Staphylococcus Aureus Induces Osteoclastogenesis via the NF-κB Signaling Pathway

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
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Background: Osteomyelitis is one of the refractory diseases encountered in orthopedics, while *Staphylococcus aureus* (*S. aureus*) is the most common causative organism in osteomyelitis. However, the precise mechanisms underlying the bone loss caused by *S. aureus* infection have not been well defined. Here, we investigated the effect of *S. aureus* on osteoclast differentiation and the probable molecular mechanism.

Material/Methods: RAW 264.7 cells were treated for 5 days with live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate. Then, the formation of osteoclast-like cells and resorption pits was observed, and the expression of osteoclast-specific genes (TRAP, MMP-9, cathepsin K, CTR and Atp6v0d2) was detected by real-time PCR. Moreover, key proteins in the signaling pathway associated with osteoclast differentiation were detected with Western blot.

Results: The data showed that live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate induced osteoclast formation, promoted bone resorption, and increased the expression of osteoclast-specific genes in a dose-dependent manner in the absence RANKL. In addition, we found that the *S. aureus*-induced osteoclastogenesis was related to the degradation of IκB-α, phosphorylation of NF-κB p65, and increased expression of NFATc1. Thus, we used JSH-23 to inhibit NF-κB transcriptional activity. The effect of the *S. aureus*-induced osteoclastogenesis and the expression of osteoclast-specific genes and NFATc1 were inhibited, which indicated that the NF-κB signaling pathway plays a role in *S. aureus*-induced osteoclastogenesis.

Conclusions: This study demonstrated that *S. aureus* induces osteoclastogenesis through its cell wall compound and secretion of small soluble molecules, and the NF-κB signaling pathway plays a role in this process.

MeSH Keywords: **NF-kappa B p52 Subunit • Osteoclasts • Osteomyelitis • *Staphylococcus Aureus***

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Background

Osteomyelitis is an infectious disease of the bones, which is characterized by severe inflammation and progressive bone destruction [1]. With the increase of open fractures and orthopedic procedures, the incidence of osteomyelitis is significantly increasing [2]. However, due to lack of effective management, osteomyelitis causes serious morbidity and causes a great deal of physical and psychological damage to patients [3]. *Staphylococcus aureus* (*S. aureus*) is the most common causative organism in osteomyelitis [4,5], and accounts for approximately 80% of all osteomyelitis cases [6]. *S. aureus* infection usually leads to excessive bone destruction and results in bone defects [7,8], but the precise mechanisms underlying the bone loss caused by *S. aureus* infection are not well understood.

Bone is a dynamic organ, which is constantly remodeled by osteoblasts (mediating bone formation) and osteoclasts (mediating bone resorption) [9], and the balance between bone formation and bone resorption plays a major role in the maintenance of bone mineralization [10]. As to the mechanisms of bone loss caused by *S. aureus* infection, a large number of studies focused on the direct effects of *S. aureus* on bone formation [11]. It is clear that *S. aureus* reduces osteogenic differentiation from marrow mesenchymal stem cells [12] and induces cell apoptosis and death of osteoblasts [13,14]. Moreover, *S. aureus* inhibits osteoblast proliferation and bone mineralization by producing virulence factors: staphylococcal protein A, Panton-Valentine leukocidin, and coagulase [15].

However, with respect to bone resorption, the direct effects of *S. aureus* on osteoclasts have been poorly studied. Some studies have explored the direct effect of *S. aureus* capsular material [16–18] or its components (such as lipoteichoic acid [19,20], peptidoglycan [21,22]) on osteoclasts, but the results are controversial due to the different methods and osteoclast precursors used in the experiments. Moreover, as the most common pathogenic bacteria in osteomyelitis, *S. aureus* is able to synthesize a large number of virulence factors, except for the cytotoxic components of the cell wall, and the effects of *S. aureus* on osteoclasts are caused by the combination of these virulence factors. Therefore, we took RAW 264.7 cell line as osteoclast precursors, which is internationally recognized and widely used in studies of osteoclast differentiation [23]. We treated RAW 264.7 cells with live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate to investigate the effects of *S. aureus* on osteoclastogenesis. In addition, we explored the underlying mechanism of *S. aureus* -induced osteoclastogenesis, and the results indicated that *S. aureus* promoted osteoclastogenesis through its cell wall compound and secretion of small soluble molecules, and we found that the NF-κB signaling pathway plays a role in this process.

Material and Methods

Chemicals

Penicillin-Streptomycin solution and high-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (USA); fetal bovine serum (FBS) was purchased from Gibco (USA); acid phosphatase leukocyte kit (TRAP, 387A) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma-Aldrich Co. (USA). JSH-23 (Cat. No. S7351) was from Selleck Chemicals; soluble receptor-activated nuclear factor κB ligand (RANKL) was obtained from R&D Systems (USA); and the GeneJET RNA purification kit was purchased from Thermo Scientific (USA). PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) were purchased from Takara Bio Co., Ltd. (Dalian, China). Oligonucleotide primer sets were synthesized by AuGCT DNA-SYN Biotechnology Synthesis Lab Co., Ltd. (Beijing, China). Immobilon Western Chemiluminescent HRP substrate and polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Germany). Antibodies against IκB-α, NF-κB p65, Akt, p38, ERK1/2, JNK, NFATc1, and GAPDH, or the phosphorylated form of NF-κB p65, Akt, p38, ERK1/2, JNK, and HRP-linked rabbit IgG antibody were purchased from Cell Signaling Technology (USA). IL-1a (Interleukin-1 alpha) mouse *in vitro* SimpleStep ELISA™ kit, Mouse TNF alpha SimpleStep ELISA® kit and IL-6 (Interleukin-6) mouse ELISA kit were purchased from Abcam (England).

S. aureus culture

The *S. aureus* (ATCC43300) culture was performed following the protocols reported in our previous study [15]. In brief, *S. aureus* was grown overnight in tryptic soy broth at 37°C with shaking, and then *S. aureus* was harvested by centrifugation and resuspended in phosphate-buffered saline (PBS). The tablet colony counting method was used to count the number of *S. aureus* in suspension. Inactivated *S. aureus* was prepared by exposing the counted live *S. aureus* to a temperature of 95°C for 20 min. The *S. aureus* filtrate was prepared by culturing live *S. aureus* in high-glucose Dulbecco's modified Eagle's medium in the upper compartment of a Transwell bicameral chamber with a 0.4-mm pore size polycarbonate filter (Corning, New York, USA), which permits small soluble molecules produced by *S. aureus* to penetrate into the lower compartment, and the concentration of *S. aureus* filtrate was roughly determined by BCA protein assay kit (Beyotime, Shanghai, China).

Cell culture

RAW 264.7 mouse monocytes/macrophage cell line (TIB-71; ATCC) was used as osteoclast precursors, the cells were grown in high-glucose DMEM, supplemented with 10% fetal bovine

serum and 1% Penicillin-Streptomycin solution, and in a humidified atmosphere of 95% air and 5% CO_2 at 37°C; the media were changed every 3 days.

Cell viability assay (MTT assay)

RAW 264.7 cells were plated in 96-well plates at a density of 5×10^3 cells/well with 10 replicates in each group and cultured overnight, and then the cells were treated with live *S. aureus* (multiplicity of infection (MOIs) of 5: 1, 10: 1, 20: 1 and 40: 1 CFU per cell), inactivated *S. aureus* (MOIs of 10: 1, 20: 1 and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10 and 20 µg/ml), and equal volume of PBS (control) for 1, 3, and 5 days; the media and stimuli were changed every 3 days. Note that live *S. aureus* infected RAW264.7 cells for 24 h, and then were inactivated with sensitive antibiotics, and RAW264.7 cells were cultured to a predetermined time. After culturing at indicated days, the cells were added with 10 µl MTT solution and incubated at 37°C for 4 h in the dark. Then, the media were removed, and the plates were added with 100 µl dimethyl sulfoxide solution and shaken for 10 min. Subsequently, the optical density (OD) values of every well were detected with a microplate reader (Model 680, BIO-RAD, USA) at 570 nm.

In vitro osteoclastogenetic assays

To differentiate into osteoclasts, RAW 264.7 cells were seeded in 96-well plates at a density of 5×10^3 cells/well with 5 duplicates in each group and cultured overnight. The cells were treated with inactivated *S. aureus* (MOIs of 10: 1, 20: 1 and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10, and 20 µg/ml), RANKL (100 ng/ml) and PBS (control) for 5 days; the media and stimuli were replaced every 3 days. For live *S. aureus* infection, the cells were cultured in DMEM with no antibiotics and stimulated with live *S. aureus* (MOIs of 5: 1, 10: 1, 20: 1, and 40: 1 CFU per cell) for 24 h. To verify the role of NF-κB, JSH-23 (20 µM) was added 1 h ahead of stimuli, and left for 24 h. After that, live *S. aureus* was removed with sensitive antibiotics, and the cells were cultured for another 4 days. After culturing for a total of 5 days, the cells were fixed and stained using a TRAP staining kit according to the manufacturer's instructions. TRAP-positive and multi-nucleated (≥3 nuclei) cells in every well were counted as osteoclast-like cells.

Bone resorption assay

RAW 264.7 cells were seeded in 24-well Corning® Osteo Assay Surface Multiple Well Plates (Corning, USA) at a density of 5×10^4 cells/well with 4 duplicates in each group and cultured overnight. Then, the cells were treated with inactivated *S. aureus* (MOIs of 10: 1, 20: 1, and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10 and 20 µg/ml), RANKL (100 ng/ml), and PBS (control) for 5 days; the media and stimuli were

replaced every 3 days. For live *S. aureus* infection, the cells were cultured in DMEM with no antibiotics and stimulated with live *S. aureus* (MOIs of 5: 1, 10: 1, 20: 1 and 40: 1 CFU per cell) for 24 h. To verify the role of NF-κB, JSH-23 (20 µM) was added 1 h ahead of stimuli, and left for 24 h. After that, live *S. aureus* were removed with sensitive antibiotics, and the cells were cultured for another 4 days. After culturing for 5 days, the media and cells were removed, and the areas of resorption pits were photographed by an inverted microscope and analyzed with Scion image software (Scion Corp., USA).

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

RAW 264.7 cells were seeded in 6-well plates at a density of 1×10^5 cells/well and cultured overnight. Then, the cells were treated with inactivated *S. aureus* (MOIs of 10: 1, 20: 1, and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10, and 20 µg/ml), RANKL (100 ng/ml) and PBS (control) for 5 days; the media and stimuli were replaced every 3 days. For live *S. aureus* infection, the cells were cultured in DMEM with no antibiotics and stimulated with live *S. aureus* (MOIs of 5: 1, 10: 1, 20: 1, and 40: 1 CFU per cell) for 24 h. After that, live *S. aureus* was removed with sensitive antibiotics, and the cells were cultured for another 4 days. After culturing at indicated days, the cells were harvested and the total RNA was isolated with the GeneJET RNA purification kit according to the manufacturer's procedure, and the concentration of total RNA was measured with a microspectrophotometer (NanoDrop 2000, Thermo Scientific, USA). Total RNA of 500 ng was used to synthesize to cDNA by reverse transcription using PrimeScript™ RT reagent kit with gDNA Eraser. Real-time PCR was performed in CFX96 Real-time System (BIO-RAD, USA) with a SYBR® Premix Ex Taq™. Reactions were initiated by incubation at 94°C for 5 min, and PCR (94°C for 30 s, 60°C (58°C for GAPDH) for 34 s and 72°C for 30 s) was performed for 40 cycles; all reactions were performed in triplicate. Relative quantities of the tested genes were normalized to GAPDH mRNA, and the normalized data are expressed using the comparative $2^{-\Delta\Delta CT}$ method. The primer sets used in experiments are shown in Table 1.

Western blot

RAW 264.7 cells were cultured in 25-cm^2 cell culture flasks; when the cells covering the bottom of the flasks were about 80%, the culture media were changed, and the cells were cultured for another 1 h in the cell incubator. After that, the cells were stimulated with live *S. aureus* at a MOI of 20: 1 for 0, 5, 10, 20, 40, and 60 min. For NFATc1 measurement, RAW 264.7 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and infected with live *S. aureus* at a MOI of 20: 1 for 24 h, after which, *S. aureus* was removed with sensitive antibiotics and the cells were cultured for 0, 1, and 2 days. To explore the relationship between

Table 1. Primer sequences used in this study.

| Target gene	Forward (5'-3')	Reverse (5'-3')
TRAP	GCGACCATTGTTAGCCACATACG	CGTTGATGTCGCACAGAGGGAT
MMP9	GCTGACTACGATAAGGACGGCA	GCGGCCCTCAAAGATGAACGG
Cathepsin K	AGCAGAACGGAGGCATTGACTC	TTTAGCTGCCTTTGCCGTGGC
Calcitonin receptor	TGGTGCGGCGGGATCCTATAAGT	AGCGTAGGCGTTGCTCGTCG
ATP6v0d2	ACGGTGATGTCACAGCAGACGT	CCTCTGGATAGAGCCTGCCGCA
GAPDH	CCCAGAAGACTGTGGATGG	CAGATTGGGGGTAGGAACAC
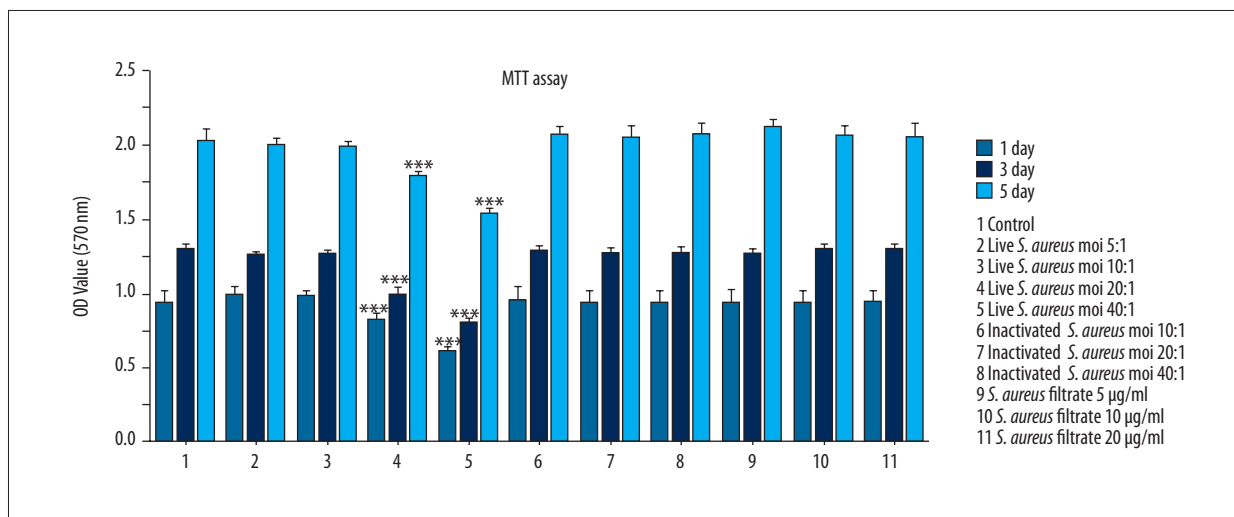
Figure 1. Effects of live *S. aureus*, inactivated *S. aureus,* and *S. aureus* filtrate on the cell viability of RAW 264.7 cells. RAW 264.7 cells were cultured in 96-well culture plates and treated with live *S. aureus* (MOIs of 5: 1, 10: 1, 20: 1, and 40: 1 CFU per cell), inactivated *S. aureus* (MOIs of 10: 1, 20: 1, and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10, and 20 μg/ml), and PBS (control). Cell proliferation was evaluated by MTT assay on days 1, 3, and 5. Data are shown as means ±SD, *** P<0.001, compared with control (PBS).

NF-κB and NFATc1, JSH-23 was added 1 h ahead of live *S. aureus*, and left for 24 h. At the indicated time, the cells were harvested and lysed using high-efficiency RIPA tissue/cell lysis solution (Solarbio, Beijing, China). After centrifugation, the proteins in the supernatant were collected. Subsequently, protein concentration was measured by a BCA protein assay kit (Beyotime, Shanghai, China), and protein samples were mixed with the sample buffer and boiled for 5 min. After that, protein samples (20 μg) were loaded onto 8–12% polyacrylamide gels, transferred to PVDF membranes subsequently blocked with 5% skimmed milk or BSA TBS (0.05% Tween-20) for 1 hat room temperature, and then were probed with specific antibodies against total ERK1/2, p38, JNK, NF-κB p65, IκB-α, Akt, GAPDH, and NFATc1, or the phosphorylated form of ERK1/2, p38, JNK, NF-κB p65, and Akt. Detection was carried out using HRP-linked rabbit IgG antibody, followed by ECL Western blotting detection reagents, and the densitometer scans of the blots were performed using the Universal Hood 2 Electrophoresis Imaging Cabinet (Bio-Rad, USA).

Data analysis

Each experiment was repeated in triplicate and similar results were obtained. Results are expressed as the mean ±SD. SPSS 19.0 software was used for statistical analysis, and the statistical significance was determined by one-way analysis of variance (ANOVA) with LSD/Student-Newman-Keul test; and p<0.05 was considered statistically significant.

Results

Effects of live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate on cell viability

The cytotoxic effects of live *S. aureus*, inactivated *S. aureus,* and *S. aureus* filtrate on RAW 264.7 cells were measured by MTT assay. As shown in Figure 1, the MOIs of inactivated *S. aureus*
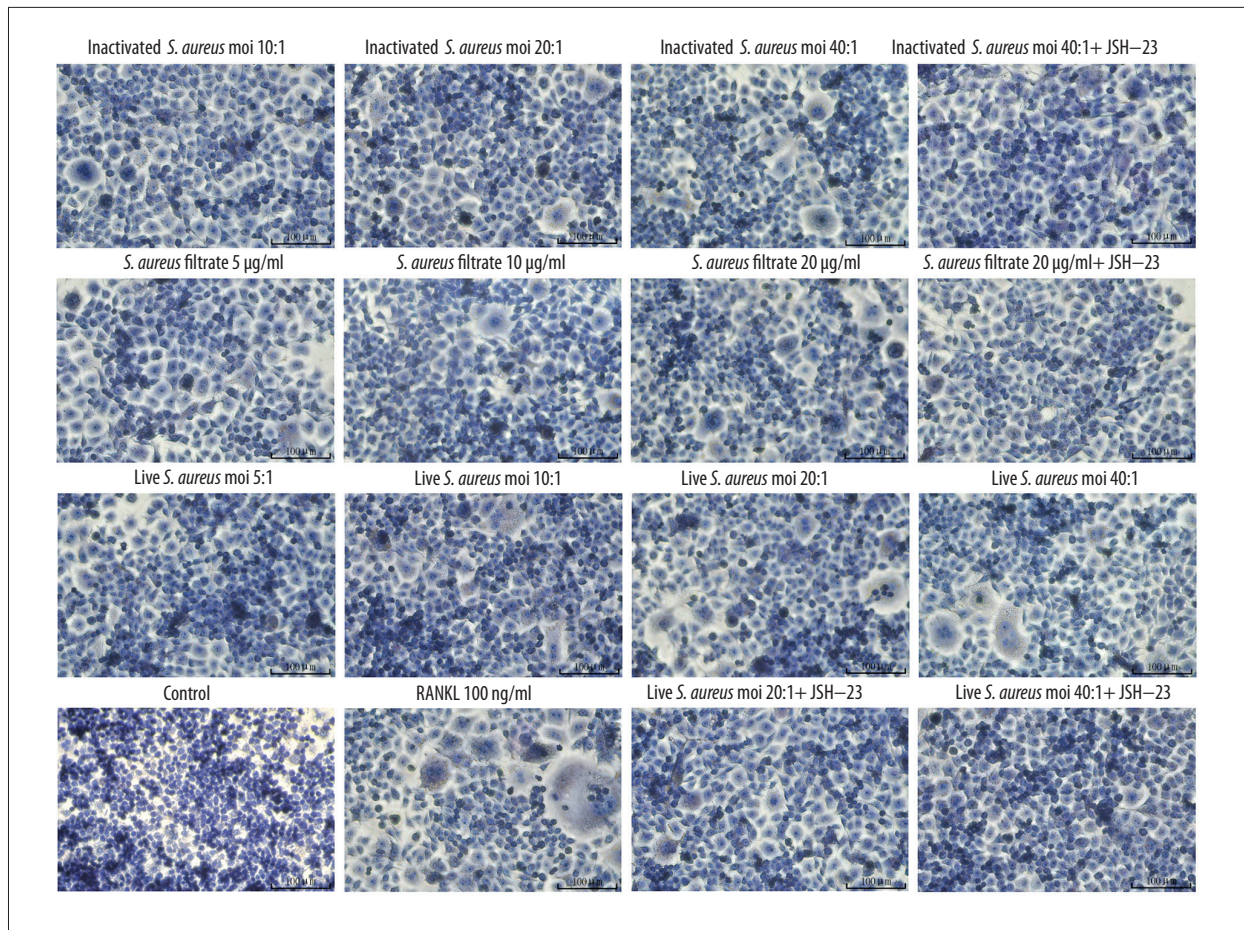
Figure 2. Effects of *S. aureus* on the osteoclast-like cell formation from RAW 264.7 cells. RAW 264.7 cells were cultured in 96-well culture plates and treated with live *S. aureus* (MOIs of 5: 1, 10: 1, 20: 1, and 40: 1 CFU per cell), inactivated *S. aureus* (MOIs of 10: 1, 20: 1, and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10, and 20 μg/ml), JSH-23(20 μM), RANKL (100 ng/ml), and PBS (control) for 5 days. Then, the cells were stained for TRAP and photographed. The positive expression of TRAP showed as red particles in the cytoplasm, and the nuclei were stained blue. Images shown above were magnified 200 times. As shown in the images, with the increase of MOIs of live *S. aureus* and inactivated *S. aureus*, and the concentrations of *S. aureus* filtrate, the number of osteoclast-like cell (TRAP-positive and ≥3 nuclei) increased gradually. However, when treated with JSH-23, the formation of osteoclast-like cells induced by live *S. aureus,* inactivated *S. aureus* and *S. aureus* filtrate was obviously reduced.

and concentrations of *S. aureus* filtrate used in experiments and live *S. aureus* with MOIs of 5: 1 and 10: 1 CFU per cell showed no obvious cytotoxicity to RAW 264.7 cells. However, live *S. aureus* with MOIs of 20: 1 and 40: 1 CFU per cell inhibited the proliferation activity of RAW264.7 cells.

S. aureus induced the formation of osteoclast-like cells from RAW 264.7 cells.

The excessively increased osteoclast activity is closely related to a variety of osteolytic diseases, such as rheumatoid arthritis, osteomyelitis, and periodontitis [24]. Therefore, we explored the effect of *S. aureus* on osteoclast formation through 3 aspects of live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate. As shown in Figures 2 and 3, after a 5-day culture, RANKL

significantly promoted the formation of osteoclast-like cells. Live *S. aureus*, inactivated *S. aureus,* and *S. aureus* filtrate promoted the formation of osteoclast-like cells in a dose-dependent manner in the absence of RANKL. However, when pretreated with JSH-23, the formation of osteoclast-like cells induced by live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate was obviously decreased. Collectively, *S. aureus* induced the formation of osteoclast-like cells through its cell wall compound and secretion of small soluble molecules, but JSH-23 inhibited this induction effect.

S. aureus promoted the formation of resorption pits in the absence of RANKL

Since *S. aureus* promoted the formation of osteoclast-like cells derived from RAW 264.7 cells, and because bone resorption is
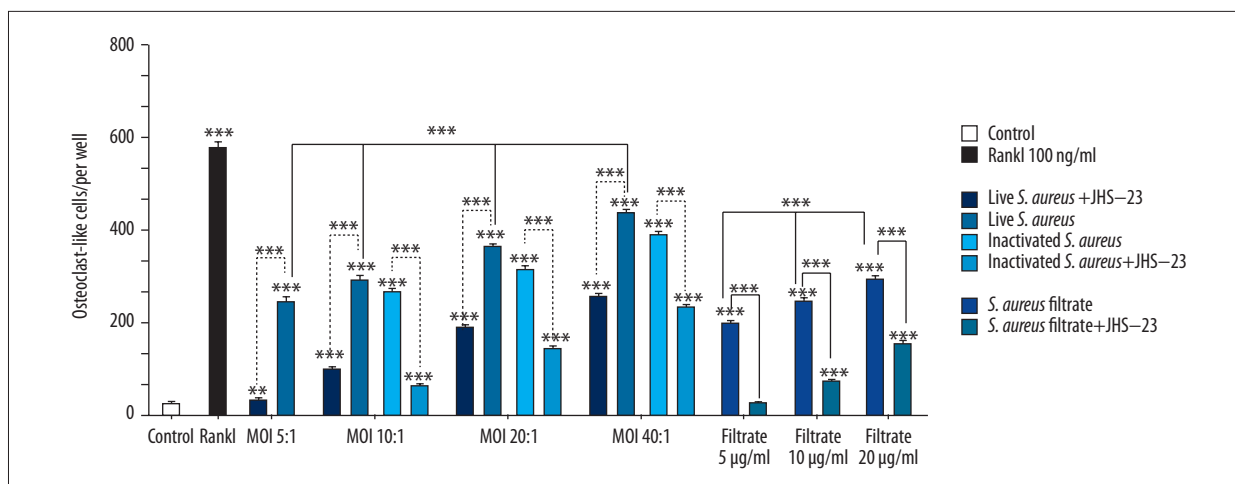
Figure 3. Results of statistical analysis of osteoclast-like cells in each experimental group. RAW 264.7 cells were treated with live *S. aureus*, inactivated *S. aureus*, *S. aureus* filtrate, JSH-23, RANKL, and PBS for 5 days. Then, the cells were stained for TRAP, and the number of osteoclast-like cells (TRAP-positive and ≥3 nuclei) in every well was counted. Data are shown as means ±SD. One-way ANOVA was used to compared among groups. For the groups with statistical significance, LSD/Student-Newman-Keul test was used to compare the 2 groups, "*" just above the column indicated the comparison with the control group, while the other "*" indicates the comparison between the 2 groups, ** $p<0.01$, *** $P<0.001$.

the most important feature that undoubtedly identifies mature osteoclasts [25], we then evaluated the bone resorption activity of *S. aureus*-induced osteoclast-like cells from RAW 264.7 cells. As shown in Figures 4 and 5, after a 5-day culture, resorption pits were clearly observed in RANKL, live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate groups (Figure 4). The area of resorption pits induced by RANKL was the largest (Figures 4, 5); and live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate induced the formation of resorption pits in a dose-dependent manner in the absence of RANKL ((Figures 4, 5). However, when pretreated with JSH-23, the formation of resorption pits induced by live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate was obviously decreased (Figures 4, 5). Collectively, *S. aureus* promoted bone resorption through its cell wall compound and secretion of small soluble molecules, but JSH-23 inhibited this promotion effect.

S. aureus increased the mRNA expression levels of osteoclast-specific genes in RAW 264.7 cells

Osteoclasts originated from hematopoietic cells of the monocyte/macrophage family [26] and RAW 264.7 cells have been shown to differentiate into mature osteoclasts when treated with RANKL [26,27]. Even in the absence of RANKL, LIGHT, IL-6 and TNF-α can induce RAW264.7 cells to differentiate into mature osteoclasts [28]. To verify the effect of *S. aureus* in promoting osteoclastogenesis in RAW264.7cells, we measured the mRNA expression levels of osteoclast-specific genes, such as TRAP, MMP-9, cathepsin K, CTR, and Atp6v0d2. As shown in Figure 6, RAW 264.7 cells themselves had the mRNA expression of TRAP, MMP-9, cathepsin K, CTR, and Atp6v0d2. Moreover,

live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate increased the mRNA expression of osteoclast-specific genes in RAW 264.7 cells in a dose-dependent manner. However, when JSH-23 was added, the expression of osteoclast-specific genes induced by live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate were obviously decreased.

S. aureus induced osteoclast differentiation through the activation of NF-κB and the increased expression of NFATc1

It was reported that NF-κB [29], 3 mitogen-activated protein kinases (MAPKs, including p38, ERK and JNK) [30], and PI-3K/Akt [31] signaling pathways are involved in osteoclast differentiation. To elucidate the signaling pathways by which *S. aureus* promotes osteoclast differentiation, we measured the protein expression levels of these signaling pathways and NFATc1 in RAW 264.7 cells stimulated by live *S. aureus*. As shown in Figure 7, the degradation of IκBα occurred at 10 min after live *S. aureus* treatment (Figure 7A, 7C), while the phosphorylation of NF-κB p65 rose markedly at 5 and 10 min (Figure 7A, 7D). Moreover, proteins in the signaling pathways of Akt and MAPKs showed no obvious change when treated with live *S. aureus* (Figure 7A).

The nuclear factor of activated T cells (NFATc1) is an important transcription factor in osteoclast differentiation; the induction of NFATc1 is a hallmark event in the cell terminal determination of osteoclasts [32,33]; and the presence of NFATc1 in osteoclast precursors could promote them to differentiate into mature osteoclasts in the absence of RANKL [33]. Thus, we
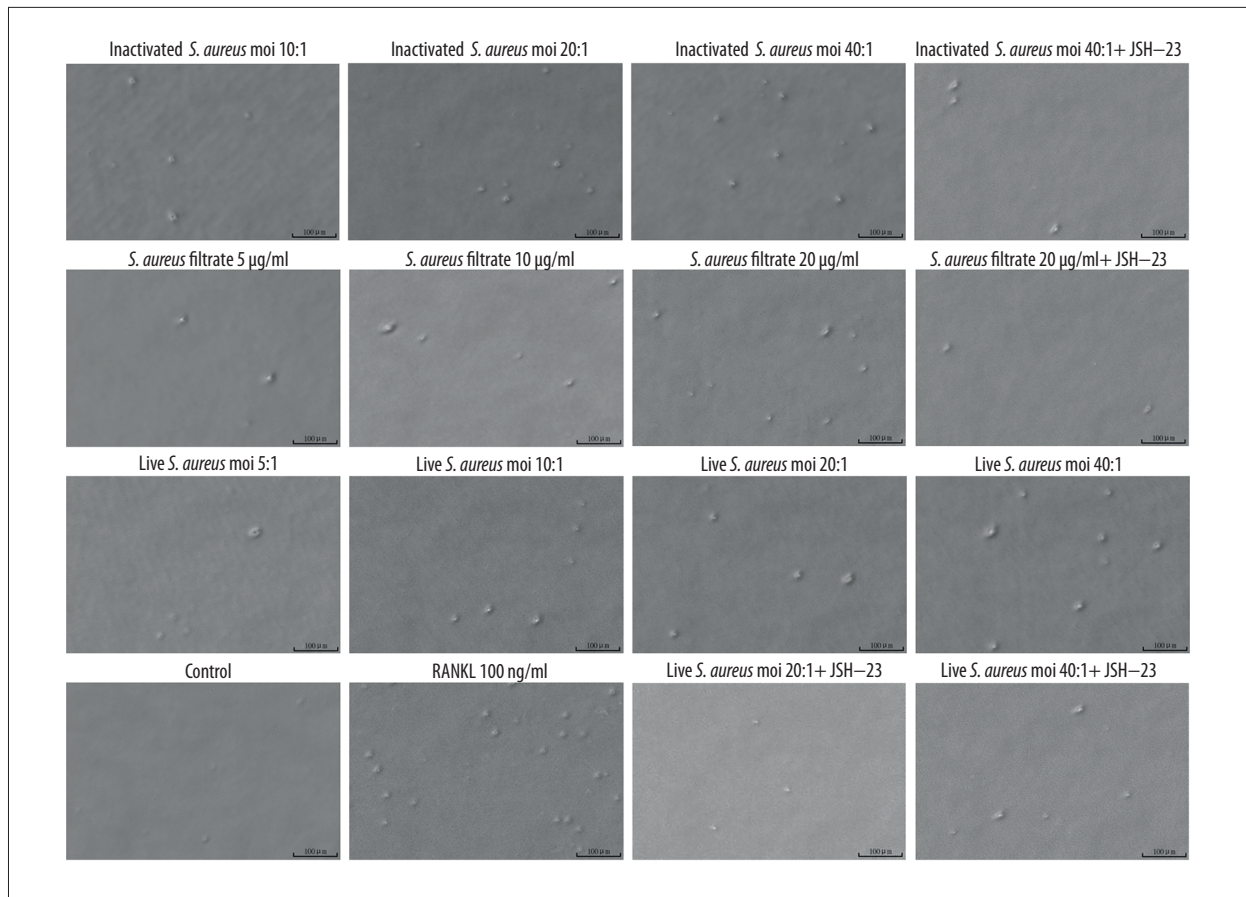
Figure 4. Effects of *S. aureus* on the bone resorption activity of osteoclasts derived from RAW 264.7 cells. RAW 264.7 cells were seeded in COAS plates and incubated with live *S. aureus* (MOIs of 5: 1, 10: 1, 20: 1, and 40: 1 CFU per cell), inactivated *S. aureus* (MOIs of 10: 1, 20: 1, and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10, and 20 μg/ml), JSH-23(20 μM), RANKL (100 ng/ml), and PBS (control) for 5 days. After that, the cells were removed and resorption pits were observed and photographed under the same microscope. Images shown above were magnified 200 times. As shown in the images, with the increase of MOIs of live *S. aureus* and inactivated *S. aureus*, and the concentrations of *S. aureus* filtrate, the area and the number of resorption pits increased gradually. However, when treated with JSH-23, the area and the number of resorption pits induced by live *S. aureus*, inactivated *S. aureus*, and *S. aureus* filtrate were obviously reduced.

examined the expression of NFATc1 in RAW 264.7 cells treated with live *S. aureus* for 0, 1, 2, and 3 days, and the results showed that *S. aureus* significantly promoted the expression of NFATc1 on days 2 and 3 (Figure 7B, 7E). However, when pretreated with JSH-23, the increased expression of NFATc1 was inhibited (Figure 7B, 7E). These findings suggest that *S. aureus* may induce osteoclast differentiation through the NF-κB signaling pathway, which subsequently activates NFATc1 and determined osteoclast differentiation.

Discussion

Mature osteoclasts are derived from hematopoietic cells of the monocyte/macrophage family [26]. Recent studies have found that macrophage-colony stimulating factor (M-CSF) and

receptor activator of NF-κB ligand (RANKL) play an important role in the process of osteoclast differentiation. M-CSF promotes the survival of osteoclast precursors and osteoclasts [34,35], and induces RANK expression in osteoclast precursors [36], which combines with RANKL and initiates intracellular signaling pathways to regulate osteoclast differentiation [37]. In addition, RAW 264.7 cells express RANK and can differentiate into mature osteoclasts induced by RANKL [27], and therefore play an important role in studies of osteoclast formation and function *in vitro* [23].

Here, we comprehensively studied the effect of *S. aureus* on osteoclast differentiation, and found that *S. aureus* induced osteoclast differentiation and promoted bone resorption in a dose-dependent way, which was verified in live *S. aureus*, inactivated *S. aureus,* and *S. aureus* filtrate. This means that *S.*
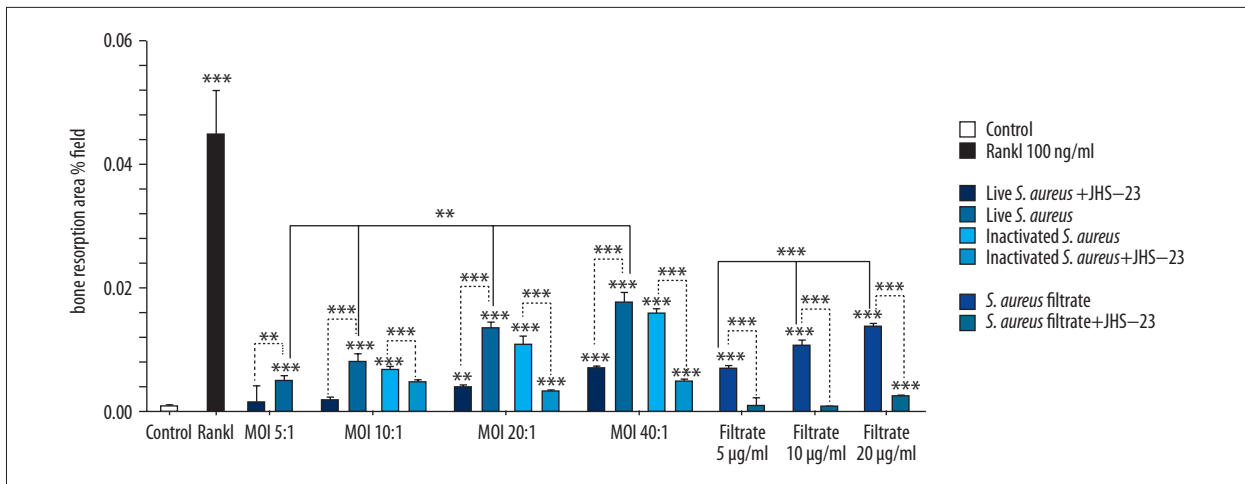
Figure 5. Results of statistical analysis of resorption pits in each experimental group. RAW 264.7 cells were treated with live *S. aureus*, inactivated *S. aureus*, *S. aureus* filtrate, JSH-23, RANKL, and PBS for 5 days. Then, the cells were removed, and the resorption pits were photographed and analyzed with Scion image software. Data indicated the ratio of the cumulative area of resorption pits divided by the entire image area in the same field of view, and the data are shown as means ±SD. One-way ANOVA was used to compared among groups. For the groups with statistical significance, the LSD/Student-Newman-Keul test was used to compare between the 2 groups. "*" just above the column indicates the comparison with the control group, while other "*" indicates the comparison between the 2 groups, ** p<0.01, *** P<0.001.
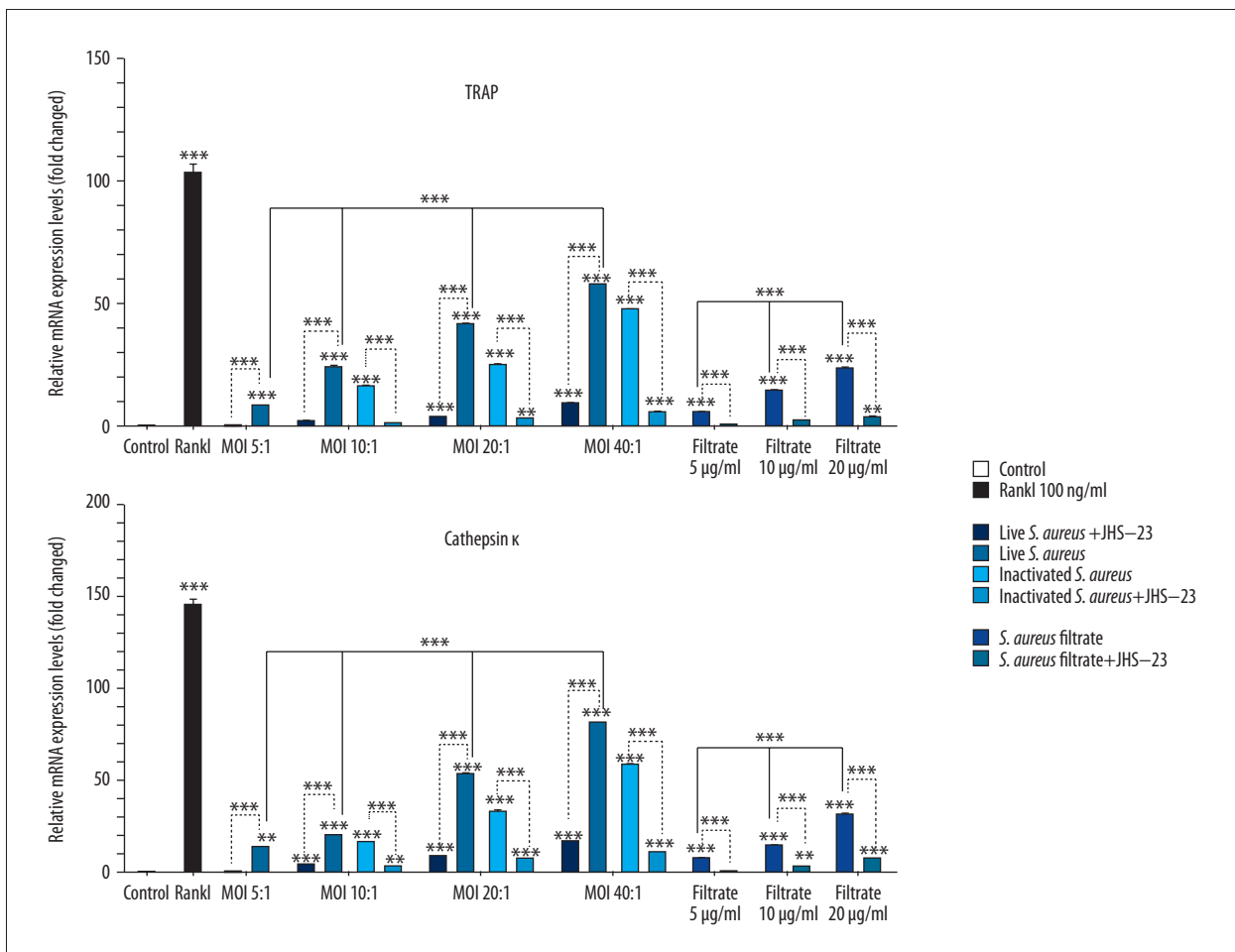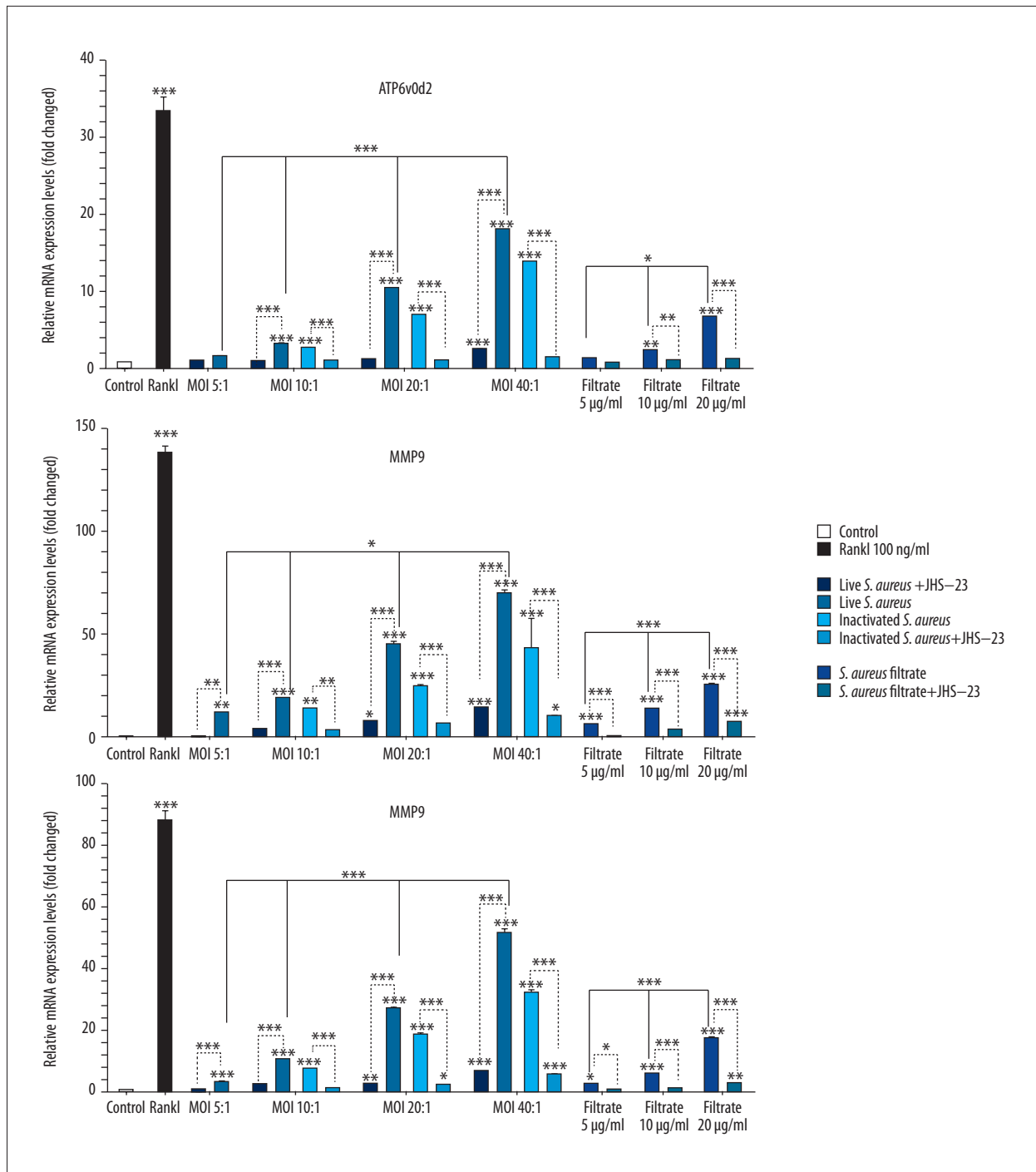
Figure 6. Effects of *S. aureus* on the mRNA expression levels of osteoclast-specific genes. RAW 264.7 cells were cultured with live *S. aureus* (MOIs of 5: 1, 10: 1, 20: 1, and 40: 1 CFU per cell), inactivated *S. aureus* (MOIs of 10: 1, 20: 1, and 40: 1 CFU per cell), *S. aureus* filtrate (concentrations of 5, 10, and 20 μg/ml), RANKL (100 ng/ml), and PBS (control) for 5 days. The expression of osteoclast-specific genes, such as TRAP, MMP-9, Cathepsin K, CTR, and ATP6v0d2, was measured by real-time PCR, and the results were normalized to the expression of GAPDH. Data are shown as means ±SD. One-way ANOVA was used to compare differences among groups. For the groups with statistical significance, the LSD/Student-Newman-Keul test was used to compare the 2 groups. "*" just above the column indicates the comparison with the control group, while other "*" indicates the comparison between the 2 groups, * P<0.05, ** P<0.01, *** P<0.001.
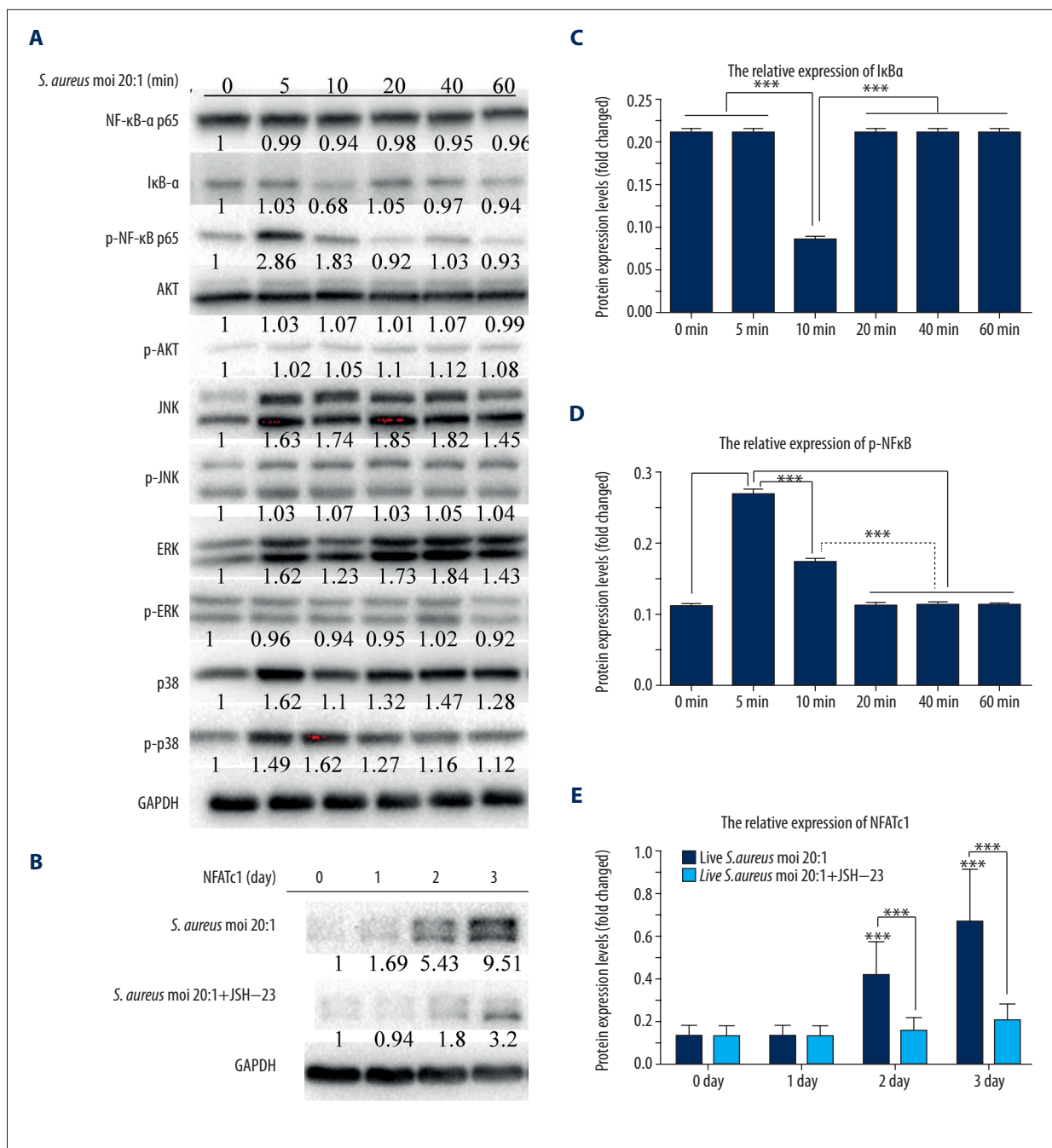
Figure 7. Effects of *S. aureus* on the protein expression levels of NF-κB, MAPKs, AKT signaling pathways, and NFATc1. RAW 264.7 cells were treated with live *S. aureus* at a MOI of 20: 1 for the indicated times (0, 5, 10, 20, 40, and 60 min and 1, 2, and 3 days). Cells were lysed for Western blot analysis with specific antibodies against total ERK1/2, p38, JNK, NF-κB p65, IκB-α, Akt, GAPDH, and NFATc1, or the phosphorylated form of p38, ERK1/2, JNK, Akt, and NF-κB p65. Relative levels of these proteins were quantified by densitometric analysis and normalized to GAPDH. (**A**) and (**B**) shows the electrophoresis of the corresponding proteins. (**C**) Fold changes in IκB-α levels. (**D**) Fold changes in phospho-NFκB p65 levels. (**E**) Fold changes in NFATc1 levels. All experiments were performed at least 3 times. "*" just above the column indicates the comparison with 0 day, while other "*" indicates the comparison between the 2 groups, *** P<0.001.

aureus promotes osteoclast differentiation through its cell wall compound and secretion of small soluble molecules. Our results are in agreement with the results of some scholars [17], but in contrast with the results of some other studies [38,39]. The main reason for these different results may be the difference in osteoclast precursors used in the experiments; they induce bone marrow cells with M-CSF and RANKL to obtain osteoclast precursors, but the osteoclast precursors they have obtained are early osteoclast precursors, committed osteoclast precursors, or mature osteoclasts, which have not been identified. In addition, the time of action and concentrations of M-CSF and RANKL undoubtedly affect the formation of osteoclast precursors. Live *S. aureus* acting on different stages of osteoclast precursors may yield different results. Here, we used RAW 264.7 cells as osteoclast precursors, which are widely used in studies of osteoclast formation and function *in vitro*, and this may be considered a more reasonable choice. Since bone is a dynamic organ, the balance between bone formation and bone resorption plays a major role in the maintenance of bone mineralization [10]. When we study the mechanisms of osteolytic diseases, we should not only study the effect of pathogenic factors on bone formation, but also explore the influence of pathogenic factors on bone resorption. When the effect of bone resorption is stronger than that of bone formation, bone destruction will arise. Our study found that the increase and activation of osteoclast differentiation induced by *S. aureus* infection is also an important cause of the formation of infectious bone defects, and the treatment methods of inhibiting osteoclast differentiation and activation may provide some help for the treatment of osteomyelitis.

In addition, we explored the molecular mechanism of *S. aureus*-induced osteoclast differentiation. RANKL, mainly derived from osteocytes [40], plays an important role in osteoclast differentiation. The binding of RANKL to RANK triggers a sequence of intracellular signal transduction pathways, including NF-κB and MAPKs, as well as PI-3K/AKT signaling pathways, which evokes the activation and autoamplification of NFATc1 and determines the terminal differentiation of osteoclasts. Moreover, NF-κB induces the initial activation of NFATc1 [33] and can induce osteoclast differentiation in the absence of RANKL [41]. In the present study, we found that the degradation of IκBα and phosphorylation of NF-κB in RAW 264.7 cells stimulated with *S. aureus*, and the changes of these 2 proteins have a certain relevance in time, but proteins in MAPKs and PI-3K/AKT signaling pathways showed no obvious change. We also found that the high expression of NFATc1 occurred on days 2 and 3 after *S. aureus* treatment. These results indicate that *S. aureus* activates NF-κB, which subsequently activates NFATc1 and

promotes osteoclast differentiation in the absence of RANKL. Thus, we used JSH-23 to inhibit the activity of NF-κB and repeated some experiments. We found that the formation of osteoclast-like cells and resorption pits was decreased, and the expression of NFATc1 and osteoclast-specific genes, such as TRAP, MMP-9, Cathepsin K, CTR, and ATP6v0d2, were inhibited, which suggests that the activation of NF-κB plays a role in *S. aureus*-induced osteoclast differentiation. In addition, some researchers found that NF-κB plays an important role in the expression of inflammatory cytokines [42], and the NF-κB signaling pathway was activated in the process of *S. aureus* infecting osteoblasts. Combined with our experimental results, the evidence suggests that inhibitors of the NF-κB signaling pathway can be useful in the treatment of osteomyelitis. However, the mechanism by which *S. aureus* induces NF-κB activation needs additional research.

The autoamplification of NFATc1 in the final stage of osteoclast differentiation regulates the expression of many osteoclast-specific genes, such as TRAP [33], MMP-9 [43], cathepsin K, CTR, and Atp6v0d2 [44,45]. In our research, we found that the increased expression of NFATc1 occurred at 2 days and 3 days after *S. aureus* stimulation, and the mRNA expression levels of TRAP, MMP-9, Cathepsin K, and Atp6v0d2 in RAW 264.7 cells treated with *S. aureus* were increased in a dose-dependent manner. However, when added with JSH-23, an inhibitor of NF-κB activation, the expression of NFATc1 was inhibited, and the mRNA expression of osteoclast-specific genes was also decreased, which indicates that *S. aureus* activated NF-κB, which subsequently activated NFATc1 and promoted osteoclast differentiation.

Conclusions

Our results demonstrate that *S. aureus* promotes osteoclast differentiation and bone resorption through its cell wall compound and secretion of small soluble molecules, and the activation of NF-κB plays a major role in this process. Our research provides a molecular basis for understanding the pathogenesis of *S. aureus*-mediated osteomyelitis, which indicates that *S. aureus* infection not only inhibits bone formation, but also enhances bone resorption, and the formation of infectious bone defect is the effect of these 2 aspects.

Conflicts of interest

The authors have declared that no competing interests exist.

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