

Original article

Expression and significance of myeloid differentiation factor 88 in marrow dendritic cells in asthmatic rats with cigarette smoke exposure

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Keywords: smoking; myeloid differentiation factor 88; dendritic cells; asthma; lymphocyte activation

Background Smoking causes frequent asthma attacks, leading to a rapid decline in lung function in patients with asthma, and it can also reduce the therapeutic effect of glucocorticoids in patients with asthma. Therefore, the present study aimed to investigate the effect of cigarette smoke on the expression of myeloid differentiation factor 88 (MyD88) in marrow dendritic cells (DCs) in asthmatic rats, and to explore the molecular mechanism of cigarette smoke exposure on asthma by DCs.

Methods Forty Wistar rats were randomly divided into the following groups: control, smoke exposure, asthma, and asthma combined with smoke exposure. The animal model was established, and then rat bone marrow-derived DCs were collected. Additionally, rat spleen lymphocytes and bone marrow-derived DCs were cultured together for mixed lymphocyte responses. Interferon (IFN)-gamma and interleukin (IL)-4, IL-10, and IL-12 expressions were determined by enzyme-linked immunosorbent assay (ELISA). MyD88 expression was determined by Western blotting. The proliferation of lymphocytes was examined with methyl thiazolyl tetrazolium (MTT) colorimetric assay.

Results MyD88 expression was decreased in the asthma combined with smoke exposure group compared to the asthma group ($P < 0.01$), and IL-10 and IL-12 expressions were decreased in the asthma combined with smoke exposure group compared to control group ($P < 0.01$). In addition, DCs stimulating activity on allogeneic lymphocytes were significantly decreased in the smoke exposure combined with asthma group compared to the control and asthma groups ($P < 0.01$). After allogeneic mixed lymphocyte responses, IL-4 expression was increased and IFN-gamma was decreased in the asthma group and the asthma combined with smoke exposure group compared to control group ($P < 0.01$). IL-4 expression was increased and IFN-gamma was decreased in the asthma combined with smoke exposure group compared to the asthma group ($P < 0.01$). The study also showed that MyD88 expression was positively correlated with IL-12 and IFN-gamma expressions and the activity of lymphocytes ($P < 0.01$), and negatively correlated with IL-4 expression ($P < 0.01$).

Conclusions Smoking aggravates asthma by weakening immunological mechanism. MyD88-dependent pathways may play a role in the immunological balance and activation of lymphocytes.

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Cigarette smoke exposure, a widespread environmental factor, can alter the immune response of the lungs to allergens, but the mechanism is unclear. Toll-like receptors (TLRs) are important innate immune pattern recognition receptors (PRRs) expressed on the surface of dendritic cells (DCs). They identify pathogen-associated molecular patterns, activate the innate immune system by myeloid differentiation factor 88 (MyD88)-dependent pathways, and release many inflammatory factors, which play a key role in the immunological mechanism of asthma.¹⁻³

However, few studies have addressed the direct effect of cigarette smoke on lymphocyte activity and the T helper cell (Th)1/Th2 balance induced by DCs. Therefore, we designed the present study to investigate the relationship between MyD88 protein with lymphocyte activation and Th1/Th2 balance. We measured MyD88 protein, interleukin (IL)-10, and IL-12 expressions in bone marrow-derived DCs, and interferon (IFN)-gamma and IL-4 expressions produced by allogeneic mixed

lymphocyte responses in asthmatic rats after exposure to cigarette smoke.

METHODS

Rats and groups

Forty specific-pathogen-free (SPF) male Wistar rats (6–8 weeks old) were obtained from the Laboratory Animal Center of Shanxi Medical University, weighing 180–200 g. The rats were kept in SPF animal research facilities and received food and water *ad libitum* and all procedures performed on the animals were in compliance with the

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Chinese Council of Animal Care Guidelines (approved by Shanxi Medical University Animal Care Committee). These rats were randomly divided into four groups: control, smoke exposure, asthma, and asthma combined with smoke exposure. Ten rats were allocated to each group.

Preparation of model

The asthma model was established based on a previously explained method.⁴ Briefly, rats in the asthma group were actively immunized by intraperitoneal injection of 100 µg ovalbumin (OVA, Sigma, USA), adsorbed in 400 µg alum in 0.2 ml 0.9% sterile saline on days 1 and 7. Then the rats were exposed to an aerosol spray of 1% OVA in 0.9% sterile saline for 30 minutes each day, six days per week, for a total of eight weeks. Rats in the smoke exposure group were exposed to smoke of 10 commercial unfiltered cigarettes for one hour each day, six days per week, for a total of eight weeks. The smoke exposure box was 91 cm × 59 cm × 62 cm and the procedure was the same as the previous method.⁵ The rats in the asthma combined with smoke exposure group were given both OVA and cigarette smoke exposure like the asthma and smoke exposure group. Rats in the control group were sensitized and challenged with phosphate-buffered saline (PBS).

Isolation, culture, and identification of rat bone marrow-derived DCs

Bone marrow was prepared from the tibia and femur bones of Wistar rats. The bones were placed in a 100-mm dish with 70% alcohol for 30 minutes, then transferred into a fresh dish with Roswell Park Memorial Institute (RPMI) medium. Both ends of the bones were cut with scissors and the bone marrow was flushed into a new dish with a syringe and a 19-gauge needle using 5–10 ml RPMI medium. Red blood cells were lysed with a lysis buffer containing ammonium chloride (0.83%). The remaining bone marrow cells were washed twice. Bone marrow cells were resuspended in culture medium supplemented with recombinant rat granulocyte monocytes colony-stimulating factor (rrGM-CSF) (10 ng/ml) and recombinant rat IL-4 (10 ng/ml) and plated on six-well plates in 3 ml culture medium per well at a density of 1×10^6 /ml. On day 3, fresh culture medium and cytokines were added. Between days 3 and 4, cell aggregates attached to the surface of dishes were observed. On days 5 and 6, the whole medium was removed and replaced by fresh medium and cytokines (GM-CSF only). Lipopolysaccharide (LPS) was then added and cultures were cultivated for 2 days.

Isolation and culture of splenic lymphocytes

Spleens (2×10^8 cells per spleen) were dissociated with a wire mesh and lymphocyte separation liquid was added. Cells were then washed and centrifuged at 1000 r/min for 10 minutes. The lymphocytes were cultured in RPMI-1640 medium containing 10% fetal bovine serum at 37°C in a 5% CO₂ humidified incubator for 2 hours. Finally, the suspended cells were collected.

Allogeneic mixed lymphocyte responses

Collected bone marrow-derived DCs and lymphocytes were co-cultured in triplicates in a round-bottomed 96-well plate in a total volume of 200 µl/well at a DCs/T-cell ratio of 2:1 for 72 hours, and 25 µl (5 mg/ml) MTT was added to the cells for 4 hours. The reaction was terminated by acidification with isopropanol. The *A* value was detected at a 570-nm wavelength.

Western blotting of MyD88 protein

DCs were homogenized in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L sodium chloride, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethanesulfonyl fluoride, 1% Triton X-100, 1% deoxycholic acid sodium, 0.1% SDS, 5 µg/ml aprotinin, and 5 µg/ml leupeptin) at 4°C, vortexed, and centrifuged at 14 000 r/min at 4°C for 30 minutes and then 50 µg protein was subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was then blocked, probed with anti-rat MyD88 (Santa Cruz, USA) and then with the appropriate secondary antibody conjugated to HRP, and visualized with DAB.

Cytokine measurement

After the cell supernatant was collected, the enzyme-linked immunosorbent assay (ELISA) technique was used for detecting IL-10 and IL-12 (R&D Systems, USA) antigens of the samples by following the manufacturer's instructions. In addition, IFN-γ and IL-4 (R&D Systems) concentrations in the culture supernatants after allogeneic mixed lymphocyte responses were determined by ELISA according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software (SPSS Inc., USA). The data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to compare the mean in each group, and the SNK-*q* test was used for intergroup comparison. A *P* < 0.05 was considered statistically significant.

RESULTS

Establishment of rat model

In the asthma and asthma combined with smoke exposure groups, there was evidence of shortness of breath, irregular respiratory rhythm, a slight tremor of the limbs, irritability, cough, and a reduction in activity. In addition, slow response, erect hair, and poor color were also sometimes observed. There were no signs of asthma in control group. Moreover, inflammatory cell infiltration was observed in the peribronchiolar area of the smoke exposure, asthma, and asthma combined with smoke exposure groups from H&E-stained tissue sections and the thickness of total bronchial wall and smooth muscle in the smoke exposure, asthma, and asthma combined

with smoke exposure groups was larger than in control group (Figure 1).

Observation and identification of rat bone marrow-derived DCs

Under an inverted phase contrast microscope, we observed that most cells were adherent and a cell colony appeared on day 2. The suspended cells were wiped off on day 3. The cell colony gradually became large. LPS was added and the culture continued to be cultivated. Most cells were suspended and a small amount of cells were adherent, but cells could be taken off from the wall by blowing gently. There were many dendrites of uniform size in rat bone marrow-derived DCs, but there was no significant difference between the groups. OX-62, a specific marker expressed in DCs, and major histocompatibility complex (MHC)-II were detected by flow cytometry, indicating that the cells were DCs.

Effect of cigarette smoke exposure on MyD88 protein in rat bone marrow-derived DCs

There were no differences in MyD88 protein between the control and asthma groups. The smoke exposure group had lower levels of MyD88 protein compared to those in the control group. Moreover, after cigarette smoke exposure, MyD88 expression was decreased in the asthma group compared to that in control group (Figure 2).

Effect of cigarette smoke exposure on IL-10 and IL-12 protein releases by rat bone marrow-derived DCs

IL-10 protein expression was increased and IL-12 protein expression was decreased in the asthma group compared to those in the control group. However, there were no differences in IL-10 protein expression between the control group and the smoke exposure group. IL-10 and IL-12 expressions were decreased in the asthma combined with smoke exposure group compared to that in the asthma group (Figures 3 and 4).

Effect of cigarette smoke exposure on allogeneic mixed lymphocyte responses

The asthma group had significantly higher DC stimulation activity on allogeneic lymphocytes than the

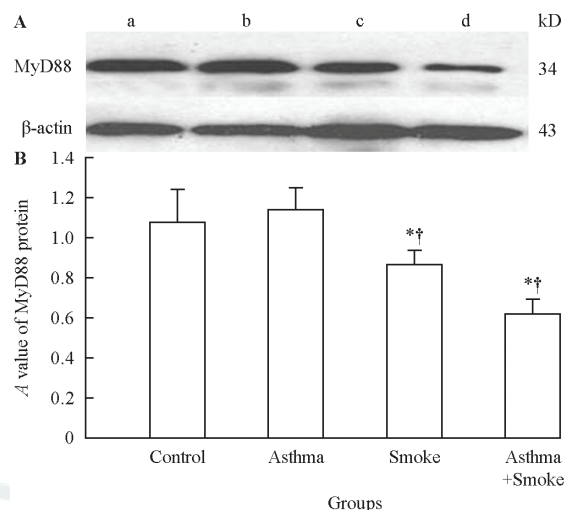


Figure 2. Expression of MyD88 protein examined by Western blotting in DCs of rats (A): a: control group; b: asthma group; c: smoke exposure group; and d: asthma combined with smoke exposure group. MyD88 protein values are presented as the relative ratio of MyD88 protein to β-actin (B). Data represent mean ± standard error from 10 samples per group for Western blotting analysis. * $P < 0.01$ compared to the asthma group. † $P < 0.01$ compared to the control group.

control group, but there were no differences between the control and smoke exposure groups. Surprisingly, the DC stimulation activity on allogeneic lymphocytes was significantly decreased in the smoke exposure combined with asthma group compared to both the control and asthma groups (Figures 5–7).

In addition, we found a change in cytokines related to Th1/Th2 balance. IL-4 expression was increased and IFN-γ expression was significantly decreased in the asthma, smoke exposure, and asthma combined with smoke exposure groups compared to those in the control group.

Correlation analysis

MyD88 expression was positively correlated with IL-12 and IFN-γ expressions and the activity of lymphocytes, and negatively correlated with IL-4 expression. IL-10 expression was negatively correlated with IL-12 expression (Figure 8).

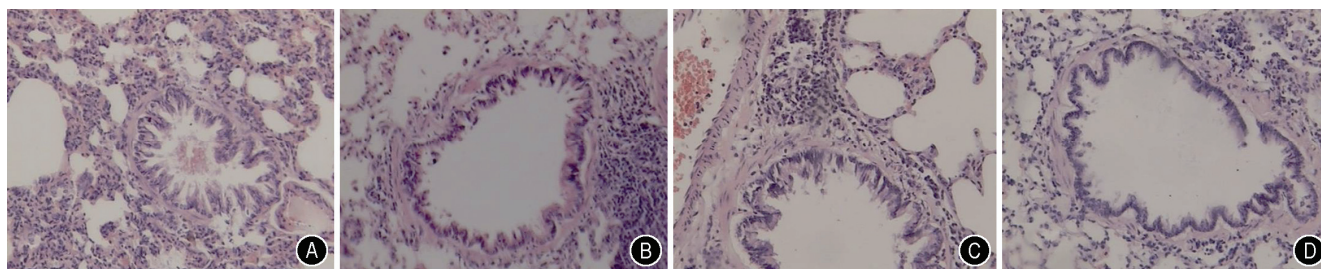


Figure 1. The changes of lung pathology was observed under microscopy in four groups. A: Normal airway of rats (HE, original magnification ×100). B: Smooth muscle proliferation and inflammatory cell infiltration, such as lymphocytes and neutrophils, in the smoke exposure rats (HE, original magnification ×100). C: Smooth muscle proliferation and inflammatory cell infiltration, such as lymphocytes and eosinophils, in the asthma rats (HE, original magnification ×100). D: Smooth muscle proliferation and inflammatory cell infiltration, such as lymphocytes, eosinophils, and neutrophils, in the asthma combined with smoke exposure rats (HE, original magnification ×100).

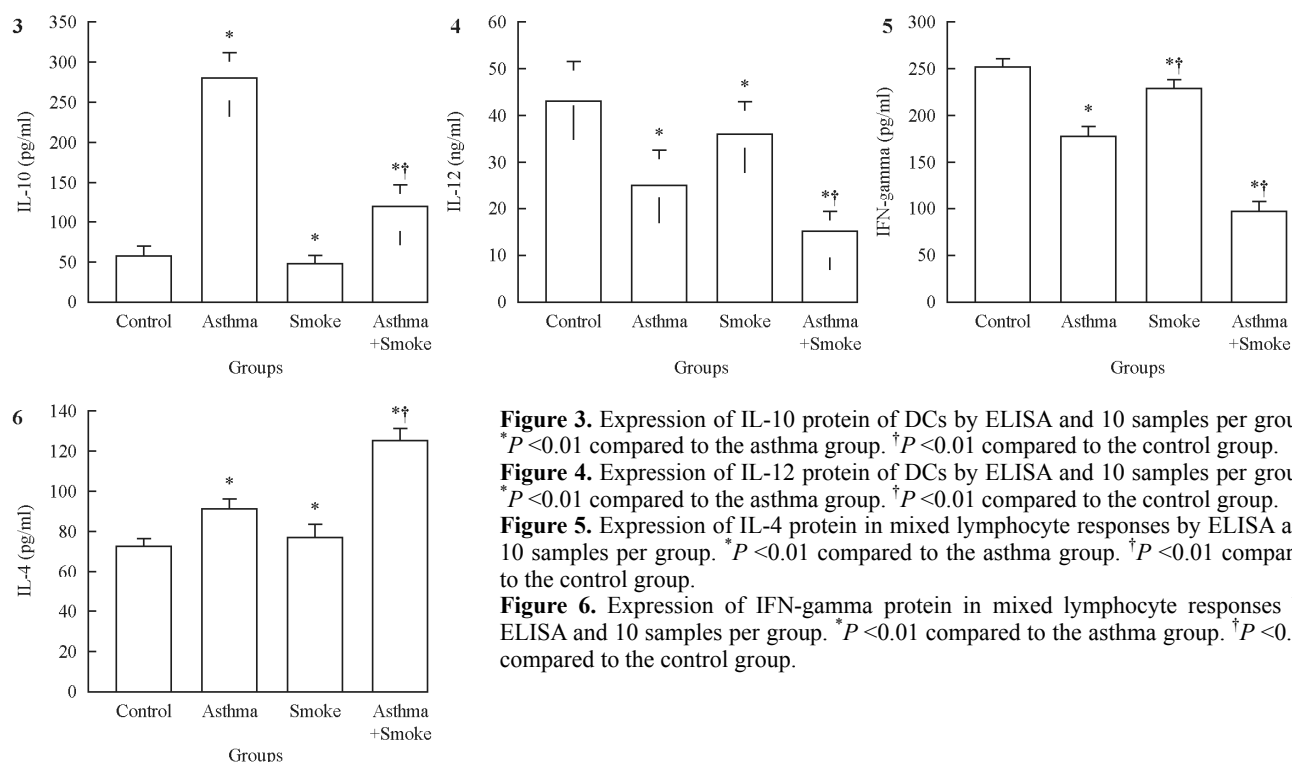


Figure 3. Expression of IL-10 protein of DCs by ELISA and 10 samples per group. * $P < 0.01$ compared to the asthma group. † $P < 0.01$ compared to the control group.

Figure 4. Expression of IL-12 protein of DCs by ELISA and 10 samples per group. * $P < 0.01$ compared to the asthma group. † $P < 0.01$ compared to the control group.

Figure 5. Expression of IL-4 protein in mixed lymphocyte responses by ELISA and 10 samples per group. * $P < 0.01$ compared to the asthma group. † $P < 0.01$ compared to the control group.

Figure 6. Expression of IFN-gamma protein in mixed lymphocyte responses by ELISA and 10 samples per group. * $P < 0.01$ compared to the asthma group. † $P < 0.01$ compared to the control group.

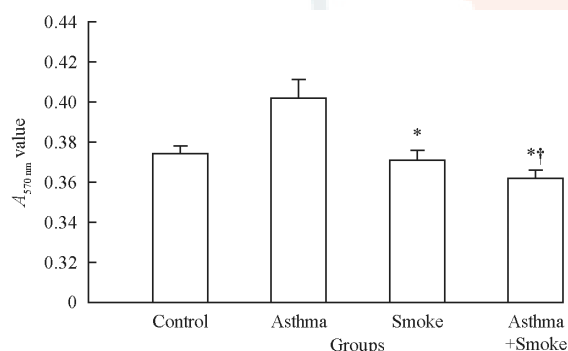


Figure 7. Mixed lymphocyte responses induced by DCs. DCs stimulation activity on allogeneic lymphocytes was presented as $A_{570\text{ nm}}$ value. * $P < 0.01$ compared to the asthma group. † $P < 0.01$ compared to the control group.

DISCUSSION

Cigarette smoke is considered as the main source of indoor air pollution and can damage the respiratory tract in various ways. Asthmatic smokers have more serious clinical symptoms, more frequent acute exacerbation, and

more hospital visits compared to non-smokers with asthma. Moreover, smoking also affects lung function in patients with asthma, such as a faster decline in forced expiratory volume in one second and peak expiratory flows. In addition, smoking increases the clearance of theophylline and reduces the reactivity of glucocorticoids.^{6,7} For cigarette smoke, some harmful compositions contained in tobacco are responsible for aggravating and altering airway immune responses in asthma, but the relevant mechanism is not completely understood. DCs initiate airway allergic inflammation and mediate immune tolerance. It is well known that surface PRRs expressed in DCs adjust to the function of DCs by identifying pathogen-related patterns and affect T-cell activation and proliferation.⁸ Therefore, smoking may regulate immune responses in asthma by controlling function of DCs to relieve airway inflammation.

DCs identify antigens, change them into proteolytic peptides, and present them to lymphocytes. They constitute a powerful defense network in airway

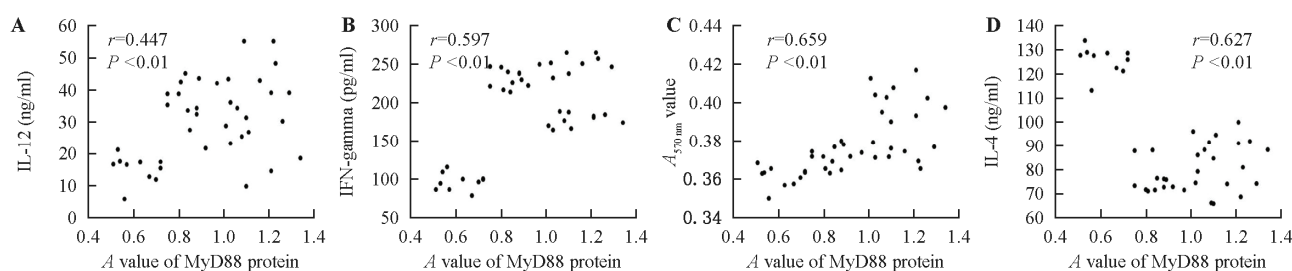


Figure 8. The expression of MyD88 in DCs, presented as the relative ratio of MyD88 protein to β -actin, correlates to IL-12 (A). The expression of MyD88 in DCs correlates to IFN-gamma (B). The expression of MyD88 in DCs correlates to DCs stimulation activity on allogeneic lymphocytes, presented as $A_{570\text{ nm}}$ value (C). The expression of MyD88 in DCs correlates to IL-4 (D). The figure presents data from four groups and each point depicts the result from one rat. Statistical calculation performed using Spearman's test.

epithelium and the lungs. Moreover, they activate lymphocytes to affect the development of airway inflammation and play an important role in the pathogenesis of asthma.⁹ In recent years, some studies have indicated that DCs provide activation and proliferation signals for T cells via MHC-II and costimulatory molecules expressed on the surface of DCs,^{10,11} as well as provide polarization and differentiation signals via secreting some soluble cell factors such as IL-4, IL-10, IL-12, and Prostaglandin E₂.¹²

Some studies have shown that IL-10 inhibits the release of IgE,¹³ prevents eosinophilic inflammation from gathering in the airway to reduce hyperresponsiveness and promote apoptosis.¹⁴ IL-10 also inhibits the synthesis and release of all inflammatory factors such as IFN-gamma, IL-2, IL-6, and IL-12. IL-12 is the key factor that affects Th1 polarization; it maintains the Th1 reaction by promoting T cells and NK cells to produce IFN-gamma.¹⁵ It also inhibits Th2 function and secretion of Th2 cytokines.¹⁶ Therefore, IL-10 and IL-12 are considered as the main cytokines that can adjust Th1/Th2 balance in the pathophysiology of asthma. In our study, we found that the ability of DCs secreting IL-10 and IL-12 was impaired in the rat model with asthma, and this impairment was further exacerbated by exposure to smoking. It might be one of the immunological mechanisms that smoking aggravates airway inflammation in asthma. In our study, we also found that IL-10 expression was negatively correlated with IL-12 expression, indicating that endogenous IL-10 inhibits IL-12 expression, which is consistent with many previous studies.¹⁷

The essence of mixed lymphocyte responses is a proliferation reaction of lymphocytes. It results from the phenomenon that antigen-presenting cells can activate lymphocytes and induce an immune response. In general, most DCs are immature and reside in non-lymphoid peripheral tissues. The ability of immature DCs to activate lymphocytes *in vitro* is weak, while the ability to take in and process antigens is powerful. When immature DCs encounter antigens or inflammatory stimulation, they migrate from non-lymphoid peripheral tissues to subprime lymphatic organization and gradually become mature. Mature DCs can express high levels of MHC-I, MHC-II, costimulatory molecules, collaborative stimulatory molecules (CD₈₀, CD₈₆), adhesion molecules (CD₄₀, ICAM-1), integrin, and characteristic markers (CD_{1a}, CD_{11c}, CD₈₃). They also secrete many cytokines such as IL-12, IL-1, IL-6, and IL-8 and tumor necrosis factor (TNF)-alpha. In our study, the proliferation ability of lymphocytes by DCs-dependent increased after exposure to OVA, but it was decreased after exposure to smoking. Therefore, smoking can inhibit the proliferation ability of lymphocytes by DCs-dependent.

IFN-gamma is only produced by Th1 cells and IL-4 is only produced by Th2 cells,¹⁸ and they often restrained

each other. Therefore, IFN-gamma/IL-4 ratio can be on behalf of Th1/Th2 balance. IFN-gamma can reduce the synthesis of IgE, activate mononuclear macrophages effectively, and promote its killing capacity. IL-4 can stimulate native T cell differentiation towards Th2 cells, induce B cell proliferation and differentiation towards plasma cells, which can produce IgE that is involved in the occurrence and development of asthma allergic inflammation.¹⁹ In the present study, we found that the function of lymphocytes was abnormal in the secretion of IFN-gamma and IL-4 in the asthmatic rat model and it was further exacerbated by exposure to smoking. Therefore, cigarette smoke can deteriorate airway immunity and aggravate airway inflammation in asthma.

Generally, PRRs can identify pathogen-associated molecular patterns that exist in pathogens, and antigens are conducted and presented to lymphocytes. The signaling pathway that is activated by PRRs can control expression of genes involved in various immune responses. Eventually, effector molecules are activated and innate immune and acquired immune responses are initiated to defend against infection by pathogens. It was recently discovered that TLR belongs to the PRR family, and it plays a very important role in innate immunity. Currently, there are 13 members in the TLR family. Although different TLRs recognize different pathogen-associated molecular patterns (PAMPs), MyD88 is a vital molecule that can transmit the signal by almost all TLR-signaling pathways.²⁰ It can recruit IL-1 receptor-related protein kinase through a death structure domain, and make signal transmit by signal molecules such as tumor necrosis-related factor 6 (TRAF6), transforming growth factor-beta-activated protein kinase (TAK1), and TAK1 binding protein 1 and 2 (TAB1, TAB2), and finally activate nuclear factor-kB²¹ or activator protein-1 by promoting mitogen activated protein kinases²² such as p38 and c-Jun N-terminal kinase. Eventually, it leads to production of inflammatory cytokines such as IL-1, IL-6, IL-8, and IL-12 and costimulatory molecules such as CD₈₀ and CD₈₆ which activate the immune system.²³ In addition, MyD88 plays a critical role in heat shock protein (HSP) 60 signal transduction, and HSP60 activates DCs through MyD88-dependent pathway.²⁴ A study has showed that MyD88 takes part in mediating STAT3 phosphorylation and it is very important in regulating infection.²⁵

We observed that MyD88 expression was decreased in asthmatic rats after exposure to smoke, which demonstrated that antigen recognition of DCs was abnormal. Therefore, cigarette smoke may inhibit antigen recognition of DCs. It has been shown that CD₄₀-CD_{40L} pathways play a key role in producing IL-12.²⁶ In our study, IL-12 expression was reduced when MyD88 expression was reduced, and MyD88 expression was positively correlated with IL-12 expression in rat bone marrow-derived DCs. This suggested that the effect of smoke on the expression of IL-12 may be partly induced

by MyD88-dependent toll-like signaling pathways. However, IL-10 and MyD88 expression were not significantly correlated, and therefore, the effect of smoke on IL-10 may be induced by other ways. Further research is required to determine this mechanism.

In conclusion, cigarette smoke inhibits MyD88, IL-10, and IL-12 expressions in rat bone marrow-derived DCs, reduces the capacity of proliferation of DCs activating lymphocytes, and undermines the function on antigen recognition and presentation of DCs. In addition, MyD88-dependent toll-like signaling pathways may affect the immunological balance and activation of lymphocytes in asthma after exposure to cigarette smoke. All these changes may worsen the airway pathology of asthma. Further research on DCs in asthma with cigarette smoke exposure will hopefully provide a new direction for therapy of patients with asthma who are often exposed to cigarette smoke.

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