

# *Staphylococcus aureus* Protein A induces osteoclastogenesis via the NF- $\kappa$ B signaling pathway

LI-RONG REN<sup>1</sup>, HAI WANG<sup>2</sup>, XIAO-QING HE<sup>2</sup>, MU-GUO SONG<sup>2</sup>, XUE-QIU CHEN<sup>2</sup> and YONG-QING XU<sup>2</sup>

<sup>1</sup>Department of Traumatology, The Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650101;

<sup>2</sup>Department of Orthopedic Surgery, Kunming General Hospital of Chengdu Military Command, Kunming, Yunnan 650032, P.R. China

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**Abstract.** *Staphylococcus aureus* (*S. aureus*) is the most common organism causing osteomyelitis, and *Staphylococcus aureus* protein A (SpA) is an important virulence factor anchored in its cell wall. However, the precise mechanisms underlying the bone loss caused by SpA have not been well understood. The present study aimed to investigate the effect of SpA on osteoclast differentiation, and the probable mechanism was investigated. Raw264.7 cells were treated with SpA in the absence or presence of receptor-activated (NF)- $\kappa$ B ligand for 5 days, and morphological and biochemical assays were used to assess osteoclastogenesis and explore the underlying mechanisms. Data demonstrated that SpA induced osteoclast differentiation and promoted bone resorption in a dose-dependent manner in the absence or presence of RANKL. In addition, the expression of osteoclast-specific genes, such as the tartrate resistant acid phosphatase, matrix metalloproteinase-9, cathepsin K, calcitonin receptors and d2 isoform of the vacuolar ATPase Vo domain, were enhanced by SpA. Furthermore, the SpA-induced osteoclast differentiation was associated with the degradation of inhibitor of  $\kappa$ B- $\alpha$ , phosphorylation of NF- $\kappa$ B p65 and increased expression of nuclear factor of activated T-cells. However, by treatment with JSH-23, an NF- $\kappa$ B inhibitor, the formation of osteoclast-like cells and resorption pits was significantly reduced, and the expression of osteoclast-specific genes was also inhibited. Collectively, in the present study SpA induced osteoclast differentiation, promoted bone resorption, and the NF- $\kappa$ B signaling pathway was involved in this process.

## Introduction

*Staphylococcus aureus* (*S. aureus*) is the most common causative organism in osteomyelitis (1,2), which is characterized by severe inflammation and progressive bone destruction (3). *S. aureus* infection often causes excessive bone destruction and leads to the formation of bone defect (4,5). However, the precise mechanisms underlying the bone loss caused by *S. aureus* infection is not well understood.

Bone is a dynamic organ that is constantly remodeled throughout life, and this physiological process is tightly regulated by osteoblasts (mediating bone formation) and osteoclasts (mediating bone resorption) (6). The balance between bone formation and bone resorption serves a great role in the maintenance of bone shape and mineralization (7). However, under the condition of bone infection, the balance is destroyed, and because of this, much research on the mechanism of bone defect infected by *S. aureus* focuses on bone formation (8). It is clear that *S. aureus* suppresses osteogenic differentiation of marrow mesenchymal stem cells (9) and inhibits osteoblast proliferation (10). In addition, *S. aureus* can be internalized by osteoblast (11,12) and subsequently induces osteoblast death (13). However, with respect to the bone resorption, previous studies have demonstrated that the surface-associated material (SAM) (14) and Surface-Associated Proteins (15,16) of *S. aureus* stimulate osteoclast formation and enhance bone resorption, but the active moiety in the SAM is unknown.

Mature osteoclasts are multinucleated cells, deriving from hematopoietic cells of the monocyte/macrophage family (17). Current studies have demonstrated that macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor (NF)- $\kappa$ B ligand (RANKL) serves an important role in the process of osteoclast differentiation. M-CSF promotes the survival of osteoclast precursors and osteoclasts (18,19) and induces RANK expression in osteoclast precursors (20). While RANKL is a key osteoclastogenic cytokine, the binding of RANKL to its receptor RANK recruits tumor necrosis factor receptor-associated factor 6, resulting in the activation of NF- $\kappa$ B, phosphatidylinositol 3-kinase (PI-3K)/Akt, p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (21), which are involved in the activation of c-Fos, activator protein 1 (AP-1), microphthalmia transcription factor (MITF) and PU.1 (22). In the nucleus, the recruitment

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*Correspondence to:* Professor Yong-Qing Xu, Department of Orthopedic Surgery, Kunming General Hospital of Chengdu Military Command, 212 Dagan Road, Kunming, Yunnan 650032, P.R. China  
E-mail: xyqkmzzy@163.com

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of activated NF- $\kappa$ B and nuclear factor of activated T-cells (NFATc) 2 in the promoter of NFATc1 initiates the early activation of NFATc1, which subsequently complexes with MTF, AP-1, PU.1 and cAMP response element-binding protein to induce the expression of osteoclast-specific genes (23), such as acid-resistant acid phosphatase (TRAP), matrix metalloproteinase-9 (MMP-9), cathepsin K, calcitonin receptors (CTR), d2 isoform of the vacuolar ATPase Vo domain (Atp6v0d2) and  $\beta_3$  integrin (23).

*Staphylococcus aureus* protein A (SpA) which is expressed by the majority of *S. aureus* is an important virulence factor anchored in the staphylococcal cell wall (24), which interacts with a large number of human immunoglobulins and exists in a membrane-associated and secreted form. It is reported that when SpA binds to osteoblasts it induces cell apoptosis and death (13,25,26) inhibiting bone formation and mineralization (10,27). However, the direct effect of SpA on osteoclasts has not been reported. In the present study, the effect of SpA on osteoclast differentiation and bone resorption was investigated and the underlying mechanisms was explored for the first time, to the best of our knowledge. Results demonstrated that SpA induced osteoclast differentiation and promoted bone resorption in the absence and presence of RANKL, and that the NF- $\kappa$ B signaling pathway serves an important role in this process.

## Materials and methods

**Materials.** The SpA was purchased from Sino Biological (Beijing, China); fetal bovine serum (FBS) was purchased from Gibco; Thermo Fisher Scientific Inc. (Waltham, MA, USA); penicillin-streptomycin solution and high-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from HyClone; GE Healthcare (Chicago, IL, USA). Soluble RANKL was obtained from R&D Systems Inc. (Minneapolis, MN, USA); JSH-23 was from Selleck Chemicals (Houston, TX, USA); acid phosphatase leukocyte kit (TRAP, 387A) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). A GeneJET RNA purification kit was purchased from Thermo Fisher Scientific Inc.; A PrimeScript<sup>TM</sup> RT reagent kit with gDNA Eraser (Perfect Real Time) and SYBR Premix Ex Taq<sup>TM</sup> II (Tli RNase H Plus) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Oligonucleotide primer sets were synthesized by Augct DNA-Syn Biotechnology Co., Ltd. (Beijing, China). Antibodies against ERK1/2 (cat. no. 9926), p38 (cat. no. 9926), JNK (cat. no. 9926), NF- $\kappa$ B p65 (cat. no. 8242), inhibitor of  $\kappa$ B- $\alpha$  (I $\kappa$ B- $\alpha$ ; cat. no. 4812), protein kinase (Akt; cat. no. 4691), GAPDH (cat. no. 8884) and NFATc1 (cat. no. 8032), or the phosphorylated form of p38 (cat. no. 9910), ERK1/2 (cat. no. 9910), JNK (cat. no. 9910), Akt (cat. no. 4060), and NF- $\kappa$ B p65 (cat. no. 3033) were purchased from Cell Signaling Technology, Inc. (Danvers, MA USA); Immobilized Western Chemiluminescent horseradish peroxidase (HRP) Substrate and polyvinylidene difluoride (PVDF) membranes were purchased from Merck KGaA. A mouse tumor necrosis factor (TNF)- $\alpha$  SimpleStep ELISA<sup>®</sup> kit (cat. no. ab208348), an interleukin-1 (IL)-1 $\alpha$  mouse *in vitro* SimpleStep ELISA<sup>TM</sup> kit (cat. no. ab199076), and an IL-6 mouse ELISA kit (cat. no. ab46100) were purchased from Abcam (Cambridge, UK).

**Cell culture.** The Raw264.7 mouse monocytes/macrophage cell line (TIB-71; American Type Culture Collection, Manassas, VA, USA) was used as osteoclast precursors, which can differentiate into osteoclast-like cells in the presence of RANKL (28). The cells were grown in high-glucose DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin solution, in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C; media were changed every 3 days.

**Cell viability assay (MTT assay).** Raw264.7 cells were plated in 96-well plates at a density of 1x10<sup>4</sup> cells/well with 10 replicates in each group and cultured for 24 h. Cells were induced with SpA (concentrations of 50, 100, 200, 400, 800 and 1,600 ng/ml) and equal volume of phosphate buffered saline (PBS) alone, as control for 1, 3 and 5 days; media and stimuli were changed every 3 days. After culturing for the above indicated days, 10  $\mu$ l MTT solution was added to the cells and cells were incubated at 37°C for 4 h away from light. Subsequently, media were removed, and 100  $\mu$ l dimethyl sulfoxide was added to the plates and cells were agitated at room temperature for 10 min. Subsequently, the optical density (OD) values of every well were detected with a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm.

**In vitro osteoclastogenetic assays.** To differentiate into osteoclasts, Raw264.7 cells were seeded in 96-well plates at a density of 1x10<sup>4</sup> cells/well with 5 duplicates in each group and cultured at 37°C for 24 h. Subsequently cells were stimulated with SpA (concentrations of 50, 100, 200, 400 and 800 ng/ml), SpA + RANKL (50 ng/ml), SpA+JSH-23 (20  $\mu$ M), RANKL (100 ng/ml) or equal volumes of PBS (control) at 37°C for 5 days; media and stimuli were replaced every 3 days. Following this, cells were fixed and stained using the TRAP staining kit according to the manufacturer's protocol. All cells in every well were counted, and TRAP-positive multi-nucleated ( $\geq$ 3 nuclei) cells were counted as osteoclast-like cells.

**Bone resorption assay.** Raw264.7 cells were seeded in 24-well Corning<sup>®</sup> Osteo Assay Surface Multiple Well Plates (Corning Inc., Corning, NY, USA) at a density of 5x10<sup>4</sup> cells/well with 4 duplicates in each group and cultured at 37°C for 24 h. Subsequently, cells were stimulated with SpA (concentrations of 50, 100, 200, 400 and 800 ng/ml), SpA+ RANKL (50 ng/ml), SpA+JSH-23 (20  $\mu$ M), RANKL (100 ng/ml) and equal volumes of PBS (control) for 5 days; media and stimuli were changed every 3 days. After 5 days, media and cells were removed, and the areas of resorption pits were imaged by an inverted microscope and analyzed with Scion image software version 4.0.3.2 (Scion Corp., Frederick, MD, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Raw264.7 cells were seeded in 6-well plates at a density of 1x10<sup>5</sup> cells/well and cultured for 24 h. Subsequently, cells were stimulated with SpA (concentrations of 50, 100, 200, 400 and 800 ng/ml), SpA+ RANKL (50 ng/ml), RANKL (100 ng/ml) or equal volumes of PBS (control) for 5 days; media and stimuli were changed every 3 days. After 5 days, cells were harvested and total RNA was isolated with a GeneJET RNA purification kit according to the manufacturer's protocol, and the concentration of total RNA was measured with a

Table I. 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	TGGTGCGGGCGGATCCTATAAGT	AGCGTAGGCGTTGCTCGTCG
ATP6v0d2	ACGGTGATGTCACAGCAGACGT	CCTCTGGATAGAGCCTGCCGCA
GAPDH	CCCAGAAGACTGTGGATGG	CAGATTGGGGGTAGGAACAC

TRAP, acid-resistant acid phosphatase; MMP, matrix metalloproteinase; ATP6v0d2, d2 isoform of the vacuolar ATPase Vo domain.

micro spectrophotometer (Thermo Fisher Scientific Inc., NanoDrop 2000). Total RNA of 500 ng was used to synthesize cDNA by reverse transcription using PrimeScript™ RT reagent kit with gDNA Eraser. PCR was performed in a CFX96 Real-time system (Bio-Rad Laboratories, Inc.) with a SYBR® Premix Ex Taq™. Reactions were initiated by incubation at 94°C for 5 min, and PCR [94°C for 30 sec, 60°C (58°C for GAPDH) for 34 sec, 72°C for 30 sec] was performed for 40 cycles; all reactions were performed in triplicate. Relative quantities of the tested genes were normalized to GAPDH, and the normalized data were expressed using the comparative  $2^{-\Delta\Delta C_q}$  method (29). Primers used in this study are presented in Table I.

**Western blot analysis.** Raw264.7 cells were cultured in 25 cm<sup>2</sup> cell culture flasks; when cells reached 80% confluency, the culture medium was replaced, and cells were cultured at 37°C for another 1 h in the cell incubator. Following that, cells were stimulated with SpA (400 ng/ml) for 0, 5, 10, 20, 40 and 60 min, and for NFATc1 measurement, Raw264.7 cells were treated with SpA (400 ng/ml) or JSH-23+ SpA (400 ng/ml) for 0, 1, 2 and 3 days. At the indicated time point, cells were harvested and lysed using high efficiency tissue/cell lysis solution (Beijing Solarbio Science and Technology Co, Ltd., Beijing, China), and were followed by centrifugation at 4°C and 13,800 x g for 15 min, proteins in the supernatant were collected. Subsequently, protein concentrations were measured by Bicinchoninic Acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China), and protein samples were mixed with the sample buffer and boiled at 95°C for 5 min. Following that, protein samples (20 µg) were loaded onto 8-12% polyacrylamide gels, transferred to PVDF membranes and subsequently blocked with 5% skimmed milk or bovine serum albumin (Beijing Solarbio Science and Technology Co, Ltd., Beijing, China)-TBS (0.05% Tween-20) for 1 h. Following that, membranes were probed with specific antibodies against total ERK1/2 (1:1,000), p38 (1:1,000), JNK (1:1,000), NF-κB p65 (1:1,000), IκB-α (1:500), Akt (1:1,000), GAPDH (1:2,000) and NFATc1 (1:1,000), or the phosphorylated form of p38 (1:1,000), ERK1/2 (1:1,000), JNK (1:1,000), Akt (1:1,000) and NF-κB p65 (1:1,000), and were incubated at 4°C for 12 h. Detection was carried out using a HRP-linked rabbit IgG antibody (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc. Danvers, MA USA) at room temperature for 1 h, followed by an enhanced chemiluminescence western blotting detection

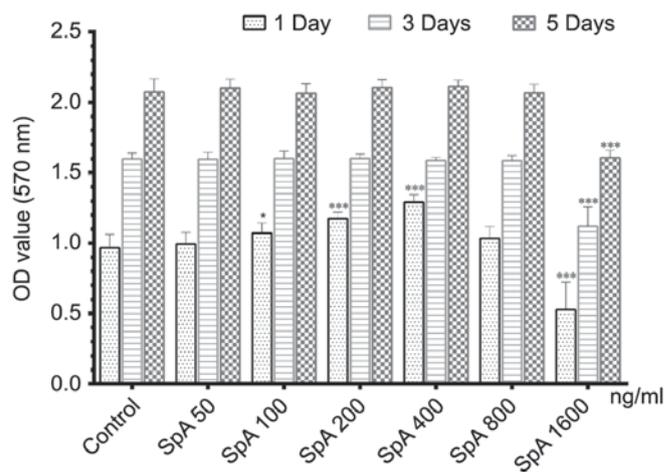


Figure 1. Effect of SpA on the cell viability of Raw264.7 cells treated with different concentrations of SpA (50, 100, 200, 400, 800 and 1600 ng/ml) and PBS alone (control), on days 1, 3 and 5. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05, \*\*\*P<0.001, vs. control. SpA, *Staphylococcus aureus* protein A; OD, optical density.

reagent at room temperature for 1 min. Densitometry of the blots were performed using the Bio-Rad Universal Hood 2 Electrophoresis Imaging Cabinet (Bio-Rad Laboratories, Inc.), and were analyzed by Bio-Rad Image Lab Software version 5.2.1 (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Each experiment was performed in triplicate and similar results were obtained. Data are expressed as the mean  $\pm$  standard deviation. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used to perform the statistical analysis, and the statistical significance was determined by one-way analysis of variance followed by the Least Significant Difference/Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of SpA on cell viability.** The cytotoxic effect of SpA on the Raw264.7 cells was measured by MTT assay. As depicted in Fig. 1, SpA promoted cell proliferation on the first day under the concentration of 100-400 ng/ml, but this effect disappeared on days 3 and 5. Overall, SpA had no cytotoxicity to Raw264.7 cells below the concentration of 800 ng/ml.

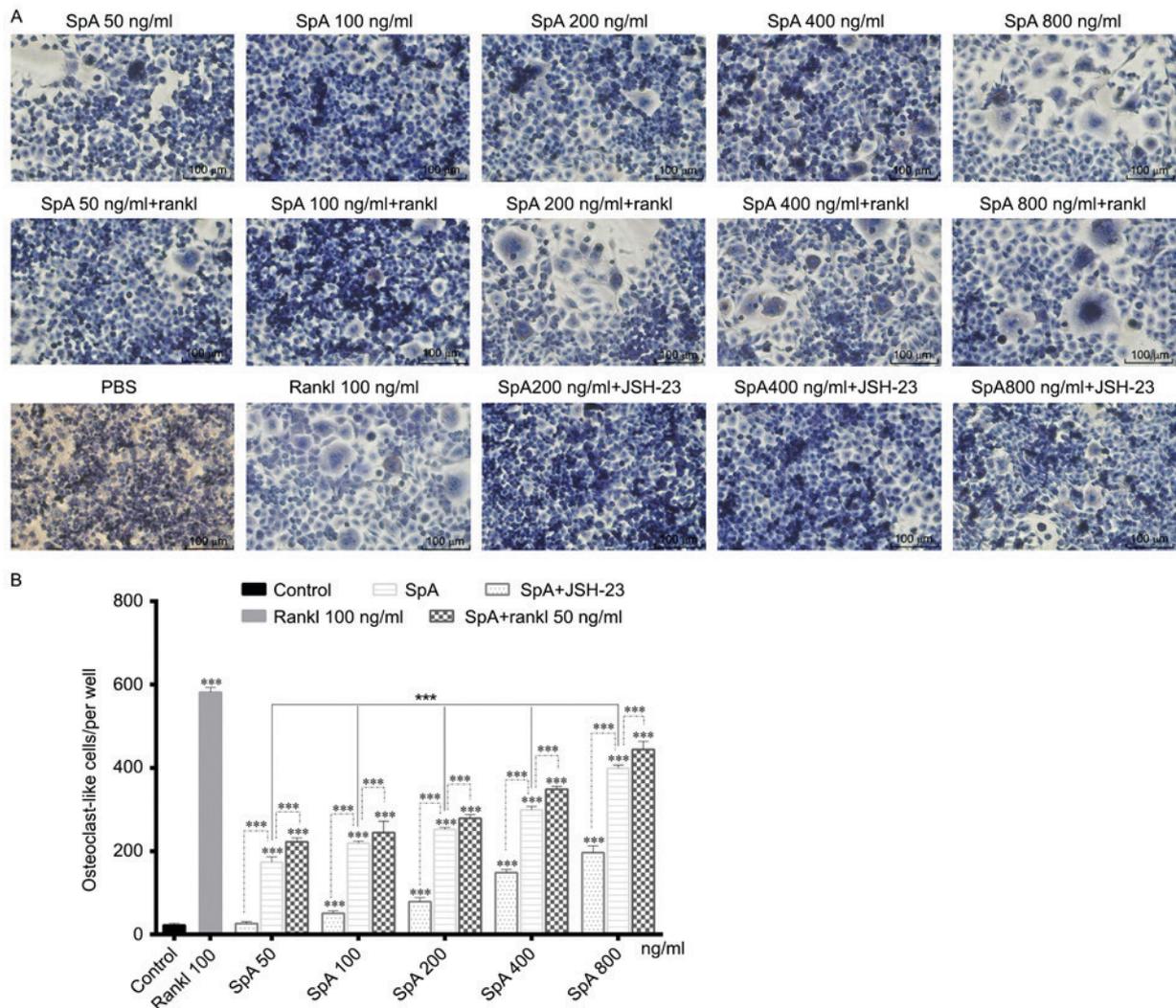


Figure 2. Effect of SpA (50–800 ng/ml), SpA+ RANKL (50 ng/ml), SpA + JSH-23 (20  $\mu$ M), RANKL (100 ng/ml) and PBS (control) for 5 days on the osteoclast-like cell formation deriving from Raw264.7 cells. Magnification,  $\times 200$ . (A) Representative photomicrographs and (B) quantification of the number of osteoclast-like cells [TRAP-positive and multinucleated ( $\geq 3$  nuclei)] in every well. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\*\* $P < 0.001$ , vs. control. SpA, *Staphylococcus aureus* protein A; RANKL, receptor activator of nuclear factor  $\kappa$ B-ligand; TRAP, acid-resistant acid phosphatase.

However, when the concentration was raised to 1,600 ng/ml, the cell proliferation activity was significantly inhibited. Since SpA did not show any toxic effect on cell viability up to 800 ng/ml, concentrations of 50–800 ng/ml were used in the following experiments.

*SpA induces the formation of osteoclast-like cells from RAW264.7 cells in the absence or presence of RANKL.* SpA is an important virulence factor of *S. aureus*, the effect of SpA on osteoclast differentiation was explored. Raw264.7 cells were grown in 96-well plates and stimulated with various concentrations of SpA in the absence or presence of RANKL for 5 days. SpA significantly promoted the formation of osteoclast-like cells in a dose-dependent manner in the absence of RANKL, but the number of osteoclast-like cells formed was lower than that of RANKL-induced (Fig. 2A and B). As reported previously, Raw264.7 cells stimulated by RANKL alone could differentiate into mature osteoclasts (28), however, Staphylococcal lipoteichoic acid, another virulence factor of *S. aureus*, inhibits osteoclast differentiation in the

presence of RANKL (30). Therefore, the induction effect of SpA on osteoclast differentiation in the presence of RANKL was also examined (Fig. 2A). However, SpA did not inhibit the formation of osteoclast-like cells in the presence of RANKL, and the number of osteoclast-like cells was higher than that without RANKL under the same concentration (Fig. 2B), which indicated that RANKL enhanced the promotion effect of SpA on osteoclast differentiation. In addition, when treated with JSH-23, an inhibitor of NF- $\kappa$ B activation, the formation of osteoclast-like cells induced by SpA was inhibited (Fig. 2).

*SpA promotes the formation of resorption pits in the absence or presence of RANKL.* Since SpA promoted the formation of osteoclast-like cells derived from Raw264.7 cells, and bone resorption is the feature that identifies mature osteoclasts (31), the bone resorption activity of osteoclast-like cells induced by SpA from Raw264.7 cells was evaluated next. In the bone resorption assay, Raw264.7 cells were grown in COAS plates and stimulated with various concentrations of SpA and

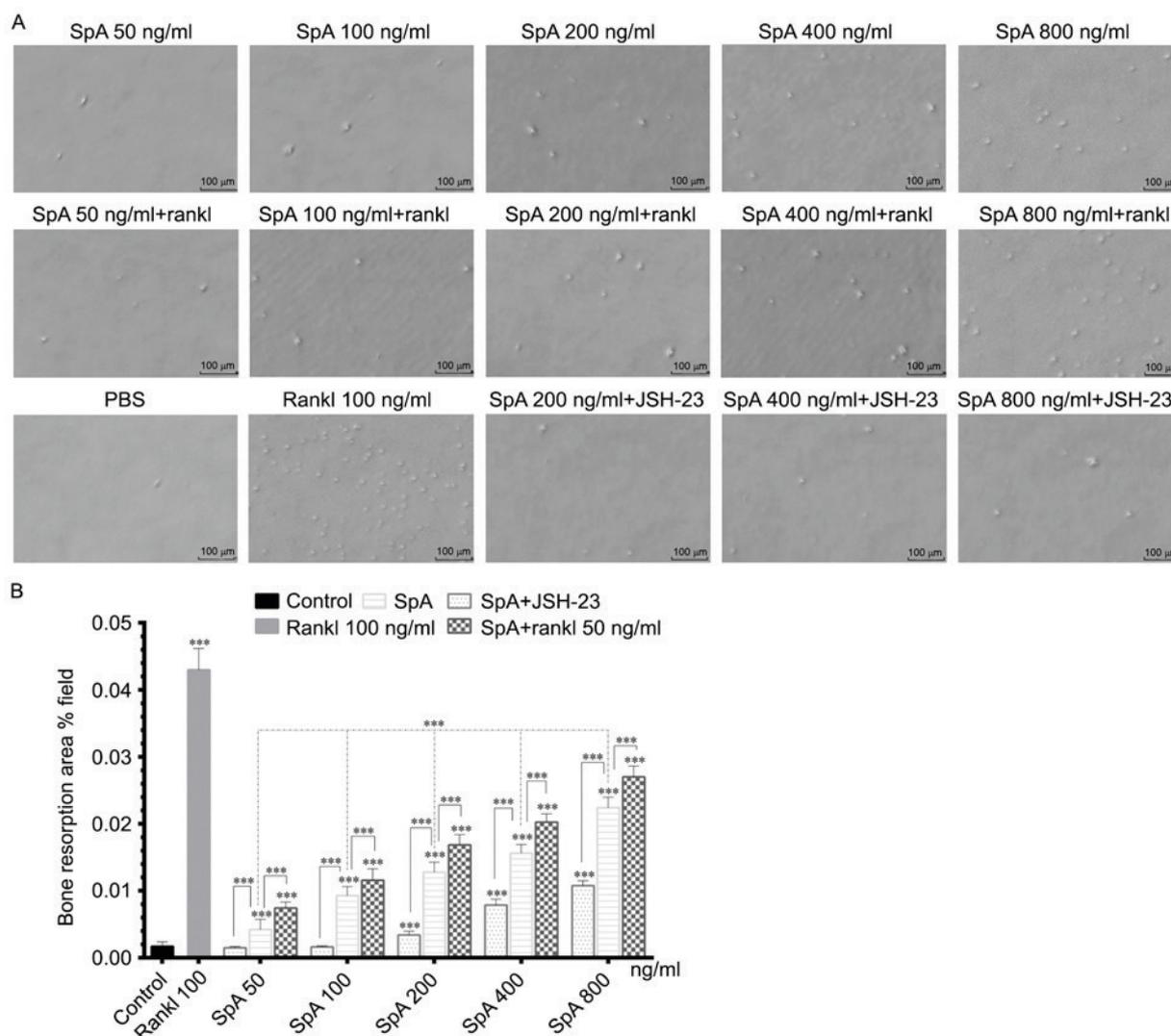


Figure 3. Effect of SpA, JSH-23 and RANKL on the bone resorption activity of osteoclasts derived from Raw264.7 cells. Magnification, x200. (A) Representative photographs and (B) quantification of the bone resorption activity of osteoclasts. The images (magnification, x200) were collected at a predetermined time point. Data are presented as the ratio of resorption area occupying the same image area and are presented as the mean  $\pm$  standard deviation, \*\*\* $P$ <0.001, vs. control. SpA, *Staphylococcus aureus* protein A; RANKL, receptor activator of nuclear factor  $\kappa$ B-ligand.

RANKL for 5 days. As illustrated in Fig. 3, SpA induced the formation of resorption pits in a dose-dependent manner in the absence or presence of RANKL (Fig. 3A and B), and the area of resorption pits with RANKL were higher than that without RANKL under the same SpA concentration (200, 400 and 800 ng/ml; Fig. 3B). However, when treated with JSH-23, the formation of resorption pits induced by SpA was decreased (Fig. 3). Collectively, these results suggested that SpA induced the formation of osteoclasts with bone resorption activity, and this effect was inhibited by JSH-23.

*SpA increases the mRNA expression levels of osteoclast-specific genes in Raw264.7 cells.* Osteoclasts originated from hematopoietic cells of the monocyte/macrophage family (17), and Raw264.7 cells have been demonstrated to differentiate into mature osteoclasts treated with RANKL alone (17,32). mRNA expression levels of osteoclast-specific genes, such as TRAP, MMP-9, cathepsin K, CTR and Atp6v0d2 were measured. As demonstrated in Fig. 4, SpA increased the mRNA expression levels of TRAP, MMP-9, cathepsin K, CTR and Atp6v0d2 in

a dose-dependent manner, and these expression levels were higher in the presence of RANKL rather than when treated with SpA alone. However, when JSH-23 was added, the mRNA expression levels of osteoclast-specific genes induced by SpA were inhibited (Fig. 4).

*NF- $\kappa$ B signaling pathway holds a role in SpA-induced osteoclast differentiation.* It was reported that, three mitogen-activated protein kinase (MAPK; including p38, ERK and JNK) (33), NF- $\kappa$ B (21,34) and PI-3K/Akt (23,35) signaling pathways have been involved in osteoclast differentiation. To elucidate the signaling pathways by which SpA promoted osteoclast differentiation, the protein expression of NFATc1 and the above-mentioned signaling pathways was measured in Raw264.7 cells stimulated by SpA. As illustrated in Fig. 5, the degradation of I $\kappa$ B $\alpha$  occurred 10 min after SpA treatment (Fig. 5A and B), while the phosphorylation of NF- $\kappa$ B increased markedly at 5 and 10 min (Fig. 5A and C). Furthermore, proteins in the signaling pathways of Akt and MAPKs showed no obvious change when treated with SpA

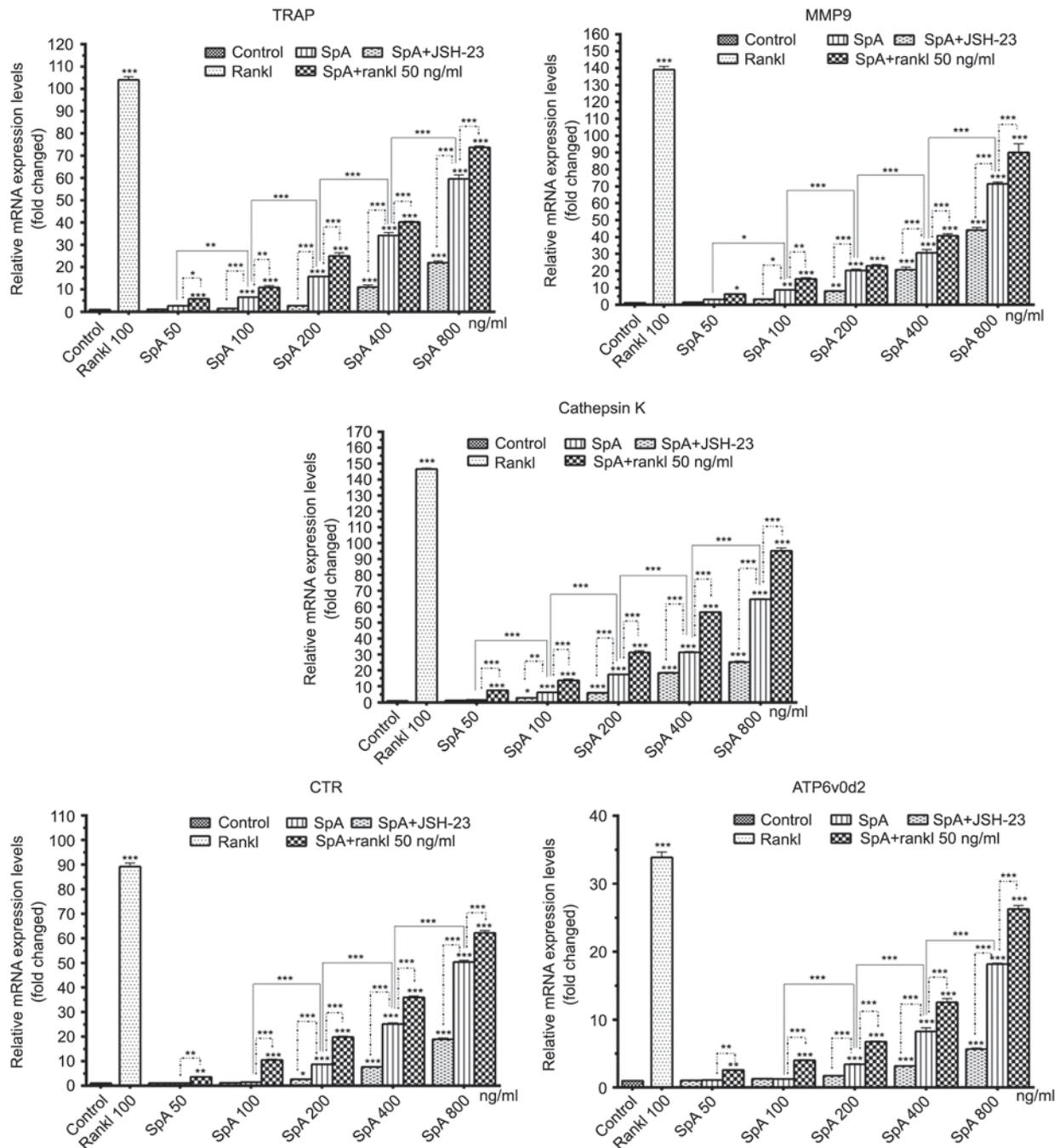


Figure 4. Effect of SpA (50-800 ng/ml), RANKL and JSH-23 on the mRNA expression levels of osteoclast-specific genes (TRAP, MMP-9, Cathepsin K, CTR and ATP6v0d2). Data are presented as the mean  $\pm$  standard deviation, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. control. SpA, *Staphylococcus aureus* protein A; RANKL, receptor activator of nuclear factor  $\kappa$ B-ligand; TRAP, acid-resistant acid phosphatase; MMP, matrix metalloproteinase; CTR, calcitonin receptor; ATP6v0d2, d2 isoform of the vacuolar ATPase Vo domain.

(Fig. 5A). Because of this, the culture medium was supplemented with JSH-23, which is an inhibitor of NF- $\kappa$ B (36,37). Subsequently, Raw264.7 cells were treated with SpA and the formation of osteoclast-like cells and resorption pits was decreased; the expression levels of osteoclast-specific genes were inhibited too, which indicated that NF- $\kappa$ B signaling pathway was involved in the SpA-induced osteoclast differentiation (Fig. 5A and C).

NFATc1 is an important transcription factor in osteoclast differentiation; the induction of NFATc1 is a hallmark in the cell terminal determination of osteoclasts (38,39); and

its presence in osteoclast precursors could promote their differentiation into mature osteoclasts in the absence of RANKL (39). Thus, the expression of NFATc1 in Raw264.7 cells treated with SpA for 1, 2 and 3 days was examined, and the results demonstrated that SpA significantly promoted the expression of NFATc1 on days 2 and 3 (Fig. 5D and E), but JSH-23 inhibited the expression of NFATc1 induced by SpA. These findings suggested that SpA may induce osteoclast differentiation through NF- $\kappa$ B activation, which subsequently activates NFATc1 determining osteoclast differentiation.

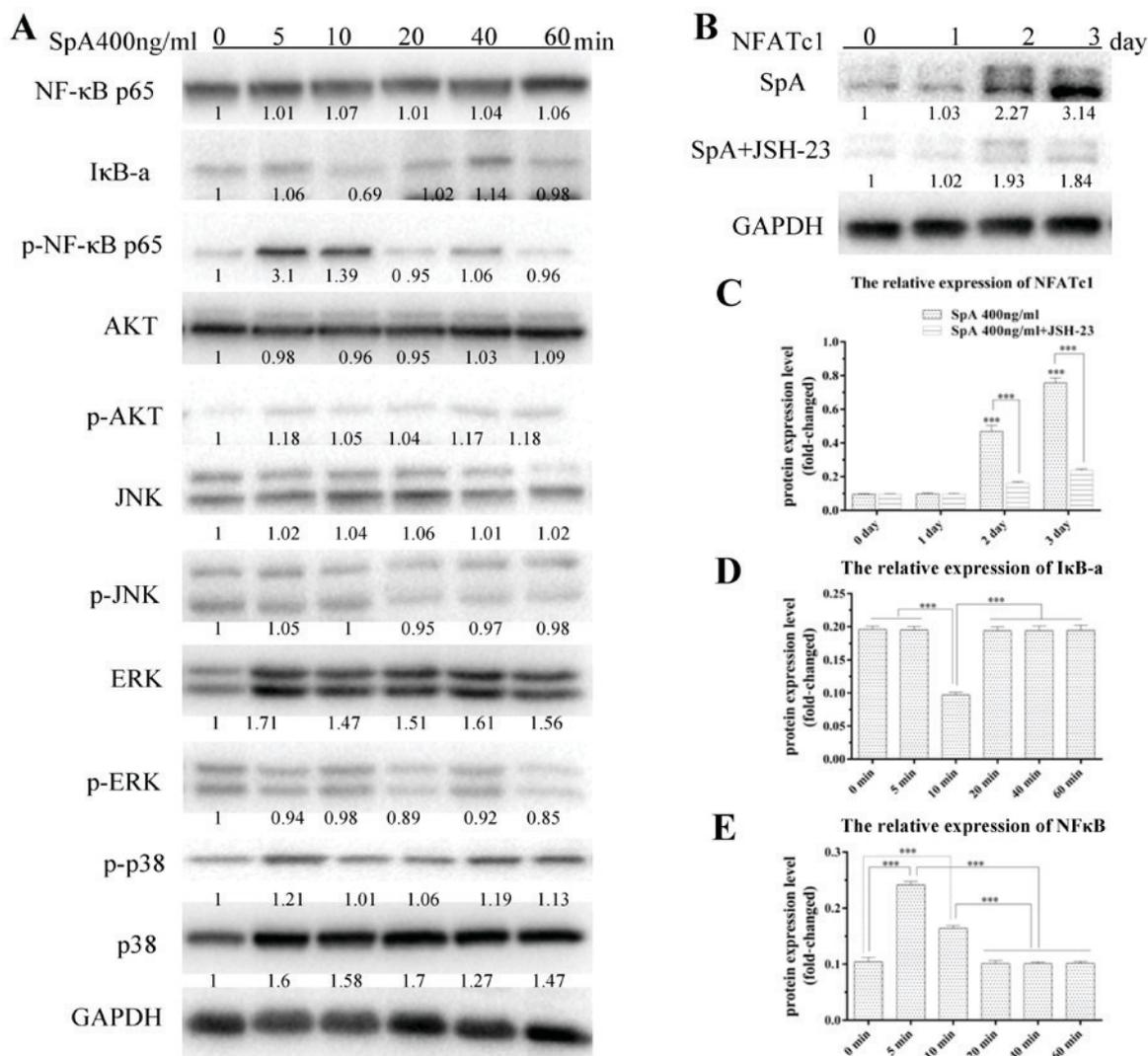


Figure 5. Effects of SpA on the NF- $\kappa$ B, MAPK and AKT signaling pathways and NFATc1 protein expression levels. Raw264.7 cells were treated with SpA (400 ng/ml) and SpA+JSH-23 for the indicated times (0, 5, 10, 20, 40, 60 min and 1, 2, 3 days). (A) Representative western blot images of NF- $\kappa$ B, MAPK and AKT signaling pathway-associated protein expression levels. (B) Representative western images and (C) quantification of NFATc1 protein expression levels. Quantification of (D) I $\kappa$ B- $\alpha$ , and (E) NF- $\kappa$ B protein expression levels. GAPDH served as an internal control. All experiments were performed at least three times. Data are presented as the mean  $\pm$  standard deviation, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. control. SpA, *Staphylococcus aureus* protein A; RANKL, receptor activator of nuclear factor  $\kappa$ B-ligand; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminus kinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B- $\alpha$ , inhibitor of I $\kappa$ B; Akt, protein kinase B; NFATc1, nuclear factor of activated T-cells; p, phosphorylated.

## Discussion

In the present study, the effect of SpA on osteoclastogenesis and the potential underlying mechanisms were investigated. It was identified that SpA induced osteoclast differentiation, promoted bone resorption and increased the expression of osteoclast-specific genes, such as TRAP, MMP-9, cathepsin K, CTR and ATP6v0d2, in a concentration-dependent manner. In addition, the effect of SpA-induced osteoclastogenesis was enhanced in the presence of RANKL. Moreover, it was demonstrated that the NF- $\kappa$ B signaling pathway may serve a role in SpA-induced osteoclastogenesis.

Raw264.7 cells express RANK and can differentiate into mature osteoclasts induced by RANKL (32), and therefore, serve an important role in studies of osteoclast formation and function *in vitro* (40). This is why Raw264.7 cells were chosen in this study as osteoclast precursors. However, Mendoza Bertelli *et al* (41) obtained osteoclast precursors by using

M-CSF to induce bone marrow cells from BALB/c, C57BL/6 or *tnfr1*<sup>-/-</sup> mice, which is another method of obtaining osteoclast precursors. They obtained a similar result, where SpA induced osteoclast differentiation and promoted bone resorption. However, Staphylococcal lipoteichoic acid, another toxic component of the cell wall of *S. aureus*, inhibited osteoclast differentiation in the presence of RANKL (30). This phenomenon may be associated with the different receptors and structures of these two virulence factors. Lipoteichoic acid is recognized by toll-like receptor (TLR) 2 (42), and stimulation by TLRs inhibits osteoclast differentiation (43), but SpA can bind to a variety of ligands, such as the TNF receptor-1 (TNFR-1) (44), the epidermal growth factor receptor (EGFR) (45) and Fc region of IgG (46), and different ligands may trigger different intracellular signaling pathways. Furthermore, Trouillet-Assant *et al* (47) have demonstrated that live *S. aureus* inhibits osteoclastogenesis. In their studies, murine bone marrow cells were infected with live *S. aureus*

only for 2 h, then the cells were induced with M-CSF and RANK-L and differentiated into activated macrophages instead of osteoclasts. This result may have been as a result of a too short infection time and with the extension of infection time, live *S. aureus* may promote osteoclast formation. In addition, although mature osteoclasts are derived from hematopoietic cells, they can be directly differentiated from the monocyte/macrophage family. This means that if they had treated bone marrow cells with M-CSF to obtain monocytes and infected them with live *S. aureus* after that, different conclusions may have been obtained. Furthermore, in the present study, although SpA induced the formation of osteoclast with bone resorption activity, compared with RANKL, the induction effect was weaker, because the formed number of osteoclast-like cells and the area of resorption pits stimulated with SpA were lower than those treated with RANKL, which emphasizes that, RANKL, may be a necessary and sufficient cytokine, serving a great role in osteoclast differentiation.

In the present study, it was demonstrated that the degradation of  $\kappa$ B- $\alpha$  and phosphorylation of NF- $\kappa$ B in Raw264.7 cells occurred under the stimulation of SpA, but proteins in MAPKs and PI-3 K signaling pathways presented no obvious change, which indicated that the NF- $\kappa$ B signaling pathway might be involved in SpA-induced osteoclast differentiation. Additionally, JSH-23 was used to inhibit NF- $\kappa$ B activation, and it was identified that the formation of osteoclast-like cells and resorption pits induced by SpA was obviously decreased, and the expression of osteoclast-specific genes (TRAP, MMP-9, cathepsin K, CTR and ATP6v0d2) were inhibited too, which confirmed that the NF- $\kappa$ B signaling pathway was involved in the SpA-induced osteoclast differentiation. As reported previously, NFATc1 serves a decisive role in the terminal differentiation of osteoclasts (39), and NF- $\kappa$ B induces the initial activation of NFATc1 (39), which evokes the auto-amplification of NFATc1 and determines its sufficient role in osteoclast differentiation (48). Therefore, the expression of NFATc1 was measured and it was demonstrated that its high expression occurred 2 and 3 days after SpA stimulation. However, when JSH-23 was added, the expression of NFATc1 was decreased, which suggested that SpA promotes osteoclast differentiation by activating the NF- $\kappa$ B signaling pathway, which subsequently activates NFATc1 and induces osteoclast differentiation. As Mendoza Bertelli *et al* (41) mentioned, SpA induces osteoclastogenesis via TNFR1 and EGFR, whereas Claro *et al* (26) demonstrated that SpA binding to TNFR-1 activates NF- $\kappa$ B and induces the release of IL-6, and Yi *et al* (49) demonstrated that EGFR cross-talks with RANK to induce osteoclast differentiation. Based on the above studies, whether SpA activates NF- $\kappa$ B by TNFR-1 and EGFR, and then activates NFATc1, which subsequently promotes osteoclast differentiation, requires further experimentation.

In conclusion, the present study indicated that SpA induces osteoclast differentiation and promotes bone resorption *in vitro*, that the NF- $\kappa$ B signaling pathway serves a role in this process, and that these findings provide a molecular basis in order to understand the pathogenesis of *S. aureus*-mediated osteomyelitis.

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