


## RESEARCH ARTICLE

# Bindarit Reduces Bone Loss in Ovariectomized Mice by Inhibiting CCL2 and CCL7 Expression *via* the NF- $\kappa$ B Signaling Pathway

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**Objective:** To investigate the changes in proinflammatory cytokines and chemokines, namely, C-C motif ligand (CCL) 2 and CCL7, in postmenopausal osteoporosis (PMOP) and to develop a new drug, bindarit (Bnd), for PMOP in an ovariectomized (OVX) mouse model.

**Methods:** Bone marrow macrophages (BMMs) from the femurs of five women with PMOP and five premenopausal women without osteoporosis were detected by RNA sequencing. BMMs from mice were differentiated into osteoclasts and treated with a synthetic inhibitor of CCL2 and CCL7, Bnd, or 17 beta estradiol (E<sub>2</sub>). Mouse BMMs were differentiated into osteoclasts with or without Bnd for 7 days and analyzed by RNA sequencing. Osteoblasts of mice were induced to undergo osteoblastogenesis and treated with Bnd. OVX mice were treated with E<sub>2</sub> or Bnd after surgery. The protein and mRNA expression of CCL2 and CCL7 was detected using immunostaining and qPCR, respectively, in OVX and aged mice and in cells cultured *in vitro*. Osteoclast formation was detected using a tartrate-resistant acid phosphatase (TRAP) assay *in vitro* and *in vivo*. Alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN) were detected using immunostaining to evaluate osteogenesis. Microcomputed tomography was conducted to analyze trabecular bone parameters, the structure model index, bone mineral density and other variables. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway-related protein phosphorylation of IKK $\alpha$ / $\beta$  (p-IKK $\alpha$ / $\beta$ ) and p-NF $\kappa$ B p65 was examined using western blotting.

**Results:** CCL2, CCL7 and their receptor of C-C chemokine receptor-2 (CCR2), and the NF- $\kappa$ B signaling pathway, were significantly increased in women with PMOP. CCL2 and CCL7 protein and mRNA expression was increased in OVX mice and aged female mice, but the increases were attenuated by E<sub>2</sub> and Bnd. E<sub>2</sub> and Bnd effectively inhibited osteoclastogenesis and the protein expression of CCL2 and CCL7 both *in vitro* and *in vivo* and reduced bone loss in OVX mice. Bnd did not affect the mineralization of osteoblasts directly *in vitro* but reduced bone turnover *in vivo*. p-IKK $\alpha$ / $\beta$  and p-NF $\kappa$ B p65 levels were increased in BMMs of mice after differentiation into osteoclasts but were significantly decreased by Bnd.

**Conclusion:** The proinflammatory cytokines and chemokines CCL2, CCL7 and CCR2 were correlated with PMOP. Bnd attenuated the increases in CCL2 and CCL7 levels to affect osteoporosis in OVX mice *via* the NF $\kappa$ B signaling pathway. Thus, Bnd may be useful as a new therapeutic for the prevention of PMOP.

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**Key words:** Bindarit; MCP-1; MCP-3; Osteoclastogenesis; Osteoporosis

## Introduction

Postmenopausal osteoporosis (PMOP) results in osteoporotic fracture, which is associated with a low quality of life, high mortality and economic and societal burdens.<sup>1</sup> Inflammatory bone diseases frequently exhibit imbalances in the regulation of bone resorption and formation, which lead to excessive bone resorption.<sup>2</sup> PMOP is regarded as the product of an inflammatory disease exhibiting many characteristics of an organ-limited autoimmune disorder that is triggered by estrogen deficiency and created by chronic mild decreases in T cell tolerance.<sup>3-5</sup> Estrogen deficiency can increase the expression of many inflammation-related genes; in contrast, estrogen can act as an anti-inflammatory agent to suppress proinflammatory and preosteoclastic cytokines.<sup>6</sup> Osteoclast activity is closely related to inflammation,<sup>7</sup> and inflammation is correlated with PMOP.<sup>8-10</sup> Modulation of inflammatory substrates may be an additional therapy for bone loss in PMOP.<sup>7,11</sup> However, there have been few studies on the effects of anti-inflammatory drugs on PMOP.

C-C motif ligand (CCL) 2 (also known as monocyte chemoattractant protein (MCP)-1) and CCL7 (MCP-3) are primarily secreted by monocytes/macrophages and mediate monocyte recruitment and inflammation.<sup>12</sup> C-C chemokine receptor 2 (CCR2), the major receptor of CCL2 and CCL7, is upregulated in ovariectomized (OVX) mice and determines osteoclast behavior.<sup>13</sup> Estrogen downregulates CCL2,<sup>14-16</sup> and some studies have considered the chemokine CCL2 as an indicator of estrogen signaling.<sup>16</sup> CCL2 is elevated in osteoporosis patients and may be a novel predictor.<sup>17</sup> The regulatory effect of estrogen on CCL2 and CCL7 is controversial,<sup>18</sup> and research on the effects of estrogen on CCL2 and CCL7 in patients with PMOP is lacking. Receptor activator for nuclear factor- $\kappa$ B ligand (RANKL) induces the expression of many chemokines, including CCL7,<sup>19</sup> an osteoclastogenic factor.<sup>20</sup> CCL7 can directly and dramatically enhance osteoclast formation.<sup>2</sup> The nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway is the key signaling pathway of osteoclast formation. Osteoclasts are differentiated from monocyte/macrophage lines and are key cells in osteoporosis because of excessive bone absorption. Excessive pathological secretion of many chemokines may potently stimulate bone resorption and local osteolysis.<sup>2,17</sup> The expression of CCL2 and CCL7 in bone marrow macrophages (BMMs) of patients with PMOP is not clear.

Although estrogen levels correlate with bone homeostasis, estrogen replacement therapy is not ideal for PMOP because of its high risk/benefit ratios.<sup>21</sup> Selective inflammatory cytokine inhibitors may be developed as new therapeutic agents for PMOP.<sup>5,6</sup> Bindarit (Bnd) is a safe and well-tolerated phase II anti-inflammatory small molecule<sup>22,23</sup> that inhibits the synthesis of CCL2, CCL7 and CCL8.<sup>11,22,24</sup> Bnd

prevents chronic inflammation in various animal models, such as bone cancer, Chikungunya virus infection, and diabetes-associated periodontitis models.<sup>25</sup> PMOP is the product of an inflammatory disease, and patients with PMOP generally suffer from inflammatory diseases at the same time. Therefore, inhibition of inflammation may benefit both inflammatory diseases and PMOP. The effects of the CCL2 and CCL7 inhibitor Bnd on PMOP remain unknown. We hypothesized that CCL2 and CCL7 levels are increased in BMMs of patients with PMOP and that upregulation of CCL2 and CCL7 promotes further monocyte recruitment. Estrogen and Bnd may inhibit the expression of CCL2 and CCL7 in BMMs. This inhibition may reduce inflammation and benefit bone metabolism.

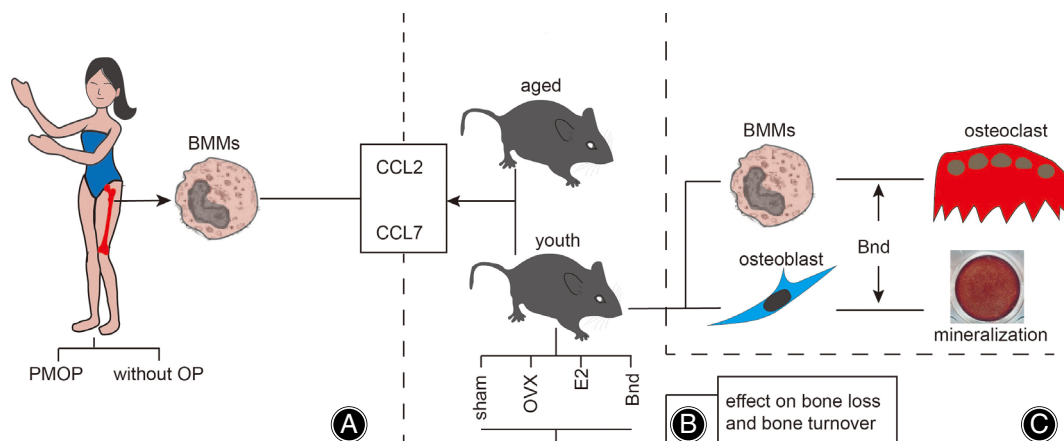
Therefore, the present study investigated: (i) the transcriptomes of BMMs between women with PMOP and premenopausal women without osteoporosis, especially with regard to the expression of the proinflammatory cytokines and chemokines CCL2 and CCL7; (ii) the serum concentrations of CCL2 and CCL7 and the mRNA levels of CCL2 and CCL7 in BMMs of OVX mice (a classic PMOP model) and aged mice as well as changes following estradiol (E<sub>2</sub>) and Bnd administration in OVX mice; and (iii) whether Bnd treatment inhibits osteoclastogenesis and benefits bone loss *via* modulation of inflammatory substrates (Fig. 1).

## Materials and Methods

This study was approved by the Human Research Ethics Committee of the Third Affiliated Hospital of Southern Medical University (approval number: 2020-ethical review-016). Informed consent was obtained from the donors. All animal experiments were performed with the approval of the Institutional Animal Care and Ethics Committee of Southern Medical University.

### Human BMMs

BMMs were collected from the Third Affiliated Hospital of Southern Medical University and aseptically purified immediately *via* centrifugation using monocyte isolation solution (Ficoll-Paque PLUS, GE Healthcare, Chicago, IL, USA) and immunomagnetic selection (CD14 MicroBeads human, MACS, Miltenyi Biotec™, Bergisch Gladbach, Germany) following the manufacturer's instructions.<sup>26</sup> The following inclusion criteria were used: (i) female patients who underwent femoral intramedullary nail fixation; and (ii) premenopausal adult women without osteoporosis ( $n = 5$ , T score of bone mineral density  $> -1.00$ ) or women with PMOP ( $n = 5$ , T score  $< -2.50$ ). The following exclusion criteria were used: (i) comorbidities such as diabetes or nephropathy; (ii) tumors; (iii) local femoral infection; or (iv) other conditions that may affect BMM extraction or the



**Fig. 1** Detailed illustration of the experiment.

(A) BMMs were collected from women and analyzed using RNA-seq to examine the expression of CCL2 and CCL7. (B) CCL2 and CCL7 protein and mRNA levels were examined in aged and young mice, and OVX mice were treated with E2 and Bnd to examine their effects on bone loss and bone turnover. (C) BMMs and osteoblasts were extracted from mice and treated with Bnd during differentiation.

accuracy of the analyses. All patients signed a consent form. There were no significant differences in height or body mass index (BMI) between the two groups ( $t = 0.63$  and  $1.08$ ,  $P = 0.55$  and  $0.31$ ; Figs. 2A, B), but the differences in age and T scores were significant ( $t = 8.37$ ,  $9.88$ , both  $P < 0.001$ ; Fig. 2C, D).

#### Mouse OVX Model and Treatment

Adult mice (aged 10 weeks and weighing 220–230 g, C57BL/6J) were assigned to a sham surgery group, OVX group, OVX + 17 beta-estradiol (E2) group and OVX + Bnd group. The OVX mice underwent bilateral ovariectomy, while the sham group underwent only ovarian exposure surgery and excision of fat tissue equivalent to the ovary volume, as described in previous literature.<sup>27</sup> During the operation, the mice were anesthetized, and the skin of the lower back was prepared. After a dorsal midline incision of ~15 mm was made, the incision was pulled to the left, and a small incision of ~10 mm was cut through the abdominal wall. The fat was pulled out using forceps, and the ovary was exposed. After the ovary or fat tissue equivalent to the ovary volume (sham group) was excised, the abdominal wall was sutured with absorbable sutures. After the other ovary or fat tissue was removed, the incisions were sutured, and each mouse was intramuscularly injected with penicillin for three consecutive days (80,000 units/mouse).<sup>27</sup> The mice in the OVX + E2 group were subcutaneously injected in the neck with E2 (E808987-1g, Macklin, Shanghai, China, 2.4  $\mu\text{g}/100 \mu\text{l}/4$  days) dissolved in peanut oil, while those in the OVX + Bnd group received intraperitoneal Bnd (200  $\mu\text{g}/\text{g}/\text{day}$ )<sup>28</sup> in 0.5% methyl cellulose (vehicle) (M29249-100G, Meryer, Shanghai, China) 1 week after surgery. The other groups received vehicle intraperitoneally. Mice were

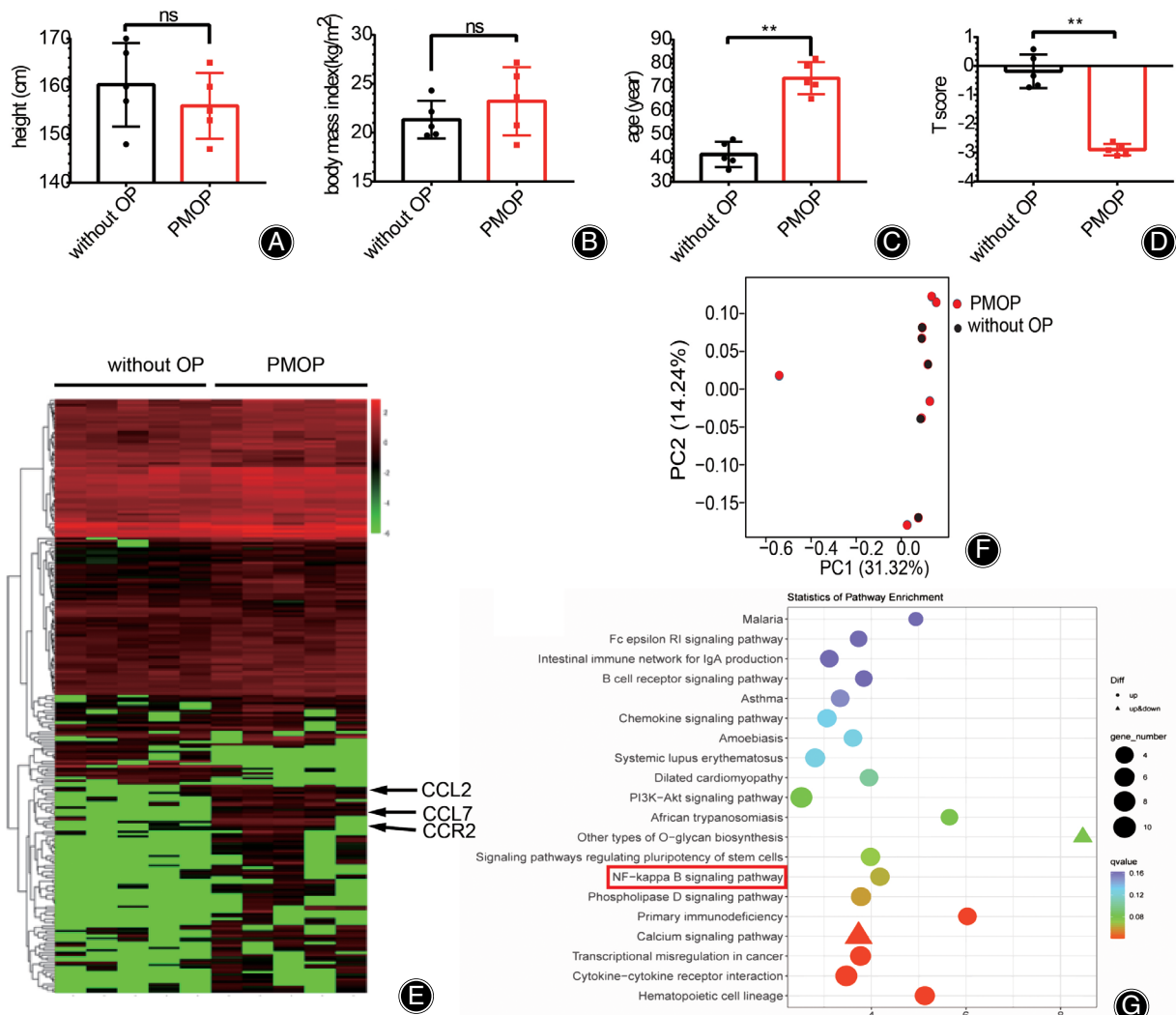
harvested 0, 15, 30, 45, and 75 days after surgery. Sixteen-month-old aged mice (C57BL/6J) were also harvested.

#### Mouse BMMs

BMMs were obtained from the removed humerus, femur and tibia bones of C57BL/6J mice. All mice were female wild-type C57BL/6J mice in this experiment (adult: 10 weeks old; aged: 16 months old). BMMs were separated in a gradient of monocyte isolation solution (P3770-2  $\times$  200 mL/KIT, Beijing, Solarbio, China) and then purified *via* immunomagnetic selection (EasySep™ Mouse Monocyte Isolation Kit, Stemcell Technologies, Vancouver, Canada).

#### RNA-Seq

Total RNA was analyzed using a NanoDrop instrument and an Agilent 2100 bioanalyzer (Thermo Fisher, Waltham, MA, USA). mRNA was enriched, and cDNA was synthesized. A cDNA library was obtained by PCR enrichment. The library was tested using a Qubit 2.0 for preliminary quantification and using qPCR to determine the effective concentration ( $>2$  nM). RNA-seq was performed for the group of women without osteoporosis ( $n = 5$ ) and the group of women with PMOP ( $n = 5$ ), which contained five biological replicates, or for the control group ( $n = 1$ ) and the Bnd group ( $n = 1$ ) with an Illumina NovaSeq sequencing system (Biomarker Technologies Corporation, Beijing, China). Clean data were obtained by filtering the offline data, and mapped data were obtained *via* sequence comparison with the specified reference genome. Differential expression analysis was performed, and functional annotation and functional enrichment were performed for the differentially expressed genes (DEGs). The differential gene expression analysis was performed with the DESeq package in R software, and the DEGs were selected based on thresholds of a fold change  $\geq 2$  and an



**Fig. 2** The levels of CCL2, CCL7 and NF- $\kappa$ B pathway members were increased in BMMs from women with PMOP. (A–D) Differences in height (A) and BMI (B) were not significant between premenopausal adult women without osteoporosis ( $n = 5$ ) and postmenopausal women with PMOP ( $n = 5$ ) ( $t = 0.63$  and  $1.08$ ,  $P = 0.55$  and  $0.31$ ), but differences in age (C) and T score (D) were significant ( $t = 8.37$ ,  $9.88$ , both  $P < 0.001$ ). (E) Heatmap of RNA-seq data showing that the levels of CCL2, CCL7 and their receptor CCR2 were higher in women with PMOP than in premenopausal women without osteoporosis. (F) A principal component analysis (PCA) plot was generated by using the expression information of each sample. A shorter distance between samples indicates more similar expression. (G) Scatter plot of the KEGG pathway enrichment results for the DEGs showing that the NF- $\kappa$ B signaling pathway was markedly upregulated. Independent  $t$  test; ns, not significant; \* $P < 0.05$ , \*\* $P < 0.001$ .

adjusted  $p$ -value  $< 0.01$ . Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEGs was conducted by the hypergeometric distribution test.<sup>29</sup>

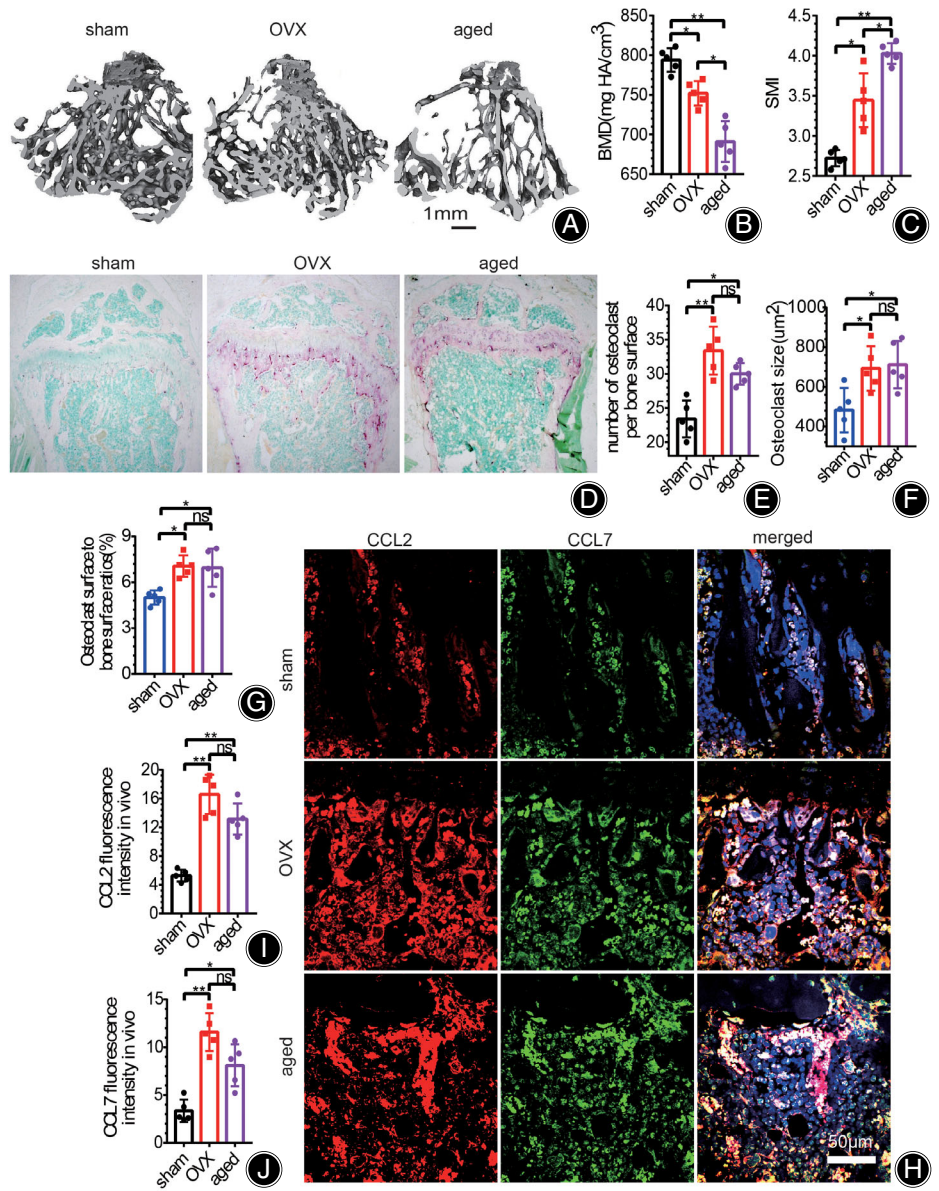
### Mouse Osteoclast Differentiation

BMMs from mice were cultured in  $\alpha$ -MEM Basic containing 10% fetal bovine serum (FBS) (10,099–141, Gibco, Big Cabin, OK, USA), macrophage colony-stimulating factor (M-CSF) (Z03275-10, Genscript, Nanjing, China, 50 ng/ml) and RANKL (CJ94-10, Novoprotein, Suzhou, China, 100 ng/mL)<sup>30</sup> with or without Bnd (130, 641-38-2, Nanjing Chemlin Chemical

Industry Co., Nanjing, China, 200  $\mu$ M). The BMMs were induced to differentiate into osteoclast-like cells in culture medium for 3, 5 or 7 days or differentiated in hydroxyapatite-coated pore plates (3989, Corning, NY, USA) for 7 days.

### Mouse Osteoblast Culture

Osteoblasts were isolated and purified from the cranial bones of 24- to 48-hour-old mice. The osteoblasts were cultured in osteogenic medium with 10% FBS and Bnd (200  $\mu$ M) when the cell density reached 70%. The cells were harvested on day 3, day 7 or day 21.



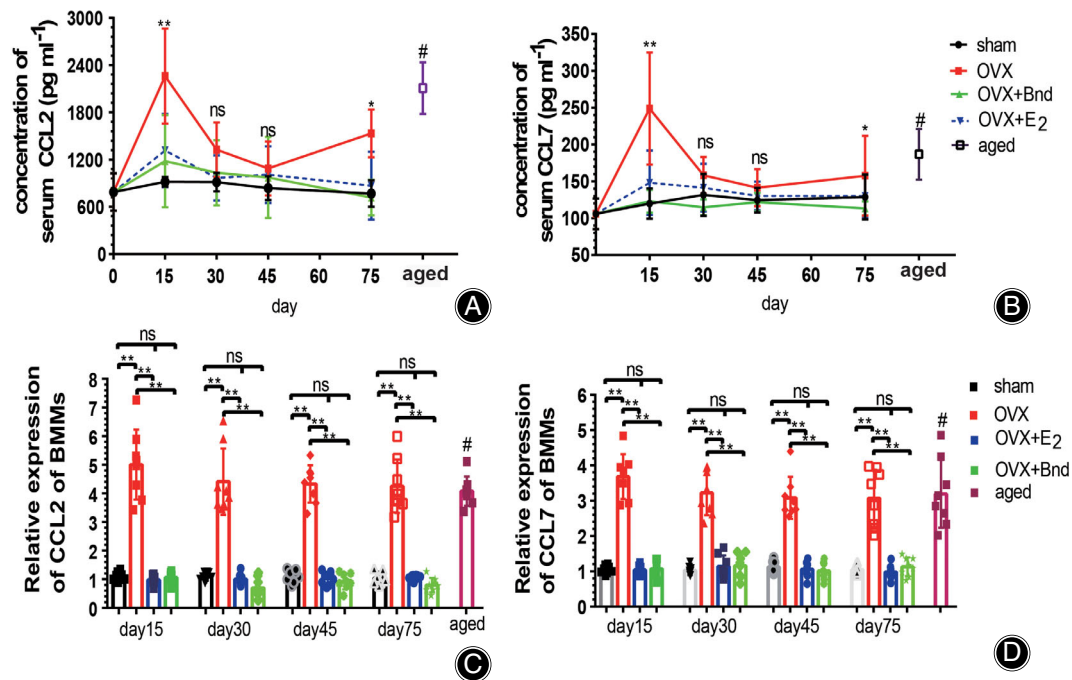
#### Cell Counting kit-8 (CCK-8) Assay

Mouse BMMs or osteoblasts were seeded into 96-well plates at a density of 5000 cells and treated with Bnd (0, 100, 200, 500, or 1000  $\mu$ M) 24 hours later. After 24 hours of incubation, 10  $\mu$ L of CCK-8 solution (C6005, NCM Biotech Co., Ltd., China) was added, and the cells were incubated at 37°C for 2 h. The absorbance was measured at 450 nm.

#### qPCR

Total RNA from fresh mouse BMMs was extracted *via* RNA microextraction (RaPure Total RNA Micro Kit, Magentec, Dongpu, China). cDNA was synthesized from 150  $\mu$ g of total RNA using HiScript II Q TR SuperMix for qPCR (+gDNA

Wiper) (R223-01, Vazyme, Nanjing, China). qPCR was then performed using ChamQ™ SYBR qPCR Master Mix (Q321-01 Vazyme, Nanjing, China) following the manufacturer's instructions. The guanine phosphoribosyl transferase (*Hprt*) gene for mice was used as an endogenous control.<sup>31</sup> All genes were compared using the comparative threshold cycle (Ct) method for relative quantification. Sangon Biotech (Shanghai Sangon Biotech, China) designed and synthesized the primers for CCL2 (sense 5'-TTTTTGTCCACCAAGCTCAAGAG-3', antisense 5'-TTCTGATCTCATTGGTTCCGA-3'), CCL7 (sense 5'-ACAAAAGATCCCCAAGAGGAAT-3', antisense 5'-TCTTGAAGATAACAGCTTCCCA-3'), and *Hprt* (sense 5'-AGGCCA-GACTTTGTTGGAT-3', antisense 5'-TGCGTCATCTTAGCCTT-3').



**Fig. 4** CCL2 and CCL7 levels were increased in OVX and aged female mice. (A, B) The serum concentrations of CCL2 and CCL7 were increased in OVX mice on days 15 and 75, but the increases were attenuated by E2 and Bnd (all  $P < 0.005$ ). However, the differences were not significant on days 30 and 45 among the sham, OVX, E2 and Bnd groups (all  $P \geq 0.13$ ). The serum concentrations of CCL2 and CCL7 in aged mice were higher than those in the other groups (all  $P < 0.05$ ) except for the OVX group on days 15 and 75 (both  $P \geq 0.09$ ). (C, D) The mRNA expression of CCL2 and CCL7 in BMMs was increased in OVX mice at all time points and in aged mice compared to sham mice (all  $P < 0.01$ ); however, E2 and Bnd attenuated these increases significantly. The mRNA expression in the aged group was not different from that in the OVX group but was higher than that in the sham, E2 and Bnd groups. One-way ANOVA and Tukey's test; ns, not significant; \* $P < 0.01$ , \*\* $P < 0.001$ .

### Mouse Specimen Processing

After intraperitoneal treatment, all mice were euthanized. Blood was collected, and serum was preserved after centrifugation. The humerus, femur and tibia bones were exposed and dissected free of soft tissue. The tibiae were routinely fixed using 4% paraformaldehyde at 4°C for 24 h and then decalcified in 15% EDTA (pH 7.4) for 14 days. The tissues were embedded in paraffin or optimal cutting temperature compound, and 5 or 10  $\mu\text{m}$  sagittal oriented sections were prepared for histological analyses.

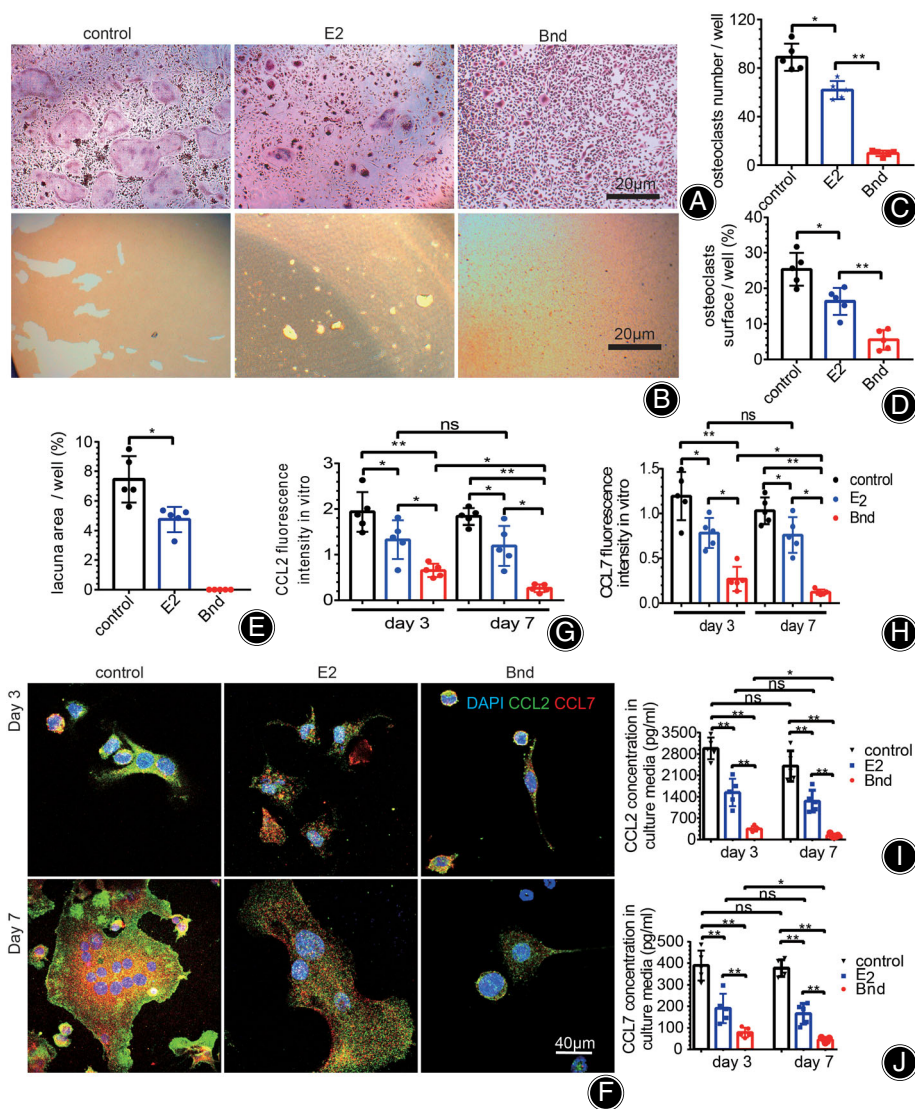
### $\mu\text{-Ct}$

Quantitative histomorphometric analysis of the proximal tibiae was performed using microcomputed tomography ( $\mu\text{-CT}$ ) ( $\mu\text{42}$ , Scanco Medical, Zurich, Switzerland) before decalcification. The scanning parameters were 55 kVp and 145  $\mu\text{A}$  using 10  $\mu\text{m}$  resolution. Only trabecular bone was selected in each selected slice, and the bone cortex was removed by an artificial method. Trabecular bone parameters, including the trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N), bone volume over total volume (BV/TV), structure model index (SMI) and bone mineral density (BMD) were analyzed using

Scanco software (built-in software) from the fully merged growth plates of the proximal tibia and extending for 100 slices distally, with a threshold of 300, according to the specifications of the American Society for Bone and Mineral Research (ASBMR) histomorphometry nomenclature committee.<sup>32</sup> In addition, 3D models were analyzed.

### Histological Staining

Cell plates or histological sections were stained with a tartrate-resistant acid phosphatase (TRAP) staining solution (G1492-4  $\times$  20 mL, Solarbio, Beijing, China) for 40 min (cell plate) or 20 min (slides) followed by hematoxylin or methyl green following the manufacturer's instructions. Positive multinucleated osteoclasts ( $\geq 3$  nuclei) were observed and counted.<sup>13,33</sup> Immunohistochemically stained slices from the proximal epiphyses and growth plates of the proximal tibiae were analyzed.<sup>13,22</sup> The osteoclast numbers and osteoclast surface areas were assessed using ImageJ (USA). Alkaline phosphatase (ALP) staining (C3206, Beyotime, Haimen, China) and staining of mineralized nodules with alizarin red (G1452, Solarbio, Beijing, China) were performed following the manufacturers' instructions. The proximal tibiae were analyzed using a 1500 $\times$ 1800- $\mu\text{m}$  region of interest (ROI)



150 μm from the growth plate in a grid-like pattern along the ROI.<sup>34</sup> Five fields of each slide and five slides were randomly selected for analysis.<sup>35</sup>

#### Immunofluorescence and Immunohistochemistry

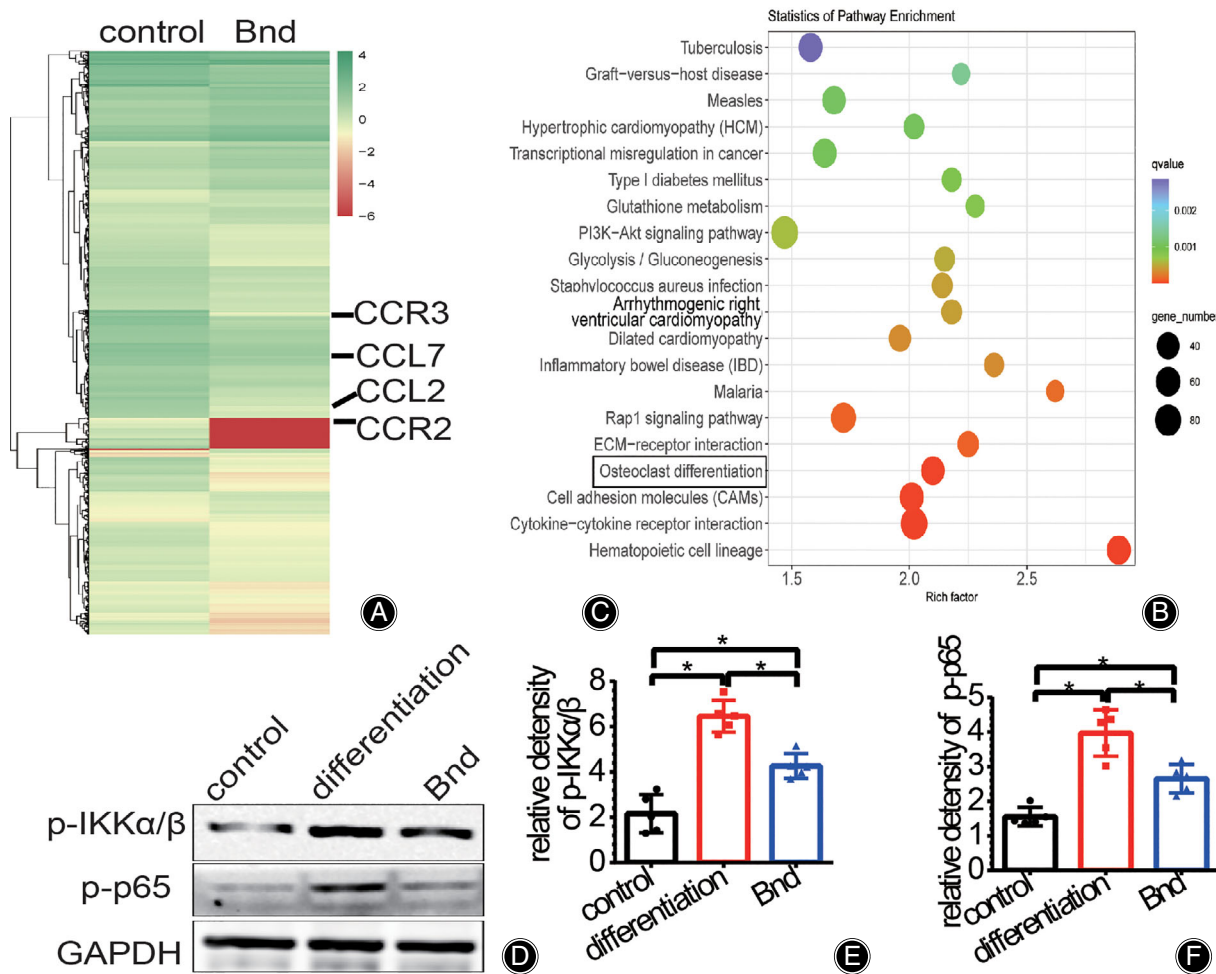
Confocal culture dishes (φ10 mm) or slides were incubated with anti-runt-related transcription factor 2 (RUNX2) (ab76956, Abcam, Cambridge, UK, 1:100) and anti-osteocalcin (DF12303, Affinity, Cincinnati, OH, USA, 1:100) at 4°C for ~12 h. The samples were incubated with secondary antibodies at 37°C for 40 min (1:200 or 1:400). Immunofluorescence images were obtained using confocal scanning microscopy (FV1200, Olympus, Tokyo, Japan) after sealing with DAPI, and the immunohistochemical slides were incubated with DAB for 1 to 4 min and stained with hematoxylin for 1 min. The immunostained areas were quantified using ImageJ (USA).

#### Western Blot Analysis

Total proteins were separated using SDS-PAGE to detect NF-κB signaling pathway proteins. The proteins were transferred onto nitrocellulose membranes, blocked with 5% non-fat milk for 2 h, incubated with primary antibodies (1:1000; Cell Signaling Technology, Danvers, MA, USA) overnight, and incubated with secondary antibodies for 2 h. Immunoreactivity was developed with enhanced chemiluminescence reagent (Super ECL Detection Reagent, Yeasen, China) and visualized by autoradiography. The western blot data were analyzed using GENE Sys V1.5.2.0.

#### Elisa

The levels of CCL2 and CCL7 in serum or culture medium were measured with ELISA kits (YXL21858-96T, YXL22324-96T, Yuannuo, China) according to the manufacturer's instructions. The absorbance was measured at



**Fig. 6** Bnd inhibited osteoclasts via the NF $\kappa$ B signaling pathway. (A) Heatmap of RNA-seq data showing that CCL2, CCL7, CCR2 and CCR3 levels were decreased in the Bnd group. (B) Scatter plot of KEGG pathway enrichment results for the DEGs showing that osteoclast differentiation was increased markedly in the Bnd group. (C–E) p-IKK $\alpha$ / $\beta$  and p-p65 protein expression was significantly increased in osteoclast precursors 7 days after induction (IN) ( $P = 0.008$ ), but Bnd (200  $\mu$ M) attenuated this increase ( $n = 5$  per group) ( $P = 0.037$ ). Equal loading was confirmed by assessment of GAPDH. Bnd inhibited osteoclastogenesis via the NF $\kappa$ B signaling pathway by inhibiting the synthesis of CCL2 and CCL7. All differences were tested using one-way ANOVA and Tukey's test; ns, not significant; \* $P < 0.01$ , \*\* $P < 0.001$ .

450 nm with a microplate reader (Synergy™ HTX multimode reader, Gene, China). The optical density value was calculated based on the standard curve. The concentration was then multiplied by the dilution factor.

#### Outcome Measures

**CCL2 and CCL7:** CCL2/MCP-1 and CCL7/MCP-3 are potent chemokines for monocytes and other immune cells and are primarily secreted by monocytes, fibroblasts and other cell types. CCL2 and CCL7 were detected using ELISA and immunofluorescence, and their mRNA levels were detected using qPCR.

**NF- $\kappa$ B signaling pathway:** The NF- $\kappa$ B signaling pathway is a critical regulator of immunity, differentiation, stress responses and apoptosis and directly and dramatically

enhances osteoclastogenesis. Members of this pathway were detected using western blot analysis.

**Osteoclasts:** Osteoclasts are giant cells that differentiate from monocytes and resorb bone. Osteoclasts were detected using TRAP staining.

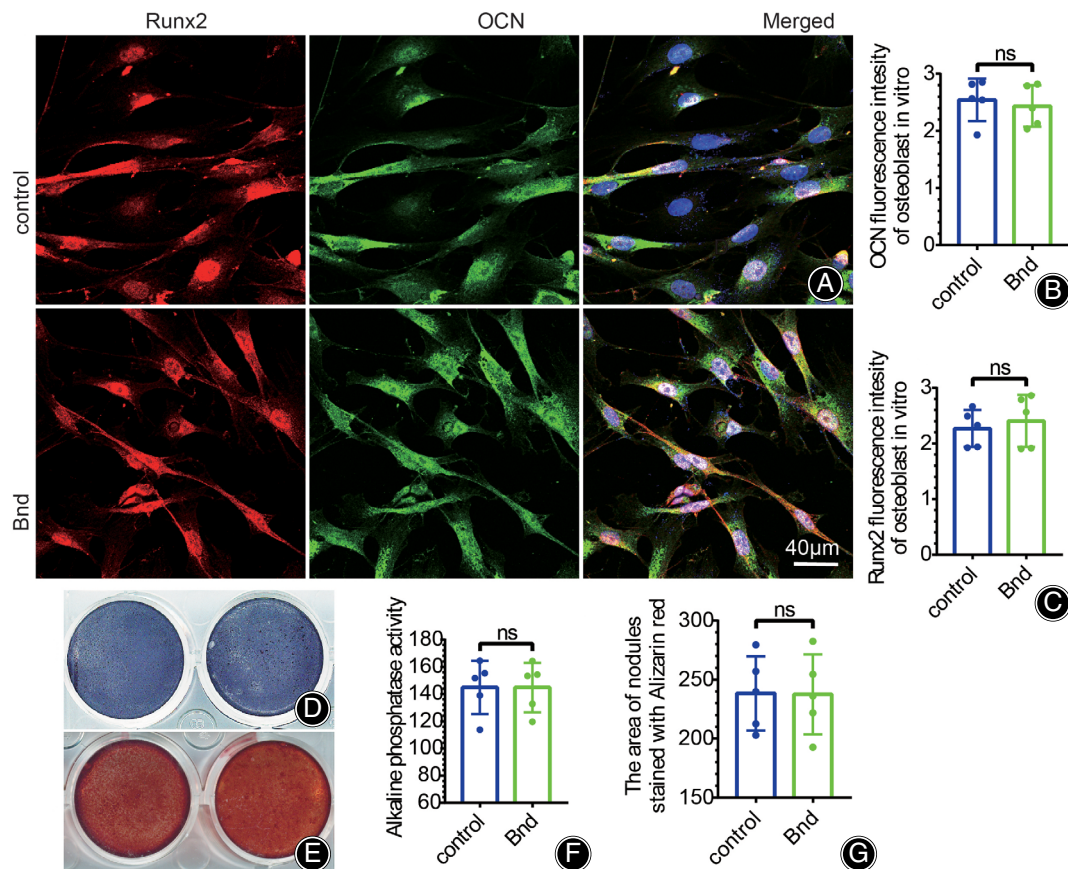
**Runx2 and osteocalcin (OCN):** Runx2 and OCN are the main markers of osteogenesis and were detected using immunostaining.

**Proximal tibiae:** Quantitative histomorphometry was conducted using  $\mu$ -CT. The assessed variables included Tb.Th, Tb.Sp, Tb.N, BMD and others.

#### Statistical Analysis

Statistical analyses were performed using SPSS (version 17.0, Chicago, IL, USA), and a  $P$  value of 0.05 was considered to





**Fig. 7** Bnd did not inhibit osteogenesis. (A–C) Runx2 (B) and OCN (C) levels were not different between the control and Bnd groups (200 μM) (B) on day 7 ( $n = 5$  per group). (D–G) ALP activity on day 9 (D, F) and mineralized nodule staining by alizarin red on day 21 (E, G) were not significantly different between the control and Bnd groups ( $n = 5$  per group). Independence  $t$  test; ns, not significant.

indicate significance. Independent-sample  $t$  tests were used for pairwise comparisons between the two groups for variables with homogenous variance. One-way ANOVA was used to compare the measurement data between groups, and Tukey's test was used for multiple comparisons. A minimum of three independent experiments were performed in triplicate for each assay.

## Results

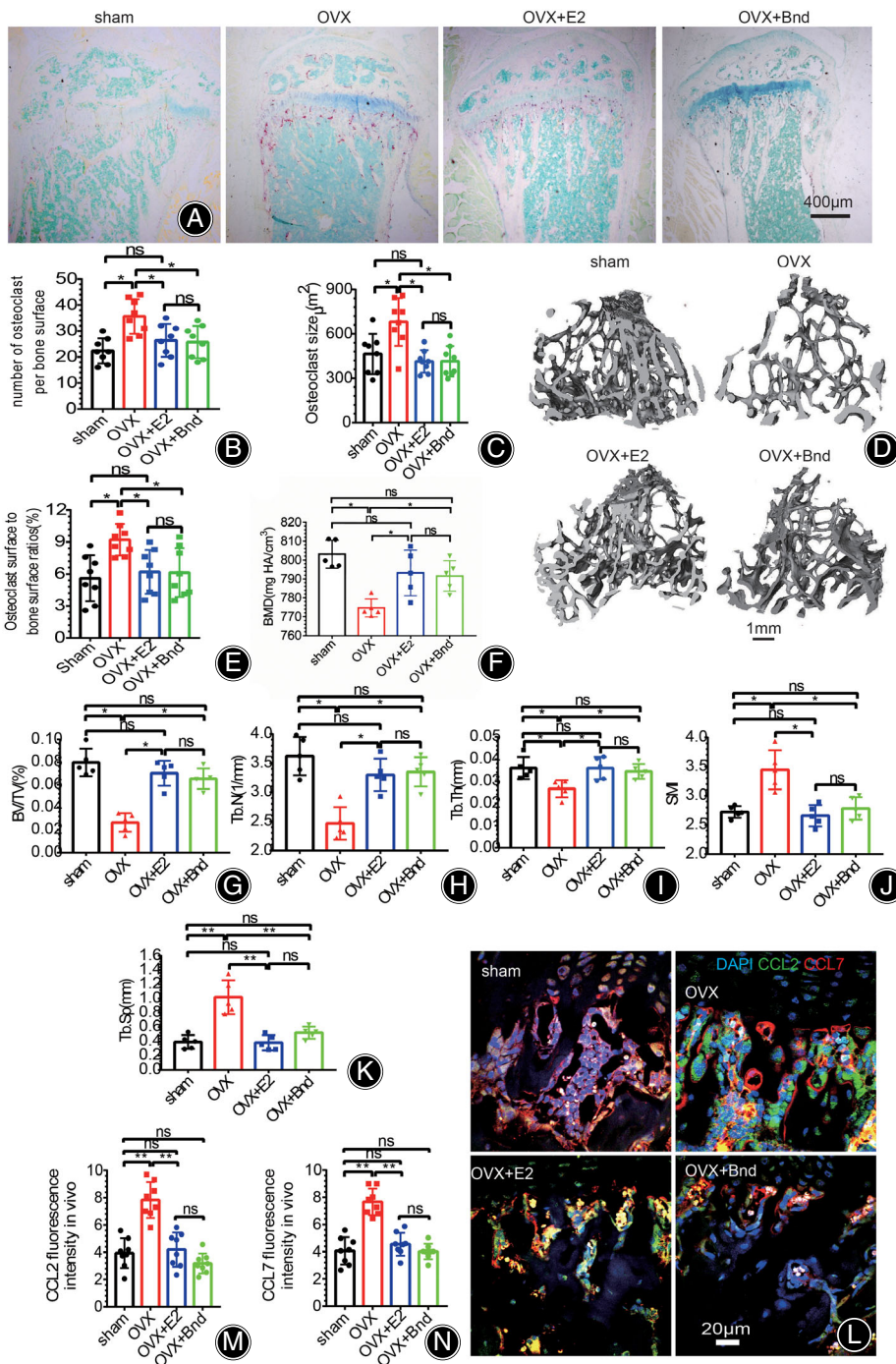
### *The Levels of CCL2, CCL7 and NF-κB Pathway Members Were Increased in BMMs from Women with PMOP*

We performed RNA-seq of BMMs from the femurs of adult premenopausal women without osteoporosis or women with PMOP to identify DEGs. CCL2 and CCL7 levels were higher in women with PMOP than in adult premenopausal women without osteoporosis, and the levels of CCR2 were significantly increased (Fig. 2E, F). The scatter plot of the KEGG pathway enrichment of the DEGs shows that the NF-κB signaling pathway was markedly upregulated (Fig. 1G). Other

signaling pathways, such as malaria and chemokine signaling pathways, were also upregulated, but signaling pathways such as the O-glycan biosynthesis and calcium signaling pathways were downregulated (Fig. 2G). RNA-seq showed that CCL2, CCL7 and CCR2 were highly expressed and that the NF-κB signaling pathway was markedly upregulated in women with PMOP.

### *CCL2 and CCL7 Levels Were Increased in OVX Mice and Aged Female Mice*

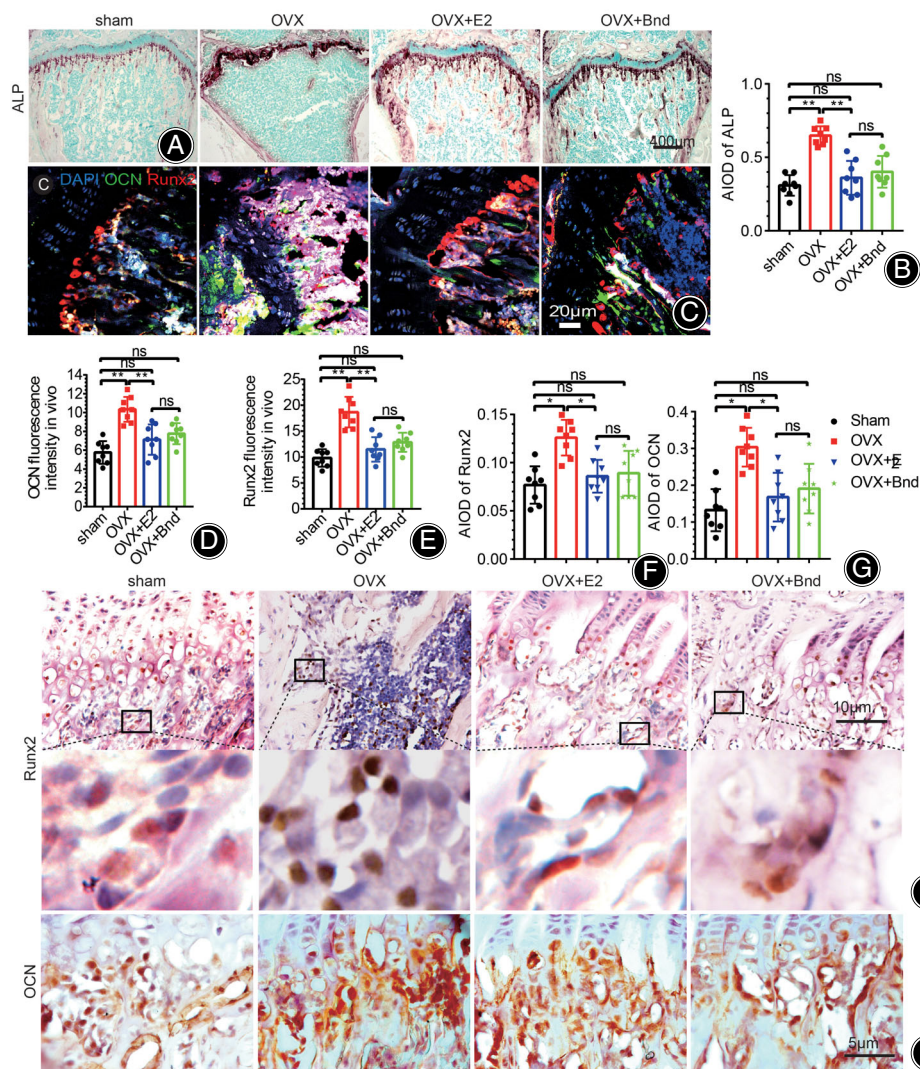
We established an OVX mouse model and verified the expression of CCL2 and CCL7. OVX mice (75 days after surgery) and aged female mice were confirmed to be osteoporotic using  $\mu$ CT (Fig. 3A), with lower BMD values (Fig. 3B), higher SMI values (Fig. 3C), more TRAP staining (Fig. 2D), more (Fig. 3E) and larger (Fig. 3F) osteoclasts, and greater osteoclast surface areas (Fig. 3G) than sham mice. The levels of CCL2 and CCL7, which were detected using immunofluorescence, were increased significantly in the proximal tibiae of OVX mice on day 75 and in aged female mice (Fig. 3H–J) compared to sham mice. We hypothesized that the serum



**Fig. 8** Bnd reduced bone loss in OVX mice by inhibiting CCL2 and CCL7 expression. (A) Representative TRAP staining of the proximal tibia ( $n = 8$  per group). (B–C, E) E2 and Bnd significantly inhibited the increases in the number and size of osteoclasts and the osteoclast surface area-to-bone surface area ratio after ovariectomy in mice (all  $P < 0.01$ ). (D) Representative 3D  $\mu$ -CT of the proximal tibia ( $n = 5$  per group). (F–K) The BMD (F), BV/TV (G), Tb.N (H), and Tb.Th (I) values decreased significantly after ovariectomy, and the SMI (J) and Tb.Sp (K) values were obviously increased in the OVX group (all  $P \leq 0.01$ ); however, E2 and Bnd significantly improved these indicators to reduce bone loss. (L–N) CCL2 and CCL7 expression in the growth plate (L) was increased significantly after ovariectomy and was inhibited by E2 and Bnd (all  $P < 0.01$ ). All differences were tested using one-way ANOVA and Tukey's test; ns, not significant; \* $P < 0.01$ , \*\* $P < 0.001$ .

concentrations of CCL2 and CCL7 would be increased in OVX and aged mice. The measured concentrations of CCL2 and CCL7 were significantly higher in OVX mice on days 15 and 75 and in aged mice compared to sham mice (Fig. 4A, B), but the differences in CCL2 and CCL7 levels between sham and OVX mice on days 30 and 45 were not significant (Fig. 4A, B). Many studies have reported contradictory effects of estrogen on CCL2.<sup>14,22</sup> We hypothesized

that the serum concentration would not reflect the true expression of CCL2 and CCL7 in BMMs of OVX mice. Therefore, we detected the mRNA expression of CCL2 and CCL7. The mRNA levels of CCL2 (Fig. 4C) and CCL7 (Fig. 4D) were increased in the OVX mice on days 15, 30, 45 and 75 and in aged mice compared to sham mice. The mRNA levels of CCL2 (Fig. 4C) and CCL7 (Fig. 4D) in aged mice were higher than those in sham mice but were not



**Fig. 9** Bnd reduced bone turnover in OVX mice.

(A-B) The expression of ALP, which was significantly higher after OVX on day 75 than in the sham group, was lower in the OVX + E2 and OVX + Bnd groups than in the OVX group. (C-E) Runx2 and OCN levels were detected using immunofluorescence and were found to be increased in the OVX group and decreased in the E2 and Bnd groups. (F-I) Runx2 and OCN levels were detected using immunohistochemistry and were found to be increased in the OVX group. However, the increases were attenuated by E2 and Bnd. The results indicated that E2 and Bnd significantly reduced bone turnover in OVX mice. All differences were tested using one-way ANOVA and Tukey's test; ns, not significant; \* $P < 0.01$ . \*\* $P < 0.001$ .

different from those in OVX mice (all  $p > 0.05$ ). The expression of CCL2 and CCL7 was increased in OVX mice and aged female mice.

#### ***E<sub>2</sub>* and Bnd Inhibited Osteoclastogenesis by Decreasing CCL2 and CCL7 Levels**

We examined the effects of E<sub>2</sub> (100 nM) and Bnd (200 µM) on osteoclastogenesis *in vitro*. E<sub>2</sub> significantly inhibited osteoclastogenesis (Fig. 5A-D), reducing the number of small osteoclasts, while Bnd significantly inhibited osteoclastogenesis, resulting in the presence of only a few giant cells and small osteoclasts ( $n = 5$ ) (Fig. 5A, C, D) compared to the situation in the control group. Although the resorption lacunae were large in the control group, there were smaller resorption lacunae in the E<sub>2</sub> group and no resorption lacunae in the Bnd group (Fig. 5B, E). CCL2 and CCL7 were expressed in the cytoplasm or in the media of BMMs or osteoclasts after induction on days 3 and 7, but

their expression was significantly inhibited by E<sub>2</sub> and Bnd (Fig. 5F-J). CCL2 and CCL7 levels were further reduced by Bnd on day 7 compared to day 3 in both the cytoplasm and media (Fig. 5G-H). E<sub>2</sub> and Bnd reduced osteoclastogenesis by significantly inhibiting CCL2 and CCL7 expression. Cell viability in BMMs (Fig. S1A) and osteoblasts (Fig. S1B) was not different among the groups treated with the various concentrations of Bnd and the control group except at 500 µM.

#### ***Bnd Inhibited Osteoclast Differentiation via the NF-κB Signaling Pathway***

Mouse BMMs were differentiated into osteoclasts with or without Bnd for 7 days. RNA-seq verified decreases in CCL2, CCL7 and their receptors CCR2 and CCR3 (Fig. 6A). The scatter plot of KEGG pathway enrichment shows the CompBio analysis results for the 20 signaling pathways with the largest variations in mRNA levels related to the presence or absence of Bnd (Fig. 6B). KEGG pathway enrichment of

Bnd-related DEGs showed that osteoclast differentiation was decreased (Fig. 6B). The NF- $\kappa$ B signaling pathway is a key pathway in osteoclast differentiation, so its role in CCR2-related osteoclastogenesis was examined.<sup>13</sup> NF $\kappa$ B signaling pathway members were detected and analyzed *via* western blotting of differentiated mouse BMMs *in vitro* (Fig. 6C, D, E). p-IKK $\alpha$ / $\beta$  and p-p65 protein expression in osteoclasts or osteoclast precursors increased 7 days after differentiation, but Bnd significantly attenuated this increase (Fig. 6C, D, E). Bnd significantly inhibited osteoclastogenesis *via* the NF $\kappa$ B signaling pathway, the classic signaling pathway of osteoclastogenesis.

#### **Bnd Did Not Inhibit Osteogenesis Directly *in vitro***

Because Bnd inhibited osteoclastogenesis, we examined whether Bnd affected osteoblast mineralization directly. Osteoblasts were treated with or without Bnd in an osteogenic differentiation cocktail. The expression of Runx2 and OCN was analyzed using immunofluorescence staining and was not significantly different between the control group and the Bnd group on day 7 *in vitro* (Fig. 7A–C). ALP activity (Fig. 7D, F) on day 9 and mineralized nodule staining with alizarin red on day 21 (Fig. 7E, G) were not significantly different between the control group and the Bnd during osteogenic differentiation induction *in vitro*. These results showed that Bnd did not inhibit osteoblast mineralization directly *in vitro*.

#### **Bnd Reduced Bone Loss in OVX Mice Via Inhibition of CCL2 and CCL7**

We verified the effects of Bnd in mice. OVX mice were treated with Bnd until 75 days after surgery. The osteoclasts were detected using TRAP (Fig. 8A). Bnd obviously reduced the number (Figure, 8B) and size (Fig. 8C) of osteoclasts and the osteoblast surface area-to-bone surface area ratio (Fig. 8E). Bone phenotypes were examined using  $\mu$ -CT (Fig. 8D). OVX mice exhibited the lowest BMD (Fig. 8F), BV/TV (Fig. 8G), Tb.N (Fig. 8H), and Tb.Th (Fig. 8I) values and the highest SMI (Fig. 8J) and Tb.Sp (Fig. 8K) values. Treatment with E<sub>2</sub> and Bnd significantly ameliorated the ovariectomy-related changes (Fig. 8D–K). The serum concentrations of CCL2 and CCL7 were lower in the E<sub>2</sub>- and Bnd-treated mice on days 15 and 75 than in the OVX and aged mice (Fig. 4A, B), and the mRNA levels of CCL2 and CCL7 were lower in the E<sub>2</sub>- and Bnd-treated mice at all time points than in the OVX and aged mice (Fig. 4C, D). The expression of CCL2 and CCL7 was analyzed using immunofluorescence (Fig. 8L–N). CCL2 (Fig. 8M) and CCL7 (Fig. 8N) levels increased significantly after ovariectomy, but these increases were inhibited by E<sub>2</sub> and Bnd (Fig. 8L–N) on day 75. Thus, Bnd and E<sub>2</sub> reduced bone loss in OVX mice *via* inhibition of CCL2 and CCL7.

#### **Bnd Reduced Bone Turnover in OVX Mice**

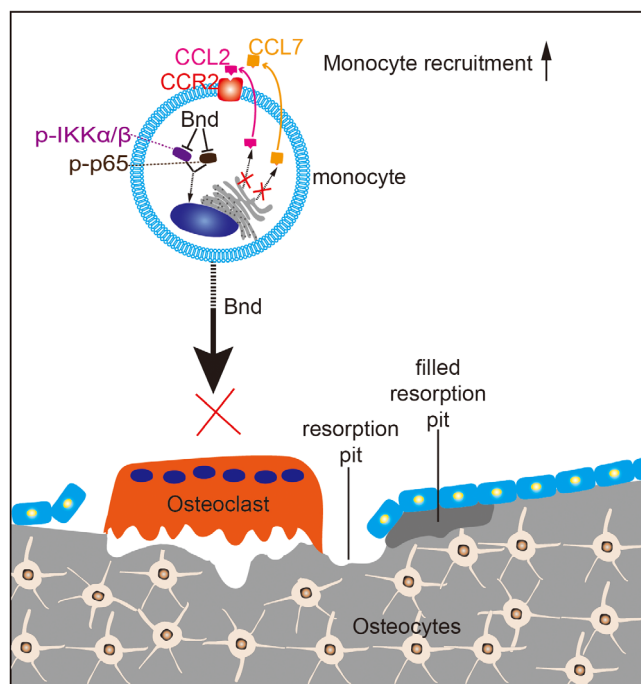
Some indicators of osteogenesis were detected to examine bone turnover. The expression of ALP was significantly

higher after ovariectomy on day 75 than in the sham group and lower in the OVX + Bnd group than in the OVX group (Fig. 9A, B). Runx2 and OCN were detected using immunofluorescence and immunohistochemistry and were increased in the OVX group and decreased in the E<sub>2</sub> and Bnd groups (Fig. 9C–I). These results indicated that Bnd significantly reduced bone turnover in OVX mice.

## **Discussion**

### **CCL2, CCL7 and CCR2 Levels were Increased in PMOP, and Inhibition of these Molecules Exerted anti-PMOP Effects**

We found, for the first time, that the levels of CCL2, CCL7 and CCR2 were increased in BMMs from women with PMOP and OVX and aged female mice. The expression of CCL2 was much higher than that of CCL7. We further demonstrated that E<sub>2</sub> obviously attenuated the increases in CCL2 and CCL7 *in vitro* and *in vivo*, which is consistent with the findings of some studies<sup>16</sup> but contrary to the findings of other studies on cancer.<sup>18</sup> The anti-PMOP effects of an inhibitor of CCL2 and CCL7 synthesis, Bnd, were examined in OVX mice. Bnd inhibited osteoclastogenesis *in vitro* and significantly reduced bone loss in OVX mice. Bnd obviously inhibited osteoclastogenesis without directly affecting



**Fig. 10** Action mechanism of Bnd.

Bnd inhibits the synthesis of CCL2 and CCL7 *via* the NF $\kappa$ B signaling pathway and therefore prevents monocytes from differentiating into osteoclasts. Bnd may be useful as a new therapeutic for the prevention of PMOP.

osteogenic mineralization *in vitro*. We confirmed that the expression of CCL2 and CCL7 was significantly reduced during the process of BMM differentiation into osteoclast progenitors (OCPs) after Bnd treatment. Bnd may inhibit osteoclastogenesis *via* the NF $\kappa$ B signaling pathway to exert its anti-PMOP effect (Figs 1 and 10).

### ***E<sub>2</sub> Reduced CCL2 and CCL7 Expression in BMMs and Osteoclastogenesis***

PMOP, which severely impacts elderly women, is accompanied by inflammation and inflammatory diseases. Bone loss resulting from estrogen withdrawal becomes increasingly serious. Estrogen has many complex functions *in vivo*, such as anti-inflammatory functions. Estrogen withdrawal causes many changes in postmenopausal women, such as upregulation of the inflammatory chemokine CCL2.<sup>17,36</sup> Chemokines such as CCL2 and CCL7 participate in multiple inflammatory diseases. Estrogen deficiency increased CCL2 and CCL7 levels in the present study, especially in BMMs, which can differentiate into osteoclasts. E<sub>2</sub> attenuated the increases in CCL2 and CCL7 *in vivo* and *in vitro*, which might have been partially responsible for its anti-PMOP effects (Figs 1 and 10).

### ***Bnd Inhibits Many Inflammatory Diseases and May Be Beneficial in PMOP***

CCL2 and its receptor determine the behavior of osteoclasts.<sup>13</sup> CCL7 is considered to be associated with bone-resorptive osteoclasts.<sup>2</sup> CCL2 and CCL7 share the same receptor, and CCR2, CCL2 and CCL7 expression levels are consistent in many cases.<sup>37,38</sup> Many inflammatory diseases ranging from atherosclerosis to diabetes-associated periodontitis also severely affect the health of elderly women. Bnd is a small anti-inflammatory molecule that inhibits the synthesis of inflammatory chemokines such as CCL2, CCL7 and CCL8.<sup>22,24</sup> Many animal experiments have shown that Bnd has a good anti-inflammatory effect. In addition, Phase II clinical trials have shown that Bnd is well tolerated and has favorable safety.<sup>23</sup> We examined the anti-PMOP effects of Bnd in OVX mice and found that Bnd inhibited osteoclastogenesis (Figs 1 and 10) and reduced bone loss and bone turnover.

### ***Limitations***

There were some limitations of the present study. First, the in-depth mechanism by which estrogen modulates the secretion of CCL2 and CCL7 was not investigated. There has been no research on the coordination or consistency of CCL2 and CCL7. Further experimental studies are needed to confirm the conclusions of our study. Clinical trials should be performed to confirm whether Bnd or other inflammatory inhibitors play anti-osteoporosis roles *via* inhibition of inflammation.

### ***Conclusion***

The present study demonstrated that estrogen withdrawal promoted the expression of CCR2 and its ligands CCL2 and CCL7 and that inhibition of CCL2 and CCL7 synthesis *via* administration of Bnd alleviated PMOP in mice. Osteogenesis was not affected directly by Bnd. Therefore, it is important to investigate PMOP from the perspective of inflammatory factors and monocyte chemotaxis.

### ***Acknowledgment***

We thank Biomarker Technologies Corporation, Beijing, China for their assistance in sample sequencing.

### ***Approval of Institutional Review Board***

This study was approved by the Human Research Ethics Committee of the Third Affiliated Hospital of Southern Medical University (approval number: 2020-ethical review-016). Informed consent was obtained from the donor. All animal experiments were carried out with the approval of the Institutional Animal Care and Ethics Committee of Southern Medical University.

### ***Supporting Information***

Additional Supporting Information may be found in the online version of this article on the publisher's web-site:

**Fig. S1** Cell viability after the treatment of Bnd. Cell viability in BMMs (A) and osteoblasts (B) after various concentrations of Bnd. The results were analyzed with one-way ANOVA. \**P* < 0.05, \*\**P* < 0.001 *vs.* the control group; ns: not significant (*n* = 8).

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