

# A Common Inhibitory Receptor for Major Histocompatibility Complex Class I Molecules on Human Lymphoid and Myelomonocytic Cells

By Marco Colonna,\* Francisco Navarro,† Teresa Bellón,‡  
Manuel Llano,‡ Pilar García,‡ Jacqueline Samaridis,\* Lena Angman,\*  
Marina Cella,\* and Miguel López-Botet‡

From the \*Basel Institute for Immunology, Basel CH-4005, Switzerland; and †Hospital Universitario de la Princesa, 28006 Madrid, Spain

## Summary

Natural killer (NK) cell-mediated lysis is negatively regulated by killer cell inhibitory receptors specific for major histocompatibility complex (MHC) class I molecules. In this study, we characterize a novel inhibitory MHC class I receptor of the immunoglobulin-superfamily, expressed not only by subsets of NK and T cells, but also by B cells, monocytes, macrophages, and dendritic cells. This receptor, called Ig-like transcript (ILT)2, binds MHC class I molecules and delivers a negative signal that inhibits killing by NK and T cells, as well as  $Ca^{2+}$  mobilization in B cells and myelomonocytic cells triggered through the B cell antigen receptor and human histocompatibility leukocyte antigens (HLA)-DR, respectively. In addition, myelomonocytic cells express receptors homologous to ILT2, which are characterized by extensive polymorphism and might recognize distinct HLA class I molecules. These results suggest that diverse leukocyte lineages have adopted recognition of self-MHC class I molecules as a common strategy to control cellular activation during an immune response.

Natural killer (NK) cells lyse transformed or virally infected cells that have lost or downregulated expression of self-MHC class I molecules (1). This recognition of “missing self” is mediated by inhibitory receptors that deliver a negative signal upon specific interaction with class I ligands (2–5). In humans, p58, p70, and p70/p140 killer cell inhibitory receptors (KIRs)<sup>1</sup> belong to the Ig-superfamily (SF), and recognize distinct polymorphic determinants of HLA-C, -B, and -A molecules, respectively (6). The CD94/NKG2A heterodimer belongs to the C-type lectin SF and recognizes cells expressing a broad range of HLA-A, -B, and -C molecules (7–9) as well as the nonclassical class I molecule HLA-G (10–12). However, KIRs and CD94/NKG2A do not entirely explain the class I specificities of several NK cell clones, suggesting the presence of yet unknown inhibitory MHC class I receptors (6, 10, 11).

Recently, we have identified new members of the Ig-SF, called Ig-like transcript (ILT)1, ILT2, and ILT3 (13, 14).

<sup>1</sup>Abbreviations used in this paper: DC, dendritic cell; ILT, Ig-like transcript; ITIM, immunoreceptor tyrosine-based inhibitory motifs; KIR, killer cell inhibitory receptor; RBL, rat basophilic leukemia; rADCC, reverse antibody-dependent cell-mediated cytotoxicity; SF, superfamily; TSST, toxic shock syndrome toxin.

Their homology to KIRs and their location on human chromosome 19 in close linkage with KIRs has suggested that some of these molecules may be inhibitory class I receptors. ILT1 is expressed on NK cells, but lacks cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that may mediate negative signaling (15). ILT3 is not expressed on NK cells and does not bind to MHC class I molecules (14). ILT2 remains a potential candidate, since it is characterized by a cytoplasmic tail containing ITIMs and is expressed by NK cells (13).

In an attempt to identify novel NK inhibitory class I receptors distinct from KIRs, we have now obtained an mAb, termed HP-F1, which is specific for ILT2. Using this mAb, we show that ILT2 is expressed not only by NK and T cells, but also by B and myelomonocytic cells. ILT2 binds class I molecules and delivers a negative signal that inhibits killing by NK and T cells and  $Ca^{2+}$  mobilization in B and myelomonocytic cells triggered via the B cell receptor and HLA-DR. We also find that myelomonocytic cells express ILT2 homologs, which are significantly diverse and might recognize distinct HLA class I molecules. Thus, diverse leukocyte lineages express inhibitory class I receptors, which may modulate thresholds of cellular activation.

## Materials and Methods

**Cells.** C1R and 721.221 are MHC class I-deficient EBV-transformed human B cell lines (16, 17). HLA-B\*2702-, -B\*2705-, -B\*5101-, -A\*0301-, -B\*0702-, -Cw\*0301- and -G1-721.221 are HLA-class I transfectants in 721.221. All of these cells were grown in RPMI/10% FCS. NKL is an NK cell line (18), which was grown as previously described (10). NK cell clones and T cell clones were derived from PBMCs and cultured as previously described (19). Monocytes were prepared from PBMCs by adhesion to plastic (14). Dendritic cells (DCs) were derived either from CD34+ hematopoietic precursors cultured in GM-CSF and TNF- $\alpha$  for 10 d (20) or from purified monocytes (21–23). Macrophages were obtained by culturing monocytes for 10 d in 6-well plates at a concentration of  $10^6$  cells/ml in RPMI with 20% human serum and supplemented with 1 ng/ml M-CSF.

**Monoclonal Antibody and Cytotoxicity Assays.** The mAb HP-F1 was raised by immunizing BALB/c mice against the NKL cell line. Hybridoma supernatants were screened for the capacity of reconstituting NKL-mediated lysis against HLA-B\*5101 and HLA-G1 transfectants in 721.221. Cytotoxicity assays, reverse antibody-dependent cell-mediated cytotoxicity (rADCC), and control mAbs [HP-1F7 (anti-MHC class I, IgG1), C218 (anti-CD56, IgG1) and HP-3B1 (anti-CD94, IgG2a)] have been previously described (9, 10).

**Antibodies and FACS<sup>®</sup> Staining.** PBMCs were stained with the HP-F1 mAb followed by either FITC- or PE-conjugated goat anti-mouse IgG1 and counter-stained with anti-CD56-PE, anti-CD3-PE, anti-TCR- $\alpha/\beta$ -FITC, anti-TCR- $\gamma/\delta$ -FITC, anti-CD19-PE, and anti-CD14-PE (Becton Dickinson and Co., Mountain View, CA). DCs derived from CD34+ hematopoietic precursors were double-stained with anti-HLA-DR (L243, IgG2a, American Type Culture Collection, Rockville, MD) and HP-F1 mAbs. DCs derived from purified monocytes were double-stained with anti-CD1a (IgG2b, PharMingen, San Diego, CA) and HP-F1 mAbs. Stained cells were analyzed by flow cytometry on a FACStar<sup>®</sup> Plus (Becton Dickinson) using the LYSYS II software.

**Transfections and Immunoprecipitations.** ILT2 cDNA subcloned into pCDNA3 (Invitrogen Corp., Carlsbad, CA) was transiently transfected into COS7 cells by DEAE-Dextran, as previously described (9). Immunoprecipitations from NKL and transfected COS cells were as previously described (9). In brief, cells were <sup>125</sup>I-labeled and lysed, and then lysates were subjected to immunoprecipitation with the HP-F1 mAb or with culture supernatant of X63 murine myeloma. Immunoprecipitates were analyzed by standard SDS-PAGE. N-linked glycans were removed from immunoprecipitates using N-glycosidase F (Boehringer Mannheim, Mannheim, Germany).

**Production of ILT2 Soluble Protein.** To obtain a soluble form of ILT2, we produced a fusion protein of the ILT2 extracellular domain and human IgG1 constant regions, using the myeloma-based expression system (24, 25). The nucleotide sequence encoding ILT2 extracellular region was amplified from cloned plasmid DNA by PCR using the oligonucleotides GGGATCGGTACCGACGCCATGACCCCCATCCTCACGGTC and GGGATCAAGCTTATACTTACCGTGCCTTCCCAGACCACT as sense and antisense primers, respectively. The sense primer contained the native ILT2 start codon. The antisense primer contained a HindIII restriction site (underlined), a splice donor sequence, and six ILT2 codons preceding the transmembrane domain. The ~1,400-bp amplified product was gel purified and ligated into the expression vector pCD4-Hg1 (24). The vector contains the exons encoding hinge, CH2, and CH3 regions of the human IgG1 gene and

the guanosine phosphotransferase gene, which confers resistance to mycophenolic acid. The ILT2-IgG1 plasmid was transfected into J558L mouse myeloma cells by electroporation and cells were cultured in DMEM supplemented with 10% FCS, 2 mM l-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50  $\mu$ g/ml kanamycin. After 2 d of culture, selective medium containing 4  $\mu$ g/ml mycophenolic acid (Calbiochem-Novabiochem, La Jolla, CA) and 125  $\mu$ g/ml xanthine (Sigma Chemical Co., St. Louis, MO) were added and incubation at 37°C was continued until resistant colonies appeared. Clones were screened for production of soluble IgG fusion proteins by a two-site sandwich ELISA. HP-F1 mAb was employed as capture antibody, while horseradish peroxidase-conjugated goat anti-human IgG antibody was used as detection antibody. Producer clones were expanded, diminishing the FCS content to 2%. For purification of the fusion protein, culture supernatant was concentrated and adsorbed over a recombinant protein A column (Repligen, Cambridge, MA). After washing with PBS with 0.02% sodium azide, bound fusion protein was eluted with 0.1 M glycine-HCl, pH 2.65. 1 ml fractions were collected in test tubes containing 100  $\mu$ l 2 M Tris-HCl, pH 8, pooled, and dialyzed against PBS. Purified protein was then concentrated, sterile filtered, and kept frozen.

**Serotonin Release.** Rat basophilic leukemia (RBL) cells were transfected with ILT2 cDNA subcloned into pCDNA3 (Invitrogen Corp.) by electroporation, and stable transfectants were selected in G418-containing medium. ILT2-transfected and untransfected RBL cells ( $10^6$ /ml) were pulsed for 3 h at 37°C in MEM/Earle's salts/10% FCS containing 2  $\mu$ Ci/ml [<sup>3</sup>H]serotonin (Dupont-NEN, Boston, MA). Cells were washed, incubated at 37°C for 1 h, washed again, resuspended at  $5 \times 10^6$ /ml, and plated in 50  $\mu$ l aliquots in 96-well flat-bottomed plates precoated with 20  $\mu$ g/ml of anti-TNP mouse IgE (Sigma Chemical Co.) alone or in combination with 20  $\mu$ g/ml of HP-F1 [whole antibody or F(ab')<sub>2</sub> fragments] or 20  $\mu$ g/ml of HP-1F7 [whole antibody or F(ab')<sub>2</sub> fragments], as controls. After 1 h at 37°C the reaction was stopped with 150  $\mu$ l of cold medium, and then 100  $\mu$ l of supernatants was collected from each well and radioactivity was measured. Serotonin release was calculated according to the formula: % serotonin release = ([cpm sample] - [cpm spontaneous release]) / ([cpm total] - [cpm spontaneous release]).

**Immunoblottings.** Cells ( $3-4 \times 10^7$ ) were incubated for 10 min at 37°C in 1 ml of RPMI alone or with 0.03% H<sub>2</sub>O<sub>2</sub>/100  $\mu$ M sodium orthovanadate (pervanadate), which inhibits phosphatase activity and thus increases protein tyrosine phosphorylation. Cells were then lysed at 4°C in lysis buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM sodium orthovanadate, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin). Lysates were precleared for 2 h at 4°C on protein G sepharose beads and incubated overnight at 4°C with HP-F1 mAb followed by protein G sepharose beads for 2 h. Precipitates were washed 3 $\times$  with lysis buffer, resolved by 8% SDS-PAGE under nonreducing conditions, and transferred to nitrocellulose membranes (Hybond-C super; Amersham Corp., Arlington Heights, IL). After blocking with PBS with 0.1% Tween 20 and 5% nonfat dry milk, the membranes were incubated with anti-SHP-1 polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL). Immunoblotted proteins were visualized by chemiluminescence using the enhanced chemiluminescence detection reagents (Amersham Corp.). Membranes were stripped of antibodies by incubation for 30 min at 55°C in 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, according to

the protocol supplied by Amersham Corp. Stripped blots were probed after blocking with anti-SHP-2 rabbit antiserum (Santa Cruz Biotechnology) or with anti-SHIP rabbit antiserum (provided by Mark Coggeshall, Ohio State University, Columbus, OH).

**Measurement of Cytosolic Calcium in B Cells and Monocytes.** EBV-transformed B cell lines and monocytes were loaded with Indo-1 AM (Sigma Chemical Co.) as previously described (26). B cells were then incubated for 5 min on ice either with anti-MHC class I mAb HP-1F7 or with the HP-F1 mAb (200  $\mu$ l of culture supernatant). 2.5 ng of mouse anti-human IgG were added and incubation was continued for 15 min on ice. After washing with medium, cells were shifted at 37°C for 15 min followed by addition of 10  $\mu$ g of F(ab')<sub>2</sub> goat anti-mouse IgG as cross-linker. Cells were analyzed on a flow cytometer (FACS® Vantage; Becton Dickinson) to detect Ca<sup>2+</sup> fluxes. Only live (based on forward scatter criteria) and Indo-1-loaded cells (based on 405 nM vs. 525 nM emission spectra) were included in the analysis. Ca<sup>2+</sup> mobilization triggered in monocytes by the anti-class II mAb 3.8B1 was determined as previously described (14).

**Reverse Transcriptase-PCR and Oligonucleotide Primers.** Oligo-dT-primed cDNAs were prepared from NK cells, T cells, monocytes, macrophages, and DCs as previously described (27). ILT2, ILT4, and ILT5 were amplified from the above cDNAs by PCR, cloned into pCR2.1 (Invitrogen Corp.) and sequenced. Amplification primers were as follows: sense, ATGACCCCATCCT-CACGGTCCTG; antisense, CTGGGCTAGTGGATGGCCAG. PCR and cloning conditions were as previously described (27). ILT5 allelic forms were amplified by reverse transcriptase (RT)-PCR, cloned, and sequenced from a pool of bone marrow cells derived from 51 donors (Clontech, Palo Alto, CA), as well as from DCs derived from a single donor.

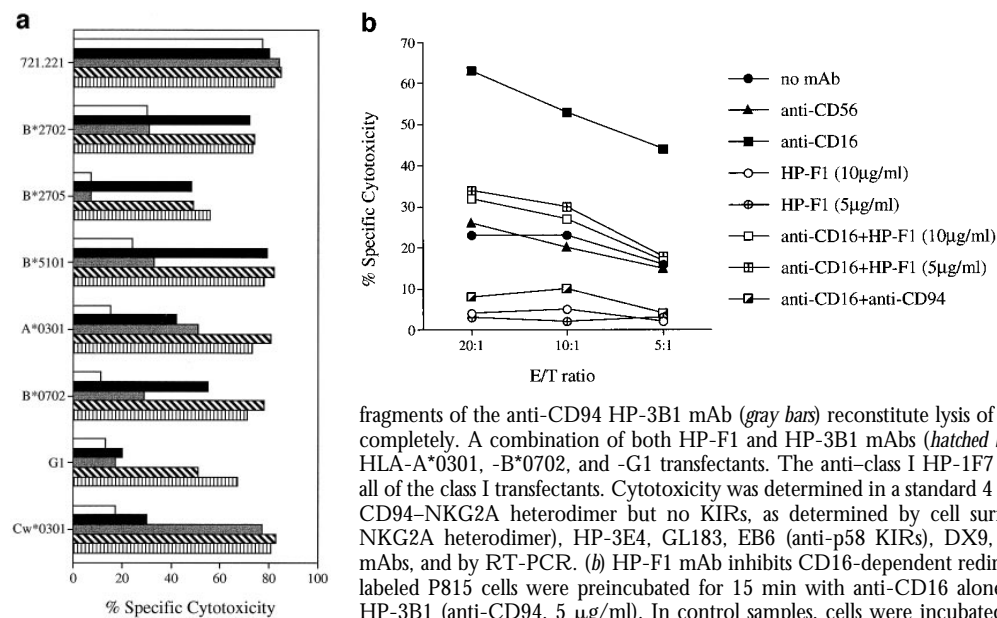
## Results and Discussion

**The HP-F1 mAb Recognizes a Novel Inhibitory MHC Class I Receptor Encoded by ILT2.** KIRs and CD94/NKG2A re-

ceptors do not completely explain class I recognition by a number of NK cell clones (6, 10). This is illustrated by a representative NK cell line, called NKL (Fig. 1 a). NKL-mediated lysis of the class I deletion mutant 721.221 is inhibited by transfection of HLA-B\*2702, -B\*2705 and -B\*5101 genes into 721.221, although NKL does not express a p70 KIR specific for these HLA-B molecules. NKL-mediated lysis is also inhibited by HLA-A\*0301, -B\*0702, -Cw\*0301 and -G1 transfected cells. This inhibition is not entirely accounted for by the CD94-NKG2A heterodimer expressed by NKL, since cytolysis of HLA-A\*0301, -B\*0702, and -G1 transfectants is only partially reconstituted by an anti-CD94 mAb. In an attempt to find additional inhibitory receptor(s) on NKL, we generated anti-NKL mAbs and selected those capable of restoring lysis of class I transfectants. Among them, the HP-F1 mAb reconstituted cytolysis of HLA-B\*2702, -B\*2705, and -B\*5101 transfectants and, in combination with an anti-CD94 mAb, restored lysis of HLA-A\*0301, -B\*0702, and -G1 transfectants (Fig. 1 a).

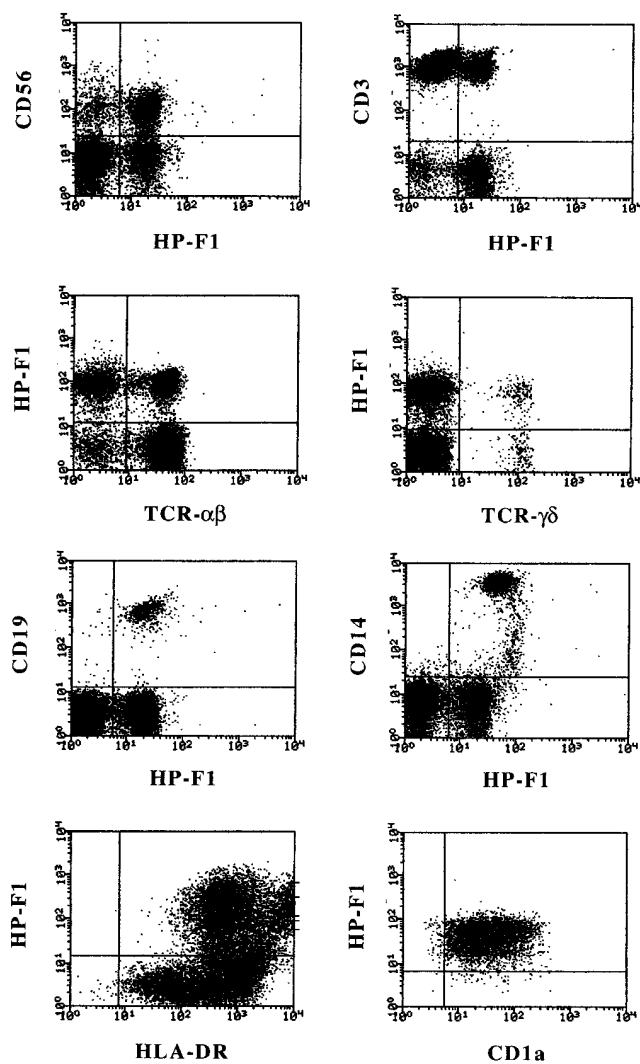
Further demonstration that the HP-F1 mAb recognizes an inhibitory receptor on NKL was obtained by rADCC experiments (Fig. 1 b) (28). The FcR<sup>+</sup> P815 murine mastocytoma cell line was efficiently killed by NKL in the presence of an anti-CD16 mAb, which binds the FcR on target cells and the triggering receptor CD16 on effector cells. When the HP-F1 mAb was added in the assay together with the anti-CD16 mAb, lysis was reduced. Thus, under these conditions, HP-F1 appears to mimic the ligand of an inhibitory receptor, resulting in blocking of CD16 stimulation.

In PBMCs, the HP-F1 antigen was detected not only on a fraction of NK cells,  $\alpha/\beta$  and  $\gamma/\delta$  T cells (as previously observed for KIRs) but, strikingly, also on almost all CD19<sup>+</sup> B cells and CD14<sup>+</sup> monocytes (Fig. 2). In addi-



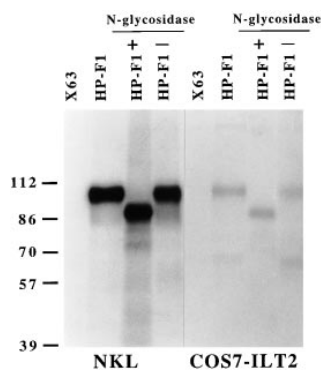
**Figure 1.** (a) The HP-F1 mAb reconstitutes NK cell-mediated lysis of HLA class I transfectants. Lysis of the class I-negative mutant cell line 721.221 by NKL is inhibited upon transfection of HLA-B\*2702, -B\*2705, -B\*5101, -A\*0301, -B\*0702, -Cw\*0301, and -G1 alleles in 721.221 (white bars), regardless of the presence of a control antibody (C218, anti-CD56, IgG1). F(ab')<sub>2</sub> fragments of the HP-F1 mAb (black bars) completely reconstitute lysis of HLA-B\*2702, -B\*2705, and -B\*5101 transfectants. F(ab')<sub>2</sub>

fragments of the anti-CD94 HP-3B1 mAb (gray bars) reconstitute lysis of the HLA-Cw\*0301 transfectant almost completely. A combination of both HP-F1 and HP-3B1 mAbs (hatched bars) is necessary to reconstitute lysis of HLA-A\*0301, -B\*0702, and -G1 transfectants. The anti-class I HP-1F7 mAb (vertical bars) reconstitutes lysis of all of the class I transfectants. Cytotoxicity was determined in a standard 4 h <sup>51</sup>Cr-release assay. NKL expresses the CD94-NKG2A heterodimer but no KIRs, as determined by cell surface staining with Z199 (anti-CD94-NKG2A heterodimer), HP-3E4, GL183, EB6 (anti-p58 KIRs), DX9, 5.133 (anti-p70 and -p70/140 KIRs) mAbs, and by RT-PCR. (b) HP-F1 mAb inhibits CD16-dependent redirected lysis of P815 cells by NKL. <sup>51</sup>Cr-labeled P815 cells were preincubated for 15 min with anti-CD16 alone, with HP-F1 (5–10  $\mu$ g/ml), or with HP-3B1 (anti-CD94, 5  $\mu$ g/ml). In control samples, cells were incubated with medium, with anti-CD56 mAb (IgG1), or with HP-F1 (5–10  $\mu$ g/ml). After incubation, NKL cells were added at different effector/target ratios. HP-F1 significantly reduced CD16-mediated lysis of P815 cells, mimicking the ligand of an inhibitory receptor. HP-F1 mAb also reduced the low basal lysis of P815 by NKL in the absence of anti-CD16. Similar results were obtained with the anti-CD94 mAb.



**Figure 2.** The HP-F1 antigen is expressed on CD56<sup>+</sup> NK cells (23–77% in six different donors),  $\alpha/\beta$  T cells (3–28%),  $\gamma/\delta$  T cells (16–50%), CD19<sup>+</sup> B cells, CD14<sup>+</sup> monocytes, HLA-DR<sup>high</sup> DCs derived from CD34<sup>+</sup> precursors, and CD1a<sup>+</sup> DCs derived from monocytes under appropriate culture conditions. Macrophages derived in vitro from purified monocytes also expressed the HP-F1 antigen, whereas neutrophils were HP-F1<sup>-</sup> (data not shown).

tion, the HP-F1 mAb stained HLA-DR<sup>high</sup> DCs derived from CD34<sup>+</sup> hematopoietic precursors, as well as DCs and macrophages derived from purified monocytes under appropriate culture conditions (Fig. 2 and data not shown). In immunoprecipitation experiments from different cell types, the HP-F1 mAb detected a prominent band of ~110 kD under both nonreducing (not shown) and reducing conditions, and of ~90 kD after *N*-deglycosylation of the immunoprecipitates (Fig. 3, left). Its cellular distribution along with biochemical analyses indicated that the HP-F1 antigen differs from previously identified KIRs but is similar to a recently cloned candidate inhibitory receptor, termed ILT2 (13). The predicted ILT2 glycoprotein is characterized by an extracellular region of four Ig-SF domains and a cytoplasmic tail containing four tyrosine-based motifs similar to



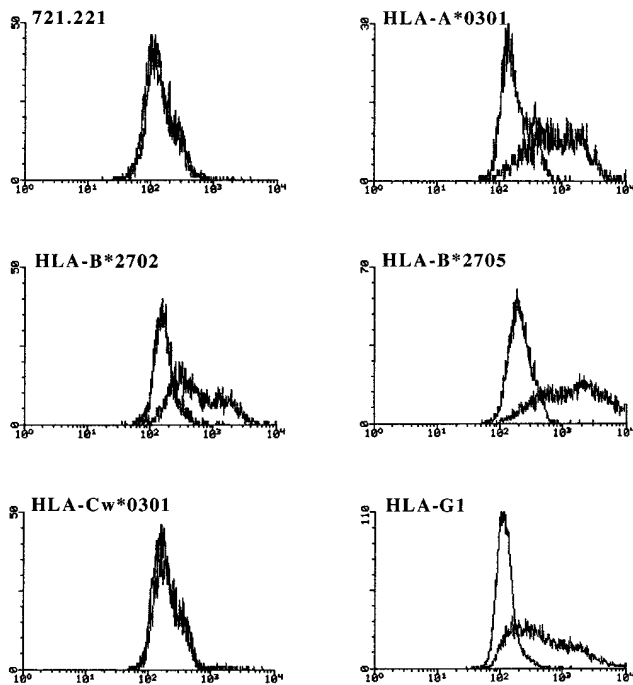
**Figure 3.** The HP-F1 $\gamma$  receptor is an ~110-kD monomeric glycoprotein. (Left) Immunoprecipitation from <sup>125</sup>I-labeled NKL cells with HP-F1 mAb yields a protein which runs as a 110-kD band in SDS-PAGE under reducing conditions. After treatment with *N*-glycosidase, the molecular mass is reduced to ~90 kD. (Right) Identical electrophoretic patterns were obtained by SDS-PAGE analysis of immunoprecipitates from ILT2-transfected COS7 cells. No bands were detected using the culture supernatant from X63 murine myeloma in control experiments.

the ITIMs of KIRs that recruit SHP-1 phosphatase upon phosphorylation (29–33). Specific staining (not shown) and immunoprecipitation (Fig. 3, right) of ILT2-transfected COS cells with the HP-F1 mAb demonstrated that the HP-F1 antigen does indeed correspond to the ILT2-encoded glycoprotein.

**ILT2 Binds MHC Class I Molecules, Delivers a Negative Signal that Is Sufficient to Inhibit Cellular Activation, and Recruits SHP-1 Phosphatase.** To provide direct evidence that ILT2 is a receptor for HLA-class I molecules, we analyzed binding of a soluble ILT2-IgG1 fusion protein to class I transfectants in 721.221 by flow cytometry. HLA-B\*2702, -B\*2705, -A\*0301, -G1, and -Cw\*0301 transfectants and 721.221 cells were incubated with ILT2-IgG1 and stained with anti-human IgG antibody. As shown in Fig. 4, ILT2 bound HLA-A, -B, and -G1 transfectants but not HLA-Cw3 transfectants or 721.221 cells. These data provide formal demonstration that ILT2 is a receptor for HLA-class I molecules, apparently with a broad specificity.

To further demonstrate that ILT2 delivers a negative signal and is sufficient to inhibit cell activation triggered via a stimulatory receptor, ILT2 cDNA was stably transfected in the RBL cell line, which constitutively expresses the FcR for IgE (FcεRI; reference 34). As shown in Fig. 5, ILT2-transfected RBL cells released serotonin when triggered through the FcεRI with mouse IgE bound to a plastic surface. Conversely, serotonin secretion was clearly inhibited by cocross-linking of FcεRI with ILT2 using mouse IgE and the HP-F1 antibody [or its F(ab')<sub>2</sub> fragments] coated on plastic. These results indicate that recruitment of ILT2 to a stimulatory receptor by antibody ligation can inhibit cellular activation.

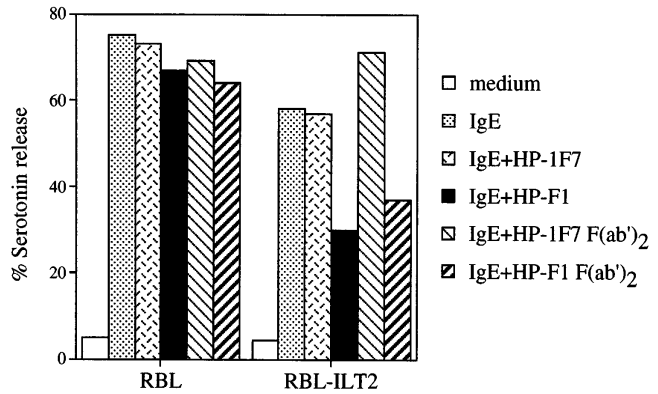
It has been shown that negative signaling through KIRs is mediated by recruitment of SHP phosphatases upon tyrosine phosphorylation of cytoplasmic ITIMs (29–33), whereas it does not involve association of SHIP phosphatase (35). The cytoplasmic tail of ILT2 contains four tyrosine-based motifs similar to those found in KIR, although only one of them fits the V/I-x-Y-x-x-L motif that has been proposed to be required for SHP-1 binding (36). To determine whether ILT2 recruits SHP phosphatases, ILT2 was immunoprecipitated from NKL cells and from the B cell line C1R either



**Figure 4.** ILT2 soluble protein binds HLA-A, -B, and -G1 transfectants. HLA-B\*2702, -B\*2705, -A\*0301, -G1, and -Cw\*0301 transfectants in 721.221 and untransfected cells were incubated with a soluble ILT2-IgG1 fusion protein, followed by a PE-labeled goat anti-human IgG antibody. Binding was assessed by FACS<sup>®</sup> analysis. HLA class I expression of the transfectants was determined in the same experiment by FACS<sup>®</sup> analysis using the w6/32 mAb (IgG2a; American Type Culture Collection). MFI were as follows: 721.221, 121; HLA-B\*2702, 2128; -B\*2705, 3045; -A\*0301, 3854; -G1, 1084; -Cw\*0301, 1582. The binding pattern did not correlate with the level of class I expression on the transfectants.

unstimulated or stimulated with pervanadate, which has been shown to induce substantial tyrosine phosphorylation of cellular substrates (37). Immunoprecipitates were immunoblotted with anti-SHP-1, anti-SHP-2 and anti-SHIP antibodies. As shown in Fig. 6, ILT2 was associated with SHP-1 after treatment with pervanadate, whereas no association was detected with SHP-2 and SHIP (data not shown).

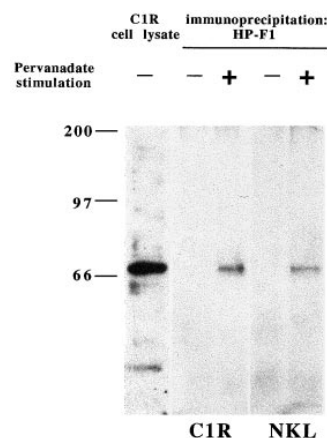
**ILT2 Inhibits Superantigen-dependent T Cell-mediated Cytotoxicity.** We next tested whether ILT2 mediates functional inhibition in cell types other than NK cells. CD8<sup>+</sup> T cells kill MHC class II<sup>+</sup> APCs pulsed with bacterial superantigens, which bind the variable region of the TCR- $\beta$  chain ( $V\beta$ ) on T cells. We investigated whether this superantigen-dependent cell-mediated cytotoxicity is inhibited by interaction between ILT2 on T cells and class I on APCs. HP-F1<sup>+</sup> T cells were sorted and cloned; T cell clones were then selected for expression of  $V\beta$ 2, which binds the toxic shock syndrome toxin-1 (TSST-1) superantigen, and for lack of KIR expression. T cell-mediated cytotoxicity was tested against either 721.221 cells or an HLA-B\*2705 transfectant of 721.221 pulsed with different concentrations of TSST-1. Both target cells expressed equivalent levels of MHC class II molecules. Results from a representative clone are shown in Fig. 7. The OKT8-24 T cell



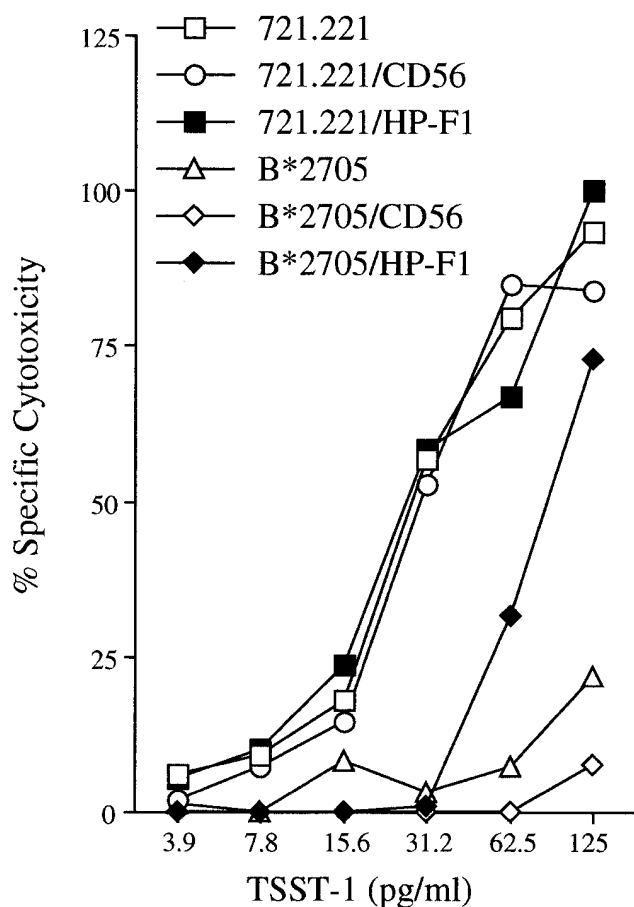
**Figure 5.** Inhibition of IgE-induced serotonin release in ILT2-transfected RBL cells. Transfected and control cells were stimulated with purified mouse IgE (20  $\mu$ g/ml) alone and in combination with either HP-F1 [20  $\mu$ g/ml of whole antibody or F(ab)<sub>2</sub> fragments] or with the isotype-matched antibody HP-1F7 [20  $\mu$ g/ml of whole antibody or F(ab)<sub>2</sub> fragments] immobilized on plastic. The percentage of serotonin release, as compared to total and spontaneous release, was determined after 1 h at 37°C. The expression of ILT2 on transfected RBL cells was assessed by indirect immunofluorescence with HP-F1 mAb (not shown).

clone efficiently killed TSST-1-coated 721.221, whereas cytolytic activity was significantly reduced against TSST-1-pulsed HLA-B\*2705 transfectants. Cytolysis of TSST-1-coated HLA-B\*2705 transfectants was partially reconstituted in the presence of F(ab)<sub>2</sub> fragments of the HP-F1 mAb. These results indicate that ILT2-class I interaction, like KIR-class I interaction, inhibits superantigen-dependent T cell-mediated cytotoxicity and may explain previous reports of KIR<sup>-</sup> T cells inhibited by HLA-B27 molecules (38).

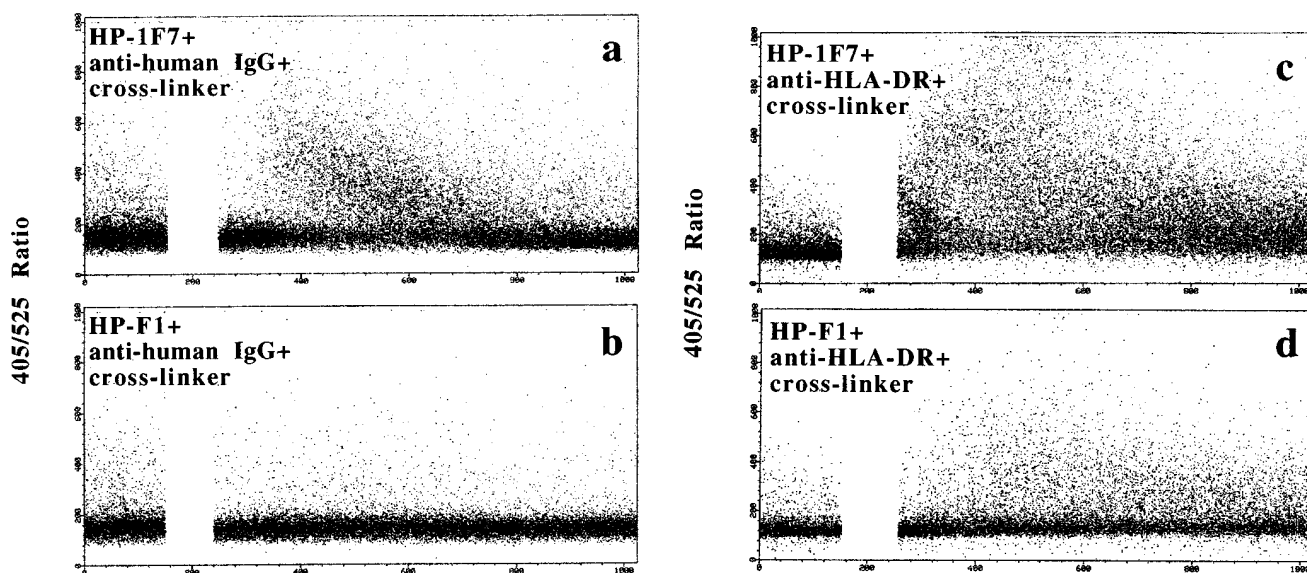
It is noteworthy that although all of the tested HP-F1<sup>+</sup>/p70 KIR<sup>-</sup> T cell and NK cell clones derived from different donors were inhibited by HLA-B\*2705, the inhibitory function of ILT2 was not always confirmed by reconstitution of cytotoxicity with the HP-F1 mAb. In addition, the HP-F1 mAb did not inhibit many NK cell clones in rADCC (data not shown). It is possible that ILT2 is characterized by structural heterogeneity and that the HP-F1 mAb efficiently recognizes only some ILT2 isoforms. Al-



**Figure 6.** ILT2 is associated with SHP-1. NKL cells and the EBV-transformed B cell line C1R were incubated for 10 min at 37°C either with medium alone or with pervanadate. ILT2 was then immunoprecipitated with the HP-F1 mAb and immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-SHP-1 antibody. Whole cell lysate is included as a positive control. SHP-1 was recruited to ILT2 after cell stimulation with pervanadate.



**Figure 7.** ILT2-class I interaction inhibits TSST-1-mediated T cell cytotoxicity. HP-F1<sup>+</sup> T cell clones were generated from the peripheral blood of a healthy donor and selected for expression of TCR-Vβ2 and for lack of KIR expression. T cell clones were then tested in a 4-h <sup>51</sup>Cr-release assay for cytotoxicity against the class I-negative B lymphoblastoid



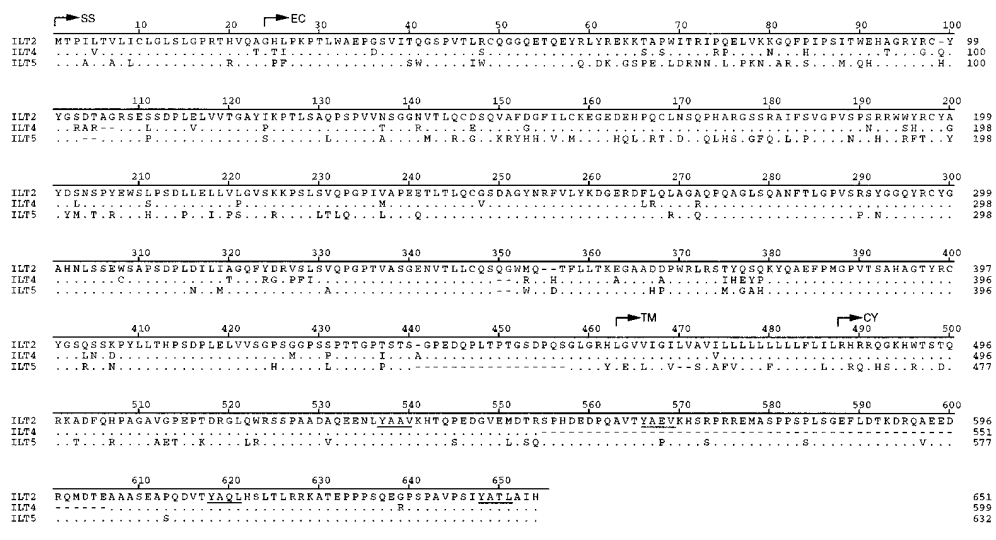
**Figure 8.** Intracellular Ca<sup>2+</sup> mobilization induced by anti-human IgG antibodies in the EBV-B cell line C1R (a) is inhibited upon crosslinking with ILT2 (b). Similarly, the increased [Ca<sup>2+</sup>]<sub>i</sub> triggered through HLA-DR by the 3.8 B1 mAb in monocytes (d) and dendritic cells (not shown) is downregulated, although to a lesser extent, when ILT2 is coligated with HLA-DR (d). HP-1F7 is an isotype-matched control antibody.

ternatively, ILT2 expression may be modulated after activation of NK and T cells or by in vitro culture conditions.

**ILT2 Inhibits Ca<sup>2+</sup> Mobilization Triggered via the B Cell Antigen Receptor and HLA-DR in B And Myelomonocytic Cells.** To explore the regulatory role of ILT2 in B cell activation, we tested whether ILT2 can inhibit Ca<sup>2+</sup> mobilization triggered via the B cell antigen receptor. Cocross-linking of ILT2 with surface IgG induced a complete extinction of the increased [Ca<sup>2+</sup>]<sub>i</sub> triggered through surface IgG in the EBV-transformed B cell line C1R (Fig. 8, a and b), while only a slight reduction was detected in peripheral B cells (data not shown). Since C1R has a very low level of class I expression, the HP-F1 mAb might be able to compete more effectively with self-class I molecules for binding with ILT2 and to recruit it to the B cell receptor in cross-linking experiments. Similarly, we obtained a moderate downregulation of Ca<sup>2+</sup> mobilization triggered through HLA-DR in monocytes, macrophages, and DCs (Fig. 8, c and d, and data not shown). Together, these results demonstrate that ILT2 can modulate signaling in B cells and myelomonocytic cells. However, the physiological functions regulated by class I-ILT2 interactions in these cell types have yet to be defined.

**Myelomonocytic Cells Express ILT2 Homologs (ILT4 and ILT5) that Are Significantly Diverse.** To investigate whether HP-F1 recognizes structurally distinct isoforms of ILT2, we amplified, cloned, and sequenced ILT2 cDNA from NK, T, B, and myeloid cells derived from one individual. By

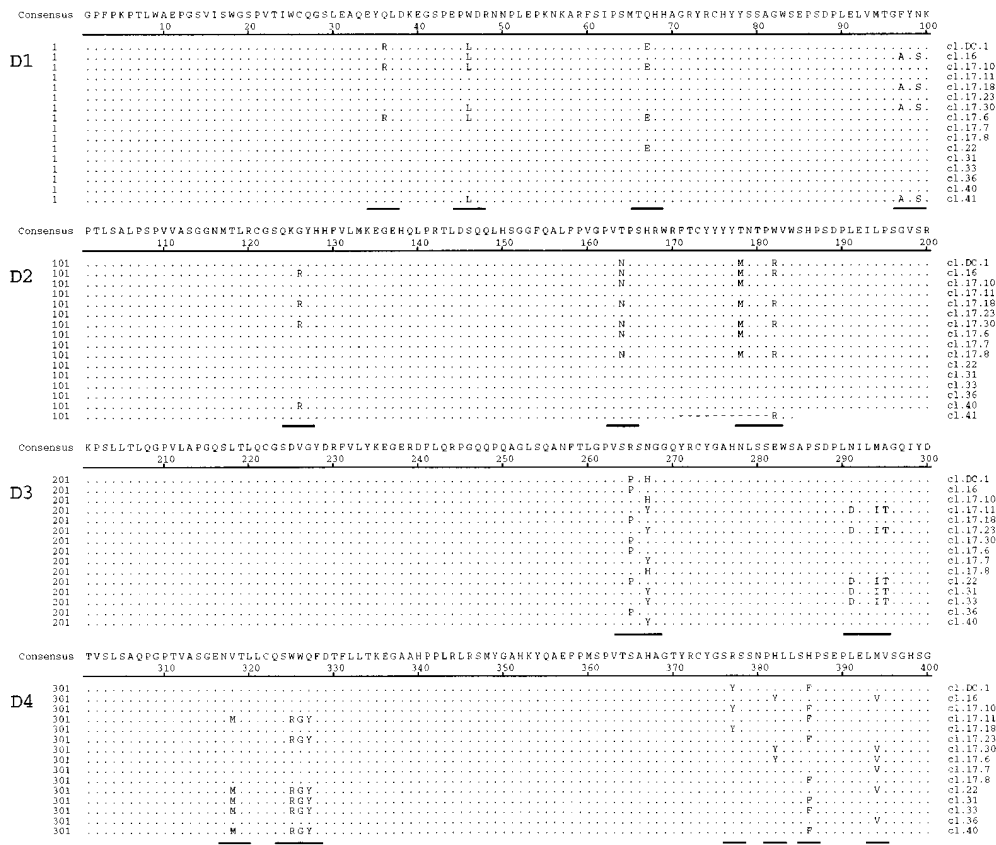
cell line 721.221 cells or 721.221 cells stably transfected with HLA-B\*2705, in the presence of serial dilutions of TSST-1 at an effector/target ratio of 20:1. The T cell clone OKT8-24 killed 721.221 in the presence of TSST-1, but did not kill B\*2705-transfected cells. F(ab')<sub>2</sub> fragments of the HP-F1 mAb partially restored the lysis, while F(ab')<sub>2</sub> fragments of the isotype-matched anti-CD56 mAb had no effect.



**Figure 9.** Alignment of ILTs with four Ig-SF extracellular domains. The alignment was generated by the Clustal method using Lasergene analysis software. (DNA-STAR, Inc., Madison, WI). Amino acid sequences were aligned with ILT2. Amino acid variants are indicated. Gaps (*dashes*) were introduced to maximize homologies. Amino acids are numbered on the right side. SS, signal sequence; EC, extracellular domain; TM, transmembrane domain; CY, cytoplasmic domain.

this approach we identified one ILT2 variant with six amino acid differences (most likely an allelic form) and two alternatively spliced forms, one of which encoded a molecule with a truncated cytoplasmic tail lacking ITIMs. (ILT2a, ILT2b, and ILT2c are available EMBL/GenBank/ DDBJ under accession numbers AF009005, AF009006, and AF009007, respectively.) These variants were not selectively expressed in different cell types. Importantly, cDNA amplification from myelomonocytic cells yielded

two cDNA clones that were more diverse, called ILT4 and ILT5, also encoding glycoproteins characterized by four extracellular Ig-SF domains and ITIM-containing cytoplasmic tails (Fig. 9). These glycoproteins are homologous to ILT2 and map to human chromosome 19, but are not recognized by the HP-F1 mAb and are selectively expressed on myelomonocytic cells as assessed by RT-PCR (data not shown). These results suggest that ILT2 may be the prototype and most broadly expressed member of a family of



**Figure 10.** Diversity of the extracellular region of ILT5. 15 variants of ILT5 were identified from a pool of bone marrow cells derived from 51 donors, while only 1 variant (clone DC.1) was detected from a single donor. Amino acid variants are clustered in variable regions (*underlined*). ILT5-cl41 is prematurely truncated as a consequence of an alternative splicing which generates a stop codon. D1–D4, extracellular domains.

structurally and functionally similar receptors with different MHC specificities and tissue distribution.

Analysis of structural variability of ILT2, ILT4, and ILT5 in the population revealed that ILT5, as compared to ILT2 and ILT4, is characterized by a striking diversity. Amplification and sequencing of ILT5 cDNA from a pool of cells derived from different individuals yielded 15 distinct cDNAs, whereas only 1 ILT5 cDNA clone was amplified from a single donor. This result suggests that ILT5 may be extensively polymorphic, with amino acid variants clustered in several distinct regions of the extracellular domains (Fig. 10). Genomic studies will be required to establish whether all of the cloned ILT5 variants are encoded by different alleles of the same locus, or by different loci. We also identified 6 alternatively spliced forms of ILT4 and ILT5 cDNAs, some of which encode glycoproteins with a short cytoplasmic tail that lacks ITIMs, and one cDNA, termed ILT6, which lacks transmembrane and cytoplasmic regions, and may encode a soluble receptor. (ILT4, ILT5, and ILT6 cDNAs are available from EMBL/GenBank/DDBJ under the following accession numbers: ILT4-cl1, AF000574; ILT4-cl17, AF011566; ILT4-cl26, AF011565; ILT5-cl16, AF000575; ILT5-cl17.6, AF009634; ILT5-cl17.7, AF009635; ILT5-cl17.8, AF009636; ILT5-cl17.10, AF009632; ILT5-cl17.11, AF009633; ILT5-cl19, AF009637; ILT5-cl22, AF009638; ILT5-cl31, AF009639; ILT5-cl33, AF009640; ILT5-cl36, AF009641; ILT5-cl40, AF009642; ILT5-cl41, AF009644; ILT5-cl6, AF009643; ILT5-cl17.18, AF031554; ILT5-cl17.23, AF031555; ILT5-

cl17.30, AF031556; ILT5-clDC.1, AF031553; and ILT6, AF014923.

**Concluding Remarks.** Our results demonstrate that inhibitory MHC class I receptors are not restricted to NK cells and T cell subsets, but at least one such receptor is found in all cell types involved in immune responses. Although the role of inhibitory receptors for MHC class I molecules in the regulation of NK and T cell cytotoxicity is well established, their function in B and myelomonocytic cells is intriguing. In B cells, ILT2-class I interactions might regulate the B cell triggering threshold, ensuring that B cells are activated only upon encountering the specific antigen. Interestingly, ILT2 is present in almost all peripheral B cells, but is weakly expressed or undetectable in tonsillar B cells (data not shown), suggesting that ILT2 may be expressed by B cells only before antigenic stimulation, whereas it may be downregulated in activated B cells. In myelomonocytic cells, ILT2 may modulate the inflammatory response to microbial challenges, thereby regulating cytokine production and preventing damage of normal tissue. In addition, ILT2 may be involved in the control of cytotoxicity by monocyte-macrophages, enabling them to recognize tumor cells that have lost expression of class I molecules (39–41). Finally, since inhibitory receptors define a threshold that must be overcome by stimulatory signals to trigger cell activation, an alteration of this regulatory mechanism may contribute to the pathophysiology of chronic inflammatory autoimmune disorders.

---

We thank Roberto Biassoni, Daniel Geraghty, Charles T. Lutz, and Peter Parham for the kind gift of HLA-class I transfectants; Mark Coggeshall, Lorenzo and Alessandro Moretta, and Lewis L. Lanier for mAbs; Kerry S. Campbell for helpful discussions; Mark Dessing and Annette Pickert for expert technical advice in FACS<sup>®</sup> and Ca<sup>2+</sup> flux analyses; and Siamak Bahram, Kerry S. Campbell, Susan Gilfillan, Antonio Lanzavecchia, Ton Rolink, and Raul Torres (Basel Institute for Immunology) for reviewing the manuscript.

The Basel Institute for Immunology was founded and is supported by Hoffmann-La Roche Ltd., Basel, Switzerland. The work at Hospital de la Princesa is supported by grants SAF96/0335 (Plan Nacional I+D) and PL950062 (European Commission). M. Llano is the recipient of a fellowship from the Fundacion Rich.

Address correspondence to Marco Colonna, Basel Institute for Immunology, 487 Grenzacherstrasse, CH-4005 Basel, Switzerland. Phone: 41-61-605-1393; FAX: 41-61-605-1364; E-mail: [colonna@bii.ch](mailto:colonna@bii.ch) or Miguel López-Botet, Servicio de Inmunología, Hospital de la Princesa, Diego de León 62, 28006 Madrid, Spain. Phone: 34-1-520-2370; FAX: 34-1-309-2496; E-mail: [mlbotet/princesa@hup.es](mailto:mlbotet/princesa@hup.es)

*Received for publication 14 July 1997 and in revised form 19 August 1997.*

## References

1. Ljunggren, H.G., and K. Karre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today*. 11:237–244.
2. Yokoyama, W.M., and W.E. Seaman. 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annu. Rev. Immunol.* 11:613–635.
3. Lanier, L.L., and J.H. Phillips. 1996. Inhibitory MHC class I receptor on NK cells and T cells. *Immunol. Today*. 17:86–91.
4. Raulet, D.H., and W. Held. 1995. Natural killer cell receptors: the offs and ons of NK cell recognition. *Cell*. 82:697–700.
5. Moretta, A., C. Bottino, M. Vitale, D. Pende, R. Biassoni, M.C. Mingari, and L. Moretta. 1996. Receptors for HLA class-I molecules in human natural killer cells. *Annu. Rev. Immunol.* 14:619–648.
6. Lanier, L.L. 1997. Natural killer cells: from no receptors to too many. *Immunity*. 6:371–378.
7. Phillips, J.H., C. Chang, J. Mattson, J.E. Gumperz, P. Parham, and L.L. Lanier. 1996. CD94 and a novel associated



- protein (94AP) form a NK cell receptor involved in the recognition of HLA-A, HLA-B, and HLA-C allotypes. *Immunity*. 5:163–172.
8. Lazetic, S., C. Chang, J.P. Houchins, L.L. Lanier, and J.H. Phillips. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J. Immunol.* 157:4741–4745.
  9. Carretero, M., C. Cantoni, T. Bellón, C. Bottino, R. Biassoni, A. Rodriguez, J.J. Perez-Villar, L. Moretta, A. Moretta and M. López-Botet. 1997. The CD94 and NKG2-A C type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. *Eur. J. Immunol.* 27: 563–567.
  10. Perez-Villar, J.J., I. Melero, F. Navarro, M. Carretero, T. Bellón, M. Llano, M. Colonna, D.E. Geraghty, and M. López-Botet. 1997. The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1. *J. Immunol.* 158:5736–5743.
  11. López-Botet, M., J.J. Perez-Villar, M. Carretero, A. Rodriguez, I. Melero, T. Bellón, M. Llano, F. Navarro. 1997. Structure and function of the CD94 C-type lectin receptor complex involved in recognition of HLA class I molecules. *Immunol. Rev.* 155:165–174.
  12. Söderström, K., B. Corliss, L.L. Lanier, and J.H. Phillips. CD94/NKG2 is the predominant inhibitory receptor involved in recognition of HLA-G by decidual and peripheral blood NK cells. *J. Immunol.* 159:1072–1075.
  13. Samaridis, J., and M. Colonna. 1996. 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.
  14. Cella, M., C. Dohring, J. Samaridis, M. Dessing, M. Brockhaus, A. Lanzavecchia, and M. Colonna. 1997. A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing. *J. Exp. Med.* 185:1743–1751.
  15. Thomas, M.L. 1995. Of ITAMs and ITIMs: turning on and off the B cell antigen receptor. *J. Exp. Med.* 181:1953–1956.
  16. Zemmour, J., A.M. Little, D.J. Schendel, and P. Parham. 1992. The HLA-A,B “negative” mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. *J. Immunol.* 148: 1941–1948.
  17. Shimizu, Y., D.E. Geraghty, B.H. Koller, H.T. Orr, and R. DeMars. 1988. Transfer and expression of three cloned human non-HLA-A,B,C class I major histocompatibility complex genes in mutant lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA.* 85:227–231.
  18. Robertson, M.J., K.J. Cochran, C. Cameron, J.M. Le, R. Tantravahi, and J. Ritz. 1996. Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. *Exp. Hematol.* 24:406–415.
  19. Cella, M., A. Longo, G.B. Ferrara, J.L. Strominger, and M. Colonna. 1994. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J. Exp. Med.* 180:1235–1242.
  20. Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Banchereau. 1992. GM-CSF and TNF- $\alpha$  cooperate in the generation of dendritic Langherans cells. *Nature (Lond.)*. 360: 258–261.
  21. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor  $\alpha$ . *J. Exp. Med.* 179:1109–1118.
  22. Bender, A., M. Sapp, G. Schuler, R.M. Steinman, and N. Bhardwaj. 1996. Improved methods for the generation of dendritic cells from non proliferating progenitors in human blood. *J. Immunol. Methods.* 196:121–135.
  23. Romani, N., D. Reider, M. Heuer, S. Ebner, E. Kampgen, B. Eibl, D. Niederwieser, and G. Schuler. 1996. Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J. Immunol. Methods.* 196:137–151.
  24. Traunecker, A., F. Oliveri, and K. Karjalainen. 1991. Myeloma based expression system for production of large mammalian proteins. *Trends Biotechnol.* 9:109–113.
  25. Döhning, C., and M. Colonna. 1996. Human natural killer cell inhibitory receptors bind to HLA class I molecules. *Eur. J. Immunol.* 26:365–369.
  26. Valitutti, S., M. Dessing, and A. Lanzavecchia. 1993. Role of cAMP in regulating cytotoxic T lymphocyte adhesion and motility. *Eur. J. Immunol.* 23:790–795.
  27. Colonna, M., and J. Samaridis. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science (Wash. DC)*. 268:405–408.
  28. Perez-Villar, J.J., I. Melero, A. Rodriguez, M. Carretero, J. Aramburu, S. Sivori, A.M. Orenge, A. Moretta, and M. López-Botet. 1995. Functional ambivalence of the Kp43 (CD94) NK cell-associated surface antigen. *J. Immunol.* 154:5779–5788.
  29. 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.
  30. Olcese, L., P. Lang, F. Vely, A. Cambiaggi, D. Marguet, M. Blery, K.L. Hippen, R. Biassoni, A. Moretta, L. Moretta, J.C. Cambier, and E. Vivier. 1996. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J. Immunol.* 156:4531–4534.
  31. Campbell, K.S., M. Dessing, M. López-Botet, M. Cella, and M. Colonna. 1996. Tyrosine phosphorylation of a human killer inhibitory receptor recruits protein tyrosine phosphatase 1C. *J. Exp. Med.* 184:93–100.
  32. 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.
  33. Binstadt, B.A., K.M. Brumbaugh, C.J. Dick, A.M. Scharenberg, B.L. Williams, M. Colonna, L.L. Lanier, J.-P. Kinet, R.T. Abraham, and P.J. Leibson. 1996. Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation. *Immunity*. 5:629–638.
  34. Daeron, M., S. Latour, O. Malbec, E. Espinosa, P. Pina, S. Pasmans, and W.H. Fridman. 1995. The same tyrosine-based inhibition motif, in the intracytoplasmic domain of Fc gamma RIIb, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. *Immunity*. 3:635–646.
  35. Ono, M., H. Okada, S. Bolland, S. Yanagi, T. Kurosaki, and J.V. Ravetch. 1997. Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell*. 90:293–301.
  36. Burshtyn, D.N., W. Yang, T. Yi, and E.O. Long. 1997. A novel phosphotyrosine motif with a critical amino acid at position -2 for the SH2 domain-mediated activation of the ty-

- rosine phosphatase SHP-1. *J. Biol. Chem.* 272:13066–13072.
37. O'Shea, J.J., D.W. McVicar, T.L. Bailey, C. Burns, and M.J. Smyth. 1992. Activation of human peripheral blood T lymphocytes by pharmacological induction of protein-tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA.* 89:10306–10310.
38. Phillips, J.H., J.E. Gumperz, P. Parham, and L.L. Lanier. 1995. Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. *Science (Wash. DC)*. 268:403–405.
39. Philip, R., and L.B. Epstein. 1986. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself,  $\gamma$ -interferon and interleukin-1. *Nature (Lond.)*. 323:86–89.
40. Ortaldo, J.R., L.H. Mason, B.J. Mathieson, S.M. Liang, D.A. Flick, and R.B. Herberman. 1986. Mediation of mouse natural cytotoxic activity by tumour necrosis factor. *Nature (Lond.)*. 321:700–702.
41. Feinman, R., D. Henriksen-DeStefano, M. Tsujimoto, and J. Vilcek. 1987. Tumour necrosis factor is an important mediator of tumor cell killing by human monocytes. *J. Immunol.* 138: 635–640.