

# MKP-1 is a Negative Regulator of *Staphylococcus aureus*-Induced Inflammatory Gene Expression in Macrophages

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**Received date:** February 13, 2026, **Accepted date:** March 26, 2026

**Citation:** Bechill MP, Franco JH, Worth RG, Wooten RM, Liu Y, Pan ZK. MKP-1 is a Negative Regulator of *Staphylococcus aureus*-Induced Inflammatory Gene Expression in Macrophages. J Cell Signal. 2026;7(2):56–63.

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## Abstract

*Staphylococcus aureus* is a non-motile Gram-positive bacterium that exhibits antibiotic resistant forms (i.e. MRSA, VRSA), which continue to represent a major source of nosocomial infection. Alternative therapeutics are urgently needed to control the overactive host response in *S. aureus*-associated sepsis. However, the therapeutic molecular targets for sepsis remain poorly understood. We aim to identify these molecular targets to better understand the complex host response and to spur the development of novel therapeutics. MKP-1 plays a critical role in negatively regulating *S. aureus*-induced inflammatory cytokine production in macrophages. We found that *S. aureus* can induce MKP-1 expression *in vitro*. Knocking out MKP-1 results in significantly higher levels of TNF $\alpha$  and IL-1 $\beta$  gene expression during *S. aureus* infection of bone marrow-derived macrophages (BMDMs). Pharmacological induction of MKP-1 by rolipram produces significantly decreased levels of TNF $\alpha$ . The mechanism of MKP-1 expression by *S. aureus* requires Toll-like receptor 2 (TLR-2). Together, these findings indicate that MKP-1 is a negative regulator during *S. aureus* infection.

**Keywords:** MKP-1, DUSP-1, *S. aureus*, Sepsis, Inflammatory cytokines, TNF $\alpha$

## Introduction

Sepsis continues to be a major obstacle for the US healthcare system, with approximately 660,000 cases reported in 2000 [1]. An analysis of health data collected by the Centers for Disease Control and Prevention showed that the US experienced 240,000 deaths from sepsis in 2022 [2]. The overall age adjusted mortality rate for sepsis was found to be 89 per 100,000 people [2]. Treatment consists of broad-spectrum antibiotics; however, antibiotic-resistant infections represent a significant cause of sepsis [3]. Therefore, novel therapeutics are urgently needed to control the overactive host response in sepsis.

Approximately half of all bacterial sepsis cases are due to infection by a Gram-positive bacterium [4]. Of these

cases, 20.5% result from *Staphylococcus aureus* infection; an additional 10.2% are due to Methicillin resistant *S. aureus* (MRSA) [4]. Another antibiotic resistant strain is Vancomycin resistant *S. aureus*, which represents a growing cause of nosocomial infection [5,6]. *S. aureus* is a non-motile Gram-positive bacterium that is responsible for causing pneumonia, skin and soft tissue infections, endocarditis, osteomyelitis, and sepsis. During sepsis, *S. aureus* induces Toll-like receptor 2 (TLR-2) to provoke an exaggerated inflammatory response [7]. In the innate immune system, TLR-2 functions as a pattern recognition receptor against common bacterial antigens [7]. Activation of TLR-2 leads to downstream production of inflammatory cytokines, such as TNF $\alpha$  and IL1- $\beta$  [8].

Inflammation is necessary for clearing bacterial infections, but it can be detrimental if left unregulated. Therefore, termination

of inflammation is a tightly controlled part of the host immune response [9]. Mitogen-activated protein kinase phosphatase-1 (MKP-1) is a critical negative regulator of inflammation [10,11]. Both proinflammatory cytokines (e.g., TNF $\alpha$ ) and MKP-1 are expressed in response to TLR activation [10]. Proinflammatory cytokines cause inflammation by stimulating immune function, while MKP-1 dampens the immune response by inhibiting proinflammatory mediators (i.e., p38, ERK, and JNK) [12,13]. *In vitro* studies have shown that increased TNF $\alpha$  leads to greater MKP-1 expression, which allows for a carefully regulated immune response [13]. However, this negative feedback system is less effective against *S. aureus* infection, which can induce an exaggerated inflammatory response that is unaffected by host anti-inflammatory mechanisms. In our study, we aim to determine if *S. aureus* can elicit MKP-1 production *in vivo*.

Rolipram is a phosphodiesterase 4 (PDE-4) inhibitor which has been shown to upregulate MKP-1 expression in mouse macrophage cell-lines treated with lipopolysaccharide (LPS) [14,15]. PDE-4 inhibitors selectively degrade secondary messengers of the inflammatory response, such as cAMP [16]. Rolipram induced upregulation of MKP-1 was associated with a dose-dependent decrease in TNF $\alpha$  [14,15].

Based on previously published work, we hypothesize that MKP-1 plays a similar role in an *in vivo* *S. aureus* infection model. We performed experiments to demonstrate that *S. aureus* can induce MKP-1 expression in RAW 264.7 macrophages and primary bone marrow-derived macrophages (BMDMs). To investigate the function of MKP-1 in macrophages after *S. aureus* infection, we verified that the absence of MKP-1 impacts the expression of proinflammatory genes. Additionally, the pharmacological induction of MKP-1 negatively regulates TNF $\alpha$  transcription in macrophages after *S. aureus* infection. Finally, we demonstrated that TLR-2 is responsible for initiating signaling mechanisms that ultimately lead to MKP-1 induction.

## Materials and Methods

### Bacterial strains and culture

A clinical isolate of *S. aureus* strain USA300 was generously provided by Dr. Randall Worth and was characterized as previously described [17]. *Escherichia coli* K12 (substrain W3110; ATCC 27325) was generously provided by Dr. R. Mark Wooten. Bacteria were cultured in Bacto™ Tryptic Soy Broth (Becton, Dickinson and Company; Sparks, MD) in a 250mL sterile, plain bottom, vented closure PETG Nalgene flask (Thermo Scientific; Rochester, NY) overnight at 37°C and 200 RPM.

### Bacterial quantitation

Overnight culture was pelleted, suspended in 1x DPBS (Life Technologies; Grand Island, NY), and absorbance was read

at OD:600 using a SmartSpec™ 3000 (Bio-Rad; Hercules, CA). Absorbance of 0.8 at OD:600, whereby *S. aureus* = (2x10<sup>8</sup> CFU/mL) and *E. coli* = (6x10<sup>8</sup> CFU/mL).

### Mice

Mice were maintained in the animal facilities of the University of Toledo Health Science Campus. All experiments were performed in accordance with and approved by the University of Toledo IACUC. Wildtype and MKP-1 knockout C57BL/6 mice were purchased from the NCI mouse repository (Frederick, MD).

### Cell culture

RAW 264.7 macrophages were cultured in DMEM (1x)+GlutaMAX™-I media (GIBCO, Life Technologies; Grand Island, NY) containing 4.5 g/L D-Glucose, without sodium pyruvate and supplemented with 10% US Qualified FBS Lot # 1365352 (GIBCO, Life Technologies; Grand Island, NY) and 1% Penicillin-Streptomycin (5,000 U/mL) (GIBCO, Life Technologies; Grand Island, NY). Primary bone marrow-derived macrophages (BMDMs) were differentiated and isolated as previously described [18]. RAW and primary macrophages were seeded in culture media overnight prior to experimentation.

### Macrophage infection assay

Macrophages were seeded into 6-well or 24-well tissue culture treated plates at a final concentration of 2.00x10<sup>6</sup> per well or 2.00x10<sup>5</sup> per well, and they were allowed to adhere overnight at 37°C in a 5% ambient CO<sub>2</sub> incubator. Media was removed from the plated macrophages and bacteria was added at an MOI of 100. The plates were then gently centrifuged at 300g for 5 minutes, then returned to a CO<sub>2</sub> incubator for 1 hour to allow internalization of bacteria. After 1 h the cells were washed twice with room temperature 1x DPBS (Life Technologies; Grand Island, NY) to remove any extracellular bacteria. After the second wash, fresh pre-warmed media was added to the macrophages for the duration of the experiment, and the cells were returned to the incubator. At each experimental time point, cells were washed once with 4°C 1x DPBS to remove any residual media. Cells were suspended in 100  $\mu$ L 4°C 1x DPBS and lysed with RIPA Lysis and Extraction buffer (Pierce, Thermo Scientific; Rockford, IL) supplemented with 1x halt protease and phosphatase inhibitor cocktail (Pierce, Thermo Scientific; Rockford, IL) for western blot samples, or RLT buffer for qPCR samples. The samples were vortexed vigorously and stored at -80°C for later analysis.

### Ligand stimulation of macrophages

Peptidoglycan (PGN) (InvivoGen; San Diego, California) and lipoteichoic acid (LTA) (InvivoGen; San Diego, California) derived from *S. aureus* were suspended according to the

manufactures recommended instructions and stored at a final concentration of 200 µg/mL or 5 mg/mL aliquots respectively at -20°C until use. Peptidoglycan or lipoteichoic acid were added to macrophages.

### Rolipram treatment of macrophages

Rolipram (MP Biomedicals, LLC; Illkirch, France) was suspended according to the manufactures recommended instructions and stored at -20°C until use. For each experiment an aliquot was thawed and suspended in pre-warmed experimental media to a final concentration ranging from 0.1–100 µM.

### Propidium iodide assay

Macrophages were infected as previously described [14]. Cells were stained with propidium iodide nucleic acid stain (Molecular Probes, Life Technologies; Grand Island, NY) following the manufactures protocol and fluorescence read on a Tali® instrument. The ratio of non-fluorescent to fluorescent cells was used to determine percent viability.

### Total internal reflection fluorescence microscopy

Raw 264.7 Cells were seeded at  $2 \times 10^5$  cells per well in 2 mL volumes on 24 mm round cover glass (Fisher Scientific; Pittsburgh, PA), within clear plastic 6-well tissue culture plates (Corning; Fisher Scientific; Pittsburgh, PA) and incubated overnight at 37°C in 5% CO<sub>2</sub>. The following day, macrophages adhering to coverslips were infected with *S. aureus* and incubated at 37°C in 5% CO<sub>2</sub> for 1 h. After incubation, the coverslip was washed three times twice with room temperature 1x DPBS (Life Technologies; Grand Island, NY) aspirating between washes. The cover slip was placed on a mounting plate and 1 mL of fresh pre-warmed experimental media was added. The mounting plate and coverslip were then placed within an environmental chamber (Live Cell, Pathology Devices Inc, Westminster, MD) that maintained cells at 37°C, humidified, and contained 5% CO<sub>2</sub> atmosphere. Bright-field images were acquired at 5 min intervals using a 40x, 1.35 numerical aperture (NA), Olympus objective on a Total Internal Reflection Fluorescence (TIRF) microscope (Olympus; Shinjuku, Tokyo). Images were processed using MetaMorph (for Olympus Premier image analysis software version 7.5.6.0) and exported as an AVI video file.

### Western blot

The cell lysates were thawed on ice, cleared of cellular debris, and total protein quantified using the BCA protein assay (Pierce, Thermo Scientific; Rockford, IL). Sample were run Bolt® Bis-Tris plus 4–12% precast polyacrylamide gradient gels in 1x Bolt® MES SDS running buffer (Life Technologies; Grand Island, NY) at 165V for 36 min. Protein was transferred using the iBlot® gel transfer system (Life Technologies; Grand

Island, NY) and blocked for 1 h in Odyssey® blocking buffer (LI-COR; Lincoln, Nebraska). The membrane was incubated with primary antibody overnight, washed using 1x 0.2% DPBST, incubated for 1 h in IRDye secondary antibodies (LI-COR; Lincoln, Nebraska), washed three times, and then scanned using the Odyssey® infrared imaging system (LI-COR; Lincoln, Nebraska). Images were analyzed using Image studio V2.1 (LI-COR; Lincoln, Nebraska).

### Quantitative polymerase chain reaction

Total mRNA was isolated with the RNeasy mini kit (Qiagen; Venlo, Limburg, Germany). Total mRNA quantity and purity was analyzed using a NanoDrop 2000 spectrophotometer (Thermo scientific; Rochester, NY). Reverse transcription was performed using the QuantiTect reverse transcription kit (Qiagen; Venlo, Limburg, Germany). cDNA was diluted 1:10 and added to FastStart essential DNA green master mix (Roche Diagnostics Corporation; Indianapolis, IN). qPCR was performed on the LightCycler96 (Roche Diagnostics Corporation; Indianapolis, IN). All qPCR data was analyzed using LightCycler96 software version 1.1.0.1320 (Roche Diagnostics Corporation; Indianapolis, IN).

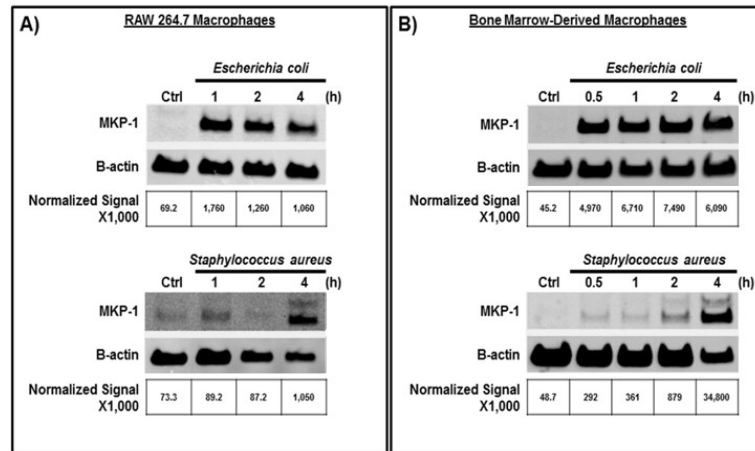
### Graphing and statistical analyses

Data was analyzed on a personal computer using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Statistical significance was determined using either GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA), Microsoft Excel 2010 (Microsoft Corporation), or SPSS Statistics Version 21 (IBM; Armonk, NY). Datasets were evaluated using a one-way ANOVA with Tukey's post-hoc test. P-values  $\leq 0.05$  were considered statistically significant, with experiments completed in triplicate.

## Results

### *S. aureus* can induce MKP-1 expression in macrophages.

We first verified the published literature showing that macrophage cell-lines upregulate MKP-1 in response to bacterial infection. Mouse RAW 264.7 macrophages were infected with *S. aureus* or *E. coli* for 0.5–4 h. Coinciding with previous findings, *E. coli* infection of RAW 264.7 macrophages induced MKP-1 protein expression after 0.5h from infection and expression was maintained through the 4 h time point (**Figure 1A**). *S. aureus* induced maximal MKP-1 expression at 4 h post infection (**Figure 1A**). MKP-1 induction was similar between cell-lines and primary BMDMs when infected with *S. aureus* or *E. coli* (**Figure 1B**). Taken together, the results confirm that MKP-1 is induced during bacterial infection of macrophage cell-lines and BMDMs. We were able to show similar kinetics of MKP-1 induction in both types of macrophages.



**Figure 1.** *S. aureus* can induce MKP-1 expression in RAW 264.7 macrophages and BMDMs from C57BL/6 mice. (A) RAW 264.7 cells or (B) BMDMs were infected with *E. coli* or *S. aureus* (MOI = 100). Macrophages were lysed at indicated times post-infection and western blot performed.

To alleviate the concern that an MOI of 100 may kill the macrophages, cell viability was monitored. Membrane integrity was not affected up to 6h post infection when examined by propidium iodide staining (Figure S1). Additionally, *S. aureus* infected RAW 264.7 macrophages were visualized by bright-field images collected using TIRF (Figure S1). Macrophages were found to be adherent after infection, although cell rounding was observed starting at 13–14 h post infection. Therefore, we focused on data acquired after a maximum of 4 h of infection to avoid potential nonspecific effects of cell toxicity on MKP-1 regulation and activity.

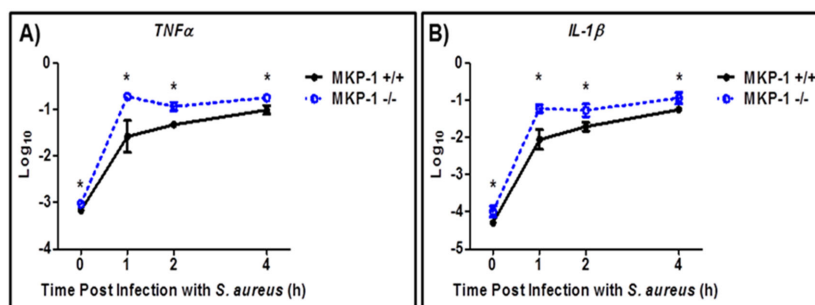
### MKP-1 knockout BMDMs produce significantly higher levels of inflammatory gene expression during *S. aureus* infection

Next, we examined inflammatory cytokine production during *S. aureus* infection in macrophages lacking MKP-1. We infected BMDMs from wild type and MKP-1 knockout mice. We detected

significantly higher levels of TNF $\alpha$  and IL-1 $\beta$  gene transcripts in MKP-1 knockout BMDMs at all-time points (Figures 2A and 2B). These results suggest that MKP-1 serves as a negative regulator of inflammatory cytokine production *in vivo*.

### Pharmacological induction of MKP-1 significantly decreases TNF $\alpha$ expression in RAW 264.7 macrophages during *S. aureus* infection

We've previously shown that in *S. aureus* infected RAW 264.7 macrophages, pretreatment with 10  $\mu$ M Rolipram was shown to induce MKP-1 [14]. Rolipram exhibited peak MKP-1 mRNA expression after a 1 h pretreatment [14]. In our current study, we pretreated RAW 264.7 macrophages with 0.1–100  $\mu$ M rolipram for 1 h. This resulted in a dose-dependent increase in MKP-1 mRNA and a decrease in TNF $\alpha$  mRNA (Figure 3A). These findings suggest that *S. aureus*-induced TNF $\alpha$  production can be inhibited by rolipram pretreatment in a dose dependent manner, via the induction of MKP-1.



**Figure 2.** Knockout of MKP-1 results in significantly elevated (A) TNF $\alpha$  and (B) IL-1 $\beta$  mRNA expression during *S. aureus* infection (MOI 100). All significant values (\* $P \leq 0.05$ ) were compared to the wild-type at the same time point. Data shown as mean  $\pm$  SEM ( $n=3$  total); each experiment was performed in triplicate.

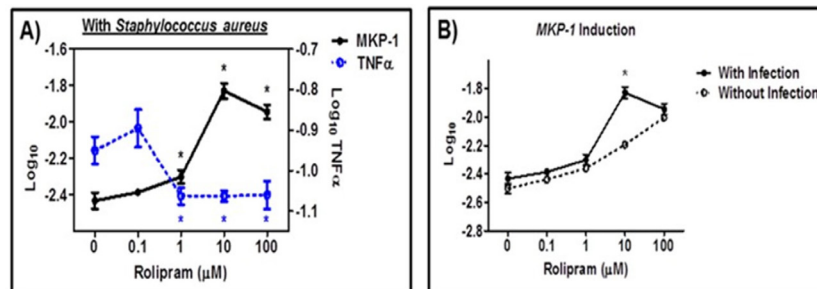
We observed a significant effect when we compared MKP-1 transcript levels in infected RAW 264.7 macrophages pretreated with 10  $\mu$ M rolipram compared to uninfected pretreated macrophages (**Figure 3B**). This suggests the possibility of an additive effect on MKP-1 induction between rolipram (10  $\mu$ M) and infection with *S. aureus*.

### TLR-2 ligands are sufficient for MKP-1 induction and TLR-2 is required for maximal MKP-1 induction by *S. aureus*

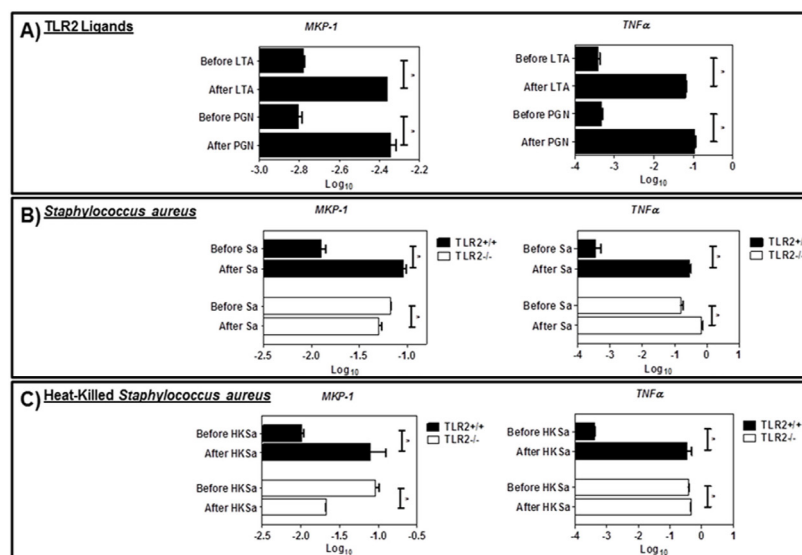
To evaluate if MKP-1 induction occurs through TLR signaling, we first determined whether TLR-2 ligands could elicit MKP-1 production. Lipoteichoic acid (LTA) and peptidoglycan (PGN)

are two common TLR-2 ligands derived from *S. aureus* [19]. When treated with LTA and PGN for 1 h, BMDMs exhibited significantly increased MKP-1 mRNA expression (**Figure 4A**). This suggests that TLR-2 ligands derived from *S. aureus* are sufficient for MKP-1 induction.

To study these effects in the context of a live infection rather than purified subunits, we next compared *S. aureus*-induced MKP-1 production in BMDM derived from wild-type and TLR-2 deficient C57BL/6 mice. *S. aureus* was able to elicit MKP-1 induction in wild-type macrophages; however, in the absence of TLR-2 there was no induction of MKP-1 mRNA (**Figure 4B**). This data suggests that TLR-2 is required for *S. aureus*-induced MKP-1 expression in macrophages.



**Figure 3.** RAW 264.7 macrophages pretreated with Rolipram exhibited (A) increased MKP-1 mRNA expression, and decreased TNF $\alpha$  mRNA expression. (B) Rolipram-induced MKP-1 mRNA expression was increased during *S. aureus* infection (MOI 100). All significant values ( $*P \leq 0.05$ ) were compared to (A) 0  $\mu$ M Rolipram or (B) similar doses with or without infection; each experiment was performed in triplicate.



**Figure 4.** MKP-1 and TNF $\alpha$  mRNA expression was increased (A) in response to pretreatment with LTA and PGN. In wild-type BMDMs, (B,C) MKP-1 and TNF $\alpha$  were increased in response to live (Sa) and heat killed *S. aureus* (HKSa). In TLR-2 knockout BMDMs, (B,C) MKP-1 expression was decreased in response to Sa and HKSa infection. TNF $\alpha$  expression was minimally increased in response to Sa and HKSa infection. All significant values ( $*P \leq 0.05$ ) were compared to the wild-type at 4 h post infection. Data shown as mean  $\pm$  SEM and are one representative experiment (n=3 total); each experiment was performed in triplicate.

An alternative hypothesis is that MKP-1 induction by *S. aureus* is due to secreted bacterial factors rather than surface ligands. To evaluate this hypothesis, we infected BMDMs with heat-killed *S. aureus*. As with the live bacterial infection, heat-killed *S. aureus* induced MKP-1 production in wild-type, but not the TLR-2 knockout BMDMs (**Figure 4C**). In addition to MKP-1, we also examined TNF $\alpha$  induction in wild-type and TLR-2 knockout BMDMs. BMDMs infected with live or heat-killed bacteria produced increased TNF $\alpha$  mRNA in response to bacterial infection in both wild-type and TLR-2 knockout cells (**Figures 4B** and **4C**).

## Discussion

We determined that MKP-1 can be induced by *S. aureus* infection in RAW 267.4 cells and BMDMs. TLR-2 signaling is necessary for MKP-1 induction after *S. aureus* infection. Taken together, we have identified that MKP-1 may be an important target for future development of novel therapeutics for *S. aureus* associated sepsis. These medications are needed against quickly expanding drug-resistant *S. aureus* infections.

In our first aim, we determined that MKP-1 is induced by *E. coli* and *S. aureus* infection in RAW 264.7 macrophages. Previous work identified that MKP-1 can be elicited by macrophage cell-lines in response to Gram-negative bacteria [11]. However, it was unclear if Gram-positive bacteria also elicited MKP-1 in an *in vivo* setting. We demonstrated that both live and heat-killed *S. aureus* elicit MKP-1 mRNA expression in BMDMs. Interestingly, the maximal production of MKP-1 protein was observed 4 h after *S. aureus* infection. This is of note because MKP-1 has generally been described as an immediate early protein, reaching maximal expression in 15–60 min *in vitro* [10]. We showed maximal MKP-1 protein induction occurring 30 min after *E. coli* infection and at 4 h post infection with *S. aureus*. This may suggest that the kinetics by which macrophages regulate inflammatory cytokines (e.g., TNF $\alpha$ ) are different depending on the infection. Also, our use of a 100  $\mu$ M MOI infection may explain the delay in peak MKP-1 expression because higher titers will result in a larger initial inflammatory response. Future studies, such as a MKP-1 reporter assay, could determine differences in MKP-1 promoter activation in response to different bacterial infections. Taken together, we have identified that *S. aureus* can elicit MKP-1 production and that kinetics of production differ depending on the infection type.

In our second aim, we verified that MKP-1 functions as a negative regulator of *S. aureus*-induced inflammatory cytokines in BMDMs. By increasing MKP-1 expression using rolipram, we demonstrated that the increase coincided with a reciprocal decrease in TNF $\alpha$  mRNA levels. This suggests an inverse relationship between MKP-1 and inflammatory gene expression. We sought to further establish this relationship

using a second line of evidence. Experiments using BMDMs derived from MKP-1 knockout mice illustrated that in the absence of MKP-1, higher levels of TNF $\alpha$  mRNA are produced. Additional studies using MKP-1 overexpressing cells can provide further evidence that MKP-1 downregulates inflammatory processes during *S. aureus* infection. By tempering the production of cytokine gene transcripts, MKP-1 likely has a role in preventing sepsis from *S. aureus* infections.

In our third aim, we determined that TLR-2 is necessary for MKP-1 induction after *S. aureus* infection in BMDMs. Previous studies have shown that TLRs are involved in MKP-1 expression, however, the requirement for specific receptors has not been well-established [20]. We demonstrated that TLR-2 ligands (i.e., PGN and LTA) are sufficient to induce MKP-1 mRNA expression. Using live or heat-killed *S. aureus* infection in wild-type and TLR-2 knockout BMDMs, we demonstrated that TLR-2 activation (i.e., via bacterial surface ligands) is necessary for maximal MKP-1 mRNA induction. Without TLR-2, BMDMs were unable to upregulate MKP-1 in response to infection. In both TLR-2 wild-type and knockout BMDMs, TNF $\alpha$  was upregulated after infection. This suggests that while MKP-1 is reliant on TLR-2 activation, TNF $\alpha$  can be produced in response to activation of other pattern recognition receptors. Overall, the results of specific aim 3 suggest that TLR-2 ligands are sufficient for MKP-1 induction and TLR-2 is required for maximal MKP-1 induction by *S. aureus*.

Unlike in our rolipram experiment, increased MKP-1 mRNA expression wasn't associated with decreased TNF $\alpha$  mRNA expression. This highlights *S. aureus*' unique capacity to promote a dysregulated inflammatory response that overpowers host anti-inflammatory mechanisms. Only when MKP-1 is overexpressed by rolipram, is the host anti-inflammatory response able to attenuate *S. aureus*-induced TNF $\alpha$  expression. An important limitation of the study was the focus on macrophage cell-lines and BMDMs. Additional studies examining rolipram's efficacy in an *in vivo* *S. aureus* sepsis mouse model would better show the effectiveness of MKP-1 induction during systemic infection.

In conclusion, the high global burden of sepsis is of increasing concern. By understanding the body's anti-inflammatory mechanisms, we could develop better strategies for treating sepsis. This has never been more pertinent than today as antibiotic-resistant bacteria continue to represent a major obstacle to healthcare systems. The data presented illustrates that MKP-1 is induced in a TLR-2 dependent fashion and functions to negatively regulate *S. aureus*-induced inflammatory cytokine production in BMDMs. This insight provides additional avenues of study as well as potential therapeutic targets for sepsis treatment.

We've shown that rolipram-induced MKP-1 expression is associated with decreased TNF $\alpha$  mRNA expression. This

provides further evidence that medications that upregulate MKP-1, such as corticosteroids, are potent inflammatory mediators [13]. While MKP-1 inducers show promise, current therapeutics are characterized by systemic side effects [21]. Corticosteroids are potent anti-inflammatory medications, but they are associated with metabolic dysfunction (e.g., hyperglycemia, hyperlipidemia) [22]. Rolipram is not associated with metabolic dysfunction, however, it carries a significant risk of nausea and headache [21]. Newer PDE-4 inhibitors (e.g., Roflumilast, Apremilast) are associated with less side effects and are currently approved for autoimmune conditions, such as COPD and severe psoriasis. These medications warrant further consideration as possible adjuncts for sepsis treatment considering their ability to increase MKP-1 and improved side effect profile.

## Acknowledgements

We thank Dr. Travis R. Taylor for his technical and intellectual advice during the early stages of the research project.

## Author Contributions

MB and ZP conceived of the research project. RW, MW, and ZP provided input on experimental design. RW and YL provided bacteria strains and animal resources, respectively. MP conducted the experiments and statistical analysis. MP and JF contributed to writing and editing the manuscript.

## Conflicts of Interest

The authors report no conflicts of interest in regard to the published article.

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