Transplantation and tolerance

Research Article

IL-12/IL-18-preactivated donor NK cells enhance GVL effects and mitigate GvHD after allogeneic hematopoietic stem cell transplantation

Yuan Song*1,3,4, Bo Hu*1,3,4, Yonghao Liu*1,3,4, Ziqi Jin1,3,4, Yinsheng Zhang1,3,4, Dandan Lin1,3,4, Ying Zhu1,3,4, Lei Lei1,3,4, Huanle Gong1,3,4, Yu Mei2, Huey Yee Teo2, Depei Wu1,3,4 and Haiyan Liu2

1 Institute of Blood and Marrow Transplantation, Soochow University, Suzhou, China
2 Immunology Programme, Life Sciences Institute and Department of Microbiology and Immunology, National University of Singapore, Singapore, Singapore
3 Collaborative Innovation Center of Hematology, Soochow University, China
4 Jiangsu Institute of Hematology, The First Affiliated Hospital of Soochow University, Suzhou, China

Adoptive transfer of donor NK cells has the potential of mediating graft-versus-leukemia (GVL) effect while suppressing acute graft-versus-host-disease (aGVHD) during allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, these beneficial effects are limited by the transient function of adoptively transferred NK cells. Previous studies demonstrate that cytokine-induced memory-like NK cells that are preactivated by IL-12, IL-15, and IL-18 have enhanced effector functions and long life span in vivo. Here, we investigated the effects of IL-12/18-preactivated and IL-12/15/18-preactivated donor NK cells on GVL and aGVHD in a murine model of allo-HSCT. We found that both IL-12/18- and IL-12/15/18-preactivated NK cells mediated stronger GVL effect than control NK cells mainly due to their elevated activation/cytotoxicity and sustained proliferative potential. Interestingly, we observed that although both IL-12/18- and IL-12/15/18-preactivated NK cells significantly inhibited severe aGVHD, only the IL-12/18-preactivated NK cells maintained the beneficial effect of donor NK cells on mild aGVHD. The IL-12/15/18-preactivated NK cell infusion accelerated aGVHD in the fully-mismatched mild aGVHD model. Our results demonstrated that IL-12/18-preactivated NK cells displayed sustained and enhanced GVL functions, and could mitigate aGVHD despite the severity of the disease. IL-12/18-preactivated donor NK cell infusion may be an effective and safe adoptive therapy after allo-HSCT.

Keywords: GVHD · GVL · IL-15 · Memory-like · NK cells
Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is one of the most effective ways of treating leukemia and other malignant diseases [1]. During allo-HSCT, donor T cells can mediate graft versus leukemia (GVL) responses, while they can also cause graft-versus-host disease (GVHD) which is one of the major complications after allo-HSCT [2–4]. New therapeutic strategies are needed to up-regulate GVL effect while suppress GVHD responses.

NK cells represent a key component of innate lymphoid cells and provide defense against microbial infection and malignant transformation by direct cytotoxicity and cytokine production [5, 6]. Previous studies have demonstrated that donor NK cells have the potential to ablate leukemia [7–9], and reduce acute GVHD (aGVHD) by killing host antigen-presenting cells (APCs) [10] or suppressing donor alloreactive T cells [11, 12]. Additionally, animal studies demonstrate that donor NK cells can facilitate engraftment by inhibiting the alloreactivity of host T cells [13, 14]. Despite their potential beneficial role in allo-HSCT, donor NK cells derived from the hematopoietic stem cells may be functionally impaired due to their immaturity and insufficient education [15–17]. Thus, adoptively transfer of mature donor NK cells has been employed to provide the immunotherapeutic benefits for allo-HSCT. However, maintaining their proliferative potential and effector function in vivo is still a critical challenge for NK cell therapy [18].

Although traditionally considered as members of innate branch, NK cells are also shown to ‘remember’ prior certain stimulations like antigens [19, 20], cytomegalovirus [21, 22], or cytokines [23–25]. Memory-like NK cells can be generated through combined IL-12, IL-15, and IL-18 cytokine preactivation in vitro for both human and murine NK cells [23, 25]. The preactivated murine NK cells exhibit superior functionality with high IFN-γ production upon restimulation [23, 25]. Moreover, these memory-like NK cells can still be detected in vivo three months after adoptive transfer [23, 25]. Intriguingly, human NK cells have similar properties following overnight culture with IL-12, IL-15, and IL-18 [25]. Thus, cytokine-induced memory-like NK cells might be an effective strategy to ensure long-lasting NK cell effector functions in vivo. Indeed, adoptive transfer of IL-12/15/18-preactivated NK cells into tumor-bearing mice displayed potent therapeutic antitumor effects [26]. Recently, a phase I study of adoptively transferred cytokine-induced memory-like NK cells has demonstrated sustained anti-leukemia responses in patients with relapsed or refractory AML [27]. Therefore, cytokine-induced memory-like NK cells may possess long-lasting effector function in vivo, which can be harnessed in the treatment of leukemia patients. However, the safety of such therapy could be complicated by the induction of aGVHD after allo-HSCT. Adoptively transfer of donor-derived IL-15/4-1BBL-activated donor NK cells contributed to aGVHD, likely through upregulation of activating receptor expression and inflammatory cytokine production [28]. Therefore, the effect of IL-12/15/18-preactivated NK cell infusion on GVL and GVHD after allo-HSCT needs further investigation.

Recently, Huber et al. have demonstrated IL-12/15/18-preactivated NK cells decreased aGVHD lethality meanwhile maintained GVL effects [29]. Although they found these memory-like NK cells delayed tumor progression, single dose of delivery could not prolong the survival of recipient mice. However, the questions remain as to whether the increase in the frequency of NK cell infusions or other combinations of cytokine pre-treatment could enhance the GVL effect of memory-like NK cells, or whether the effect on aGVHD is dependent on its severity. We screened different combinations of cytokine treatments in vitro and found that both IL-12/18- and IL-12/15/18-preactivated NK cells had potent memory-like effector functions. We then investigated the effects of single and multiple infusions of IL-12/18- and IL-12/15/18-preactivated donor NK cells in murine allo-HSCT models. Our results demonstrated that three-time infusions of IL-12/18- and IL-12/15/18-preactivated NK cells mediated stronger GVL effect than control NK cells and prolonged survival mainly due to their elevated activation/cytotoxicity and sustained proliferative potential. Interestingly, we observed that although IL-12/15/18-preactivated NK cells significantly inhibited severe aGVHD which was consistent with the results in the study by Huber et al., only the IL-12/18-preactivated NK cells maintained the beneficial effect of donor NK cells in mild aGVHD. The adoptive transfer of IL-12/15/18-preactivated NK cells exhibited accelerated aGVHD compared with the control NK cells in the fully-mismatched mild aGVHD model. Our results suggested that while both IL-12/18- and IL-12/15/18-preactivated NK cells displayed sustained GVL functions, IL-12/15/18-preactivated NK cells could be superior in their ability to inhibit aGVHD. IL-12/18-preactivated donor NK cell infusion may be an effective and safe adoptive therapy after allo-HSCT.

Results

Both IL-12/18 and IL-12/15/18-preactivated NK cells exhibit memory-like properties in vitro

In order to determine the effects of cytokine treatments, NK cells were enriched from T-cell depleted BMCs and expanded by IL-2, then washed and treated with IL-12, IL-15, IL-18 alone or combination of IL-12/15, IL-15/18, IL-12/18, or IL-12/15/18 (Fig. 1 and Supporting Information Fig. 1). The purity of the cytokine-preactivated NK cells was comparably high (over 95%, Fig. 1A and Supporting Information Fig. 1A). NK cells treated with IL-12/18 and IL-12/15/18 had increased CD11b expression (Fig. 1B and Supporting Information Fig. 1B) and maintained the high-level expression of CD43 (Fig. 1C and Supporting Information Fig. 1C), indicating a mature status. While NKG2D and Nkp46 expressions were not greatly influenced by the cytokine treatments (Fig. 1D, E and Supporting Information Fig. 1D, 1E), CD25 expression was significantly up-regulated in IL-12/18 and IL-12/15/18-preactivated NK cells (Fig. 1F and Supporting Information Fig. 1F). The components of the cytoplasmic granules,
Figure 1. IL-12/18- and IL-12/15/18-preactivated NK cells exhibited memory-like properties with low-dose IL-12/15 stimulation. Donor derived NK cells were generated from the bone marrow cells of C57BL/6 mouse and expended by human IL-2 for 5 days. To generate cytokine preactivated NK cells, NK cells were washed and treated with the combination of IL-12/18 or IL-12/15/18. The IL-2-expanded NK cells were used as the control NK cells. For the flow cytometry assay, IL-12/18-, IL-12/15/18-preactivated NK cells and control NK cells were washed three times to remove preactivating cytokines or high concentration of IL-2 (5000 IU/mL) and rested for four days with low concentration of IL-2 (100 IU/mL). Four days later, these NK cells were restimulated with IL-12 + IL-15 for 16 h and the phenotypes were detected by flow cytometry (n = 3). (A) the purity of the NK cells. The expressions of (B) CD11b, (C) CD43, (D) NKG2D, (E) Nkp46, (F) CD25, (G) Granzyme B, (H) Perforin, (I, J) IFN-γ were detected by flow cytometry. (K) Cytotoxicity of NK cells was evaluated by killing assay. Upon restimulation, the expressions of (L) NKG2D, (M) CD25, (N) IFN-γ were detected by flow cytometry. Data shown are the representative of at least three experiments with 3 repeats for each experiment. All summary graphs display mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 determined by two-tailed unpaired Student’s t-test (A-I) or one-way ANOVA followed by Bonferroni posttest (L-N). The gating strategy used in this figure is shown on Supporting Information Fig. 4A.

The expressions of CD11b and CD43 (data not shown) of the IL-12/18- and IL-12/15/18 pretreated NK cells remained high. The expressions of the activation marker NKG2D and CD25 were up-regulated in both groups upon restimulation (Fig. 1L and M). Most strikingly, IFN-γ productions were greatly increased in both IL-12/18- and IL-12/15/18-preactivated NK cells upon restimulation (Fig. 1N). Overall, our results demonstrated that both IL-12/18-preactivated NK cells possessed similar abilities to upregulate activation marker expression and IFN-γ production with no change in cytotoxicity against tumor targets in vitro.

To further confirm whether the IL-12/18- and IL-12/15/18-preactivated NK cells could exhibit memory-like properties, the preactivated NK cells were restimulated with low dose IL-12/15 after resting for 4 days. Upon restimulation, the purity, the expressions of CD11b and CD43 (data not shown) of the IL-12/18- and IL-12/15/18 pretreated NK cells remained high. The expressions of the activation marker NKG2D and CD25 were up-regulated in both groups upon restimulation (Fig. 1L and M). Most strikingly, IFN-γ productions were greatly increased in both IL-12/18- and IL-12/15/18-preactivated NK cells upon restimulation (Fig. 1N). Overall, our results demonstrated that both IL-12/18-preactivated NK cells possessed similar abilities to upregulate activation marker expression and IFN-γ production with no change in cytotoxicity against tumor targets in vitro.

IL-12/18-preactivated donor NK cells have enhanced GVL effect after allo-HSCT

To address the GVL effect of IL-12/18-preactivated donor NK cells in vivo, we established a murine model by intravenously
Figure 2. IL-12/18- and IL-12/15/18-preactivated donor NK cells have enhanced GVL effect during allo-HSCT. The cytokine-preactivated NK cells and control NK cells were prepared according to the method described in Fig. 1. (A) Tumor burdens of recipients were measured using in vivo BLI on day 15 post transplantation to evaluate the GVL effect of single infusion of donor NK cells. On the day of transplantation, host mouse (BALB/c) received A20-luc⁺/yfp plus donor BMCs from CD45.2-C57BL/6 mouse, as well as donor IL-12/18- or IL-12/15/18-preactivated NK cells or control NK cells from CD45.1-C57BL/6 mouse. (B) Quantification of BLI images from panel A (n = 4–6). (C) The progression of leukemia was monitored by in vivo BLI on day 20 post triple injection of cytokine-preactivated NK cells. Host mouse (BALB/c) received A20-luc⁺/yfp plus donor BMCs from CD45.2-C57BL/6 mouse. donor IL-12/18- or IL-12/15/18-preactivated NK cells or control NK cells from CD45.1-C57BL/6 mouse were transferred 3 times by intravenous injection on day 0, 7 and 14. 2 mice in the group of no NK infusion died of leukemia before day 20. (D) Quantification of BLI images from panel C (n = 4–5). (E) Host mouse (BALB/c) received A20 cells and BMCs from donor mouse (CD45.2-C57BL/6) on day 0. Tumor-bearing recipients received IL-12/18- or IL-12/15/18-pretreated NK cells or control NK cells from CD45.1-C57BL/6 mouse three times on day 0, day 7 and day 14 post transplantation. The survival of recipient mice (n = 7–9 mice per group) were monitored. The data shown are the representative of three experiments. All summary graphs displayed mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 determined by one-way ANOVA followed by Bonferroni posttest (B and D), and statistical analysis of survival was performed using log-rank test (E).

Injecting luciferase (lus⁺)/yfp-expressing B-cell lymphoma (A20) cells on the day of transplantation. Control NK cells, IL-12/18- or IL-12/15/18-preactivated NK cells (5 × 10⁶ cells/mouse) were adoptively transferred into hosts on day 0. Since single infusion of human IL-12/15/18-preactivated NK cells were previously shown to exhibit enhanced GVL ability [26, 27], we next assessed tumor growth by in vivo BLI on day 15 after transplantation (Fig. 2A and B). Strikingly, despite no therapeutic effect of the adoptively transferred NK cells was observed in terms of prolonged survival, single injection of control NK cells, IL-12/18- or IL-12/15/18-preactivated NK cells indeed reduced tumor burdens. Then control NK cells, IL-12/18- or IL-12/15/18 preactivated NK cells were infused three times on day 0, day 7 and day 14 respectively post transplantation (Fig. 2C and D). We assessed the tumor progression by in vivo BLI on day 20 post transplantation. IL-12/18-preactivated NK cells significantly decreased the tumor expansion compared to control NK or no NK infusion group. IL-12/15/18-preactivated NK cell infusion showed decreased tumor growth with no significant difference compared with the control NK group. To investigate the effect of multiple infusions of NK cells on the survival of recipient mice, we also established a GVL model using A20 cells. Compared to transplant alone, donor NK cell infusion significantly prolonged the survival of recipient mice (Fig. 2E). Moreover, both IL-12/18- and IL-12/15/18-preactivated NK cells displayed more profound therapeutic effects compared with control NK cells, while IL-12/15/18-preactivated NK cells were
We examined the percentages of infused cytokine-preactivated increased proliferation and reduced apoptosis in vivo. IL-12/18-preactivated donor NK cells display activated immune phenotypes in vivo after allo-HSCT

To explore the mechanism of the anti-leukemia effects of the IL-12/18-preactivated donor NK cells in vivo, we cultured the donor NK cells from congenic CD45.1 mice and transferred them into host mice on day 0. On day 7 post transplantation, activation and maturation markers of transferred NK cells from spleen of recipient mice were analyzed by flow cytometry (Fig. 3). IL-12/18- and IL-12/15/18-preactivated NK cells expressed higher levels of maturation markers CD11b and CD43 in vivo (Fig. 3A). Both the percentages and absolute numbers of cells positive for activation markers NKG2D and CD25 of the IL-12/18- and IL-12/15/18-preactivated NK cells were significantly higher compared with control NK cells (Fig. 3A). Despite the preactivated NK cells only displayed slightly increased percentage of Nkp46, the absolute numbers of Nkp46 positive cytokine-preactivated NK cells were significantly higher than that of control NK cells (Fig. 3A).

We next assessed the effector functions of the infused donor NK cells in different organs of the recipient mice. Both IL-12/18- and IL-12/15/18-preactivated NK cells produced significantly higher level of IFN-γ in spleen, liver and lung compared with control NK cells (Fig. 3B). Additionally, IL-12/15/18-preactivated NK cells expressed significantly increased level of granzyme B and perforin in spleen, liver and lung compared with control NK cells whereas only slightly higher levels of granzyme B and perforin were detected in IL-12/18-preactivated NK with only statistical significance in the lung (Fig. 3B). To further evaluate whether the preactivated NK cells could induce enhanced cytotoxicity after adoptive transfer, we assessed the expression of extracellular CD107a on donor NK cells from spleen, liver and lung of host mice. Similar to their killing capacities analyzed before transfer (Fig. 1K), the all-transferred donor NK cells expressed similar levels of CD107a regardless of preactivation (Fig. 3C), indicating similar cytotoxicity of these NK cells in vivo. Collectively, our data demonstrated that IL-12/18-preactivated memory-like donor NK cells, similar to IL-12/15/18-preactivated NK cells, exhibited a more mature and activated phenotype in vivo after adoptive transfer. However, the individual cytotoxicity of preactivated NK cells may not be different from the control NK cells.

IL-12/18-preactivated donor NK cells display increased proliferation and reduced apoptosis in vivo

We determined the percentages of cytokine-preactivated donor NK cells and found they were significantly higher compared with those of control NK cells in spleen, liver and lung of the recipient mice (Fig. 4A). To determine whether the higher percentages of cytokine-preactivated NK cells were associated with increased proliferation, we labeled donor NK cells with CFSE before infusion (Fig. 4B). The proliferation of IL-12/18- and IL-12/15/18-preactivated NK cells was significantly increased compared with that of control NK cells in spleen, liver and lung of host mice shown by CFSE labeling and quantitative analysis of the proliferation index on day 4 post transplantation (Fig. 4B). We also investigated the rate of apoptosis among transferred donor NK cells in spleen, liver and lung of host mice (Fig. 4C). After adoptive transfer, early apoptosis of IL-12/15/18-preactivated NK cells was significantly decreased in spleen and liver, and IL-12/18-preactivated NK cells had decreased early apoptosis in liver compared with the control NK cells. Our results suggested that both the increased proliferation and reduced early apoptosis of the IL-12/18- and IL-12/15/18-preactivated NK cells might result in their higher percentages in different organs of the recipient mice thereby contributing to their therapeutic enhancement in experimental GVL models.

IL-12/18-preactivated NK cells maintain the protective effect in both severe and mild aGVHD models

To examine the role of IL-12/18- and IL-12/15/18-preactivated NK cells in aGVHD, murine aGVHD model was established with MHC-mismatched C57BL/6 donor mice and lethally irradiated BALB/c hosts. To generate the fully-mismatched mild aGVHD model, BALB/c recipient mice received lethal irradiation with a dose of 750cGy (divided into two doses of 375cGy with 4h interval). Single infusion of control NK cells and IL-12/18-preactivated NK cells on day 0 after transplantation significantly prolonged survival of recipient mice compared with BMT alone (Fig. 5A). In striking contrast, single infusion of IL-12/15/18-preactivated NK cells had no beneficial effect in suppressing aGVHD, and the survival of the hosts was significantly shortened compared with the mice that received IL-12/18-preactivated or control NK cell infusion (Fig. 5A). Mild aGVHD model was also established in haplo-identical HSCT by administrating C57BL/6 splenocytes and BMCs into B6D2F1 recipient mice (Supporting Information Fig. 2). We found that single infusion of control donor NK cells, IL-12/18- or IL-12/15/18-preactivated NK cells significantly prolonged the survival of the recipient mice with no difference among the three groups. To further evaluate the aGVHD/GVL combined effect of the preactivated NK cells in the same mild aGVHD model, we established the aGVHD+GVL model by injecting luciferase (luc+)/yfp-expressing B-cell lymphoma (A20) cells, donor splenocytes and BMCs (C57BL/6) into host mice (BALB/c). Similar to the results of the haplo-identical HSCT model, single infusion of control NK cells, IL-12/18- or IL-12/15/18-preactivated NK cells significantly prolonged the survival of the recipient mice with no difference among the three groups (Supporting Information Fig. 3). Therefore, single infusion of pre-activated NK cells after transplantation did not significantly provide survival benefit over control donor NK cells.
Figure 3. IL-12/18- and IL-12/15/18-preactivated NK cells exhibit activated phenotypes in vivo after allo-HSCT. The cytokine preactivated NK cells and control NK cells were prepared according to the method described in Fig. 1. Tumor-bearing mice (BALB/c) received control NK cells or cytokine-induced memory-like NK cells from CD45.1-C57BL/6 mouse on the day of transplantation (n = 4–5). (A) At day 7, adoptively transferred IL-12/18- or IL-12/15/18-preactivated NK cells expressed higher levels of activation and maturation markers, including NKG2D, NKp46, CD11b, CD43 and CD25 in the spleen. (B) IL-12/18 (dashed line)- or IL-12/15/18 (solid line)-preactivated NK cells produced increased levels of IFN-γ, granzyme B and perforin compared with control NK cells (dotted line) in the spleen, liver and lung of recipient mice. (C) Percentage of CD107a positive NK cells after adoptive transfer of IL-12/18- or IL-12/15/18-preactivated NK cells or control NK cells. The data shown are the representative of three experiments with 4–5 samples per experiment. All summary graphs displayed mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 determined by one-way ANOVA followed by Bonferroni post-test. The gating strategy used in this figure is shown on Supporting Information Fig. 4B.
We then performed triple infusions of donor NK cells on day 0, 7, 14 after transplantation. Three infusions of control NK cells further prevented aGVHD, while IL-12/18-preactivated NK cells were not as effective as the control NK cells, but still prolonged survival compared with BMT alone (Fig. 5B). When IL-12/15/18-preactivated NK cells were infused into host mice three times, they even had the trend to exacerbate aGVHD (Fig. 5B). These results suggest that while IL-12/18-preactivated NK cells could maintain the suppressive effect of donor NK cells on aGVHD, IL-12/15/18-preactivated NK cells may exacerbate the disease, especially when infused multiple times in fully-mismatched conditions.

In order to evaluate whether infusion of donor NK cells influenced the alloreactivity of donor T cells thereby resulted in distinct effects on aGVHD. MLR assay was performed on day 14 after transplantation (Fig. 5C). Both IL-12/18-preactivated and control NK cell infusion significantly reduced the alloresponses of donor T cells, whereas infusion of IL-12/15/18-preactivated NK cells had no effect on the alloreactivity of donor T cells (Fig. 5C), indicating the loss of the suppressive effect of the donor NK cells. We further explored the effect of the transferred NK cells on donor T cell proliferation and cytokine production. Donor splenocytes (C57BL/6) were labelled with CFSE and transferred into host mice (BALB/c). CFSE expression and cytokine production of the donor T cells were analyzed on day 7 after transplantation (Fig. 5D and E). We found that IL-12/15/18-preactivated NK cells significantly enhanced the proliferation of donor-derived CD4+ T cells compared to control NK cells or IL-12/18-preactivated NK cells in the spleen and liver (Fig. 5D). Furthermore, IL-12/15/18-preactivated NK cells could also increase the numbers of IFN-γ- or TNF-α-producing CD4+ and CD8+ T cells in the spleen in this mild aGVHD model (Fig. 5E). These results suggest that IL-12/15/18-preactivated NK cells could promote the proliferation and inflammatory cytokine production of the donor T cells, which may facilitate the onset of aGVHD.

A recent study reported that IL-12/15/18-preactivated NK cells suppressed the progress of aGVHD by inhibiting donor T cell proliferation in a murine model of allo-HSCT [29]. In their model, host mice received BMT alone all died within 7 days, which was a more severe aGVHD than our model (Fig. 5A and B). We then established a severe aGVHD model by lethal irradiation with a...
Figure 5. IL-12/18-preactivated NK cells maintain the protective effect of donor NK cells in both severe and mild aGVHD models. The cytokine preactivated NK cells and control NK cells were prepared according to the method described in Fig. 1. Donor NK cells were generated from CD45.1-C57BL/6 mouse. Splenocytes and BMCs derived from CD45.2-C57BL/6 mouse were used to establish aGVHD model for graph A to D. For graph E and F, splenocytes from CD45.1-C57BL/6 mouse and BMCs from CD45.2-C57BL/6 mouse were used to establish aGVHD. (A) The survival of recipient mice treated with single infusion of IL-12/18- or IL-12/15/18-preactivated NK cells or control NK cells in a mild aGVHD model \((n = 8)\). (B) The survival for triple infusions of IL-12/18- or IL-12/15/18-preactivated NK cells or control NK cells in a mild aGVHD model \((n = 6)\). (C) Alloreactivity of donor T cells was analyzed by MLR assay on day 14 post transplantation \((n = 3–5)\). (D and E) Host mice (BALB/c) received lethal TBI (750 cGy; 2 doses of 375cGy with 4-h interval) from a 137Cs source. CFSE-labelled splenocytes from CD45.1-C57BL/6 mouse and BMCs from CD45.2-C57BL/6 mouse were injected into host mouse to induce mild aGVHD model. 7 days later, the proliferation (D) and the cytokine production of donor T cells from the spleen, liver and lung were detected. (F) Severe aGVHD model was established and donor NK cells were adoptively transferred into hosts once on the day of transplantation. The survival of recipients was monitored \((n = 7–8)\). (G) The percentages and absolute numbers of CD4+ T cells and CD8+ T cells in the spleen and liver. The expression of T-bet on the CD4+ T cells and CD8+ T cells derived from spleen and liver \((n = 5)\). The data shown are the representative of three experiments with 3–8 samples per experiment. All summary graphs displayed mean ± SD. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) determined by one-way ANOVA followed by Bonferroni posttest (C, D, E and G), and statistical analysis of survival was done using log-rank test (A, B and F). The gating strategy used for panel G was shown on Supporting Information Fig. 4C. The gating strategy used for panel D and E is shown on Supporting Information Fig. 4D.
single dose of 750cGy, and the host mice received BMT alone died within 8 days (Fig. 5F). Donor NK cells were infused into the recipients on the day of transplantation. Consistent with the previous report, we observed that the infusion of IL-12/18- or IL-12/15/18-preactivated NK cells significantly prolonged survival of host mice compared with BMT alone or control NK cell infusion (Fig. 5F). Further analysis showed that IL-12/18- or IL-12/15/18-preactivated NK cell infusion significantly reduced the number of CD4+ and CD8+ T cells, as well as Th1 and Tc1 cells in the spleen and liver (Fig. 5G). Therefore, these results demonstrated that IL-12/18-preactivated NK cells suppressed aGVHD regardless of the severity, while IL-12/15/18-preactivated NK cells only exhibited beneficial effects in a severe aGVHD model. Therefore, infusion of IL-12/18-preactivated NK cells might be a safer and better choice for adoptive therapy after allo-HSCT.

Discussion

Although donor NK cell infusion may benefit allo-HSCT in many aspects including improvement of engraftment [13], suppression of GVHD [11, 30] and enhancement of GVL effect [7, 8, 31], all the merits of this approach are severely limited by their transient effector function after adoptive transfer [32]. In this regard, Cooper et al. [23] reported that IL-12/15/18-preactivated NK cells obtained the ability to produce increased IFN-γ upon restimulation for up to four months after adoptive transfer. Therefore, cytokine preactivation before infusion may amplify and sustain the beneficial effects of NK cells during allo-HSCT. In the current study, we found that IL-12/18-preactivated NK cells had comparable memory-like phenotypes and enhanced GVL effects as IL-12/15/18-preactivated NK cells. Although both IL-12/18- and IL-12/15/18-preactivated NK cells suppressed aGVHD in a severe aGVHD model, only IL-12/18-preactivated NK cells maintained the protective effect of donor NK cells in mild aGVHD, suggesting IL-12/18-preactivated NK cell infusion as a safer and better choice for adoptive therapy after allo-HSCT.

The enhanced antitumor activity of murine IL-12/15/18-preactivated NK cells was first demonstrated in a murine tumor model [26]. The sustained high expression of IFN-γ and perforin in these cytokine-pretreated NK cells was required for the antitumor activity. Similar to murine NK cells, human IL-12/15/18-preactivated NK cells have been recently shown to have memory-like properties, including increased IFN-γ production, enhanced proliferation and high expression of IL-2 receptor [25]. A phase I clinical trial using cytokine-induced memory-like NK cells was conducted and demonstrated these memory-like NK cells had enhanced GVL effects post allo-HSCT [27]. Previous studies demonstrated that IL-15 was not required for memory-like NK cell generation [23, 25]. Our results also demonstrated that IL-12/18-preactivated NK cells and IL-12/15/18-preactivated NK cells exhibited similar GVL effects in vivo. Consistent with previous studies [23, 26, 27, 33], we showed that IL-12/18- and IL-12/15/18-preactivated NK cells both produced high amount of IFN-γ in response to restimulation with cytokines in vitro. They displayed enhanced IFN-γ production and increased expression of granzyme B and perforin after adoptive transfer. Furthermore, we found that the expressions of NK cell activation markers, CD11b, CD43, NKG2D and Nkp46, were also enhanced in IL-12/18- and IL-12/15/18-preactivated NK cells. Despite of their activated phenotypes, IL-12/18- and IL-12/15/18-preactivated NK cells did not have increased cytotoxicity compared with control NK cells when they were examined for their killing capacity against A20 targets before (Fig. 1K) or 7 days post adoptive transfer (data not shown). However, cytokine pretreatment promoted the proliferation of NK cells in vivo since IL-12/18- and IL-12/15/18-preactivated NK cells were found at higher numbers in spleen, liver and lung of host mice after adoptive transfer (Fig. 4). The early apoptosis in vivo was also inhibited in cytokine-pretreated NK cells (Fig. 4B). Therefore, our results suggested that the enhanced effector function of IL-12/18- and IL-12/15/18-preactivated NK cells in vivo could be dependent on their increased IFN-γ production, rapid proliferation and prolonged survival.

In our murine GVL models, three infusions of IL-12/18- or IL-12/15/18-preactivated NK cells on day 0, day 7 and day 14 significantly prolonged host survival compared with IL-2 expanded control NK cells. It should be noted that we did not observe any therapeutic effects of single infusion of IL-12/18- or IL-12/15/18-preactivated NK cells on day 0 (data not shown), which was different from the results of the previous tumor studies [26, 27]. One of these studies also used IL-2-expanded NK cells for cytokine-pretreatment [26], so the discrepancy in the therapeutic effects of single infusion might not be due to the method of generating cytokine-pretreated NK cells. Recent studies proved that memory-like NK cells required the presence of CD4+ T cells expressing IL-2 to maintain their long-term antitumor activity [26, 33]. Low dose rhIL-2 was used in one study [27] and sub-lethal dose of radiation was applied in the other [26], while lethal irradiation was used in our models. The lack of IL-2 and the host CD4+ T cells in our study may result in the poor therapeutic effect of the single infusion of memory-like NK cells. This discrepancy between our results and the previous studies may indicate IL-2 indeed plays an important role in sustained effector function of cytokine-induced memory-like NK cells after adoptive transfer.

Donor NK cell infusion has been shown to inhibit aGVHD [11]. Indeed, we found that both IL-12/18- and IL-12/15/18-preactivated NK cells suppressed aGVHD in a severe aGVHD model, only IL-12/18-preactivated NK cells maintained the protective effect of donor NK cells in mild aGVHD. Our results of severe aGVHD model were in line with a recent study that revealed the suppressive effect of IL-12/15/18-preactivated NK cells in aGVHD [29]. Our results showing differential effects of IL-12/18- and IL-12/15/18-preactivated NK cells on mild aGVHD were very intriguing. In a recent clinical trial, IL-15/4-1BBL-activated donor NK cell infusion contributed to aGVHD [28]. They reported 5 transplant recipients experienced aGVHD following the NK cell infusion. There have been also reports of murine studies indicating donor NK cells may aggravate aGVHD [34–37]. Therefore, precautions need to be taken when using activated donor NK cells in allo-HSCT.
IL-12/15/18-preactivated NK cells might regulate aGVHD in at least three aspects. First, they may mediate suppression of aGVHD through the direct killing of activated donor alloreactive T cells [11, 30]. The increased binding between NKG2D and NKG2D ligand rendered donor T cells sensitive to NK cells mediated lysis [38–40]. Second, with upregulated CD25 expression, they may compete with Treg cells for IL-2 thus promote aGVHD. It has been shown that IL-12/15/18-preactivated NK cell infusion resulted in the reduction of Treg cells [29, 33]. Furthermore, previous reports showed IL-12/15/18-preactivated NK cells were resistant to the regulation by Treg cells due to their down regulation of TGF-β receptor [41]. Finally, NK cells might regulate T cell killing through the enhanced production of inflammatory cytokines [42, 43], such as IFN-γ and TNF-α, thereby augment the severity of aGVHD. Considering the possible roles of IL-12/15/18-preactivated NK cells during allo-HSCT, we propose that during the severe aGVHD, the inflammation induced by irradiation regimen significantly upregulates the expression of the activating receptors on IL-12/15/18-preactivated NK cells and the activating ligands on donor allo-reactive T cells. Therefore, IL-12/15/18-preactivated NK cells predominantly mediated the lysis of donor allo-reactive T cells to inhibit aGVHD. In contrast, in a mild aGVHD, the mild inflammation may not be able to induce high-level expression of the activating ligands on donor T cells. IL-12/15/18-preactivated NK cells might promote the proliferation and cytokine production of donor T cells through increased inflammatory cytokine release thereby augment T cell-mediated aGVHD. Indeed, our results showed that the proliferation and cytokine production of donor T cells were significantly upregulated after IL-12/15/18-preactivated NK cell infusion. However, IL-12/15/18-preactivated NK cells did not lose the beneficial effect of the donor NK cells on mild aGVHD in haplo-identical model (Supporting Information Fig. 2). This could be due to the reduced alloreactivity of the donor T cells in this model. Intriguingly, both IL-12/18- and IL-12/15/18-preactivated NK cells extended the survival of recipient mice in combined aGVHD+GVL model (Supporting Information Fig. 3). One possible mechanism is that the promoting effect of IL-12/15/18-preactivated NK cells on aGVHD could be alleviated in the presence of the tumor cells, which most of their effector molecules are targeted at. Thus, both inflammatory conditions, donor T cell alloresponses, and tumor burdens may influence the effect of IL-12/15/18-preactivated NK cells on aGVHD, which could be difficult to determine in clinical settings. Meanwhile, IL-12/18-preactivated NK cells showed protective effects on aGVHD in all models we have performed, which might be a safer choice in clinical donor NK cell infusions. The absolute numbers of NKG2D or NKp46 positive IL-12/15/18-preactivated NK cells in spleen were significantly higher than those among IL-12/18-preactivated NK cells after adoptive transfer (Fig. 3A). This difference might be associated with their sensitivity to the intensity of inflammation thereby accounted for their differential roles in aGVHD. Nevertheless, the detailed mechanism for the function of cytokine-induced memory-like donor NK cells still needs further investigation.

In summary, our results demonstrated IL-12/18- and IL-12/15/18-preactivated NK cells exhibited similar memory-like phenotypes in vitro and comparable GVL effects in vivo. Their enhanced GVL effect might be due to their increased IFN-γ production, rapid proliferation and prolonged survival. Interestingly, we found that both IL-12/18- and IL-12/15/18-preactivated NK cells suppressed aGVHD in a severe aGVHD model, only IL-12/18-preactivated NK cells maintained the protective effect of donor NK cells in all the mild aGVHD models performed. Therefore, IL-12/18-preactivated NK cells may be a safer and more therapeutically effective choice over IL-12/15/18-preactivated NK cells as the adoptive therapy during allo-HSCT.

Materials and methods

Mice

Specific pathogen free C57BL/6 (H2Kb) and BALB/c (H2Kd) mice (aged 6–8 weeks) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). CD45.1-C57BL/6 (H2Kb) mice were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All mice were female and maintained in specific pathogen free conditions and in accordance with the guidelines approved by the Institutional Laboratory Animal Care and Use Committee of Soochow University.

Cell lines

A20 (H2Kd) lymphoma cell line was purchased from American Type Culture Collection (Manassas, VA). Luciferase-expressing A20 cells were generated by lentiviral system. Stable luciferase-expressing A20 cells were sorted by flow cytometry (BD FACS Aria III, BD Bioscience, San Jose, CA), and cultured with RPMI 1640 medium supplemented with 10% FBS.

GVL and GVHD model

GVL model: BALB/c recipient mice received lethal TBI (750 cGy: 2 doses of 375cGy with 4 h interval) from a 137Cs source. Three hours later, 5 × 10⁶ bone marrow cells (BMGs) from C57BL/6 plus 1 × 10⁶ A20 lymphoma cells or 5 × 10⁶ A20-luc⁺/yfp cells were intravenously injected into lethally irradiated BALB/c recipient. Fully-mismatched mild aGVHD model: BALB/c recipient mice received lethal TBI (750 cGy: two doses of 375cGy with 4 h interval) from a 137Cs source. Three hours later, 1 × 10⁷ BMGs and 5 × 10⁶ splenocytes from C57BL/6 mice were intravenously injected into lethally irradiated BALB/c recipient. Fully-mismatched severe aGVHD model: BALB/c recipient mice received lethal TBI (one dose of 750cGy) from a 137Cs source. Three hours later, 1 × 10⁷ BMGs and 5 × 10⁶ splenocytes from C57BL/6 were intravenously injected into lethally irradiated BALB/c recipient. Haplo-identical mild aGVHD model: B6D2F1 recipient mice received lethal TBI
(925cGy) from X-Ray. Three hours later, 1 × 10^7 BMCs and 7.5 × 10^7 splenocytes from C57BL/6 were intravenously injected into lethally irradiated B6D2F1 recipient. The body weights of recipients and aGVHD clinical scores were assessed every three days. aGVHD-GVL model: BALB/c recipient mice received lethal TBI (650cGy) from X-Ray. Three hours later, 1 × 10^7 BMCs, 3 × 10^7 splenocytes from C57BL/6 and 2 × 10^6 luciferase-expressing A20 lymphoma cells were intravenously injected into lethally irradiated BALB/c recipient.

**NK cell culture and stimulation**

The NK cells were generated from the bone marrow cells of CD45.1-C57BL/6 or CD45.2-C57BL/6 mouse and cultured according to the method described previously [13]. After five days of cell culture, NK cells were harvested and washed three times with large volumes of RPMI 1640, then resuspended with RPMI 1640 plus 10% FBS. NK cells were treated with IL-12 (10 ng/ml, Peprotech, Rocky Hill, NJ) and IL-18 (50 ng/ml, MBL, Japan), or IL-12, IL-15 (10 ng/ml, Xiamen Special Treasure Biological Engineering, Xiamen, China) and IL-18 for 16 h. Then the purity and phenotype of NK cells were measured by FACS (BD Canto II, BD Bioscience, San Jose, CA). The purity of the NK cells (CD3−NK1.1+) was >95% in all groups. NK cells were washed three times with PBS before adoptive transfer. The IL-2-expended NK cells were used as control NK cells. For adoptive transfer, 5 × 10^6 NK cells were injected into each recipient mouse through the tail vein. In the experiments of restimulation, control NK cells, IL-12/15/18-pretreated NK cells were washed three times to remove preactivating cytokines. Then they were cultured in low concentration of IL-2 (100 IU/ml, Beijing Four Rings Biopharmaceutical, Beijing, China) for 4 or 8 days. Rested NK cells were restimulated with IL-12 (10 ng/ml) plus IL-15 (10 ng/ml) for 16 h then washed three times with RPMI 1640 and stained for FACS analysis (BD Canto II, BD Bioscience, San Jose, CA).

**Biomimetic imaging**

5 × 10^6 luciferase-expressing A20 lymphoma cells were adoptively transferred into lethally irradiated BALB/c mice along with bone marrow cells derived from C57BL/6 mice on the day of transplantation. For GVL effect assessment by biomimetic imaging (BLI), mice were anesthetized by injecting i.p. 10% chloral hydrate then injected i.p. with 100 μl of 150 μg/ml D-luciferin (Gold Biotechnology, St. Louis, MO). Five minutes later, mice were imaged using Xenogen, IVIS 100 Biomimetic Imaging System (Caliper Life Sciences, Hopkinton, MA) to determine the level of tumor burdens.

**CFSE labeling and in vivo proliferation analysis**

Donor NK cells were labeled with a 5 mM Cell Trace CFSE Cell Proliferation kit (Invitrogen, Waltham, MA) as described previously [13]. Cells were transferred into tumor-bearing recipient mice. Four days later, cell suspensions from spleen, liver and lung were prepared and stained. FACS analysis allowed gating on individual CFSE generations, and the proliferations of transferred donor NK cells were analyzed. The proliferation index of transferred donor NK cells was calculated using FlowJo software (Tree Star, Ashland, OR).

**Cytotoxicity assay**

NK cells (effectors) were labeled with CFSElow (0.5 μM) and A20 cells (targets) were labeled with CFSEhi (2 μM). Then effector and target cells were mixed and the primary CFSE intensity was detected at the starting point of the experiment using FACS (BD Canto II, BD Bioscience, San Jose, CA). After 4-h culture, the final CFSE intensity of the mixed cells was detected. Results are expressed as percentage of cytotoxicity, using the formula: percentage of cytotoxicity = [CFSEhi/CFSElow (0 h) − CFSEhi/CFSElow (4 h)] × 100%.

**Mixed lymphocyte reaction (MLR)**

Responder splenocytes from aGVHD recipients and stimulator splenocytes from donor BALB/c mice were made into single cell suspension. Stimulator cells were treated with mitomycin C (50 μg/ml, Sigma, St. Louis, MO) for 30 min at 37°C and washed three times with RPMI 1640. Then responders and stimulators were cocultured for 3 days at a final concentration of 0.5 × 10^6/ml, pulsed with tritiated thymidine (1 μCi/well), (Shanghai Institute of Physics, Chinese Academy of Sciences, Shanghai, China) 16–18 h prior to harvest, and counted on a beta plate reader (Perkin Elmer Instruments, Meriden, CT).

**Flow cytometric analysis**

Single cell suspensions from spleen, liver and lung were obtained according to the methods previously described [44] and analyzed using flow cytometry. The antibodies used: PE-CF594-anti-mouse-CD3e (145-2C11), PerCP-Cy5.5-anti-mouse-CD45.1 (A20), APC-Cy7-anti-mouse-CD11b (M1/70), PerCP-Cy5.5-anti-mouse-NK1.1 (PK136) were purchased from BD Bioscience (San Diego, CA); purified anti-mouse-CD16/32 (93), APC-anti-mouse-CD43 (S11), PE-anti-mouse-NKp46 (29A1.4), APC-anti-mouse-CD25 (PC61), FITC-anti-mouse-NKG2D (C7), PE-anti-mouse-IFN-gamma (XMG1.2), PE/Cy7-anti-mouse TNF-α (MP6-XT22) were purchased from Biologic (San Diego, CA); PE-Cy7-anti-mouse-Granlyme B (NGZB), APC-anti-mouse-Perforin (eBioOMAK-D) were purchased from ebioscience (San Diego, CA). Flow cytometric analysis were performed using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA) and the Flowjo software (Tree Star, Ashland, OR). The gating strategy used in this paper was displayed on Supporting Information Fig. 4.
Statistical analysis

One-way ANOVA was used to determine statistically significant differences among 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). P value < 0.05 was considered statistically significant (*), less than 0.01 or 0.001 was shown as ** or *** respectively.

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References

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Abbreviations: aGVHD: acute GVHD · Allo-HSCT: allogeneic hematopoietic stem cell transplantation · APCs: antigen-presenting cells · BMCs: bone marrow cells · GVHD: graft-versus-host disease · GVL: graft versus leukemia

Full correspondence: Dr. Haiyan Liu, Immunology Programme, Life Sciences Institute and Department of Microbiology and Immunology, National University of Singapore, Singapore 117456, Singapore
Fax: +65-67782684
e-mail: micliuh@nus.edu.sg

Additional correspondence: Dr. Depei Wu, Institute of Blood and Marrow Transplantation, Department of Hematology, Jiangsu Institute of Hematology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, P. R. China
e-mail: wudepei@medmail.com.cn

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