Rapid Communication

Role of Interleukin-15 in the Development of Human CD56+ Natural Killer Cells From CD34+ Hematopoietic Progenitor Cells

By Ewa Mrózek, Paul Anderson, and Michael A. Caligiuri

Human natural killer (NK) cells are bone marrow (BM)-derived CD2−CD16−CD56+ large granular lymphocytes (LGL) that lack CD3 yet contain the T-cell receptor (ζ-TCR). NK cells provide requisite interferon-γ (IFN-γ) during the early stages of infection in several experimental animal models. A number of studies have shown that human CD3−CD56− NK cells can be obtained from BM-derived CD34+ hematopoietic progenitor cells (HPCs) cultured in the presence of interleukin-2 (IL-2) and an allogeneic feeder cell layer, or IL-2 and other hematopoietic growth factors such as the c-kit ligand (KL). The failure to detect the IL-2 gene product within the BM stroma and the presence of NK cells in IL-2-deficient mice suggested that cytokines other than IL-2 may participate in NK cell differentiation from HPCs in vivo. IL-15 is a cytokine which, while lacking any sequence homology to IL-2, can activate cells via the IL-2 receptor. Here we show that human BM stromal cells express the IL-16 transcript, and supernatants from long-term BM stromal cell cultures contain IL-15 protein. In vitro, CD3−CD56+ NK cells can be obtained from 21-day cultures of CD34+ HPCs supplemented with IL-15 in the absence of IL-2, stromal cells, or other cytokines. The addition of the KL to these cultures had no effect on the differentiation of the CD3−CD56+ cytotoxic effector cells, but greatly enhanced their expansion. The majority of these cells lack CD2 and CD16, but do express ζ-TCR. Similar to NK cells found in peripheral blood, the CD2−CD16−CD56+ NK cells grown in the presence of IL-15 were found to be potent producers of IFN-γ in response to monocyte-derived cytokines. Thus IL-15, like KL, appears to be produced by BM stromal cells. IL-15 can induce CD34+ HPCs to differentiate into CD3−CD56+ NK cells, and KL can amplify this. Therefore, IL-15 may be a physiologically relevant ligand for NK cell differentiation in vivo.

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purified from MNC by panning, using flasks coated with anti-CD34 monoclonal antibody (MoAb) (Applied Immune Sciences, Santa Clara, CA) or by affinity chromatography with the Ceprate device (Cellpro, Bothel, WA), following the manufacturer’s instructions. The enriched cells were next stained with anti-CD34-PE, anti-CD2-FITC (Becton Dickinson, San Jose, CA) and anti-CD16-FITC (Caltag Laboratories, San Francisco, CA) and sorted for the CD34+CD2-CD16- (CD34+LIN-) population on a FACStar Plus cell sorter (Becton Dickinson). Purity was always ≥97%.

Long-term suspension culture of CD34+ HPCs. Sorted CD34+LIN- HPCs were cultured in 96-well U-bottomed microplates at a concentration of 2 × 10⁴ cells in 200 μL of RPMI 1640 (GIBCO, Grand Island, NY), 10% heat-inactivated human AB (HAB) serum (C-Six Diagnostics, Mequon, WI), and antibiotics. The experimental cultures were supplemented with 100 ng/mL of recombinant (r) IL-15 and/or 100 ng/mL of rKL (Immunex Corp, Seattle, WA). For one set of experiments, CD34+LIN- HPCs were cultured in 230 μL/mL of rIL-2 (Hoffman LaRoche, Nutley, NJ; specific activity of 1.53 × 10⁶ U/mg) and 100 ng/mL of rKL. Plates were incubated at 37°C in 5% CO₂ humidified atmosphere for 21 days. At day 7, day 14, and every 3 days thereafter, half of the culture medium was replaced with fresh medium containing 10% HAB and the same concentration of growth factors. After 21 days, the cells were enumerated and analyzed for morphology, cytotoxicity, phenotype, and cytokine production.

Assessment of immunophenotype, cytotoxicity, and proliferation.
The cells generated in 21-day cultures were first incubated with nonreactive mouse Ig for 10 minutes on ice and then stained with directly conjugated MoAbs: NKH-1(anti-CD56)-PE (Coulter Immunology, Hialeah, FL) and anti-CD16-RTC and/or Leu-5b(anti-CD2)-FITC and/or Leu-4(anti-CD3)-RTC (Becton Dickinson). Background fluorescence was determined on cells stained with directly conjugated nonreactive MoAbs (Becton Dickinson). Cells were analyzed using an MNC gate on a FACScan with the Lysis II software program (Becton Dickinson). Cytoplasmic staining for \( \zeta \)-TCR on digitonin-permeabilized CD56\(^+\) BM-derived NK cells was performed as previously described.\(^6\) Cytotoxic activity of cultured cells was determined by a standard \(^{51}\)Cr-release cytotoxicity assay against the NK-sensitive K562 and NK-resistant COLO 205 cell lines as previously described,\(^6\) at an effector-to-target ratio of four. Cell proliferation was measured by methyl-[\(^{3}H\)] thymidine incorporation\(^6\) and cell enumeration was performed by vital dye exclusion using a standard hemocytometer. Results for cytotoxicity and proliferation assays represent the mean ± SE of triplicate wells, and a Student's paired t-test was used where indicated.

**BM stromal cell cultures.** Fresh BM MNC were suspended in 75-cm\(^2\) tissue culture flasks at a concentration of 0.5 to 1.0 \( \times 10^{6} \) cells/mL in minimum essential \( \alpha \)-medium with 15% fetal calf serum

![Image of flow cytometry plots](image-url)
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**RESULTS**

Human IL-15 and IL-2 gene products in human stromal cells. We analyzed human BM stromal cell cultures for the presence of the IL-2 and IL-15 gene products. As shown in Fig 1, transcript for IL-15 was detected in stromal cells from three of three individuals tested by RT-PCR, whereas transcript for IL-2 was not detected by RT-PCR. Further, supernatants collected from these cultures were concentrated

(GIBCO) and $1 \times 10^{-8}$ mol/L hydrocortisone (Upjohn, Irving, TX) according to the method of Wetzler et al.\textsuperscript{16} Cultures were maintained at 37°C in humidified 5% CO₂. Medium was replaced and nonadherent cells removed every 7 days. Cultures reached confluence between 4 and 6 weeks, at which time they were washed twice to remove nonadherent cells and then treated with 0.25% trypsin (GIBCO) for 10 minutes to remove the adherent layer for RNA extraction. Under these conditions the adherent layer was found to contain 3% to 5% monocytes/macrophages. Culture supernatants obtained immediately after initiating the process of adherent cell removal were collected and stored at -70°C.

**Measurement of cytokine production.** CD34⁺ BM HPCs, CD³⁺CD56⁺ NK cells, and 21-day cultures of CD56⁺LIN⁻ NK cells were each sorted (>97% purity) by flow cytometry using a FACStar Plus (Becton Dickinson) and plated at 50,000 cells/well in RPMI with 10% HAB. After overnight incubation in the absence of any cytokines, cells were co-cultured with 100 ng/mL IL-15 and 10 U of rIL-12 (Genetics Institute Inc, Cambridge, MA; specific activity 4.5 x 10⁵ U/mg). After 72 hours supernatants were collected and frozen at -70°C until assayed simultaneously. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used for measuring granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) (R & D Systems, Minneapolis, MN), and IFN-γ (GIBCO). Supernatants from BM stromal cell cultures were thawed and concentrated 10-fold using Centrifugal concentrators (Amicon, Beverly, MA). RPMI 1640 with 10% HAB and RPMI 1640 with 10% fetal bovine serum (FBS) were used as negative controls and were also concentrated 10-fold. All samples were then assayed for IL-15 protein using our human IL-15 ELISA,\textsuperscript{22} with a sensitivity of 10 pg/mL. Identical samples were assayed for human IL-15 by a commercially available ELISA (BioSource International, Camarillo, CA) with a sensitivity of 6 pg/mL, and were assayed for IL-2 by ELISA (Endogen, Inc, Cambridge, MA) with a sensitivity of <6 pg/mL.

**Immunoblotting analysis for the expression of ζ-TCR protein.** Western analysis for ζ-TCR was performed as previously described.\textsuperscript{17} Briefly, after 3-week cultures of HPCs in the presence of IL-15 and KL or IL-2 and KL, sorted CD56⁺LIN⁻ cells were solubilized in NP-40 lysis buffer (1% NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 50 mmol/L Tris, pH 8.0) for 30 minutes on ice, then centrifuged for 30 minutes at 12,000g. Supernatants were diluted 1:1 with sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1 mol/L Tris HCl, pH 6.8, 0.02% bromphenol blue), separated on 10% SDS polyacrylamide gels, transferred to nitrocellulose, and probed with the anti-ζ-TCR MoAb TIA-1. Controls included CD³⁺CD56⁺ NK cells sorted from PB (positive), and the COLO 205 cell line (ATCC, Rockville, MD; negative).
10-fold and tested for the presence of IL-15 and IL-2 protein by ELISA. After correction for concentration, IL-15 protein was detected in two of the three samples at a concentration of 3.7 pg/mL and 1.6 pg/mL, respectively, while undetectable in the third sample and in negative controls. In contrast, IL-2 protein was not found in any of the samples by an ELISA that could have detected <0.6 pg/mL in these concentrated supernatants.

Effect of IL-15 and KL on the generation of NK cells from CD34+ HPCs. A purified population of CD34+ HPCs was cultured for 21 days in the presence of KL alone, IL-15 alone, or a combination of KL and IL-15. The results are summarized graphically in Fig 2A-C. Cells cultured for 21 days in medium alone neither expanded nor differentiated into CD56+ NK cells (not shown). CD34+ HPCs cultured with KL had a 5.7 ± 0.6-fold increase in absolute cell number but did not develop CD56 surface antigen expression or LGL morphology. CD34+ HPCs cultured for 21 days with IL-15 alone had minimal (1.1 ± 0.3-fold) increase in absolute cell number, yet did acquire CD56 antigen expression (~40% to 85%), and LGL morphology. CD34+ HPCs cultured for 21 days in the presence of IL-15 and KL showed a 6.5 ± 0.7-fold increase in cell number with ~40% to 85% of cells expressing CD56 with LGL morphology. Thus, while IL-15 alone can induce CD34+ HPCs to express CD56 surface antigen and develop LGL morphology during 21 days of culture, IL-15 and KL are required for differentiation and significant expansion of the CD56+ NK population. This was confirmed in follow-up studies where CD34+ HPCs were cultured under different conditions and assayed for proliferation on days 1, 7, and 21 by [3H]-thymidine incorporation. As shown in Fig 3, by day 7 there was a significant difference in [3H]-TdR incorporation by cells cultured in IL-15 alone compared to cells cultured with KL or KL and IL-15 (P < .0005).

CD56 antigen expression was an excellent predictor of NK cytotoxic activity. CD34+ HPCs cultured for 21 days in IL-15 alone or IL-15 and KL showed an equivalent amount of cytotoxic activity against K562 tumor cell targets when corrected for cell number (Fig 4A). The same was true of the NK-resistant target, COLO 205 (not shown). In contrast, no cytotoxic activity was seen from CD34+ HPCs cultured for 21 days in KL alone.

The vast majority (>95%) of CD56+ cells generated in 21-day culture of HPCs in the presence of IL-15, with or without KL, had LGL morphology with high-density surface expression of CD56 (CD56bright), and all lacked expression of CD34. Lineage marker analysis reproducibly showed that the BM-derived CD56bright cells did not express CD2 or CD16 surface antigens (Fig 4B), in contrast to the majority of PB NK cells.5,23 The CD56brightLIN- phenotype is similar to that observed for CD34+ HPCs cultured in the presence of IL-2,4,5,6,24

ζ-TCR protein expression in the CD56+ LIN- BM-derived NK cells. ζ-TCR is found in PB NK cells and is biochemically and functionally linked to CD16.25,27 ζ-TCR protein expression was analyzed in CD56+ LIN- cells derived from CD34+ HPCs of three different donors, cultured for 21 days in KL + IL-15. ζ-TCR was also analyzed in CD56+ LIN- cells derived from CD34+ HPCs of three different donors, cultured for 21 days in KL + IL-2. Both populations were found to express ζ-TCR protein by immunoblot analysis (Fig 5) and on flow cytometric analysis of permeabilized cells (not shown). CD56+ LIN- NK cells cultured in KL and IL-2 appeared to express less ζ-TCR than that detected in CD56+ LIN- NK cells cultured in KL and IL-15.

Cytokine production by CD56+ LIN- BM-derived NK cells. To date, the cytokine production by CD56+ LIN- NK cells derived from CD34+ HPCs has not been investigated. Costimulation of PB NK cells with monocyte-derived cytokines IL-12 and IL-15 provides an optimal stimulus for NK cell production of INF-γ, TNF-α, and GM-CSF.15 CD56+ LIN- cells were sorted from 21-day cultures of CD34+ HPCs in IL-15 and KL. CD3+ CD56+ LIN- PB NK cells and CD34+ BM HPCs were also sorted from fresh blood and BM, respectively. Cells were next incubated overnight in medium alone and then costimulated with rIL-12 and rIL-15 for 72 hours. Supernatants were collected and assayed for cytokine production by ELISA. Results are displayed in Table 1 and show that CD56+ LIN- BM-derived NK cells produced significant amounts of INF-γ as well as moderate amounts of TNF-α and GM-CSF, compared with PB NK cells. In contrast, freshly isolated CD34+ HPCs produced minimal amounts of all three cytokines.

**DISCUSSION**

A number of studies have now demonstrated that NK cells can be generated in vitro from CD34+ human BM HPCs in LTBM with IL-2,7,23 Endogenous IL-2 is thought to be only transiently produced by antigen-activated T cells, which themselves express a high-affinity IL-2R and require the lymphocytotrophic hormone for clonal expansion.7 Further, NK cells can develop in the absence of T cells or IL-2,7,11 but not in the absence of IL-2Ry.12,13 Collectively these data suggest that IL-2 is probably not required and not sufficiently available for NK cell differentiation in vivo, and that other cytokines which use IL-2Ry are likely to be important in this process. Recently, human IL-15 has been cloned from...
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Fig 4. (A) Cytotoxic activity of CD34+ HPCs cultured for 21 days in medium alone, IL-15 alone, KL alone or combination of IL-15 and KL. The effector-to-target ratio was 4. Results represent the mean ± SE for five separate experiments. (B) Phenotypic characterization of cells generated from a 21-day culture of CD34+ HPCs in the presence of both IL-15 and KL. Results displayed are representative of 10 separate experiments.

a human BM stromal cell line, and has been shown to use the IL-2Rβ and IL-2Rγ protein subunits for binding and signal transduction in T cells and NK cells. Despite lacking any sequence homology with IL-2, IL-15 has thus far been shown to share virtually identical bioactivities with IL-2. Moreover, IL-15 transcript is widely expressed in a number of tissues. These data led us to hypothesize that IL-15 could be involved in human NK cell differentiation from BM-derived HPCs, and the current study was performed to test this hypothesis.

Several novel observations have been made in this report. First, we were able to show that long-term cultures of human BM stromal cells obtained from normal individuals constitutively express transcript for human IL-15 and secrete IL-15 protein into culture supernatants. We have not identified the specific cellular components of BM stroma responsible for IL-15 production. However unstimulated CD14+ monocytes, comprising ~5% of cultures, do not produce detectable IL-15 protein, suggesting that other stromal cells are likely to be involved. We were unable to detect IL-2 mRNA in BM-derived stromal cells using RT-PCR, consistent with an earlier report, and we did not find IL-2 protein in concentrated stromal cell supernatants. Thus, IL-15 is a ligand that not only binds to components of the IL-2R, but is produced within the marrow stroma, making it a more physiologically relevant ligand for the IL-2R thought to be constitutively expressed on CD34+ hematopoietic progenitors. Definitive proof of a role for IL-15 in NK cell development will have to await gene disruption studies in the mouse.

Results presented here demonstrate for the first time that IL-15 and KL are the only two cytokines required for the differentiation and expansion of the CD56Lin NK cells from CD34+ HPCs. Others have shown that BM stromal cells produce both the membrane-bound and soluble isoforms of KL transcript. Thus, it seems plausible that the CD34+ HPC subset which differentiates into a CD56+ NK cell could coexpress IL-2Rβγ and c-kit protein receptors either constitutively or early in their differentiation pathway, receiving coactivation signals from their respective ligands produced by the human stromal cells. IL-15 and KL have relatively distinct functions in NK cell development from CD34+ HPCs. In the absence of IL-2, IL-15 appears to be essential for CD56+ antigen expression, LGL morphology, and functional differentiation. Given the low NK precursor frequency previously reported within a CD34+ HPC subset, and our results demonstrating a modest expansion of CD56+ NK cells from CD34+ HPCs in the presence of IL-15 alone, it is likely that IL-15 can induce some proliferation in the absence of KL. In contrast, KL alone has virtually no influence on NK cell differentiation from CD34+ HPCs, yet can clearly amplify the expansion of the CD56+ NK population in the presence IL-15. Therefore, IL-15 may serve primarily to promote differentiation of CD34+ HPCs that are already committed as NK cell precursors, whereas KL may serve to amplify this response. Additionally, IL-15 may serve to promote less committed HPC populations toward NK cell differentiation. Further elucidation of the effects IL-15 may have on this pathway are likely to require the identification and characterization of the CD34+ HPC that is committed to NK cell differentiation.

The synergy seen between IL-15 and KL in promoting NK cell differentiation and expansion from CD34+ HPCs in vitro is supported by our earlier identification of an NK cell subset found in PB which is unique among human lymphocytes in its constitutive expression of IL-2R and c-kit. Alone, KL functions as a survival factor for the c-kit+ NK cells, and significantly enhances IL-2- or IL-15–induced NK cell proliferation (and W.E. Carson, M.A. Caligiuri, unpublished observation, July 1994). Interestingly, this subset of c-kit+ PB NK cells appears to be less differentiated than the majority of NK cells because they maintain a high proliferative potential and either lack CD16 or express it in low density. The more abundant CD2+CD16+CD56dim PB
NK cells neither express c-kit nor show significant \(^{3}H\)-TdR incorporation in the presence of IL-2 or IL-15. Thus, it appears that the minor c-kit\(^{-}\)CD2\(^{-}\)CD16\(^{-}\)CD56\(^{\text{bright}}\) PB NK cell subset is immediately differentiated between the BM-derived population and the more abundant c-kit\(^{-}\)CD2\(^{-}\)CD16\(^{-}\)CD56\(^{\text{dim}}\) NK population.

The absence of CD2 antigen surface expression on the BM-derived NK cells cultured in the presence of IL-15 is in striking contrast to virtually all CD56\(^{-}\) NK cells found in PB. However, a CD2\(^{-}\)CD16\(^{-}\)CD56\(^{\text{bright}}\) NK cell of maternal origin has been identified within the human decidua during early pregnancy, and the same cell can be found within fetal liver (L.L. Lanier, personal communication, December 1995). These data suggest that additional differentiation factor(s) are likely to be important prior to NK cell migration into the circulation. It is possible that undefined thymocyte-derived cytokines required for high-density CD2 expression on T cells are also found in BM stroma and are required for CD2 expression on PB NK cells. The presence of a CD2\(^{-}\)CD16 CD56\(^{-}\) NK cell subset in PB suggests that acquisition of CD16 may occur in the peripheral circulation during subsequent steps of NK cell maturation.

Expression of the \(\zeta\)-TCR protein has not previously been analyzed in CD56\(^{\text{Lin}}\) BM-derived NK cells. CD2 and CD16 are both NK cell activation antigens, and we have previously shown that CD16 is biochemically and functionally linked to \(\zeta\)-TCR in NK cells. Therefore, it was somewhat surprising to find that the CD56\(^{\text{Lin}}\) NK cells derived from CD34\(^{+}\) HPCs in the presence of IL-15 or IL-2 do indeed express the \(\zeta\)-TCR protein in the absence of these activation antigens. Their expression does appear lower than the CD2\(^{-}\)CD56\(^{\text{bright}}\) NK cells in PB which either lack CD16 or express it in low density. Nonetheless, this observation lends support to the recent report that other NK cell molecules such as the target recognition structure p58 can associate with \(\zeta\)-TCR, and may indeed be expressed on the BM-derived NK cells.

A number of experimental animal models have demonstrated that NK cells have a pivotal role in providing IFN-\(\gamma\) during infections and septic shock. Interestingly, the current report provides the first documentation that the CD56\(^{\text{Lin}}\) NK cells derived from CD34\(^{+}\) HPCs can produce substantial amounts of IFN-\(\gamma\) in response to two monocyte-derived cytokines, IL-12 and IL-15, quite comparable to that produced by mature PB NK cells. The abundant production of IFN-\(\gamma\) at this presumably early stage of NK cell differentiation, ie, in the absence of CD2 and CD16 surface antigen expression, would lend support to this function being highly conserved and hence possibly more critical during the early innate immune response to infection.

In summary, this study provides the first in vitro evidence of a possible role for IL-15 in human NK cell development. We identified the IL-15 gene product in human BM stromal cells that lack IL-2, and demonstrated that CD56\(^{\text{Lin}}\) NK cells can be derived from CD34\(^{+}\) HPCs in the presence of IL-15 and the absence of IL-2. The synergy between IL-15 and IL-2 in expanding the CD56\(^{-}\) NK population is consistent with IL-2R and c-kit expression on a circulating subset of NK cells normally found in PB. Morphology, CD56 and \(\zeta\)-TCR expression, cytotoxicity, and cytokine production of these cells are all most consistent with the NK cell lineage. The existence of the CD2\(^{-}\)CD16 CD56\(^{\text{bright}}\) phenotype in certain fetal and adult organ tissues suggests this cell may be fully differentiated in some instances. However, the acquisition of CD2 and CD16 on the vast majority of PB NK cells is likely to be important prior to NK cell migration into the circulation. It is possible that undefined thymocyte-derived cytokines required for high-density CD2 expression on T cells are also found in BM stroma and are required for CD2 expression on PB NK cells. The presence of a CD2\(^{-}\)CD16 CD56\(^{-}\) NK cell subset in PB suggests that acquisition of CD16 may occur in the peripheral circulation during subsequent steps of NK cell maturation.

### Table 1. Cytokine Production by CD34\(^{+}\) HPCs, CD56\(^{\text{Lin}}\) BM-Derived NK Cells, and CD3 CD56\(^{\text{bright}}\) PB NK Cells

<table>
<thead>
<tr>
<th>Cytokine(^{a})</th>
<th>CD34(^{+}) HPCs</th>
<th>CD56(^{\text{Lin}}) BM NK</th>
<th>CD56(^{\text{bright}}) PB NK</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-(\alpha)</td>
<td>8 ± 7.4</td>
<td>46.6 ± 16.1</td>
<td>269.3 ± 20.3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>11 ± 1.8</td>
<td>284.4 ± 67.3</td>
<td>734 ± 207</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>28.1 ± 6.5</td>
<td>3,520 ± 760</td>
<td>5,650 ± 650</td>
</tr>
</tbody>
</table>

\(^{a}\) Reported in pg/mL, following 72-hour costimulation with 3 ng/mL of rhuL-12 and 100 ng/mL of rhuL-15. Cytokine production in the absence costimulation was not detectable.
cells suggests that additional, as of yet undefined factors are required for the complete differentiation of PB NK cells from BM precursor cells.

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