

# Expression and pathological significance of CC chemokine receptor 7 and its ligands in the airway of asthmatic rats exposed to cigarette smoke

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**Background:** Cigarette smoking aggravates the symptoms of asthma, leading to the rapid decline of lung function. Dendritic cells (DCs) and lymphocytes are considered initiating and promoting factors for the airway inflammation reactions of asthma. In addition, activation of CC chemokine receptor 7 (CCR7) by chemokine (C-C motif) ligand (CCL) 19 and 21 promotes DCs and T cells migration to lymphoid tissues during inflammation. We aimed to examine how cigarette smoke affects the expression of CCR7 in the lungs of asthmatic rats and explore the signaling mechanism linking CCR7 expression to exacerbation of symptoms.

**Methods:** Forty Wistar rats were randomized to four groups: control, asthma, smoke exposure, and asthma with smoke exposure groups. A rat asthma model was established by intraperitoneal ovalbumin injection. CCR7 expression was examined with immunohistochemistry and western blotting. The number of airway DCs was determined by OX62 immunohistochemistry. Interferon (INF)- $\gamma$ , interleukin (IL)-4, CCL19, and CCL21 expression levels in blood and bronchioalveolar lavage fluid (BALF) were determined by enzyme-linked immunosorbent assays (ELISAs).

**Results:** Tissue CCR7 expression, peripheral blood and BALF CCL19 and CCL21 concentrations, and the number of airway DCs were significantly higher in the asthma with smoke exposure group than the asthma group ( $P < 0.01$ ). In addition, INF- $\gamma$  expression was decreased and IL-4 increased in the asthma and asthma with smoke exposure groups compared with the control group ( $P < 0.01$ ), and in the asthma with smoke exposure group compared with the asthma group ( $P < 0.01$ ). Expression of CCR7 correlated negatively with INF- $\gamma$  expression in peripheral blood and BALF ( $P < 0.01$ ), and positively with the airway DCs and IL-4 expression in the peripheral blood and BALF ( $P < 0.01$ ).

**Conclusions:** Cigarette smoking may aggravate asthma symptoms by attenuating immunity, possibly through CCR7-mediated DCs aggregation in lung tissue.

**Keywords:** Asthma; smoking; CC chemokines; lymphocyte activation; dendritic cells (DCs)

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## Introduction

Asthma incidence is strongly related to air pollution levels, and cigarette smoke is among the most common indoor air pollutants. Cigarette smoke exposure alters immune response allergens in the lungs and is regarded as a major risk factor for asthma pathogenesis and exacerbation (1-3). A hyperactive inflammatory response in lung tissue is a defining characteristic of asthma. Recent studies have shown that lymphocytes and dendritic cells (DCs) play important roles in the development of asthma. Chemokine receptor 7 (CCR7) is involved in the process of DCs and T cell migration to lymphoid tissues and subsequent induction of the immune response (4-6). However, few studies have analyzed the direct effect of cigarette smoke on CCR7 expression, the T helper cell (Th)1/Th2 balance, and the immune inflammatory response in asthma.

This study was aimed to shed light on the relationship between lung CCR7 expression, Th1/Th2 balance and immune response in asthma rat models with or without cigarette smoke exposure.

## Methods

### *Animals*

Male Wistar rats (n=40) were provided by the Shanxi Medical University Center of Laboratory Animals, and were housed in specific-pathogen-free (SPF) environment. All experimental procedures were approved by Animal Care Committee of Shanxi Medical University, and completed in compliance with Chinese Council of Animal Care Guidelines. These rats were randomized to four groups (n=10 each): the control group, asthma group, smoke exposure group, and asthma with smoke exposure group.

### *Modeling of asthma in rats*

Asthma was modeled in rats as previously described (7). Briefly, the rats in the asthma groups (with and without subsequent smoke exposure, n=20) were sensitized by two intraperitoneal injections (100 µg per dose) of ovalbumin (OVA, Sigma, St. Louis, MO, USA) adsorbed in 400 µg alum and dissolved in 0.2 mL normal saline (sensitization phase). Injections were separated by 1 week. Rats were then exposed to an aerosol containing 1% OVA for 30 minutes per day, 6 days each week, for up to 8 weeks (challenge phase). The rats in the smoke exposure groups (with and without prior asthma induction, n=20) were exposed to the

smoke of ten unfiltered cigarettes (of a commercial product) for 1 hour per day, 6 days each week, for up to 8 weeks in a 91×59×62 cm<sup>3</sup> box using a previous method (8). All rats in the control group were sensitized and challenged with sterile normal saline.

### *Western blotting of CCR7*

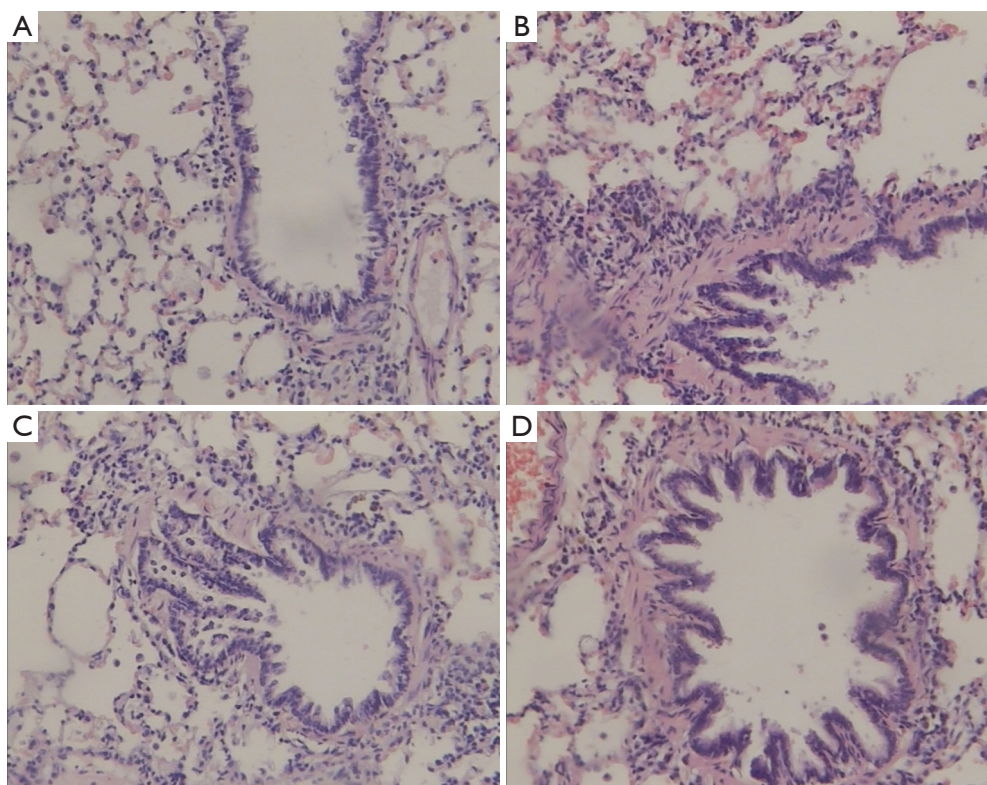
Lung tissues were homogenized in radio immunoprecipitation assay (RIPA) lysis buffer and the protein levels were determined with bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, Rockford, USA). The total protein was separated on 10% SDS-PAGE gels at 30 µg per lane and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA). Membranes were blocked in 5% non-fat dry milk, incubated with a 1:1,000 primary anti-rat CCR7 antibody (Abcam, Cambridge, USA) overnight at 4 °C, and then with a corresponding secondary anti-mouse or anti-rabbit IgG peroxidase (1:5,000) (Santa Cruz Biotechnology, Dallas, USA) at room temperature for 45 min. Anti-β-actin was employed as the gel loading control (1:1,000, Santa Cruz Biotechnology, Dallas, USA). The bands were visualized by autoradiography and quantified by densitometry. The measurements were normalized to β-actin. All experiments were repeated three times.

### *Immunohistochemistry of CCR7 and OX62*

For immunohistochemical staining, paraffin embedded lung tissue samples were treated with xylene and ethyl alcohol, followed by incubation in 0.3% methanol/H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidases. Antigen retrieval was performed by boiling the mounted sections in 10 mM citrate (pH 6.0) solution for 20 min. The sections were then incubated overnight at 4 °C with primary antibodies. The two-step technique was used for visualization, with 3,3'-diaminobenzidine (DAB) and 0.02% H<sub>2</sub>O<sub>2</sub> as chromogens. The sections were counterstained with hematoxylin. Primary antibodies for immunohistochemistry included anti-rat CCR7 (Santa Cruz) and anti-rat OX62 (Santa Cruz).

### *Cytokine measurement of peripheral blood and BALF*

After serum and BALF supernatant were collected, enzyme-linked immunosorbent assays (ELISAs) were used for measuring the levels of IL-4, INF-γ, CCL19, and CCL21



**Figure 1** Lung pathohistology in the asthma and smoke-exposure groups. (A) normal airway of rats (HE,  $\times 100$ ); (B) infiltration of inflammatory cells, such as neutrophils and lymphocytes, and smooth muscle proliferation in the smoke exposure groups (HE,  $\times 100$ ); (C) infiltration of inflammatory cells, such as eosinophils and lymphocytes, and smooth muscle proliferation in the asthma groups (HE,  $\times 100$ ); (D) infiltration of inflammatory cells, such as eosinophils, neutrophils and lymphocytes, and smooth muscle proliferation in the asthma with smoke exposure group rats (HE,  $\times 100$ ). HE, hematoxylin and eosin.

according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

### Statistical analysis

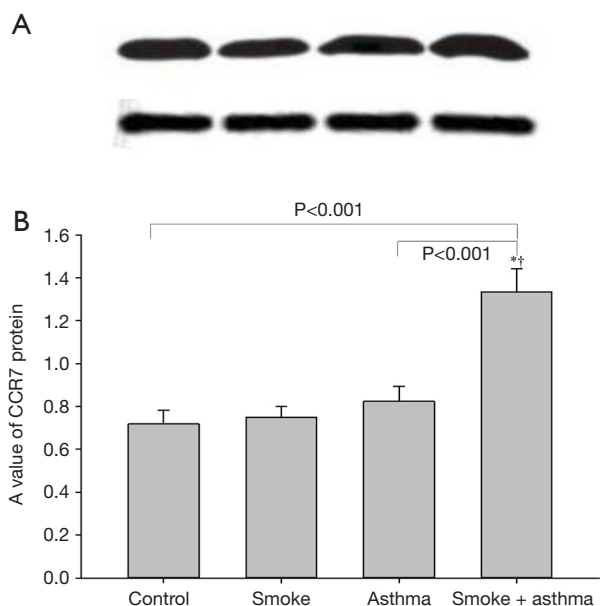
Statistical analysis was analyzed using SPSS 13.0 software (SPSS Inc., USA). The data are existed in form of mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means among groups, followed by the SNK-q test for pair-wise comparisons. P values below 0.05 were considered statistically significant.

## Results

### Modeling of asthma in rats

All rats in the asthma group and the asthma with smoke

exposure group exhibited irregular respiratory rhythm, limb tremor, cough, and poor activity. Retarded reaction and pale color hair were occasionally noted. These manifestations were not seen in the control group. In addition, hematoxylin and eosin (H&E)-staining revealed inflammatory infiltration in the peribronchiolar area of the asthma, smoke exposure, and asthma with smoke exposure groups. Moreover, the thickness of bronchial wall or the smooth muscle layer was higher in the asthma, smoke exposure, and asthma with smoke exposure groups compared with the control group (*Figure 1*). In addition, we also measured the cell classification of alveolar lavage fluid. The results showed that the number of neutrophils in the asthma group was higher than that in the control group. The total number of white blood cells and neutrophils in the asthma with smoke exposure group was increased compared with the asthma group.



**Figure 2** Elevated expression of CCR7 protein in lung tissue of smoke-exposed asthmatic rats. (A) Sample western blot control group (control); smoke exposure (smoke) group; asthma (asthma) group; asthma with smoke exposure group (smoke + asthma). CCR7 protein values are presented relative to  $\beta$ -actin (the gel loading control); (B) densitometry. Data presented as mean  $\pm$  SD from 10 samples per group. \*,  $P < 0.01$  vs. asthma group; †,  $P < 0.01$  vs. control group. CCR7, CC chemokine receptor 7; SD, standard deviation.

#### Effect of cigarette smoke exposure on CCR7 protein in the airway

Both the smoke exposure and asthma groups exhibited slightly higher levels of CCR7 protein in lung tissue than the control group, while the asthma with smoke exposure group exhibited significantly elevated lung tissue CCR7 expression compared with all other groups (Figures 2,3).

#### Effect of cigarette smoke exposure on number of DCs in the airway

OX-62 is a relatively specific antigen expressed by rat DCs, and was used as a marker to count the DCs population in airway tissue. The DCs number did not differ between the control and smoke exposure groups, while the asthma group showed more DCs than did the control group. Moreover, the number of DCs was higher in the asthma with smoke exposure group compared with the control group (Figure 4).

#### Effect of cigarette smoke exposure on CCL19 and CCL21 protein in peripheral blood and BALF

There were no differences in CCL19 and CCL21 protein expression levels in BALF among control, smoke exposure, and asthma groups, but these levels were significantly higher in the asthma with smoke exposure group compared with the control and asthma groups. There were no differences in CCL19 and CCL21 protein expression levels in peripheral blood between control and smoke exposure groups, while expression levels were significantly higher in asthma and asthma with smoke exposure compared with the control group (Figures 5,6).

#### Effect of cigarette smoke exposure on $INF-\gamma$ and IL-4 protein in the peripheral blood and BALF

We also found changes in cytokine concentrations related to Th1/Th2 balance in both peripheral blood and BALF.  $INF-\gamma$  was lower and IL-4 higher in both peripheral blood and BALF of the asthma group compared with the control group. In addition,  $INF-\gamma$  expression was lower and IL-4 significantly higher in the asthma with smoke exposure group compared with the asthma group (Figures 7,8).

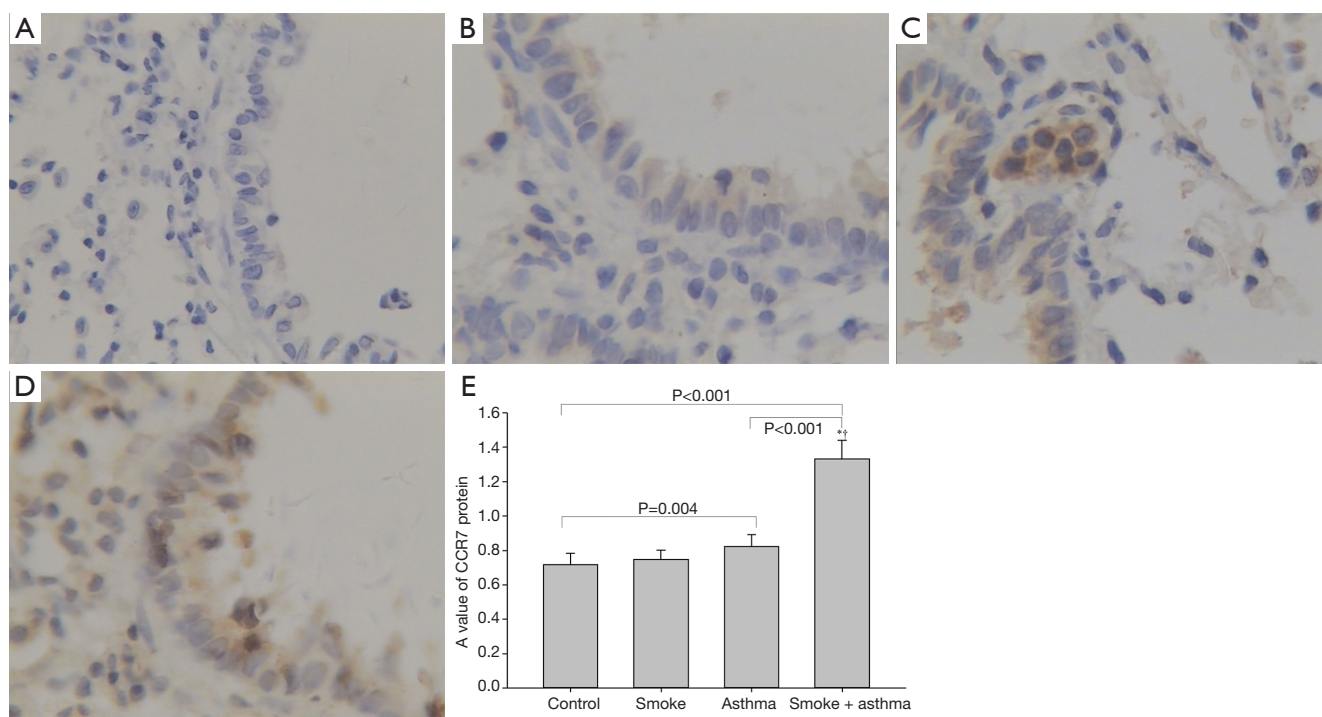
#### Correlation analysis

CCR7 expression in airway tissue was positively correlated with the number of DCs as well as with IL-4 expression in peripheral blood and BALF, and negatively correlated with  $INF-\gamma$  in peripheral blood and BALF (Figure 9).

#### Discussion

Chemokines are a group of cytokines with similar structures and chemotactic functions. They promote the migration of inflammatory cells such as lymphocytes, monocytes, neutrophils, and DCs by interacting with corresponding chemokine receptors, and play important roles in both immune function and inflammatory mechanisms of allergic diseases (9). CCR7 is a 7-transmembrane domain, G-protein-coupled receptor mainly localized on the surfaces of dendritic, initial B, T, and Treg cells that induces asymmetric intracellular signaling pathways for directional migration. CCL19 and CCL21 are the major chemokine ligands of CCR7. In addition, CCL21 is expressed by high endothelial venules (HEVs) in lymph nodes and by lymphatic endothelial cells in most non-lymphoid tissues (10).





**Figure 3** Expression of CCR7 protein examined by immunohistochemistry in the airway of rats (IHC, original magnification  $\times 400$ ). (A) control group, (B) smoke exposure group, (C) asthma group, (D) asthma with smoke exposure group; (E) densitometry. Data presented as mean  $\pm$  SD from 10 samples per group. \*,  $P < 0.01$  vs. asthma group; †,  $P < 0.01$  vs. control group. CCR7, CC chemokine receptor 7; SD, standard deviation; IHC, immunohistochemistry.

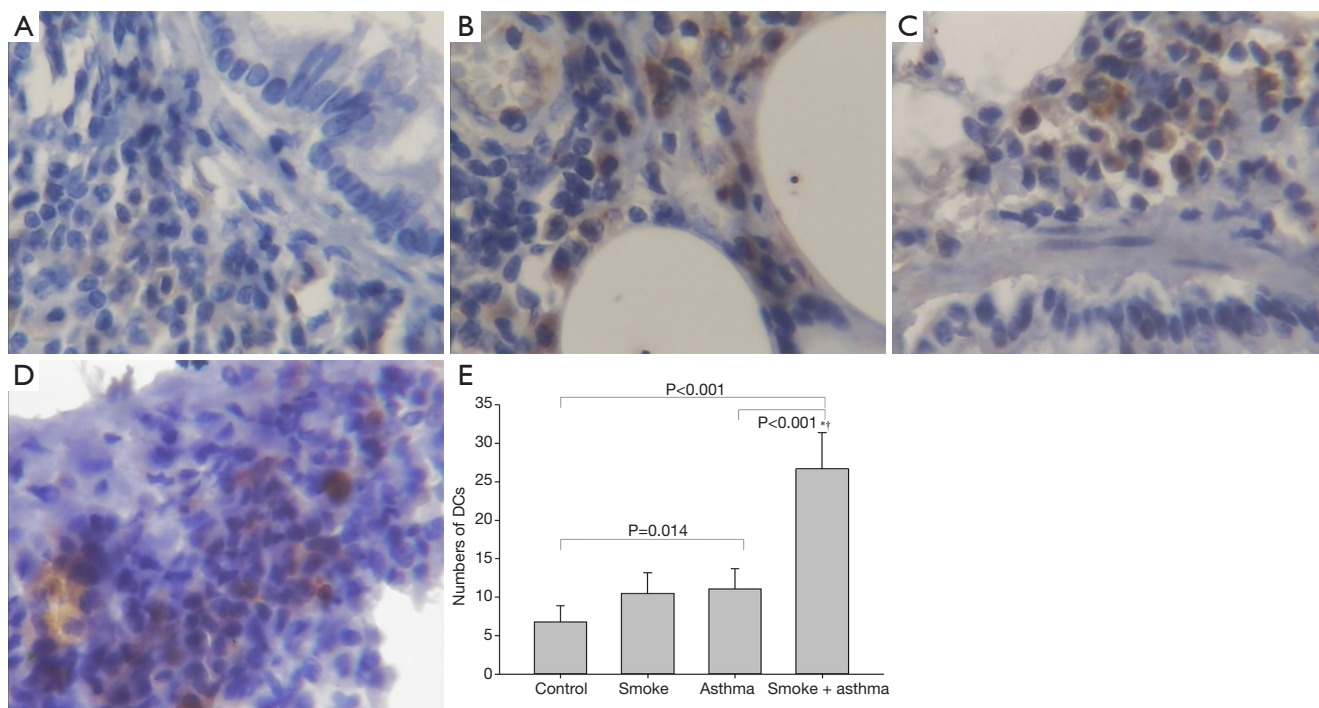
There is no substantial difference in CCR7 affinity between CCL19 and CCL21, but they induce distinct intracellular signals (11). While CCL19/CCR7 signaling may be involved in the proliferation of smooth muscles in the airway (12), CCL21/CCR7 signaling is a more potent directional cue for DCs and lymphocyte migration than CCL19/CCR7 signaling (13). In the present study, we the expression levels of CCR7 and its ligands were shown to be increased in asthmatic rats after exposure to smoke, suggesting that smoke exposure exacerbates immune cell infiltration in asthma.

Many clinical studies found smoking has deleterious effects on lung function, such as a rapid reduction in forced expiratory volume in one second and reduced peak expiratory flow as well as lowered responsiveness to routine asthma treatment (14,15). Moreover, asthmatic smokers are also more likely to develop severe asthma (16,17). It was known that asthma is a heterogeneous airway disorder with different phenotypes (18-20) among which asthma with smoke exposure is considered a special one (21). The purpose of our designed experiment is to further explain the

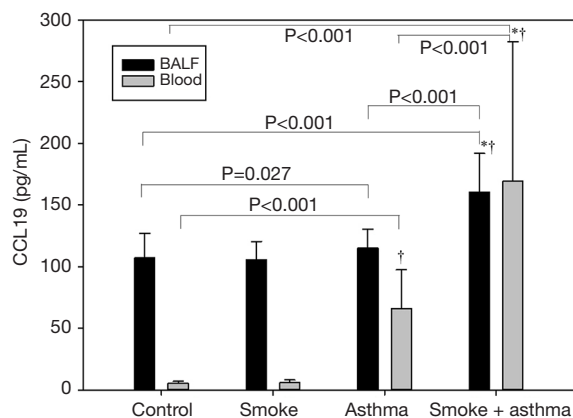
clinical features of asthmatic smokers.

DCs are the predominant antigen-presenting cells in the mucosa of the respiratory tract (22). After infection and early inflammation, immature DCs infiltrate inflammatory sites through chemotaxis, where they identify and transform antigens into proteolytic peptides, and present them to lymphocytes. Thus, DCs build up an efficient defense network in the airway. In this process, immature DCs gradually become mature, and their function changes significantly with upregulated expression of the costimulatory molecules CD80, CD86, and CCR7 (23). Recent studies also have showed that DCs trigger activation and proliferation of T-lymphocytes through costimulatory molecules and MHC-II expressed on cell surface; in addition, they initiate T-cell polarization and differentiation signals via secretion of soluble cell factors such as prostaglandin E2 and IL-6, IL-12 (24). All of these factors play important roles in the pathogenesis of asthma.

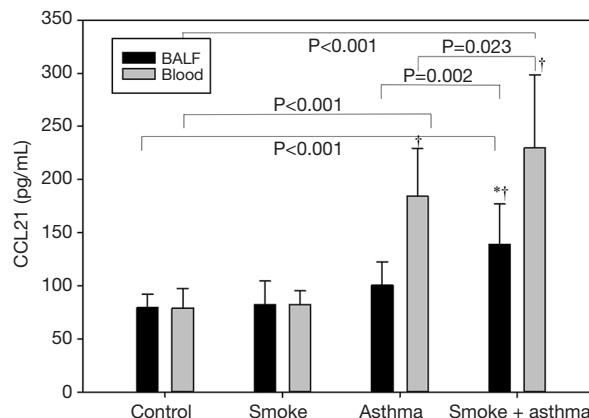
Cigarette smoke exposure can damage airway structure through various processes and affect airway immune function. It was demonstrated that airway Th2 cell function,



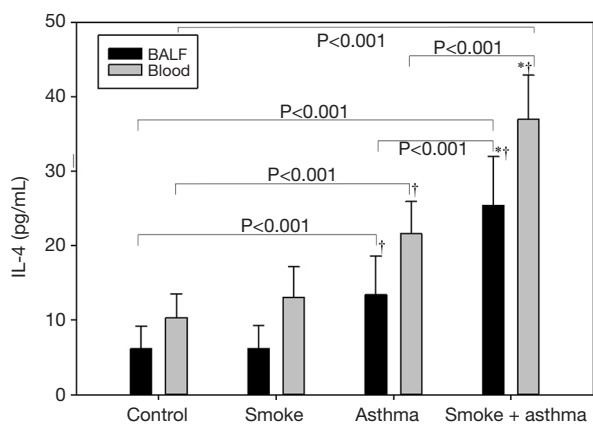
**Figure 4** Elevated DCs number in airway tissues of rats. Numbers of DCs in rat airway were determined by immunohistochemistry for OX-62 (original magnification  $\times 400$ ). Cells with cytoplasm and membranes stained brown and yellow were defined as DCs. (A) control group, (B) smoke exposure group, (C) asthma group, (D) asthma with smoke exposure group, (E) number of DCs. Data presented as mean  $\pm$  SD from 10 samples per group. \*,  $P < 0.01$  vs. asthma group; †,  $P < 0.01$  vs. control group. DCs, dendritic cells; SD, standard deviation.



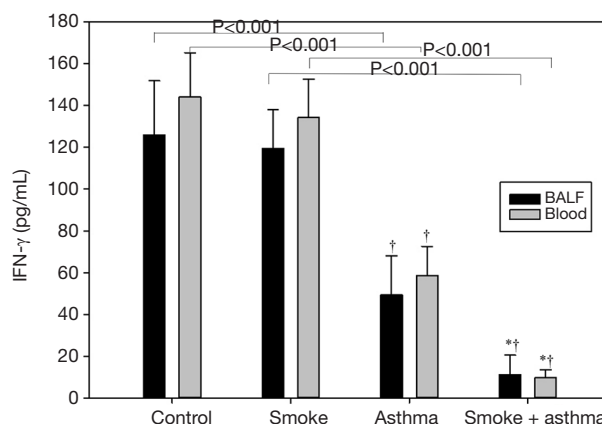
**Figure 5** Elevated CCL19 protein in peripheral blood and BALF of smoke-exposed asthmatic rats. CCL19 was measured by ELISA. Data are presented as the mean  $\pm$  SD. \*,  $P < 0.01$  vs. asthma group; †,  $P < 0.01$  vs. control group. CCL, chemokine (C-C motif) ligand; BALF, bronchioalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay.



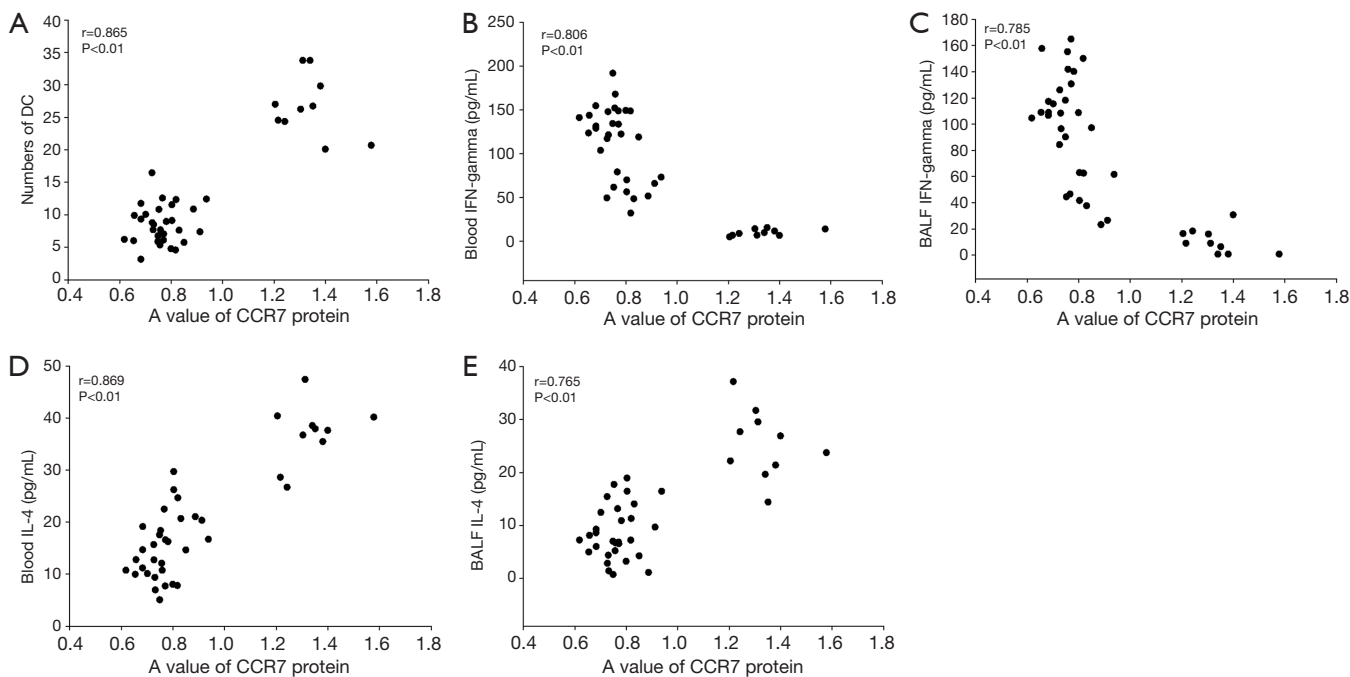
**Figure 6** Elevated expression of CCL21 protein in peripheral blood and BALF of asthmatic and smoke-exposed asthmatic rats, as determined by ELISA. Data are presented as mean  $\pm$  SD. \*,  $P < 0.01$  vs. asthma group; †,  $P < 0.01$  vs. control group. CCL, chemokine (C-C motif) ligand; BALF, bronchioalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.



**Figure 7** Elevated IL-4 protein in peripheral blood and BALF of asthmatic and smoke-exposed asthmatic rats, as measured by ELISA. Data are mean  $\pm$  SD of 10 samples per group. \*,  $P < 0.01$  vs. asthma group; †,  $P < 0.01$  vs. control group. BALF, bronchioalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; IL, interleukin



**Figure 8** Reduced expression of INF- $\gamma$  in peripheral blood and BALF of asthmatic and smoke-exposed asthmatic rats as measured by ELISA. Data are presented as mean  $\pm$  SD. \*,  $P < 0.01$  vs. asthma group; †,  $P < 0.01$  vs. control group. BALF, bronchioalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; INF, interferon.



**Figure 9** Positive correlation of lung CCR7 expression with tissue DCs number as well as blood and BALF IL-4 concentrations, and negative correlation with peripheral blood and BALF INF- $\gamma$  concentrations. CCR7 expression estimated by western blotting with  $\beta$ -actin as the internal gel loading control, DCs number by OX-62 immunostaining, and chemokine levels by ELISA. (A) Expression of CCR7 in lungs vs. DCs number; (B) expression of CCR7 in lungs vs. blood INF- $\gamma$  concentration; (C) expression of CCR7 in lungs vs. BALF INF- $\gamma$  concentration; (D) expression of CCR7 in lungs vs. blood IL-4 concentration; (E) expression of CCR7 in lungs vs. BALF IL-4 concentration. Statistical calculation performed using Spearman's test. Each data point depicts the result from one rat. CCR7, CC chemokine receptor 7; BALF, bronchioalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; DCs, dendritic cells; INF, interferon; IL, interleukin.

neutrophil infiltration, inflammatory cytokine levels (such as that of tumor necrosis factor, TNF) are enhanced by smoke exposure (25,26). In the present study, we observed that DCs were increased around the airway in asthmatic rats and that this response was amplified after exposure to smoke. DCs may be involved in the inflammatory response of airways in asthma after exposure to smoke. Moreover, we found the number of DCs around the airway positively correlated with CCR7 expression, so we speculate that cigarette smoke exposure can upregulate the number of DCs and then generate a range of immunoreactions through CCR7-mediated chemotaxis.

INF- $\gamma$  is secreted by Th1 cells, as is IL-4 by Th2 cells, exclusively. These cytokines may modify expansion of other cell types, thereby regulating the Th1/Th2 balance (27,28). INF- $\gamma$  can effectively activate mononuclear macrophages, enhance macrophage cytotoxic capacity, and reduce the synthesis of IgE (29). IL-4 can induce B cell proliferation, and stimulate differentiation of B cells towards IgE-generating plasmacytes, and promote native T cell differentiation towards Th2 cells involved in the inflammation response in allergic asthma (30). In the present research, we revealed abnormal secretion of both INF- $\gamma$  and IL-4 in peripheral blood and BALF of asthmatic rats, which was further aggravated with exposure to smoking. Therefore, we speculated that cigarette smoke may interfere with the airway immune balance and worsen the airway inflammation in asthmatics. We observed that CCR7 expression was negatively correlated with INF- $\gamma$  expression and positively correlated with IL-4 expression, suggesting that the effects of smoke on Th1/Th2 balance may be partly induced by CCR7. Further study is required to explore the precise mechanisms.

In conclusion, cigarette smoke exacerbated CCR7 expression, increased the number of DCs in airway tissue, and altered both systemic and airway expression levels of IL-4 and INF- $\gamma$  in asthmatic rats. In addition, smoking enhance blood and BALF levels of the CCR7 ligands CCL19 and CCL21. Collectively, these results indicate that exposure to cigarette smoke affects the immune balance and immune response in asthma. All these changes may aggravate airway immunopathology. Hopefully further study on the signaling pathways induced by CCR7 activation in asthma with cigarette smoke exposure will show a new direction for treatment of these patients.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* All experimental procedures were approved by Animal Care Committee of Shanxi Medical University (No.2014-kp-8) and completed in compliance with Chinese Council of Animal Care Guidelines.

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