The expression and function of programmed death-ligand 1 and related cytokines in neutrophilic asthma

Yin-Ying Ren[1,2#], He-Ting Dong[1#], Jian-Yi Liao[3#], Hui-Ming Sun[1], Ting Wang[1], Wen-Jing Gu[1], Xin-Xing Zhang[1], Yong-Dong Yan[1], Wei Ji[1], Zheng-Rong Chen[1], Can-Hong Zhu[1]

[1]Department of Respiratory Disease, Children's Hospital of Soochow University, Suzhou, China; [2]Department of Pediatrics, Xi'an Fourth Hospital, Xi'an, China; [3]Department of Cardio-Thoracic Surgery, Children's Hospital of Soochow University, Suzhou, China

Contributions: (I) Conception and design: CH Zhu, ZR Chen; (II) Administrative support: ZR Chen; (III) Provision of study materials or patients: YY Ren; (IV) Collection and assembly of data: HM Sun, WJ Gu, XX Zhang, YD Yan, W Ji; (V) Data analysis and interpretation: YY Ren, HT Dong; (VI) Drafting of manuscript: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Can-Hong Zhu; Zheng-Rong Chen. Department of Respiratory Disease, Children's Hospital of Soochow University, Suzhou 215003, China. Email: Canhongzhu@sina.com; chenzhengrong@suda.edu.cn.

Background: Programmed death-ligand 1 (PD-L1) is an important immune checkpoint inhibitor. Recent studies suggest that the PD-L1-mediated pathway may be a promising target in allergic asthma. However, the mechanism by which PD-L1 represses neutrophilic asthma (NA) remains unclear. In this study, we examined correlations between the expression of PD-L1 and the production of T helper cell type 1 (Th1), T helper cell type 2 (Th2), and T helper cell type 17 (Th17) cells in pediatric patients with NA and a mouse model.

Methods: The clinical samples of 26 children with asthma and 15 children with a bronchial foreign body were collected over a period of 12 months by the Children's Hospital of Soochow University. An experimental mouse model of asthma was established to study NA. An enzyme-linked immunoassay (ELISA) was used to assess soluble PD-L1 (sPD-L1) and cytokines [e.g., interleukin (IL)-4, IL-6, interferon gamma (IFN-γ), IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF)] in bronchoalveolar lavage fluid (BALF).

Results: NA patients had significantly higher levels of sPD-L1, IL-6, IL-17, and GM-CSF in their BALF than non-NA and control patients ($P<0.05$). In a murine model of asthma, the positive rate and fluorescence intensity of PD-L1 in the NA group and the immunoglobulin G (IgG)-treated NA group were higher than in the PD-L1 antibody (Ab)-treated NA group and the phosphate-buffered saline (PBS) control group ($P<0.05$). In the plasma and the BALF of the NA group and the IgG-treatment NA group, the levels of IL-17, IL-4, tumor necrosis factor alpha (TNF-α), and granulocyte colony-stimulating were higher than those in the PBS control group ($P<0.05$). The histopathological examination of lung tissues from all mice groups showed that a large number of inflammatory cells were found around the airway in the NA group and the IgG-treatment group.

Conclusions: PD-L1 may contribute to the Th17/IL-17 immune response, which is associated with neutrophilic inflammation and asthma. A PD-L1 blockade reduces pulmonary neutrophils and mucus production.

Keywords: Soluble programmed death-ligand 1 (sPD-L1); neutrophilic asthma (NA); murine asthmatic model; T helper cell type 17/interleukin-17 immunity imbalance (Th17/IL-17 immunity imbalance); cytokines

Submitted Sep 24, 2021. Accepted for publication Nov 29, 2021.
doi: 10.21037/atm-21-5648
View this article at: https://dx.doi.org/10.21037/atm-21-5648

Introduction

Bronchial asthma is a common chronic respiratory disease. It is defined by a history of respiratory symptoms that vary over time and in intensity, and a variable expiratory airflow limitation (1,2). Asthma effects approximately 300 million individuals worldwide, and its prevalence is increasing in many developing countries (3). There are approximately 30 million asthmatic patients in China, of whom 1/3 are children (4). The prevalence of asthma in children is increasing (5).

The pathogenesis of asthma is complex. It has been shown that skewed T helper cell type 2 (Th2) cell responses and decreased T helper cell type 1 (Th1) cell responses are important mechanisms mediating asthma. Th2 cells release immunoglobulin E (IgE) and interleukins (ILs), including IL-4, IL-5, and IL-13, to activate eosinophils, mast cells, innate lymphoid cells, and other inflammation cells. They also reduce Th1 cell immune responses and decrease interferon gamma (IFN-γ) secretion, which weaken the inhibition of the Th2 immune response and cause chronic airway inflammation (6,7). Notably, approximately 50% of asthma patients do not have skewed Th2 cell immune responses. These patients do not display significant eosinophilic airway inflammation (8). Recent studies have classified asthma into two broad types: (I) eosinophilic asthma (EA); and (II) non-EA (NEA). Neutrophilic asthma (NA) is an important inflammatory phenotype of NEA. NA is associated with disease severity, airflow limitation, and steroid resistance. Its underlying mechanism is still uncertain.

T helper cell type 17 (Th17) cells represent a distinct subset of inflammatory cells characterized by a high level of production of IL-17. Th17 cells have been implicated in the immunopathology of both asthma and chronic obstructive pulmonary disease. Th17 effector cells recruit neutrophils into the airway (9,10). Th17 cells and their-related cytokines (e.g., IL-17, IL-21, and IL-22) contribute to the development of severe asthma or refractory asthma. IL-17 family cytokines also target innate immune cells and epithelial cells to produce granulocyte colony-stimulating factor (G-CSF) and IL-8 (11,12). They can also recruit and activate neutrophilic granulocytes to the airway to mediate severe inflammation (13).

Lymphocyte activation and differentiation are modulated by co-stimulatory molecules provided by antigen presenting cells (APCs). Programmed death-ligand 1 (PD-L1) is expressed on resting B cells, T cells, macrophages, and dendritic cells (DCs) (14). PD-L1 regulates T cell-mediated immune responses by acting as both a T cell co-stimulator and co-inhibitor (15,16). Recent studies have shown that PD-L1 has an important function in allergies and asthma, but the data on the function of PD-L1 and related cytokines of Th cells in NA have been limited and the definitive mechanism underlying PD-L1 in NA is unclear. we found that PD-L1 and related cytokines were linked with NA in both murine models and clinical research. We sought to study the expression levels of soluble PD-L1 (sPD-L1) in the bronchoalveolar lavage fluid (BALF) of children with NA, and analyze its correlation with the expression of Th1, Th2, and Th17-type cytokines. Further, using neutralizing antibody (Ab) specific to PD-L1 and an experimental NA mouse model, we examined the immune mechanism of PD-L1 in NA, and its relationship to Th1, Th2, and Th17 cells. Some patients with NA respond poorly to inhaled high dose of corticosteroid treatment, PD-L1 Ab might be a useful immunotherapeutic treatment of NA. We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5648).

Methods

Human subjects

Samples from 26 asthma patients were collected from the Department of Respiratory Medicine of the Children's Hospital of Soochow University from October 2015 to November 2016. The diagnosis and severity of an asthma attack were determined according to the Global Initiative for Asthma guidelines (17). The patients were allocated into a NA group or non-NA group based on the amounts of neutrophils in their BALF. Each group comprised 13 patients. Patients with ≥48% neutrophils and ≤1% eosinophils in their BALF were diagnosed with NA, while the others were diagnosed with non-NA. Fifteen children who had been hospitalized for bronchial foreign body treatment were enrolled in the control group. The inhalation time was <1 day. The patients had to have no personal or family allergic disease history or history of lung infection within the last 6 months. The study was approved by the Institutional Human Ethics Committee of the Children's Hospital of Soochow University (No. 2014009), and written consent was obtained from the parent/guardian of each child participating in this study. All procedures performed in this study involving human participants were

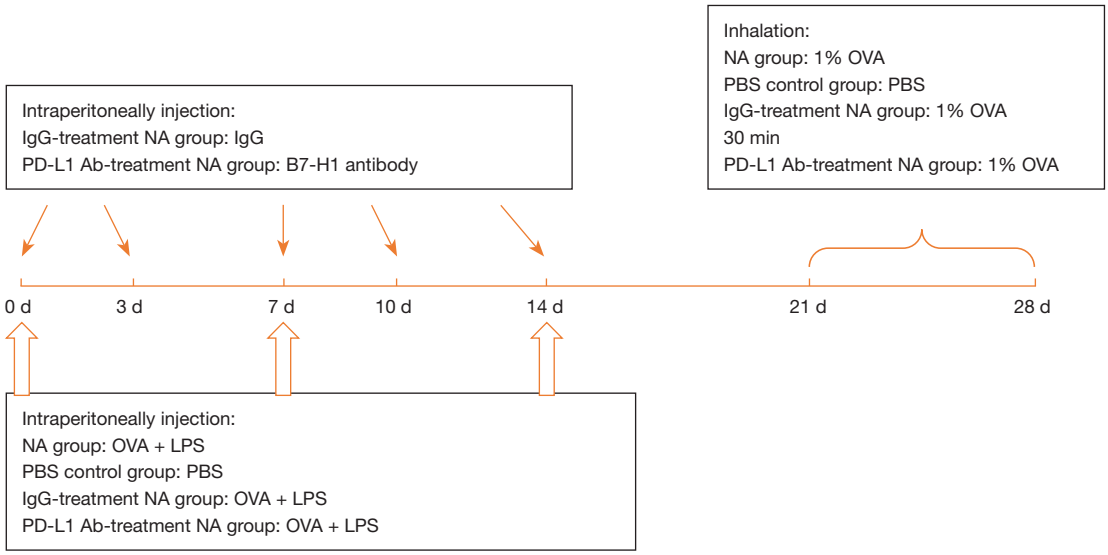

Figure 1 Experimental timeline. NA group, PBS control group, IgG-treated NA group and PD-L1 Ab-treated NA group. NA, neutrophilic asthma; PBS, phosphate-buffered saline; IgG, immunoglobulin G; PD-L1, programmed death-ligand 1; Ab, antibody; OVA, ovalbumin; LPS, lipopolysaccharides.

in accordance with the Declaration of Helsinki (as revised in 2013).

Specimen collection

The BALF was collected from the asthmatic children by bronchoalveolar lavage within 24 to 48 hours of hospitalization. To qualify, each specimen had to meet the following criteria: the percentage of recovery of the BALF had to be >40%, and red blood cells had to be <20%. The BALF of the control group was collected in a similar manner.

Mice

A total of 22 specific-pathogen-free BALB/c mice (females, aged 6–8 weeks old, weighing 18–20 g) were purchased from the JOINN Laboratories, Suzhou, China. The mice were housed in barrier cages under controlled environmental conditions (12/12 h light/dark cycle, 55%±5% humidity, 23±1 ℃). They had free access to pelleted food and pure water. Animal experiments were performed under a project license (No. SUDA20200510A02) granted by the Ethics Committee for Animal Use of Soochow University, in compliance with European Community Guidelines for the care and use of animals (18).

Experimental groups

The mice were randomly divided into the following four groups using a random number table: the NA group (n=6), the phosphate-buffered saline (PBS) control group (n=4), the immunoglobulin G (IgG)-treatment NA group (n=6), and the PD-L1 Ab-treated NA group (n=6). The NA group, the IgG-treatment NA group, and the PD-L1 Ab-treated NA group were sensitized with 0.2 mL ovalbumin (OVA) (Sigma-Aldrich, Los Angeles, California), which contained 20 μg of OVA and 10 μg of lipopolysaccharides (LPS) in PBS, and was intraperitoneally injected on days 0, 7, and 14. The IgG-treated NA mice and the PD-L1 Ab-treated NA mice were intraperitoneally injected with 20 μg of IgG and B7-H1 Ab on days 0, 3, 7, 10, and 14 after the OVA injection. The PBS control mice were intraperitoneally injected with PBS on the same days and inhaled PBS in a similar manner. From day 21, these mice were challenged with aerosolized 1% OVA daily for 7 consecutive days (see *Figure 1*).

Specimen collection

Twenty-four hours after the last inhalation of OVA, the mice were anesthetized and then killed. Blood was retro-orbitally collected from the mice, and anticoagulated with ethylenediaminetetraacetic acid. The right lung of

each mouse was ligated and removed for histological and flow cytometric analyses. The left principal bronchus was cannulated with a polyethylene tube. A total of 1.5 mL of BALF was collected.

Lung histology of mice model

The right lungs were first perfused with 4% paraformaldehyde in 0.1 M PBS, fixed with formalin, and embedded with paraffin after dehydration. The 3-μm paraffin sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) to measure neutrophil infiltration and mucus production, respectively. Other paraffin sections were incubated with the rat anti-PD-L1 monoclonal Ab (mAb) at 4 ℃ overnight after antigen repair. After incubation with an anti-rat IgG Ab conjugated with horseradish peroxidase, the specimens were developed using the diaminobenzidine coloration method.

Enzyme-linked immunoassay (ELISA) for cytokines

The concentrations of sPD-L1, IL-4, IL-6, IFN-γ, IL-17, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the BALF supernatants collected from patients were measured using ELISA kits (R&D, USA) in accordance with the manufacturer's instructions. The concentrations of the proinflammatory cytokines of IL-4, IL-6, IL-17, IFN-γ, tumor necrosis factor alpha (TNF-α), and G-CSF in the plasma and the BALF of the mice were assessed using ELISA kits (R&D, USA) in accordance with the manufacturer's instructions.

BALF cytology

The pellets of BALF were resuspended in 100 μL PBS and pipetted onto glass slides to count the total cell numbers. The cell differential count was performed using Wright-Giemsa staining. Four hundred cells were counted under a light microscope. The results are expressed as the total cell number $\times 10^4$/mL.

Flow cytometric analysis

The cells were freshly isolated from the mice lungs following digestion with Collagenase type I (Worthington Biochemical, USA) and DNase I (Worthington Biochemical, USA) for 90 min at 37 ℃. Single-cell suspension was collected and incubated with Erythrocyte lysate (Biolegend, USA) for 5 min at 4 ℃. After incubation, the cells were washed twice with PBS, and incubated with the anti-mouse CD16/32 Fc block (Biolegend, USA) at 25 ℃ for 15 min. The following fluorochrome-labeled Abs were obtained from Biolegend, USA: FITC-CD45, PE/Cy7-CD11C, APC/Cy7-CD11b, APC-F4/80, and PB-MHC-II. The cells were fixed with antibodies, and each sample was allocated to the experimental group or the control group. The experimental group cells and control group cells were incubated with PE-PD-L1 and PE-Rat IgG2a, respectively at 4 ℃ for 20–30 min. The cells were washed twice with PBS, resuspended, and analyzed by flow cytometry (Beckman Coulter Gallios, USA).

Statistical analysis

Measurement data with a normal distribution are expressed as mean ± standard deviation (SD). The chi-square test was applied to the numerical data. A one-way or multiple analysis of variance (ANOVA) was used to compare the cell classifications and cytokines among different groups. Spearman's correlation analysis was used to examine the correlation of cytokines in the BALF of NA patients. The statistical analysis was performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) using the rank-sum test and a one-way ANOVA. Values of $P<0.05$ were considered statistically significant.

Results

Medical history of cases

All 26 asthma patients had symptoms of coughing and wheezing, and 10 of the patients had fever (38.5%), 6 had shortness of breath (23.1%), and 3 had dyspnea (11.5%). There was no significant difference in the age and sex ratio among the three groups ($P>0.05$; see *Table 1*).

BALF cell classification

The percentages of lymphocytes and neutrophils in the BALF of NA patients were significantly higher than those of non-NA and control patients ($P>0.05$). The percentage of eosinophils in the BALF of non-NA patients was significantly higher than that of NA and control patients ($P<0.05$; see *Table 2*).

Table 1 Demographic characteristics

Parameters	NA patients	Non-NA patients	Control patients	χ^2/Z	P
Age (year), $\bar{x}$ ± SD	4.35±2.34	4.76±2.85	4.62±2.19	0.173	0.971
Sex (male/female)	8/5	11/2	8/7	3.188	0.203
Fever, n (%)	6 (46.15)	4 (30.76)	–	0.650	0.420
Shortness of breath, n (%)	4 (30.77)	2 (15.38)	–	0.867	0.352
Dyspnea, n (%)	2 (15.38)	1 (7.69)	–	0.377	0.539

NA, neutrophilic asthma; SD, standard deviation.

Table 2 BALF cell classification counts ($\bar{x}$ ± SD)

Parameters	NA patients	Non-NA patients	Control patients	χ^2	P
Neutrophils (%)	60.38±11.0[ab]	11.69±5.38	9.13±4.14	26.88	<0.001
Eosinophils (%)	0.15±0.38[a]	6.08±10.52[b]	0.67±0.98	7.82	0.020
Lymphocytes (%)	7.31±4.15[ab]	3.08±3.38	2.73±3.13	11.37	0.003
Phagocytes (%)	32.15±9.75[ab]	72.54±23.28[b]	87.53±5.45	25.49	<0.001

[a], <0.05 compared to the non-NA patients; [b], <0.05 compared to the control patients. BALF, bronchoalveolar lavage fluid; SD, standard deviation; NA, neutrophilic asthma.

Comparison of cytokines in the BALF of the three groups

NA patients had significantly higher levels of sPD-L1 (27.61±33.35 ng/L), IL-6 (24.19±20.43 ng/L), and IL-17 (17.12±11.46 ng/L) in their BALF than non-NA and control patients (P<0.05). Additionally, the NA patients had significantly elevated BLAF GM-CSF compared to the non-NA and control patients (P<0.05). Conversely, non-NA patients had higher IL-4 concentrations (24.42±36.96 ng/L) but lower IFN-γ (34.42±27.37 ng/L) than NA and control patients (P<0.05). There was no significant difference in sPD-L1, IL-6, and IL-17 in the BALF between non-NA and control patients, and there was no significant difference in IL-4 and IFN-γ between NA and control patients (see *Figure 2*). These data suggest that sPD-L1 is associated with increases of IL-17 and IL-6 in the BLAF of NA patients.

The linear correlation analysis of cytokines in the BALF of NA patients

To further confirm these results, we performed a linear regression analysis to examine linear correlations between the concentration of sPD-L1 and GM-CSF, IL-6, IFN-γ, and IL-17. In the NA patients, the concentration of sPD-L1 was significantly correlated with the level of GM-CSF, IL-6, IFN-γ, and IL-17 in the BALF (r=0.879, P<0.001; r=0.718, P=0.006; r=0.791, P=0.001; r=0.726, P=0.005), but was not correlated with IL-4 (r=–0.349, P=0.242). IL-17 level was positively correlated with IL-6 and GM-CSF in the BALF (r=0.890, P<0.001; r=0.817, P=0.001), but was not correlated with IL-4 (r=–0.029, P=0.924; see *Figure 3*).

The syndromes in the mouse model

To examine the role of PD-L1 in mediating NA, we used an asthmatic mouse model. Asthmatic syndromes were observed in the mice induced by OVA after a 10–15 min aerosol challenge on days 22 to 28. The asthmatic syndromes of the NA mice and the IgG-treated NA mice, such as cough, tachypnea, nodding breathing, and urine incontinence, were significantly more severe than those of the PBS control mice. The clinical symptoms of the NA treated mice with PD-L1 Ab were markedly alleviated compared to those of the IgG-treated mice. The semi-quantitative score analysis showed that the mean [interquartile range] of the NA mice of 2 [2–2] was significantly higher than that of the PBS control mice of 0 [0–1] (P<0.001). The semi-quantitative score of the PD-L1 Ab-treated NA group of 1 [1–2] was significantly lower than that of the NA asthma group (P=0.003).

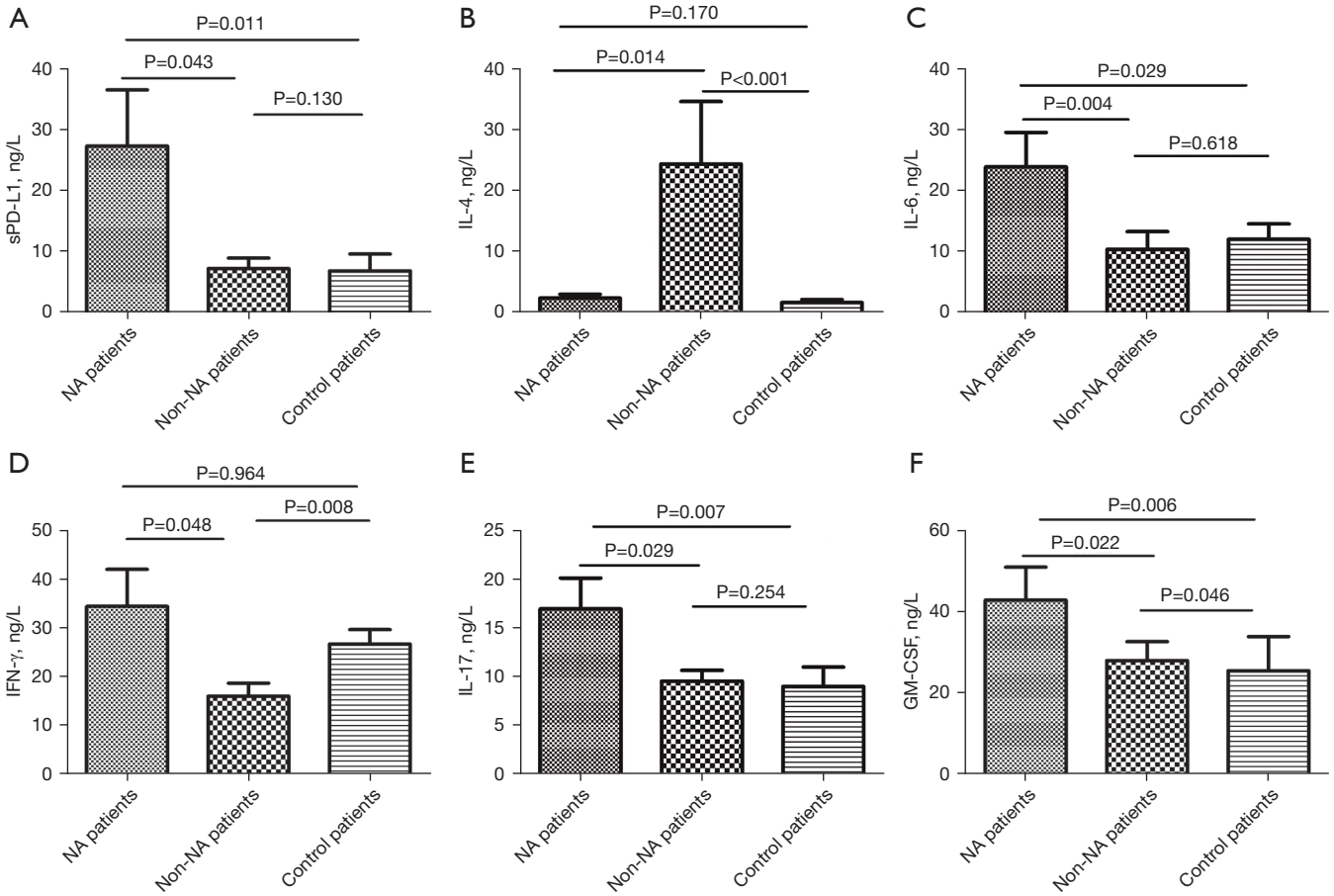

Figure 2 Concentrations of cytokines (A) sPD-L1, (B) IL-4, (C) IL-6, (D) IFN-γ, (E) IL-17, and (F) GM-CSF in the BALF of the three groups. sPD-L1, soluble programmed death-ligand 1; IL, interleukin; IFN-γ, interferon gamma; GM-CSF, granulocyte-macrophage colony-stimulating factor; BALF, bronchoalveolar lavage fluid; NA, neutrophilic asthma.

Cytology in the BALF in the mouse model

The recovery ranged from 81% to 98% of the BALF introduced. As *Table 3* shows, the NA mice and the IgG-treated NA mice showed hypercellularity compared to the PBS control mice and the PD-L1 Ab-treated NA mice. The total numbers of cells and the percentages of neutrophils were significantly higher in the NA mice and the IgG-treated NA mice than the PBS control mice and the PD-L1 Ab-treated NA mice. Additionally, the total number of cells and the percentage of neutrophils in the PD-L1 Ab-treated NA mice were significantly higher than those in the PBS control mice. There was no significant difference in eosinophils among the four groups of mice.

Flow cytometric analysis of PD-L1 in lungs

The percentages of PD-L1-positive cells in the lungs were significantly higher in the NA mice and the IgG-treated NA mice than the PD-L1 Ab-treated NA mice ($P<0.05$). No significant difference was found between the PBS control mice and the PD-L1 Ab-treated NA mice (see *Figure 4*). These data suggest that PD-L1 plays an important role in mediating the development of NA.

Comparison of cytokines in the plasma and BALF of the experimental mice

As *Table 4* shows, the production of IL-17, IL-4, TNF-α,

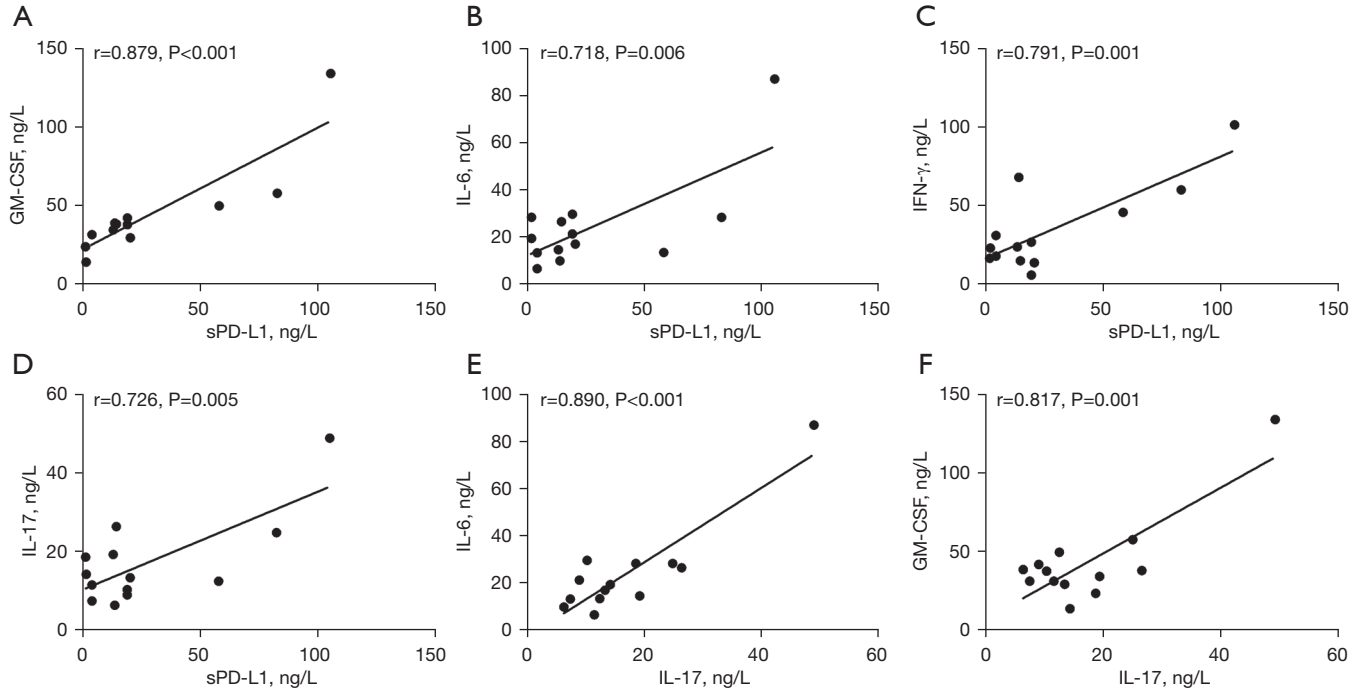

Figure 3 Linear correlation analysis of the NA groups. (A) Linear correlation between the sPD-L1 and GM-CSF; (B) linear correlation between the sPD-L1 and IL-6; (C) linear correlation between the sPD-L1 and IFN-γ; (D) linear correlation between the sPD-L1 and IL-17; (E) linear correlation between the IL-17 and IL-6; (F) linear correlation between the IL-17 and GM-CSF. NA, neutrophilic asthma; GM-CSF, granulocyte-macrophage colony-stimulating factor; sPD-L1, soluble programmed death-ligand 1; IL, interleukin; IFN-γ, interferon gamma.

Table 3 Cellular profile in the BALF in the different groups ($\bar{x}$ ± SD)

Experimental groups	Total cells (×10^4/mL)	Eosinophils (%)	Neutrophils (%)	Lymphocytes (%)	Macrophagocytes (%)
NA mice	306.3±135.7[b]	4.8±1.47	11.4±2.05[b]	36.1±5.94[b]	48.4±8.73[b]
PBS control mice	36.9±11.4	3.0±1.66	1.5±2.95	19.5±13.67	76.0±9.94
IgG-treated NA mice	290.0±96.1[b]	2.6±0.80	10.0±1.45[b]	37.2±7.16[b]	50.3±7.19[b]
PD-L1 Ab-treated NA mice	159.6±41.0[abc]	3.8±0.88	7.3±1.67[abc]	47.2±13.95[b]	41.7±13.6[b]
χ^2	14.071	6.201	15.23	8.076	10.751
P	0.003	0.102	0.002	0.044	0.013

[a], <0.05 compared to the NA mice; [b], <0.05 compared to the PBS control mice; [c], <0.05 compared to the IgG-treated mice. BALF, bronchoalveolar lavage fluid; SD, standard deviation; NA, neutrophilic asthma; PBS, phosphate-buffered saline; IgG, immunoglobulin G; PD-L1, programmed death-ligand 1; Ab, antibody.

and G-CSF was significantly more downregulated in the blood plasma of the PD-L1 Ab-treated NA mice than the NA mice and the IgG-treated NA mice (P<0.05). The PD-L1 Ab-treated NA mice had significantly higher concentrations of IL-17, IL-4, TNF-α, and G-CSF in their plasma than the PBS control mice (P<0.05). Notably, the PBS control mice had higher concentrations of IFN-γ in their plasma than the other three groups (P<0.05). There was no significant difference in the concentrations of IL-6 in the plasma among the four groups (see *Table 3*). Thus, the results suggest that PD-L1 Ab treatment decreases airway inflammation by restricting Th2 and Th17-cell responses.

We also examined the cytokines in the BALF of the experimental mice. The NA mice had higher levels of IL-17 (72.59±9.50 pg/mL), IL-4 (32.26±4.73 pg/mL), G-CSF (240.97±184.04 pg/mL), IL-6 (13.55±0.58 pg/mL), and TNF-α (309.43±53.15 pg/mL) (P<0.05; see *Figure 5*) than the PBS control mice. The levels of IL-17, IL-6, and G-CSF were significantly lower in the PD-L1 Ab-treated mice than the NA mice (P<0.05, *Figure 5*). Further, the production of IL-4 (28.81±1.99 pg/mL), G-CSF (205.47±13.51 pg/mL), and TNF-α (286.43±34.65 pg/mL) was significantly more decreased in the BALF of the PD-L1 Ab-treated mice than the BALF of the untreated NA mice (see *Figure 5*). However, PD-L1 Ab treatment did not affect the level of IFN-γ production in the BLAF (see *Figure 5*). There was no significant difference in the production of IL-6 and IL-17 between the PBS control mice and the PD-L1 Ab-treated NA mice (see *Figure 5*). These findings suggest that PD-L1 increases IL-17, IL-4, and G-CSF in the BLAF of the NA mice, thereby contributing to airway inflammation.

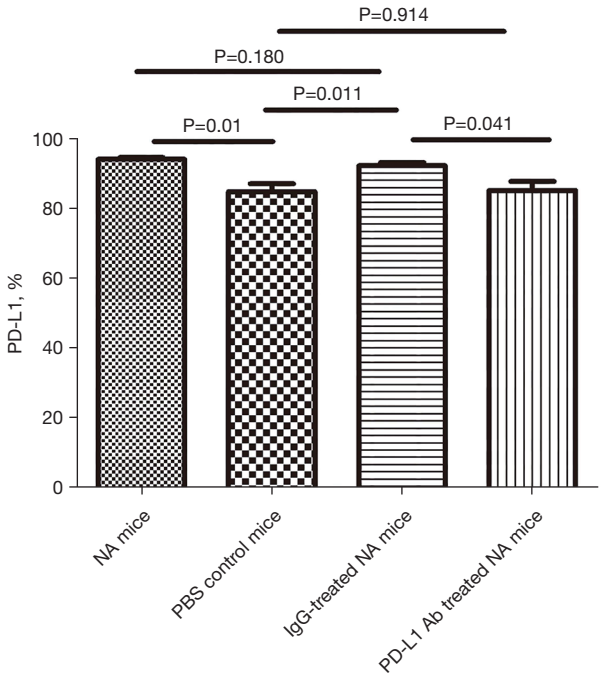

Figure 4 Percentage of PD-L1 in the lungs of different groups. PD-L1, programmed death-ligand 1; NA, neutrophilic asthma; PBS, phosphate-buffered saline; IgG, immunoglobulin G; Ab, antibody.

PD-L1 Ab treatment reduced pulmonary inflammation and suppressed mucus production

Finally, to investigate the effects of PD-L1 Ab treatment on airway inflammation and mucus production in the lung tissue, we examined pulmonary pathology using H&E and PAS staining. The mice treated with OVA + LPS or with OVA + LPS + IgG exhibited more severe airway inflammation, mucus cell metaplasia, and had higher percentages of neutrophils and lymphocytes than those treated with PD-L1 Ab (see *Figure 6A*,*6B*). As *Figure 6A* shows, the neutrophil infiltration in the lung tissues of mice treated with PD-L1 Ab was significantly reduced. Notably, PD-L1 Ab treatment also significantly decreased mucus production in the lung tissue of the mice (see *Figure 6B*). The immunohistochemical studies showed that PD-L1-positive cells were increased in the NA mice and the IgG-treated NA mice (see *Figure 6C*). As *Figure 6C* shows, PD-L1 was decreased in the lung tissue of the PD-L1 Ab-treated mice.

Table 4 Comparison of cytokines in the plasma of the four mice groups (IL-4, IL-6, IL-7, IFN-γ, TNF-α, and G-CSF) ($\bar{x}$ ± SD)

Experimental groups	IL-17 (pg/mL)	IFN-γ (pg/mL)	IL-4 (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	G-CSF (pg/mL)
NA mice	132.3±17.10	396.3±53.32	110.0±7.38	66.7±4.85	776.9±148.57	633.3±57.22
PBS control mice	97.9±3.79	502.4±43.97	67.2±15.19	53.9±21.69	466.5±57.36	467.2±14.99
IgG-treated NA mice	125.0±8.16	364.8±27.74	105.2±9.90	63.4±5.47	723.6±58.84	595.4±40.22
PD-L1 Ab-treated NA mice	113.3±9.58	348.1±44.54	93.3±11.23	64.1±8.58	650.2±61.55	532.8±56.43
χ^2	15.631	12.292	14.429	1.703	15.403	16.123
P	0.001	0.006	0.002	0.636	0.002	0.001

IL, interleukin; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; G-CSF, granulocyte colony-stimulating factor; SD, standard deviation; NA, neutrophilic asthma; PBS, phosphate-buffered saline; IgG, immunoglobulin G; PD-L1, programmed death-ligand 1; Ab, antibody.

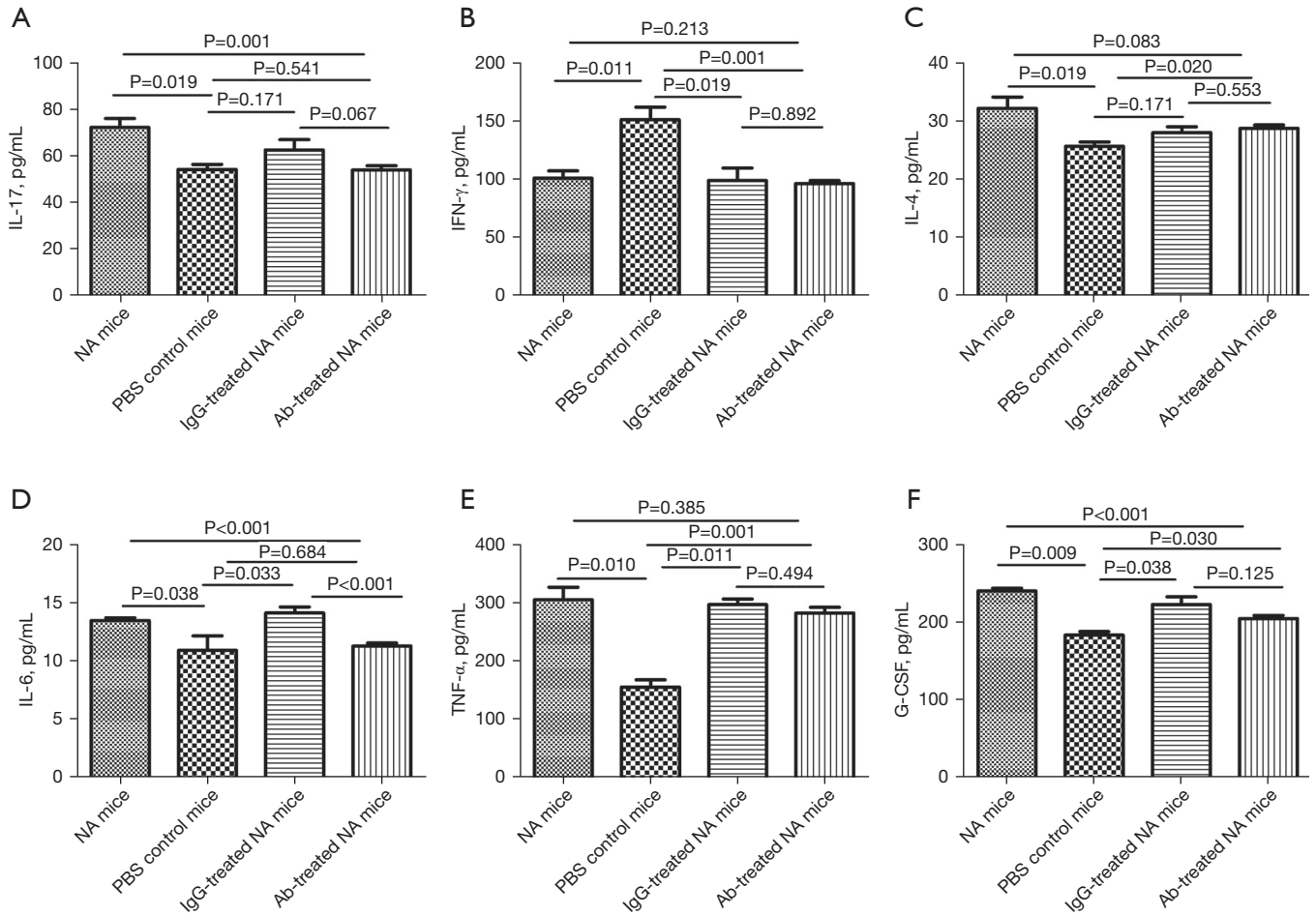

Figure 5 Concentrations of cytokines (A) IL-17, (B) IFN-γ, (C) IL-4, (D) IL-6, (E) TNF-α, and (F) G-CSF in the BALF of the different mice groups. IL, interleukin; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; G-CSF, granulocyte colony-stimulating factor; BALF, bronchoalveolar lavage fluid; NA, neutrophilic asthma; PBS, phosphate-buffered saline; IgG, immunoglobulin G; Ab, antibody.

Discussion

Immunological factors are thought to play important roles in mediating asthma. Research has shown that an imbalance of Th1/Th2 cells may be a major contributor to the development of asthma (19,20). Th2 cells release IL-4 to further enhance the differentiation of $CD4^+$ Th precursors into Th2-like cells. Th2 cells also promote B cell responses to produce IgE. Together, the Th2 cell responses and B cell-produced IgE combine to recruit eosinophils and other inflammatory cells to the airway. This process leads to immediate hypersensitivity and airway inflammation, which causes bronchial hyperresponsiveness and airway structural remodeling (21-23). Th1-derived cytokine IFN-γ, which can induce the activation of macrophages and generate immunoglobulin, is mainly characterized by its cytotoxic effects (24). IFN-γ can antagonize IL-4 by repressing Th2 cell responses and IgE production. Augmenting Th1 immune responses may effectively reduce chronic inflammatory reactions mediated by Th2 cell responses (25). In the present study, we found that IL-4 in the BALF of non-NA patients was significantly higher than that of NA and control patients. Notably, IFN-γ in the BALF of non-NA patients was significantly lower than that of NA and control patients. These findings support previous results that non-NA patients, who primarily suffered from EA, had imbalanced Th1/Th2 responses, but highly activated Th2 cell responses. In our clinical study, we also found that there was no significant difference in the IL-4 and IFN-γ in

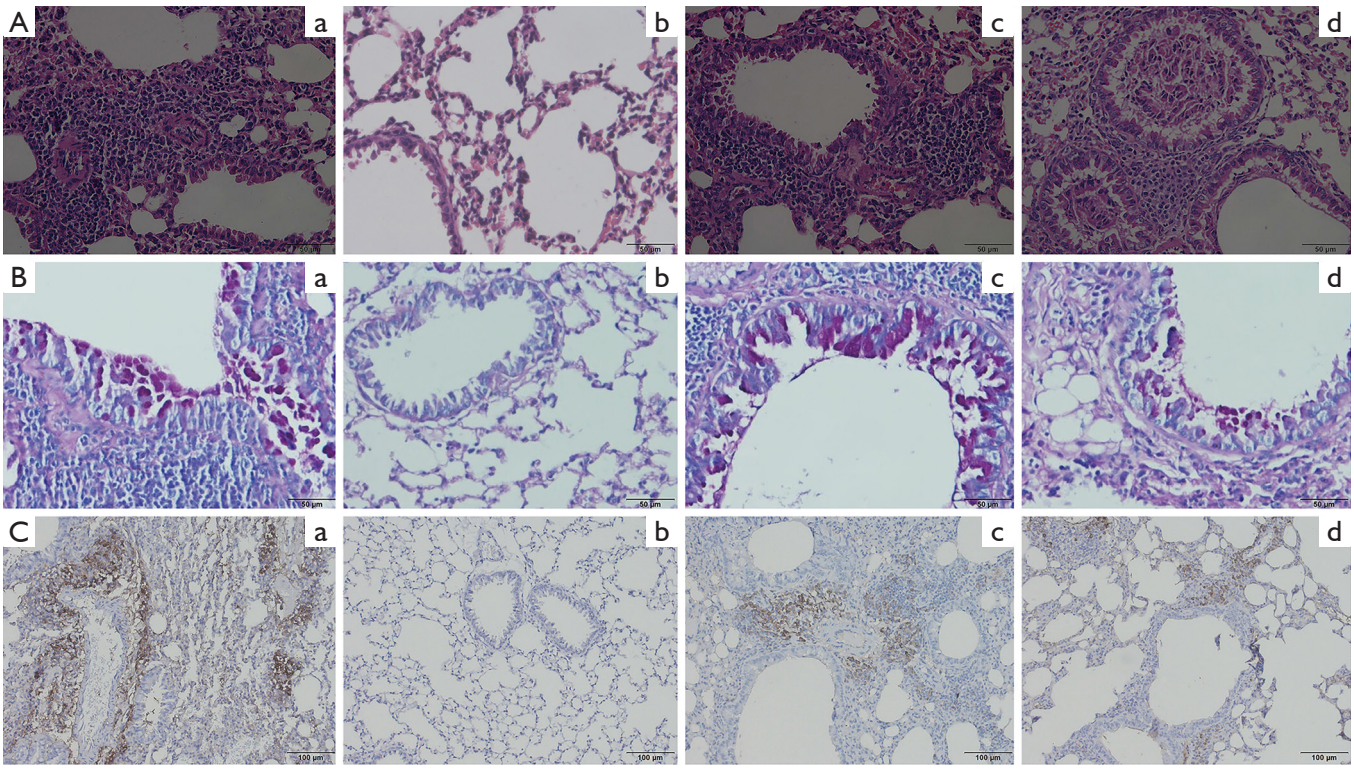

Figure 6 Histological Analysis of Lung Sections of different groups. (A) The lung tissues of different groups were stained with H&E (original magnification ×400, scale bars =50 μm); (B) the lung tissues of different groups were stained with PAS to examine mucous secretion (original magnification ×400, scale bars =50 μm); (C) PD-L1-positive cells in various groups by immunohistochemistry detection (original magnification ×200, scale bars =100 μm). a: NA mice; b: PBS control mice; c: IgG-treated NA mice; d: PD-L1 Ab-treated NA mice. H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; PD-L1, programmed death-ligand 1; NA, neutrophilic asthma; PBS, phosphate-buffered saline; IgG, immunoglobulin G; Ab, antibody.

the BALF between NA patients and control patients. This implies that the imbalance of Th1/Th2 cells may not be a major contributor to the development of NA.

Previous studies have shown that an imbalance between Th17/Treg and/or Th17/Th2 cells is related to NA, severe asthma, and refractory asthma (2,26). In severe asthmatic patients, imbalanced Th17/Treg or Th1/Th17 responses recruit and activate neutrophils into the airway (26-28). Th1 inflammatory cytokines are also effect in NA. IFN-γ, a signature Th1 cytokine, and its level was reported to be increased in BALF from severe asthmatic patients (20). Liu *et al.* (29) uncovered that Th2/Th17-low asthma that was associated with NA. And they also found that the levels of IL-1 α, IL-6, IL-8, G-CSF, and GM-CSF increased in the group of Th2/Th17-low patients. Type 2 cytokine suppression improved TH17 responses in a preclinical model of allergen-induced asthma. Th17 cells and neutrophilic inflammation increased in the lung by neutralization of IL-4 and/or IL-13 (11). Th17 cells mainly produce IL-17A, IL-17F, IL-6, IL-22, GM-CSF, and other cytokines. They cause potent proinflammation responses by recruiting and activating neutrophils (13). High level of IL-17A in serum is an independent risk factor for severe asthma that impacts neutrophil- airway smooth muscle remodeling and hypercontractility (30). IL-17 is a signature cytokine of Th17 cells that promotes IL-8 production by airway epithelial cells and smooth muscle cells. IL-8 is a chemokine which recruit neutrophils and granulocytes. As a neutrophil-specific chemotactic factor, IL-8 can recruit neutrophils into the airway. Additionally, IL-17 can stimulate airway cells to produce high levels of IL-6 and G-CSF, which stimulate neutrophil development and granulopoiesis to induce neutrophilic responses (31-33). Th1, Th2 and Th17 immunity may cooperate to enhance asthma severity or interact to suppress the activity of on another (30).

Clinical studies have shown that the levels of IL-17A and Thl7 cells in the sputum of NA patients are significantly higher than those in the sputum of EA patients, and are correlated with the percentage of neutrophils in sputum, which suggests that Thl7 cells mediate airway inflammation by IL-17A (34). Zhao *et al.* developed a mouse model by exposing OVA-induced asthmatic mice to LPS (35). Neutrophil-predominant airway inflammation was observed *in vivo* in these mice with increases of airway hyperresponsiveness (AHR) and increases of Th17 cells and IL-17A levels in the BALF. In our clinical study, we found that NA patients had significantly higher levels of sPD-L1, IL-6, IL-17, and G-CSF than non-NA and control patients, and lower IL-4 levels than non-NA patients. In NA patients, the level of IL-17 was positively correlated with G-CSF and IL-6. Thus, IL-17 may induce the secretion of G-CSF and IL-6 and serve as a mediator that induces NA. These data suggest that augmented Th17 cell responses are associated with neutrophilic inflammation and asthma. These responses may be a major mechanism in the development of NA in patients.

Eisenbarth *et al.* (36) found that in the mouse model, high dose of LPS during intranasal OVA priming resulted in Th1 cells inflammatory response dominated by neutrophils. And the level of IFN-γ also increased in high dose LPS-exposed mice. Lajoie *et al.* (37) found that IL-17A and Th2 cytokine levels were elevated in a severe asthmatic murine model. This suggests that Th17 and Th2 cells are related to severe asthmatic inflammation. IL-17A may increase neutrophilic inflammation. Cosmi *et al.* (31) described a new type of memory Th17/Th2 cells in the circulating blood of humans that appear to be significantly increased in patients with chronic severe asthma. These Th17/Th2 lymphocytes may originate from allergen-specific T lymphocytes on IL-4 stimulation. Cosmi *et al.* hypothesized that an IL-4-rich microenvironment may encourage allergen-specific Th17 lymphocytes to switch to Th17/Th2 phenotypes. Preclinical studies have shown that exposing young and adult mice to a combination of diesel exhaust particle (DEP) and house dust mite (HDM) provokes an exaggerated Th2 and mixed Th2/Th17 cell-mediated inflammatory response in the airways (38). In our study, we found that after treatment with OVA + LPS or with OVA + LPS + IgG, the lungs of the mice displayed more severe airway inflammation. The total number of cells and the percentage of neutrophils were significantly. We also found that the levels of IL-4, IL-17, G-CSF, and TNF-α in the plasma and the BALF of the NA mice were significantly higher than those in the PBS control mice, but the level of IFN-γ in the plasma and the BALF was lower than that in the control group. Additionally, despite blocking the PD-L1 signaling pathway, there was no significant difference in the eosinophil levels in the BALF between the groups. Combined with others' observations, our findings indicate that dysregulated T cell responses, including Th1, Th2, and Th17 cell responses, are associated with the development of NA. Further, a decrease in Th1 cells and the hyper-activation of Th2/Th17 responses may lead to neutrophil aggregation.

It is well known that the B7 family plays a major role in providing co-stimulatory or co-inhibitory signals for T cells (39). PD-L1 has important regulatory functions in T cell-mediated immune responses and innate immune inflammatory responses. The interaction between PD-1 and PD-L1 leads to protection against self-reactivity. PD-L1 plays a critical immunoregulatory role in the chronicity of autoimmune diseases, tumors, graft rejection, and infectious diseases (40,41). The mechanism of PD-1 and PD-L1 inhibitory signaling also plays a crucial role in neurodegenerative diseases. PD-1/PD-L1 interaction plays protective role in autoimmunity diseases such as multiple sclerosis and rheumatoid arthritis (42). However, its express high levels in tumor cells and result in T cell suppression by interfering with T cell receptor signal transduction, which protect cancer cells from attack of immune response (43). Studies have also shown that PD-L1/programmed death-1 (PD-1) inhibits the proliferation and activation of CD4 T and CD8 T cells and decreases the levels of IL-2 and IFN-γ (44,45). Conversely, some studies have suggested that naïve T cells are primed or trapped by co-stimulatory PD-L1 signals. Singh *et al.* (14) found that combining PD-1 from invariant natural killer T (iNKT) cells with PD-L1 from DC greatly enhanced levels of IL-4 production and decreased levels of IFN-γ. Akbari *et al.* (46) found that a significantly greater reduction in eosinophils and macrophages in the BALF led to a reduction in AHR of the PD-L1$^{-/-}$ mice compared to the WT BALB/c mice after OVA sensitization and challenge. Further, PAS staining showed that the PD-L1$^{-/-}$ mice had less cell infiltration and did not produce mucus. Mcalees *et al.* (47) found that blocking PD-L1 significantly increased the severity of AHR in the HDM-exposed C3H/HeJ mice compared to the IgG-control mice. They observed that the PD-1/PD-L1 blockade enhanced pulmonary neutrophilia and IL-17A production without affecting the Th2 cytokine synthesis of IL-4, IL-5, and IgE levels *in vivo*. They also cultured naive T cells under Th1^{-}, Th2^{-}, or Th17^{-}polarizing conditions

in the presence of IgG-Fc or rPD-L1. Similar to the $Th1^-$ and $Th17^-$ polarized cells restimulated in the presence of rPD-L1, the presence of PD-L1-Ig during polarization, decreased cytokine production of both Th1 (IFN-γ) and Th17 (IL-17A and IL-17F) cells. Conversely, the presence of rPD-L1 during Th2 polarization did not significantly affect IL-5 and IL-13 production. They observed that the PD-1/PD-L1 axis constrains the development of severe allergic asthma by targeting Th17-cell activity.

In the present study, we found that the PD-L1-blocked mice had reduced pulmonary neutrophils and mucus production compared to the NA mice and the IgG-control NA mice. Further, the levels of pulmonary neutrophils and mucus production in the PD-L1 Ab-treated mice were higher than those in the PBS control mice. The concentrations of IL-17, IL-4, G-CSF, and TNF-α in the plasma and IL-17, IL-6, and G-CSF in the BALF of the PD-L1 Ab-treated NA mice were significantly lower than those of the NA mice. The concentration of IFN-γ in the plasma and the BALF of the PD-L1 Ab-treated mice was lower than that in the PBS control mice; however, there was no significant difference between the NA mice and the IgG-control NA mice. We found that PD-L1 Ab treatment decreased pulmonary neutrophil infiltration and airway inflammation by restricting Th2 and Th17 cell responses. Conversely, the PD-L1 Ab treatment did not significantly affect the Th1 cell responses of the NA mice. PD-L1 Ab treatment has been shown to decrease neutrophil infiltration by inhibiting the function of Th2/Th17 in the NA mice, partially blocking the progression of asthma and reducing airway inflammation. However, this blocking effect is not complete. This may be due to the APCs that exist on other co-stimulatory molecules of the B7 family, such as B7-1, B7-2, B7-H2, and B7 -H3, which are also involved in T and B lymphocyte activation and immune responses. Notably, it is possible that PD-L1 Ab could be applied to ameliorate NA in patients. However, the exact molecular signaling pathways responsible for the production of these cytokines and co-stimulatory molecules remain largely unknown. Thus, we will increase our sample sizes in future studies to gain further insights into the mechanisms driving Th cell differentiation and co-stimulatory molecules to improve our understanding of NA.

This study had some limitations. First, while we analyzed the cytokines in the BALF of pediatric patients, we did not collect plasma samples; however, doing so in a future study would enable comparisons to be made. We also intend to design experiments *in vitro* to verify the findings of our study. Finally, our clinical cases and mice model sample size was relatively small; however, due to our desire to reduce the use of animals, we chose to limit the number of animals to 4–6 animals per group for this asthma study. Research with larger sample sizes needs to be conducted in the future to gather further evidence to support our findings.

Conclusions

In conclusion, our results suggested that PD-L1 may contribute to the Th1/Th2/Th17 immune response, which is associated with neutrophilic inflammation and asthma, and affects Th17 and its related cytokines (IL-17, IL-6, G-CSF). In NA, a PD-L1 blockade may decrease neutrophil infiltration and mucus production by inhibiting the function of Th2/Th17.

Acknowledgments

Funding: This work was supported by the following foundations: (I) The National Natural Science Foundation of China (Grant to WJ, No. 81570016); (II) The National Science Foundation of China (Grant to ZRC, No. 81771676 and 81970027); (III) The Health Department Project of Jiangsu Province, China (Grant to ZRC, No. Q201403); (IV) Science and Technology Plan Projects of Suzhou, Jiangsu Province, China (Grant to ZRC, No. SYS201350).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-5648

Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-5648

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-5648). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Human Ethics Committee

of the Children's Hospital of Soochow University (No. 2014009), and written consent was obtained from the parent/guardian of each child participating in this study. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The animal experiments were performed according to the European Community Guidelines for the Care and Use of Animals and approved by the Ethics Committee for Animal Use of Soochow University (No. SUDA20200510A02).

References

1. Xu P, Wang L, Chen D, et al. The application of proteomics in the diagnosis and treatment of bronchial asthma. Ann Transl Med 2020;8:132.
2. Vroman H, van den Blink B, Kool M. Mode of dendritic cell activation: the decisive hand in Th2/Th17 cell differentiation. Implications in asthma severity? Immunobiology 2015;220:254-61.
3. Global Initiative for Asthma. A pocket guide for physicians and nurses updated 2015. Available online: https://ginasthma.org/
4. Kong XY. Problems of prevention and treatment in children with asthma. Journal of Applied Clinical Pediatrics 2006;21:1043-4.
5. Hong JG. The revision points for Chinese guideline of childhood asthma. Journal of Clinical Pediatrics 2014;32:101-3.
6. Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nat Med 2012;18:693-704.
7. Gu W, Zhang X, Yan Y, et al. B7-H3 participates in the development of Asthma by augmentation of the inflammatory response independent of TLR2 pathway. Sci Rep 2017;7:40398.
8. Douwes J, Gibson P, Pekkanen J, et al. Non-eosinophilic asthma: importance and possible mechanisms. Thorax 2002;57:643-8.
9. Nakae S, Komiyama Y, Nambu A, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. Immunity 2002;17:375-87.
10. Fossiez F, Djossou O, Chomarat P, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 1996;183:2593-603.
11. Choy DF, Hart KM, Borthwick LA, et al. TH2 and TH17 inflammatory pathways are reciprocally regulated in asthma. Sci Transl Med 2015;7:301ra129.
12. Alcorn JF, Crowe CR, Kolls JK. TH17 cells in asthma and COPD. Annu Rev Physiol 2010;72:495-516.
13. Newcomb DC, Peebles RS Jr. Th17-mediated inflammation in asthma. Curr Opin Immunol 2013;25:755-60.
14. Singh AK, Stock P, Akbari O. Role of PD-L1 and PD-L2 in allergic diseases and asthma. Allergy 2011;66:155-62.
15. Goldberg MV, Maris CH, Hipkiss EL, et al. Role of PD-1 and its ligand, B7-H1, in early fate decisions of CD8 T cells. Blood 2007;110:186-92.
16. Wang Q, Yang AG, Wen WH. Synergistic effect of costimulatory molecule B7-H1 on tumor immunity. Chinese Journal of Cellular and Molecular Immunology 2013;4:437-40.
17. Bateman ED, Hurd SS, Barnes PJ, et al. Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J 2008;31:143-78.
18. The European Parliament and the Council of the European Union. Directive 2010/63/Eu of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union 2010. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF
19. Parulekar AD, Diamant Z, Hanania NA. Role of T2 inflammation biomarkers in severe asthma. Curr Opin Pulm Med 2016;22:59-68.
20. Raundhal M, Morse C, Khare A, et al. High IFN-γ and low SLPI mark severe asthma in mice and humans. J Clin Invest 2015;125:3037-50.
21. Holgate ST. Innate and adaptive immune responses in asthma. Nat Med 2012;18:673-83.
22. Robinson D, Humbert M, Buhl R, et al. Revisiting Type 2-high and Type 2-low airway inflammation in asthma: current knowledge and therapeutic implications. Clin Exp Allergy 2017;47:161-75.
23. Rogala B, Bozek A, Gluck J, et al. Prevalence of IgE-

mediated allergy and evaluation of Th1/Th2 cytokine profiles in patients with severe bronchial asthma. Postepy Dermatol Alergol 2015;32:274-80.
24. Diao M, Min J, Guo F, et al. Effects of salbutamol aerosol combined with magnesium sulfate on T-lymphocyte subgroup and Th1/Th2 cytokines of pediatric asthma. Exp Ther Med 2017;13:117-20.
25. de Araújo CC, Marques PS, Silva JD, et al. Regular and moderate aerobic training before allergic asthma induction reduces lung inflammation and remodeling. Scand J Med Sci Sports 2016;26:1360-72.
26. Hou X, Wan H, Ai X, et al. Histone deacetylase inhibitor regulates the balance of Th17/Treg in allergic asthma. Clin Respir J 2016;10:371-9.
27. Chakir J, Shannon J, Molet S, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. J Allergy Clin Immunol 2003;111:1293-8.
28. Hansbro PM, Kaiko GE, Foster PS. Cytokine/anti-cytokine therapy - novel treatments for asthma? Br J Pharmacol 2011;163:81-95.
29. Liu W, Liu S, Verma M, et al. Mechanism of TH2/TH17-predominant and neutrophilic TH2/TH17-low subtypes of asthma. J Allergy Clin Immunol 2017;139:1548-1558.e4.
30. Evasovic JM, Singer CA. Regulation of IL-17A and implications for TGF-β1 comodulation of airway smooth muscle remodeling in severe asthma. Am J Physiol Lung Cell Mol Physiol 2019;316:L843-68.
31. Cosmi L, Liotta F, Maggi E, et al. Th17 cells: new players in asthma pathogenesis. Allergy 2011;66:989-98.
32. Zhao J, Lloyd CM, Noble A. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. Mucosal Immunol 2013;6:335-46.
33. Schinnerling K, Aguillón JC, Catalán D, et al. The role of interleukin-6 signalling and its therapeutic blockage in skewing the T cell balance in rheumatoid arthritis. Clin Exp Immunol 2017;189:12-20.
34. Liao J. Relationship between Th17 cells and neutrophilic airway inflammation in childhood neutrophilic asthma. Nanning: Guangxi Medical University, 2014.
35. Zhao S, Jiang Y, Yang X, et al. Lipopolysaccharides promote a shift from Th2-derived airway eosinophilic inflammation to Th17-derived neutrophilic inflammation in an ovalbumin-sensitized murine asthma model. J Asthma 2017;54:447-55.
36. Eisenbarth SC, Piggott DA, Huleatt JW, et al. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002;196:1645-51.
37. Lajoie S, Lewkowich IP, Suzuki Y, et al. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. Nat Immunol 2010;11:928-35.
38. Acciani TH, Brandt EB, Khurana Hershey GK, et al. Diesel exhaust particle exposure increases severity of allergic asthma in young mice. Clin Exp Allergy 2013;43:1406-18.
39. Lombardi V, Singh AK, Akbari O. The role of costimulatory molecules in allergic disease and asthma. Int Arch Allergy Immunol 2010;151:179-89.
40. Dong H, Chen X. Immunoregulatory role of B7-H1 in chronicity of inflammatory responses. Cell Mol Immunol 2006;3:179-87.
41. Waisman A, Yogev N. B7-H1 and CD8+ Treg: the enigmatic role of B7-H1 in peripheral tolerance. Eur J Immunol 2009;39:1448-51.
42. Kuol N, Stojanovska L, Nurgali K, et al. PD-1/PD-L1 in disease. Immunotherapy 2018;10:149-60.
43. Sun Y, Li L, Wu Y, et al. PD-1/PD-L1 in cardiovascular disease. Clin Chim Acta 2020;505:26-30.
44. Dong H, Zhu G, Tamada K, et al. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 1999;5:1365-9.
45. Keir ME, Butte MJ, Freeman GJ, et al. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008;26:677-704.
46. Akbari O, Stock P, Singh AK, et al. PD-L1 and PD-L2 modulate airway inflammation and iNKT-cell-dependent airway hyperreactivity in opposing directions. Mucosal Immunol 2010;3:81-91.
47. McAlees JW, Lajoie S, Dienger K, et al. Differential control of CD4(+) T-cell subsets by the PD-1/PD-L1 axis in a mouse model of allergic asthma. Eur J Immunol 2015;45:1019-29.

(English Language Editor: L. Huleatt)

Cite this article as: Ren YY, Dong HT, Liao JY, Sun HM, Wang T, Gu WJ, Zhang XX, Yan YD, Ji W, Chen ZR, Zhu CH. The expression and function of programmed death-ligand 1 and related cytokines in neutrophilic asthma. Ann Transl Med 2021;9(23):1727. doi: 10.21037/atm-21-5648

