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Bypassing Nk Cell Tuning by Targeting Diacylglycerol Kinase Zeta, A Distal Regulator of Signaling

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Abstract
NK cells are part of the innate immune system, and play an important role in viral and tumor defense. Improving natural killer (NK) cell function could be beneficial for enhancing anti-tumor or anti-viral responses. However, efforts to improve NK cell function by disrupting negative regulators that target proximal signaling pathways paradoxically results in less responsive NK cells. This is often attributed to their ability to tune their responsiveness. In this thesis, I found that NK cells are extremely sensitive to loss of inhibitory ligand or mediators of inhibitory signaling. Using adoptive transfer and mixed chimera models, I found that MHC class I expression is necessary both in cis and trans for NK cells to possess full functionality. Furthermore, using an acute model of genetic targeting, I found that temporal ablation of SHP-1 was sufficient to drive hyporesponsiveness, and the loss of even a single allele of SHP-1 had profound effects on NK cell responses. However, the data also showed that tuned NK cells could still be stimulated to respond via analogs of secondary messengers of signaling, suggesting that NK cell tuning targets proximal signaling pathways.

To improve NK cell function but avoid NK cell tuning, I targeted a distal negative regulator of signaling. I found that genetic deletion of diacylglycerol kinase zeta (DGKζ), a negative regulator of diacylglycerol-mediated signaling, enhances NK cell function due to its distal position in the signaling cascade. Upon activating receptor stimulation, NK cells from mice lacking DGKζ display increased cytokine production and cytotoxicity in an ERK-dependent manner. This enhancement of NK cell function is NK cell-intrinsic and developmentally independent. Importantly, DGKζ deficiency does not affect inhibitory NK cell receptor expression or function. Thus, DGKζ KO mice display enhanced clearance of a TAP-deficient tumor. I therefore propose that enzymes that negatively regulate distal signaling pathways such as DGKζ represent novel targets for augmenting the therapeutic potential of NK cells.

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REGULATOR OF SIGNALING

Dedication page

To my brother, Enbin, who keeps me firmly grounded.

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ABSTRACT

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NK cells are part of the innate immune system, and play an important role in viral and tumor defense. Improving natural killer (NK) cell function could be beneficial for enhancing anti-tumor or anti-viral responses. However, efforts to improve NK cell function by disrupting negative regulators that target proximal signaling pathways paradoxically results in less responsive NK cells. This is often attributed to their ability to tune their responsiveness. In this thesis, I found that NK cells are extremely sensitive to loss of inhibitory ligand or mediators of inhibitory signaling. Using adoptive transfer and mixed chimera models, I found that MHC class I expression is necessary both in cis and trans for NK cells to possess full functionality. Furthermore, using an acute model of genetic targeting, I found that temporal ablation of SHP-1 was sufficient to drive hyporesponsiveness, and the loss of even a single allele of SHP-1 had profound effects on NK cell responses. However, the data also showed that tuned NK cells could still be stimulated to respond via analogs of secondary messengers of signaling, suggesting that NK cell tuning targets proximal signaling pathways.

To improve NK cell function but avoid NK cell tuning, I targeted a distal negative regulator of signaling. I found that genetic deletion of diacylglycerol kinase zeta (DGKζ), a negative regulator of diacylglycerol-mediated signaling, enhances NK cell function due to its distal position in the signaling cascade. Upon activating receptor stimulation, NK cells from mice lacking DGKζ display increased cytokine production and cytotoxicity in an ERK-dependent manner. This enhancement of NK cell function is NK cell-intrinsic and developmentally independent. Importantly, DGKζ deficiency does not affect inhibitory NK cell receptor expression or function. Thus, DGKζ KO mice display enhanced clearance of a TAP-deficient tumor. I therefore propose that enzymes that negatively regulate distal signaling pathways such as DGKζ represent novel targets for augmenting the therapeutic potential of NK cells.

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CHAPTER 1: Introduction

Natural killer cells and innate immunity

The immune system is the result of constant evolutionary pressure on an organism to protect itself from illness. Indeed, there are a large variety and number of internal and external threats that exist, and the immune system has had to construct a number of different defenses against them. Broadly speaking, the immune system can be divided into two arms, the innate and the adaptive. The former consists of a group of cells that are capable of responding to threats quickly, often by engulfing or trapping foreign material and are also important in recruiting and activating other immune cells at the site of disease. Some examples of innate immunity include natural killer (NK) cells, dendritic cells (DCs), macrophages, and mast cells. Many of their functions are carried out through pattern recognition receptors that are encoded in the germline of each organism. These receptors allow for rapid recognition of a broad range of pathogens. They are therefore often considered our first line of defense.

In contrast, the adaptive immune system consists of T and B cells whose responses are more specific. The adaptive immune system is able to achieve this specificity due to a series of error-prone but deliberate genetic rearrangements that provide a diverse array of cells that have different repertoires. However, due to this mechanism of development and formation, the initial frequency of adaptive immune cell that can respond to a particular threat is extremely low. Upon

contact with their cognate antigen, the adaptive immune cell is able to proliferate quickly to increase their numbers, and eventually overcome the threat. Following this response, some of these cells are then kept in a memory “reserve”, which allows the organism to fight off subsequent reinfection in a shorter period of time. However, the low initial frequency of adaptive immune cells for a specific target implies that the adaptive immune system requires time in order to expand and fight off the disease; the innate immune system is thus involved in both buying this time by keeping the pathogen growth in check, as well as promoting the recruitment and activation of the adaptive system if necessary.

NK cells fall under a subset of innate immune cells called the innate lymphoid cells (ILCs). ILCs are subdivided according to the cytokines they produce and the transcription factors they express. For example, ILC group I cells (which include NK cells) are typically associated with transcription factors such as Tbet and Eomes and are capable of producing the cytokines TNFα and IFNγ upon stimulation (1). This diversity of function in the ILCs has often been compared to the adaptive helper T cell. However, compared to the helper T cell, ILCs, as innate immune cells, are capable of rapid response to stimuli.

More specifically, NK cells were found by Rolf Kiessling et al. who reported that a subset of cells isolated from naive mouse spleen was able to display rapid *in vitro* killing of target cell lines, and thus gave rise to their nomenclature (2). Eventually, the existence of the NK cell was finally narrowed down to the subset of cells that expressed the receptors CD49b (DX5), NCR1

(NKp46) and in C57/BL6 mice, the receptor KLRB1 (NK1.1). To differentiate them from NKT cells, an exclusion gate consisting of CD3 or CD4 and CD8 is also often used. While a similar exclusion gating strategy is used in humans, NK cells are instead marked by their expression of the receptors CD16 and CD56 and form about 10% of the lymphocytes in human peripheral blood.

The anti-tumor ability of NK cells has now been confirmed by multiple *in vivo* studies, and reports have shown that NK cells utilize an abundance of cytotoxic granules to kill their targets (3, 4). However, research has also shown that the cytokine production capacity of NK cells is important in viral defenses (5, 6). In particular, their ability to produce high amounts of IFNγ is critical in inhibiting viral replication and boosting innate as well as adaptive immune responses (7).

This capacity to kill target cells and produce large quantities of cytokine is also a hallmark of other immune cell types like the activated $CD8^+$ T cell. What truly sets NK cells apart is their ability to detect the loss of immunological “self”. One of the key determining factors of cellular immunological identity is the expression of Major Histocompatibility Complex (MHC) Class I. This molecule is present on a large majority of cells in the body, and it presents peptides on the cell surface that are from proteins normally expressed within the cell. In particular, $CD8^+$ T cells rely on the presentation of aberrant antigens by MHC Class I to recognize the transformation of normal cells (by viral or carcinogenic methods) and thereby target the transformed cell for killing (8). Hence, one

common method utilized by virus-infected or transformed cells is the downregulation of MHC Class I to evade detection by $CD8^+$ T cells. NK cells are capable of detecting this downregulation of class I, and are therefore complementary to $CD8^+$ T cells in protecting the host from such threats. The importance of NK cells is demonstrated in humans bearing genetic mutations that result in impaired NK cell development or function; they are more vulnerable to infection by viruses that downregulate MHC Class I, such as the herpesviruses and papillomaviruses (9).

This unique function of NK cells, first postulated by Ljunggren et al., is now known as the “missing self hypothesis” (10). It is now recognized that missing self recognition is made possible by the array of germline encoded NK inhibitory receptors that are able to bind to MHC Class I and enable NK cells to detect fluctuations in surface class I levels on target cells. In humans, many of these receptors fall under the Killer-cell Immunoglobulin-like Receptors (KIRs), while in mice they largely fall under the Ly49 receptor family.

Aside from these inhibitory receptors, NK cells also express a variety of activating receptors, which assist them in understanding their surroundings. The inhibitory and activating receptors combined form the NK cell receptor repertoire, and their expression is an integral part of NK cell development and function.

NK cell development and the NK cell receptor repertoire

The vast majority of NK cells develop in the bone marrow, with smaller tissue resident subsets that potentially develop *in situ* in the thymus and liver(11, 12). In both humans and mice, the expression of the beta chain of the IL-2 receptor (CD122) denotes a critical stage in the development of NK cells. This enables the NK progenitors to be responsive to IL-15, which has been shown to be important in the expansion, survival and further maturation of NK cells (13, 14). Not only has IL-15 deficiency been shown to affect NK cell numbers *in vivo*, IL-15 overexpression results in overproliferation of NK cells (15). Oddly enough, the expression of the high-affinity IL-15 receptor alpha is critical in maintaining NK cell numbers, but is most important on bone marrow DCs; it is thought that they are responsible for cross presentation of IL-15 to developing NK cells in the bone marrow (16). Once they become IL-15 responsive, NK cell progenitors proceed by expressing the activating receptors NKG2D and NK1.1, and it is at this point that NK cells are thought to begin their education on their MHC Class I environment. This education is performed by bone marrow stromal cells, and is meant to instruct NK cells on their host's specific immunological self. The hypothesis surrounding early marrow education of NK cells is supported by observations of NK cell developmental assays *in vitro* (17).

In order to understand the surrounding MHC class I, the immature NK cells must begin to express their inhibitory receptors. Unfortunately, due to the homology between the receptors, studying the mechanistic details of Ly49

acquisition has proven to be challenging. However, we do know that not all NK cells will express an inhibitory receptor, as only a fraction of NK cells will express each receptor. While this fraction only stabilizes in adult mice, it remains consistent between WT control mice. Much like the immunoglobulin genes and TCR alpha locus, the Ly49 receptors also predominantly undergo allelic exclusion (18, 19). Intriguingly, the data further suggest that the expression of inhibitory receptors is a stochastic process (20). Furthermore, the fraction of NK cells coexpressing two or more receptors is similar to the expected result when applying the product rule, suggesting that the receptors are independently acquired (18). Finally, reports have shown that the process is sequential; there is a specific timeframe for an individual NK cell to acquire each inhibitory receptor, leading to stable expression of that particular receptor for the life of the NK cell (21). These data combined paint a picture of how diversity of surface receptor expression on NK cells is acquired and maintained, in order to create a repertoire of NK cells that are capable of recognize different threats.

Further hints to the inhibitory receptor acquisition process can be found throughout the literature. As alluded to before, bone marrow stroma can instruct developing NK cells; the MHC class I expression pattern of the host shapes the receptor repertoire of its NK cells (17, 21). More strikingly, mutations in the signal activation cascade have demonstrated that activating signals are important in NK cell inhibitory receptor acquisition. For example, inhibitory receptor expression is mildly defective in NK cells that are missing Syk and Zap 70, and is largely abrogated in NK cells from PLCγ2 knockout (KO) mice (22, 23). Conversely, loss

of inhibitory signal mediators (like the inhibitory phosphatase SHP-1) appears to increase Ly49 receptor expression (24). Thus, receptor acquisition in NK cells appears to be a multifaceted phenomenon, and gives rise to a vast array of NK cell "subsets" that have a variegated expression of potential receptors. In humans, a recent study showed that there might be as many as 30,000 different NK cell surface phenotypes in a single healthy donor (25). However, more work remains to be done in understanding the NK cell receptor repertoire.

The murine NK cell inhibitory receptors

Of the Ly49 family of receptors that have been sequenced, 13 of them are inhibitory(26). However, only 4 of them are often discussed, and thus will be within the scope of this thesis. Additionally, one other inhibitory receptor, the NKG2A/CD94 complex is also often brought up in literature, and has therefore been added to the list as well (Table 1.1).

Receptor	Ligand
Ly49A	H2-M3, H2-D^d
Ly49C	H2-K^d, H2-K^b
Ly49I	H2-K^d, H2-K^b, H2-D^k(?)
Ly9G2	H2-D^d
NKG2A/CD94	$Qa1^b$

Table1.1: Inhibitory receptors on murine NK cells

As the table above shows, the large majority of ligands for the inhibitory receptors are MHC Class I molecules, from both the non-classical and the classical families. Many of the Ly49 inhibitory receptors are promiscuous, and combined with their variegated expression patterns, it enables NK cells to have a greater coverage of the potential MHC class I haplotype of the host animal (27). In general, all the inhibitory receptors express an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in their cytoplasmic domain (NKG2A itself does not, but it is expressed as a heterodimer with CD94 which does) (28). Upon ligation of the inhibitory receptor, the ITIM gets phosphorylated, likely by a Src kinase (29). The phosphorylated ITIM is then able to associate with inhibitory phosphatases like the Src Homology region 2 (SH2) domain-containing inositol 5'-phosphatase (SHIP) or SH2 domain-containing phosphatase (SHP-1). This brings the inhibitory phosphatases in close proximity to the surface to attenuate proximal signaling. Thus, it came as no surprise when mutations affecting the expression or function of the inhibitory phosphatases SHP-1 and SHIP were shown to affect the ability of the inhibitory receptors to inhibit killing (24, 30). Mechanistically, it has also been shown that the expression of the relevant MHC class I molecule on a target cell recruits the relevant inhibitory receptor into the pre-activation synapse. This draws in the inhibitory phosphatases toward the cell-cell interface, resulting in attenuated activation signals and cessation of the killing machinery (31). Thus, inhibitory receptors play an important role in determining the outcome

of cell-cell interactions in NK cells, and are crucial in reducing the potential for NK cell mediated autoimmunity.

The murine NK cell activating receptors

Unlike the inhibitory receptors, many of the NK cell activating receptors are expressed on a majority of NK cells. The exceptions are Ly49D and Ly49H, which are expressed on roughly half of all mature NK cells (32). The sequences of the activating receptors quickly revealed that many had short cytoplasmic tails with no intrinsic signaling capacity, and would require adaptor proteins to transmit their signals. For the sake of discussing activating signals in NK cells, it is therefore more useful to broadly divide the activating NK cell receptors in the mouse into 3 major groups based on their signaling modules (28)(Table 1.2).

Receptor	Signal adaptor	Signaling motif
NK1.1	FCεRIγ	ITAM
CD16	FCεRIγ	ITAM
NKp46	CD3ζ	ITAM
Ly49D	DAP12	ITAM
Ly49H	DAP12	ITAM
NKG2D	DAP10	YNIM
CD244 (2B4)	SAP	ITSM (on 2B4)

Table 1.2: Activating receptors on murine NK cells

Receptors that utilize ITAM mediated signaling

NK cells constitutively express 3 major ITAM-bearing adaptors of signaling: FCεRIγ, DAP12, and CD3ζ. Both FCεRIγ and DAP12 have a single ITAM in their cytoplasmic tails, whereas CD3ζ has three. Unsurprisingly, the signal transduction pathways downstream of the receptors utilizing these adaptors are similar to that of T and B cells, whose specialized receptors also signal using ITAM motifs. Upon engagement of their associated receptors, these ITAMs are phosphorylated by Src kinase family members like Fyn (33). The phosphorylated tyrosines on the ITAMs are then able to bind to the tyrosine

kinases Zap-70 and Syk, which then proceed to engage further mediators of the activation cascade, including PI3K and Vav (28).

The NKG2D pathway

NKG2D is expressed in both mice and humans, and pairs with both itself and two units of DAP10. A large area of interest in NKG2D research has been on the regulation and expression of its ligands. Not only are NKG2D ligands upregulated during viral transformation, but the expression of ligands like Rae1 (murine) and MICA/B (human) have also been linked to the activity of E2F transcription factors, which are regulated by cell cycle machinery. Thus, researchers have linked the expression of NKG2D ligands to cellular hyperproliferation, a hallmark of tumorigenesis, which makes NKG2D an attractive target for cancer immunosurveillance.

In mice, a shorter transcript of NKG2D is generated by alternative splicing, and can associate with DAP12. However, the most common form of the NKG2D receptor exists as a hexamer, and each of the four DAP10 units in the hexamer has a single YNIM signal motif in the cytoplasm (34, 35). Like the ITAM bearing adaptors, the YNIM signal motif in DAP10 is phosphorylated by Src family kinases upon ligation of NKG2D with cognate ligand. However, this process does not seem to activate the Syk family kinases, but instead may directly recruit Grb2/Vav1 or PI3K to transduce signals further downstream.

The SAP dependent pathway

CD244, also known as 2B4, is a member of the SLAM family of receptors. It contains an Immunoreceptor tyrosine-based switch motif (ITSM) which, when phosphorylated, associates with SH2 domains of proteins like SAP and EAT2 (28). However, while 2B4 is classed as an activating receptor in many studies, the data also demonstrate that certain conformations of 2B4 prefer to bind to phosphatases like SHP1 instead, which could indicate a role for 2B4 in inhibitory signals (36). The ligand for 2B4 is CD48, which is expressed on other hematopoietic cells; upon engaging its ligand, 2B4 is phosphorylated by Fyn on its ITSMs. The fact that CD48 is expressed on many hematopoietic cell subsets (including other NK cells) also suggests the potential for NK cells to provide costimulation for each other. Indeed, blocking 2B4 interactions during *in vitro* NK culture results in decreased proliferation. Furthermore, loss of 2B4 costimulation *in vivo* also results in poorer NK cell responses (36). More recently, a study was able to show that SAP signaling was responsible for regulating both Vav1 and SHIP, and resulted in increased conjugate formation in NK cells (37).

NK cell activating signals – Beyond the receptors

The complexity of proximal signaling pathways deriving from the array of activating receptor-adaptor pairs serves as a foreshadowing for some of the difficulties in studying NK cell signaling. For example, NK cells express a large number of the Src family kinases: including Fyn, Lyn, Lck and Src itself. Models of genetic loss of these molecules has shown modest phenotypes in NK cells, suggesting some redundancy in their function (29, 33, 38). In another surprising yet similar example, knocking out both Syk and Zap70 in NK cells attenuates but does not abrogate their development or function completely (22).

Fortunately, as our discussion proceeds to signals that are distal to the different activating receptors that were previously listed, their activation pathways actually begin to overlap. They converge not only with each other, but also onto pathways that have been well studied in other cell types. PI3K is listed as a target protein downstream in two out of three pathways of activation, and Vav is a target in all three. Furthermore, work by a previous graduate student from our lab provided confirmation of the convergence in signaling; NK cells lacking SLP-76, an adaptor molecule that is critical in T cell signal transduction, were unable to respond to signals through the three groups of activating receptors (32). However, the same study also provided a slight twist to the comparison between NK cells and T cells: two different proximal activating receptor complexes were reported in NK cells, and loss of either impaired but did not abrogate NK cell responses. Research on T cells has demonstrated that one of the most crucial

factors downstream of SLP-76 activation is the activation of PLCγ. Thus, similar to the findings in SLP-76 deficient mice, NK cells from mice lacking PLCγ2 were also severely defective in their ability to kill targets and produce cytokines, reiterating the importance of this pathway in NK cell function (23, 39).

Aside from the aforementioned studies, much the information we have regarding the distal activation pathways relies on studies from other immune cell types like T cells. While the following discussion probably holds true in NK cells as well, it is entirely possible that future research may uncover more intricate details that are unique to NK cells as well.

In T cells, the primary effect of PLCγ activation is the cleaving of phosphatidylinositol 4,5-bisphosphate (PIP_2) into two secondary messengers: inositol 1,4,5-trisphosphate (IP_3) and Diacylglycerol (DAG) (40, 41). Both of these secondary messengers play separate roles in the activation cascade, and will be briefly touched upon in the subsections to follow.

IP_3 Signaling

As mentioned prior, IP_3 is one of the second messengers formed by activation of PLCγ. One function of IP_3 is to serve as a precursor for the formation of other higher order inositol messenger molecules via the activity of inositol kinases and phosphatases. The effects of inositol messengers include the activation of the GTPase GAP1, and potentially even AKT(42). However, the

primary focus of research into IP_3 function has been on its interactions with its receptor located on the endoplasmic reticulum (ER). Binding of IP_3 to the receptor releases Ca^{2+} that has been stored by the cell in the ER, which then proceeds to activate store operated calcium (SOC) channels and allows for entry of extracellular calcium into the cytoplasm through the plasma membrane. Recent work has indicated that in immune cells, CRACM1 (Orai1) is the major SOC channel, with STIM1 being the ER-located calcium sensor that activates Orai1. SOC release and Orai1 function leads to sharp increases in calcium flux, which activates kinases like JNK and transcriptional regulators like NFAT and NFκB (43). In particular, NFAT requires high concentrations of Ca^{2+} (and corresponding calcineurin activity) in order to remain in the nucleus to perform its transcriptional functions. NFAT is critical for immune cell development, survival and cytokine production (44), and cements the importance of IP_3 signaling in immune cells.

DAG signaling in activation

DAG mediated signaling has been shown to activate a wide variety of different proteins, including PKCs, Ras-GRPs, and Munc-13 protein family members (45). However, in T cell activation, studies of DAG signaling have focused on three pathways that are affected by DAG regulation: AKT, MEK and PKCθ (46-48). The binding of DAG to PKCθ is important for the activation of

NFκB through the MALT1/Carma1/BCL10 complex and leads to increased cell survival and production of T helper cytokines (45, 49). PKCθ also promotes the functions of the Ras-GRPs, which in turn leads to the AKT and MEK pathways. Upon binding to DAG, Ras-GRP combines with Sos, which binds to as well as activates Ras (49). Ras in turn targets the Raf-1/MEK/Erk pathway which has been shown to lead to increased AKT/mTOR function (47). Furthermore, phosphorylated ERK also leads to AP-1 activation (50), and allows for the cooperative binding of AP-1 and NFAT to the promoter sites of cytokines such as IL-2 (49, 51). This is thus one method by which the IP_3 and DAG signaling arms combine to initiate the "activated" status of an immune cell.

NK cell responsiveness – A concert of inhibition and activation

NK cell activation is therefore formed by the synergy between the inhibitory and activating receptors. Two potential scenarios can explain how an NK cell might target a cell for killing. Firstly, a target cell might experience an increase in activation ligand expression, like a rapidly dividing tumor cell that increases its expression of Rae1. The NKG2D receptor on a surveying NK cell would thus recognize this increase in NK cell activation signals, and respond against the tumor. Alternatively, the loss of MHC class I on a herpes virus infected cell would be seen as a decrease in inhibitory signals on the NK cell, and would therefore also evoke a response.

To recognize this fluctuation in either inhibitory or activating receptor signaling, NK cells must therefore calibrate signaling according to some baseline equilibrium. The system of calibration should include the following components:

A) Enable an NK cell to understand how many inhibitory and activating receptors it expresses at baseline.

B) Be flexible enough to accommodate inhibitory and activating receptors that may never see ligand (e.g. an inhibitory receptor for a different MHC haplotype).

C) Be able to shut down any potential for autoimmunity, like an NK cell that does not express any inhibitory receptors.

Thus, we arrive at a fundamental disagreement that is still evolving in the NK cell field today. It is accepted that NK cells tune their responsiveness according to their environments. This idea is supported by numerous observations:

1) NK cells that express more inhibitory receptors that recognize self-MHC are more responsive than those that have fewer. (52).

2) NK cells from MHC Class I deficient backgrounds are less responsive than WT controls. However, transferring NK cells from a MHC class I deficient environment to a WT environment restores their responsiveness (53, 54).

3) NK cells that have lost SHP-1 or SHIP signaling are less likely to respond than their WT counterparts (24, 30, 55).

However, the mechanism behind this tuning is still not understood. Two major approaches to tuning have been proposed: Arming versus disarming

NK cell tuning – Arming versus disarming

NK cell arming proposes that when an NK cell that expresses an inhibitory receptor that recognizes self-MHC Class I, it receives a final maturation signal and becomes functionally competent. In this process, a negative signal somehow promotes the final maturation of an NK cell (56). This observation is supported by the phenomenon, henceforth referred to as “licensing”, where an NK cell that expresses an inhibitory receptor for self MHC is more likely to respond than one that does not (57). An additional subclass of the arming phenomenon suggests that it is induced by cis interactions with self MHC on the same NK cell itself (58).

In contrast, NK cell disarming is the idea that NK cells are capable of killing targets as soon as they exit development. However, when faced with unopposed activating signals, they are disarmed and maintain an “anergic” like state. This hypothesis is supported by experiments where WT NK cells were transferred into MHC Class I deficient hosts(53) became less responsive than those that were transferred into WT hosts.

While these ideas are not necessarily mutually exclusive, the debate over NK cell tuning has not abated, especially because of increasing interest in using NK cells as a therapeutic tool.

NK cells in the clinic

The ability of NK cells to target virally-infected and neoplastic cells has led to major interest in using them for therapy. Early trials have shown safety and preliminary efficacy in NK cell-based adoptive immunotherapy to treat hematological malignancies (59). More recently, researchers have focused on methods to improve NK function *in vivo*. These approaches can broadly be divided into three categories: 1) expansion and activation of autologous NK cells to improve their responsiveness (60); 2) by mismatching of ligand expression on patient cells to killer immunoglobulin-like receptor (KIR) expression on donor NK cells (61); and 3) genetic or molecular augmentation of NK cell signaling machinery in donor cells (62).

However, with our latest understanding of NK cell tuning, it is reasonable to assume that the NK cells used in therapy may end up being unresponsive as they adapt to their environments. Indeed, multiple early trials have shown limited long-term efficacy of activated NK cells in the treatment of patients (63, 64). While many potential explanations exist for their failure as a therapy, the need to understand the mechanism behind NK cell tuning or the ability to circumvent this phenomenon will still be relevant to improving therapeutic outcomes. Thus, the rest of this thesis will be broadly divided into three sections – In chapter two, I will attempt to further dissect the potential differences between the arming and disarming hypothesis. Chapter three will cover an investigation into circumventing tuning by targeting a distal regulator of signaling, diacylglycerol

kinase zeta (DGKζ). The last chapter shall be a discussion of future directions that can be derived from this body of work.

CHAPTER 2: NK cells tune their proximal signaling pathways in response to changes in MHC Class I Expression

Introduction

Initially, researchers hypothesized that the lack of inhibitory ligand for NK cells in MHC class I deficient mice would either prevent the development of NK cells, or predispose them toward causing autoimmunity *in vivo*. To date, three major models of mice lacking MHC Class I expression exist: beta-2-microglobulin deficient (B2M KO), Tap1 deficient (TAP1 KO), and H2-K^b/H2-D^b doubly deficient mice (KbDb DKO). In general, MHC Class I is assembled in the ER as a heterodimer of a polymorphic heavy chain and the light chain B2M. The association between the chains is fairly loose, but can be stabilized by the loading of a peptide into the binding cleft by the protein Tap(8). Thus, B2M KO and Tap1 KO deficient mice are low in surface expression of many classical and non-classical MHC Class I molecules (65, 66). In contrast, KbDb DKO mice have a targeted mutation to prevent expression of the heavy chains of the classical MHC Class I molecules which are encoded by the H-2 locus (67). They therefore lack surface expression of classical MHC Class I but can still express non-classical molecules. All three mice models have normal NK cell numbers, but no overt NK immunopathology, which came as a surprise to NK cell biologists.

The next series of experiments demonstrated that the NK cells from B2M deficient mice were less likely to kill MHC class I deficient blasts as compared to WT NK cells (68, 69). These studies confirmed that NK cell function was instructed by class I MHC molecules. Further research indicated that this education was conducted by cells from both the radioresistant as well as hematopoietic lineages (70). This conclusion was backed by a study on mismatched allogeneic chimeras, which also suggested that NK cells become tolerant of cells from both host and donor bone marrow origin (71). One potential trivial explanation for all these observations would be that NK cells from these mice were not expressing the correct activating receptors, and therefore could not kill their targets. This hypothesis was rejected when mice that expressed a transgenic form of the viral MCMV protein m157 (m157-tg) were found to have NK cells expressing the Ly49H receptor, which recognizes m157. Furthermore, the data from this study showed that the Ly49H cells were hyporesponsive to activating signals from both Ly49H as well as NK1.1, and resulted in susceptibility to MCMV infection. In contrast, the Ly49H- cells were similar to WT controls in responding to stimuli (72, 73). This indicated that only the NK cells that were self-reactive were tuning.

However, inhibitory signals are not just important for downregulation of NK cell self-reactivity. They have also been found to increase NK cell activation. As mentioned in chapter 1, the key finding in this regard was the observation of NK cell licensing, where NK cells that expressed a self-binding inhibitory receptor

were more likely to produce cytokine and, in one experiment, kill target cells as compared to NK cells that did not have an inhibitory receptor for self (57).

More recently, studies also showed that NK cell tuning was not restricted to development, as NK cells were able to increase or decrease their responsiveness according to the changes in the MHC Class I environment surrounding them (53, 54). Further hints to the NK cell tolerance process came in the form of a study by Sun and Lanier, which suggested that the tuning mechanism in NK cells relied on an unstable form of equilibrium. They found that viral infection was sufficient to break tolerance mechanisms (74) in mixed chimeras made from WT and MHC Class I deficient cells, which resulted in rejection of the MHC Class I deficient donor cells in a chimeric situation that was previously stable. This data was later reinforced by another paper which found that cytokine treatment was sufficient to reverse NK cell anergy (4).

Hence, unlike adaptive cells that undergo a selection process that eliminates highly self-reactive cells, NK cells seem to avoid causing autoimmune pathology by tuning their responsiveness. While the studies listed above have been illuminating, they fail to address the mechanism behind NK cell tuning. Specifically, all the studies involving mixed bone marrow chimeras with MHC class I deficient cells have failed to address if there were baseline differences between the cells from MHC Class I deficient, or MHC class I sufficient origin (70, 74). Indeed, in the data from those papers, mice with mixed bone marrow donors showed some residual activity against MHC Class I deficient cells (70). In light of reports that the tolerance in mixed chimeras can be broken by viral infection, it is

potentially possible that this “tolerance” could be a game of numbers, where enough class I deficient cells are being produced for WT NK cells to kill without affecting the MHC class I deficient HSCs.

Another interesting dimension to the phenomenon of NK tuning was discovered in the report describing licensing by Kim et al (57). In a surprising twist, they also suggested that SHP-1, a major phosphatase downstream of the inhibitory receptors, was not involved in licensing. While their data was statistically sound, their approach stood out due to a major caveat. They used NK cells from the motheaten viable (me^v) mouse, which has germline defects in SHP-1 function. Given the ability of NK cells to tune rapidly, as well as their expression of multiple phosphatases (SHP-1, SHP2 and SHIP), it is conceivable that NK cells from these germline deficient mice were already tuned to the loss of SHP-1. Furthermore, the me^v mouse has major inflammatory syndromes due to the loss of SHP-1 in multiple immune cell lineages, which could complicate the interpretation of said result (75, 76).

Thus, I was interested in investigating the mechanisms of tuning in MHC Class I deficient NK cells, and more specifically examining the role of SHP-1 in NK cell licensing.

Results

Ly49C binds to MHC Class I in Cis and licenses NK cells in C57/BL6 mice

In order to study tuning in NK cells, I first wanted to confirm the previous reports of licensing. Thus, I attempted to identify Ly49C$^+$ NK cells in B6 mice. A commercially available antibody for Ly49C/I was reported to largely stain for Ly49I (77), and therefore I tried staining using a monoclonal anti-Ly49C antibody (4LO-3311). The initial protocols I tested gave rise to an unexpected result, where Ly49C stained more distinctly in MHC class I deficient cells than WT cells (Fig. 1A). One potential explanation for the difference was that cis binding of MHC Class I to Ly49C on WT NK cells was preventing binding of the 4LO-3311 antibody (78). To test if there was masking of the epitope, I therefore stripped the WT NK cells of MHC Class I using an acidic buffer. The buffer was able to remove surface MHC Class I from a majority (>90%) of WT NK cells (Fig. 1B, top). I found that stripping off MHC Class I from the surface of WT NK cells was sufficient to improve the Ly49C stain (Fig. 1B, bottom).

In the interest of stimulating NK cells without resorting to acidic treatments, I tested other staining protocols for Ly49C. Eventually, using a different staining method, I was able to reliably identify Ly49C$^+$ NK cells in a WT C57/BL6 mouse (Fig 1C). Utilizing this protocol, I was also able to show that WT Ly49C$^+$ NK cells were not only more likely than Ly49C$^-$ NK cells to produce

cytokine, but they also had an increased tendency to degranulate *in vitro* as well (Fig. 1, D and E).

To confirm that licensing in WT Ly49C$^+$ NK cells was due to interactions with MHC Class I ligand, I stimulated NK cells from MHC Class I deficient mice. Similar to previously published data, I showed that in MHC class I deficient mice, Ly49C expression did not appear to increase the likelihood of NK cells to degranulate (Fig. 2A). Similar trends were also observed for cytokine production (data not shown). Additionally, through these experiments I also confirmed that MHC Class I deficient cells had a trend toward decreased responsiveness as compared to WT controls (Fig. 2, A-C). However, the differences between WT controls and B2M KO mice were abrogated when the cells were stimulated with PMA and Ionomycin (Fig 2, D and E).

NK cells are acutely dependent on SHP-1 concentration

I was now ready to proceed to investigate the role of SHP-1 in licensing mature NK cells. I analyzed a mouse that had inducible deletion of floxed alleles of SHP-1 using the ER-CreT2 system to delete floxed alleles of SHP-1. As a control, I treated both a WT B6 mouse, as well as a heterozygous SHP-1 floxed ER-CreT2 mouse. Unexpectedly, we still observed a major defect in NK cell function in these mice (Fig. 3) despite an overall short course of gene deletion. Based on the preliminary results, each allele of SHP-1 deleted saw the difference

in responsiveness between Ly49C$^+$ and Ly49C$^-$ NK cells appear to decrease (Fig 3). The most surprising finding was that NK cell responses were severely affected even in the SHP-1 heterozygous mouse, suggesting haploinsufficiency was occurring (Fig 3, middle row).

NK cell tuning may have both cell intrinsic and cell extrinsic components

Looking at these data, it suggested that mature NK cells could still alter their licensing status. However, I wanted to utilize a different model to see if the difference between licensed Ly49C$^+$ and unlicensed Ly49C$^-$ NK cells was actually developmentally independent. Thus, I turned to the adoptive transfer model that was previously reported (53). In concordance with published work, I found that the WT NK cells were indeed reducing their responsiveness in a MHC class I deficient background as compared to WT control hosts (Fig. 4, A and B). However, the percentage yield of adoptively transferred cells was small (Fig. 4C), and made it technically unfeasible to determine the differences between the Ly49C$^+$ and Ly49C$^-$ NK cells.

I proceeded to investigate if tuning, licensing or both were cell intrinsic. I made mixed chimeras using bone marrow from WT and MHC Class I deficient donors. Initially, I made mixed chimeras using WT and B2M KO cells, but was surprised to find that in these mixed chimeras, cells from the B2M KO background appeared to increase in H2-K^b expression (Fig 4D). The preliminary

results also showed that the NK cells of B2M KO origin in these chimeras did not have a difference in responsiveness than those of WT origin (Fig. 4E). I therefore turned to the K^bD^b DKO mice, but was unable to get reliable engraftment of cells from the K^bD^b DKO background to perform the analysis on licensed versus unlicensed cells (Fig. 4F). Statistical analysis on total NK cell responses indicated that there was no difference between any of the groups (Fig. 4G).

Discussion

In this chapter, I attempted to uncover the mechanisms underlying NK cell licensing and tuning. I found that the Ly49C receptor on WT B6 NK cells binds to MHC Class I in cis, consistent with data showing that Ly49A also binds to H2-D^d in cis in transgenic D^d mice (58). I showed that the Ly49C receptor licenses NK cells in mice on a C57/BL6 background, not only in terms of cytokine production, but also their ability to degranulate. Furthermore, in attempting to determine the molecular basis of NK cell licensing, I found that deletion of a single SHP-1 allele was sufficient to profoundly affect mature NK cell responsiveness. My data show that using B2M KO cells in a mixed environment restores a low level of expression of MHC class I molecules on their surface.

Previous reports of the NK cell inhibitory receptor binding MHC Class I in cis have specifically targeted Ly49A, and showed using multiple mutant forms of Ly49A that a flexible stalk region was required for cis binding (79). The cis binding ability of the other Ly49s was postulated in numerous papers due similarities in their crystal structures, but there has not been an association of any of the other Ly49s with their cognate MHC Class I reported (78, 80). The binding of the Ly49 receptors to cognate MHC Class I molecules in cis requires the same binding site as trans binding (79). Initially this was shown to be an important step in regulating the responsiveness of self-recognizing NK cells by sequestering excess inhibitory receptor (81). However, a recent article also showed that cis binding was necessary for the receptor based education of NK

cells but dispensable for the actual inhibition of NK cell function (58). In a somewhat serendipitous fashion, my initial failure to distinctly stain for Ly49C has provided more support for the association of Ly49C with MHC Class I molecules in cis, and thus indirectly supports its role in educating NK cells on a C57/BL6 background.

Further validation of the role of Ly49C in educating NK cells, was in confirmation that Ly49C$^+$ NK cells from WT mice were licensed. The phenomenon of licensing is thought to be a compensatory mechanism for NK cells expressing self-recognizing inhibitory receptor to be able to activate against other cells with mismatched self MHC ligand appropriately (82). Additionally, most of the data regarding licensed NK cells has focused on IFNγ production (57), while details regarding its effect on degranulation are not as well appreciated. Indeed, Ly49C$^+$ NK cells from a WT C57/BL6 mouse were shown to be able to produce more cytokine in the aforementioned study. In comparison, another study found that degranulation of licensed versus unlicensed NK cells was too unstable to report significant differences (52). My data demonstrate that degranulation is also more likely in a licensed Ly49C$^+$ NK cell, and that this difference is abrogated upon the loss of inhibitory receptor ligand.

These experiments were performed with the aim to study the molecular mechanism behind licensing, and one key approach that I had in mind was to acutely deplete SHP-1 from NK cells. Previous studies on the role of inhibitory phosphatases either utilized germline deficient mice, or a mouse that expressed

a Cre driven by the NKp46 promoter, neither of which would rule out developmental defects. Furthermore, the NK cells that had lost SHP-1 would also have plenty of time to tune if possible. Thus, they all arrived at similar conclusions, that NK cells lacking inhibitory phosphatases SHP-1 or SHIP were hyporesponsive (24, 30, 55, 75). By utilizing an ER-CreT2 system, my goal was to reduce the potential for inflammatory disease to occur (as compared to the me^{v} mouse), reduce the time allowed for NK cells to retune their function, as well as bypass potential developmental requirements for SHP-1 in NK cell function. Surprisingly, I found that the deletion of a single allele of SHP-1 was sufficient to induce NK cells into a hypofunctional state, which suggests that NK cells are acutely sensitive to the concentration of SHP-1. Due to this technical limitation, it remains to be seen if SHP-1 is involved in licensing. However, my preliminary data suggests that it could be a possibility, as loss of two alleles appeared to diminish the difference between $Ly49C^{+}$ and $Ly49C^{-}$ NK cells more than loss of a single allele. Regardless, the sensitivity of NK cells to SHP-1 loss could be an important finding for clinical purposes, both in terms of the potential effects of genetic variation, as well as in the context of therapies attempting to target inhibitory phosphatase activity in NK cells.

The final thrust of this chapter was a study into whether licensing or tuning could be driven by developmentally independent or cell intrinsic factors. In the case of the former adoptive transfer model, I was able to recapitulate previous results, but was unable to examine the different NK cell subsets due to small return of cells. The latter question was also bogged down by technical difficulties,

as data from the mixed chimeras proved to be hard to analyze convincingly. The increase in H2-K^b expression on B2M KO cells could be explained by trogocytosis of MHC Class I by the Ly49 receptors on NK cells. However, the enhancement in expression of H2-K^b was observed in other immune cells of the B2M KO background, including $CD3e^+$ T cells (data not shown). One other explanation for the low level of H2-K^b expression by the B2M KO cells is the passive acquisition of B2M by the donor cells from either WT competitor bone marrow or the surrounding WT host cells. Regardless, this increase in surface MHC Class I expression is concerning, as many experimental results in the literature with similar mixed chimeric or adoptive transfer situations have utilized B2M KO animals and cells, potentially coloring some of the interpretations in these reports (53, 54, 70). The increase in H2-K^b expression on the B2M KO cells could also explain my data showing no difference in NK responsiveness between the WT competitor and B2M KO derived cells. The recovery of H2-K^b on the surface of NK cells could potentially aid in their tuning, or perhaps even be sufficient for licensing these NK cells, thereby restoring their functionality.

Unlike the data from B2M KO mixed chimeras, the chimeras made using WT and KbDb DKO mixed donor bone marrow showed a trend suggesting that full functional maturation of NK cells may require MHC Class I on both the NK cells themselves as well as on host non-hematopoietic cells. As expected, the largest difference in mean was observed between the WT NK cells in the WT host and the KbDb DKO NK cells in the KbDb DKO host. However, the variable rates of reconstitution, and the high variability in the data from the *in vitro*

stimulation would preclude making any conclusions from these data alone. One possibility to explain the variability in engraftment is the location of the animal housing facility used by the lab. As mentioned in the introduction, viral infection has been shown to break NK cell tolerance mechanisms. The animal facility, while maintained as an SPF facility, could have variable rates of normally benign infections. To solve this quandary, it may either require a cleaner facility than our lab currently has access to, or placing our chimeras on some form of prophylactic treatment against infections.

In summary, these results have merely skimmed the surface of the mechanism of tuning, and highlighted the technical difficulties of working with the MHC Class I deficient models in NK cells. Far more work remains to be done, and I shall discuss this in further detail in chapter 4.

Figures

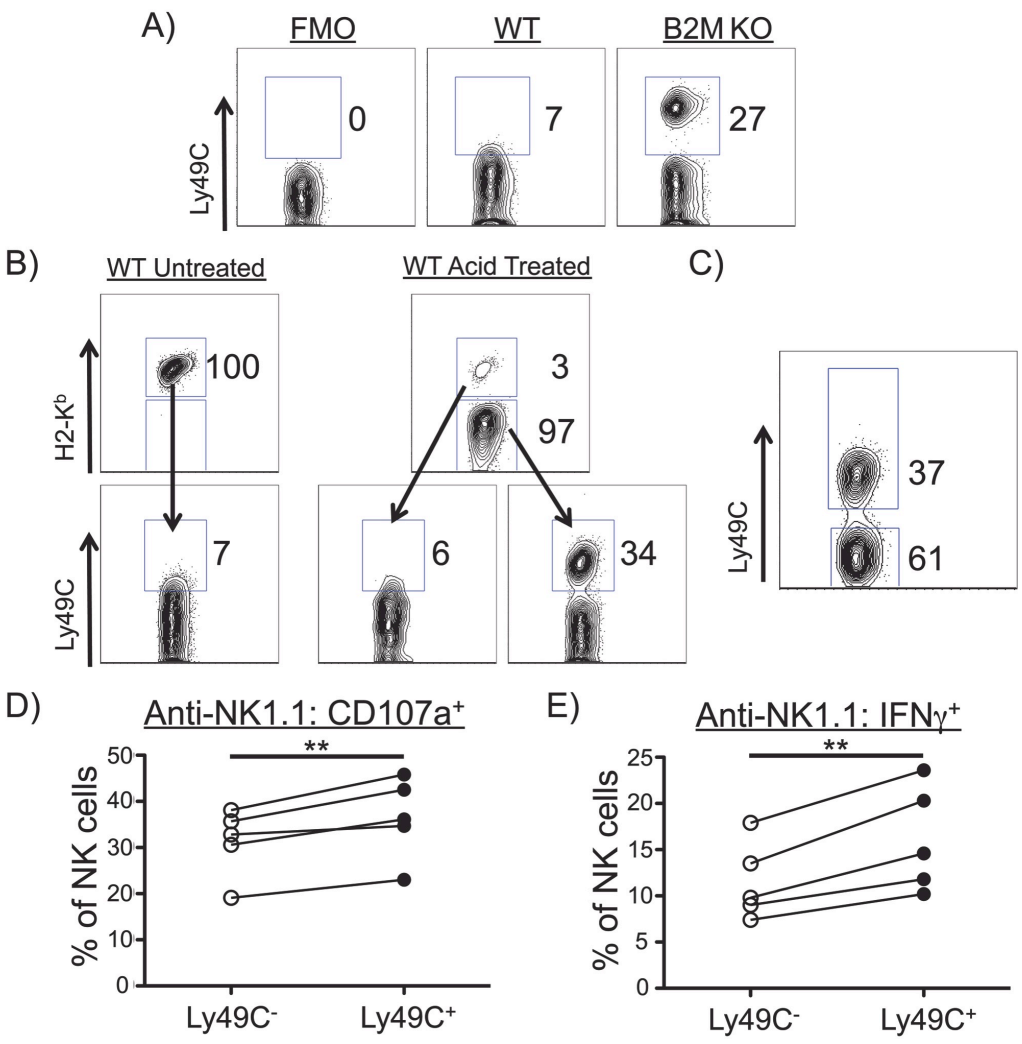


Figure 1: Ly49C binds to MHC Class I in cis, and increases NK cell responsiveness in C57/BL6 mice

A) WT NK cells were either stained with a full minus one (FMO) control or a fluorochrome-conjugated Ly49C (4LO-3311) antibody (WT). NK cells from a B2M KO were also stained with the Ly49C antibody as part of the experiment (far right). Data is representative of 2 separate experiments, N= 2. B) WT splenocytes were treated with an acid buffer and their NK cells were stained for the presence of MHC Class I and Ly49C (right column). Untreated cells were stained as a negative control (left column). Data is representative of at least 3 separate experiments. C) WT NK cells were incubated with Ly49C supernatant followed by a fluorochrome-conjugated secondary antibody. Data shown is representative of at least 2 separate experiments. WT splenocytes were stimulated with platebound anti-NK1.1, and the ability of $Ly49C^+$ NK cells or $Ly49C^-$ NK cells to D) degranulate and E) produce IFNg in response was measured. N = 5, Data shown is pooled from at least 3 separate experiments.

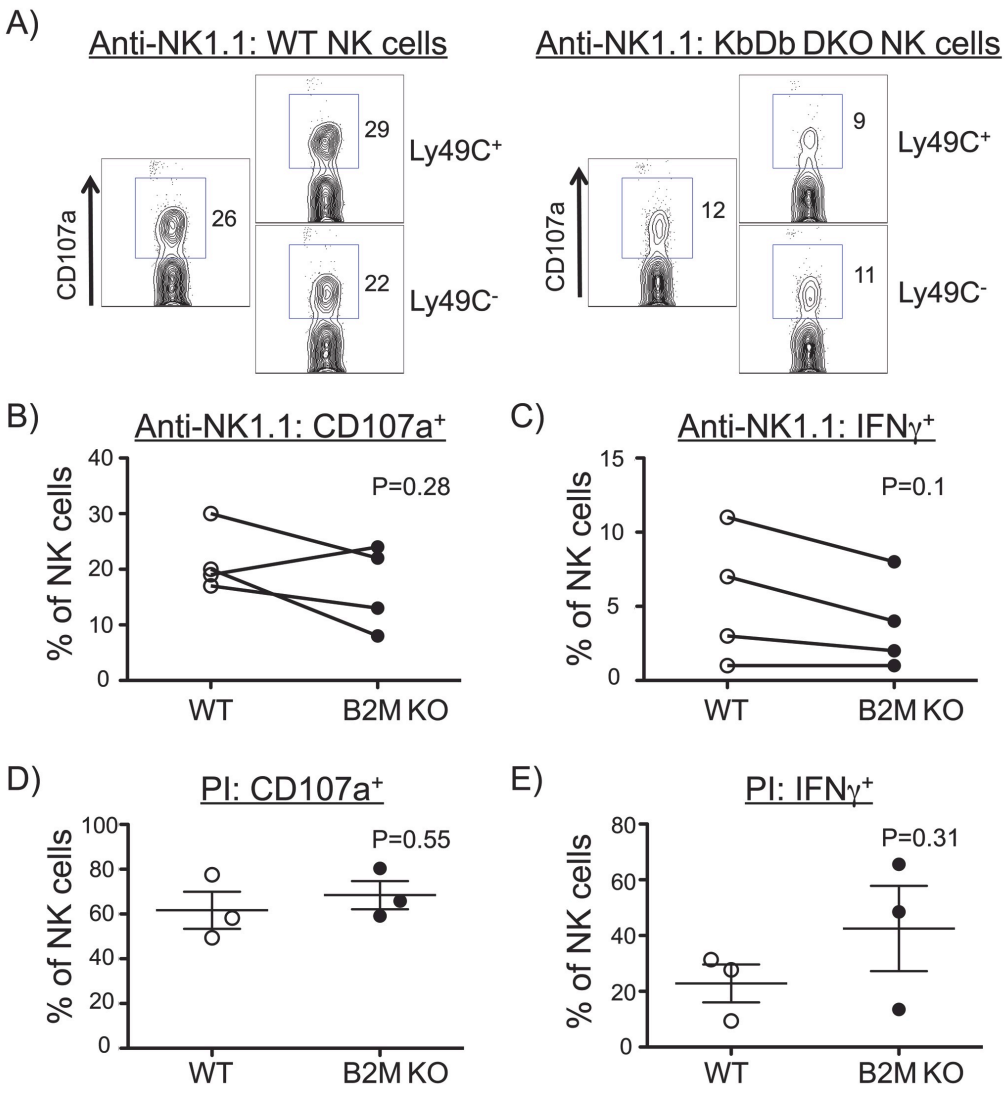


Figure 2: NK cells from MHC Class I deficient mice are less likely to respond to proximal signals as compared to WT controls.
A) NK cells from WT (left) or KbDb$^{-/-}$ (right) hosts were stimulated using platebound anti-NK1.1 and gated on either all NK cells (first and third column), or Ly49C^{+} versus Ly49C^{-} NK cells (second and fourth columns). The propensity of each subset to degranulate was measured by the incorporation of an anti-CD107a antibody. Data shown is representative of 2 independent experiments, N=2. Splenocytes from WT or B2M$^{-/-}$ mice were stimulated with platebound anti-NK1.1 and the ability of NK cells to B) degranulate and C) produce IFNg was measured via flow cytometery. Data is from 4 independent experiments, N = 4. Percentage of NK cells that D) degranulated and E) produced IFNg after stimulation with PMA and Ionomycin as controls in the experiments from B) and C). N=3.

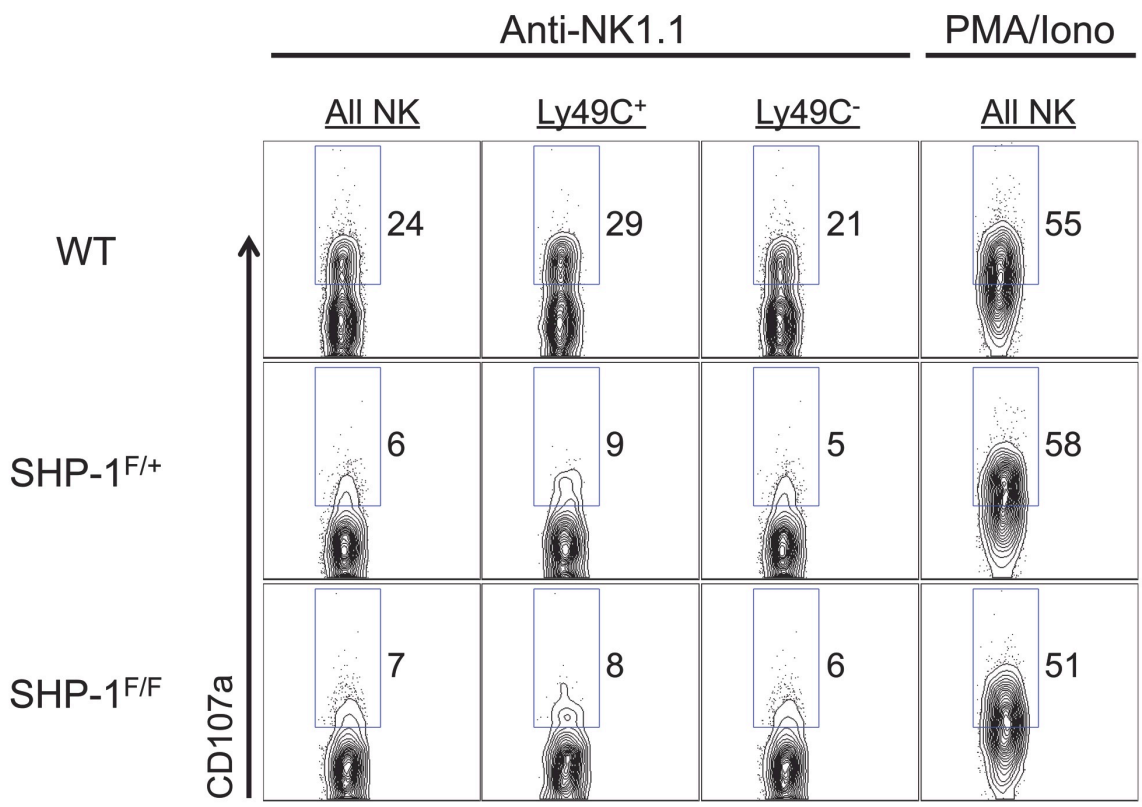


Figure 3: SHP-1 haploinsufficiency results in NK cell hyporesponsiveness
A WT C57/BL6, a heterozygous ER-CreT2/SHP-1 floxed ($SHP1^{F/+}$) and a homozygous ER-CreT2/SHP-1 floxed ($SHP1^{F/F}$) mouse were treated with tamoxifen to induce deletion of the floxed alleles in mature NK cells. 5 days after the end of treatment, splenocytes were harvested and stimulated with either platebound anti-NK1.1, or PMA and Ionomycin (far right). The ability of $Ly49C^+$ NK cells, $Ly49C^-$ NK cells, or all NK cells (all NK) to degranulate was analyzed by flow cytometery. N=1

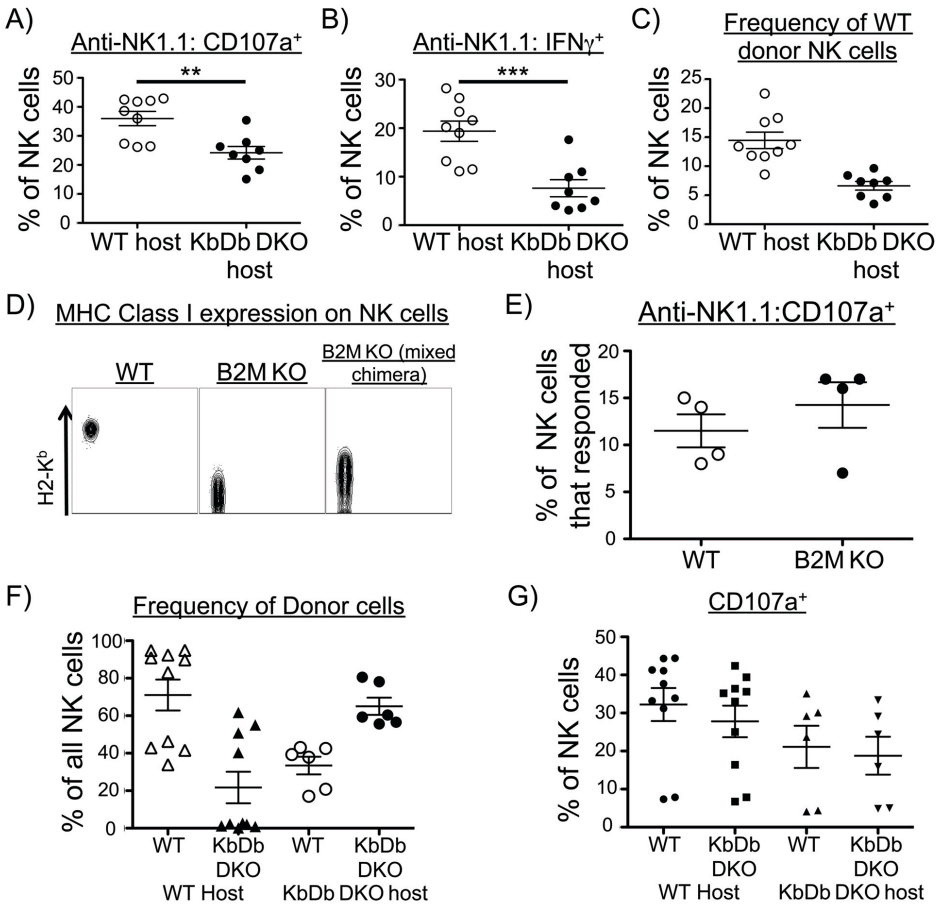


Figure 4: Expression of MHC Class I in cis and trans is important for NK cell tuning.

WT NK cells were adoptively transferred into sublethally irradiated WT or KbDb$^{-/-}$ hosts. 7 days post transfer, NK cells were stimulated with platebound anti-NK1.1 antibody, and the percentage of donor NK cells that A) degranulated or B) produced IFNg is shown as a dot plot. C) Graphical representation of WT donor percentages in the NK cell compartment for the experiments from A) and B). Data shown is pooled from 2 independent experiments, N=8. D) WT and B2M$^{-/-}$ donor bone marrow were mixed at a 1:1 ratio and injected into WT hosts. The expression of H2-K^{b} on NK cells of B2M$^{-/-}$ origin in the mixed bone marrow chimera (right), on WT, or on B2M KO control NK cells are shown (left and center). Data is representative of 2 independent experiments, N=4. E) Splenocytes from WT and B2M$^{-/-}$ mixed bone marrow chimeras from D) were stimulated using platebound anti-NK1.1 antibody, and the percentage of NK cells of WT or B2M$^{-/-}$ origin that responded by degranulating is depicted as a scatter plot. Data is pooled from 2 independent experiments, N=4. F) WT or KbDb$^{/-}$ lethally irradiated hosts were injected with a 1:1 mixture of WT and KbDb$^{/-}$ bone marrow to create mixed bone marrow chimeras. The fraction of NK cells of WT or KbDb$^{/-}$ origin in each chimera is represented as a scatter plot. G) Splenocytes from the mixed bone marrow chimeras from F) were stimulated with platebound anti-NK1.1 antibody, and the fraction of cells that responded by degranulating is depicted as a scatter plot. Data is pooled from 2 independent experiments, N= 6 or 10 per group.

CHAPTER 3: Diacylglycerol kinase zeta negatively regulates NK cell function

Introduction

As seen from the data from chapter 2 (Fig. 2, D and E, Fig. 3), as well as in the published literature, increasing NK cell function by targeting an inhibitory phosphatase paradoxically results in less responsive NK cells (24, 30, 55). One potential explanation is that removing a negative regulator of signaling from NK cells obstructs their function. Alternatively, it is possible that loss of the inhibitory receptor signaling pathway induces NK cells to tune and reduce their responsiveness. A third, non-mutually exclusive explanation is that altering the proximal signaling pathway triggers NK cell hyporesponsiveness. To shed some light on these hypotheses, I decided to target a negative regulator of signaling that was downstream of the activating pathway instead.

As mentioned in chapter 1, manipulation of NK cell activating receptor-mediated signaling is complicated by the variety of signaling modules utilized by NK cell activating receptors. Previous attempts at examining the role of proximal activating signals in NK cells have involved extensive effort, often with conflicting results (22, 83). Fortunately, the distal activation pathway of all the receptors converge upon SLP-76 and PLCγ. NK cells that lack either of these signaling molecules are hyporesponsive to all three groups (ITAM, SAP, YNIM) of activating receptor stimulation (23, 32).

The formation of DAG downstream of PLCγ activation is an important secondary messenger that triggers activation of the AKT, MEK and NFκB pathways. After the activation of an immune cell, one major pathway for the removal of excess DAG from the signal cascade entails its phosphorylation into phosphatidic acid (PA). This reaction is catalyzed by a family of enzymes known as the Diacylglycerol Kinases (DGKs) (49, 84).

The family of DGKs consists of ten different proteins that all share the common substrate DAG. They express a common catalytic domain that enables them to phosphorylate DAG and perform their function. However, the ten DGKs are further subdivided into five classes according to the other domains that they contain. These additional domains are crucial to the differences in cellular localization and activation of each DGK; For example, Type I DGKs have a calcium sensitive domain, while type II DGKs have domains that can interact with inositol's (85). Most tissue types express multiple DGKs, but in lymphocytes, only three DGKs have been reported: DGKα, DGKδ, and DGKζ. The function of DGKδ in lymphocytes is unclear, but multiple roles for DGKα and DGKζ have been reported in the literature (45). As a type I DGK, DGKα expresses a calcium sensitive recoverin homology domain, and its activity is thought to be controlled by calcium flux in the cell. In comparison, DGKζ, a type IV DGK, expresses domains that enable it to interact with trafficking proteins and translocate to the plasma membrane (84).

Both DGKα and DGKζ have been extensively studied in the context of T cell signaling. Indeed, T cells lacking either form of DGK have enhanced ERK activation downstream of the TCR(86), experience hyperproliferation and are less likely to become anergic (87). More specifically, DGKα has been shown to regulate a DAG gradient at the cell-cell interface, which is important for the polarization of the MTOC and formation of multivesicular bodies (88, 89). In comparison, DGKζ is important for determining the overall threshold of T cell activation, by converting an analog signal (receptor ligand interactions) to a digital response(86, 90). In line with these findings, DGKζ deficient $CD8^+$ T cells are more likely to produce cytokine, and become effector memory cells upon viral challenge. Developmentally, both iNKT and T cell selection is impaired in mice lacking both forms of DGK (91, 92), and increased regulatory T cell selection in DGKζ KO mice has been reported by a previous graduate student in our lab (48).

Other than T cells, the loss of DGKζ has also been studied in other immune cell types like macrophages and mast cells. In contrast to its negative regulatory role in T lymphocytes, DGKζ deficiency in macrophages results in reduced cytokine production, and impaired responses to infection by *Toxoplasma gondii* (46). Similarly, DGKζ KO mast cells also show decreased function *in vivo*, and a decreased tendency to degranulate upon stimulation with IgE *in vitro*.

While DAG metabolism and function has not been studied in NK cells, one clue to the importance of DAG signaling is that the NK cells can be activated by phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation. In fact, PMA

and ionomycin has been shown to overcome the hyporesponsive NK cell phenotype (Fig. 3) (23, 30, 32). Given that PMA and ionomycin mimic PLCγ activation by acting as a diacylglycerol (DAG) analog and inducing Ca^{2+} flux respectively, these data suggest that NK signal tuning occurs proximal to PLCγ activation. Thus, I hypothesized that targeting an enzyme that negatively regulates activating receptor-mediated signaling distal to PLCγ would not allow NK cells to tune to their increased signaling capacity and hence, make NK cells hyperresponsive to activating receptor stimulation.

In this chapter, I provide data that indicates genetic ablation of DGKζ, a negative regulator of DAG-mediated signaling, leads to hyperresponsive NK cells in a cell-intrinsic and developmentally-independent manner. Thus, enzymes that negatively regulate distal activating receptor signaling pathways such as DGKζ may represent novel targets for augmenting the therapeutic potential of NK cells.

Results

DGKζ-deficient NK cells are hyperresponsive to activating receptor stimulation in an NK cell-intrinsic and developmentally independent manner

NK cells from WT and DGKζ KO mice were stimulated through multiple cell surface activating receptors. Notably, the development of NK cells was largely similar between WT and DGKζ KO mice with regards to inhibitory receptor expression, activating receptor expression, and maturity (Fig. 5 A-C). Upon activation through three distinct activating receptor families (ITAM-dependent: NK1.1, Ly49D; costimulatory-like: NKG2D; SAP-dependent: 2B4), an increased fraction of DGKζ KO NK cells degranulated and produced IFNγ compared to WT NK cells (Fig. 5D, 5E). Importantly, IFNγ production downstream of cytokine activation (IL-12 + IL-18) or by PMA/ionomycin was similar between DGKζ KO and WT NK cells (Fig. 5F). Increased NK cell function was also observed in DGKζ KO mice treated with Poly I:C, which mimics a viral infection and primes NK cell responses through type I interferons (Fig. 5G). Thus, DGKζ deficiency enhances NK cell function even in an inflammatory environment.

In T cells, both DGKζ and DGKα deficiency have been reported to enhance signals downstream of the TCR (86). Thus I wanted to see if DGKα KO NK cells were also more likely to respond to stimulation with platebound antibody. I found that DGKα KO NK cells were as likely to respond to stimulation

as WT controls, suggesting that DGKζ had a larger role to play in NK cell activation (Fig. 6).

In order to test if DGKζ KO NK cells were indeed more likely to respond to actual target cells, I wanted to directly examine their cytotoxicity capabilities. Thus, WT and DGKζ KO NK cells were expanded in IL-2 to create lymphokine-activated killer (LAK) cells. Similar to freshly isolated NK cells, an increased proportion of DGKζ KO LAKs degranulated and produced IFNγ upon activating receptor stimulation (Fig. 7A). Moreover, this correlated with an increased ability of DGKζ KO LAKs to kill and produce IFNγ upon co-culture with the NK cell-sensitive tumor cell line, YAC-1 (Fig. 7B, 7C).

Since DGKζ KO mice display increased activation in multiple hematopoietic lineages, it was conceivable that the hyperresponsive phenotype of NK cells was not NK cell-intrinsic. To test this possibility, bone marrow (BM) chimeric mice were created by BM transplantation of WT competitor BM mixed with WT control or DGKζ KO BM. Upon stimulation of NK cells from these mice, NK cells in the WT competitor/WT control BM chimeric mice were similarly responsive, while NK cells of DGKζ KO origin in the WT competitor/DGKζ KO BM chimeras were significantly more responsive than the WT competitor controls (Fig. 8A, 8B). Next, to test whether the hyperresponsive phenotype of DGKζ KO NK cells was independent of altered NK cell development, I acutely deleted DGKζ from NK cells using a Tamoxifen-inducible Cre (Cre-ERT2) system. NK

cells from Tamoxifen-treated mice bearing floxed alleles of DGKζ were more responsive to activating receptor stimulation than Cre^+ WT controls (Fig. 8C, 8D). Together, these data suggest that DGKζ deficiency enhances NK cell responsiveness to activating receptor stimuli in an NK cell-intrinsic and developmentally independent manner.

DGKζ KO NK cells are hyperresponsive secondary to enhanced DAG signaling and ERK activation

DGKζ is thought to remove DAG from the activation signaling pathway. In other immune cells, DAG can activate at least 3 distinct downstream signaling pathways: ERK, AKT, and NFκB. To test which of these pathways are activated in NK cells downstream of DAG, I stimulated NK cells with PMA alone and examined the activation of these 3 signaling pathways. In line with previous reports, PMA activation resulted in large increase phosphorylation of ERK (pERK), AKT (pAKT) and IκBα (pIκBα) in T cells. In comparison, NK cells stimulated with PMA had a similarly large increase in pERK, but a more modest increase in pAKT or pIκBa levels. suggesting that DAG more selectively activates the ERK signaling pathway in NK cells (Fig. 9A, 9B). Consistent with this finding, anti-NK1.1 antibody-stimulated DGKζ KO NK cells displayed increased levels of pERK with no difference in pAKT or IκB degradation compared to WT NK cells (Fig. 9 C-E).

Next, to test whether the enhancement in pERK was responsible for the increased function of DGKζ KO NK cells, I treated anti-NK1.1 antibody-activated NK cells with the MEK inhibitor U0126. The degranulation of WT NK cells was inhibited by U0126 in a dose-dependent manner, suggesting that pERK was important for NK cell degranulation. Although DGKζ KO NK cells were also inhibited by U0126 in a dose-dependent manner, they required higher levels of U0126 to attenuate their ability to degranulate (Fig. 9F). Together with increased pERK activation, these data suggest that that the enhanced responsiveness of DGKζ KO NK cells was secondary to augmented pERK levels.

DGKζ KO NK cells are licensed and exhibit increased clearance of TAP-deficient tumor cells *in vivo*.

One unique function of NK cells is their ability to recognize reduced MHC class I expression (missing self) on target cells through their inhibitory receptors. To test whether DGKζ deficiency affected the function of inhibitory receptors, I first stimulated WT or DGKζ KO NK cells with either anti-NK1.1, or a combination of anti-NK1.1 and anti-Ly49G2. As expected, NK cells that did not express the Ly49G2 receptor were not inhibited by the addition of the anti-Ly49G2 antibody. In contrast, Ly49G2 expressing NK cells were less likely to respond to anti-NK1.1 upon the addition of anti-Ly49G2 antibody. More importantly, the % inhibition by the stimulation of the inhibitory Ly49G2 receptor was similar between WT and

DGKζ KO NK cells, suggesting that DGKζ deficiency did not affect inhibitory receptor function. To further test this hypothesis, I examined whether DGKζ KO NK cells displayed intact licensing, a process in which an NK cell that expresses an inhibitory receptor for self-MHC I (e.g., Ly49C in B6 mice) is more likely to respond than one that does not. Both WT and DGKζ KO NK cells that expressed $Ly49C^{+}$ were more likely to respond to activating receptor stimulation than their $Ly49C^{-}$ counterparts, suggesting that licensing and therefore inhibitory receptor signaling was intact in these mice. Additionally, both $Ly49C^{+}$ and $Ly49C^{-}$ DGKζ KO NK cells were hyperresponsive compared to their WT subset counterparts, suggesting that licensing was not the major cause of NK cell hyperreponsiveness in DGKζ KO mice (Fig. 10 A and B).

Another potential explanation for the increase in responsiveness in DGKζ KO NK cells is that they fail to tune correctly. To test this hypothesis, I adoptively transferred both WT and DGKζ KO NK cells into sublethally irradiated WT and MHC Class I deficient hosts. Like WT cells, the DGKζ KO NK cells in the MHC Class I deficient host were less responsive than those in the WT hosts (Fig, 10C).

To test whether the enhanced NK cell function led to better anti-tumor responses *in vivo*, I tested the ability of DGKζ KO mice to clear a TAP-deficient (MHC I low) tumor. RMA (TAP-sufficient) and RMA-S (TAP-deficient) tumor cells were differentially labeled and injected at a 1:3 ratio into WT or DGKζ KO hosts. 18 hours after injection into hosts, RMA-S cells are preferentially killed by NK cells, leading to a decreased RMA-S:RMA ratio. Despite harboring a reduced

number of NK cells (data not shown), the RMA-S:RMA ratio was significantly reduced in DGKζ KO compared to WT mice, suggesting that DGKζ KO NK cells were better at clearing tumor cells than WT NK cells (Fig. 10D).

Discussion

The ability of NK cells to quickly tune their activation threshold to the environment has been a major obstacle in maintaining the functionality of NK cells for clinical purposes. NK cells that lack enzymes that negatively regulate proximal signaling pathways (SHP-1 and SHIP) tune their responsiveness, which results in NK cells with diminished function (24, 30). Similarly, NK cells subjected to an MHC I-deficient environment also leads to hyporesponsiveness (4). In each of these situations, NK cells are normally responsive to PMA and ionomycin, suggesting that the tuning process occurs upstream of PLCγ activation. My data support a model where NK cell tuning acts proximal to the generation of DAG, suggesting that therapeutic targeting of distal negative regulators of NK cell activation can be a means for improving clinical efficacy. In particular, my data from this chapter illustrates that DGKζ is one example of a distal negative regulator in NK cells that can be targeted to improve NK cell responses, likely through the enhancement of ERK signals downstream of the activating receptors (Fig, 11). These data combined support the data from T lymphocytes indicating that the DGKs attenuate responses through the TCR.

One piece of data that came as a surprise was that DGKα deficiency did not affect the functionality of NK cells. Given previous reports that DGKζ and DGKα are important in T cells, I predicted that DGKα loss would also have enhanced NK cell function. However, a previous report demonstrated that

DGKζ had a dominant role in restraining T cell activation compared to DGKα, which could account for the difference in phenotype(86). Additionally, DGKα has been shown to be calcium dependent (84), and perhaps the relatively short assay used in a majority of this chapter may not be sufficient to capture the phenotype of DGKα NK cells. Furthermore, DGKα is known to play a much larger role in maintaining the polarization of the T cell immune synapse. It would therefore be logical hypothesize that it might play similar roles in NK cells as well. If so, testing the ability of DGKα cells to form conjugates with target cells and deliver lytic granules to their intended targets could prove to be more enlightening. In particular, NK cells have been shown to serially kill targets. One might predict that the loss of DGKα could have repercussions on the activity of serial killers.

One of the objectives of my studies was to understand the underlying biochemistry that was affected by the loss of DGKζ in NK cells. In particular I wanted to test if the functions of DGKζ in NK cells was through its modulation of DAG metabolism. Preliminary retroviral transduction studies appear to suggest that mutating the kinase domain of DGKζ affected its ability to restrict NK cell responses, however, the intermediate phenotype of the kinase dead DGKζ mutant as compared to the empty vector and WT DGK controls gave me reason to consider the potential for other functions of DGKζ (Appendix I). I thus wanted to see if I could biochemically assay for the components of the DGK reaction with DAG. Due to the many different potential acyl chains on DAG, direct measurement of DAG levels in cells is a process that has yet to be optimized for

primary cells (93). Instead, I attempted to test a commercially available protocol for measuring total PA levels, with the prediction that compared to WT LAKs, DGKζ deficient LAKs would have decreased production of PA downstream of activation signals. Unfortunately, I was unable to show that there was any formation of phosphatidic acid downstream of receptor stimulation in LAKs (Appendix II). Thus, at this point it is impossible to conclude if DGKζ has other functions aside from recycling DAG levels in NK cells. Based on its sequence, one potential secondary role it could have is in acting as a scaffold protein. DGKζ has been reported to bind to intracellular trafficking proteins like Sorting Nexin-27, as well as associate with PKCs (94, 95), and bringing these proteins together could potentially be important in degranulation and cytokine production.

Despite this setback, my other data also implicate the role of DGKζ in removing excess DAG from NK cells to terminate activation signals. DGKζ deficient NK cells experience an excess phosphorylation of ERK pathway, and require higher concentrations of the MEK inhibitor U0126 to attenuate their function. ERK has been previously shown to be critical for NK cell function(96). However, ERK activation alone is insufficient to drive NK cell responses, as IL-2 stimulation also drives ERK phosphorylation, but is not sufficient to induce NK cells to degranulate or produce cytokine(97). In that respect, it seems probable that pERK is acting as an amplifier for NK cell responses. This idea is reinforced by my observation that the enhancement in DGKζ KO NK function is in both maximal response and sensitivity.

In this chapter, we also examined two of the other major signaling pathways known to be affected by DAG signaling in T cells. We find that indeed, stimulation through the DAG analogue PMA causes T cells to increase in pAKT and pIκB, which are mediators of the AKT and the NFκB pathways respectively. However, in comparison, these two pathways are less activatable by PMA in NK cells, suggesting the dominance of ERK in dictating immediate NK cell responses.

Clinically speaking, not only does targeting DGKζ for NK cell therapies bypass the potential for tuning, it also has two major benefits as compared to convention methods that target the inhibitory receptor. Firstly, targeting the inhibitory receptor has the potential for altering the NK cells specificity. The loss of inhibitory receptor signaling could mean that NK cells might begin to target self. In the case of a downstream negative regulator like DGKζ, its effects can only be potentiated if the NK cell receives a net positive activation signal in the first place. Secondly, the removal of negative regulators that are directly associated with MHC class I-binding inhibitory receptors such as SHP-1 and SHIP abrogates the ability of NK cells to conduct missing-self recognition. In comparison, my data show that inhibitory receptor function and missing-self recognition are intact in DGKζ deficient NK cells.

Lastly, the key position of DGKζ, in cytotoxic T cell and NK cell function highlights its potential as a specific target for clinical purposes in anti-tumor responses (98). A small molecule inhibitor for the type I DGKs has been reported

in the literature (99), though its solubility in aqueous solutions is limited. It is possible that a combination of cellular and pharmaceutical therapies aimed at enhancing cytotoxic functions of both T and NK cells by targeting the DGKs can therefore be achieved in the future.

Figures

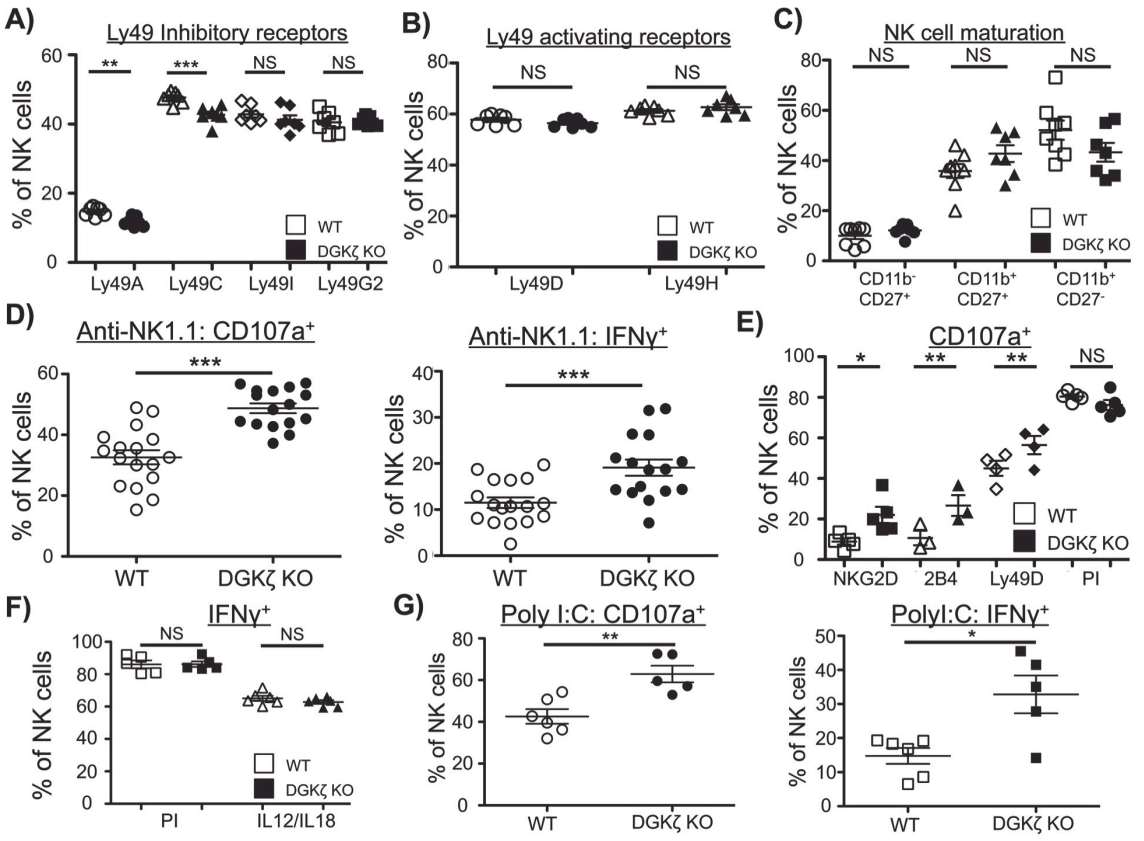


Figure 5 :DGKζ-deficient NK cells exhibit enhanced function downstream of activating receptors.

The proportion of NK ($CD3^-$ $NK1.1^+$) cells expressing A) inhibitory and B) activating Ly49 receptors, and C) CD27/CD11b in WT and DGKζ KO cells is shown. N=7. D) Splenocytes from WT and DGKζ KO mice were stimulated with plate-bound anti-NK1.1 antibody. The proportion of NK cells labeled with anti-CD107a (left) and intracellular IFNγ antibody (right) is shown. N=17. E) Splenocytes from WT and DGKζ KO mice were stimulated with plate-bound antibodies against the indicated activating receptors or with PMA/Ionomycin (PI). The proportion of NK cells incoporating anti-CD107a antibody is shown. N=4-5 per condition. F) Splenocytes from WT and DGKζ KO mice were stimulated with PMA/ionomycin or with IL-12 and IL-18. The proportion of NK cells expressing intracellular IFNγ is shown. N=5-6 per condition G) Splenocytes from polyI:C-treated WT and DGKζ KO mice were stimulated with plate-bound anti-NK1.1 antibodies. The proportion of NK cells labeled with anti-CD107a (left) and intracellular IFNγ antibody (right) is shown. N=5. *,** and *** represent statistical significance of $p<0.05$, $p<0.01$, and $p<0.001$ by Students' t-test, respectively. NS = not significant. Data shown are compiled from 2 separate experiments are shown in A-C and E, and from at least 3 separate experiments for figures D, F and G.

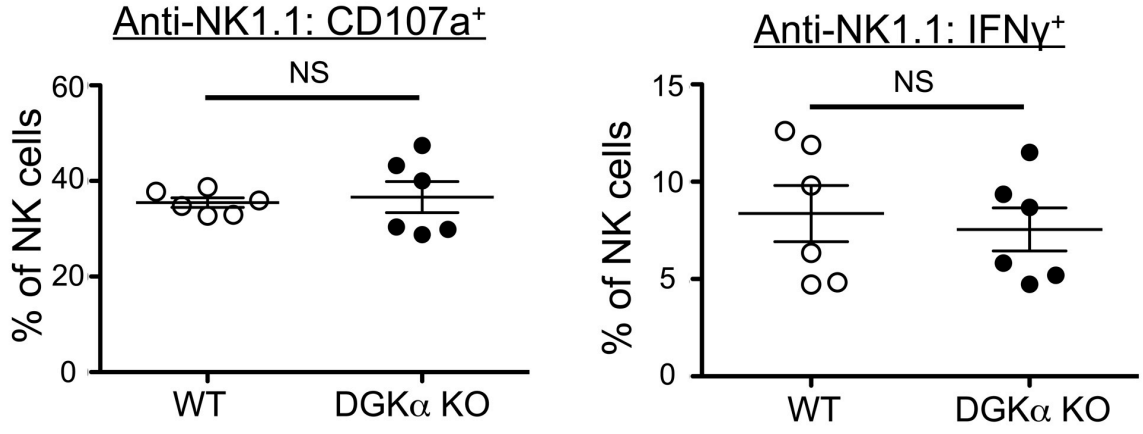


Figure 6: DGKa deficiency does not enhance NK cell responsiveness.
Splenocytes from WT or DGKa KO mice were stimulated with platebound anti-NK1.1, and the ability of NK cells to degranulate (left) or produce IFNg (right) was analyzed via flow cytometry. N=6. NS = Not significant by Student's T test.

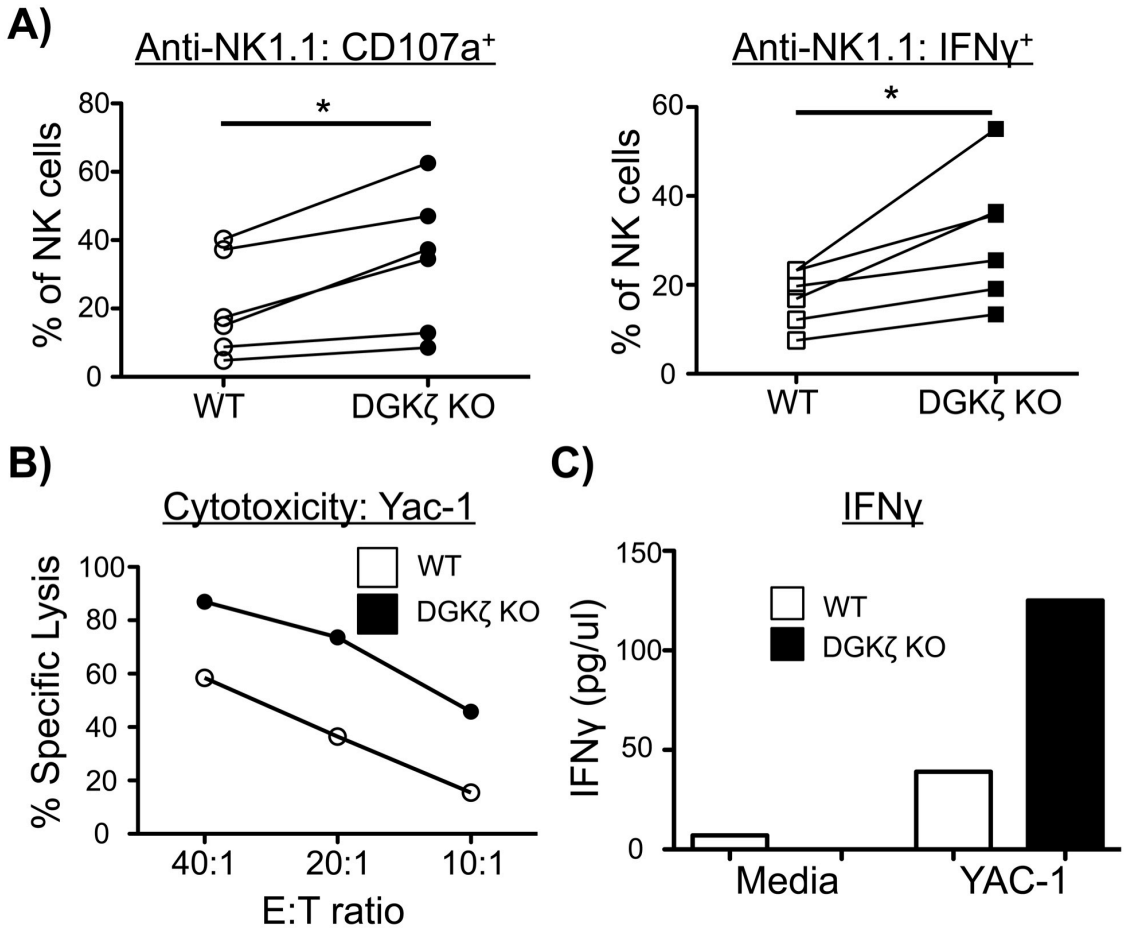


Figure 7: DGKζ KO LAKs display increased cytotoxicity and cytokine production upon interaction with tumor cells.

A) WT or DGKζ KO LAKs were stimulated with plate-bound anti-NK1.1 antibodies. The proportion of NK cells labeled with anti-CD107a (left) and intracellular IFNγ antibody (right) is shown. Data from 6 independent experiments is shown (*P<0.05 by Student's t-test). N=6 B) WT or DGKζ KO LAKs were co-cultured with YAC-1 cells at the indicated E:T ratios and % specific lysis was determined 4 hours later. C) WT or DGKζ KO LAKs were plated with or without YAC-1 cells at a 1:1 ratio for 24 hours. IFNγ content in the cell-free supernatants was determined by ELISA. One representative of N=3 independent experiments is shown for B and C.

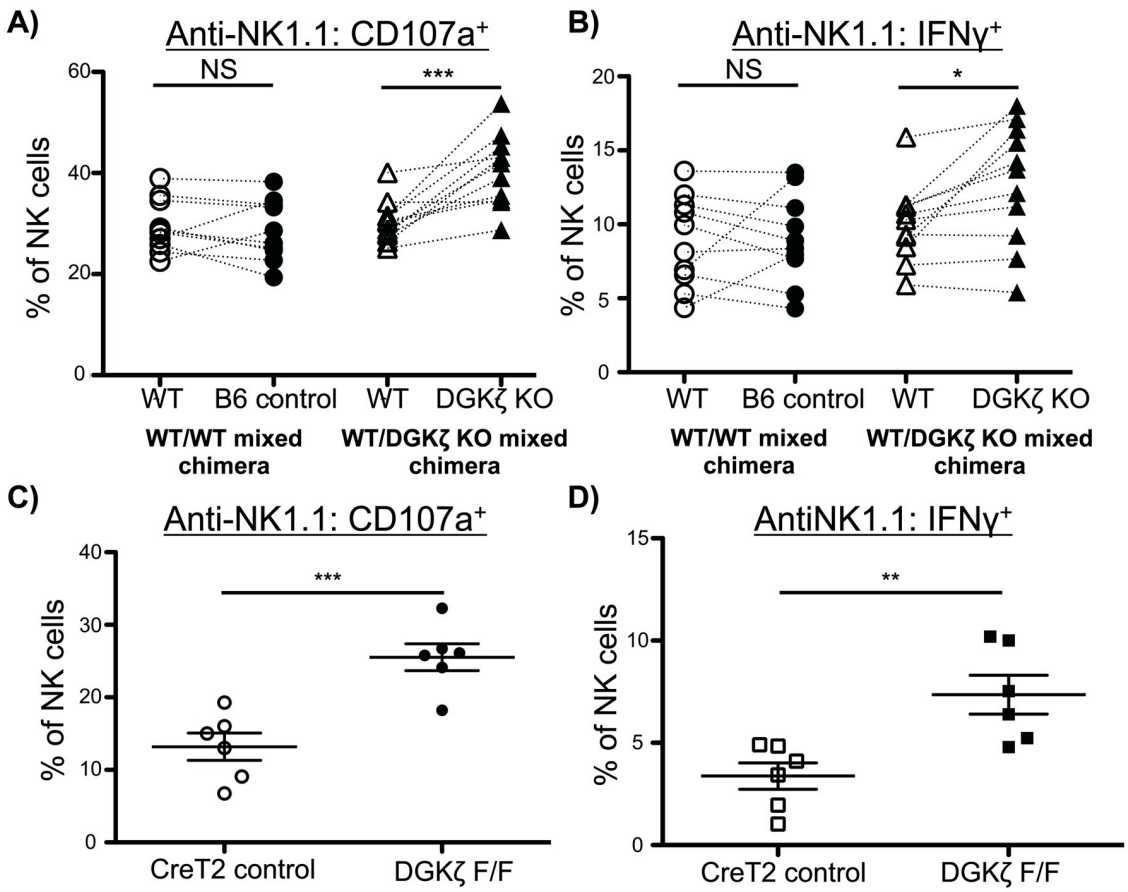


Figure 8: DGKζ KO NK cells are hyperreponsive in a cell-intrinsic and developmentally independent manner.

A) Splenocytes from WT/WT control and WT/DGKζ KO mixed BM chimeras were stimulated with plate-bound anti-NK1.1 antibody. The proportion of NK cells labeled with anti-CD107a and B) intracellular IFNγ antibody is shown. Cells derived from either the B6 control BM or DGKζ KO bone marrow were paired with cells that were of WT competitor BM origin within the same mouse. N=11. C) Splenocytes from Tamoxifen-treated DGK$\boldsymbol{\zeta}^{F/F}$ Rosa26-YFP ERCreT2 or Rosa26-YFP ERCreT2 control mice were stimulated with plate-bound anti-NK1.1 antibody. The proportion of NK cells ($CD3^{-}DX5^{+}NKp46^{+}YFP^{+}$ lymphocytes) labeled with anti-CD107a and D) intracellular IFNγ antibody is shown. N=6. *,** and *** represent a statistical significance by students' t-test of $P<0.05$, $P<0.01$ and $P<0.001$ respectively. NS= not significant. All data shown in this figure is compiled from at least 3 independent experiments.

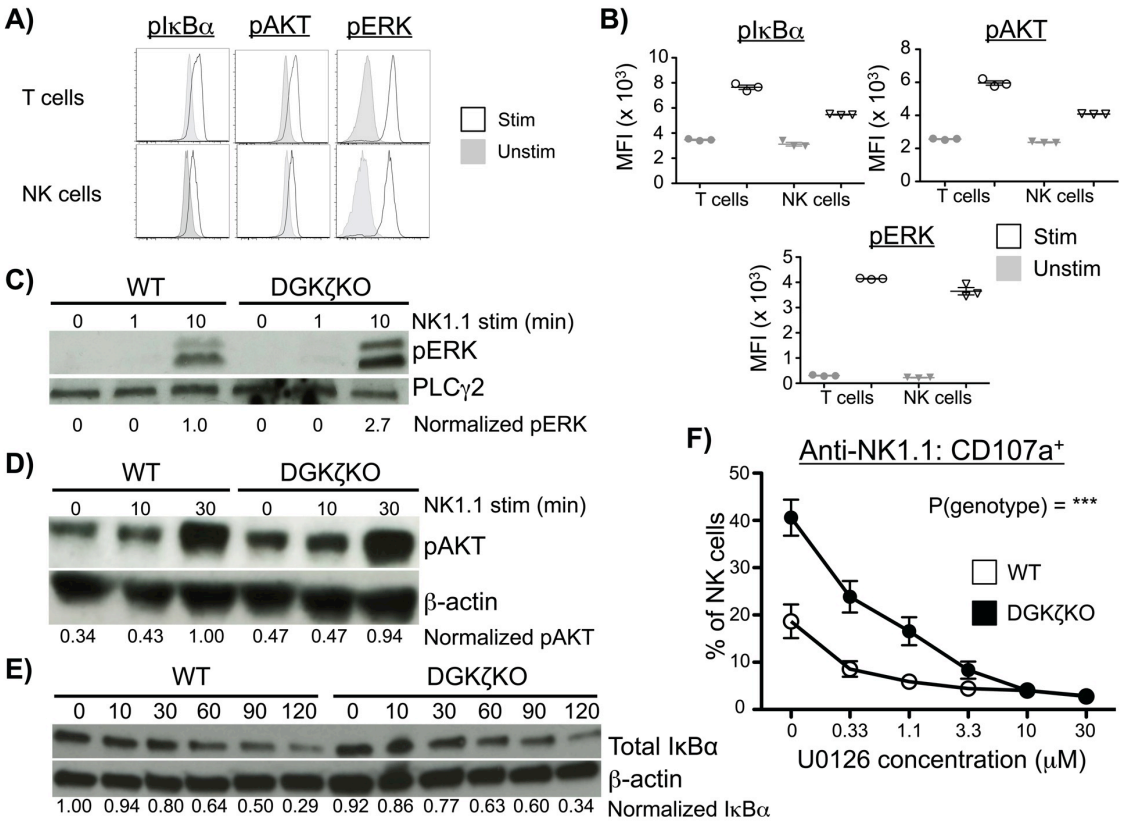


Figure 9: Enhanced ERK signaling is associated with the hyperresponsiveness of DGKζ KO NK cells.

A) Splenocytes from WT or DGKζ KO mice were stimulated with PMA for 5 minutes followed by flow cytometric analysis of pIkBa, pERK, and pAKT expression by T cells ($CD4^+$ or $CD8^+$) and NK cells ($CD4^-CD8^-NK1.1^+$). Representative histograms and B) mean fluorescence intensities are shown. One representative of 2 independent experiments is shown, N=6. C) LAKs were left unstimulated or stimulated with anti-NK1.1 antibody for the indicated duration. Cell lysates were analyzed for pERK, D) pAKT, or E) IκB by Western blot analysis. Total PLCγ2 or b-actin was used as a loading control. All bands were normalized to their respective loading control, and values for pERK and pAKT were then divided by the maximum WT response, while values for total IkBa were divided by the unstimulated WT. F) WT or DGKζ KO splenocytes were stimulated with plate-bound PK136 antibody in the presence of various concentrations of the MEK inhibitor U0126. The proportion of NK cells incorporating anti-CD107a antibody is shown N=3-5 per concentration. NS = not significant by Students' T test, *** = P<0.001 by 2 way-ANOVA. Data is representative (in C-E) or complied from (F) at least 3 independent experiments.

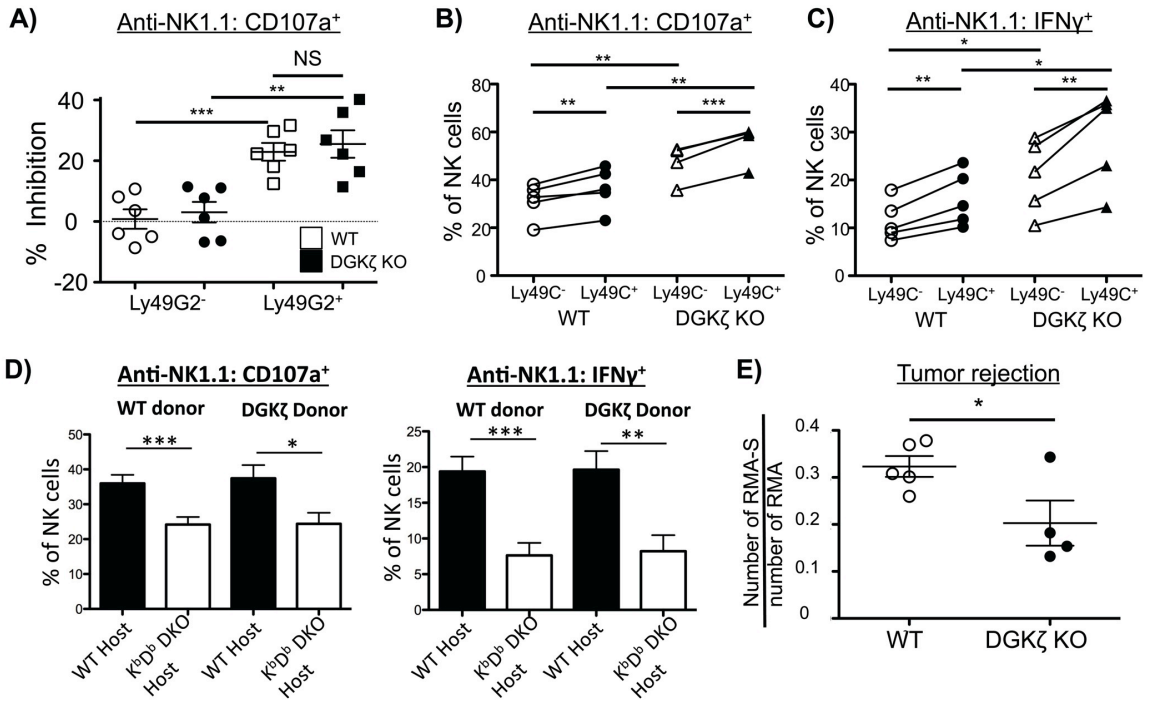


Figure 10: DGKζ KO NK cells are licensed and more effectively clear TAP-deficient tumor cells.

A) Splenocytes from WT and DGKζ KO mice were stimulated with either plate-bound anti-NK1.1 antibody + anti-Ly49G2 antibody, or anti-NK1.1 antibody alone. The % inhibition by the addition of the Ly49G2 antibody on the incorporation of anti-CD107 by $Ly49G2^-$ or $Ly49G2^+$ NK cells is shown by scatter plot. Data shown is compiled from 2 independent experiments N=6 B) Splenocytes from WT and DGKζ KO mice were stimulated with plate-bound anti-NK1.1 antibody. The proportion of $Ly49C^+$ versus $Ly49C^-$ NK cells incoporating anti-CD107a and C) intracellular IFNγ antibody (right) is shown. Data shown is compiled from 2 independent experiments, N=5. D) Sublethally irradiated WT or KbDb DKO hosts were injected with congenically disparate WT and DGKζ KO splenocytes. 7 days after adoptive transfer, splenocytes were harvested and stimulated with plate bound anti-NK1.1. The responsiveness of WT and DGKζ KO NK cells in each host was measured by $CD107^+$ (left) and IFNγ production (right) was measured by flow cytometery. Data shown is pooled from 2 independent experiments, N=7. E) WT or DGKζ KO mice were injected i.v. with a mixture of CFSE-labeled RMA and CelltraceViolet-labeled RMAS-S tumors. 18 hours later, splenocytes were analyzed for the presence of residual tumor cells via flow cytometry. The ratio of RMA-S versus RMA within each WT or DGKζ KO mouse was calculated and shown as a scatter plot. One representative experiment of 2 independent experiments is shown. *,** and *** represent a statistical significance by students' t-test of $P<0.05$, $P<0.01$ and $P<0.001$ respectively.

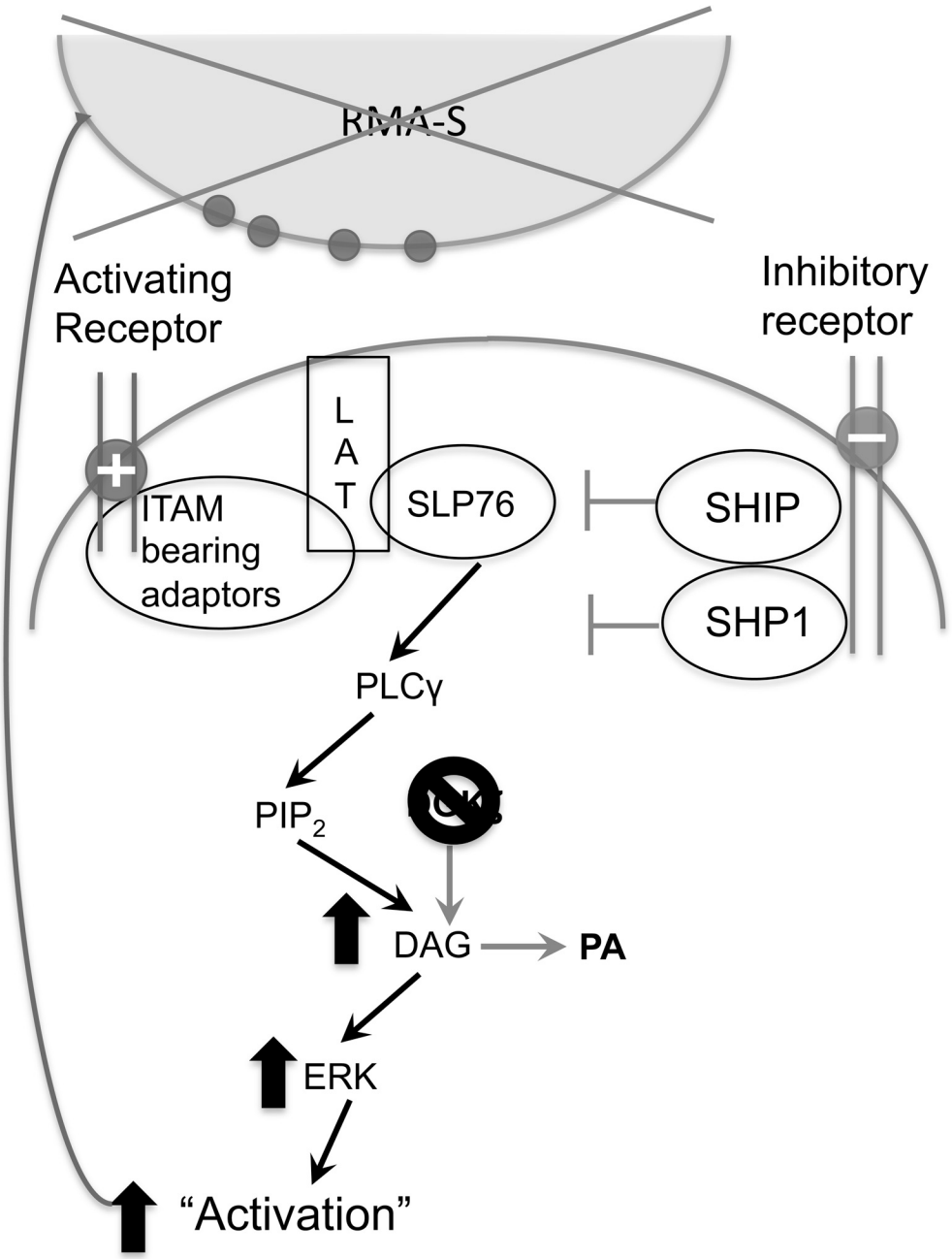


Figure 11: DGKζ deficiency enhances NK cell function without affecting inhibitory receptor signaling.
A summary figure demonstrating how DGKζ deficiency enhances pERK levels in NK cells, resulting in increased function and tumor rejection, while preserving inhibitory receptor signaling and missing self recognition.

CHAPTER 4: Discussion

Overview

In this thesis, I examine the nature of tuning in NK cells. In chapter 2, I find that NK cells are likely to be tuned by both cell intrinsic and cell extrinsic expression of MHC Class I. They are also inherently sensitive to the total cellular SHP-1 levels, and tune rapidly (<7days post gene deletion). I also confirmed that NK cell tuning can be bypassed using PMA and Ionomycin stimulation, suggesting that the downstream activation pathways are intact in tuned NK cells. In chapter 3, I demonstrate that knowledge of the proximal nature of NK cell tuning allows us to modulate NK cell responsiveness by targeting a distal negative regulator of signaling DGKζ. NK cells lacking DGKζ are more likely to respond to stimuli through their activating receptors, but are just as likely to respond to cytokines or PMA and Ionomycin. DGKζ KO NK cells are hyperfunctional due to the excess activation of the ERK pathway, and are more likely to reject MHC Class I tumors than their WT counterparts.

However, many questions still remain in the field of NK cell tuning. One striking finding is the dependence of NK cells on SHP-1 expression. The haploinsufficiency of SHP-1 in NK cells confirms the importance of inhibitory signaling to NK cell function. It would be interesting to see if this haploinsufficient phenotype also occurs in NK cells lacking one allele of the other inhibitory phosphatases like SHIP and SHP-2. Additionally, this data suggests that NK cells

could be uniquely sensitive to SHP-1 concentrations in the cytoplasm. If so, one potentially interesting experiment is to overexpress SHP-1, and see how NK cell function is affected. My usage of the ER-CreT2 system to delete the gene also shows that mature NK cells are still capable of detecting differences in SHP-1 levels, and supports previous findings on germline SHP-1 and SHIP deficiency (24, 30, 55). I also show that while these cells are hyporesponsive to activating receptor stimulus, they are still responsive to PMA and ionomycin. Indeed, other studies of tuned NK cells have also shown that “hyporesponsive” NK cells respond as well as WT controls not just to PMA and Ionomycin, but also IL-12 and IL-18 (23, 32).

The idea that NK cells are still normally responsive to cytokine has made the overall *In vivo* importance of tuning uncertain. Experiments have shown that unlicensed NK cells (that have reduced responsiveness *in vitro*) are the primary responders in certain viral infections(82). The same report also found that NK cell licensing normalized the *in vitro* responsiveness of NK cells that expressed self recognizing inhibitory receptors to those that did not, but only when the NK cells were challenged with targets that expressed ligands for the inhibitory receptors. This suggested that tuning (or licensing) is important to improve NK cell responses during missing self recognition (100). In that respect, understanding the molecular mechanism of tuning could unlock the key to improving NK cell function against MHC class I low targets. Another potential hypothesis for the importance of tuning involves the prevention of autoimmunity by NK cells that do not express the correct inhibitory receptors.

Regrettably, I was unable to identify the molecular mechanism of tuning. However, based on my data and that of others, it is likely that NK cell tuning is focused on the proximal signaling pathway. One potential mechanism for induced chronic activation could be a positive feedback loop on the inhibitory pathway. The Src kinase LCK has been shown to phosphorylate the inhibitory receptors, and may induce the recruitment of the inhibitory phosphatase SHP-1 to the plasma membrane where it can attenuate signaling (29). Furthermore, SHP-1 function has also been shown to increase in response to PA, which is a byproduct of activation signals(45). However, this explanation does not account for the acute defect in NK cell function upon deletion of a negative regulator (24, 30, 55).

NK cell tuning is also thought to be controlled at a transcriptional level. Mice bearing a mutation in NKp46 revealed a potential role for Helios in the tuning of NK cells (101). Helios is a member of the Ikaros family of transcription factors; in particular Ikaros itself has been shown to calibrate lymphocyte responses(102). Ikaros and its family member Aiolos are also important in the development and maturation of NK cells respectively (103, 104). While the precise mechanism of Helios involvement in NK cell tuning is unknown, it is possible that Helios could bind to Ikaros or Aiolos, and thereby participate in NK cell tuning as well.

Another potential mechanism for tuning could be the downregulation of proximal signaling molecules shared by the three activating receptor signaling pathways. This is supported by the observation that tuned NK cells are

hyporesponsive to stimuli through multiple receptors, not just the receptor that is chronically stimulated (72). In preliminary data that I have not discussed here, I have found that prolonged stimulation through the activating receptor reduces the protein levels of proximal signaling mediators, which could be a mechanic by which tolerance is induced. A signaling strength dependent negative feedback loop on proteins in the activating signal cascade could also serve as a reason why NK cells appear to have dependence on MHC class I expression both in cis and trans (chapter 2) (58, 105, 106). It could also serve as an explanation for the rapid downregulation of NK cell responses upon loss of inhibitory phosphatase activity.

All three options are not mutually exclusive, and could play important roles in NK cell tuning. Fundamentally, solving the mechanism of tuning will be crucial in calibrating cellular therapies. Chimeric antigen receptors (CARs) have been effective in clearing tumor burden from patients, but clinical data has also shown that CAR therapy can cause a massive cytokine release syndrome (107). If tuning can be translated to other cell types, it could potentially help alleviate the effects of this cytokine storm. On the other hand, patients that have undergone allogeneic bone marrow transplantation of bone marrow have also reported an upregulation of NKG2 receptors on their NK cells, indicating the occurrence of a tuning phenomenon(108). Thus, by targeting tuning in these patients, prolonged retention of NK missing self function could be achieved, which would enhance graft versus tumor effects. On a similar note, CAR therapy is also being translated into NK cells, as NK cells do not require MHC matching in order to

activate against tumors (109). However, for prolonged function against the cancer cells, it is likely that these CAR NK cells will need to be edited to prevent them from tuning.

In the third chapter of this thesis, the phenotype of the DGKζ KO mice confirms what has hitherto been a theoretical proposition: it is possible to improve NK cell responses by targeting negative regulators of signaling. Before this body of work, previous reports had only shown that NK cells would adapt by becoming less functional (24, 30, 32, 55), or regain lost functionality (53, 54). However, the study of DGKζ KO NK cells has also added new dimensions to NK cell development and function.

One unexpected finding in DGKζ KO NK was their surface receptor phenotype. NK cells lacking PLCγ2 or SLP-76 cannot propagate activating signals, and have severely decreased inhibitory Ly49 receptor acquisition (23, 32); whereas B2M KO mice which have unopposed activating signals during development have increased fraction of NK cells expressing the Ly49 receptors (17, 110). Based on this information, and the hyperactive phenotype of the DGKζ KO NK cells, I predicted that they would have increased levels of inhibitory receptor expression, much like the B2M KO mice. However, I found that these mice had comparable expression of the inhibitory NK cell receptors as compared to WT controls. Overall, this discovery increases the attractiveness of DGKζ as a target for modulating NK cell function without affecting their ability to target missing self. However, these findings also suggested that calcium flux might play

a larger role than DAG levels in affecting receptor acquisition. Alternatively, it might also be possible that like iNKT cells, the only the deletion of both DGKα and DGKζ will uncover defects in NK cell development(111).

The discovery that DGKζ KO NK cells are more activatible than WT NK cells begets a number of questions. Firstly, if the other two isoforms of DGK (α and δ) expressed in lymphocytes have roles to play in NK cell development or function. My preliminary data on the DGKα mice suggest that it does not have a role to play in the activation of fresh NK cells, whereas DGKδ has not been studied in lymphocytes yet. Furthermore, based on data from T cells, it is likely that studies on DGKα function should focus on synapse formation and MTOC polarization. It could be possible that DGKα deficiency may affect the ability of NK cells to serially kill target cells. Another intriguing possibility is that through its calcium sensitive EF hand domain, DGKα may also play a larger role than DGKζ in Ly49 receptor acquisition.

In contrast, not much has been reported regarding DGKδ in immune lymphocytes. Domain analysis of DGKδ indicates that it has a PH domain, which has been shown to promote its translocation to the plasma membrane upon PMA stimulation (84). Additionally, the PH domain is known to weakly bind to other phosphoinositols, which suggests that DGKδ might have a role to play in the recycling of DAG to the plasma membrane.

Compared to T cells however, DGKζ KO NK cells display hyperactive ERK, and less activation of pathways like pAKT and the NFκB pathway. My data

on the effects of the non-competitive MEK inhibitor U0126 on both WT and DGKζ KO NK cells suggests that ERK is not saturating under normal conditions. In both WT and in the DGKζ KO mice, NK cells are still highly sensitive to the addition of U0126, which highlights its importance in modulating NK cell activation compared to the other pathways downstream of DAG. This observation begs the question: Why ERK? Perhaps due to the rapid nature of NK cell responses or the time course of assays used to stimulate them in this thesis, the role of the other pathways like AKT and NFκB were not fully appreciated. Indeed, the slight reduction in total NK cell numbers in DGKζ KO mice may point to an expansion or survival defect, both of which are also downstream of AKT and NFκB activation.

Like the NK cells in this thesis, $CD8^+$ T cells that are DGKζ deficient have been reported to increase their cytokine production and degranulation; however, the same paper also highlighted a defective expansion in the memory T cell compartment (112). NK cell memory is now also a major point of discussion in the field, and NK cells that have seen viral expansion are more likely to respond to rechallenge(113). It would therefore be interesting to study NK cell memory in the context of DGKζ deficiency. One would predict that the NK cells in DGKζ KO mice would be less likely to expand during rechallenge.

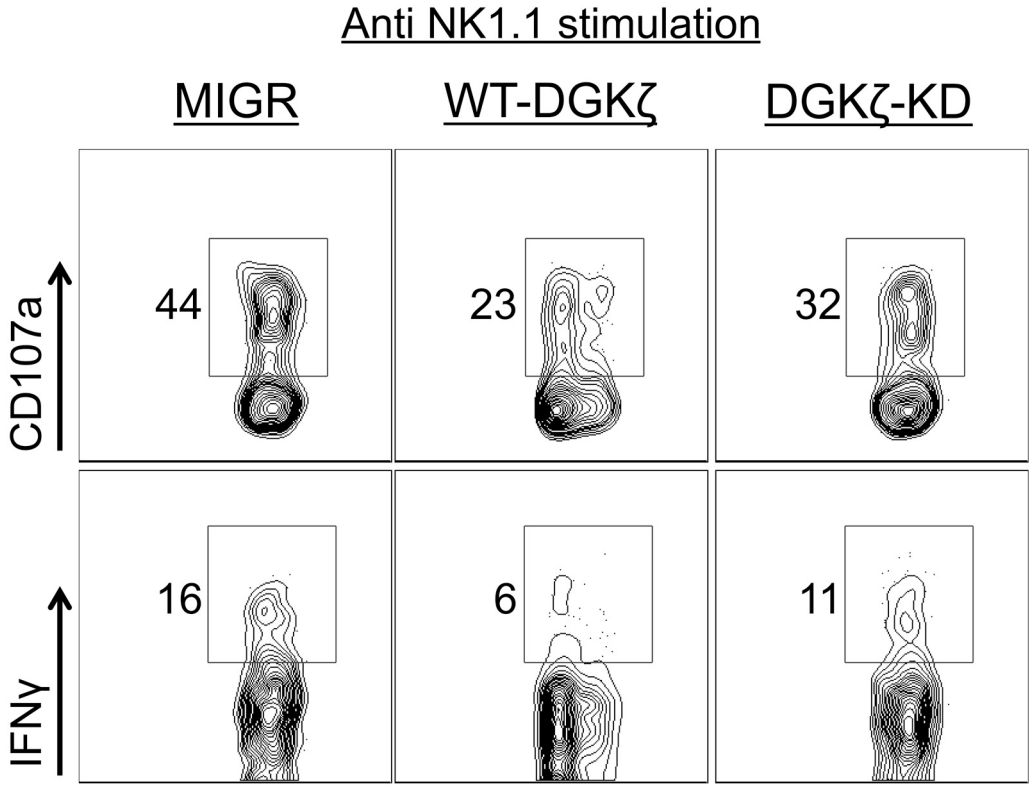
A related field of study would involve the role of PA in NK cells. Very little is known about PA metabolism in immune cells. However, it comes as a surprise that stimulation through the NK receptors did not appear to increase PA levels.

Due to technical limitations of the PA assay, I was unable to test all the conditions that I would have liked. It is probable that the kinetics of PA formation or NK cell stimulation were not captured by the assay. One approach that could help answer this question is the use of fluorescent tags that can measure DAG or PA levels via live FRET imaging (93). This technology, once implemented in primary NK cells, could also be useful in determining which checkpoints of the NK cell cytotoxic process is affected by localize DAG concentrations (114).

On a more clinical note, DGKζ is being studied as a potential target for improving anti-tumor responses in adoptive T cell therapy. My data also highlight its importance in restraining NK cell activation, and provide further support for the utility of modulating DGKζ activity in immune cells. More specifically, perhaps a combination therapy targeting DGKζ in T and NK cells (for example using a small molecule inhibitor) could prove beneficial, and provide a broader spectrum of coverage against both MHC Class I expressing and MHC class I low tumor targets.

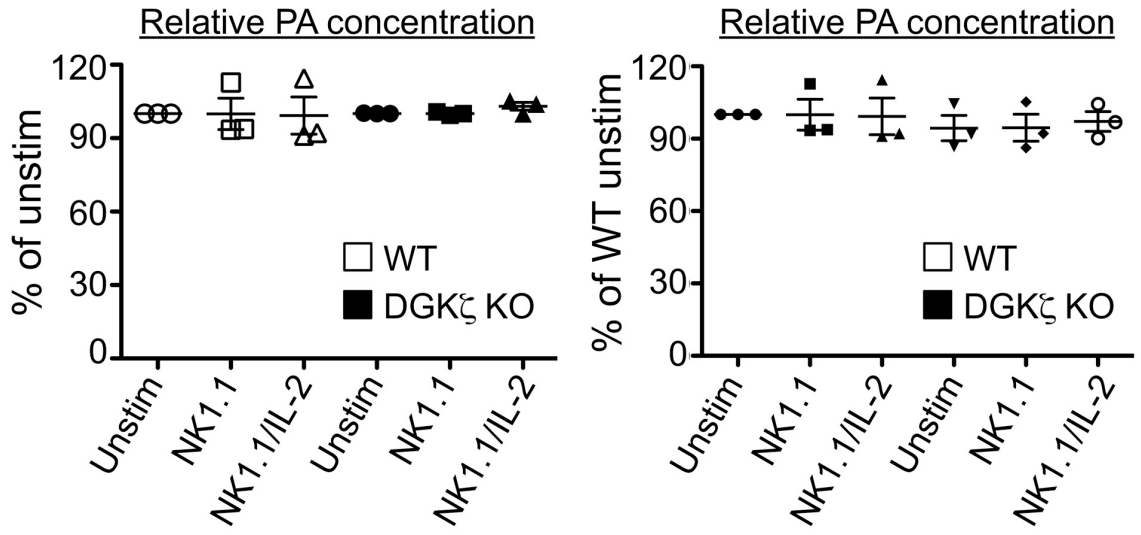
In any case, I believe that the findings of this thesis have added some perspective to bypassing the phenomenon of NK cell tuning. Importantly, it shows proof of concept that negative regulators of signaling in NK cells can be genetically altered to improve their function, and may guide others toward improving NK cell therapies.

APPENDIX I



Appendix I: The kinase domain of DGKζ is important for its function
A) DGKζ KO bone marrow was retrovirally transduced with empty MIGR, WT-DGKζ, or DGKζ that had a point mutation in its kinase domain to stop its catalytic activity (DGKζ-KD). The transduced bone marrow was then injected into lethally irradiated hosts. 8 weeks after injection, splenocytes were harvested and stimulated with platebound anti-NK1.1 antibody. The ability of NK cells expressing the viral proteins (marked by GFP expression) to degranulate (top) and produce IFNg (bottom) was analyzed by flow cytometery. N=2

APPENDIX II



Appendix II: DGKz deficiency may affect total PA levels, but receptor stimulation does not appear to affect PA levels in LAKs.
WT, or DGKz KO LAKs were stimulated with soluble anti-NK1.1, or soluble NK1.1 and IL-2, and then total cell lysate was analyzed for PA levels. The value of PA in each sample was normalized to either unstim controls (left) or WT unstim control only (right).

APPENDIX III: Materials and methods

Mice

B2M KO, KbDb DKO, C57BL/6 (B6) and B6.SJL mice were purchased from The Jackson Laboratory, Taconic Biosciences or Charles River Laboratories. DGKα KO DGKζ KO and DGKζ$^{F/F}$ mice were described previously (46, 48, 86). SHP-1$^{F/F}$ mice were bought from The Jackson laboratory and bred to ER-CreT2/Rosa-26-flox-stop-YFP reporter mice. CD45.1/45.2 heterozygous mice were created by breeding B6 mice to B6.SJL mice. Mice were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

Flow cytometry, cell sorting, and data analysis

Abs for flow cytometry were purchased from BD Pharmingen (San Diego, CA), Biolegend (San Diego, CA), eBioscience (San Diego, CA), or Molecular Probes, Invitrogen (Carlsbad, CA). Ly49C (4LO-3311) antibody was obtained from the UCSF cell culture facility. Flow cytometry and FACS were performed with an LSR II, FACS Canto, or a FACS Aria cell sorter (BD Biosciences). Data were analyzed with Flowjo software (Tree Star) and Prism (Graphpad), and all scatter plots have mean and SEM depicted. All flow data are pregated on live lymphocyte singlets, NK cells are $CD3^{-}DX5^{+}NKp46^{+}$ are $CD4^{-}CD8^{-}NK1.1^{+}DX5^{+}$ unless otherwise stated. For phosphoflow, splenocytes from WT or DGKζ KO mice were stimulated with 100 ng/ml PMA and incubated at 37°C for 5 minutes. Cells were

fixed with 1.5% PFA and permeabilized with methanol before being stained for flow cytometric analysis.

NK cell functional assays

$DX5^+$ cells from B6 or DGKζ KO mice were enriched by MACs (Miltenyi Biotech) and expanded in hIL-2 (1000 U/ml) in tissue culture media for at least 5 days to create LAKs. LAKs were used for assays after resting in fresh cytokine-free media. For NK cell activation, freshly isolated splenocytes or LAKs were cultured together with anti-CD107a antibody, hIL-2 (1000U/ml) and Monensin for 6 hours in tissue culture plates that were pre-coated with antibodies against NK cell activating receptors (20 μg/ml, overnight at 4°C unless otherwise specified). Cells were analyzed for anti-CD107a antibody and intracellular IFNγ by flow cytometry. In some experiments, splenocytes were preincubated with the specified concentration of the MEK inhibitor U0126 for 30 min both before and during stimulation. In experiments involving inhibitory receptor function, anti-Ly49G2 antibody (20 μg/ml) was added to some wells that also contained anti-NK1.1 antibody. % inhibition was then calculated by the following formula: (% of NK cells that responded to anti-NK1.1 - % of NK cells that responded to anti-NK1.1 + anti-Ly49G2) / (% of NK cells that responded to anti-NK1.1)

A luciferase expressing YAC-1 cell line was co-cultured with LAKs at varying E:T ratios for 4 hours for bioluminescent cytotoxic assays as previously described (115). Luciferase activity was detected via an IVIS Lumina II imaging system and

% specific lysis was calculated by the following formula: (minimum - test well) / (minimum – maximum) x 100%. To measure cytokine production after co-culture with YAC-1 cells, LAKs were co-cultured at a 1:1 ratio with either no targets or YAC-1 target cells for 24h in LAK media. The IFNγ content in cell-free supernatants was determined by ELISA (Biolegend).

Adoptive transfers and mixed BM chimeras

In chapter 2, splenocytes (~50 × 10^6 cells) from WT B6.SJL mice were injected i.v. into sublethally irradiated (6.0 Gy) WT B6 or KbDb DKO hosts. 7 days after transfer, the spleens from host animals were harvested for functional analysis. BM (5 × 10^6 cells) from B2M KO or KbDb DKO mice were mixed with CD45.1/45.2 heterozygous competitor BM (5 × 10^6 cells) and injected i.v. into lethally irradiated B6.SJL congenic host mice (9.5 Gy). Splenocytes were taken from the BM chimeras between 9-12 wk later for functional analysis.

In chapter 3, BM (5 × 10^6 cells) from control B6 or DGKζ KO mice were mixed with CD45.1/45.2 heterozygous competitor BM (5 × 10^6 cells) and injected i.v. into lethally irradiated B6.SJL congenic host mice (9.5 Gy). Splenocytes were taken from the BM chimeras between 9-12 wk later for functional analysis.

Acute deletion of DGKζ floxed alleles using ERCreT2

SHP1$^{F/F}$ Rosa26-YFP ERCreT2, DGKζ$^{F/F}$ Rosa26-YFP ERCreT2 or control Rosa26-YFP ERCreT2 mice were treated with tamoxifen for 5 days as previously

described (116). 1 week after the end of treatment, splenocytes were removed for functional analysis.

Retroviral transduction of BM

Retroviral transductions were performed as previously described(86). Briefly, DGKζ KO donors were injected with 5-fluorouracil. 5 days after injection, BM from these mice were harvested and incubated with viral particles that had either the empty vector MIGR, WT- DGKζ, or DGKζ KD mutant isoform. The transduced bone marrow was then injected into lethally irradiated hosts (9.5 Gy), and 8 weeks after injection the splenocytes were taken for functional analysis.

Assay for PA concentration

LAKs (5×10^6 cells) were made as described above. They were then stimulated using soluble anti-NK1.1 with or without the addition of IL-2 for 15 minutes. The cells were then lysed and analyzed according to the instructions in the Total Phosphatidic Acid Assay kit (from Cayman Chemicals).

Western blot analysis

MACS-enriched splenic $DX5^+$ NK cells (pERK) or LAKs were rested for 2-4 hours, and stimulated with soluble isotype control Ab or PK136 Ab (30 μg/ml) for the indicated times. The cells were then lysed in 1% Ipegal in Tris-buffered saline with protease/phosphatase inhibitors (protease inhibitor cocktail solution [Roche,

Sigma]), and the proteins were resolved by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). The phosphorylation of ERK1/2 (Thr202/Tyr204), total IkBα (Ser32, and pAKT (Ser473) were analyzed by Western blotting. Total PLCγ2 or beta-actin was used as a loading control. All antibodies were from Cell Signaling (Danvers, MA), except for anti-beta-actin-HRP antibody (Sigma)

In vivo tumor challenge

RMA and RMA-S cells were labeled with CFSE and CellTrace violet, respectively and injected i.v. at a 1:3 ratio (20 × 106 cells total) into WT or DGKζ KO mice. 18 hours after injection, spleens were harvested from these mice and the presence of tumor cells was analyzed by flow cytometry.

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