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Regulatory $\gamma\delta$ T cells induced by G-CSF participate in acute graft-versus-host disease regulation in G-CSF-mobilized allogeneic peripheral blood stem cell transplantation

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Abstract

Background: The immunomodulatory effects of granulocyte colony-stimulating factor (G-CSF) on T cells result in a low incidence of acute graft-versus-host disease (aGVHD) in G-CSF-mobilized allogeneic peripheral blood stem cell transplantation (G-PBSCT). However, the exact mechanism remains unclear. Regulatory $\gamma\delta$ T cells ($\gamma\delta$ Tregs), characterized by the presence of TCR $\gamma\delta$ and Foxp3, have aroused great concern in the maintenance of immune tolerance. We hypothesized that $\gamma\delta$ Tregs might involve in the immunomodulatory effects of G-CSF mobilization.

Methods: The expression and immunomodulatory function of $\gamma\delta$ Treg subsets in peripheral blood of donors before and after G-CSF treatment in vivo and in vitro were evaluated by flow cytometry and CFSE assays. To investigate the effects of $\gamma\delta$ Tregs on aGVHD, the association between $\gamma\delta$ Treg subsets in grafts and aGVHD in recipients was estimated.

Results: The proportions of V δ 1Tregs, CD27⁺V δ 1Tregs and CD25⁺V δ 1Tregs were significantly increased in peripheral blood after G-CSF treatment in vivo. $\gamma\delta$ Tregs could be generated in vitro by stimulating with anti-TCR $\gamma\delta$ in the presence of G-CSF. The immune phenotype, proliferation suppression function, and cytokine secretion of G-CSF-induced $\gamma\delta$ Tregs were similar to that of transforming growth factor- β (TGF- β)-induced $\gamma\delta$ Tregs. The clinical data demonstrated that the proportion of CD27⁺V δ 1Tregs in grafts was significantly lower in the patients who experienced aGVHD than in those who did not develop aGVHD ($P=0.028$), and the proportions of other $\gamma\delta$ Treg subsets in grafts did not differ significantly between the two groups. The best cutoff value for CD27⁺V δ 1Treg proportion in grafts in prediction of aGVHD was 0.33%, with an area under the curve value of 0.725 ($P=0.043$). Eight patients (26.7%) were classified as the low-CD27⁺V δ 1Treg group (<0.33%), and 22 patients (73.3%) as the high-CD27⁺V δ 1Treg group ($\geq 0.33\%$). The incidence of aGVHD was higher in the low-CD27⁺V δ 1Treg group than in the high-CD27⁺V δ 1Treg group (75.0% versus 22.7%, $P=0.028$).

Conclusions: G-CSF could induce the generation of $\gamma\delta$ Tregs in vivo and in vitro, and $\gamma\delta$ Tregs might participate in aGVHD regulation in G-PBSCT.

Keywords: Acute graft-versus-host disease, Allogeneic peripheral blood stem cell transplantation, Granulocyte colony-stimulating factor, Regulatory $\gamma\delta$ T cells

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Background

Nowadays granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cell transplantation (PBSCT) has been more widely applied than bone marrow transplantation (BMT) due to its faster engraftment and practicability [1]. Although G-CSF-mobilized allogeneic PBSCT (G-PBSCT) contains more mature T cells, neither the incidence nor the severity of acute graft-versus-host disease (aGVHD) is higher compared with BMT [2, 3]. The protective effects of G-CSF against aGVHD might result from the immunoregulatory effects of G-CSF on T cells, including inhibiting T cell proliferation, polarizing T cells from the Th1 to Th2 phenotype, switching T cell cytokine secretion profile, and inducing CD4⁺CD25⁺Foxp3⁺T cells (regulatory T cells, Tregs) [4–7]. Recent studies have shown that Tregs with immunosuppressive function are not just confined to CD4⁺ T cells but also exist in CD8⁺ T and $\gamma\delta$ T cell populations [8–11].

Regulatory $\gamma\delta$ T cells ($\gamma\delta$ Tregs), characterized by the presence of TCR $\gamma\delta$ and a high level of Foxp3 expression, are a novel subset of $\gamma\delta$ T cells with immunosuppressive effects [12–14]. $\gamma\delta$ Tregs exist at very low frequencies in peripheral blood, and can be induced from peripheral blood mononuclear cells (PBMCs) *in vitro* in the presence of antigen stimulation and cytokines (transforming growth factor (TGF)- β 1 and interleukin (IL)-2) [12, 14]. Recent studies have demonstrated that reduced numbers of $\gamma\delta$ Tregs are correlated with the development of autoimmune diseases [12, 15, 16]. In addition, it has been confirmed that prophylactic infusion of $\gamma\delta$ Tregs could reduce the incidence of GVHD in a mouse model [16]. Thus, $\gamma\delta$ Tregs might be a new therapeutic target in autoimmune diseases. Our previous study has documented that G-CSF might change the distribution and clonality of the T cell receptors (TCRs) on $\gamma\delta$ T cells, and this alteration might play a role in mediating GVHD in G-PBSCT [17]. Based on these results, we hypothesize that a possible mechanism of G-CSF inducing immune tolerance in G-PBSCT is that G-CSF induces $\gamma\delta$ Tregs in grafts. To verify this hypothesis, we investigated the effects of G-CSF on $\gamma\delta$ Tregs *in vivo* and *in vitro*, and explored the role of $\gamma\delta$ Tregs in aGVHD in G-PBSCT recipients.

Methods

Samples

Peripheral blood (PB) was obtained from 30 healthy stem cell donors (13 female, 17 male; median age 33 years, range 12–56 years) before treatment and on the 5th day of treatment with G-CSF (Filgrastim, subcutaneous injection of 5 μ g/kg/day; Kirin Brewery Co, Tokyo, Japan). G-CSF-primed PB (G-PB) grafts were harvested on the 5th day of treatment. The corresponding patients were 30

acute leukemia undergoing G-PBSCT from HLA-identical sibling donors. All donors and patients were willing to accept the trial after being informed, and all samples were obtained with consent. All of the procedures were conducted according to the guidelines of the Medical Ethics Committees of the Health Bureau of the Guangdong Province of China. This study was approved by the Ethics Committee of Nanfang Hospital and the Medical School of Jinan University.

Flow cytometric analysis of $\gamma\delta$ Tregs

$\gamma\delta$ T cells in humans can be divided into two major groups, V δ 1 and V δ 2 T cells, depending on δ -chain usage [18, 19]. $\gamma\delta$ Tregs have been reported to be characterized by the presence of TCR $\gamma\delta$ and Foxp3 expression with a CD27^{high}CD25^{high} phenotype [12, 14]. Therefore, $\gamma\delta$ Tregs (Foxp3⁺ $\gamma\delta$ T cells) were classified as V δ 1Tregs (Foxp3⁺V δ 1 T cells) and V δ 2Tregs (Foxp3⁺V δ 2 T cells) in this study. FITC-conjugated anti-human TCR $\gamma\delta$ (B1) and V δ 2 (B6), PE-conjugated anti-human CD25 (M-A251), PE-Cy7-conjugated anti-human CD3 (UCHT1), PerCP/Cy5.5-conjugated anti-human CD27 (M-T271), Alexa Fluor 647-conjugated anti-human Foxp3 (259D/C7) and the respective isotype control antibodies were purchased from BD Pharmingen. FITC-conjugated anti-human V δ 1 (TS 8.2) was obtained from Thermo Scientific. Purified no azide/low endotoxin mouse anti-human CD3 (UCHT1) and purified mouse anti-human TCR $\gamma\delta$ (B1) were purchased from BD Pharmingen.

$\gamma\delta$ Treg subsets in PB before and after G-CSF *in vivo* treatment were analyzed by flow cytometry based on the above markers. PBMCs were first isolated from PB samples of healthy donors before and after G-CSF *in vivo* treatment by Ficoll-Hypaque density gradient centrifugation. Freshly isolated PBMCs were suspended in phosphate-buffered saline (PBS) containing 1% bull serum albumin. For the staining of surface antigens, cells were incubated with FITC-, PE-, PerCP/Cy5.5- and PE-Cy7-conjugated mAbs or their isotype controls for 30 min at 4 °C, respectively. For intracellular staining, cells were fixed and permeabilized with Perm/Fix solution, and finally stained with anti-Foxp3 Alexa Fluor 647 or isotype control according to the manufacturer's instructions. Cells were assayed by BD FACS Canto™ II (BD Biosciences), and the acquired data were analyzed with FlowJo Software. Flow cytometric results were represented as percent positive.

Induction of $\gamma\delta$ Tregs *in vitro*

$\gamma\delta$ T cells were first sorted by magnetic activated cell sorting (MACS, Miltenyi Biotec) from PBMCs of healthy stem cell donors, and then cultured in complete RPMI 1640 medium (supplemented with 2 mM L-glutamine,

10 mM HEPES, 10% fetal calf serum and 200 IU/ml recombinant human IL-2) in 6-well culture plates pre-coated with immobilized anti-human TCR $\gamma\delta$ (1 $\mu\text{g/ml}$) for 12 days. In some groups, G-CSF (15 $\mu\text{g/ml}$) and/or TGF- β (2 ng/ml) were added at the beginning of the culture and replenished every 3 days. The culture system was grouped based on the differences in added cytokines, including (1) G-CSF, (2) TGF- β , (3) G-CSF + TGF- β and (4) blank control [(1)–(3) induction experimental groups]. After 3, 6, 9, and 12 days of culture, cells in the induction and control groups were harvested and analyzed by flow cytometry and real-time quantitative polymerase chain reaction (RQ-PCR). The percentages of $\gamma\delta$ Treg subsets were analyzed by flow cytometry. The expression levels of Foxp3, CD25, CTLA-4, GITR, STAT3, and Pias3 genes were examined by RQ-PCR.

RQ-PCR

RNA was extracted from $\gamma\delta$ T cells in the induction and control groups according to the manufacturer's protocol (Trizol, Invitrogen, USA), and transcribed into the first single-stranded cDNA with random hexamer primers, using reverse transcriptase of the Superscript II Kit (Gibco, USA). The quality of cDNA was confirmed by reverse transcription PCR for β_2 microglobulin ($\beta_2\text{M}$) gene amplification. The primer sequences of Foxp3, CD25, CTLA-4, GITR, STAT3, and Pias3 genes were listed in Table 1. RQ-PCR was performed in a volume of 20 μl containing 1 μl cDNA, 0.5 μM of each primer and $2.5 \times \text{RealMasterMix}$ 9 μl (Tiangen, China). After the initial denaturation at 95 $^\circ\text{C}$ for 2 min, 45 cycles consisting of 95 $^\circ\text{C}$ 15 s, 60 $^\circ\text{C}$ 60 s and 82 $^\circ\text{C}$ 1 s for plate reading were performed using MJ Research DNA Engine Opticon

2 PCR cycler (BIO-RAD, USA). The relative mRNA expression levels of relative gene in each sample were calculated according to the comparative cycle time method [17, 20].

Cytokine measurements

The concentrations of interferon (IFN)- γ , IL-4, IL-10, IL-17, and TGF- β 1 in the culture supernatant of three induction and control groups at days 3, 6, 9, and 12 were evaluated by Luminex analysis using Procarta Cytokine Assay Kits (Affymetrix) according to the manufacturer's instructions [21, 22].

Carboxyfluorescein diacetate (CFSE)-based suppression assay

CFSE assays to measure CD4⁺ T cell proliferation were performed as previously described [10, 12]. Autologous CD4⁺ T cells sorted by MACS (Miltenyi Biotec) were labeled with CFSE and used as responder cells. MACS-sorted $\gamma\delta$ T cells before and after G-CSF treatment in vivo, or $\gamma\delta$ T cells in the induction and control groups were used as effector cells. Responder cells (2×10^5 /well) were cultured alone or in combination with effector cells at a ratio of 1:1 in 12-well round-bottom plates pre-coated with anti-CD3 (1 $\mu\text{g/ml}$) in a final volume of 1 ml for 5 days. CFSE-unlabeled autologous CD4⁺ T cells were used as negative control, and CFSE-labeled CD4⁺ T cells alone were used as positive control. In the analysis of proliferation suppression effect of $\gamma\delta$ T cells in the induction and control groups, to study the role that TGF- β played in the proliferation suppression of responder cells, CD4⁺ T cells were pretreated with TGF- β receptor inhibitor (10 $\mu\text{mol/l}$; LY364947, Cayman Chemical) for 1 h prior to the addition of effector cells in some experiments. After 5 days incubation, cells were harvested and analyzed by flow cytometry by gating on the CFSE-labeled cells, in which 10,000 events were collected. Data were analyzed with FlowJo Software. The percentages of divided responder T cells were compared.

Cell counting kit-8 (CCK-8) assay

Cytotoxicity was measured with the CCK-8 assay kit (Dojindo, Japan). Sorted $\gamma\delta$ T cells before and after G-CSF in vivo treatment, or $\gamma\delta$ T cells in the induction and control groups were used as effector cells. Leukemia cell lines (Molt4, HL-60, and K562) or lymphoma cell lines (Raji and Daudi) were used as target cells. The effector cells were then mixed in a ratio of 1:1 with the target cells. A total of 10,000 cells were plated into each well of a 96-well plate, in which 10 μl CCK-8 was added to 100 μl of culture medium. The cells were subsequently incubated for 2–4 h at 37 $^\circ\text{C}$, and the absorbance was

Table 1 PCR primers of target genes and $\beta_2\text{M}$ gene

Primer	Sequence
Foxp3-for	5'-CTGACCAAGGCTTCATCTGTG-3'
Foxp3-back	5'-ACTCTGGGAATGTGCTGTTTC-3
CD25-for	5'-ACCCTGAATTTGGCCTGCACTA-3'
CD25-back	5'-GACCTCCATCCCTTCTCCCTCT-3'
CTLA-4-for	5'-GCCCTGCACTCTCTGTTTTT-3'
CTLA-4-back	5'-GGTTGCCGCACAGACTTCA-3'
GITR-for	5'-ACCCAGTTCGGGTTTCTCAC-3'
GITR-back	5'-CCAGATGTGAGTCCAAGC-3'
STAT3-for	5'-CAGCAGCTTGACACACGGTA-3'
STAT3-back	5'-AAACACCAAAGTGGCATGTGA-3'
Pias3-for	5'-CTGGGCGAATTAAGCACATGG-3'
Pias3-back	5'-AAAGCGTCGTCGGTAAAGCTC-3'
$\beta_2\text{M}$ -for	5'-TACACTGAATTCACCCCCAC-3'
$\beta_2\text{M}$ -back	5'-CATCCAATCCAATGCGGCA-3'

measured at 450 nm. All the experiments were performed in triplicate.

Statistical analysis

All data were analyzed using SPSS software, version 17.0. Results were represented as medians. Statistical significances for differences between the pre-G-CSF-treated and G-CSF-treated groups were assessed by the Wilcoxon matched pair test. Statistical significance among the induction and control groups was assessed by Kruskal–Wallis Test, and Bonferroni correction was used for pairwise comparisons. The cutoff value for $\gamma\delta$ Treg subsets in predicting aGVHD was determined using the receiver operating characteristics (ROC) curve analysis. Binary logistic regression analysis was used to estimate the association between G-PB graft cell subsets and aGVHD/relapse in recipients. aGVHD/relapse in recipients was considered as the dichotomous dependent variable, and the proportions of G-PB graft cell subsets were considered as the covariates. aGVHD was graded according to the literature [23]. $P < 0.05$ was considered statistically significant.

Results

G-CSF induces $\gamma\delta$ Tregs in vivo

To investigate the effects of G-CSF on $\gamma\delta$ Tregs, the proportions of total $\gamma\delta$, V δ 1, and V δ 2 T cells were first compared in PB from 30 donors before and after G-CSF treatment in vivo. The results demonstrated that there was an increase in V δ 1 T cell proportion ($P = 0.002$), and a decrease in the proportions of V δ 2 and total $\gamma\delta$ T cells after in vivo administration of G-CSF ($P < 0.001$, $P = 0.008$), compared with that before G-CSF treatment (Fig. 1c). The V δ 1Treg proportion was significantly increased ($P = 0.007$), and the V δ 2Treg proportion displayed a decreased tendency ($P = 0.088$) after G-CSF treatment in vivo. The proportion of $\gamma\delta$ Tregs did not significantly differ between the two groups ($P = 0.910$) (Fig. 1a–c). Additionally, we compared the CD27 and CD25 phenotypes in the $\gamma\delta$ Treg subsets, and observed a significant increase in the proportions of CD27⁺V δ 1Tregs and CD25⁺V δ 1Tregs after in vivo administration of G-CSF ($P = 0.003$, $P = 0.044$; Fig. 1a, b, d).

G-CSF induces $\gamma\delta$ Tregs in vitro

To investigate whether G-CSF could induce $\gamma\delta$ Tregs in vitro, MACS-sorted $\gamma\delta$ T cells were cultured in the presence of anti-TCR $\gamma\delta$ and G-CSF, and compared with TGF- β -induced $\gamma\delta$ Tregs. As shown in Fig. 2a, b, sorted $\gamma\delta$ T cells from the PBMCs of healthy donors rarely expressed Foxp3. After culture with anti-TCR $\gamma\delta$ plus G-CSF or TGF- β , the $\gamma\delta$ Treg population was

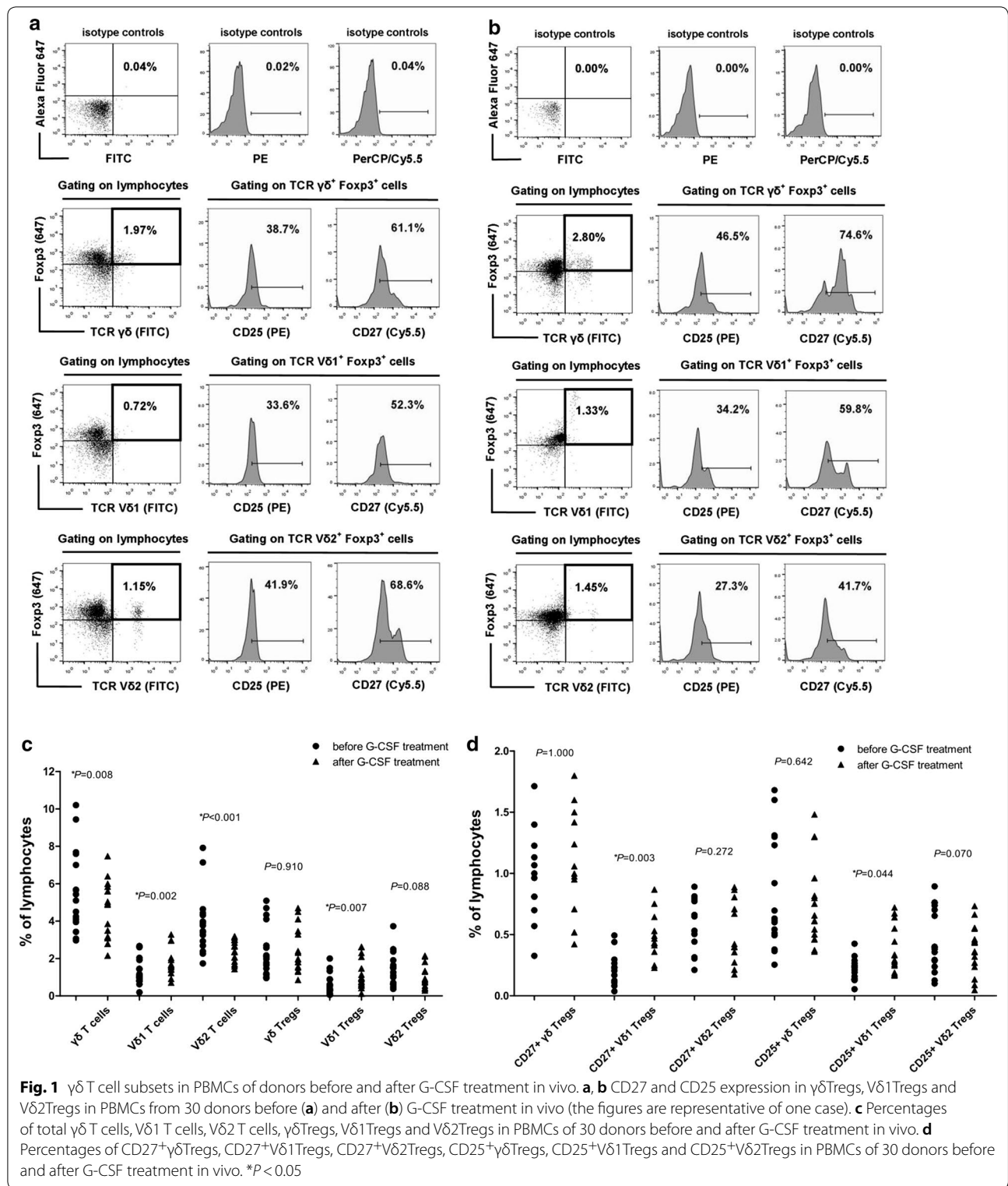
significantly increased. The expression of the $\gamma\delta$ Treg population reached its peak (approximately 30% or more) at the 9th day of culture and declined slightly afterwards. Therefore, the 9th day of culture was chosen as the observation point for evaluating the expression and function of $\gamma\delta$ Tregs.

First, the proportions of the $\gamma\delta$ Treg subsets on day 9 were compared among the control and three induction groups. Compared with $\gamma\delta$ T cells without induction, the proportions of $\gamma\delta$ Tregs and V δ 1Tregs were significantly increased following G-CSF, TGF- β , or G-CSF + TGF- β induction ($P = 0.008$, $P = 0.008$) (Fig. 2f). The proportions of $\gamma\delta$ Tregs and V δ 1Tregs in the G-CSF group were similar to that in the TGF- β and G-CSF + TGF- β group ($P = 0.873$, $P = 0.336$; $P = 0.728$, $P = 0.309$). The V δ 2Treg proportion did not significantly differ among the G-CSF, TGF- β , G-CSF + TGF- β and control groups ($P = 0.083$) (Fig. 2f). The CD25 and CD27 expression in the $\gamma\delta$ Treg subsets were then examined. Following G-CSF and/or TGF- β induction, the proportions of CD25⁺ $\gamma\delta$ Tregs and CD27⁺ $\gamma\delta$ Tregs were significantly increased ($P = 0.008$, $P = 0.010$) (Fig. 2c, g). These differences were mainly found in the V δ 1 T cells. The proportions of CD25⁺V δ 1Tregs and CD27⁺V δ 1Tregs were significantly increased after G-CSF and/or TGF- β induction ($P = 0.006$, $P = 0.005$) (Fig. 2d, g), whereas there was no significant difference in the proportions of CD25⁺V δ 2Tregs and CD27⁺V δ 2Tregs among the four groups ($P = 0.268$, $P = 0.206$) (Fig. 2e, g).

G-CSF induces $\gamma\delta$ T cells to express related molecules and cytokines

$\gamma\delta$ Tregs have been characterized to express a series of regulatory-related molecules, such as Foxp3, CD25, CTLA-4, and GITR [12, 13]. To compare differences in the expression of regulatory-related molecules in the induction and control groups, the expression of regulatory-related molecules at day 9 was analyzed by RQ-PCR. As illustrated in Fig. 3a, Foxp3 mRNA expression was greatly increased after G-CSF and/or TGF- β induction ($P < 0.001$), which was consistent with the higher proportion of $\gamma\delta$ Tregs in the induction groups. The Foxp3 expression in the G-CSF group was similar to that in the TGF- β group ($P = 0.951$), but it was lower than that in the G-CSF + TGF- β group ($P = 0.034$). The $\gamma\delta$ T cells in the induction and control groups had similar CD25, CTLA-4, and GITR expressions ($P = 0.094$, $P = 0.541$, and $P = 0.865$).

STAT3 is reported to promote the instability of natural Tregs and limit the generation of induced Tregs [24, 25]. Pias3 is the protein inhibitor of activated STAT3 [25]. Consequently, we speculated that STAT3-Pias-axis might affect the regulation of $\gamma\delta$ Tregs, and



compared the expression of STAT3 and Pias3 genes at day 9 in the induction and control groups. Pias3 expression was increased ($P=0.001$), and STAT3 expression

was decreased after G-CSF and/or TGF- β induction ($P<0.001$) (Fig. 3b, c). Pias3 expression demonstrated no significant difference among the three induction groups

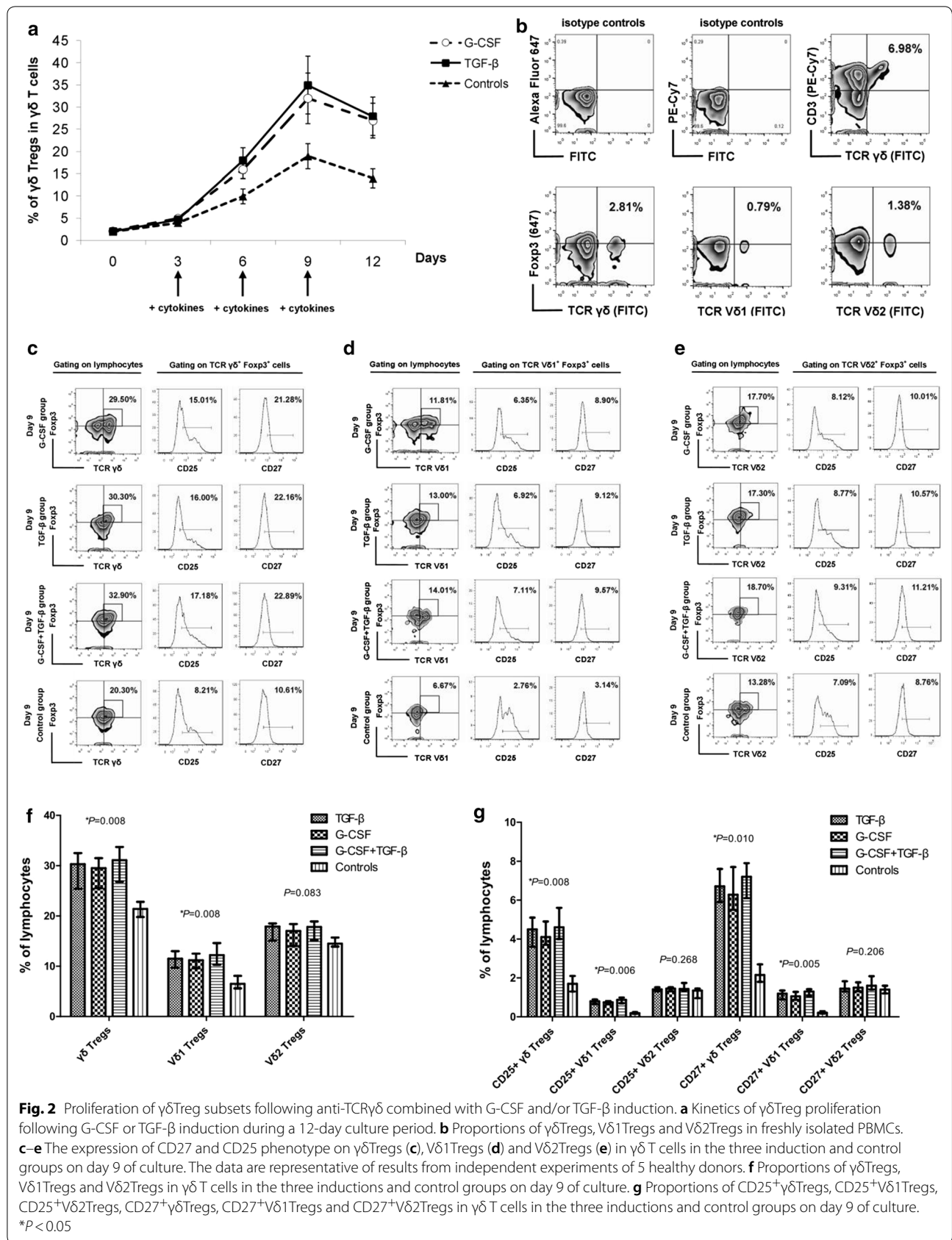
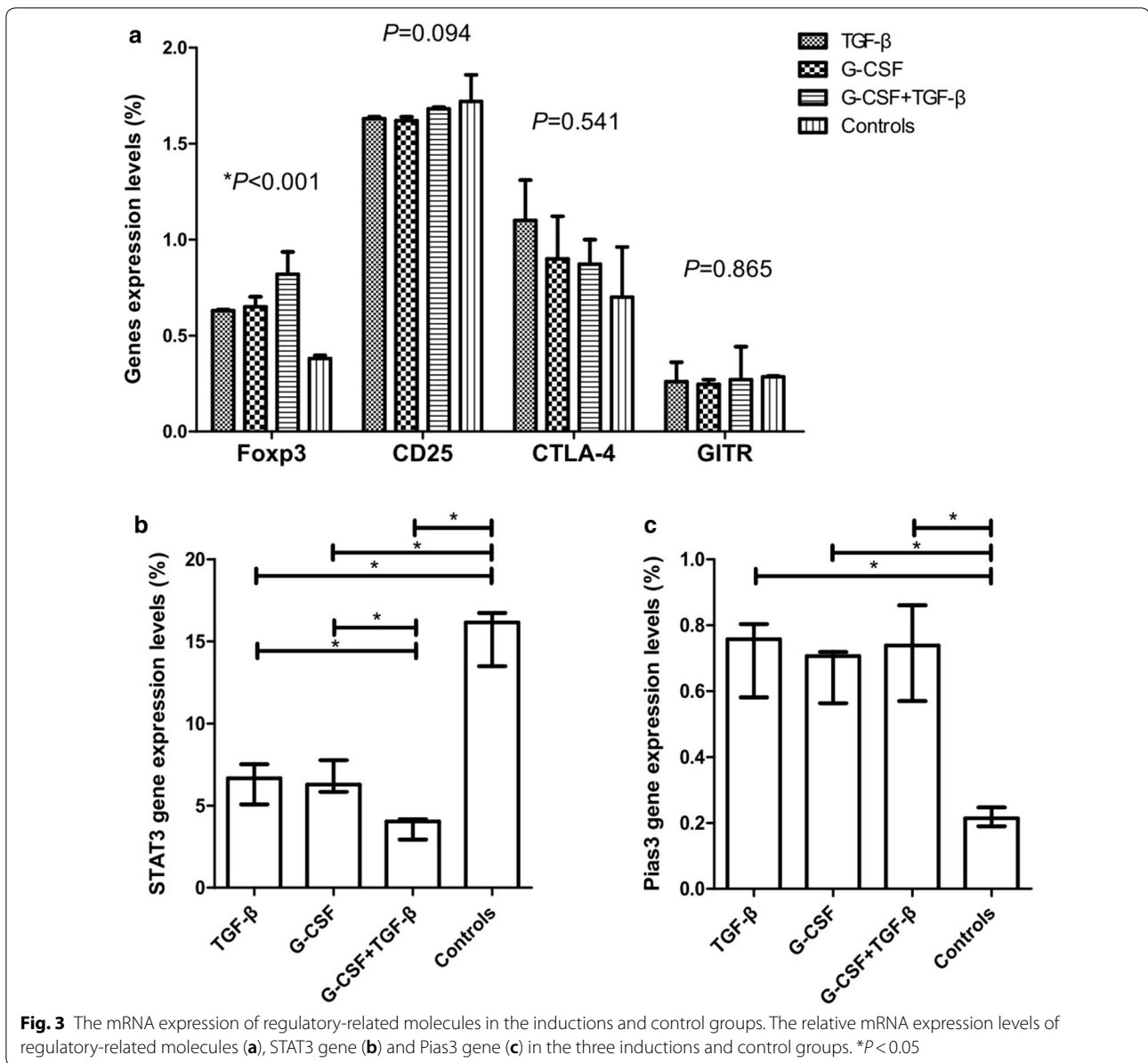


Fig. 2 Proliferation of $\gamma\delta$ Treg subsets following anti-TCR $\gamma\delta$ combined with G-CSF and/or TGF- β induction. **a** Kinetics of $\gamma\delta$ Treg proliferation following G-CSF or TGF- β induction during a 12-day culture period. **b** Proportions of $\gamma\delta$ Tregs, V δ 1Tregs and V δ 2Tregs in freshly isolated PBMCs. **c-e** The expression of CD27 and CD25 phenotype on $\gamma\delta$ Tregs (**c**), V δ 1Tregs (**d**) and V δ 2Tregs (**e**) in $\gamma\delta$ T cells in the three induction and control groups on day 9 of culture. The data are representative of results from independent experiments of 5 healthy donors. **f** Proportions of $\gamma\delta$ Tregs, V δ 1Tregs and V δ 2Tregs in $\gamma\delta$ T cells in the three inductions and control groups on day 9 of culture. **g** Proportions of CD25 $^+$ $\gamma\delta$ Tregs, CD25 $^+$ V δ 1Tregs, CD25 $^+$ V δ 2Tregs, CD27 $^+$ $\gamma\delta$ Tregs, CD27 $^+$ V δ 1Tregs and CD27 $^+$ V δ 2Tregs in $\gamma\delta$ T cells in the three inductions and control groups on day 9 of culture. * $P < 0.05$



($P=0.806$). The STAT3 expression in the G-CSF group was similar to that in the TGF- β group ($P=0.839$), but higher than that in the G-CSF + TGF- β group ($P=0.019$).

Moreover, the concentrations of IFN- γ , IL-4, IL-10, IL-17, and TGF- β 1 in the culture supernatant at day 9 were compared among the control and induction groups. Supernatant cytokine measurements demonstrated that the concentration of IFN- γ was significantly decreased following G-CSF, TGF- β , or G-CSF + TGF- β induction ($P=0.023$). $\gamma\delta$ T cells in the control group produced higher amounts of IFN- γ than $\gamma\delta$ T cells in the G-CSF, TGF- β , and G-CSF + TGF- β groups ($P=0.019$, $P=0.006$, and $P=0.014$, respectively). A similar productions of

TGF- β , IL-4, IL-10, and IL-17 were detected in $\gamma\delta$ T cells in the three induction and control groups ($P=0.125$, $P=0.299$, $P=0.292$, and $P=0.331$, respectively) (Fig. 4).

G-CSF-induced $\gamma\delta$ T cells suppress CD4⁺ T cell proliferation and have certain cytotoxic effects on tumor cells

The proliferation suppressive and cytotoxic effects of sorted $\gamma\delta$ T cells before and after G-CSF treatment in vivo were evaluated by CFSE and CCK-8 assays. The results demonstrated that sorted $\gamma\delta$ T cells before and after treatment exerted certain suppression on the proliferation of CD4⁺ T cells, whereas sorted $\gamma\delta$ T cells after G-CSF treatment had a higher suppressive effect

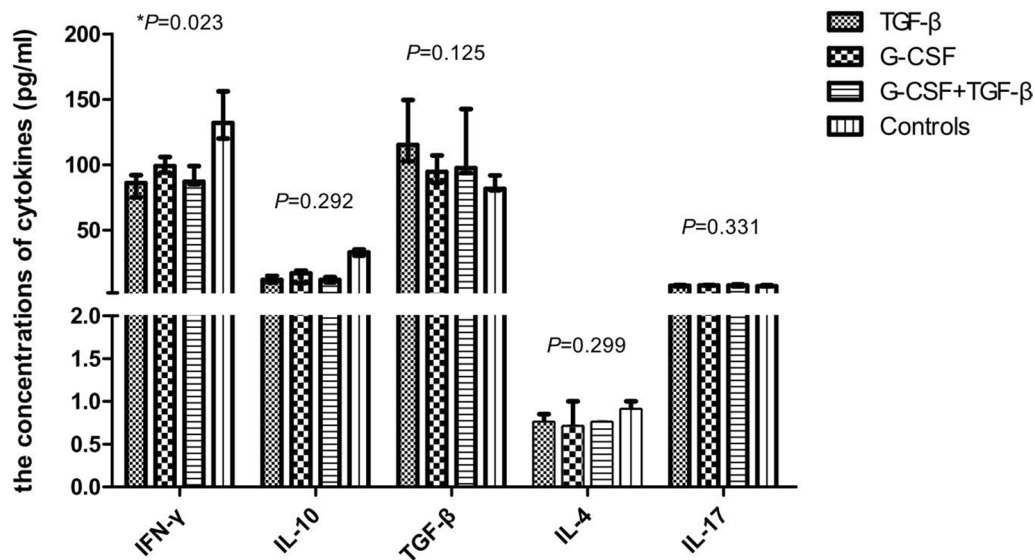


Fig. 4 The concentrations of cytokines in the supernatant of the three induction and control groups. * $P < 0.05$

on $CD4^+$ T cells than that before treatment ($P = 0.002$, Fig. 5a, b). In addition, the proliferation suppressive and cytotoxic effects of $\gamma\delta$ T cells in the induction and control groups were compared. As shown in Fig. 6a, b, G-CSF and/or TGF- β -induced $\gamma\delta$ T cells had a similar suppressive effect on $CD4^+$ T cells, which was a more significant suppressive effect than that of $\gamma\delta$ T cells in the control group ($P < 0.001$). This result was in accordance with the above finding that $\gamma\delta$ T cells in the induction groups expressed a higher proportion of $\gamma\delta$ Tregs than that in the control group. We then used TGF- β receptor inhibitor to pre-treat $CD4^+$ T cells and found that the suppressive effect could be reversed to some extent in the TGF- β and G-CSF + TGF- β group, whereas G-CSF combined with TGF- β receptor inhibitor could also have a certain suppressive effect. Moreover, the cytotoxic effects of $\gamma\delta$ T cells on leukemia cell lines (Molt4, HL-60, and K562) and lymphoma cell lines (Raji and Daudi) were not influenced by G-CSF, TGF- β , and G-CSF + TGF- β induction compared with the control group ($P = 0.116$, $P = 0.538$, $P = 0.617$, $P = 0.355$, and $P = 0.288$, respectively) (Fig. 7).

The proportion of $CD27^+V\delta 1$ Tregs in grafts negatively correlates with aGVHD in G-PBSCT recipients

To explore the effects of $\gamma\delta$ Tregs in grafts on aGVHD, 30 patients with acute leukemia in first complete remission (18 female, 12 male; median age 31 years, range 14–47 years) undergoing G-PBSCT from HLA-matched sibling donors were enrolled in this study. The primary diseases included acute myelocytic leukemia (AML, $n = 18$) and acute lymphoblastic leukemia (ALL, $n = 12$). The AML patients received busulfan (Bu) + cyclophosphamide (CY), and the ALL patients had total body irradiation (TBI) + CY conditioning regimen. Cyclosporine A plus methotrexate were administered in all of the 30 patients for GVHD prophylaxis. The 30 patients all achieved hematopoietic reconstitution. Of the 30 patients, 11 cases experienced aGVHD (6 with grade I and 5 with grade II) post-transplantation. Skin, gut and liver involvement was seen in 8, 2 and 1 patients, respectively. With a median follow up of 869 (range 147–981) days post-transplantation, 4 patients relapsed, 23 survived, and 7 died.

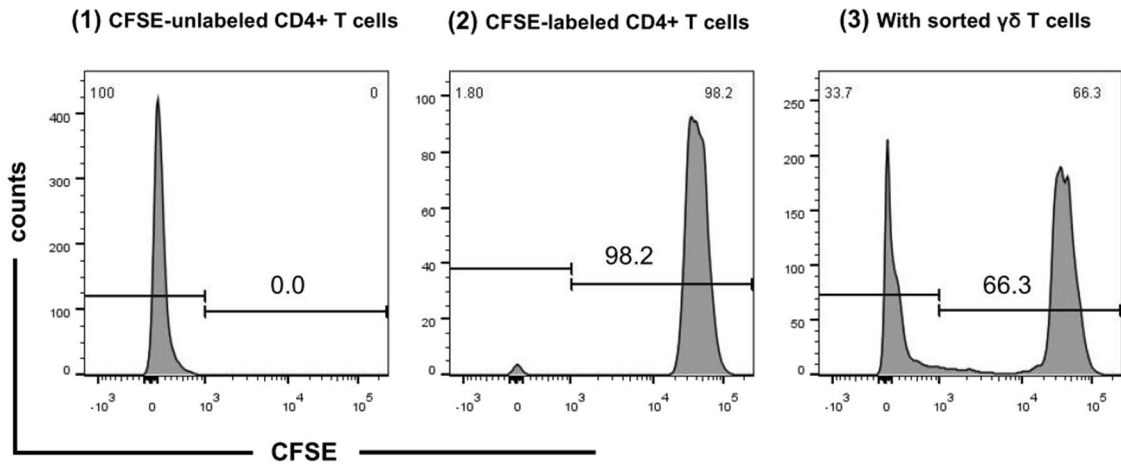
We first analyzed the effects of $\gamma\delta$ Treg subsets in grafts ($\gamma\delta$ Tregs, $V\delta 1$ Tregs, $V\delta 2$ Tregs, $CD27^+\gamma\delta$ Tregs, $CD27^+V\delta 1$ Tregs, $CD27^+V\delta 2$ Tregs, $CD25^+\gamma\delta$ Tregs, $CD25^+V\delta 1$ Tregs and $CD25^+V\delta 2$ Tregs) on the occurrence of aGVHD in G-PBSCT recipients. The results

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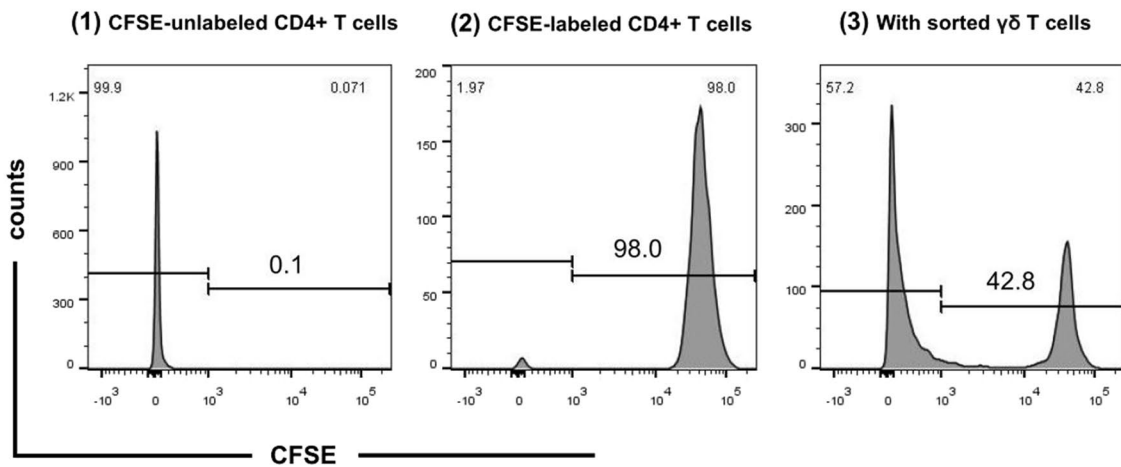
Fig. 5 Sorted $\gamma\delta$ T cells before and after G-CSF treatment in vivo exerted proliferation suppressive effects. **a** Proliferation of CFSE-labeled autologous peripheral blood $CD4^+$ T cells (responders) co-cultured with sorted $\gamma\delta$ T cells (effectors) before [(1)–(3)] and after [(4)–(6)] G-CSF treatment in vivo. (1), (4): negative controls (CFSE-unlabeled $CD4^+$ T cells); (2), (5): positive controls (CFSE-labeled $CD4^+$ T cells); (3), (6): experimental groups (CFSE-labeled $CD4^+$ T cells + sorted $\gamma\delta$ T cells). The data are representative of results from 10 healthy donors. **(B)** The scatter plot reflected the effect of sorted $\gamma\delta$ T cells before and after G-CSF treatment in vivo on the proliferation rates of CFSE-labeled $CD4^+$ T cells. * $P < 0.05$

a

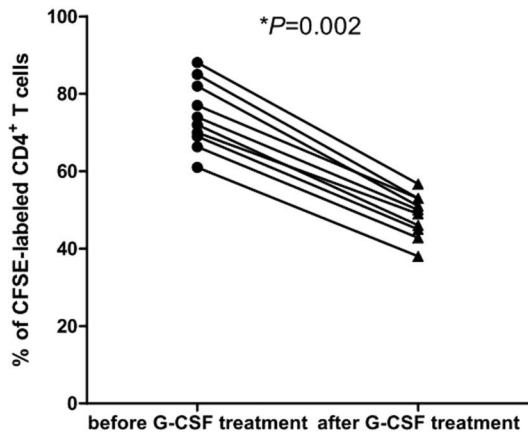
Before G-CSF treatment



After G-CSF treatment



b



showed that the proportion of CD27⁺Vδ1Tregs in grafts was significantly lower in the patients who experienced aGVHD than in those who did not develop aGVHD ($P=0.028$), and the proportions of other $\gamma\delta$ Treg subsets in grafts did not differ significantly between the two groups (all $P>0.05$) (Fig. 8a). Then we performed the ROC curve analysis for the optimal cutoff point of CD27⁺Vδ1Treg proportion in grafts in prediction of aGVHD. The most discriminative cutoff value of CD27⁺Vδ1Treg proportion in grafts was 0.33% with an area under the curve value of 0.725 ($P=0.043$, Fig. 8b). The sensitivity and specificity were 89.5 and 45.5%, respectively. According to the results of ROC analysis, we used the CD27⁺Vδ1Treg proportion in grafts 0.33% as the cutoff value. In terms of this cutoff value, 8 patients (26.7%) were classified as the low-CD27⁺Vδ1Treg group ($<0.33\%$), and 22 patients (73.3%) as the high-CD27⁺Vδ1Treg group ($\geq 0.33\%$). The incidence of aGVHD was higher in the low-CD27⁺Vδ1Treg group than in the high-CD27⁺Vδ1Treg group (75.0% versus 22.7%, $P=0.028$). In addition, there was no significant association between CD27⁺Vδ1Treg proportion and age, gender and disease type.

The association between other cell subsets in G-PB grafts and aGVHD in recipients was also analyzed. The median proportions of CD3⁺ T cells, CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, CD19⁺B cells, CD3⁻CD56⁺NK cells of lymphocytes in G-PB grafts were 70.45, 33.5, 27.1, 14.2 and 9.6%, respectively. G-PB graft cell subsets (CD3⁺T cells, CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, CD19⁺B cells, CD3⁻CD56⁺NK cells and $\gamma\delta$ Treg subsets) were divided into two groups based on their medians. Simple effect analysis of binary logistic regression showed that the proportion of CD27⁺Vδ1Tregs in G-PB grafts was negatively associated with the incidence of aGVHD in recipients ($P=0.023$, $r_s=-0.563$). Multivariate analysis also demonstrated that the higher proportion of CD27⁺Vδ1Tregs in G-PB grafts indicated a lower incidence of aGVHD in G-PBSCT recipients ($P=0.027$, odds ratio=0.033, 95% confidence interval 0.002–0.680). Nevertheless, the proportions of other cell subsets in grafts had no significant association with the incidence of aGVHD (all $P>0.05$). In addition, we

analyzed the association between $\gamma\delta$ Tregs subsets in grafts and relapse in recipients. There was no association between the proportions of $\gamma\delta$ Treg subsets in grafts and leukemia relapse in recipients (all $P>0.05$).

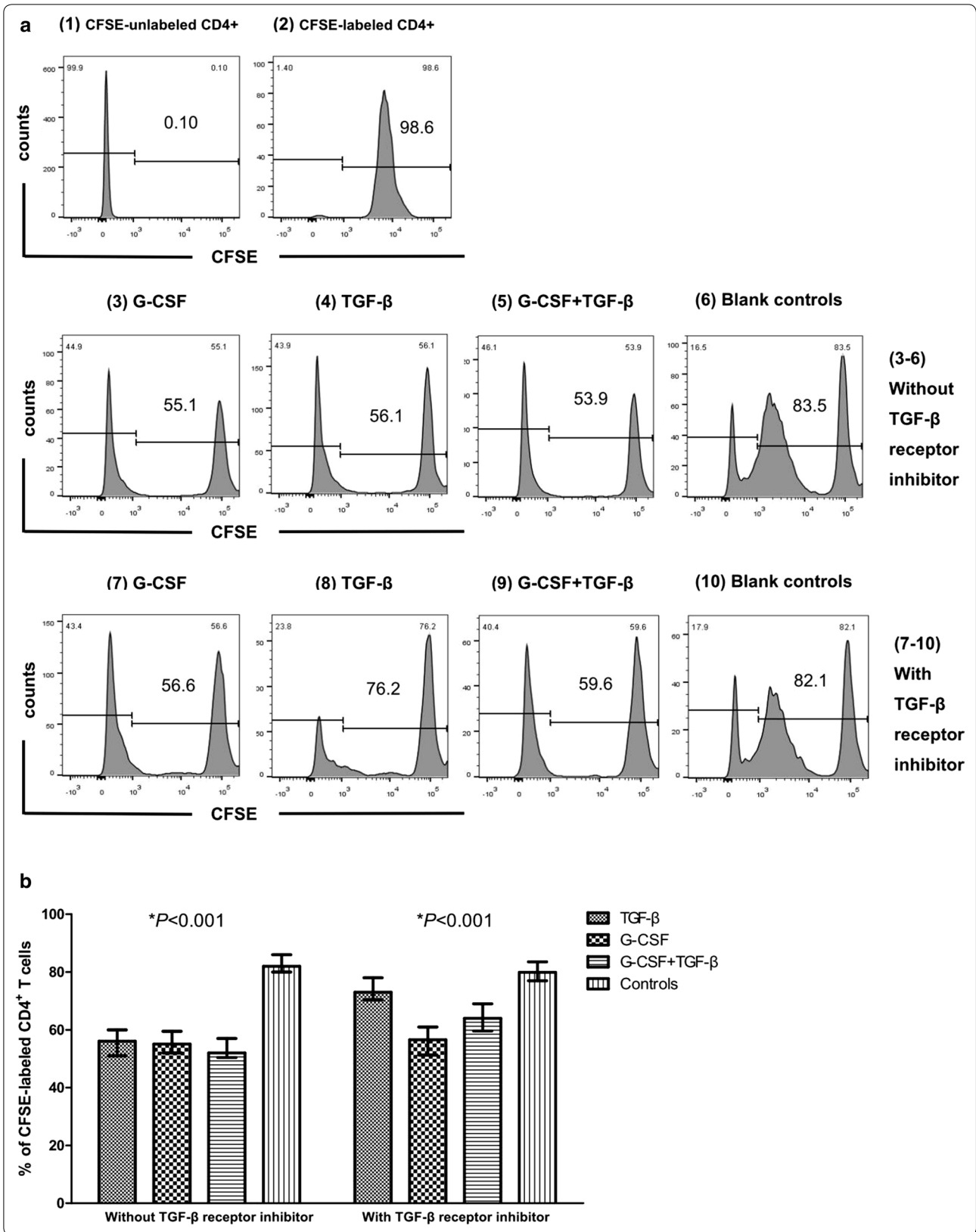
Discussion

G-CSF-induced immune tolerance has been widely studied in G-PBSCT, but to our knowledge, this is the first report from the perspective of $\gamma\delta$ Tregs. A few studies have suggested that $\gamma\delta$ Tregs are implicated in the pathogenesis of GVHD [15, 16]. Our previous study has demonstrated that G-CSF might induce immune tolerance by influencing the clonality of TCRs on $\gamma\delta$ T cells [17]. In this study, we investigated the effects of G-CSF on $\gamma\delta$ Tregs and explored the role of $\gamma\delta$ Tregs in aGVHD in G-PBSCT recipients. Our results suggested that G-CSF induced the generation of $\gamma\delta$ Tregs in vivo and in vitro, and the higher proportion of CD27⁺Vδ1Tregs in grafts was associated with a lower incidence of aGVHD in G-PBSCT recipients. Therefore, we hypothesized that G-CSF inducing immune tolerance in G-PBSCT might be associated with the increase of $\gamma\delta$ Tregs in grafts.

Under normal physiological conditions, $\gamma\delta$ Tregs exist at very low frequencies in PB, and they are decreased in PB in some autoimmune diseases [12, 15]. Li et al. [12] reported that $\gamma\delta$ Tregs could be induced from PBMCs in vitro by stimulating with anti-TCR $\gamma\delta$ in the presence of TGF- β 1. In this study, we found that $\gamma\delta$ Tregs could not only be induced in vitro by stimulating with anti-TCR $\gamma\delta$ in the presence of G-CSF but also in vivo by G-CSF alone. In addition, the immune phenotype, proliferation suppression function, and cytokine secretion of G-CSF-induced $\gamma\delta$ Tregs were similar to that of TGF- β -induced $\gamma\delta$ Tregs. The JAK/STAT3 signaling pathway might contribute to direct effects of G-CSF on T cells [24–26]. The interaction between G-CSF and its receptor promoted the phosphorylation of JAK proteins, which led to the phosphorylation and homodimerization of STAT3. The homodimerized STAT3 translocated to the nucleus and promoted the transcription of Pias3, resulting in enhancing Treg survival [24–26]. Our preliminary study found that STAT3 expression was decreased and Pias3 expression was increased after G-CSF treatment. Therefore, we speculated that G-CSF might induce $\gamma\delta$ Tregs by

(See figure on next page.)

Fig. 6 Anti-TCR $\gamma\delta$ -stimulated $\gamma\delta$ T cells exert suppressive effects on CD4⁺ T cell proliferation. **a** The proliferation of CFSE-labeled autologous peripheral blood CD4⁺ T cells (responders) co-cultured with anti-TCR $\gamma\delta$ -stimulated $\gamma\delta$ T cells on day 9 (effectors) without [(3)–(6)]/with [(7)–(10)] TGF- β receptor inhibitor. (1) negative control (CFSE-unlabeled responders); (2) positive control (CFSE-labeled responders); (3)–(10) experimental groups; (3), (7): CFSE-labeled responders + G-CSF-induced $\gamma\delta$ T cells; (4), (8): CFSE-labeled responders + TGF- β -induced $\gamma\delta$ T cells; (5), (9): CFSE-labeled responders + (G-CSF + TGF- β)-induced $\gamma\delta$ T cells; (6), (10): CFSE-labeled responders + only anti-TCR $\gamma\delta$ stimulated $\gamma\delta$ T cells. The data are representative of results from independent experiments of 5 healthy donors. **b** The bar reflected the effect of anti-TCR $\gamma\delta$ -stimulated $\gamma\delta$ T cells in the three inductions and control groups at day 9 of culture on the proliferation rates of CFSE-labeled CD4⁺ T cells. * $P<0.05$



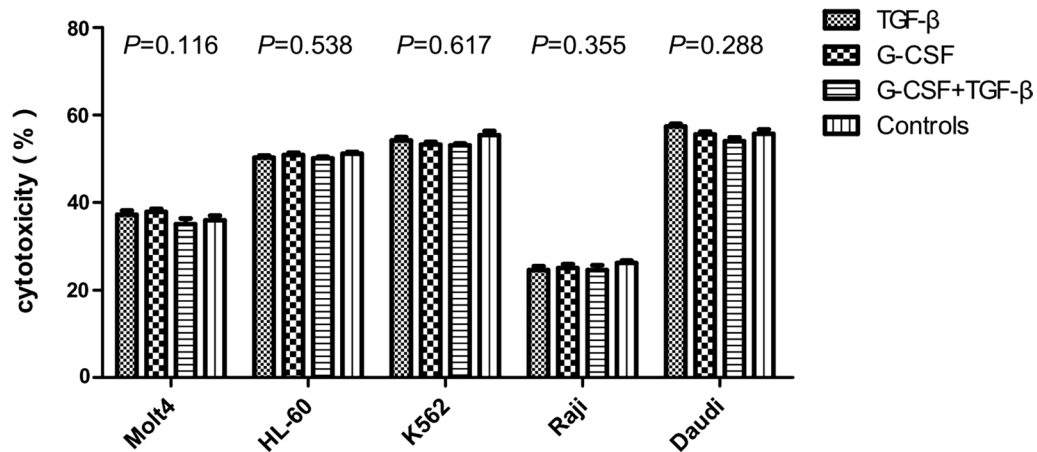


Fig. 7 The cytotoxic effects of induced $\gamma\delta$ T cells on leukemia and lymphoma cell lines. The cytotoxic effects of $\gamma\delta$ T cells on leukemia cell lines (Molt4, HL-60, and K562) and lymphoma cell lines (Raji and Daudi) were not influenced by G-CSF, TGF- β , and G-CSF + TGF- β induction compared with the control group ($P=0.116$, $P=0.538$, $P=0.617$, $P=0.355$, and $P=0.288$, respectively)

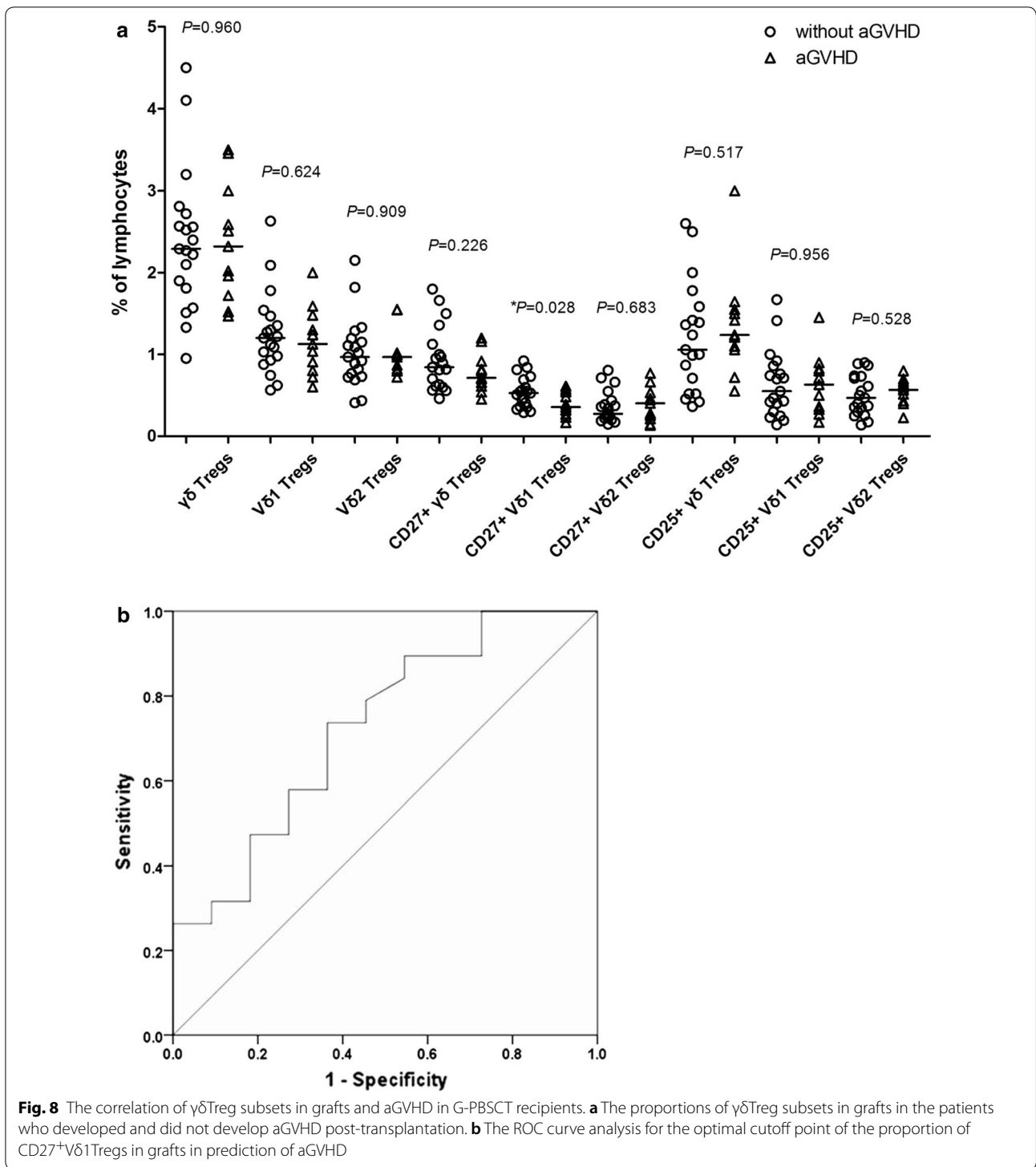
increasing Pias3 expression and inhibiting STAT3 expression, which required further experimental verification. To explore whether G-CSF and TGF- β had synergistic effects in inducing $\gamma\delta$ Tregs, sorted $\gamma\delta$ T cells were cultured in vitro in the presence of G-CSF and TGF- β . Surprisingly, the combination of the two did not have a synergistic effect in inducing $\gamma\delta$ Tregs. The reason of the result was not clear and needed further investigation. Interestingly, we observed that the proportion of $\gamma\delta$ Tregs in the control group could reach up to 20%, which was consistent with Hua's recent finding that V δ 1 T cells could be stimulated by anti-human TCR V δ 1 mAb in the absence of exogenous TGF- β 1 [13]. We suggested that the results might be due to the effect of autocrine TGF- β 1. The similar concentrations of TGF- β 1 in the culture supernatant of three induction and control groups supported our suggestion. Additionally, as soluble cytokine TGF- β in culture might be involved in the proliferation suppression of the CD4 responder cells, we used TGF- β receptor inhibitor to pre-treat CD4⁺ T cells and found that the suppression effect could be reversed to some extent in TGF- β group, suggesting that cytokine TGF- β might also participate in the course. However, the suppression effect was not reversed in the G-CSF group, which indicated that G-CSF-induced $\gamma\delta$ Tregs might have the proliferation suppressive effect on CD4⁺ T cells through the non-dependent TGF- β pathway.

$\gamma\delta$ T cells consist of two major subsets in humans: V δ 1 and V δ 2. V δ 2 T cells predominate in PB, and V δ 1 T cells are frequently found in mucosal and intraepithelial tissue [27]. The two subsets are similar in many aspects, such as activation and expansion, but they have considerable differences in inherent gene expression. V δ 2 T cells tend

to have a more inflammatory cell phenotype, and V δ 1 T cells have a more regulatory cell phenotype [28, 29]. In this study, we divided $\gamma\delta$ Tregs into the V δ 1Tregs and V δ 2Tregs groups according to δ -chain usage, and analyzed the proportions of $\gamma\delta$ Treg subsets before and after G-CSF treatment in vivo. Our study demonstrated that the V δ 1Treg proportion was found to be increased in PB after G-CSF treatment in vivo. This result indicated that V δ 1 T cells had a more marked conversion to Foxp3 expression after G-CSF treatment.

$\gamma\delta$ T cells have been shown to exert major histocompatibility complex -unrestricted natural cytotoxicity against different types of tumors such as neuroblastoma, esophageal carcinoma, and some subsets of lymphoma and leukemia [30–32]. Whether $\gamma\delta$ Tregs have a cytotoxic effect on tumor cells remains unknown. Our study demonstrated that the proportion of $\gamma\delta$ Tregs was significantly increased following G-CSF and/or TGF- β induction. The cytotoxic effects of $\gamma\delta$ T cells on leukemia and lymphoma cell lines in the three induction and control groups did not significantly differ, indicating that $\gamma\delta$ Tregs induced by G-CSF and/or TGF- β in vitro also had certain cytotoxic effects. Meanwhile, the clinical data demonstrated that the proportions of $\gamma\delta$ Tregs in grafts had no significant association with leukemia relapse, suggesting that $\gamma\delta$ Tregs in grafts had no effect on the function of graft-versus-leukemia.

Recent studies have demonstrated that $\gamma\delta$ Tregs might play an important role in tumor immunity and autoimmunity [12, 33, 34], but their role in GVHD remains undefined. Hu et al. first reported that co-transplantation of $\gamma\delta$ Tregs with human PBMCs in NOD/SCID mice could reduce the incidence of GVHD [16]. Subsequently,



this group team demonstrated that the percentages of $\gamma\delta$ Tregs were associated with the incidence and severity of chronic GVHD in patients undergoing allo-HSCT [15]. In this study, our results demonstrated that the proportion of $CD27^+ V\delta 1$ Tregs in grafts was negatively

associated with the incidence of aGVHD in G-PBSCT recipients. It was reported that G-CSF could also expand other regulatory cell subsets like $CD4^+ Foxp3^+$ Tregs and myeloid-derived suppressor cells, which might contribute to the amelioration of aGVHD [35, 36]. Therefore, we

deemed that G-CSF could affect not only Tregs but also $\gamma\delta$ Tregs, and G-CSF inducing $\gamma\delta$ Tregs in grafts might be an important way of G-CSF inducing immune tolerance. Although further prospective studies involving larger sample size are needed to validate the exact $\gamma\delta$ Treg graft dose that protects from aGVHD, our results strongly suggest $\gamma\delta$ Tregs might be used as an evaluating indicator and therapeutic target for aGVHD in the future.

Conclusions

To our best knowledge, we firstly demonstrated that G-CSF could induce the generation of $\gamma\delta$ Tregs in vivo and in vitro. And lower incidence of aGVHD in G-PBSCT recipients might be in partly due to higher proportion of CD27⁺V δ 1Tregs after G-CSF mobilization. This finding might provide a theoretical basis and feasibility for employing $\gamma\delta$ Tregs as a potential target for the prevention and treatment of aGVHD.

Abbreviations

G-CSF: granulocyte colony-stimulating factor; PBSCT: peripheral blood stem cell transplantation; BMT: bone marrow transplantation; G-PBSCT: G-CSF-mobilized allogeneic PBSCT; aGVHD: acute graft-versus-host disease; Tregs: regulatory T cells; $\gamma\delta$ Tregs: regulatory $\gamma\delta$ T cells; PBMCs: peripheral blood mononuclear cells; TGF- β 1: transforming growth factor- β 1; IL-2: interleukin-2; TCRs: T cell receptors; PB: peripheral blood; G-PB: G-CSF-primed PB; PBS: phosphate-buffered saline; MACS: magnetic activated cell sorting; RQ-PCR: real-time quantitative polymerase chain reaction; β_2 M: β_2 microglobulin; IFN- γ : interferon- γ ; CFSE: carboxyfluorescein diacetate; CCK-8: cell counting kit-8; ROC: receiver operating characteristics; AML: acute myelocytic leukemia; ALL: acute lymphoblastic leukemia; Bu: busulfan; CY: cyclophosphamide; TBI: total body irradiation.

Authors' contributions

LX and XLW designed and performed the research, analyzed the data and wrote the manuscript; DQ, LG, HL and ZYJ performed experiments, analyzed and interpreted the data; ZPF, FH, and JS assisted in experiments and collected the clinical data; QFL and YQL designed and supervised the research, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its additional files).

Consent for publication

Not applicable.

Ethics approval and consent to participate

All donors and patients were willing to accept the trial after being informed, and all samples were obtained with consent. All of the procedures were conducted according to the guidelines of the Medical Ethics Committees of the Health Bureau of the Guangdong Province of China. This study was approved by the Ethics Committee of Nanfang Hospital and the Medical School of Jinan University.

Funding

This work was supported by National Natural Science Foundation of China (No. 81770150, No. 81300445, No. 81401315, No. 81270647, No. 81200388, No. 81470349, No. 81000231, No. U1401221), Outstanding Youths Development Scheme of Nanfang Hospital, Southern Medical University (No. 2015J003), Natural Science Foundation of Guangdong Province (No. 2014A030310171, No. S2012010009299, No. 2014A030313380), Project of the Zhujiang Science & Technology Star of Guangzhou City (No. 201806010029, No. 2013J2200046), and Fundamental Research Funds for the Central Universities (No. 21617465).

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 18 March 2018 Accepted: 16 May 2018

Published online: 25 May 2018

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