TLR2-ERK signaling pathway regulates expression of galectin-3 in a murine model of OVA-induced allergic airway inflammation

Yunxiang Lv (yunxianglv@126.com)
The First Affiliated Hospital of Bengbu Medical College
Guiyun Jiang
The First Affiliated Hospital of Bengbu Medical College
Yanru Jiang
The First Affiliated Hospital of Bengbu Medical College
Caiqiu Peng
The First Affiliated Hospital of Bengbu Medical College

Research Article

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Abstract

Background: Toll-like receptor 2 (TLR2) and galectin-3 (Gal-3) mediate the pathological process of asthma, but the underlying mechanism is not fully understood. We hypothesized that TLR2 pathway may regulate expression of Gal-3 in allergic airway inflammation.

Methods: Wild-type (WT) and TLR2$^{-/-}$ mice were sensitized on day 0 and challenged with ovalbumin (OVA) on days 14–21 to establish a model of allergic airway inflammation, and were treated with U0126. Airway inflammation was analyzed by hematoxylin-eosin (HE) and Periodic Acid-Schiff (PAS) staining; cytokines and anti-OVA immunoglobulin E (IgE) were tested by ELISA; and related protein expression in lung tissues was measured by western blot.

Results: We found that the expression levels of TLR2 and Gal-3 markedly increased concomitantly with airway inflammation after OVA induction, while TLR2 deficiency significantly alleviated airway inflammation and reduced Gal-3 expression. Moreover, the expression levels of phosphorylated mitogen-activated protein kinases (p-MAPKs) were significantly elevated in OVA-challenged WT mice, while TLR2 deficiency only significantly decreased phosphorylated extracellular signal-regulated kinase (p-ERK) levels. Although blockade of ERK by U0126 had no effect on OVA-induced TLR2 activation, TLR2 deficiency significantly reduced p-ERK expression, suggesting that TLR2 is an upstream signal molecule of ERK. Furthermore, U0126 treatment significantly alleviated allergic airway inflammation and decreased Gal-3 levels in OVA-challenged WT mice, but had no further effect in OVA-challenged TLR2$^{-/-}$ mice. We further demonstrated that TLR2 regulates Gal-3 expression through the ERK pathway in S. aureus-infected macrophages in vitro.

Conclusions: Our findings showed that the TLR2-ERK signaling pathway regulates Gal-3 expression in a murine model of allergic airway inflammation.

Background

Allergic asthma is a chronic inflammatory disease driven by IL-4-, IL-5-, and IL-13-producing Th2 cells or ILC2s. Its hallmark features are eosinophilia, mucus hypersecretion, bronchial hyperresponsiveness (BHR), IgE production, and susceptibility to exacerbations [1]. As a member of Toll-like receptors (TLRs) family, TLR2 is universally regarded as a key regulator for the activation of Th2 immune response in allergic airway inflammation [2, 3]. However, despite the increasing advances in our knowledge of the role of TLR2 in allergic airway diseases, the underlying mechanism of TLR2 regulation in allergic airway inflammation is still elusive.

Mitogen-activated protein kinases (MAPKs) are ubiquitously expressed signal enzymes involved in almost all aspects of asthmatic inflammatory network [4]. Three distinct MAPK pathways explored in mammalian cells include the extracellular signal-regulated kinase (ERK), the c-Jun amino-terminal kinase (JNK), and the p38 MAPK pathway, which are involved in regulation of inflammatory cell proliferation,

differentiation, migration, apoptosis, extracellular matrix protein secretion, cytokine production, and airway smooth muscle cell contraction [4]. Previous studies have shown that inhibition of MAPKs significantly reduces OVA-induced allergic airway inflammation, including inflammatory cells infiltration, cytokines production, mucus secretion, and airway hyperresponsiveness [5, 6]. Moreover, recent evidence has revealed that TLRs are involved in the regulation of allergic airway inflammation through the MAPK signaling pathways [7, 8]. However, the cross talks between TLR2 and MAPKs, as well as the potential mechanism, have not been fully described in allergic airway diseases.

Galectin-3 (Gal-3) is a member of a β-galactoside-binding lectin family that is expressed by most tissues, including the lung, all types of epithelia, and inflammatory cells [9]. There is increasing evidence for the proinflammatory effect of Gal-3 in various inflammatory disease models, including allergic disorders such as asthma [10] and atopic dermatitis [11]. On the one hand, increased expression of Gal-3 in inflammatory cells has been shown in allergic airways [10]. On the other hand, ovalbumin (OVA)-challenged Gal-3-deficient mice exhibited significantly decreased airway eosinophil infiltration, mucus production, airway hyperresponsiveness, and Th2 cytokines secretion compared with the wild-type (WT) mice [12]. The expression levels of TLR2 and Gal-3 were significantly elevated in *Toxoplasma gondii*–infected macrophages, suggesting the potential correlation between TLR2 and Gal-3 in macrophages [13]. Furthermore, the increased levels of TLR2 and Gal-3 were associated with reactive periapical inflammation in periapical granulomas [14]. These results suggest that TLR2 may be involved in the regulation of Gal-3 in inflammatory cells.

In the present study, we hypothesized that the TLR2 pathway may regulate Gal-3 expression in allergic airway inflammation. We thus assessed the roles of TLR2 and investigated the underlying mechanism in a mouse model of OVA-induced allergic airway inflammation.

Methods

Cell culture

RAW264.7 cells (a mouse macrophage cell line obtained from Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Inc., St. Louis, MI, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin (Sigma Inc.), and 100 mg/mL streptomycin (Sigma Inc.) at 37°C in a humidified atmosphere with 5% CO_2.

S. aureus infection

S. aureus (strain NCTC8325; a gift from Professor Sun, Department of Microbiology and Immunology, School of Life Sciences, University of Science and Technology of China, Hefei, China) proliferated overnight in 10 mL Luria–Bertani (LB) broth with kanamycin at 37°C. *S. aureus* was washed with chilled

nonpyrogenic saline, resuspended in nonpyrogenic saline at an OD650 of 0.1, and then kept on ice until infection. *S. aureus* was diluted to reach a multiplicity of infection (MOI) of 10:1 (bacterium: cell) in DMEM for 1 h as in our previous study [15].

RNA interference

Specific small interfering RNAs targeting TLR2 were purchased from Shanghai GenePharma Corporation. TLR2 siRNA (sense: *5′-GGAACAGAGUGGCAACAGUTT-3′* and antisense: *5′-ACUGUUGCCACUCUGUUCCTT-3′*) and Control siRNA (sense: *5′-UUCUCCGAACGUGUCACGUTT-3′* and antisense: *5′-ACGUGACACGUUCGGAGAATT-3′*) were used. Adherent cells were transfected with siRNAs by applying Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Knockdown efficiency was determined by western blot analysis.

Immunocytochemistry

RAW264.7 cells (5×10^5 cells/well) were seeded into glass-bottomed dishes with 1 mL DMEM for 12 h and were washed three times with phosphate buffer saline (PBS) to remove nonadherent cells. After being transfected with TLR2 siRNA or treated with U1026 for 2 h as in the previous study [16], the cells were infected with *S. aureus* (MOI: 10) at 37°C for 1 h in antibody-free DMEM. The cells were then fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) for 10 min, washed with PBS five times, and then blocked with 5% donkey serum for 30 min at room temperature to inhibit nonspecific immunoreactivity. Next, the cells were incubated overnight at 4°C with primary goat anti-mouse Gal-3 antibody at 1:100, rinsed three times in PBS, and incubated with FITC anti-goat secondary antibody (Jackson Immuno Research Lab, West Grove, PA, USA) at a 1:200 dilution. The cells were counterstained with DAPI (Sigma-Aldrich). Images were captured under a Zeiss LMS880 confocal microscope.

Animals

Female C57BL/6 mice (WT, 6-8 weeks, 20-25 g) were obtained from the Shanghai Laboratory Animal Center. TLR2 deficient ($TLR2^{-/-}$) mice on C57BL/6 background were granted by Dr. ZG. Tian (Institute of Immunology, School of Life Sciences, University of Science and Technology of China). WT and $TLR2^{-/-}$ were housed under specific pathogen-free conditions at the Department of Laboratory Animals Center. All of the animal experiments were strictly conducted under protocols approved by the Institutional Animal Care and Use Committee of the Bengbu Medical College.

Experimental protocols and treatment

The protocol applied to establish OVA-induced allergic airway disease in mice was in accordance with a previous study [17]. Briefly, the mice were sensitized by intraperitoneal injection of 10 μg OVA (Chicken Egg OVA, Grade V; Sigma) and 1 mg aluminum potassium sulfate (Sangon Biotech, Shanghai, China) in 0.5 mL saline on day 0, then challenged for 30 min per day with 1% aerosolized OVA on days 14–20. Control mice were saline-sensitized and challenged with nebulized saline solution. On days 14–20, the WT and $TLR2^{-/-}$ mice from the U0126-treated group were pretreated with specific MAPK/ERK inhibitor U0126 (30 mg/kg, Sigma-Aldrich) or vehicle (polyethylene glycol (PEG), Sigma-Aldrich) intraperitoneally, diluted in 0.1 mL sterile saline 30 min prior to OVA challenge as described previously [5]. All of the mice were sacrificed after the last challenge; on the left lung, bronchoalveolar lavage and subsequent differential cell counting and enzyme-linked immunosorbent assay (ELISA) were performed, while the right lung was used for histopathological analysis and western blot.

Histological analysis

Lung tissues were fixed in 4% paraformaldehyde. After that the paraffin-embedded tissues were sectioned (5-μm thickness) and stained with hematoxylin/eosin (H&E) or periodic acid-Schiff (PAS) to measure inflammatory cellular infiltration and mucus productions, respectively. The quantitative analysis of peri-bronchial inflammation in H&E-stained lung slices was performed by determining the number of rings of inflammatory cells around bronchial, and the mucus productions quantification was accomplished by assessing the number of PAS^{+} cells in the airway by the perimeter of the basement membrane (Pbm) as our previous study [18].

Bronchoalveolar lavage fluid (BALF) and cellular analysis.

BALF was collected from the left lung with 0.5 mL of ice-cold PBS and was centrifuged at 700 g at 4°C for 5 min. After that the cell pellets harvested from BALF were resuspended in 200 μL PBS. Total cells were counted in a hemocytometer, and differential cell count was determined by staining cytospins of BALF samples with Wright Stain solution (Sigma). At least 200 cells were counted for each mouse. The supernatants were stored at -80℃for ELISA analysis.

ELISA

The secretion of IL-4, IL-5, and IL-13 in BALF and OVA-specific IgE in serum were measured by ELISA using specific kits from Cusabio (Wuhan, China). The levels of Gal-3 in BALF and serum were tested by ELISA kits from Bio-Swamp (Wuhan, China) in accordance with the protocols from the manufacturer. The sensitivity for IL-4, IL-5, IL-13, and Gal-3 was 0.39 pg/mL, 7.8 pg/mL, 7.8 pg/mL, and 0.078 ng/mL, respectively.

Western blot

The lung tissues were homogenized using a homogenizer with RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phosphatase inhibitor PhosSTOP (Roche). Total protein was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% nonfat milk and then incubated with primary antibodies. including anti-p-ERK/ERK (1:1000), anti-p-p38/p38, anti-p-JNK/JNK, and anti-TLR2 (1:1000) (Cell Signaling Technology Inc., Beverly, MA, USA), anti-galectin-3 (1:500) (Santa Cruz Biotechnology, CA, USA), and anti-GAPDH (1:2000) (KANGCHEN Biotech, Shanghai, China). The membranes were subsequently incubated with HRP-conjugated anti-rabbit IgG (1:2000) (Promega, Madison, WI, USA) and polyclonal rabbit anti-mouse IgG (Dako, Copenhagen, Denmark), and all blots were detected with enhanced chemiluminescence (ECL; Thermo Scientific). For quantitative analysis, intensity of protein bands was determined using ImageJ 1.38 software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using SPSS 16.0. All data were expressed as the mean ± standard deviation of triplicate samples and were representative of at least three separate experiments. Independent-sample *t* test or one-way ANOVA with a post-hoc Bonferroni test was used for all statistical analyses. P values <0.05 were considered significant.

Results

TLR2 promotes allergic airway inflammation in OVA-induced mice

To detect the roles of TLR2 in allergic airway inflammatory disease, WT and $TLR2^{-/-}$ mice were sensitized and challenged with OVA as illustrated in Fig. 1A. We found that the expression level of TLR2 protein was significantly elevated in OVA-challenged WT mice compared with control mice (Fig. 1B, C). Concomitantly, there was a significant increase in inflammatory cells recruiting to the peribronchial regions and PAS^{+} cells surrounding the airway in OVA-challenged WT mice as compared with control mice (Fig. 1E–G). Expectedly, histological staining revealed that fewer inflammatory cells were recruited into the peribronchial regions in OVA-challenged $TLR2^{-/-}$ mice than in OVA-challenged WT mice (Fig. 1D, E). Concurrently, PAS^{+} cells surrounding the airway were significantly decreased in OVA-challenged $TLR2^{-/-}$ mice compared with OVA-challenged WT mice (Fig. 1F, G).

$TLR2^{-/-}$ reduces the invasion by inflammatory cells and the production of inflammatory cytokines in BALF, and the production of anti-OVA IgE in serum

We counted the number of inflammatory cells and measured Th2 cytokines in BALF. The numbers of total cells, eosinophils, lymphocytes, macrophages, and neutrophils in the allergic mice were significantly higher in BALF of OVA-challenged WT mice than in BALF of control mice (Fig. 2A–E). Moreover, the production of IL-4, IL-5, and IL-13 in BALF of the WT allergic mice was also significantly increased compared with that of the control mice (Fig. 2F–H). Compared with the WT mice, TLR2$^{-/-}$ produced a significant decrease in released inflammatory cells, especially eosinophils in BALF (Fig. 2A–E). Concurrently, TLR2$^{-/-}$ significantly reduced the secretion of IL-4, IL-5, and IL-13 in BALF (Fig. 2F–H). Similarly, the production of anti-OVA IgE in the serum of OVA-challenged TLR2$^{-/-}$ mice was significantly decreased compared with that of OVA-challenged WT mice (Fig. 2I).

TLR2$^{-/-}$ decreases the expression of Gal-3 and phosphorylated ERK in allergic mice

We next examined whether TLR2 could mediate the MAPK signal pathway in allergic airway inflammation. We further assessed the association between TLR2 and MAPKs activation. The present study showed that the expression levels of phosphorylated MAPKs (p-ERK, p-p38, and p-JNK) were significantly increased in OVA-challenged WT mice compared with control mice (Fig. 3A, B). However, only the expression level of p-ERK, but not that of p-p38 or p-JNK, was significantly lower in OVA-challenged TLR2$^{-/-}$ mice in comparison with OVA-challenged WT mice (Fig. 3A, B). To examine whether TLR2 influences the production of Gal-3, we measured the expression level of Gal-3 in the lung, BALF, and serum. The results showed that the expression level of Gal-3 protein in the lung was significantly higher in OVA-challenged WT mice than in control mice, while such an increase was markedly abrogated in OVA-challenged TLR2$^{-/-}$ mice (Fig. 3C). Moreover, consistent findings of Gal-3 production were also observed in serum and BALF (Fig. 3D, E).

Inhibition of ERK reduces airway inflammation in OVA-induced WT mice but has no additional effects in OVA-induced TLR2$^{-/-}$ mice

U0126—an inhibitor of ERK—was used to further testify the link between TLR2 and ERK signal in the regulation of allergic airway inflammation as shown in Fig. 4A. The present findings revealed that treatment with U0126 had no significant influence on the expression of TLR2 in OVA-challenged WT mice (Fig. 4B, C). We also observed reduced counts of inflammatory cells in the peribronchial regions (Fig. 4D, E) and PAS^{+} cells in airways (Fig. 4F, G) in OVA-challenged WT mice treated with U0126. Furthermore, administration of U0126 did not further ameliorate airway inflammatory cells recruitment and histopathological manifestations, which were similar to those in vehicle-treated TLR2$^{-/-}$ mice (Fig. 4D–G).

U0126 reduces the invasion by inflammatory cells and the production of Th2 cytokines in BALF, and serum anti-OVA IgE levels in OVA-challenged WT mice, but has no additional effect in OVA-challenged TLR2$^{-/-}$ mice

We further compared the invasion by inflammatory cells and the production of Th2 cytokines in BALF and anti-OVA IgE in serum between WT and TLR2$^{-/-}$ mice. The number of inflammatory cells and the secretion levels of IL-4, IL-5, and IL-13 in BALF significantly decreased in OVA-challenged WT mice treated with U0126 (Fig. 5A–H). However, the decrease was not observed in OVA-challenged TLR2$^{-/-}$ treated with U0126 (Fig. 5A–H). Similarly, we also found that U0126 significantly reduced the production of anti-OVA IgE in serum in OVA-challenged WT mice (Fig. 5I), but had no additional effects in OVA-challenged TLR2$^{-/-}$ mice (Fig. 5I).

U0126 decreases Gal-3 expression in OVA-challenged WT mice but has no additional effect in OVA-challenged TLR2$^{-/-}$ mice

We next assessed whether the TLR2-ERK pathway could regulate Gal-3 expression in allergic mice. We revealed that the expression level of p-ERK significantly decreased in both OVA-induced WT and TLR2$^{-/-}$ mice after the treatment with U0126 (Fig. 6A, B). Additionally, the expression level of p-ERK was significantly lower in OVA-induced TLR2$^{-/-}$ mice than in OVA-induced WT mice (Fig. 6A, B). Importantly, the expression level of Gal-3 in OVA-induced WT mice was significantly reduced by U0126, but U0126 did not further reduce Gal-3 expression in TLR2$^{-/-}$ mice compared with that in WT mice (Fig. 6A, B). Moreover, similar results were observed in the production of Gal-3 in serum (Fig. 6C) and BALF (Fig. 6D).

Knockdown of TLR2 decreases Gal-3 and phosphorylated ERK expression in *S. aureus*-infected macrophages

We assessed whether TLR2 could regulate Gal-3 expression by the ERK pathway in *S. aureus*-infected macrophages. The expression level of TLR2 after the treatment with TLR2 siRNA was significantly lower than that after Control siRNA (Fig. 7A, B). When cells were infected by *S. aureus*, the expression levels of phosphorylated MAPKs (p-ERK, p-p38, and p-JNK) were significantly increased. However, TLR2 siRNA significantly decreased only the expression levels of p-ERK, but not those of p-P38 or p-JNK. Meanwhile, *S. aureus* infection significantly increased the expression level of Gal-3, while TLR2 siRNA significantly reversed it (Fig. 7C, D). Furthermore, similar changes were also found in *S. aureus*-infected Raw264.7 cells by immunocytochemistry analysis (Fig. 7E).

TLR2 regulates Gal-3 expression by the ERK signaling pathway in *S. aureus*-infected macrophages

To testify our findings, we further used U0126 in *S. aureus*-infected macrophages. U0126 significantly reduced not only the expression level of p-ERK but also that of Gal-3 in *S. aureus*-infected Raw264.7 cells (Fig. 8A, B). However, U0126 did not further reduce Gal-3 expression in the TLR2 siRNA group compared with the Control siRNA group in *S. aureus*-infected macrophages (Fig. 8A, B). Moreover, similar results were observed in *S. aureus*-infected Raw264.7 cells by immunocytochemistry analysis (Fig. 8C). These findings demonstrated that TLR2 regulated Gal-3 expression by the ERK signaling pathway in *S. aureus*-infected macrophages.

Discussion

The present study showed that TLR2 deficiency significantly alleviated airway inflammation and decreased the expression level of Gal-3 in the murine model. Although blockade of ERK by U0126 had no effect on OVA-induced TLR2 activation, TLR2 deficiency significantly reduced OVA-induced phosphorylation of ERK. Furthermore, treatment with U0126 showed significant resolution of allergic airway inflammation and decreased the expression level of Gal-3 in OVA-challenged WT mice, but had no further effect on reduction of allergic airway inflammation and the expression of Gal-3 in OVA-challenged $TLR2^{-/-}$ mice. Meanwhile, U0126 therapy significantly decreased Gal-3 expression in OVA-challenged WT mice, but such a decrease was not further observed in $TLR2^{-/-}$ mice. Similarly, we further demonstrated that TLR2 regulates Gal-3 expression through the ERK pathway in *S. aureus*-infected macrophages. Our novel findings show that the TLR2-ERK signaling pathway regulates Gal-3 expression in a murine model of allergic airway inflammation.

The hallmark of asthma is the imbalance of Th1/Th2 response [1]. TLRs play a pivotal role in regulating Th2 inflammatory response through recognizing environmental allergens, microorganisms, or microbial products [19]. To date, TLR2 has been acknowledged as the most related to the initiation of allergic airway inflammation [2, 3]. The studies focusing on the roles of TLR2 in allergic airway disease have yielded different results. Although some studies have shown that TLR2 activation could result in the inhibition of allergic inflammation [20, 21], other studies have revealed that activation of TLR2 could promote allergic Th2 inflammation in a mouse model [22-24]. Furthermore, on the one hand, the expression level of TLR2 in patients with asthma was significantly elevated compared with that in healthy subjects [25]. On the other hand, patients with asthma who eventually died showed increased expression level of TLR2 [26]. In line with these findings, our present studies revealed a significant increase in TLR2 expression in OVA-challenged WT mice, and this increase was accompanied by enhanced allergic inflammation, including higher eosinophils, monocytes, macrophages, and neutrophils; higher Th2-related IL-4, IL-15, and IL-13 in BALF; higher anti-OVA IgE level in serum; and more mucus metaplasia in the airway. However, such OVA-induced airway inflammation was significantly reduced in OVA-challenged $TLR2^{-/-}$ mice. These results demonstrate that TLR2 is involved in the regulation of

allergic airway inflammation, and blockade of TLR2 may bring therapeutic benefits to patients with allergic airway inflammatory diseases.

Although previous studies have testified that TLR2 is involved in the regulation of Th2 response in allergic airway inflammation, the underlying mechanism of TLR2 regulation of allergic airway inflammation has not yet been fully elucidated [19]. Increasing evidence has shown the proinflammatory effect of Gal-3 in various inflammatory disease models including allergic asthma [10] and atopic dermatitis [11]. The expression level of Gal-3 in inflammatory cells is significantly increased in allergic airways [10]. Moreover, OVA-challenged Gal-3-deficient mice exhibit significantly reduced airway eosinophil infiltration, mucus production, airway hyperresponsiveness, and Th2 cytokines secretion compared with WT mice [12]. The expression levels of TLR2 and Gal-3 are significantly elevated in *Toxoplasma gondii*–infected macrophages, suggesting the potential correlation between TLR2 and Gal-3 in macrophages [13]. Additionally, the increased levels of TLR2 and Gal-3 are associated with reactive periapical inflammation in periapical granulomas [27]. Therefore, we further evaluated Gal-3 expression in allergic mice. Expectedly, the expression levels of Gal-3 in the lung, serum, and BALF in OVA-challenged WT mice were significantly elevated relative to those in control mice, which is consistent with previous studies [10]. Furthermore, the activation of TLR2 was also observed in OVA-challenged WT mice. These results further confirmed the potential correlation between TLR2 and Gal-3 in inflammatory response as previous studies had indicated [13, 14]. Importantly, we found that the expression level of Gal-3 in OVA-challenged TLR2$^{-/-}$ mice was significantly reduced compared with that in OVA-challenged WT mice. Furthermore, we also found that the expression level of Gal-3 was significantly increased in *S. aureus*-infected macrophages, and knockdown of TLR2 significantly reversed this trend. These findings suggest that TLR2 can regulate Gal-3 expression, which indicates that TLR2 is an upstream molecule of Gal-3 in inflammatory disease.

A question arises as to how TLR2 mediates Gal-3 expression and participates in allergic airway inflammation. Previous studies have confirmed that MAPKs are involved in almost all aspects of asthmatic inflammatory network [4]. Furthermore, inhibition of MAPKs significantly restored OVA-induced allergic airway inflammation, including inflammatory cells infiltration, cytokines production, mucus secretion, and airway hyperresponsiveness [5, 6]. Moreover, recent evidence has demonstrated that TLRs are involved in the regulation of allergic airway inflammation through the MAPK signal pathways [7, 8]. However, the cross talks between TLR2 and MAPKs, as well as the potential mechanism in allergic airway diseases, have not been clarified. We thus assessed the expression level of phosphorylated MAPKs in OVA-induced WT and TLR2$^{-/-}$ mice. The results showed that the expression levels of p-ERK, p-JNK, and p-p38 were significantly elevated in OVA-challenged WT mice compared with control mice, which is consistent with previous studies [8, 28-30]. In an OVA-induced male mouse model, TLR2 deficiency significantly reduced the expression of p-ERK, p-JNK, and p-p38 [8]. However, in the present study, only the expression level of p-ERK, but not that of p-p38 or p-JNK, was significantly decreased in OVA-challenged TLR2$^{-/-}$ mice relative to OVA-challenged WT mice, suggesting that TLR2 may be involved in allergic airways through the ERK signaling pathway, which is consistent with previous studies [29, 30]. The

difference between the two studies may arise from the sex differences of the included mice, as our allergic airway inflammatory model was established based on female mice as previously indicated [29, 30]. Furthermore, in various murine disease models, there is differential activation of MAPK pathways between male and female mice [31–33]. Therefore, these results suggest that hormonal differences may induce the activation of different MAPK pathways in mice with allergic airway inflammation.

To further demonstrate whether the TLR2-ERK signaling pathway is involved in the regulation of allergic airway inflammation through the control of Gal-3, U0126—a specific ERK inhibitor—was used for the treatment of allergic airway inflammation in mice. Treatment with U0126 showed significant resolution of allergic airway inflammation in OVA-challenged WT mice, as shown previously [5], but had no further effect on reduction of allergic airway inflammation in OVA-challenged $TLR2^{-/-}$ mice. These results further suggest that TLR2 regulates allergic airway inflammation through the ERK signaling pathway. To further address the problem whether the activation of ERK can regulate endogenous Gal-3 expression or not, an inflammatory model of *S. aureus*-infected macrophages were used in vitro. Although previous studies have demonstrated that elevated Gal-3 could induce the activation of ERK in tumor cells [34, 35], recent studies have shown that cardiotrophin-1 increases endogenous Gal-3 expression through the ERK pathway and that blockade of ERK signaling decreases Gal-3 expression in human cardiac fibroblasts [36]. Similarly, decreased expression levels of endogenous Gal-3 were observed in U0126-treated OVA-induced WT mice, suggesting that the ERK signaling pathway was able to directly mediate Gal-3 expression in OVA-induced allergic mice. Moreover, we found that the inhibition of ERK resulted in the decrease of Gal-3 expression in *S. aureus*-infected macrophages. Our results further confirm that the ERK pathway can regulate Gal-3 expression. These findings suggest that there may be a positive feedback regulation between ERK and Gal-3. However, administration of U0126 did not further reduce the expression levels of Gal-3 in OVA-challenged $TLR2^{-/-}$ mice. These findings suggest that the TLR2-ERK signaling pathway can induce allergic airway inflammation through the activation of Gal-3 expression.

Conclusions

Overall, the TLR2-ERK signaling pathway triggers allergic airway inflammation via enhancing Gal-3 expression in an OVA-challenged murine model. Moreover, targeting the TLR2-ERK pathway promotes resolution of allergic airway inflammation. Therefore, the TLR2-ERK-Gal-3 pathway may provide novel therapeutic approaches for allergic airway diseases.

Abbreviations

BALF Bronchoalveolar lavage fluid; BHR bronchial hyperresponsiveness; ERK extracellular signal-regulated kinase; Gal-3 galectin-3; HE hematoxylin-eosin; Ig E immunoglobulin E; JNK c-Jun amino-terminal kinase; MAPK mitogen-activated protein kinases; OVA ovalbumin; PAS Periodic Acid-Schiff; TLR2 Toll-like receptor; WT wide-type.

Declarations

Acknowledgements

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization: YX Lv; Methodology and Performance of experiments: GY Jiang, Yanru Jiang and Caiqiu Peng; Writing original draft preparation: YX Lv; Funding acquisition: YX Lv; All members contributed to the manuscript revision, read, and approved the submission.

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Ethics Approval and Consent to Participate

This work was approved from the ethics committee of Bengbu Medical College. The animal experiments were complied with The National Institutes of Health Guide for the Care and Use of Laboratory Animals. We confirmed this study is reported in accordance with ARRIVE guidelines for the reporting of animal experiments. Consent to participate is not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Figures

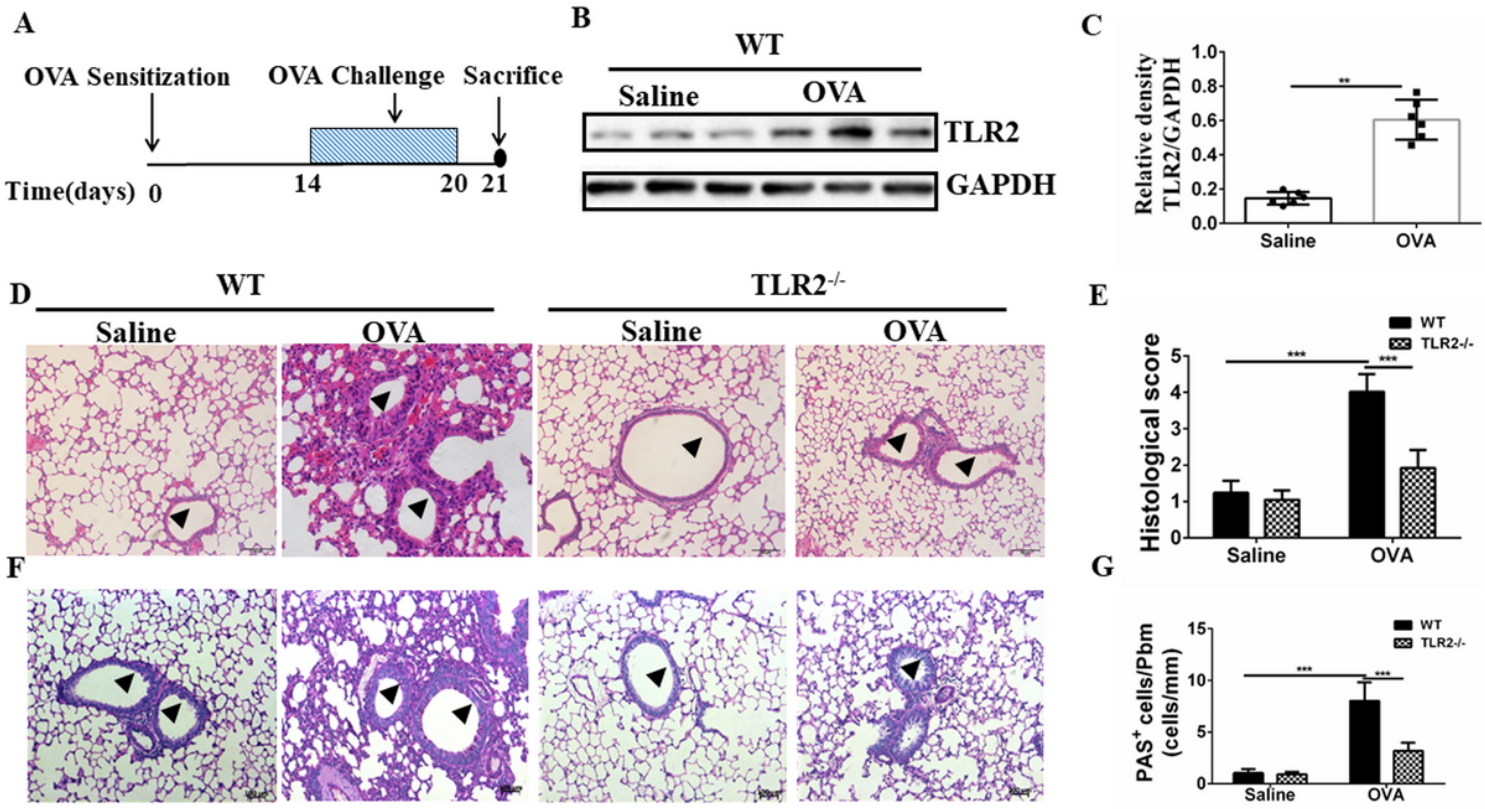

Figure 1

TLR2 promotes allergic airway inflammation in OVA-induced mice. (A) Experimental protocol of establishment of allergic airway disease model in C57BL/6 WT and TLR2$^{-/-}$mice. (B) Detection of TLR2 protein expression in the lung of WT mice by Western blot. (C)Relative ratio of TLR2 to GADPH. (D) Representative H&E-staining of lung sections from C57BL/6 and TLR2$^{-/-}$ mice. Arrow represents bronchia, Bars: 100 μm. (E) Histological scoring of lung inflammatory in mice. (F) Representative periodic acid-Schiff (PAS) staining of lung sections from C57BL/6 and TLR2$^{-/-}$ mice. Bars: 100 μm. (G) Quantification

of PAS^{+} cells in the airways. Data were expressed as the means ±SD of 6 mice per group. $^{**}P<0.01$, $^{***}P<0.001$.

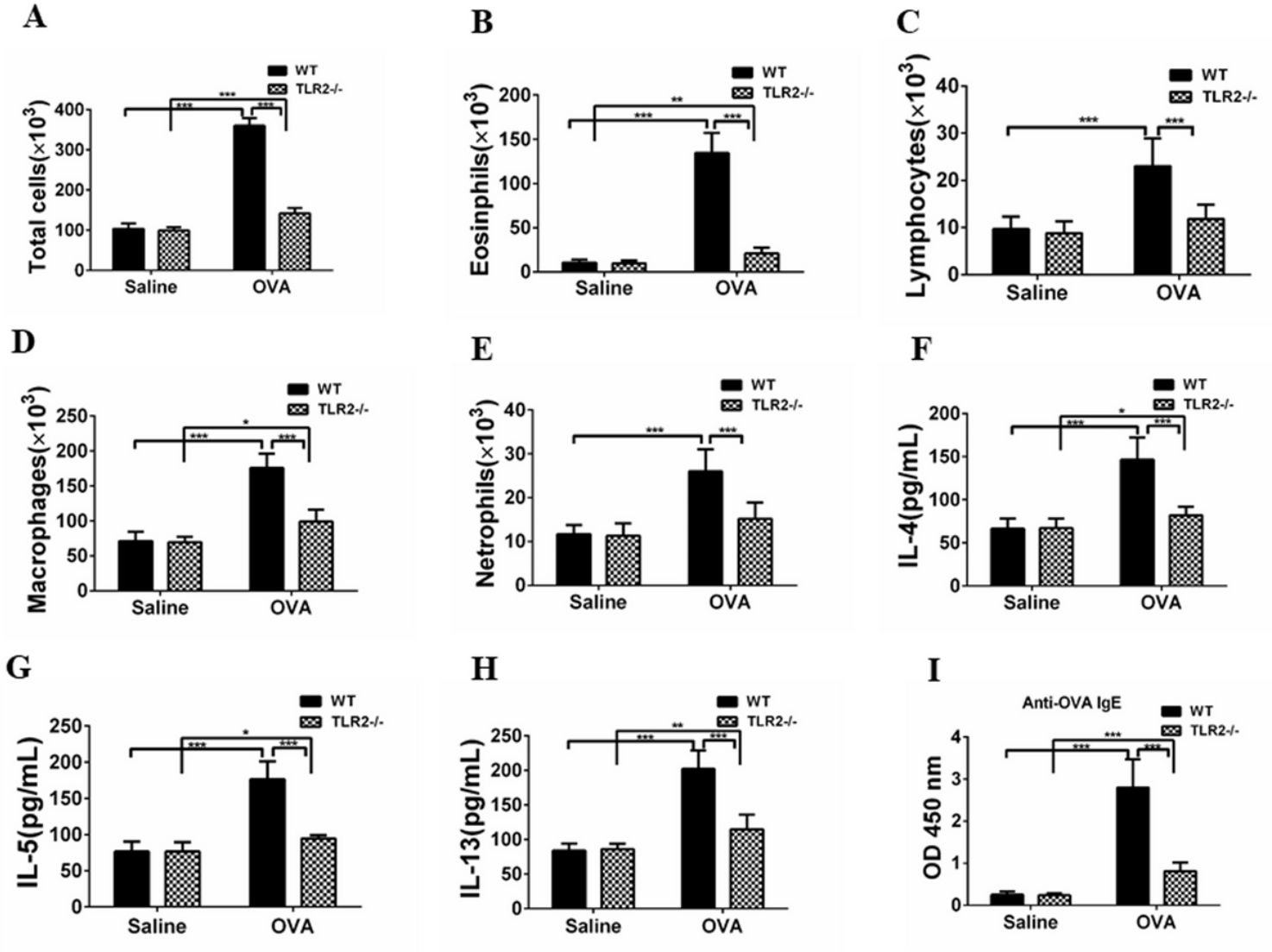

Figure 2

TLR2$^{-/-}$ reduces the invasion of inflammatory cells and the production of inflammatory cytokines in BALF, and the production of the anti-OVA IgE in serum. (A) Total cell counts in BALF of WT and TLR2$^{-/-}$ mice. (B-E) differential cell counts in BALF of WT and TLR2$^{-/-}$ mice. (F-H) Secretion levels of IL-4, IL-5 and IL-13 in BALF of WT and TLR2$^{-/-}$ mice. (I) Secretion level of anti-OVA IgE in serum. Data were expressed as the means ±SD of 6 mice per group. $^{***}P<0.001$.

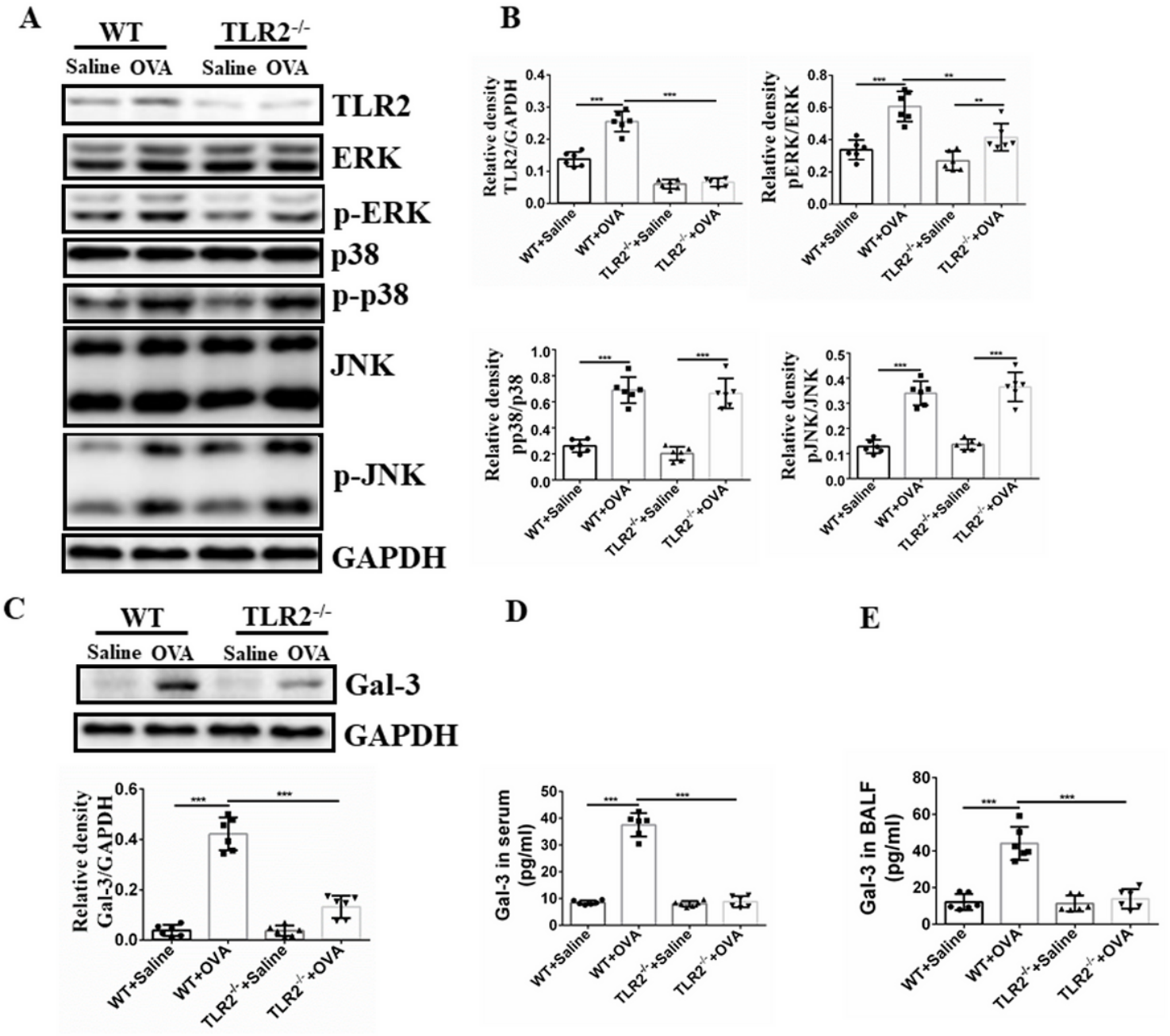

Figure 3

TLR2$^{-/-}$ decreases Galectin-3 and phosphorylated ERK expression in allergic mice. (A) Detection of TLR2, p-ERK/ERK, p-p38/p38 and p-JNK/JNK protein expression in the lung of WT and TLR2$^{-/-}$ mice by Western blot. (B) Relative ratio of TLR2 to GAPDH, p-ERK to ERK, p-p38 to p38 and p-JNK to JNK. (C) Relative expression level of Gal-3 in the lung of WT and TLR2$^{-/-}$ mice. (D) The level of Gal-3 in serum of WT and TLR2$^{-/-}$mice. (E) The level of Gal-3 in BALF of WT and TLR2$^{-/-}$mice. Data were expressed as the means ±SD of 6 mice per group. $^{**}P$ <0.01, $^{***}P$ < 0.001.

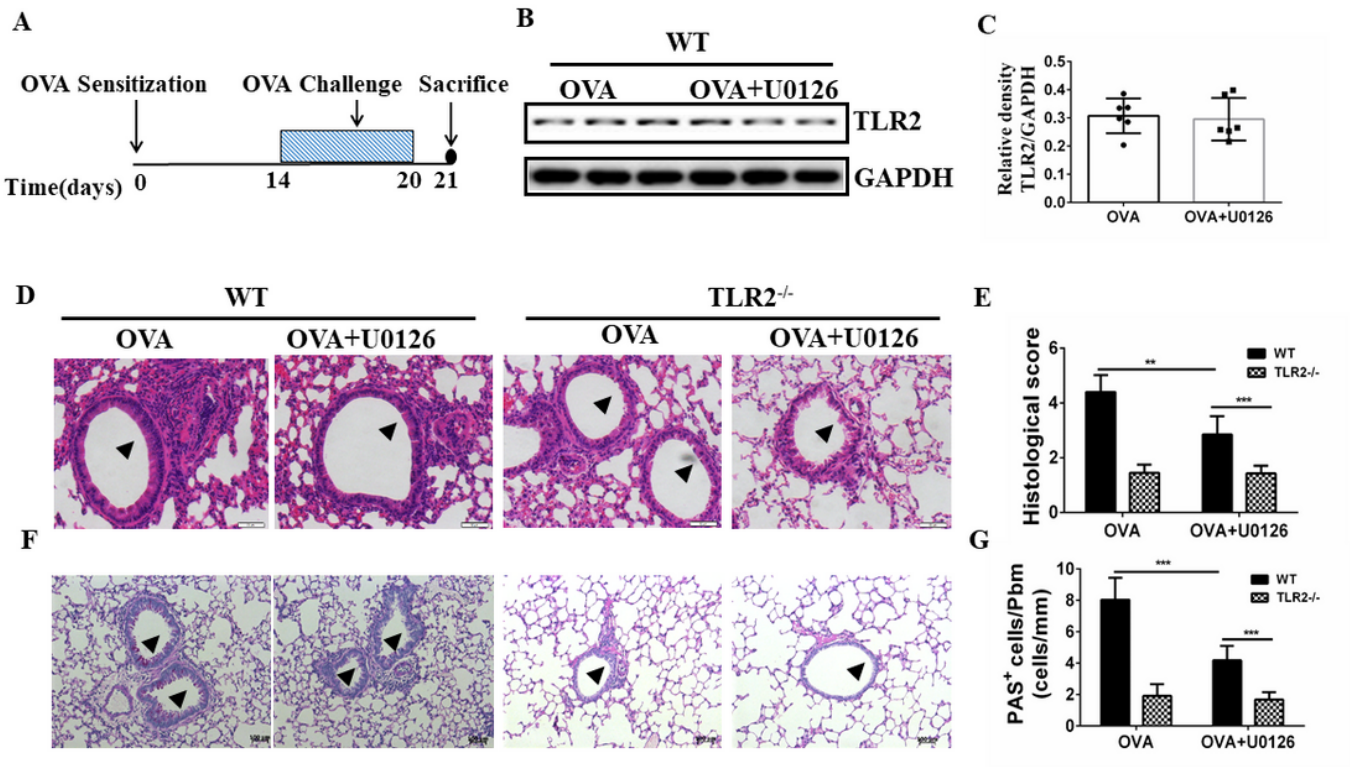

Figure 4

Inhibition of ERK reduces airway inflammation in OVA-induced WT mice but have no further effects on that in OVA-induced TLR2$^{-/-}$ mice. (A) Protocol of administration of U0126 during establishing allergic airway disease model. (B) Measurement of TLR2 protein expression in the lung of OVA-challenged WT mice treated with U0126 or not. (C) Relative ratio of TLR2 to GAPDH. (D) Representative H&E-staining of lung sections from C57BL/6 and TLR2$^{-/-}$ mice treated with U0126 or not. Arrow represents bronchia, Bars: 50 μm. (E) Histological scoring of lung inflammatory in mice. (F) Representative periodic acid-Schiff (PAS) staining of lung sections from C57BL/6 and TLR2$^{-/-}$ mice treated with U0126 or not. Bars: 100 μm. (G) Quantification of PAS^{+} cells in the airways. Data were expressed as the means ±SD of 6 mice per group. $^{**}P<0.01$, $^{***}P<0.001$.

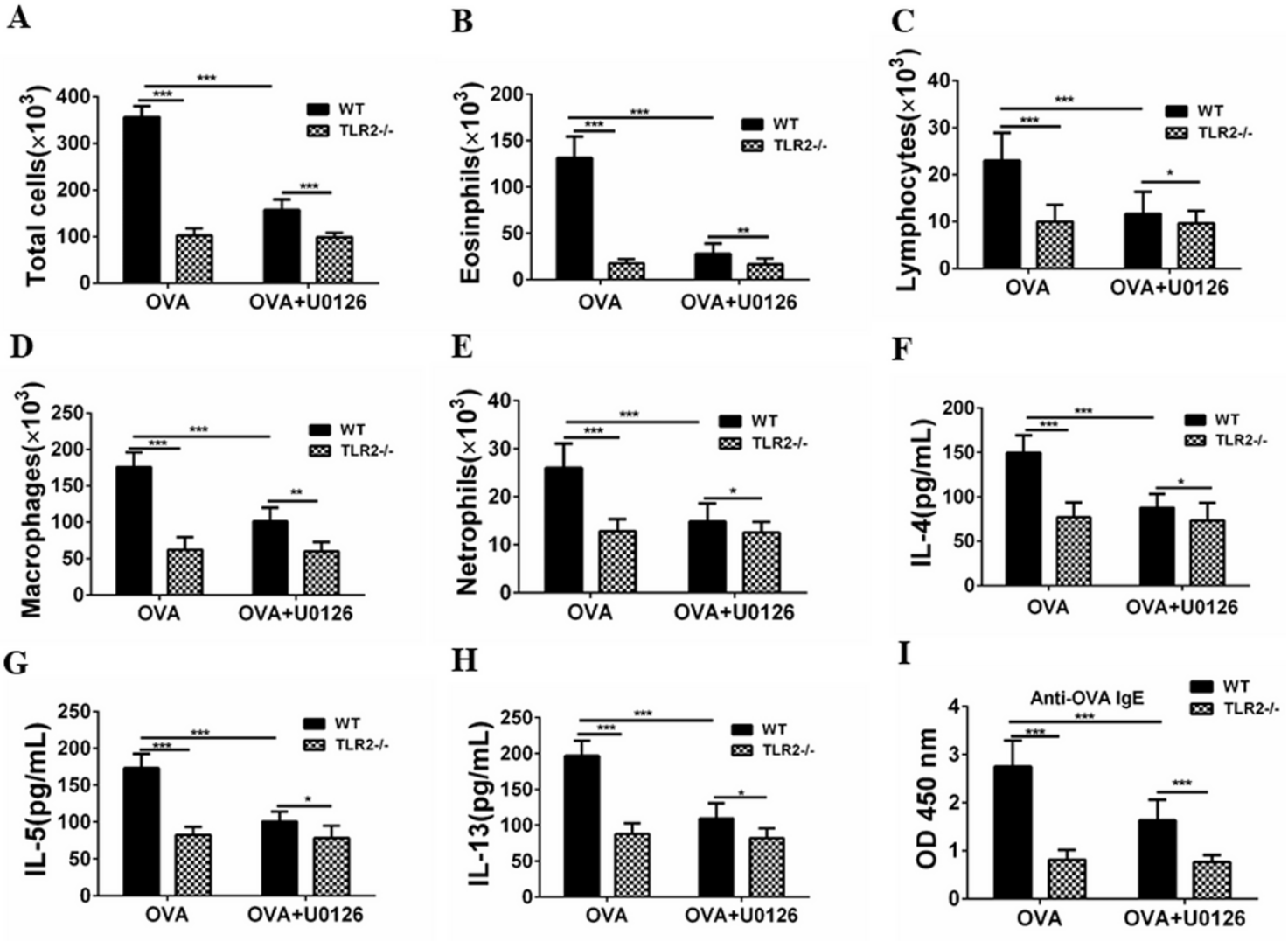

Figure 5

U0126 reduces the invasion of inflammatory cells and the production of Th2 cytokines in BALF, and serum anti-OVA IgE levels in OVA-challenged WT mice but had no further effect on that in OVA-challenged TLR2$^{-/-}$ mice. (A) Total cell counts in BALF of WT and TLR2$^{-/-}$ mice treated with U0126 or not. (B-E) differential cell counts in BALF of WT and TLR2$^{-/-}$ mice treated with U0126 or not. (F-H) Secretion levels of IL-4, IL-5 and IL-13 in BALF of WT and TLR2$^{-/-}$ mice treated with U0126 or not. (I) Secretion level of anti-OVA IgE in serum. Data were expressed as the means ±SD of 6 mice per group. *P <0.05, **P <0.01, ***P < 0.001.

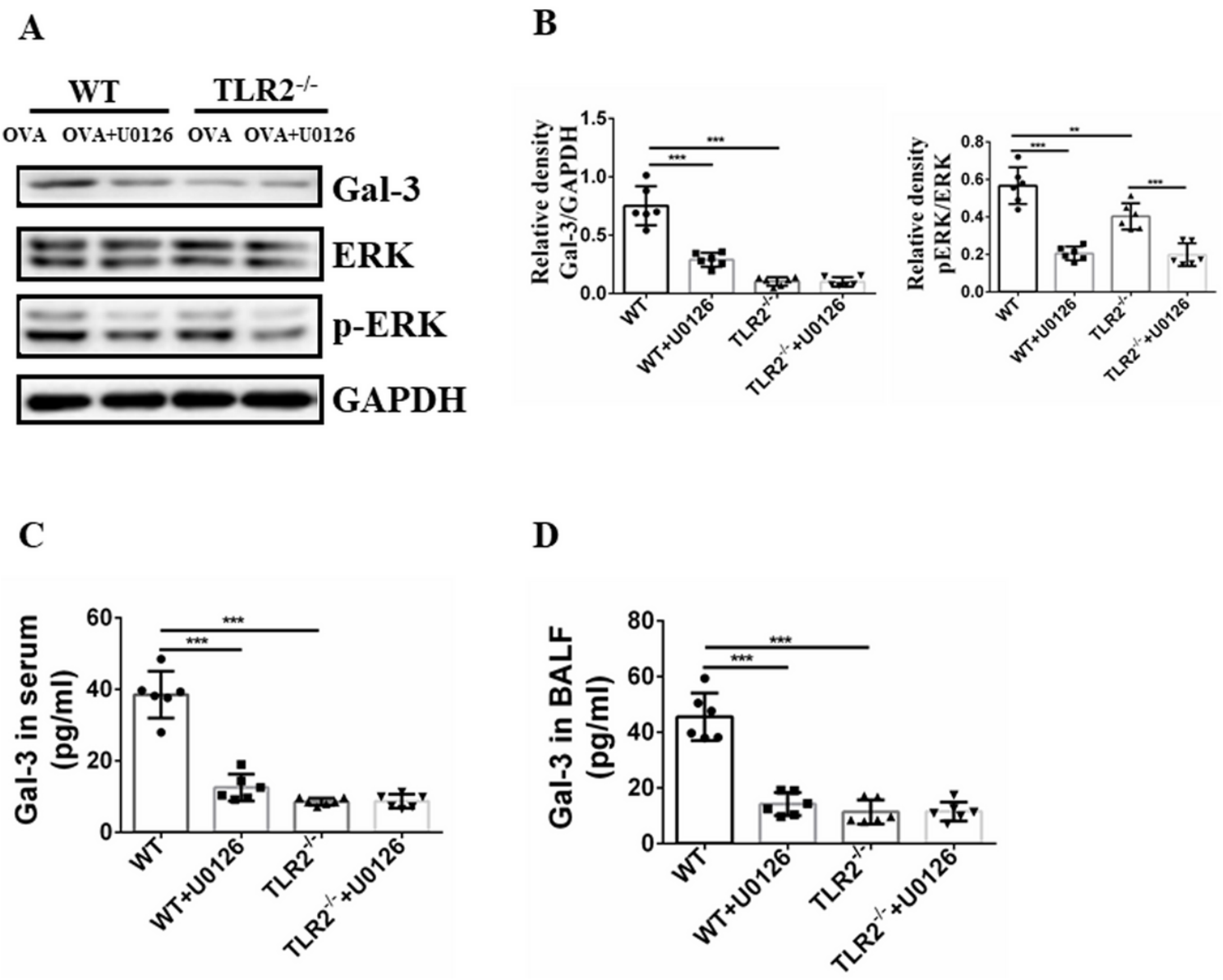

Figure 6

U0126 decreases Gal-3 expression in OVA-challenged WT mice but had no further effect on that in OVA-challenged TLR2$^{-/-}$ mice. (A) Measurement of Gal-3, p-ERK/ERK protein expression in the lung of WT and TLR2$^{-/-}$ mice treated with U0126 or not by Western blot. (B) Relative ratio of Gal-3 to GAPDH, p-ERK to ERK. (C) The level of Gal-3 in serum of WT and TLR2$^{-/-}$ mice treated with U0126 or not. (D) The level of Gal-3 in BALF of WT and TLR2$^{-/-}$ mice treated with U0126 or not. Data were expressed as the means ±SD of 6 mice per group. $^{**}P$ <0.01, $^{***}P$ < 0.001.

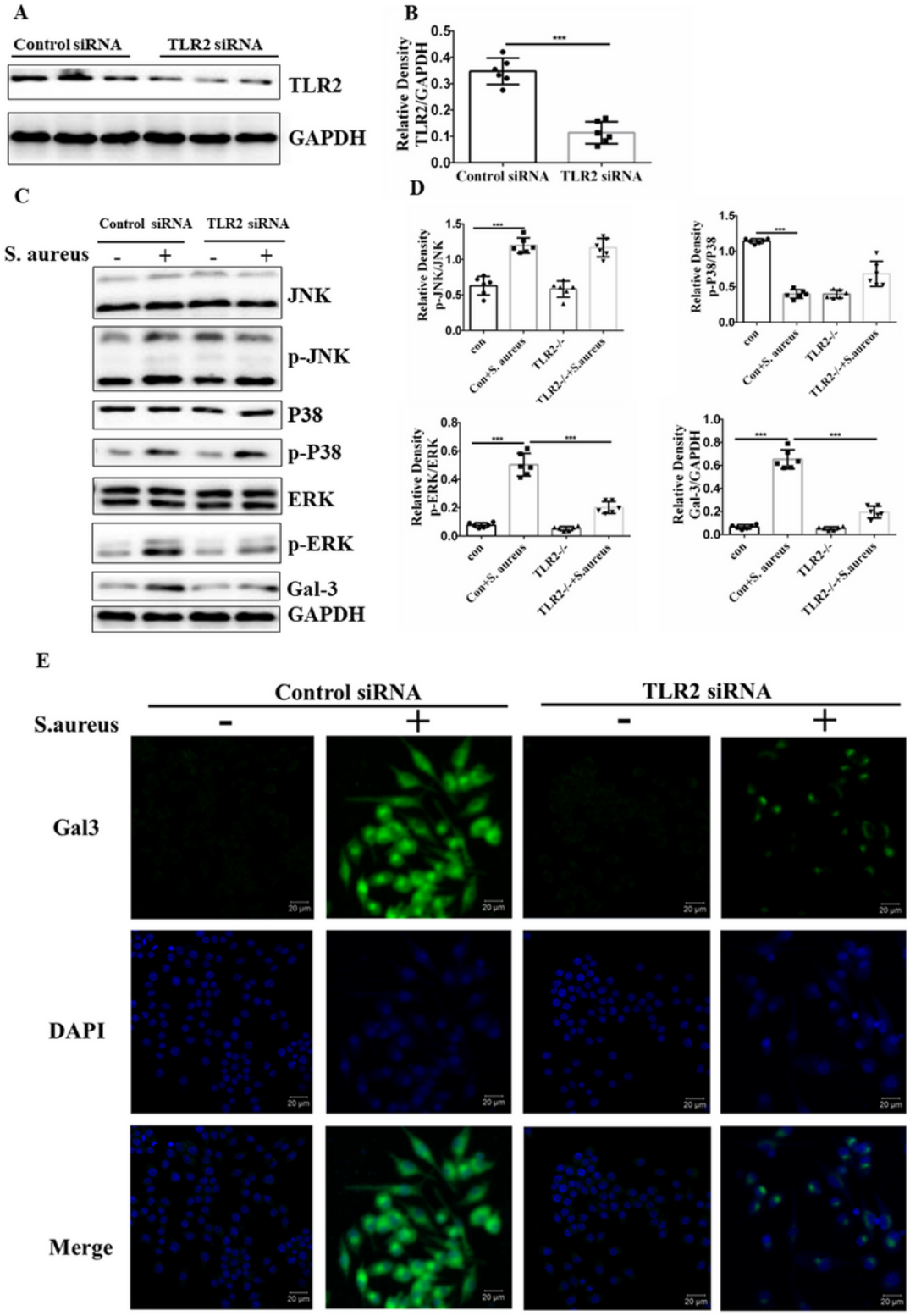

Figure 7

TLR2 siRNA reduces Gal-3 and phosphorylated ERK expression in S. aureus-infected macrophages. (A) Cells were transfected with control siRNA orTLR2 siRNA and then infected with *S. aureus* for 1 h. The TLR2 expression was measured by Western

blot. (B) The relative TLR2 expression. (C) The expression levels of p-JNK, p-ERK, p-P38 and Gal-3 were measured by Western blot. (D) The relative proteins expression. (E)The expression levels of Gal-3 in cells were measured by Immunocytochemistry. Scale bars = 20 mm. ***$P < 0.001$.

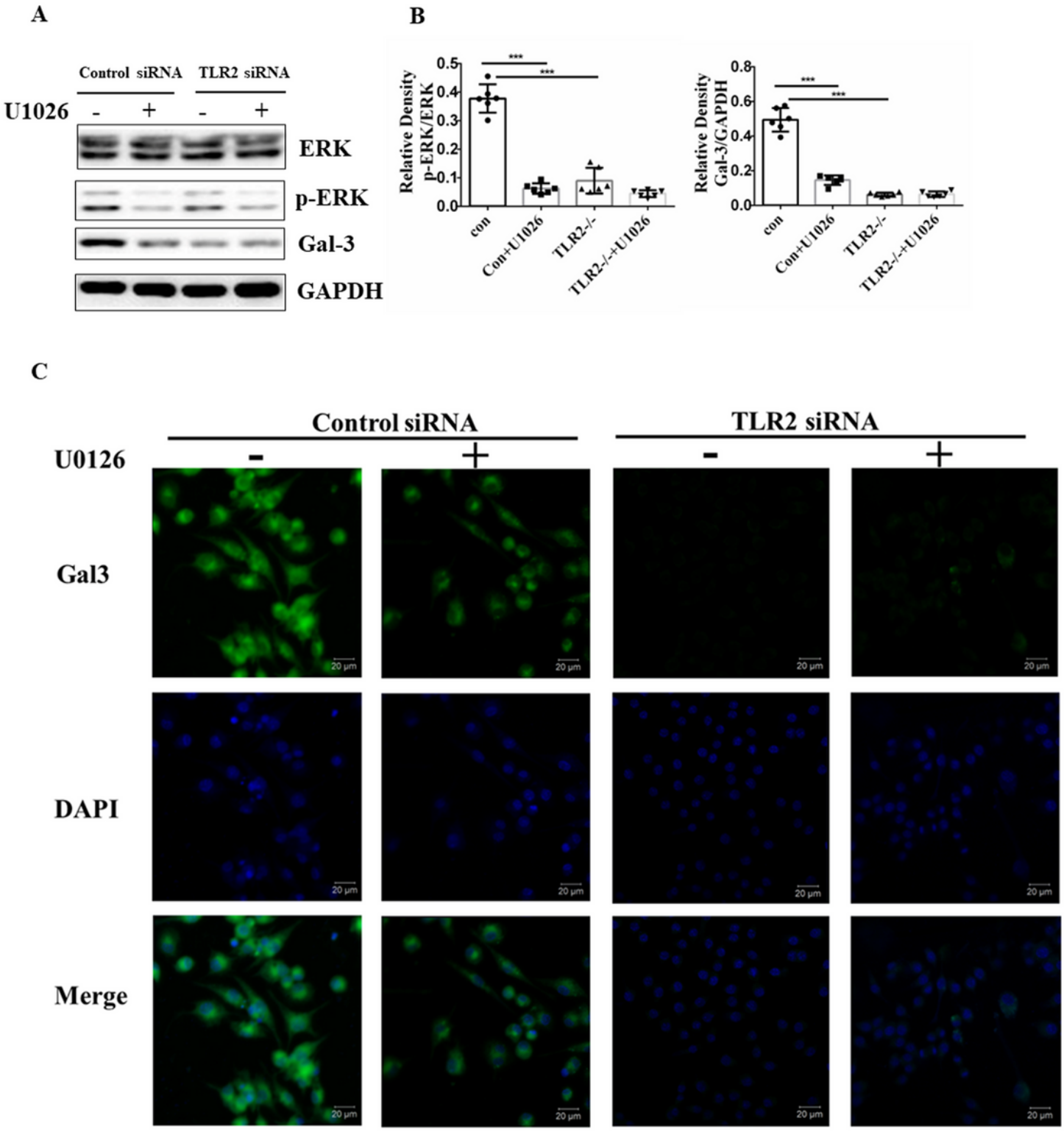

Figure 8

TLR2 regulates Gal-3 expression by ERK pathway in *S. aureus*-infected macrophages. (A) Cells were transfected with control siRNA orTLR2 siRNA and then were pretreated with U0126 for 2h, cells were infected with *S. aureus* for 1 h. The p-ERK and Gal-3 expression were measured by Western blot. (B) The relative proteins expression. (C) The expression levels of Gal-3 in cells were measured by Immunocytochemistry. Scale bars = 20 mm. ***$P < 0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig7ERKGAPDH.jpg
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- Fig7PERKGAPDH.jpg
- Fig7PERKPJNKGAPDH.jpg
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- Fig7gal3GAPDH.jpg
- Fig7jnkp38GAPDH.jpg
- Fig7jnk.jpg
- Fig8ERKPERK.jpg
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- Fig3.Gal3GAPDH.jpg
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- Fig6.Gal3jpg.jpg
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