Activated Natural Killer Cells and Interleukin-2 Promote Granulocytic and Megakaryocytic Reconstitution After Syngeneic Bone Marrow Transplantation in Mice

By Anne K. Siefer, Dan L. Longo, Christie Lee Harrison, Craig W. Reynolds, and William J. Murphy

Purified populations of natural killer (NK) cells were obtained from mice with severe combined immune deficiency (SCID). SCID spleen cells were cultured and activated with recombinant human interleukin-2 (rhIL-2) in vitro. The activated NK cells were then transferred with syngeneic BALB/c bone marrow cells (BMC) and rhIL-2 into lethally irradiated syngeneic recipients to determine their effect on long-term hematopoietic reconstitution. On analysis, the transfer of rhIL-2-activated NK cells along with BMC resulted in significant increases in splenic and BM hematopoietic progenitor cells when compared with those for mice not receiving NK cells. Histologic and flow cytometric analysis showed a marked increase in granulocytic and megakaryocytic lineage cells present in the spleens of the mice receiving activated NK cells. Analysis of the peripheral blood indicated that the transfer of activated NK cells with BMC also significantly improved platelet and total white blood cell counts, with increases in segmented neutrophils. Erythroid recovery was not affected. Finally, lethally irradiated mice receiving activated NK cells and rhIL-2 along with limiting numbers of syngeneic BMC showed a marked increase in survival rate. These results show that the use of populations enriched for activated NK cells after syngeneic BM transplantation (BMT) has a profound enhancing effect on engraftment primarily affecting megakaryocytic and granulocytic cell reconstitution. Therefore, the transfer of activated NK cells and rhIL-2 may be of clinical use to promote hematopoietic reconstitution after BMT.

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washed again with HBSS, and supernatants were collected. All supernatants were then centrifuged at 1,600 rpms for 6 minutes. Pellets were then resuspended in an appropriate volume of media, and cells were counted and collected. A total of 50,000 U rhlL-2 in 0.2 mL PBS IP once a day on days 0, 1, and 2 after BMC transfer. The groups consisted of irradiation only, BMC only, BMC with rhlL-2, and BMC with rhlL-2 and NK cells. On day 7 after BMT, mice were reinfected with NK cells and rhlL-2 IP. IL-2 was administered on days 7, 8, and 9. Mice not receiving IL-2 were injected with 0.2 mL of HBSS. All experiments were assayed with 3 to 4 mice/group and were performed 3 to 6 times with a representative experiment being shown.

**Assay for in vitro hematopoiesis.** BMC and SC from mice 12 days after BMT were washed and resuspended in Iscove’s modified Dulbecco’s medium (IMDM) with 15% FBS, 1% L-glutamine, 2 x 10^{-7} mol/L 2-mercaptoethanol, and antibodies (complete IMDM). SC were plated at 1 x 10^5 cells/plate, and BMC were plated at 1 x 10^5 cells/plate. Both SC and BMC were plated with 0.35% Sea Plaque (FMC BioProducts, Rockville, MD) in 35-mm x 10-mm plates with grid (NUNC, Naperville, IL). Colony growth was stimulated with optimal doses of the growth promoting cytokines recombinant mouse granulocyte-macrophage colony-stimulating factors (GM-CSF; 10 ng/mL) and recombinant mouse IL-3 (10 ng/mL), supplied by the Biological Response Modifiers Program Repository (NCI-FCRDC). Plates were incubated at 37°C, 5% CO_2, and 100% humidity for 7 days. The plates were then examined for colonies, where greater than 50 cells constituted a colony.

**In vitro depletion of ASGM1^+ cells.** NK cells were cultured and harvested, as described above, and divided into 2 groups. The cells were cultured for 30 minutes on ice with anti-ASGM1 (Wako Pure Chemical Industries Ltd, Richmond, VA) at a dilution of 1:50 (1 x 10^7 c/mL) or with HBSS alone. The cells were then washed and resuspended in HBSS containing 1,000 U/mL of rhlL-2. Both groups of cells were then incubated at 37°C for 45 minutes with baby rabbit complement (a kind gift from Dr Dennis Taub, NCI-FCRDC) at a dilution of 1:12 (1 x 10^7 c/mL). The cells were then washed 2 times with IL-2-containing media. The cells were analyzed by flow cytometric analysis to determine that greater than 90% of the ASGM1^+ cells were removed by antibody and complement treatment. The cells were then injected into mice without readjusting the cell concentration.

**Histology.** On days 11, 15, and 19 after BMT, spleen tissues from 3 mice/group were fixed in 10% formalin and paraffin-embedded, and 4-μm sections were cut and stained with hematoxylin and eosin.

**Analysis of peripheral blood (PB).** For analysis of PB, blood was collected from the lateral tail vein of the mice using EDTA as an anticoagulant. Complete blood cell counts were performed with a Coulter counter (Coulter), and differential cell counts were performed by microscopic examination of Wright’s stained PB smears (MetPath, Inc, Rockville, MD).

### Table 1. Phenotype of SCID NK Cells Cultured With rhlL-2

<table>
<thead>
<tr>
<th>Cell Surface Marker</th>
<th>% Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsiAco GM1</td>
<td>85</td>
</tr>
<tr>
<td>Thy-1,2</td>
<td>87</td>
</tr>
<tr>
<td>CD3</td>
<td>0</td>
</tr>
<tr>
<td>CD4</td>
<td>0</td>
</tr>
<tr>
<td>CD8</td>
<td>0</td>
</tr>
<tr>
<td>Surface Ig</td>
<td>0</td>
</tr>
<tr>
<td>MAC-1</td>
<td>0</td>
</tr>
<tr>
<td>FcR</td>
<td>76</td>
</tr>
</tbody>
</table>

SCID spleen and BM cells cultured for 4 to 7 days with 1,000 U/mL of rhlL-2. Flow cytometric analysis using FITC- or biotinylated MoAbs under conditions described in Materials and Methods.

<table>
<thead>
<tr>
<th>Effector E/T Ratio</th>
<th>% Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c SC</td>
<td>100:1</td>
</tr>
<tr>
<td>rhlL-2-activated SCID NK cells</td>
<td>25:1</td>
</tr>
<tr>
<td></td>
<td>13:1</td>
</tr>
<tr>
<td></td>
<td>6:1</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
</tr>
</tbody>
</table>

Varying numbers of effector cells were incubated with 5 x 10^7 ^{51}Cr-labeled NK-sensitive YAC-1 tumor target cells for 4 hours. Supernatants were collected and counted on a γ radiation counter as described in the Materials and Methods.
ACTIVATED NK CELLS PROMOTE MARROW ENGRAFTMENT

Table 3. Effect of rhIL-2—Activated NK Cells on Marrow Engraftment After Syngeneic BMT

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatment After Irradiation</th>
<th>Total Cellularity (&lt;10^6)</th>
<th>Cytokines*</th>
<th>Media Alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen A†</td>
<td>BM alone</td>
<td>10.2 ± 3.0</td>
<td>12.6 ± 1.9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Spleen A†</td>
<td>BM + rhIL-2†</td>
<td>16.2 ± 0.0</td>
<td>7.75 ± 1.0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Spleen A†</td>
<td>BM + NK + rhIL-2§</td>
<td>14.7 ± 0.4</td>
<td>19.6 ± 2.9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>BM</td>
<td>BM alone</td>
<td>4.1 ± 0.5</td>
<td>7.0 ± 1.7</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>BM</td>
<td>BM + rhIL-2†</td>
<td>2.9 ± 1.8</td>
<td>12.5 ± 0.7</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>BM</td>
<td>BM + NK + rhIL-2§</td>
<td>6.4 ± 1.0</td>
<td>30.3 ± 5.1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Spleen B†</td>
<td>BM + rhIL-2</td>
<td>17.4 ± 3.5</td>
<td>169.0 ± 21.7</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Spleen B†</td>
<td>BM + NK + rhIL-2</td>
<td>20.3 ± 2.1</td>
<td>223.0 ± 14.9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Spleen B†</td>
<td>BM + ASGM1+ cells + rhIL-2§</td>
<td>12.8 ± 6.0</td>
<td>46.5 ± 6.0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

* SC and BMC were placed with IL-3 and GM-CSF as described in Materials and Methods.
† Representative of five to six experiments with 3 to 4 mice/group. Colony assay performed 12 days after BMT. Mice in experiment A were administered 850 cGy, and mice in experiment B were administered 800 cGy.
‡ 50,000 U of rhIL-2 administered IP days 0, 1, 2, 7, 8, and 9.
§ 2 × 10^7 NK cells administered IP on days 0 and 7, and 50,000 U of rhIL-2 administered IP on days 0, 1, 2, 7, 8, and 9.
∥ Values significantly (P < .001) greater than those for mice receiving either BMC or BMC with rhIL-2.
# Values significantly (P < .001) lower than those for mice receiving BMC and rhIL-2 or BMC, NK cells, and rhIL-2.

Survival studies. Recipient BALB/cJ mice were exposed to 137Cs γ irradiation source. The mice received 950 cGy total irradiation. Mice in some groups received 2 × 10^9 BALB/c BMC IV. Mice in other groups received either BMC and 50,000 U rhIL-2 or BMC, rhIL-2, and 2 × 10^7 NK cells. In some experiments mice received 2 × 10^7 NK cells with rhIL-2 without BMC. Mice were then observed for several weeks, and moribund mice were killed. Three experiments were performed with 6 to 10 mice/group, with a representative experiment being shown.

Statistical analysis. A Student's t-test was performed to determine if values were significantly different (P < .001). A Wilcoxon rank-sum test was performed on the survival studies to determine if treatments were significantly different (P < .05).

RESULTS

NK cells cultured in vitro with rhIL-2 maintain a homogeneous phenotype. SC and BMC from SCID mice were cultured in vitro with rhIL-2 for 7 to 10 days to obtain pure populations of NK cells. SCID SC and BMC were used to culture NK cells because SCID mice have no T or B cells because of a defect in their recombines system and cannot rearrange their T-cell receptor or Ig genes.9,10 However, SCID mice have normal NK cells and NK cell function.10 Because of a change in previous culture conditions to allow for the generation of large numbers of NK cells, the cultured cells were then analyzed with respect to phenotype and function. Through flow cytometric analysis, it was determined that the cultured NK cells were 87.6% Thy-1 positive and were negative for CD3, CD4, and CD8 surface markers. The cells were greater than 85% positive for ASGM1, greater than 75% positive for the Fc receptor for Ig (FcR), and negative for the macrophage marker, MAC-1 (Table 1). These results are consistent with the phenotype of activated NK cells.8,10,12 The lytic capability of the cells was also determined in a 51Cr-release cytotoxicity assay. The activated NK cells were found to be highly lytic against the NK-sensi-

tive tumor target, YAC-1 (Table 2), which is consistent with the phenotypic analysis showing that the majority of the cultured cells are activated NK cells.

Activated NK cells promote marrow engraftment of syngeneic BMC in lethally irradiated recipients. The activated NK cells were then adoptively transferred along with BMC into lethally irradiated syngeneic recipients on day 0 to determine their effect on engraftment. NK cells were then administered again on day 7. rhIL-2 was also administered daily for 3 days after each injection of the activated NK cells for the NK cells to maintain their function in vivo. Some mice received BMC alone, others received BMC and rhIL-2, whereas others received BMC, rhIL-2, and NK cells. Previous studies had shown that NK cells alone, IL-2 alone, and NK cells plus IL-2 could not reconstitute hematopoietic cells was determined from the BM and spleen. The results showed that the transfer of rhIL-2—activated NK cells along with BMC resulted in significant (P < .001) increases in splenic and BMC hematopoietic colony formation and in an increase in total hematopoietic progenitor content when compared with those for mice in groups not receiving NK cells (Table 3). To ascertain if the hematopoietic effects of the transferred cells were indeed due to NK cells, ASGM1+ cells were removed before cell transfer by antibody and complement. The results showed that the transfer of ASGM1+ cells with BMC actually resulted in lower hematopoietic engraftment in the recipients as evidenced by the lower numbers of hematopoietic progenitor cells in the spleen (Table 3, experiment B). Thus, it appears that the transfer of activated NK cells along with BMC can promote short-term marrow engraftment after syngeneic BMT.

Histologic examination of the spleen also showed an increase of hematopoietic progenitor cells present in recipi-

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Fig 1. Histology of the spleens of mice receiving either BMC alone (A) or BMC with rhIL-2–activated NK cells (B). Histology was performed 12 days after BMT as described in the Materials and Methods. Note the increased cellularity (B) and megakaryocytic precursors (C) in the spleens of mice receiving IL-2–activated NK cells and BMC. Representative of 3 mice/group and three experiments.
Fig 2. 8C5 content of the spleens from mice in different groups, 15 days after syngeneic BMT as determined by flow cytometric analysis as described in the Materials and Methods. (A and B) Mice receiving $5 \times 10^5$ BMC. (C) The group receiving $5 \times 10^5$ BMC and 50,000 U rhIL-2. (D) The group receiving $5 \times 10^5$ BMC, 50,000 U rhIL-2, and $2 \times 10^7$ activated NK cells. Representative of 3 to 4 mice/group and three experiments.

Fig 3. Platelet levels (k/cmm) in mice receiving $5 \times 10^5$ BMC injected IV (○). Other mice received BMC and 50,000 U rhIL-2 injected IP (●). Mice in other groups received BMC, rhIL-2, and $2 \times 10^7$ NK cells injected IP (▲). The points that lack error bars have standard errors that are smaller than the size of the symbol. (#) Values are significantly ($P < .001$) greater than those for recipients of BMC alone and for BMC and IL-2. (*) Values are significantly ($P < .001$) greater than those for recipients of BMC alone.
further suggest that NK cells promote hematopoiesis in syngeneic BMT primarily affecting granulocytic and megakaryocytic lineages.

Transfer of activated NK cells in syngeneic BMT improves PB hematologic parameters. Analysis of the PB complete blood counts and differentials indicated that the transfer of activated NK cells with BMC significantly improved platelet counts as well as white blood cell (WBC) counts. Not only were the platelet precursors (megakaryocytes) seen in the spleen, but a significant increase \((P < 0.001)\) in platelet counts in the PB was observed on day 15 after BMT (Fig 3). A significant increase \((P < 0.001)\) in WBC count in the PB was also observed on day 14 after BMT (Fig 4). The WBCs were then stained to determine their phenotype. A significant increase \((P < 0.001)\) in segmented cells was noted on day 14 after BMT (Fig 5). Hemoglobin levels and hematocrit were not significantly affected. Additionally, the transfer of irradiated NK cells did not affect long-term hematopoietic reconstitution (data not shown), in agreement with our previous report. Thus, the transfer of activated NK cells after syngeneic BMT results in the enhanced recovery of PB platelets and granulocytes.

**NK cells promote animal survival when transferred with limiting numbers of BMC.** We then wished to determine if the activated NK cells were sufficiently active at promoting hematopoiesis in vivo to permit hematopoietic reconstitution and survival of lethally irradiated mice administered limiting numbers of BMC. All BALB/c mice given 950 R of \(\gamma\) radiation without BMC died. Additionally, all mice receiving NK cells and IL-2 without BMC also died, which is comparable with radiation controls indicating that the cultured SCID cells were not contributing a source of hematopoietic stem cells (data not shown). This is in agreement with previous experiments that showed that the transfer of NK cells without BMC did not result in short-term hematopoietic recovery in irradiated recipients. Administration of \(2 \times 10^5\) BALB/c BMC rescued 20% of the irradiated recipients. Addition of IL-2–activated NK cells to the BMC rescued two-thirds of the irradiated mice. Thus, mice receiving NK cells had significantly \((P < 0.05)\) better survival rates than those not receiving NK cells (Fig 6). Interestingly, there were also improved survival rates in the mice receiving BMC and rhIL-2; however, the magnitude of the survival advantage associated with the use of rhIL-2 alone did not reach statistical significance.

**DISCUSSION**

The data presented here indicate that the transfer of rhIL-2–activated NK cells along with BMC and rhIL-2 into lethally irradiated syngeneic mice promotes granulocytic and megakaryocytic reconstitution.

The mechanism by which rhIL-2–activated NK cells promote hematopoietic reconstitution is not yet clear. The stimulatory effects on hematopoiesis may be due to their ability to produce cytokines such as granulocyte-macrophage col-
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It was recently reported that systemic administration of IL-11 also promoted granulocytic and megakaryocyctic reconstitution in mice after syngeneic BMT. Experiments are currently being performed to determine if activated NK cells can produce IL-11. GM-CSF and G-CSF have been shown to enhance granulocyte regeneration and also to accelerate granulocyte maturation. We have previously shown that activated NK cells produce these cytokines. The data reported here support this finding because there was an increase in granulocyte precursors present in the spleens and the increased levels of WBCs were noted in the PB of mice receiving NK cells. In a murine model, IL-6 administration increases platelet recovery after radiotherapy. Other recent studies have determined that GM-CSF and IL-6 promote megakaryocyte colony formation in vitro and in vivo. Our data support these findings because an increase in megakaryocytes in the spleen and increased platelet counts were noted in the blood of irradiated mice receiving BMC plus NK cells. Therefore, the production of GM-CSF and IL-6 by the NK cells (as previously demonstrated) may be promoting platelet recovery. In a clinical setting, systemic administration of these cytokines may produce toxic side effects. Therefore, the possible advantage of using NK cells in a clinical setting may be the reduction of the toxic side effects because of the local release of adequate amounts of the cytokines in the sites where the cytokines act. This type of cellular immunotherapy might then be more advantageous than systemic administration of pharmacologic doses of these cytokines.

Because NK cells possess hematopoietic growth-promoting effects as well as antitumor activity, it would be worthwhile to explore their use in a clinical setting such as ABMT. The problems associated with ABMT, including immune deficiency and recurrence of the cancer, may be decreased with the use of activated NK cells. Clinically, activated NK cells could be used in ABMT to increase engraftment and to remove residual tumor. Additionally, because NK cells show antiviral properties, the use of these cells may be beneficial in the prevention of cytomegalovirus infection in the setting of allogeneic BMT. Experiments are underway to examine the effect of NK cells on the removal of tumor in tumor-bearing mice after BMT.

There have been contradictory reports involving the effect of activated NK cells on hematopoiesis. In part, this may be due to differences in the assay systems and to the fact that many NK cell preparations contain contaminating populations of T cells or the difference between allogeneic and syngeneic NK cells on BM, or the transplant conditions (suboptimal BM → NK cells help; optimal BM → NK cells suppress). Our studies were performed with highly purified populations of activated NK cells. It may be difficult to isolate pure populations of NK cells in humans due to the paucity of NK cells in the PB. Therefore, clinical studies might require the use of lymphokine activated killer (LAK) cells, which contain both T and NK cells. Experiments for examining the role of LAK cells in syngeneic BMT are underway. This is an important issue, because studies using human LAK cells showed inhibitory effects on hematopoiesis in vitro. However, a recent study has shown that the transfer of LAK cells was not deleterious to hematopoietic reconstitution and survival rates after syngeneic BMT in mice. These results then suggest that adoptive immunotherapy may be of clinical use when ABMT is used in the treatment of cancer.

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