Inappropriate Recruitment and Activity by the Src Homology Region 2 Domain-Containing Phosphatase 1 (SHP1) Is Responsible for Receptor Dominance in the SHIP-Deficient NK Cell

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Inappropriate Recruitment and Activity by the Src Homology Region 2 Domain-Containing Phosphatase 1 (SHP1) Is Responsible for Receptor Dominance in the SHIP-Deficient NK Cell

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We have previously demonstrated that the NKR repertoire is profoundly disrupted by SHIP deficiency. This repertoire disruption is characterized by receptor dominance where inhibitory signals from 2B4 repress killing of complex targets expressing MHC class I and activating ligands. In this study, we examine the molecular basis of receptor dominance in SHIP−/− NK cells. In this study, we show that in SHIP−/− NK cells there is a pronounced bias toward the 2B4 long isoform. We have also characterized signaling molecules recruited to 2B4 in SHIP−/− NK cells. Interestingly, we find that ~10- to 16-fold more Src homology region 2 domain-containing phosphatase 1 (SHP1) is recruited to 2B4 in SHIP−/− NK cells when compared with wild type. Consistent with SHP1 overrecruitment, treatment with sodium orthovanadate or a novel inhibitor with micromolar activity against SHP1 restores the ability of SHIP−/− NK cells to kill Rae1+/− RMA and M157+ targets. These findings define the molecular basis for hyporesponsiveness by SHIP-deficient NK cells. The Journal of Immunology, 2007, 179: 8009–8015.

The process by which an NK cell recognizes a target cell and delivers a sufficient signal to trigger target lysis is determined by an array of inhibitory and activating receptors on the cell surface. NK discrimination of self from altered self involves inhibitory receptor recognition of MHC class I (MHC-I) molecules (1) and non-MHC ligands like CD48 and Clr-1b (2–5). NK recognition of infected or damaged cells (altered self) is coordinated through stress-induced ligands (e.g., MHC class I chain-related protein, MHC class I chain-related protein B, Rae1, H60, Mult1) or virally encoded ligands (e.g., m157, hemagglutinin) recognized by various activating receptors, including NKG2D, Ly49H, and NKp46/Ncr1 (6–10).

The process of initial target cell recognition and the recruitment of appropriate downstream signaling molecules to the NK synapse is carefully coordinated for the NK cell to effectively kill the target. Although many of the key players in the process are known, the manner in which these disparate steps and pathways are coordinated is less well-understood (11–13). NK-activating receptors, such as NKG2D and Ly49H, upon ligand engagement are able to bind DNAX activating protein 10 or DNAX activating protein 12 molecules that contain an ITAM or YxxM motif (14, 15). This then allows for the recruitment of various effectors of cell signaling, including the Src- and Syk-related protein tyrosine kinases that subsequently lead to the activation of more distal effector pathways such as the PI3K and MAP/ERK pathways (11, 16–18).

Inhibitory receptors that engage self-ligands can oppose activation of these pathways through the recruitment of various Src homology 2 domain-containing phosphatases to their ITIM. These include Src homology region 2 domain-containing phosphatase (SHP) 1 and SHP2, which are responsible for the removal of tyrosine phosphates (19–22) and the inositol phosphatase SHIP (23), which is responsible for the removal of the 5′ phosphate from PI(3,4,5)P3 (24, 25).

2B4 is a member of the signal lymphocyte activation molecule (SLAM) family of receptors (26, 27). It functions through the recognition of another SLAM family member, CD48, that is ubiquitously expressed on cells of the hemopoietic system (28, 29). 2B4 has a complex role in NK cell function and physiology that remains an active area of investigation. Depending on the context, 2B4 has been shown to act as both an inhibitory and activating receptor (2, 27, 30–36). This is likely due, at least in part, to the ability of 2B4 to differentially recruit various downstream effectors of cell signaling. Under different signaling contexts and in different species, 2B4 can recruit SLAM-associated protein, Ewing’s sarcoma’s/FLI1-activated transcript-2, FynT, SHP1, PI3K, and SHIP (27, 37–40). How the differential recruitment of these signaling entities is controlled is not completely understood. However, which molecules are recruited and thus which signal is propagated following CD48 engagement may be influenced by the ratio of 2B4 isoforms expressed in the NK cell. Two 2B4 isoforms have been identified in mice: short (2B4S) and long (2B4L), that were

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Abbreviations used in this paper: MHC-I, MHC class I; SHP, Src homology region 2 domain-containing phosphatase; WT, wild type; LAK, lymphokine-activated killer; NaOVO4, sodium orthovanadate; NMR, nuclear magnetic resonance; IP, immunoprecipitate; PTP, protein tyrosine phosphatase; DdFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; WCL, whole cell lysate; SLAM, signal lymphocyte activation molecule; SAP, SLAM-associated protein; EAT, Ewing’s sarcoma’s/FLI1-activated transcript; s, singlet; br s, broad singlet; d, doublet; m, multiplet.

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proposed to have activating and inhibitory signaling capacities, respectively (32, 41). Although the exact function of these two isoforms remains to be defined, it is feasible that the different intracellular domains within these isoforms could recruit different effectors of cell signaling. 2B4 could also mediate different signaling outcomes through changes in the availability or recruitment of different signaling molecules. For instance, it has been shown that there are diminished levels of the SAP protein in immature human NK cells. The lack of this key activating molecule in the cell appears to lock 2B4 into an inhibitory signaling mode (36). In other SLAM family members, namely CD150, there is evidence that the presence or absence of SAP can regulate the binding of both SHP1 and SHIP to the immunoreceptor-based tyrosine switch motifs of this receptor (42).

We have previously demonstrated that the NKR repertoire is highly disrupted by SHIP deficiency (23, 43). This repertoire disruption leads to receptor dominance by 2B4 such that inhibitory signals from 2B4 repress killing of complex targets (43). In this study, we define the molecular basis for 2B4’s dominance of key NK-activating receptors for both stress-induced and virally encoded NK-activating ligands.

**Materials and Methods**

**Animals**

SHIP−/− mice were previously created in our laboratory (23) and were maintained by intercrossing SHIP−/− mice (F10 to the C57BL/6j background). All Western blot and tumor cytology studies were performed with SHIP−/− and wild-type (WT) littermates between 6 and 9 wk of age. All studies were performed in accordance with the guidelines and approval of the Institutional Animal Certification and Use Committee at the University of South Florida.

**Lymphokine-activated killer (LAK) cultures and cytolyis assays**

NK cells were magnetically enriched from whole splenocytes using the Miltenyi Mouse NK Cell Enrichment kit and an Automac (Miltenyi Biotec) per the manufacturer’s instructions. Magnetically enriched cells were plated at 2 × 10⁶ cells/ml in the presence of 2000 U/ml human rIL-2 (Proleukin). Nonadherent cells were removed on day 2 and medium replaced as necessary. On day 7, a standard 4-h chromium release assay was performed. Briefly, target cells were loaded with 100 μCi of ³¹CrCl₂ × 10⁶ cells for 60 min at 37°C. The target and LAK cells were then incubated together at 37°C for 4 h in the presence of inhibitors or medium alone. Supernatants were collected and measured for radioactivity on a gamma counter (Wizard 1470; PerkinElmer). Cells being present. In all instances, no cell death or chromium release above background was observed in the presence of the inhibitors.

**Western blots and immunoprecipitates (IPs)**

For Western blots and IPs, whole cell lysates were made from sorted SHIP−/− or WT NK cells or LAK cells as indicated. For LAK cells, 7-day LAK cultures were prepared as described above; the purity of the LAK cells at the end of the 7 days was 90–95%. For freshly isolated nonstimulated NK cells, spleens were removed from SHIP1−/− and WT littermates. Whole splenocytes were prepared from the spleen, RBC lysed and Fe blocked with anti-CD16/32 (BD Biosciences). The cells were stained with NK.1.1 FITC, TCRβ PE, and 4,6-diamidino-2-phenylindole (DAPI). NK cells were then sorted on the basis of NK.1.1⁺, TCRβ⁺, and DAPI⁻ on a FACS Aria (BD Biosciences). Cells were 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 inhibitors. For Western blots, equal cell equivalents for SHIP1−/− and WT lysates were resolved on a 4–12% Bis-Tris gel (Invitrogen Life Technologies) and transferred to an ECL membrane (Amersham). Blots were blocked with 5% nonfat milk-PBS-T. Primary Abs were used at varying concentrations: p110 (1:1000; Cell Signaling), p85 (1:1000; Cell Signaling), EAT-2 (0.5 μg/ml; a gift of A. Veilleux, Clinical Research Institute of Montreal, Montreal, Canada), SHP1 (1:500; BD Transduction Laboratories), SHIP2 (1:1000; 2B4-HA), and wild-type (WT). The appropriate anti-IgG HRP secondary was used and resolved with the Super Signal HRP detection system (Pierce). Quantification was performed using Imagequant software (GE Healthcare). The integrated density value is calculated by area × (mean density – background), to assure that areas of differing size did not skew quantitation bands were delineated by boxes of the same area between samples that would be directly compared (i.e., SHP1 between SHIP−/− and WT). For fluorochrome-tagged secondary Abs, the appropriate anti-IgG conjugated to an Alexa Fluor 488 or 680 (Invitrogen Life Technologies) was used. For IPs, cells were lysed in modified TNE lysis 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 inhibitors. Lysates were precleared with protein G-Septarose beads at 4°C for 60 min. Cleared lysates were then incubated with anti-2B4 Ab (BD Biosciences) for 60 min at 4°C while rocking. Protein G beads (eBioscience) were then added to the lysates for 60 min at 4°C while rocking, after which the protein G beads were washed six times with cold TNE buffer. IPs were then resolved by SDS-PAGE and blotted as described.

**Protein tyrosine phosphatase (PTP) inhibition assay**

PTP activity was measured using the fluorogenic 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, Invitrogen Life Technologies) as the substrate. Each reaction contained 25 mM HEPES, 50 mM NaCl, 0.05% Triton X-100, 1 mM DTT, 20 mM DiFMUP, 10 mM microcystin LR, 20 mM GST-PTP, and 10 μl of test compound or DMSO (solvent) in a total reaction volume of 100 μl in black 96-well plates. The reaction was initiated by addition of DiFMUP, and the incubation time was 30 min at room temperature. The DiFMUP–fluorescence signal was measured at an excitation of 355 nm and an emission of 460 nm with a plate reader (Victor2 1420; PerkinElmer Wallac). IC₅₀ was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activity. For IC₅₀ determination, eight concentrations of NSC119910 at a one-third dilution (0.5 log) were tested. The ranges of NSC119910 concentrations used in each PTP assay were determined from preliminary trials. Each experiment was performed either in triplicate or duplicate, and IC₅₀ data were derived from at least two independent experiments. The curve-fitting program Prism 4 (GraphPad Software) was used to calculate IC₅₀ values.

**Statistical analysis**

Statistical analysis was done using GraphPad Prism. The statistical test that was used was a Student two-tailed t test (n = 3 except where a greater n is indicated). Results were considered significant with a value of p < 0.05.

**Results**

**2B4 and SHP expression in SHIP-deficient NK cells**

We previously found that 2B4 levels are increased on the surface of SHIP−/−/− NK cells (43). To determine whether this increase is due to increased expression of 2B4, rather than increased surface deposition, we blotted whole cell lysates (WCL) prepared from sorted SHIP−/− and WT NK cells, that were not stimulated, for the presence of 2B4 (Fig. 1A). This analysis reveals, consistent with
our previous FACS analysis, that steady state levels of 2B4 are increased in SHIP$^{-/-}$ NK cells. We also find that the ratio of 2B4S to 2B4L is skewed toward the long isoform (2B4L) in the SHIP$^{-/-}$ NK cell relative to WT NK cells. In addition to 2B4, WCL were blotted for SHP1 and SHP2 (Fig. 1, B and C). This revealed that like 2B4, SHP1 is overexpressed in SHIP$^{-/-}$ NK cells as compared with WT. In contrast, SHP2 levels are consistently comparable between SHIP$^{-/-}$ and WT NK cells.

**Increased recruitment of SHP1 to 2B4 in SHIP-deficient NK cells**

Due to the overexpression of 2B4, the bias toward the 2B4L isoform, and SHP1 overexpression, we hypothesized that there might be a qualitative change in signals emanating from 2B4 in SHIP$^{-/-}$ NK cells. To examine this possibility, we prepared 2B4 IPs from sorted SHIP$^{-/-}$ and WT NK cells that were nonstimulated (Fig. 2). Given the increase of SHP1 in SHIP$^{-/-}$ NK cells, we explored the recruitment of it as well as SHP2 to 2B4 (Fig. 2A). In these blots, we see a substantial increase in the recruitment of SHP1 to 2B4 in the SHIP$^{-/-}$ NK cell as compared with WT NK cells. However, no change was seen in SHP2 recruitment to 2B4 between SHIP$^{-/-}$ and WT NK cells. These blots were subsequently stripped and reprobed for 2B4. We were then able to quantitate the amount of SHP1, SHP2, and 2B4 present in these IPs. This allowed us to compare the relative amount of each of these proteins present in the IPs (Fig. 2B). Through this comparison, we were able to show that there is ~2-fold more 2B4 in the SHIP$^{-/-}$ 2B4 IPs compared with WT. This 2-fold greater amount of 2B4 in the SHIP$^{-/-}$ NK cell was expected due to the fact that, as we have previously shown, there is an ~2-fold increase in the amount of 2B4 on the surface of SHIP$^{-/-}$ NK cells as measured by flow cytometry (43). Therefore, if equal cell equivalents were loaded, we would expect ~2-fold more 2B4 in the IPs of SHIP$^{-/-}$ NK cells as compared with WT IPs. We were also able to show that in the SHIP$^{-/-}$ IPs there is at least a 10-fold increase in SHP1 recruitment, so although there is more 2B4 in SHIP$^{-/-}$ IPs there is dramatically more SHP1. We also performed this same analysis of SHP1 recruitment to 2B4 using a chemiluminescent secondary and a Licor Odyssey imager. This allowed us once again to quantitate the amount of SHP1 recruited to 2B4. Through this technique, we were able to reconfirm our SHP1 finding showing that there is ~16 times more SHP1 recruited to 2B4 in the SHIP$^{-/-}$ NK compared with the WT.

Given the key role of SHIP in opposing PI3K signaling, we blotted for the PI3K subunits p110 and p85 (Fig. 2, D and E). In both instances, we see a small but consistent increase in the association of 2B4 with both PI3K subunits in SHIP$^{-/-}$ NK cells. This change likely reflects increased 2B4 expression in SHIP-deficient NK cells, rather than preferential recruitment of these PI3K subunits. EAT-2 has been proposed to be a key mediator of the 2B4 inhibitory pathway (39), and therefore we also blotted 2B4 IPs for EAT-2 with LAK cells where we saw no appreciable difference (Fig. 2F).

Given that our functional assays of 2B4’s impact on NK cytolytic function are performed with LAK cells (see Figs. 4 and 5), we also examined SHP1 and SHP2 recruitment to 2B4 receptor complexes in SHIP$^{-/-}$ and WT LAK cells. As was observed with freshly isolated NK cells, there is a dramatic increase in the recruitment of SHP1 to 2B4 in activated SHIP$^{-/-}$ NK cells compared with WT where SHP2 remains equal in the same cells (Fig. 3A). Once again, we were able to quantify the amount of SHP1, SHP2, and 2B4 present in the 2B4 IPs. This finding agreed with the finding in resting NK cells that even though there is a 2-fold increase in 2B4 expression in the SHIP$^{-/-}$ NK cell, the increased recruitment of SHP1 is much greater. Taken together, the analysis of both resting and activated NK cells suggests that 2B4 dominance of activating receptors and the hyporesponsiveness of SHIP$^{-/-}$ NK cells could be due to an inappropriate degree of SHP1 recruitment to 2B4 receptor complexes.
capacity of SHIP to our surprise we observed that NaOV treatment increased the
cells. This finding indicates phosphatase inhibition can restore the
We have consistently observed this supernormal killing of RMA
while also expanding the capacity of SHIP-deficient NK cells to
mediate efficient killing (Fig. 4A). Importantly, we consistently
A
recruitment to 2B4 (Fig. 4A). We find that the addition of 100 μM
NaOV to SHIP
NaOV to SHIP
SHP1, SHP2, and 2B4 levels were quantified using Imagequant software
and compared in a bar graph, showing that SHP1 is dramatically overre-
cruited to 2B4 in the SHIP
NKG2D killing. Nonetheless, we consistently observe
in SHIP-deficient NK cells (43), the ability of NaOV to restore
Killing by SHIP
target cells via NKG2D,

Inhibition of SHP1 activity restores SHIP−/− NK cytolytic function
To further test the hypothesis that inappropriate recruitment of SHP1 to 2B4 is locking SHIP−/− NK cells into a hyporesponsive state, we tested several novel low m.w. compounds that have the ability to inhibit the phosphatase activity of SHP1 at micromolar levels. These compounds were identified during a screen for SHP2 inhibitors (45). We screened six compounds with predicted micromolar activity against SHP1 and 2. Of these six compounds, we identified one, NSC119910 (Fig. 5A), which was effective in restoring the cytolytic capacity of SHIP−/− NK cells. The selectivity of this compound was tested in vitro against SHP1, SHP2, and PTP1b (Fig. 5B). In these experiments, we were able to show that NSC119910 is ~10-fold more selective to SHP1 and ~100-fold more selective to SHP2 than a very closely related tyrosine phosphatase PTP1b.

We next tested the ability of NSC119910 to restore killing in the
SHIP−/− NK cell. The effective in vitro dose at which NSC119910 was able to restore SHIP−/− cytotoxicity was determined in a dose titration experiment. Through this, 67.32 μM of NSC119910 was used for all subsequent standard ⁵¹Cr-release assays. The addition of NSC119910 significantly restored killing of Rae1+ RMA cells as well as parental RMA targets by SHIP−/− NK cells, while it had no effect on the cytolytic activity of WT NK cells against Rae1+ targets (Fig. 5C). The addition of NSC119910 to LAK cells had no effect on the expression levels of NKG2D (Fig. 5E). We have also performed these experiments with m157+
BaF3 targets. As shown in Fig. 5, the addition of NSC119910 also increased SHIP−/− NK killing of m157+ targets. Although the increase was not as dramatic as we observed with NKG2D-mediated cytolyis, this increase has been observed consistently in multiple cytolyis assays with the m157+ BaF3 targets. These tyrosine phosphatase inhibition studies when paired with our biochemical determination of inappropriate SHP1 recruitment to 2B4 in SHIP−/− NK cells provides a mechanistic rationale for the hyporesponsiveness of SHIP−/− NK cells.

Discussion

Previously, we have shown that SHIP deficiency leads to an NKR repertoire disruption such that 2B4 acts as a dominant inhibitory receptor (43). In this study, we have extended these findings to identify a molecular mechanism responsible for 2B4 receptor dominance in SHIP−/− NK cells. We have previously shown that there is significant overrepresentation of 2B4 on the surface of SHIP−/− NK cells. We have extended this by demonstrating that in the SHIP-deficient NK cell there is not only more surface deposition of 2B4, but also significantly more 2B4 protein expressed by SHIP−/− NK cells. We have also determined that when compared with the WT NK cell, there is a bias in the SHIP−/− NK cell toward the 2B4L isoform. We examined the various signaling molecules that are recruited to 2B4 in SHIP−/− NK cells. We found that there is a small increase in the PI3K subunits p110 and p85 that is most likely attributable to increased 2B4 expression. We have also identified that there is no demonstrable difference in either SHP2 or EAT-2 recruitment to 2B4. Furthermore, we have identified that there is a small increase in the PI3K subunits p110 and p85 that is most likely attributable to increased 2B4 expression. We were able to reverse the effect of the SHP1 overrecruitment by inhibiting its enzymatic activity using either a broad-acting tyrosine phosphatase inhibitor (NaOV) or a more selective SHP inhibitor (NSC119910). These results have led us to hypothesize that SHIP deficiency leads not only to 2B4 receptor dominance, but 2B4L bias, as well as altered inhibitory signaling within the SHIP−/− NK cell. We have developed a model incorporating the key differences that exist within 2B4 signaling in the SHIP−/− and WT environment (Fig. 6).
Given that SHIP is a key inhibitor of the PI3K pathway, we initially considered the possible overrecruitment of the PI3K subunits, p110 and p85, to 2B4 might be responsible for the qualitative change in 2B4 function in SHIP-deficient NK cells. Given the inhibition of SHP1 was able to restore killing in SHIP<sup>−/−</sup> NK cells to WT levels, it stands to reason that PI3K does not play a major role in rendering SHIP<sup>−/−</sup> NK cells hypo-responsive. PI3K could still play a subtle and indirect role in 2B4 receptor dominance. As is detailed in Fig. 6, in the absence of SHIP, PI3K activity may not need to be increased, but rather its unopposed activity could potentially alter 2B4 expression and contribute to receptor dominance. Previous studies have identified AP-1-binding sites in the promoter of 2B4 (46). PI3K can trigger nuclear translocation of AP-1 via activation of protein kinase C-ε (47), and thus unopposed PI3K activity at 2B4 could potentially increase 2B4 expression and/or bias isoform usage toward 2B4L.

2B4 has proven to have a somewhat complex role in NK biology with in vitro and in vivo experiments indicating both activating and inhibitory roles in NK function (2, 27, 30–36). This disparity has been attributed, to some extent, to the various signaling adaptors that can potentially associate with the immunoreceptor-based tyrosine switch motif of 2B4 (27, 37–40). Both SHP1 and SHP2 have been shown to be recruited to 2B4 in certain contexts (35, 37, 40) and are also key regulators of inhibitory signaling for MHC-I receptors on NK cells. In this study, we identified a ~10- to 16-fold increase in SHPI1 recruitment to 2B4 in the SHIP<sup>−/−</sup> NK cell. This is a key finding given that we have previously shown that the surface expression of 2B4 is increased only ~2-fold in the SHIP<sup>−/−</sup> NK cell compared with the WT NK cell (43). There is clearly a qualitative change in the 2B4 receptor complexes such that a much larger proportion of 2B4 molecules associate with SHP1 in the absence of SHIP expression. 2B4 has up to four tyrosine residues in its cytoplasmic tail that can be phosphorylated and recruit downstream signaling molecules (37). Both SHIP and SHP1 have Src homology 2 domains that can bind overlapping phosphoryrosines in 2B4 (37). Thus, in the SHIP-deficient NK cell there is likely greater access to 2B4 by SHP1. This dramatic increase of SHP1 at 2B4 receptor complexes could alter the balance of signaling in the SHIP-deficient NK cell. Importantly, 2B4 has been shown to be recruited to the NK synapse (48, 49). Therefore, the increased presence of SHP1 at the NK synapse in SHIP<sup>−/−</sup> NK cells is likely to terminate activating signals before they propagate to more distal effectors required for NK function. It is also important to note that the overrecruitment of SHP1 in LAK cells is likely to be occurring in the presence of 2B4 CD48 receptor ligand interactions. It has been previously shown that NK cells ubiquitously express CD48 (50), thereby allowing a 2B4 CD48 interaction to occur in LAK cultures. In fact, it has been shown that the 2B4 CD48 interaction is important in inhibiting NK fratricide, indicating that this interaction is not only theoretically possible but is actually a key interaction within NK cells (51). Therefore, when we are examining 2B4 in our LAK cells, it is probable that we are examining the receptor in an engaged state.

In this study, we used two phosphatase inhibitors: first, a broad-acting phosphatase inhibitor NaOV and second, a more specific SHP inhibitor NSC119910. We used both of these compounds in an attempt to counteract SHP1 overrecruitment and thereby restore killing by the SHIP<sup>−/−</sup> NK cells to WT levels. NaOV was able to successfully restore killing by SHIP<sup>−/−</sup> NK cells of Rae1<sup>+</sup> cells to WT levels. Interestingly, the killing of RMA parental cells by SHIP<sup>−/−</sup> NK cells was also significantly increased. We propose that this increase in SHIP<sup>−/−</sup> killing results from the underrepresentation of other inhibitory receptors, for MHC-I ligands, in SHIP<sup>−/−</sup> NK cells, that would prevent WT killing of MHC-I<sup>+</sup> targets that lack activating ligands. Therefore, when inhibitory dominance of 2B4 is released by phosphatase blockade this presumably enables supernormal killing of MHC-I<sup>+</sup> targets that lack activating ligands. Although it is possible that the use of the broad-acting NaOV is having an effect on other phosphatases in the SHIP<sup>−/−</sup> NK cell, it is unlikely due to the fact that we have previously shown that 2B4 is dominant inhibitory receptor in the SHIP<sup>−/−</sup> NK cell. Our results with the BaF3 targets are less clear but nonetheless provocative. Most importantly, we see a consistent increase in SHIP<sup>−/−</sup> cytotoxicity in the presence of NaOV reconfirming the ability of phosphatase blockade to increase cytotoxicity of the hypo-responsive SHIP<sup>−/−</sup> NK cell against BaF3 and m157<sup>+</sup> cells. Importantly, when we later tested the more specific NSC119910 we did not see the same increase of killing of m157<sup>+</sup> cells by WT LAK cells indicating that the increase we see with NaOV is due to non-SHIP1-related effects.

The use of the more specific SHP inhibitor NSC119910 was able to confirm our initial findings that the blockade of SHP1-mediated inhibition restores killing by SHIP<sup>−/−</sup> NK cells. In the killing of RMA and Rae1<sup>+</sup> cells, we see a very dramatic and significant increase in killing by the SHIP<sup>−/−</sup> cells. Although we do see an increase in the killing of RMA cells by WT NK cells in the presence of NSC119910, this could be due to blocking of 2B4-independent inhibitory signals. It is likely that in the WT NK environment, the enhancement we see for killing of syngeneic parental cells that lack the activating ligand is due to inhibition of SHP1 and/or SHP2 recruited to MHC receptors, rather than 2B4. It is important to note that in the WT environment it may be possible to have 2B4-independent mechanisms at work, where in the SHIP<sup>−/−</sup> NK cell it is 2B4-dependent mechanisms rendering the cells hypo-responsive (43).

Our demonstration that killing of tumor targets by SHIP-deficient NK cells can exceed that of WT NK cells suggests an approach to potentially enhance NK killing of tumors in vivo, and potentially in the absence of NK-activating ligand expression. We propose that dual or tandem inhibition of SHP1 and SHP1 might be used to temporarily increase NK clearance of tumor cells. The first step would be to inhibit SHIP to create an NK compartment that is overly dependent on one or a limited number of inhibitory receptors, for MHC-I ligands, in tumor cells. The first step would be to inhibit SHIP to create an NK compartment that is overly dependent on one or a limited number of inhibitory receptors, for MHC-I ligands, in tumor cells. The first step would be to inhibit SHIP to create an NK compartment that is overly dependent on one or a limited number of inhibitory receptors, for MHC-I ligands, in tumor cells. The first step would be to inhibit SHIP to create an NK compartment that is overly dependent on one or a limited number of inhibitory receptors, for MHC-I ligands, in tumor cells. The first step would be to inhibit SHIP to create an NK compartment that is overly dependent on one or a limited number of inhibitory receptors, for MHC-I ligands, in tumor cells. The first step would be to inhibit SHIP to create an NK compartment that is overly dependent on one or a limited number of inhibitory receptors, for MHC-I ligands, in tumor cells. The first step would be to inhibit SHIP to create an NK compartment that is overly dependent on one or a limited number of inhibitory receptors, for MHC-I ligands, in tumor cells.

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**Disclosures**

The authors have no financial conflict of interest.
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