

JNK–TLR9 signal pathway mediates allergic airway inflammation through suppressing melatonin biosynthesis

Abstract: Toll-like receptors (TLRs) play pivotal role in the pathogenesis of allergic airway diseases such as asthma. TLR9 is one of the most extensively studied TLRs as an approach to treat asthma. In this study, we investigated the role of TLR9 in the allergic airway inflammation and the underlying mechanism. Wild-type (WT) mice and TLR9^{−/−} mice were sensitized and challenged with OVA to establish allergic airway disease model. We found that the expression of TLR9 was elevated concomitantly with airway inflammation post-OVA challenge, and TLR9 deficiency effectively inhibited airway inflammation, including serum OVA-specific immunoglobulin E (IgE), pulmonary inflammatory cell recruitment, mucus secretion, and bronchoalveolar lavage fluid (BALF) inflammatory cytokine production. Meanwhile, the protein expression of hydroxyindole-o-methyltransferase (HIOMT) in lung tissues, the level of melatonin in serum, and BALF were reduced in OVA-challenged WT mice, while these reductions were significantly restored by TLR9 deficiency. Additionally, we showed that although TLR9 deficiency had no effect on OVA-induced phosphorylation of JNK, inhibition of JNK by specific inhibitor SP600125 significantly decreased OVA-induced expression of TLR9, suggesting that JNK is the upstream signal molecular of TLR9. Furthermore, SP600125 treatment promoted resolution of allergic airway inflammation in OVA-challenged WT mice, but not further ameliorated allergic airway inflammation in OVA-challenged TLR9^{−/−} mice. Similarly, SP600125 significantly restored the protein expression of HIOMT and the level of melatonin in OVA-challenged WT mice, while such effect was not further enhanced by TLR9 deficiency. Collectively, our results indicated that JNK–TLR9 signal pathway mediates allergic airway inflammation through suppressing melatonin biosynthesis.

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Introduction

Prolonged or nonresolving inflammation underpins the pathogenesis of allergic airway disease [1]. Numerous factors are suggested to contribute to the development or persistence of allergic airway inflammation, of which Toll-like receptors (TLRs) are the most prominent factors because of their role as the host's first line of defense against invading pathogens and aeroallergens [2, 3]. However, the underlying mechanism how TLRs regulate allergic airway inflammation is little known.

TLRs are reported to mediate the recognition of pathogen-associated molecular patterns (PAMPs) and play a critical role in both innate and adaptive immunity [4]. Till now, by means of gene knockout or agonists, considerable effort has been devoted to reveal the potential immunomodulatory effect of TLRs [5, 6]. Despite structural similarities, TLRs are demonstrated to cause different immune effects. For example, TLR3 and TLR4 were considered to contribute to the initiation or propagation of inflammation [7, 8], while TLR7, TLR8, and TLR9 exhibit surprising immunoregulatory activity [6, 9]. Therefore,

modulators have been developed to target TLRs for the therapeutic potential, with a particular focus on TLR9. CpG oligodeoxynucleotides, which are TLR9 ligands, have been used in the treatment of allergic airway diseases such as asthma in animal models [10] and even in placebo-controlled phase II study [9, 11, 12]. Nevertheless, achieving inflammation control is still an important goal in the management of allergic airway disease. Our previous study has suggested that inactivation of JNK decreases the expression of TLR9 and in turn inhibits OVA-induced allergic airway inflammation [13]. However, the mechanism how TLR9 regulates allergic airway inflammation is still lacking, and characterization of TLR9-mediated signaling might provide alternative approaches of therapeutic manipulation.

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized by the pineal gland or extra-pineal tissues that exerts various biological effects, especially anti-inflammatory and immunomodulatory functions [14, 15]. Melatonin is synthesized from tryptophan (Trp) by a highly conserved pathway involving the conversion of serotonin successively by two key enzymes such as N-acetyltransferase (NAT)

and HIOMT [16]. Recent studies have demonstrated that exogenous melatonin inhibits mucus secretion and reduces pro-inflammatory cytokine production accompanying by decreasing MAPK phosphorylation in asthmatic animals [17]. Moreover, a considerable amount of evidence shows that exogenous administration of melatonin is able to modulate of TLR-mediated inflammation [18–21]. However, the cross talk between the TLRs system and endogenous melatonin in allergic airway disease has not yet been elucidated.

This study was aimed to investigate the underlying mechanism how TLR9 regulates allergic airway inflammation, and reveal whether TLR9-mediated signaling has specific effect on melatonin biosynthesis during allergic airway diseases.

Materials and methods

Animal

Female C57BL/6 mice (Shanghai Laboratory Animal Center) and TLR9-deficient (TLR9^{-/-}) mice on C57BL/6 background (kind gifts from Dr. ZG. Tian, Institute of Immunology, School of Life Sciences, University of Science and Technology of China) were housed under specific pathogen-free conditions. All animal experiments were in accordance with the Committee on the Ethics of Animal Care and Use of University of Science and Technology of China.

Experiment protocols and treatment

The protocol used to establish the model of allergic airway disease in mice was according to the previous report [22]. Briefly, 6- to 8-week-old mice were divided into six groups: wild-type (WT) control, WT-OVA, WT-OVA-SP (SP600125), TLR9^{-/-} control, TLR9^{-/-}-OVA, and TLR9^{-/-}-OVA-SP. The mice were sensitized by intraperitoneal injection of 10 µg OVA (Sigma, St. Louis, MO, USA) complexed with 1 mg potassium aluminum sulfate (Sangon Biotech, Shanghai, China) in 0.5 mL of saline on day 0, then challenged for 30 min per day with 1% aerosolized OVA on days 14–17. Control mice were saline-sensitized and challenged with nebulized saline solution. Two hours before allergen challenge, two groups of mice (WT-OVA-SP and TLR9^{-/-}-OVA-SP) were injected intraperitoneally with 30 mg/kg SP600125. All mice were sacrificed 24 hr after the last challenge, the left lung was subjected to perform bronchoalveolar lavage and subsequent differential cell counting and ELISA analysis, and the right lung of each mouse was used for histopathological analysis and further Western blotting assays ($n = 6$ per group).

Bronchoalveolar lavage fluid (BALF) and cellular analysis

Immediately after sacrifice, bronchoalveolar lavage was performed thrice by lavage the left lung with 0.5 mL of ice-cold PBS. The collected BALF was centrifuged at 700 g at 4°C for 5 min, and 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 cytopins of BALF samples with Wright Stain solution (Sigma). At least 200 cells were counted for each mouse.

Histological analysis

After the BALF samples were collected, the right lung tissues of each mouse were fixed in 4% paraformaldehyde. Subsequently, the paraffin-embedded tissues were sectioned into 5 µm and stained with hematoxylin/eosin (H&E) or periodic acid-Schiff (PAS) to analyze the inflammation and mucus productions, respectively. The quantitative analysis of peri-bronchial inflammation in H&E-stained lung slices was performed by assessing the number of rings of inflammatory cells around bronchial [1], and the mucus productions quantification was accomplished by dividing the number of PAS⁺ cells in the airway by the perimeter of the basement membrane (Pbm) [5].

Enzyme-linked immunosorbent assay (ELISA)

The levels of IFN-γ in the BALF and OVA-specific IgE in the serum were measured by ELISA using specific kits from Cusabio (Wuhan, China), and the levels of IL-4 and IL-13 in BALF were measured by ELISA kits from Cloud-Clone Crop (Wuhan, China) according to the protocols from the manufacturer. The detection limit for IL-4, IL-13, and IFN-γ was 3.1 pg/mL, 3.5 pg/mL, and 3.9 pg/mL, respectively. We used the ELISA kit from Cusabio (Wuhan, China) for the measurement of melatonin in serum and BALF, and this assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to melatonin has been precoated onto a microplate. After adding 50 µL each of dilutions of standard, blank, and samples, a competitive inhibition reaction is launched between biotin-labeled melatonin and unlabeled melatonin with the precoated specific melatonin antibody. The incubation is stopped by washing the unbound conjugate three times. Next, avidin conjugated to horseradish peroxidase is added to each well and incubated for 30 min. The amount of HRP conjugate is reverse proportional to the concentration of melatonin in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of melatonin in the sample. The minimum detectable dose of melatonin is less than 4.94 pg/mL.

Western blotting analysis

The lung tissue was homogenized (1/5 w/v) using a homogenizer with RIPA reagent containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phosphatase inhibitor PhosSTOP (Roche). Protein concentration was determined by the microbicinchoninic acid assay (BCA, Pierce). Equal amounts of proteins (25 µg/lane) were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated with 5% nonfat milk for 1 hr and then with primary antibodies overnight at 4°C. The following primary antibodies were used: p-JNK

(Signaling Technology Inc., Beverly, MA, USA), JNK (Signaling Technology Inc.), TLR9 (Abcam, Boston, MA, USA), NAT (Sigma-Aldrich, St. Louis, MO, USA), HIOMT (Abcam), and GAPDH (KANGCHEN Biotech, Shanghai, China). For quantitative analysis, intensity of protein bands was determined using Image J 1.38× software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using SPSS 19. Data were expressed as means $\pm$ S.D. Statistical comparison of TLR9 expression was performed using independent sample *t*-test. The differences among the other groups were analyzed using one-way ANOVA with a post hoc Bonferroni tests. *P*-values <0.05 were considered to be significant.

Results

To investigate the role of TLR9 in allergic airway disease, WT and TLR9^{-/-} mice were sensitized and challenged with OVA as illustrated in Fig. 1A. We found a significant increase of protein level of TLR9 in OVA-challenged WT mice as compared to that of control mice (Fig. 1B,C). Concomitantly, inflammatory cells recruiting to the peri-bronchial regions (Fig. 1D,E) and PAS⁺ cells surrounding the airway (Fig. 1F, G) were markedly increased in OVA-challenged WT mice as

compared to that of control mice. Interestingly, while challenged with OVA, TLR9^{-/-} mice exhibited a significant decrease of inflammatory cell recruitment (Fig. 1D,E) and airway intensity of PAS staining (Fig. 1F,G) as compared to that of WT mice. Taken together, these results suggested that TLR9 activation is accompanied by airway inflammation, and TLR9 deficiency diminishes OVA-induced allergic airway inflammation.

In view of the potent pro-resolving effect of deletion of TLR9 in murine allergic airway inflammation, we compared the plasma level of OVA-specific IgE, the influx of inflammatory cells into BALF, and Th1 and Th2 cytokine levels from WT mice with those from TLR9^{-/-} mice post-OVA challenge. As we had expected, at day 4 postchallenge, TLR9^{-/-} mice showed significantly lowered OVA-induced IgE level as compared to that of WT mice, although it remained higher than those in the control mice (Fig. 2A). Similarly, the OVA-induced increase of the number of total cells, eosinophils, monocytes, neutrophils, and lymphocytes in BALF of WT allergic mice was completely abolished in TLR9^{-/-} mice (Fig. 2B–F). Furthermore, ELISA analysis showed that OVA challenge led to markedly increased level of IL-4, IL-13 in BALF of WT allergic mice, while such increase was notably attenuated in TLR9^{-/-} mice (Fig. 2G–H). Importantly, the level of Th1 cytokine IFN- γ induced by OVA was also considerably reduced in TLR9^{-/-} mice (Fig. 2I).

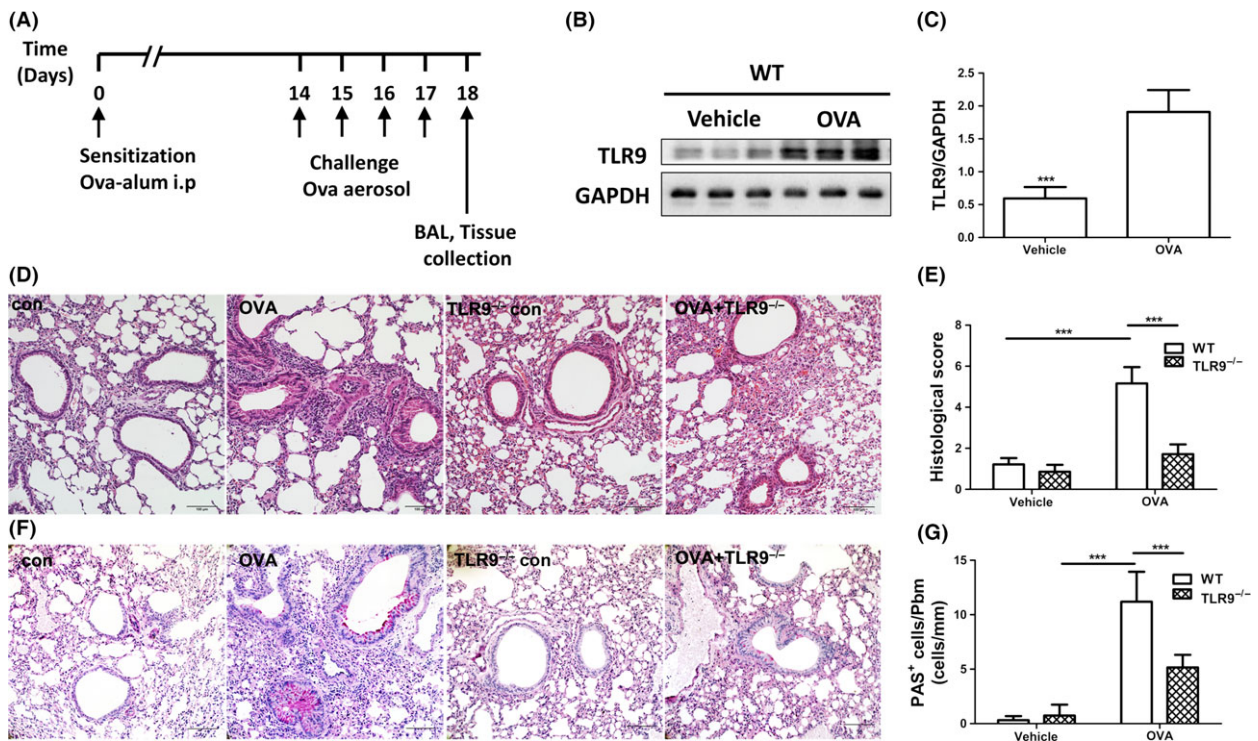


Fig. 1. TLR9^{-/-} mice showed significantly reduced airway inflammation and goblet cell hyperplasia. (A) Experimental protocol of establishing allergic airway diseases model in wild-type mice and TLR9^{-/-} mice. (B) TLR9 protein expression in lung tissues of OVA-challenged WT mice and WT control mice was evaluated by Western blot. (C) The relative level of TLR9 in the lung. (D) Representative H&E-stained sections of lungs from WT and TLR9^{-/-} mice. (E) Histological scoring of lung inflammatory of WT and TLR9^{-/-} mice. (F) Representative periodic acid-Schiff (PAS) staining of lung sections from WT and TLR9^{-/-} mice. (G) Quantification of PAS⁺ cells in the airway of WT and TLR9^{-/-} mice. Gels were representative of three different experiments. Data were expressed as the means $\pm$ S.D. of 6 mice per group, ****P* < 0.001.

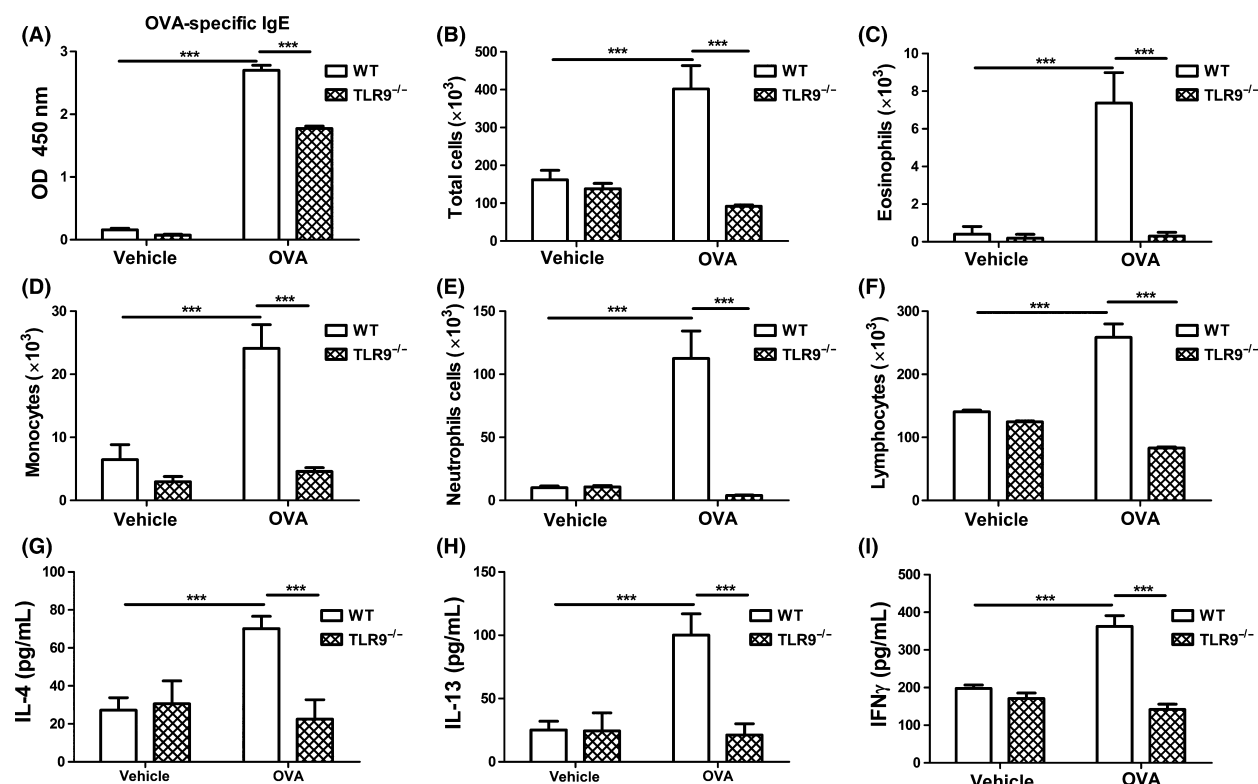


Fig. 2. TLR9 deficiency promoted resolution of allergic airway inflammation. (A) Serum levels of OVA-specific IgE. (B–F) Total and differential cell counts in BALF. (G–I) Productions of IL-4, IL-13, and IFN- γ in BALF. Data were expressed as the means $\pm$ S.D. of 6 mice per group, *** $P < 0.001$.

A question that arises from our study is how TLR9 regulated allergic airway inflammation. Exogenous melatonin has been reported to inhibit the asthmatic responses in a murine model of OVA-induced allergic asthma [23]. Whether endogenous melatonin has the same protective effect and was regulated by TLR9 is still unknown. Our present study demonstrated that OVA challenge obviously reduced the protein expression of HIOMT and the levels of melatonin in serum and BALF (Fig. 3A,B,D,E) in WT mice, while this reduction was significantly attenuated in TLR9^{-/-} mice (Fig. 3A,B,D,E). However, both OVA challenge and TLR9 deficiency had no effect on the protein expression of NAT (Fig. 3A,C). These results suggested that TLR9 is involved in the pathogenesis of allergic airway disease via regulating endogenous melatonin biosynthesis, and this effect was mainly mediated by HIOMT. Additionally, we showed that OVA challenge induced increased activity of phosphorylated JNK in the lung tissues of WT mice, while TLR9 deficiency had no effect on this OVA-induced expression of p-JNK (Fig. 3F, G). Considering that OVA challenge concomitantly increased TLR9 expression as demonstrated above (Fig. 1B,C), it is reasonable to speculate that JNK signaling molecule may be the upstream of TLR9 pathway, or JNK has no effect on TLR9 signal.

Therefore, SP600125, an inhibitor of JNK was used to further clarify the relationship of JNK and TLR9 signal in the controlling of allergic airway inflammation as illustrated in Fig. 4A. The current study demonstrated that

administration of SP600125 significantly decreased the OVA-induced expression of TLR9 in WT mice (Fig. 4B, C). Meanwhile, the OVA-challenged WT mice treated with SP600125 showed marked reduction in inflammatory cells in the peri-bronchial regions (Fig. 4D,E) and fewer airway PAS⁺ cells (Fig. 4F,G). Treatment of OVA-challenged TLR9^{-/-} mice with SP600125 not further ameliorated airway inflammatory responses and histopathological manifestations, which is similar to that of vehicle-treated TLR9^{-/-} mice (Fig. 4D,E,F,G). It was obvious to conclude that JNK–TLR9 pathway mediates allergic airway inflammation.

Moreover, we observed that inhibition of JNK resulted in a dramatic reduction in the level of plasma OVA-specific IgE compared with that in WT vehicle-treated OVA-challenged mice (Fig. 5A). Furthermore, upon OVA challenge, SP600125 treatment led to reduction in the number of total and differential inflammatory cells in BALF (Fig. 5B–F), productions of IL-4, IL-13, and IFN- γ in BALF (Fig. 5G–I) in comparison with that in WT vehicle-treated group. However, treatment of OVA-induced TLR9^{-/-} mice with SP600125 did not further decreased the level of serum OVA-specific IgE, the influx of inflammatory cells into BALF, and Th1 and Th2 cytokine levels, when compared with vehicle-treated TLR9^{-/-} mice (Fig. 5A–I).

Finally, we evaluated whether JNK–TLR9 pathway was involved in the regulation of melatonin biosynthesis. The results showed that the phosphorylation level of JNK in

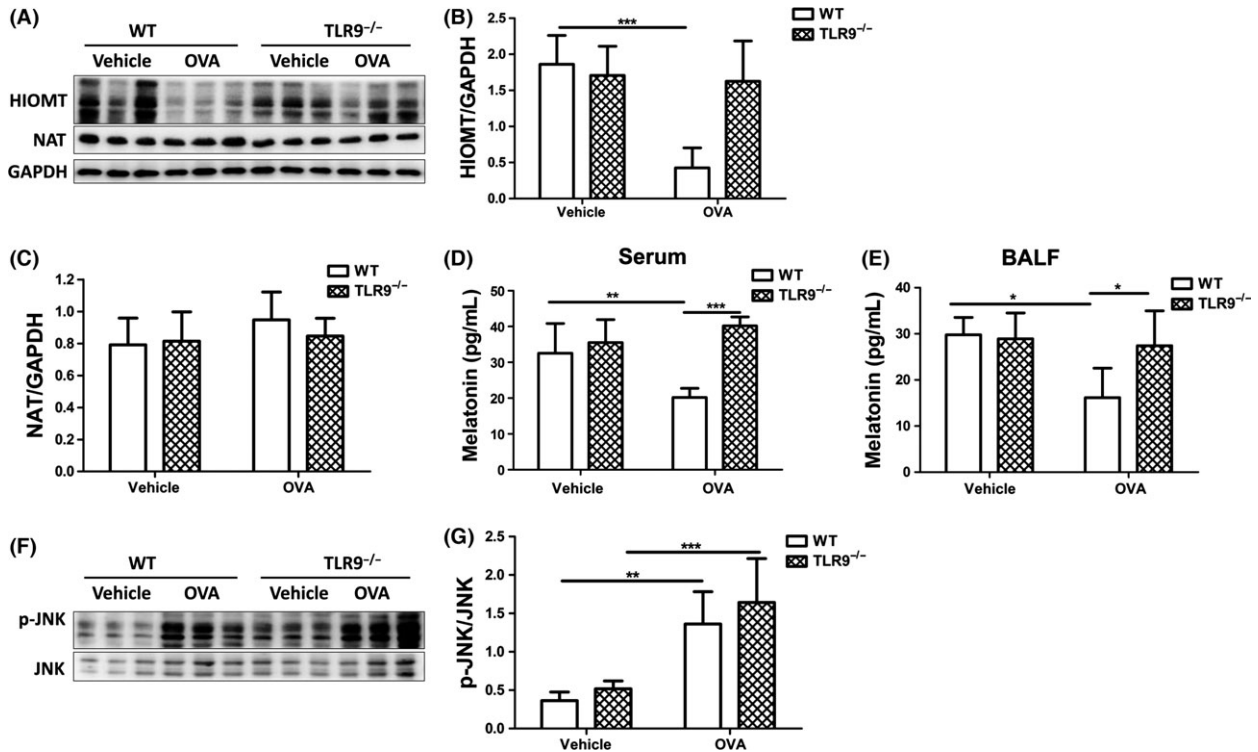


Fig. 3. Deletion of TLR9 restored melatonin biosynthesis while not affected OVA-induced expression of p-JNK. (A) Western blot analysis of the expression of HIOMT, NAT, and GAPDH in the lung tissues. (B, C) The relative levels of HIOMT and NAT in the lung tissues. (D) ELISA analysis of plasma melatonin. (E) ELISA analysis of BALF melatonin. (F) The expression of p-JNK and JNK in the lung tissues. (G) The relative level of p-JNK. Gels were representative of three different experiments. Data were expressed as the means $\pm$ S.D. of 6 mice per group. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$.

both OVA-challenged WT and TLR9^{-/-} mice was significantly decreased by SP600125, but SP600125 not further decreased the expression of p-JNK in TLR9^{-/-} mice as compared to that of WT mice (Fig. 6A,B). Additionally, the expression of HIOMT, the level of melatonin in serum, and BALF in OVA-challenged WT mice were significantly increased by SP600125 as compared to that of vehicle-treated WT mice (Fig. 6A,C,E,F). However, while challenged with OVA, the expression of HIOMT and the level of melatonin in serum and BALF in SP600125-treated TLR9^{-/-} mice were not further increased, which were comparable to that of vehicle-treated TLR9^{-/-} mice (Fig. 6A,C,E,F).

Discussion

The present study demonstrated that, in the mouse model, deletion of TLR9 effectively inhibited the allergic airway inflammation and concomitantly restored the protein expression of HIOMT and the level of melatonin. Although TLR9 deficiency had no effect on OVA-induced phosphorylation of JNK, inhibition of JNK by SP600125 significantly decreased the OVA-induced expression of TLR9. Additionally, SP600125 administration promoted resolution of allergic airway inflammation and restored the biosynthesis of melatonin in OVA-challenged WT mice, while such effect was not further enhanced by TLR9 deficiency. These findings linked for the first time the

activation of JNK–TLR9 signal pathway with the biosynthesis of melatonin and the propagation of allergic airway inflammation.

During the last decade, many researchers have been focused on the role of TLRs in the immune control of the airways inflammation such as asthma [24]. Redirecting an allergic Th2 response by triggering a Th1 response via TLR regulation has been considered as an approach to treat asthma [24]. Till now, TLR9 is one of the most extensively studied TLRs in this respect. However, the role of TLR9 as a novel therapeutic target for allergic airway disease is still controversial. Synthetic TLR9 ligands (CpG ODNs) are shown to be beneficial in various animal models of asthma and even in a number of early human clinical trials [11, 12, 25], while CpG ODNs are also reported to activate TLR9 and cause lung inflammation [26]. In fact, consistent with our previous report and the study of Carmen Maldonado-Berna that high expression of TLR9 is found in asthma [13, 27], the present study demonstrated that pronounced elevated expression of TLR9 was accompanying with lung inflammation exacerbation in WT mice after OVA challenges. However, such OVA-induced inflammation including inflammatory cells around bronchial, mucus production in the airways, IgE level in the plasma, total and differential inflammatory cell infiltration in the BALF, and pro-inflammatory cytokine production such as IL-4 and IL-13 was significantly attenuated in OVA-challenged TLR9^{-/-} mice. Both IL-4 and

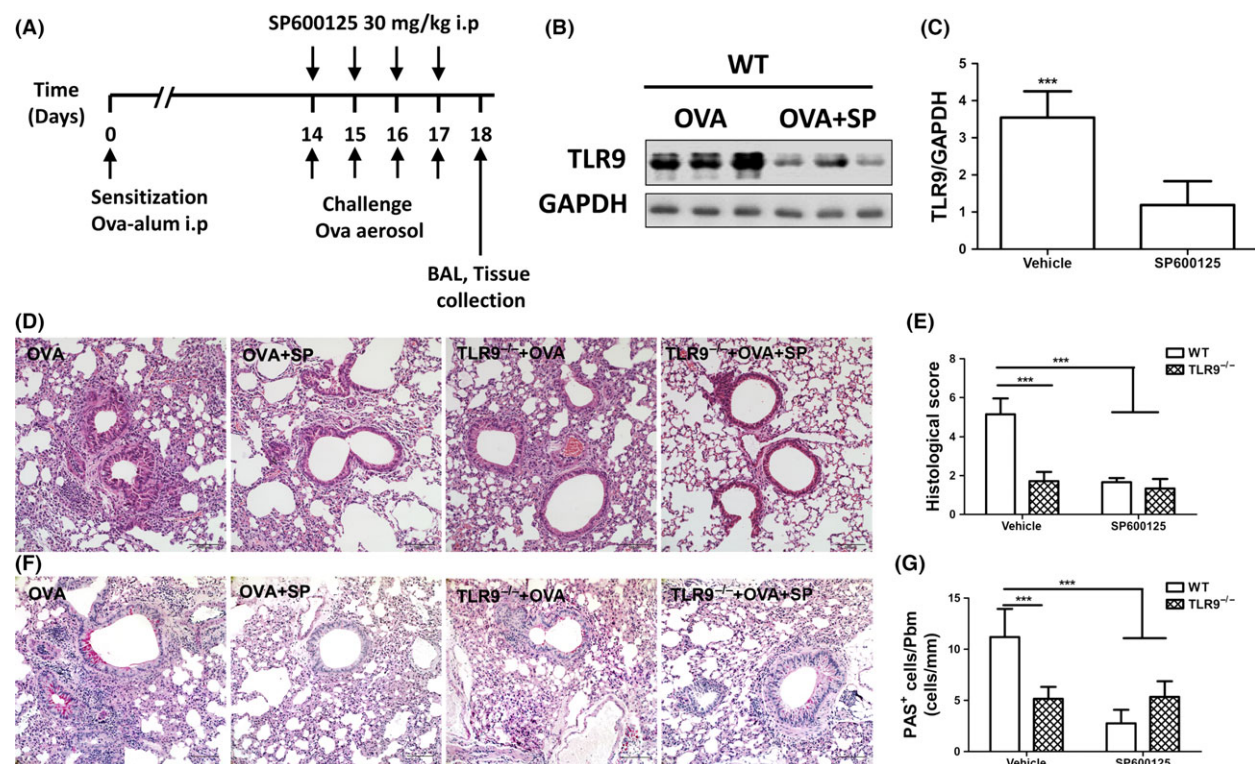


Fig. 4. SP600125 not further ameliorated airway inflammation and goblet cell hyperplasia in OVA-challenged TLR9^{-/-} mice. (A) Experimental protocol of SP600125 treatment in wild-type mice and TLR9^{-/-} mice during establishing allergic airway diseases model. (B) Protein expression of TLR9 in lung tissues of OVA-challenged WT mice treated with SP600125 or not. (C) The relative level of TLR9 in the lung. (D) Representative H&E-stained sections of lungs from OVA-challenged WT and TLR9^{-/-} mice treated with SP600125 or not. (E) Histological scoring of lung inflammatory of SP600125 treated or not treated WT and TLR9^{-/-} mice post-OVA challenge. (F) Representative periodic acid-Schiff (PAS) staining of lung sections from OVA-challenged WT and TLR9^{-/-} mice treated with SP600125 or not. (G) Quantification of PAS⁺ cells in the airway of SP600125 treated or not treated WT and TLR9^{-/-} mice post-OVA challenge. Gels were representative of three different experiments. Data were expressed as the means $\pm$ S.D. of 6 mice per group, ****P* < 0.001.

IL-13 are demonstrated to contribute to airway obstruction, IL-4 is the most important Th2-type cytokine in inducing isotype switching to IgE in B lymphocytes [28], while IL-13 is reported to induce goblet cell metaplasia and mucus hypersecretion [29]. Additionally, a surprising observation in the current study was that the level of IFN- γ , a Th1-type cytokine was significantly increased after OVA challenge and that TLR9 deficiency abrogated this induction. Therefore, our findings suggested that TLR9 acts as a pro-inflammatory factor that mediates the allergic airway inflammation, and deletion of TLR9 does not exert its effect simply by redirecting the Th2 immune response toward a Th1 response.

However, there are few studies concerning the mechanism how TLR9 mediates allergic airway inflammation. Melatonin has been reported to have important immunomodulating effects in allergic diseases [15, 23, 30, 31]. We and others have reported that saliva or serum melatonin levels in patients with asthma were significantly lower than those in healthy controls [32–34]. Exogenous replenishment of melatonin significantly inhibits airway inflammation [17, 30]. In line with these studies, our current study showed that OVA challenge significantly decreased the protein expression of HIOMT as well as the level of melatonin in both serum and BALF, while such effect was abolished by

deletion of TLR9. Although melatonin has been reported to modulate the TLRs-mediated immune responses [19, 20, 35], our study is the first to propose that TLR9 signaling regulate endogenous melatonin biosynthesis. Furthermore, our previous works have shown that exogenous addition of melatonin significantly ameliorates OVA-induced airway inflammation, while blocking the effect of endogenous melatonin by its receptor antagonist luzindole exacerbates OVA-induced airway inflammation as compared to vehicle-treated mice [36, 37], indicating that both exogenous and endogenous melatonin directly regulate airway inflammation. Therefore, our present study suggested that endogenous melatonin synthesis regulated by TLR9 signal may play an important role in the allergic airway diseases.

Activation of JNK is accumulating reported to be involved in the TLRs-mediated airway inflammation [13, 38, 39]. Our present study demonstrated that OVA challenges increased the phosphorylation level of JNK in WT mice lung tissues, while deletion of TLR9 had no effect on OVA-induced activation of JNK. On the other hand, inhibition of JNK by SP600125 significantly decreased the OVA-induced TLR9 expression, which was consistent with our previous study [13]. Therefore, it was quite clear that JNK was the upstream molecular of TLR9 signal from our observation.

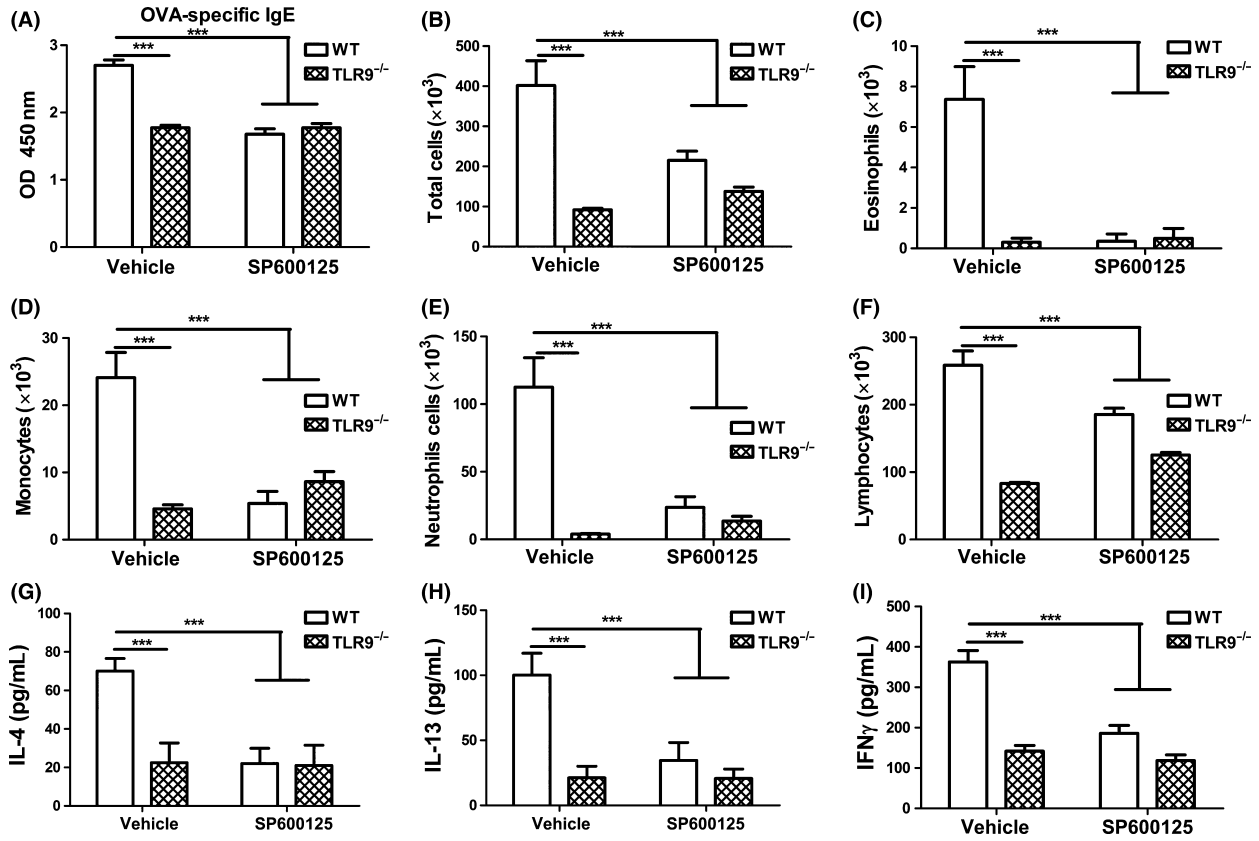


Fig. 5. SP600125 not further promoted resolution of allergic airway inflammation in OVA-challenged TLR9^{-/-} mice. (A) OVA-specific IgE level was detected in serum samples. (B–F) Total and differential cell counts in BALF. (G–I) Cytokine levels of IL-4, IL-13, and IFN-γ in BALF were measured by ELISA. Data were expressed as the means ±S.D. of 6 mice per group, ****P* < 0.001.

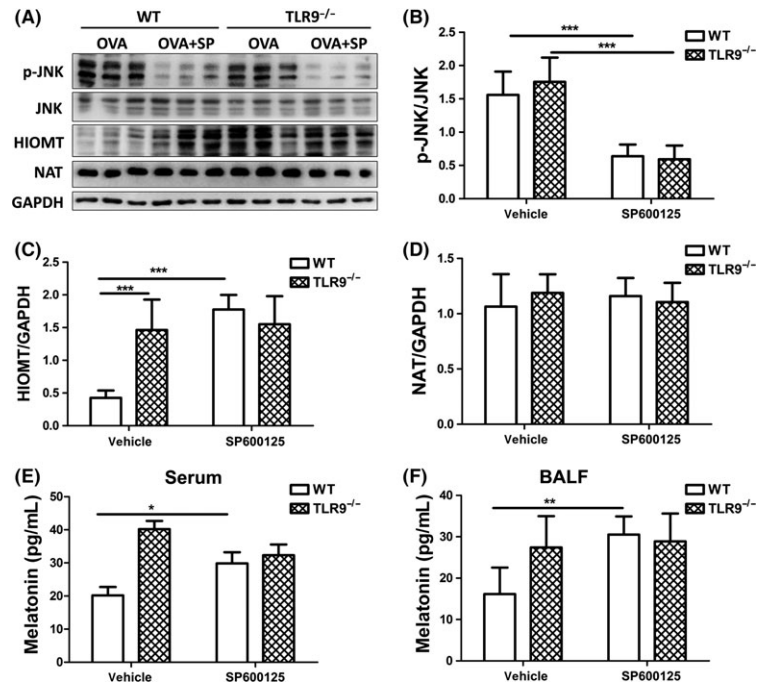


Fig. 6. SP600125 not further restored melatonin biosynthesis in OVA-challenged TLR9^{-/-} mice. (A) Western blot analysis of the expression of p-JNK, JNK, HIOMT, NAT, and GAPDH in the lung tissues. (B–D) The relative levels of p-JNK, HIOMT, and NAT in the lung tissues. (E) Plasma melatonin measured by ELISA. (F) BALF melatonin measured by ELISA. Gels were representative of three different experiments. Data were expressed as the means ±S.D. of 6 mice per group. **P* < 0.05, ***P* < 0.001, ****P* < 0.001.

As a major subgroup of MAPKs, JNK has been frequently reported to participate in allergen-induced experimental airway inflammation and remodeling [40, 41], and specific inhibitor SP600125 was demonstrated to reduce allergic airway inflammation [42, 43]. Consistent with these results, our findings also showed that SP600125 notably inhibited airway inflammation after 4 days continuous OVA challenges. However, we further found that administration of SP600125 in TLR9^{-/-} mice showed analogous effects on lung inflammation during OVA stimulation in comparison with that in vehicle-treated TLR9^{-/-} group. Thus, it is likely that JNK–TLR9 signaling pathway contributed to allergic airway inflammation in OVA-induced animal model.

Additionally, MAPK has been demonstrated to participate in the melatonin biosynthesis [16]. Consistent with the study that inhibition of P38 MAPK has an enhancing effect on melatonin production [44], our study showed that inhibition of JNK by specific inhibitor SP600125 profoundly increased HIOMT protein expression and melatonin level in OVA-challenged WT mice. However, our result was not in line with the report that inhibition of JNK reduced the activity of NAT in pineal [45]. Although we found that SP600125 had no effect on the expression of NAT in lung tissue, this may be due to the mechanism of extra-pineal melatonin synthesis is different from that in pineal. Additionally, we found that SP600125 not further increased the melatonin production in TLR9^{-/-} mice as compared to that of vehicle-treated TLR9^{-/-} mice. Thus, our study suggested that JNK–TLR9 signal pathway regulates melatonin biosynthesis mainly by HIOMT.

In summary, the present results suggested that activation of JNK–TLR9 signal pathway acts as negative signal to regulate the protein expression of HIOMT and subsequent melatonin production in response to OVA challenge, and this endogenous melatonin production controlled by JNK–TLR9 may regulate airway inflammation.

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Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

W.H.M designed and carried out most of the experiments, analyzed the data, and wrote the manuscript. S.Q.Y. and F.L performed the bronchoalveolar lavage. Z.S.H. performed ELISA assays. S.P.T helped to establish the murine model of allergic airway disease. L.Y.J helped to

design the experiment. L.R.Y helped to design and to coordinate the experiment.

References

1. KOLTSIDA O, KARAMNOV S, PYRILLOU K et al. Toll-like receptor 7 stimulates production of specialized pro-resolving lipid mediators and promotes resolution of airway inflammation. *EMBO Mol Med* 2013; **5**:762–775.
2. EDWARDS MR, BARTLETT NW, HUSSELL T et al. The microbiology of asthma. *Nat Rev Microbiol* 2012; **10**:459–471.
3. PALM NW, ROSENSTEIN RK, MEDZHITOV R. Allergic host defences. *Nature* 2012; **484**:465–472.
4. HENNESSY EJ, PARKER AE, O'NEILL LA. Targeting Toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov* 2010; **9**:293–307.
5. CAMATEROS P, TAMAOKA M, HASSAN M et al. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. *Am J Respir Crit Care Med* 2007; **175**:1241–1249.
6. XIRAKIA C, KOLTSIDA O, STAVROPOULOS A et al. Toll-like receptor 7-triggered immune response in the lung mediates acute and long-lasting suppression of experimental asthma. *Am J Respir Crit Care Med* 2010; **181**:1207–1216.
7. REUTER S, DEHZAD N, MARTIN H et al. TLR3 but not TLR7/8 ligand induces allergic sensitization to inhaled allergen. *J Immunol* 2012; **188**:5123–5131.
8. LI DQ, ZHANG L, PFLUGFELDER SC et al. Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4-dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways. *J Allergy Clin Immunol* 2011; **128**:1318–1325.
9. BEEH KM, KANNIEN F, WAGNER F et al. The novel TLR-9 agonist QbG10 shows clinical efficacy in persistent allergic asthma. *J Allergy Clin Immunol* 2013; **131**:866–874.
10. JAIN VV, KITAGAKI K, BUSINGA T et al. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *J Allergy Clin Immunol* 2002; **110**:867–872.
11. CASALE TB, COLE J, BECK E et al. CYT003, a TLR9 agonist, in persistent allergic asthma—a randomized placebo-controlled Phase 2b study. *Allergy* 2015; **70**:1160–1168.
12. KLIMEK L, WILLERS J, HAMMANN-HAENNI A et al. Assessment of clinical efficacy of CYT003-QbG10 in patients with allergic rhinoconjunctivitis: a phase IIb study. *Clin Exp Allergy* 2011; **41**:1305–1312.
13. WU HM, FANG L, SHEN QY et al. SP600125 promotes resolution of allergic airway inflammation via TLR9 in an OVA-induced murine acute asthma model. *Mol Immunol* 2015; **67**:311–316.
14. LIU YJ, MENG FT, WANG LL et al. Apolipoprotein E influences melatonin biosynthesis by regulating NAT and MAOA expression in C6 cells. *J Pineal Res* 2012; **52**:397–402.
15. CALVO JR, GONZALEZ-YANES C, MALDONADO MD. The role of melatonin in the cells of the innate immunity: a review. *J Pineal Res* 2013; **55**:103–120.
16. GUPTA BB, SPESSERT R, VOLLRAH L. Molecular components and mechanism of adrenergic signal transduction in mammalian pineal gland: regulation of melatonin synthesis. *Indian J Exp Biol* 2005; **43**:115–149.
17. SHIN IS, PARK JW, SHIN NR et al. Melatonin inhibits MUC5AC production via suppression of MAPK signaling in human airway epithelial cells. *J Pineal Res* 2014; **56**:398–407.

18. HUANG SH, CAO XJ, WEI W. Melatonin decreases TLR3-mediated inflammatory factor expression via inhibition of NF-kappa B activation in respiratory syncytial virus-infected RAW264.7 macrophages. *J Pineal Res* 2008; **45**:93–100.
19. XIA MZ, LIANG YL, WANG H et al. Melatonin modulates TLR4-mediated inflammatory genes through MyD88- and TRIF-dependent signaling pathways in lipopolysaccharide-stimulated RAW264.7 cells. *J Pineal Res* 2012; **53**:325–334.
20. KANG JW, KOH EJ, LEE SM. Melatonin protects liver against ischemia and reperfusion injury through inhibition of toll-like receptor signaling pathway. *J Pineal Res* 2011; **50**:403–411.
21. NDUHIRABANDI F, LAMONT K, ALBERTYN Z et al. Role of toll-like receptor 4 in melatonin-induced cardioprotection. *J Pineal Res* 2016; **60**:39–47.
22. LOCKE NR, ROYCE SG, WAINSWRIGHT JS et al. Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. *Am J Respir Cell Mol Biol* 2007; **36**:625–632.
23. SHIN IS, PARK JW, SHIN NR et al. Melatonin reduces airway inflammation in ovalbumin-induced asthma. *Immunobiology* 2014; **219**:901–908.
24. BEZEMER GF, SAGAR S, van BERGENHENEGOUWEN J et al. Dual role of Toll-like receptors in asthma and chronic obstructive pulmonary disease. *Pharmacol Rev* 2012; **64**:337–358.
25. VOLLMER J, KRIEG AM. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv Drug Deliv Rev* 2009; **61**:195–204.
26. KNUEFERMANN P, BAUMGARTEN G, KOCH A et al. CpG oligonucleotide activates Toll-like receptor 9 and causes lung inflammation in vivo. *Respir Res* 2007; **8**:72.
27. SANCHEZ-ZAUCO N, Del RIO-NAVARRO B, GALLARDO-CASAS C et al. High expression of Toll-like receptors 2 and 9 and Th1/Th2 cytokines profile in obese asthmatic children. *Allergy Asthma Proc* 2014; **35**:34–41.
28. KASHIWADA M, LEVY DM, McKEAG L et al. IL-4-induced transcription factor NFIL3/E4BP4 controls IgE class switching. *Proc Natl Acad Sci USA* 2010; **107**:821–826.
29. WILLS-KARP M, LUYIMBAZI J, XU X et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998; **282**:2258–2261.
30. MARTINS E Jr, LIGEIRO DE, OLIVEIRA AP et al. Melatonin modulates allergic lung inflammation. *J Pineal Res* 2001; **31**:363–369.
31. SHIN IS, SHIN NR, PARK JW et al. Melatonin attenuates neutrophil inflammation and mucus secretion in cigarette smoke-induced chronic obstructive pulmonary diseases via the suppression of Erk-Sp1 signaling. *J Pineal Res* 2015; **58**:50–60.
32. FEI GH, LIU RY, ZHANG ZH et al. Relationships between melatonin and cortisol and the status of disease in patients with bronchial asthma. *Chin J Tuberc Respir Dis* 2003; **26**:679–682.
33. GUMRAL N, CALISKAN S, OZGUNER F et al. Melatonin levels and enzymatic antioxidant defense system decrease in blood of patients with bronchial asthma. *Toxicol Ind Health* 2009; **25**:411–416.
34. FEI GH, LIU RY, ZHANG ZH et al. Alterations in circadian rhythms of melatonin and cortisol in patients with bronchial asthma. *Acta Pharmacol Sin* 2004; **25**:651–656.
35. KANG JW, LEE SM. Melatonin inhibits type I interferon signaling of toll-like receptor 4 via heme oxygenase-1 induction in hepatic ischemia/reperfusion. *J Pineal Res* 2012; **53**:67–76.
36. QIAN ZX, LIU RY, ZHOU L. Effects of melatonin and its receptor antagonist luzindole on airway inflammation and remodeling in a mouse asthma model. *Chin J Respir Crit Care Med* 2006; **5**:117–121.
37. ZHOU L, QIAN ZX, LI F et al. The effect of melatonin on the regulation of collagen accumulation and matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 mRNA and protein in a murine model of chronic asthma. *Chin J Tuberc Respir Dis* 2007; **30**:527–532.
38. BACHAR O, ADNER M, UDDMAN R et al. Toll-like receptor stimulation induces airway hyper-responsiveness to bradykinin, an effect mediated by JNK and NF-kappa B signaling pathways. *Eur J Immunol* 2004; **34**:1196–1207.
39. ADHIKARY G, SUN Y, PEARLMAN E. C-Jun NH2 terminal kinase (JNK) is an essential mediator of Toll-like receptor 2-induced corneal inflammation. *J Leukoc Biol* 2008; **83**:991–997.
40. HASALA H, ZHANG X, SAARELAINEIN S et al. c-Jun N-terminal kinase mediates constitutive human eosinophil apoptosis. *Pulm Pharmacol Ther* 2007; **20**:580–587.
41. BENNETT BL. c-Jun N-terminal kinase-dependent mechanisms in respiratory disease. *Eur Respir J* 2006; **28**:651–661.
42. NATH P, EYNOTT P, LEUNG SY et al. Potential role of c-Jun NH2-terminal kinase in allergic airway inflammation and remodelling: effects of SP600125. *Eur J Pharmacol* 2005; **506**:273–283.
43. EYNOTT PR, XU L, BENNETT BL et al. Effect of an inhibitor of Jun N-terminal protein kinase, SP600125, in single allergen challenge in sensitized rats. *Immunology* 2004; **112**:446–453.
44. MAN JR, RUSTAEUS S, PRICE DM et al. Inhibition of p38 mitogen-activated protein kinase enhances adrenergic-stimulated arylalkylamine N-acetyltransferase activity in rat pinealocytes. *Endocrinology* 2004; **145**:1167–1174.
45. CHANSARD M, IWAHANA E, LIANG J et al. Regulation of cAMP-induced arylalkylamine N-acetyltransferase, Period1, and MKP-1 gene expression by mitogen-activated protein kinases in the rat pineal gland. *Brain Res Mol Brain Res* 2005; **139**:333–340.