RESEARCH ARTICLE

Vitamin D receptor activated by vitamin D administration alleviates *Mycobacterium tuberculosis*-induced bone destruction by inhibiting NFκB-mediated aberrant osteoclastogenesis

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Abstract
Clinically, bone destruction caused by *Mycobacterium tuberculosis* was serious especially in patients with vitamin D (VD) deficiency. However, the role of VD in *M. tuberculosis*-induced bone destruction remains clear. In this context, we investigate the role of VD and vitamin D receptor (VDR) in the *M. tuberculosis*-induced bone destruction. First, we infected RAW264.7 and bone marrow-derived macrophages (BMMs) with *Mycobacterium bovis* Bacillus Calmette-Guérin (*M. bovis* BCG) in vitro. Then, we activated VDR through VD administration. TRAP and FAK staining, bone resorption assays, immunofluorescence staining, qPCR, and western blot were carried out. In vivo, the *M. tuberculosis*-induced osteolytic model on the murine skull was established and the μCT and histological analyses were performed. We found that VDR and TRAP were upregulated in bone tuberculosis tissue and proved that *M. tuberculosis* infection promoted osteoclastogenesis in RAW264.7 and BMMs. VD could inhibit osteoclasts differentiation, fusion, and bone resorption dose-dependently. However, when VDR was knocked down, the inhibitory effect of VD on osteoclasts disappeared. In mechanism, activation of VDR inhibits the phosphorylation of IκB α, thereby inhibiting NFκB signaling pathway and alleviating osteoclastogenesis. Furthermore, in the skull osteolysis model, VD administration reduced osteolysis, but not in VDR−/− mice. Our study, for the first time, demonstrates that activation of VDR by VD administration inhibits *M. tuberculosis*-induced bone destruction. Our results reveal that VD and VDR are potential therapeutic targets for...
1 | INTRODUCTION

Globally, tuberculosis remains to be the most severe infections with an estimated amount of 10 million in 2018. The increasing incidence of drug-resistant tuberculosis has shown an alarming trend that 3.4% of the new cases and 18% of the previous ones are multidrug-resistant tuberculosis.\(^1\) The clinical challenges of Mycobacterium tuberculosis osteoarticular tuberculosis have been highlighted by a recent International Consensus Meeting on Musculoskeletal Infection.\(^2\) As the most common type of extrapulmonary tuberculosis, osteoarticular tuberculosis is mainly characterized by bone destruction, which is caused by aberrant osteoclasts activation in response to M. tuberculosis infection.\(^3\) Aberrant osteoclasts activation by M. tuberculosis infection caused an imbalanced bone remodeling that favored bone resorption and it was reported that M. tuberculosis-induced osteoclasts in calvariae.\(^4\) Furthermore, it is confirmed that osteoclasts are overactivated in the osteoarticular tuberculosis lesions from a rabbit model.\(^5\)

Bone destruction caused by osteoarticular tuberculosis is gradually being valued by clinicians. Recently, the clinicians find that vitamin D (VD) deficiency is related to susceptibility to M. tuberculosis infection, and we discovered that M. tuberculosis-induced bone destruction was more serious in patients with VD deficiency.\(^6\) However, the effect of VD on the aberrant osteoclasts activation in osteoarticular tuberculosis is still not clear.

Vitamin D, a fat-soluble vitamin, is primarily involved in maintaining mineral ion homeostasis. The biologically active VD, 1,25(OH)\(_2\)D\(_3\) has been demonstrated as a major regulator of cell growth, migration, differentiation, and immune response modulation.\(^10\) It has been revealed that 1,25(OH)\(_2\)D\(_3\) control immune responses from macrophages for early M. tuberculosis clearance through the vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily.\(^12\) It was reported that VDR expression was upregulated by the interaction of the pathogen-associated molecular patterns (PAMPs) on M. tuberculosis and toll-like receptor 2 (TLR2) on monocytes.\(^15\) These evidences convincingly indicated the importance of both 1,25(OH)\(_2\)D\(_3\) and VDR for tuberculosis.

Osteoclasts derived from the monocyte/macrophage lineage precursors are specialized for bone resorption. Its differentiation, activity, and survival are mainly initiated and regulated by two indispensable cytokines, macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). During osteoclasts formation, RANKL and M-CSF activate nuclear factor-κB (NFκB) and mitogen-activated protein kinase (MAPK) signaling pathways.\(^5,18\) Then, it activates downstream c-Fos and Jun to constitute activator protein-1 (AP-1) and further induces the cascade amplification and nuclear translocation of nuclear factor of activated T cell, cytoplasmic 1 (NFATc1) which is crucial for the cell fate determination of osteoclasts. Subsequently, downstream osteoclast-specific genes including tartrate-resistant acid phosphatase (TRAP), cathepsin K (CtsK), and matrix metalloproteinase 9 (MMP9) are activated and then, secreted to degrade the organic bone matrix.\(^19\) During osteoclastogenesis, NFκB is proved to play an important role. NFκB exist in both cytoplasmic and nuclear compartments. In the base condition, NF-κB is held inactive in the cytoplasm by inhibitor of κB (IκB) proteins. Upon the IκB phosphorylation, IκB releases the inhibition of NF-κB, and then NFκB translocates to the nucleus to promote gene transcription by binding to NFκB response elements. A number of studies have identified that inhibition of IκB phosphorylation can inhibit osteoclastogenesis and bone resorption.\(^20,21\)

In our study, we investigate the role of VDR in M. tuberculosis-induced bone destruction in vivo and in vitro. For the first time, we demonstrate that activation of VDR by VD administration inhibits M. tuberculosis-induced aberrant osteoclasts activation by blocking IκB α phosphorylation. Our results reveal that VD and VDR are potential therapeutic targets for M. tuberculosis-induced bone destruction, and are of great clinical significance for the development of new therapeutic strategies.

2 | MATERIALS AND METHODS

2.1 | Materials

Dulbecco’s Modified Eagle’s Medium (DMEM), α-minimum essential medium (α-MEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) solution were purchased from Hyclone (Thermo Fisher Scientific, USA). RANKL and M-CSF were purchased from R&D Systems (Minneapolis, USA). 1,25(OH)\(_2\)D\(_3\) with purity >95% was purchased from Sigma-Aldrich (NY, USA) and stored at −20°C in the light-protected condition. The TRAP staining kit was purchased from Sigma-Aldrich (St. Louis, USA). The Actin Cytoskeleton and Focal
Adhesion (FAK) Staining Kit was purchased from Millipore (Darmstadt, Germany). Osteo Assay Stripwell Plate was purchased from Corning (NY, USA). RIPA Buffer, SDS-PAGE Loading Buffer, and SDS-PAGE Gel Kit were purchased from ComWin Biotech (Beijing, China). Anti-GAPDH (bs-0755R) antibody was purchased from Bioss (Beijing, China). Anti-NFκB p65 (BS9879M) antibody was purchased from Bioworld Technology (USA). Anti-phospho-NF-κB p65 (3033S), anti- IκB α (4812S), anti-phospho-IκB α (2859S), anti-c-Fos (2250S), anti- VDR (12550S), and anti- phospho- NF-κB p65 (3033S) antibodies were purchased from CST (Boston, MA, USA). Anti- CtsK (ab187647), anti-MMP9 (ab228402), and anti-rACP5 (ab238033) antibodies were purchased from Abcam (Cambridge, UK). HRP- conjugated anti- rabbit secondary antibody (A0208) was purchased from Beyotime Biotechnology (Shanghai, China). Goat Anti- Rabbit IgG H&L (Alexa Fluor 488) (ab150077) and Goat Anti- Mouse IgG H&L (Alexa Fluor 647) (ab150115) were purchased from Abcam (Cambridge, UK).

2.2 | Immunofluorescence staining

Human bone samples were collected from patients undergoing Curettage of spinal tuberculosis with and without VD deficiency and non-tuberculosis patients with ethical approval and according to the Army Medical University and Human Tissue Act ethical procedures. Then Immunofluorescence staining was performed for TRAP, CtsK, and VDR. The tissues were sliced into 2-mm sections. The tissue sections were treated with 0.1% of Triton X-100 for 5 minutes and then, blocked with 10% of goat normal serum for at least 30 minutes. Then, dilute the primary antibodies (anti-TRAP, anti-CtsK, and anti-VDR) with 1:200 in blocking buffer to incubate the sections for 1 hour at 37°C. Goat Anti-Rabbit IgG H&L (Alexa Flor 488) (ab150077) and Goat Anti-Mouse IgG H&L (Alexa Fluor 647) (ab150115) were purchased from Abcam (Cambridge, UK). The fluorescence signal was observed under a fluorescence microscope.

2.3 | Mice

Four to 6-week-old male C57BL/6 mice were obtained from the animal center of the Army Medical University. BMMs were extracted from the bone marrow of the femurs and tibias and cultured by α-MEM with M-CSF (25 ng/mL) for 72 hours to use. VDR knockout (KO) mice were obtained from the Department of Gastroenterology, West China Hospital, Sichuan University. All mice were raised under specific-pathogen-free (SPF) environment in the animal center of the Army Medical University with 12 hours dark/light cycles and constant temperature. When mice were 3 weeks old, the genotype was identified by quantitative real-time polymerase chain reaction (qPCR). All experiment involving animals were approved by the Ethics Committee of the Army Medical University and were carried out based on its guidelines.

2.4 | Cell culture

BMMs were incubated for 72 hours in complete α-MEM (10% FBS and 1% P/S) supplemented with 25 ng/mL of M-CSF. The murine monocyte/macrophage cell line, RAW264.7, was obtained from American Type Culture collection (ATCC) and cells were grown in complete DMEM. All cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C. To induce RAW264.7 and BMMs into osteoclasts, cells were seeded at a density of 2 × 10^3/well in 96-well plates and 1 × 10^6/well in 6-well plates supplemented with 50 ng/mL of RANKL and 25 ng/mL of M-CSF for 72 hours. VDR knockdown was performed by being transfected with a VDR-specific siRNA purchased from GenePharma (Jiangsu, China) and lipofectamine 3000 purchased from Sigma-Aldrich (St. Louis, USA) for 4 hours before cells were induced. 1,25(OH)2D3 was mixed with M-CSF and RANKL at the beginning of the induction. Mycobacterium bovis Bacillus Calmette-Guérin (M. bovis BCG) was purchased from Gene Optimal (Shanghai, China) and it was used to infect cells at a multiplicity of infection (MOI) of 10:1 in the second day of induction for 4 hours. After that, cells were cultured by complete medium with 20 µg/mL of gentamicin to kill the extracellular bacteria.

2.5 | Cell counting kit-8

We tested the cytotoxicity of 1,25(OH)2D3 in RAW264.7 and BMMs by cell counting kit-8 (CCK-8). Cells were seeded in 96-well plates at a density of 2 × 10^3/well and were treated with 0.1, 10, and 1000 nmol/L 1,25(OH)2D3 accompanied with RANKL and M-CSF for 72 hours. After that, CCK-8 solution (10 µL/well) was added to each well, then cells were incubated in the dark for 1 hour. Finally, the absorbance at 450nm was measured on a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA).

2.6 | TRAP staining assay

First, cells were rinsed by phosphate-buffered saline (PBS), then fixed with 4% paraformaldehyde for 20 minutes at 37°C. The staining solution was mixed according to the
manufacturer’s instructions. The cells were then incubated for at least 2 hours at 37°C in the dark until TRAP-positive cells were observed under the microscope in the control group. TRAP-positive cells with more than three nuclei were identified as mature osteoclasts.

2.7 | FAK staining assay

First, 96-well plates were rinsed by PBS and then, were fixed with 4% paraformaldehyde at 37°C for 20 minutes. Then, cells were washed twice with 1x wash buffer (PBS containing 0.05% Tween-20). After that, cells were permeabilized with 0.1% of Triton X-100 in PBS for 5 minutes at room temperature. Cells were washed twice again and blocked with 1% of BSA for 30 minutes at room temperature. Next, cells were incubated with TRITC-conjugated Phalloidin for 1 hour at 37°C and then, washed three times with 1× wash buffer. The nuclei counterstaining was performed with DAPI for 5 minutes at room temperature, and cells were washed three times again. Finally, the fluorescence images were visualized by fluorescent microscopy (Nikon Ti-E imaging system, Tokyo, Japan).

2.8 | Pit formation assay

RAW264.7 cells were seeded into Osteo Assay surface plates, and induced according to the experimental requirements. Sodium hypochlorite solution was used to remove cells and images were captured using a light microscope (DMI 6000B; Leica Microsystems, scale bar, 200 µm). The relative resorption area was measured by ImageJ (ImageJ 1.46r, Wayne Rasband, National Institutes of Health, USA).

2.9 | Quantitative RT-PCR

The total RNA was extracted using TRIzol buffer and concentrations were measured by NanoDrop ND-1000 Microplate Reader (Thermo Fisher Scientific, Loughborough, UK). A260/A280 and A260/A230 were used to assess the purity of RNA. Complementary DNA was synthesized from 2 µg total RNA using the reverse transcriptase kit (TAKARA). For real-time PCR, 1 µL of cDNA was mixed with the SYBR green super mix and PCR primer pair. GAPDH was used as the reference gene in this experiment and the expression of CtsK, VDR, c-Fos, NFκB, MMP9, and NFATc1 was measured. All PCR primers were synthesized by Sangon Biotech (Shanghai, China). The nucleotide sequences of all the primers were described in Table S1.

2.10 | Western blot

First, cells were washed by precooled PBS and then, lysed with RIPA buffer containing protease inhibitor on ice for 30 minutes. At least 30 µg of proteins were subjected to 10% of SDS-PAGE gels by electrophoresis and then, transferred onto 0.2 µm polyvinylidene fluoride membranes. Then, the membranes were blocked with 5% skim milk in TBST for 2 hours. After blocking, the membranes were incubated with specific primary antibodies against GAPDH, β-actin, CtsK, IκB α, p-IκB α, VDR, c-Fos, NFκB p65, p-NFκB p65, MMP9, Vinculin, and NFATc1 at 4°C overnight. The next day, the membranes were washed by TBST three times and then, incubated with HRP-conjugated secondary antibodies for 1.5 hours at room temperature and next washed by TBST three times again. Finally, the membranes were exposed by the ChemiDoc Touch Imaging System (Bio-Rad, CA, USA) using ECL luminous fluid.

2.11 | M. tuberculosis-induced murine calvarial osteolysis model

A total of 18 8-week-old C57/BL6 male mice were randomly distributed into three equal groups: vehicle (injection with PBS), M. bovis BCG (injection with 1 × 10⁶ CFU/mouse), and 1,25(OH)₂D₃ (M. bovis BCG treatment and injection with 0.5 µg/g 1,25(OH)₂D₃). Besides, we set up the fourth group: six 8-week-old VDR KO mice (M. bovis BCG treatment and injection with 0.5 µg/g 1,25(OH)₂D₃). We performed the injection (PBS, M. bovis BCG, 1,25(OH)₂D₃) subcutaneously on the surface of the skull after anesthesia every 2 days altogether seven times according to the above scheme. Mice were sacrificed and the calvariae were dissected for micro-computed tomography (μCT) scan (Bruker μCT Skyscan 1272 system, Kontich, Belgium) and the 3D structures were reconstructed by CTvox software. After that, all calvariae were decalcified in ethylenediaminetetraacetic acid (EDTA) for 2 weeks at room temperature. Hematoxylin and eosin (H&E) staining and TRAP staining were then performed according to the manufacturer’s instructions and sections were observed using a light microscope.

2.12 | Statistical analyses

All reported data are representative of three independent experiments. Data are exhibited as means ± standard deviation. The differences between the two groups were analyzed by two-tailed Student’s t test in GraphPad Prism software. The shown p values indicate the calculated significance: ns (not significant; P > .05), #/## (significant;
Osteoarticular tuberculosis caused by *M. tuberculosis* is characterized by bone loss due to aberrant osteoclasts activation. To confirm it, we collected bone tissues from patients with and without osteoarticular tuberculosis. The results of the immunofluorescence staining for TRAP and CtsK showed that *M. tuberculosis* infection increased the proportion of TRAP-positive cells, suggesting osteoclast activation (Figure 1A, Figure S1E).

*M. tuberculosis* is an intracellular bacterium that survives and replicates within cells through sheltering from the host defenses of macrophages. First, to confirm that *M. tuberculosis* can be phagocytosed by macrophages, we infected RAW264.7 cells with *M. bovis* BCG under MOI 10 for 4 hours, and then killed the extracellular bacteria with gentamicin. The Ag85B antibody, a marker antigen in different types of mycobacteria, was used for the immunofluorescence staining. The results showed that there was an obvious Ag85B positive signal in the cytoplasm of RAW264.7 cells (Figure 1B). The above results suggest that macrophages can phagocytose *M. tuberculosis*.

To study the effect of *M. tuberculosis* infection on osteoclastogenesis in vitro, RAW264.7 and BMMs were treated with RANKL and M-CSF for 72 hours. Cells were infected by *M. bovis* BCG according the above protocol. TRAP staining results showed that TRAP-positive multinucleated osteoclasts increased significantly after *M. tuberculosis* infection (Figure 1C). In addition, qPCR and western blot analysis were performed for c-Fos and NFATc1. The expression of these two markers was obviously upregulated in the infected group compared with the control one (Figure 1D,F). Furthermore, the FAK staining and pit formation assay were performed, respectively, to explore the effects of *M. tuberculosis* infection on osteoclasts fusion and bone resorption activity. FAK staining showed a significant increase in the size and number of actin rings after *M. tuberculosis* infection (Figure 1E).

The pit formation assay on collagen plates showed that osteoclastic bone resorption activity was obviously promoted under *M. tuberculosis* infection (Figure 1G). The expression of functional markers CtsK and MMP9 in mature osteoclasts was extremely upregulated with the treatment of *M. tuberculosis* (Figure 1H,I). The above results indicated that *M. tuberculosis* infection markedly promoted RANKL-induced osteoclastogenesis. Furthermore, qPCR and western blot analysis were carried out to explore the expression of VDR during osteoclastogenesis. The results revealed that both macrophages and mature osteoclasts showed low expression of VDR. It is worth noting that *M. tuberculosis* apparently upregulated the VDR expression (Figure S1A).

### 3.2 1,25(OH)2D3 dose-dependently inhibits *M. tuberculosis*-induced aberrant osteoclasts activation

We found that *M. tuberculosis*-induced bone destruction was more serious in patients with VD deficiency in clinic. On the basis of the clinical observation, the expression of VDR and TRAP was detected by immunofluorescence staining on osteoarticular tuberculosis from patients with or without VD deficiency. The result confirmed that *M. tuberculosis* infection increased the expression of VDR in TRAP-positive osteoclasts (Figure 2A). The above observation indicated the *M. tuberculosis* infection increased not only the number but also the VDR expression of osteoclasts. However, VD deficiency showed lower VDR expression level but more TRAP-positive osteoclasts, indicating that VD deficiency caused more severe aberrant osteoclastogenesis (Figure 2A).

To investigate the effect of 1,25(OH)2D3 on aberrant osteoclastogenesis induced by *M. tuberculosis*, RAW264.7 and BMMs were treated with RANKL and M-CSF for 72 hours. All groups were infected by *M. bovis* BCG with MOI 10 for 4 hours in the second day, and then treated with gentamicin to kill the extracellular bacteria. At the same time, 1,25(OH)2D3 was added into the culture medium at the final concentration of 10^{-6}, 10^{-8}, 10^{-10} \text{mol/L}, respectively. First, the cytotoxicity of 1,25(OH)2D3 was tested by CCK-8 and it showed no cytotoxicity to RAW264.7 and BMMs (Figure S1B). Then TRAP staining was performed, showing that 1,25(OH)2D3 prominently suppressed osteoclasts formation in dose-dependent manner (Figure 2B). Also, qPCR and western blot analysis of c-Fos and NFATc1 were performed. Consistently, the mRNA expression of c-Fos and NFATc1 was repressed by 1,25(OH)2D3 significantly (Figure 2C,D). Next, we studied the effect of 1,25(OH)2D3 on osteoclasts fusion. FAK staining was performed and the result indicated that both the size and number of actin rings were markedly decreased by 1,25(OH)2D3 dose-dependently (Figure 2E). To further explore the effect of 1,25(OH)2D3 on osteoclasts function, pit formation assay was carried out on collagen plates. Quantification analysis for the pit area revealed that 1,25(OH)2D3 apparently inhibited osteoclastic bone resorption activity dose-dependently (Figure 2F). Furthermore, qPCR and western blot analyses of CtsK and MMP9 showed an inhibitory effect of 1,25(OH)2D3 on osteoclasts function (Figure 2G,H). The above results indicate that 1,25(OH)2D3 has a robust inhibitory effect on *M. tuberculosis*-stimulated aberrant osteoclastogenesis dose-dependently, and 10^{-6} \text{mol/L} is the most considerable concentration in our study.
**FIGURE 1** *Mycobacterium tuberculosis* infection promotes osteoclasts differentiation, fusion, and function. 

A, Immunofluorescence staining for tartrate-resistant acid phosphatase (TRAP) and Cathepsin K (CtsK) of the bone tissues from patients with and without osteoarticular tuberculosis. Scale bars, 100 µm. 

B, Immunofluorescence staining for Ag85B of RAW264.7 cells infected by *Mycobacterium bovis* Bacillus Calmette-Guérin (*M. bovis* BCG). 

C, TRAP staining of negative control group, receptor activator of nuclear factor-κB ligand (RANKL) group, and *M. bovis* BCG infection group in both RAW264.7 and bone marrow-derived macrophages (BMMs). Scale bars, 200 µm. 

D, Relative mRNA expression of c-Fos and nuclear factor of activated T cell, cytoplasmic 1 (NFATc1) in negative control group, RANKL group, and *M. bovis* BCG infection group in both RAW264.7 and BMMs. 

E, FAK staining of negative control group, RANKL group, and *M. bovis* BCG infection group in both RAW264.7 and BMMs. 

F, Western blot analysis of c-Fos and NFATc1 in negative control group, RANKL group, and *M. bovis* BCG infection group in both RAW264.7 and BMMs. 

G, Pit formation assay of negative control group, RANKL group, and *M. bovis* BCG infection group in RAW264.7 cells. Scale bars, 200 µm. 

H, Relative mRNA expression of CtsK and matrix metalloproteinase 9 (MMP9) in negative control group, RANKL group, and *M. bovis* BCG infection group in both RAW264.7 and BMMs. 

I, Western blot analysis of CtsK and MMP9 in negative control group, RANKL group, and *M. bovis* BCG infection group in both RAW264.7 and BMMs.
FIGURE 2 1,25(OH)₂D₃ dose-dependently inhibits *Mycobacterium tuberculosis*-induced aberrant osteoclast activation. A, Immunofluorescence staining for TRAP and VDR of the bone tissues from patients without osteoarticular tuberculosis, spine tuberculosis patients with and without vitamin D (VD) deficiency. Scale bars, 100 µm. B, TRAP staining of negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. Scale bars, 200 µm. C, Relative mRNA expression of c-Fos and NFATc1 in negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. Scale bars, 100 µm. D, Western blot analysis of c-Fos and NFATc1 in negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. Scale bars, 200 µm. E, FAK staining of negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. F, Pit formation assay of negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. G, Relative mRNA expression of CtsK and MMP9 in negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. H, Western blot analysis of CtsK and MMP9 in negative control group, RANKL group, and VD group in both RAW264.7 and BMMs.

3.3 | The effect of 1,25(OH)₂D₃ on osteoclastogenesis depends on VDR

To determine whether the effect of 1,25(OH)₂D₃ on osteoclasts activation depends on VDR, the expression of VDR was knocked down by siRNA (Figure S1C). First, qPCR and western blot results showed that 1,25(OH)₂D₃ obviously promoted the expression of VDR. And we effectively attenuated the VDR expression by VDR siRNA transfetion as expected, even adequate 1,25(OH)₂D₃ failed to elevate VDR expression to a normal level (Figure 3A). Then, osteoclastic marker genes, c-Fos, NFATc1, CtsK, and MMP9 were detected by qPCR and western blot as well. The findings indicated that knocking down of VDR apparently suppressed the effect of 1,25(OH)₂D₃ on osteoclasts differentiation and maturation (Figure 3B,C). The above results confirmed our hypothesis that 1,25(OH)₂D₃ inhibited *M. tuberculosis*-activated aberrant osteoclastogenesis depending on VDR.

3.4 | Activated VDR suppressed *M. tuberculosis*-induced osteoclastogenesis through inhibiting NFκB phosphorylation

It was suggested that activation of monocyte TLRs by *M. tuberculosis* increases NFκB activity.⁹ To investigate whether *M. tuberculosis* has the same effect on osteoclasts, qPCR and western blot were carried out to explore the expression of NFκB during osteoclastogenesis. The results confirmed that *M. tuberculosis* infection significantly upregulated the NFκB expression (Figure S1A). Furthermore, it has been demonstrated that VDR activation could suppress inflammatory NFκB activity in macrophages.²⁷ We first measured the alteration of VDR and NFκB expression by qPCR and western blot. The results displayed that 1,25(OH)₂D₃ could intensively activate VDR expression both in RAW264.7 and BMMs at the concentration of 10⁻⁶ mol/L (Figure 4A,B). The immunofluorescence staining also showed that 1,25(OH)₂D₃ treatment obviously increased the fluorescence intensity of VDR in the cytoplasm (Figure S1D). Correlative, NFκB was repressed by the activation of VDR and the expression of NFκB showed markedly upregulated when VDR was knocked down by siRNA, indicating that VDR inhibited NFκB during osteoclastogenesis (Figure 4C).

To further investigate the underlying mechanism, RAW264.7 and BMMs were treated with RANKL and M-CSF for 72 hours, and then cells were starved without FBS for 12 hours. After that, cells were treated by RANKL and M-CSF with or without 1,25(OH)₂D₃ (10⁻⁶ mol/L) for 15, 30, and 60 minutes, respectively. Western blot was performed for NFκB p65, p-NFκB p65, IκB α, and p-IκB α. It is well known that IκB α binds to the p65/p50 heterodimer to block its nuclear translocation.²² Our results showed that 1,25(OH)₂D₃ did not affect the total amount of IκB α but downregulated p-IκB α thus apparently decreasing the ratio of p-IκB α/IκB α. Consequently, decreased degradation of IκB α arrested the p65/p50 heterodimer nuclear translocation and suppressed the phosphorylation of p65 (Figure 4D). Furthermore, the immunofluorescence staining for NFκB p65 was performed. The results showed that nuclear translocation of NFκB p65 was extremely increased when induced by RANKL, while 1,25(OH)₂D₃ apparently repressed this process. Importantly, nuclear translocation of NFκB p65 was increased again when VDR was knocked down (Figure 4E). The above results implied that 1,25(OH)₂D₃ inhibited *M. tuberculosis*-induced aberrant osteoclastogenesis through VDR-IκB α interaction.

3.5 | Activation of VDR signaling by 1,25(OH)₂D₃ suppressed osteoclastogenesis and prevented *M. tuberculosis*-induced bone loss in vivo

Since we demonstrated that activation of VDR inhibited *M. tuberculosis*-induced aberrant osteoclastogenesis in vitro, we next tested the effect of 1,25(OH)₂D₃ on *M. tuberculosis*-induced osteolysis in vivo. The *M. tuberculosis*-induced osteolytic model on the murine skull has been established previously in male C57/B6J mice with or without VDR KO. We injected *M. bovis* BCG with or without 1,25(OH)₂D₃ subcutaneously on the calvariae. The calvarial bones were dissected 2 weeks after injection and analyzed by μCT scanning.
FIGURE 3  Vitamin D receptor (VDR) silencing apparently hampered the effect of 1,25(OH)2D3 on osteoclastogenesis. A, Relative mRNA and protein expression of VDR in RANKL group, VD group, and VDR silence group in BMMs. "#" stands by the degree of deviation between RANKL group and VD group. "*" stands by the degree of deviation between VD group and VDR silence group. B, Relative mRNA expression of c-Fos, NFATc1, CtsK, and MMP9 in RANKL group, VD group, and VDR silence group in BMMs. C, Western blot analysis of c-Fos, NFATc1, CtsK, and MMP9 in RANKL group, VD group, and VDR silence group in BMMs.

FIGURE 4  Activated VDR suppressed Mycobacterium tuberculosis-induced osteoclast activation through inhibiting IkB phosphorylation. A, Relative mRNA expression of VDR and NFκB in negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. B, Western blot analysis of VDR and NFκB in negative control group, RANKL group, and VD group in both RAW264.7 and BMMs. "#" stands by the degree of deviation between negative control group and RANKL group. "*" stands by the degree of deviation between RANKL group and VD group. C, Western blot analysis of NFκB in RANKL group, VD group, and VDR silence group in BMMs. "#" stands by the degree of deviation between RANKL group and VD group. "*" stands by the degree of deviation between VD group and VDR silence group. D, Western blot analysis of IkB α, p- IkB α, p65, and p-p65 at 15, 30, and 60 minutes in control group and VD group in both RAW264.7 and BMMs. E, Immunofluorescence staining for p65 during osteoclastogenesis treated by VD with and without VDR silence.
and 3D image reconstruction immediately. The results show obvious pitting from *M. tuberculosis*-induced osteoclast resorption on the calvariae of the control mice. As expected, the bone loss was obviously prevented in the 1,25(OH)₂D₃ group but not in the VDR KO group (Figure 5A). Quantitative analyses further indicated that bone volume/total tissue volume (BV/TV) was obviously decreased in the *M. tuberculosis*-treated group compared with the vehicle group but increased in the 1,25(OH)₂D₃ group. It is worth noting that the protective effects of 1,25(OH)₂D₃ were diminished in the VDR⁻/- group (Figure 5A). Consistent with the μCT results, H&E staining showed obvious cell infiltration and bone destruction in *M. bovis* BCG-treated group, but were rarely detected in 1,25(OH)₂D₃ group. And, 1,25(OH)₂D₃ showed no protective effect in the VDR⁻/- group (Figure 5B). Furthermore, TRAP staining was performed, and we detected massive TRAP-positive cells in *M. bovis* BCG-treated group compared to the control one. 1,25(OH)₂D₃ apparently reduced the number of TRAP-positive cells only with the existence of VDR (Figure 5C). Totally, the above results suggested that 1,25(OH)₂D₃ exhibited protective effects on *M. tuberculosis*-induced osteolysis through activation of VDR in vivo.

### DISCUSSION

Bone remodeling is a dynamic process by maintaining a subtle equilibrium between osteoblastic bone formation and osteoclastic bone resorption, and imbalanced bone homeostasis results in pathological inflammatory bone diseases. Correlative studies have shown that *M. tuberculosis* treatment induces abnormal osteoclasts activation and causes bone resorption. However, studies on the treatment of tuberculosis progress slowly, but multidrug-resistant tuberculosis developed rapidly. It has been reported that VD strengthened the capacity of macrophages to kill bacteria.
by several antibacterial peptides. But the effect of VD on *M. tuberculosis*-induced active osteoclastogenesis is still unknown.

In this study, RAW264.7 and BMMs were infected with *M. bovis* BCG and 1,25(OH)₂D₃ was used when induced by M-CSF and RANKL. The results indicated that *M. tuberculosis* infection promoted the VDR expression of macrophages and osteoclastogenesis. Then 1,25(OH)₂D₃ treatment activated the VDR signaling pathway and displayed an inhibitory effect on *M. tuberculosis*-induced osteoclastogenesis. Furthermore, the *M. tuberculosis*-induced osteolytic model on murine calvariae was established to explore the effect of 1,25(OH)₂D₃ on bone resorption in vivo. And, the result showed a protective effect for 1,25(OH)₂D₃ against *M. tuberculosis*-induced bone resorption.

It was reported that macrophages upregulated the NFκB expression to enhance the phagocytosis of *M. tuberculosis* and promote the expression and secretion of downstream cytokines. In this study, we demonstrated that macrophages engulfed *M. bovis* BCG and upregulated the expression of VDR and NFκB. At the same time, osteoclasts-specific genes including CtsK, c-Fos, MMP9, and NFATc1 were markedly promoted. TRAP and FAK staining showed more multinucleated osteoclasts and the pit formation assay showed stronger bone resorption activation in the infected group. Thus, we considered that *M. tuberculosis* treatment targeted at NFκB which initiated the expression of c-Fos and NFATc1 to facilitate both differentiation and function of osteoclasts.

Several in vitro studies suggested that activation of VDR effectively inhibited osteoclasts differentiation and function. It also existed the opposite viewpoint that osteoclastogenesis is not influenced by the absence of VDR. In our study, VDR expression was proved to be significantly promoted with *M. tuberculosis* infection which provided a basis for us to study the effect of 1,25(OH)₂D₃ on *M. tuberculosis*-stimulated osteoclastogenesis. We discovered that 1,25(OH)₂D₃ was a strong agonist for VDR and activated VDR to repress osteoclastic marker genes including CtsK, c-Fos, MMP9, and NFATc1 in a dose-dependent manner. Thus, we preliminarily concluded that activation of VDR by 1,25(OH)₂D₃ suppressed osteoclasts differentiation and maturation. Both TRAP and FAK staining showed reduced multinucleated osteoclasts and the pit formation assay showed attenuated bone resorption activation in the 1,25(OH)₂D₃ group. It indicated that activated VDR inhibited osteoclasts fusion and function. Importantly, all the effects mentioned above were attenuated when VDR was knocked down. The above results hinted that both 1,25(OH)₂D₃ and VDR were indispensable to fight against *M. tuberculosis*-induced bone resorption.

Previous evidence showed that TLR-2 activation by binding of the PAMPs on *M. tuberculosis* leads to upregulated expression of the VDR in monocytes which plays a critical role in modulating monocyte and macrophage activities. Therefore, qPCR and western blot analysis were carried out to explore the expression of VDR during osteoclastogenesis. It was reported that VDR is not expressed by mature osteoclasts which are in line with our results: mature osteoclasts displayed prominent lower VDR level compared to RAW264.7 cells-derived macrophages. Differently, both BMM-derived macrophages and mature osteoclasts showed low expression of VDR. It is worth noting that *M. tuberculosis* apparently upregulated the VDR expression.

Then, we investigated the underlying mechanism between VDR signaling pathway and osteoclastogenesis. As a member of the dominant pathway for osteoclastogenesis, NFκB resides in the cytosol steadily but rapidly enters the nucleus upon stimulation with RANKL. It has been illustrated that 1,25(OH)₂D₃ arrests p65/p50 nuclear translocation. And we found that 1,25(OH)₂D₃ had no effect on the total amount of IκB α, but significantly downregulated p-IκB α thus decreasing the ratio of p-IκB α/IκB α. As a result, reduced degradation of IκB α suppressed the phosphorylation of p65 and arrested the p65/p50 heterodimer nuclear translocation. In total, 1,25(OH)₂D₃ activates VDR to inhibit *M. tuberculosis*-induced aberrant osteoclasts activation through the IκB α/p65 signaling pathway. Furthermore, the *M. tuberculosis*-induced osteolytic model on murine calvariae was established to investigate the in vivo effect of 1,25(OH)₂D₃. We discovered that *M. tuberculosis* treatment caused an obvious bone loss on the calvariae and 1,25(OH)₂D₃ had a protective effect against it as expected. While the protective effect of 1,25(OH)₂D₃ was not observed in the VDR KO group. All the discoveries from the animal model were consistent with the experiment in vitro which provided further evidence for our conclusion.

However, there existed some limitations in our study as well. First, due to biosafety concerns and limitation of experimental conditions, we used *M. bovis* BCG instead of H37Rv strain as the subject of infection. What’s more, more osteolytic models on different osteoarticular parts such as the spine need to be completed. Finally, the concentration of 1,25(OH)₂D₃ needs further exploration for the clinic.

5 | CONCLUSIONS

The present study demonstrates that *M. tuberculosis* infection causes excessive activation of osteoclastogenesis and VDR is significantly upregulated. Activation of VDR by 1,25(OH)₂D₃ attenuates *M. tuberculosis*-induced aberrant osteoclasts differentiation, fusion, and bone resorption activity in vitro and in vivo through IκB α/p65 signaling pathway. As the multidrug-resistant tuberculosis becomes more serious, new drug treatments are urgently needed. Our data imply the
potential value of 1,25(OH)₂D₃ as a new drug in preventing and improving osteoarticular tuberculosis.

DISCLOSURE
The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS
J. Deng completed the literature search, experiments, data analyses, figures, and writings; Y. Yang completed parts of experiments and figures; Z. Zhang and J. Xu performed the study design; J. He completed the tissue collection; Z. Zhang, J. Xu, F. Luo, and Z. Xie edited and revised the manuscript.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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