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Brief Definitive Report

Generation of Lytic Natural Killer 1.1\(^+\), Ly-49\(^-\) Cells from Multipotent Murine Bone Marrow Progenitors in a Stroma-free Culture: Definition of Cytokine Requirements and Developmental Intermediates

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Summary

We have developed a stroma-free culture system in which mouse marrow or thymus cells, known to be enriched for lymphoid progenitors, can be driven to generate natural killer (NK) cells. Culture of lineage marker (Lin)\(^-\), c-kit\(^+\), Sca2\(^+\), interleukin (IL)-2/15R\(^b\) (CD122\(^-\)) marrow cells in IL-6, IL-7, stem cell factor (SCF), and flt3 ligand (flt3-L) for 5–6 d followed by IL-15 alone for an additional 4–5 d expanded the starting population 30–40-fold and gave rise to a virtually pure population of NK1.1\(^+\), CD3\(^-\) cells. Preculture in IL-6, IL-7, SCF, and flt3-L was necessary for inducing IL-15 responsiveness in the progenitors because the cells failed to significantly expand when cultured in IL-15 alone from the outset. Although culture of the sorted progenitors in IL-6, IL-7, SCF, and flt3-L for the entire 9–11-d culture period caused significant expansion, no lytic NK1.1\(^+\) cells were generated if IL-15 was not added, demonstrating a critical role for IL-15 in NK differentiation. Thus, two distinct populations of NK progenitors, IL-15 unresponsive and IL-15 responsive, have been defined. Similar results were obtained with Lin\(^-\), CD44\(^+\), CD25\(^+\), c-kit\(^+\) lymphoid progenitors obtained from adult thymus. The NK cells generated by this protocol lysed the NK-sensitive target YAC-1 and expressed markers of mature NK cells with the notable absence of Ly-49 major histocompatibility complex (MHC) receptors. However, despite the apparent lack of these inhibitory MHC receptors, the NK cells generated could distinguish MHC class I\(^1\) from class I\(^2\) syngeneic targets, suggesting the existence of novel class I receptors.

NK cells express two families of receptors, which in the murine system are known as NKR-P1 and Ly-49 (1). The NKR-P1 molecule is expressed on all NK cells of certain mouse strains and may play a role in triggering NK cytotoxicity. Conversely, the Ly-49 receptors, which recognize class I MHC molecules, are expressed on subsets of NK cells and inhibit NK-mediated lysis. Despite increasing knowledge of NK cell function and target cell recognition, differentiation of NK cells from hematopoietic stem cells is poorly understood. NK cells are known to be bone marrow derived, and early work suggested an important role for the marrow microenvironment in generating mature lytic NK cells (2). To dissect the steps of NK differentiation, several in vitro systems have been established that allow the development of lytic NK cells from CD34\(^+\) human cells (3–6) or unseparated rodent bone marrow (reviewed in reference 7). However, in many of these systems, the starting populations were heterogeneous, containing pluripotent stem cells and progenitors at different stages of development. In addition, the NK cells that developed were not fully characterized, especially with respect to the expression and function of class I MHC receptors.

Although IL-2 has frequently been used to support NK development, the failure to detect the IL-2 gene product within bone marrow stroma (8) and the presence of NK cells in IL-2\(^-\) mice (9) strongly suggests that cytokines other than IL-2 participate in NK cell differentiation in vivo. The newly described cytokine IL-15 has been shown to use both the common \(\gamma\) chain (\(\gamma\c\)) and IL-2R \(\beta\) as components of its receptor (10), and it is produced by bone marrow stromal cell cultures (8). In addition, IL-15 supports development of NK cells from human CD34\(^+\) stem cells and murine fetal thymic cells (8, 11) and causes terminal differ-
entiation of immature, nonlytic NK 1.1+ cells found in the spleen of marrow ablated mice (12).

In the present report, we describe an in vitro culture system yielding virtually pure populations of lytic NK 1.1+ cells from lineage (Lin−), c-kit+, and Sca2+ multipotent progenitors in the absence of stroma. The data show that a mixture of early acting cytokines and IL-15 play sequential and important roles in the differentiation of NK cells from these marrow progenitors.

Materials and Methods

Animals. 7–12-wk C57BL/6 mice were bred at The University of Texas Southwestern Medical Center (Dallas, TX) and used for bone marrow progenitor experiments. 4–5-wk (C57BL/6 × BALB/C)F1 mice (Jackson Labs, Bar Harbor, ME) were used for thymic progenitor experiments.

mAbs. Except as noted below, all mAbs and their isotype controls were obtained from Pharmingen (San Diego, CA). Anti-gp49B1 FITC (B231.1) was the gift of Dr. H. K. Katz (Harvard University, Boston, MA), anti-Ly-49G2 (4D11) and anti-Ly-49D (12A8) were provided by Dr. J. R. O’Riordan (National Cancer Institute, Frederick, MD), anti-Ly-49A (JR-9-318) was the gift of Dr. J. R. O’Riordan (Institut Pasteur, Paris, France), and anti-Sca2 hybridoma supernatant was provided by Dr. G. Spangrude (University of Utah, Salt Lake City, UT). Goat anti-rat γT red (Southern Biotechnology Assoc., Birmingham, AL) or streptavidin-Red670 (GIBCO BRL, Gaithersburg, MD) was used to detect some primary antibodies.

Cell Preparation, Isolation, and Analysis of Precursor Cells. Cell suspensions of bone marrow and thymocytes were prepared as previously described (13, 14). Staining was performed on ice in PBS/0.5% EDTA at 3 × 106 cells/ml. For three-color sorting of bone marrow progenitors, cells were incubated with anti-FCγRI/III/II (2.4G2) and then with a mixture of lineage-specific biotinylated antibodies: anti-B220 (RA3-6B2), anti-CD2 (RM2-5), anti-B230/240 (PK136) followed by streptavidin magnetic beads (Miltenyi Biotech Inc., Auburn, CA). After washing, the cells were passed over a Cs column (Miltenyi Biotech Inc.) to remove Lin+ cells. The Lin-depleted population was then stained with streptavidin-R-PE, c-kit (2B8) PE, and Sca2 (thymic shared antigen [TSA]1) FITC. Lin−R ε670−, c-kit−PE+, Sca2 FITC− cells of lymphoid and blast size were then sorted on a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) using Lysys II software. The identification and isolation of CD4a+ (CD44+, CD25−, c-kit+, Lin−) has been previously described (14).

For four-color analysis of marrow progenitors, cells were stained sequentially with anti-Sca2 hybridoma supernatant, goat anti-rat γ Texas red, 10% normal rat serum, the cocktail of biotinylated antibodies: anti-B220 (RA3-6B2), anti-CD2 (RM2-5), anti-Gr1 (RB6-8C5), anti-CD11b (M1/70), and anti-NK1.1 (PK136) followed by streptavidin magnetic beads (Miltenyi Biotech Inc., Auburn, CA). After washing, the cells were passed over a Cs column (Miltenyi Biotech Inc.) to remove Lin+ cells. The Lin-depleted population was then stained with streptavidin-R-PE, c-kit (2B8) PE, and Sca2 (thymic shared antigen [TSA]1) FITC. Lin−R ε670−, c-kit−PE+, Sca2 FITC− cells of lymphoid and blast size were then sorted on a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) using Lysys II software. The identification and isolation of CD4a+ (CD44+, CD25−, c-kit+, Lin−) has been previously described (14).

Results and Discussion

Isolation and Characterization of a Lin−, c-kit+, Sca2+ Bone Marrow Population. We previously identified a multipotent bone marrow population characterized by Ly6 (Scal)+, Lin−, c-kit (CD117)+, CD43hi, Fall-3hi, Sca2 (TSA1)−, AA40, and R h123hi with both lymphoid and myeloid repopulating abilities in vivo (13). Upon adoptive transfer, this population also gives rise to N K cells (13). To define a population capable of giving rise to NK cells in vitro, a marker more restricted to the lymphoid lineage was sought. TSA-1, also known as Sca2 (19), was previously found to delineate a population of Lin−, Thy-1+, HSA (CD24)+ bone marrow cells enriched for lymphoid (T and B cell) repopulating ability, but essentially devoid of pluripotent stem cells (20). Because NK cells may share a common progenitor with T and B cells (21) and may indeed derive from an NK/T precursor (11, 22), we hypothesized that a marrow population expressing Sca2 may also contain NK progenitors. Thus
Table 1. Expansion and Generation of N K Cells from Lin−, c-kit+, Sca2+ Bone Marrow Progenitors Cultured in IL-6, IL-7, SCF, and flt3-L ± IL-15

| Primary culture | Secondary culture | Total |
|-----------------|-------------------|-------|
| Cytokines       | Yield*            | Cytokines | Yield* | Yield* | N K 1.1+ |
| IL-15           | 0.3               | IL-15     | 1.0    | 0.3    | ND      |
| 6/7/SCF/flt3-L  | 9.4               | 6/7/SCF/flt3-L | 8.7    | 81.8   | 2       |
| 6/7/SCF/flt3-L  | 9.4               | IL-15     | 3.8    | 35.7   | 85      |

Sorted progenitors were cultured in the two-part culture system as described in Materials and Methods. N K 1.1 expression was assessed using PE-labeled anti-N K 1.1 mAb (PK136) from Pharmingen. The data are representative of six experiments.

*Yield represents the fold increase in total cell number.

**Total yield is the product of the yield in the primary and secondary cultures.

Over the input numbers of cells, but these cells did lyse the N K-sensitive tumor YAC-1 (Fig. 1). It is possible that a small number of the progenitors are truly capable of responding to IL-15 alone. We do not favor this hypothesis because cell surface expression of IL-2/15R β was not detected on the sorted population (Fig. 2 B), and the β chain is usually required for signal transduction (24). Alternatively, this growth may represent contamination by rare mature N K cells.

Although the sorted marrow progenitor cells failed to expand significantly when cultured in IL-15 only, when cultured for 5–6 d in IL-6, IL-7, SCF, and flt3-L and then placed in IL-15 alone for an additional 4–5 d, significant expansion occurred (Table 1), and the majority of cells generated were N K 1.1+ and lytic (Table 1 and Fig. 1). If the cultures were maintained in the original cocktail instead of switching to IL-15, significant expansion again occurred, but no lytic activity and few, if any, N K 1.1+ cells were detectable. Together these data indicate that culture of sorted Lin−, c-kit+, Sca2+ progenitors in the early acting cytokines causes expansion and primes at least some cells within this population to respond to IL-15 alone. The identity of other cells generated in the presence of the cytokine cocktail and absence of IL-15 is still being explored, but a small fraction (~13%) are CD19+, dgm−, indicative of immature B cells, whereas a larger fraction (~63%) express high levels of CD11b. Thus, it appears that this population may be multipotential. The data also point to a critical role for IL-15 in N K differentiation because culture of the multipotential progenitors, “primed” by early cytokines in IL-15 only, generated a population of predominantly N K 1.1+ cells.

**Figure 1.** Lytic activity of Lin−, c-kit+, Sca2+ cells cultured with a cocktail of early acting cytokines ± IL-15. Sorted cells were cultured in the indicated cytokines as described in Materials and Methods. Cytotoxic activity was assessed on YAC-1 tumor cells. The data are representative of six experiments.

**Figure 2.** Expression of IL-15R α messenger RNA and IL-2/15R β on Lin−, c-kit+, Sca2+ progenitors and an IL-15-responsive population derived by culture of the progenitors in IL-6, IL-7, SCF, and flt3-L. (A) IL-15R α message levels were determined with R NA isolated from sorted Lin−, c-kit+, Sca2+ progenitors in two experiments (lanes 1 and 2) and from a population of splenic N K 1.1 cells (45% N K 1.1+ by flow cytometry) derived by culture of spleen cells in 500 U/ml of recombinant human IL-2 for 4 d (lane 3). (B) IL-2/15R β expression was examined by flow cytometry on gated Lin−, c-kit+, Sca2+ cells using PE-labeled anti-IL-2/15R β mAb TM-β1. The data are representative of three experiments. (C) Sorted Lin−, c-kit+, Sca2+ cells, cultured for 5 d in IL-6, IL-7, SCF, and flt3-L, were stained for IL-2/15R β expression using FITC-labeled TM-β1. Shaded curves represent staining with TM-β1, whereas open curves represent staining with isotype control mAbs. The data are representative of two experiments.

"1" Culture "2" Culture
- IL-15
- 6/7/SCF/flt3-L
- 6/7/SCF/flt3-L

% Specific Lysis

E : T Ratio
- 5:1
- 2.5:1
- 1:2.5
- 0.6:1
- 0.3:1
Similar experiments were performed using immature thymic progenitors (Lin-\(\text{^2}\), CD44\(\text{^1}\), CD25\(\text{^2}\), c-kit\(\text{^1}\), CD4\(\text{^lo}\)) previously shown to generate T, B, and NK cells (14, 23). When these cells were cultured with IL-15 alone, all cells died within 72 h (Table 2). However, like the marrow progenitor population, when this thymic population was cultured in a cocktail of early acting cytokines and then switched to culture with IL-15 only, significant expansion was observed and a virtually pure population of NK1.1\(\text{^1}\) cells was generated. In initial experiments, the primary culture was performed in IL-3, IL-6, IL-7, and SCF because this mixture is known to maintain and expand lymphoid progenitors (14). Because IL-3 is a T cell–derived cytokine that should not be required for NK differentiation, we hypothesized that the NK progenitors would develop in the absence of IL-3. Indeed, culture in IL-6, IL-7, IL-15, SCF, and flt3-L for 13 d generated large numbers of an 86% pure population of NK1.1\(\text{^+}\) cells whereas culture in the absence of IL-15 failed to give rise to NK1.1\(\text{^+}\) cells but did result in significant expansion (Table 2).

Expression of IL-15R\(\text{a}\) and IL-2/15R\(\text{b}\) by Sorted Population and Developmental Intermediates. The ability of IL-6, IL-7, SCF, and flt3-L to induce IL-15 responsiveness in sorted Lin-\(\text{^-}\), c-kit\(\text{^+}\), Sca2\(\text{^+}\) cells suggested that this mixture of cytokines may be inducing expression of functional IL-15 receptors. Sorted Lin-\(\text{^-}\), c-kit\(\text{^+}\), Sca2\(\text{^+}\) bone marrow cells were thus examined for IL-15R\(\text{a}\) expression by PCR and IL-2/15R\(\text{b}\) expression by FACS\(\text{®}\) analysis. Interestingly, the freshly sorted cells did express transcripts of IL-15R\(\text{a}\) (Fig. 2 A), but they failed to show cell surface expression of IL-2/15R\(\text{b}\) (Fig. 2 B). Furthermore, preliminary evidence has indicated surface expression of the γc by a small proportion of these cells (data not shown). These data suggest that the failure of sorted Lin-\(\text{^-}\), c-kit\(\text{^+}\), Sca2\(\text{^+}\) cells to expand significantly in IL-15 alone is most likely due to the absence of IL-2/15R\(\text{b}\) expression. We, therefore, anticipated that culture of Lin-\(\text{^-}\), c-kit\(\text{^+}\), Sca2\(\text{^+}\) cells in IL-6, IL-7, SCF, and flt3-L would induce IL-2/15R\(\text{b}\) expression. Indeed, after primary culture, ~13% of the progenitors expressed IL-2/15R\(\text{b}\) (Fig. 2 C), but not NK1.1\(\text{^+}\) (data not shown). Upon further culture in IL-15, the vast majority of the NK1.1\(\text{^+}\) cells generated also expressed IL-2/15R\(\text{b}\) (data not shown). These data thus suggest that IL-2/15R\(\text{b}\) expression may precede that of NK1.1, and its acquisition may be a critical event in NK cell differentiation. This idea is supported by the absence of NK cells in IL-2/15R\(\text{b}^-\)/IL-15 mice (25) and isolation of an IL-2/15R\(\text{b}^+\), NK1.1\(\text{^+}\) population from SCID bone marrow that can give rise to lytic NK1.1\(\text{^+}\) cells upon culture in IL-15 (our unpublished data).

Cell Surface Profile of In Vitro Generated NK Cells. The NK cells generated by culture of marrow progenitor cells were

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Ly-49 profile of NK cells generated from culture of Lin-\(\text{^-}\), c-kit\(\text{^+}\), Sca2\(\text{^+}\) progenitors. Expression of NK1.1 was analyzed by setting a "live" gate based on forward and side scatter of the cells, whereas expression of the reminder of the markers was analyzed on NK1.1\(\text{^+}\), live cells. Shaded curves represent staining with the NK1.1 or Ly49 mAbs, whereas open curves represent staining with isotype control mAbs. The data are representative of four separate experiments.
phenotypically quite similar to mature IL-2–activated splenic NK cells (12, 26, 27). They were CD3−, CD11b−, FcγRII/III/II−, IL-2/15Rβ−, c-kit−, and 2B4− (data not shown) and lysed the NK-sensitive target YAC-1 (Fig. 1). Interestingly, they also expressed gp49B1, an inhibitory receptor of the Ig superfamily shared by mast cells (28). However, they failed to express the Ly-49 family of MHC receptors (Fig. 3). In four experiments, expression of Ly-49A, C, I, and D was undetectable or <3% over isotype controls, whereas mature C57BL/6 splenic NK1.1+ cells are 20% Ly-49A+, 50% Ly-49C/I+, and 50% Ly-49D+. Expression of Ly-49G2 was slightly more variable ranging from 2–8% over isotype controls, whereas nearly 50% of splenic NK cells express this receptor. We have been unable to ascertain whether the in vitro–derived NK cells truly express low levels of Ly-49G2 or whether this antibody simply shows nonspecific binding. In any case, these data suggest that NK cells acquire expression of NK1.1 before expression of Ly-49 receptors. The factors required for induction of Ly-49 molecules are unknown, but it is clear that IL-15 alone is not sufficient.

Cytotoxic Activity of In Vitro–generated NK Cells Against C127+ and Class I– Tumor Cells. It has been demonstrated that interaction of Ly-49 receptors with their appropriate MHC class I ligands sends an inhibitory signal to NK cells and prevents NK-mediated lysis of the class I+ target (1). However, despite the failure to express significant levels of Ly-49 MHC receptors, these in vitro–derived NK cells differentiated class I+ from class I− syngeneic tumor cells. They lysed class I+ Tap-deficient RMA-S cells but failed to lyse RMA, the class I− parent of RMA-S (Fig. 4). That this difference in lysis was due to the absence of class I on the surface of RMA-S is supported by the observation that the culture-derived NK cells failed to lyse Q11, a class I− Tap-2 transfectant of RMA-S (16).

In summary, we have developed a stroma-free culture system capable of generating lytic NK1.1+ cells from a defined multipotential progenitor population contained in the bone marrow. This system has allowed identification of a putative (NK1.1−) developmental intermediate in NK differentiation, characterized by expression of IL-2/15Rβ and the ability to respond to IL-15. One or all of a mixture of cytokines including IL-6, IL-7, SCF, and flt3-L was shown to be critical in reaching this developmental stage. This study also provided additional evidence for the critical role that IL-15 plays in NK differentiation, because no NK1.1+ cells were detected in its absence. IL-15 was not, however, capable of inducing expression of the Ly-49 family of MHC molecules, suggesting that some stimulus necessary to induce Ly-49 expression was missing from this culture system. Finally, these data suggested that development of lytic activity and expression of NK1.1 can precede expression of Ly-49 molecules; however, despite the absence of these receptors, the in vitro–derived NK cells could distinguish class I− from class I+ syngeneic tumor cells, suggesting the existence of novel class I receptors.
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