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## Original Article

# Reconstitution and clinical significance of T cell subsets in the early stage after related HLA-mismatched peripheral blood hematopoietic SCT without T-cell depletion *in vitro*

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**Abstract:** Related HLA-haploidentical HSCT has been applied more and more recently, but the reconstitution of T lymphocyte subsets and its clinical significance in patients received related HLA-haploidentical non T-cell depleted *in vitro* high-dose peripheral blood hematopoietic SCT (RHNT-PSCT) are incompletely defined. In the present study of our RHNT-PSCT, we found that in non-aGVHD group, CD3<sup>+</sup> T lymphocyte recovered to normal levels gradually between 60 and 90 days, and the recovery of CD4<sup>+</sup> T lymphocyte was retarded significantly, CD4<sup>+</sup>/CD8<sup>+</sup> ratio was apparently inverted. Whereas, the ratio of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells was significantly lower in aGVHD group than in healthy control group and non-aGVHD group, and also in grade III-IV aGVHD patients than in grade I-II aGVHD patients. Meanwhile, we observed the level of interleukin-10 (IL-10) gradually increased in serum of patients without aGVHD, but decreased in III-IV aGVHD patients significantly. Spearman correlation analysis showed that serum IL-10 level was negatively correlated with the grade of aGVHD. These results suggest that the reconstitution of peripheral blood T lymphocyte subsets is good, and dynamic detection of Treg cells and serum IL-10 level might predict aGVHD in the early stage after our RHNT-PSCT.

**Keywords:** Allo-HSCT, HLA-mismatched, lymphocyte subsets, regulatory T-cell, acute GVHD

## Introduction

Recently, related HLA-haploidentical HSCT (RH-HSCT) has been widely used for malignant hematological diseases. Acute GVHD (aGVHD) and infection are major problems after RH-HSCT, and impact the survival and quality of life of patients. These are closely related with the recovery of T lymphocyte subsets [1-3]. However, the reconstitution of T lymphocyte subsets are incompletely defined, and it remains controversial whether regulatory T cell (Treg cell) plays a key role in immune dysfunction of aGVHD after RH-HSCT [4-6]. A few studies concerned Treg cells after RH-HSCT [4, 7-9], and fewer studies addressed related HLA-haploidentical non T-cell depleted *in vitro* peripheral blood hematopoietic SCT (RHNT-PSCT). As we know, collected donor's peripheral blood stem cell (PBSC) graft contains too

much matured T lymphocytes, so it would affect the engraftment and increase the risk of GVHD occurrence after RHNT-PSCT [10, 11]. To overcome above shortcomings of RHNT-PSCT, we designed and gradually improved a unique RHNT-PSCT protocol which was mainly characterized with infusing unmanipulated high-dose related HLA-mismatched donors' PBSC *ex vivo*. This study focuses on dynamically analyzing the recovery of T lymphocyte subsets, and primarily investigating the relationship between the change of Treg cells and the occurrence of aGVHD in the early stage after our RHNT-PSCT.

## Data and methods

### Subjects

A total of 35 patients undergoing RHNT-PSCT in our center from July 2011 to May 2013 were

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enrolled in this study. There were 19 males and 16 females, at median age of 27 years (4-49 years). All patients with hematologic malignancies were in CR1. HLA locus was matched between related donor and recipient: 3/6 matched in 23 cases and 4/6 matched in 12 cases. If patients did not suffer from aGVHD after transplantation, anticoagulated peripheral blood and serum were collected at +30, +60 and +90 days after transplantation. If patients suffered from aGVHD after transplantation, anticoagulated peripheral blood and serum were detected immediately. Simultaneously, anticoagulated peripheral blood of 20 healthy controls with matched age and gender was collected as the control group. The protocols were performed in accordance with ethical standards of Ethics Committee of First Affiliated Hospital of Xinjiang Medical University in China. All subjects signed informed consent. This study was approved by the Ethics Committee of First Affiliated Hospital of Xinjiang Medical University in China (approval No. 20101203).

### *Myeloablative conditioning regimen, peripheral blood hematopoietic stem cells (PBSCs) collection and infusion*

A total of 35 patients were subjected to the following myeloablative conditioning regimen: Ara-C + busulfan + cyclophosphamide + anti-human thymocyte immunoglobulin: briefly, intravenous infusion of Ara-C 2-4 g/m<sup>2</sup> (days-9, -8), busulfan 3.2 mg/kg·d (days-7~-5), cyclophosphamide 60 mg/kg·d (days-3, -2) and Me-CCNU 250 mg/m<sup>2</sup> orally once on day -3; along with rATG (rabbit thymoglobuline 2.5 mg/kg per day; Sang Stat, Lyon, France, now marketed by Genzyme, Cambridge, MA) intravenously for 4 consecutive days (days-4~-1). Granulocyte-colony stimulating factor 7-10 µg/kg·d was used to mobilize peripheral blood mononuclear cells. The infusion numbers of mononuclear cells (MNC) was  $(14.65 \pm 3.04) \times 10^8$ /kg, CD34<sup>+</sup> cells was  $(10.25 \pm 3.42) \times 10^6$ /kg and CD3<sup>+</sup> cells was  $(4.8 \pm 1.21) \times 10^8$ /kg.

### *Diagnosis, prevention and treatment of aGVHD*

Enhanced GVHD prevention program was utilized as follows: intravenous infusion of cyclosporin A 2.5 mg/kg·d (continuous administration for 24 hours, 200-400 ng/ml), or intravenous infusion of tacrolimus 0.02 mg/kg·d (continuous administration for 24 hours, 7-15 ng/

ml); intravenous infusion of methotrexate 15 mg/m<sup>2</sup> (+1 day) and 10 mg/m<sup>2</sup> (+3, +6, +11 days); take orally mycophenolate mofetil 1,000 mg/d (+1-+100 days, at twice); intravenous injection of dexamethasone 5 mg/day (+1-+30 days, at twice), prednisone 30 mg/d (+31 days beginning, gradually decrement, until 60 days); anti-CD25 monoclonal antibody 20 mg/d (01 day, +2 day). The diagnosis and grade of aGVHD were in accordance with Seattle diagnostic criteria.

### *Main reagents and equipment*

There were FACS Lysing Solution Erythrocyte Lysate (BD, USA), mouse anti-human monoclonal antibody CD25, CD4, CD3, CD8, intracellular antibody Foxp3 and isotype control Mouse IgG1, Foxp3 Special rupture fixative, and human interleukin-10 (IL-10) enzyme linked immunosorbent assay (ELISA) kit (eBioscience, USA).

### *Detection methods of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells*

5 µl CD4-Percp, 5 µl CD3-APC and 5 µl CD25-FITC were added in two FACS tubes, isotype control tube and experimental tube. Fixation, rupture of membrane and intracellular staining were conducted in strict accordance with reagent instructions. During intracellular staining, 5 µl Foxp3-PE and 5 µl IgG1-PE were respectively added in the experimental tube and isotype control tube. Lymphocyte populations were selected on the FSC-SSC scatter plot. CD4-positive cells were selected using CD4/SSC gating, and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cells were analyzed.

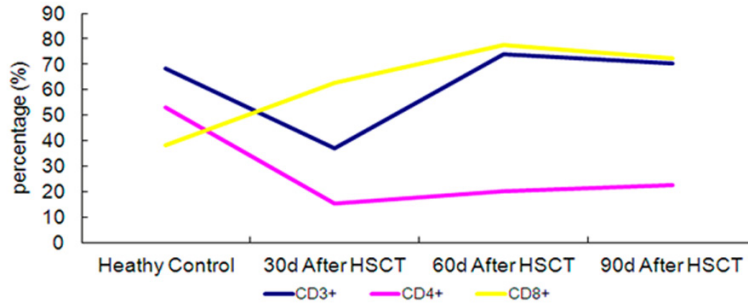
### *Determination of serum IL-10*

Serum IL-10 concentration was detected with ELISA kit. The protocols were performed in accordance with the instruction. Blank and negative controls were set to ensure the reliability of detection results. Minimum value of IL-10 was 0.78 pg/ml.

### *Statistical analysis*

The data were analyzed using SPSS 13.0 software, and expressed as mean ± SD. Data, which obey a normal distribution, were compared among multiple groups using analysis of

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**Figure 1.** Dynamic alterations in peripheral blood T lymphocyte subsets in patients without aGVHD group after transplantation. CD3<sup>+</sup> and CD8<sup>+</sup> T lymphocytes gradually increased at 30 days after transplantation. The recovery of CD4<sup>+</sup> T lymphocytes was relatively slow.

variance. Intergroup pairwise comparison was done using LSD. For heterogeneity of variance, intergroup difference was compared using rank sum test. Correlation was analyzed utilizing Spearman correlation test. A value of  $P < 0.05$  was considered statistically significant.

### Results

#### *Hematopoietic reconstitution and aGVHD occurrence after transplantation*

In this study, transplantation was successful in 35 patients. Patients engrafted to absolute neutrophil counts exceeded  $0.5 \times 10^9/L$  in a median time of  $[(15.6 \pm 3.88)$  days]. The patient's platelet counts exceeded  $20 \times 10^9/L$  in a median time of  $[(18.18 \pm 4.88)$  days]. Of 35 patients, 16 patients suffered from aGVHD within 100 days after transplantation, including 11 patients with grade I-II aGVHD and 5 patients with grade III-IV aGVHD. 14 patients were recovered apparently after treatment, and 2 patients died of grade III-IV aGVHD at +102 and +87 days after transplantation.

#### *Peripheral blood T lymphocyte subsets in the normal control group and patients without aGVHD at 30, 60 and 90 days after transplantation*

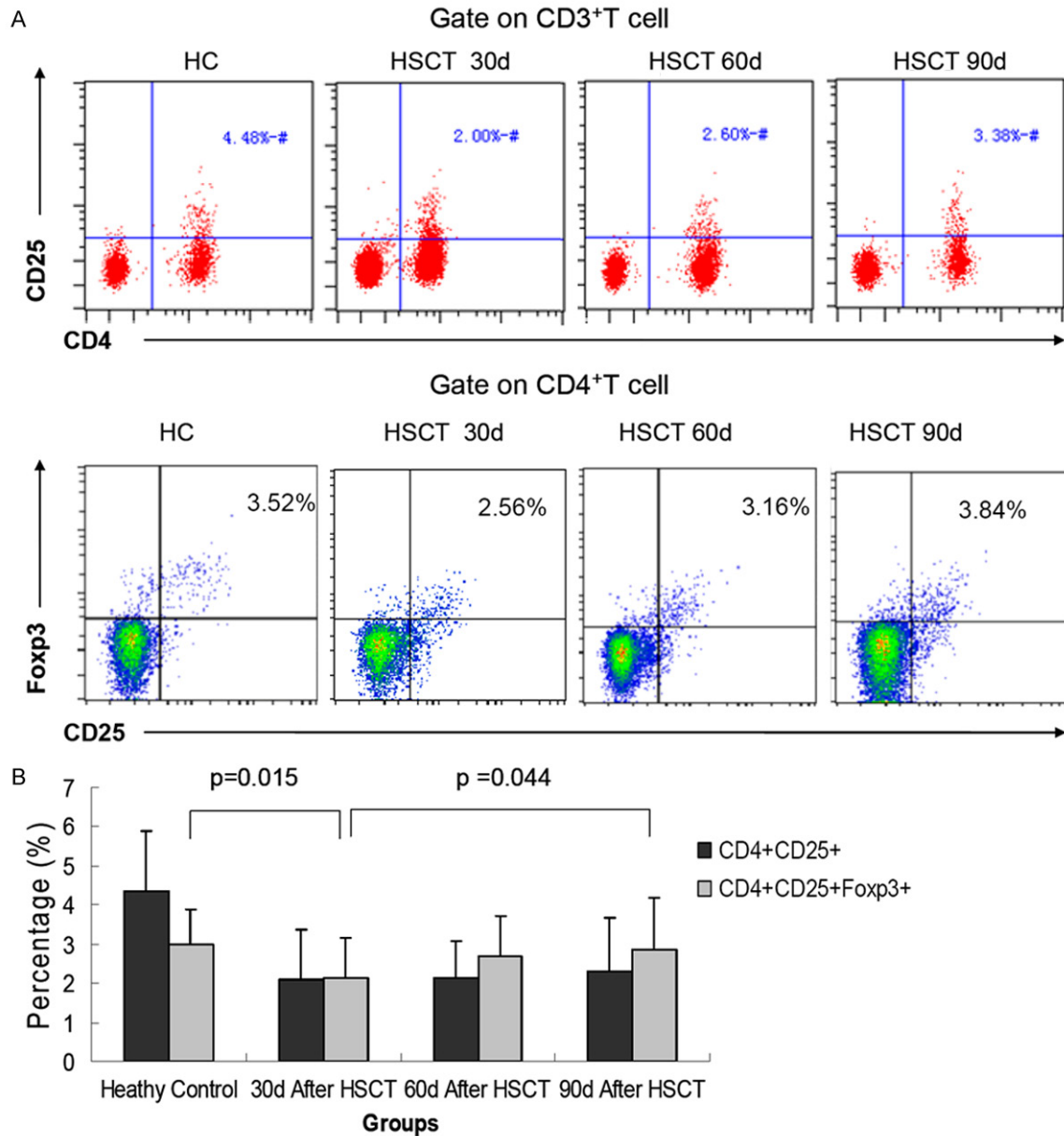
CD3<sup>+</sup> T lymphocyte percentage was lower at 30 days after transplantation compared with healthy control group ( $P = 0.000$ ) and recovered to normal levels at 60 and 90 days ( $P = 0.267$ ,  $P = 0.076$ ) (60 days vs. 30 days:  $P = 0.000$ ; 90 days vs. 30 days:  $P = 0.000$ ) CD4<sup>+</sup> T lymphocyte percentage was significantly lower at 30, 60 and 90 days after transplantation

compared with healthy control group ( $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$ ) and no significant difference was observed among 30-, 60- and 90-day transplantation groups ( $P > 0.05$ ). CD8<sup>+</sup> T lymphocyte percentage was higher at 30, 60 and 90 days after transplantation compared with healthy control group ( $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$ ) and there were no significant differences among 30, 60 and 90 days after transplantation ( $P > 0.05$ ). CD4/

CD8 ratio was significantly inverted at 30, 60 and 90 days after transplantation ( $P < 0.01$ ; **Figure 1**). CD4<sup>+</sup> CD25<sup>+</sup> T lymphocyte percentages were  $[(3.09 \pm 1.27)\%$ ,  $(2.42 \pm 1.09)\%$ ,  $(2.32 \pm 1.35)\%$ ] at 30, 60 and 90 days after transplantation, respectively, and no significant difference was detectable as compared with healthy control group ( $3.27 \pm 0.81\%$ ) ( $P = 0.994$ ,  $P = 0.053$ ,  $P = 0.073$ ). Moreover, there was no significant difference in CD4<sup>+</sup> CD25<sup>+</sup> T lymphocyte ratio among 30-, 60- and 90-day transplantation groups ( $P > 0.05$ ). The ratio of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells in CD4<sup>+</sup> T lymphocytes in peripheral blood of patients at 30, 60 and 90 days after transplantation were  $[(2.15 \pm 1.02$ ,  $2.69 \pm 1.04$ ,  $2.86 \pm 1.31)\%$ ], slowly increased. Compared with healthy control group and 90 days after transplantation, the ratio of Treg cells at 30 days was relatively lower ( $P = 0.015$ ,  $P = 0.044$ ; **Figure 2**).

#### *Peripheral blood T lymphocyte subsets in patients occurred aGVHD after transplantation*

Significant differences in CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocyte percentage were detected between aGVHD group and healthy control group ( $P = 0.001$ ,  $P = 0.000$ ,  $P = 0.001$ ). There were no significant differences in CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocyte percentage between aGVHD and non-aGVHD groups (30 days after transplantation) ( $P = 0.998$ ,  $P = 0.179$ ,  $P = 0.941$ ). CD4<sup>+</sup> CD25<sup>+</sup> T lymphocyte ratio  $[(1.95 \pm 1.14)\%$ ] and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cell ratio  $[(1.75 \pm 0.99)\%]$  were obviously lower in the aGVHD group than in healthy control group ( $P = 0.002$ ,  $P = 0.001$ ) and non-GVHD group ( $P = 0.038$ ,  $P = 0.006$ ) (**Figure 3**).



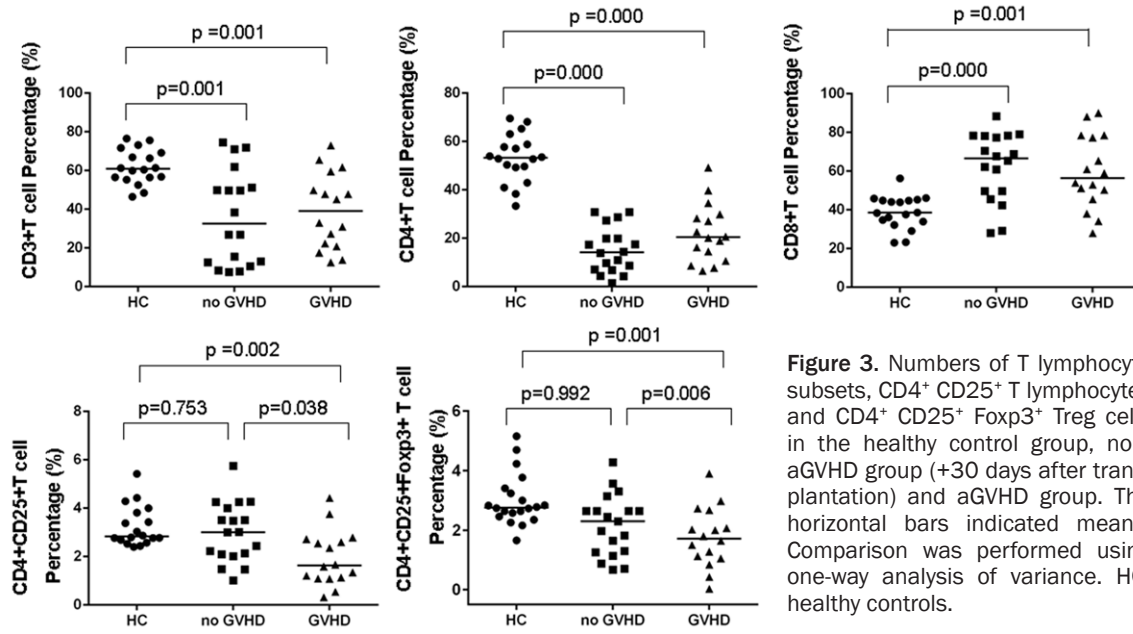
**Figure 2.** Changes in CD4<sup>+</sup> CD25<sup>+</sup> T lymphocytes and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells in patients without aGVHD at various time points after transplantation. Subtypes of CD3<sup>+</sup> T cells in peripheral blood were detected by flow cytometry. Cells were first gated on CD3<sup>+</sup> T cells and then on CD4<sup>+</sup> CD25<sup>+</sup> T cells. A. The representative flow cytometric dot-plots of CD4<sup>+</sup> CD25<sup>+</sup> T cells and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells in the blood of the control, 30-day, 60-day, and 90-day groups. Numbers within each quadrant indicated percentages of cells within each dot-plot. B. Comparisons of the percentages of CD4<sup>+</sup> CD25<sup>+</sup> T and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells in the peripheral blood of these groups. No significant difference was detected between 30-, 60- and 90- day transplantation groups and healthy control group ( $P > 0.05$ ).

*Peripheral blood T lymphocyte subsets in patients with I-II and III-IV aGVHD*

We further analyzed the differences in the percentages of CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocytes, CD4<sup>+</sup> CD25<sup>+</sup> T lymphocytes and CD4<sup>+</sup> CD25<sup>+</sup>

Foxp3<sup>+</sup> Treg cells in patients with grade I-II and III-IV aGVHD. Results demonstrated that the differences in CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocytes ratios between patients with grade I-II and III-IV aGVHD were not statistically significant ( $P = 0.773$ ,  $P = 0.687$ ,  $P = 0.075$ ,  $P = 0.165$ ).

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**Figure 3.** Numbers of T lymphocyte subsets, CD4<sup>+</sup> CD25<sup>+</sup> T lymphocytes and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells in the healthy control group, non-aGVHD group (+30 days after transplantation) and aGVHD group. The horizontal bars indicated means. Comparison was performed using one-way analysis of variance. HC: healthy controls.

However, the percentages of CD4<sup>+</sup> CD25<sup>+</sup> T lymphocytes [(0.96 ± 0.54)%] and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells [(1 ± 0.64)%] in grade III-IV aGVHD group were significantly lower than CD4<sup>+</sup> CD25<sup>+</sup> T lymphocytes [(2.4 ± 0.19)%] and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells [(2.09 ± 0.96)%] in grade I-II aGVHD group [(P = 0.018, P = 0.039)]. The percentages of CD4<sup>+</sup> CD25<sup>+</sup> T lymphocytes and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells between grade I-II aGVHD group and non-aGVHD group were no significant differences (P = 0.523, P = 0.142; **Figure 4**).

### Dynamic changes in serum IL-10 levels in patients with and without aGVHD after transplantation

Serum IL-10 level gradually increased at 30 days [(4.03 ± 0.9) pg/ml], 60 days [(4.6 ± 0.74) pg/ml] and 90 days [(5.07 ± 1.48) pg/ml] in non-aGVHD group, but was still lower than in healthy control group [(8.99 ± 2.97) pg/ml] (P = 0.000, P = 0.000, P = 0.000). There was no statistical significance among 30, 60 and 90 days after transplantation (P > 0.05). In the early stage after transplantation, IL-10 concentration [(3.76 ± 0.58) pg/ml] was lower in grade I-II aGVHD group than in healthy control group (P = 0.000), but no significant difference was observed between grade I-II aGVHD and non-aGVHD groups (P = 0.404). Serum IL-10 level [(2.65 ± 0.86) pg/ml] was significantly lower in

grade III-IV aGVHD group than in non-aGVHD group (P = 0.003) and healthy control group (P = 0.000), although lower than in grade I-II aGVHD group, but there was no statistical significance (P = 0.053; **Figure 5**).

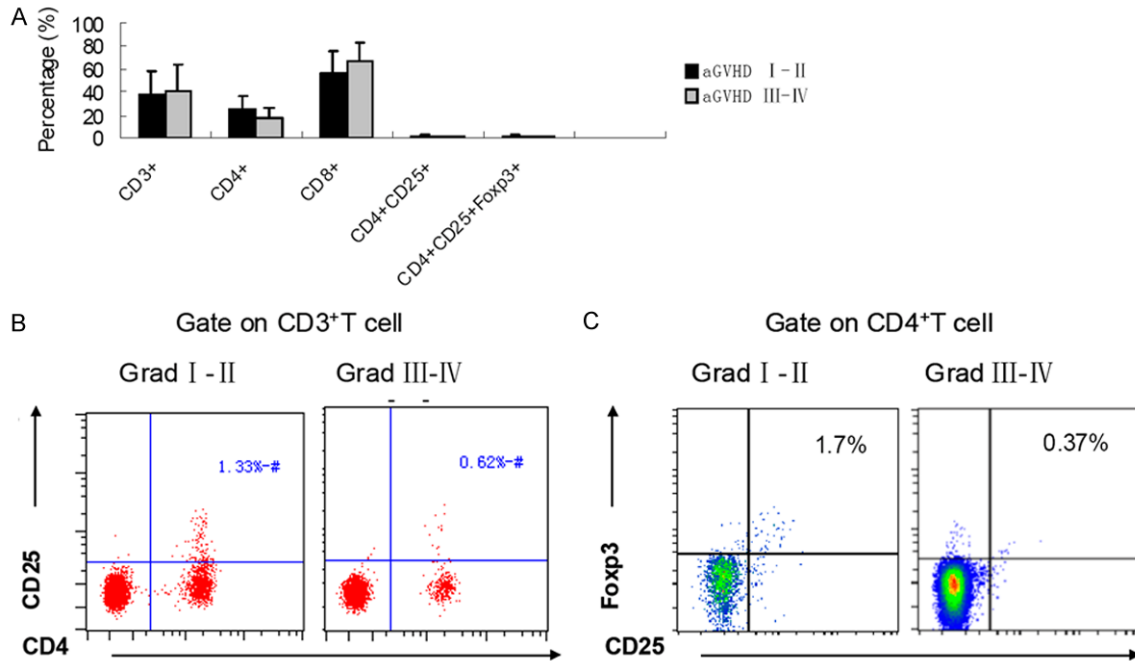
### Correlation of IL-10 level and aGVHD severity

Serum IL-10 level was negatively correlated with the severity of aGVHD in patients after transplantation (P = 0.000, r = -0.594; **Figure 6**).

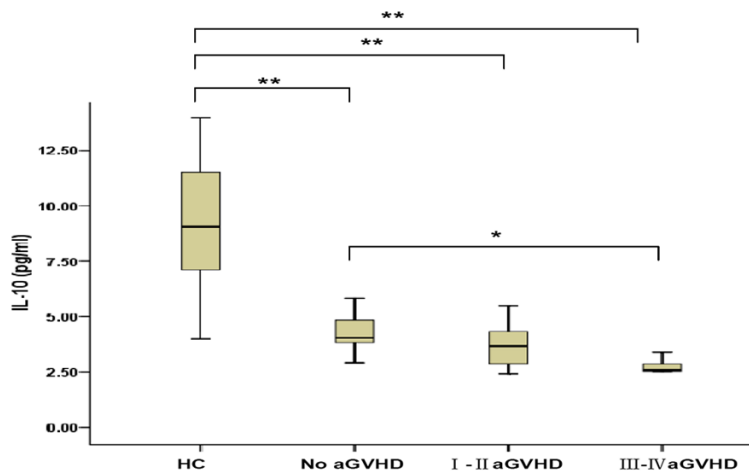
## Discussion

Up to now, peripheral blood as a graft to undergo RH-HSCT reported was mainly depletes T cells ex-vivo, although obtained great progress in GVHD, but relapse of hematological malignant disease and infection are still the main problem affecting the survival of patients [12-15]. Since 2000, Lu [16] and Huang [17] et al. performed related HLA haploidentical non TCD *in vitro* combined G-mobilized bone marrow and PBSC transplantation (RHNT-BPSCT) and obtained good outcomes. Bartolomeo et al. [18] conducted related non TCD ex-vivo haploid granulocyte colony-stimulating factor-mobilized bone marrow transplantation (RHNT-BMT) in 80 patients with malignant blood disease using intensive myeloablative conditioning regimen, and also obtained good outcomes. Nevertheless, a few studies concerned related HLA

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**Figure 4.** Peripheral blood T cell subsets in patients with various grades of aGVHD after transplantation. A. No statistical significance in the percentages of T cells, CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in grade I-II and grade III-IV aGVHD patients ( $P > 0.05$ ). B. ratio of CD4<sup>+</sup> CD25<sup>+</sup> T cells in grade I-II and grade III-IV aGVHD patients. C. ratio of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells in grade I-II and grade III-IV aGVHD patients. The number of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells was obviously decreased in patients with grade III-IV aGVHD.



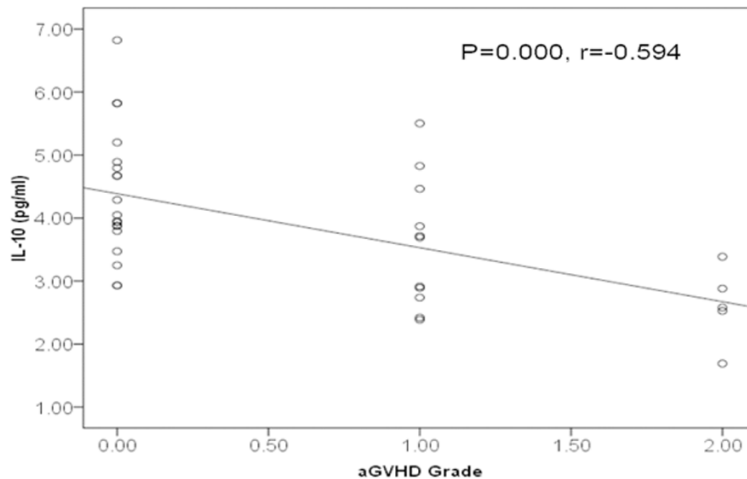
**Figure 5.** Serum IL-10 levels in the control, aGVHD and non-aGVHD groups. \* $P < 0.05$ , \*\* $P < 0.01$ , between groups after transplantation.

haploidentical non TCD *in vitro* HSCT taking G-mobilized peripheral blood as a graft [19-22]. From 2003, we designed and gradually improved a unique RHNT-PSCT protocol, that is: ① Infusing-mobilized high dose donor's peripheral blood mononuclear cells and CD34<sup>+</sup> cells without T-cell depletion *in vitro*; ② Ara-c,

Busulfa and Cyclophosphamide (ABU/CY2) + rabbit anti-human thymocyte immunoglobulin (rATG) was used as myeloablative conditioning regimen; ③ Cyclosporin A/tacrolimus + short-course methotrexate + mycophenolate mofetil + anti-CD25 monoclonal antibody + glucocorticoid served as enhanced GVHD prophylaxis regimen; ④ Intensive prevention and control measures against bacteria, fungi and viruses. Our previous research has shown that clinical curative effect is good [19, 23]. Compared with Huang's Beijing RHNT-BPSCT

protocol, [17] the biggest feature of our RHNT-PSCT protocol is to infuse 2 or 3 loci HLA-mismatched donors' high-dose G-mobilized PBSC without TCD *in vitro*. In the present study, infused number of mononuclear cells and CD34<sup>+</sup> cells were  $(14.65 \pm 3.04) \times 10^8/\text{kg}$  and  $(10.25 \pm 3.42) \times 10^6/\text{kg}$  respectively, and it

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**Figure 6.** Correlation of IL-10 and aGVHD grade. Spearman correlation analysis shows that in aGVHD patients, IL-10 concentrations were correlated negatively with the grade of aGVHD ( $r = -0.594$ ,  $P = 0.000$ ).

also contained high dose CD3<sup>+</sup> T cells apparently. No reports came down to the infusion of such a high dose PBSC without T-cell depletion *in vitro* for RH-HSCT. In this study, we focused on the reconstitution and clinical significance of T lymphocyte subsets of patients underwent our RHNT-PSCT. On the one hand, we longitudinally observed the recovery of T lymphocyte subsets in patients without aGVHD after transplantation, on the other hand, we horizontally compared the differences in Treg cells between aGVHD and non-aGVHD groups.

Results from this study demonstrated that CD3<sup>+</sup> and CD8<sup>+</sup> T lymphocytes cell in peripheral blood of patients without aGVHD recovered to normal levels at 60 and 90 days after transplantation. However, the recovery of CD4<sup>+</sup> T cells delayed obviously. Chang et al. [24]. Compared immune reconstitution of RHNT-BPSC and HLA-matched transplantation, found that the number of T cell subsets was lower in the haploidentical recipients group than in the HLA-matched group at 90 days after transplantation, especially, the number of CD4<sup>+</sup> and CD4<sup>+</sup> naïve T cells was obviously diminished. Our previous study also confirmed that the recovery of CD4<sup>+</sup> cells was identical between haploidentical and matched patients, and no difference in CD4<sup>+</sup> T cells was seen between patients at 1 year after transplantation and healthy controls which may be associated with high-dose PBSC infusion [19].

CD4<sup>+</sup> Treg cells have been shown to prevent and treat GVHD in recent studies [4, 25]. Of them, CD4<sup>+</sup> CD25<sup>+</sup> Treg cells are major components in above kind of cells. Foxp3 has been shown to specifically express in CD4<sup>+</sup> CD25<sup>+</sup> T cells, which is necessary for cell differentiation, development and maintaining function, and can be considered as a reliable mark for Treg cells [2]. Numerous studies verified that Foxp3 expression or the number of Foxp3<sup>+</sup> Treg cells in donor graft was strongly correlated with the occurrence of aGVHD [4, 15, 25]. Xhaard et al. [8]

demonstrated that total initial memory Treg cell subsets increased in 185 patients at 3-24 months after transplantation, but were still lower than healthy population within 2 years, and Treg cells were better reconstructed at 3 months after hematopoietic stem cell transplantation with low-intensity preconditioning taking peripheral blood as a graft. Fujioka et al. [9] proved that the ratio of CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T-cells: CD4<sup>+</sup> T-cells was significantly lower in aGVHD patients than that in non-aGVHD patients, and moreover, this specific ratio was the most significant value among all other possible lymphocyte-associated ratios and absolute cell counts. They considered that the frequency of CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T-cells might predict the incidence of aGVHD at 2 weeks after HLA-mismatched allogeneic HSCT which included patients receiving nonmyeloablative or myeloablative conditioning regimen, bone marrow or PBSCs as a graft. Above data primarily indicated that CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells might be contribute to restricting excessive immune response of effector T cells and maintaining dynamic equilibrium between them. In this study, our results showed that the ratio of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells (2.15 ± 1.02) % was lower at 30 days after transplantation in patients without aGVHD compared with the control group significantly, but gradually increased to (2.86 ± 1.31)% in 90 days after transplantation. Our previous clinical results of our RHNT-PSCT protocol demonstrated that the

incidence of severe aGVHD is lower, the transplant related mortality (TRM) and relapse rate is not high, and the overall survival is good [19, 23]. There was no significant difference in the percentage of CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T cells between aGVHD and non-aGVHD groups at 30 days after transplantation. However, the number of CD4<sup>+</sup> CD25<sup>+</sup> T cells, especially CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells, was apparently lower in aGVHD group than in non-aGVHD group and control group. CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells even could not be detected in patients with grade III-IV aGVHD. Compared with Fujioka et al.'s study, all patients in this study received myeloablative conditioning regimen and anti-CD25<sup>+</sup> mAb in GVHD prophylaxis regimen, Treg cells evidently delayed at 15 days, but better reconstituted at 30 days in patients without aGVHD after transplantation, the reason might be that anti-CD25<sup>+</sup> mAb affected CD4<sup>+</sup> lymphocytes recovery partially. However, CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells decreased dramatically in patients with severe aGVHD, indicates that the lower Treg cells at 30 days after transplantation possibly predicts the occurrence of aGVHD? In fact, other previous studies [1, 4, 26] also demonstrated that the number of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells after bone marrow + peripheral blood or bone marrow transplantation was strongly associated with the occurrence and the level of severity of aGVHD after allogeneic hematopoietic stem cell transplantation.

To prove it further, we observed serum IL-10 concentration change after transplantation. Usually, Treg cells immune suppression response by secreting IL-10 and transforming growth factor- $\beta$  mainly. IL-10 down regulates the proliferation and differentiation of effector T lymphocytes, and is considered a negative regulator for the occurrence of aGVHD [27-29]. In the present study, IL-10 level slowly increased at 30-90 days in non-aGVHD group, but were lower than in control group yet, which demonstrates that the number of Treg cells was restored to normal at 90 days after transplantation, but their functions might be defective. IL-10 levels were significantly lower in grade III-IV aGVHD group than in non-aGVHD group, and were correlated with the severity of aGVHD negatively. The change trends of IL-10 level and Treg cell number were consistent. When aGVHD occurred, the number of Treg cells reduced and serum IL-10 level also diminished, which fur-

ther indicate that rapid reconstruction of Treg cells after transplantation possibly decreased the risk of aGVHD. Our results strongly suggest that Treg cells, especially CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells, and IL-10 also might be the specific predictors for early forecast or even diagnosis of aGVHD after our RHNT-PSCT protocol.

In summary, using our RHNT-PSCT protocol, the reconstitution of peripheral blood T lymphocyte subsets in early stage is good. Treg cells, especially CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells, also play an important role in the occurrence and development of aGVHD, and there was a negative correlation between IL-10 and aGVHD, indicates that Treg cells and IL-10 also might be the specific predictors for early forecast of aGVHD after our RHNT-PSCT. However, what's the detailed mechanism and how about the immune network developed in our unique RHNT-PSCT are still unknown. We will get a multi-center study and gather large-sample to investigate it further.

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### Disclosure of conflict of interest

None.

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