

Role of TLR-NF- κ B Signaling Pathway in Pathogenesis of Allergic Rhinitis in Rats and Intervention with *Tripterygium wilfordii* Hook

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Abstract

To investigate the role of TLR-NF- κ B signaling pathway in pathogenesis of allergic rhinitis (AR) in rats and intervention mechanism with *Tripterygium wilfordii* Hook (TWH). 80 Wistar rats were randomly divided into 4 groups: normal control group (N); allergic rhinitis model group (M); allergic rhinitis+lipopolysaccharide group (LPS); TWH intervention group (TWH). Changes of nasal mucosa tissues and inflammatory cell infiltration were observed by hematoxylin and eosin (HE) staining, while neutrophil and eosinophil were counted under high power microscope. Expression of interleukin-4 (IL-4), interferon gamma (IFN- γ), and immunoglobulin E (IgE) was measured with immunohistochemical analysis. Realtime-PCR and Western-blot were used to evaluate expression of toll-like receptor (TLR4) and nuclear factor κ B (NF- κ B). The results indicated that both group M and LPS developed allergic rhinitis symptoms and distinct inflammatory injury of nasal mucosa. Eosinophil count was significantly higher in M group than in others ($P<0.05$), while neutrophil count was significantly higher in LPS group than in others ($P<0.05$). Expression of IL-4 in M group was significantly higher than group N, LPS, and TWH ($P<0.05$). IFN- γ expression significantly increased in group LPS compared with M, N and TWH ($P<0.05$). The IgE expression was decreased significantly in LPS and TWH group compare with the M group ($P<0.05$). TLR4 and NF- κ B levels were significantly higher in LPS and lower in TWH group than M groups ($P<0.05$). In conclusion, LPS and TWH can regulate the relative balance between TH1 and TH2 in the pathogenesis of AR, and inhibits infiltration of eosinophil and expression of IgE, by regulating the levels of TLR4 and NF- κ B.

Keywords: Allergic rhinitis; TLR-NF- κ B Signaling pathway; TH1/TH2; *Tripterygium wilfordii* hook

Introduction

Pathogenesis of Allergic Rhinitis (AR) is mainly a type I allergic reaction featured by increase of IgE resulted from Th1/Th2 imbalance. Rebalancing the Th1/Th2 is a critical method to prevent and treat AR. With living condition for human beings increasingly improved, morbidity of AR is higher than before. The present theory of health hypothesis suggests that, immune reactions caused by infection (by bacteria or viruses) during period of child development could be effective at decreasing the morbidity of allergic diseases [9,11,16,17]. And therefore, application of properly low or non-infective stimuli may develop protective ability against allergic diseases in our bodies. In another way, we can relatively reduce the expression of Th2 cytokines via increasing the expression of Th1, and rebalance Th1/Th2 in AR pathogenesis [4,10].

Besides Th1/Th2 imbalance above, Toll-like receptor (TLR) is markedly related to pathogenesis of AR. TLR can non-specifically bind the pathogen-relevant molecules, starting signaling transduction and then activating NF- κ B, which indicates that the starting inflammatory responses and immune responses are triggered by the TLR-NF- κ B pathway.

Tripterygium wilfordii is a twining vine in the botanical family Celastraceae, which is featured by its effects of immunosuppression, anti-inflammation, anti-tumor and anti-bacteria. It could treat the

autoimmune diseases effectively, such as asthma and rheumatoid arthritis. *Tripterygium wilfordii* hook (TWH) is the major anti-inflammatory component extracted from *Tripterygium wilfordii*, preserved the immunosuppressive effects of crude drug *Tripterygium wilfordii*, and removed a lot of toxic components [5,7].

In the present study, Rat AR model received LPS was utilized to induce TLR4 and NF- κ B expression in nasal mucosa tissues. Then the expression of Th1 cytokine, IFN- γ and Th2 cytokine, IL-4 was evaluated and ratio between the two cytokines was calculated. All these together were intended to investigate the dual effects of inflammatory reactions and immune responses of TLR-NF- κ B signaling pathway in AR pathogenesis and the potential mechanism of intervention with TWH against allergic diseases.

Materials and Methods

Animals and trial grouping

Eighty healthy Wistar rats, aged 6-8 weeks, clean, weight 120-150g were purchased from laboratory animal center, Fujian medical university. The Wistar rats were divided randomly into 4 groups, 20 rats each group, including 1) Normal control group (N group n=20); 2) Allergic rhinitis model group (M group n=20), no treatment; 3) allergic rhinitis model+LPS 20 μ g/100 μ L (LPS group, n=20) 4) allergic rhinitis model+TWH (30 mg/kg/d) intervention group (TWH group, n=20). The above group was treated once a day and for 7 days. The present study was approved by the Ethics Committee of the Fujian Medical University.

Modeling method

The rats were bred adaptively for 1 week after divided from the animal center. Primary sensitization was then given to all groups except the control group. The mixture of albumin (egg white) (OVA) 0.3 mg and Al(OH)₃ 30 mg (in 1ml saline) was injected to rats on the day 1, 3, 5, 7, 9, 11, and 13 to sensitize them. Then on the day 15, the OVA (10 mg/100 μ l) 50 μ l was applied by dripping into each nostril everyday and for 7 days successively, to stimulate and enhance the sensitization. N group: saline was used instead of OVA for intraperitoneal injection and nostril dripping.

Preparation of nasal mucosa tissues

All rats were injected intraperitoneally with 10% chloral hydrate (3 ml/kg) for anaesthesia after the last nostril dripping. Then their hearts were exposed, and saline and 4% polyoxymethylene were perfused into left ventricle. Nasal cavity was split along with the right middle line, and the nasal mucosa tissue was peeled off for immunohistochemical staining, mRNA and protein detection.

HE Staining

Nasal mucosa samples were fixed with 4% polyoxymethylene, then paraffin embedding within 24 hours. HE staining was employed to observe morphological changes of nasal mucosa tissue under optical microscope, and 5 high power fields for counting of neutrophil and eosinophil.

Immunohistochemical Staining

Immunohistochemical SP staining was used to detect the expression of IL-4, FN- γ , IgE incubating with specific antibodies (IL-4 1:200, IFN- γ 1:200, IgE 1:200, Santa Cruze, USA). PBS buffer instead of primary antibody for negative control. Image-Pro Plus 6.0 image analysis software was used to determine average optical density.

RNA extraction and hemi-quantitative RT-PCR

To evaluate transcriptional situations of the TLR4, NF- κ B mRNA in the samples, series of real time-PCR assay were performed. The specific primers for TLR4 and NF- κ B were listed as following: TLR-4, F5'-CAGAACTTCAGTGGCTGGA-3', R5'-ATTTTGTCTCCACAGCCAC C-3', 180 bp in length; NF- κ B, F5'-GCTCGG CTGAATGAATCTACCA-3', R5'-GTCTCCACGTATTTCCGCAACT-3', 168 bp in length. In parallel, we selected the individual β -actin as the internal control (F 5'-CCTTCCGTGTTCTACCCC-3', R5'-GCCCAAGATGCCCTTCAG-3', 130 bp in length). With an RNAsimple Total RNA Kit (TIANGEN, China), total cellular RNA was prepared. Reverse transcription was performed using SuperScriptTM III First-Strand Synthesis System (Invitrogen, USA) as the manufacturer's protocol. 2 μ l of RT reaction products were amplified by PCR in a volume of 50 μ l under the following conditions: 94°C for 40s, 60°C for 30s and 72°C for 30s. After electrophoresis on 1.5% agarose gel, the gel images of each PCR product were digitally captured with a CCD camera and analyzed with the NIH Imager beta version 2. Relative transcriptional values of each factor in hemi-quantitative RT-PCR are presented as a ratio of the signal value of the specific PCR product and that of the individual β -actin.

Western blot

The sample lysates were separated by 15% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After blocking with 5% defatted milk in PBST (phosphate buffered saline, pH 7.6, containing 0.05% Tween-20) overnight at 4°C, the membranes were incubated with 1:1000 TLR-4 specific monoclonal antibody ab95563 (Cambridge, UK) and 1:1000 goat monoclonal antibody anti-NF- κ B N8523 (Sigma-Aldrich, USA) for 2 h at room temperature, and then incubated with 1:2000 horseradish peroxidase (HRP)-conjugated anti-rat IgG (Santa Cruz, USA). The reactive signals were visualized by ECL kit (PE Applied Biosystems, USA).

Statistic analysis

Quantitative analysis of immunoblot images was carried out using computer-assisted software Image Total Tech (Pharmacia, USA). Briefly, the image of immunoblot was scanned with Typhoon (Pharmacia, USA) and digitalized, saved as TIF format. The values of each target blot were evaluated. All data are presented as the mean \pm SD. Statistical analysis was performed using the t-test. Probabilities of less than 0.05 were considered to be statistically significant.

Results

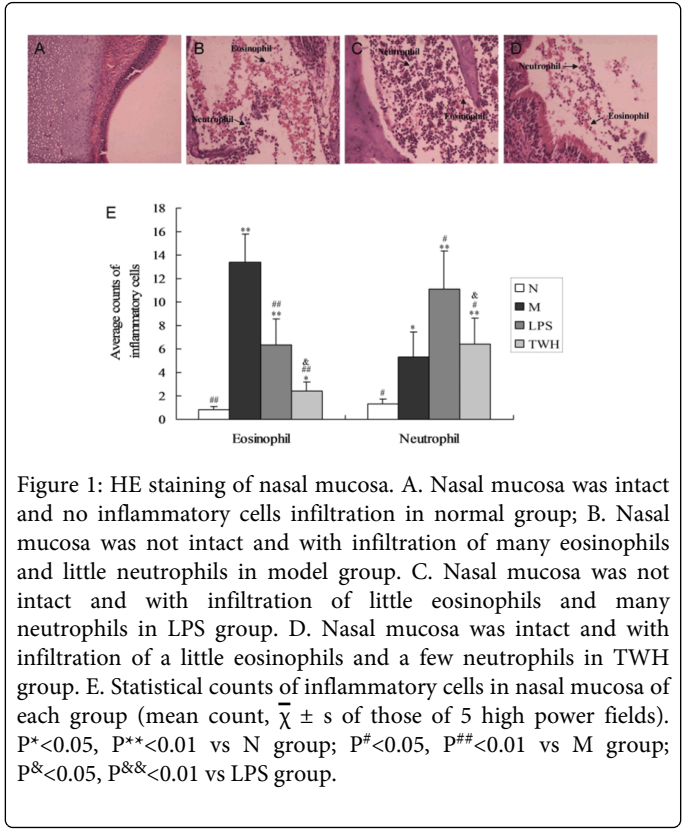
Inflammatory cell counting

Morphological changes of nasal mucosa tissue were observed under the optical microscope. The results indicated that nasal membrane of nasal mucosa tissue was intact in N group, a few plasma cells and eosinophils were observed under mucosa (Figures 1A-1D and Table 1). In model group, the nasal mucosa tissue was swelled, structure loosed, cilia lodged and lost, mucous gland proliferated, a lot of inflammatory cells, infiltrated under mucosa and nostril. After treatment with LPS, LPS group was infiltrated by massive neutrophils and lymphocytes. The treatment of the TWH could improve the morphological of mucosa tissue and inflammatory cells, and even achieve the similar status of normal rats (Figure 1E and Table 1).

Expression of IFN- γ , IL-4, and IgE

IFN- γ , IL-4, and IgE were mainly expressed in pseudostratified ciliated columnar epithelium and gland cells in tunica propria of mucosa, with the staining of brown. Image-Pro Plus 6.0 image analysis software was used to determine average optical density of IFN- γ , IL-4, and IgE in all groups.

In Table 2, the mean optical density of IgE was significantly lower in group LPS and TWH than in group M (both $P < 0.01$), which indicated that expression of IgE in AR model was decreased when treated with LPS and TWH. But the IgE level in group LPS and TWH was also higher significantly than N group (both $P < 0.01$). The mean optical density of IFN- γ was significantly higher in group LPS and TWH than in group M ($P < 0.01$ and $P < 0.05$, respectively), which indicated that expression of IFN- γ in AR model was further enhanced after 7 days of LPS and TWH stimulation. The mean optical density of IL-4 was significantly lower in LPS and TWH group than in M group (both $P < 0.01$), but also higher than the group N.



Group	n	Average Count of Eosinophil	Average Count of Neutrophil
N	20	0.81 ± 0.32 ^{###}	1.33 ± 0.41 [#]
M	20	13.41 ± 2.37 ^{**}	5.32 ± 2.16 [*]
LPS	20	6.35 ± 2.18 ^{**} , ^{##}	11.07 ± 3.25 ^{**} , [#]
TWH	20	2.44 ± 0.76 [*] , ^{##} , ^{&}	6.39 ± 2.23 ^{**} , [#] , ^{&}

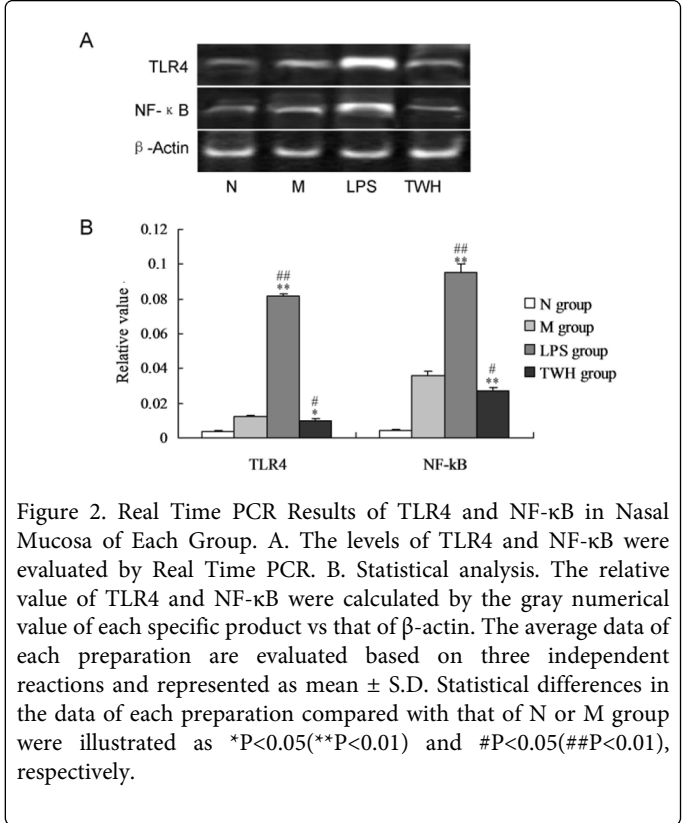
Table 1: counts of inflammatory cells in nasal mucosa of each group (mean count, $\pm s$ of those of 5 high power fields). $P^* < 0.05$, $P^{**} < 0.01$ vs N group; $P^\# < 0.05$, $P^{\#\#} < 0.01$ vs M group; $P^\& < 0.05$, $P^\&\& < 0.01$ vs LPS group.

Group	n	IFN-γ	IL-4	IgE
N	20	0.0937 ± 0.0093 [#]	0.1062 ± 0.0039 [#]	0.0682 ± 0.0015 [#]
M	20	0.1223 ± 0.0100 [*]	0.4339 ± 0.0097 [*]	0.3866 ± 0.012 [*]
LPS	20	0.1950 ± 0.0143 ^{**} , ^{##}	0.1496 ± 0.008 [*] , ^{##}	0.122 ± 0.0047 ^{**} , ^{##}
TWH	20	0.1452 ± 0.0123 ^{**} , ^{##} , ^{&}	0.1209 ± 0.0016 [*] , ^{##}	0.1547 ± 0.0044 ^{**} , ^{##}

Table 2: Average Optical Density of IFN-γ, IL-4, and IgE in Each Group ($\bar{x} \pm s$). $P^* < 0.05$, $P^{**} < 0.01$ vs N group; $P^\# < 0.05$, $P^{\#\#} < 0.01$ vs M group; $P^\& < 0.05$, $P^\&\& < 0.01$ vs LPS group.

mRNA transcription of TLR4 and NF-κB

For the mRNA detection, the rats in the LPS group could transcript the TLR4 and NF-κB effectively, and the level was higher significantly compared with the M group (Figure 2 and Table 3) ($P < 0.01$). Contrarily, the TLR4 and NF-κB level of TWH group was decreased significantly when treating the AR model with WTH. And the level was lower significantly compared with the M group ($P < 0.05$) (Table 3).

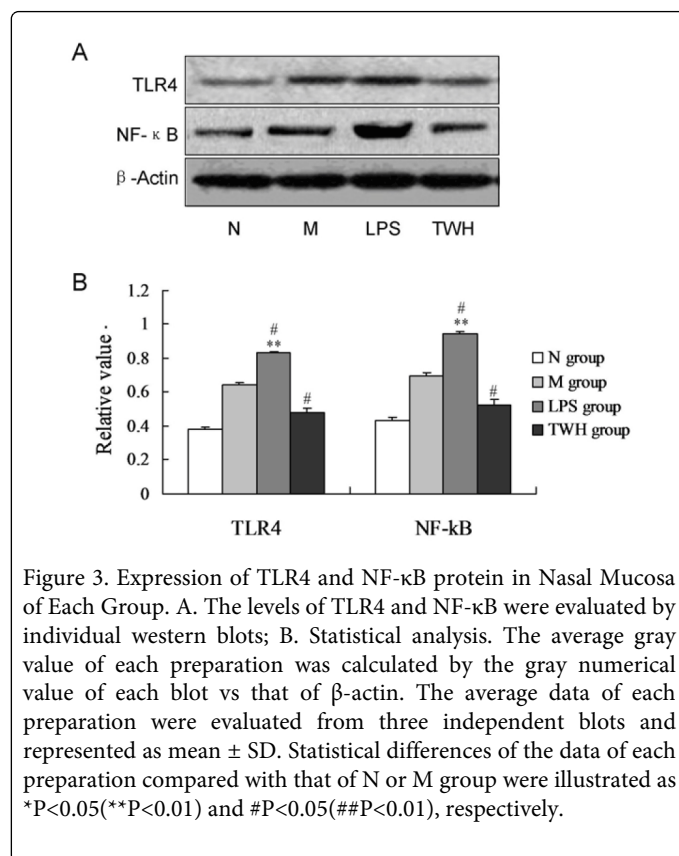


Group	n	TLR4	NF-κB
N	20	0.003925 ± 0.000446 [#]	0.004222 ± 0.0008266 ^{##}
M	20	0.012202 ± 0.0008552 [*]	0.036088 ± 0.0020988 ^{**}
LPS	20	0.081614 ± 0.003031 ^{**} , ^{##}	0.095346 ± 0.005034 ^{**} , [#]
TWH	20	0.009696 ± 0.0014482 ^{**} , [#] , ^{&}	0.027193 ± 0.002127 [*] , [#] , ^{&}

Table 3: Real Time PCR Results of TLR4 and NF-κB in Nasal Mucosa of Each Group ($\bar{x} \pm s$). $P^* < 0.05$, $P^{**} < 0.01$ vs N group; $P^\# < 0.05$, $P^{\#\#} < 0.01$ vs M group; $P^\& < 0.05$, $P^\&\& < 0.01$ vs LPS group.

Expression of TLR4 and NF-κB protein

Western blotting was used to determine relative expression value of TLR4 and NF-κB. Quantity One image analysis software was used to determine mean gray value of TLR4 and internal reference of GAPDH. The results showed that the expression of TLR4 was significantly higher in LPS group compared with other groups (Figure 3 and Table 4) ($P < 0.05$); Expression of NF-κB was also higher significantly in LPS group compared with other groups ($P < 0.01$). The expression of TLR4 and NF-κB was lower significantly compared with other groups, but also higher than the N group ($P > 0.05$) (Table 4).



Group	n	TLR4	NF-κB
N	20	0.419203 ± 0.03823#	0.447752 ± 0.0333546#
M	20	0.616091 ± 0.025088*	0.748121 ± 0.034314*
LPS	20	0.888861 ± 0.032909**,#	0.913275 ± 0.031064**,#
TWH	20	0.517669 ± 0.027594*,#,&	0.615351 ± 0.025816*,#,&

Table 4. Relative Expression Value of TLR4 and NF-κB in Nasal Mucosa of Each Group ($\bar{x} \pm s$). P* <0.05 , P** <0.01 vs N group; P# <0.05 , P## <0.01 vs M group; P& <0.05 , P&& <0.01 vs LPS group.

Discussion

Allergy Rhinitis and its impact on Asthma (ARIA) shows that global morbidity of AR is approximately 20% [1]. It severely affected people's quality of life and work efficiency. Further, AR complicated with asthma will be more difficult to be controlled. Level of serum IgE is usually higher in patients with AR or asthma, and increase of total serum IgE is somewhat correlated with severity of the diseases [12]. To some extent, the increase of serum IgE may be used to evaluate changes or severity of the diseases. Pathogenesis of AR is very complicated, which is now mainly accepted as type I allergic reaction. AR features mainly by increase of IgE in serum and nasal mucosa resulted from Th1/Th2 imbalance [6].

Pathogenesis of AR involves network reactions among a great many inflammatory factors. CD4⁺ T cells play essential role in allergic inflammatory reactions. During inflammation of AR, CD4⁺ T cells will differentiate into Th2 cells that will then produce Th2 cytokines

such as IL-4, 5, 6, 9, 10, 13, and 14. IL-14 (also named IgE) is so special that it is the master cytokine promoting B lymphocytes to produce IgE [13]. With the participation of IL-5 and IL-6, IL-14 can regulate B lymphocytes to transform to produce IgE, mainly mediating humoral immunity and being critical element in pathogenesis of AR. Th1 cells of helper T cells mainly secrete Th1 cytokines such as IL-2, IFN-γ, and TNF-α. IFN-γ can inhibit production of IgE, mediating mainly cellular immunity. IFN-γ and IL-4, the essential Th cytokines in pathogenesis of AR, play the most important role in IgE production [18]. Regulating the balance between Th1 cytokines and Th2 cytokines (mainly IFN-γ/IL-4) is critical to prevent and treat AR.

In the present study, LPS was used to bind TLR4 to form LPS/LBP/CD14 compound that would successively activate IL-1 receptor associated kinase (IRAK), TNF receptor-associated factor 6 (TRAF-6), and MAPKKK family via myeloid differentiation protein (MyD88), which will finally activates NF-κB-inducing kinase (NIK) and IκB kinases (IKK). As a result of above, suppression upon NF-κB will be removed and then, transferred into the nucleus to induce synthesis and release of Th1 cytokines. Our research showed a certain concentration of LPS could bind TLR4 specifically to further activate NF-κB transcription associated genes, stimulating a lot of Th1 cytokines (such as IFN-γ) to be released, resulting in enhanced Th1 immunity and relatively inhibiting the release of Th2 cytokine, IL-4, which means LPS could significantly increase the expression of TLR and promote the activation of NF-κB. Then increased IFN-γ release and reduced IL-4 release will increase ratio of TH1/TH2, correct TH1/TH2 imbalance in pathogenesis of AR, and relatively reduce the level of IgE, finally. This suggests that during the early stage of AR, proper TLR agonist be effective to activate TLR-NF-κB pathway. TLR-NF-κB pathway will rebalance TH1/TH2 and reduce the synthesis of IgE, preventing and treating AR earlier.

To treat AR, cortisol is usually used to control inflammation and Histamine H1 Receptor Antagonists to inhibit release of granular medium by mast cells. But both of them can cause toxic or side effects [8]. However, traditional Chinese drugs are mostly used as assistant for the reason that they have commonly complicated components and their mechanisms are usually unknown. *Tripterygium wilfordii* hook is a twining vine in the botanical family Celastraceae, which is featured by its effects of immunosuppression, anti-inflammation, anti-tumor and anti-bacteria. It is effective for treating autoimmune diseases, such as asthma and rheumatoid arthritis. However, its toxic and side effects should not be neglected [3]. *Tripterygium wilfordii* hook (TWH) is the first kind of Chinese traditional patent medicine developed domestically. It is the major anti-inflammatory component extracted from *Tripterygium wilfordii*, with immunosuppressive effect of crude drug *Tripterygium wilfordii* preserved and a lot of toxic components are removed [14]. TWH is used by ophthalmologists to prevent and treat inflammation in eye after implanting artificial lens and immunorejection response after cornea transplantation. Consistent with other experimentations, TLR4 corresponding mRNA was significantly reduced resulting in decreased expression of protein TLR4 in TWH intervention group. Moreover, the expression of NF-κB was also down-regulated, syntheses of Th cytokines were decreased, level of IgE was significantly lower than that in group M, which remarkably improving inflammatory manifestations in nose.

At present, many other treatments are still expected to be developed, such as specific cytokine regulators, antagonists against inflammatory medium, and gene therapy targeted at pathogenic genes. As a pattern recognizing receptor, TLR plays an important role in

activating innate immunity and inducing adaptive immunity. It is a key factor associating innate immunity and adaptive immunity. It is another way preventing and treating allergic diseases to use specific nontoxic TLR agonistes to stimulate Th1 cells mediated cellular immunity, inhibit function of Th2 cells and correct humoral immunity mainly by Th2 cells, or immune deviation [2,14,15]. As to patients of AR complicated with asthma, in view of down-regulating expression of TLR and inhibiting NF- κ Bp50 transplamtational cytokines, using less toxic and more purified TWH may get treatment costs and toxic and side effects reduced, promoting the development of our national medicine.

Declaration

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