Staphylococcus aureus regulates secretion of interleukin-6 and monocyte chemoattractant protein-1 through activation of nuclear factor kappaB signaling pathway in human osteoblasts

ABSTRACT

Objective: Activation of nuclear factor kappaB by diverse bacteria regulates the secretion of chemokines and cytokines. *Staphylococcus aureus (S. aureus)*-infected osteoblasts can significantly increase the secretion of interleukin-6 and monocyte chemoattractant protein-1. The aim of this study was to investigate whether *S. aureus* can activate nuclear factor kappaB in human osteoblasts, and whether the activation of nuclear factor kappaB by *S. aureus* regulates the secretion of interleukin-6 and monocyte chemoattractant protein-1. **Methods:** Immunoblot and electrophoretic mobility shift assay were used to detect the degradation of IκBa and activation of nuclear factor kappaB in human osteoblasts in response to *S. aureus*, respectively. Enzyme-linked immunosorbent assay was used to measure the secretion of interleukin-6 and monocyte chemoattractant protein-1 in the supernatants. Lastly, carbobenzoxyl-l-leucinyl-l-leucinyl-l-leucinal, an inhibitor of the nuclear factor kappaB, was used to determine if activation of nuclear factor kappaB by *S. aureus* in human osteoblasts regulates the secretions of interleukin-6 and monocyte chemoattractant protein-1. **Results:** Our results for the first time demonstrated that *S. aureus* can induce the degradation of IκBa and activation of nuclear factor kappaB in human osteoblasts in a time and dose-dependent manner. In addition, inhibition of nuclear factor kappaB by carbobenzoxyl-l-leucinyl-l-leucinyl-l-leucinal suppressed the secretion of interleukin-6 and monocyte chemoattractant protein-1 in the supernatants of *S. aureus*-infected human osteoblasts in a dose-dependent manner. **Conclusions:** These findings suggest that *S. aureus* can activate nuclear factor kappaB in human osteoblasts, and subsequently regulate the secretion of interleukin-6 and monocyte chemoattractant protein-1. The nuclear factor kappaB transcription factor regulates a number of genes involved in a wide variety of biological processes. Further study of the effects of nuclear factor kappaB activation on *S. aureus*-infected human osteoblast may provide us new insights into discovery of the immune mechanisms in osteomyelitis.

Keywords: *Staphylococcus aureus*; osteoblasts; NF-kappaB; interleukin-6; chemokine CCL2.

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INTRODUCTION

Osteomyelitis is a severe infection of bone tissue, which results in progressive inflammatory destruction of bone. *Staphylococcus aureus* is known as an important causative organism in osteomyelitis,[1,2] which accounts for approximately 80% of all human cases.[2] Recent works have demonstrated that *S. aureus*-infected osteoblasts can secrete a number of cytokines and chemokines to mediate the immune responses in osteomyelitis.[3-6] However, to date, the pathogenesis of immune responses in *S. aureus*-induced osteomyelitis is still poorly understood.

The activation of nuclear factor kappaB (NF-κB) signaling pathway to produce inflammatory mediators plays an essential role in the host response to pathogenic organisms.[7] NF-κB is a dimeric protein composed of members of the Rel / NF-κB family, and NF-κB dimers are retained into the cytoplasm by the inhibitory protein IκBa.[8] Different stimuli can induce the phosphorylation of IκBa, and its subsequent ubiquitination and degradation.[9] Freed NF-κB can translocate into the nucleus, leading to the transcriptional activation of NF-κB-dependent genes,[10] such as chemokines and cytokines genes.[11] Previous studies have demonstrated that NF-κB in eukaryocyte could be activated by diverse bacteria, such as *Porphyromonas gingivalis*,[12] *Streptococcus pyogenes*,[13,14] or *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG).[15]

However, it is not clearly defined whether *S. aureus* infection can induce the activation of NF-κB in human osteoblasts. Interleukin-6 (IL-6) is a pleiotropic cytokine and plays important role in the regulation of immune response and inflammation,[16] which controls the development of both humoral and cell-mediated immune responses[17] and block the suppressive activity of regulatory CD4+CD25+ regulatory T-cells,[18] thereby enabling the progression of immune responses. Monocyte chemoattractant protein (MCP)-1 is a small cytokine belonging to the CC chemokine family that is also known as CCL2 (c-c chemokine ligand 2),[19] which can induce the recruitment of macrophages and activated T-lymphocytes into areas of inflammation or infection, and sustain inflammatory responses by maintaining the activated status of such cells.[20] The previous studies have confirmed that *S. aureus*-infected human osteoblasts can significantly augment MCP-1 and IL-6 secretion *in vitro*,[3,4] *in vivo* and in clinical human osteomyelitic lesions.[21,22] It is unknown, however, the mechanism of IL-6 and MCP-1 immune responses to infection of *S. aureus*. Thus, in our present study, we investigated whether *S. aureus* can activate NF-κB signaling pathway, and whether such activation regulate the secretion of IL-6 and MCP-1 in human osteoblasts.

MATERIALS AND METHODS

Bacteria, human osteoblasts and culture conditions

S. aureus UAMS-1 (ATCC 49230), a human osteomyelitis clinically isolated strain[23] was used in this study. *S. aureus* ATCC 49230 was obtained from the American Type Culture Collection. *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 4,300 × g at 4°C and washed twice in 5 mL of Hank's Balanced Salt Solution. The pellets were then resuspended in 5 mL of growth medium lacking antibiotics and antimycotics.

The SV40 human osteoblasts (SV40 hOBs, ATCC) were obtained from the American Type Culture Collection. These cells were previously characterized as authentic osteoblasts.[24] The cells were seeded in 25-cm^2 flasks and incubated at 37°C in 5% CO_2 with DMEM/F12 (Gibco) supplemented with 0.3 mg/mL G418 (Sigma) and 10% fetal bovine serum (HyClone). Then, the cells were propagated as described by the manufacturer. Once osteoblasts reached approximately 80% confluency, cells were trypsinized (0.025% trypsin-0.01% EDTA), washed in medium and seeded into 6-well plates. Two to three hours before the addition of bacteria, the cells were washed twice with PBS and then incubated with an assay medium (growth medium without antibiotics and antimycotics) and infected as described in the following section.

Infection assay

To investigate the IκBa degradation and NF-κB activation following infection, 2×10^6 cells/well were exposed to *S. aureus* for the designated times and at the indicated multiplicity of infections (MOI). To investigate the secretion of IL-6 and MCP-1 in the supernatant of human osteoblasts infected with *S. aureus*, 2×10^6 cells/well were infected with *S. aureus* at an MOI of 250 (250:1 bacteria/osteoblasts) or without *S. aureus* (controls) for 1 h. Following infection, the cells were washed with PBS and grown in DMEM/F12 supplemented with 0.3 mg/mL G418 and 10% fetal bovine serum for 24 h at 37°C in a 5% CO_2 atmosphere. To determine whether *S. aureus*-activated NF-κB signaling pathway regulates the IL-6 and MCP-1 secretion in the supernatants of human osteoblasts, 2×10^6 cells/well were pretreated with carbobenzoxyl-l-leucinyl-l-leucinyl-l-leucinal (Z-LLL-al) (Sigma), an inhibitor of NF-κB,[12] for 1 h, and then washed, added bacteria at an MOI of 250. Z-LLL-al is also named the proteasome inhibitor MG132 which can inhibit NF-κB formation and degradation of its inhibitor I-κB.[25]

Immunoblot assay

After infection, cells were then washed once 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 phenylmethylsulfonyl fluoride (PMSF), 50 mmol/L NaF and 0.1 mmol/L sodium vanadate. The lysates were centrifuged at 15,000 × g for 15 min at 4°C and the concentrations of the protein were determined with the Bradford assay (Bio-Rad, Hercules, CA). Whole cell lysates were boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) for 5 min. The protein extracts (50 μg each) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane (Pall Gelman Laboratory, Ann Arbor, MI). The membranes were blocked with 10% nonfat dried milk in Tris-buffered saline (TBS) for 1 h at room temperature, washed with TBST (Tris buffered-saline Tween-20) for 3 × 5 min, and incubated with rabbit polyclonal anti-human IκBa (Cell Signaling Technology, Danvers, MA) at a dilution of 1:200 for 45 min at room temperature. After hybridization with primary antibody, the membrane was washed with TBST three times. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cell Signaling Technology) at a dilution of 1:10,000 in TBST for 30 min at room temperature and washed with TBST three times, and the antigens were detected with an enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ, USA) and exposed to photographic film.

Electrophoretic mobility shift assay (EMSA)

After infection, nuclear extracts were prepared using reagents from the NE-PER extraction system (Pierce, Rockford, IL), as recommended by the manufacturer's instructions. Then the nuclear extracts were used to test for NF-κB protein/DNA binding by a DIG Gel-Shift Kit (Roche, Germany). The sequences for NF-κB were 5'-AGTTGAGGGGACTTTCCCAGGC-3' (sense) and 5'-GCCTGGGAAAGTCCCCTCAACT-3' (antisense). Briefly, equal molar of complementary NF-κB oligonucleotides were mixed in TEN-buffer [10 mM Tris–Cl, 1 mM EDTA, and 0.1 M NaCl (pH 8.0)] and incubated at 95°C for 10 min. After slowly cooling down to room temperature, double-stranded (ds) oligonucleotides were diluted with TEN-buffer to a final concentration of 3.85 pmol/μL. Labeling reaction was done in a final volume of 20 μL containing 1 μL ds oligonucleotides, 9 μL water, 4 μL 5 × labeling buffer, 4 μL $CoCl_2$ solution, 1 μL DIG-ddUTP and 1 μL terminal deoxynucleotide transferase. The reaction mixture was incubated at 37°C for 15 min and chilled on ice immediately. Labeling reaction was stopped by 2 μL 0.2 M EDTA (pH 8.0). The labeled NF-κB oligonucleotides were diluted to 0.155 pmol/μL by adding 3 μL double distilled water. For gel shift analysis, a small aliquot was further diluted to 15.5 fmol/μL. In the gel shift assay, 5 μg nuclear extract were incubated with 4 μL binding buffer [5 mM EDTA, 50 mM (NH4)2SO4, 5 mM dithiothreitol (DTT), 1% Tween 20 (v/v), 150 mM KCl, 100 mM Hepes (pH 7.6)], 1 μL poly (dI-dC) and 1 μL poly-L-lysine. Double distilled water was added to a final reaction volume of 18 μL. To confirm the specificity of NF-κB protein/DNA binding by competition assay, we added 100-fold excess unlabeled NF-κB oligonucleotides (cold probes) in the reaction mixture. After 5 min incubation at room temperature, 2 μL DIG-labeled NF-κB -oligonucleotides were added into the reaction mixture and incubated for another 30 min. After adding 5 μL loading buffer, the reaction mixture was fractionated in a 6% native polyacrylamide gel in 0.5 × TBE buffer and electroblotted onto Hybond-N+ membranes (Amersham Biosciences). After blotting, the membrane was baked at 120°C for 30 min and rinsed in washing buffer [0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20 (pH 7.5)] for 5 min, and then incubated for 30 min in blocking solution (1% blocking reagent in maleic acid buffer). Following incubation with anti-DIG-AP (1:10,000) in blocking solution for 30 min, the membrane was washed 2 × 15 min in washing buffer and equilibrated in detection buffer [0.1 M Tris–HCl, 0.1 M NaCl (pH 9.5)] for 5 min. Chemiluminescence signals were detected with CSPD as the substrate in X-ray film.

IL-6 and MCP-1 secretion assay

After infection, the culture supernatants were collected and centrifuged (10,000 × g, 5 min). Then IL-6 and MCP-1 in the culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits for human IL-6 or MCP-1 (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Each sample was assayed in duplicate and absorbance was determined at 450 nm.

Data analysis

All assays were repeated in three independent experiments. Data are presented as mean ± SD. Statistical analysis among groups was performed by ANOVA and SNK test using the SPSS17.0 software package. Statistically significant values were defined as $p < 0.05$.

RESULTS

S. aureus induced IκBa degradation and NF-κB activation in human osteoblasts in a time-dependent manner.

In order to relate the kinetics of infection to the stability of IκBa in human osteoblasts, human osteoblasts were infected with *S. aureus* at an MOI of 250 for indicated times. The infected cell lysates showed a quick IκBa degradation after 15 min and with a nearly loss of immunoreactivity at 30 min, and nearly removed the prior levels at 60 min. However, uninfected cells had stable levels of IκBa expression (Figure 1A).

To correlate the degradation of IκBa with the nuclear translocation of NF-κB in human osteoblasts, EMSAs were used to investigate the nuclear translocation of NF-κB. As shown in Figure 1B, nuclear proteins from the cells infected with *S. aureus* stimulated NF-κB DNA binding activity to rise at 30 min, reached the maximal level at 45 min, nearly loss at 90 min at an MOI of 250, whereas nuclear proteins

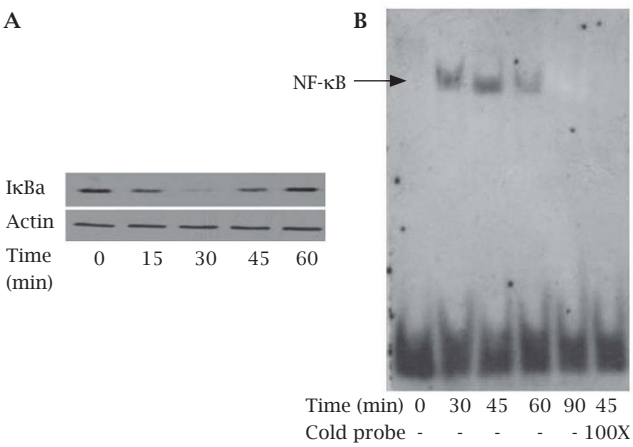

Figure 1: Time-dependent activation of NF-κB signaling pathway by *Staphylococcus aureus* in human osteoblasts. Human osteoblasts were either uninfected (0 min) or infected with *Staphylococcus aureus* at a multiplicity of infections (MOI) of 250 for the indicated times. Immunoblot demonstrated cytosoic IκBa degradation **(A)** and EMSA showed nuclear NF-κB activation **(B)** in human osteoblasts. The exposed film is representative of results from three independent experiments.

from uninfected cells nearly failed to display binding activity to DNA. To confirm the specificity of the DNA protein interaction, a 100-fold excess of cold probes was used as a competitor to inhibit the binding activity of NF-κB at 45 min after infection. As shown in Figure 1B, cold probes completely inhibited the DNA binding activity of NF-κB, indicating that the increased binding is specific to the NF-κB-binding sequence. Collectively, these findings suggest that *S. aureus* can induce activation of NF-κB in human osteoblasts in a time-dependent manner.

S. aureus induced IκB degradation and NF-κB activation in human osteoblasts in a dose-dependent manner to determine the effect of the infectious dose on IκBa degradation, human osteoblats were infected with *S. aureus* for 30 min at the indicated MOIs. *S. aureus*-infected cells demonstrated IκBa degradation substantially increased from MOI of 25 to 500 at 30 min (Figure 2A). To further determine the infectious doses on the roles of NF-κB DNA binding activity in human osteoblasts, NF-κB DNA binding activity from cells infected with *S. aureus* at 45 min quickly increased from an MOI of 25 to 250 (Figure 2B), but started to decline when the MOI reached to 500. These results indicate that *S. aureus* can induce activation of NF-κB in human osteoblasts in a dose-dependent manner. Activation of NF-κB signaling pathway by *S. aureus* regulated the secretion of IL-6 and MCP-1 in human osteoblats.

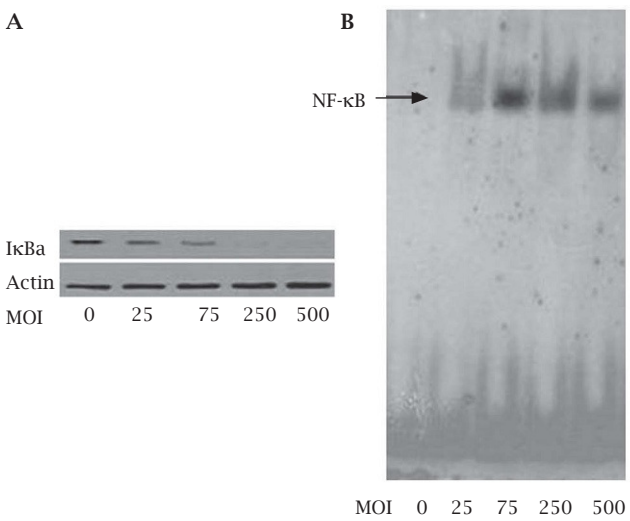

Figure 2: Dose-dependent activation of NF-κB signaling pathway by *Staphylococcus aureus* in human osteoblasts. Human osteoblasts were either uninfected (0 min) or infected with *Staphylococcus aureus* at the indicated multiplicity of infections (MOI). Immunoblot demonstrated cytosoic IκBa degradation at 30 min postinfection **(A)** and EMSA showed nuclear NF-κB activation at 45 min postinfection **(B)** in human osteoblasts. The exposed film is representative of results from three independent experiments.

S. aureus-infected human osteoblasts can significantly augment IL-6 and MCP-1 secretion at 24 h postinfection *in vitro*.[3,4] Therefore, in our present study, we examined the supernatants of human osteoblasts at 24 h postinfection of *S. aureus* to determine if activation of NF-κB signaling pathway by *S. aureus* regulates the secretion of IL-6 and MCP-1 in human osteoblasts. As shown in Figure 3, uninfected cells (controls) could secrete IL-6 (0.22 ng/mL) and MCP-1 (0.84 ng/mL), respectively. However, the secretion of IL-6 and MCP-1 in the supernatants of human osteoblasts infected with *S. aureus* at an MOI of 250 for 1 h was significantly

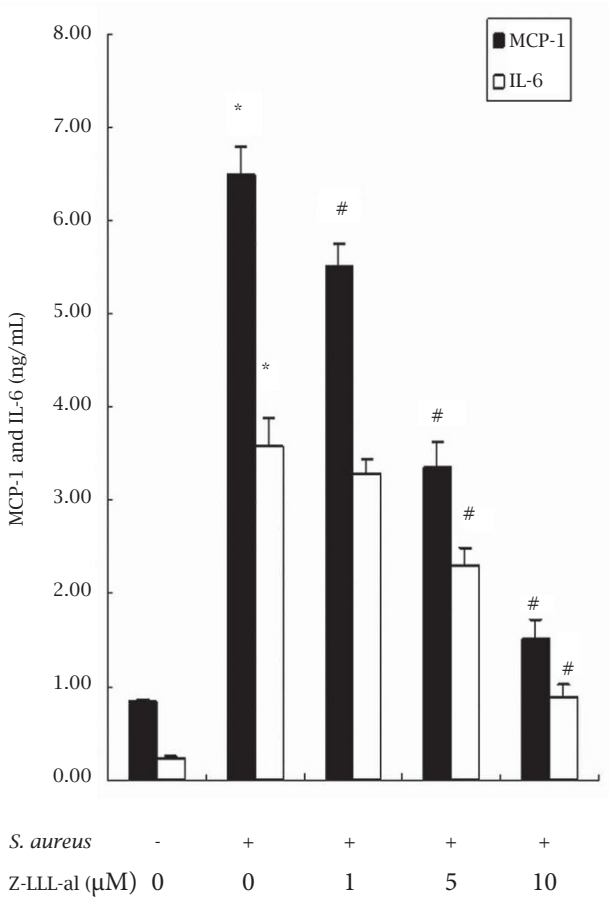

Figure 3: Effect of carbobenzoxyl-l-leucinyl-l-leucinyl-l-leucinal (Z-LLL-al), an inhibitor of NF-κB, on the secretion of MCP-1 and IL-6 in human osteoblasts infected with *Staphylococcus aureus.* Human osteoblasts were uninfected or infected with *Staphylococcus aureus* (*S. aureus*) at a multiplicity of infections (MOI) of 250 for 1 h or pretreated with Z-LLL-al at different concentrations for 1 h, then infected with *S. aureus* at an MOI of 250 for 1 h. Following infection, the cells were washed and grown in the culture medium for 24 h, and then the culture supernatants were collected. IL-6 and MCP-1 in the culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits. Data are presented as mean ± SD from three independent experiments. Statistical analysis comparing groups was performed by ANOVA and SNK test.
* $p < 0.05$, cells infected with *S. aureus versus* controls, # $p < 0.05$, cells infected with *S. aureus versus* cells pretreated with different concentrations of Z-LLL-al prior to infection of *S. aureus.*

enhanced to 3.58 ± 0.31 ng/mL ($p < 0.05$) and 6.48 ± 0.30 ng/mL ($p < 0.05$), compared with controls, respectively. In addition, Figure 3 also shows that the secretion of Il-6 and MCP-1 in the supernatants of human osteoblasts infected with *S. aureus* were suppressed by the addition of Z-LLL-al (1 to 10 μM) in a dose-dependent manner, and IL-6 secretion was reduced from 3.58 ± 0.31 to 3.27 ± 0.18, 2.30 ± 0.20 ($p < 0.05$), 0.89 ± 0.13 ng/mL ($p < 0.05$) and MCP-1 secretion was reduced from 6.48 ± 0.30 to 5.52 ± 0. 22 ($p < 0.05$), 3.36 ± 0.26 ($p < 0.05$), 1.52 ± 0.19 ng/mL ($p < 0.05$) at 1, 5, 10 μM Z-LLL-al, respectively.

DISCUSSION

This study shows that *S. aureus* can activate NF-κB in human osteoblasts in a time and dose-dependent manner and *S. aureus*-activated NF-κB signaling pathway in human osteoblasts regulates the secretion of IL-6 and MCP-1.

The nuclear translocation of NF-κB is regulated by the cytoplasmic inhibitory species IκBa via its binding to the nuclear localization sequence of p65; degradation of IκBa triggers the activation of NF-κB.[8,9] In our current study, activation of NF-κB by *S. aureus* in human osteoblasts has been demonstrated by two different methods. First, our immunoblot experiments demonstrated that degradation of cytoplasmic IκBa in *S. aureus*-infected human osteoblasts occurred in a time and dose-dependent manner (Figures 1A and 2A). Second, correlated IκBa degradation with NF-κB DNA binding activity, our findings by EMSA confirmed that nuclear proteins from *S. aureus*-infected human osteoblasts could stimulate the DNA binding activity of NF-κB in a time and dose-depenent manner (Figures 1B and 2B).

In addition, we demonstrated that *S. aureus*-activated NF-κB signaling pathway in human osteoblasts regulates the secretion of IL-6 and MCP-1. As shown in Figure 3, human osteoblats infected with *S. aureus* at MOI of 250 significantly augment IL-6 ($p < 0.05$) and MCP-1 ($p < 0.05$) secretion compared with controls, respectively. These results are consistent with previous studies suggesting that *S. aureus* could stimulate osteoblasts to secrete high levels of interleukin-6 and monocyte chemoattractant protein-1.[3,4] Furthermore, Figure 3 also demonstrated that the secretion of IL-6 and MCP-1 in the supernatants of *S. aureus*-infected human osteoblats were significantly suppressed by the addition of Z-LLL-al, an inhibitor of NF-κB,[12] in a dose-dependent manner, suggesting that *S. aureus*-activated NF-κB signaling pathway in human osteoblasts regulates the secretion of IL-6 and MCP-1. These findings are consistent with previous studies suggesting that NF-κB signaling pathway can regulate the secretion of IL-613 and MCP-1.[26,27] Indeed, the overall infectious process that human osteoblasts were exposed to *S. aureus* clearly involved a variety of converging signaling transduction pathway; it is possible that *S. aureus* may activate another signaling pathway to regulate the secretion of IL-6 and MCP-1.

NF-κB is the predominantly modulated transcription factor functioning in immune and inflammatory response to microbial ligands by mediating signaling from the Toll-like receptors (TLRs).[8,28] TLRs can recognize bacterial cell components such as M protein,[29] lipoteichoic acid,[30] LPS, peptidoglycans and lipopeptides, flagella and bacterial DNA.[28,31,32] The different TLRs recognize different ligands in a pathogen-associated molecular pattern. It has been reported that a number of these TLR homologues are present on osteoblasts, such as TLR2,[33,34] TLR4,[34,35] TLR5,[36] and TLR9.[37] We are now in the process of exploring if the pathogen associated molecular pattern between TLRs of *S. aureus*-infected human osteoblasts and *S. aureus* cell components is a predominant transcriptional factor that regulates the activation of NF-κB.

In conclusion, our results demonstrate for the first time that *S. aureus* can activate NF-κB in human osteoblasts in response to *S. aureus* infection, and activation of NF-κB by *S. aureus* regulates the secretion of IL-6 and MCP-1 in human osteoblasts. The NF-κB transcription factor regulates a number of genes involved in a wide variety of biological processes.[38] Further investigation of the effects of *S. aureus* on activation of NF-κB in human osteoblast may provide us with more insight into the regulation of the immune mechanisms in osteomyelitis. More understanding about the pathogenesis of osteomyelitis may provide new therapeutic targets for the treatment of osteomyelitis.

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