



# Vitamin D alleviates acute graft-versus-host disease through promoting the generation of Foxp3<sup>+</sup> T cells

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**Background:** Acute graft-versus-host disease (aGVHD) is a medical complication which may result in significant morbidity and mortality after transplantation. The aim of this study investigated the therapeutic effect and underlying mechanism of 1,25-dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) in the treatment of aGVHD.

**Method:** An aGVHD model was built by transferring splenocytes of B6 mice into B6D2F1 mice. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> was added to evaluate the protective function to aGVHD; the phenotype and cytokine expression profile of spleen cells from the aGVHD model were determined using flow cytometry 2 weeks after the model is established.

**Result:** Administration of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> significantly slowed aGVHD progression and improved survival of B6D2F1 recipients of grafted B6 splenocytes. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment also resulted in an increased number of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) but decreased the number of CD4<sup>+</sup>IL-4<sup>+</sup> cells. *In vitro* analysis demonstrated that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> directly increased forkhead box P3 (Foxp3) and IL-10 expression and enhanced the function of induced Tregs (iTregs).

**Conclusions:** This analysis indicated that the effect of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> is mediated in part by improving the number of Tregs. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> administration thus represents a viable approach for treating aGVHD.

**Keywords:** Vitamin D (VD); acute graft-versus-host disease (aGVHD); regulatory T cell (Treg); forkhead box P3 (Foxp3)

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## Introduction

Vitamin D (VD) refers to a group of fat-soluble secosteroids that increase intestinal absorption of calcium, magnesium, and phosphate and exhibit numerous other biological effects (1). The most important compounds in VD in humans are VD<sub>3</sub> (also known as cholecalciferol) and VD<sub>2</sub> (ergocalciferol), which can be obtained via diet or supplements (2,3).

Acute graft-versus-host disease (aGVHD) is a medical complication which may result in significant morbidity and mortality after allogeneic hematopoietic stem cell transplantation (4) and organ transplantation (5). Currently, limited success had been made for the strategies to treat or even control aGVHD (6). To simulate the lymphopenic but delayed pathogenic state of graft-versus-host reaction during aGVHD, spleen cells derived from parental origin were injected into F1 mice which may generate donor CD8<sup>+</sup>

cytotoxic T lymphocytes specific for host spleen cells in 2 weeks (7).

The traditional view of 1,25-dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) is a crucial role in maintaining calcium homeostasis and modulating calcium metabolism. The role of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in immune responses to infection has become an exciting topic of research (8,9). 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to regulate cellular proliferation, differentiation, apoptosis, and innate and adaptive immunity (10) in several diseases, such as inflammatory bowel disease (11), diabetes (12), food allergies (13), and tumors (14). However, whether 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> exerts a therapeutic effect against aGVHD had not been determined. Therefore, we examined the regulative ability of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in protecting aGVHD. Surprisingly, our results consistently demonstrated that administration of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> significantly slowed aGVHD progression and improved survival of B6D2F1 recipients of grafted B6 splenocytes. *In vitro* studies proved that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> induces the differentiation of regulatory T cells (Tregs), which regulate, at least in part, immune hemostasis in aGVHD. The results of the study emphatically proved that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment could be useful to prevent aGVHD or other autoimmune disease (15).

## Methods

### Animals

C57BL/6 (H-2K<sup>b</sup>) and B6D2F1 (H-2K<sup>b/d</sup>) mice (male, 8 weeks old) were obtained from the Animal Resources Center, Nanjing Medical University. The mice were housed with standard rodent diet and water provision. Relevant legal and ethical requirements were followed carefully according to the protocol (number NMU08-092), which was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

### Fluorescence-activated cell sorting (FACS) analysis

Spleen samples were obtained from recipient mice on the indicated days after cell transplantation. Cells were stained with surface antibody markers before analyzed by FACS. For forkhead box P3 (Foxp3) staining, cells were fixed, permeabilized, and finally stained with Foxp3. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 0.05  $\mu$ g/mL) and ionomycin (0.5  $\mu$ g/mL) for 5 hours, and brefeldin A (5  $\mu$ g/mL) for four hours under 5% CO<sub>2</sub> and 37 °C

environment. The stimulated cells were collected, fixed, and permeabilized (85-88-8824-00, eBioscience, San Diego, CA, USA) and stained with FACS-targeted antibodies. Mouse-specific monoclonal antibodies used for flow cytometry included CD8 (APC), CD4 (PE-cy7), CD25 (APC-cy7), IFN- $\gamma$  (APC), TNF- $\alpha$  (BV421), IL-4 (BV421), TGF- $\beta$  (AF488), IL-10 (PE), H-2K<sup>b</sup> (APC), and H-2K<sup>d</sup> (PE) purchased from BioLegend, and Foxp3 (APC), CD19 (FITC) purchased from BD Pharmingen.

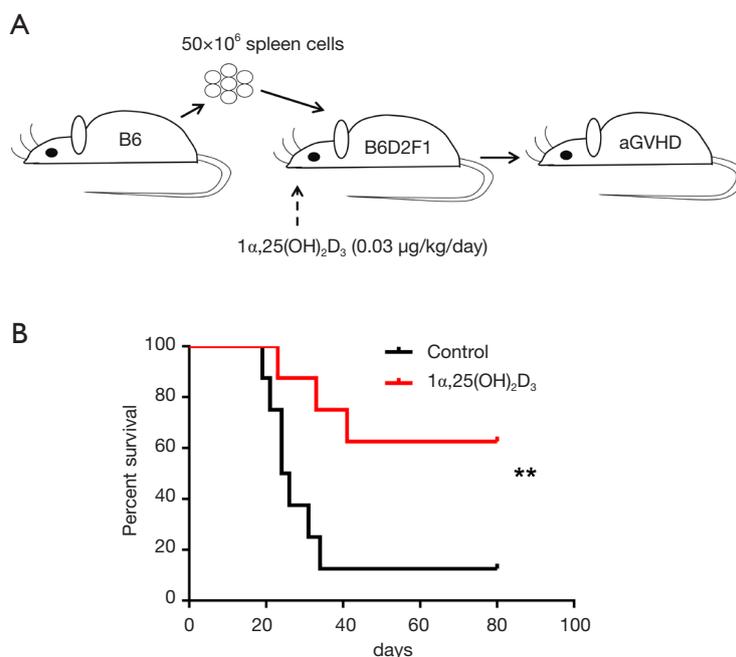
### Development of mouse aGVHD models

aGVHD was induced in normal, unirradiated B6D2F1 mice on the same day by intravenous injection of 5 $\times$ 10<sup>7</sup> B6 mice derived spleen cells, as reported previously (16). Two weeks later, the mice were sacrificed, and splenocytes were stained with anti-mouse-H-2K<sup>b</sup> and anti-mouse-H-2K<sup>d</sup> to identify the donor and host cells and indicated cell markers (BioLegend, San Diego, CA, USA). Based on the aGVHD model, mice were divided into four groups: control group, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> group, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> + IgG group, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> + PC61 group. Mice in 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> + PC61 group and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> + IgG were injected intraperitoneally with PC61 (250 mg/mouse/day) and IgG (250 mg/mouse/day), respectively for 7 days before they were injected with 50 $\times$ 10<sup>6</sup> B6 cells. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (0.03  $\mu$ g/kg/day) (740551, Sigma-Aldrich, St. Louis, MO, USA) was administered intragastrically for 4 weeks (2 weeks before the aGVHD model was established and 2 weeks after establishment).

### Naïve T cell isolation and CD4<sup>+</sup> Treg generation

Spleen cells from B6 mice were derived, and CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells were sorted using a magnetic naïve CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec, Bergisch-Gradbach, Germany). The purity of CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells was tested through FACS and found to be at >98% purity before cell culture.

The above cells were cultured in 48-well plates and stimulated for 3 days with the addition of anti-mouse-CD3/CD28 labeled beads (the ratio of bead to cell is 1:5) in conjunction with IL-2 (20 IU/mL) and TGF- $\beta$  (10 ng/mL). 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> M) was added in some experiments at the beginning of the culture. The culture medium contained RPMI 1640 medium, 100 U/mL penicillin, 100 mg/mL streptomycin, 10 mM HEPES (Invitrogen Life Technologies, Carlsbad, CA, USA), and 10% heat-inactivated fetal calf serum (Hyclone, Chicago, IL, USA).



**Figure 1**  $1\alpha,25(\text{OH})_2\text{D}_3$  protected against aGVHD in a mouse model. B6D2F1 mice were injected intravenously with  $50 \times 10^6$  B6 spleen cells.  $1\alpha,25(\text{OH})_2\text{D}_3$  ( $0.03 \mu\text{g}/\text{kg}/\text{day}$ ) was administered intragastrically for 4 weeks (2 weeks before the aGVHD model was established and 2 weeks after establishment). (A) Protocol for *in vivo*  $1\alpha,25(\text{OH})_2\text{D}_3$  administration; (B) effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  on the survival of aGVHD mice. Kaplan-Meier survival curves depict the percentage of living mice ( $n=8$  for each group). \*\*,  $P<0.01$ .  $1\alpha,25(\text{OH})_2\text{D}_3$ ,  $1,25$ -dihydroxyvitamin  $\text{D}_3$ ; aGVHD, acute graft-versus-host disease.

### *In vitro* suppression assay

B6 derived  $\text{CD4}^+\text{CD25}^-$  T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA), and cultured with  $\text{CD4}^+$  induced Tregs (iTregs) as well as irradiated dendritic cells and anti-CD3 for 3 days at different ratios. The suppressive ability of iTregs was tested through flow cytometry.

### Statistical analysis

Results are shown as the mean  $\pm$  standard error of the mean (SEM). All analysis were performed using Stata software (version 11.0). P values less than 0.05 (two-tailed) was considered statistically significant.

## Results

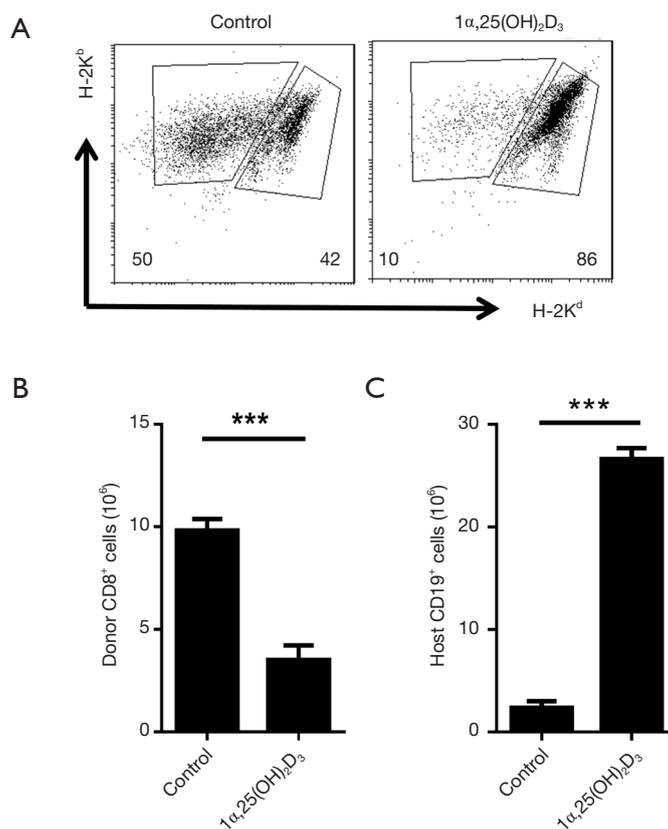
### $1\alpha,25(\text{OH})_2\text{D}_3$ protects against aGVHD in a mouse model

To investigate the protective effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  during the pathogenesis of aGVHD,  $50 \times 10^6$  B6 spleen cells were

injected intravenously into B6D2F1 mice (Figure 1A). Survival was monitored daily.  $1\alpha,25(\text{OH})_2\text{D}_3$  administration significantly prolonged survival ratio compared with the model group (Figure 1B). Additionally, improvement of the kidney injury was also observed in aGVHD mouse model with  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment (Figure S1).

### *Infusion of $1\alpha,25(\text{OH})_2\text{D}_3$ markedly suppresses donor cell engraftment and prevents host cell depletion in aGVHD mice*

To assess the effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  administration on the development of aGVHD, mice were sacrificed on day 14 after the establishment of aGVHD, and donor and host cells in the spleen were identified by flow cytometry following H-2K<sup>d</sup> and H-2K<sup>b</sup> staining (donor cells: H-2K<sup>b+/d-</sup>, host cells: H-2K<sup>b+/d+</sup>). Donor cells comprised almost 50% of the cells in the Control group (17). Interestingly,  $1\alpha,25(\text{OH})_2\text{D}_3$  injection suppressed donor cell engraftment (50% to 10%). The primary pathologic characteristic of aGVHD is a reduction of lymphocytes from the host origin (18). What's



**Figure 2** Infusion of  $1\alpha,25(\text{OH})_2\text{D}_3$  markedly suppresses donor cell engraftment and prevents host cell depletion in aGVHD mice. Two weeks after aGVHD model was established, lymphocyte subsets were analyzed through flow cytometry. (A) Representative plots for donor and host cells in the spleen; (B) total numbers of donor CD8<sup>+</sup> cells and host CD19<sup>+</sup> cells are indicated. Data are shown as mean  $\pm$  SEM from six independent experiments ( $n=8$  for each group). \*\*\*,  $P<0.001$ .  $1\alpha,25(\text{OH})_2\text{D}_3$ , 1,25-dihydroxyvitamin D<sub>3</sub>; aGVHD, acute graft-versus-host disease; SEM, standard error of the mean.

more, although B6 spleen cells transfer led to the lower number of the total number of spleen cells and host cells in F1 mice,  $1\alpha,25(\text{OH})_2\text{D}_3$  presented strong ability to preserve the proportion of host cells and decrease the expansion of donor cells (Figure 2A).

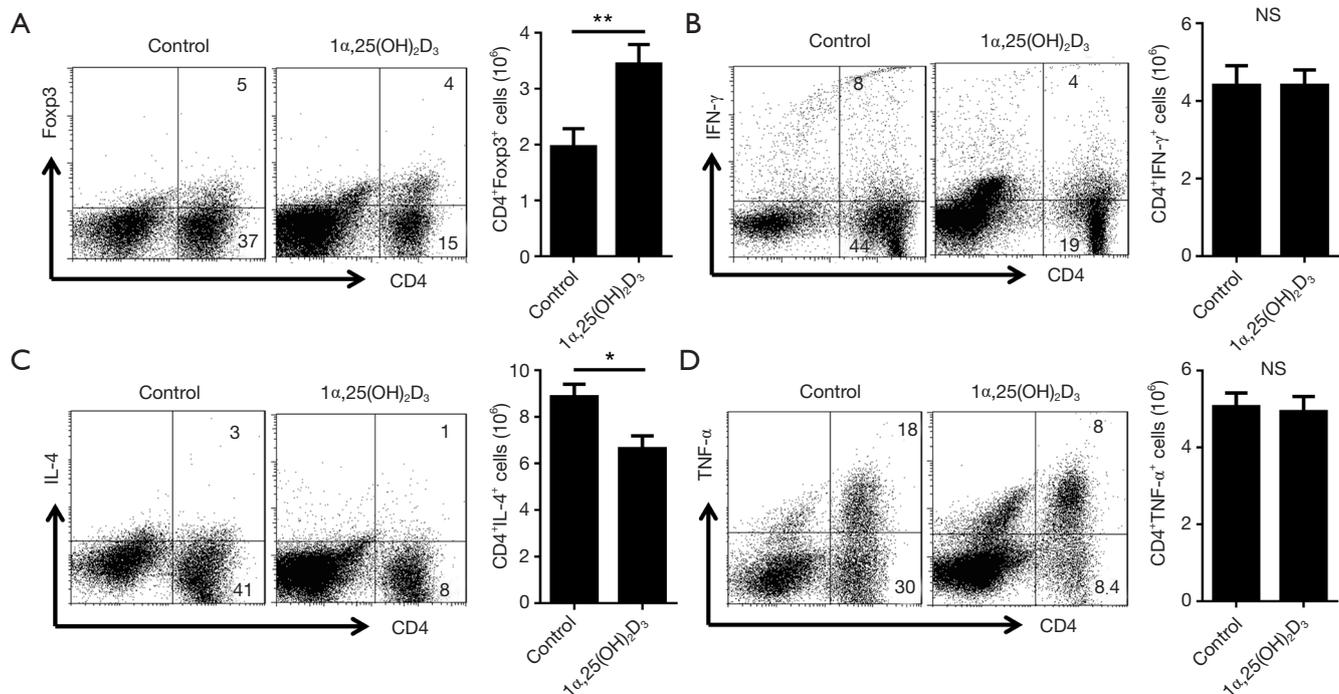
Donor CD8<sup>+</sup> cells are important in initiating the pathogenesis of aGVHD in B6-to-F1 control mice (19). We, therefore, tested the frequencies of the donor as well as host cells in the Control and  $1\alpha,25(\text{OH})_2\text{D}_3$  groups.  $1\alpha,25(\text{OH})_2\text{D}_3$  markedly suppressed donor CD8<sup>+</sup> cells expansion as well as CD4<sup>+</sup> cells (Figure 2B,S2). We analyzed the killing effect of donor CD8<sup>+</sup> cells as it is another characteristic of aGVHD (19). As shown in Figure 2C,  $1\alpha,25(\text{OH})_2\text{D}_3$  almost wholly prevented the aGVHD-associated host B cells reduction while without  $1\alpha,25(\text{OH})_2\text{D}_3$  administered, dramatically B cell decline was observed. Thus, our data demonstrate

that  $1\alpha,25(\text{OH})_2\text{D}_3$  suppresses the expansion and cytotoxic effects of donor CD8<sup>+</sup> cells against host CD19<sup>+</sup> cells.

#### ***1α,25(OH)<sub>2</sub>D<sub>3</sub> increases Foxp3 but reduces cytokine expression in vivo***

Next, we examined Foxp3 expression in  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated and -untreated mice. Administration of  $1\alpha,25(\text{OH})_2\text{D}_3$  led to increasing number of spleen lymphocytes in  $1\alpha,25(\text{OH})_2\text{D}_3$  group mice, which is almost twice the control (data not shown). Therefore, the absolute number of Foxp3-expressing CD4<sup>+</sup> cells increased, whereas no difference was observed in the percentage of Foxp3<sup>+</sup> T cells in the spleen (Figure 3A).

Previous studies suggested the production of inflammatory cytokine contributes to aGVHD induction



**Figure 3**  $1\alpha,25(\text{OH})_2\text{D}_3$  increased the expression of Foxp3 and reduced cytokine expression *in vivo*. Representative dot plots and absolute numbers of (A) CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, (B) CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells, (C) CD4<sup>+</sup>IL-4<sup>+</sup> T cells, and (D) CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup> T cells were determined by flow cytometry. Bars show the mean  $\pm$  SEM of six independent experiments (n=8 for each group). \*, P<0.05; \*\*, P<0.01.  $1\alpha,25(\text{OH})_2\text{D}_3$ , 1,25-dihydroxyvitamin D<sub>3</sub>; Foxp3, forkhead box P3; SEM, standard error of the mean; NS, no significant difference.

(20,21). So, we evaluated various cytokines expression in CD4<sup>+</sup> T cells with or without  $1\alpha,25(\text{OH})_2\text{D}_3$  administration.  $1\alpha,25(\text{OH})_2\text{D}_3$  administration led to down-regulated cytokine expression such as IFN- $\gamma$ , IL-4, and TNF- $\alpha$  from CD4<sup>+</sup> cells (Figure 3B,C,D). Also, the absolute number of CD4<sup>+</sup>IL-4<sup>+</sup> T cells declined following  $1\alpha,25(\text{OH})_2\text{D}_3$  administration, whereas no difference was observed in the total number of CD4<sup>+</sup> cells expressing IFN- $\gamma$  and TNF- $\alpha$ . In addition, we also found  $1\alpha,25(\text{OH})_2\text{D}_3$  could decrease the percentage as well as the absolute number of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells (Figure S3).

#### ***1 $\alpha,25(\text{OH})_2\text{D}_3$ increases Foxp3 and IL-10 expression and regulatory function during iTreg generation***

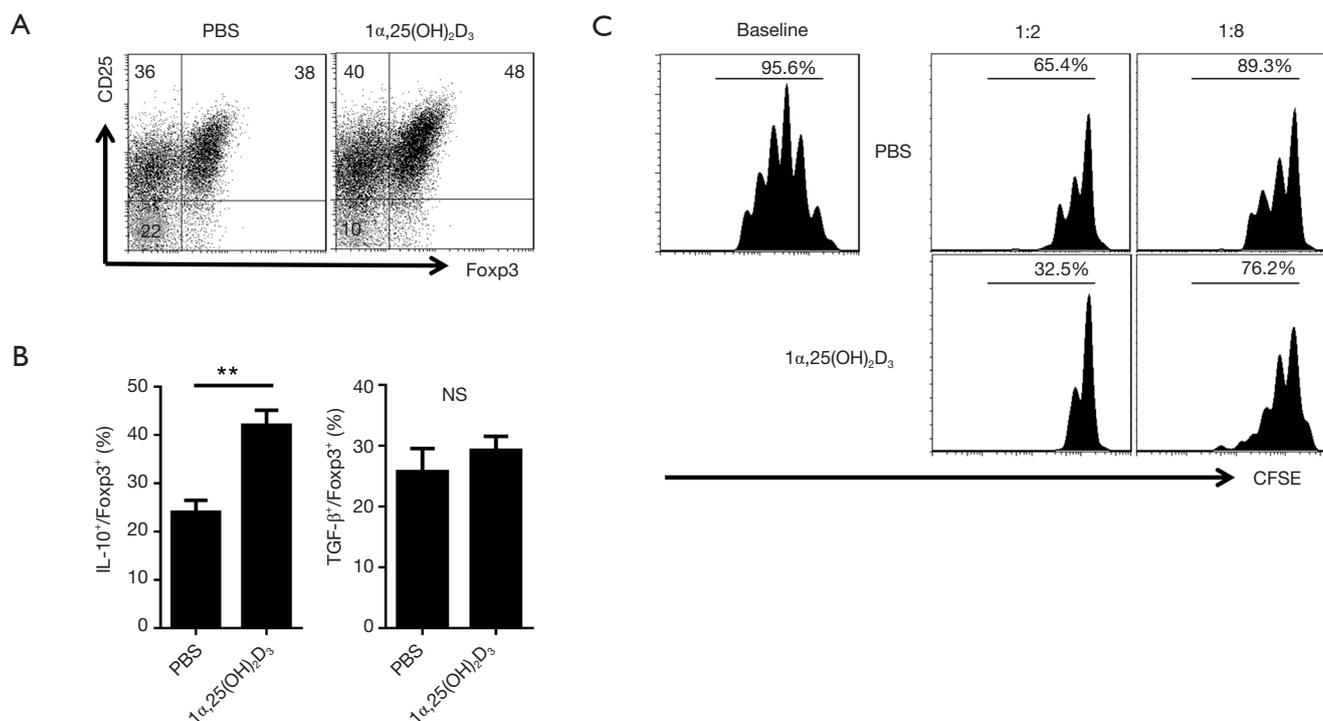
To evaluate the regulatory effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  on the role of Tregs, we first examined Foxp3 expression in  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated and -untreated iTreg subsets. iTregs treated with  $1\alpha,25(\text{OH})_2\text{D}_3$  exhibited higher Foxp3 expression on day three compared with the control cells treated only with

phosphate-buffered saline (PBS) (Figure 4A).

Next, we examined the expression of various cytokines in  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated and -untreated iTreg subsets, as Tregs are known to express anti-inflammatory cytokines such as IL-10 and TGF- $\beta$  to suppress effector T cell expansion. As expected,  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment resulted in increased IL-10 expression; however, no significant change was observed in TGF- $\beta$  expression (Figure 4B). We then directly compared the suppressive effects of both iTreg subsets *in vitro*. Compared with PBS-treated iTregs, T cell proliferation was suppressed in co-culture with  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated iTregs.

#### ***Tregs mediate 1 $\alpha,25(\text{OH})_2\text{D}_3$ -associated protection against aGVHD***

Finally, a monoclonal antibody against CD25 (CD25MoAb or PC61) was used to abolish the function of Tregs *in vivo* (22,23). Meanwhile, mice in  $1\alpha,25(\text{OH})_2\text{D}_3$  + PC61 group and  $1\alpha,25(\text{OH})_2\text{D}_3$  + IgG were injected intraperitoneally with



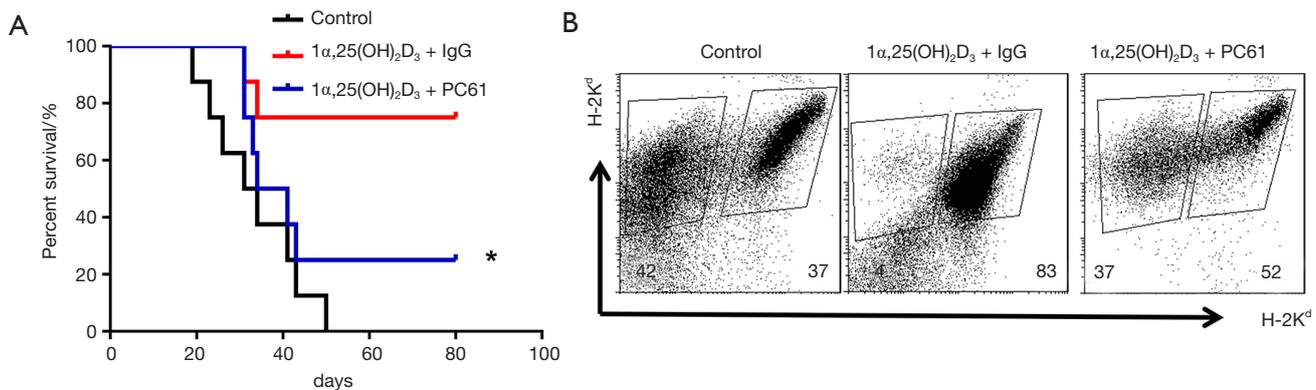
**Figure 4** 1 $\alpha$ ,25(OH) $_2$ D $_3$  enhanced Foxp3 and IL-10 expression and regulatory function during iTreg generation. iTregs were cultured from naïve CD4<sup>+</sup>CD62<sup>+</sup>CD25<sup>-</sup> T cells with anti-CD3/CD28 beads (the ratio of bead to cell is 1:5), IL-2 (20 IU/mL), and TGF- $\beta$  (10 ng/mL) for 3 days. (A) Representative plots gated on CD4<sup>+</sup> cells for CD25<sup>+</sup>Foxp3<sup>+</sup> expression by iTregs treated with PBS or 1 $\alpha$ ,25(OH) $_2$ D $_3$  (10<sup>-7</sup>M); (B) the percentages IL-10<sup>+</sup> and TGF- $\beta$ <sup>+</sup> cells among Foxp3<sup>+</sup> cells on day 3. Data are expressed as mean  $\pm$  SEM of five independent experiments; (C) representative histogram plots of T cell expansion after co-culture for 3 days with PBS- or 1 $\alpha$ ,25(OH) $_2$ D $_3$ -treated iTreg subsets in the presence of anti-CD3. \*\*, P<0.01. 1 $\alpha$ ,25(OH) $_2$ D $_3$ , 1,25-dihydroxyvitamin D $_3$ ; Foxp3, forkhead box P3; iTregs, induced regulatory T cells; SEM, standard error of the mean; PBS, phosphate-buffered saline; CFSE, carboxyfluorescein succinimidyl ester; NS, no significant difference.

PC61 (250 mg/mouse/day) and IgG (250 mg/mouse/day), respectively for 7 days before they were injected with 50 $\times$ 10<sup>6</sup> B6 cells. After 14 days, the effect of 1 $\alpha$ ,25(OH) $_2$ D $_3$  on aGVHD was generally examined by flow cytometry, which showed that the protective ability of 1 $\alpha$ ,25(OH) $_2$ D $_3$  was substantially reversed (Figure 5A), consistent with the results of survival analyses shown in Figure 5B.

## Discussion

In this study, we demonstrated that the administration of 1 $\alpha$ ,25(OH) $_2$ D $_3$  reduced the severity of aGVHD in a mouse model. 1 $\alpha$ ,25(OH) $_2$ D $_3$  promoted the expansion of Foxp3-expressing Treg populations and inhibited IFN- $\gamma$  expressing Th1 and IL-4 expression Th2 cells *in vivo* during the development of aGVHD.

Several studies have reported that VD has positive effect on the treatment of aGVHD and Tregs are critical regulators of aGVHD (19,24). Silva *et al.* reported that treatment with VD appears to be effective, safe and inexpensive for the management of patients with chronic GVHD (25). Rosenblatt *et al.* also demonstrated that VD may have an important role in the prevention and treatment of aGVHD by hindering the maturation of DCs and inducing Foxp3 expressing Treg populations. Zahran *et al.* reported a strong association between the percentage of Treg phenotypes and VD level in term and preterm pregnant women with VD deficiency (26). Gorman *et al.* reported an increased proportion of Tregs in VD-treated mice (27). Present data had proved that 1 $\alpha$ ,25(OH) $_2$ D $_3$  successfully increased the number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells both *in vitro* and *in vivo*, in agreement with previous studies



**Figure 5** Tregs mediated 1 $\alpha$ ,25(OH) $_2$ D $_3$ -associated aGVHD protection. aGVHD model was established as mentioned above, PC61 or control IgG were injected intraperitoneally. (A) Survival of aGVHD mice in different groups (n=8 for each group); (B) representative plots for donor and host cells. \*, P<0.05. Tregs, regulatory T cells; 1 $\alpha$ ,25(OH) $_2$ D $_3$ , 1,25-dihydroxyvitamin D $_3$ ; aGVHD, acute graft-versus-host disease.

indicating that VD increases Treg populations *in vivo*. In mice injected with PC61, the effect of 1 $\alpha$ ,25(OH) $_2$ D $_3$  was diminished, confirming that upregulation of Foxp3 $^+$  T cells is an important mechanism for 1 $\alpha$ ,25(OH) $_2$ D $_3$  against aGVHD.

*In vitro* experiments found that 1 $\alpha$ ,25(OH) $_2$ D $_3$  directly enhanced Foxp3 and IL-10 expression as well as the suppressive effects of Tregs 3 days after Treg induction. However, additional studies are needed to elucidate the mechanism by which 1 $\alpha$ ,25(OH) $_2$ D $_3$  regulates the differentiation of iTregs. Such research should also consider other immune cells, including antigen-presenting cells, which exhibit significant cross-talk with Tregs. Dauletbaev *et al.* demonstrated that VD regulates inflammatory macrophages via downregulation of IL-8 expression (28). In addition, VD was shown to reduce IL-22 expression in innate lymphoid cells (29), and Vanherwegen *et al.* reported that VD modulates the function of dendritic cells to improve Tregs in glucose metabolism (30), which may indirectly cause the rise of iTregs.

Several previous studies have suggested that increased cytokine production contributes to the pathogenesis of aGVHD. We demonstrated that administration of 1 $\alpha$ ,25(OH) $_2$ D $_3$  modulates the expression of inflammatory cytokines involved in the pathogenesis of aGVHD, such as IFN- $\gamma$ , IL-4, and TNF- $\alpha$ . These results suggest that VD exerts potent anti-inflammatory activity via suppression of other Th cells. Our data thus indicate that 1 $\alpha$ ,25(OH) $_2$ D $_3$  protects against aGVHD in part via modulation of cytokine expression.

In conclusion, we found that 1 $\alpha$ ,25(OH) $_2$ D $_3$  improves the clinical course of aGVHD in a murine model. 1 $\alpha$ ,25(OH) $_2$ D $_3$  induces the expansion of Treg populations and suppresses that of pro-inflammatory T cells. PC61 injection abolished the protective effect of 1 $\alpha$ ,25(OH) $_2$ D $_3$  against aGVHD. In addition, 1 $\alpha$ ,25(OH) $_2$ D $_3$  inhibits the proliferation of T cells *in vivo*, which is closely related to the engraftment of donor cells. These data suggest that 1 $\alpha$ ,25(OH) $_2$ D $_3$  is a promising therapeutic candidate for preventing and treating aGVHD.

### Acknowledgments

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### Footnote

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are

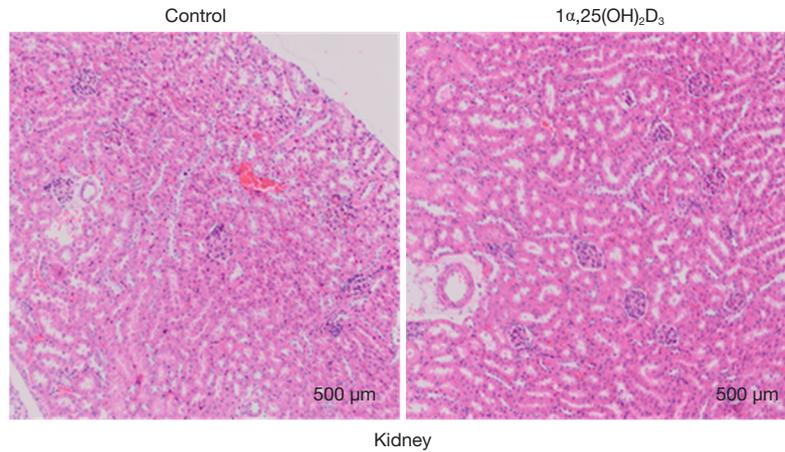
appropriately investigated and resolved. Relevant legal and ethical requirements were followed carefully according to the protocol (number NMU08-092), which was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

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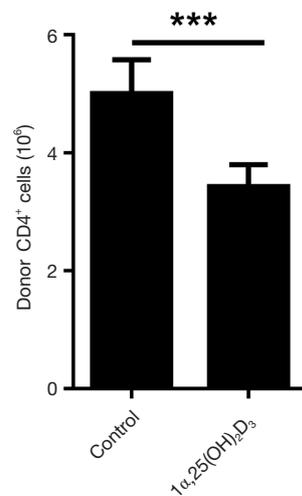
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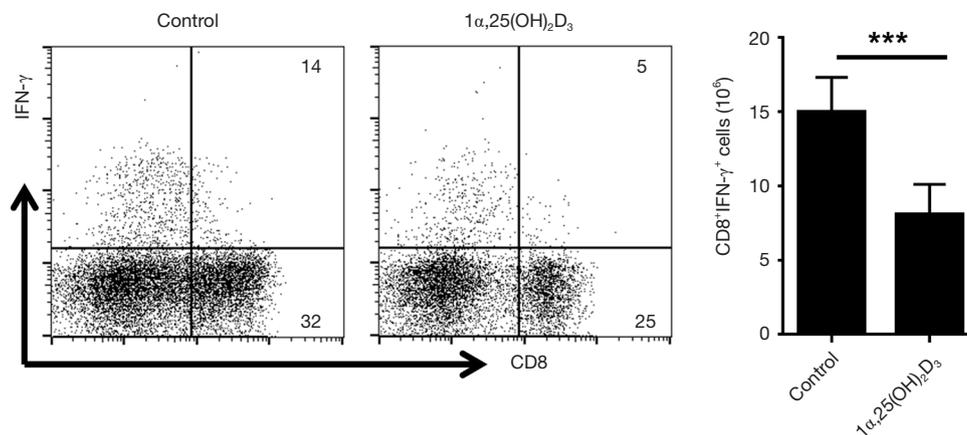
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**Figure S1**  $1\alpha,25(\text{OH})_2\text{D}_3$  alleviates the kidney injury in aGVHD mouse model. Representative images of mouse Kidney injury (hematoxylin and eosin staining, 200 $\times$ ) by light microscopy.  $1\alpha,25(\text{OH})_2\text{D}_3$ , 1,25-dihydroxyvitamin D<sub>3</sub>; aGVHD, acute graft-versus-host disease.



**Figure S2** Infusion of  $1\alpha,25(\text{OH})_2\text{D}_3$  markedly suppresses donor cell engraftment and prevents host cell depletion in aGVHD mice. Total numbers of donor CD4<sup>+</sup> cells are indicated. Data are shown as mean  $\pm$  SEM from six independent experiments (n=8 for each group). \*\*\*, P<0.001.  $1\alpha,25(\text{OH})_2\text{D}_3$ , 1,25-dihydroxyvitamin D<sub>3</sub>; aGVHD, acute graft-versus-host disease; SEM, standard error of the mean.



**Figure S3**  $1\alpha,25(\text{OH})_2\text{D}_3$  decreased the percentage and absolute number of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells *in vivo*. Representative dot plots and absolute numbers of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells were determined by flow cytometry. Data are shown as mean  $\pm$  SEM of six independent experiments (n=8 for each group). \*\*\*, P<0.001.  $1\alpha,25(\text{OH})_2\text{D}_3$ , 1,25-dihydroxyvitamin D<sub>3</sub>; SEM, standard error of the mean.