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Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) is required for full activation of Ras/ERK in many cytokine and growth factor receptor signaling pathways. In contrast, SHP-2 inhibits activation of human NK cells upon recruitment to killer cell Ig-like receptors (KIR). To determine how SHP-2 impacts NK cell activation in KIR-dependent or KIR-independent signaling pathways, we employed knockdown and overexpression strategies in NK-like cell lines and analyzed the consequences on functional responses. In response to stimulation with susceptible target cells, SHP-2-silenced NK cells had elevated cytolytic activity and IFN-γ production, whereas cells overexpressing wild-type or gain-of-function mutants of SHP-2 exhibited dampened activities. Increased levels of SHP-2 expression over this range significantly suppressed microtubule organizing center polarization and granzyme B release in response to target cells. Interestingly, NK-target cell conjugation was only reduced by overexpressing SHP-2, but not potentiated in SHP-2-silenced cells, indicating that conjugation is not influenced by physiological levels of SHP-2 expression. KIR-dependent inhibition of cytotoxicity was unaffected by significant reductions in SHP-2 levels, presumably because KIR were still capable of recruiting the phosphatase under these limiting conditions. In contrast, the general suppressive effect of SHP-2 on cytotoxicity and cytokine release was much more sensitive to changes in cellular SHP-2 levels. In summary, our studies have identified a new, KIR-independent role for SHP-2 in dampening NK cell activation in response to tumor target cells in a concentration-dependent manner. This suppression of activation impacts microtubule organizing center-based cytoskeletal rearrangement and granule release. The Journal of Immunology, 2009, 183: 7234–7243.

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1 Abbreviations used in this paper: NKIS, NK immune synapse; KIR, killer cell Ig-like receptor; MHC-I, MHC class I; MTOC, microtubule organizing center; SHP-2, src homology region 2-containing protein tyrosine phosphatase-2; shRNA, small hairpin RNA; WT, wild type.

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pathways (36). As previously mentioned, SHP-2 can inhibit cellular activation through recruitment to a number of inhibitory receptors (e.g., KIRs, CD31, CTLA-4), where the phosphatase is thought to dephosphorylate key players of cellular activation (37, 38). In sharp contrast, SHP-2 is also well known to function as an activator of the Ras/ERK signaling cascade downstream of many receptor tyrosine kinases (e.g., EGF, PDGFR) and cytokine receptors (e.g., IL-2) (39–44). In this context, SHP-2 may mediate activation of this pathway either by inhibiting the Src kinase inhibitor Csk, allowing for Src-dependent activation of Ras/ERK (45), or by inhibiting RasGAP (46, 47), which catalyzes the transition from GTP-bound, active Ras to GDP-bound, inactive Ras. SHP-2-mediated activation of Ras/ERK may also involve inhibition of Sprotty proteins, a small family of molecules involved in the negative regulation of Ras (48).

Tight regulation of SHP-2 function is important for human health, since too much or too little SHP-2 can be detrimental to cellular development and function. Severe gain-of-function SHP-2 mutations are associated with cancer (e.g., juvenile myelomonocytic leukemia, acute myelogenous leukemia) (49–51). Less severe gain-of-function mutations cause Noonan syndrome, a fairly common autosomal dominant disorder typified by an irregular face, short stature, cardiac abnormalities, and an increased cancer risk (52, 53). Many of the abnormalities associated with Noonan syndrome can be linked to the inappropriate overproliferation of cells during development. Noonan syndrome mutations are found throughout the protein sequence, although most map to the N-terminal SH2 domain (e.g., Y63C, E76D, Q79P) or phosphatase domain (e.g., I282V, N308D) (52, 54–56). The vast majority of these gain-of-function mutations disrupt the interaction between the N-terminal SH2 domain and the phosphatase domain, which constitutively suppresses catalytic activity of wild-type SHP-2 (36).

Our previous work utilized dominant-negative SHP-2 to demonstrate the role of SHP-2 in KIR-dependent inhibition of NK cell function (27, 31). In the current report, we extended our studies of SHP-2 in NK cells by performing small hairpin RNA (shRNA) knockdown and overexpression of SHP-2 to address the role(s) of the phosphatase in KIR-dependent and KIR-independent processes. Our findings demonstrate that SHP-2 is an inhibitor of both cytolytic activity and IFN-γ secretion by NK cells and that this function is independent of the role of SHP-2 in KIR signaling.

Materials and Methods

Cells and culture

All cell culture was performed at 37°C in a 5% CO2 humidified atmosphere. The IL-2-dependent NK-like cell lines KHYG-1 (provided by Dr. Masato Yagita, Kijano Hospital, Osaka, Japan through the Japan Health Science Research Resources Bank, no. JCRB0156) and NKL (a gift from Dr. Marco Colonna, Washington University, St. Louis, MO) were maintained in α-MEM medium (In vitrogen) containing 10% heat-inactivated FBS (HyClone), 2% nonessential amino acids (Mediatech), 100 U/ml penicillin (Mediatech), 100 μg/ml streptomycin (Mediatech), 1 mM sodium pyruvate (Sigma-Aldrich), 200 μM myoinositol (Sigma-Aldrich), 2.5 μg/ml folic acid (Sigma-Aldrich), 1× nonessential amino acids (Mediatech), 100 μg/ml 2-ME (Fishier Scientific), and supplemented with 2% culture supernatant of J558L cells transfected with the human IL-2 gene (a gift from Dr. Antonio Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland). Cells were passed with fresh IL-2 and medium every 3–4 days. The IL-2-dependent NK-like cell lines KHYG-1 (provided by Dr. Marco Colonna, Washington University, St. Louis, MO) were maintained in α-MEM medium (In vitrogen) containing 10% heat-inactivated FBS, 2% nonessential amino acids (Mediatech), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM HEPES (Fisher Scientific), and 50 μg/ml 2-ME. The 721.221 cells were passed into fresh medium every 3–4 days.

Since a subset of KHYG-1 cells endogenously express KIR3DL1 (57), the parental KHYG-1 cells were sorted for lack of this receptor using the DX9 mAb (BD Pharmingen). These sorted cells stably lacked KIR3DL1 for at least 1 mo in culture. KHYG-1 cells lacking the endogenous KIR3DL1 were used for all experiments.

Retroviral construct generation

Four SHP-2 shRNAs were designed using Oligoengine software (www. oligoengine.com); shRNA no. 1 targeting gattaagcaacagttggtt, in N-terminal SH2 domain; a gift from Dr. B. Neel, Ontario Cancer Institute, Ontario, Canada and Dr. F. David, previously of Beth Israel Deaconess Medical Center, Boston, MA), no. 2 (gaactctttgggaanaa, in C-terminal SH2 domain), no. 3 (caggaagcaatcaagac, in C-terminal SH2 domain), and no. 4 (gaggaagcaatcatcag, just preceding the phosphatase domain).

To generate double-stranded shRNA, 3 μg of sense and 3 μg of antisense strands incorporating an intervening hairpin and terminal BgIII and HincIII restriction site overhangs were annealed together and ligated into psiScreen retro.neo or psiScreen retro.puro vectors (Oligoengine) according to the manufacturer’s instructions. SHP-2-WT cDNA (a gift from Dr. Benjamin Neel through Addgene; plasmid no. 8329) was mutared to the Noonan syndrome missense mutations, E76D and N308D (54), using the QuickChange site-directed mutagenesis kit (Stratagene) and cloned into pBMN-ires-EGFP vector (a gift from Dr. G. Nolan, Stanford University, Stanford, CA). KIR3DL1-WT and -YF have been described previously (27). All manipulations and duplication of pSuperior and pBMN plasmids were done at 30°C in Stbl2 bacteria (Invitrogen) to prevent recombination at retroviral long terminal repeats.

Retroviral transduction

The retroviral transduction protocol was previously described (58, 59). Briefly, 4 μg of each pSuperior or pBMN-ires-EGFP construct was transacted with Lipofectamine and Plus reagent (Invitrogen) into the retroviral packaging cell line, Phoenix-ampho (also a gift from Dr. G. Nolan). After 2 days, viral supernatant was harvested and used to transduce NK cell lines with Lipofectamine and Plus reagent. Cells transduced with SHP-2 shRNAs were selected with 1.25 ng/ml G418 and/or 2.5 μg/ml puromycin for 5 days (starting 2 days after viral infection). SHP-2 protein levels decreased over time after selection, becoming stably knocked down 7 days after antibiotic selection was completed. Cells were used for a maximum of 40 days after drug selection. Data from multiple transduced NK cell populations are shown for all shRNA experiments and some overexpression experiments.

Immunoblotting and protein quantification

KHYG-1 cells were mock-treated or stimulated with pervanadate for 10 min, and then lysed for 30 min in lysis buffer containing 20 mM Tris (pH 7.4) and 1% Triton X-100 (Pierce) as described (60). To generate whole cell lysates, 5 × 105 NK cells were washed once with HBSS, boiled in 3× SDS sample buffer for 5 min and probe-sonicated for 10 s. Samples were separated in 10% SDS-PAGE gels under reducing conditions and transferred onto polyvinylidene fluoride membranes (Millipore). After transfer, membranes were air-dried, reactivated with methanol, washed once with distilled H2O and blocked (5% nonfat milk in TBS) before use. Blocked membranes were incubated initially with mouse anti-GAPDH mAb (Chemicon International), mouse anti-SHP-2 mAb, and rabbit anti-SH-1 pAb (Santa Cruz Biotechnology), washed in PBS 0.1% Tween 20, followed by probing with anti-mouse-IR dye-680 and anti-rabbit-IR dye-800 Abs (LI-COR Biosciences). Blots were washed with PBS/0.1% Tween 20, and then with PBS alone. Proteins were visualized with an Odyssey infrared imaging scanner (LI-COR Biosciences) by scanning at 700 and 800 nm. Fluorescence of GAPDH, SHP-1, and SHP-2 bands were quantified using Odyssey software.

Direct cytotoxicity assay

KHYG-1 or NKL cells (harvested on day 1 or 2 after IL-2 stimulation, respectively) were tested for direct cytolytic activity against 721.221 cells in a 51Cr-release assay in 200 μl of medium per well (complete α-MEM lacking IL-2). 721.221 targets were labeled with 100 μCi of 51Cr (PerkinElmer) in 200 μl of 10% heat-inactivated FBS for 60–90 min at 37°C, washed three times with RPMI 1640, and resuspended in complete α-MEM lacking IL-2. Labeled targets and NK cells were mixed in V-bottom 96-well plates (Costar; 1 × 105 targets/well), pelleted at 1300–1500 rpm for 3 min, and incubated for 2–5 h at 37°C and 7% CO2 atmosphere. Plates were then centrifuged and 100 μl of culture medium was removed from each well and gamma-counted. Spontaneous release at total release of 51Cr were determined by incubating target cells in medium alone or containing 1% Triton X-100, respectively. Each assay condition was always performed at least in triplicate. The percentage of specific

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target cell lysis was determined as: [(average cpm experimental release – average cpm spontaneous release)/(average cpm total release – average cpm spontaneous release)] × 100.

IFN-γ assay

NK cells (2.5–5 × 10^5) were stimulated for 24 h in a flat-bottom 96-well plate with an equal number of 721.221 cells, plate-bound Abs, or medium alone. All Abs were preabsorbed to plates at 25°C for 3 h or overnight at 4°C in 100 μl of Ab coating buffer (E Bioscience) per well at the following concentrations: 0.5–1 μg/ml anti-NKp44 (3-43.13, a gift from Dr. M. Colonna), 0.5–1 μg/ml anti-NKG2D (R&D Systems), or 1 μg/ml anti-Ddit1 (a subunit of LFA-1; BioLegend). Following stimulation with targets, medium, or Abs, culture supernatants were harvested and then tested for the presence of IFN-γ by ELISA according to the manufacturer’s instructions (E Bioscience).

Conjugation assay

Up to 3 × 10^6 NK and target cells were washed once with OPTI-MEM serum-free medium (Invitrogen) and resuspended in the same medium containing 4 μM Cell Tracker Blue (Invitrogen) or 5 μM Cell Tracker Orange (Invitrogen), respectively. Cells were stained for at least 10 min at 37°C in the dark, pelleted, and incubated in fresh OPTI-MEM for at least 15 min more. Following staining, cells were resuspended in cold α-MEM and kept on ice. NK and target cells (1–5 × 10^5) were combined in prechilled FACS tubes on ice at a 1:1 ratio, pelleted briefly (<1 min) at 500 rpm, and either immediately fixed with 200 μl of cold 0.5% PFA (0 min of conjugation) or moved to 37°C water bath for 5 or 10 min. Pellets were resuspended by vigorous agitation, and 3–10 × 10^5 cells were analyzed by FACS. The percentage of NK cells in conjugates was determined as: average [% conjugates at t min/(% conjugates at t min + % unconjugated NK cells at t min)] − average [% conjugates at 0 min/(% conjugates at 0 min + % unconjugated NK cells at 0 min)]. All time points were assayed in triplicate, and only samples in which the E/T ratio was similar (±0.25) to control cells were compared. In live cell assays, cells were treated as above, except that NK cells were stained with 0.4 μM CTGreen (Invitrogen). Instead of fixing cells following centrifugation, cell pellets were moved to 37°C for 3, 5, 10, 15, 30, or 60 min, when they were resuspended by vigorous flicking and immediately analyzed by FACS.

Granzyne B ELISPOT

A granzyme B ELISPOT kit (R&D Systems) was used to measure the secretion of granzyme B from NK cells, NKL or KHYG-1 cells alone or NKL or KHYG-1 cells (61) in the IL-2-dependent human NK-like cell line KHYG-1 (61). As summarized in Fig. 1B, all four shRNAs decreased SHP-2 protein levels to varying degrees, with nos. 1 and 4 being the most effective. Cotransduction of shRNAs nos. 1 and 4 further improved the knockdown of SHP-2 to <10% of wild-type levels (Fig. 1C, and D). This combination was used in all subsequent experiments unless otherwise indicated, and cells expressing these constructs will be referred to as SHP-2-silenced cells.

SHP-2 silencing was specific and shRNA-dependent, since cells transfected to express empty pSuperior vector had similar SHP-2 levels as the untransduced control cells (Fig. 1C), and the levels of another closely related phosphatase, SHP-1, were not significantly altered (Fig. 1E). The expression of SHP-2 shRNAs led to a similar loss of SHP-2 in two other NK-like cell lines, NKL and NK-92 (Fig. 1F and data not shown, respectively). In contrast, SHP-2 levels were greatly increased when cells were transduced to express wild-type (WT) or gain-of-function Noonan mutants of SHP-2 (E76D or N308D), but not empty retroviral expression vector (pBMN) alone (Fig. 1, C and F).

SHP-2 inhibits NK cell cytolytic activity and IFN-γ production in response to tumor target cells

To determine the effect of SHP-2 knockdown on NK cell function, we first compared untransduced (control) and SHP-2-silenced cells in a direct cytotoxicity assay against an EBV-transformed MHC-I-deficient B cell line, 721.221 (62). Notably, SHP-2-silenced KHYG-1 and NKL cells killed the target cells significantly better than did control cells (Fig. 2). The enhanced cytotoxicity was observed with distinct SHP-2 shRNAs, demonstrating that the impact on cytotoxicity was not due to off-target effects of individual shRNAs (Fig. 2A and data not shown). Furthermore, this increased cytolytic activity correlated with decreasing levels of SHP-2 in a concentration-dependent manner (Fig. 2, A and B). This phenotype was highly reproducible across multiple independent transductions and was specific to the SHP-2 shRNAs, since the empty vector control did not significantly impact upon cytolytic activity (Fig. 2E). In accordance with the increased cytotoxicity seen in SHP-2-silenced cells, overexpression of WT SHP-2 decreased the killing of 721.221 targets in a concentration-dependent manner compared with untransduced cells or compared with cells transfected with vector alone (Fig. 2C–F). Overexpression of gain-of-function
mutants of SHP-2 (E76D or N308D that are commonly found in Noonan syndrome) further decreased cytolytic activity (Fig. 2, E and F). Taken together, these data indicate that SHP-2 restrains the cytolytic activity of two distinct NK cell lines in a concentration-dependent manner.

We attempted to knock down SHP-2 in primary human NK cells by introducing these shRNA constructs with retroviral transduction and with the Amaxa Nucleofector system. Primary NK cells transduced/nucleofected with SHP-2 shRNAs, however, never survived drug selection. Cell death was not due to nucleofection alone or drug toxicity, since cells transfected with pMax control vector (Amaxa) survived and primary NK cells demonstrated the same drug sensitivity as KHYG-1 cells (data not shown). These data suggest that acute silencing of SHP-2 in human primary NK cells may be toxic.

We next addressed whether cytokine production by the NK-like cell lines could be impacted by varying levels of SHP-2. IFN-γ, a type 1 cytokine, is readily secreted by NK cells after activation (18). The 721.221 target cells were used to stimulate either parental NKL or KHYG-1 cells or the same lines expressing either SHP-2 shRNAs or SHP-2 cDNAs, and then the resulting IFN-γ production was quantified by ELISA (Fig. 3, A and B). Silencing...
of SHP-2 significantly increased IFN-γ production as compared with untransduced control NK cells, while the overexpression of SHP-2-WT or -E76D significantly suppressed the production of IFN-γ (Fig. 3, A and B). Therefore, similar to the effect on cytotoxicity, SHP-2 also suppressed IFN-γ production in a concentration-dependent manner in response to encounter with transformed target cells.

Conjugation with tumor targets results in the ligand engagement of multiple receptors on the NK cell surface, and coengagement of both adhesion receptors (e.g., LFA-1) and activating receptors (e.g., NKp44 and NKGD2) results in optimal NK activation (5, 63). LFA-1 is an integrin that is important in mediating adhesion of NK cells to target cells (64). NKp44 and NKGD2 are activating receptors that promote IFN-γ production through the DAP12/Src family kinase/Syk family kinase cascade (65) and DAP10/Pi3K/Grb2-mediated signaling (3), respectively. To determine whether SHP-2 acts through one or all of these specific signaling pathways, we stimulated SHP-2-silenced and SHP-2 overexpressing KHYG-1 cells with plate-bound Abs against each receptor alone or in combination and measured IFN-γ production by ELISA (Fig. 3C). Surprisingly, in contrast to the collective engagement of multiple activating receptors that occurs when NK cells are stimulated by target cells, alterations in SHP-2 levels did not influence IFN-γ production in response to stimulation through Ab-mediated engagement of the NKp44, NKGD2, and/or LFA-1 receptors.

**KIR-dependent inhibition of cytotoxicity is intact in SHP-2-silenced cells**

A wealth of data indicate that the recruitment of SHP-1 and SHP-2 to KIR blocks the tyrosine kinase-mediated activation events in NK cells. Previously, we demonstrated that transiently overexpressing catalytically inactive SHP-2 (DN-SHP-2) in NK-92 cells significantly suppressed KIR-mediated inhibition of cytotoxicity (27). To determine whether KIR function was similarly affected in SHP-2-silenced cells, KHYG-1 cells were transduced to express catalytically inactive SHP-2 (DN-SHP-2) in NK-92 cells, and/or LFA-1 receptors.

KIR-dependent inhibition of cytotoxicity is intact in SHP-2-silenced NK cells. Untransduced control (parent) and SHP-2-silenced (shRNA) KHYG-1 cells transduced to express WT (A) or an ITIM mutant of KIR3DL1 (YF, B) were compared in a direct cytotoxicity assay for their ability to kill 721.221 target cells either expressing (ligand) or lacking (no ligand) the KIR3DL1 ligand, HLA-B51. The percentage of specific 51Cr released due to target cell lysis was quantified (% specific release) at three different NK-to-target cell ratios (E:T ratio). The mean ± SD of triplicate samples from one representative experiment of 10 is shown. KIR levels were unaffected by SHP-2 shRNA expression (data not shown). C, Compilation of data from A and B. The mean ± SD of 2.5:1 E:T ratio data from 10 separate experiments are compared. For each experiment, the data were first divided by the “control vs no ligand” sample (arbitrarily set to 100%) to generate the percentage of cytotoxicity. *p ≤ 0.05 and **p ≤ 0.01 when comparing the bracketed conditions. D, Control and SHP-2-silenced (shRNA) KHYG-1 cells transduced to express KIR3DL1-WT or -YF were treated with pre-ordinate for 10 min, lysed with 1% Triton X-100, and immunoprecipitated with anti-CD56, and then with anti-KIR3DL1 (D39) mAbs. Immunoprecipitates were separated by SDS-PAGE and immunoblotted (IB) for SHP-2 and KIR. Expression levels in whole cell lysates (WCL) are shown in the first lane of each blot. H chain (HC) is also indicated.
DN-SHP-2, however, KIR-dependent inhibition of cytotoxicity was intact in SHP-2-silenced cells expressing either WT or YF receptors (Fig. 4A–C). Moreover, the degree of inhibition by KIR engagement in control vs SHP-2-silenced cells expressing KIR-WT (50.8 vs 44.0%, p = 0.32; n = 10 experiments) or KIR-YF (31.2 vs 33.4%, p = 0.76; n = 10 experiments) was essentially indistinguishable. To clarify our current and previous findings, we performed immunoprecipitation of KIR from lysates of pervanadate-stimulated cells and found that a detectable amount of the remaining SHP-2 was still recruited to phosphorylated KIR3DL1-WT or -YF in the SHP-2-silenced cells (Fig. 4D). Consequently, we conclude that either SHP-2 is dispensable for KIR-mediated inhibition of cytotoxicity or only very low levels of SHP-2 protein are necessary to mediate effective inhibitory KIR signaling. Despite the considerable degree of SHP-2 silencing using our currently available tools, we cannot distinguish between these two possibilities.

Impact of changing levels of SHP-2 on conjugation to target cells

We hypothesized that SHP-2 may suppress NK cell cytotoxicity by diminishing their capacity to conjugate with target cells. To test this hypothesis, we analyzed the capacity of NK cells with varying levels of SHP-2 to bind to 721.221 target cells using two-color FACS analysis (Fig. 5). Knockdown of SHP-2 with shRNAs nos. 1 and 4 alone or in combination, however, did not significantly impact the conjugation of KHYG-1 cells to 721.221 target cells (Fig. 5, B and C). Similarly, SHP-2 silencing did not potentiate the degree of conjugation of NKL cells to 721.221 target cells (Fig. 5D). Results were similar when conjugates were fixed just before FACS analysis (Fig. 5) or analyzed freshly over a time course of 60 min (data not shown). These data demonstrate that the enhanced cytotoxicity observed in SHP-2-silenced cells is independent of effects on adhesion. Surprisingly, in contrast to the effect observed in SHP-2-silenced cells, overexpression of WT SHP-2 in KHYG-1 or NKL cells significantly decreased the extent of conjugation to 721.221 target cells (Fig. 5, C and D). Therefore, the impact of SHP-2 on target cell conjugation is only evident when the phosphatase is expressed at higher than physiological levels.

**SHP-2 inhibits granzyme B release**

NK cells can kill tumor or virus-infected cells through the targeted exocytosis of lytic granules, which releases proteins that compromise the target cell membrane (e.g., perforin) and activate caspase-mediated apoptosis (e.g., granzyme B). The blockade of vesicle exocytosis with inhibitors or mutations in the exocytic machinery decreases target cell killing (66). Similarly, loss of perforin or granzymes either blocks or decreases the killing of target cells, respectively (67, 68). We hypothesized that SHP-2 might suppress granule release in NK cells, which would thereby dampen cytolytic activity. To test this hypothesis, SHP-2-silenced, control, and SHP-2 overexpressing NK cells were incubated with or without 721.221 target cells for 4 h, and the resulting granzyme B release was quantified by ELISPOT (Fig. 6). Substantially greater spontaneous granzyme B release was observed in KHYG-1 cells as compared with NKL cells (Fig. 6A), which is consistent with a previous report that KHYG-1 cells have prelocked granules (69). Nonetheless, following stimulation with target cells, granzyme B release was significantly elevated in both NK cell lines as compared with unstimulated cells (Fig. 6A). Consistent with our cytotoxicity results, SHP-2 silencing significantly enhanced the target cell-mediated release of granzyme B, while overexpression of WT or Noonan mutants of SHP-2 significantly suppressed granzyme B release in both cell lines (Fig. 6, B and C). These data indicate that

**FIGURE 5.** Conjugation of NK cells to target cells is not consistently affected by changes in SHP-2 levels. A, The percentage of NK cells bound to targets was determined using a fixed cell conjugation assay (see Materials and Methods). KHYG-1 and 721.221 cells were mixed and pelleted briefly. Cells were incubated together for 0 or 10 min, fixed, and the percentage of NK cells in conjugates was determined by FACS. A representative dot plot of FACS data comparing the incidence of conjugates between 721.221 cells (y-axis) and control (left panels) or SHP-2-silenced (shRNA nos. 1 and 4; right panels) KHYG-1 cells (x-axis) at 0 and 10 min of incubation is shown. The percentage of cells within each quadrant is displayed and incidence of KHYG-1/721.221 conjugates appears in the upper right quadrant. B, Comparison of conjugation to 721.221 target cells between control KHYG-1 cells and those expressing SHP-2 shRNAs nos. 1, 4, or 1 and 4 in combination in a fixed cell conjugation assay. The mean ± SD of triplicate samples from one representative experiment out of two is shown. C, The mean ± SD of the percentage of control NK cells in conjugates at 5–10 min for untransduced parent (control), SHP-2 silenced (shRNA), and SHP-2 overexpressing (SHP-2 WT) KHYG-1 (C) and NKL cells (D) are shown. For each experiment, the data were first divided by the percentage of NK cells conjugated for the 5 min untransduced parent NK cell sample (arbitrarily set to 100%) to generate the percentage of control conjugation. The mean was computed from five separate experiments for KHYG-1 and seven separate experiments for NKL cells. *, p ≤ 0.05 and **, p ≤ 0.01.

SHP-2 suppresses cytolytic granule release in response to target cell conjugation.

**SHP-2 expression diminishes MTOC polarization toward the target cell**

We next hypothesized that the SHP-2-mediated suppression of granzyme B release could be caused by altered polarization of the cytolytic apparatus toward the target cells. To kill target cells, NK cells first polarize the cytoskeleton toward the target cell, which moves the cytolytic granules to the NK immune synapse (NKIS) for release at the interface with the target cell membrane (8). Cytolysis can be abolished by blocking cytoskeletal rearrangement with drugs or inhibitors (55, 70, 71), and genetic mutations in NK cells that prevent granule polarization toward the target cell can severely decrease cytotoxicity (66). Therefore, we analyzed MTOC polarization in NK-target cell conjugates, since MTOC polarization provides a defined focal point for measurement and is a critical initiating event for orienting the cytolytic granules toward the target cell (8). SHP-2-silenced and SHP-2 overexpressing (WT
or E76D) NKL cells were conjugated with 721.221 target cells for 40 min, then fixed and stained for the NKIS (F-actin), granules (perforin), and the MTOC (pericentrin). The conjugates were imaged by fluorescence microscopy, and the average distances between the MTOC and the center of the NKIS were then measured within the NK cells (Fig. 7A). Within each population, we observed a wide distribution in MTOC distance, since each population encompassed a heterogeneous group of NK cells at various stages of conjugation. Although all three populations of NK cells exhibited a similar range in MTOC distances (1–13 μm), the mean MTOC distance was significantly shorter in SHP-2-silenced cells as compared with SHP-2-overexpressing cells (Fig. 7D). A concentration response relationship was observed in which a greater MTOC-NKIS distance was significantly shorter in SHP-2-silenced cells as compared with SHP-2-overexpressing cells (Fig. 7D). A concentration-dependent shift toward more cells exhibiting greater MTOC-NKIS distance was observed: shRNA-silenced < SHP-2-WT-expressing < SHP-2-E76D-expressing NK cells. These data demonstrate that SHP-2 suppresses polarization of the cytolytic machinery toward the NKIS in a concentration-dependent manner.

Discussion

In this study, we describe a previously unknown KIR-independent role for the SHP-2 phosphatase in suppressing the general responsiveness of NK cells toward tumor target cells. Using shRNA silencing and overexpression of WT and gain-of-function SHP-2 Noonan mutants in NK-like cell lines, we found that SHP-2 suppresses both cytolytic activity and cytokine production in a concentration-dependent manner. This role for SHP-2 was observed in two distinct NK-like cell lines, indicating that SHP-2 likely plays a general role in suppressing the activation potential of NK cells. Furthermore, SHP-2 silencing increased cytotoxicity even when KIR were engaged (Fig. 4), indicating that the depletion of cellular levels of SHP-2 reduces the general NK cell activation threshold in a KIR-independent manner. Since knockdown and overexpression of SHP-2 affected MTOC polarization and granzyme B release in both NK cell lines tested, we conclude that SHP-2 is playing an inhibitory role at the level of pathways controlling cytoskeleton rearrangement and granule exocytosis. Alternatively, we observed suppressed conjugation when SHP-2 was overexpressed, but no effect when SHP-2 was silenced. This result indicates that SHP-2 may also inhibit the pathways controlling adhesion to target cells, but only when overexpressed at nonphysiological concentrations.

SHP-2, through control of FAK and Rho, has been shown to both promote and inhibit remodeling of the actin cytoskeleton (72). Therefore, we postulate that SHP-2 suppresses NK function through blocking the polarization of the cytoskeleton in response to target cell conjugation. Our data demonstrating greater impacts when expressing the gain-of-function Noonan mutants in most assays also suggest that SHP-2 catalytic activity plays a role in the
Our previous work using dominant-negative SHP-2 constructs identified a role for SHP-2 in KIR-dependent inhibition of NK cell activation (27). However, results from those studies were not definitive since overexpressed dominant-negative SHPs would competitively block the binding of any other inhibitory effector molecules to phosphorylated ITIMs of KIR. Using shRNA-mediated silencing of SHP-2 in our present study, we were surprised to still observe potent inhibition through a mutant KIR (YF) that was previously shown to rely solely on SHP-2 recruitment for function (Fig. 4), B and C). Our biochemical evidence, however, showed that SHP-2 can still be recruited to tyrosine phosphorylated KIR in these shRNA-transduced cells, despite significant reductions in expression of the phosphatase (Fig. 4D). Therefore, it is plausible that only minimal levels of SHP-2 protein are required to mediate KIR inhibition. Our previous work showed that SHP-2 is constitutively, albeit weakly, associated with unphosphorylated KIR (27), which may reflect a mechanism by which low cellular levels of SHP-2 can still efficiently contribute to inhibitory function by the receptor. However, we cannot at this time rule out the possibility that SHP-2 is dispensable for KIR-mediated inhibition of cytotoxicity and that another effector enzyme is contributing to KIR-mediated inhibition, particularly inhibition by the YF mutant, which cannot recruit SHP-1. Note that we used NK cells that lacked KIR expression for all experiments assessing general NK cell functions (Figs. 2, 3, and 5–7), further demonstrating that the SHP-2 effects are KIR-independent.

We also found that higher SHP-2 levels reduced IFN-γ responses by NK cells in response to target cells, but not in response to crosslinking of the ITAM-dependent activating receptor NKp44, the DAP10-dependent receptor NKG2D, or the adhesion receptor LFA-1 with plate-bound Abs (Fig. 3). Although the phosphoprotein targets of SHP-2-dependent suppression of NK cell activation are unknown, these data suggest that the ITAM- or DAP10-mediated receptor signaling pathways are not directly impacted. Multiple NK cell receptors, including adhesion and activating receptors, are engaged upon target cell conjugation, and these receptors activate a wide array of signaling pathways and biological events. We attempted coengaging both activating and adhesion receptors to mimic target cell binding, but IFN-γ production in response to coengagement was still not significantly impacted by changes in SHP-2 levels (Fig. 3C). It must be stressed, however, that Ab-mediated engagement is biophysically distinct from the dynamic receptor-ligand engagement occurring during target cell conjugation. The NKIS is known to functionally segregate signaling molecules at the target cell interface (8, 10), which may not be mimicked by immobile plate-bound Abs. The disparate results also provide further evidence that SHP-2 imparts significant impact on NKIS-directed cytoskeletal rearrangement in response to target cell conjugation (as in Fig. 7), whereas Ab-mediated stimulation may not require such a directed response and may therefore be insensitive to alterations in SHP-2 levels. We cannot rule out the possibility that an inhibitory receptor other than KIR is facilitating SHP-2-mediated inhibition, but if such a receptor exists, it would be independent of MHC-I ligand, which is lacking on the 721.221 target cells used in our experiments. Dissection of the receptor pathways and biochemical events through which SHP-2 is acting will be the focus of our future studies.

We were surprised that NK-target cell conjugation was not enhanced by SHP-2 silencing in either cell line (Fig. 5), since this phosphatase has been reported to modulate integrin-dependent adhesion in other cell types (73, 74). In contrast to SHP-2 silencing, SHP-2 overexpression significantly reduced conjugation of KHYG-1 and NKL cells to target cells. These results indicate that physiologic levels of SHP-2 are not negatively influencing target cell conjugation, whereas these levels are blunting cytotoxicity, IFN-γ production, MTOC polarization, and granzyme B release. The literature describing impacts of SHP-2 on integrin functions contains inconsistencies, which may be due to the expression of dominant-negative or truncated forms of SHP-2 in many of these studies (75, 76). Dominant-negative SHP-2 lacks catalytic activity, but may still act as an adaptor to recruit SHP-2 substrates/binding partners without dephosphorylating them. Furthermore, some early studies used SHP-2 knockout mouse embryonic fibroblasts in which a truncated form of SHP-2 protein lacking the N-terminal SH2 domain was still expressed (77), thereby potentially disrupting the autoinhibited state to generate a gain-of-function mutation suppression of cytotoxicity (Fig. 2), granule release (Fig. 6), and MTOC polarization (Fig. 7).
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(78). In view of these findings, these early studies may have established mutations with unanticipated biological impacts. In our studies, we generated a battery of NK cells expressing a range of SHP-2 concentrations from <10% of normal (shRNA expression) through >500% of normal expression of either WT or gain-of-function forms of the phosphatase to demonstrate a significant impact only under overexpressed conditions. We conclude that SHP-2 does not suppress NK cell adhesion to susceptible tumor target cells under physiological expression conditions.

Overall, this report demonstrates a novel, KIR-independent role for SHP-2 as a general inhibitor of NK cell responsiveness. SHP-2 suppressed MTOC polarization and granyme B release in response to target cells, and this directly correlated with decreased cytolytic activity in a concentration-dependent manner. In support of previous observations by Wülfing et al. (11), we show a direct connection between MTOC polarization and cytotoxicity by NK cells. MTOC polarization is required for target-directed granule release, indicating that SHP-2 likely suppresses granyme B release indirectly due to an earlier (more upstream) block of MTOC polarization. Additionally, this work indicates that therapeutic agents that limit SHP-2 levels or catalytic function may be beneficial to stimulate NK cell activity in patients with cancer or viral infections. Furthermore, our data with the gain-of-function mutants suggest that NK cells may exhibit suppressed function in Noonan syndrome patients, where these mutations where originally identified.

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Disclosures

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