

ORIGINAL ARTICLE

Knockdown of TNFR1 Suppresses Expression of TLR2 in the Cellular Response to *Staphylococcus aureus* Infection

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Abstract—Osteomyelitis is a common manifestation of invasive *Staphylococcus aureus* infection characterized by widespread bone loss and destruction. Phagocytes possess various receptors to detect pathogens, including the Toll-like receptors (TLRs). Previous studies have demonstrated that the *S. aureus* protein SpA binds directly to pre-osteoblastic cells via tumor necrosis factor receptor-1 (TNFR-1). In our present study, we investigated the relationship between TLR2 and TNFR-1 in *S. aureus*-infected osteoblasts. Our results showed that cell viability decreased, and apoptosis, expression of TLR2, and the secretion of inflammatory cytokines (TNF- α and IL-6) increased with increasing concentrations of *S. aureus*. The JNK pathway was also activated in response to *S. aureus* infection. Knockdown of TNFR1 not only inhibited the JNK pathway but also reduced TLR2 protein and RANKL levels in *S. aureus*-infected cells. Inhibition of the JNK pathway reduced the protein level of TLR2 and reduced TNF- α and IL-6 secretion in *S. aureus*-infected cells.

KEY WORDS: tumor necrosis factor receptor-1; Toll-like receptor 2; *Staphylococcus aureus*; osteomyelitis; JNK pathway.

INTRODUCTION

Osteomyelitis is a common manifestation of invasive *Staphylococcus aureus* infection characterized by widespread bone loss and destruction [1]. Bone is a dynamic structure that is in a continuous process of remodeling, which is primarily driven by two cell types, osteoblasts,

which deposit bone matrix, and osteoclasts, which remove it [2]. The balance of absorption and deposition of bone matrix facilitated by these two cell types determines the structure, density, and strength of bone tissue.

SpA, a central extracellular virulence factor that is covalently linked to the peptidoglycan coat of *S. aureus*, has been shown to bind to the Fc region of IgG [3] and also to tumor necrosis factor receptor 1 (TNFR1) [4], among other important cellular targets. Claro *et al.* demonstrated that the *S. aureus* SpA protein also binds directly to pre-osteoblastic cells via TNFR1. Binding alone induced apoptosis in cultured osteoblasts, inhibited mineral deposition, and induced expression of the protein RANKL, a protein expressed by osteoblasts that is critical for the initiation of bone resorption [5].

TNFR1 binding by SpA causes the activation of nuclear factor kappa B (NF- κ B), which then translocates to the nucleus and promotes the expression of IL-6 [6], a promoter of inflammation and bone resorption [7]. Indeed, conditioned media from osteoblasts bound by SpA has been shown to activate pre-osteoclastic cells [8]. The native

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ligand for TNFR1, tumor necrosis factor alpha (TNF- α), also activates apoptosis via strong activation of the c-Jun N-terminal protein kinase (JNK) and, to a lesser extent, the extracellular signal-regulated kinase (ERK) pathways [9], each of which have complex effects on the decision-making processes governing cell survival in multicellular organisms [10].

In a complementary pathway activated by infection, phagocytes and other cell types, including osteoblasts, have various receptors that bind to molecular components characteristic of microbial pathogens. The primary sensors of the innate immune system response to bacterial infections in humans are the Toll-like receptors (TLRs). These receptors provide a significant level of specificity to the innate immune system by binding a wide variety of components from infectious organisms, known as pathogen-associated molecular patterns [11], and integrating these signals into a coherent, anti-microbial cellular response. This process also activates the JNK pathway and ultimately releases inflammatory mediators, including interleukin (IL)-6, IL-8, TNF- α , and IL-1 β [12].

TLRs, TNFR1, and the pathways they affect are critical drivers of the host response to *S. aureus* infection during osteomyelitis. Therefore, in our present study, we investigated the relationship between these two receptors in *S. aureus*-infected osteoblasts to shed light on the complex molecular interactions driving the behavior of osteoblasts in response to infections of bone tissue.

Materials and Methods

Bacterial Culture

S. aureus 6850 (ATCC 53657), a wild-type isolate from a patient with osteomyelitis [13], was used in this study. Bacterial cultures were grown at 37 °C in tryptic soy broth (TSB) with shaking. Cells were resuspended in phosphate-buffered saline (PBS) after harvesting and washed via centrifugation for 5 min at 15,000 \times g. Cultures in PBS were adjusted to 1 \times 10⁹ cells/ml in each case.

Cell Culture and Treatment

MC3T3-E1 is an osteoblast precursor cell line isolated from the mouse (*Mus musculus*) cranium. Culture conditions for the MC3T3-E1 cell line have been described previously [8]. MC3T3-E1 cells were cultured with various concentrations of *S. aureus* (MOI of 10, 20, 40, 80, and 100) for 24 h, and uninfected cells were incubated for 24 h as a control.

To study the role of the JNK pathway and TLR2, MC3T3-E1 cells were pretreated with 25 μ M SP600125 (JNK inhibitor, Sigma Aldrich, St. Louis, Missouri, USA), an equal volume of 0.5 % (v/v) DMSO or a blocking antibody against TLR2 (100 ng/ml Alexis Bio-chemicals, San Diego, CA, USA) for 1 h before *S. aureus* infection at an MOI of 100. This MOI was selected based on previous studies and our initial findings [14, 15].

Lentivirus-Based Small-Hairpin RNA Transfection

TNFR1 expression levels were knocked-down by transfecting MC3T3-E1 cells with lentiviral vectors carrying small-hairpin RNAs (shRNAs) targeting TNFR1 using the murine Amaxa Cell Line Nucleofector kit R (Lonza, Cologne, Germany) [8]. The two small-hairpin RNAs used were TTNFR1-shRNA1 GCAGGGTCTTTCTGAGAGAA and TNFR1-shRNA2 CCCGAAGTCTACTCCATCATT. pLKO.1 (Sigma) was used as the lentiviral vector. The recombinant lentivirus was produced by cotransfection of HEK293 cells with two helper vectors, pCMV and pMD.G, and the target vector pLKO.5-puro-shRNA. MC3T3-E1 cells were then infected with recombinant lentivirus for 48 h. Both nontransfected cells and cells transfected with pLKO.1-GFP were cultured for 24 h as a control or were infected with *S. aureus* (MOI= 100) for 24 h.

Cell Viability Determinations

The viability of cells exposed to *S. aureus* was determined using the CCK-8 assay (Beyotime, Shanghai, China) according to the manufacturer's instructions. MC3T3-E1 cells were seeded in 96-well culture plates (Corning Inc., Corning, New York, USA) at 1 \times 10⁴ cells/well. Cultured cells were then incubated with different concentrations of *S. aureus* at 37 °C in a humidified incubator with 5 % atmospheric CO₂. Ten microliters of CCK-8 solution was added to each well after 24 h of culture, and plates were incubated for 1 h using the same conditions. Plate absorbance at a wavelength of 450 nm was determined with a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Annexin V-FITC/PI Apoptosis Detection

MC3T3-E1 cells were cultured with various concentrations of *S. aureus*. Apoptosis was calculated by flow cytometry followed by annexin V-FITC/PI double-staining assay.

MC3T3-E1 cells (1.5×10^5 cells/cm²) were plated in one six-well plate for each bacterial MOI investigated (10, 20, 40, 80, and 100). Cells were mixed with *S. aureus* for 24 h after an overnight incubation at 37 °C. An annexin V-FITC apoptosis detection kit (Sigma Aldrich) was used for annexin V-FITC staining according to the manufacturer's directions. Cells treated with trypsin/EDTA for 1 min were centrifuged, then re-suspended in 100 µl of 1× binding buffer at 1×10^6 cells/ml, and the mixtures was transferred to a 5-ml FACS tube containing 10 µl of propidium iodide (PI) and 5 µl of annexin V-FITC. Following incubation in the dark for 30 min at 25 °C, 400 µl of 1× binding buffer were added. Then, flow cytometry was immediately carried out, and data acquisition and analysis were performed with a FACScalibur flow cytometer and CELLQuest software. Annexin V and PI (−) cells were viable, while cells that were annexin V (+) and PI (−) or annexin V and PI (+) were either early- or late-stage apoptotic, respectively.

Quantitative RT-PCR Analysis

MC3T3-E1 cells (1.5×10^5 cells/cm²) were plated in one six-well plate for each bacterial MOI investigated (10, 20, 40, 80, and 100). Cells were mixed with *S. aureus* for 24 h after an overnight incubation at 37 °C. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from each sample according to the manufacturer's directions. The concentration and quality of the resulting RNA were evaluated using spectrophotometry, and a 260/280 ratio of ≥ 1.8 was determined to be adequate. First-strand cDNA was generated using a transcription system (Promega, Madison, WI, USA). To amplify TLR2 from first-strand cDNA, we used forward 5'-CAC TGG GGG TAA CAT CGC TT-3' and reverse 5'-GCT GAC TTC ATC TAC GGG CA-3' primers. To amplify TNFR1, we used forward 5'-AAC ACC GTG TGT AAC TGC CA-3' and reverse 5'-CTC TTT GAC AGG CAC GGG AT-3' primers. To amplify GAPDH, we used forward 5'-GTG ATG GGT GTG AAC CAC GA-3' and reverse 5'-GTC AGA TCC ACG ACG GAC AC-3' primers.

Transcript quantitation was performed using fluorescence-based, real-time quantitative PCR on an ABI PRISM 7500 sequence detection system (Applied Biosystems) and a DyNAmo™ SYBR Green_qPCR kit (MJ Research Inc., Waltham, MA, USA). The comparative ddCt method was used for relative quantitation of mRNA quantities [16]. The reaction conditions for all primers were

95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s for 40 cycles. Each qPCR analysis was repeated at least three times.

Western Blotting

Protein levels were analyzed with western blotting using a standard procedure [17]. Briefly, cultured cells were washed with cold PBS, lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na₃VO₄, 0.5 % NP40, 1 % Triton X-100, 1 mmol/L PMSF, and a protease inhibitor cocktail), and the lysate was cleared by centrifugation at 14,000×g for 15 min at 4 °C. Aliquots containing 25–40 µg of protein were resolved in 8–12 % polyacrylamide gels and transferred to nitrocellulose membranes. Blots were blocked in blocking buffer (TBS containing 5 % nonfat dry milk and 1 % Tween 20) for 1 h at room temperature and incubated in the indicated primary antibodies in blocking buffer for 2 h at room temperature or overnight at 4 °C. After incubation in the appropriate secondary antibodies, blots were developed by enhanced chemiluminescence (Amersham Life Science) and autoradiography. The following antibodies were purchased from Abcam (Cambridge, England) and diluted as indicated in parentheses: Anti-TLR2 (1/200), Anti-JNK1+JNK2 (pT183+pY185) (1/1000), Anti-JNK1/2 (1/500), Anti-c-Jun (phospho S63) (1/5000), Anti-c-Jun (1/1000), Anti-TNF Receptor I (1/1000), and Anti-b-actin (1/2500). A goat horseradish peroxidase-conjugated polyclonal to rabbit IgG was diluted 1/2000 for the secondary antibody. β-Actin served as loading control.

ELISA

The concentration of TNF-α and IL-6 in conditioned media and RANKL from cell lysates was determined using commercially available sandwich ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's directions.

Statistical Analysis

Data are representative of at least three independent experiments. Results were expressed as the mean ± standard deviation. Data were analyzed by one-way ANOVA (SPSS 17.0) and $p < 0.05$ was judged to be statistically significant.

RESULTS

***S. aureus* Reduced Cell Viability and Induced Apoptosis**

We studied the effects of *S. aureus* infection on MC3T3-E1 by culturing the cell line with various concentrations of bacteria (MOI=0 [control], 10, 20, 40, 80, and 100) for 24 h. Cell viability, as determined with a CCK-8 assay, is presented in Fig. 1a. Increasing concentrations of bacteria slowly reduced assay

absorbance, indicating a reduction in viability for MC3T3-E1, and a significant difference was reached at an MOI of 40. In Fig. 1b, apoptosis levels were assessed by flow cytometry, followed by an annexin V-FITC/PI double-staining assay. The percentage of cells undergoing apoptosis climbed to significance at an MOI of 20 and then reached 33.2 % at an MOI of 100, a magnitude in agreement with the viability results. These data demonstrated that apoptosis was driving cell loss in response to *S. aureus* exposure.

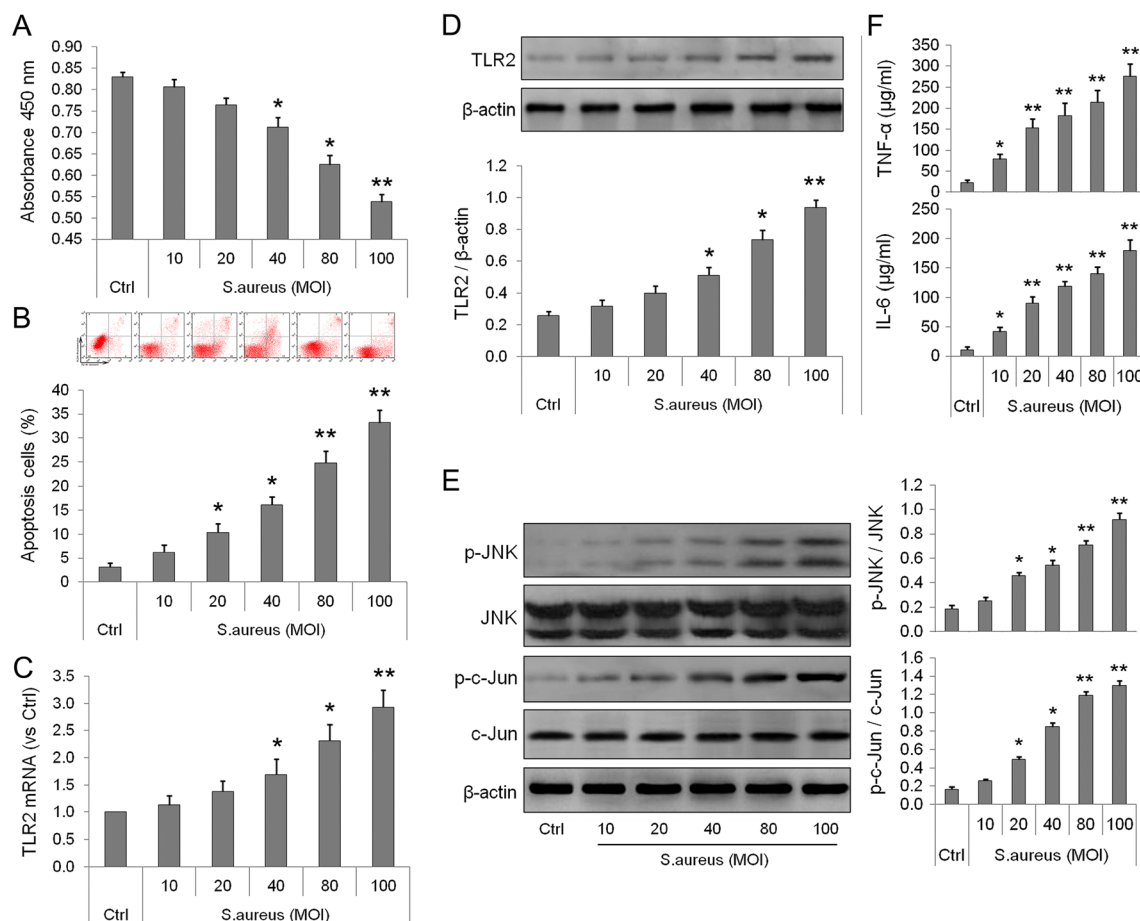


Fig. 1. TNFR1 and TLR2 expression during *S. aureus* infection. MC3T3-E1 cells were cultured with *S. aureus* at an MOI of 10, 20, 40, 80, or 100 for 24 h, while the control group (*Ctrl*) was cultured for 24 h without treatment. **a** Cell viability was determined with the CCK-8 assay. **b** Apoptosis was determined by flow cytometry followed by an annexin V-FITC/PI double staining assay. **c** TLR2 mRNA was detected with qRT-PCR, normalized to GAPDH expression, and depicted as a fold-change relative to the *Ctrl* group. **d** TLR2 protein was detected with western blotting. Histograms indicate the relative densities of bands normalized to β-actin. **e** Protein levels of p-JNK, JNK, p-c-Jun, and c-Jun were analyzed by western blotting. Results of densitometric analysis were normalized to the respective unphosphorylated protein. **f** Conditioned media were harvested, and TNF-α and IL-6 concentrations were assessed using ELISAs. Data are presented as the mean of triplicate experiments. Asterisks represent significant differences between the control group (*Ctrl*) and *S. aureus*-treated MC3T3-E1 cells (**p* < 0.05, ***p* < 0.01).

S. aureus Upregulated TLR2 mRNA and Protein Expression

As MC3T3-E1 cell loss in response to *S. aureus* infection was driven primarily by apoptosis, we determined the expression and condition of key proteins typically involved in such cellular responses. We first assayed TLR2 mRNA expression using qRT-PCR (Fig. 1c) and TLR2 protein levels using western blotting (Fig. 1d). These experiments demonstrated that TLR2 expression at both the RNA and protein level became significantly elevated in response to a *S. aureus* MOI of 40.

S. aureus Activated the JNK Pathway

We also assessed the expression level and phosphorylation of key proteins involved in the JNK pathway, each of which are involved in cell survival and apoptotic decision making. The effect of *S. aureus* exposure on the phosphorylation status of JNK and c-Jun in the MC3T3-E1 cell line was analyzed by western blotting, and the results of densitometric analysis of phosphorylated forms were normalized to their respective unphosphorylated proteins (Fig. 1e). In response to *S. aureus* infection, phosphorylation levels of the JNK and c-Jun proteins were

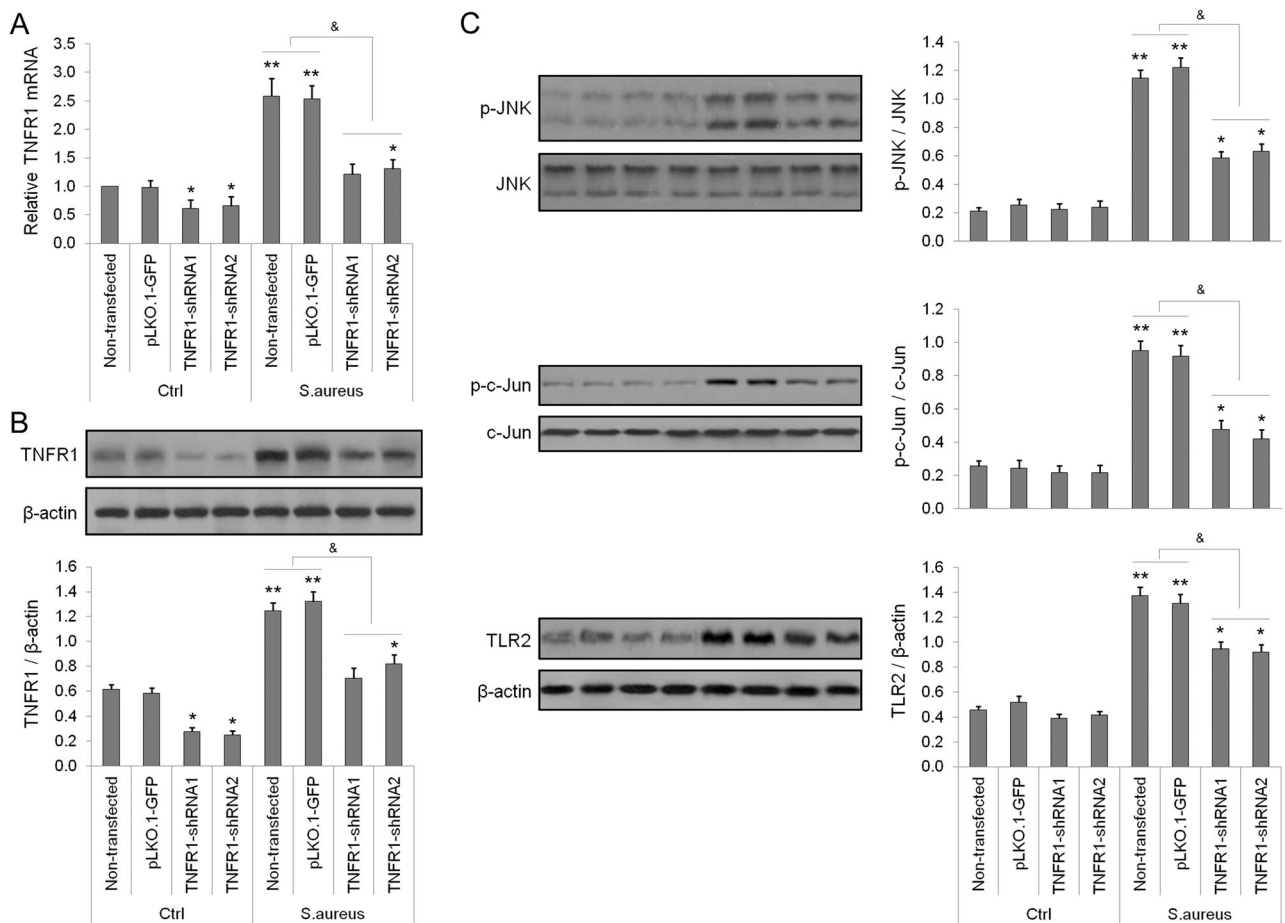


Fig. 2. Cellular response to TNFR1 knockdown. TNFR1 expression was knocked down by infecting MC3T3-E1 cells with lentiviral vectors carrying shRNAs targeting TNFR1 (shRNA1 or shRNA2) for 48 h. The non-transfected cells or infected with pLKO.1-GFP lentivirus served as controls. Non-transfected and transfected pLKO.1-GFP or shRNAs cells were cultured for 24 h (*Ctrl*) or infected with *S. aureus* (MOI = 100) for 24 h. **a** TNFR1 mRNA was detected by qRT-PCR, normalized to GAPDH expression, and presented as the fold-change relative to the *Ctrl*. **b** TNFR1 protein was detected with western blotting. Histograms indicate the relative densities of bands normalized to β -actin. **c** Protein levels of p-JNK, JNK, p-c-Jun, c-Jun, and TLR2 were analyzed with western blotting. The results of densitometric analysis were normalized to the respective unphosphorylated protein or β -actin. * $p < 0.05$ and ** $p < 0.01$ vs. the non-transfected in *Ctrl* group; & $p < 0.05$.

significantly elevated. As each of these proteins is activated by phosphorylation, the JNK pathway appears to have been activated.

S. aureus Promoted the Secretion of IL-6 and TNF- α

TNF- α binding to TNFR1 promotes apoptosis, while IL-6 promotes inflammation and bone loss. Therefore, we assessed TNF- α and IL-6 concentrations in conditioned media via ELISA (Fig. 1f). Expression of both factors was significantly elevated even at the lowest MOI of 10, as would be expected from the activation the JNK pathway.

Knockdown of TNFR1 Inhibited the JNK Pathway

To further assess the effect of TNFR1 on cellular responses to *S. aureus* infection, expression of this protein was knocked-down by infection of MC3T3-E1 cells with lentiviral vectors carrying shRNAs targeting TNFR1 (shRNA1 or shRNA2) for 48 h. In Fig. 2a, *S. aureus* infection dramatically elevated TNFR1 mRNA expression ($p < 0.01$), while the presence of both shRNAs significantly reduced the mRNA expression of TNFR1 in either the presence or absence of *S. aureus*. A nearly identical pattern was observed at the level of TNFR1 protein (Fig. 2b). While shRNA1 and shRNA2 did not affect the phosphorylation of JNK, c-Jun, or the expression of TLR2 in the

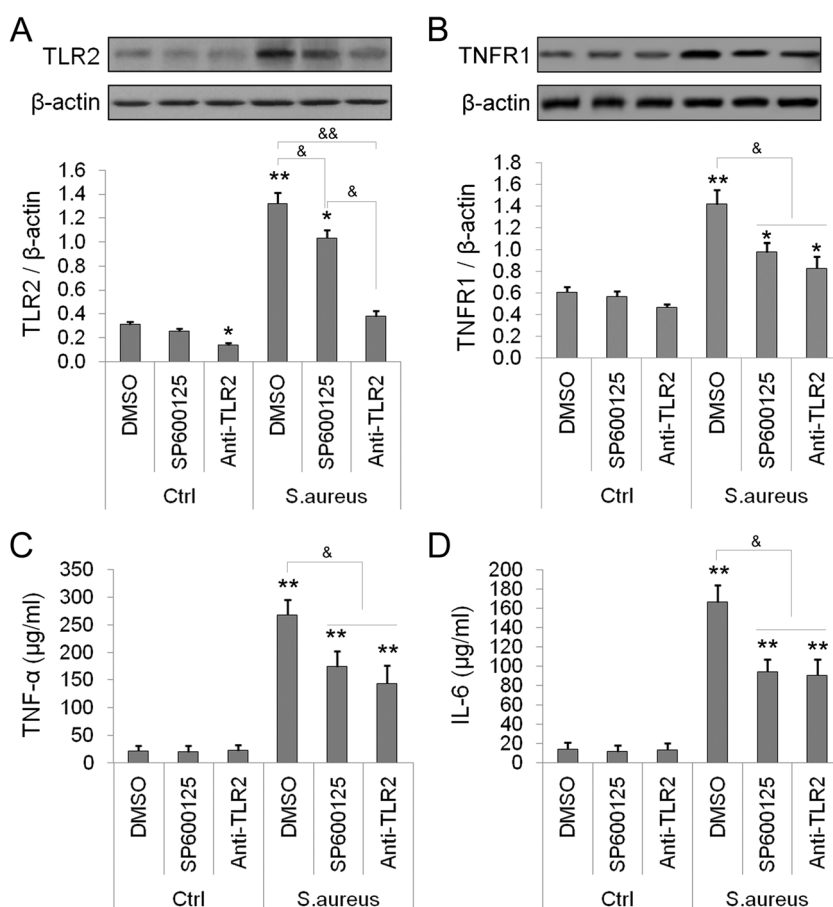


Fig. 3. Inhibition of TLR2 and JNK. MC3T3-E1 cells were pretreated with 25 μ M of SP600125 or an equal volume of 0.5 % (v/v) DMSO as a control and blocking antibody against TLR-2 (100 ng/ml) for 1 h. Cells were cultured for 24 h (Ctrl) or infected with *S. aureus* at an MOI of 100 and incubated for 24 h. **a**, **b** The protein levels of TLR2 (**a**) and TNFR1 (**b**) were detected by western blotting. Histograms indicate the relative densities of bands normalized to β -actin. **c**, **d** Conditioned media were harvested, and TNF- α (**c**) and IL-6 (**d**) concentrations were assessed with ELISAs. Data are presented as the mean of triplicate experiments. * $p < 0.05$ and ** $p < 0.01$ vs. the DMSO in Ctrl group; & $p < 0.05$; && $p < 0.01$.

S. aureus-free control, these small-hairpin RNAs significantly reduced the phosphorylation of all three proteins and the expression of TLR2 ($p < 0.05$) in the presence of *S. aureus*. These shRNAs were effective at reducing TNFR1 protein expression, which then produced downstream effects on the activation of the JNK pathway and TLR2 expression (Fig. 2c). Therefore, expression of TLR2 in the presence, but not the absence, of *S. aureus* was responsive to a feedback loop tied to TNFR1 signaling.

Inhibition of JNK or TLR2 Reduced the Levels of IL-6, TNF- α , and TNFR1 Induced by *S. aureus*

To determine whether TLR2, the JNK pathway, and IL-6, TNF- α , and TNFR1 expression are tied together in a common pathway, we pretreated MC3T3-E1 cells with 25 μ M of SP600125, an inhibitor of the JNK protein, or a blocking antibody against TLR2 prior to exposure to *S. aureus*. SP600125 and the blocking antibody significantly reduced the expression of TLR2 and TNFR1 in the presence of *S. aureus* ($p < 0.05$) with the blocking antibody dropping TLR2 expression down to control levels (Fig. 3a, b). TNF- α and IL-6 concentrations were also suppressed in the presence of *S. aureus* by both SP600125 and the blocking antibody against TLR2 (Fig. 3c, d). These results demonstrated that both the JNK pathway and TLR2 signaling were involved in the cellular response, i.e., cytokine expression and the upregulation of TLR2. However, neither SP600125 nor the anti-TLR2 antibody completely blocked the elevated expression of cytokines to *S. aureus* infection, suggesting that other pathways were also involved. Notably, the anti-TLR2 antibody did bring TLR2 expression levels down to control levels, suggesting a robust connection between TLR2 activation via antigen binding and expression.

Knockdown of TNFR1 Reduced RANKL Levels

The protein RANKL is a central molecule promoting a balance between bone formation by osteoblasts and resorption by osteoclasts. To assess the behavior of this molecule in our system, we assessed the expression of RANKL in response to shRNA1, SP600125, and the blocking antibody against TLR2. Figure 4 demonstrates that shRNA1 significantly reduced the expression of RANKL in the presence of *S. aureus* down to control levels when applied alone, or in the presence of SP600125 or the TLR2 blocking antibody, demonstrating that expression of RANKL is directly linked to activation of TNFR1.

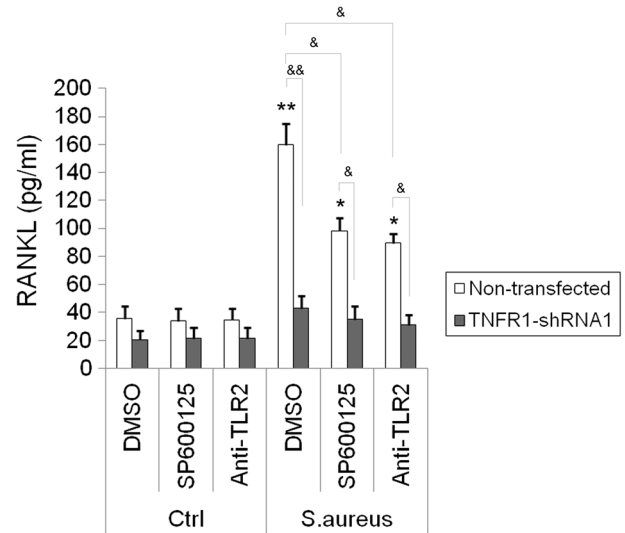


Fig. 4. RANKL expression and the inhibition of TLR2 and JNK. Non-transfected cells or cells transfected with TNFR1-shRNA1 were exposed to 25 μ M SP600125, an equal volume of 0.5% (v/v) DMSO, or a blocking antibody against TLR-2 (100 ng/ml) for 1 h. Cells were then cultured for 24 h (Ctrl) or infected with *S. aureus* at an MOI of 100 and incubated for 24 h. RANKL concentrations in cells were assessed by ELISA analysis. * $p < 0.05$ and ** $p < 0.01$ vs. the non-transfected cells with DMSO treatment in Ctrl group; & $p < 0.05$; && $p < 0.01$.

DISCUSSION

Our current study demonstrated that *S. aureus* exposure caused cellular apoptosis in the osteoblast precursor cell line MC3T3-E1. These cells also experienced concomitant, dose-dependent elevation of TLR2, IL-6, TNF- α , and TNFR1 expression, which would either directly or indirectly induce apoptosis [18–20]. The TLR pathways connecting infection detection and cellular responses indicated by cytokine expression are channeled through the MyD88 and IRAK4 proteins and then into several independent mitogen-activated protein kinase (MAPK) shunts, including ERK and JNK [21]. These pathways then proceed to the gene expression level where they affect cellular survival decision-making and other cellular responses to infection, such as cytokine production.

In the present study, we used a complex agent, live *S. aureus* bacteria, to provoke cellular responses in the MC3T3-E1 cell line. A wide variety of different bacterial antigens, including SpA, were present to provoke signals through a variety of different regulatory cascades. SpA has been shown to bind to

TNFR1 and then activate both NF- κ B and MAPKs. For example, Zhang *et al.* [22] demonstrated that phosphorylation of NF- κ B, c-Jun, and ERKs was suppressed in TNFR1-null osteoclast precursors treated with RANKL. Claro *et al.* [8] also demonstrated activation of NF- κ B and release of IL-6 by SpA exposure of the MC3T3-E1 cell line. Our results demonstrated that knockdown of TNFR1 with shRNA suppressed the activation (phosphorylation) of the JNK pathway in MC3T3-E1 cells, but not down to pre-infection levels. This demonstrated that, although the MAPK pathways were active in infection signaling in this pre-osteoblastic cell line, other pathways were also directing downstream responses to infection. Future studies will focus on identifying and elucidating the role of SpA and other known ligands on the cellular responses to infection signaling.

Comprehensive network mapping of TLR signaling has identified at least seven positive and seven negative feedback loops [21]. The ERK pathway, among others in this complex regulatory network, has also been identified as a potential negative feedforward control for apoptosis through the Nur77 protein and TLR2. A feedforward loop allows for a set expression response independent of the magnitude of the input. Rather, the output of the control circuit is dependent on the fold increase in the signal [23]. Such control circuits allow for the detection of a signal in a noisy background that, for example, may be present when detecting infection signals in the extracellular environment.

We demonstrated the activity of such feedback loops, as the elevated expression of TLR2 in response to infection was partially suppressed by TNFR1 knockdown in cells exposed and fully suppressed by antibody against TLR2 itself. In one known positive feedback loop, TLR2 is a transcriptional target of NF- κ B. Bonnard *et al.* [24] demonstrated that TNF dramatically induces TLR2 mRNA, and murine embryonic fibroblasts deleted for *relA*, the gene encoding NF- κ B, do not express detectable TLR2 mRNA. Therefore, any reduction in signaling inputs that reduces the amount of NF- κ B entering the nucleus will also reduce the expression of TLR2 and potentially the strength of signal detection through the TLR system. This same antibody to TLR2 also suppressed TNF- α and IL-6 expression, but not to pre-infection levels, again demonstrating the presence of alternative activating pathways.

We demonstrated the importance of the JNK pathway in pre-osteoblast infection signaling by

inhibiting the JNK protein with SP600125. This chemical also suppressed TNF- α , IL-6, and, to a lesser extent, TLR2 expression, but again not down to un-induced levels. Notably, 25 μ M SP600125 is an highly effective suppressor of the phosphorylating abilities of the proteins JNK1, JNK2, and JNK3 [25]. Therefore, specific inhibition of any particular protein cannot be linked to a particular regulatory pathway based upon these results.

In summary, we demonstrated that TLR2 and TNFR1 expression are linked through a global cellular response to infection.

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COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no competing interests.

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