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Original Article

Excessive IL-4 environment enhances osteoclastogenesis and modulates inflammatory cell differentiation in bone loss associated with food allergic enteropathy

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Abbreviations:
BALB, BALB/cA; BM, bone marrow; CN, casein; EW, egg white; EMT, effector memory T; KikGR, Kikume Green-Red; μCT, microcomputed tomography; MLN, mesenteric lymph nodes; RAG2KO, recombination-activating gene 2 knock-out; RNA-seq, RNA-sequencing; RT-qPCR, Quantitative reverse transcription –PCR; Tr1, type 1 regulatory T

ABSTRACT

Background: Severe Th2 inflammatory diseases, including food allergy, are known to be associated with osteoporosis. However, while IL-4 inhibits osteoclast differentiation, detailed mechanisms of osteoporosis caused under IL-4-excessive environments remain unclear.
Methods: OVA23-3 mice are transgenic mice expressing OVA-specific T-cell receptors and develop significant IL-4-producing T-cell responses resulting in food-allergic enteropathy associated with osteoporosis when fed an egg white (EW) diet. This enteropathy is characterized by phases of inflammation and desensitization; bone loss develops during the inflammatory phase with the onset of allergic enteropathy and is maintained during the desensitization phase when the enteropathy is alleviated by immunological tolerance induction by continuous EW-feeding. We used this model to elucidate the mechanism of food antigen-induced osteoporosis, particularly in an IL-4-dominant environment.
Results: During the inflammatory phase, EW-feeding promoted osteoclastogenesis with increased mast cells, suppressed by administering anti-IL-4 antibody to the model. This finding suggests a critical role for IL-4 in the induction of osteoclastogenesis, which may be associated with mast cells and eosinophils over-differentiation and lead to osteoporosis. However, during the desensitization phase, the bone loss mechanism switched to high metabolic bone turnover, maintaining osteoclast activity despite amelioration of the enteropathy by continuous EW feeding. The increased number of IL-10-producing Tregs from mesenteric lymph nodes may reduce osteoclastogenesis during the desensitization phase, but did not suppress osteoporosis.
Conclusions: The present study provides an old perspective on a poorly understood mechanism of osteoporosis in severe allergies, suggesting the importance of maintaining bone health in allergic patients, including food allergies.

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Introduction

Secondary osteoporosis can be triggered by several inflammatory diseases, such as inflammatory bowel diseases and obesity, and also possibly by Th2-mediated diseases,[1,2] including allergies.[3,4] Although the relationship between allergy and osteoporosis is gaining attention,[5,6] a detailed mechanism remains to be elucidated since IL-4, a major cytokine in allergic responses, is implied to suppress bone loss.[7,8]

OVA23-3 mice are transgenic mice expressing OVA-specific T-cell receptors. When these mice are fed a diet containing egg white (EW), they develop food-allergic enteropathy and bone loss.[9–11] This enteropathy is characterized by an inflammatory phase and a desensitization phase. During the inflammatory phase, which begins approximately 9 days after the start of EW feeding, the mice lose weight due to acute enteropathy that develops within an IL-4-dominant environment.[11,12] With continued EW feeding, the mice acquire immunological tolerance and enter the desensitization phase, which begins approximately 28 days after the start of EW feeding, during which they recover from the enteropathy. However, the bone loss persists through the desensitization phase despite the recovery of the enteropathy due to the induction of OVA-specific Tregs. This enteropathy and osteoporosis have also been observed in OVA-sensitized BALB/cA (BALB) mice,[11] indicating the generality of OVA23-3 mice model.

Previous studies of EW diet-fed (EW-fed) OVA23-3 mice have shown that immune responses against OVA in the mesenteric lymph nodes (MLN) are an essential part of the primary mechanism linking the enteropathy and osteoporosis[9,11] and have suggested critical roles of IL-4 in inducing osteoporosis during the inflammatory phase.[11] During the desensitization phase, MLN-derived effector memory T cells ($CD4^{+}CD44^{hi}CD62L^{lo}$) (EMT cells) migrate to the bone marrow (BM), where they promote bone loss. Importance of MLN are confirmed by MLN excision, which mitigates this bone loss.[11] However, although we have indicated that IL-4 can induce osteoclast differentiation with IL-1β,[11] it remains unclear how bone metabolism is regulated in an IL-4-dominant environment and how bone loss develops and is maintained until the desensitization phase.

Here, we analyzed the bone metabolism by bone histomorphometry analysis underlying the osteoporosis develops in recombination-activating gene 2 knock-out (RAG2KO)/OVA23-3 (RAG23-3) crossed BALB mice (RAG23-3/BALB mice) to attenuate allergen-specific responses to OVA. RAG23-3 mice are a source of OVA-specific T cells without any other T cells and B cells when crossed with BALB mice, and RAG23-3/BALB showed similar characteristics with OVA23-3 mice when fed a diet containing EW.[11] We successfully found that increased osteoclastogenesis in an IL-4-dominant environment during the inflammatory phase leads to bone loss. Anti-IL-4 injection elucidated that IL-4 is responsible for the bone loss by increasing the number of osteoclasts with inflammatory cells, particularly mast cells and eosinophils. The enteropathy was re-evaluated to exhibit an eosinophilic with mast cell activation. However, with continuous EW feeding, the mechanism of bone loss changes during the desensitization phase, and bone loss is maintained by a high metabolic bone turnover despite improvement in the enteropathy caused in EW diet-fed mice. Our study highlights the importance of maintaining bone health in individuals with severe food allergy.

Methods

Mice

RAG23-3 and OVA23-3 mice were kindly provided by S. Habu (Tokai University School of Medicine, Tokyo, Japan). In the present study, F1 mice were generated by crossing RAG23-3 mice with BALB mice (CLEA Japan, Inc., Tokyo, Japan) (RAG23-3/BALB). Kikume Green-Red (KikGR) mice were crossed with the RAG23-3 mice to produce RAG23-3 × KikGR mice. Only male mice were used in all experiments. All mice were housed under specific pathogen-free conditions at The University of Tokyo and fed an OVA-free diet (CE-2 diet, CLEA Japan Inc., Tokyo, Japan) until the start of the experiments. All animal experiments were approved by the Animal Use Committee of the Faculty of Agriculture at The University of Tokyo (approval numbers: P18-027, P18-090, P19-087, and P19-091) and performed in accordance with relevant guidelines and regulations. All methods are reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

Diets and collection of tissues from food-allergic model mice

To induce allergic enteropathy, mice were fed an EW diet or casein (CN)-based control diet (Funabashi Farm Co., Chiba, Japan) for 9 days or 28 days. All mice were euthanized by cervical dislocation or cardiac puncture under deep anesthesia with isoflurane (Fujifilm Wako Pure Chemical, Osaka, Japan), and samples of MLN, spleen, and BM were collected and used to prepare single-cell suspensions for further analysis. OVA-specific IgE titer in the sera collected by cardiac puncture was analyzed by ELISA (Supplementary Materials**).** Quantitative reverse transcription—PCR (RT-qPCR) analysis of the cells was performed according to the method described in Supplementary Materials and the sequences of primers used for RT-qPCR are listed in Supplementary Table 1. The jejunum tissues were collected for histological analysis (Supplementary Materials). The right tibia was collected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, Fujifilm Wako Pure Chemical) and used for microcomputed tomography (μCT) using either RmCT (Rigaku, Tokyo, Japan) or ScanXmate (Comscantecno, Yokohama, Japan). In some experiments, the right femur was collected for bone histomorphometry analysis as follows. For details on the analysis of bone mass, see Supplementary Materials.

Bone histomorphometry analysis

Mice were injected subcutaneously with a 1 μg/μL solution of tetracycline (Sigma—Aldrich, St. Louis, MO, USA) or calcein (Sigma—Aldrich) (final dose, 10 μL/g body weight) [both solutions diluted with PBS (serum-free) buffer] at 4 days (tetracycline) or 2 days (calcein) before analysis. The corrected right femur was fixed in 70% ethanol for the subsequent processing and the femur sections were stained using Villanueva Bone Stain. Sample preparation and bone histomorphometry analysis were performed by Ito Bone Morphometry Laboratory (Niigata, Japan). The measured histomorphometric parameters[13] are listed in Supplementary Table 2.

Flow cytometry

Flow cytometry analysis was done by FACSVerse [Becton Dickinson and Company (BD), Franklin Lakes, NJ, USA] and FlowJo

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software (BD). Details for cell staining and antibodies used in the analysis are in Supplementary Materials.

Intracellular cytokine staining

RAG23-3/BALB mice fed the CN or EW diet for 9 days. The isolated cells from the BM of the mice were resuspended at 2.5×10^6 cells/2 mL in complete RPMI 1640 containing 10% fetal calf serum and then stimulated with ionomycin (2 μg/mL, Sigma—Aldrich), phorbol myristate acetate (10 ng/mL, Sigma—Aldrich), and brefeldin A (40 μg/mL, Sigma—Aldrich) in 48-well flat-bottomed plates (Falcon, New York, NY, USA) at 37°C for 4 h. The cells were then stained with antibodies for flow cytometry analysis, see Supplementary Materials.

T cell characterization and Treg isolation

In this study, we defined Tregs as $CD4^+$ T cells expressing Foxp3 molecule. However, when we isolated Tregs from tissues, we used CD25 as a marker of regulatory T cells and confirmed the percentage of the $Foxp3^+$ cells in the $CD4^+CD25^{hi}$ fraction. $CD4^+CD25^{hi}$ fraction as a Treg population ($CD25^{hi}$Tregs) from EW- or CN-fed RAG23-3/BALB mice, was sorted by using FACSAria II cell sorter (BD) for further expression analysis and RNA-seq analysis. Detailed RNA-seq analysis was described in Supplementary Materials. To sort BM Kik-Red Tregs from RAG23-3 × KikGR mice, $CD4^+$ cells were enriched by using a magnetic-activated cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany), and then Kik-Red and Kik-Green $CD4^+hCD2^+(Foxp3^+)$ cells were sorted by using FACSAria II cell sorter.

Analysis of cell migration

On 24 h before euthanization, EW or CN diet fed RAG23-3 × KikGR mice were anesthetized with isoflurane (Viatris, Canonsburg, PA, USA) under aseptic conditions, and their MLN was surgically exposed and irradiated with violet light (3 min per section of MLN divided into 3 sections, 435 nm LED; Prizmatix, Holon, Israel). During surgery, abdominal tissues were bathed with warm saline to prevent them from drying, and mice that had recovered from anesthesia and surgery were kept in a clean, warm cage. Kik-Red cells in the BM and spleen were identified as MLN-derived cells.

Anti-IL-4 Ab treatment

Anti-IL-4 Ab (clone 11B11, Bio X Cell, Lebanon, NH, USA) or isotype control IgG (control: Bio X Cell) diluted to 4 mg/mL with PBS was injected into each mouse (1 mg) intravenously via the tail vein on the day before and on 7 days after the start of EW or CN diets feeding. In some experiment, these Abs were injected on the day before and on 6 days after the start of experimental diets.

$Ly6G^+$ neutrophils depletion experiment

RAG23-3/BALB mice were fed with a diet containing CN or EW for 9 days. Anti-Ly6G Ab (BioXcell) or isotype control Ab (BioXcell) was administered by intravenous injection (1 mg/250 μL/mice) every two days starting the day before the administration of the experimental diet. Experimental groups were created as follows; CN: CN diet + isotype Ab, EW: EW diet + isotype Ab, CA: CN diet + anti-Ly6G Ab, EA: EW diet + anti-Ly6G Ab.

Statistical analysis

Two groups were compared by using Welch's *t*-test (Microsoft Excel). Groups more than three were compared using Tukey's honestly Significant difference (HSD) Test or Dunnett's test [R (4.3.1)]. *p*-values below 0.05 were considered significant. All data in the graphs are shown as mean ± SD.

Results

Bone loss develops due to increased osteoclast numbers during the inflammatory phase and is maintained by a high bone metabolic turnover during the desensitization phase

RAG23-3/BALB mice fed the EW diet for 9 (inflammatory phase) or 28 days (desensitization phase) and showed weight loss and the aggravation of the enteropathy during the inflammatory phase and their recovery until the desensitization phase (Fig. 1A-C). Significant ($p < 0.05$) bone loss was observed in the EW-fed compared with the control group on both day 9 and 28 (Fig. 1D). These data confirm previous results obtained in OVA23-3 mice.[9,11]

To clarify the bone metabolism of bone loss, we performed a bone morphometry analysis in EW- or CN-fed RAG23-3/BALB mice (the experimental schedule and the body weight changes shown in Supplementary Fig. 1). Osteoclast number (N.Mu.Oc/BS) was significantly ($p < 0.05$) increased in the EW diet compared to CN diet group during the inflammatory phase, but the difference was gone during the desensitization phase (Fig. 1E). Compared to the CN diet group, osteoblast number (N.Ob/BS) was decreased in the EW diet group during the inflammatory phase and increased during the desensitization phase, although they were not statistically significant (Fig. 1E). During the inflammatory phase, there were no significant differences in bone formation rate (BFR/BS), mineral apposition rate (MAR), or bone resorption rate (BRs.R) between the EW diet and CN diet groups; however, during the desensitization phase, there were significant increases in all three of these parameters in the EW diet compared with CN diet group ($p < 0.05$, 0.01, and 0.05, respectively), indicating that both of bone formation and resorption were increased. Also during the desensitization phase, significant decrease ($p < 0.05$) in trabecular number (Tb.N) and increase ($p < 0.05$) in trabecular separation (Tb.Sp) were observed in the EW diet compared with CN diet group, indicating the development of a sparse bone structure. Since there was no significant difference in osteoclast count between the two groups despite the increased bone resorption during the desensitization phase (Fig. 1E), we assessed the capability of the bone resorption of osteoclasts by measuring erosion depth. We found a significant ($p < 0.01$) increase in erosion depth in the EW diet compared to CN diet group, during both phases. These results indicated that osteoporosis is initiated by increased osteoclast numbers during the inflammatory phase and that the mechanism is altered during the desensitization phase; a high bone metabolic turnover maintains bone loss.

Excessive IL-4 environment promotes differentiation of inflammatory cells during the inflammatory phase

Inflammatory cells, including myeloid cells such as eosinophils and mast cells, are involved in the pathogenesis of allergy. Since these cells are also known to be involved in osteoporosis,[14] we next analyzed the inflammatory cells profile in MLN and BM in this model (Supplementary Fig. 2). In MLN, the percentage of $c\text{-}kit^+Fc\varepsilon RI^+$ cells (as mast cells and their progenitor) in whole cells was significantly ($p < 0.05$) increased in the EW diet compared to

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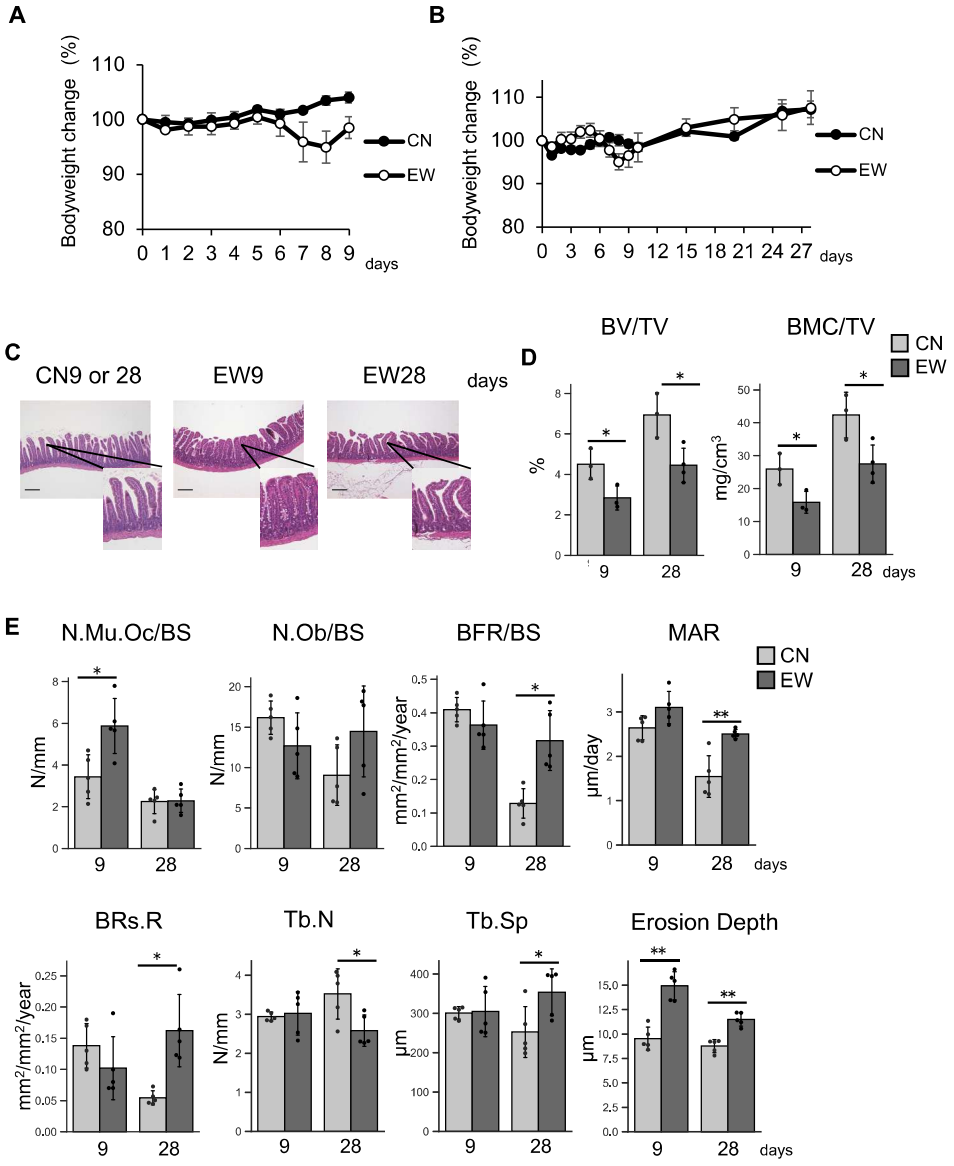

Fig. 1. Bone loss develops due to increased osteoclast numbers during the inflammatory phase and is maintained by a high bone metabolic turnover during the desensitization phase. RAG2KO/OVA23-3 × BALB/cA (RAG23-3/BALB) mice were fed with egg white (EW) or a casein (CN)-based control diet for 9 or 28 days. (**A, B**) Body weight relative to that on day 0 during feeding of EW diet for 9 days (inflammatory phase) (**A**) and that for 28 days (desensitization phase) (**B**). Body weights were measured daily until day 9 and every 5 days thereafter. (**C**) Panels of hematoxylin and eosin stained jejunum from the CN-fed mice on day 28 (left), or from the EW-fed mice after feeding for 9 days (center) or 28 days (right). Scale bars show 200 μm. (**D**) The bone mass of the right tibia measured through Micro-computed tomography (μCT). Data for bone volume fraction (BV/TV) and bone mineral content (BMC/TV) are shown. (**E**) Bone morphometry results regarding multinucleated osteoclast count (N.Mu.Oc/BS), osteoblast count (N.Ob/BS), the number of bone trabeculae (Tb.N), and the width of the spaces between trabeculae (Tb.Sp). Parameters for evaluating osteogenic function (bone formation rate, BFR/BS; mineral apposition rate: MAR) and bone resorption (bone resorption rate: BRs.R; Erosion Depth) are shown. Each experiment was performed twice, and representative data are shown except Fig. 1**(E)**. Individual points indicate the values for individual mice; the bar shows the mean ± SD for each group (n = 3–5 per group). Statistical analyses were performed using Welch's *t*-test (*$p < 0.05$, **$p < 0.01$).

CN diet group during the inflammatory phase, and the percentage of $CD11b^+SiglecF^+Ly6G^-$ cells (as eosinophils) in whole cells significantly ($p < 0.05$) increased during the desensitization phase (Fig. 2A, B). In BM, the percentages of $c\text{-}kit^+Fc\varepsilon RI^+$ cells, $CD11b^+SiglecF^+Ly6G^-$ cells, and $CD11b^+SiglecF^-Ly6G^+$ cells (as neutrophils) in whole cells were also significantly ($p < 0.05$, 0.01, and 0.01, respectively) increased only during the inflammatory phase (Fig. 2A-C). The percentage of $CD11b^+F4/80^+$ cells (macrophages and their progenitors) in both MLN and BM was significantly increased in the EW debt compared to CN diet group during both phases (Fig. 2D). Absolute cell number data followed the same patterns (Supplementary Fig. 3). These results indicate that during the inflammatory phase, increases in inflammatory cell populations, particularly those of $c\text{-}kit^+Fc\varepsilon RI^+$ mast cells and $CD11b^+SiglecF^+Ly6G^-$ eosinophils, contribute to the development of allergic intestinal inflammation and bone loss. Conversely, the contribution of these inflammatory cells decreases during the desensitization phase, other than $CD11b^+F4/80^+$ macrophages.

Significant induction of inflammatory cells in the inflammatory phase, we examined cytokine environment in both tissues. The expression levels of mRNA for inflammatory cytokines, such as IL-17, IL-6, and tumor necrosis factor-α (TNF-α), which contribute to

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bone loss,[14] and immunosuppressive cytokines, IL-10, were analyzed. However, they are comparable between CN-fed and EW-fed mice during the inflammatory and desensitization phase (Supplementary Fig. 4). Whereas, among Th2 cytokines (IL-4 and IL-5) responsible for allergic inflammation, we found a significant ($p < 0.05$) increase in *Il4* expression during the inflammatory phase in the EW diet compared to CN diet group in both MLN and BM (Fig. 2E), indicating the development of excessive IL-4 environment during the inflammatory phase but not the desensitization phase, especially in BM. *Il5* promoting mainly eosinophils differentiation, increased in the EW diet group, but not significant during the inflammatory phase and the increase was not observed during the desensitization phase (Supplementary Fig. 4). We previously reported that $CD4^+$ cells in MLN from EW-fed mice produce excess IL-4 in response to OVA, but the major IL-4 producers in BM were $CD4^-$ cells.[11] Here, we indicated that in BM, both $lineage^+$ and $lineage^-$ cells, but not including NK^+ cells, produced IL-4 (Fig. 2F), and that $CD11b^+F4/80^+$ cells, rather than $CD11b^+Ly6G^+$ cells, were the primary producers of IL-4 among $lineage^+$ cells (Fig. 2G). The results also suggest that $CD11b^+F4/80^+$ cells, which can produce IL-4, promote inflammation during both the inflammatory and desensitization phases.

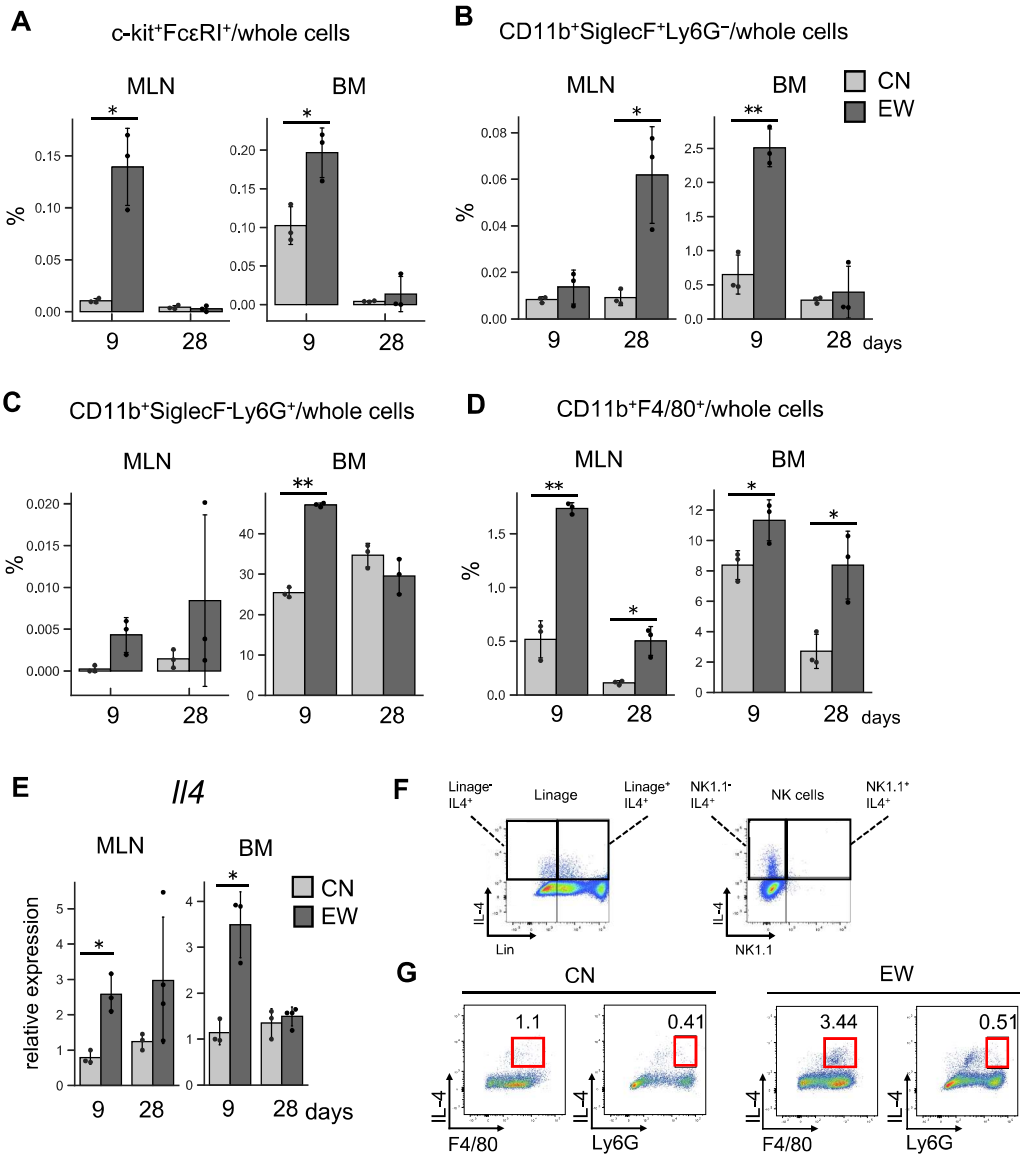

Fig. 2. Excessive IL-4 environment triggers bone loss with inflammatory cells during the inflammatory phase. RAG2KO/OVA23-3 × BALB/cA (RAG23-3/BALB) mice were fed with egg white (EW) or a casein (CN)-based control diet for 9 or 28 days. (**A-D**) The percentages of mast cells ($FcεRI^+$c-kit^+ cells) (**A**), eosinophils ($CD11b^+SiglecF^+Ly6G^-$ cells) (**B**), neutrophils ($CD11b^+SiglecF^-Ly6G^+$ cells) (**C**), and macrophages ($CD11b^+F4/80^+$ cells) (**D**), relative to whole cells in mesenteric lymph nodes (MLN) and bone marrow (BM) were measured by flow cytometry. (**E**) mRNA expression of IL-4 (*Il4*) in whole cells of MLN and BM during the inflammatory or desensitization phase was measured through RT-qPCR. (**F**) left: IL-4–producing cells among lineage-positive (+) or -negative (−) cells. right: IL-4–producing cells among natural killer (NK1.1) cells. (**G**) RAG23-3/BALB mice were fed with EW or a CN-based control diet for 9 days. IL-4–producing cells within the $CD11b^+F4/80^+$ and $CD11b^+Ly6G^+$ cell populations are shown. Each experiment was performed twice, and representative data are shown. Individual points indicate the values for individual mice; the bar shows the mean ± SD for each group (n = 3–5 per group). Statistical analyses were performed using Welch's *t*-test (*$p < 0.05$, **$p < 0.01$).

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Tregs induced during the desensitization phase produce IL-10 in the BM, but not in MLN

Roles of Tregs are reported as a suppressive regulator or an activator in the bone as well as MLN.[15–17] Therefore, we first compared the ratio of $CD4^+$T cells and Tregs induced during the inflammatory and desensitization phase by setting the day of sacrifice on the same day as shown in Figure 3A and Supplementary Fig. 5 [CN diet group, CN; the group fed the CN-diet for 19 days, followed by 9 days of EW feeding, E9 (inflammatory phase); and the group fed EW diet for 28 days, E28 (desensitization phase)]. This setting was conducted to decrease the difference caused by the difference of the day of sacrifice, considering the number of T cells is lower in BM than in MLN.

The ratio, not number, of $CD4^+$ T cells in MLN decreased significantly over time (Fig. 3B, Supplementary Fig. 6A), suggesting tolerant responses of OVA-specific T cells throughout the experimental period. However, in the BM, the ratio of $CD4^+$ T cells in the whole cells in E9 group was significantly increased compared to those in the CN and E28 group, responding to the changes of the inflammatory status like increases in the inflammatory cells. The percentage and number of Tregs ($Foxp3^+/CD4^+$ cells) in MLN was significantly ($p < 0.01$) increased in E28 groups compared to CN and E9 groups (Fig. 3C, Supplementary Fig. 6B). In the BM, there was a significant ($p < 0.01$) increase in the percentage ($Foxp3^+/CD4^+$ cells) but not in the number of cells in the E28 compared to the CN and E9 groups, which was influenced by the decrease in the number of $CD4^+$ T cells in the E28 group (Supplementary Fig. 6A). Throughout the experimental period, the proportion of Tregs in $CD4^+$ cells was increased in BM, like in MLN, but continuous bone loss occurred during the desensitization phase.

RNA-seq data of $CD25^{hi}$Tregs [around 80% of cells are $Foxp3^+$ (Supplementary Fig. 7)], implies that IL-10 is a specific molecule in both MLN and BM Tregs (Supplementary Fig. 8). Thus, we focused on mRNA expression of *Il10* in CN, E9, and E28 groups. In BM, *Il10* expression level in $CD25^{hi}$ Tregs in E28 group tended to be twice compared to those in E9 and CN group (Fig. 3D). We also found that IL-10-producing $CD25^{hi}$ Tregs tended to increase in E28 compared to that in E9 group in BM, and the level in E9 was lower than in CN groups (Fig. 3E). IL-10 mRNA expression and production in $CD25^{hi}$ Tregs in MLN was lower in E9 and E28 than in CN groups (Fig. 3D, E), indicating that fewer IL-10-producing Tregs were induced under the EW-fed food-allergic condition than in the steady state in MLN. However, in MLN, there were also IL-10-producing $CD4^+CD25^-$ cells, which may include $CD25^{lo}Foxp3^+$ Tregs or $Foxp3^-$IL-10 producing type 1 regulatory T (Tr1) cells (Supplementary Fig. 9). Assuming IL-10-producing Tregs migration from MLN to BM, we used RAG23-3 × KikGR mice and also found that the expression levels of *Il10* were higher in Tregs that had migrated from the MLN to BM (Kik-Red) than in those that had migrated from other organs or in those that initially resided in BM (Kik-Green cells) in E28 group. In contrast, no difference was observed between Kik-Red and Green Tregs in E9 group (Fig. 3F). This suggests that IL-10 production by Tregs in BM could be influenced more by cells that have migrated from MLN during the desensitization phase (E28) than the inflammatory phase (E9). Since IL-10 is reported as an inhibitory cytokine against osteoporosis that inhibits osteoclast differentiation, these results suggest

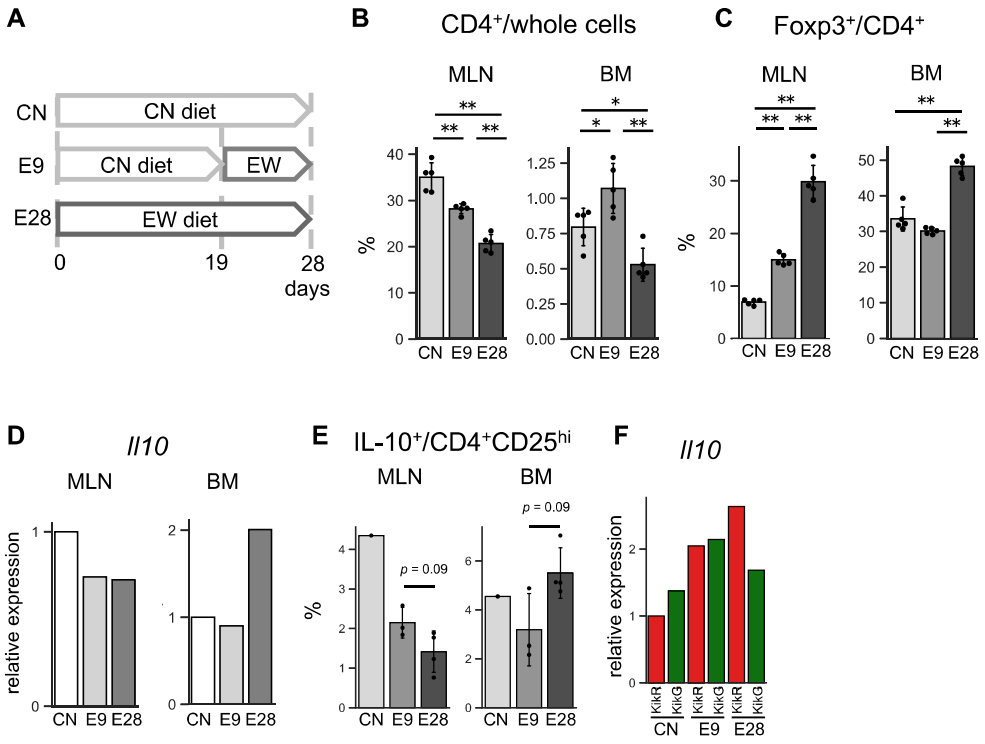

Fig. 3. Treg population expands during the desensitization phase, producing IL-10 in BM. (**A**) RAG2KO/OVA23-3 × BALB/cA (RAG23-3/BALB) mice were fed with EW or CN diet as follows: control group (CN); CN diet for 28 days, inflammatory phase group (E9); 19 days of CN diet followed by 9 days of EW diet feeding, desensitization phase group (E28); EW diet for 28 days. (**B, C**) The percentage of $CD4^+$ T cells relative to whole cells (**B**) and $Foxp3^+$Tregs relative to $CD4^+$ T cells (**C**) of mesenteric lymph nodes (MLN) and bone marrow (BM) were analyzed by flow cytometry. (**D**) mRNA expression of IL-10 (*Il10*) in $CD4^+CD25^{hi}$ Tregs of MLN and BM during the inflammatory or desensitization phase was measured through RT-qPCR. (**E**) The percentage of IL-10-producing $CD4^+CD25^{hi}$ cells was measured by flow cytometry. (**F**) *Il10* expression of purified Kik-Red (KikR) or Kik-Green (KikG) $Foxp3^+$Tregs was measured through RT-qPCR. Each experiment was performed twice, and representative data are shown. Individual points indicate the values for individual mice; the bar shows the mean ± SD for each group (n = 3–5 per group). Statistical analyses were performed using Welch's *t*-test (E) or Tukey HSD test (B, C) (*$p < 0.05$, **$p < 0.01$).

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that IL-10-producing Tregs in E28 in BM during the desensitization phase might contribute to the inhibition of osteoclast differentiation, thus regulating numbers of osteoclasts (Fig. 1E).

Excessive IL-4 environment promotes osteoclastogenesis during the inflammatory inflammatory

In OVA23-3 mice, we confirmed that the administration of anti-IL-4 Ab suppresses bone loss during the inflammatory phase, but not during the desensitization phase.[11] This finding suggests that IL-4 contributes to the pathogenesis of osteoporosis[12,18] especially during inflammatory phase, although IL-4 is generally considered a cytokine that inhibits osteoclast differentiation.[7] Therefore, we examined the effects of anti-IL-4 Ab administration on bone metabolism and the degree of inflammation in the intestine and bone in RAG23-3/BALB mice during the inflammatory phase to clarify the roles of IL-4. The mice that received EW and anti-IL-4 Ab (EW/Ab) showed results similar to those we reported previously,[11] including weight regain, amelioration of allergic enteropathy, and bone loss (Fig. 4A-C) compared to the mice that received EW and the control Ab (EW). Bone morphometry analysis showed that osteoclast number (N.Mu.Oc/BS) was significantly ($p < 0.01$) decreased in the EW/Ab compared to EW group (Fig. 4D). Trabecular number (Tb.N) was significantly ($p < 0.01$) increased and trabecular separation (Tb.Sp) was significantly ($p < 0.01$) decreased in the EW/Ab compared to EW group, indicating that bone structure was improved in the EW/Ab. However, other parameters [osteoblast number (N.Ob/BS), bone formation rate (BFR/BS), mineral apposition rate (MAR), and bone resorption rate (BRs.R)] were comparable between the EW and EW/Ab groups. Together, these results show that excessive IL-4 environment aggravates the differentiation and activation of osteoclasts, causing osteoporosis that occurs with food-allergic enteropathy.

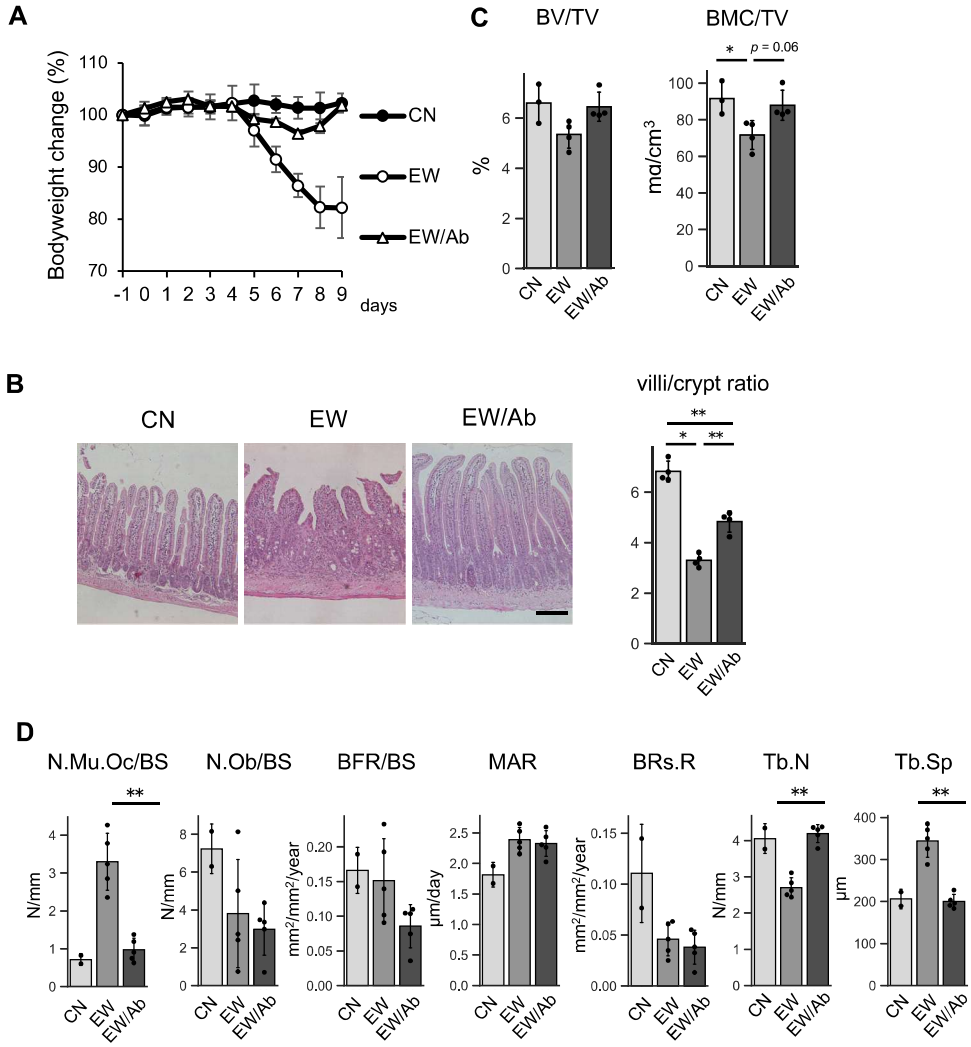

Fig. 4. IL-4 promotes osteoclast differentiation and contributes to osteoporosis during the inflammatory phase. RAG2KO/OVA23-3 × BALB/cA (RAG23-3/BALB) mice were fed with egg white (EW) or a casein (CN)-based control diet for 9 days. (**A**) Body weight relative to that on day −1 after feeding EW or CN diet for 9 days is shown. During the experimental period, anti-IL-4 (for EW/Ab group) or control IgG Ab (for CN and EW group) was injected intravenously on days −1 and 6. (**B**) Panels of hematoxylin- and eosin-stained jejunum from the CN group (left), EW group (center), and EW/Ab group (right). Scale bars show 200 μm. The bar graph shows the ratio of villi length to crypt depth (villi/crypt ratio), which exhibits the severity of inflammation of jejunum tissue. (**C**) Bone mass was measured through micro-computed tomography (μCT) of the right tibia on day 9; data regarding bone volume fraction (BV/TV) and bone mineral content (BMC/TV) are shown. (**D**) Bone morphometry results regarding multinucleated osteoclast count (N.Mu.Oc/BS), osteoblast count (N.Ob/BS), the number of bone trabeculae (Tb,N), and the width of the spaces between trabeculae (Tb.Sp). Parameters for evaluating osteogenic function (bone formation rate, BFR/BS; mineral apposition rate: MAR) and bone resorption (bone resorption rate: BRs.R) are also shown. Each experiment was performed twice, and representative data are shown except Fig. 3**(D)**. Individual points indicate the values for individual mice, and the bars show the mean ± SD for each group [**(D)**, n = 2 (CN), n = 5 (EW, EW/Ab)] or [**(A–C)**, n = 3 (CN), n = 4 (EW, EW/Ab)]. Statistical analyses were performed using Welch's *t*-test **(D)** or Tukey HSD test **(B, C)** (*$p < 0.05$, **$p < 0.01$).

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In inflammatory cells in MLN, only CD11b$^+$SiglecF$^+$Ly6G$^-$ (eosinophils) (Fig. 5A) and CD11b$^+$F4/80$^+$ (macrophages and their progenitor) (Fig. 5B) populations showed significant decreases ($p < 0.01$, 0.05) in the EW/Ab compared to EW group. Also, the number of mast cells in the jejunum tissues (Supplementary Fig. 10) was significantly decreased in the EW/Ab compared to EW group. In BM, the c-kit$^+$FcεRI$^+$ (mast cells and their progenitor) population decreased significantly (Fig. 5C), and the CD11b$^+$SiglecF$^+$Ly6G$^-$ populations (eosinophils) (Fig. 5A) tended to decrease in the EW/Ab compared to EW group, but did not in CD11b$^+$F4/80$^+$(macrophages and their progenitor) and CD11b$^+$SiglecF$^-$Ly6G$^+$ (neutrophils) (Fig. 5B, D). These reductions of inflammatory cells suggest a remarkable contribution of mast cells, and probably eosinophils, in excessive IL-4-dependent increase of osteoclast number. IL-4 suppresses Treg differentiation in MLN during the inflammatory phase.[12] Anti-IL-4 Ab treatment tended to increase the percentage of Foxp3$^+$Tregs ($p = 0.07$) within the CD4$^+$ cell population and significantly increased the absolute number of Foxp3$^+$Tregs ($p < 0.01$) in MLN in the EW/Ab group compared to EW group (Fig. 5E). However, there were no significant differences in the ratio or number of Foxp3$^+$Tregs in BM between the EW/Ab and EW groups (Fig. 5E), suggesting that Foxp3 expression is not regulated by excessive IL-4 environment in BM during the inflammatory phase, in contrast to the Treg differentiation promoted in MLN.

These results indicate that IL-4 promotes bone loss by regulating osteoclast differentiation during the inflammatory phase. This suggests that myeloid cells, mast cells and particularly eosinophils, may be important, with less impact from regulation of Treg differentiation since Foxp3$^+$Tregs in BM did not increase by inhibition of IL-4 (Fig. 5E).

Discussion

In this study, by using RAG23-3/BALB mice, we further investigated the mechanism of bone loss associated with allergic enteropathy in an IL-4-dominant environment under EW feeding. As a result, we clarified for the first time that IL-4 causes bone loss by promoting osteoclast differentiation with an increase in inflammatory cells, particularly mast cells in the BM during the inflammatory phase when intestinal inflammation is exacerbated. Our additional important finding is that even during the desensitization phase, when the enteropathy recovers by continuous antigen feeding, the mechanism of bone metabolism switches to the high bone metabolic turnover and maintains the bone loss. Also, IL-10-producing Tregs in BM during the desensitization phase were suggested to play a role in reducing the number of osteoclasts. Still, these IL-10-producing Tregs might not be fully effective in inhibiting bone loss.

IL-4 is a cytokine generally reported to suppress osteoclast formation.[7] However, the present study shows that by administrating anti-IL-4 Ab, IL-4 promotes osteoclast differentiation during the inflammatory phase, resulting in bone loss in food-allergic enteropathy. IL-4 overexpression in lck-IL-4 mice itself has been reported to cause osteoporosis.[1] In addition, recently, in clinical cases, bone loss has been reported to occur in a single-gene mutant

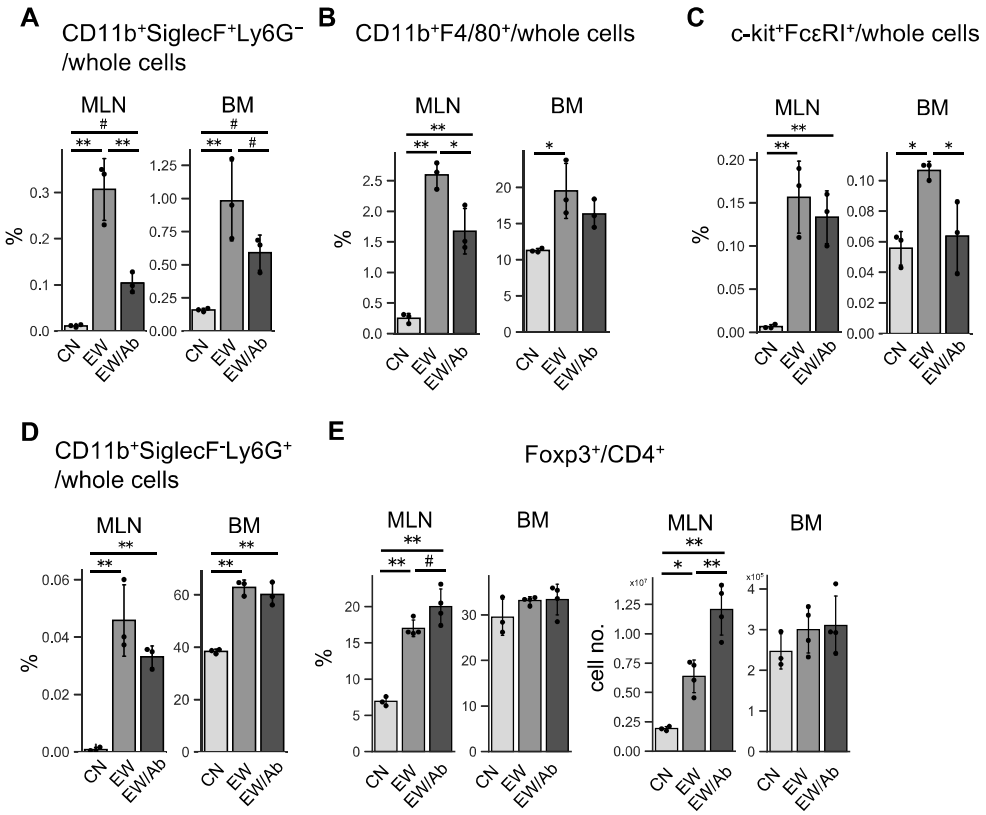

Fig. 5. IL-4 regulates mast cell population among the inflammatory cells. RAG2KO/OVA23-3 × BALB/cA (RAG23-3/BALB) mice were fed with egg white (EW) or a casein (CN)-based control diet for 9 days. During experimental period, anti-IL-4 (for EW/Ab group) or control IgG Ab (for CN and EW group) was injected intravenously on days −1 and 6. The percentages of eosinophils (CD11b$^+$SiglecF$^+$Ly6G$^-$ cells) (**A**), macrophages (CD11b$^+$F4/80$^+$ cells) (**B**), mast cells (FcεRI$^+$c-kit$^+$ cells) (**C**), and neutrophils (CD11b$^+$SiglecF$^-$Ly6G$^+$ cells) (**D**), relative to whole cells in MLN and BM were measured by flow cytometry. (**E**) The percentage (left) and cell number (right) of Foxp3$^+$ Tregs in mesenteric lymph nodes (MLN) and bone marrow (BM) were assessed through flow cytometry. Individual points indicate the values for individual mice, and the bars show the mean ± SD for each group (n = 3). Statistical analyses were performed using Tukey HSD test ($^\#p < 0.1$, $^*p < 0.05$, $^{**}p < 0.01$).

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disorder characterized by the overexpression of genes downstream of IL-4 signaling, such as STAT3 and STAT6.[19,20] Thus, bone loss occurs under IL-4-excessive environment, however it remains unclear whether IL-4 acts directly on osteoclasts to promote their differentiation and bone resorption. One possible factor to induce osteoporosis under IL-4-excessive environment is inflammatory cell differentiation and enhancement of their function by overproduction of IL-4[11]. Moreover, excessive T cell activation via IL-4R/STAT6 signaling and diminished Treg suppressive function are considered to be the other one.[21] These studies strongly suggest that excessive Th2 cell activation affects bone loss.[1,11] Therefore, further investigation into the relationship between T cell activation and bone loss associated with severe intestinal inflammation may provide deeper mechanistic insights and enhance our understanding of the mechanisms of bone loss in our mouse model. This mechanism of IL-4-dependent osteoporosis can be reproduced in a food allergic-enteropathy model of inbred BALB mice since we have confirmed that almost all of the pathogenesis, including bone loss, shown in RAG23-3/BALB and OVA23-3 mice is comparable to that of BALB mice enteritis model, sensitized with alum and OVA followed by EW feeding.[11,22]

Our results suggest that under IL-4-excessive environment, inflammatory cells may be indispensable for the development and maintenance of osteoporosis. Mast cells are prominent candidates among them because administration of anti-IL-4 Ab significantly reduced bone loss as well as intestinal inflammation, and the percentage of mast cells was simultaneously suppressed in both bone marrow and intestinal tissue. In the role of mast cells for inducing osteoporosis, some reports have been reported as one of the key contributors to osteoporosis among the allergic inflammation.[5] Experiments using mast cell-deficient mice have shown that absence of mast cells leads to a significant reduction in bone loss.[23] Moreover, recent studies have also shown that mast cells contribute to bone loss through mainly histamine and through IL-6, or TNF-α.[24–26] Because the level of the intestinal histamine increased in EW-fed OVA23-3 mice,[9] mast cells are possible to contribute to the bone loss. Regarding the other inflammatory cells, their proportion and number increased in BM, some of which also increased in MLN, although the levels of mRNA expression of inflammatory cytokines (i.e. *Il17*, *Il6*, and *Tnf*) were equivalent between CN-fed and EW-fed mice during the inflammatory phase. Recently, it has been reported that the interaction between mast cells and the other myeloid cells through chemical mediators enhance the pathophysiological relevance in allergic patients.[27] Thus, in our study, mast cells may also orchestrate the activation of inflammatory cells via chemical mediators, leading to the specific pathogenesis, such as osteoporosis as well as enteropathy in EW-fed mice.

For other inflammatory cells, recent reports have also implicated their association with bone loss in allergy.[14,28,5] In our study, $CD11b^+$ cells mainly produce IL-4 in the BM of EW-fed RAG23-3/BALB mice and are considered to be actively involved in bone loss. However, when we performed an inhibition study on the neutrophil population ($CD11b^+Ly6G^+$ cells), which has a particularly high percentage of cells in the BM, no recovery from bone loss was observed, suggesting that this fraction is unlikely to be primarily involved so far (Supplementary Fig. 11). Regulation of macrophages function and differentiation by IL-4 may promote bone resorption,[29] although we were unable to inhibit macrophage function with anti-CSF1R Abs during the long-term experiment. However, the percentages of macrophages continuously and significantly increased in EW-fed compared to CN-fed mice during the experimental period of our study. Regarding eosinophils, we observed an increase in BM during the inflammatory phase (Fig. 2B), and elevated *Il5* expression in BM suggests that eosinophil differentiation was also being promoted in BM (Supplementary Fig. 4). Administering an anti-IL-4 Ab inhibited the increase in eosinophils, albeit not significantly (Fig. 5A). Although reports describing the role of eosinophils in osteoporosis are fewer than those in mast cells, an increase in eosinophils in the BM in an IL-4-excessive environment in EW-fed RAG23-3/BALB mice seems to contribute to bone loss. An increase in eosinophils in the BM could play a role in inducing the enteropathy. Elevated mRNA expression of *Il4*, *Il13*, and *Il15*, as well as a significant increase in *Prg2* (Supplementary Fig. 12A) and the accumulation of eosinophils in the villi of the jejunum of EW-diet fed mice (Supplementary Fig. 12B), suggest that the pathogenesis of the enteropathy may show eosinophilic gastrointestinal diseases.[30] The slightly higher *Ccl11* expression in EW-diet fed mice may be involved in the migration of eosinophils.[30] Undetectable serum levels of OVA-specific IgE (Supplementary Fig. 12C) during the inflammatory phase suggest that the enteropathy is induced by an IgE-independent mechanism, which is also characteristic of eosinophilic gastrointestinal diseases as well as FPIES. Hence, mast cells and, probably eosinophils which are involved in type 2 inflammatory responses that exacerbate the enteropathy, may play a role even in the bone loss, either individually or sometimes cooperatively.[30]

Tregs in the BM of EW-fed RAG23-3/BALB mice, producing IL-10, might have reduced osteoclast number during the desensitization phase. However, bone loss did not recover during the desensitization phase. Whereas the number of Tregs in BM was comparable between EW- and CN-fed mice, it rather decreased as an average during the desensitization phase compared to the inflammatory phase. These results suggest that the suppressive function of IL-10-producing Tregs is insufficient for inhibiting bone loss, even if the Tregs successfully functioned in inhibiting osteoclast differentiation. We have previously shown that effector memory T cells in MLNs promote bone loss by migrating from MLN to BM[11]; in EW-fed OVA23-3 mice, and almost all of the Tregs which were induced in an antigen-specific manner expressing Foxp3 molecule were effector memory T cells.[12] In addition, the number of Tregs migrating from MLN to BM during the desensitization phase was significantly increased in the EW-fed mice (Supplementary Fig. 13). These findings suggest that the mechanisms underlying effector memory T cells-mediated bone loss during the desensitization phase require further investigation, relative to Tregs.

We have confirmed that nutritional factors are not responsible for bone loss in EW-fed OVA23-3 mice.[10] Therefore, we can describe that bone loss is maintained by unbalanced bone metabolism of continuous remodeling of bone formation by osteoblasts and bone resorption by osteoclasts. The changes in bone metabolism from a significant increase in the number of osteoclasts during the inflammatory phase to a high bone metabolic turnover during the desensitization phase against continuous EW stimulation throughout of this study are crucial for the persistent bone loss in the EW-fed RAG23-3/BALB model. The detailed reason why the changes in bone metabolism occurred in our study is probably related to the changes in immune responses from inflammation to desensitization, but remains unknown. However, our findings on the mechanism of continuous bone loss during the desensitization phase are one of the most outstanding achievements of this study, which is maintained by high bone metabolic turnover. The bone resorptive function was increased in the EW-fed group, confirming the accelerated bone resorption by a smaller number of osteoclasts than in the CN-fed group. This leads to bone loss during the desensitization phase, although the mechanism remains unclear. Bone loss due to high bone metabolic turnover is also a mechanism of osteoporosis often observed in postmenopausal women with

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reduced estrogen levels, and it is thought to mainly promote bone resorption. Moreover, T cell expansion is involved in estrogen deficiency-induced bone loss.[31] Taken together, excessive osteoclast differentiation associated with activated T cells may be a target for the treatment to prevent osteoporosis in food-allergic enteropathy.

This study highlights the need for a deeper understanding into the role of IL-4 in allergic inflammation and bone metabolism. Given that the factors contributing to bone loss may vary during the transition from the inflammatory phase to the desensitization phase, this study provides important insights to explore the mechanisms by which bone loss occurs under IL-4-excessive conditions and persists independently of the allergic inflammation mitigation. The present study strongly suggests the importance of maintaining bone health in severe allergies, including food allergies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2025.11.007.

Conflict of interest

HNA reports that financial support was provided by Meiji Holdings. HNA reports a relationship with Meiji Holdings that includes: FY is an employee of Meiji Holdings, and funding grants. SH reports a relationship with Meiji Holdings that includes: funding grants and non-financial support. The rest of the authors have no conflict of interest.

Authors' contributions

HNA designed and supervised the study and HNA and KS contributed to the conceptualization of the study. KS and HNA wrote the manuscript. KS, TH, MT, SU, SN, KN, RI, AF, KT, FY, and HNA performed experiments, analyzed the data, and created the figures. AI performed bone morphometry analysis. MH, SK, SM, MT, NS, and SH provided important advice and suggestions during discussions regarding the study. All authors discussed and interpreted the data.

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