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The IL-12 Signature: NK Cell Terminal CD56^{high} Stage and Effector Functions¹

Matthew J. Loza and Bice Perussia²

We report that human peripheral NK cells expressing high CD56 levels (CD56^{high}) are terminally differentiated cells indistinguishable from mature NK cells recently activated in the presence of IL-12, and not a functionally distinct NK-cell subset or progenitors to mature CD56^{low} NK cells. CD56^{high} NK cells coexpress all differentiation Ags constitutive or inducible in mature (CD56^{high}) NK cells, except CD16, present at lower level than on most mature NK cells. Also, activation markers, activating receptors and adhesion molecules, and most inducible receptors are expressed exclusively and constitutively and are inducible at higher levels on CD56^{high} than on CD56^{low} NK cells. Consistent with their activated phenotype, many CD56^{high} NK cells are cycling and mediate heightened effector functions (proliferation, IFN-γ and IL-10 but not IL-13 production) in response to IL-12 and other NK cell-specific stimuli. Conversely, IL-12 induces on CD56^{low} NK cells all markers constitutively expressed on the CD56^{high} NK cells, concomitantly preventing the IL-2 (and IL-15)-inducible expression of Nkp44 and CD16 re-expression after immune complex-induced down-modulation, and CD56^{+/low} NK cells acquire a CD56^{high} NK cell phenotype in short term in vitro culture with IL-12. The significance of these findings to the NK cell-mediated regulation of immune responses and NK cell development is discussed. The Journal of Immunology, 2004, 172: 88–96.

The major role of IL-12 in NK-cell biology possibly rests on the regulation of their terminal development from phenotypically and functionally immature CD161^{−}CD56^{−} type 2 cytokine^{+} to mature CD56^{+}IFN-γ^{+} cells expressing all NK cell markers (1). However, its primary role in the NK cell-mediated effects as innate effectors and regulators of adaptive immune responses depends on its concomitant direct effects on functions and phenotype of peripheral CD56^{+} NK cells. These include: synergy with IL-2 to enhance IFN-γ production regulating, in a transcription-independent fashion, IFN-γ mRNA stability (2); induction of proliferation in CD56^{−} NK cells reverted to a resting state after cytokine-induced activation (3); enhancement of cytotoxicity (4) and granulogenesis (5); and modulated expression of NK cell differentiation Ags via transcriptional regulation of genes for selected molecules (e.g., CD161) specialized in binding target cell ligands, antagonizing, in this context, the effects of other cytokines like IL-2 (6).

Two NK cell populations, CD56^{bright}{³} (7) and CD56^{+}CD16^{−} (8), detectable in low proportions in freshly separated PBL, have been proposed to represent functionally unique or possibly more immature cells (7, 8). However, in light of the direct effects of IL-12 and other cytokines and ligands on phenotype and functions of mature NK cells and the fact that immature NK cells are CD56^{−} (1, 9, 10), the possibility should be considered that these populations contain mature NK cells, the functions or phenotype of which have been recently modulated in vivo. This is relevant to understanding NK cell biology and important on a clinical standpoint to define any possible significance of NK cell populations that, like the CD56^{bright} NK cells, are detected at increased proportions in vivo during IL-2 therapy (11) or in affected tissues in pathological inflammatory conditions like rheumatoid arthritis (12). Additionally, in the mouse, CD56 is not expressed (13), and reagents specific for CD16 (and not the FcγRII exclusively expressed on myeloid and B cells; Refs. 14 and 15) are lacking, and NK cell detection can rely only on expression of CD161 (expressed on both immature and mature NK cells), CD94 (16), and the Ly49 family members (reviewed in Ref. 17), functionally equivalent to, but structurally distinct from human killer Ig-like receptors (KIR){⁴} (reviewed in Ref. 18). Thus, conclusive definition of functions and phenotype of human NK cells expressing CD56 and CD16 levels different from those on most mature NK cells, as addressed here, is also essential to guide interpretation of preclinical studies manipulating NK cells in animal models.

Materials and Methods

Lymphocyte isolation and culture conditions

PBL were separated from healthy adult and neonatal (umbilical cord) peripheral blood (19). When indicated, sheep E treated with 2-aminoethylisothiouronium bromide (E_{AET}) were used to enrich most mature CD2^{+} T and NK cells (20). Immature CD56^{−} NK cells were purified from the E_{AET} cell fraction after depletion of leukocytes expressing FcγR upon adherence to immune complex monolayers (EA) (20). To induce CD16 downmodulation, PBL were depleted of B cells (E_{AET} rosetting) and let adhere on EA monolayers (EA^{+}), from which, after a 2-h incubation at 37°C, nonadherent cells were discarded, and the adherent ones recovered. Cultures of the different cell populations were for the indicated time periods in the presence of the indicated cytokines and anti-TNF-α mAb to prevent the otherwise massive NK cell death in cultures with IL-12 (10).

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¹ Based on antigen density, the CD3^{−}CD161^{+} NK cells expressing relatively high or relatively lower CD56 levels are referred to here as CD56^{high} and CD56^{low}, respectively. However, for correctness, we do maintain the term CD56^{high}, referring to the results with the same cells in reports that have used this term.

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⁴ Abbreviations used in this paper: CyC, CyChrome; E_{AET}, sheep E treated with 2-aminoethylisothiouronium bromide; IFL, immunofluorescence; KIR, killer Ig-like receptor; ^high, expressing relatively high levels; ^low, expressing positive, but relatively low levels.

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Surface phenotyping and cell sorting
The fluorochrome- or biotin-labeled CD2, CD3, CD5, CD16, CD56, CD161, NKp46, KIR3DL1, HLA-DR mAb used for surface phenotyping or cell sorting (immunofluorescence (IFL) flow cytometry, according to our published protocols) were as previously reported (1, 9). mAb to NKp46 (3.43.13) and CD244 (HD2) (2B4, a member of the CD2 family) (21) were gifts of M. Colonna; CD45RO and CD26L mAb were from Caltag Laboratories (Burlingame, CA), and the CD162R mAb (PEN5, clone 5H10.21.5) was from Beckman Coulter (Fullerton, CA). Viable cells, gated based on light scatter characteristics, were analyzed on an XL-MCL automated analytical flow cytometer or sorted on an EPICS Elite flow cytometer (Beckman Coulter). Listmode data were analyzed with the WinMDI Flow Cytometry Application (J. Trotter). When sorting Ag-expressed on restaining and analysis of cell aliquots immediately after sorting (not shown).

Cytokine detection
Cytokine accumulation in cells stimulated maximally with PMA (2 × 10⁻⁹ M) plus A23187 (0.2 μg/ml) plus IL-2 (10² U/ml), or specifically with the indicated cytokines and target cells, was detected by intracellular IFL using previously described anti-IL-13 (PE), -IFNa-γ (PE), -TNF-α (PE), -IL-10 (PE) mAb. Conditions (22) and mAb (23) were according to our established protocols. For IL-13 detection in CD3⁺ CD161⁺ CD56⁻ immature NK cells, (CD3⁺ CD5⁺ CD16⁺ CD56⁻)-biotin and CD161 (nonlabeled) mAb detected with CyC-labeled streptavidin and FITC-labeled goat anti-mouse Ig were used to stain E₆L A⁺ PBL and analysis was performed on gated CyC⁺ FITC⁻ cells. Autologous E₆L CD56⁺ and CD56⁻ NK cells were analyzed in parallel. ELISA for IL-13 detection was as previously reported (10).

Cell proliferation
Lymphocytes were cultured for the indicated times in the different conditions after addition of BrdU (60 μM) (24) at the start of culture. BrdU⁺ cells were detected with FITC-anti-BrdU mAb (BD PharMingen, San Diego, CA; IFL) on cells fixed and permeabilized as for intracellular cytokine detection (22), except that they were incubated (30 min, 37°C) with 300 μg/ml DNase I after the permeabilization step (modified from Ref. 25) and washed before adding the anti-BrdU mAb. When indicated, the G₁-S phase transition cell cycle inhibitor mimosine was added to the cultures as reported (22).

Cytotoxicity assays
This was performed, using THP-1 target cells and the indicated mAb in 4-h ⁵¹Cr release redirected cytotoxicity according to published protocols (26).

Results
IL-12-induced modulation of NK cell differentiation Ags
To determine whether IL-12 and IL-15 modulate phenotype and functions of peripheral mature CD161⁺ CD56⁻ NK cells, the expression of functionally relevant differentiation Ags was analyzed on NK cells from 5-day cultures of freshly isolated PBL with these monokines, single or combined (Fig. 1). As expected, CD56 expression was higher on CD3⁺ CD161⁺ total NK cells from cultures with IL-15 or IL-12 than without monokines; the CD161 levels were highest after culture with IL-12; and CD16 expression was decreased on the CD3⁺ CD56⁻ mature NK cells after culture with IL-12, and almost undetectable when IL-15 was also added. The activating receptor NKp46 and the two costimulatory/adhesion molecules CD2 and CD244 (2B4) had highest expression on cells from cultures with IL-12, both IL-15 and IL-12, and IL-12 alone, respectively. KIR expression (KIR3DL1 reported) was moderately increased compared with that on cells freshly isolated or cultured without exogenous monokines, only after culture with IL-15. The proportion of NK cells expressing CD62L (L-selectin), an adhesion molecule involved in migration through high endothelial venules, was increased in cultures with IL-12, whereas CD162R (PEN5), a potential ligand for CD62L (27), was lost by most NK cells from cultures with both IL-15 and IL-12, but not significantly modulated during culture with IL-12 alone. Identical results were obtained using IL-2 instead of IL-15, and cultures for shorter times gave similar results, especially CD16 down-regulation, although less apparent (not shown). Longer culture times were not analyzed, given the uncontrollable intervention of complicating factors like cell death, especially in cultures with IL-12 (1) and proliferation in response to IL-15, maximal at days 5–7 (not shown).

NKp44, reportedly undetectable on freshly ex vivo-derived NK cells and inducible on the same cells after a 10-day culture with IL-2 (28), was expressed at high levels on NK cells from a 5-day culture of PBL with IL-2 and IL-15, alone or combined (Fig. 2A), with induced expression detectable within 15 h (Fig. 2B, top). Induction of NKp44 expression by IL-15- and IL-2- was significantly inhibited by IL-12 (Fig. 2A), and, once induced upon culture with IL-2 (or IL-15) alone, it was down-regulated on further culture in the presence of IL-12 (Fig. 2B, bottom). This down-modulation was moderate or complete, respectively, depending on the presence or absence of IL-2 during secondary culture. Among other cytokines that affect NK cell functions (IL-4, IL-13, IL-18,
FIGURE 2. Effect of IL-12 on NKp44 expression. A and B, NKp44 expression was analyzed on gated CD3^-CD56^+ lymphocytes, as in Fig. 1, after culture of PBL with the cytokines indicated at top and right for 6 days (A) or 15 h (B, top), or after 3-day secondary culture after the 5-day culture with IL-2 alone (B, bottom). Here, and in all figures, the numbers in the panels are percent positive cells, background subtracted. C. Redirected cytotoxicity assays were performed with THP-1 target cells and mAb to the indicated Ags, using as effector cells PBL after 5-day culture with the indicated cytokines. Each experiment is representative of at least three.

IFN-α, IFN-γ, TNF-α), only IL-18 and IFN-γ induced low level NKp44 expression in a small proportion of NK cells, and IL-4, IL-18, and IFN-γ only mildly attenuated its IL-2-induced expression, with effects significantly lesser than those of IL-12 (not shown).

Although most other activating and costimulatory/adhesion molecules were up-regulated in response to IL-12 (alone or with IL-15), CD16 was expressed at significantly lower levels after culture with IL-12. However, it was capable of triggering the highest cytotoxicity levels in redirected cytotoxicity (Fig. 2C), confirming that the expression level of activating receptors in IFL does not necessarily correlate with their ability to trigger cytotoxicity when engaged by excess ligand. NKp44, although expressed at lower levels after culture with IL-12 plus IL-15 than with IL-15 alone, triggered in both conditions cytotoxicity at levels similar to those of NKp46, expressed at higher levels in the former condition.

Activated phenotype of CD56^+^{high} NK cells

The phenotype of mature CD56^+ NK cells after short term culture with IL-12 and IL-15 was similar to that reported for CD56^{high} NK cells (summarized and referenced in Table I, reporting also the results on IL-12-induced modulation of all NK cell differentiation Ags tested here and, with similar outcome (not shown), also in NK cells from 10-day cocultures of PBL with the RPMI-8866 B-lymphoblastoid cell line; Ref. 29). This included high CD56, CD62L, and CD2 levels, and low-to-null CD16 levels. The CD56^+^{low} NK cells in freshly isolated adult PBL (Fig. 3A) were mostly CD16^-, although a minor proportion was consistently CD16^+ (9 ± 3%, n = 7), similar to that in neonates (14 ± 8%, n = 5, p > 0.13). The proportions of CD56^+^{high} cells within the CD56^+ NK cells or total PBL were similar in adult (8 ± 4% and 0.8 ± 0.7%, respectively, n = 18) and neonatal (7 ± 4% and 0.9 ± 0.3%, respectively, n = 9) cells. Consistent with previous reports (8, 30), the CD56^+^{high} NK cells were mostly CD16^- (adult, 72 ± 14%, n = 7; neonate, 79 ± 4%, n = 5) or CD16^+^{low}.

Comparison of the relative expression levels of the Ags modulated by IL-12 (four-color IFL) indicated higher levels of CD2, CD161, CD244, and NKp46, detectable in CD56^+^{high} and CD56^+^{low} NK cells in freshly isolated PBL, in the CD56^+^{high} cells (Fig. 3). NKp44, undetectable on CD56^+^{low} NK cells, was

### Table I. Expression and modulation of NK cell differentiation Ags

<table>
<thead>
<tr>
<th>Ag</th>
<th>Modulation of Expression^a</th>
<th>CD56^+^{low}</th>
<th>CD56^+^{high}</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activating receptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD16</td>
<td>↓</td>
<td>-</td>
<td>+/low</td>
<td>F, (30)</td>
</tr>
<tr>
<td>NKp44</td>
<td>↑</td>
<td>+</td>
<td>+/low</td>
<td>F</td>
</tr>
<tr>
<td>NKp46</td>
<td>↑</td>
<td>-</td>
<td>+/high</td>
<td>F</td>
</tr>
<tr>
<td>Inhibitory receptors</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>+high</td>
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</tr>
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<td>-/+</td>
<td>-/+</td>
<td>F, (45)</td>
</tr>
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<td>Costimulatory/adhesion molecules</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td>+/low</td>
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</tr>
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<td>-</td>
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<td>NS</td>
</tr>
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<tr>
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<td>↑</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>F, (27)</td>
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<tr>
<td>CD244</td>
<td>↑</td>
<td>+</td>
<td>+</td>
<td>F</td>
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</table>

^a Modulation of expression of the indicated antigens on NK cells after culture with IL-2 (IL-15) + IL-12, including any induced, direct, or indirect effect. Up arrow, increased; down arrow, decreased; equal sign, unchanged.

^b Expression of the indicated antigens on CD3^-CD56^+^{low} and CD56^+^{high} NK cells in freshly isolated adult PBL. +, positive; −, negative; +/−/low and +/−/high, relatively low and high compared with the other population; +/+, discrete + and + populations detected.

^c F, figures in this article; NS, data reported here as not shown; +, IL-2 or IL-15-induced expression of NKp44 was attenuated by IL-12.
The activation markers CD25 (IL-2Rα) and HLA-DR, undetectable on CD56<sup>+</sup> lower NK cells, were also expressed at low levels on most CD56<sup>+</sup> higher NK cells, and minor proportions of CD56<sup>+</sup> higher NK cells expressed high HLA-DR levels. Expression of these activation markers, often linked to ongoing or occurred proliferation, suggested the possibility that a portion of the CD56<sup>+</sup> higher cells might be actively proliferating. Indeed, ~30% CD56<sup>+</sup> higher NK cells within freshly isolated PBL cultured for 3 days without exogenous stimuli and with BrdU added on day 0, were BrdU<sup>+</sup> (Fig. 4). A small cell proportion also incorporated BrdU in the presence of mimosine, suggesting that such cells were in S phase before culture. These results indicate that freshly ex vivo-derived CD56<sup>+</sup> higher NK cells contain actively cycling cells, likely as a result of recent in vivo activation. These data do not contrast previous reports demonstrating no detectable [<sup>3</sup>H]TdR incorporation on days 3–4 of culture of CD56<sup>+</sup> higher NK cells in the absence of exogenous stimulus (8, 11), because analysis of proliferation at the single-cell level after adding BrdU at the start of culture allows one to detect active cell proliferation that may wane quickly and thus be undetectable, at a population level, after culture without stimuli. Many of the CD56<sup>+</sup> higher NK cells that did not proliferate autonomously did so in response to IL-12 or low IL-2 doses, whereas almost all proliferated in response to high IL-2 doses. CD56<sup>+</sup> lower NK cells did not proliferate in the absence of exogenous stimulation, and lower percentages of these cells than of CD56<sup>+</sup> higher cells proliferated in response to IL-2 or IL-12.

The combined findings of active proliferation, high proliferative response to IL-12, and activated phenotype confirms that peripheral blood CD56<sup>+</sup> higher NK cells are recently activated cells.

**CD16 re-expression after down-modulation**

CD56<sup>+</sup> higher NK cells, unlike most mature NK cells, have characteristically low CD16 expression. The contention that the CD56<sup>+</sup> higher CD16<sup>+</sup> phenotype is stable derives from the observation that CD16 expression is not inducible on CD56<sup>+</sup> higher CD16<sup>+</sup> NK cells upon culture with IL-2 (8). Although experiments (not shown) confirmed these data, low level CD16 expression was induced in isolated CD56<sup>+</sup> higher CD16<sup>+</sup> NK cells after a 5-day culture with IL-2 and gamma-ray-irradiated autologous PBMC (TNF-α and IL-12-neutralizing mAb added to prevent cell death; Ref. 1) with low proportions of cells expressing relatively higher levels (Fig. 5A). Thus, factors other than IL-2, related to the interaction with accessory cells, can and are necessary to induce NK cell CD16 expression on CD16<sup>+</sup> NK cells. Taking this into account, we tested the possibility that the low CD16 expression on the CD56<sup>+</sup> higher NK cells might result, in part, from in vivo activation of cells previously CD16<sup>+</sup> by ligands and cytokines modulating FcyR expression (e.g., immune complexes (20), and IL-12; Ref. 6). For this, we analyzed CD16 re-expression after its down-modulation had been induced by immune complexes on cells CD16<sup>+</sup> (Fig. 5, B and C). Provided IL-12 was present at low levels in most CD56<sup>+</sup> higher cells; it was induced at low levels in some CD56<sup>+</sup> lower NK cells (Fig. 2) but rapidly induced to high levels on most CD56<sup>+</sup> higher NK cells within 15 h of stimulation with IL-2 or IL-15 (not shown). The CD56<sup>+</sup> higher cells contained significantly higher proportions of CD62L<sup>+</sup> cells (61 ± 16%), with expression density higher than that on the positive CD56<sup>+</sup> lower population (15 ± 7%, n = 11, p < 1 × 10<sup>-5</sup>). Conversely, the proportions of CD162R<sup>+</sup> cells were significantly higher among the CD56<sup>+</sup> lower (57 ± 3%) than the CD56<sup>+</sup> higher (12 ± 8%, n = 4, p < 0.002) NK cells. Similar proportions of CD56<sup>+</sup> lower and CD56<sup>+</sup> higher NK cells coexpressed CD62L and CD162R (6 ± 5% and 3 ± 2%, respectively, n = 4) and expressed neither (32 ± 6% and 29 ± 17%, respectively, n = 4). A fraction of CD56<sup>+</sup> higher, but not CD56<sup>+</sup> lower NK cells, higher among adult than neonatal NK cells, also expressed CD45RO (Fig. 3C).

**FIGURE 4.** Proliferative capability of CD56<sup>+</sup> higher and CD56<sup>+</sup> lower NK cells within freshly separated PBL. The percentages of BrdU<sup>+</sup> cells were determined in gated CD3 (PE-Texas Red) CD56 (PE)<sup>+</sup>CD16 (CyC)<sup>+</sup> (top) and CD3<sup>+</sup> CD56<sup>+</sup> higher (bottom) NK cells from a 3-day culture of freshly isolated adult PBL in medium without (none) or with added the indicated cytokines (or mimosine, control) and BrdU on day 0. Experiment representative of three.
FIGURE 5. IL-12-mediated modulation of CD16 expression. A, CD3−CD56<sup>+</sup>CD16<sup>−</sup> cells were separated on day 0 (three-color IFL, cell sorting) from freshly isolated lymphocytes (top, gated regions highlighted) and cultured for 5 days in the presence of CFSE-labeled autologous irradiated PBMC and IL-2 (see Materials and Methods). At this time (bottom), CD56 and CD16 expression was analyzed (density plots at right) within gated CD3− cells (left histogram). B and C, PBL from two adult donors were depleted of B cells (E<sub>AET</sub> rosetting, top) or untreated (bottom) or allowed to adhere on EA monolayers (EA°), from which nonadherent cells were discarded and the adherent ones recovered after a 2-h incubation at 37°C (top). Both samples were then cultured for the indicated times in the presence of the indicated cytokines (anti-TNF-α-mAb added to all cultures). CD16 expression was analyzed (three-color IFL) on the derived cells gated on CD3 (PE)−CD161 (CyC)<sup>+</sup> cells. Each experiment is representative of two.

Cytokine production by CD56<sup>+</sup>CD16<sup>−</sup> and CD56<sup>+</sup>CD16<sup>+</sup> NK cells

Most adult peripheral blood NK cells produce TNF-α and low levels of IFN-γ, whereas variable or low proportions produce high levels IFN-γ or IL-13, respectively (10). Immature CD56<sup>−</sup> NK cells produce no IFN-γ and higher TNF-α levels, and most produce IL-13 (1, 9). To determine whether CD56<sup>+</sup> NK cells produce cytokines like activated, mature NK cells, IFN-γ and TNF-α production in response to various stimuli was analyzed at the single cell level in CD56<sup>+</sup> and CD56<sup>−</sup> NK cells in the same PBL population, freshly isolated or after a 15-h culture with IL-2 and IL-12 (Fig. 6). The IFN-γ levels accumulated on maximal (PMA plus Ca<sup>2+</sup> ionophore plus IL-2) stimulation were similar in CD56<sup>+</sup> and CD56<sup>−</sup> NK cells (27 ± 26%) and CD56<sup>+</sup> NK cells (30 ± 31%, n = 7) in freshly isolated PBL but were higher in the latter when stimulated after a 15-h culture with IL-12. On maximal stimulation, CD56<sup>+</sup> NK cells produced higher levels of TNF-α than CD56<sup>−</sup> cells, whereas after a 15-h culture with IL-12 greater proportions of CD56<sup>+</sup> than CD56<sup>−</sup> cells produced TNF-α at higher levels. Also, after 15-h culture with IL-12, CD56<sup>+</sup> NK cells produced higher levels of IFN-γ and TNF-α than the CD56<sup>−</sup> ones in the absence of additional stimulation. In fresh PBL, target cells (K562) induced IFN-γ production in a small proportion of CD56<sup>+</sup> but not CD56<sup>−</sup> NK cells; consistent with only ~25% CD16<sup>+</sup> cells in the CD56<sup>+</sup> population, CD16 engagement induced a higher proportion of CD56<sup>+</sup> than CD56<sup>−</sup> NK cells to produce IFN-γ. In cells from 15-h culture with IL-12, both K562 and CD16 engagement induced IFN-γ accumulation similar to that induced upon maximal stimulation, i.e., greater than observed without stimulation, in CD56<sup>+</sup> NK cells, whereas IFN-γ accumulation in CD56<sup>−</sup> NK cells after culture was maximal regardless of stimulation.

Immature CD56<sup>−</sup> NK cells lose ability to produce IL-13 as they become mature CD56<sup>+</sup> cells. As predicted if CD56<sup>+</sup> NK cells derive from CD56<sup>−</sup> NK cells, analysis at the single-cell level

FIGURE 6. Cytokine production by CD56<sup>+</sup> and CD56<sup>−</sup> NK cells. Intracellular accumulation of IFN-γ (top) and TNF-α (bottom) was analyzed (intracellular IFL), after a 5-h stimulation with the stimuli indicated on top, within gated CD3/CD5 (FITC), CD56 (CyC)<sup>+</sup>, and CD56<sup>−</sup> NK cells in adult PBL freshly isolated (left) or cultured for 18 h with IL-2 and IL-12 (right). Experiment representative of three.
detected no IL-13 accumulation in CD56<sup>+</sup><sub>high</sub> NK cells (Fig. 7, A and B) and no IL-13 was detectable by ELISA in culture supernatants from the same cells after purification (Fig. 7A), whereas IL-13 accumulation and production were confined, as expected (1, 10), to the CD56<sup>−</sup> NK cells.

Upon maximal stimulation, almost no IL-10 was detected in viable CD56<sup>−</sup>low or CD56<sup>+</sup><sub>high</sub> NK cells in freshly isolated PBL, whereas IFN-γ accumulated at similar levels in both populations. Consistent with previous reports associating IL-10 production with dying cells in NK and T cells (1, 23), IL-10 and IFN-γ, but not IL-13, accumulated at low levels in CD3<sup>+</sup> particles with high side scatter characteristics of lymphocytes undergoing apoptosis. In PBL cultured with IL-12 for 15 h, a small portion of the NK cells, produced detectable IL-10 levels (Fig. 7C), whereas most NK cells produced low levels IL-10 after an 8-day culture with IL-12 (Fig. 7D), with concomitantly increased CD56 expression (not shown).

**Discussion**

We report that, within CD56<sup>+</sup> NK cells in freshly separated lymphocytes, these CD56<sup>+</sup><sub>high</sub> have a phenotype identical with that of mature (CD3<sup>−</sup>CD56<sup>+</sup>) NK cells that have been stimulated with combinations of IL-15 (or IL-2) and IL-12 (Table I), including the highest expression of activating receptors (NKp44 and NKp46), costimulatory receptors and adhesion molecules (CD2, CD62L, CD244), and the inhibitory receptor CD94, with concomitantly lowered expression of CD16 and CD162R. Also, unlike resting CD56<sup>−</sup>low and like activated NK cells, the CD56<sup>+</sup><sub>high</sub> NK cells in freshly isolated PBL expressed IL-2Ra (CD25), CD45RO, HLA-DR, and NKp44; responded to IL-12 with fast kinetics; and proliferated in the absence of exogenous IL-2 and IL-15. These results strongly support the conclusion that, rather than an immature or distinct specialized subset, CD56<sup>+</sup><sub>high</sub> NK cells are terminally differentiated cells that, within the general pool of mature CD56<sup>+</sup> NK cells, have been recently activated in vivo. Known progenitors to CD56<sup>+</sup> NK cells, including CD34<sup>+</sup> hemopoietic precursors (31), Lin<sup>−</sup> leukocytes, and CD161<sup>+</sup> CD56<sup>−</sup> NK cells (10), are all found at higher proportions in neonatal than adult blood. The similar proportions of CD56<sup>+</sup><sub>high</sub> NK cells in neonatal and adult lymphocytes further detract from the hypothesis (7, 8) that they are immature progenitors to CD56<sup>−</sup><sub>low</sub> NK cells.

As determined here and consistent with our previous reports (1, 10), immature CD3<sup>−</sup>CD161<sup>+</sup>CD56<sup>−</sup> NK cells produce mainly type 2 cytokines (IL-5 and IL-13), relatively high GM-CSF and TNF-α levels, and no IFN-γ. Instead, mature CD3<sup>−</sup>CD161<sup>+</sup>CD56<sup>−</sup>low NK cells produce low-to-null type 2 cytokines, low-to-high IFN-γ levels, and relatively moderate GM-CSF and TNF-α levels. CD56<sup>+</sup><sub>high</sub> NK cells have a functional phenotype consistent with that of terminally differentiated, activated NK cells. On maximal, nonspecific stimulation, they produce IFN-γ levels similar to those produced by CD56<sup>−</sup><sub>high</sub> NK cells; IL-10; GM-CSF, and TNF-α levels lower than those produced by the immature CD56<sup>−</sup> NK cells; and no type 2 cytokines. However, upon specific stimulation (e.g., IL-12, NKp46, K562, CD16) and like CD56<sup>+</sup> NK cells cultured with IL-12, CD56<sup>+</sup><sub>high</sub> NK cells produced higher levels of IFN-γ and TNF-α than CD56<sup>−</sup><sub>low</sub> NK cells. The immediate responsiveness of CD56<sup>+</sup><sub>high</sub> NK cells to IL-12 is consistent with the report of IFN-γ and TNF-β levels higher in culture supernatants of purified CD56<sup>+</sup><sub>high</sub> than of CD56<sup>−</sup><sub>low</sub> NK cells (32). The similar cytokine production by CD56<sup>+</sup><sub>high</sub> and CD56<sup>−</sup><sub>low</sub> NK cells in response to nonspecific, maximal stimulation before culture with IL-12 confirms that CD56<sup>+</sup><sub>high</sub> NK cells are not constitutively capable of producing higher cytokine levels than the CD56<sup>−</sup><sub>low</sub> cells but, having the potential to produce IFN-γ and TNF-α similar to that of the CD56<sup>−</sup><sub>low</sub> cells, respond more rapidly to selected specific stimuli.

The present data confirm our previous reports (1, 10) that maximal IL-13 production, exclusive of IFN-γ, is restricted to immature CD56<sup>−</sup> NK cells, with almost no CD56<sup>+</sup><sub>high</sub> or CD56<sup>−</sup><sub>low</sub> NK cells producing IL-13 on maximal stimulation. They dismiss a previous conclusion that, based on analysis at the population level disregarding minor but detectable contaminant CD56<sup>−</sup> NK cells among the sorted CD56<sup>+</sup> population, proposed that the CD56<sup>+</sup><sub>high</sub> NK cells are the sole or major producers of IL-13 (32). Based on the analysis at the single-cell level reported here, the low (0–80 pg/ml) IL-13 amounts detected, in that report, in 72-h supernatants from CD56<sup>+</sup><sub>high</sub> cells, compared with the 10<sup>3</sup>-fold higher levels determined here to be produced in 15 h by CD56<sup>−</sup>
NK cells, are accounted for by IL-13 produced by few contaminating CD56<sup>−</sup> cells. Instead, IL-10 is produced mostly by CD56<sup>+</sup>CD16<sup>+</sup> NK cells at low levels within 18 h in response to IL-2 and IL-12, most prominently in apoptosing cells. After longer (8-day) culture with IL-12, most NK cells produced detectable IL-10 and concomitantly expressed increased CD56 levels. These results may explain why IL-10, accumulating in only minimal proportions of viable NK cells, is detectable in culture supernatants of CD56<sup>+</sup> NK cells stimulated with IL-12 (32). The same occurs in T cells, with IL-10 and IL-13 detected exclusively in CD56<sup>+</sup> and CD56<sup>−</sup> cells, respectively, and induced and lost, respectively, concomitant to induced CD65 expression (33).

The reportedly low cytotoxicity levels of the CD56<sup>+</sup> high NK cells (30) might reflect loss of cytotoxic granules in recently activated NK cells, rather than CD56<sup>+</sup> high NK cells being a separate specialized (32) or immature subset mediating cytokine-dependent functions and almost no cytotoxicity (8), in contrast to CD56<sup>−</sup> low cells mediating primarily cytotoxic functions. Absence of cytotoxic granules in many, but not all, CD56<sup>+</sup> high NK cells (30) and significant cytotoxicity levels mediated by these cells within 15 h of stimulation with IL-2 (8) are inconsistent with CD56<sup>+</sup> high NK cells being as developmentally immature as CD16<sup>+</sup>CD56<sup>−</sup> NK cells. Indeed, the latter do not express mature NK cell differentiation Ags and activating receptors and do not mediate granule exocytosis-dependent cytotoxicity on short term stimulation (34) but contain functional granules (35). Rather, this phenotype is consistent with degranulation of mature NK cells following recent triggering in vivo.

The observation that CD56<sup>+</sup> high NK cells are indistinguishable from mature NK cells activated by IL-15 (or IL-2), IL-12, and NK cell triggering stimuli (e.g., immune complex) supports the conclusion that these cells are generated in vivo after similar stimulation and explains their presence at sites of inflammation. CD56<sup>+</sup> high NK cells are found in the affected synovial tissues in rheumatoid arthritis (12), together with the monokines IL-12 (36) and IL-15 (37), and systemic immune complexes. CD62L expression on CD56<sup>+</sup> high NK cells explains their presence in lymph nodes, where they may produce IFN-γ and drive effective cellular immune responses (38). Interestingly, expression of CD162R, detectable on CD56<sup>−</sup> low but not CD56<sup>+</sup> high NK cells, is inversely regulated by IL-15 plus IL-2 compared with CD62L, a potential ligand for CD162R. The significance of this reciprocal expression is to be determined.

The anatomical site(s) for in vivo NK cell activation to become CD56<sup>+</sup> high remain(s) to be defined. Unlike T and B cells, minimal NK cell numbers are retained in the spleen, explaining their lower proportions in spleen vs peripheral blood in mice and humans. During murine CMV infection in mice, the number of splenic NK cells increases in correlation with and depending on IL-12 and IL-18 levels (39). In humans, transit through the spleen, or through capillaries and postcapillary venules containing monokines released as a result of inflammation from surrounding tissues, may induce mature NK cell activation and CD56<sup>+</sup> high phenotype. The activated, CD56<sup>+</sup> high NK cells, induced to express CD62L, may subsequently accumulate in lymph nodes, where they can affect the adaptive arm of immune responses via IFN-γ release. IL-10, produced as the cells die, likely limits the ongoing cellular response while promoting plasma cell generation, thus providing Ab for future protective immunity. The possibility will need to be considered that, among the CD56<sup>+</sup> low CD16<sup>+</sup> NK cells, those that express CD62L (15% ± 7%, n = 11), possibly acquired in a fashion similar to that discussed above, may also transit to the lymph nodes and complete the transition to CD56<sup>+</sup> high cells under inflammatory conditions in which monokines are produced. Alternatively, the CD56<sup>+</sup> lowCD16<sup>+</sup> NK cells not expressing CD62L may acquire the CD56<sup>+</sup>CD16<sup>+</sup>low phenotype on interaction with immune complexes bound to the FcγR of the peripheral monocytes and be simultaneously triggered by monocyte-released monokines (IL-12, IL-15, IL-18). Although CD16 expression is down-modulated in response to IL-12 and immune complexes, the combined stimulation via CD16 engagement and monokines would result in CD62L expression, increased CD56 expression, among the other NK cell receptors described here, increased sensitivity to IL-2 and IL-12, and thus increased IFN-γ production and proliferative response. Simultaneous CD94 (if not other KIR) engagement, expected in most NK cells concomitant to CD16 engagement, may, however, limit their accumulation.

A major characteristic of CD56<sup>+</sup> high NK cells is their reduced expression of CD16. Consistent with the above scenario, and in agreement with reports of CD16 down-modulation and re-expression in response to IL-12 (6) or immune complex (40, 41) stimulation, this CD16<sup>+</sup>low phenotype likely reflects, at least in part, a mechanism by which mature CD56<sup>+</sup> high NK cells are removed from circulation, so as to prevent effector activity (42). NK cell re-expression to IL-12 parallels the effect of PD-1 engagement by MHC class I molecules (40, 41), which facilitates regulatory CD16 engagement, expected in most NK cells concomitant to CD16 engagement, may, however, limit their accumulation. A major characteristic of CD56<sup>+</sup> high NK cells is their reduced expression of CD16. Consistent with the above scenario, and in agreement with reports of CD16 down-modulation and re-expression in response to IL-12 (6) or immune complex (40, 41) stimulation, this CD16<sup>+</sup>low phenotype likely reflects, at least in part, a mechanism by which mature CD56<sup>+</sup> high NK cells are removed from circulation, so as to prevent effector activity (42). NK cell re-expression to IL-12 parallels the effect of PD-1 engagement by MHC class I molecules (40, 41), which facilitates regulatory CD16 engagement, expected in most NK cells concomitant to CD16 engagement, may, however, limit their accumulation.
On the basis of the definition of the latest stages of T cell development, we proposed that terminally differentiated, activated CD56+ T cells might be equivalent to regulatory T_{reg} cells (33). Like T_{reg} (42) and CD56+ T cells, CD56+high NK cells produce IL-10, express CD25, rapidly proliferate in response to IL-2, and have activated phenotype. T_{reg} cells are reportedly likely generated in vivo in nonpathological conditions in response to self-Ags. The same may be the case for CD56+high NK cells, in that the ligands for the triggering receptors expressed on most NK cells (e.g., MIC-A/B for NKG2D; Ref. 43) are surmised to be self proteins, either constitutively expressed or induced on stress. A thorough study of the factors (stromal and accessory cell interactions, soluble factors, temporal windows of opportunity) modulating CD16 and KIR expression during development in the bone marrow and periphery is needed to define which mechanisms are responsible for accumulation of CD16+ and KIR− CD56+high NK cells in the periphery and to guide rational approaches to the ex vivo generation and manipulation of these cells for possible therapeutic intervention.

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References


