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KIR2DL5 Can Inhibit Human NK Cell Activation Via Recruitment of Src Homology Region 2-Containing Protein Tyrosine Phosphatase-2 (SHP-2)\(^1\)

Sei-ichi Yusa, Tracey L. Catina, and Kerry S. Campbell\(^2\)

Human NK cells use class I MHC-binding inhibitory receptors, such as the killer cell Ig-like receptor (KIR) family, to discriminate between normal and abnormal cells. Some tumors and virus-infected cells down-regulate class I MHC and thereby become targets of NK cells. Substantial evidence indicates that the mechanism of KIR-mediated inhibition involves recruitment of the protein tyrosine phosphatases, Src homology 2-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2, to two phosphorylated cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). KIR2DL5 is a type II member of the KIR2D family with an atypical extracellular domain and an intracytoplasmic domain containing one typical ITIM and one atypical ITIM sequence. Although KIR2DL5 structure is expressed by \(\approx 50\%\) of humans and is conserved among primate species, its function has not been determined. In the present study, we directly compared functional and biochemical properties of KIR2DL5, KIR3DL1 (a type I KIR with two ITIMs), and KIR2DL4 (the only other type II KIR, which has a single ITIM) in a human NK-like cell line. Our results show that KIR2DL5 is an inhibitory receptor that can recruit both SHP-1 and SHP-2, and its inhibitory capacity is more similar to that of the cytoplasmic domain of KIR2DL4 than KIR3DL1. Interestingly, inhibition of NK cell cytotoxicity by KIR2DL5 was blocked by dominant-negative SHP-2, but not dominant-negative SHP-1, whereas both dominant-negative phosphatases can block inhibition by KIR3DL1. Therefore, the cytoplasmic domains of type II KIRs (2DL4 and 2DL5) exhibit distinct inhibitory capacities when compared with type I KIRs (3DL1), due to alterations in the canonical ITIM sequences. \(\text{The Journal of Immunology, 2004, 172: 7385–7392.}\)

\(\text{N}a\)tural killer cells are a subset of lymphocytes that play an important role in innate immunity by directing cytoytic attack toward class I MHC (MHC-I)-deficient tumor cells and virus-infected cells (1, 2). NK cells also contribute to acquired immunity by secreting various cytokines, including IFN-\(\gamma\) (1, 2). NK cell activation is controlled by a balance between activating and inhibitory receptors (3). Although T and B cells are activated by single clonotypic surface Ag receptors, activation of NK cells is derived from a combination of numerous positive signals generated by many receptors engaging with ligands on target cell surfaces, including CD16, activating forms of killer cell Ig-like receptors (KIRs)\(^3\), activating forms of CD94/NKG2 dimers, NKG2D, and natural cytotoxicity receptors (NKP30, NKP44, and NKP46). These potent activating receptors associate noncovalently with dimers of accessory chains, named TCR-\(\zeta\), FcεRI-\(\gamma\), DAP10, or DAP12, which contain the immunoreceptor tyrosine-based activation motif or a YxxM motif for positive signaling (4, 5). Conversely, NK cell activation is abrogated by MHC-I-binding inhibitory receptors that have a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (6). Inhibitory KIRs are the major classical MHC-I-binding inhibitory receptors expressed on human NK cells and a subset of T cells (7). Engagement of these inhibitory receptors with appropriate MHC-I ligands has been shown to block natural cytotoxicity. Ab-dependent cellular cytotoxicity (8, 9), and adhesion toward target cells (10). Thus, inhibitory KIRs are important not only for tumor surveillance, but also for immunological tolerance to discriminate between normal and abnormal cells.

ITIM sequences are found in many inhibitory receptors, and the critical role of ITIM sequences on negative signaling has been well established (11–12). Phosphorylated ITIM sequences recruit Src homology region 2 (SH2)-containing negative effector phosphatases, such as SH2-containing protein tyrosine phosphatase-1 (SHP-1), SHP-2, and SH2-containing inositol 5’-phosphatease (SHIP). Substantial evidence indicates that SHP-1 is involved in KIR-mediated inhibition, including SHP-1 binding and activation by KIR ITIM phosphopeptides, recruitment of SHP-1 to tyrosine-phosphorylated KIRs in NK cells, and blockade of KIR inhibition with a dominant-negative (DN) form of SHP-1 (8, 9, 13–19). In addition, we have recently used similar approaches to demonstrate that KIR inhibition in human NK cells also involves recruitment of SHP-2 (20). In contrast, KIR inhibition does not appear to involve SHIP (17). Thus, both SHP-1 and SHP-2 protein tyrosine phosphatases (PTP) play roles in inhibitory KIR function. However, how KIRs selectively use these phosphatases and whether they function through dephosphorylation of distinct substrates are unknown. KIR2DL5 (2DL5) is a recently described member of the...
KIR family, belonging to the type II KIR2D subgroup, which contains one other member named KIR2DL4 (2DL4) (21, 22). KIR2DL5 is structurally related to 2DL4, showing 79% amino acid sequence homology. Both receptors have a distinct configuration of extracellular Ig-like domains (D0–D2) and a longer cytoplasmic tail than that of classical type I inhibitory KIRs. KIR2DL5 has two tyrosines in the cytoplasmic domain, no charged amino acids in the transmembrane domain, and is found in 50% of all humans, while 2DL4 is the only long KIR that has a single cytoplasmic tyrosine, a positively charged transmembrane arginine, and is encoded by a gene found in virtually all individuals (22, 23). Interestingly, both 2DL5 and 2DL4 are highly conserved among rhesus monkeys, chimpanzees, and humans, suggesting their physiological importance in NK cell functions (24–26). The ligand of 2DL4 is thought to be the nonclassical class I MHC molecule, HLA-G, which is normally expressed only on fatally derived trophoblast cells that invade the maternal decidua in pregnant women (27). In contrast, the ligand specificity and function of 2DL5 have not been addressed to date, although the structural similarities with 2DL4 suggest similar ligand recognition patterns. Despite the cytoplasmic ITIM, 2DL4 was shown to function as an activating receptor to selectively produce IFN-γ, apparently through association with an undefined transmembrane accessory protein (28, 29). Interestingly, mutation of the charged residue in the transmembrane domain of 2DL4 converted it from an activating to an inhibitory receptor, probably due to dissociation from the putative activating accessory signaling protein (30). In addition, the cytoplasmic domain of 2DL4 was shown to inhibit NK cell cytolysis when fused with extracellular and transmembrane domains of KIR3DL1 (3DL1), which appears to be due to exclusive recruitment of SHP-2, but not SHP-1 (31). These results suggest that 2DL4 may play both activating and inhibitory roles in NK cell biology, although only activating function has been demonstrated for the full-length wild-type receptor, which is not hindered by the cytoplasmic ITIM (28–30). The function of 2DL5, however, has not been tested.

The two tyrosine-based sequences in the cytoplasmic domain of 2DL5 are characteristic of a classical N-terminal ITIM and another sequence similar to the immunoreceptor tyrosine-based switch motif (ITSM; T/SxxYxxV/I). The ITSM sequence has been shown to exist in several human immunoreceptors, including 2B4, signaling lymphocyte activation molecule (SLAM), CD84, and Ly-9 (32, 33), and has been shown to recruit both the SLAM-associated protein (SAP) (otherwise known as SH2-containing adapter protein 1A (SH2D1A)) and SHP-2 (34, 35). Although 2B4 recruits both proteins when expressed in nonlymphoid cells, all published experiments have failed to detect SHP-2 binding to 2B4 in NK cells (35, 36), even in the absence of SAP (37). Because SHP-2 is also involved in KIR function, it is of interest to determine whether the ITSM-like sequence of 2DL5 affects SHP-2 recruitment and inhibitory function of the receptor in human NK cells.

In this study, we directly compared the inhibitory function and PTP recruitment capacities of the cytoplasmic domains of the classical KIR, 3DL1, with 2DL5 and 2DL4 using a chimeric receptor approach. We defined the importance of two ITIM sequences for optimal KIR inhibition, because 2DL5 inhibition seems to be weaker than that of 3DL1. Our results indicate that 2DL5 functions as an inhibitory receptor, sharing similar inhibitory capacity with that of the cytoplasmic domain of 2DL4. Using cytotoxicity and conjugation formation assays and DN forms of phosphatases, we demonstrate that different inhibitory KIRs have qualitatively distinct inhibitory capacities, which cannot be entirely predicted from a strictly biochemical approach.

Materials and Methods
Cell culture and Abs
The IL-2-dependent NK-like cell line, NK-92 (a gift from C. Lutz, University of Iowa, Iowa City, IA), was cultured, as previously described (31). Cells were passaged with fresh IL-2 every 4 days. The murine mastocytoma, P815, was cultured, as previously described (31). Abs were purified with protein G from the hybridomas for the anti-3DL1 mAb, DX9, and the anti-CD56 mAb, B159.5.2, which were kindly provided by L. Lanier (University of California, San Francisco, CA) and B. Perussia (Thomas Jefferson University, Philadelphia, PA), respectively. The anti-FLAG mAb, M2, was purchased from Sigma-Aldrich (St. Louis, MO). Anti-SAP polyclonal Abs raised against the entire recombinant protein were obtained from Exalpha (Boston, MA).

cDNA constructs
KIR cDNA constructs were ligated into the retroviral expression vector, pBMN-NoGFP (green fluorescence protein), derived from pBMN-ires-EGFP (enhanced green fluorescence protein) (38), using BamHI, XhoI, and NotI restriction sites, as previously described (31). The 3DL1 cDNA (NKAT; GenBank accession number, L41269) was obtained from M. Colonna (Washington University, St. Louis, MO). The deletion mutant of 3DL1 (27P2A), which was truncated before the first ITIM and lacks most of the cytoplasmic domain, was described previously (31). To generate the chimeric 3DL1/L5 receptor, the cytoplasmic domain was amplified by PCR from the human 2DL1 (AF580003), kindly provided by C. Vilches (Inmunologia, Hospital Universitario Puerta de Hierro, Madrid, Spain) and cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego, CA) using the following primers: sense containing 5′-GAC GAC TGT TCA ACC ATT CC-3′, antisense containing 5′-GAT CAC GCT CGG GTA TTT AAT TAT TAT ATG GAA GAT-3′, and an antisense primer (BspHI), 5′-AGG TTC GGT GGG GGT GCC AGT-3′.

The function of 2DL5, however, has not been tested.
not be used as targets to engage 3DL1, because background inhibition resulted from HLA-A*2702 binding by the CD85 inhibitory receptor on NK-92 cells (data not shown).

Conjugate assay

P815 cells were cultured with fresh medium 1 day before assay. Also 1 day before assay, NK-92 cells were restimulated with fresh medium containing human IL-2, and the cell numbers of cultured NK-92 cells were adjusted to 8 × 10⁶ cells/ml in a 75-cm² flask, because cell density and its activation status influence the optimal conjugation. P815 and NK-92 cells were incubated for 30 min at 2 million/ml with prewarmed (37°C) medium containing either CellTracker Orange CMTMR (Molecular Probes, Eugene, OR) at 2 μM or CellTracker Green CMFDA (Molecular Probes) at 0.2 μM, respectively. The stained cells were washed, resuspended, incubated with prewarmed medium for 30 min at 2 million/ml, and washed again. P815 cells were incubated at room temperature with anti-KIR3DL1 mAb (DX9; 2 μg/ml). Conjugate formation was performed, as previously described (20), using a FACScan analyzer (BD Biosciences).

Pervanadate treatment and cell lysis

NK cells were washed three times in HBSS (Life Technologies), stimulated with pervanadate (100 μM), and lysed for 30 min on ice in 1 ml of 1% Triton X-100 lysis buffer per sample, as previously described (31). Lysates were cleared by centrifugation at 20,800 × g for 15 min at 4°C.

Immunoprecipitation and immunoblotting

Lysates were precleared twice for 30 min each at 4°C with protein G-coupled agarose (Upstate Biotechnology, Lake Placid, NY). CD56 and 2B4 or KIR were sequentially immunoprecipitated for 90 min at 4°C with B159.5.2 and anti-2B4 or DX9 mAbs (5 μg/sample precoupled to 30 μl of protein G-agarose), as performed previously (31), separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and 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). SAP was detected by rabbit polyclonal anti-SAP Abs (1/1,000), and secondarily with HRP-coupled protein G (1/10,000; Calbiochem).

Vaccinia virus infections

Recombinant vaccinia virus preparations generated with the plasmid pSC65 containing cDNA of wild-type SHP-1 or DN-SHP-1 (C455S) (9) were kindly provided by E. Long (National Institutes of Health, Rockville, MD). Vaccinia virus recombinants were prepared with recombinant vacinia virus encoding DN-SHP-1 (C455S) cDNA was previously described (20, 39). Aliquots of the recombinant virus preparations were dispersed by water-bath sonication and stored at −70°C before use. The titer of viral stocks was determined by plaque assay, as described (39) (wild-type SHP-1, 1 × 10⁶ PFU/ml; C455S SHP-1, 0.25 × 10⁶ PFU/ml; C455S SHP-2, 1 × 10⁶ PFU/ml). NK-92 cells were infected with the recombinant vaccinia virus, as described previously (31, 40). Vaccinia virus infections were monitored for SHP-1 or SHP-2 protein expression by immunoblot analysis of the infected cells. Viability of the infected cells was monitored by trypan blue staining before mixing with target cells (>95% viable).

Results

Inhibitory function of the cytoplasmic domain of 2DL5

To examine the potential inhibitory function of the 2DL5 cytoplasmic domain, we generated a chimeric receptor construct (3DL1/L5) in which the cytoplasmic domain of 2DL5, including the N-terminal ITIM- and C-terminal ITSM-like sequences, was fused to the extracellular and transmembrane domains of 3DL1 (Fig. 1, A and C). We previously used this chimeric receptor approach to establish the inhibitory potential of the 2DL4 cytoplasmic domain with the 3DL1/L4 fusion construct (Fig. 1, A and C) (31). These chimeric receptors allowed us to directly compare inhibitory properties of the 2DL5 cytoplasmic domain with those of 3DL1 and 2DL4 in the context of a common extracellular domain (3DL1). A truncated receptor, in which we deleted the 3DL1 cytoplasmic domain from just before the N-terminal ITIM (3DL1/272Δ), was used as a negative control (Fig. 1A). All of these receptors were expressed on the surface at similar levels in retrovirus-transduced NK-92 cells (Fig. 1B).

We first tested the inhibitory capacity of the transduced KIR using a redirected cytotoxicity assay against the murine mastocytoma cell line, P815. P815 expresses FcγRII/III that can interact with the Fc portion of IgG mAbs bound to the surface of NK cells, thereby effectively making them surrogate ligands in redirected cytotoxicity assays.

Ab engagement of the 3DL1/L5 chimera strongly inhibited target cell lysis to a degree that was nearly comparable to that of 3DL1 (Fig. 2A and Table I). The inhibition through 3DL1/L5 was similar to that through engagement of 3DL1/L4, whereas cytotoxicity was not affected by engagement of either CD56 or the control 3DL1/272Δ receptor (Fig. 2A), demonstrating the fidelity of this assay system. Therefore, despite the ITSM-like sequence, the cytoplasmic domain of 2DL5 can inhibit NK cell cytotoxicity to almost the same extent as a classical KIR.

The initial stage of NK cell-mediated cytotoxicity is the formation of stable conjugates between target and effector cells, which can be disrupted if inhibitory KIR engage with the target cell (10). Therefore, we performed a comparative analysis of the impacts of engaging 3DL1, 3DL1/L5, 3DL1/L4, and 3DL1/272Δ on the interaction of NK-92 cells with P815 cells using two-colored flow cytometry. We found that the wild-type 3DL1 significantly reduced the conjugate formation between NK-92 and P815 cells (Fig. 2B). However, the inhibitory capacities of the cytoplasmic domains of 2DL5 and 2DL4 were weaker than that of 3DL1 (Fig. 2B). As we have previously observed (31), engagement of 3DL1/272Δ reproducibly enhanced the conjugate formation, which may be due to the adhesion resulting from the multiple KIR/Ab/Fc receptor interactions (Fig. 2B, right panel). Thus, the cytoplasmic domains of 2DL5 and 2DL4 can only weakly inhibit target cell conjugate formation, despite the strong inhibition of target cell cytotoxicity by all three KIR cytoplasmic domains. These results indicate that differences in KIR ITIM configuration can more readily affect the capacity to inhibit adhesion to target cells than it can influence inhibition of cytotoxicity responses.

To determine the tyrosine sequence contributing to KIR2DL5 inhibition, the ITIM tyrosine in 3DL1/L5 was mutated to phenylalanine to create the mutant chimeric receptor, named 3DL1/L5/1F, and expressed individually on the cell surface. The mutant chimer was expressed in the NK-92 cell line by retroviral transduction and tested for inhibitory capacity in the redirected cytotoxicity assay. As shown in Fig. 2C, mutation of the ITIM completely abolished inhibitory capacity of the 2DL5 cytoplasmic domain. This result indicates that the ITSM-like sequence alone cannot elicit inhibitory function. Our previous work has already demonstrated that KIRs containing only the membrane-proximal ITIM (mutant 3DL1/YF or 3DL1/L4) can elicit strong inhibitory function through recruitment of SHP-2 (20, 31). Therefore, we did not mutate the ITSM-like tyrosine, because the remaining ITIM (VTYQL) would create a receptor that is virtually identical with those previously tested (especially 3DL1/L4).

The KIR2DL5 cytoplasmic domain recruits SHP-1 and SHP-2

We next examined whether the 2DL5 cytoplasmic domain could recruit SHP-1 and/or SHP-2 upon phosphorylation of the tyrosine residues. Both SHP-1 and SHP-2 have been shown to be recruited to classical KIRs to mediate inhibition (8, 9, 13–18, 20). In contrast, we have previously shown that the single ITIM of 2DL4 mediates strong inhibition of cytotoxicity, despite only binding SHP-2, but not SHP-1 (31). Therefore, both 3DL1 and 3DL1/L5 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. 3, upper left panel). Interestingly, the tyrosine-phosphorylated 3DL1/L5 chimera coimmunoprecipitated both SHP-1 and SHP-2 (left panels in Fig. 3), even though the inhibition of conjugate formation by 3DL1/L5 was weaker than that of 3DL1. As previously shown, both SHP-1 and SHP-2 were coimmunoprecipitated with tyrosine-phosphorylated 3DL1 (left panels in Fig. 3), while control CD56 immunoprecipititates did not bind either PTP (right panels). In contrast, we were unable to detect the recruitment of either PTP to the phosphorylated mutant 3DL1/L5, FY receptor (data not shown), indicating that binding depends upon the membrane-proximal ITIM sequence. We also tested whether these phosphorylated receptors could recruit other effector enzymes that have potential inhibitory function and an SH2 domain to bind phosphorylated ITIMs. Neither Csk (COOH-terminal Src kinase) nor SHIP was detectably recruited to phosphorylated forms of either 3DL1 or 3DL1/L5 in NK-92 cells (data not shown). These results suggest that 2DL5 inhibits NK cell activation through the PTP-mediated inhibitory pathway, like other classical KIRs possessing two canonical ITIMs.

**DN-SHP-2 blocks inhibition of natural cytotoxicity by 3DL1/L5**

To directly address the functional importance of either SHP-1 or SHP-2 in 3DL1/L5-mediated inhibition, we next tested whether expression of DN forms of the PTPs (catalytically inactive) could abolish KIR inhibitory function. Vaccinia virus-mediated expression of either DN-SHP-1 or DN-SHP-2 has been shown to block inhibition through classical KIRs, including 3DL1, which provides convincing evidence for involvement of both PTPs in this process (8, 9, 41, 42). Although SHP-1 and SHP-2 were both able to bind the cytoplasmic domain of 2DL5 (as was true for 3DL1), our conjugate formation study indicated that the inhibitory capacity of 3DL1/L5 was similar to that of 3DL1/L4 (SHP-2-only binding) (31). Therefore, we compared the impacts of treating NK-92 cells with recombinant vaccinia virus preparations expressing DN-SHP-1, wild-type SHP-1, or DN-SHP-2 on 3DL1/L5 inhibitory function. Receptor-transduced NK-92 cells were infected with purified vaccinia preparations at PFU titers that produced robust levels of the exogenous PTP (10- to 50-fold above endogenous) (20, 31) (data not shown). These doses of vaccinia expressing both DN-SHP-1 and DN-SHP-2 routinely block 3DL1 inhibitory function in our hands (20, 31). In a redirected cytotoxicity assay against P815 target cells, inhibition through 3DL1/L5 was virtually unchanged by expression of wild-type SHP-1 (Fig. 4). In contrast, expression of DN-SHP-2 completely abrogated wild-type 3DL1-mediated inhibition (Fig. 4), providing evidence that SHP-2 is directly recruited to KIR2DL5 during inhibitory signaling in vivo. Interestingly, DN-SHP-1 did not effectively block the inhibition by 3DL1/L5 (Fig. 4), even though our biochemical studies showed that SHP-1 could bind to 3DL1/L5 (Fig. 3). These results indicate that 2DL5 inhibition of NK cell activation is more dependent upon SHP-2 than SHP-1. This is an intriguing observation, indicating that the phosphorylated tyrosine-based motifs of 3DL1/L5 create a docking site that dominantly recruits SHP-2 to mediate inhibition.
The ITSM-like sequence of 2DL5 does not bind SAP. Because the cytoplasmic domain of 2DL5 contains an ITSM-like sequence, which is a known docking site for both SHP-2 and SAP, we next tested whether SAP binds to phosphorylated 3DL1/L5 or influences the role of SHP-2 in 2DL5 function. First, 3DL1/L5 and 2B4 were immunoprecipitated from pervanadate-treated NK-92 cells and tested for SHP-2 and SAP recruitment by immunoblotting. Pervanadate treatment stimulated robust tyrosine phosphorylation of 3DL1/L5 and 2B4 (Fig. 5A). Immunoprecipitates of tyrosine-phosphorylated 3DL1/L5 coprecipitated SHP-2, but SAP binding was not detected (Fig. 5A). Importantly, SAP was also not detected in immunoprecipitates of the phosphorylated mutant 3DL1/L5.FY receptor, in which only the ITSM-like tyrosine is intact (data not shown). In contrast, tyrosine-phosphorylated 2B4 coimmunoprecipitated SAP, but not SHP-2 (Fig. 5A), as previously described (35, 36). Control CD56 immunoprecipitates did not contain either protein (Fig. 5A).

We next tested whether overexpression of exogenous SAP could influence the inhibitory function of 3DL1/L5. In these experiments, SAP was overexpressed at levels of 5- to 10-fold above cells and tested for SHP-2 and SAP recruitment by immunoblotting. Pervanadate treatment stimulated robust tyrosine phosphorylation of 3DL1/L5 and 2B4 (Fig. 5A). Immunoprecipitates of tyrosine-phosphorylated 3DL1/L5 coprecipitated SAP, but SHP-2 binding was not detected (Fig. 5A). Importantly, SAP was also not detected in immunoprecipitates of the phosphorylated mutant 3DL1/L5.FY receptor, in which only the ITSM-like tyrosine is intact (data not shown). In contrast, tyrosine-phosphorylated 2B4 coimmunoprecipitated SAP, but not SHP-2 (Fig. 5A), as previously described (35, 36). Control CD56 immunoprecipitates did not contain either protein (Fig. 5A).

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Table 1. Statistical analysis of the capacities of mutant KIR3DL1 receptors to inhibit cytotoxicity

<table>
<thead>
<tr>
<th>Receptors</th>
<th>No. of Experiments</th>
<th>Mean Percentage of Control Cytotoxicity when Engaged with Anti-KIR3DL1 (+ SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3DL1</td>
<td>4</td>
<td>26.9 (12.5)*</td>
</tr>
<tr>
<td>3DL1/L5</td>
<td>6</td>
<td>34.9 (8.8)*</td>
</tr>
<tr>
<td>3DL1/L4</td>
<td>4</td>
<td>43.3 (10.8)*</td>
</tr>
<tr>
<td>3DL1/272PA</td>
<td>4</td>
<td>99.5 (6.2)*</td>
</tr>
</tbody>
</table>

*Statistical analysis was performed on cytotoxicity data from multiple experiments by converting the data at 10:1 E:T ratio within each experiment to percentage of control cytotoxicity (without Ab). Within each experiment, the duplicate or triplicate data points in the absence of Ab 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 percentage of control cytotoxicity (SD ranged from 2.8 to 4.4), and Student’s t test was performed to compare the aggregate data points from Ab-untreated and anti-KIR3DL1-treated conjugation conditions for each transduced cell line. Statistical analysis was performed on the Excel X program for Macintosh (Microsoft). *p < 0.05 compared with nontreated controls.
The full-length KIR2DL5 can inhibit NK cell activation

Despite the strong inhibitory capacity of the 2DL4 cytoplasmic domain in isolation (30, 31), substantial evidence indicates that 2DL4 is an activating receptor due to a charged transmembrane residue that appears to mediate association with a transmembrane accessory protein (28–30). Therefore, it was important to address the function of the full-length 2DL5 protein. For these experiments, the full-length 2DL5 was modified with an N-terminal FLAG epitope tag and introduced into NK-92 cells by retroviral transduction (Fig. 6, A and B). FLAG-2DL4 expression was always lower than FLAG-2DL5, as demonstrated in Fig. 6B. Engagement of the full-length 2DL5 with anti-FLAG mAb modestly, but reproducibly suppressed redirected cytotoxicity (Fig. 6C), while engagement of KIR2DL4 weakly potentiated cytotoxicity (Fig. 6C), as has been previously reported (28–30). Collectively, our results indicate that 2DL5 is an inhibitory receptor in human NK cells.

Discussion

It is well established that SHP-1 is recruited to phosphorylated ITIMs on the cytoplasmic domains of classical type I KIRs. A number of studies confirm that phosphorylation of both ITIMs is required for SHP-1 recruitment (8, 9, 13–16, 18, 31, 43). In contrast, our results (20, 31) and the work of Bruhs et al. (43) show that phosphorylation of the N-terminal ITIM of KIRs is essential and sufficient for strong inhibitory function that correlates with SHP-2 recruitment. It is of interest in the present study that 2DL5 showed weaker inhibitory capacity than 3DL1 (Table I), especially in conjugation assays (Fig. 2), even though both receptors recruited both SHP-1 and SHP-2 (Fig. 4), while DN-SHP-2 did block, indicating that 2DL5 predominantly uses a SHP-2-dependent inhibitory pathway. Our results further demonstrate that two canonical ITIMs are critical for strong inhibitory function that correlates with SHP-2 recruitment. It is of interest in the present study that 2DL5 showed weaker inhibitory capacity than 3DL1 (Table I), especially in conjugation assays (Fig. 2), even though both receptors recruited both SHP-1 and SHP-2 (Fig. 4), while DN-SHP-2 did block, indicating that 2DL5 predominantly uses a SHP-2-dependent inhibitory pathway. Our results further demonstrate that two canonical ITIMs are critical for SHP-2-dependent inhibitory function. Thus, the atypical ITSM-like sequence in 2DL5 seems to reduce the avidity of SHP-1 binding that is necessary for competent inhibitory function. Evidence of an inhibitory role for SHP-2 catalytic activity in 2DL5 function is further supported by our recent demonstration that direct fusion of the SHP-2 catalytic domain to the intracytoplasmic interface of 3DL1 results in a functional inhibitory receptor (20). Interestingly, as shown in Fig. 2C, the inhibitory KIR cytoplasmic domains that function predominantly through SHP-2 (2DL5, 2DL4, and mutant forms of 3DL1; Figs. 3 and 4) (20, 31) exhibit substantially reduced capacity to disrupt target cell conjugation when compared with 3DL1, which functions through both SHP-1

FIGURE 5. The ITSM-like sequence of KIR2DL5 does not recruit SAP. A, 3DL1/L5-transduced NK-92 cells were treated with pervanadate for 10 min and lysed with 1% Triton X-100, and sequential immunoprecipitation were prepared with anti-CD56, anti-2B4, and then anti-3DL1 mAbs. Each lane represents immunoprecipitation from a lysate of 40 million cells. Samples were analyzed on 15% SDS-PAGE gels under reducing conditions. Sequential immunoblotting analysis was performed with antiphosphotyrosine, anti-SHP-2, and anti-SAP. B, Redirected cytotoxicity assay using 3DL1/L5-NK-92 cells secondarily transduced with either vector alone (pBMN-IRES-EGFP (GFP); middle panel) or SAP in the same vector (right panel). The stable double-transduced NK-92 cells were tested for their abilities to lyse P815 in the absence of mAb (C) or in the presence of 1 μg/ml anti-3DL1 mAb (DX9; ○) or the anti-CD56 mAb (B159.5.2; open cross) as a control. Results are representative of six independent experiments.

FIGURE 6. Engagement of FLAG-tagged full-length KIR2DL5 can inhibit NK cell cytotoxicity. A, Schematic representations of the full-length KIR2DL5 constructs. FLAG-tag is shown as III. D0 and D2 are highly conserved Ig-like domains in the KIR family. TM and CY represent transmembrane and cytoplasmic domains, respectively. Tyrosine-based ITIM motif (VTYAQL) and ITSM-like sequence of 2DL5 (TMYMEL) are marked in the cytoplasmic domains. B, Flow cytometric analysis of 2DL5-transduced cells. The NK-92 cell line was transduced with the constructs shown as in A with retrovirus. The data show staining with anti-FLAG (M2; filled line) and PE-labeled anti-mouse IgG as a secondary alone (dashed line). C, Redirected cytotoxicity assay using NK-92 cells transduced with the KIR2DL5 receptor. The stable transduced NK-92 cells were tested for their abilities to lyse P815 in the absence of mAb (C) or in the presence of 1 μg/ml anti-FLAG mAb (M2; ○) or the anti-CD56 mAb (B159.5.2; open cross) as a control. Results are representative of three independent experiments, showing identical patterns of inhibition.
and SHP-2 (20). This result suggests that conjugation may be inhibited better by SHP-1 (or both phosphatases together) than by SHP-2 alone.

The SAP-binding ITSM sequence has recently been described in several human immunoreceptors, including 2B4, SLAM, CD84, Ly-9, and NTB-A (32, 33). Although these CD2 family members mainly exhibit activating functions, evidence indicates that engagement of some of them can inhibit cell activation in the absence of SAP expression. However, the exact consensus sequence of the ITSM is not yet established, because another receptor, CRACC/CSI, does not bind SAP despite the existence of two ITSM-like sequences (44). Similarly, our experiments failed to detect SAP binding to 3DL1/L5 by immunoprecipitation, and overexpression of SAP did not affect the inhibitory function of 3DL1/L5 in NK-92 cells, indicating that SAP does not contribute to the unique inhibitory function of 2DL5. Another important incidental finding is that we could not detect SHP-2 binding to 2B4 when it was phosphorylated in NK-92 cells (Fig. 5A). Although 2B4 has been shown to bind SHP-2 in the absence of SAP in COS-7 or BaF3 cells (35, 45, 46), SHP-2 recruitment to 2B4 has never been demonstrated in human NK cells, including primary human NK cells (36, 37), YT cells (35), and NK-92 cells (Fig. 5A), or even in the absence of SAP in NK cells derived from X-linked lymphoproliferative disease patients (37).

The function of 2DL4 was originally thought to be inhibitory in NK cells, because it has an ITIM in the cytoplasmic domain and binds the nonclassical MHC-I molecule, HLA-G (47, 48). However, it has become apparent that 2DL4 is an activating receptor, which strongly induces IFN-γ production when engaged (28–30). In contrast, when only the cytoplasmic domain of 2DL4 was tested in isolation, it inhibited NK cell cytotoxicity (30, 31). Because of the high homology between the two members of the type II KIR subfamily (79% amino acid identity) (49), 2DL5 and 2DL4, we examined the inhibitory capacity of the full-length 2DL5 appended with an N-terminal FLAG epitope tag in NK-92 cells. The full-length 2DL5 suppressed cytotoxicity, demonstrating that 2DL5 is an inhibitory receptor. Although we cannot exclude binding of 2DL5 to an activating accessory protein that is not expressed in NK-92 cells, the absence of a charged amino acid in the 2DL5 transmembrane domain makes this possibility unlikely. Thus, evidence indicates that 2DL5 is an inhibitory receptor, while 2DL4 is an activating receptor within the type II KIR2D subgroup of human NK cells. The weak inhibitory capacity of the full-length 2DL5 compared with the chimera, 3DL1/L5, may be due to weaker affinity of the FLAG-specific Ab compared with that of DX9, anti-KIR3DL1 mAb. Alternatively, unique D0–D2 structural orientations of 2DL4 and 2DL5 or mAb-binding orientation of the N-FLAG motif may prevent optimal engagement in the redirected cytotoxicity conjugates when compared with engagement of the 3DL1 extracellular domain by DX9 mAb. In accordance with our results, hemoglobin-tagged 2DL4 with a mutation in the transmembrane arginine demonstrated only weak inhibition when engaged with anti-hemoglobin-tag Ab (30).

Human NK cells use inhibitory lectin-like and Ig-like receptors, including CD94/NKG2A and KIRs, to recognize MHC-I molecules and block responses toward normal cells. CD94/NKG2A engages with the nonclassical HLA-E, while the majority of KIR family members are specific for subsets of HLA-A, -B, or -C allotypes (50). KIR genes are differentially inherited among individuals in the human population (51). A model for KIR gene expression has been proposed, in which two major expression profiles were defined as A and B haplotypes. Individuals exhibiting the group A haplotype have six KIR genes, including 2DL1, 2DL3, 3DL1, and 2DS4, while the group B KIR haplotypes are more variable in their organization, including at least one additional gene (51). Although the 2DL4 gene is present in almost all individuals and is reportedly transcribed in all NK cells, 2DL5 belongs to the B haplotype and is clonally expressed by a subset of NK cells in those individuals (22). It is tempting to speculate that the 2DL5 gene derives from 2DL4, because the exon-intron genomic DNA organization of 2DL5 is similar to that of 2DL4 (49), they share 79% amino acid homology, and they have the same configuration of extracellular Ig-like domains and a long cytoplasmic tail (49). Furthermore, it is interesting that the C-terminal tyrosine in the cytoplasmic domain of 2DL4 of pygmy chimpanzee, common chimpanzee, and rhesus monkey is in the context of SxxYxxL, similar to the TyYxXL of 2DL5, suggesting related evolution and/or functional importance. Our results demonstrate that the lack of the charged transmembrane residue in 2DL5 allows it to function as an inhibitory member of the type II KIR2D subfamily. Our data in this report and our previous analysis of 2DL4 (31), however, demonstrate that the atypical nature of or complete loss of the C-terminal ITIM of type II KIR2D receptors establishes a SHP-2-selective docking site. Thus, it is tempting to speculate that the classical inhibitory KIRs, such as 3DL1, seems to have acquired SHP-1-binding capacity to enhance or insure signaling in NK cells that express both SHP-1 and SHP-2 when they obtained two typical ITIMs in the cytoplasmic domain. Whether KIRs use distinct PTPs to inhibit different activation signaling pathways or whether SHP-1 and SHP-2 have redundant roles in inhibition are still unknown and await further analysis.

The ligand specificities of the type II KIR2D receptors remain to be clarified. The ligand specificity of 2DL5 has not been reported to date. Although two groups have reported that HLA-G serves as a ligand for 2DL4 (47, 48, 52), two other groups have disputed this ligand specificity (53, 54). Nonetheless, the type II KIR2D receptors exhibit a unique Ig domain architecture (D0 and D2) that borrows from the conserved Ig domains of the KIR3D subfamily (possessing D0, D1, and D2), but is distinct from those of the type I KIR2D subfamily (consisting of D1 and D2 domains). Because D1 and D2 domains contribute the structural elements that recognize the classical HLA-A, -B, and -C molecules (55–57), the D0–D2 orientation implies distinct ligand specificity for 2DL4 and 2DL5. Although it is unclear whether they recognize the same ligand, our results demonstrate that 2DL5 transduces negative signals, while 2DL4 is an activating member of this type II KIR2D subfamily.

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