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Staphylococcus aureus increases Prostaglandin E₂ secretion in cow neutrophils by activating TLR2, TLR4, and NLRP3 inflammasome signaling pathways

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Introduction: In clinical settings, dairy cows are often attacked by pathogenic bacteria after delivery, especially *Staphylococcus aureus* (*S. aureus*). Neutrophils have long been regarded as essential for host defense against *S. aureus*. Prostaglandin E₂ (PGE₂) can additionally be used as an inflammatory mediator in pathological conditions to promote the repair of inflammatory injuries. However, whether *S. aureus* can promote the accumulation of PGE₂ after the infection of neutrophils in cows and its mechanism remain unclear. Lipoprotein is an important immune bioactive ingredient of *S. aureus*.

Methods: In this study, the changes in neutrophils were monitored in dairy cows infected with wild-type *S. aureus* (SA113) and an *S. aureus* lipoprotein-deficient strain (Δlgt); meanwhile, we established whether pattern recognition receptors mediate this process and whether *S. aureus* lipoproteins are necessary for causing the release of PGE₂ from cow neutrophils.

Results: The results showed that Δlgt was less effective than SA113 in inducing the production of IL-1 β , IL-6, IL-8, IL-10, and PGE₂ within neutrophils; furthermore, TLR2, TLR4, and NLRP3 receptors were found to mediate the inducible effect of lipoprotein on the above inflammation mediators and cytokines, which depended on MAPK and Caspase-1 signaling pathways. In addition, TLR2, TLR4, and NLRP3 inhibitors significantly inhibited PGE₂ and cytokine secretion, and PGE₂ was involved in the interaction of *S. aureus* and neutrophils in dairy cows, which could be regulated by TLR2, TLR4, and NLRP3 receptors. We also found that *S. aureus* was more likely to be killed by neutrophils when it lacked lipoprotein and TLR2, TLR4, and NLRP3 were involved, but PGE₂ seemed to have no effect.

Discussion: Taken together, these results suggest that lipoprotein is a crucial component of *S. aureus* in inducing cytokine secretion by neutrophils as well as killing within neutrophils, which could be accomplished by the accumulation of PGE₂ by activating MAPK and the Caspase-1 signaling pathways through TLR2, TLR4, and NLRP3 receptors. These results will contribute to a better understanding of the interaction between *S. aureus* and host immune cells in dairy cows.

KEYWORDS

Staphylococcus aureus, Prostaglandin E₂, neutrophils, toll-like receptor 2, toll-like receptor 4, NLR pyrin domain-containing 3

Introduction

In veterinary clinical practice, *Staphylococcus aureus* (*S. aureus*) is one of the main bacteria that cause infectious diseases in dairy cows, such as mastitis, vaginitis (Petit et al., 2010), and endometritis (Zhao et al., 2014). Neutrophils play a role in innate immunity and are vital for the clearance of *S. aureus* (Dsa et al., 2022). Unlike other phagocytes such as monocytes and macrophages, neutrophils are fully capable of killing gram-positive bacteria such as *S. aureus* (Kevin and DeLeo, 2012). Therefore, neutrophils are the most prominent immune cells, which can effectively kill *S. aureus* in the immune system (Amulic et al., 2012). Prostaglandin E₂ (PGE₂) performs an indispensable function in endometrial inflammatory harm (Tingting et al., 2018). It builds up in pathogen-infected endometrial tissues and increases the expression of pro-inflammatory proteins and damage-associated molecular patterns (DAMPs), which increased the degree of the inflammatory response (Tingting et al., 2018). However, the mechanisms underlying the role of PGE₂ accumulation in *S. aureus*-infected cow neutrophils remain unclear.

The innate immune system plays a crucial role in the host's defense against infections brought on by *S. aureus*, a well-known gram-positive bacterium. Notably, many virulence elements of *S. aureus* are directed toward factors of the innate immune system (Kessel et al., 2014). Toll-like receptor 2 (TLR2) is a class I transmembrane receptor that recognizes lipophosphatidic acid (LTA) and peptidoglycan (PGN) of *S. aureus*, thereby mediating the innate immune inflammatory response (Nakanishi et al., 2008). *S. aureus* lipoproteins are ligands for the major TLR2 receptor in cell wall components (Hashimoto et al., 2006a,b). The transcription factors such as mitogen-activated protein kinase (MAPK) signaling pathways are activated when TLR2 receptors are stimulated by *S. aureus*, which promotes the secretion of many pro-inflammatory mediators (Sheldon et al., 2014; Tingting et al., 2019). It was found that neutrophils express TLR2 and Toll-like receptor 4 (TLR4), and brain hemorrhage is not only dependent on TLR2 receptors but also requires TLR4 receptors. Increased expression of TLR2 and TLR4 receptors is linked to functional deficits within 3 months of a brain hemorrhage. TLR4 receptors are the first TLRs identified in mammals that are homologous to *Drosophila* and are the main receptors recognized by lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria (Rodríguez-Yáñez et al., 2012). TLR2 and TLR4 are the two widely studied receptors that regulate the phagocytic killing of gram-positive and gram-negative bacteria by polymorphonuclear neutrophils (PMNs), respectively (Prince et al., 2011). It is believed that the IL-1 β produced by the NLRP3-inflammasome activates Caspase-1, which causes cyclooxygenase-2 (COX-2) to be upregulated and PGE₂ to be produced more often (Catley et al., 2003). It was demonstrated that not only TLR2 is involved in the defense of *S. aureus* infection in mouse peritoneal macrophages but also TLR4 and the NLR pyrin domain-containing 3 (NLRP3) inflammasome can be activated by *S. aureus* (Wu et al., 2020). However, it is not clear how cow neutrophils recognize *S. aureus*.

Among the PGs, PGE₂ is mostly related to inflammation and is acknowledged to be present in large amounts in inflammatory exudates. In particular, PGE₂ is present in higher concentrations during inflammation compared to other PGs, and it is involved in all responses leading to the typical symptoms of inflammation, including fever, edema, and pain (Wan et al., 2018). PGE₂ production and biosynthesis, through inflammation, are exceptionally regulated by COX-2 and microsomal PGE synthase (mPGES)-1 and are induced by a variety of pro-inflammatory factors, which include interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) (Pecchi et al., 2009). Preliminary laboratory discovery shows that the use of TLR2, TLR4, and NLRP3-deficient mice in tests revealed that these proteins are involved in macrophage PGE₂ secretion in response to *S. aureus* (Wu et al., 2020). However, it is still unclear whether PGE₂ accumulation in cow neutrophils infected with *S. aureus* is regulated by TLR2, TLR4, or NLRP3 receptors.

In this study, we found how *S. aureus* lipoprotein and host inflammatory bodies of TLR2, TLR4, and NLRP3 affected neutrophils' ability to secrete PGE₂ in cows with *S. aureus* infections.

Materials and methods

Reagents, chemicals, and antibodies

Fetal bovine serum (FBS), RPMI1640 medium, and PBS (Hyclon, Logan, UT); dairy cow peripheral blood neutrophil isolation kit (Hao Yang, Tian Jing, China); MH broth (Oxoid, Hampshire, UK); TLR2 receptor inhibitor (C29, MCE, China), TLR4 receptor inhibitor (TAK-242, MCE, China), NLRP3 receptor inhibitor (MCC950, MCE, China); COX-2 inhibitor (CAY10404), mPGES-1 inhibitor (MF63) (Cayman Chemical, MI, United States); gentamicin sulfate (Promega, Wisconsin, United States); Bovine IL-1 β , IL-10, IL-8 ELISA Kits (Kingfisher, Biotech); Bovine IL-6 ELISA Kit (R&D Systems, California, United States); PGE₂ ELISA Kit (Cayman Chemical, MI, United States); M-PER mammalian protein extraction reagent, Halt protease inhibitor, pre-stained protein ladders, blocking buffer, starting block T20 (TBS), LIVE/DEAD Bac Light Bacterial Viability Kits (Thermo Fisher Scientific); SDS-PAGE loading buffer (Takara, Shiga, Japan); Pierce BCA Protein Assay Kit (Rockford, United States); SDS-PAGE gel electrophoresis kit (Solarbio, Beijing, China); Axy Prep Multisource Total mRNA Miniprep Kit (Axygen Scientific, Union City, United States); Primer Script RT Master Mix (Takara); SYBR Green Master (Rox) (Roche, Basel, Switzerland). All primers were synthesized by Generay (Shanghai, China).

Bacterial strains and animals

Prof. Friedrich Götz (Mikrobielle Genetik, Universität Tübingen, Germany) kindly contributed to the *S. aureus* SA113 wild-type strain (WT; ATCC 35558) and an *S. aureus*

SA113 isogenic mutant *lgt::ermB* (Δlgt) deficient in lipoprotein maturation (Stoll et al., 2005). At a constant temperature of 37°C for 16 h, all bacterial strains were grown in MH broth to an optical density of 2.0 at 600 nm. The 4~6-year-old Holstein cows were provided by the Veterinary College of Inner Mongolia Agricultural University.

Isolation of peripheral blood neutrophils

Jugular vein blood was collected from healthy Holstein cows. The cells were isolated using a bovine peripheral blood neutrophil extraction kit. A measure of 4 mL of separation solution I from the kit was added to a 15-mL centrifuge tube, and then 3 mL of the fresh anticoagulant was slowly added. The solution was centrifuged horizontally for 25 min at 2,000 rpm/min. The cells in the white layer at the interface of the separation solution were collected and put into a 50 mL centrifuge tube. In total, 2 mL of separation fluid II was increased to the new 15 mL centrifuge tube, and the collected white layer cells were slowly added to it. It was centrifuged horizontally at 2,000 rpm/min for 40 min. The supernatant was thrown out, the cells were collected, and 2 mL of RBC lysate was added for 2 min before being neutralized with a PBS solution three times the volume and centrifuged for 5 min at 800 g. Red blood cells that were still present were cleaved once more and then re-suspended in RPMI 1640 medium with 10% FBS before being counted and put in a 6-well plate (Ciliberti et al., 2021).

Experimental treatments

Dairy cow neutrophils were placed 2×10^6 cells per well on 6-well culture plates at 37°C in a 5% CO₂ cell culture incubator. Two strains of *S. aureus* SA113 and Δlgt were, respectively, infected with cells at 6×10^6 (MOI 3:1) and 2×10^7 (MOI 10:1). The infected cells were cultivated for 1 h at 37°C in an incubator, and gentamicin sulfate with a final concentration of 100 µg/ml was added to kill the extracellular *S. aureus*. In the TLR2 (C29, 10^{-5} M), TLR4 (TAK-242, 10^{-5} M), and NLRP3 (MCC950, 10^{-5} M) inhibitor groups, TLR2, TLR4, and NLRP3 inhibitors were added 4 h before *S. aureus* addition. In the COX2 (CAY10404, 10^{-5} M) and mPGES-1 (MF63, 10^{-5} M) inhibitor groups, the inhibitor was added 1 h in advance.

ELISA

Different cytokines were separately tested according to the instructions provided with each ELISA assay kit, the corresponding standard curves were plotted, and the cytokine samples' concentrations were calculated. A total of three biological replicates were performed.

Western blot analysis

Cells were treated with an M-PER reagent, and total protein extraction was carried out on the ice. According to

TABLE 1 List of major antibodies.

Antibody name	Dilution ratio	Producer
COX-2	1:1,000	Abcam, Cambridge, UK
mPGES-1	1:1,000	Cayman Chemical, MI, USA
Phospho-ERK	1:1,000	Cell Signaling Technology,
ERK	1:1,000	Beverly, MA
Phospho-p38	1:1,000	Ditto
p38	1:1,000	Ditto
Phospho-JNK	1:1,000	Ditto
JNK	1:1,000	Ditto
β-actin	1:1,000	Ditto
Pro-caspase-1 + p10 + p12	1:1,000	Abcam, Cambridge,
TLR2, TLR4	1:200	UKNovus Biologicals, USA
NLRP3	1:200	Novus Biologicals, USA

the manufacturer's instructions, concentration measurements and protein denaturation were carried out. A measure of 20 µg of full protein on each lane was divided by SDS-PAGE (12%) electrophoresis at 80 V, and the protein was transferred to polyvinylidene fluoride membranes at 25 V for 30 min. The membranes were blocked with TBST containing 3% BSA for 4 h at room temperature. The primary antibody is diluted to the appropriate concentration and incubated at 4°C for 14 h the dilution ratio of primary antibody is shown in Table 1. The membranes were washed five times with TBST for 5 min, each after incubation. The secondary antibody was incubated at room temperature for 1 h (1:3,000). The membranes were, then, subjected to three 20 min TBST washes after incubation, and after that exposed to electrochemiluminescence film and Western blot detection tools. Using ImageJ, band densities were measured.

Real-time RT-PCR analysis

After *S. aureus*-infected neutrophils, total RNA was isolated. Total mRNA extraction and reverse transcription were performed. The polymerase chain reaction conditions were as follows: 50°C, 2 min; 95°C, 10 min; 95°C, 15 s; and 60°C, 60 s, for 40 cycles. The annealing temperature was 58°C. The primers used in this study are listed in Table 2. The results were calculated using the $2^{-\Delta\Delta Ct}$ calculation method.

Microscopical examination of neutrophil cytotoxicity to bacteria

To verify the effect of PGE2, TLR2, TLR4, and NLRP3 on phagocytosis, neutrophils were cultured in 35 mm glass-bottomed Petri dishes (1×10^5 cells/dish). Cells were pretreated, or not treated, with a selective mPGES-1 inhibitor MF63, a TLR2 receptor inhibitor C29, a TLR4 receptor inhibitor TAK-242, and an NLRP3 receptor inhibitor MCC950. The cell membrane is, then, labeled

TABLE 2 Primer sequences for RT-qPCR.

Gene name	Sequences(5'-3')	Accession number
<i>β-actin</i>	F:5'-CCAAGGCCAACCGTGAGAAGAT-3' R:5'-CCACGTTCCGTGAGGATCTTCA-3'	NM_173979.3
<i>TLR2</i>	F:5'-ATGATGCTGCCATTCTGATTCT-3' R:5'-CTCCAGGTAGGTCTTGGTGTTC-3'	NM_174197.2
<i>TLR4</i>	F:5'-AGGTAGCCCAGACAGCATTTC-3' R:5'-AGCGAGTGGAGTGGTTCATAA-3'	NM_174198.6
<i>NLRP3</i>	F:5'-TCCCTGACCAGACTCTACTTG-3' R:5'-GTGGGTGAGATTCTGATTTGTA-3'	NM_001102219.1
<i>COX-2</i>	F:5'-GGTGCCTGGTCTGATGATGT-3' R:5'-GATTAGCCTGCTTGTCTGGAAC-3'	XM-007115297.3
<i>mPGES-1</i>	F:5'-ATGGTACACACCGTGGCATA-3' R:5'-CACAACTCAAAGGGCCATC-3'	XM-027556544.1

with SYTO9. *S. aureus* SA113 or ΔIgt strain (MOI 30:1) was labeled with PI for 6 h and then fixed with 4% paraformaldehyde. Laser confocal microscopy is used to capture images (400 X). Images of different samples were collected under the same conditions.

Statistical analysis

All data had been analyzed with the use of GraphPad Prism 5 and are expressed as the mean \pm standard deviation (SD). Statistical significance was evaluated by the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or two-way ANOVA (Bonferroni's post-test), as appropriate. Differences with $P \leq 0.05$ were considered statistically significant (* $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$).

Results

Staphylococcus aureus lipoprotein is an important protein that can stimulate the secretion of PGE₂ and cytokines by dairy cow neutrophils, as well as activate TLR2, TLR4, and NLRP3 receptors and the MAPK and Caspase-1 signaling pathways

Whether lipoprotein can induce the secretion of PGE₂ and cytokines after the infection of neutrophils by *S. aureus* is unclear. Moreover, the activation of TLR2, TLR4, and NLRP3 receptors and their mediated MAPK and Caspase-1 signaling pathways are still unclear. To analyze the effects of *S. aureus* on the secretion of PGE₂ and cytokines in neutrophils, the activation of TLR2, TLR4, and NLRP3 receptors, and the mediated MAPK and Caspase-1 signaling pathways, ELISA was used to detect the secretion of PGE₂, IL-1 β , IL-10, IL-6, and IL-8. The phosphorylation levels of

ERK, JNK, P38, and Caspase-1 were detected by Western blotting. TLR2, TLR4, and NLRP3 receptors' mRNA levels were detected by qRT-PCR and Western blotting.

The consequences indicated that, in contrast with those produced by way of SA113 *S. aureus*, ΔIgt *S. aureus* induced low levels of PGE₂, IL-1 β , IL-6, IL-10, and IL-8 secretion into neutrophil supernatants after infection of neutrophils for 9 h at MOI 3:1 and MOI 10:1 ($P < 0.01$, $P < 0.001$, Figures 1A–E). In MAPK and Caspase-1 pathways, the phosphorylation levels of ERK and P38 decreased in 15 min, 30 min, 60 min, and 120 min after infection with neutrophils by *S. aureus* ΔIgt compared with *S. aureus* SA113. Phosphorylation levels of Caspase-1 also decreased at 3 h, 6 h, and 9 h (Figures 1F–J). qRT-PCR results showed that compared with the SA113 *S. aureus*, the expression of TLR2, TLR4, and NLRP3 receptor genes in ΔIgt *S. aureus* was impaired at both MOI 3:1 and MOI 10:1, which was consistent with the results of the Western blotting ($P < 0.001$, Figures 1K–Q).

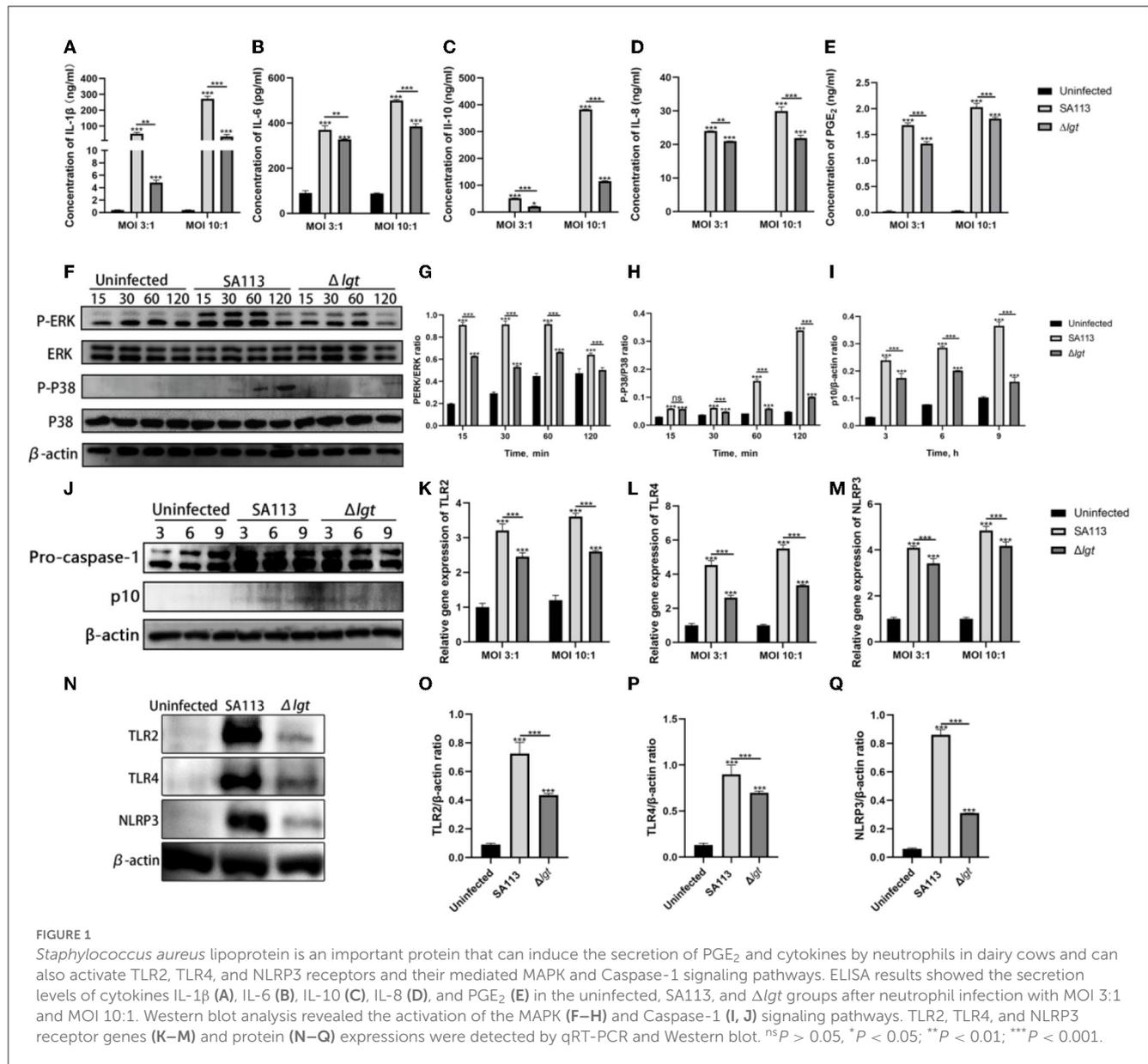
COX-2 and mPGES-1 expressions in *S. aureus*-infected neutrophils and lipoproteins are essential

The precise functions of lipoproteins in the expression of COX-2 and mPGES-1 in neutrophils following *S. aureus* infection are still unknown. COX-2 and mPGES-1 mRNA levels were determined by qRT-PCR, and their protein levels were examined by Western blotting (extracts from *S. aureus*-infected neutrophils).

The qRT-PCR results showed that in MOI 3:1, for COX-2 and mPGES-1, the SA113 *S. aureus* and ΔIgt *S. aureus* mRNA expression levels at 4 h did not differ significantly ($P > 0.05$, Figures 2A, C). COX-2 is the same thing at 12 h, but at 8 h, there is substantial mRNA expression of COX-2 and mPGES-1 ($P < 0.001$, Figures 2A, C). Furthermore, mPGES-1 mRNA expression was the same at 12 h ($P < 0.001$, Figure 2C); in MOI 10:1, there was no discernible variation in COX-2 mRNA expression levels between SA113 *S. aureus* and ΔIgt *S. aureus* at 4 h but high COX-2 mRNA expression at 8 h and 12 h ($P < 0.001$, Figure 2B). In addition, mPGES-1 mRNA expression was higher at 4 h, 8 h, and 12 h ($P < 0.01$, $P < 0.001$, Figure 2D). The COX-2 and mPGES-1 protein expressions increased 6 h and 12 h after infection with SA113 *S. aureus* (MOI 10: 1) by the Western blot analysis ($P < 0.001$, Figure 2E). This was in contrast to that in cells infected with the ΔIgt strain or uninfected controls. These findings show that *S. aureus* lipoproteins are essential for inducing the expression of COX-2 and mPGES-1 in neutrophils.

The secretion of PGE₂ and cytokines mediated by neutrophils depends on TLR2, TLR4, and NLRP3 receptors

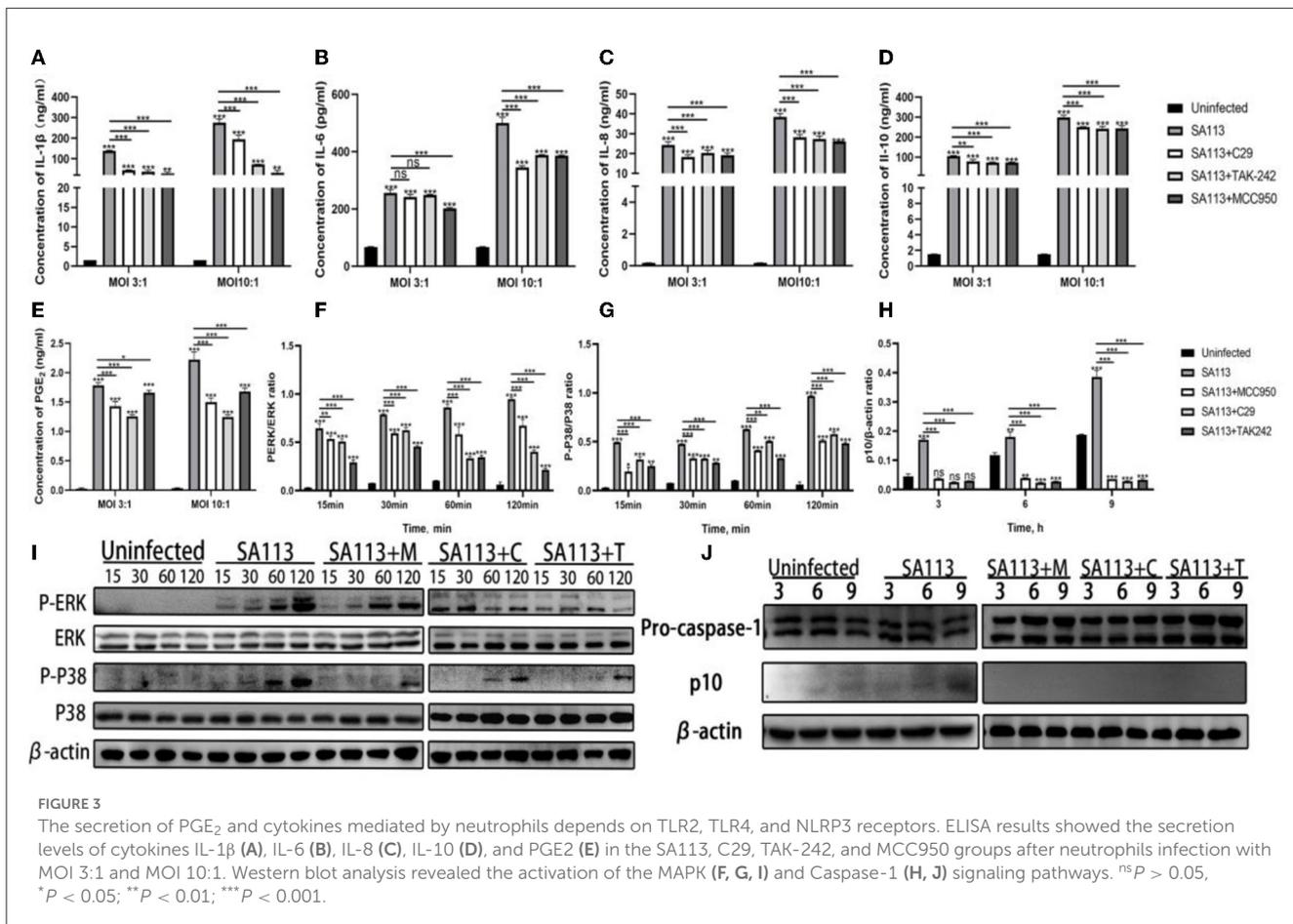
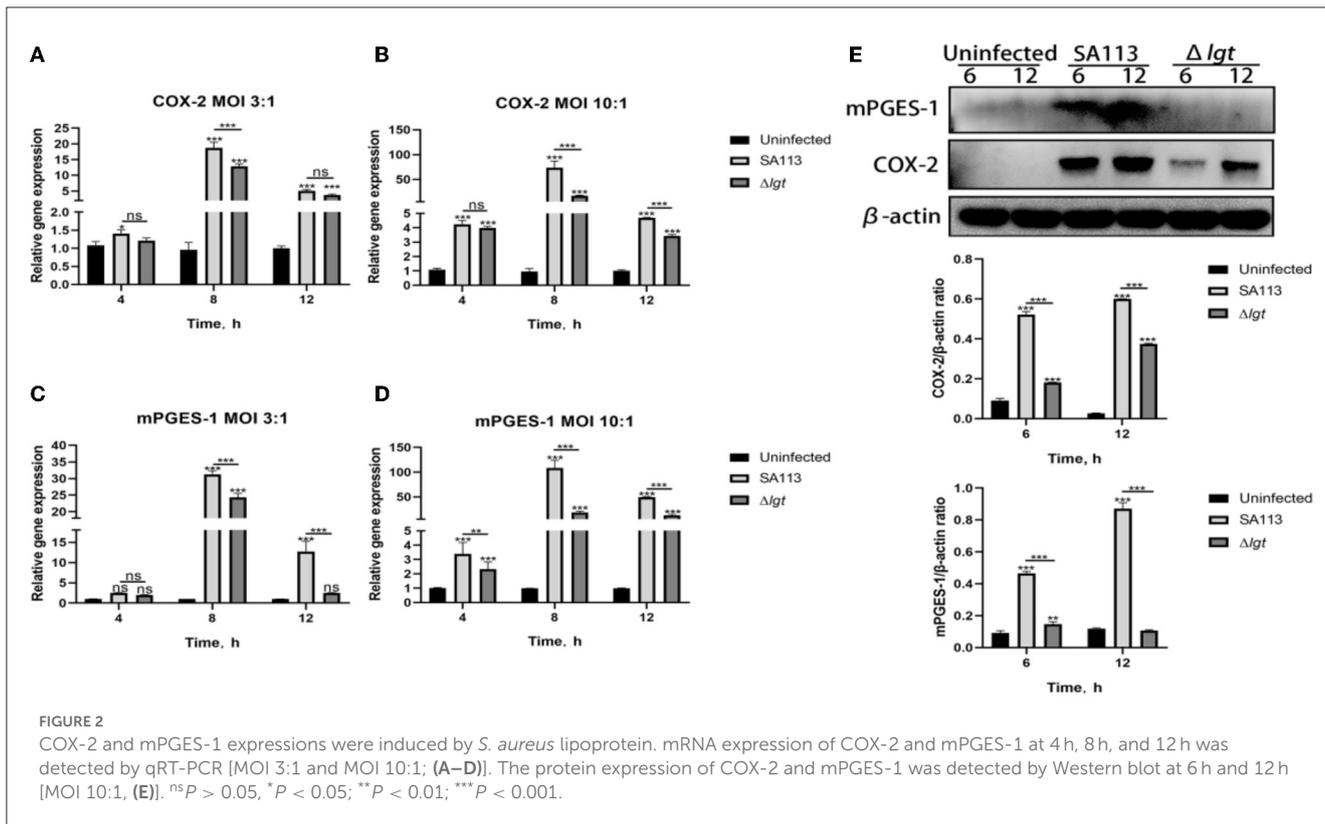
The above experiments indicated that *S. aureus* could not only activate the receptors of TLR2, TLR4, and NLRP3 in neutrophils but also induce the secretion of PGE₂ and cytokines. Therefore, to better verify whether TLR2, TLR4, and NLRP3 receptors can

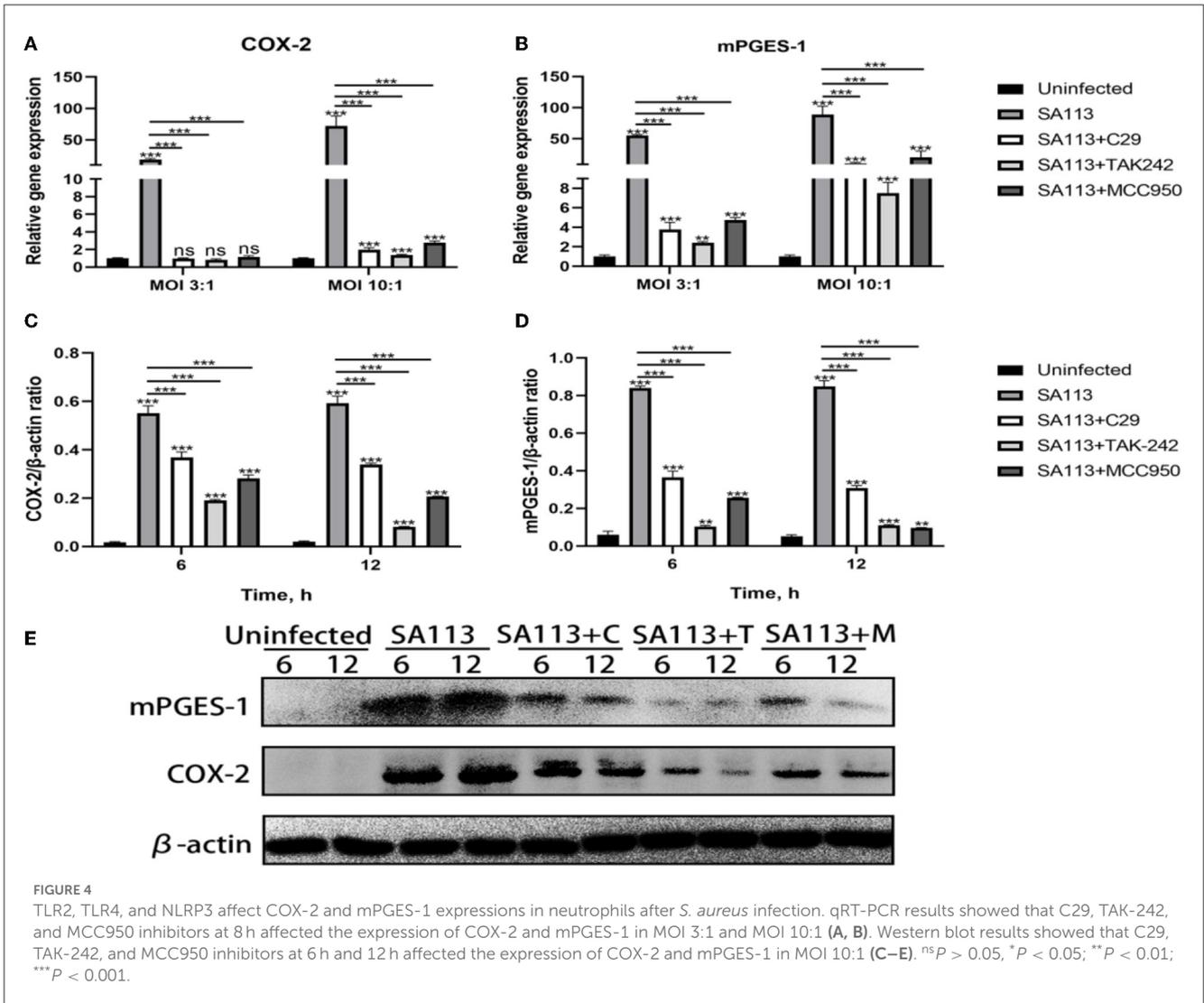


affect the secretion of PGE₂ and cytokines, we introduced TLR2 (C29), TLR4 (TAK-242), and NLRP3 (MCC950) receptor inhibitors for verification. ELISA results showed that compared with the C29, TAK-242, and MCC950 inhibitor groups, the SA113 group induced higher levels of PGE₂, IL-1β, IL-10, IL-6, and IL-8 secretion at MOI 3:1 and MOI 10:1 (*P* < 0.001, Figures 3A–E) but except for IL-6 and PGE₂ (Figures 3B, E) at MOI 3:1. At MOI 3:1, there was no discernible variation in IL-6 secretion in the SA113 group contrasted with the C29 and TAK-242 inhibitor groups (*P* > 0.05, Figure 3B). Western blot was used to detect the TLR2, TLR4, and NLRP3-mediated MAPK and Caspase-1 signaling pathways, and it was found that the inhibitor group was significantly inhibited contrasted with the SA113 group (*P* < 0.001, Figures 3F–J). These results demonstrate that the secretion of PGE₂ and cytokines mediated by neutrophils depends on TLR2, TLR4, and NLRP3 receptors.

TLR2, TLR4, and NLRP3 affect COX-2 and mPGES-1 expression in neutrophils after *S. aureus* infection

To verify the effect of TLR2, TLR4, and NLRP3 receptors on the COX-2, mPGES-1 gene, and protein, we pretreated neutrophils with C29, TAK-242, and MCC950 inhibitors and measured the expression of COX-2, mPGES-1 gene, and protein with qRT-PCR and Western blotting. qRT-PCR results showed that compared with the C29, TAK-242, and MCC950 inhibitor groups, the SA113 group induced higher level expression of COX-2 and the mPGES-1 at MOI 3:1 and MOI 10:1 (*P* < 0.001, Figures 4A, B). qRT-PCR and Western blot results were in agreement (*P* < 0.001, Figures 4C–E). These findings support the hypothesis that COX-2 and mPGES-1 expression in *S. aureus*-infected neutrophils is regulated by TLR2, TLR4, and the NLRP3 inflammasome.





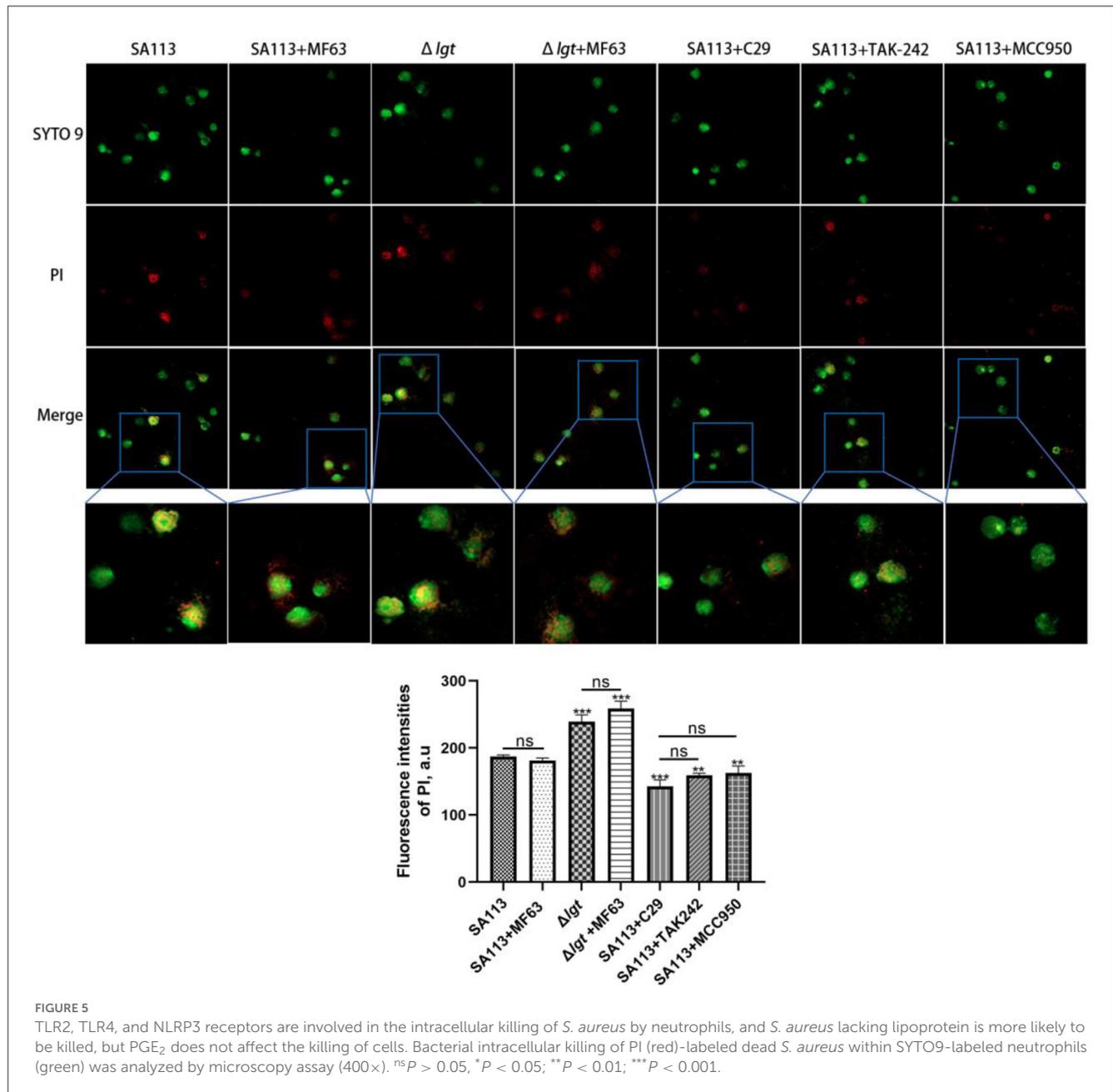
TLR2, TLR4, and NLRP3 receptors are involved in the intracellular killing of *S. aureus* by neutrophils, and *S. aureus* lacking lipoprotein is more likely to be killed, but PGE₂ had no effect on cell killing

In this study, we analyzed whether TLR2, TLR4, and NLRP3 receptors were involved in the killing ability of neutrophils against *S. aureus*, whether the lack of lipoprotein in *S. aureus* would affect this ability, and what effect PGE₂ had on it. We introduced MF63, a PGE₂ synthetase mPGES-1 inhibitor, to block the production of PGE₂ in neutrophils after infection before testing the killing power. TLR2, TLR4, and NLRP3 inhibitors were also introduced to pretreat cells. In comparison to SA113 *S. aureus*, infection with Δ*lgt* resulted in low levels of bacterial survival in neutrophils (*P* < 0.001). The addition of the MF63 inhibitor group did not affect this process (*P* > 0.05). However, the number of *S. aureus* deaths decreased significantly in the C29 (*P* < 0.001), TAK-242 (*P* < 0.01), and MCC950 (*P* < 0.01) inhibitor groups compared with the SA113 group (Figure 5). These consequences reveal that inhibition

of TLR2, TLR4, and NLRP3 receptors on neutrophils affected their killing ability against *S. aureus*, but PGE₂ did not affect cell killing. Lipoprotein plays a vital role in the survival of *S. aureus*.

Prostaglandin E₂ can affect the secretion of pro-inflammatory factors, anti-inflammatory factors, and chemokines in neutrophils of cows infected with *S. aureus*

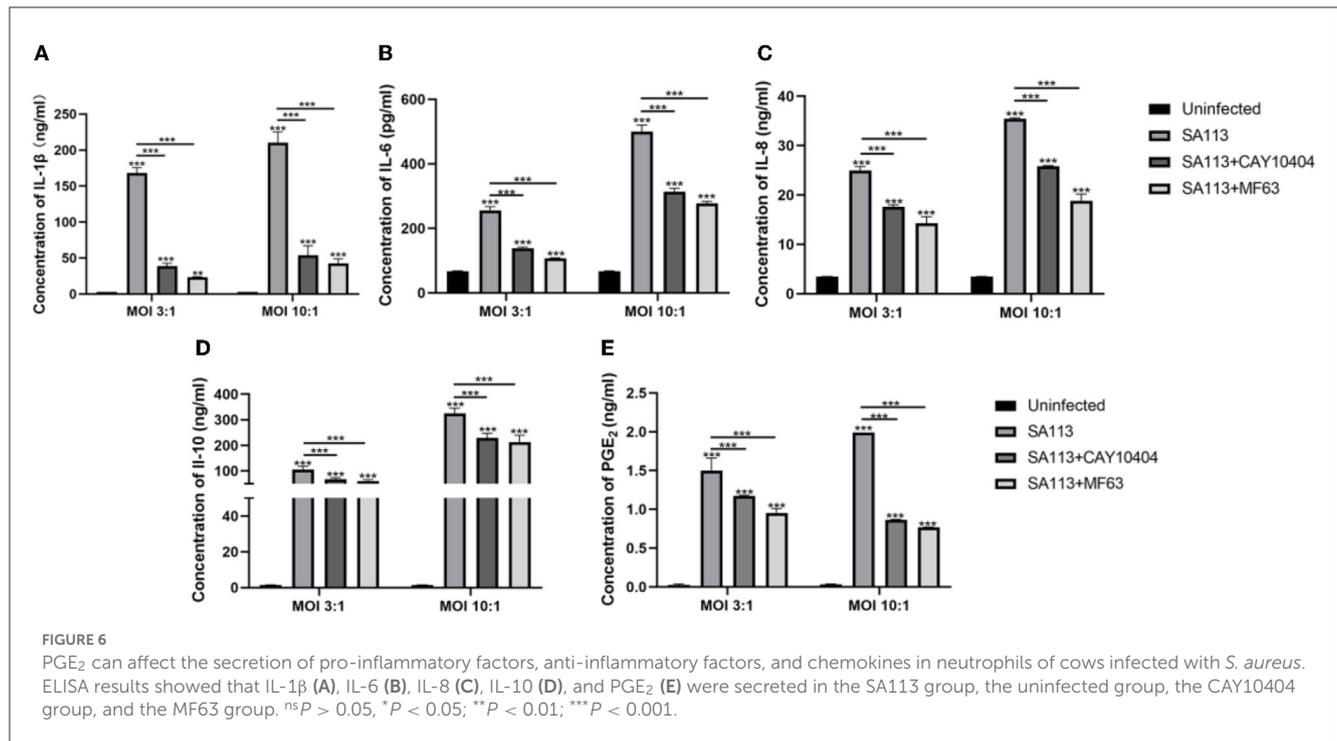
To explore the role of PGE₂ as an inflammatory mediator and other pro-inflammatory factors, anti-inflammatory factors, and chemokines, we introduced PGE₂ synthetase COX-2 (CAY10404) and mPGES-1 (MF63) inhibitors to block the production of PGE₂ in neutrophils after infection. ELISA results showed that the SA113 group induced higher levels of IL-1β, IL-6, IL-8, IL-10, and PGE₂ secretion than the uninfected CAY10404 and MF63 groups (*P* < 0.001, Figures 6A–E).



Discussion

Staphylococcus aureus (*S. aureus*) is a highly viable and widely distributed opportunistic pathogen (Mohammed et al., 2018). *S. aureus* causes economically valuable diseases in cows, including endometritis, vaginitis, and mastitis, which generate significant losses in the cow industry (Petit et al., 2010; Zhao et al., 2014; Shijin, 2022). Neutrophils are the first line of defense against bacterial pathogens that break through the epithelial barrier. Within minutes of bacterial invasion, neutrophils are drawn to the infection site in response to soluble substances such as chemokines and cytokines, where they ingest the pathogens and kill them (Guerra et al., 2017). PGE₂ is one of the prostaglandins most closely associated with various inflammatory diseases (Pecchi et al., 2009) and

induces inflammatory cytokines that mediate tissue harm at some stages in bacterial invasion (Tingting et al., 2018, 2019). Microbial membrane components of bacteria are generally identified by using TLRs, along with TLR2 and TLR4, which are activated through LPS and lipoproteins and are imperative factors for initiating innate immune responses such as inflammation (Langmann, 2015). NOD-like receptors also play a vital role in the infection of various pathogens and the process of intrinsic immunity, for example, NLRP3 (Singh and Jha, 2018). Therefore, we investigated whether PGE₂ accumulation in neutrophils of *S. aureus*-infected cows is correlated with TLR2, TLR4, and NLRP3 receptors. As expected, we found that *S. aureus* promotes the secretion of PGE₂ by activating TLR2, TLR4, and NLRP3 inflammasome signaling pathways in the neutrophils of cows. We also provide evidence that *S. aureus*



is an important protein for its survival and activation of the inflammatory response.

Hayashi et al. (2010) found that TLR2 mediated the pro-inflammatory response to atherogenesis after *Porphyromonas gingivalis* infection, including the aggregation of macrophages and the release of inflammatory factors, for example, IFN- γ , IL-6, IL-1 β , TNF- α , and IL-1 β . Babelova et al. (2009) reported that dimeric glycans stimulate the mRNA expression of NLRP3 and pro-IL-1 β through TLR2 and TLR4. Bauernfeind et al. (2009) found that the activation of TLR2, TLR3, TLR4, and TLR7 can activate the NLRP3 inflammasome in macrophages and Caspase-1. Therefore, we detected the expression of TLR2, TLR4, and NLRP3 receptor genes, proteins, and their associated signaling pathways, MAPK and Caspase-1, in dairy neutrophils infected by two strains of *S. aureus* SA113 and Δ lgt. And PGE₂, IL-1 β , IL-10, IL-6, and IL-8 secretion. We found that SA113 *S. aureus* induced higher levels of PGE₂, IL-1 β , IL-6, IL-10, and IL-8 secretion compared to neutrophils from cows infected with Δ lgt *S. aureus* or uninfected groups (Figures 1A–E). Furthermore, *S. aureus* SA113 induced higher levels of TLR2, TLR4, and NLRP3 receptor genes, and protein expression (Figures 1K–Q), and their mediated activation of MAPK, Caspase-1 signaling pathways (Figures 1F–J). The aforementioned findings indicate that the *S. aureus* lipoprotein component is one of the important components of neutrophil activation in *S. aureus*-infected cows. It induces not only the synthesis and secretion of neutrophils' PGE₂, IL-1 β , IL-10, IL-6, and IL-8 but also the expression of neutrophil TLR2, TLR4, and NLRP3 receptor genes and the activation of these three receptor-mediated related MAPK and Caspase-1 signaling pathways.

To verify the above experimental results, we added TLR2, TLR4, and NLRP3 receptor inhibitors to prove whether the secretion of

PGE₂, IL-6, IL-10, IL-1 β , and IL-8 is mediated by TLR2, TLR4, and NLRP3 receptors. Impaired phosphorylation of MAPK and Caspase-1 signaling pathways indicated that TLR2, TLR4, and NLRP3 receptor inhibitors were effective (Figures 3F–J). Compared with the SA113 group, the C29, TAK-242, and MCC950 groups induced neutrophils to secrete PGE₂, IL-6, IL-1 β , IL-10, and IL-8 levels decreased (Figures 3A–E). It is suggested that TLR2, TLR4, and NLRP3 receptors on dairy neutrophils mediate the secretion of PGE₂ and the secretion of cytokines IL-1 β , IL-10, IL-6, and IL-8 after *S. aureus* infection of cow neutrophils. Our findings are consistent with those of previous studies (Stenzel et al., 2008; Müller et al., 2010).

Prostaglandin E₂ is a recognized pro-inflammatory medium, mainly produced by COX-2 and mPGES-1 (Samuelsson et al., 2007). Here, we discovered that, compared to cells infected with *S. aureus* SA113, the *S. aureus* Δ lgt induced low levels of COX-2 and mPGES-1 expression in neutrophils (Figures 2A–E). mPGES-1 and COX-2 expressions were also impaired in C29, TAK-242, and MCC950 inhibitor group neutrophils (Figures 4A–E). These findings show that COX-2 and mPGES-1 expressions in *S. aureus*-infected neutrophils are dependent on *S. aureus* lipoprotein. In Addition, the presence of TLR2, TLR4, and NLRP3 in neutrophils was crucial for *S. aureus*-induced COX-2 and mPGES-1 expressions. This result is consistent with the previous research of Jindi et al. in mouse peritoneal macrophages (Wu et al., 2020).

Staphylococcus aureus is a highly adaptive gram-positive pathogen (Gorwitz et al., 2008). It can cause serious illnesses such as pneumonia, endocarditis, and bacteremia by infecting different organs. The innate immunity of the human body sends out expert phagocytic cells in response to bacterial infection, such as neutrophils and macrophages, to engulf the invading pathogens

and kill them (Thammavongsa et al., 2015). The literature reports that *S. aureus* can survive and proliferate in specialized phagocytes such as neutrophils, macrophages, and monocytes, and this phenomenon may be one of the reasons for the spread of *S. aureus* in the host (Horn et al., 2017). In this study, we examined the killing function of neutrophils against *S. aureus*. The result found that compared with *S. aureus* SA113 infected cells, *S. aureus* Δ lgt is more easily killed by neutrophils. To determine whether PGE₂ and TLR2, TLR4, and NLRP3 receptors affect this effect, we introduced MF63, the main synthetase mPGES-1 inhibitor of PGE₂, and TLR2 (C29), TLR4 (TAK-242), and NLRP3 (MCC950) receptor inhibitors to observe the killing effect of neutrophils on *S. aureus*. We found that the addition of the MF63 group had no significant effect on neutrophil-killing of *S. aureus*, but the addition of C29, TAK-242, and MCC950 significantly inhibited this effect. We also found that *S. aureus* Δ lgt had decreased survival in neutrophils compared to *S. aureus* SA113 (Figure 5). These findings advise that PGE₂ no longer has an effect on bacterial intracellular killing, and *S. aureus* lipoprotein plays a role in cell survival.

Neutrophils can regulate the immune response through the production of the cytokines IL-12 and IL-10 and the lipid mediators PGE₂ and LTB₄, as reported by Balderramas et al. This regulation may be in a pro-inflammatory or anti-inflammatory mode (Balderramas et al., 2014). PGE₂ has been reported to play a key role in human endometrial repair by regulating the expression of connective tissue growth factor and IL-8 (Maybin et al., 2011). It has been shown that PGE₂ induces IL-6 production (Subedi et al., 2017). IL-1 β promotes RA synovial infiltration and increases PGE₂ release, which, in turn, promotes IL-1 β synthesis (Nilsson et al., 2017). IL-10 reduces the secretion of PGE₂ in osteoarthritic synovial fibroblasts and has a protective effect on articular cartilage (Botha-Scheepers et al., 2008; Järvinen et al., 2008). PGE₂ also inhibits dendritic cell and macrophage maturation and antigen presentation via EP2/EP4 receptors and increases IL-10 production while decreasing IL-12, TNF-, and IL-1 production (Harizi et al., 2001). So is there a link between PGE₂ and these cytokines in *S. aureus*-infected neutrophils? The above reports suggest a possible synergistic or antagonistic relationship between PGE₂ and IL-6, IL-8, IL-1 β , and IL-10. Therefore, we added MF63, an inhibitor of mPGES-1, and CAY10404, an inhibitor of COX-2, to neutrophils from *S. aureus*-infected cows to investigate whether blocking the secretory pathway of PGE₂ would lead to changes in the secretion of IL-6, IL-10, IL-1 β , and IL-8. The results found that the secretion of PGE₂ (Figure 6E) and IL-1 (Figure 6A), IL-6 (Figure 6B), IL-8 (Figure 6C), and IL-10 (Figure 6D) cytokines were restricted when MF63 inhibitor and CAY10404 inhibitor were added, suggesting that there may be a synergistic relationship between PGE₂ and IL-1 β , IL-8, IL-6, and IL-10 in neutrophils of *S. aureus*-infected cows and that they jointly exert immunomodulatory effects.

We demonstrate an association between PGE₂ and PRR-mediated innate immune responses of cow neutrophils to *S. aureus*. TLR2, TLR4, and NLRP3 receptors are involved in the synthesis and secretion of PGE₂ in *S. aureus*-infected cow neutrophils. In this process, we unexpectedly found that *S. aureus* lipoprotein is an important protein in activating the inflammatory response

and the survival of *S. aureus*. Nevertheless, it is still uncertain whether PGE₂ and its analogs can be utilized as a medication or an immunomodulator to guard against infectious disorders brought on by *S. aureus*. Therefore, more research is required, particularly medical testing.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

All animal experiments were performed according to the regulations of the Administration of Affairs Concerning Experimental Animals in China. The experimental protocol was approved by the Animal Welfare and Research Ethics Committee of the Inner Mongolia Agricultural University (Approval ID: NND202103).

Author contributions

YQ, XJ, YJ, WM, BL, SZ, YS, and JC: preparation, creation, and presentation of the published study, specifically writing the initial draft (including substantive translation). KZ: methodology, data analysis, and original draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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