Original Article

Effect of 1,25-Dihydroxyvitamin D_3 on Regulatory T Cells in Ovariectomized Mice*

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Abstract

Objective To investigate the correlation between regulatory T (Treg) cells and postmenopausal osteoporosis and the antiosteoporotic effect of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] in relation to Treg cells.

Methods Fifty female BALB/c mice were randomly divided into five groups: the basal control (BAS), Sham, ovariectomy (OVX), OVX+diethylstilbestrol (OVX+DES), and OVX+1,25$(OH)_2D_3$. Tibias were harvested and processed with decalcification for quantitative bone histomorphometry. Femurs were stained by immunohistochemistry to detect Foxp3 protein expression. Spleens were used to detect Treg and Foxp3 gene expression by flow cytometry and quantitative RT-PCR, respectively.

Results In comparison with the Sham group, a significant decrease was found in the OVX group in such indices as trabecular bone volume/total tissue area (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th). 1,25$(OH)_2D_3$ and DES partly prevented the decrease in BV/TV, Tb.N, Tb.Th in OVX mice. Treg cell number, Foxp3 mRNA expression in spleen and Foxp3 protein expression in femur significantly decreased in the OVX-treated group compared with those in the sham group. 1,25$(OH)_2D_3$ and DES significantly increased Treg cell number and Foxp3 expression. Treg cells and Foxp3 gene expression were related to bone histomorphometric parameters.

Conclusion The decrease in Treg cell numbers is relevant to the postmenopausal osteoporosis. The antiosteoporosis of 1,25$(OH)_2D_3$ is related to regulatory T cells.

Key words: 1,25-dihydroxyvitamin D_3; Osteoporosis; Regulatory T cells; Ovariectomy

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INTRODUCTION

Osteoporosis is the most widespread bone disease which is characterized with a low bone mineral density and the deterioration of bone microarchitecture and may lead to bone fragility fractures[1-2]. Skeletal homeostasis depends on the balance between bone formation and bone resorption. Bone loss often occurs when bone resorption exceeds bone

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formation. Recent studies have shown that immune activation is associated with bone loss[3-4].

$CD4^+CD25^+$ regulatory T (Treg) cells, as a T cell subgroup, play an important role in induction and maintenance of peripheral immune tolerance. Treg cells can suppress a variety of immune cells through different mechanisms, including direct contact, secreting inhibitory cytokines, inhibiting autologous T cells reaction[5-6]. Many studies have confirmed that impaired Treg cells are closely related to human diseases such as autoimmune, infectious and allergic disease and even cancer development[7-9]. Recent studies have shown that Treg cells inhibit osteoclast differentiation[10]. A study by Zaiss et al. showed that Treg cells could control bone resorption and preserve bone mass in an ovariectomy-induced bone loss model[11]. Additionally, Treg cells protect against local and systemic TNF-mediated bone loss[12]. Therefore, Treg cells may play an important role in preventing osteoporosis.

1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], an active metabolite of vitamin D, not only plays a vital role in bone and calcium metabolism, but also modulates the immune system. Studies showed that $1,25(OH)_2D_3$ increased the expansion and the frequency of Treg cells[13]. $Foxp3^+CD4^+$ T cells significantly increased in mice treated with oral $1,25(OH)_2D_3$[14-15]. However, it is unclear whether the preventive effect of $1,25(OH)_2D_3$ against postmenopausal osteoporosis is associated with Treg cells.

MATERIALS AND METHODS

Reagents

$1,25(OH)_2D_3$ was purchased from Shanghai Roche Pharmaceuticals Ltd. (Shanghai, China). Diethylstilbestrol (DES) was obtained from Hefei Jiulian Pharmaceuticals Company (Hefei, China). Silver nitrate and dibutyl phthalate were purchased from Xilong Chemical Company (Shantou, China). Methyl methacrylate was from Beijing Chemical Factory (Beijing, China). Calcein and toluidine blue were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-anti-mouse CD4, phycoerythrin (PE)-anti-mouse CD25 and PE-Cy5-anti-mouse CD127 were from eBioscience (San Diego, CA, USA). Goat anti-mouse Foxp3 was obtained from Ray Biotech (Norcross, GA, USA).

Animals and Treatments

Fifty female BALB/c mice of eight weeks old were provided by the Animal Center of Guangdong Medical College (Zhanjiang, China). The mice were acclimatized for 2 weeks to local vivarium conditions at temperature of 24-26 °C and a humidity of 70% with free access to water and a pelleted commercial natural diet containing 1.33% calcium and 0.95% phosphorus. Thereafter, the mice were divided into baseline (BAS) group (n=10), sham operation (Sham) group (n=10) and ovariectomy (OVX) treatment group (n=30). The OVX mice were further divided into vehicle treatment group (OVX, n=10), diethylstilbestrol (0.1 mg/kg·d) treatment group (OVX+DES, n=10) and $1,25(OH)_2D_3$ (0.1 μg/kg·d) treatment group [OVX+$1,25(OH)_2D_3$, n=10]. The drugs were given by oral gavage for 90 d. Body weight of the mice was recorded weekly. The mice were sacrificed at day 0 for basal control. The mice in other groups were killed 90 d later. The left tibias were collected for the bone histomorphometric analysis in undecalcified sections. Left femurs were taken to detect Foxp3 protein expression by immunohistochemistry. Spleens were divided into two parts; one was detected for Treg quantity by flow cytometry and the other was detected for Foxp3 gene expression by quantitative RT-PCR. The animals were treated in accordance with the Guide for Care and Use of Laboratory Animals by the National Committee of Science and Technology of China. The experimental protocol was approved by the Institutional Animal Use and Care Review Board of Guangdong Medical College.

Static Histomorphometric Measurement and Calculations

Left tibias were removed and bone marrow cavities were exposed using low speed saw (Buehler Ltd., USA). The proximal tibial metaphysis (PTM) was fixed in 10% phosphate-buffered formalin for 24 h, dehydrated in an increasing series of alcohol, defatted in xylene, and embedded into methyl methacrylate. The frontal PTM tissue was cut into 5 μm thick sections with the RM2155 hard tissue microtome (Leica AG, Wetzlar, Germany). The 5 μm sections were stained with either silver nitrate or toluidine blue for static histomorphometric measurements. A semi-automatic digitizing image analysis system (OsteoMetrics, GA, USA) was used for quantitative bone histomorphometry measurement. To avoid primary spongiosa, the measurement site on the bone sections was the PTM between 1 and 3 mm distal to the growth plate-epiphyseal junction. Static measurement

parameters included total tissue area (TV), trabecular area (BV), trabecular bone surface (BS), and osteoclast number (Oc.N). According to relevant formula, the following parameters were calculated, including trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and osteoclast number/trabecular perimeter (Oc.N/Tb.Pm). All histomorphometric parameters were calculated in accordance with the published study[16].

Flow Cytometric Analysis

For surface marker analysis, 1×10^6 cells were collected from mice spleens, and cells were stained using FITC-anti-mouse CD4, PE-anti-mouse CD25, PE-Cy5-anti-mouse CD127, or IgG isotype-matched control antibodies. After incubation for 30 min at 4 °C, the cells were analyzed on a FACS Calibur (Becton Dickinson, USA).

Quantitative Real-time PCR

Total RNA was isolated from spleen cells using TRIZOL® Reagent (Invitrogen, UAS). In brief, splenic cells were produced by teasing the organs through a sterile nylon mesh and lysed using red blood cell lysing buffer. Then cells were washed twice with PBS and centrifuged at 400 g for 5 min. Real-time RT-PCR analysis of Foxp3 mRNA levels was performed using One Step SYBR® Prime Script™ RT-PCR kit (TaKaRa, China) according to the manufacturer's instructions. The following primers were designed for real-time RT-PCR: β-actin, forward 5'-TGACGTGGACATCCGC AAAG-3' and reverse 5'-CTGGAAGGTGGACAGCGAGG -3'. Foxp3: forward 5'-CTGACCAAGGCTTCATCTGTG -3', and reverse 5'-ACTCTGGGAATGTGCTGTTTC-3'. All the primers were synthesized by Shanghai Shengong Biotechnology Co. (Shanghai, China). Amplification conditions were as follows: 42 °C for 5 min, 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. The relative quantification of Foxp3 mRNA was determined by calculating the values of $2^{-\Delta\Delta CT}$, with each sample being normalized to the expression level of β-actin. The experiment was repeated in triplicate.

Immunohistochemistry

The expression of Foxp3 proteins in femur was analyzed by immunohistochemistry as described previously[12]. Briefly, formalin-fixed decalcified femurs were paraffin-embedded and sectioned at a thickness of 5 μm. All sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed in PBS (pH 7.2). The sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) and then incubated overnight at 4 °C with primary antibody (goat anti-mouse Foxp3 antibody), followed by incubation with streptavidin/HRP-conjugated secondary antibody (Fujian Maixin Biosynthesis Biotechnology Co.). The conventional streptavidin/peroxidase method was used to develop signals, and sections were stained by *3*, *3'-diaminobenzidine* (*DAB*) and counterstained with hematoxylin. The sections incubated with secondary antibody in the absence of primary antibody served as negative controls. Images were processed with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD).

Statistical Analysis

Data were presented as the mean±standard deviation and analyzed using SPSS software (version 13.0, SPSS). One-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test, was adopted for multiple-group comparison. Bivariate correlations were performed using Spearman correlations. $P<0.05$ was considered statistically significant.

RESULTS

Histomorphometry of Cancellous Bone

Static histomorphometric measurements of PTM were illustrated in Table 1 and Figure 1. Compared with the Sham group, a significant decrease was found in the OVX group in such indices as BV/TV, Tb.N and Tb.Th ($P<0.01$). In addition, significantly increased Tb.Sp and Oc.N/Tb.Pm were observed in the OVX-treated group, compared with the Sham group ($P<0.01$). 1,25(OH)$_2$D$_3$ and DES partly prevented the decrease in BV/TV, Tb.N, Tb.Th, as well as the increase in Tb.Sp, Oc.N/Tb.Pm in the OVX mice ($P<0.01$) (Figure 1, Table 1).

Effect of 1,25(OH)$_2$D$_3$ on Tregs in Spleen

Compared with the Sham group, the number of Treg cells was significantly decreased in the OVX group ($P<0.05$). Treg cell number in the DES group and 1,25(OH)$_2$D$_3$ group was higher than that in the OVX group ($P<0.05$). The number of Treg cells in the DES group was slightly higher than that in the 1,25(OH)$_2$D$_3$ group, but there was no statistically significant difference ($P>0.05$) (Figure 2).

Table 1. Effect of $1,25(OH)_2D_3$ on Static Parameters of the Right Proximal Tibial Metaphysis in Ovariectomized Mice

Parameters	BV/TV (%)	Tb.Th (μm)	Tb.N (/mm)	Tb.Sp (μm)	Oc.N/Tb.Pm (/mm)
BAS	1.04±0.4	20.51±1.81	0.51±0.19	2154.84±739.13	0.33±0.09
Sham	1.39±0.34	18.89±3.40	0.73±0.12	1372.78±230.38	0.34±0.11
OVX	0.27±0.09**	12.95±3.05**	0.21±0.03**	4866.75±604.78**	0.78±0.18**
OVX+$1,25(OH)_2D_3$	0.78±0.12$^{\#\#}$	19.92±3.40$^{\#\#}$	0.39±0.03$^{\#\#}$	2549.49±233.28$^{\#\#}$	0.38±0.16$^{\#\#}$
OVX+DES	0.91±0.17$^{\#\#}$	21.29±3.28$^{\#\#}$	0.44±0.11$^{\#\#}$	2387.66±633.11$^{\#\#}$	0.41±0.13$^{\#\#}$

Note. $^{**}P<0.01$ *vs* Sham; $^{\#\#}P<0.01$ *vs* OVX; BV: trabecular bone volume; TV: total tissue area; Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular separation; Oc.N: osteoclast number; Tb.Pm: trabecular perimeter.

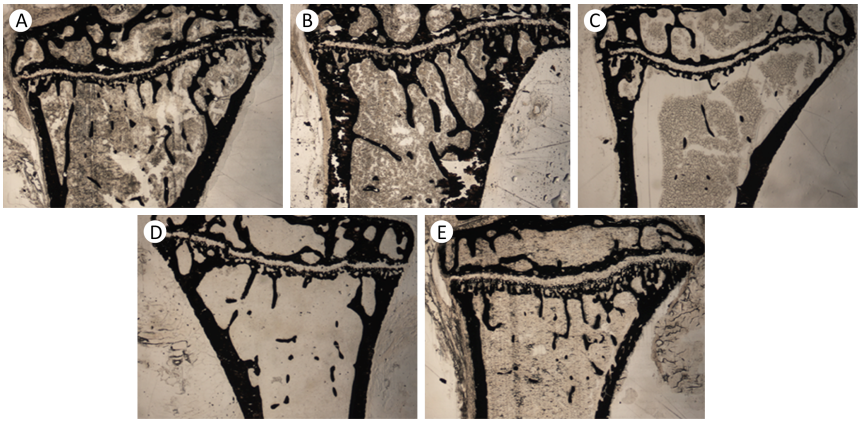

Figure 1. Microphotograph of the right proximal tibial metaphysic. The undecalcified proximal tibial metaphyseal sections were cut and stained with silver nitrate. (A) BAS, (B) Sham, (C) OVX, (D) OVX+ $1,25(OH)_2D_3$, (E) OVX+DES. (original magnification ×40).

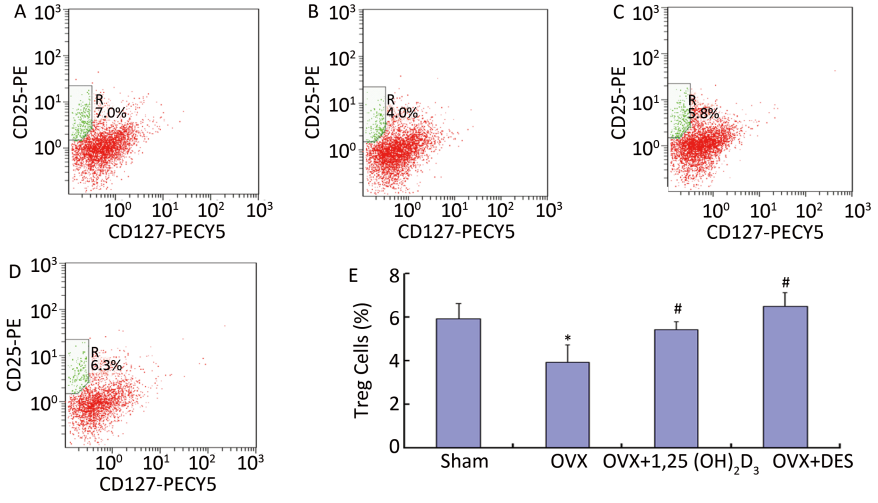

Figure 2. Effect of $1,25(OH)_2D_3$ on Treg cells in spleen. 1×10^6 cells collected from mice spleens were stained using FITC-anti-mouse CD4, PE-anti-mouse CD25, PE-Cy5-anti-mouse CD127, or IgG isotype-matched control antibodies. After incubated for 30 min, the cells were analyzed on a FACS Calibur. (A) Sham, (B) OVX, (C) OVX+$1,25(OH)_2D_3$, (D) OVX+DES, (E) Percentages of Treg cells in different groups. $^{*}P<0.05$ *vs.* Sham group; $^{\#}P<0.05$ *vs.* OVX treatment group.

Effect of 1,25(OH)$_2$D$_3$ on Foxp3 mRNA in Spleen

Foxp3 is the key transcription factor controlling Treg cells development and function, and is a marker for Treg cells[6]. Our results showed that the expression of Foxp3 in the OVX group was significantly lower than that in the Sham group (P<0.01). As compared with the OVX group, Foxp3 mRNA expressions in the 1,25(OH)$_2$D$_3$ and DES groups were significantly increased (P<0.01) (Figure 3).

Effect of 1,25(OH)$_2$D$_3$ on the Expression of Foxp3 Protein in Femur

Furthermore, we detected the expression of Foxp3 in femur by immunohistochemical analysis. As expected, Foxp3 expression in the OVX group was significantly lower than that in the Sham group. Foxp3 expressions in the 1,25(OH)$_2$D$_3$ and DES groups were significantly increased compared with its expression in the OVX group (Figure 4).

Correlation Analysis

There was a positive correlation between Treg cell frequency and such indices as BV/TV, Tb.Th, Tb.N, and an inverse relationship between Treg cell frequency and Tb.Sp, Oc.N/Tb.Pm. Meanwhile, a significant positive correlation is found between Foxp3 gene expression and such indices as BV/TV, Tb.Th, Tb.N, and an inverse relationship exists between Foxp3 gene expression and Tb.Sp, Oc.N/Tb.Pm (Table 2).

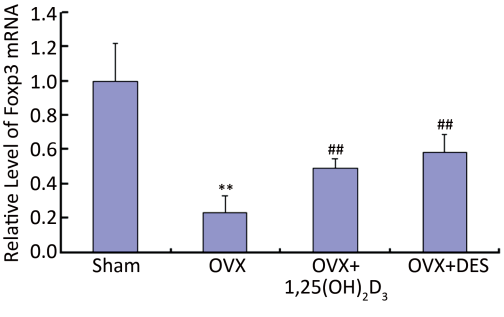

Figure 3. Effect of 1,25(OH)$_2$D$_3$ on Foxp3 mRNA in spleen. The expression levels of Foxp3 mRNA in spleen were measured by real time PCR, with β-actin as an internal control. $^{**}P$<0.01 *vs.* Sham group; $^{\#\#}P$<0.01 *vs.* OVX treatment group.

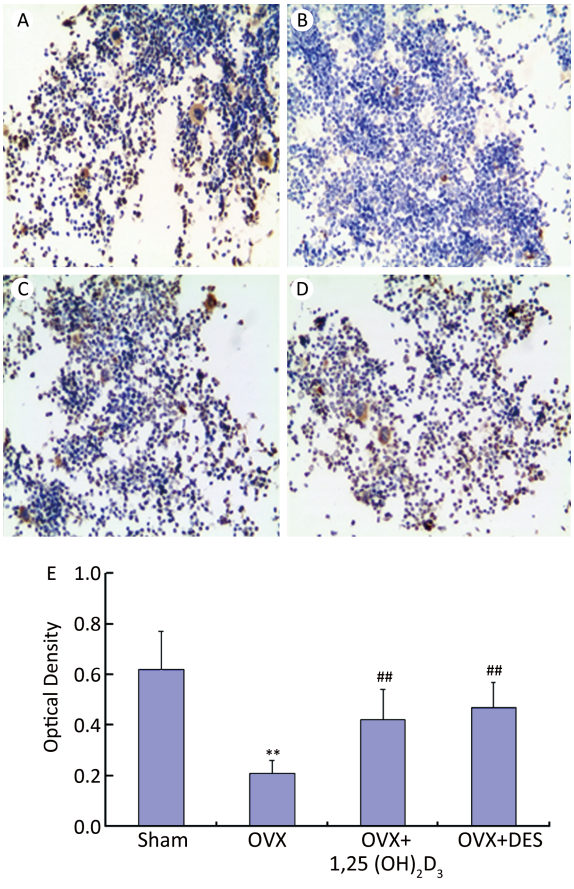

Figure 4. Effect of 1,25(OH)$_2$D$_3$ on the expression of Foxp3 protein in femur. Foxp3 protein in sections was identified with goat anti-mouse Foxp3 antibody and stained with 3,3'-diaminobenzidine (DAB) substrate. Images were processed with Image-Pro Plus 6.0 software. (A) Sham, (B) OVX, (C) OVX+ 1,25(OH)$_2$D$_3$, (D) OVX+DES, (E) Average optical density of sections in different groups. $^{**}P$<0.01 *vs.* Sham group; $^{\#\#}P$<0.01 *vs.* OVX treatment group. (original magnification ×200).

Table 2. Correlation of Treg cells, Foxp3 Gene Expression and Bone Histomorphometric Parameters

Parameters	BV/TV (%)	Tb.Th (μm)	Tb.N (/mm)	Tb.Sp (μm)	Oc.N/ Tb.Pm (/mm)
Treg cells	0.9	0.675	0.78	-0.77	-0.767
Foxp3 expression	0.923	0.641	0.781	-0.773	-0.698

DISCUSSION

Treg cells are a population of $CD4^+$ T cells with immunosuppressive activity and play an indispensable role in maintaining immune homeostasis[17]. Treg cells can suppress the function of other T cells to limit excessive immune responses that are deleterious to the host. Alterations in the number and function of Treg cells have been implicated in several autoimmune diseases such as multiple sclerosis, active rheumatoid arthritis, and type 1 diabetes[18-20].

In recent years, postmenopausal osteoporosis has been proposed to be a chronic inflammatory disease[21]. Treg cells as a kind of immune regulator seem to play an important role in regulating development of the disease. Studies have shown that Treg cells inhibit osteoclast formation by expressing CTLA-4, which binds to B7-1 and B7-2 on the surface of mononuclear osteoclast precursors, impairing their differentiation to osteoclasts[22].

The fork-head transcription factor, Foxp3 is expressed specifically in Treg cells and has been shown to regulate their development and function[6-7]. Zaiss et al. have found that FoxP3-Tg mice developed higher bone mass and were protected from OVX-induced bone loss. Treg cells can control bone resorption *in vivo* and preserve bone mass during physiologic and pathologic bone remodeling[11]. A study by Luo et al showed that Treg cells suppressed osteoclast differentiation and bone resorption by secreting IL-10 and TGF-β_1. DES enhanced the suppressive effects of Treg cells on osteoclast differentiation and bone resorption by stimulating IL-10 and TGF-β_1 secretion. Therefore, Treg cell-derived IL-10 and TGF-β_1 are likely involved in the regulation of DES on bone metabolism[23]. Our results have shown that bone mass in OVX mice significantly decreased. Meanwhile, Treg cells in spleen and femur also reduced. DES treatment increased bone mass and the number of Treg cells in OVX mice. These results demonstrated that Treg cells might be involved in the development of postmenopausal osteoporosis.

It is known that $1,25(OH)_2D_3$ facilitates calcification by promoting calcium absorption by the intestine and modulating the secretion of parathyroid hormone. In addition, studies have shown that $1,25(OH)_2D_3$ inhibits bone absorption and increases osteoblast activity[24]. $1,25(OH)_2D_3$ treatment can increase bone mass in osteopenic OVX rats[25]. In recent years, $1,25\ (OH)_2D_3$ immune regulating function has been paid greater attention. Research confirmed that $1,25(OH)_2D_3$ could increase the ratio of Treg cells in spleen of mice, inhibit acute rejection after islet transplantation, and effectively prolong grafts' survival in the host[26]. $1,25(OH)_2D_3$ and its analogue can reduce the incidence of type 1 diabetes in mice and damaging of $CD4^+$Th1 cells, increase the Treg cells in pancreas and lymph nodes, reduce the number of the apoptosis of pancreatic islet B cells and promote the peripheral T lymphocyte apoptosis to alleviate autoimmune reaction, which protects islet B cell[26]. Studies have shown that $1,25(OH)_2D_3$ binds to the vitamin D receptor on $CD4^+$T cells, promotes the Foxp3 gene expression, and strengthens the inhibitory function of regulatory T cells[27-28].

In this study, we found that $1,25(OH)_2D_3$ could partly prevent bone loss in OVX mice and increase Treg cell numbers in spleen and femur. In addition, the number of Treg cells and Foxp3 gene expression in spleen were associated with bone morphologic index. Therefore, antiosteoporotic effect of $1,25(OH)_2D_3$ may be related to Treg cells.

In summary, our results showed that Treg cells were involved in postmenopausal osteoporosis and the antiosteoporotic effect of $1,25(OH)_2D_3$ was related to Treg. This may represent a potential new mechanism of antiosteoporosis of $1,25(OH)_2D_3$, and Treg cells may be used as a therapeutic target against postmenopausal osteoporosis.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XU Dao Hua, CUI Liao. Performed the experiments: LIU Jun Chen, ZHOU Chen Hui, ZHANG Xue, CHEN Yan, XU Bian Lian. Analyzed the data: LIU Jun Chen, ZHOU Chen Hui. Contributed reagents/materials/analysis tools: XU Dao Hua. Wrote the paper: XU Dao Hua, LIU Jun Chen, CUI Liao.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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