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HLA Class I Molecules Regulate IFN-γ Production Induced in NK Cells by Target Cells, Viral Products, or Immature Dendritic Cells through the Inhibitory Receptor ILT2/CD85j

Esther Morel and Teresa Bellón

Recent advances support an important role for NK cells in determining immune responses beyond their cytolytic functions, which is supported by their capacity to secrete several cytokines and chemokines. In particular, NK-derived IFN-γ has proven to be fundamental in shaping adaptive immune responses. Although the role of inhibitory NK receptors (iNKR) in the regulation of cytotoxicity has been widely explored, their involvement in the control of cytokine production has been scarcely analyzed. Specifically, no data are available referring to the role of the iNKR ILT2/CD85j in the regulation of IFN-γ secretion by NK cells. Published data support a differential regulation of cytotoxicity and cytokine expression. Thus, formal proof of the involvement of HLA class I in regulating the production of cytokines through binding to ILT2/CD85j has been missing. We have determined the response of human NK-92 and primary human ILT2/CD85j+ NK cells from healthy donors to target cells expressing or not HLA class I. We found specificities of HLA class I-mediated inhibition of IFN-γ mRNA expression, protein production, and secretion consistent with the specific recognition by ILT2/CD85j. We also found inhibition of IFN-γ production by ILT2/CD85j+ T cells in response to superantigen stimulation. Furthermore, ligation of ILT2/CD85j inhibited the production of IFN-γ in response to poly(I:C), and blocking of ILT2/CD85j-HLA class I interactions increased the secretion of IFN-γ in NK/immature dendritic cell cocultures. The data support a role for self HLA class I in the regulation of IFN-γ secretion at the mRNA and protein levels by interacting with the iNKR ILT2/CD85j. *The Journal of Immunology*, 2008, 181: 2368–2381.

Natural killer cells provide an essential first line of defense against virus-infected and transformed cells. These cells identify their targets through a set of activating and inhibitory receptors, and cell activation is the result of the dynamic balance between activating and inhibitory signals. NK cells lyse target cells by two basic mechanisms: Ab-dependent cell cytotoxicity (ADCC) and natural cytotoxicity, for which a variety of receptors have been identified. Some of these receptors (NKp30, NKp46, and NKp44) are NK specific and are referred to as natural cytotoxicity receptors. A series of additional activating receptors expressed on NK cells and other cell types have been described (1). Ligands for most activating receptors are thought to have broad distribution in different cell lineages. In general, NK cells “sense” the health state of cells through the recognition of several MHC products. In particular, NK cells have the ability to monitor the integrity of MHC expression on target cells through different NKR specific to classical and nonclassical MHC class I molecules (2). Specific recognition of class I allotypes by inhibitory NKR (iNKR) represses NK cell-mediated cytotoxicity triggered by activating receptors and prevents autoreactivity against normal cells. Although NKR were first described in NK cells, they are also expressed by subsets of CTLs (3). The existence of multiple receptor families and multiple receptors within a family accounts for the diversity within an individual’s NKR repertoire.

In humans, NKR fall into two structurally distinct groups. The first group consists of type I transmembrane proteins with Ig domains; these include killer cell Ig-like receptors (KIRs) and Ig-like transcripts (ILTs), also called leukocyte Ig-like receptors (LIRs/LILRs) or CD85 Ags. The second group includes type II transmembrane proteins containing C-type lectin-like domains and comprises CD94/NKG2 heterodimers, which belong to the killer lectin-like receptor family (2). Inhibitory and activating receptors with similar structural features have been described within these three protein families. Although the function of HLA class I-specific activating receptors is still being debated, it is well established that inhibitory receptors regulate NK cell cytotoxic activity (4) and modulate the threshold of activation of T cells (3, 5). The molecular basis for the delivery of the inhibitory signal is common to all of them: tyrosine residues within immunoreceptor tyrosine-based inhibition motifs in their cytoplasmic regions become phosphorylated under HLA ligation and recruit SHP-1 phosphatase, which is responsible for the abrogation or attenuation of activating signals (6). CD94/NKG2A heterodimers constitute inhibitory receptors that specifically recognize the nonclassical HLA class I molecule HLA-E, whose expression is dependent on the availability of HLA class I H chain leader peptides. In this way, CD94/NKG2A senses the overall class I level of a putative target cell by the expression of HLA-E. The Ig-like receptors are encoded by genes clustered in a region known as leukocyte receptor complex in chromosome 19 (7). Among them, KIRs exhibit fine specificities for HLA class I...
allootypes and can distinguish between HLA-A, -B, and -C molecules. In contrast, those ILT/LIR/LIR/CD85 family members whose ligands are known (ILT2/LIR-1/LIRLB1/CD85j and ILT4/LIR-2/LIRLB2/CD85d) are broadly reactive toward HLA-A, -B, and -C allootypes, and nonclassical HLA-E, -G, and -F molecules, and similarly to CD94/NKG2A, allowing NK cells to survey for overall class I expression. Specifically, the ILT2/LIR-1/LIRLB1/CD85j inhibitory receptor (which will be referred to as ILT2/CD85j in the rest of this article) has been found to interact with several classical and nonclassical class I molecules (8–10) and with UL18, a human CMV protein homologous to HLA class I, through its N-terminal Ig domain (11). Surface plasmon resonance studies suggest that ILT2/CD85j binds with a higher affinity to HLA-G than to classical MHC class I (12) and with a very high affinity to UL18 (10). Upon ligand binding, ILT2/CD85j is able to inhibit cytolysis in NK cells (8), and published data support a role for ILT2/CD85j in modulation of CD3/TCR-mediated activation of CD4+ and CD8+ T lymphocytes (8, 13–17). In contrast to CD94/NKG2 heterodimers and KIRs, expression of ILTs is not restricted to NK and T cells, and these genes have a broader distribution in different leukocyte lineages (8, 18). Inhibitory effects have also been shown in B lymphocytes (8) and in myeloid cells (19, 20).

NK cell responses are mediated by two major effector functions: direct cytolysis of target cells, and production of chemokines and cytokines such as IFN-γ, GM-CSF, and TNF-α (21). Studies in both murine and human systems have established the importance of IFN-γ in the polarization of immune responses. Through the secretion of IFN-γ, NK cells participate in the shaping of the adaptative immune response. Moreover, it has been shown that NK cell-derived IFN-γ has a key role in polarization toward Th1 immune responses in the lymph nodes (22). In contrast, it is known that the interaction of NK cells with dendritic cells (DCs) results in a bi-directional modulation of the activity of both cell types that involves, among other functions, IFN-γ production by NK cells (23, 24). Furthermore, it has been demonstrated in mice models that IFN-γ secreted by NK cells is critical for CTL generation against certain tumors (25, 26).

Although NK/ligand interactions have been widely studied from the point of view of their consequences in the cytokytic activity of NK cells and CTLs, the regulation of cytokine secretion by iNKRs is less well-documented. Although it is generally assumed that SHP-1-coupled iNKRs can regulate or even prevent cytokine secretion in T cells upon interaction with immature DC (iDC), as well as the ability of HLA class I ligands to inhibit IFN-γ secretion in T cells upon challenge by superantigens. We herein provide formal proof of the regulation of IFN-γ production by ILT2/CD85j at the mRNA and protein level in NK cells by interaction with its natural ligands in target cells. These data suggest that in addition to control the threshold of cell activation in T cells when stimulated to secrete IFN-γ through Ag recognition, the recognition of HLA class I through the ILT2/CD85j inhibitory receptor regulates production of IFN-γ by NK cells in various situations, including the interaction with iDCs and the recognition of viral products.

**Materials and Methods**

**Cells and Abs**

The NK-92 human cell line (40) was grown in MEM-α medium (plus ribonucleosides and deoxyribonucleosides) (Cambrex) with 10% heat-inactivated FCS (Cambrex), 1% sodium pyruvate (Cambrex), 1% nonessential amino acids (Cambrex) and 200 U/ml rh-IL-2 (PeproTech). The NK.3.3 human cell line (41) was grown in RPMI 1640 medium (Cambrex) supplemented with 10% (v/v) heat-inactivated FCS, 1% (v/v) Killer Cult (42), and 100 U/ml rh-IL-2. The HLA class I-defective B cell line 721.221, and the transfectedants 221.Cw07 and 221.B*2705, were a generous gift from Dr. Parham (Stanford University School of Medicine, Stanford, CA). The HLA-G transfectant 221.G1*0101 cell line (43) contains a mutation in the HLA-G1 signal peptide that does not support HLA-E expression on the cell surface and will be referred to as 221.G1 throughout the rest of this article. The 221.G1*0101 cell line (43) was a gift from Dr. Geraghty (University of Washington, Seattle, WA). These cell lines were grown in RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated FCS. 221.281 cell line (43) was grown in RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated FCS. Primary NK cells were purified from freshly isolated PBMCs by negative selection using a human NK cell isolation kit from Miltenyi Biotec, according to manufacturer’s instructions, and cultured overnight with 50–100 U/ml rh-IL-2 before being used. For generation of iDCs, CD14+ cells were purified from PBMCs using CD14 human Microbeads (Miltenyi Biotec) and cultured in the presence of rh-GM-CSF (500 U/ml) and rh-IL-4 (500 U/ml) (PeproTech). After 3 days of culture, fresh medium containing rh-GM-CSF and rh-IL-4 was added, and the incubation prolonged for 2 additional days to obtain cells characterized by the CD14+CD80+DCSIGN+ phenotype corresponding to iDCs. Flow cytometry analysis of both isolated subsets (NK cells and monocytes) showed that cell purity was at least 95%.

The mAbs used were: HP-3B1 (anti-CD94), HP-1F7 (anti-HLA class I), HP-F1 (anti-ILT2/CD85j), HP-3E4 (anti-KIR2DL1/DS1/DS4), and CD7, kindly provided by Dr. López-Botet (Universitat Pompeu Fabra, Barcelona, Spain); Z199 (anti-NKG2A), C218 (anti-CD56), and KD1 (anti-CD16), kindly provided by Dr. Moretta (University of Genoa, Genoa, Italy); DX9 (anti-KIR3DL1), kindly provided by Dr. L. Lanier (Cancer Research Institute, University of California, San Francisco, CA); 5.133 (anti-KIR3DL2), kindly provided by Dr. M. Colonna (Washington University School of Medicine, St. Louis, MO). Anti-IFN-γ-PE, anti-TNF-α-PE, and anti-Nkp30 were purchased from Miltenyi Biotec. CD56-FITC, CD85J-FITC (anti-ILT2-IFITC), CD69-PE, CD3-PerCP, and CH-L (anti-KIR2DL2/2DL3/2DS2) were purchased from BD Biosciences. FITC-conjugated polyclonal rabbit anti-mouse Fab(‘)2 was purchased from Dako, Cytomation and allophycocyanin-conjugated goat anti-mouse IgG (H+L) was purchased from Molecular Probes.

The study was approved by the Ethics Committee of Hospital La Paz and conducted according to Helsinki declaration principles.

**Flow cytometry**

Flow cytometry analyses were performed in a FACSCalibur cytometer using the CellQuestPro software (BD Biosciences). Surface immunofluorescence staining was conducted following standard procedures as described (8).

**Cytotoxicity assays**

Cytotoxicity mediated by ILT2/CD85j- and/or CD94/NKG2A-expressing NK cells (pretreated or not with poly(IC)) or the NK-92 cell line was
IFN-γ REGULATION BY HLA CLASS I-ILT2/CD85J INTERACTIONS

In a standard 4-h 51Cr-release assay (8). In brief, 721.221, 221.Cw07, 221.B*2705, 221.G1m, and 221.AEH cell lines or human allogeneic iDCs were labeled with 51Cr (AB) and used as targets in the presence or absence of saturating concentrations of specific mAbs HP-F1 (anti-ILT2/CD85j), HP-1F7 (anti-HLA class I), HP-3B1 (anti-CD94), or Z199 (anti-NKG2A) or combinations of anti-CD94 F(ab\(^\prime\))\(_2\) (clone HP-3B1), anti-ILT2 F(ab\(^\prime\))\(_2\) (clone HP-F1), and control F(ab\(^\prime\))\(_2\) mAbs.

In the re-directed killing (rADCC) assays, NK-92 and NK3.3 cell lines or isolated human NK cells were tested against the FR\(^\text{b}^\prime\) murine mastocytoma cell line P815 preincubated with saturating amounts (5 μg/ml) of mAbs anti-CD7, anti-NKp30, and anti-CD16 alone or in combination with a mAb anti-ILT2/CD85j. Assays were conducted in triplicate using U-bottom 96-well microtiter plates, and specific lysis was calculated as 100 × (specific release [cpm] – spontaneous release [cpm]/maximum release [cpm]) × (100 – spontaneous release [cpm]). In all cases, spontaneous release was <20% of the maximum lysis.

Coculture of NK-92 and PBMCs with NK targets and cytokine production determination

Effector and target cells were mixed at a 2:1 or 1:1 (effector:target) ratio in sterile U-bottom 96-well microtiter plates at 37°C. NK-92 (1.5 × 10\(^5\)) and NK targets (7.5 × 10^4 to 1.5 × 10^5) were cocultured in MEM-α medium with 10% FCS, 10% horse serum, 1% sodium pyruvate, 1% essential aminoacids, and 100–200 U/ml rh-IL-2. In blocking experiments, NK-92 effector cells were preincubated with anti-ILT2/CD85j mAb. Alternatively, HLA class I blocking was performed by pretreating the target cell line with anti-HLA class I mAb for 20 min at 37°C before coculture. In PBMCs assays, IL-2-activated effector (1.5 × 10^5) and target cells (1.5 × 10^5 or 7.5 × 10^5) were incubated in RPMI 1640 with 10% FCS and 100 U/ml rh-IL-2. When indicated, 1 U/ml of rh-IL-12 (Pepro- Tech) and/or 100 ng/ml of brefeldin A (Sigma-Aldrich) were added to effector cells to obtain control samples for cytokine production. NK-92 and isolated NK cells were incubated alone and with the NK-sensitive 221.Cw07 cell line in the absence of mAbs to obtain negative and positive control samples for the secretion of IFN-γ. IDCs were incubated alone or in the presence of 1 μg/ml LPS to obtain control samples for the production of IL-12. After a 20-h incubation period, culture supernatants were harvested and frozen at −80°C. The concentrations of IFN-γ and IL-12 were measured in cell-free supernatants by flow cytometry using human IFN-γ and human IL-12p70 CBA Flex Set Kits (BD Biosciences).

Cytokine assays were conducted as described above at different (NK:IDC) ratios.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism software (Graph-Pad). The nonparametric Mann-Whitney U test for independent samples was used to analyze differences between the percentage of cells producing IFN-γ upon stimulation with 721.221 and HLA class I transfectants. p values of less than or equal to 0.05 were considered statistically significant.

Results

NK-92 cells express functional ILT2/CD85j

A few reports address the involvement of ILT2/CD85j in the regulation of NK cell cytotoxicity (8, 43, 44), but no data are available about the regulation of cytokine production in NK cells by interaction of ILT2/CD85j with HLA class I ligands. The NK-92 cell line has been widely used to investigate the regulation of IFN-γ production in NK cells under several conditions. We explored the possibility of using it as a model cell line to study the involvement of ILT2/CD85j in this cell line leads to an inhibition of cytotoxicity as has been described for primary NK cells and other NK cell lines (8, 43, 44). For this purpose we used the HLA class I-defective lymphoblastoid cell line 721.221 and three HLA class I transfectants of the same cell line expressing HLA-B*B2705, HLA-G1, and HLA-Cw7, respectively. We chose HLA-B*B2705 and HLA-G1 as both have been described as ligands of ILT2/CD85j (8), and it has been reported that NK-mediated lysis of 721.221 is inhibited by transfection of HLA-B*B2705 or HLA-G1 and that this inhibition is accounted for by the ILT2/CD85j receptor in the absence of other iKIRs (8). To analyze just the result of ILT2/CD85j ligation, we used the transfectant of a mutant HLA-G1 protein (which will be referred to as 221.G1m throughout the manuscript) whose signal peptide does not support HLA-E expression on the cell membrane; thus, CD94/NKG2A receptor is not involved in the recognition of 221.G1m by effector cells. We used the 221.Cw07 transfectant as a control HLA class I-expressing target cell line, as HLA-Cw07 has been reported not to interact with ILT2/CD85j (9, 36). More, it does not contribute an HLA-E binding peptide to make a

The concentration of IFN-γ was measured by flow cytometry using a CBA Flex Set Kit.

For cytotoxicity assays, the 721.221, 221.B*2705, 221.AEH, and P815 cell lines were used as targets against NK-92, NK3.3, or isolated NK cells as described above. To study poly(C) stimulated cytotoxicity, human NK cells were treated for 24 h with 100 μg/ml of poly(C) before the encounter with targets.

NK/DC coculture

NK-92 and purified NK cells were cultured in RPMI 1664 medium containing 10% FCS and 100 U/ml rh-IL-2, in 96-well round-bottom microtiter plates in the presence of allogeneic iDCs. For receptor blocking studies, saturating amounts (10 μg/ml) of anti-CD94 F(ab\(^\prime\))\(_2\) (clone HP-3B1), anti-ILT2 F(ab\(^\prime\))\(_2\) (clone HP-F1), and control F(ab\(^\prime\))\(_2\) mAbs were added to effector cells for 15 min at 37°C before contact with targets and throughout the assays. To analyze cytokine production, NK cells were seeded at 1.5 to 1 × 10^5 cells/well, and cocultures were conducted at a 2:1 (effector:target) ratio. NK-92 and isolated NK cells were incubated alone and with the NK-sensitive 721.221 cell line in the absence of mAbs to obtain negative and positive control samples for the secretion of IFN-γ. IDCs were incubated alone or in the presence of 1 μg/ml LPS to obtain control samples for the production of IL-12. After a 20-h incubation period, culture supernatants were harvested and frozen at −80°C. The concentrations of IFN-γ and IL-12 were measured in cell-free supernatants by flow cytometry using human IFN-γ and human IL-12p70 CBA Flex Set Kits (BD Biosciences).

Cytokine assays were conducted as described above at different (NK:IDC) ratios.
CD94/NKG2 ligand, so HLA class I expression in the 221.Cw07 transfectant is not detected by the inhibitory receptor CD94/ NKG2A either (36, 37). As it is shown in Fig. 1B, expression of HLA-B*2705 or HLA-G1 on target cells inhibited cell lysis by NK-92 cells, and this inhibition was reverted to the same extent by the presence of HLA class I mAb was added, IFN-γ production was analyzed by intracellular flow cytometry in CD56+ cells from cocultures of effector and target cells (not shown). We next wondered whether the differences detected in intracellular IFN-γ production could be relevant to the amount of cytokine secreted into the medium. To analyze this question, we measured the concentration of cytokine secreted by NK-92 cells cocultured with target cells expressing different HLA class I allotypes. As shown in Fig. 2C, NK-92 cells were stimulated by HLA class I-deficient 721.221 target cells to secrete IFN-γ, and the amount of cytokine secreted was noticeably lower when target cells expressed ILT2/CD85j ligands but was not diminished by the presence of HLA-Cw07. As the 721.221 lymphoblastoid cell line and its transfectants do not produce IFN-γ even upon stimulation with PMA plus ionomycin (data not shown), the amount of cytokine detected in cocultures must correspond to IFN-γ secreted by NK-92 cells.

To further confirm ILT2/CD85j involvement in the inhibition of cytokine production, we performed the stimulation experiments with the target cell lines 721.221 and 221.B*2705 in the presence of saturating amounts of blocking mAbs anti-ILT2/CD85j or anti-HLA class I and analyzed IFN-γ production in CD56+ cells by intracellular flow cytometry. In the experiment shown in Fig. 2D, the expression of HLA-B*2705 on target cells inhibited the production of the cytokine by 80% as compared with the production induced by the HLA class I-deficient 721.221 in the presence of a control isotype mAb anti-CD7. In contrast, when a blocking anti-HLA class I mAb was added, IFN-γ expression was only inhibited by 18%, and coincubation of effector and target cells in the presence of blocking Abs anti-ILT2/CD85j partially restored the production of IFN-γ cells leading to an inhibition of only 45%. These effects may be attributed to the abrogation of the interaction between ILT2/CD85j and HLA class I and further support the involvement of ILT2/CD85j in regulation of the synthesis of IFN-γ by NK cells.

ILT2/CD85j HLA class I ligands down-regulate IFN-γ mRNA levels in NK-92 cells

IFN-γ mRNA expression and protein synthesis are not always correlated (45, 46). To test whether down-regulation of cytokine production was due to a down-regulation of IFN-γ mRNA levels, we measured IFN-γ gene expression levels in NK-92 cells cocultured with 721.221 target cells expressing or not ILT2/CD85j HLA ligands. As mentioned previously, 721.221 lymphoblastoid cells do not produce IFN-γ in basal cell culture conditions or upon PMA stimulation. Nonetheless, IFN-γ mRNA levels were measured by intracellular flow cytometry. Preliminary experiments were performed to test IFN-γ production by target and effector cells, in which we verified that the 721.221 cell line and HLA transfectants were unable to synthesize IFN-γ, even upon PMA and ionomycin treatment, although CD69 expression was induced in response to the stimulation (data not shown). Nonetheless, containing with CD56 mAb was performed in all the experiments so the analysis could be directed to NK-92 cells. As shown in Fig. 2A, upon incubation with 721.221 cells, a variable percentage of NK-92 cells responded by making IFN-γ, a response not seen in the absence of target cells, and a statistically significant reduction of the percentage of cells producing IFN-γ was detected when NK-92 cells were challenged with cells expressing the ILT2/CD85j ligands HLA-B*2705 or HLA-G1. In most experiments, the response was inhibited by more than 50% by the expression of the ILT2/CD85j ligands HLA-B*2705 or HLA-G1 on target cells (Fig. 2, A and B). In contrast, HLA-Cw07 expression by target cells was unable to inhibit the production of the cytokine, which is in accordance with the lack of recognition of this allele by nNKRs in NK-92 cells. Similar results were obtained when TNF-α production was analyzed by intracellular flow cytometry in CD56+ cells from cocultures of effector and target cells (not shown). We next wondered whether the differences detected in intracellular IFN-γ production could be relevant to the amount of cytokine secreted into the medium. To analyze this question, we measured the concentration of cytokine secreted by NK-92 cells cocultured with target cells expressing different HLA class I allotypes. As shown in Fig. 2C, NK-92 cells were stimulated by HLA class I-deficient 721.221 target cells to secrete IFN-γ, and the amount of cytokine secreted was noticeably lower when target cells expressed ILT2/CD85j ligands but was not diminished by the presence of HLA-Cw07. As the 721.221 lymphoblastoid cell line and its transfectants do not produce IFN-γ even upon stimulation with PMA plus ionomycin (data not shown), the amount of cytokine detected in cocultures must correspond to IFN-γ secreted by NK-92 cells. 

FIGURE 1. The NK-92 cell line expresses functional ILT2/CD85j on the cell membrane. A, Flow cytometry analysis of the NK-92 human cell line phenotype. Indirect immunofluorescence was performed with anti-CD56, anti-CD16, anti-ILT2/CD85j, anti-KIR mix (anti-KIR2DL2/2DL3/2DS2, anti-KIR2DL1/2DS1/2DS4, anti-KIR3DL1 and anti-KIR3DL2), anti-CD94, and anti-NKG2A mAbs. A secondary FITC-conjugated rabbit anti-mouse F(ab’)2 was used. Staining with control IgG (gray histograms) and with specific mAbs (white histograms) is shown. B, Cytoxicity mediated by the NK-92 cell line was measured in a 4-h 51Cr-release assay at various ratios against the NK-sensitive 721.221, 221.B*2705, 221.Cw07, and 221.G1m cell lines in the presence or absence of specific mAbs anti-ILT2/CD85j, anti-HLA class I, and anti-CD94. Assays were performed in triplicates.

ILT2/CD85j ligands inhibit synthesis of IFN-γ by NK-92 cells

It has been shown that the encounter with target cells promotes IFN-γ production in NK cells (27). To study whether ILT2/CD85j regulates IFN-γ production by NK cells, a flow cytometric approach was used to assess the frequency of NK-92 cells secreting IFN-γ following coincubation with target cells. NK-92 cells were cultured with 721.221, 221.B*2705, 221.G1m, and 221.Cw07 cell lines, and the synthesis of IFN-γ was assessed by intracellular flow cytometry. Preliminary experiments were performed to test IFN-γ production by target and effector cells, in which we verified that the 721.221 cell line and HLA transfectants were unable to synthesize IFN-γ, even upon PMA and ionomycin treatment, although CD69 expression was induced in response to the stimulation (data not shown). Nonetheless, containing with CD56 mAb was performed in all the experiments so the analysis could be directed to NK-92 cells. As shown in Fig. 2A, upon incubation with 721.221 cells, a variable percentage of NK-92 cells responded by making IFN-γ, a response not seen in the absence of target cells, and a statistically significant reduction of the percentage of cells producing IFN-γ was detected when NK-92 cells were challenged with cells expressing the ILT2/CD85j ligands HLA-B*2705 or HLA-G1. In most experiments, the response was inhibited by more than 50% by the expression of the ILT2/CD85j ligands HLA-B*2705 or HLA-G1 on target cells (Fig. 2, A and B). In contrast, HLA-Cw07 expression by target cells was unable to inhibit the production of the cytokine, which is in accordance with the lack of recognition of this allele by nNKRs in NK-92 cells. Similar results were obtained when TNF-α production was analyzed by intracellular flow cytometry in CD56+ cells from cocultures of effector and target cells (not shown). We next wondered whether the differences detected in intracellular IFN-γ production could be relevant to the amount of cytokine secreted into the medium. To analyze this question, we measured the concentration of cytokine secreted by NK-92 cells cocultured with target cells expressing different HLA class I allotypes. As shown in Fig. 2C, NK-92 cells were stimulated by HLA class I-deficient 721.221 target cells to secrete IFN-γ, and the amount of cytokine secreted was noticeably lower when target cells expressed ILT2/CD85j ligands but was not diminished by the presence of HLA-Cw07. As the 721.221 lymphoblastoid cell line and its transfectants do not produce IFN-γ even upon stimulation with PMA plus ionomycin (data not shown), the amount of cytokine detected in cocultures must correspond to IFN-γ secreted by NK-92 cells. To further confirm ILT2/CD85j involvement in the inhibition of cytokine production, we performed the stimulation experiments with the target cell lines 721.221 and 221.B*2705 in the presence of saturating amounts of blocking mAbs anti-ILT2/CD85j or anti-HLA class I and analyzed IFN-γ production in CD56+ cells by intracellular flow cytometry. In the experiment shown in Fig. 2D, the expression of HLA-B*2705 on target cells inhibited the production of the cytokine by 80% as compared with the production induced by the HLA class I-deficient 721.221 in the presence of a control isotype mAb anti-CD7. In contrast, when a blocking anti-HLA class I mAb was added, IFN-γ expression was only inhibited by 18%, and coincubation of effector and target cells in the presence of blocking Abs anti-ILT2/CD85j partially restored the production of IFN-γ cells leading to an inhibition of only 45%. These effects may be attributed to the abrogation of the interaction between ILT2/CD85j and HLA class I and further support the involvement of ILT2/CD85j in regulation of the synthesis of IFN-γ by NK cells.
Figure 2. Expression of ILT2/CD85j ligands on target cells inhibits IFN-γ production induced by the 721.221 NK target cell line in NK-92 cells. Human NK-92 cells were incubated alone or with 721.221, 221.B*2705, 221.G1m, or 221.Cw07 cell lines at a 2:1 (effector:target) ratio. A, Cells were incubated for 6 h in the presence of brefeldin A and collected for extracellular staining with CD56-FITC, fixed, and permeabilized for intracellular staining with anti-IFN-γ-PE mAb. The NK-92 cell line was stimulated with PMA plus ionomycin (PMA/Io) as a positive control. IFN-γ production by NK-92 cells (CD56+) was analyzed by intracellular flow cytometry. Upper panel, A representative experiment. Lower graph, The mean percentage of CD56+ IFN-γ-positive cells and SEs of the mean corresponding to at least four independent experiments. Statistical analysis of the data was performed using the Mann-Whitney U test. *, denotes *p < 0.05; n.s., no significative differences. B, Cells were treated as described for A. Graph shows the percent inhibition achieved by each transfectant relative to the stimulation by the parental cell line 721.221. The results are represented as mean values and SDs corresponding to at least four independent experiments. C, Human NK-92 cells were incubated alone or with NK targets for 20 h. After this time, culture supernatants were harvested and the concentration of IFN-γ was measured by flow cytometry using a CBA Flex Set Kit. Graph shows nanograms of IFN-γ per milliliter of culture supernatant per 10^6 NK-92 cells cultured with the different stimuli depicted in the figure. PMA/Io stimulation was used as a positive control. Bars represent mean values of triplicate cultures in one representative experiment. D, Percent of IFN-γ-positive CD56+ cells was determined as described for A, after coculture of effector and target cells in the presence of specific mAbs anti-ILT2/CD85j, anti-HLA class I, or a control IgG1 mAb anti-CD7. The percent of CD56+ IFN-γ-positive cells (upper panel) and the percent of NK-92 cells inhibited by HLA-B*2705 (lower panel) in the presence of specific Abs relative to the stimulation by 721.221 are shown. Mean values of triplicate cultures are shown. In all cases, percent inhibition was calculated as 1 - (% IFN-γ-positive NK-92 cells following incubation with 221 transfectants/ IFN-γ-positive NK-92 cells following incubation with 221 parental) × 100.

Figure 3. IFN-γ mRNA levels induced by target cells in the NK-92 cell line are down-regulated by the expression of ILT2/CD85j ligands on target cells. Human NK-92 cells were incubated alone or in the presence of 721.221, 221.B*2705, 221.G1m, and 221.Cw07 target cell lines for 3 h at a 2:1 (effector:target) ratio. After this time, cells were collected and total RNA was isolated. IFN-γ mRNA was quantified by real-time RT-PCR as described in Materials and Methods. PMA plus ionomycin treatment was used as a positive control of gene transcription. Results of a representative experiment are shown as the ratio between IFN-γ and CD94 mRNA molecules (upper panel). Lower panel, Percent inhibition of IFN-γ mRNA expression in NK-92 cell line after coculture with HLA-I transfectants relative to the induction by the parental HLA class I-deficient cell line 721.221. Percent inhibition was calculated as 1 - (relative IFN-γ mRNA following NK-92 incubation with 221 transfectants/relative IFN-γ mRNA following NK-92 incubation with 221 parental) × 100. A representative experiment of three performed with similar results is shown.

ILT2/CD85j HLA class I ligands down-regulate IFN-γ production by peripheral blood NK and T cells

Although the experiments described above led us to the conclusion that the ILT2/CD85j receptor is able to regulate the levels of IFN-γ secreted by NK cells, limiting the approach is the use of a cell line derived from long-term culture in high doses of IL-2. It seemed important to investigate ILT2/CD85j function in a more natural effector cell population. In addition, a distinctive feature of NK cell biology is the ability to work early in the immune response; therefore, we investigated the ability of ILT2/CD85j receptor to regulate IFN-γ production in short-term cultured peripheral blood NK cells from healthy donors. Freshly isolated NK cells are poor
IFN-γ producers in response to 721.221 lymphoblastoid cells (29, 36). Moreover, ILT2/CD85j cell surface expression is highly variable in NK and T cells from different individuals (see Refs. 44, 47 and our own unpublished data). Thus, freshly isolated PBMCs from selected donors, in which a defined subpopulation of CD3−/CD56+ cells expressing high levels of ILT2/CD85j could be detected by flow cytometry were cultured 1–3 days in the presence of 50 U/ml IL-2 to prime cytokine secretion and make NK cells susceptible to stimulation by 721.221 cells. These cells were further incubated with 721.221 NK targets or HLA class I transfecteds for six additional hours in the presence of brefeldin A. IFN-γ-producing cells were analyzed by intracellular flow cytometry. Although control samples demonstrated negligible amounts of IFN-γ staining, upon coculture with NK targets, we could detect IFN-γ production in a small proportion of total PBLs (defined by forward (FSC) and side light scatter (SSC) criteria), which was inhibited by the expression of the ILT2/CD85j ligands HLA-B*2705 and HLA-G1 on the membrane of target cells. HLA-Cw07 also inhibited the synthesis of IFN-γ, although to a much lower extent.

**FIGURE 4.** ILT2/CD85j inhibits IFN-γ production by peripheral blood-derived NK and T lymphocytes. PBMCs from healthy donors were cultured in RPMI 1640 plus 10% FCS and 25–50 U/ml rh-IL2 for 1–3 days before being incubated alone or with 721.221, 221.B*2705, 221.G1m, and 221.Cw07 cell lines for six additional hours at a 1:1 (effector:target) ratio in the presence of brefeldin A. A, Cells were collected for extracellular staining and incubated with either CD3-PerCP and CD56-FITC or CD3-PerCP and CD85j-FITC mAbs, fixed, and permeabilized for intracellular staining with anti-IFN-γ-PE mAb. A representative experiment showing the percent of IFN-γ-positive PBLs (selected according to forward and side light scatter criteria), IFN-γ-positive NK (CD3−/CD56+) cells, and IFN-γ-positive CD3+ ILT2+ cells is shown. B, Cell cultures were collected for extracellular staining with anti-KIR3DL1 mAb followed by washing and labeling with allophycocyanin-conjugated goat anti-mouse IgG. Then, cells were stained with CD3-PerCP and CD85j-FITC mAbs, fixed, and permeabilized for intracellular staining with anti-IFN-γ-PE mAb. Left panel, shows flow cytometry analysis of IFN-γ-positive CD3+ ILT2− KIR3DL1− cells in a representative experiment. Right panel, shows the percent of CD3− ILT2− KIR3DL1− cells inhibited by different HLA class I molecules in n = 7 (HLA-B*2705), n = 4 (HLA-G1), and n = 4 (HLA-Cw07) independent experiments. C, PBMCs were stimulated with target cell lines preincubated with 10 ng/ml SEB, and stained as described for B. Left panel, shows analysis of IFN-γ-positive CD3+ ILT2− KIR3DL1− cells in a representative experiment. Right panel, shows the percent of CD3− ILT2− KIR3DL1− cells inhibited by different HLA class I molecules in n = 8 (HLA-B*2705), n = 6 (HLA-G1m), and n = 6 (HLA-Cw07) independent experiments. In all cases, percent inhibition was calculated as 1 − (% IFN-γ-positive CD3− ILT2− KIR3DL1− or CD3+ ILT2+ KIR3DL1− cells following incubation with 221 transfectants/% IFN-γ-positive CD3− ILT2− KIR3DL1− or CD3+ ILT2+ KIR3DL1− cells following incubation with 221 parental) × 100.
analysis of the results revealed that only CD3+CD56− NK cells were stimulated to produce IFN-γ by coculture with 721.221 (not shown). Analysis of this subpopulation (which contained a 40% ILT2/CD85j+), 25% CH-L+, and 10% KIR3DL1+ cells) revealed that only 15% of these cells were stimulated by 721.221 cells to produce IFN-γ, and ~76% of this production was inhibited by HLA-B*2705 whereas HLA-G1 inhibited about a 50%. In contrast, IFN-γ production was inhibited by only 20% when target cells expressed HLA-Cw07, in accordance with a lower expression of KIR2DL2/L3 in this donor. Although only 10% of the NK cells were KIR3DL1+, the higher inhibition achieved by HLA-B*2705 (as compared with HLA-G1) could be attributed to its additional recognition by this iNKR. We next focused our analysis on ILT2/CD85j+CD3− cells (Fig. 4A, right panel). Although ILT2/CD85j is also expressed by B lymphocytes within the CD3− subpopulation, CD3−CD56− cells were not induced to synthesize IFN-γ by 721.221 target cells (data not shown); therefore, the CD3−ILT2+ cells producing this cytokine within the PBL gate should be NK cells. A total of 60% of the CD3−ILT2+ cells were NK cells according to CD56 expression; nevertheless, only 12% were induced to produce IFN-γ by stimulation with 721.221 targets. Further flow cytometry analysis of this donor showed that only 5% of CD3−ILT2/CD85j+ cells were KIR3DL1+, consistent with the more equal levels of inhibition achieved by both 221.G1m and 221.B*2705 transfectants in this subpopulation (67 and 72% inhibition, respectively). These results support the notion that self HLA class I molecules can regulate IFN-γ production, CD3+cells producing this cytokine within the PBL gate should be NK cells. A total of 60% of the CD3+ILT2+ cells were NK cells according to CD56 expression; nevertheless, only 12% were induced to produce IFN-γ by stimulation with 721.221 targets. Further flow cytometry analysis of this donor showed that only 5% of CD3−ILT2/CD85j+ cells were KIR3DL1+, consistent with the more equal levels of inhibition achieved by both 221.G1m and 221.B*2705 transfectants in this subpopulation (67 and 72% inhibition, respectively). These results support the notion that self HLA class I molecules can regulate IFN-γ production in primary NK cells by interaction with the iNKR ILT2/CD85j.

A variable proportion of ILT2/CD85j+ NK cells can express KIR3DL1, which is another iNKR for HLA-B*2705. Therefore, in the following set of experiments, we used four-color flow cytometry to rule out the possible contribution of KIR3DL1 to the inhibitory effect observed with 221.B*2705 transfectant. Fig. 4B shows the results obtained in NK cells from a representative donor and summary data from independent experiments performed with PBMCs from various individuals. Inhibition of IFN-γ production in gated CD3+ KIR3DL1+ ILT2/CD85j+ cells was detected in all the experiments performed when 221.B*2705 were used as stimulatory cells. In most cases, the inhibition delivered by the interaction with 221.B*2705 transfectant was higher than that observed when 221.G1m cells were used to stimulate the cultures. In some experiments we detected a modest inhibition by HLA-Cw07, which could be in accordance with low levels of expression of KIR2DL2/3 in effector cells (data not shown).

Several reports analyzed the contribution of ILT2/CD85j to the regulation of IFN-γ production in T cells, although the direct contribution of self HLA class I ligands was not addressed (13, 15, 17). Thus, we also measured IFN-γ production in CD3+ILT2/CD85j+ cells from selected donors for comparative purposes. To stimulate T lymphocytes, 721.221 cells and transfectants were preincubated with SEB, washed, and used as APCs as described (35). Fig. 4C shows that a considerable inhibition of cytokine synthesis was also detected in T cells when APCs expressed HLA-B*2705 in all donors tested. In sharp contrast, the introduction of HLA-G1 in the system did not always lead to a substantial inhibition of the cytokine production. Surprisingly, stimulation of the synthesis of IFN-γ was detected in T cells from several donors when HLA-G1 was expressed on APCs (Fig. 4C, right panel). Altogether, these data support a role for ILT2/CD85j in the regulation of the production of IFN-γ by NK cells and a different level of regulation in NK and T cells.

iNKRs down-modulate IFN-γ secretion induced by TLR3 ligands in fresh NK cells

It has been recently published that NK cells can participate in the innate immune response against viral infections not only by the killing of infected cells, but also through the direct recognition of viral products by TLRs. In particular, NK cells can sense the presence of dsRNA through TLR3 expressed on their cell surface (48, 49), and it has been reported that suboptimal doses of IL-12 can synergize with the dsRNA analog poly(I:C) to induce the secretion of IFN-γ (49, 50).

To test whether ILT2/CD85j could also regulate the production of cytokine stimulated by TLR3 ligands, we used anti-ILT2/CD85j mAb-coated tissue culture wells to mimic receptor ligation and stimulated NK-92 cells with poly(I:C) and suboptimal doses of IL-12. In this system, ILT2/CD85j was not able to down modulate IFN-γ secretion induced by poly(I:C), despite the high levels of expression of ILT2/CD85j in NK-92. The experiment was repeated three times with identical results (data not shown). To test whether the same was true with primary NK cells, identical experiments were performed with purified NK cells from various donors containing at least 25% of ILT2/CD85j+ cells and cultured overnight with 100 U/ml rh-IL-2. We found that the amount of IFN-γ secreted into the culture medium upon poly(I:C) stimulation in the presence of suboptimal doses of IL-12 was highly variable between donors. However, costimulation of ILT2/CD85j receptor by immobilized mAb down-regulated cytokine secretion in five of six NK cultures tested (mean inhibition 41.9 ± 17.5%). Fig. 5A shows data obtained with two representative donors. It is notable that in some donors, suboptimal doses of IL-12 (1 U/ml) were able to induce the secretion of substantial amounts of IFN-γ in the presence of IL-2, and that in these cases, ILT2/CD85j ligation was able to down-regulate cytokine secretion to almost background levels (see Fig. 5A, NK donor 2). We next wondered whether other iNKRs were also able to diminish the production of cytokine stimulated by TLR3 ligands. Therefore, the same experimental approach was followed to test the ability of the iNKRs CD94/NKG2A and KIR3DL1 to regulate the production of IFN-γ. The cross-linking of CD94/NKG2A reduced the production of IFN-γ in the four donors tested (mean inhibition 44.3 ± 10.7%). Similar results were obtained when KIR3DL1 was cross-linked in NK from three donors selected for the expression of this iNKR (mean inhibition 52 ± 29.4%). The results obtained for each receptor with two representative donors are shown in Fig. 5A. Several groups have reported that TLR3 agonists are also able to enhance NK cell cytotoxicity (50). Therefore, the capability of iNKRs to regulate this function was also analyzed. While, although to variable levels, IFN-γ production was always enhanced by poly(I:C) treatment, in some donors the stimulation with poly(I:C) was not able to enhance the cytotoxicity of primary NK cells against 721.221 or transfectants. Nonetheless, we analyze the effect of iNKRs in those NK populations responding to poly(I:C) stimulation with enhanced cytotoxicity. As shown in Fig. 5B, in these donors the addition of blocking anti-ILT2 mAb did not affect the cytotoxic function against 721.221 or 21.AEH cell lines, but further enhanced NK cell cytotoxicity against 221.B*2705 transfectants, as expected, and also when NK cell cytotoxicity had been stimulated by poly(I:C). In the same way, the addition of blocking mAb against CD94/NKG2A increased NK cell cytotoxicity against 21.AEH cells and also further enhanced the killing capability of poly(I:C)-pretreated NK cells. Altogether, these results suggest that ILT2/CD85j and other iNKRs may interfere with activating signals delivered by TLR ligands in NK cells.
In a developing immune response, IFN-γ production induced by NK cells without 1 U/ml of rh-IL-12 and 100 KIR3DL1 mAbs for 20 h in the presence of 100 U/ml of rh-IL-2 and with control IgG1, HP-F1 (anti-ILT2), Z199 (anti-NKG2A), or DX9 (anti-ILT2/CD85j) mAbs for 20 h in the presence of 100 U/ml of rh-IL-2 and with medium alone at different E:T (effector:target) ratios. NKp30 (upper panels) and NK-92 (lower panel) cell lines were cultured in flat-bottom 96-well microplates precoated with rh-IL-2 and with or without 1 U/ml of rh-IL-12 and 100 μg/ml of poly(I:C). After this time, supernatants were harvested and the concentration of IFN-γ was measured by flow cytometry using a CBA Flex Set Kit. Assays were performed in triplicate. Graphs show concentration of IFN-γ per 10^5 purified NK cells. Data from representative experiments are shown. Flow cytometry histograms show the expression of ILT2, CD94/NKG2A, or KIR3DL1 in isolated human NK cells used in each experiment. B, Cytotoxicity mediated by purified NK cells pretreated (discontinuous lines) or not (continuous lines) for 24 h with 100 μg/ml of poly(I:C) was measured in a 4-h 51Cr-release assay against the FcγR-positive P815 murine mastocytoma cell line P815 preincubated with saturating amounts (5 μg/ml) of the indicated mAbs (anti-CD7, anti-NKp30, anti-CD16, and anti-ILT2) or in medium alone at different E:T (effector:target) ratios. NKp30 (upper panels) and NK-92 (lower panel) cell lines were cultured in flat-bottom 96-well microplates precoated with 5 μg/ml anti-CD7, anti-NKp30, or anti-CD16 mAbs combined or not with anti-ILT2 mAb in the presence of 100 U/ml of rh-IL-2. After 20 h, culture supernatants were harvested for analysis of IFN-γ concentration by flow cytometry using a CBA Flex Set Kit. Graphs show nanograms of IFN-γ per milliliter of culture supernatant per 10^5 NKp30 or NK-92 cells from two representative experiments.

 associations to CD3ζ adaptor molecule to transduce activating signals. It has been described that by binding to ILT2/CD85j receptor on effector cells, HLA class I molecules can reduce cytotoxicity-inducing activation signals delivered by CD16 (8), which also associates to CD3ζ. Thus, we hypothesized that ILT2/CD85j could also down-regulate activating signals delivered through NKp30 in NK cells. A first set of experiments was performed in which NK cell lines were used as effectors in rADCC assays. As NK-92 cells do not express CD16, the NK3.3 (CD16−ILT2/CD85j+) cell line was included for comparative purposes. As shown in Fig. 6A, CD16-dependent lysis of FcγR-positive P815 target cells was down-regulated by ILT2/CD85j cross-linking in NK3.3, as expected. The same extent of inhibition was obtained in NKp30-dependent lysis, although NKp30 was less efficient in stimulating cytotoxicity. Inhibition of NKp30-dependent lysis by ILT2/CD85j ligation was also observed when NK-92 cells were used as effector cells. NK-92 and NK3.3 cell lines were then stimulated by immobilized mAbs, and IFN-γ secreted to the medium was measured in 20 h culture supernatants. NKp30 was more potent than CD16 in inducing the secretion of IFN-γ in NK3.3, and both stimuli were inhibited by cross-linking with ILT2/CD85j (Fig. 6B). In NK-92 cells, NKp30-induced IFN-γ secretion was inhibited upon cross-linking of ILT2/CD85j and NKp30 receptors in three independent experiments performed (mean inhibition 73 ± 34%), in sharp contrast to the results observed with poly(I:C) stimulation.

FIGURE 6. IFN-γ production induced by NKp30 is inhibited by ILT2/CD85j ligation in NK-92 and NK3.3 cell lines. A, Cytotoxicity mediated by NK3.3 (upper panels) and NK-92 (lower panel) cell lines was measured in triplicate in a 4-h 51Cr-release assay against the FcγR-positive P815 murine mastocytoma cell line P815 preincubated with saturating amounts (5 μg/ml) of the indicated mAbs (anti-CD7, anti-NKp30, anti-CD16, and anti-ILT2) or in medium alone at different E:T (effector:target) ratios. B, NKp30 (upper panels) and NK-92 (lower panel) cell lines were cultured in flat-bottom 96-well microplates precoated with 5 μg/ml anti-CD7, anti-NKp30, or anti-CD16 mAbs combined or not with anti-ILT2 mAb in the presence of 100 U/ml of rh-IL-2. After 20 h, culture supernatants were harvested for analysis of IFN-γ concentration by flow cytometry using a CBA Flex Set Kit. Graphs show nanograms of IFN-γ per milliliter of culture supernatant per 10^5 NKp30 or NK-92 cells from two representative experiments.

ILT2/CD85j inhibits the secretion of IFN-γ induced by NKp30 in NK cells

In a developing immune response, IFN-γ and TNF-α are secreted during the NK-DC cross talk that results in maturation or elimination of iDCs. The natural cytotoxicity receptor NKp30 has been shown to play a critical role in the induction of cytokine production by NK cells (23, 24). It has been suggested that some iNKRs could participate in the regulation of this process (23), but the putative involvement of ILT2/CD85j remains unexplored. NKp30 associated to CD3ζ adaptor molecule to transduce activating signals. It has been described that by binding to ILT2/CD85j receptor on effector cells, HLA class I molecules can reduce cytotoxicity-inducing activation signals delivered by CD16 (8), which also associates to CD3ζ. Thus, we hypothesized that ILT2/CD85j could also down-regulate activating signals delivered through NKp30 in NK cells. A first set of experiments was performed in which NK cell lines were used as effectors in rADCC assays. As NK-92 cells do not express CD16, the NK3.3 (CD16−ILT2/CD85j+) cell line was included for comparative purposes. As shown in Fig. 6A, CD16-dependent lysis of FcγR-positive P815 target cells was down-regulated by ILT2/CD85j cross-linking in NK3.3, as expected. The same extent of inhibition was obtained in NKp30-dependent lysis, although NKp30 was less efficient in stimulating cytotoxicity. Inhibition of NKp30-dependent lysis by ILT2/CD85j ligation was also observed when NK-92 cells were used as effector cells. NK-92 and NK3.3 cell lines were then stimulated by immobilized mAbs, and IFN-γ secreted to the medium was measured in 20 h culture supernatants. NKp30 was more potent than CD16 in inducing the secretion of IFN-γ in NK3.3, and both stimuli were inhibited by cross-linking with ILT2/CD85j (Fig. 6B). In NK-92 cells, NKp30-induced IFN-γ secretion was inhibited upon cross-linking of ILT2/CD85j and NKp30 receptors in three independent experiments performed (mean inhibition 73 ± 34%), in sharp contrast to the results observed with poly(I:C) stimulation.

FIGURE 5. ILT2/CD85j, CD94/NKG2A, and KIR3DL1 modulate IFN-γ production induced by poly(I:C) in primary NK cells. A, Peripheral blood purified NK cells were stimulated overnight with 100 U/ml of rh-IL-2 and then cultured in flat-bottom 96-well microplates precoated with control IgG1, HP-F1 (anti-ILT2), Z199 (anti-NKG2A), or DX9 (anti-KIR3DL1) mAbs for 20 h in the presence of 100 U/ml of rh-IL-2 and with or without 1 U/ml of rh-IL-12 and 100 μg/ml of poly(I:C). After this time, supernatants were harvested and the concentration of IFN-γ was measured by flow cytometry using a CBA Flex Set Kit. Assays were performed in triplicate. Graphs show concentration of IFN-γ per 10^5 purified NK cells. Data from representative experiments are shown. Flow cytometry histograms show the expression of ILT2, CD94/NKG2A, or KIR3DL1 in isolated human NK cells used in each experiment. B, Cytotoxicity mediated by purified NK cells pretreated (discontinuous lines) or not (continuous lines) for 24 h with 100 μg/ml of poly(I:C) was measured in a 4-h 51Cr-release assay against 721.221, 221.B+2705, or 221.AEH cell lines in the absence (Δ) or presence of anti-ILT2 (○), or anti-NKG2A (□) mAbs at different (effector:target) ratios. Assays were performed in triplicates. Two representative experiments and expression of ILT2 and CD94/NKG2A in effector cells are shown.
degree of inhibition even when an elevated proportion (80%) of purified NK cells from the donor were expressing high levels of cell surface ILT2/CD85j. Nonetheless, the same degree of inhibition of CD16-stimulated cytotoxic activity was observed (Fig. 7A). Similar results were obtained when IFN-γ was measured in cultured supernatants from purified short-term cultured peripheral blood NK cells stimulated by NKp30 or CD16. In these experiments, we consistently found a small degree of inhibition by ILT2/CD85j cross-linking that never exceeded 30% (Fig. 7, B and C). The discrepancy between the inhibition of IFN-γ production in the NK cell lines (NK-92 and NK3.3) and primary NK cells may reflect the heterogeneity of polyclonal cell populations regarding to the expression of NKp30, CD16, and ILT2/CD85j.

ILT2/CD85j cooperates with CD94/NKG2A to modulate IFN-γ production induced in NK cells by encounter with iDCs

The data obtained in NK-92 cells suggest that ILT2/CD85j might regulate the IFN-γ secretion induced by NKp30, and the results in peripheral blood purified NK cells support a mild but consistent inhibition of NKp30-induced IFN-γ production through ILT2/CD85j ligation. To analyze the role of ILT2/CD85j in this process more thoroughly, we challenged NK effector cells with monocyte derived-iDCs, as NKp30 has been shown to be the key player for IFN-γ induction in this system (23, 24). According to published data, maturation of myeloid DCs appears to be efficiently promoted in autologous and heterologous systems equally in a process strictly dependent on cytokine secretion by NK cells (22, 23). The major NK cell population promoting maturation of DC is represented by KIR-NKG2A<sup>+</sup> NK clones (51). According to its phenotype, the NK-92 cell line seemed to be a good model to set up experiments with allogenic iDCs. It has been suggested that CD94/NKG2A might be involved in the regulation of the production of IFN-γ by NK cells (24, 52). To eliminate the putative contribution of NKG2A, we masked CD94/NKG2A receptors by preincubation of NK cells with a blocking Ab anti-CD94. To avoid nonspecific effects due to binding to FcγRs expressed in NK cells and iDCs, F(ab’)2 were used. In addition, effector cells were preincubated with F(ab’)2 of a blocking mAb anti-ILT2/CD85j or F(ab’)2 from a control IgG. As shown in Fig. 8A, allogenic iDCs from the first donor (iDC1) stimulated NK-92 cells preincubated with anti-CD94, although to a lower extent than 721.221 cells, and the addition of a blocking Ab anti-ILT2/CD85j further allowed the stimulation of IFN-γ secretion. When iDCs from a second donor (iDC2) were used, the amount of cytokine released in the presence of a blocking anti-CD94 mAb was comparable to that found in the supernatants from cocultures with 721.221 target cells and the blocking of ILT2/CD85j resulted in a noticeable increase in the secretion of IFN-γ. The same results were obtained when short-term cultured primary NK cells (60% ILT2<sup>+</sup>) were exposed to allogenic iDCs from a third donor. In this case, the concentration of IFN-γ secreted in the presence of anti-CD94 was increased by 2-fold upon additional preincubation of NK cells with anti-ILT2/CD85j mAb (Fig. 8A, lower graph). In addition, increased maturation of DC as assessed by expression of CD83 was detected in the presence of anti-ILT2 (data not shown). As IL-12 might stimulate the production of IFN-γ by NK cells, we measured IL-12 p70 in the same culture supernatants. We did not detect any increase in IL-12 production by iDCs cocultured with autologous or allogenic NK cells during 20 h, as compared with the cytokine secreted by iDCs alone, whereas LPS treatment of iDCs induced a strong secretion of IL-12 (not shown).

To further dissect the contribution of ILT2/CD85j and CD94/NKG2A to this process, NK cells expressing or not ILT2/CD85j were purified from different donors and stimulated with allogenic iDCs, in the presence of F(ab’)2 from either CD94 or ILT2/CD85j blocking mAb, and the combination of both. The same experiments were performed with NK-92 cells as well. As shown in Fig. 8B, the production of IFN-γ was substantially increased in NK-92 cells only by the simultaneous presence of both anti-CD94 and ILT2/CD85j mAb. This result proved the complementary role played by both receptors in the recognition of iDCs. The same kind of outcome was obtained with purified ILT2/CD85j-expressing NK cells from some donors (Fig. 8A; donor NK7). In other ILT2/CD85j<sup>+</sup> polyclonal primary NK cell populations, blocking of CD94 alone resulted in improved IFN-γ production, but the release of cytokine to the medium was further increased by the additional masking of ILT2/CD85j-HLA class I interactions in all donors tested. The results obtained in five independent experiments performed with polyclonal NK cell populations containing variable proportions of ILT2/CD85j<sup>+</sup> cells are summarized in Fig. 8C. On the contrary, when NK cells with low or no expression of ILT2/CD85j were used in the same experiments, the secretion of IFN-γ augmented when anti-CD94 blocking Abs were used but it was not
Further increased by the combination with anti-ILT2/CD85j Abs (Fig. 8B, lower panels). As it has been described that NK cells are able to edit the DC population by killing iDCs (51), we analyzed whether ILT2/CD85j could also be involved in the regulation of iDC cytotoxicity. Cytotoxicity experiments were performed in which NK-92 cells or purified NK cell populations were tested for their ability to kill iDCs in the absence or presence of blocking Abs against CD94, ILT2/CD85j, and the combination of both. As shown in Fig. 8D, masking ILT2/CD85j-HLA class I interactions slightly increased the cytotoxic activity of the NK-92 cell line or NK cell populations containing ILT2/CD85j+ cells, against iDCs. However, in the presence of a blocking mAb anti-CD94, a higher cytolytic activity against allogeneic iDCs was obtained, which was never further increased by the simultaneous addition of anti-ILT2/CD85j and anti-CD94 mAb. Fig. 8D shows two representative experiments of five performed with the NK-92 cell line and three performed with ILT2/CD85j+ primary short-term cultured NK cells with similar results. When ILT2/CD85j+ NK cells were used, anti-CD94/CD85j mAb did not affect the lysis of iDCs. These data indicate that although ILT2/CD85j may contribute to the downregulation of iDC lysis by NK cells, this process is mainly controlled by CD94/NKG2A recognition of HLA-E, in agreement with previously reported data (51). On the contrary, the interactions of HLA class I molecules with ILT2/CD85j seem to be more relevant in the regulation of the secretion of IFN-γ during the NK-iDC cross talk.

**Discussion**

NK cells are important components of the innate immune system, and they participate in the early response against pathogens or malignant transformed cells through their cytolytic activity and via the secretion of chemokines and cytokines (21). Several recent findings highlight noncytolytic, cytokine, or chemokine-driven functions of NK cells and implicate them as major regulators of different immune and nonimmune processes in vivo (53). Amid the cytokines produced by NK cells, IFN-γ-secreted during the innate phase has been shown of great importance for maturation of DCs and shaping of consequent immune responses (22, 24). Although it is generally assumed that the major CD56bright CD16− subpopulation of peripheral blood NK cells is mainly responsible for cytokine production (54), Anfossi and colleagues (29) demonstrated that the dichotomy between cytolytic NK cells and cytokine producing CD56bright NK cells does not apply when freshly isolated NK cells were stimulated with tumor cells. By using freshly isolated human peripheral blood mononuclear cell preparations and a multi-parameter flow cytometric assay, these authors showed that most, if not all, NK cells that are cytolytic and/or produce IFN-γ upon K562 or Ab-coated target stimulation belong to the CD56dim subset, whereas NK cells that readily respond to IL-12 or IL-15 belong to the CD56bright NK cell subset. The authors propose to define CD56dim and CD56bright NK cell subsets as “target cell responsive” and “cytokine responsive,” respectively.
Variegated expression of inhibitory receptors for MHC class I confers specificity to individual NK cells. Specificity might also be provided by the engagement of an array of activating receptors, many of which signal through different pathways. How inhibitory receptors intersect different signaling cascades is not fully understood (55). It is generally assumed that iNKR down-regulate NK effector functions; however, there is evidence that divergent signals lead to either cytotoxicity or cytokine secretion in NK cells, and even different synapses lead to one effector function or the other (56). It has been shown that NK cell cytolytic function and cytokine secretion can occur in the same cells but do not precisely correlate (29). Moreover, perforin expression and IFN-γ production can be largely segregated also in CD8+ T cells (57). The regulation of cytolytic activity by iNKR has been widely explored, whereas only a few reports address the involvement of iNKR in cytokine secretion. KIR2DL2/3 and KIR3DL1 have been shown to inhibit IFN-γ production upon binding to HLA ligands in NK and T cells (27, 35, 36, 58), and CD94/NKG2A has been involved in the regulation of TNF-α and IFN-γ (37–39, 58).

A few reports have addressed the involvement of the iNKR ILT2/CD85j in the regulation of the cytolytic activity of peripheral NK cells or NK cell clones (8, 43, 44), and some evidence has been reported supporting regulation of cytolytic activity and IFN-γ secretion by ILT2/CD85j ligation in T cells (8, 13–15, 17). However, there is no information referring to interactions with natural HLA class I ligands of ILT2/CD85j in T cells, and no studies addressing the role of ILT2/CD85j in the regulation of IFN-γ production by NK cells are available. Therefore, an analysis of HLA class I involvement in cytokine production and release through binding to ILT2/CD85j was missing.

We have explored the capability of ILT2/CD85j to shape IFN-γ release by NK cells in three physiological situations known to trigger cytokine release: upon interaction with target cells, in response to viral products such as TLR3 ligands, and during the NK/iDC cross talk.

Our data reveal that interaction with target cells is able to up-regulate IFN-γ mRNA expression levels in NK cells and that this induction was specifically hampered by target expression of ILT2/CD85j HLA class I ligands. Moreover, we have found that ILT2/CD85j HLA class I ligands are able to down-regulate IFN-γ protein synthesis and secretion in NK-92 and in primary NK cells in response to target cells. Additionally the expression of ILT2/CD85j HLA class I ligands on APCs was able to down-regulate the production of IFN-γ induced by superantigens in T cells. In these experiments, we used HLA-Cw7, which is not recognized by ILT2/CD85j (19, 36), and two HLA class I molecules that have been shown to trigger inhibitory signals through ILT2/CD85j: HLA-B*2705 and HLA-G1 (8). Surface plasmon resonance studies suggest that the nonclassical HLA-G1 molecule binds ILT2/CD85j with more affinity than HLA-B molecules (12). However, in the experiments performed with primary cells, we have consistently found strong inhibition through binding to HLA-B*2705, whereas HLA-G1 was less efficient in triggering inhibition signals.

In this sense, it is of note that challenge with 221.G1m cells was hampered in primary NK cells, whereas CD94/NKG2A was not able to modulate cytokine secretion in NK cells from the same donor in some assays performed with unfractionated PBMCs, whereas 221.B*2705 always inhibited cytokine production (see Fig. 4). We have used 721.221 transfectants of three HLA class I molecules that do not support HLA-E cell surface expression (36, 43, 59). In the absence of HLA-E, neither CD94/NKG2A nor CD94/NKG2C could be involved in the recognition of these cell lines. Since KIR3DL1 is the only iKIR specific for HLA-B*2705, we addressed the analysis to ILT2+KIR3DL1+ cells, as no iKIR has been implicated in the recognition of HLA-G. Nonetheless, it has been described that HLA-G1 can stimulate IFN-γ secretion through binding to KIR2DL4 (60), which is transcribed in all NK and T cells expressing KIRs (61). It is possible that positive signals triggered by KIR2DL4 (or another as yet undescribed activating receptor) in T cells counterbalance the inhibitory signal delivered by ILT2/CD85j in these donors. It is noteworthy that most ILT2/CD85j+ T cells are KIR− but express KIR2DL4 (47). In this regard, it has been described that homozygous individuals for KIR2DL4/9A genotype are not able to express the protein on the cell surface of NK cells (62, 63). Interestingly, one of the donors in which activation was observed by 221.G1m was homozygous for KIR2DL4.2 (E. Morel and T. Bellón, unpublished data). Additional mechanisms might control the expression of this receptor in T cells or, alternatively, different HLA-G-specific activating receptors may trigger cytokine production in T cells. The results obtained upon T cell stimulation with SEB-preincubated 221.B*2705 suggest that classical HLA class I molecules down-regulate TCR-driven cytokine secretion upon interaction with ILT2/CD85j. However, based on results obtained upon stimulation with SEB-preincubated 221.G1m it is tempting to speculate that other HLA-G-specific receptors expressed on T cells may override the inhibitory signal delivered upon binding to classical HLA class I molecules. Nevertheless, HLA-G-ILT2/CD85j interactions might be relevant in some physiological or pathological situations. HLA-G has a limited distribution pattern restricted to trophoblast cells, which do not transcribe HLA-A or HLA-B class I alleles. It has been proposed that HLA-G plays a major role in maternal-fetal immune tolerance through ligation of ILT2/CD85j expressed by uterine immune cells (12). In humans, over 85% of these cells are NK cells during the first weeks of pregnancy (53). In contrast, it has been proposed that HLA-G expressed in trophoblast cells has an activating role upon interacting with decidual NK cells, in which HLA-G stimulated IFN-γ production (64). It is tempting to speculate that HLA-G interaction with ILT2/CD85j modulates the secretion of cytokine to keep optimal levels of IFN-γ, which has been shown to be essential for normal placentation (53). Moreover HLA-G expression may be induced in other cell types in response to inflammation, transformation, and infection (65). In particular, it has been described that HLA-G is induced in CMV infection. Interestingly, ILT2/CD85j expression is also enhanced in NK and T cells of human CMV chronically infected individuals (66).

It has been recently shown that NK cells can also be directly activated to secrete IFN-γ by viral products (48–50). We tested whether the iNKR ILT2/CD85j could also modulate this effector response triggered by TLR3. Although we did not find any inhibitory effect in NK-92 cell line, we consistently detected a diminished secretion of IFN-γ in response to poly(I:C) when the ILT2/CD85j receptor was stimulated in primary NK cells. Similarly, other iNKR such as CD94/NKG2A and KIR3DL1 were able to down-regulate the secretion of IFN-γ induced by poly(I:C) in primary NK cells, whereas CD94/NKG2A was not able to modulate poly(I:C)-induced cytokine secretion in NK-92 cells (data not shown). The lack of inhibitory response in NK-92 cells could be ascribed to the strong stimulation of IFN-γ production in these cells; however, this explanation seems unlikely as 721.221 target cells also stimulated the production of high amounts of IFN-γ, which was inhibited by HLA ligands. Although it is possible that the immunobilized Ab did not precisely mimic the interaction with HLA, a different explanation could be that, as the initial signal transduction machinery used by TLR3 (50) is different from that initiated by target synapses, the point where ILT2/CD85j and other iNKR-driven inhibition intersects with signals delivered by TLR3 could be hampered specifically in the NK-92 cell line.
or, alternatively, in fully activated NK cells represented by NK-92 cells. The opposite has already been described for KIR2DL4-induced IFN-γ secretion, which is down-regulated by CD94/NKG2A in activated but not in resting NK cells (34). Experiments in which ILT2/CD85j- and CD94/NKG2A-specific ligands down modulated the cytotoxic activity stimulated by poly(I:C) further support a role for iNKR in the modulation of activating signals induced by viral products.

The bidirectional cross talk between NK and iDC in the course of the immune response is another situation in which an important induction of IFN-γ release by NK cells takes place. During this process, NKP30 receptor signaling seems to have a pivotal role. Ferlazzo and colleagues (23) showed that activated NK cells exposed to iDCs released amounts of IFN-γ comparable to those induced by interaction with targets, and that anti-NKP30 blocking Abs inhibited IFN-γ secretion in NK clones and polyclonal NK cells. Later, Vitale and colleagues (24) demonstrated that NK-dependent DC maturation is mediated by TNF-α and IFN-γ released upon engagement of the NKP30 receptor. These authors did not find differences between the production of cytokine in autologous and allogeneic NK/iDC mixed cultures, although the engagement of KIRs seemed to weaken the ability of NK cells to induce DC maturation; the involvement of ILT2/CD85j in the process was not addressed. In a first approach to this issue, we explored NKP30-driven cytotoxicity and cytokine secretion in two different NK cell lines in which a consistent inhibition of both effector functions by ILT2/CD85j receptor was found. In particular, IFN-γ production was inhibited by 80% in NK-92 cells. However, only modest inhibitions of ~20% were found in primary NK cells, even if donors with high expression levels of ILT2/CD85j were selected. Again, it is possible that the immobilized Ab did not precisely mimic the interaction with HLA. An alternative explanation is that in polyclonal cell populations, the production of IFN-γ triggered in CD16+ILT2+ or NKP30+ILT2 primary NK cell clones is masking the inhibition delivered by ILT2/CD85j in CD16+ILT2+ or NKP30+ILT2 cells. In this sense, it has been described that ILT2/CD85j+ NK cells express lower levels of NKP30 receptor (66).

The inhibitory function of ILT2/CD85j was also explored in a cellular experimental setting in which NK cells were cocultured with iDCs. As CD94/NKG2A has been suggested to regulate IFN-γ secretion during NK/iDC interactions (24), in a first approach, an anti-CD94 blocking Ab was always added to the system. In this situation, masking ILT2/CD85j-HLA class I recognition with an anti-ILT2/CD85j blocking Ab further stimulated the secretion of IFN-γ by NK-92 and short-term cultured NK cells. In these experiments, NK cells were preincubated with blocking Abs before the addition of iDCs, and these reagents were maintained in the culture media during the assay. As iDCs also express ILT2/CD85j on the cell surface, a contribution of ILT2/CD85j blocking iDC activation could be argued, as IFN-γ secretion by NK cells is strongly up-regulated by IL-12 produced by iDCs. In contrast, NK production of IFN-γ has been described in the absence of IL-12 (67). In our experimental setting, we did not detect any increase in IL-12 production by iDCs cocultured with NK cells for 20 h, as compared with the cytokine secreted by iDCs alone. To analyze the contribution of each receptor to the regulation of cytokine production, NK/iDC coculture experiments were conducted in which NK cells were preincubated with either CD94 or ILT2/CD85j blocking Abs or the combination of both. When these experiments were performed with NK-92 cells which homogeneously express CD94/NKG2A and ILT2/CD85j, we found that neither anti-CD94 nor anti-ILT2/CD85j mAb alone were able to increase substantially the production of IFN-γ upon encounter with iDCs. When polyclonal populations of short-term cultured NK cells were tested in the same kind of experiments, two patterns of response were found. The first one was similar to that observed in NK-92 cells, in which the simultaneous blocking of both receptors was necessary to improve the production of cytokine. The second pattern corresponded to those cultures in which an anti-CD94 mAb alone was able to promote IFN-γ production but the secretion was further increased by the simultaneous addition of anti-ILT2/CD85j and anti-CD94 mAbs. This complementary effect of anti-CD94 and anti-ILT2 mAbs was always found when IFN-γ was measured in the supernatants of cocultures containing ILT2+ NK cells but not when ILT2+ NK cell populations were used. On the contrary, when the cytotoxic activity against iDCs was evaluated, the addition of anti-ILT2 together with anti-CD94 mAbs was never able to further increase the cytolytic activity of ILT2+ NK cell cultures against iDCs above that obtained with anti-CD94 alone. A similar behavior was reported in ILT2+CD8− T cells in which, while blocking ILT2/CD85j engagement had little effect on cytotoxicity, it increased IFN-γ production (17) and it is reminiscent of that observed in KIR2DL2+CD4+ T cells (68), in which KIR2DL2 binding to its ligand HLA-Cw3 was not able to prevent degranulation, but it was able to inhibit late signaling events such as the synthesis of IFN-γ. As ILT2/CD85j recognizes a broad spectrum of HLA class I alleles, our results strongly support a role for the engagement of ILT2/CD85j by self HLA class I ligands in the regulation of IFN-γ release by NK cells during the encounter with iDCs. Nonetheless, as both receptor and ligands are expressed in both cell types, a down-regulation of DC effector functions by ILT2/CD85j ligation of self HLA expressed on NK cells cannot be ruled out.

IFN- γ plays a fundamental role during inflammation, tumor immunity, the shaping of Th1 responses, and autoimmune disorders (69). The specificity of ILT2/CD85j is reminiscent of that described for CD94/NKG2A, since both receptors survey for the overall expression of a broad range of HLA class I molecules. However, ILT2/CD85j, unlike CD94/NKG2A, also recognizes HLA-B alleles that lack a leader peptide allowing surface expression of HLA-E molecules. IFN-γ, a dominant cytokine generated during innate and adaptive phases of the immune response, up-regulates the expression of HLA class I molecules and, therefore, the expression of iNKR ligands. If iNKR-transduced negative signals overrode activating signals, IFN-γ could impair effector functions through the up-regulation of HLA class I. This possibility is supported by evidence that IFN-γ can facilitate viral evasion of NK killing by up-regulating HLA-expression on infected cells (70). It has been hypothesized that IFN-γ would up-regulate the expression of HLA-E, which upon interaction with CD94/NKG2A in tumor infiltrating CTLs would impair tumor rejection (71). This hypothesis could be extended to ILT2/CD85j recognition of HLA class I molecules and a variety of effector functions performed by different cell lineages expressing ILT2/CD85j and would explain some negative results obtained with IFN-γ therapy in vivo.

In contrast, our results suggest that by up-regulating a variety of HLA class I ligands for ILT2/CD85j, IFN-γ may provide a negative-feedback regulation for its own production in a variety of situations. This mechanism would down-modulate ongoing inflammatory responses, and alteration of this regulatory mechanism could contribute to the pathophysiology of chronic inflammatory or autoimmune disorders. In this sense, it is of note that NK and CD8 T cells are the main IFN-γ producers in response to superantigens (72), and that, in T lymphocytes, ILT2/CD85j is almost exclusively expressed by the CD8 T-cell subpopulation in healthy individuals (our own unpublished results). Moreover ILT2/
CD85j+CD8− cells predominate over KIR+CD8− cells within the IFN-γ secreting T cells (73).

In conclusion, our data formally demonstrate the regulation of the production of IFN-γ by NK and T cells upon HLA class I recognition by the inhibitory receptor ILT2/CD85j and strongly suggest that, in addition to increasing the threshold of activation of immune cells, ILT2/CD85j might participate in a regulatory feedback mechanism leading to the maintenance of optimal cytokine levels or termination of immune responses. Moreover, our data suggest by the first time that iNKRs may control NK cell functions stimulated by TLRs.

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Disclosures
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