Regulatory B cells promote graft-versus-host disease prevention and maintain graft-versus-leukemia activity following allogeneic bone marrow transplantation

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ABSTRACT
Regulatory B cells (Bregs) are involved in the pathogenesis of graft-versus-host disease (GVHD). However, whether Bregs can alleviate acute GVHD without compromising graft-versus-leukemia (GVL) effects remains unclear. Here, we evaluated the role of Bregs in acute GVHD and GVL activity in both a mouse model and a clinical cohort study. In the acute GVHD mouse model, co-transplantation of Bregs prevents onset through inhibiting Th1 and Th17 differentiation and expanding regulatory T cells. In the GVL mouse model, Bregs contributed to the suppression of acute GVHD but had no adverse effect on GVL activity. In the clinical cohort study, a higher dose of Bregs in allografts was associated with a lower cumulative incidence of acute GVHD but not with increased risk of relapse. Our data demonstrate that Bregs can prevent acute GVHD and maintain GVL effects and suggest that Bregs have potential as a novel strategy for acute GVHD alleviation.

Introduction
Allogeneic stem cell transplantation (allo-SCT) is limited by the major lethal complication of graft-versus-host disease (GVHD). Prophylaxis and treatment of GVHD have been restricted to either functional inactivation of donor T cells using immunosuppressive drugs, or depletion of donor T cells. These strategies might lead to significant toxicities of the immunosuppressants, delayed immune reconstitution, a high rate of opportunistic infections, and increased risk of hematological malignant relapse. Therefore, several regulatory cells, including CD4+CD25+ regulatory T cells (Tregs), myeloid-derived suppressor cells, and mesenchymal stromal cells (MSCs), have been investigated to elucidate whether these cells could be used for prevention or therapy of GVHD. Although several studies using a mouse bone marrow transplant (BMT) model have identified Tregs, myeloid-derived suppressor cells, and MSCs as available strategies for GVHD alleviation, none of them can be routinely applied in clinic.

Regulatory B cells (Bregs) are a newly described subset of B cells that appears to play important roles in autoimmunity. Human Bregs were enriched in both transitional (CD24hiCD38hi) and memory (CD24hiCD27+) B cells; in mice, they are enriched within the CD1dhiCD5+CD19high subpopulation. In 2006, Rowe et al. reported that host B cells produce interleukin (IL)-10 following total body irradiation and attenuate acute GVHD after allogeneic BMT. Le Huy et al. observed that donor-derived B10 cells have a suppressive role in Scleroderma-like chronic GVHD development. In addition, the quantity and quality impairment of Bregs was also observed in human chronic GVHD. However, it remains unclear whether Bregs could alleviate acute GVHD while maintaining graft-versus-leukemia (GVL) effects. Here, we show that the immunosuppressive potential of Bregs can be used to suppress acute GVHD without compromising the effects of GVL activity based on results from a mouse model and a clinical cohort study.

Results
Bregs significantly reduce the severity of acute GVHD in a mouse model
To test whether Bregs can be implemented as cellular GVHD therapy following BMT, we used a classical GVHD mouse model (B6 into BALB/c). Lethally irradiated BALB/c mice were transplanted with 5 × 10^6 B6 TCD-BM cells with or without 3 × 10^6 B6 spleen T cells. BMT recipients were injected i.v. in the tail with either Bregs (3 × 10^6) or phosphate-buffered saline immediately after the transplant. As expected, BALB/c mice receiving B6 TCD-BM alone developed no sign of GVHD whereas all control BALB/c mice receiving B6 donor TCD-BM + spleen T cells died of GVHD (Fig. 1A). In contrast, injection of Bregs inhibited GVHD in T cell recipients, with 20% more...
of them surviving without severe GVHD (Fig. 1A). This survival was accompanied by a lower clinical score in Bregs recipients (Fig. 1B). We performed histological examination of GVHD target organs (liver, skin, intestine, and colon) at 1 and 2 weeks after transplantation. There was a significant reduction in pathological damage in all sites of Bregs recipients compared with control mice (Fig. 1C, 1D). Thus, injection of Bregs had a salutary effect on the severity of acute GVHD.

**Bregs modulate Th cell balance**

GVHD is characterized by the differentiation of T cells present in the graft. Excessive production of cytokines such as IL-1β, IL-6, IL-17, and IFNγ by differentiated T cells can lead to an inflammatory response and cause damage to several host tissues in GVHD. To examine whether Bregs could regulate Th cell response, we analyzed Th cell subsets in peripheral blood, bone marrow, and spleen of recipients on the indicated days after transplantation. Flow cytometry analysis showed that compared with control, IL-4-positive CD4+ T cells derived from spleen significantly increased in the Breg injection group at the early stage after transplantation (Fig. 2A). We also detected substantially lower serum levels of Th1- and Th17-related cytokines (TNF-α and IFNγ) in the Breg injection group (Fig. 2B).

The expression of RORγt and T-bet, which is the key transcription factor of Th17 and Th1 cell differentiation, was decreased in blood and bone marrow CD4+ T cells in the Breg injection group (Fig. S1). On the other hand, the expression of the Th2-specific gene GATA3 in splenic CD4+ T cells was significantly higher than in the controls (Fig. 2C). Breg injection markedly raised the Th2/Th1 and Th2/(Th1+Th17) ratios in peripheral blood, bone marrow, and spleen (Fig. 2D). Taken together, these data suggest that the polarization of T cells from Th1 to Th2 may be the primary cause of Breg-mediated GVHD inhibition.

**Bregs attenuate GVHD via regulatory Tregs**

While playing key roles in suppressing autoimmunity and in maintaining immune homeostasis, Tregs can reduce the severity of GVHD. Therefore, we wondered whether the effect of Bregs on GVHD resided in their effect of promoting Tregs and quantified the frequencies and absolute numbers of Foxp3-expressing CD4+ T cells in peripheral blood on the indicated days after transplantation. Recipients injected with Bregs showed a significantly higher frequency of Foxp3+ cells (Fig. 3A). Ratios of Treg/Th1 and Treg/(Th1+Th17) also were significantly increased in Breg injection recipients compared with the control group on day 7 (Fig. 3B). Therefore, these data suggest that the effect of Bregs on GVHD prevention is associated with Tregs.

To ascertain the protective functions of Bregs in GVHD target organs, the expression of T-bet, GATA3, RORγt, and Foxp3 in the skin, intestine, colon, liver, and lung were assessed by immunohistochemical staining. Microscopy analysis revealed that the number of T-bet+ and RORγt+ cells was...
significantly reduced when Bregs were injected. However, the number of cells expressing GATA3 in lung from the Breg injection group was increased significantly ($p < 0.05$) compared with that of the controls (Fig. 3C). In addition, the Foxp3-expressing cells also increased in the Breg injection group (Fig. 3C). Taken together, the results indicate that Bregs significantly inhibited T-bet and ROR$\gamma$ expression and expanded the Treg population in GVHD target organs of recipient mice.

**Breg preserves GVL activity after BMT**

To evaluate the impact of Breg injection on the preservation of GVL activity, we challenged BALB/c recipients with host-type GFP$^+$ acute myeloid leukemia cells (H-2d) to mimic residual leukemia in patients receiving allogeneic BMT. All mice transplanted with TCD-BM and challenged with leukemia cells died from leukemia (Fig. 4A). In vivo injection of spleen T cells prolonged the survival of mice transplanted with TCD-BM and challenged with leukemia cells (Fig. 4A), indicating a potent GVL effect. However, CD4$^+$ T cells failed to eliminate leukemia, and these mice eventually died from severe GVHD and leukemia. In contrast, Breg injection from the day of transplantation had no adverse effect on the control of leukemia and prolonged the survival of leukemia cell-challenged mice receiving TCD-BM + spleen T cells. The mice injected with Bregs showed less associated GVHD clinical features (Fig. 4B) and weight loss (Fig. 4C) than other leukemia injection groups. A representative dot plot for GFP$^+$ leukemia cells was showed (Fig. 4D). About 60% GFP$^+$ leukemia cells were found in the leukemia injection group on day 14. In contrast, the Breg and T cell injection limited the leukemia cells to 10−20% (Fig. 4E). Autopsy showed spleen and liver enlargement and indicated signs of controlled leukemia in BMT recipients, confirming the GVL effect (Fig. 4F, 4G). Therefore, Breg injection had no adverse effect on GVL activity.

**Correlation of Breg in allografts with acute GVHD, but not relapse**

All but one subject (who died of infection) exhibited hematopoietic recovery after transplantation. Patients were classified into two groups according to the median dose of Breg in allografts ($1.63 \times 10^7$ Breg cells infused/kg). These groups had
equivalent subject and donor characteristics, including the conditioning regimens, except that cases in the high-dose Breg group received a higher dose of CD34+ cells ($p = 0.044$), CD19+ cells ($p < 0.001$), naïve B cells ($p < 0.001$), and memory B cells ($p = 0.031$) (Table 1). The cumulative, 100-d incidence of acute GVHD grades II–IV in the low-dose Breg group (Group A, 39%; 95% confidence interval (CI), 23–55%) was significantly higher than in the high-dose Breg group (Group B, 16%; 95% CI, 4–28%; $p = 0.038$; Fig. 5A). In multivariate analysis, infusion of $> 1.63 \times 10^7$ Breg cells infused/kg decreased the risk of grades II to IV acute GVHD (hazard ratio (HR) = 0.379, 95% CI = 0.146–0.988, $p = 0.047$).

After a median follow-up of 206 d (range 186–252) for surviving patients, the 1-y cumulative incidences of relapse and NRM were 3% (95% CI, 0–7%) and 11% (95% CI, 3–19%), respectively. The 1-y probabilities of disease-free survival (DFS) and overall survival (OS) were 86% (95% CI, 77–95%) and 86% (95% CI, 77–95%), respectively. There was no significant difference in relapse, NRM, DFS, and OS between patients receiving high-dose Bregs and those receiving low-dose Bregs (Table 2, Fig. 2B–2E). Multivariate analyses adjusted for variables in Table 1, including other B cell subsets, showed that there were no effects of Bregs in allografts on relapse, NRM, DFS, or OS.

**Discussion**

Bregs show significant immunosuppressive capacity in vitro and in vivo and for this reason, these cells have been tested for treating severe autoimmune diseases. In the present study, we observed for the first time that co-transplantation of Bregs significantly ameliorated effective T cell functions by modulating Th cell balance and Tregs and preventing acute GVHD.
without abrogating the GVT effects in an allogeneic BMT mouse model. In addition, our clinical study also demonstrated that Bregs could alleviate acute GVHD and have no effects on relapse. These data suggest that Bregs have potential as a novel strategy for acute GVHD alleviation.

Acute GVHD is mediated by donor-derived T cells. On activation with alloantigens expressed on host tissues, donor-derived naive CD4+ T cells differentiate into Th cell subsets of effector T cells, such as Th1 and Th17, expressing distinct sets of transcriptional factors and cytokines, which mediate organ-specific GVHD. In this study, we found that co-transplantation of donor-derived Bregs led to a significantly higher ratio of Th2/Th1+Th17 compared with the control group (Fig. 2). Previous work has demonstrated that Th2 cells can alleviate acute GVHD in a mouse model. In vitro and in vivo experiments also have shown that Bregs can limit Th1 and Th17 differentiation. The data reported by others and observed in our study suggest that Bregs can prevent acute GVHD via modulating Th cell balance, which is in agreement with other studies. The results of both of those earlier studies suggested that downregulating Th1 and Th17 cells could ameliorate GVHD.

The effects of Tregs in inhibiting acute GVHD have been reported in several previous studies. Li et al. demonstrated that induced Tregs specific for HY miHAs are highly effective in controlling GVHD in an Ag-dependent manner while sparing the GVL effect. In the present study, we showed that compared with the control group, co-transplantation of donor-derived Bregs significantly increased the percentages of Tregs in skin, intestine, colon, liver, and lung (Fig. 3). Considering the evidence that Bregs could induce and/or maintain Tregs, our results suggest that Bregs may ameliorate acute GVHD by inducing Tregs in vivo. In addition, given the plasticity between Th17 and Treg, another hypothesis, maybe more relevant, would be a polarization of T cells from Th17 to Treg.

The finding that donor-derived Bregs can successfully prevent acute GVHD led us to investigate the effects of this subset on GVL effects. Interestingly, we found a significantly lower percentage of GFP+ leukemia cells and longer survival of mice

Figure 4. Bregs preserve the (T)cell-mediated GVL effect while inhibiting GVHD. Lethally irradiated BALB/c recipients were transplanted with B6 TCD-BM, with or without T cells, and challenged or not with leukemia cells (5 × 10^7/mouse). Bregs were injected at the time of transplantation. (A) Survival was monitored over time. (B) Recipient mice were assessed every 2 d for clinical severity of GVHD; clinical scores are shown. (C) Mean percentage initial body weight of recipients over the first 30 d. (D) A representative dot plot for GFP+ leukemia cells. (E) GFP+ leukemia cells in peripheral blood and bone marrow were counted by flow cytometry on days 7 and 14. (F) On day 14, mice from different groups were killed, and autopsy was performed to observe the enlargement of liver (upper panel) and spleen (lower panel). (G) Comparable mouse general physical status was found in the Breg injection group and bone marrow-transplanted group. From left to right, TCD-BM + T cells + leukemia cells, TCD-BM + T cells, TCD-BM, TCD-BM + T cells + leukemia cells, TCD-BM + leukemia cells, TCD-BM. Results are representative of three independent experiments with three mice per group per experiment. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
in the Breg group compared with those in the Tcon group (Fig. 4). These results suggest that co-transplantation of donor-derived Bregs modulates the function of alloreactive T cells and prevents GVHD without impairing the GVL effects of Tcon. Previously, Endinger et al.9 confirmed that Treg could preserve GVL effects. Therefore, further exploitation of mechanisms associated with GVHD before allografting (Table 2 and Fig. 5). Overall, the results of the human studies further support the detailed Breg-related mechanisms in preventing GVHD and maintaining GVL activity.

More recently, Sarvaria et al.34 demonstrated that Bregs are present in both naive and transitional B cell compartments of cord blood and can suppress T cell proliferation and effector function through IL-10 production as well as cell-to-cell contact involving CTLA-4. They also reported a protective role for cord blood-derived Bregs against GVHD development in cord blood recipients. The role of Bregs in acute GVHD was further elucidated by Chakupurakal et al.35 In agreement with these studies, we found that a high dose of Bregs decreased the incidence of grades II–IV acute GVHD but had no negative effects on relapse after unmanipulated allografts (Table 2 and Fig. 5). Overall, the results of the human studies further support the results obtained using a mouse model: that Bregs alleviate GVHD without compromising GVL activity.

The present study had several limitations. First, the more detailed Breg-related mechanisms in preventing GVHD and maintaining GVL effects remain to be elucidated. Second, the follow-up time of the cases in our clinical cohort study was short. A multicenter study with large sample and a long-term follow-up in allo-SCT settings, including ISD and unrelated donor, to investigate the effects of Breg on GVHD and relapse should be performed.

In summary, our findings demonstrated a role for Bregs in inhibiting Th1 and Th17 cells and modulating Tregs that contributed to preventing acute GVHD without compromising the GVL effects. Therefore, further exploitation of mechanisms

### Table 1. Patient and donor characteristics.

| Characteristics                          | Total patients | Low-dose Breg group | High-dose Breg group | p value |
|------------------------------------------|----------------|---------------------|----------------------|---------|
| Patient Number                          | 74             | 37                  | 37                   | NS.     |
| Median age (range), years                | 32 (7–64)      | 33 (7–52)           | 32 (8–64)            | NS.     |
| Median weight (range), kg                | 62 (26–98)     | 65.5 (46–95)        | 20 (54.1%)           | 0.231   |
| Male sex, n (%)                          | 46 (62.2%)     | 26 (70.3%)          | 20 (54.1%)           | 0.151   |
| Diagnosis, n (%)                         | 0.48           | 0.48                | 0.48                 |         |
| AML                                      | 32 (43.2%)     | 12 (32.4%)          | 20 (54.1%)           |         |
| ALL                                      | 28 (37.8%)     | 15 (40.5%)          | 13 (35.1%)           |         |
| MDS                                      | 9 (12.2%)      | 7 (18.9%)           | 2 (5.4%)             |         |
| Others                                   | 5 (6.8%)       | 3 (8.1%)            | 2 (5.4%)             |         |
| Low                                      | 7 (9.5%)       | 2 (5.4%)            | 5 (13.5%)            | 0.481   |
| Intermediate                             | 65 (87.8%)     | 34 (91.9%)          | 31 (83.8%)           |         |
| High                                     | 2 (2.7%)       | 1 (2.7%)            | 1 (2.7%)             |         |
| Conditioning regimen                     |                |                     |                      |         |
| MA                                       | 74 (100%)      | 37 (100%)           | 37 (100%)            | NS.     |
| No of HLA-A,B, DR mismatched             | 0.74           | 0.74                | 0.74                 | 0.533   |
| 0                                        | 11 (14.9%)     | 4 (10.8%)           | 7 (18.9%)            |         |
| 2                                        | 7 (9.5%)       | 3 (8.1%)            | 4 (10.8%)            |         |
| 3                                        | 56 (75.7%)     | 30 (81.1%)          | 26 (70.3%)           |         |
| Donor-recipient sex match, n (%)         | 0.48           | 0.48                | 0.48                 |         |
| Male–male                                | 33 (44.6%)     | 18 (38.6%)          | 15 (40.5%)           | 0.748   |
| Male–female                              | 20 (20.7%)     | 9 (24.3%)           | 11 (29.7%)           |         |
| Female–male                              | 15 (20.3%)     | 8 (21.6%)           | 7 (18.9%)            |         |
| Female–female                            | 6 (8.1%)       | 2 (5.4%)            | 4 (10.8%)            |         |
| Donor–recipient relation, n (%)          | 0.265          | 0.265               | 0.265                |         |
| Sibling–sibling                          | 27 (36.5%)     | 15 (40.5%)          | 12 (32.4%)           |         |
| Parent–child                             | 33 (44.6%)     | 18 (48.6%)          | 15 (40.5%)           |         |
| Child–parent                             | 13 (17.6%)     | 4 (10.8%)           | 9 (24.3%)            |         |
| Others                                   | 1 (1.4%)       | 0 (0%)              | 1 (2.7%)             |         |
| ABO matched, n (%)                       | 0.286          | 0.286               | 0.286                |         |
| Matched                                  | 28 (37.8%)     | 16 (43.2%)          | 12 (32.4%)           |         |
| Major mismatched                         | 20 (27.0%)     | 7 (18.9%)           | 13 (35.1%)           |         |
| Minor mismatched                         | 19 (25.7%)     | 9 (24.3%)           | 10 (27.0%)           |         |
| Bidirec mismatched                       | 7 (9.5%)       | 5 (13.5%)           | 2 (5.4%)             |         |
| Cell compositions in allografts          |                |                     |                      |         |
| Infused nuclear cells 10^6/kg            | 7.69 (2.2–11.08) | 7.98 (3.57–13.72) | NS.                |         |
| Infused CD34+ cells 10^6/kg              | 2.80 (0.67–7.66) | 2.53 (0.67–5.67) | 3.43 (1.06–7.66) | 0.044   |
| Infused CD3+ cells 10^6/kg               | 2.43 (1.00–7.98) | 2.29 (1.00–3.77) | 2.64 (1.16–7.98) | 0.104   |
| Infused CD4+ cells 10^6/kg               | 1.40 (0.38–4.32) | 1.32 (0.38–2.47) | 1.56 (0.58–4.32) | 0.099   |
| Infused CD8+ cells 10^6/kg               | 0.74 (0.18–2.73) | 0.72 (0.28–1.44) | 0.82 (0.18–2.73) | 0.350   |
| Infused CD14+ cells 10^6/kg              | 1.57 (0.39–7.48) | 1.61 (0.39–3.35) | 1.51 (0.58–7.48) | 0.725   |
| Ratio of CD4+ /CD8+ in BM allografts     | 1.14 (0.47–5.91) | 1.01 (0.48–2.46) | 1.28 (0.47–5.91) | 0.077   |
| Infused CD19+ cells 10^6/kg              | 0.37 (0.13–1.17) | 0.27 (0.13–0.86) | 0.47 (0.23–1.17) | <0.001  |
| Infused naive B cells 10^6/kg            | 0.23 (0.03–0.88) | 0.19 (0.03–0.51) | 0.34 (0.08–0.88) | <0.001  |
| Infused memory B cells 10^6/kg           | 0.10 (0.02–0.84) | 0.09 (0.02–0.36) | 0.10 (0.03–0.84) | 0.031   |

Abbreviations: Breg = regulatory B cells; NS. = no significance; AML = acute myeloid leukemia; ALL = acute lymphoblastic leukemia; MDS = myelodysplastic syndrome; DRI = disease risk index; HLA = human leukocyte antigen; BM = bone marrow.
underlying the separation of GVHD and GVL activity by Bregs may improve transplantation outcomes with allo-SCT and facilitate the potential use of Bregs in the clinic, which could enhance the safe use of allografts.

Materials and methods

**Mice**

Female C57BL/6 (B6; H-2b) and BALB/c (H-2d) mice were purchased from Beijing Vital Laboratory Animal Technology Company, Ltd. (Beijing, China). The mice were maintained in specific pathogen-free conditions and fed sterilized water and food at the animal facility of Peking University People’s Hospital. Mice were between 8 and 10 weeks of age at the start of the experiments. All experiments were performed according to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

**GVHD and GVL model**

BALB/c mice received 8 Gy total body irradiation from a $^{60}$Co source for 8 min. On day 0, recipient mice were transplanted

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**Figure 5.** Outcome of allogeneic stem cell transplantations in the cohort of patients. (A) Grades II–IV acute GVHD, (B) relapse, (C) non-relapse mortality, (D) disease-free survival, and (E) overall survival. Patients receiving a high dose of Bregs in allografts (more than or equal to $1.63 \times 10^7$ Breg cells infused/kg) ($n = 37$); green line; subjects receiving a high dose of Bregs in allografts (less than $1.63 \times 10^7$ Breg cells infused/kg) ($n = 37$); blue line.
with $5 \times 10^6$ T cell-depleted bone marrow (TCD-BM) cells and $3 \times 10^6$ spleen T cells from B6 donors. T cell depletion was performed with anti-CD5 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocols, as previously reported.\textsuperscript{36} CD3$^+$CD4$^+$CD25$^+$ spleen T cells were purified using a FACSAria flow cytometer (BD Biosciences). Donor cells were injected through the tail vein. In GVL experiments, $5 \times 10^5$ GFP-expressing acute myeloid leukemia cells (H-2d),\textsuperscript{37} kindly provided by Professor Yue-Ying Wang of Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine, were given along with the transplantation. The development of leukemia was monitored by counting the percentage of peripheral blood GFP$^+$ cells (3$\times$10$^6$) were immediately transferred into the appropriate recipient mice by tail vein injection.

### Breg generation and enrichment

For adoptive transfer experiments, donor mice were treated with granulocyte colony-stimulating factor (G-CSF, 2 mg daily i.p.) for 10 d. These donor mice were killed at 14 d after G-CSF treatments. Splenic CD19$^+$ B cells were isolated from mice by positive selection using magnetic beads (Miltenyi) with an obtained purity $\geq 95$%. CD1d$^{-}\text{hi}$CD5$^+$ Breg cells were purified (95–98%) using a FACSAria flow cytometer (BD Biosciences). After purification, CD1d$^{-}\text{hi}$CD5$^+$ Breg cells ($3 \times 10^6$) were immediately transferred into the appropriate recipient mice by tail vein injection.

### Evaluation of GVHD and histopathological analysis

After BMT, animals were weighed every 2 d, and the degree of systemic GVHD was assessed by a clinical scoring system including five clinical parameters (weight loss, posture, activity, fur ruffling, and skin integrity), as published previously.\textsuperscript{38} For histopathological analysis, left lung, liver, intestine, colon, and skin specimens of recipients were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin. Two slides/organisms were evaluated and scored by a pathologist blinded to experimental group using a scoring system described previously.\textsuperscript{39} Immunohistochemical analysis of specimens were performed using GATA-3 (Cell Signaling Technology), T-bet, ROR$\gamma$t, and Foxp3 (Bioss) antibodies.

### Flow cytometry assay

At designated time points, recipient mice were killed and organs collected. Single-cell suspensions were obtained from the blood, spleen, and bone marrow of mice. For detection of cytokine production, cells were stimulated for 6 h with 2$\mu$L/mL leukocyte activation cocktail (BD, GolgiPlug$^\text{TM}$, Catalog No. 550583) before being stained for flow cytometry analysis. Cells were assayed for cytokine production by intracellular flow cytometry, and the following antibodies were used: CD3- BV510 anti-mouse, CD4$^+$-APC-CY7 anti-mouse, CD8$^+$-PerCP anti-mouse, CD25- PE-Cy7 anti-mouse, interferon-γ (IFN$\gamma$)-FITC anti-mouse, interleukin-4 (IL-4)-PE anti-mouse, IL-17- BV421 anti-mouse (BD Biosciences), Foxp3-Alexa Fluor 487 (eBioscience); and GATA3-PE-CFS94 anti-mouse, ROR$\gamma$t- BV421 anti-mouse, and T-bet-PE anti-mouse (BD Biosciences). Intracellular cytokine staining was performed using a BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), and the intracellular staining of Foxp3 was performed with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Data were acquired with a LSRFortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar).

### Cytokine measurement

Mouse serum was collected on days 7 and 14 after BMT, and the levels of cytokines were measured using Multi-Analyte Flow Assay Kit, according to the manufacturer's instructions (BioLegend).

### Patients and transplant protocol

Between January 2016 and March 2016, 74 patients who underwent an allo-SCT in our center entered this study (Table 1). All patients and their donors provided an
informed consent for biologic research purposes. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Peking University. Diagnosis and grading of acute and chronic GVHD were performed as described by physicians in charge of the patients who were not aware of the results of laboratory tests. Unmanipulated haploidentical allografts and HLA-identical sibling donor transplantation were performed according to the protocols reported previously by our group. In brief, the conditioning regimen for the haploidentical donor group (HID, n = 63) was as follows: cytarabine (4 g/m² per day) IV on days −10 to −9; busulfan (3.2 mg/kg per day) IV on days −8 to −6; cyclophosphamide (1.8 g/m² per day), IV on days −5 to −4; methyl chloride hexamethylene urea nitrate (Me-CCNU) (250 mg/m² per day), orally once on day −3; and ATG (2.5 mg/kg per day; Sang Stat,Lyon, France) IV on days −5 to −2. Patients in the human leukocyte antigen identical sibling donor transplantation group (ISD, n = 11) received hydroxy-carbamide (80 mg/kg) orally on day −10 and a lower dose of cytarabine (2 g/m² per day) on day −9, but otherwise, an identical regimen to the HID patients without ATG was used. GVHD prophylaxis regimen consisted of mycophenolate mofetil, cyclosporine A, and short-term methotrexate.

Human Breg phenotype

Immune cell phenotyping of G-CSF-stimulated bone marrow grafts and peripheral blood stem cell grafts was performed in all 74 patients. Anti-human fluorochrome-conjugated antibodies for Breg analysis included: CD45-V500 (BD Biosciences), CD19-APC (BioLegend), CD19-APC-Cy7 (BioLegend), CD20-PerCP-Cy7 (BioLegend), CD24-PE-Cy7 (BD Biosciences), CD27-V450 (BD Biosciences), CD38-APC (BD), and CD38-FITC (BD). B cell subsets (including Bregs), T cell subgroups, and CD34⁺ cells were identified as described previously. A representative dot plot for B cell subsets was showed in Fig. S2.

Statistics

Laboratory data analysis was performed using Prism software. Survival comparisons were performed using the log-rank test. Other differences between experimental groups were analyzed using two-tailed unpaired Student tests.

For the patient analyses, comparisons were made with the χ² statistic for categorical variables and the Mann–Whitney test for continuous variables. Cumulative incidence curves were used in a competing risk setting, with relapse treated as a competing event to calculate non-relapse mortality (NRM) probabilities, and with death from any cause as a competing risk for GVHD, engraftment, and relapse. Time to GVHD was defined as the time from transplantation to the onset of GVHD of any grade. Probabilities of survival were estimated with the Kaplan–Meier method. All variables in Table 1 were included in the univariate analysis; then, only variables with p < 0.1 were included in a Cox proportional hazards model with time-dependent variables. Unless otherwise specified, p values were based on two-sided hypothesis tests. α was set at 0.05. Most analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

Contribution: X.-J.H. designed the study; Y.-J.C. and Y. H. collected data; Y.H., Y.-J.C., and X.-J.H. analyzed the data and drafted the manuscript; and all authors contributed to data interpretation, manuscript preparation, and approval of the final version.

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