Donor Natural Killer (NK1.1+) Cells Do Not Play a Role in the Suppression of GVHD or in the Mediation of GVL Reactions After DLI

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ABSTRACT

Donor regulatory T cells (CD3+ αβ T-cell receptor [TCR]+) derived from the repopulating host thymus have been shown to be primarily responsible for suppression of GVHD following DLI therapy in murine BMT models. However, natural killer (NK) T cells also have regulatory properties, and a role for NK T cells in suppression of GVH reactivity has not been completely excluded. NK cells may also contribute to the graft-versus-leukemia (GVL) effect associated with DLI therapy. In this study, we used a murine BMT model (C57BL/6 into AKR) to study whether depletion of donor NK cells had any impact on the suppression of GVH reactivity after DLI or on the DLI-induced GVL effect against acute T-cell leukemia. Depletion of donor NK cells was accomplished in vivo by giving DLI-treated bone marrow chimeras multiple injections of anti-NK1.1 monoclonal antibody (MoAb). The chimeras treated with anti-NK1.1 MoAb had significantly fewer splenic NK1.1+ cells than nontreated chimeras, and splenocytes from anti-NK1.1-treated mice were deficient in the ability to generate lymphokine-activated lytic activity. Results presented here showed that NK-cell depletion had no effect on the suppression of GVH reactivity after DLI. When DLI-treated chimeras were challenged with an acute T-cell leukemia, NK-cell depletion had no discernible effect on GVL reactivity. These preclinical data suggest that donor NK cells do not have a significant role in the suppression of GVHD after DLI or in the mediation of GVL reactivity induced by DLI.

KEY WORDS

Donor leukocyte infusion • NK cells • Bone marrow transplantation • Graft-versus-leukemia effect • Graft-versus-host disease

INTRODUCTION

Delayed donor leukocyte infusions (DLI) after allogeneic bone marrow transplantation (BMT) have induced dramatic graft-versus-leukemia (GVL) effects in patients treated for relapse of disease [1]. Despite the fact that DLI has been used clinically for more than 10 years, the immune effector cells involved in GVL reactivity have not been well characterized. Both T cells and natural killer (NK) cells have been implicated in GVL reactions after DLI [2-4]. The relative importance of these 2 cell populations likely depends on multiple factors, including the genetic disparity between the donor and recipient as well as the type of tumor targeted for therapy.

One of the more remarkable observations regarding DLI therapy is that whereas graft-versus-host disease (GVHD) is still a problem after DLI, less severe GVHD has typically been observed in the posttransplantation setting than would be anticipated if comparable numbers of donor T cells were given at the time of BMT. The decreased risk for developing severe GVHD after DLI has been attributed to avoidance of conditioning-related toxicity [5,6], decreased numbers of host antigen-presenting cells to stimulate GVHD [7], and the reestablishment of immune regulatory mechanisms that suppress T-cell alloreactivity [8]. In previous studies, we demonstrated that Thy1+ αβ T-cell receptor (TCR)+ cells (CD4+8+ and CD4+8−) suppress GVH reactivity after DLI in mice [8]. We are currently investigating whether other phenotypic markers can further distinguish these immunosuppressive cells from nonsuppressive populations of T cells. NK T cells have been found to suppress GVH reactivity [9,10], and they are comprised of cells including αβTCR+CD4+ and αβTCR+CD4− cells [11]. Thus, the possibility exists that at least one subpopulation of the Thy1+ αβTCR+ regulatory T cells identified in our previous studies is comprised of NK T cells.

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In the current study, we used a spontaneously arising AKR leukemia as a model of acute T-cell leukemia to investigate the role of donor NK cells in GVL and in the suppression of GVHD after DLI. Transplant recipients were treated with an anti-NK1.1 monoclonal antibody (MoAb) to deplete donor NK cells in vivo. Donor NK cells did not contribute to the GVL effect of DLI against this tumor. In vivo depletion of NK1.1+ cells also did not increase the severity of GVHD, indicating that CD4+ or CD4− NK T cells do not play a role in the suppression of GVH reactivity after DLI.

**Materials and Methods**

**Mice**

AKR/J (H-2b, Thy1.1, NK1.1+), C57BL/6 (H-2b, Thy1.2, NK1.1+), and B6.PL-Thy1b+ (H-2b, Thy1.1+) mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 4 to 6 weeks of age. The animals were cared for in the Medical College of Wisconsin Animal Resource Center, which is accredited by the American Association for the Accreditation of Laboratory Animal Care.

**Leukemia**

The tumor used in these studies is an acute T-cell leukemia that spontaneously arose in a male AKR mouse. The leukemia, designated as M2, was passaged in vivo, harvested from the spleens of inoculated AKR mice, and frozen as a stock. Vials of the frozen stock were thawed for transplantation experiments, and the same stock of leukemia that spontaneously arose in a male AKR mouse was used to minimize experimental variability. The percentages of NK1.1+ splenocytes, spleen and thymus cells. GVH reactivity is significantly suppressed when DLI is given at this time point in the C57BL/6-into-AKR model [8].

Due to the aggressiveness of the AKR M2 leukemia, mice were challenged with different doses of leukemia after DLI. This same tumor-challenge approach has been used in previous studies to assess GVL reactivity [12,13]. BM chimeras were injected IV on day 35 after BMT with indicated numbers of M2 leukemia cells.

**Phenotypic Analysis and Cytolytic Assays**

To assess the effectiveness of in vivo NK-cell depletion, splenocytes of individual anti-NK1.1 treated and nontreated BM chimeras were collected on day 28 or day 56 after BMT. Red cells were eliminated by hypotonic lysis. The cells were stained with a combination of anti-CD3-fluorescein isothiocyanate and anti-NK1.1-phycocerythin MoAbs (BD PharMingen, San Diego, CA) and analyzed by flow cytometry to determine the percentages of NK1.1+ cells. For each sample, 5 × 10⁶ events were collected for analysis.

Effectiveness of NK-cell depletion was confirmed by performing NK/lymphokine-activated killer (LAK) cytolytic assays as previously described [14]. Briefly, splenocytes collected for phenotypic analysis were cultured for 3 days in complete DMEM [15] containing 10% fetal bovine serum (FBS) and 500 U/mL human recombinant interleukin (IL)-2 (ID Labs Inc, London, Ontario, Canada). Following culture, the cells were washed twice with DMEM by centrifugation, resuspended in complete DMEM containing 5% FBS, counted, and placed in quadruplicate U-bottom microtiter wells with 2500 ¹⁵Cr-labeled NK/LAK-sensitive YAC-1 murine lymphoma cells at effector:target ratios ranging from 200:1 to 6:1. The effector and target cells were cocultured for 3 hours, then supernatants were collected from each well for γ scintillation counting. Maximum and spontaneous release values were obtained by culturing target cells alone in detergent or culture medium, respectively. The percent lysis was calculated by the following formula:

\[
\frac{cpm\ experimental - cpm\ spontaneous\ release}{cpm\ maximum\ release - cpm\ spontaneous\ release} \times 100
\]

**Statistics**

Survival curves were compared using the log-rank test. The percentages of NK1.1+ splenocytes, spleen and thymus plants by intravenous (IV) injection with 10⁷ C57BL/6 T-cell–depleted BM cells.

For in vivo donor NK-cell depletion, BM chimeras were treated intraperitoneally (IP) with anti-NK1.1 MoAb (clone PK 136, ATCC) beginning on day 24 after BMT; for a total of 6 to 10 injections (500 µg per injection). Specifically, anti-NK1.1 MoAb was given on days 24, 26, 30, 32, 36, and 38 for a total of 6 injections or with additional injections on days 42, 44, 48, and 52 for a total of 10 injections. In some experiments, mice were depleted in vivo of Thy1.2+ cells by treating BM chimeras with IP injections of anti-Thy1.2 MoAb (clone 30-H12) on days 24 and 26 after BMT.

DLI was given on day 28 after BMT as a single IV injection of 3 × 10⁷ C57BL/6 or Thy1 congenic B6.PL-Thy1b+ spleen cells. GVH reactivity is significantly suppressed when DLI is given at this time point in the C57BL/6-into-AKR model [8].

Statistics

Survival curves were compared using the log-rank test. The percentages of NK1.1+ splenocytes, spleen and thymus...
results

Day Post-BMT n Treatment* % NK1.1+ Splenocytes (±SD) NK1.1+ Cells, x10^5 (±SD)

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* B6/AKR BM chimeras were treated or not treated with anti-NK1.1 MoAb (see “Materials and Methods” for details). Some chimeras were given DLI on day 28 after BMT, as indicated. Mice were killed for analysis on either day 28 or day 56 after BMT (ie, 2 days after the second anti-NK1.1 MoAb injection or 4 days after the last of 10 injections, respectively). Percentages and absolute numbers of splenic NK1.1+ cells were determined by flow cytometric analysis. The data are the combined results of 3 sets of experiments.

†P < .05 compared with day 28 non–anti-NK1.1–treated chimeras.
‡P < .05 compared with day 28 non–anti-NK1.1–treated chimeras receiving BM transplants only (no DLI).
§P < .05 compared with day 56 DLI-treated chimeras.

NK T Cells Do Not Play a Role in the Suppression of GVH Reactivity After DLI

A series of experiments was done to assess whether donor NK1.1+ cells suppress GVH reactivity after DLI. Two previous observations led us to undertake these experiments. First, NK cells have been shown to contribute to GVH reactivity after allogeneic BMT [14,16]. Second, NK T cells have been shown by others to be immunosuppressive [17], and in the current studies we detected small percentages (0.2%-0.3%) of NK T cells (NK1.1/CD3 double positive) in the spleens of BM chimeras (data not shown). We have previously demonstrated that there are populations of thymus-derived Thy1+ αβ TCR+ regulatory cells that suppress GVH reactivity after DLI [8]. Thus, T cells expressing the NK1.1 antigen were candidates for regulatory T cells in our model.

For these experiments, BM chimeras given a DLI were treated with a 6-injection regimen (days 24-38 after BMT) of anti-NK1.1 MoAb (Figure 3). For comparison, 2 other groups of BM chimeras were included in these experiments. One group was given DLI with no anti-NK1.1 MoAb. Historically, these mice have developed relatively mild GVHD [8]. A second group was treated with in vivo–depleting anti-Thy1.2 MoAb prior to DLI to eliminate donor regulatory T cells. This group was included in the experiment to demonstrate that DLI can induce severe GVHD if immunoregulatory T cells are eliminated [8]. For DLI treatment in these experiments, B6.PL-Thy1.2 MoAb spleen cells (Thy1.1+) were given so that the infused donor T cells would not be depleted by residual MoAb in anti-Thy1.2–treated chimeras. If NK1.1+ T cells function as regulatory T cells after DLI, we anticipated that GVHD would be more severe in anti-NK1.1–treated chimeras than in nontreated chimeras. However, survival of the NK1.1-depleted chimeras was not significantly different from that of non–NK1.1-depleted chimeras (Figure 3A). Body weight curves of these 2 groups were also similar (P > .05) (Figure 3B). All 3 groups
of BM chimeras lost body weight during the first 2 weeks after DLI. Whereas body weights of the anti-NK1.1–treated and nontreated chimeras plateaued, the Thy1.2-depleted chimeras continued to lose body weight and died. Similar results were obtained in experiments in which the 10-injection regimen of anti-NK1.1 MoAb was used (data not shown).

These data indicated that donor NK cells are not involved in the suppression of GVH reactivity after DLI.

**Depletion of Donor NK Cells In Vivo Did Not Alter the DLI-Induced GVL Effect Against a T-Cell Leukemia**

To examine whether donor NK-cell depletion in vivo had an effect on GVL reactivity induced by DLI, the following 3 groups of BM chimeras were challenged with $10^3$, $10^4$, or $10^5$ AKR M2 leukemia cells: (a) a group given BM only (no DLI), (b) a group treated with DLI, and (c) a group treated with anti-NK1.1 MoAb and DLI. For anti-NK1.1 MoAb treatment, the mice were given a 10-injection regimen (days 24-52 after BMT) to encompass the time period during which mice that received transplants with T-cell-depleted BM begin to die of tumor progression after challenge [12]. DLI was given on day 28 after BMT and challenge with leukemia 1 week later (day 35). AKR recipients of T-cell–depleted BM only (no DLI) died of tumor progression, with the exception of 2 mice challenged with $10^3$ leukemia cells (Figure 4). Thus, the lethal dose for this leukemia in AKR mice that received transplants with B6 BM is fewer than $10^2$ cells. Treatment with DLI induced a significant GVL effect, as indicated by long-term survival rates of ≥75% at all 3 doses of leukemia (Figure 4). Based on observations made at necropsy, the majority of deaths in these mice were attributed to GVH reactivity and not tumor progression. At all 3 doses of leukemia, the overall survival of NK1.1-depleted chimeras given DLI was not significantly different from that of nondepleted chimeras given DLI (Figure 4), indicating that treatment of BM chimeras with anti-NK1.1 MoAb had no impact on GVL reactivity.

**DISCUSSION**

In the current study, we investigated the role of donor NK cells in suppression of GVHD after DLI and whether depletion of NK cells altered DLI-induced GVL reactivity against a murine T-cell leukemia. To eliminate donor-derived NK cells in vivo, a MoAb directed to the NK1.1 antigen (clone PK136) was employed [18]. NK1.1+ cells were significantly depleted after in vivo treatment with anti-NK1.1 MoAb, and the elimination of donor NK cells did not affect suppression of GVHD after DLI or have a discernible impact on DLI-induced GVL reactivity.

We were interested in looking at the effect of NK1.1 depletion on GVH reactivity after DLI to determine whether immunoregulatory cells that suppress GVH reactivity after DLI [8] express the NK1.1 antigen (clone PK136) was employed [18]. NK1.1+ cells were significantly depleted after in vivo treatment with anti-NK1.1 MoAb, and the elimination of donor NK cells did not affect suppression of GVH after DLI or have a discernible impact on DLI-induced GVL reactivity.

We were interested in looking at the effect of NK1.1 depletion on GVH reactivity after DLI to determine whether immunoregulatory cells that suppress GVH reactivity after DLI [8] express the NK1.1 antigen. At least 2 populations of Thy1+ αβ TCR+ cells (CD4−8− and CD4+8+) play important roles in the suppression of GVH reactivity after DLI [8]. Immunosuppressive NK1.1+ T cells expressing these same markers have been described by others and have been shown to suppress GVH reactivity both in vitro and in vivo [9,10]. These observations led us to investigate whether NK1.1+ cells play a role in the suppression of GVH reactivity after DLI. The data indicated that cells expressing the NK1.1 antigen do not have any role in the suppression of DLI-induced GVH reactivity (Figure 3 and data not shown). These results have led us to focus our efforts on looking at other cell surface markers, such as CD25, to further distinguish the ‘Thy1+ αβ TCR’ regulatory T cells from other T cells.
Similar to the clinical data from Jiang and coworkers [4], suppressed NK function as well as reduced numbers of NK cells were observed in mouse BM chimeras given DLI (Figure 2B and Table). We did not look at NK-cell function past day 56 after BMT, but at approximately 100 days after BMT mice given DLI had significantly reduced numbers of NK1.1+ cells compared to mice not given a DLI (data not shown). Because similar reduced numbers of NK cells were observed in both anti-NK1.1–treated and nontreated mice, it appears that the DLI treatment had a long-term effect on NK1.1+-cell numbers. The biologic significance of reduced splenic NK-cell numbers, if any, is not known.

Due to the relatively mild GVH reactivity in the DLI model used for these studies, we were unable to assess the role of NK cells as GVH effectors after DLI. For this assessment, a DLI model in which stronger GVH reactivity occurs would be needed. Perhaps a model in which regulatory T cells have been depleted to allow for the development of GVHD after DLI could be employed to study the role of NK cells as GVH effectors.

Donor T cells are essential mediators in the GVL effect against AKR T-cell leukemias after DLI [12]. Thus, it is not surprising that donor NK cells did not play a significant role in the GVL effect against an aggressively growing acute T-cell leukemia (Figure 4). Our results confirm previous work by Blazar et al. [19], who showed that NK cells were not the major DLI-induced GVL effectors against either EL4, another murine T-cell leukemia, or C1498, a murine acute myeloid leukemia. However, because others have demonstrated that NK cells can mediate GVL reactions...
after allogeneic BMT [20,21] as well as after DLI [22], the role of NK cells in the GVL effect after DLI may depend on the type of tumor or other unknown factors. The AKR leukemia used in our studies is highly aggressive, with a lethal dose of less than 100 cells. Because the DLI inoculum used in our studies contained fewer than 10⁶ donor NK cells (data not shown), it appeared unlikely that these relatively small numbers of DLI-derived NK cells would contribute to GVL reactivity. Rather, we anticipated that any contribution of NK cells to a GVL effect would originate from the endogenous donor NK cells. Perhaps greater numbers of adoptively transferred NK cells or treatment with IL-2–activated NK cells might enhance NK-mediated GVL reactivity in our model, as suggested by data from other investigators [23-25]. In other studies, we generated data suggesting that NK cells can negatively influence GVL reactions after allogeneic BMT [26]. Due to the strength of DLI-induced GVL reactivity, experiments in the current study could not assess whether donor NK cells may actually suppress GVL reactivity after DLI.

In the clinical setting, the contribution of NK cells to GVL reactivity after DLI has been unclear. T cells are important to the GVL effect, but it is not known whether NK cells can contribute to the elimination of tumor cells after DLI, or perhaps even suppress GVL reactions after DLI. Relatively high numbers of NK cells have been found in the peripheral blood of patients early after allogeneic BMT [2,27], suggesting that these cells would be present at the time of DLI to potentially mediate a GVL effect. Jiang et al. investigated immune function in 2 patients who achieved complete remission after DLI for the treatment of relapsed chronic myeloid leukemia, and they found that there was a period of depressed NK-cell function, which eventually recovered [4]. Interestingly, the recovery of NK function coincided with the disappearance of disease, suggesting that NK cells may have been involved in the GVL effect. However, the true role that NK cells play in GVL reactions after DLI in humans continues to be unknown.

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REFERENCES


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**Figure 4.** Depletion of donor NK cells did not compromise GVL reactivity after DLI. AKR recipients received transplants of B6 BM and were then randomized to receive no further treatment (BM only), or to be given DLI with 3 x 10⁷ B6 donor spleen cells at 28 days post-BMT. The indicated groups of BM chimeras were treated with 10 injections of anti-NK1.1 MoAb starting on day 24 post-BMT (see "Materials and Methods" for injection schedule). One week after DLI (day 35), the mice were challenged with (A) 10², (B) 10³, or (C) 10⁴ AKR M2 leukemia cells. The survival curves are the combined results of 2 independent experiments.


