NEMO-binding domain peptide ameliorates inflammatory bone destruction in a *Staphylococcus aureus*-induced chronic osteomyelitis model

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Abstract. Osteomyelitis, which is characterized by progressive inflammatory bone destruction and resorption, is a difficult-to-treat infection. *Staphylococcus aureus* (*S. aureus*) is one of the major causes of this disease. This pathogenic microorganism possesses several characteristics, which facilitate its involvement in the occurrence and progression of osteomyelitis. A cell-permeable peptide inhibitor of the IκB kinase complex, the nuclear factor (NF)-κB essential modulator-binding domain (NBD) peptide, has been reported to block osteoclastogenesis and may be considered a potential strategy for preventing inflammatory bone resorption. However, it remains to be determined as to whether the NBD peptide can regulate inflammation and bone resorption in *S. aureus*-induced osteomyelitis. In order to investigate the role of NBD in *S. aureus*-induced osteomyelitis, the present study obtained the NBD peptide, and confirmed that it inhibited receptor activator of NF-κB ligand-induced osteoclastogenesis *in vitro*. Subsequently, a bone defect was generated and *S. aureus* was injected into the mandible of experimental animals, in order to establish an *in vivo* osteomyelitis model. The present study analyzed the following three experimental groups: Untreated, treated with debridement, and treated with debridement plus NBD peptide administration. The results revealed that treatment with the NBD peptide reduced the bone defect in a 3-dimensional manner, and reduced bone resorption. To the best of our knowledge, the present study is the first to demonstrate that, in a model of osteomyelitis caused by *S. aureus*, the NBD peptide serves a role in inhibiting osteolysis and promoting bone remodeling in the direction of osteogenesis. The effects were better than those produced by debridement alone, thus suggesting that it may have promising therapeutic potential in osteomyelitis.

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Introduction

Osteomyelitis is a bone infection, which is mainly caused by microorganisms and accompanied by bone destruction. Bacteria bind to the bone and induce acute inflammation, after which, immune cells release cytokines and chemokines that regulate bone metabolism (1). *Staphylococcus aureus* (*S. aureus*) is the most common bacterial species involved. This microorganism has several characteristics that facilitate its role as a common pathogen in human osteomyelitis (2), and there are a series of extracellular and cell-associated factors contributing to its virulence (3). It has been reported that infection of cultured osteoblasts with *S. aureus* prevents proliferation, induces apoptosis and inhibits mineralization. In addition, *S. aureus* increases receptor activator of nuclear factor (NF)-κB ligand (RANKL) expression and decreases osteoprotegerin expression in osteoblasts; these effects are likely to promote osteoclast formation and function (4,5). Osteoclasts are generated from myeloid progenitors produced in the bone marrow. They are released into the vasculature and serve critical roles in the processes of bone resorption and destruction, which occur in osteomyelitis (6-9). Osteomyelitis requires multimodal, appropriate therapy. The goal is to clear the infection thorough debridement and appropriate antibiotic treatment. Recently, gene-targeted therapy has become increasingly popular, encouraging investigation of the mechanisms underlying this disease.

There are several important mechanisms involved in the process of bone remodeling during bone-associated disease. In particular, the NF-κB transcription factor has garnered

increasing attention (10-12). A recent study suggested that the NF-κB pathway may be considered a pharmacological target for the modulation of chronic inflammation-induced bone resorption (13). The NF-κB family consists of several members, including p50, p52, RelA/p65, RelB, c-Rel, NF-κB1/p105 and NF-κB2/p100; as well as the inhibitory subunits IκBα, IκBβ and IκBγ (14). NF-κB dimers are activated via regulation of the inhibitory proteins, of which IκBα is the most widely investigated (15). IκBα is phosphorylated at N-terminal serine residues by a large IκB kinase (IKK) complex. The predominant IKK complex is comprised of two catalytic subunits, IKK1 (also known as IKKα) and IKK2 (also known as IKKβ), as well as a regulatory subunit, IKKγ [also known as NF-κB essential modulator (NEMO)] (14). The N-terminal, α-helical region of NEMO associates with a hexapeptide sequence at the distal carboxyl terminus of IKK2 and IKK1, termed the NEMO-binding domain (NBD) (15). It has been reported that a short cell-permeable peptide containing the NBD sequence disrupts the association of NEMO with IKK2, blocks NF-κB activation, and ameliorates bone resorption and inflammation in several animal models (16). In addition, disturbing the binding of NEMO to IKK2 with NBD peptides inhibits osteoclastogenesis *in vitro* (16). NBD peptides can block NF-κB activation *in vivo*, reduce osteoclast recruitment and bone erosion, and ameliorate the pathological severity of inflammation (17). However, to the best of our knowledge, no previous studies have been conducted to investigate the therapeutic potential of NBD peptides in *S. aureus*-induced osteomyelitis.

Given the central role of the IKK complex in osteoclastogenesis and inflammatory osteolysis, it was hypothesized that the NBD peptide may prevent the absorption of bone by arresting osteoclastogenesis in an osteomyelitis model. To test this hypothesis, the NBD peptide was applied *in vitro* and *in vivo*, the formation and function of osteoclasts cultured on bone slices in the presence of the NBD peptide were studied, and bone mineral density and morphology in a rabbit mandible *S. aureus* osteomyelitis model were analyzed. This study was conducted in order to investigate the potential role of the NBD decoy peptide in an osteomyelitis model.

Materials and methods

NBD peptides. NBD peptides have been described previously (16,18). A cell-permeable, wild type NBD peptide (DRQIKIWFQNRRMKWKK-TALDWS-WLQTE) was provided by GL Biochem (Shanghai) Ltd. (Shanghai, China) as lyophilized powder. Immediately prior to use, 20 mM stock solutions of the NBD peptide were prepared in dimethyl sulfoxide (DMSO). Stocks were diluted in culture medium to the final concentration required.

Cell culture. RAW264.7 cells [American Type Culture Collection (ATCC) no. TIB-71; Cell Bank of Chinese Academy of Sciences, Shanghai, China] were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in an atmosphere containing 5% CO_2. Fresh media were added every 2 days. To study osteoclast formation, RAW264.7 cells were cultured in complete α-minimum Eagle's medium (α-MEM; Gibco; Thermo Fisher Scientific, Inc.) in the presence of NBD peptide at concentrations of 0 or 20 μM (18), and simultaneously stimulated with soluble recombinant mouse RANKL (100 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) (19). The media were replaced every 2 days.

Osteoclast formation. RAW264.7 cells grown as aforementioned were cultured in the presence of soluble RANKL (100 ng/ml). After 4 days of culture, cells were fixed with 4% formaldehyde at room temperature for 10 min and histostained for tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (cat. no. 387A; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), according to the manufacturer's protocol and as previously described (20,21). Cells were also immunostained with fluorescein isothiocyanate-labeled phalloidin (F-actin, 5 μg/ml; cat. no. P5282-1MG; Sigma-Aldrich; Merck KGaA) for 60 min, and with 4',6-diamidino-2-phenylindole (1 μg/ml; Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. Cells were analyzed using a Nikon microscope (Nikon Corporation, Tokyo, Japan) under x10 magnification, and images were captured and analyzed with NIS-Elements F2.20 (Nikon Eclipse 80i; Nikon Corporation). $TRAP^+$ multinucleated cells, containing >3 nuclei, were counted as osteoclasts. The average number of nuclei in osteoclasts was detected from the immunostaining images.

Pit formation assay. Bovine femoral cortical bone (22), purchased fresh from a butcher's shop, was cut into 0.1 cm slices, which were cleaned by ultrasonication in sterile distilled water at 40 kHz for 30 min at room temperature, autoclaved at 120˚C and 0.4 MPa, and immersed in DMEM. The medium was changed three times every 30 min, and the slices were stored in DMEM at 4˚C. Prior to each experiment, bone slices were preheated at 37˚C for 1 h in 48-well plates containing 1 ml α-MEM. Subsequently, RAW264.7 cells stimulated with RANKL were added, with or without NBD peptide. After 4 days, the cells on the slices were fixed with 4% formaldehyde at room temperature for 10 min and observed under scanning electron microscopy (SEM; SU8010FE-SEM; Hitachi, Ltd., Tokyo, Japan). Subsequently, cells were removed by ultrasonication at 40 kHz for 30 min at room temperature and resorption lacunae were observed under the electron microscope. The total area of bone lacunae on the slices was quantified using NIS Elements-AR software (Nikon Corporation).

Chronic osteomyelitis model. New Zealand white rabbits were provided by the Animal Experiment Center of Zhejiang University (Hangzhou, China). All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. Briefly, male rabbits (n=6/group; age, 4 months; weight, 2.5-3.0 kg) were selected for the osteomyelitis model (23). Rabbits were housed in stainless steel cages under controlled conditions (temperature, 23±2˚C; relative humidity, 55±10%; ventilation, >10 times/h; 12-h light/dark cycle). All animals had free access to food and water throughout the acclimation and experimentation periods, and were maintained according to the Animal Experiment Center of Zhejiang University. Animals were anesthetized with Sumianxin II (10 mg/kg, 0.2 ml/kg; Dunhua Shengda Animal Medicine Co.,

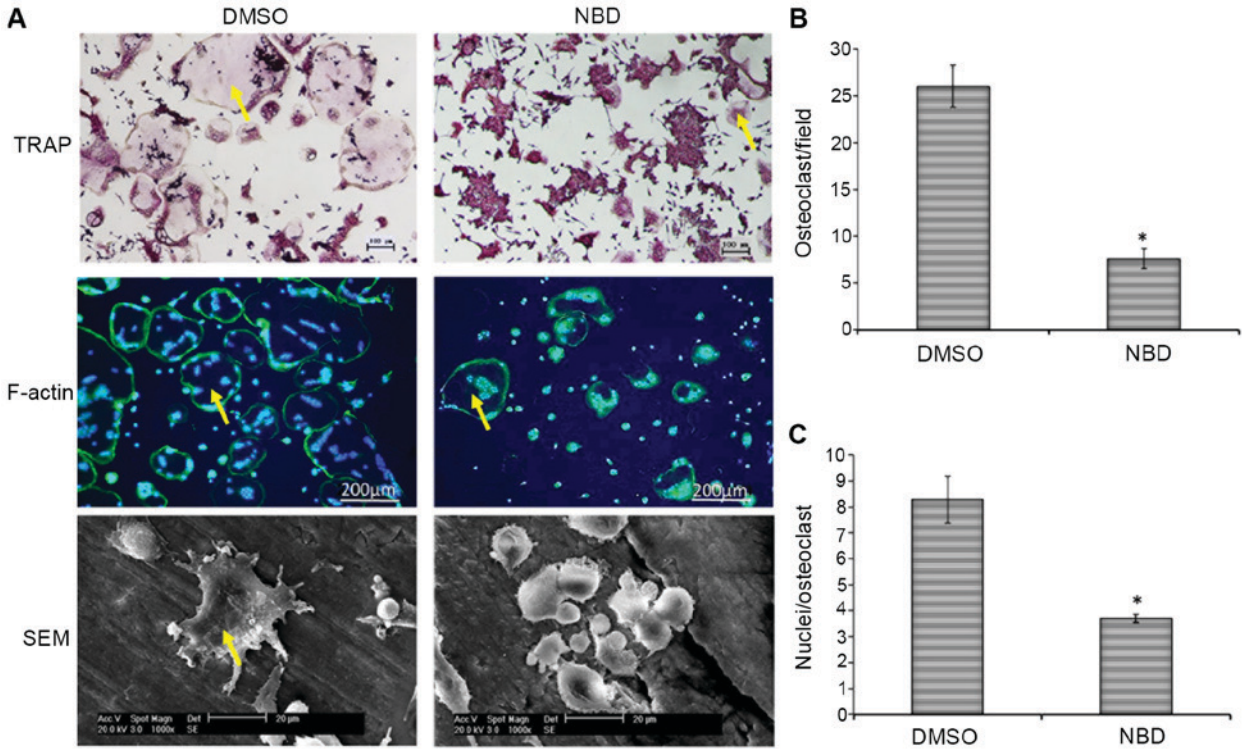

Figure 1. NBD peptide inhibits RANKL-induced osteoclast formation and function *in vitro*. (A) RAW 264.7 cells were incubated in the presence of RANKL (100 ng/ml) on plates or bone slices. DMSO or NBD peptide (20 μM) were added simultaneously with RANKL. After 4 days, cells were fixed and stained for TRAP and F-actin (green), and counterstained with DAPI (blue) (F-actin images, x20 magnification; TRAP images, x10 magnification). Yellow arrows indicate multinucleated osteoclasts with >3 nuclei. In addition, bone slices were washed with PBS, fixed and osteoclasts were visualized by scanning electron microscopy. (B) Number of TRAP$^+$ multinucleated osteoclasts (>3 nuclei/osteoclast). (C) Mean number of nuclei per osteoclast. Data are presented as the means ± standard error of the mean of three independent experiments. *P<0.05 vs. the DMSO group. DMSO, dimethyl sulfoxide; NBD, nuclear factor-κB essential modulator-binding domain; SEM, scanning electron microscopy; TRAP, tartrate-resistant acid phosphatase.

Ltd., Dunhua, China; also known as xylazine hydrochloride), and a bone defect (8x8x3 mm) was created at the lateral inferior border of the mandible beside the middle joint. Subsequently, 100 μl 5% sodium morrhuate containing 5.0x10^7 cfu/ml *S. aureus* (ATCC no. 25923; Department of Microbiology of Zhejiang University) was injected into the bone defect, which was then sealed with bone wax. After 6 weeks, rabbits were separated into the following three groups: i) Untreated controls, ii) treated with debridement surgery, and iii) treated with debridement surgery plus 500 μg/kg NBD peptide, which was injected into the defect area. During debridement, dead, damaged and infected tissue was removed from the bone defect. A total of 4 and 8 weeks following surgery, rabbits were sacrificed and specimens were harvested.

Dual energy X-ray and cone beam computed tomography (CBCT). A total of 4 and 8 weeks following surgery, rabbits were euthanized and bone mineral density (BMD) was examined in the surgical area by dual energy X-ray absorptiometry (24). In addition, bone morphology was analyzed by radiographic analysis using CBCT (25) (NewTom 3G QR-DVT9000; NewTom, Verona, Italy).

Statistical analysis. *In vitro* experiments were conducted three times to obtain the mean and standard error from independent experiments. *In vivo* experiments were conducted on six animals/group. Statistical analysis was performed using SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA) by one-way analysis of variance with Tukey's post hoc test for comparisons between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

NBD peptide inhibits RANKL-induced osteoclast formation in vitro. RAW264.7 cells were treated with DMSO or the NBD peptide and were stimulated with RANKL *in vitro*, in order to induce osteoclastogenesis (Fig. 1A). Addition of the NBD peptide resulted in a 71±5.6% reduction in the number of osteoclasts formed (P<0.05; Fig. 1B). In addition, there was a 55±1.5% reduction in the average number of nuclei per osteoclast (P<0.05; Fig. 1C). Osteoclast morphology in bone slices was analyzed by electron microscopy. Consistent with the TRAP staining results, the number and size of osteoclasts were reduced in the NBD group compared with in the DMSO group (Fig. 1A).

NBD peptide hinders bone erosion caused by osteoclasts in vitro. Osteoclast function *in vitro* was measured according to lacunae area on bone slices using scanning electron microscopy (Fig. 2A). There was a 95±3.7% reduction in total lacunae area in the NBD group compared with in the DMSO group (P<0.05; Fig. 2B).

NBD peptide ameliorates bone resorption and reduces the size of the bone defect in an in vivo model of mandibular osteomyelitis. A bone defect was created at the lateral inferior border of the

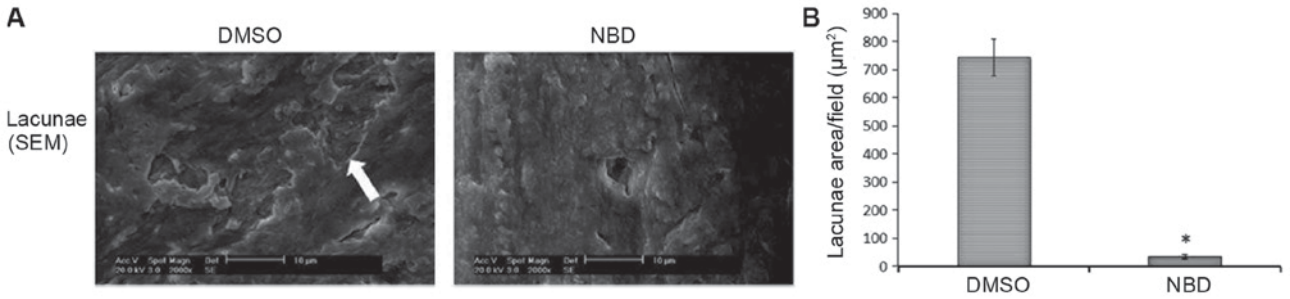

Figure 2. NBD peptide hinders bone erosion *in vitro*. (A) Resorption lacunae on bone slices were visualized by SEM. A white arrow indicates a bone lacuna. (B) Areas of bone lacunae per field were quantified using NIS Elements-AR software. Data are presented as the means ± standard error of the mean of three independent experiments. *P<0.05 vs. the DMSO group. DMSO, dimethyl sulfoxide; NBD, nuclear factor-κB essential modulator-binding domain; SEM, scanning electron microscopy.

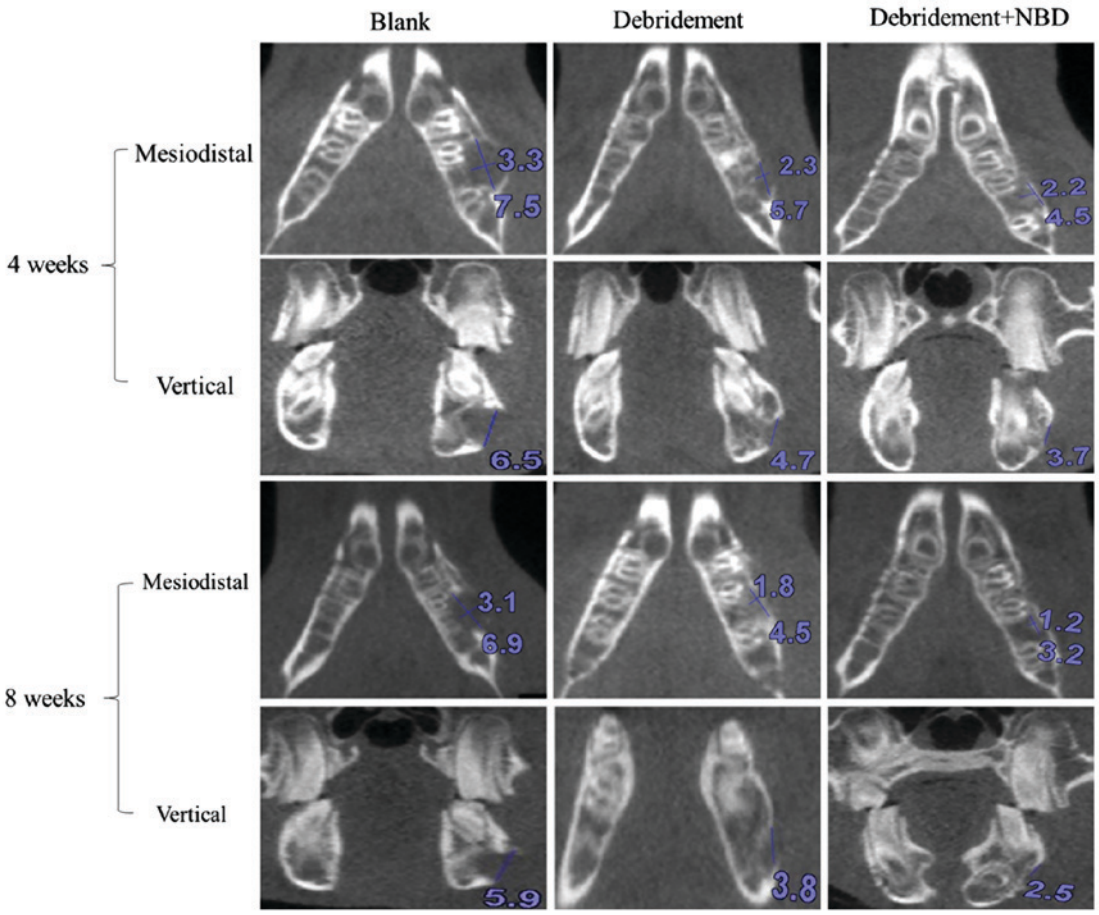

Figure 3. NBD peptide ameliorates bone resorption in an *in vivo* model of mandibular osteomyelitis, as shown by CBCT. Briefly, 100-μl *Staphylococcus aureus* suspension ($5.0x10^8$ cfu/ml) was injected into the bone defect area created at the lateral inferior border of the mandible beside the middle joint of rabbits, which was then sealed with bone wax. After 6 weeks, osteomyelitis was confirmed to be established by observing redness and swelling in the surgical field of rabbits. Rabbits were divided into three experimental groups: Untreated, treated with debridement, and treated with debridement plus 500 μg/ml NBD peptide. After 4 and 8 weeks, bone morphology was analyzed by CBCT. Each value represents the mean for 6 mice/group. The length and width of the bone defect shown in the figure is presented in mm. CBCT, cone beam computed tomography; NBD, nuclear factor-κB essential modulator-binding domain.

mandible beside the middle joint of laboratory rabbits, and a 100-μl suspension of *S. aureus* ($5.0x10^8$ cfu/ml) was injected into the bone defect. After 6 weeks, redness and swelling in the surgical field of rabbits were observed, thus confirming that osteomyelitis had been established. Then animals were divided into three experimental groups: Untreated, treated with debridement, and treated with debridement plus NBD peptide. After another 4 and 8 weeks, BMD and bone morphology of the surgical area were analyzed by dual energy X-ray absorptiometry and CBCT. As shown in Fig. 3, the size of the bone defect was gradually decreased in a 3-dimensional manner at 4 and 8 weeks in the debridement plus NBD peptide group compared with in the debridement group, as determined by CBCT.

Dual energy X-ray absorptiometry revealed that there were no significant differences in BMD between the three different time-points in the control group (P>0.05; Fig. 4A). Conversely,

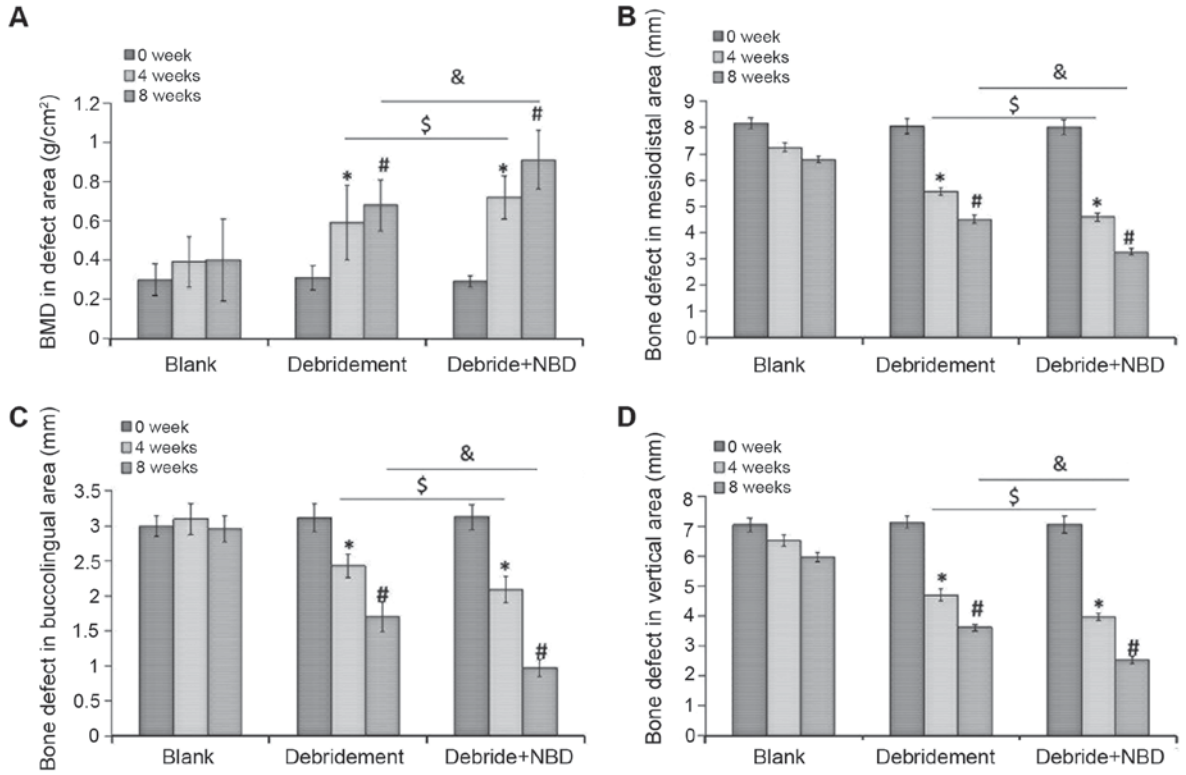

Figure 4. NBD reduces bone defects in mandibular osteomyelitis. (A) After 4 and 8 weeks of treatment, the BMD in the surgical area was examined by dual energy X-ray absorptiometry. (B-D) Bone morphology in three different dimensions (mesiodistal, buccolingual and vertical) was analyzed by cone beam computed tomography. Data are presented as the means ± standard error of the mean (n=6 rabbits/group). *P<0.05 vs. the untreated group at 4 weeks; #P<0.05 vs. the untreated group at 8 weeks; $P<0.05 vs. the debridement group at 4 weeks; &P<0.05 vs. the debridement group at 8 weeks. BMD, bone mineral density; NBD, nuclear factor-κB essential modulator-binding domain.

BMD was increased by 51±6.1 and 85±2.2% at 4 and 8 weeks, respectively, in the debridement group when compared with the baseline value at week 0 (P<0.05; Fig. 4A). Notably, additional 18±4.5 and 42±5.1% increases in BMD were observed in the debridement plus NBD peptide group when compared with the debridement group at 4 and 8 weeks, respectively (P<0.05; Fig. 4A).

The bone defects were also analyzed in three different dimensions by CBCT. Similar to the BMD results, the control group exhibited no significant differences in the 3-dimensional areas of the bone defect at the three time-points analyzed (P>0.05 Fig. 4B-D). Conversely, the bone defect area was decreased by 23.3±2.1, 21.6±2.5 and 28±2.6% in the mesiodistal, buccolingual and vertical directions, respectively, at 4 weeks; and by 36.7±3.1, 32.6±2.5 and 39.2±2.9%, respectively, at 8 weeks in the debridement group (P<0.05; Fig. 4B-D). Notably, injection with the NBD peptide resulted in increased inhibition of bone resorption, with an additional 10±1.1, 20±3.1 and 11.4±2.2% reduction in bone defect areas at 4 weeks; and 15±3.6, 34.6±3.1 and 18.3±4.5% reductions at 8 weeks, when compared with debridement alone (P<0.05) (Fig. 4B-D).

Discussion

The therapeutic potential of the NBD peptide in osteomyelitis has received relatively little attention; however, NBD has been reported to modulate inflammation and osteoclastogenesis (18). The present study demonstrated that the NBD peptide may ameliorate the progression of osteomyelitis in an *S. aureus*-induced mandibular osteomyelitis model. The results suggested that the NBD peptide reduced RANKL-induced osteoclast formation and function *in vitro*, and bone resorption in an *in vivo* model of osteomyelitis. The possible mechanism underlying these effects is that the NBD peptide suppresses synthesis of the IKK complex by disrupting the association of IKKγ/NEMO with IKKα and IKKβ, and further blocks the activation of NF-κB, which in turn inhibits the formation of osteoclasts in an *S. aureus*-induced mandibular osteomyelitis model (Fig. 5).

To investigate the role of NBD in RANKL-induced osteoclastogenesis, the cell-permeable, wild type NBD peptide (DRQIKIWFQNRRMKWKK-TALDWS-WLQTE) was synthesized as previously reported (10). This study used RAW264.7 cells, which can be induced to differentiate into mature mouse osteoclasts (26), to study the influence of the NBD peptide on osteoclastogenesis. Treatment with the NBD peptide exhibited significant activity regulating the effects of RANKL stimulation, as demonstrated by the significantly reduced formation of osteoclasts revealed by TRAP staining and F-actin immunostaining, and by the significantly smaller number of osteoclasts and average number of nuclei observed per cell. The area per osteoclast was also smaller in the NBD group when compared with the control group, as determined by SEM. The osteoclast bone-resorbing activity was also reduced when the NBD peptide was tested *in vitro*, and the lacuna area measured by SEM was smaller in the NBD group than in the control group. These results demonstrated that the synthesized NBD peptide inhibited osteoclast formation and resorption.

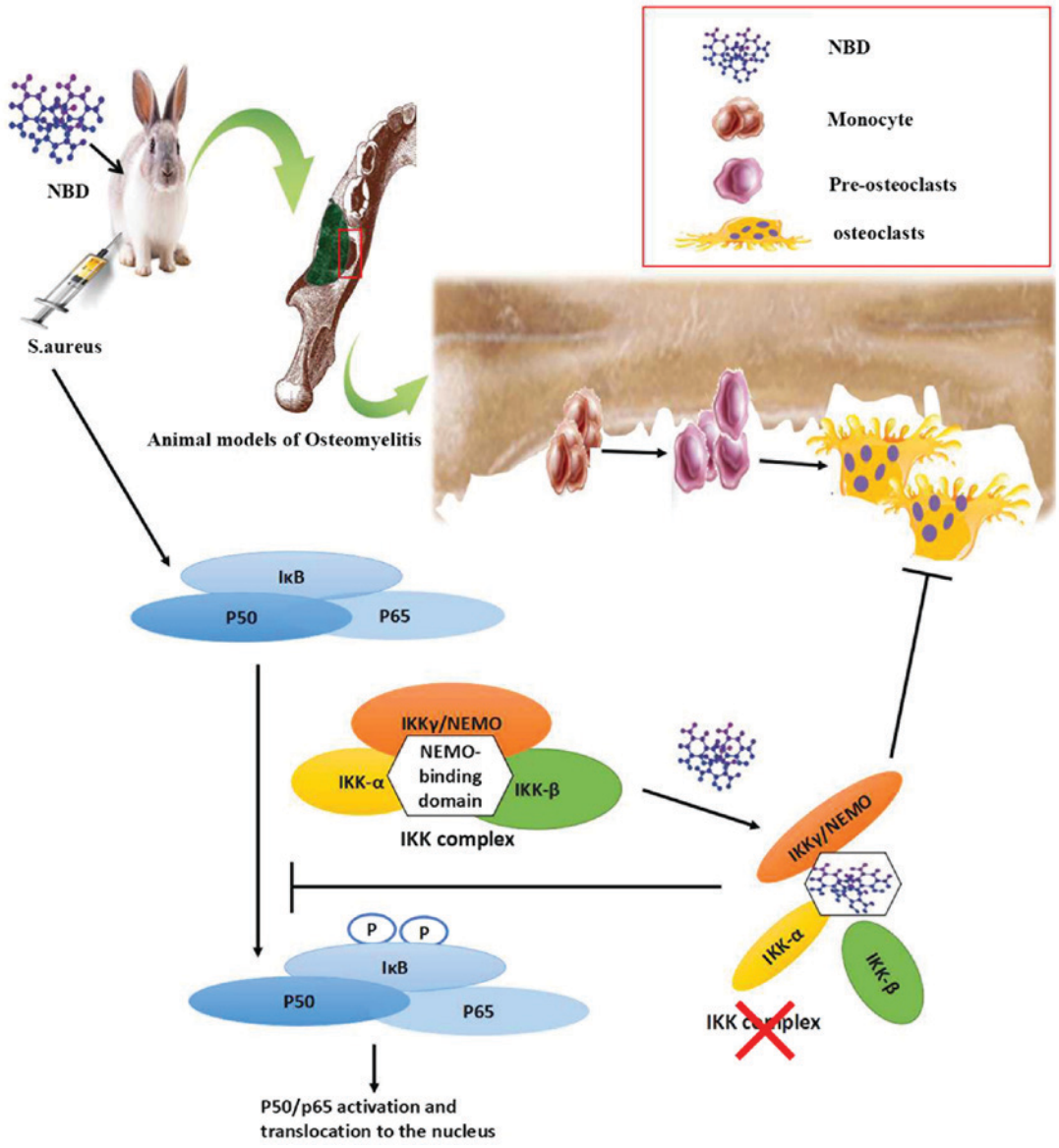

Figure 5. Schematic diagram of the mechanisms underlying inhibition of osteoclast formation and bone resorption by NBD peptide in *Staphylococcus aureus*-induced chronic osteomyelitis. NBD peptide may disrupt the association of IKKγ/NEMO with IKKα and IKKβ and block activation of NF-κB, which in turn inhibits the formation of osteoclasts. IKK, IκB kinase; NBD, NEMO-binding domain; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB.

Several lines of evidence obtained through the present study indicated that the NBD peptide may prevent the progression of osteomyelitis *in vivo*. The osteomyelitis model was established and animals were divided into three experimental groups: Untreated, treated with debridement, and treated with debridement plus NBD peptide. Treatment with the NBD peptide increased BMD in the area of the bone defect, inhibited bone resorption activity and restored bone tissue in a 3-dimensional manner when compared with the untreated and debridement groups at 4 and 8 weeks. These findings suggested that the NBD peptide ameliorated osteomyelitis induced by *S. aureus* by modulating bone metabolism.

NF-κB is an inducible transcription factor that regulates hundreds of genes involved in important physiological and pathological processes. Although NF-κB can activate various genes, those involved in inflammatory responses and osteoclastogenesis are increasingly considered to be the most important activation targets of this transcription factor. This study suggested that systemic administration of the NBD peptide may not only inhibit the formation of osteoclasts in *S. aureus*-induced osteomyelitis, but may also modulate the function of osteoclasts. This is in agreement with previous observations on osteolysis in an osteoarthritis model (16). Notably, the majority of original studies on osteomyelitis focused on the effects of *S. aureus* on osteoblasts. However, the interaction of *S. aureus* with osteoclasts, the only cells known to degrade bone, has often been overlooked. As key cells during bone infection, osteoclasts are not well equipped to kill bacteria and can become a reservoir of bacterial pathogens; this possibility requires further attention (27). The results of the present study demonstrated that debridement alongside administration of the NBD peptide into the bone defect area inhibited osteoclast activity and bone destruction, thus indicating that it may be considered an effective therapy that could be applied in osteomyelitis in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL performed the majority of the experiments. ZX and YW designed the experiments. HX, YS and QJ prepared the figures and conducted statistical analysis. XZ contributed to the animal experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University. All surgical procedures and euthanasia were performed in a manner that minimized animal suffering.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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