Human natural killer cells: a unique innate immunoregulatory role for the CD56<sup>bright</sup> subset

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During the innate immune response to infection, monocyte-derived cytokines (monokines), stimulate natural killer (NK) cells to produce immunoregulatory cytokines that are important to the host’s early defense. Human NK cell subsets can be distinguished by CD56 surface density expression (ie, CD56<sup>bright</sup> and CD56<sup>dim</sup>). In this report, it is shown that CD56<sup>bright</sup> NK cells produce significantly greater levels of interferon-γ, tumor necrosis factor-β, granulocyte macrophage–colony-stimulating factor, IL-10, and IL-13 protein in response to monokine stimulation than do CD56<sup>dim</sup> NK cells, which produce negligible amounts of these cytokines. Further, qualitative differences in CD56<sup>bright</sup> NK-derived cytokines are shown to be dependent on the specific monokines present. For example, the monokine IL-15 appears to be required for type 2 cytokine production by CD56<sup>bright</sup> NK cells. It is proposed that human CD56<sup>bright</sup> NK cells have a unique functional role in the innate immune response as the primary source of NK cell–derived immunoregulatory cytokines, regulated in part by differential monokine production. (Blood. 2001;97:3146-3151)

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Materials and methods

Cell culture reagents and antibodies

Human NK cells and macrophages were cultured in RPMI-1640 with 10% human serum (C-6 Diagnostics, Mequon, WI) and antibiotics. Recombinant human IL-12 was provided by Genetics Institute (Cambridge, MA); rIL-15 was a gift from Immunex (Seattle, WA); and rIL-18 was a gift from BASF Bioresearch (Worcester, MA); and rIL-1β was purchased from Peprotech (Rocky Hill, NJ). PMA and ionomycin (calcium salt) were obtained from Calbiochem (La Jolla, CA). Anti-CD56-phycocerythrin (PE) (NKH1), CD16-fluorescein isothiocyanate (FITC) monoclonal antibodies, and isotype controls were purchased from Coulter (Miami, FL).

Purification of human NK cell subsets and macrophages

Human NK cells were isolated from fresh normal donor leukopacks (American Red Cross, Columbus, OH) as previously described1,12 or with RosetteSep NK cell cocktail (StemCell Technologies, Vancouver, BC) according to the manufacturer’s directions. NK cells were stained with anti-CD56-PE or control PE, and subsets were purified based on CD56 cell-surface density by FACS (Elite Flow Cytometer; Coulter) as previously described.6 Cells were routinely greater than 98% pure by post-FACS cytometric analysis of CD56 and CD16, as shown in Figure 1. Human macrophages were isolated by adherence and gentle scraping (more than 85% CD14+ by flow cytometry).

NK cell monokine and PMA–ionomycin stimulation

Purified NK cells (5 × 10⁴ cells/well) were stimulated with combinations of IL-12 (10 ng/mL), IL-15 (100 ng/mL), IL-18 (100 ng/mL), IL-1β (10 ng/mL), PMA (20 ng/mL), or ionomycin (5 μM), and cell-free culture supernatants were harvested at 72 hours. Cell culture supernatants were assayed for IFN-γ, IL-10, GM-CSF, TNF-α, IL-13, IL-5 (Endogen, Woburn, MA), and TNF-β (R&D Systems, Minneapolis, MN) protein in duplicate enzyme-linked immunosorbent assay (ELISA) wells. Results represent the mean ± SEM of 3 or more donors.

Quantitation of cytokine transcripts by real-time RT-PCR

FACS-purified CD56 bright and CD56 dim NK cells (1 × 10⁵) were either immediately lysed for RNA (Qiagen RNeasy lysis buffer; Qiagen, Valencia, CA) or cultured at 1 × 10⁶ cells/well with recombinant monokines. Cells were harvested at 24 hours and lysed with 300 μL RNA lysis buffer. Total cellular RNA was isolated (Qiagen RNeasy Mini-kits; Qiagen) and cDNA was generated with random hexamer primers and MMLV-RT according to the manufacturer’s recommendations (Gibco Life Technologies, Rockville, MD). cDNA was then used as a template for real-time polymerase chain reaction (PCR).

Real-time quantitative reverse transcription (RT)-PCR is a novel method to accurately measure amplified target copy number through the use of a dual-labeled fluorogenic probe.22 Real-time PCR reactions for human IFN-γ, GM-CSF, and IL-10 transcripts were performed as previously described and as multiplex reactions with primer and probe sets specific for the cytokine transcript of interest and an internal control (rRNA, 18s; PE Applied Biosystems, Foster City, CA).18 cDNA from PHA-activated human lymphocytes was used as a positive control for cytokine transcripts, and murine cDNA (P815 cell line) was used as a negative control. Reactions were performed using an ABI prism 7700 sequence detector (Taqman; PE Applied Biosystems), and data were analyzed with the Sequence Detector version 1.6 software to establish the PCR cycle at which the fluorescence exceeded a set threshold, C_T, for each sample. Data were analyzed according to the comparative C_T method, as previously described, using internal control (18s) transcript levels to normalize differences in sample loading and preparation. Results are semiquantitative and represent the n-fold difference of transcript levels in a particular sample compared to calibrator cDNA (for these experiments, cDNA samples of resting NK cells from each donor). Results are expressed as the mean ± SEM of triplicate reaction wells.

NK cell and macrophage co-cultures

Purified CD56 bright and CD56 dim NK cells (1.0 × 10⁵) were co-cultured with autologous macrophages (1.0 × 10⁵) as previously described2 and stimulated with 10 μg/mL lipopolysaccharide (LPS; serotype 0127 B8; Sigma, St Louis, MO) for 72 hours.

Statistical analysis

Statistical analysis was performed using the Student paired t test; P < .05 was considered significant.

Results

CD56 bright NK cells produce abundant type 1 and type 2 cytokines compared to CD56 dim NK cells

We stimulated sorted resting CD56 bright and CD56 dim NK cells (Figure 1) with the recombinant monokines IL-12, IL-15, IL-18, and IL-1β alone and in combination with IL-12 or IL-15. To examine the stimulation of NK cells independent of monokine receptor expression, NK cell subsets were also activated with phorbol esters (PMA) plus ionomycin. The CD56 bright subset produced significantly more of the type 1 cytokines IFN-γ and TNF-β than CD56 dim NK cells cultured under identical conditions after stimulation with monokines or PMA plus ionomycin (Figure 2). CD56 bright NK cells co-stimulated with IL-18 plus IL-12...
produced the most IFN-γ protein (Figure 2A), whereas stimulation with IL-18 plus IL-15 or IL-1b plus IL-15 induced the highest levels of TNF-β protein production.

NK cell production of IL-10, a type 2 cytokine, was only detected after co-stimulation with IL-12 plus IL-15 (Figure 3A), with CD56bright NK cells producing in excess of 25-fold more IL-10 protein than CD56dim cells. Interestingly, other monokine combinations, including IL-12 plus IL-18, failed to elicit any production of IL-10 from either subset. Modest amounts of IL-13, another cytokine produced by committed Th2 cells, were detected in cultures of CD56bright NK cells stimulated with IL-15 plus IL-18 or IL-1β, and this production was always significantly greater than in CD56dim NK cells (Figure 3B). We did not detect any NK cell production of IL-5 protein in response to monokine or PMA plus ionomycin stimulation (data not shown; sensitivity less than 2 pg/mL).

Thus, CD56bright NK cells produce high levels of 2 principal type 1 cytokines, IFN-γ and TNF-β, after stimulation with monokines or phorbol esters plus ionomycin. However, only specific combinations of monokines that included IL-15 as a co-stimulus induced IL-10 or IL-13 protein in cultures of CD56bright NK cells, suggesting that production of these type 2 cytokines by human NK cells requires specific monokine (eg, IL-15)-induced signaling.

CD56bright NK cells produced significantly more of the macrophage-activating GM-CSF than CD56dim NK cells in response to all monokine or PMA plus ionomycin stimulation (data not shown; sensitivity less than 2 pg/mL).

![Figure 2. NK cell production of type 1 cytokines IFN-γ and TNF-β.](image)

![Figure 3. CD56bright NK cells produce the type 2 cytokines IL-10 and IL-13.](image)

![Figure 4. GM-CSF production by human NK cell subsets.](image)
positive stimuli (Figure 4). IL-15 was the only monokine that induced GM-CSF protein without a co-stimulus (sensitivity less than 15 pg/mL). The combination of IL-15 plus IL-18 was the most potent monokine stimulus for GM-CSF production, similar to TNF-β. Unlike other NK-derived cytokines, stimulation with PMA plus ionomycin induced the highest levels of GM-CSF protein from CD56bright NK cells. CD56bright NK cell production of the pro-inflammatory cytokine TNF-α, after culture with monokines or PMA plus ionomycin, was modest (less than 300 pg/mL) and somewhat variable, but it was consistently greater than in the CD56dim NK cell subset (data not shown).

Cytokine transcript levels in NK cell subsets

To determine whether NK cell subsets have differential baseline expression of cytokines not detectable by ELISA that might account for observed differences in protein production, we measured transcript levels of 3 primary NK-derived cytokines in resting and monokine-activated NK cell subsets by real-time quantitative RT-PCR. Resting subsets lacked any detectable expression of IL-10 (data not shown), but both CD56bright and CD56dim NK cell subsets expressed equal amounts of IFN-γ and GM-CSF transcript (data not shown; n = 3). Therefore, there is no detectable difference in the baseline production of these cytokine transcripts by resting NK cell subsets. After 24 hours of monokine stimulation, the CD56bright NK subset produced higher levels of IFN-γ and GM-CSF transcript than the CD56dim NK subset, consistent with their production of the respective proteins (Figure 5A,B).

CD56bright NK cells produce significantly more IFN-γ than CD56dim NK cells when cultured with LPS-activated macrophages

Gram-negative bacteria-derived LPS stimulate macrophages to secrete a number of monokines, including IL-1, IL-12, and IL-15.22-24 We have previously shown that the production of monokines by LPS-activated macrophages induces IFN-γ production by human NK cells in vitro.25 To determine the subset of NK cells responsible for IFN-γ production after monokine activation by LPS, purified CD56bright and CD56dim NK cells were co-cultured with LPS-stimulated autologous macrophages (Figure 6). Similar results obtained with recombinant monokines, CD56bright NK cells produced 8-fold more IFN-γ protein than CD56dim cells (n = 7). Thus, CD56bright NK cells are the primary producers of IFN-γ in response to both recombinant and endogenous monokines.

Discussion

Collectively, our results reveal that CD56bright human NK cells are the primary source of NK-derived immunoregulatory cytokines, including IFN-γ, TNF-β, IL-10, IL-13, and GM-CSF, whereas the CD56dim NK cell subset consistently produces significantly less of these cytokines in vitro. The results confirm our earlier observations of CD56bright NK IFN-γ production26 but also provide evidence for a more generalized property that can be attributed to this distinct human NK cell subset. Although the possibility exists that differences in cytokine production may be attributed to differential monokine receptor expression, density, or both, the activation of NK cell subsets with phorbol esters plus ionomycin, which is not dependent on monokine receptor activation, resulted in significantly greater production of IFN-γ, TNF-β, and GM-CSF by CD56bright NK cells. Furthermore, LPS, a bacterial component recognized by host innate immune effector cells, indirectly induced CD56bright NK cells to produce much greater IFN-γ than CD56dim NK cells when co-cultured with macrophages. Therefore, the CD56bright NK subset has a significantly higher capacity for cytokine production than the CD56dim subset.

Peritt et al22 recently reported the differentiation of human NK cells into NK1 and NK2 subsets by generating NK cell clones after 8-day in vitro culture under type 1– or type 2–inducing conditions. After stimulation with PMA plus ionomycin, NK1 cells produced IFN-γ and TNF-β but also produced IL-10, a type 2 cytokine, and NK2 cells produced IL-5 and IL-13. However, because of the clear role of NK cells as efficient producers of cytokines early in the innate immune response to infection long before clonal responses are mounted, it is unlikely that prolonged peripheral NK cell differentiation is necessary in vivo. In this report we show that freshly isolated CD56bright NK cells produce abundant type 1 and type 2 cytokines immediately after monokine stimulation, consistent with their known in vivo role. Hence, though we do not exclude the possibility for subsets of human NK cells that produce type 1 or type 2 cytokines, our data suggest that these subsets would be found within the resting CD56bright NK cell population.

Additional data presented here indicate that the qualitative and quantitative production of monokines after host infection are likely...
Figure 6. CD56bright NK cell co-cultured with LPS-activated macrophages produce abundant IFN-γ. Resting CD56bright and CD56dim NK cells (1 × 10⁶/well) were co-cultured with autologous macrophages (1 × 10⁶/well) or LPS for 72 hours, and cell culture supernatants were assayed for IFN-γ protein. CD56bright NK cells co-cultured with LPS-activated macrophages (Mø + LPS) produced significantly more IFN-γ protein than CD56dim NK cells (6439 ± 2579.16 pg/mL vs 791 ± 289.2 pg/mL; P < .05; n = 7). No IFN-γ protein was detected in co-cultures without LPS (Mø) or in cultures of LPS-activated NK cells or macrophages (data not shown). Indicates CD56dim; ■. CD56bright.

Figure 7. CD56bright and CD56dim NK cell subsets exhibit differential receptor profiles and innate immune functions. Many of the receptors and functions of CD56bright (red cell) and CD56dim (blue cell) NK cells are schematized in this figure. CD56brightCD16dim/neg NK cells produce abundant immunoregulatory cytokines (some of which are depicted here) and exhibit potent LAK activity but are less effective mediators of ADCC and natural cytotoxicity. By contrast, CD56dimCD16bright NK cells produce low levels of NK-derived cytokines and are potent mediators of ADCC, LAK activity, and natural cytotoxicity. CD56dim NK cells have a less granular activity, and natural cytotoxicity. The authors cultured CD34⁺Lin⁻⁺ hematopoietic progenitors with FL ± IL-21 and FL + IL-15 ± IL-21. IL-21, in addition to IL-15 and FL, induced the differentiation of CD56⁺CD16⁺ NK cells, which, by flow cytometric analysis, appear to be CD56dimCD16⁺. FL plus IL-21 alone did not induce NK cell differentiation, whereas stimulation with FL plus IL-15 resulted in the expected population of CD56brightCD16neg NK cells. The discovery of this cytokine, which can, in combination with IL-15, induce differentiation of CD56dimCD16⁺ NK cells, supports a hypothesis whereby human CD56bright and CD56dim NK cells are terminally differentiated cell types that develop within the bone marrow under the influence of differential growth factors, such as IL-15 and IL-21. Further studies are required to investigate definitively the developmental relation between CD56bright and CD56dim NK cells. It will be interesting to determine the role of...
IL-21/IL-21R in the development of human NK cell subsets and NK receptors (e.g., CD16 and KIR).

As we continue to develop immunotherapeutic strategies that target human NK cells, such as the selective expansion of CD56bright NK cells with low-dose IL-2 treatment of malignancies,\textsuperscript{8,35} and human immunodeficiency virus,\textsuperscript{36} it will be important to understand the functional differences between these human NK cell subsets. Knowledge of the distinct functional attributes of CD56bright (e.g., immunoregulatory cytokine production) and CD56dim (e.g., cytotoxicity and antibody-dependent cellular cytotoxicity) human NK cell subsets and the factors involved in their development and expansion may enable us to design strategies that preferentially activate that subset with the greatest therapeutic potential for a particular disease.

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References

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