Donor NK Cells and IL-15 Promoted Engraftment in Nonmyeloablative Allogeneic Bone Marrow Transplantation

Bo Hu, Guangming Bao, Yinsheng Zhang, Dandan Lin, Yan Wu, Depei Wu and Haiyan Liu

*J Immunol* published online 13 July 2012
http://www.jimmunol.org/content/early/2012/07/13/jimmunol.1103199

Why *The JI*?

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Donor NK cells could promote engraftment by suppressing host alloreactive responses during allogeneic bone marrow transplantation (allo-BMT). The biological activity of NK cells could be significantly enhanced by IL-15. The current study attempted to evaluate the effect of donor NK cells and IL-15 administration on engraftment and immune reconstitution in a murine nonmyeloablative allo-BMT model. Mice infused with donor NK cells and treated with IL-15 during nonmyeloablative allo-BMT resulted in increased donor engraftment compared with either treatment alone. The number of donor-derived cell subsets also increased in the spleen of the recipient mice with combination treatment. The allosecrecy to donor type Ags was significantly reduced in the recipient mice with donor NK cell infusion and IL-15 treatment, which was manifested by decreased proliferation and IL-2 secretion of splenocytes from recipient mice in response to donor type Ags in MLR and decreased capacity of the splenocytes killing donor type tumor targets. We subsequently exposed recipient mice to reduced irradiation conditioning and showed that donor NK cell infusion and hydrodynamic injection-mediated IL-15 expression could synergistically promote donor engraftment and suppressalloreactivity during nonmyeloablative allo-BMT. Infusion of CFSE-labeled donor CD45.1+ NK cells demonstrated that IL-15 could enhance the infused donor NK cell proliferation and function in vivo. IL-15 treatment also promoted donor bone marrow-derived NK cell development and function. Thus, donor NK cell infusion and IL-15 treatment could synergistically promote the engraftment and the development of donor-derived cell subsets and suppress the host allosecrecy in a murine nonmyeloablative allo-BMT model. The Journal of Immunology, 2012, 189: 000–000.

NK cells are a subset of lymphocytes that play critical roles in both innate and adaptive immune responses and provide defense against microbial infection and malignant transformation (4, 5). In allo-BMT, host NK cells mediate acute rejection of hematopoietic stem cells. Donor NK cell, however, could ablate leukemia, favor engraftment by killing host alloreactive T cells, and protect host from graft-versus-host disease (GVHD) by eliminating host dendritic cells or suppressing donor alloreactive T cells (6, 7). Donor NK cells’ allosecrecy is triggered by a mismatch between MHC class I-specific inhibitory receptors on donor cells and the MHC ligands on host cells (8). Donor NK cells derived from the hematopoietic stem cells may be functionally defective because of immaturity or immunosuppression. Therefore, adoptively transfer of mature donor NK cells has been proposed to be beneficial by providing both a cytolytic and immune regulatory effects. It has been shown that donor NK cell infusion could promote engraftment during nonmyeloablative allo-BMT (6, 9).

Although the adoptive donor NK cell therapy may exert many beneficial effects including promoting engraftment during allo-BMT, it will be important to ensure that the in vitro-activated NK cells do not lose their proliferative potential and cytolytic function in vivo, as has been shown for adoptively transferred T cells (10). IL-15 has been shown to be the major NK cell homoeostatic cytokines. IL-15 displays extensive biological activities, stimulating the activation and proliferation of macrophages, NK cells, T cells, and B cells (11, 12). It has been shown that, in the bone marrow microenvironment, NK cells differentiation requires the actions of the stromal cell-derived IL-15 (13). Many studies have also indicated IL-15 is the critical cytokine necessary for NK cell development in vivo (14–17). Barao et al. (18) have shown that hydrodynamic delivery of human IL-15 cDNA resulted in accelerated and marked NK cell expansion in multiple organs following BMT. On the basis of the significant role of IL-15 in NK
cell development and function, we hypothesize that IL-15 may enhance the effects of donor NK cells promoting engraftment in nonmyeloablative allo-BMT through either by prolonging the transferred donor NK cell survival or by facilitating the development of donor NK cells.

In the current study, we assessed the effects of donor NK cells and IL-15 treatment on donor engraftment in a murine nonmyeloablative allo-BMT model. By adoptively transferring CFSE-labeled CD45.1+ donor NK cells and IL-15 treatment, we further elucidated the role of IL-15 on the infused donor NK cells and donor bone marrow-derived NK cells during nonmyeloablative allo-BMT.

Materials and Methods

Mice
C57BL/6 (H-2b, CD45.2), C57BL/6 (H-2b, CD45.1), and BALB/c (H-2d) mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Mice were kept in a specific pathogen-free facility in microisolator cages, and experiments were performed when the mice were between 7 and 9 wk of age. All animal protocols were approved by the Institutional Laboratory Animal Care and Use Committee at Soochow University.

Cell lines and reagents
EL4 (H-2b), a murine lymphoblast cell line was maintained in complete RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA). Anti-Thy1.2 mAb (30H12) and rabbit complement were purchased from eBioscience (San Diego, CA). Recombinant human (rh)IL-15 was purchased from Xiamen Special Treasure Biological Engineering (Xiamen, China). rhIL-2 was obtained from Beijing Four Rings Bio-Pharmaceutical (Beijing, China).

Cell preparation
Bone marrow cell (BMC) suspensions were prepared by gently releasing cells from the backbones, femurs, and tibiae into Dulbecco’s PBS solution with a mortar and pestle, filtering through a mesh filter to remove particulates, and washed with PBS twice. Spleen cells were prepared by gently crushing the tissues to release the cells. Preparations were filtered to remove debris and washed twice in PBS before resuspending in RPMI 1640 complete medium. Livers were perfused with PBS and processed into single-cell suspensions, and lymphocytes were separated on a Percoll (GE Healthcare, Piscataway, NJ) gradient. RBCs were lysed. Cell counts were performed on a Coulter Z1 cell counter (Beckman Coulter, Brea, CA).

Generation of activated NK cells
The activated NK cells were generated according to the method described previously (19). Briefly, splenocytes and BMCs from C57BL/6 mice were depleted of T cells by treatment with anti-Thy1.2 mAb (30H12) and rabbit complement. T cell-depleted samples were cultured in RPMI 1640 medium supplemented with 10% FBS (Invitrogen Life Technologies), 100 U/ml penicillin/streptomycin, 2 mM l-glutamine, 10 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 2.5 $\times$ 10^{-5} M 2-ME containing 1000 IU/ml rhIL-2 at 1–2 $\times$ 10^6 cells/ml for 6 d at 37˚C and 5% CO_2. At day 3, nonadherent cells were transferred to new flasks, and all cells were fed with 50% conditioned medium, 50% new RPMI 1640 medium supplemented with 10% FBS, and rhIL-2. NK cells generated from C57BL/6 mice contain over 95% NK1.1+CD3- cells.

RT-PCR
mRNA was prepared from the spleen cells using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 2 $\mu$g mRNA using reverse transcriptase (Fermentas, Glen Burnie, MD) and oligo(dT) primers. The synthesized first-strand cDNA (2 $\mu$l) was amplified by means of the

FIGURE 1. Donor NK cell infusion and IL-15 treatment promoted donor engraftment and immune reconstitution during nonmyeloablative allo-BMT. Sublethally irradiated BABL/c mice were reconstituted with 1 $\times$ 10^6 C57BL/6 bone marrow cells with or without donor NK cell (5 $\times$ 10^6) infusion and 1 $\mu$g/d IL-15 administration on days 1 through 7. (A) Recipient mice were euthanized on day 60, and the donor engraftment was analyzed by flow cytometry. (B) Statistical analysis of the donor engraftment was shown. The percentages (C) and absolute numbers (D) of donor-derived cell subsets in the spleens of the recipient mice were calculated and shown. The donor-derived cells were identified as H2k b/H2K d, and the cell subsets were analyzed by CD19+ for B cells, CD3ε+CD4+ for CD4+ T cells, CD3ε+CD8+ for CD8+ T cells, CD3ε+NK1.1+ for NK cells, and CD11b+ for macrophages. Compared with BMT alone group, all the treatment groups showed statistically significant differences for all cell subsets. The statistical analysis between the three treatment groups was shown in the graph. Values were expressed as mean ± SD. The experiments were performed with five mice per group. The data shown are the representative of three experiments. *p < 0.001.
PCR using 20 pmol of each primer specific for murine IL-15 with 2.5 U rTaq (Fermentas) in a total volume of 50 μl reaction buffer consisting of 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂. The specific primers used were as follows: IL-15 subcloned into pcDNA3.1 vector; sense, 5′-GG-TACCATGAAAATTTTGAAACCA-3′; and antisense, 5′-CTCGAGT-TAGGACGTGTTGATGAAACA-3′. The products were resolved on 1.5% agarose gels and visualized by staining with ethidium bromide.

Hydroydynamic IL-15 gene transfer

The coding region of IL-15 was amplified by RT-PCR and inserted into a mammalian expression vector, pcDNA3.1 (Invitrogen), to generate pcDNA3.1 IL-15. The structure of all plasmid constructs was verified by restriction enzyme mapping and nucleotide sequencing. Plasmid DNA was purified by Maxi-prep Kit (Axygen, Union City, CA). For hydrodynamic gene transfer (HGT), the recipient mice were injected i.v. with 25 μg of the recombinant plasmid in a total of 1 ml saline solution within 5 s using a 23-gauge needle 24 h before transplantation.

MLR

To prepare responder spleen cells, spleen cells were harvested from the host mice and made into single-cell suspension, and RBCs were lysed. Stimulator cells were prepared from single-cell suspensions of spleens from C57BL/6 mice and irradiated (20 Gy). Responders and stimulators were cultured at a final concentration of 0.5 × 10⁶/ml, pulsed with tritiated thymidine (1 mCi/well) (Shanghai Institute of Physics, Chinese Academy of Sciences, Shanghai, China) 16–18 h prior to harvesting, and counted on a beta plate reader (PerkinElmer Instruments, Meriden, CT). Four individual wells were analyzed per data point.

Cytotoxicity assay

Alloreactive cytotoxic T cell activity was determined by using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Fitchburg, WI) with responding cells after a 72-h MLR using splenocytes 2 mo after BMT. The assay is based on the measurement of lactate dehydrogenase that is released upon cell lysis. Briefly, the mixed splenocytes were resuspended in RPMI 1640 medium supplemented with 10% FCS and mixed with EL4 target cells in U-bottom 96-well microplates at various E:T ratios in triplicates. Microplates were incubated at 37°C and 5% CO₂ for 4 h and spun for 4 min at 250 × g to pellet cells. Supernatant (50 μl) was collected from each well and added to 50 μl reconstituted substrate mix for 30 min in the dark at room temperature. Enzymatic reaction was stopped by adding stop solution. Absorbance was recorded at 490 nm. Spontaneous release was determined from wells with targets only and total release from wells with targets plus 1% Triton X-100. Results are expressed as percentage of cytotoxicity, using the formula: percentage of cytotoxicity = (experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous) × 100%.

Quantitation of IL-2 levels by MTT assay

Supernatants (50 μl) were removed from a three day MLR mentioned above. The IL-2 levels of the supernatants were estimated by the proliferation of IL-2-dependent T cell line CTLL-2 (5 × 10⁶; 50 μl). When CTLL-2 cells in negative control wells were all dead (~20–22 h), proliferation was determined by using MTT assay according to the manufacturer’s instructions. IL-2 concentration was determined by using rhIL-2 as a standard (300 IU/ml), 3-fold dilutions to 3 U/ml. Results are expressed as IU-2 U (IU/ml) in triplicates.

Flow cytometric analysis

Anti-murine CD16/CD32 FcR block (2.4G2) and all of the following Abs against murine Ags were purchased from BD Biosciences (Franklin Lakes, NJ): PE- or FITC-conjugated anti-CD3e (145-2C11), CD4 (RM4-5), CD8α (53-6.7), NK1.1 (PK136), CD11b (M1/70), CD19 (1D3), H-2Kd (AF6-88.5), H-2Kd (SF1-1.1), CD45.1 (A20), and CD45.2 (104). All stainings were performed in FACS buffer (1× PBS, 1% BSA, and 0.1% NaN₃) at room temperature. Enzymatic reaction was stopped by adding stop solution. Absorbance was recorded at 490 nm. Spontaneous release was determined from wells with targets only and total release from wells with targets plus 1% Triton X-100. Results are expressed as percentage of cytotoxicity, using the formula: percentage of cytotoxicity = (experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous) × 100%.

Values were expressed as mean ± SD. The experiments were performed with five mice per group. The assays were done in quadruplicates. The data shown are the representative of three experiments. *p < 0.05, **p < 0.001.
staining for 30 min at 4°C. The flow cytometric results were analyzed with FACSscan (BD Biosciences) using CellQuest software. The transferred NK cells were sorted by anti-CD45.1 staining using a BD FACSaria III cell sorter (BD Biosciences). The sorted NK cells were stimulated with anti–NK-46 Ab (eBioscience) 25 μg/ml for 24 h. Intracellular staining was performed after the cells were incubated with 10 μg/ml brefeldin A for 4 h. Cells were fixed with 4% paraformaldehyde and permeabilized with BD Perm/Wash buffer (BD Biosciences) and stained for IFN-γ.

CFSE labeling and proliferation analysis

Donor NK cells were labeled with a 5 μM CellTrace CFSE Cell Proliferation kit (Invitrogen) in PBS with 2% FCS for 10 min at 37°C. The labeling reaction was quenched by addition of cold RPMI 1640 medium with 10% FCS, and cells were washed twice with PBS with 2% FCS to remove excess CFSE. FACS analysis allowed gating on individual CFSE generations, and the proliferations of transferred donor NK cells were analyzed. The proliferation index was calculated using FlowJo software (FlowJo, Ashland, OR).

Immunohistochemistry

Liver tissues fixed in 10% phosphate-buffered formalin (pH 7.4) were dehydrated in 100% ethanol and embedded in paraffin wax at 58°C. The paraffin sections were stained with a polyclonal rabbit Ab to murine IL-15 (Abcam, Boston, U.K.). The IL-15 Ab was diluted in PBS (pH 7.4) and applied at concentrations of 1:1000 at 37°C for 30 min. Endogenous peroxidase activity was blocked with 3% H2O2 and methanol. Avidin and biotin pretreatment was used to prevent endogenous staining. The secondary Ab was biotinylated goat anti-rabbit IgG used at 1:2000 dilution (Abcam). Color development was performed with the aminoethylcarbazole detection kit from Ventana Medical Systems (Beijing Biosynthesis Biotechnology, Beijing, China).

Murine model of nonmyeloablative allo-BMT

Female BALB/c recipient mice received nonmyeloablative doses (700 or 600 cGy) of TBI from a 137Co source. Mice in some groups received an HGT injection 24 h before TBI. Irradiation was followed by the infusion of 1.0 × 105 C57BL/6 BMCs i.v. with or without activated NK cells from C57BL/6 mice (5 × 106 cells i.v.). Some groups of mice received further i.p. injection of 1 μg rhIL-15 once a day from days 1 to 7. Mice received gentamicin sulfate oral suspension (Ryen Pharma, Henan, China) in drinking water starting 7 d before the transplant. Mice were monitored and weighed weekly.

Statistical analysis

One-way ANOVA was used to determine statistically significant differences between more than two experimental groups. Unpaired Student t tests were used to determine statistically significant differences between two experimental groups. Data were analyzed using GraphPad Prism 5 software for Windows (GraphPad Software, San Diego, CA). A p value <0.05 was considered statistically significant.

Results

Donor NK cell infusion and IL-15 treatment promoted donor engraftment and immune reconstitution in nonmyeloablative allo-BMT

Donor NK cells were generated from C57BL/6 (H-2b) mice and expanded in vitro. Over 95% of the cells were NK1.1+CD3− cells. To evaluate the role of donor NK cell infusion and IL-15 administration in transplantation outcomes, the recipient BALB/c (H-2d) mice that received donor bone marrow cells were also infused with donor-activated NK cells or treated with IL-15 or both. After 2 mo, engraftment of the donor hematopoietic stem cells was determined by flow cytometric analysis. The donor engraftment was dramatically enhanced with donor NK cell infusion from 0.00 to 52.44% (Fig. 1A). The IL-15 treatment also promoted the donor engraftment to the similar level (Fig. 1A). The group conditioned with both donor NK cell infusion and IL-15 administration resulted in almost complete donor engraftment.
The variations within each experimental group were shown in Fig. 1B. Therefore, the combination of donor NK cell infusion and IL-15 treatment could significantly promote donor engraftment during nonmyeloablative allo-BMT.

The immune reconstitutions were measured by flow cytometric analysis of all major cell subsets in the spleens 2 mo after nonmyeloablative allo-BMT. The results showed that spleens of the recipient mice with donor NK cell infusion and IL-15 treatment consisted predominantly of donor-derived cells, including CD4+ T cells, CD8+ T cells, B cells, NK cells, and macrophages (Fig. 1C, 1D). Donor NK cell infusion or IL-15 treatment alone could also increase the number of donor-derived cells in the host spleens compared with the group of BMT alone (Fig. 1C, 1D). These results demonstrated that donor NK cell infusion and IL-15 administration resulted in enhanced donor engraftment during nonmyeloablative allo-BMT.

To further evaluate whether host alloresponses could be suppressed by donor NK cell infusion and IL-15 treatment during nonmyeloablative allo-BMT, MLR, IL-2 production assay and killing assay against allogeneic tumor targets were performed (Fig. 2A–C). Donor NK cell infusion or IL-15 treatment alone significantly reduced the host alloreactivity in terms of allospecific proliferation, IL-2 production and killing of allogeneic tumor targets. Interestingly, reduction of host T cells for ~40% by donor NK infusion or IL-15 treatment alone (Fig. 2D) resulted in over 70% suppression of the host alloreactivity, suggesting that donor NK cell infusion or IL-15 treatment not only decreased the percentage of host T cells but also suppressed their alloreactivity. The combination treatment further reduced the host alloreactivity to the base level. The remaining 10% host T cells in the combination treatment group did not confer any alloreactivity. Taken together, these data indicated that the host alloresponse could be significantly suppressed by donor NK cell infusion and IL-15 treatment in the nonmyeloablative allo-BMT.

Donor NK cell infusion and IL-15 hydrodynamic injection promoted donor engraftment synergistically during nonmyeloablative allo-BMT

To determine whether NK cell infusion and IL-15 treatment could promote the engraftment synergistically in nonmyeloablative allo-BMT, we modified the conditioning regimen to allow for the lower percentage of donor engraftment with NK cell infusion alone. Female BALB/c recipient mice received reduced dose (600 cGy) of TBI and were given donor BMT cells with or without donor NK cell infusion. Some recipient mice also received hydrodynamic injection of plasmid DNA encoding IL-15 gene 24 h before TBI (Fig. 3A). As shown in Fig. 3B, liver tissues from mice that received hydrodynamic injection showed a higher level of IL-15 expression compared with those of control mice (Fig. 3B). Therefore, the HGT could successfully mediate the expression of IL-15 in vivo. The NK cell infusion alone only slightly enhanced donor engraftment with the reduce intensity conditioning regimen, whereas IL-15 HGT alone significantly promoted donor engraftment to ~20 to ~50% (Fig. 3C, 3D). IL-15 HGT alone also resulted in greater donor-derived reconstitution in the recipients compared to BMT alone (Fig. 3E, 3F). Furthermore, the combination treatment of NK infusion and IL-15 HGT synergistically promoted donor engraftment to >95%, and the immune reconstitutions of all cell lineages were also synergistically enhanced (Fig. 3C–F).

The host alloreactivity was further evaluated using the same method described before (Fig. 4). NK cell infusion or IL-15 HGT could significantly reduce the host alloreactivity by not only de-

![FIGURE 4](http://www.jimmunol.org/) Donor NK cell infusion and IL-15 HGT suppressed the host alloresponses during nonmyeloablative allo-BMT. On day 60 post-nonmyeloablative allo-BMT as described in Fig. 3, MLR were performed with splenocytes from host mice as responder cells and irradiated (20 Gy) splenocytes from C57BL/6 mice as stimulator cells. Responders and stimulators were cultured at a final concentration of 0.5 × 10^6/ml for 3 d. (A) Cells were pulsed with tritiated thymidine (1 μCi/well), and the proliferations of the responder cells were determined. (B) Supernatants were used to measure the IL-2 production during the MLR. (C) The responder cells from the MLR were used as effector cells, and their allokilling capacity of EL-4 (H2b) targets was measured using CytoTox 96 nonradioactive cytotoxicity assay kit. (D) The percentages of host CD4+ and CD8+ T cells were analyzed by flow cytometry. Values were expressed as mean ± SD. The experiments were performed with five mice per group. The assays were done in quadruplicates. The data shown are the representative of three experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
creasing the number of remaining host T cells but also suppressing their alloreactivity. The host T cells’ killing of syngeneic tumor targets was also measured, and they showed similar killing capacity between the different treatment groups (data not shown), suggesting that mainly the allokilling activity was suppressed. The combination treatment further suppressed the host alloreactivity to the base level. Therefore, donor NK cell infusion and IL-15 HGT synergistically promoted donor engraftment possibly through suppressing host alloresponses.

**IL-15 treatment promoted the proliferation and function of the adoptively transferred donor NK cells in vivo**

To investigate the effect of IL-15 on the adoptively transferred donor NK cells, we labeled the activated donor NK cells from congeneric CD45.1 mice with CFSE and infused during nonmyeloablative allo-BMT. Day 7 post-BMT, the proliferation of transferred donor NK cells from spleen, liver, and bone marrow were analyzed by flow cytometry. The percentages of infused donor NK cells were significantly higher with IL-15 treatment in all the organs examined (Fig. 5A). The proliferation of the transferred donor NK cells was also greatly promoted by IL-15 treatment shown by CFSE labeling and quantitative analysis of the proliferation index (Fig. 5A, 5B). The absolute numbers of adoptively transferred donor NK cells in the spleen, liver, and bone marrow were also increased in the recipient mice with IL-15 administration (Fig. 5B). These data demonstrated that IL-15 could promote the proliferation and expand the transferred donor NK cells in vivo.

To further dissect the phenotypic characteristics of the transferred NK cells, the expressions of NKG2D, NKp46, CD43, and CD11b on transferred donor NK cells were analyzed by flow cytometry day 7 post-BMT (Fig. 5C). The expressions of the activation markers, NKG2D and NKp46, on the transferred NK cells were both upregulated when combined with IL-15 HGT in vivo.

**FIGURE 5.** IL-15 treatment could promote the proliferation and function of adoptively transferred donor NK cells in vivo. Donor NK cells (CD45.1+, H-2b+) were CFSE labeled and infused during nonmyeloablative allo-BMT with or without IL-15 HGT. The experiments were performed day 7 post-BMT. (A) The FACS profiles of transferred CFSE-labeled NK cells in recipient spleen, liver, and bone marrow. (B) The absolute numbers and the proliferative index of adoptively transferred donor NK cells were calculated and shown. (C) The expressions of NKG2D, NKp46, CD43, and CD11b on the transferred NK cells in the spleen, liver, and bone marrow. (D) The production of IFN-γ by the transferred NK cells with anti-NKp46 stimulation. Values were expressed as mean ± SD. The experiments were performed with five mice per group. The data shown are the representative of three experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
the spleen and liver. The expressions of the maturation markers, CD43 and CD11b, were increased in the liver, and CD11b was also upregulated in the spleen with IL-15 HGT. To study the activation capacity of those transferred NK cells, the infused donor NK cells were sorted with CD45.1 staining and stimulated with anti-NKp46 Ab (Fig. 5D). IFN-γ production was detected by intracellular staining and NK cells sorted from the mice combined with IL-15 HGT produced significantly higher amount of IFN-γ, suggesting that when combined with IL-15 treatment, transferred NK cells could respond better to activation mediated by NKp46.

The time-course studies analyzing the percentage and number of transferred NK cells on day 7, 20, and 40 post-BMT showed that the amounts of infused NK cells were significantly higher in the spleen, liver, and bone marrow at all the time points when combined with IL-15 treatment (Fig. 6A). Even on day 40, there were still significant amount of transferred NK cells present in all three organs when combined with IL-15, whereas the transferred NK cells alone almost diminished in vivo. However, the expressions of the activation and maturation markers on the transferred NK cells were similar on day 40 with or without IL-15 treatment (Fig. 6B), except that NKG2D expression was higher in the spleen, and NKp46 was upregulated in the bone marrow when combined with IL-15 treatment. These data demonstrated that IL-15 could promote the proliferation and function of the transferred NK cells in vivo early after BMT, whereas IL-15 mainly sustained the survival of the transferred NK cells and may not have impact on their activation capacity in the later phase post-BMT.

**FIGURE 6.** The quantitative and phenotypic dynamics of the infused donor NK cells with or without IL-15 treatment. (A) The percentage and number of transferred NK cells on days 7, 20, and 40 post-BMT in the spleen, liver, and bone marrow. (B) The expressions of NKG2D, NKp46, CD43, and CD11b on the transferred NK cells in the spleen, liver, and bone marrow day 40 post-BMT. Values were expressed as mean ± SD. The experiments were performed with five mice per group. The data shown are the representative of three experiments. **p < 0.01, ***p < 0.001.
The donor bone marrow-derived NK cells were also sorted day 7 post-BMT, and the production of IFN-γ was analyzed upon anti-NKp46 stimulation (Fig. 7D). The donor bone marrow-derived NK cells sorted from the mice with IL-15 treatment produced significantly higher amount of IFN-γ upon activation. Taken together, these results suggested that NK cell infusion and IL-15 treatment could significantly promote donor bone marrow-derived NK cell development and function, which may further facilitate donor engraftment during nonmyeloablative allo-BMT.

**Discussion**

The data presented in this paper indicated that the combination treatment of donor NK cells and IL-15 could synergistically promote donor engraftment during nonmyeloablative allo-BMT. Donor NK cells infusion has been suggested as a cellular therapy in allo-BMT, and the production of IFN-γ was analyzed upon anti-NKp46 stimulation (Fig. 7D). The donor bone marrow-derived NK cells sorted from the mice with IL-15 treatment produced significantly higher amount of IFN-γ upon activation. Taken together, these results suggested that NK cell infusion and IL-15 treatment could significantly promote donor bone marrow-derived NK cell development and function, which may further facilitate donor engraftment during nonmyeloablative allo-BMT.

Clinical data suggested that engraftment of donor NK cells correlated with lessened risks of relapse but not with GVHD after nonmyeloablative conditioning in allo-BMT (3). The potential effect of donor NK cells on engraftment during nonmyeloablative allo-BMT still needs further exploration in vivo.

Our previous work demonstrated activated donor NK cells could play an important role in promoting engraftment by suppressing host alloreactive responses in a nonmyeloablative allo-BMT model (6). Despite their important roles, the lifespan and the further expansion of the transferred donor NK cells in vivo is limited (7). IL-15 is a pleiotropic cytokine that plays a crucial role in NK cells development, homeostasis, and survival (22). It shares biological functions and receptor components with IL-2. IL-2 is frequently administered after the adoptive transfer of T cells to support their in vivo survival. However, previous studies have shown that IL-2 can cause substantial systemic toxicity and promote the expansion of regulatory CD4+ T cells (23, 24). By contrast, the administration of IL-15 has proven to be well tolerated with only a minor increase in the absolute numbers of regulatory T cells (25). It is therefore important to investigate whether the combination treat-
function of promoting donor NK cell development, proliferation, and donor NK cells could induce donor T cell tolerance while promoting donor engraftment. Using the reduced intensity conditioning and IL-15 HGT, we also showed that the effect of combining two treatments was synergistic. The mechanism of this synergistic effect could be due to the interplay between donor NK cells and IL-15. Our data have demonstrated that IL-15 could promote the proliferation of transferred donor NK cells and possibly prolonged their survival in vivo (Fig. 5), resulting in increased number of donor NK cells in the recipient mice. Previous studies and our data also showed that IL-15 could also facilitate NK cell function (26, 27). Therefore, IL-15 treatment could significantly promote the effect of adoptively transferred donor NK cells to suppress host alloresponses. In contrast, IL-15 could also promote donor NK cell development during nonmyeloablative allo-BMT (Fig. 6). It could be one of the mechanisms for IL-15 to promote donor engraftment because donor-derived NK cells may exert suppressive effects on host T cells. During combination treatment, the effect of IL-15 on promoting donor engraftment could be enhanced through acting on both adoptively transferred and bone marrow-derived donor NK cells.

Our previous studies demonstrated that the suppressive effect of donor NK cells on the recipient alloreactive T cells was partially dependent on perforin-mediated killing mechanism. Recent data have demonstrated that IL-15 could augment the cytotoxic effector molecules (TRAIL and perforin) expression of NK cells (28, 29). Thus, further studies are needed to determine whether IL-15 could enhance the effects of donor NK cells on the promotion of engraftment by augmenting the cytotoxic functions of the transferred donor NK cells.

It is of interest to note that IL-15 administration may also affect T cells other than NK cells. Studies indicated that IL-15 administration posttransplantation could safely and effectively enhance the reconstitution of CD8+ T, NK, and NKT cells in recipients of an allogeneic T cell-depleted BMT (26). Promoting donor T cell reconstitution, as we have shown in the current study, by IL-15 treatment as well as donor NK cell infusion may raise the concern of causing GVHD. It has been demonstrated that donor NK cells could mediate GVHD reduction by inhibiting activated, alloreactive T cells while retaining a graft-versus-tumor effect (7). Therefore, donor NK cells could mediate donor T cell tolerance while promoting its reconstitution. IL-15 may have the same effect, through its function of promoting donor NK cell development, proliferation, and survival. However, IL-15 has been suggested to aggravate GVHD during allo-BMT (30–32). Therefore, further studies for the role of IL-15 in GVHD is warranted before its clinical administration and targeted IL-15 delivery to NK cells may be exploited in the future.

In conclusion, donor NK cell infusion and IL-15 administration could synergistically promote donor engraftment and reconstitution of all donor-derived cell subsets as well as suppress the host alloresponses in a nonmyeloablative allo-BMT model. The results provide a promising way to promote donor engraftment during allo-BMT without involving systemic and nonspecific suppression of the immune system.

Acknowledgments

We thank Dr. Xiao Yu and Lixiang Zhao (Soochow University, Suzhou, China) and members of the Haifan Liu laboratory for helpful discussions and review of this manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017


