



Original Article

The influence of zoledronate and teriparatide on gamma delta T cells in mice



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Abstract *Background/purpose:* Few studies have investigated the possibility that bisphosphonate-related osteonecrosis of the jaw (BRONJ) might reflect an immune response; however, gamma delta T cells have been shown to significantly decline in the blood of BRONJ patients. Additionally, there have been some reports of teriparatide usage for the treatment of BRONJ. In this study, we compared the effects of zoledronate and teriparatide on lymphocyte populations and inflammatory cytokine production in mice.

Materials and methods: Thirty female ICR mice were divided into three groups (n = 10 each): a vehicle, a zoledronate, and a teriparatide group. Drugs were administered for 8 weeks in each group. Lymphocytes in the blood and thymus were analyzed and femurs were used for histological observation and lymphocytes analysis of bone marrow. Cytokines were measured in separated serum using Milliplex[®] multiplex immunoassay analysis.

Results: Zoledronate decreased the T cell number in the bone marrow. Additionally, serum levels of interleukin (IL)-2, IL-7, IL-12, IL-15 and RANTES, which are cytokines that affect T cell activation, differentiation and/or proliferation, were significantly lower in zoledronate treated mice. Conversely, teriparatide treatment induced an increase in gamma delta T cells in peripheral blood.

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Conclusion: Gamma delta T cells in the bone marrow are expected to decrease with zoledronate treatment and increase with teriparatide treatment. If BRONJ involves a loss of gamma delta T cells in the circulation or bone marrow, then the increase in gamma delta T cells that is induced by teriparatide may account for its ability to resolve BRONJ.

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Introduction

Nitrogen-containing bisphosphonates (NBPs), which are synthetic analogs of pyrophosphate, are an effective treatment for osteoporosis, hypercalcemia of malignancy, osteolytic lesions in multiple myeloma, and bone metastases from solid tumors, including breast cancer and hormone-independent prostate cancer.^{1,2} NBPs have a high affinity for bone minerals³ and accumulate in high concentrations in bones.⁴ They are selectively taken up by osteoclasts and strongly inhibit bone resorption by inducing apoptosis in osteoclasts.⁵ However, serious side effects such as bisphosphonate-related osteonecrosis of the jaw (BRONJ) have been reported with the use of NBPs.⁶ BRONJ appears as a painful lesion of exposed necrotic bone in the mandible or maxilla that fails to heal within 8 weeks.⁷ It has been reported that BRONJ is limited to the jaw bone and is not observed in other bones. The cause of BRONJ is poorly understood, but NBPs have been shown to reduce bone remodeling⁸ and angiogenesis,⁹ and thus the suppression of bone turnover and jaw angiogenesis resulting from NBPs has been proposed as the underlying mechanism of BRONJ.

BRONJ is thought to be an inflammation process. Although innate immunity has a critical role in this inflammation process, few studies have investigated the possibility that BRONJ might reflect an immune response. For example, lymphocytes are potent inducers of immune responses, but few studies have been carried out to understand the association between BRONJ and lymphocytes. However, in 2013, an intriguing association between loss of V γ 9V δ 2 T cells, which are a major subpopulation of human $\gamma\delta$ T cells, following NBP therapy and development of BRONJ was reported.¹⁰ In that study, six immunocompromised patients, who further underwent a significant loss of V γ 9V δ 2 T cells following NBP therapy, all experienced BRONJ. Conversely, Kunzmann reported that V γ 9V δ 2 T cells were activated by NBPs¹¹ with a consequent release of cytokines such as interferon γ (IFN- γ), which might contribute to the acute-phase reaction that is commonly observed following the first infusion of a NBP.

Apart from NBPs, there are some other types of anti-osteoporosis drugs, one of which is Teriparatide (recombinant human parathyroid hormone-(1-34) (PTH)-(1-34)). PTH is the major hormone that regulates bone remodeling. Although continuous infusion of high doses of PTH has catabolic effects, intermittent administration of PTH (iPTH) at low doses is anabolic for bone formation. Teriparatide is an anabolic agent that has been shown to increase both bone mass and bone strength.¹² The PTH receptor (PPR) is mainly expressed in the bone and kidney. In the bone, PPR is

expressed in osteoclasts¹³ but is mainly expressed in cells of osteoblastic lineage such as osteocytes.¹⁴ Osteocytes are necessary for iPTH anabolic effects.¹⁴ Treatment with iPTH activates the Wnt signaling pathway in osteoblasts by suppressing sclerostin production by osteocytes.^{15–20} The PPR is also expressed by T cells,²¹ and these cells are required for iPTH to exert its bone anabolic effect.^{21–23} The bone anabolic response to iPTH is blunted in the absence of T cells and is restored by adoptive transfer of these cells.^{21,22} T cells mediate the iPTH anabolic effect on bone through the upregulation of Wnt10b on their surface.²² Wnt10b interacts with osteoblasts, thereby upregulating Wnt pathway signaling and inducing bone formation.

The above findings indicate that these two anti-osteoporosis drugs have different mechanisms for modulation of bone metabolism and the immune system. Interestingly, there have also been some reports of teriparatide use in the treatment of BRONJ.^{24–26}

Based on the above findings, we hypothesized that NBPs would induce immune alteration. We further hypothesized that this alteration might be associated with lymphocytes including $\gamma\delta$ T cells, and that teriparatide might also have some effects on the immune system.

Therefore, in this study, we analyzed lymphocyte populations, including $\gamma\delta$ T cells, in mice treated with zoledronate (a NBP) or teriparatide, and we determined the effects of zoledronate and teriparatide on inflammatory cytokine production.

Materials and methods

Ethics statement

The animal study was approved by the Animal Ethical Committee of Okayama University. All experiments in this study regarding the use of laboratory animals were performed in accordance with the guidelines of Okayama University.

Reagents

Zoledronate was purchased from Santa Cruz Biotechnology (Dallas, TX) and teriparatide was purchased from Bachem AG (Bubendorf, Switzerland).

Mice

Thirty female ICR mice at 12 weeks of age were obtained from CLEA Japan (Tokyo, Japan) and were divided into three

groups (n = 10 each): vehicle group, zoledronate group, and teriparatide group. In the vehicle group, physiological saline was administered intraperitoneally. In the zoledronate group, zoledronate was administered intraperitoneally. The dose of zoledronate was set at 200 $\mu\text{g}/\text{Kg}/\text{day}$ and 3 days/week. In the teriparatide group, teriparatide was administered intraperitoneally. The dose of teriparatide was set at 40 $\mu\text{g}/\text{Kg}/\text{day}$ and 5 days/week. Mice were housed under standard conditions and food and water were available *ad libitum*. The doses used were the minimal dose at which effects were confirmed in the preliminary study. Drugs were administered for 8 weeks in each group. This administration period was also confirmed in the preliminary study.

At the end of the administration period, ether anesthesia was induced and blood was collected from the caudal vena cava. The thymus and the femurs were then collected for lymphocyte and bone analysis.

Histological observation

In each group, the right femur was collected from mice and was immediately fixed in 4% phosphate-buffered formaldehyde solution for 24 h. Fixed tissues were demineralized using 10% ethylenediaminetetraacetic acid for 30 days, dehydrated in the conventional manner and embedded in paraffin. Sections (5 μm -thick) were stained with hematoxylin and eosin (HE) and immunohistochemical staining was performed using an anti-mouse CD3 antibody (ab5690 Abcam Ltd., Cambridge, UK).

Flow cytometry

Flow cytometric studies were performed on blood and thymus using a FACSria (BD Franklin Lake, NJ). The cells

were labeled with fluorescein isothiocyanate-, phycoerythrin-cyanine-7-, allophycocyanin-, phycoerythrin-, brilliant violet 421-, allophycocyanin-, phycoerythrin-anti-mouse CD45 (BioLegend, San Diego, CA), CD3e (BioLegend), CD19 (eBioscience, San Diego, CA), CD49b (eBioscience), CD4 (eBioscience), CD8a (BioLegend), and TCR γ/δ (BioLegend), respectively. Data analysis was conducted using FlowJo version 7.5 software (Tree Star, Inc., Ashland, OR).

Biochemical analysis of mouse serum samples

Blood samples were centrifuged in the conventional manner to separate serum. A total of 32 cytokines were then measured in this serum using the Milliplex[®] multiplex immunoassay analysis (Merck Millipore Darmstadt, Germany).

Statistical analysis

Unless otherwise stated, all values are presented as means \pm SD of measurements from three independent experiments. Data were analyzed by one-way ANOVA for multiple group comparison. Tukey's HSD test was used to specify differences between groups. A p-value of less than 0.05 was considered significant.

Results

CD3+ cells in the femur bone marrow

We first analyzed expression of the pan T cell marker CD3 in the bone marrow of the femurs of the three mouse groups of vehicle (Fig. 1a), zoledronate (Fig. 1b) and teriparatide

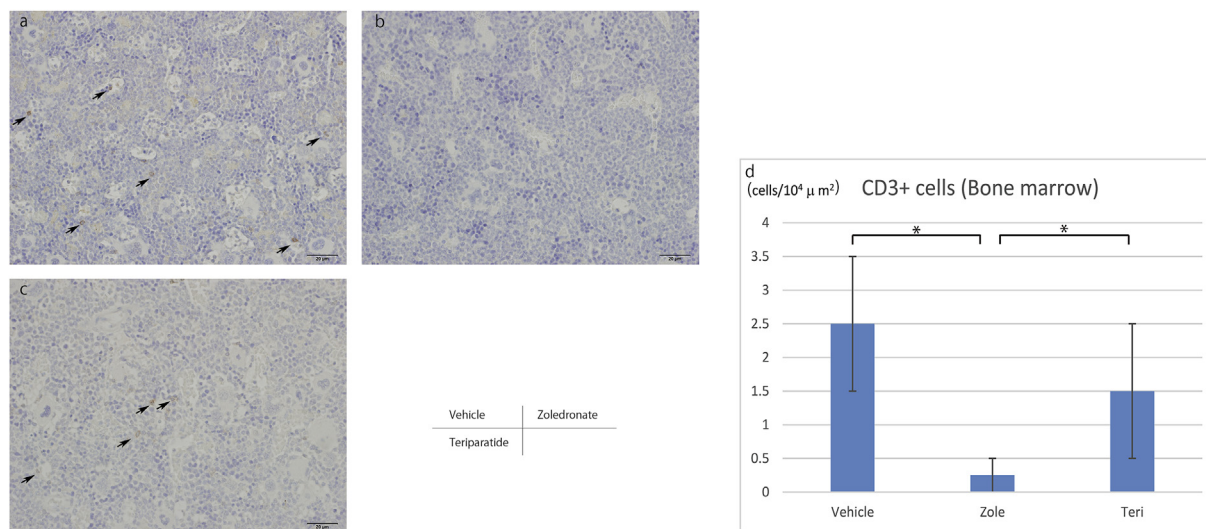


Figure 1 Analysis of T (CD3+) cells in the bone marrow of treated mice. (a–c). Immunohistochemical staining of CD3+ cells in the three mouse groups. Paraffin sections of bone marrow from the vehicle (a), zoledronate (b) and teriparatide (c) mouse groups were immunohistochemically stained for CD3+ cells (=T cells). (Scale bar = 20 μm). There appeared to be fewer CD3+ cells in the zoledronate group than in the other two groups. Arrows point to CD3+ cells. (d). Quantification of the CD3+ cell number in Fig. 1a–c. The Y-axis shows the number of CD3+ cells in every 100 μm square of bone marrow. The X-axis shows the vehicle group, the zoledronate group (Zole) and the teriparatide group (Teri). Data are expressed as the mean cell numbers of each group. Bars represent the standard deviation. The cell number in the zoledronate group was significantly different from that of the vehicle group and the teriparatide group. (*P < 0.05; Tukey's HSD test).

(Fig. 1c) treatment. Immunohistochemical staining suggested that zoledronate reduced the number of CD3+ cells in the bone marrow.

CD3+ cell numbers in the stained tissues were then counted and are shown in Fig. 1d. The mean number of CD3+ cells in every 100 μm^2 of bone marrow was 2.5, 0.25 and 1.5 in the vehicle, zoledronate and teriparatide groups, respectively. These results showed that zoledronate significantly reduced T cell number in the mouse bone marrow compared to the other two groups.

Flow cytometric analysis

Cells obtained from blood were then analyzed using flow cytometric analysis for the expression of cell surface markers. T cells were defined as CD45+/CD19-/CD3+ cells. B cells were defined as CD45+/CD19+ cells. Double-positive (DP) T cells were defined as CD45+/CD19-/CD3+/CD4+/CD8+ cells. Double-negative (DN) T cells were defined as CD45+/CD19-/CD3+/CD4-/CD8-cells. $\gamma\delta$ T cells were defined as CD45+/CD3+/CD4-/CD8-/TCR $\gamma\delta$ + cells.

Analysis of blood cells

The percentage of T cells in the total blood lymphocytes was 43%, 43% and 32% in the vehicle group, the zoledronate group and the teriparatide group, respectively. Administration of zoledronate induced neither an increase nor a decrease in the T cell population in blood compared to the

control. However, the T cell population was significantly lower in the teriparatide group than in the other two groups (Fig. 2a).

The percentage of B cells in the total blood lymphocytes was 43%, 43% and 57% in the vehicle group, the zoledronate group and the teriparatide group respectively. The percentage of B cells in the teriparatide group was significantly higher than that in the vehicle and zoledronate groups (Fig. 2b).

The percentage of $\gamma\delta$ T cells in the total blood lymphocytes was 2.1%, 2.4% and 4.4% in the vehicle group, the zoledronate group and the teriparatide group respectively. Administration of zoledronate did not induce any increase or decrease in the $\gamma\delta$ T cell population in blood. However, the $\gamma\delta$ T cell population in blood was significantly higher in the teriparatide group than in the other two groups (Fig. 2c).

Thus, zoledronate treatment had no influence on T cell, B cell or $\gamma\delta$ T cell populations in blood; however, teriparatide treatment decreased the T cell population and increased the B cell and $\gamma\delta$ T populations in blood compared to the other two groups.

Analysis of thymus cells

We next analyzed the percentage of DP T cells, DN T cells, SP T cells and $\gamma\delta$ T cells in thymus T cells. The percentage of DP cells was 16%, 13% and 36% in the vehicle group, the zoledronate group and the teriparatide group, respectively (Fig. 2d); the percentage of DN cells was 1.5%, 1.8% and 2.3%, respectively (Fig. 2e), that of SP cells was 83%, 86%

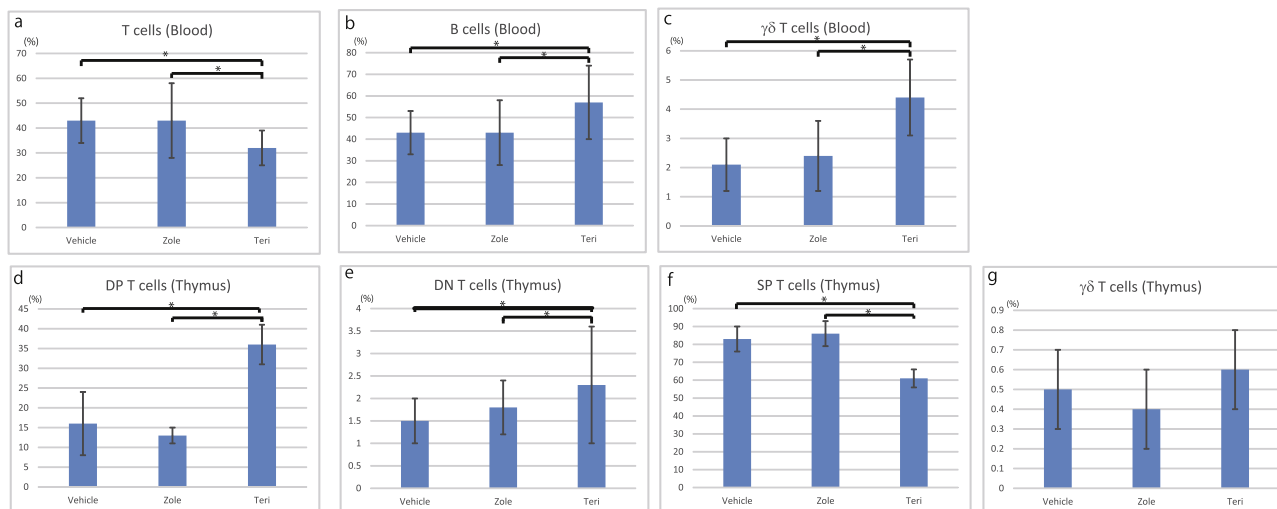


Figure 2 Analysis of T cell populations in the blood and the thymus of treated mice. Lymphocyte sub-populations in the blood and thymus of the three mouse groups were analyzed using FACS. (a–c). The Y-axis shows the percentage of the total blood lymphocytes that were (a) CD45+/CD19-/CD3+ cells (= T cells), (b) CD45+/CD19+ cells (= B cells) and (c) CD45+/CD3+/CD4-/CD8-/TCR $\gamma\delta$ + cells (= $\gamma\delta$ T cells). Data are expressed as the mean percentage for each group; the vehicle group, the zoledronate group (Zole) and the teriparatide group (Teri). Bars represent the standard deviation. The percentages of T cells, B cells and $\gamma\delta$ T cells in the teriparatide group were significantly different from those of the vehicle and the zoledronate groups. (* $P < 0.05$; Tukey's HSD test). (d–g) The Y-axis shows the percentage of the total T cells in the thymus that were (d) CD45+/CD3+/CD4+/CD8+ cells (= DP cells), (e) CD45+/CD3+/CD4-/CD8-cells (= DN T cells), (f) CD45+/CD3+/CD4-/CD8-or CD45+/CD3+/CD4-/CD8+ cells (= SP T cells), or (g) CD45+/CD3+/CD4-/CD8-/TCR $\gamma\delta$ + cells (= $\gamma\delta$ T cells). Data are expressed as the mean percentage for each group; the vehicle group, the zoledronate group (Zole) and the teriparatide group (Teri). Bars represent the standard deviation. The percentages of DP T cells, DN T cells and SP T cells in the teriparatide group were significantly different from those of the vehicle and the zoledronate groups. (* $P < 0.05$; Tukey's HSD test).

and 61%, respectively (Fig. 2f) and that of $\gamma\delta$ T cells was 0.5%, 0.4% and 0.6%, respectively (Fig. 2g).

Administration of zoledronate induced no increase or decrease in DP, DN or SP T cell populations in the thymus compared to the control. However, in the teriparatide group, the percentage of the DP and DN T cell populations was significantly higher than that of the other two groups and the percentage of the SP T cell population was significantly lower than that of the other two groups. Since the number of $\gamma\delta$ T cells in the thymus was very small, it was not possible to detect significant differences in $\gamma\delta$ T cells in the thymus among these 3 groups. In summary, teriparatide increased DP T cells and the DN T cell population and decreased the SP cell population in the thymus.

The immunohistochemical stainings of femur bone marrow and these flow cytometric analyses showed that zoledronate decreased T cells in the bone marrow but did not influence T cell, $\gamma\delta$ T cell or B cell populations in the blood. However, teriparatide decreased the T cell population and increased the $\gamma\delta$ T cell and B cell populations in blood. In thymus cells, zoledronate had no influence on DP T cell-, DN T cell- or SP T cell-populations; however, teriparatide increased DP T cell- and DN T cell-, and decreased SP T cell-populations.

Biochemical analysis of mouse serum samples

The levels of serum cytokines were measured after the 8-week administration period of vehicle, zoledronate or teriparatide to mice. The levels of IL-2, IL-7, IL-12, IL-15 RANTES(CCL-5) and tumor necrosis factor (TNF) α were

significantly lower in the zoledronate group than in the vehicle group or the teriparatide group (Fig. 3).

Discussion

Bone and the immune system are frequently discussed together and numerous studies have been performed under the understanding that bone disease and immunology are linked. For example, T and B cells are now implicated in the pathological bone loss associated with a variety of conditions, including ovariectomy-induced bone loss²⁷ hyperparathyroidism,²⁸ periodontal infection,²⁹ and rheumatoid arthritis (RA).³⁰ Regarding T cells, promotion of T cell activation ultimately leads to receptor activator of nuclear factor kappa-B ligand (RANKL) and TNF α secretion.³¹

However, few studies have been undertaken to determine the association between NBP exposure and immune cells, and no study has been performed to investigate the effect of teriparatide as a treatment for BRONJ viewed from the standpoint of immune cells.

Recently, $\gamma\delta$ T cells have been discussed in association with NBP exposure and two different types of results were reported; one result was that NBPs decreased $\gamma\delta$ T cells in human BRONJ patients¹⁰ and the second result was that NBPs activated $\gamma\delta$ T cells in acute-phase reaction patients.¹¹ The antigenic specificity of individual T cells is achieved through a heterodimeric complex that is comprised of two receptor chains and is referred to as the T cell receptor (TCR). Complexes that are comprised of a heterodimerized α and β chain are the most common TCRs and T cells bearing these complexes are referred to as $\alpha\beta$ T cells. However, $\gamma\delta$ T cells express TCRs that are comprised

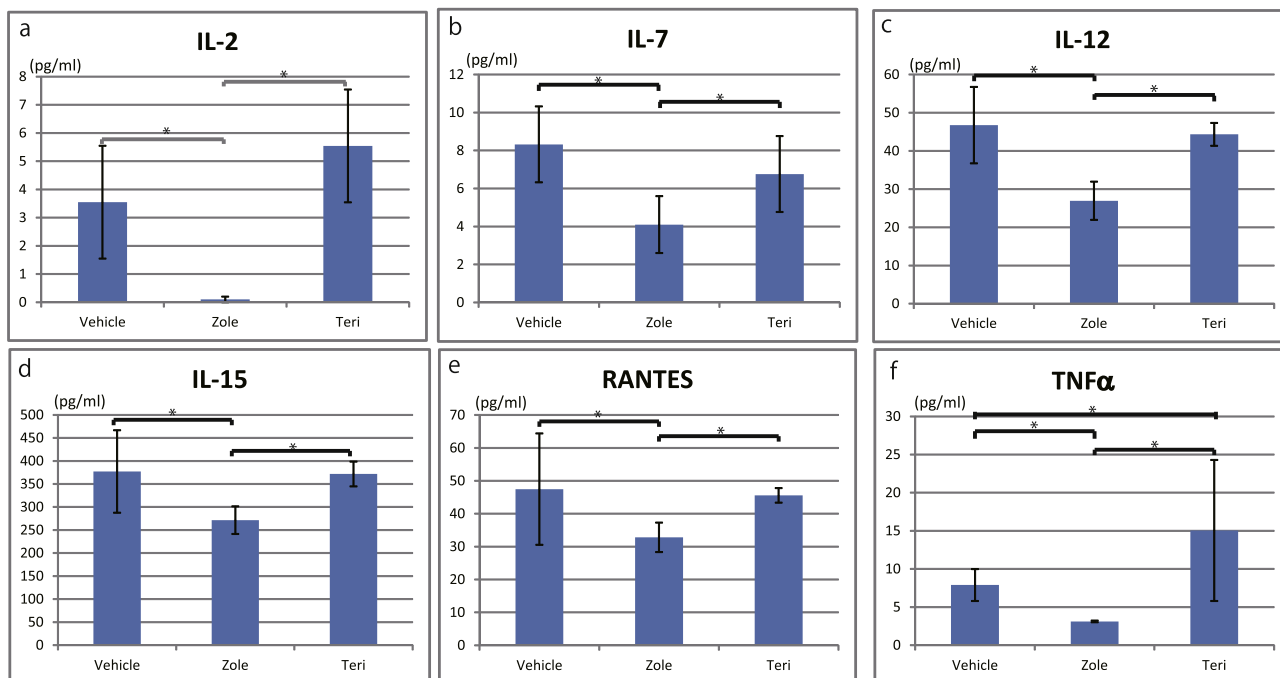


Figure 3 Analysis of cytokine levels in the serum of treated mice. Cytokine levels in serum samples from the three mouse groups were analyzed. The Y-axis indicates the serum level of (a) IL-2, (b) IL-7, (c) IL-12, (d) IL-15, (e) RANTES and (f) TNF α . Data are expressed as the mean values of each group; the vehicle group, the zoledronate group (Zole) and the teriparatide group (Teri). Bars represent the standard deviation. (*P < 0.05; Tukey's HSD test).

of a γ chain paired with a δ chain. $\gamma\delta$ T cells are functionally distinct from $\alpha\beta$ T cells and their TCR-specificity is directed almost exclusively towards nonpeptide antigens.

Regarding intermittent parathyroid hormone (iPTH) administration and T cells, the anabolic actions of iPTH are achieved through the induction of Wnt10b in T cells. This induction leads to osteoblast proliferation and differentiation, and activation of quiescent lining osteoblasts. Osteoblast life-span is increased by suppression of apoptosis and down-modulation of the Wnt receptor antagonist sclerostin that is produced by osteocytes.^{21,22,32}

Our results showed that neither the proportion of CD3+ T cells nor the proportion of $\gamma\delta$ T cells in mouse blood changed with zoledronate treatment. These results appear to differ from the reported loss of V γ 9V δ 2 T cells following NBP therapy and development of BRONJ,¹⁰ or from the report that V γ 9V δ 2 T cells are activated by NBPs in the case of an acute-phase reaction.¹¹ However in our case, although the mice were treated with zoledronate, these mice did not have any necrotic bone, they were not diagnosed as BRONJ, and their conditions were not those of an acute-phase reaction situation. Additionally, the immune system of the mouse and the human are not exactly the same and therefore a direct comparison may not be possible. The cytokine analyses indicated that zoledronate reduced the serum levels of IL-2, IL-7, IL-12, IL-15 and RANTES. These cytokines have effects on T cell activation, differentiation and proliferation.^{33,34} These results regarding the effects of zoledronate on T cell-related cytokines are consistent with the loss of T cells in the bone marrow that was observed following zoledronate treatment. Moreover, TNF α levels were decreased by zoledronate treatment, suggesting that zoledronate inhibited TNF α secretion by reducing T cell activation. This loss of TNF α suggests the inhibition of osteoclast activation that is believed to be the pharmacological effect of NBPs.

In addition, our results also showed that teriparatide (iPTH) treatment resulted in an increase in the $\gamma\delta$ cell population in blood. In the thymus, teriparatide increased DP and DN T cells and decreased SP T cells. Because DN T cells are precursors of $\gamma\delta$ T cells, the increase in DN T cells in the thymus as a result of teriparatide exposure might suggest that these DN T cells differentiate into $\gamma\delta$ T cells and are linked to the increased number of $\gamma\delta$ cells in the blood of teriparatide-treated mice. To our knowledge, this is the first report of analysis of $\gamma\delta$ cell populations following NBP and teriparatide treatment of mice.

Furthermore, if BRONJ is related to a loss of $\gamma\delta$ T cells in the circulation, the increase in $\gamma\delta$ T cells following teriparatide treatment may account for its treatment effects on BRONJ. Some BRONJ model mice have been reported^{35,36}; however, we could not easily establish such model mice without using immunosuppressants, including cortical hormones. For this reason, in this study, we analyzed only wild type mice. However, BRONJ model mice should be studied in the near future if the model mice can be successfully established without immunosuppressants.

We believe that our study will provide some new information that will lead to an understanding of how teriparatide treatment affects BRONJ.

Although the exact basis by which $\gamma\delta$ T cells protect against BRONJ and a direct cause-effect relationship remains

to be established, this study opens a new window into the potential importance of the immune-skeletal interface.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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