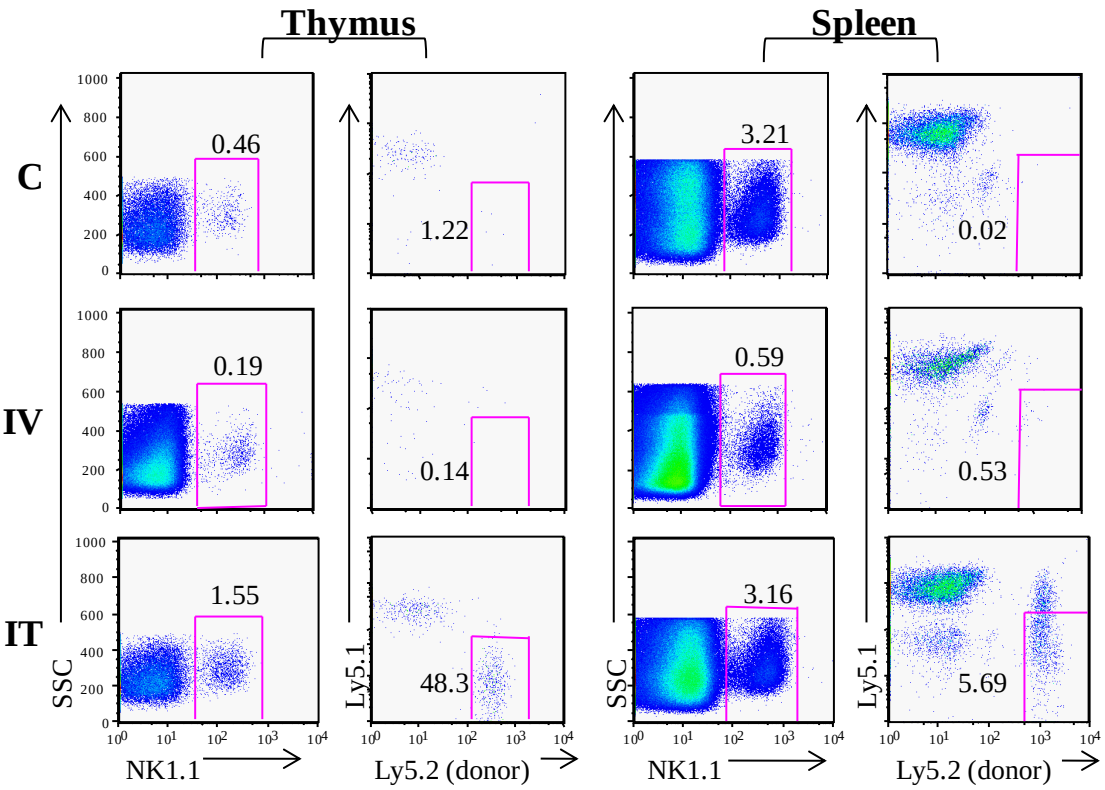
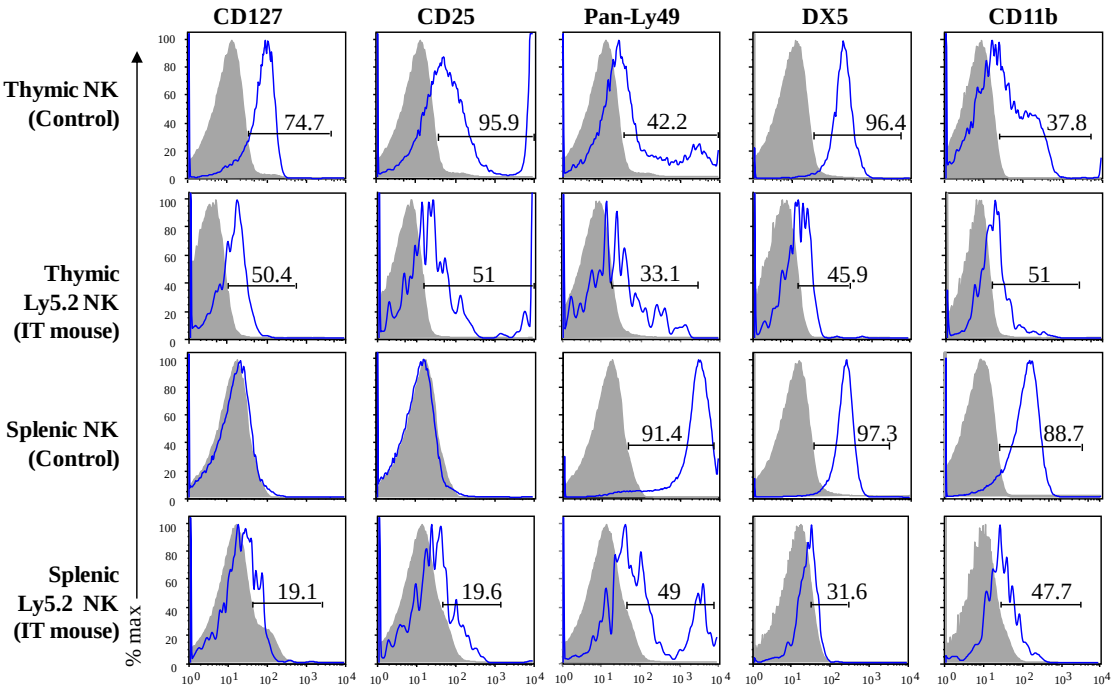

Thymus
Spleen
C
IV
IT
0.46
1.22
3.21
0.02
0.19
0.14
0.59
0.53
1.55
48.3
3.16
5.69
SSC
Ly5.1
NK1.1
Ly5.2 (donor)

Figure 4. DN1 CD122$^-$NK1.1$^-$ thymocytes differentiate into NK cells with a unique phenotype. DN1 CD122$^-$NK1.1$^-$ thymocytes from *Rag1*$^{-/-}$ Ly5.2 mice were transferred into irradiated *Rag1*$^{-/-}$ Ly5.1 mice intrathymically (IT). Donor (Ly5.2$^+$) NK cells from IT mice were compared to NK cells from unmanipulated (non-irradiated) littermates 32 days post transfer. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated antibody. Data are representative of two experiments.


CD127
CD25
Pan-Ly49
DX5
CD11b
Thymic NK (Control)
Thymic Ly5.2 NK (IT mouse)
Splenic NK (Control)
Splenic Ly5.2 NK (IT mouse)
% max
74.7
95.9
42.2
96.4
37.8
50.4
51
33.1
45.9
51
91.4
97.3
88.7
19.1
19.6
49
31.6
47.7

CHAPTER 4

IN VITRO DEVELOPMENT OF THYMIC NK CELLS FROM DN1 CD122^{-}NK1.1^{-} THYMOCYTES

Early studies performed by T cell biologists showed that when DN1 cells were cultured with OP9 stromal cells transfected with Delta-like 1, T cell markers were detected on developing cells by day 6. When this same population was cultured with control OP9 cells, the emerging cells expressed NK1.1 [33]. The limitation to this significant finding was the lack of any further characterization of these NK1.1^{+} cells in terms of phenotype or function. Here, we aimed to determine whether *in vitro* generated cells that differentiate from DN1 cells resemble thymic or conventional splenic NK cells and whether these cells were functional.

In vitro differentiation of NK1.1^{+} cells from thymic DN1 progenitors

Due to low donor-derived NK cell numbers in adoptive transfer experiments, we were unable to perform more detailed experiments with this approach. To further examine the events that take place during NK differentiation of the putative thymic progenitors, we instead utilized an *in vitro* system in which thymic progenitors were co-cultured with OP9 stromal cells in the presence of cytokines. Sorted DN1 CD122^{-}NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice were seeded onto a confluent monolayer of irradiated OP9 stromal cells and cultured in IL7, Flt3L, SCF, and IL15. At different time points, we visually and microscopically examined wells for growth (Figure 5). After 10 days of co-culture, we visualized a colony of cells at the center of most wells, but by day 16 cells appeared in areas devoid of the OP9 monolayer. To assess whether these cells had differentiated after 19 days in culture, we pooled growth-positive wells identified by visual inspection and analyzed surface expression of NK1.1 via flow cytometry. Results showed that by day 16, most cells were NK1.1^{+} (Figure 6A). Kinetic analysis showed the

absence of $NK1.1^+$ cells on day 4 but $NK1.1^+$ cells were readily detected on day 7, and continued to increase on day 10 (Figure 6B) . Thereafter, the percentage of $NK1.1^+$ cells was relatively stable. CD122 expression paralleled that of NK1.1 expression. Together, these results suggest that DN1 $CD122^-NK1.1^-$ thymocytes contained progenitors capable of developing into $NK1.1^+$ cells in an *in vitro* system with OP9 stromal cells and cytokines.

Precursor frequency of the DN1 population

To determine the frequency at which an $NK1.1^+$ cell was differentiated, we performed a limiting dilution analysis. Sorted *Rag1*$^{-/-}$ DN1 $CD122^-NK1.1^-$ thymocytes were seeded in wells at different cell densities in 24-well replicates. After 19 days, all growth-positive wells were individually collected and cells were surface stained and analyzed via flow cytometry. Dead or dying cells were excluded with propidium iodide (PI) staining. Results showed that approximately 80-90% of the cells in all growth-positive wells were $NK1.1^+$. Although the level of NK1.1 expression was somewhat variable, the NK1.1 mean fluorescent intensity (MFI) was independent of seeding dose (Figure 7A). For example, cells seeded at 300 cells/well expressed higher levels (MFI) of NK1.1 compared to cells seeded at 400 and 500 cell/well. In addition, when all growth-positive wells from one seeding dose were compared to each other, expression level of NK1.1 varied from well to well (Figure 7B). This data suggest that we could use the frequency of wells with $NK1.1^+$ cells, regardless of the level of NK1.1 expression, as a reliable outcome in limiting dilution analysis. If we assume that the cells being titrated are randomly and independently distributed among all wells, then the number of cells that

differentiated into NK1.1^{+} cells found in each well should follow the Poisson distribution. By graphing the number of cells per well versus the log of the frequency of negative cultures, we were able to demonstrate a frequency of 0.24% with a 95% confidence interval of 0.3% to 17% (Table I and Figure 8). These results indicate that one out of every 416 sorted DN1 CD122^{-}NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice is an NK cell precursor that can differentiate into and NK1.1^{+} cell *in vitro*.

In vitro generated NK1.1^{+} cells have a unique phenotype

In vivo studies have delineated tentative developmental stages for conventional splenic NK cells based on marker correlation but developing NK cells have not been directly observed to undergo the putative phenotypic changes that characterize each stage [16, 20]. Here, we were able to directly follow the kinetics of NK cell development from progenitor stage to NK1.1-expressing cells. Briefly, sorted *Rag1*$^{-/-}$ thymocytes were seeded at a concentration of 1000 cells per well on a monolayer of irradiated OP9 stromal cells in the presence of cytokines. At different time points, equal number of wells were pooled, stained with a panel of antibodies and analyzed via six-color flow cytometry. We used a seeding concentration of 1000 cells per well to insure that each well would have differentiating NK1.1^{+} cells (from an average of 1-2 progenitors) and chose to pool wells for analysis due to the small number of cells available. Again, as described for the intrathymic transfer studies, the staining patterns for each marker were somewhat different but we used the percent of positive cells (above isotype control staining) as a convenient (although imprecise) means to compare and describe the various staining profiles. Phenotypic analysis of the developing cells after 4 days of co-culture showed

most markers were absent, except CD117 (cKit) and CD25 (IL2Rα) which were expressed at low levels (Figure 9). Although the cells lacked most markers on day 4, including NK1.1 and CD122 (Figures 6, 9A), more than 30% had differentiated into NK1.1 expressing cells by day 7, all of which co-expressed CD122. The number of $NK1.1^+$ cells increased to more than 85% by day 19 (Figures 6, 9B). Taken together, these data strongly suggest that the cells were differentiating into $NK1.1^+$ cells rather than representing outgrowth of contaminating mature NK cells because NK1.1 was not initially expressed and markers associated with NK cells were primarily expressed only after a week of *in vivo* culture.

To further analyze the kinetics of development in the *in vitro* generated cells, we compared the phenotypic results on $NK1.1^+$ cells from different culture time points to each other, expressed as a percentage of $NK1.1^+$ cells (Figure 10). As compared to other markers, both CD122 and NK1.1 were expressed relatively early with ready detection on day 7, where approximately 30% of total cells express both markers (Figures 6, 9A). Interestingly, CD44 was present throughout the entire culture time, with more than 99% of the cells expressing it (Figure 10). Developing cells also failed to express CD94 on day 4, but more than 80% of $NK1.1^+$ cells had up-regulated this receptor by day 7. By contrast, NKG2D was poorly expressed on day 7, and instead was expressed by essentially all NK cells on day 10. The acquisition of CD94 and NKG2D on developing NK cells after CD122 and NK1.1 are expressed supports previous findings by other colleagues [8, 20, 21]. With the acquisition of NKG2D on day 10, followed by the subsequent expression of Ly49 on day 13, it appeared that the *in vitro* generated NK cells progressed from stage II of development to sate III during this window of time. Again,

limited by the number of cells, we were unable to assess specific Ly49 expression with mono-specific anti-Ly49 antibodies during this temporal analysis. Only a fraction of the cells expressed Ly49s. However, unlike conventional splenic NK cells, the *in vitro* generated cells lacked DX5 expression, and CD43 and CD11b were detected only after about 16 days in culture. Interestingly, low levels of CD127 was detected on the *in vitro* cultured NK cells. While 10% to 25% of the cells were $CD127^{low}$ on day 10, there were more than 50% $NK1.1^{+}CD127^{+}$ cells by day 19 (Figures 9, 10). Overall, these studies have allowed us to follow the sequential development of putative NK cells from DN1 thymocytes which generally recapitulates developmental stages of developing NK cells in the BM.

Comparison between *in vitro* generated cells and freshly isolated NK cells

Our studies on the kinetics of development suggested that *in vitro* generated $NK1.1^{+}$ cells were either conventional NK cells arrested between stage II and stage IV of the developmental process or that they differentiated into $NK1.1^{+}$ cells resembling thymic NK cells. Inasmuch as our adoptive transfer studies suggested that the sorted DN1 cells gave rise to $NK1.1^{+}$ cells that more closely resembled thymic NK cells, we sought to further determine if our *in vitro* differentiated $NK1.1^{+}$ cells resembled thymic NK cells. Here, we compared expression of surface markers on *in vitro* derived $NK1.1^{+}$ cells after 19 days in culture with that of freshly isolated thymic and splenic NK cells (Figure 11). Similar to thymic $CD127^{+}$ NK cells, less than half of the *in vitro* generated cells had low expression of Ly49 receptors, CD11b, and CD62L. Further detailed analysis of the Ly49 receptors confirmed these data because splenic NK cells readily expressed Ly49A,

Ly49C, Ly49D, Ly49G2, and Ly49H, some of which are expressed on >50% of cells. On the contrary, while the largest pool of thymic NK cells expressed Ly49G2, less than 20% expressed any other of these receptors, (Figure 12). *In vitro* generated cells lacked Ly49C and Ly49G2 and less than 10% expressed low levels of Ly49A, Ly49D, Ly49F, or Ly49H (Figure 12). Kinetic analysis showed that CD25 was up-regulated in enriched thymic progenitors during the first 4 days of culture and expression was then constant through day 19 (Figures 9, 10). Surprisingly, as noted above (Figure 4), CD25 was also detected in thymic NK cells from *Rag1*$^{-/-}$ mice with essentially all cells expressing it at intermediate to high levels (Figure 11). Studies done on splenic and thymic NK cells from C57BL/6 mice showed CD25 was not present on either NK cell type, suggesting CD25 is selectively expressed on thymic cells from *Rag1*$^{-/-}$ mice (data not shown). Finally, and most importantly, the majority of *in vitro* generated NK cells uniformly expressed CD127, a marker of thymic NK cells [39], albeit at lower levels than thymic NK cells. Overall, the phenotypic comparisons between all three cells types suggest that *in vitro* generated NK1.1^{+} cells more closely resemble thymic, and not conventional splenic NK cells.

Unlike thymic NK cells, the *in vitro* NK1.1^{+} cells did not express the integrin DX5 or CD69. However, a slight modification in the culture protocol was informative. Briefly, thymic progenitors were allowed to differentiate into NK1.1-expressing cells for 19 days under standard culture conditions with OP9 cells and cytokines, at which point cells were harvested, washed, and incubated in low-dose (LD) IL15 alone for 36 hrs. Interestingly, culture in LD IL15 alone was associated with the up-regulation of DX5 and CD69 and the down-regulation of CD11b (Figure 13), resulting in a phenotype strikingly

similar to thymic NK cells (Figure 11). Thus, this modification resulted in *in vitro* differentiated NK cells that now very closely resembled thymic NK cells.

In vitro generated cells are functional

Due to phenotypic similarity to thymic NK cells, we further sought to characterize the *in vitro* generated NK1.1^{+} cells by comparing their function to freshly isolated thymic NK cells. Functional analysis for the production and secretion of cytokines, along with the ability to degranulate were assessed by both target cell-free and cell-dependent stimulation assays, respectively. For these studies, we used *in vitro* differentiated NK cells cultured under standard conditions with OP9 cells and cytokines for 19 days. Cytometric Bead Array (CBA) results showed that at this point, the cells were secreting several cytokines and chemokines, including monocyte chemotactic protein-1 (MCP-1), IFNγ, TNF, and IL6, without further stimulation (Figure 14). These results suggest that the *in vitro* NK1.1^{+} cells were in a pre-activated state due to their culturing environment, thus making them incomparable to freshly isolated splenic and thymic NK cells.

To address this, we differentiated *Rag1*$^{-/-}$ DN1 CD122^{-}NK1.1^{-} thymocytes in standard culture conditions for 19 days, at which point, cells were collected, washed and "rested" in low-dose (LD) IL15 alone. Every 12 hours, we pooled cells and re-cultured them in either media alone (non-stimulated) or media supplemented with IL12+IL18 for a period of eight hours. Results showed that *in vitro* generated cells reached a state of "rest" only after being in the LD IL15 culture for 36 hours, at which point they ceased to secrete all tested cytokines (Figure 15 and data no shown). In addition, cells did not lose the ability to secrete certain cytokines upon IL12+IL18 stimulation at any time point,

even after reaching their resting state. Finally, once removed from their standard culturing conditions and placed in LD IL15 alone, cells altered their phenotype to further resemble thymic NK cells more closely (Figure 13). Together, these results suggest that subtle changes in culture may resemble, but not necessarily recapitulate, the dynamically changing environment that occurs during *in vivo* cell development.

To assess whether the resting *in vitro* generated NK cells were functional, we performed a target cell-free stimulation assay with IL12+IL8 or plate bound anti-NK1.1 (clone PK136) for 8 hours. Intracellular flow cytometry results were compared to those from sorted splenic and thymic NK cells that were cultured in LD IL15 for 16 hours (Figure 16). These *in vivo* differentiated NK cells were not cultured (rested) longer due to a lower yield at later time points. Pilot studies showed there were no changes in phenotypic markers or activation during this incubation period (data not shown). While the majority of cells, regardless of *in vivo* or *in vitro* differentiation, produced a large amount of IFNγ when stimulated with IL12+IL18, less than 30% were capable of responding to PK136 (Figure 16). Moreover, the amount of IFNγ detected in PK136 cultures was approximately one log lower than that produced when stimulated with IL12+IL18. These results suggest that the *in vitro* generated NK cells are functional and can be stimulated to produce IFNγ, especially when stimulated in an ITAM-independent manner.

To more broadly compare the function of LD IL15 rested *in vitro* differentiated $NK1.1^+$ cells to rested splenic and thymic NK cells, we once again used a mouse inflammation CBA to quantitatively detect a wider panel of secreted cytokines. As expected from the intracellular staining assay, all cell types secreted a large amount of

IFNγ when stimulated with IL12+IL18, but only the latter responded as well, if not better, upon PK136 stimulation. Interestingly, TNFα was produced by thymic and *in vitro* NK cells upon stimulation with both IL12+IL18 and PK136, whereas splenic NK cells produced TNFα only with PK136 stimulation. IL6 was detected only after IL12+IL18 stimulation but MCP-1 was not detected at any point (Figure 17). These results further indicate that the *in vitro* generated NK cells can be activated with various stimuli to functionally resemble thymic NK cells.

Finally, to determine if the *in vitro* generated NK cells were capable of responding to target cells, we co-incubated the three cell types with YAC-1 target cells. In a similar manner, thymic and *in vitro* NK cells degranulated upon target encounter (Figure 18). While there was a difference in degranulation between splenic NK cells and thymic and *in vitro* NK cells, it may not be biologically significant. Overall, phenotypic and functional analysis show the similarities between thymic and *in vitro* generated NK cells, and together they share differences from their splenic counterpart.

Discussion

Adoptive transfer studies showed thymic $CD127^+$ NK cells developed from DN1 $CD122^-NK1.1^-$ progenitors, although further analysis was limited due to the low recovery yield of $Ly5.2^+NK1.1^+$ cells 32 days post transfer. To compensate for the experimental limitations imposed by *in vivo* studies, we cultured the enriched thymic progenitors from *Rag1*$^{-/-}$ mice in an *in vitro* system conducive for precursor development. Early *in vitro* studies showed that $NK1.1^+$ cells can differentiate from ELP when cytokine such as SCF, IL7, Flt3L, and IL15 are added to the culture [22]. However, it was later shown that

developing ELPs need to come in direct contact with BM stromal cells to acquire a phenotype that resembles conventional splenic NK cells, thereby expressing molecules such as Ly49 receptors [23-25] .

In these studies, we confirmed that OP9 stromal cells and all cytokines were necessary for the growth of $NK1.1^+$ wells, resulting in a low to negative yield of $NK1.1^+$ cells if any component was excluded during the culture (data not shown). For the most part, we seeded irradiated OP9 cells in 96 well U-bottom plates 24 hours before co-culture to allow them to settle to the bottom of the well and form a confluent monolayer essential for progenitor development. Microscopic images showed the progenitors made direct contact with the OP9 cells for the first 8-9 days of culture, but by day 10, the center of the well was devoid of the stromal cells and instead was populated by the developing cells (Figures 5,10). This suggested that OP9 cells were essential for the early stages of development, while the cytokines were important for any further maturation during later time points.

In addition, it was crucial that the OP9 stromal cells were in a healthy condition before and during co-culture. Surprisingly, a simple change in FBS (from Biomeda to Sigma) resulted in a morphological change in OP9 cells which affected their ability to adhere to the bottom of the well, thereby leading to early cell death. Without the OP9 monolayer, the sorted DN1 $CD122^-NK1.1^-$ thymocytes did not receive the proper cell-to-cell signaling required for differentiation (data not shown). After replacing the new Sigma FBS with Biomeda FBS, we were once again able to detect $NK1.1^+$ cells after 7 days in culture (data not shown and Figure 6B). This data suggest that direct and

constant interaction between healthy stromal cells and DN1 CD122^{-}NK1.1^{-} thymocytes is necessary for the generation of NK1.1^{+} cells.

Besides showing that OP9 cells and cytokines are essential for *in vitro* NK1.1^{+} differentiation, our results also showed that the cultured cells did not represent an outgrowth of mature NK cells since these do not require OP9 cells to proliferate. This was further confirmed by the absence of any detectable CD122 and NK1.1-expressing cells after 4 days in culture. By assessing marker expression at different time points we were also able to follow the gradual acquisition of markers associated with developing NK cells, which would not have been possible if there was a contaminating mature NK population.

Unlike their conventional counterparts in the spleen, little is known about thymic NK cells. While our *in vitro* differentiated cells resembled thymic NK cells and not conventional NK cells, there were some subtle differences. For example, when we analyzed the *in vitro* generated cells after being in culture for 19 days, we were unable to detect DX5 and CD69 expression, markers typical of thymic NK cells. It was only after we optimized the culture by "resting" the cells in LD IL15 alone that the *in vitro* cells up-regulated DX5 and CD69, a marker generally associated with cell activation, while down-regulating CD11b. In addition, subtle differences in the expression levels of Ly49 molecules in thymic and *in vitro* generated NK cells may be due to stromal factors that regulate Ly49 expression that are difficult to recapitulate *in vitro* [24]. For example, irradiation may alter stromal molecules or the MHC on OP9 cells could affect Ly49 expression or acquisition. These *in vitro* studies perhaps reflect a dynamically changing *in vivo* cytokine environment during normal development. Regardless, the studies

described here provide future opportunities to dissect the influence of differing cytokine environments and stromal components on thymic NK cells and their development.

Moreover, functional studies showed that unlike their splenic counterpart, thymic and *in vitro* generated NK cells are less efficient at degranulating upon target cell encounter, yet more efficient at secreting certain cytokines such as TNF. Although the exact function of this NK cell subset has yet to be elucidated, we can speculate that it may produce a specific cytokine or chemokine upon activation which has not been identified. The recent discovery of the NK-22 subset in mucosal tissues which can secrete IL22, IL26 and leukemia inhibitor factor upon IL23 exposure, suggests that different NK cell subsets may have differing roles [58]. While the function of these cells may include protecting mucosal sites by constraining inflammation during bacterial infection, the specific role of $CD127^+$ thymic NK cells is not yet clear. Future studies looking at the effect of depleting these cells *in vivo* will help us further examine their function within the thymus and other tissues.

While our studies demonstrate that thymic-like NK cells can differentiate from DN1 $CD122^-NK1.1^-$ cells, more work is needed to determine if a single progenitor cell in the thymus could give rise to this particular $CD127^+$ NK cell subset. In addition, it has yet to be determined whether these progenitors are pre-committed to this specific NK lineage once they home to the thymus or if experimental constraints allowed preferential development of thymic NK cells. Future studies where the DN1 population is further dissected will help us answer some of these questions.

To our knowledge, our studies were the first to attempt to determine the progenitor frequency in which a thymic precursor can give rise to an NK1.1-expressing

population by limiting dilution analysis. Since the putative DN1 CD122$^-$NK1.1$^-$ progenitor population that differentiates into thymic NK cells has yet to be determined, we lack the point of reference to compare our findings. While one out of every 416 DN1 CD122$^-$NK1.1$^-$ thymocytes may seem like a low frequency, it should be noted that this precursor population is negatively defined by lack of marker expression (CD4, CD8, CD117, CD122, NK1.1), rather than the expression of markers associated with high progenitor frequency. Nonetheless, it should also be emphasized that we enriched for a thymic NK cell precursor by using *Rag1*$^{-/-}$ thymi and sorted for the DN1 CD122$^-$NK1.1$^-$ population which represents approximately 0.0073% of C57BL/6 (WT) thymocytes (data not shown). Future experiments to enrich for the putative DN1 CD122$^-$NK1.1$^-$ progenitor, perhaps the CD117$^+$ population mentioned in Chapter 3, may be informative to markedly increase precursor frequency to about 1/10. Nonetheless, these studies show that this *in vitro* approach is feasible for the differentiation of thymic progenitors to CD127$^+$NK1.1$^+$ cells that phenotypically and functionally resemble thymic NK cells.

Figure 5. Differentiating thymic precursors can be visualized by day 10. Sorted DN1 $CD122^{-}NK1.1^{-}$ thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and IL7, Flt3L, SCF, and IL15. Wells were visually and microscopically examined for growth on different days. Top panels are at 4X magnification, while bottom panels are at 20X magnification. Data are representative of more than three experiments

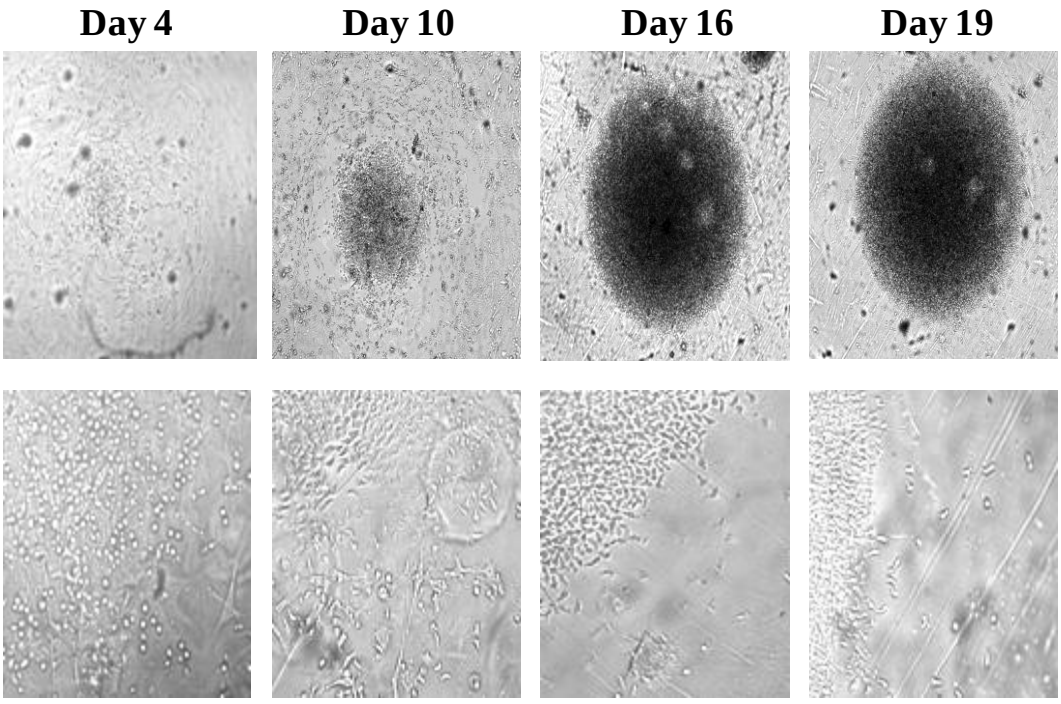
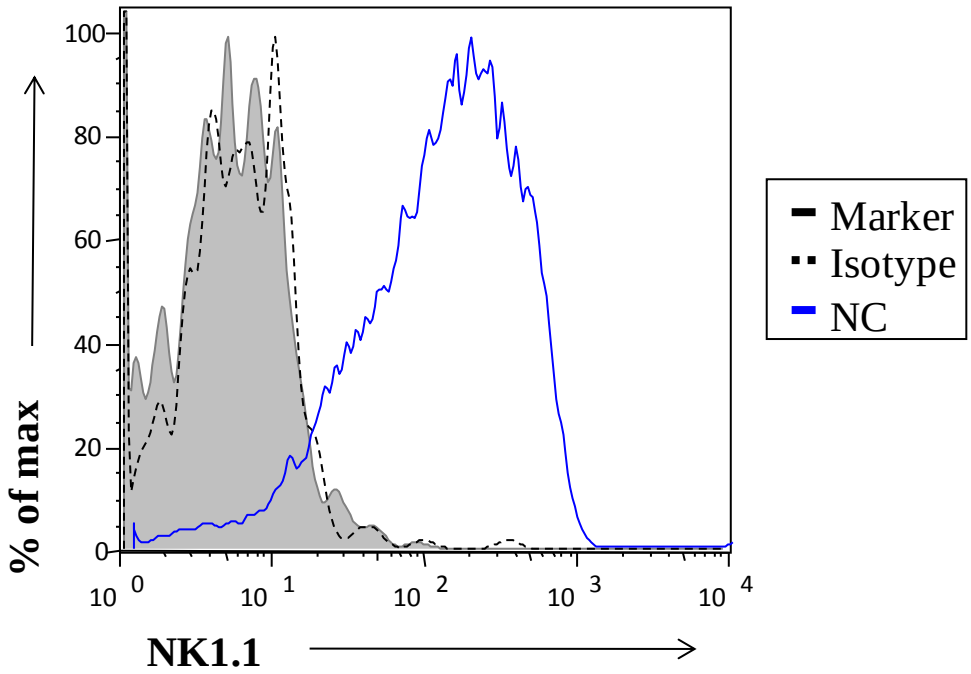

Day 4
Day 10
Day 16
Day 19

Figure 6. Thymic progenitors can differentiate into NK 1.1^{+} cells *in vitro*. Sorted DN1 CD122^{-}NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and cytokines. A) Growth positive wells by visual inspection were pooled, stained, and analyzed for expression of NK1.1 after 19 days in culture. For the negative controls, cells were either left unstained (gray-filled histograms) or stained using the appropriate isotype antibody (black dotted histogram). Cells were gated based on lymphocyte population by scatter parameters. B) Kinetic analysis of NK1.1 and CD122 expression on lymphocyte population, gated by scatter parameters. At varying culture periods, wells were examined for the indicated markers as described in Figure 6A. Data are representative of at least three experiments.

A


100
80
60
40
20
0
% of max
10 0
10 1
10 2
10 3
10 4
NK1.1
Marker
Isotype
NC

B

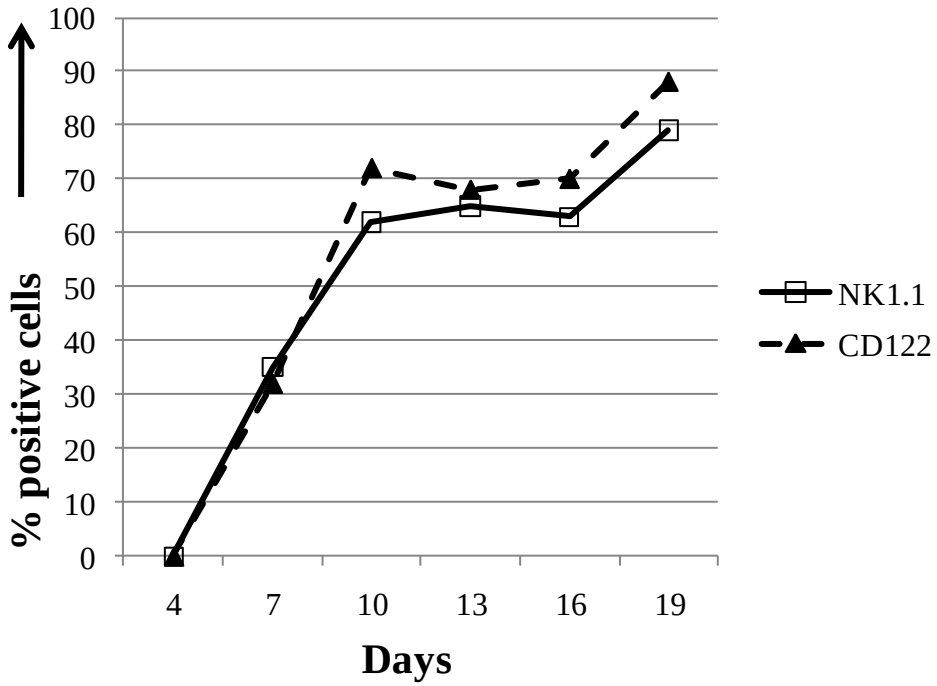

100
90
80
70
60
50
40
30
20
10
0
% positive cells
4
7
10
13
16
19
Days
NK1.1
CD122

Figure 7. All visually growth positive wells were NK1.1^{+}. Sorted DN1 CD122^{-} NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and cytokines at limiting cell densities for 19 days. Growth-positive wells were analyzed for NK1.1 expression by flow cytometry. Cells were gated on the lymphocyte population. (A) Histograms from one representative well for each seeding dose are shown. (B) Histograms show all growth-positive wells for progenitors seeded at 300 cells/well. Data represent three experiments.

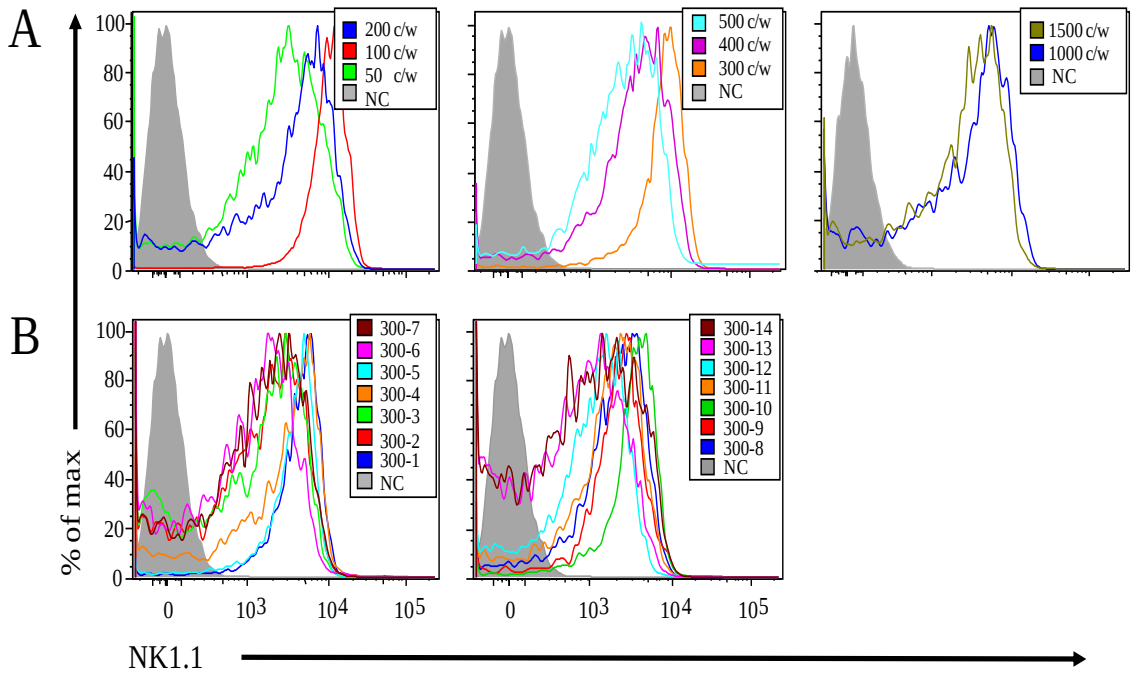

A
B
% of max
NK1.1
200 c/w
100 c/w
50 c/w
NC
500 c/w
400 c/w
300 c/w
NC
1500 c/w
1000 c/w
NC
300-7
300-6
300-5
300-4
300-3
300-2
300-1
NC
300-14
300-13
300-12
300-11
300-10
300-9
300-8
NC
0
10^3
10^4
10^5

Table I and Figure 8. Limiting dilution analysis (LDA) of DN1 CD122$^-$ NK1.1$^-$ thymocytes to determine progenitor frequency. Sorted DN1 CD122$^-$ NK1.1$^-$ thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and cytokines at limiting cell densities for 19 days. Growth-positive wells were analyzed for NK1.1 expression by flow cytometry. The frequency of growing cells was determined using Poisson distribution : Y=(e-m)(mr)/r!, where *Y* is the expected fraction of wells with *r* precursors when there is a mean of *m* precursors per well. Growth-negative wells are plotted as a function of the number of sorted thymocytes added per well using the Graphpad Prism software. The frequency is 0.24%. Standard error is 0.027% and 95% confidence interval is 0.3% to 0.17%. Data represent three experiments.

Cell/Well	# of positive wells	Fraction of negative wells
50	3/24	0.875
100	5/24	0.792
200	5/24	0.792
300	14/24	0.417
400	17/24	0.292
500	17/24	0.292
1000	21/24	0.125

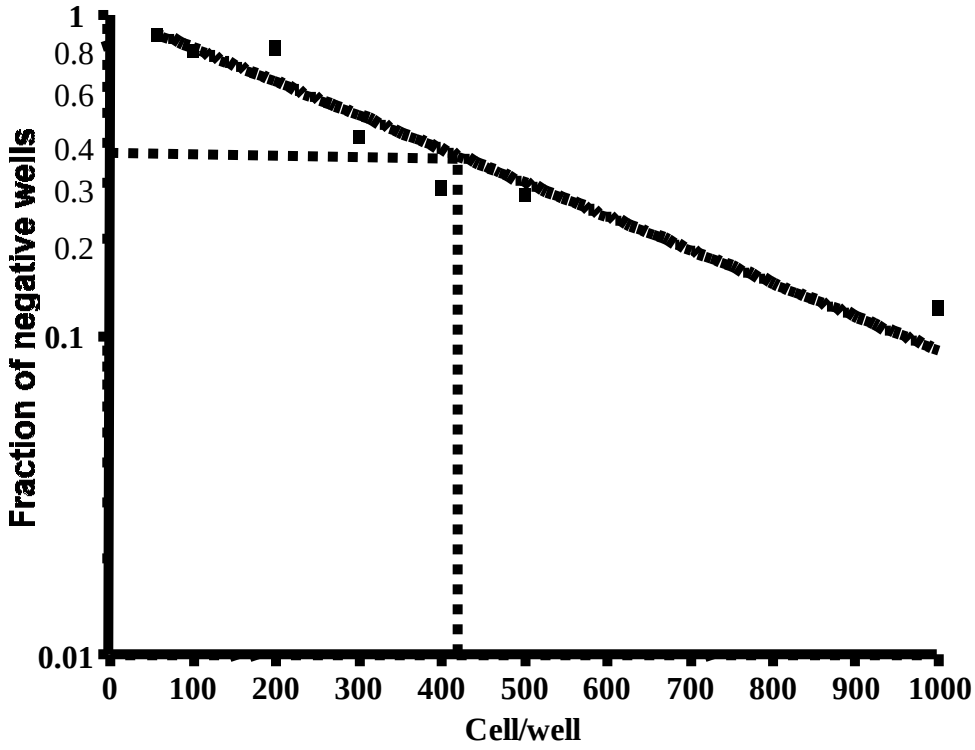


Figure 9. Phenotypic profile of cells generated *in vitro* from thymic progenitors. Cells cultured *in vitro* were pooled on different days and assessed for marker expression via flow cytometry. Black histograms represent cells stained with appropriate isotype controls and colored-line histograms correspond to different days of analysis. Early (A) and late (B) marker acquisition is shown. Expression on gated NK1.1^{+} cells is shown, except for all markers on day 4 and NK1.1 expression on different days, which were gated on the lymphocyte population. Data are representative of 3-5 experiments.

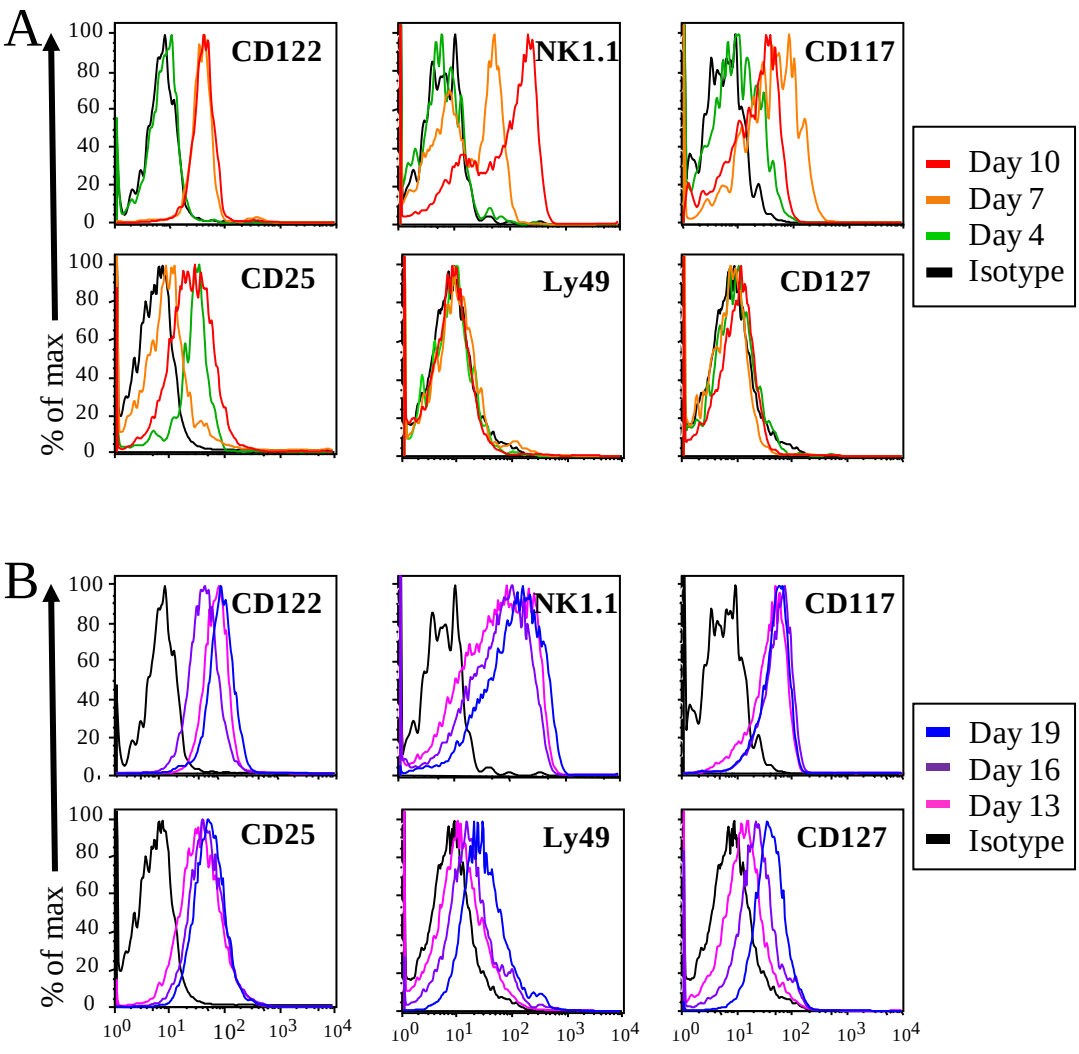

A
CD122
NK1.1
CD117
CD25
Ly49
CD127
% of max
Day 10
Day 7
Day 4
Isotype
B
CD122
NK1.1
CD117
CD25
Ly49
CD127
% of max
Day 19
Day 16
Day 13
Isotype

Figure 10. Developmental kinetic profile of NK1.1^{+} cells generated *in vitro* from thymic progenitors. Sorted DN1 CD122^{-} NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and cytokines at limiting cell densities for 19 days. Cells were pooled on different days and assessed for marker expression via flow cytometry. Developmental kinetics of sorted thymocytes as a function of days versus the percentage of NK1.1^{+} cells expressing the given marker. Data are representative of 3-5 experiments.

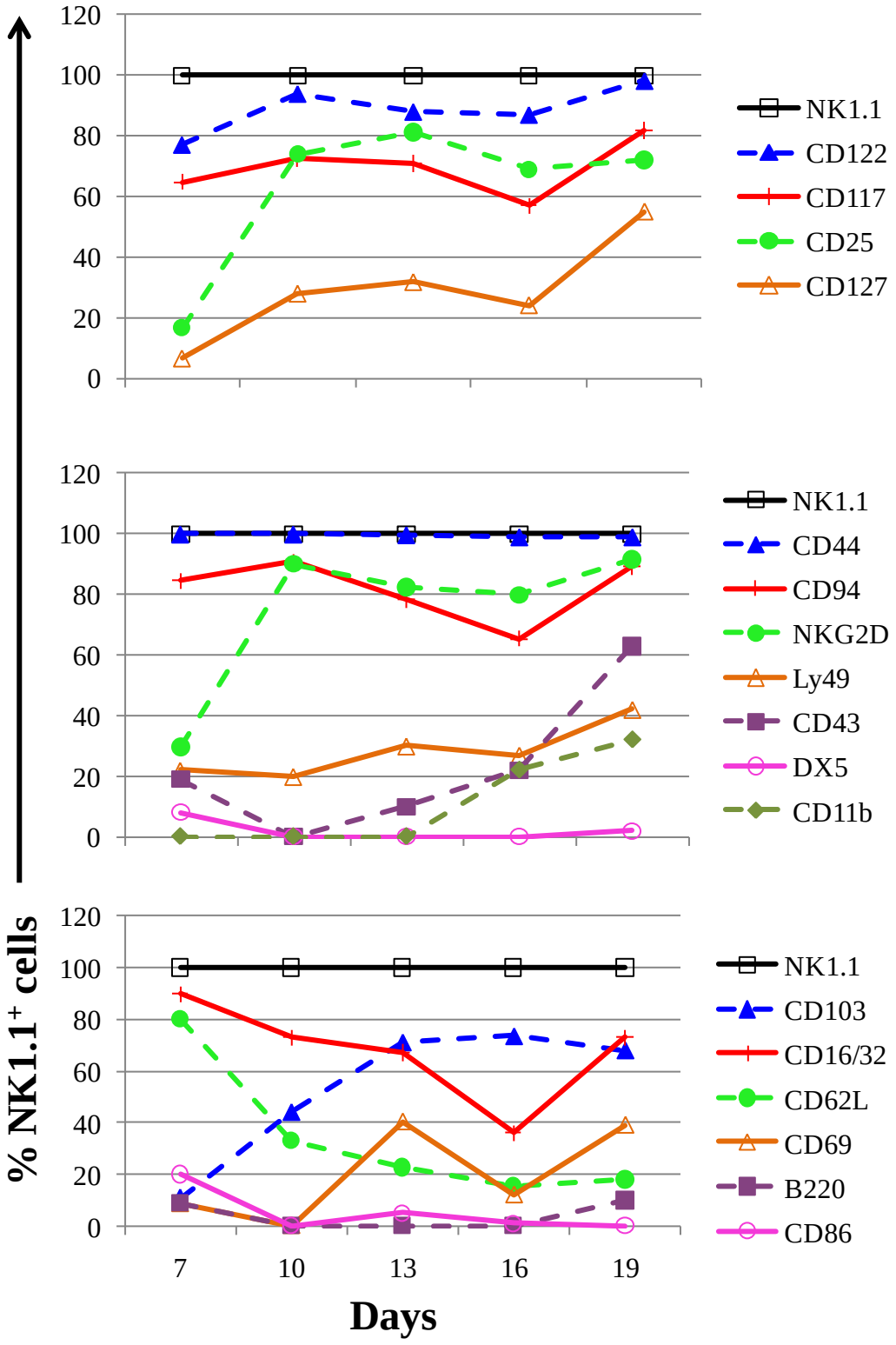

120
100
80
60
40
20
0
NK1.1
CD122
CD117
CD25
CD127
NK1.1
CD44
CD94
NKG2D
Ly49
CD43
DX5
CD11b
NK1.1
CD103
CD16/32
CD62L
CD69
B220
CD86
% NK1.1+ cells
7
10
13
16
19
Days

Figure 11. *In vitro* developed NK1.1^{+} cells resemble those found in the thymus and not conventional splenic NK cells. Cells from indicated tissues of *Rag1*$^{-/-}$ mice, along with *in vitro* differentiated NK cells were analyzed by cytometry. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated antibody. *In vitro* generated NK cells were compared to freshly isolated splenic and thymic NK cells. Gated NK1.1^{+} cells are shown. Data are representative of 5 experiments.

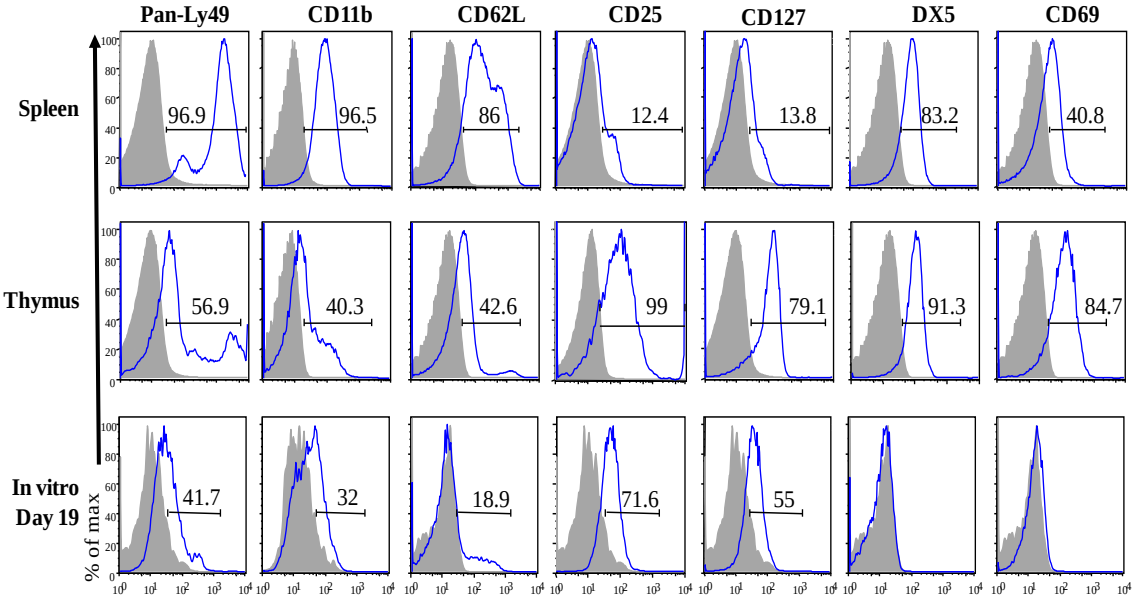

Pan-Ly49
CD11b
CD62L
CD25
CD127
DX5
CD69
Spleen
Thymus
In vitro Day 19
% of max
96.9
96.5
86
12.4
13.8
83.2
40.8
56.9
40.3
42.6
99
79.1
91.3
84.7
41.7
32
18.9
71.6
55

Figure 12. Ly49 receptors expressed in splenic, thymic and *in vitro* derived NK cells. Cells from indicated tissues of *Rag1*$^{-/-}$ mice, along with *in vitro* differentiated NK cells were analyzed by cytometry. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated Ly49 antibody. *In vitro* generated NK cells were compared to freshly isolated splenic and thymic NK cells. Gated NK1.1^{+} cells are shown. Data are representative of 4 experiments.

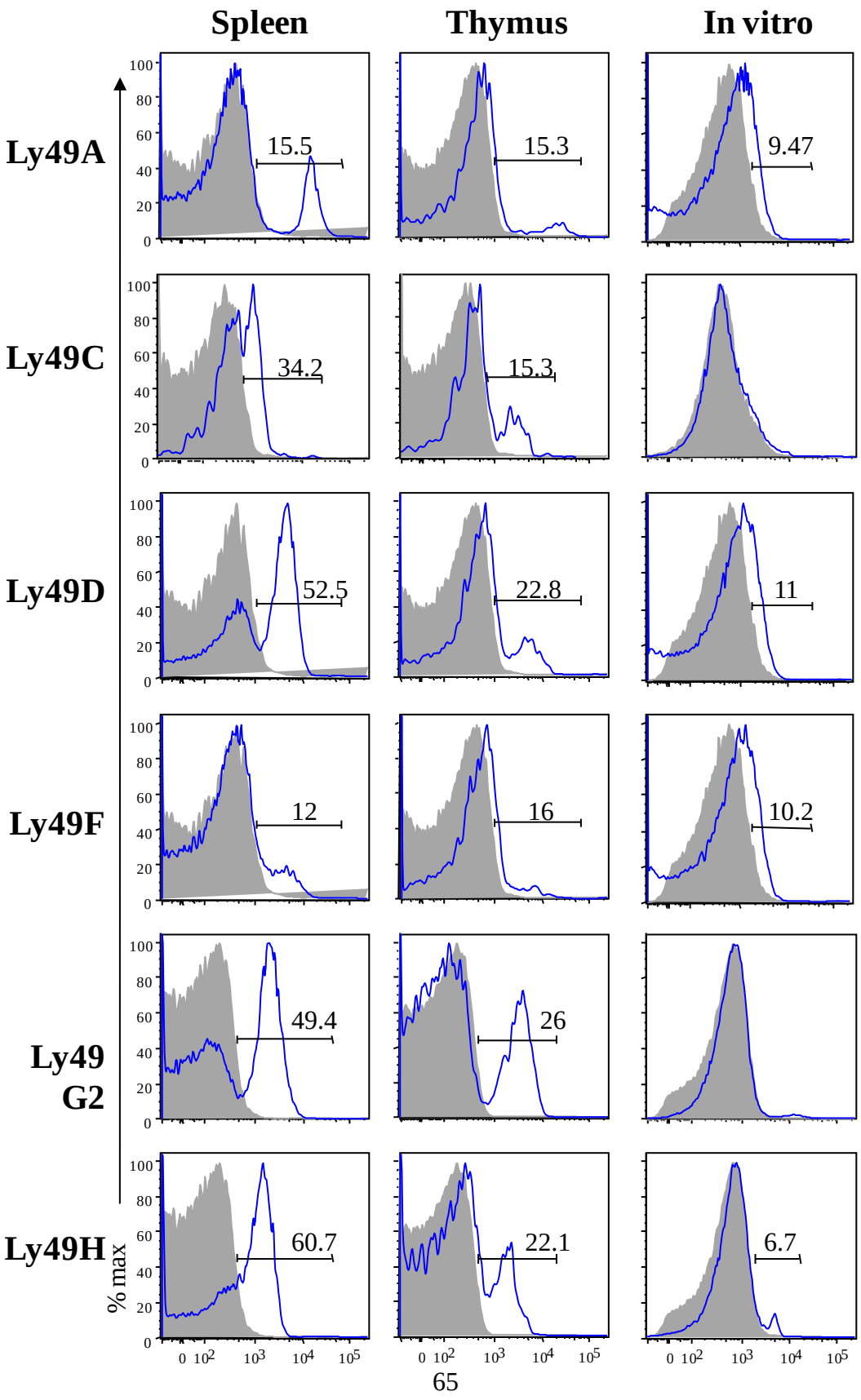

Spleen
Thymus
In vitro
Ly49A
Ly49C
Ly49D
Ly49F
Ly49 G2
Ly49H
15.5
15.3
9.47
34.2
15.3
52.5
22.8
11
12
16
10.2
49.4
26
60.7
22.1
6.7
% max
65

Figure 13. *In vitro* generated NK1.1^{+} cells closely resemble thymic NK cells after a change in culture condition. The phenotype of *in vitro* generated NK cells in culture for 19 days (top panel) was compared to that of cells that were removed from culture after 19 days and rested in LD IL15 for 36 hrs (bottom panel). Gated NK1.1^{+} cells are shown. Data are representative of 2 experiments.

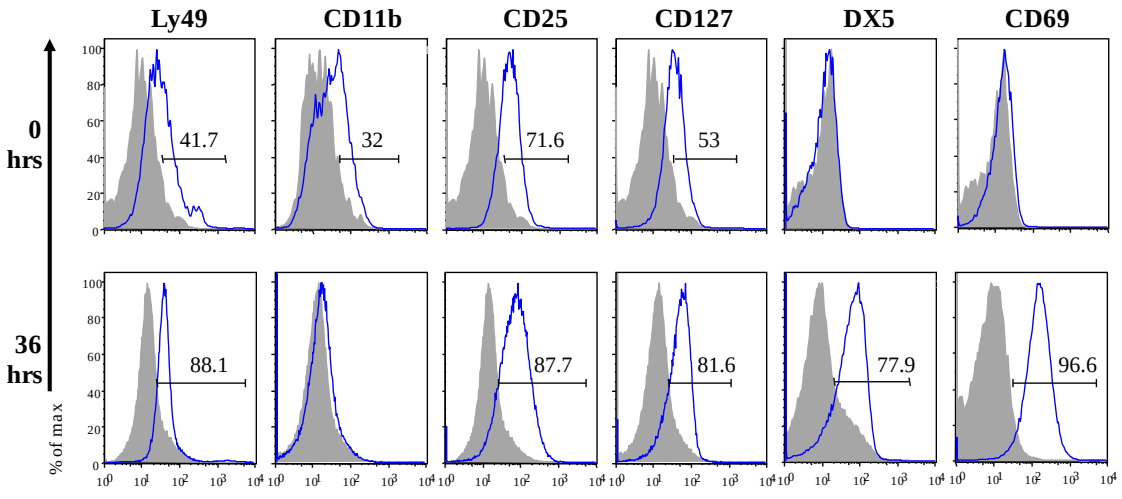

Ly49
CD11b
CD25
CD127
DX5
CD69
0 hrs
36 hrs
% of max
41.7
32
71.6
53
88.1
87.7
81.6
77.9
96.6

Figure 14. *In vitro* generated NK1.1^{+} cells are in an activated state. Cytokine production from i*n vitro* differentiated NK cells grown in culture for 19 days was compared to that produced by freshly isolated *Rag1*$^{-/-}$ splenic and thymic NK cells. Sorted NK1.1^{+} cells were stimulated with IL12+IL18 or plate-bound anti-NK1.1 for 8 hours. Supernatants were collected and cytokines secreted were assessed by cytometric bead array (CBA). All experimental conditions were done in triplicates. Data are representative of 3 experiments

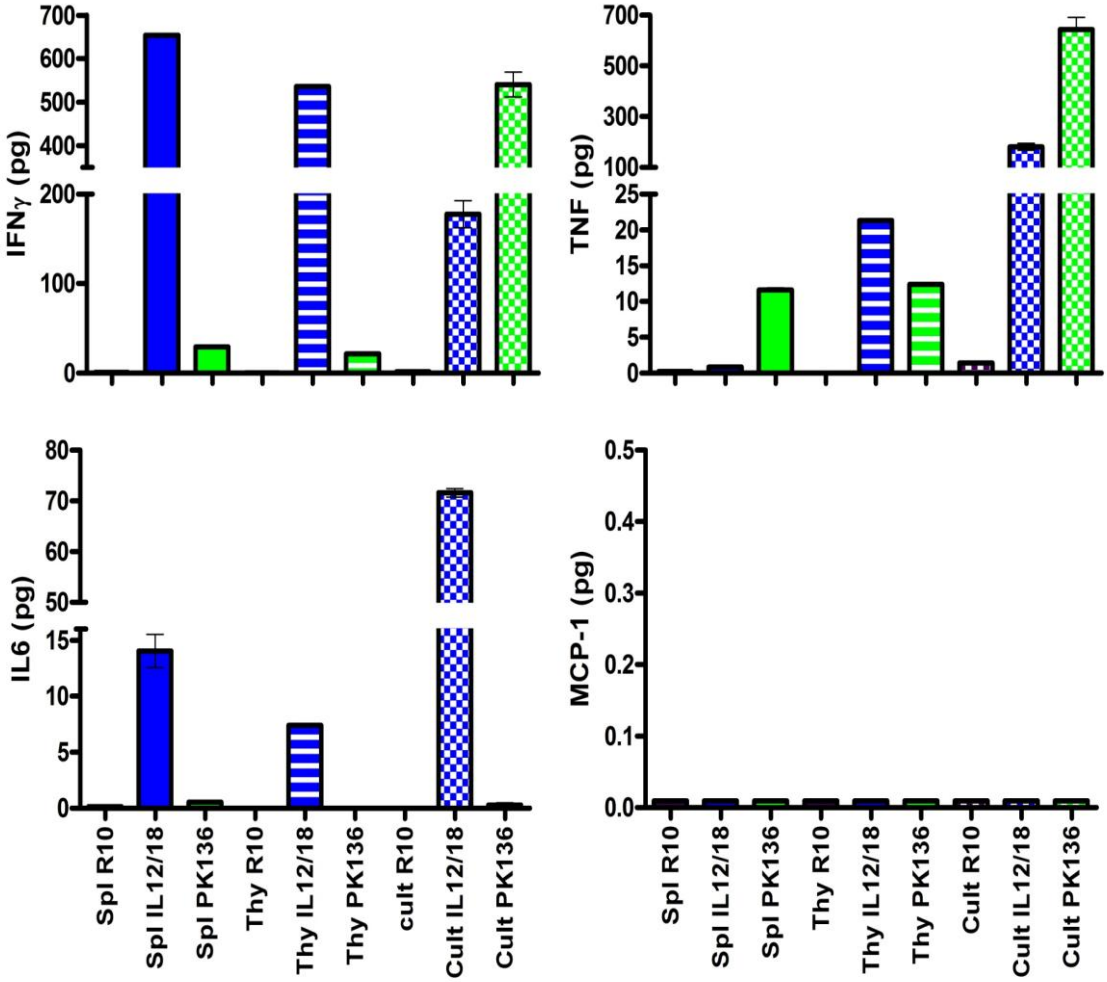

IFNγ (pg)
700
600
500
400
200
100
0
TNF (pg)
700
500
300
100
25
20
15
10
5
0
IL6 (pg)
80
70
60
50
15
10
5
0
MCP-1 (pg)
0.5
0.4
0.3
0.2
0.1
0.0
Spl R10
Spl IL12/18
Spl PK136
Thy R10
Thy IL12/18
Thy PK136
cult R10
Cult IL12/18
Cult PK136

Figure 15. *In vitro* generated NK1.1^{+} cells reach a "resting" state after culturing in LD IL15 alone. Sorted DN1 CD122^{-} NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and cytokines for 19 days. Cells were then pooled, washed with PBS and seeded in LD IL15. Every 12 hours, we collected cells and cultured them in either R10 or R10 supplemented with IL12+IL18 for an additional 8 hours. Supernatants were collected and cytokine secretion was assessed by CBA. Data are representative of 2 experiments.

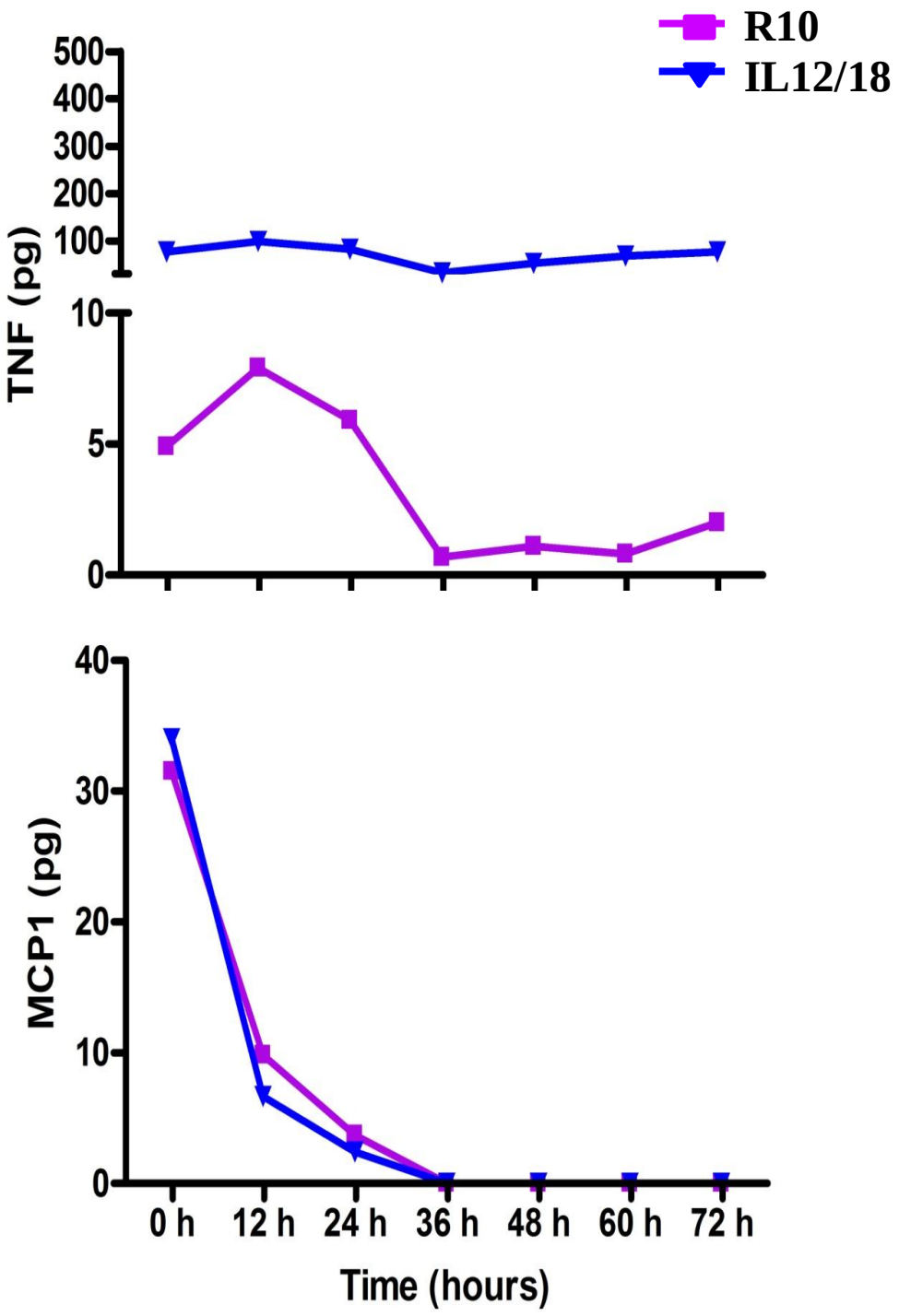

R10
IL12/18
500
400
300
200
100
10
5
0
TNF (pg)
40
30
20
10
0
MCP1 (pg)
0 h
12 h
24 h
36 h
48 h
60 h
72 h
Time (hours)

Figure 16. *In vitro* generated NK1.1^{+} cells are functional. The function of i*n vitro* differentiated NK cells grown in culture for 19 days then rested in LD IL15 was compared to the function of *Rag1*$^{-/-}$ splenic and thymic NK cells cultured in LD IL15. Sorted NK1.1^{+} cells were stimulated with IL12+IL18 or plate-bound anti-NK1.1 for 1hr, Brefeldin A was added and cells were further incubated for 7 hours. IFNγ production was measured by intracellular flow cytometry. For negative controls, cells were either not stained (gray-filled histograms) or not stimulated (black-dotted histograms). All histograms are gated on NK1.1^{+} cells. Data are representative of 2 experiments.

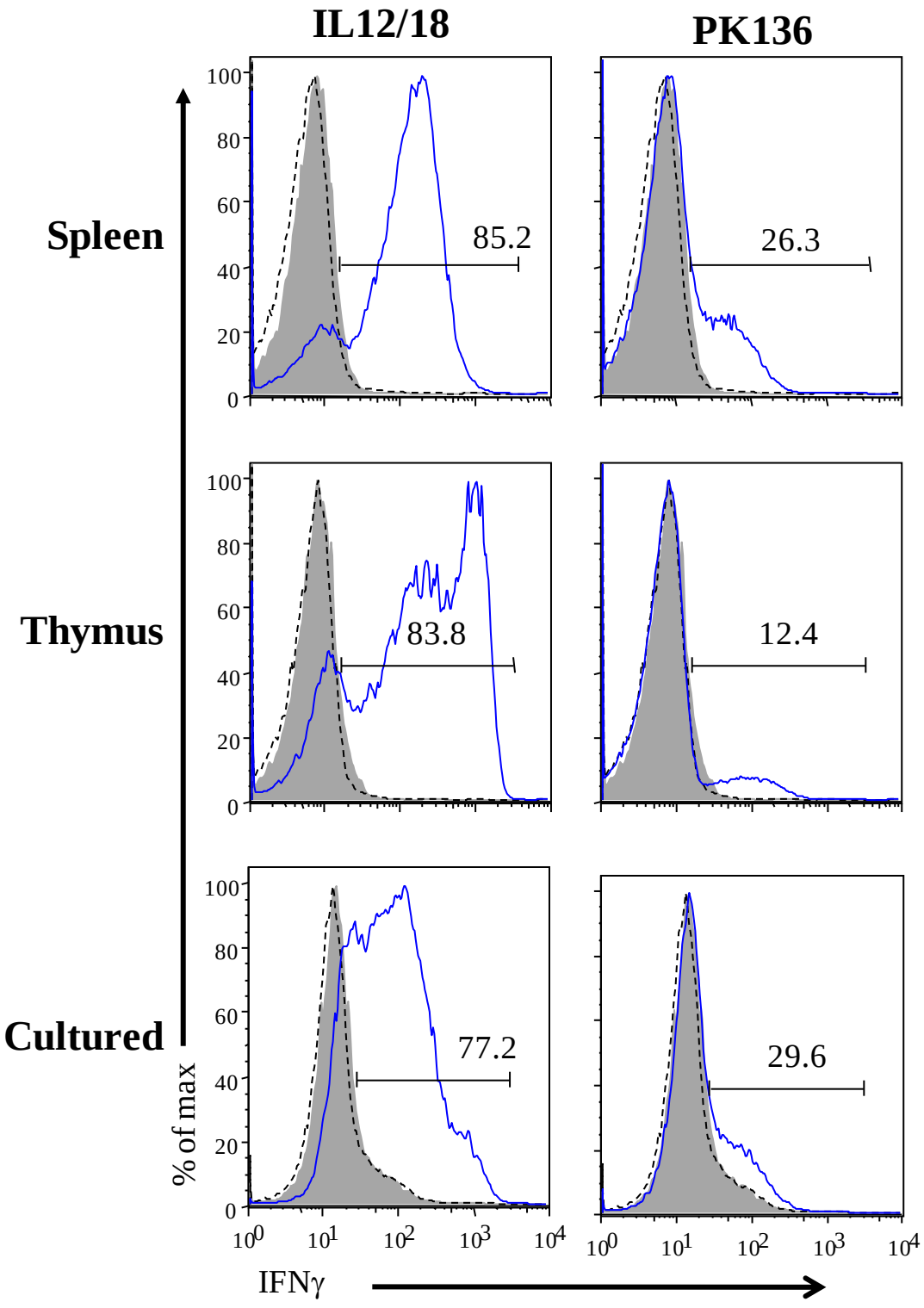

IL12/18
PK136
Spleen
Thymus
Cultured
85.2
26.3
83.8
12.4
77.2
29.6
% of max
IFNγ

Figure 17. *In vitro* generated NK1.1^{+} cells secrete cytokines when activated. The function of i*n vitro* differentiated NK cells grown in culture for 19 days then rested in LD IL15 was compared to the function of *Rag1*$^{-/-}$ splenic and thymic NK cells cultured in LD IL15. Sorted NK1.1^{+} cells were stimulated with IL12+IL18 or plate-bound anti-NK1.1 for 8 hours. Cytokines secreted were assessed by cytometric bead array. All experimental conditions were done in triplicates. Data are representative of 2 experiments.

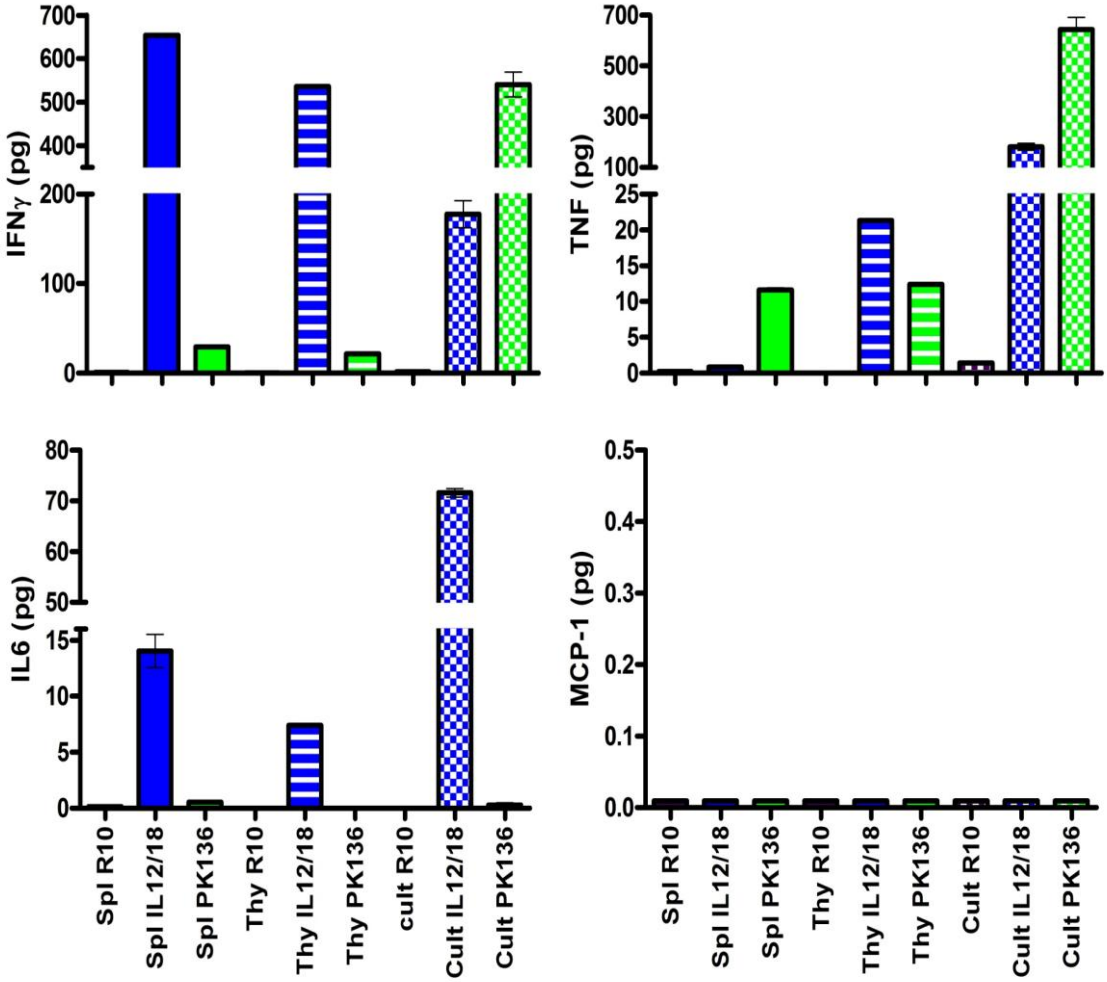
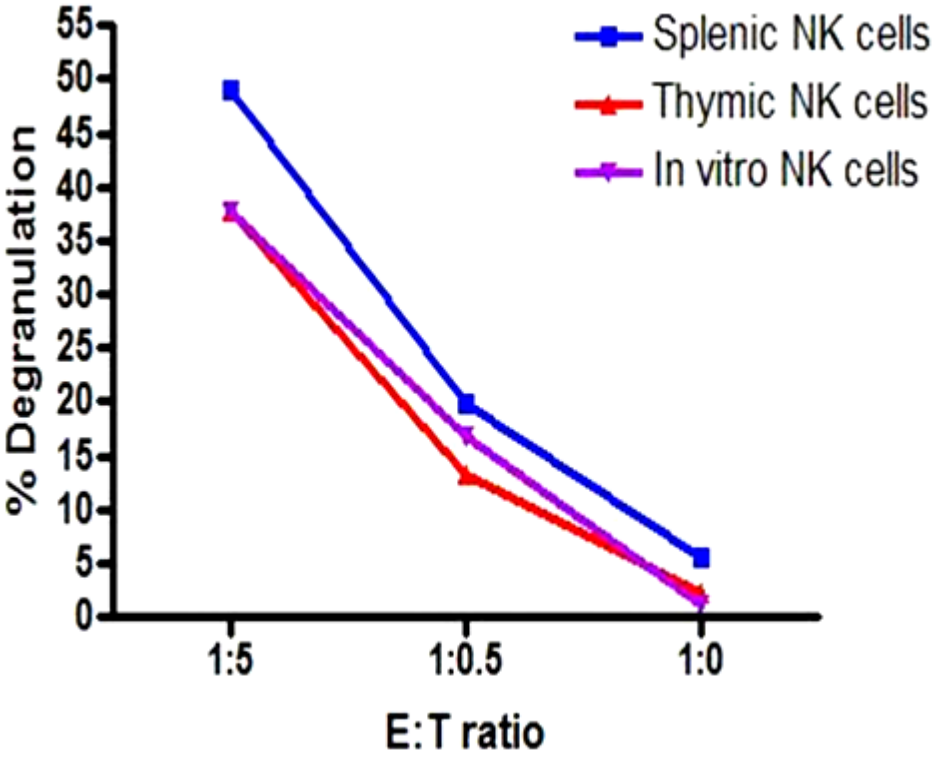

IFNγ (pg)
700
600
500
400
200
100
0
TNF (pg)
700
500
300
100
25
20
15
10
5
0
IL6 (pg)
80
70
60
50
15
10
5
0
MCP-1 (pg)
0.5
0.4
0.3
0.2
0.1
0.0
Spl R10
Spl IL12/18
Spl PK136
Thy R10
Thy IL12/18
Thy PK136
cult R10
Cult IL12/18
Cult PK136
Spl R10
Spl IL12/18
Spl PK136
Thy R10
Thy IL12/18
Thy PK136
Cult R10
Cult IL12/18
Cult PK136

Figure 18. *In vitro* generated NK cells resemble the function of thymic NK cells.

The function of i*n vitro* differentiated NK cells grown in culture for 19 days then rested in LD IL15 was compared to the function of *Rag1*$^{-/-}$ splenic and thymic NK cells cultured in LD IL15. Sorted NK1.1^{+} cells were co-cultured with YAC-1 target cells for 2 hours. CD107 degranulation upon target encounter was measured by flow cytometry. All experimental conditions were done in triplicates. Data are representative of 2 experiments.


Splenic NK cells
Thymic NK cells
In vitro NK cells
% Degranulation
55
50
45
40
35
30
25
20
15
10
5
0
1:5
1:0.5
1:0
E:T ratio

CHAPTER 5

GENE PROFILES AND THE IDENTIFICATION OF OTHER NOVEL RECEPTORS

Our *in vivo* and *in vitro* studies indicate that DN1 CD122$^-$NK1.1$^-$ thymocytes harbor a putative population which can differentiate into NK1.1$^+$ cells. These cells phenotypically resemble thymic NK cells. Thus far, most of our studies have assessed surface molecule expression with the use of flow cytometry. This approach has allowed us to follow the sequential acquisition of many markers. While both the *in vitro* generated and thymic NK cells were shown to be NK1.1$^+$CD127$^+$Ly49^{low}CD11b^{low}, a more detailed analysis must be done to further understand what makes these cells unique. Herein, we attempt to further compare *in vitro* generated NK cells, thymic NK cells, and splenic NK cells by gene expression profiling.

Gene expression profiling of splenic, thymic, and *in vitro* generated NK cells

To further examine the characteristics that make the *in vitro* generated and thymic NK cells distinct, we compared their gene expression to that of their splenic counterpart. Briefly, DN1 CD122$^-$NK1.1$^-$ thymocytes were cultured with OP9 stromal cells and cytokines for 19 days. At this time, all wells were pooled, stained, and sorted based on NK1.1$^+$ expression. While essentially all cells expressed NK1.1, it was essential we excluded any cells that could potentially alter our results. In parallel experiments, we harvested spleen and thymus from *Rag1*$^{-/-}$ mice, made single cell suspensions and enriched for the NK1.1$^+$ cells via cell sorting. Total RNA was isolated and gene expression profiling was performed using genome-wide oligonucleotide microarrays (see Methods). Total RNA from 3 different experiments was pooled for target synthesis and three target samples were pooled and hybridized to each chip, resulting in three chips for each cell type.

By utilizing the Partek® Genomics Suite™, microarray chips were normalized and unsupervised hierarchical clustering of the data grouped all three samples together (Figure 19). PCA (principal component analysis) mapping showing the global analysis of the genome, suggested that there was good concordance of the results between replicate samples in each group. This was essential since triplicate gene chips were not only going to be compared to each other, but to other triplicate chips from the remaining samples.

Due to multiple sample comparison, we applied the Bonferroni correction to further normalize gene profiles. Genes with a significant Bonferroni p-value of less than 0.02 were graphed in a heat map and lists indicating their fold change were created (Figure 20 and Table II). Results showed that *in vitro* generated NK cells had a significant fold increase in granzyme D, granzyme E, and granzyme G expression. In addition, *in vitro* NK cells had approximately 6 times more lymphotoxin A (LTα) and tumor necrosis factor ligand. While surface expression showed that these cells were similar, transcript profiles suggest there may be some subtle differences created by the *in vitro* culture conditions.

To further examine the difference between splenic and thymic NK cells, we mapped genes with a Bonferroni p-value of less than 0.02 (Figure 21 and Table III). As expected, we were able to detect a 4 fold difference in CD127 expression in thymic NK cells, where splenic gene expression was down. Surprisingly, we detected integrin alpha E (CD103), IL2 receptor alpha chain (IL2Rα, CD25), and cadherin 1 at a 25, 40, and 97 fold increase in thymic NK cells respectively. However, splenic NK cells had higher expression of Fc receptor IgG2b (CD32) and CD86. Together, these results not only

validate the our functional assays where splenic NK cells were more efficient at degranulating, perhaps due to CD32 expression, but also suggest that thymic NK cells are even more unique in their phenotype, expressing CD25 and CD103, than conventional NK cells.

Finally, we compared the gene expression profile of splenic NK cells to that of thymic and *in vitro* NK cells combined (Figure 22 and Table IV) . By filtering genes out with a Bonferroni p-value of 0.01, we were able to detect many of the similar genes already described. Results showed that splenic NK cells had higher KLRG1, CD32, and CD86 transcript expression. However, both thymic and *in vitro* NK cells expressed more CD127, CD25, CD103, LTα, and granzymes D and E. To verify these results, we stained for some of the surface receptors and analyzed them via flow cytometry (Figure 23). Results confirmed the gene expression profiles, but future intracellular flow and RT-PCR experiments will be done to validate other transcripts found to be significant. These results further support our hypothesis that DN1 $CD122^{-}NK1.1^{-}$ thymocytes can differentiate into $NK1.1^{+}$ cells that very closely resemble thymic NK cells, and not conventional splenic NK cells.

Novel marker expression is specific to NK cells differentiated in a thymic environment

While extracellular CD25 was detected on *in vivo*, *in vitro*, and thymic NK cells during previous experiments (Figures 4, 9-11, 13, 24), the presence of CD103 transcripts in thymic NK cells was an interesting finding. To determine if the CD103 protein was expressed on the cell surface, we once again differentiated DN1 $CD122^{-}NK1.1^{-}$

thymocytes into NK1.1^{+} cells *in vivo* and *in vitro* (Figure 24). Adoptive transfer experiments showed about half of Ly5.2^{+}NK1.1^{+} (donor-generated) cells found in the thymus of IT transferred mice, expressed CD103. Although the same percent of cells also expressed CD25, there was no correlation between the two surface molecules (data not shown). In addition, both CD103 and CD25 detection on thymic Ly5.2^{+}NK1.1^{+} cells was significantly lower than their control unmanipulated counterparts (Figures 23, 24). This may be a direct consequence from irradiating the host mice. Regardless, these results suggest that thymic progenitors can generate CD103^{+} NK cells.

To investigate the kinetics of CD103 acquisition, we cultured DN1 CD122^{-} NK1.1^{-} thymocytes *in vitro* with OP9 stromal cells and cytokines. Once again, differentiating cells were harvested at certain time points and their phenotypic profiles were assessed via flow cytometry (Figure 10). Results showed that while less than 10% of NK1.1^{+} cells expressed low levels (MFI) of CD103 on day 7, we were able to detect a higher MFI on more than 60% NK1.1^{+} cells on day 13 (Figure 10 and data not shown). This expression was shown to be stable since NK cells were still CD103^{+} on day 19. This suggest that CD103 expression is acquired during the early stages of development, more specifically during stage II. Furthermore, by altering the culture conditions we were able to show that essentially all (96%) *in vitro* generated NK1.1^{+} cells expressed CD103 at similar detection levels as thymic NK cells (Figures 23, 24). Taken together, these results once again verify that the *in vitro* generated NK cells very closely resemble thymic NK cells.

Expression of CD127^{+} NK cells in the absence of CD25 and CD103

To examine whether CD25 or CD103 influence the generation of thymic NK cells, we looked for CD127^{+}NK1.1^{+} cells in different strains of mice including B6, CD103$^{-/-}$ on a B6 background, *Rag1*$^{-/-}$, and CD25$^{-/-}$ on a *Rag1*$^{-/-}$ background. As expected, less than 15% of splenic NK cells in B6 and *Rag1*$^{-/-}$ mice express CD127, while essentially all thymic NK cells are CD127^{+}. Similar results were obtained in CD103$^{-/-}$ mice. Surprisingly, we detected CD127 at a lower MFI on less than half of thymic NK1.1^{+} cells in CD25$^{-/-}$*Rag1*$^{-/-}$ mice (Figure 25A).

Although the percent of NK cells that expressed CD127 was comparable between B6, CD103$^{-/-}$, and *Rag1*$^{-/-}$ mice, and lower on CD25$^{-/-}$*Rag1*$^{-/-}$ mice, their absolute numbers varied significantly (Figure 25B). Compared to WT B6 mice, a larger number of CD127^{+} NK cells were detected in the thymus of CD103 KO (knockout) mice, approximately 1.5 times more cells. However, CD127 was detected in only one tenth (1/10) of thymic NK cells in *Rag1*$^{-/-}$ mice, compared to WT. Furthermore, while a smaller percent of thymic NK cells from CD25$^{-/-}$*Rag1*$^{-/-}$ mice were positive, their absolute number was 4 times greater than that in thymic NK cells from *Rag1*$^{-/-}$ mice. Together, these results suggest that CD103 expression in a WT background, and CD25 in a RAG background are not necessary for thymic NK cell development, but may be important for the export of these cells.

Thymic NK cells in the periphery

CD103 has been shown to be expressed in T lymphocytes and dendritic cells (DCs) that reside in the intestinal mucosa [59, 60]. Since FACS analysis and gene expression profiles showed that thymic NK cells also expressed abundant CD103, we assessed whether this NK subset also had the potential to travel to the intestinal mucosa (Figure 26). Results showed that although the percent of NK cells in the small and large intestine (SI and LI, respectively) was low, these cells were still detectable. A population of $NK1.1^+$ cells in both areas of the intestine were $CD127^+$, while only those in the small intestine were $CD103^+$. A more in-depth phenotypic analysis of $NK1.1^+$ cells found in the small intestine showed these cells resembled thymic NK cells in that they expressed CD25 and low levels of Ly49 and CD11b (Figure 27). These preliminary studies may indicate that a small population of thymic NK cells have the potential to emigrate from the thymus and seed other tissues, perhaps partially guided by the CD103 integrin.

Discussion

While phenotype analysis was essential to our kinetics experiments, we further characterized both thymic and *in vitro* generated NK cells by examining their genome-wide expression profiles and comparing them with that of splenic NK cells. To ensure the RNA isolated from sorted $NK1.1^+$ cells represented a whole population and not just one mouse, we pooled RNA from 3 independent experiments. Due to the small amount of RNA, the Washington University Gene Core Facility performed two rounds of linear amplification, creating a target synthesis for hybridization to Affymetrix 430 v2.0 GeneChips, which offers complete coverage of over 39,000 transcripts on a single array [61]. Three of these target samples were then pooled and hybridized to each chip.

We assessed statistical significance by using the q value of false discover rate (qFDR) based on the Bonferroni correction (Bonferroni p-value), which corrects for multiple test samples. In this study, we defined the qFDR< 0.01 or <0.02 to be statistically significant. Although the Bonferroni correction is usually overly stringent for microarray data analysis, there will still be a 1-2% chance that a single false positive will be present if the p-value cutoff is 0.01 to 0.02. Regardless, we were able to identify several distinct gene transcripts present in either spleen, thymus, or *in vitro* NK cells.

When we compared the gene expression profile of *in vitro* NK cells to the profile of thymic NK cells, we were able to detect significantly higher amounts of granzymes and LTα transcripts in the *in vitro* population. Whereas most granzymes are structurally related serine proteases, they differ in their substrate specificity [5]. In mice, four granzyme orthologues can be found, including A, B, K, and M. While it has been shown that the murine genome encodes several other granzymes (D, E, F, G, L, and N), little is known about them [62]. Recent studies have shown that granzymes D-G were expressed on granulated metrial gland (GMG) cells, which belong to the NK lineage, located in the decidua and metrial gland during mid-gestation in mice [63]. In this study, expression of the genes for granzymes D-G was found to be developmentally regulated in murine GMG cells during pregnancy. These granzymes were shown to be expressed in late gestation, in contrast to the mid-gestational expression of granzyme A. Furthermore, expression of granzymes D-G was shown to be up-regulated by both IL2 and IL15 in *in vitro* culture experiments. These finding suggests different roles for granzymes D-G compared with granzyme A.

The fact that transcripts for granzymes D, E, and G were found in *in vitro* cells but not in thymic NK cells indicates that the culturing conditions influenced their expression. When culturing our cells, we add a high dose of IL15 to push NK lineage commitment, proliferation, and survival. In addition to IL15, studies have shown that stromal cells support HSC differentiation via LTα engagement [19], which could also explain why *in vitro* NK cells had a higher number of transcripts. In any case, these experiments were carried out prior to discovering that *in vitro* NK cells are in a pre-activated state at day 19 of culture. Further gene expression microarrays will need to be done on "rested" *in vitro* NK cells for a fair comparison to thymic NK cells.

When the gene expression profile of splenic NK cells was compared to the thymic NK cells, we were able to detect a 40-fold increase in CD25 expression in thymic NK cells. Although gene expression profiles validated our phenotypic results in regards to CD25 expression on thymic NK cells, we still do not know the basis for this difference at the moment. The expression of this IL2R on thymic NK cells from *Rag1*$^{-/-}$ mice, but not on WT B6 mice, suggest it might be specific to this strain.

Interestingly, we also detected a 26-fold increase in CD103 expression in thymic NK cells. Also termed integrin $\alpha_E\beta_7$, CD103 was first identified through its selective expression on more than 90% of CD8 T cells, approximately 40-50% of CD4$^+$ T lymphocytes, and a subset of DCs, all of which are found in the intestinal mucosa [64-67]. The gastrointestinal tract is exposed to continuous antigenic challenges including food antigens, bacterial antigens of the normal bacterial flora and pathogens. The intestinal immune system therefore must be able to defend against these pathogens while maintaining tolerance to the normal bacterial flora and food antigens.

Studies have shown that T cells in intestinal mucosa undergo a shift in expression of cell surface adhesion molecules upon stimulation [68]. Also, transforming growth factor-β1 (TGF-β1) has been shown to induce expression of $\alpha_E\beta_7$ on almost half of the peripheral T lymphocytes during *in vitro* cultures [60]. While CD103 binds to E-cadherin [69, 70], and possibly to other ligands [71], it has been shown to mediate T cell adhesion to epithelial cells [72, 73] . In addition, this integrin has been shown to transmit an outside-in signal that enhances T cell proliferation and induces redirected lysis of FcR-bearing target cells [59]. Furthermore, there is a decrease in the number of gut IEL's, particularly of the TCR $\alpha\beta^+$ $CD8^+$ subset in $CD103^{-/-}$ mice, suggesting that its expression may be involved in either the generation or maintenance of this IEL subset [74]. Together, these studies indicate that the expression of CD103 may influence thymic NK cells to emigrate from the thymus and seed mucosal tissues, such as the gut. Here, these cells may have the potential to carry out a specific function that may differ from the effector function seen in their splenic counterparts.

The detection of these two novel receptors (CD25 and CD103) prompted us to question whether either molecule affected or influenced the development of thymic $CD127^+$ NK cells. Because CD25 was only expressed by thymic NK cells in *Rag1*$^{-/-}$ mice, we backcrossed these two strains. Although a smaller percent of thymic NK cells were shown to express CD127 in this strain, their absolute number was 4 times greater than in *Rag1*$^{-/-}$ mice. In a similar situation, $CD103^{-/-}$ mice had an higher absolute number of $CD127^+$ NK cells in the thymus compared to WT B6 mice. This suggests that while CD25 or CD103 may not have an effect on thymic NK cell development, it may influence the export of these cells from the thymus.

Although our knowledge of thymic NK cell development, receptor repertoire, and function is still in its infancy, we have shown that these cells express novel receptors that may give us clues as to their specific effector function. Herein, we have shown that our method of gene expression profiling is a feasible and reliable method to characterize splenic, thymic, and *in vitro* NK cells in a more in-depth manner. Along with phenotypic and functional analysis, gene expression profiles further confirmed that the *in vitro* generated NK cells closely resemble thymic NK cells, both of which are distinct from their splenic counterpart.

Figure 19. PCA map of splenic, thymic, and *in vitro* generated NK cells. NK1.1^{+} cells from spleen and thymus of *Rag1*$^{-/-}$ mice, along with *in vitro* differentiated NK cells were sorted, RNA was isolated and gene expression was assessed by genome-wide microarrays. PCA mapping is a global analysis of the genome and not of any gene in particular. Samples that are close together are similar across the whole genome. Each chip represents 3 pooled samples independently sorted from 10-20 mice.

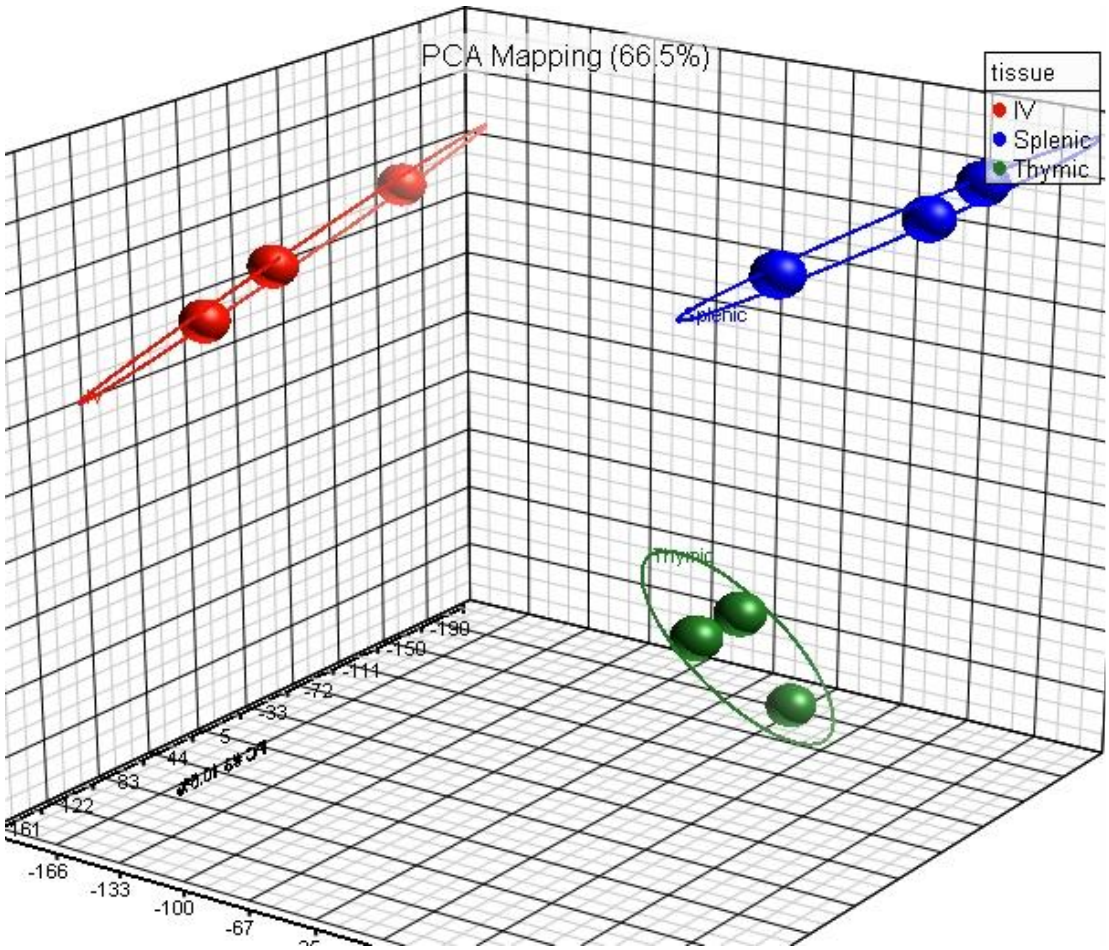

PCA Mapping (66.5%)
tissue
IV
Splenic
Thymic
Splenic
Thymic
-190
-150
-111
-72
-33
5
44
83
122
161
-166
-133
-100
-67

Figure 20. Gene expression profiles of thymic and *in vitro* generated NK cells. NK1.1^{+} cells were generated by using the *in vitro* system described above. After 19 days in culture, all growth positive wells were pooled and, along with thymic NK1.1^{+} cells from *Rag1*$^{-/-}$ mice, were sorted using Dako MoFlo. RNA was isolated using the Qiagen RNeasy Micro Kit, quantified and submitted to the Multiplex Gene Analysis Core to be used in an Affymetrix GeneChip® microarray. Transcript expression was analyzed via Partek® Genomic Suite™ and adjusted with a Bonferroni p-value of less than 0.02. Each cell type was done in triplicate. Data are representative of three gene chips.

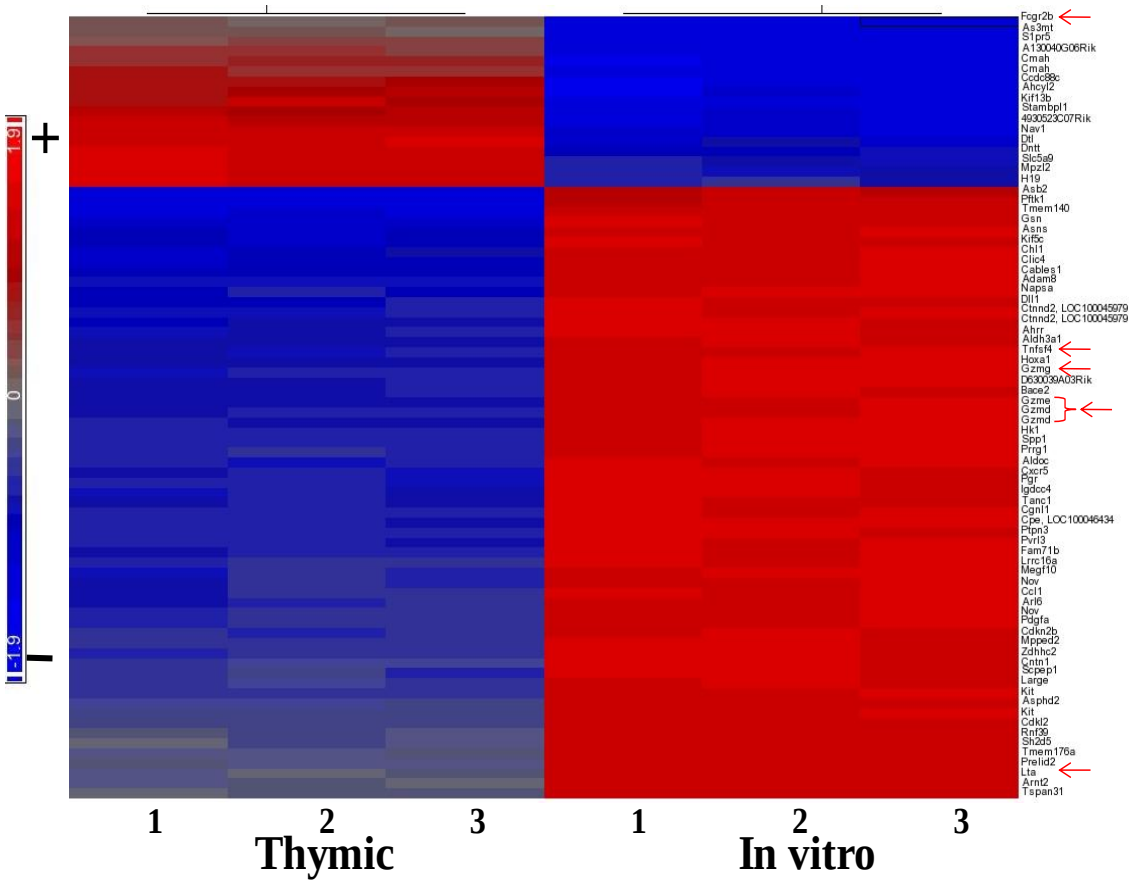
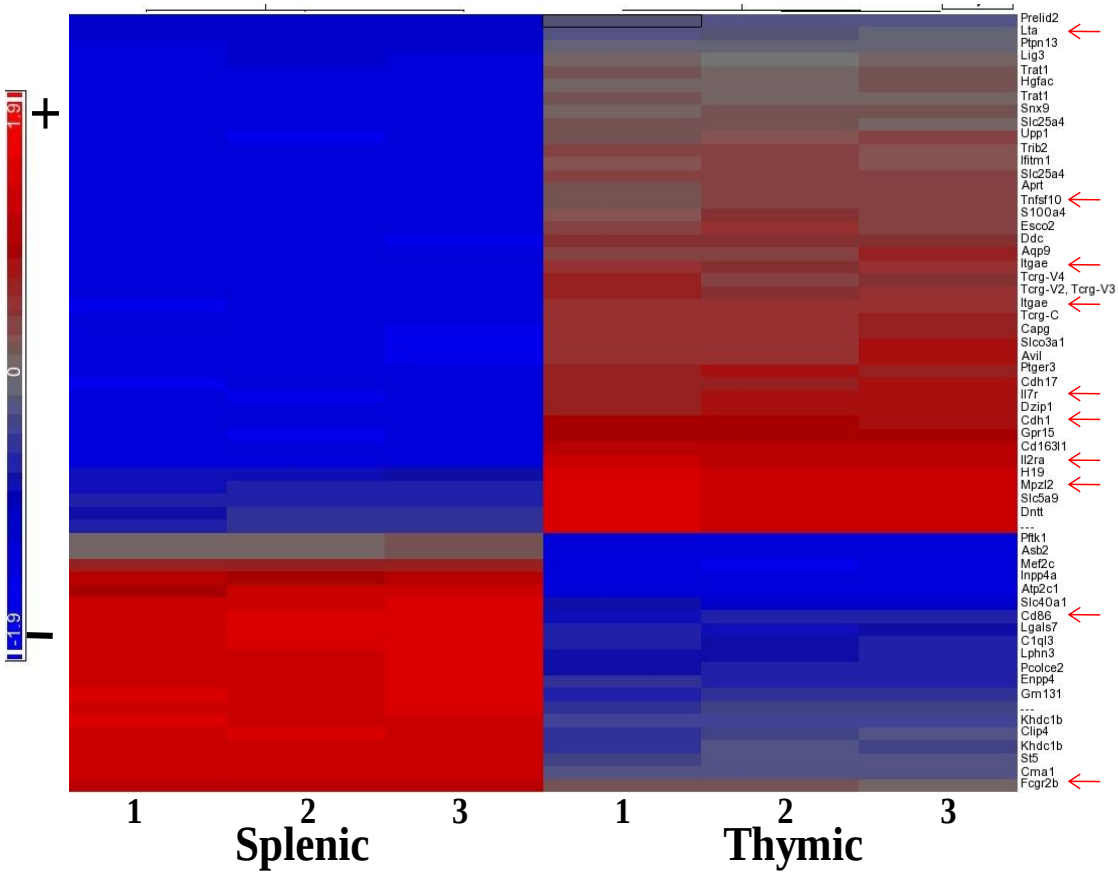

+
1.91
0
-1.19
-
Fcgr2b
As3mt
S1pr5
A130040G06Rik
Cmah
Cmah
Cdc88c
Ahcyl2
Kif13b
Stambpl1
4930523C07Rik
Nav1
Dtl
Dntt
Slc5a9
Mpzl2
H19
Asb2
Pftk1
Tmem140
Gsn
Asns
Kif5c
Chl1
Clic4
Cables1
Adam8
Napsa
Dll1
Ctnnd2, LOC100045979
Ctnnd2, LOC100045979
Ahrr
Aldh3a1
Tnfsf4
Hoxa1
Gzmg
D630039A03Rik
Bace2
Gzme
Gzmd
Gzmd
Hk1
Spp1
Prrg1
Aldoc
Gpcr5
Pgr
Igdcc4
Tanc1
Cgnl1
Cpe, LOC100046434
Ptpn3
Pvrl3
Fam71b
Lrrc16a
Megf10
Nov
Ccl1
Arl6
Nov
Pdgfa
Cdkn2b
Mpped2
Zdhhc2
Cntn1
Scpep1
Large
Kit
Asphd2
Kit
Cdkl2
Rnf39
Sh2d5
Tmem176a
Prelid2
Lta
Arnt2
Tspan31
1
2
3
1
2
3
Thymic
In vitro

Table II. List of gene expression profiles of thymic and *in vitro* generated NK cells. Microarray data from freshly isolated thymic NK cells and *in vitro* differentiated NK cells (day 19) were analyzed with Partek® Genomic Suite™. Genes were filtered by adjusting the Bonferroni p-value to less than 0.02. Each cell type was done in triplicate. Data are representative of three gene chips.

Gene Symbol	Gene Title	p-value(IV vs. Thymic)	bonferroni(p-value(IV vs. Thymic))	Fold-Change(IV vs. Thymic)	Fold-Change(IV vs. Thymic) (Description)
Prelid2	PRELI domain containing 2	3.48E-10	1.57E-05	11.6529	IV up vs Thymic
Adam8	a disintegrin and metallopeptidase domain 8	9.62E-10	4.34E-05	16.4309	IV up vs Thymic
Aldh3a1	aldehyde dehydrogenase family 3, subfamily A1	2.19E-09	9.90E-05	13.6493	IV up vs Thymic
Asphd2	aspartate beta-hydroxylase domain containing 2	2.31E-09	0.000104093	4.96388	IV up vs Thymic
Mpped2	metallophosphoesterase domain containing 2	2.33E-09	0.000104921	14.2896	IV up vs Thymic
Gzme	granzyme E	2.51E-09	0.000113173	186.844	IV up vs Thymic
Pftk1	PFTAIRE protein kinase 1	2.87E-09	0.000129375	18.5244	IV up vs Thymic
Zdhhc2	zinc finger, DHHC domain containing 2	3.26E-09	0.000147165	7.22219	IV up vs Thymic
Gzmd	granzyme D	4.95E-09	0.000223127	115.629	IV up vs Thymic
Ccdc88c	coiled-coil domain containing 88C	9.46E-09	0.000426487	-4.68531	IV down vs Thymic
Cables1	CDK5 and Abl enzyme substrate 1	9.61E-09	0.000433637	9.74955	IV up vs Thymic
Gzmd	granzyme D	9.69E-09	0.000436865	104.992	IV up vs Thymic
Asb2	ankyrin repeat and SOCS box-containing 2	9.93E-09	0.000448075	38.42	IV up vs Thymic
Cpe /// LOC1(	carboxypeptidase E /// similar to carboxypeptidase E	1.01E-08	0.000454161	39.6519	IV up vs Thymic
Ahrr	aryl-hydrocarbon receptor repressor	1.32E-08	0.000597495	71.2702	IV up vs Thymic
Cdkl2	cyclin-dependent kinase-like 2 (CDC2-related kinase)	1.36E-08	0.000614903	5.28959	IV up vs Thymic
Fcgr2b	Fc receptor, IgG, low affinity IIb	1.55E-08	0.000697174	-13.271	IV down vs Thymic
Ctnnd2 /// LC	catenin (cadherin associated protein), delta 2 /// simil	1.57E-08	0.000709612	105.613	IV up vs Thymic
Lta	lymphotoxin A	2.82E-08	0.00127359	6.05208	IV up vs Thymic
Tmem140	transmembrane protein 140	2.85E-08	0.00128545	4.97292	IV up vs Thymic
S1pr5	sphingosine-1-phosphate receptor 5	3.10E-08	0.00139734	-106.404	IV down vs Thymic
Tanc1	tetratricopeptide repeat, ankyrin repeat and coiled-co	3.57E-08	0.00161182	8.90591	IV up vs Thymic
Pgr	progesterone receptor	3.93E-08	0.00177282	69.5983	IV up vs Thymic
Ctnnd2 /// LC	catenin (cadherin associated protein), delta 2 /// simil	3.97E-08	0.00178968	18.9016	IV up vs Thymic
Ptpn3	protein tyrosine phosphatase, non-receptor type 3	4.26E-08	0.00191924	6.56061	IV up vs Thymic
Kif5c	kinesin family member 5C	4.34E-08	0.00195679	7.47177	IV up vs Thymic
Cxcr5	chemochine (C-X-C motif) receptor 5	4.43E-08	0.00200013	22.8956	IV up vs Thymic
Nav1	neuron navigator 1	5.00E-08	0.00225723	-9.74379	IV down vs Thymic
Igdcc4	immunoglobulin superfamily, DCC subclass, member	5.16E-08	0.0023259	7.80927	IV up vs Thymic
Hk1	hexokinase 1	5.80E-08	0.00261705	8.26247	IV up vs Thymic
Pdgfa	platelet derived growth factor, alpha	6.09E-08	0.00274561	6.20889	IV up vs Thymic

Gene Symbol	Gene Title	p-value(IV vs. Thymic)	bonferroni(p-value(IV vs. Thymic))	Fold-Change(IV vs. Thymic)	Fold-Change(IV vs. Thymic) (Description)
Arnt2	aryl hydrocarbon receptor nuclear translocator 2	6.44E-08	0.00290414	8.51067	IV up vs Thymic
Cntn1	contactin 1	7.95E-08	0.00358442	29.8758	IV up vs Thymic
Tnfsf4	tumor necrosis factor (ligand) superfamily, member 4	9.61E-08	0.0043351	6.2596	IV up vs Thymic
Pvrl3	poliovirus receptor-related 3	9.99E-08	0.00450558	13.7189	IV up vs Thymic
Dll1	delta-like 1 (Drosophila)	1.02E-07	0.00461436	19.1459	IV up vs Thymic
Kit	kit oncogene	1.13E-07	0.00509222	5.96802	IV up vs Thymic
Fam71b	family with sequence similarity 71, member B	1.13E-07	0.00510716	22.8387	IV up vs Thymic
Nov	nephroblastoma overexpressed gene	1.22E-07	0.00548038	51.6892	IV up vs Thymic
Dntt	deoxynucleotidyltransferase, terminal	1.29E-07	0.00580708	-188.363	IV down vs Thymic
H19	H19 fetal liver mRNA	1.38E-07	0.00623555	-39.9155	IV down vs Thymic
Arl6	ADP-ribosylation factor-like 6	1.40E-07	0.0063088	9.26583	IV up vs Thymic
Gzmg	granzyme G	1.49E-07	0.00670014	19.8177	IV up vs Thymic
Nov	nephroblastoma overexpressed gene	1.50E-07	0.00675806	58.2651	IV up vs Thymic
Napsa	napsin A aspartic peptidase	1.50E-07	0.00676267	5.85097	IV up vs Thymic
Hoxa1	homeo box A1	1.71E-07	0.00769617	12.5426	IV up vs Thymic
Megf10	multiple EGF-like-domains 10	1.80E-07	0.00812599	8.90418	IV up vs Thymic
Cgnl1	cingulin-like 1	1.98E-07	0.0089306	4.57113	IV up vs Thymic
Cdkn2b	cyclin-dependent kinase inhibitor 2B (p15, inhibits Cl	2.09E-07	0.00944262	9.41069	IV up vs Thymic
Spp1	secreted phosphoprotein 1	2.09E-07	0.0094474	126.437	IV up vs Thymic
Tspan31	tetraspanin 31	2.37E-07	0.0107078	4.19839	IV up vs Thymic
Chl1	cell adhesion molecule with homology to L1CAM	2.42E-07	0.0109296	14.3121	IV up vs Thymic
Rnf39	Ring finger protein 39 (Rnf39), mRNA	2.47E-07	0.0111554	13.8966	IV up vs Thymic
Sh2d5	SH2 domain containing 5	2.59E-07	0.011698	6.43186	IV up vs Thymic
Gsn	gelsolin	2.65E-07	0.011971	6.35693	IV up vs Thymic
Tmem176a	transmembrane protein 176A	2.66E-07	0.0119974	5.66456	IV up vs Thymic
Mpzl2	myelin protein zero-like 2	2.69E-07	0.0121482	-155.799	IV down vs Thymic
Cmah	cytidine monophospho-N-acetylneuraminic acid hyd	2.71E-07	0.0122037	-23.5037	IV down vs Thymic
Prrg1	proline rich Gla (G-carboxyglutamic acid) 1	3.14E-07	0.0141606	9.78567	IV up vs Thymic
Clic4	chloride intracellular channel 4 (mitochondrial)	3.31E-07	0.0149136	4.09815	IV up vs Thymic
Kif13b	kinesin family member 13B	3.63E-07	0.0163764	-6.55086	IV down vs Thymic
Ccl1	chemokine (C-C motif) ligand 1	4.30E-07	0.0193864	4.76996	IV up vs Thymic

Figure 21. Gene expression profiles of splenic and thymic NK cells. NK1.1^{+} cells from spleen and thymus in *Rag1*$^{-/-}$ mice were enriched by FACS sorting. RNA was isolated using the Qiagen RNeasy Micro Kit, quantified and submitted to the Multiplex Gene Analysis Core to be used in an Affymetrix GeneChip® microarray. Transcript expression was analyzed via Partek® Genomic Suite™ and adjusted with a Bonferroni p-value less than 0.02. Each cell type was done in triplicate. Data are representative of three gene chips.


+
1.9
0
-1.9
-
Prelid2
Lta
Ptpn13
Lig3
Trat1
Hgfac
Trat1
Snx9
Slc25a4
Upp1
Trib2
Ifitm1
Slc25a4
Aprt
Tnfsf10
S100a4
Esco2
Ddc
Aqp9
Itgae
Tcrg-V4
Tcrg-V2, Tcrg-V3
Itgae
Tcrg-C
Capg
Slco3a1
Avil
Ptger3
Cdh17
Il7r
Dzip1
Cdh1
Gpr15
Cd163l1
Il2ra
H19
Mpzl2
Slc5a9
Dntt

Pftk1
Asb2
Mef2c
Inpp4a
Atp2c1
Slc40a1
Cd86
Lgals7
C1ql3
Lphn3
Pcolce2
Enpp4
Gm131

Khdc1b
Clip4
Khdc1b
St5
Cma1
Fcgr2b
1
2
3
1
2
3
Splenic
Thymic

Table III. List of gene expression profiles of splenic and thymic NK cells.

Microarray data from freshly isolated splenic and thymic NK cells was analyzed with Partek® Genomic Suite™. Genes were filtered by adjusting the Bonferroni p-value to less than 0.02. Each cell type was done in triplicate. Data are representative of three gene chips.

Gene Symbol	Gene Title	p-value(Splenic vs. Thymic)	bonferroni(p-value(Splenic vs. Thymic))	Fold-Change(Splenic vs.	Fold-Change(Splenic vs. Thymic) (Description)
Trib2	tribbles homolog 2 (Drosophila)	2.40E-09	0.000108189	-9.17896	Splenic down vs Thymic
Itgae	integrin alpha E, epithelial-associated	3.04E-09	0.000136885	-25.604	Splenic down vs Thymic
Prelid2	PRELI domain containing 2	7.17E-09	0.000323564	-4.39996	Splenic down vs Thymic
Cdh1	cadherin 1	1.46E-08	0.000657312	-97.4662	Splenic down vs Thymic
Capg	capping protein (actin filament), gelsolin-like	2.39E-08	0.00107982	-10.6809	Splenic down vs Thymic
Khdc1b	KH domain containing 1B	3.28E-08	0.00147784	8.01497	Splenic up vs Thymic
Il2ra	interleukin 2 receptor, alpha chain	3.41E-08	0.00153685	-40.3814	Splenic down vs Thymic
Tcrg-V2 /// T	T-cell receptor gamma, variable 2 /// T-cell receptor gamma	3.78E-08	0.00170656	-77.1815	Splenic down vs Thymic
Ifitm1	interferon induced transmembrane protein 1	3.85E-08	0.00173447	-14.4452	Splenic down vs Thymic
Slc5a9	solute carrier family 5 (sodium/glucose cotransporter), mer	4.66E-08	0.00210236	-21.9033	Splenic down vs Thymic
Lgals7	lectin, galactose binding, soluble 7	5.68E-08	0.00256358	3.59848	Splenic up vs Thymic
C1ql3	C1q-like 3	6.26E-08	0.00282178	18.8079	Splenic up vs Thymic
Ptger3	prostaglandin E receptor 3 (subtype EP3)	6.54E-08	0.0029512	-81.4617	Splenic down vs Thymic
Tcrg-V4	Non-functional transcript for T-cell receptor gamma from a	6.90E-08	0.00311181	-40.1804	Splenic down vs Thymic
Pftk1	PFTAIRE protein kinase 1	8.12E-08	0.00366399	5.30597	Splenic up vs Thymic
S100a4	S100 calcium binding protein A4	8.13E-08	0.00366763	-7.85387	Splenic down vs Thymic
Enpp4	ectonucleotide pyrophosphatase/phosphodiesterase 4	8.78E-08	0.00395777	3.21908	Splenic up vs Thymic
H19	H19 fetal liver mRNA	8.89E-08	0.00400967	-52.9919	Splenic down vs Thymic
St5	suppression of tumorigenicity 5	9.32E-08	0.00420476	13.2568	Splenic up vs Thymic
Ddc	dopa decarboxylase	1.02E-07	0.00458103	-7.31516	Splenic down vs Thymic
Tcrg-C	T-cell receptor gamma, constant region, mRNA (cDNA clo	1.12E-07	0.00506375	-8.12482	Splenic down vs Thymic
Snx9	sorting nexin 9	1.16E-07	0.0052477	-2.48627	Splenic down vs Thymic
Gm131	gene model 131, (NCBI)	1.21E-07	0.00545548	5.29128	Splenic up vs Thymic
Khdc1b	KH domain containing 1B	1.47E-07	0.00662003	10.6311	Splenic up vs Thymic
Lig3	ligase III, DNA, ATP-dependent	1.52E-07	0.00683919	-1.25198	Splenic down vs Thymic
Cd163l1	CD163 molecule-like 1	1.65E-07	0.0074535	-47.5565	Splenic down vs Thymic
Itgae	integrin alpha E, epithelial-associated	1.67E-07	0.00753965	-27.9126	Splenic down vs Thymic
Atp2c1	ATPase, Casequestering	2.16E-07	0.00972891	1.59763	Splenic up vs Thymic
Slc25a4	solute carrier family 25 (mitochondrial carrier, adenine nucl	2.16E-07	0.0097393	-2.85623	Splenic down vs Thymic
Ptpn13	protein tyrosine phosphatase, non-receptor type 13	2.16E-07	0.00974317	-2.38986	Splenic down vs Thymic
Mef2c	myocyte enhancer factor 2C	2.19E-07	0.00988084	3.65496	Splenic up vs Thymic

Gene Symbol	Gene Title	p-value(Splenic vs. Thymic)	bonferroni(p-value(Splenic vs. Thymic))	Fold-Change(Splenic vs.	Fold-Change(Splenic vs. Thymic) (Description)
Inpp4a	inositol polyphosphate-4-phosphatase, type I	2.25E-07	0.010128	4.00662	Splenic up vs Thymic
Pcolce2	procollagen C-endopeptidase enhancer 2	2.25E-07	0.0101307	7.89679	Splenic up vs Thymic
Lphn3	latrophilin 3	2.30E-07	0.0103754	6.38926	Splenic up vs Thymic
Dzip1	DAZ interacting protein 1, mRNA (cDNA clone MGC:1067	2.34E-07	0.0105413	-7.18577	Splenic down vs Thymic
Fcgr2b	Fc receptor, IgG, low affinity IIb	2.35E-07	0.0106091	5.14781	Splenic up vs Thymic
Avil	advillin	2.40E-07	0.0108159	-11.3216	Splenic down vs Thymic
Hgfac	hepatocyte growth factor activator	2.43E-07	0.0109654	-10.235	Splenic down vs Thymic
Esco2	establishment of cohesion 1 homolog 2 (S. cerevisiae)	2.56E-07	0.0115362	-5.02129	Splenic down vs Thymic
Aprt	adenine phosphoribosyl transferase	2.59E-07	0.0116767	-1.55998	Splenic down vs Thymic
Asb2	ankyrin repeat and SOCS box-containing 2	2.60E-07	0.0117472	8.25701	Splenic up vs Thymic
Trat1	T cell receptor associated transmembrane adaptor 1	2.61E-07	0.0117737	-14.0037	Splenic down vs Thymic
Cd86	CD86 antigen	2.61E-07	0.0117872	6.6907	Splenic up vs Thymic
Cdh17	cadherin 17	2.72E-07	0.0122846	-4.02989	Splenic down vs Thymic
Trat1	T cell receptor associated transmembrane adaptor 1	2.79E-07	0.0125971	-17.3496	Splenic down vs Thymic
Mpzl2	myelin protein zero-like 2	2.95E-07	0.0133162	-144.209	Splenic down vs Thymic
Cma1	chymase 1, mast cell	2.97E-07	0.0134081	12.1289	Splenic up vs Thymic
Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10	3.19E-07	0.0143968	-5.68166	Splenic down vs Thymic
Aqp9	aquaporin 9	3.20E-07	0.01443	-13.3654	Splenic down vs Thymic
Clip4	CAP-GLY domain containing linker protein family, member	3.21E-07	0.0144902	6.3033	Splenic up vs Thymic
Dntt	deoxynucleotidyltransferase, terminal	3.28E-07	0.0147721	-88.0689	Splenic down vs Thymic
Gpr15	G protein-coupled receptor 15	3.58E-07	0.0161391	-33.0305	Splenic down vs Thymic
Upp1	uridine phosphorylase 1	3.59E-07	0.0161833	-15.3293	Splenic down vs Thymic
Lta	lymphotoxin A	3.62E-07	0.016322	-3.23428	Splenic down vs Thymic
Slco3a1	solute carrier organic anion transporter family, member 3a1	3.76E-07	0.0169366	-5.3453	Splenic down vs Thymic
Slc40a1	solute carrier family 40 (iron-regulated transporter), membe	3.79E-07	0.0171035	186.675	Splenic up vs Thymic
Slc25a4	solute carrier family 25 (mitochondrial carrier, adenine nucl	4.17E-07	0.0188265	-2.95871	Splenic down vs Thymic
Il7r	interleukin 7 receptor	4.30E-07	0.0193838	-4.73807	Splenic down vs Thymic

Figure 22. Gene expression profiles of splenic vs. thymic and *in vitro* NK cells. Along with *in vitro* generated cells, NK1.1^{+} cells from spleen and thymus in *Rag1*$^{-/-}$ mice were enriched by FACS sorting. RNA was isolated using the Qiagen RNeasy Micro Kit, quantified and submitted to the Multiplex Gene Analysis Core to be used in an Affymetrix GeneChip® microarray. Transcript expression was analyzed via Partek® Genomic Suite™ and adjusted with a Bonferroni p-value of less than 0.01. Each cell type was done in triplicate. Data are representative of three gene chips.

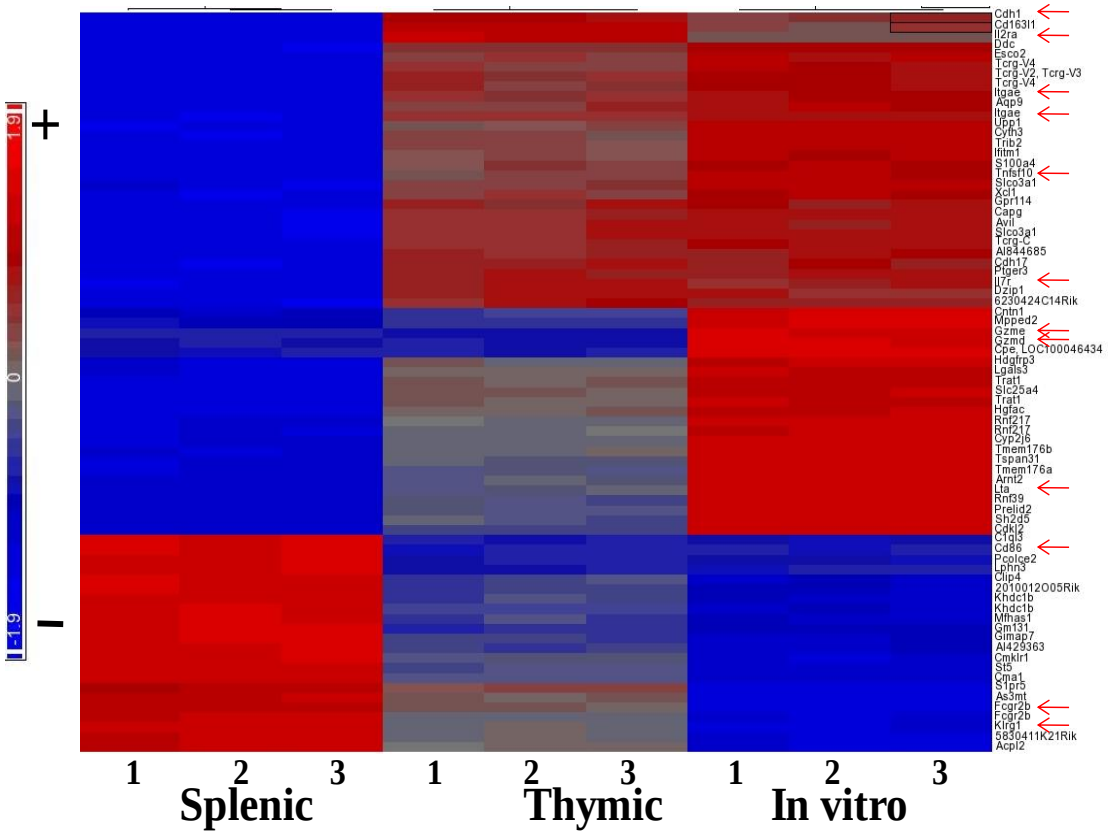

+
−
1.9
0
-1.9
Cdh1
Cd163l1
Il2ra
Ddc
Esco2
Tcrg-V4
Tcrg-V2, Tcrg-V3
Tcrg-V4
Itgae
Aqp9
Itgae
Upp1
Cyth3
Trib2
Ifitm1
S100a4
Tnfsf10
Slco3a1
Xcl1
Gpr114
Capg
Avil
Slco3a1
Tcrg-C
AI844685
Cdh17
Ptger3
Il7r
Dzip1
6230424C14Rik
Cntn1
Mpped2
Gzme
Gzmd
Cpe, LOC100046434
Hdgfrp3
Lgals3
Trat1
Slc25a4
Trat1
Hgfac
Rnf217
Rnf217
Cyp2j6
Tmem176b
Tspan31
Tmem176a
Arnt2
Lta
Rnf39
Prelid2
Sh2d5
Cdkl2
C1ql3
Cd86
Pcolce2
Lphn3
Clip4
2010012O05Rik
Khdc1b
Khdc1b
Mfhas1
Gm131
Gimap7
AI429363
Cmklr1
Sf3
Cma1
S1pr5
As3mt
Fcgr2b
Fcgr2b
Klrg1
5830411K21Rik
Acpl2
1 2 3 1 2 3 1 2 3
Splenic
Thymic
In vitro

Table IV. List of gene expression profiles of splenic vs. thymic and *in vitro* NK cells. Microarray data from freshly isolated splenic and thymic NK cells, along with *in vitro* generated NK1.1^{+} cell, was analyzed with Partek® Genomic Suite™. Genes were filtered by adjusting the Bonferroni p-value to less than 0.01. Each cell type was done in triplicate. Data are representative of three gene chips

Gene Symbol	Gene Title	p-value(Splenic vs. Thymic and IV)	bonferroni(p-value(Splenic vs. Thymic and IV))	Fold-Change(Splenic vs. Thymic and	Fold-Change(Splenic vs. Thymic and IV) (Description)
Prelid2	PRELI domain containing 2	8.14E-11	3.67E-06	-15.0199	Splenic down vs Thymic and IV
Trib2	tribbles homolog 2 (Drosophila)	3.09E-10	1.39E-05	-14.9219	Splenic down vs Thymic and IV
Itgae	integrin alpha E, epithelial-associated	7.31E-10	3.30E-05	-35.2207	Splenic down vs Thymic and IV
Fcgr2b	Fc receptor, IgG, low affinity IIb	3.08E-09	0.000139059	18.7532	Splenic up vs Thymic and IV
Lta	lymphotoxin A	5.12E-09	0.000231098	-7.95663	Splenic down vs Thymic and IV
Ifitm1	interferon induced transmembrane protein 1	5.29E-09	0.000238668	-25.0774	Splenic down vs Thymic and IV
Khdc1b	KH domain containing 1B	5.64E-09	0.000254285	11.2356	Splenic up vs Thymic and IV
Cdkl2	cyclin-dependent kinase-like 2 (CDC2-related kinase)	7.12E-09	0.000320919	-4.99377	Splenic down vs Thymic and IV
Capg	capping protein (actin filament), gelsolin-like	7.57E-09	0.000341369	-12.0206	Splenic down vs Thymic and IV
Mpped2	metallophosphoesterase domain containing 2	1.01E-08	0.000457174	-6.05698	Splenic down vs Thymic and IV
Cdh1	cadherin 1	1.06E-08	0.000479963	-65.367	Splenic down vs Thymic and IV
St5	suppression of tumorigenicity 5	1.10E-08	0.000496283	24.5465	Splenic up vs Thymic and IV
Arnt2	aryl hydrocarbon receptor nuclear translocator 2	1.13E-08	0.000509517	-11.9572	Splenic down vs Thymic and IV
S100a4	S100 calcium binding protein A4	1.39E-08	0.000625074	-11.0242	Splenic down vs Thymic and IV
Tcrg-V4	Non-functional transcript for T-cell receptor gamma fro	1.53E-08	0.000689656	-61.3122	Splenic down vs Thymic and IV
Hgfac	hepatocyte growth factor activator	1.65E-08	0.000744498	-23.5965	Splenic down vs Thymic and IV
Slc25a4	solute carrier family 25 (mitochondrial carrier, adenine	1.74E-08	0.000783515	-4.00041	Splenic down vs Thymic and IV
Trat1	T cell receptor associated transmembrane adaptor 1	1.85E-08	0.000834063	-49.1643	Splenic down vs Thymic and IV
Khdc1b	KH domain containing 1B	2.02E-08	0.000911086	17.3515	Splenic up vs Thymic and IV
Ddc	dopa decarboxylase	2.11E-08	0.00095175	-9.41272	Splenic down vs Thymic and IV
C1ql3	C1q-like 3	2.32E-08	0.00104832	20.0711	Splenic up vs Thymic and IV
Ptger3	prostaglandin E receptor 3 (subtype EP3)	2.52E-08	0.00113661	-87.4124	Splenic down vs Thymic and IV
Rnf217	ring finger protein 217	2.84E-08	0.00128111	-5.70585	Splenic down vs Thymic and IV
S1pr5	sphingosine-1-phosphate receptor 5	2.86E-08	0.00128768	60.1964	Splenic up vs Thymic and IV
Gm131	gene model 131, (NCBI)	2.87E-08	0.00129293	6.27374	Splenic up vs Thymic and IV
Tspan31	tetraspanin 31	2.93E-08	0.00132115	-5.83873	Splenic down vs Thymic and IV
Cma1	chymase 1, mast cell	3.00E-08	0.00135395	23.9137	Splenic up vs Thymic and IV
Fcgr2b	Fc receptor, IgG, low affinity IIb	3.14E-08	0.00141693	7.12664	Splenic up vs Thymic and IV
Tcrg-C	T-cell receptor gamma, constant region, mRNA (cDNA	3.27E-08	0.001476	-9.3038	Splenic down vs Thymic and IV
Klrg1	killer cell lectin-like receptor subfamily G, member 1	4.55E-08	0.00205386	8.77128	Splenic up vs Thymic and IV
Itgae	integrin alpha E, epithelial-associated	4.71E-08	0.00212216	-35.3661	Splenic down vs Thymic and IV

Gene Symbol	Gene Title	p-value(Splenic vs. Thymic and IV)	bonferroni(p-value(Splenic vs. Thymic and IV))	Fold-Change(Splenic vs. Thymic and IV)	Fold-Change(Splenic vs. Thymic and IV) (Description)
Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10	4.72E-08	0.00213069	-7.94824	Splenic down vs Thymic and IV
Esco2	establishment of cohesion 1 homolog 2 (S. cerevisiae)	5.10E-08	0.00230217	-6.24167	Splenic down vs Thymic and IV
Clip4	CAP-GLY domain containing linker protein family, mer	5.16E-08	0.00232524	8.73458	Splenic up vs Thymic and IV
Il2ra	interleukin 2 receptor, alpha chain	5.29E-08	0.00238741	-19.5933	Splenic down vs Thymic and IV
Tmem176a	transmembrane protein 176A	6.35E-08	0.00286176	-6.75411	Splenic down vs Thymic and IV
Cyp2j6	cytochrome P450, family 2, subfamily j, polypeptide 6	6.94E-08	0.00313053	-5.42158	Splenic down vs Thymic and IV
Sh2d5	SH2 domain containing 5	7.93E-08	0.00357765	-7.14509	Splenic down vs Thymic and IV
Pcolce2	procollagen C-endopeptidase enhancer 2	8.30E-08	0.00374488	8.28812	Splenic up vs Thymic and IV
Avil	advillin	8.38E-08	0.00377963	-12.269	Splenic down vs Thymic and IV
Gzme	granzyme E	8.59E-08	0.00387608	-12.2871	Splenic down vs Thymic and IV
Rnf39	Ring finger protein 39 (Rnf39), mRNA	9.10E-08	0.00410641	-14.8089	Splenic down vs Thymic and IV
Lphn3	latrophilin 3	9.75E-08	0.00439664	6.3922	Splenic up vs Thymic and IV
Cdh17	cadherin 17	1.08E-07	0.00486911	-4.09546	Splenic down vs Thymic and IV
Mfhas1	malignant fibrous histiocytoma amplified sequence 1	1.10E-07	0.00494284	4.0778	Splenic up vs Thymic and IV
Cyth3	cytohesin 3	1.17E-07	0.00526901	-4.49723	Splenic down vs Thymic and IV
Cd86	CD86 antigen	1.17E-07	0.00528419	6.57584	Splenic up vs Thymic and IV
Dzip1	DAZ interacting protein 1, mRNA (cDNA clone MGC:	1.19E-07	0.00535876	-6.77538	Splenic down vs Thymic and IV
Gzmd	granzyme D	1.25E-07	0.00563329	-10.988	Splenic down vs Thymic and IV
Acpl2	acid phosphatase-like 2	1.25E-07	0.00565892	11.1177	Splenic up vs Thymic and IV
Cmklr1	chemokine-like receptor 1	1.26E-07	0.00568846	19.924	Splenic up vs Thymic and IV
Slco3a1	solute carrier organic anion transporter family, member	1.26E-07	0.00569767	-5.71533	Splenic down vs Thymic and IV
Cntn1	contactin 1	1.32E-07	0.00596771	-14.8933	Splenic down vs Thymic and IV
Tcrg-V4	Non-functional transcript for T-cell receptor gamma fro	1.48E-07	0.00668834	-59.2102	Splenic down vs Thymic and IV
Lgals3	lectin, galactose binding, soluble 3	1.51E-07	0.00681172	-8.04885	Splenic down vs Thymic and IV
Xcl1	chemokine (C motif) ligand 1	1.54E-07	0.00695875	-4.76577	Splenic down vs Thymic and IV
Cd163l1	CD163 molecule-like 1	1.74E-07	0.00784922	-27.539	Splenic down vs Thymic and IV
Slco3a1	solute carrier organic anion transporter family, member	1.75E-07	0.00787127	-6.57655	Splenic down vs Thymic and IV
Rnf217	ring finger protein 217	1.85E-07	0.00832372	-6.43805	Splenic down vs Thymic and IV
Il7r	interleukin 7 receptor	2.01E-07	0.00905475	-4.62333	Splenic down vs Thymic and IV

Figure 23. Extracellular staining verifies gene chip results. Cells from indicated tissues of *Rag1*$^{-/-}$ mice, along with cells differentiated *in vitro* (day 19) were stained with a panel of antibodies and analyzed by flow cytometry. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated antibody. *In vitro* generated NK cells were compared to freshly isolated splenic and thymic NK cells. Gated NK1.1^{+} cells are shown. N.D. signifies no data available. Data are representative of 3 experiments.

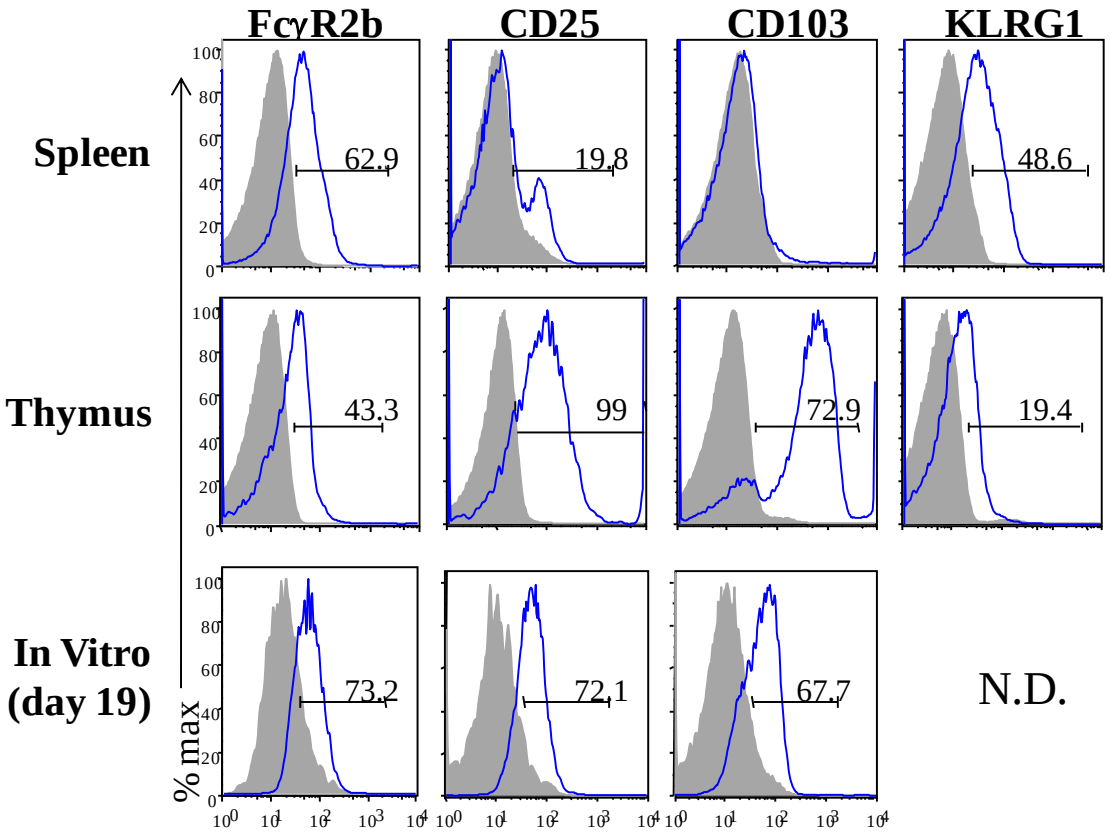

FcγR2b
CD25
CD103
KLRG1
Spleen
Thymus
In Vitro (day 19)
62.9
19.8
48.6
43.3
99
72.9
19.4
73.2
72.1
67.7
N.D.
% max

Figure 24. DN1 CD122$^-$NK1.1$^-$ thymocytes generate CD103$^+$ NK cells in a thymic microenvironment. DN1 CD122$^-$NK1.1$^-$ thymocytes from *Rag1*$^{-/-}$ Ly5.2 mice were transferred into irradiated *Rag1*$^{-/-}$ Ly5.1 mice intrathymically (IT). 32 days post transfer, cells were harvested from the spleen and thymus from host mice. In addition DN1 CD122$^-$NK1.1$^-$ thymocytes were cultured *in vitro* for 19 days and rested in IL15 alone for 36 hours. All cells were stained and analyzed via flow cytometry. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated antibody. Gated NK1.1$^+$ cells are shown. Data are representative of 2 experiments.

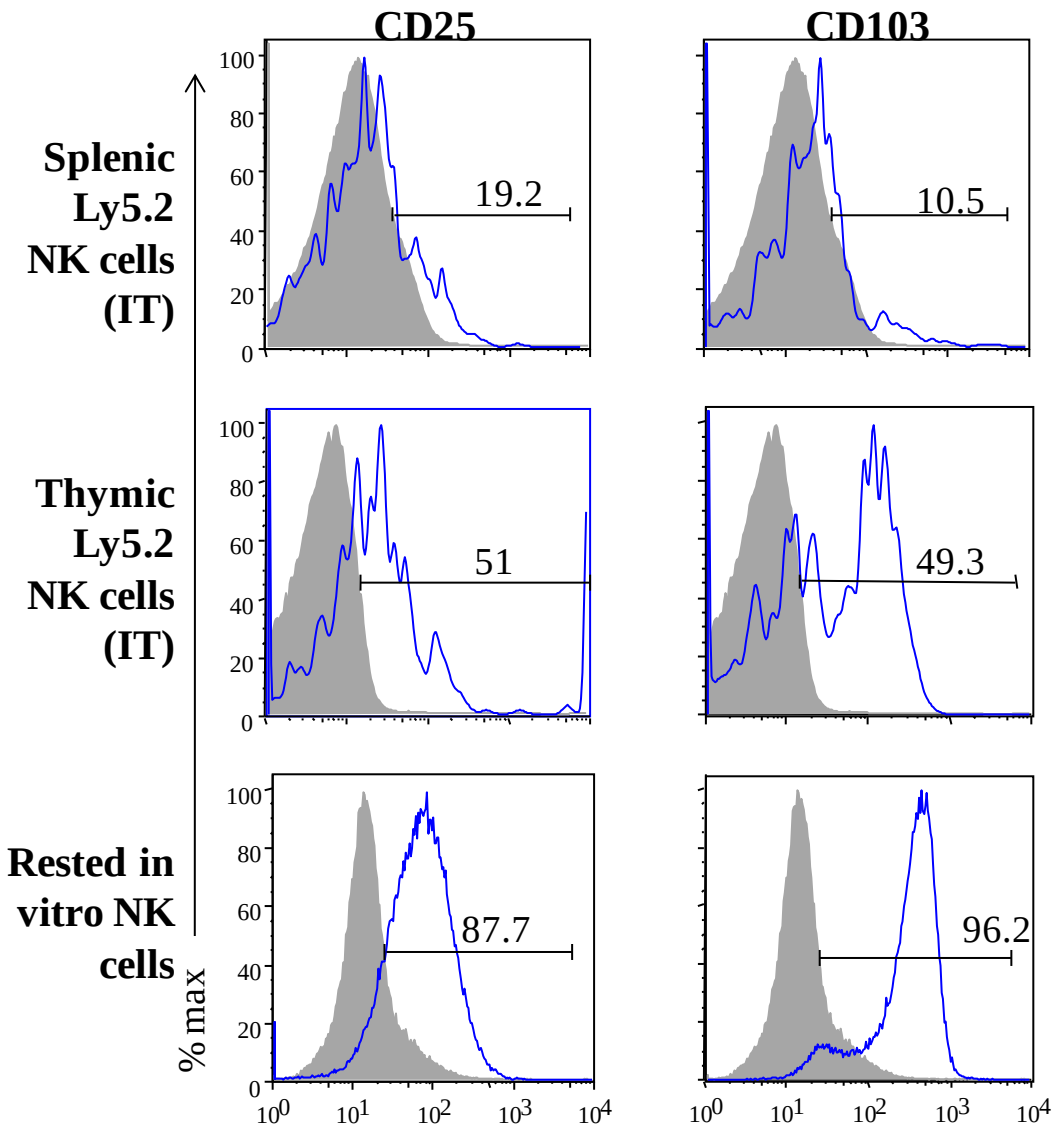

CD25
CD103
Splenic Ly5.2 NK cells (IT)
Thymic Ly5.2 NK cells (IT)
Rested in vitro NK cells
% max
19.2
10.5
51
49.3
87.7
96.2
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4

Figure 25. CD127^{+} NK cells are present in CD25- and CD103- deficient mice. Cells from indicated tissues of B6, CD103$^{-/-}$, *Rag1*$^{-/-}$, and CD25$^{-/-}$*Rag1*$^{-/-}$ mice were harvested and assessed for CD127^{+} NK cells by flow cytometry. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated antibody. The absolute value was calculated using the parameters specified in Flowjo. Gated NK1.1^{+} cells are shown. Data are representative of 2 experiments.

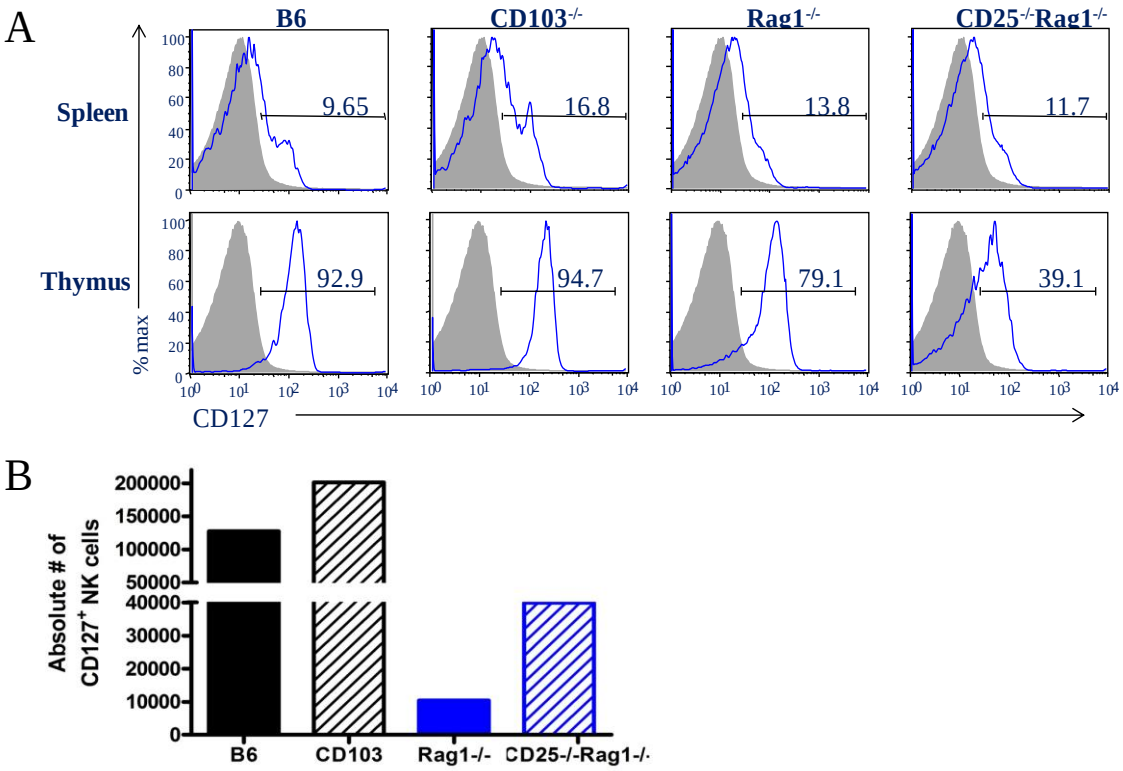

A
B6
CD103-/-
Rag1-/-
CD25-/-Rag1-/-
Spleen
Thymus
% max
9.65
16.8
13.8
11.7
92.9
94.7
79.1
39.1
CD127
B
Absolute # of CD127+ NK cells
B6
CD103
Rag1-/-
CD25-/-Rag1-/-

Figure 26. CD127^{+} NK cells are present in the intestinal mucosa. Cells from the small and large intestine of *Rag1*$^{-/-}$ mice were analyzed by flow cytometry. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated antibody. Gated NK1.1^{+} cells are shown. Data are representative of 2 experiments.

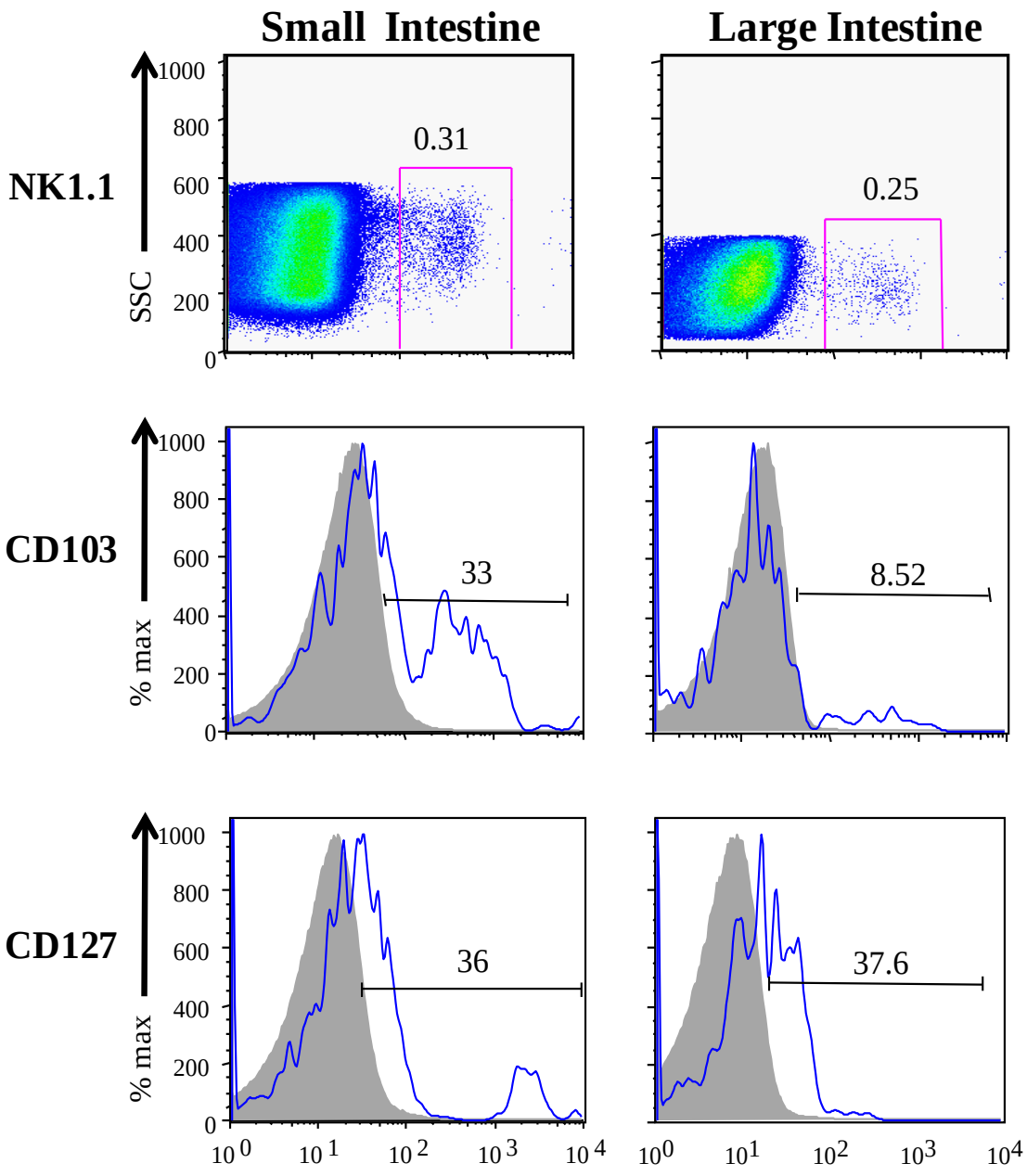
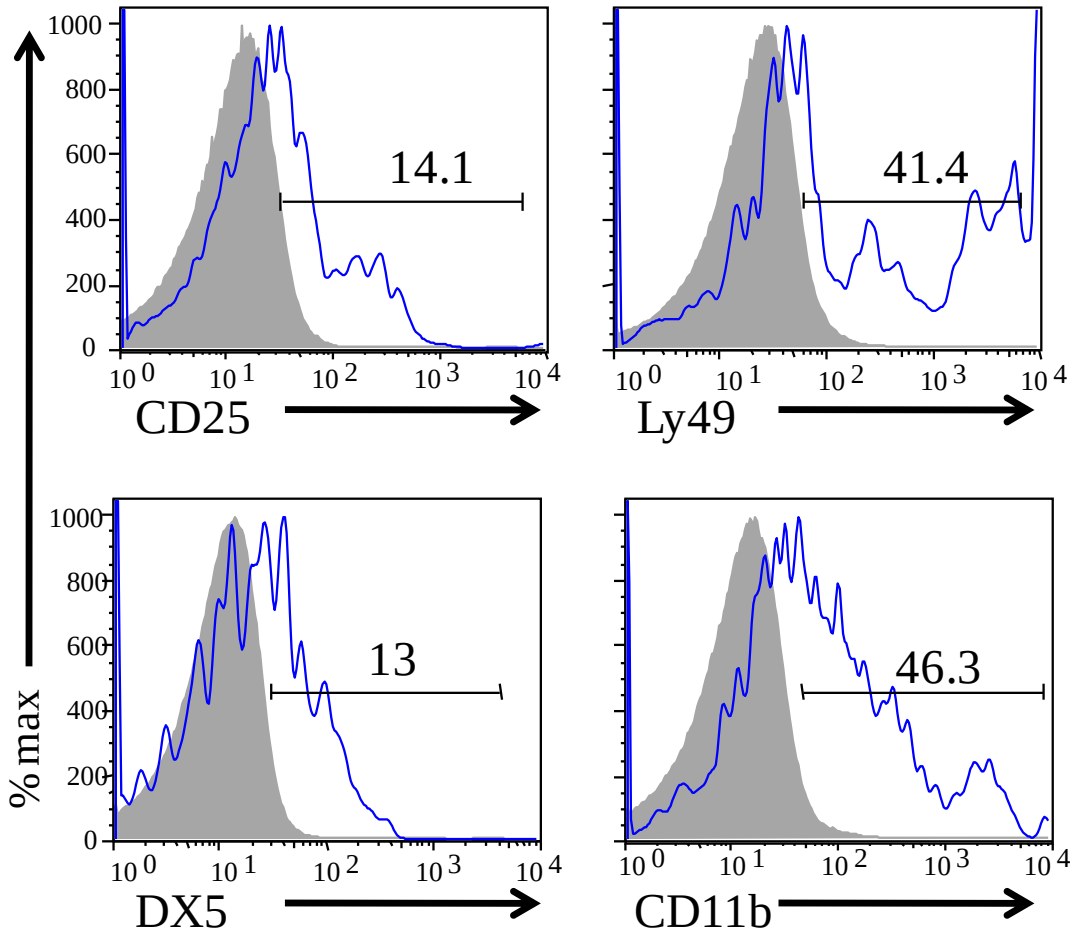

Small Intestine
Large Intestine
NK1.1
CD103
CD127
SSC
% max
0.31
0.25
33
8.52
36
37.6
1000
800
600
400
200
0
10^0
10^1
10^2
10^3
10^4

Figure 27. CD127^{+} NK cells in the small intestine resemble thymic NK cells. Cells from the small intestine of *Rag1*$^{-/-}$ mice were stained with a panel of antibodies and analyzed by flow cytometry. Gray-filled histograms represent cells stained with an isotype control and blue-line histograms represent cells stained with the indicated antibody. Gated NK1.1^{+} cells are shown. Data are representative of 2 experiments. representative of 5 experiments.


% max
1000
800
600
400
200
0
14.1
CD25
41.4
Ly49
13
DX5
46.3
CD11b

CHAPTER 6

DISCUSSION

Our studies showed that highly purified DN1 CD122$^-$NK1.1$^-$ thymocytes from *Rag1*$^{-/-}$ mice can differentiate into NK1.1$^+$ cells. The emerging NK population is unlikely due to contaminating mature NK cells that grew out in the subsequent *in vitro* cultures for a number of reasons: 1) The purified DN1 CD122$^-$NK1.1$^-$ population from *Rag1*$^{-/-}$Ly5.2 mice can give rise to cells with NK cell markers when adoptively transferred into sub-lethally irradiated *Rag1*$^{-/-}$Ly5.1 congenic recipients *in vivo*. 2) Although NK1.1 and CD122 are among the earliest markers on developing NK cells, they were not expressed at the earliest time points during our kinetic analysis, indicating that the cells acquired these markers during differentiation. 3) Kinetic analysis showed the gradual and sequential acquisition of markers associated with developing NK cell subsets. If these cultured cells represented outgrowth of mature NK cells, we would have expected that they expressed mature markers characteristic of later developmental stages early in the culture period. 4) Acquisition of the NK cell markers did not occur unless the cultures had all cytokines and OP9 cells, whereas mature NK cells can proliferate with IL15 alone. 5) Finally, the number of cells expressing the various NK cell markers cannot be accounted for by proliferation of a small contaminating pool of mature NK cells which typically have a 24 hour doubling time *in vitro*. For example, on day 4 we failed to detected NK1.1$^+$ cells, while on day 7, 35% of the cells were NK1.1$^+$. Taken together, these considerations strongly support that DN1 CD122$^-$NK1.1$^-$ thymocytes can differentiate into cells with NK markers *in vivo* and *in vitro*.

Unlike their conventional counterparts in the spleen, little is known about thymic NK cells. While the vast majority of splenic NK cells originate and develop in the BM, early studies have shown the existence of a T/NK bipotential progenitor in the thymus

[27-29]. Recent studies showed a similar T/NK bipotential progenitor may be present in the BM, based on *in vitro* differentiation but thymic NK cells did not develop upon adoptive transfer, unlike our results [75]. This suggest that these BM precursors lack the signals to develop into thymic NK cells. On the other hand, T/NK bipotential precursors are most commonly found within the fetal thymus, thus the possibility of thymic NK cells developing within the thymus of an adult mouse has been suggested [27]. Furthermore, previous studies did not characterize the $NK1.1^{+}$ cells that developed *in vitro* in the absence of Notch signaling, and thymic NK cells were described subsequently; their relationship to thymic NK cells was not investigated.

Here, our adoptive transfer studies strongly suggest that bipotential progenitors exist in the adult thymus which can differentiate into thymic NK cells rather than conventional NK cells. Taking into account that NK cells do not require gene rearrangement for their receptors, unlike T and B cell receptors, we opted to perform all of our experiments using *Rag1*$^{-/-}$ mice on a C57BL/6 background. This approach was supported by a recent publication indicating that thymic NK cells are not derived from thymocytes having undergone antigen receptor rearrangement [43]. Pre-sort analysis showed that only a very small percent of thymocytes were in the DN1 ($CD44^{+}CD25^{low/neg}$) stage of development, while most cells were arrested in the DN2/DN3 stage due to the lack of RAG genes. After sorting DN1 $CD122^{-}NK1.1^{-}$ thymocytes, we injected them intrathymically into *Rag1*$^{-/-}$Ly5.1 congenic recipients. Host mice had to be sub-lethally irradiated since we were unable to detect any $Ly5.2^{+}$ NK cells during our pilot studies with transfer into unmanipulated mice. Our *in vivo* studies also showed that thymic cells transferred intravenously failed to develop. This may be a

direct consequence of the inability of these cells to develop in other tissues, such as the spleen, or the failure to home back to the thymus for development, or both. Regardless, when the DN1 population was directly injected into the thymus of host mice, we not only detected donor NK1.1^{+} cells in the thymus, but also in the spleen. The presence of donor NK cells in the spleen may suggest that they develop in the thymus and have the potential to migrate and seed other sites. A second, though not mutually exclusive, explanation is that at some stage during development, a small percent of cells may escape the thymic environment, seed the spleen, and finish their maturation. Although further studies will be required to distinguish among these possibilities, our current studies indicate that the adult murine thymic DN1 population harbors progenitors that develop within the thymic environment into NK1.1^{+} cells that resemble thymic NK cells.

While the differentiated cells from our adoptive transfer experiments were too few for detailed analysis, we were able to study the *in vitro* differentiated cells in greater detail. Early studies examining the heterogeneity of the thymic DN1 population showed that T cells or NK cells could be generated *in vitro* using either OP9 cells with or without the expression of the Notch ligand Delta-like 1 (OP9-DL1), respectively. These studies showed that after six days of DN1/OP9 co-culture, NK1.1-expressing cells could be detected [33]. A recent study indicates that BM-derived precursors can also differentiate into NK1.1^{+} cells with a similar *in vitro* system [75]. However, in either case, the NK cells generated from this progenitor population were not further characterized.

In a similar manner, we seeded our sorted DN1 population on irradiated OP9 cells and added Flt3L, SCF, IL15, and IL7 to create an environment conducive for NK differentiation. By pooling wells on different days, we were able to delineate the kinetics

of developmental markers. Within 7 days, our sorted population had differentiated into $CD122^{+}NK1.1^{+}$ cells. The acquisition of other markers seem to be following the stages proposed by Kim and colleagues, where CD94, NKG2D, Ly49, CD43 and CD11b are sequentially up-regulated following CD122 and NK1.1 expression [20]. However, low Ly49 and CD11b MFI, together with the early CD25 and late CD127 expression, made the *in vitro* differentiated $NK1.1^{+}$ cells distinct from conventional BM NK differentiation. Regardless of the percent of cells that expressed each marker, we were able to detect the acquisition and the uniform increment of expression, as compared to isotype controls, of the positive population as time progressed. These studies suggest that conventional splenic and thymic NK cells undergo similar initial stages during development.

For the most part, the DN1 $CD122^{-}NK1.1^{-}$ cells were cultured with OP9 cells and cytokines for approximately 21-30 days, although the OP9 cells were no longer necessary after 13 days. From day 19 to 25, there was no phenotypic change in the *in vitro* differentiated $NK1.1^{+}$ cells. Cell numbers quickly declined if cells were left in culture conditions for more than 25 days, thus most experiments were done on day 19. Our studies show that these *in vitro* differentiated cells have a phenotype that resemble thymic NK cells, thus differing from conventional splenic NK cells. This was further validated when we modified the *in vitro* culture conditions and incubated the cells in IL15 alone which allowed up-regulation of DX5 and CD69, as well as CD127 itself to levels remarkably similar to thymic NK cells. Thus, the DN1 cells differentiated *in vitro* into $NK1.1^{+}$ cells more closely resembling thymic NK cells.

Moreover, our studies yielded an unexpected difference with respect to CD25 that we and others did not find expressed on wild type thymic NK cells [39, 55]. The basis

for this difference is not clear at the moment but at minimum, it appears to be another marker selectively expressed on thymic NK cells. Interestingly, gene chip profiles verified that CD25 mRNA was 40- and 20- fold higher in thymic and *in vitro* NK cells, respectively, than in their splenic counterparts.

In addition to CD25, microarray profiling also showed CD103 mRNA was expressed 26-fold higher on thymic NK cells. Thus far, CD103 has been shown to be expressed on $CD8^+$ and $CD4^+$ T cells in the intestinal mucosa [64-66]. It is also expressed on lymphocytes in the genitourinary tract and lungs [76-79]. To our knowledge, it has never been shown to be expressed on any NK subset. The expression of this integrin on thymic NK cells may perhaps indicate that these cells differentiate in the thymus but ultimately emigrate to mucosal tissues, such as gut or lungs. While functional results showed that both thymic and *in vitro* generated NK cells have the capacity to produce cytokines and displayed degranulation when exposed to NK sensitive targets, a more specific role in other tissues is not out of the question. Further studies which will determine if this unique NK subset is present in mucosal tissues will allow us to determine if these cells have a different effector function than splenic NK cells, such as a tolerogenic role.

Although CD127 is thought to be a good marker to identify thymic NK cells, it has been shown to be expressed transiently in other lymphocytes [80]. IL7Rα down-regulation, which is induced by IL7 signaling, has been proposed to serve as a mechanism for T cells to receive pro-survival signals [81]. Cells signaled by IL7 transiently down-regulate surface expression of IL7Rα and thus become unresponsive to the continued presence of IL7. This behavior explains how activated T cells undergo

rapid expansion, increasing their numbers to almost half of the total number of T cells during some acute infections [82]. Upon activation, T cells respond to other cytokines such as IL2 and down-regulate IL7Rα and IL7 consumption, presumably in order to preserve the naïve repertoire that is critically dependent on IL7. A similar phenomenon is believed to exist between DN and double positive (DP) thymocytes where the DN population of lymphocyte precursors is critically dependent on IL7 to give rise to double positive (DP) thymocytes [83], or in this case thymic NK cells. The transient expression of CD127 suggest that it is a non-reliable marker for identifying NK cells generated in the thymus in other tissues of the periphery. However, our studies show that CD103 is exclusively expressed in thymic NK cells and *in vitro* differentiated NK cells. While we were able to detect CD103 by day 7, the majority of *in vitro* NK cells expressed it by day 13. This expression was stable in that we were able to detect it consistently at later time points. Furthermore, our studies showed that all $CD127^+$ NK cells were CD103-positive, yet not all $CD103^+$ NK cells expressed CD127 (data not shown). With the transient expression of CD127 and the constant expression of CD103, we propose that the latter is a better identification marker for thymic NK cells.

Taken together, our results suggest that DN1 $CD122^-NK1.1^-$ thymocytes harbor a population that utilizes the thymic stroma to differentiate into NK1.1 expressing cells. Both adoptive transfer and *in vitro* experiments showed that these cells have a unique phenotype including the expression of CD127, CD25, and CD103. While these cells were capable of lysing target cells, they were more efficient at secreting cytokines such as TNF. Nonetheless, the studies described here provide future opportunities to dissect the function of thymic NK cells and their development.

CHAPTER 7

FUTURE DIRECTIONS

By utilizing the methods described here, our understanding of NK cell development and differences between NK cell subsets has grown expansively. The idea that T/NKPs in the thymus of adult mice could develop into a unique NK cell was confirmed with adoptive transfer experiments. Although our return yield was slightly lower than expected, *in vitro* studies allowed us to further study the DN1 population and their association with thymic NK cells. However, many questions remain regarding thymic NK cell development and function that merit further investigation.

Determine if thymic precursors are committed or influenced to become thymic NK cells

Our studies showed that a small population of DN1 thymic progenitors are destined to become thymic NK cells rather than conventional NK cells. A question that remains is whether they acquire this commitment once they reside in the thymus or if experimental constraints allowed preferential development of thymic NK cells. In order to address these issues, further dissection of the DN1 progenitor pool will be necessary.

First, we must identify the putative progenitor population within the DN1 stage of development that leads to the generation of thymic NK1.1^{+} cells. Studies have shown that the DN1 thymic population is heterogeneous [84]. To further refine the analysis of DN subsets, the expression or absence of CD117 has been included in certain studies [33, 85]. The level of expression of CD117 by DN thymocytes is very broad and, depending on staining strategy, spans two to three logs of fluorescence intensity (data not shown). Whereas T lineage cells in the DN1 subset are CD117^{bright}, other non-T lineage precursors, including those for dendritic cells and NK cells, express intermediate levels of

CD117 [84]. We will therefore, attempt to sub-sort the DN1 $CD122^{-}NK1.1^{-}$ thymocytes according to CD117 expression and culture them in the *in vitro* conditions described above. A quick phenotypic analysis will indicate which population generates $CD127^{+}NK1.1^{+}$ cells. Furthermore, we will re-address progenitor frequency by culturing the specific population in limiting numbers. Current results show that one out of every 416 DN1 $CD122^{-}NK1.1^{-}$ thymocytes generates an $NK1.1^{+}$ population , but it should be noted that this precursor population is negatively defined by lack of marker expression (CD4, CD8, CD117, CD122, NK1.1), rather than the expression of markers associated with high progenitor frequency. Perhaps by further enriching our starting population, we may be able to increase precursor frequency to about 1/10.

Once the putative precursor population is identified, we can differentiate them *in vitro* with either our current culture conditions or with different factors that could potentially alter the fate of the progenitors. Currently, we seed the progenitors on OP9 BM stromal cells and add IL7, SCF, Flt3L, and IL15. Our attempts to differentiate the progenitors in a stroma-free culture, using only cytokines have been unsuccessful (data not shown). For that reason, we will attempt to change the stromal cell line while keeping all other factors constant. Early studies have shown that the AFT024 stromal cell line supports murine HSCs by providing a positive stem cell regulator via delta-like/preadipocyte factor-1 [86]. These cells not only represent the liver micro-environment, but have also been immortalized with temperature sensitive SV40T antigen, which allows them to grow only at 33°C while inhibiting their proliferation at 37°C. Results could yield one of three options: 1) The progenitors could give rise to $CD127^{+}NK1.1^{+}$ cells that resemble thymic NK cells, indicating that these DN1 precursors

are precommitted to the thymic NK subset early on. 2) The progenitors could differentiate into NK1.1^{+} cells that do not resemble thymic NK cells, suggesting the progenitors are committed to the NK lineage but not the CD127^{+} NK subset. These results could signify that the thymic progenitors are influenced by their environment to express a certain receptor repertoire. 3) Finally, progenitors may not express NK1.1^{+} at any time point, indicating the stromal cells are not providing the signals necessary for NK development. Regardless, these results will give us the opportunity to further understand the factors involved in NK lineage commitment.

While these *in vitro* results are possible, it will not be feasible to differentiate this putative population *in vivo*. Our current protocol for adoptive transfer includes harvesting the thymus from 80-100 *Rag1*$^{-/-}$ mice to obtain an appropriate number of DN1 CD122^{-}NK1.1^{-} progenitors. If this population is further sub-sorted, we will need to increase the number of mice used to collect enough of the putative progenitors that will generate thymic-like NK1.1^{+} cells. For this reason, we will attempt to answer these questions in an *in vitro* system.

Examine transcripts that correlate with the developmental stages of NK cells

In addition to surface marker acquisition, it will be important to assess specific transcription factors (TF) involved in the differentiation process. Because the *in vitro* generated NK cells were shown closely resemble thymic NK cells, we can follow the expression of TF as they develop. Thus far, we have initiated these studies by culturing the DN1 CD122^{-}NK1.1^{-} progenitors *in vitro* for 4, 8, and 13 days. We enriched NK1.1^{+} cells on days 8 and 13 by doing a high-purity sort with MoFlow, while all differentiating

cells were collected on day 4. Total RNA was isolated and target synthesis for hybridization to Affymetrix 430 v2.0 GeneChips was performed with two rounds of linear amplification. Total RNA from 3 different experiments was pooled for target synthesis and three target samples were pooled and hybridized to each chip, resulting in three chips for each cell type. Although we have received the gene profiles, we have not performed detailed analysis of the transcription factors.

Since our studies suggest that these cells follow a similar differentiation process as BM derived NK cells, we expect to see expression of the most common NKP TFs during the early stages of development, such as PU.1, Ets-1, Ikaros, and Id2. PU.1 has been implicated in early NK cell development since deficiency in this transcription factor affects expression of the CD127 and the Flt3 receptor [87-89]. Compared to WT, $PU.1^{-/-}$ fetal liver HSC generated reduced numbers of NKPs when transferred into alymphoid $Rag2^{-/-}$ x $\gamma_c^{-/-}$ mice [90]. Similarly, deficiency in Ets-1 results in the absence of NK cells in the bone marrow, spleen, and LN, suggesting NKPs are also reduced or absent [91]. Furthermore, mice that are deficient in the zinc-finger transcription factor Ikaros display severe defects in the development of all lymphoid cell lineages including NK cells, which may be due to the diminished expression of Flt3, CD122, and CD117 [92-94]. Finally, the inhibitors of DNA binding (Id2) have been shown to heterodimerize with E-box transcription factors, thereby titrating them out and inhibiting their transcriptional activity. Since E-box transcription factors are critical mediators of T and B lymphopoiesis, their inhibition by Id2 generates NK cell lineage committed NKPs [95, 96]. Together these transcription factors have been shown to affect NKPs.

During later stages of development, such as day 8 or day 13, we expect to see different transcription factors than those found in earlier stages of development. For the most part, immature NK cells have been found to express Gata-3, T-bet, and IRF-2, whereas mature NK cells express CEBP-γ, MEF, and MITF [13]. Gata-3 is has been shown to be necessary for the proper expression of CD11b, CD43, and Ly49s on NK cells along with CD127 on thymic NK cells [97]. It has been found to have a contradictory function in NK cells compared to T cells, promoting IFN-γ production by mature NK cells [97]. In addition, Gata-3 has been associated with T-bet, which has similar affects on CD11b and CD43 expression [98]. NK cells that develop in the absence of T-bet have comparable cytotoxicity as their normal counterparts, but are unable produce INFγ after stimulation. A study done by Taki and colleagues shows that IRF-2 also plays an important role in NK cell development in the BM and is also required for proper CD11b and CD43 expression [99]. Results also show that mice deficient in IRF-2 had a reduced number of splenic NK cells that had an immature phenotype, lacking DX5 and Ly49 expression as well as expressing low levels of CD11b and CD43. This study also found that although these cells seemed immature, they were found to have normal cytotoxicity against sensitive targets. While we expect to see GATA-3 and T-bet gene up-regulation, we suspect IRF-2 will not be detectable.

Unlike TFs involved in immature NK cells, TFs in mature NK cells are important for proper effector functions. While an absence of CEBP-γ, MEF, and MITF in these cells has been shown to not have a negative impact on development and differentiation, this deficiency has been implicated in a reduction of cytotoxicity and cytokine production [13]. The absence of these TFs leads to a reduction in perforin and granzyme expression,

both of which are necessary to carry out an appropriate effector function [13]. Since our results suggest the *in vitro* generated NK cells are functional, we suspect these TFs will be expressed.

By using the gene profiling method, we will be able to take a closer look at the TFs that are involved in the developmental process. Because most of these TFs were shown to be expressed during BM development, we expect to find some differences. While we have initiated the process by doing the microarray, further studies that will verify these results, such as quantitative RT-PCR, are still pending.

Determine whether NK cells in the GALT are derived from thymic DN1 CD122$^-$ NK1.1$^-$ progenitors

Human and mouse studies have shown that different NK subsets are present within the gut-associated lymphoid tissue (GALT) including the intraepithelial lymphoid compartment, intestinal lamina propria, Peyer's patches, and mesenteric lymphoid nodes [100, 101]. Analysis of NK cells found within the intestinal mucosa revealed the presence of a unique subpopulation that was NKp46$^+$CD127$^+$Ly49$^-$NK1.1$^{+/-}$, along with a second population that resembled conventional NK cells [102]. While very little is known about their receptor repertoire and effector function, the developmental origin of these cells has yet to be determined.

To assess whether the NKp46$^+$CD127$^+$Ly49$^-$NK1.1$^+$ cells are thymic-derived NK cells, we will repeat the adoptive transfer experiments but expand our search for the donor-differentiated NK cells. Briefly, DN1 CD122$^-$NK1.1$^-$ thymic progenitors from *Rag1*$^{-/-}$Ly5.2 mice will be enriched via FACS sorting. These cells will be intrathymically

injected into irradiated congenic *Rag1*$^{-/-}$Ly5.1 mice. Based on our previous results, cells will be allowed to differentiate *in vivo* for 35-45 days, at which point, we will harvest spleen, thymus, lungs, small intestine, and large intestine. We will look for the presence of Ly5.2^{+} NK cells by staining with both anti-NK1.1 and anti-NKp46 antibodies. To further characterize these cells, we will look at their phenotypic profile by staining the previously described surface molecules.

Taking our recent CD103 discovery into consideration, we expect to find Ly5.2^{+} NK cells in mucosal tissues. Whether these cells are NKp46^{+}NK1.1^{-} or NKp46^{+}NK1.1^{+} will be further investigated upon phenotypic analysis. Positive results will suggest that the DN1 CD122^{-}NK1.1^{-} progenitors differentiate in the thymus, then guided by the CD103 integrin, along with some other unknown factors, emigrate from the thymus and homes to mucosal tissues. We can speculate that CD103 is involved in the thymic NK cell migration since our results showed an increase in numbers of CD127^{+} NK cells in the thymus of CD103$^{-/-}$ mice. Further validation as to whether it is directly involved in homing to mucosal tissues would need to be done by assessing the presence or absence of these cells in the lungs or intestinal mucosa of CD103$^{-/-}$ mice. However, negative results may suggest a number of things: 1) The presence of Ly5.2^{+}CD127^{-} NK cells, with a similar thymic phenotype could suggest these cells developed in the thymus, but upon homing to the tissue, altered their phenotype to better suit the environment. 2) The presence of Ly5.2^{-}CD127^{+} NK cells would indicate these cells are not the same subset as those derived from the thymus. Together, these future studies will perhaps provide an insight as to whether thymic NK cells have a specific role in not only the thymus, but in the mucosal tissues as well.

Do thymic NK cells carry out a specific function

NK cells are often thought of as killing machines that zone in on a target, bind, and lyse all within a matter of minutes. The discovery of NK subsets has shown this to be an erroneous idea. Since NK cells can circulate in the blood and are found in secondary lymphoid tissue and non-lymphoid tissue, they may have a role in systemic immunosurveillance against various pathogens and tumors [103]. However, studies have shown that a subset of uterine NK cells in both humans and mice contribute and sustain important changes in the maternal placental bed during the first half of gestation, mainly by secreting vascular endothelial growth factor (VEGF) [104].

In addition, the mucosal NK subset, termed NK-22, that was recently described has been shown to express both IL22 transcripts and proteins [58, 105]. IL22, which belongs to the IL10 family of cytokines, has been shown to protects the epithelial cell barrier in the gut and other mucosal tissues from pathogens [106, 107]. Unlike conventional NK cells that carry out their function by detecting targets cells that have down-regulated their MHC [108], these mucosal NK cells are highly responsive to IL23 in both human and mouse [58]. Studies have shown this NK-22 subset provides a protective barrier against intestinal *C. rodentium* infection in *Rag2*$^{-/-}$ mice [109]. Perhaps the tolerogenic effect that both uterine and intestinal NK cells have, may provide the clues necessary to determine the specific effector function of thymic NK cells.

Once we determine whether thymus-derived NK cells have the potential to home to the gut, we can further study their effector function by applying some of the same methods described above. Since studies showed that *Rag* KO mice are resistant to *C. rodentium* infection due to NK cells in the mucosa, we can utilize *Rag1*$^{-/-}\gamma_c^{-/-}$ mice to

address specific function. Due to their lack of T, B, and NK cells, we suspect these mice will succumb to the infection. Once this has been established, we can adoptively transfer $CD127^+$ NK cells and assess the mice for weight loss and survival. Studies examining the role of naive $CD4^+CD45RB^{hi}$ T cells in irritable bowel syndrome suggest approximately $5x10^5$ cells is an appropriate number to see any type of results, therefore, we will start with this number of $CD127^+$ NK cells and transfer them IP. These studies could result in two possible outcomes: 1) The mice will survive, suggesting that $CD127^+$ NK cells have a tolerogenic role. 2) The mice will succumb to infection, suggesting either that $CD127^+$ NK cells are not responsible for repopulating the mucosa, thus providing no source of protection, or that they need the presence of other NK subsets to effectively protect the mice.

While this is only one source of infection, we could repeat these experiments using a several KO mice including the $IL7^{-/-}$ and $CD103^{-/-}$, in addition to us depleting the cells with specific antibodies. Furthermore, we could infect mice with a number of different pathogens and, once again, assess the phenotype and function of $CD127^+CD103^+$NK cells present in the thymus and perhaps in the intestinal mucosa as well. Taken together, these studies will allow us to further examine the potential role of thymic NK cells during a pathogen infection. However, it is essential that we first determine whether these NK cells are permanent residence of the thymus, or whether they have the potential to home to other tissues.

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