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*J Immunol* 2004; 173:5349-5354; doi: 10.4049/jimmunol.173.9.5349

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Cutting Edge: Engagement of CD160 by its HLA-C Physiological Ligand Triggers a Unique Cytokine Profile Secretion in the Cytotoxic Peripheral Blood NK Cell Subset

Aliz Barakonyi,1* Magali Rabot, * Anne Marie-Cardine, † Maryse Aguerre-Girr, *, Beata Polgar, * Valérie Schiavon, † Armand Bensussan,3,4† and Philippe Le Bouteiller3,4*  

CD160 is an Ig-like activating NK cell receptor expressed on the majority of circulating NK cells. This population corresponds to the nonproliferating, highly cytolytic, CD56dimCD16+ subset. CD160 engagement by HLA-C molecules mediates cytotoxic function. In this study, we report that upon specific activation by the physiological ligand HLA-C, or Ab cross-linking, CD160+ peripheral blood NK cells produce IFN-γ, TNF-α, and IL-6. This unique CD160-mediated cytokine production differs from the one observed after CD16 engagement whose expression is also restricted to the CD56dim cytotoxic NK cell subset. As already reported for the CD160-mediated cytotoxic effector function, CD160-mediated cytokine production by peripheral blood-NK cells is negatively controlled by the killer Ig-like receptor CD158b. Thus, the CD160 receptor represents a unique triggering surface molecule expressed by cytotoxic NK cells that participates in the inflammatory response and determines the type of subsequent specific immunity. The Journal of Immunology, 2004, 173: 5349–5354.

Natural killer cells constitute a subset of lymphocytes that play a role in innate immunity directed against virally infected or tumor cells (1). NK cells use a combination of inhibitory and activating receptors expressed at their cell surface to mediate target cell killing and cytokine release upon interaction with specific ligands (2). Yet, only few human activating NK cell receptors have been shown to induce cytokine production upon specific engagement. CD158d induces IFN-γ production in resting and activated NK cells (3). Signaling via CD16, a low-affinity FcγRIII responsible for Ab-dependent cellular cytotoxicity, triggers the production of several cytokines and chemokines (4). Incubation of activated NK cells with anti-NKp30 or anti-NKp46 mAb led to IFN-γ production (5). In contrast, human NKG2D activating receptor is apparently unable to produce cytokines when triggered by specific mAbs (5, 6).

The BY55 NK cell receptor, recently designated CD160, is expressed by circulating CD56dimCD16brighthCD3− NK cells, which constitute the majority of peripheral blood (PB)−NK cells (7). The CD56dim NK cell subset is more naturally cytotoxic and produces less abundant cytokines than the CD56bright subset following activation by monocytes (8). The CD56dim NK cell subset also expresses a specific pattern of chemokine receptors and adhesion molecules (8). Such phenotype is characteristic of terminally differentiated effector cells (9). CD160+ NK cells have a high cytotoxic activity potential, do not proliferate in response to IL-2 (7), and mediate cell lysis upon interaction with HLA-C (10). The CD160 receptor appears unique: it is encoded by a gene located on human chromosome 1, it is a GPI-anchored molecule (11) and its cell surface expression is down-modulated by NK cell activation mediated by cytokines including IL-2 and IL-15 (data not shown). As described for the killer cell Ig-like inhibitory receptors (KIRs), CD160 is also expressed by γδ T cells, and a subset of the αβ CD8+ T cell (12, 13).

In this report, we studied the cytokine secretion produced by the cytotoxic effector PB-NK cell subset upon specific engagement of the CD160 receptor. To this end, we activated CD160 by HLA-C-expressing cells or by Ab-mediated cross-linking. We show that CD160 is a NK cell activating receptor with the unique property of inducing high amounts of IFN-γ as well as TNF-α and IL-6 production.

1 This work was supported by grants from INSERM (to A.Be., P.L.B.), Association pour la Recherche contre le Cancer (to A.Be.), Etablissement Français des Greffes (to A.Be.), and by research fellowships to A.Ba. (INSERM, Fondation pour la Recherche Médicale), B.P. (Association pour la Recherche sur le Cancer), and M.R. (Ministère de la Recherche).

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5 Abbreviations used in this paper: PB, peripheral blood; KIR, killer cell Ig-like inhibitory receptor; CBA, cytometric bead array; NCR, natural cytotoxic receptor.

*Correspondence. Received for publication June 21, 2004. Accepted for publication August 19, 2004. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Materials and Methods

Cells

Effector cells were the CD16+ NK92 line cultured with IL-2 for several days, and fresh human PB-NK cells isolated from normal donors by MACS negative selection (Miltenyi Biotec, Auburn, CA). PB-NK purity was shown to be >90% CD3- CD56+ by flow cytometry, and >80% of purified PB-NK were CD16+. Two variants of the K562 cell line were used as target cells. One variant (K562class-I+) expressed HLA-C (10) whereas the other one (K562; American Type Culture Collection, Manassas, VA) did not. K562-HLA-Cw5 transfectants (K562-Cw5) were obtained by transfection of HLA-Cw5 cDNA in K562 MHC class I-negative parental cells.

Abs and flow cytometry analysis

mAbs used included CL1-R2 anti-CD160 (IgG1), B5Y5 anti-CD160 (IgM), B1.23.2 anti-HLA-B/C (IgG2a), referred to here as anti-HLA-C, produced in our laboratories, and PE-conjugated 3G8 anti-CD16 (IgG1), GL183 anti-CD158b (IgG1), anti-CD3, or anti-CD56 (Beckman Coulter, Fullerton, CA), and anti-NKG2D clone 149810 (R&D Systems, Mountain View, CA). For single staining flow cytometry analysis, cells were incubated with PE-Cy5-conjugated B5Y5 mAb or with each of the PE-conjugated mAbs. For the NKG2D staining, cells were incubated with anti-NKG2D mAb followed by PE-conjugated F(ab')2 goat anti-mouse IgG1 Ab (Cinisience, Montrouge, France). For double staining, cells were incubated with PE-Cy5-conjugated B5Y5, followed by PE-conjugated anti-CD56, -CD3, -CD16, -CD158b mAbs, or by anti-NKG2D mAb followed by PE-conjugated F(ab')2 goat anti-mouse IgG1 Ab, PE-Cy5-IgM or PE-IgG (Beckman Coulter) were used as isotype controls. Samples were analyzed on an EPICS XL4C flow cytometer (Beckman Coulter).

Receptor-specific mAb-mediated cross-linking

Cross-linking of CD160, NKG2D, CD16, or CD158b receptors on PB-NK cells was performed at the final concentration of 1–10 μg/ml for 16 h at 37°C in 5% CO2. IgG1 isotype control was also used at the same conditions. IL-2 (100 U/ml) was added during the incubation time. Supernatants were collected at 80°C until further analysis.

NK cells and CD160 ligand-expressing cell cocultures

NK92 or PB-NK cells were incubated alone or coincubated either with K562class-I+, K562, or K562-Cw5 at a ratio of 10:1 during 4 h (NK92) or 16 h (PB-NK) at 37°C in the presence or not of blocking concentrations of B1.23.2 or CL1-R2 mAbs, or Ig-isotype controls. IL-2 (100 U/ml) was added during the incubation times.

Intracellular TNF-α detection

NK92 cells treated as above were washed, fixed in 2% paraformaldehyde, permeabilized with 0.1% saponin for 10 min, stained by PE-conjugated anti-

Cytokine measurement by cytometric bead array (CBA)

The Th1/Th2 CBA kit (BD Biosciences, Mountain View, CA) was used for simultaneous measurement of IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ, according to the manufacturer’s instructions (14). Analysis was made on a FACScalibur flow cytometer (BD Biosciences) using CellQuest (BD Biosciences). The mean fluorescence was compared with standard curves and cytokine concentrations (picograms per milliliters) calculated by using the CBA software provided (BD Biosciences). IL-2 measurements were excluded from analysis because the culture medium in which NK cells were incubated during the different assays always contained IL-2.

Statistics

Statistical analyses were performed using either the two-tailed Student t test or the Student paired t test with p < 0.05 defined as significant.

Results and Discussion

HLA-C expressing K562 target cell lines trigger cytokine production by PB-NK cells

Because engagement of CD160 by HLA-C triggered cytotoxicity of the NK92 cell line (10), we investigated whether TNF-α production could be also obtained in this cell line expressing a high amount of CD160. Intracellular expression of this cytokine was evaluated by flow cytometry in NK92 cocultured with HLA-C-expressing K562 target cells (K562class-I+). We found that such coculture stimulated TNF-α production, as compared with the moderate secretion of this cytokine by NK92 cultured alone (Fig. 1A). Absence of TNF-α production by K562class-I+ alone indicated that TNF-α release was produced solely by NK92. Furthermore, addition in the culture medium of HLA-C-masking mAb B1.23.2 on target cells resulted in a mean 74% decrease of TNF-α production by NK92 (Fig. 1A). These results indicate that HLA-C was capable to trigger TNF-α secretion from NK92.

Next, we evaluated whether cytokytic PB-NK could also produce TNF-α upon specific HLA-C-mediated triggering. PB-NK were cocultured for 16 h with either K562class-I+, K562-Cw5 transfectant, which both express HLA-C molecules at their cell surface, or K562 which is completely MHC class I

![Figure 1](http://www.jimmunol.org/)
negative (Fig. 1B). Using the CBA kit and flow cytometry, TNF-α and four other Th1/Th2 cytokines were measured in the cell-free supernatant fluid (Table I). When PB-NK from different donors were cocultured with K562classI or K562-Cw5, a large amount of IFN-γ, TNF-α, and IL-6 was detected (Table I) while neither IL-4 nor IL-10 was found (data not shown). By comparison, PB-NK cocultured with class I-negative K562 produced very low amounts of IFN-γ and only marginal amounts of TNF-α and IL-6, not significantly different from those observed when PB-NK cells were cultured alone (Table I). No spontaneous cytokine release was ever produced when K562 or K562classI were cultured alone (data not shown). However, we should mention that PB-NK from some donors produced cytokines when cocultured with K562, as already reported (15). This suggested that MHC class I-independent activating receptors could be also involved. Altogether, these data indicate that HLA-C recognition by the cytotoxic PB-NK cell subset could trigger specific cytokine secretion.

Specific engagement of CD160 by its physiological ligand HLA-C results in IFN-γ, TNF-α, and IL-6 production by PB-NK

The only identified HLA-C-dependent activating receptor present on NK92 is CD160 (10). Therefore, we investigated whether this receptor triggered specific cytokine secretion by PB-NK upon engagement with HLA-C. PB-NK were cocultured with K562classI, in the presence of blocking concentrations of mAbs to either CD160 or HLA-C, or of Ig-isotype controls (Table II). Masking HLA-C ligand or CD160 receptor by their specific mAbs significantly diminished the IFN-γ, TNF-α, and IL-6 production. These results show that this PB-NK cytokine production is mainly attributable to CD160-HLA-C interaction. However, for an unknown reason, the use of B1.23.2 anti-HLA-C mAb did not significantly inhibit IL-6 secretion.

Ab cross-linking of CD160 expressed by cytotoxic PB-NK triggers a unique cytokine production profile different from the one obtained after CD16 or NKG2D engagement

We then compared the cytokine profile produced by CD160 triggering with the one obtained upon CD16-activating receptor engagement, whose expression is also restricted to the cytotoxic NK cell subset. The activating natural cytotoxic receptors (NCR) and CD244 coreceptor were excluded from this comparison as they are equally distributed on both cytotoxic and noncytotoxic PB-NK lymphocytes (16). NKG2D activating receptor triggering was used as negative control for its inability to mediate cytokine production by itself on human NK cells (5, 6). The results indicate that CD160-mAb cross-linking leads PB-NK to produce the same pattern of cytokine release, namely high levels of IFN-γ, and lower amounts of TNF-α and IL-6, but no IL-4 or IL-10 (Fig. 2), as the HLA-C physiological ligand triggering (Table I). The use of an isotype-matched control Ig did not result in such secretion. Next, we analyzed the cytokine production after cross-linking of the CD16 receptor with the specific 3G8 mAb. This triggered both IFN-γ and TNF-α production but not IL-6 (Fig. 2). Importantly, whereas the amount of TNF-α was almost comparable after CD160 or CD16 engagement, the production of IFN-γ mediated by CD16 cross-linking was ~30-fold less than the secretion obtained after CD160 engagement. As expected, Ab cross-linking of NKG2D did not trigger significant cytokine production. These data further demonstrate that Ab cross-linking of the CD160 receptor on cytotoxic PB-NK cells results in a unique cytokine profile similar to that observed after interaction with HLA-C. It should be of note that IL-6 production by a cytotoxic NK cell subset, upon triggering of activating receptors, has not been reported yet and may be of importance if we consider the recent report showing that some tumor-infiltrating lymphocytes produced high concentrations of

Table I.  IFN-γ, TNF-α, and IL-6 production by PB-NK cells cocultured with HLA-C-expressing K562 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>185 ± 183</td>
<td>16 ± 15</td>
<td>16 ± 16</td>
</tr>
<tr>
<td>NK/K562classI</td>
<td>29,441 ± 4,988</td>
<td>334 ± 116 (p = 0.02)</td>
<td>620 ± 145 (p = 0.02)</td>
</tr>
<tr>
<td>NK/K562</td>
<td>296 ± 106</td>
<td>19 ± 9</td>
<td>33 ± 23</td>
</tr>
<tr>
<td>NK/K562-Cw5</td>
<td>20,089 ± 8,718</td>
<td>389 ± 179 (p = 0.02)</td>
<td>689 ± 367 (p = 0.03)</td>
</tr>
</tbody>
</table>

* Purified PB-NK cells were cultured alone (NK) or cocultured with K562classI, K562, or K562-Cw5 cells. During coincubation, cell densities were 10⁶ cells/ml (PB-NK) and 10⁵ cells/ml (K562 cells) and the PB-NK/K562 coculture ratio was 10:1. After 16 h, culture supernatants were collected and cytokine concentrations measured by CBA, as described in Materials and Methods. The paired Student t test was used for statistical analysis. Results were compared to the NK group and expressed as mean ± SE of five independent experiments performed with different donors which were selected according to the absence of cytokine production when PB-NK were cocultured with K562.

Table II.  Anti-CD160 and HLA-C blocking mAbs prevent production of IFN-γ, TNF-α, and IL-6 by PB-NK cocultured with K562classI

<table>
<thead>
<tr>
<th>Type</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>186 ± 183</td>
<td>18 ± 15</td>
<td>20 ± 15</td>
</tr>
<tr>
<td>NK/K562classI + IgG1</td>
<td>23,054 ± 11,052</td>
<td>200 ± 44</td>
<td>414 ± 96</td>
</tr>
<tr>
<td>NK/K562classI + anti-CD160 mAb</td>
<td>1,083 ± 441 (p = 0.04)</td>
<td>58 ± 10 (p = 0.01)</td>
<td>143 ± 46 (p = 0.03)</td>
</tr>
<tr>
<td>NK/K562classI + IgG2a</td>
<td>16,125 ± 4,941</td>
<td>215 ± 54</td>
<td>478 ± 101</td>
</tr>
<tr>
<td>NK/K562classI + anti-HLA-C mAb</td>
<td>1,149 ± 415 (p = 0.01)</td>
<td>64 ± 9 (p = 0.01)</td>
<td>370 ± 57</td>
</tr>
</tbody>
</table>

* Purified PB-NK cells were cultured alone (NK) or cocultured with K562classI in the presence of anti-CD160 or anti-HLA-C mAbs at blocking concentrations or Ig-isotype controls. During coincubation, cell densities were 10⁶ cells/ml (PB-NK) and 10⁵ cells/ml (K562) and the PB-NK/K562 coculture ratio was 10:1. After 16 h of incubation, culture supernatants were collected and cytokine concentrations measured by CBA, as described in Materials and Methods. The Student t test was used for statistical analysis. Results were compared to the control NK/K562classI + IgG groups and are expressed as mean ± SE of four independent experiments performed with different donors. Values of p of NK + IgG1 and NK + IgG2a controls compared to NK groups (data not shown) were always ≥0.2.
IL-6 to counteract the anti-lymphokine-activated killer activity of tumor cell TGF-β1 (17).

**Inhibition of CD160-mediated NK cell cytokine production by the CD158b inhibitory receptor**

Activation of NK cells is dependent on activating receptors that are functionally silenced by inhibitory receptors, including the KIRs that recognize different allelic groups of HLA-A, -B, or -C molecules. We previously reported that the cytotoxic activity triggered upon CD160 engagement was inhibited by the coengagement of the CD158b inhibitory receptor (10). We thus investigated whether inhibitory receptors also controlled CD160-mediated cytokine production. We used PB-NK from donors who express variable percentages of the cell population bearing the CD158b inhibitory receptor. We analyzed cell surface expression of CD160, as well as CD158b, NKG2D and other NK cell markers by flow cytometry on freshly isolated purified PB-NK. Fig. 3A shows the results obtained with one representative donor. A major subset of PB-NK expresses CD160, whereas all of them are CD56dim, CD3-, and CD16+ (Fig. 3A, upper panels). Whereas the whole PB-NK population is NKG2D+, only a subset expresses the CD158b inhibitory receptor. Double staining confirms that CD160+ PB-NK were CD3−, and mostly CD56dim and CD16+ (Fig. 3A, lower panels). In addition, we found that only subpopulations of CD160+ cells also expressed CD158b (Fig. 3A, lower panels). As expected, we found that mAb-mediated cross-linking of CD160 and not of the NKG2D receptor led to the production of IFN-γ, TNF-α, and IL-6 (Fig. 3B). In addition, the co-cross-linking of both the CD158b inhibitory receptor and CD160 significantly reduced IFN-γ and IL-6 production. Such reduction did not occur when an isotype-matched control Ab was substituted to CD158b mAb. Similar results were obtained with five different PB-NK donors that contained variable percentages (≈8–30%) of the CD158b+ NK subset among the purified PB-NK cells. As only a subpopulation of PB-NK expressed CD158b, this may explain why the down-modulation of cytokine secretion was only partial in our experiments. One can speculate that other KIRs, which interact with different HLA alleles, may also contribute to such control of CD160-inducing cytokine production and thus participate in NK cell tolerance in normal physiological situation. We also examined whether NKG2D co-engagement could synergize with CD160 to produce an increased stimulatory signal. We found that the simultaneous cross-linking of NKG2D and of CD160 activating receptors did not result in a cumulative positive signal compared with the stimulation through the CD160 receptor alone (Fig. 3B). This confirms previous results showing that human NKG2D triggering by specific mAb cross-linking did not induce activation of cytokine secretion (5). However, stimulation of activated PB-NK cells with plastic-bound recombinant MHC class I chain-related protein A or UL16-binding protein physiological ligands triggers GM-CSF and IFN-γ production (5).

The CD160 receptor, whose expression is restricted to the effector cytokotic CD56dimCD16bright PB-NK cell subset, acts as a unique MHC class I-dependent activating receptor capable of promoting cytokine secretion upon specific ligation. Indeed, the CD160 major ligand, HLA-C, is constitutively expressed, which differs from the inducible self-ligands or pathogen-induced ligands of the other NK triggering receptors expressed on both cytotoxic and noncytotoxic NK lymphocyte subsets. NKG2D is unable to trigger by itself IFN-γ production in humans (18). The recently described CD155 and CD112 ligands of the DNAX accessory molecule-1 DNAM-1 (CD226) coactivating receptor are also mostly expressed in stressed tissues (1). NCR ligands are non-MHC molecules (18). In contrast to the above-mentioned receptors, CD16 is present only on the effector cytokotic PB-NK lymphocyte subset and its ligand is the Fc portion of IgG. In addition, stimulatory KIRs and CD94/NKG2C activating receptor that are only expressed by a subset of cytotoxic PB-NK lymphocytes also interact with constitutive HLA class I molecules (including HLA-C for the former) and have short cytoplasmic domains with no known signaling motif (4). Furthermore, these activating receptors associate with adaptor molecules to initiate signaling (4), which differs from the CD160 GPI-anchored cell surface molecule (11). CD244 NK cell receptor provides a costimulatory signal to other activating receptors including NCR or NKG2D (19).

Data from this study strongly suggest that stimulation of the CD160 receptor on NK cells can lead to the enhancement of both innate (through specific cell killing) and adaptive (through...
The signals that transform a circulating resting NK cell into an activated cytokine-secreting cell in vivo are not fully understood. This mainly depends on the outcome of signals derived from activating and inhibitory receptors upon engagement by their specific ligands. Knowing that CD158a/CD158b inhibitory receptors engage HLA-C molecules on target cells, we hypothesize that the level of expression of HLA-C may be a key factor to trigger either the KIR or CD160 receptors. When the level of HLA-C is normal, KIR inhibitor receptor engagement would control CD160. In contrast, when the level of expression of HLA-C is down-modulated, KIR receptors might no longer be efficiently engaged, allowing the activating function of the CD160 receptor to take place. We need additional data to validate this hypothesis.

In conclusion, this study, combined with a previous one (10), demonstrates that the functional activation of the CD160 NK cell receptor by the HLA-C physiological ligand initiates both cytotoxicity and cytokine production after optimal receptor triggering. The unique signaling pathway likely used by CD160 for mediating the activating effector functions to limit viremia and tumor burden or pathogen-infected cells remains to be determined.

**References**


