Differentiation of NK1.1+, Ly49+ NK Cells from flt3+ Multipotent Marrow Progenitor Cells

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*J Immunol* 1999; 163:2648-2656; 
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To delineate factors involved in NK cell development, we established an in vitro system in which lineage marker (Lin)$^-$, c-kit$^+$, Sca2$^+$ bone marrow cells differentiate into lytic NK1.1$^+$ but Ly49$^-$ cells upon culture in IL-7, stem cell factor (SCF), and flt3 ligand (flt3L), followed by IL-15 alone. A comparison of the ability of IL-7, SCF, and flt3L to generate IL-15-responsive precursors suggested that NK progenitors express the receptor for flt3L. In support of this, when Lin$^-$, c-kit$^+$, flt3$^+$ or Lin$^-$, c-kit$^+$, flt3$^+$ progenitors were utilized, 3-fold more NK cells arose from the flt3$^+$ than from the flt3$^-$ progenitors. Furthermore, NK cells that arose from flt3$^+$ progenitors showed an immature NK1.1$^{dim}$, CD2$^-$, c-kit$^+$ phenotype as compared with the more mature NK1.1$^{bright}$, CD2$^{+/−}$, c-kit$^-$ phenotype displayed by NK cells derived from flt3$^-$ progenitors. Both progenitors, however, gave rise to NK cells that were Ly49 negative. To test the hypothesis that additional marrow-derived signals are necessary for Ly49 expression on developing NK cells, flt3$^+$ progenitors were grown in IL-7, SCF, and flt3L followed by culture with IL-15 and a marrow-derived stromal cell line. Expression of Ly49 molecules, including those of which the MHC class I ligands were expressed on the stromal or progenitor cells, as well as others of which the known ligands were absent, was induced within 6–13 days. Thus, we have established an in vitro system in which Ly49 expression on developing NK cells can be analyzed and possibly experimentally manipulated. The Journal of Immunology, 1999, 163: 2648–2656.

Materials and Methods

Animals

C57BL/6 or (C57BL/6 × DBA/2)F1, mice 7–12 wk old, bred at University of Texas Southwestern Medical Center, were used as the source of bone marrow progenitors. CB17.SCID mice obtained from Taconic Farms (Germantown, NY) were used as recipients for in vivo repopulation studies. The SCID mice were irradiated with 750 rad using a Mark I irradiator (J. L. Shepherd and Associates, San Fernando, CA) and were maintained under pathogen-free conditions with acidified water containing antibiotics (200 mg neomycin sulfate, 200 mg sulfamethoxazole, 50 mg trimethoprim, and 5.2 mg polymixin B in 500 ml H$_2$O, pH 2.5, all from Sigma, St. Louis, MO) for 2 wk followed by acidified water only for the remaining 1–2 wk.
**Monoclonal Abs**

Except as noted below, all mAbs and their isotype controls were obtained from Pharmingen (San Diego, CA). Anti-Ly49G2D (4D11) and anti-Ly49D (4E5) were provided by Dr. J. Ortaldo (National Cancer Institute, Frederick, MD), anti-Ly49A (JR9-318) was the gift of Dr. J. Roland (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 β-Texas Red (Southern Biotechnology Associates, Birmingham, AL) or streptavidin-Red670 (Life Technologies, Grand Island, NY) was used to detect some primary Abs.

**Stromal cells**

OP9 stromal cells (25) were the kind gift of Dr. J.C. Zúñiga-Pflu¨cker (University of Toronto, Toronto, Ontario, Canada). The cells were passaged weekly in MEB (Life Technologies) containing 200 nM glutamine, 100 µg/ml streptomycin sulfate, and 100 U/ml penicillin.

**Cell preparation, isolation, and analysis of precursor cells**

Lin−, c-kit+, Sca2+ progenitors were isolated by a combination of magnetic bead depletion of Lin+ cells and cell sorting as previously described (24). Lin−, c-kit+, flt3− or flt3+ progenitors were isolated in a similar fashion. Analysis of cultured cells was performed by sequential staining with anti-FeViIII (2.4G2), a biotinylated mAb, and finally streptavidin-Red670 plus a FITC- and/or PE-conjugated mAb.

**In vitro culture conditions**

Sorted Lin−, c-kit+, Sca2−; Lin−, c-kit+, flt3−; or Lin−, c-kit+, flt3+ marrow cells were cultured in 96-well U-bottom plates (Falcon, San Jose, CA) at 10,000 cells/well in 0.2 ml of complete RPMI (RPMI 1640 containing 10% FBS, 100 µg/ml streptomycin sulfate, 100 U/ml penicillin, 1 mM sodium pyruvate, 2 mM glutamine, and 1/10 nonessential amino acids) and a mixture of 0.5 µg/ml murine IL-7 (PeproTech, Rocky Hill, NJ), 30 ng/ml mouse SCF (BioSource, Camarillo, CA), and 100 U/ml murine flt3L (a gift from Dr. D. Rennick at DNAX, Palo Alto, CA). The cells were refed (25) and maintained as described above. Donor type cells were detected with anti-Fcγ FITC (PharMingen).

In vivo repopulation studies

Lymphocyte- and blast-sized (LB) Lin−, c-kit+, flt3−, or flt3+ progenitors were sorted from (C57Bl/6 × DBA/2J)F1 bone marrow as described above. The percentage of each population in whole bone marrow was calculated (% = fraction of LB cells × fraction of Lin− cells in LB gate × fraction of c-kit−, flt3− or c-kit+, flt3+ cells in LB gate × 100). The cells were washed two times with PBS and resuspended such that the equivalent of 1.4 × 107 whole bone marrow cells for each progenitor was contained in 0.75 ml. CB17.Scid mice were irradiated with 175 rad 0.5–1 h before injection. The mice were then injected with 0.75 ml of cells via the tail vein and maintained as described above. Donor type cells were detected with anti-Kb FITC (PharMingen).

**Western blot analysis**

IL-2 cultured SCID NK cells (1–2 × 106 cells) or NK cells derived from Lin−, c-kit+, Sca2+ progenitor cells (107 cells) were lysed in a HNTG lysis buffer (10% glyceral, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM MgCl2) with 1% Triton X-100 for 30 min on ice. Cleared lysates were separated on a 4–20% SDS-PAGE gel after addition of 2% sample buffer (50 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, and 5% 2-ME). Separated proteins were transferred onto a nitrocellulose membrane. Western analysis was done after blocking the membrane in TBST (20 mM Tris and 500 mM NaCl) with 3% nonfat dry milk for 30 min. Primary anti-CD94-2 Ab (26) was added at a dilution of 1:200 in TBST for 1 h at room temperature, followed by the secondary Ab (donkey anti-rabbit IgG-HRP conjugate, 1:1000 dilution, Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h. The membrane was developed in enhanced chemiluminescence developing solution (Amersham Pharmacia Biotech). A CD94-GST fusion protein generated in yeast was used as a control. Lysates from T cell lines EL-4 and Bw5147 did not show CD94 expression (data not shown).

**Results**

Flt3L potently induces IL-15 responsiveness in Lin−, c-kit−, Sca2+ progenitors

We previously identified a population of multipotent progenitors in mouse bone marrow characterized as Lin− (B220, Gr1, Mac1+, CD2, Ter119, and NK1.1), c-kit−, Sca2−, which gives rise to NK1.1+ cells in vitro (24). Although culture of these progenitors in mIL-15 alone led to generation of small numbers of lytic NK1.1+ cells, primary culture in a mixture of early acting cytokines, including IL-6, IL-7, SCF, and flt3L, for 5 days before secondary culture in mIL-15 significantly enhanced by 10- to 30-fold the number of NK cells generated. We inferred from these data that primary culture of the progenitor cells in early acting cytokines gave rise to a population of IL-15-responsive progenitors that subsequently gave rise to NK cells on exposure to IL-15. Indeed, we previously demonstrated that, whereas the starting population of progenitors was IL-2/15Rβ−, 5–15% of the cells following the primary culture were sorted and placed in culture in IL-15, only the IL-2/15Rβ+ precursor cells gave rise to NK cells (data not shown).

Table 1. Flt3L plays a critical role in expanding IL-15-responsive NK precursors

<table>
<thead>
<tr>
<th>Primary Culture Cytokines</th>
<th>Yield in mIL-15</th>
<th>% NK1.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF</td>
<td>1.6 ± 1.0</td>
<td>44, 84, ND</td>
</tr>
<tr>
<td>flt3L</td>
<td>1.2 ± 0.7</td>
<td>72, 86, ND</td>
</tr>
<tr>
<td>7/SCF</td>
<td>1.7 ± 0.9</td>
<td>68, 87, ND</td>
</tr>
<tr>
<td>7/flt3L</td>
<td>4.3 ± 0.8</td>
<td>88, 86, 60</td>
</tr>
<tr>
<td>7/SCF/flt3L</td>
<td>4.0 ± 1.1</td>
<td>76, 91, 76</td>
</tr>
</tbody>
</table>

* Sorted Lin−, c-kit−, Sca2− progenitors were cultured for 5 days in the indicated primary culture cytokines. On day 5, the cells generated, which are referred to as NK precursors, were harvested, washed, and replated in mIL-15 for 6 days (secondary culture). On day 6, the cells were harvested, counted, and stained for NK1.1 expression.

**Table 1. Flt3L plays a critical role in expanding IL-15-responsive NK precursors**

- **Results**

Flt3L potently induces IL-15 responsiveness in Lin−, c-kit−, Sca2+ progenitors

We previously identified a population of multipotent progenitors in mouse bone marrow characterized as Lin− (B220, Gr1, Mac1+, CD2, Ter119, and NK1.1), c-kit−, Sca2−, which gives rise to NK1.1+ cells in vitro (24). Although culture of these progenitors in mIL-15 alone led to generation of small numbers of lytic NK1.1+ cells, primary culture in a mixture of early acting cytokines, including IL-6, IL-7, SCF, and flt3L, for 5 days before secondary culture in mIL-15 significantly enhanced by 10- to 30-fold the number of NK cells generated. We inferred from these data that primary culture of the progenitor cells in early acting cytokines gave rise to a population of IL-15-responsive progenitors that subsequently gave rise to NK cells on exposure to IL-15. Indeed, we previously demonstrated that, whereas the starting population of progenitors was IL-2/15Rβ−, 5–15% of the cells following the primary culture were sorted and placed in culture in IL-15, only the IL-2/15Rβ+ precursor cells gave rise to NK cells (data not shown).

It was not clear from this analysis, however, which of the early acting cytokines was critical for expanding and/or inducing the differentiation of an IL-15-responsive cell that subsequently could give rise to NK1.1+ cells on culture in IL-15.

Early experiments in which IL-6 was or was not included indicated that, whereas IL-6 did increase cell yields during the primary culture, the yield of NK cells was lower overall. We hypothesize that this effect was due to the ability of IL-6 to stimulate differentiation and proliferation of non-NK progenitors. Therefore, subsequent cytokine comparisons were performed using only IL-7, SCF, and flt3L.

Progenitors were cultured for 5 days with SCF alone, flt3L alone, IL-7 + SCF, IL-7 + flt3L, or IL-7 + SCF + flt3L. To determine the relative ability of these various cytokine combinations to expand and/or induce the differentiation of an IL-15-responsive precursor, the cells were then washed and replated in mIL-15 for an additional 6 days. The fold expansion in mIL-15 on a per-cell basis was then calculated for each condition (Table 1). Neither SCF nor flt3L alone generated cells that responded efficiently to IL-15. Although the addition of IL-7 to SCF did not appear to have an effect, the addition of IL-7 to flt3L enhanced the ability of the generated population to respond to IL-15 (p = 0.10, Mann-Whitney test (27)). This observation suggests that IL-7 may play an important role in the generation of IL-15-responsive NK cells.
precursors, at least for cells grown in the absence of SCF. However, cells cultured in IL-7 + flt3L were 2.5-fold better at responding to IL-15 than were cells grown in IL-7 + SCF. This difference, while not highly significant (p = 0.05, Mann-Whitney test), also suggests an important role for flt3L in NK development. In fact, culture in IL-7 + flt3L stimulated generation of a population of cells on day 5 that responded more efficiently to IL-15 than those populations generated by all the other cytokine combinations. Although the addition of SCF to IL-7 + flt3L did not improve the ability of the generated cells to respond to IL-15, it did improve the overall yield of NK cells, largely because of the additional proliferation induced during the primary culture before culture in IL-15 (data not shown). Taken together, these data imply that IL-7 and flt3L specifically expand or induce differentiation of IL-15-responsive NK precursors from Lin−, c-kit+, Sca2+ progenitors. SCF has an additive effect on the overall expansion of this population of cells, but it does not affect the frequency with which IL-15-responsive NK precursors are generated.

Identification of flt3 receptor-positive and -negative fractions in Lin−, c-kit+ murine bone marrow

It has been reported that flt3L−/− mice lack NK cells, whereas IL-7−/− or IL-7Rα−/− mice show minimal defects in NK development (23, 28, 29). We chose, therefore, to focus initially on the role of flt3L in NK development. The ability of the Lin−, c-kit+, Sca2+ population to respond to flt3L suggested that this population may express the receptor for flt3L (flt3). A four-color flow cytometry analysis revealed that 20–30% of Lin−, c-kit+, Sca2+ cells express flt3 (Fig. 1). A similar proportion of the total Lin−, c-kit+ population also expresses flt3. Because of the greater ease with which three-color as compared with four-color sorting can be performed, we chose to analyze the NK potential of LB Lin−, c-kit+, flt3+ vs Lin−, c-kit+, flt3− populations, which represent respectively ~0.2% and 1.0–1.5% of whole bone marrow. Subsequent experiments in which Lin−, c-kit+, Sca2+, flt3+ or flt3− populations were isolated, however, yielded similar results (data not shown).

Lin−, c-kit+, flt3+ cells show greater NK potential than do Lin−, c-kit+, flt3− cells in vitro

Lin−, c-kit+, flt3+ and Lin−, c-kit+, flt3− cells were cultured in IL-7, SCF, and flt3L for 5 days followed by IL-15 alone for an additional 6 days. Significantly, flt3+ progenitors gave rise to, on average, 3-fold more NK cells on a per-cell basis than did flt3− progenitors (Fig. 2A). This was due in large part to the fact that the population of NK precursors generated by culture of flt3+ progenitors in IL-7, SCF, and flt3L responded more efficiently to IL-15 on a per-cell basis to generate NK1.1+ cells than did the population of NK precursors generated in the same manner from flt3− progenitors (Fig. 2B). To control for differences in NK generation due to stimulation of the flt3+ but not flt3− progenitors with flt3L itself in the primary culture, we attempted to culture both populations with IL-7 and SCF alone followed by culture in IL-15. The flt3+ population gave rise to similar numbers of NK cells as when IL-7, SCF, and flt3L were utilized (data not shown). Surprisingly, however, the flt3+ population failed in two separate experiments to survive with IL-7 and SCF alone (data not shown). This suggests that somehow signals sent through flt3 are critical for the survival of Lin−, c-kit+, flt3+ cells. SCF does, however, augment proliferation of these flt3+ cells in the presence of flt3L, because SCF + IL-7 + flt3L stimulated ~2-fold more proliferation of Lin−, c-kit+, flt3− cells than IL-7 and flt3L alone (data not shown).

Characterization of NK cells generated in vitro from Lin−, c-kit+, flt3+ and Lin−, c-kit+, flt3− progenitors

Mature peripheral NK cells are NK1.1bright, CD2+, and flt3− (Ref. 30 and J. Klem and V. Kumar, unpublished observations). They are largely c-kit−, although a small fraction is positive for this receptor (30). Interestingly, not only did flt3+ progenitors give rise to more NK cells, the cells derived from these progenitors more closely resembled mature NK cells than did the cells derived from flt3− progenitors. NK cells derived from flt3− progenitors lysed the prototypical NK target YAC-1 as well as or better than NK cells derived from flt3+ progenitors (data not shown). In addition, NK cells derived from both progenitors expressed NK1.1+. However, the level of expression of the NK1.1 receptor on NK cells generated from the flt3− progenitors was much lower than that on NK cells generated from the flt3+ cells (Fig. 3). Furthermore, the NK cells derived from flt3− progenitors failed to express the lymphoid marker CD2 and continued to express c-kit. A significant fraction of the flt3− progenitor-derived NK cells, on the other hand, did express CD2, and the cells were NK1.1bright and c-kitdim. Both populations of NK cells were negative for flt3.

Although the NK cells derived from flt3− progenitors displayed an overall more mature phenotype than NK cells derived from...
give rise to somewhat distinct NK1.1+ cells in vitro than Lin−, c-kit+, flt3+ cells. Lin−, c-kit+, flt3+ and Lin−, c-kit+, flt3− progenitors were isolated from C57BL/6 bone marrow as indicated in Materials and Methods and plated in IL-7, SCF, and flt3L for 5 days followed by mIL-15 alone for an additional 6 days. In A, the number of NK cells generated per progenitor was calculated by multiplying the total cell yield after culture in IL-7, SCF, and flt3L and mIL-15 for 5 days followed by mIL-15 alone for an additional 6 days. In A, the number of NK cells generated per progenitor was calculated by multiplying the total cell yield after culture in IL-7, SCF, and flt3L and mIL-15 by the percentage of NK1.1+ cells in the final population. In B, the number of NK cells generated per NK precursor was then calculated by multiplying the fold increase in cell number in mIL-15 by the percentage of NK1.1+ cells. The results represent the mean ± SEM from four independent experiments.

flt3− progenitors, both types of NK cells completely lacked expression of any Ly49 MHC receptors known to be expressed on mature peripheral NK cells (Fig. 6 and data not shown). Despite this, both populations were able to distinguish class I+ targets from class I− targets (data not shown), a property we had previously found in the Ly49− NK cells derived in vitro from Lin−, c-kit+, Sca2+ progenitors (24). That is, the in vitro-derived NK cells lysed the class I-deficient target RMA-S but failed to lyse the class I+ target RMA. On the basis of similar observations for KIR-negative human NK cells and fetal liver-derived murine NK cells, it seems likely that the receptor responsible for this activity in developing NK cells is CD94/NKG2 (26, 31). In support of this, CD94 can be detected in lysates of NK cells derived from Lin−, c-kit+, flt3− progenitors. Shaded curves represent staining with isotype control Abs; open curves represent staining with the indicated Abs. NK1.1 expression was examined on live gated cells, whereas expression of CD2, c-kit, and flt3 was examined on gated NK1.1+ live cells. The data are representative of staining patterns observed in two to six independent experiments.

The ability of Lin−, c-kit+, flt3+ and Lin−, c-kit+, flt3− cells to give rise to NK cells in vivo

To determine whether the ability of flt3+ and flt3− progenitors to give rise to somewhat distinct NK1.1+ cells in vitro could be recapitulated in vivo under more physiologic conditions, Lin−, c-kit+, flt3+ or flt3− bone marrow progenitors were sorted from (C57BL/6 × DBA.2)F1 mice (H-2d) and injected into 175 rad-treated CB17.SCID mice (H-2b). Semiallogeneic donor cells were utilized so that their presence in the host could be detected with Abs to H-2b MHC. The absence of T cells in the host prevents rejection of the allogeneic marrow cells, and NK-mediated rejection of (H-2b/d)F1 bone marrow cells by parental H-2b NK cells has been shown to be insignificant (32). It should be noted that the numbers of flt3− and flt3+ progenitor cells injected per mouse were 30,000 and 120,000, respectively. The relative number of each population utilized was calculated from the relative ratios of flt3+ and flt3− cells in normal adult marrow (i.e., 1:4). The mice were then sacrificed on day 27 or 36 after cell transfer. Data from these two time points were pooled. Despite the fact that four times as many flt3− cells were injected, the total number of splenic NK cells generated in mice injected with flt3− cells was 2-fold higher than the number in mice injected with flt3+ cells. Thus, the differences in the NK-generating activity in flt3+ vs flt3− cells in
in vitro could be reproduced in vivo as well. However, NK cells from both sets of mice expressed a completely mature phenotype: NK1.1bright, CD2+, c-kit−. Additionally, near-normal percentages of Ly49+ cells were detected (Table II). Thus, under more physiologic conditions both types of progenitors give rise to NK cells with a mature phenotype, although less efficiently from the flt3− population.

Culture of Lin−, c-kit+, flt3− cells on bone marrow-derived stroma can induce Ly49 expression

Because both flt3+ and flt3− progenitor cells gave rise to Ly49+ NK cells in vivo, we reasoned that their inability to generate Ly49+ cells in vitro could be due to inadequacies of the culture system. It is known that NK development in vivo is bone marrow dependent (33). We hypothesized, therefore, that additional signals from bone marrow-derived stromal cells may be necessary for complete NK maturation, including Ly49 induction. To provide an environment conducive to lymphoid development, we chose to utilize a stromal cell line, OP9, derived from the calvaria of a B6C3F2-op/op mouse deficient in the production of M-CSF (25). The haplotype of this stromal cell line is H-2k (data not shown). Lin−, c-kit+, flt3− and Lin−, c-kit+, flt3+ progenitors from C57BL/6 mice, grown for 5 days in IL-7, SCF, and flt3L, were thus plated on confluent monolayers of this OP9 stroma in the presence of human IL-15 or high-dose human IL-2. Cells were then washed and plated at 200,000–500,000 per well in 24-well plates containing a confluent monolayer of OP9 stroma in the presence of 150 ng/ml of human IL-15 or 5000 IU/ml human IL-2. Cells were stained as described in Materials and Methods for NK1.1 and CD2. The numbers indicated in each quadrant represent the percentage of positive cells minus the percentage of cells staining nonspecifically with isotype control mAbs. The data are representative of two separate experiments.

Table II. In vivo NK potential of Lin−, c-kit+, flt3+ and Lin−, c-kit+, flt3− progenitors

<table>
<thead>
<tr>
<th>Progenitor</th>
<th>Splenic Cellularity (×10^5)</th>
<th>No. of NK1.1+ Cells/ Spleen (×10^5)</th>
<th>% of NK1.1+ Cells Expressingb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD2</td>
<td>c-kit</td>
</tr>
<tr>
<td>flt3− (n = 6)</td>
<td>818 ± 127</td>
<td>6.4 ± 1.1</td>
<td>91.6 ± 1.4</td>
</tr>
<tr>
<td>flt3− (n = 5)</td>
<td>248 ± 55.8</td>
<td>3.1 ± 0.5</td>
<td>94.6 ± 1.0</td>
</tr>
</tbody>
</table>

* Lin−, c-kit+, flt3+, and Lin−, c-kit+, flt3− progenitors were sorted from C57BL/6 × DBA/2F1 bone marrow. Approximately 30,000 flt3+ or 120,000 flt3− cells (equivalent to 1.4 × 10^6 whole bone marrow cells) were injected per irradiated (175 rad) CB17 SCID mouse. The mice were maintained in pathogen-free conditions with acidified water and antibiotics as indicated in Materials and Methods. 1 wk followed by acidified water only for the remaining 1–2 wk. Mice were sacrificed on day 27 or 36. Cell counts were performed on the spleen and based on this number and the percentage of cells staining positive for NK1.1 PE (PharMingen), the number of NK1.1+ cells/spleen was calculated. NK1.1+ cells were gated and the percentage of cells expressing CD2 (mAB RM2-5), c-kit/CD117 (mAB 2B8), Ly49G2 (mAB 4D11), Ly49C/I (mAB 5E6), Ly49A (mAB JR9-318), and Ly49D (mAB 4E5) was determined. Values ± SEM are indicated for mice from two experiments.

b Values indicated represent the mean ± SEM of two to six mice.  

Figure 5. Culture of Lin−, c-kit+, flt3+ or flt3− cells on OP9 stroma induces expression of CD2. Lin−, c-kit+, flt3− cells were cultured for 5 days in IL-7, SCF, and flt3L. The cells were then washed and plated at 200,000–500,000 per well in 24-well plates containing a confluent monolayer of OP9 stroma in the presence of human IL-15 or 5000 IU/ml human IL-2. Cells were stained as described in Materials and Methods for NK1.1 and CD2. The numbers indicated in each quadrant represent the percentage of positive cells minus the percentage of cells staining nonspecifically with isotype control mAbs. The data are representative of two separate experiments.
and still others express both molecules. No Ly49A (JR9-318 positive) or Ly49D (4E5 positive) molecules (36, 37) were detected at this time point (data not shown). With longer culture on stromal cells, very small numbers of JR9-318- and 4E5-reactive cells (1.0% of NK1.1 cells) were noted; however, the fraction of 4D11- and 1F8-reactive cells did not increase substantially (data not shown). In the same experiments, NK cells that differentiated in the absence of stroma failed to express Ly49 molecules (Fig. 6, B and D).

**Generation of Ly491⁺ NK1.1⁺ cells from Lin⁻, c-kit⁻ progenitors**

Although coculture of Lin⁻, c-kit⁻, flt3⁺ progenitors with OP9 stroma did yield NK1.1⁺, Ly49⁺ cells, the frequency of Ly49⁺ cells generated in vitro was low. Because in vivo transfer of this population gave rise to near-normal numbers of Ly49⁺ NK cells (Table II), we hypothesized that interactions with other cells, in addition to stroma, may be important for generation of NK1.1⁺, Ly49⁺ cells. Therefore, we decided not to separate flt3⁻ or flt3⁺ cells from the Lin⁻, c-kit⁻ marrow population. Thus, Lin⁻, c-kit⁻ cells were cultured with IL-7, SCF, and flt3L for 5 days as usual, followed by culture with IL-2 on OP9 stroma. The numbers indicated in each quadrant represent the percentage of positive cells minus the percentage of cells staining nonspecifically with isotype control mAbs. The data are representative of four separate experiments.

as expected, Ly49⁺ cells were generated only when the cells were cultured with stroma. However, as compared with cultures initiated with Lin⁻, c-kit⁻, flt3⁺ cells, a substantially greater number of Ly49⁺ cells was generated. As with the expression of NK1.1, Ly49 molecules were first detected 3 days after culture with stroma, and the numbers increased rapidly to peak at 10 days postculture. Ly49 receptors reactive with four distinct Abs, including 4D11, 1F8, JR9-318, and 4E5, could be detected. The time course analysis revealed that some Ly49 molecules could be detected earlier than others. Thus, Ly49 receptors recognized by the mAbs 4D11 and 1F8 were noted as early as 3 days after culture on stroma, whereas Ly49 receptors detected by JR9-318 and 4E5 were not noted until day 6. While the frequency of 4D11⁺ and 1F8⁺ cells at later time points approached that seen on peripheral C57BL/6 NK cells, the frequency of 4E5⁺ and JR9-318⁺ cells did not. The reason for this discrepancy is not yet clear, but possible explanations are explored in **Discussion**.

It is important to point out that contamination of the sorted progenitor population with mature NK cells cannot account for the Ly49⁺ NK cells observed. First, the sorted progenitor cells are cultured for 5 days in IL-7, SCF, and flt3L and are then split into two groups. Both groups are cultured in IL-2 without any stroma; hence, there is no reason to believe that stroma is
necessary for survival of any contaminating mature NK cells. In support of this, when Thy1.2+ progenitor cells were deliberately contaminated with 0.5% or 2.5% mature Thy1.1+ NK cells in the presence or absence of stroma (data not shown), the pattern of Ly49 expression is selective. For example, although 40–60% of NK cells in the periphery express Ly49G2 and an approximately equivalent percentage express Ly49D, 15- to 30-fold more Ly49G2+ than Ly49D+ cells are noted in our in vitro system (Table III). Third, under the standard assay conditions, Ly49+ cells are not generated from flt3- progenitors (Fig. 6C). There is no reason to believe that NK1.1+ contaminants would occur selectively in cultures of flt3- progenitor cells, especially as mature NK cells are flt3-.

**Discussion**

We present here a culture system in which NK cell differentiation from multipotent stem cells to fully mature NK cells can be recapitulated. On the basis of these studies, we propose a two-step model of NK cell differentiation (Fig. 7). In the first step, multipotent stem cells containing NK cell progenitors are driven to become IL-2/15Rα+ NK precursors under the influence of several early acting cytokines, especially flt3L. We hypothesize that this effect is due to induction of the IL-2/15Rβ (CD122) chain on a fraction of the progenitor cells. This step can occur in the absence of stroma (at least in vitro). In the second step, the NK precursors, so generated, give rise to mature Ly49-expressing NK cells on being exposed to IL-2/15. In this step, the induction of Ly49 molecules but not NK1.1 is strictly dependent on the presence of stromal cells. Using this two-part culture system, we have investigated the mechanisms responsible for NK cell differentiation.

Data from multiple sources have emerged showing that two cytokines are essential for NK cell differentiation: IL-15 and flt3L. The importance of IL-15 in NK cell differentiation has been documented in several previous studies from our laboratory and (24, 38) and others (8). It is further supported by the absence of NK cells in mice with targeted deletion of IL-15Rα (39) and IL-15 (40).

Likewise, the importance of flt3L in NK development has been suggested by several recent reports. First, NK1.1+ cells are missing in flt3L−/− mice (23). Second, administration of recombinant flt3L induces a striking increase in the absolute number of mature nonactivated NK cells within various tissues (21, 22). This increase in NK cell numbers may provide a possible additional explanation for the potent antitumor effect of in vivo-administered flt3L (41–43), which was previously attributed solely to an increase in dendritic cells (44).

Although such observations suggest a significant potential for flt3L in therapy of both cancer and viral infection through expansion of NK cells, they do not address the mechanism by which this occurs. The data presented in this paper show that flt3L acts by expanding and/or inducing the differentiation of a progenitor population into IL-15-responsive precursors capable of giving rise to NK cells on in vitro culture in IL-15 (Fig. 7). The progenitor on which flt3L exerts its effect is identified in the mouse as Lin−, c-kit+, flt3+. Somewhat surprisingly, in light of data from the flt3L−/− mice, the corresponding Lin−, c-kit+, flt3− population also gave rise to NK, albeit less efficiently than the flt3+ population (Fig. 2). An explanation for this discrepancy may lie in the phenotype of NK cells derived from each progenitor. NK cells derived from flt3+ progenitors showed a less mature NK1.1dim,
CD2\(^{-}\), c-kit\(^{-}\) phenotype than NK cells derived from flt3\(^{+}\) progenitors, which were NK1.1\(^{+}\), CD2\(^{+}\), c-kit\(^{+}\). It is possible that in flt3L\(^{-/-}\) mice, Lin\(^{-}\), c-kit\(^{-}\), flt3\(^{-}\) cells inefficiently give rise to NK cells. As these NK cells express quite dim levels of NK1.1, it could appear that flt3L\(^{-/-}\) mice lack NK1.1\(^{bright}\) cells as previously reported (23). It would be interesting to further phenotype any NK cells that develop in these mice for expression of CD2 and c-kit.

It should be noted, however, that immature NK1.1\(^{dim}\), CD2\(^{-}\), c-kit\(^{-}\) NK cells do not exist in the spleens of normal mice in vivo. Such NK cells may represent a developmental artifact that occurred because in vitro the flt3\(^{-}\) progenitors did not progress through a normal developmental pathway important for the up-regulation of NK1.1 and CD2 and down-regulation of c-kit. We therefore assessed the NK potential of flt3\(^{+}\) and flt3\(^{-}\) progenitors in vivo. Significantly, both flt3\(^{+}\) and flt3\(^{-}\) progenitors gave rise to NK cells with a mature CD2\(^{+}\), c-kit\(^{+}\), NK1.1\(^{bright}\) phenotype, albeit less efficiently from the flt3\(^{-}\) population. Thus, signals necessary to up-regulate NK1.1 and CD2 and to down-regulate c-kit were provided to the flt3\(^{-}\) progenitors in vivo. One possible explanation for the ability of flt3\(^{-}\) cells to give rise to mature NK cells in vivo but not in vitro is that flt3\(^{+}\) and flt3\(^{-}\) cells are in the same lineage pathway, with flt3\(^{-}\) cells being more immature than flt3\(^{+}\) cells. Thus, in vivo but not in vitro, flt3\(^{-}\) cells receive sufficient signals to complete their normal developmental pathway. In support of this, preliminary data have suggested that NK1.1\(^{+}\) cells are detectable in the peripheral blood of mice injected with flt3\(^{-}\) progenitors earlier than they are detectable in mice injected with flt3\(^{+}\) progenitors. Furthermore, flt3\(^{-}\) cells have a greater capacity than flt3\(^{+}\) cells to self-renew following serial transfers into irradiated recipients, again suggesting that flt3\(^{-}\) cells are more immature than flt3\(^{+}\) cells (N. S. Williams and V. Kumar, unpublished data). Because flt3L\(^{-/-}\) mice lack NK1.1\(^{bright}\) cells, it is possible that flt3\(^{-}\) progenitors normally must differentiate into flt3\(^{+}\) cells before entering the NK differentiation pathway and that interaction of flt3L with flt3\(^{-}\) cells is essential for the development of mature NK cells. In support of this, we found in vitro that flt3L was absolutely essential for the survival of Lin\(^{-}\), c-kit\(^{-}\), flt3\(^{-}\) cells. These cells failed to survive when cultured in IL-7 and SCF alone. Together, these data reinforce the hypothesis that flt3L plays a critical role in NK cell differentiation and suggest an explanation for the NK cell-deficient phenotype of flt3L\(^{-/-}\) mice.

Although we have not pursued the observation here, it was interesting to note that IL-7 also seemed to play an important role in the development of IL-15-responsive precursors from Sca1\(^{+}\) progenitors. This is in contrast to the observation that IL-7R\(^{α-/-}\) mice show no defects in NK cells. It is possible that in vivo, signaling through this receptor can be compensated for by other cytokines not present in our in vitro system. On the other hand, IL-7R\(^{α-/-}\) mice were found to have a 3-fold decrease in the absolute number of NK cells. It is therefore a formal possibility that IL-7 can exert its effects, at least on NK cells, independently of the IL-7Rα receptor. We are currently examining Lin\(^{-}\), c-kit\(^{-}\), flt3\(^{-}\) NK progenitors for the presence of the IL-7Rα-chain in an effort to better understand the role of IL-7 in NK cell development.

In addition to allowing maturation of NK cells from both flt3\(^{+}\) and flt3\(^{-}\) populations to a NK1.1\(^{bright}\), CD2\(^{+}\), c-kit\(^{+}\) stage, in vivo transplantation also allowed for expression of Ly49 molecules on these NK cells. We show here for the first time that this environment can be recreatned in vitro by growing flt3\(^{+}\) progenitors in IL-7, SCF, and flt3L and then placing them on a confluent monolayer of the bone marrow stromal cell line, OP9, in the presence of IL-15 or IL-2. The NK cells generated expressed Ly49 molecules reactive with three distinct Ly49 Abs, 4D11 (Ly49G2), 1F8 (Ly49C/I/H), and 5E6 (Ly49C/I). It is tempting to speculate, on the basis of the larger percentage of cells reactive with 1F8 vs 5E6, that Ly49H may be expressed on these cells, but definitive evidence of this awaits more extensive analysis. It is clear, however, that at least three distinct Ly49\(^{+}\) subsets are generated, because two-color analysis of 4D11 vs 1F8 revealed the presence of two single-positive populations in addition to a double-positive population.

Because the frequency of Ly49\(^{+}\) cells was lower than that observed on mature peripheral NK cells, we speculated that additional cellular interactions may be necessary for inducing the normal frequency of Ly49 molecules. Therefore, we utilized a less refined Lin\(^{-}\), c-kit\(^{-}\) progenitor population to initiate the cultures. Interestingly, this strategy did yield a higher frequency of Ly49\(^{+}\) cells for reasons we do not yet understand. Using this protocol, Ly49 molecules reactive with four distinctive Abs were detectable. In addition, the time course analysis performed revealed that Ly49 molecules reactive with the mAbs 4D11 and 1F8 appeared at least 3 days earlier than molecules reactive with the mAbs 4E5 and JR9-318. Such findings mirror observations made of the onset of Ly49 expression on NK cells isolated from neonatal spleens. In these studies, expression of Ly49G2, as detected with 4D11, preceded expression of Ly49A (45, 46). It is tempting to speculate that subsets of Ly49 molecules may be expressed with different kinetics, although definitive evidence of such regulation awaits further analysis of individual Ly49 gene expression in developing NK cells. Finally, it is significant that at the earliest time points examined, individual Ly49\(^{+}\) NK cells could express more than one Ly49 molecule (Fig. 6E and data not shown). Several models have been put forth to explain regulation of the repertoire of Ly49 molecules in NK cells. In one model, NK cells stochastically express individual Ly49 molecules one at a time and continue to do so until an interaction of sufficient magnitude between an expressed Ly49 and self-class I MHC occurs (47). In a second model, individual NK cells begin to express one, two, or multiple Ly49 molecules at one time. Those NK cells that express at least one self-MHC-reactive Ly49 molecule but not so many self-reactive receptors as to be insensitive to virus- or tumor-induced changes in class I are then selected for expansion (47). Our data at this point do not allow us to distinguish between these two hypotheses. Neither model seems to adequately explain our observations, because we observe certain Ly49 molecules being expressed in concert (i.e., 4D11- and 1F8-reactive molecules), whereas others require additional time before detection (i.e., 4E5- and JR9-318-reactive molecules). Furthermore, the majority of Ly49\(^{+}\) NK cells fail to express Ly49A, the Ly49 molecule known to bind the H-2\(^{b}\) MHC of the OP9 stroma (48). Both models would predict the need for at least one self-reactive Ly49 receptor for survival and/or expansion. In this regard, it is interesting to note that both Ly49C/I (T. George and M. Bennett, unpublished data) and Ly49G2 (N. S. Williams and V. Kumar, unpublished data) can receive weak negative signals from H-2\(^{b}\). If there are true kinetic differences in the expression pattern of the Ly49 molecules, with Ly49C/I and Ly49G2 being expressed before Ly49A and Ly49D, it is possible that the weak signals sent by H-2\(^{b}\) through Ly49G2 or Ly49C/I are sufficient in many cases to shut off further Ly49 expression. In this regard it is important to note that the progenitors remain in close proximity to the stromal cells during their entire development in vitro. Weak signals may be more effective here than in vivo, where developing NK cells may interact only briefly with stromal cells. These and other issues relating to the development of the Ly49 repertoire can now be addressed by utilizing progenitor and stromal cells of different MHC types and by interrupting contact of the developing NK cells with the stroma in this in vitro culture system.
In conclusion, our experiments have demonstrated a role for flt3L in expanding and/or inducing the differentiation of Lin−, c-kit+ flt3+ progenitors into cells capable of responding to IL-15 to give rise to NK cells. The steps of NK differentiation from the flt3+ progenitor can be largely mimicked in vitro by culture with IL-7, SCF, and flt3L followed by culture with IL-15. However, induction of Ly49 is critically dependent on additional signals provided by bone marrow-derived stromal cells.

Acknowledgments

We thank Ted George for help with in vivo experiments, Angie Moubley and Bonnie Darnell for all aspects of cell sorting, and Dr. John Schatzle for helpful discussions.

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