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Original Article

Polydatin ameliorates *Staphylococcus aureus*-induced mastitis in mice via inhibiting TLR2-mediated activation of the p38 MAPK/NF-κB pathway

Kang-feng JIANG[1], Gan ZHAO[1], Gan-zhen DENG[1], Hai-chong WU[1], Nan-nan YIN[1], Xiu-ying CHEN[1], Chang-wei QIU[1], Xiu-li PENG[2, *]

[1]*Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China;* [2]*Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction, Ministry of Education, Huazhong Agricultural University, Wuhan 430070, China*

Abstract

Recent studies show that Polydatin (PD) extracted from the roots of *Polygonum cuspidatum* Sieb, a widely used traditional Chinese remedies, possesses anti-inflammatory activity in several experimental models. In this study, we investigated the anti-inflammatory effects of PD on *Staphylococcus aureus*-induced mastitis in mice and elucidated the potential mechanisms. In mice with *S aureus*-induced mastitis, administration of PD (15, 30, 45 mg/kg, ip) or dexamethasone (Dex, 5 mg/kg, ip) significantly suppressed the infiltration of inflammatory cells, ameliorated the mammary structural damage, and inhibited the activity of myeloperoxidase, a biomarker of neutrophils accumulation. Furthermore, PD treatment dose-dependently decreased the levels of TNF-α, IL-1β, IL-6 and IL-8 in the mammary gland tissues. PD treatment also dose-dependently decreased the expression of TLR2, MyD88, IRAK1, IRAK4 and TRAF6 as well as the phosphorylation of TAK1, MKK3/6, p38 MAPK, IκB-α and NF-κB in the mammary gland tissues. In mouse mammary epithelial cells (mMECs) infected by *S aureus in vitro*, pretreatment with PD dose-dependently suppressed the upregulated pro-inflammatory cytokines and signaling proteins, and the nuclear translocation of NF-κB p65 and AP-1. A TLR2-neutralizing antibody mimicked PD in its suppression on *S aureus*-induced upregulation of MyD88, p-p38 and p-p65 levels in mMECs. PD (50, 100 μg/mL) affected neither the growth of *S aureus in vitro*, nor the viability of mMECs. In conclusion, PD does not exhibit antibacterial activity against *S aureus*, its therapeutic effects in mouse *S aureus*-induced mastitis depend on its ability to down-regulate pro-inflammatory cytokine levels via inhibiting TLR2-mediated activation of the p38 MAPK/NF-κB signaling pathway.

Keywords: mastitis; *Staphylococcus aureus*; Polydatin; dexamethasone; anti-inflammation; cytokines; TLR2; MyD88; p38 MAPK/NF-κB signaling pathway

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Introduction

Mastitis, a disease that affects humans and animals worldwide, occurs most commonly in the postpartum period[1, 2]. Mastitis is characterized by inflammation of the mammary gland tissues due to infection by etiologic microorganisms, such as the Gram-positive bacterium, *Staphylococcus aureus*[3].

S aureus, an opportunistic bacterial pathogen, is the most prevalent pathogen in mastitis in both humans and animals[4]. One of the characteristics of *S aureus* is that it evades and influences the host immune defensive system and avoids some antibacterial agents by hiding in host cells. Thus, once mammary gland tissues are infected by *S aureus*, it is extremely difficult to control and eliminate the infection[5].

It has been well established that recognition of the infection through activation of pattern recognition receptors (PRRs) is necessary for initiating the immune response when mammary glands are infected by *S aureus*[6]. PRRs are considered crucial in the survival of the pathogen, and they recognize pathogen-associated molecular patterns (PAMPs)[7, 8]. TLR2, one of the PRRs, plays a key role in the innate immune response to microbial infection[9], and its activation leads to the release of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6

*To whom correspondence should be addressed.
E-mail pengxiuli666@163.com
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and IL-8[10]. Myeloid differentiation factor 88 (MyD88) is the primary adaptor protein in the TLR2 signaling pathway and is essential for the induction of pro-inflammatory cytokines triggered by TLR2[11]. Most of the TLRs, including TLR2, recruit MyD88 to their receptor complex, as do members of the interleukin-1 receptor family. Subsequently, MyD88 recruits interleukin-1 receptor-associated kinase 1 (IRAK1), IRAK4, and then TNF receptor-associated factor 6 (TRAF6), resulting in the activation of the NF-κB pathway, which regulates the expression of inflammatory genes[12, 13]. In addition to the NF-κB pathway, TLR2 and IL-1Rs activate the mitogen-activated protein kinase (MAPK) pathway, in which a kinase cascade leads to the activation of c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK) and the p38 subunit of MAPKs[14]. JNK/ERK/p38 MAPK phosphorylate the transcription factor AP-1, which leads to the release of pro-inflammatory cytokines[15].

Polydatin (PD), extracted from the roots of *Polygonum cuspidatum* Sieb, is widely applied in traditional Chinese remedies[16]. Some studies have indicated that PD can improve heart function and ameliorate renal injury through anti-oxidative and anti-inflammatory effects[17, 18]. However, there are no reports concerning the effects of PD on mastitis, and the mechanism involved is not clear. Accordingly, a mouse model of *S aureus* mastitis and mouse mammary epithelial cells (mMECs) were used in this research, and the study investigated the anti-inflammatory effects of PD on *S aureus*-induced mastitis and elucidated the potential mechanisms.

Materials and methods

Reagents

Polydatin was obtained from the National Institutes for Food and Drug Control (purity >98%, HPLC; Beijing, China) (Figure 1). Mouse TNF-α, IL-1β and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from ImmunoWay Biotechnology (Newark, DE, USA). The mouse IL-8 ELISA kit was purchased from Cusabio (Wuhan, China). The nuclear extraction kit, TransAM NF-κB p65 assay kit and TransAM AP-1 ELISA kit were purchased from Active Motif (Carlsbad, CA, USA). The myeloperoxidase (MPO) determination kits were provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). Primary antibodies against TLR2, MyD88, IRAK1, IRAK4, TRAF6, TAK1, p-TAK1, MKK3/6, p-MKK3/6, MAPKs, NF-κB, IκBα, p-IκBα and β-actin and the HRP-conjugated goat anti-rabbit antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemical reagents were reagent grade.

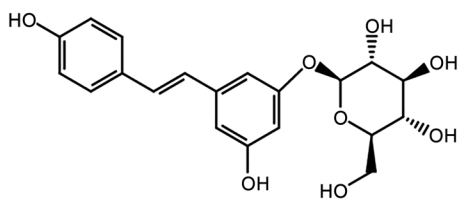

Figure 1. The chemical structure of PD.

S aureus strains and culture

S aureus strains (ATCC 25923) were purchased from the American Type Culture Collection (ATCC, MD, USA) and cultured in Luria-Bertani (LB) broth (Oxoid, Hampshire, UK) at 37 °C and 180 rounds per minute.

Animals and experimental design

Sixty adult female postpartum and lactating BALB/c mice (6–8 weeks old, weighing 35–40 g) were obtained from Wuhan Institute of Biological Products Co Ltd (Wuhan, China). All procedures were performed according to the Guide for the Care and Use of Laboratory Animals established by the US National Institutes of Health and approved by the Ethical Committee on Animal Research at Huazhong Agricultural University. The mice were housed in a room maintained at 24±1 °C with 40%–80% humidity. All animals received food and water *ad libitum*.

PD was dissolved in dimethyl sulfoxide (DMSO) and diluted in Dulbecco's modified Eagle's medium (DMEM). *S aureus* cultures were maintained in 100 mL of LB broth at 37 °C and 180 rounds per minute until they reached the stationary phase. Subsequently, the bacteria were centrifuged and then washed twice with phosphate-buffered saline (PBS, pH 7.0). The collected cell pellet was resuspended in 1 mL of PBS at 3.1×10^{10} CFU/mL. The method for establishing the *S aureus*-induced mastitis model was described previously[19]. Briefly, 100 μL of *S aureus* suspension (10^9 CFU) was injected into two abdominal mammary glands to induce mastitis. Then, 24 h after *S aureus* infection, three intraperitoneal injections of PD or DEX were given every 8 h at doses established in a previous study[20]. The mice were randomly divided into four groups as follows.

1. Control group (CG): The mice were treated with 100 μL of PBS as a vehicle control.
2. *S aureus* group (SG): The mouse model of *S aureus* mastitis without drug intervention.
3. PD groups: 24 h after *S aureus* infection, the mouse model of *S aureus* mastitis was intraperitoneally administered PD at 15, 30 and 45 mg/kg.
4. Dexamethasone administration group (DEX group): 24 h after *S aureus* infection, the mouse model of *S aureus* mastitis was intraperitoneally administered DEX at 5 mg/kg.

Then, 8 h after the last treatment with PD or DEX, all the mice were euthanized with sodium pentobarbital, and the mammary gland tissues were collected and stored at -80 °C for further research.

Cell culture and treatment

Primary mouse mammary epithelial cells were prepared as previously described[21]. Mammary gland tissues were aseptically harvested from normal pregnant BALB/c mice and minced into paste. The homogenized tissues were digested with a collagenase I/II/trypsin mixture at 37 °C and then centrifuged. The fatty layer and cell pellets were resuspended in DMEM/F12 with 10% FBS. The suspension was centrifuged again and filtered through a 40 μm pore-size filter, and the

cells were then cultured at 37 °C with 5% CO_2. The primary mouse mammary epithelial cells were identified using specific antibodies against cytokeratin 18.

Mouse mammary epithelial cells (mMECs) were pretreated for 1 h with PD at final concentrations of 25, 50 and 100 μg/mL. PD-untreated cells containing DEX (100 μg/mL) were used as a positive control. Subsequently, *S aureus* at a multiplicity of infection (MOI) of 10 was added to the cells. After 8 h of incubation at 37 °C, the cell supernatant and cells were collected for further study.

Cell viability assay

The effect of PD on mMEC viability was evaluated with an MTT assay. mMECs were incubated in the presence or absence of various concentrations of PD (25, 50 and 100 μg/mL) and DEX (100 μg/mL) for 24 h. Next, 20 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h. After the supernatants were removed and the formazan was dissolved with 150 μL of DMSO in each well, the optical density (*OD*) value was measured at 570 nm on a microplate reader (Bio-Rad Instruments, Hercules, CA, USA).

Histopathological analysis

Mammary gland tissues were harvested and fixed with 10% formalin. Then, the tissue sections were embedded in paraffin and sectioned to 4-μm thickness. After deparaffinization and dehydration, the sections were stained with hematoxylin and eosin (H&E). The pathological changes were observed using a microscope (Olympus, Japan).

MPO activity assay

Mammary gland tissues were homogenized with reaction buffer (*w/v*, 1/9), and MPO activity was determined using an MPO test kit according to the manufacturer's protocols.

ELISA assay

The effects of PD on the levels of *S aureus*-induced pro-inflammatory cytokines were determined in mammary gland tissues and mMECs. Mammary gland tissues were homogenized and centrifuged, and the supernatant was collected. The supernatants of the mMECs with different treatments were also collected. The levels of TNF-α, IL-1 β, IL-6 and IL-8 in these supernatants were measured with ELISA kits, which were used according to the manufacturer's instructions. Finally, the *OD* value of each well was read at 450 nm with an automatic microplate reader (Thermo Scientific Multiskan MK3, USA).

TransAM NF-κB and AP-1 activation assays

A TransAM NF-κB p65 kit and a TransAM AP-1 Family kit were used to determine the concentrations of NF-κB p65 and AP-1 in nuclear protein extracts obtained from mMECs via a colorimetric DNA-binding ELISA, which was performed according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR) assays

Total RNA was extracted from the mammary gland tissues and mMECs using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. The concentration and purity of the extracted RNA were evaluated with Q5000 (Quawell Technology, USA). Total RNA (1 μg) was reverse-transcribed into first-strand DNA (cDNA) using a PrimeScript RT-PCR kit according to the manufacturer's instructions (Takara). The levels of TNF-α, IL-1β, IL-6, IL-8 and TLR2 messenger RNA (mRNA) were measured with quantitative real-time polymerase chain reaction (qRT-PCR). PCR reactions were carried out using SYBR green Plus reagent kit (Roche, Swissland) in a 25 μL reaction. Each sample was run as three replicates. The primers used in the study are listed in Table 1. The thermal cycler parameters were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 60 °C for 30 s. The mRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ comparative method and analyzed by normalization to with GAPDH mRNA expression.

Western blot analysis

Total protein was extracted from the mMECs with RIPA reagent according to the manufacturer's recommended pro-

Table 1. Primers used for qPCR.

Name	Primer sequence (5′–3′)	GenBank accession number	Product size (bp)
TLR2	F: TTTGCTCCTGCGAACTCC R: GCCACGCCCACATCATTC	NM_011905.3	267
TNF-α	F: CTTCTCATTCCTGCTTGTG R: ACTTGGTGGTTTGCTACG	NM_013693.3	198
IL-1β	F: CCTGGGCTGTCCTGATGAGAG R: TCCACGGGAAAGACACAGGTA	NM_008361.4	131
IL-6	F: GGCGGATCGGATGTTGTGAT R: GGACCCCAGACAATCGGTTG	NM_031168.1	199
IL-8	F: GGTGAAGGCTACTGTTGG R: CTGGAGTCCCGTAGAAAA	NM_011339.2	234
GAPDH	F: CAATGTGTCCGTCGTGGATCT R: GTCCTCAGTGTAGCCCAAGATG	NM_001289726.1	124

tocol (Biosharp, China). The total protein concentration was measured with a BCA protein assay kit (Thermo Scientific, MA, USA). Equal amounts of protein were separated with 10% SDS polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) membranes, and blocked in 5% skim milk in TBST for 2 h. Subsequently, the membranes were incubated at 4 °C overnight with primary antibodies against TLR2, MyD88, IRAK1, IRAK4, TRAF6, TAK1, p-TAK1, MKK3/6, p-MKK3/6, MAPKs, NF-κB, IκBα, p-IκBα and β-actin (1:1000 dilutions). β-Actin was used as a control. After incubation with the secondary antibodies (1:5000 dilutions) for 2 h at 25±1 °C, protein levels were detected with an enhanced chemiluminescence reagent, and the intensities were quantified using Image J gel analysis software.

Statistical analyses

All the data in this study are presented as the mean±SEM and were analyzed with SPSS19.0 (IBM). The differences between groups were assessed using one-way ANOVA with the LSD multiple comparison test and Student's *t*-test. Statistical significance was defined as $P<0.05$.

Results

Effects of PD on *S aureus* growth

To investigate whether PD had antibacterial activity against *S aureus*, the growth curve of *S aureus* at different concentrations of PD was determined. Our results suggested that PD had little effect on *S aureus* growth (Figure 2).

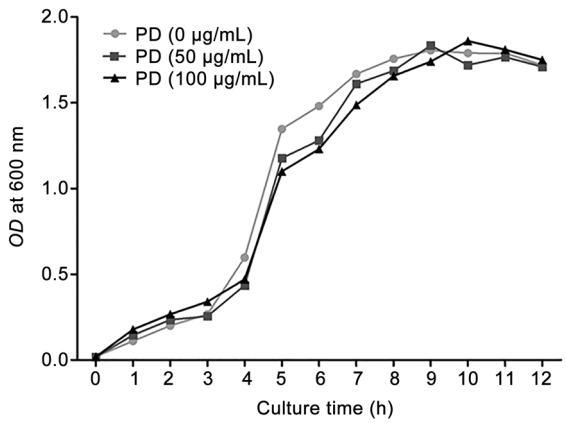

Figure 2. The effects of PD on *S aureus* growth. *S aureus* was cultured in LB with various concentrations of PD (0, 50, 100 μg/mL). The *OD* value was measured every 1 h.

Effects of PD on *S aureus*-induced histopathological changes

There were no histopathological changes in the control group (Figure 3A). In the *S aureus* group, the lobules of the mammary gland were not complete, and a large number of inflammatory cells were observed in the mammary gland tissues. Moreover, the acini of the mammary glands were also damaged (Figure 3F). However, these histopathological changes were alleviated in the PD groups and DEX group, and the lobules were complete and the number of inflammatory cells decreased (Figure 3B–3E).

Effects of PD on MPO activity

As an important indicator of neutrophil accumulation in the mammary gland tissues, MPO activity was measured in the present study. The results are shown in Figure 3G. We observed that infection with *S aureus* dramatically increased MPO activity in mammary gland tissues relative to the control group. However, these increases were suppressed by PD in a dose-dependent manner.

Cell integrity and cell viability assay

Cytokeratin 18 is widely used to identify the integrity of mammary gland epithelial cells[19]. In our study, mMECs were pretreated with a blue fluorophore to observe the cell nucleus, and cytokeratin 18 was labeled with a green fluorophore to identify cell integrity (Figure 4A). The cytotoxicity of PD to mMECs was assessed with an MTT assay. The results showed that cell viability was not affected by PD treatment (25, 50 and 100 μg/mL) (Figure 4B).

Effects of PD on inflammatory cytokines

Many studies have shown that the production of pro-inflammatory cytokines plays an essential role in inflammatory responses; thus, we investigated whether PD could inhibit the secretion of TNF-α, IL-1β, IL-6 and IL-8 in *S aureus*-induced mastitis. The results are shown in Figures 5A and 5B. The TNF-α, IL-1β, IL-6 and IL-8 levels in the *S aureus* group were significantly increased relative to the control group. However, these increases were significantly inhibited by PD and DEX. With increasing doses of PD, the effect was more pronounced. Additional studies performed on mMECs produced the same results as those observed in the mammary gland tissues (Figures 5A and 5B).

Effects of PD on TLR2 expression

TLR2 plays a pivotal role in the *S aureus*-induced inflammatory response[22]. To explore whether PD could affect TLR2, the expression of TLR2 was measured with qPCR and Western blotting. The expression of TLR2 was significantly higher in the *S aureus* group than in the control group. However, PD significantly inhibited these increases in a dose-dependent manner (Figure 6).

Effects of PD on the MyD88-MAPK signaling pathway

To further investigate the anti-inflammatory mechanism of PD, Western blotting was performed to measure the expression of proteins in the MyD88-MAPK signaling pathway in tissues. Compared with the control group, the *S aureus* group exhibited significantly increased MyD88, IRAK1, IRAK4, TRAF6, p-TAK1, p-MKK3/6 and MAPK expression. However, PD significantly blocked the increase in MyD88, IRAK1, IRAK4 and TRAF6 levels and the phosphorylation of TAK1, MKK3/6 and p38 but did not influence the level of p-JNK or p-ERK (Figure 7). To further confirm the results, the same

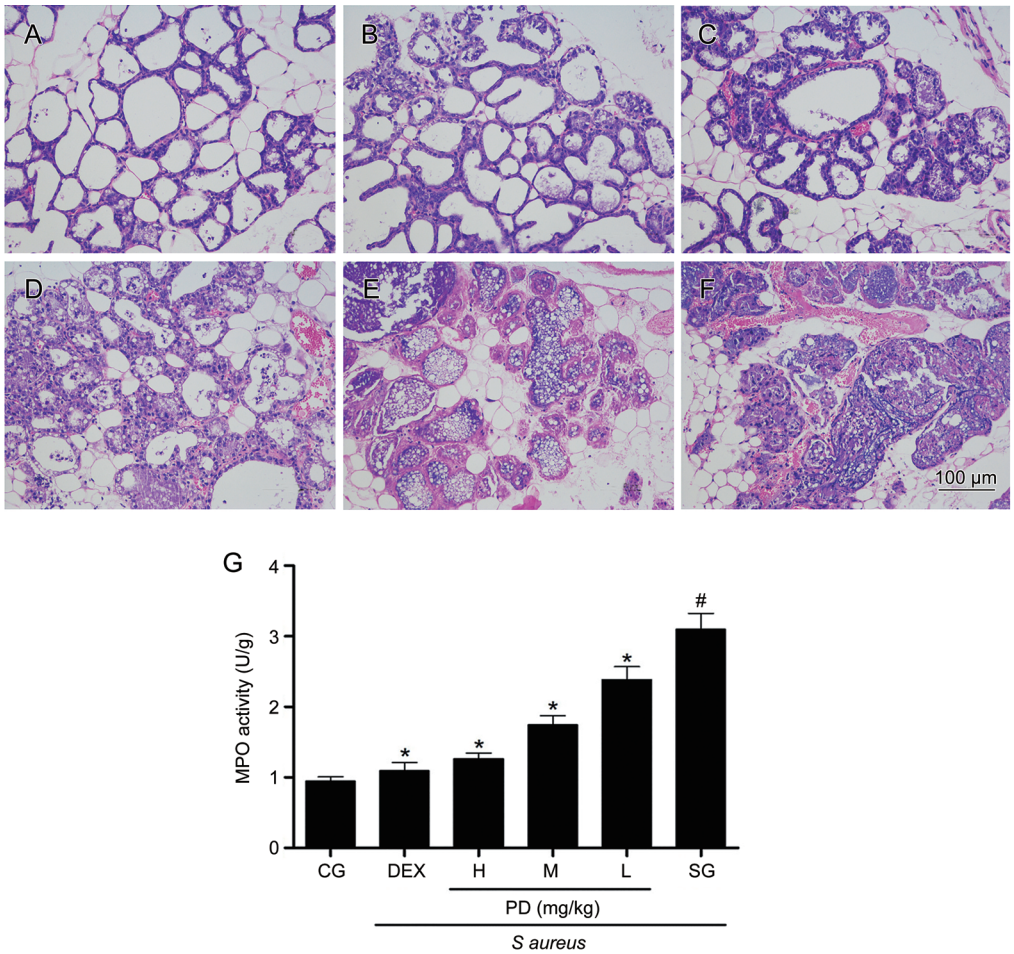

Figure 3. The effects of PD on *S aureus*-induced mammary gland injury. Mammary gland tissues were harvested 8 h after the last treatment with PD or DEX. (A) Control group. (B) Dexamethasone group. (C–E) PD (45, 30, 15 mg/kg) groups and (F) *S aureus* group. (G) MPO activity assay. CG is the control group; SG is the *S aureus* group; DEX is the dexamethasone group; and H, M and L are the PD administration groups, with doses of 45, 30 and 15 mg/kg, respectively. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

study was performed using mMECs. The results were consistent with those obtained using the tissues (Figure 8).

Effects of PD on the NF-κB signaling pathway

NF-κB is a crucial mediator in the inflammatory response and can also be activated by TLR2[23]. The levels of p-IκBα and p-p65 were significantly increased in the *S aureus* group relative to the control group. In contrast, their levels were greatly decreased in the PD treatment group (Figure 9).

Effects of PD on the nuclear translocation of NF-κB p65 and AP-1

To study the effects of PD on the nuclear translocation of NF-κB p65 and AP-1, we used TransAM assays to measure the concentrations of NF-κB p65 and AP-1 in nuclear protein extracts, as described previously[24]. The nuclear translocation of both NF-κB p65 and AP-1 was markedly enhanced after inoculation with *S aureus* in the *S aureus* group. However, pretreatment with PD significantly attenuated these increases (Figure 10).

Effects of PD were mediated through TLR2

Additionally, to confirm whether the anti-inflammatory effects of PD were mediated through TLR2, mMECs were pretreated with a TLR2-neutralizing antibody (1 μg/mL, InvivoGen) or PD (100 μg/mL) for 1 h and then incubated with *S aureus* for 8 h. The protein levels of MyD88, p-p38 and p-p65 were determined by Western blotting. The results showed that the TLR2-neutralizing antibody significantly decreased *S aureus*-induced MyD88, p-p38 and p-p65 levels, and a similar effect was observed for PD, indicating that the anti-inflammatory effects of PD were mediated through TLR2 (Figure 11).

Discussion

Polydatin (PD), which is extracted from the roots of *Polygonum cuspidatum* Sieb, has been widely used in the clinical treatment of various diseases, including LPS-stimulated ALI in rats[20]. In addition, it has also been reported to exert anti-inflammatory effects in RAW 264.7 cells[25]. However, few reports have focused on the effects of PD in *S aureus*-induced mastitis. In

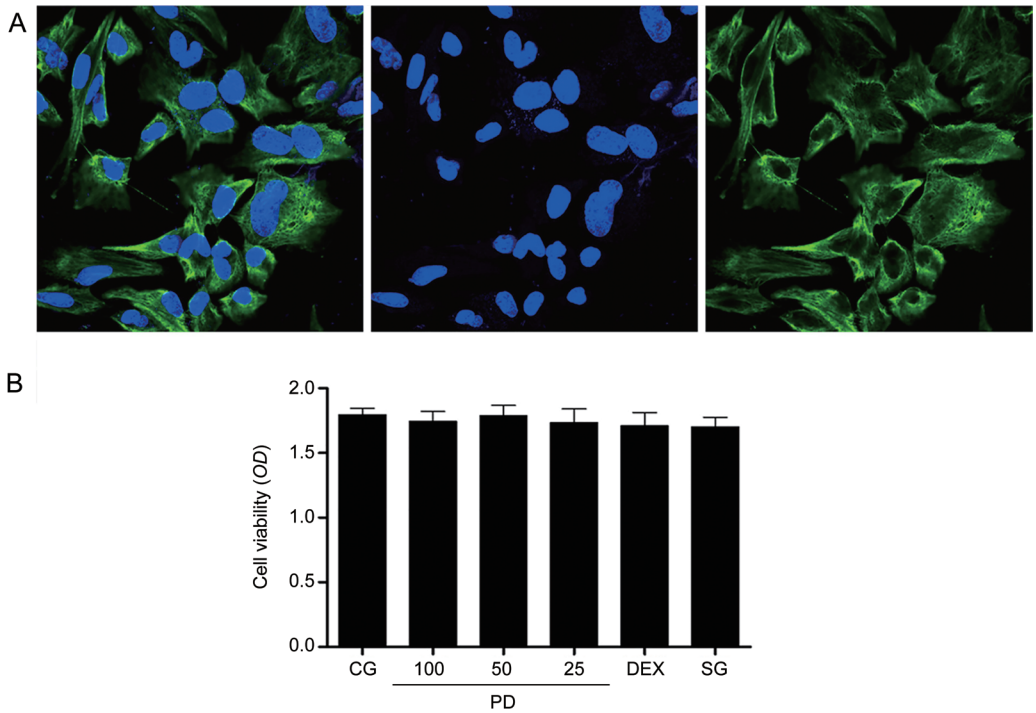

Figure 4. Mouse mammary epithelial cell identification and cell viability. (A) Mouse mammary epithelial cells (mMECs) were pretreated with a fluorophore to observe their integrity. Cytokeratin 18 was labeled with a green fluorophore, and the cell nucleus was labeled by a blue fluorophore. (B) The effects of PD on the viability of mMECs. The cells were cultured with different concentrations of PD (25, 50 and 100 μg/mL). Cell viability was determined with an MTT assay. The data are presented as the mean±SEM.

the present study, we investigated the anti-inflammatory effects of PD on *S aureus*-induced mastitis *in vivo* and *in vitro*.

S aureus, a Gram-positive bacterium, is one of the major pathogens that causes mastitis[4]. The intramammary instillation of *S aureus* is a well-known model of mastitis in mice[19]. In our study, it was observed in the histopathological analysis that PD clearly suppressed the infiltration of inflammatory cells and reversed the mammary structural damage. Moreover, MPO is a biomarker of neutrophil influx into tissues and reflects the number of neutrophils in mammary gland tissues[26]. The MPO assay results showed that PD significantly inhibited MPO activity in *S aureus*-induced mastitis. Moreover, an antibacterial test was also performed to explore whether PD has antibacterial activity against *S aureus*. The results showed that PD did not exert its anti-inflammatory effects by inhibiting bacterial proliferation, indicating that an additional anti-inflammatory mechanism exists.

S aureus infection leads to the overwhelming release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8, which play pivotal roles in the initiation and regulation of inflammatory responses[27]. These pro-inflammatory cytokines also participate in the recruitment of neutrophils and the promotion of MPO activity. In this study, the TNF-α, IL-1β, IL-6 and IL-8 levels in tissues and mMECs were significantly lower in the PD group than in the *S aureus* group. The results were also in accordance with the MPO results. Moreover, PD at 45 mg/kg resulted in anti-inflammatory effects similar to those of dexamethasone. These results revealed that PD exerted anti-inflammatory effects by regulating pro-inflammatory cytokine levels in *S aureus*-induced mastitis.

TLR2, a key immune receptor in the innate immune system, was found to be activated by the *S aureus* cell wall components peptidoglycan and lipoteichoic acid, and TLR2 expression increased significantly after infection[28]. Recent studies have also shown that TLR2 plays a very important role in inflammatory responses by regulating MyD88, leading to the activation of MAPKs and NF-κB and the subsequent production of pro-inflammatory cytokines[29]. MyD88 is a signaling adaptor protein that is critical in the immune response against a wide range of microbial pathogens[30] and is necessary for the phosphorylation and activation of IRAK1 through the recruitment of IRAK4[31]. Activated IRAK is released from the receptor complex and interacts with TRAF6 and TAB2 (TAK1 binding protein 2), which mediates the translocation of TRAF6 and TAB2 to the cytosol and ultimately results in the activation of TAK1[32, 33]. Subsequently, activated TAK1 phosphorylates MAPK kinase 3/6 (MKK3/6), leading to the activation of MAPKs and NF-κB[33, 34]. MAPKs have been reported to play a central role in *S aureus*-induced inflammation and can also activate AP-1, which acts similarly to NF-κB p65 to promote the expression of pro-inflammatory cytokines[35]. To further understand the molecular mechanism through which PD exerts its anti-inflammatory effects, we explored the TLR2-mediated activation of the MAPK/NF-κB signaling pathway.

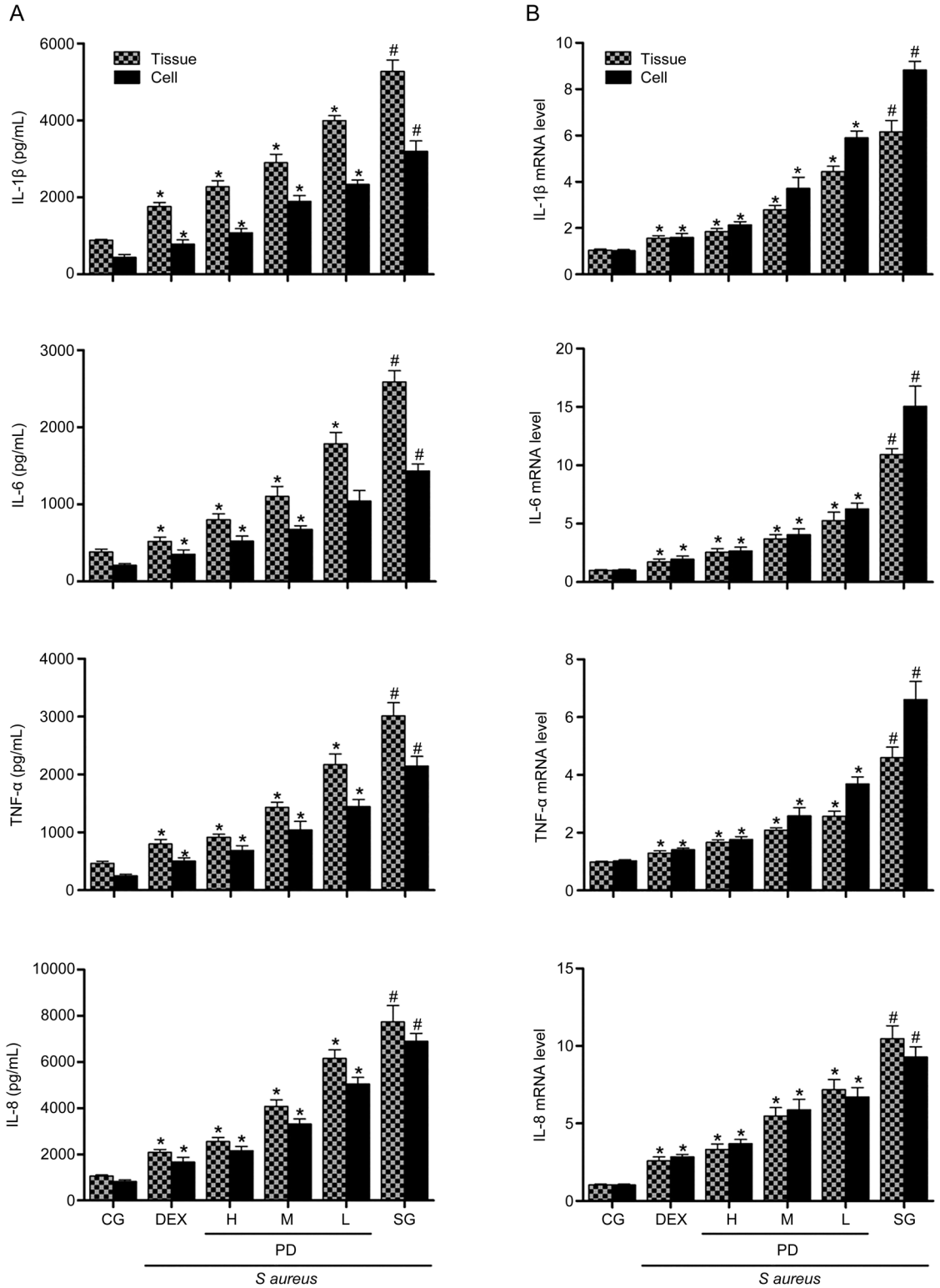

Figure 5. The effects of PD on the production of pro-inflammatory cytokines. (A) The levels of the cytokines TNF-α, IL-1β, IL-6 and IL-8 induced by *S aureus* were determined with an ELISA. (B) The *S aureus*-induced expression of the cytokines TNF-α, IL-1β, IL-6 and IL-8 was determined with qPCR. GAPDH was used as a control. CG is the control group; SG is the *S aureus* group; DEX is the dexamethasone group; and H, M and L are the PD administration groups, with doses of 45, 30 and 15 mg/kg per animal, respectively, and 100, 50 and 25 μg/mL in the cells, respectively. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

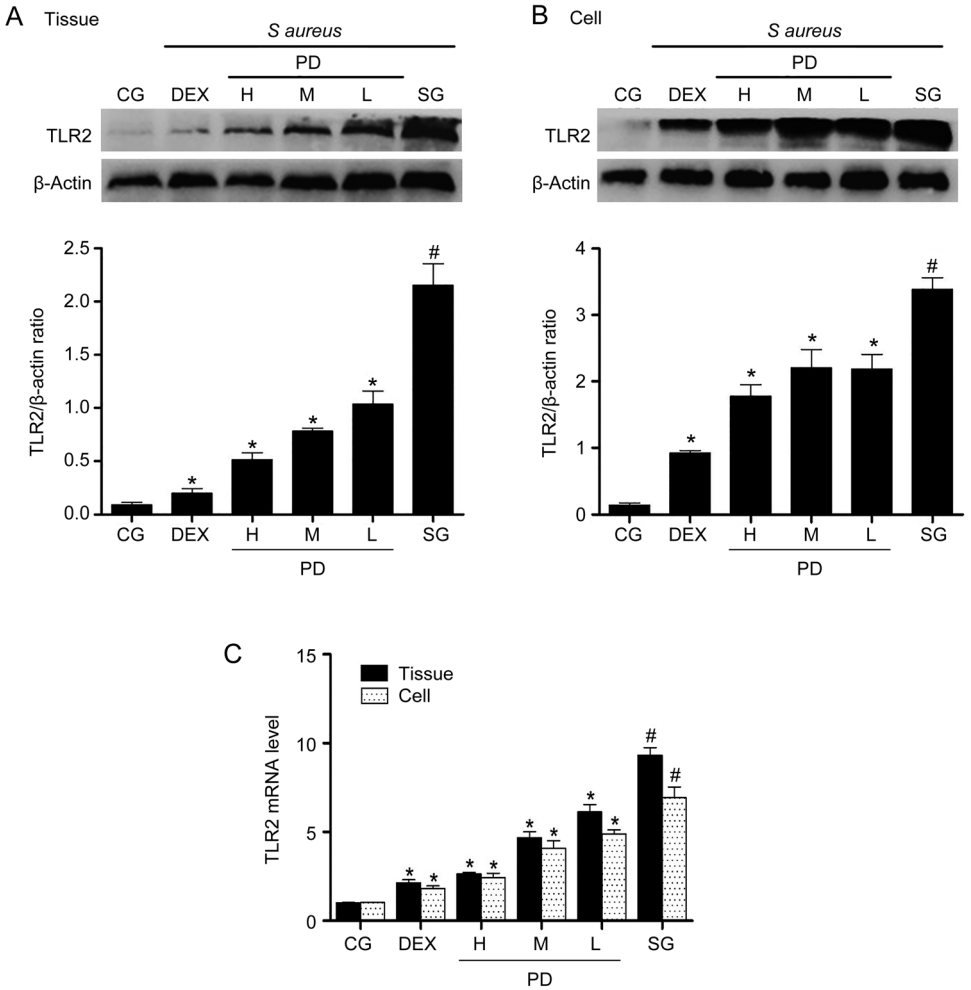

Figure 6. The effect of PD on TLR2 expression. (A) The protein expression of TLR2 in tissues was measured with Western blotting. (B) The protein expression of TLR2 in mMECs. β-Actin was used as a control. (C) The mRNA expression of TLR2 in tissues and mMECs was determined with qPCR. GAPDH was used as a control. CG is the control group; SG is the *S aureus* group; DEX is the dexamethasone group; and H, M and L are the PD administration groups, with doses of 45, 30 and 15 mg/kg per animal, respectively, and 100, 50 and 25 μg/mL in the cells, respectively. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

Our results suggested that PD significantly inhibited the expression of TLR2, MyD88, IRAK1, IRAK4, TRAF6, p-TAK1 and p-MKK3/6. Consequently, pretreatment with PD significantly suppressed the activation of p38 MAPK, AP-1, IκB-α and NF-κB but did not influence the level of p-JNK or p-ERK, which is consistent with previous research[36]. Additionally, we further confirmed the role of TLR2 in *S aureus*-induced mastitis using a TLR2-neutralizing antibody; the results were identical to those for PD and indicated that the anti-inflammatory effects of PD were mediated through TLR2.

In conclusion, the results demonstrated that PD exerted anti-inflammatory effects in *S aureus*-induced mastitis by inhibiting the TLR2-mediated activation of the p38 MAPK/NF-κB signaling pathway. The findings of this study indirectly suggest that PD may be an effective drug in the treatment of *S aureus*-induced mastitis.

Acknowledgements

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Author contribution

Kang-feng JIANG, Gan ZHAO, and Gan-zhen DENG conceived and designed the experiments; Kang-feng JIANG, Hai-chong WU, and Xiu-ying CHEN performed the experiments; Kang-feng JIANG and Nan-nan YIN analyzed the data; and Xiu-li PENG helped to draft the manuscript. All authors read and approved the final manuscript.

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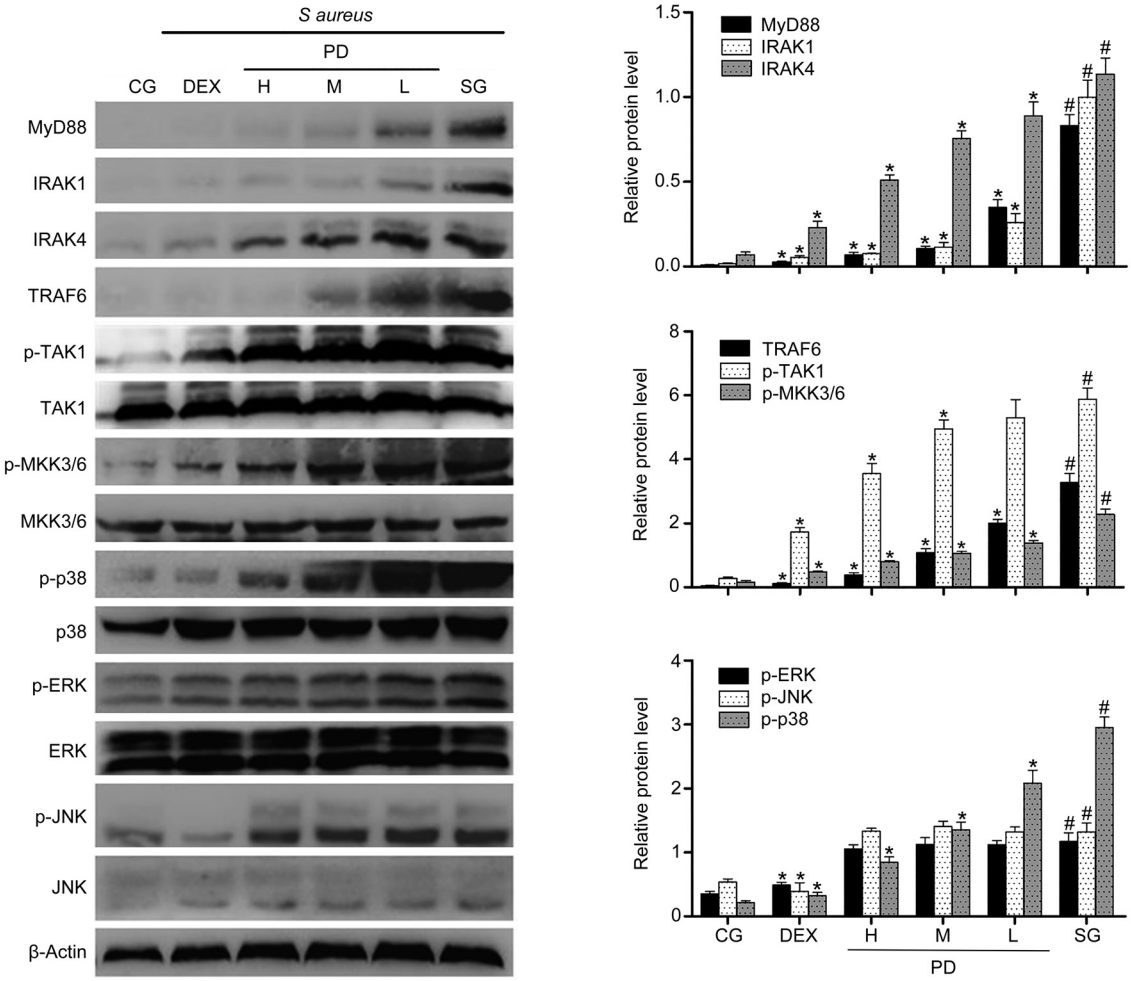

Figure 7. The effects of PD on the TLR2-mediated activation of the MAPK signaling pathway in tissues. The expression of MyD88, IRAK1, IRAK4, TRAF6, TAK1, MKK3/6 and MAPK protein in tissues was determined using a Western blot. β-Actin was used as a control. CG is the control group; SG is the *S aureus* group; DEX is the dexamethasone group; and H, M and L are the PD administration groups, with doses of 45, 30 and 15 mg/kg per animal, respectively, and 100, 50 and 25 μg/mL in the cells, respectively. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

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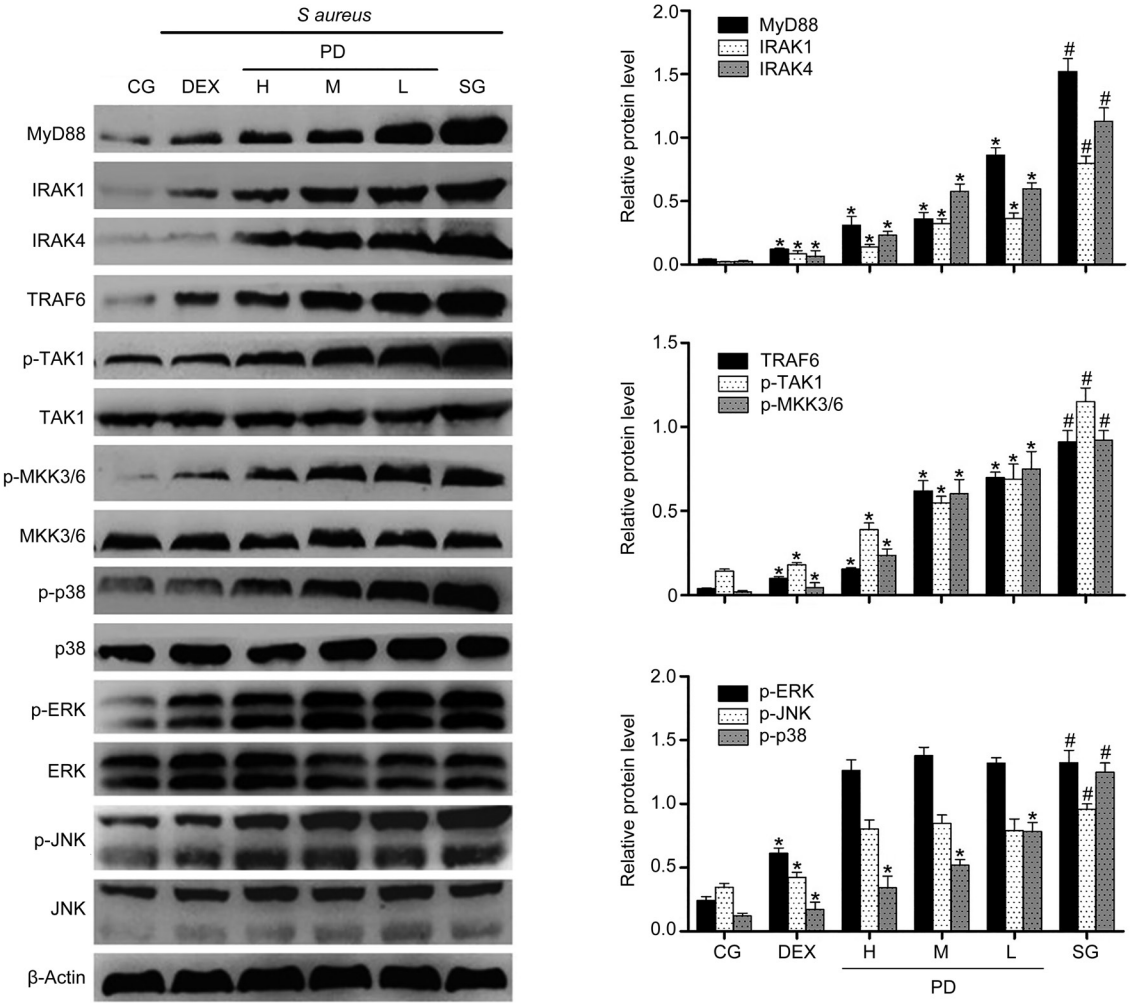

Figure 8. The effects of PD on the TLR2-mediated activation of the MAPK signaling pathway in mMECs. The expression of MyD88, IRAK1, IRAK4, TRAF6, TAK1, MKK3/6 and MAPK protein in mMECs was determined using Western blotting. β-Actin was used as a control. CG is the control group; SG is the *S aureus* group; DEX is the dexamethasone group; and H, M and L are the PD administration groups, with doses of 45, 30 and 15 mg/kg per animal, respectively, and 100, 50 and 25 μg/mL in the cells, respectively. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

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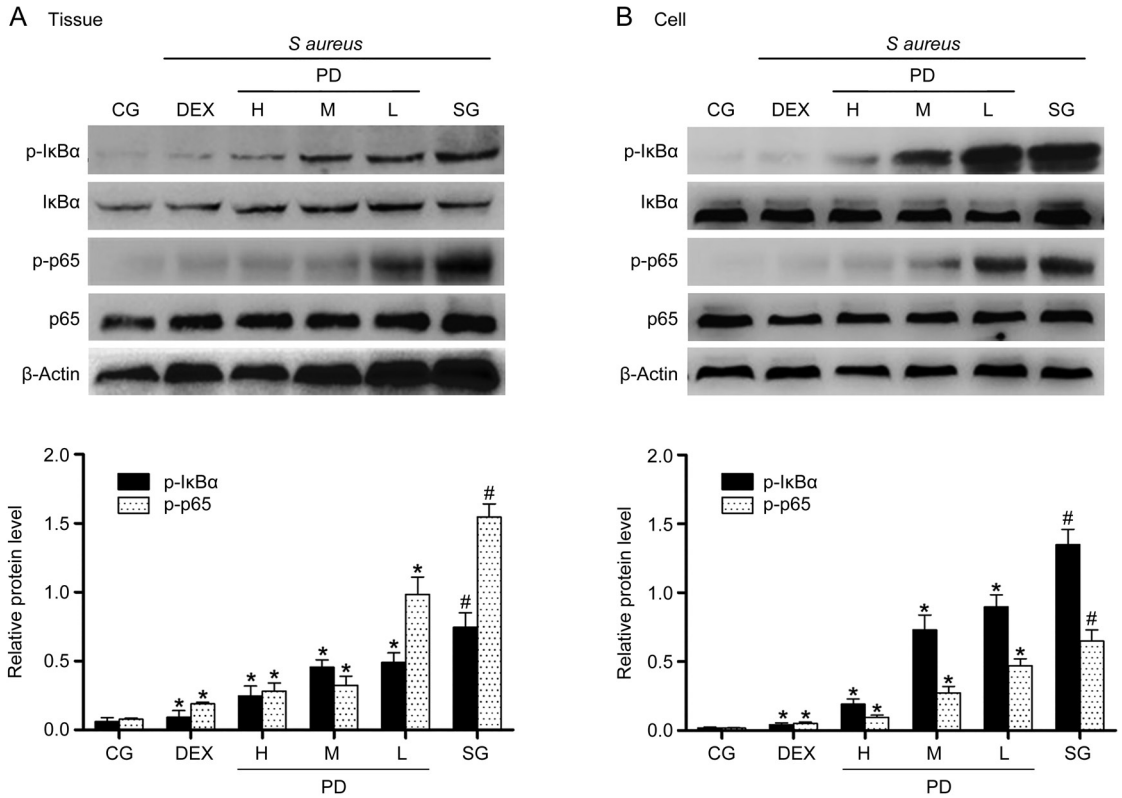

Figure 9. The effects of PD on the NF-κB signaling pathway. (A) The expression of IκBα and p65 protein in tissues. (B) The expression of IκBα and p65 protein in mMECs. β-Actin was used as a control. CG is the control group; SG is the *S aureus* group; DEX is the dexamethasone group; and H, M and L are the PD administration groups, with doses of 45, 30 and 15 mg/kg per animal, respectively, and 100, 50 and 25 μg/mL in the cells, respectively. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

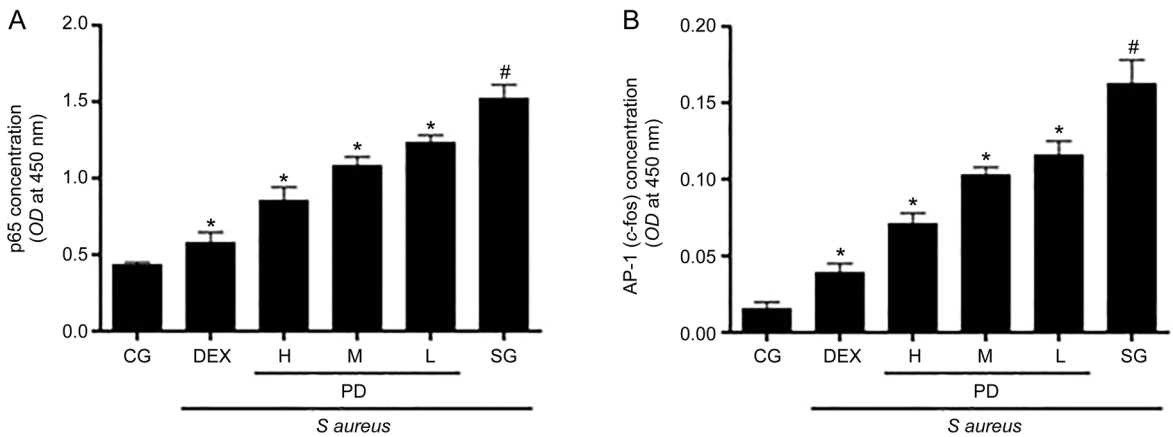

Figure 10. The effects of PD on the nuclear translocation of NF-κB p65 and AP-1. The mMECs were pretreated with or without PD for 1 h and were subjected to *S aureus* for an additional 8 h. The concentrations of NF-κB p65 and AP-1 in nuclear protein extracts from the cells were measured using TransAM assays. (A) The concentration of NF-κB p65 in nuclear protein extracts from mMECs. (B) The concentration of AP-1 in nuclear protein extracts from mMECs. CG is the control group; SG is the *S aureus* group; DEX is the dexamethasone group; and H, M and L are the PD administration groups, with doses of 100, 50 and 25 μg/mL, respectively. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

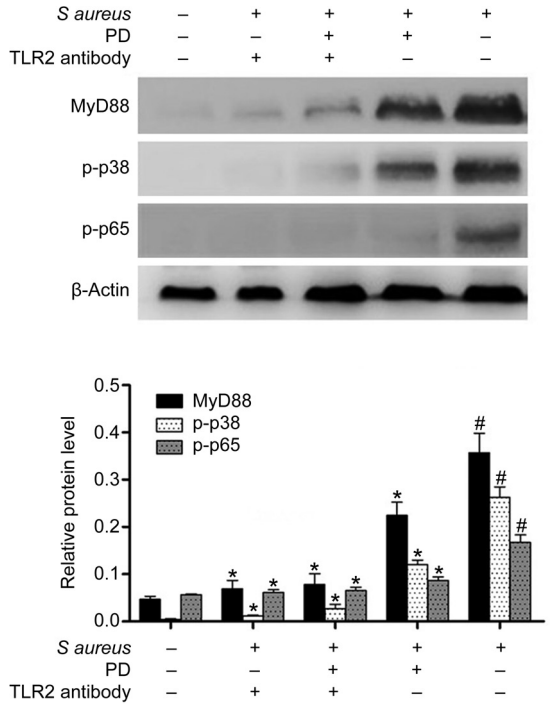

Figure 11. The effects of PD were mediated through TLR2. The mMECs were pretreated with a TLR2-neutralizing antibody (1 μg/mL) or PD (100 μg/mL) for 1 h and then incubated with *S aureus* for 8 h. The levels of MyD88, p-p38 and p-p65 were determined by Western blotting. The data are presented as the mean±SEM. $^{*}P<0.05$ *vs* SG. $^{\#}P<0.05$ *vs* CG.

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