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TLR2 regulates allergic airway inflammation through NF-κB and MAPK signaling pathways in asthmatic mice

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Abstract. – OBJECTIVE: To investigate the role of toll-like receptor 2 (TLR2) in asthmatic mouse model and its possible signal transduction pathways.

MATERIALS AND METHODS: Mice were divided into three groups: TLR2$^{-/-}$ asthma mouse model group (n=10), C57BL/6 asthma mouse model group (n=10) and control group (n=10). Mice were sensitized and stimulated with ovalbumin (OVA) to establish the asthmatic mouse model. The unilateral bronchoalveolar lavage fluid (BALF) was collected and centrifuged to separate cells, and the cells were classified and counted via smear test under a microscope. Part of the lung tissues on the other side was taken for hematoxylin-eosin (HE) staining to observe the histopathological change in lung tissues. The remaining lung tissues on the other side were taken to detect the messenger ribonucleic acid (mRNA) expression levels of interleukin-4 (IL-4), IL-5 and IL-13 via reverse transcription-polymerase chain reaction (RT-PCR). The levels of nuclear factor-κB (NF-κB) p65, phosphorylated (p)-NF-κB p65, p-IκBα, extracellular signal-regulated kinase (ERK)1/2, p-ERK1/2, Jun N-terminal kinase (JNK), p-JNK, p38 mitogen-activated protein kinase (MAPK), p-p38 MAPK, IL-4, IL-5 and IL-13 were detected via enzyme-linked immunosorbent assay (ELISA). The protein expressions of NF-κB p65, p-NF-κB p65, p-IκBα, ERK1/2, p-ERK1/2, JNK, p-JNK, p38 MAPK, and p-p38 MAPK were detected using the immunohistochemical method.

RESULTS: HE staining showed that the infiltration degree of inflammatory cells in perivascular tissues in TLR2$^{-/-}$ asthma group was reduced compared with that in C57BL/6 asthma group. Results of electron microscopy showed that the ultrastructural changes in alveolar type I epithelial cells in mice in TLR2$^{-/-}$ asthma group was significantly alleviated. In BALF in TLR2$^{-/-}$ asthma group, the numbers of eosinophils and lymphocytes were significantly decreased, but the number of macrophages was significantly increased compared with those in C57BL/6 asthma group. Results of RT-PCR and ELISA revealed that the mRNA and protein expression levels of IL-4, IL-5, and IL-13 in lung tissues of mice in TLR2$^{-/-}$ asthma group were significantly decreased compared with those in C57BL/6 asthma group. Besides, results of ELISA and immunohistochemistry revealed that the protein expressions of NF-κB p65, p-NF-κB p65, p-IκBα, ERK1/2, JNK, p38 MAPK, p-ERK1/2, p-JNK, and p-p38 MAPK in lung tissues of mice in TLR2$^{-/-}$ asthma group were significantly decreased compared with those in C57BL/6 asthma group.

CONCLUSIONS: TLR2 is involved in the occurrence and development of experimental asthmatic airway inflammation. TLR2 gene knockout in asthmatic mice can alleviate the airway inflammation, whose mechanism may be that the allergic airway inflammation of asthmatic mice is alleviated through inhibiting NF-κB and MAPK signaling pathways.

Key Words:
Bronchial asthma, TLR2, Inflammation, NF-κB, MAPK.

Introduction

Bronchial asthma is one of the most common chronic respiratory diseases in childhood, mainly manifested as airway hyper-responsiveness, reversible airflow limitation, mucus hyper-secretion and remodeling of airway structure[1, 2]. Its morbidity rate shows an ever-increasing trend around the world. In China, the incidence rate of childhood asthma is about 2.5% and that of severe childhood asthma is also increased significantly, seriously threatening the physical and psychological health of children[3]. However, the pathogenesis of asthma is not fully clear yet, so

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the in-depth research on the pathogenesis of asthma is of very important theoretical and practical significance for the prevention and treatment of asthma. At present, it is believed that bronchial asthma is a chronic airway inflammatory disease involving a variety of inflammatory cells and mediators, but its exact pathogenesis remains unclear[4,5]. Currently, asthma is considered to be a T helper 2 (TH2) cell-mediated inflammatory disease[6]. Cluster of differentiation 4 $(CD4)^+$ TH2 cells can produce unique pro-inflammatory cytokines, such as interleukin-4 (IL-4), IL-5 and IL-13, so they are considered to be the core in the development and amplification of allergic asthma[7]. Eosinophilic inflammation of airway is related to the acute exacerbation of asthma[8]. There are abundant macrophages in lungs, which are cells exposed to antigens first and posing a threat to homeostasis. Therefore, macrophages occupy a central position in the innate immune of lungs[9]. Toll-like receptor 2 (TLR2) is a family member of TLRs, which is expressed the most widely in the family of TLRs and exists extensively in plants, insects, mammals and human[10]. TLR2 is a type I transmembrane protein, which, as a pattern recognition receptor, can recognize and bind to several pathogen-associated molecular patterns (PAMPs) and trigger a series of signal transduction, thus leading to the release of inflammatory mediators, initiating the innate immune response, removing the invasive pathogenic microorganisms, and playing an important role in the innate immune defense[10,11]. What's more, TLR2 can also activate the acquired immune response, thereby acting as a bridge between the innate and adaptive immune systems. In recent years, researches have found that TLR2 also plays an important role in allergic diseases, such as bronchial asthma, but its mechanism of action is not fully clear yet[12]. In this work, mice with TLR2 gene knockout were used as objects of study and were sensitized and stimulated with ovalbumin (OVA) as an allergen to establish the asthmatic mouse model. The role of TLR2 in asthmatic mouse model and its possible mechanism of action were observed, so as to provide new ideas for further revealing the pathogenesis of asthma and searching new possible targets for prevention and treatment.

Materials and Methods

Experimental Animals and Modeling

Male $TLR2^{-/-}$ mice ($B6.129\text{-}Tlr2^{tmIkirNJU}$) and C57BL/6 mice aged 5-6 weeks old weighing 18-22 g were purchased from Model Animal Research Center of Nanjing University. Establishment of asthma model[13]: each mouse was sensitized via intraperitoneal injection of 100 μg OVA and 200 μL aluminum hydroxide suspension twice (once at 0 d and 7 d, respectively). At 14 d, 15 d, 16 d, 17 d, 18 d, 19 d and 20 d, mice were stimulated with 1% OVA aerosol in a self-made aerosol inhalation box for 30 min once per day. In control group, mice were given normal saline, instead of OVA, and the rest of the process was the same as that in model group. Mice in this experiment were divided into three groups: $TLR2^{-/-}$ asthma model group (n=10), C57BL/6 asthma model group (n=10) and control group (n=10). This study was approved by the Animal Ethics Committee of The Second People's Hospital of Shen County Animal Center.

Collection of Bronchoalveolar Lavage Fluid (BALF) and Cell Classification and Counting

After mice were anesthetized, they were fixed on the operation table under a supine position. The neck and chest skin were disinfected with 70% medical alcohol. The neck skin was cut, the sternum was cut off to expose the trachea, and the right main bronchus was ligated. An about 0.2 cm-long incision was made obliquely on the proximal part of trachea, the medical indwelling needle was inserted into an about 1 cm-deep proximal part, and the incision was ligated and fixed using silk threads. Next, the left lung was lavage using cold phosphate-buffered saline (PBS) for 3 times (0.5 cm/time)[14]. A total of 1 mL BALF was fully recycled, centrifuged and smeared using the centrifugation-smear machine, fixed and stained using the modified Wright method. At least 500 cells were counted and classified on each slide.

Lung Tissue Sections and Hematoxylin-Eosin (HE) Staining

Part of the lung tissues of mice on the right side was taken, fixed with 10% paraformaldehyde for 48 h, taken and cut into 0.2 cm-thick tissue blocks, followed by dehydration via gradient alcohol at room temperature, transparency via xylene and paraffin embedding. Then, blocks were cut into 3 μm-thick sections and dried. After HE staining for 5 min, sections were washed with warm water (about 50°C), blued for 5 min, stained with 0.5% eosin for 20-30 s, and sealed with neutral resin. Finally, sections were observed and photographed under a microscope.

Electron Microscopy

After mice were executed, lung tissues (1 mm^3) were immediately taken, fixed in 2.5% glutaraldehyde, fixed again with 1% osmic acid, embedded in dehydrated epoxy resin, and cut into ultra-thin sections (1 μm). The sections were stained via uranium lead, and the alveolar type I epithelial ultrastructure was observed under an electron microscope.

Ribonucleic Acid (RNA) Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Part of the right lung tissues of mice was taken, frozen and ground in a grinding bowl. After 1 mL TRIzol was added and fully mixed, the mixture was placed in ice water for 10 min. Then, 200 μL chloroform were added, and the mixture was shaken for 15 s and let stand at room temperature for 2 min, followed by centrifugation at 12,000 g for 15 min at 4°C. The upper-layer aqueous solution was carefully transferred into a new 1.5 mL centrifuge tube, and added with 500 μL isopropanol. The mixture was mixed evenly and let stand on ice for 10 min, followed by centrifugation at 12000 g for 15 min at 4°C. The supernatant was discarded, and 1 mL 75% pre-cooled ethanol prepared by diethylpyrocarbonate (DEPC)-double distilled water was added to wash the sediment, followed by centrifugation at 12,000 g for 5 min at 4°C. The supernatant was discarded, and 1 mL absolute ethyl alcohol was added to wash the sediment, followed by centrifugation at 12,000 g for 5 min at 4°C. After the supernatant was discarded, the sediment was dried at room temperature. RNA sediment was dissolved using RNase-free water until 50 μL sediment were left, and RT-PCR was performed after quantification. The operation was repeated 3 times for each sample. After the reaction was finished, the amplification curve and melting curve were plotted, and the relative quantification of messenger RNA (mRNA) level was performed using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-Linked Immunosorbent Assay (ELISA)

Fresh right lung tissues were taken, added with normal saline (2 mL normal saline for 1 g tissue) at 4°C for homogenate, and centrifuged at 2500 rpm for 10 min. The supernatant was taken to detect the levels of pro-inflammatory cytokines (including IL-4, IL-5, and IL-13), NF-κB signaling pathway related factors (including NF-κB p65, p-NF-κB p65, p-IκBα), and MAPK signaling pathway related factors (including ERK1/2, p-ERK1/2, JNK, p-JNK, p38 MAPK, p-p38 MAPK) via ELISA in strict accordance with the instructions.

Immunohistochemistry

Paraffin-embedded sections of lung tissues of mice were dewaxed via xylene and dehydrated via gradient ethanol. 3% H_2O_2 was used to block the endogenous peroxidase activity. Sections were immersed in the citrate buffer (pH 6.0) and warmed in a microwave oven and boiled for 5 min after boiling for antigen retrieval. 50 μL 5% bull serum albumin (BSA) blocking solution were added, and the primary antibody of NF-κB signaling pathway related factors (including NF-κB p65, p-NF-κB p65, p-IκBα) and MAPK signaling pathway related factors (including ERK1/2, p-ERK1/2, JNK, p-JNK, p38 MAPK, p-p38 MAPK) were incubated and placed in a refrigerator at 4°C overnight. On the next day, the secondary antibody was added for incubation, followed by color development (dark brown) via diaminobenzidine (DAB). After air-drying, sections were sealed using neutral resin, and observed under an optical microscope.

Statistical Analysis

Statistical product and service solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for analysis. All data were presented as mean ± standard deviation ($\bar{x} \pm s$). Unpaired two-tailed Student's *t*-test was used for the comparison of intergroup differences. $p < 0.05$ suggested that the difference was statistically significant.

Results

TLR2 Knockout Alleviated the Histopathological Inflammatory Manifestations in Asthmatic Mice

In C57BL/6 asthma group, the bronchial wall was thickened with lumen stenosis, a large amount of mucus, even red mucus plugs, could be seen in the lumen, and there was infiltration of a large number of inflammatory cells (such as lymphocytes, eosinophils and neutrophils) around the bronchi and blood vessels. Pathological sections of lung tissues of mice in $TLR2^{-/-}$ asthma group showed that, compared with those of mice in C57BL/6 asthma group, the infiltration of inflammatory cells around the bronchi and blood vessels was significantly alleviated, the bronchial lumen

was intact and there were no necrosis and exfoliation of epithelial cells in TLR2$^{-/-}$ asthma group. Pathological sections of lung tissues in control group revealed that the bronchial and alveolar structures were complete, bronchial cilia were arranged neatly, bronchial mucous epithelium was intact, and there was no significant inflammatory cell infiltration. It could be seen under the electron microscope that mitochondria of alveolar type I epithelial cells in mice in C57BL/6 asthma group were swelling with blurred cristae, and the Golgi complex was less developed, with chromatin aggregation. The above ultra structural changes in alveolar type I epithelial cells in mice in TLR2$^{-/-}$ asthma group were significantly alleviated (Figure 1).

TLR2 Knockout Reduced the Inflammatory Cells in BALF

In BALF in C57BL/6 asthma group, the numbers of eosinophils and lymphocytes were significantly increased, but the number of macrophages was significantly decreased compared with those in control group. In BALF in TLR2$^{-/-}$ asthma group, the numbers of eosinophils and lymphocytes were significantly decreased, but the number of macrophages was significantly increased compared with those in C57BL/6 asthma group. The number of eosinophils in TLR2$^{-/-}$ asthma group was increased significantly compared with that in control group, but there were no statistically significant differences in the numbers of lymphocytes and macrophages compared with those in control group. There was no statistically significant difference in neutrophil count in BALF among three groups of mice (Figure 2A).

TLR2 Knockout Reduced the Expression of Pro-Inflammatory Cytokines (IL-4, IL-5 and IL-13) in Lung Tissues

The mRNA expressions of IL-4, IL-5, and IL-13 in lung tissues of mice in C57BL/6 asthma group, TLR2$^{-/-}$ asthma group and control group were detected via RT-PCR. Compared with those in C57BL/6 asthma group, the mRNA levels of IL-4, IL-5, and IL-13 in TLR2$^{-/-}$ asthma group were significantly decreased, and the differences were statistically significant. However, the levels were still higher than those in control group, and the differences were statistically significant. ELISA detection showed that compared with those in C57BL/6 asthma group, the protein expressions of IL-4, IL-5, and IL-13 in lung tissues of mice in TLR2$^{-/-}$ asthma group were significantly decreased, and the differences were statistically significant (Figure 2B and C).

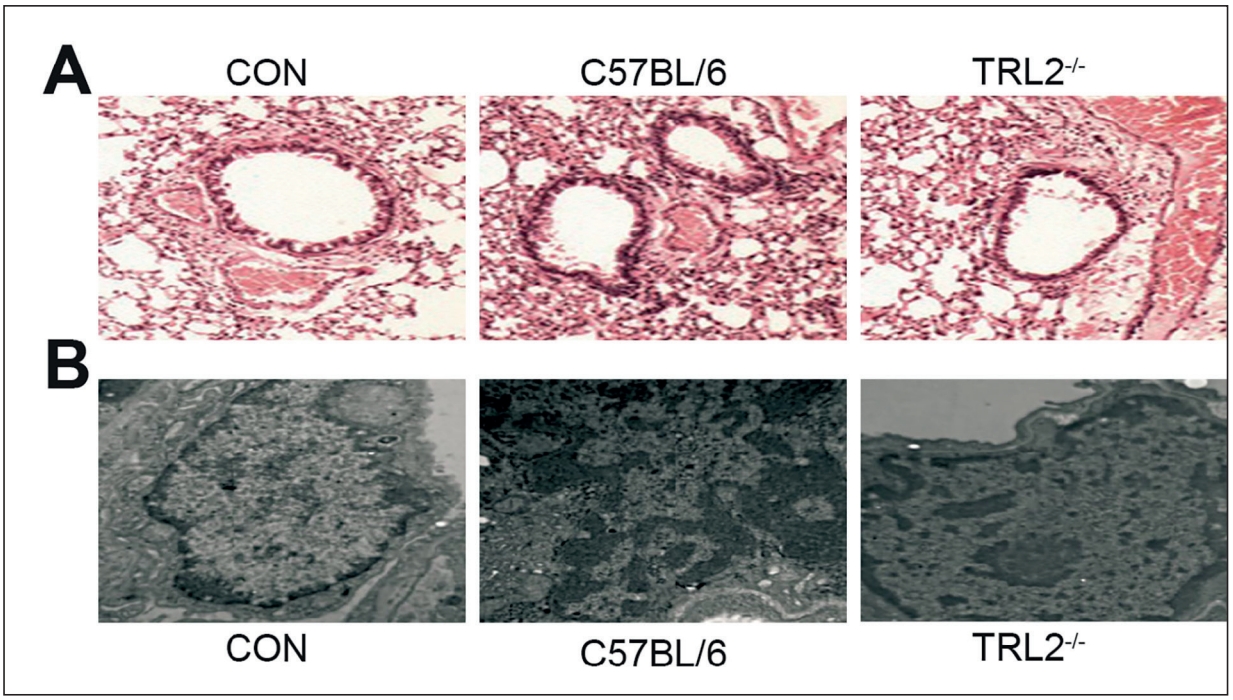

Figure 1. TLR2 knockout alleviates the histopathological inflammatory manifestations in asthmatic mice. ***(A)*** The representative images of hematoxylin and eosin (HE) staining (Magnification: 100 ×). ***(B)*** The representative images of electron microscope (Magnification: 100,000 ×).

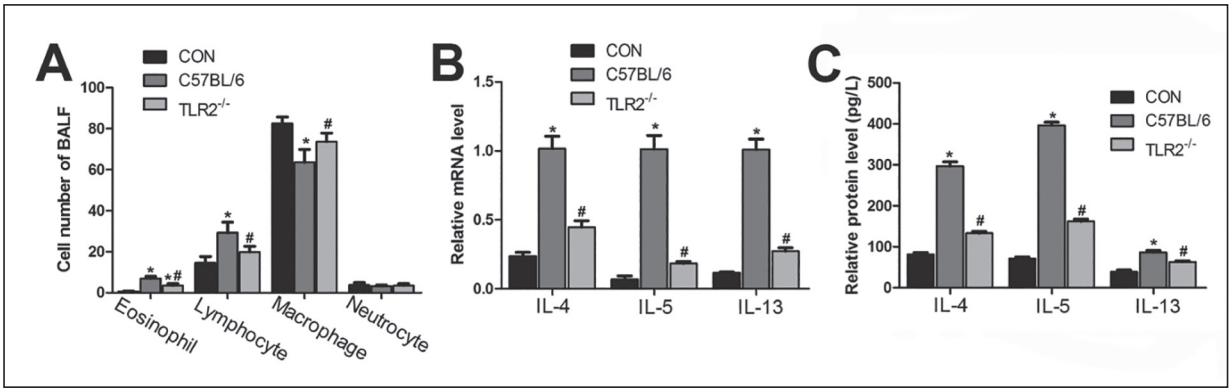

Figure 2. TLR2 knockout reduces the inflammatory cells and pro-inflammatory cytokines. ***(A)*** Analysis of inflammatory cells in BALF. ***(B)*** Analysis of mRNA levels of IL-4, IL-5, IL-13 by RT-PCR. (***C)*** Analysis of the activity of IL-4, IL-5, IL-13 by ELISA. $^{*}p < 0.05$ *vs.* CON group, $^{\#}p < 0.05$ *vs.* C57BL/6 group.

Effect of TLR2 Knockout on NF-κB Signaling Pathway in Mouse Lungs

Results of ELISA showed that compared with those in control group, the levels of NF-κB p65, p-NF-κB p65, and p-IκBα in lungs of mice in C57BL/6 asthma group were significantly increased. Compared with those in C57BL/6 asthma group, the levels of NF-κB p65, p-NF-κB p65, and p-IκBα in lungs of mice in TLR2$^{-/-}$ asthma group were significantly decreased, but levels of the above indexes were still higher than those in normal group. The above change trends of proteins were further confirmed *via* immunohistochemical results (Figure 3).

Effect of TLR2 Knockout on MAPK Signaling Pathway in Mouse Lungs

Results of ELISA showed that the levels of ERK1/2, JNK, p38 MAPK, p-ERK1/2, p-JNK, and p-p38 MAPK in lungs of mice in C57BL/6 asthma group were significantly increased compared with those in control group. Compared with those in C57BL/6 asthma group, levels of the above indexes in lungs of mice in TLR2$^{-/-}$ asthma group were significantly decreased, but they were still higher than those in normal group. The above change trends of proteins were further confirmed via immunohistochemical results (Figure 4).

Discussion

TLRs play important roles in innate immunity and acquired immunity, which can balance the immune response and induce the immune tolerance. Among them, TLR2 is the most widely-expressed member in TLR family on the surface of immune cells, such as macrophages, neutrophils, monocytes and dendritic cells. In recent years, it has been found that TLR2 is also expressed in lymphocytes, which is confirmed in both human and mouse. It has been clear that the occurrence or acute exacerbation of asthma is associated with the infection of a variety of pathogens[15,16], so it is speculated that the occurrence or acute exacerbation of asthma after pathogen infection is closely related to the TLR2-mediated intracellular signal transduction mechanism. However, the role of TLR2 in the occurrence and development of asthma is unclear in existing experimental research results. In this experiment, in order to confirm whether TLR2 is involved in regulating the development process of experimental asthmatic airway inflammation, the most commonly used method was applied. Mice were sensitized and stimulated with OVA as an allergen to establish the asthmatic mouse model. TLR2$^{-/-}$ mice and genetically matched C57BL/6 mice were used as objects of study. After stimulation via OVA atomization for 7 d, mice in C57BL/6 asthma model showed significant pruritus in the head and face and deepened and accelerated breathing, and they were quiet and moved less or excited and restless, with manifestations of acute exacerbation of asthma, such as back arching, forelimb raising and gatism. This showed that the asthma model was successfully established. Mice in C57BL/6 asthma group, TLR2$^{-/-}$ asthma group and control group were taken as the research objects. The experimental results showed that the histopathological inflammatory manifestations of mice in TLR2$^{-/-}$ asthma group, and the

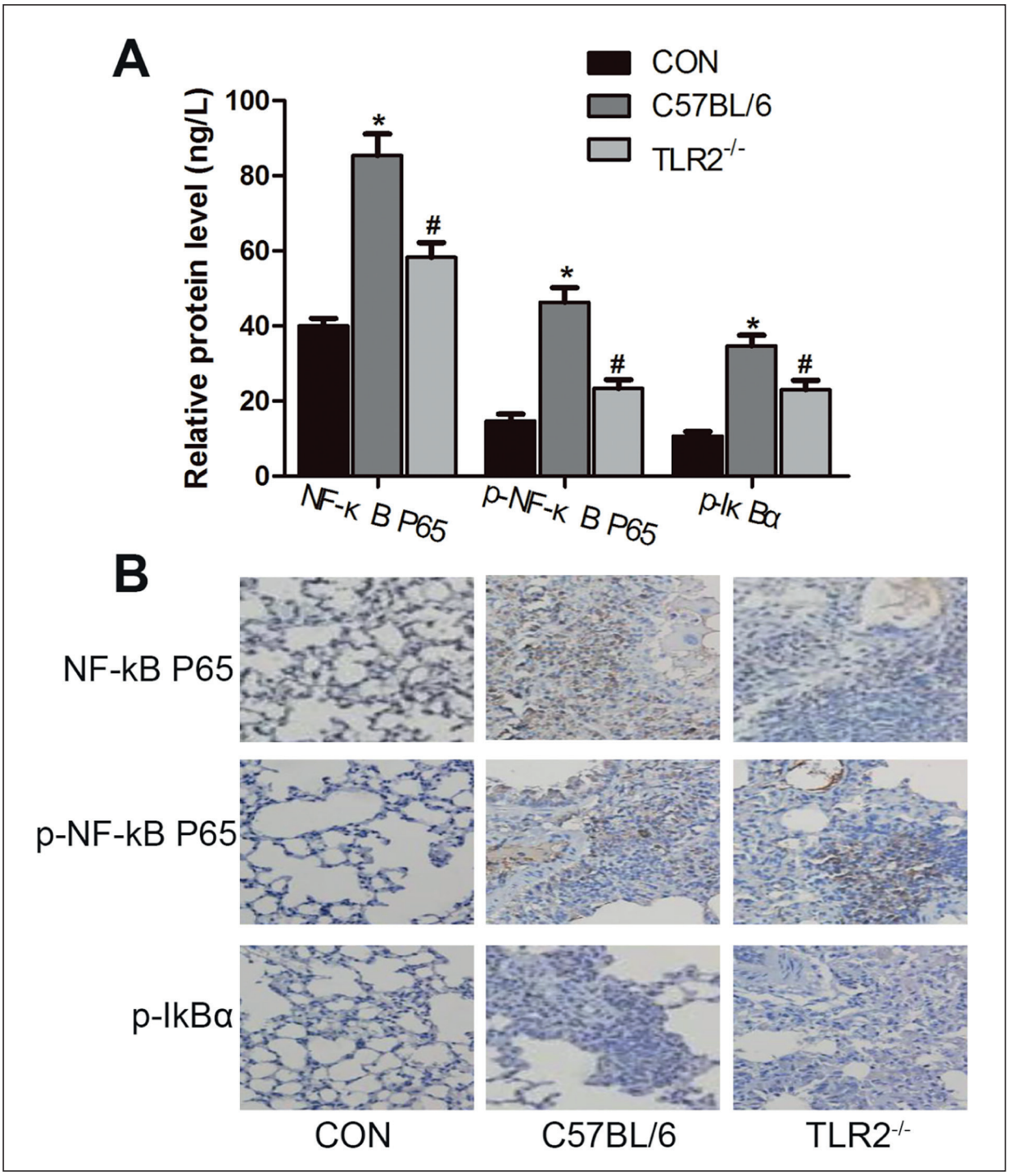

Figure 3. Effect of TLR2 knockout on NF-κB signaling pathway. ***(A)*** Analysis of the activity of NF-κB P65, p-NF-κB P65, p-IκBα by ELISA. ***(B)*** The representative images of immunohistochemistry (Magnification: 100 ×). $^{*}p < 0.05$ *vs.* CON group, $^{\#}p < 0.05$ *vs.* C57BL/6 group.

mRNA expressions of inflammatory cytokines (IL-4, IL-5, and IL-13) in BALF and lung tissues were significantly alleviated or decreased compared with those of mice in C57BL/6 asthma group, indicating that TLR2 is involved in the development process of airway inflammation of asthmatic mice induced by OVA sensitization and stimulation. After TLR2 gene knockout, histological examination showed that the eosinophilic inflammation of airway of asthmatic mice was

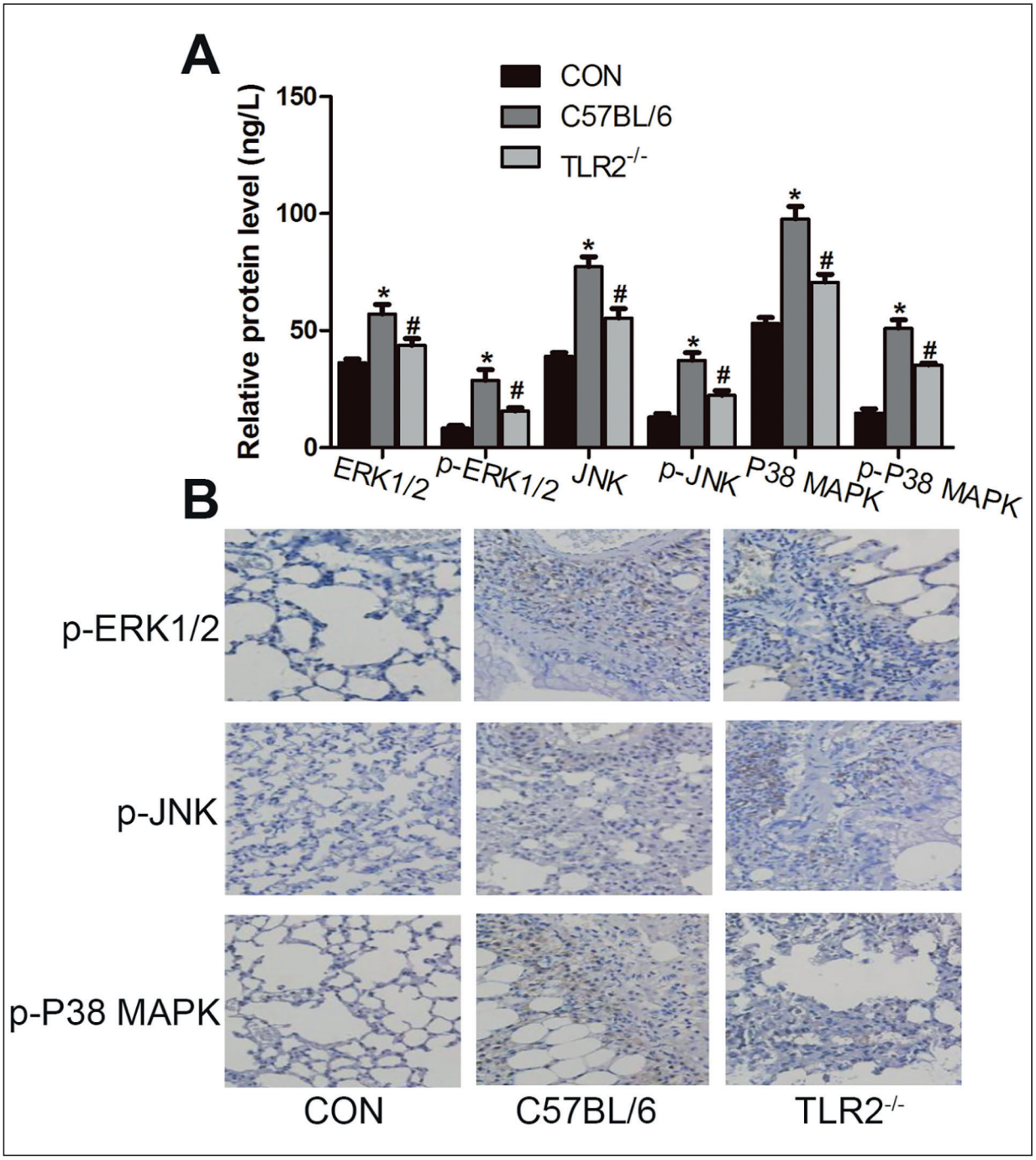

Figure 4. Effect of TLR2 knockout on MAPK signaling pathway. ***(A)*** Analysis of the activity of ERK1/2, JNK, P38 MAPK, p-ERK1/2, p-JNK and p-P38 MAPK by ELISA. **(B)** The representative images of immunohistochemistry (Magnification: 100×). $^{*}p < 0.05$ *vs.* CON group, $^{\#}p < 0.05$ *vs.* C57BL/6 group.

significantly alleviated, and the mucus secretion was reduced. Cytological examination showed the decreased eosinophils, lymphocytes, etc., but increased macrophages in BALF. Finally, the expression levels of TH2 cytokines (IL-4, IL-5, and IL-13) in lung tissues were decreased. Therefore, TLR2 knockout can relieve the OVA-induced airway inflammation of experimental asthma. It has been reported in clinical studies that in the acute attack of asthma, TLR2 mRNA expressions in the airway epithelial cells and outer layer of large and small airways in patients with fatal asthma

are significantly higher than those in control group[17]. In another clinical study[18], the TLR2 mRNA expression level was detected via RT-PCR in sputum specimens induced by isotonic saline. We showed that the TLR2 mRNA expression level in asthma patients in acute attack or stable period was significantly upregulated compared with that in control group, and the level was higher in acute attack. The TLR2 mRNA level in patients with virus infection detected was higher than that in patients without virus detected, suggesting that TLR2 signal transduction plays an important role in the regulation of airway inflammation induced by viral infection, which activates the acquired immune response by the innate immune response pathway, linking the innate and acquired immune systems.

Duan et al[19] thought that inhibiting the phosphatidylinositol-3 kinase (PI3K) signaling pathway in asthmatic mouse model can suppress the production of TH2 factor, eosinophil infiltration, mucus production and airway hyper-responsiveness, which may be a potential therapeutic target for allergic airway inflammation. Their data also showed that inhibiting the ERK signaling pathway may have a potential therapeutic effect on allergic airway inflammation, and the inhalation of p38-α MAPK antisense oligonucleotide (p38-α-ASO) may have a potential therapeutic effect on asthma and other allergic lung diseases. Chiou et al[20] found that Der p2 binds to TLR2 in primary cultured human arterial smooth muscle cell (ASMC), activating the MyD88 signaling pathway, increasing the NF-κB and ERK1/2 activation and inducing the high expression of pro-inflammatory factors. Der p2 can increase the ERK1/2 phosphorylation and e-Fos expression via stimulating ASMC, thus it is inferred that Der p2 induces asthma through the MyD88 signaling pathway. In addition, Kim et al[21] and Agrawal et al[22] indicated that STAT3 and JNK are associated with airway inflammation in asthmatic mouse model. In order to further explore the anti-inflammatory response mechanism in TLR2$^{-/-}$ asthmatic mouse model induced by OVA sensitization and stimulation, namely the role of TLR2 in the development process of asthmatic airway inflammation, the expressions of NF-κB p65, p-NF-κB p65, p-IκBα, ERK1/2, JNK, p38 MAPK, p-ERK1/2, p-JNK, and p -p38 MAPK in lung tissues of mice were detected via ELISA and immunohistochemical method. Results of the study showed that the expressions of the above proteins in lungs of mice in C57BL/6 asthma group were significantly increased, while those in TLR2$^{-/-}$ asthma group were significantly decreased compared with those in C57BL/6 asthma group; the differences were statistically significant. Our experimental findings indicate that the pro-inflammatory effect of TLR2 is related to the NF-κB and MAPK signaling pathways.

Conclusions

We suggest that TLR2 is involved in the occurrence and development of experimental asthmatic airway inflammation. TLR2 gene knockout in asthmatic mice can alleviate the airway inflammation, whose mechanism may be that the allergic airway inflammation of asthmatic mice is alleviated through inhibiting NF-κB and MAPK signaling pathways. TLR2 antagonists may be one of the ways to prevent the allergen from inducing the airway hyper-responsiveness and prevent its developing into asthma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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