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Original Paper

Staphylococcal Protein A, Panton-Valentine Leukocidin and Coagulase Aggravate the Bone Loss and Bone Destruction in Osteomyelitis

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Key Words
Osteomyelitis • Staphylococcus aureus • Protein A • Panton-Valentine leukocidin • Coagulase

Abstract
Background/Aims: Osteomyelitis is a debilitating infectious disease of the bone which is predominantly caused by *Staphylococcus aureus (S. aureus)*. The purpose of this study was to investigate the role of the *S. aureus* virulence factors, *i.e.* protein A (SpA), Panton-Valentine leukocidin (PVL) and coagulase (Coa) on osteomyelitis. ***Methods:*** The effect of SpA, PVL and Coa on osteoblasts was studied through the following aspects including osteoblast proliferation, apoptosis, bone formation, bone mineralization and RANK-L expression. *S. aureus* overexpressing PVL, SpA or Coa was constructed and used to study the role of PVL, SpA and Coa, respectively. *S. aureus* silencing PVL, SpA or Coa was also constructed and used for reversing verification. Osteoblast proliferation was detected by MTT tetrazolium dye reduction assay. Apoptosis was determined by Annexin V-FITC staining. The levels of pro-caspase 3, cleaved-caspase 3, pro-caspase 9 and cleaved-caspase 9 were detected by western blot. Bone formation markers including collagen I, osteopontin and osteocalcin were detected by real time RT-PCR. Alkaline phosphatase activity was measured by adding p-nitrophenyl phosphate as a phosphatase substrate. Von kossa stain and alizarin red stain were applied for determining phosphate and calcium deposition, respectively. The RANK-L expression was tested by ELISA. ***Results:*** PVL, SpA and Coa inhibited osteoblast proliferation, induced osteoblast apotosis, prohibited bone formation and mineralization and upregulated RANK-L expression. ***Conclusions:*** PVL, SpA and Coa play a critical role on bone loss and bone destruction of osteomyelitis.

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Introduction

Bone development and homeostasis is achieved by a strict balance between bone formation by osteoblasts and resorption by osteoclasts [1]. During the process of bone formation, alkaline phosphatase and type I collagen are expressed at the early stage, both of which are important for bone matrix deposition and mineralization [2, 3]. When fully differentiated, mature osteoblasts also produce regulators of matrix mineralisation including osteocalcin and osteopontin [4, 5]. Osteoblasts then eventually produce receptor activator of nuclear factor (NF)-κB ligand (RANKL) which acts as a signal for the recruitment, proliferation and activation of osteoclasts which initiates the resorption phase [6].

Bone is a sterile organ system, however, bacteria can reach the healthy bone by direct inoculation, haematogenous spread or from a contiguous focus of infection [7]. The invasion of pathogenic bacteria to the healthy bone leads to osteomyelitis, which is associated with significant morbidity and mortality [8]. Osteomyelitis may be acute or chronic and affect joints, long bones, vertebrae and almost any other bone. It is characterised by progressive bone destruction, severe inflammation and bone neoformation [9, 10]. Treatment of osteomyelitis includes antimicrobial therapy, debridement with management of resultant dead space and stabilization of bone [11]. However, the treatment is often unsuccessful due to the continued emergence of antibiotic-resistant strains [12]. Besides, the infected nidus that harbours sessile matrix-protected pathogens is impermeable to antibiotics [13].

A broad range of bacterial species have been isolated from osteomeylitis, among which *Staphylococcus aureus* (*S. aureus*) is the main offender [14]. *S. aureus* has been identified in 38% to 67% of culture-positive cases [15]. *S. aureus* is a gram-positive bacterium that lives as part of the normal microflora on the skin and mucous membranes of humans and animals [16, 17]. It permanently colonizes the moist squamous epithelium of the anterior nares of approximately 20% of the human population and is transiently associated with the rest [18]. The success of *S. aureus* as an opportunistic pathogen is primarily due to its ability to produce a large number of virulence factors, including adherence factors such as staphylococcal protein A (SpA) and exoproteins such as Panton-Valentine leukocidin (PVL), coagulase (Coa), a-haemolysin, hyaluronidase and so on [19-21]. In this study, we investigated the role of SpA, PVL and Coa in osteomyelitis. A better understanding of the mechanisms leading to bone infection may help to improve treatment or develop novel therapies of osteomyelitis.

Materials and Methods

Bacterial strain and culture condition

S. aureus 6850 (ATCC 53657) was used in this study. It was grown in tryptic soy broth (TSB) at 37°C with shaking. Bacteria were harvested and washed by centrifugation at 15,000 g for 5 min and finally re-suspended in phosphate buffered saline (PBS). *S. aureus 6850* suspensions were adjusted to 1×10^9 cells/ml for all experiments. The bacterial supernatant was prepared by growing *S. aureus 6850* in 5 ml TSB at 37°C with shaking for 12-14 h and pelleted for 10 min at 3000 g. Supernatants were sterile-filtered through a Millex-GP filter unit (0.22 um; Millipore, Bedford, MA, USA) and used for the experiments.

Plasmid constructions

lukF and lukS genes which coding the PVL, SpA gene and Coa gene were amplified by PCR using *S. aureus* genomic DNA as template. The PCR primers were listed in Table 1. PCR fragments were digested with *Xba I* and *Xho I*, and cloned between *Xba I* and *Xho I* in pcDNA3.1(+) vector (invitrogen, Carlsbad, CA, USA) to give pcDNA3.1(+)-PVL, pcDNA3.1(+)-SpA and pcDNA3.1(+)-Coa, respectively. Plasmids were transformed into *S. aureus 6850* by electroporation using gene pulser^R II electroporation system (Bio-Rad, Hercules, CA) described as previously protocol [22].

To inactive the PVL, SpA and Coa expression, allelic replacement was used. The procedure was performed as preciously description [23, 24]. Briefly, two fragments of about 800bp that flanked the left side and the right side of the sequence targeted for deletion were amplified by PCR. The two fragments

were digested by the same restriction enzyme and fused by ligation. The fused fragment was further cloned into the shuttle plasmid pMAD (invitrogen, Carlsbad, CA, USA) which contains a temperature-sensitive origin of replication and an erythromycin resistance gene [25]. The resulting plasmid was transformed into *S. aureus 6850* by electroporation. Disruption the targeted gene was achieved by homologous recombination. Erythromycin-sensitive white colonies, which no longer contained the pMAD plasmid, were tested by PCR to confirm the gene replacement. *S. aureus* silencing PVL, SpA or Coa were referred to as PVL (-), SpA(-) and Coa(-), respectively.

Table 1. Primers used in plasmid construction. The restriction enzyme recognition sites are underlined

Primer	Sequence(5' to 3')
lukF+lukS - F	CTCTCTAGATTGTTTGGTAAT
	GAACGGGTTTTTTCG
lukF+lukS - R	CTCCTCGAGAGTGAATGCCCT
	TATTTAAATAATCCGCCC
SpA - F	GCGTCTAGAATGTTGAAAAAG
	AAAAACATTA
SpA - R	CCGCTCGAGTTATAGTTCGCG
	ACGACG
Coa - F	CGCTCTAGAAGCTTATTTACA
	TGGGAT
Coa - R	CCGCTCGAGTTATTTTGTTAC
	TCTAGGC

Cell culture and infection

The mouse clonal MC3T3-E1 pre-osteoblastic cell line (ATCC, Middlesex, UK) was used in this study. It is a common cell line and is used routinely for the assessment of osteoblast function [26, 27]. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10% (v/v) foetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 2% penicillin-streptomycin solution and 1% L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) in a 5% carbon dioxide (CO_2) atmosphere at 37°C. The media was replaced every 3-4 days and after confluency, cells were harvested using trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) and re-suspended in medium.

MC3T3-E1 was infected with *S. aureus 6850* as previously described [28, 29]. Briefly, MC3T3-E1 was infected with a multiplicity of infection (MOI) of 100. After 3h cells were washed and lysostaphin (20mg/ml) was added for 30 min to lyse all extracellular *S. aureus 6850*, then fresh culture medium was added to the cells. The washing, lysostaphin and medium exchange was repeated every 2-3 days to remove all extracellular staphylococci, which might have been released from the infected cells.

MTT cell proliferation assay

Overnight cultures of *S. auerus* were harvested, washed and fixed in 4.8% formaldehyde. *S. aureus* were centrifuged at 15,000 g for 5 minutes and re-suspended in a-MEM at 1×10^9 cells/ml. MC3T3-E1 cells were plated at 5×10^5 cells per well and incubated with fixed *S. aureus* (1×10^9) or comparative *S. aureus* supernatant at 37°Cwith 5% CO_2 for 96 h. Cell proliferation was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, Sigma) assay as described previously with minor modifications [30]. Briefly, The cultures were incubated with the MTT at a final concentration of 0.5 mg/mL for 3 h at 37°C. MTT is cleaved by an enzyme in the respiratory chain in mitochondria if the cell is viable, thereby generating MTT formazan, a purple, highly visible product. After purple precipitate is visible, a detergent solution was added to lyse the cells and solubilize crystals of formazan. The samples were read at a wavelength of 570 nm. All assays were performed in triplicate. Un-infected MC3T3-E1 cells were seeded and cultured as a control.

Apoptosis detection

Apoptosis was assayed using an Annexin V-FITC/PI Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer guidelines with minor modifications as described [31]. MC3T3-E1 cells (1×10^6) treated with formaldehyde fixed *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or *S. aureus* supernatant for 24 h were collected, washed with PBS twice and incubated with Annexin V-FITC/PI in the dark for 15 min at RT. Then the cells were washed and analyzed by flow cytometry. Un-infected osteoblasts were used as negative control. Cells in the lower right quadrant represented apoptosis and in the upper right quadrant represented necrosis or post apoptotic necrosis [32].

The levels of pro-caspase 3, cleaved-caspase 3, pro-caspase 9 and cleaved-caspase 9 were detected by western blot. MC3T3-E1 cells treated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or *S. aureus* supernatant for 24 h were collected and lysed in RIPA buffer containing 1× protease inhibitor cocktail on ice for 10 minutes. Lysates were centrifuged at 13,000×g for

20 min and the supernatants were used. Proteins were separated on a 10% sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) gel and transferred to polyvinyl difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) for 1 h. After blocking, the membranes were incubated with primary antibodies (Cell Signaling, MA, USA) against pro-caspase 3, cleaved-caspase 3, pro-caspase 9 or cleaved-caspase 9 at 4°C over night, respectively. The membranes were washed and then incubated with horseradish peroxidase (HRP) secondary antibody. Detection of the target proteins on the membranes was performed using the ECL Western Blotting Detection Reagents (Thermo Scientific Pierce, Rockford, IL, USA). Gyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) used as a loading control was also determined in each sample.

Table 2. Primers used in real time RT-PCR analysis

Primer	Sequence(5' to 3')
collagen I - F	TCTCCACTCTTCTAGTTCCT
collagen I - R	TTGGGTCATTTCCACATGC
osteopontin - F	GGCATTGCCTCCTCCCTC
osteopontin - R	GCAGGCTGTAAAGCTTCTCC
osteocalcin - F	TCTGACAAACCTTCATGTCC
osteocalcin - R	AAATAGTGATACCGTAGATGCG
GAPDH - F	CACCATGGAGAAGGCCGGGG
GAPDH - R	GACGGACACATTGGGGGTAG

Real Time RT-PCR analyses of bone formation markers expression

The expression of bone formation markers including collagen I, osteopontin and osteocalcin were detected by real time RT-PCR. RNA was isolated from MC3T3-E1 cells treated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or *S. aureus* supernatant on days 0, 7, 14 and 21 respectively. Isolation was carried out by using Trizol (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized by using random primers, oligo primers and PrimeScript Reverse Transcriptase (Takara, Dalian, China). The mRNA levels of type I collagen, osteopontin and osteocalcin were determined by the ABI PRISM 7300 System (Applied Biosystems, Foster City, California, USA) using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. As endogenous controls for normalization of the amount of RNA in the reaction and for reverse transcription efficiency, the cDNA of GAPDH was measured in parallel separate amplification reactions. Negative controls (blanks, no RNA) were included to discard contamination. The final results were determined from three independent assays. The relative quantification of target RNA was achieved by the comparative threshold cycle (C_T) method and the target C_T number were normalized to GAPDH. Primers for real time RT-PCR were listed in Table 2.

Measurement of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured by adding p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow when dephosphorylated by ALP. Briefly, after 7-day infection, MC3T3-E1 cells were lysed with lysis buffer containing 0.1 M Na acetate, 2% Triton X-100 and 10mM pNPP and incubated in the dark for 1 h at 37°C. The reaction was stopped using 0.3 M NaOH (Sigma-Aldrich, St. Louis, MO, USA) and read at a wavelength of 540 nm. All assays were performed in triplicate.

Staining the phosphates and calcium-rich deposits on MC3T3-E1

MC3T3-E1 cells were grown on cover slips in a six-well plate. von kossa stain and alizarin red stain were applied for determining phosphate and calcium deposition, respectively. Phosphate deposition was detected after 7-day infection, while calcium deposition was detected after 21-day infection. For von kossa stain, MC3T3-E1 cells were fixed with 4% paraformaldehyde, washed by PBS and further treated with 5% silver nitrate solution in the dark at 37°C for 30 minutes. Silver nitrate solution was then completely washed away and the cells were exposed to a bright light for 15 min to develop color. For alizarin red stain, after fixation, MC3T3-E1 cells were incubated with 2% alizarin red S stain solution for 30 min at RT and then the dye was completely washed away. The cover slips were analyzed using 200× bright field microscopy. Staining was quantified by leeching the cells of the dye and reading absorbance at 540 nm.

Quantification of RANK-L

MC3T3-E1 cells (1×10^6cells/well) were seeded on six well plates and incubated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or *S. aureus* supernatant for 24 hours.

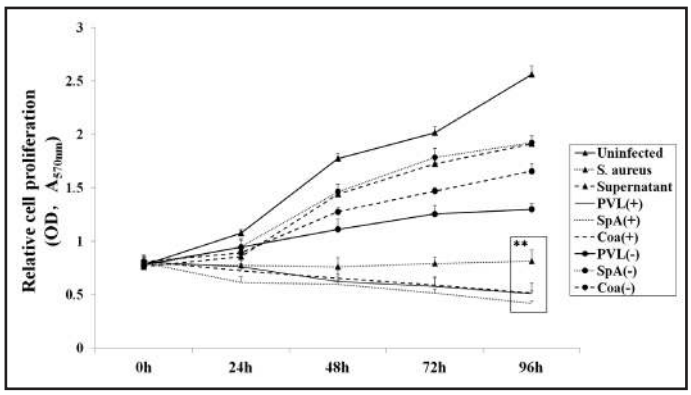

Fig. 1. *S. aureus* inhibits osteoblast proliferation. The proliferation of MC3T3-E1 cells treated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant was detected using the MTT assay at 0h, 24 h, 48 h, 72 h and 96 h. All values are the means ± SD of three replicates, $^{**}p<0.01$ vs uninfected.

Then the culture media was removed, the cells were lysed in 200 ml RIPA buffer. The lysate was centrifuged at 10,000 g for 2 minutes at 4°C. RANK-L was detected using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

Statistical analysis

All data were expressed as means ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. ANOVA with *F* test or Scheffe's *post-hoc* multiple comparison test was used to determine statistical significance between treatment groups, when appropriate. Statistical differences were considered significant at the $^{*}P < 0.05$ or $^{**}P < 0.01$ level.

Results

S. aureus inhibits osteoblast proliferation

In order to determine the effect of *S. aureus*, PVL, SpA and Coa on osteoblast survival or proliferation, MTT tetrazolium dye reduction assay was carried out. *S. aureus* were fixed to maintain bacterial cell integrity yet stunt their growth. This could prevent loss of essential nutrients necessary for osteoblast growth and proliferation. We investigated the ability of MC3T3-E1 cells to proliferate in the presence of *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa) or supernatant for 96 h. Uninfected osteoblasts proliferated normally over the 96 h period. The osteoblasts growth slowed down by addition of the *S. aureus* supernatant or *S. aureus* silencing PVL, SpA or Coa. However, the difference did not reach significance (Fig. 1). Addition of *S. aureus* or *S. aureus* overexpressing PVL, SpA or Coa prevented osteoblasts from proliferation significantly compared to the uninfected osteoblasts ($P<0.01$) (Fig. 1). The proliferation inhibition of *S. aureus* overexpressing PVL, SpA or Coa group was even stronger than that of the *S. aureus* group.

S. aureus induces osteoblast apoptosis

Apoptosis was first determined by measuring the amount of FITC-Annexin V binding following incubation of MC3T3-E1 cells with formalin-treated *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant after a 24 h period. Apotosis was rarely found in the uninfected osteoblasts (<5%). Compared to the uninfected osteoblasts, addition of *S. aureus* supernatant slightly increased the Annexin V binding (<10%), while addition of *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa) induced apoptosis (Fig. 2A). The ratio of osteoblast apoptosis between the *S. aureus* and the supernatant group was statistically different (Fig. 2B, $P<0.05$).

We next investigated the amount of pro-caspase 3, cleaved-caspase 3, pro-caspase 9 and cleaved-caspase 9 following incubation of MC3T3-E1 cells with formalin-treated *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant after a 24 h period. In the uninfected osteoblasts, caspase 3 cleavage and caspase 9 cleavage were minimal. No statistical difference of the pro-caspase 3, cleaved-caspase 3, pro-caspase 9 or cleaved-caspase 9 expression was found between the *S. aureus* silencing PVL, SpA or Coa

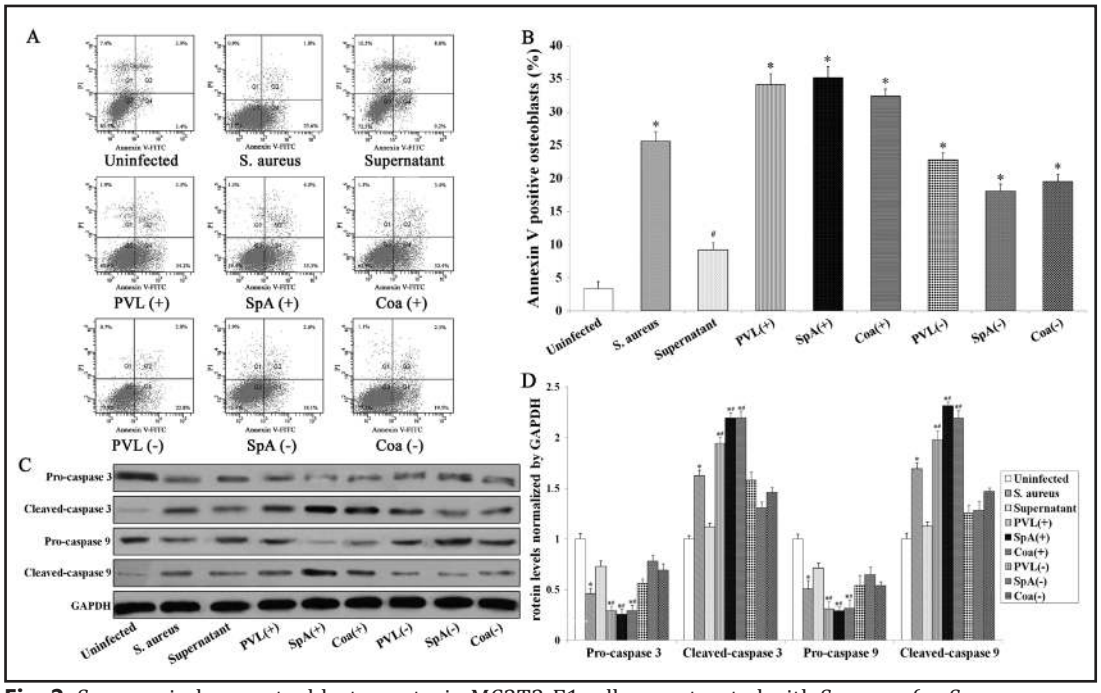

Fig. 2. *S. aureus* induces osteoblast apoptosis. MC3T3-E1 cells were treated with *S. aureus* (or *S. aureus* over-expressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant for 24h. (A) To measure Annexin V, treated osteoblasts were re-suspended in 100μl of FITC-Annexin V antibody. Suspensions were incubated in the dark for 15 min at RT and analysed by flow cytometry. (B) Quantification of the percentage of Annexin V positive MC3T3-E1 cells subjected to different treatments. (C) Western blot analysis of caspase 3 and caspase 9. (D) Quantitative analysis of caspase 3 and caspase 9 protein levels normalized by GAPDH. All values are the means ± SD of three replicates, $^{*}p<0.05$ vs uninfected, $^{\#}p<0.05$ vs *S. aureus.*

group and the uninfected osteoblasts. Similar situation was also found in the supernatant group. However, after incubation with *S. aureus* or *S. aureus* overexpressing PVL, SpA or Coa, the amount of pro-caspase 3 and pro-caspase 9 decreased significantly ($p<0.05$), while the amount of cleaved-caspase 3 and cleaved-caspase 9 increased significantly ($p<0.05$) (Fig. 2C, 2D). Compared to the *S. aureus* group, the amount of pro-caspase 3 and pro-caspase 9 decreased significantly ($p<0.05$), while the amount of cleaved-caspase 3 and cleaved-caspase 9 increased significantly ($p<0.05$) in the *S. aureus* overexpressing PVL, SpA or Coa group (Fig. 2C, 2D).

S. aureus inhibits bone formation markers expression

Osteoblasts secrete proteins including collagen I, osteopontin and osteocalcin to form extracellular matrix. To investigate the effect of *S. aureus*, PVL, SpA and Coa on the osteogenesis, the expression levels of collagen I, osteopontin and osteocalcin were detected by real time RT-PCR at 0d, 7d, 14d, and 21d. The gene expression was normalized to GAPDH expression and depicted as fold change *vs* uninfected group at 0d. Collagen I is an early stage marker of osteogenesis that is typically expressed at day 7. The collagen I was increased about 2-fold in the uninfected osteoblasts at day 7. Compared to the uninfected MC3T3-E1, the expression of collagen I at day 7 was significantly lower in the MC3T3-E1 cells which were treated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant ($p<0.01$, Fig. 3A). The collagen I expression gradually decreased back to baseline at day 14 and 21 in the uninfected cells. There was no difference of collagen I expression in all the groups both at day 14 and day 21.

Osteopontin is a mid stage markers of osteogenesis. The osteopontin was increased about 18-fold in the uninfected osteoblasts at day 14. Compared to the uninfected group,

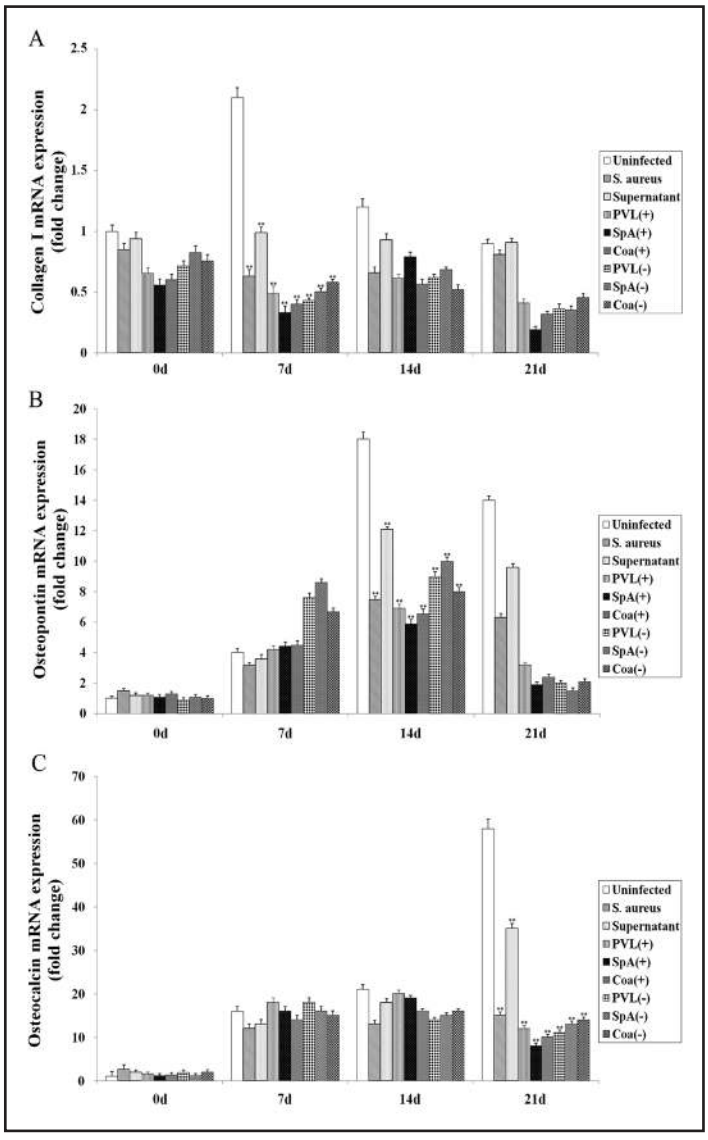

Fig. 3. MC3T3-E1 cells were treated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant and incubated for 21 days. The mRNA expression of collagen I, osteopontin and osteocalcin was detected with RT-PCR at 0d, 7d, 14d, and 21d. The mRNA expression of collagen I (A), osteopontin (B) and osteocalcin (C) was normalized to GAPDH expression and depicted as fold change vs Uninfected group at 0d. All values are the means ± SD of three replicates, $^{**}p<0.01$ vs uninfected.

the expression of osteopontin at day 14 was significantly lower in the *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant group ($p<0.01$, Fig. 3B). Compared to the uninfected group, no statistical difference was found in the expression of osteopontin at day 7 in *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant group. Similar situation was also found at day 21.

Osteocalcin is a late stage marker of osteogenesis. Expression of osteocalcin was greatly increased at day 21 in the uninfected cells (at almost 60-fold). Addition of *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant did not cause the great increase of osteocalcin expression at day 21 and resulted in significantly difference of osteocalcin expression between the uninfected group and the *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant group ($p<0.01$, Fig. 3C). Compared to the uninfected group, no statistical difference was found in the expression of osteocalcin at day 7 in *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant group. Similar situation was also found at day 14.

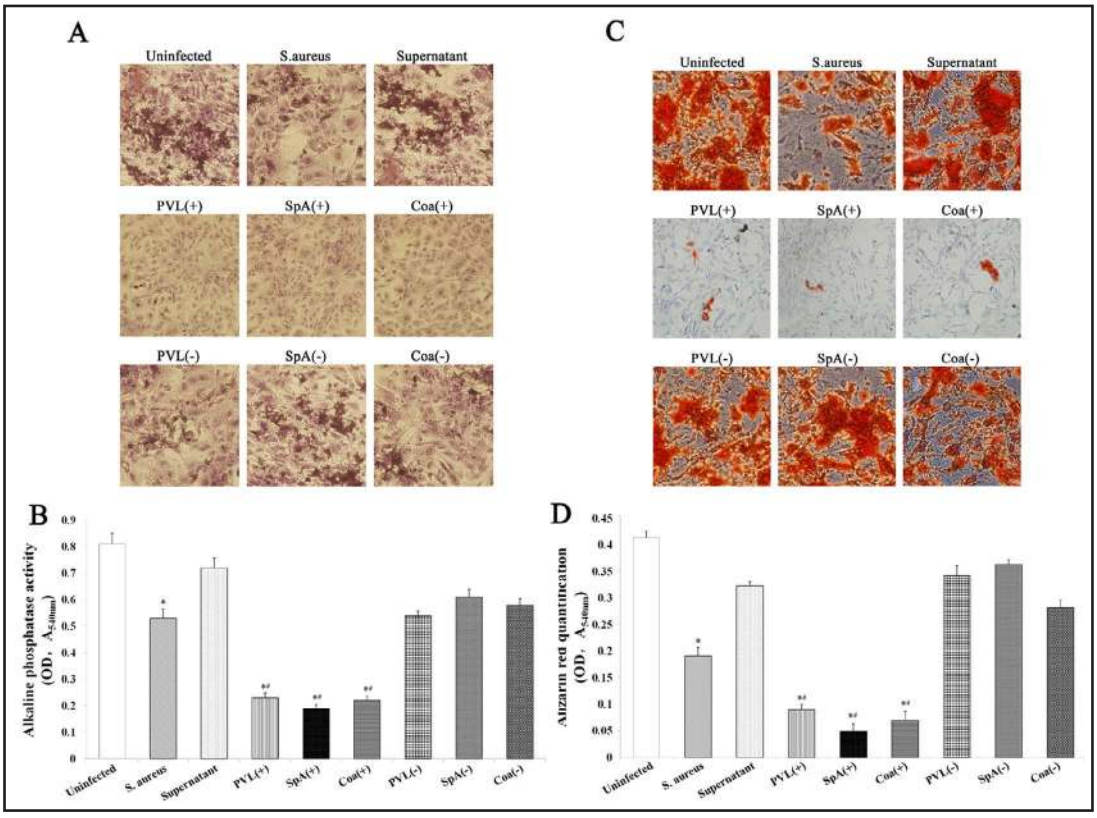

Fig. 4. *S. aureus* prevents osteoblast mineralisation. MC3T3-E1 cells were treated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant and incubated for 21 days. (A) At the 7 day infection, von kossa stain was added to the osteoblasts to determine phosphate deposition. (B) At the 7 day infection, osteoblasts were lysed with 1 ml of lysis buffer containing a substrate for alkaline phosphatase (0.1 M Na acetate, 2% Triton X-100 and 10mM p-nitrophenol phosphate) and incubated in the dark for 1 hour at 37°C, and reading absorbance at 540 nm. (C) Following a 21 day infection, alizarin red stain (2%) was added to the osteoblasts. (D) Staining was quantified by leeching the cells of the dye and reading absorbance at 540 nm. Representative images for both stains were obtained using 200× bright field microscopy. All values are the means ± SD of three replicates, $^{*}p<0.05$ vs uninfected, $^{\#}p<0.05$ vs *S. aureus.*

S. aureus prevents osteoblast mineralisation

The calcification and mineralisation of bone matrix which facilitated by osteoblasts are essential for the strength and rigidity of the skeletal system. To estimate the osteoblastic mineralisation and calcification, staining for phosphates (von Kossa) at day 7 and for calcium deposition (Alizarin red) at day 21 were performed. Representative images of von kossa stain and alizarin red stain were obtained by brightfield microscopy and shown in Fig. 4A and 4C, respectively. Uninfected MC3T3-E1 showed signs of both phosphate and calcium deposition. Compared to the uninfected MC3T3-E1, the phosphate deposition of *S. aureus* overexpressing PVL, SpA or Coa was decreased greatly (Fig. 4A), the calcium deposition of *S. aureus* (*S. aureus* overexpressing PVL, SpA or Coa) was significantly decreased (Fig. 4C). Quantification of the calcium deposition (alizarin red staining) supported the results ($p<0.05$, Fig. 4D). What's more, compared to the *S. aureus* group, the calcium deposition was significantly decreased in the *S. aureus* overexpressing PVL, SpA or Coa group (Fig. 4D, $p<0.05$).

ALP is an enzyme found in osteoblasts and pivotal for the bone mineralization. The ALP activity was determined in this study. Compared to the uninfected MC3T3-E1 cells, the ALP activity was decreased significantly in the *S. aureus* (*S. aureus* overexpressing PVL, SpA or

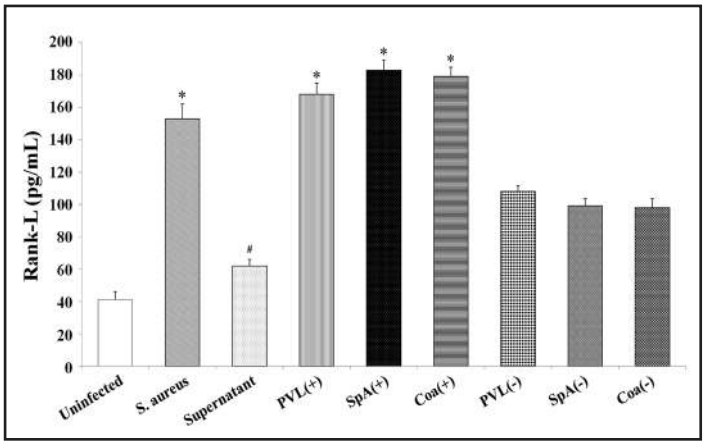

Fig. 5. *S. aureus* induces RANK-L in osteoblasts. MC3T3-E1 cells were treated with *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant for 24h. Two hundred micro litres of the RIPA buffer was added to each well resulting protein was removed and centrifuged at 10,000 g for 2 minutes. RANK-L was detected using an ELISA kit. All values are the means ± SD of three replicates, $^{*}p<0.05$ vs uninfected, $^{\#}p<0.05$ vs *S. aureus.*

Coa) group (p<0.05, Fig. 4B). The ALP activity of *S. aureus* overexpressing PVL, SpA or Coa group was significantly lower than that of the *S. aureus* group (p<0.05, Fig. 4B).

S. aureus induces RANK-L in osteoblasts

RANK-L which expressed on osteoblasts is a key molecule involved in bone remodeling. To investigate the effect of *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant on RANK-L expression, quantitative analysis was performed by ELISA. Uninfected MC3T3-E1 cells had a low level of RANK-L expression. The expression of RANK-L was increased in *S. aureus* (or *S. aureus* overexpressing PVL, SpA or Coa or silencing PVL, SpA or Coa), or supernatant group. And the difference between the uninfected group and the *S. aureus* or *S. aureus* overexpressing PVL, SpA or Coa group reached statistically significant (p<0.05, Fig. 5). The RANK-L expression of *S. aureus* group was significantly higher than that of supernatant group (p<0.05, Fig. 5).

Discussion

Osteomyelitis is a debilitating infectious disease of the bone which is predominantly caused by *S. aureus*. It is characterised by progressive bone loss or bone destruction. Currently the mechanism of bone loss or bone destruction in osteomyelitis is poorly understood. In this study, we investigated the role of three common *S. aureus* virulence factors including SpA, PVL and Coa on osteomyelitis.

SpA is a typical member of the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs). It binds to a variety of ligands including the Fc region of IgG, von Willebrand factor (vWF), tumour necrosis factor receptor-1 (TNFR-1), the Fab-heavy chains of the Vh3 subclass and the epidermal growth factor receptor (EGFR) [33-37]. It is an important virulence factor of *S. aureus* for its mediation of colonization. PVL is a kind of exotoxin secreted by *S. aureus.* It is classified as a bicomponent cytolysin (LukF-PV and LukS-PV) and hetero-oligomerize to form a pore. PVL is cytotoxic toward leukocytes [38]. Coa is an enzyme secreted by *S. aureus.* It directly binds to prothrombin and leads to plasma clot [39]. The effect of SpA, PVL and Coa on osteoblasts was studied through the following aspects including osteoblast proliferation, apoptosis, bone formation, bone mineralization and RANK-L expression. *S. aureus* overexpressing PVL, SpA or Coa was constructed and used to study the role of PVL, SpA and Coa, respectively. *S. aureus* silencing PVL, SpA or Coa was also constructed and used for reversing verification.

Our study demonstrated that *S. aureus* inhibited osteoblast proliferation and induced osteoblast apotosis. The proliferation inhibition was aggravated by *S. aureus* overexpressing PVL, SpA or Coa and the proliferation was almost unaffected by *S. aureus* silencing PVL, SpA or Coa. The results indicated that SpA, PVL and Coa might trigger a signal transduction

pathway that inhibits osteoblast proliferation. Significant apoptosis was observed in both *S. aureus* overexpressing PVL, SpA or Coa group and *S. aureus* silencing PVL, SpA or Coa group. The apoptosis was more serious in the presence of PVL, SpA or Coa. These results suggested that other *S. aureus* proteins besides PVL, SpA and Coa might induce apoptosis. Further experiment showed that the cleaved-caspase 3 and cleaved-caspase 9 expressed highly in *S. aureus* overexpressing PVL, SpA or Coa group. Caspase-9 is an initiator caspase and links to the mitochondrial death pathway of apoptosis [40]. It interacts with caspase 3. The activation of both caspase 3 and caspase 9 in the presence of PVL, SpA or Coa indicating that PVL, SpA or Coa triggered mitochondrial death pathway.

Bone formation is typically characterised by the sequential expression of a series of bone formation markers including collagen I, osteopontin and osteocalcin. The expression of collagen I, osteopontin and osteocalcin were decreased significantly in both *S. aureus* overexpressing PVL, SpA or Coa group and *S. aureus* silencing PVL, SpA or Coa group in this study. The results suggested that bone formation was disrupted in these situations. Other proteins beside PVL, SpA or Coa may inhibit the osteogenesis. Mineralisation is a process where phosphate and calcium becomes deposited in bone [41]. This gives the bones additional strength and rigidity. Our study showed that mineralization (phosphate and calcium deposition) was inhibited significantly in the *S. aureus* overexpressing PVL, SpA or Coa group, while phosphate and calcium deposition formed normally in *S. aureus* silencing PVL, SpA or Coa group. Besides, the ALP activity was also decreased significantly in the *S. aureus* (*S. aureus* overexpressing PVL, SpA or Coa) group. These indicated that bone mineralization had been inhibited by PVL, SpA or Coa.

RANK-L is a membrane protein of osteoblasts. It stimulates differentiation in osteoclasts and involves in bone resorption [42]. A significant increase in RANK-L expression was found in *S. aureus* overexpressing PVL, SpA or Coa group, The increase in RANKL can trigger osteoclast-induced bone resorption and bone destruction. This may also lead to bone loss in osteomyelitis. Furthermore, *S. aureus* silencing PVL, SpA or Coa had no affect on the RANK-L expression, which revealed that PVL, SpA and Coa were the important virulence factors of *S. aureus* in osteomyelitis.

In summary, we evaluated the role of PVL, SpA or Coa on osteomyelitis by constructing *S. aureus* overexpressing PVL, SpA or Coa and *S. aureus* silencing PVL, SpA or Coa. PVL, SpA and Coa inhibited osteoblast proliferation, induced osteoblast apotosis, prohibited bone formation and mineralization and triggered osteoclast-induced bone resorption and bone destruction by upregulated RANK-L expression. PVL, SpA and Coa play a critical role on bone destruction and bone loss of osteomyelitis.

Conflict of Interests

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Erratum

The original article by Jin et al., entitled "Staphylococcal Protein A, Panton-Valentine Leukocidin and Coagulase Aggravate the Bone Loss and Bone Destruction in Osteomyelitis" [Cell Physiol Biochem 2013;32:322-333 (DOI: 10.1159/000354440)], failed to discuss and cite previous work and to acknowledge the original authors for identifying.

The authors wish to point out that Staphylococcus aureus protein A has previously been shown to interact with osteoblasts. As a result of this interaction osteoblasts fail to proliferate, mineralize or express critical osteogenic markers collagen type I, osteopontin or osteocalcin when engaged with protein A. In addition, the interaction resulted in apoptosis and increased recruitment of the bone resorbing cells, osteoclasts. These interactions were identified by another group and published in 2011 and 2012 (1, 2, respectively). The authors of the current article failed to discuss or cite this previous work and acknowledge that due credit must go to the original authors for identifying this interaction.

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