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Human KIR2DL5 Is an Inhibitory Receptor Expressed on the Surface of NK and T Lymphocyte Subsets

Ernesto Estefanía,* Raquel Flores,† Natalia Gómez-Lozano,* Helena Aguilar,† Miguel López-Botet,† and Carlos Vilches2*

Human NK cells, by means of a repertoire of clonally distributed killer cell Ig-like receptors (KIR), survey the expression of individual self HLA class I molecules, which is often altered in infections and tumors. KIR2DL5 (CD158F) is the last identified KIR gene and, with KIR2DL4, constitutes a structurally divergent lineage conserved in different primate species. Research on KIR2DL5 has thus far been limited to its genetic aspects due to a lack of reagents to detect its product. We report here the identification and characterization of the receptor encoded by KIR2DL5 using a newly generated specific mAb that recognizes its most commonly expressed allele, KIR2DL5A*001. KIR2DL5 displays a variegated distribution on the surface of CD56dim NK cells. This contrasts with the expression pattern of its structural homolog KIR2DL4 (ubiquitous transcription, surface expression restricted to CD56bright NK cells) and resembles the profile of KIR recognizing classical HLA class I molecules. Like other MHC class I receptors, KIR2DL5 is also found in a variable proportion of T lymphocytes. KIR2DL5 is detected on the cell surface as a monomer of ~60 kDa that, upon tyrosine phosphorylation, recruits the Src homology region 2-containing protein tyrosine phosphatase-2 and, to a lesser extent, Src homology region 2-containing protein tyrosine phosphatase-1. Ab-mediated cross-linking of KIR2DL5 inhibits NK cell cytotoxicity against murine FcγR+ P815 cells. KIR2DL5 is thus an inhibitory receptor gathering a combination of genetic, structural, and functional features unique among KIR, which suggests that KIR2DL5 plays a specialized role in innate immunity. The Journal of Immunology, 2007, 178: 4402–4410.

The NK cells contribute to the innate immunological surveillance against infections and tumors but, in contrast with T and B lymphocytes, they do not express Ag-specific receptors encoded by combinatorially rearranged genes (1). Instead, human NK lymphocytes discriminate between healthy and potentially dangerous cells by means of a variety of receptors that enable them to recognize pathogen-derived or stress-induced molecules and through inhibitory receptors that confer them the ability to survey the physiological expression of certain self molecules (2–6). Best known of the latter type of molecules that behave as markers of cell health for NK lymphocytes are those encoded at the MHC, the expression of which is often altered in infected cells and tumors (7–9).

Multiple complementary strategies and families of receptors have evolved in mammals to achieve surveillance of MHC class I expression (10–14). In humans, inhibitory members of the killer cell Ig-like receptor (KIR) family are distributed clonally in NK cells, thus enabling these to sense the individual expression levels of the subsets of HLA alleles for which each of those receptors is specific (14): KIR2DL1, which recognizes HLA-C alleles having lysine in position 80; KIR2DL2 and KIR2DL3, specific for HLA-C alleles with the alternative asparagine 80 residue; KIR3DL1, for HLA-B alleles with isoleucine or threonine in the same position; and KIR3DL2, a receptor for the alleles HLA-A*03 and A*11. In the nomenclature of KIR, 2D/3D indicates the number of Ig-like domains, whereas L denotes the presence of large cytoplasmatic tails with ITIMs that mediate cell inhibition through recruitment of the Src homology region 2-containing protein tyrosine phosphatase-1 (SHP-1) and Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) (3, 15–18).

The KIR gene cluster codes for additional receptors having uncertain functions (14, 19). Among the latter are: activating homologs of the HLA-B- and HLA-C-specific inhibitory KIR (10, 11), designated with an S that refers to their short ITIM-lacking tails (KIR2DS1–KIR2DS5 and KIR3DS1); and others having more distantly related structures (KIR3DL3, KIR3DP1, KIR2DL4 (a receptor for the MHC class Ib molecule HLA-G) and KIR2DL5) (19–24).

KIR2DL4 and KIR2DL5 are the only members of a gene lineage coding for KIR with a D0–D2 organization of the Ig-like domains, which distinguishes them from all other KIR2Ds, having domains of the D1–D2 type (14, 25). The two genes also share a frame shift in their last exons, which extends their coding regions 31 codons beyond those of other KIR2DL4. Despite the similar gene organization of KIR2DL4 and KIR2DL5, their predicted primary structures are only 79% identical (23). Moreover, distinctive genetic, structural, and functional features separate the two genes. In particular, KIR2DL4 is conserved in most humans (26–28); surface expression appears to be restricted to the KIR CD56bright minority of NK cells, although transcription and intracellular expression of KIR2DL4 in a functional state have been detected in all NK cells (24, 29–33). Furthermore, KIR2DL4 induces cytokine
secretion but has an inhibitory potential; it has not been fully established how its signaling motifs (one ITIM, one transmembrane arginine residue, and the ability to associate with the FcεRIγ chain) contribute to those two functions (24, 34–37).

Much less is known about KIR2DL5. In contrast with KIR2DL4, its gene is found in only a fraction of individuals in each population (23, 26), and it is represented in the human genome by two genes, KIR2DL5A and KIR2DL5B, that show 99.5–99.7% identity in their coding sequences (27). KIR2DL5 has alleles with mRNA transcripts that are clonally distributed in NK and T lymphocytes and ones that are apparently not transcribed, the most common of which are, respectively, KIR2DL5A*001 and KIR2DL5B*002 (23, 27). Finally, KIR2DL5 is predicted to encode a purely inhibitory receptor, according to its signaling motifs (two ITIMs, the second one having a noncanonical TxYxxL sequence, and a transmembrane region lacking charged amino acid residues) and to the behavior of cells transfected with tagged or chimerical KIR2DL5 constructs (23, 38).

Investigation in KIR2DL5 has thus far focused on its genetic aspects due to a lack of reagents to detect its putative product. In this study, we report the identification and characterization of the receptor encoded by KIR2DL5 by means of a newly generated specific mAb.

Materials and Methods
cDNA constructs, cells, and transfectants
KIR2DL5-Ig fusion protein The exons coding for the extracellular region of KIR2DL5A*001 (Ig-like domains and stem) were PCR amplified from a cDNA clone (23) with primers Fl48Nhel (5′-cagggggtcagctcatggtgcagtgccagaa-3′) and R1064dBanHI (5′-gagttcgcagttcagttcagttcagttcag-3′). The PCR product was digested with the restriction endonucleases Nhel and BanHI, gel purified, and inserted, in frame with the Fc portion of human IgG1, into the Cd5neg1 vector, a gift from Dr. Eric Long (National Institute of Allergy and Infectious Diseases-National Institutes of Health, Bethesda, MD), reference (39). The nucleotide sequences of all constructs used herein were verified using dye-labeled dideoxy terminators and a 3100-Avant automated DNA sequencer (Applied Biosystems). The plasmid containing the KIR2DL5-Ig construct was transiently transfected into human embryonic kidney-derived (HEK)-Biosystems). The plasmid containing the KIR2DL5-Ig construct was transfected into human embryonic kidney-derived HEK-293T cells by the calcium phosphate method (40).

For Western blot experiments, polyclonal UP-R1 mAb and paramagnetic beads coated with an anti-mouse IgG mAb (Dynabeads Pan Mouse IgG; Dynal), as per the manufacturer’s instructions. A KIR2DL5-negative NK cell subpopulation was purified from the remaining UP-R1-depleted PBMC by negative selection with EasySep (StemCell Technologies). To isolate double-positive KIR2DL5+KIR3DL1+ NK cells, we first performed a selection with the UP-R1 mAb and, after expansion, with DX9. The selected NK cell subpopulations were expanded as described (43).

Generation of a KIR2DL5-specific mAb A soluble KIR2DL5-Ig fusion protein was generated as previously reported for other KIR (39), and it was purified from the culture medium of transiently transfected HEK-293T cells by affinity chromatography with protein G-Sepharose (Amersham Biosciences). Immunization of BALB/c mice with the fusion protein, generation of hybridomas, and ELISA screening of supernatants for reactivity with the KIR2DL5-Ig immunogen were done as described (43).

Abs and flow cytometry
The anti-FLAG M2 and negative control MOPC21 (mouse IgG1) mAbs were purchased from Sigma-Aldrich. The 12CA5 mAb, recognizing the HA epitope (44), was kindly provided by Dr. José Aromburu (University Pompeu Fabra, Barcelona, Spain); DX9, specific for KIR3DL1 (45), by Dr. Lewis Lanier (University of California, San Francisco, CA); CH-L anti-KIR2DL2L3/S2 (46), by Dr. Silvano Ferrini (Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy); 5.133 anti-KIR3DL1/2 plus KIR2DS4 (47), by Dr. Marco Colonna (Washington University, St. Louis, MO); and both Z199 anti-NKGA2 (48) and C218 anti-CD56 (49), by Dr. Alessandro Moretta (University of Genoa, Genoa, Italy). The HP-MA4 (anti-KIR2DL1/KIR2DS1) mAb has been described elsewhere (50). For single-color flow cytometry experiments, binding of these mAbs was revealed by incubation with sheep Fl (50), anti-mouse IgG-FITC (Sigma-Aldrich) or goat Fl (50), anti-mouse IgG-PE (Jackson ImmunoResearch Laboratories).

For two- and three-color flow cytometry experiments, the UP-R1 and the isotype-matched negative control MOPC21 mAbs were labeled with Alexa Fluor 488 (Molecular Probes-Invitrogen), according to the manufacturer’s instructions. The PE-labeled anti-CD8 mAb MEM-31 was purchased from Immunotoools; the anti-CD3-TC S4.1 clone, from Caltag; anti-CD56-RD1 clone N901 and the anti-CD4-DR1 clone SFC127/4D11, from Beckman Coulter; and the anti-KIR2DL1 DX9-PE, from Miltenyi Biotec. For analysis of KIR2DL5 expression, we used PBMC of voluntary donors whose KIR genotypes had been determined using a published method (51). All donors gave their informed consent for these studies, which were approved by the Ethical Committee of Clinical Investigation of our center. Flow cytometry analysis was made in an Epics XL apparatus and was represented with the EXPO-32 software (Beckman Coulter). Immunoblots were probed with an HRP-coupled anti-phosphotyrosine mAb (Zymed), and with anti-SHP-1 and anti-SHP-2 rabbit polyclonal Abs (Santa Cruz Biotechnology).

Immunoprecipitation and immunoblotting
Polyclonal UP-R1+ and UP-R1-depleted NK cells were surface labeled with 125I by using Iodo-Gen reagent (Pierce). Cells were then lysed in 1% Triton X-100, 20 mM HEPES, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 1 mM EGTA and, after two preclearings with protein G-Sepharose 4B Fast Flow (Sigma-Aldrich), immunoprecipitated sequentially with the MOPC21 (negative control mouse IgG1) and anti-2DL5 UP-R1 mAbs. One aliquot of the UP-R1 immunoprecipitate was subjected to deglycosylation with peptide N-glycosidase F (PNGaseF; New England Biolabs). Immunoprecipitates were run in 8% SDS-PAGE and autoradiographed using Hyperfilm-MP films (Amersham Biosciences).

For Western blot experiments, polyclonal UP-R1+ NK cells, either treated or untreated with 1 mM sodium pervanadate for 15 min at 37°C, were lysed and immunoprecipitated sequentially with MOPC21, UP-R1, and HP-MA4 mAbs. Aliquots of each immunoprecipitate were run in 10%
The UP-R1 mAb recognizes a subpopulation of CD56-negative control.

Table I. The UP-R1 mAb recognizes a subpopulation of CD56-negative control.

<table>
<thead>
<tr>
<th>Donors expressing the KIR2DL5 gene</th>
<th>UP-R1-stained cells% of PBL</th>
<th>% of CD56</th>
<th>KIR Genotype</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% of PBL</td>
<td>% of CD56</td>
<td>2DL5</td>
</tr>
<tr>
<td>C11</td>
<td>1.52</td>
<td>5.76</td>
<td>A*001</td>
</tr>
<tr>
<td>C25</td>
<td>0.56</td>
<td>5.18</td>
<td>A*001</td>
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<td>0.56</td>
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<td>A*001</td>
</tr>
<tr>
<td>C176</td>
<td>3.15</td>
<td>6.28</td>
<td>A*001</td>
</tr>
<tr>
<td>C185</td>
<td>2.22</td>
<td>7.78</td>
<td>A*001</td>
</tr>
<tr>
<td>C238</td>
<td>0.46</td>
<td>3.58</td>
<td>A*001</td>
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<td>Donors lacking KIR2DL5-gene expression</td>
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<td></td>
</tr>
<tr>
<td>C135</td>
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<td>0.01</td>
<td>B*002d</td>
</tr>
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<td>0.00</td>
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<td>B*002d</td>
</tr>
<tr>
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<td>B*006d</td>
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<td>H335-H1b</td>
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<td>0.10</td>
<td>+</td>
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<tr>
<td>C136</td>
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<td>+</td>
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<td>0.00</td>
<td>0.01</td>
<td>+</td>
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</table>

* The background staining of an isotype-matched negative control mAb is subtracted from the percentage of UP-R1-stained cells.

† The framework genes KIR3DL2 and KIR3DL3, present in all donors, are not represented.

‡ KIR2DS5, despite its name, is not an activating counterpart of KIR2DL5, but a member of a different KIR gene lineage—that encoding HLA-C-specific KIR with Ig-like domains of the D1-D2 type (54).

§ KIR2DL5*002 and the novel KIR2DL5*006 (unpublished) are nonexpressed alleles, as assessed by lack of RNA transcripts detectable by RT-PCR (55).

Cytotoxicity assays against mouse mastocytoma P815 cells were conducted as previously described (52). Briefly, polyclonal KIR2DL5* KIR2DL1* NK cells were incubated with 51Cr-labeled P815 cells, either in culture medium alone or in the presence of each of the following Abs: anti-CD56 C218 (negative control); anti-3DL1 DX9; and anti-2DL5 UP-R1. Each assay was performed in triplicate, in two independent experiments. After 4 h in culture, the percentage of specific lysis was calculated. The spontaneous release of 51Cr was always <20% of the maximum release.

Results

A novel mAb recognizes specifically the product of the KIR2DL5 gene

A soluble form of KIR2DL5 was produced by transfecting transiently HEK-293T cells with a cDNA encoding the extracellular region of the receptor fused to the Fc fragment of human IgG1 (39). The KIR2DL5-Ig fusion protein was affinity purified from culture supernatants and used for mice immunization. The UP-R1 clone, secreting a mAb of the IgG1 subclass, was selected by screening the hybridomas derived from immunized mice for reactivity against the immunogen.

To verify that the reactivity of the UP-R1 mAb with the fusion protein was due to recognition of the KIR2DL5 extracellular region, instead of other epitopes of the fusion protein, we transfected HEK-293T cells with a cDNA of KIR2DL5 labeled in its N terminus with a FLAG tag. Flow cytometry analyses using UP-R1 and the anti-FLAG Ab M2 showed that KIR2DL5-FLAG is expressed on the surface of transfected cells and that it is recognized by the UP-R1 mAb (Fig. 1). Furthermore, this Ab does not cross-react with HEK-293T cells overexpressing each of three different alleles of KIR2DL4 (Fig. 1 and results not shown), the receptor closest to KIR2DL5 in terms of amino acid sequence (79% identity) and configuration of the Ig-like domains (D0-D2 instead of D1-D2). We also analyzed cells transfected with plasmids encoding KIR with other types of Ig domain organization: KIR2DL1, as a representative of KIR2DL5 with D1-D2 domains; and three KIR2DL5.
with D0-D1-D2 domains: KIR3DL1; KIR3DL2; and the structurally divergent KIR3DL3. The UP-R1 mAb recognized neither of the KIR2DL1 and KIR3DL2 transfectants, but showed some weak cross-reactivity with cells overexpressing FLAG-tagged KIR3DL1 and KIR3DL3 (fluorescence intensity, ~1–6% of that obtained with an anti-FLAG mAb; Fig. 1). Such cross-reactivity is not seen in NK cells expressing naturally the latter genes, as will be shown in the following paragraphs.

**KIR2DL5 is expressed constitutively on the surface of resting NK and T lymphocyte subsets**

Transcripts of KIR2DL5 have been detected previously in NK and T cell clones derived from peripheral blood (23), but the lack of suitable reagents precluded the analysis of its surface expression. To assess for the expression of a natural KIR2DL5 gene product on human lymphocytes and for its reactivity with UP-R1, we performed flow cytometry on PBMC freshly isolated from donors with diverse KIR genotypes (Table I and Fig. 2A). UP-R1-stained lymphocytes were detected in all donors having a KIR2DL5A*001 allele, in proportions ranging between 0.46 and 3.15% (3.58–7.78% of CD56+ cells). By contrast, UP-R1 binding was undetectable in individuals that lacked a KIR2DL5 gene or carried nontranscribed KIR2DL5B alleles, regardless of the presence or absence of other KIR genes (Table I and Fig. 2A). Because we had observed weak cross-reactivity of UP-R1 with cells transfected with other KIR, and anti-KIR mAbs tend to stain expanded NK cells more brightly than resting cells, we verified the specificity of the UP-R1 mAb on NK cells expanded in vitro from donors with different KIR genotypes (Fig. 2B and results not shown). Subpopulations of UP-R1+ NK cells were seen in donors having KIR2DL5A*001, but not in subjects without this gene, including one (H318P) who expressed KIR3DL1*002, the allele that showed weak UP-R1 cross-reactivity in transfected cells. Lack of cross-reactivity of UP-R1 with KIR3DL1 in NK cells was further assessed by two-color flow cytometry using that mAb in combination with the anti-KIR3DL1 reagent DX9 (Fig. 2C). These results constitute the first direct proof that the KIR2DL5 gene encodes a protein constitutively expressed on the membrane of human lymphocytes, and they also demonstrate the specificity of the UP-R1 Ab for KIR2DL5.

To determine which lymphocyte subpopulations express KIR2DL5 on their membrane, we undertook three-color flow cytometry analyses of PBMCs isolated from several donors. The majority of UP-R1-stained cells were found in the NK cell fraction defined by the CD56+CD3− phenotype (Fig. 3A). As is the case for other MHC-specific NK cell receptors (29, 52), only a proportion of NK cells expressed KIR2DL5 (range of 10 donors, 2.50–10%), which is consistent with the clonal distribution suggested by previous analysis of the KIR2DL5 mRNA (23). The NK cells expressing KIR2DL5 belonged in all cases to the CD56bright subpopulation, whereas the minority of cells with the CD56−bright phenotype were not stained by the Ab (Fig. 3A). The low proportion of CD56bright NK cells among circulating lymphocytes makes it difficult to assess the possible expression of a given surface molecule.
in a minority of them using flow cytometry of unsorted PBMC. To rule out the possibility of overlooking a low number of CD56\textsuperscript{bright} cells expressing KIR2DL5, we isolated fresh NK cells from PBMC by negative selection and re-evaluated their staining pattern with UP-R1. This confirmed that KIR2DL5 expression in circulating NK cells is restricted to the CD56\textsuperscript{dim} subpopulation (Fig. 3), as has been shown for other KIR (33), with the remarkable exceptions of KIR2DL4 (31, 32, 34) and KIR3DL3 (41, 55). Because only CD56\textsuperscript{bright} cells transcribe KIR3DL3, their lack of staining with UP-R1 is also relevant with regard to the specificity of the mAb, which cross-reacted weakly with the KIR3DL3 transfectant.

To characterize the distribution of KIR2DL5 with regard to that of other KIR, we performed double staining experiments of NK cells, using UP-R1 and individual mAbs recognizing different other KIR. In these assays, we always identified subpopulations coexpressing both KIR2DL5 and each of those KIR, besides others expressing only either marker (Fig. 2C and results not shown). This indicates that the distribution of KIR2DL5 in NK clones is, as for most other KIR, random rather than coordinated with the latter. This prompted a question on the existence of NK cells expressing KIR2DL5 in isolation. To address this issue, we performed three-color flow cytometry analyses in which PBMCs were stained with anti-CD3, anti-KIR2DL5 UP-R1, and a mixture of five mAbs specific for different KIR (including all of 3DL1/L2, 2DL1–L3, 2DS1/2S2, and 2DS4), and for NKG2A (Fig. 4). Consistent with the known frequencies of expression of the different NKR and combinatorial rules, most NK cells (defined in these experiments as CD3\textsuperscript{−} NKR\textsuperscript{−}) were pan-KIR/NKG2A\textsuperscript{−} KIR2DL5\textsuperscript{−}, followed in frequency by pan-KIR/NKG2A\textsuperscript{+} KIR2DL5\textsuperscript{−} cells. However, a discrete minority of pan-KIR/NKG2A\textsuperscript{+} KIR2DL5\textsuperscript{−} cells could also be defined (~0.5–1% of the NKR\textsuperscript{−} cells in Fig. 4A) and was corroborated in NK cells expanded from one donor (Fig. 4B), meaning that some NK cells do express KIR2DL5 segregated from other KIR and from NKG2A. Given the low frequency of this subpopulation, it is conspicuous in flow cytometry only when high numbers of PBMCs are analyzed.

Expression of KIR2DL5 was also detected on subpopulations of T lymphocytes, yet at lower proportions than on NK cells (<0.1%–0.8% of CD3\textsuperscript{−} cells) and with noticeable variability between different subjects (Fig. 5 and results not shown). A discrete CD3\textsuperscript{+} KIR2DL5\textsuperscript{+} subpopulation was visible in some individuals having KIR2DL5A\textsuperscript{∗}001, but not in others (e.g., C202 and C180 vs C11). KIR2DL5\textsuperscript{+} T lymphocytes expressed preferentially the
CD56 molecule, but a proportion of them was CD56\(^{-}\). The vast majority of KIR2DL5\(^{+}\) T lymphocytes was confined within the CD8\(^{-}\) subpopulation, whereas CD4\(^{+}\) KIR2DL5\(^{+}\) T cells could not be demonstrated. We cannot exclude, however, the existence of CD4\(^{+}\)-KIR2DL5\(^{+}\) T lymphocytes in numbers below the sensitivity threshold of our assays.

Biochemical characterization of KIR2DL5

To characterize biochemically KIR2DL5, we performed an SDS-PAGE separation of UP-R1 immunoprecipitates obtained from KIR2DL5\(^{+}\) cells. These were purified from PBMC using UP-R1 and paramagnetic beads coated with a human anti-mouse IgG mAb (Fig. 6). After expansion of the NK cells in vitro, the proteins expressed on their surface were radioactively labeled with \(^{125}\)I, and lysates were subjected to immunoprecipitation and subsequent SDS-PAGE. A single band with a relative mobility of \(~60\) kDa was immunoprecipitated from KIR2DL5\(^{+}\), but not from KIR2DL5-depleted NK cells isolated from the same donor (Fig. 6). Coincident results were obtained by comparing immunoprecipitates obtained from KIR2DL5A\(^{\text{FLAG}}\)-transfected and untransfected Ba/F3 cells and from a KIR2DL5\(^{-}\) clone (not shown). After N-deglycosylation with PNGaseF, the immunoprecipitated KIR2DL5 migrated at an approximate \(M_\text{r}\) of 45 (Fig. 6), a value that is similar to the predicted size (38.4) of the mature peptide encoded by an mRNA containing all of the KIR2DL5 exons (23). Thus, >25% of the KIR2DL5 mass appears to derive from N-linked carbohydrates. No differences between the electrophoretic mobilities of UP-R1 immunoprecipitates treated under either reducing or nonreducing conditions were appreciated (not shown), indicating that KIR2DL5 is probably expressed as a monomer on the cell surface.

NK cell cytotoxicity of FcR\(^{+}\) P815 cells is inhibited by UP-R1 cross-linking of KIR2DL5, which, upon phosphorylation, recruits the SHP-1 and SHP-2 phosphatases

To test the ability of KIR2DL5 to modulate the NK cell cytotoxicity, polyclonal KIR2DL5\(^{+}\) KIR3DL1\(^{-}\) NK cells were obtained by expansion after sequential purification with UP-R1, DX9, and paramagnetic beads. Those cells were then studied in cytotoxicity assays against the FcR\(^{+}\) murine mastocytoma cell line P815. Cross-linking of KIR2DL5 with UP-R1 inhibited the spontaneous lysis of P815 at every tested E:T ratio (Fig. 7), in comparison with the cultures in which no Abs, or an anti-CD56 mAb, were added.
The degree of inhibition achieved with UP-R1 (17–32% of the maximum lysis value) was similar to that induced by the KIR3DL1-specific Ab DX9 (Fig. 7).

To identify the possible mechanisms by which KIR2DL5 inhibits NK cell cytotoxicity, we investigated by immunoblot its binding to SHP-1 and SHP-2, the protein-tyrosine phosphatases that mediate the inhibitory effect of other KIR. As a control, we studied the binding of those phosphatases to KIR2DL1 in the same cells (Fig. 8A). Treatment of the NK cells with sodium pervanadate induced a strong tyrosine phosphorylation of both KIR2DL5 and KIR2DL1 (Fig. 8B), and the recruitment of SHP-1 and SHP-2 to each of the hyperphosphorylated KIR (Fig. 8, C and D). However, the behavior of the two receptors was not identical: whereas both bound comparably to SHP-1 (Fig. 8, C and E), KIR2DL5 appeared to recruit SHP-2 more efficiently than KIR2DL1 (Fig. 8, D and E).

Discussion

KIR were discovered thanks to seminal studies showing that the observed allospecificity of certain NK cell clones correlated with the distribution of the surface molecules recognized by a reduced number of mAbs (52). The identification and characterization of KIR as inhibitory receptors for HLA validated definitely the missing-self model as a way in which human NK cells contribute to innate immunity (7, 10, 11). The discovery of KIR also constitutes an example of classical genetic research, in which investigation of a functional phenomenon is culminated by the isolation of the responsible gene. Investigation on KIR, however, soon proceeded in the opposite direction, given that the molecular cloning of the genes encoding the inhibitory KIR specific for HLA-A, -B, and -C was accompanied and followed by the unexpected isolation of mRNAs and, more recently, DNAs derived from a multiplicity of additional KIR genes, the role of which is still intriguing (19, 20, 23). Among the KIR genes identified by studies of genomic DNA, only KIR2DL5 appeared to be functional from the beginning (23); whereas another two genes, KIR3DL3 and KIR3DP1, seemed initially nontranscribed pseudogenes, a vision that has been questioned by the isolation of their mRNA in certain cells or donors (41, 55, 56).

Whether new KIR genes identified in genomic or cDNA clones actually code for proteins expressed in human leukocytes remains in many cases unknown, due to a lack of reagents capable of detecting those gene products. By using a newly generated specific mAb, we have obtained here the first direct proof that one such gene, KIR2DL5, encodes a physiologically expressed protein. We have shown that the KIR2DL5 product is a glycoprotein detectable on the membrane of some human lymphocytes without previous stimulation. In particular, it is expressed in a fraction of blood NK cells with the CD56dim phenotype and in a variable proportion of circulating T lymphocytes.

The observed pattern of KIR2DL5 expression is consistent with the clonal distribution previously reported for its mRNA in NK and T cell clones of one donor (23). The frequency of NK clones expressing KIR2DL5 mRNA found in that donor was at least 2-fold higher than the proportions of UP-R1+ NK cells we have seen in five individuals, which could be explained, among other reasons, by variability among donors or by KIR2DL5 favoring the survival of the NK clones that express it. In support of the first possibility is the fact that the proportion of NK cells (and, even more noticeably, T lymphocytes) expressing KIR2DL5 indeed varies among different individuals (Table I and Figs. 2–5). Such form of variability is common to other KIR, in which it appears to be determined in part by allelic polymorphism of the receptors and by the modulating effect of their HLA ligands (57). However, sequence polymorphism is unlikely to be a contributing factor in the case of KIR2DL5A*001, because the promoter region of this allele appears to be conserved (Refs. 19 and 53 and GenBank sequence submission AY320039, by D. E. Geraghty et al.). It is thus more likely that the variable frequency of use of KIR2DL5 depends either on the accompanying repertoire of NK cell receptors and ligands (including the unknown KIR2DL5 ligand), or on other genetic or environmental factors; alternatively, it might be entirely stochastic. We have also shown that KIR2DL5 cross-linking is capable of inhibiting the NK cell cytotoxicity against a xenogeneic FcRγ+ target cell, an ability that likely involves the recruitment of both SHP-1 and SHP-2 phosphatases. The inhibitory effect and the binding of both phosphatases to KIR2DL5 in cells expressing naturally the receptor are in agreement with previous predictions based on the KIR2DL5 primary structure (23) and on the behavior of NK-92 cells transduced with FLAG-tagged- or chimerical molecules containing the KIR2DL5 cytoplasmic tail (38). The higher proportion of SHP-2 bound by KIR2DL5 in comparison with KIR2DL1 we have observed here (Fig. 8), and the behavior of the

FIGURE 7. UP-R1 inhibits the lysis of the P815 mastocytoma by KIR2DL5+ NK cells. Positively selected KIR2DL5+ KIR3DL1+ NK cells (97.8% UP-R1−, 99.7% DX9−) were incubated at different E:T ratios with the murine FcR− cell line P815, either alone or in the presence of mAbs directed against CD56, KIR3DL1 (DX9), and KIR2DL5 (UP-R1), in a standard cytotoxicity assay. Each combination was assayed in triplicate, and the average value and SEM are represented; results are representative of two experiments.

FIGURE 8. Phosphorylated KIR2DL5 recruits the SHP-1 and SHP-2 phosphatases. UP-R1-selected NK cells (A), either with or without a pretreatment with pervanadate (PVN), were immunoprecipitated sequentially with mouse IgG1 (isotype-matched negative control), UP-R1 and HP-MA4 mAbs. Equivalent fractions of each immunoprecipitate were subjected to 10% SDS-PAGE, membrane transferred in triplicate and probed with Abs specific for phosphotyrosine (B), SHP-1 (C), and SHP-2 (D). After partial stripping, the membrane shown in D was reprobed with the anti-SHP-1 Ab (E).
to NK cells through SHP-1 and, to a lesser extent, SHP-2; KIR2DL5, in which the last proportion is reversed; KIR2DL4, capable of activating and inhibiting NK cells through recruitment of FceRy, SHP-2, and, possibly, other unknown mediators; and ones that, through the ITAM-containing protein DAP12, activate NK cells. A fifth functional category might be constituted by KIR3DL3, a receptor with a single ITIM located within an intermediate length cytoplasmic tail (22).

KIR2DL5 thus gathers a unique and intriguing combination of features. Like KIR2DL4, it is a structurally divergent and ancient receptor conserved in several primates, as compared with most other human KIR, which evolved rapidly after the separation of our species and its closest living relatives, the chimpanzees, from a common ancestor (25). In contrast, the variable presence of KIR2DL5 in the genome, its inhibitory function and its variated pattern of expression in cytotoxic CD56dim NK cells are features that resemble more closely those of the evolutionarily newer human KIR that recognize classical HLA class I molecules. By analogy with these KIR, it can be speculated that the role of NK cells expressing KIR2DL5 could be to survey the expression of an unknown ligand that is down-regulated in pathological situations. This concept is consistent with the existence of an NK subpopulation expressing KIR2DL5 but lacking every other inhibitory KIR and NKG2A, as we have shown here.

On the other hand, the tight association of KIR2DL5A*001 with haplotypes that lack KIR2DL1 and, instead, encode more activating KIR (2DS1, 2DS5, and 3DS1; Refs 19, 23, and 26) can be seen as a counterbalance that provides supplementary inhibition to an excessively activating combination of genes. Against this view, it can be argued that KIR2DL5 most likely predates those activating KIR in evolution (58) and that it may hardly operate as a general, non-specific inhibitor of NK cells, given that we have shown that only subsets of these express the receptor. The availability of a specific reagent to detect KIR2DL5 should help clarify what this receptor recognizes, how it contributes to natural and adaptive immunity, and how its presence and absence in the genome influence human health.

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4410 IDENTIFICATION AND CHARACTERIZATION OF HUMAN KIR2DL5 RECEPTOR


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