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*Original Article*

# Peripheral blood Th9 cells reconstitution and its relationship with acute graft-versus-host disease after matched-sibling peripheral blood hematopoietic stem cell transplantation

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**Abstract:** T helper type 9 (Th9) cells have recently been identified as a new effector T cell subset. This study is to analyze the reconstitution of Th9 cell after matched sibling peripheral blood hematopoietic stem cell transplantation (MS-PBSCT) and the relationship between Th9 cell and acute graft-versus-host disease (aGVHD). Flow cytometry and ELISA were used to analyze the percentages of Th9 cell, levels of IL-9, TGF-β, IFN-γ, and IL-4. The results showed that for patients without aGVHD, Th9 cells recovery started from day 60 after transplantation and reached normal level on day 90. Serum TGF-β, IL-4, and IFN-γ reached normal levels on day 60, 60, and 90 post transplantation respectively. The serum IL-9 recovery is slower than that of IFN-γ and IL-4. For patients suffering from aGVHD, they had declined Th9 cell numbers, lower IL-9 and TGF-β levels, but higher serum IFN-γ level when compared with those without aGVHD after transplantation. Serum IFN-γ/IL-9 ratios increased linearly with grades of aGVHD. In conclusion, Th9 cells recovery is delayed after MS-PBSCT in patients with aGVHD but early in patients without aGVHD, indicating quick immune reconstitution of Th9 cells and IL-9 after MS-PBSCT may promote the immune tolerance.

**Keywords:** Allogeneic peripheral blood hematopoietic stem cell transplantation (allo-PBSCT), acute graft-versus-host disease (aGVHD), Th9 cells, IL-9

## Introduction

At present, allogeneic hematopoietic stem cell transplantation (allo-HSCT) is one of the best treatment options for some malignant hematological diseases [1, 2]. It is accepted that T cell function recovery after allo-HSCT is very important. Some T-cell subsets reconstitution is important for incidence of infection, and some T-cell subsets activation, proliferation, and differentiation are considered determining factors for GVHD development after allo-HSCT [3]. aGVHD has been considered a Th1-type disease based on the predominance of cytotoxic T lymphocyte (CTL)-mediated pathology and the increased production of Th1-type cytokines, including IFN-γ, TNF-α [4, 5]. However, Th17 cells have also been implicated in the induction of aGVHD [6]. Recent studies have shown that $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells play indispensable roles in the maintenance of immune tolerance after allo-HSCT [7, 8]. In our previous study [9], we found that, in the early stages after our HLA-haploidentical PBSCT, the $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells may predict aGVHD, and regular monitoring of it is crucial for understanding the clinical manifestations of patients. In addition to the Treg cells, Th9 cells are newly identified $CD4^+$ T cells subset who play key roles in the immune responses. When stimulated by TGF-β together with IL-4, Th0 cells would differentiate into Th9 cells. When used separately, TGF-β alone would cause Treg development, while IL-4 would induce Th2 cell differentiation

**Table 1.** Characteristics of patients and donors

| | N (%) |
|---|---|
| Patient age, y (median, range) | 32 (23-55) |
| Donor age, y (median, range) | 38 (20-53) |
| Patient sex | |
| Male | 15 (48.39%) |
| Female | 16 (51.61%) |
| Donor sex | |
| Male | 12 (38.71%) |
| Female | 19 (61.29%) |
| Hematological disease | |
| Acute lymphoid leukemia | 9 (29.03%) |
| Acute myeloid leukemia | 14 (45.16%) |
| Chronic myeloid leukemia | 6 (19.35%) |
| Myelodysplastic syndrome | 2 (6.45%) |
| Disease stage | |
| Standard-risk | 17 (54.84%) |
| High-risk | 14 (45.16%) |
| Donor blood type match | |
| Match | 18 (58.07%) |
| Mismatch | 13 (41.94%) |
| Median infused values | |
| $CD34^+$, $10^6$/kg (median, range) | 5.61 (1.2-11.4) |
| MNC, $10^8$/kg (median, range) | 10.49 (7.29-14.77) |
| Acute GVHD (aGVHD) | |
| Grades I-II | 9 (29%) |
| Grades III-IV | 2 (6.45%) |
| No aGVHD | 20 (64.52%) |
| Acute GVHD onset, day after PBSCT (median, range) | 47 (23-91) |

[10-12]. Studies have shown Th9 cells primarily secrete IL-9 to mediate the immune response in several diseases, such as asthma, autoimmune diseases, and parasitic infections [13-15]. IL-9 is associated with impaired Th1 immune response in patients with tuberculosis, due to decreased IL-12 production and limited APC function [16]. The role of Th9 cells in the pathogenesis of GVHD remains to be clarified. In solid organ transplantation models, IL-9 expression by iTregs has been shown to promote graft tolerance through the recruitment of immunosuppressive mast cells [17]. A recent study has shown that, Th9 cells not only continue to differentiate in the presence of rapamycin, but also maintain the cytokine phenotype, generating high levels of IL-9 *in vivo*, inhibiting the IFN-γ-driven allo-reactivity [18]. However, the role of Th9 cells in the pathogenesis of aGVHD after allo-HSCT for patients is still unknown. In this study, the reconstitution of Th9 cells and the changes in Th9-related cytokines after MS-PBSCT, were analyzed. The relationship between Th9 cells and aGVHD was also preliminary explored.

## Patients, materials and methods

### *Patients and healthy individuals*

Totally 31 patients undergoing allo-PBSCT were included in this study, who were admitted to our center from January 2012 to June 2015. Patients who relapsed or died in the first 5 months after PBSCT were excluded. Basic characteristics of patients and donors were presented in **Table 1**. If no aGVHD occurred after transplantation, peripheral blood and serum samples were collected from each patient, on days 30, 60, 90, and 150 after transplantation; when aGVHD occurred after transplantation, peripheral blood sampling was performed immediately. Cell percentages and cytokine levels in patients with aGVHD were compared with those in patients without aGVHD at day 30 after transplantation. On the other hand, peripheral blood samples were also collected from 20 age- and gender-matched healthy control (HC) subjects. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of the First Affiliated Hospital of Xinjiang Medical University (approval No. 20130411-07). We confirm that all methods were performed in accordance with the ethical guidelines of the First Affiliated Hospital of Xinjiang Medical University.

### *Conditioning regimen and aGVHD prophylaxis*

31 patients who underwent MS-PBSCT were subjected to the modified Bu/Cy conditioning regimen: intravenous infusion of 2 g/$m^2$/d Ara-C (days -9 and -8), intravenous infusion of 3.2 mg/kg/d busulfan for 4 days (days -7 to -5), and intravenous infusion of 60 mg/kg/d cyclo-

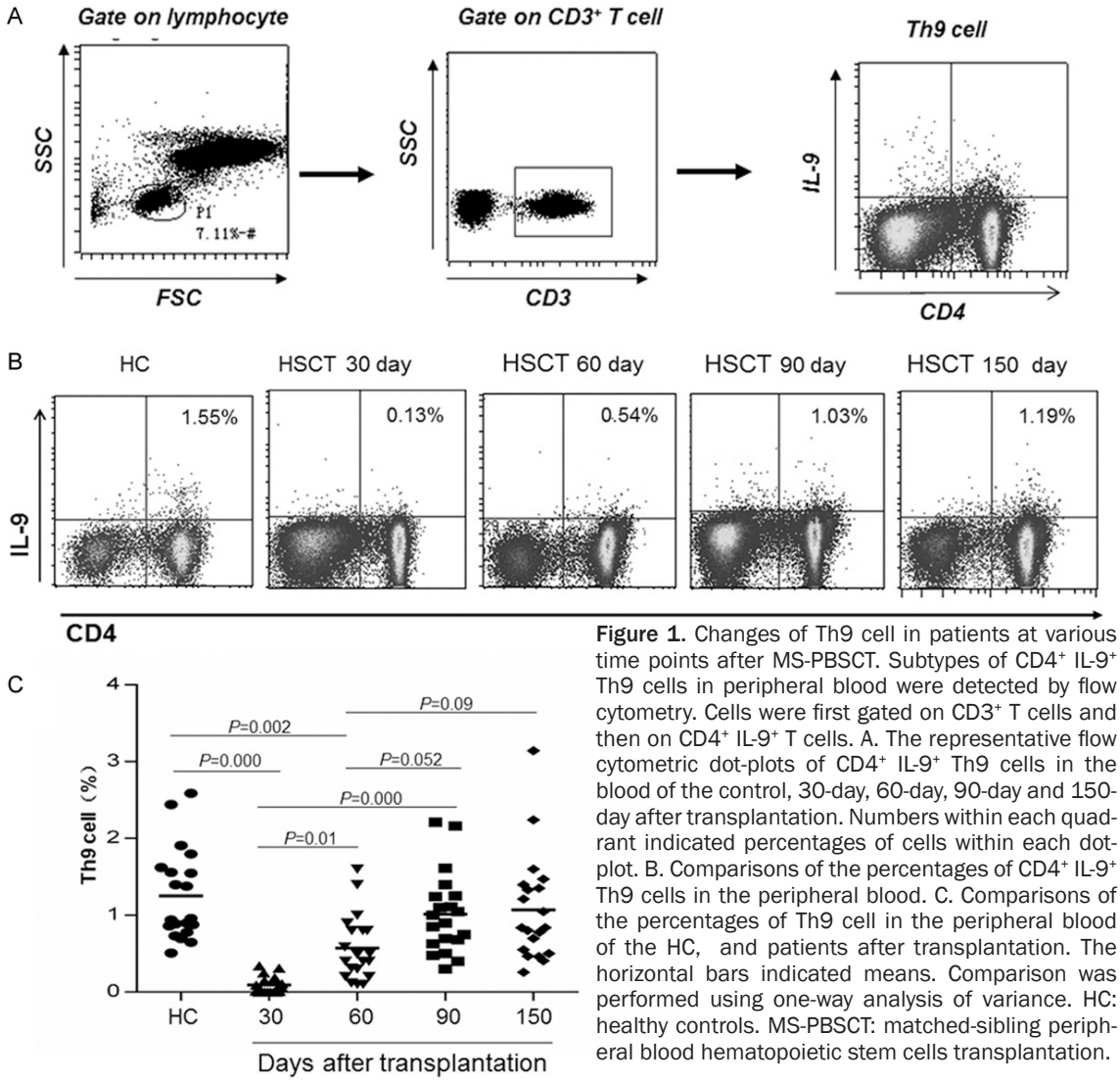


**Figure 1.** Changes of Th9 cell in patients at various time points after MS-PBSCT. Subtypes of $CD4^+$ $IL\text{-}9^+$ Th9 cells in peripheral blood were detected by flow cytometry. Cells were first gated on $CD3^+$ T cells and then on $CD4^+$ $IL\text{-}9^+$ T cells. A. The representative flow cytometric dot-plots of $CD4^+$ $IL\text{-}9^+$ Th9 cells in the blood of the control, 30-day, 60-day, 90-day and 150-day after transplantation. Numbers within each quadrant indicated percentages of cells within each dot-plot. B. Comparisons of the percentages of $CD4^+$ $IL\text{-}9^+$ Th9 cells in the peripheral blood. C. Comparisons of the percentages of Th9 cell in the peripheral blood of the HC, and patients after transplantation. The horizontal bars indicated means. Comparison was performed using one-way analysis of variance. HC: healthy controls. MS-PBSCT: matched-sibling peripheral blood hematopoietic stem cells transplantation.

phosphamide (days -3 and -2). All the patients received cyclosporine A (CsA) + short methotrexate (MTX) + mycophenolatemofetil (MMF) for aGVHD prophylaxis: intravenous infusion of 2.5 mg/kg/d CsA (day -5 to day +30, continuous administration for 24 h) and oral administration of 3-5 mg/d CsA (day +30 to day +100), adjusted the concentration to 200-400 ng/ml; intravenous infusion of 15 mg/$m^2$ MTX (day +1) and 10 mg/$m^2$ MTX (day +3, +6, and +11); oral administration of 500 mg/d MMF (day +1 to +30).

*Flow cytometry*

Peripheral blood mononuclear cells (PBMCs) were isolated with the Ficoll-Hypaque density centrifugation (Amersham Biosciences, Amersham, Buckinghamshire, UK). PBMCs were suspended at a density of $2\times10^6$ cells/ml in the Roswell Park Memorial Institute (RPMI) media 1640 supplemented with GlutaMAX (Gibco, Grand Island, NY, USA). Then $2\times10^6$ PBMCs were seeded onto the 9-well plate, and stimulated with 50 ng/ml phorbolmyristate acetate (PMA), 1 μM ionomycin (Sigma), and 500 ng/ml monensin (all from Sigma, St. Louis, MO, USA), for 4 h.

For the intracellular staining of cytokines, cells were first stained for the CD3 and CD4 surface antigen, treated with Fix/Per (Fix/Per buffer; eBiosciences, San Diego, CA, USA), and then stained for IL-9, according to the manufacturer's instructions. Fluorescence was detected with a FACS Calibur flow cytometer, and the data were analyzed with the Flow Jo software (Tree Star Inc., Ashland, OR, USA).

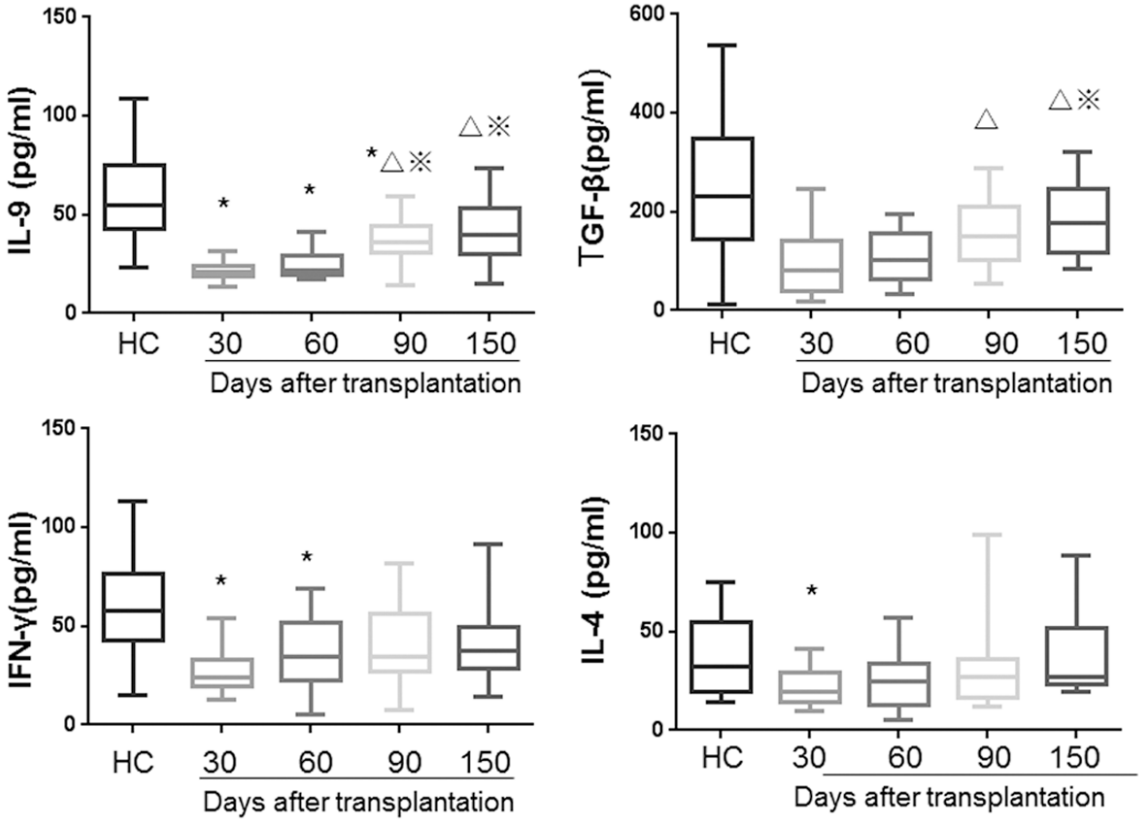


**Figure 2.** Serum cytokine levels in patients without aGVHD after transplantation. Serum levels of IL-9, IFN-γ, TGF-β, and IL-4 in the patients from the MS-PBSCT group (on days 30, 60, 90, and 150 after transplantation), as well as in the healthy control (HC) subjects, were detected with ELISA. Compared with the HC group, $^{*}P < 0.05$; compared with day 30 after transplantation, $^{\Delta}P < 0.05$; compared with the day 60 after transplantation, $^{※}P < 0.05$.

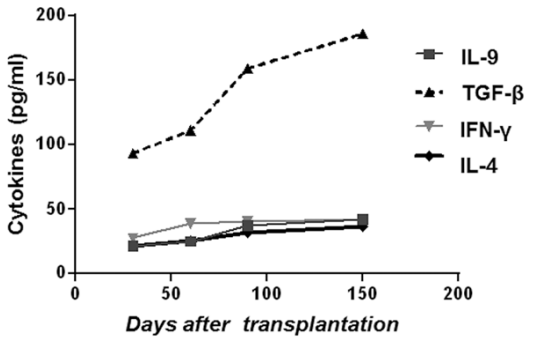


**Figure 3.** Dynamic change of IL-9, IFN-γ, TGF-β, and IL-4 in patients without aGVHD. IL-9 and TGF-β levels were significantly increased and then gradually recovered after transplantation.

*Enzyme-linked immunosorbent assay (ELISA)*

Serum samples were collected from the PBSCT patients and healthy controls. Serum levels of IL-9, TGF-β, IFN-γ, and IL-4 were determined with the ELISA kits (eBioscience), according to the manufacturer's instructions.

*Statistical analysis*

Data were expressed as mean ± SD. SPSS13.0 software package was used for statistical analysis. Logarithmic transformation was performed for data not following normal distribution. One-way analysis of variance (ANOVA) was used for group comparison, with homogeneous variances, while the rank sum test was used for non-homogeneous variances. *Spearman* rank correlation analysis was used for correlation analysis. $P < 0.05$ was considered statistically significant.

## Results

*Patients*

All patients achieved a sustained and stable donor engraftment For the 31 patients receiving MS-PBSCT, hematopoietic reconstitution was analyzed. For all the patients, the absolute neutrophil counts in the engrafted patients exceeded $0.5\times10^9$/L in a median time of

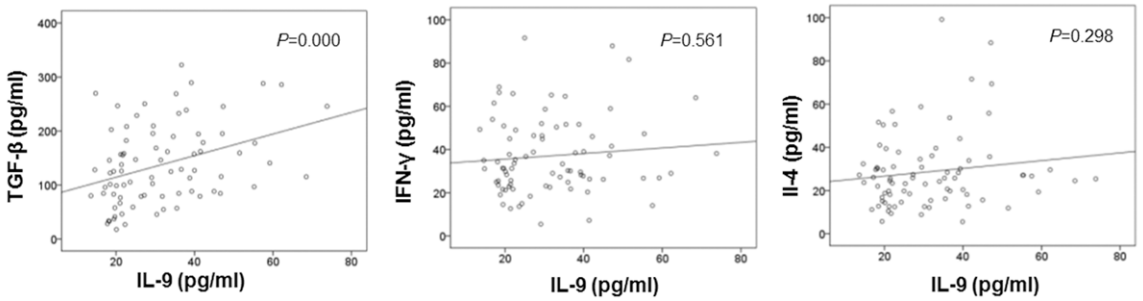


**Figure 4.** Correlation analysis of cytokines in patients without aGVHD after transplantation. Data was analyzed with Spearman correlation analysis. IL-9 was positively correlated with the concentration of TGF-β (*P*=0.000, r=0.381), but not IFN-γ (*P*=0.561) and IL-4 (*P*=0.298).

14.56±2.76 d, and the platelet counts exceeded $20\times10^9$/L in a median time of 14.16±3.67 d. All the patients had no serious bacteria, viruses and fungi infection after transplantation. Among the 31 patients, 11 patients had aGVHD and 20 patients had no aGVHD. The median day of onset of aGVHD was 47 (range, 23 to 91). Of the patients with aGVHD, including 9 patients with grades I-II aGVHD and 2 patients with grades III-IV Agvhd (**Table 1**).

*Peripheral blood Th9 cells reconstitution and Serum cytokine levels recovery after transplantation after transplantation*

There were 20 patients without aGVHD within 150 days after MS-PBSCT. Blood samples were collected from these patients on days 30, 60, 90, and 150 after MS-PBSCT. Th9 cells in the peripheral blood were analyzed with flow cytometry, and the results were compared between the healthy control (HC) and MS-PBSCT patients. Our results showed that the circulating $CD4^+$ $IL\text{-}9^+$ Th9 cell ratios in the patients were significantly lower on day 30 (0.09±0.11%) and 60 (0.57±0.42%) compared with HC (1.25±0.6%) after transplantation (both $P < 0.01$; **Figure 1**). However, $CD4^+$ $IL\text{-}9^+$ Th9 cell ratios of patients returned to normal on days 90 (*day 90 vs HC, P=0.859*), and maintained the same level on days 150 (*day 150 vs HC, P=0.998*) after transplantation (**Figure 1**). To determine the serum levels of cytokines in these patients, ELISA was performed. As shown in **Figures 2** and **3**, compared with HC, the serum levels of IL-9, TGF-β, IFN-γ, and IL-4 were significantly decreased on days 30 after transplantation. For the serum level of IL-9, a gradual increasing trend was observed along with the days 30, 60, and 90 after transplantation (all significantly lower than the HC, *P*<0.01), while normal serum IL-9 level was reached on day 150 after transplantation (compared with the HC, *P*=0.082). For the serum level of TGF-β, IFN-γ and *IL-4* normal levels were observed on day 60, 90 and 60 after transplantation (*P*=0.245 for TGF-β; *P*=0.069 for IFN-γ and *P*=0.325 for IL-4). These results suggest that, the serum levels of IL-9 are significantly decreased after transplantation, which are recovered a little slowly than IFN-γ, TGF-β and IL-4 (**Figures 2**, **3**). Spearman analysis showed that, the serum level of IL-9 was positively related to TGF-β (r=0.381, *P*=0.000), however IL-9 was not related to IFN-γ or IL-4 (**Figure 4**).

*Peripheral blood Th9 cells, related cytokines are decreased in patients with aGVHD*

The Th9 cells and related cytokines in the patients (n=11) with and without aGVHD (day 30, n=20) (aGVHD+ and aGVHD-, respectively) were next investigated. Our results showed that, the percentage of Th9 cells in aGVHD- patients were significantly higher than the aGVHD+ patients (*P*=0.018). In 5 aGVHD+ patients, it is even hard to detect Th9 cells in the Peripheral blood. However, the percentage of Th9 cells in the aGVHD+ and aGVHD- patients were both lower than the healthy control ($P < 0.01$) (**Figure 5A**). For the cytokine detection, the IL-9 levels in both the aGVHD+ and aGVHD- patients were significantly lower than the HC group (both $P < 0.01$). Moreover, the IL-9 level was generally higher in the aGVHD- patients, compared with the aGVHD+ patients (*P*=0.034, **Figure 5B**). In the early stage after transplantation, The TGF-β concentration was higher in the aGVHD- patients (93.21±62.08 pg/ml) than the aGVHD+ patients (45.22±19.81 pg/ml)

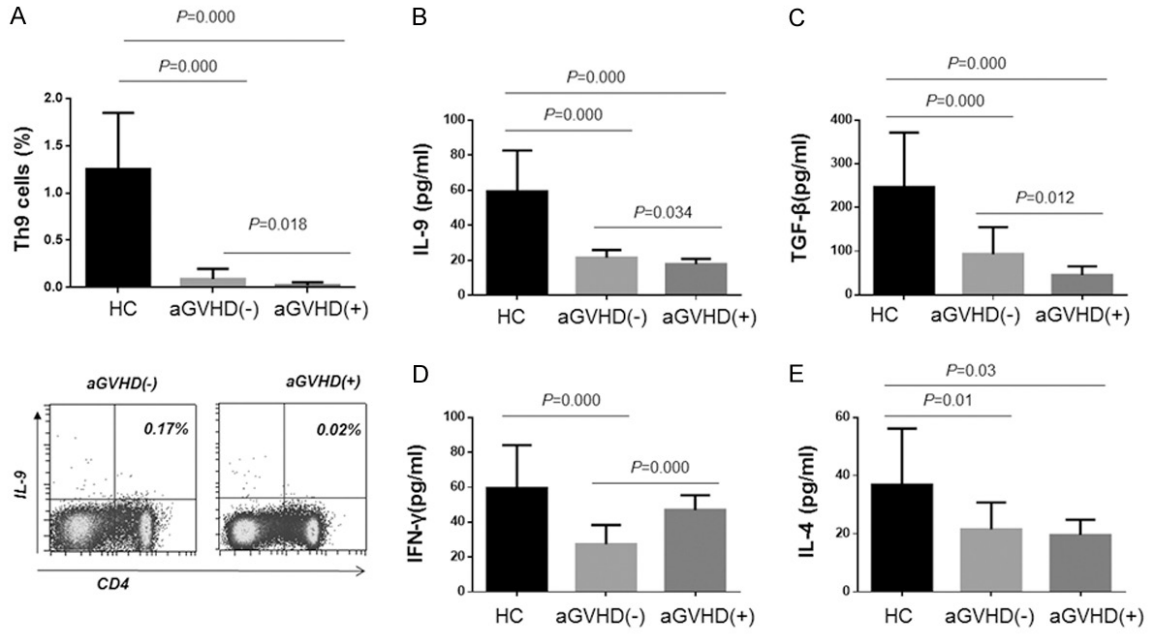


**Figure 5.** Th9 cell percentage and associated cytokine levels in patients with or without aGVHD after transplantation. (A) Th9 cell percentage in the patients with or without aGVHD. Serum levels of IL-9 (B), TGF-β (C), IFN-γ (D), and IL-4 (E) in the patients with or without aGVHD after transplantation were detected with ELISA. In the aGVHD(+) group, IL-9 and TGF-β concentration was lower in the aGVHD(+) group than in aGVHD(-) group, but IFN-γ concentration was higher in the aGVHD(+) group.

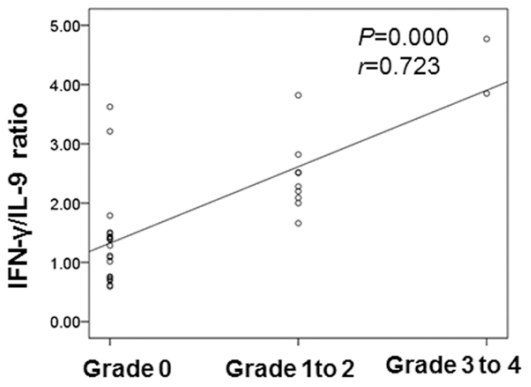


**Figure 6.** Correlation between the IFN-γ/IL-9 ratio and aGVHD grade. Correlation between the IFN-γ/IL-9 ratio and aGVHD grade was analyzed by the Spearman correlation analysis. Patients without aGVHD (grade 0), patients with mild aGVHD (grade 1 to 2), patients with sever aGVHD (grade 3 to 4). IFN-γ/IL-9 ratio was positively correlated with the severity of aGVHD ($P$=0.000, $r$=0.723).

($P$=0.012; **Figure 5C**), which were both lower than the healthy control ($P$ < 0.01). However, the level of IFN-γ was higher in the aGVHD+ patients (46.97±8.39 pg/ml) than the aGVHD- patients (27.33±10.98 pg/ml) ($P$ < 0.01; **Figure 5D**), aGVHD+ patients showed no significant difference compared with the healthy control. Furthermore, the IL-4 concentration in the aGVHD+ and aGVHD- patients showed no significant differences, which was however lower than the healthy control (both $P$ < 0.05; **Figure 5E**). In addition, the IFN-γ/IL-9 ratio in the aGVHD+ patients (2.77±0.96) was significantly higher than the aGVHD- patient (1.36±0.79) ($P$ < 0.01). Taken together, these results suggest that the IFN-γ level is elevated, while Th9 cells, the IL-9 and TGF-β levels are declined in the aGVHD patients.

*Correlation between IFN-γ/IL-9 ratio and aGVHD severity*

Among these cytokines, serum IFN-γ/IL-9 ratio was positively correlated with the severity of aGVHD after transplantation ($P$=0.000, $r$= 0.723; **Figure 6**). Therefore, it is possible that the reconstitution of IL-9 in the early stage after transplantation might be due to the lower rate of aGVHD.

## Discussion

After allogeneic stem cell transplantation, the recovery of immune system is always a slow and incomplete process. The innate immunity often reconstitutes quickly following transplan-

tation, and adaptive B- and T-cell lymphopoiesis may be compromised for years. The thymus is the primary site for T-cell development. When peripheral T-cell population is severely depleted, the renewed thymic activity can contribute to T-cell reconstitution, producing naïve CD4 helper, CD8 cytotoxic effector, and $CD4^+$ $CD25^+$ Treg cells [19]. And immune reconstitution following transplantation is closely linked to the development of acute graft-versus-host disease (aGVHD), which influences infectious complications [20]. In this study, blood Th9/IL-9 levels were monitored following MS-PBSCT for hematologic malignance to explore its role in the immune reconstitution. Our results showed that, the Th9 cells and IL-9 levels were significantly increased and then gradually recovered on day 90 and day 150 after transplantation and the patients with aGVHD showed lower IL-9 levels than the patients without, indicating that aGVHD may impair Th9 cell reconstruction.

Twenty years ago, the first helper T cell was found to secrete IFN-γ. From then on, further investigation showed that the vast helper T cell family has been steadily expanded by the IL-4-secreting Th2 cells, immune regulating Treg cells, and so on. Currently, Th9 cells have been also recognized as a new helper T cell subtype. Like Th9 cells in mice, human Th9 cells could produce IL-9, but not IL-10 [21]. IL-9 has been thought to be a Th2 cytokine for a long time, because it promotes the allergic inflammation and is associated with various Th2 responses [10, 11]. In addition, IL-9 could be secreted by different inflammatory cells to mediate inflammation responses, including T cells, B cells, Mast cells, and neutrophils [22, 23]. IL-9 is also part of the inflammation response of Th17 cells and Treg cells, regulating the immune functions [24, 25]. Despite the various roles of IL-9, Th9 cells are primarily pro-inflammatory cells functioning in the allergic inflammation and autoimmune diseases [25, 26]. Th9 cells have also been shown to be able to induce protective antitumor immunity by eliciting tumor-specific CTL response [27], but the role of Th9 cells in graft rejection has not been thoroughly studied [28], especially for MS-PBSCT. In our study, we found that, the chemotherapy and immunosuppressive treatments before HSCT severely damaged the immune system in the patients. It took 60 days before the Th9 cells began to show restoring signs, but no normal levels were reached until day 90 after transplantation.

IL-9 secreted by Th9 cells is a multi-functional cytokine, which can up-regulate or down-regulate the immune function. Studies have shown IL-9, IL-4, IL-5, and other Th2 cell cytokines participate in the immune response of CD8-deficient mice. In the major histocompatibility complex class II disparate model, heart allografts from IL-9 transgenic donors suffer from acute rejection, whereas grafts from wild-type donors do not. The expression of IL-9 mRNA has been detected during the immune rejection [29]. Moreover, after skin grafting, IL-9 will increase the $Foxp3^+$ Treg cell immune suppression functions, suppressing the host immune responses [30]. Our results showed that, the serum IL-9 levels were consistent with the Th9 cell numbers. Serum IL-9 level was increased on day 30 after transplantation, and reached to normal levels until day 150 after transplantation. Our previous study has shown that, the peripheral blood hematopoietic cell-transplanted patients show early Treg cell restoration, which is able to stimulate the immune tolerance. Treg cells down-regulate the immunity through secreting TGF-β and IL-10. Among the Treg cell secreted cytokines, TGF-β has been attracting considerable interest with the regards of the immunologic outcomes after allo-HSCT [30, 31]. Moreover, in this study, TGF-β recovered quickly after transplantation. Furthermore, for the correlation analysis, IL-9 was also positively correlated with TGF-β after MS-PBSCT. Although IL-9 cannot improve the Treg cell growth and proliferation, it could increase the $Foxp3^+$ Treg cell-induced suppression of $CD4^+$ T cells [32]. TGF-β can stimulate the secretion of IL-9 by $CD4^+$ $CD25^+$ T cells, and also stimulate the differentiation of Th0 cells into Th9 cells [33]. Our results showed that in patients without aGVHD, Th9 cells, IL-9 and TGF-β gradually recovered after transplantation, which may better avoid the occurrence of aGVHD. Therefore, we hypothesize that TGF-β and IL-9 may mediate the immune tolerance by improving the function of Treg cells after transplantation. IL-9 might serve as a protective cytokine following the MS-PBSCT. The IFN-γ and IL-4 levels were also monitored and analyzed in these 20 patients after transplantation. For the serum level of IL-4 and IFN-γ, normal levels were observed on day 60 and 90 after transplantation, the serum levels of IL-9 recovered a little slowly than TGF-β and IL-4. These results suggest that may prove that Th9

cell development requires the presence of TGF-β and IL-4 [10, 11].

Further studies show that, the IFN-γ level is elevated, while the IL-9 and TGF-β levels are declined in the aGVHD patients. Host alloreactivity pre-transplantation therapeutic drug treatments increased the inflammatory Th1-related cytokines of IL-2 and IFN-γ, which may play a key role in aGVHD by amplifying immune responses. Meanwhile, the Th2 cytokines of IL-4 has the opposite effect against the Th1 responses [34-37]. Our results found that the patients suffering from aGVHD had significantly higher blood IFN-γ level, as well as lower IL-9 and TGF-β levels, than those without aGVHD. There is debate on the effects of Th2 cytokines after transplantation. Chen et al. [38] have found that, the Th2 cytokines of IL-4 and IL-10 are increased in the patients suffering from aGVHD after transplantation. However, another study has found that IL-4 levels are higher in the aGVHD+ patients than the aGVHD- patients [39]. We found out that no significant differences in IL-4 were observed between these patients. In addition, the IFN-γ/IL-9 ratio was positively correlated with the disease severity, which may be associated with the occurrence and development of aGVHD. Our results suggest a complicated cytokine network responsible for maintaining and preventing aGVHD after transplantation. Other than the abnormal Th1/Th2 pattern, aGVHD+ patients also exhibited irregular Th1/Th9 patterns as well. Therefore, it is important to monitor the IL-9, TGF-β, and IFN-γ levels to detect early symptoms of aGVHD. Therefore, in the early stage of transplantation, if IL-9 and TGF-β levels are not increased whereas IFN-γ level is significantly increased, aGVHD might more easily occur. As samples were obtained at predefined time points, it is necessary to further study and large number of patients to explore the role of Th9 /IL-9 in acute or chronic GVHD.

In conclusion, our results show that the faster immune reconstitution of Th9 cells and related cytokines IL-9, TGF-β may promote the formation of immune tolerance after MS-PBSCT. Additionally, the increase of IL-9 and TGF-β in the early phase after transplantation may lower the risk of aGVHD. IL-9 may also be used as a biomarker to predict the development of aGVHD in the future. Therapeutic treatments targeting on the Th9/IL-9 profile may increase the immune tolerance and provide alternative treatment options for aGVHD. These findings might help understanding the roles of Th9 cells in MS-PBSCT, as well as the aGVHD after transplantation, in clinic.

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## Disclosure of conflict of interest

None.

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