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IL-12-Dependent Inducible Expression of the CD94/NKG2A Inhibitory Receptor Regulates CD94/NKG2C⁺ NK Cell Function

Andrea Sáez-Borderías,* Neus Romo,* Giuliana Magri,† Mónica Gumá,* Ana Angulo,‡ and Miguel López-Botet**††

The inhibitory CD94/NKG2A and activating CD94/NKG2C killer lectin-like receptors specific for HLA-E have been reported to be selectively expressed by discrete NK and T cell subsets. In the present study, minor proportions of NK and T cells coexpressing both CD94/NKG2A and CD94/NKG2C were found in fresh peripheral blood from adult blood donors. Moreover, CD94/NKG2A surface expression was transiently detected upon in vitro stimulation of CD94/NKG2C⁺ NK cells in the presence of irradiated allogeneic PBMC or rIL-12. A similar effect was observed upon coculture of NKG2C⁺ NK clones with human CMV-infected autologous dendritic cell cultures, and it was prevented by an anti-IL-12 mAb. NKG2A inhibited the cytolytic activity of NKG2C⁺ NK clones upon engagement either by a specific mAb or upon interaction with a transfectant of the HLA class I-deficient 721.221 cell line expressing HLA-E. These data indicate that beyond its constitutive expression by an NK cell subset, NKG2A may be also transiently displayed by CD94/NKG2C⁺ NK cells under the influence of IL-12, providing a potential negative regulatory feedback mechanism.

Natural killer cells participate in the innate immune response against microbial pathogens and tumors, exerting cytotoxicity and cytokine production. Triggering of NK cell effector functions depends on the integration of signals delivered by an array of inhibitory and activating receptors. Some inhibitory receptors are engaged by self MHC class I molecules preventing NK cell reactivity against normal autologous cells. Loss of inhibitory receptors and/or expression of ligands for activating receptors render tumors and infected cells susceptible to the NK cell attack (1–4). The human NK cell receptor (NKR) repertoire is defined by the different combinations of receptors acquired by individual NK clones along differentiation, and it is conditioned by the genomic diversity at the killer Ig-like receptor (KIR) locus (5, 6).

Different NKR gene families (i.e., NKG2, KIR, and Ly49) encode for pairs of homologous activating and inhibitory molecules that may interact with the same ligand. Among them, the NKG2A and NKG2C C-type lectin molecules are expressed as heterodimers coupled to CD94, forming receptors with opposite functions (7, 8). The inhibitory NKG2A molecule contains cytoplasmic ITIMs that recruit SHP-1 and SHP-2 tyrosine phosphatases (9). In contrast, NKG2C is coupled through the DAP12/KARAP adaptor to a tyrosine kinase-dependent pathway, triggering NK cell effector functions (10). Both receptors specifically recognize HLA-E, which presents peptides derived from the leader sequence of other HLA class I molecules (11–13); the affinity of their interaction depends on the sequence of the HLA-E-bound nonamers and appears higher for CD94/NKG2A (14–16). According to recent structural analyses, the CD94 subunit plays a pivotal role in the interaction between CD94/NKG2A and the HLA-E/peptide complex (17, 18). In mice, CD94/NKG2 receptors are conserved and recognize the Qa1b class Ib molecule, which presents as well MHC class I-derived peptides (19, 20).

CD94/NKG2A and CD94/NKG2C are displayed by subsets of human NK cells, γδ and αβ T lymphocytes (7, 8, 21). Differences in the expression pattern of other NK cell receptors have been noticed comparing NKG2A⁺ and NKG2C⁺ NK cells (22, 23). CD94/NKG2A appears detectable at relatively early stages of NK cell differentiation, preceding the expression of KIR (24, 25); in contrast, this inhibitory receptor may be displayed by T cells with an effector/memory phenotype and is inducible in vitro under the influence of some cytokines (26–28). Little information is currently available on the expression of NKG2C during NK and T cell differentiation (24, 29). In this regard, increased numbers of circulating NKG2C⁺ NK cells have been associated to a positive serology for human cytomegalovirus (HCMV) in healthy individuals and aviremic HIV-1-infected patients (22, 23, 30). Furthermore, an expansion of CD94/NKG2C⁺ cells upon in vitro interaction with HCMV-infected fibroblasts was reported (31). Taken together, these observations support the hypothesis that NKG2C⁺
cells may play a role in the response to HCMV, and that the life-long persistent viral infection may shape the NKR repertoire. Functional characterization of CD94/NKG2 receptors has been reported in NK and T cell subsets selectively bearing NKG2A or NKG2C (9, 10, 32–34). However, these genes may be cotranscribed at the clonal level in some NK and T cells (35, 36), and both proteins have been detected together at the surface of decidual and peripheral blood CD56<sup>+</sup> NK cells (37). The functional implications resulting from coexpression at the single cell level of activating and inhibitory NKR specific for the same ligand are unknown.

In the present study, minor NK and T cell subsets coexpressing CD94/NKG2A and CD94/NKG2C were found in fresh blood samples from adult donors. Moreover, we provide evidence that, under the influence of IL-12, NKG2A may be transiently displayed by CD94/NKG2C<sup>+</sup> NK cells, inhibiting their cytolytic activity against target cells bearing HLA-E. Such an expression pattern is reminiscent of that observed for the CD28 and CTLA-4 leukocyte receptors, where the inhibitory molecule induced upon cell stimulation serves as a negative regulatory feedback mechanism (38).

Materials and Methods

Subjects

Peripheral blood samples were obtained in EDTA tubes by venous puncture from 195 adult individuals, including 104 men and 91 women (age range, 18–79 years; mean ± SD, 49.2 ± 17.2 years; median age, 50 years). Written informed consent was obtained, and the study protocol was approved by the Comité de Ética e Investigación Clínica-Instituto Municipal d’Assistência Sanitaria.

Antibodies

HP-3B1(IgG2a) anti-CD94 mAb and anti CD94 F(ab’)<sub>2</sub> fragments were prepared as previously described (39). Z199 (IgG2b) anti-NKG2A mAb was provided by Dr. A. Moretta (University of Genova, Italy) and was conjugated to FITC (Sigma-Aldrich). HP-3E4 anti-KIR2DL1/S1/S3 and HP-F1 anti-ILT2 were generated in our laboratory and have been previously described (40). 5.133 anti-KIR3DL2 was provided by Dr. Marco Colonna. Dx9 anti-KIR3DL1 mAb was provided by Dr. Lewis Lanier (University of California at San Francisco, San Francisco, CA). CH-L anti-KIR2DL2/S2L3 was provided by Dr. Silvano Ferrini (University of Genova, Italy). 3A1 (IgG2b) anti-CD7 and 9E10 (IgG1) anti-myc hybridoma have been described (41, 42), and the 20C2 (IgG1) anti-IL-12 hybridoma was obtained from the American Type Culture Collection. Anti-NKG2C (MAb 13813B, IgG2b) and anti-NKG2C-PE mAb were from R&D Systems. Anti-CD3-PerCP and anti-CD56-allophycocyanin were from BD Pharmingen. Indirect immunofluorescence analysis was conducted with a FITC-tagged F(ab’)<sub>2</sub> rabbit anti-mouse Ig Ab (Dako) or allophycocyanin-labeled goat anti-mouse Ig (BD Pharmingen).

Immunofluorescence and flow cytometry analysis

Immunofluorescence staining was performed according to the protocols detailed below, and samples were analyzed by flow cytometry (FACS) (BD LSR; BD Biosciences). For immunochemical analysis, whole blood samples were incubated with anti-NKG2A-FITC; subsequently, samples were incubated with anti-NKG2C-PE (R&D Systems), anti-CD3-PerCP, and anti-CD56-allophycocyanin (BD Pharmingen). After washing, erythrocytes were lysed with FACS lysis buffer (BD Biosciences) and cells were resuspended in PBS. For indirect immunofluorescence staining of fresh PBMC, cells were isolated by centrifugation on Ficoll-Hyphaque (Axis-Shield) and incubated with unlabeled Abs (HP-3E4, 5.133, Dx9, CH-L, and HP-F1) followed, after washing, by allopococyanin-labeled goat anti-mouse Ig, and NKG2A-FTTC, NKG2C-PE, and CD3-PerCP staining.

For indirect immunofluorescence staining of polyclonal NK cell populations and NK clones, samples were incubated with the individual unlabeled Abs followed, after washing, by a FITC-tagged F(ab’)<sub>2</sub> rabbit anti-mouse Ig Ab (Dako). Cells were treated with propidium iodide (Sigma-Aldrich) to exclude dead cells during flow cytometry analysis; appropriate isotype-matched control mAbs were used to assess nonspecific binding.

Cell cultures

The RPMI-8866 B lymphoma cell line and the 721.221 (221) HLA class I-deficient EBV-transformed B lymphoblastoid cell line (LCL) were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (10 μg/ml), referred to as complete medium. The HLA-E-positive 721.221-AEH (221-AEH) cell line (221 cells with a construct in which the leader sequence of the HLA-E*0101 allele was replaced by that of HLA-A2 (43); 221-AEH cells were cultured in complete medium supplemented with 300 μg/ml hygromycin B (Calbiochem). PBMC were isolated by centrifugation on Ficoll-Hyphaque (Axis-Shield), to obtain polyclonal-activated NK cell populations, PBMC (1.5 × 10<sup>6</sup> cells/ml) were cocultured in complete medium with irradiated (40 Gy) RPMI-8866 or 221-AEH cells (0.5 × 10<sup>5</sup> cells/ml) in 24-well plates. After 8–10 days cells were harvested, washed, and incubated with an anti-CD3 mAb followed by complement lysis with rabbit sera (Sera Laboratories International) to remove T cells, as described (39). Purified CD3<sup>+</sup> CD56<sup>+</sup> NK cell populations (>98%) were expanded in the presence of rIL-2 (400 U/ml). Polyclonal NK cells generated with 221-AEH cells were >92% NKG2C<sup>+</sup> and are referred to as NKG2C<sup>+</sup>. Alternatively, NKG2C<sup>−</sup> NK cell polyclonal populations were also generated by sorting of NKG2C<sup>+</sup> cells from PBMC and stimulation at 2 × 10<sup>6</sup> cells/ml with rIL-2 (1000 U/ml), 2 μg/ml PHA, irradiated (40 Gy) allogeneic PBMC (4.5 × 10<sup>5</sup> cells/ml), and RPMI-8866 (9 × 10<sup>5</sup> cells/ml). NK cell polyclonal populations generated with rIL-12, NKG2A<sup>−</sup> and NKG2C<sup>-</sup> contained NKG2A<sup>−</sup> and NKG2C<sup>−</sup> cells, and were subjected to a negative selection incubating with anti-NKG2C Ab followed by sheep anti-mouse IgG DynaBeads (Dynal Biotech), as recommended by the manufacturer. The resulting NKG2C<sup>−</sup> NK cell populations contained NKG2A<sup>+</sup> cells as well as some NKG2A<sup>−</sup> NKG2C<sup>−</sup> cells and are referred to as NKG2A<sup>+</sup> NKG2C<sup>−</sup> polyclonal NK cells. CD94/NKG2C<sup>−</sup> and CD94/NKG2A<sup>−</sup> NK cell clones were derived from fresh NKG2C<sup>−</sup> or NKG2A<sup>−</sup> PBL sorted by flow cytometry (FACS) (Adjacent SE, BD Biosciences). As described (44), cells were cultured under limiting dilution conditions in 96-well plates with irradiated (40 Gy) allogeneic PBMC (1.5 × 10<sup>5</sup> cells/ml) and RPMI-8866 cells (0.3 × 10<sup>5</sup> cells/ml), in the presence of rIL-2 (1000 U/ml) and 2 μg/ml PHA (Sigma-Aldrich), and were restimulated every 15 days with irradiated feeder cells and rIL-2. In some assays, polyclonal NK cells or NK clones were restimulated with irradiated (40 Gy) PBMC and RPMI-8866 cells, rIL-15 (30 ng/ml), rIL-12 (20 ng/ml), or rIFNγ (20 ng/ml) (PeproTech) in the presence of rIL-2 (400 U/ml).

Virus stock preparation

Stocks of HCMV strain TB40E (45) (kindly provided by Christian Sinzger, Institute for Medical Virology, University of Tübingen, Tübingen, Germany) were prepared by infecting MRC-5 cells at low multiplicity of infection. Infected cell supernatants were harvested when maximum cytopathic effect was reached and cleared of cellular debris by centrifugation at 1750 × g for 10 min. Virus was pelleted twice through a sorbitol cushion (20% w-sorbitol in TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl)) by centrifugation 90 min at 27,000 × g at 15°C. Pelleted virus was resuspended in DMEM supplemented with 3% FCS and titrated by standard plaque assays on MRC-5 cells.

HCMV infection of dendritic cells (DC)

After Ficoll-Hyphaque separation of PBMC, monocytes were obtained by positive selection of CD14<sup>+</sup> cells using magnetic separation (Miltenyi Biotec). To differentiate into DC, CD14<sup>+</sup> cells were plated at 1 × 10<sup>6</sup> cells/ml and cultured in the presence of IL-4 (25 ng/ml) and GM-CSF (50 ng/ml) (PeproTech). After 6 days, cells were CD14<sup>+</sup> CD1a<sup>−</sup> CD83<sup>−</sup> as assessed by immunofluorescence staining. As previously described (46), monococyte-derived DC (moDC) were cultured overnight in 24-well plates (4 × 10<sup>5</sup> cells/well) with the TB40E HCMV strain (multiplicity of infection of 100). After incubation, DC were washed and incubated in 48-well plates (3 × 10<sup>5</sup> cells/well) with NK cells (3 × 10<sup>5</sup> cells/well) in a final volume of 400 μl; cells were harvested after 5 days for analysis. In some experiments, anti-IL-12 or anti-myc hybridoma supernatants were added (1/5 dilution) at the beginning of the coculture. To test IL-12 production, supernatants of mock-treated or HCMV-infected moDC were harvested 48 h postinfection and analyzed by a human IL-12 (p70) ELISA test (Bender MedSystems).

Cytotoxicity assays

As previously described (44), the cytolytic activity of NK clones against the murine FcγRII<sup>+</sup> P185 mastocytoma cell line was analyzed in a 4-h <sup>51</sup>Cr-release reverse Ab-dependent cell-mediated cytotoxicity.
were set up in triplicate and specific lysis was calculated as described (47).

face HLA-E bound to an HLA class I leader sequence (43). All assays compared with the NKG2C^+ /H11001^+ examples of different distribution patterns of NKG2A^+/H11001^+ CD56^−/H11001^+ T cell subsets (b). Numbers correspond to the proportions of stained cells. The distribution of CD56^dim^ and CD56^bright^ subsets was assessed in NKG2C^+ /NKG2A^+ populations (c). The expression of KIR and ILT2 by NKG2C^+ /NKG2A^+ cells was also studied in five different individuals and compared with the NKG2C^+ /NKG2A^− subset; a representative distribution pattern is shown in d.

Results

NKG2A and NKG2C are coexpressed by subsets of peripheral blood NK and T cells

To study the distribution of CD94/NKG2A and CD94/NKG2C receptors, a multicolor flow cytometry analysis was conducted in peripheral blood samples from a population of adult individuals (n = 195). Fig. 1 presents representative examples of the different distribution patterns observed. The proportions of NK (CD3^−/CD56^+) and T cells (CD3^+/CD56^−/CD3^+^/CD56^−) stained by NKG2A^+ and/or NKG2C^+ specific mAbs are shown in Table I. Although most NK cells were either NKG2A^−/NKG2C^−, or NKG2A^+ /NKG2C^+ variable numbers of double-positive NKG2C^−/NKG2A^− NK cells and T cells were detectable in different donors, indicating that both receptors may be expressed together at the cell surface in vivo. In contrast to a previous report (37), NKG2C^+ /NKG2A^− cells were found among both the CD56^dim^ and CD56^bright^ NK cell subsets (Fig. 1C) and, predominantly, within the CD56^+ T cell subset (Fig. 1B). No significant correlation between the proportions of double-positive NKG2C^+ /NKG2A^+ NK and T cells was substantiated.

Whether double-positive NKG2A^+ /NKG2C^+ cells bear additional inhibitory receptors for HLA class I molecules appeared a relevant question, considering that the NKG2C^+ population has been reported to contain higher proportions of KIR^+ /ILT2^+ cells than NKG2A^+ cells (22). To directly address this point, a multicolor analysis of NKG2C^−/NKG2A^+ cells was performed in PBMC from five donors, assessing the expression of KIRs (using a mixture of anti-KIR mAbs) and ILT2 (LIR-1) receptors specific for HLA class I molecules on NK cells. Variable proportions of CD3^+ /NKG2C^−/NKG2A^+ cells were stained by anti-KIR (mean ± SD, 48 ± 15%) and anti-ILT2 (mean ± SD, 39 ± 14%) mAbs; similarly, a subset of CD3^−/NKG2C^+ /NKG2A^+ NK cells displayed KIR (mean ± SD, 26 ± 4%) or ILT2 (mean ± SD, 32 ± 36%). Remarkably, a fraction of double-positive cells that did not express KIR or ILT2 was clearly identified (Fig. 1D), whereas most NKG2C^+ /NKG2A^+ cells were stained by the combination of ILT2- and KIR-specific mAbs (Fig. 1D). It is of note that as homologous activating and inhibitory KIRs could not be discriminated by specific mAbs, coexpression of NKG2A with inhibitory KIRs could not be precisely defined. The detection of both KIR^+ and KIR^− NKG2A^+ /NKG2C^+ cells was consistent with the observation that NKG2A and NKG2C coexpression is not restricted to the CD56^bright^ population, previously reported to be predominantly KIR^−.

Table I. Proportions of NK (CD3^−/CD56^+) and T cells (CD3^+/CD56^−/CD3^+^/CD56^−) stained by NKG2A^+ and/or NKG2C^+ specific mAbs

<table>
<thead>
<tr>
<th>Subset</th>
<th>CD3^−CD56^+</th>
<th>CD3^+CD56^−</th>
<th>CD3^+CD56^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKG2A^+</td>
<td>34.2 ± 13.2 (6.1–69.8)</td>
<td>15.5 ± 15.8 (0–83.4)</td>
<td>2.9 ± 2.1 (0.2–16)</td>
</tr>
<tr>
<td>NKG2C^+</td>
<td>15.4 ± 14.7 (0.2–84.2)</td>
<td>9.6 ± 11.9 (0.5–84.2)</td>
<td>0.8 ± 1 (0.01–8.6)</td>
</tr>
<tr>
<td>NKG2A^−/NKG2C^+</td>
<td>3.2 ± 2.5 (0.5–18.2)</td>
<td>2.4 ± 6.1 (0–48.4)</td>
<td>0.1 ± 0.3 (0–1.9)</td>
</tr>
</tbody>
</table>

* Multicolor flow cytometry analysis carried out in whole blood from adult donors (n = 195).

* Numbers correspond to the proportions of stained cells. Mean ± SD (range).
Surface NKG2A expression is inducible upon CD94/NKG2C NK cell activation

In previous studies we observed that NKG2C and NKG2A were occasionally found together at the surface of in vitro-activated NK cells. We addressed whether coexpression of both NKG2 receptors resulted from the expansion of double-positive cells or, alternatively, was induced upon cell activation. To this end, polyclonal NKG2C+H11001 and NKG2A+H11001 NK cell populations, with the latter including also a subset of NKG2A+H11002 NKG2C+H11002 cells, were generated as described in Materials and Methods and their phenotype was analyzed upon in vitro stimulation. After a 5-day coculture with irradiated allogeneic feeder cells (PBMC and RPMI-8866) and rIL-2, substantial proportions of NKG2A+H11001 cells were detected among the NKG2C+H11001 polyclonal population; in contrast, NKG2A+H11001 cells remained NKG2C+H11002 (Fig. 2). To more precisely assess whether NKG2A expression was inducible upon in vitro stimulation, ruling out the outgrowth of double-positive NK cells, NKG2C+H11001 cells were sorted and cloned under limiting dilution conditions in the presence of irradiated feeder cells and rIL-2. After expansion for at least 1 mo, which required periodical restimulation, substantial proportions of NKG2C+H11001 NK clones displayed variable levels of NKG2A (Fig. 3A); under the same experimental conditions, NKG2A+H11001 clones remained NKG2C+H11002 (Fig. 3B). Sequential phenotypic analysis revealed that expression of NKG2A in NKG2C+H11001 clones was unstable, progressively decreasing along in vitro culture in the presence of rIL-2 and becoming detectable again upon restimulation with irradiated feeder cells (Fig. 4A).

To evaluate the relative contribution of feeder cells in the induction of NKG2A expression, NKG2C+H11001 clones were restimulated with irradiated PBMC or/and RPMI-8866 cells. Our results indicated that only incubation with PBMC was sufficient to induce NKG2A expression, whereas RPMI-8866 cells enhanced the effect (Fig. 4B).

IL-12 induces NKG2A expression on NKG2C+H11001 NK cells

Previous reports described that NKG2A expression was inducible in T cells by IL-12 stimulation (28) and upon TCR-dependent activation in the presence of IL-15 or TGFβ (26, 27). Moreover, increased proportions of NKG2A+H11001 lymphocytes were observed in NK cell populations cultured in the presence of IL-12 (48). To evaluate whether any of these cytokines accounted for the inducible expression of NKG2A in NKG2C+H11001 cells, polyclonal
NKG2C⁺ NK cell populations and NKG2C⁺ clones, cultured in rIL-2 containing medium, were treated with rIL-15, rTGF-β, or rIL-12. As shown in Fig. 5, NKG2A was up-regulated in both NKG2C⁺ polyclonal populations and clones upon incubation with rIL-12, whereas no effect of the other cytokines was substantiated (data not shown). NKG2A expression appeared also induced in the NKG2A⁺ NKG2C⁺ subset, whereas none of these stimuli promoted NKG2C expression in NKG2A⁺ NK cells (Fig. 5).

NKG2A expression is inducible in NKG2C⁺ NK cells stimulated with HCMV-infected autologous DC

To address whether NKG2A expression may be inducible in the context of the NK-DC crosstalk during the innate immune response to infections, we used HCMV-infected DC as an experimental model. To this end, immature moDC were infected as described with the TB40E HCMV strain (46). The proportions of infected moDC identified by detection of the IE1 Ag ranged between 25% and 95%, and cells remained CD83⁺ after infection. Mock-treated and HCMV-infected moDC were cocultured either with autologous polyclonal NKG2C⁺ NK populations or clones. Under these conditions, NKG2A became detectable on the surface of NKG2C⁺ cells stimulated with infected but not mock-treated moDC (Fig. 6A). The effect could be markedly inhibited by an anti-IL-12 Ab, further supporting a central role of the cytokine. To directly check the presence of IL-12 in the system, mock-treated and virus-infected moDC were plated alone at the same concentrations employed for NK cell cocultures, and supernatants were harvested after 48 h. Consistent with their ability to induce NKG2A expression, IL-12 was detectable by ELISA only in the supernatants of infected moDC (Fig. 6B). These data support that
endogenous IL-12 secretion may up-regulate the expression of the NKG2A inhibitory receptor in the NKG2C+ population during the innate immune response to HCMV infection.

Engagement of the CD94/NKG2A receptor inhibits the cytolytic activity of CD94/NKG2C+ NK clones

The implications resulting from coexpression at the clonal level of inhibitory and activating receptors specific for the same ligand depend on their signaling capacity. Our results suggested that the inducible expression of NKG2A in NKG2C+ NK clones might constitute a negative feedback regulatory mechanism. To assess the inhibitory function of CD94/NKG2A in double-positive NKG2C+ NKG2A+ NK clones, cells were first tested in redirected lysis assays against the P815 mastocytoma cell line in the presence of mAbs specific for NKG2A, NKG2C, and CD94 or control anti-CD7 mAb (E/T ratio of 2:1). Three representative examples of the different patterns of response observed are shown.

To further examine the inhibitory role of NKG2A in NKG2C− cells, double-positive NK clones were tested in cytotoxicity assays against the .221-AEH cell line that displays surface HLA-E in the absence of classical HLA class I molecules, and was generated by stable transfection of the 721.221 cell line with a construct in which the leader sequence of the HLA-E*0101 allele was replaced by that of HLA-A2 (43). NKG2A−NKG2C+ and NKG2C+NKG2A− clones were also comparatively studied in these assays. Cytotoxicity mediated by double-positive NK clones, but not by NKG2C+NKG2A− cells, against .221-AEH cells appeared lower than that detected against wild-type .221 cells (Fig. 8A), but was restored in the presence of anti-CD94 mAb F(ab′)2 (Fig. 8B). These data support that the inducible CD94/NKG2A inhibitory receptor may regulate the response of CD94/NKG2C+ NK cells by engaging HLA-E bound to leader sequence peptides from other HLA class I molecules (11–13); it is of note that CD94/NKG2A has a higher affinity for HLA-E than does CD94/NKG2C (14–16).

Discussion

The inhibitory CD94/NKG2A and activating CD94/NKG2C receptors are constitutively displayed by discrete NK cell subsets (1, 22). In the present report we provide evidence supporting that, under the influence of IL-12 stimulation, CD94/NKG2A is also transiently inducible in NK cells bearing the homologous CD94/NKG2C-activating receptor. Moreover, our results indicate that CD94/NKG2A is functional, regulating the response of CD94/NKG2C+ cells against targets bearing HLA-E, the natural ligand shared by both lectin-like receptors. This situation is reminiscent of the activation-dependent expression of CTLA-4 on T lymphocytes, which counteracts CD28-mediated costimulation (38). By analogy, it is conceivable that acquisition of the NKG2A inhibitory receptor may play a physiological role, providing a reversible regulatory feedback mechanism to control the activation of NKG2C+ cells. The higher affinity of CD94/NKG2A for HLA-E likely favors its competition with CD94/NKG2C for ligand engagement, as described for the CD28/CTLA-4 pair. This would enhance the efficiency of the regulatory mechanism, compensating the lower surface levels of the inhibitory receptor transiently expressed by activated CD94/NKG2C+ cells. In NKG2C− NK cells the surface levels of the activating receptor appeared rather stable, whereas NKG2A expression decreased after stimulation, predictably reaching a threshold avidity for HLA-E peptide complexes unable to counteract the triggering signals. A clear correlation between the expression levels of NKG2A and the inhibition of cytotoxicity in redirected lysis assays was noticed when a panel of NK clones were tested in parallel.
CD94/NKG2A expression was previously shown to be inducible in T cells stimulated with IL-12 (28) or upon TCR-dependent activation under the influence of IL-15 or TGFB (26, 27). Our results indicate that incubation with rIL-12 alone promoted NKG2A expression by NKG2C+ polyclonal NK cell populations and clones, whereas IL-15 and TGFB had no detectable effect. It is of note that stimulation of resting purified NK cells with rIL-12 alone induced NKG2A expression, thus suggesting that secretion of the cytokine during an inflammatory response is sufficient to promote this regulatory mechanism controlling the activation of CD94/NKG2C+ cells. Information about the mechanisms that regulate transcription of the different NKG2 genes is limited. The NKG2A promoter has been partially characterized and shown to be under the control of GATA3 (49). Further studies are required to define how constitutive and inducible NKG2A expression are differentially regulated, and whether IL-12R signaling via STAT-4 plays a direct role in the process. On the other hand, the possibility that NKG2C expression may be inducible in NKG2A+ cells activated in response to other stimuli cannot be excluded.

It is presently accepted that individual NK cells acquire along differentiation various combinations of inhibitory receptors specific for MHC class I molecules, including NKG2A. The NKR repertoire of an individual will be ultimately conditioned by the variability of the inherited KIR haplotypes (25). NKG2A becomes detectable during NK cell differentiation at earlier stages than KIR molecules and is constitutively expressed by a NK cell subset (1, 25, 29). As compared with NKG2C+ cells, the NKG2A+ subset tends to include lower proportions of KIR+ and ILT2+ (LIR-1, CD85j) and higher expression levels of natural cytotoxicity receptors (NKp30 and NKp46) (22, 23). The inducible expression pattern of NKG2A in NKG2C+ cells described in this study reveals that the NKR repertoire may be transiently altered during NK cell activation depending on the influence of IL-12. From a practical standpoint, this should be carefully taken into account when performing functional studies on CD94/NKG2C+ cells. Phenotypic studies of PBMC indicated that a subset of NKG2C+NKG2A+ NK cells are KIR-ILT2+, thus suggesting that NKG2A expression may play a central inhibitory role in this NKG2C+ population. On the other hand, the function of the inducible NKG2A molecule may be overlapping with that of other inhibitory receptors for class I molecules (i.e., KIRs and ILT2) expressed by NKG2C+ cells. In this regard, our previous functional studies (40, 47) indicated that CD94/NKG2A and ILT2 may contribute in a complementary way to repress NK cell function, whereas in clones coexpressing CD94/NKG2A and KIR the inhibitory function of the latter may be dominant. Thus, it can be predicted that the inducible expression of NKG2A may have an additive effect with other receptors in some NKG2C+ NK cells, contributing to establish the inhibitory threshold, while being redundant in cells that are effectively repressed by appropriate inhibitory KIR-HLA class I interactions.

NK cells have been reported to interact with DC, regulating their survival and function (50–52). The KIR NKG2A+ cell subset was shown to kill autologous immature DC, whereas up-regulation of HLA-E expression protected mature DC by engaging the CD94/NKG2A inhibitory receptor (53). It is of note that IL-12 has been reported to be pivotal in regulating the NK-DC crosstalk (54). Our results support that IL-12 secretion by cells of the myelomonocytic lineage may play a dual role, activating NK cell functions and concomitantly inducing the expression of the NKG2A inhibitory receptor on NKG2C+ cells, thus contributing to prevent their potential autoreactivity. This regulatory mechanism may be particularly relevant in the context of the NK-DC crosstalk established during the innate immune response to some infections (55). In this regard, increased numbers of circulating CD94/NKG2C+ NK cells were previously associated to a positive serology for HCMV (22, 23). Moreover, an expansion of NKG2C+ populations was observed upon coculture with HCMV-infected fibroblasts (31), suggesting that this subset may be involved in the defense against the viral infection. In the present study we provide data supporting that endogenous IL-12 secretion in HCMV-infected moDC cultures induced NKG2A expression in NKG2C+ cells and thus might modulate their response against infected cells. Paradoxically, this effect might provide an advantage for the virus, which down-regulates classical HLA class I expression while maintaining surface HLA-E (56); in this scenario, the induced CD94/NKG2A expression could reinforce the immune evasion mechanism.

IL-12 was detectable by ELISA in supernatants of HCMV-infected moDC cultures and, moreover, expression of NKG2A by NKG2C+ NK clones was blocked by an anti-IL-12 mAb. Whether IL-12 is secreted by the HCMV-infected moDC or by the fraction of cells that remain uninfected is being studied. The discrepancy with a previous report showing that IL-12 was undetectable by intracellular staining in HCMV-infected DC cultures (57) may be explained by technical differences (i.e., cytokine detection assays).

It has been recently reported that decidural and peripheral blood CD56bright cells may coexpress NKG2A and NKG2C (37). In the present study, we observed that minor subsets of peripheral blood NK cells, including CD56bright and CD56dim, as well as T lymphocytes, mainly CD56+, displayed both NKG2A and NKG2C at the cell surface; the proportions of these subsets varied widely in different donors. Whether they correspond to NK cells that have transiently acquired NKG2A in vivo and/or they represent a distinct subset stably expressing both receptors is uncertain.

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References


