

## Original Article



# Interleukin-10-Producing B Cells Help Suppress Ovariectomy-Mediated Osteoporosis

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### Conflict of Interest

The authors declare no potential conflicts of interest.

## ABSTRACT

Osteoporosis is prevalent in elderly women and it may cause dental implant failure. In particular, estrogen deficiency in postmenopausal women leads to higher rates of osteoporosis prevalence. Immune cell-mediated effects involving the development of osteoporosis have been studied previously; however, the role of IL-10-producing regulatory B (B10) cells in osteoporosis is largely unclear. Here, we examined the role of B10 cells in osteoporosis. C57BL/6 mice were subjected to ovariectomy (OVX). Fifteen weeks after OVX surgery, the first molar of the right maxillary was extracted, and twenty-four weeks after OVX surgery, serous progression of osteoporosis was observed in the alveolar bone. Moreover, the proportion of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>high</sup> regulatory B cells, B10, and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells from the spleen of OVX mice decreased during the progression of osteoporosis, compared to controls. In contrast to regulatory cells, IL-17-producing Th (Th17) cell levels were increased in OVX mice. Adoptive transfer of B10 cells to OVX mice led to a decrease in Th17 cell abundance and inhibited the development of osteoporosis in the alveolar bone from OVX mice. Thus, our results suggest that B10 cells may help suppress osteoporosis development.

**Keywords:** Osteoporosis; Ovariectomy; Breg cells; Treg cells; Th17 cells; Adoptive transfer

## INTRODUCTION

Osteoporosis is a metabolic bone disorder (1,2) that globally affects >200 million people (3). It is characterized by microarchitectural changes such as decreased bone mass, which increases susceptibility to bone fractures (4). The incidence of osteoporosis increases with age and occurs most frequently in postmenopausal women because of considerably reduced estrogen levels associated with menopause, which indicates that estrogen deficiency is a crucial risk factor for the development of osteoporosis (5-7). In animal models, ovariectomy (OVX) has been used to induce osteoporosis (8).

The contribution of immune cells to the development of OVX-mediated osteoporosis has been shown in previous studies (9,10). T cells are the main contributors to bone loss in estrogen-deficient mice, as OVX increases the production of pro-inflammatory cytokines by T cells to levels that induce increased generation of osteoclasts (11,12). In T cell immunity,

### Abbreviations

B10, IL-10-producing regulatory B; Breg, regulatory B; BV/ToV, bone volume per total volume; CT, computed tomography; OVX, ovariectomy; PMA, phorbol myristate acetate; RANKL, receptor activator of NF- $\kappa$ B ligand; Th17, IL-17-producing Th; Tra Number, trabecular number; Tra Sep, trabecular separation; Tra Thick, trabecular thickness.

### Author Contributions

Data curation: Zhang W, Lim SM, Xu L; Formal analysis: Zhang W; Investigation: Wang Y, Zhang W, Xu L, Jin JO; Methodology: Wang Y, Lim SM, Xu L, Jin JO; Supervision: Jin JO; Writing - original draft: Wang Y, Jin JO; Writing - review & editing: Jin JO.

IL-17-producing Th (Th17) cells have controlling effects in inflammatory and other bone diseases (13,14). Th17 cells have been implicated in the pathogenesis of rheumatoid arthritis in mice in which IL-17 deficiency and blockade of IL-17 reduce disease progression (15). Moreover, Th17 cells have been shown to directly control the induction of osteoporosis in OVX mice (9).

Regulatory immune cells can suppress inflammatory bone diseases (13,16). Tregs can exert considerable immunosuppressive effects and inhibit the development of inflammatory diseases (17). Several recent studies confirmed that Treg cells contribute to the inhibition of osteoclastogenesis and bone resorption (18,19), and depletion of Treg cells can exacerbate collagen-induced arthritis in mice (20). Moreover, adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>T cells shows promising results regarding protection from rheumatoid arthritis (21,22). In addition, Tregs have been shown to directly inhibit osteoclastogenesis by suppressing the expression of receptor activator of NF- $\kappa$ B ligand (RANKL) and M-CSF, leading to increased bone volume (19,23).

Recent studies have found that regulatory B (Breg) cells can contribute to the suppression of inflammation and support the differentiation of Tregs (24,25). IL-10-producing regulatory B (B10) cells, a subset of Breg cells, counteract inflammatory diseases such as collagen-induced arthritis and colitis (26). More importantly, B10 cells alleviate pathogen- or immune stimulator-mediated periodontal inflammation and bone loss (27,28). Although B10 cells exhibit anti-inflammatory effects, the question of whether B10 cells help suppress osteoporosis remains to be answered. We hypothesized that B10 cells may help prevent osteoporosis development in OVX mice, and thus, we conducted experiments to assess the effects of B10 cells on osteoporosis.

## MATERIALS AND METHODS

### Mice

C57BL/6 mice (6–8 wk old) were purchased from Shanghai Public Health Clinical Center, China. The mice were housed under specific pathogen-free conditions at 20°C–22°C and 50%–60% humidity; water and a standard rodent chow diet were provided *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of Shanghai Public Health Clinical Center (approval No. 2018-A050-01), and all experiments were performed according to the respective guidelines. Mice were euthanized by CO<sub>2</sub> inhalation.

### Abs

Isotype control Abs (IgG1, IgG2a, and IgG2b), CD1d (1B1), CD5 (53-7.3), CD19 (1D2/CD19), CD4 (GK1.5), CD25 (3C7), anti-FoxP3 (MF-14), anti-IL-10 (JES5-16E3), and anti-IL-17A (TC11-18H10.1) were purchased from BioLegend (San Diego, CA, USA).

### Flow cytometry analysis

Cells were stained with the Fc-block Abs for 15 min (BioLegend). Fluorescence-conjugated Abs were then added and incubated on ice for 20 min. After washing with PBS, the 0.2×10<sup>5</sup> cells were analyzed on FACS Fortessa (Becton Dickinson, Franklin Lakes, NJ, USA) using FlowJo 8.6 software (Tree Star, San Diego, CA, USA). Isotype control Abs were used for determination of negative cells. Cellular debris and dead cells were excluded by forward- and side-scatter gating and DAPI (Sigma-Aldrich, St. Louis, MO, USA) staining.

### OVX and molar tooth extraction

Mice were shaved, and an incision was made to the abdomen after anesthesia by intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine solutions. After the ovarian fat pad was removed from the incision site, the oviduct was ligated using sterilized thread, and each ovary was removed using a single cut. Control mice were subjected to the same procedures but without ovary removal. Tooth extraction was performed in all mice 15 weeks after surgery. The first right maxillary molar tooth (M1) was carefully luxated using two 18-gauge needles as levers, with the aid of a 2.5-fold magnifying lens.

### Stimulation of B cells and isolation of B10 cells

B10 cells were purified from stimulated B cells using a Breg cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, B cells from mouse spleen were pre-enriched and stimulated using *Porphyromonas gingivalis* LPS (5 µg/mL; InvivoGen, San Diego, CA, USA) and CpG-ODN (1 µM; Sangon Biotech, Shanghai, China) for 24 h. Phorbol myristate acetate (PMA, 50 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (500 ng/mL, Sigma-Aldrich) were then added to the enriched B cell culture medium for the last 5 h of stimulation. B10 cells were isolated using a B10 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and were then transferred intravenously into OVX and control mice.

### Adoptive transfer of B10 cells

C57BL/6 mice were subjected to OVX or sham surgery. All mice underwent extraction of the first molar of the right maxillary during surgery. B10 cells were isolated from naïve mice and intravenously transferred to OVX mice 15, 18, and 21 wk after surgery at a density of  $1 \times 10^6$  B10 cells per mouse. The mice were euthanized 24 wk after surgery. Thereafter, the right maxilla was dissected for subsequent micro-computed tomography (CT).

### Intracellular cytokine staining

As described previously (29–31), splenocytes were incubated with monensin solution (BioLegend) for 4 h. Cells were stained with surface Abs and were then fixed and permeabilized using Cytofix/Cytoperm buffer (eBioscience, San Diego, CA, USA). After washing with Perm/Wash buffer (eBioscience), the cells were incubated with anti-cytokine Abs in Perm/Wash buffer for 30 min at room temperature. Staining was blocked using Fc blocking Ab, and isotype control IgG were used as negative controls in all experiments. Dead cells were gated out by the Zombie Violet Fixable Viability Kit (BioLegend).

### ELISA

Concentrations of IL-17 in mouse sera were measured in triplicate using an ELISA kit (BioLegend).

### Micro-CT

Using a micro-CT scanner equipped with a custom software package (Skyscan 1176; Bruker, Billerica, MA, USA), bone specimens were scanned at 70 kVp and 114 µA, at high resolution (9 µm slice thickness), and in three planes. A region of interest distal to the remaining second molar tooth was selected and highlighted on cross-sectional images of each bone specimen. After scanning, three-dimensional images of the region of interest were produced. The bone volume as a proportion of total tissue volume in the region of interest was used as a measure of bone density and was calculated for all treatment groups. Additional trabecular measurements included trabecular thickness (Tra Thick), trabecular separation (Tra Septra), and trabecular number (Tra Number). Total bone volume was calculated automatically using micro-CT software.

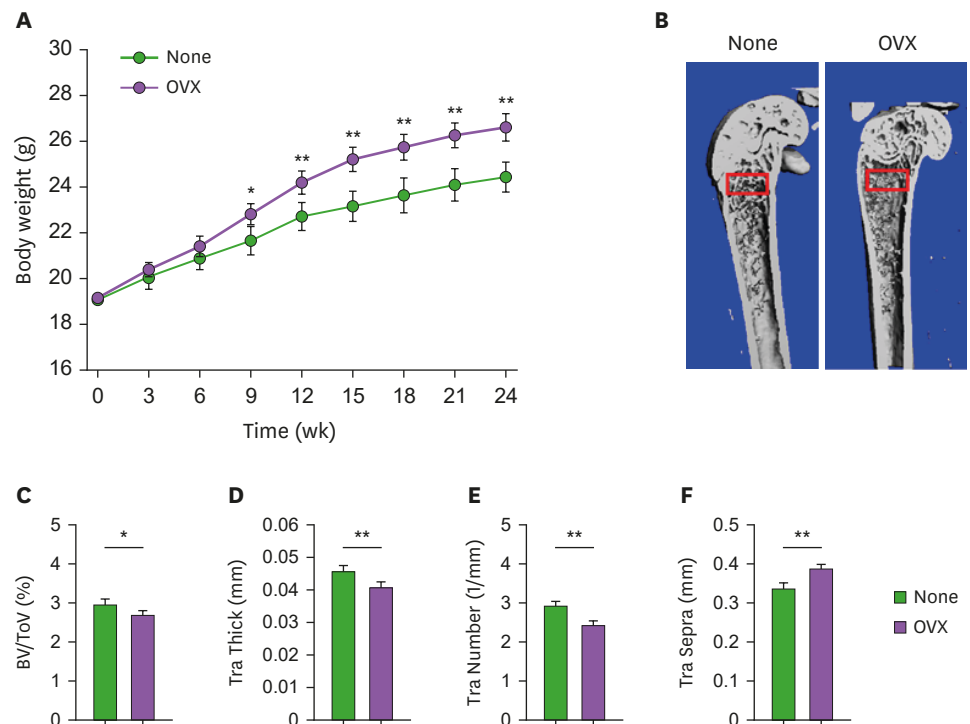
### Statistical analyses

Data are shown as means $\pm$ SEM. A 1- or 2-way ANOVA (Tukey multiple comparison test) and the Mann-Whitney *t*-test were used for analysis of the data sets. The *p*-values <0.05 were considered as statistically significant.

## RESULTS

### Ovariectomy promoted osteoporosis in femur

To evaluate the role of B10 cells in osteoporosis, we first established osteoporosis in mice by OVX. Ovaries were surgically removed from C57BL/6 mice, and changes in body weight were observed for 24 wk. As shown in **Fig. 1A**, the body weight of mice was significantly increased after OVX surgery compared to controls. Proportions of bone volume per total volume (BV/ToV) in femur was significantly decreased in OVX mice compared to that in control mice 24 wk after OVX, which indicated the bone density in the femur of OVX mice was lower than control mice (**Fig. 1B and C**). In addition, Tra Thick (**Fig. 1D**) and Tra Number (**Fig. 1E**) in the femur were also substantially decreased in OVX mice compared to control mice. Tra Septra (**Fig. 1F**) in femur was substantially higher in OVX mice than the controls (**Fig. 1F**). Thus, these results indicated that OVX promoted osteoporosis in mice.



**Figure 1.** Induction of OVX-mediated osteoporosis in C57BL/6 mice. (A) Changes in body weight after OVX (n=6 per group). (B) Micro-CT image of femurs 24 wk after surgery. (C) Proportions of BV/ToV in femur. (D) Tra Thick in the femur is shown. (E) Tra Number in the 1 mm of the femur. (F) Tra Septra in femur is shown (n=6, 3 independent samples from 2 experiments).

\**p*<0.05, \*\**p*<0.01.

### Regulatory immune cells were decreased in OVX mice

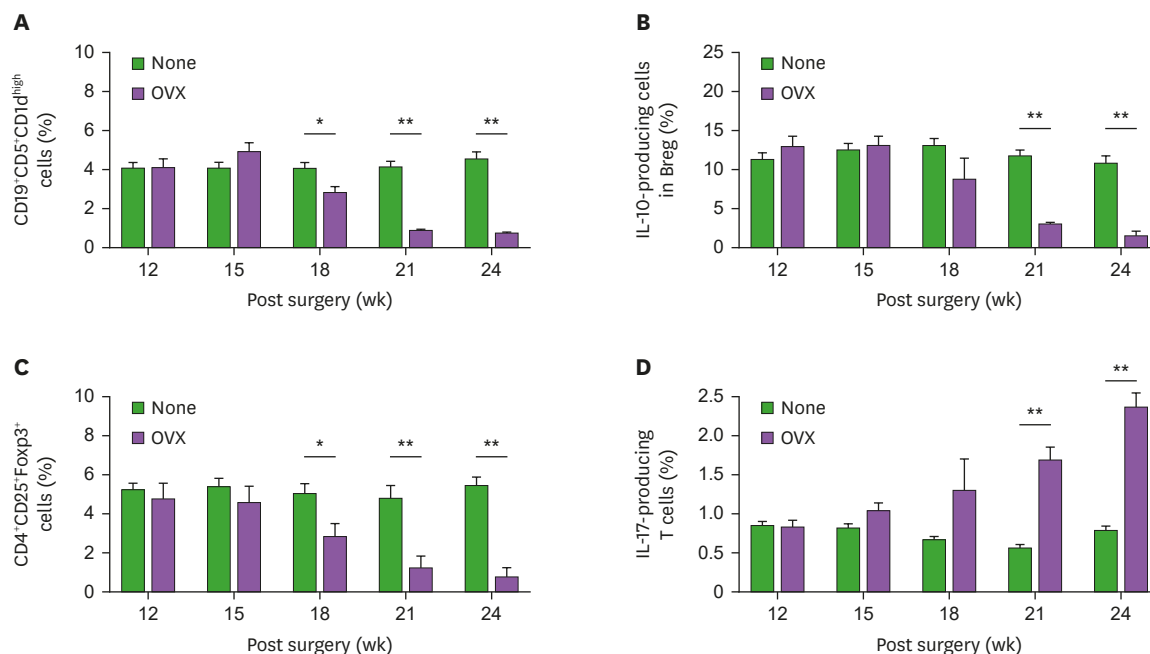
We subsequently examined alterations in regulatory immune cell proportions in OVX mice and found that after OVX, the proportion of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>high</sup> Breg cells in the spleen decreased over time (Fig. 2A and Supplementary Fig. 1). Moreover, B10 cells in the spleen were also significantly decreased 21 and 24 wk after OVX (Fig. 2B and Supplementary Fig. 2). Consistent with previous studies, the proportions of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells were also substantially reduced in OVX mice (Fig. 2C and Supplementary Fig. 3). In contrast to regulatory immune cells, Th17 cells were substantially increased in OVX mice (Fig. 2D and Supplementary Fig. 4). Thus, regulatory immune cells, especially Breg and B10 cells, appear to contribute to the development of osteoporosis in OVX mice.

### Adoptive transfer of B10 cells prevented the expansion of Th17 cells in OVX mice

We further tested the effects of B10 cell transfer on inflammatory reactions in OVX mice. B10 cells were adoptively transferred to OVX mice 15, 18, and 21 wk after OVX surgery (Fig. 3A). Twenty-four wk after surgery, the proportions of Breg and B10 cells in the spleen were substantially increased following transfer of B10 cells (Fig. 3B and C). In addition, the proportion of Th17 cells in the spleen and serum concentrations of IL-17 were substantially decreased following adoptive transfer of B10 cells into OVX mice (Fig. 3D and E). These data suggested that adoptive transfer of B10 cells downregulated Th17 immune responses in OVX mice.

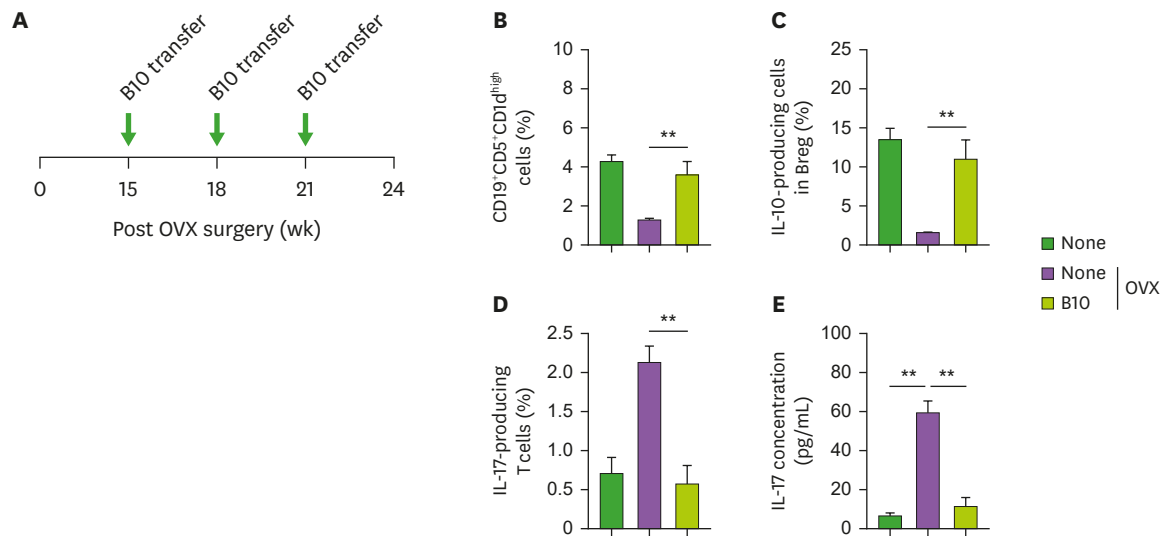
### B10 cell transfer alleviated osteoporosis development in OVX mice

Because adoptive transfer of B10 cells prevented Th17 immune responses, we subsequently examined whether B10 cell transfer would reduce the progression of osteoporosis in OVX



**Figure 2.** Alteration of immune cell percentages in the spleen during progression of osteoporosis in OVX mice. C57BL/6 mice were euthanized 12, 15, 18, 21, and 24 weeks after OVX, and spleen cells were analyzed by flow cytometry. (A) Proportion of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>high</sup> Breg cells. (B) Proportion of IL-10-producing cells in Breg cells. (C) CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cell percentages in the spleen. (D) Proportion of IL-17-producing CD4 T cells in the spleen. The means of six independent samples are shown (n=6, 3 mice for 2 experiments).

\*p<0.05, \*\*p<0.01.



**Figure 3.** Adoptive transfer of B10 cells suppressed IL-17 production in OVX mice. (A) B10 cells were adoptively transferred into C57BL/6 mice 15, 18, and 21 weeks after OVX. (B) CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>high</sup> Breg cell proportions in the spleen 24 wk after OVX. (C) Proportion of IL-10-producing cells in Breg cells. (D) Proportion of IL-17-producing CD4 T cells. (E) Serum concentration of IL-17 as measured by ELISA. The means of 6 independent samples are shown (n=6, 3 mice for 2 experiments). \*\*p<0.01.

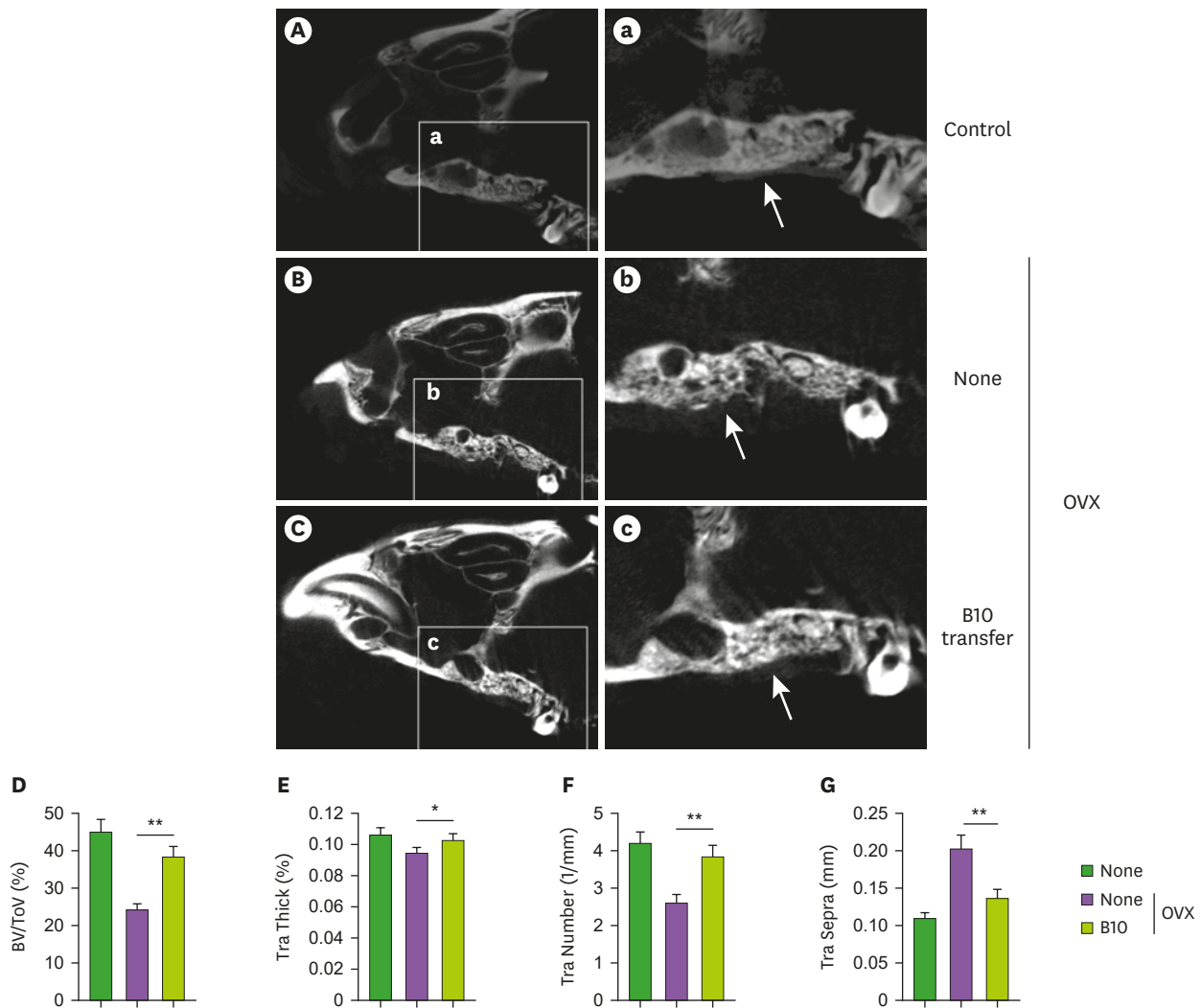
mice. Twenty-four weeks after OVX surgery, mice subjected to adoptive transfer of B10 cells exhibited no decrease in alveolar bone density (**Fig. 4A-D**). Moreover, reduction of trabecular thickness and number in alveolar bone was inhibited by B10 cell transfer into OVX mice (**Fig. 4E and F**), and the increased levels of Tra Septra by OVX were significantly reduced after transfer of B10 cells (**Fig. 4G**). Thus, these results suggested that adoptive transfer of B10 cells prevented the development of osteoporosis in OVX mice.

## DISCUSSION

Regulatory immune cells are known to counteract inflammatory diseases (21,22,24,26), and Treg-mediated anti-inflammatory effects have been promising in terms of treatment of inflammation and diseases affecting bone mass in animals and humans (21,22). In the current study, we found that numbers of B10 cells, a subset of Breg cells, were decreased in OVX mice, which indicated that B10 cells may contribute to the development of osteoporosis. Moreover, adoptive transfer of B10 cells into OVX mice suppressed the progression of osteoporosis. These results suggested that, consistent with Tregs, B10 cells also affect the development of osteoporosis.

We found that the number of both Treg and Breg cells decreased during the development of osteoporosis in OVX mice, while Th17 cells increased. The effect of Tregs on inflammatory and bone-affecting diseases has been studied extensively, the delicate balance of Treg-Th17 cells is essential in bone health, as Tregs exhibit protecting effect, whereas Th17 cells involve in bone loss (32); however, the Breg cell type was defined only relatively recently, and its role remains to be further investigated (19). Although both Treg and Breg cells suppress immune system activation (21,24), Breg cells help control T cell-dependent inflammatory responses and induction of Treg differentiation (25). Moreover, CD19<sup>+</sup>CD25<sup>high</sup> human Breg cells induced increasing of both the cell number and percentage of Tregs, while decreased





**Figure 4.** B10 cell transfer inhibited the progression of osteoporosis in OVX mice. (A to C) Representative micro-CT 2D cross section of extraction sockets and adjacent molar teeth in (A) control, (B) none transferred OVX and (C) B10 transferred OVX (left-whole samples, white line marks the region of interest; right-higher magnifications of the extraction region, white arrows point out the extraction sites). (D) Proportion of BV/ToV in extraction alveolar bone. (E) Tra Thick is shown. (F) Tra Number in 1 mm is shown. (G) Tra Septra in alveolar bone (n=6, 3 independent samples from 2 experiments).

\*p<0.05, \*\*p<0.01.

Th17 cells population in an *in vitro* conculture system (33). Therefore, transfer of B10 cells into OVX mice may not only suppress Th17 immunity but also induce Treg differentiation. In this study, we focused on the inhibition of osteoporosis by B10 cell transfer; thus, our results do not clearly reveal whether B10 cell transfer can help recover decreased Treg cell levels in OVX mice. Further studies regarding the relationship between B10 and Treg cells during osteoporosis in OVX mice would thus be required.

It has shown that progression of osteoporosis by OVX exhibited increases of CD19 cells in the spleen and bone marrow (34,35), however the function of the CD19 cells, whether it contributes the development or prevention of osteoporosis, has not been explored. Since B cells increasingly infiltrated in the inflamed tissue, especially autoimmune tissues (36,37), it may involve in the development of osteoporosis. In contrast to the increased number of

total B cells, we found that the percentage of B10 cells was decreased in OVX mice. Since the total number of B cells increased in OVX mice, the number of B10 cells would have decreased relatively. We will further study the changes in inflammatory B cells and B10 cells during development of osteoporosis by OVX.

Increased serum levels of inflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  have potent effects in the induction of osteoclastic bone loss (16). Inflammatory cytokines contribute to osteoclastic bone loss, and effector T cell-mediated cytokines suppress osteoclastogenesis (13,15). IFN- $\gamma$  is a marker cytokine of Th1 cells and inhibits the formation of osteoclasts (38), and neutralization of interferon  $\gamma$  promotes differentiation of osteoclasts (39). In addition, Th2-mediated IL-4, IL-5, and IL-13 also function as potential inhibitors of osteoclastogenesis (40-42). In contrast to Th1 and Th2 cytokines, IL-17 has been shown to be a critical factor in the pathogenesis of bone loss, osteoporosis, and inflammatory diseases (13-15). The main source of IL-17 is CD4 T cells, which are termed Th17 cells (13). Compared with different types of effector T cells, Th17 cells have been shown to directly contribute to disease onset in estrogen-deficient osteoporosis (43,44). In addition, neutralization of IL-17 by Ab treatment prevents the progression of osteoporosis in OVX mice (44). In the current study, we also found that the proportion of Th17 cells in the spleen increased in a time-dependent manner in OVX mice; however, this effect was reduced by adoptive transfer of B10 cells. Thus, B10 cells may inhibit the differentiation of Th17 cells in OVX mice, thereby decelerating the progression of osteoporosis.

Dental implantation is a common treatment for edentulous patients (45,46). However, accumulating evidence suggests higher failure risks of implant installation in low-density bone tissues (47,48). We found that transfer of B10 cells prevented the development of osteoporosis in the alveolar bone, indicating that B10 cell adoptive transfer may potentially reduce the failure rate of dental implant installation in patients with osteoporosis. Further studies are required to test whether B10 cell transfer improves the efficiency of dental implants in osteoporosis in rats and mice.

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## SUPPLEMENTARY MATERIALS

### Supplementary Figure 1

Changes of Breg cell percentage in the spleen after OVX. CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>+</sup> Breg cells in spleen were analyzed by flow cytometry 12, 15, 18, 21, and 24 wk after OVX.

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**Supplementary Figure 2**

Changes of B10 cell percentage in the spleen after OVX. Intracellular IL-10 producing CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>+</sup> cells in spleen were analyzed by flow cytometry 12, 15, 18, 21, and 24 wk after OVX.

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**Supplementary Figure 3**

Alteration of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cell percentage in the spleen after OVX. Intracellular expression of FoxP3 and surface expression of CD25 were analyzed in spleen CD4 T cells by flow cytometry 12, 15, 18, 21, and 24 wk after OVX.

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**Supplementary Figure 4**

Change of Th17 cell percentage in the spleen after OVX. Intracellular IL-17 producing cells in spleen CD4 T cells were analyzed by flow cytometry 12, 15, 18, 21, and 24 wk after OVX.

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