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Original Article

Relationship between toll-like receptor 7 and indoleamine 2, 3-dioxygenase in mice with asthma and the effect of interferon-γ on asthma

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Abstract: Objective: To explore the relationship between toll-like receptor 7 (TLR7) and indoleamine 2, 3-dioxygenase (IDO) in airway intraepithelial dendritic cells of mice with house dust mite asthma and the treatment effects of interferon-γ (IFN-γ) at different concentrations on the asthma. Methods: Fifty mice were randomly assigned into a normal group, model group, and three IFN-γ groups (modeled mice treated with IFN-γ at 100 U/mL, 300 U/mL, and 500 U/mL). Airway responsiveness, pathological changes in lung tissues, in peripheral blood and in bronchoalveolar lavage fluid (BALF), as well as mRNA and protein levels of TLR-7 and IDO in airway intraepithelial dendritic cells of mice were detected. Results: Compared with the normal group, the total number of leukocytes in BALF, the number of eosinophils, neutrophils, lymphocytes and macrophages, and levels of IL-4, IL-13, and IL-22 were elevated, while mRNA and protein levels of TLR-7, IDO declined in the other groups. Compared with the model group, the IFN-γ groups had significantly reduced numbers of leukocytes, eosinophils, and lymphocytes, and declined levels of IL-4, IL-13, and IL-22 in BALF, as well as elevated mRNA and protein levels of TLR-7, and IDO. There was a significant positive correlation between the mRNA and protein expressions of TLR-7 and IDO in airway intraepithelial dendritic cells. Conclusion: The mRNA and protein expression of TLR7 and IDO were down-regulated and positively correlated in airway intraepithelial dendritic cells of mice with house dust mite asthma. Additionally, intervention with IFN-γ at different concentrations can improve asthma in mice.

Keywords: House dust mite asthma, airway intraepithelial dendritic cells, toll-like receptor 7 (TLR7), indoleamine 2, 3-dioxygenase (IDO), IFN-γ

Introduction

Bronchial asthma, a chronic airway inflammatory disease, with a rising incidence globally, it seriously impacts patients' quality of life, and dust mites are the predominant (80%) allergens [1, 2]. The main pathological features of asthma are inflammation and airway hyperresponsiveness. The most potent and effective antigen-presenting cells are airway intraepithelial dendritic cells, and Thl/Th2 cell imbalance is the dominant pathogenesis of asthma [3, 4]. The lack or weakening of immune tolerance in the body will lead to an increase in Th2 cells, thereby leading to airway hyper-responsiveness and inflammation [5-8].

Toll-like receptors (TLRs) are pattern recognition receptors. There are 10 members in TLR family, named TLR1-10 [9]. The activation of TLR7 plays a significant role in the occurrence and development of allergic and autoimmune diseases. The inflammatory cytokines produced by the activation of TLR7 can mediate the Th1-dominant immune response, which corrects the dominance of Th2 cells in asthma patients, thus alleviating asthma symptoms [10-12]. Indoleamine 2, 3-dioxygenase (IDO), a rate-limiting enzyme in the catabolism of tryptophan, is highly expressed in normal immune organs. Inhibition of IDO can lead to an increase in Th2 cytokines and airway eosinophils, thereby promoting airway inflammation. Besides, the metabolites of IDO can enhance immune tolerance and reduce the inflammatory response [13-15].

IFN-γ, as a major cytokine of Th1 cells, shows a regulatory effect on the inflammatory response

after asthma attack [16, 17]. IFN-γ can also promote the production of Th1 cells, inhibit the recruitment of Th2 cells and the release of Th2 cytokines, as well as reduce the production of IL-4, IL-13, and IL-22. IFN-γ is a common inducer of IDO, and this pathway is regulated by a variety of pro-inflammatory cytokines [18-20]. These pro-inflammatory cytokines are regulated by downstream products of the TLR signal transduction pathway, suggesting that IDO may be associated with TLR7. Different concentrations of IFN-γ can affect the expression level of IDO and TLR7 and regulate immune tolerance to various degrees. In this study, a mouse model of house dust mite asthma was established and treated with IFN-γ at 100 U/mL, 300 U/mL, and 500 U/mL, respectively, to observe the airway responsiveness, related inflammatory factors, and expression of TLR7 and IDO, so as to investigate the relationship between TLR7 and IDO and the treatment effects of IFN-γ at different concentrations on asthma.

Materials and methods

Animals

Fifty healthy specific pathogen-free BALB/c female mice (6-8 weeks old, (21±3) g in weight) were purchased from the Experimental Animal Center, and housed at a relative humidity of 70% and a temperature of 25°C. This study was approved by the Ethics Committee of Shandong Provincial Chest Hospital.

Grouping and establishment of the mouse model

The mice were randomly assigned into 5 groups, with 10 mice in each group: normal group (normal mice), model group (mouse model of house dust mite asthma), as well as 100 U/mL, 300 U/mL, and 500 U/mL IFN-γ groups (mouse model of asthma treated with IFN-γ at 100 U/mL, 300 U/mL, and 500 U/mL before challenge, respectively).

The mouse model of house dust mite asthma was established as follows. The mice were sensitized with 0.2 mL mixture of house dust mite antigen and aluminum hydroxide (using a ratio of 20 μg/2 mg) by intraperitoneal injection on the first and tenth day of housing; meanwhile, the same amount of normal saline was given to the normal group. Seven days after the injection, a mixture of 20 μg of house dust mite antigen and 50 μL of normal saline was applied for challenge, once a day for seven consecutive days. The normal group was challenged using 50 μL of normal saline. Mice in the 100 U/mL IFN-γ group inhaled 100 U/mL of aerosol IFN-γ for 30 min before each challenge; mice in the 300 U/mL IFN-γ group and the 500 U/mL IFN-γ group were respectively treated with 300 U/mL IFN-γ and 500 U/mL IFN-γ by the same methods.

Detection of airway responsiveness

Twenty-four hours after the final challenge, the mice were intraperitoneally injected with 100 mg/kg of 1% sodium pentobarbital for anesthesia. Then, the tracheal cannula was inserted and fixed. The mice were placed in a supine position, and the exposed trachea was separated for a subsequent inverted T-shaped incision at the 3-4th cartilage ring. The tracheal cannula was inserted into the trachea and tied. The external jugular vein of mice was then separated for the insert of a venous indwelling needle into the internal jugular vein. Thereafter, the mice were placed in a supine position in a closed plethysmography box for the analysis of lung function, with assisted ventilation by an animal ventilator, with respiratory rate 90 times/min, and tidal volume 5 mL/kg. Lastly, the mice were intravenously administered saline, recorded for the airway resistance as a base value, then sequentially injected with 0.5, 1, 2, 4 and 8 μg of methacholine (MCh), and measured for airway resistance (Raw) and pulmonary dynamic compliance (Cdyn) after each injection,. Every injection was performed when the indicators returned to normal levels. The lung function of mice was evaluated by comparing the changes in Raw and Cdyn in each group.

Collection of peripheral blood, tissue specimens and bronchoalveolar lavage fluid (BALF)

The mice were sacrificed, with chest open and exposure of trachea and lungs. The hilum of the right lung was clamped, and 1.5 mL of 1*PBS was injected from the trachea to lavage the left lung. The lavage was repeated twice, and the recovered lavage fluid was centrifuged at 2,000 rpm and 4°C for 10 min. The supernatant was frozen at -80°C, as well as the right lung. After filtration, 0.1 mL of the lavage fluid was taken

and counted for the white blood cells, so as to calculate the proportion of eosinophils, neutrophils, lymphocytes, and macrophages. The ratio of each kind of cells multiplied by the total number of white blood cells was the number of the corresponding cells in BALF.

H&E staining

The lung tissue of the mice was fixed, dehydrated, waxed, embedded, and serially sectioned into slices of 5 μm in thickness. After a water bath at 45°C, the slices were baked at 60°C for an hour, and dewaxed with the use of xylene. Conventional H&E staining was performed after hydration. The morphology changes of the lung tissues of each group were observed with the use of an optical microscope (XP-330, Shanghai Bingyu, China).

Isolation, culture, and identification of dendritic cells in BALF

The precipitate obtained by irrigating was resuspended in 3 mL of normal saline, centrifuged at 2,000 rpm and 4°C for 10 min, washed for 3 times, and then resuspended and incubated in a Petri dish with RPMI1640 medium for 2 h in an incubator of 5% CO_2 at 37°C. Then, the sample was gently aspirated for supernatant, removed for non-adherent cells, and resuspended in RPMI1640 medium containing 10% fetal bovine serum, with the cell concentration adjusted to 10^5 cells/mL, and cultured suspension cells were collected. Freshly prepared dendritic cells of 10^6 cells/mL were added to 1.5 mL EP tubes, washed with PBS twice at 2,000 rpm, for 5 min each time, and added with 1 μg of FITC-labeled anti-CD80, CD86, and MHCII. Thereafter, 0.2 μg of FITC-labeled IgG solution was added to the 1 μg PE-labeled CD11c control tube, incubated at 4°C for 30 min, washed with 1*PBS twice at 2,000 rpm for 5 min. After the cells were mixed, flow cytometry was used for detection and analysis.

ELISA

Peripheral blood and lung tissue homogenates of mice were taken and tested following the instruction of ELISA kit (Ebioscience, Thermo Fisher, USA). After the dissolution of the standard substance, 100 μL of the washing solution was added to the reaction plate to prepare a standard curve. Then, 10 μL of sample and 40 μL of diluent were added into the sample wells. The coated plate was sealed and incubated at 37°C for 30 min. Thereafter, the sample was washed, dried, added with 50 μL of biotinylated antibody, and incubate at 37°C for 30 min; the sample was again washed, dried, added with 50 μL of chromogenic agent A and 50 μL of chromogenic agent B, gently mixed, and developed at 37°C for 15 min. Immediately after color development, 50 μL of stop solution was added. The OD value at 450 nm of each well was measured by a microplate reader (BioTek Synergy 2, BioTek, USA), and a standard curve was drawn based on the OD value to analyze the contents of IL-4 (ab100770, Abcam, UK), IL-13 (ab79277, Abcam, UK), and IL-22 (ab18499, Abcam, UK) in serum. The results are expressed in pg/mL.

qRT-PCR

Airway intraepithelial dendritic cells of each group were collected and washed twice with 1*PBS. The total cellular RNA was extracted with Trizol to determine the purity and concentration. The RNA sample was inverted into cDNA using a reverse transcription kit (11939823001, Sigma-Aldrich, USA). The qRT-PCR was performed using 1 μL cDNA as a template, 0.5 μL of PCR forward primer, 0.5 μL of PCR reverse primer, 5 μL of (2×) SYBR® Premix Ex Taq™ II, and 3 μL of ddH_2O, with a total reaction system of 10 μL. The qRT-PCR reaction procedures were as follows: pre-denaturation at 95°C for 4 min, followed by 35 cycles at 94°C for 30 sec and 60°C for 30 sec. The dissolution curve was set and the reaction was extended at 72°C for 5 min. The qRT-PCR primers were reduced glyceraldehyde-phosphate dehydrogenase (GAPDH), TLR7, and IDO, which were synthesized by Sangon Biotech, Shanghai, China. See **Table 1**. The mRNA levels of TLR7 and IDO were analyzed using the $2^{-\Delta\Delta Ct}$ method. The formula is as follows: $\Delta Ct = \Delta Ct_{\text{remaining group}} - \Delta Ct_{\text{control group}}$, $\Delta C_t = Ct_{\text{target gene}} - Ct_{GAPDH}$. Ct refers to the number of amplification cycles when the real-time fluorescence intensity reaches the threshold. The experiment was repeated 3 times independently.

Western blot

The airway intraepithelial dendritic cells were washed twice with 1*PBS, lysed on ice, and

Table 1. Primer sequence of qRT-PCR

Name	Sequence
TLR-7	Forward: 5'-ATGTGGACACGGAAGAGACAA-3'
	Reverse: 5'-ACCATCGAAACCCAAAGACTC-3'
IDO	Forward: 5'-TGGCGTATGTGTGGAACCG-3'
	Reverse: 5'-CTCGCAGTAGGGAACAGCAA-3'
GAPDH	Forward: 5'-AGGTCGGTGTGAACGGATTTG-3'
	Reverse: 5'-GGGGTCGTTGATGGCAACA-3'

Note: TLR-7, toll-like receptor 7; IDO, indoleamine 2,3-dioxygenase; GAPDH, glyceraldehyde-phosphate dehydrogenase.

centrifuged at 13,000 rpm for 2 min to collect the supernatant. The BCA kits (P0012S) were from Beyotime, Shanghai, China. Supernatant 160 μL was taken, mixed with 40 μL of 5*SDS loading buffer, then heated in boiling water bath for 10 min, and subjected to SDS-PAGE gel electrophoresis. The protein was then blocked in 5% skim milk at room temperature for 2 h and incubated with primary antibodies at room temperature for 2 h. The primary antibodies were all from Abcam, UK, including rabbit polyclonal antibody TLR7 (ab24184, 1/1,000), IDO (ab74562, 1/1,000), and GAPDH (ab181602, 1/10,000). After that, the protein was washed with TBST 3 times, 5 min for each time, and incubated with secondary antibody goat anti-rabbit IgG (ab6721, 1/2,000, Abcam, UK) at room temperature for 2 h. Then, the protein was again washed with PBS 3 times, for 5 min each time. The Nitrocellulose membrane was placed in a gel imager, overlaid with a developer, photographed by the Bio-Rad image analysis system (The ChemiDoc MP, BIO-RAD, USA), and analyzed for gray value using Quantity One software.

Statistical analyses

Data were processed using SPSS 21.0 (SPSS, Inc, Chicago, IL, USA). The measurement data were expressed as mean ± SD. Comparison among groups was carried out with the use of one-way ANOVA plus pairwise comparison post-hoc Bonferroni test. The difference of $P<0.05$ was considered statistically significant.

Results

Airway responsiveness and pathological changes of lung tissues

The raise of Raw and the decline of Cdyn with increasing concentration of MCh were observed in the normal group, model group, 100 U/mL IFN-γ group, 300 U/mL IFN-γ group and 500 U/mL IFN-γ group (**Figure 1A** and **1B**). With the increasing concentration of MCh, the raise of Raw and the decline of Cdyn were more significant in the other groups as compared with the normal group, (all $P<0.05$). The differences in the raise of Raw and the decline of Cdyn between the model group and IFN-γ groups were not significant (all $P>0.05$). The results showed that the mouse models of house dust mite asthma were successfully constructed, and different concentrations of IFN-γ had no significant effect on the airway responsiveness of mice.

H&E staining was performed on lung tissues of mice (**Figure 1C**). In the normal group, the lung tissue and alveolar structure were clearly visible, and the structure of the alveolar wall was intact, and there was no inflammatory cell infiltration. In the model group, the lung tissue structure was severely damaged; the alveolar wall was significantly thickened, and a large amount of inflammatory cell infiltration developed. In the three IFN-γ groups, the structure of alveolar wall showed different degrees of thickening, and there was obvious inflammatory cell infiltration, but the pathological changes were significantly decreased as compared with the model group.

Inflammatory response

Compared with the normal group, the total number of leukocytes, eosinophils, neutrophils, lymphocytes and macrophages in the BALF of the other groups were elevated (all $P<0.05$). Compared with the model group, the total number of leukocytes, eosinophils, and lymphocytes in BALF of IFN-γ groups were declined (all $P<0.05$), but the differences in the number of neutrophils and macrophages were not significant (both $P>0.05$); similar results were found when comparing the 100 U/mL IFN-γ group with 300 U/mL IFN-γ group and 500 U/mL IFN-γ group. Our results showed that different concentrations of IFN-γ intervention reduced the total number of leukocytes, and number of eosinophils and lymphocytes, but had no effect on the number of neutrophils and macrophages. See **Figure 2A**.

The levels of inflammatory factors IL-4, IL-13 and IL-22 in the peripheral blood of mice were

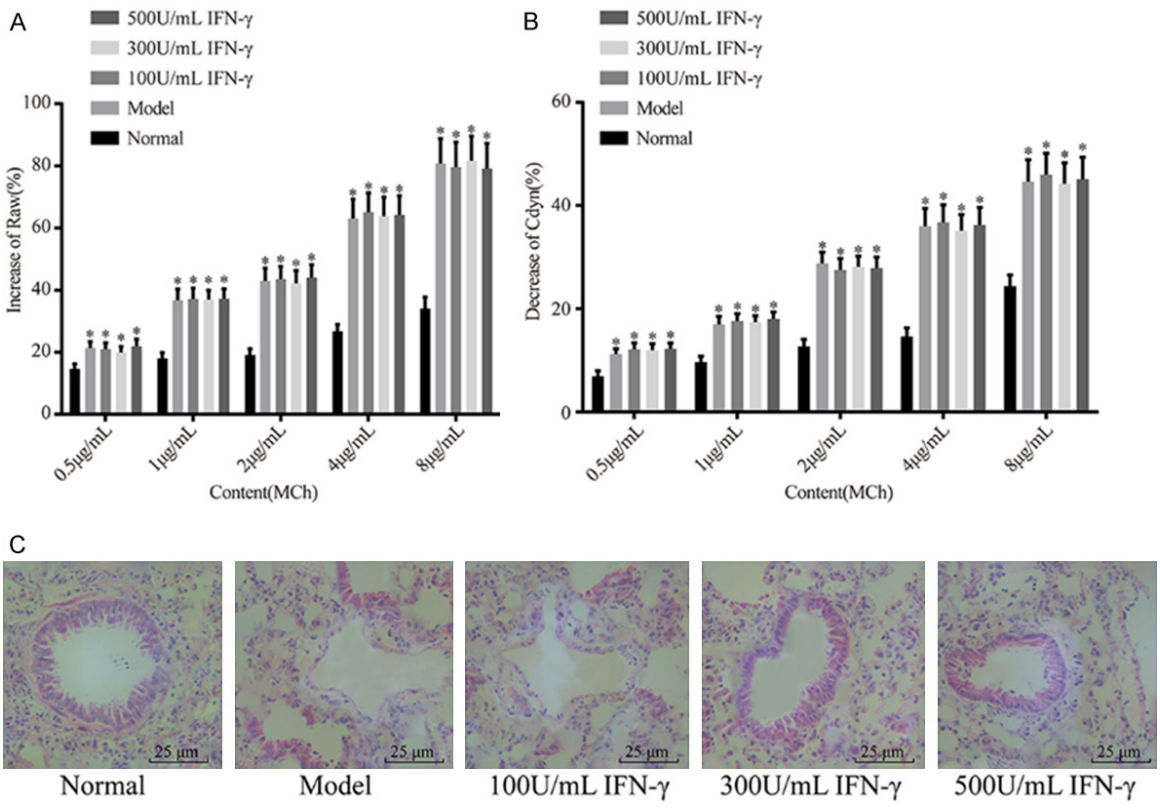

Figure 1. Raw, Cdyn and H&E staining of each group of mice. A: Comparison of the increase in Raw between each group; B: Comparison of the decrease in Cdyn between each group; C: H&E staining of each group (400×). Compared with the Normal group, *P<0.05. Raw: airway resistance; Cdyn: pulmonary dynamic compliance.

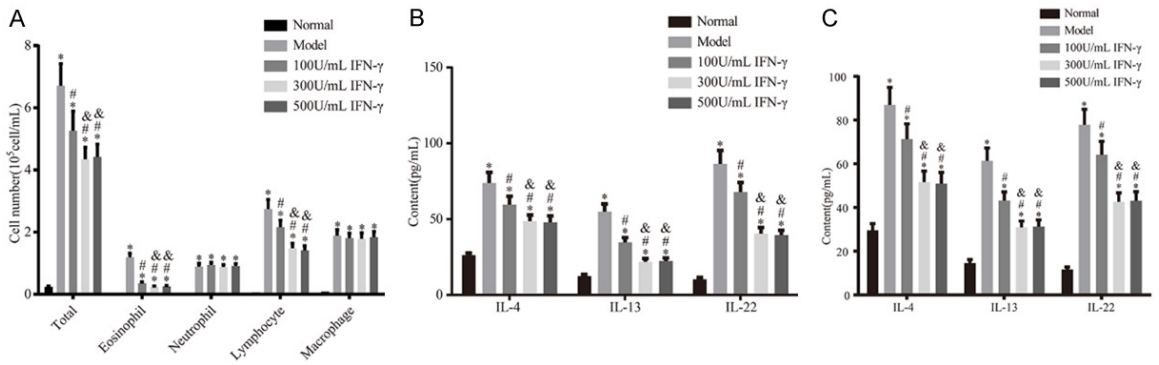

Figure 2. Measure of white blood cells, and inflammatory factors in airway intraepithelial dendritic cells and peripheral blood. A: Classification and count of white blood cells in bronchoalveolar lavage fluid; B: Inflammatory factors in peripheral blood; C: Inflammatory factors in bronchoalveolar lavage fluid. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the 100 U/mL IFN-γ group, &P<0.05. IFN-γ: interferon-γ; IL: interleukin.

detected by Elisa (**Figure 2B**), which showed reduced levels when comparing the normal group with the other groups (all P<0.05). The levels of IL-4, IL-13, and IL-22 in the peripheral blood were decreased in the IFN-γ groups as compared with the model group (all P<0.05). Similar results were found in the 300 U/mL and 500 U/mL IFN-γ groups when comparing with the 100 U/mL IFN-γ group (all P<0.05), while the differences between the 300 U/mL IFN-γ group and the 500 U/mL IFN-γ group were not significant (all P>0.05).

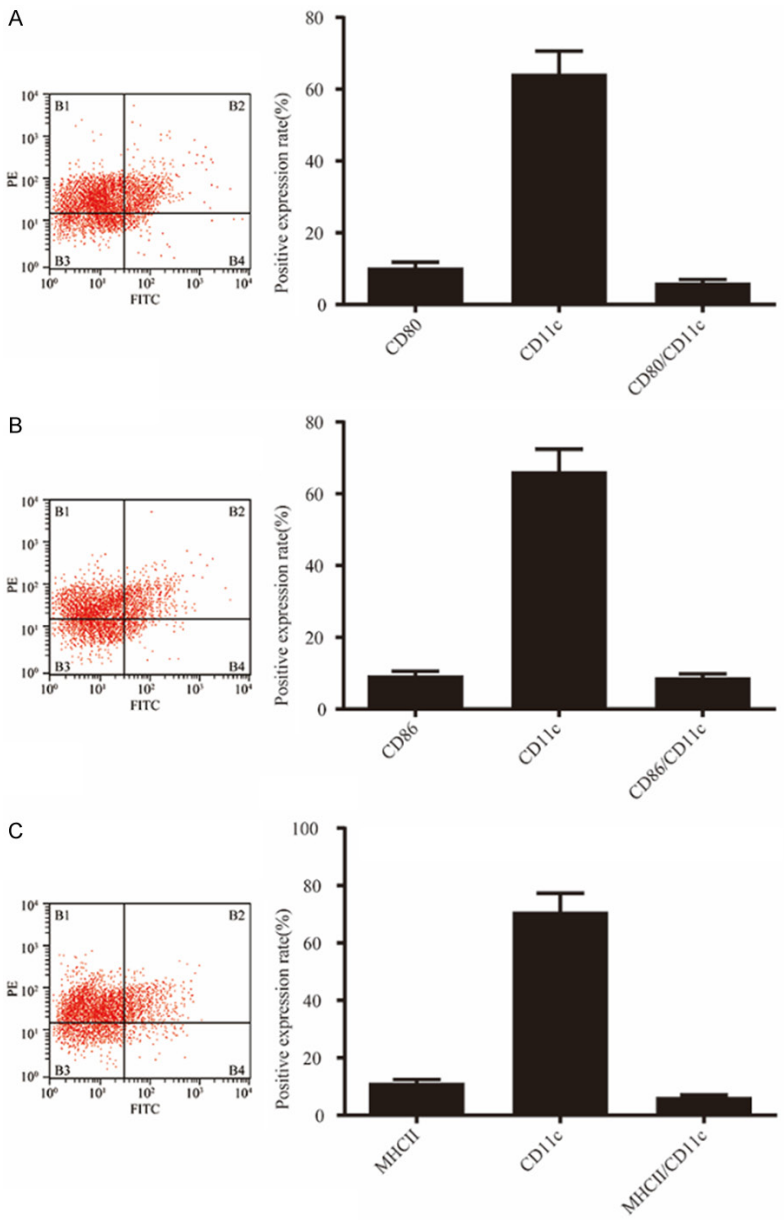

Figure 3. Molecular markers in airway intraepithelial dendritic cells. A: Expression of CD11c and CD80; B: Expression of CD11c and CD86; C: Expression of CD11c and MHCII. The quadrants of CD11c expression are B1 and B2; the quadrants of CD80, CD86, and MHCII expressions are B2 and B4.

The levels of inflammatory factors IL-4, IL-13 and IL-22 in BALF in the other groups were elevated as compared with the normal group (all P<0.05). The opposite results were observed when IFN-γ groups were compared with the model group, and when the 300 U/mL and 500 U/mL IFN-γ groups were compared with the 100 U/mL IFN-γ group (all P<0.05). See **Figure 2C**.

Identification of airway intraepithelial dendritic cells

The positive expression of molecular markers CD11c, CD80, CD86, and MHCII on the surface of airway intraepithelial dendritic cells was detected by flow cytometry (**Figure 3**). The results were pairwise compared between CD11c and CD80, CD86 and MHCII. When CD11c was highly expressed, other factors were lowly expressed, and co-expression also showed significantly lower levels. The results showed that the intraepithelial dendritic cells of each group were successfully isolated and cultured.

Expression of TLR-7 and IDO in airway intraepithelial dendritic cells

The mRNA levels of TLR-7 and IDO in airway intraepithelial dendritic cells of each group were detected by qRT-PCR. Compared with the normal group, the mRNA levels of TLR-7 and IDO in the airway intraepithelial dendritic cells were decreased in the other groups (all P<0.05) The opposite results were observed when IFN-γ groups were compared with the model group, and when the 300 U/mL and 500 U/mL IFN-γ groups were compared with the 100 U/mL IFN-γ group (all P<0.05). See **Figure 4A**.

Compared with the normal group, the protein levels of TLR-7 and IDO in the airway intraepithelial dendritic cells were decreased in the other groups (all P<0.05). The opposite results were observed when IFN-γ groups were compared with the model group, and when the 300 U/mL and 500 U/mL IFN-γ groups were com-

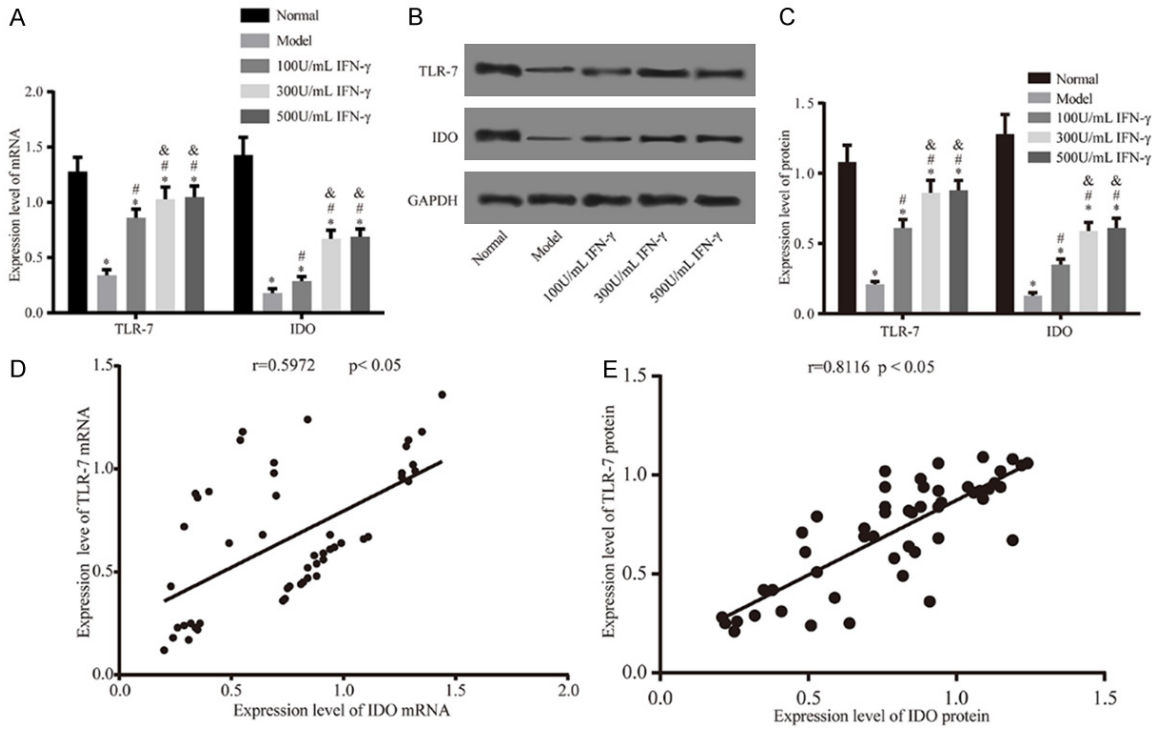

Figure 4. Expression of TLR-7 and IDO in airway intraepithelial dendritic cells. A: mRNA levels of TLR-7 and IDO; B: Protein bands; C: Protein levels of TLR-7 and IDO; D: Linear-regression analysis of TLR-7 and IDO mRNA; E: Linear-regression analysis of TLR-7 and IDO protein. Compared with the normal group, $^{*}P<0.05$; compared with the model group, $^{\#}P<0.05$; compared with the 100 U/ml IFN-γ group, $^{\&}P<0.05$. TLR7: toll-like receptor 7; IDO: indoleamine 2, 3-dioxygenase, IFN-γ: interferon-γ; GAPDH: glyceraldehyde-phosphate dehydrogenase.

pared with the 100 U/mL IFN-γ group (all P<0.05). See **Figure 4B** and **4C**.

Linear-regression analysis showed that there was a significant positive correlation between mRNA levels of TLR-7 and IDO in airway intraepithelial dendritic cells, and a similar result was found in the analysis of protein levels of TLR-7 and IDO. See **Figure 4D** and **4E**.

Discussion

Bronchial asthma is a worldwide disease affecting human health. In recent years, the incidence of asthma in children has increased year by year [21, 22]. Globally, 80% of asthma patients' allergens are from house dust mites. Therefore, we established a mouse model of house dust mite asthma to explore the related pathogenesis and possible treatment strategy.

IFN-γ is a major cytokine of Th1 cells and plays an important role in asthma. Aerosol inhalation of low-dose IFN-γ has a pronounced effect on asthma, and high doses of IFN-γ have an effect on promoting the inflammatory response of asthma [23-27]. Therefore, in this study, we treated mice with house dust mite asthma with IFN-γ at 100 U/mL, 300 U/mL, and 500 U/mL to test the airway responsiveness of each group and to detect the levels of inflammatory factors IL-4, IL-13 and IL-22 in peripheral blood and BALF of the mice. The results showed that different concentrations of IFN-γ did not improve airway responsiveness, but significantly inhibited the contents of inflammatory factors, and the inhibitory effects of IFN-γ at 300 U/mL and 500 U/mL were better than that of IFN-γ at 100 U/mL, while there was no significant difference between 300 U/mL and 500 U/mL. We speculate that low-dose IFN-γ inhalation may have a significant improvement in the inflammatory response of asthma. When IFN-γ reaches a certain dose, the inhibitory effect on inflammatory response is optimal. Intervention with IFN-γ above this dose will reduce the inhibitory effect on the inflammatory response and may even promote inflammation in the body. The present study provided further understanding of the IFN-γ intervention on asthma.

TLR7 as a member of the TLR family, promotes the activation and expression of downstream genes after its activation, thereby promoting the transcription of genes related to immunoregulation. The transcription products reduce the body's inflammatory response by mediating the expression of inflammatory factors [28-30]. IDO as a rate-limiting enzyme for catabolism of tryptophan, is highly expressed in normal immune organs, and has an important impact on immune tolerance. IDO can reduce the inflammatory response of the airways by promoting immune tolerance of the body [31, 32]. We isolated and identified airway intraepithelial dendritic cells from mice in the normal, model, 100, 300, and 500 U/mL IFN-γ treated groups, and detected the mRNA and protein expression of TLR7 and IDO in each group by qRT-PCR and Western blot. The results showed that the mRNA and protein expression of TLR7, IDO were decreased in the other groups as compared with the normal group while the opposite results were observed when the IFN-γ groups were compared with the model group, and when the 300 U/mL and 500 U/mL IFN-γ groups were compared with the 100 U/mL IFN-γ group. Linear regression analysis showed significant positive correlations between mRNA and protein expressions of TLR7 and IDO. In summary, IFN-γ at 100 U/mL, 300 U/mL, and 500 U/mL had no significant effect on airway responsiveness in mice with asthma, but significantly reduced the inflammatory response. Furthermore, IFN-γ at 300 and 500 U/mL were more effective than that at 100 U/mL.

The mRNA and protein expression of TLR7 and IDO in airway intraepithelial dendritic cells of mice was significantly decreased, and there was a significant positive correlation between mRNA and protein expressions of TLR7 and IDO. The reason may be that Th1/Th2 cells are imbalanced in mice with asthma, resulting in a significant decline in the expression of TLR7, which is dominant in Th2 cells. The decline in TLR7 expression resulted in decreased expression of the associated gene IDO. Additionally, 100, 300 and 500 U/mL IFN-γ intervention can elevate the mRNA and protein expression of TLR7 and IDO. As an regulator of IDO, IFN-γ can induce the expression of IDO, thereby reducing the body's inflammatory response. The elevated expression of IDO promotes the expression of TLR7.

IFN-γ at 100, 300, and 500 U/mL had significant relief effects in mice with asthma. However, there was no significant difference between 300 and 500 U/mL IFN-γ. We speculate that low concentrations of IFN-γ can promote the expression of IDO and TLR7, thereby regulating the expression of downstream factors, inhibiting the expression of inflammatory factors, and alleviating the inflammatory response. With the raise of IFN-γ concentration, the induction on IDO is saturated, and the excessive atomization treatment of IFN-γ may cause new inflammatory reaction and damage to the body. Therefore, the optimal dose of IFN-γ intervention therapy remains to be studied.

Disclosure of conflict of interest

None.

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