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Regulation of Natural Killer Cells: SHIP-1, 2B4, and Immunomodulation by Lenalidomide

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by

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Dedication

I dedicate this work...

To my family...I have awesome parents who have provided me with love, support, and motivation. Thank you for always believing in me and for being there. To my brothers: James, Ronny, Chris, and Paul who helped shape me into a strong and compassionate woman. I love you guys. And finally, to my adorable nephews, James and Jaiden, whom I love more than I could ever describe. Please never, ever, ever give up on your dreams. Accept life's challenges as opportunities, not hurdles. It will all be worth it someday, I promise.

To my friends...I have been blessed with amazing people in my life. Thank you for all of your encouragement, love, and understanding. It may never really make sense when I say, "I have to feed my cells", but you were always there for me. My life is *truly* enriched by all of you. Thank you for your friendship.

"Perfection is a state in which things are the way they are, and are not the way they are not. Life is perfect."

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ABSTRACT

Natural Killer cells (NK) are critical components of the innate immune system. Often referred to by their morphology, these large granular lymphocytes (LGLs) are bone marrow-derived lymphocytes and can be found throughout the body. NK cells reside in the liver, lymph nodes, spleen, thymus, and mucosal-associated lymphoid tissues (MALT). Importantly, NK cells also circulate throughout the blood where they function as surveyors of the body and are armed to eliminate malignant, infected, damaged, or foreign cells.

NK cells function by a dual receptor system. That is, NK receptors are broadly categorized as inhibitory or activating. It is a fine balance, or lack thereof, that dictates the function of an NK cell. Unlike their T and B cell adaptive counterparts, NK cell receptors (NKR) are germline encoded and do not undergo gene rearrangement. NKRs are expressed in a variegated but overlapping fashion such that different cell subsets in the NK compartment elaborate different combinations of activating and inhibitory NKR. Varying the array of NKRs used by each subset increases the potential specificities of the NK compartment, while retaining tolerance to self. Thus, a diverse and balanced NK cell

receptor repertoire (NKRR) is extremely important in order for this lineage to respond to various immunologic challenges and to do so in a normal, effective manner.

As we have previously shown, aberrations in the expression of NKRs or downstream signaling can lead to severe immune deficiency, as observed in SHIP-deficient mice. We also showed that in the absence of SHIP-1, 2B4 becomes highly upregulated, functioning as a dominant inhibitory receptor and rendering the SHIP-1-deficient NK cell unresponsive to complex tumor targets. Traditionally MHC-I inhibitory ligands are largely responsible for the regulation of NK function. However, we show here that 2B4, which mediates MHC-I-independent inhibition, is required for formation of a normal NKRR, NK homeostasis, and effector functions. Moreover, in the absence of 2B4 and SHIP-1, NK cells have improper licensing, or education. In addition to SHIP-1 and 2B4 we show that the nature of the MHC-I ligands also play a significant role in repertoire formation, NK effector functions, and NK cell education.

As described above, NK cells are critical components of the immune system. Understanding how NK cell biology and function are regulated, or affected in the context of pathology is of high significance. NK function is often severely impaired in a diseased state, and more importantly, NK cells are frequently adversely affected by the treatments themselves. Here we sought out to determine the effects of an immunomodulating drug, lenalidomide, on the biology and function of healthy NK cells. Lenalidomide is a unique drug that displays immune enhancing functions yet can be cytotoxic to tumor cells. However, lenalidomide treatment can result in immune suppression and severe cytopenia, and has the ability to impair NK viability. We show here that if used in combination with cytokine treatment (e.g. IL-2 or IL-15), many of these negative affects can be overcome.

Furthermore, we show that lenalidomide treatment results in what appears to be an NK activating phenotype with a down-modulation of inhibitory KIRs and upregulation of CD16. Lenalidomide also leads to a sustained and robust activation of STAT5 and consequential increase in perforin and granzyme B. Finally, we find that treatment with lenalidomide in combination with IL-2 or IL-15 enhances the expression of IL-Rβ and IL-2Rγ chains, a presumed mechanism of action, which may provide a positive feedback loop. These findings have important clinical application. We propose that using lenalidomide in combination with IL-15 can augment its immune activating effects, while minimizing unwanted cytopenias.

Chapter 1: Background

Natural Killer Cells

Natural killer (NK) cells are bone marrow derived lymphocytes that belong to the innate branch of the immune system. They mediate cytolysis of malignant, infected, stressed or damaged self-cells, and allogeneic cells. Moreover, NK cells produce large amounts of cytokines and chemokines, and function to shape early anti-viral responses. Natural Killer cells, as their name implies, were identified and subsequently named for their "natural" ability to kill. That is, unlike T and B lymphocytes, NK cells are able to kill a target cell without prior immunization. A process referred to as "natural cytotoxicity" [1]. This killing occurs spontaneously, or what originally appeared to be non-specifically, and does not require clonal proliferation or antigen receptor gene rearrangement. It is quite common within the immune system (and most areas of study) for cells, proteins, etc, to be named based off their function (i.e. Antigen Presenting cells). However, NK cells are not merely killers. These cells play important roles in adaptive and innate immune regulation as well as being critical in the clearance of viral infections [2].

The Discovery of NK Cells. It has been over 30 years since the discovery of the Natural Killer cell. When examining cell-mediated cytotoxicity against tumor cells [3-7] and virally infected cells [8-10], several groups reported what was referred to at the time as unexplained "background" killing . It was observed that tumor cells and viral infections

from non-immunized animals, as well as normal donors, could be eliminated at levels similar to what was seen with immunized animals and cancer patients. Based on the knowledge regarding immunity and cancer immunology at the time (1975), T and B lymphocytes were responsible for the rejection of tumors and virally infected cells. Studies of virally induced tumors [11, 12] implicated a thymus-derived lymphocyte in the elimination of these tumors [12, 13]. In vivo and in vitro studies provided further support that the tumor regression or killing was indeed T cell mediated [14, 15]. According to a T-cell mediated model of tumor destruction, athymic and/or nude mice, which lack T cells, should have unrestrained tumor growth. However, unimpeded growth of tumors in the absence of T cells was never observed in these models [16, 17], suggesting that another cell type is mediating tumor cytolysis. There was now a large amount of data demonstrating "spontaneous" T cell-independent cell-mediated cytotoxicity that could not be ignored. Taken together, these findings eventually led to the coining of the terms "natural killing" and "natural cytotoxicity" [1, 18].

Although there was now a better explanation of the disturbing "background" killing, the idea of NK cells as a distinct subset was resisted. Instead, NK cells were defined as "null" cells, which meant they were non-B, non-T, non-phagocytic and non-adherent cells that are largely Fc receptor positive, mostly complement receptor negative, and have low affinity to form rosettes with sheep erythrocytes [19-21]. A few years later, Timonen and colleagues correlated their morphological appearance and function to that of large granular lymphocytes [22]. Seminal studies demonstrating that NK cell activity was associated with in vivo resistance to tumor growth[18], and the use of NK cells in cancer immunotherapy, particularly IL-2 activated NK cells (lymphokine activated killer cells-

LAK cells) [23, 24] provided evidence that these cells were important players in the immune system. The presence (and relevance) of NK cells remained somewhat controversial up until the late 1980's, but by then NK cells became generally considered a unique population having distinct functional, morphological, and phenotypical characteristics.

Missing Self Hypothesis. Following the discovery of the NK cell, considerable progress was made characterizing this cell type in the context of its lytic mechanisms[19, 25] and regulation by cytokines and other soluble factors[26, 27]. However, the nature of target cell recognition and the basis of selectivity and specificity remained elusive. It was clear that NK cells preferred (certain) malignant, virally infected, undifferentiated, and allogeneic cells [28], but the molecular event(s) that transformed a cell into an 'NK sensitive' target or an 'NK resistant' target remained elusive. The 'Missing Self Hypothesis', ingeniously presented by Klas Karre as part of his doctoral thesis, proposed that NK cytolysis of a target cell could be triggered by a decrease or absence of host major histocompatibility class I molecules (MHC-I) on the surface of a target cell. In other words, NK recognition of MHC class I molecules, unlike T cell-MHC-I interactions results in inhibition of the NK cell.

In addition to "the mystery of the NK cells themselves," a major impetus for pursuing the missing self hypothesis was derived from a perplexing question asked many years earlier regarding hybrid resistance and bone marrow graft and/or tumor rejection [29]. Hybrid resistance refers to the rejection of parental strain bone marrow cells by NK cells that are F_1 hybrids from two inbred parental strains and this was proposed to be regulated by the MHC molecules themselves [30]. Simply put, A x B (F_1) mice progeny reject (B x B)

or (A x A) parental bone marrow, where A and B refer to MHC-I haplotype [31]. It was evident by the late 1970's that tumors and bone marrow grafts can be rejected in a T cell-independent, NK cell-dependent manner and MHC-I plays a role, but a major question remained as to *how* NK cells rejected the grafts [32, 33]. Ironically, the only thing researchers could agree on was that NK cells were not at all influenced by target cell expression of MHC-I. As Karre wrote his thesis he asked not what NK and T cells have in common, but rather, what are the differences between these two cell types when it comes to target recognition? He found a common theme: targets that were insensitive to T cells were sensitive to NK cells, and vice versa. Considering this, Karre's model predicted the expression of NK receptors that were specific for MHC-I and engagement between these molecules resulted in inhibition of the NK cell.

There was literature that supported his hypothesis, but to address this experimentally, Karre created several MHC-I mutant cell lines. His results were in close agreement with his hypothesis: cell lines lacking MHC-I were highly susceptible to NK cytolysis and this was inversely proportional to the amount of MHC-I expressed on the surface of the target cell. Even more stunning, when they moved this to an in vivo mouse model, mice receiving MHC-I-deficient lymphoma cells failed to develop palpable tumors. When mice were injected with MHC-I^+ lymphoma cells, but depleted of NK cells using an anti-NK1.1 antibody, this effect was completely abrogated [34, 35].

Parallel studies provided support and confirmation [36, 37]. Eventually technology caught up with Karre's hypothesis. Introduction of an H-$2D^d$ transgene into C57BL6 (H-$2D^b$) mice resulted in acquired resistance, or rejection, of C57BL lymphoma grafts and B6 bone marrow cells [38, 39]. Taken together, these studies and others [40] demonstrated that

NK cells utilize an MHC-I-dependent system whereby they are prevented to attack self or normal cells, but have the ability to detect and lyse non-self, or cells that have down-regulated MHC-I.

NK Cell Recognition. NK cells distinguish normal healthy cells from non-healthy, or foreign cells by way of a sophisticated repertoire of receptors [41]. These receptors are broadly categorized as 'inhibitory' or 'activating'. Unlike T cells and B cells, where their activation, proliferation, and function is driven by a single antigen receptor, NK cells assimilate signals coming from a multitude of receptors that may be classified as adhesion or co-stimulatory molecules on other cells. Initial engagement of an NK cell with a potential target cell results in NK inhibitory and activating receptor interactions with ligands present on the other cell. An integration of signals downstream of the different NK receptors occurs and the NK cell will either detach and move away, or stay and respond. If an NK cell remains, cytosolic granules containing pre-stored pools of perforin and granzymes will reorganize and be prepared for release. Further, cytokines will be transcribed and secreted[41].

In the case of NK recognition and action (or no action), there are several different scenarios that can occur (Fig. 1). In a normal or healthy state virtually all cells express MHC-I molecules, which are recognized by NK inhibitory receptors. Some exceptions to this are erythrocytes, which lack MHC-I [42], and the central nervous system, which mostly has low levels of MHC-I [43] and embryonic and immature hematopoietic cells[44]. In the absence of MHC-I, NK receptors that recognize ubiquitous, or constitutive molecules may function to inhibit the NK cell. Such examples include, 2B4 (mice)[45], NKRP1-d[46], LAIR1[47], Klrg1[48], Siglec-7[49], and others[50, 51]. The inhibitory signal transmitted to the NK

cell sets the threshold and sufficient activating signals must occur in order for an NK cell to kill and/or produce cytokines. Importantly, activation signals may come in the form of cytokines or interferons, or cell-cell contact. Often times during transformation, cellular stress, or infection, MHC-I molecules become down-regulated[52, 53] and/or activating ligands become up-regulated[54-57]. This skews the balance and triggers activation of the NK cell. It is important to mention, although not depicted below, in addition to detecting self and non-self, NK cells are capable of recognizing pathogen components directly (discussed later).

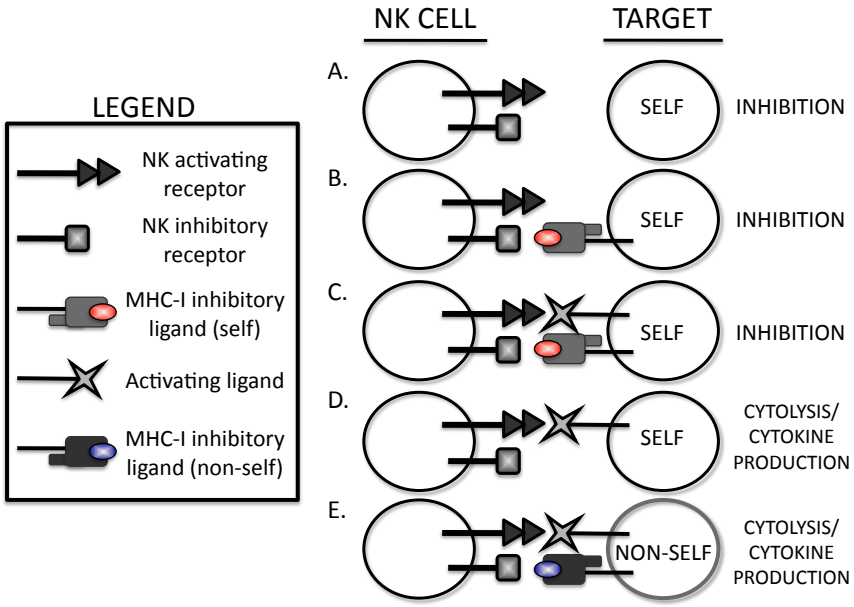


Figure 1. NK target cell recognition and outcomes. The presence of ligands on the target cell directly affects the outcome of NK-target cell interactions. A) A normal healthy cell may lack expression of MHC-I and activating ligands and therefore no response is provoked. B) Commonly a cell will express MHC-I, but will not express activating ligands. Like the above situation, the NK cell will not respond. C) In the case where a target cell expresses MHC-I, but may also express activating ligands (at low

levels), the MHC-I-NK inhibitory receptor interaction sets the threshold and the NK cell will be inhibited. D) During infection, stress, or malignant transformation MHC-I molecules are down-regulated. This alters the balance and the NK cell will become activated E) During transplantation and bone marrow grafts foreign cells are recognized by the NK cell as 'non-self' and thereby eliminated.

NK Receptors. NK function is directly influenced by the receptors expressed on the surface of the NK cell. NK receptors are broadly categorized as inhibitory and activating. Because there are several different NK receptors that are inhibitory or activating, it is useful to further categorize them by their ligand recognition. Simply, there are specific receptors for MHC-I and MHC-I-like molecules and also receptors categorized as MHC-independent receptors. Further, there are both inhibitory and activating members that bind MHC-I. This is also the case for MHC-independent recognition. Thus, at any given time an NK cell is integrating multiple signals in order to effectively deal with potential harm, all while preventing autoreactivity.

MHC-I and MHC-like Recognition. Karre and colleague's pioneering work quickly led to the discovery that NK cells are actively inhibited by MHC-I. The 'Missing Self Hypothesis' predicted the presence of inhibitory receptors, however, this theory wasn't fully substantiated until the discovery of the first MHC-I specific inhibitory NK receptor[58]. There are three distinct receptor systems utilized by NK cells to recognize MHC-I molecules: Ly49s, KIRs, and CD94/NKG2 heterodimers. Rodents and humans have structurally distinct receptors for direct recognition of MHC-I. Mice utilize the lectin-like Ly49 family of receptors[59], while humans have killer cell immunoglobulin-like

receptors, or KIRs[60]. This is a perfect example of functional convergent evolution: different species independently evolving similar traits to perform the same function[61]. These MHC-specific receptors are encoded by two gene complexes on different chromosomes: the natural killer complex (NKC), which is located on mouse chromosome 6 and human chromosome 12[62], and the leukocyte receptor complex (LRC) on human chromosome 19[63]. Besides Ly49s and KIRs, rodents and humans share a receptor system that recognizes MHC-I in an indirect manner[64 65, 66]. The lectin-like heterodimeric receptors, CD94 with NKG2A, NKG2C, NKG2E or NKG2F, recognize HLA-E (humans)[67] or Qa-1b (mice)[66], which are non-classical MHC-I molecules and therefore this interaction is termed MHC-like recognition.

The Ly49s and the KIRs. Although there are two structurally distinct receptor families mediating the same function, there are some very striking similarities between the Ly49s and the KIRs. Both gene complexes, the NKC and the LRC are rapidly evolving[68, 69], highly polymorphic[70, 71], have undergone duplication many times, and are variable in different individuals in regards to their terms of sequence and whether they are expressed or not [69]. Moreover, both the Ly49s and KIRs are expressed in a clonal fashion and employ similar mechanisms of signal transduction[72, 73]. Even the MHC ligands they recognize, which are not direct orthologues[74], are also rapidly evolving and highly polymorphic, likely to maintain a functionally matched repertoire[75].

The Ly49 family of receptors has both activating and inhibitory members. Ly49A, which is the prototypical Ly49 receptor, was the first MHC-I specific inhibitory receptor identified on mouse NK cells[58]. The Ly49 family of receptors comprises a possible 23

members (*Ly49a* to *Ly49w*), which are encoded by a complex of genes located on the distal mouse chromosome 6 (NKC)[76]. Ly49 receptors belong to the C-type lectin family of proteins and are expressed at the cell surface strictly as transmembrane disulphide-bonded homodimers. Each chain is composed of a C-type lectin-like domain (CTLD), which is connected to the cell membrane by an alpha-helix stalk region[77]. Classically, CTLD domains are carbohydrate binding protein domains where the 'C' stands for calcium binding[78]. However, unlike other members in this protein family, the Ly49s recognize carbohydrate-independent epitopes on the MHC-I molecule and do not require calcium [79].

Inhibitory Ly49s have immunoreceptor tyrosine inhibitory motifs, or ITIMs, in their cytoplasmic domains[73]. There are several inhibitory Ly49 receptors, but Ly49A, Ly49G2 and Ly49C/I are the best characterized in terms of ligand binding and function[79-82]. Classically, ITIMs are involved in cellular inhibition. Upon inhibitory Ly49 engagement with its ligand, specific tyrosine residues of the ITIM are phosphorylated and subsequent recruitment of tyrosine and inositol phosphatases occurs [83]. The end result of this signaling cascade is inhibition of NK cytolytic function.

Based off functional data or the presence of an ITIM, of the 23 Ly49 members, 13 are inhibitory. Of the remaining Ly49 receptors, 8 are predicted to be activating[77]. Typical activating members include, Ly49D and Ly49H[84-86]. Ly49H is very important for the clearance of murine cytomegaloviral (CMV) infections by NK cells since mice lacking Ly49H have uncontrolled viral titers and markedly higher levels of morbidity caused by CMV infection[87]. It was later shown that Ly49H directly recognizes m157, a virally encoded protein expressed by infected cells during the early phases of infection[88,

[89]. Activating Ly49s lack ITIMs, but instead have charged amino acids in their transmembrane (TM) domains, which allow them to non-covalently associate with transmembrane homodimeric signaling adaptors, such as DNAX-activating protein of 12kD (DAP12)[90]. Each polypeptide chain of the signaling adaptor (DAP12) has an immunoreceptor tyrosine activating motif (ITAM). Interaction of a Ly49 activating receptor with its ligand results in phosphorylation of ITAMs, *syk* tyrosine kinase, and several other substrates[91]. Initiation of this signaling cascade leads to NK cytolysis and activation of effector functions.

Like the Ly49s, there are inhibitory and activating KIRs. There are 18 KIR genes clustered on human chromosome 19q13.42 (LRC)[63]. KIRs are members of the immunoglobulin superfamily (IgSF) of proteins and are only found in primates; there is no evidence of rodents expressing KIRs. Other IgSF members, such as the ILTs, leukocyte-associated inhibitory receptors (LAIRs), and the NK activating receptor, NKp46 also reside in this complex[71]. Existing mainly as monomeric type I transmembrane glycoproteins, KIRs are characterized by the number of extracellular Ig domains (2D or 3D) and by the length of their cytoplasmic tail[92]. For example, long-tailed KIRs (2DL or 3DL) contain ITIMs and are inhibitory. In contrast, short-tailed KIRs (2DS or 3DS) do not have ITIMs and instead send stimulatory signals to the NK cell by way of associated signaling adaptors, namely DAP12 (synonymous with the activating Ly49 receptors)[83]. Thus, the nomenclature of KIRs provides useful information regarding their structure and function. For example, KIR2DL1 has 2 extracellular Ig domains and a long cytoplasmic ITIM domain. The basic signaling mechanisms employed by the KIRs are identical to the Ly49 system.

Inhibitory KIRs recognize MHC-I molecules, or human leukocyte antigens (HLA)-A, HLA-B, and HLA-C, with HLA-C being the most committed to interaction with KIRs: all HLA-C allotypes interact with a KIR and HLA-C interaction with KIRs is fixed. That is, every human being has *at least* one HLA-C molecule that is recognized by a KIR[75]. Only a third of HLA-B alleles are known to interact with KIRs, and HLA-A appears to be the least committed to binding KIRs[75]. An interesting feature of KIRs is that multiple HLA-allotypes can be recognized by the same KIR. For instance, KIR2DL1 and KIR2DS1 can bind HLA-Cw2, 4, 5, 6, and 15; while KIR2DL2 and KIR2DL3 both recognize HLA-Cw1, 3, 7, and 8. Like the KIRs, HLA molecules are highly polymorphic. In the case of HLA-C, amino acid residue 80 appears to be critical[75]. Until this understanding it wasn't clear *how* KIRs were recognizing HLA. We now know that KIRs bind regions of HLA molecules that *are not* polymorphic and this explains how KIRs are capable of recognizing many different HLA allotypes[92]. Although variable for the different Ly49s and their interaction with MHC-I[93, 94], the MHC associated peptide is critical for KIR interaction[95].

Characterization of NK activating KIRs and their ligands has lagged behind the inhibitory receptors, and the precise role for MHC-specific activating receptors is still debated. There are activating KIRs that bind the same HLA molecules as the inhibitory KIRs, albeit activating KIRs (and other MHC-I specific activating receptors) generally bind the target ligand with lower affinity[96, 97]. Moreover, often times activating and inhibitory KIRs with the same Class I specificity are not expressed on the same cell[98]. The presence of MHC-specific activating KIRs may be useful if the target cell down-regulates the inhibitory allele, but retained the stimulatory allele. This cell would then

become sensitized to lysis by the NK cell. During viral infection there are high levels of interferons, which up-regulate the expression of MHC-I[99]. Thus, activating KIRs may preferentially function to detect high levels of MHC-I. Alternatively, these stimulatory KIRs specific for MHC may have evolved as a mechanism to detect virally infected cells. There are known viral-specific MHC-I decoy molecules that presumably were intended to inhibit the NK cell, but perhaps the NK cell had pressure to evolve a method of detecting these viral molecules[100]. It has also been proposed that activating KIR may be involved with NK maturation. However, a few findings challenge this idea: mice that lack DAP12 have functional NK cells [101] and further, activating receptors are expressed *after* inhibitory receptors during development[102].

CD94/NKG2. CD94/NKG2 are C-type lectin heterodimers, which are encoded by genes located in the NKC[64]. Like the KIRs and the Ly49s, there are activating and inhibitory members. Unlike the KIRs and Ly49s, however, the CD94/NK92 heterodimers recognize MHC-I indirectly. Specifically, the CD94/NKG2 receptor recognizes peptides processed from the leader sequences of classical MHC-I molecules, rather than the mature Class I molecule itself. These peptides are bound into the groove of a nonclassical class Ib molecule and are HLA-E (humans)[67] or Qa-1 (mice)[66]. CD94 has a short cytoplasmic domain lacking any apparent signaling capabilities and functions as the "common" subunit of these receptor pairs. Dimerization of CD94 with an NKG2 partner is required for cell surface expression and signaling[103]. CD94 can bind with NKG2A, C, E, or F isoforms, with the combination of CD94/NKG2A being the only inhibitory pair. Importantly, NKG2D also belongs to this family; however, NKG2D does not pair with

CD94 nor does it recognize MHC-I molecules (although it recognizes molecules homologous to MHC. NKG2D will be discussed later). In humans, the activating heterodimer, CD94/NKG2C, associates with DAP12 via a charged amino acid residue in the TM domain of NKG2C[104]. In mice, however, this interaction is dependent on a charged residue in CD94, not NKG2C[105]. Human CD94/NKG2A and CD94/NKG2C can both recognize HLA-E[106], which means like the KIRs, there are inhibitory and activating members with identical MHC specificity. This also occurs in the mouse. Again, the inhibitory pair tends to dominate the activating pair since the CD94/NKG2C binds Qa-1 with lower affinity[107].

MHC-I-independent Recognition

2B4. For many years following the discovery of NK cells it was thought that NK inhibition was strictly mediated by MHC-dependent receptors. However, there are now numerous reports demonstrating that NK inhibition (and activation) is largely regulated by receptors that recognize ligands other than MHC molecules[45, 108-110]. A prominent NK receptor falling under this category is 2B4.

2B4 (CD244) belongs to the signaling lymphocytic activation molecule (SLAM)-related receptor family[111]. There are several members in this family, including but not limited to, SLAM, CD48, Ly9, CD84, NK-T and B-antigen (NKTB-A), and CD2-like receptor activating cytotoxic cells (CRACC). These receptors belong to a larger family of proteins, the CD2 superfamily of immunoglobulin domain containing molecules[112]. SLAM-related receptors, and their ligands, are expressed on a wide variety of hematopoietic and non-hematopoietic cells, implicating them in diverse functions from

cellular activation, costimulation, cell-cell adhesion, and importantly, immune tolerance[113-116].

2B4 is expressed on NK cells, γδ−T cells, a subset of αβ−CD8$^+$ T cells, and other leukocytes[111]. 2B4 recognizes and binds it ligand, CD48, with high affinity and CD48 is expressed on virtually all cells of the hematopoietic system[117]. Structurally, 2B4 has a V-like (variable) and C-like (constant) Ig domain in its extracellular region. As a member of the SLAM family of receptors, 2B4 possesses four immunoreceptor tyrosine-based switch motifs (ITSMs) in its cytoplasmic tail. An ITSM, distinct from an ITAM (which recruits syk), or an ITIM (which recruits SHP-1 and other phosphatases such as SHIP) recruits SAP, or SLAM-associated protein and other proteins involved with cellular activation *or* inhibition[112]. ITSMs are known to bind several SH2-domain containing kinases, phosphatases, and adaptors. Engagement of 2B4 on mouse NK cells results in phosphorylation of all four ITSMs. It has been shown that subsequent recruitment of FynT and further 2B4 phosphorylation occurs in a SAP-dependent manner. This leads to downstream activation of Vav1 and c-CBL[118]. Despite extensive work to elucidate the signaling mechanisms downstream of 2B4, there remains a great deal of mystery around how this receptor exerts its opposing functions.

Although 2B4 was first discovered as an activating receptor[119-121], it has since been shown that 2B4 functions as an inhibitory receptor in mice. This has been unequivocally demonstrated using 2B4 knockout mice, which have normal NK numbers and cytolytic function against certain tumor targets, but have significantly higher killing against CD48 expressing targets when compared to 2B4 WT mice[122, 123]. However, 2B4 *is* an activating receptor in humans[124, 125]. The reason for this difference between mice

and man is not completely clear, especially since human 2B4 also recognizes CD48 and has an identical four ITSMs in its cytoplasmic domain. However, there are a few explanations for the dichotomous functions of human and mouse 2B4. One explanation is that two isoforms of 2B4 exist in mice, which are generated by alternative splicing[126]. The only differences between the two murine isoforms are found in the cytoplasmic region: a long form (2B4-L) that has four tyrosine residues in the cytoplasmic tail; and a short form (2B4-S), which has only one tyrosine residue[126]. It has been shown that these isoforms have distinct functions regarding cell activation or cell inhibition in mice, with 2B4-L mediating cellular inhibition via the recruitment of SHP-2, while 2B4-S mediates cell activation in a SAP-dependent manner.[127] Interestingly, the human *2B4* gene also produces two isoforms, however, unlike the mouse 2B4, both isoforms produce intracytoplasmic domains with four tyrosine residues[128]. Based off previous findings, this would suggest an inhibitory function of human 2B4, although this is typically not the case. Another study suggests that the inhibitory/activating role of 2B4 may be regulated by the different signaling molecules recruited to the ITSMs, and these molecules may be expressed at different levels in mouse NK cells[129]. Specifically, recruitment of SAP to 2B4 imparts cytotoxicity, but recruitment of EWS-activated transcript-2 (EAT-2) or Eat-2-related transducer (ERT) results in NK cell inhibition[130]. Importantly, EAT-2 and ERT can mediate global inhibition of NK cells since deletion of these molecules results in an increased ability to produce IFN-γ in response to triggering of NK receptors: CD16, NKG2D, Ly49D, and 2B4. One explanation for the differences in signaling potentials of these very related molecules is that SAP, but not EAT-2 or ERT, can recruit FynT kinase via an SH3 domain located in SAP[131, 132] and this recruitment is required for SAP-

mediated activation of NK cells by 2B4 triggering[118, 133]. EAT-2 and ERT genes are both functional in mice, however, only EAT-2 appears to be functioning in humans[130].

2B4 is a very important receptor on NK cells and other lymphocytes. An intriguing study done recently demonstrates an unappreciated role for 2B4. 2B4 positively regulates NK cell-mediated control of T cells in persistent viral infections[134]. Using an LCMV model of persistent viral infection, this group showed that in the absence of 2B4 (using 2B4 KO mice), NK cells from these mice lysed highly activated $CD8^+$-LCMV specific T cells. Due to NK-mediated cytolysis of these activated T cells, clearance of the viral infection was delayed and there were immunopathologies, such as a major increase in naïve CD8 T cells and splenomegaly. These impairments were reversed when NK cells were depleted from $2B4^{-/-}$ mice, confirming an NK-dependent defect. Moreover, the deficiency in viral-specific $CD8^+$ T cells was not due to the absence of 2B4 on T cells[134]. NK cells have long been known for their direct anti-viral activities, however, this study highlights a newer, more regulatory role for NK cells in the control of virus specific CD8 T cells. It also underscores previously proposed notions that 2B4 functions in immune tolerance[116].

Many of the MHC-I independent inhibitory NK receptors recognize self-molecules that have broad expression. This positions these receptors in the establishment or maintenance of self-tolerance. This is exceptionally apparent in individuals with a mutation in TAP2, or Transporter associated with antigen processing-2, a protein required for proper peptide presentation and stable MHC-I cell surface expression[135]. These patients have normal numbers of NK cells and the NK cells are capable of killing tumor cells, albeit to a lower extent. Still, the NK cells are capable of self-tolerance in the

absence of MHC-I[108, 136, 137]. Further, about 10% of NK cells lack MHC-specific inhibitory receptors; molecules such as 2B4 may prevent auto-reactivity under this circumstance. Finally, during NK development, cytotoxic functions precede the expression of MHC-specific inhibitory receptors. These developing cells have the potential for autoaggression, yet, NK cells maintain tolerance and are not autoreactive. Evidence suggests that MHC-independent receptors, such as 2B4, are expressed earlier in development than their MHC-specific counterparts, and thus may function to maintain tolerance during these times[45]. Interestingly, this last study was done using human NK cells, where 2B4 classically functions as an activating receptor. The stipulation here being that these immature NK cells were deficient in SAP. Taken together, these studies stress the importance of this molecule in the biology of NK cells and demonstrate the need to further define a precise role of 2B4 in the development and functions of NK cells (and other immune cells) and the acquisition of the NKRR.

NKG2D. NK group 2 member D (NKG2D) is a prototypical NK activating receptor. It belongs to a subfamily of the C type lectin-like receptors and like other members of this family, the gene encoding NKG2D is located in the NKC on human chromosome 12 and mouse chromosome 6[62]. NKG2D is expressed by virtually all NK cells, a large fraction NKT cells, most γδTCR T cells, all $CD8^+$ T cells, macrophages, and αβ CD4 T cells in certain pathological states [54, 138-140]. NKG2D exists as a disulphide-linked homodimer and upon ligand recognition and binding, NKG2D associates with two DAP12 or DAP10 dimers, forming a hexameric structure[141]. Two isoforms of NKG2D exist in the mouse, NKG2D short (NKG2D-s) and NKG2D long (NKG2D-l). NKG2D-s

can bind both adaptors, while NKG2D-l can only bind DAP10[142, 143]. There are structural differences in the TM domain of human NKG2D, which restricts it to pair exclusively with DAP10[144]. DAP12 contains an ITAM in the cytoplasmic domain, which is phosphorylated by members of the Src family of kinases, after which recruitment of SH2-domain containing kinases, Syk and/or ZAP70 occurs[145, 146]. Unlike DAP12, DAP10 contains a YINM motif similar to what is found in CD28 or CTLA-4. YINM motifs, when phosphorylated, offer potential binding sites for Grb2, PI-3K, and Shc[147-149]. The ability of DAP12 and DAP10 to recruit several different signaling molecules allows NKG2D to carry out diverse functions.

A unique property of NKG2D is that it is capable of mediating the destruction of tumor cells and virally infected cells by recognizing multiple different ligands. Although NKG2D is well conserved between humans and rodents, NKG2D ligands differ between the two species and vary in structure. While seemingly disparate, NKG2D ligands share several common features: all known NKG2D ligands are distant structural homologs of MHC-I molecules[150-152]. However, unlike true MHC-I, they do not bind antigenic peptides or associate with β2-microglobulin. Another shared feature of NKG2D ligands is that they are expressed at relatively low levels on normal healthy cells, but become upregulated during stress, infection, or malignant transformation[153]. Thus, NKG2D mediates "induced self". Human NKG2D recognizes MICA and MICB, or MHC-I chain related protein A, -B[54] and UL16 binding proteins (ULBPs), cell surface proteins that bind human CMV[55]. Mouse NKG2D recognizes a family of retinoic acid early inducible proteins (Rae1)[138, 154]. It also binds to the closely related histocompatibility antigen 60 (H60) glycoproteins, and the murine UL-16-binding protein-like transcript 1

(MULT1)[138]. Most NKG2D ligands are expressed at very low levels on normal tissues, but can be upregulated during stress. Because of the negative implications that can occur by expression of these ligands (e.g. autoimmunity), regulation of these ligands occurs at the transcriptional and/or post-transcriptional levels and is tightly controlled.

In addition to 2B4 and NKG2D, There are numerous NK receptors that recognize ligands that are not MHC-I molecules. The previous and following discussions (due to a lack of space) only scratch the surface. A family of receptors that deserve discussion are the natural cytotoxic receptors, or the NCRs. NKp46, NKp44 and NKp30 trigger NK cell activation upon ligand recognition or antibody cross-linking and therefore function as activating receptors. Expression of NCRs, especially NKp46, is specific to the NK compartment, with a few known exceptions[155-157]. NKp46 and NKp30 are expressed by all human NK cells, resting and activated, while NKp44 expression is (mostly) limited to IL-2 activated NK cells, and thus has been proposed as a marker for activated NK cells[158, 159]. Despite extensive study of the NCRs, the exact ligands for these receptors remain unknown. It is clear, however, that these receptors are involved with the recognition of pathogen components, such as viral hemagglutinins and Mycobacterium bovis bacillus Calmette-Guérin (BCG)[160-162]. It has been shown by several groups that the expression of NCRs correlates with NK function and absence, or inhibition of NCRs has a severe negative impact on NK cytotoxic functions and disease progression[163-167].

NK Cell Effector Functions. NK cells have two main functions: they lyse target cells and produce soluble mediators (cytokines and chemokines). These functions help them to control the early stages of infections, promote antigen presentation, recruit other immune cells, and regulate the development of the adaptive immune response.

***Granule Exocytosis*.** NK cell mediated death is rapid and efficient. There are two main pathways by which NK cells initiate apoptosis of their targets: the granule exocytosis pathway or ligation of death receptors.

The most common pathway is granule exocytosis. Cytotoxic lymphocytes contain specialized compartments, secretory lysosomes, which are dual function organelles combining the degradative function of lysosomes with the capacity to undergo regulated exocytosis[168]. These secretory lysosomes contain important cytolytic mediators (among other molecules), namely perforin and granzymes. Perforin, or cytolysin, produces pores in the membranes of target cells. It was considered for a long time that perforin was solely responsible for the lysis of target cells, although it was never ruled out that there may be other molecules contributing to cell death[169]. Either way, the exact role of perforin remains controversial. Without a doubt perforin is a critical component of secretory lysosomes and the granule exocytosis pathway in cytotoxic lymphocytes[170]. It can induce death on it's own and has also been shown to facilitate the transfer of granzymes into the target cell. Granzymes are a family of closely related serine proteases that are expressed by cytotoxic T cells and NK cells. There are 5 granzymes in humans (A, B, H, K, M) and 10 functional granzymes in mice (A-G, K, M, N)[171, 172]. Granzymes

A and B are the most abundant and the most well studied. Granzyme B, in particular because it initiates caspase-dependent cell death, whereas Granzyme A can mediate caspase independent apoptosis[173, 174]. The actual killing of a target cell is rapid and can occur within 20 minutes. It is a multistep process that is well orchestrated and highly regulated: step one involves NK cell binding to the target cell, which is mediated by interaction of LFA-1 integrins on the NK cell with ICAM-1 molecules present on the target cell. This induces a talin-dependent polarization of the actin cytoskeleton[175] and translocation of the lytic granules. Finally, the granules fuse with the target cell membrane and perforin and granzymes are released. The complex formed between an NK cell and a target cell is often referred to as the "lytic immune synapse". LFA-1, actin, and talin form a ring, which provides stability to the interaction and functions as the scaffold for the assembly of inhibitory and stimulatory signaling complexes[176]. An important feature of NK cells, and one that lends them their 'ready-to-go' state is that these granules are formed during development and unlike T cells, synthesis of new molecules is not required. This is also what allows NK cells to act as a first line defense and control infections until the adaptive immune system is ready.

Death Receptor System. NK cells express ligands on their surface that when bound with the respective receptor present on the target cell will induce death of that target cell. This system, which is used throughout the body as a method of immune homeostasis and regulation, is called the death receptor system and functions as a complementary system to granule exocytosis in cytotoxic lymphocytes. For example, NK cells express FasL, TNF-α, and TRAIL, members of the TNF-like family of molecules

that when bound to their receptors will initiate caspase-dependent apoptosis [177-181]. NK death receptor-mediated induction of apoptosis has been shown to be important for the destruction of tumors[182-184], graft rejection[185], virally infected cells[186], and even NK-mediated death of DCs[187]. Interestingly, IFN-γ production by NK cells can induce the expression of TRAIL by NK cells[183], and IFN-γ also up-regulates the expression of Fas on certain tumor cells[188].

ADCC. NK cells carry out target killing either "naturally" through direct recognition of the target cell by NK receptors, or through a process known as antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC is an adaptive immune function and typical ADCC involves activation of NK cells by antibody coated target cells[189]; however, neutrophils and eosinophils can also perform ADCC. ADCC by NK cells is mediated by the low affinity Fcγ receptor (FcγRIIIa), which binds immune-complexed IgG. This interaction between CD16 and IgG activates the NK cells to kill and/or produce cytokines since CD16 associates with the γ-chain signaling adaptor containing an ITAM[190]. ADCC is an important NK mediated function in cancer immunotherapy and has shown success with monoclonal antibody treatments against: CD20 (NHL, CLL), Her2 (breast), EGFR (metastatic colon) GD2 (multiple tumor types-somewhat tumor specific), and several others[191-195]. NK-ADCC function is also highly influential in combating certain infections, especially HIV[196].

Immunoregulatory Functions of NK Cells. NK cells are not merely killers. Cytokine and chemokine production by NK cells puts them in a powerful position to

regulate *both* adaptive and innate immune functions. NK cells are found throughout the body, including the skin, liver, pancreas, mucosal tissues, and lungs, and during infection or high levels of inflammation, NK cells are even recruited to the CNS and joints. The major functions of NK cells in these locations are to amplify the immune response and kill infectious agents directly. NK cells are also found in the uterus and the placenta, where they have a major role in the fetal-maternal interface[197]. The distribution of NK cells is not a static process and during an infection as NK cell number increase in one location, they decrease in another, indicating that most NK cells are indeed derived from the bone marrow and spleen.

It is easy to divide human NK cells into functionally distinct subsets based on their expression of CD56 and CD16. The predominate NK subset found circulating throughout the blood are $CD56^{dim}CD16^{bright}$ (~90% of blood NK); NK cells present in the peripheral lymphoid organs are $CD56^{bright}CD16^{-/dim}$ (<10% of blood NK). $CD56^{dim}$ NK cells are highly cytolytic and produce relatively low levels of cytokines[198]. On the other hand, $CD56^{bright}$ NK cells produce robust amounts of cytokines upon proinflammatory cytokine stimulation, are highly proliferative, and have low cytolytic potential[199, 200]. There are also differences in the expression of inhibitory and activating receptors between these two subsets. The cytolytic $CD56^{dim}$ cells tend to express KIRs, but lack expression of inhibitory CD94/NKG2A, and the $CD56^{bright}$ NK cells lack KIR expression, but express high levels of CD94/NKG2A. Both subsets express activating receptors, NKG2D, NKP46, and NKp30, but there are differences in the expression of chemokine receptors. In line with their presence in secondary lymphoid organs, the $CD56^{bright}$ NK express homing receptors, CCR7, CD62L, and CXCR3, which are markers for adhesion

and migration and trafficking[201, 202]. These subsets are specific to humans. There has yet to be a discovery of a murine CD56 homolog, however, mouse NK cells can be classified based on their expression of CD11b, CD27, CD127, and B220[203-205]. Like human NK subsets, mouse NK subsets also have disparities in their cytotoxic and immunoregulatory functions. In contrast to resting T lymphocytes, NK cells constitutively express receptors for a multitude of monokines[206]. A characteristic that allows them to rapidly and spontaneously respond to various stimuli produced by other immune cells.

In line with the immunoregulatory (and immune surveillance) role of NK cells, they are widely distributed throughout the body and have different functions depending on the location and state of health [207]. NK cell subsets (both human and mouse) also have different distribution patterns within the various anatomical locations suggesting specialized functions between the subsets. In normal human lymph nodes NK cells are present at significant levels in perifollicular T cells zones. There, NK cells are activated by T cell production of IL-2, which in turn stimulates the production of IFN-γ by NK cells. This localization of $CD56^{bright}$ NK to the perifollicular regions provides a possible interaction with resident and incoming DCs. The proximity of NK to DC provides IL-12 and IL-15 in vivo, which can further stimulate NK IFN-γ secretion and activation[208-210]. The NK-DC interaction is an important one, and has been shown by many to be a two-way street. The first study demonstrating DC-NK cross-talk in a tumor setting was done using a non-immunogenic in vivo mesothelioma model where they showed DC activation of NK cells was required for NK killing of MHC-I negative tumors. Importantly, depletion of NK cells also resulted in a loss of the anti-tumor effects indicating that NK cells are important for activation of DC and other immune cells[211]. A later study showed

a similar involvement of NK-DC for the elimination of MHC-deficient tumors; however, this study worked out a pathway demonstrating IFN-γ production by NK was responsible for IL-12 production by DC. The NK-DC interaction eventually led to a DC-mediated activation of CD8 T cells. Thus, NK cells can promote protective CD8 T cell responses to tumors by providing stimulation to DCs[212]. IFN-γ production by NK cells has also been shown to induce a T_H1 polarization, and this effect is abrogated by TGF-β [213, 214].

The NK-DC pair is not only important for tumor immunity, but also for anti-viral responses. NK cells and DCs are both involved in resistance to viral infections[215, 216] and IFN-α/β, IL-12, and IL-15 stimulate NK anti-viral responses [217]. Upon virus infection, DCs (conventional and IFN-producing, plasmacytoid) are major producers of IL-12 and IFN-α/β. Production of these cytokines by DCs enhances the NK IFN-γ response and cytotoxicity and thus the clearance of the viral infection by NK cells. An interesting regulatory function of NK cells is that they can also kill DCs that fail to undergo proper maturation ("DC editing") and the NK activating receptor, NKp30, is responsible for the lysis of these immature DCs (iDC)[218]. NK cells have been (and are still) used for immunotherapeutic purposes due to their outstanding ability to lyse tumor cells; however, perhaps NK cells should also be harnessed for their ability polarize adaptive immune responses via DCs.

SHIP-1

SH2 (Src homology 2)-containing inositol phosphatase-1 (SHIP-1) was initially identified for its role in erythropoietin (EpoR) signaling in hematopoietic cell lines [219]. In 1996 SHIP-1 was independently cloned by five groups based on its ability to interact with the protein-tyrosine binding domain (PTB) of SH2-containing sequence protein (Shc), the SH3 domain of growth factor receptor-bound protein 2 (Grb2), and the FcγRIIB receptor[220-224]. SHIP was also shown to have enzymatic properties and could hydrolyze the 5' phosphate of phosphatidylinositol-3,4,5-phosphate (PIP3) and inositol-1,3,4,5-tetrakisphosphate (IP4) [220, 221, 223, 224]. Because of SHIP's ability to modulate PIP3 and IP3 levels, SHIP is positioned to regulate cell viability, differentiation, proliferation, and effector functions[225-227].

Structure and Functional Domains. SHIP-1 is a large (145kD) multi-domain protein with the capacity to regulate many cellular functions. There is an SH2 domain in the amino terminus of SHIP-1, a central 5' inositol phosphatase domain (IP domain), two NPXY motifs and several polyproline rich motifs (PxxP) located in the carboxy terminal end of the protein (Fig. 2).

***SH2 domain*.** SH2 domains are conserved protein regions that specifically bind phosphorylated tyrosine residues[228]. Thus, the SH2 domain of SHIP-1 allows it to bind phospho-tyrosines present in signaling molecules, adaptors, growth factor receptors, and immune receptor cytoplasmic signaling chains [229-231]. Namely, SHIP-1 can be directly recruited to FcγRIIB on B cells[220], the IgE receptor, FcγRI, on mast cells[232], IL-4R[233], fms-like tyrosine kinase receptor-3 receptor (flt-3 R)[234] on hematopoietic cells, and

several NK receptors (discussed in more detail later)[129, 235, 236]. The SH2 domain of SHIP-1 has been shown to bind phosphorylated tyrosines of tyrosine phosphatase, SHP-2 and Src family kinase, Lyn[237, 238]. SHIP-1 is also indirectly recruited to receptor signaling complexes by its association with adaptor proteins (i.e. Shc, Grb2, and DOK3)[239]. Association of SHIP with receptor signaling motifs and/or adaptor and scaffolding proteins stabilizes SHIP at the membrane where its function is enhanced.

5' Inositol Phosphatase Domain. Inositol phospholipid signaling is involved in many cellular processes[225-227]. After recruitment to the plasma membrane, SHIP can then hydrolyze PI(3,4,5)P_3 to generate PI(3,4)P_2, and by doing so, SHIP attenuates different PI-3K effector pathways[226, 227]. The 5' inositol phosphatase domain of SHIP-1 specifically recognizes a phosphate group, PO_4, which is positioned at the D3 location of the inositol ring. SHIP's enzymatic activity is limited to the phosphoinositides, PIP3 and IP4, which are converted to PI(3,4)P_2 and I(1,3,4)P_4, respectively[224]. Contrarily, PI-3K generates PIP3 by adding a phosphate group to the 3' position of PI(4,5)P_2[240]. Careful and precise balance of these opposing enzymatic activities is necessary to control the levels of PIP3 since this molecule plays a key role in recruiting pleckstrin homology (PH) domain containing proteins such as AKT[241-244].

By decreasing levels of available PIP3, SHIP can directly negatively regulate AKT, bruton's tyrosine kinase (Btk), and phospho lipase C gamma (PLC-γ) signaling. Upon production of PIP3 by PI-3K, AKT translocates to the plasma membrane via its PH domain. AKT is subsequently phosphorylated at two key residues, threonine 308 and serine 473, which when phosphorylated, AKT is said to be activated[242, 245, 246]. Activated

AKT phosphorylates and inactivates pro-apoptotic proteins, such as BAD and inhibits the intrinsic apoptotic pathway[247]. This finding and a role for SHIP in the negative regulation of proliferation and survival is substantiated by our findings demonstrating increased levels of the anti-apoptotic molecule, Bcl-2 (a target of BAD), and enhanced proliferation and survival of several hematolympoid cell compartments in SHIP-deficient mice[248-251]. Btk, a Tec family kinase is affected in a similar way. Like AKT, Btk is a downstream of PI-3K and becomes recruited and activated by increased levels of PIP3. Btk when anchored at the membrane is in close proximity to PLC-γ and subsequently phosphorylates and activates PLC-γ. However, low levels of PIP3 results in decreased activation of Btk and ultimately a block in the influx of extracellular calcium due to decreased levels of activated PLC-γ [252].

***NPXY and PxxP Motifs*.** SHIP-1 has two NPXY motifs at its carboxy terminus where (N) represents arginine, (P) for proline, (X) is any amino acid, and (Y) is tyrosine[253], NPXY motifs are recognized by proteins containing a PTB or phosphotyrosine-interacting domain (PID) [254]. Upon activation of SHIP, NPXY motifs are phosphorylated at the tyrosine residue (NPXpY), which forms a binding site for PTB domains present in Shc, DOK1, and DOK2[131, 255, 256]. There are also a number of proline rich (PxxP) motifs (possibly 8) located throughout the carboxy terminus of SHIP and allow interaction with (Src homology 3) SH3 domain containing proteins[253]. Importantly, these proline-rich motifs have been shown to be critical for the phosphatase activity of SHIP-1[257].

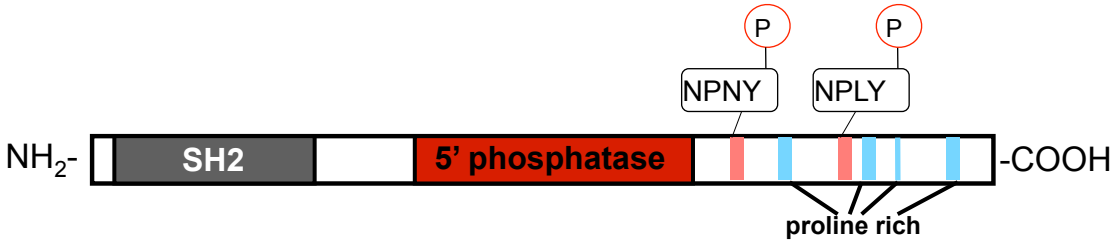


Figure 2. Structure of SHIP. Full-length SH2 domain containing 5' inositol phosphatase is a 145 kDa protein primarily expressed in the hematopoietic system.

SHIP and NK Cells. When one considers phosphatase signaling in NK cells, the tyrosine phosphatases, SHP-1 and SHP-2 are the most thoroughly studied[83]. However, the inositol phosphatase, SHIP also has an important role in NK cells as demonstrated previously by our group and in the present study [250, 258]. Original studies of SHIP did not implicate a role for this phosphatase in NK cells specifically, but the finding that SHIP can be recruited to ITIMs[259] eventually led to the exploration of SHIP in NK cells. The first studies, however, demonstrated a role for SHIP in the negative regulation of activating receptors, not ITIM bearing inhibitory receptors[235, 260]. This group was investigating the involvement of Shc in NK natural cytotoxicity and ADCC. They found that Shc becomes phosphorylated upon CD16 (FcR) engagement and forms a complex with Grb2 and SHIP (and CD16 phospho zeta chain). However, overexpression of a mutant Shc resulted in reduced Ca^{2+} influx by a PLC-γ dependent manner, which was attributed to an increase in SHIP recruitment to Shc-CD16 receptor complexes[260]. This group followed up these findings by showing that SHIP rapidly and transiently translocates to lipid rafts upon CD16 (FcR) stimulation where it functions to suppress ADCC. Overexpression of SHIP led to a reduction CD16-mediated killing and this negative regulation is dependent on 5'

inositol phosphatase activity of SHIP [235]. Consistent with these findings, Parihar *et al* found a reduction in CD16-activation induced cytokine production when WT SHIP was overexpressed, and this too was dependent on SHIP's enzymatic domain [261]. Together these studies reveal that SHIP has an important function in NK receptor mediated killing and cytokine production.

In addition to a role for SHIP in NK receptor signaling, our lab has discovered a more crucial role for SHIP in the NK cell [250]. We found that SHIP knock out (KO) mice have an over representation of key NK receptors, Ly49A and C/I. Remarkably, the SHIP$^{-/-}$ mouse could fully accept a bone marrow transplant from a MHC mismatched mouse[250], and this was due to the overrepresentation of these inhibitory Ly49 receptors. Further studies expanded our understanding of SHIP in the NK cell by showing that SHIP is recruited to Ly49B, KLRG1, and 2B4[129, 236, 262]. In recent reports we found that in the absence of SHIP, 2B4 functions as a dominant inhibitory receptor[258] and this is due to inappropriate recruitment of SHP-1. This latter finding is likely due to the fact that in the absence of SHIP, SHP-1 gets over recruited as a compensatory mechanism. This study here will attempt to build off our earlier studies and delve deeper into the specific molecular role SHIP plays in the NK cell and how 2B4, a "SHIP regulated receptor" also contributes to NK function and receptor repertoire formation.

The role for SHIP has extended beyond mouse studies and in vitro mechanisms of cytotoxicity. Trotta *et al* went on to define a role for SHIP in the two human NK subsets, CD56^{bright} and CD56^{dim} [263]. CD56^{bright} are traditionally the cytokine producers, while the CD56^{dim} NK cells have robust cytolytic function[264]. Moreover, these subsets are also found in distinct locations. This group found that SHIP is differentially expressed by the

two subsets with the $CD56^{bright}$ NK cells having significantly less SHIP, and the cytolytic $CD56^{dim}$ NK cells having high levels of SHIP. They went on to show that SHIP levels decrease in response to monokine stimulation and overexpression of SHIP resulted in a decreased production of IFN-γ in response to stimulation by monokines[263]. However, it has also been shown that a subset of human NK cells from patients with chronic HIV infection has elevated levels of SHIP and lowered perforin levels. Unlike NK cells from normal donors, this subset of cells has been shown to become functionally anergic in chronic HIV infection[265].

Lenalidomide

Thalidomide. Lenalidomide (Len) is an analog of thalidomide. Thalidomide (α-(N-phthalimidoglutarimide) is a synthetic derivative of glutamic acid and was first synthesized in the early 1950s[266]. Thalidomide has a tragic history: it was originally intended as an anti-convulsant to treat epileptic patients, but it wasn't very effective for this. However, thalidomide was quite good as a sedative and as an anti-emetic and so was given to pregnant women during their first trimester of gestation to treat morning sickness. A few years later, two independent physicians from different sides of the globe linked thalidomide to devastating congenital malformations [267, 268]. It was quickly banned due to these discoveries. The United States Food and Drug Administration (FDA) actually never approved thalidomide, but not because of its teratogenic effects (this was unknown at the time), but because thalidomide has other adverse side effects such as deep vein thrombosis, neuropathy, and unwanted sedation. There are over 30 proposed

mechanisms for the teratogenic effects of thalidomide. These proposed mechanisms are not mutually exclusive and it is very likely there are working in parallel or synergistically[269], suggesting that thalidomide (and other IMiDs) have multiple targets. The chemistry of thalidomide has shed some light on the differential effects of this drug. Thalidomide exists as a racemic mixture of R(+) and S(-) enantiomers (Fig. 3). That is, the two isoforms are mirror images of one another having identical chemical composition, yet they are not superimposable. The thalidomide enantiomers have a segregation of function: the S(-) isoform has been associated with the teratogenic effects of thalidomide, while the R(+) isoform is said to be responsible for the sedative effects[270, 271].

A serendipitous finding by an Israeli dermatologist, Jacob Sheskin, put the spotlight back on thalidomide. Dr. Sheskin was treating a patient with erythema nodosum leprosum (ENL) who was having sleep difficulties. Dr. Sheskin recalled the sedative effects of thalidomide and so gave this patient the drug. Amazingly after just one dose of thalidomide, the patient's fever, night sweats, and lesions were dramatically improved[272]. ENL is a potentially life threatening complication of leprosy treatment. It is an immune-mediated inflammatory disease with the major pathology being antibody-antigen complexes that deposit in the skin, but ENL can affect many organ systems. There is a resulting local activation of complement and inflammation with an infiltration of neutrophils[273]. T cells and macrophages can be found in the skin where levels of IFN-γ and TNF-α mRNA are detected. There is also increased levels of TNF-α in the serum of some patients[274, 275]. Understanding the pathophysiology of this disease led researchers to understand that thalidomide is an anti-inflammatory and immunomodulatory agent. After

a multi-thousand-person study (that included only men) conducted by the World Health Organization (WHO), thalidomide was eventually approved in 1998 to treat ENL, but of course with very strict guidelines for obtaining the drug[276]. A partial explanation for thalidomide's anti-inflammatory effects came when it was discovered that thalidomide inhibits monocyte-derived TNF-α and does so by enhancing the degradation of TNF-α mRNA[277, 278]. Thalidomide was then used in several open-labeled studies where increased TNF-α production was associated with disease[279].

Judah Folkman was among the first researchers to link angiogenesis, or the formation of new blood vessels with tumor growth and metastasis. His laboratory also discovered that thalidomide has anti-angiogenic properties[280]. This was the main impetus to use thalidomide in the treatment of cancers. About a half decade later, thalidomide had remarkable success in treating advanced or refractory multiple myeloma (MM)[281]. MM is an incurable B cell malignancy where an increase in bone marrow vascularization, or bone marrow microvessel density (MVD) is associated with poor prognosis. A link between thalidomide and decreased bone marrow MVD was not shown by these authors, but it was later demonstrated that only patients responding to thalidomide had a decrease in the bone marrow MVD, thus indicating angiogenesis as a therapeutic target in MM[282]. This clinical finding has since been confirmed and many subsequent studies have shown that thalidomide has multiple mechanisms of action in MM including inhibition of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL-6 and TNF-α[283]. Thalidomide (and Len) inhibits NF-κB-mediated expression of intracellular adhesion molecule -1 (ICAM-1)[284, 285]. This disruption is an important mechanism of thalidomide action in MM and other cancers. Inhibition of NK-kB has also

been shown to lend direct anti-tumor effects through the down-regulation of anti-apoptotic molecules[286] and activation of caspases[287]. This list is not exhaustive, however, despite the many benefits of thalidomide, its use is severely limited because of the negative side effects. To this end, thalidomide became the lead compound in a drug discovery program to produce more potent immunomodulatory drugs, and so, IMiDs were born.

Immunomodulatory Drugs. Shortly after discovering that thalidomide has anti-angiogenic properties, attempts were made to create compounds with similar features. IMiDs are structurally and functionally analogous to thalidomide (Fig X). This class of drugs represents a promising new class of immunomodulators for the treatment of inflammatory, autoimmune, and malignant diseases. There were actually four IMiDs developed, but only two of them, CC-5047 (Actimid®; pomalidomide) and CC-5013 (Revlimid®; lenalidomide) have shown clinical stamina[288]. Len has been far more successful in the clinic compared to pomalidomide[289]. The initial goal when generating the second generation IMiDs was to enhance the anti-TNF-α function of thalidomide, which was achieved[290, 291]. Improved anti-angiogenic, anti-tumor, and immunomodulatory/immune enhancing effects were also desired, all while striving for a safer toxicity profile. Indeed the neurotoxicity seen with thalidomide is very rare in patients receiving Len treatment[292]. IMiDs (including thalidomide) are currently being investigated in over 1000 clinical trials; over 400 of those involve Len (clinicaltrials.gov search “thalidomide”, “lenalidomide”, “pomalidomide”).

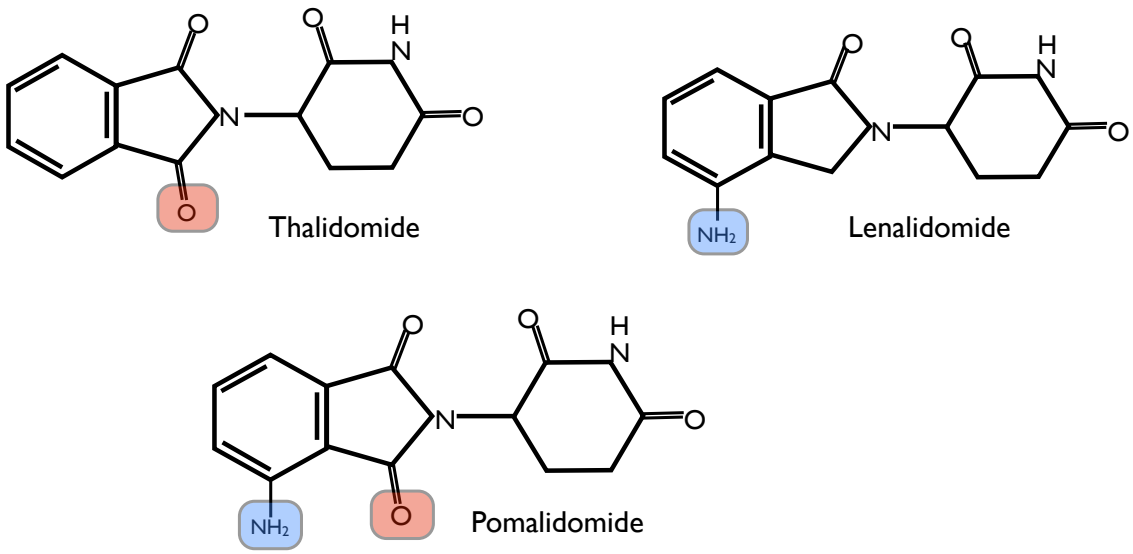


Figure 3. Chemical structures of thalidomide and its analogues, lenalidomide and pomalidomide. Lenalidomide, a second generation IMiD, was created using thalidomide as a template by adding an amino group (highlighted in blue) to the 4th carbon of the phthaloyl ring and removal of a carbonyl group. Pomalidomide, generated in a similar manner, also has an amino group added to the 4th carbon, but the carbonyl group is left in tact (highlighted in red).

Lenalidomide Functions. As intended, lenalidomide and pomalidomide do have superior anti-TNF activity compared to thalidomide. Additionally, these compounds appear to have more robust immunomodulatory properties, specifically T cell co-stimulation (pomalidomide is even more effective at T cell co-stimulation compared to Len). However, there is still some concern regarding toxicity. Myelosuppression is the major dose-limiting side effect. The multiple clinical benefits of lenalidomide make it an outstanding drug, but also make it difficult to organize and interpret the data. Moreover, the effects of lenalidomide are often conflicting and controversial with variances in the

results depending on the cell type being studied, the stimulus being received, and the pathological state. A simple approach is to categorize the data based on three broad mechanisms studied: Anti-angiogenic, anti-neoplastic, and immunomodulatory/immune enhancing.

Anti-angiogenesis. Angiogenesis is the growth of new blood vessels from existing vessels and is critical for tumor growth and metastasis [293]. A characteristic feature of tumor cells is their ability to attract or promote the generation of new blood vessels. Moreover, without new vasculature tumors cannot grown beyond a critical size or metastasize to another organ. Thus, inhibition of angiogenesis provides a strategy for tumor arrest[294]. Proper activation, proliferation, migration, maturation, and adhesion of the endothelial cells (EC) is required for angiogenesis[295]. Thalidomide is most noted for its anti-angiogenic effect in multiple myeloma, while lenalidomide (and pomalidomide) is far better as an immunomodulator. Nonetheless, a few studies have demonstrated that lenalidomide has anti-angiogenic potential and this may be applicable to MM and other malignancies. The anti-angiogenic effects of lenalidomide are independent of the immunomodulatory effects and involve inhibition of EC migration, not proliferation[296]. One proposed mechanism for inhibited EC migration, and ultimately angiogenesis was decreased VEGF and bFGF-induced AKT phosphorylation[297]. Using an in vivo model in the absence of tumors, the direct effects on angiogenesis were explored. As the authors mention, this implies that Len is working directly on EC migration and is not inhibiting any growth factors that may be produced by the tumors. The ability to inhibit VEGF and bFGF signaling has significant implications that extend beyond angiogenesis since these

molecules are also involved with up-regulation of pro-inflammatory cytokines such as IL-6[298].

Abnormal angiogenesis has also been implicated in MDS and leukemias. MDS are a heterogeneous group clonal stem cell disorders characterized by ineffective hematopoiesis and a variable risk for transformation to AML[299]. The overproduction of proinflammatory cytokines and growth factors has been implicated in the pathology of MDS. There have been reports of modestly increased bone marrow MVD in MDS, as well as expression of VEGF, bFGF, and angiogenin in the bone marrow and/or plasma of MDS and AML patients[300-303]. However, the in vivo or in vitro effects, if any, of Len on angiogenesis in these diseases has yet to be clearly defined. Delineation of a role for Len as an anti-angiogenic factor as lagged behind its function as an anti-neoplastic agent and immunostimulatory drug. This is likely because several more specific and more potent anti-angiogenic therapies already exist.

***Anti-neoplastic*.** A unique feature of Len is that it can target malignant, or cancerous cells while mostly sparing normal cells. The anti-neoplastic function of Len is variable and can result in cell cycle arrest or cell death via apoptosis. This disparity may be in part due to the levels of p21 and the mutational status of p53[304]. Len upregulates p21 (and other cyclin-dependent kinase inhibitors), which results in decreased cyclin-dependent kinase activity and reduced phosphorylation of retinoblastoma proteins, which causes G_0/G_1 arrest. In a study using MM cell lines and primary MM cells from patients, the authors showed that cell growth inhibition occurred because Len caused downregulation of IL-6 and could inhibit proliferation of drug resistant cells. Importantly,

the combination of Len and dexamethasone (Dex) enhanced the anti-MM effect[304]. IL-6 is an important cytokine for the growth of tumor cells in vivo and in vitro[305, 306], and the ability of Len (and other IMiDs) to inhibit IL-6 is a very relevant anti-cancer feature of this class of drugs. Also in MM cells, Len can activate caspase-3, -8, and -9 in certain cell lines and caspase-8 in both MM lines and patient samples. Treatment with an inhibitor of caspase-8, but not caspase-9 abrogated the pro-apoptotic effects, suggesting a caspase-8-dependent mechanism[307]. This caspase-dependent pathway is likely due to downregulation of the transcription factor, nuclear factor kappa-B (NF-κB) by Len. NF-κB results in the activation of anti-apoptotic proteins, FLIP (FLICE-inhibitory protein) and cellular inhibitor of apoptosis protein 2 (cIAP2), which have both been shown to inhibit caspase-8 activation[307-310].

In a model of non-Hodgkin's lymphoma (NHL), Len was capable of inducing cell death and growth inhibition of lymphoma cells[311]. This was an interesting study and these authors demonstrated disparate effects of the drug in vitro and in vivo. Although in vitro these NHL cells underwent cell death or arrest, there were no direct anti-tumor effects observed in vivo. However, using a severe combined immune deficient mice (SCID) xenograft model of NHL they showed a strong anti-tumor response when Len was followed by treatment with rituximab, a monoclonal antibody that targets CD20, an antigen expressed by lymphoma cells. This anti-tumor effect was completely abrogated when NK cells were depleted[311], suggesting NK-mediated tumor clearance, although experiments to rule out (or in) a CD16-dependent activation (ADCC) of NK cells was not demonstrated in this study. The mechanism of cell death and growth arrest in vitro was also not investigated.

Lenalidomide is also capable of inducing apoptosis in tumor cells as a result of modulating the surrounding microenvironment. This is especially true for MM where the bone marrow stromal cells (BMSC) have been shown time and time again to support MM survival and even confer drug resistance. Specifically, Len downregulates VEGF, bFGF, TGF-β, TNF-α, IL-10, and IL-6, molecules that are involved in the cooperation of MM cell growth[306]. In addition to BMSC and epithelial cells in the microenvironment, osteoclasts also have an essential role in MM pathogenesis[312]. A large percent of MM patients have osteolytic bone disease, which is due to an imbalance in the osteoblast and osteoclast activity as a result of increased osteoclast stimulating factors produced by MM cells[313]. MM cells also release factors that suppress osteoblast differentiation, which leads to reduced osteoblast numbers and decreased bone formation[314]. Len has been shown to interfere with osteoclast differentiation and function, while bortezomib can induce osteoblast function in vitro and in vivo in MM patients[315]. This drug combination results in new bone formation. Indeed, Len and bortezomib are currently used in combination to treat MM and have shown great success[316].

The scenario of Len in MDS is complicated and multifaceted, characteristics inherent to this group of disorders. The major clinical challenge among lower risk MDS patients (with or without chromosomal deletions) is refractory anemia caused by ineffective erythropoiesis, which is a hallmark of this disease[317]. Len hit the fast track to approval in 2005 because of its high response rates in lower-risk, transfusion-dependent MDS patients where a significant percent of patients had improved erythropoiesis and cytogenetic responses. The efficacy of Len to restore erythropoiesis and induce transfusion independence is greatest when there is an isolated 5q deletion. However,

patients with a normal karyotype also respond well, and patients that have other karyotypic abnormalities do respond to Len although with a lower frequency [318, 319]. Despite clinical responses, the major limiting factors are severe neutropenia and thrombocytopenia, which occur in the majority of patients receiving high dose monotherapy Len. Something that has been debated is whether clinical responses are due to the immunomodulatory effects of Len, or if Len has direct cytotoxic function on abnormal progenitor cells present in MDS BM. Very likely both of these features of Len contribute to the responses.

The commonly deleted region (CDR) of MDS del(5q) is a 1.5Mb interstitial region containing 44 genes[320]. A few studies (discussed below) have demonstrated genes present in this CDR to be essential for MDS pathogenesis and response to Len treatment. For example, RPS14, an integral component of the 40S ribosomal subunit when partially deleted (by RNAi or using a genetic mouse model) reproduces a similar phenotype observed in human MDS[321, 322]. Moreover, RPS14 expression is decreased compared to normal controls in lower-risk MDS patients and upon treatment with Len, RPS14 expression becomes up regulated[321, 323]. Although RPS14 is not the target of Len, these data provide strong evidence that this protein is involved in the pathogenesis of del(5q) MDS. Our group has shown del(5q) MDS clones are sensitive to Len and this is due to allelic haploinsufficiency of dual specificity phosphatases, Cdc25C and PP2A, genes also located in the CDR of chromosome 5. Treatment of Len induces a G_2 arrest and apoptosis in del(5q) clones, but has no effect in non-del(5q) cells. In fact, using siRNA to reduce the levels of PP2A and Cdc25c in non- del(5q) MDS BM cells we could sensitize these cells to Len, providing strong evidence that these phosphatases are involved in the

response of 5q MDS patients to Len treatment[324]. An earlier study investigating the molecular basis for response to Len implicates SPARC (secreted protein acidic and rich in cysteine) and activin A in the pathogenesis of the 5q clone. Treatment of del(5q) (and normal BM) cells with Len had a global effect on gene expression with down regulation of several genes and up regulation of others. Of interest, SPARC was consistently up regulated upon treatment with Len (in MDS *and* normal cells) and is found in the CDR of chromosome 5. SPARC functions as a tumor suppressor protein and is anti-proliferative, anti-adhesive, and anti-angiogenic[325]. Like our study, allelic haploinsufficiency of SPARC is a proposed mechanism of how Len selectively targets the 5q clones. A very recent study has implicated SPARC in the growth arrest of medulloblastoma cells[326]. Although Len treatment was not involved with this study, the authors did show that the G_2/M arrest induced by exogenous SPARC was dependent on p21 and cdc25C, two proteins modulated by Len. Moreover, expression of SPARC also results in decreased STAT3 activation, an effect observed in our lab (Fortenbery, unpublished data) and other labs. Addition of constitutively active STAT3 overcame the induction of G_2/M arrest mediated by SPARC. Together these data, and others[327] provide support for one of the pathways controlled by Len that leads to its anti-tumor activity. Whether a STAT3-dependent mechanism occurs in del(5q) MDS BM progenitor cells remains unknown. Another active area of investigation is p53 and its role in Len treatment. MDS and AML patients with (del)5q frequently have *TP53* mutations[328, 329]. What's more is that del(5q) MDS clones have resistance to treatment with Len[330]. A recent study investigating the efficacy and safety of high dose Len in AML and high-risk MDS patients found a striking similarity: a high frequency of patients enrolled in this study had a *TP53* mutation and

moreover, the presence of this mutation had significant association with Len resistance[329]. In fact, only two cases with *TP53* mutations responded to treatment. This study also reported that there were no differences between patients having an isolated del(5q) and patients with complex cytogenetic patterns, which implicates a direct anti-tumor effect of Len even in non-del(5q) individuals[329]. Our group has also made an attractive connection between Len, RPS14, p53, MDM2, and PP2A (manuscript in press, Oncogene). When ribosomal integrity is disrupted, free ribosomal proteins are released which bind and trigger degradation of MDM2[331]. Consequently, p53 becomes activated and p53 mediated genes are transcribed and/or cells undergo apoptosis. In our study, we demonstrate that p53 is overexpressed by erythroid precursors from del(5q) MDS BM and reduced MDM2 expression accompanies elevated p53 expression. More importantly, we Len acts to stabilize MDM2, which accelerates p53 degradation. When we investigated the biochemical and molecular events associated with this process we find that Len-mediated inhibition of PP2A results in hyperphosphorylation of inhibitory serine-166 and serine-186 residues on MDM2, and displaces binding of RPS14 to suppress MDM2 auto-ubiquitination. When we overexpressed PP2A (to simulate haplosufficiency) we observed drug resistance. In fact, BM specimens from del(5q) MDS patients that were resistant to Len had overexpressed PP2Acα accompanied by restored accumulation of p53 in erythroid precursors. These findings indicate that Len restores MDM2 functionality in the 5q- syndrome to overcome p53 activation in response to nucleolar stress (manuscript in press, Oncogene). Collectively, these findings suggest that Len has multiple targets. Discussed below are the newest findings that eloquently

demonstrate Len and thalidomide's ability to directly bind and inhibit an E3 ligase, cereblon[269, 332, 333]. This is one of the proposed mechanisms of teratogenecity.

Immunomodulatory Functions of Lenalidomide

Anti-inflammatory. Lenalidomide's ability to kill or inhibit tumor cell growth all while enhancing different components of the immune system may explain how patients rapidly respond to treatment and show sustained responses, respectively. Inflammation is now well understood to underlie many diseases, especially cancer. Len is a potent anti-inflammatory compound and has the ability to inhibit TNF-α and IL-6, IL-12, IL-1β, and GM-CSF, IL-10 by CD3-stimulated T cells or PBMC[291, 334, 335]. A reduction of inflammatory cytokines especially, TNF-a and IL-6 is a proposed mechanism of action in MM and MDS, and there is a good amount of evidence supporting this[283, 304, 306, 336-338]. However, the ability of Len to modulate cytokine production varies greatly depending on cell type and stimulus received. For example, Len can enhance the production of TNF-α, monocyte chemoattractant protein-1 (MCP-1), and IFN-γ by DCs co-stimulated in vitro with GM-CSF or IL-2. Increased cytokine production was shown to augment NK-mediated ADCC[339]Although in contrast to what others have found, this ability of Len to enhance TNF-α and MCP-1 by DCs provides a protective effect and aids in the recruitment and activation of neutrophils, macrophages, T cells, B cells, and NK cells. Increased activity of the transcription factor, AP-1 may be partially responsible for the increased production of cytokines by T cell lines, however, the precise molecular

mechanisms governing the differential effects induced by Len on other cell types remain undiscovered[340].

T cell Modulation. Although Len does not have the apparent ability to directly induce T cell proliferation, it can very powerfully amplify co-stimulated T cells and overcome CTLA-4 mediated inhibitory signals via phosphorylation of CD28 [340, 341]. Len also induces the production of Th_1 cytokines, IL-2 and IFN-γ, Th_2 cytokines, IL-4 and IL-10[340 342]. More recently, Len has been shown to enhance antigen-specific $CD8^+$ T cell responses from healthy donors and patients. This has major clinical applications regarding the use of Len as an adjuvant in peptide-based, peptides, or tumor lysate vaccines[343]. These authors also demonstrated an upregulation of cytolytic mediators, perforin and granzyme, which provides support for our data. Immune dysfunction and suppression are hallmarks of cancer and aging. Multiple T cell abnormalities exist in CLL patients, such as increased ZAP-70 and CD3ζ signaling, which results in chronic and aberrant activation of T cells[344]. Len is not only capable of restoring T cell subset numbers (CD4, CD8, and Treg) and function (production of TNF-α, IL-2, IFN-γ) to normal in CLL patients that respond to Len treatment[344], but Len can also reverse immunological synapse defects, another hallmark of CLL[345]. An interesting study recently showed that Len has differential effects on T cells isolated from young (21-40 years) and old (≥65 years) individuals. Like other groups, they confirmed that Len induces IL-2 and IFN-γ production by T cells; however, its immunomodulating effects are more potent in elderly people. These results provide rationale to use Len as a method to improve immunity in the older population[346].

Lenalidomide in NK Cells. NK cells are major effectors of the innate immune response. NK cell function is severely impaired in many types of malignancies, namely MDS, MM, and CLL, diseases that have clinical responses to lenalidomide[347-349]. As described above, the mechanisms of action of Len in these diseases includes anti-angiogenesis, alteration of the BM microenvironment, direct anti-tumor effects, and enhancement/modulation of T cell responses. However, an undeniable attribute of Len is its ability to enhance NK cells. NK cells can kill or produce cytokines. Of the two functions, their cytolytic ability is more thoroughly studied in the context of Len. A unanimous finding is that Len can up-regulate the expression of CD16 (FcR), which mediates ADCC[189]. Enhanced ADCC function is the best-defined effect of Len on NK cells and has been shown by several groups to translate to enhanced killing of tumor cells in vitro[311, 349-352].

Most of the focus of Len and NK cell activation has been on MM and lymphomas (NHL and CLL). Len is used in combination with different therapies, which has proven to be most successful. However, in the case of MM, the use of Dex in combination with Len negates the immunostimulatory effects produced by Len[353]. Although NK numbers are increased in patients receiving this drug combination, cytotoxic functions by NK cells is largely impaired. This was attributed to a Dex-dependent decrease in IL-2 production by T cells[353]. As is the case with Len and T cells (e.g. Len cannot directly stimulate T cells- it is more of a co-stimulator), Len also does not directly activate NK cells. T cell-produced IL-2 has been implicated in the stimulatory effects of Len on NK cells[354]. The question then becomes, *how* does the combination of IL-2 and Len result in hyperactivated NK cells? We sought out to define the molecular mechanisms governing

this effect. We argue that understanding this in NK cells will allow future treatments to be tailored to enhance this function (e.g. Len in combination with cytokine therapy).

Cereblon. Based on the multitude of effects and the disparities observed with Len when different cell types are studied, it seems obvious that there must be multiple targets for this drug. However, despite extensive studies little is known regarding the direct target of Len (and thalidomide) and their precise molecular mechanisms. There have been over 30 proposed mechanisms of action for thalidomide and Len's teratogenecity alone, each one providing evidence to support its claim[355]. One explanation is the hypothesized >20 (some say over 100) hydrolysis products and metabolites that may form from the metabolism of these drugs[356-358].

Recently, thalidomide and Len were shown to bind and inhibit cereblon (CRBN) [386, 387]. CRBN is abundantly expressed in the hippocampus and neocortex of the brain and is important for normal human nervous system development and function[359, 360]. It has been shown to function as a regulator of voltage-gated ion channels in neuronal synapses, which is likely how CRBN contributes to memory and learning[361]. CRBN is also an E3 ligase[332, 362] and forms a functional E3 ubiquitin ligase complex with damaged DNA binding protein 1 (DDB1), Cullin-4A (CUL4A), and regulator of cullins 1 (ROC1), with CRBN and DDB1 being functionally associated[332, 363]. Proteins are commonly polyubiquitinated as a form of post-translational modification and as a method of normal protein degradation and turnover. This CRBN-DDB1-CUL4A-ROC1 complex ubiquitinates several different proteins and by an unknown mechanism, inhibits the production of FGF-8, which regulates many developmental processes, such as limb

development and auditory vesicle formation[332]. In an earlier study, thalidomide treatment caused a reduction of fibroblast growth factor 8 (FGF-8) in rabbit (a thalidomide sensitive species), but not rat embryos (insensitive to thalidomide teratogenecity)[364]. Using chicken embryos, a well-established model for studying the teratogenic effects of thalidomide, Knobloch *et al* found that thalidomide induced the expression of bone morphogenic proteins (BMPs)[365]. Interestingly, in mice, BMPs have been shown to inhibit the expression of FGF-8[366]. Therefore, CRBN seems to be the link between these developmental proteins and in some part, thalidomide's teratogenecity.

A role for CRBN in antimyeloma efficacy and myelosuppression has been suggested recently. Deletion of CRBN from human myeloma cells was cytotoxic and the cells that survived CRBN deficiency became resistant to lenalidomide[333]. Moreover, using MM1.S myeloma cell lines, the authors showed that the presence or absence of CRBN was directly linked to Len sensitivity or resistance, respectively[333].

CRBN also directly binds to and is a negative regulator of amp-activated protein kinase (AMPK), which is a serine-threonine protein kinase that regulates pathways involved in cell growth, apoptosis, and protein, lipid, and carbohydrate metabolism[367]. How CRBN's reported functions contribute to the diverse effects of Len are not clear. Important questions remaining are: what are other targets of CRBN? Do other E3 ligases share sequence homology to CRBN and may then also be targets for Len and thalidomide? What is the expression of CRBN in other hematopoietic and immune cells? Can CRBN binding explain the immune enhancing effects? Answering these questions will undoubtedly drive this field forward and allow the generation of safer, more effective analogues.

CHAPTER 2
Regulation of NK Homeostasis, Function, and Repertoire Formation: SHIP-1, 2B4, and MHC-I

A note to reader

This work has been previously published in the Journal of Immunology, Fortenbery *et al.*[368] and has been reproduced here with permission from the publisher.

Introduction

Natural killer cells express invariant receptors that are broadly categorized as activating or inhibitory. These receptors enable responses to viral infections, tumor cells, allogeneic grafts and damaged cells[369, 370]. A fine balance, or lack thereof, of stimulatory and inhibitory signals transmitted via these receptors that dictates the function of an NK cell. Thus, a diverse and balanced NK receptor repertoire (NKRR) is extremely important in order for this lymphocyte compartment to respond to various immunological challenges and to do so in a normal, effective manner. Aberrations in the expression of NK receptors (NKR) or the downstream signaling can lead to severe immune deficiency, as observed demonstrated in our in SHIP-deficient$^{-/-}$ mice[250, 258, 371]. Upon engagement of an NK activating receptor with its ligand, or cross-linking the receptor with an antibody, a functionally competent NK cell will produce cytolytic mediators e.g. perforin, granzymes and/or cytokines, such as γ-IFN. NKR are expressed in a variegated but overlapping fashion such that different cell subsets in the NK compartment elaborate different combinations of activating and inhibitory NK receptors. Varying the array of NKR used by each subset increases the potential specificities of the NK compartment,

while retaining tolerance to self [372]. Therefore, it is essential to understand how this repertoire diversity is created, maintained or remodeled.

The acquisition of NKR expression is considered a semi-stochastic process with the representation of an individual NKR largely determined by the relative strength of its promoter[373]. This probabilistic model for NKR expression is based on inherent promoter features[373] as further supported by the demonstration that a genomic Ly49A transgene recapitulates the representation of Ly49A in the NK compartment[374]. However, there is a wealth of evidence that the NKRR is not solely determined by relative promoter strength as the presence or absence of NKR ligands, such as major histocompatibility complex class I (MHC-I), as well as mutations in intracellular signaling molecules can significantly impact the representation and expression of NKR[250, 258, 371, 375, 376]. Thus, differential effects on the proliferation and/or turnover of NK subsets provide a secondary layer of regulation that determines the final composition of the NKRR. Defining the ligands, receptors and signaling molecules that constitute these secondary regulation pathways is required to fully understand how the NKRR is formed and thus how it might be manipulated to better control malignancy, and infection and inflammatory diseases.

A major extrinsic influence on NKRR formation are ligands encoded by MHC class- I genes[377-379]. The influence of MHC-I on the NKRR presumably reflects education of the developing NK cell to avoid reactivity against self. There are two models that have been proposed for the MHC-specific education of the murine NKRR: The sequential activation model and the two-step selection model[380]. The first model proposes that a developing NK cell sequentially acquires, or activates, Ly49 genes and it does so in a seemingly random fashion. Once the NK cell expresses a Ly49 gene, expression is

maintained throughout the life of that NK cell. The NK cell acquires expression of a sufficient number of self-reactive inhibitory receptors to establish an inhibitory signaling threshold that prevents inappropriate killing of normal host cells. Thus, interaction of inhibitory receptors with host MHC signals the NK cell to terminate further expression of Ly49 genes and complete the maturation process. In the alternative model a developing NK cell acquires a fully formed repertoire at an initial stage of development by a stochastic process, but can undergo two possible types of selection. NK cells that have at least one self-specific Ly49 inhibitory receptor are positively selected for, or NK cells that express multiple self-specific Ly49 receptors are selected against to avoid accumulation of cells in the compartment that have too great of an inhibitory threshold[380].

In addition to influencing formation of the NKRR, MHC-I interactions with self-specific Ly49 or Killer Immunoglobulin-like Receptor (KIR) are also necessary for efficient NK function through a process referred to alternatively as licensing[381], or disarming[382], or tuning[383]. For example, a Ly49A receptor that has high affinity for H-2^d molecules can "license" or tune an NK cell's function in H-2^d haplotype mice[381]. In the absence of these interactions, others argue that the NK cell is disarmed. The recent demonstration that NK cells also express inhibitory and activating SLAM family receptors for ubiquitous self-ligands encoded outside the MHC locus[122], such as 2B4, is likely to increase the complexity of NK education by self-ligands and their receptors.

The signal transduction pathways that regulate NKRR formation are incompletely defined. PI3K[384] and SHIP[250] are recruited to inhibitory NKR upon MHC-I engagement and are therefore able to control activation of AKT/PKB in NK cells. This suggests a role for inositol phospholipid signaling in the regulation of the NKRR by

allowing differential turnover of various NK cell subsets (indirect effects) and directly by alteration of NKR gene transcription by transcription factors that are distal mediators of PI3K signaling. The former was shown to be the case in SHIP$^{-/-}$ mice where a specific NK subset that dominated the compartment also exhibited decreased turnover[250]. We have also shown that SHIP functions in NK cells to prevent certain inhibitory receptors from dominating the NKRR. We found that on a C57BL/6 background (H-2^{b}) SHIP-deficiency leads to a number of signaling and gene expression perturbations that culminate in an NK cell being hyporesponsive to complex tumor targets that express both MHC-I and an activating ligand, RMA-Rae1^{+} and or BaF-m157^{+}[258, 371]. These disruptions include increased surface expression of 2B4 and the tyrosine phosphatase, SHP1, and inappropriate recruitment and activity of SHP1 at 2B4. Thus, a signaling environment where the inhibitory mode of the 2B4 receptor dominates is created,, and rendering SHIP$^{-/-}$ NK cells are rendered hyporesponsive.

To examine whether 2B4 receptor dominance might also be responsible for the NKRR receptor repertoire disruption we observe in SHIP-deficient mice and whether another receptor might dominate cytolytic function in the absence of 2B4, we created 2B4$^{-/-}$SHIP$^{-/-}$ mice on an H-2^{b} background. As anticipated from our previous studies, 2B4 deficiency restores the ability of SHIP$^{-/-}$ NK cells to kill via NKG2D; however, we find that both 2B4 and SHIP are required for formation of a normal NK cell repertoire. SHIP is required for the induction of γ-IFN production upon stimulation of all NK activating receptors examined in this study (NKp46, NKG2D and NK1.1), but 2B4 regulates or restrains this induction only when NK1.1 is stimulated. Moreover, we demonstrate a novel role for SHIP and 2B4 in lineage-restricted expression of Ly49B, which is

normally restricted to myeloid cells. Intriguingly, we also find that MHC haplotype can overcome the negative impact of 2B4 over-expression on SHIP$^{-/-}$ NK cytolytic function, since deregulated 2B4 expression by SHIP$^{-/-}$ H-2^{d} NK cells does not compromise their ability to lyse MHC matched targets or MHC mismatched targets, indicating that MHC haplotype has a critical role in the cytolytic competency of SHIP-deficient NK cells. Significantly, H-2^{d} NK cells, similar to H-2^{b} NK cells, display a highly disrupted NKR. Thus, the interplay of SHIP, 2B4 and MHC influences NKRR formation, γ-IFN production, and cytolytic function in the NK compartment.

Results

Discordance of cytolytic function and IFN-γ induction in SHIP$^{-/-}$, 2B4$^{-/-}$SHIP$^{-/-}$ and 2B4$^{-/-}$ NK cells. To assess whether 2B4 deficiency influences homeostasis, repertoire formation, cytolysis and IFN-γ induction in SHIP-deficient NK ells, we generated 2B4$^{-/-}$SHIP$^{-/-}$ mice on an H-2^{b} background. 2B4$^{-/-}$SHIP$^{-/-}$ mice exhibit the same pathologies previously reported in SHIP mice including splenomegaly, weight loss, and a crystalline pneumonia that culminates in their demise at 8-10 wk old. As previously reported, we continue to observe a significant increase in peripheral NK cell numbers in SHIP-deficient mice; however, this expansion is dependent on 2B4, because 2B4$^{-/-}$SHIP$^{-/-}$ mice have normal peripheral NK cell numbers comparable to that of 2B4SHIP$^{+/+}$ and 2B4$^{+/+}$SHIP$^{+/+}$ controls (Fig. 4).

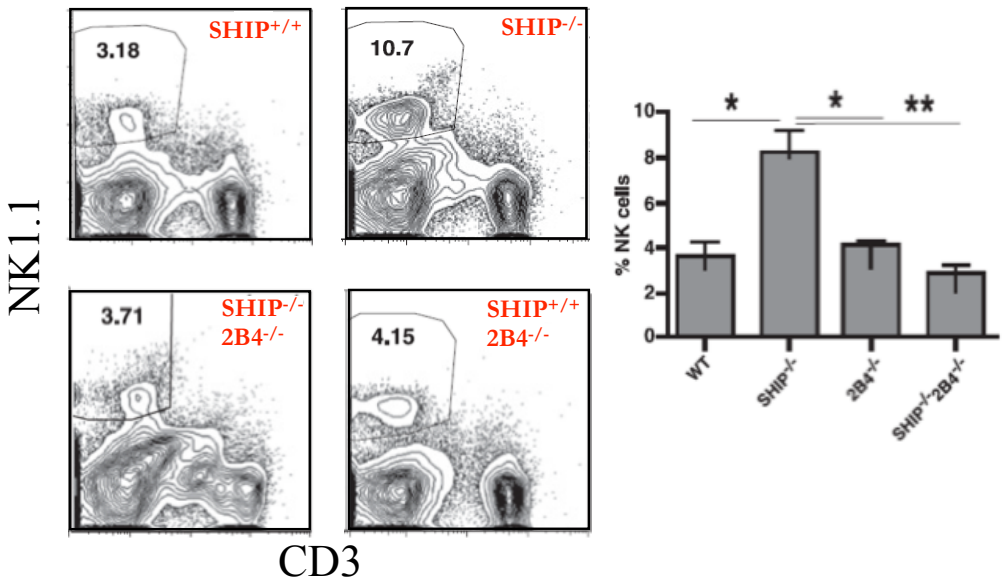
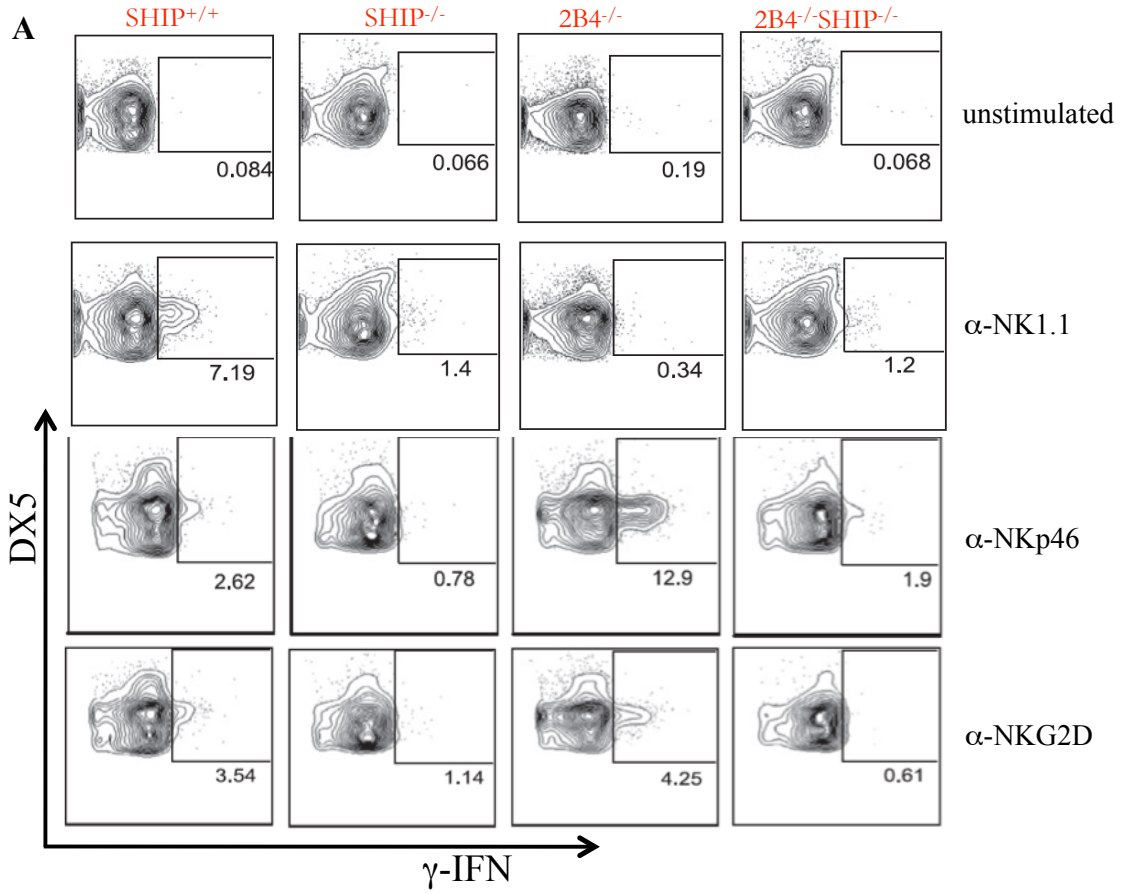


Figure 4. SHIP and 2B4 are required for NK homeostasis. Representative NK1.1 versus CD3 contour plots of splenocytes from the indicated genotype and bar graph indicating the mean percent of splenic NK cells for each genotype (n = 5 per genotype). Results were considered significant when $p<0.05$ (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$). Statistical analyses was performed using Graphpad Prism Software and the Student's two-tailed paired t test.

The interaction of self-specific NK inhibitory Ly49 receptors with MHC has been shown to endow murine and human NK cells with cytolytic competence, a process alternatively referred to as NK licensing[381] or disarming[382]. The functional competence of NK cells can be assessed by intracellular flow cytometric detection of IFN-γ production by freshly isolated splenocytes following Ab mediated cross-linking of an activating receptor such as NK1.1, NKp46, or NKG2D[381]. Analysis of the frequency of IFN- γ producing NK cells following engagement by plate-bound anti-NK1.1, -NKG2D, or –NKp46 revealed that SHIP NK cells have defective IFN- γ production relative to WT controls, which is consistent with their previously reported cytolytic effector function defect[250, 258]. However, the absence of 2B4 expression in SHIP-deficient NK cells did not

restore IFN- γ induction by any NK-activating receptor tested, including NK1.1, NKp46, and NKG2D. Surprisingly, we find that 2B4 also plays a prominent role in induction of IFN- γ by NK activating receptors because $2B4^{-/-}$ NK cells exhibit significantly reduced induction after NK1.1 engagement (Fig. 5A, B) and supernormal induction following NKp46 engagement 5A,C). NKG2D induction of IFN-g is normal in 2B4-/- NK cells (Fig 5A,D). The supernormal induction of IFN-γ is likely due to the increased frequency of NKp46-expressing cells present in the $2B4^{-/-}$ NK compartment (see below). However, NK1.1 levels are normal in $2B4^{-/-}$ NK cells, implying that signals from this CD28 receptor facilitate induction of IFN-γ.



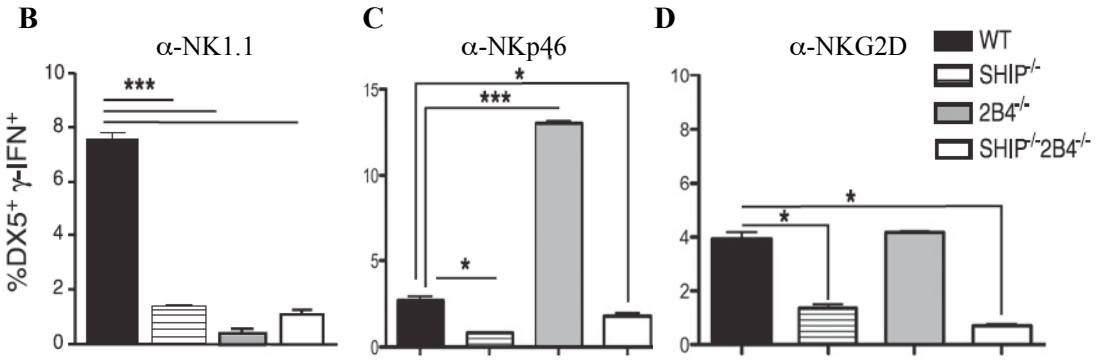


Figure 5. Defective IFN-γ production in NK cells that lack expression of SHIP, 2B4 or both. (A) Representative DX5 vs. γ-IFN contour plots (back gated on $DX5^{+}TCR\beta^{-}$) of splenocytes Representative NK1.1 vs. γ-IFN contour plots of NK cells exposed to wells coated with 0, 1, or 10μg of αντια-NK1.1 (PK136), α-NKp46, or α-NKG2D (clone PK136). Genotypes of the NK cells and the percentage of $\gamma\text{-IFN}^{+}$ NK cells are indicated. (B-D) Percentage of licensed $\gamma\text{-IFN}^{+}$ NK cells in each genotype analyzed based on γ-IFN production in response to NK activating receptor cross-linking of NK1.1. (B) NK1.1 (10μg) (C) 1μg anti-NKp46 (50μg) (D) 10μg anti-NKG2D (25μg). Results were considered significant when $p<0.05$ (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$). Statistical analyses was performed using Graphpad Prism Software and the Student's two-tailed paired t test.

The above results demonstrate that 2B4 deficiency restores normal homeostatic control to the peripheral NK compartment, but does not restore the ability of key NK activating receptors, including NKG2D, to induce IFN-γ production. To determine whether 2B4 could restore cytolytic function we then assessed the ability of NK cells from $2B4^{-/-}SHIP^{-/-}$ mice to lyse $RMA/Rae1^{+}$ targets as compared with NK cells from WT ($2B4^{+/+}SHIP^{+/+}$), $2B4^{-/-}$, and $SHIP^{-/-}$ mice. Cytolysis of $RMA/Rae1^{+}$ targets was measured in a standard 4 h ^{51}Cr release assay with different E:T ratios, using IL-2 activated NK cells (Fig 6). In fact, we find that 2B4 deficiency restores SHIP NK killing to WT levels at all E:T ratios tested. Thus, 2B4 expression compromised SHIP NK cytolysis of complex targets that express MHC-I with an NKG2D ligand. However, these results

indicate the functional competence of NK cells, as measured by IFN-γ production is not inextricably linked to cytolytic competency.

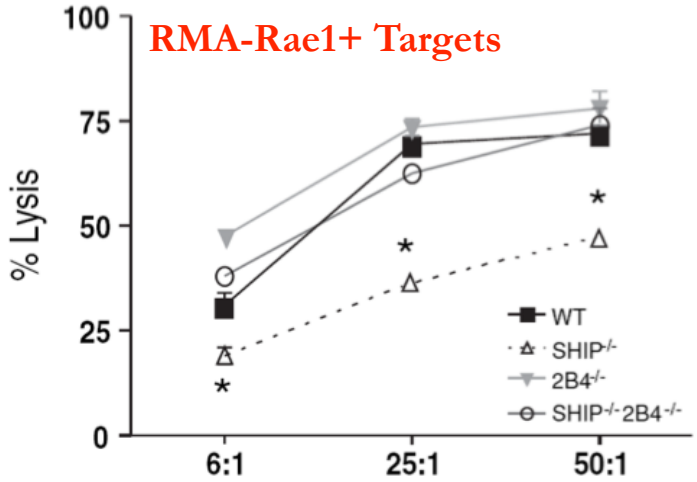


Figure 6. Inhibitory dominance by 2B4 in BL6 SHIP$^{-/-}$ NK cells. NK cells were magnetically enriched from splenocytes of mice of the indicated genotypes by AutoMACS depletion of B, T, and myeloid cells and then cultured in 2000U/mL of human recombinant IL-2 for 7 days. Cytolysis of RMA/Rae1^{+} transfectants was then assessed by a 4-h ^{51}Cr release assay at the indicated E:T ratios. %Specific lysis= 100 x X [(experimental release-spontaneous release) / (maximum release-spontaneous release)]. These studies are representative of three independent experiments. Killing for all three genotypes was significantly greater than SHIP$^{-/-}$ at all E:T ratios tested. Results were considered significant when $p<0.05$ (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$). Statistical analyses was performed using Graphpad Prism Software and the Student's two-tailed paired t test.

2B4 and SHIP are mutually required for a normal NK receptor repertoire.

SHIP deficient NK cells have a highly disrupted NKRR with under representation of many NK receptors and severe over expression of 2B4 (Fig 7B). We then asked whether 2B4-deficiency might also lead to a normal receptor repertoire in the peripheral NK compartment of SHIP-deficient mice. To determine how SHIP and 2B4 contribute to the acquisition of the NKRR we did flow cytometry on splenocytes from naïve mice, first

gating on NK1.1^{+}Lin^{-} cells and then analyzing the percent expression of the indicated receptors (Fig. 7). These data are depicted as bar graphs where the representation of each NK receptor in the compartment is normalized to that of WT controls analyzed in parallel with each of the three mutant genotypes (2B4$^{-/-}$SHIP$^{+/+}$, SHIP$^{-/-}$2B4$^{-/-}$, SHIP$^{-/-}$2B4$^{+/+}$) (Fig. 7A-C). With the exception of 2B4, all "% of normal" values refer to the frequency of NK cells in the indicated mutant after normalization to WT. The 2B4 "% of normal" values refer to mean fluorescence intensity (MFI) or surface density after normalization to WT controls.

Contrary to what was observed with cytolytic function, we find that the NK repertoire of 2B4$^{-/-}$SHIP$^{-/-}$ mice remains severely disrupted, with 12 of 14 NK receptors showing a significant alteration in either their representation or surface density (Fig. 7A). However, we also observe a significant degree of repertoire disruption in 2B4$^{-/-}$ mice with 9 out of 14 NK receptors significantly altered as compared to WT mice (Fig. 7C). Thus, expression of both 2B4 and SHIP is required for the normal development of the NK receptor repertoire. Because SHIP is recruited to 2B4 in NK cells[385] and can influence the role of 2B4 in cytolytic function[258, 371], it is possible that 2B4 and SHIP interact in a signaling pathway that also promotes NK repertoire formation. Thus, NK receptors whose expression is altered in a similar manner by 2B4, SHIP, or combined 2B4/SHIP deficiency are then potentially regulated by signaling pathways controlled by a 2B4:SHIP complex**.** However, certain NK receptors exhibit different patterns of disruption by 2B4- and SHIP-deficiency (Fig.7B,C). For example, 2B4 deficiency leads to increased representation of key activating receptors like NKp46 and DNAM-1 in the NK compartment, but only in the context of SHIP competency (Fig. 7A,C). On the contrary,

there are significantly fewer SHIP-deficient NK cells that express NKp46 and NKG2D, two key NK activating receptors that are supposedly expressed by virtually all NK cells (Fig 7B). Up-regulation of these two key activators could account for the improved killing of tumor targets by $2B4^{-/-}$ NK cells that we and others have observed[123]. Thus, 2B4 and SHIP have overlapping, and distinct effects on the NK receptor repertoire.

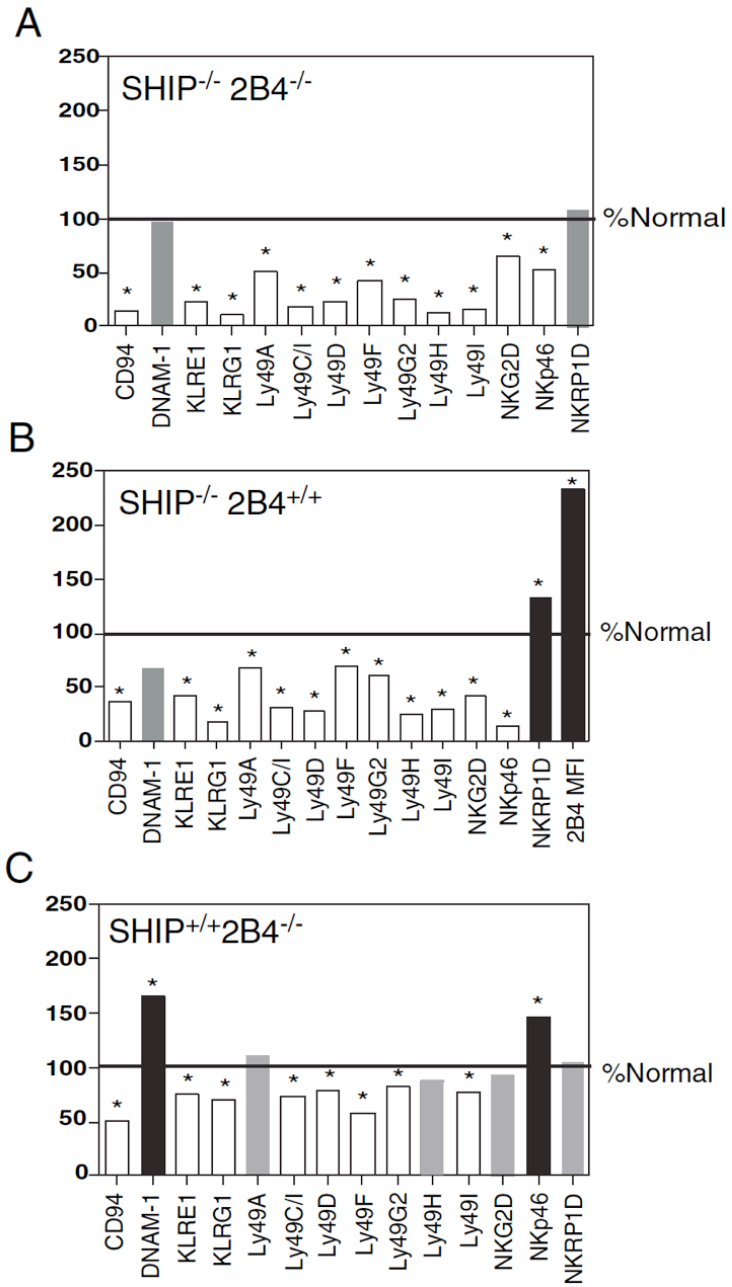


Figure 7. Normalized representation of each NK receptor (NKR) in the peripheral NK compartment (A) $2B4^{-/-}SHIP^{-/-}$, (B) $SHIP^{-/-}$ and (C) $2B4^{-/-}$ mice. All % and MFI for NK receptors were determined after gating on $NK1.1^{+}Lin^{-}$ splenocytes of 6-8 week old adult mice (Lin panel: IgM, CD3, TCR-β, Gr1, CD11c). In order to estimate the percentage of NKR^{+} cells in the NK compartment, positive NKR gates were set at ≥95% of $NK1.1^{+}Lin^{-}$ cells staining positive for an isotype control stain performed on an equal mixture of null and WT splenocytes. Representation of individual NKR in splenic $SHIP^{-/-}$, $SHIP^{-/-}2B4^{-/-}$, and $2B4^{-/-}$ NK cells are presented after normalization to WT. The % of normal was calculated as follows: = (%NKR^{+} $SHIP^{-/-}$genotype "X"/ %NKR^{+} $SHIP^{+/+}$) x 100 for each indicated NKR. Where "X" represents the genotypes indicated (e.g. SHIP, 2B4 or SHIP 2B4 double knockout). For the 2B4 receptor, % normal was calculated in the same manner except that MFI was used rather than %NKR^{+}. White, black and grey bar graphs represent % normal values that are significantly lower, higher, and/or unchanged in the $SHIP^{-/-}$, $SHIP^{-/-}2B4^{-/-}$, and $2B4^{-/-}$ NK compartment as compared to WT, respectively. Results were considered significant when $p<0.05$ (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$). Statistical analyses was performed using Graphpad Prism Software and the Student's two-tailed paired t test.

SHIP and 2B4 are required to prevent lineage inappropriate expression of Ly49B by NK cells. In addition to the NK-associated receptors analyzed above, we also examined the role of SHIP and 2B4 on Ly49B expression. Ly49B and Ly49Q are the sole members of the Ly49 gene family that are not expressed by NK cells, but instead are restricted to myeloid lineage cells[262]. Like the Ly49A and C receptors we have previously found to be regulated by SHIP, Ly49B is also a promiscuous MHC-I receptor that does not exhibit precise specificity for a given MHC haplotype[385]. SHIP is also recruited to Ly49B[262] making it a strong candidate for a *SHIP-regulated* receptor. Analysis of Ly49B expression revealed that it is expressed on a significant proportion of $SHIP^{-/-}$ NK cells in either an $H\text{-}2^{b}$ (C57BL/6) background (Fig. 8A) or an $H\text{-}2^{d}$ (B10.D2) background (Fig. 8B) in both the spleen and BM, while virtually no Ly49B expression was detected on WT NK cells from both haplotypes, consistent with the findings of Gays *et al*[262]. We performed Ly49B blocking studies using hybridoma supernatants or purified antibody

against Ly49B^{262} in H-2^{d} SHIP$^{-/-}$ and WT in ^{51}Cr release assays. Blocking of Ly49B did not significantly increase or decrease cytolysis by SHIP$^{-/-}$ LAK cells (data not shown). These in vitro assays indicate that deregulated Ly49B expression may not influence SHIP$^{-/-}$ NK effector functions. Whether there are functional consequences for NK cells in vivo that arise owing to lineage-inappropriate expression of Ly49B will require the development of Ly49B$^{-/-}$ mice and thus remain to be determined.

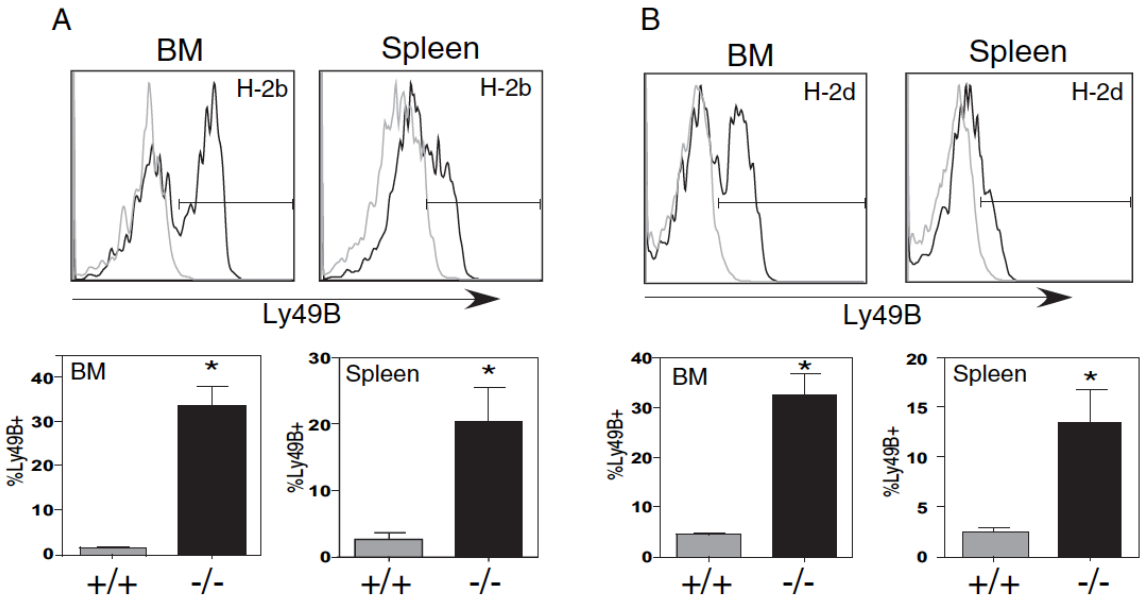


Figure 8. SHIP is required to limit the expression of Ly49B in the NK compartment *of H-2b and H-2d mice.* Spleen and bone marrow of (A) H-2^{b} mice and (B) H-2^{d} mice. Gates for Ly49B^{+} in SHIP$^{-/-}$ (black histogram) or SHIP$^{+/+}$ (gray histogram), were based on gating beginning at the 95th percentile of an rIgG1 isotype control. Statistical analysis of the frequency of Ly49B expression in H-2^{b} and H-2^{d} SHIP$^{-/-}$ vs. SHIP$^{+/+}$ mice is shown by bar graphs under the respective histogram. Results were considered significant when $p<0.05$ ($*p<0.05$) as determined by the student's t test using Graphpad Prism software for analysis.

2B4$^{-/-}$ NK cells from the bone marrow and spleen also express Ly49B at significant levels (Fig. 9A). This finding suggests a direct role for 2B4 in lineage-specific regulation of Ly49 receptors. Not surprisingly, BM and spleen NK cells from SHIP$^{-/-}$2B4^{-}

$^{/-}$ NK cells also express high levels of Ly49B (Fig. 9B). Whether there are *in vivo* functional consequences for NK cells due to aberrant expression of Ly49B is not known at this time.

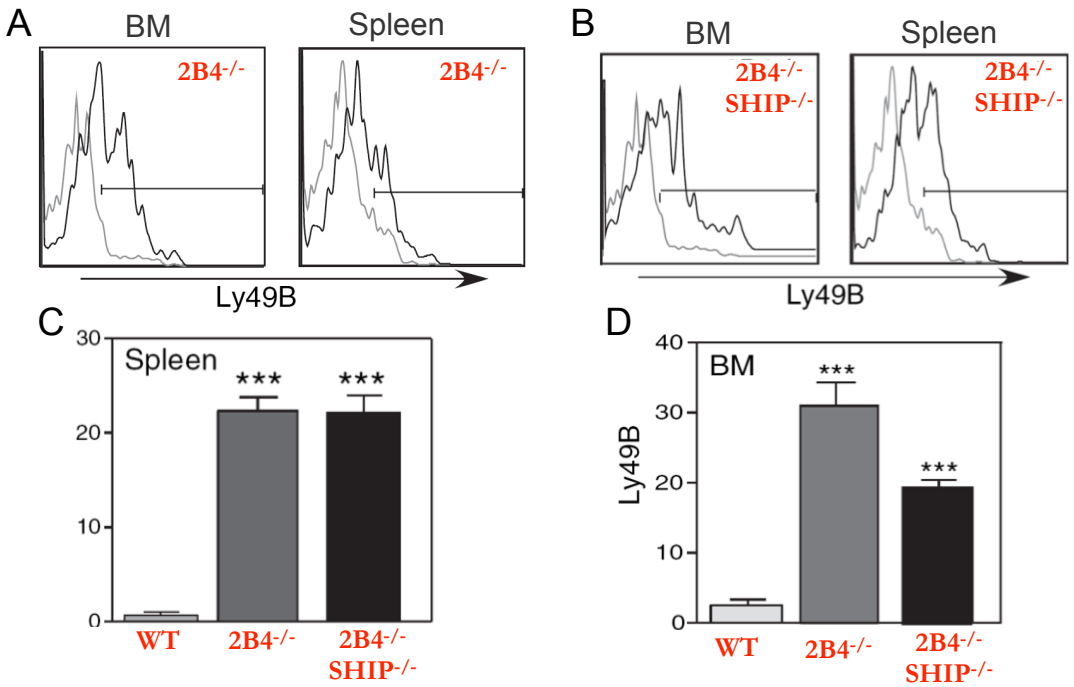


Figure 9. 2B4 is also required to prevent lineage inappropriate expression of Ly49B. Spleen and bone marrow of (A) 2B4$^{-/-}$ and (B) 2B4$^{-/-}$SHIP$^{-/-}$ mice. Black histograms are Ly49B staining while grey histograms are isotype control stains. Statistical analysis of the frequency of Ly49B expression in the (C) bone marrow and (D) Spleen in the following three genotypes: 2B4$^{+/+}$SHIP$^{+/+}$(WT), 2B4$^{-/-}$SHIP$^{-/-}$ and 2B4$^{-/-}$ SHIP$^{+/+}$ ***p<0.0001 represents significance of Ly49B in the indicated mutants versus WT NK cells.

SHIP$^{-/-}$ H-2^{d} NK cells are cytolytically competent despite a disrupted repertoire and overexpression of dominant inhibitory receptors. As with 2B4, we find that the expression or representation of most Ly49 receptors is abnormal in the peripheral NK compartment of H-2^{b} SHIP$^{-/-}$ mice (Fig. 7). To determine whether variation in MHC haplotype might alter how the repertoire is disrupted in SHIP$^{-/-}$ NK

cells, we assessed the NK repertoire perturbation in SHIP$^{-/-}$ mice with an H-2^{d} haplotype known to have a unique effect on the representation of certain Ly49 receptors. For example, surface expression of Ly49A is downmodulated in the presence of high-affinity MHC-I ligands in the H-2^{d} locus[378]. Analysis of the peripheral NK repertoire in SHIP$^{-/-}$ mice with an H-2^{d} haplotype (Fig. 10A) indicated that the repertoire perturbation in these mice is essentially identical to that of SHIP$^{-/-}$ H-2^{b} mice, with the exception of Ly49A, NKp46, and DNAM-1. We find that Ly49A is significantly overexpressed in H-2^{d} SHIP$^{-/-}$ NK cells relative to NK cells of WT littermates (Fig. 10A, B). Consistent with the MHC independence of its ligand, the surface density of 2B4 is abnormally high in the peripheral NK compartment of SHIP$^{-/-}$ H-2^{d} mice, similar to what we observed on an H-2^{b} background. The frequency of NKp46^{+} NK cells is not significantly reduced as it is in H2^{b} SHIP$^{-/-}$ NK cells (Fig. 10A), whereas DNAM-1 is highly overexpressed in H-2^{d} SHIP$^{-/-}$ NK cells as compared with H-2^{d} WT controls (Fig 10A).

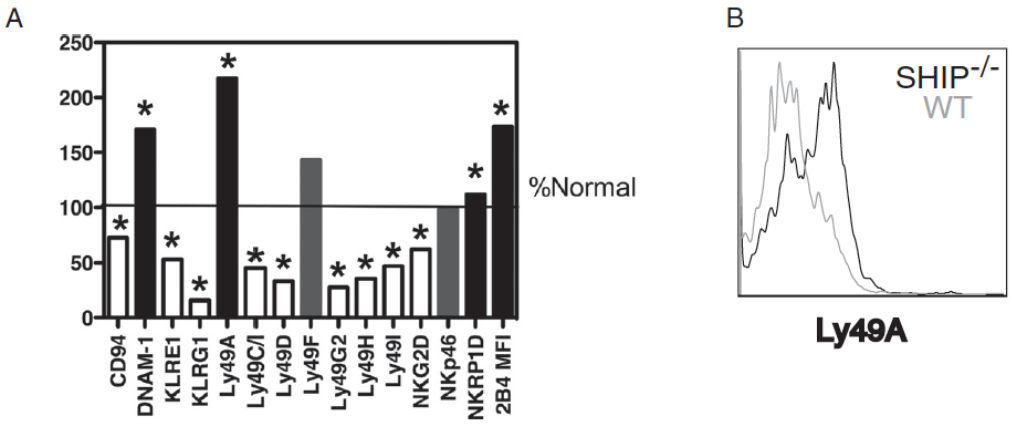


Figure 10. H-2^{d} NK receptor repertoire and Ly49A overexpression. (A) Normalized representation of the NKRR in SHIP$^{-/-}$ splenic H-2^{d} NK cells (n=6 mice for each genotype). Representation of individual NKR in splenic SHIP$^{-/-}$ NK cells are presented after normalization to WT. The % of normal = (%NKR^{+} SHIP$^{-/-}$ / %NKR^{+} SHIP$^{+/+}$) x 100 for each indicated NKR. For Ly49A and 2B4 receptors, % normal was calculated in the same manner except that MFI was used rather than %NKR^{+}. White, black and grey bar graphs represent % normal values that are significantly lower, higher, and/or unchanged

in the SHIP$^{-/-}$ NK compartment as compared to WT, respectively. *$p<0.05$ as determined using the student's t-test. (B) Representative Ly49A staining on NK1.1^{+}CD3^{-} splenic NK cells on WT and SHIP$^{-/-}$ H-2^{d} backgrounds.

As shown above (Fig. 10), Ly49A is downregulated in the presence of H-2^{d}. According to a newly proposed quantitative regulation model of NK education[383], high expression of Ly49A by NK cells in an MHC-I background with a strong educating impact should reduce the threshold required for NK cell activation. To assess whether inappropriate expression of 2B4 or Ly49A might alter cytolytic function in H-2^{d} SHIP$^{-/-}$ NK cells, we compared the ability of SHIP$^{-/-}$ and WT H-2^{d} NK cells to kill H-2^{d} (self) and H-2^{b} (missing self/non-self) tumor targets. We find that SHIP$^{-/-}$ H-2^{d} NK cells have normal cytolytic activity against two different H-2^{d} MHC-I matched tumor targets, BCL1 and A20 (Fig. 11A, B). We find that overexpression of 2B4 does not compromise H-2^{d} SHIP$^{-/-}$ NK cytolytic function as it does on an H-2^{b} background. Thus, Ly49A overexpression by SHIP$^{-/-}$ H-2^{d} NK cells does not function as a dominant inhibitory receptor and impair cytolysis. In fact, we find that H-2^{d} SHIP$^{-/-}$ NK cells exhibit supernormal cytolytic activity against MHC mismatched H-2^{b} tumor targets, RMA cells (Fig. 11C). However, this finding is not the case for SHIP$^{-/-}$ NK cells with an H-2^{b} haplotype, because these cells exhibit impaired cytolytic function against the MHC-I mismatched targets, A20 and BCL-1 (Fig 12). The increased expression of the DNAM-1 activating receptor in SHIP$^{-/-}$ H-2^{d} NK cells could potentially account for their normal or enhanced cytolytic function against MHC-matched and mismatched targets, respectively. Thus, MHC haplotype can modulate the effect of SHIP on the NK repertoire and its impact on effector function, such that SHIP-deficiency in certain MHC-I mismatched contexts can promote supernormal cytolytic activity by NK cells.

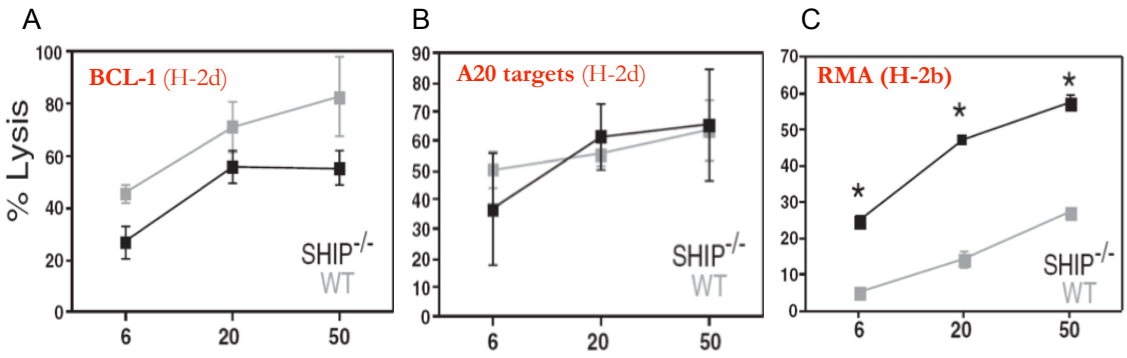


Figure 11. 2B4 is not a dominant inhibitory receptor in H-2^{d} SHIP$^{-/-}$ NK cells where a strong licensing receptor is overexpressed. H-2^{d} SHIP$^{-/-}$ and WT NK cytolysis of (A) BCL1 (H-2^{d}) lymphoma targets, (B) A20 (H-2^{d}) lymphoma targets and (C) RMA (H-2^{b}) lymphoma targets at various E:T ratios, 6:1; 20:1; 50:1. Cytolysis was analyzed in a standard 4-hr ^{51}Cr release assay. Each analysis is representative of at least 2-3 independent experiments. *p<0.05 as determined by the student's t test. Statistical analyses was performed using Graphpad Prism Software

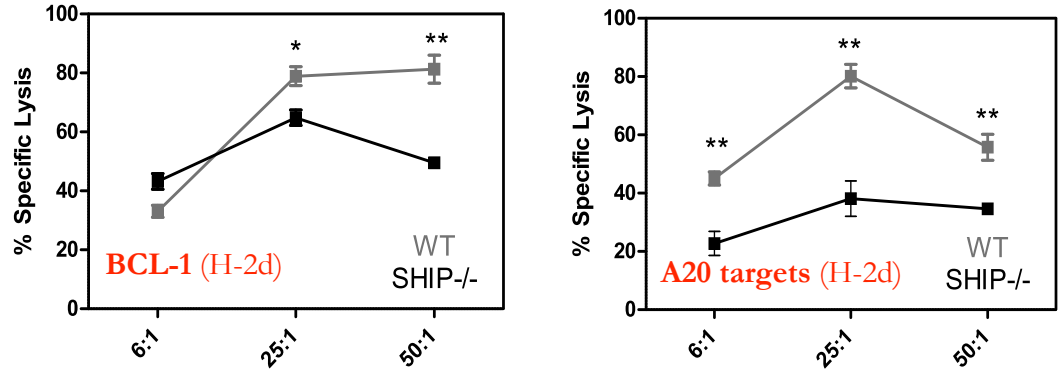


Figure 12. H-2^{b} SHIP-deficient NK cells have defective killing against allogeneic targets. H-2^{b} SHIP$^{-/-}$ and WT NK cytolysis of BCL1 (H-2^{d}) lymphoma targets and A20 (H-2^{d}) lymphoma targets at various E:T ratios, 6:1; 25:1; 50:1. Cytolysis was analyzed in a standard 4-hr ^{51}Cr release assay. Each analysis is representative of at least 2-3 independent experiments. *p<0.05 and **p<0.005 as determined by Student's t test.

NK cells from H-2^{d} mice have impaired IFN-γ induction. Our analysis of 2B4$^{-/-}$SHIP$^{-/-}$ NK cells indicated discordance in cytolytic function and IFN-γ induction. We then wanted to determine whether the normal or supernormal cytolytic capacity of H-2^{d}

SHIP$^{-/-}$ NK cells could also result in the restoration of normal or above-normal IFN-γ induction from key NK activating receptors. To investigate this, we primed the mice with polyinosinic-polycytidylic acid (day -1) and harvested the spleens on day 0. To induce IFN-γ production, we cross-linked NK1.1 (Fig.13 and 14B) or NKG2D (Fig. 13 and 14C) using plate-bound mAbs. We measured the production of IFN-γ by intracellular flow, and we show here that SHIP-deficient NK cells from H-2^{d} mice have a markedly impaired ability to induce IFN-γ from both NK activating receptors (Fig.14). This finding is consistent with the discordance of cytolytic function and IFN-γ induction in 2B4$^{-/-}$ SHIP$^{-/-}$ NK cells, and it indicates that although in certain genetic contexts (2B4 deficiency, H-2^{d} haplotype) SHIP-deficient NK cells can have normal or supernormal cytolytic function, they nonetheless remain poor producers of IFN-γ in response to engagement of major NK activating receptors. We can conclude that SHIP expression is a uniform and essential requirement for this NK effector function.

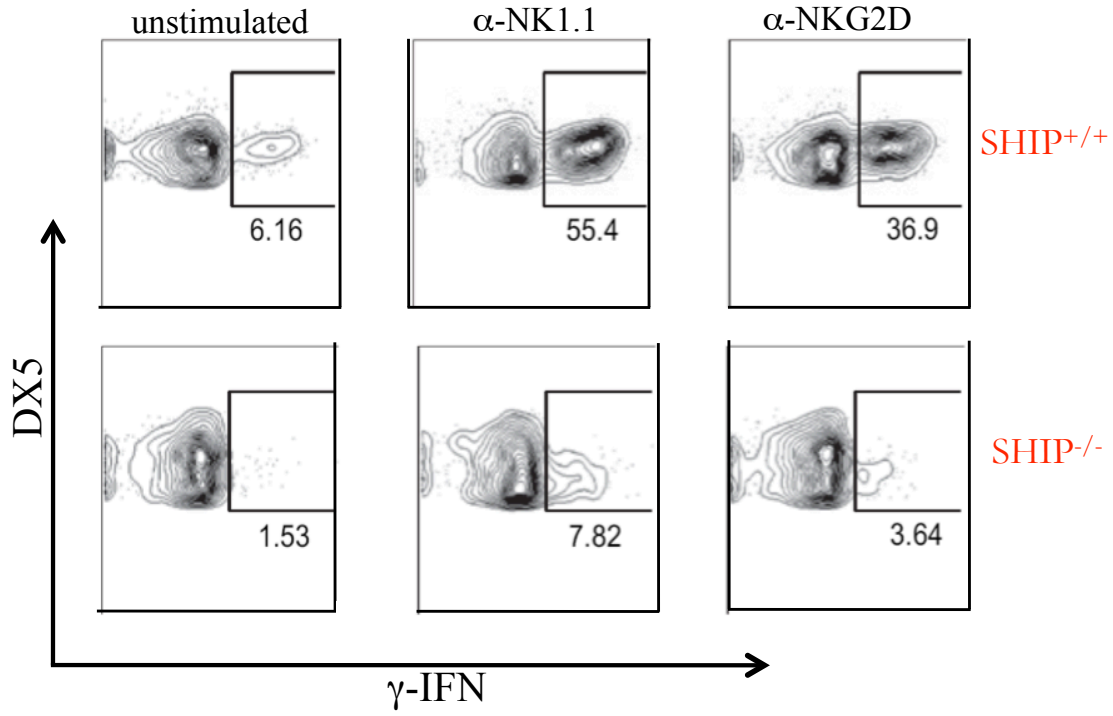


Figure 13. H-2^{d} NK cells exhibit impaired ability to produce γ-IFN. Representative DX5 vs. γ-IFN contour plots (back gated on DX5^{+}TCRβ^{-}) of whole spleen cells either unstimulated (PBS) or stimulated with 50μg of plate-bound α-NK1.1 (clone PK136) or 50μg of plate-bound α-NKG2D (A10).

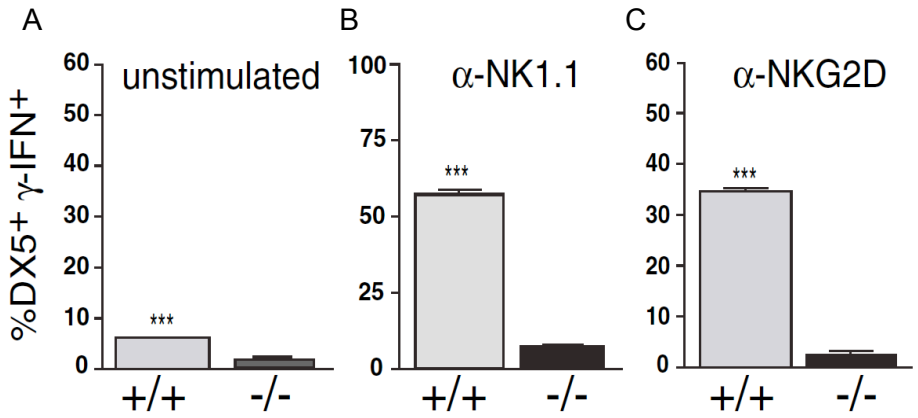


Figure 14. Statistical analysis of *licensed* NK cells from SHIP$^{+/+}$ and SHIP$^{-/-}$ mice with an H-2^{d} haplotype. Graphs represent data from Fig 13. (A) unstimulated (B) NK1.1 and (C) NKG2D ***P<0.0001 compared to WT control. Representative of at least 3 independent experiments.

Discussion

In this study, we provide genetic evidence that the interaction of SHIP with both 2B4 and MHC-I loci is required for the acquisition of a normal repertoire of inhibitory and activating receptors, normal cytolytic function, and induction of IFN-γ production by key NK activating receptors. Consistent with our previous study showing that blockade of CD48 on complex MHC-I+Rae1+ targets restored normal cytolysis to SHIP-deficient NK cells[258], the cytolytic defect of SHIP-deficient NK cells is corrected by 2B4 deficiency. Cytolytic competence against Rae1+ target cells by $2B4^{-/-}$ $SHIP^{-/-}$ and $2B4^{-/-}$ NK cells occurs despite a highly disrupted NKRR. However, $SHIP^{-/-}2B4^{-/-}$ and H-2d $SHIP^{-/-}$ NK cells exhibit defective induction of IFN-γ, although they exhibit normal or supernormal cytolytic capacity. This defect in IFN-γ induction can result from decreased expression of NKp46 and NKG2D in $SHIP^{-/-}$ NK cells; however, NK1.1 receptor expression levels are normal or elevated on $SHIP^{-/-}$ NK cells[250], indicating that SHIP actually is required downstream of certain NK activating receptors for efficient induction of IFN-γ. Surprisingly, we find that 2B4 restrains induction of IFN-γ in response to engagement of the NK activating receptor NKp46, but not NK1.1. These findings demonstrate that IFN-γ induction and cytolytic competence are regulated by distinct mechanisms in NK cells and that both SHIP and 2B4 play a prominent role in IFN-γ production by NK cells by modulating activating receptor expression and/or signaling pathways downstream of these receptors.

The finding that 2B4 limits the expression of key activating receptors, NKp46 and DNAM-1, is attractive and may explain why we and Vaidya *et al*[123] observed enhanced

killing by 2B4$^{-/-}$ NK cells. These data suggest that 2B4 may be necessary to limit the expression of these activating receptors. In addition, we find that SHIP and 2B4 are required to prevent the lineage-inappropriate expression of Ly49B, the polyspecific MHC-I receptor that is normally restricted to myeloid cells[262]. This finding establishes a previously unappreciated role for SHIP and 2B4 in maintaining restricted expression of immune receptors. The in vivo functional consequences of inappropriate Ly49B expression in the NK cell compartment of SHIP$^{-/-}$, SHIP$^{-/-}$2B4$^{-/-}$, and 2B4$^{-/-}$ mice remains unknown, but certainly merits further investigation.

We also find that the effects of SHIP on repertoire formation and cytolytic function is influenced by the composition of MHC-I ligands, because a potent educating or licensing receptor, Ly49A, is overexpressed by SHIP-deficient NK cells in the presence of its high-affinity H-2^{d} ligand. Surprisingly, cytolytic function is not found to be defective in SHIP$^{-/-}$ H-2^{d} NK cells, suggesting that the increased educating or licensing capacity of Ly49A in H-2^{d} SHIP$^{-/-}$ NK cells could counteract inhibitory signals resulting from overexpression of 2B4; however, H-2^{d} NK cells have defective IFN-γ induction. The normal or supernormal cytolytic activity we observe with H-2^{d} SHIP$^{-/-}$ NK cells could also be due to the normal levels of NKp46 expression or increased expression of DNAM-1, respectively, that we observe in H-2^{d} SHIP$^{-/-}$ NK cells. Based on our findings, we suggest that the IFN-γ induction assay may not always be a valid surrogate for NK cytolytic competence, particularly for analysis of signal transduction mutations that can affect NK function. Others have also observed a similar discordance of IFN-γ induction and cytolysis in Bcl10$^{-/-}$ NK cells[386].

The interaction of SHIP and 2B4 influences signaling pathways that determine the

cytolytic function of mature NK cells in both humans and mice[371, 385]. Our findings suggest that 2B4:SHIP signaling could also play a role in early NK development to promote the efficient acquisition of NK receptors that sense MHC-I ligands. In fact, expression of 2B4[116, 387] and SHIP precedes expression of the Ly49 and CD94/NKG2 receptors in NK development. The early expression of 2B4 in developing NK cells may be necessary to achieve self-tolerance until a properly diverse Ly49 and CD94/NKG2 repertoire is acquired. Consistent with this hypothesis, McNerney *et al.*[388] found that 2B4 promotes self tolerance by mature murine NK cells in rodents, and Sivori *et al.*[45] showed that 2B4 inhibitory signals limits cytolysis by NK cells that have yet to acquire KIR expression. This putative function of 2B4 in developing NK cells could be particularly important in preventing inappropriate NK cytolysis in the BM compartment, because immature NK cells have been shown to acquire cytolytic activity prior to acquisition of Ly49 receptors and KIR receptors[45, 387]. Consistent with this hypothesis, the ligand for 2B4, CD48, is ubiquitously expressed in the developing hematopoietic system providing nearly constant interaction of developing NK cells with a tolerizing signal in the form of CD48. The exception to this is the primitive subset of hematopoietic stem cells (HSCs) that lack CD48 expression[389]. Presumably developing NK cells do not co-occupy the endosteal niche where primitive HSC reside, although this merits direct analysis.

A role for 2B4 in maintaining self-tolerance in early NK cells and a lack of CD48 expression by HSCs would enable the NK lineage to modulate hematopoiesis by lysis of $CD48^-$ HSCs. Because NK cells are the only cytolytic lymphocyte that develops in the BM, this role for 2B4 would provide a means for NK cells to mediate negative feedback on HSCs and thus lymphoid output. The plausibility of such a mechanism is further

suggested by evidence that syngeneic HSC function can be limited by NK cells in vivo[390]. Thus, a 2B4: SHIP complex could potentially play a role in such a lymphoid feedback pathway. Consistent with this hypothesis, CD48- HSCs inappropriately accumulate in the BM of SHIP$^{-/-}$ mice[248]. An additional role for the 2B4:SHIP complex in NK tolerance toward APCs in secondary lymphoid tissues is also a distinct possibility. Evidence for this role includes the inappropriate expansion of the dendritic cell compartment in the lymph nodes (LNs) of SHIP$^{-/-}$ mice[249], increased expression of SHIP in a subset of LN NK cells that lack KIR, but express 2B4[263], and that 2B4 has inhibitory function in human LN NK cells[45].

How 2B4 signals promote NK tolerance toward CD48+ targets in the absence of MHC-I inhibitory receptors, and trigger the acquisition of a full repertoire of MHC-I receptors, remains to be defined. 2B4 can directly or indirectly recruit a wide variety of signaling molecules via its four immunoreceptor tyrosine-based switch motifs. To date, the following signaling components have been shown to be recruited to 2B4: PLC-g, LAT, Grb2, SAP, Fyn, EAT2, PI3K, SHP1, SHP2, and SHIP[129, 130, 371, 391]. Thus, signaling complexes at 2B4 have the biochemical capacity to impact a wide variety of distal signaling pathways that can control NK cell survival, proliferation, and/or gene expression. Because 2B4 is recruited to the NK synapse and SHIP can attenuate PI3K activity from other receptors in *trans*[392], a 2B4:SHIP complex also has the potential to limit PI3K activity originating at other NK receptors. In fact, immature KIR^{-} NK cells can kill autologous cells, including APCs, via PI3K-mediated pathways downstream of NKp46 and NKp30[45, 393], and 2B4 limits this activity. Thus, *trans* activity of SHIP at 2B4 to oppose PI3K activity at other receptors could be an essential feature of NK tolerance

signaling. The expansion of HSCs in BM and APCs in the LNs of SHIP$^{-/-}$ mice is consistent with this possibility[249].

The NKRR is regulated by elements intrinsic and extrinsic to the NK lineage. Several studies have shown that external signals received by NK cells from self-ligands, such as MHC-I, can influence the repertoire through differential effects on NK subset survival and/or proliferation[394, 395]. In addition, NK intrinsic signaling pathways influence NKR expression, including 2B4, via cis-acting sequences present in receptor promoters[373, 374, 396, 397] and by activation of transcription factors that bind to these sites[398, 399]. The challenge for biologists studying NK cells is to better understand how integration of these extrinsic and intrinsic pathways determines the final composition of the NKRR. Co-expression of 2B4 and SHIP prior to MHC-I receptor expression suggests that 2B4:SHIP complexes are uniquely positioned to play such a role. For example, the *trans* activity of SHIP from 2B4 could oppose PI-3K activity at MHC-I receptors and thus limit the survival or proliferation of NK subsets expressing these MHC-I receptors. Consistent with this possibility, PI-3K can be recruited to MHC-I inhibitory receptors to activate AKT. SHIP acting in *trans* from 2B4 or in *cis* from the same MHC-I receptors could limit PI-3K/AKT survival signals, and thereby prevent inappropriate expansion of such NK subsets. SHIP can also be recruited directly to MHC-I receptors; therefore, SHIP may also limit these subsets in *cis* [262, 310, 385]. This *cis* activity may be important when MHC-I receptors are in a genetic background where high-affinity MHC-I ligands are also present, as suggested by the overexpression of Ly49A in SHIP$^{-/-}$ H-2^{d} mice.

In addition to limiting expansion of specific subsets of NK cells, it is also possible that 2B4:SHIP complexes influence intrinsic pathways that determine NK receptor expression

and effector function. For example, activation of transcription factors known to act on promoters for NK receptors and/or 2B4 (e.g., Ets, NF-κB, CREB) is influenced by 2B4 engagement[397] and signaling molecules recruited to 2B4 (e.g., Fyn, SHIP, PI3K)[400]. Consistent with this hypothesis, 2B4 expression is deregulated in $SHIP^{-/-}$ NK cells[258], whereas $Fyn^{-/-}$, $SHIP^{-/-}$, and $PI3K^{-/-}$ mutants all exhibit profound disruptions of their MHC-I NK receptor repertoires[375, 376]. The receptor expression changes created by SHIP or 2B4 deficiency are likely to cause some of the alterations in effector function that we observe in $SHIP^{-/-}$ and $2B4^{-/-}$ NK cells.

However, independent of these receptor expression changes, 2B4: SHIP complexes also appear to influence signaling pathways that promote NK effector functions. For example, $SHIP^{-/-}$ NK cells are unable to trigger IFN-γ induction in response to NK1.1 engagement despite normal or increased surface density of NK1.1. This finding suggests a role for SHIP in promoting IFN-γ expression via generation of its product PI(3,4)P2, which along with PI(3,4,5)P3 is a critical second messenger for the PI3K pathway. This is consistent with a recent report showing that SHIP promotes rather than inhibits, macrophage effector function via generation of PI(3,4)P2[401]. Thus, the uniform defect in IFN-γ induction that we observed for SHIP-deficient NK cells, whether 2B4-deficient or of different MHC haplotypes, demonstrates an absolute requirement for SHIP in the induction of IFN-γ production by major NK activating receptors. These findings reveal a pivotal role for the interaction of SHIP and 2B4 in the regulation of the NKRR, cytolytic function, and IFN-γ production.

Materials and Methods

Mice. All H-2^{b} repertoire analyses described herein are derived from analysis of SHIP$^{+/+}$ and SHIP$^{-/-}$ mice derived from intercrosses of SHIP$^{+/-}$ mice F10XC57BL6/J mice. SHIP$^{-/-}$2B4$^{-/-}$ were generated by intercrossing C57BL/6 2B4$^{-/-}$ mice with our SHIP$^{+/-}$ mice (2B4$^{-/-}$ were kindly provided by J.D. Schatzle). The 2B4$^{-/-}$SHIP$^{-/-}$ genotype of the offspring from these matings was confirmed by flow cytometry of viable cells and PCR analysis of genomic DNA. SHIP$^{-/-}$ H-2^{d} mice were generated by crossing SHIP$^{+/-}$ mice to the B10.D2 (H-2^{d}) strain. The progeny of these initial crosses were then backcrossed once more to the H-2^{d} congenic strain to obtain SHIP$^{+/-}$ males and females homozygous for the H-2^{d} haplotypes. H-2^{d} homozygous SHIP$^{-/-}$ males and females were identified and their SHIP$^{+/-}$ progeny intercrossed to generate WT and SHIP$^{-/-}$ progeny for NK repertoire studies on the H-2^{d} haplotypes. All NK repertoire analyses were performed with mice between 6 to 9 weeks of age. All studies were performed in accordance with the guidelines and approval of the Institutional Animal Certification and Use Committee (IACUC) at the University of South Florida.

Flow cytometry. Anti-CD16/32 was co-incubated with the samples to block Fc receptor binding. Antibodies used for staining included: NK1.1(PK136) (mIgG2a); CD3ε and TCRβ; Ly49A(A1) and Ly49C/I(5E6) (mIgG2a,κ); Ly49F(HBF-719) and Ly49I(YLI-90) (mIgG1,κ); Ly49G2(4D11) and CD94(18d3) (rIgG2a,κ) were obtained from BD Pharmingen (San Jose, CA). 2B4(244F4) (rIgG2a,κ); Ly49H(3D10) (mIgG1); Ly49D(4E5) (rIgG2a,κ); C7 (hIgG1) were purchased from eBioscience (San Diego, CA). The anti-Anti-KLRE1(7E8)[402] (rIgG1); -NKRP1D(2D12) (mIgG2a), Ly49B(2G4A1)[262] (rIgG1), and -KLRG1[236] antibodies were previously described and are conjugated to

biotin and revealed with SA-APC as described here. Ly49B(2G4) (rIgG1) was produced in the laboratory of by Colin Brooks. KLRG1. Samples were acquired on a FACS Calibur and analyzed using FlowJo8. Dead cells were excluded from the analysis following cytometer acquisition of staining data based on exclusion of the 7AAD dye.

Cytotoxicity assays. Cytolysis of RMA, RMA-Rae1^{+}, A20, and Bcl-CL1 targets was measured in a standard 4-h ^{51}chromium release assay. Briefly, on day 7 of NK culture, target cells were loaded with 100 μCi of ^{51}Cr per10^{6} cells for 60 min at 37°C. The target and NK (effector) cells were then incubated together in a sterile U bottom 96 well plate at 37°C for 4-5 h. The total volume in each well was 200μL. After the 4-5 h incubation, 100μL of supernatant was collected and measured for radioactivity on a gamma counter (Wizard 1470; PerkinElmer). %Specific lysis= 100 x X [(experimental release-spontaneous release) / (maximum release-spontaneous release)]. Statistics were calculated with Prism software using the Student's t test.

Cytokine assay. To stimulate NK cells, 4-6 million splenocytes from naïve mice were incubated with antibody-coated 6- well plates for 5-6 hours at 37^{o}C in the presence of GolgiPlug (BD Biosciences). Plates were coated with anti-NK1.1 (PK136), or anti-NKG2D (A10), or anti-NKp46/NCR1 for 2 hours at 37^{o}C or overnight at 4C. Spleens were harvested on day 0 and put into single cell suspension by passing through a 70μM cell strainer. Red blood cells were lysed with ACK buffer for 5 minutes at room temperature. Cells were washed with cold PBS and resuspended in RPMI 1640 supplemented with 10% FBS, 1% Penicillin-Streptomycin, 1% L-glutamine, 1% sodium

pyruvate, and 1% non-essential amino acids. After the 5-6 hour incubation, the cells were harvested, Fc receptor blocked, and stained for DX5, TCRβ and γ-IFN, with the latter stain performed following cell permeabilization and fixation. For H-2^d licensing assays only, mice were injected intraperitoneally with 70μg of Poly I:C on day –1 and spleens were harvested on day 0.

CHAPTER 3: Lenalidomide Combined with IL-2 or IL-15 Results in Robust Activation of STAT5 and Subsequent Increase in Cytolytic Mediators and Natural Cytotoxicity

Introduction

IMiDs, or immunomodulatory drugs, are a series of compounds developed by using the thalidomide backbone as the lead compound[288]. Lenalidomide (Len) is a second generation IMiD and is currently approved to treat Myelodsyplastic Syndromes (MDS) and Multiple Myeloma (MM)[318, 403, 404]. There are also several (>400) ongoing clinical trials investigating the use of Len to treat hematological malignancies as well as solid tumors, autoimmune diseases, and inflammatory conditions (clinicaltrials.gov search "lenalidomide"). There are many proposed mechanisms of action, although the target(s) of Len, and its parent compound thalidomide, remain unknown. The side effects with Len are not as severe as with thalidomide, but Len is still associated with negative side effects, such as thrombosis, pulmonary embolus, and hepatoxicity. Additionally, there is bone marrow toxicity resulting in severe neutropenia and thrombocytopenia, with myelosuppression being the major dose-limiting toxicity of this drug[319, 405].

The effects of Len vary greatly depending on the cell type being studied and the stimulus being received. It is quite effective at treating transfusion-dependent anemia in low-risk myelodysplastic syndrome (MDS), for which it has been shown to target the abnormal (5q) progenitors. Our group has demonstrated that haploinsufficiency of serine/threonine phosphatases PP2A and Cdc25c sensitize del(5q) MDS clones to Len. In fact, silencing these proteins in non-del(5q) MDS progenitor cells using siRNA imparted

a similar sensitivity to Len and subsequent arrest and apoptosis[324]. These findings suggest that Cdc25c and PP2A play an important role in the mechanism of lenalidomide in del(5q) MDS. There are also many other studies implicating genes located in the commonly deleted region (CDR) of chromosome 5 in the pathogenesis of MDS and treatment using Len[322, 325, 406]. However, these findings cannot explain the immunomodulatory and immune enhancing effects seen with Len in other cell types and pathological conditions.

Natural Killer (NK) cells are a major component of the innate immune system. As their name implies, they are effective killers of malignant, virally infected, and stressed cells. Killing by NK cells is carried out by the introduction of cytolytic proteins, such as perforin and granzymes into the target cell. Perforin inserts itself into the target cell, forming a pore in the membrane thereby directly killing the cell[170]. Perforin is also involved with the transport of granzymes into the cell. Granzymes, which are serine proteases, are released into the target cell thereby inducing apoptosis by caspase cleavage and activation[172]. In addition to cytolytic functions, NK cells are potent producers of several cytokines and chemokines, which position them as regulators of both the innate and adaptive arms of the immune system[216]. NK cell function is regulated by a dual receptor system. That is, they possess inhibitory and activating receptors, where the inhibitory receptor sets the threshold for whether an NK cell will kill, produce cytokines, both, or neither. The expression of NK receptors directly affects the function of an NK cell[41].

Len can upregulate CD16 and enhance Natural Killer (NK) cell Antibody Dependent Cell cytotoxicity (ADCC) in vitro[311, 349-352]. In addition to ADCC, NK cells

can be activated to kill without the requirement of antibody production by B cells. This function is termed 'natural cytotoxicity' and is mediated by direct binding of other NK activating receptors (e.g. NKG2D, 2B4, KIRs) with their target ligands. NK cells are important players against cancer, and if manipulated properly they can be harnessed to eliminate hematological as well as solid tumors[407-410]. Much of the regulation of NK cell cytotoxicity also applies to cytotoxic T lymphocytes (CTL)[411], and thus we propose that understanding the molecular mechanisms of Len in NK cells will undoubtedly provide some insight into how this drug impacts CTLs. The precise molecular mechanisms governing Len's effects on CTLs also remain undiscovered.

NK cells require several cytokines for their development, activation, and survival[412, 413]. Specifically, members of the IL-2 family of cytokines are extremely imperative, and in certain cases, critical for the development of functional NK cells. IL-2 and IL-15 are prime examples. These cytokines belong to the IL-2 family of cytokines and signal through a common receptor system where they share the IL-2Rβ and IL-Rγ chains. The common gamma chain (γ_c), is shared by all members in the IL-2 family of cytokines[414]. Upon receptor stimulation by cytokines (e.g. IL-2 or IL-15), cytoplasmic portions of the IL-2R are phosphorylated and subsequent recruitment and activation of Janus Activated Kinase-3 (JAK3) occurs. JAK3 then phosphorylates STAT5, or STAT5 is directly recruited to the phosphorylated IL-2R chains via its SH2 domain. Phosphorylation of STAT5 is required for it homodimerization, nuclear translocation, and DNA binding[415, 416]. Of relevance, IL-2 and/or IL-15 activation of STAT5 can result in perforin, granzyme B, and IL-2R expression[415, 417, 418]. In the present study, we examined the impact Len has on NK cell viability and proliferation, the phenotype and the receptors

expressed, as well as cytotoxic functions, including natural cytotoxicity and cytokine production. There has only been one other study (to our knowledge[419]) that has explored the effects of Len on normal healthy NK cells. This study had important findings that are in agreement with our data here. However, the authors did not assess the molecular mechanisms involved. We sought out to do a thorough investigation of how Len modulates NK cell function and biology. We find that many receptors are unaffected by treatment with Len, however, NKp46 and two inhibitory KIRs become downregulated. In support of other studies[351], we also find CD16 to be upregulated upon treatment. Importantly, natural cytotoxicity is enhanced with concomitant increases in granule mobilization and granzyme B and perforin expression. This upregulation of cytotoxicity is likely due to the fact that we find a sustained and robust activation of STAT5. This effect is enhanced when we treat the cells with exogenous IL-2 or IL-15. IL-Rβ and IL-2Rγ chains are also increased at the surface level and are presumably involved in a positive feedback loop. These finding have important clinical application since IL-15 is critical for NK development and function, and for the proliferation of memory CD8 T cells[414, 420, 421]. We provide what we feel is rationale to further study the combination of Len with IL-15 in order to enhance the immunomodulatory and activating properties of this drug, while ideally eliminating some of the immune suppressive side effects seen with Len monotherapy.

Results

The effect of lenalidomide on NK viability, proliferation, and cell cycle.

Lenalidomide is an emerging drug with much emphasis and focus placed on it over the last few years. Despite intensive work, there remains a lot of mystery and conflicting results associated with this drug. It has been previously reported that Len has differential effects depending on the cell type being studied and the stimulus received. In certain instances it can induce apoptosis[304], in others, Len causes G_0/G_1[307] or G_2 arrest [324]. Considering these findings we sought to determine what effect, if any, Len had on cell cycle and viability of NK cells. In our early studies we employed the NK cell line, YT. YT cells provided a valuable tool with reproducible results and easy manipulability. We found that Len does not induce apoptosis of YT cells, even at a high dose (20μM; low dose: 5μM, data not shown) (Fig. 15). However, Len does severely impair cell proliferation (Fig. 16) and cell cycle progression in G_0/G_1 (Fig. 17) and by 14 days of treatment 100% of cells are arrested.

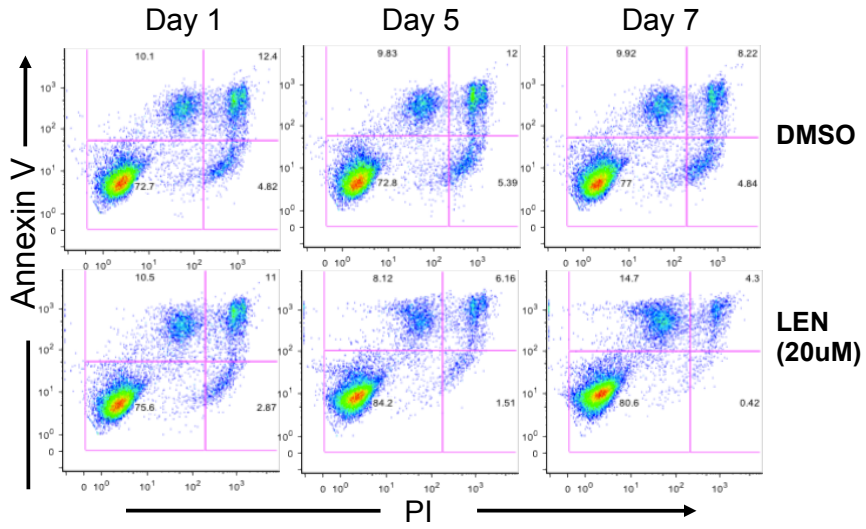


Figure 15. Lenalidomide effect on YT cell viability. The YT NK cell line was treated with a 20μM lenalidomide or DMSO for 1, 5, or 7 days and apoptosis was

measured using Annexin V and PI staining. This is a representative of 3 independent experiments.

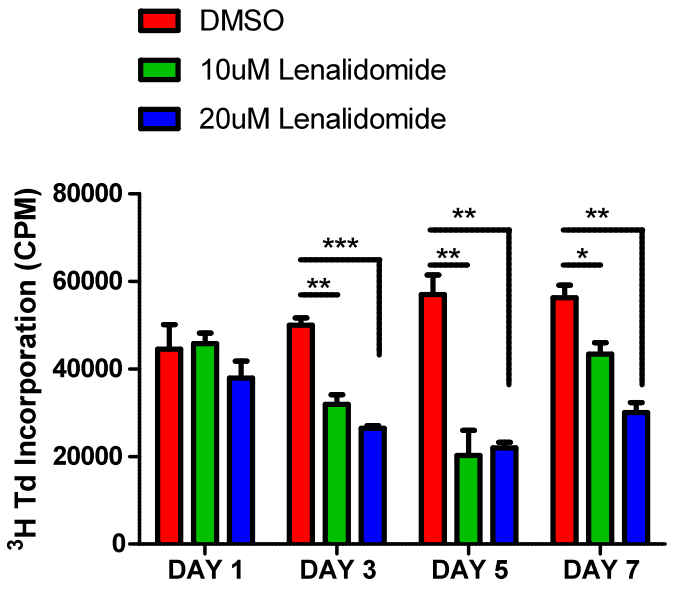


Figure 16. YT cell proliferation is impaired by lenalidomide treatment. YT cells were cultured in the presence of 10μM or 20μM Len for 1, 3, 5, or 7 days or DMSO and proliferation of YT cells was determined by quantifying overnight incorporation of [^{3}H] thymidine (1.0 μCi per well) Results are expressed as the mean counts per minute (cpm) of triplicate wells plus or minus the SD. Statistical analyses was performed using GraphPad Prism Software and the Student's two-tailed paired t test. Results were considered significant when $p<0.05$ (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$).

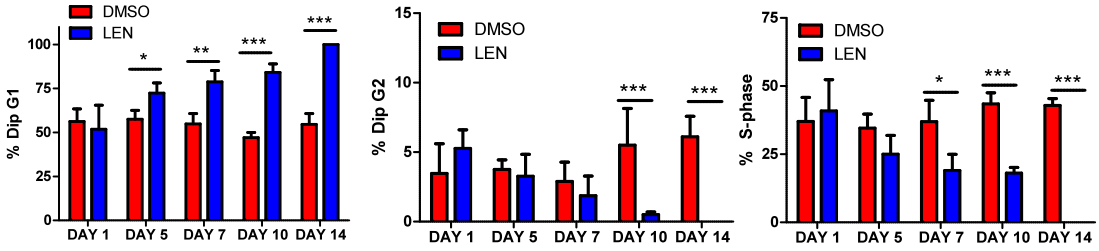


Figure 17. Lenalidomide induces G0/G1 YT cell cycle arrest. YT cells were cultured with 20μM Len or DMSO for up to 14 days. Cell cycle analysis was assessed by PI staining and Modfit software was used to analyze the data. Data from at least 3 independent experiments were pooled. Error bars represent the mean +/-SD from pooled experiments. Statistical significance was determined using Student's t test (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$).

A unique feature of YT NK cells is their IL-2 independence, a feature that other NK cell lines and primary NK cells are not privy to[422]. Based on previous data implicating a role for Len-induced T cell-derived IL-2 in the activation of NK cells[354], we asked what effect exogenous IL-2 in combination with Len would have on YT proliferation. Addition of exogenous IL-2 overcame proliferation impairment caused by Len. In fact, proliferation was restored to levels greater than the DMSO control group (Fig. 18), indicating Len can synergize with IL-2 to enhance NK cell proliferation. Not surprisingly, cell cycle progression was also rescued by IL-2 (Fig. 19). Primary unstimulated human NK cells are not actively proliferating. Thus, when we measured proliferation (CFSE) and cell cycle (PI) in primary human NK cells, we observed no difference between Len treated and DMSO treated cells (Fig. 20). Given Len's ability to "co-stimulate" T cells, we anticipated perhaps an induction in cell cycle or proliferation, but this was never realized. In line with the general concept of chemotherapeutics, Len appears to be mostly specific for rapidly dividing cells.

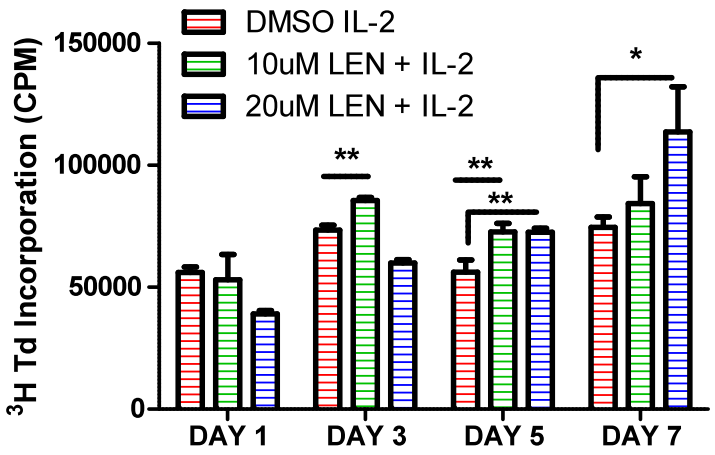


Figure 18. IL-2 restores proliferation defects caused by lenalidomide. YT cells were cultured in the presence of 10μM or 20μM Len or DMSO plus hrIL-2 (100U/mL) for 1, 3, 5, or 7 days and proliferation of YT cells was determined by quantifying overnight incorporation of [^{3}H] thymidine (1.0 μCi per well). Results are expressed as the mean counts per minute (cpm) of triplicate wells +/- SD. Statistical analyses was performed using Graphpad Prism Software and the Student's two-tailed paired t test. Results were considered significant when $p<0.05$ (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$).

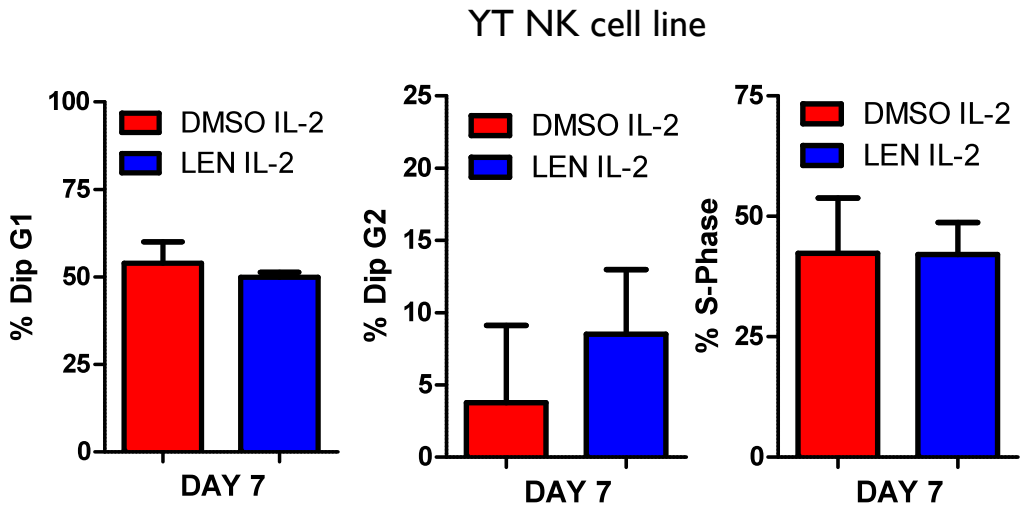


Figure 19. YT Cell cycle arrest is overcome by exogenous IL-2. YT cells were cultured with 20μM Len or DMSO for 7 days in the presence of hrIL-2 (100U/mL). Cell cycle analysis was assessed by PI staining and Modfit software was used for data analysis. Data from at least 3 independent experiments were pooled. Error bars represent the mean +/-SD from these pooled experiments.

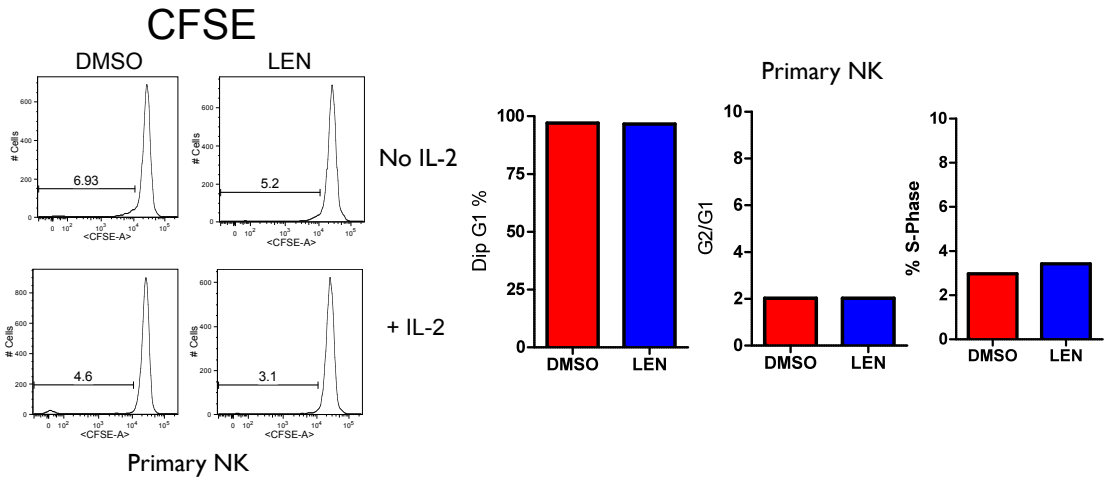


Figure 20. Human primary NK cell proliferation and cell cycle are not affected by lenalidomide. Human primary NK cells from healthy donors were FACS sorted based on the expression of CD56+CD3- and cultured with 10μM Len or DMSO for 7 days in the presence or absence of IL-2. Cell cycle analysis was assessed by PI staining and Modfit software was used for data analysis. This is representative of 2-3 independent experiments.

We also assessed PBMC cell viability and whether addition of exogenous cytokines can affect Len's impact. Interestingly, after 7 days of treatment there was no major difference in viability between DMSO and Len treated groups (Fig. 21); however, after 14 days of treatment we found that Len induced a substantial amount of apoptosis. This effect was partially restored by the addition of exogenous IL-2, but we saw a major improvement in cell viability when the cells were treated with IL-15 (Fig. 22). These data provide impetus for investigating how cytokines (specifically IL-2 or IL-15) combined with Len can further exploit Len's immune enhancing properties, while helping to eliminate unwanted cytopenias and immune suppression.

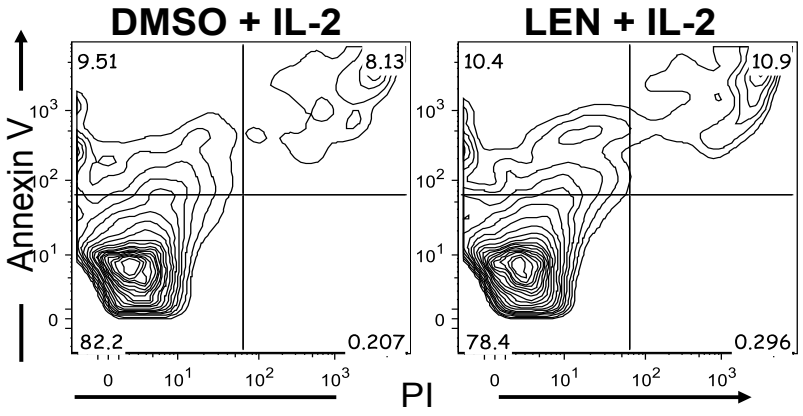


Figure 21. 7 days of treatment with lenalidomide does not induce apoptosis of healthy PBMC. PBMCs from healthy donors were cultured in the presence of hrIL-2 (100U/mL) and treated with a 20μM Len or DMSO for 7 days. Apoptosis was measured using Annexin V and PI staining by flow cytometry. This is a representative of at least 3 independent experiments.

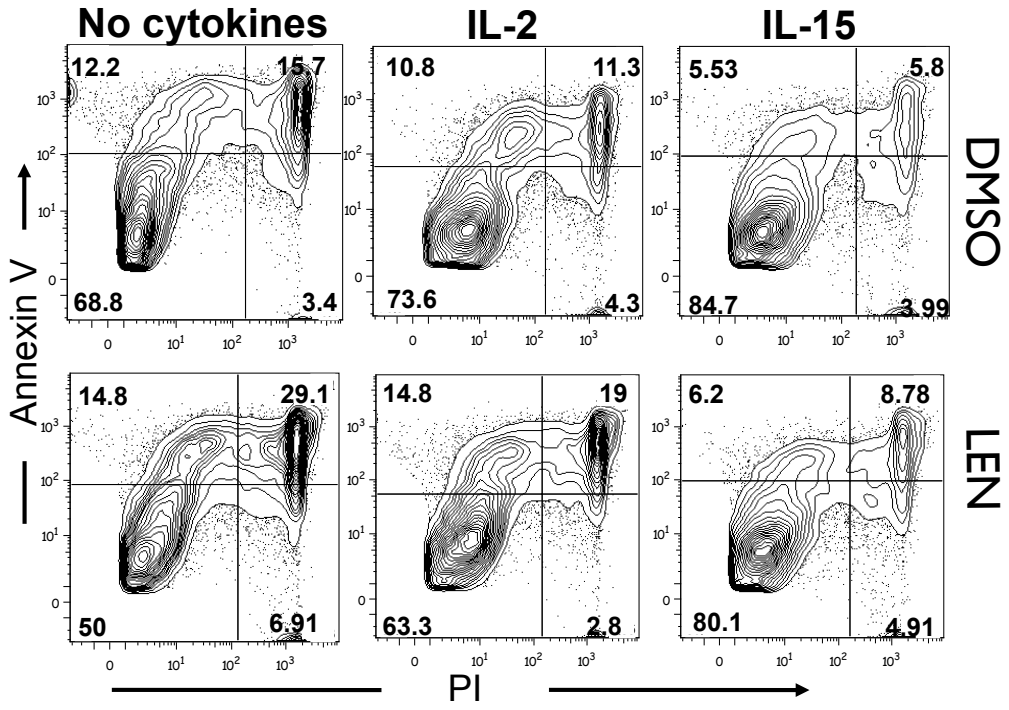


Figure 22. IL-15 rescues PBMC viability after long-term treatment with lenalidomide. PBMCs from healthy donors were treated with 10μM Len or DMSO for 14 days either without exogenous cytokines or with the addition of IL-2 (100U/mL) or IL-15 (10ng/mL) and apoptosis was measured using Annexin V and PI staining. This is representative of at least 3 independent experiments.

Lenalidomide selectively modulates the expression of key NK receptors. Len can modulate the expression of CD56[350, 351], a marker used to define the human NK cell subsets[264]. We examined PBMC isolated from healthy donors and found that Len induces $CD56^{bright}$ expression in a time-dependent manner (Fig. 23). Because there are many cell types present in a PBMC culture, we wanted to eliminate the possibility of an indirect effect. Therefore, we FACs sorted NK cells and treated them directly with Len. There is an identical upregulation of CD56 on primary sorted NK cells from healthy donors (Fig. 24), indicating that Len has a direct effect on CD56 expression by NK cells. The possibility that $CD56^{bright}$ NK cells are surviving longer, or proliferating in response to Len was also a consideration; however, as measured previously (Fig. 20), ex vivo NK cells are not actively proliferating when treated with (or without) Len, nor do they undergo apoptosis after 7 days, thereby ruling out $CD56^{bright}$ proliferation or $CD56^{dim}$ death. It should be mentioned that we do not see any major changes in the percentages of NK cells, NKT cells, or $CD3^+$ T cells upon treatment with Len (Fig. 23, gates are drawn around the two subsets and total NK population). The molecular mechanisms governing CD56 upregulation, and the functional implications, if any, have not been investigated.

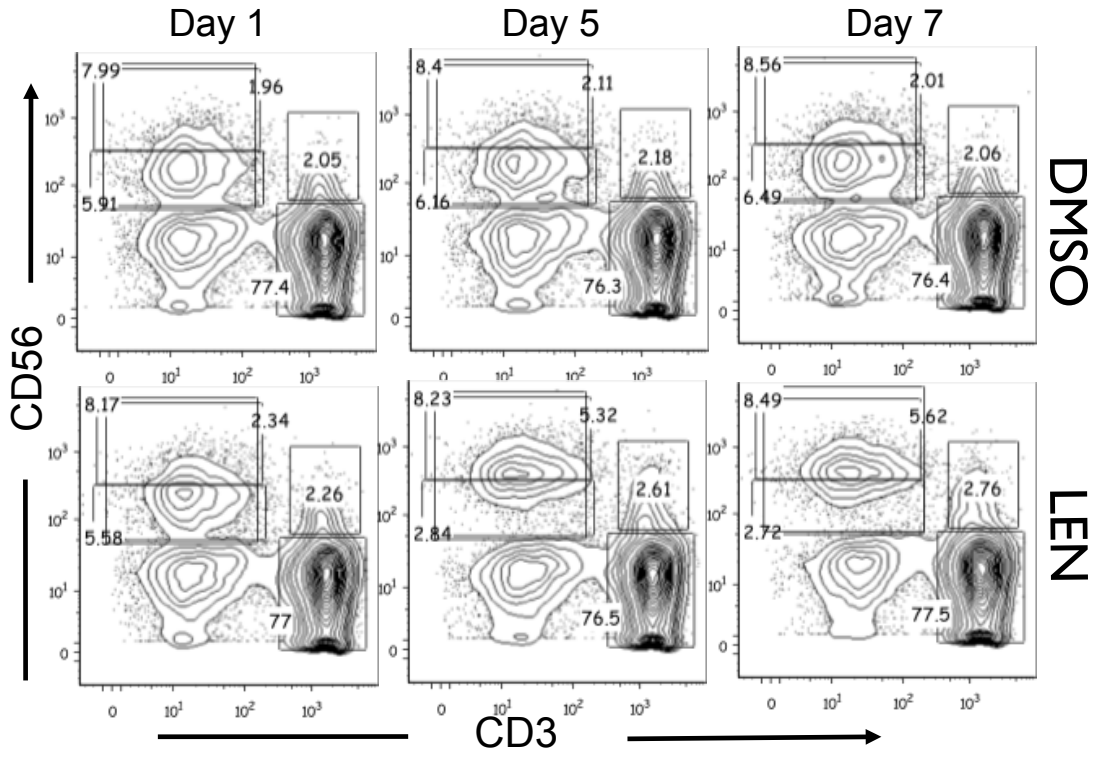


Figure 23. CD56 expression on PBMC is upregulated by Lenalidomide. PBMCs from healthy donors were treated with a 10μM Len or DMSO for 1, 5, or 7 days and were then stained for the expression of CD56. Viable cells were gated by on the exclusion of DAPI or another viability marker (e.g. 7-AAD). NK cells were identified as $CD3^{-}CD56^{+}$; NKT cells were considered $CD56^{+}CD3^{+}$ (no further phenotypic analysis was done); and $CD56^{-}CD3^{+}$ cells were considered T cells (no further phenotypic analysis was done). $CD56^{dim}$ and $CD56^{bright}$ NK are designated by gates drawn around those populations. These data are representative of more than 10 independent experiments.

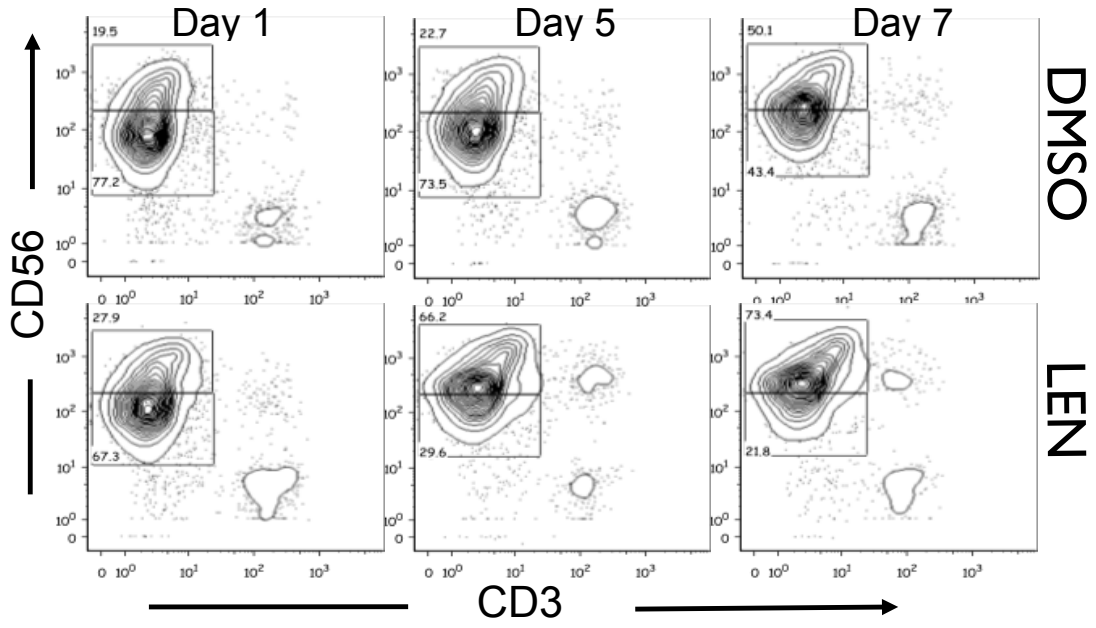
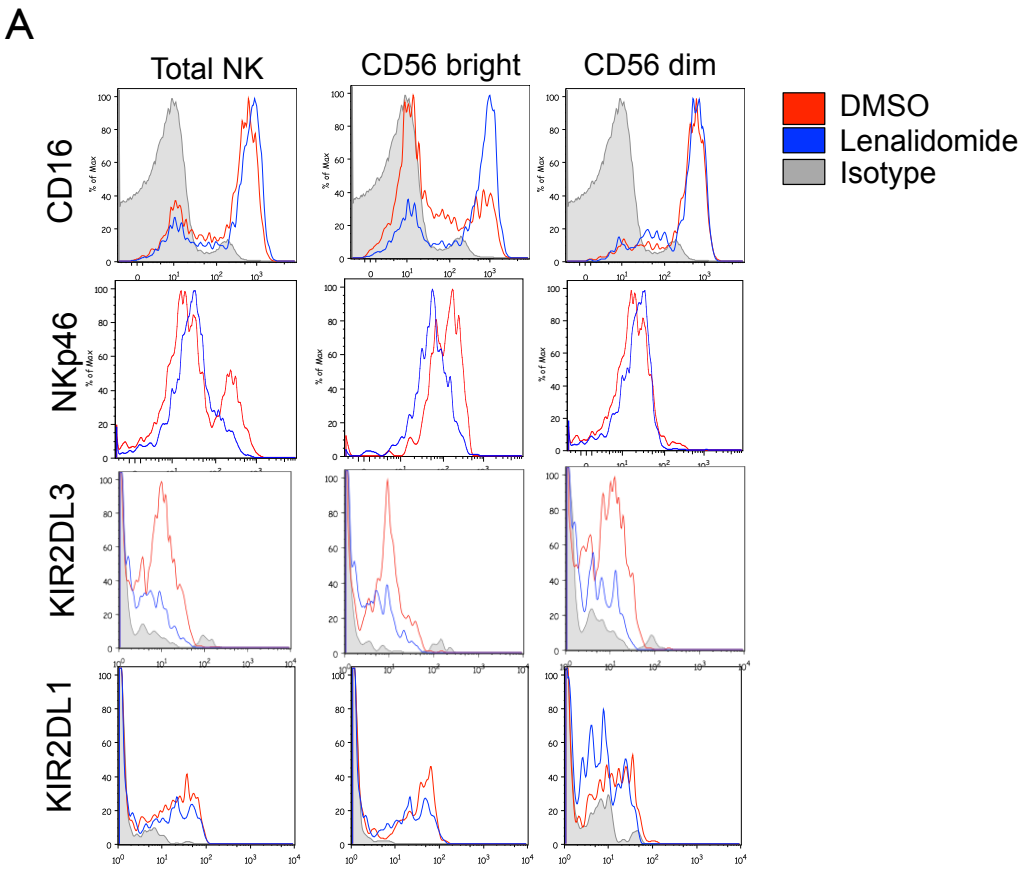


Figure 24. Lenalidomide acts directly on NK cells to induce CD56 expression. NK cells from healthy donors were FACS sorted based on the expression of $CD56^{+}CD3^{-}$ and cultured with 10μM Len or DMSO for 1, 5, or 7 days in the presence of hrIL-2 (100U/mL). NK cells were assessed for their expression of CD56 by flow cytometry. These data are representative of more than 5 independent experiments.

NK activating and inhibitory receptor expression has a direct impact on the function of an NK cell[370]. Len can modulate the expression of receptors by NK cells and other cell types[350, 419]. Therefore we did a comprehensive analysis of Len's impact on NK receptor expression. We find that several key NK receptors are affected (Fig. 25A,B). Like others, we find CD16 expression is increased and it appears to be specific to NK cells that are also $CD56^{bright}$ [351]. NKp46, a key NK activating receptor, is down-modulated in the $CD56^{bright}$ population, but unchanged on $CD56^{dim}$ cells (Fig 25A). We looked at two inhibitory KIRs, KIR2DL1 and KIR2DL3, which were both reduced. KIR2DL3 being more profoundly decreased and KIR2DL1 being only modestly affected

(Fig. 25A). Len had no major effect on the expression of NKG2D, 2B4, NKG2C, NKp30, NKp44, or DNAM-1 (Fig. 25B and data not shown).



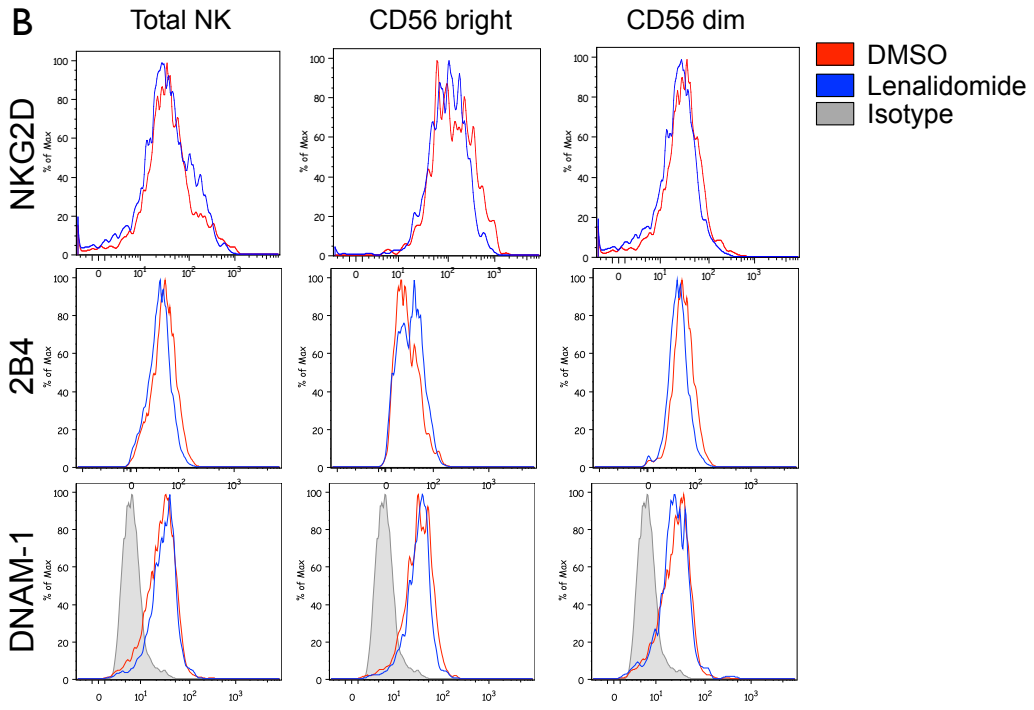


Figure 25. Lenalidomide differentially affects the expression of key NK receptors. PBMCs from normal healthy donors were treated with a 10uM lenalidomide or DMSO for 7 days and were then examined for the expression of several NK receptors. NK cells were gated based on their expression of CD56. NKR were then analyzed for their differential expression in the two NK subsets. Shown are: (A) CD16, NKp46, KIR2DL3, KIR2DL1 (B) NKG2D, 2B4, DNAM-1, Not shown: NKp30, NKp44, NKG2C. These data are representative of more than 10 independent experiments.

Inhibition of AKT by lenalidomide results in down-modulation of NKp46.

Len can affect multiple signaling pathways (our data not shown, and [286]). Len can inhibit AKT activation as measured by phosphorylation levels (Fig. 26A). There is no difference in total AKT protein levels, or p85 levels, the catalytic subunit of PI-3K suggesting a specific inhibition of AKT activation (Fig. 26A). Further experiments to investigate the mechanism for NKp46 downregulation revealed that the PI-3K/AKT pathway in part regulates NKp46 expression as supported by chemical inhibition of PI-3K using wortmannin, which resulted in decreased expression of NKp46 (Fig. 26B). We also used a MEK inhibitor (U0126) to rule out the ERK pathway and as a negative control. To

substantiate these findings, we infected YT cells with vaccinia viral vectors expressing a constitutively active PI-3K (CAp110) or a dominant negative PI-3K (DNp110) and treated the cells with Len for four days. Cells expressing DNp110 had a considerable inhibition of NKp46 as anticipated based off our wortmannin experiment, while the active p110 restored expression and overcame any inhibitory effects of Len (Fig. 26C). Whether this pathway is also responsible for the alteration of other NK receptors remains unknown.

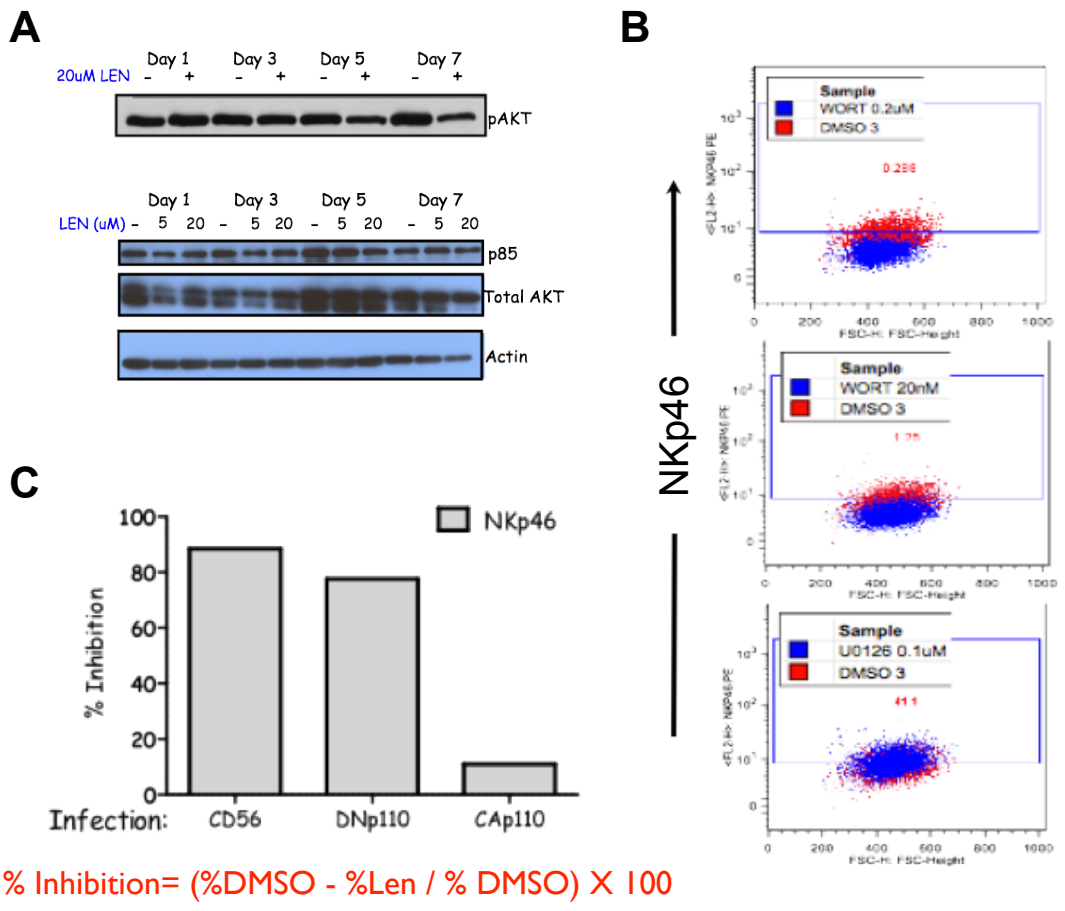


Figure 26. Lenalidomide-induced down-modulation of NKp46 is partly mediated by PI-3K. (A) YT cells were treated with 5μM or 20μM of Len or DMSO for 1, 3, 5, or 7 days and activation of AKT was measured by western blot using antibodies specific for phosphorylated serine 473 or threonine 308 (phospho threonine 308 shown). (B)

Wortmannin (PI-3K inhibitor) was added to YT cells at 0.2μM and 20nM for 4 days after which expression of NKp46 was measured by flow cytometry. The MEK inhibitor, U0126 was used as a negative control and DMSO was always used as a vehicle control. (C) YT cells were infected with vaccinia viral vectors expressing a CD56 irrelevant control, DNp110, or CAp110 and cells were treated with Len for 4 days. After which, cells were harvested and stained for NKp46 in order to measure percent inhibition of NKp46 expression ((% Inhibition= (%DMSO - %Len / % DMSO) X 100)). The CD56 control group was done to establish the percent of NKp46 inhibition seen when treated with Len alone. Data are representative of 4 independent experiments.

Lenalidomide has Differential Effects on NK Cytokine Production. NK cells are potent producers of several cytokines, which play a role in immune regulation, tumor destruction, and activation of the adaptive and innate arms of the immune system. Lenalidomide and its parent compound, thalidomide, are well known for their inhibitory effects on cytokine and growth factor production, especially IL-6 and TNF-a[291, 304]. Further, Len has differential effects on IFN-γ secretion depending the cell type studied and the context of the experiments[334, 339, 344]. Not surprisingly, we find that Len leads to a dramatic decrease in IL-6 and TNF-α production by NK cells, as well as IFN-γ. We find no major change in IL-2 secretion by NK cells when treated with Len (Fig. 27).

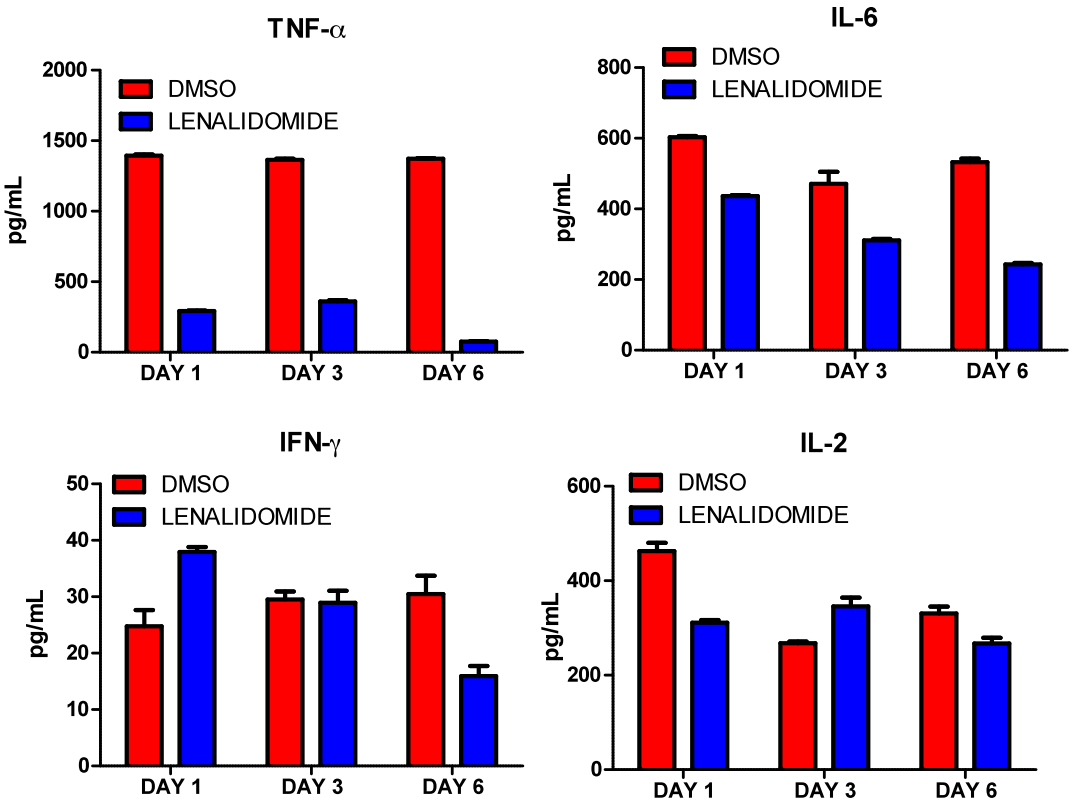


Figure 27. Modulation of NK cytokine production by lenalidomide. Human primary NK cells from healthy donors were FACS sorted based on the expression of $CD56^{+}CD3^{-}$ and cultured with 10μM Len or DMSO for 1, 5, or 7 days in the presence of hrIL-2 (100U/mL). Cell culture supernatants were harvested and measured for the secretion of IL-6, TNF-α, IFN-γ, and IL-2 using a cytometric bead array flow cytometric assay (BD Biosciences). Error bars represent the mean +/- SD of three independent experiments. Experiment is a representative of 5 different donors.

Enhanced NK cytotoxicity is due to increased production of cytolytic mediators. Attractive immune modulating features of Len include its ability to co-stimulate T cells[340] and enhance NK cell mediated ADCC[351, 354]. We find that NK natural cytotoxicity is also enhanced against several tumor targets (Fig. 28) and this improved killing is associated with an increase in granule mobilization as measured by flow cytometric staining of CD107a (Fig. 29).

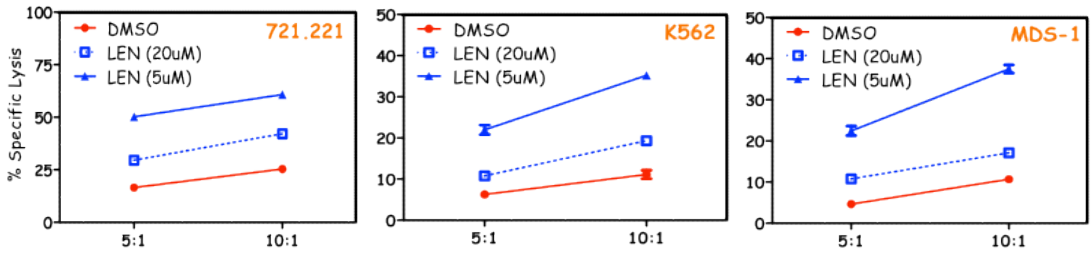


Figure 28. Natural cytotoxicity is enhanced by lenalidomide. Cytotoxicity of 3 different tumor targets, 721.221, K562, and MDS-1, was measured after primary NK cells were cultured in the presence of 5μM or 20μM Len or DMSO for 7 days. NK effectors were tested at two different E:T ratios (5:1 and 10:1 as indicated on x-axis). Statistical analysis was performed using GraphPad Prism software. The statistical test used was a Student two-tailed t test. Results were considered significant when $p<0.05$ (* $p< 0.05$; ** $p< 0.005$; *** $p< 0.0005$).

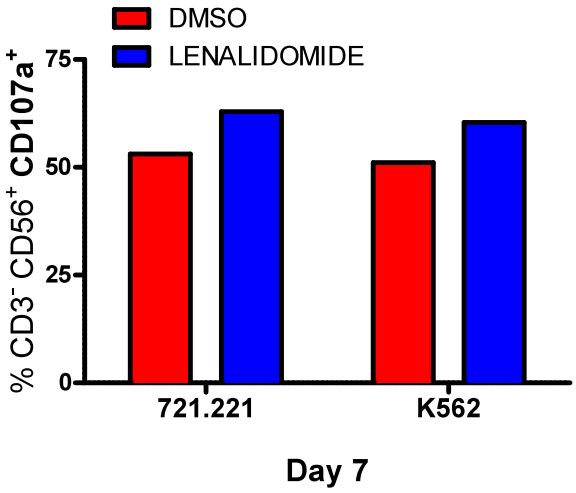


Figure 29. Lenalidomide treatment results in increased Granule mobilization upon tumor stimulation. Purified NK cells were cultured in 10μM lenalidomide or DMSO for 7 days. NK cells were then co-incubated with 721.221 or K562 tumor targets at a 1:1 ratio for 5 hours. Anti-CD107a antibody was added at the beginning of the culture; Golgi Stop and Golgi Plug (BD Biosciences) were added 30 minutes into the co-incubation. Cells were harvested and stained for expression of $CD3^{-}CD56^{+}CD107a^{+}$. This is representative of at least 3 independent experiments.

Cytolysis is enhanced as well as granule mobilization, however, we see a disparity between the amount of killing and the increase in CD107a staining, with the killing being disproportionately higher than granule mobilization. This difference may be explained by the sensitivity of the two assays, but we argued that Len might also stimulate expression of perforin and/or granzyme B[343]. We found that upon treatment both mRNA and protein levels are increased (Fig. 30), which provides an explanation as to why we see such an enhancement in cytolysis.

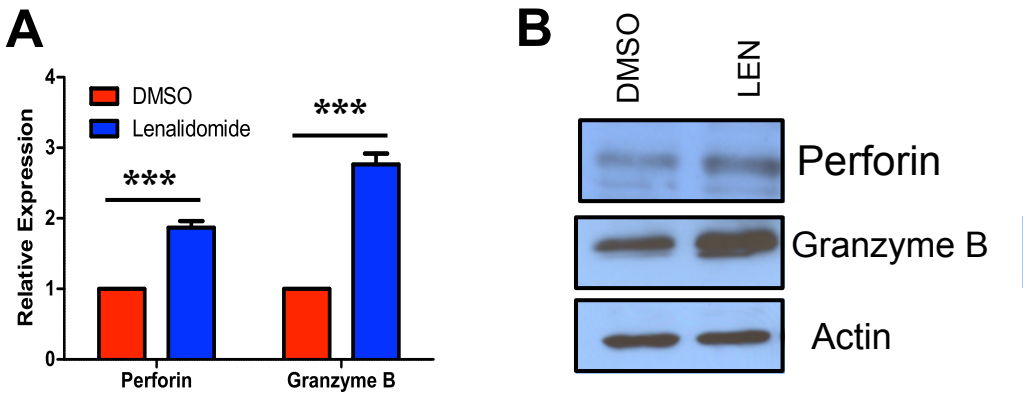


Figure 30. Lenalidomide upregulates the expression of cytolytic mediators in NK cells from healthy donors. (A) Primary NK cells from healthy donors were cultured in the presence of 10μM Len or DMSO for 7 days. mRNA expression of perforin and granzyme B was calculated by the ΔΔCt method where DMSO treated cells were the experimental control and the housekeeping gene GAPDH was the internal control. Error bars represent the mean +/- SD of three independent experiments. Statistical analysis was performed using GraphPad Prism software. The statistical test used was the Student's two-tailed t test. Results were considered significant when $p<0.05$ (*** $p< 0.0005$). (B) Expression of perforin and granzyme B was measured by Western blot. Human primary NK cells from healthy donors were purified using the human NK negative enrichment kit (Stem Cell Technologies) and cultured with 10μM Len or DMSO for 7 days in the presence of hrIL-2 (100U/mL). Whole cell equivalents of 750,000 cells were used. Whole cell lysates were prepared and Western blots performed for the indicated protein; blots were subsequently stripped and reprobed for actin as a loading control.

Lenalidomide combines with IL-2 and IL-15 and activates STAT5. Perforin expression is regulated by IL-2R signaling, and specifically STAT5 activation[418]. Considering this, we next asked if Len may also activate STAT5. We found that PBMCs treated with Len for 7 days have robust activation of STAT5 (Fig. 31A). This effect was augmented when the cells were treated with exogenous IL-2 (Fig 31A). It is known that Len can induce IL-2 production by T cells, so we reasoned that the increase in STAT5 phosphorylation in PBMCs could in part be due to the presumed additional IL-2 in the culture. To address this, we sorted NK cells from healthy donors and treated with them with IL-2 and Len. Because IL-15 shares a common receptor with IL-2 and because it is a potent NK stimulating cytokine, we also checked the effect of IL-15 in combination with Len. We find that both IL-2 and IL-15 synergize with Len to activate STAT5 (Fig 31B).

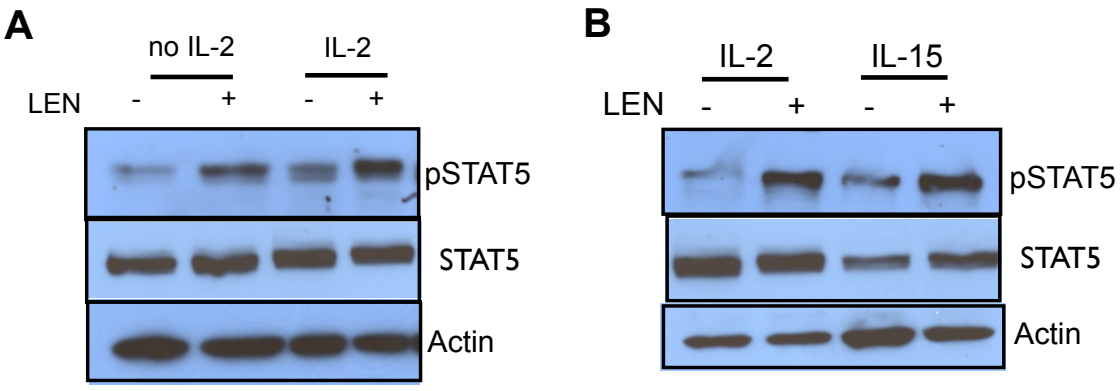


Figure 31. STAT5 activation in PBMC and primary NK from healthy donors. (A) PBMCs from healthy donors were treated with 10μM Len or DMSO for 7 days without additional cytokines or with 100U/mL of hrIL-2. (B) FACS sorted NK cells from healthy donors were treated with 10μM lenalidomide or DMSO for 7 days with 100U/mL IL-2 or 10ng/mL IL-15. Whole cell lysates were prepared and Western blots performed for the indicated protein; blots were probed for actin and total STAT5 as loading controls. Whole cell equivalents of 750,000 cells were used.

IL-2Rβ and IL-2Rγ chains are upregulated upon treatment with lenalidomide. STAT proteins have multiple levels of regulation[423]. To investigate the mechanism by which Len results in a robust and sustained activation of STAT5 we considered our previous findings regarding Len's role in the modulation of other receptors. We looked at the surface expression of IL-2Rβ and γc chains to determine whether increased surface expression of IL-2R could be a contributing factor to the enhanced STAT phosphorylation. We find that IL-2Rβ expression is mostly unaffected by treatment with Len and IL-2 combined, but quite dramatically increases with IL-15 and Len (Fig. 32). Contrarily, IL-2Rγ expression appears to be mostly affected by Len when used in combination with IL-2, not IL-15 (Fig. 32). Others have also shown similar divergent effects of IL-2 and IL-15 cytokines on IL-2Rβ and γc subunit expression[417].

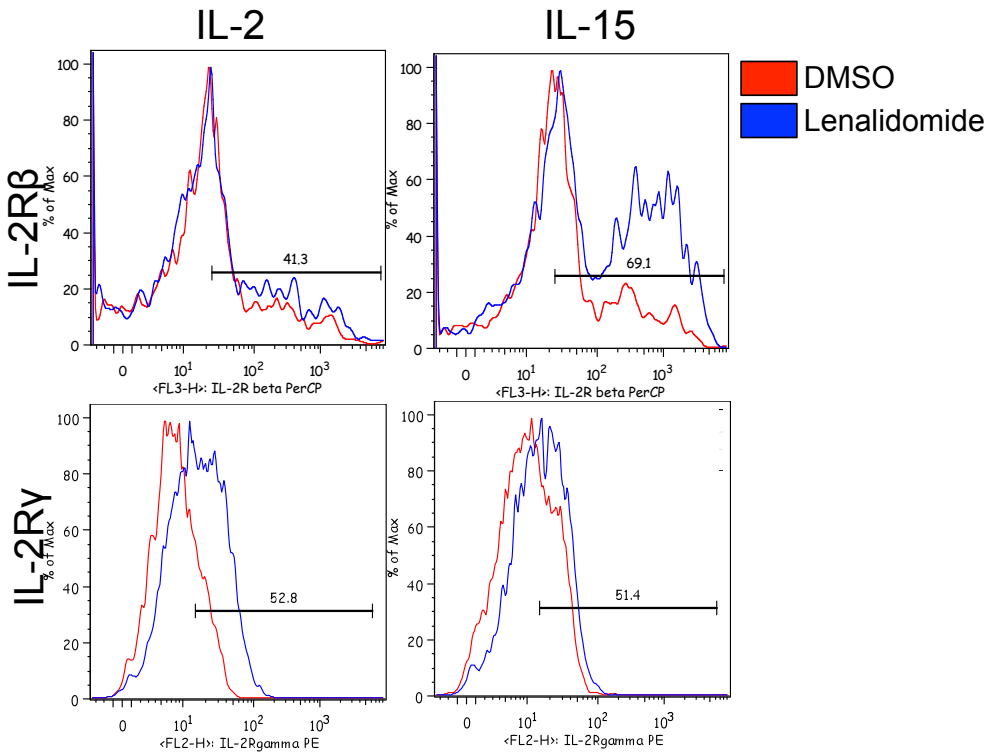


Figure 32. Differential effects of Len on IL-2R subunits. PBMCs from healthy donors were treated with 10μM lenalidomide or DMSO for 7 days with 100U/mL of IL-2 or 10ng/mL of IL-15. Cells were stained and gated based on their expression of $CD3^{-}CD56^{+}$ and then analyzed for IL-2 beta or gamma chains by flow cytometry.

The effect of lenalidomide on NK cells: A Model. Based on our findings here, we propose that lenalidomide, by itself, or in combination with IL-2 or IL-15 can amplify and sustain STAT5 activation. This in turn results in increased expression of IL-2R subunits and a presumed positive feedback loop. The overall increase in IL-2R and sustained STAT5 activation leads to an upregulation of cytolytic mediators, perforin and granzyme B, and consequently, enhanced cytotoxicity (Fig. 33). This model explains why we do not see an increase, or alteration in IFN-γ since NK cytokine production is largely mediated by the PI-3K and MAPK pathways[424], which are actually decreased in our cells when treated with Len (data not shown).

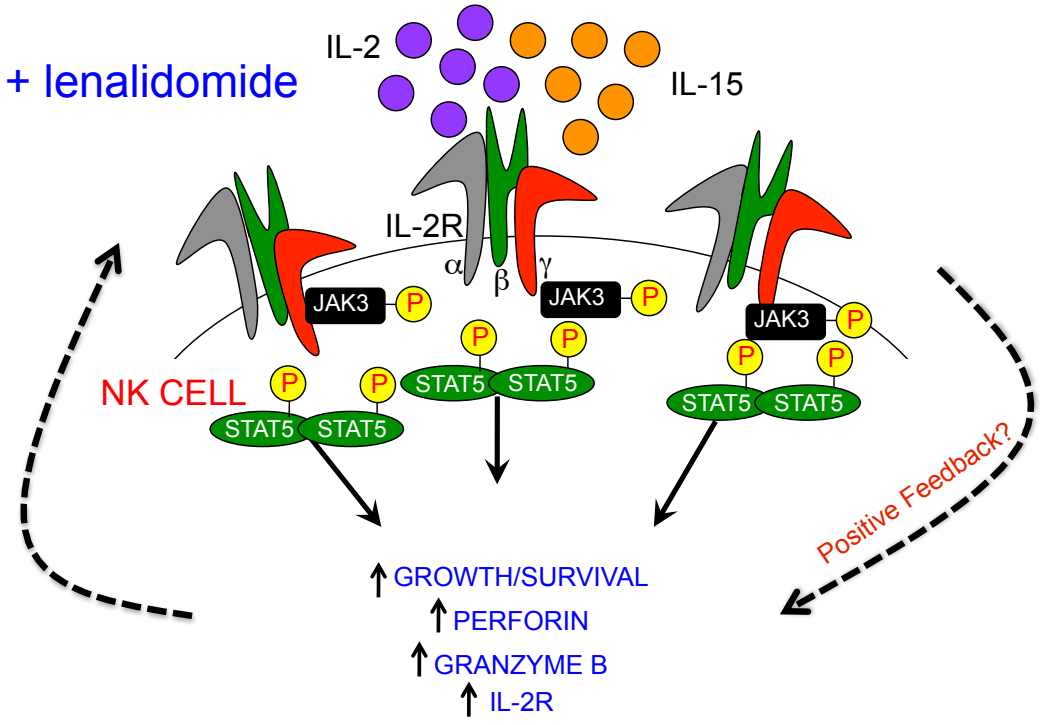


Figure 33. Proposed model of Len-mediated NK activation. Under normal conditions, IL-2 and IL-15 bind to the IL-2R and propagate signals to activate JAK3 and

subsequently, STAT5. Phosphorylated STAT5 homodimerizes and translocates into the nucleus where it regulates the transcription of many target genes involved in NK cell development, growth, survival, and activation. In the presence of lenalidomide, there is a sustained STAT5 activation and consequent upregulation of cytolytic mediators and importantly IL-2R, which in turn enhance cytolysis and contribute to a positive feedback loop maintaining NK activation, respectively.

Discussion

Lenalidomide is a fascinating drug that possesses great potential to treat cancer and other immune-mediated diseases. There have been numerous studies on its efficacy in hematological as well as solid malignancies[318, 351, 425-427]. A major limitation, however, is that the target(s) and precise molecular mechanisms of lenalidomide remain unknown. We set out to gain a better understanding and perform a comprehensive study of how this drug impacts NK cells from healthy donors. Our goal is to identify possible treatments that may be combined with Len in effort to exploit its immune enhancing properties. We identified an important pathway that is activated by Len, especially when combined with IL-2 or IL-15. Specifically, we find that NK cells have improved natural cytotoxic function against several tumor targets and augmentation of killing is likely due to increases in perforin and granzyme B. In contrast to what we see with cytolysis, we do not find that NK cytokine pro-inflammatory cytokine production is increased. Upon treatment with Len, there is a robust and sustained activation of STAT5, which presumably functions to promote transcription of perforin and granzyme B and IL-2R subunits since we see a concomitant increase in IL-2Rβ and γ_c signaling chains. This is one of our proposed mechanisms of how NK cells when treated with Len and IL-2 or IL-15 maintain their activation of STAT5.

Cytokines govern many immune functions and multiple levels of regulation are required in order to maintain a controlled state. There must also be tight regulation of the signaling that occurs downstream of cytokine receptors, such as with JAK-STAT signaling[423]. Here we did not investigate the many possible mechanisms of STAT5 regulation, but in agreement with our data, Len has previously been linked to enhanced JAK-STAT signaling, not only in NK cells (here and[428]), but also in T cells[428] and erythroid cells[426]. The unifying theme is that Len treatment results in hyperactive JAK-STAT signaling and does so by inhibiting different negative regulators of this pathway. For example, Len can inhibit CD45 in T cells and erythroid cells, which results in T cell activation and improved Ag-specific responses, and rescued erythropoietic differentiation, respectively[343, 429]. CD45, a protein tyrosine phosphatase (PTP), is highly expressed in the hematopoietic compartment and is a negative regulator of JAK activation[430]. Early studies showed that MDS patients have impaired erythropoietin (Epo)-induced STAT5 signaling[431], which is restored upon treatment with Len[406, 429, 432]. Most recently, Len was shown to improve functional responses of anergic T cells in MDS patients and restore T cell subset homeostasis[433]. Interestingly, the patients that had erythropoietic responses also had desired T cell responses, suggesting a shared mechanism of Len between these two cell types. Whether CD45 inhibition and activation of the JAK-STAT pathway was responsible for these effects was not investigated or mentioned[433]. Further, $CD45^{-/-}$ mice have markedly increased NK cell numbers and when CD45-null NK cells are stimulated with IL-2 they exhibit significantly higher killing compared to $CD45^{+/+}$ NK cells[434]. Whether CD45 is also inhibited in our studies was not investigated however.

Another mechanism of Len-rescued STAT5 signaling was recently demonstrated. This group showed that Len treatment resulted in decreased expression of SOCS1, also a negative regulator of JAK-STAT signaling [428]. Importantly, downregulation of SOCS1 correlated with response to Len and immune enhancement against MM cells[428]. Collectively, these studies offer a strong linkage between Len and JAK-STAT signaling. A question, however, remains as to how Len regulates this pathway.

Recently it has been shown that thalidomide and lenalidomide can bind to and inhibit cereblon, an E3 ligase[332]. It has also been shown that STATs and JAKs are regulated by ubiquitin modification and E3 ligases[423]. In line with this, it would be exciting to know whether this could be a mechanism in NK cells when treated with Len. The role, if any, of cereblon in NK cells is unknown. This protein is best studied in the central nervous system where it is connected to mild mental retardation[360, 362]. Cereblon is also required for normal myeloid cell growth and survival and deletion of the protein is toxic to this cell compartment[333]. This latter finding is what links Len to the severe myelosuppression seen in the majority of patients. Inhibition of an ubiquitin ligase could explain the multitude of effects observed with treatment of IMiDs, but inhibition of CRBN may not provide the answer alone. Thalidomide binds the C-terminus of CRBN at 104 amino acids, which is the most highly conserved region of the protein[332]. Investigation into whether other E3 ligase complex subunits share this sequence similarity would provide insight into whether or not thalidomide or Len have the potential to inhibit other E3 ligases. Proteosome-mediated protein degradation is an important mechanism of removing unnecessary or damaged proteins. Moreover, this process is essential for cell cycle, growth survival, and differentiation. Assessment of

cereblon protein expression in NK cells and then subsequent deletion (by siRNA for example) in the presence of Len would provide valuable information into whether this protein is also responsible for the observed effects in NK cells.

When PBMC are treated with Len in the absence of cytokines for long term (14 days), there is substantial cell death. Addition of IL-2 is capable of restoring this to some degree, however, IL-15 results in a much more pronounced effect, suggesting this cytokine in combination with Len may restore some of the cytopenias seen with lenalidomide monotherapy. Moreover, although IL-2 has been used for immunotherapy, there have been variable degrees of success[191, 435]. IL-15 is absolutely required for NK development, while IL-2 is indispensible[414, 421, 436]. Moreover, IL-15 induces proliferation of $CD8^+$ memory T cells, $CD4^+$ T cells and B cells[414, 420, 421]. There have been numerous in studies using IL-15 to augment immune function to eliminate tumors[437-442] and reviewed here[442]. There are also several ongoing clinical trials using IL-15 in order to activate NK and CTLs to fight different cancers (clinicaltrials.gov; search "IL-15"). Our findings suggest that IL-15 and Len may synergize to activate the immune system and provide another potential use for lenalidomide in the treatment of malignancies.

Materials and Methods

Cells and reagents. The human NK cell line, YT, was a kind gift from Eric Long. YT cells were maintained in RPMI 1640 plus 12.5% fetal bovine serum (FBS) and 1% pen-strep and L-glutamine. K562, 721.221, and MDS-1 target cells were maintained in RPMI supplemented with 10% FBS and 1% pen-strep and L-glutamine. PBMC were

obtained from buffy coats from healthy donors (Florida Blood Services). All cells were grown in a humidified 37C incubator, 5% CO_2. PBMCs were isolated by Ficoll-plaque plus density gradient per manufactures instructions (GE Healthcare). In the experiments where only NK were used, NK cells were either FACs sorted (viable CD56+ CD3- cells) using a FACS Aria cell sorter (BD Biosciences) or enriched using a negative NK cell enrichment kit from Stem Cell Technologies. Purity was ≥95% (data not shown). PBMCs and NK cells were cultured in RPMI 1640 supplemented with 10% FBS and the following: 1mM HEPES, 50μM β2 mercaptoethanol and 1% of the following: non essential amino acids (Gibco), sodium pyruvate (Gibco), pen-strep (Gibco) and L-glutamine (Gibco). Recombinant human IL-2 was added at 100U/mL and recombinant human IL-15 (R&D Systems) was added at 10ng/mL, where indicated. Lenalidomide was obtained from Colene and was kindly provided by the laboratory of Dr. Alan List. Lenalidomide was dissolved in DMSO at a 10mM concentration and further diluted to working concentrations in warm media before adding to cells. Len was added every day or every other day. DMSO was added at an equal volume in all experiments.

Cell cycle and apoptosis. Cells were washed twice in 1X PBS before performing experiments. For cell cycle, 7.5e5 cells were resuspended in 500μL ice cold PBS. Cells were pipetted well and vortexed gently to promote a single cell suspension. 1.5mL of ice cold 100% ethanol was added while gently vortexing cells. Cells were fixed overnight at -20C. The next day, or up to 1 week later, the cells were washed twice in 1X PBS and resuspended in a mixture containing propidium iodine (1mg/mL), RNAse (10mg/mL) and PBS. Staining took place in the dark at room temp for 2-3 hours or overnight at 4C.

Cells were acquired on a FACs Calibur (BD Biosciences) and analyzed using Modfit Software using manual analysis (Verity Software House). To measure apoptosis, cells were stained with Annexin V FITC and PI in Annexin V Binding Buffer (BD Biosciences) according to manufacture's instructions. Cells were acquired on a FACS Calibur and data was analyzed using FlowJo software (TreeStar).

Proliferation and CFSE. YT cells treated with indicated concentrations of lenalidomide +/- IL-2 were cultured in a 96-well U bottom plates (Corning Life Sciences, Acton, MA) for 1, 3, 5, or 7 days. After the final day of culture, proliferation of YT cells was determined by quantifying overnight incorporation of [^{3}H] thymidine (1.0 μCi per well; MP Biomedicals, Irvine, CA). Results are expressed as the mean counts per minute (cpm) of triplicate wells plus or minus the SD. For primary NK cell proliferation using CFSE, sorted NK cells were treated with CFSE (Invitrogen) per manufacture's instructions and cultured for 1, 5, or 7 days in the presence of 10uM lenalidomide. Proliferation was measured by acquisition of cells on the LSRII.

Flow cytometry of human NK receptors. Prior to antibody staining, cells were counted and washed in 1X PBS. Antibodies were added in FACs buffer, which consisted of 1mM HEPES and 3% bovine serum albumin (BSA) in 1X PBS. Human IgG was co-incubated with the samples for 10 min on ice to block Fc receptors. The following antibodies were obtained from BD Biosciences: CD3 FITC or PE, CD16 FITC, NKG2D PE, NKp44 PE and NKp30 PE, IL-2Rgamma PE and IL-2R beta PerCP eFluor 750, CD107a PE or

PECy7, and IFN-g APC or eFluor 450. The following antibodies were obtained from ebioscience: CD56 APC or FITC, 2B4 PE, DNAM-1 APC; and R&D Systems: NKp46 PE, KIR2DL1 FITC and KIR2DL3 FITC. Viable cells were gated using 7AAD (BD Bioscience) or DAPI. Samples were acquired on the Calibur or the LSRII and data was analyzed using FlowJo software (TreeStar).

Chromium release assays. Cytolysis of K562, 721.221, and MDS-1 targets was measured in a standard 5-h ^{51}Cr release assay. Briefly, on day 7 of NK culture, target cells were loaded with 100 μCi of ^{51}Cr per10^6 cells for 60 min at 37°C. The target and NK (effector) cells were then incubated together in a sterile U bottom 96 well plate at 37°C for 5 h The total volume in each well was 200μL. After the 5 h incubation, 100μL of supernatant was collected and measured for radioactivity on a gamma counter (Wizard 1470; PerkinElmer). Statistics were calculated with GraphPad Prism software (GraphPad Software, La Jolla, CA) using the Student two-tailed *t* test.

Cytokine production. Culture supernatants from PBMC or NK cultures were saved and stored at -80C until use. Cytokines were measured using cytometric bead array (BD Biosciences) and flow cytometry. IL-6, TNF-α, IFN-γ, and IL-2 were measured.

Western blotting. Cells were harvested, washed in ice cold 1X PBS, and lysed for 30 min on ice in a modified TNE buffer consisting of 50 mM Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaOV, 1 mM NaF, and protease inhibitor cocktails I and II (Sigma and Pierce). Protein lysates were quantified using the

Bradford protein assay and a spectrophotometer. Alternatively, cell equivalents for DMSO and Len treated lysates were resolved on a 10-12% Tris Acrylamide gel and transferred to a PVDF membrane (BioRad). Blots were blocked with 5% nonfat milk- in a Tris Buffered Salt (TBS) and 0.05% Tween 20 (sigma). Primary Abs were used at varying concentrations: Perforin and Granzyme B 1:500 (Santa Cruz Biotechnology); Total and phospho STAT5 1:2500 (Cell Signaling); Actin 1:5000 (Sigma). The appropriate anti-IgG HRP secondary was used and resolved with the West Pico HRP detection system (Pierce).

RNA isolation, cDNA synthesis, and Real Time PCR. Cells were harvested, centrifuged, and resuspended in Trizol Reagent (Sigma). RNA was isolated per manufacture's instructions. Alternatively, RNA was isolated using the RNeasy kit (Qiagen). RNA concentration was measured using a Nanodrop Spectrophotometer (ThermoScientific) and 1μg RNA was used for reverse transcription (RT) (iScript cDNA synthesis kit, BioRad). A "no RT" control was used for all experiments to confirm the absence of DNA contamination. After reverse transcription, 1μL of cDNA was used for each reaction during quantitative real-time RT-PCR (IQ SYBR Green Supermix, BioRad). All samples were done in triplicate and reactions were conducted in a 96-well spectrofluorometric thermal cycler (CFX96 realtime sytem, BioRad). Fluorescence was monitored during every PCR cycle at the annealing step. The PCR conditions were as follows: 95°C for 3 min followed by 40 cycles of 95°C, 15 s; 60°C–62°C, 30 s and 95°C, 1 min. A melting curve was added to every run for quality assessment starting at 55°C and ramping at 0.5°C for 80 repeats. All genes were analyzed through the $2^{-\Delta\Delta Ct}$ method

following previously described calculations[443]. The following primers were used: Perforin forward 5'CAGCACTGACACGGTGGAGT 3' ; Perforin Reverse- 5'GTCAGGGTGCAGCGGG 3'; Granzyme B forward- 5' TCCTAAGAACTTCTCCAACGACATC 3' ; Granzyme B reverse- 5' GCACAGCTCTGGTCCGCT 3' ; Actin forward- 5' TGGCACCCAGCACAATGAA-3'; Actin reverse- 5' CTAAGTCATAGTCCGCCTAGAAGCA3'.

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About the Author

Nicole Fortenbery graduated from Florida Atlantic University in 2005 with a B.S. in Microbiology. After graduation she began working in the laboratory of Dr. Richard Riley at the University of Miami, Miller School of Medicine in Miami Florida where she studied the dysregulation of B cell development in senescent mice. It was here she decided to pursue her love of immunology and research and next entered the Cancer Biology PhD program at the University of South Florida at Moffitt Cancer Center. Nicole began her graduate school tenure in the laboratory of Dr. William Kerr studying Natural Killer cells and the regulation of their receptors and cytotoxic functions. She published this work in The Journal of Immunology in 2010. After 3 years in Dr. Kerr's lab Nicole moved to Dr. Wei's lab where she worked on several projects and was active in assisting in manuscript and grant preparation. Here she wrote a book chapter on Neutrophils and was co-author on five manuscripts. Her dissertation work in Dr. Wei's lab involved investigating the mechanism of action of lenalidomide in Natural Killer Cells. After graduate school Nicole plans to continue in the academic setting, with a focus on neuroimmunology and clinical research.