

Original Article

Attachment of *Staphylococcus aureus* is required for activation of nuclear factor kappa B in human osteoblasts

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Nuclear factor kappa B (NF- κ B) plays a prominent role in the pathogenesis of infectious diseases. *Staphylococcus aureus* (*S. aureus*), which can attach to and invade human osteoblasts, is the most common causative agent of osteomyelitis. To determine whether *S. aureus* can activate NF- κ B in human osteoblasts and explore the possible factors of activation in response to infection, we used flow cytometry, enzyme-linked immunosorbent assay, immunoblots, and electrophoretic mobility shift assays to quantify the invasion of bacteria, to measure the interleukin-6 (IL-6) of culture supernatants, and to investigate the I κ B α degradation and NF- κ B activation in human osteoblasts. Moreover, we explored the possible factors responsible for the activation of NF- κ B by preventing *S. aureus* from physically touching human osteoblasts or inhibiting the invasion of *S. aureus* into human osteoblasts under co-culture conditions, by incubating proteinase K-treated or ultraviolet-killed *S. aureus* with human osteoblasts and by treating human osteoblasts with peptidoglycan (PGN) or lipoteichoic acid (LTA). We found that *S. aureus* induced the I κ B α degradation and NF- κ B activation, which could regulate IL-6 secretion in the culture supernatants of human osteoblasts in response to infection. In addition, the maximal I κ B α degradation and NF- κ B activation in human osteoblasts occurred prior to the maximal invasion of *S. aureus*. It was the attachment not invasion or the secreted soluble factor(s), PGN, LTA of *S. aureus*, that could induce the I κ B α degradation and NF- κ B activation in human osteoblasts. These results indicated that *S. aureus* can activate NF- κ B in human osteoblasts and that the attachment of *S. aureus* is required for this activation in response to infection.

Keywords *Staphylococcus aureus*; human osteoblasts; attachment; nuclear factor kappa B; osteomyelitis

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Introduction

Osteomyelitis is an inflammation of bone associated with abnormal bone remodeling and massive bone resorption. The Gram-positive organism *Staphylococcus aureus* (*S. aureus*) is the most common causative agent of osteomyelitis accounting for ~80% of all human cases [1]. Recent studies have demonstrated that *S. aureus* can attach to and invade osteoblasts [2,3]. The attachment and invasion of bacteria to host cells is thought to involve numerous pathogen–host cell interactions [4]. We focused on further revealing the mechanism of pathogenesis in osteomyelitis and reevaluating the cellular pathophysiology of this disease process. However, little is known about the interaction of *S. aureus* and cultured osteoblast in response to infection.

Nuclear factor kappa B (NF- κ B) is a dimeric protein composed of members of Rel/NF- κ B family, and NF- κ B dimers are retained into the cytoplasm by inhibitory protein I κ B α [5,6]. Different stimuli determine I κ B α phosphorylation, the subsequent ubiquitination, and degradation [6]. Free NF- κ B translocates into the nucleus to initiate the transcriptional activation of NF- κ B-dependent genes [7] including chemokines, cytokines, adhesion molecules, and growth factors [8].

Recently, studies have demonstrated that NF- κ B in eukaryocytes could be activated by diverse bacteria, such as *Porphyromonas gingivalis* [9], *Streptococcus pyogenes* [10,11], and *Mycobacterium bovis* bacillus Calmette–Guerin (BCG), in response to infection [12]. In addition, bacterial invasion is an important process in the pathogenesis of infectious diseases in which NF- κ B plays a prominent role [4]. The invasion of *Shigella flexneri* into HeLa cells induced the DNA-binding activity of NF- κ B [13] and the invasion of *S. aureus* into bovine endothelial cells was also associated with the active state of NF- κ B [4]. To date, it is not clear whether *S. aureus* can induce the activation of NF- κ B in osteoblasts in response to infection. Here, we

hypothesize that *S. aureus* may activate NF- κ B in human osteoblasts and the invasion of *S. aureus* may be required for this activation in response to infection.

In the present study, we quantified the invasion of *S. aureus* into human osteoblasts in response to infection, observed the state of NF- κ B in cultured human osteoblasts, and explored the possible factors responsible for the activation of NF- κ B. Our results provided further insight into the pathogenesis of infection between osteoblasts and *S. aureus*.

Materials and Methods

Bacteria, human osteoblasts, and culture conditions

The *S. aureus* UAMS-1 (ATCC 49230), which is a human osteomyelitis strain isolated clinically [14], was used in this study. The ATCC 49230 was obtained from the American Type Culture Collection (Manassas, USA). The *S. aureus* cells were grown overnight (16 h) in 5 ml of tryptic soy broth (Oxoid, Basingstoke, UK) in a shaking water bath at 37°C. The bacteria were harvested by centrifugation for 10 min at 4300 g at 4°C and washed twice in 5 ml of Hanks' balanced salt solution (HBSS). The pellets were then resuspended in 5 ml of Dulbecco's modified eagle medium (DMEM)/F12 medium (Invitrogen, Carlsbad, USA). The *S. aureus* were labeled with 5-(6)-carboxy fluorescein diacetate succinimidylester (CFSE) (Molecular Probes, Eugene, USA) as previously described [15] to quantify the invasion of labeled bacteria by flow cytometry. Briefly, bacteria (1×10^9 /ml) were washed twice with sterile phosphate-buffered saline (PBS), suspended in 1 μ g/ml CFSE in PBS, incubated for 20–30 min under constant shaking at 37°C, and washed three times with PBS prior to use.

The SV40 human osteoblasts (SV40 hOBs, ATCC) used in this work were previously characterized as authentic osteoblasts [16]. The cells were seeded in 25-cm² flasks and incubated at 37°C in 5% CO₂ with DMEM/F12 medium supplemented with 0.3 mg/ml G418 (Sigma, St. Louis, USA) and 10% fetal bovine serum (FBS; HyClone, Logan, USA). The cells were propagated according to the manufacturer's instruction. Once osteoblasts reached ~80% confluency, cells were trypsinized [0.025% trypsin–0.01% ethylenediaminetetraacetic acid (EDTA)], washed in DMEM/F12 medium with 0.3 mg/ml G418 and 10% FBS, and seeded into 6- and 12-well plates. Two to 3 h before the addition of bacteria, the cells were washed with PBS twice and then incubated with DMEM/F12 medium with 10% FBS and infected as described in the following section.

Bacteria invasion assay

The invasion of *S. aureus* into human osteoblasts was quantified using flow cytometry analysis as described

previously [15,17]. Briefly, osteoblasts were plated at 5×10^4 cells/well in 12-well tissue culture plates and exposed to CFSE-labeled *S. aureus* at a multiplicity of infection (MOI) of 250 for different designated times. Since *S. aureus* invasion into osteoblasts is dependent on the polymerization of actin microfilaments and clathrin-dependent receptor-mediated endocytosis [2,3], we further confirmed the role of microfilaments and clathrin-coated pit pathway by flow cytometry. Briefly, cells were pre-treated with 5 μ g/ml cytochalasin D (Sigma) or 0.6 mM monodansylcadaverine (MDC, Sigma) for 30 min, which are inhibitors of microfilaments and clathrin-coated pits pathway, respectively [2], and then exposed to *S. aureus* at an MOI of 250 for 2 h. After infection, the cell cultures were washed three times with 4 ml HBSS and incubated for 3 h in 4 ml of DMEM/F12 medium containing 25 μ g/ml gentamicin to kill the remaining extracellular *S. aureus* before suspension for 1 min in trypsin. The suspended cells were washed twice with ice-cold flow cytometry buffer (PBS containing 1% fetal serum) and resuspended in 1 ml flow cytometry buffer. To eliminate signals from extracellular *S. aureus*, 0.4% trypan blue solution (Sigma) was added directly to a final concentration of 0.2% before analysis. The samples were analyzed on a FACS Calibur (Becton–Dickinson, San Jose, USA) by gating on eukaryotic cells based on forward and side scatter, and the cell-associated fluorescence of 10,000 cells/sample was measured in fluorescence channel 1 (FL1-H) to detect CFSE fluorescence. To obtain an estimate of bacteria invasion, the percentage of CFSE-positive cells was calculated by the mean fluorescence intensity of these cells to obtain the uptake index.

Infection assay

To investigate I κ B α degradation and NF- κ B activation in human osteoblasts in response to infection, 2×10^6 cells/well in six-well tissue culture plates were infected with *S. aureus* at an MOI of 250 for the designated time.

To further detect the possible factors responsible for this activation, cells were grown in the lower compartment of a Transwell bicameral chamber with a 0.4- μ m-pore size polycarbonate filter (Corning, New York, USA), and *S. aureus* were added to the upper chamber, which could prevent the physically touching between osteoblasts and *S. aureus*. In addition, cells were pre-treated with 5 μ g/ml cytochalasin D or 0.6 mM MDC for 30 min to prevent the invasion of *S. aureus* into human osteoblasts [2,3], then cells were infected with *S. aureus* at an MOI of 250 for different designated times.

To confirm the role of *S. aureus* attachment on the activation of NF- κ B, cells were incubated with proteinase K-treated *S. aureus* as previously described [18–20] with minor changes or with ultraviolet (UV)-killed *S. aureus* (irradiated under UV light for 5 min) at an MOI of 250 for

the designated time. The treatments of *S. aureus* with proteinase K were as follows: briefly, proteinase K (Sigma) (500 μ g in 0.5 ml of 50 mM Tris, pH 7.5) was added to bacterial pellets obtained from 25 ml of *S. aureus* (centrifuged at 4300 g for 10 min). The enzymatic reactions were carried out at 37°C for 45 min with rotatory mixing at 24 rpm and terminated by addition of 30 μ l of 0.1 M phenylmethylsulfonyl fluoride (PMSF, Sigma). Cells were sedimented at 4300 g for 10 min. To arrest any residual enzymatic activities, bacterial pellets were again treated with PMSF for 5 min at 4°C followed by four washes with HBSS; the pellets were then resuspended in DMEM/F12 medium.

To investigate whether peptidoglycan (PGN, Sigma) and lipoteichoic acid (LTA, Sigma), which are two main cell wall components of *S. aureus*, are involved in the activation of NF- κ B, cells were treated with these two reagents at 10 μ g/ml [21–23] for different designated times.

Immunoblot analyses

After infection, osteoblasts were washed in PBS and lysed in cold buffer containing 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1 mM PMSF, 50 mM NaF, and 0.1 mM sodium vanadate. The lysates were centrifuged at 15,000 g for 15 min, and the concentration of protein lysates was determined with the Bradford assay kit (Bio-Rad, Hercules, USA).

The protein extracts (50 μ g each) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Pall Gelman Laboratory, Ann Arbor, USA). The membranes were blocked with 10% non-fat dried milk in Tris-buffered saline for 1 h prior to exposure to rabbit polyclonal anti-I κ B α antiserum (Cell Signaling Technology, Danvers, USA) at 1:200 for 45 min. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cell Signaling Technology, Inc.) at 1:10,000 for 30 min, and the antigens were detected with enhanced chemiluminescence western blotting detection reagents (Amersham Life Science, Inc., Piscataway, USA).

Electrophoretic mobility shift assay

After infection, nuclear extracts were prepared using reagents from the NE-PER extraction system (Pierce, Rockford, USA) as recommended by the manufacturer's instructions. A 5 μ g sample of nuclear extracts was used to test for NF- κ B protein/DNA binding using a LightShift EMSA kit (Pierce) with a biotin 3'-end-labeled NF- κ B oligonucleotide (sense, 5'-AGTTGAGGGGACTTTCCCAGG C-3'; antisense, 5'-GCCTGGGAAAGTCCCCTCAACT-3) (Invitrogen). Briefly, the double-stranded NF- κ B oligonucleotide was end-labeled with biotin on the 3'-end, then

nuclear extracts were incubated with a biotin end-labeled NF- κ B oligonucleotide for 20 min at room temperature to allow DNA/protein bind. For competition assay, a 100-fold molar excess of unlabelled NF- κ B oligonucleotide (cold probe) was pre-incubated for 5 min at room temperature with nuclear extracts before the addition of the labeled NF- κ B oligonucleotide. The reaction products were electrophoresed onto native 6% polyacrylamide gels and transferred to a Hybond-N⁺ membrane (Amersham and Pharmacia Biotech, Piscataway, USA). Transferred DNAs were cross-linked to the membrane at 120 mJ/cm² and detected using a streptavidin–horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to X-ray film (XAR-5; Amersham Life Science, Inc.) and developed using a Kodak film processor.

Enzyme-linked immunosorbent assay

To further investigate whether *S. aureus* can activate NF- κ B in human osteoblasts and whether *S. aureus*-activated NF- κ B signaling pathway modulates the cytokines secretion, enzyme-linked immunosorbent assay (ELISA) was carried out to measure interleukin-6 (IL-6) secretion in culture supernatants. Briefly, 2 \times 10⁶ cells/well were pre-treated with 50 μ M SN50, a special inhibitor of NF- κ B [4], for 1 h prior to the infection of *S. aureus*. After infection, cells were washed with PBS and grown in DMEM/F12 medium supplemented with 0.3 mg/ml G418 and 10% FBS at 37°C for 24 h in a 5% CO₂ atmosphere, then the culture supernatants were collected by centrifuging at 10,000 g for 5 min and measured using ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Statistical analysis

All assays were repeated in three independent experiments. The statistical analysis among groups was performed by ANOVA and SNK test using the SPSS 17.0 software package. The results were expressed as the mean \pm SD. The statistically significant values were defined as $P < 0.05$.

Results

Staphylococcus aureus invaded human osteoblasts

Staphylococcus aureus can attach to and invade human osteoblast [2,3]. We quantified the invasion of CFSE-labeled *S. aureus* into osteoblasts using flow cytometry analysis as described in the 'Materials and Methods' section. Results showed that the invasion of *S. aureus* (uptake index) increased in a time-dependent manner following infection at an MOI of 250. Using 3146.45 at 30 min as the reference value, it was observed that the

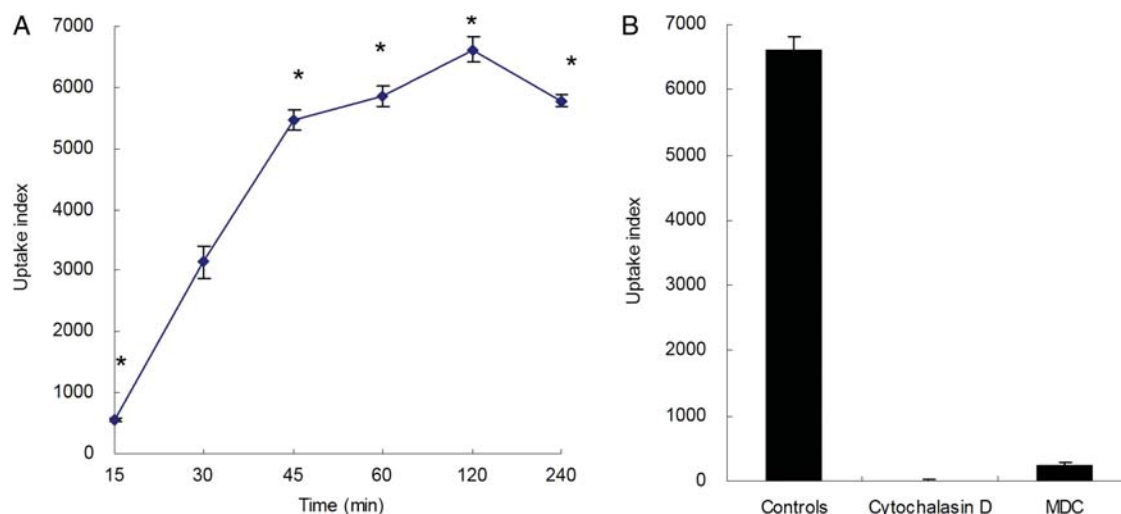


Figure 1 Quantification of invasion of *S. aureus* into human osteoblasts (A) The SV40 human osteoblasts were exposed to *S. aureus* ATCC 49230 labeled with 1 μ g/ml CFSE at an MOI of 250 for the indicated time. (B) The SV40 human osteoblasts were pre-treated by cytochalasin D (5 μ g/ml) or MDC (0.6 mM) for 30 min or without pre-treatment of cytochalasin D and MDC (controls) and exposed to CFSE-labeled *S. aureus* ATCC 49230 at an MOI of 250 for 2 h. After infection, cells were washed, treated with gentamicin, and quantified for the invasion of bacteria by flow cytometry as described in the 'Materials and Methods' section. The uptake index is defined as the total number of invasive bacteria. The data are presented as mean \pm SD ($n = 3$). * $P < 0.05$ vs. value at 30 min.

uptake index reached 1.74 ($P < 0.05$), 1.86 ($P < 0.05$), 2.1 ($P < 0.05$), and 1.84 folds at 45, 60, 120, and 240 min, respectively [Fig. 1(A)]. In addition, 5 μ g/ml cytochalasin D and 0.6 mM MDC could inhibit the invasion by 98.81 and 96.63%, respectively [Fig. 1(B)].

Staphylococcus aureus activated NF- κ B in human osteoblasts in response to infection

We determined the stability of I κ B α in human osteoblasts after infection with *S. aureus*. Uninfected cells had stable I κ B α expression levels and infected cells experienced I κ B α degradation after 15 min, and the level of I κ B α was dramatically decreased at 30 min [Fig. 2(A)].

To correlate the degradation of I κ B α with the nuclear translocation of NF- κ B, electrophoretic mobility shift assays (EMSA) were carried out to investigate the nuclear translocation of NF- κ B. As shown in Fig. 2(B), nuclear proteins from uninfected cells barely displayed binding activity to DNA containing a consensus NF- κ B-binding sequence. However, the DNA-binding activity of NF- κ B was stimulated at 30 min in *S. aureus*-infected cells and reached the maximal level at 45 min. To confirm the specificity of DNA-protein interaction, a 100-fold excess of cold probes was used as a competitor to inhibit the binding activity of NF- κ B at 45 min after infection. As shown in Fig. 2(B), cold probes completely inhibited the binding activity of NF- κ B.

Staphylococcus aureus-activated NF- κ B regulated IL-6 secretion in the culture supernatants of human osteoblasts

IL-6 secretion in culture supernatants of human osteoblasts, which were pre-treated with 50 μ M SN50 for 1 h, then

infected with *S. aureus* at an MOI of 250, was significantly suppressed by 38.87% ($P < 0.05$) compared with that in cells infected with *S. aureus* (Fig. 3). This result not only demonstrates that *S. aureus* could activate NF- κ B in human osteoblasts, but also indicates that *S. aureus*-activated NF- κ B signaling pathway could regulate the IL-6 secretion in culture supernatants in response to infection.

Attachment, not secreted soluble factor(s) or invasion, of *S. aureus* could activate NF- κ B in human osteoblasts

To eliminate the possibility that *S. aureus* secretes a soluble factor(s) that could activate NF- κ B in human osteoblasts, cells were indirectly co-cultured with *S. aureus* in a Transwell bicameral chamber which prevented *S. aureus* from physically touching cells. The immunoblot analysis and the EMSA showed that the secreted soluble factor(s) of *S. aureus* did not induce I κ B α degradation [Fig. 4(A)] and NF- κ B activation [Fig. 4(B)].

To explore whether the invasion or attachment of *S. aureus* can activate NF- κ B in human osteoblasts, cells were pre-treated with inhibitor cytochalasin D (5 μ g/ml) or MDC (0.6 mM) for 30 min, then infected with *S. aureus*. Previous experiments demonstrated that these two inhibitors could decrease the invasion of *S. aureus* by 99% and 96% compared with control, respectively [3]. As indicated in Fig. 5(A,B), there was no difference ($P > 0.05$) for I κ B α degradation at 30 min post-infection between *S. aureus*-infected cells with and without pre-treatment of inhibitor cytochalasin D (5 μ g/ml) or MDC (0.6 mM) for 30 min. Solely cytochalasin D or MDC did not interfere with I κ B α degradation in human osteoblasts ($P > 0.05$) compared with uninfected cells. NF- κ B activation in

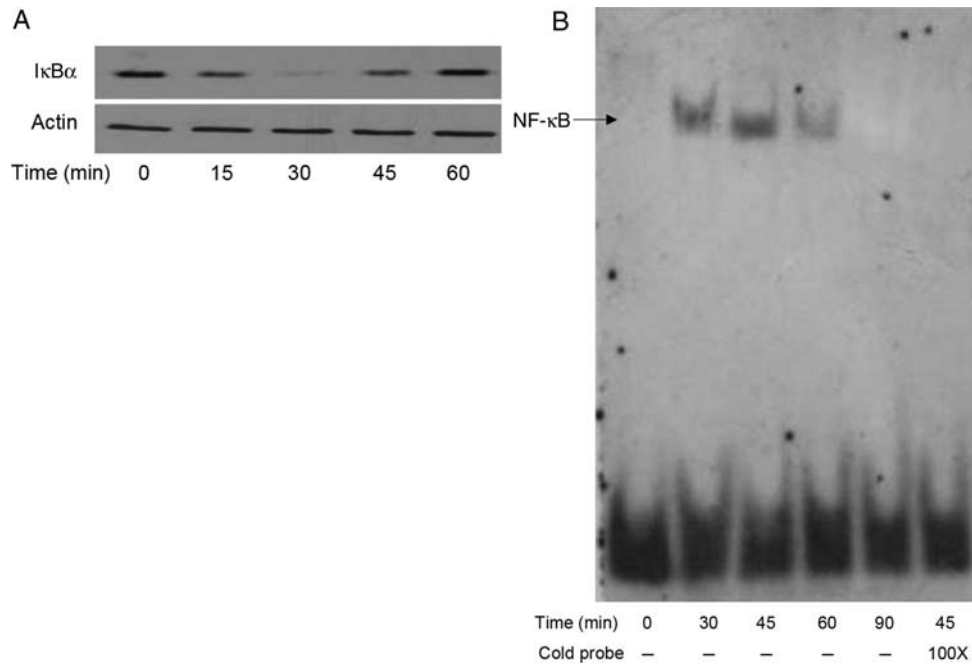


Figure 2 I κ B α degradation and NF- κ B activation in human osteoblasts in response to kinetic infection with *S. aureus* The SV40 human osteoblasts were either uninfected (0 min) or infected with *S. aureus* ATCC 49230 at an MOI of 250 for the indicated time. Immunoblot assay shows the kinetic degradation of cytosolic I κ B α (A) and the EMSA demonstrates NF- κ B activation of nuclear proteins (B) in human osteoblasts. The data are representative of results from three independent experiments.

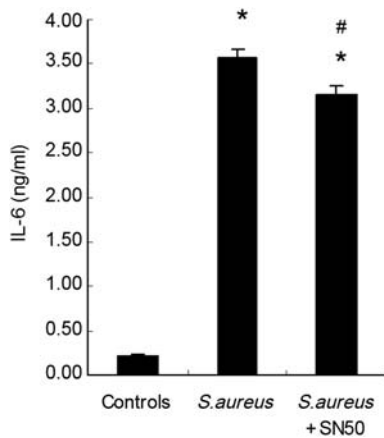


Figure 3 Measurements of IL-6 secretion in the supernatants of human osteoblasts The SV40 human osteoblasts were uninfected (controls) or infected with *S. aureus* ATCC 49230 alone or pre-treated with 50 μ M SN50 for 1 h, then infected with *S. aureus* ATCC 49230 at an MOI of 250 for 1 h. SN50 is an inhibitor of NF- κ B. ELISA was carried out to measure IL-6 secretion in culture supernatants at 24 h post-infection. Data are presented as mean \pm SD from three independent experiments performed in duplicate. * P < 0.05 vs. controls and # P < 0.05 vs. *S. aureus*.

aureus-infected human osteoblasts with pre-treatment of cytochalasin D (5 μ g/ml) or MDC (0.6 mM) could be clearly observed at 45 min post-infection by the EMSA [Fig. 5(C)]. To further confirm that the attachment of *S. aureus* can activate NF- κ B in human osteoblasts, proteinase K was used to extract the bacterial surface proteins

involved in the attachment [18–20]. As shown in Fig. 6(A), I κ B α degradation in human osteoblasts incubated with proteinase K-treated *S. aureus* was dramatically reduced by 72.7% (P < 0.05) compared with cells infected by *S. aureus* at 30 min following incubation. EMSA showed that NF- κ B activation in human osteoblasts was also sharply reduced at 45 min following incubation with proteinase K-treated *S. aureus* [Fig. 6(B)]. In addition, UV was used to kill bacteria maintaining the integrity of the surface structures, including those structures that bound and interacted with osteoblast surface [24]. Immunoblot analysis showed that when human osteoblasts were incubated with UV-killed *S. aureus*, I κ B α degradation occurred at 15, 30, and 45 min following incubation (P < 0.05) compared with controls [Fig. 7(A)]. NF- κ B activation in human osteoblasts was also observed at 30 and 45 min following incubation with UV-killed *S. aureus* [Fig. 7(B)].

In summary, the results indicate that the attachment, not secreted soluble factor(s) or invasion, of *S. aureus* could activate NF- κ B in human osteoblasts.

PGN and LTA were not involved in the activation of NF- κ B in human osteoblasts

To investigate whether PGN and LTA of *S. aureus* were involved in the activation of NF- κ B in human osteoblasts, cells were treated with PGN or LTA at 10 μ g/ml. Figure 8(A) clearly shows that I κ B α degradation did not occur in 1 h following the treatment of PGN or LTA.

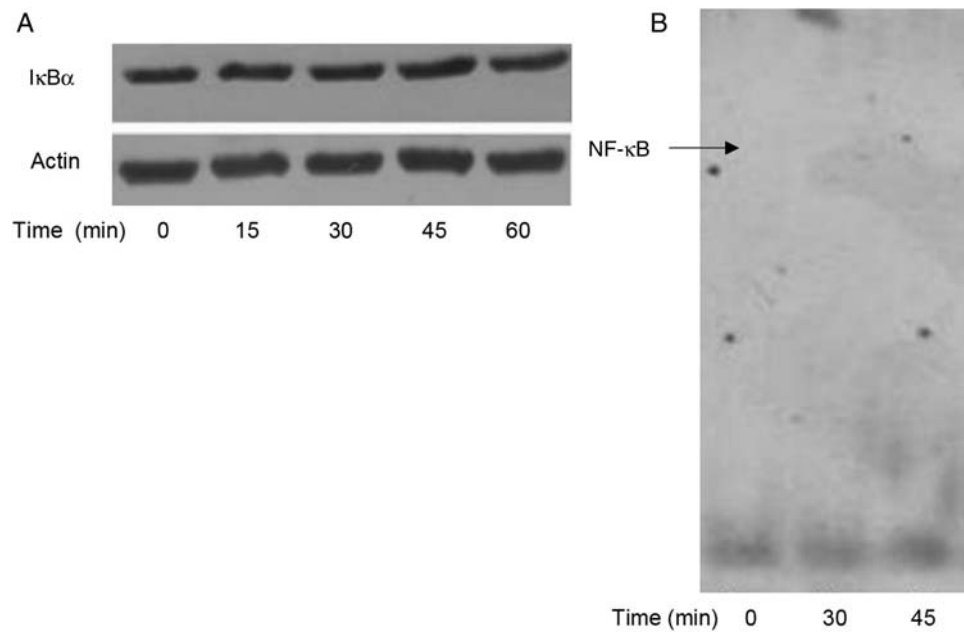


Figure 4 The effect of soluble factor(s) *S. aureus* secreted on the activation of NF-κB in human osteoblasts The SV40 human osteoblasts were indirectly co-cultured with *S. aureus* ATCC 49230 in a Transwell bicameral chamber at an MOI of 250 or cultured alone (0 min) for the indicated time. The immunoblot demonstrates the degradation of cytosolic IκBα (A) and the EMSA shows NF-κB activation of nuclear proteins (B) in human osteoblasts. The data are representative of results from three independent experiments.

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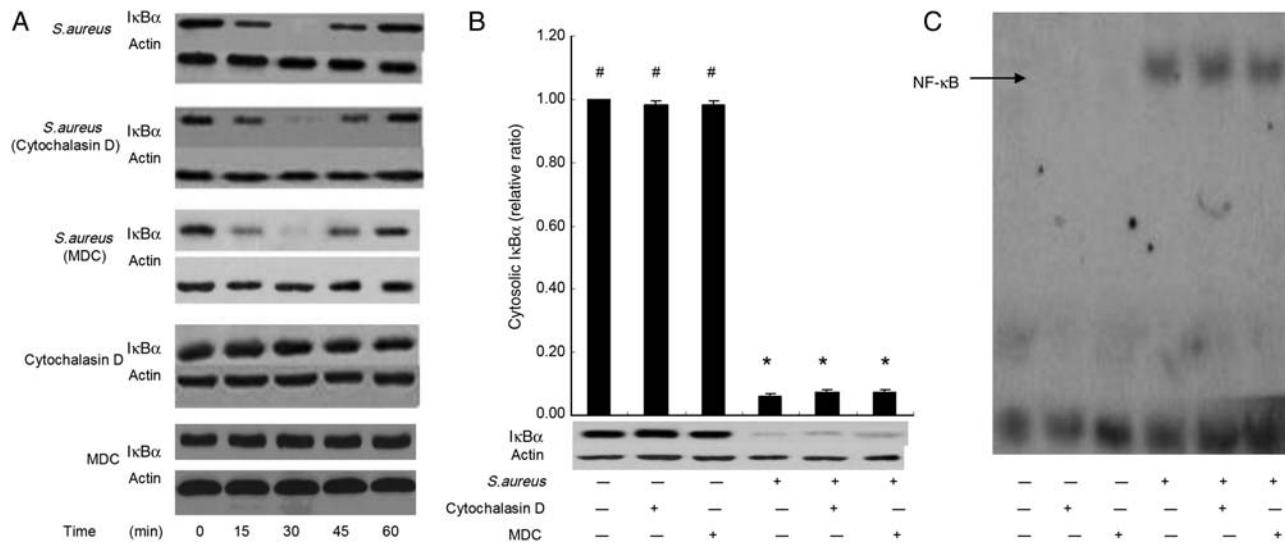


Figure 5 The effect of bacterial invasion or attachment on the activation of NF-κB in human osteoblasts The SV40 human osteoblasts were infected with *S. aureus* ATCC 49230 at an MOI of 250 or pre-treated with cytochalasin D (5 μg/ml) or MDC (0.6 mM) for 30 min prior to infection, solely treated with cytochalasin D (5 μg/ml) or MDC (0.6 mM), or uninfected. (A) Immunoblot assay shows the degradation of cytosolic IκBα at the indicated times. (B) Densitometric analysis shows the degradation of cytosolic IκBα at 30 min following infection or treatment. The cytosolic protein α-actin is used as a control for equal protein loading. The data are presented as mean ± SD ($n = 3$) as the relative ratio of the target protein in infected or treated cells to those of uninfected controls. * $P < 0.05$ vs. uninfected and # $P < 0.05$ vs. *S. aureus*. (C) The EMSA demonstrates NF-κB activation of nuclear proteins at 45 min following infection or treatment. The data are representative of results from three independent experiments.

NF-κB activation was not observed at 45 min following the treatment of PGN or LTA [Fig. 8(B)], either. These results imply that PGN and LTA of *S. aureus* are not involved in the activation of NF-κB in human osteoblasts.

Discussion

This study shows that *S. aureus* can activate NF-κB in human osteoblasts in response to infection. Moreover, it is the attachment of *S. aureus* that is required for this activation.

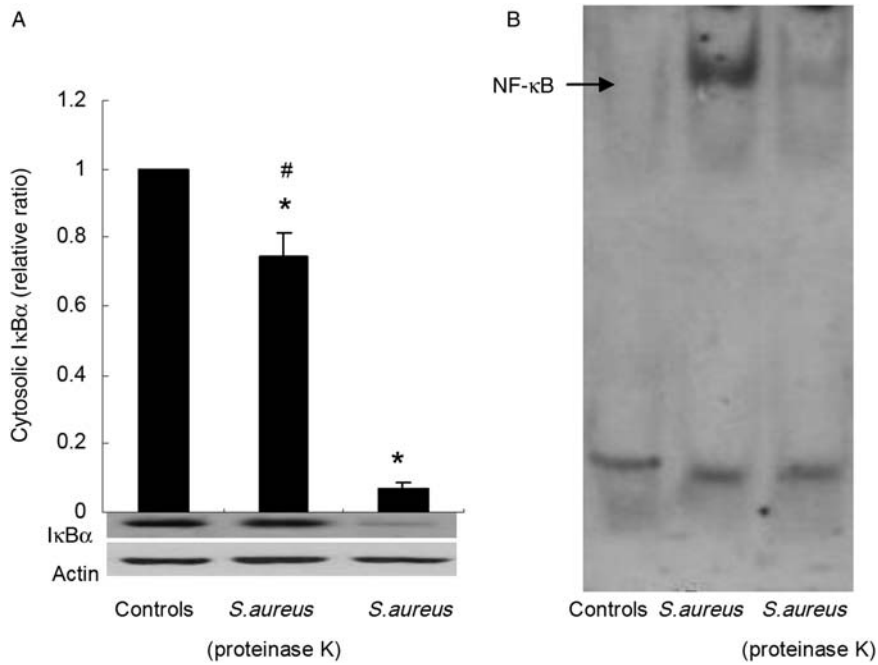


Figure 6 The effect of proteinase K-treated *S. aureus* on the activation of NF-κB in human osteoblasts The SV40 human osteoblasts were infected with *S. aureus* ATCC 49230 or proteinase K-treated *S. aureus* ATCC 49230 at an MOI of 250 or uninfected (controls). (A) Immunoblot assay and densitometric analysis show the degradation of cytosolic IκBα at 30 min post-infection. The cytosolic protein α-actin was used as a control for equal protein loading. The data are presented as mean ± SD ($n = 3$) as the relative ratio of the target protein in infected cells to those of uninfected controls. * $P < 0.05$ vs. controls and # $P < 0.05$ vs. *S. aureus*. (B) EMSA demonstrates the NF-κB activation of nuclear proteins at 45 min post-infection. The data are representative of results from three independent experiments.

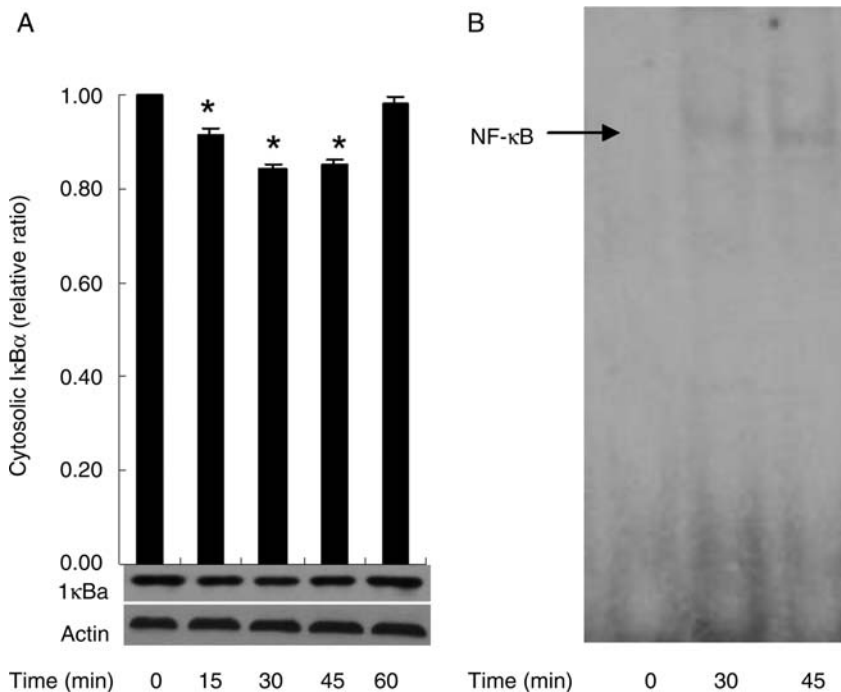


Figure 7 The effect of UV-killed *S. aureus* ATCC 49230 on the activation of NF-κB in human osteoblasts The SV40 human osteoblasts were either incubated with UV-killed *S. aureus* ATCC 49230 at an MOI of 250 or cultured alone (controls, 0 min). (A) Immunoblot assay and densitometric analysis show the degradation of cytosolic IκBα at the indicated time. The cytosolic protein α-actin was used as a control for equal protein loading. The data are presented as mean ± SD ($n = 3$) as the relative ratio of the target protein in incubated cells to those of controls. * $P < 0.05$ vs. controls. (B) EMSA demonstrates the NF-κB activation of nuclear proteins at the indicated time. The data are representative of results from three independent experiments.

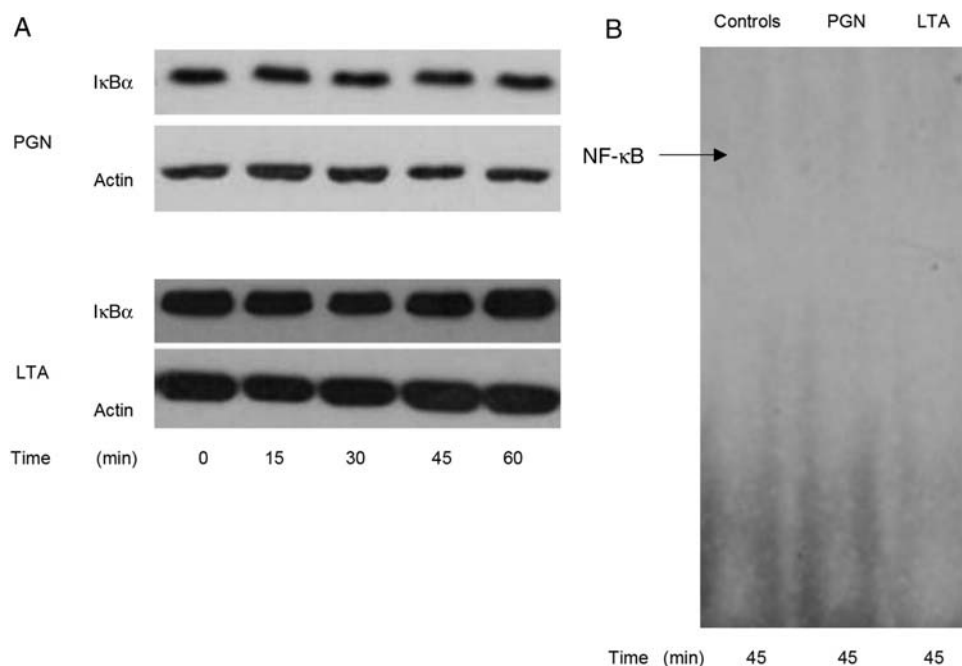


Figure 8 Effect of PGN or LTA on the activation of NF- κ B in human osteoblasts The SV40 human osteoblasts were untreated (controls, 0 min) or treated with PGN (10 μ g/ml) and LTA (10 μ g/ml). (A) Immunoblot assay shows the degradation of cytosolic I κ B α at the indicated times. (B) EMSA demonstrates the NF- κ B activation of nuclear proteins at 45 min after incubation. The data are representative of results from three independent experiments.

The nuclear translocation of NF- κ B is regulated by the cytoplasmic inhibitory factor I κ B α via its binding to the nuclear localization sequence of p65, and I κ B α degradation triggers the activation of NF- κ B [5,6]. Free NF- κ B translocates into the nucleus to initiate transcriptional activation of NF- κ B-dependent genes [7]. In the current study, evidence that *S. aureus* can activate NF- κ B in human osteoblasts in response to infection was demonstrated by three different methods: the immunoblot experiments demonstrated that the degradation of cytoplasmic I κ B α in human osteoblasts infected with *S. aureus* occurred in a time-dependent manner [Fig. 2(A)]; findings by EMSA confirmed that nuclear proteins from human osteoblasts infected with *S. aureus* could stimulate NF- κ B DNA-binding activity [Fig. 2(B)]; and results of ELISA further confirmed that *S. aureus* could activate NF- κ B in human osteoblasts and that *S. aureus*-activated NF- κ B signaling pathway regulated IL-6 secretion in culture supernatants, following the infection (Fig. 3).

We also confirmed that the attachment of *S. aureus* was required for the activation of NF- κ B in human osteoblasts. First, the maximal activation of NF- κ B occurred prior to the maximal invasion of *S. aureus* in human osteoblasts. Since the quantitative analysis of bacterial invasion into non-professional phagocytes is an important pre-requisite to unravel the molecular details of pathogen-to-host cell interaction [15], in the present study, we used flow cytometry analysis with CFSE-labeled *S. aureus* as previously

described [15,17] to quantify the kinetic invasion of *S. aureus* into human osteoblasts and to investigate the inhibitory effect of cytochalasin D or MDC on the invasion of *S. aureus* into human osteoblast. Our results indicated that the invasion of *S. aureus* into human osteoblasts increased for approximately 120 min following infection and that cytochalasin D (5 μ g/ml) and MDC (0.6 mM) could inhibited the invasion of *S. aureus* into human osteoblasts by 99.81% and 96.63%, respectively (Fig. 1). These results are consistent with prior results of maximal invasion of *S. aureus* into human osteoblasts observed at 2 h and inhibitory effects of 5 μ g/ml cytochalasin D and 0.6 mM MDC on the invasion of *S. aureus* into human osteoblasts demonstrated by 99.8 and 96%, respectively, using antibiotic protection assay [3]. However, this maximal invasion time did not correlate with the maximal I κ B α degradation which almost completed at 30 min post-infection [Fig. 2(A)] and the maximal NF- κ B DNA-binding activity which occurred at 45 min post-infection [Fig. 2(B)], indicating that I κ B α degradation might occur prior to *S. aureus* invasion and that NF- κ B activation as a requirement for enteroinvasion appeared improbable. Second, our data from the indirect co-culture of the human osteoblasts and *S. aureus* in a Transwell bicameral chamber not only excluded the possibility of soluble factors secreted by the *S. aureus* to activate NF- κ B in human osteoblasts, but also implied that the possibility of invasion and/or attachment of *S. aureus* to activate NF- κ B in human osteoblasts

(Fig. 4). However, *S. aureus*-derived exotoxins, one of the *S. aureus*-secreted soluble factors, have the ability to induce NF- κ B activation in murine bone marrow macrophages [25]. This discrepancy may reflect differences between professional and non-professional phagocytes. Third, results by using human osteoblasts with/without pretreatment of cytochalasin D or MDC confirmed that the attachment, but not invasion, of *S. aureus* is required for the activation of NF- κ B in human osteoblasts (Fig. 5). Our results are consistent with the studies that showed the attachment of group A streptococci accounting for the induction of NF- κ B activation in human respiratory epithelial cells [11] and the invasion of *Salmonella dublin* or *Salmonella typhimurium* was not required for the activation of NF- κ B in epithelial cells [26]. These reports contrast other findings showing the invasion of *Shigella flexneri* is required for the activation of NF- κ B in HeLa cells [13] and the invasion of *S. aureus* into bovine endothelial cells is associated with the active state of NF- κ B [4]. The individual and complex microorganism–host cell interactions in pathogenesis of infectious diseases may account for these differences in the activation of NF- κ B. Moreover, our experiments that the activation of NF- κ B in cells incubated with proteinase K-treated was lower than that in *S. aureus*-infected cells further confirmed that the attachment of *S. aureus* is required for the activation of NF- κ B. It is also noted that the activation of NF- κ B in human osteoblasts could be slightly induced by proteinase K-treated *S. aureus* (Fig. 6) and it is a possible reason that proteinase K could not remove all the bacterial surface proteins involved in the attachment of bacteria to human osteoblasts. Furthermore, UV-killed cells of *S. aureus* could activate NF- κ B in human osteoblasts, albeit at lower levels (Fig. 7). This finding indicates that the interaction of a bacterial ligand with an osteoblast receptor results in an increased activation of NF- κ B and active bacterial gene expression may be required for the maximal activation of NF- κ B. These results provide further evidence that the attachment of *S. aureus* has the ability to induce cell-contact-dependent NF- κ B activation. Similar results show that UV-killed *S. aureus* could induce NF- κ B activation in murine bone marrow macrophages [25] and mitogen-activated protein kinase activation in osteoblasts [24].

Finally, our experiments further excluded the possibility that PGN and LTA of *S. aureus* were involved in the activation of NF- κ B in human osteoblasts (Fig. 8); these results are in contrast to previous reports that LTA [27] and PGN [28–30] can modulate the NF- κ B signaling pathway by recognizing microbial ligands of host cells. These discrepancies may reflect differences in the interacting patterns between the microorganism and host cell.

In conclusion, our results demonstrate for the first time the activation of NF- κ B in human osteoblasts in response

to infection with *S. aureus* and that the attachment of *S. aureus* is required for this activation of NF- κ B. The NF- κ B transcription factor regulates a number of genes involved in a wide variety of biological processes [31]. Further investigation of the effects on human osteoblasts that activated NF- κ B in response to *S. aureus* will allow us to reveal the pathophysiological mechanisms of osteomyelitis.

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