

Correlation of PD-1/PD-L1 Signaling Pathway with Treg/Th17 Imbalance from Asthmatic Children

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Keywords

Asthma · PD-1/PD-L1 pathway · Treg cells · Th17 cells · Immune imbalance

Abstract

Background: The balance between T helper 17 (Th17) and regulatory T cells (Treg) is a new paradigm in asthma pathogenesis, but no therapeutic targets could modulate the Th17/Treg balance specifically for asthma. Since previous studies have shown the programmed cell death-1 (PD-1)/PD-ligand 1 (PD-L1) pathway is critical to immune homeostasis in this disease, we hypothesized that the PD-1/PD-L1 pathway might be involved in the regulation of Treg/Th17 imbalance in asthmatic children. **Methods:** The percentage of Treg and Th17 cells and the expression of PD-1 and PD-L1 were detected by flow cytometry in children with asthma and healthy controls. CD4⁺ T cells were stimulated with Th17 and Treg differentiating factors, and treated with anti-PD-1. Then cells were harvested and measured for Th17 and Treg percentages and Foxp3 and RORγt levels using RT-PCR. **Results:** We observed an inverse correlation between the percentages of Treg and Th17 cells, and the expression of PD-1 and PD-L1 in the two subsets also changed in the mild persistent and moderate to

severe persistent groups compared with healthy controls. In vitro, administration of anti-PD-1 could decrease Th17 percentages and RORγt mRNA, and increase Treg percentages and Foxp3 mRNA in CD4⁺ T cells of children with asthma in the mild persistent and moderate to persistent groups. Additionally, the role played by anti-PD-1 in regulating Treg/Th17 balance was further confirmed in an asthmatic mouse model. **Conclusion:** Alteration of the PD-1/PD-L1 pathway can modulate Treg/Th17 balance in asthmatic children. Treatment with anti-PD-1 posed protective effects on asthma models, providing a novel theoretical target for asthma.

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Introduction

Bronchial asthma, also known as asthma, is the most common chronic disease of the airway in childhood, which seriously threatens children's health and growth. It attracts global public health concern, due to annually

Edited by: E. Padovan, Basel. X.X. and J.-M.L. contributed equally to this work.

increasing morbidity [1, 2]. Despite great progress in the prevention and treatment of asthma with the comprehensive promotion of the Global Initiative for Asthma (GINA) guidelines, asthma still cannot be cured, resulting in heavy burdens to patients and society [3]. However, the pathogenesis of asthma is very complex and has not yet been fully understood. As a critical component of lymphocytes, CD4⁺ T cells have been reported to be involved in various asthma immunological processes [4]. Naive CD4⁺ cells could differentiate into T helper cells (including Th1, Th2, and Th17) and regulatory T cells (Treg) to execute their immunological function [5]. In past investigations, immunocyte imbalances, particularly of Th1 and Th2 cells, have been credited as the classical immune theory in asthma, but it still cannot explain its pathogenesis exactly [6, 7]. Moreover, it is still a big challenge for asthma to convert the concept of Th1/Th2 into clinical practice without deleterious side effects, although the regulation of Th1/Th2 imbalance has been widely accepted as a major cause for asthmatic disease [8]. There is evidence that some asthmatic patients did not achieve the desired effects after applied cell targeting therapy [9], and nonatopic asthmatic patients also presented a non-Th2 type airway inflammation [10]. Recently, the discovery of Treg/Th17 cell imbalance has also enhanced the additional layers of complexity to better understanding the etiology of asthma [11]. Imbalances of Th17/Treg cells have been demonstrated in patients with allergic asthma, and were shown to be associated with moderate to severe asthma [12, 13]. Choy et al. [14] have identified Th2-high, Th17-high, and Th2/-17-low 409 clusters of patients with asthma, and combination therapies targeting both Th2 and Th7 pathways could provide additional efficacy over single inhibition. In addition, the elevated plasma IL-17 and IL-22, as well as the circulating Th17 cells are important features of allergic asthma and the Th17-driven immunity is paralleled with the severity of asthma, as indicated by Zhao et al. [15]. More importantly, a disruption in the balance between Th17 and Treg cells has been reported as a crucial factor in the development of autoimmune diseases, including asthma Th17 cells, consisting of a definitive lineage of proinflammatory Th cells, which is a potent pathogenic factor in autoallergic diseases, and in particular, asthma [16]. In contrast, Tregs have anti-inflammatory properties and play key roles in the maintenance of autoimmune tolerance [17]. Treg and Th17 cells have a close relation with each other, which is important for maintaining the homeostasis of the immune system [18], while the changes in the function or in the number

of the two cell types may lead to different diseases, including asthma [12, 19]. Therefore, reversing the imbalances may provide a new direction for the treatment of asthma.

Programmed cell death-1 (PD-1) and its ligand PD-L1, as members of CD28/B7 protein family, participate in the transmission of the T lymphocyte co-stimulatory signal and act as a key element in regulating T-cell immune response and T-cell-mediated peripheral immune tolerance [20, 21]. Furthermore, previous studies found that the PD-1/PD-L1 signaling pathway is closely correlated with autoimmune diseases, such as systemic lupus erythematosus [22], nonobese diabetes [23], experimental allergic encephalomyelitis [24], and rheumatoid arthritis [25]. Thus, targeting the PD-1/PD-L1 signaling pathway has become a new insight for investigating the treatment of autoimmune diseases [20]. A previous study conducted by Wang et al. [26] suggested that PD-1 was involved in the modulation of Treg function in patients with melanoma. Notably, the Treg/Th17 imbalance has been observed in pre-eclamptic rats, owing to the change in the PD-1/PD-L1 pathway [27]. However, few studies have focused on this relationship in asthma.

In the study of McAlees et al. [28], the PD-1/PD-L1 axis was believed to limit asthma severity not by altering the magnitude of the underlying Th2-type immune response but by constraining Th17 cell activity, which suggested that severe allergic asthma might be linked to a defective PD-1/PD-L1 regulatory axis in some individuals. Therefore, our study aims to explore the correlation of PD-1/PD-L1 signaling pathway with Treg/Th17 imbalance in peripheral blood of children with asthma, in order to further understand the molecular immunological pathogenesis of asthma and investigate the potential therapeutic target.

Materials and Methods

Study Subjects

A total of 182 children aged 3~12 years (mean age: 7.8 ± 0.6 years) were hospitalized and treated at the respiration department of Dongying People's Hospital from December 2013 to December 2016 (116 males and 66 females). According to the Global Strategy for Asthma (GINA) management and prevention criteria in 2009 [29], all patients were divided into mild intermittent ($n = 61$), mild persistent ($n = 65$), and moderate to severe persistent ($n = 56$) groups. Exclusion criteria were as follows: wheezing, cough, shortness of breath, and chest tightness caused by other diseases; patients recently using glucocorticoid drug; patients diagnosed with allergic diseases; patients with a history of asthma or taking anti-asthma drugs. In addition, 42 healthy children aged 3~14 years (25

males and 17 females; mean age, 8.2 ± 0.8 years) with no history of infection and no family allergic diseases were examined in our hospital and were enrolled as the normal control group. This study was carried out with the permission of the ethics committee of Dongying People's Hospital and all study subjects and their guardians signed written informed consents.

Asthma-Related Index Collection

In the early morning, the fasting blood sample was taken to conduct a routine blood test for counting eosinophils and neutrophils. With 30 min standing at room temperature, whole blood was centrifuged at 2,500 rpm for 10 min to separate serum, and the total serum immunoglobulin E (IgE) level was detected using an Elecsys 2010 electrochemiluminescence analyzer (Boehringer Mannheim Canada Ltd., Dorval, QC, Canada). The CHEST HI-101 (Chest Co. Ltd., Kobe, Japan) was used to detect the percentage of the forced expiratory volume in the first second (FEV₁) of the predicted value (FEV₁%) and the ratio of FEV₁ versus forced vital capacity (FVC). In addition, the childhood asthma control test (C-ACT) was used for monitoring asthma control [30].

Extraction of Peripheral Blood Mononuclear Cells

A total of 3 mL of fasting peripheral venous blood was extracted from children with asthma and healthy individuals, and heparin was used for anti-freezing. Ficoll density gradient centrifugation was carried out to separate peripheral blood mononuclear cells (PBMCs). RPMI-1640 medium containing 10% fetal calf serum was used to suspend PBMCs until 3 mL and adjust the cell number into 2×10^6 cells/mL.

Isolation and Culturing of CD4⁺ T Cells

The peripheral venous blood samples were freshly obtained from each subject and collected into a heparinized vacutainer. Afterwards, CD4⁺ T cells were separated using a RosetteSep human CD4⁺ T cell enrichment cocktail (StemCell Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions. The purity of CD4⁺ T cell isolates was >97% as measured by flow cytometry (FACScalibur; Becton Dickinson, NJ, USA). CD4⁺ T cells were cultured in 96-well plates with 5 µg/mL plate bound anti-CD3 (Biolegend, San Diego, CA, USA). Cells were aliquoted into 5×10^5 cells/well with complete RPMI culture medium (Sigma-Aldrich, St. Louis, MO, USA) containing L-glutamine and 10% fetal bovine serum (Gibco, Grand Island, NY, USA) supplemented with 100 µg/mL streptomycin (Gibco), 100 U/mL penicillin (Gibco), and 5 µg/mL antiCD28 (R&D Systems, Minneapolis, MN, USA).

PD-1 Blockade Assay

The cultured CD4⁺ T cells were stimulated into Treg by adding 100 µg/mL IL-2, 10 ng/mL TGF-β1, 10 mg/mL anti-IFN-γ, and 10 mg/mL anti-IL-4, and into Th17 by adding 10 ng/mL IL-6, 5 ng/mL TGF-β1, 10 µg/mL anti-IFN-γ, and 10 µg/mL anti-IL-4 (all BioLegend, San Diego, CA, USA) in cell cultures. Lastly, 10 µg/mL anti-PD-1 (BioLegend) were added in cell cultures. Cells were incubated at 37 °C and 5% CO₂ for 72 h.

Flow Cytometric Analysis

Before the detection of Th17 cells in CD4⁺ T cells, cells were stimulated with 50 ng/mL PMA (Sigma-Aldrich) and 1 µg/mL ionomycin (Sigma-Aldrich) in the presence of Brefeldin A (BD

Pharmingen, USA) at 37 °C for 4 h. PBMC (2×10^6 cells/mL) and harvested CD4⁺ T cell suspension was placed in a sterile centrifuge tube and mixed with CD4-FITC mAb (0.2 mg/mL), CD25-PE mAb (0.2 mg/mL), CD127-PE-Cy5.5 mAb (0.5 mg/mL), PD-1-APC mAb (0.2 mg/mL), and PD-L1-PE-Cy7mAb (0.5 mg/mL) (eBioscience, San Diego, CA, USA), followed by a supplement of proper staining buffer, and incubated for 30 min at 4 °C. With the addition of a flow staining buffer (2 mL), the suspension was centrifuged at 2,000 rpm for 10 min and washed once. The cells were collected, mixed with Foxp3-PE mAb (0.5 mg/mL) and IL-17A-PE mAb (0.5 mg/mL), shaved, and the final volume adjusted to 100 µL. The cells were incubated at room temperature in dark for 30 min. Then, the cell solution was mixed with 2 mL staining buffer, centrifuged at 2,000 rpm for 10 min, and washed twice. After the supernatant was abandoned, cells were suspended in 300 µL 4% paraformaldehyde and detected using flow cytometry (Becton Dickinson).

RT-PCR

Total RNA was extracted from CD4⁺ T cells with TRIzol reagents (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RT-PCR was performed according to the protocol of One Step SYBRH PrimeScript™ RT-PCR Kit II (TaKaRa, Shiga, Japan) and 1 mg purified RNA was provided to bring to 20 mL volume. Each target was measured in triplicate and normalized to the level of β-actin. The PCR primers of Foxp3 and RORγt used in our study have been described previously [31].

Experimental Animals and Treatments

Male Balb/c mice (4 weeks of age and free of specific pathogens) were immunized as previously reported (PMID: 24785327). Briefly, mice were immunized intraperitoneally with 25 µg of OVA (Sigma-Aldrich) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL, USA) on days 1 and 15. Then, mice were challenged for 30 min via the airway with OVA (5% OVA) each day on days 21–23 consecutively. Collectively, the mice were categorized into three groups ($n = 10$ in each group): control group, asthma group, and Anti-PD-1 group. Mice in the control group received the same schedule for sensitization and challenge with an equivalent amount of phosphate buffered saline instead of OVA. In the Anti-PD-1 group, mice were injected intraperitoneally with anti-PD-1 (200 µg/mouse; clone RMP1–14) each day from day 18 to 20 consecutively. The left lobe of the lung was used to prepare a single-cell suspension for flow cytometry for Treg and Th17 cells as described previously [32].

Statistical Analysis

All data were analyzed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Baseline data of the phenotypes of patient populations are presented as median. The Kruskal-Wallis test and Mann-Whitney U test were used to analyze differences between groups. Other measurement data in vitro were expressed as mean ± standard deviation (SD). For measurement data, comparison between two groups was analyzed by the Student *t* test, and comparison among multiple groups was analyzed using ANOVA. The enumeration data were represented as frequency, and the comparison was analyzed by χ^2 test. The correlation analysis was carried out using the Pearson method. The statistical significance was set at a *p* value less than 0.05.

Table 1. Comparison of general information between children with asthma and healthy controls

	Healthy controls	Mild intermittent	Mild persistent	Moderate to severe persistent	<i>p</i>
Age, years	9.0 (5–13)	9.0 (5–12)	9.0 (6–12)	9.0 (5–11)	0.934
Gender (M/F)	25/17	38/23	44/21	34/22	0.483
Height, cm	136.9 (103.6–161.8)	134.9 (105.8–154.5)	132.6 (108.9–157.0)	131.5 (107.3–144.6)	0.145
Weight, kg	30.9 (103.6–161.8)	32.8 (11.8–47.3)	30.7 (13.0–56.4)	28.5 (9.1–57.7)	0.584
Total IgE, IU		331.4 (26.5–569.4)	817.2 (58.7–2,205.3)*	1,208.8 (12.6–3,615.6)*, #	<0.001
Neutrophils, <i>n</i> /μL		48.7 (3.95–97.7)	53.6 (0.3–86.3)	52.0 (1.9–104.2)	0.341
Eosinophils, <i>n</i> /μL		1.12 (0.4–3.9)	2.4 (0.5–6.1)*	3.26±1.54*, #	<0.001
C-ACT		24.4 (16.5–33.8)	22.0 (14.5–29.8)*	18.6 (12.6–24.7)*#	<0.001
FEV ₁ (% predicted)		83.4 (49.1–112.9)	82.4 (50.4–113.4)	65.7 (38.9–181.2)*	<0.001
FEV ₁ /FVC, %		83.5 (70.1–101.0)	81.7(62.5–101.1)	70.8 (50.4–101.6)*	<0.001

* $p < 0.05$, compared with mild intermittent group; # $p < 0.05$, compared with mild persistent group.

Results

Comparison of General Information between Children with Asthma and Healthy Controls

As shown in Table 1, no significant differences were found in gender, age, and weight between children with asthma in different severity degrees and healthy controls (all $p > 0.05$), suggesting that the groups were comparable. There was no difference among children with asthma with different degrees of severity with respect to neutrophils ($p > 0.05$). In addition, the total IgE levels and eosinophil counts were obviously higher in the mild and moderate to severe persistent groups than in the mild intermittent group (all $p < 0.05$), which were even higher in the moderate to severe persistent group than in the mild persistent group ($p < 0.05$). However, C-ACT, FEV₁ (% predicted), and FEV₁/FVC (%) were significantly lower in the mild persistent and moderate to severe persistent groups than in the mild intermittent group (all $p < 0.05$), while they were lower in the moderate to severe persistent group than in the mild persistent group ($p < 0.05$).

Comparison of the Percentage of Treg and Th17 Cells in Peripheral Blood and Cultured CD4⁺ T Cells between Children with Asthma and Healthy Controls

As shown in Figure 1a–c, the proportion of CD4⁺CD25⁺CD127^{low} Treg, CD4⁺CD25⁺Foxp3⁺ Treg, and CD4⁺IL-17A⁺ Th17 cells did not differ between the mild intermittent group and healthy controls (all $p > 0.05$). Moreover, the percentages of CD4⁺CD25⁺CD127^{low} Treg and CD4⁺CD25⁺Foxp3⁺ Treg cells in the mild persistent and moderate to severe persistent groups were

clearly lower than in the mild intermittent group and healthy controls, while they were lower in the moderate to severe persistent group than in the mild persistent group (all $p < 0.05$), which was the exact opposite to CD4⁺IL-17A⁺ Th17 cells (all $p < 0.05$). In addition, we compared the percentages of Th17 and Treg in vitro between children with asthma and healthy controls, and the percentage of CD4⁺CD25⁺CD127^{low} Treg and CD4⁺IL-17A⁺ Th17 cells in cultured CD4⁺ T cells had the same tendency in peripheral blood (Fig. 1e, f). Furthermore, the Treg/Th17 ratio was decreased in the mild persistent and moderate to severe persistent groups compared to healthy controls (both $p < 0.01$), and the moderate to severe persistent group had a significantly lower Treg/Th17 ratio than the mild persistent group ($p < 0.05$), but no statistical difference was observed between the mild intermittent and normal control groups ($p > 0.05$; Fig. 1d).

Comparison of the Expression of PD-1 and PD-L1 on Treg and Th17 Cells in Peripheral Blood between Children with Asthma and Healthy Controls

Flow cytometry was carried out to determine the percentages of PD-1⁺ Treg and PD-L1⁺ Th17 in peripheral blood in children with asthma and healthy controls (Fig. 2). There were no differences in the percentages of PD-1⁺ Treg and PD-L1⁺ Th17, as well as PD-L1⁺ Treg and PD-1⁺ Th17, between healthy controls and the mild intermittent group ($p > 0.05$). A marked elevation of the percentages of PD-1⁺ Treg and PD-1⁺ Th17 was demonstrated in the mild persistent and moderate to persistent groups in comparison with healthy controls and the mild inter-

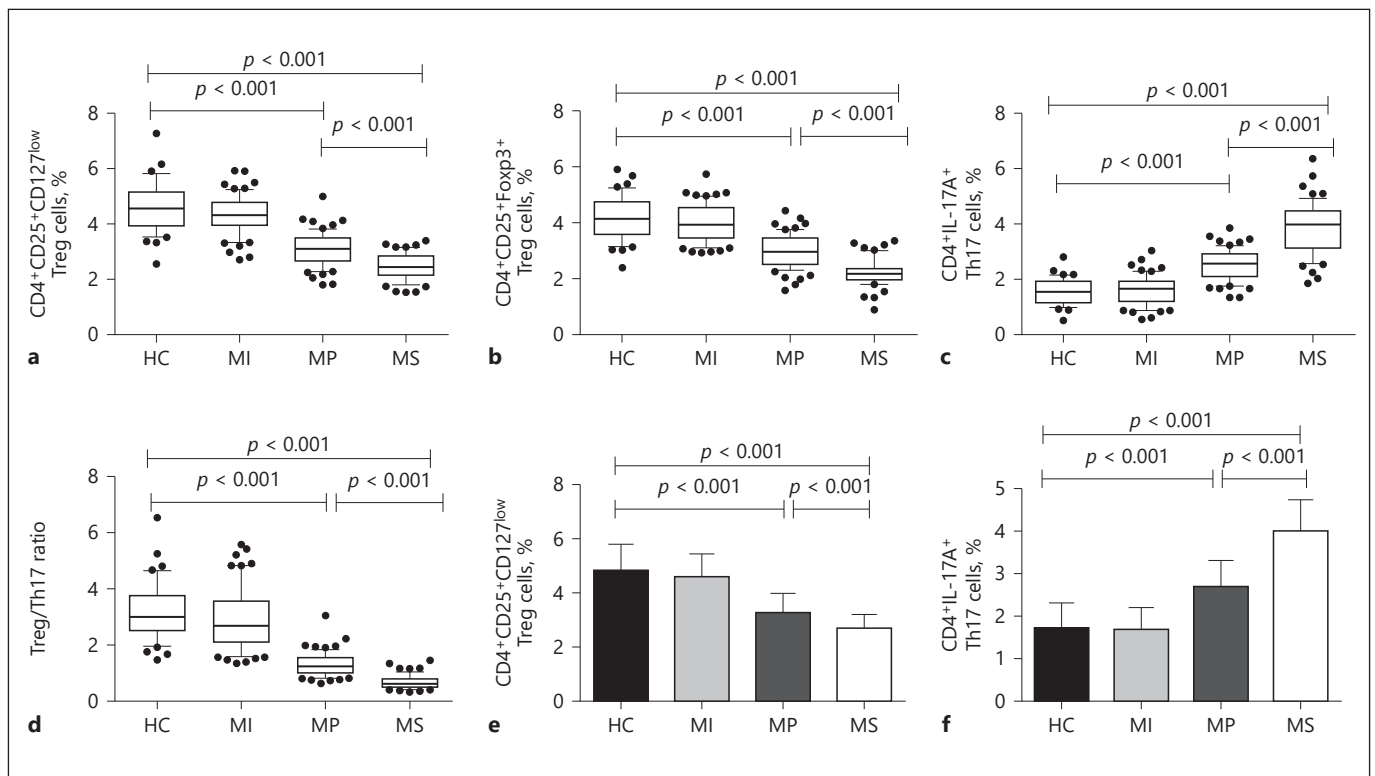


Fig. 1. The percentage of Treg and Th17 cells in peripheral blood between children with asthma and healthy controls. **a–d** Comparison of the percentage of Treg (**a**, **b**) and Th17 (**c**) cells and Treg/Th17 ratio (**d**) between children with asthma and healthy controls in peripheral blood. **e–f** Comparison of the percentage of Treg (**e**) and Th17 (**f**) cells between children with asthma and healthy controls in cultured CD4⁺ T cells. HC, healthy controls; MI, mild intermittent; MP, mild persistent; MS, moderate to severe persistent.

mittent group (all $p < 0.05$), while the percentage of PD-1⁺ Treg and PD-1⁺ Th17 in the moderate to severe persistent group was higher than that in the mild persistent group ($p < 0.05$). On the contrary, the percentages of PD-L1⁺ Treg and PD-L1⁺ Th17 were significantly reduced in the mild persistent and moderate to severe persistent groups when compared with healthy controls and the mild intermittent group (all $p < 0.05$), while they were lower in the moderate to severe persistent group than in the mild persistent group ($p < 0.05$), as exhibited in Figure 3.

Comparison of PD-1 and PD-L1 Expressions in Treg and Th17 Cells between Children with Asthma and Healthy Controls

As shown in Figure 4, healthy controls showed no significant differences in percentages of PD-1⁺ Treg and PD-1⁺ Th17 as well as percentages of PD-L1⁺ Treg and PD-L1⁺ Th17 (all $p > 0.05$). Comparisons among asthmatic children with different degrees of severity revealed that the PD-1⁺ Treg percent was notably higher than the PD-

1⁺ Th17 percent with the severity of asthma (all $p < 0.05$), whereas the PD-L1⁺ Treg percent was significantly lower than the PD-L1⁺ Th17 percent with asthma severity (all $p < 0.05$).

Correlation Analysis

Pearson correlation analysis was used to analyze the clinical information of asthmatic children and expressions of PD-1 and PD-L1 in Treg and Th17 cells. As shown in Table 2, percentages of PD-1⁺ Treg and PD-1⁺ Th17 were in a positive correlation with total IgE and eosinophils, and in a negative correlation with C-ACT, FEV₁ (% predicted), and FEV₁/FVC (%) (all $p < 0.05$). In addition, percentages of PD-L1⁺ Th17 and PD-L1⁺ Treg were negatively associated with total IgE and eosinophils, and positively linked to C-ACT, FEV₁ (% predicted) and FEV₁/FVC (%) (all $p < 0.05$), while both the percentages of PD-1⁺ Treg and PD-L1⁺ Th17 as well as PD-1⁺ Th17 and PD-L1⁺ Treg had no relation to neutrophils (both $p > 0.05$).

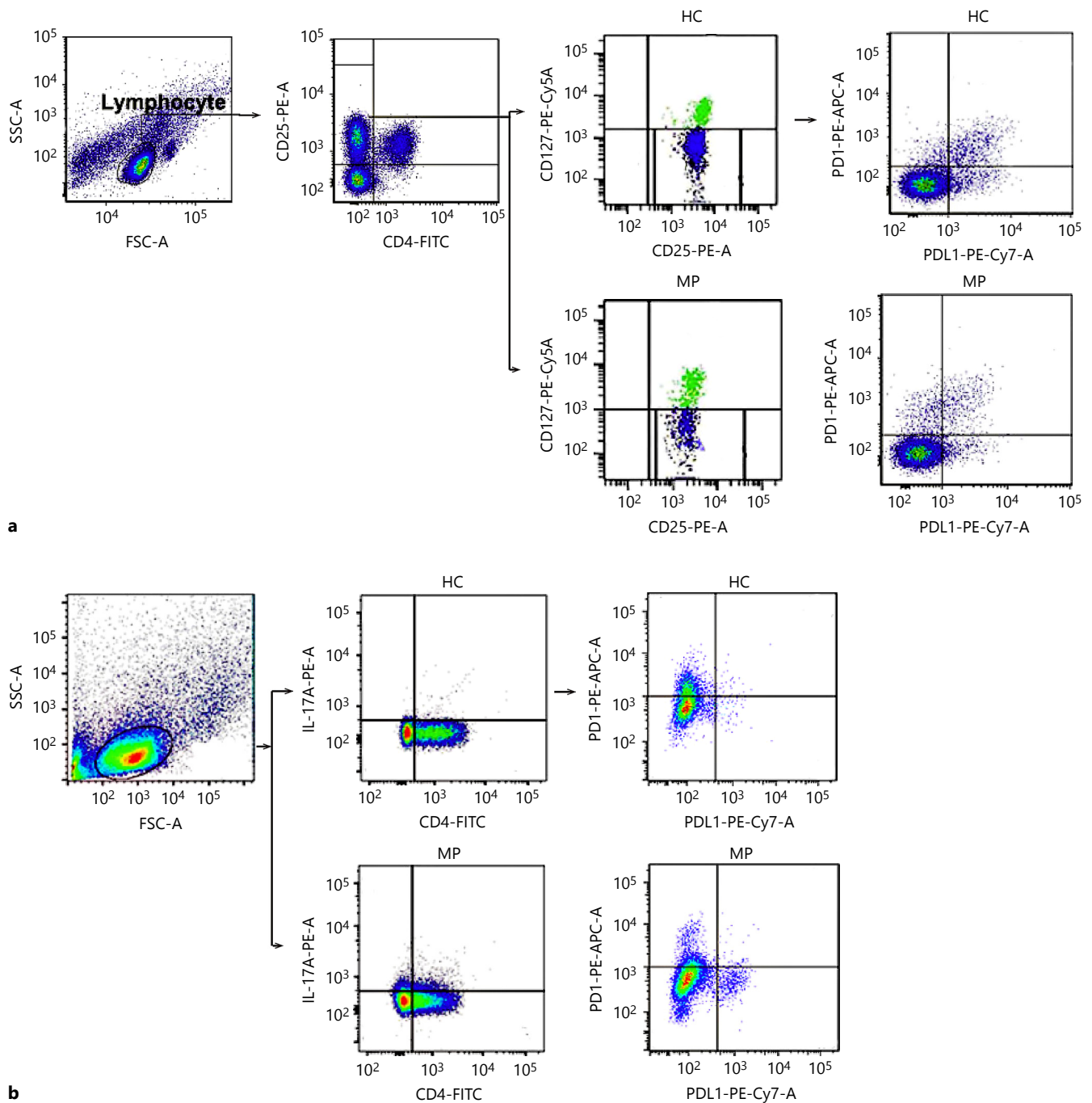


Fig. 2. Gating strategies and repertoire for flow cytometry to detect expressions of PD-1 and PD-L1 in Treg and Th17 cells. **a** flow cytometry analyses of peripheral blood Treg cells stained with mAbs to CD4, CD25, and CD127. Dot plots for PD-1-APC and PD-L1-PE-Cy7 staining of Treg cells were made. **b** flow cytometry analyses of peripheral blood Th17 cells stained with mAbs to CD4 and IL-17A. Dot plots for PD-1-APC and PD-L1-PE-Cy7 staining of Th17 cells were made. HC, healthy controls, MP, mild persistent.

Fig. 3. The percentage of PD-1 and PD-L1 in Treg and Th17 cells in peripheral blood between children with asthma and healthy controls. Data are presented as median (range). An elevation of the percentages of PD-1⁺ Treg and PD-1⁺ Th17 and a reduction of the percentages of PD-L1⁺ Treg and PD-L1⁺ Th17 were demonstrated in the mild persistent and moderate to persistent groups. HC, healthy controls; MI, mild intermittent; MP, mild persistent; MS, moderate to severe persistent.

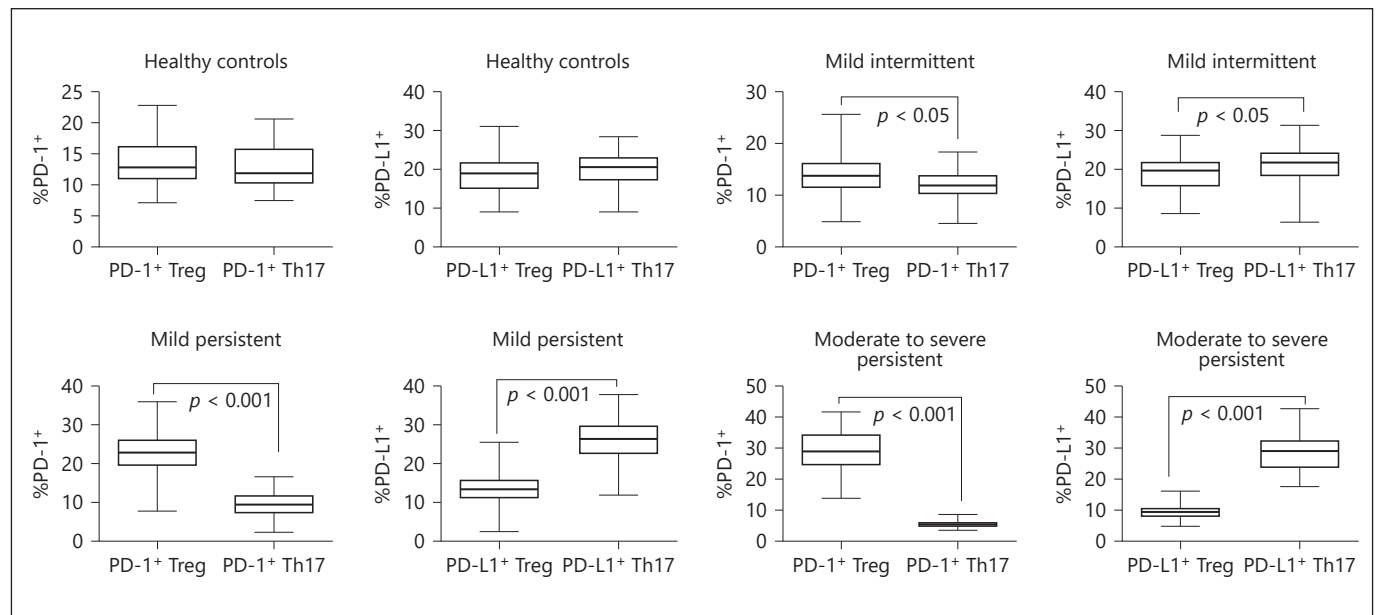
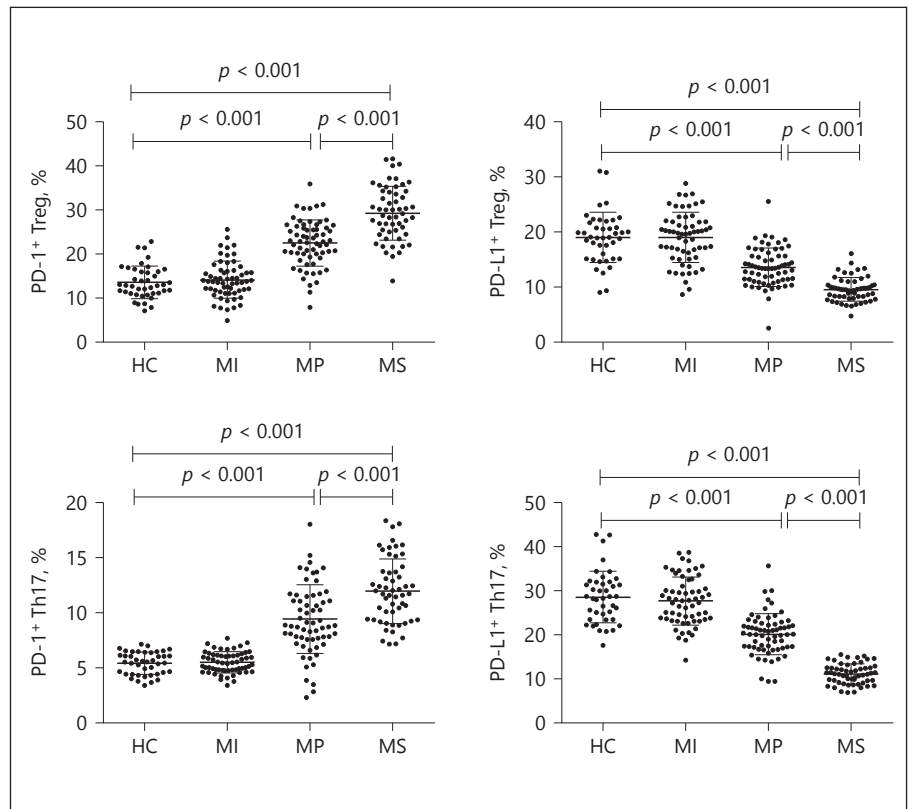


Fig. 4. Comparison of PD-1 and PD-L1 expressions in Treg and Th17 cells in peripheral blood between children with asthma and healthy controls. Data are presented as median (range). The PD-1⁺ Treg percent was higher than the PD-1⁺ Th17 percent with the severity of asthma, whereas the PD-L1⁺ Treg percent was lower than the PD-L1⁺ Th17 percent with asthma severity.

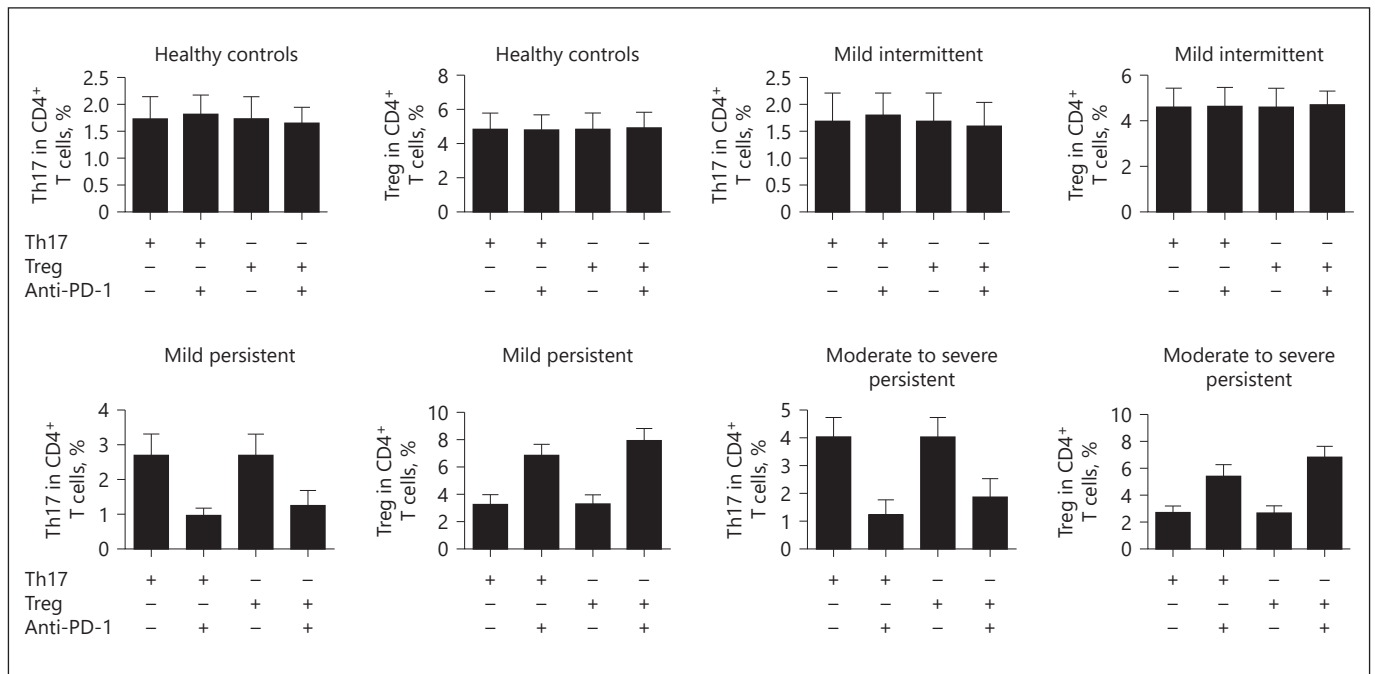


Fig. 5. Effect of anti-PD-1 treatment on differentiated Th17 and Treg in vitro from CD4⁺ T-cell cultures in children with asthma and healthy controls. Data are presented as the mean $\pm$ standard deviation (SD). There were significant reductions in Th17 percentages and increments of Treg percentages in CD4⁺ T cell cultures from children with asthma in the mild persistent and moderate to persistent groups after treatment with anti-PD-1.

Table 2. Correlation analysis of clinical information of asthmatic children and expressions of PD-1 and PD-L1 on Treg and Th17 cells

	PD-1+ Treg		PD-1+ Th17		PD-L1+ Treg		PD-L1+ Th17	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Total IgE	0.394	<0.001	0.385	<0.001	-0.413	<0.001	-0.321	<0.001
Neutrophils	0.061	0.411	0.038	0.607	0.030	0.692	0.070	0.332
Eosinophils	0.435	<0.001	0.369	<0.001	-0.407	<0.001	-0.260	<0.001
C-ACT	-0.458	<0.001	-0.448	<0.001	0.441	<0.001	0.415	<0.001
FEV ₁ (% predicted)	-0.439	<0.001	-0.336	<0.001	0.330	<0.001	0.221	0.003
FEV ₁ /FVC, %	-0.449	<0.001	-0.333	<0.001	0.406	<0.001	0.278	<0.001

Effect of Anti-PD-1 Treatment on the Differentiation of Treg and Th17 in vitro

We examined the effect of anti-PD-1 treatments on the differentiation of Th17 and Treg from CD4⁺ T cell cultures from children with asthma and healthy controls. As seen in Figure 5, there were significant reductions of Th17 percentages and increments of Treg percentages in CD4⁺ T cell cultures from children with asthma in the mild persistent and moderate to persistent groups after treatment with anti-PD-1 in a culture condition favoring Th17 or

Treg differentiation (all $p < 0.05$). Interestingly, we found that there were no differences in Th17 percentages and Treg percentages measured from CD4⁺ T cells of healthy controls and the mild intermittent group after treatment with anti-PD-1 under Th17 and Treg conversion (all $p > 0.05$). All of these data indicate that anti-PD-1 was able to decrease the differentiation of Th17 and induce differentiation of Treg in vitro in CD4⁺ T cells from children with asthma.

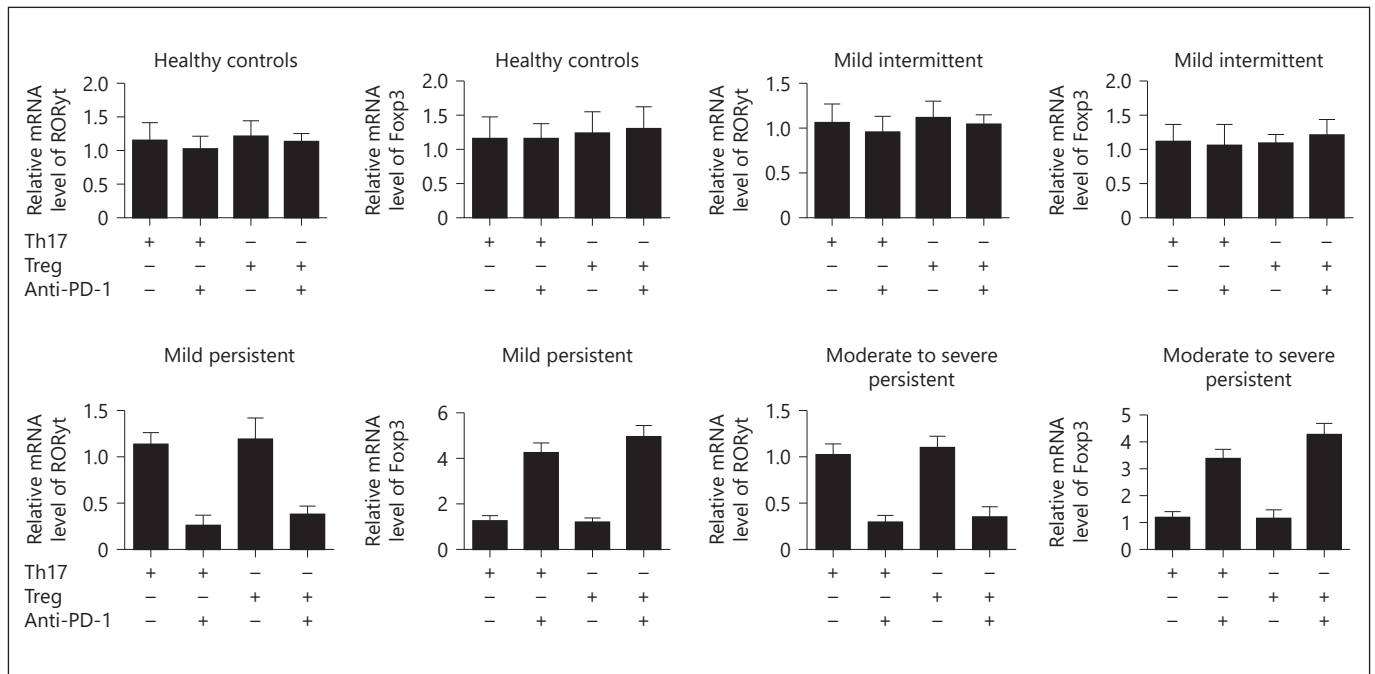


Fig. 6. Effect of anti-PD-1 treatment on the mRNA levels of Foxp3 and RORyt from CD4⁺ T-cell cultures in children with asthma and healthy controls. Data are presented as the mean $\pm$ standard deviation (SD).

Effect of Anti-PD-1 Treatment on Th17 and Treg Functions in vitro

We also detected the mRNA levels of Foxp3 and RORyt, which are important transcriptional regulators that regulate Treg and Th17 cell differentiation and function. As seen in Figure 6, we found that anti-PD-1 treatments could reduce RORyt mRNA expressions significantly in the mild persistent and moderate to persistent groups in a culture condition favoring Th17 or Treg differentiation ($p < 0.05$). Conversely, Foxp3 mRNA levels increased significantly after treatment with anti-PD-1 in the mild persistent and moderate to persistent groups ($p < 0.05$). However, anti-PD-1 treatment failed to change RORyt and Foxp3 expressions in healthy controls and the mild intermittent group under Th17 and Treg conversion (all $p > 0.05$).

Effect of Anti-PD-1 Treatment on the Differentiation of Treg and Th17 Cells in Asthma Mouse Models

To determine whether anti-PD-1 treatment was associated with changes in Treg/Th17 expression in asthma mice, we identified Treg and Th17 cells via flow cytometry of lymphocytes from lung tissue. As seen in Figure 7, the proportion of Treg cells was significantly lower while the proportion of Th17 cells was significantly higher in

mice in the asthma group compared with the control group; thus, the Treg/Th17 ratio was significantly lower in the asthma group (all $p < 0.05$). However, anti-PD-1 treatment of asthmatic mice resulted in a significantly higher proportion of Treg cells and a significantly lower proportion of Th17 cells than in the asthma group, resulting in a higher ratio of Treg/Th17 cells (all $p < 0.05$).

Discussion

In our study, we found that asthmatic children in the mild persistent and moderate to severe persistent groups showed a decreased percentage of CD4⁺CD25⁺CD127^{low} Treg cells and an increased percentage of CD4⁺IL-17A⁺ Th17 cells, and the ratio of Treg/Th17 was decreased, leading to an elevation in Th17 cells and a concomitant reduction in Treg cells, especially in the moderate to severe persistent group, suggesting that a break in the Treg/Th17 balance may exist and play an important role in children with persistent asthma [12, 33]. This was similar to what has been revealed by Shi et al. [12], who also identified an enhanced Th17 cell response, the absence of Tregs, and an imbalance of Th17/Treg levels to be correlated with the incidence and development of moderate to severe asthma.

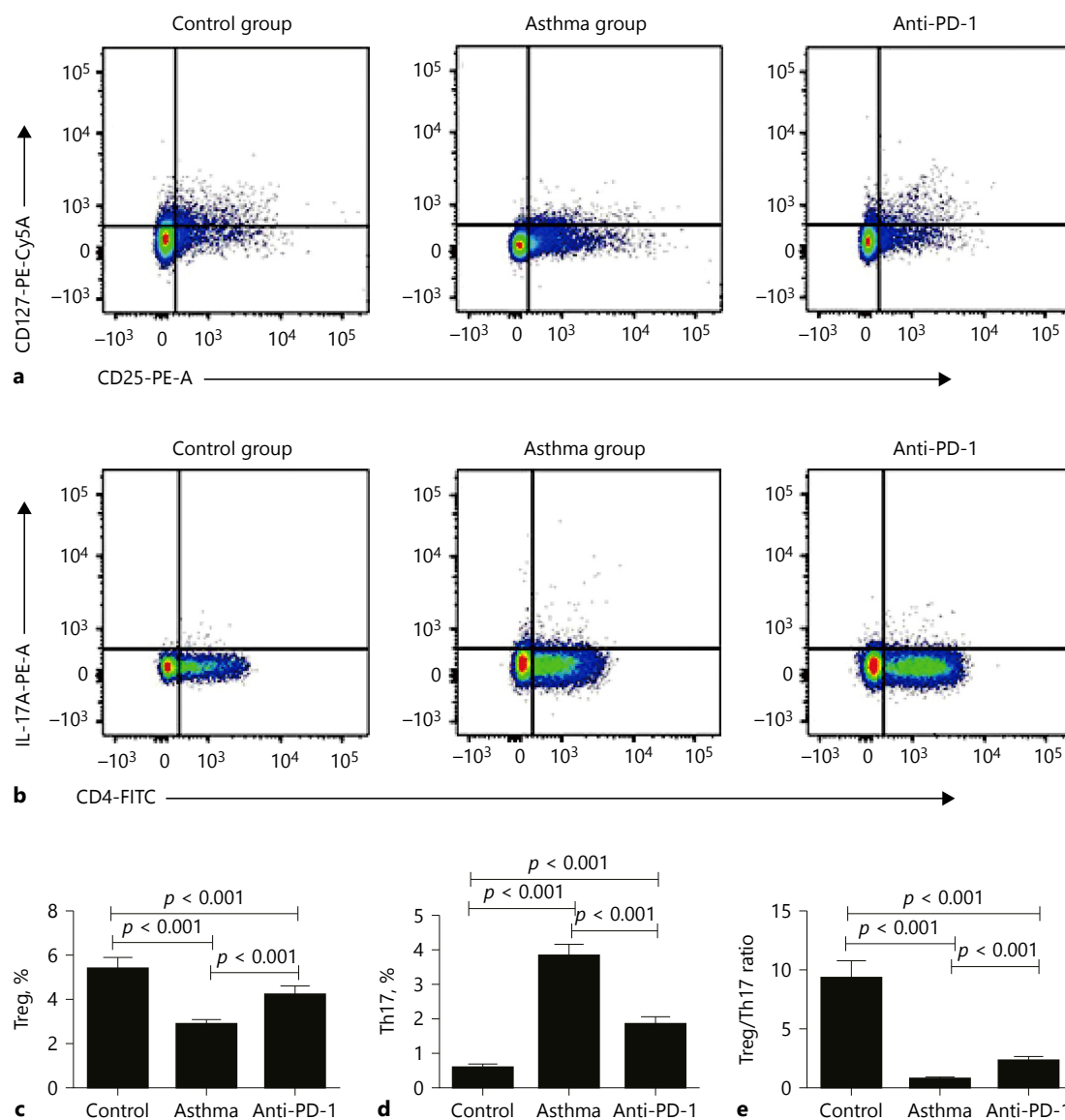


Fig. 7. Percentages of Th17 and Treg cells in lung tissues of mice. Data are presented as the mean $\pm$ standard deviation (SD). **a, b** Flow chart of Treg (**a**) and Th17 (**b**) cells in the lung tissues. **c–e** Percentages of Treg (**c**) and Th17 (**d**) cells and Treg/Th17 ratio (**e**) isolated from lung tissues of control, asthmatic, and anti-PD-1 mice.

More importantly, the young asthmatics in the mild persistent and moderate to severe persistent groups were presented with abnormally elevated PD-1⁺ Treg and a significant reduced PD-L1⁺ Treg. Such a situation might be partially caused by the abnormality of the PD-1/PD-L1 signaling pathway, resulting in the deficiency of Treg quantity. To the best of our knowledge, PD-1 and its li-

gand (PD-L1) are widely accepted as the negative regulatory co-stimulatory molecules involved in the regulation of T-cell immune response and induce body immune tolerance, ultimately having impacts on the immune regulation of various diseases, including asthma [34–36]. As previous studies reported, the PD-1/PD-L1 signaling pathway was considered crucial for immune ho-

meostasis through the promotion of Treg development and the inhibition of effector T (such as Th17) cell responses [27]. In this regard, the interactions between PD-1 and PD-L1 may exert functions to achieve a balanced immune response partially through Treg and Th17 immunity [37]. Therefore, the upregulated expression of PD-1, an inhibitory co-receptor, could interact with PD-L1 to suppress the activity of Treg cells toward blocking cells in the G0/G1 phase, and thereby attenuating Treg cell proliferation, cytokine secretion, and cytotoxic capacity [20, 38, 39]. In the study by Radziejewicz et al. [40], it was found that the PD-1/PD-L1 signaling pathway in patients with chronic hepatitis C virus negatively regulates Treg cell quantity through inhibiting STAT-5 phosphorylation, leading to a decrease in Treg cell proliferation. On the other hand, compared with healthy controls, asthmatic children from the mild persistent and moderate to severe persistent groups demonstrated a significant increase in the percentage of PD-1⁺ Th17 and an obvious decrease in the percentage of PD-L1⁺ Th17, suggesting that the abnormality of the PD-1/PD-L1 pathway was associated with the increase of Th17 cell quantity. As suggested by D'Addio et al. [41], the PD-L1 blockade could not only result in the deficiency of Treg quantity and a lower Treg/Th17 cell ratio, but also induce Treg cell apoptosis and a shift to a higher frequency of Th17 cells, thereby breaking immune tolerance. Along these lines, downregulation of PD-1 and upregulation of PD-L1 in Th17 cells will cause a decrease in the inhibitory effect of PD-1/PD-L1 signaling, thus increasing cell proliferation, as well as cytokine secretion and the viability of Th17 cells, consequently leading to the excess activation of Th17 cells in asthma. Consistent with our results, McAlees et al. [28] confirmed a relation of Th17 cells with PD-1/PD-L1 in allergic asthma. To be specific, a defective PD-1/PD-L1 might be linked to asthma, since the PD-1/PD-L1 could limit asthma severity by constraining Th17 cell activity. The importance of intracellular versus extracellular expression of PD-1 as a possible discriminating marker within the CD4⁺CD25⁺ fraction for Tregs rather than activated effector cells has been presented and discussed by Raimondi et al. [42]. Our results showed that anti-PD-1 could decrease Th17 percentages and RORγt mRNA expression, but increase Treg percentages and Foxp3 mRNA levels in CD4⁺ T cells of children with asthma, which in accordance with previous studies highlights the regulation of PD-1/PD-L1 signaling in Treg/Th17 balance. As shown by previous studies, PD-1 was expressed in Tregs and negatively regulated CD4⁺CD25⁺Foxp3⁺ Tregs function by pre-

venting STAT-5 phosphorylation in chronic hepatitis C patients [37]. Therefore, we hypothesized that Tregs was inhibited by PD-1 and subsequent IL-2-driven STAT-5 phosphorylation in asthma. Besides, the asthmatic children had a significantly increased PD-1⁺ Treg percentage compared with the PD-1⁺ Th17 percentage, and an obvious decreased PD-L1⁺ Treg percentage, in contrast to PD-L1⁺ Th17 percentage, while no significant differences were found in healthy controls. This provides a possible explanation that in an inflammatory environment, Treg cells seems to be more sensitive than Th17 cells with respect to the regulation of the PD-1/PD-L1 signaling pathway, and the decreased Treg cells play a leading role in Treg/Th17 immune imbalance, which remains to be further explored.

Furthermore, an increase in total IgE and eosinophils as well as a reduction in C-ACT, FEV₁, and FEV₁/FVC were discovered in asthmatic children. IgE, a kind of immunoglobulin, mainly mediates immediate allergy and participates in the airway inflammation and airway remodeling of obstructive airway diseases [43]. As for eosinophils, they play a central role in the establishment and outcome of bronchial inflammation in asthma [44]. During the treatment of asthma, the ACT score is an effective and simple tool for monitoring and evaluating asthma, which could help patients to evaluate the control level of asthma [45], that is, the worse the condition is, the lower the C-ACT score is. In addition, pulmonary function tests were clinically performed to assess the airflow limitation, mainly including FEV₁ (% predicted) and FEV₁/FVC (%) [46, 47]. In the current study, Pearson correlation analysis pointed out that the increased proportion of PD-1⁺ Treg and PD-L1⁺ Th17 cells and the decreased proportion of PD-1⁺ Th17 and PD-L1⁺ Treg cells in peripheral blood shared a correlation with total IgE, eosinophils, C-ACT, FEV₁, and FEV₁/FVC, further illustrating that alteration of the PD-1/PD-L1 pathway may mediate Treg/Th17 imbalance to affect the lung function and airway inflammation of asthmatic children.

To sum up, our finding showed that an altered PD-1/PD-L1 signaling pathway may favor an increase in the numbers of Th17 cells, and lead to a decrease in Tregs, which significantly breaks the Treg/Th17 balance to affect the incidence and development of young asthmatics. Hence, the PD-1/PD-L1 signaling pathway may become a new immunological target for the treatment of asthma.

Acknowledgments

We would like to thank all the people who contributed to this work.

Disclosure Statement

The authors have no conflict of interest to declare.

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