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

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RECEIVED MARCH 9, 2013; REVISED JULY 7, 2014; ACCEPTED JULY 15, 2014. DOI: 10.1189/jlb.3A0313-136RRR

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-β1, which are markers of macrophage alternative activation. In response to Pdcd4 RNA in NR8383 cells, the mRNA expression of markers Fizz1, Ym1/2, arginase-1, and TGF-β1 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-β1 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 airflow 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, PCR=radioimmunoprecipitation assay, RNAi=RNA interference, shRNA=short hairpin RNA, SSH=subtractive hybridization, Ym1/2=chitinase 3-like protein 3/4

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

References:

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involved in inflammatory processes. One group reported that Pdcdf-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 Pdcdf participating in the development of inflammatory disease remain to be elucidated. Our following experiments after SSH revealed that alveolar macrophages can express Pdcdf protein, suggesting that Pdcdf 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-β1 [19–21].

In the present study, we induced AIPI in E3 rats, evaluated the difference of Pdcdf expression between control and AIPI rats, and tried to identify the role of Pdcdf in airway remodeling and macrophage alternative activation. To the best of our knowledge, this is the first report to show that Pdcdf 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 μg OVA/PBS (1 mg/mL), once daily for 7 days, whereas rats from the control group were administered 50 μg NC-shRNA plasmid in 50 μL saline, served as control and AIPI groups; 50 μg NC-shRNA plasmid in 50 μL saline, named as AIPI + sR-nc group; and 50 μg Pdcdf-shRNA3 plasmid in 50 μL saline, named as AIPI + sR-Pdcdf group. Rats were killed on Day 21. The lungs in each group were lavaged with 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 μl 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 Pdcdf (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 1 h. The slides were washed with PBS and mounted.

Overexpression of Pdcdf with recombinant plasmid in NR8383 cells
For the construction of the pEGFP-Pdcdf recombinant plasmid, rat Pdcdf cDNA was amplified and subcloned into the pEGFP-C1 vector at the Xhol and HindIII sites. NR8383 cells were seeded at 8 × 104/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 μg pEGFP-Pdcdf recombinant plasmid (pEGFP-Pdcdf group) and pEGFP-C1 empty vector (pEGFP group) by using 8 μl 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.

Pdcdf knockdown with RNAi in vitro and in vivo
Four target sequences of the Pdcdf gene for RNAi are: GCCATTCAGT-CAGTGAGAGATG, named as Pdcdf-shRNA1; GCCGTTGAGAGAGGCTT-TAGCA, named as Pdcdf-shRNA2; GCGGAGATGTGAAAGGGATTG, named as Pdcdf-shRNA3; and GGGACAGTGATTGACCAAAAT, named as Pdcdf-shRNA4. The sequence of the NC-shRNA is GTTCCTCGAAGCCTGT-CACGT. These sequences were inserted into the PGPU6/GFP/Neo RNAi plasmid. NR8383 cells were transfected with these RNAi plasmids, and mRNA expression of Pdcdf was detected by RT-qPCR. Pdcdf-shRNA3 was found as the most efficient to knock down Pdcdf expression. Then, NR8383 cells were transfected with Pdcdf-shRNA3 (sR-Pdcdf 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 μl saline, served as control and AIPI groups; 50 μg NC-shRNA plasmid in 50 μL saline, named as AIPI + sR-nc group; and 50 μg Pdcdf-shRNA3 plasmid in 50 μL saline, named as AIPI + sR-Pdcdf group. Rats were killed on Day 21. The lungs in each group were lavaged with 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 μl 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.
Arginase activity was expressed as units/milligram protein.

The Arginase activity assay was performed as follows: the reaction mixture was transferred to microplates in duplicates, and absorbance was measured at 540 nm with an ELISA reader (Thermo Electron, MA, USA). The bands of interest were visualized with the SuperSignal West Pico Trial Kit (Thermo Fisher Scientific, Waltham, MA, USA) and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and performed with lung tissue, and SSH was performed with lung tissue, and

**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 (Zhuanhe Joincare Biosciences, China), and then, an equal amount of the denatured protein (20 µg for Pdcd4 and 80 µg 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. Briefly, 400 µL perchloric acid and 50 µL 10% hydrochloric acid were added, which were then incubated for 5 min at room temperature. Then, 50 µL percoll and 50 µL 10% paraformaldehyde 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 µL 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 µg/µL hydroxyproline, and the result was expressed as pg/µg protein.

**Statistical analysis**

Data were expressed as mean ± 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 ≤ 0.05.

**RESULTS**

Pdcd4 was up-regulated in lung, spleen, and blood cells from AIPI rats

The AIPI model was induced with OVA in E3 rats. SSH was performed with lung tissue, and Pdcd4 was identified as a dif-
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 demonstrated 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 AIPI rats compared with the control ones (Fig. 1D).

The regulation of Pdcd4 expression influenced markers of macrophage alternative activation in vitro

Pdcd4 protein expression in alveolar macrophages and the difference of Pdcd4 mRNA expression in BALF cells prompted us to investigate whether Pdcd4 can exert functions in macrophages. As macrophages resemble an M2 phenotype in asthma, it is essential to clarify whether Pdcd4 affects macrophage alternative activation. The rat alveolar macrophage cell line NR8383 cells were transfected with Pdcd4 recombinant plasmid. Fizz1, Ym1/2, arginase-1, and TGFβ1 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 Pdcd4 (Fig. 2A and B), arginase-1 and TGFβ1 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-12b 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 Pdcd4 in NR8383 had no effect on arginase-1 activity (Fig. 2I).

Then, we wanted to know whether down-regulation of Pdcd4 can influence the markers of macrophage alternative activation. Pdcd4-shRNA3 down-regulated Pdcd4-level efficiently in a pretest (Fig. 3A) and was transfected into NR8383. Then, the M2 marker expression was suppressed markedly after Pdcd4 knockdown (Fig. 3D–G), and arginase-1 activity decreased significantly, accompanied by Pdcd4 knockdown (Fig. 3I). Similarly to the Pdcd4 up-regulation result, down-regulation of Pdcd4 expression in NR8383 produced no change in iNOS and IL-12b mRNA level (Fig. 3H and I).

RNAi of Pdcd4 suppressed macrophage alternative activation in vivo

We then extended the observation to determine whether Pdcd4 influenced macrophage activation in vivo. Pdcd4-
shRNAs (AIPI+/sR-Pdcd4 group) and NC-shRNA (AIPI+/sR-nc group) were applied to AIPI rats through nasal cavity installation. Immunohistochemical results showed a decrease of Pdcd4 expression in alveolar macrophages in the AIPI+/sR-Pdcd4 group (Fig. 4A). To compare with the AIPI+/sR-nc group, Pdcd4 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-Pdcd4 rats (Fig. 4B and C). Lavaged lung tissue showed a tendency of decreased Pdcd4 mRNA expression after Pdcd4 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-Pdcd4 group (Fig. 5A). However, Pdcd4 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-β1 in BALF cells and found that their expressions were decreased significantly after Pdcd4 RNAi (Fig. 5B–E), whereas iNOS mRNA expression in BALF cells showed no difference between the AIPI+/sR-Pdcd4 group and the AIPI+/sR-nc group (Fig. 5F). TGF-β1 mRNA level decreased markedly accompanied by Pdcd4 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-Pdcd4 group (Fig. 5H).

RNAi of Pdcd4 in vivo alleviated airway remodeling in AIPI rats

Furthermore, the association between Pdcd4 function and airway remodeling was evaluated. RNAi of Pdcd4 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-Pdcd4 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-Pdcd4 group compared with the AIPI+/sR-nc group (Fig. 6C). Furthermore, the epithelium mucus secretion was less in the AIPI+/sR-Pdcd4 group than in the AIPI+/sR-nc group (Fig. 6A, bottom, and D). Moreover, the mass of hydroxyproline in lung tis-

Figure 3. Effects of Pdcd4 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 ± sem (n=3 for each group). (B) Pdcd4 mRNA expression after Pdcd4 RNAi in NR8383 cells. (C) Pdcd4 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-β1; H, iNOS; I, IL-12). (J) Arginase-1 activity assessment of NR8383 cells after RNAi. Values are the mean ± 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.

Journal of Leukocyte Biology
Volume 96, December 2014
www.jleukbio.org
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 Pdcd4. Although the cell type expressing Pdcd4 in spleen and blood cells was not definite, it is plausible that Pdcd4 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 Pdcd4 in regulating macrophage alternative activation and airway remodeling.

First, we up-regulated Pdcd4 expression in rat macrophage cell line NR8383 cells by transfecting cells with the pEGFp-Pdcd4 recombinant plasmid. Partially consistent with our expectation, overexpression of Pdcd4 increased mRNA expressions of arginase-1 and TGF-β1, indicating a relationship between Pdcd4 and M2 markers. Unfortunately, overexpression of Pdcd4 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 Pdcd4 failed to up-regulate the expression of some M2 markers.

Accompanied with the down-regulation of Pdcd4 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 Pdcd4 expression. However, the expression of M1 macrophage markers, never fluctuating with the expression of Pdcd4, indicates that Pdcd4 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 Pdcd4 and M2 markers indicates that the higher expression of Pdcd4 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 Pdcd4 and M2 markers reveals the association between Pdcd4 and airway remodeling. To gain further insights into its biological functions, we suppressed Pdcd4 expression in AIPI rats via shRNA technology. Our in vivo RNAi result showed that lung eosinophil infiltration, collagen deposition, and mucus secretion decreased after the interference of Pdcd4, as well as the expression of M2 markers in BALF cells. These observations support that Pdcd4 can affect markers of macrophage alter-
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-β1 [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 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, ×20 for H&E; ×40 for Masson and PAS. Original scale bars, 50 μm. (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 ± SEM (n=8 for each group). *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 7. Pdcd4-C/EBP-β interactions determined by coimmunoprecipitation. (A) NR8383 cells were lysed, and protein extracts were immunoprecipitated (IP) with anti-Pdcd4 or anti-C/EBP-β antibodies. (B) Protein extracts of NR8383 cells were immunoprecipitated with anti-Pdcd4 or anti-PPARγ 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 Pdcd4 is difficult to build, owing to lack of both the rationality and experimental evidence. So we have performed coimmunoprecipitation between Pdcd4 and other molecules. The preliminary result showed that Pdcd4 bound to C/EBP-β, a potential transcription factor for macrophage polarization, rather than PPAR-γ, a metabolism-related transcriptional factor, which implied that Pdcd4 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 Pdcd4 expression can regulate markers of macrophage alternative activation and airway remodeling. Although the functions and mechanisms of Pdcd4 in tumor promotion are well documented, the functions of Pdcd4 in other diseases have been poorly described. Our findings may provide a clue for more investigations in Pdcd4 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 histochimistry 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.

ACKNOWLEDGMENTS
This work was supported by a National Natural Science Foundation of China grant (No. 81170017), a China Postdoctoral Science Foundation grant (2012M521754), the Fundamental Research Funds for the Central Universities (DXYX111000117; xjj2017), and Xi’an Jiaotong University Scientific Fund (xjj2011021). We thank Yan Han, Qihan Ning, Fujun Zhang, Liesu Meng, and Jing Xu for expert assistance. We thank Jie Ma and Dongmin Li for helpful discussions and productive critiques.

DISCLOSURES
The authors declare no conflict of interest.

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Zhong et al. The role of Pdcd4 in macrophage alternative activation


KEY WORDS: programmed cell death 4 · asthma · M2 macrophage
Pdcd4 modulates markers of macrophage alternative activation and airway remodeling in antigen-induced pulmonary inflammation

Bo Zhong, Xudong Yang, Qingzhu Sun, et al.

*J Leukoc Biol* published online August 5, 2014
Access the most recent version at doi:10.1189/jlb.3A0313-136RRR

**Supplemental Material**
http://www.jleukbio.org/content/suppl/2014/07/17/jlb.3A0313-136RRR.DC1

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