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SHP-1- and Phosphotyrosine-Independent Inhibitory Signaling by a Killer Cell Ig-Like Receptor Cytoplasmic Domain in Human NK Cells[1]

Sei-ichi Yusa, Tracey L. Catina, and Kerry S. Campbell[2]

Killer cell Ig-like receptors (KIR) are MHC class I-binding immunoreceptors that can suppress activation of human NK cells through recruitment of the Src homology 2-containing protein tyrosine phosphatase-1 (SHP-1) to two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains. KIR2DL4 (2DL4; CD158d) is a structurally distinct member of the KIR family, which is expressed on most, if not all, human NK cells. 2DL4 contains only one ITIM in its cytoplasmic domain and an arginine in its transmembrane region, suggesting both inhibitory and activating functions. While 2DL4 can activate IFN-γ production, dependent upon the transmembrane arginine, the function of the single ITIM of 2DL4 remains unknown. In this study, tandem ITIMs of KIR3DL1 (3DL1) and the single ITIM of 2DL4 were directly compared in functional and biochemical assays. Using a retroviral transduction method, we show in human NK cell lines that 1) the single ITIM of 2DL4 efficiently inhibits natural cytotoxicity responses; 2) the phosphorylated single ITIM recruits SHP-2 protein tyrosine phosphatase, but not SHP-1 in NK cells; 3) expression of dominant-negative SHP-1 does not block the ability of 2DL4 to inhibit natural cytotoxicity; 4) surprisingly, mutation of the tyrosine within the single ITIM does not completely abolish inhibitory function; and 5) this correlates with weak SHP-2 binding to the mutant ITIM of 2DL4 in NK cells and a corresponding nonphosphorylated ITIM peptide in vitro. These results reveal new aspects of the KIR-inhibitory pathway in human NK cells, which are SHP-1 and phosphotyrosine independent. *The Journal of Immunology,* 2002, 168: 5047–5057.

N atural killer cells constitute, on average, 10–15% of PBL from healthy individuals, and are found in peripheral tissues, including the spleen, liver, peritoneal cavity, and placenta in humans (1). NK cells modulate the adaptive and innate immune systems by secreting various cytokines (2), and can also spontaneously lyse a variety of abnormal cells altered by virus infection or malignant transformation through either natural cytotoxicity or CD16-mediated Ab-dependent cell-mediated cytotoxicity (3). NK cells target those abnormal cells that have down-regulated MHC class I (MHC-I)[3] molecules, which are cell surface proteins normally expressed on nearly every cell of the body. NK cells recognize this loss by expressing several inhibitory receptors that bind MHC-I, including the lectin-like dimers, CD94/NKG2A (CD159a) (4) and the rodent lymphocyte Ag 49 complex (Ly-49)

receptors (5), as well as the Ig superfamily receptors, including human Ig-like transcript 2/leukocyte Ig-like receptor 1 (CD85j) (6) and killer cell Ig-like receptors (KIRs; CD158) (7, 8).

KIRs constitute a family of slightly polymorphic receptors with distinct MHC-I-binding profiles for classical HLA-A, HLA-B, and HLA-C molecules. Most KIRs with two Ig domains (KIR2DL) recognize subsets of the HLA-C allotypes, whereas those expressing three domains (KIR3DL) recognize HLA-A and HLA-B (8). 2DL4 is a unique receptor family member that does not recognize classical HLA molecules, but instead reportedly recognizes the nonclassical HLA-G (9, 10). HLA-G is normally expressed only on fetal-derived trophoblast cells that invade the maternal decidua in pregnant women (11).

Most inhibitory KIR have two cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM) with the consensus sequence of (I/V)xYxx(L/V) (12) that are critical for inhibitory function. ITIM sequences are found in many inhibitory receptors. Phosphorylated ITIM tyrosine residues recruit Src homology 2 (SH2)-containing protein tyrosine phosphatases (such as SHP-1, SHP-2, and SH2-containing inositol 5′-phosphatase (SHIP)) to mediate inhibition. Considerable experimental evidence indicates that the SHP-1 protein tyrosine phosphatase is involved in KIR inhibition. This evidence includes SHP-1 binding and activation by KIR ITIM phosphopeptides (12–15) and recruitment of SHP-1 to tyrosine-phosphorylated KIR in NK cells (13–20). Furthermore, KIR inhibition is prevented by expression of catalytically inactive SHP-1 in NK cells (16, 18), and by comparison, severely impaired inhibitory function of ITIM-containing Ly-49A receptors has been observed in NK cells from SHP-1-deficient motheaten mice (21). To date there is no report suggesting involvement of SHIP in KIR-inhibitory function (17). In contrast, phosphopeptides corresponding to the two ITIMs of KIR have also been shown to bind SHP-2 protein tyrosine phosphatase from cell lysates (14, 17), and chimeric forms of KIR expressed in B cells and mast cells were shown to recruit

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[3] Abbreviations used in this paper: MHC-I, MHC class I; EGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell Ig-like receptor; HC, H chain; SH2, Src homology 2; SHIP, SH2-containing inositol 5′-phosphatase; SHP, SH2-containing protein tryosine phosphatase.

SHP-2 after cross-linking with activating receptors (22). Although the role of SHP-2 in KIR function is poorly defined, particularly in NK cells, these findings suggest that SHP-2 may also contribute to inhibition.

In addition to unique HLA-G-binding specificity, 2DL4 (CD158d) is a structurally distinct member of the KIR family designated as a type II KIR (23). The 2DL4 is reportedly the only KIR expressed on most, if not all, human NK cells. HLA-G recognition suggests that 2DL4 acts as an inhibitory receptor to block maternal NK cell-mediated attack of the fetus. 2DL4 possesses a number of unique structural elements, which include 1) an extracellular domain consisting of D0 and D2 Ig-like domains (a feature shared only by KIR2DL5) (23), 2) a cytoplasmic domain possessing only a single ITIM sequence, and 3) a transmembrane domain containing a charged arginine residue (24). By analogy, activating forms of KIR with truncated cytoplasmic domains that lack functional ITIMs also exist and associate noncovalently with a homodimer of the activating accessory protein DAP12 via similar basic transmembrane residue in the activating KIR and an acidic transmembrane residue in DAP12 (3, 25–27). Therefore, the presence of both an ITIM and a basic transmembrane residue suggests that 2DL4 may have both inhibitory and activating functions. A recent report demonstrated that the transmembrane arginine enabled the induction of IFN-γ upon cross-linking 2DL4 in resting NK cells (28). However, nothing is known about the function of the single ITIM of 2DL4.

In this study, we examined the inhibitory functions of the 2DL4 cytoplasmic domain. To study this domain in isolation, we generated a chimeric receptor of the 2DL4 cytoplasmic domain fused to the extracellular and transmembrane domains of another KIR, named KIR3DL1 (3DL1). Retroviral transduction in NK cell lines allowed us to directly compare the functional and biochemical properties of this chimeric receptor (3DL1/L4) containing a single ITIM with that of 3DL1, which contains two ITIMs. To our surprise, both receptors exhibited similar capacity to inhibit cytotoxicity, even though 3DL1/L4 recruited only SHP-2, while 3DL1 recruited both SHP-1 and SHP-2. In addition, we found that mutation of the only ITIM tyrosine in 3DL1/L4 did not completely eliminate inhibitory function. Further analysis revealed that the mutant ITIM could still weakly bind SHP-2 in a phosphotyrosine-independent manner.

Materials and Methods

Cells and culture

All cell culture was performed at 37°C in 7% CO_2 humidified atmosphere. The IL-2-dependent NK-like cell lines, NK3.3 (a gift from J. Kornbluth, St. Louis University School of Medicine, St. Louis, MO) and NK-92 (a gift from C. Lutz, University of Iowa, Iowa City, IA), were maintained in α-MEM medium (Life Technologies, Rockville, MD) containing 12.5% FBS (HyClone Laboratories, Logan, UT), 12.5% horse serum (Life Technologies), 2 mM L-glutamate (Life Technologies), 100 μg/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 μM 2-ME (Fisher, Pittsburgh, PA), 2 mM folic acid (Sigma-Aldrich, St. Louis, MO), 20 mM myoinositol (Sigma-Aldrich), and supplemented with 2% culture supernatant of J558L cells transfected with the human IL-2 gene (NK-92; kindly provided by A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland) or with 15% Lymphocult (NK3.3; Biotest Diagnostics, Danville, NJ). Cells were passed with fresh IL-2 or Lymphocult every 4 days. The murine mastocytoma, P815, was cultured in DMEM medium (Life Technologies) containing 10% FBS (HyClone Laboratories), 2 mM L-glutamate (Life Technologies), 100 μg/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), and 50 μM 2-ME (Fisher).

KIR cDNA constructs

KIR cDNA constructs were ligated into the bicistronic retroviral expression vector, pBMN-IRES-EGFP (29) (generously provided by G. Nolan, Stanford University, Stanford, CA), to produce recombinant retrovirus for generation of NK cell lines with stably integrated cDNA. A 1.35-kb cDNA fragment encoding human 3DL1 (NKAT3) was obtained from M. Colonna (Washington University, St. Louis, MO) and subcloned into the pBMN-IRES-EGFP vector using *Bam*HI and *Not*I restriction sites. To generate the chimeric 3DL1/L4 receptor, a 1.15-kb DNA fragment encoding human 2DL4 cDNA (KIR103, AF002981) in pcDNA3 (Invitrogen, San Diego, CA) was provided by M. Colonna, and the cytoplasmic domain was amplified by PCR using the following primers: sense containing *Bsp*HI site (underlined), 5′-CTGCTG<u>TCATGA</u>ACCAAGAGCCTGC-3′; antisense containing *Xho*I site, 5′-ACTGTA<u>CTCGAG</u>CCTTCAGATTCCAGCTGCTG-3′. To make the 3DL1/L4 chimeric receptor, a cDNA encompassing the extracellular and transmembrane domains of 3DL1 cDNA was amplified by PCR using the sense primer (*Bam*HI), 5′-TCGACT<u>GGATCC</u>ACCATGTCGCTCATGGTCGTCAGCATG-3′, and the antisense primer (*Bsp*HI), 5′-AGGCTCTTGGTT<u>CATGA</u>CAGCAGCAT-3′. The *Bam*HI-*Bsp*HI fragment of 3DL1 and the *Bsp*HI-*Xho*I fragment of 2DL4 were cloned into the pBMN-IRES-EGFP vector. To mutate the tyrosine residue in the ITIM motif of 2DL4 (3DL1/L4/Y277F), delete the sequence after the ITIM (3DL1/L4/Y277F/282HΔ), delete the sequence just before the ITIM (3DL1/L4/272PΔ), and remove the majority of the cytoplasmic domain (KIR3DL1/255EΔ), the 3DL1/L4 chimeric cDNA was used as a template for PCR, and the following oligonucleotide primers were used, respectively: Y277F sense, 5′-AGGAGGTGACATTCGCACAGTTGGATCA-3′; Y277F antisense, 5′-TGATCCAACTGTGCGAATGTCACCTCCT-3′; 282HΔ antisense (*Not*I), 5′-GATCA<u>GCGGCCGC</u>TCAGTGATCCAACTGTGCG-3′; 272PΔ antisense (*Not*I), 5′-ATCGA<u>GCGGCCGC</u>TAAGGGTCTTGTTCATCAGAGT-3′; 255EΔ antisense (*Not*I), 5′-TAGACT<u>GCGGCCGC</u>TATTTGTTGGAGCACCAGAG-3′. All PCR was performed using Platinum Pfx DNA polymerase (Life Technologies), and the integrity of all constructs was confirmed by sequencing in the Fox Chase Cancer Center Automated DNA Sequencing Facility (PE Applied Biosystems, Shelton, CT). The cDNA encoding mouse IgM H chain (HC) in pBMN-IRES-EGFP was kindly provided by R. Hardy (Fox Chase Cancer Center, Philadelphia, PA).

Retroviral transduction into NK-92 or NK3.3 cell lines

The packaging cell line, Phoenix-Amphotropic (provided by G. Nolan), was transfected with the pBMN-IRES-EGFP vector containing one of the KIR genes or a control gene using Lipofectamine Plus reagent (Life Technologies). Supernatants of these transfected cells grown in serum-free Opti-MEM medium (Life Technologies) for 2 days were cocultured with NK-92 or NK3.3 cell lines for 8 h in the presence of Lipofectamine Plus reagent, and then complete α-MEM medium containing IL-2 was added for 3 days before sorting. At that time, depending upon the cell types and genes used, ~1–10% of the infected NK cells efficiently expressed enhanced green fluorescent protein (EGFP), as assessed by cytofluorometry. The transduced NK cells were sorted for expression of EGFP or 3DL1 (3DL1-specific PE-conjugated DX9 mAb; BD PharMingen, San Diego, CA) in the Fox Chase Cancer Center Cell Sorting Facility. These sorted cells stably and coordinately expressed both genes for at least 1 mo. Uniform exogenous 3DL1 expression in >95% of the transduced cell population was confirmed at the time of every experiment using PE-conjugated DX9 mAb by flow cytometric analysis on a FACScan analyzer (BD Biosciences, Mountain View, CA). Expression of EGFP and exogenous mouse IgM HC had no effect on growth or cytotoxicity responses in NK-92 cells (data not shown). In some experiments, KIR constructs were expressed in a modified pBMN vector in which the internal ribosome entry site (IRES) and EGFP DNA sequences were removed (pBMN-NOEGFP). This vector resulted in even greater stability of expression for longer periods of time. Cytotoxicity, KIR-mediated inhibition, and growth rates were identical in NK-92 cells transduced with recombinant retrovirus generated using either vector (data not shown).

Redirected cytotoxicity assay

NK-92 cells were cultured with fresh IL-2-containing medium on the day before assay, unless redirected cytotoxicity was induced by addition of Abs directed toward NKp46 or 2B4. NK cell lines were tested for natural cytolytic activity against the FcγRII/III+ P815 murine mastocytoma cell line in a 4-h ^{51}Cr release assay in 200 μl medium/well (complete α-MEM medium lacking IL-2 or Lymphocult). The P815 target cells (2 million) were labeled with 100 μCi ^{51}Cr (5 mCi/ml; stock product 2030B; NEN Life Science, Boston, MA) in 200 μl 100% FBS for 90 min and incubated

with NK cells in V-bottom 96-well plates (Costar, Cambridge, MA). Spontaneous release and maximal release of ^{51}Cr were determined by incubating target cells in medium alone or 1% Triton X-100, respectively. Each assay condition was always performed in triplicate. The percentage of specific lysis was determined as follows: ((mean cpm experimental release − mean cpm spontaneous release)/(mean cpm maximal release − mean cpm spontaneous release)) × 100. For activation through specific receptors, anti-NKp46 (9E2; provided by M. Colonna) or anti-2B4 (158; provided by M. Colonna) mAb (10 μl hybridoma supernatant/well) were mixed with P815 cells in V-bottom 96-well plates, and they were incubated for 5 min before effector cell addition. To engage KIRs, purified anti-3DL1 mAb, DX9 (purchased from BioSource (Camarillo, CA) or purified from the hybridoma kindly provided by L. Lanier (University of California, San Francisco, CA)), was added at 1 μg/ml or at the indicated concentrations. The anti-CD56 mAb, B159.5.2 (purchased from BD PharMingen or purified from the hybridoma kindly provided by B. Perussia (Thomas Jefferson University, Philadelphia, PA)), was used as a control at 1 μg/ml.

Pervanadate treatment and cell lysis

Pervanadate treatment of cells was performed, as previously described (13). NK cells (up to 80 million/sample) were washed three times in HBSS (Life Technologies), resuspended in 1 ml/sample HBSS, and preincubated for 10 min at 37°C. Cells were then incubated for 5, 10, or 15 min without (0 min) or with pervanadate (100 μM Na$_3$VO$_4$ plus 100 μM H$_2$O$_2$) and lysed for 30 min on ice in 0.5–1 ml/sample of lysis buffer, containing 1% Triton X-100 (Surfact-Amps; Pierce, Rockford, IL), 150 mM NaCl (Fisher), 10 mM Tris-HCl (pH 7.5) (Fisher), 2 mM Na$_3$VO$_4$ (from 100× stock boiled 5 min before addition), 0.4 mM EDTA (Fisher),10 mM sodium fluoride (Sigma-Aldrich), 1 mM Pefabloc (Roche, Indianapolis, IN), and 1 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor (Sigma-Aldrich). Lysates were cleared of nuclear/cytoskeletal components by centrifugation at 20,800 × g for 15 min at 4°C.

Immunoprecipitation and immunoblotting

Lysates were precleared for 30 min at 4°C with protein G-coupled agarose (30 μl 50% slurry per sample; Upstate Biotechnology, Lake Placid, NY) and then precleared again for 30 min at 4°C with control Ab (mouse mAb, IgG)-coupled protein G-agarose. CD56 and KIRs were sequentially immunoprecipitated for 90 min at 4°C with B159.5.2 and DX9 mAbs (2.5 μg/ each sample precoupled to 30 μl protein G-agarose). All immunoprecipitates were washed five times with ice-cold 0.1% Triton X-100 buffer (same components as lysis buffer) and resuspended in Laemmli reducing sample buffer (10% DTT). Immunoprecipitated samples were boiled for 3 min before separation on discontinuous 10% SDS-PAGE. Proteins were electrophoretically transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and blocked with 5% skim milk (ACME, Salt Lake City, UT) in TBST buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20). Blocked membranes were probed either directly with HRP-coupled 4G10 mAb (anti-phosphotyrosine, 1:10,000; Upstate Biotechnology) or initially with rabbit polyclonal anti-SHP-1 (1 μg/ml; Upstate Biotechnology) or anti-SHP-2 (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) Abs and secondarily with HRP-coupled protein G (1:10,000; Calbiochem, La Jolla, CA). Immunoblotted proteins were visualized by chemiluminescence using the ECL detection reagents (Amersham, Arlington Heights, IL) or SuperSignal (Pierce). Membranes were stripped of Ab by incubating for 30 min at 50°C in 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7), according to a protocol supplied by Amersham. Stripped blots were reprobed after blocking. For whole cell lysates, 0.5 × 10^6 cells were washed with PBS, directly resuspended in Laemmli reducing sample buffer, and probe sonicated for 7 s before addition to SDS-PAGE.

Vaccinia virus infections

Purified recombinant vaccinia virus preparations generated with the plasmid pSC65 containing cDNA of mouse SHP-1 or SHP-1 with the cysteine residue 453 mutated to serine, as previously described (18), were kindly provided by E. O. Long (National Institutes of Health, Rockville, MD). NK cell infection was performed as previously described (30). Aliquots of the recombinant virus preparations were dispersed by water-bath sonication and stored at −70°C before use. The titer of virus stocks was determined by plaque assay, as described (31) (wild-type SHP-1, 1 × 10^{10} PFU/ml; C453S SHP-1, 3 × 10^9 PFU/ml). NK-92 cells were stimulated with human IL-2 containing complete α-MEM medium 1 day before the vaccinia virus infection. NK-92 cells (>95% viable) were washed three times with the prewarmed infection medium (0.5% BSA, 1× nonessential amino acids (Life Technologies), 2 mM L-glutamine, 2% human rIL-2 supernatant (as described above) in RPMI 1640) and resuspended in the prewarmed in-

fection medium at 10^6 cells per infection point. Cells were incubated with virus at 37°C in a 5% CO$_2$ incubator for 1 h, washed once with 10 ml complete α-MEM medium, resuspended in 1 ml IL-2 containing complete α-MEM medium per experimental point, and incubated for 2 h to allow for SHP-1 expression. Infected and mock-infected control cells were simultaneously plated as effector cells in ^{51}Cr release cytotoxicity assays, as described above. Vaccinia virus infections were monitored for SHP-1 protein expression by immunoblot analysis of the infected cells within the experiment. Viability of the cells infected was monitored by trypan blue staining at each experimental point.

Peptides

The tyrosine-phosphorylated (p) and nonphosphorylated KIR-derived peptides, CEEVTYAQLDH (YAQL), CEEVT(p)YAQLDH (pYAQL), CDTI LYTELPN (YTEL), and CDTIL(p)YTELPN (pYTEL), were purchased from Sigma-Genosys (The Woodlands, TX). Peptides were synthesized by Sigma-Genosys with a carboxyl-terminal amide and purified by reverse-phase HPLC. Purity was determined to be >95%, as assessed by analytical HPLC and mass spectrometry. The YAQL peptides correspond to N-terminal ITIM of 3DL1 or single ITIM of 2DL4, while the YTEL peptides coincide with C-terminal ITIM of 3DL1. Peptides were coupled to SulfoLink coupling gel (Pierce) for 1 h at room temperature (pH 8.5), according to the manufacturer's instructions. Coupling efficiency from the supernatant was determined by monitoring OD$_{280}$ absorbance. Peptide beads (15 μg/precipitation) were used in precipitation assays immediately after coupling, as described above.

Results

Establishment of NK cell lines expressing functional KIR

Studies of mutant KIR are hampered by the inability to transfect human NK cell lines or primary clones with standard mammalian expression vectors. Therefore, we sought to establish a retroviral transduction system to introduce exogenous cDNAs and still maintain functional attributes of the cell line. To this end, we used the bicistronic retroviral vector, pBMN-IRES-EGFP, in combination with Phoenix Amphotropic packaging cells to generate recombinant retrovirus. pBMN-IRES-EGFP is capable of generating retrovirus that can coordinately express a cloned cDNA and EGFP simultaneously in transduced cell lines through the incorporation of an intervening IRES sequence. In experiments testing retroviral transduction of several transformed human NK-like cell lines, the human NK-92 lymphoma line demonstrated the highest susceptibility to transduction (5–12% of IL-2-stimulated cells).

The NK-92 cell line is an IL-2-dependent NK-like cell line. Notably, NK-92 does not express FcγRIII (CD16), allowing the use of intact Abs instead of F(ab′)$_2$ to manipulate surface receptors. NK-92 serves as an excellent model system to study KIR functions, because it also exhibits strong target cell cytotoxicity and expresses no endogenous 3DL1, allowing the 3DL1 cDNA-transduced cells to be specifically stained with anti-3DL1 mAb, DX9, by flow cytometry (as in Fig. 1C). Importantly, transduction of NK-92 cells using this system does not affect growth or cytotoxicity responses (data not shown). The transduced NK-92 cells stably expressed cDNA products for at least 1 mo using pBMN-IRES-EGFP and for >3 mo using retrovirus generated with a modified version of the plasmid that lacks the IRES and EGFP elements (data not shown), which was used in some studies. No differences in biological or biochemical results were observed in the presence or absence of EGFP.

To examine the potential inhibitory function of the 2DL4 cytoplasmic domain in isolation from this receptor's charged transmembrane segment, we generated a chimeric receptor construct in which the cytoplasmic domain of 2DL4, including the single ITIM, was fused with the extracellular and transmembrane domains of 3DL1 (Fig. 1, A and B). This allowed us to directly compare inhibitory properties of the 2DL4 cytoplasmic domain with those of 3DL1 and avoid any positive influences from the 2DL4 transmembrane domain. Several additional constructs were
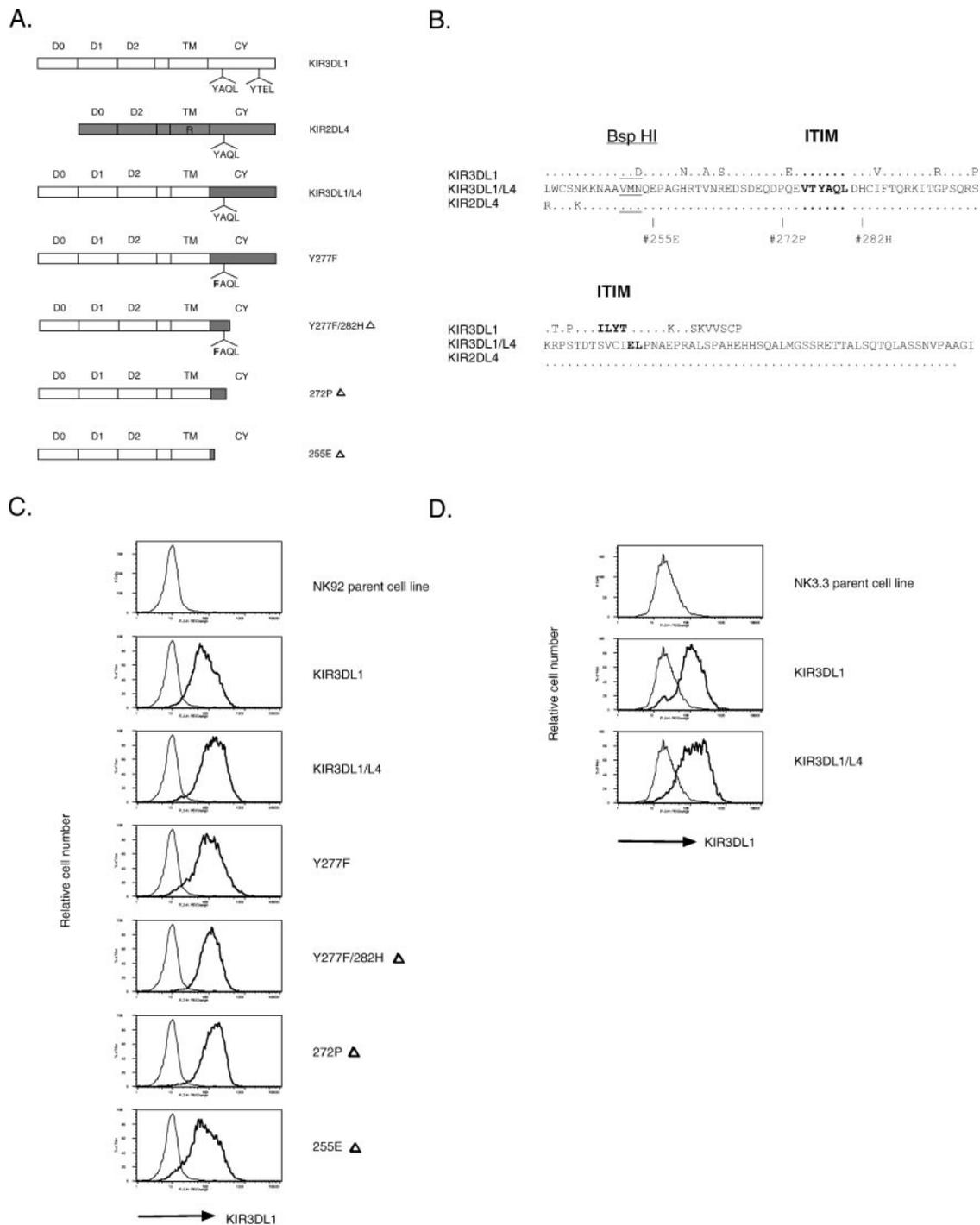
FIGURE 1. Map of gene constructs used in these studies and establishment of stable transduced NK cell lines. *A*, Schematic representations of the various chimeric constructs between 3DL1 (open boxes) and 2DL4 (shaded boxes) that were generated. D0, D1, and D2 are highly conserved Ig-like domains in the KIR family. TM and CY represent transmembrane and cytoplasmic domains, respectively. Tyrosine-based ITIM motifs (YAQL and YTEL) are marked in the cytoplasmic domains. Some of the constructs possess the mutant ITIM tyrosine (Y277F). These genes were introduced into a retrovirus-based plasmid vector, pBMN-IRES-EGFP. *B*, Amino acid sequence of the cytoplasmic domains of the KIR constructs. The 3DL1 and 2DL4 were fused at the common methionine in the cytoplasmic domain by introducing a *Bsp*HI restriction site (underlined), which encodes the amino acids VMN. The dots indicate identity of either 3DL1 or 2DL4 to the chimera. Boldface sequences represent the positions of ITIMs. The last amino acids of KIR3DL1/255EΔ, 3DL1/L4/272PΔ, and 3DL1/L4/282HΔ are labeled with #. *C*, Flow cytometric analysis of 3DL1-positive transduced cells. The 3DL1-negative NK cell line, NK-92, was introduced with those various constructs shown as in *A* by retroviral transduction. The data show staining for 3DL1 with PE-labeled DX9 mAb and background staining of the 3DL1-negative parent cell line (thin lines). *D*, Flow cytometric analysis of NK3.3 cells transduced with either 3DL1 or 3DL1/L4 chimera cDNA. The data show staining with PE-labeled DX9 mAb.

generated to study the effect of ITIM mutation (Y277F) and deletion of various portions of the cytoplasmic domain (Y277F/282HΔ, 272PΔ, 255EΔ) on the inhibition (Fig. 1, *A* and *B*). These

receptors were expressed on the surface at similar levels in retrovirus-transduced NK-92 cells (Fig. 1*C*) and another IL-2-dependent human NK cell line, NK3.3 (Fig. 1*D*).
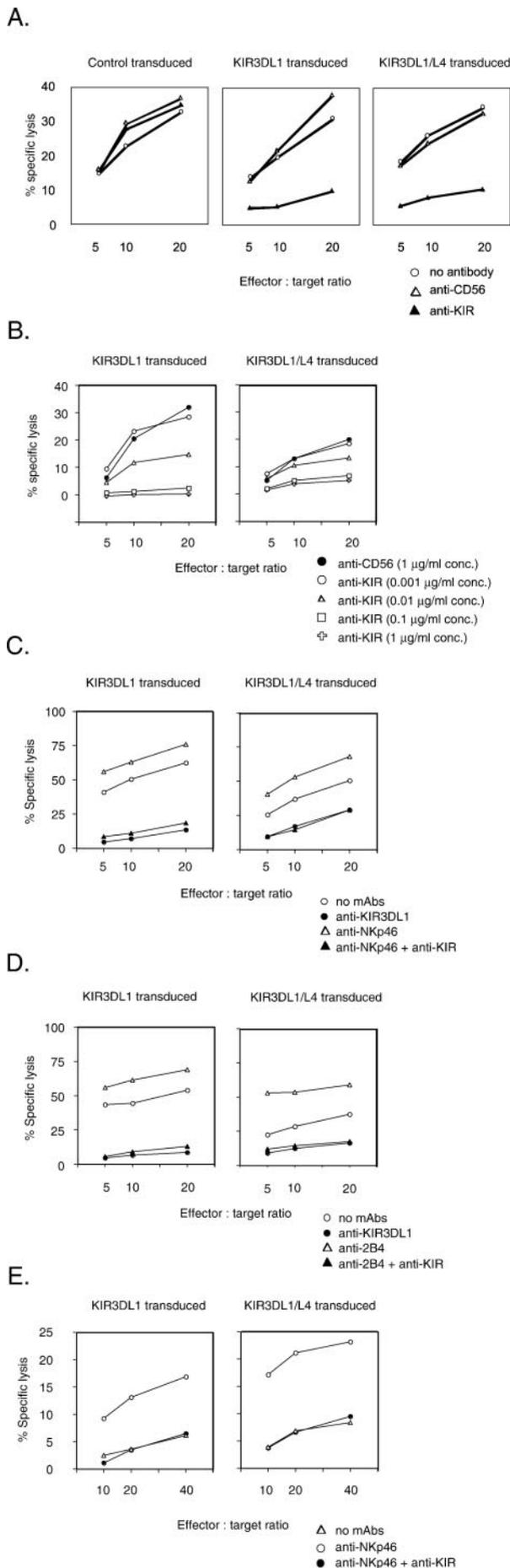
Strong inhibition of natural cytotoxicity by the single ITIM of 2DL4

We tested the inhibitory capacity of the transduced KIR receptors using a redirected cytotoxicity assay against the murine mastocytoma cell line, P815. P815 expresses FcγRII/III that can interact with the Fc portion of mAbs bound to the surface of NK cells, thereby effectively making them surrogate ligands in redirected cytotoxicity assays. HLA-B*2702-expressing 721.221 cells could not be used as targets to engage 3DL1, because background inhibition resulted from HLA-B*2702 binding by the Ig-like transcript 2/leukocyte Ig-like receptor 1 on NK-92 cells (data not shown).

The wild-type 3DL1- and 3DL1/L4-transduced NK-92 cells were tested for their cytotoxicity against P815 in the presence of anti-3DL1 or Ab toward the negative control surface molecule, CD56 (Fig. 2A). Interestingly, 3DL1/L4 chimera with only a single ITIM strongly inhibited the cell lysis to a level comparable with that of 3DL1 with two ITIMs. Cytotoxicity of control IgM-transduced NK-92 cells was not affected in the presence of anti-KIR mAbs (Fig. 2A, *left panel*), demonstrating fidelity of the assay system. The inhibition by both KIRs also demonstrates comparable dose dependency toward anti-3DL1 mAb (Fig. 2B).

NK-92 cells express several endogenous activating receptors, such as NKp46, NKp44, and 2B4 (data not shown). Therefore, we tested whether 3DL1/L4 chimera inhibits the immunoreceptor tyrosine-based activation motif (ITAM)-dependent activation mediated by NKp46 or the ITAM-independent activation mediated by 2B4. As shown in Fig. 2, *C* and *D*, cytotoxicity of NK-92 cells was strongly augmented by anti-NKp46 or anti-2B4 mAbs, and comparable suppression of activation was observed when combined with anti-3DL1 mAb to engage either 3DL1 (*left panel*) or 3DL1/L4 (*right panel*). These results indicated that the two ITIMs of 3DL1 and one ITIM of 2DL4 can comparably block activation of cytotoxicity mediated by either ITAM-dependent or ITAM-independent pathways. To confirm our observations in another NK cell line, both receptors were transduced into the NK3.3 cell line (Fig. 1D), and cytotoxicity of NK3.3 against P815 was modestly induced by anti-NKp46. Again, coengagement of NKp46 with either 3DL1 or 3DL1/L4 chimera (Fig. 2E) inhibited the cytotoxicity by NKp46 to an equivalent extent in NK3.3.

FIGURE 2. Comparable inhibition of cytotoxicity by engagement of either 3DL1 or 3DL1/L4. *A*, Redirected cytotoxicity assay using NK-92 cells transduced with mouse IgM HC (*left panel*), 3DL1 (*middle panel*), or 3DL1/L4 chimera (*right panel*) constructs. The stable transduced NK-92 cells were tested for their abilities to lyse P815 in the presence of 1 μg/ml anti-3DL1 mAb (DX9; ▲) or the anti-CD56 mAb (B159.5.2; △) as a control. *B*, Comparable dose-dependent inhibition was observed upon engaging 3DL1 (*left panel*) or 3DL1/L4 (*right panel*) using the anti-3DL1 mAb in NK-92-transduced cells. The anti-CD56 mAb was used as a control (●) at the maximal dose. *C*, The activation of cytotoxicity through NKp46 was efficiently blocked by the single ITIM of 2DL4 in NK-92-transduced cells. Redirected cytotoxicity assay was performed in the presence of 1 μg/ml anti-3DL1 mAb alone (●) or 10 μl supernatant from the anti-NKp46 mAb hybridoma (9E2) alone (△) or in combination with the same concentrations of both mAbs (▲). *D*, The enhanced activation by 2B4 was blocked by the single ITIM of 2DL4 in NK-92-transduced cells. A total of 10 μl supernatant from the anti-2B4 mAb hybridoma (158) was added per well of the redirected cytotoxicity assay, as described in *C*. *E*, The single ITIM of 2DL4 efficiently inhibited the activation by NKp46 in another NK cell line, NK3.3. The NK3.3 cell line was transduced with either 3DL1 (*left panel*) or 3DL1/L4 (*right panel*) constructs. NK3.3 cytotoxicity was induced in the presence of anti-NKp46 mAb (○) and inhibited when combined with anti-3DL1 mAb (●) as compared with the lack of cytotoxicity in the absence of Abs (△).

SHP-2 is selectively recruited to the 2DL4 cytoplasmic domain

The preceding results indicated that the single ITIM of 2DL4 is effective in mediating inhibition. SHP-1 has been shown to be recruited to classical KIR in human NK cells via binding of its SH2 domains to the phosphorylated ITIM motifs (12–19). In contrast, although SHP-2 binds to KIR ITIM phosphopeptides in vitro or phosphorylated KIR cytoplasmic domains expressed in RBL-2H3 mast/basophil-like and A20 B cell lines (14, 17, 22), no binding of SHP-2 to KIR has been reported in intact NK cells.

To identify the phosphatase that binds the single ITIM of 2DL4 in human NK cells, both 3DL1 and 3DL1/L4 were immunoprecipitated from pervanadate-treated NK-92 cells and tested for SHP-1 and SHP-2 recruitment by immunoblotting. Pervanadate treatment stimulated robust tyrosine phosphorylation of both receptors (Fig. 3A). Immunoprecipitates of tyrosine-phosphorylated 3DL1 (Fig. 3A, *left panels*) coprecipitated SHP-1 and SHP-2 phosphatase, while control CD56 immunoprecipitates did not bind either (Fig. 3A, *right panels*). Interestingly, the tyrosine-phosphorylated 3DL1/L4 chimera coimmunoprecipitated only SHP-2 (Fig. 3A, *lower left panel*). SHP-1 binding to 3DL1/L4 was undetectable even when wild-type SHP-1 was overexpressed by retroviral transduction in the 3DL1/L4-transduced NK-92 cells (data not shown). The 3DL1- or 3DL1/L4-transduced NK3.3 cells were also tested for phosphatase recruitment (Fig. 3B). Again, only SHP-2 is recruited to tyrosine-phosphorylated 3DL1/L4 chimera in NK3.3 cells. We also tested whether these phosphorylated receptors could recruit other effector enzymes that have potential inhibitory function and an SH2 domain to bind the phosphorylated ITIM. Neither Csk (COOH-terminal Src kinase) nor SHIP was detectably recruited in intact cells to either 3DL1 or 3DL1/L4 chimera in NK-92 cells by immunoblot analysis (data not shown).

Transient expression of dominant-negative SHP-1 by vaccinia virus does not block inhibition by the 2DL4 cytoplasmic ITIM

To further support the lack of SHP-1 involvement in 3DL1/L4 inhibition, dominant-negative SHP-1 (C453S) was introduced into both KIR-transduced cells using vaccinia virus. Expression of C453S SHP-1 upon recombinant vaccinia virus infection has been shown to block inhibition of cytotoxicity mediated by classical KIR (16, 18). Because expression of the protein of interest varies with viral dose, multiple concentrations of PFU/cell were tested.

To monitor the exogenous SHP-1 protein expression, infected or uninfected cell lysates were tested by Western blot analysis (Fig. 4A). The optimal multiplicity of infection of virus containing either wild-type SHP-1 or C453S SHP-1 was identified as 10 PFU/cell, which produced a 50- to 100-fold increased expression of SHP-1 protein (Fig. 4A). As expected, the 3DL1-mediated inhibition of cytotoxicity was abolished upon overexpression of C453S SHP-1 at this optimal multiplicity of infection (Fig. 4B, *upper panels*). In contrast, the suppression by 3DL1/L4 was not affected by this extensive overexpression of C453S SHP-1 (Fig. 4B, *lower panels*), demonstrating that inhibition by 3DL1/L4 is independent of SHP-1, which correlates with our biochemical data. Overexpression of wild-type SHP-1 had no effect on inhibition by either KIR.

Modest inhibitory function is retained after mutation of the single ITIM tyrosine of 3DL1/L4

To confirm that inhibition by 3DL1/L4 is mediated through the single ITIM domain, we mutated the ITIM tyrosine (tyrosine 277 to phenylalanine) to prevent phosphorylation and presumably eliminate the negative signaling. Surprisingly, engagement of the mutated KIR (3DL1/L4/Y277F) reproducibly resulted in partial inhibition of cytotoxicity (Fig. 5A). This result raised several possibilities: 1) 3DL1/L4 associates at the cell surface with another inhibitory receptor expressed in NK-92 cells; 2) modest inhibition is mediated through a portion of the cytoplasmic domain other than the ITIM; or 3) an effector enzyme such as SHP-2 still binds the ITIM of 2DL4 in the absence of phosphotyrosine. To address these possibilities, most of the cytoplasmic domain of 3DL1 was deleted (KIR3DL1/255EΔ) (Fig. 1A), and NK-92 cells expressing this construct were tested for cytotoxicity. Upon KIR engagement, this tailless form showed no inhibition, as shown in Fig. 5B, suggesting that inhibitory receptor multimerization involving the extracellular and/or transmembrane domains was not contributing to the inhibition. Next, the cytoplasmic domain was deleted immediately before (272PΔ) or after (282HΔ) the mutated ITIM of 2DL4 (Fig. 1A). Interestingly, 3DL1/L4/Y277F/282HΔ still retained the slight inhibition of cytotoxicity that we had seen in 3DL1/L4/Y277F, while 3DL1/L4/272PΔ (lacking the ITIM) did not. A cumulative analysis of data from multiple experiments demonstrated statistically significant inhibition for 3DL1, 3DL1/L4, and 3DL1/
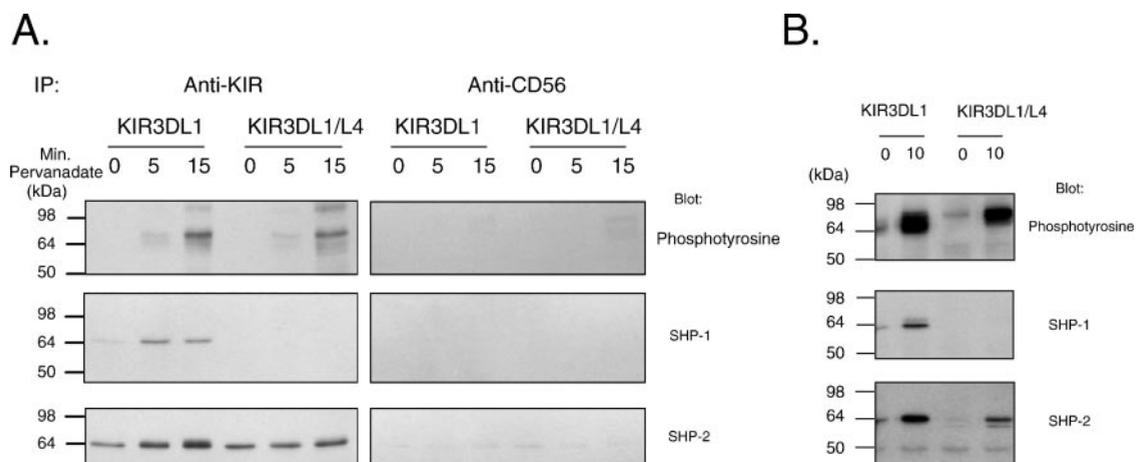

FIGURE 3. Tyrosine-phosphorylated 3DL1 binds SHP-1 and SHP-2, while phosphorylated 3DL1/L4 binds only SHP-2. Receptor-transduced NK-92 cells (*A*) or NK3.3 cells (*B*) were treated with pervanadate for various times and lysed with 1% Triton X-100, and sequential immunoprecipitates were prepared with anti-CD56 and then anti-3DL1 mAbs. Each lane represents immunoprecipitation from a lysate of 40 million cells. Samples were analyzed on 10% SDS-PAGE gels under reducing conditions. Sequential immunoblot analysis was performed with anti-phosphotyrosine, anti-SHP-1, and anti-SHP-2.
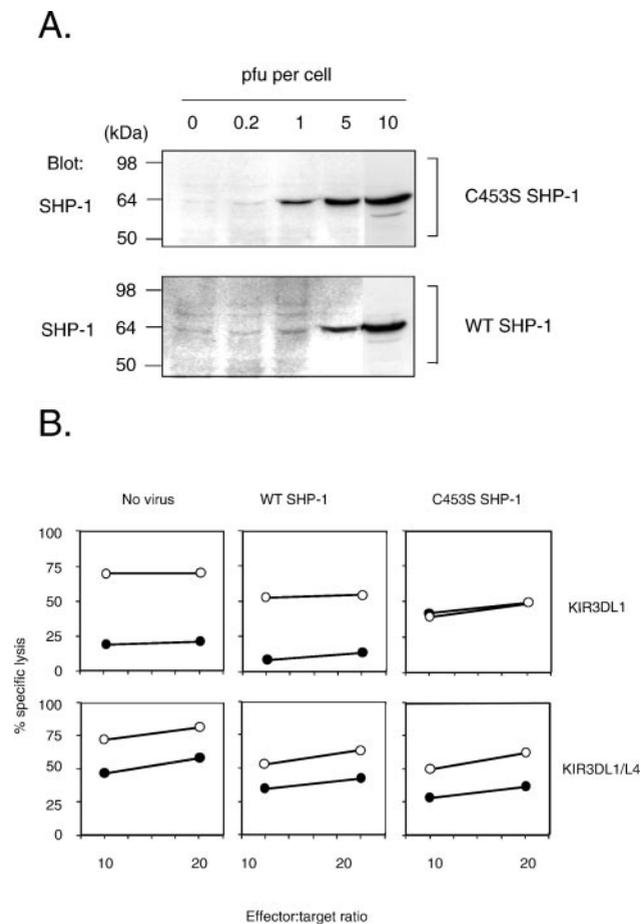
A.

B.

FIGURE 4. Transient expression of the dominant-negative form of SHP-1 blocks 3DL1 inhibition, but not 3DL1/L4 inhibition. *A*, Expression of the exogenous wild-type or dominant-negative, C453S, form of SHP-1 using recombinant vaccinia viruses. NK-92 cells were infected with the indicated recombinant virus preparations at different PFU concentrations. The whole cell lysates (0.5 million cells/lane) were separated by 10% SDS-PAGE gels under reducing conditions and probed with anti-SHP-1. *B*, Redirected cytotoxicity assay using P815 as a target. Recombinant vaccinia viruses encoding the SHP-1 preparations used in *A* were used to infect NK-92 cells that stably express the KIR receptors by retroviral transduction. The 3DL1-expressing NK-92 cells (*upper panels*) and 3DL1/L4-expressing NK-92 cells (*lower panels*) were infected with recombinant vaccinia virus preparations at 10 PFU/cell. Aliquots of infected cells were used in a redirected cytotoxicity assay in the presence of either anti-CD56 (○) or anti-3DL1 (●) mAbs (1 μg/ml).

L4282HΔ, but not for either 3DL1/255EΔ or 3DL1/L4/272PΔ (Table I). This result demonstrates that the mutant ITIM is the critical element that transduces modest inhibition, and that the ITIM of 2DL4 can transduce negative signaling in the absence of phosphorylated tyrosine. We further found that the inhibition of cytotoxicity elicited by the mutated 3DL1/L4/Y277F was not blocked by vaccinia-driven expression of C453S SHP-1, further indicating that its inhibitory function is also SHP-1 independent (Fig. 6).

Weak SHP-2 binding to the nonphosphorylated ITIM of 2DL4 in intact cells and in vitro

Because we had already found that SHP-2 is selectively recruited to the single ITIM of 2DL4, SHP-2 binding to the Y277F mutant chimera was tested by biochemical analysis. To assess the occurrence of weak SHP-2 binding, immunoprecipitates were prepared from more cells than previously (80 million cells/sample). As pre-

viously demonstrated, the single ITIM tyrosine of the wild-type 2DL4 cytoplasmic domain was phosphorylated after the pervanadate treatment, and SHP-2 recruitment to the KIR was significantly elevated upon phosphorylation (Fig. 7A, *second* and *third lanes*). Importantly, SHP-2 still associated modestly with the wild-type and tyrosine mutant receptors in untreated cells (Fig. 7A, *lower panel*, *first* and *second lanes*). The Y277F mutant form of 3DL1/L4 was not phosphorylated upon pervanadate treatment, as expected, and SHP-2 association was not enhanced (data not shown). As expected, we did not detect SHP-1 binding to either receptor (data not shown).

These results suggested the unexpected possibility that SHP-2 may be binding to its cognate ITIM domain in a phosphorylation-independent manner. This may represent a novel SH2 interaction with either this particular ITIM sequence or additional flanking sequences. To discriminate this possibility, we focused on in vitro interactions with the ITIM using corresponding synthetic KIR ITIM peptides. Among human KIR, the amino acid sequences surrounding the two tandem ITIMs are highly conserved. The N-terminal and C-terminal ITIM sequences on 3DL1 are VTYAQL (YAQL) and IVYTEL (YTEL), respectively. The single ITIM of 2DL4 also corresponds to VTYAQL. Nonphosphorylated and phosphorylated peptides corresponding to both ITIMs and short flanking sequences were used as affinity matrices to adsorb SHP-1 and SHP-2 phosphatases from NK-92 cell lysates. As previously reported (12–14, 17), SHP-1 bound significantly to both phosphorylated ITIM peptides, whereas binding to the nonphosphorylated ITIM peptides was undetectable (Fig. 7B, *upper panel*). However, SHP-2 bound strongly to the YAQL peptides whether phosphorylated or not (Fig. 7B, *lower panel*, *first* and *second lanes*). However, detectable SHP-2 binding to the C-terminal YTEL peptide required phosphorylation of the ITIM tyrosine. Collectively, these results indicate that SHP-2 interacts with both the phosphorylated and nonphosphorylated N-terminal ITIM corresponding to that found in 2DL4. SHP-1 binding to the phosphorylated YAQL peptide, but not phosphorylated 3DL1/L4 receptor, is inconsistent with our immunoprecipitation results, but correlates well with previous reports (15, 22). Although SHP-1 can bind efficiently to small peptides corresponding to either of these phosphorylated ITIM domains (13, 14, 18), larger peptides containing both KIR ITIMs and the intervening sequence cannot bind SHP-1 unless both ITIMs are phosphorylated (15). This indicates that the intervening sequence alters accessibility of the SHP-1 SH2 domains to the phosphorylated YAQL sequence unless the YTEL ITIM is also phosphorylated.

Discussion

We have used a retroviral transduction system to study the functional and signal transduction properties of the cytoplasmic domain of 2DL4 in NK-like cell lines. The classical KIR, 3DL1, which contains two ITIM domains, was directly compared with a chimeric form of 3DL1 in which the cytoplasmic domain was substituted with that of 2DL4, possessing a single ITIM (3DL1/L4). Interestingly, the inhibition of NK cell cytotoxicity was comparable between both receptors, despite the difference in ITIM number. This equivalent inhibition resulted, despite the fact that the classical inhibitory KIR having two ITIMs recruits both SHP-1 and SHP-2, while 2DL4 having only a single ITIM exclusively binds SHP-2. We also observed an intriguing modest inhibition of cytotoxicity by a mutant form of the 2DL4 cytoplasmic domain in which the only ITIM tyrosine is substituted with phenylalanine. Surprisingly, this requires the mutant ITIM and correlates with weak SHP-2 binding to the nonphosphorylated receptor ITIM, whereas SH2 domain interactions with ITIMs normally require
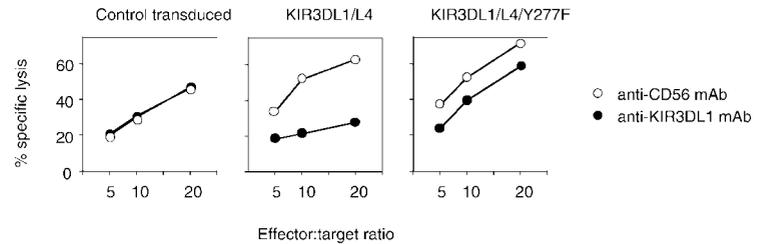
A.

B.

FIGURE 5. Mutation of tyrosine 277 to phenylalanine (Y277F) within the single ITIM of the 2DL4 cytoplasmic domain still retains some inhibition. *A*, NK-92 cells stably transduced with mouse IgM (*left panel*), 3DL1/L4 (*middle panel*), or 3DL1/L4/Y277F (*right panel*) were tested for their cytotoxicity against P815 in the presence of either anti-CD56 (○) or anti-3DL1 (●) mAbs (1 μg/ml). The slight inhibition by Y277F transfectants was highly reproducible in more than 10 experiments. *B*, The cytoplasmic domain of 3DL1/L4 was almost entirely deleted after glutamic acid 255 (KIR3DL1/255EΔ) or deleted just before (3DL1/L4/272PΔ) or after (3DL1/L4/Y277F/282HΔ) the mutated ITIM (as in Fig. 1*B*). These transduced NK-92 cells were tested for their cytotoxicity against P815 as a target in the presence of anti-CD56 (○) or anti-3DL1 (●) mAbs.
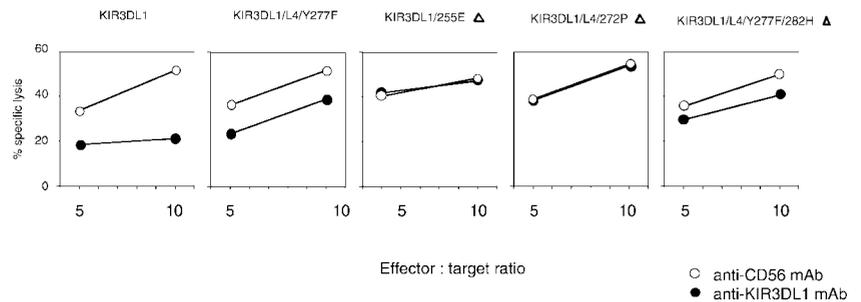
phosphorylation of the tyrosine residue. These results suggest that SHP-2 recruitment by KIR can provide the necessary protein tyrosine phosphatase activity that can substitute for SHP-1 to mediate efficient inhibition of NK cell-activating receptors. In addition, SHP-2 appears to be weakly constitutively associated with the nonphosphorylated 2DL4 cytoplasmic domain, and presumably to the corresponding domain on all inhibitory KIR.

The 3DL1/L4 chimeric molecules strongly inhibited the cytotoxicity of NK-92 against P815 (Fig. 2), indicating that 2DL4 has potent inhibitory capacity. Importantly, the two ITIMs of 3DL1 and the single ITIM of 2DL4 equally inhibited cytotoxicity in our experiments (Fig. 2). Although NK-92 cells show high cytotoxicity aginst P815 cells, the activating receptors that mediate NK-92 lysis of P815 cells are unknown. Therefore, we also demonstrated that these two forms of KIR block specific activating receptors, NKp46 and 2B4, in a redirected cytotoxicity assay. NKp46 was previously shown to couple to the ITAM-containing transmem-

brane adapter protein, TCRζ (32), while the signaling mechanisms used by 2B4 to enhance NK cell cytotoxicity are not well defined, but appear to involve the linker for activation of T cells (LAT) adapter (33–35). In addition, 3DL1/L4 inhibited cytotoxicity in NK3.3 cells, which demonstrates that this is not a unique property in the NK-92 cell line (Fig. 2*E*). Collectively, the 2DL4 cytoplasmic domain has an equivalent ability to inhibit several activating pathways with only a single ITIM, as compared with the tandem ITIMs found on typical inhibitory KIR (3DL1).

Our results build upon numerous previous publications, demonstrating that the two ITIMs (I/VxYxxL/V) of classical inhibitory KIR (KIR3DL and type I KIR2DL) (23) are important to recruit SHP-1 via its tandem SH2 domains (13–15, 17, 18, 22). By studying the functions of 3DL1 and 2DL4 expressed in their native

Table I. *Statistical analysis of the capacities of KIR3DL1/L4 receptors to inhibit cytotoxicity from multiple experiments[a]*

		Control Cytotoxicity (%)	
Receptors	No. of Experiments	Mean anti-CD56 (±SD)	Mean anti-KIR3DL1 (±SD)
KIR3DL1/L4	4	100 (3.0)	34.1 (8.6)*
Y277F	7	100 (2.6)	74.8 (5.3)*
282H	2	100 (4.8)	84.3 (7.6)*
272P	2	100 (1.1)	96.6 (3.3)
255E	6	100 (1.6)	99.1 (10.5)

[a] Statistical analysis was performed on cytotoxicity data from multiple experiments by converting the data at 10:1 E:T ratio within each experiment to the percentage of control cytotoxicity (with anti-CD56 Ab). Within each experiment the triplicate data points for anti-CD56 Ab treatment of each cell line were averaged, and the average was designated 100% of control cytotoxicity. Every data point for each transduced cell line within each experiment was converted to this percentage of control cytotoxicity, and Student's *t* test was performed to compare the aggregate data points from anti-CD56-treated and anti-KIR3DL1-treated conjugation conditions for each transduced cell line. Statistical analysis was performed on the JMP statistical analysis program for Macintosh (version 3; SAS Institute, Cary, NC).

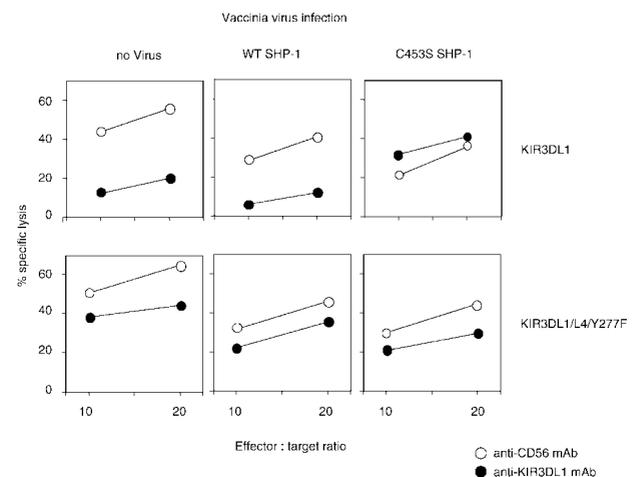*, *p* < 0.05 compared to anti-CD56-treated controls.

FIGURE 6. The 2DL4 cytoplasmic domain with a phenylalanine mutation at the ITIM tyrosine still has the capacity to inhibit NK cell cytotoxicity in the presence of dominant-negative SHP-1. NK-92 cells expressing 3DL1 (*upper panels*) or 3DL1/L4/Y277F (*lower panels*) were tested for their cytotoxicity against P815 in the presence of anti-CD56 (○) or anti-3DL1 (●) mAbs (1 μg/ml) after infection with recombinant vaccinia virus containing wild-type or C453S SHP-1 cDNAs at 10 PFU/cell.
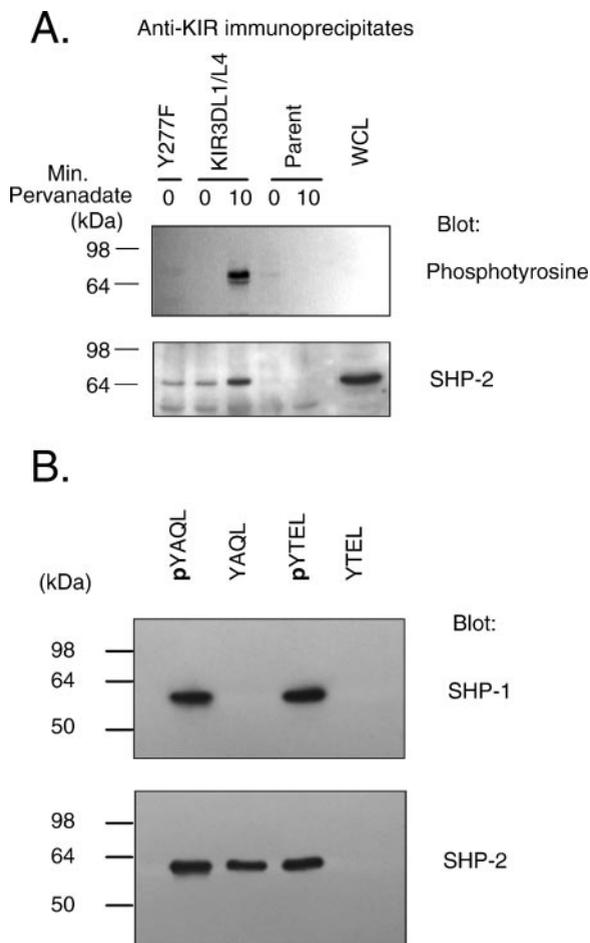
FIGURE 7. SHP-2 can bind weakly to the Y277F mutant ITIM as well as nonphosphorylated YAQL ITIM peptide. *A*, NK-92 cells transduced with 3DL1/L4 having either the intact ITIM or a mutated ITIM (Y277F) and the parent cells were treated with pervanadate for 10 min, lysed with 1% Triton X-100, and immunoprecipitated with anti-3DL1 mAb (DX9). Sequential immunoblot analysis was performed with anti-phosphotyrosine and anti-SHP-2. Each lane represents immunoprecipitation from a lysate of 80 million cells. Pervanadate treatment did not result in phosphorylation of the Y277F mutant or enhanced association with SHP-2 (data not shown). WCL, Whole cell lysate. *B*, Nonphosphorylated tyrosine peptides corresponding to the 2DL4 ITIM (YAQL) do not bind SHP-1, but can bind SHP-2. NK-92 cells were lysed in 1% Triton X-100 buffer (15 million cells/sample), and lysates were precipitated with peptides coupled to SulfoLink gel. Precipitates were separated by 10% SDS-PAGE and subjected to sequential immunoblot analysis with anti-SHP-1 and anti-SHP-2. YAQL corresponds to the N-terminal ITIM shared by both 3DL1 and 2DL4, while YTEL is a peptide corresponding to the C-terminal ITIM of 3DL1.

cellular context, the NK-92 cell line, our results confirm and extend some of the SHP-1 and SHP-2 recruitment patterns previously observed by Bruhns et al. (22) in B cell and mast/basophil-like cell lines. In our studies, the classical KIR, 3DL1, recruited SHP-1 phosphatase (15) (Fig. 3*A*), as reported with other classical KIRs (13, 18), while the single ITIM of 2DL4 did not recruit detectable levels of SHP-1 in intact NK cells (Fig. 3*A*), despite strong ITIM tyrosine phosphorylation by pervanadate treatment. Instead, SHP-2 was recruited to the single ITIM of 2DL4 in intact cells (Fig. 3*A*), suggesting that SHP-2 contributes to this inhibition.

The involvement of SHP-2 in inhibitory function of 2DL4 can be further supported by our findings, as follows. First, C453S SHP-1 blocked the inhibition by 3DL1, but not 3DL1/L4 (Fig. 4*B*),

thereby ruling out a role for SHP-1 in inhibition through the 2DL4 cytoplasmic domain. This phosphatase dead form of SHP-1 has the ability to compete with and block function of both endogenous SHP-1 and SHP-2 on classical KIR due to high-affinity binding mediated by tandem SH2 interactions with bisphosphorylated ITIMs, resulting in the strong inhibition of 3DL1. Thus, we provide both biochemical and dominant-negative evidence that SHP-1 cannot bind effectively to the single ITIM of 2DL4. Second, the cytotoxicity against P815 was still reduced by 2DL4, in which the single tyrosine residue within the ITIM is mutated to phenylalanine (Fig. 5*A*), and we observed coordinate weak binding of SHP-2, but not SHP-1, to this mutant cytoplasmic domain of 2DL4 (Fig. 7*A*). Third, significant amounts of SHP-2 bound to nonphosphorylated YAQL peptide, but not nonphosphorylated YTEL peptide in vitro (Fig. 7*B*). SHP-2 binding to the phosphorylated YAQL was already described by Vely et al. (17) and Olcese et al. (14), but their group showed no binding of nonphosphorylated YAQL peptide to SHP-2 from RBL-2H3 cells. The discrepancy between their results and ours might be attributing to the cell type used or the peptide sequences. Their peptides contained five additional KIR amino acids on the N-terminal side and minor sequence differences from another KIR.

KIR engagement was previously shown to decrease the phosphorylation of ζ signaling chain, ZAP-70, phospholipase C-γ2, and SLP-76 during Ab-dependent cell-mediated cytotoxicity, which had previously been attributed to SHP-1 recruitment (16, 36). Although the precise contribution of SHP-1 to NK cell inhibitory function is still unknown, it is not surprising that the other protein tyrosine phosphatase, SHP-2, might substitute for these activities on 2DL4. These findings establish a model in which classical KIR, having two ITIMs use SHP-1 and SHP-2, while 2DL4 possesses single ITIM, uses SHP-2 to mediate inhibitory function. SHP-2 has been shown to serve as a positive regulator in many receptor systems (37, 38), but roles in negative signaling have also been described for receptors such as CTLA4 (39), FcγRIIb (40), and ciliary neutrophic factor receptor (41). The possibility that additional SH2-containing effector enzymes contribute to the inhibition by the single ITIM of 2DL4, however, remains to be addressed.

It is noteworthy that, unlike other KIRs, 2DL4 is reportedly ubiquitously expressed on all NK cells (42), as well as by every individual person (43), indicating that it is very important for NK cell function. In addition, 2DL4 is highly conserved not only in pygmy chimpanzee (*Bonobo*) (44), but also in rhesus monkey (*Macaca mulatta*) (45), suggesting that 2DL4 serves an important role in primates. Important insights regarding the functions of 2DL4 have emerged from several recent findings. 2DL4 is reported to be a receptor for HLA-G (9), which is normally expressed only on fetal trophoblast cells that invade the maternal decidua (11), suggesting that it plays an inhibitory role. In contrast, 2DL4 was shown to selectively augment IFN-γ production, but not cytotoxicity, when engaged in resting NK cells (28), suggesting 2DL4 is an activating receptor. The importance of IFN-γ production by NK cells in decidual development during pregnancy has recently been reported (46). This IFN-γ stimulatory function was attributed to an unknown interacting protein binding through the transmembrane arginine of 2DL4. Our observations indicate that the inhibitory capacity of 2DL4 may selectively contribute to the lack of cytotoxicity stimulated by the full receptor, and we are currently exploring this possibility.

SHIP is a SH2 domain-containing inositol polyphosphate 5-phosphatase that was shown to mediate inhibition upon binding the phosphorylated ITIM of FcγRIIB1 receptor (47, 48). SHIP recruitment to inhibitory KIR has never been reported, and we have not detected SHIP binding to the cytoplasmic domains of

3DL1 or 2DL4 in intact cells (data not shown). Interestingly, it has been shown that KIR ITIMs recruit only SHP-1, when both SHP-1 and SHIP are available (49). These findings clearly indicate that individual inhibitory receptors choose specific effector enzymes to mediate inhibition. In the present study, we found that SHP-2, in addition to SHP-1, can be recruited to the cytoplasmic domain of the classical 3DL1 (Fig. 3), indicating that either phosphatase can contribute if quantities of one of the enzymes is limiting. In view of the identical sequence surrounding the first ITIM of many classical KIR and that of 2DL4, our results also imply that phosphorylation of only the YAQL tyrosine of classical KIR results in the selective recruitment of SHP-2, while either SHP-1 or SHP-2 can bind when both ITIM tyrosines become phosphorylated.

Our observations of weak inhibition by the Y277F mutant form of 2DL4 have precedence in the literature. A modest inhibition of Ca^{2+} mobilization was previously reported in response to B cell receptor coligation with a mutant FcγRIIB1 in which the ITIM tyrosine was changed to alanine (50), and murine Ly-49A-bearing mutant ITIM (Y to F) was also able to inhibit IL-2 secretion in the A20 B cell line (51), suggesting that the nonphosphorylatable mutant ITIM may still interact to some degree with phosphatases. Our observations of weak SHP-2 binding to both 3DL1/L4/Y277F and nonphosphorylated YAQL peptide suggest that this may indeed be the case.

The present study on the single ITIM of 2DL4 uncovered new aspects of the inhibitory pathway in human NK cells, which are SHP-1 independent and phosphotyrosine independent. The results further imply that SHP-2 may be weakly associated with all non-phosphorylated KIR in a constitutive manner, thereby contributing to initial stages of KIR function before phosphorylation of the ITIMs, followed by elevated high avidity recruitment of both tyrosine phosphatases after ITIM phosphorylation.

Acknowledgments

References

1. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol. 47:187.*
2. Perussia, B. 1991. Lymphokine activated killer cells, NK cells, and cytokines. *Curr. Opin. Immunol. 3:49.*
3. Cerwenka, A., and L. L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nat. Immun. 1:41.*
4. Braud, V. M., D. S. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature 391:795.*
5. Takei, F., J. Brenman, and D. L. Mager. 1997. The Ly-49 family: genes, proteins and recognition of class I MHC. *Immunol. Rev. 151:67.*
6. Yokoyama, W. M. 1997. What goes up must come down: the emerging spectrum of inhibitory receptors. *J. Exp. Med. 186:1803.*
7. Trowsdale, J. 2001. Genetic and functional relationships between MHC and NK receptor genes. *Immunity 15:363.*
8. Long, E. O., D. F. Barber, D. N. Burshtyn, M. Faure, M. Peterson, S. Rajagopalan, V. Renard, M. Sandusky, C. C. Stebbins, N. Wagtmann, and C. Watzl. 2001. Inhibition of killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunol. Rev. 181:223.*
9. Rafagopalan, S., and E. O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med. 189:1093.*
10. Ponte, M., C. Cantoni, R. Biassoni, A. Tradori-Cappai, G. Bentivoglio, C. Vitale, S. Bertone, A. Moretta, L. Moretta, and M. C. Mingari. 1999. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc. Natl. Acad. Sci. USA 96:5674.*
11. Loke, Y. W., and A. King. 1997. Immunology of human placental implantation: clinical implications of our current understanding. *Mol. Med. Today 3:153.*
12. Burshtyn, D. N., W. Yang, T. Yi, and E. O. Long. 1997. A novel phosphotyrosine motif with a critical amino acid at position −2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. *J. Biol. Chem. 272:13066.*
13. Campbell, K. S., M. Dessing, M. Lopez-Botet, M. Cella, and M. Colonna. 1996. Tyrosine phosphorylation of a human killer inhibitory receptor recruits protein tyrosine phosphatase 1C. *J. Exp. Med. 184:93.*
14. Olcese, L., P. Lang, F. Vely, A. Cambiaggi, D. Marguet, M. Blery, K. L. Hippen, R. Biassoni, A. Moretta, L. Moretta, et al. 1996. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J. Immunol. 156:4531.*
15. Fry, A. M., L. L. Lanier, and A. Weiss. 1996. Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. *J. Exp. Med. 184:295.*
16. Binstadt, B. A., K. Brumbaugh, C. J. Dick, A. M. Scharenberg, B. L. Williams, M. Colonna, L. L. Lanier, J. P. Kinet, R. T. Abraham, and P. J. Leibson. 1996. Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation. *Immunity 5:629.*
17. Vely, F., S. Olivero, L. Olcese, A. Moretta, J. E. Damen, L. Liu, G. Krystal, J. C. Cambier, M. Daeron, and E. Vivier. 1997. Differential association of phosphatases with co-receptors bearing immunoreceptor tyrosine-based inhibition motifs. *Eur. J. Immunol. 27:1994.*
18. Burshtyn, D. N., A. M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J. P. Kinet, and E. O. Long. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitory receptor. *Immunity 4:77.*
19. Burshtyn, B. N., A. S. Lam, M. Weston, N. Gupta, P. A. M. Warmerdam, and E. O. Long. 1999. Conserved residues amino-terminal of cytoplasmic tyrosines contribute to the SHP-1-mediated inhibitory function of killer cell Ig-like receptors. *J. Immunol. 162:897.*
20. Vyas, Y. M., K. M. Mehta, M. Morgan, H. Maniar, L. Butros, S. Jung, J. K. Burkhardt, and B. Dupont. 2001. Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated non-cytolytic and cytolytic interactions. *J. Immunol. 167:4358.*
21. Nakamura, M. C., E. C. Niemi, M. J. Fisher, L. D. Shultz, W. E. Seaman, and J. C. Ryan. 1997. Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase. *J. Exp. Med. 185:673.*
22. Bruhns, P., P. Marchetti, W. H. Fridman, E. Vivier, and M. Daeron. 1999. Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptor. *J. Immunol. 162:3168.*
23. Vilches, C., R. Rajalingam, M. Uhrberg, C. M. Gardiner, N. T. Young, and P. Parham. 2000. KIR2DL5, a novel killer-cell receptor with a D0–D2 configuration of Ig-like domains. *J. Immunol. 164:5797.*
24. Cantoni, C., S. Verdiani, M. Falco, A. Pessino, M. Cilli, R. Conte, D. Pende, M. Ponte, M. S. Mikaelsson, L Moretta, and R. Biassoni. 1998. P49, a putative HLA class I-specific inhibitory NK receptor belonging to the immunoglobulin superfamily. *Eur. J. Immunol. 28:1980.*
25. McVicar, D. W., L. S. Taylor, P. Gosselin, J. Willette-Brown, A. I. Mikhael, R. L. Geahlen, M. C. Nakamura, P. Linnemeyer, W. E. Seaman, S. K. Anderson, et al. 1998. DAP12-mediated signal transduction in natural killer cells. *J. Biol. Chem. 273:32934.*
26. Tomasello, E., C. Cant, H.-J. Buhring, F. Vely, P. Andre, M. Seiffert, A. Ullrich, and E. Vivier. 2000. Association of signal-regulatory protein β with KARAP/DAP12. *Eur. J. Immunol. 30:2147.*
27. Lanier, L. L., B. C. Corliss, J. Wu, C. Leong, and J. H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activation NK cells. *Nature 391:703.*
28. Rajagopalan, S., and E. O. Long. 2001. Induction of IFN-γ production but not cytotoxicity by the killer cell Ig-like receptor 2DL4 (CD158d) in resting NK cells. *J. Immunol. 167:1877.*
29. Kinsella, T. M., and G. P. Nolan. 1996. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther. 7:1405.*
30. Rajagopalan, S., and E. O. Long. 2000. Use of vaccinia virus for functional gene transfer in natural killer cells. *Natural Killer Cell Protocols: Cellular and Molecular Methods, Methods in Molecular Biology*, Vol. 121. K. S. Campbell and M. Colonna, eds. Humana, Totowa, NJ, p. 265.
31. Moss, B., and P. L. Earl. 1998. Expression of proteins in mammalian cells using vaccinia viral vectors. *Current Protocols in Molecular Biology*, Vol. 3. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. Wiley, New York, p. 16.15.1.
32. Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta, and A. Moretta. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J. Exp. Med. 187:2065.*
33. Chuang, S. S., P. R. Kumaresan, and P. A. Mathew. 2001. 2B4 (CD244)-mediated activation of cytotoxicity and IFN-γ release in human NK cells involves distinct pathways. *J. Immunol. 167:6210.*
34. Bottino, C., R. Augugliaro, R. Castriconi, M. Nanni, R. Biassoni, L. Moretta, and A. Moretta. 2000. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. *Eur. J. Immunol. 30:3718.*

35. Watzl, C., C. C. Stebbins, and E. O. Long. 2000. NK cell inhibitory receptors prevent tyrosine phosphorylation of the activation receptor 2B4 (CD244). *J. Immunol. 165:3545.*

36. Binstadt, B. A., D. D. Billadeau, D. Jevremovic, B. L. Williams, N. Fang, T. Yi, G. A. Koretzky, R. T. Abraham, and P. J. Leibson. 1998. SLP-76 is a direct substrate of SHP-1 recruited to killer cell inhibitory receptors. *J. Biol. Chem. 273:27518.*

37. Huyer, G., and D. R. Alexander. 1999. Immune signalling: SHP-2 docks at multiple ports. *Curr. Biol. 9:R129.*

38. Tamir, I., J. M. Dal Porto, and J. C. Cambier. 1999. Cytoplasmic protein tyrosine phosphatases SHP-1 and SHP-2: regulators of B cell signal transduction. *Curr. Opin. Immunol. 12:307.*

39. Lee, K. M., E. Chuang, M. Griffin, R. Khattri, D. K. Hong, W. Zhang, D. Straus, L. E. Samelson, C. B. Thompson, and J. A. Bluestone. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science 282:2263.*

40. Koncz, G., I. Pecht, J. Gergely, and G. Sarmay. 1999. Fcγ receptor-mediated inhibition of human B cell activation: the role of SHP-2 phosphatase. *Eur. J. Immunol. 29:1980.*

41. Symes, A., N. Stahl, S. A. Reeves, T. Farruggella, T. Servidei, T. Gearan, G. Yancopoulos, and J. S. Fink. 1997. The protein tyrosine phosphatase SHP-2 negatively regulates ciliary neurotrophic factor induction of gene expression. *Curr. Biol. 7:697.*

42. Valiante, N. M., M. Uhrberg, H. G. Shilling, K. Lienert-Weidenbach, K. L. Arnett, A. D'Andrea, J. H. Phillips, L. L. Lanier, and P. Parham. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity 7:739.*

43. Uhrberg, M., N. M. Valiante, B. P. Shum, H. G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L. L. Lanier, and P. Parham. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity 7:753.*

44. Rajalingam, R., M. Hong, E. J. Adams, B. P. Shum, L. A. Guethlein, and P. Parham. 2001. Short KIR haplotypes in pygmy chimpanzee (*Bonobo*) resemble the conserved framework of diverse human KIR haplotypes. *J. Exp. Med. 193: 135.*

45. Hershberger, K. L., R. Shyam, A. Miura, and N. L. Letvin. 2001. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J. Immunol. 166:4380.*

46. Ashkar, A. A., and B. A. Croy. 2001. Functions of uterine natural killer cells are mediated by interferon γ production during murine pregnancy. *Semin. Immunol. 13:235.*

47. Ono, M., S. Bolland, P. Tempst, and J. V. Ravetch. 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. *Nature 383:263.*

48. Nadler, M. J. S., B. B. Chen, J. S. Anderson, H. H. Wortis, and B. G. Neel. 1997. Protein-tyrosine phosphatase SHP-1 is dispensable for FcγRIIB-mediated inhibition of B cell antigen receptor activation. *J. Biol. Chem. 272:20038.*

49. Ono, M., H. Okada, S. Bolland, S. Yanagi, T. Kurosaki, and J. V. Ravetch. 1997. Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell 90:293.*

50. D'Ambrosio, D., K. L. Hippen, S. A. Minskoff, I. Mellman, G. Pani, K. A. Siminovitch, and J. C. Cambier. 1995. Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by FcγRIIB1. *Science 268:293.*

51. Motoda, K., M. Takata, K. Kiura, I. Nakamura, and M. Harada. 2000. SHP-1/ immunoreceptor tyrosine-based inhibition motif-independent inhibitory signalling through murine natural killer cell receptor Ly-49A in a transfected B-cell line. *Immunology 100:370.*