Biol Blood Marrow Transplant 25 (2019) 1–11

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Biology

Quantity and Quality Reconstitution of NKG2A$^+$ Natural Killer Cells Are Associated with Graft-versus-Host Disease after Allogeneic Hematopoietic Cell Transplantation

Li-Juan Hu[1], Xiang-Yu Zhao[1], Xing-Xing Yu[1,2], Meng Lv[1], Ting-Ting Han[1], Wei Han[1], Xiao-Jun Huang[1,2,*]

[1] Beijing Key Laboratory of Hematopoietic Stem Cell Transplantation, Peking University People's Hospital & Peking University Institute of Hematology, Beijing, China
[2] Peking-Tsinghua Center for Life Sciences, Beijing, China

Article history:
Received 16 May 2018
Accepted 6 August 2018

Key Words:
Haplo-HSCT
GVHD
NK cells
NKG2A
T cells

A B S T R A C T

The immune mechanism underlying graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (HSCT) remains unclear. Natural killer (NK) cells play a crucial role in mediating pathogen-specific immunity and are the first donor-derived lymphocytes reconstituted post-HSCT. However, NK cells vary at different stages after HSCT. Here, we found that the absolute NKG2A$^+$ subset cell counts and the percentages of NKG2A$^+$ among NK cells were significantly reduced in GVHD patients after HSCT compared with those from non-GVHD patients. Moreover, the reduction in NKG2A$^+$ NK cells in post-HSCT GVHD patients was ascribed to increased apoptosis and a decreased proliferation capacity while retaining a strong graft-versus-leukemia effect. In vitro assays showed that co-culture of T cells with NKG2A$^+$ NK cells significantly reduced IFN-γ secretion by T cells and increased IL-4 secretion. Moreover, the CD25 expression level was decreased, whereas the number of cells with the CD4$^+$CD25$^+$FOXP3$^+$ phenotype was increased. In addition, the NKG2A$^+$ NK cells induced T cell apoptosis and decreased T cell proliferation during the co-culture process. Importantly, NKG2A$^+$ NK cells mainly regulated activated but not resting T cells. In vivo assays showed that the serologic IL-10 level was evidently lower in GVHD than in non-GVHD patients, whereas the IL-1β, IFN-γ, and tumor necrosis factor-α levels were higher in GVHD patients. Furthermore, the NKG2A$^+$ NK cell ratio from GVHD patients was markedly increased by the presence of exogenous IL-10 but not by other cytokines. In contrast, the NKG2A$^+$ cell ratio from non-GVHD patients was not increased by IL-10. Therefore, post-HSCT GVHD may be ascribed to the reduced induction of NKG2A$^+$ NK cells by IL-10, which further overactivates T cells.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment strategy for hematologic malignancies [1-6]. However, graft-versus-host disease (GVHD) is a common complication after allo-HSCT [7-10], resulting from the activation, amplification, and secretion of numerous inflammatory factors related to donor alloreactive T cells that damage host tissues and organs, mainly in the gastrointestinal tract, liver, and skin [11]. Notably, natural killer (NK) cells represent the first donor-derived lymphocyte population to recover after allo-HSCT and generally are observed within the first month after allo-HSCT [12]. NK cells mediate a strong graft-versus-leukemia (GVL) effect and play an important role in GVHD [13]. Previous studies have demonstrated that donor NK cells have the potential to reduce acute GVHD (aGVHD) by killing host antigen-presenting cells [14]. Adoptively transferred donor NK cells may exert a GVL effect while suppressing aGVHD during allo-HSCT [15]. Our previous study showed that patients with higher T/NK cell ratios were associated with high incidence rates of aGVHD and chronic GVHD after transplantation [16]. These in vivo findings revealed an important role for NK cells in GVHD. However, because of their heterogeneity, NK cell subsets vary at each stage after HSCT. Importantly, the specific function of each NK cell subset in GVHD development is unclear.

NK cells express a series of immune receptors that identify relevant ligands on target cells and maintain the immune balance between NK cell activation and tolerance [17]. NKG2A, which is an inhibitory receptor belonging to the C-type lectin-like superfamily, recognizes HLA-E, which is a nonclassic MHC class I molecule. Early reconstituted NK cells exhibit a more immature phenotype and express NKG2A at a level of

Financial disclosure: See Acknowledgments on page 10.

* Correspondence and reprint requests: Xiao-Jun Huang, Peking University People's Hospital, Peking University Institute of Hematology, No.11 Xizhimen South Street, Beijing 100044, China.
E-mail address: huangxiaojun@bjmu.edu.cn (X.-J. Huang).

https://doi.org/10.1016/j.bbmt.2018.08.008
1083-8791/

approximately 90% compared with the approximately 50% level expressed in healthy donors [18]. Therefore, NKG2A$^+$ NK cells have been proposed to play a crucial role during the early stage of GVHD after transplantation. Previous studies have shown that the number of NKG2A$^+$ cells is decreased in patients with chronic GHVD after HSCT [19]. However, the relationship between NKG2A$^+$ NK cells and aGVHD has not been characterized. In addition, the role of NKG2A$^+$ cells in aGVHD disease progression and the mechanism underlying NKG2A$^+$ cell immunoregulation have not been clearly explained.

In this study we used peripheral blood from GVHD and non-GVHD paired specimens and healthy donors to address the underlying mechanism by which NKG2A$^+$ NK cells regulate T cells after HSCT.

METHODS

Study Design

Two patient cohort studies, including a prospective cohort (A) and a paired cohort (B), were included herein. Cohort A included 59 consecutive patients aged 15 to 60 years with acute myeloid leukemia, acute lymphoid leukemia, chronic myeloid leukemia, or myelodysplastic syndrome who underwent non-T cell–depleted haploidentical HSCT from May 2015 to May 2017 at Peking University Institute of Hematology. The exclusion criteria included severe heart, kidney, or liver disease; HLA-matched transplants; and second transplantation. The characteristics of the 59 patients are presented in Table 1. In addition, in the first evaluation of the prospective cohort (cohort A) at day 30 post-HSCT, none of the enrolled patients exhibited aGVHD. According to the subsequent status of GVHD occurrence or nonoccurrence after day 30 post-transplantation, patients were grouped into the with or without aGVHD groups.

Cohort B enrolled 3 subsets for the in vitro assays: GVHD subjects, non-GVHD subjects, and donors. Transplant recipients were identified from subjects (n = 180) who had acute leukemia and received a haploidentical HSCT from April 19, 2017 to August 30, 2017 and from December 1 to 31, 2017 at Peking University Institute of Hematology. Fifteen patients who developed GVHD and 15 cases of non-GVHD patients were enrolled. All 30 patients were acute leukemia patients in their first complete remission [20], including acute lymphoid leukemia and acute myeloid leukemia. The group-matching criteria included age at HSCT (±5 years), pre-HSCT chemotherapy cycles (±1 cycle), HSCT disease status, and post-transplant time (±1 day) [21,22]. All specimens were collected before steroid treatment. The use of immunosuppressive drugs for GVHD prophylaxis was the same in all patients, and no steroids were used. None of the clinical characteristics, including a history of Epstein-Barr virus or cytomegalovirus infection and antiviral therapy with ganciclovir, showed significant differences between the GVHD and non-GVHD subjects (Table 2).

This study was approved by the Clinical Ethics Review Committee at Peking University Institute of Hematology and was performed in accordance with the Declaration of Helsinki. Donors and recipients provided written informed consent before entry into the study.

Table 1
Patient Characteristics from the Prospective Study (Cohort A)

Variable	GVHD Group	Non-GVHD Group	*P*
Gender of patients, male/female	20/11	16/12	.5620
Median age at transplantation, yr (range)	31 (15-48)	35 (15-63)	.3110
Diagnosis, n			.1244
Acute myeloid leukemia	16	13	
Acute lymphoblastic leukemia	13	7	
Chronic myelocytic leukemia	0	1	
Myelodysplastic syndrome	2	7	
Disease status at transplantation, n			.8769
Standard risk	27	24	
High risk	4	4	
Conditioning, n			.1300
BU/CY	0	2	
BU/CY+ATG	31	26	
HLA-A, -B, -DR mismatched, n			
3/6	18	18	
4/6	1	2	
5/6	7	3	
Donor–recipient sex matched, n			.3536
Female–male	4	6	
Others	22	17	
Donor–recipient relationships, n			.6229
Father	12	8	
Sibling	7	6	
Child	7	9	
Donor–recipient blood type, n			.3624
Matched	15	14	
Major mismatched	3	3	
Minor mismatched	8	4	
Major and minor mismatched	0	2	
aGVHD grade, n			
I-II	27		
III-IV	4		
Median onset of aGVHD, days (range)	29 (12-64)		
Organ involvement of GVHD, n			
Skin	16		
Intestinal	11		
Skin and intestinal	4		
Donor/recipient CMV serostatus +/+, n	31/31	28/28	1.0000
History of CMV reactivation, n	23	19	.5915
CMV reactivation treated with ganciclovir, n	20	17	.7133
Steroid administration, n	16	13	.6908
Immunosuppressant, n	31	28	1.0000

BU indicates busulfan; CY, cyclophosphamide; ATG, antithymocyte globulin; CMV, cytomegalovirus.

Transplantation Protocols

Donor selection, HLA typing, graft harvesting, and conditioning therapy were performed as previously reported [5,20,22-25]. GVHD prophylaxis mainly combined methotrexate, cyclosporine A, and mycophenolate mofetil. Cyclosporine A (2.5 mg/kg, every 12 hours, iv) was used from day −9, and the trough concentration was adjusted to 150 to 250 ng/mL. This regimen was switched to oral administration when the patient's bowel function returned to normal. From day −9, .5 g of mycophenolate mofetil was administered orally every 12 hours, tapered to half until day +60, and discontinued thereafter. After graft infusion a dose of 15 mg/m^2 of methotrexate was administered iv on day +1, and a dose of 10 mg/m^2 was administered on days +3, +6, and +11 [26].

Sample Collection

For prospective cohort A patient blood samples were collected on days 30 and 90 after allo-HSCT. Patients suffering from aGVHD within 90 days after transplantation were assigned to the aGVHD group, whereas those without any signs of aGVHD were identified as non-GVHD patients. For the paired GVHD and non-GVHD patients, patients were considered "with aGVHD" or "without aGVHD" based on clinical findings at each blood collection time point. All patients were alive at the time when their peripheral blood was collected. The evaluation of aGVHD was performed at weekly intervals by applying the modified Keystone and National Institutes of Health criteria [27,28]. Peripheral blood mononuclear cells (PBMCs) from each sample were freshly isolated by density gradient centrifugation (Pancoll human; Pan-Biotech Tianjin, China) and analyzed by flow cytometry (fluorescent activated cell sorter) within 24 hours.

Flow Cytometric Analyses

Surface marker staining for the following markers was performed in PBS at 4°C for 30 minutes following Fc blocking: CD3 (Becton Dickinson, UCHT1), CD56 (Becton Dickinson, NKCM16), CD4 (Becton Dickinson, L200), CD8 (Becton Dickinson, RPA-T8 CA, USA), CD335 (NKp46) (Becton Dickinson, 9E2/NKp46), CD16 (Becton Dickinson, 3G8), CD57 (Becton Dickinson, NK-1), DNAM1 (Becton Dickinson, DX11), NKG2C (CD159c) (Milteny Biotec, REA205 Germany), NKG2A (CD159a) (Milteny Biotec, REA110), NKG2D (Becton Dickinson, 1D11), Bcl-2 (Becton Dickinson, Bcl-2/100), Ki-67 (Becton Dickinson, 35/Ki-67), and CD25 (Becton Dickinson, 2A3). Intracellular cytokine and FOXP3 staining were performed in Cytoperm buffer (Becton Dickinson) with monoclonal antibodies against IL-2 (eBioscience, MQ1-17H12), IL-4 (eBioscience, 8D4-8), IFN-γ (Becton Dickinson, B27), and FOXP3 (eBioscience, PCH101 CA, USA).

Cytotoxicity Assays

PBMCs were cultured in RPMI medium with 10% FCS supplemented with 1000 IU/mL of IL-2 (Beijing Double-Crane Pharmaceutical Co., Ltd., Beijing, China) for 10 to 14 hours for use in both the spontaneous and IL-2-stimulated NK cytotoxicity assays. The cytotoxicity of NK cells was determined using CD107α expression against the MHC class I–deficient human

Table 2
Patient Characteristics from the Paired Cohort Study (Cohort B)

Variable	GVHD Group	Non-GVHD Group	*P*
Gender of patients, male/female	12/3	9/6	.2320
Median age at transplantation, yr (range)	31 (7-55)	31 (6-56)	.9050
Diagnosis, n			.1244
Acute myeloid leukemia	9	10	
Acute lymphoblastic leukemia	6	5	
Disease status at transplantation, n			1.0000
Standard risk	15	15	
Conditioning			1.0000
BU/CY+ATG	15	15	
HLA-A, -B, -DR mismatched, n			1.0000
3/6	14	14	
5/6	1	1	
Donor–recipient sex matched, n			.5428
Female–male	1	2	
Others	14	13	
Donor–recipient relationships, n			.1347
Father	8	5	
Sibling	2	7	
Child	5	3	
Donor–recipient blood type, n			.4655
Matched	10	9	
Major mismatched	2	0	
Minor mismatched	2	4	
Major and minor mismatched	1	2	
aGVHD grade			
I-II	11		
III-IV	4		
Median onset of aGVHD, days (range)	28 (23-36)		
Organ involvement of GVHD, n			
Skin	6		
Intestinal	5		
Skin and intestinal	4		
Donor/recipient CMV positive, n	15/15	15/15	1.0000
History of CMV reactivation, n	7	8	1.0000
CMV reactivation treated with ganciclovir, n	7	6	.7047
Steroid administration, n	0	0	1.0000
Immunosuppressant, n	15	15	1.0000

erythroleukemia K562 cell line as a target at an effector-to-target ratio of 5:1 for 4 hours. GolgiStop (.7 μL/mL; BD Biosciences) was added after 1 hour. These cells were not tested for mycoplasma. The percentages of CD107α expression and IFN-γ production by the NK and NKG2A$^+$ cells were measured using the Pharmingen Intracellular Staining kit (BD Pharmingen, San Diego, CA).

Suppression Assays

NK cells, NKG2A$^+$ NK cells, and CD3$^+$CD56$^-$ T cells were purified by flow cytometry–based sorting (BD Aria). T cells from healthy donors were stimulated at a density of 1×10^5 cells per well with Human T-Activator CD3/CD28 Dynabeads (Gibco) in the presence or absence of sorted autologous NK or NKG2A$^+$ cells from GVHD-negative patients at a 1:1 ratio of NK cells or NKG2A$^+$ cells to T cells. Cytokine suppression was determined by analysis of intracellular cytokine staining of CD4$^+$ and CD8$^+$ T cells stimulated for 72 hours with Human T-Activator CD3/CD28 Dynabeads in the presence or absence of autologous purified NK or NKG2A$^+$ cells. The T cells were restimulated with Cell Stimulation Cocktail (00-4975; eBioscience) for 5 to 6 hours. After surface staining the cells were fixed with Cytofix/Cytoperm buffer (Becton Dickinson). Intracellular cytokine staining was performed in Cytoperm buffer with monoclonal antibodies against IL-4 (8D4-8; eBioscience) and IFN-γ (B27; Becton Dickinson). Samples were acquired on the FACSCanto II (Becton Dickinson), and data were analyzed with the FlowJo software.

Statistical Considerations

Statistical significance was determined by 2-way analysis of variance for comparisons among subsets. Subject variables were compared using the Mann-Whitney U test for continuous variables. Significance is indicated within the figures, and n.s. indicates nonsignificant differences ($P > .05$). The analyses were performed using GraphPad Prism 6.0, and $P < .05$ were considered significant.

RESULTS

NKG2A$^+$ NK Cells Were Significantly Reduced in GVHD Patients after HSCT

In cohort A we collected peripheral blood samples from patients on days 30 and 90 after haploidentical HSCT to investigate the NK cell reconstitution phenotype after HSCT and to detect quantitative changes in NK cells using flow cytometry. None of the patients exhibited aGVHD at day 30. According to the subsequent GVHD status (the occurrence or not of GVHD by day 30 post-transplant), patients were grouped into the with or without aGVHD groups. The NK cell percentages and absolute cell counts in the GVHD patients at day 30 were significantly lower than those in the patients without GVHD (Figure 1A,B). We also analyzed the expression of other NK cell receptors (NKG2A, NKG2D, CD25, CD122, DNAM1, NKp30, and NKp46) and found that the absolute cell counts of NKG2A$^+$ and the percentage of NKG2A$^+$ among NK cells were significantly reduced in the GVHD group (Figure 1C,D). In addition, NKp46 expression was significantly reduced in the GVHD group, but no differences in the NKp30, NKG2D, CD25, CD122, and DNAM1 expression levels in NK cells were observed between the GVHD and non-GVHD specimens (Supplementary Figure S1). However, analysis of the day 90 samples showed no significant differences between the NK and NKG2A$^+$ NK cells (Figure 1E-H) or in other molecular phenotypes between the GVHD and non-GVHD groups (Supplementary Figure S2). In addition, we analyzed the NKG2D, CD25, CD122, DNAM1, NKp30, and NKp46 expression levels in NKG2A$^+$ NK cells from the day 30 and 90 samples but found no significant differences between the GVHD and non-GVHD groups (Supplementary Figures S3 and S4).

To verify the changes in NK cells and the NKG2A$^+$ subset in patients with GVHD after HSCT, we performed an in vivo assay using paired specimens from the GVHD and non-GVHD patients in cohort B. Compared with those in the non-GVHD group, the percentages and absolute cell counts of the NK and NKG2A$^+$ subset cells were significantly decreased in the GVHD patients (Figure 2). Moreover, the CD56^{bright} cells were significantly reduced but the CD56^{dim} cells exhibited no significant changes in the GVHD patients. Furthermore, the percentage of NKG2A$^+$ NK cells among the CD56^{dim} cells was obviously decreased, but no significant difference was observed among the CD56^{bright} cells.

The expression of other receptors and molecules (CD122, DNAM1, NKG2D, NKp46, NKG2C, and CD57) in the NK cells was comparable between the 2 subsets (Supplementary Figure S5). This finding was identical to the results obtained for the prospective cohort A. Importantly, the numbers and absolute cell counts of the NK and NKG2A$^+$ NK cells were increased in the GVHD patients in complete recovery compared with those of the GVHD patients in partial recovery or the active stage (Supplementary Figure S6).

As reported, NK cells comprise 2 main subsets, CD56^{bright} and CD56^{dim} cells [29,30]. We analyzed the distribution of CD56^{bright} and CD56^{dim} cells and the percentages of NKG2A$^+$ NK cells among the CD56$^{bright/dim}$ cells in the 2 cohorts. We found that the percentage of CD56^{bright} cells was significantly reduced in the GVHD patients, whereas the absolute cell counts of CD56^{dim} cells exhibited no significant changes (Figure 3A-D). These results were consistent with those reported in previous studies. Furthermore, the percentage of NKG2A$^+$ NK cells among the CD56^{dim} cells was obviously decreased, but no significant difference was observed among the CD56^{bright} cells (Figure 3E-H). Approximately 90% of the CD56^{bright} cells of GVHD and non-GVHD patients expressed

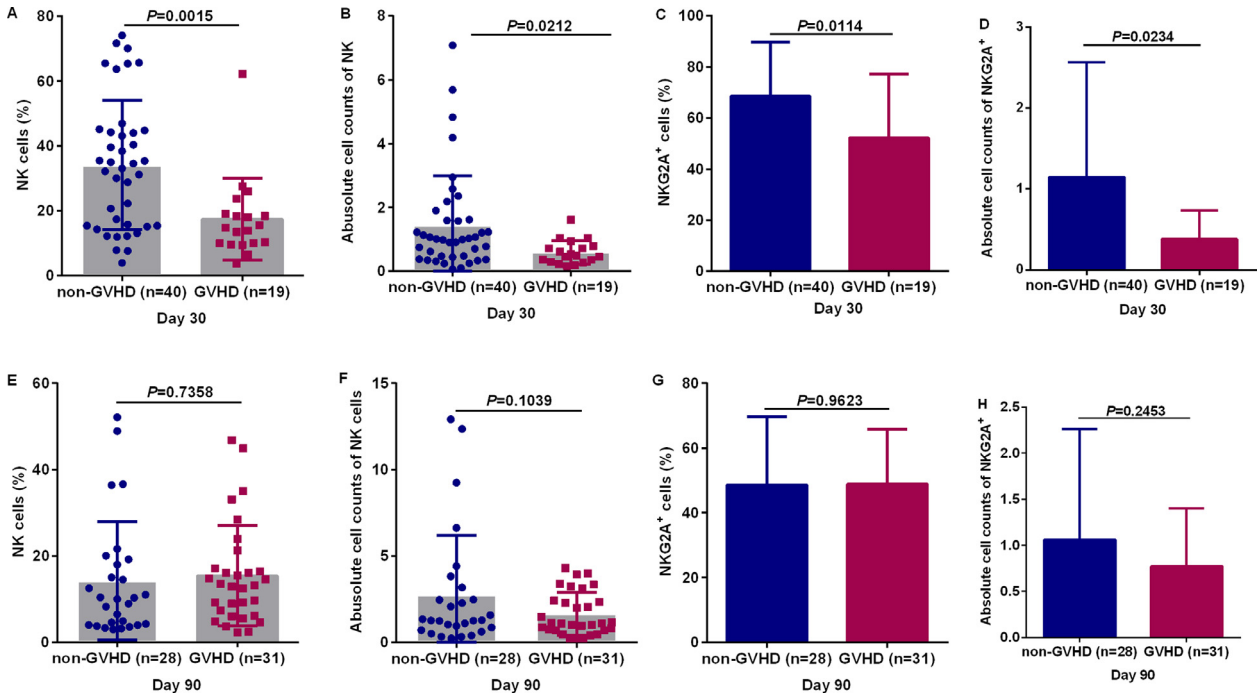

Figure 1. Comparison of $CD3^{-}CD56^{+}$ and $NKG2A^{+}$ NK cells in patients suffering from aGVHD versus patients without events. In prospective cohort A peripheral blood was collected from the patients at days 30 and 90 post-HSCT and subjected to analysis of reconstitution of $CD3^{-}CD56^{+}$ NK cells and other NK cell ratio phenotypes using flow cytometry. On day 30 post-HSCT the aGVHD patients exhibited significantly lower NK and $NKG2A^{+}$ NK cell levels than the non-GVHD patients at both the percentage level (A and C) ($P < .05$) and the absolute cell count level (B and D) ($P < .05$). On day 90 post-HSCT the NK cell ratios and absolute cell counts were similar between the aGVHD and non-GVHD subgroups (E and F). The $NKG2A^{+}$ subset among the NK cells and the absolute cell counts were comparable between the aGVHD and non-GVHD subgroups (G and H).

the NKG2A receptor. In conclusion, our results not only describe the relationship between $CD56^{bright/dim}$ cells and GVHD but also further demonstrate the distribution of NKG2A cells among the 2 subgroups in the GVHD group, which is improvement over existing reports.

$NKG2A^{+}$ NK Cells in GVHD Patients Showed Decreased Proliferation Levels While Retaining a Strong GVL Effect

Next, we cultured the PBMCs extracted from the paired specimens to detect $NKG2A^{+}$ subset cell proliferation. $NKG2A^{+}$ cell proliferation was decreased in the GVHD patients (Figure 4A,B). We also investigated changes in the killing ability of $NKG2A^{+}$ subset cells in these 2 groups. After co-culture of PBMCs with the leukemia cell line K562 for 4 to 6 hours, the cytokine IFN-γ level secreted by the $NKG2A^{+}$ cells was not decreased in the GVHD patients compared with the level in the non-GVHD patients (Figure 4C,D), and no significant difference was found in CD107α expression (Figure 4E,F). These results suggested that the killing ability of $NKG2A^{+}$ cells from the GVHD patients did not weaken and maintained a strong GVL effect.

$NKG2A^{+}$ NK Cells Inhibited Activation of Alloreactive T Cells In Vitro

The ability of $NKG2A^{+}$ cells to suppress alloreactive T cells suggested that patients with $NKG2A^{+}$ cells might have reduced immune rejection responses. To verify the effect of $NKG2A^{+}$ subsets on T cells, NK and $NKG2A^{+}$ cells sorted from the non-GVHD patients were co-cultured with $CD3^{+}$ T cells sorted from the donors. Then, we evaluated the killing effect of $NKG2A^{+}$ cells on resting and activated T cells after co-culture for 4 to 6 hours. We found that apoptosis was increased in the activated T cell and Ki-67 expression was decreased, whereas these parameters were unchanged in the resting T cells (Figure 5). To investigate the inhibitory effect of $NKG2A^{+}$ cells on T cells, cytokine secretion from T cells was examined after co-culture for 72 hours. Compared with the levels in singly cultured T cells, the IFN-γ cytokine levels secreted from the co-cultured T cells were significantly reduced, and IL-4 expression was increased (Figure 6). Moreover, CD25 expression was decreased, and the number of cells with the $CD4^{+}CD25^{+}FOXP3^{+}$ phenotype was increased (Figure 7). Importantly, the $NKG2A^{+}$ mainly regulated activated T cells but not resting T cells. These results suggested that the $NKG2A^{+}$ NK cell subset induced T cell apoptosis and decreased T cell proliferation. In addition, the $NKG2A^{+}$ NK cells suppressed cytokine secretion by T cells and inhibited T cell activation.

IL-10 Level Was Positively Correlated with NKG2A Expression Status

We also evaluated the cytokine expression status in the sera of the paired GVHD and non-GVHD specimens. The IL-10 level was significantly lower in the GVHD patient sera than in the non-GVHD patient sera, whereas the IL-1β, IFN-γ, and tumor necrosis factor-α levels were higher in the GVHD group (Figure 8A). In addition, when the GVHD patients were in complete recovery, the IL-1β, IFN-γ, and tumor necrosis factor-α expression levels were decreased and the IL-10 level was increased in the active GVHD samples (Figure 8B). Because NK cell development depends on cytokines, we analyzed whether changes in IL-10 affected the development of NK cells and the $NKG2A^{+}$ cell subset. We subsequently preincubated PBMCs from GVHD and non-GVHD patients with IL-2, IL-10, or IL-15 stimulation for 72 hours to evaluate the possibility that NKG2A expression was regulated by IL-2, IL-10, or IL-15. We found that the percentages of NKG2A NK cells from GVHD patients

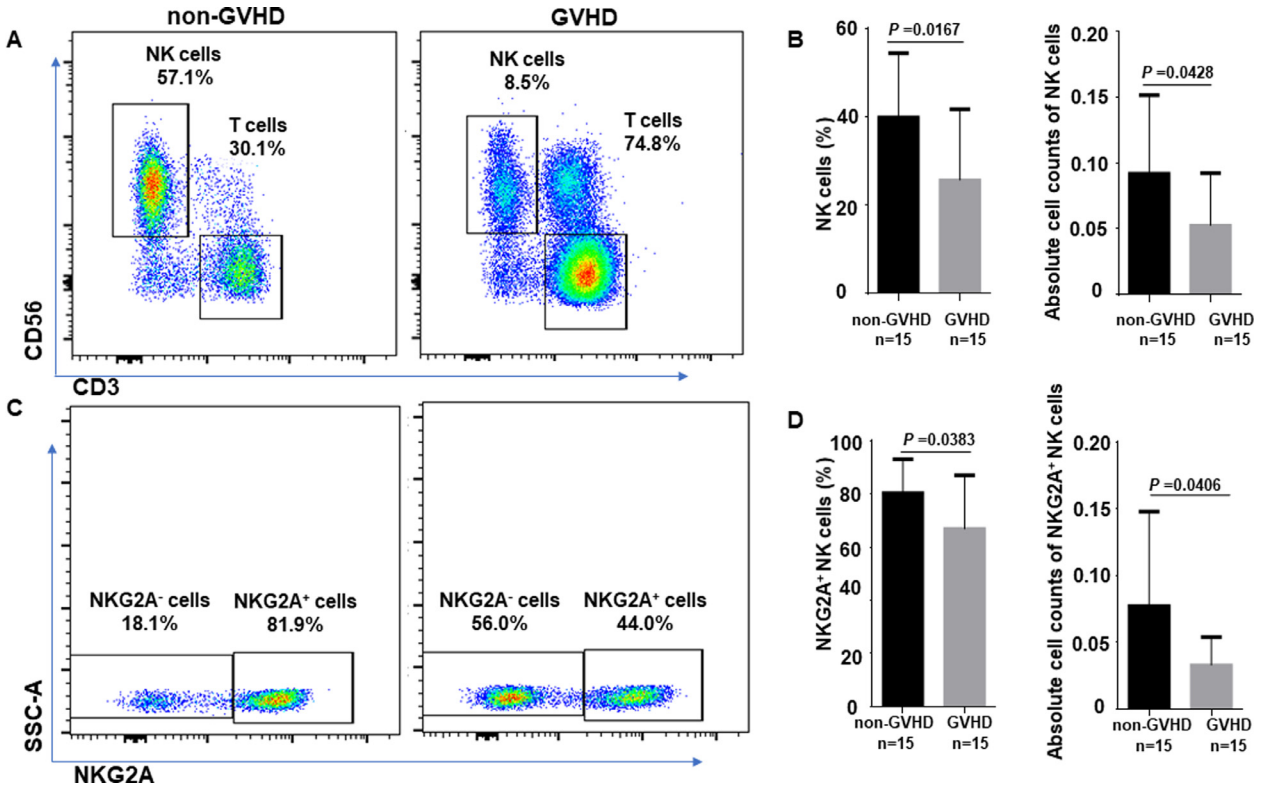

Figure 2. The frequency and absolute cell counts of NK and NKG2A$^+$ NK subset cells were decreased in GVHD patients. In independent cohort B peripheral blood was collected from post-HSCT GVHD and non-GVHD patients and subjected to analysis of NK cell reconstitution using flow cytometry. (A) Flow cytometry revealed that the percentage of NK cells was lower in the GVHD patients than in the non-GVHD patients. (B) The percentage and absolute NK cell count were significantly lower in the GVHD patients (n = 15) than in the non-GVHD patients (n = 15, $P < .05$). (C) Flow cytometry analysis revealed that the percentage of NKG2A+ subset cells among NK cells was lower in the GVHD patients than in the non-GVHD patients. (D) The absolute NKG2A+ subset cell count and percentage among NK cells were significantly lower in the GVHD patients (n = 15) than in the non-GVHD patients (n = 15, $P < .05$).

were markedly upregulated by the presence of exogenous IL-10 but not by the other cytokines (Figure 9A). In addition, no significant differences were observed in these cytokines in the non-GVHD specimens (Figure 9B).

DISCUSSION

Our results presented herein demonstrated a novel finding that the quantity and quality of NKG2A$^+$ NK cells reconstituted after allo-HSCT were associated with GVHD. In vitro

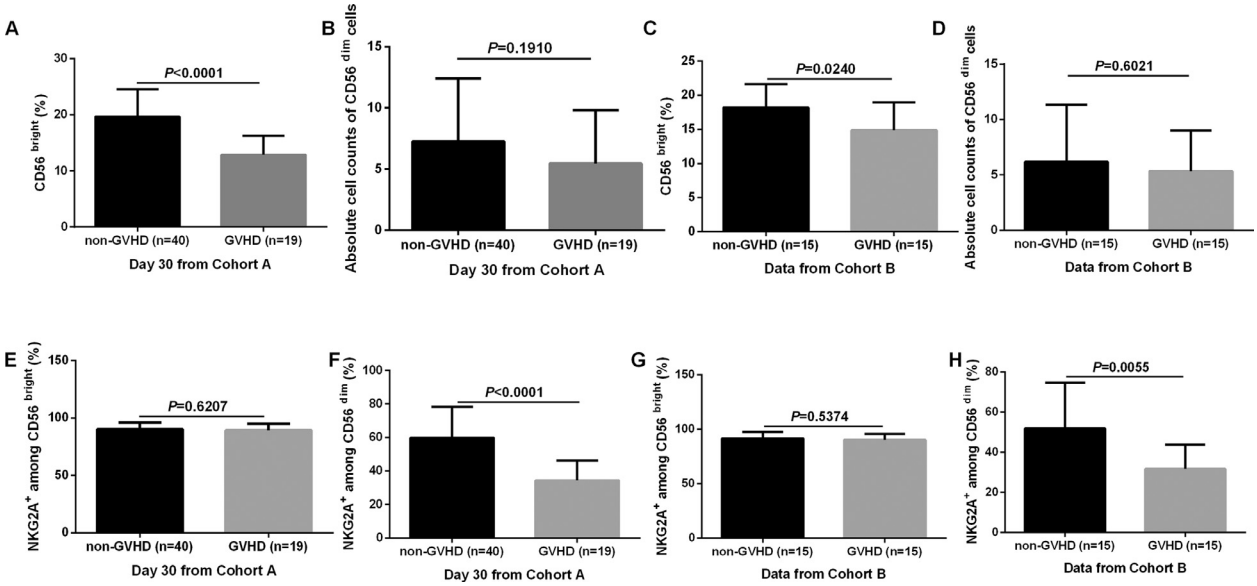

Figure 3. The distribution of CD56^{bright}, CD56^{dim}, and percentage of NKG2A$^+$ NK cells among CD56$^{bright/dim}$ cells in GVHD patients. We divided NK cells into 2 subpopulations, CD56^{bright} and CD56^{dim} cells, and in cohort A we analyzed the data from patients on day 30 post-HSCT. (A and C) Data from the 2 cohorts showed that the aGVHD patients exhibited significantly lower CD56^{bright} than the non-GVHD patients at the percentage level form ($P < .05$), but the absolute cell counts of CD56^{dim} cells had no significant changes ($P > .05$) (B and D). (E and G) Data from the 2 cohorts showed that compared with non-GVHD patients, the percentage of NKG2A$^+$ cells among CD56^{bright} cells in GVHD patients had no significant changes ($P > .05$), but it was decreased obviously among CD^{dim} cells ($P < .05$) (F and H).

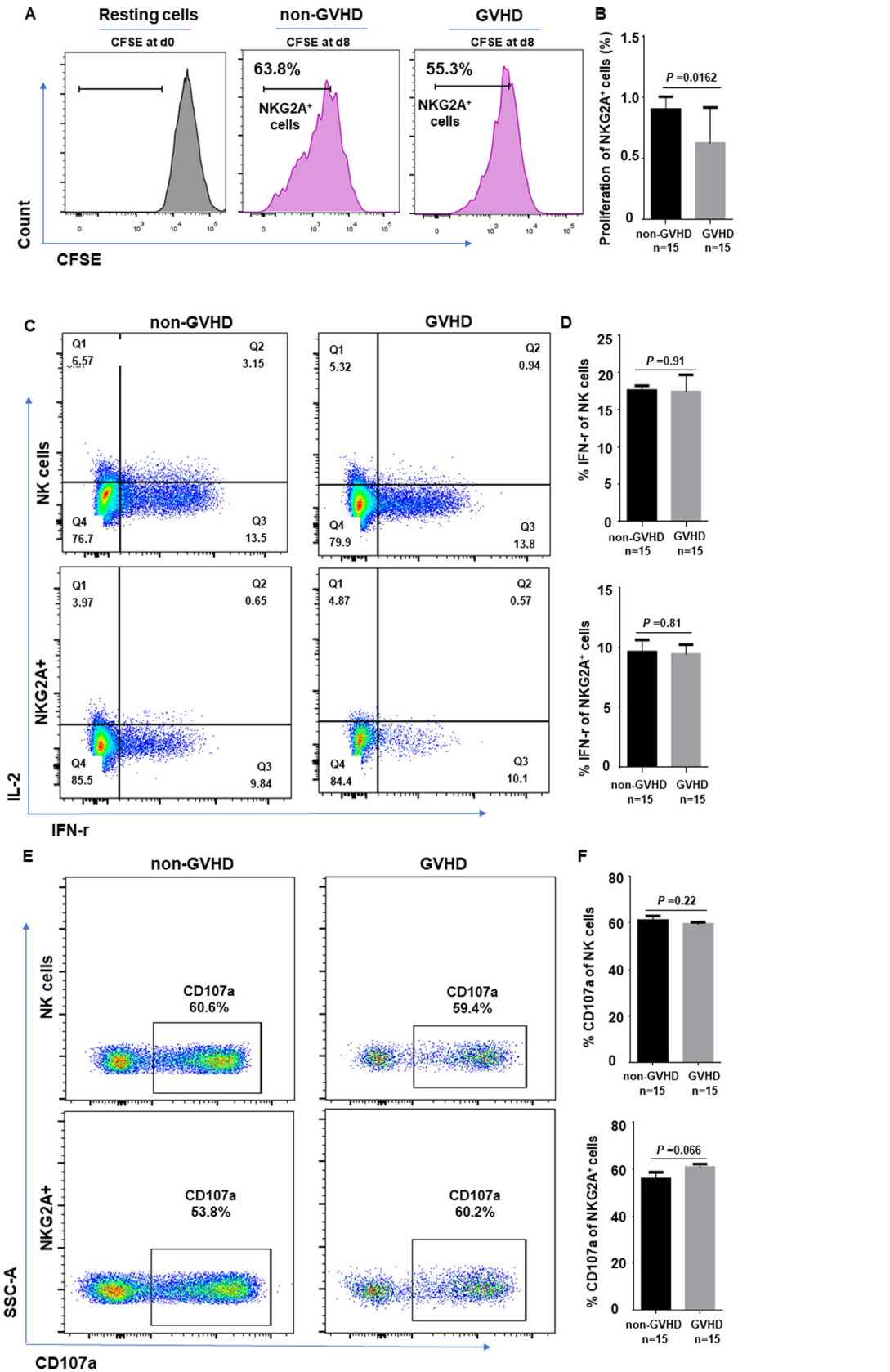

Figure 4. NKG2A$^+$ NK cells in GVHD patients displayed increased apoptosis and a decreased proliferation capacity while retaining a strong GVL effect. (A and B) PBMCs from the paired cohorts were labeled with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured for 7 days. NKG2A$^+$ cell proliferation was decreased in the GVHD patients ($P < .05$). (C and D) After co-culture of PBMCs with the leukemia cell line K562 for 4 hours, the cytokine IFN-γ level secreted by NKG2A$^+$ cells was comparable between the GVHD patients and non-GVHD patients ($P > .05$). (E and F) After co-culture of PBMCs with the leukemia cell line K562 for 4 hours, the CD107α expression level was comparable between the GVHD patient and non-GVHD patient NKG2A+ cells ($P > .05$).

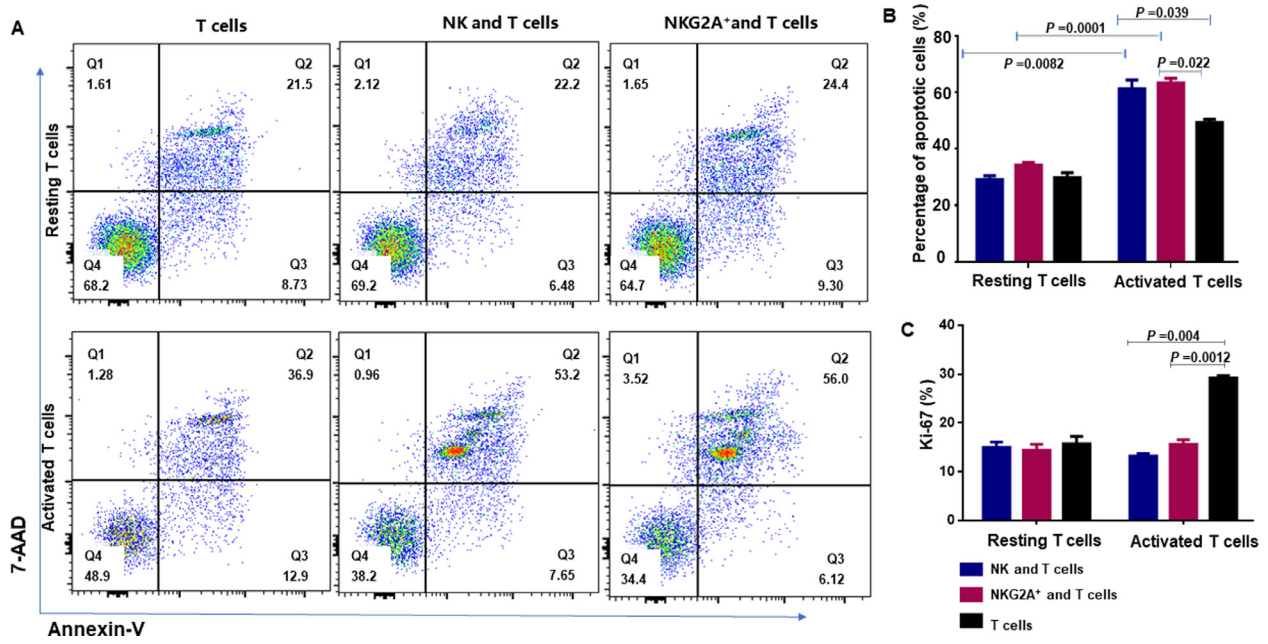

Figure 5. The NKG2A$^+$ cells played a role in the killing effect mainly for activated but not resting T cells. (A) NKG2A$^+$ NK cells was sorted from the non-GVHD patients, and T cells were sorted from the healthy donors. The T cells were activated or not using T-Activator CD3/CD28 Dynabeads. Flow cytometry analysis indicated that apoptosis was increased in the activated T cells co-cultured with the NKG2A$^+$ NK cells, but this phenotype was not observed in the resting T cells. (B) Quantitative histogram showing the increase in apoptosis in the activated T cells, whereas the Ki-67 expression level was decreased. (C) These phenomena were not observed in the resting T cells.

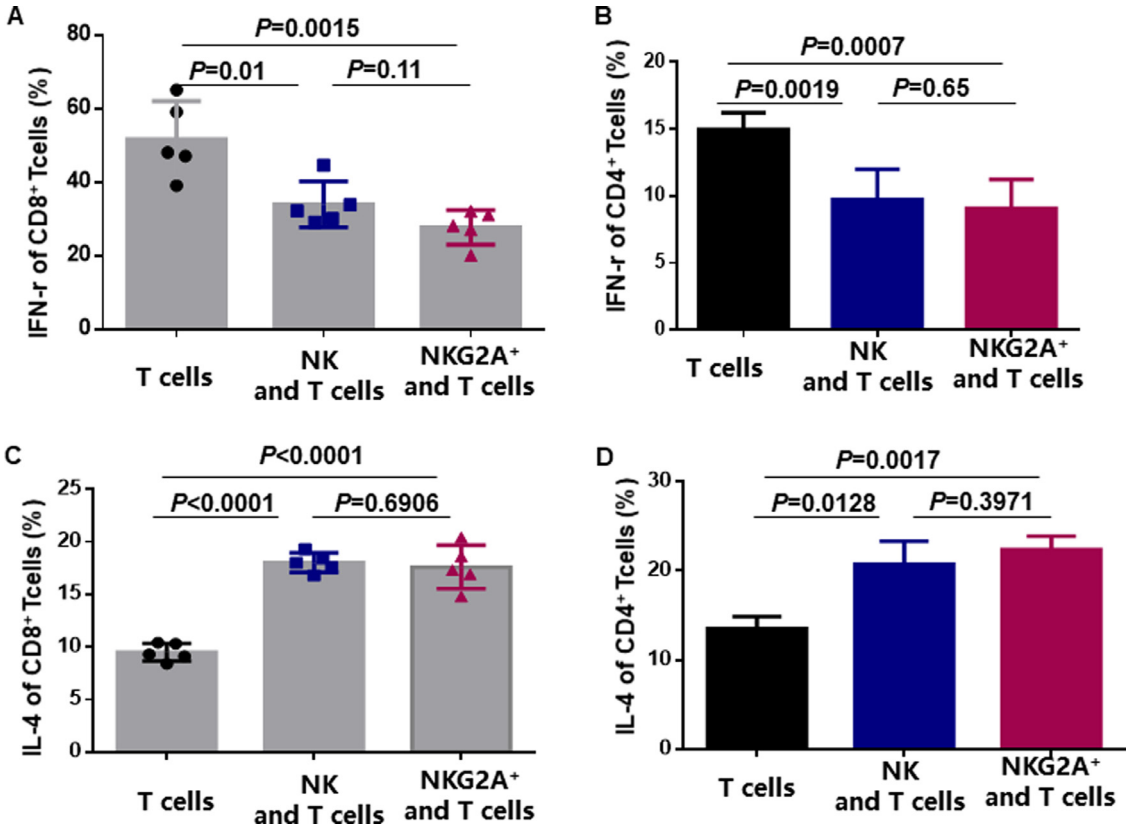

Figure 6. The expression levels of the T cell cytokines IFN-γ and IL-4 were changed by co-culture with NK or NKG2A$^+$NK cells. NK and NKG2A$^+$ cells sorted from the non-GVHD patients (n = 5) were co-cultured for 72 hours with CD3$^+$ T cells sorted from the healthy donors (n = 5) and subjected to analysis. The T cells were activated using T-Activator CD3/CD28 Dynabeads. (A) Activated CD8$^+$ T cells exhibited lower IFN-γ expression in the presence of NK or NKG2A$^+$ NK cells. (B) Activated CD4$^+$ T cells exhibited lower IFN-γ expression in the presence of NK or NKG2A$^+$ NK cells. (C) Activated CD8$^+$ T cells exhibited higher IL-4 expression in the presence of NK or NKG2A$^+$ NK cells. (D) Activated CD4+ T cells exhibited higher IL-4 expression in the presence of NK or NKG2A+ NK cells.

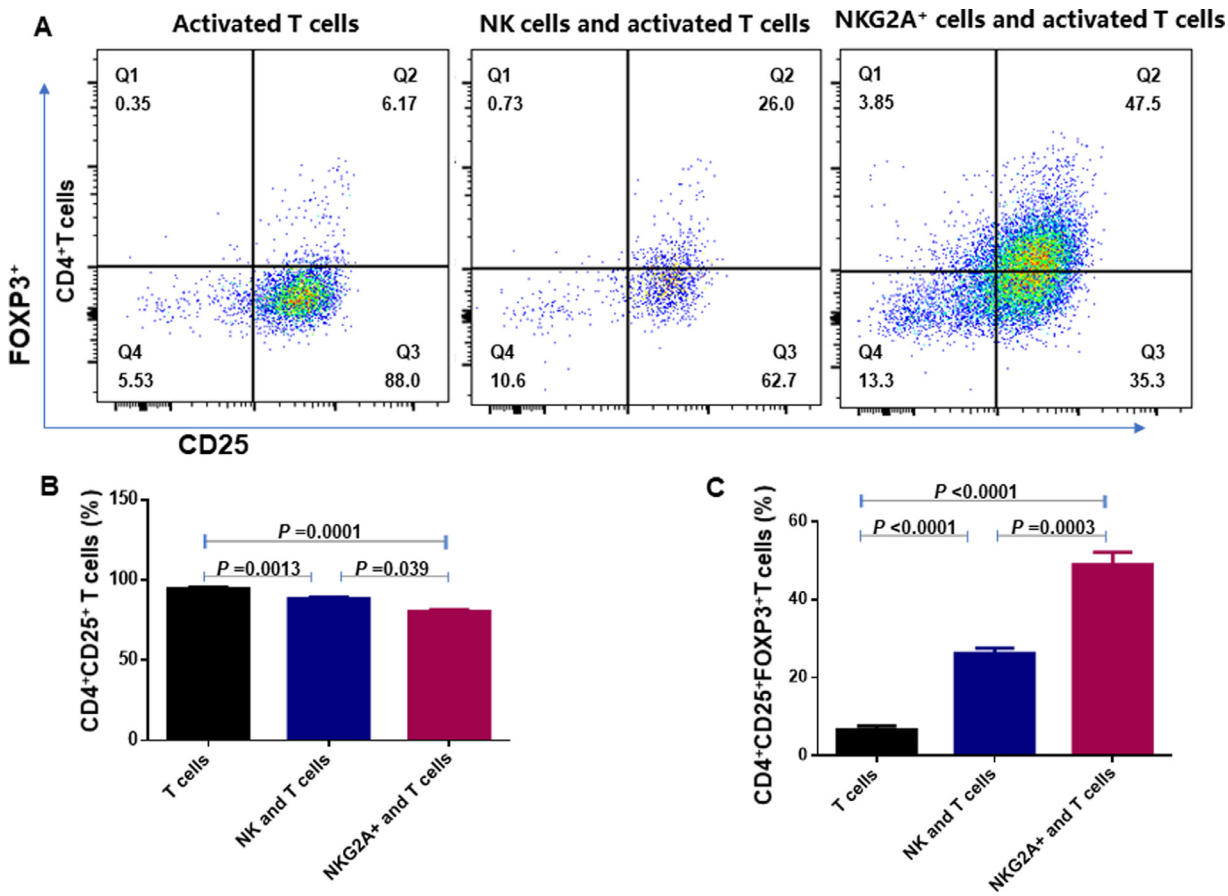

Figure 7. NK or NKG2A$^+$ NK cells exhibited decreased CD25 expression and an increased number of cells with the CD4$^+$CD25$^+$FOXP3$^+$ phenotype in the activated T cell analysis. NK and NKG2A$^+$ cells sorted from the non-GVHD patients (n = 5) were co-cultured with CD3$^+$ T cells sorted from the healthy donors (n = 5) and subjected to CD25 and FOXP3 expression analysis. The T cells were activated using T-Activator CD3/CD28 Dynabeads. (A) Flow cytometry showed that activated T cells expressed lower CD25 levels in the presence of NK or NKG2A$^+$ NK cells, whereas the percentage of cells with the CD4$^+$CD25$^+$FOXP3$^+$ phenotype was increased. (B) Quantitative histogram showing that activated T cells expressed lower CD25 levels in the presence of NK or NKG2A$^+$ NK cells, whereas the percentage of cells with the CD4$^+$CD25$^+$FOXP3$^+$ phenotype was increased (C).

experiments suggested that NKG2A$^+$ NK cells might prevent aGVHD by regulating T cell immune functions. First, we found that the percentages and absolute numbers of NK and NKG2A$^+$ subset cells in patients with GVHD after transplantation were reduced in the prospective cohort and then confirmed this finding in the paired cohort study. In vitro assays showed that NKG2A$^+$ NK cells inhibited T cell proliferation and activation as well as secretion of the inflammatory factor IFN-γ, promoted T cell apoptosis, and increased the number of cells with the CD4$^+$CD25$^+$FOXP3$^+$ phenotype.

Previous studies showed that NK cells had important regulatory functions that could modulate adaptive T cell responses via cytokine release, cytotoxicity, or promotion of antigen cross-presentation to T cells [13,31-34]. In a major mismatch murine model of transplantation, Murphy et al. [35] demonstrated that NK cells transferred together with non-T cell–depleted bone marrow cells without splenocytes did not induce GVHD. Interestingly, activated NK cells prevented the development of GVHD in mice that received splenocytes and invariably led to the death of mice injected with bone marrow cells and splenocytes alone [12]. Furthermore, Olson et al. [36] reported that mice receiving donor NK and T cells showed improved survival and decreased GVHD scores when compared with controls receiving donor T cells alone. Moreover, in the presence of NK cells, donor T cells exhibited less proliferation, lower CD25 expression, and decreased IFN-γ production. Accumulated evidence suggested that donor-derived NK cells might attenuate GVHD by eliminating recipient antigen-presenting cells or donor-derived alloreactive T cells [37,38]. These studies demonstrated that NKG2A$^+$ subsets had some regulatory impact on T cells in in vitro systems after allo-HSCT. Indeed, our study confirmed these findings and found that after co-culture with NK cells, T cell proliferation, CD25 expression, and IFN-γ secretion were reduced compared with those in T cells cultured alone. Because of the variable characteristics of NK cells reconstituted post-transplant, the major NK cell subsets reconstituted at each stage after HSCT were different. Therefore, we speculate that although NK cells regulate the function of T cells as a whole, the primary roles of each subset need to be further explored.

As mentioned above, evidence for the recovery of NK cell receptor subsets after stem cell transplantation is scarce. In a prospective study Kordelas et al. [19] monitored the frequencies of inhibitory receptor CD94/NKG2A expression in NK cells after the allo-HSCT course for 1 year and related the results to the occurrence of severe aGVHD or chronic GVHD in a cohort of 26 patients undergoing allo-HSCT. This study showed that the NKG2A receptor continuously decreased in the first year after allo-HSCT and that the NKG2A$^+$ subset was reduced in chronic GVHD patients. However, the study did not identify a

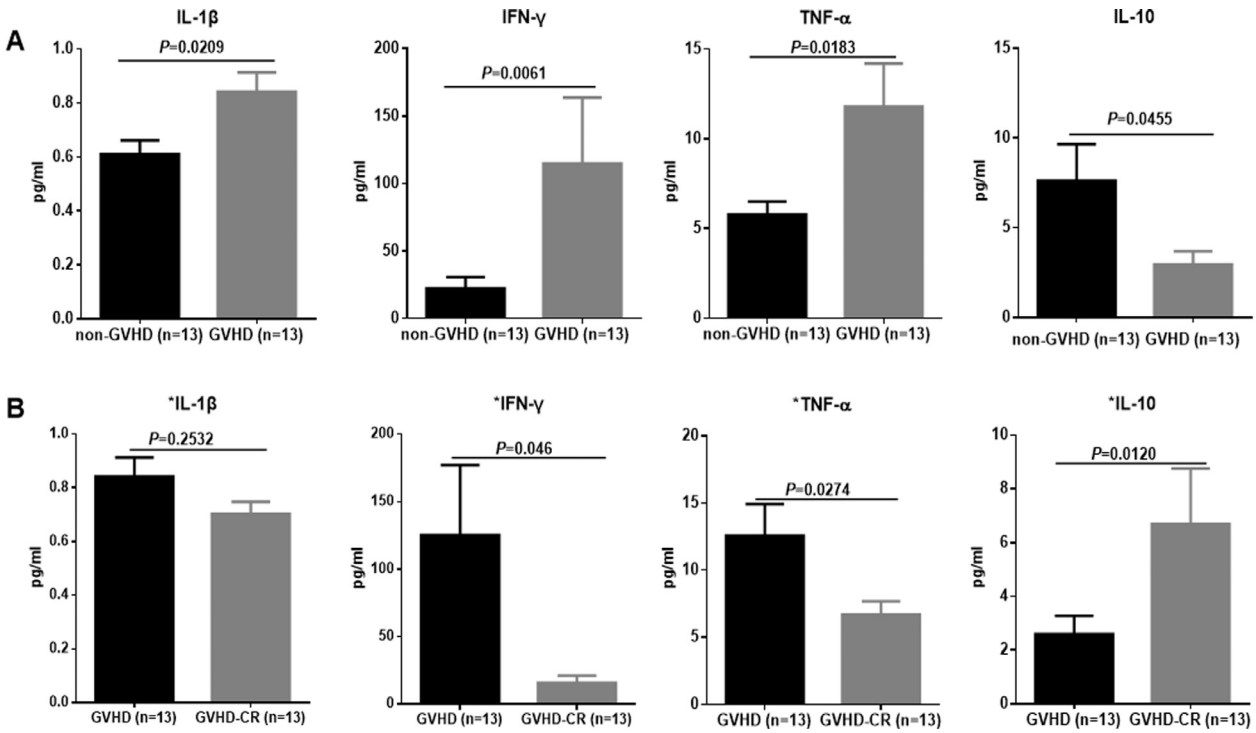

Figure 8. The cytokine expression phenotype was different between the GVHD and non-GVHD patients. Cytokines were detected in the sera of paired GVHD and non-GVHD specimens measured in duplicate via Meso Scale technology using a V-PLEX Custom Human Cytokine kit (Meso Scale Discovery). (A) The GVHD subgroup serum IL-1β, IFN-γ, and tumor necrosis factor-α levels were higher than those of the non-GVHD subgroup, whereas the GVHD subgroup serum IL-10 level was significantly lower. (B) Compared with the active GVHD samples, the serum IL-1β, IFN-γ, and tumor necrosis factor-α levels were decreased, whereas the IL-10 level was increased in GVHD patients in the complete recovery (CR) condition.

relationship between the NKG2A$^+$ subset and aGVHD, and functional experiments to elucidate the role of NKG2A$^+$ cells during the development of chronic GVHD were also lacking [19]. In our study we found a decreased number of NKG2A$^+$ subset cells in patients suffering from aGVHD in a prospective study. Importantly, we confirmed this result using paired GVHD and non-GVHD specimens. Moreover, the number of NKG2A$^+$ subsets was increased in GVHD patients in a complete recovery status compared with that of GVHD patients in partial recovery. Furthermore, co-culture of T cells and NKG2A$^+$ cells in vitro revealed that T cell proliferation, CD25 expression, and IFN-γ production were reduced compared with those in T cells cultured alone.

Our results suggested that NKG2A$^+$ cells might prevent the occurrence of GVHD by inhibiting activated alloreactive T cells. We also found that exogenous addition of IL-10 increased the

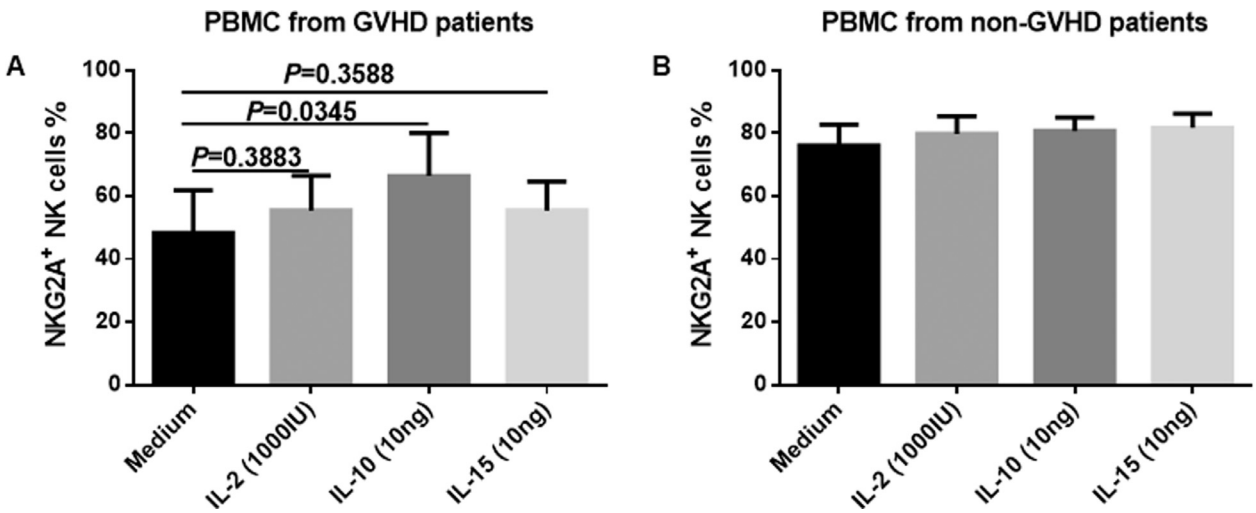

Figure 9. The soluble plasma IL-10 level was closely associated with increased NKG2A expression in GVHD patient–derived NK cells. PBMCs from the GVHD and non-GVHD patients were preincubated with IL-2, IL-10, or IL-15 for 72 hours and subjected to analysis. (A) The percentage of NKG2A$^+$ NK cells from the GVHD patients was markedly increased under stimulation by exogenous IL-10 but not by stimulation with IL-2 or IL-15. (B) The non-GVHD patient-derived NKG2A$^+$ NK cell ratio was stable when stimulated by IL-10, IL-2, or IL-15.

number of NKG2A$^+$ subset cells in the peripheral blood. This result suggested the potential of increasing the number of NKG2A$^+$ cells via intervention to prevent the occurrence of GVHD. Because the patient groups were small in this study, more studies with a larger numbers of patients are warranted to further elucidate the role of NK cell receptors in allo-HSCT. Moreover, the specific NK receptor recovery and impact on alloreactive T cells after allo-HSCT should be analyzed in more detail in larger patient cohorts after allo-HSCT. The role of HLA-E as the cognate ligand for NKG2A should be considered in this context. In the in vitro system involving co-culture of T cells with NK and NKG2A$^+$ cells, the number of CD4$^+$CD25$^+$FOXP3$^+$ phenotype cells was increased; however, whether these cells were regulatory T cells could not be confirmed. Further experiments are needed to confirm the functions of these cells and further clarify the mechanism by which NKG2A$^+$ cells regulate T cells and thus inhibit the occurrence of GVHD. Mouse models may help elucidate the crosstalk between NKG2A and other immune cells during early reconstitution after allo-HSCT with respect to the prevention and relapse of GVHD.

Overall, herein we observed reduced proportions and absolute cell counts of NK and NKG2A$^+$ subset cells in patients with aGVHD after allo-HSCT. The causative association between NK cell numbers, the NKG2A$^+$ subset, and GVHD remains debatable. Based on our results, we are tempted to speculate that reduced proportions of NKG2A$^+$ subset cells in patients after allo-HSCT are associated with aGVHD because of their interplay with the patient's donor-derived alloreactive T cells.

ACKNOWLEDGMENTS

Financial disclosure: Supported by grants from the National Natural Science Foundation of China (grant nos. 81670166 and 81530046), Innovative Research Groups of the National Natural Science Foundation of China (grant no. 81621001), the Beijing Talents Fund (grant no. 2015000021223ZK26), the Beijing Municipal Science & Technology Commission (grant no. Z171100001017098), the Project of Health Collaborative Innovation of Guangzhou City (grant no. 201704020214), and the Scientific Research Foundation for Capital Medicine Development (grant nos. 2018-2-4084 and 2016-1-4082).

Conflict of interest statement: There are no conflicts of interest to report.

SUPPLEMENTARY DATA

Supplementary material associated with this article can be found, in the online version at doi:10.1016/j.bbmt.2018.08.008.

REFERENCES

1. Xu L, Chen H, Chen J, et al. The consensus on indications, conditioning regimen, and donor selection of allogeneic hematopoietic cell transplantation for hematological diseases in China—recommendations from the Chinese Society of Hematology. *J Hematol Oncol*. 2018;11:33.
2. Gratwohl A, Pasquini MC, Aljurf M, et al. One million haemopoietic stem-cell transplants: a retrospective observational study. *Lancet Haematol*. 2015;2:e91–e100.
3. Passweg JR, Baldomero H, Bader P, et al. Use of haploidentical stem cell transplantation continues to increase: the 2015 European Society for Blood and Marrow Transplant activity survey report. *Bone Marrow Transplant*. 2017;52:811–817.
4. Lv M, Huang X. Fighting against hematological malignancy in China: from unique system to global impact. *Sci China Life Sci*. 2015;58:1183–1190.
5. Wang Y, Liu QF, Xu LP, et al. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. *Blood*. 2015;125:3956–3962.
6. Wang Y, Wang HX, Lai YR, et al. Haploidentical transplant for myelodysplastic syndrome: registry-based comparison with identical sibling transplant. *Leukemia*. 2016;30:2055–2063.
7. Bunting MD, Varelias A, Souza-Fonseca-Guimaraes F, et al. GVHD prevents NK-cell-dependent leukemia and virus-specific innate immunity. *Blood*. 2017;129:630–642.
8. Gaballa A, Norberg A, Stikvoort A, et al. Assessment of TREC, KREC and telomere length in long-term survivors after allogeneic HSCT: the role of GvHD and graft source and evidence for telomere homeostasis in young recipients. *Bone Marrow Transplant*. 2018;53:69–77.
9. Remberger M, Afram G, Sundin M, et al. High incidence of severe chronic GvHD after HSCT with sibling donors. A single center analysis. *Bone Marrow Transplant*. 2016;51:1518–1521.
10. Kosmoliaptsis V, Joris MM, Mallon DH, et al. Physiochemical disparity of mismatched HLA class I alloantigens and risk of acute GVHD following HSCT. *Bone Marrow Transplant*. 2015;50:540–544.
11. Ghimire S, Weber D, Mavin E, Wang XN, Dickinson AM, Holler E. Pathophysiology of GvHD and Other HSCT-Related Major Complications. *Front Immunol*. 2017;8:79.
12. Simonetta F, Alvarez M, Negrin RS. Natural killer cells in graft-versus-host-disease after allogeneic hematopoietic cell transplantation. *Front Immunol*. 2017;8:465.
13. Ghadially H, Ohana M, Elboim M, et al. NK cell receptor NKp46 regulates graft-versus-host disease. *Cell Rep*. 2014;7:1809–1814.
14. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097–2100.
15. Song Y, Hu B, Liu Y, et al. preactivated donor NK cells enhance GVL effects and mitigate GvHD after allogeneic hematopoietic stem cell transplantation. *Eur J Immunol*. 2017;48:670–682.
16. Chang YJ, Zhao XY, Huang XJ. Effects of the NK cell recovery on outcomes of unmanipulated haploidentical blood and marrow transplantation for patients with hematologic malignancies. *Biol Blood Marrow Transplant*. 2008;14:323–334.
17. Sun C, Xu J, Huang Q, et al. High NKG2A expression contributes to NK cell exhaustion and predicts a poor prognosis of patients with liver cancer. *Oncoimmunology*. 2017;6:e1264562–12.
18. Bjorklund AT, Schaffer M, Fauriat C, et al. NK cells expressing inhibitory KIR for non-self-ligands remain tolerant in HLA-matched sibling stem cell transplantation. *Blood*. 2010;115:2686–2694.
19. Kordelas L, Steckel NK, Horn PA, Beelen DW, Rebmann V. The activating NKG2C receptor is significantly reduced in nk cells after allogeneic stem cell transplantation in patients with severe graft-versus-host disease. *Int J Mol Sci*. 2016;17:1797.
20. Wang Y, Chang YJ, Xu LP, et al. Who is the best donor for a related HLA haplotype-mismatched transplant? *Blood*. 2014;124:843–850.
21. Wacholder S, Silverman DT, McLaughlin JK, Mandel JS. Selection of controls in case-control studies. III. Design options. *Am J Epidemiol.*. 1992;135:1042–1050.
22. Shi MM, Kong Y, Song Y, et al. Atorvastatin enhances endothelial cell function in posttransplant poor graft function. *Blood*. 2016;128:2988–2999.
23. Huang XJ, Zhu HH, Chang YJ, et al. The superiority of haploidentical related stem cell transplantation over chemotherapy alone as postremission treatment for patients with intermediate- or high-risk acute myeloid leukemia in first complete remission. *Blood*. 2012;119:5584–5590.
24. Wang Y, Wu DP, Liu QF, et al. In adults with t(8;21)AML, posttransplant RUNX1/RUNX1T1-based MRD monitoring, rather than c-KIT mutations, allows further risk stratification. *Blood*. 2014;124:1880–1886.
25. Yan CH, Liu DH, Liu KY, et al. Risk stratification-directed donor lymphocyte infusion could reduce relapse of standard-risk acute leukemia patients after allogeneic hematopoietic stem cell transplantation. *Blood*. 2012;119:3256–6322.
26. Huang XJ, Liu DH, Liu KY, et al. Haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant*. 2006;38:291–297.
27. de Koning C, Plantinga M, Besseling P, Boelens JJ, Nierkens S. Immune reconstitution after allogeneic hematopoietic cell transplantation in children. *Biol Blood Marrow Transplant*. 2016;22:195–206.
28. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease. I. The 2014 Diagnosis and Staging Working Group report. *Biol Blood Marrow Transplant*. 2015;21:389–401.
29. Dulphy N, Haas P, Busson M, et al. An unusual CD56(bright) CD16(low) NK cell subset dominates the early posttransplant period following HLA-matched hematopoietic stem cell transplantation. *J Immunol*. 2008;181:2227–2237.
30. Beziat V, Duffy D, Quoc SN, et al. CD56brightCD16+ NK cells: a functional intermediate stage of NK cell differentiation. *J Immunol*. 2011;186:6753–6761.

31. Crome SQ, Lang PA, Lang KS, Ohashi PS. Natural killer cells regulate diverse T cell responses. *Trends Immunol*. 2013;34:342–349.
32. Artis D, Spits H. The biology of innate lymphoid cells. *Nature*. 2015;517:293–301.
33. Gasteiger G, Rudensky AY. Interactions between innate and adaptive lymphocytes. *Nat Rev Immunol*. 2014;14:631–639.
34. Ghadially H, Horani A, Glasner A, et al. NKp46 regulates allergic responses. *Eur J Immunol*. 2013;43:3006–3016.
35. Murphy WJ, Bennett M, Kumar V, Longo DL. Donor-type activated natural killer cells promote marrow engraftment and B cell development during allogeneic bone marrow transplantation. *J Immunol*. 1992;148:2953–2960.
36. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood*. 2010;115:4293–4301.
37. Ruggeri L, Mancusi A, Burchielli E, et al. Natural killer cell recognition of missing self and haploidentical hematopoietic transplantation. *Semin Cancer Biol*. 2006;16:404–411.
38. Locatelli F, Pende D, Mingari MC, et al. Cellular and molecular basis of haploidentical hematopoietic stem cell transplantation in the successful treatment of high-risk leukemias: role of alloreactive NK cells. *Front Immunol*. 2013;4:15.