Increased Type 1 Immune Response in the Bone Marrow Immune Microenvironment of Patients with Poor Graft Function after Allogeneic Hematopoietic Stem Cell Transplantation

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A B S T R A C T
Poor graft function (PGF) is a severe complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The question of whether the bone marrow (BM) immune microenvironment is involved in the pathogenesis of PGF remains unresolved. In total, 10 patients with PGF, 30 matched patients with good graft function after allo-HSCT, and 15 healthy donors were enrolled in this nested case-control study. The Th1, Th2, Tc1, Tc2, and active phenotypes were analyzed by flow cytometry. IFN-γ and IL-4 levels in BM plasma were evaluated using cytometric beads assay. Relative to other subjects, patients with PGF had significantly higher proportions of stimulated CD4+ and CD8+ T cells that produced IFN-γ (Th1 and Tc1 cells) but notably decreased proportions of IL-4-producing T cells (Th2 and Tc2 cells), resulting in a shift of the IFN-γ/IL-4 ratio towards a type 1 response and an elevated percentage of activated CD8+ T cells. Changes in IFN-γ and IL-4 levels in BM plasma were consistent with the cellular results. Our results suggest that dysregulated T cell responses may contribute to the occurrence of PGF after HSCT.

INTRODUCTION
Allogeneic (allo) hematopoietic stem cell transplantation (HSCT) is regarded as an effective treatment for patients with hematological malignancies; for such patients, hematopoietic recovery is crucial for successful transplantation [1]. Poor graft function (PGF) characterized by pancytopenia renders patients susceptible to life-threatening infections and bleeding, thereby remaining a success-limiting complication after allo-HSCT [2–6], particularly in HLA-mismatched cases [7]. Our previous studies showed that PGF patients had defective perivascular cells and endothelial progenitor cells in the bone marrow (BM) microenvironment [8,9], which may be involved in the occurrence of PGF. Nonetheless, the exact mechanisms underlying PGF remain incompletely elucidated.

The BM microenvironment in which hematopoietic stem cells (HSCs) reside plays a fundamental role in HSCs maintenance: here, cellular components provide signals that regulate the self-renewal, differentiation, and quiescence of HSCs [10,11]. In addition to stromal cells, the BM hosts various mature immune cell types, including T cells, B cells, dendritic cells, and macrophages, constituting the immune microenvironment that regulates HSCs hematopoiesis and maintaining homeostasis [11,12]. Observations in an allogeneic BM transplantation mouse model have indicated that the T cells in the BM microenvironment differ from those in the peripheral blood in terms of surface receptors, cytokine secretion, and immune functions [13]. Clinical and experimental approaches have suggested that the T cells in the BM immune microenvironment modulate HSCs hematopoiesis mainly by secreting cytokines, such as hematopoiesis-promoting cytokines, including IL-3, IL-7,
granulocyte-macrophage-colony stimulating factor [14], and inflammatory cytokines, including IFN-γ and TNF-α [15,16]. Depending on the immune response and the amount of cytokine secretion, the immune microenvironment also has suppressive effects on hematopoiesis. Studies have demonstrated that in aplastic anemia (AA), a BM failure syndrome, patients’ dysregulated CD8⁺ cytotoxic T cells and CD4⁺ T cells, including Th1 cells, Th2 cells, regulatory T cells, and Th17 cells, along with elevated production of cytokines, including IFN-γ and TNF-α, in the BM microenvironment suppress proliferation and induce apoptosis in HSCs [17-22]. Therefore, we speculate that the occurrence of PGF, most likely in the form of AA, may also be associated with dysregulated immune responses in the BM microenvironment.

To confirm our hypothesis, we used flow cytometry to analyze components of the BM microenvironment in human BM samples, including subtypes of CD4⁺ T cells and CD8⁺ T cells. In addition, we assessed whether allo-HSCT recipients with PGF differed from allo-HSCT recipients without PGF and healthy donors (HD) with respect to the levels of the aforementioned components of the BM immune microenvironment.

**MATERIALS AND METHODS**

**Patients and Controls**

In this nested case-control study, cases were identified from patients who underwent allo-HSCT for hematologic malignancies between February 1, 2015, and July 18, 2015 at Peking University Institute of Hematology. The study only included patients with acute leukemia before allo-HSCT, since the research suggests increased risk of graft failure in AA, chronic leukemia, and myelodysplastic syndrome [23]. A total of 10 patients who had developed PGF after allo-HSCT were included; for each PGF case, 3 matched patients with good graft function (GGF) (n = 30) were randomly selected from the same cohort at the time when PGF occurred. These control cases were matched using the following criteria: age at HSCT (±1 years), underlying disease, pre-HSCT cycles of chemotherapy (±1 cycle), and disease status at HSCT (“risk-set sampling”) [24]. Characteristics of the PGF patients and GGF controls are summarized in Table 1. BM samples from 10 male and 5 female HD were used as healthy controls. Donor ages ranged from 21 to 48 years (median, 41 years). This study

**Table 1**

| Characteristics                  | PGF (n = 10) | GGF (n = 30) | P Value
|----------------------------------|--------------|--------------|----------
| BM evaluated time, after HSCT, median (range), d | 94 (53-375) | 94.5 (24-561) | .3585    |
| Blood cell count                 |              |              |          |
| WBC, median (range), ×10⁹/L      | 1.95 (3-6.5) | 4.67 (2.64-9.83) | .0009    |
| ANC, median (range), ×10⁹/L      | 1.39 (1-6)   | 2.72 (0.89-5.42) | .9966    |
| Hb, median (range), g/L          | 83.5 (67-90) | 118 (85-165) | <.0001   |
| PLT, median (range), ×10⁹/L      | 22.5 (4-53)  | 146.5 (31-252) | <.0001   |
| Age at HSCT median (range), yr   | 30 (4-53)    | 26 (7-51)    | .0871    |
| Gender (male/female)             | 6/4          | 20/10        | >.9999   |
| Underlying disease               |              |              |          |
| AML                              | 5            | 15           |          |
| ALL                              | 5            | 15           |          |
| Status at HSCT                   |              |              |          |
| Standard-risk                    | 5            | 11           |          |
| High-risk                        | 5            | 19           |          |
| Source of stem cell              |              |              | >.9999   |
| G-BM and G-PB                    | 10           | 28           |          |
| G-PB                             | 0            | 2            |          |
| Transplanted total nucleated cell dose, median (range) ×10⁹/kg | 7.75 (4.98-9.29) | 7.77 (5.32-13.81) | .6192    |
| Transplanted CD34⁺ cell dose, median (range), ×10⁹/kg | 2.04 (1.09-2.74) | 2.37 (0.85-6) | .0622    |
| Donor match                      |              |              |          |
| HLA-identical unrelated donor    | 0            | 2            | .7538    |
| HLA-identical sibling donor      | 3            | 9            |          |
| HLA-partially matched related donor | 7     | 19           |          |
| Sex mismatch                     |              |              | .6006    |
| Female to male                   | 3            | 7            |          |
| Female to male                   | 0            | 2            |          |
| Male to female                   | 4            | 8            |          |
| Male to male                     | 3            | 13           |          |
| ABO mismatch                     |              |              | .5430    |
| No                               | 2            | 12           |          |
| Minor                            | 3            | 6            |          |
| Major                            | 5            | 12           |          |
| Pre-HSCT cycles of chemotherapy  | 4 (3-6)      | 4 (1-11)     | .2608    |
| Conditioning                     |              |              | >.9999   |
| BU/CY                            | 3            | 9            |          |
| BU/CY-ATG                        | 7            | 21           |          |
| History of aGVHD                 | 6            | 15           | .7209    |
| Target organ- skin/intestine/liver | 5/3/1     | 13/4/1       | .5015    |
| Degree I-II/III-IV               | 5/1          | 13/2         | >.9999   |
| History of CMV reactivation      | 9            | 16           | .0599    |

Allo-HSCT, allogeneic hematopoietic stem cells transplantation; PGF, poor graft function; GGF, good graft function; BM, bone marrow; WBC, white blood cells; Hb, hemoglobin; PLT, platelet; HLA, human leukocyte antigen; CMV, cytomegalovirus; ANC indicates absolute neutrophil cell; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; G-BM, recombinant human granulocyte colony-stimulating factor–primed bone marrow allo-grafts; G-PB, recombinant human granulocyte colony–stimulating factor–primed peripheral blood allo-grafts; Bu, busulfan; Cy, cyclophosphamide; ATG, antithymocyte globulin; aGVHD, acute graft-versus-host disease.

1 Group matching criteria included age at HSCT (±1 years), pre-HSCT cycles of chemotherapy (±1 cycle), underlying disease and disease status at HSCT. For each PGF case, 3 GGF controls were randomly selected from the same cohort at which the PGF occurred (“risk-set sampling”).

2 The blood cell counts were higher than the criterion of PGF since they had to accept RBC or PLT transfusions and rh-CSF administration to avoid infection, anemia, and bleeding.
cytometric analyses were performed using a BD LSR Fortessa instrument (BD legend, San Diego, CA). After incubation, RBCs were lysed and white blood cells PE (3D12) (BD Biosciences, San Jose, CA), and HLA-DR-APC-Cy7 (L243) (Bio-following directly conjugated mouse antihuman monoclonal antibodies
Lymphocyte subpopulations were determined by separate MNCs from 8 mL BM obtained by puncturing the posterior superior Surface Immunophenotyping

As indicated in Table 1, allo-HSCT patients with PGF and allo-HSCT patients with GGF did not significantly differ with respect to demographic or clinical characteristics, including age, gender, underlying disease, disease status before transplant, median time from diagnosis to transplantation, source of stem cells, dose of transplanted total nucleated cells, dose of CD34+ cells, donor HLA match, sex/ABO mismatch, pre-HSCT chemotherapy cycles, conditioning, history of GVHD, or history of CMV (P > .05 for all comparisons).

Blood and BM Cellularity

The hemogamms of the PGF patients exhibited significant pancytopenia relative to the hemograms of GGF controls. Evaluations of blood in the PGF and GGF groups revealed dramatically lower median levels of WBC (1.95 × 10^3/L) versus...
4.67 × 10⁹/L, P < .0005), Hb (83.5 g/L versus 118 g/L, P < .0001), and PLT (22.5 × 10⁹/L versus 146.5 × 10⁹/L, P < .0001) in the former group relative to the latter group (Table 1). The blood cell counts were higher than the criterion of PGF since they had to accept RBC or PLT transfusions and rhG-CSF administration to avoid infection, anemia, and bleeding. Therefore, the counts of absolute neutrophil cell were not significantly different between PGF and GGF groups (1.39 × 10⁹/L versus 2.72 × 10⁹/L, P > .05).

The BMMNCs count was also significantly lower in the PGF group than in the GGF and HD groups (.396 × 10⁹/L versus 2.5 × 10⁹/L versus 2.625 × 10⁹/L, P < .005) (Supplementary Table S1).

**Blood Lymphocyte Subsets of BMMNCs**

The median percentages and absolute quantities of T lymphocyte subpopulations in BMMNCs from PGF patients, GGF patients, and normal controls are provided in Supplementary Table S1. Significant lymphopenia was observed in the PGF group. Lymphocyte percentages in the PGF, GGF, and HD groups were similar; thus, this lymphopenia was primarily caused by an overall reduction in

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**Figure 1.** Immunophenotypic analysis of Tc1, Tc2, Th1, and Th2 cells. The lymphocytic population was initially defined using a forward (FSC) and side scatter (SSC) gate (A). CD4⁺ T cells and CD8⁺ T cells were then gated based on the CD8 antigen (B). Representative fluorescence-activated cell sorting analysis of CD8⁺ IFN-γ⁺ Th1 cells and CD8⁺ IL-4⁺ Tc2 cells from a PGF patient (C), a GGF patient (D), an HD, and (E) CD8⁺ IL-4⁺ Th2 cells and CD8⁺ IL-4⁺ Tc2 cells from a PGF patient (F), a GGF patient (G), and an HD (H) are provided.
BMMNCs. For almost all lymphocyte subpopulations, percentages in the PGF and GGF groups were comparable; therefore, the reduced values of all lymphocyte subpopulations in the PGF group were attributable to the overall reduction in lymphocytes. Only the percentage of activated CD8$^+$ T cells in the BM immune microenvironment was significantly higher among PGF patients than among GGF patients. For CD4$^+$ and CD8$^+$ subsets, activation was demonstrated by the surface expression of HLA-DR. With the exception of activated CD4$^+$ T cells and CD8$^+$ T cells, absolute quantities of cell subsets were significantly lower among PGF patients than among healthy donors. Compared with healthy donors, PGF and GGF patients had lower levels of naïve phenotypes but higher levels of effector phenotypes for both CD4$^+$ T cells and CD8$^+$ T cells; in addition, the percentage of effector memory CD4$^+$ T cells was also elevated.

**Levels of Th1/Th2 Cytokines in BM Plasma**

BM plasma levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines from PGF patients, GGF patients, and HD controls were measured by CBA. As indicated in Figure 2, plasma IFN-γ levels were significantly higher in the PGF and GGF groups than in the HD group (14.395 pg/mL and 14.57 pg/mL versus 10.73 pg/mL, respectively, $P < .05$ for both comparisons) but did not differ between PGF and GGF patients. The levels of plasma IL-4 in PGF patients were lower than GGF and HD groups notably (0 pg/mL and 4.995 pg/mL versus 2.8 pg/mL, respectively, $P < .05$ for both comparisons).

**Balance between Type 1/Type 2 Cytokine-Producing Cells**

The percentages of stimulated CD4$^+$ and CD8$^+$ T cells that produced IFN-γ and IL-4 were analyzed using flow cytometry, following the gating strategies depicted in Figure 1. CD3$^+$CD8$^+$IFN-γ$^+$, CD3$^+$CD8$^+$IL4$^+$, CD3$^+$CD8$^+$IL4$^{-}$ cells were identified as Th1, Th2, Tc1, and Tc2 cells, respectively [34]. As indicated in Figure 3, the percentage of Th1 cells was higher for PGF patients than for HD (27.9% versus 21.7%, $P < .05$). A significantly higher proportion of Tc1 cells was found in PGF patients than in GGF patients (43.3% versus 22.8%, $P < .05$). The percentage of Th2 cells was higher for the GGF group than for the PGF and HD groups (4.2% versus 1.2% versus 2.3%, $P < .05$). The frequency of Tc2 cells was dramatically reduced in PGF patients compared with GGF patients and HD (.6% versus 2.0% versus 2.0%, $P < .0001$).

The type 1/type 2 immune response ratio was calculated using the Th1/Th2 and Tc1/Tc2 ratios. Comparisons of PGF patients with GGF patients and HD revealed that PGF

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**Figure 2.** IFN-γ (A) and IL-4 (B) levels in BM plasma, as measured by cytometric beads assay. The PGF patients exhibited significantly elevated plasma IFN-γ levels compared with HD and decreased plasma IL-4 levels compared with GGF patients and HD.

**Figure 3.** The percentages of Th1 (A) and Th2 (B) cells among CD4$^+$ T cells and Tc1 (D) and Tc2 (E) cells among CD8$^+$ T cells. Th1/Th2 (C) and Tc1/Tc2 (F) ratios are indicated. Statistical analyses were performed using the Mann-Whitney U test.
patients had significantly greater Th1/Th2 (25.82 versus 7.51 versus 6.91, P < .005) and Tc1/Tc2 ratios (72.44 versus 10.23 versus 14.57, P < .0001).

**DISCUSSION**

In this nested case-control study, we demonstrated for the first time that CD4+ and CD8+ T cells were polarized towards type 1 cytokine secretions in PGF patients relative to GGF patients and HD. In particular, among PGF patients, we observed an elevated IFN-γ level in BM plasma and increased proportions of Th1 and Tc1 cells (IFN-γ—producing CD4+ and CD8+ T cells, respectively). Moreover, PGF patients exhibited significantly decreased IL-4 levels in BM plasma and proportions of Th2 and Tc2 cells (IL-4—producing CD4+ and CD8+ T cells, respectively). Consequently, the Th1/Th2 and Tc1/Tc2 ratios were dramatically elevated among PGF patients relative to GGF patients and HD. In addition, the active CD8+ T cell phenotype was present at higher proportions in the PGF group than in the GGF and HD groups, whereas the active CD4+ T cell phenotype was more common in the PGF group than in the HD group (but not the GGF group). Compared with HD, PGF and GGF patients had lower levels of naïve CD4+ and CD8+ T cells and higher levels of effector memory CD4+ T cells.

Although we found aberrant T cell responses in the BM microenvironment of PGF patients, the mechanisms by which these abnormalities damage hematopoietic precursor cells or other cells in the BM microenvironment remain unclear. Analogously to PGF, AA is a BM failure syndrome characterized by BM and peripheral blood pancytopenia [35]. Emerging evidence suggests that dysregulated T cell responses play essential roles in the pathogenesis of AA [32,36]. Giannakoulas et al. reported that relative to controls, untreated or refractory acquired AA patients had significantly higher proportions of stimulated Th1 and Tc1 cells that produced IFN-γ but no differences in Th2 or Tc2 cells, resulting in a shift in the IFN-γ/IL-4 ratio towards a type 1 response [22]. Researchers have also found that the Th1 transcription factor T-bet is upregulated in AA patients [37]. Functionally, these dominant Th1 and Tc1 clones that secrete IFN-γ in active AA are capable of lysing autologous CD34+ cells and inhibiting hematopoietic colony formation by these cells [38]. Therefore, we speculated that the elevated IFN-γ in the BM microenvironment of PGF patients might adversely affect the quantity and function of HSCs, leading to the occurrence of PGF after allo-HSCT. We are aware, however, that further functional studies are needed to elucidate the direct effects of these dysregulated immune cells on hematopoietic precursor cells or the other cellular components of BM microenvironment and their underlying signal pathways in the future.

Our results indicate that both CD4+ and CD8+ T cells are primed in vitro towards a type 1 response in cases of PGF. The in vitro priming of T cells was further demonstrated by the cytokine levels of BM plasma, which exhibited elevated IFN-γ levels and decreased IL-4 levels and accompanied by the increased percentages of CD4+ T and CD8+ T cells expressing the activation marker HLA-DR in the BM microenvironment. The CD8+ HLA-DR+ T cells regarded as activated cytotoxic T lymphocytes have previously been detected in patients with severe AA, and it has been suggested that the increased quantity and enhanced function of these cells play a central role in the pathogenesis of the disease [32,39]. Differences among PGF patients, GGF patients, and HD with respect to naïve and effector memory T cells could be due to immune reconstitution after allo-HSCT [40].

We postulate that an initial antigenic stimulation, such as GVHD or a viral infection after allo-HSCT, may polarize CD4+ T cells towards a type 1 response, which activate the cytotoxic CD8+ cells and lead to hematopoietic precursor cells destruction [29,41,42]. Recent murine studies have provided evidence that BM stromal cells, such as osteoblasts and sinusoidal endothelial cells, could also be targets of GVHD mediated by CD4+ donor T cells via the Fas/FasL pathway [43]. Meanwhile, various redox reactions may also shift the balance between type 1 and type 2 immune responses [44,45]. Thus, it is plausible that deficits in the BM microenvironment due to aberrant responses by these T cells may aggravate BM failure.

In addition, other immune cells, such as macrophages, dendritic cells, regulatory T cells, and natural killer cells [11], also play important roles in the formation of the HSCs microenvironment and the regulation of hematopoiesis. Further studies to investigate whether the alteration of these immune cells were quantitatively and functionally involved in the occurrence of PGF will be performed.

In conclusion, this nested case-control study provides evidence that both CD4+ and CD8+ T cells were polarized towards a type 1 cytokine response in patients with PGF, suggesting that the aberrant T cell responses in the BM immune microenvironment may hamper hematopoietic recovery after allo-HSCT. Although requiring further functional validation, our study offers a guide for functionally evaluating the immune microenvironment during the occurrence of PGF, thereby providing insights into how the BM immune microenvironment is dysregulated in patients with PGF. These findings may eventually be used to produce recommendations regarding the treatment of PGF after allo-HSCT. For the PGF patients without hematological improvement after etiological delineation, persistent immune activation may exist and pointed immunosuppressive therapy may be effective. The concrete method for application should be on the basis of large-scale clinical trials.

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**SUPPLEMENTARY DATA**

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