Vitamin D alleviates acute graft-versus-host disease through promoting the generation of Foxp3$^+$ T cells

Chuangye Ni$^1$, Xiaojie Gan$^1$, Xu Li$^1$, Han Sun$^2$, Zhen Chen$^2$, Hao Lu$^1$

$^1$Hepatobiliary Center, The First Affiliated Hospital, Nanjing Medical University, Nanjing 210029, China; $^2$Department of Articular Orthopaedics, The Third Affiliated Hospital of Soochow University, Changzhou 213003, China

**Contributions:** (I) Conception and design: H Lu; (II) Administrative support: C Ni, X Gan; (III) Provision of study materials or patients: X Li, H Sun, Z Chen; (IV) Collection and assembly of data: X Li, H Sun, Z Chen; (V) Data analysis and interpretation: C Ni, X Gan; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*These authors contributed equally to this work.

**Correspondence to:** Hao Lu. Hepatobiliary Center, The First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China. Email: luhao@njmu.edu.cn.

**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 D3 (1$\alpha$,25(OH)$_2$D$_3$) in the treatment of aGVHD.

**Method:** An aGVHD model was built by transferring splenocytes of B6 mice into B6D2F1 mice. 1$\alpha$,25(OH)$_2$D$_3$ 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)$_2$D$_3$ significantly slowed aGVHD progression and improved survival of B6D2F1 recipients of grafted B6 splenocytes. 1$\alpha$,25(OH)$_2$D$_3$ treatment also resulted in an increased number of CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) but decreased the number of CD4$^+$IL-4$^+$ cells. In vitro analysis demonstrated that 1$\alpha$,25(OH)$_2$D$_3$ 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)$_2$D$_3$ is mediated in part by improving the number of Tregs. 1$\alpha$,25(OH)$_2$D$_3$ 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)

Submitted Aug 12, 2019. Accepted for publication Nov 05, 2019.

doi: 10.21037/atm.2019.11.102

**View this article at:** http://dx.doi.org/10.21037/atm.2019.11.102

**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$_3$ (also known as cholecalciferol) and VD$_2$ (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$^+$
cytotoxic T lymphocytes specific for host spleen cells in 2 weeks (7).

The traditional view of 1,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) is a crucial role in maintaining calcium homeostasis and modulating calcium metabolism. The role of 1α,25(OH)₂D₃ in immune responses to infection has become an exciting topic of research (8,9). 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ exerts a therapeutic effect against aGVHD had not been determined. Therefore, we examined the regulatory ability of 1α,25(OH)₂D₃ in protecting aGVHD. Surprisingly, our results consistently demonstrated that administration of 1α,25(OH)₂D₃ significantly slowed aGVHD progression and improved survival of B6D2F1 recipients of grafted B6 spleenocytes. In vitro studies proved that 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ treatment could be useful to prevent aGVHD or other autoimmune disease (15).

Methods

Animals

C57BL/6 (H-2Kb) and B6D2F1 (H-2Kb/d) 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 μg/mL) and ionomycin (0.5 μg/mL) for 5 hours, and brefeldin A (5 μg/mL) for four hours under 5% CO₂ 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-γ (APC), TNF-α (BV421), IL-4 (BV421), TGF-β (AF488), IL-10 (PE), H-2Kb (APC), and H-2Kd (PE) purchased from BioLegend, and Foxp3 (APC), CD19 (FITC) purchased from BD Pharmpingen.

Development of mouse aGVHD models

aGVHD was induced in normal, unirradiated B6D2F1 mice on the same day by intravenous injection of 5×10⁶ B6 mouse derived spleen cells, as reported previously (16). Two weeks later, the mice were sacrificed, and splenocytes were stained with anti-mouse-H-2Kb and anti-mouse-H-2Kd 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, 1α,25(OH)₂D₃ group, 1α,25(OH)₂D₃ + IgG group, 1α,25(OH)₂D₃ + PC61 group. Mice in 1α,25(OH)₂D₃ + PC61 group and 1α,25(OH)₂D₃ + 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 50×10⁶ B6 cells. 1α,25(OH)₂D₃ (0.03 μg/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+ Treg generation

Spleen cells from B6 mice were derived, and CD4⁺CD62L⁻CD25⁻ T cells were sorted using a magnetic naïve CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch-Gradbach, Germany). The purity of CD4⁺CD62L⁻CD25⁻ 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-β (10 ng/mL). 1α,25(OH)₂D₃ (10⁻⁷ 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).
In vitro suppression assay

B6 derived CD4+CD25− T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA), and cultured with 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 ± 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

1α,25(OH)2D3 protects against aGVHD in a mouse model

To investigate the protective effect of 1α,25(OH)2D3 during the pathogenesis of aGVHD, 50×10^6 B6 spleen cells were injected intravenously into B6D2F1 mice (Figure 1A). Survival was monitored daily. 1α,25(OH)2D3 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 1α,25(OH)2D3 treatment (Figure S1).

Infusion of 1α,25(OH)2D3 markedly suppresses donor cell engraftment and prevents host cell depletion in aGVHD mice

To assess the effect of 1α,25(OH)2D3 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-2Kd and H-2Kb staining (donor cells: H-2Kb/d−, host cells: H-2Kb/d+). Donor cells comprised almost 50% of the cells in the Control group (17). Interestingly, 1α,25(OH)2D3 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
more, although B6 spleen cells transfer led to the lower number of the total number of spleen cells and host cells in F1 mice, 1α,25(OH)₂D₃ presented strong ability to preserve the proportion of host cells and decrease the expansion of donor cells (Figure 2A).

Donor CD8⁺ 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 1α,25(OH)₂D₃ groups. 1α,25(OH)₂D₃ markedly suppressed donor CD8⁺ cells expansion as well as CD4⁺ cells (Figure 2B,S2). We analyzed the killing effect of donor CD8⁺ cells as it is another characteristic of aGVHD (19). As shown in Figure 2C, 1α,25(OH)₂D₃ almost wholly prevented the aGVHD-associated host B cells reduction while without 1α,25(OH)₂D₃ administered, dramatically B cell decline was observed. Thus, our data demonstrate that 1α,25(OH)₂D₃ suppresses the expansion and cytotoxic effects of donor CD8⁺ cells against host CD19⁺ cells.

1α,25(OH)₂D₃ increases Foxp3 but reduces cytokine expression in vivo

Next, we examined Foxp3 expression in 1α,25(OH)₂D₃-treated and -untreated mice. Administration of 1α,25(OH)₂D₃ led to increasing number of spleen lymphocytes in 1α,25(OH)₂D₃ group mice, which is almost twice the control (data not shown). Therefore, the absolute number of Foxp3-expressing CD4⁺ cells increased, whereas no difference was observed in the percentage of Foxp3⁺ T cells in the spleen (Figure 3A).

Previous studies suggested the production of inflammatory cytokine contributes to aGVHD induction
1α,25(OH)₂D₃ increased the expression of Foxp3 and reduced cytokine expression in vivo. Representative dot plots and absolute numbers of (A) CD4⁺Foxp3⁺ T cells, (B) CD4⁺IFN-γ⁺ T cells, (C) CD4⁺IL-4⁺ T cells, and (D) CD4⁺TNF-α⁺ T cells were determined by flow cytometry. Bars show the mean ± SEM of six independent experiments (n=8 for each group). *, P<0.05; **, P<0.01. 1α,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Foxp3, forkhead box P3; SEM, standard error of the mean; NS, no significant difference.

(20,21). So, we evaluated various cytokines expression in CD4⁺ T cells with or without 1α,25(OH)₂D₃ administration. 1α,25(OH)₂D₃ administration led to down-regulated cytokine expression such as IFN-γ, IL-4, and TNF-α from CD4⁺ cells (Figure 3B,C,D). Also, the absolute number of CD4⁺IL-4⁺ T cells declined following 1α,25(OH)₂D₃ administration, whereas no difference was observed in the total number of CD4⁺ cells expressing IFN-γ and TNF-α. In addition, we also found 1α,25(OH)₂D₃ could decrease the percentage as well as the absolute number of CD8⁺IFN-γ⁺ T cells (Figure S3).

1α,25(OH)₂D₃ increases Foxp3 and IL-10 expression and regulatory function during iTreg generation

To evaluate the regulatory effect of 1α,25(OH)₂D₃, on the role of Tregs, we first examined Foxp3 expression in 1α,25(OH)₂D₃-treated and -untreated iTreg subsets. iTregs treated with 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃-treated and -untreated iTreg subsets, as Tregs are known to express anti-inflammatory cytokines such as IL-10 and TGF-β to suppress effector T cell expansion. As expected, 1α,25(OH)₂D₃ treatment resulted in increased IL-10 expression; however, no significant change was observed in TGF-β 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 1α,25(OH)₂D₃-treated iTregs.

Tregs mediate 1α,25(OH)₂D₃-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 1α,25(OH)₂D₃ + PC61 group and 1α,25(OH)₂D₃ + IgG were injected intraperitoneally with
Figure 4 1α,25(OH)₂D₃ enhanced Foxp3 and IL-10 expression and regulatory function during iTreg generation. iTregs were cultured from naïve CD4⁺CD62⁺CD25⁻ T cells with anti-CD3/CD28 beads (the ratio of bead to cell is 1:5), IL-2 (20 IU/mL), and TGF-β (10 ng/mL) for 3 days. (A) Representative plots gated on CD4⁺ cells for CD25⁺Foxp3⁺ expression by iTregs treated with PBS or 1α,25(OH)₂D₃ (10⁻⁷ M); (B) the percentages IL-10⁺ and TGF-β⁺ cells among Foxp3⁺ cells on day 3. Data are expressed as mean ± SEM of five independent experiments; (C) representative histogram plots of T cell expansion after co-culture for 3 days with PBS- or 1α,25(OH)₂D₃-treated iTreg subsets in the presence of anti-CD3. **, P<0.01. 1α,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 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.

Discussion

In this study, we demonstrated that the administration of 1α,25(OH)₂D₃ reduced the severity of aGVHD in a mouse model. 1α,25(OH)₂D₃ promoted the expansion of Foxp3-expressing Treg populations and inhibited IFN-γ 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 1α,25(OH)₂D₃ successfully increased the number of CD4⁺Foxp3⁺ cells both in vitro and vivo, in agreement with previous studies.
Figure 5  Tregs mediated 1α,25(OH)2D3-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; 1α,25(OH)2D3, 1,25-dihydroxyvitamin D3; aGVHD, acute graft-versus-host disease.

indicating that VD increases Treg populations in vivo. In mice injected with PC61, the effect of 1α,25(OH)2D3 was diminished, confirming that upregulation of Foxp3+ T cells is an important mechanism for 1α,25(OH)2D3 against aGVHD.

In vitro experiments found that 1α,25(OH)2D3 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 1α,25(OH)2D3 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 1α,25(OH)2D3 modulates the expression of inflammatory cytokines involved in the pathogenesis of aGVHD, such as IFN-γ, IL-4, and TNF-α. These results suggest that VD exerts potent anti-inflammatory activity via suppression of other Th cells. Our data thus indicate that 1α,25(OH)2D3 protects against aGVHD in part via modulation of cytokine expression.

In conclusion, we found that 1α,25(OH)2D3 improves the clinical course of aGVHD in a murine model. 1α,25(OH)2D3 induces the expansion of Treg populations and suppresses that of pro-inflammatory T cells. PC61 injection abolished the protective effect of 1α,25(OH)2D3 against aGVHD. In addition, 1α,25(OH)2D3 inhibits the proliferation of T cells in vivo, which is closely related to the engraftment of donor cells. These data suggest that 1α,25(OH)2D3 is a promising therapeutic candidate for preventing and treating aGVHD.

Acknowledgments

Funding: This work was supported by the National Natural Science Fund Outstanding Youth Fund 81522020, 863 Young Scientists Special Fund (No. 2015AA020932) and National Natural Science Fund 91442117 and 81571564 in China and the foundation of Jiangsu Collaborative Innovation Center of Biomedical Functional materials and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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.

References

25. Zahran AM, Zharan KM, Hetta HF. Significant correlation between regulatory T cells and vitamin D status in term


Cite this article as: Ni C, Gan X, Li X, Sun H, Chen Z, Lu H. Vitamin D alleviates acute graft-versus-host disease through promoting the generation of Foxp3+ T cells. Ann Transl Med 2019;7(23):748. doi: 10.21037/atm.2019.11.102
**Figure S1** 1α,25(OH)₂D₃ alleviates the kidney injury in aGVHD mouse model. Representative images of mouse kidney injury (hematoxylin and eosin staining, 200x) by light microscopy. 1α,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; aGVHD, acute graft-versus-host disease.

**Figure S2** Infusion of 1α,25(OH)₂D₃ markedly suppresses donor cell engraftment and prevents host cell depletion in aGVHD mice. Total numbers of donor CD₄⁺ cells are indicated. Data are shown as mean ± SEM from six independent experiments (n=8 for each group). ***, P<0.001. 1α,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; aGVHD, acute graft-versus-host disease; SEM, standard error of the mean.

**Figure S3** 1α,25(OH)₂D₃ decreased the percentage and absolute number of CD₈⁺ IFN-γ⁺ T cells in vivo. Representative dot plots and absolute numbers of CD₈⁺ IFN-γ⁺ T cells were determined by flow cytometry. Data are shown as mean ± SEM of six independent experiments (n=8 for each group). ***, P<0.001. 1α,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; SEM, standard error of the mean.