

Pdcd4 modulates markers of macrophage alternative activation and airway remodeling in antigen-induced pulmonary inflammation

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ABSTRACT

Pdcd4 has been known as a tumor-suppressor gene initially and is up-regulated during apoptosis. Surprisingly, we found that Pdcd4 was differentially expressed in the lung from E3 rats with AIPI, an animal model for asthma, but the precise role of Pdcd4 in AIPI still remained to be defined. In the present study, we first evaluated the expression of Pdcd4 in lung from control and AIPI rats with RT-qPCR, Western blot, and immunohistochemistry. Then, we investigated the effects of intervention of Pdcd4 on markers of macrophage alternative activation and airway remodeling. Upon challenging E3 rats with OVA, Pdcd4 was up-regulated in lung tissue with AIPI. Immunohistochemistry results showed that alveolar macrophages and airway epithelia expressed Pdcd4 protein. Overexpression of Pdcd4 in the rat alveolar macrophage cell line, NR8383 cells, increased the mRNA expression of arginase-1 and TGF- β 1, which are markers of macrophage alternative activation. In response to Pdcd4 RNAi in NR8383 cells, the mRNA expression of markers Fizz1, Ym1/2, arginase-1, and TGF- β 1 was decreased significantly. In addition, Pdcd4 RNAi in AIPI rats led to a decrease of the mRNA expression of Fizz1, Ym1/2, arginase-1, and TGF- β 1 in BALF cells. Finally, knockdown of Pdcd4 suppressed airway eosinophil infiltration, bronchus collagen deposition, and mucus production. Overall, these results suggest that Pdcd4 may be worthy of further investigation as a target for macrophage alternative activation and

airway remodeling in allergic pulmonary inflammation. *J. Leukoc. Biol.* 96: 000–000; 2014.

Introduction

Asthma is a chronic inflammatory disorder of the airways, and airway remodeling is the cardinal feature [1]. Airway remodeling refers to airway wall structure changes, mainly including deposition of bronchus collagen, increase of mucus secretion, hyperplasia, and hypertrophy of airway smooth muscles and angiogenesis [2]. It has been confessed that airway remodeling is present in each form of asthma at all stages of disease progression, even before the development of the airway inflammation process [3]. Ample evidence indicates that airway remodeling contributes to the development and persistence of air-flow obstruction and decreased lung function [4]. As populations become more “Westernized” and urbanized, the prevalence of asthma has increased dramatically in the past few decades, and researchers conjecture that asthma will affect ~400 million people worldwide by 2025 [5]. Thus, deciphering the cellular and molecular mechanisms responsible for airway remodeling would be crucial for the identification of potential targets for future intervention of asthma.

To learn more about the molecular mechanisms of airway remodeling, we performed SSH to analyze the differentially expressed genes in lung of E3 rats with and without AIPI in our previous study [6]. It was observed that the *Pdcd4* gene was differentially expressed in AIPI, suggesting that *Pdcd4* may play a potential role in the disease progression. *Pdcd4* is also called a death up-regulation gene in rats. Its gene is mapped to chromosome 1q55 in rat genome [7]. Pdcd4 is originally identified as a tumor-related gene in mouse epidermal carcinoma cells and consistently down-regulated in a variety of human cancers [8–14]. Very few reports indicate that Pdcd4 is

Abbreviations: AIPI=antigen-induced pulmonary inflammation, BALF=bronchoalveolar lavage fluid, eIF4=eukaryotic initiation factor-4, Fizz1=found in the inflammatory zone, NC-shRNA=negative control short hairpin RNA, OVA=ovalbumin, PAS=Periodic acid-Schiff, Pdcd4=programmed cell death 4, PPAR- γ =peroxisome proliferator-activated receptor γ , qPCR=quantitative PCR, RIPA=radioimmunoprecipitation assay, RNAi=RNA interference, shRNA=short hairpin RNA, SSH=suppression-subtractive hybridization, Ym1/2=chitinase 3-like protein 3/4

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involved in inflammatory processes. One group reported that *Pdcd4*-deficient mice are resistant to autoimmune encephalomyelitis and diabetes [15]. A recent study has shown that the polymorphism within the promoter of the *PDCD4* gene is associated with severe asthma in children [16]. However, the precise mechanisms responsible for *Pdcd4* participating in the development of inflammatory disease remain to be elucidated. Our following experiments after SSH revealed that alveolar macrophages can express *Pdcd4* protein, suggesting that *Pdcd4* may participate in the biological activity of alveolar macrophages.

Macrophages have a plastic gene expression phenotype that changes depending on the biochemical signals of the local milieu. They are polarized to an alternative-activation (or M2) phenotype under the Th2 cytokine milieu, exemplified by asthma [17, 18]. These macrophages participate in tissue repair and airway remodeling through expressing such M2 markers, such as Fizz1, chitinase-like protein Ym1/2, arginase-1, and TGF- β 1 [19–21].

In the present study, we induced AIPI in E3 rats, evaluated the difference of *Pdcd4* expression between control and AIPI rats, and tried to identify the role of *Pdcd4* in airway remodeling and macrophage alternative activation. To the best of our knowledge, this is the first report to show that *Pdcd4* plays roles in airway remodeling.

MATERIALS AND METHODS

Rats

E3 rats were bred and kept in a specific pathogen-free animal house. Rats, aged from 8 to 12 weeks, in each group were age- and sex-matched in the experiments. Animal study protocols were approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University (Permission No. 2009–12).

Induction of AIPI in rats

Rats were sensitized on Day 0 by i.p. injection of 1 mg OVA (Sigma-Aldrich, St. Louis, MO, USA), emulsified in 50 mg Al(OH)₃ (Pierce Biotechnology, Rockford, IL, USA) in a total volume of 1 mL PBS; on Day 14, the rats were subjected to intranasal challenge of 100 μ L OVA/PBS (1 mg/mL), once daily for 7 days, whereas rats from the control group were administered OVA/Al(OH)₃ for sensitization and PBS for challenge. On Day 21, the rats were anesthetized with diethyl ether and killed. Lung tissues were removed and stored at -80°C until used. The lungs were lavaged or not, depending on the experiment demands. The blood cells and spleens were collected for RNA isolation and RT-qPCR.

Overexpression of *Pdcd4* with recombinant plasmid in NR8383 cells

For the construction of the pEGFP-*Pdcd4* recombinant plasmid, rat *Pdcd4* cDNA was amplified and subcloned into the pEGFP-C1 vector at the *Xho*I and *Hind*III sites. NR8383 cells were seeded at 8×10^5 /well in six-well plates, cultured in F-12 K medium (Sigma-Aldrich) containing L-glutamine and sodium bicarbonate with 20% heat-inactivated FBS (HyClone, Logan, UT, USA), and transfected with 2 μ g pEGFP-*Pdcd4* recombinant plasmid (pEGFP-*Pdcd4* group) and pEGFP-C1 empty vector (pEGFP group) by using 8 μ L FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) as a carrier. Twenty-four hours after the transfection, the cells were harvested for RNA and protein isolation as described below. Each experiment was

performed in triplicate. Data shown are the averages of three separate experiments.

Pdcd4 knockdown with RNAi in vitro and in vivo

Four target sequences of the *Pdcd4* gene for RNAi are: GCGATTCAGT-CAGTGAGAATG, named as *Pdcd4*-shRNA1; GCGTTTGAGAAGACGT-TAACA, named as *Pdcd4*-shRNA2; GCGGAGATGTTAAGGGATTG, named as *Pdcd4*-shRNA3; and GGGACAGTGATGAGCACAAAT, named as *Pdcd4*-shRNA4. The sequence of the NC-shRNA is GTTCTCCGAACGTGT-CACGT. These sequences were inserted into the PGPU6/GFP/Neo RNAi plasmid. NR8383 cells were transfected with these RNAi plasmids, and mRNA expression of *Pdcd4* was detected by RT-qPCR. *Pdcd4*-shRNA3 was found as the most efficient to knock down *Pdcd4* expression. Then, NR8383 cells were transfected with *Pdcd4*-shRNA3 (sR-*Pdcd4* group) and NC-shRNA (sR-nc group) for three more times. After 24 h transfection, the cells were harvested for RNA and protein isolation. Each experiment was conducted in triplicate.

RNAi in vivo was performed as described previously [22]. Briefly, 32 E3 rats were intranasally instilled with saline or plasmids on Days 13 and 17 during the period of AIPI induction. The rats were divided randomly into four groups: 50 μ L saline, served as control and AIPI groups; 50 μ g NC-shRNA plasmid in 50 μ L saline, named as AIPI + sR-nc group; and 50 μ g *Pdcd4*-shRNA3 plasmid in 50 μ L saline, named as AIPI + sR-*Pdcd4* group. Rats were killed on Day 21. The lungs in each group were lavaged by instillation and withdrawal of 2 mL ice-cold PBS for three times through the tracheal route, and BALF cells were collected. After centrifugation (1000 rpm for 10 min), the cell pellet from BALF was resuspended in 1 mL PBS. Resuspension fluid of 30 μ L was used to determine the total cell numbers by using a hemocytometer. Suspension smear was prepared and stained with Wright-Giemsa staining. The eosinophils, macrophages, and lymphocytes were counted microscopically. The residue of the resuspension fluid was centrifuged again, and the cellular pellet was used to isolate total RNA.

Then, another 32 rats were treated as above without collecting BALF. Lung tissues were homogenized in RIPA lysis buffer (Beyotime, Beijing, China) for protein extraction, Western blot, and arginase activity analysis.

Lung immunohistochemistry and histochemical staining

Immunohistochemistry was performed with the Non-Biotin HRP Detection System (Zhongshan, Beijing, China). The sections were incubated with goat polyclonal antibody to rat *Pdcd4* (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1:100 dilution at 4°C overnight. Then, the slides were incubated with the poly-HRP-conjugated anti-goat IgG (Zhongshan) at 37°C for 30 min. Finally, the 3,3'-diaminobenzidine staining kit (Zhongshan) was used for color development. Images of stained sections were obtained through an optical microscope (Olympus BX51; Olympus, Tokyo, Japan), equipped with a digital camera (Olympus PD71; Olympus).

Lung tissues were stained with H&E to observe pathological changes, as described previously [23]. Masson staining was performed for the detection of bronchus collagen fibers stained blue in rat lung tissues by using a method described previously [24]. The scale of the blue color was determined by Image-Pro Plus 6 (Media Cybernetics, Rockville, MD, USA). The relative collagen area was calculated as a ratio of mean bronchus wall/mean airway lumen.

PAS staining was performed to detect bronchus mucus secretion, as described previously [25]. We selected at least three medium-sized bronchioles on each slide and calculated the ratio of PAS-positive cells with total airway epithelia. The mean of the ratios on each slide was further calculated to represent mucus secretion.

RNA quantitation

RNA isolation and reverse transcription were performed using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and an RT kit (RevertAid; Fermentas Life Science International, Ontario, Canada). RT-qPCR was performed using SYBR Premix Ex Taq II (TaKaRa, Japan) in an IQ5 real-time

TABLE 1. Information of Primers for RT-qPCR

Gene name	Sequence, 5'–3'	Annealing temperature, °C	Product size, bp
<i>Rattus norvegicus</i> Pdc4	AACTATGATGATGACCAGGAGAAC GCTAAGGACACTGCCAACAC	62	200
Ym1/2	CCACCAGGGAAGTTCACAGG TTGTTGTCCTTGAGCCACTGAG	57	191
Fizz1	AGGTCAAGGAACCTCTAGCCCATC AAGCCACAAGAACAACCACTAGC	62	139
Arginase-1 iNOS	ACTGTGGTGCTGGGTGGAG GCGGAGTGTTGATGTCAGTGTG	54 60	118 124
TGF- β 1	AAGAGACGCACAGGCAGAG CAGGCACACGCAATGATGG GGACTACTACGCCAAAGAAG	57	294
IL-12b	TCAAAAAGACAGCCACTCAGG TGCACCGAGATGTTTGTACCAGA	54	92
GAPDH	TTGCGAAGCACCCCTGGAAG CGGCAAGTTCAACGGCACAG	60	148
U6	GAAGACGCCAGTAGACTCCACGAC CTCGCTTCGGCAGCACA AACGCTTCACGAATTTGCGT	60	89

PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The total PCR system included 0.2 μ L cDNA, 0.5 μ L each primer, 5 μ L 2 $\times$ SYBR Premix, and 3.8 μ L H₂O. The changes of RNA transcripts were calculated by the $2^{-\Delta\Delta\text{comparative threshold}}$ method with rat GAPDH for lung tissues and U6 for NR8383 cells as internal controls. The information of primers, annealing temperature, and product size is depicted in Table 1.

Western blot

Lung tissues and NR8383 cells were lysed with RIPA. All of the lysates were centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant was kept. Protein concentrations were measured by the bicinchoninic acid protein assay kit (Zhuhai Joicare Biosciences, China), and then, an equal amount of the denatured protein (200 μ g for Pdc4 and 80 μ g for β -actin) was separated on SDS-PAGE and transferred onto nitrocellulose membranes, which were then incubated with corresponding primary antibodies (Santa Cruz Biotechnology) and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The bands of interest were visualized with the SuperSignal West Pico Trial Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Arginase activity assay

Arginase-1 activity was measured in rat lung tissue and NR8383 cell lysates in the presence of urea, which is a conversion product of L-arginine, according to the procedure described previously [26], with some modifications. Briefly, 400 μ g protein dissolved in 50 μ L RIPA was added into 5 μ L 10 mM MnCl₂ and 50 μ L 0.5 M L-arginine (pH 9.7). After incubated at 37°C for 60 min, the reaction was stopped with 400 μ L H₂SO₄ (96%)/H₃PO₄ (85%)/H₂O (1/3/7, v/v/v). Then, 20 μ L 0.8% α -isonitrosopropiophenone (Sigma-Aldrich), dissolved in pure ethanol, was added to the tubes, which were heated for 30 min at 95°C. After cooling, 200 μ L of each reaction mixture was transferred to microplates in duplicates, and absorbance was measured at 540 nm with an ELISA reader (Thermo Electron, Vantaa, Finland). Arginase activity was expressed as units/milligram protein (1 U was defined as the amount of enzyme that catalyzed the formation of 1 μ M urea/min).

Analysis of hydroxyproline in lung tissue

Lung tissue was weighed and scissored into small pieces. Two milliliters of 1 M HCL was added to the sample in a glass test tube. The tubes were tightened carefully and incubated at 120°C for 6 h. After cooling, the spec-

imen was transferred to a 15 mL tube. Then, pH was adjusted to six, and the volume was adjusted to 5 mL. After centrifugation at 12,000 g for 15 min, 25 μ L citric acid buffer (pH 6.0) and 50 μ L chloramine-T were added to 25 μ L fluid, and the mixture was incubated for 5 min at room temperature. Then, 50 μ L perchloric acid and 50 μ L 10% paradime thylaminobenzaldehyde were added, which were then incubated for 5 min at room temperature and another 5 min at 100°C, respectively. At last the absorbance of 100 μ L sample was read at 260 nm with an ELISA reader (Thermo Electron). The concentration of hydroxyproline was calculated by comparison with a standard curve of 6.25–100 μ g/ μ L hydroxyproline, and the result was expressed as pg/ μ g protein.

Coimmunoprecipitation

NR8383 cells were lysed with 120 μ L cell lysate (Beyotime Institute of Biotechnology, Shanghai, China) and incubated at 0°C for 30 min. All of the lysates were centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was kept. The protein was added with corresponding immunoprecipitation antibodies (Santa Cruz Biotechnology) and incubated at 4°C for 14 h. Then, 20 μ L protein A/G agarose was added to the sample for per- μ g antibody, and the mixture was incubated at 4°C for 4 h. After centrifugation at 3000 rpm for 5 min, the pellets were added with 800 μ L cell lysate and washed five times. Last, 30 μ L of the denatured protein was used for Western blot analysis.

Statistical analysis

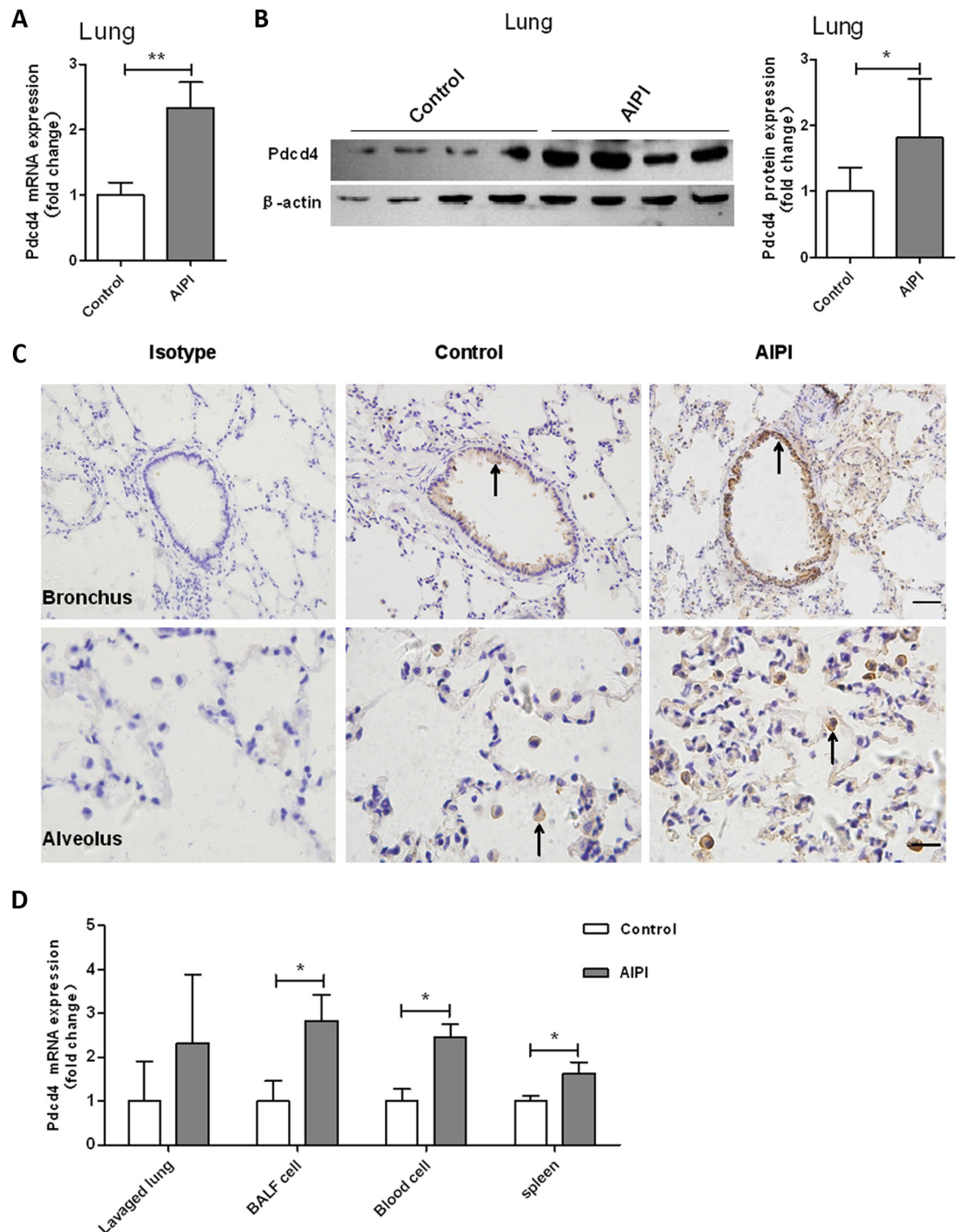
Data were expressed as mean $\pm$ SEM. The statistical analysis of differences between experimental groups was performed by Student's *t*-test. Comparisons among three or more groups were performed using the one-way ANOVA, followed by Tukey's multiple comparison tests. Statistical significance was set at $P \leq 0.05$.

RESULTS

Pdc4 was up-regulated in lung, spleen, and blood cells from AIP rats

The AIP model was induced with OVA in E3 rats. SSH was performed with lung tissue, and *Pdc4* was identified as a dif-

Figure 1. Comparison of *Pdcd4* expression in AIPI and control groups. E3 rats were sensitized with OVA and challenged with PBS (Control) or OVA (AIPI). On Day 21, the rats were killed, and the lung, spleen, and blood cells were collected. (A) *Pdcd4* mRNA expression in rat lung tissue without being lavaged, measured by RT-qPCR ($n=9$ for each group). Transcript level was normalized to GAPDH. (B) *Pdcd4* protein expression in rat lung tissue without being lavaged as assessed by Western blot. (C) Representative immunohistochemical images of *Pdcd4* expressed in rat airway epithelia and alveolar macrophages. Lungs were stained with anti-*Pdcd4* antibody. The arrows represent the expression of *Pdcd4* in cells stained brown. (Upper) Original magnification, $\times 40$; (lower) original magnification, $\times 100$; original scale bars, $50\ \mu\text{m}$. (D) *Pdcd4* mRNA expression in lavaged rat lung, BALF cells, blood cells, and spleen ($n=8$ for each group). Levels of significance were calculated by Student's *t*-test. Values are shown as the mean $\pm$ SEM. * $P < 0.05$, and ** $P < 0.01$.



ferentially expressed gene in AIPI [6]. Then, RT-qPCR, Western blot, and immunohistochemistry were performed to measure the expression of *Pdcd4* in the lung from AIPI and control rats. **Figure 1** illustrates the results obtained in these experiments. Lung tissue, without being lavaged from AIPI rats, exhibited an elevated level of *Pdcd4* expression compared with those from control rats (Fig. 1A and B).

Next, immunohistochemical staining was used to visualize *Pdcd4* protein expression in the lung. The results demon-

strated that *Pdcd4* was expressed in airway epithelia and alveolar macrophages (Fig. 1C). To identify the cell type that made the difference of *Pdcd4* expression, we compared *Pdcd4* expression in lavaged lung and BALF cells from AIPI and control rats with RT-qPCR. Results showed that *Pdcd4* mRNA expression in BALF cells increased significantly in the AIPI group (Fig. 1D). Increased *Pdcd4* expression was also observed in the lavaged lung, but this did not achieve statistical significance (Fig. 1D). Furthermore, it should be noted that *Pdcd4*

was up-regulated dramatically in spleen and blood cells from AIP rats compared with the control ones (Fig. 1D).

The regulation of Pdc4 expression influenced markers of macrophage alternative activation in vitro

Pdc4 protein expression in alveolar macrophages and the difference of *Pdc4* mRNA expression in BALF cells prompted us to investigate whether Pdc4 can exert functions in macrophages. As macrophages resemble an M2 phenotype in asthma, it is essential to clarify whether *Pdc4* affects macrophage alternative activation. The rat alveolar macrophage cell line NR8383 cells were transfected with *Pdc4* recombinant plasmid. *Fizz1*, *Ym1/2*, *arginase-1*, and *TGF- β 1* mRNA level was analyzed with RT-qPCR, and arginase activity was determined by measuring arginine-derived urea in the cell extracts. As depicted in Fig. 2, to accompany the overexpression of Pdc4 (Fig. 2A and B), *arginase-1* and *TGF- β 1* mRNA level was markedly up-regulated (Fig. 2E and F), whereas no such result was obtained in *Fizz1*, *Ym1/2* expression (Fig. 2C and D), or in M1 markers *iNOS* and *IL-12 β* expression (Fig. 2G and H). Arginase

activity was already present in the nontreated cells, as extracts obtained from these cells were able to convert L-arginine to urea (Fig. 2I). Unexpectedly, overexpression of Pdc4 in NR8383 had no effect on arginase-1 activity (Fig. 2I).

Then, we wanted to know whether down-regulation of Pdc4 can influence the markers of macrophage alternative activation. Pdc4-shRNA3 down-regulated Pdc4-level efficiently in a pretest (Fig. 3A) and was transfected into NR8383. Then, the M2 marker expression was suppressed markedly after Pdc4 knockdown (Fig. 3D–G), and arginase-1 activity decreased significantly, accompanied by Pdc4 knockdown (Fig. 3J). Similarly to the Pdc4 up-regulation result, down-regulation of Pdc4 expression in NR8383 produced no change in *iNOS* and *IL-12 β* mRNA level (Fig. 3H and I).

RNAi of Pdc4 suppressed macrophage alternative activation in vivo

We then extended the observation to determine whether *Pdc4* influenced macrophage activation in vivo. Pdc4-

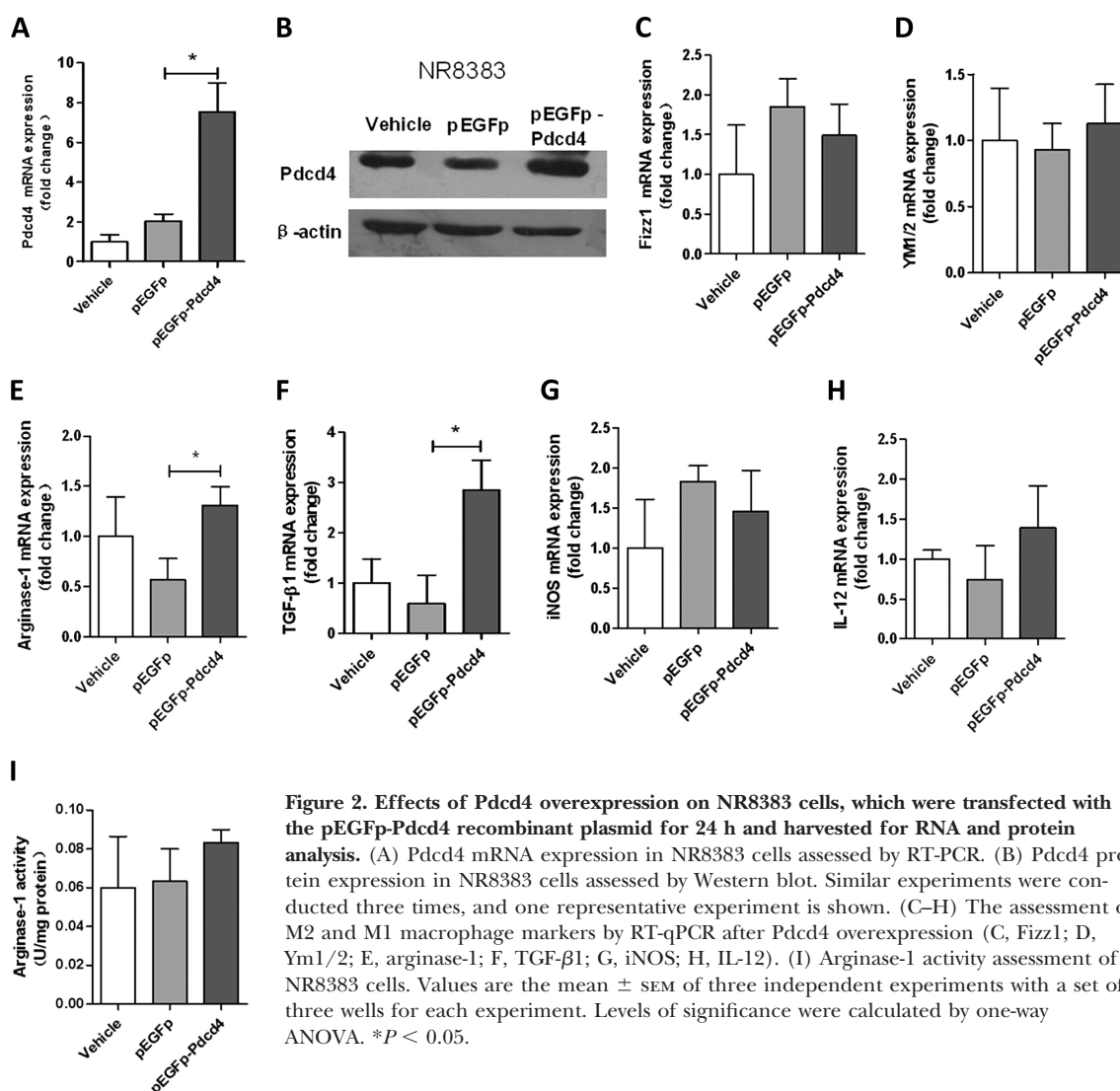


Figure 2. Effects of Pdc4 overexpression on NR8383 cells, which were transfected with the pEGFP-Pdc4 recombinant plasmid for 24 h and harvested for RNA and protein analysis. (A) Pdc4 mRNA expression in NR8383 cells assessed by RT-PCR. (B) Pdc4 protein expression in NR8383 cells assessed by Western blot. Similar experiments were conducted three times, and one representative experiment is shown. (C–H) The assessment of M2 and M1 macrophage markers by RT-qPCR after Pdc4 overexpression (C, *Fizz1*; D, *Ym1/2*; E, *arginase-1*; F, *TGF- β 1*; G, *iNOS*; H, *IL-12*). (I) Arginase-1 activity assessment of NR8383 cells. Values are the mean $\pm$ SEM of three independent experiments with a set of three wells for each experiment. Levels of significance were calculated by one-way ANOVA. * $P < 0.05$.

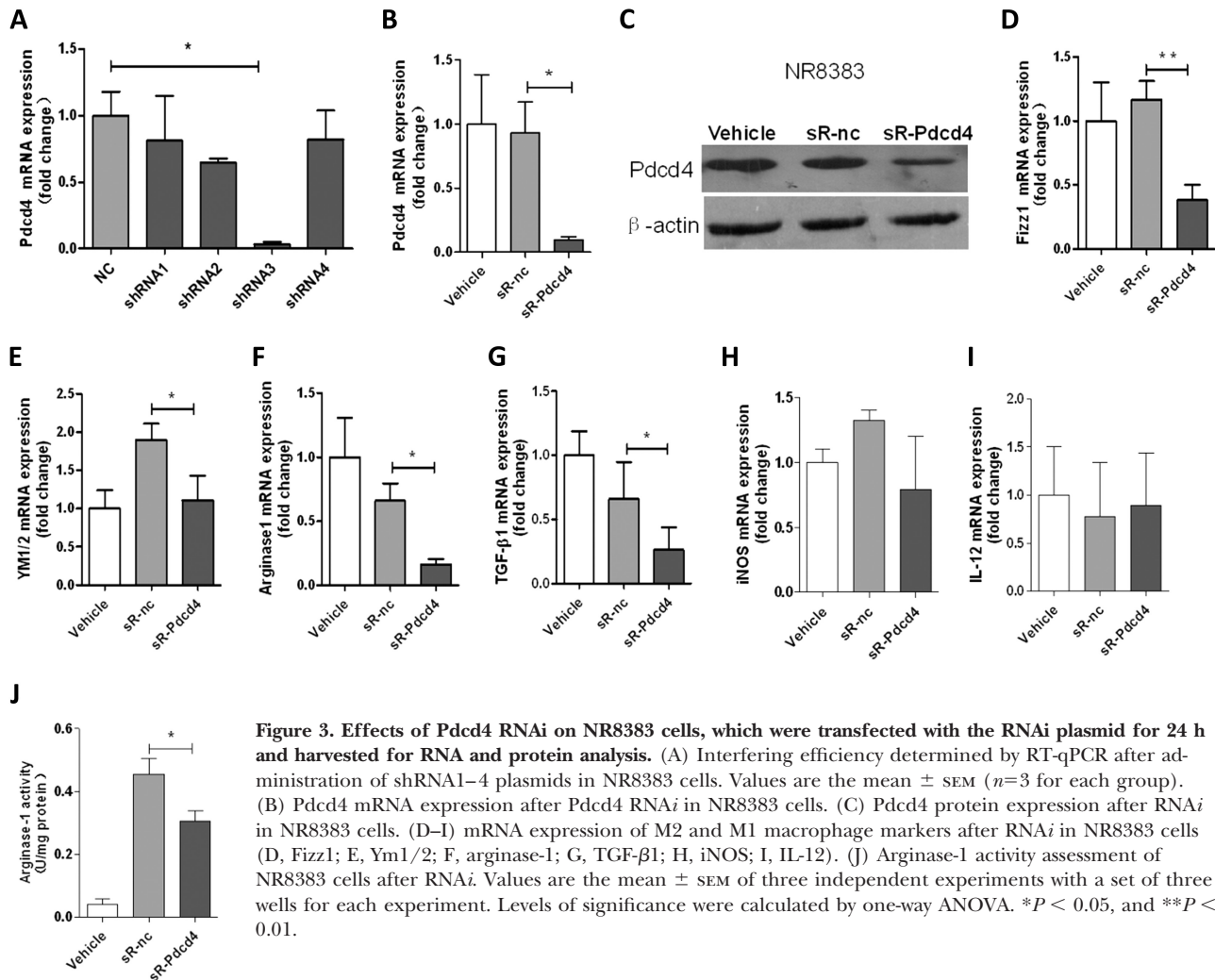


Figure 3. Effects of Pdc4 RNAi on NR8383 cells, which were transfected with the RNAi plasmid for 24 h and harvested for RNA and protein analysis. (A) Interfering efficiency determined by RT-qPCR after administration of shRNA1–4 plasmids in NR8383 cells. Values are the mean $\pm$ SEM ($n=3$ for each group). (B) Pdc4 mRNA expression after Pdc4 RNAi in NR8383 cells. (C) Pdc4 protein expression after RNAi in NR8383 cells. (D–I) mRNA expression of M2 and M1 macrophage markers after RNAi in NR8383 cells (D, Fizz1; E, Ym1/2; F, arginase-1; G, TGF- β 1; H, iNOS; I, IL-12). (J) Arginase-1 activity assessment of NR8383 cells after RNAi. Values are the mean $\pm$ SEM of three independent experiments with a set of three wells for each experiment. Levels of significance were calculated by one-way ANOVA. * $P < 0.05$, and ** $P < 0.01$.

shRNA3 (AIPI+sR-Pdc4 group) and NC-shRNA (AIPI+sR-nc group) were applied to AIPI rats through nasal cavity installation. Immunohistochemical results showed a decrease of Pdc4 expression in alveolar macrophages in the AIPI + sR-Pdc4 group (Fig. 4A). To compare with the AIPI + sR-nc group, Pdc4 expression also decreased significantly in BALF cells at the mRNA level and in lung tissue without being lavaged at the protein level from AIPI + sR-Pdc4 rats (Fig. 4B and C). Lavaged lung tissue showed a tendency of decreased Pdc4 mRNA expression after Pdc4 RNAi but no statistical significance (Fig. 4D). Then, we analyzed different cell-type ratios in BALF and found that the eosinophil ratio decreased dramatically in the AIPI + sR-Pdc4 group (Fig. 5A). However, Pdc4 knockdown did not influence other asthmatic indices, such as lung/body-weight ratio, total cell number in BALF, total IgE and OVA-specific IgG1 level in serum, or concentration of NO in serum (Supplemental Fig. 1). Next, we determined the mRNA level of *Fizz1*, *Ym1/2*, *arginase-1*, and *TGF- β 1* in BALF cells and found that their expressions were decreased significantly after Pdc4 RNAi (Fig. 5B–E), whereas iNOS mRNA expression in BALF cells showed no difference between the AIPI + sR-Pdc4 group and the AIPI + sR-nc

group (Fig. 5F). *TGF- β 1* mRNA level decreased markedly accompanied by Pdc4 knockdown, even in lavaged lung tissue (Fig. 5G). As the protein mass from BALF cells was not enough for arginase activity analysis, we used lung tissue without being lavaged to detect arginase-1 activity, which was decreased notably in the AIPI + sR-Pdc4 group (Fig. 5H).

RNAi of Pdc4 in vivo alleviated airway remodeling in AIPI rats

Furthermore, the association between Pdc4 function and airway remodeling was evaluated. RNAi of Pdc4 in vivo played no roles in the inflammation infiltration and pathological score of lung tissue (Fig. 6A, top, and B). However, less collagenation was observed in the AIPI + sR-Pdc4 group than in other groups (Fig. 6A, middle). The mean bronchus wall/mean airway lumen ratio, representing the collagen mass around airways, decreased significantly in the AIPI + sR-Pdc4 group compared with the AIPI + sR-nc group (Fig. 6C). Furthermore, the epithelium mucus secretion was less in the AIPI + sR-Pdc4 group than in the AIPI + sR-nc group (Fig. 6A, bottom, and D). Moreover, the mass of hydroxyproline in lung tis-

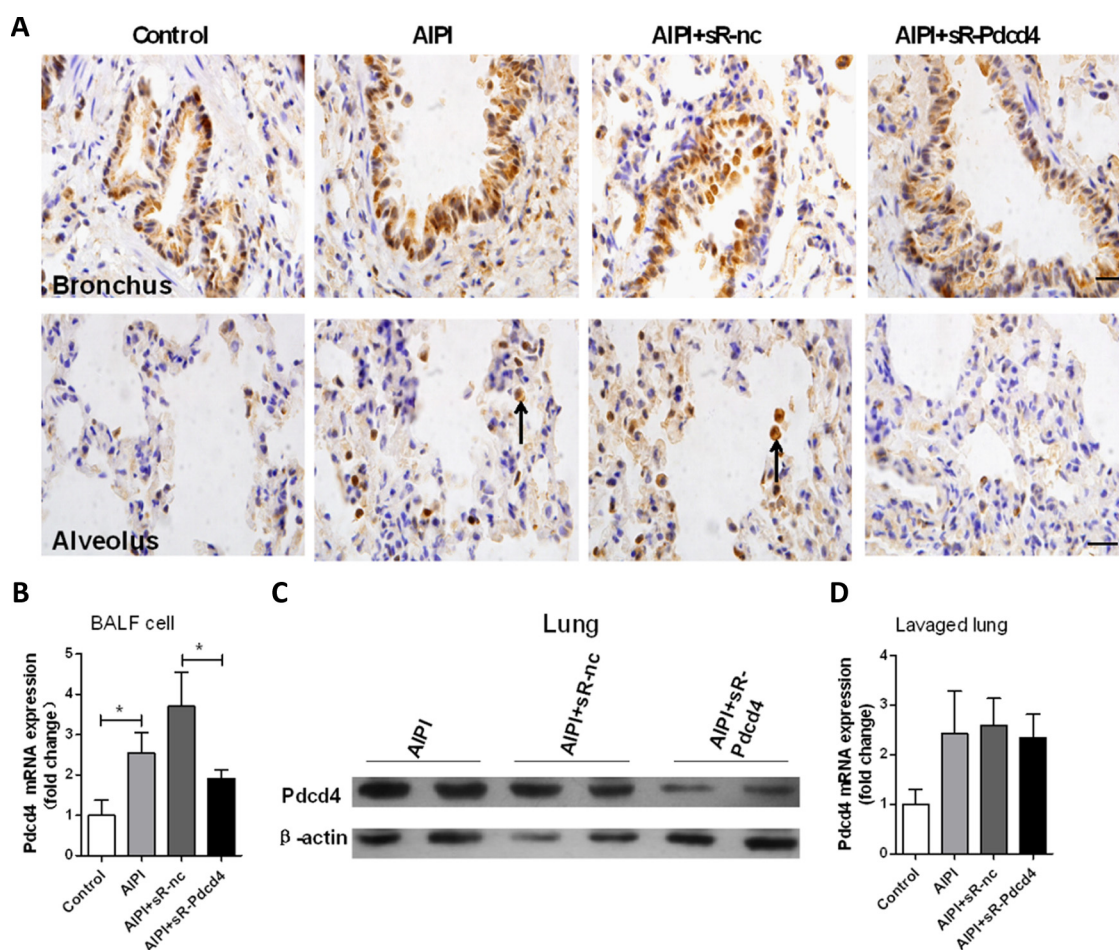


Figure 4. Efficiency of *Pdcd4* RNAi on AIPi rats. The AIPi model was treated with saline (Control and AIPi), NC-shRNA plasmid (AIPi+sR-nc), or *Pdcd4*-shRNA3 plasmid (AIPi+sR-*Pdcd4*) on Days 13 and 17 during the period of AIPi induction. Rats were killed on Day 21. (A) Representative immunohistochemical images of *Pdcd4* expressed in rat airway epithelia and alveolar macrophages. (B) Interfering efficiency in rat BALF cells, determined by RT-qPCR after *Pdcd4* RNAi in vivo. (C) *Pdcd4* protein expression in lung tissue without being lavaged after *Pdcd4* RNAi. (D) *Pdcd4* mRNA expression in lavaged lung after *Pdcd4* RNAi. The results are expressed as mean $\pm$ SEM ($n=8$ for each group). * $P < 0.05$.

sue, which was often used to reflect the synthesis of collagen, decreased significantly after RNAi of *Pdcd4* (Fig. 6E).

***Pdcd4* was found to bind to C/EBP- β in NR8383 cells**

To analyze the downstream signaling events of *Pdcd4*, we performed coimmunoprecipitation between *Pdcd4* and other molecules in NR8383 cells. The result showed that *Pdcd4* bound to C/EBP- β (Fig. 7A) rather than PPAR- γ (Fig. 7B), which implied that *Pdcd4* may influence macrophage alternative activation via the C/EBP- β signal pathway.

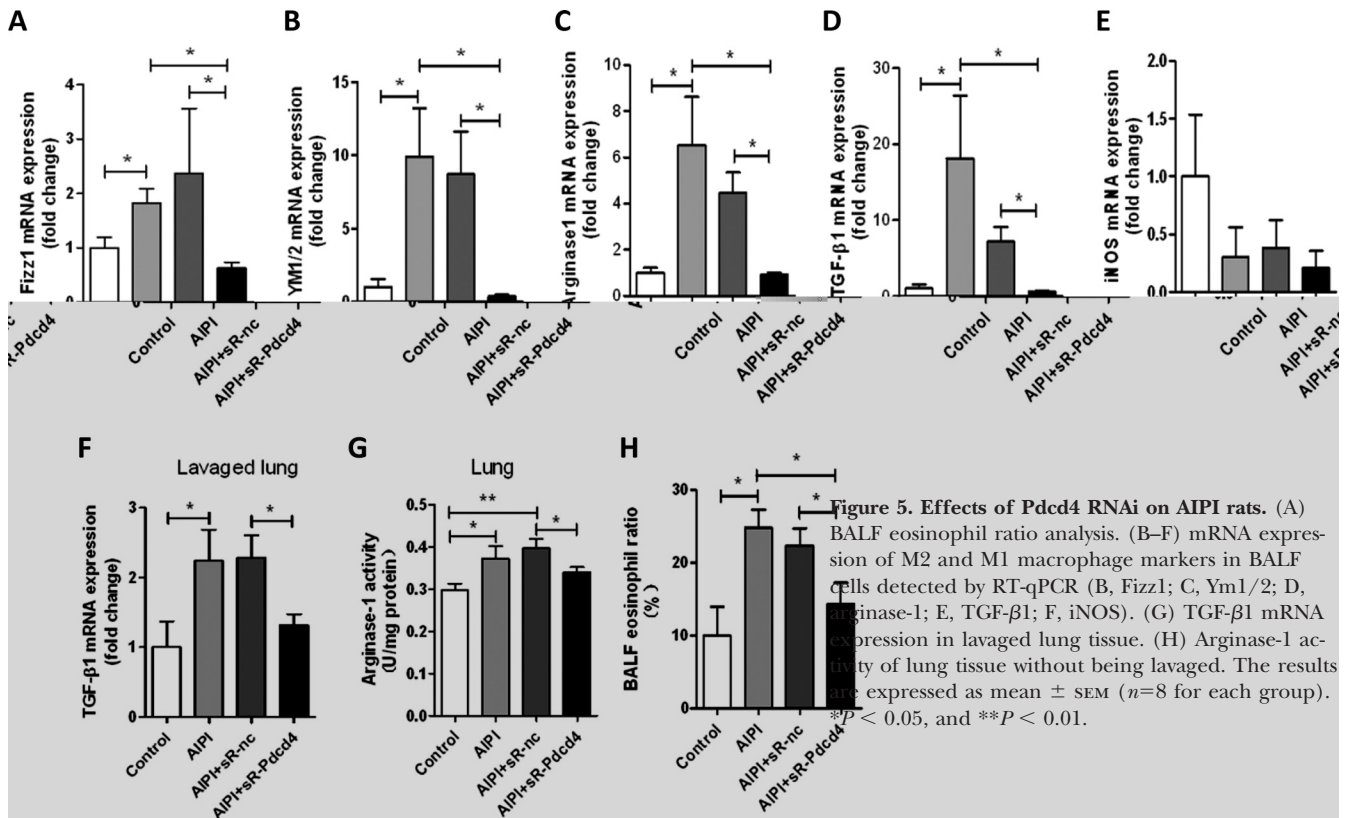
DISCUSSION

Over the past decade, >100 candidate genes that are associated with asthma have been identified [27]. Despite the progress made in asthma genetics, much room for identification of asthma candidate genes remains [28]. We have identified *Pdcd4* as a differentially expressed gene in our AIPi model by

SSH. In the current study, we analyzed the expression of *Pdcd4* in control and AIPi model rats, as well as the effect of *Pdcd4* RNAi on NR8383 cells and AIPi rats.

Initially, we evaluated *Pdcd4* expression in lung tissue by RT-qPCR and Western blot. *Pdcd4* was up-regulated significantly in the lung without being lavaged from AIPi rats. Hitherto, among various lung diseases, lung cancer has been found to be closely related to *Pdcd4*, whereas *Pdcd4* is down-regulated in primary lung tumors, and the loss of *Pdcd4* expression may correlate with tumor progression [10]. Our data show that *Pdcd4* is involved in the pathogenesis of allergic pulmonary inflammation. Contrary to the down-regulation of *Pdcd4* in lung cancer, *Pdcd4* was found to be up-regulated in AIPi.

Immunohistochemistry revealed that *Pdcd4* was localized in airway epithelia and alveolar macrophages. In a previous study, Northern blot analysis has shown that mouse lung tissue can express *Pdcd4* [29], but the cell type expressing *Pdcd4* is unknown at that time. Our study first revealed that in lung tissue, *Pdcd4* expression is localized to airway epithelia and alveolar



macrophages of rats. Moreover, spleen tissue and blood cells from AIPi rats showed a more elevated expression of *Pdc4*. Although the cell type expressing *Pdc4* in spleen and blood cells was not definite, it is plausible that *Pdc4* may, in part, play a potential role in the physiological activity of macrophages. During allergic asthma, alveolar macrophages are often activated in the alternative way [30, 31] and orchestrate the process of airway remodeling, including collagen deposition and goblet cell hyperplasia [30–32]. So, for the present study, we selected to focus specifically on the role of *Pdc4* in regulating macrophage alternative activation and airway remodeling.

First, we up-regulated *Pdc4* expression in rat macrophage cell line NR8383 cells by transfecting cells with the pEGFP-*Pdc4* recombinant plasmid. Partially consistent with our expectation, overexpression of *Pdc4* increased mRNA expression of *arginase-1* and *TGF-β1*, indicating a relationship between *Pdc4* and M2 markers. Unfortunately, overexpression of *Pdc4* had no effect on *Ym1/2*, *Fizz1* expression, or arginase-1 activity. Published data [33] and our work demonstrated that NR8383 cells expressed abundant M2 markers but a trace of M1 markers, suggesting that NR8383 cells are biased to the M2 phenotype per se. The high basal level of M2 markers in NR8383 cells may be a mechanism that could partially explain the reason why exogenous *Pdc4* failed to up-regulate the expression of some M2 markers.

Accompanied with the down-regulation of *Pdc4* expression in NR8383 cells, the expression of *Fizz1*, *Ym1/2*, *arginase-1*, and *TGF-β1* decreased correspondingly, which indicates that the

alternative activation of macrophages can be switched by the change of *Pdc4* expression. However, the expression of M1 macrophage markers, never fluctuating with the expression of *Pdc4*, indicates that *Pdc4* cannot switch the classical activation of macrophages. To be specific, *Fizz1*, *Ym1/2*, *arginase-1*, and *TGF-β1* have been identified as typical markers of M2 macrophages, which are necessary for M2 macrophages to fulfill their function. *Fizz1* functions as a factor for eosinophil recruitment in asthmatic mice [34]; *TGF-β1* induces epithelial-mesenchymal transition [35] and smooth muscle proliferation [36]; *Ym1/2* can act as an eosinophil chemoattractant [37] and involves tissue repairing [38]; and *arginase-1* can promote the production of proline [25], which is the raw material for collagen biosynthesis. All of these factors are involved in airway remodeling.

In other words, the correspondence between *Pdc4* and M2 markers indicates that the higher expression of *Pdc4* can promote the function of M2 macrophages, and in asthma, this means promoting eosinophil infiltration, collagen deposition, and mucus secretion and concurring changes, leading to airway remodeling. So, the correspondence of *Pdc4* and M2 markers reveals the association between *Pdc4* and airway remodeling. To gain further insights into its biological functions, we suppressed *Pdc4* expression in AIPi rats via shRNA technology. Our in vivo RNAi result showed that lung eosinophil infiltration, airway collagen deposition, and mucus secretion decreased after the interference of *Pdc4*, as well as the expression of M2 markers in BALF cells. These observations support that *Pdc4* can affect markers of macrophage alter-

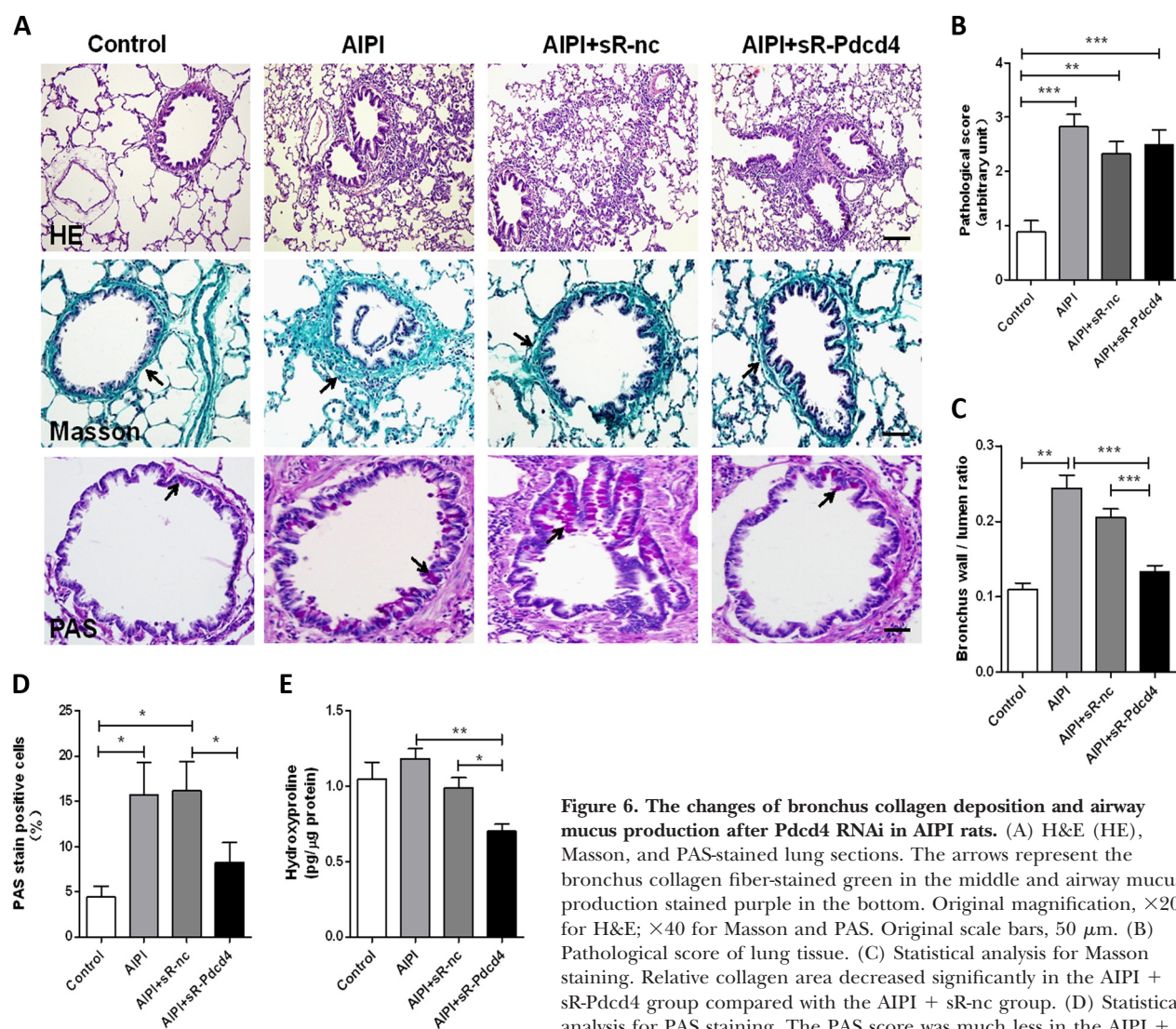


Figure 6. The changes of bronchus collagen deposition and airway mucus production after *Pdcd4* RNAi in AIPi rats. (A) H&E (HE), Masson, and PAS-stained lung sections. The arrows represent the bronchus collagen fiber-stained green in the middle and airway mucus production stained purple in the bottom. Original magnification, $\times 20$ for H&E; $\times 40$ for Masson and PAS. Original scale bars, 50 μ m. (B) Pathological score of lung tissue. (C) Statistical analysis for Masson staining. Relative collagen area decreased significantly in the AIPi + sR-*Pdcd4* group compared with the AIPi + sR-nc group. (D) Statistical analysis for PAS staining. The PAS score was much less in the AIPi + sR-*Pdcd4* group than the AIPi + sR-nc group. (E) Analysis of hydroxyproline in lung tissue. The mass of hydroxyproline decreased significantly in the AIPi + sR-*Pdcd4* group compared with AIPi + sR-nc and AIPi groups. The results of statistical analysis are expressed as mean $\pm$ SEM ($n=8$ for each group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

native activation in vivo and that *Pdcd4* can influence airway remodeling.

Moreover, *Pdcd4* shRNA in vivo reduced the ratio of BALF eosinophil, indicating that after the interference of the *Pdcd4* gene, lung eosinophil infiltration decreased significantly. There have been reports demonstrating that alternative-activation macrophages exacerbate eosinophil airway recruitment [39–41]. In view of this fact, it seems plausible to speculate that *Pdcd4* RNAi attenuated macrophage alternative activation and then, in turn, restrained lung eosinophil infiltration. A substantial body of experimental proof documents that the primary role of eosinophils in asthma is their effect on airway remodeling as a result of the secretion of crucial profibrotic cytokine TGF- β 1 [42–45]. As markers of macrophage alternative activation and eosinophil infiltration decreased simultaneously, it is fairly well reasoned to have observed less bronchial collagen deposition and epithelial mucus secretion after *Pdcd4*

RNAi in vivo. In line with these findings, it has been observed that in colon cancer cells, *Pdcd4* could down-regulate the urokinase-type plasminogen activator receptor, which promotes degradation of extracellular matrix components [46], and in breast cancer cells, *Pdcd4* overexpression could increase the secretion of the tissue inhibitor of metalloproteinase 2 [47]. These facts demonstrate that *Pdcd4* definitely has the function of inducing tissue remodeling. Our findings support *Pdcd4* as a novel factor for the modulation of airway remodeling.

The next crucial issue is the underlying molecular mechanism of *Pdcd4* to affect macrophage alternative activation. It has been known that diverse factors, such as cytokines, signal molecules, and transcriptional factors, might modulate macrophage polarization [20]. On the other hand, *Pdcd4* plays versatile roles through different molecular mechanisms [48]. A prototypical function of *Pdcd4*, for example, is that *Pdcd4*

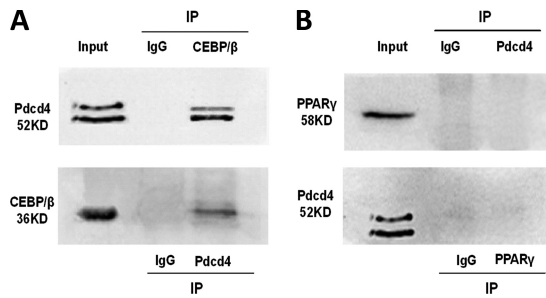


Figure 7. Pdc4-C/EBP- β interactions determined by coimmunoprecipitation. (A) NR8383 cells were lysed, and protein extracts were immunoprecipitated (IP) with anti-Pdc4 or anti-C/EBP- β antibodies. (B) Protein extracts of NR8383 cells were immunoprecipitated with anti-Pdc4 or anti-PPAR- γ antibodies. Analyses of the crude protein extracts (Input) demonstrate comparable expression levels of the proteins in the different samples.

binds to eIF4A and suppresses helicase activity by preventing the association of eIF4A and eIF4G, which results in an inhibition of translation [49]. So far, the relationship between macrophage alternative activation and well-known functions of Pdc4 is difficult to build, owing to lack of both the rationality and experimental evidence. So we have performed coimmunoprecipitation between Pdc4 and other molecules. The preliminary result showed that Pdc4 bound to C/EBP- β , a potential transcription factor for macrophage polarization, rather than PPAR- γ , a metabolism-related transcriptional factor, which implied that Pdc4 may influence macrophage alternative activation via the C/EBP- β signal pathway.

In summary, the cellular and molecular mechanisms underlying airway remodeling in asthma are not fully understood. Among the various factors involved in the pathogenesis of remodeling, we focused on macrophage alternative activation. We found that the changes of Pdc4 expression can regulate markers of macrophage alternative activation and airway remodeling. Although the functions and mechanisms of Pdc4 in tumor promotion are well documented, the functions of Pdc4 in other diseases have been poorly described. Our findings may provide a clue for more investigations in Pdc4 and suggest a new option for asthma treatment.

AUTHORSHIP

B.Z. carried out part of the animal and cell experiments and drafted the manuscript. X.Y. and Q.H. performed the animal experiments. Q.S. and C.J. completed the histochemistry experiments. L.L. executed the cell culture. X.L. performed the molecular biological experiments. J.T. carried out analysis of hydroxyproline and part of the molecular biological experiments. W.H. contributed to the design of the study and performed the statistical analysis. H.L. and N.G. participated in the molecular biological experiments. S.L. designed the study and participated in drafting the manuscript. All authors read and approved the final manuscript.

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DISCLOSURES

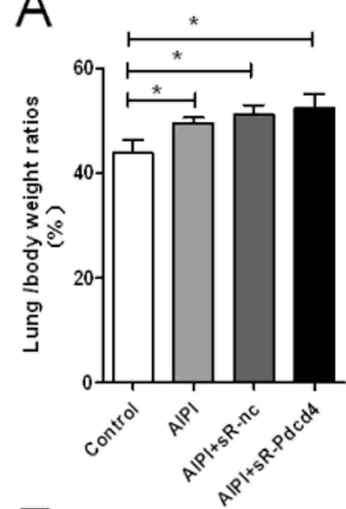
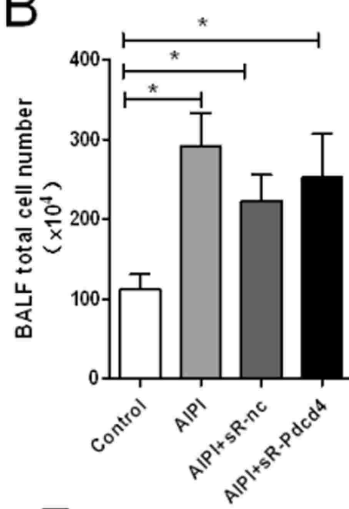
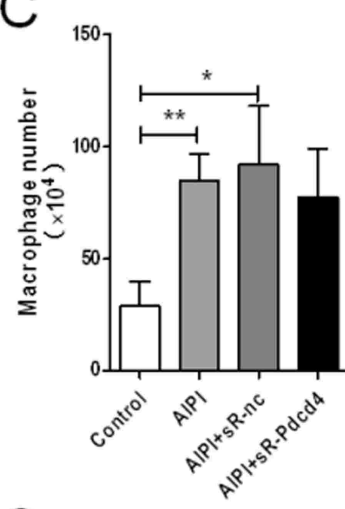
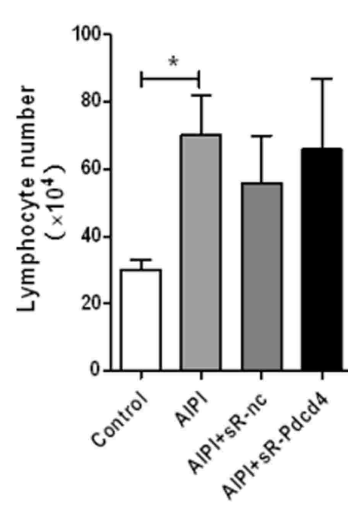
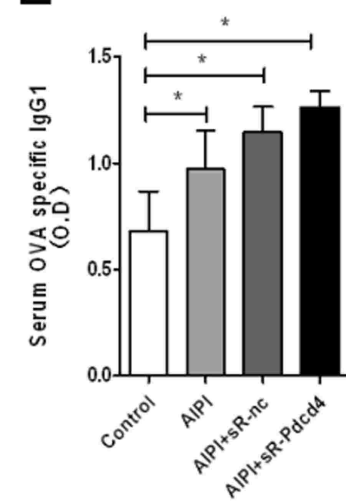
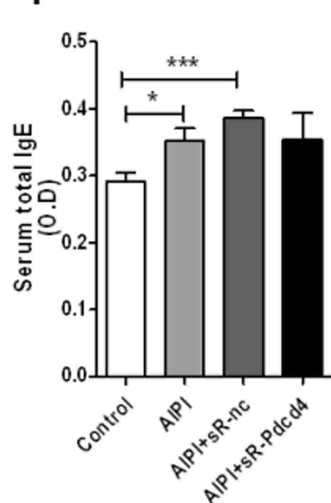
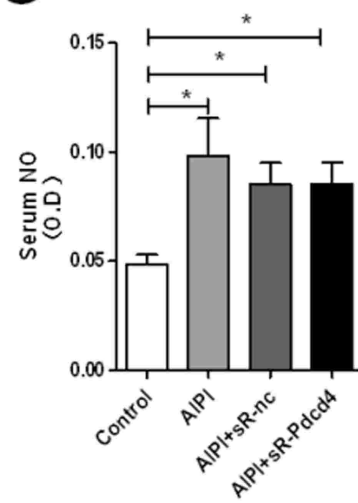
The authors declare no conflict of interest.

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KEY WORDS:
programmed cell death 4 · asthma · M2 macrophage

A**B****C****D****E****F****G**

Supplemental Figure legend

AIPI parameters in rats after Pdc4 RNAi. **A**, Lung/body weight ratio. **B**, Total cell number count in BALF. **C**, Macrophage number count in BALF. **D**, Lymphocyte number count in BALF. **E**, Serum OVA specific IgG1. **F**, Serum total IgE. **G**, Serum NO. The results are expressed as mean $\pm$ SEM (n = 8 for each group). *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.