The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 15 2005

Killer Cell Ig-Like Receptor-Dependent Signaling by Ig-Like Transcript 2 (ILT2/CD85j/LILRB1/LIR-1)[1]

Sheryl E. Kirwan; ... et. al

J Immunol (2005) 175 (8): 5006–5015.
https://doi.org/10.4049/jimmunol.175.8.5006

Killer Cell Ig-Like Receptor-Dependent Signaling by Ig-Like Transcript 2 (ILT2/CD85j/LILRB1/LIR-1)[1]

Sheryl E. Kirwan and Deborah N. Burshtyn[2]

Inhibitory killer cell Ig-like receptors (KIR) signal by recruitment of the tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 to ITIM. In the present study, we show that, surprisingly, KIR lacking ITIM are able to signal and inhibit in the human NK cell line NK92, but not in mouse NK cells. Signaling by mutant KIR is weaker than the wild-type receptor, does not require the transmembrane or cytoplasmic tail of KIR, and is blocked by overexpression of a catalytically inactive Src homology region 2 domain-containing phosphatase-1 molecule. We also demonstrate that mutant KIR signaling is blocked by Abs, which disrupt the interaction between KIR and human leukocyte Ag-C or Abs, which block the interaction between Ig-like transcript 2 (ILT2) and the α3 domain of HLA class I molecules. Thus, although ILT2 expressed in NK92 is insufficient to signal in response to human leukocyte Ag-C alone, ILT2 can signal in a KIR-dependent manner revealing functional cooperation between receptors encoded by two distinct inhibitory receptor families. *The Journal of Immunology,* **2005, 175: 5006–5015.**

Natural killer cells are large granular lymphocytes of the innate immune system that can recognize and eliminate cells which fail to express self-MHC class I (MHC-I)[3] molecules. Susceptibility to NK-mediated lysis due to down-regulation of MHC-I expression occurs during virus infection and malignancy (1, 2). NK cells detect a cell that has down-modulated MHC-I expression by virtue of cell surface inhibitory receptors that bind classical and nonclassical MHC-I molecules. Individual NK cells express both activating and inhibitory receptors, which together determine the target cell specificity (3, 4). Activating receptors trigger tyrosine kinases that initiate signaling cascades, leading to degranulation of the NK cells and/or gene transcription (5). The hallmark of inhibitory receptors is the presence of the ITIM consensus sequence I/L/V/SxYxxL/V (6). ITIMs mediate the inhibitory signal by their phosphorylation-dependent recruitment of a tyrosine phosphatase with Src homology (SH)2 domains, SH region 2 domain-containing phosphatase (SHP)-1 or SHP-2. The SH2 domain-mediated recruitment of SHP-1 or SHP-2 stimulates the catalytic activity of the phosphatase, which then targets phosphorylated intermediates required for activation (7, 8). In general, mutation of the ITIM produces a nonfunctional receptor (9–15).

Human NK cells express a complex mixture of inhibitory receptors that fall into two broad families: the paired Ig-like receptor superfamily and those related to C-type lectins (4) that have unique and overlapping functions. Killer cell Ig-like receptors (KIR) are prototypic members of the paired Ig-like receptor superfamily expressed by NK cells and a subset of T lymphocytes. Inhibitory KIR on NK cells prevent lysis of normal healthy cells by recognition of classical MHC-I proteins. KIR bind to polymorphic determinants in the α1 domain of HLA-A, -B, or -C. HLA-C is believed to be particularly important for the NK cell response because all allotypes of HLA-C are KIR ligands, and the majority of cloned NK cells recognize HLA-C (16). In the case of KIR, both the receptors and the ligands are highly polymorphic (16). CD94/NKG2A is a relatively nonpolymorphic inhibitory receptor belonging to the C-type lectin family that recognizes the nonclassical class I molecule HLA-E (17–19). The expression of HLA-E on the cell surface is dependent on signal peptides derived from classical and other nonclassical MHC-I molecules, essentially allowing all cells with classical MHC-I molecules to express some HLA-E. Recently, CD94/NKG2A has been suggested to be an important inhibitory receptor for NK interactions with dendritic cells promoting maturation of dendritic cells (20, 21) and may play a role in recognition of cells that naturally express nonclassical MHC-I molecules (HLA-G and HLA-E), such as placental trophoblasts (22). CD94/NKG2A is expressed on the majority of NK cells derived from peripheral blood in certain individuals and is believed to account for their NK self-tolerance (23).

Ig-like transcript 2 (ILT2), also known as CD85j, LIR-1, and LILRB1, is an inhibitory receptor belonging to the Ig superfamily expressed on a wide range of immune cells, possibly providing inhibitory signals to multiple components of the host's immune system (24–26). ILT2 is predominantly expressed on B lymphocytes, monocytes, and dendritic cells but is also present on a subset of NK cells and T lymphocytes. ILT2 has four Ig domains and contains ITIMs in its cytoplasmic tail that inhibit cellular responses by recruiting SHP-1 (15, 25). ILT2/LIR-1 was cloned based on its interaction with the human cytomegalovirus UL18 gene product, a MHC-I homologue (25). ILT2 has also been shown to recognize a wide range of both classical and nonclassical MHC-I molecules by interaction with the relatively nonpolymorphic α3 region of class I MHC proteins (25–28). Surface plasmon resonance studies suggest that ILT2 binds with a higher affinity to HLA-G (which is predominantly expressed by placental trophoblasts) than to classical MHC-I (29) and with very high affinity to

Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada

Received for publication December 13, 2004. Accepted for publication July 28, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported part by a grant from the Canadian Institutes for Health Research (CIHR) and the Alberta Heritage Foundation for Medical Research. D.N.B. is a Heritage Research Scholar, a CIHR Scholar, and a recipient of the Lougheed Fellowship. S.E.K. is a recipient of a Province of Alberta Graduate Fellowship.

[2] Address correspondence and reprint requests to Dr. Deborah Burshtyn, Department of Medical Microbiology and Immunology, 659 Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, T6G 2S2 Canada. E-mail address: burshtyn@ulberta.ca

[3] Abbreviations used in this paper: MHC-I, MHC class I; SH, Src homology; SHP, SH region 2 domain-containing phosphatase; KIR, killer cell Ig-like receptor; ILT2, Ig-like transcript 2; EFGP, enhanced GFP; PDGF, platelet-derived growth factor receptor; PDGFR, PDGF receptor; MOI, multiplicity of infection; DN, dominant negative; ADCC, Ab-dependent cellular cytotoxicity.

0022-1767/05/$02.00

UL18 (28). Based on these observations, it has been suggested that ILT2 functions in NK cells as a MHC-I inhibitory receptor with broad specificity.

It is believed that processes during NK development ensure that NK cells express a complement of inhibitory receptors sufficient to recognize autologous MHC-I (23). The resulting individual NK cells may express one or multiple KIR and/or CD94/NKG2A and/or ILT2. Both ILT2 and CD94/NKG2A are able to provide broad reactivity for a large subset of MHC-I alleles. However the frequency of peripheral blood NK cells that express ILT2 and CD94/NKG2A varies among individuals (23, 26, 27). In addition, ILT2 on NK cells is lower than on myeloid and B cells (25, 26). Although ILT2 function has been intensively studied in T cells (30–38), there is only one report examining ILT2 function in NK clones (27). In the study of Vitale et al. (27), one clone was shown to be inhibited by several HLA-A and B alleles dependent on ILT2, but this inhibition was also dependent on CD94/NKG2A with the exception of HLA-A1. A single clone expressing ILT2 in the absence of CD94/NKG2A or known KIR was shown to be inhibited by HLA-G through ILT2 and exhibited a slight reduction in lysis of HLA-C-expressing cells. Others have shown that ILT2 expressed on primary NK cells is involved in inhibition via HLA-G (39–41). To our knowledge, there is presently little evidence to suggest that the level of ILT2 in most primary NK cells can protect target cells expressing classical MHC-I in the absence of another inhibitory receptor. Therefore, it is curious why the frequency of ILT2 expression is variable and why it is prevalent only at relatively low levels on peripheral NK cells. Here we present studies with ITIM-deficient KIR, which reveal the contribution of ILT2 to KIR signaling. These results suggest that coexpression of ILT2 with KIR in human NK cells may compensate for weak interactions between particular KIR and MHC-I.

Materials and Methods

Cells and Abs

The NK92 cell line was obtained from Dr. E. Long (National Institutes of Health, Bethesda, MD) and purchased from American Type Culture Collection (CRL-2407). NK92 cells were cultured in 50% Myelocult H5100 (StemCell Technologies) and 50% Iscove's medium with 10% FBS (HyClone), 50 μM 2-ME, and 2 mM L-glutamine (Invitrogen Life Technologies) supplemented with 100 U/ml human rIL-2 (TECIN; Biological Resources Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute-Frederick Cancer Research and Development Center). NK92 cells were transfected with KIR2DL1 fused to GFP (NK92/KIR2DL1-GFP) and KIR2DL1 TR-GFP (NK92/KIR2DL1TR-GFP) using the vector pBSRαEN and subcloned (42). The clone expressing full-length KIR2DL1-enhanced GFP (EGFP) chimera (KIR-GFP) was previously described and encodes the entire KIR2DL1 sequence linked to EGFP by the amino acids GSIAT (43). The clone expressing truncated KIR (KIR2DL1TR-GFP) was constructed such that the coding sequence is truncated just upstream of the membrane proximal ITIM at residue 276 and linked to EGFP by the amino acids PVAT, as described previously (42). Both NK92/KIR2DL1-GFP and NK92/KIR2DL1TR-GFP were maintained in NK92 medium supplemented with 100 U/ml rIL-2 and 0.5 mg/ml geneticin (Invitrogen Life Technologies). The cell line YTS was maintained in Iscove's medium containing 15% FBS (HyClone), 50 μM 2-ME, and 2 mM L-glutamine.

Mouse NK cells were isolated from the spleens of C57BL/6 mice as previously described and cultured with 1000 U/ml rIL-2 (10). Primary human NK cells were isolated from whole blood by magnetic separation using StemSep (Stem Cell Technologies). Collection of blood and experimentation has been approved by the Health Research Ethics Board at the University of Alberta. Where indicated, day 1 human NK cells were depleted for KIR2DL1/S1 and sorted for ILT2 low or high expression. The target cell lines 721.221, 221-Cw3, 221-Cw4, 221-Cw7, and 221-Cw15 cells were obtained from P. Parham (Stanford University, Stanford, CA). 721.221 cells were maintained in Iscove's with 10% FBS (Invitrogen Life Technologies) and 2 mM L-glutamine. The transfectants were maintained in the same medium supplemented with 0.5 mg/ml geneticin.

Anti-CD158a mAb EB6 specific for KIR2DL1/S1 (IgG1) and anti-NKG2A clone Z199 (IgG2b) were purchased from Beckman Coulter. Clarified MOPC-104E ascites (control IgM) was purchased from Sigma-Aldrich. Anti-CD85j Ab HP-F1 specific for ILT2 (26) was kindly provided by M. Lopez-Botet (Universitat Pompen Fabra, Barcelona, Spain). Anti-CD158a clone HP-3E4 (IgM) ascites (44) was provided by Dr. E. Long. W6/32 (IgG2a), a pan-HLA-reactive Ab, and L243, an anti-HLA-DR Ab (IgG2a), were purified from culture supernatants using protein A-Sepharose. $F(ab')_2$ fragments of W6/32 were generated by pepsin digestion. FITC- and PE-conjugated goat anti-mouse IgG were purchased from Cedarlane Laboratories. Where required for functional experiments, Abs were dialyzed into Dulbecco's PBS to remove azide. Directly conjugated Abs HP-3E4-FITC (anti-KIR2DL1/S1; BD Biosciences), DX27-FITC (anti-KIR2DL2/L3/S2; BD Biosciences), DX9-FITC (anti-KIR3DL1; BioLegend), GHI/75-CyChrome (anti-ILT2/CD85j; BD Biosciences), and isotype-matched control directly conjugated Abs were used in multicolored flow cytometry analysis of purified human NK cells from each donor.

Constructs and recombinant vaccinia viruses

The double tyrosine-to-phenylalanine mutant 2DL1.Y^2F was generated by site-directed mutagenesis of KIR2DL1 in the vector pSport using the QuikChange (Stratagene) method. Primers used to generate 2DL1.Y^2F were 5′-GATATCATCGTGTTCACGGAACTTCC-3′ and its reverse complement, followed by 5′-CCTCAGGAGGTGACATTCACACAGTT GAATC-3′ and its reverse complement. The 2DL1.HRK truncation mutant was generated by PCR using the forward primer Sal-KIRECfwd (5′-GT GGACATGTCGCTCTTGTTCGTC-3′) that introduced the *Sal*I cloning site and the reverse primer KIR-HRKstoprev (5′-GCGGCCGCTCACTT GCGATGAAGGAG-3′) that introduced both the stop codon and the *Not*I cloning site and TA cloned into pGEMT (Promega). The KIR.PDGF mutant was generated by overlapping PCR. The first step was to amplify the extracellular region of KIR with an overlap into platelet-derived growth factor receptor (PDGFR) region using the primers Sal-KIRECfwd (as above) and the reverse primer KIR.PDGFRoverlap (5′-CGTGTCCTGGC CCACAGCGTGCAGGTGTCGGGGGTT-3′). The PDGFR transmembrane to the 3′-PDGFR end were amplified from the plasmid pDisplay (Invitrogen Life Technologies) with the primers PDGFR-TMfwd (5′-GCT GTGGGCCAGGACACG-3′) and PDGFR-TMrev (5′-GCGGCCGCCTA ACGTGGC-3′). These purified PCR products were combined and amplified by PCR with the primers Sal-KIRECfwd and PDGFR-TMrev. The PCR product was ligated into pGEMT and sequenced. The extracellular region of KIR and the transmembrane of PDGFR were fused together with no linker. The *Sal*I-*Not*I fragments containing the constructs KIR2DL1.Y^2F, KIR2DL1.HRK, and KIR2DL1.PDGF were subcloned into pSC65 with a modified multiple cloning site to include *Sal*I and *Not*I (herein denoted as pSC66). We obtained from the cDNA of ILT2 from Dr. E. Long (named MIR.CL7-pCMV-Sport) and subcloned the *Sal*I-*Not*I insert into pSC66.

All constructs within pSC66 and the empty vector pSC66 alone were recombined with vaccinia strain WR as described previously (45). Vaccinia viruses encoding KIR2DL1 and DN.SHP1 have been described previously, named cl42 and HCP453S, respectively (46, 47). All recombinant vaccinia viruses were propagated in TK^- cells, released from the cells by sonication, and enriched by spinning through a 36% sucrose cushion. Titers in plaque-forming units were determined in TK^- cells.

Infection with recombinant vaccinia viruses

NK92 or mouse NK cells were washed into Iscove's medium supplemented with 2 mM L-glutamine, 1× nonessential amino acids, 0.2% BSA, and 100 U/ml rIL-2. The cells were infected at the indicated multiplicity of infection (MOI) with vaccinia virus at 37°C with 5% CO_2. All experiments involving vaccinia virus infection were conducted in the presence of cytosine β-D-arabinofuranoside-HCl (Sigma-Aldrich) at a final concentration of 40 μg/ml to prevent replication of viral DNA.

Cytolysis assay

After virus infection, mouse NK cells, human NK cells, or NK92 cells were washed, counted, and diluted to the appropriate concentrations in warm assay medium (Iscove's medium with 5% FBS and 2 mM L-glutamine) with 100 U/ml rIL-2. Cytosine β-D-arabinofuranoside-HCl was maintained at 40 μg/ml throughout the experiment. Cytolysis was measured by chromium release as follows: Target cells were labeled with ^{51}Cr sodium chromate (NEN), washed three times in warm assay medium, diluted to the appropriate concentration of 2500 cells/well, plated with effector cells in triplicate, and incubated at 37°C with 5% CO_2 for 4 h. For Ab-blocking experiments, NK cells were preincubated in twice the final concentration of intact Ab or W6/32 $F(ab')_2$ fragments for 5 min at room temperature and

then mixed 1:1 with target cells. Chromium release was quantified for 50 μl of supernatant incorporated into 150 μl of scintillation fluid and analyzed in a 1450 Microbeta Trilux (Wallac). ^{51}Cr release was calculated as: percent lysis = 100 × (mean sample release − mean spontaneous release)/(mean total release − mean spontaneous release). For assays using mouse NK cells, the chromium-labeled target cells were preincubated in 1 μg/ml L243 for 20 min, washed once, and plated.

Results

Signaling by truncated KIR-EGFP chimeras in transfected NK92 cells

NK92 cells were stably transfected with KIR-EGFP chimeras named KIR2DL1-GFP and KIR2DL1TR-GFP (42). KIR2DL1-GFP has EGFP fused to the C terminus of the full-length receptor, and KIR2DL1TR-GFP has EGFP fused in frame just upstream of the membrane proximal ITIM (Fig. 1*A*). The cell lines express KIR-GFP and KIR TR-GFP at comparable levels (Fig. 1*B*). KIR2DL1-GFP provided strong inhibition of lysis of target cells expressing its ligand HLA-Cw15 (Fig. 1*C*). However, we also observed ~30% drop in specific lysis with the ITIM-deficient receptor KIR2DL1TR-GFP, albeit the reduction in lysis was much less compared with the full-length construct (Fig. 1*C*). To ensure the reduction in lysis was due to the specific interaction of KIR with Cw15, we performed experiments in the presence of a blocking anti-KIR Ab, HP-3E4. The presence of the anti-KIR Ab brought Cw15 target lysis to a level similar to lysis of the target cells with the control MHC-I, HLA-Cw3 (Fig. 1*D*). These experiments suggest that ITIM-deficient KIR can signal in NK92 cells and that the inhibition requires the interaction between the KIR and its ligand, MHC-I. This interaction is likely also sensitive to the allele or amount of HLA-C because we did not observe this with 721.221 cells transfected with Cw4 (42) that do not express as highly as Cw15.

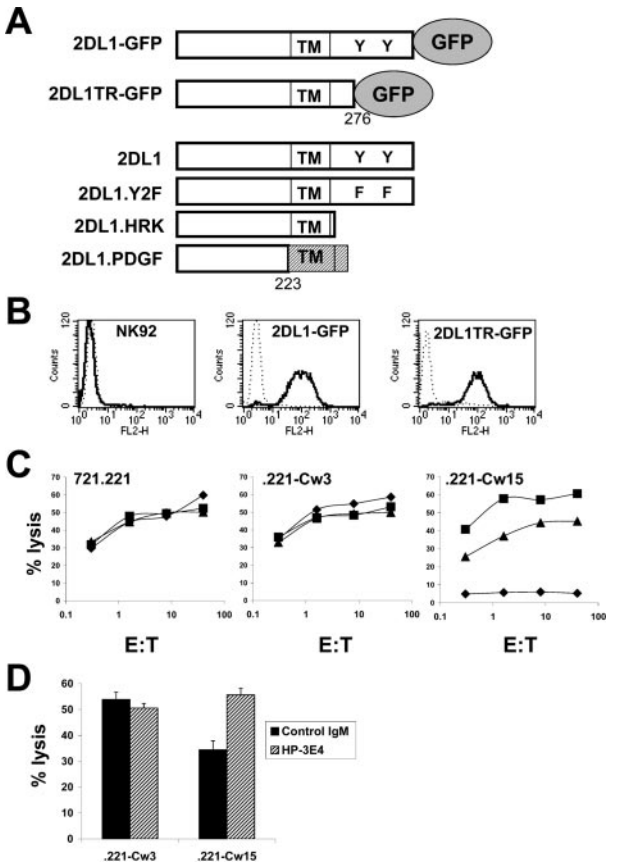

FIGURE 1. Signaling by a truncated KIR in stably transfected NK92 cells. *A*, Schematic diagram of wild-type KIR (2DL1) and mutant KIR constructs used in this study. TM stands for transmembrane region, GFP for enhanced GFP, and the hatched box represents the transmembrane region and short tail from the PDGR. The numbers 276 and 223 of 2DL1TR-GFP and 2DL1.GFP, respectively, indicate the final amino acid of KIR2DL1 that is present in each chimeric receptor. *B*, The KIR2DL1 expression levels were determined by flow cytometry for NK92 cells (*left*), NK92/2DL1-GFP (*middle*), and NK92/2DL1TR-GFP (*right*). The isotype control is indicated by the dotted line and the anti-KIR2DL1 mAb EB6 by the solid line. *C*, Cytolysis of ligand-positive or -negative cells. Cytolysis by NK92 (■), NK92/2DL1-GFP (◆), or NK92/2DL1TR-GFP (▲) with the indicated target cell line was determined in a standard chromium release assay. *D*, Ab blocking of KIR function. Cytolysis assays were performed with NK92/2DL1TR-GFP in the presence of control IgM (■) or anti-KIR Ab HP-3E4 (▨). The E:T shown is 15:1.

ITIM-deficient KIR signals in NK92 but not mouse NK cells

We have published previously that KIR of another specificity carrying point mutations of the ITIM were unable to function in mouse NK cells and KIR2DL1 lacking the ITIM region and does not function in the human YTS cell line (8, 10, 11). Therefore, we considered the possibility that the EGFP moiety was responsible for the inhibitory signal we observed in the stable cell lines. To test the ability of untagged ITIM-deficient KIR2DL1 to signal in NK92 cells and mouse NK cells, we compared the function of KIR2DL1 and a double tyrosine to phenylalanine substituted KIR2DL1, named 2DL1.Y^{2}F (Fig. 1*A* and *Materials and Methods*) using recombinant vaccinia virus. Similar to our previous observations with KIR2DL3 (10), 2DL1.Y^{2}F did not inhibit Ab-dependent cellular cytotoxicity (ADCC) by mouse NK cells (Fig. 2, *A* and *B*). However, when expressed in NK92 cells using recombinant vaccinia viruses, the mutant receptor 2DL1.Y^{2}F also reduced lysis of HLA-Cw15 cells, although not quite to the same extent as the wild-type receptor (Fig. 2, *C* and *D*). We observed this same result with another KIR (KIR2DL3) that recognizes a different allele of HLA-C; a double tyrosine to phenylalanine mutant 2DL3.Y^{2}F expressed in NK92 cells by vaccinia virus inhibited killing of target lines bearing HLA-Cw3 showing that this phenomenon in not specific to KIR2DL1 (data not shown).

The extracellular domains of KIR are sufficient for inhibition by KIR in NK92 cells

It has been suggested that KIR with phenylalanine substitutions of the ITIM tyrosines signal weakly by recruitment of SHP-2 to the mutated ITIM (48). However, our KIR2DL1TR-GFP chimera completely lacks the ITIM sequences, suggesting a cryptic ITIM is not essential for the secondary signaling pathway. Therefore, to further delineate what region of the receptor was required to signal in NK92 cells, we generated a KIR that was truncated just after the transmembrane domain, 2DL1.HRK, and a chimera of the extracellular region of KIR2DL1 fused to the transmembrane and short tail of the PDGFR, 2DL1.PDGF (Fig. 1*A*). These receptors were also introduced into NK92 by the vaccinia virus expression system. Again, inhibition of lysis was observed when the NK92 cells were infected with the virus expressing wild-type KIR2DL1, 2DL1.Y^{2}F, as well as 2DL1.HRK and 2DL1.PDGF (Fig. 3*A*). The amount of inhibition by the truncated and membrane swapped receptors was not as pronounced as 2DL1.Y^{2}F in any of the experiments performed; however, the level of expression of the truncated receptors was always much lower than the 2DL1 or 2DL1.Y^{2}F. In the experiment shown, a higher MOI was used for the truncated receptors to attempt to compensate for the apparent expression defect (Fig. 3*B*).

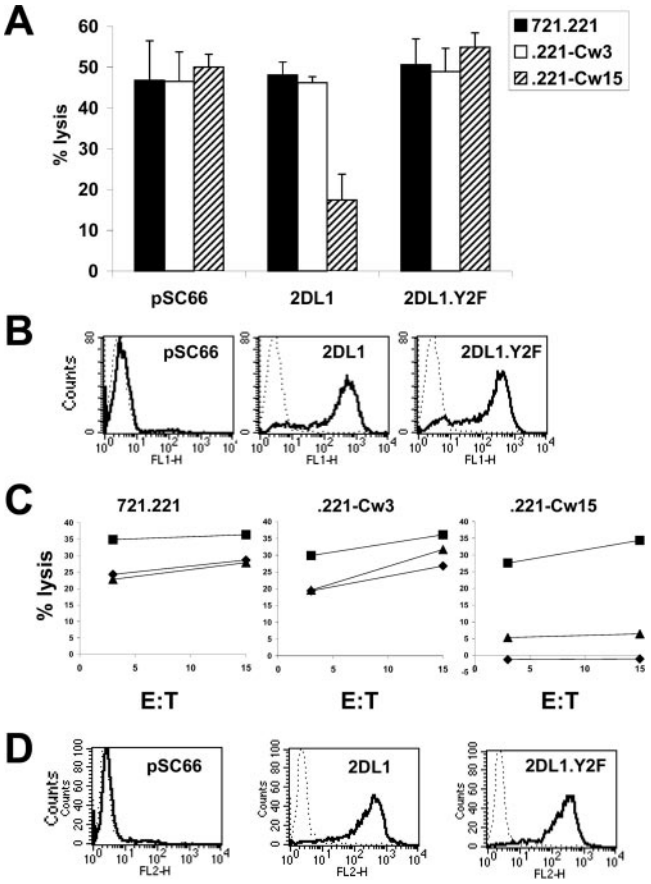

FIGURE 2. Comparison of ITIM-deficient KIR function in NK92 and mouse NK cells using recombinant vaccinia virus. *A*, Function of wild-type and mutant KIR2DL1 in mouse cells. Mouse NK cells were infected for 3 h with recombinant vaccinia virus to express 2DL1 (MOI of 10) or 2DL1.Y^2F (MOI of 35) or with the control virus pSC66 (MOI of 35). Following infection, the cells were washed, counted, and plated for the cytolysis assay with the indicated target cells that had been coated with L243 Ab to induce activation of killing by ADCC. The E:T shown is 12:1. *B*, Corresponding analysis by flow cytometry of KIR2DL1 expression on effector cells used in *A*. Isotype control Ab is indicated with the dotted line and anti-KIR mAb EB6 with the dark line. *C*, NK92 cells were infected for 2 h with vaccinia viruses encoding 2DL1 (◆), 2DL1.Y^2F (▲), or the pSC66 (■). The MOI were 10, 20, and 20, respectively. *D*, Corresponding analysis by flow cytometry of KIR2DL1 expression on effector cells used in *C*. Isotype control is indicated with the dotted line and anti-KIR mAb EB6 with the dark line.

Catalytically inactive SHP-1 reverts ITIM-deficient KIR signaling

We have previously shown that inhibition by wild-type KIR expressed in NK92 can be blocked by overexpression of catalytically inactive SHP-1 (47). Catalytically inactive SHP-1 is believed to act as a dominant negative (DN) by competing for association with the receptor through its SH2 domains. To test whether DN-SHP-1 would also interfere with signaling by ITIM-deficient KIR, we coexpressed catalytically inactive SHP-1 with the wild-type or ITIM-deficient 2DL1 in NK92 cells, using recombinant vaccinia virus. To normalize the degree of infection, we coinfected with vaccinia virus recombined with the empty vector pSC66. In this case, the wild-type and mutant receptors exhibit very similar levels of expression, illustrating that 2DL1.HRK is very similar in potency to 2DL1.Y^2F. Catalytically inactive DN-SHP-1 reverted inhibition by wild-type KIR2DL1, 2DL1.Y^2F, and 2DL1.HRK (Fig. 4*A*). Fig. 4*B* shows that the coinfection with virus expressing DN-SHP-1 did not alter the level of receptor on the cell surface. Therefore, the ability of catalytically inactive SHP-1 to revert inhibition by even the completely truncated KIR suggested that another protein with binding sites for SHP-1, or a highly related molecule such as SHP-2, is involved in the inhibition.

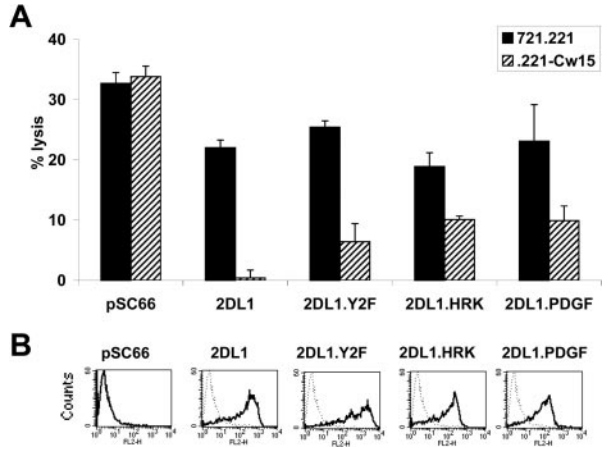

FIGURE 3. The extracellular domains of KIR are sufficient for inhibition in NK92 cells. *A*, NK92 were infected for 2 h with pSC66 (MOI of 20) or with recombinant vaccinia viruses to express 2DL1 (MOI of 10), 2DL1.Y^2F (MOI of 10), 2DL1.HRK (MOI of 20), and 2DL1.PDGF (MOI of 20). Cytolysis was measured in a standard chromium release assay and the E:T ratio shown is 20:1. *B*, Corresponding analysis by flow cytometry for expression of the KIR2DL1 extracellular domains. The isotype control is indicated with the dotted line and anti-KIR mAb EB6 with the dark line.

The role of ILT2 in signaling by ITIM-deficient KIR

To explain our observations, we considered the possibility that other known receptors with ITIMs might be involved in ITIM-deficient KIR signaling. Such a receptor would need to be

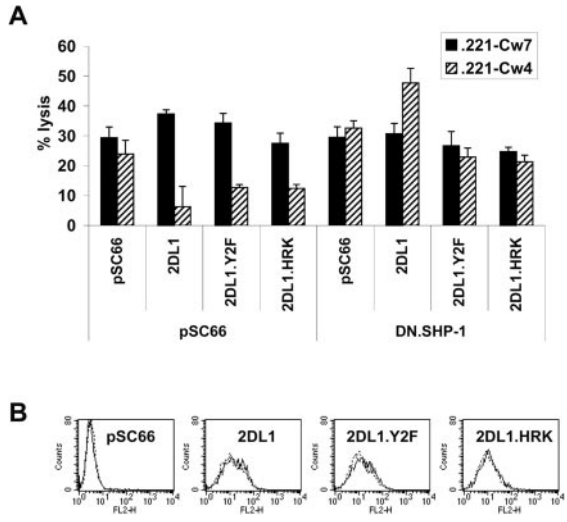

FIGURE 4. ITIM-deficient KIR signaling in the presence of catalytically inactive SHP-1. *A*, NK92 cells were infected for 2 h to express 2DL1 (MOI of 12.5), 2DL1.Y^2F (MOI of 12.5), or 2DL1.HRK (MOI of 20) plus either pSC66 or DN.SHP-1 (MOI of 12.5). Cytolysis was measured in a standard chromium release assay. The E:T ratio shown is 36:1. *B*, Corresponding analysis by flow cytometry of KIR levels with EB6 Ab for the cells used in *A*. The solid line is coinfection with pSC66, and the dashed line is coinfection with DN-SHP-1.

expressed by NK92 but not mouse NK cells. In addition, we considered the observation that similar point mutations and truncations render the 2DL1 receptor nonfunctional when stably expressed in another human NK-like line, YTS (11). Therefore, we compared the expression levels of other ITIM-containing receptors on NK92 and YTS. NK92, but not YTS, express both CD94/NKG2A and ILT2, making these receptors candidates for contributing to inhibition (Fig. 5). To determine the physiologically relevant levels of these receptors, we also compared them to those expressed on ex vivo IL-2-activated NK cells. The level of ILT2 is higher on NK92 than the primary NK cells, whereas CD94/NKG2A is higher on many primary NK cells than on NK92.

To address whether CD94/NKG2A or ILT2 was contributing to inhibition by ITIM-deficient KIR, we performed Ab reversal experiments on NK92 cells. Inhibition by KIR2DL1 and 2DL1.Y^{2}F was reverted in the presence of anti-2DL1 mAb HP-3E4 (Fig. 6*A*). The complete reversal by anti-KIR indicates the importance of the receptor/ligand interaction between a KIR and MHC-I. Inhibition by KIR2DL1 and 2DL1.Y^{2}F was not affected in the presence of anti-NKG2A mAb Z199. Therefore, HLA-E recognition does not appear to contribute to the inhibition observed in NK92 cells. Inhibition through 2DL1.Y^{2}F was reverted in the presence of HP-F1, a mAb recognizing ILT2, although HP-F1 did not reverse inhibition through wild-type KIR2DL1. Anti-MHC-I Ab W6/32 binds to the α3 region of MHC-I and prevents ILT2 from binding to MHC-I but does not affect binding of KIR to MHC-I. Ab blocking by W6/32 showed the same trend as HP-F1. We then performed the similar Ab blocking experiments on the NK92 stable lines expressing full-length and truncated KIR (Fig. 7). Inhibition with full-length KIR was only reverted in the presence of anti-KIR Ab HP-3E4 (Fig. 7*A*). Blocking ILT2 or CD94/NKG2A had no effect, even though they are both expressed on this KIR2DL1-GFP (Fig. 7*B*). The inhibition through KIR2DL1TR-GFP was fully reverted in the presence of HP-3E4 (Figs. 7*A* and 1*D*). Blocking the ILT2-MHC-I interaction with either HP-F1 or W6/32 also reverted the observed inhibition of KIR2DL1TR-GFP. Importantly, CD94/NKG2A is not playing a role in KIR2DL1TR-GFP inhibition because Z199 does not revert inhibition. In fact, fortuitously CD94/NKG2A is not present on this clone at all (Fig. 7*B*). These results further indicate that inhibition through ITIM-deficient KIR requires both an interaction of the extracellular region of KIR with its MHC-I ligand, as well as an interaction between ILT2 and the α3 domain of MHC-I.

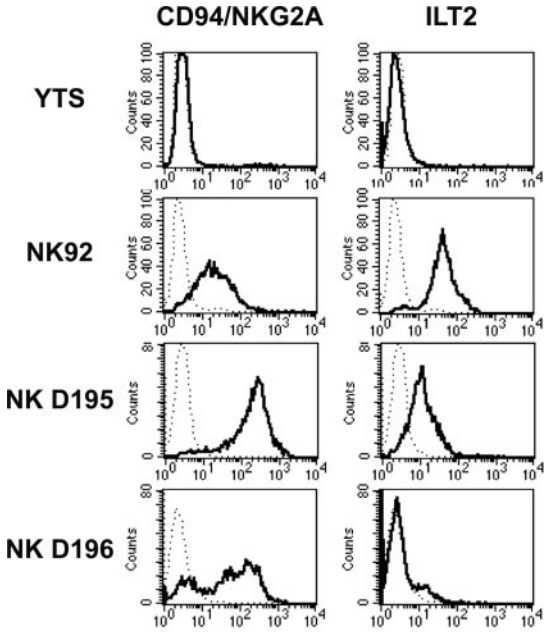

FIGURE 5. Profiles of inhibitory receptors on NK cells. IL-2 activated NK cells were derived from two donors (see *Materials and Methods*) to compare with YTS and NK92 cells by flow cytometry. CD94/NKG2A was detected with Z199 and ILT2 with HP-F1. Background levels with secondary Ab alone are indicated by dotted lines, and specific Ab staining is indicated by the solid line.

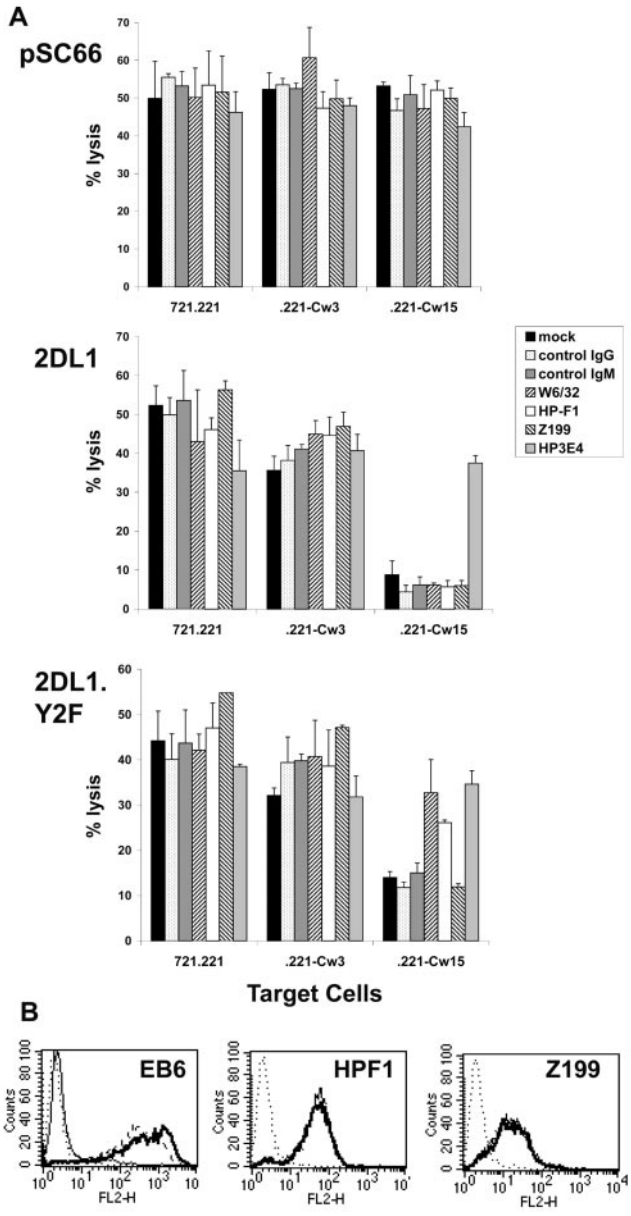

FIGURE 6. Ab blocking of mutant KIR signaling. *A*, NK92 cells were infected with recombinant vaccinia virus as before with either pSC66 (*top panel*), 2DL1 (*middle panel*), or 2DL1.Y^{2}F (*bottom panel*). The effector cells were preincubated with the Abs indicated in the figure legend at the following concentrations: control IgG 5 μg/ml; control IgM ascites (367 μg/ml); anti-MHC-I W6/32 (10 μg/ml); anti-ILT2 HP-F1 (1/50 dilution); anti-NKG2A Z119 (1 μg/ml); and anti-KIR HP-3E4 ascites (1/100 dilution). The cytolysis assay was performed as before with the target cell line indicated on the *x*-axis at an E:T of 12:1. *B*, The surface expression of KIR2DL1 (EB6), ILT2 (HP-F1), and CD94/NKG2A (Z199) was determined by flow cytometry for NK92/pSC66 (thin), 2DL1 (thick), and 2DL1.Y^{2}F (dashed). Secondary Ab alone is shown with a dotted line.

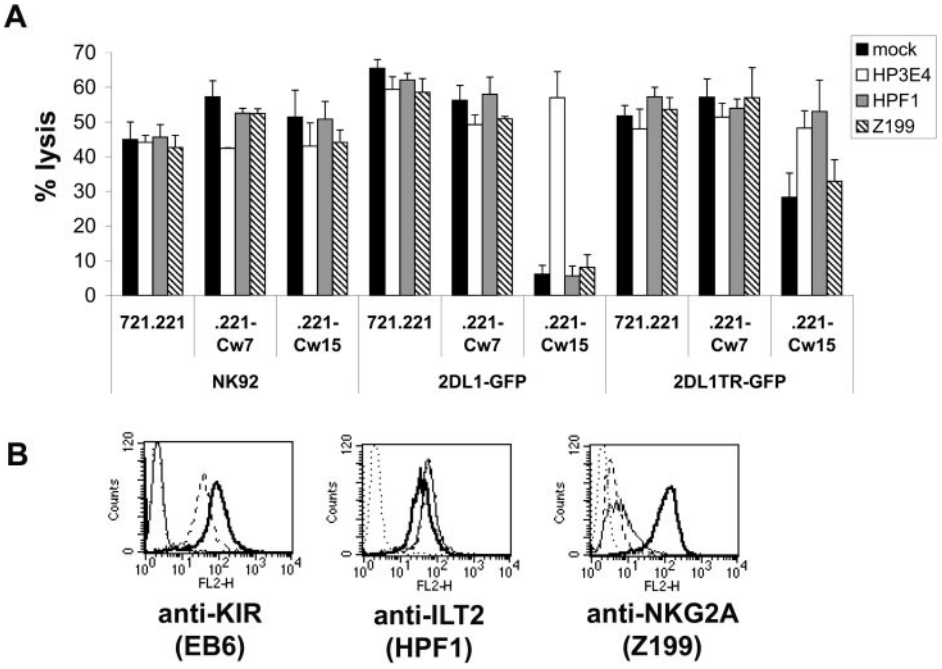

FIGURE 7. Ab blocking of NK92 stable lines expressing wild-type and mutant KIR. *A*, Cytolysis by NK92, NK92/2DL1-GFP, or NK92/2DL1TR-GFP with the indicated target cell line was determined in a standard chromium release assay with the blocking Abs anti-ILT2 HP-F1 (1/50 dilution), anti-NKG2A Z119 (1 μg/ml), and anti-KIR HP-3E4 ascites (1/100 dilution). *B*, The surface expression of KIR2DL1 (EB6), ILT2 (HP-F1), and CD94/NKG2A (Z199) was determined by flow cytometry for NK92 (thin), NK92/2DL1-GFP (thick), and NK92/2DL1TR-GFP (dashed). Secondary Ab alone is shown with a dotted line.

Human-derived NK cell expression of ILT2 and recognition of HLA-C

The ability of ILT2 to contribute to recognition of HLA-C in NK92 cells suggests this might occur in primary NK cells. To determine whether KIR and ILT2 are coexpressed on human NK cells, we performed two-color flow cytometry. Freshly derived human NK cells from four healthy donors were isolated and purified by magnetic separation and their CD16/CD56 profiles determined (Fig. 8*A*). We then used a mixture of anti-KIR Abs to assess the overall levels of coexpression of ILT2 and a KIR. ILT2 and KIR are coexpressed on human NK cells at ratios varying from 6.1 to 30.1% depending on the donor tested (Fig. 8*B*). We assessed coexpression of individual KIR specificities and found that individual KIR also shows coexpression with ILT2 (data not shown).

The frequent coexpression of a KIR and ILT2 in primary NK cells raises a question of whether or not the amount of ILT2 in these cells was enough to contribute to KIR signals. Therefore, we tested whether mutant KIR signals in ILT2 expressing primary NK cells. We isolated NK cells from D187, depleted KIR2DL1/S1 positive cells, and then sorted into ILT2 high and low populations

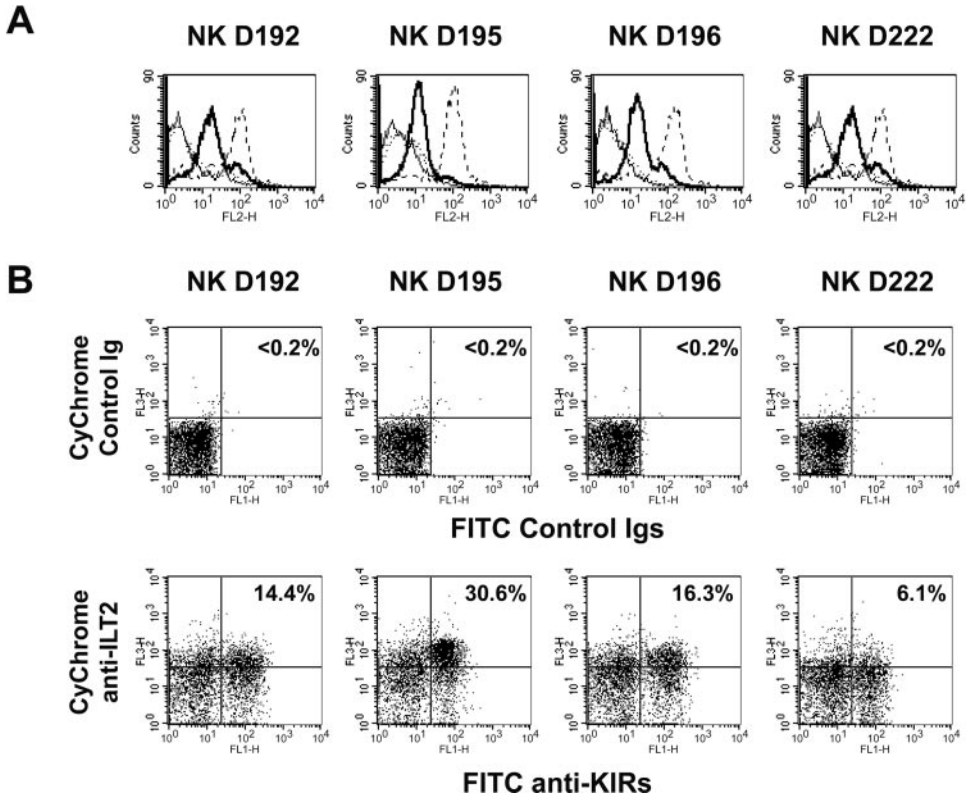

FIGURE 8. Two color expression profiles of KIR and ILT2 on various human NK cells. Freshly isolated NK cells derived from four donors (see *Materials and Methods*) were stained for various receptors. *A*, NK cells were stained with anti-CD3 (thin black line), anti-CD16 (thin dashed line), and anti-CD56 (thick black line) Abs. Background levels (isotype-matched control Abs) are shown as the thin dotted black line. *B*, NK cells were costained with either FITC- and CyChrome-coupled isotype-matched control Abs (*top panels*) or a combination of FITC-coupled anti-KIR Abs (anti-KIR2DL1/S1, anti-KIR2DL2/L3/S2, and anti-KIR3DL1) and CyChrome-coupled anti-ILT2 Abs (*bottom panels*). Percentages shown indicate the amount of coexpression of KIR and ILT2 (two color events).

before expanding these cells in culture. On day 11 using recombinant vaccinia virus, we expressed KIR2DL1 or ITIM-deficient KIR (Fig. 9*A*) in these ILT2 low or high NK populations. Both KIR2DL1 and ITIM-deficient KIR caused a reduction in lysis with target cells expressing Cw15 (Fig. 9*B*). The ITIM-deficient KIR signal appears stronger in the ILT2 high NK population than in the ILT2 low population. Inhibition through wild-type KIR2DL1 was only revertible in the presence of anti-KIR blocking Ab HP3E4. However, ITIM-deficient KIR signaling was blocked both with HP3E4 and F(ab')$_2$ of W6/32 that bind MHC-I in the α3 region where ILT2 binds.

To further clarify the role of ILT2 in recognition of HLA-C, we generated an ILT2 recombinant virus and used it to express ILT2 in mouse NK cells that do not express ILT2, the mouse equivalent PIR-B, or human CD94/NKG2A. Expression of ILT2 in these cells (Fig. 10*A*) resulted in direct recognition of MHC-I-positive targets (Fig. 10*B*). Unfortunately, the viral vector system was not amenable to comparing high and low amounts of ILT2 in the mouse NK cells because at shorter infection times or with lower MOIs many cells remained negative for ILT2 expression. To confirm that higher amounts of ILT2 would result in direct recognition of HLA-C in human cells, we performed similar experiments in NK92 cells, overexpressing ILT2 with recombinant vaccinia virus (Fig. 10*C*). A cytolysis assay with these cells showed that overexpression of ILT2 results in direct recognition of both HLA-C transfectants compared with MHC-I negative cells (Fig. 10*D*).

Discussion

In the present study, we have shown that KIR without ITIMs retain considerable inhibitory capacity when expressed in NK92 cells or ex vivo IL-2 activated human NK cells but not mouse NK cells. We have shown that ITIM-deficient KIR inhibition is independent of the KIR transmembrane and cytoplasmic tail. Similar to wild-type KIR, ITIM-deficient KIR inhibition is also completely reverted by the coexpression of catalytically inactive SHP-1. Using Abs to block the interaction between KIR and MHC-I, we have shown that both wild-type and ITIM-deficient KIR require the KIR ligand-specific interaction. However, inhibition through ITIM-deficient KIR also requires the interaction between the α3 domain of MHC-I and ILT2, as Abs against either the α3 region of MHC-I or ILT2 reverted the inhibition. Together these data indicate that ILT2 can signal in a manner that is dependent on a KIR-MHC-I interaction.

There are several possible mechanisms that could explain how KIR invokes a signal through ILT2. It is possible that KIR influences signaling through ILT2 due to the ability of KIR to cause MHC-I clustering at the interface between effector and target cells (49, 50). It has been reported that the affinity of KIR2DL1 for HLA-Cw4 is 2-fold that of ILT2 in vitro (29). Therefore, it is possible that the KIR-MHC-I interaction drives clustering of MHC-I, which then provides a high density of binding sites for ILT2 at the interface between a NK cell and target cell. This would be especially true if there are significantly more KIR molecules on the cell surface than ILT2. KIR and ILT2 may also engage the same MHC-I molecule. Spatially, this is feasible in a manner similar to how CD4 or CD8 can bind to the same MHC molecule as the TCR. The binding site of KIR with two Ig domains is formed at the junction between the two Ig domains and interacts with the α1 helix on the top of the MHC-I molecule (51, 52). On the other hand, ILT2 has four Ig domains and is predicted to extend further out from the cell membrane than KIR (28). The first two Ig domains of ILT2 confers the binding and interacts with the α3 region of MHC-I and β_2-microglobulin (28, 53, 54). The site on MHC-I bound by ILT2 overlaps with the CD8 binding site; however, the interaction of ILT2 with MHC-I has been proposed to be more similar to CD4 binding MHC-II than CD8 binding MHC-I (28). In support of the possibility that KIR and ILT2 can bind simultaneously, in vitro binding studies have shown KIR2DL1 and ILT2

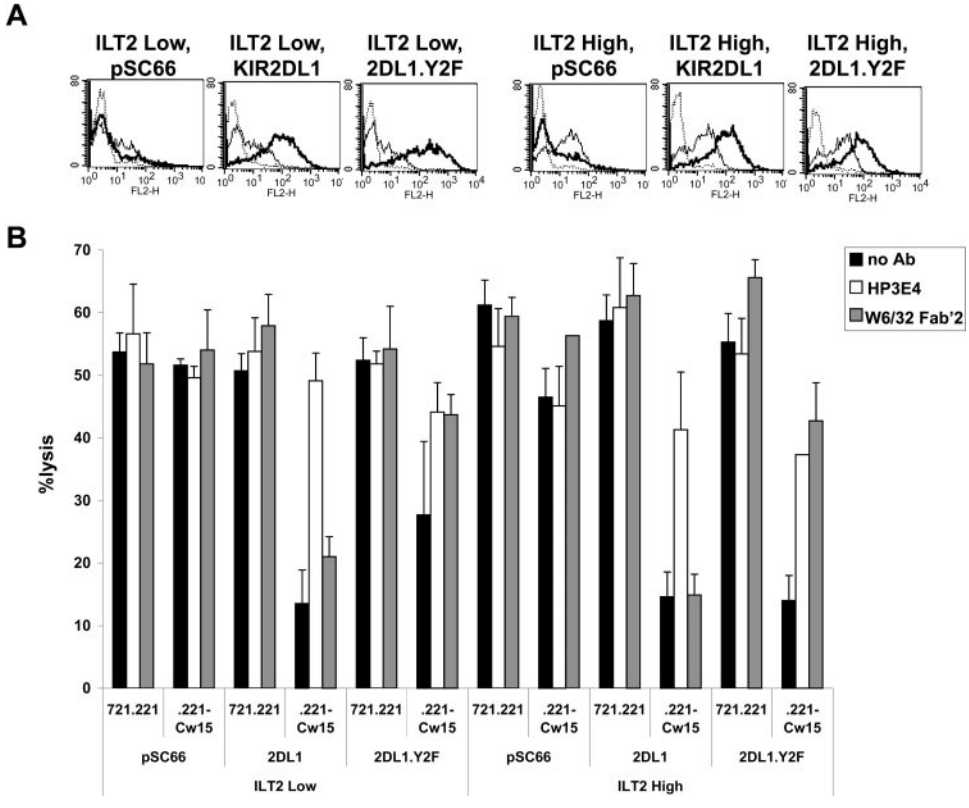

FIGURE 9. Inhibition of killing by ILT2 low or ILT2-enriched human NK cells with wild-type and mutant KIR. *A*, ILT2 low and ILT2-enriched human NK cells were infected with recombinant vaccinia viruses pSC66, KIR2DL1, or 2DL1.Y2F at a MOI of 20 for 2.5 h. Flow cytometry histogram plots showing secondary Ab alone (dotted black line), KIR2DL1 expression (thick black line), and ILT2 expression (thin black line). *B*, The cytolysis by these cells was measured in a standard chromium release assay and the E:T ratio shown is 15:1. In cases where no error bar appears, it is because the error is too small to be visible.

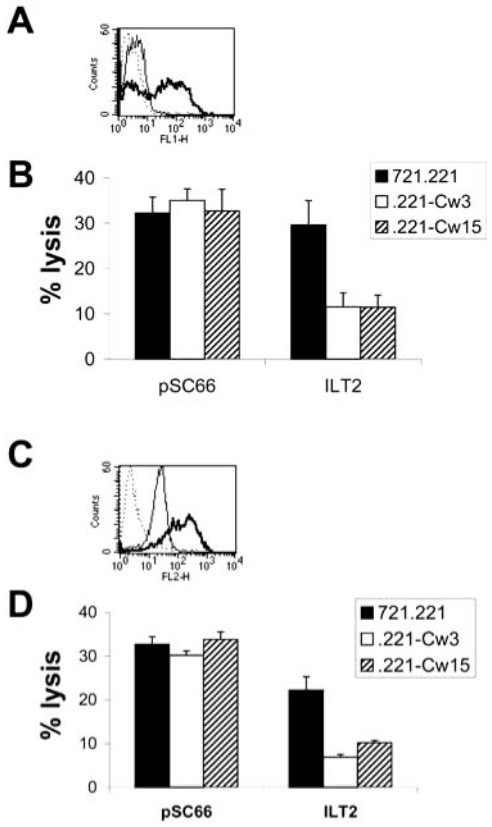

FIGURE 10. Higher expression of ILT2 on mouse NK cells and NK92 cells leads to direct recognition of HLA-C. *A*, Mouse NK cells were infected with pSC66 or recombinant vaccinia virus expressing ILT2 at a MOI of 3 for 2.5 h. Flow cytometry histogram plot showing secondary Ab alone (dotted black line), ILT2 expression on mNK cells infected with pSC66 (thin black line), or expression of ILT2 with recombinant vaccinia virus (thick black line). *B*, Corresponding cytolysis assay showing infected mouse NK cells incubated with target cells precoated with 1.0 μg/ml L243 to induce ADCC. *C*, NK92 cells were either infected with pSC66 or recombinant vaccinia virus expressing ILT2 at a MOI of 15 for 2 h. Flow cytometry histogram plot showing secondary Ab alone (dotted black line), surface ILT2 expression on NK92 cells infected with pSC66 (thin black line), or overexpression of ILT2 with recombinant vaccinia virus (thick black line). *D*, Cytolysis was measured in a standard chromium release assay and the E:T ratio shown is 20:1.

do not compete for interaction with MHC-I (29). In these studies, the binding was additive; however, it remains possible that when the receptors are in the membrane, the binding to MHC-I is cooperative. Thus, KIR binding to MHC-I might better expose the ILT2 binding site. A final possibility is that ILT2 forms a complex with KIR before association with HLA-C. Based on our observations, such a complex would only require the extracellular domains of KIR and would be disrupted by anti-MHC-I mAb W6/32 binding to the ligand. In any event, the KIR-dependent inhibitory signaling by ILT2 is reminiscent of CD8 coreceptor function in activation of T cells.

The ability of KIR to signal in response to HLA-C in the absence of ILT2 has been well established in model systems without ILT2 such as KIR expressed in YTS and in mouse NK cells (10, 11, 43, 55). In line with this, we did not observe any effect of Abs to ILT2 or the α3 domain of MHC-I on wild-type KIR2DL1 signal in response to Cw15. However, the KIR2DL1 interaction with C2 HLA-C molecules is known to be quite strong (56, 57). It is possible that ILT2 could improve the signaling through a wild-type KIR when the affinity of the KIR for a specific MHC-I was suboptimal. The contribution of ILT2 would have been overlooked in previous studies as it would be completely blocked by anti-KIR Abs. Although we have observed mutant KIR signaling in primary NK cells from several donors, in some cases the inhibition was similar in the ILT2 high and low subsets but blockable by W6/32 (data not shown). This suggests that yet another inhibitory receptor may also perform the same function.

One study has previously established that ILT2 and KIR expression overlap in a large subset of NK cells in one individual (27). In fact, we have observed KIR-ILT2 coexpression on >30% of peripheral NK cells of one donor (Fig. 8). For NK cells derived from peripheral blood, the variation in the frequency of ILT2-positive cells between individuals ranges from 17 to 75% (26, 39, 40), and in our donor set we have seen as low as 6% (Fig. 8). This degree of variability supports the idea that ILT2 expression is also involved in recognition of polymorphic MHC-I, as opposed to solely HLA-G. KIR haplotype diversity is second only to MHC and inheritance of KIR and MHC loci are not linked, leading to enormous variability in the combination of KIR and MHC molecules that an individual can inherit (16). In contrast to the mouse system in which Ly49 molecules are down modulated in the presence of high-affinity H-2 ligands (reviewed in Ref. 58), little is understood regarding how the threshold for inhibition is established in human NK cells. However, this is emerging as an important feature in innate resistance. For example, the affinity of KIR2DL3 for the C1 group of HLA-C alleles is much less than KIR2DL2 (56, 57) and possessing a NK repertoire with the KIR2DL3/C1 combination has recently been reported to have implications for resistance to hepatitis C (57, 59). In conjunction with this possibility, Carr, Pando, and Parham have observed polymorphic residues of KIR3DL1 to have an impact on the interaction with HLA-B (personal communication), suggesting cooperative signaling with ILT2 could apply to certain HLA-B/KIR3DL combinations as well.

The level of ILT2 on NK92 and in primary NK cells is relatively low and, on its own, is insufficient to mediate recognition of HLA-C alleles expressed in 721.221 cells. This is similar to what has been reported for an ex vivo NK clone (27). However, overexpression of ILT2 in NK92 or mouse NK cells leads to an interaction with HLA-C that is sufficient to mediate inhibition in the absence of KIR. This suggests the amount of ILT2 on NK cells is held below the threshold that would allow direct recognition of most MHC-I alleles. The level of ILT2 on NKL cells has been shown to provide inhibition in response to certain HLA-A and -B alleles (60), and we have determined that overexpression of ILT2 alone in NK92 or in mouse NK cells can signal in response to Cw3, Cw4, and Cw15 (Fig. 10 and data not shown). Therefore, cells that express too much ILT2 would be similar to those that express CD94/NKG2A, i.e., lack specificity for individual MHC-I molecules. Our results suggest that by tightly regulating the level of ILT2 in peripheral NK cells, ILT2 could cooperate with KIR to increase the functional range of KIR, while still allowing KIR to dictate the specificity for classical MHC-I. Future studies examining NK clone sensitivity to HLA-C should include examination of ILT2 as well as KIR and CD94/NKG2A.

Acknowledgments

We thank the blood donors for their contribution, Catharine Compston, George Zahariadis, and Melissa Steward for drawing blood, and Deborah Merriam for general technical support. We also thank Dorothy Rutkowski for help with cell sorting. We thank Drs. Kevin Kane, Miguel Lopez-Botet, Eric Long, and Peter Parham for providing reagents and Dr. Paul Leibson for advice on purifying vaccinia virus. We also thank

Dr. Peter Parham for communicating results and Drs. Kevin Kane and Troy Baldwin for comments on the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Orange, J. S., M. S. Fassett, L. A. Koopman, J. E. Boyson, and J. L. Strominger. 2002. Viral evasion of natural killer cells. *Nat. Immunol.* 3: 1006–1012.
2. Bubenik, J. 2004. MHC class I down-regulation: tumour escape from immune surveillance? *Int. J. Oncol.* 25: 487–491.
3. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 19: 197–223.
4. Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. *Annu. Rev. Immunol.* 17: 875–904.
5. Lanier, L. L. 2003. Natural killer cell receptor signaling. *Curr. Opin. Immunol.* 15: 308–314.
6. Burshtyn, D. N., and E. O. Long. 1997. Regulation through inhibitory receptors: lessons from natural killer cells. *Trends Cell Biol.* 7: 473–479.
7. McVicar, D., and D. N. Burshtyn. 2001. Intracellular signaling by the killer cell immunoglobulin-like receptors and Ly49. *Sci. STKE* 75: RE1.
8. Stebbins, C. C., C. Watzl, D. D. Billadeau, P. J. Leibson, D. N. Burshtyn, and E. O. Long. 2003. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol. Cell. Biol.* 23: 6291–6299.
9. Bruhns, P., F. Vely, O. Malbec, W. H. Fridman, E. Vivier, and M. Daeron. 2000. Molecular basis of the recruitment of the SH2 domain-containing inositol 5-phosphatases SHIP1 and SHIP2 by fcγRIIB. *J. Biol. Chem.* 275: 37357–37364.
10. Burshtyn, D. N., A. S. Lam, M. Weston, N. Gupta, P. A. 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: 897–902.
11. Fassett, M., D. M. Davis, M. M. Valter, G. B. Choen, and J. Strominger. 2001. Signaling at the inhibitory natural killer cell immune synapse regulates lipid raft polarization but not class I MHC clustering. *Proc. Natl. Acad. Sci. USA* 98: 14547–14552.
12. 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: 295–300.
13. 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: 673–684.
14. Ortaldo, J. R., R. Winkler-Pickett, J. Willette-Brown, R. L. Wange, S. K. Anderson, G. J. Palumbo, L. H. Mason, and D. W. McVicar. 1999. Structure/function relationship of activating Ly-49D and inhibitory Ly-49G2 NK receptors. *J. Immunol.* 163: 5269–5277.
15. Bellon, T., F. Kitzig, J. Sayos, and M. Lopez-Botet. 2002. Mutational analysis of immunoreceptor tyrosine-based inhibition motifs of the Ig-like transcript 2 (CD85j) leukocyte receptor. *J. Immunol.* 168: 3351–3359.
16. Vilches, C., and P. Parham. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu. Rev. Immunol.* 20: 217–251.
17. Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet, and D. E. Geraghty. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci. USA* 95: 5199–5204.
18. Braud, V. M., D. S. J. Allan, C. A. O'Callaghan, K. Söderström, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, L. L. Lanier, and A. J. McMichael. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795–799.
19. Borrego, F., M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187: 813–818.
20. Della Chiesa, M., M. Vitale, S. Carlomagno, G. Ferlazzo, L. Moretta, and A. Moretta. 2003. The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. *Eur. J. Immunol.* 33: 1657–1666.
21. Vitale, M., M. Della Chiesa, S. Carlomagno, C. Romagnani, A. Thiel, L. Moretta, and A. Moretta. 2004. The small subset of $CD56^{bright}CD16^{-}$ natural killer cells is selectively responsible for both cell proliferation and interferon γ production upon interaction with dendritic cells. *Eur. J. Immunol.* 34: 1715–1722.
22. Lopez-Botet, M., M. Llano, F. Navarro, and T. Bellon. 2000. NK cell recognition of non-classical HLA class I molecules. *Semin. Immunol.* 12: 109–119.
23. 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: 739–751.
24. Samaridis, J., and M. Colonna. 1997. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur. J. Immunol.* 27: 660–665.
25. Cosman, D., N. Fanger, L. Borges, M. Kubin, W. Chin, L. Peterson, and M. L. Hsu. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* 7: 273–282.
26. Colonna, M., F. Navarro, T. Bellón, M. Llano, P. García, J. Samaridis, L. Angman, M. Cella, and M. López-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med.* 186: 1809–1818.
27. Vitale, M., R. Castriconi, S. Parolini, D. Pende, M. L. Hsu, L. Moretta, D. Cosman, and A. Moretta. 1999. The leukocyte Ig-like receptor (LIR)-1 for the cytomegalovirus UL18 protein displays a broad specificity for different HLA class I alleles: analysis of LIR-1 + NK cell clones. *Int. Immunol.* 11: 29–35.
28. Chapman, T. L., A. P. Heikeman, and P. J. Bjorkman. 1999. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity* 11: 603–613.
29. Shiroishi, M., K. Tsumoto, K. Amano, Y. Shirakihara, M. Colonna, V. M. Braud, D. S. Allan, A. Makadzange, S. Rowland-Jones, B. Willcox, et al. 2003. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc. Natl. Acad. Sci. USA* 100: 8856–8861.
30. Ince, M. N., B. Harnisch, Z. Xu, S. K. Lee, C. Lange, L. Moretta, M. Lederman, and J. Lieberman. 2004. Increased expression of the natural killer cell inhibitory receptor CD85j/ILT2 on antigen-specific effector CD8 T cells and its impact on CD8 T cell function. *Immunology* 112: 531–542.
31. Saverino, D., F. Ghiotto, A. Merlo, S. Bruno, L. Battini, M. Occhino, M. Maffei, C. Tenca, S. Pileri, L. Baldi, et al. 2004. Specific recognition of the viral protein UL18 by CD85j/LIR-1/ILT2 on $CD8^{+}$ T cells mediates the non-MHC-restricted lysis of human cytomegalovirus-infected cells. *J. Immunol.* 172: 5629–5637.
32. Dulphy, N., C. Rabian, C. Douay, O. Flinois, S. Laoussadi, J. Kuipers, R. Tamouza, D. Charron, and A. Toubert. 2002. Functional modulation of expanded $CD8^{+}$ synovial fluid T cells by NK cell receptor expression in HLA-B27-associated reactive arthritis. *Int. Immunol.* 14: 471–479.
33. Nikolova, M., P. Musette, M. Bagot, L. Boumsell, and A. Bensussan. 2002. Engagement of ILT2/CD85j in Sezary syndrome cells inhibits their CD3/TCR signaling. *Blood* 100: 1019–1025.
34. Saverino, D., A. Merlo, S. Bruno, V. Pistoia, C. E. Grossi, and E. Ciccone. 2002. Dual effect of CD85/leukocyte Ig-like receptor-1/Ig-like transcript 2 and CD152 (CTLA-4) on cytokine production by antigen-stimulated human T cells. *J. Immunol.* 168: 207–215.
35. Dietrich, J., M. Cella, and M. Colonna. 2001. Ig-like transcript (ILT2)/leukocyte Ig-like receptor 1 (LIR1) inhibits TCR signaling and actin cytoskeleton reorganization. *J. Immunol.* 166: 2514–2521.
36. Young, N. T., M. Uhrberg, J. H. Phillips, L. L. Lanier, and P. Parham. 2001. Differential expression of leukocyte receptor complex-encoded Ig-like receptors correlates with the transition from effector to memory CTL. *J. Immunol.* 166: 3933–3941.
37. Merlo, A., D. Saverino, C. Tenca, C. E. Grossi, S. Bruno, and E. Ciccone. 2001. CD85/LIR-1/ILT2 and CD152 (cytotoxic T lymphocyte antigen 4) inhibitory molecules down-regulate the cytolytic activity of human $CD4^{+}$ T cell clones specific for *Mycobacterium tuberculosis*. *Infect. Immun.* 69: 6022–6029.
38. Saverino, D., M. Fabbi, F. Ghiotto, A. Merlo, S. Bruno, D. Zarcone, C. Tenca, M. Tiso, G. Santoro, G. Anastasi, et al. 2000. The CD85/LIR-1/ILT2 inhibitory receptor is expressed by all human T lymphocytes and down-regulates their functions. *J. Immunol.* 165: 3742–3755.
39. 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: 5674–5679.
40. Riteau, B., C. Menier, I. Khalil-Daher, S. Martinozzi, M. Pla, J. Dausset, E. D. Carosella, and N. Rouas-Freiss. 2001. HLA-G1 co-expression boosts the HLA class I-mediated NK lysis inhibition. *Int. Immunol.* 13: 193–201.
41. Riteau, B., N. Rouas-Freiss, C. Menier, P. Paul, J. Dausset, and E. D. Carosella. 2001. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. *J. Immunol.* 166: 5018–5026.
42. Standeven, L., L. M. Carlin, P. Borszcz, D. M. Davis, and D. N. Burshtyn. 2004. The actin cytoskeleton controls the efficiency of killer cell Ig-like receptors (KIR) accumulation at inhibitory natural killer cell immune synapses. *J. Immunol.* 173: 5617–5625.
43. Borszcz, P. D., M. Peterson, L. Standeven, S. Kirwan, M. Sandusky, A. Shaw, E. O. Long, and D. N. Burshtyn. 2003. KIR enrichment at the effector-target cell interface is more sensitive than signaling to the strength of ligand binding. *Eur. J. Immunol.* 33: 1084–1093.
44. Melero, I., A. Salmeron, M. A. Balboa, J. Aramburu, and M. Lopez-Botet. 1994. Tyrosine kinase-dependent activation of human NK cell functions upon stimulation through a 58-kDa surface antigen selectively expressed on discrete subsets of NK cells and T lymphocytes. *J. Immunol.* 152: 1662–1673.
45. Earl, P. L., and B. Moss. 1988. Generation of recombinant vaccinia viruses. In *Current Protocols in Molecular Biology.* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. John Wiley & Sons, New York, p. 16.17.1–16.17.
46. Wagtmann, N., S. Rajagopalan, C. C. Winter, M. Peruzzi, and E. O. Long. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity* 3: 801–809.
47. 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 inhibitor receptor. *Immunity* 4: 77–85.
48. Yusa, S.-I., T. L. Catina, and K. S. Campbell. 2002. SHP-1- and phosphotyrosine-independent inhibitory signaling by a killer cell Ig-like receptor cytoplasmic domain in human NK cells. *J. Immunol.* 168: 5047–5057.

49. Davis, D. M., I. Chiu, M. Fassett, G. B. Cohen, M. Mandelboim, and J. Strominger. 1999. The human natural killer cell immune synapse. *Proc. Natl. Acad. Sci. USA* 96: 15062–15067.
50. Davis, D. M., and M. L. Dustin. 2004. What is the importance of the immunological synapse? *Trends Immunol.* 25: 323–327.
51. Fan, Q. R., E. O. Long, and D. C. Wiley. 2001. Crystal structure of the complex between the human natural killer cell inhibitory receptors KIR2DL1 and its class I MHC ligand HLA-Cw4. *Nat. Immunol.* 2: 452–460.
52. Boyington, J. C., S. A. Motyka, P. Schuck, A. G. Brooks, and P. D. Sun. 2000. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature* 405: 537–543.
53. Willcox, B. E., L. M. Thomas, and P. J. Bjorkman. 2003. Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. *Nat. Immunol.* 4: 913–919.
54. Chapman, T. L., A. P. Heikema, A. P. West, Jr., and P. J. Bjorkman. 2000. Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). *Immunity* 13: 727–736.
55. Cohen, G. B., R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, and D. Baltimore. 1999. The selective down-regulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* 10: 661–671.
56. Winter, C. C., J. E. Gumperz, P. Parham, E. O. Long, and N. Wagtmann. 1998. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J. Immunol.* 161: 571–577.
57. Parham, P. 2004. Immunology: NK cells lose their inhibition. *Science* 305: 786–787.
58. Hoglund, P., J. Sundback, M. Y. Olsson-Alheim, M. Johansson, M. Salcedo, C. Ohlen, H. G. Ljunggren, C. L. Sentman, and K. Karre. 1997. Host MHC class I gene control of NK-cell specificity in the mouse. *Immunol. Rev.* 155: 11–28.
59. Khakoo, S. I., C. L. Thio, M. P. Martin, C. R. Brooks, X. Gao, J. Astemborski, J. Cheng, J. J. Goedert, D. Vlahov, M. Hilgartner, et al. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305: 872–874.
60. Colonna, M., H. Nakajima, F. Navarro, and M. López-Botet. 1999. A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. *J. Leukocyte Biol.* 66: 375–381.