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Regulation Of Natural Killer Cell Development And Function By Activating Receptor Signaling Pathways

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Abstract

Natural killer (NK) cells are lymphocytes of the innate immune system that recognize and eliminate virally infected and transformed cells through their release of cytotoxic granules and production of inflammatory cytokines. The balance of intracellular signals received through NK cell activating and inhibitory receptors dictates these functions and generates target cell specificity during development. Many signaling pathways downstream of activating receptors contribute to these processes, however, what pathways and what signaling proteins contribute to NK cell development and function are not fully understood

While NK cells do not possess an antigen-specific immunoreceptor, they do express a variety of germline-encoded activating and inhibitory receptors. MHC I-binding inhibitory receptors, including those of the Ly49 and KIR families, are expressed in a variegated manner, which creates ligand-specific diversity within the NK cell pool. In this thesis, I demonstrate that signals derived from activating receptors are critical for induction of Ly49 receptors/KIRs during NK cell development; activation signals through SLP-76 increased the probability of the Ly49 bi-directional Pro1 promoter to transcribe in the forward versus the reverse direction, leading to stable Ly49 receptor expression and receptor diversity in mature NK cells.

Not only does activation through SLP-76 impact NK cell development, but downstream signaling pathways also impact NK cell function. Sustained Ca2+ signaling, known as store-operated Ca2+ entry (SOCE), occurs downstream of NK cell activating receptor engagement. CD8+ T cells require SOCE for cytokine production and cytotoxicity; however, less is known about its role in NK cells. In this thesis, I use mice deficient in STIM1/2, which are required for SOCE, to examine the contribution of sustained Ca2+ signaling to NK cell function. Surprisingly, we found that while SOCE is required for NK cell IFNγ production in an NFAT-dependent manner, NK cell degranulation and tumor rejection in vivo remained intact in the absence of SOCE. Our data suggest that mouse NK cells utilize different signaling mechanisms for cytotoxicity compared to other cytotoxic lymphocytes. In summary, NK cell activating receptor signals and downstream signaling pathways contribute greatly to the development and function of NK cells, allowing them to effectively eliminate cancer and virally infected cells.

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DEDICATION

This body of work is dedicated to my sister, Jessica A. Wolff. She truly is my inspiration-- not only in life, but also in science. If not for her unique and interesting medical narrative and our shared autoimmune disease, I would *never* have pursued immunology (or science for that matter) as a career. I want to thank her for pushing me to be my best and for always being my biggest cheerleader. Jess- you are my motivation to get up everyday and go to lab. You are a wonderful big sister, and I feel lucky that we are so close. You make me want to discover new facets of immunology that will one day influence the design of therapeutic options for amazing people just like you.

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ABSTRACT

REGULATION OF NATURAL KILLER CELL DEVELOPMENT AND FUNCTION BY ACTIVATING RECEPTOR SIGNALING PATHWAYS

Jacquelyn E. Freund

Taku Kambayashi

Natural killer (NK) cells are lymphocytes of the innate immune system that recognize and eliminate virally infected and transformed cells through their release of cytotoxic granules and production of inflammatory cytokines. The balance of intracellular signals received through NK cell activating and inhibitory receptors dictates these functions and generates target cell specificity during development. Many signaling pathways downstream of activating receptors contribute to these processes, however, what pathways and what signaling proteins contribute to NK cell development and function are not fully understood

While NK cells do not possess an antigen-specific immunoreceptor, they do express a variety of germline-encoded activating and inhibitory receptors. MHC I-binding inhibitory receptors, including those of the Ly49 and KIR families, are expressed in a variegated manner, which creates ligand-specific diversity within the NK cell pool. In this thesis, I demonstrate that signals derived from activating receptors are critical for induction of Ly49 receptors/KIRs during NK cell development; activation signals through SLP-76 increased the probability of the Ly49 bi-directional Pro1 promoter to transcribe in the forward versus the reverse direction, leading to stable Ly49 receptor expression and receptor diversity in mature NK cells.

Not only does activation through SLP-76 impact NK cell development, but

downstream signaling pathways also impact NK cell function. Sustained Ca^{2+} signaling, known as store-operated Ca^{2+} entry (SOCE), occurs downstream of NK cell activating receptor engagement. $CD8^{+}$ T cells require SOCE for cytokine production and cytotoxicity; however, less is known about its role in NK cells. In this thesis, I use mice deficient in STIM1/2, which are required for SOCE, to examine the contribution of sustained Ca^{2+} signaling to NK cell function. Surprisingly, we found that while SOCE is required for NK cell IFNγ production in an NFAT-dependent manner, NK cell degranulation and tumor rejection *in vivo* remained intact in the absence of SOCE. Our data suggest that mouse NK cells utilize different signaling mechanisms for cytotoxicity compared to other cytotoxic lymphocytes. In summary, NK cell activating receptor signals and downstream signaling pathways contribute greatly to the development and function of NK cells, allowing them to effectively eliminate cancer and virally infected cells.

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CHAPTER I: Introduction

Natural Killer Cells & The Immune System

The general purpose of the mammalian immune system is to protect the body from foreign invaders. The term foreign invaders can refer to multiple entities, including inorganic materials, bodily fluids of another mammal, pollen from a daffodil, and pathogens such as bacteria, fungi, and viruses that can be disseminated through food, air particles, or liquid. The mammalian immune system has developed a complex yet comprehensive process of protecting the body and maintaining homeostasis. To do this, it uses a two-armed system: the innate immune arm and the adaptive immune arm. Both arms share multiple properties, but each has unique features in its execution. The immune system's objective is to identify "self" versus "non-self", and cells of the innate and adaptive immune system approach this objective differently, using a system of checks and balances to ensure that non-self entitles are removed [1].

The responses generated by the innate immune system are largely non-specific. Unlike the adaptive system, the innate immune system is not activated by antigen-specific receptors, but rather through the recognition of conserved features on "non-self", or foreign, entities. Cells of the innate immune system include macrophages, dendritic cells, neutrophils, mast cells, and natural killer cells [1,2]. The kinetics of the innate immune system during an immune response are rapid. These cells are first responders, as they patrol barrier surfaces such as the skin and mucosa. Furthermore, they are important cells for recognizing and often eliminating foreign threats to the body, despite responding in a relatively similar manner to different kinds of foreign threats. Often times, while this response is critical for the control of the foreign invader, clearance fails

to occur. In that case, the innate immune system provides helper signals to recruit cells of the adaptive immune system to eliminate the biological threat [1,2].

Cells of the adaptive immune system are characterized differently than those of the innate immune system because they undergo genetic recombination of cell membrane receptors that are required for the recognition of specific antigens. The two cell types of the adaptive immune system are B and T-lymphocytes. Through a developmental and activation-based process, these cells create antigen-specific recognition receptors to particular proteins or antigens. Therefore, following general activation from the innate immune system, B or T cells specific to the foreign antigen have ample time to clonally expand and eliminate the threat. What is even more unique about the adaptive immune response is its ability to create memory [3,4]. These antigen-specific lymphocytes can persist through the lifespan of an organism and remember the original threat in case it re-appears later in life.

A downside of this system is that mammals have evolved so rapidly that foreign invaders are no longer the only threat the immune system must encounter. In fact, the immune system is imperative for the fight against cancer, which is considered to be "altered self". In an elegant attempt to eliminate "non-self" and "altered-self", adaptive immune cells need to recognize "altered-self" antigens to be able to discriminate "self" from "non-self" or altered-self". Because of this need to recognize and differentiate "self" versus "non- or altered-self", the immune system has the potential to misdirect its attacks, which results in autoimmunity, the immune system's destruction of "self".

Natural killer (NK) cells are cells of the innate immune system, as they do not possess a genetically re-arranged antigen-specific receptor. NK cells comprise between

5-15% of peripheral blood lymphocytes, but also reside in the bone marrow, liver, spleen, uterus, lungs, and secondary lymphoid organs. NK cells were first described in the 1970s as large granular lymphocytes with the ability to spontaneously kill certain tumor cell lines [5]. NK cells have been described as the innate counterpart of the $CD8^+$ T-cell, the cytotoxic lymphocyte, because NK cells and $CD8^+$ T-cells utilize similar mechanisms to eliminate biological threats. Yet unlike $CD8^+$ T-cells, NK cells express a myriad of germline encoded receptors that combinatorially create specificity towards "non- or altered-self" antigens [5,6].

Not only could NK cells eliminate tumor cells, but also it was discovered early on that an F1 generation of mice between two inbred parental mouse strains were unable to accept bone marrow grafts from the parental generation. Researchers determined that the major histocompatibility I protein complex (MHC-I) was involved, but that it was not T-cell mediated [7]. This defied the previously established rules of bone marrow transplantation. Both parental MHC-I molecules were expressed in the F1 mouse, yet the bone marrow graft was still rejected. This novel finding helped transform the theory that the immune system's role was to only eliminate "non-self", and an NK cell's ability to eliminate parental tissue lacking a specific MHC-I molecule led to the concept known as the "missing-self hypothesis" [8]**.** This process is unique to NK cells and is imperative for NK cell clearance of cancerous malignancies and viral infections. While the combination of NK cell receptors creates NK cell specificity against "non- and altered-self", the fact that an NK cell can express so many of these receptors individually and in combination allows them to have a more flexible response to a stimulus than a $CD8^+$ T cell. Because of these characteristics, NK cells have not been shown to cause overt autoimmunity.

NK cell development and function interweave with each other: NK development affects function and NK cell function impacts development. One aspect of cellular biology that impacts both equally are the intracellular signaling pathways employed by NK cells. While much is already known about signaling in both NK cell development and function, key questions remain as to the contributions of intracellular signaling in specific developmental processes and function. Specifically, what signals does an NK cell need to express its large repertoire of germline encoded receptors? If there are so many, do different receptors utilize different signals from the NK cell or the environment? Lastly, do all of the signaling proteins downstream of the MHC-I binding NK cell receptors or activating receptors contribute to NK cell function against cancer or virally infected cells? My thesis work addresses these very broad questions by exploring the regulation of NK cell development and function through activating receptor-signaling pathways.

Natural Killer Cell Development

Hematopoiesis to Mature NK Cell

Both human and murine NK cells begin development in the bone marrow, deriving from hematopoietic stem cells (HSCs) and the subsequent common lymphoid progenitors (CLP) [9,10]. The following description of NK cell development is focused on murine NK cells. NK cell commitment requires the up-regulation of the interleukin (IL)-15 receptor beta chain (CD122), as IL-15 is required for NK cell differentiation and survival. The up-regulation of CD122 and the lack of lineage markers designated for other cell types marks the NK precursor stage (pNK), which is defined by the following cell surface phenotype: $Lin^{-}CD122^{+}NK1.1^{-}DX5^{-}$ [11]. Furthermore, IL-15 has been shown to be

important for cytokine priming of resting NK cells, and dendritic cells and monocytes produce this IL-15 [12-14]. Mice deficient in IL-15 or IL-15 signaling components, such as janus kinase 3 (JAK3), have reduced numbers of NK cells [10,15], whereas NK cells from mice deficient in other gamma chain-based cytokines (e.g. IL-2, IL-4, IL-7) have normal NK cell numbers and NK cell differentiation [16]. Precursor NK (pNK) cells do not yet express markers of NK cell specificity, but moreover, they do not express markers of T- or B-cell specificity (CD3 or CD19). The NK cell activating receptors NKG2D and 2B4 are expressed on pNK cells, but these receptors are not NK cell specific [16].

The subsequent stage of murine NK cell development is the immature bone marrow NK cell (iNK). This stage of development is marked by the acquisition of NK cell specific membrane proteins such as activating receptors NK1.1, NKp46, and the inhibitory receptor heterodimer CD94/NKG2A. At this stage, NK cells begin to acquire of a family of C-type lectin molecules—the Ly49 receptors. NK cells complete their maturation in both the bone marrow and the spleen with the up-regulation of the integrin CD49b, also known as DX5, and the further expression of Ly49 family members [6,17]. These are referred to as mature NK cells (mNK). The factors defining the progression from pNK to mNK cell have not been fully elucidated, but an iNK cell can be identified by its lack of the complete phenotypic and functional characteristics of mNKs. iNK cells have limited (or completely lack) Ly49 receptors and DX5 expression [17]. The acquisition of Ly49 receptors is discussed in more detail in a later introductory section.

Once a mature NK cell leaves the bone marrow to circulate to other organs, its maturation is not necessarily complete. Splenic NK cells, commonly referred to as conventional NK cells, alter their expression of the cell surface proteins CD11b and CD27. A four-stage developmental process has been described where NK cells

transition through the following stages: $CD27^{-}CD11b^{-}$ (least mature) → $CD27^{+}CD11b^{-}$→ $CD27^{+}CD11b^{+}$→ $CD27^{-}CD11b^{+}$ (most mature)[18]. From adoptive transfer experiments, most mature NK cells derive from the previously described stage of development and express higher levels of granzyme B, CD43, and activating/inhibitor receptors [18].

Factors Influencing NK Cell Development

Biological factors from the NK cell's environment, DNA transcription factors, and intracellular signaling pathways play an important role in NK cell development. For example, flt-3 ligand (Flt3L) and the lymphotoxin-beta receptor (LTβR) are vital for NK cell generation. Absence of Flt3 in mice negatively impacts NK cell numbers. Flt3 has been shown to induce CD122 expression, which makes NK cells responsive to IL-15 or IL-2. Furthermore, HSCs from a wild-type (WT) control mouse transferred into a LTβR-deficient mouse cannot support NK cell generation due to defects in the stromal cell compartment [19]**.** Signals delivered to NK progenitors via the bone marrow stroma and IL-15 are sufficient to support both NK cell survival and Ly49 receptor expression [20]. In fact, in an *in vitro* culture system in which bone marrow NK progenitors were cultured on bone marrow stromal cells, stromal cells lacking MHC- I ($\beta2m^{-/-}$ stroma) induced Ly49G2 expression on $Ly49A^{+}$-expressing transgenic progenitors over $H\text{-}2^{d}$ stromal cells (MHC-I ligand for Ly49A and Ly49G2) [21]**.** This suggests that the lack of stromal MHC-I is quite important for Ly49 acquisition

When it comes to transcription factors, the balance of Eomes and T-bet expression is as critical for NK cells as it is for T-cells. iNKs express T-bet and the death ligand TRAIL [22]. T-bet is required for the stabilization of the $TRAIL^{+}$ iNK cell phenotype

which are $DX5^-$. The T-bet transcriptional partner, Eomes, is required for the generation of $DX5^+$ NK cells. In the absence of Eomes ($Eomes^{fl/fl}$-$VavCre^+$ mice), NK cells reverted to a more "immature" $TRAIL^+$ state [22]. TRAIL positivity correlated with the developmental stages for splenic maturation described by Chissone; $CD27^{hi}CD11b^{lo}$ iNKs are $TRAIL^+$ and $CD27^{lo}CD11b^{hi}$ NKs were $DX5^+$ and $Eomes^+$ [23].

Lastly, like T-cell development, which requires signaling inputs downstream of the T-cell receptor (TCR), NK cells utilize a similar signaling cascade downstream of their activating receptors. The use of major signaling proteins has been studied in NK cell development. It was found that NK cells develop normally in mice deficient in Syk-ZAP70, DNAX-activating protein of 10 kDa (DAP10), and SAP/Fyn [24-26]. T cells require signaling pathways proximal to the TCR for development so this data suggests that NK cells do not require them or utilize redundant pathways. A distal signaling protein required for T-cell development, SLP-76, is also dispensable for the generation of bone marrow and splenic NK cells [27,28]. Slp-76 knock-out (KO) mice have increased numbers and percentages of NK cells (thought to be due to the homeostatic proliferation of cells in the absence of T-cells), which suggests that activating signaling pathways are not required for NK cell ($CD3^-NK1.1^+$ $NKp46^+DX5^+$) generation, but may have other consequences [27-29].

Ly49 Receptors

Ly49 receptors are NK cell specific receptors that play a critical role in helping shape NK cell substrate specificity. These receptors are only found in mice, but humans have functional orthologues known as killer immunoglobulin-like receptors (KIRs). The majority of Ly49 receptors expressed on NK cells have an inhibitory function. When an

NK cell gets activated, Ly49 receptors bind to their ligand, MHC-I, to inhibit NK cell function. These receptors mediate “missing-self recognition”, which as pointed out earlier is a major function of NK cells.

Ly49 receptors are homodimeric type II C-lectin proteins. They are located in the NK cell gene cluster on mouse chromosome 6 and encoded by the *Klra* gene [30]. There are 15 Ly49 receptor genes in the C57BL/6 mouse strain, which is the most common mouse strain for NK cell research, but not all are expressed as protein on the cell surface [30,31]. Since, Ly49 receptors bind to specific haplotypes of MHC-I, thus not all Ly49 receptors expressed in a specific mouse strain bind to “self” MHC [32]**.** Other commonly studied mouse strains have different numbers and genetic variants of Ly49 receptors, so as a family Ly49 receptors are polymorphic and polygenic. The receptors are germline encoded and do not undergo DNA re-arrangement to generate specificity. Nonetheless, NK cells utilize the Ly49 receptor repertoire to help dictate function. Evolutionarily, this system of Ly49 polymorphism and polygeneicity was probably pathogen-driven. Because the immune system has evolved over time to survive despite debilitating infectious agents and each species fails to express a single specificity-expressing cell to fight off foreign antigens, each species had to create a combinatorial receptor repertoire to survive.

The Ly49 family of receptors possesses inhibitory and activating receptors, and is critical in “missing-self recognition” [8,33,34]. Two Ly49 receptors (Ly49d,h) encode activating receptors and the remainder are inhibitory (Ly49q,e,f,I,g,c,a) [35]. Activating receptors Ly49D and Ly49H signal through the adaptor protein DAP12, which has an immunoreceptor tyrosine-based activation motif (ITAM) and initiates a phosphorylation cascade of downstream tyrosine kinases [36,37]. Inhibitory Ly49 receptors possess

immunoreceptor tyrosine inhibitory motifs (ITIMs) in their cytoplasmic domains. ITIMs are phosphorylated following NK cell activation, and recruit SH2-domain containing protein tyrosine phosphatases such as SHP1 to shut down activation-derived signals [38].

Ly49 receptor expression commences at the iNK cell stage; receptors are acquired in a particular order until maturation is complete around 8 weeks of age. From that point on, Ly49 receptor expression is fixed; further expression is not acquired or lost from this point on [17,39]. Ly49s are expressed heterogeneously and in a seemingly stochastic manner on an NK cell's surface [40]. NK cells express up to 6 different Ly49 receptors at the same time, but most range in the 2-3 overlapping receptors per cell [40]. Lastly, Ly49s are not the only inhibitory receptors expressed on NK cells, just as Ly49D and Ly49H are not the only activating receptors. Heterodimers of CD94 and NKG2 (CD94/NKG2) can recognize non-classical class I molecule Qa1, and can deliver inhibitory or activating signals depending on which NKG2 chain attaches [41].

Ly49 receptors are regulated both at the transcriptional and epigenetic level (Appendix I). The promoter regions of Ly49 genes are active and *cis*-acting transcription factors are responsible for protein expression. Ly49 genes have two promoter regions, but lack any type of enhancer elements [42]. Pro-2 is the promoter region directly upstream of transcriptional start site in adult NK cells. In 2001, it was found that that Ly49G contained a further upstream regulatory promoter region that was only active in immature bone marrow NK cells. This region was coined Pro-1 and is bi-directional, meaning it transcribes downstream off one strand of DNA and transcribes in reverse off of the opposite strand. Forward transcription off the Pro-1 promoter correlated with gene expression, while reverse transcription resulted in no Ly49G protein expression [43]. A Pro-1element was also found to be important for Ly49A [44].

This probabilistic switch contains many transcription factor-binding sites that were discovered to overlap (e.g. Ets-1, NFκB, AML-1, C/EBP). Simultaneous disruption of both the Ets-1 (top strand) and NFκB (bottom strand) sites on the forward or reverse strands resulted in decreased forward and reverse transcription, which was found to be totally dependent on Ets-1. AML-1 site mutation (bottom strand) also decreased transcription in both directions. Interestingly, NFκB site mutation on the top strand stopped reverse transcription, and disruption of the NFκB site on the bottom strand abrogated reverse and decreased forward Ly49 transcription. This might suggest that NFκB might be crucial for reverse transcription or plays opposing roles depending on binding site location. Furthermore, of the 3 C/EBP sites on the top DNA strand, the first two may interfere with the TATA box of the promoter, so competition between C/EBP and TATA binding protein (TBP) for binding would interfere with forward transcription [45].

Anderson et al, suggest that in immature NK cells, Pro-2 chromatin is in a closed state, and the early development transcription factors mentioned above direct the probabilistic switch, Pro-1 [45]. This may remove a suppressor complex from binding to Pro-2. Consequently when Pro-2 chromatin opens, the transcription factors required for this site are able to bind [45]. Epigenetic regulation (e.g. DNA methylation, histone acetylation) has been shown to play a role at the Pro-1 and Pro-2 Ly49 promoters. KIR genes have CpG islands, where the cytosine/guanine density is higher than rest of the genome, that are highly methylated. Promoter regions of Ly49s have a limited number of CpG islands, but it is not the quantity that necessarily matters, as a single methylated CpG can be functionally important. It was found that Ly49A and Ly49C expression correlated with an un-methylated Pro-2 region; Ly49A and Ly49C non-expressing NK cells have highly methylated Pro-2 regions. Furthermore, Ly49A Pro-2 was highly

methylated in primary fetal NK cells, where the only Ly49 receptor expressed is Ly49e. Lastly DNA methylation of Ly49A Pro-2 correlated with histone acetylation. In Ly49A$^-$ EL4 cells, Ly49A Pro-2 remained un-methylated, and it had enriched histone 3 lysine-9 (H3K9) acetylation, a mark of open chromatin [46].

Although inhibitory Ly49 receptors aid in the process of dampening an NK cell response, their expression on an NK cell is required for optimal NK cell activation as well. Ly49 receptors play an important role in the education of NK cells---the education that teaches them "self" from "non-self" or "altered-self". This education process also dictates the threshold of signaling an NK cell must achieve to be activated or homeostatically dormant. While NK cell education will be discussed in more detail in a later section, there is both positive and negative evidence to suggest that expression of self-MHC-I binding inhibitory receptors are critical in the clearance of cancer. For example, self-MHC-I binding KIR receptors (the human equivalent of Ly49 receptors) are detrimental in human neuroblastoma. Neuroblastoma cells express high levels of MHC-I, so self-KIRs are inhibited from killing the tumor. KIR blockade is being tested as a viable therapeutic option for neuroblastoma patients. On the other hand, mice lacking Ly49 receptors and NKG2/CD94 (NKC KD mice) have accelerated solid tumor growth. These mice undergo MHC-I specific immunoediting, in which both H-2K^b and H-2D^b expression is decreased in tumors of NKC KD mice, but other markers of stress ligands, such as Rae-1 and Mult1 (NKG2D ligands) are unaffected [47].

Ly49 receptors do not only play an important part in recognizing malignant cells, but they can also be important in the NK cell mediated clearance of virally infected cells. In C57BL/6 mice, NK cells express Ly49H. This activating Ly49 does not recognize MHC-I. Instead its ligand is the m157 protein of mouse cytomegalovirus (MCMV).

Therefore, C57BL/6 NK cells are resistant to this specific virus as their NK cells can effectively eliminate cells infected with the m157 protein. Other mouse strains that do not express Ly49H are highly susceptible to MCMV. Similar to the role of Ly49s in cancer, there are conflicting reports as to the importance of Ly49H to MCMV clearance. $Ly49H^+$ NK cells are specific for MCMV-m157, but a report by Orr, et al, provided data to support the idea that $Ly49R^-$ NK cells are responsible for the killing of m157-MCMV infected cells[48].

Killer Immunoglobulin-like Receptors

Human killer immunoglobulin-like receptors (KIRs) are the functional orthologues of Ly49 receptors. Like Ly49 receptors, human KIRs prevent NK cell mediated killing of normal cells, whereas distressed cells with decreased or absent MHC-I are susceptible to NK cell killing. KIRs are functionally similar to Ly49 receptors, yet instead of being C-type lectin proteins, KIRs possess extracellular immunoglobulin domains and have specificity towards HLA allotypes [49].

KIRs are polymorphic. There are multiple inhibitory and activating KIRs in the human population. Inhibitory KIRs have long cytoplasmic domains containing two ITIMs (KIR-L) and two extracellular immunoglobulin (Ig) domains. Activating KIRs (KIR-S) have shorter cytoplasmic domains, which lack ITIMs and instead pair with DAP12, the activating ITAM-containing adaptor protein. Generally, for almost every KIR-L there is a KIR-S. Although most of the ligands have been determined, there is still promiscuity in KIR binding and some ligands have not been fully defined. KIRs are up-regulated during human NK cell development and are expressed in a variegated pattern.

KIRs are also transcriptionally regulated similarly to Ly49 receptors. Like the Ly49s, there is a high level of promoter sequence homology between individual KIRs. KIRs also possess two upstream promoter regions, one that is important for stable KIR expression in mature NK cells and another that is a bi-directional promoter, like the Ly49 Pro-1. This acts as a transcriptional switch for KIR expression. A major difference between KIRs and Ly49s is the location of the bi-directional switch. For KIRs, it is the proximal promoter rather than the distal. The regulation of KIR expression from the proximal and distal regions might be very different than that of Ly49 receptors, as simultaneous activity of the distal sense strand and the proximal anti-sense strand may lead to the production of a dsRNA. This would be impossible for Ly49 receptors because of the organization of the promoter regions. It has been suggested that this dsRNA may be important for silencing the KIR locus through DNA methylation, as silent KIRs are methylated and expressed KIRs are hypo-methylated [50].

The modulation of KIR expression in treating hematological malignancies has been of interest to clinicians in the last decade, and there have been promising results in the use of KIR therapy in acute myeloid leukemia (AML) and multiple myeloma (MM). In particular, KIR mismatch following or in the absence of allo-hematopoietic stem cell transplantation has resulted in the clearance of tumor cells. The KIRs do not find or recognize their MHC-I ligand, and therefore, are generally uninhibited. These NK cells thus kill the cancer cells. This therapy, as well as KIR-blockade pharmacologics, is currently in clinical trials. Furthermore, NK cell therapies that up-regulate NK cell activation markers are being developed for NK cell based treatment options [49].

Natural Killer Cell Activation

Activating Receptors and Signaling

NK cells eliminate “non-self” or “altered self”, such as malignant tumors or virally infected cells, by cell-mediated cytotoxicity or through the controlled production of inflammatory cytokines. Cell-mediated cytotoxicity is the exocytosis of cytotoxic granules that contain perforin, granzymes, and Fas ligand. The most common inflammatory cytokine produced by NK cells is interferon-gamma (IFNγ), but NK cells can also secrete tumor necrosis factor alpha (TNFα) and granulocyte macrophage stimulating colony factor (GM-CSF). These effector functions are initiated through a process known as NK cell activation. NK cells are primarily activated through the engagement of activation receptors on their cell surface. Similar to TCR activation, this initiates a phosphorylation cascade of kinases and adaptor molecules, leading to the activation of distal secondary messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). NK cells can also be primed for activation or activated directly by cytokines, which will only be briefly discussed in this introduction [51].

NK cells uniformly express a plethora of activating receptors and all signal through diverse, but well-characterized signaling pathways. Multiple receptors signal through ITAMs, such as NK1.1, FcRγIIIa (CD16), Ly49H, Ly49D and the natural cytotoxicity receptors (NKp46, NKp30, NKp44). There are also receptors that signal through non-ITAMs, such as NKG2D, 2B4, CD2, and DNAM-1. NK1.1 and CD16 utilize the ITAM-bearing FCεRIγ adaptor. Natural cytotoxicity receptors, such as NKp46, use CD3ζ chain ITAMs, and Ly49D/H link up with the adaptor DAP12 for phosphorylation of the single ITAM motif. Upon engagement, ITAMs are tyrosine phosphorylated on their

cytoplasmic tails by Src family kinases. This recruits Syk family kinases, such as ZAP-70, which phosphorylate the downstream molecules LAT1/LAT2. LAT1/LAT2 bind to the signaling adaptor SLP-76 and SLP-76 phosphorylation leads to the cleavage of phospholipase C gamma (PLCγ) into DAG and IP_3 [51,52].

The two major non-ITAM receptors are NKG2D and CD244. NKG2D is expressed in both mice and humans and pairs with either the DAP10 or DAP12 signaling adaptor. DAP10 expresses YNIM signaling motifs on its cytoplasmic tail and DAP12 signals through ITAMs. The long form of NKG2D, NKG2D-L, associates with DAP10 in resting murine NK cells. YNIM motifs are phosphorylated by Src family kinases upon NKG2D ligation with cognate ligand. Unlike ITAM phosphorylation, which activates Syk kinases, NKG2D may directly recruit Grb2, Vav1, or PI3K [53,54]. A shorter, spliced alternative of NKG2D, NKG2D-s, associates with DAP10 and DAP12. In fact following IL-2 or polyI:C activation of NK cells, NKG2D-s is the primary transcribed isoform of NKG2D, therefore both the ITAM-mediated signaling and PI3K signaling occur [55]. NKG2D ligands are expressed at low levels in both mice and humans in different tissues, but they are heavily up-regulated following viral infection and cellular transformation as a result of cellular stress. The major ligands are the Rae1 family of proteins, MULT-1 and MIC A/C. These ligands resemble MHC-I proteins in their structure [56].

CD244, also known as 2B4, is expressed on all NK cells and associates with the SLAM-associated protein (SAP) adaptor. 2B4 binds to CD48, which is expressed on all hematopoietic cells in mice and humans. Upon activation of 2B4, Src famiy kinases phosphorylate immunoreceptor tyrosine-based switch motifs (ITSM) that recruit SAP and the Src kinase Fyn. Vav1 and PLCγ2 are also phosphorylated downstream of 2B4

activation. While it is thought that 2B4 ligation on its own is sufficient for activation, evidence has also suggested that 2B4 acts as a co-receptor. For example, NKG2D and 2B4 may operate together to induce cytotoxicity against target cells [57]. Additionally, 2B4 may have different forms, one being inhibitory and one being activating. In 1999, evidence emerged that a short form, 2B4-S, mediated lysis of p815 tumor cells while a long form, 2B4-L, led to the inhibition of that killing [58].

Distal Secondary Messengers: DAG & IP_3

Although initially described as being dispensable for NK cell activation in IL-2-expanded splenocyte cultures [27,59], subsequent studies have shown that SH2 domain containing leukocyte protein of 76kDa, SLP-76, is indeed critical for signal transduction downstream of multiple NK cell activating receptors [29,60]. SLP-76 was discovered to be a substrate of the tyrosine kinase ZAP-70 [61,62] and, furthermore, was found to be a critical signaling protein for T cell development, education, and activation [63,64]. It is an adaptor protein with multiple domains specific for protein kinase or membrane docking proteins. In particular, PLCγ (NK cells preferentially use PLCγ2 over PLCγ1[52]**)** binds to the proline rich region of SLP-76, and PLCγ activation results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and IP_3. Disruption of SLP-76 in T cells results in reduced IP_3 generation and calcium (Ca^{2+}) flux. Correspondingly SLP-76 deficient T cells have decreased ERK and NFAT activation due to the loss of DAG and IP_3-mediated signaling [64].

DAG is a direct activator of protein kinase C (PKC). Most work on DAG has been done on T cells or mast cells, but one major study of DAG and NK cells was recently published from our lab [65]. For example, mice with enhanced DAG signaling (DGKζ KO

mice) have increased T cell proliferation following TCR stimulation, are more likely to respond against LCMV infection, and have increased markers of activation [66]. DAG is important for the polarization of cytotoxic T cell microtubule-organizing center, which organizes the immune synapse, and is also critical for T cell cytotoxicity against target cells [67]. Recently, NK cells with increased DAG signaling (from DGKζ KO mice) were found to be hyper-functional, showing increased degranulation and IFNγ production following NK cell stimulation, and enhanced killing of tumor cells via an ERK-dependent mechanism [65].

IP_3, the other signaling arm of PLCγ pathway, is the second messenger required for sustained Ca^{2+} mobilization from the extracellular environment into cellular cytoplasm. IP_3 binds to IP_3R on the membrane of the endoplasmic reticulum (ER), which signals the ER to empty its intracellular stores of Ca^{2+}into the cytoplasm. This efflux of Ca^{2+}into the cytoplasm causes the stromal interacting molecules 1 & 2 (STIM1/STIM2), which act as ER Ca^{2+}sensors, to oligomerize and bind to the cellular membrane ORAI proteins, or what is referred to as the CRAC channel. It is through the CRAC channel, which is highly Ca^{2+}specific, that Ca^{2+} can mobilize into the cell very transiently. It is the continual stimulation of this pathway (which often acts as a positive feed-forward loop) that accounts for sustained Ca^{2+} signaling. Ca^{2+} signaling via this manner is also known as store-operated Ca^{2+} entry (SOCE), and is very important for maintaining local Ca^{2+} domains, activating a host of transcription factors, specifically NFAT via calcineurin [68-71]. NK cells mediate SOCE following stimulation of a variety of NK cell activating receptors [72], but Ca^{2+} signaling has only been explored lightly in NK cells and is still considered unexplored territory.

Functional Outcomes & Flexibility of NK cell Responses

NK cells, like their T-cell adaptive counterparts, are critical in the clearance of "non-self" or "altered-self", and they respond to such target cells through the intracellular signaling pathways downstream of their activating and inhibitory receptors. But critical questions remain in the NK cell field about how exactly this response is achieved, especially with the diverse assortment of both inhibitory and activating receptors an NK cell can express. T-cells learn their peripheral response in the thymus, an organ dedicated to their education. Unlike T-cells, NK cells do not develop in an organ solely devoted to fostering NK cell education or development. NK cells develop in the bone marrow, and over time, acquire their activating and inhibitory receptors, leaving it unclear how NK cell tolerance and NK cell effector response to targets are achieved.

There are three principal theories for how NK cells are educated: licensing, disarming, and tuning (rheostat model). Licensing proposes that NK cells acquire functional competence through the acquisition of inhibitory receptors. The disarming hypothesis proposes that lack of MHC-I expression renders NK cells anergic or hypo-responsive. In 2005, research from Wayne Yokoyama's laboratory found that NK cells expressing a self-binding Ly49 receptor (licensed) are more responsive to activating receptor stimulation than NK cells not expressing a self-binding Ly49 (unlicensed). Ly49C+ NK cells taken from a C57BL/6 animal ($H2^b$) readily produce more IFNγ than Ly49A+ NK cells from the same mouse following NK1.1 stimulation [33]. Ly49C and Ly49A bind $H2^b$ and $H2^d$ respectively. The licensing hypothesis has been challenged. For example, in 2010 a *Nature Immunology* paper from Lewis Lanier's lab demonstrated that unlicensed $Ly49H^+$ (which recognize the m157 protein of MCMV) NK cells are responsible for the elimination of m157-expressing target cells. These NK cells secrete

more IFNγ and a larger percentage of these cells degranulate compared to their licensed Ly49H$^+$ counterparts [73].

Lastly, the tuning hypothesis or rheostat model suggests that NK cells balance input from signals downstream of inhibitory and activating stimuli to drive a graduated response. Rejecting the licensing hypothesis, it has been shown that NK cells with no inhibitory receptors are *still* responsive to activation stimuli. Research also shows that NK cells expressing zero, one, two, and three inhibitory receptors show a gradual increase in responsiveness to stimulation the more receptors are expressed [74-76]. In further support of the tuning hypothesis, NK cells from mice deficient in MHC-I, deficient in structural components of the MHC-I protein, or are deficient for ITSM-binding phosphatases are hypo-responsive[77-81]. These NK cells are unable to signal through inhibitory receptors, and therefore, this phenomenon rejects the licensing hypothesis. Instead it favors the tuning hypothesis, which is quickly becoming more popular in the NK cell field as more is learned about activation signaling and inhibitory receptor development.

Use of NK Cells for Therapeutic Purposes

It is a very exciting time to be studying NK cell signaling, as clinicians are beginning to appreciate NK cells as a cellular immunotherapeutic option. In the last twenty years, the idea of harnessing and manipulating NK cells for therapeutic purposes (specifically in the fight against cancer) has stimulated attention. Researchers like myself predict NK cell immunotherapy to be the next frontier for cancer research. NK cells are a unique choice of cells to use for cancer-based immunotherapies because of

their ability to kill in an unprimed and non-MHC restricted state. NK cells directly kill tumor cells or kill via indirect effects, such as cytokine-priming $CD8^+$ T cells or presenting antigen to dendritic cells. Tumors not only develop mechanisms to avoid T cell recognition (down regulation of MHC-I) but can even escape NK cell recognition as well. The loss of adhesion molecules, co-stimulatory molecules, the up-regulation of MHC-I, and the expression of immunosuppressive cytokines all decrease NK cell effectiveness. This could be a caveat, but because NK cells have the ability to kill almost on demand, their use therapeutically can be manipulated in various ways for patient benefit. Currently, three major approaches are being tested for clinical purposes. They include 1) the use of cytokine-expanded/cytokine-activated autologous NK cells, 2) the adoptive transfer of KIR-mismatched allogeneic NK cells, and 3) the genetic manipulation of donor NK cells for improved NK cell function [82].

Early studies have focused on attempts to enhance autologous NK cell function through increased NK cell killing and NK cell proliferation. This was achieved through the systemic administration of NK cell activating cytokines, such as IL-2, IL-15, IL-12, IL-21, IL-18, and type I interferons [82-86]. IL-15 and IL-2 were first picks for NK cell cytokine expansion, as these are required for survival and activation respectively. These cytokine-activated lymphokine activated killer cells (LAKs) have enhanced cytotoxicity and increased cytokine production against target cell lines *in vitro,* but this has generally failed to limit tumor growth in cancer patients. Additionally, IL-2 administration to patients caused severe toxicity and induced vascular leak syndrome, dampening this direct method of treatment. IL-2 expanded LAKs were more effective against cancer when expanded *ex vivo* and adoptively transferred or when stimulated with other cytokines. Of course, toxicity problems and cytokine-induced NK cell apoptosis remain a

concern with systemic cytokine administration for autologous expansion and activation [82].

Allogeneic NK cell transplantation with KIR-mismatch has shown promise as an effective therapy for AML. While KIR-mismatched NK cells offer major benefits to patients by killing target cancer cells of mismatched MHC-I, these cells are allogeneic and will eventually be rejected by the host immune system [87,88,89]. Ideally, autologous NK cells with manipulated KIR repertoire (to support KIR-mismatch) would be feasible for long-term survival and extended killing potential. Ideas on the feasibility of this will be elaborated on in Chapter IV. Not only are primary cells being used, but also interest has pushed researchers towards allogeneic NK cell lines to execute tumor cell killing. For example, the NK-92 cell line is the first and only cell line to be entered in a clinical trial for NK cell tumor immunotherapy [90,91].

Lastly, genetic manipulation of NK cells for therapeutic purposes is less established, but creating headway in therapeutic options. NK-92 cells need IL-2 to survive and divide, but IL-2 has proved to be problematic for many reasons listed previously. To circumvent some problems, some researchers transduced NK-92 cells with the IL-2 gene to encourage survival in the absence of exogenously delivered cytokine [92,93]. Cells were able to survive and thrive for over 5 months without exogenous cytokine. This has also been shown in primary NK cells with mb-IL-15 [94]. Not only can cell lines be genetically manipulated, but primary NK cells may also be used as a vehicle for retroviral or lentiviral constructs. This leads us to the potential use of NK cell chimeric antigen receptors (CARs). Chimeric antigen receptors are single chain antibody variable fragments targeted against a certain antigen. This is linked to an intracellular signaling domain, which can contain different signaling motifs—the most tested being CD3ζ

chains that contain ITAMs. CAR-expressing T cells have been quite successful in clinical trials for the treatment of hematological malignancies, specifically against B cell lymphomas and leukemias. Many more malignancies expressing different target cancer antigens are being currently tested, but little is known about NK cell CARs. Based on pre-clinical studies of CAR-NK cells, it is predicted that they could provide targeted killing and avoid major complications of current CAR-T cell therapy [95]. This will be elaborated on in the discussion section of the dissertation.

Structure of the Thesis

While NK cell biology has been of interest in recent years due to the similar role NK cells play to $CD8^+$ effector T cells in cancer and viral immunity, fundamental gaps in our understanding about how NK cells develop, become educated, and function still remain. Signaling downstream of activating receptors is critical for NK cell effector functions, like degranulation and inflammatory cytokine production. Furthermore, it has been shown that disruption of proteins proximal to activating receptors on an NK cell's surface results in skewed NK cell development, specifically in regards to inhibitory receptor repertoire [52,96]. While NK cell biologists tend to separate the concepts of NK cell development, education, and activation, these processes can all be impacted by signals received through activating receptors. Therefore NK cell activation drives development, education and functional effector-like consequences, but what type of signals contributes to each of these processes is still unclear. This thesis aims to address the major question: What activating signals and/or signaling proteins impact NK cell development, education and function? Work presented in this thesis will clarify the role of NK cell

activation in shaping the NK cell inhibitory receptor repertoire during early NK cell development, which is critical for NK cell education and function. Additionally, this thesis shows that not all signaling pathways downstream of activating receptors are required for NK cell effector functions; there are detailed nuances in the signaling pathways that lead to different effector outcomes.

Inhibitory receptor signaling blunts NK cell activation. There is also data to suggest that expression of an inhibitory receptor that binds self-MHC-I enhances the functional capability of an NK cell (licensing). It is clear from the literature that the expression of inhibitory receptors might be important for developing NK cell education. The acquisition of self-binding MHC I inhibitory receptors may help developing NK cells mature, learn their signaling threshold, and function properly in the periphery (outside of the bone marrow). In fact, expression of Ly49/KIR receptors, major classes of inhibitory receptors in mice and humans, is critical for function of NK cells and recognition of target cells. NK cells express a diversified repertoire of these receptors, but how does a developing NK cell know how many or what types of receptors to express in order to functional in the periphery? In Chapter II, I show that NK cells receive activating signals during development, which are important for the transcription of Ly49 receptors on mouse NK cells and KIRs on human NKs. Activation through SLP-76 is critical for this process, because SLP-76 dependent signaling actively determines the direction of transcription of a bi-directional promoter of Ly49 receptor genes in immature NK cells. This suggests that during early development in the bone marrow, NK cells are activated through activating receptors and induce transcription of Ly49 or KIR genes, which allows for expression of these inhibitory receptors on an NK cell's surface, thus aiding in the creation of diverse, mature NK cells.

Activation through SLP-76 not only impacts early NK cell development, but it leads to two major signaling pathways downstream: DAG and IP_3. Research generated by another member of my lab elegantly demonstrated an important role for DAG signals, through ERK activation, in NK cell degranulation, cytotoxic killing, and IFNγ production [65]. These studies were never completely isolated from the other arm of the SLP-76 signaling bi-furcation-- IP_3 mediated signaling--which results in sustained Ca^{2+} entry. The NK cell literature on roles for sustained Ca^{2+} signaling in NK cell function is also lacking. NK cell studies have been performed in human patients with STIM1 or ORAI1 mutations, which abolish sustained Ca^{2+} entry [97,98]. These patients have problems with NK cell degranulation, target cell killing, and cytokine production but the mechanism behind this finding has yet to be revealed. The principal question I aimed to answer in Chapter III is: how does sustained Ca^{2+} signaling impact NK cell function? In Chapter III, I aim to address this question by using *in vitro* and genetic models of blocking sustained Ca^{2+}entry in NK cells and assessing their functional outcome. At odds with the data published on human patients, I show that murine NK cells are able to retain degranulation and cell-mediated killing functions in the absence of sustained Ca^{2+} entry. These effector functions might be carried out via DAG-mediated signals, perhaps through PKC activation, in the absence of sustained Ca^{2+} entry. The production of the inflammatory cytokine IFNγ was significantly reduced in the absence of sustained Ca^{2+} entry, and this was found to be due to loss of NFAT activation.

In the final chapter, Chapter IV, I discuss how my findings add to and impact NK cell biology. I propose ideas for future experiments to answer any remaining and new biological questions that my research has generated. These emerging questions will require more experimental effort to fully understand how activation-signaling influences

NK cell development and function. This work will be critical for the utilization of NK cells in clinical scenarios (e.g.adoptive NK cell therapies) and other types of NK cell-specific immunotherapy and drug targeting.

CHAPTER II: Activating receptor signals drive receptor diversity in developing natural killer cells

Work in this chapter has been published in PLOS Biology:

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Introduction

Natural killer (NK) cells are innate lymphocytes that play an important role in defense against viral infections and tumor clearance. NK cells express a wide variety of inhibitory and activating receptors, whose downstream signals integrate to dictate a functional response. For example, the Ly49 family of receptors on murine NK cells plays a key role in NK cell function. Inhibitory Ly49 receptors (e.g. Ly49A, Ly49G, Ly49C, and Ly49I) recognize major histocompatibility complex class I (MHC-I) and allow NK cells to carry out "missing-self" recognition, a process that eliminates cells with abnormally down-regulated MHC-I expression due to certain types of infection or neoplastic transformation [8,99]. Also, the activating receptor Ly49H binds to cytomegalovirus (CMV)-encoded m157 protein, aiding in the clearance of CMV-infected cells. Ly49 receptors are acquired in a sequential and variegated manner during development, which yields a diverse repertoire of NK cells with various Ly49 receptor expression patterns. Since each Ly49 receptor recognizes a subset of MHC-I alleles, the Ly49 receptor expression pattern on an individual NK cell determines its target cell specificity. Thus, unlike T and B cells that create antigen-specific diversity by genetic recombination, NK cells generate ligand-specific diversity by acquiring an assortment of inhibitory and activating receptors; however, the mechanisms that regulate NK cell receptor acquisition during development

are not well understood.

NK cells commence their acquisition of Ly49 receptors during the immature NK ($CD3\varepsilon^{-}CD122^{+}NK1.1^{+}DX5^{-}$) bone marrow (BM) stage [6,17]. Ly49 receptor genes are activated in a specific order, and each receptor possesses a developmental timeframe for the initiation of expression, which is maintained for the lifetime of the NK cell. However, once this window of opportunity passes, the NK cell can never express that Ly49 receptor [100]. Ly49 receptor expression patterns are influenced by polymorphisms in the *Ly49* locus and the MHC haplotype expressed in each strain of mouse. Thus, the fraction of NK cells expressing a particular Ly49 receptor is similar within a given mouse strain. For example, ~10% and ~50% of NK cells in C57BL/6 mice express Ly49A and Ly49G2, respectively.

Ly49 receptor gene transcription is controlled by at least two distinct promoters: Pro1, which is active in immature NK cells, and Pro2, which is critical in maintaining expression in mature NK cells [43,45,46,101]. Each Ly49 receptor possesses a unique Pro1 promoter that acts as a bi-directional switch. Transcription factors bind to Pro1 on either the positive (forward) or negative (reverse) strand in a probabilistic manner, thus determining forward or reverse transcription from this promoter. Transcription of Pro1 in the forward direction leads to activation of Pro2 [43,45]. Pro2 can be regulated through DNA methylation, and forward transcription of Pro1 is thought to remove a repressor complex, allowing for acetylation of histones and/or demethylation of DNA at the Pro2 promoter [46,102]. This promotes *Ly49* transcription in mature NK cells and the stable expression of the receptor. Pro1-mediated transcription in the reverse direction results in no Pro2 activity and therefore no Ly49 receptor expression. Thus, the proportion of NK cells

expressing a given Ly49 receptor is determined by the probability of the specific Pro1 promoter to transcribe in the forward vs. reverse direction.

Two important factors that shape the NK cell inhibitory Ly49 receptor profile are the MHC haplotype and the MHC-binding specificities of the inhibitory receptors themselves [103]. However, the mechanism by which inhibitory receptor specificity and MHC haplotype regulate NK cell receptor acquisition is unclear, especially since inhibitory receptors block (through recruitment of phosphatases such as SHP-1 and SHIP) rather than transmit signals to the NK cell. Mice with NK cells lacking SHP-1 or SHIP display increased proportions of Ly49 receptor-expressing NK cells [80,81,104,105], suggesting that inhibitory receptor-induced phosphatase activity attenuates Ly49 receptor acquisition. To explain how this might work, we hypothesized that activating receptor signals are the driving force behind inhibitory receptor acquisition. We propose that MHC-I influences the acquisition of inhibitory receptors by blocking this activating signal, which impedes expression of additional inhibitory receptors. This notion is difficult to test by deleting specific activating receptors, as NK cells express numerous activating receptors that utilize various signaling modules. Instead, we took advantage of mice lacking the adaptor molecule SH2 domain-containing leukocyte protein-76 (SLP-76). Although initially described as being dispensable for NK cell activation in IL-2-expanded splenocyte cultures [27,59], subsequent studies have shown that SLP-76 is indeed critical for signal transduction downstream of multiple NK cell activating receptors [27,29,60]. In this study, we report that activating signals downstream of SLP-76 drive the stable expression of a subset of Ly49 receptors by increasing the probability of forward Ly49 transcription from the bidirectional Pro1 promoter. Our data support a model where competing activating and inhibitory receptor signals determine the probability of Ly49

receptor expression, which ultimately shapes an appropriate inhibitory receptor repertoire during NK cell development.

Results

SLP-76-derived signals are required for optimal induction of Ly49 receptor and KIR expression by developing NK cells.

To test whether NK cell effector function following activation is dependent on SLP-76, wild-type (WT) and SLP-76 knockout (KO) NK cells were stimulated through three distinct activating receptor families (ITAM-dependent: NK1.1, Ly49H; co-stimulatory-like: NKG2D; SAP-dependent: 2B4). We found that the baseline expression level (MFI) of all activating receptors on SLP-76 KO NK cells was comparable to controls (Fig. 2.1A). Upon stimulation with antibodies against the various activating receptors, SLP-76 KO NK cells were significantly defective in degranulation (as measured by surface CD107a) compared to WT NK cells (Fig. 2.1B-C).

Given that SLP-76-mediated signals were critical for NK cell function downstream of multiple activating receptors, we next determined if SLP-76-mediated signals impacted NK cell development. The percentage and absolute number of splenic NK cells in SLP-76 KO mice were higher than WT littermate controls (Fig. 2.1D-E). This increase in NK cells was most likely a consequence of the increased availability of homeostatic cytokines due to the lack of competing T cells in SLP-76 KO mice. We also examined expression of SLP-76 in all stages of NK cell development and found that SLP-76 mRNA was highly expressed in both the early and late stages of NK cell development (Fig. 2.1F). Next, WT and SLP-76 KO NK cells were analyzed for expression of activating and

inhibitory receptors, including the Ly49 family of receptors. A strikingly significant decrease in Ly49 receptor-expressing splenic and BM NK cells was observed in SLP-76 KO mice. This included both inhibitory (Ly49A, Ly49G2, Ly49C, Ly49I) and activating (Ly49D and Ly49H) family members (Fig. 2.1G-H). The earliest acquired receptors, Ly49A and Ly49G2, were most affected by the loss of SLP-76 (~90% reduction) compared to Ly49C and Ly49I (~50% reduction). Not all MHC-I binding inhibitory receptors were reduced, as the proportion of CD94/NKG2A expressing NK cells was unaltered in SLP-76 KO mice (Fig. 2.1G).

Since Ly49 receptor acquisition can also occur at later stages of NK cell development, a maturation defect in SLP-76 KO NK cells could be responsible for the phenotype observed. To test this possibility, we assessed splenic NK cell maturation using the cell surface markers CD27 and CD11b [18] and found that NK cells were more developmentally mature in SLP-76 KO mice (increased proportion of $CD27^{-}CD11b^{+}$ NK cells (Fig. 2.1I). Moreover, Ly49 receptor expression by SLP-76 KO NK cells was decreased at every stage of splenic maturation compared to WT controls (Fig 2.2). These data show that an NK cell maturation defect was not responsible for the reduction in Ly49 receptor expression.

Killer immunoglobulin-like (KIR) receptors on human NK cells are functional orthologs of Ly49 receptors in mice. To test whether SLP-76-derived signals also contributed to KIR acquisition, we differentiated human NK cells from $CD34^{+}$ umbilical cord blood cells transduced with SLP-76 or scrambled shRNA *in vitro* for 21 days. SLP-76 shRNA transduction resulted in a decrease in SLP-76 expression, which correlated with a reduced ability to activate KIR gene expression (KIR cocktail of KIR2DL1, KIR2DL2/DL3, KIR3DL1) but not CD56 or NKp46 (Fig. 2.3). These data along with the

Ly49 receptor acquisition defect in SLP-76 KO NK cells suggest that NK cells rely on SLP-76-dependent activation signals for a MHC-I binding inhibitory receptor acquisition.

The loss of Ly49 receptor expression in SLP-76 KO NK cells is MHC-I haplotype-independent.

To obtain a more global picture of the Ly49 receptor repertoire of SLP-76 KO NK cells, we examined the co-expression pattern of inhibitory Ly49 receptors. This analysis revealed that SLP-76 KO mice have an expanded population of Ly49 receptor-negative NK cells. Although the proportion of NK cells that express Ly49C or Ly49I was reduced in SLP-76 KO mice (Fig 2.1G), there was relative preservation of Ly49C and Ly49I single-positive NK cells that did not co-express other Ly49 inhibitory receptors (Fig. 2.4A). Ly49C and Ly49I bind to MHC-I (H2-K^b) in C57BL/6 mice, as opposed to Ly49A and Ly49G2 that bind H-$2D^d$ and do not possess ligands in C57BL/6 mice. Since MHC-I interactions with Ly49 receptors shape the Ly49 repertoire [74,104-106], we wondered whether the relative preservation of Ly49C and Ly49I expression could be related to their ability to bind MHC-I in C57BL/6 mice. To test this, we bred SLP-76 KO mice to the B10.D2 mouse strain. If ligand binding were responsible for the preservation of Ly49 receptor expression, the proportion of $Ly49A^+$ and $Ly49G2^+$ NK cells would be relatively preserved in SLP-76 KO.B10.D2 mice, as B10.D2 mice express H-$2D^d$. However, we found that SLP-76 KO-B10.D2 NK cells also displayed a similarly defective Ly49 receptor repertoire as compared to SLP-76 KO NK cells on a H-2^b background (Fig. 2.4B-C). These data suggest that the Ly49 receptor repertoire defect in SLP-76 KO NK cells is independent of MHC-I haplotype.

SLP-76-derived signals regulate some, but not all, Ly49 receptors in an NK cell-intrinsic manner.

As SLP-76 is expressed in almost all hematopoietic cells, SLP-76 KO mice harbor defects in multiple hematopoietic lineages [107]. Although we predicted that SLP-76-derived signals controlled Ly49 receptor acquisition in an NK cell-intrinsic manner, it was still possible that the defects arose secondary to cell-extrinsic effects. To address this, we generated mixed BM chimeric mice using BM from congenically disparate WT and SLP-76 KO mice mixed at a 2:1 ratio. Ten to twelve weeks after reconstitution, although some variability was seen, the contribution of SLP-76 KO bone marrow to non-T cell/non-NK cells compared to NK cells was similar, suggesting that there was no significant advantage or disadvantage of SLP-76 deficiency in NK cell development (Fig. 2.5A). Consistent with our hypothesis, we found that the proportion of NK cells expressing Ly49A, Ly49G2, and Ly49I was decreased in SLP-76 KO BM compared to WT BM-derived NK cells (Fig. 2.5B). However, no differences in the proportion of Ly49C, Ly49D, and Ly49H expressing NK cells was observed between SLP-76 KO BM and WT BM-derived NK cells (Fig. 2.5C). Thus, although a subset of Ly49 receptors (Ly49A, G2, and I) was regulated in an NK cell-intrinsic manner, Ly49C, Ly49D, and Ly49H were controlled by a SLP-76-dependent, NK cell-extrinsic mechanism.

It has been published that mice deficient in MHC-I or inhibitory receptor signaling harbor increased proportions of Ly49-expressing NK cells [80,81,104,105]. We hypothesized that the proportion of Ly49 receptor-expressing NK cells is increased in such mice because activation signals are unopposed by MHC-binding inhibitory receptors during NK cell development. The NK cell-intrinsic regulation of some but not all Ly49 receptors provided us with an opportunity to test this hypothesis, as we would predict that only NK

cells expressing Ly49 receptors regulated by an NK cell-intrinsic mechanism would be increased in MHC-I-deficient mice. We examined the Ly49 receptor repertoire of MHC-I-deficient β2m KO mice and observed an increase in the proportion of NK cells expressing Ly49 receptors that are regulated in a cell-intrinsic manner (Ly49A, Ly49G2, and Ly49I). In contrast, the proportion of NK cells expressing Ly49 receptors regulated in an NK cell-extrinsic manner (Ly49C, Ly49D, and Ly49H) were the same or reduced in β2m KO mice (Fig. 2.5D). These findings suggest that the lack of inhibitory ligands (MHC-I), presumably leading to more NK cell activation, results in an increased chance of NK cells expressing cell-intrinsic Ly49 receptors.

Distinct signaling pathways upstream of SLP-76 differentially contribute to Ly49 receptor acquisition by NK cells.

In NK cells, SLP-76 can be recruited to the membrane by two independent proximal signaling complexes: one involving LAT family members (LAT1/LAT2) and the other involving ADAP [27,108]. As both SLP-76 signaling complexes are important for NK cell function downstream of activating receptors, we predicted that both LAT1/LAT2 and ADAP proteins would contribute to Ly49 receptor acquisition by NK cells. Surprisingly, we found that these SLP-76 signaling complexes differentially contributed to Ly49 receptor acquisition. A significantly decreased proportion of NK cells expressing Ly49A and Ly49I was observed in LAT1/LAT2 DKO but not in ADAP KO mice. Conversely, a significantly smaller proportion of NK cells expressing Ly49G2 was observed in ADAP KO but not LAT1/LAT2 DKO mice (Fig. 2.6A). The LAT1/LAT2/ADAP TKO mice displayed decreased proportions of all three Ly49 receptors similar to the SLP-76 KO NK

cells. The upstream ADAP and LAT pathways also differently affected NK cell-extrinsic Ly49 receptors. Ly49C was primarily driven by LAT1/LAT2-dependent signals while Ly49D and Ly49H utilized both pathways for their expression (Fig. 2.6B). These data point to a differential influence of SLP-76 upstream signaling pathways on Ly49 receptor induction during NK cell development.

SLP-76 regulates the probabilistic switch function of the Ly49G Pro1 promoter.

To test whether the reduced frequency of Ly49 receptor-expressing NK cells was due to decreased transcription, we quantified mRNA transcripts of an NK cell-intrinsic Ly49 receptor at early and late stages of NK cell development in WT and SLP-76 KO mice. We found that Ly49G2 mRNA transcripts were reduced in SLP-76 KO immature (iNK) and mature (mNK) BM subsets compared to WT controls (Fig. 2.7A). Ly49G was chosen as a model gene since this receptor is expressed on half of NK cells.

Ly49 receptor gene transcription is driven off the Pro1 promoter region in immature NK cells and the Pro2 region during maturity [43,45,46,101]. Transcription factors can bind to Pro1 on either the positive (forward) or negative strand (reverse) in a probabilistic manner, and this determines the directionality of transcription from this promoter. Forward transcription allows for stable expression of that Ly49 receptor in mature NK cells while reverse transcription leads to no Ly49 expression. To test whether SLP-76-mediated signaling affected transcription from the Pro1 promoter, forward and reverse transcripts from the Ly49G Pro1 promoter were examined. As expected, compared to WT NK cells, $DX5^-$ (BM iNK cells) and $DX5^+$ (BM mNK cells) SLP-76 KO NK cells expressed significantly reduced levels of Ly49G Pro1 forward transcripts (Fig.

2.7B). However, we also surprisingly observed that SLP-76 KO NK cells expressed increased Ly49G Pro1 reverse transcripts in iNK and mNK BM subsets compared to WT NK cells (Fig. 2.7B). These data suggest that SLP-76-mediated signaling affects Ly49 receptor acquisition in developing NK cells by promoting Pro1 forward over reverse transcription, thereby increasing the probability of NK cells to express a given Ly49 receptor.

We further investigated the accessibility of chromatin at Ly49G Pro1 and Pro2 in WT and SLP-76 KO NK cells, by performing a chromatin immunoprecipitation for histone 3 lysine 9 acetylation (H3K9Ac), an indicative marker for open/accessible chromatin. The Pro2 loci of Ly49G is CpG poor, and has been previously shown to be epigenetically regulated by H3K9Ac [109]. Based on the reduction in Ly49G2 expression in SLP-76 KO NK cells, we predicted there to be less H3K9Ac, and less Pro2-mediated transcription. H3K9Ac was observed at Pro2 of Ly49g but not at Ly49e (silenced in adult NK cells) in WT NK cells. Surprisingly, however, Ly49G Pro2 H3K9Ac was similar between WT and SLP-76 KO NK cells (Fig. 2.7C), perhaps suggesting that Pro2 chromatin accessibility is not sufficient to drive Ly49G expression in mature NK cells. Although the role of H3K9Ac at Pro1 is unknown, we found SLP-76 KO NK cells showed significantly decreased Ly49G Pro1 H3K9Ac compared to WT controls (Fig 2.7C). These data suggest that SLP-76-derived signals mainly control transcriptional activity at the Pro1 promoter and that epigenetic alterations at the Ly49 Pro1 loci may control receptor expression in mature splenic NK cells.

Discussion

Although NK cells are part of the innate immune system, NK cells exhibit many features of adaptive immune cells. Unlike T cells and B cells that create antigen specificity by genetic recombination, NK cells create diversity by expressing a seemingly "random" assortment of inhibitory and activating receptors The various combinations of expressed receptors generate ligand-specificity, allowing subsets of NK cells to respond, expand, and differentiate into memory-like cells in a ligand-specific manner, as well as create a diverse repertoire within the NK cell pool [110,111]. However, how NK cells determine which inhibitory receptors to express on their cell surface, during a narrow window of development, was largely unknown. The data presented in this manuscript support a model by which NK cell activation during development drives inhibitory receptor acquisition on immature NK cells. Our model proposes that during early NK cell development, NK cells are activated via interactions between activating receptors and their ligands expressed by BM stroma. Activation of NK cells results in a signaling cascade that promotes the transcription of different Ly49/KIR genes. The NK cell acquires these receptors until a self-binding inhibitory receptor is expressed on the cell surface and blocks the activating signal (Fig. 2.8).

Our model potentially explains how MHC-I interactions with NK cell inhibitory receptors shape the inhibitory receptor repertoire. The regulation of Ly49/KIR induction by activating receptor-derived signals provides a mechanism whereby developing NK cells can generate a ligand-specific receptor repertoire that appropriately recognizes missing self. Furthermore, the expression of a self-binding inhibitory receptor increases

the functional capacity of NK cells, a process known as licensing. Thus, our model suggests that strong activating signals during NK cell development increase the likelihood of developing more functionally active licensed NK cells that can carry out missing self-recognition.

SLP-76 KO mice harbored an increased fraction of the most mature subset of NK cells. However, the decreased proportion of Ly49 receptor-expressing NK cells in SLP-76 KO mice could not be explained by differences in maturation, since the proportion of NK cells expressing Ly49 receptors was significantly reduced at each stage of NK cell maturation in SLP-76 KO mice. Our analysis showed that the fraction of Ly49 receptor-expressing NK cells was similar among all maturation stages except for Ly49I, which was overrepresented in the most mature NK cell subset in WT mice. Interestingly, the proportion of Ly49I-expressing NK cells was relatively preserved compared to Ly49A or Ly49G2-expressing NK cells. This could be potentially explained by the increased maturation status of SLP-76 KO NK cells, since almost all Ly49I-positive NK cells in SLP-76 KO mice resided in the most mature NK cell subset. Alternatively, self-binding Ly49 receptors such as Ly49I might drive NK cell maturation, yielding more mature NK cells in SLP-76 KO mice. Further investigation is required to understand how SLP-76-derived signals and self-MHC-I binding Ly49 receptors impact NK cell maturation.

We were surprised to find that not all Ly49 receptor acquisition was intrinsically driven by SLP-76 signals in NK cells. While Ly49A, Ly49G2, and Ly49I were acquired in a SLP-76-dependent NK-cell intrinsic manner, Ly49C, Ly49D, and Ly49H were regulated in an NK cell-extrinsic manner. This suggests another cell type is necessary to generate a full Ly49 receptor repertoire. It is possible that myeloid lineage cells such as dendritic cells (DCs) may be responsible for NK cell activation that leads to Ly49 receptor

expression [112]. NK cells and DCs form stimulatory synapses, resulting in IL-12 secretion and IL-15 trans-presentation. IL-12 is critical for optimal NK cell activation by DCs, and IL-15 is required for NK cell survival and Ly49 receptor expression [113]. Since SLP-76 is critical for murine DC migration and cell-cell contact [114], the interaction of DCs with NK cells may be important for cell-extrinsic Ly49 receptor expression.

We previously reported that LAT1/LAT2 and ADAP can independently recruit SLP-76 to NK cell activation synapses [27]. As both signaling pathways are required for optimal NK cell activation, we predicted that they would equally contribute to Ly49 receptor acquisition by developing NK cells. However, LAT1/LAT2 was more important for Ly49A and Ly49I, whereas ADAP contributed to Ly49G2 expression. LAT1/LAT2 signaling pathways primarily contributed to the extrinsically regulated Ly49C and partially impacted Ly49D and Ly49H. ADAP only contributed to Ly49D and Ly49H. The Ly49 receptor phenotype of ADAP KO NK cells is supported from recently published data on SLP-76$^{ace/ace}$ mice that contain a mutation in the SH2 domain of SLP-76, where ADAP binds [115]. Perhaps, activating receptors that preferentially use either ADAP or LAT1/LAT2 to recruit SLP-76 are engaged at different times during NK cell development, leading to the expression of Ly49 receptors in a specific order. Alternatively, each Ly49 receptor Pro1 promoter may be differentially affected by the assortment of transcription factors induced by the ADAP or LAT1/LAT2 signaling pathways.

Alteration of the probabilistic switch function of the Ly49 Pro1 promoter provides a mechanism that explains how SLP-76 signaling could increase Ly49 receptor acquisition. Ly49 receptor expression has been shown to occur in a stochastic manner [21,40], and the probabilistic mechanism has been explained by differential binding of transcription factors to either forward or reverse promoter elements in the Pro1 bi-

directional promoter. Transcription factors, such as NFκB, bind this region and are inducible following activating receptor signaling. One study has explored the role of NFκB in NK cell development and Ly49 receptor expression, but the results showed only a small decrease in the proportion of Ly49 receptor-expressing NK cells [116]. Other transcription factors, such as AML and Ets-1, are expressed in NK cell progenitors prior to Ly49 receptor transcriptional initiation, but it is possible that activation induced signals and other transcription factors contribute to the stabilization of the transcriptional landscape. For example, Ca^{2+}-dependent NFAT and CREB binding sites in the Pro1 region of Ly49G2 may contribute to forward transcription. Studies examining the contribution of Ca^{2+} signaling to Ly49 receptor acquisition by NK cells are currently ongoing.

Since Pro1 forward transcription is thought to control the accessibility of Pro2 in mature NK cells, we predicted that SLP-76 KO NK cells would exhibit decreased Pro2 chromatin accessibility as measured by H3K9Ac at Ly49G Pro2. However, we found that SLP-76 KO and WT NK cells exhibited equivalent H3K9Ac at Pro2, despite decreased Ly49G transcripts in SLP-76 KO NK cells. Instead, we found that H3K9Ac at Pro1 was almost absent in SLP-76 KO NK cells, suggesting that SLP-76-derived signals impacted Pro1 accessibility. This suggests that Pro1 accessibility might be important for Ly49 receptor transcription in mature NK cells and that Pro2 accessibility alone is insufficient to drive Ly49 transcription. This is in line with a recent report proposing that in addition to being a bidirectional switch, Pro1 may act as an enhancer for Ly49 receptor expression in mature NK cells [117]. Further studies will be required to elucidate the exact role of Pro1 H3K9Ac in control of Ly49 receptor expression.

SLP-76-silenced human NK cells differentiated in culture were defective in human killer-cell immunoglobulin-like receptor (KIR) acquisition. Although the *in vitro* differentiation system may not precisely recapitulate human NK cell development *in vivo*, these data suggest that KIR acquisition might be influenced by SLP-76-dependent signals. It has recently been shown that KIR expression may be transcriptionally regulated in a manner similar to Ly49 receptors. There are promoter regions in KIRs similar to the Pro1 and Pro2 elements of *Ly49* genes; however, their relative location is inverted. A unidirectional promoter/enhancer is located upstream, and a proximal Pro1-like region near the transcriptional start site is methylated in non-expressed KIRs [118,119]. The bi-directional proximal promoter has putative binding sites for transcription factors such as Sp1 and YY1 [120]. It is thought that the antisense transcripts generated from the proximal switch produce a small RNA that is involved in the transcriptional silencing of KIRs through methylation of the proximal promoter region [121,122]. The presence of a bi-directional switch in human KIRs and murine Ly49s suggests a conserved regulatory mechanism of inhibitory receptor acquisition by NK cells. Thus, our studies on activation signals driving Ly49 and KIR expression may also shed light on mechanisms by which KIRs are acquired on human NK cells.

Further investigation is needed to determine the exact transcription factors required for inhibitory receptor acquisition, as differences in the proximal signaling pathways suggest differential regulation of the Ly49 receptors. Nevertheless, our work highlights the complexity of Ly49 regulation. The results from this study are likely to be applicable to the regulation of human KIR receptors[123-125]. Our data support a model where competing activating and inhibitory receptor signals determine the probability of inhibitory receptor expression, which ultimately shapes the inhibitory receptor repertoire

during NK cell development and creates appropriate ligand-specific diversity within the NK cell pool.

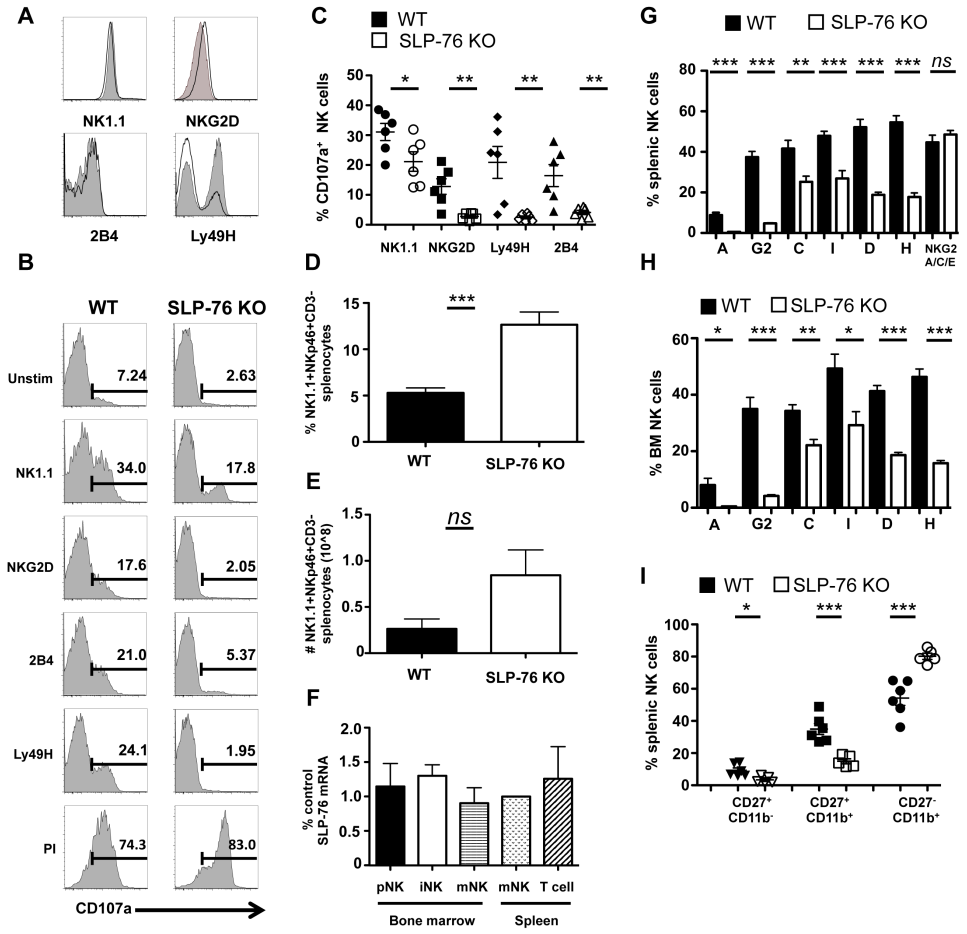


Figure 2.1 SLP-76 is important for Ly49 receptor expression and activation downstream of multiple NK cell activating receptors. (A) Representative histograms of NK cell (gated on NKp46^{+}DX5^{+} splenocytes) expression of multiple activating receptors are shown (WT: tint; SLP-76 KO: black line). (B) Representative histograms of CD107a expression by WT and SLP-76 KO NK cells post stimulation from 4 independent experiments are shown. (C) %CD107a expressing NK cells are represented as mean ± SEM of 4 independent experiments (n=6 mice). Ly49H-stimulated NK cells are gated on Ly49H^{+} cells. (D) The percent and (E) total number of splenic NK cells (NK1.1^{+}NKp46^{+} CD3ε^{-}) were calculated and represented as mean percent positive ± SEM of n = 4-5 mice/group. (F) qPCR was performed for SLP-76 (*lcp2)* and GAPDH (housekeeping gene) with RNA from sorted BM NK cells [CD3ε $^{-}$CD122^{+}NK1.1^{-} (pNK), CD3ε $^{-}$CD122^{+}NK1.1^{+}DX5^{-} (iNK), CD3ε $^{-}$NK1.1^{+}DX5^{+} (mNK)], splenic CD3ε $^{-}$NK1.1^{+}DX5^{+} (mNK) and splenic T cells (CD3ε $^{+}$). Data are from 3 experimental replicates and is graphed as % of control (splenic mNK). *p<0.05, **p<0.01, and ***p<0.001 by paired student's t test. (G) Splenic and (H) BM NK cells from WT (black bars) and SLP-76 KO (white bars) mice were assessed for expression of Ly49 inhibitory receptors (Ly49A, Ly49G2, Ly49C, Ly49I), Ly49 activating receptors (Ly49D and Ly49H), and NKG2A/C/E. The proportion of receptor-expressing NK cells from multiple mice is represented as mean percent positive ± SEM of n = 4-5 mice/group. (**I)** CD27 and CD11b expression by WT (black) and SLP-76 KO (white) NK cells is represented as percent positive ± SEM of n=5-6 mice. *p<0.05, **p<0.01, ***p<0.001, and ns = not significant by student's t test.

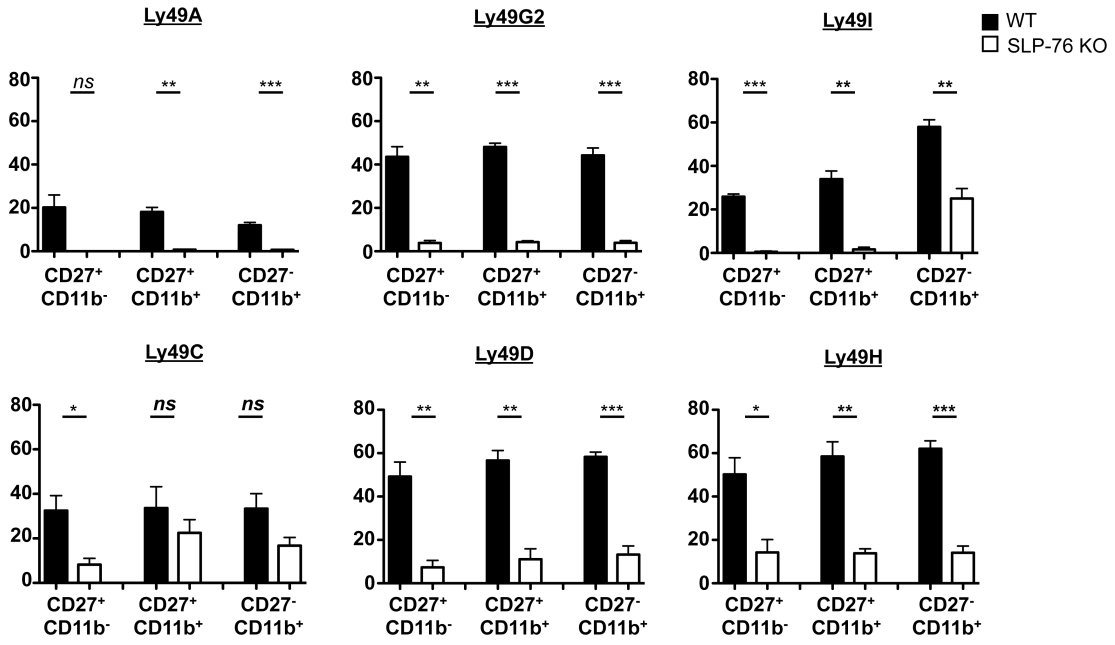


Figure 2.2. Ly49 receptor expression is reduced at all stages of NK cell splenic maturation. Ly49 receptor expression is reduced at all stages of NK cell splenic maturation. Ly49 receptor expression was assessed at three stages of splenic NK cell maturation in WT (black bars) and SLP-76 KO (white bars) mice. Maturity in the spleen evolves as follows: $CD27^{+}CD11b^{-}$ (least mature), $CD27^{+}CD11b^{+}$, $CD27^{-}CD11b^{+}$ (most mature). Data is represented as percent positive ± SEM of three independent experiments (n = 3 mice). *p < 0.05, **p < 0.01, ***p < 0.001, and ns = not significant by unpaired student's t test.

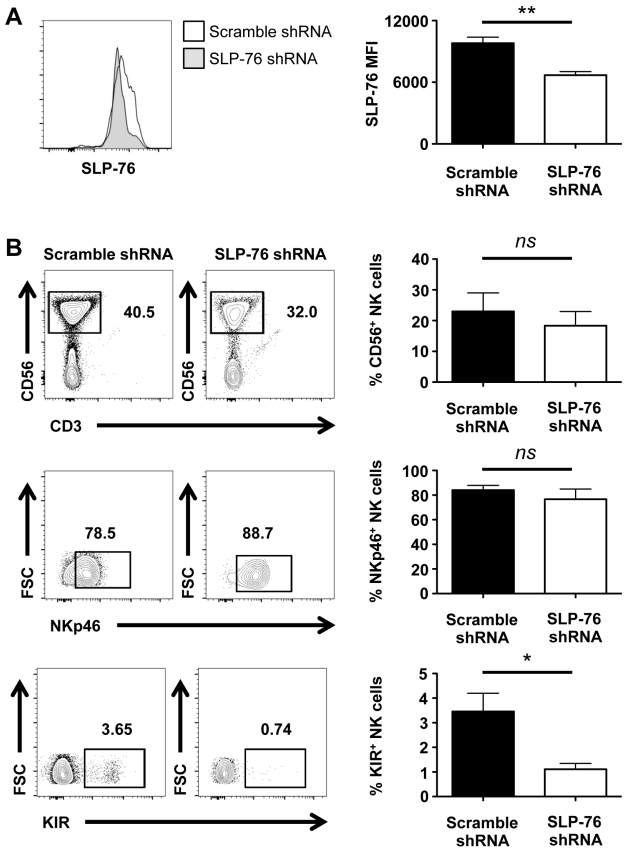


Figure 2.3. Human NK cells require SLP-76 for optimal KIR acquisition during development. (A) Knockdown of SLP-76 from differentiated human NK cells transduced with scramble (black bars) or SLP-76 shRNA (white bars) at Day 21 culture is shown. SLP-76 MFI was calculated from scramble or SLP-76 shRNA transduced donors. Data is plotted as MFI ± SEM of two independent experiments (n=5 donors over two experiments). *p < 0.05, ***p < 0.001, by paired student' s t test. (B) Representative flow plots and histograms of $CD56^{+}CD3^{-}$ $NKp46^{+}$ and KIR^{+} (KIR2DL1, KIR2DL2/DL3, KIR3DL1 antibody cocktail) NK cells are represented asmean percent positive ± SEM of two independent experiments (n=5 donors over two experiments). *p < 0.05, ***p < 0.001, by paired student' s t test. ***Experiments performed by B. Zhang, T. Lenvik, F. Cichocki.**

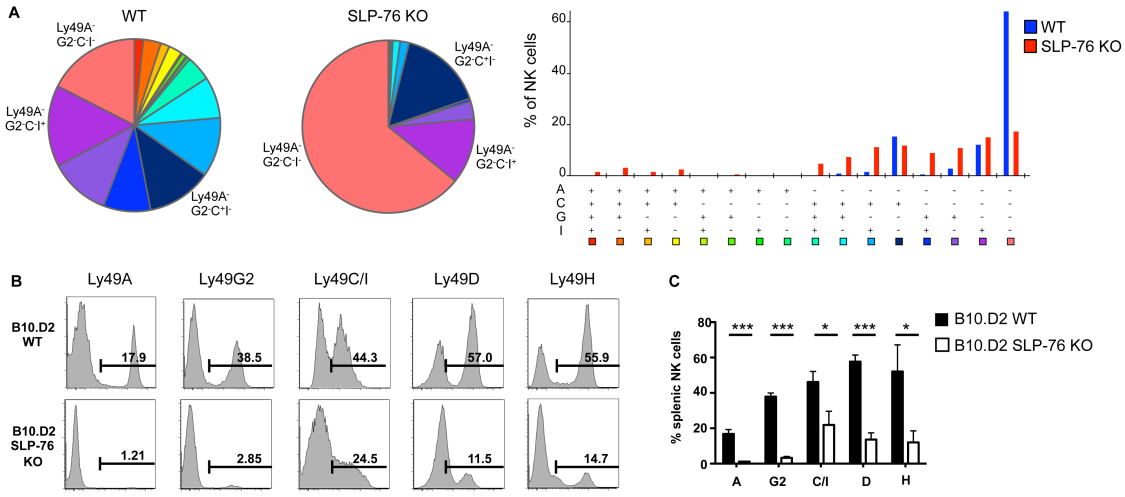


Figure 2.4. Ly49 receptor expression loss in SLP-76 KO NK cells is independent of MHC-I haplotype. (A) The co-expression pattern of inhibitory Ly49 receptor in WT vs SLP-76 KO splenic NK cells was assessed through SPICE analysis. (B) Representative histograms of Ly49 receptor expression by WT B10.D2 and B10D2.SLP-76 KO splenic NK cells are shown. (C) The proportion of Ly49 receptor-expressing NK cells from multiple WT B10.D2 (black bars) and B10D2.SLP-76 KO (white bars) mice is represented as mean percent positive ± SEM of (n = 3 mice/group). *p<0.05, ***p<0.001 by student's t test.

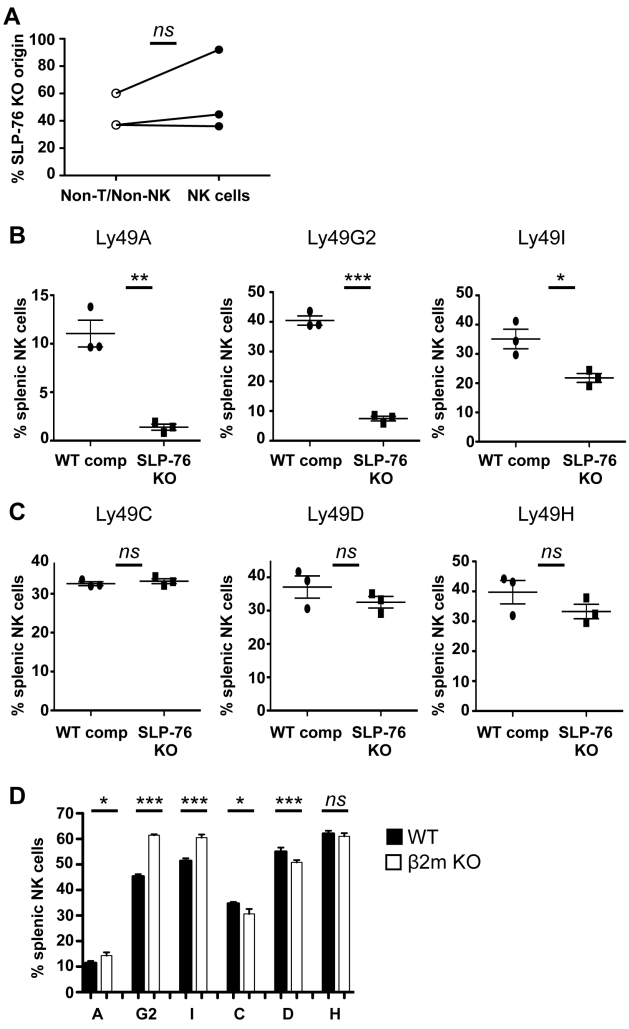


Figure 2.5. A subset of Ly49 receptors is regulated in an NK cell-intrinsic SLP-76-dependent manner. (A) The percentage of Non-T/NK cells and NK cells from WT competitor (CD45.2) was assessed in each mixed bone marrow chimeric mouse. One representative experiment of 4 experiments is plotted (n = 3 mice). ns = not significant by paired student's t test. **(B)** The proportion of Ly49 receptor-expressing NK cells derived from WT or SLP-76 KO BM from mixed BM chimeras was assessed. One representative experiment of 4 experiments is shown as mean percent positive ± SEM of n=3 mice. *p<0.05, **p<0.01, ***p<0.001, ns=not significant by paired student's t test. **(C)** Ly49 receptor expression on splenic NK cells from WT (black bars) and β2m KO (open bars) mice was assessed and represented as mean percent positive ± SEM of n = 10 mice. *p<0.05, **p<0.01, ***p<0.001, and ns = not significant by student's t test.

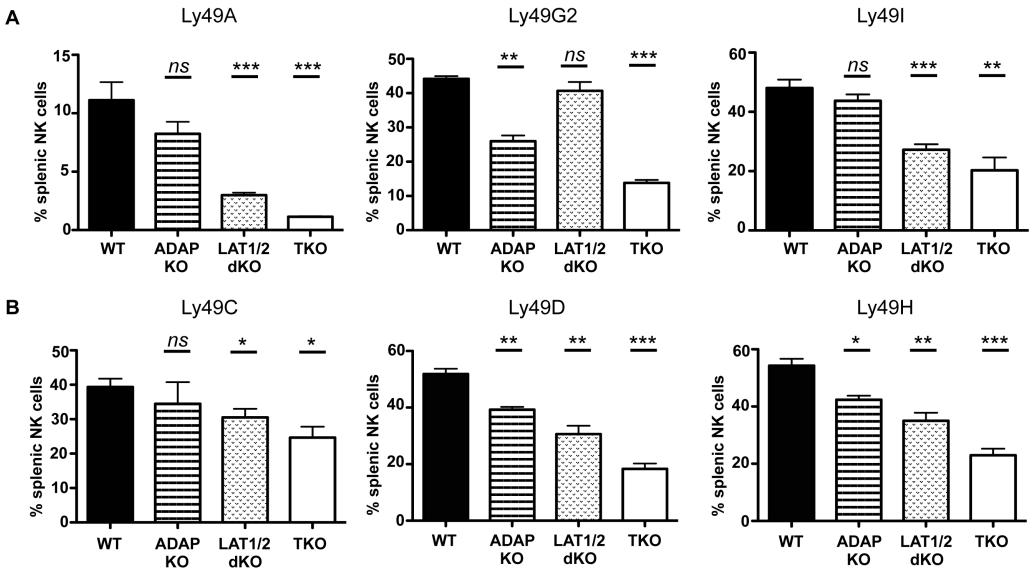


Figure 2.6. Upstream signaling pathways of SLP-76 differentially contribute to induction of Ly49 expression by NK cells. (A) Splenic NK cells from WT, ADAP KO, LAT1/LAT2 DKO, and ADAP/LAT1/LAT2 TKO NK cells were assessed for expression of NK cell-intrinsic Ly49A, Ly49G2, Ly49I and (B) NK cell-extrinsic Ly49C, Ly49D, and Ly49H. Data are represented as mean percent positive ± SEM of n=3-4 mice per genotype. *p<0.05, **p<0.01, ***p<0.001, and ns=not significant by student's t test.

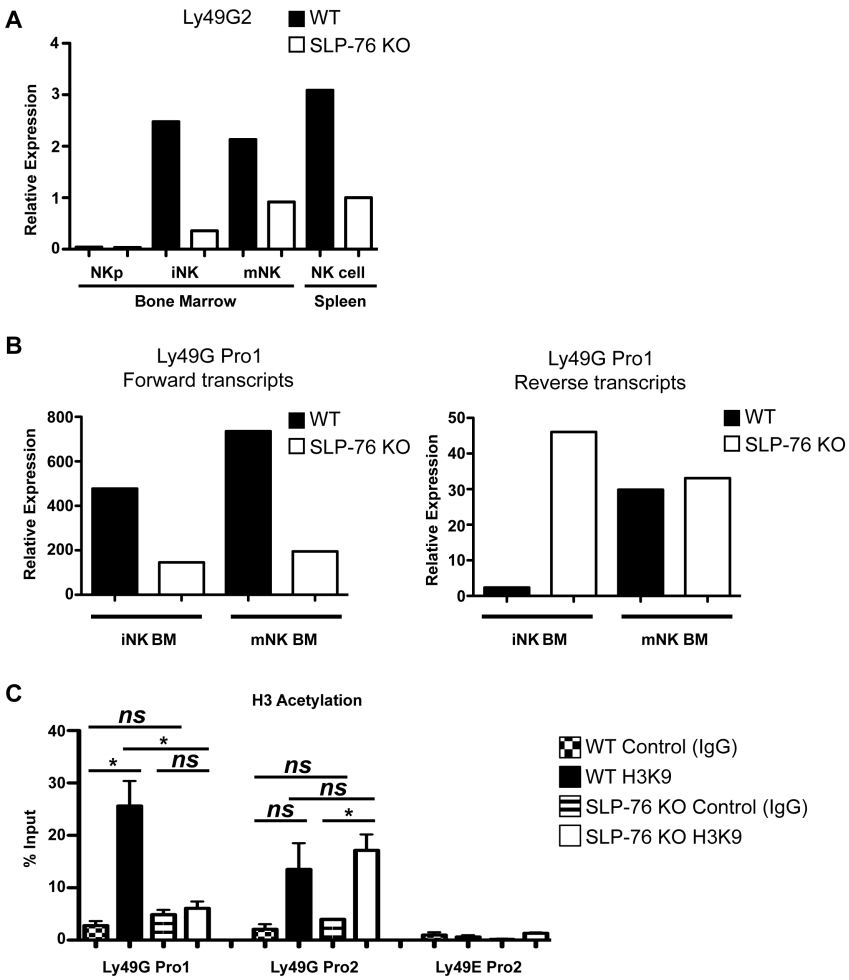


Figure 2.7. SLP-76 regulates *Ly49* gene transcription during early BM development. (A) qPCR was performed for Ly49G2 and GAPDH (housekeeping gene) with RNA from sorted BM NK cells at various developmental stages [$CD3\varepsilon^{-}CD122^{+}NK1.1^{-}$ (NKp), $CD3\varepsilon^{-}CD122^{+}NK1.1^{+}DX5^{-}$ (iNK), $CD3\varepsilon^{-}NK1.1^{+}DX5^{+}$ (mNK)] and splenic $CD3\varepsilon^{-}NK1.1^{+}DX5^{+}$ (NK). One representative of 2 independent experiments is shown. (B) qPCR for Ly49G Pro1 forward and reverse transcripts was performed on $DX5^{-}$ (iNK) and $DX5^{+}$ (mNK) BM NK cells from SLP-76 KO and littermate control mice. β-actin was used as a housekeeping gene. One representative of 4 independent experiments is shown. (C) ChIP for histone 3 lysine 9 (H3K9) acetylation was performed on splenic NK cells from WT and SLP-76 KO mice. The results were normalized as the percentage of the input (%input) from Ct values, and data are represented as mean % input ± SEM from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001, and ns=not significant by paired student's t test. ***Experiments in collaboration with H. Li, M. McCullen, and S.K. Anderson.**

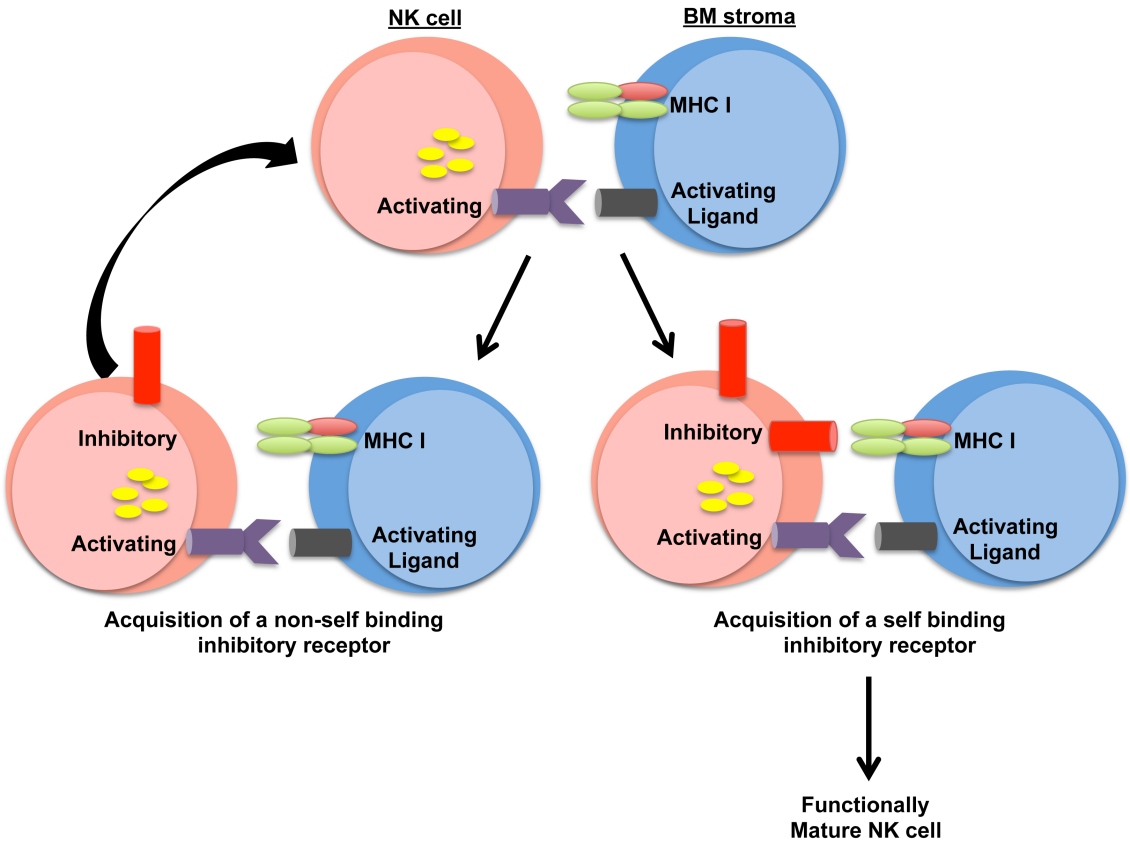


Figure 2.8. Activating receptor-mediated signaling is the driving force for Ly49 receptor induction during NK cell development. Activating receptors on developing NK cells are stimulated by their respective ligands via interactions with BM stromal cells. This interaction results in the activation of transcription factors that promote Ly49 receptor expression. If the Ly49 receptor that is acquired does not recognize self-MHC I, the NK cell continues to receive "positive" activating receptor signals, which increases the probability of the NK cell to acquire another Ly49 receptor. This process occurs until a self-MHC I binding Ly49 is acquired, which will dampen the activating receptor signal. This NK cell can then mature and migrate to peripheral tissues to perform "missing-self" recognition.

CHAPTER III: Murine natural killer cells degranulate and retain cytotoxic function without store-operated calcium entry.

Introduction

Intracellular signaling is a biochemical process that allows cells to respond to extracellular cues. Signaling is controlled by direct modification to proteins (e.g., phosphorylation) and by binding of ions such as Ca^{2+}. These modifications influence protein-protein interactions, which have broad downstream consequences for cells, including changes in transcription, cell motility, mitochondrial function, and cell death [126].

The mobilization of Ca^{2+} ions from the extracellular environment into the cytoplasm is important for immune cell activation downstream of activating immunoreceptors or G protein-coupled receptors (GPCR)s [127]. Engagement of these activating receptors leads to the phosphorylation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) into the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) [128,129]. The basal concentration of intracellular Ca^{2+} is very low (~100nM) as compared to the extracellular environment, but this quickly changes when IP_3 binds to IP_3-receptors on the endoplasmic reticulum (ER), causing the release of ER Ca^{2+} stores into the cytoplasm (raises cytoplasmic concentration to ~1uM). ER depletion dissociates Ca^{2+} ions from the ER Ca^{2+}-sensors stromal interacting molecules 1 and 2 (STIM1/2). STIM1/2 oligomerize and activate ORAI1-3 (Ca^{2+} release-activated channel; CRAC) on the plasma membrane to facilitate entry of extracellular Ca^{2+} into the cytoplasm. This process is important for sustaining of high levels of Ca^{2+} in the cytoplasm after immune activation and is referred to as store operated Ca^{2+}entry (SOCE) [69,128,130].

Sustained Ca^{2+} entry mediated through SOCE is critical for T cell specific functions. STIM1/2-deficient T cells cannot mobilize Ca^{2+} through the CRAC channel following TCR stimulation [71]. They have defects in proliferation following TCR stimulation, fail to produce IL-2 and IFNγ, and have impaired degranulation. Because of these defects, STIM1/2-deficient T cells have significantly compromised cytotoxicity against tumors [131], defective control of acute infections, and possess reduced memory T cell formation and persistence [132].

Like $CD8^+$ T cells, the key functions of NK cells are to produce inflammatory cytokines and perform cell-mediated killing. NK cells initiate Ca^{2+} signaling following activating receptor stimulation [72]. A few studies have probed the requirement of SOCE in NK cell effector function by examining rare patients with STIM1 or ORAI1 mutations [97,98]. These patients had normal frequencies of NK cells, expression of NK cell-defining markers, LFA-1 activation, and granule polarization. Similar to their T cells, these patients exhibited defective NK cell cytokine production and problems with target cell killing due to degranulation defects.

In this study, we sought to investigate the contribution of the DAG and Ca^{2+} signaling pathways to NK cell effector function. Surprisingly, we found that activation of the DAG signaling pathway alone was sufficient to induce degranulation by NK cells. Moreover, while NK cells displayed defective IFNγ production, they retained the ability to degranulate and kill target cells in the absence of SOCE. These data suggest that unlike $CD8^+$ T cells, which need sustained Ca^{2+} signaling for cytotoxicity, primary murine NK cells do not require SOCE to perform cell-mediated cytotoxicity.

Results and Discussion

Activation of the DAG signaling pathway in the absence of extracellular Ca^{2+} is sufficient to induce NK cell degranulation

PLCγ transduces its downstream signals through the activation of the DAG and Ca^{2+} signaling pathways. To individually test the contribution of these two distinct signaling pathways in NK cell activation, we stimulated primary splenic C57BL/6 (WT) NK cells with the Ca^{2+} ionophore, ionomycin (Iono), or the DAG analog, phorbol 12-myristate 13-acetate (PMA). As expected, stimulation of NK cells with PMA and Iono induced robust expression of both IFNγ and cell surface CD107a, a marker for degranulation (Fig. 3.1 A-C). Although a small fraction of NK cells stimulated with Iono alone expressed IFNγ, Iono alone did not induce any cell surface CD107a expression (Fig. 3.1 A-C). Similarly, PMA alone negligibly up-regulated IFNγ expression on NK cells. However, we unexpectedly found that PMA alone robustly induced cell surface CD107a expression by NK cells (Fig. 3.1 A-C). These data suggest that DAG signaling may be sufficient to trigger degranulation by primary NK cells.

Although PMA alone would not be expected to elevate cytoplasmic Ca^{2+} through the activation of SOCE, we wondered whether extracellular Ca^{2+} entering the cell from other membrane channels contributed to the effects of PMA-induced degranulation. To test this possibility, we stimulated WT splenocytes with PMA in the presence and absence of extracellular Ca^{2+}. The proportion of NK cells expressing CD107a in Ca^{2+}-sufficient media (2mM Ca^{2+}) and Ca^{2+}-free media cultures was similar (Fig. 3.1D). This suggests that PMA derived signals can prompt NK cell degranulation in a Ca^{2+}-independent manner.

Extracellular Ca^{2+} entry is required for IFNγ expression but not for degranulation and cytotoxicity by NK cells

To more rigorously assess the role of sustained extracellular Ca^{2+} entry in NK cell function, we stimulated WT splenocytes with plate-bound antibodies against NK cell activating receptors in the presence or absence of extracellular Ca^{2+}. Compared to NK cells stimulated in Ca^{2+}-sufficient media, the proportion of NK cells expressing IFNγ was severely reduced (80-90% inhibited) when activated in Ca^{2+}-free media (Fig. 3.2A). In contrast, the degranulation of NK cells activated in Ca^{2+}-free media was relatively preserved, ranging from a 15-40% decrease in the proportion of CD107a-expressing NK cells, depending on the activating receptor. The decrease in the percentage of NK cells expressing CD107a was not statistically significant following NK1.1 or NKG2D stimulation but was significantly reduced following 2B4, Ly49D, and PMA/ionomycin stimulation (Fig. 3.2A). Still, in general, there was relative preservation of the degranulation response compared to IFNγ production in NK cells stimulated in Ca^{2+}-free conditions.

We next tested the role of extracellular Ca^{2+} entry in NK cell function using a genetic approach. Although the initial burst of intracellular Ca^{2+} is controlled by IP_3-mediated release of Ca^{2+} from ER stores, sustained Ca^{2+} signaling requires extracellular Ca^{2+} mobilization through the CRAC channel that is controlled by the STIM1 and STIM2 proteins. We crossed $STIM1^{fl/fl}STIM2^{fl/fl}$ mice to an NK cell-cre recombinase mouse—$NKp46^{icre}$—to generate mice deficient in both STIM1 and STIM2 proteins in mature NK cells. $STIM1^{fl/fl}STIM2^{fl/fl}$-$NKp46^{icre}$ (STIM1/2 conditional double knockout; cDKO) mice displayed similar splenic NK cell percentages, numbers, and normal NK cell development compared to WT control mice (Fig. 3.3). STIM1/2 cDKO NK cells also

expressed major NK cell activating receptors (NK1.1, 2B4, NKG2D, and Ly49D) comparably to WT controls (Fig. 3.3). Similar to NK cells stimulated in Ca^{2+}-free conditions, STIM1/2 cDKO NK cells displayed significantly defective IFNγ production but intact degranulation compared to WT NK cells upon stimulation with various activating receptors and with PMA/Ionomycin (Fig. 3.2B-C). These data suggest that NK cell cytotoxic function may be intact in the absence of SOCE.

A crucial step in NK cell-mediated cytotoxicity is the polarization of the microtubule organizing center (MTOC) to the NK cell/target cell synapse [133,134]. Thus, we assessed MTOC polarization of WT and STIM1/2 cDKO NK cells when co-cultured with the NK cell-sensitive YAC-1 target cell. The polarization of the MTOC to the target cell synapse was quantitatively similar between WT and STIM1/2 cDKO NK cells (Fig. 3.2D-E). The preserved degranulation and MTOC polarization response correlated with intact cytotoxic function of STIM1/2 cDKO NK cells, as STIM1/2 cDKO NK cells killed YAC-1 target cell as effectively as WT NK cells (Fig. 3.2F). Similarly, the cytotoxicity of WT and STIM1/2 cDKO NK cells was comparable in a p815 target cell re-directed killing assay using anti-NK1.1 antibody (Fig. 3.2G).

In T cells, STIM1 and STIM2 differentially contribute to downstream effector functions. For example, STIM1-deficient T cells do not produce IL-2, IFNγ, and TNFα [71,131,132], whereas STIM2-deficient T cells only have marginally reduced inflammatory cytokine production. To test the relative contribution of STIM1 and STIM2 in NK cell function, mice with NK cells lacking either STIM1 (STIM1 cKO) or STIM2 (STIM2 cKO) were generated. Although STIM1 cKO NK cells to a large extent phenocopied STIM1/2 cDKO NK cells, STIM2 cKO NK cells displayed no statistically significant defect in IFNγ

production or degranulation (Fig. 3.4), suggesting that STIM1 is the dominant isoform in controlling SOCE during NK cell activation.

NK cells from STIM1/2 cDKO mice exhibit cytotoxic function *in vivo*

We next examined the capacity of STIM1/2 cDKO NK cells to mediate cytotoxicity and clear tumor cells *in vivo.* We first tested the ability of STIM1/2 cDKO mice to acutely clear RMA-S (TAP-deficient/MHC-I^{lo}, NK cell-susceptible) compared to parental RMA (MHC-I^{+}, NK cell resistant) tumor cells lines. We intravenously injected RMA:RMA-S cells at a 1:3 ratio into WT, STIM1/2 cDKO, or NK cell-depleted WT animals and measured how this ratio changed 18 hours post tumor challenge. As expected, the RMA:RMA-S ratio skewed significantly towards RMA cells in WT mice compared to NK cell-depleted WT mice (Fig. 3.5A). Although the RMA:RMA-S ratio was significantly higher in STIM1/2 cDKO compared to WT mice, the ratio was still significantly lower than that of NK cell-depleted WT mice, suggesting that STIM1/2 cDKO NK cells were capable of acutely killing RMA-S cells in vivo (Fig. 3.5A). To measure long-term tumor growth after tumor challenge, we subcutaneously injected equivalent numbers of RMA or RMA-s cells into WT, cDKO, and NK cell-depleted WT mice. The tumor size of RMA-S cells was smaller than RMA cells, which was normalized by NK cell depletion. Importantly, no difference in tumor size was observed between WT and cDKO mice injected with either RMA or RMA-S tumor cells, demonstrating that NK cells lacking STIM1/STIM2 limit tumor growth comparably to WT controls (Fig. 3.5B). Together with our *in vitro* results, these data strongly suggest that sustained Ca^{2+} entry through SOCE is dispensable for NK cell degranulation and cytotoxic function.

DAG-mediated signaling pathways drive NK cell degranulation in a transcription-independent manner

We next interrogated the mechanism behind why sustained mobilization of extracellular Ca^{2+} was required for NK cell IFNγ production but not for NK cell degranulation. We reasoned that IFNγ production might be defective in the absence of SOCE because the induction of IFNγ but not degranulation requires the Ca^{2+}-activated transcription factor NFAT. To test this possibility, we blocked transcription by actinomycin D and more specifically abrogated the activation of NFAT using a calcineurin inhibitor (Cyclosporine A; CsA). Indeed, while the proportion of NK cells producing IFNγ was significantly decreased in NK cells treated with actinomycin D or with CsA, these inhibitors had no effect on degranulation, suggesting that degranulation does not require transcriptional events (Fig. 3.6A).

The finding that SOCE is expendable for NK cell degranulation and cell-mediated killing is surprising, but multiple Ca^{2+}-dependent and independent pathways influence this process [65,135-140]. Since DAG-derived signals were sufficient to drive NK cell degranulation in the absence of extracellular Ca^{2+}, we sought to determine the signaling pathways downstream of DAG needed for NK cell degranulation when Ca^{2+}-dependent pathways were eliminated. DAG signals through three major downstream signaling pathways that involve the activation of ERK, AKT, and PKC. To test the relative contribution of these signaling pathways, we pharmacologically inhibited ERK (MEK inhibitor U0126), AKT (AKT 1/2 inhibitor), and PKC (pan-PKC inhibitor Gö6983) in splenocytes from WT and STIM1/2 cDKO mice that were stimulated with the anti–NK1.1 antibody. Following ERK inhibition using U0126, the proportion of STIM1/2 cDKO NK cells expressing CD107a was higher than that of WT NK cells at each concentration,

suggesting that WT NK cells rely on ERK for degranulation more than STIM1/2 cDKO NK cells (Fig. 3.6B). AKT was equally required by WT and STIM1/2 cDKO NK cells for degranulation (Fig. 3.6C). In contrast, cDKO NK cells were more sensitive to PKC inhibition by Gö6983 compared to WT NK cells (Fig. 3.6D). This shows that in the absence of sustained Ca^{2+} signaling, NK cells heavily rely on DAG-mediated PKC activation for the degranulation response.

Our data indicate that in the absence of SOCE, NK cells have the ability to degranulate and perform cell-mediated killing. Although sustained Ca^{2+} signaling is required for IFNγ production in an NFAT-dependent manner, it is surprising that NK cell-mediated killing is achievable in the absence of SOCE. This does not disqualify Ca^{2+} from being important to this process, as STIM1/2 cDKO NK cells undergo ER-mediated Ca^{2+} release, have mitochondrial retention of Ca^{2+}, and may express other Ca^{2+} channels such as TRP channels [126]. What is clear from our data is that SOCE is not required for NK cell killing as it is for $CD8^+$ cytotoxic T cells [71,132].

Our data suggest that in the absence of SOCE, DAG-mediated signals alone are sufficient to drive NK cell degranulation. PKC, which is directly activated by DAG, may be critically important for degranulation in the absence of extracellular Ca^{2+} and represents the minimal signaling requirement for NK cells to degranulate. It has been shown that DAG-dependent signals drive microtubule-organizing center (MTOC) and granule polarization in T cells [67,141,142]. That is in line with our data showing that STIM1/2 cDKO NK cells polarize their MTOC normally. Key synaptic and exocytosis proteins depend on PKC for function. PKC isozymes phosphorylates the synaptic proteins synaptotagmin, Syntaxin 4, VAMP, Munc18, and SNAP25, all which are critical for the fusing of lysosomes to the plasma membrane and release of their contents [143]. While

many of these proteins also have C2 Ca^{2+} binding domains, PKC isozymes may be the chief factor in the final steps of NK cell degranulation. It is important to note that in contrast to our mouse NK cell data, NK cells from human patients with ORAI1 and STIM1 mutations are unable to degranulate. Interestingly, however, both human and mouse NK cells do not require SOCE for the MTOC polarization process [98]. Thus, while DAG-mediated signals also drive lytic granule docking and fusion in mouse NK cells, human NK cells appear to require SOCE for these final steps of degranulation.

In summary, our data show that primary mouse NK cell degranulation and cell-mediated killing does not depend on SOCE, and furthermore, point to DAG-mediated downstream signaling molecules such as PKC as the key driver of this process. Our studies show that degranulation, a key cell biological process that has been long thought to be driven primarily by Ca^{2+} signals, can occur in the absence of sustained Ca^{2+} entry.

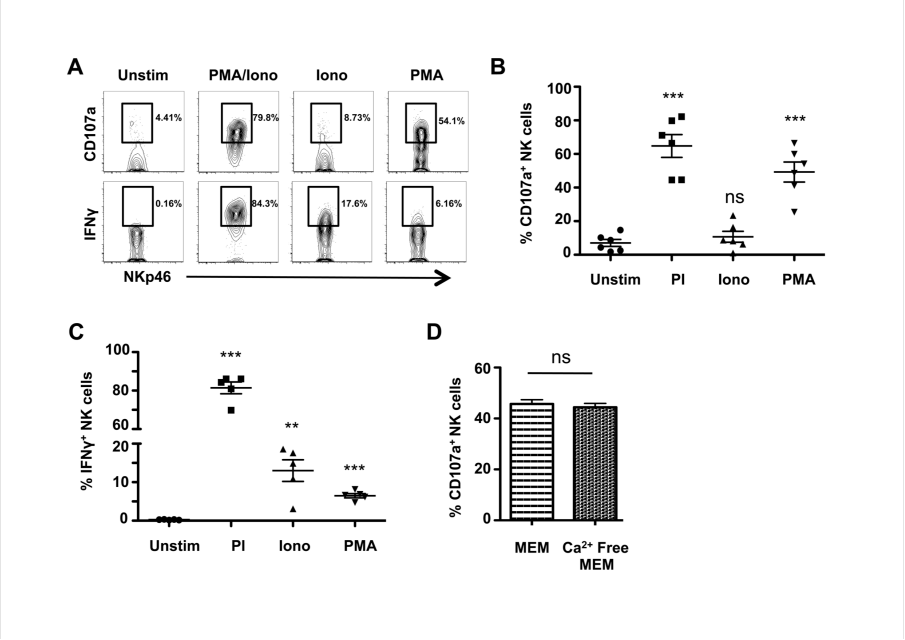


Figure 3.1. PMA-driven signals cause NK cell degranulation. (A) Splenocytes were unstimulated (unstim) or stimulated with PMA/ionomycin (PI), ionomycin alone, or PMA alone for 6 hours. Representative flow cytometry plots of NK cell ($NKp46^+DX5^+CD3^-$ or $CD4/CD8^-$) CD107a and IFNγ expression are shown. (B) The fraction of CD107a and (C) IFNγ expressing NK cells are represented as mean ± SEM of 4 independent experiments (*n*=6 mice). (D) NK cells were stimulated with PMA alone in the presence (MEM) and absence (Ca^{2+}-free MEM) of extracellular Ca^{2+}. The fraction of CD107a expressing NK cells is represented as mean ± SEM of 3 independent experiments (*n*=3-6 mice). Significance for experiments was determined by Student's t test **p<0.05, **p<0.01, ***p<0.001*, and *ns*= not significant.

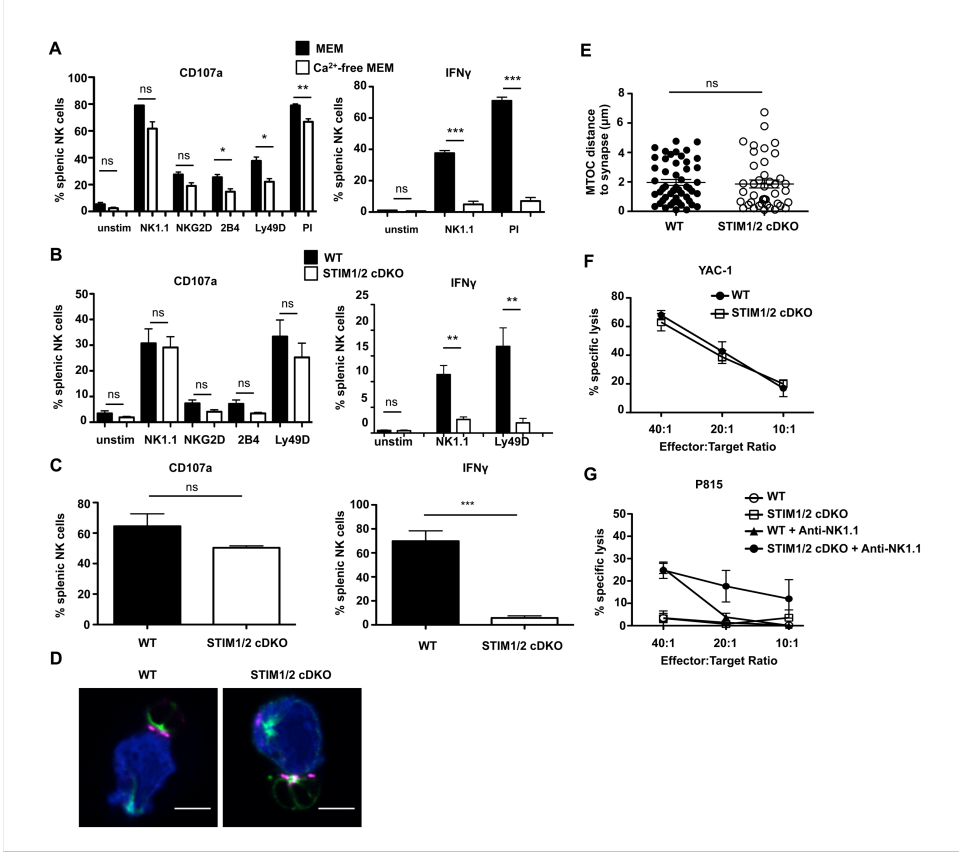


Figure 3.2. Sustained calcium signaling is critical for IFNγ production, but is not required for NK cell degranulation and cytotoxicity. (A) WT splenocytes were stimulated for 6 hours by plate-bound activating receptor antibodies in the presence (MEM) or absence (Ca^{2+}-free MEM) of extracellular Ca^{2+}. The fraction of CD107a and IFNγ-expressing NK cells are represented as mean ± SEM of 2 independent experiments (*n*=4 mice). (B) Splenocytes from WT or STIM1/2 cDKO mice were stimulated by (B) plate-bound activating receptor antibodies or (C) PMA/Ionomycin. The fraction of CD107a and IFNγ-expressing NK cells are represented as mean ± SEM of 5 independent experiments (n=4-6 mice/genotype). (D) WT or cDKO $DX5^+$ NK cells were mixed 2:1 with CMAC-labeled YAC-1 cells (blue) in a conjugate assay and assessed for MTOC polarization. Cell conjugates were stained for tubulin (green) and cathepsin D (magenta) to visualize the MTOC and lytic granules, respectively. Representative confocal microscopy images are shown. Scale bar is 5 μm. (E) NK cell MTOC distances to the immune synapse (μm) are represented as mean ± SEM of >50 YAC-1: NK cell conjugates (n=3 mice). (F) $DX5^+$ NK cells from WT (black) and cDKO (white) mice were co-cultured with $luciferase^+$ YAC-1 cells or (G) $luciferase^+$ p815 cells ± anti-NK1.1 in the presence of rIL-2 for 6 hours at various E:T ratios. % specific lysis is shown as mean ± SEM of 3 independent experiments (*n=4* mice/genotype or condition). Statistics were determined by using Student's t test $p<0.05$, $**p<0.01$, $***p<0.001$, and ns=not significant. For all plots, Ly49D-stimulated NK cells are pre-gated on $Ly49D^+$ NK cells.

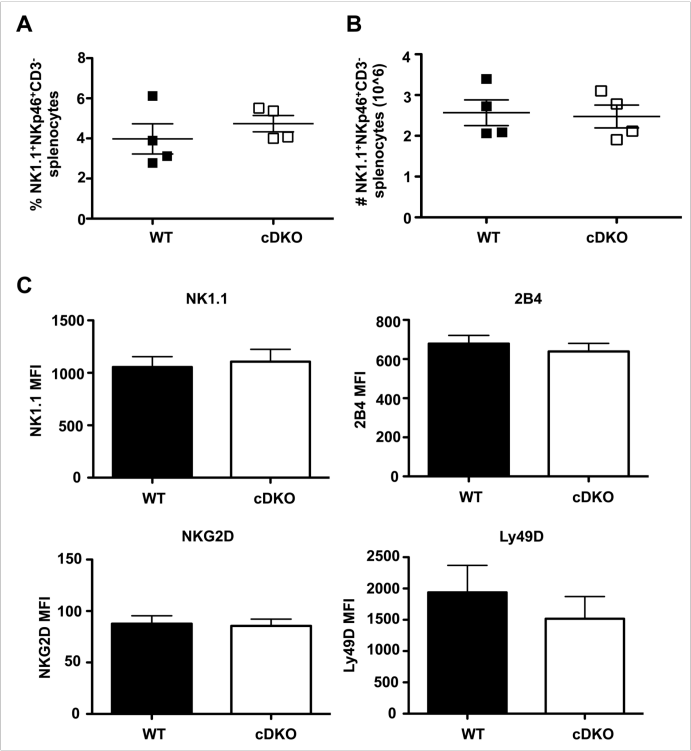


Figure 3.3. STIM1/2 cDKO and WT NK cells are phenotypically similar. (A) The percent and (B) total number of splenic NK cells ($NK1.1^+NKp46^+CD3\varepsilon^-$) from WT and STIM1/2 cDKO mice were calculated and represented as mean ± SEM of 3 independent experiments (*n*=4 mice/genotype). (C) Splenocytes were assessed for expression of NK1.1, 2B4, NKG2D, and Ly49D. The MFI of each receptor expressed on splenic NK cells ($CD3\varepsilon^-NKp46^+DX5^+$ cells) are represented as mean ± SEM of 3 independent experiments (*n*=4-5 mice/genotype).

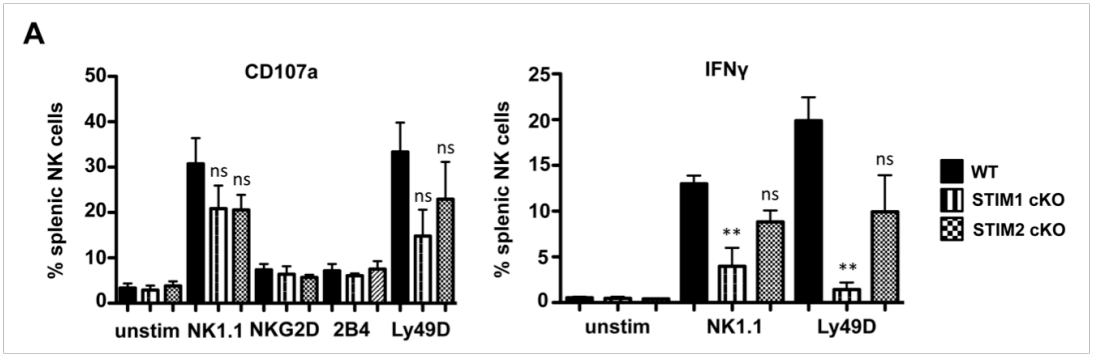


Figure 3.4. STIM1 controls SOCE in murine NK cells. (A) Splenocytes from WT, STIM1 cKO, or STIM2 cKO NK cells mice were stimulated with plate-bound activating receptor antibodies for 6 hours. The fraction of CD107a and IFNγ-expressing NK cells are represented as mean ± SEM of 3 independent experiments (*n=3-5* mice/genotype). For all plots, Ly49D-stimulated NK cells are pre-gated on Ly49D^{+} NK cells.

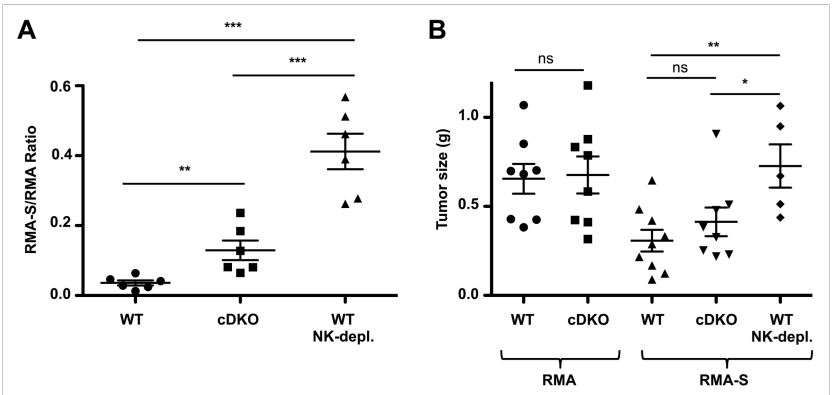


Figure 3.5. STIM1/2 cDKO mice clear MHC-I deficient tumors. (A) WT, cDKO, and NK cell–depleted WT mice were injected i.v. with a 1:3 ratio of CellTrace Violet-labeled RMA and CFSE-labeled RMAS-S cells. 18 hours post injection, splenocytes were analyzed for the presence of tumor cells by flow cytometry. The ratio of RMA-s:RMA tumor cells within each mouse was calculated. Combined data from 2 independent experiments is shown. Statistics were determined using paired student's t test ($p< 0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and ns=not significant). (B) RMA and RMA-S cells were injected s.c. into WT, cDKO, or NK cell-depleted WT mice. Tumors were isolated and weighed 11 days post injection. Data is combined from 3 independent experiments. Statistics were determined using student's t test $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and ns=not significant.

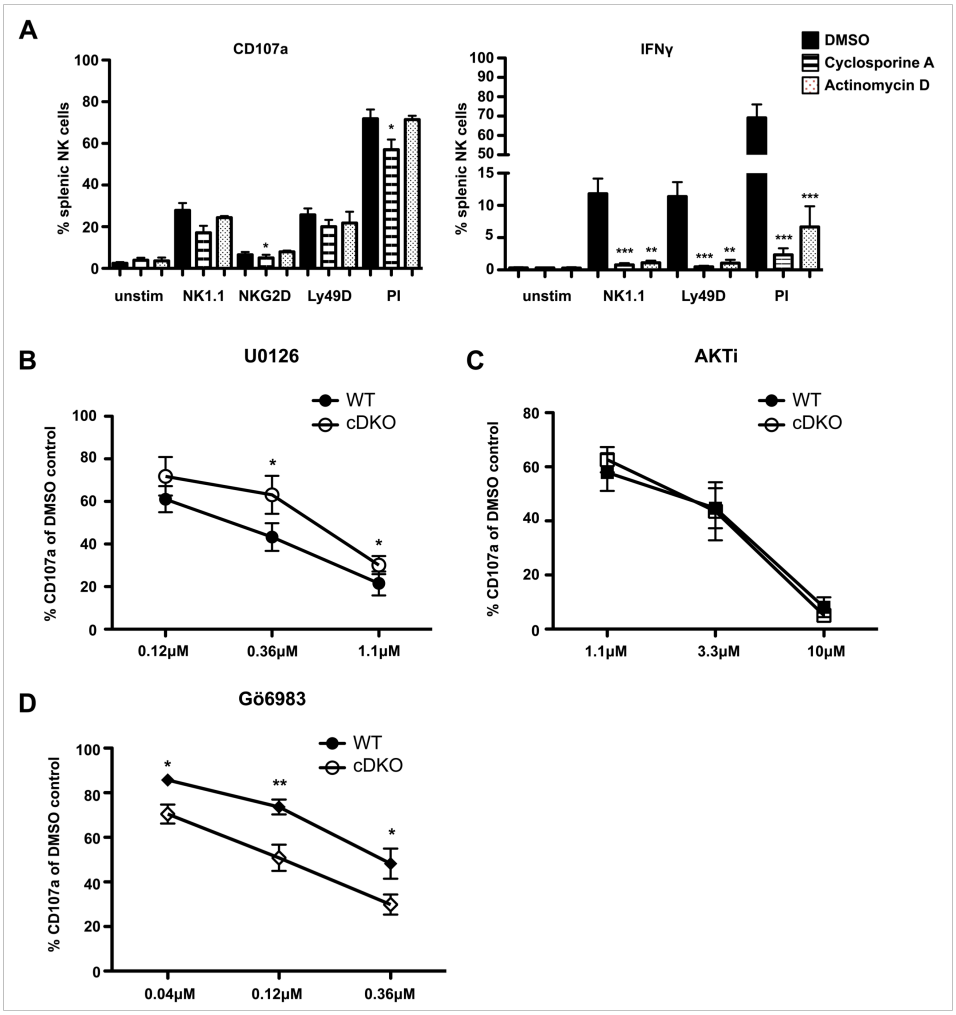


Figure 3.6. DAG-mediated signaling pathways drive NK cell degranulation in a transcription-independent manner. (A) WT splenocytes were stimulated by plate-bound activating receptor antibodies with DMSO (0.1%), CsA (1μM), or Actinomycin D (5μg/mL) for 6 hours. The fraction of CD107a and IFNγ-expressing NK cells are represented as mean ± SEM of 3 independent experiments (*n*=4-8 mice/condition). (B) Splenocytes from WT and cDKO mice were stimulated with plate bound anti-NK1.1 for 6 hours with DMSO (0.1%) or various concentrations of U0126 (MEK inhibitor), (C) AKT inhibitor (AKTi), or (D) Gö6983 (pan-PKC inhibitor). The percentage of CD107a expressing cells compared to DMSO controls (percentage of baseline) is plotted as mean ± SEM of 5 independent experiments (n=4–6 mice/concentration). Significance was determined by paired Student's t test *p<0.05, **p<0.01, ***p<0.001, and ns=not significant.

CHAPTER IV: DISCUSSION

In this thesis, I examined the contributions of NK cell activating receptor signaling pathways to NK cell development and effector function. In Chapter II, I describe a mechanism by which NK cells generate diversity and create target cell specificity. Ly49 receptors, the functional orthologs of KIRs in humans, generate vast diversity for the murine NK cell population. I determined that SLP-76-dependent activation signals are required for NK cell Ly49 receptor expression. SLP-76 proximal signaling proteins, LAT1/LAT2 and ADAP, differentially affect Ly49 family member expression. These pathways have been shown to initiate expression of different transcription factors that may ultimately affect the transcriptional landscape of the Ly49 receptor genes. SLP-76 regulates Ly49 transcription by regulating the switch function of the Pro1 bi-directional promoter region in immature NK cells. In Chapter III, I demonstrate that while NK cells require sustained Ca^{2+} entry (mediated by STIM1/2) for IFNγ production, NK cells are able to degranulate and perform cell-mediated killing without SOCE. This is a novel finding, as it has been reported that cytotoxic $CD8^{+}$ T cells and even human NK cells may need SOCE following immunoreceptor activation for cytotoxic function. While this work sheds new light on two major subjects of interest to the NK cell field, it provokes new questions that require further experimental investigation.

Different activating receptors for acquisition of different inhibitory receptors

I utilized SLP-76 KO mice, which have defective NK cell signaling, to test if Ly49 receptor acquisition during NK cell development depended on activation through NK cell activating receptors. NK cells express not only a variety of inhibitory receptors, but also a variety of activating receptors. For example, all murine NK cells express NK1.1,

NKG2D, 2B4, DNAM-1, CD2, and the natural cytotoxicity receptors NKp46 and NKp30. Even 50% of murine NK cells express the activating receptors Ly49D and Ly49H. The majority of these activating receptors are expressed early in hematopoiesis and serve not only as activating receptors, but also as protein markers for identifying NK cells by flow cytometry. Although all of the receptors listed converge on SLP-76, they differ in the adaptor molecules they use for signaling. For example, NKG2D is phosphorylated on YNIM motifs on the cytoplasmic tail of the receptor. CD16 signals using ITAM motifs of CD3 zeta chains [144]. Because of these differences in signaling adaptors, different downstream signaling pathways and transcription factors may be activated.

I showed that LAT1/LAT2 and ADAP signaling pathways contribute differentially to Ly49 receptor expression, and I suspect different NK cell activating receptors preferentially activate one pathway or the other. While both ITAM-mediated and other adaptors (e.g. SAP-mediated) can lead to *both* LAT1/LAT2 and ADAP downstream, it is possible the ADAP pathway preferentially is activated over LAT/LAT2 following stimulation with 2B4. 2B4 signals through the SAP-Fyn axis,. The src kinase Fyn is able to engage ADAP and initiate its signaling cascade [51,145], which could lead to Ly49G2 expression. Furthermore, because NK cells require 2B4 co-stimulation for maximal effector function [72,146], the NK cell may be more likely to require integrin signals, which would preferentially initiate ADAP activation through outside-in-signaling.

It is also possible that certain activating receptors are not the directing agents for determining LAT1/LAT2 vs ADAP signaling pathway usage. It could very well be a protein concentration or protein localization problem. What concentration of LAT or ADAP protein is available to bind to SLP-76 the time of stimulation? Studies examining how those pathways are preferentially activated and their relative contribution to the

Pro1 transcriptional landscape are, in my opinion, the next step to understanding SLP-76's role in Ly49 Pro1 regulation.

SLP-76 and the Pro1 bi-directional switch: Does it open up the playing field or just send the players?

In Chapter II of my dissertation, I shed light on the role of SLP-76 in regulating the bi-directional Ly49G Pro1 promoter region during early NK cell development. Pro1 is active in developing NK cells and is thought to be regulated by transcription factors that bind upstream of a TATA box region. I discovered that SLP-76 is critical for driving forward transcription off the Pro1 promoter and it blocks reverse transcription. I, and others, have generally believed that transcription factors activated downstream of SLP-76 are the regulating factors over Pro1. My finding that SLP-76 KO NK cells have reduced H3K9 at Pro1 compared to controls is the first evidence of epigenetic modifications found to control Pro1, which may suggest another role for SLP-76 in early Ly49 receptor transcription.

The Pro2 region is regulated by epigenetic modifications, including DNA methylation and histone acetylation. Interestingly, while WT and SLP-76 KO NK cells showed a similar amount of H3K9 acetylation at Pro2 (even with a 90% reduction in Ly49 expression in mature NK cells), I found H3K9 acetylation at the Ly49G Pro1 region in splenic WT NK cells and a reduction of this open chromatin mark in SLP-76 KO NK cells. This is a puzzling finding, as it has been published [46,117] that Pro2 acetylation in mature NK cells equates with open chromatin at the locus and expression of Ly49s. In 2016, Stephen Anderson's group published an elegant study challenging work done by

Colin Brook's laboratory in 2015. Anderson's group cited that the Ly49 Pro1 region is not an enhancer region for Pro2 [42,117]. Anderson's team provided evidence that Pro1 is not active in mature NK cells as Brook's group had claimed, but if not, what is the functional meaning of my H3K9 Pro1 acetylation in immature NK cells?

First, H3K9 acetylation at Ly49G Pro1 could just be an indicating marker of Ly49 protein expression. If the chromatin at Pro1 was accessible for transcription during early development, there is protein expression in mature NK cells, which may be regulated through DNA methylation rather than histone acetylation at Pro2. Secondly, our data could point to the novel finding that SLP-76 assists in opening the chromatin landscape. SLP-76 may drive the activation of a/the histone acetyltransferase that acetylates H3K9 at Pro1. If this is the case, then perhaps the role of SLP-76 activation signals is to promote epigenetic modifications of the Ly49 promoter rather than activate transcriptional programs that bind to sense and antisense DNA strands on Ly49 genes. Finally, SLP-76 dependent signals could contribute to both open chromatin landscape as well as regulating transcription factors that bind at the Pro1 promoter region. It would be difficult to block SLP-76 downstream pathways to assess transcription factors binding to Pro1 promoter regions, as there is so much redundancy from the variety of activating receptors that can be stimulated, the cytokine signaling pathways that interface or activate the pathways independently, etc. It would be easy to test SLP-76's contribution to H3K9 acetylation using an *in vitro* NK cell differentiation assay. By transducing SLP-76 shRNA or scramble shRNA into hematopoietic stem cells, NK cells could be developed *in vitro* using the OP-9 stromal cell line to generate Ly49 receptor-expressing NK cells [20]. We could then treat the developing NK cells with a histone deacetylase (HDAC) inhibitor to see if SLP-76 knockdown NK cells were able to increase expression

of Pro1 forward transcripts. Results of this experiment may reveal whether or not SLP-76 is just required for transcription factor binding to Pro1 or potentially more. If HDAC inhibition of developing NK cells resulted in an increase of Ly49G Pro1 forward transcripts, it would tell us that SLP-76 is needed for Pro1 chromatin accessibility rather than SLP-76 dependent transcription factors. HDAC inhibition may cause extensive problems for multiple loci, in which case we would want to genetically alter the Pro1 locus by ensuring constitutive histone acetylation at Pro1 and then looking for SLP-76's contribution to the transcriptional landscape.

Role of DAG and IP_3 signaling pathways in Ly49 receptor acquisition

As I have described in detail, SLP-76 is an adaptor molecule downstream of NK cell activating receptors that binds PLCγ. PLCγ hydrolyzes PIP_2 into the second messengers DAG and IP_3. Therefore, if SLP-76 is critical for Ly49 receptor acquisition during NK cell development, one or both of these downstream pathways should contribute to the expression of the Ly49 inhibitory receptors. Published work on NK cells that are deficient in a negative regulator of DAG signaling, diacylglycerol kinase zeta (DGKζ), had normal percentages of NK cells expressing inhibitory receptors of the Ly49 receptor family [65]. This data suggests that transcription factors downstream of DAG signaling may not contribute to the Ly49 Pro1 promoter region. If increased DAG-signaling does not increase Ly49 receptor-expressing NK cells, it may suggest that these percentages of Ly49 receptor-expressing NK cells are fixed *or* that IP_3 signaling and SOCE influence Ly49 receptor expression.

I bred STIM1$^{flox/flox}$STIM2$^{flox/flox}$ animals to VavCre^{+} mice to generate STIM1/2 cDKO animals in which all hematopoietic cells were deficient in STIM1 and STIM2. I was unable to use the NKp46^{icre} mice for Ly49 receptor phenotyping, as deletion of STIM1 and STIM2 would occur too late in the Ly49 receptor acquisition process (which begins at the same time NKp46 expressed on NK cells). As NK cells regulate Ly49A, Ly49G2, and Ly49I intrinsically, we were able to assess these receptors with confidence in a VavCre model. STIM1/2 cDKO NK cells had mildly reduced Ly49 receptor-expressing NK cells, but did not phenocopy SLP-76 KO NK cells (Data Not Shown). The most affected NK cell populations were Ly49A and Ly49G2 expressing cells. In particular, there was a 50% reduction in the percentage of NK cells expressing Ly49A.

The Ly49 receptors are located next to each other in an NK cell cluster on chromosome 6. Perhaps since Ly49A is the first Ly49 to be acquired on an NK cell's surface, a large activating signal is required to initiate the transcription of this family of genes. It is plausible that because all the Ly49 genes sit next to each other, one activation signal is enough to open/unwind the chromatin of this region. A large activation signal through activating receptors could result in the mobilization of extracellular Ca^{2+} into the cytoplasm. A large influx of Ca^{2+} might be a signal required for opening of the transcriptional landscape, perhaps through histone acetylation at Pro1 Ly49A. While each Ly49 has its own Pro1 promoter, there may be different histone marks that open a region of he Ly49 gene cluster vs specific promoter regions of individual Ly49 genes. It is puzzling that while SLP-76 KO NK cells have no Ly49G2 receptor expression and reduced Ly49G Pro1 forward transcripts, DGKζ $^{-/-}$ NK cells and STIM1/2 cDKO NK cells have normal Ly49G2 expression as DAG and IP_3 are the two main pathways downstream of SLP-76. Perhaps, SOCE through STIM1 and STIM2 is

not required for Ly49 receptor transcription, but rather other Ca^{2+} sources through TRPC receptors or even ER release of Ca^{2+} is enough to activate the Pro1 promoter region. One could test ER release on this process by developing NK cells *in vitro* (as proposed earlier) and using SERCA pump inhibitors to block this release. There is a chance that long-term treatment with such inhibitors would cause other channels to open. Another approach might be to knockdown IP_3R using an shRNA to see if it would inhibit Pro1 forward transcripts of Ly49G-expressing NK cells.

PKC regulation of NK cell degranulation

During my doctoral research I generated further preliminary data where the role of SOCE in NK cell degranulation is unclear. Although primary murine NK cells do not require SOCE for NK cell degranulation and cytotoxicity, IL-2 expanded NK cells, also known as LAKs, do. Following stimulation with plate-bound antibodies against NK cell activating receptors, STIM1/2 cDKO LAKs were unable to express CD107a on their cell surface. Furthermore, STIM1/2 cDKO LAKs did not kill YAC-1 target cells, even at the highest NK cell to target ratio—40:1 (Appendix II). This was a surprising finding specifically because we determined in Chapter III that MTOC and cytotoxic granule polarization) is intact in STIM1/2 cDKO NK cells. In fact, STIM1/2 cDKO LAKs also have normal MTOC polarization (Data Not Shown). Normal polarization of the MTOC but reduced killing in STIM1/2 cDKO LAKs points to the processes of granule fusing, docking, or release as a STIM1/2-dependent process in IL-2-expanded LAKs. This finding did not apply to all cytokine expanded NK cells, as polyI:C activated STIM1/2

cDKO NK cells (expanded through up-regulation of Type I interferons) preserved their ability to degranulate following activating receptor-mediated stimulation (Appendix III).

In Chapter III, I demonstrate that PMA, a chemical analog of DAG, induces the expression of CD107a on primary murine NK cells. Furthermore, inhibitor data suggests that PKC activation contributes to SOCE-independent degranulation. This finding is substantiated by previously published research on the role of PKC and T cell degranulation as well as my own preliminary experimental findings. PKC isozymes are critical for degranulation and killing of tumor cells [139,140,147,148]. Based on what is known about DAG signals driving PKC activation at the plasma membrane, I suspect that PKC might be critical for proteins critical to vesicle docking, fusion, or release[141,143]. PKC family members differ in their requirement for Ca^{2+} binding. There are three Ca^{2+}-dependent PKCs (PKCα and PKCβI and II) and multiple Ca^{2+}-independent isozymes (e.g.PKCθ and PKCδ). Blocking the Ca^{2+}-dependent PKCs using the inhibitor Gö6979, which selectively inhibits PKCα and PKCβI, blocks NK cell degranulation in primary murine NK cells (Data not shown). This might suggest that Ca^{2+}-dependent PKCs are needed for NK cell cytotoxicity, implying a role for Ca^{2+} in the process. Although it seems at odds, this finding may still be in line with my STIM1/2 cDKO NK cell data. Because STIM1/2 cDKO NK cells release Ca^{2+} from the ER stores into the cytoplasm, that Ca^{2+} could be enough to trigger Ca^{2+}-dependent PKC activation. In preliminary experiments, I found that treatment of primary murine NK cells with the IP_3 inhibitor 2-APB (2-Aminoethoxydiphenyl borate) dramatically reduced the percentage of $CD107a^+$ NK cells after stimulation through activating receptors [149].

On the other hand, DAG signaling directly activates PKC, and could be the chief driver of NK cell degranulation regardless of the presence of Ca^{2+}. PMA stimulation of

primary murine NK cells causes them to degranulate. PMA stimulation of WT LAKs does *not* induce degranulation, a finding that has stumped NK cell biologists for decades. This observation has never been given proper investigation, but when my preliminary data suggested that LAKs require sustained Ca^{2+} for degranulation, I examined if NK cells express different amounts of PKC isozymes. I originally hypothesized that LAKs might utilize Ca^{2+}-dependent PKCs for degranulation while primary NK cells utilized Ca^{2+}-independent PKCs. Surely enough, multiple PKC isozymes are down-regulated in LAKs (PKCθ,α,δ, βI) as assessed by western blot and an initial qPCR experiment (Data Not Shown), which may be why these cells are unable to degranulate upon PMA stimulation. Furthermore, without PKCs, the LAKs may not be able to initiate CD107a expression on NK cells following activating receptor stimulation, while PKC-expressing primary NK cells can. These initial observations are intriguing and might uncover differences in how NK cells use PKC-dependent pathways to initiate the degranulation of lytic granules. It is conceivable that NK cells even require PKC isozymes at different stages of target cell recognition and cytolysis than their T cell counterparts. These questions will be explored in the lab after my departure.

Applications of this research to NK cells in the clinic

The research in this dissertation adds a great deal of knowledge to the NK cell field about signaling pathways that govern both NK cell development and effector function. While the current state of the research is just starting to branch out of murine models into human NK cell work, there are many exciting ways in which my research could impact the future directions of using NK cells as therapeutics, specifically in the settings

of cancer. Although not a usable model in the clinic now, clinician scientists are able to grow fully functional NK cells outside the body from $CD34^+$ fetal cord blood. In parallel, researchers are improving ways to genetically modify NK cells using lentiviral techniques and electroporation. The research on signaling pathways that govern inhibitory receptor acquisition in early NK cell development could help design personalized KIR repertoires for patient benefit. During NK cell differentiation from $CD34^+$ cord blood, cells may be manipulated through activating or inhibiting certain signaling pathways (lentiviral transduction or commercially available inhibitors). Depending on what signals are received, this could influence the inhibitory KIR receptors that are expressed. Additionally, if a situation of KIR-negativity was required on allogeneic NK cells for re-transplantation, lentiviral knockdown of SLP-76 could be used to generate a KIR^- population.

NK cells are often re-infused into patients following bone marrow transplant (BMT) or organ transplant as a way to fight off residual cancer cells. With transplantation though, comes treatment of patients with many immunosuppressive drugs that alter major signaling pathways not only in NK cells but also T cells. This is to blunt the activation of T cells in hopes of eliminating symptoms of GVHD. These drugs and inhibitors will ultimately affect the repertoire of inhibitory receptors on NK cells, which could be problematic for tumor cell clearance or viral infection clearance. The information this dissertation research adds to the NK cell field's knowledge of influences on inhibitory receptor repertoire may guide clinicians to different types of immunosuppressants or different timings to positively impact NK cell repertoire.

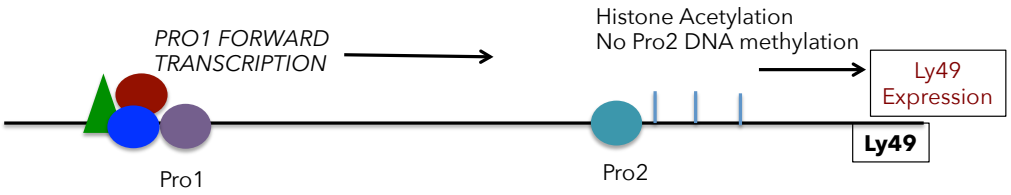
Lastly, research showing NK cell cytotoxicity in the absence of SOCE is important, novel information for the same reason as above. Immunosuppressive drugs,

like cyclosporine A, block effects of SOCE like NFAT activation. Short term, this has no impact on NK cell degranulation as it is a transcription-independent process. There is a great potential that through DAG-mediated signaling, and even in an environment with suppressed SOCE, NK cells are able to function through cell-mediated killing to eliminate target cells. This will most likely be most applicable to cancer, as inflammatory cytokine recruitment of T cells is extremely important for clearance of viral infection, but the benefits will be important for all NK cell target cells.

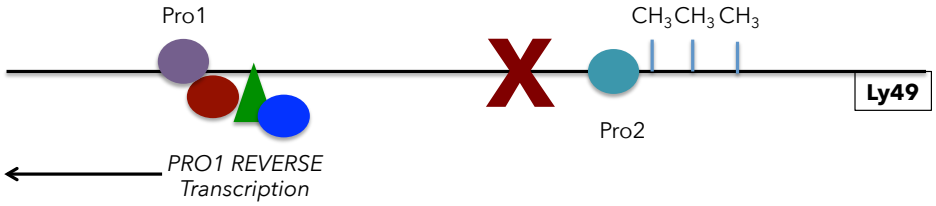
In conclusion, the work presented in this thesis demonstrates that activation signals through NK cell activating receptors initiate transcription of Ly49 or KIR inhibitory receptors on NK cells. The repertoire of inhibitory receptors is critical for NK cell function and recognition of target cells. Additionally, this work demonstrates that NK cells may not need sustained Ca^{2+} signals to perform all their important effector functions, such as degranulation. Ca^{2+}-independent signals, through DAG, are sufficient to drive NK cell degranulation and the cell-mediated killing of target cells. NK cells are still a young and mysterious cell type, so many questions remain as to how they function, what controls their development, and how can clinicians and research scientists manipulate them for patient benefit. Further studies will be required to answer remaining questions about NK cell signaling and transition our knowledge of these signaling impacts on the NK cell population into clinical therapies.

APPENDIX I

A) Ly49 Pro1 Forward Transcription- Ly49 expression

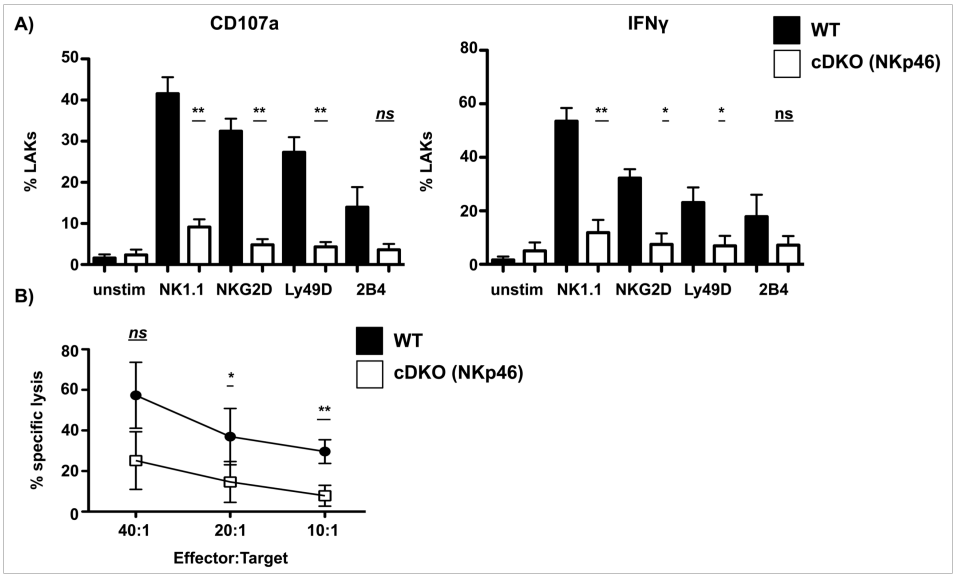


B) Ly49 Pro1 Reverse Transcription- No Ly49 expression



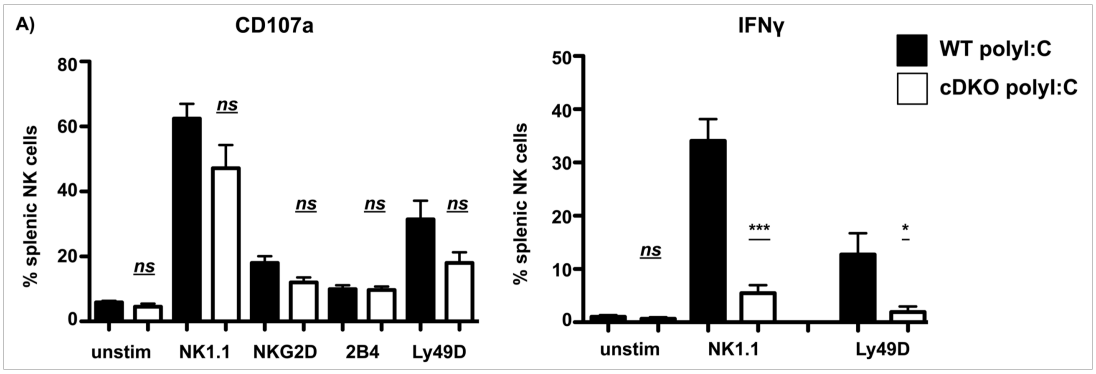
APPENDIX I: Regulation of Ly49 receptor transcription by two promoter regions. A) The Pro1 promoter region is active in immature NK cells and is a bi-directional promoter that is regulated by transcription factors. When transcription factors bind to Pro1 on the sense DNA strand, the Pro2 promoter (active in mature NK cells) opens. Histone acetylation occurs at Pro2 as well as the active demethylation of Pro2 DNA. This leads to Ly49 receptor gene expression. This is **forward transcription.** B) If transcription factors bind on the anti-sense strand in a different confirmation than what binds to the sense DNA strand, **reverse transcription** occurs. Histones at Pro2 remain unacetylated and the DNA is highly methylated, resulting in no Ly49 receptor transcription and subsequent protein expression.

APPENDIX II



APPENDIX II: IL-2-expanded NK cells (LAKs) require SOCE for degranulation and cytotoxicity. (A) WT or STIM1/2 cDKO DX5^{+} splenocytes were MACS purified and expanded *in vitro* with 1000U/mL human recombinant IL-2 for 5-6 days. LAKs were rested in IL-2-free media for 2 hours and then were stimulated for 6 hours by plate-bound activating receptor antibodies. The fraction of CD107a and IFNγ-expressing NK cells are represented as mean ± SEM of n=3-4 mice. (B) WT (black) and STIM1/2 cDKO (white) LAKs (rested for 2 hours without IL-2) were co-cultured with luciferase^{+} YAC-1 cells at various E:T ratios. % specific lysis is shown as mean ± SEM n= 3 mice/genotype or condition. Statistics were determined by using unpaired (A) or paired (B) Student's t test p<0.05, **p<0.01, ***p<0.001, and ns=not significant. Ly49D-stimulated NK cells are pre-gated on Ly49D^{+} NK cells.

APPENDIX III



APPENDIX III: Poly(I:C) activated STIM1/2 cDKO NK cells degranulate following activating receptor stimulation. (A) WT or STIM1/2 cDKO mice were i.p. injected with 250μg polyinosinic-polycytidylic acid [poly(I:C)]. 18 hours after injection, splenocytes were harvested and stimulated for 6 hours by plate-bound activating receptor antibodies. The fraction of CD107a and IFNγ-expressing NK cells are represented as mean ± SEM of n=4 mice. Statistics were determined by using Student's t test $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and ns=not significant. Ly49D-stimulated NK cells are pre-gated on $Ly49D^{+}$ NK cells.

APPENDIX IV: Materials and Methods

Mice

Mice were housed in pathogen-free conditions and treated in strict compliance with the Institutional Animal Care and Use Committee regulations at the University of Pennsylvania. In Chapter II: C57BL/6 (CD45.2^{+}), B6.SJL (CD45.1^{+}), and β2m KO mice were purchased from The Jackson Laboratory or Charles River Laboratories. LAT1/2 DKO, ADAP KO, and SLP-76 KO mice have been previously described and were bred in our facility [150-152]. SLP-76 KO mice have been ~3 times backcrossed to C57BL/6 mice due to embryonic lethality of fully backcrossed mice and thus, littermate controls were used for all experiments. LAT1/LAT2/ADAP TKO mice were generated by crossing LAT1/2 DKO mice to ADAP KO mice. SLP-76.B10D2 KO mice were generated by crossing B10.D2 mice to SLP-76 KO mice and screening for H-2^{d} alleles. All mice were sacrificed and analyzed between 10-12 weeks of age. In Chapter III: STIM1$^{flox/flox}$ STIM2$^{flox/flox}$ mice were generated and generously provided by Dr. Anjana Rao, La Jolla Institute for Allergy and Immunology (LIAI), Dr. Patrick Hogan, LIAI, and Dr. Masatsugu Oh-hora, Kyushu University, [71]. These animals were bred to NKp46$^{iCre/wt}$ transgenic mice [153]. Age-matched littermate or vendor purchased C57BL/6 (WT) control animals were used for all experiments using STIM1$^{flox/flox}$ STIM2$^{flox/flox}$ (cDKO), STIM1 cKO, and STIM2 cKO mice. Mice were sacrificed and analyzed between 8–12 weeks of age.

Reagents

For Chapter II: All reagents were purchased from Sigma-Aldrich (St. Louis, MO) and unless otherwise specified. Cytokines were purchased from Peprotech (Rocky Hill, NJ).

Antibodies (Abs) for cell stimulation were purchased from BioXcell (Malaysia) or Biolegend. Abs for flow cytometry were purchased from Biolegend, eBiosciences, BD Biosciences, and Molecular Probes. The following Ly49 receptor antibodies/clones were used: Anti-Ly49A (YE1/48.10.6), anti-Ly49G2 (4D11), anti-Ly49I (YL1-90), anti-Ly49D (4E5), anti-Ly49H (3D10), and anti-Ly49C/I (5E6) from BD Pharmigen. Anti-Ly49C (4LO3311) was purchased from the UCSF Cell Culture Facility (San Francisco, CA). For Chapter III: Antibodies for NK cell stimulations, phenotyping, and MACS purification were purchased from BD Pharmingen (San Diego, CA): NK1.1 APC (PK136), CD244.2 Biotin (2B4), purified Ly49D purified (4E5), and CD4 Pacific Blue (RM4-5); BioLegend (San Diego, CA): CD8 BV421 (53-6.7), CD49b Biotin (DX5), CD107a PE (1D4B), and Streptavidin BV421; eBioscience (San Diego, CA): CD3ε FITC (17A2), CD3ε APCeFl780 (145-2C11), NKp46 PerCPeFl710 (29A1.4), CD49b FITC (DX5), IFNγ PE-Cy7 (XMG1.2), NKG2D APC (CX5), and Ly49D APC (4E5); BioXcell: purified NKG2D (HMG2D) and purified NK1.1 (PK136); and Molecular Probes, Invitrogen (Carlsbad, CA): Live/Dead Aqua or Near-IR. Antibodies used for confocal microscopy: Rat anti-alpha tubulin (clone YL1/2, Serotec), goat anti-Cathepsin D (R&D systems AF1029), CMAC (Invitrogen), Alexa Fluor 488 donkey anti-rat IgG (Invitrogen A21208), Alexa Fluor 568 donkey anti-mouse IgG (Invitrogen A10037), and Alexa Fluor 647 donkey anti-goat IgG (Invitrogen A21447). Inhibitors: Akt Inhibitor VIII, Isozyme-Selective, Akti-1/2 was purchased from Calbiochem (Darmstadt, Germany). Gö6983 was purchased from Tocris Bioscience (Bristol, United Kingdom). U0126 was purchased from Cell Signaling (Danvers, MA). Cyclosporine A and Actinomycin D were purchased from Sigma (St. Louis, MO). Inhibitors were reconstituted following manufacturer's instructions.

Flow cytometry, cell sorting, and data analysis

Cells were stained with antibodies against cell-surface antigens and LIVE/DEAD cell stain at 4°C for 20 minutes. Intracellular staining was performed using the Cytofix/Cytoperm Fixation and Permabilization Kit (BD Pharmingen) per manufacturer instructions. Flow cytometry was performed with a FACS Canto flow cytometer (BD Biosciences), and cell sorting was performed using a FACSAria (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR) and Simplified Presentation of Incredibly Complex Evaluations (SPICE- NIAID, Bethesda, MD). Statistical analysis was performed using Prism (GraphPad, San Diego, CA) computer software.

Primary NK cell cultures and stimulations

Total splenocytes were plated in 96-well plates in NK-cell media (MEMα [Invitrogen] supplemented with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES and 1 x 10^{-5} β-ME) with human IL-2 (1,000 U/mL) on plate-immobilized (20 μg/mL) anti-NK1.1, anti-NKG2D, anti-CD244, anti-Ly49D or with soluble PMA (100 ng/mL), ionomycin (1 ug/mL) or a combination of both in the presence of monensin (eBiosciences) and anti-CD107a-PE for 6 h at 37°C. For experiments using Ca^{2+}-free media, splenocytes were plated in either Ca^{2+}-sufficient media (MEM with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES and 1 x 10^{-5} β-ME) or Ca^{2+} free media (S-MEM with 10% FBS, 1% penicillin/streptomycin, 1% L-Glutamine, 250uM EGTA, 10 mM HEPES and 1 x 10^{-5} β-ME). Following stimulation, splenocytes were assessed using flow cytometry and gated on live, singlet NK cells ($CD3\varepsilon^{-}NKp46^{+}DX5^{+}$) unless described otherwise and assessed for CD107a and intracellular IFNγ expression.

Generation of mixed BM chimeras

SLP-76 KO ($CD45.1^+$) bone marrow (BM) cells were T/NK cell depleted by CD3 and NK1.1 magnetic bead depletion (Miltenyi Biotec). T/NK-cell depleted BM cells from $CD45.1^+CD45.2^+$ wild-type (competitor) mice were mixed at a 2:1 ratio with the SLP-76 KO BM. Cells were injected intravenously into lethally irradiated (950 cGy) $CD45.2^+$ recipient mice. Mixed BM chimeric mice were analyzed by flow cytometry 10-12 weeks post injection.

RT-PCR

RNA was purified from equivalent cell numbers of sorted splenic and BM NK cells (RNeasy kit-Qiagen). cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen) kit and performed using manufacturer's instructions. Primers for SLP-76, Ly49G2, and Ly49I (Applied Biosystems) were used. The reaction was performed on the Applied Biosystems StepOnePlus Real Time PCR System (Carlsbad, CA), and ΔΔ-CT method was employed. Results were normalized to the housekeeping gene GAPDH.

RT-PCR of Ly49G2 Pro1 Transcripts

Total RNA was purified from 100,000 sorted cells with the RNeasy Micro Kit (Qiagen), and cDNA synthesis was carried out using Random Hexamer primer, Taqman Reverse

Transcription Reagents kit (Applied Biosystems) according to the manufacturer's instructions. The primers used in the Ly49-specific qRT-PCR assay were: Ly49g-Pro1 forward primer (5′-CAAGTGATCAGCCTATTCTTGTG-3′); Ly49g-Pro1 reverse primer (5′-CTTGTGTGAGTTTTGTACTTCAG -3′); Ly49g-Pro1as forward primer (5′-CACTGCCTTATATGCCTAAACAC-3′); Ly49g-Pro1as reverse primer (5′-GACTTCATGACTAGTTACTGG-3′); β-Actin forward primer (5′-CCTGGCACCCAGCACAAT-3′); and β-Actin reverse primer (5′-GGGCCGGACTCGTCATACT-3′). Reactions were carried out using the FastStart SYBR Green Master kit (Roche Diagnostics, Indianapolis, IN, USA) on the 7300 Real-Time PCR System (Applied Biosystems). The qRT-PCR was performed in duplicate and was repeated in at least three separate experiments using the following conditions. Reaction mixtures contained 12.5μL of SYBR Green master mix, 2 pmoles each of forward and reverse primers and 5 ng cDNA. Thermocycler conditions included initial denaturation at 50 and 95 °C (10 min each), followed by 40 cycles at 95 °C (15 s) and 60 °C (1 min). Melting curve analyses were performed to verify the amplification specificity. Relative quantification of gene expression was performed according to the ΔΔ-CT method using the StepOne Software 2.0 (Applied Biosystems). The results were normalized to the housekeeping gene β-Actin.

Chromatin Immunoprecipitation (ChIP)

WT and SLP-76 KO splenic NK cells (CD3ε $^{-}$NK1.1^{+}NKp46^{+}DX5^{+}) were sorted by flow cytometry, cross-linked for 10 min with 1% formaldehyde in cold PBS buffer, and subsequently quenched with 125 mM glycine for 5 min. The cells were pelleted by

centrifugation at 470× g for 10 min and washed with PBS containing protease inhibitor cocktail. After centrifugation, the supernatant was discarded, and the cell pellet was stored at −80°C. DNA shearing was performed with a chromatin immunoprecipitation enzymatic shearing kit (Chromatrap, Ashland, VA) following the manufacturer's instructions. Immunoprecipitation was performed with the True MicroChIP kit (Diagenode, Denville, NJ, USA) using a CHIP grade antibody against H3K9ac (Diagenode). A non-specific rabbit IgG was used as a negative control. All ChIP steps were performed in Eppendorf 1.5-ml DNA LoBind Tubes (Eppendorf, Hamburg, Germany). The specific primer sequences used in ChIP-qPCR were as follows: Ly49g Pro-1, forward, 5'- CCCATCAAGGACTATGTGTTTAGG-3', reverse,5'-ATGGTAAACTTCACAGATCTTAGG-3'; Ly49g Pro-2, forward, 5'-CACAGGAATCACTTCTCAGTAGA-3',reverse, 5'-ATCGAGCGCTCACATAACACTAT-3';Ly49e Pro-2, forward, 5'-GCAATTTCCTCCTTTTGCTTAGATA-3',reverse, 5'-TGGAGGGAAAAGTTGGGTGAAA-3'. The precipitated DNA fractions were quantified by real-time PCR with the FastStart Universal SYBR Green Master Kit (Roche Diagnostics, Indianapolis, IN, USA) using 7300 Real-Time PCR System; (Applied Biosystems). The results were normalized as the percentage of the input (%input) from Ct values. The experiments were repeated three times.

$CD34^+$ cell retroviral transduction and *in vitro* development

The use of all human tissue was approved by the Committee on the Use of Human Subjects in research at that University of Minnesota (Minneapolis, MN), and informed consent was obtained in accordance with the Declaration of Helsinki. Lentivirus containing either scramble control or SLP-76 shRNA in pGIPZ vectors was packaged in

293T cells using PAX2 and pMDG.2 plasmids (Open Biosystems, Lafayette, CO). A pool of four SLP-76 shRNA vectors (clones V2LHS_62885, V3LHS_364697, V2LHS_62886 and V3LHS_364699) was used. $CD34^+$ hematopoietic cells were isolated from umbilical cord blood by double-column positive selection using anti-CD34 microbeads (Miltenyi Biotec). Cells were transduced with lentivirus by spin transduction, and $CD34^+GFP^+$ cells were sorted. $CD34^+GFP^+$ cells were cultured for 21 days on the EL08-1D2 fetal stromal line [154]. The culture media and cytokines used for human NK cell differentiation are published [155].

Immunofluorescence Microscopy and MTOC Measurements

YAC-1 cells were labeled with 20 μM CMAC in serum-free RPMI (Gibco) for 30 minutes at 37 degrees, washed twice with 10% FBS RPMI, and mixed 1:2 with NK cells in polypropylene round-bottom tubes in serum-free RPMI. Conjugation was induced by centrifugation for 5 minutes. Pelleted cells were incubated together for 10 minutes, gently suspended, added to Poly-L lysine coated 12-mm coverslips to adhere for 10 minutes. Cells were subsequently fixed with 3% paraformaldehyde in PBS, quenched, permeabilized with 0.1% Triton X-100, and blocked in PBS with 0.01% saponin and 0.25% gelatin (PSG). Cells were stained with primary antibodies in PSG, washed 5 times with PSG, stained with secondary antibodies in PSG, washed an additional 5 times with PSG, washed once with milli-Q H_2O, and mounted on slides with mowiol mounting media (Sigma Aldrich). Cell conjugates were imaged using a 63x PlanApo 1.4 NA objective on an Axiovert 200 (Carl Zeiss) equipped with a spinning disk confocal system (UltraView ERS 6; Perkin Elmer). Images were collected using an ORCA-ER

camera (Hamamatsu Photonics) using Volocity acquisition software (v6.1.1; PerkinElmer). MTOC to synapse measurements were performed manually as follows: the border of the YAC-1 cell was determined on an experimental basis based on CMAC fluorescence intensity. The MTOC was defined as the pixel with the brightest intensity in the 488 channel (anti-tubulin). MTOC to synapse measurement is the distance from the MTOC pixel to one pixel within the YAC-1 border. All measurements were made with Volocity imaging software. Image preparation was performed with ImageJ.

Bioluminescent Cytotoxicity Assay

Splenocytes from STIM1/STIM2 cDKO or control mice were stained with bioyinylated anti–DX5 antibody and enriched using anti-biotin MACS beads (Miltenyi Biotec). Luciferase-expressing target cells (Yac-1 or P815) were incubated at various effector to target (E:T) ratios with $DX5^{+}$-enriched splenocytes for 6 hours at 37°C in the presence of 1000 U/mL human recombinant IL-2 as previously described [156]. For the P815 target cells, anti-PK136 (NK1.1) was added at 5 μg/mL for re-directed lysis prior to effector cell plating. Lucerifase activity was detected using the IVIS Lumina II imager, and the % specific lysis was calculated as follows: [(minimum-test condition)/(minimum-maximum)] x 100.

RMA vs RMA-S Tumor Challenges

For short-term tumor rejection assays, RMA and RMA-s cells were labeled with CFSE and Cell Trace Violet respectively and injected i.v. at a 1:3 ratio into C57BL/6, cDKO ($NKp46^{iCre/WT}$) or NK-cell depleted C57BL/6 mice. For NK depletion, C57BL/6 mice were i.p. injected with 200μg of anti-PK136 (NK1.1) 24 hours before tumor challenge. Animals

were sacrificed 18 hours post tumor cell injection and the presence of splenic tumor cells was determined by flow cytometry. The ratio of RMA-s ($CFSE^+$)/RMA (Cell Trace $Violet^+$) cells was calculated. For long-term tumor engraftment assays, 1 x 10^6 RMA or RMA-s cells were subcutaneously injected into the left abdomen of C57BL/6, cDKO ($NKp46^{iCre/WT}$), or NK-cell depleted C57BL/6 mice. Tumors were left to grow for 11 days. On Day 11, tumors were excised and weighed.

BIBLIOGRAPHY

1. Paul, W. E. *Bridging innate and adaptive immunity. Cell* **147,** 1212–1215 (2011).
2. Janeway, C. A. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20,** 197–216 (2002).
3. Jiang, H. & Chess, L. Regulation of immune responses by T cells. *N. Engl. J. Med.* **354,** 1166–1176 (2006).
4. Cooper, M. D. The early history of B cells. *Nature Publishing Group* **15,** 191–197 (2015).
5. Kiessling, R., Klein, E., Pross, H. & Wigzell, H. 'Natural' killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5,** 117–121 (1975).
6. Di Santo, J. P. NATURAL KILLER CELL DEVELOPMENTAL PATHWAYS: A Question of Balance. *Annu. Rev. Immunol.* **24,** 257–286 (2006).
7. Cudkowicz, G. Genetic control of resistance to allogeneic and xenogeneic bone-marrow grafts in mice. *Transplant. Proc.* **7,** 155–159 (1975).
8. Karre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* **319,** 675–678 (1986).
9. Kondo, M., Scherer, D. C., King, A. G., Manz, M. G. & Weissman, I. L. Lymphocyte development from hematopoietic stem cells. *Curr. Opin. Genet. Dev.* **11,** 520–526 (2001).
10. Yu, J., Freud, A. G. & Caligiuri, M. A. Location and cellular stages of natural killer cell development. *Trends in Immunology* **34,** 573–582 (2013).
11. Rosmaraki, E. E. *et al.* Identification of committed NK cell progenitors in adult murine bone marrow. *Eur. J. Immunol.* **31,** 1900–1909 (2001).
12. Fehniger, T. A. *et al.* Acquisition of Murine NK Cell Cytotoxicity Requires the Translation of a Pre-existing Pool of Granzyme B and Perforin mRNAs. *Immunity* **26,** 798–811 (2007).
13. Lucas, M., Schachterle, W., Oberle, K., Aichele, P. & Diefenbach, A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* **26,** 503–517 (2007).
14. Soudja, S. M., Ruiz, A. L., Marie, J. C. & Lauvau, G. Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity* **37,** 549–562 (2012).
15. Kennedy, M. K. *et al.* Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *Journal of Experimental Medicine* **191,** 771–780 (2000).
16. Vosshenrich, C. A. J. *et al.* Roles for Common Cytokine Receptor -Chain-Dependent Cytokines in the Generation, Differentiation, and Maturation of NK Cell Precursors and Peripheral NK Cells in Vivo. *J. Immunol.* **174,** 1213–1221 (2005).
17. Kim, S. *et al.* In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol* **3,** 523–528 (2002).
18. Chiossone, L. *et al.* Maturation of mouse NK cells is a 4-stage developmental program. *Blood* **113,** 5488–5496 (2009).

19. Wu, Q. *et al.* Signal via lymphotoxin-beta R on bone marrow stromal cells is required for an early checkpoint of NK cell development. *J.I.* **166,** 1684–1689 (2001).
20. Williams, N. S. *et al.* Differentiation of NK1.1+, Ly49+ NK Cells from flt3 + Multipotent Marrow Progenitor Cells. *J.I.* **163,** 2648–2656 (1999).
21. Roth, C., Carlyle, J. R., Takizawa, H. & Raulet, D. H. Clonal acquisition of inhibitory Ly49 receptors on developing NK cells is successively restricted and regulated by stromal class I MHC. *Immunity* **13,** 143–153 (2000).
22. Gordon, S. M. *et al.* The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. *Immunity* **36,** 55–67 (2012).
23. The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. **36,** 55–67 (2012).
24. Colucci, F. *et al.* Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. *Nat Immunol* **3,** 288–294 (2002).
25. Gilfillan, S., Ho, E. L., Cella, M., Yokoyama, W. M. & Colonna, M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol* **3,** 1150–1155 (2002).
26. Bloch-Queyrat, C. *et al.* Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn. *Journal of Experimental Medicine* **202,** 181–192 (2005).
27. May, R. M. *et al.* Murine natural killer immunoreceptors use distinct proximal signaling complexes to direct cell function. *Blood* **121,** 3135–3146 (2013).
28. Clements, J. L., Ross-Barta, S. E., Tygrett, L. T., Waldschmidt, T. J. & Koretzky, G. A. SLP-76 expression is restricted to hemopoietic cells of monocyte, granulocyte, and T lymphocyte lineage and is regulated during T cell maturation and activation. *J. Immunol.* **161,** 3880–3889 (1998).
29. Hidano, S. *et al.* Distinct regulatory functions of SLP-76 and MIST in NK cell cytotoxicity and IFN-gamma production. *International Immunology* **20,** 345–352 (2008).
30. Yokoyama, W. M., Kehn, P. J., Cohen, D. I. & Shevach, E. M. Chromosomal location of the Ly-49 (A1, YE1/48) multigene family. Genetic association with the NK 1.1 antigen. *J. Immunol.* **145,** 2353–2358 (1990).
31. Ortaldo, J. R. Ly-49 receptor expression and functional analysis in multiple mouse strains. *J. Leukoc. Biol.* 1–9 (1999).
32. Schenkel, A. R., Kingry, L. C. & Slayden, R. A. The ly49 gene family. A brief guide to the nomenclature, genetics, and role in intracellular infection. *Frontiers in Immunology* **4,** 90 (2013).
33. Kim, S. *et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436,** 709–713 (2005).
34. Bélanger, S. *et al.* Impaired natural killer cell self-education and 'missing-self' responses in Ly49-deficient mice. *Blood* **120,** 592–602 (2012).
35. Carlyle, J. R. *et al.* Evolution of the Ly49 and Nkrp1 recognition systems. *Seminars in Immunology* **20,** 321–330 (2008).
36. Gosselin, P. *et al.* Induction of DAP12 phosphorylation, calcium mobilization, and cytokine secretion by Ly49H. *J. Leukoc. Biol.* **66,** 165–171 (1999).
37. Smith, K. M., Wu, J., Baker, A. B. H., Phillips, J. H. & Lanier, L. L. Cutting Edge: Ly-49D and Ly-49H Associate with Mouse DAP12 and Form Activating Receptors. *J.I.* **161,** 7–10 (1998).
38. D'Ambrosio, D. *et al.* Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by Fc gamma RIIB1. *Science* **268,** 293–297 (1995).
39. Dorfman, J. R. & Raulet, D. H. Acquisition of Ly49 receptor expression by developing natural killer cells. *Journal of Experimental Medicine* **187,** 609–618 (1998).
40. Kubota, A., Kubota, S., Lohwasser, S., Mager, D. L. & Takei, F. Diversity of NK cell receptor repertoire in adult and neonatal mice. *J. Immunol.* **163,** 212–216 (1999).

41. Gays, F., Martin, K., Kenefeck, R., Aust, J. G. & Brooks, C. G. Multiple cytokines regulate the NK gene complex-encoded receptor repertoire of mature NK cells and T cells. *J. Immunol.* **175,** 2938–2947 (2005).
42. McCullen, M. V. *et al.* Analysis of Ly49 gene transcripts in mature NK cells supports a role for the Pro1 element in gene activation, not gene expression. **17,** 349–357 (2016).
43. Saleh, A., Makrigiannis, A. P., Hodge, D. L. & Anderson, S. K. Identification of a novel Ly49 promoter that is active in bone marrow and fetal thymus. *J. Immunol.* **168,** 5163–5169 (2002).
44. Tanamachi, D. M. *et al.* Genomic Ly49A transgenes: basis of variegated Ly49A gene expression and identification of a critical regulatory element. *J.I.* **172,** 1074–1082 (2004).
45. Saleh, A. *et al.* Identification of probabilistic transcriptional switches in the Ly49 gene cluster: a eukaryotic mechanism for selective gene activation. *Immunity* **21,** 55–66 (2004).
46. Rouhi, A., Gagnier, L., Takei, F. & Mager, D. L. Evidence for epigenetic maintenance of Ly49a monoallelic gene expression. *J. Immunol.* **176,** 2991–2999 (2006).
47. Tu, M. M. *et al.* Ly49 family receptors are required for cancer immunosurveillance mediated by natural killer cells. *Cancer Res.* **74,** 3684–3694 (2014).
48. Orr, M. T. *et al.* Ly49H signaling through DAP10 is essential for optimal natural killer cell responses to mouse cytomegalovirus infection. *J. Exp. Med.* **206,** 807–817 (2009).
49. Thielens, A., Vivier, E. & Romagné, F. NK cell MHC class I specific receptors (KIR): from biology to clinical intervention. *Current Opinion in Immunology* **24,** 239–245 (2012).
50. Davies, G. E. *et al.* Identification of bidirectional promoters in the human KIR genes. *Genes Immun.* **8,** 245–253 (2007).
51. Lanier, L. L. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* **9,** 495–502 (2008).
52. Tassi, I. *et al.* Phospholipase C-gamma 2 is a critical signaling mediator for murine NK cell activating receptors. *J. Immunol.* **175,** 749–754 (2005).
53. Lanier, L. L. DAP10- and DAP12-associated receptors in innate immunity. *Immunological Reviews* **227,** 150–160 (2009).
54. Upshaw, J. L. & Leibson, P. J. NKG2D-mediated activation of cytotoxic lymphocytes: unique signaling pathways and distinct functional outcomes. *Seminars in Immunology* **18,** 167–175 (2006).
55. Diefenbach, A. *et al.* Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol* **3,** 1142–1149 (2002).
56. Nausch, N. & Cerwenka, A. NKG2D ligands in tumor immunity. *Oncogene* **27,** 5944–5958 (2008).
57. Kwon, H.-J. & Kim, H. S. Signaling for Synergistic Activation of Natural Killer Cells. *Immune Netw* **12,** 240 (2012).
58. Schatzle, J. D. *et al.* Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. *Proceedings of the National Academy of Sciences* **96,** 3870–3875 (1999).
59. Peterson, E. J., Clements, J. L., Ballas, Z. K. & Koretzky, G. A. NK cytokine secretion and cytotoxicity occur independently of the SLP-76 adaptor protein. *Eur. J. Immunol.* **29,** 2223–2232 (1999).
60. Kim, H. S. & Long, E. O. Complementary Phosphorylation Sites in the Adaptor Protein SLP-76 Promote Synergistic Activation of Natural Killer Cells. *Science Signaling* **5,** ra49–ra49 (2012).
61. Jackman, J. K. *et al.* Molecular Cloning of SLP-76, a 76-kDa Tyrosine

Phosphoprotein Associated with Grb2 in T cells. *Journal of Biological Chemistry* **270,** 7029–7032 (2001).
62. JB, W. *et al.* Phosphorylation of SLP-76 by the ZAP-70 Protein-tyrosine Kinase Is Required for T-cell Receptor Function. *Journal of Biochemistry* **271,** 19641–19644 (1996).
63. Koretzky, G. A., Abtahian, F. & Silverman, M. A. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat Rev Immunol* **6,** 67–78 (2006).
64. Jordan, M. S. & Koretzky, G. A. Coordination of receptor signaling in multiple hematopoietic cell lineages by the adaptor protein SLP-76. *Cold Spring Harb Perspect Biol* **2,** a002501–a002501 (2010).
65. Yang, E., Singh, B. K., Paustian, A. M. S. & Kambayashi, T. Diacylglycerol Kinase ζ Is a Target To Enhance NK Cell Function. *J.I.* **197,** 934–941 (2016).
66. Zhong, X.-P. *et al.* Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nat Immunol* **4,** 882–890 (2003).
67. Quann, E. J., Merino, E., Furuta, T. & Huse, M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol* **10,** 627–635 (2009).
68. Crabtree, G. R. Calcium, Calcineurin, and the Control of Transcription. *Journal of Biological Chemistry* **276,** 2313–2316 (2001).
69. Feske, S. ORAI1 and STIM1 deficiency in human and mice: roles of store-operated Ca. *Immunological Reviews* **231,** 189–209 (2009).
70. Quintana, A. *et al.* Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation. *EMBO J.* **30,** 3895–3912 (2011).
71. Oh-Hora, M. *et al.* Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nat Immunol* **9,** 432–443 (2008).
72. Bryceson, Y. T., March, M. E., Ljunggren, H.-G. & Long, E. O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107,** 159–166 (2006).
73. Orr, M. T., Murphy, W. J. & Lanier, L. L. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nature Publishing Group* **11,** 321–327 (2010).
74. Brodin, P., Lakshmikanth, T., Johansson, S., Karre, K. & Hoglund, P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* **113,** 2434–2441 (2009).
75. Joncker, N. T., Fernandez, N. C., Treiner, E., Vivier, E. & Raulet, D. H. NK Cell Responsiveness Is Tuned Commensurate with the Number of Inhibitory Receptors for Self-MHC Class I: The Rheostat Model. *J. Immunol.* **182,** 4572–4580 (2009).
76. Brodin, P., Kärre, K. & HOglund, P. NK cell education: not an on-off switch but a tunable rheostat. *Trends in Immunology* **30,** 143–149 (2009).
77. Joncker, N. T., Shifrin, N., Delebecque, F. & Raulet, D. H. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *Journal of Experimental Medicine* **207,** 2065–2072 (2010).
78. Elliott, J. M., Wahle, J. A. & Yokoyama, W. M. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *Journal of Experimental Medicine* **207,** 2073–2079 (2010).
79. Viant, C. *et al.* SHP-1-mediated inhibitory signals promote responsiveness and anti-tumour functions of natural killer cells. *Nat Commun* **5,** 5108 (2014).
80. Lowin-Kropf, B., Kunz, B., Beermann, F. & Held, W. Impaired natural killing of MHC class I-deficient targets by NK cells expressing a catalytically inactive form of SHP-1. *J. Immunol.* **165,** 1314–1321 (2000).
81. Gumbleton, M., Vivier, E. & Kerr, W. G. SHIP1 intrinsically regulates NK cell

signaling and education, resulting in tolerance of an MHC class I-mismatched bone marrow graft in mice. *J. Immunol.* **194,** 2847–2854 (2015).
82. Cheng, M., Chen, Y., Xiao, W., Sun, R. & Tian, Z. NK cell-based immunotherapy for malignant diseases. *Cellular and Molecular Immunology* **10,** 230–252 (2013).
83. Robertson, M. J. *et al.* Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. *Clin. Cancer Res.* **5,** 9–16 (1999).
84. Boiardi, A. *et al.* Loco-regional immunotherapy with recombinant interleukin-2 and adherent lymphokine-activated killer cells (A-LAK) in recurrent glioblastoma patients. *Cancer Immunol. Immunother.* **39,** 193–197 (1994).
85. Ma, H. L. *et al.* IL-21 Activates Both Innate and Adaptive Immunity to Generate Potent Antitumor Responses that Require Perforin but Are Independent of IFN. *J.I.* **171,** 608–615 (2003).
86. Hayes, R. L. *et al.* Improved long term survival after intracavitary interleukin-2 and lymphokine-activated killer cells for adults with recurrent malignant glioma. *Cancer* **76,** 840–852 (1995).
87. Miller, J. S. *et al.* Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* **105,** 3051–3057 (2005).
88. Ruggeri, L. *et al.* Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **295,** 2097–2100 (2002).
89. Ruggeri, L. *et al.* Natural killer cell alloreactivity for leukemia therapy. *J. Immunother.* **28,** 175–182 (2005).
90. Arai, S. *et al.* Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. *Cytotherapy* **10,** 625–632 (2008).
91. Tonn, T. *et al.* Treatment of patients with advanced cancer with the natural killer cell line NK-92. *Cytotherapy* **15,** 1563–1570 (2013).
92. Nagashima, S. *et al.* Stable transduction of the interleukin-2 gene into human natural killer cell lines and their phenotypic and functional characterization in vitro and in vivo. *Blood* **91,** 3850–3861 (1998).
93. Konstantinidis, K. V. *et al.* Targeting IL-2 to the endoplasmic reticulum confines autocrine growth stimulation to NK-92 cells. *Exp. Hematol.* **33,** 159–164 (2005).
94. Imamura, M. *et al.* Autonomous growth and increased cytotoxicity of natural killer cells expressing membrane-bound interleukin-15. *Blood* **124,** 1081–1088 (2014).
95. Glienke, W. *et al.* Advantages and applications of CAR-expressing natural killer cells. *Front Pharmacol* **6,** 21 (2015).
96. Lowin-Kropf, B., Kunz, B., Schneider, P. & Held, W. A role for the src family kinase Fyn in NK cell activation and the formation of the repertoire of Ly49 receptors. *Eur. J. Immunol.* **32,** 773–782 (2002).
97. Parry, D. A. *et al.* A homozygous STIM1 mutation impairs store-operated calcium entry and natural killer cell effector function without clinical immunodeficiency. *J. Allergy Clin. Immunol.* **137,** 955–7.e8 (2016).
98. Maul-Pavicic, A. *et al.* ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 3324–3329 (2011).
99. Ljunggren, H. G. & Karre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11,** 237–244 (1990).
100. Raulet, D. H. Acquisition of Ly49 Receptor Expression by Developing Natural Killer Cells. *Journal of Experimental Medicine* 1–10 (1998).
101. Gays, F., Koh, A. S. C., Mickiewicz, K. M., Aust, J. G. & Brooks, C. G. Comprehensive analysis of transcript start sites in ly49 genes reveals an unexpected relationship with gene function and a lack of upstream promoters. *PLoS ONE* **6,** e18475 (2011).
102. Rouhi, A. *et al.* Evidence for high bi-allelic expression of activating Ly49 receptors. *Nucleic Acids Research* **37,** 5331–5342 (2009).
103. Johansson, S. *et al.* Probing natural killer cell education by Ly49 receptor

expression analysis and computational modelling in single MHC class I mice. *PLoS ONE* **4,** e6046 (2009).
104. Held, W., Dorfman, J. R., Wu, M. F. & Raulet, D. H. Major histocompatibility complex class I-dependent skewing of the natural killer cell Ly49 receptor repertoire. *Eur. J. Immunol.* **26,** 2286–2292 (1996).
105. Salcedo, M. *et al.* Altered expression of Ly49 inhibitory receptors on natural killer cells from MHC class I-deficient mice. *J. Immunol.* **158,** 3174–3180 (1997).
106. Fahlén, L., Lendahl, U. & Sentman, C. L. MHC class I-Ly49 interactions shape the Ly49 repertoire on murine NK cells. *J. Immunol.* **166,** 6585–6592 (2001).
107. Koretzky, G. A., Abtahian, F. & Silverman, M. A. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat Rev Immunol* **6,** 67–78 (2006).
108. Rajasekaran, K. *et al.* Signaling by Fyn-ADAP via the Carma1–Bcl-10–MAP3K7 signalosome exclusively regulates inflammatory cytokine production in NK cells. *Nat Immunol* **14,** 1127–1136 (2013).
109. Rouhi, A., Brooks, C. G., Takei, F. & Mager, D. L. Plasticity of Ly49g expression is due to epigenetics. *Molecular Immunology* **44,** 821–826 (2007).
110. Horowitz, A. *et al.* Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med* **5,** 208ra145–208ra145 (2013).
111. Sun, J. C., Beilke, J. N. & Lanier, L. L. Adaptive immune features of natural killer cells. *Nature* **457,** 557–561 (2009).
112. Fernandez, N. C. *et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat. Med.* **5,** 405–411 (1999).
113. Lee, G. A. *et al.* Different NK Cell Developmental Events Require Different Levels of IL-15 Trans-Presentation. *J. Immunol.* **187,** 1212–1221 (2011).
114. Luckashenak, N. A., Ryszkiewicz, R. L., Ramsey, K. D. & Clements, J. L. The Src homology 2 domain-containing leukocyte protein of 76-kDa adaptor links integrin ligation with p44/42 MAPK phosphorylation and podosome distribution in murine dendritic cells. *J. Immunol.* **177,** 5177–5185 (2006).
115. Lampe, K. *et al.* Slp-76 is a critical determinant of NK-cell mediated recognition of missing-self targets. *Eur. J. Immunol.* **45,** 2072–2083 (2015).
116. Pascal, V. & Anderson, S. B p50/p65 Affects the Frequency of. *J. Immunol.* 1–10 (2007).
117. Gays, F., Taha, S. & Brooks, C. G. The distal upstream promoter in Ly49 genes, Pro1, is active in mature NK cells and T cells, does not require TATA boxes, and displays enhancer activity. *J. Immunol.* **194,** 6068–6081 (2015).
118. Santourlidis, S. *et al.* Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. *J. Immunol.* **169,** 4253–4261 (2002).
119. Chan, H.-W., Miller, J. S., Moore, M. B. & Lutz, C. T. Epigenetic control of highly homologous killer Ig-like receptor gene alleles. *J. Immunol.* **175,** 5966–5974 (2005).
120. Li, H., Pascal, V., Martin, M. P., Carrington, M. & Anderson, S. K. Genetic control of variegated KIR gene expression: polymorphisms of the bi-directional KIR3DL1 promoter are associated with distinct frequencies of gene expression. *PLoS Genet.* **4,** e1000254 (2008).
121. Cichocki, F. *et al.* Cutting edge: KIR antisense transcripts are processed into a 28-base PIWI-like RNA in human NK cells. *J. Immunol.* **185,** 2009–2012 (2010).
122. Cichocki, F., Miller, J. S. & Anderson, S. K. Killer Immunoglobulin-Like Receptor Transcriptional Regulation: A Fascinating Dance of Multiple Promoters. *J Innate Immun* **3,** 242–248 (2011).
123. Anderson, S. K. Probabilistic bidirectional promoter switches: noncoding RNA takes control. *Mol Ther Nucleic Acids* **3,** e191 (2014).

124. Cichocki, F., Miller, J. S. & Anderson, S. K. Killer Immunoglobulin-Like Receptor Transcriptional Regulation: A Fascinating Dance of Multiple Promoters. *J Innate Immun* **3,** 242–248 (2011).
125. Uhrberg, M. Shaping the human NK cell repertoire: an epigenetic glance at KIR gene regulation. *Molecular Immunology* **42,** 471–475 (2005).
126. Clapham, D. E. Calcium Signaling. *Cell* **131,** 1047–1058 (2007).
127. Partida-Sánchez, S. *et al.* Chemotaxis and calcium responses of phagocytes to formyl peptide receptor ligands is differentially regulated by cyclic ADP ribose. *J.I.* **172,** 1896–1906 (2004).
128. Vig, M. & Kinet, J.-P. Calcium signaling in immune cells. *Nat Immunol* **10,** 21–27 (2009).
129. Rhee, S. G. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* **70,** 281–312 (2001).
130. Shaw, P. J. & Feske, S. Regulation of lymphocyte function by ORAI and STIM proteins in infection and autoimmunity. *The Journal of Physiology* **590,** 4157–4167 (2012).
131. Weidinger, C., Shaw, P. J. & Feske, S. STIM1 and STIM2-mediated Ca(2+) influx regulates antitumour immunity by CD8(+) T cells. *EMBO Mol Med* **5,** 1311–1321 (2013).
132. Shaw, P. J. *et al.* $CD4^+$ and $CD8^+$ T cell-dependent antiviral immunity requires STIM1 and STIM2. *J. Clin. Invest.* **124,** 4549–4563 (2014).
133. Mace, E. M. *et al.* Cell biological steps and checkpoints in accessing NK cell cytotoxicity. **92,** 245–255 (2014).
134. Orange, J. S. Formation and function of the lytic NK-cell immunological synapse. *Nat Rev Immunol* **8,** 713–725 (2008).
135. Lee, S. H. *et al.* RasGRP1 Is Required for Human NK Cell Function. *J.I.* **183,** 7931–7938 (2009).
136. Yu, T. K., Caudell, E. G., Smid, C. & Grimm, E. A. IL-2 activation of NK cells: involvement of MKK1/2/ERK but not p38 kinase pathway. *J.I.* **164,** 6244–6251 (2000).
137. Jiang, K. *et al.* Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. *Nat Immunol* **1,** 419–425 (2000).
138. Sauer, K. *et al.* Inositol tetrakisphosphate limits NK cell effector functions by controlling PI3K signaling. *Blood* **121,** 286–297 (2013).
139. Anel, A. Protein kinase C-θ (PKC-θ) in natural killer cell function and anti-tumor immunity. *Frontiers in Immunology* 1–12 (2012). doi:10.3389/fimmu.2012.00187/abstract
140. Aguilo, J. I., Garaude, J., Pardo, J., Villalba, M. & Anel, A. Protein Kinase C- Is Required for NK Cell Activation and In Vivo Control of Tumor Progression. *J.I.* **182,** 1972–1981 (2009).
141. Pores-Fernando, A. T., Ranaghan, M. Y. D. & Zweifach, A. No specific subcellular localization of protein kinase C is required for cytotoxic T cell granule exocytosis. *J. Biol. Chem.* **284,** 25107–25115 (2009).
142. Ma, J. S. Y. *et al.* Protein kinase Cdelta regulates antigen receptor-induced lytic granule polarization in mouse CD8+ CTL. *J.I.* **178,** 7814–7821 (2007).
143. Barclay, J. W., Morgan, A. & Burgoyne, R. D. Calcium-dependent regulation of exocytosis. *Cell Calcium* **38,** 343–353 (2005).
144. Lanier, L. L., Yu, G. & Phillips, J. H. Co-association of CD3 zeta with a receptor (CD16) for IgG Fc on human natural killer cells. *Nature* **342,** 803–805 (1989).
145. Griffiths, E. K. & Penninger, J. M. ADAP-ting TCR signaling to integrins. *Sci. STKE* **2002,** re3–re3 (2002).
146. Kim, H. S., Das, A., Gross, C. C., Bryceson, Y. T. & Long, E. O. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase.

Immunity **32,** 175–186 (2010).
147. Garcia-Lora, A., Martinez, M., Pedrinaci, S. & Garrido, F. Different regulation of PKC isoenzymes and MAPK by PSK and IL-2 in the proliferative and cytotoxic activities of the NKL human natural killer cell line. *Cancer Immunol. Immunother.* **52,** 59–64 (2003).
148. Merino, E. *et al.* Protein Kinase C- Clustering at Immunological Synapses Amplifies Effector Responses in NK Cells. *J.I.* **189,** 4859–4869 (2012).
149. Peppiatt, C. M. *et al.* 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. *Cell Calcium* **34,** 97–108 (2003).
150. Peterson, E. J. Coupling of the TCR to Integrin Activation by SLAP-130/Fyb. *Science* **293,** 2263–2265 (2001).
151. Volná, P. *et al.* Negative regulation of mast cell signaling and function by the adaptor LAB/NTAL. *Journal of Experimental Medicine* **200,** 1001–1013 (2004).
152. Clements, J. L. *et al.* Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science* **281,** 416–419 (1998).
153. Narni-Mancinelli, E. *et al.* Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. *Proceedings of the National Academy of Sciences* **108,** 18324–18329 (2011).
154. Oostendorp, R. *et al.* Stromal cell lines from mouse aorta-gonads-mesonephros subregions are potent supporters of hematopoietic stem cell activity. *Blood* 1–8 (2002).
155. Cichocki, F. & Miller, J. S. In vitro development of human Killer-Immunoglobulin Receptor-positive NK cells. *Methods Mol. Biol.* **612,** 15–26 (2010).
156. Karimi, M. A. *et al.* Measuring cytotoxicity by bioluminescence imaging outperforms the standard chromium-51 release assay. *PLoS ONE* **9,** e89357 (2014).