Th17 cells are involved in mouse chronic obstructive pulmonary disease complicated with invasive pulmonary aspergillosis

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Abstract

Background: The incidence of chronic obstructive pulmonary disease (COPD) complicated with invasive pulmonary aspergillosis (IPA) has increased in the last two decades. The mechanism underpinning susceptibility to and high mortality of COPD complicated with IPA is unclear, and the role of T helper cells 17 (Th17 cells) in the compound disease remains unknown. Therefore, this study aimed to assess the function of Th17 cells in COPD combined with IPA.

Methods: COPD, IPA, and COPD+IPA mouse models were established in male wild type C57/BL6 mice. The amounts of Th17 cells and retinoic acid-related orphan receptors γt (RORγt) were tested by flow cytometry. Then, serum interleukin (IL)-17 and IL-23 levels were detected by enzyme-linked immunosorbent assay (ELISA) in the control, COPD, IPA and COPD+IPA groups. In addition, COPD+IPA was induced in IL-17 knockout (KO) mice, for determining the role of Th17 cells in COPD+IPA.

Results: Compared with the COPD group, the COPD+IPA group showed higher amounts of blood RORγt ([35.09 ± 16.12]% *vs.* [17.92 ± 4.91]%, $P = 0.02$) and serum IL-17 (17.96 ± 9.59 pg/mL *vs.* 8.05 ± 4.44 pg/mL, $P = 0.02$), but blood ([5.18 ± 1.09]% *vs.* [4.15 ± 0.87]%, $P = 0.28$) and lung levels of Th17 cells ([1.98 ± 0.83]% *vs.* [2.03 ± 0.98]%, $P = 0.91$), lung levels of RORγt ([9.58 ± 6.93]% *vs.* [9.63 ± 5.98]%, $P = 0.49$) and serum IL-23 (51.55 ± 27.82 pg/mL *vs.* 68.70 ± 15.20 pg/mL, $P = 0.15$) showed no significant differences. Compared with the IPA group, the COPD+IPA group displayed lower amounts of blood ([5.18 ± 1.09]% *vs.* [9.21 ± 3.56]%, $P = 0.01$) and lung Th17 cells ([1.98 ± 0.83]% *vs.* [6.29 ± 1.11]%, $P = 0.01$) and serum IL-23 (51.55 ± 27.82 pg/mL *vs.* 154.90 ± 64.60 pg/mL, $P = 0.01$) and IL-17 (17.96 ± 9.59 pg/mL *vs.* 39.81 ± 22.37 pg/mL, $P = 0.02$), while comparable blood ([35.09 ± 16.12]% *vs.* [29.86 ± 15.42]%, $P = 0.25$) and lung levels of RORγt ([9.58 ± 6.93]% *vs.* [15.10 ± 2.95]%, $P = 0.18$) were found in these two groups. Finally, *Aspergillus* load in IL-17 KO COPD+IPA mice was almost 2 times that of COPD+IPA mice (1,851,687.69 ± 944,480.43 *vs.* 892,958.10 ± 686,808.80, $t = 2.32$, $P = 0.02$).

Conclusion: These findings indicate that Th17 cells might be involved in the pathogenesis of COPD combined with IPA, with IL-17 likely playing an antifungal role.

Keywords: T helper cells 17; Chronic obstructive pulmonary disease (COPD); Invasive pulmonary aspergillosis (IPA)

Introduction

Aspergillus spp. can cause an opportunistic infection in the airway and pulmonary parenchyma, known as invasive pulmonary aspergillosis (IPA) in immunocompromised hosts.[1] Chronic obstructive pulmonary disease (COPD) patients, who were once considered non-classical immunosuppressive hosts, are less prone to IPA. However, reports of COPD patients complicated with IPA have increased during the last two decades.[2,3] According to previous reports, the incidence of IPA in patients with COPD is 16.3‰.[4] Meanwhile, an incidence of IPA in critically ill COPD patients of up to 13% was found in our intensive care unit.[5] Severe COPD patients have been recognized as a susceptible population for IPA.[6,7] The mortality rate is as high as 70% to 100% for COPD patients with IPA.[4,5,7] However, the mechanism underlying susceptibility to and high mortality of COPD complicated with *Aspergillus* infection is unknown.

The immune response is the main host defense mechanism against *Aspergillus* infection. Among the pool of T cells that participate in immune response against *Aspergillus*, T helper cells 17 (Th17 cells) are perhaps the most important subset.[8] In the past two decades, studies have found that Th17 cells, with the dominant secretion of the cytokine interleukin 17 (IL-17), play an important role in the host

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response to fungal infections.[9] Retinoic acid-related orphan receptor γt (RORγt), a specific nuclear transcription factor in Th17 cells, and IL-23 play an important role in stabilizing and promoting the differentiation of Th17 cells.[10] Th17 cells are involved in inflammatory responses by secreting a variety of cytokines, including their most studied cytokine IL-17A.[11] Moreover, Th17 cells also act as an important part of inflammation during the pathogenesis of COPD.[12,13] Meanwhile, Th17 cell amounts are inversely correlated with forced expiratory volume in 1 s (FEV_1) percentage in COPD.[14,15] In addition, it was demonstrated that dysregulation in Th17 cell compartment, creating an imbalance of Th17/regulatory T cells, contributes to COPD-associated pulmonary hypertension.[16]

The above findings demonstrate that Th17 cells have critical functions in *Aspergillus* infection and COPD. However, the role of Th17 cells in COPD combined IPA remains unknown. We hypothesized that COPD has lower Th17 cell amounts when combined with *Aspergillus* infection, with Th17 cells playing a protective role in response to *Aspergillus* infection in COPD. Therefore, the present study aimed to assess the function of Th17 cells in COPD combined with IPA. To this end, we built an IPA animal model in COPD mice, and assessed the amounts of Th17 cells and their transcription factor RORγt, IL-17 and IL-23 as compared with the control, COPD and IPA groups. The specific role of IL-17 in COPD combined with IPA was evaluated in IL-17 knockout (KO) mice.

Methods

Ethical approval

Animal studies were reviewed and approved by the ethics committee of Beijing Chao-Yang Hospital, Capital Medical University (No. 2015-9-18). All animal procedures were carried out in strict accordance with the recommendations of the Chinese Laboratory Animal Requirements of Environment and Housing Facilities.

Mice

Male wild-type C57/BL mice, 6 to 8 weeks old, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). IL-17a-/- (IL-17 KO) mice in a C57BL/6 background were provided by Dr. Iwakura (University of Tokyo, Tokyo, Japan) as previously described.[17] The animals were housed in individual ventilated cages (IVC) under standard laboratory conditions with a 12/12h light/dark cycle. The full system was kept in an environmentally controlled room at $(22 \pm 1)^{\circ}C$ and $(55 \pm 5)\%$ humidity. Clean food and water were provided *ad libitum*. All mice were maintained in the Beijing Institute of Respiratory Medicine. The following five groups were set up: control, IPA, COPD, COPD+IPA, and IL-17 KO COPD+IPA groups.

COPD models

Cigarette smoke induced COPD: The mice of the COPD, COPD+IPA, and IL-17 KO COPD+IPA groups were exposed to cigarette smoke intermittently for 10 min at 120 min/day for consecutive 5 days with 2 days of rest for 16 weeks; meanwhile, the IPA and control groups were exposed to filtered air correspondingly. The experiments were performed with a nose-only inhalation generator (SIBATA technology company, Toyota, Japan), which was composed of a smoke generating device SG-300 (SIBATA) inhaled chamber and the connecting transfer pipe. Baisha cigarettes (Tar at 20 mg, 1.0 mg nicotine and 14 mg carbon monoxide) were put in the smoke generating device, and the parameters were set to reach a smoke concentration of about 2.5%.

Confirmation of COPD model establishment: Weight changes in the four groups were based on individual animals assessed separately at different times. Lung function (Flexi Vent rodent animal breath tester, SCIREQ, Montreal, PQ, Canada) was analyzed for respiratory mechanics parameters, including pulmonary compliance, airway resistance and elastic resistance.[18,19] Then, lung samples were fixed with 10% neutral buffered formalin (Sigma, St. Louis, MO, USA), paraffin embedded, sliced at 5-μm and stained with hematoxylin and eosin (H&E) for histological analysis. In combination with histological evidence of emphysema in the lung, increased airway resistance reflected successful COPD model establishment.

Establishment of COPD combined with IPA

Aspergillus fumigatus isolate 204305 (ATCC, Manassas, VA, USA) was maintained on potato dextrose agar for 5 to 7 days at 37°C. Conidia were harvested by washing the culture flask with 50 mL of sterile phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40 μm nylon membrane to remove hyphal fragments and enumerated on a hemocytometer. The final density of *Aspergillus fumigatus* spore suspension was 1×10^8/mL.

Methylprednisolone (Pfizer Pharmaceuticals Limited, New York, NY, USA) was suspended in sterile water containing 0.1% Tween 80. Doses were 100 mg/kg injected subcutaneously in 0.5 mL or less saline. This dose was given on three alternate days per week (beginning on Monday with infection on Friday) for the three-dose regimen.

COPD mice were slightly anesthetized with 1.5% pentobarbital (5 mL/kg). An incision of 0.5 to 1 cm was made along the midline of the mouse neck, and muscle tissues were separated bluntly to expose the trachea. Then, 30 μL of the previously obtained *Aspergillus fumigatus* spore suspension was intratracheally administered, followed by 10 μL filtered air to ensure all the suspension was pushed into the trachea. With histological evidence of invasive growth of *Aspergillus* in the lung, a successful COPD+IPA model establishment was confirmed.[20]

Flow cytometry for cell detection

Cells were washed and resuspended at a density of 10^7 cells/mL in fluorescent antibody (FA) buffer (Difco) and 0.1% NaN3. Fc receptors were blocked by addition of unlabeled anti-CD16/32 antibody (Fc block; BD Pharmingen, San Diego, CA, USA). After Fc receptor blocking, 0.5×10^6 to 1×10^6 cells were stained in a final volume of

100 μL for 30 min at 4°C. Fluorochrome-conjugated antibodies directed against the following antigens were obtained (BD Pharmingen) and used according to the manufacturer's instructions: anti-mouse CD3-FITC (BD Pharmingen), anti-mouse CD4-PERCP 5.5 (BD Pharmingen) and anti-mouse CD8-APCy7 (BD Pharmingen). Cells were washed twice with FA buffer and resuspended in 100 μL saline, and an equal volume of 4% formalin was added for fixation. A minimum of 20,000 events were acquired on a FACS Calibur flow cytometer (BD Biosciences, Piscataway, NJ, USA) using the Cell-Quest software (BD Biosciences, Frankin Lakes, NJ, USA). The acquired data were analyzed with the FlowJo software (Tree Star, Ashland, OR, USA).

Flow cytometry for extracellular and intracellular molecules

Prior to intracellular cytokine staining, cells were stimulated *in vitro* for 5 h with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (1 μg/mL) in the presence of brefeldin A (BD Pharmingen) to promote intracellular accumulation of cytokines. After stimulation, cells were washed twice prior to surface molecule staining. After surface molecule staining, intracellular molecules were stained using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Cells were stained with anti-mouse interferon (IFN)-γ-phycoerythrin/Cyanine 7 (Cy7), RORγT-AF647, and anti-mouse IL-17A-phycoerythrin (BD Pharmingen) according to the manufacturer's instructions. FACS Calibur flow cytometer (BD Pharmingen) was used for analysis with the Cell-Quest software (BD Pharmingen).

ELISA

Serum IL-17 and IL-23 levels were tested. First, whole blood was centrifuged at 3000 r/min for 10 min for serum preparation. Then, ELISA kits (BD Biosciences) were used to measure serum IL-17 and IL-23 levels according to the manufacturer's protocols.

Quantitative real-time polymerase chain reaction (PCR)

Fresh lung samples were removed and snap frozen in liquid nitrogen. Total RNA was extracted with RNAiso Plus (TaKaRa, Dalian, China) and reverse transcribed into complementary DNA (cDNA) with the PrimeScript RT reagent kit (TaKaRa). Quantitative real-time PCR was performed with SYBR Premix Ex Taq II reagents from TaKaRa, according to the manufacturer's instructions. PCR reactions were performed in 20 μL mixtures containing 2 μL of cDNA. The 26S RNA of *Aspergillus fumigatus* ATCC204305 was amplified using specific primers (TaKaRa) (26S rRNA-F: CCGTCTAAGTGCCCTGGAAC; 26S rRNA-R: CTTGTGCGCTATCGGTCTCC). The standard curve was drawn according to the Ct value and copy number of the standard. The Ct value in the test sample was obtained, and the concentration of *Aspergillus fumigates* 26S RNA in the sample was derived according to the standard curve.

Statistical analysis

All experiments were repeated three times, and the results were pooled. Continuous data with normal distribution were presented as mean ± standard deviation (SD) and analyzed using Student's *t* test. Non-normally distributed data were expressed as median (IQR). The Mann-Whitney *U* test was used for comparing non-normally distributed data. Multiple group comparison of normally and non-normally distributed data was performed by analysis of variance (ANOVA) and the Kruskal-Wallis *H* test, respectively. Student-Newman-Keuls (SNK) test was performed for comparison between two groups. All tests were carried out with the SPSS statistics 22.0 System Software (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

Results

Confirmation of the establishment of the animal model with COPD combined IPA

First, COPD model establishment was confirmed by individual weight recording, lung function parameters and lung histology. In the process of modeling, weights in the COPD and COPD+IPA groups were declined compared with those of the control and IPA groups [Figure 1A]. Weight change was the lowest in the IPA+COPD group, followed by the COPD and IPA groups, with the control group showing the highest values [Figure 1B].

Lung function tests showed that pulmonary compliance (0.027 ± 0.008 mL/cmH_2O *vs.* 0.016 ± 0.005 mL/cmH_2O, $t = 4.26$, $P = 0.01$) and airway resistance (Raw) (1.90 ± 0.22 cmH_2O·s/mL *vs.* 1.54 ± 0.15 cmH_2O·s/mL, $t = 3.12$, $P = 0.02$) in the COPD groups were significantly higher than those of the control group, while elastic resistance (Er) was significantly lower (34.75 ± 10.75 cmH_2O/mL *vs.* 61.89 ± 16.54 cmH_2O/mL, $t = -4.30$, $P = 0.03$) [Figure 2A–C].

Histologically, compared with the control group, COPD mice showed incomplete mucosal epithelial structure, with occasionally few alveolar fusion fractures observed. Furthermore, bronchial cilia were shorter and partially exfoliated; the alveolar wall became thinner or ruptured into larger alveoli. The alveolar structure of the IPA group was similar to that of the control group, but there were necrosis and *Aspergillus* hyphal growing in the IPA [Figure 3A and 3B]. Meanwhile, increased small airway secretions, glandular hyperplasia, large amounts of infiltrated inflammatory cells, alveolar tissue structure destruction, and emphysema formation were found in the COPD and COPD+IPA groups [Figure 3C and 3D].

Amounts of Th17 cells and RORγt in blood and lung

Compared with the COPD group, the COPD+IPA group showed higher amounts of blood RORγt ([35.09 ± 16.12]% *vs.* [17.92 ± 4.91]%, $P = 0.02$), but blood ([5.18 ± 1.09]% *vs.* [4.15 ± 0.87]%, $P = 0.28$) and lung levels of Th17 cells ([1.98 ± 0.83]% *vs.* [2.03 ± 0.98]%, $P = 0.91$) and lung level of RORγt ([9.58 ± 6.93]% *vs.* [9.63 ± 5.98]%, $P = 0.49$) showed no significant differences [Figure 4, Table 1]. Compared with the IPA group, the COPD+IPA group displayed significantly lower amounts of blood Th17 cells ([5.18 ± 1.09]% *vs.* [9.21 ± 3.56]%, $P = 0.01$) and lung Th17 cells ([1.98 ± 0.83]% *vs.* [6.29 ± 1.11]%, $P = 0.01$), while

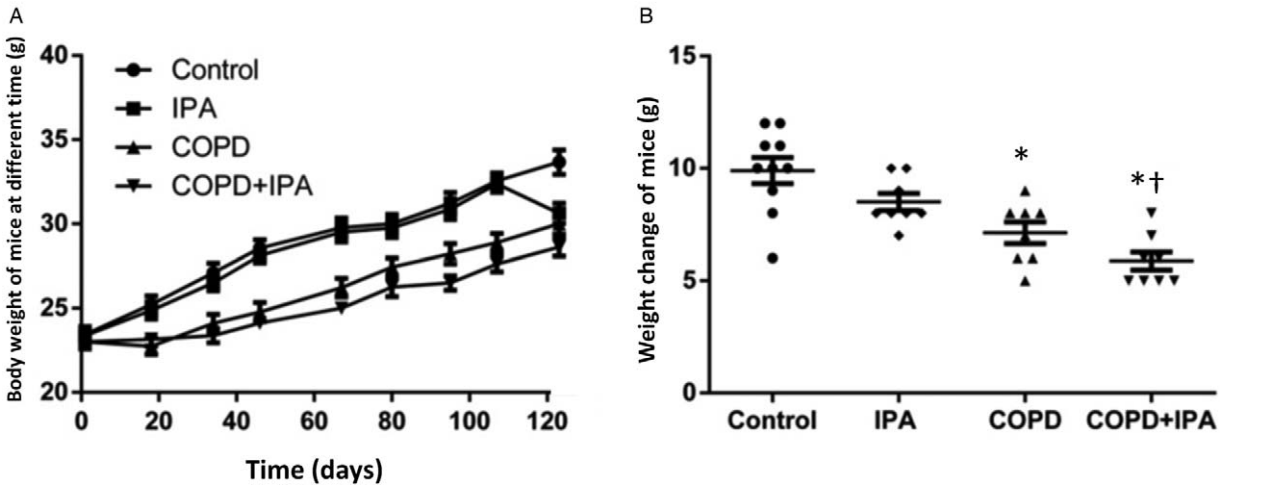

Figure 1: Body weight changes after model establishment. (A) Body weights in all four groups of mice increased with time. (B) Body weight changes in the four groups. $^{*}P < 0.05$ *vs.* control; $^{+}P < 0.05$ *vs.* IPA group. IPA: Invasive pulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease.

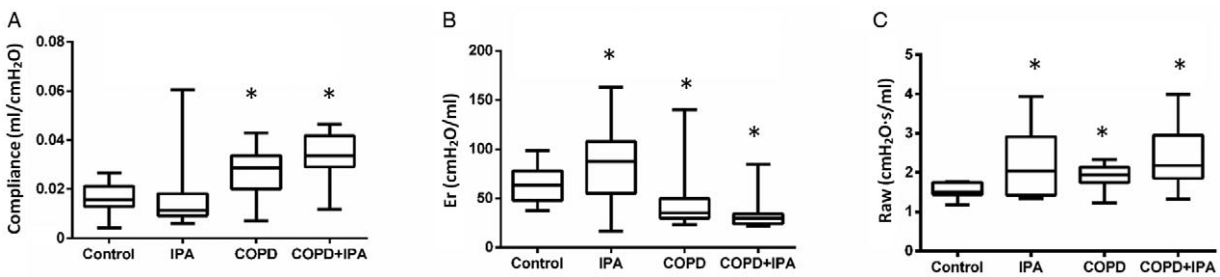

Figure 2: Lung function after model establishment. Pulmonary compliance (A), elastic resistance (Er) (B) and airway resistance (Raw) (C) in all four groups. $^{*}P < 0.05$ *vs.* Control. IPA: Invasive pulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease.

comparable blood ([35.09 ± 16.12]% *vs.* [29.86 ± 15.42]%, $P = 0.25$) and lung levels ([9.58 ± 6.93]% *vs.* [15.10 ± 2.95]%, $P = 0.06$) of RORγt were found in these two groups. Compared with the control group, the COPD group had similar levels of blood and lung Th17 cells ([4.15 ± 0.87]% *vs.* [3.43 ± 0.47]%, $P = 0.06$; [2.03 ± 0.98]% *vs.* [2.46 ± 0.88]%, $P = 0.18$, respectively) and RORγt ([17.92 ± 4.91]% *vs.* [18.41 ± 15.13]%, $P = 0.93$; [9.63 ± 5.98]% *vs.* [8.86 ± 1.24]%, $P = 0.35$, respectively). Compared with the control group, the COPD+IPA group had higher blood RORγt ([35.09 ± 16.12]% *vs.* [18.41 ± 15.13]%, $P = 0.04$), and similar levels of blood and lung Th17 cells ([5.18 ± 1.09]% *vs.* [3.43 ± 0.47]%, $P = 0.28$; [1.98 ± 0.83]% *vs.* [2.46 ± 0.88]%, $P = 0.28$) and lung RORγt ([9.58 ± 6.93]% *vs.* [8.86 ± 1.24]%, $P = 0.78$). Finally, blood ([9.21 ± 3.56]% *vs.* [3.43 ± 0.47]%, $P < 0.01$) and lung levels ([6.29 ± 1.11]% vs. [2.46 ± 0.88]%, P=0.02) of Th17 cells and blood level of RORγt ([29.86 ± 15.42]% *vs.* [18.41 ± 15.13]%, $P = 0.04$) were significantly higher in the IPA group compared with control mice [Figure 4, Table 1].

Serum IL-17 and IL-23 levels

Compared with the COPD group, the COPD+IPA group showed significantly higher amounts of serum IL-17 (17.96 ± 9.59 pg/mL *vs.* 8.05 ± 4.44 pg/mL, $P = 0.02$), while serum IL-23 (51.55 ± 27.82 pg/mL *vs.* 68.70 ± 15.20 pg/mL, $P = 0.15$) showed no significant difference. Compared with the IPA group, the COPD+IPA group displayed significantly lower amounts of serum IL-23 (51.55 ± 27.82 pg/mL *vs.* 154.90 ± 64.60 pg/mL, $P = 0.01$) and IL-17 (17.96 ± 9.59 pg/mL *vs.* 39.81 ± 22.37 pg/mL, $P = 0.02$). In comparison with the control group, the COPD group showed no significant differences in serum IL-23 (68.70 ± 15.20 pg/mL *vs.* 65.19 ± 8.93 pg/mL, $P = 0.58$) and IL-17 (8.05 ± 4.44 pg/mL *vs.* 5.24 ± 0.59 pg/mL, $P = 0.06$) amounts. The COPD +IPA group had a significantly higher IL-17 (17.96 ± 9.59 pg/mL *vs.* 5.24 ± 0.59 pg/mL, $P = 0.01$) and a similar IL-23 (51.55 ± 27.82 pg/mL *vs.* 65.19 ± 8.93 pg/mL, $P = 0.22$) when compared with the control group. Finally, serum IL-17 (39.81 ± 22.37 pg/mL *vs.* 5.24 ± 0.59 pg/mL, $P < 0.01$) and IL-23 levels (154.90 ± 64.60 pg/mL *vs.* 65.19 ± 8.93 pg/mL, $P = 0.01$) were significantly higher in the IPA group than in the control group [Figure 5, Table 2]. Compared with COPD+IPA group, IL-17 KO COPD+IPA mice had no IL-17 detected in serum.

COPD model in IL-17 KO mice and Aspergillus load comparison between IL-17 KO COPD+IPA and COPD+IPA mice

In the process of establishing a COPD model in IL-17 KO mice, animal weights in the control and IL-17 KO COPD groups increased with time, but the IL-17 KO group showed significantly lower values compared with control mice from the second week [Figure 6A]. This resulted in an

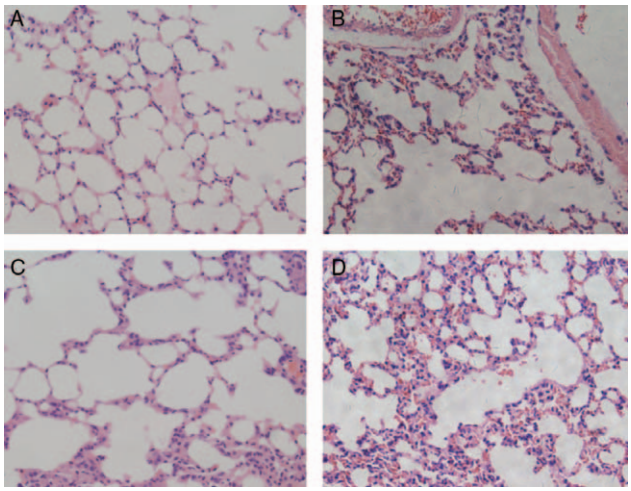

Figure 3: Histological findings of the lung tissue as examined by H&E staining. (A) The CON group showed normal lung uniform morphology with neatly arranged cilia, scattered lymphocyte infiltration at all levels of tracheobronchial space, relatively uniformed alveoli, and proper thickness of alveolar interstitial space. (B) In the IPA group, loose and small aggregates of *Aspergillus* mycelium aligned in an irregular pattern in alveoli were observed. (C) The COPD group showed detachment of the bronchial epithelium, thinner alveoli, irregular arrangement of bronchial smooth muscles, and larger alveoli fused by some parts of the fractured alveoli. (D) The COPD+IPA group showed detachment of the bronchial epithelium, irregular arrangement of bronchial smooth muscles, and loose and small aggregates of *Aspergillus* mycelium aligned in thinner alveoli. Original magnification × 400. H&E: Hematoxylin and eosin; CON: Control; IPA: Invasive pulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease.

overall decrease of body weight change in the IL-17 KO group compared with controls [Figure 6B].

Next, *Aspergillus* load was tested by culture on potato dextrose agar. While the control group showed no growth, the IL-17 KO COPD+IPA group showed *Aspergillus* colonies covering the whole plate [Figure 6C]. This was confirmed by RT-PCR. Indeed, *Aspergillus* 26S RNA load in the IL-17 KO COPD+IPA group was about two times that of the COPD+IPA mice ($1,851,687.69 \pm 944,480.43$ *vs.* $892,958.10 \pm 686,808.80$, $t = 2.32$, $P = 0.02$) [Figure 6D].

Discussion

The main strengths of this study were the establishment of a COPD+IPA model in mice for the first time, and investigating the role of Th17 cell based on this model. This enabled us to discriminate the effects of Th17 cells in COPD and/or IPA. In addition, we found that IL-17 played a protective role in IL-17 KO COPD+IPA mice by reducing fungal load. Upon infection of COPD mice with IPA, Th17 cell amounts were reduced in the blood or lung compared with control mice infected with IPA; this lower amount of Th17 cells may not be directly related to inflammation in COPD itself. Some other pathways maybe responsible for the inappropriately reduced levels of Th17 cells in COPD mice infected with IPA, which deserves further investigation.

In this study, compared with the COPD group, the COPD +IPA group showed higher amounts of blood RORγt as well as serum levels of IL-17, while blood level of Th17 cells was comparable in both groups. RORγt is the key

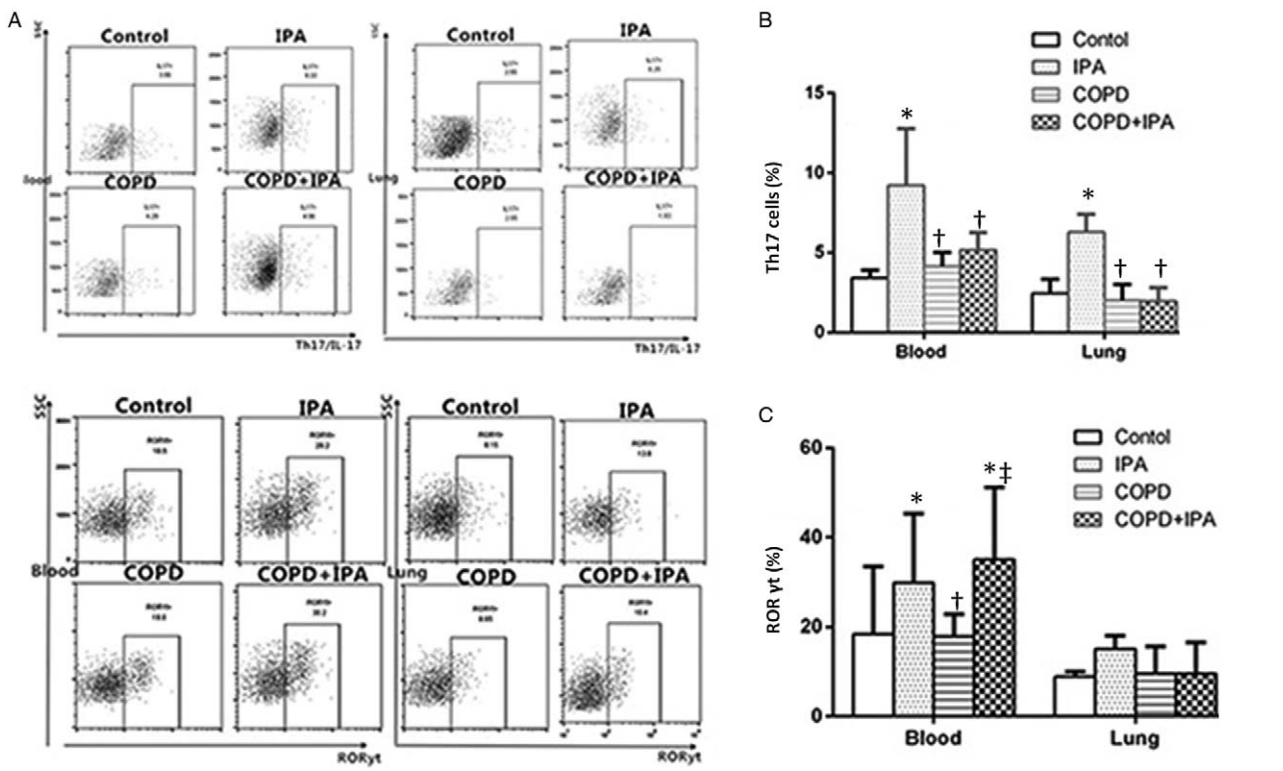

Figure 4: Blood and lung Th17 cells and RORγt amounts as analyzed by flow cytometry. (A) Flow cytometry analysis of Th17 cells and RORγt amounts in blood and lung. (B) Comparison of Th17 cells amounts in blood and lung in all four groups. (C) Comparison of RORγt amounts in blood and lung in all four groups. $^{*}P < 0.05$ *vs.* control; $^{\dagger}P < 0.05$ *vs.* IPA group; $^{\ddagger}P < 0.05$ *vs.* COPD group. Th17: T helper 17; RORγt: Retinoic acid-related orphan receptors γt; IPA: Invasive pulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease.

Table 1: Expression levels of Th17 cells and RORγt in different groups.

Variables	Sample	Control group ($n=8$)	IPA group ($n=8$)	COPD group ($n=8$)	COPD+IPA group ($n=8$)	F	P
Th17 cells (%)	Blood	3.43 ± 0.47	9.21 ± 3.56	4.15 ± 0.87	5.18 ± 1.09	15.62	<0.01
	Lung	2.46 ± 0.88	6.29 ± 1.11	2.03 ± 0.98	1.98 ± 0.83	37.80	<0.01
RORγt (%)	Blood	18.41 ± 15.13	29.86 ± 15.42	17.92 ± 4.91	35.09 ± 16.12	3.16	0.04
	Lung	8.86 ± 1.24	15.10 ± 2.95	9.63 ± 5.98	9.58 ± 6.93	2.84	0.04

Data were presented as mean ± standard deviation. Th17: T helper 17; RORγt: Retinoic acid-related orphan receptors γt; IPA: Invasive pulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease.

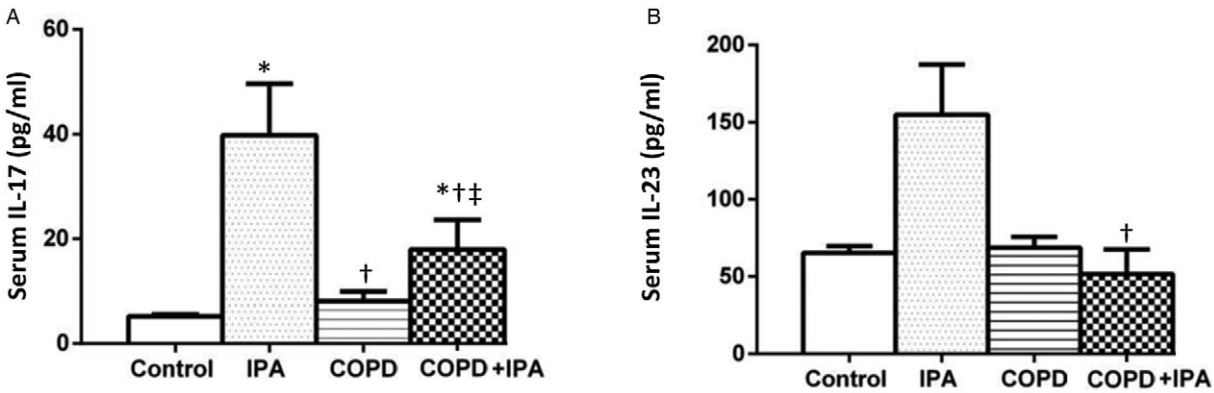

Figure 5: Serum interleukin-17 (A) and interleukin-23 (B) levels as assessed by ELISA. $^{*}P<0.05$ *vs.* control; $^{\dagger}P<0.05$ *vs.* IPA group; $^{\ddagger}P<0.05$ *vs.* COPD group. ELISA: Enzyme-linked immunosorbent assay; IPA: Invasive pulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease.

Table 2: Serum level of IL-17 and IL-23 in different groups.

Variables	Control group ($n=8$)	IPA group ($n=8$)	COPD group ($n=8$)	COPD+IPA group ($n=8$)	F	P
IL-17 (pg/ml)	5.24 ± 0.59	39.81 ± 22.37	8.05 ± 4.44	17.96 ± 9.59	12.81	<0.01
IL-23 (pg/ml)	65.19 ± 8.93	154.90 ± 64.60	68.70 ± 15.20	51.55 ± 27.82	10.69	<0.01

Data were presented as mean ± standard deviation. IL: Interleukin; IPA: Invasive pulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease.

transcription factor which directs the differentiation program of proinflammatory Th17 cells.[14] Acute exacerbations of COPD are associated with decreased CD4+ T cells, which may be related to the immunocompromised status of COPD.[15] Another study also reported that stimulated lung CD4+ T cells from COPD subjects displayed significantly impaired interferon-γ (IFN-γ) production.[16] In patients with COPD, chronic pulmonary inflammation is accompanied by the induction of defective immune responses that contribute to intermittent respiratory infections, worsening the inflammatory lung microenvironment and disease severity.[17-19] Also, reduced immune cell infiltration into the lung, along with decreased levels of the inflammatory cytokine IL-17, was also noted in a previous report.[20] Therefore, when the amount of CD4+ T cells decreased or its function was inhibited in COPD, although RORγt level increased, the blood level of Th17 cells did not increase accordingly.

Furthermore, compared with the IPA group, the COPD +IPA group showed lower levels of blood and lung Th17 cells and serum IL-23 and IL-17 amounts, although blood RORγt was similarly elevated. Several explanations for mechanism of ineffective immune reaction in COPD are possible for these findings. Firstly, immune imbalance in COPD may affect secondary immune responses against infection.[21] A previous study found that chronic exposure to smoke and high doses of influenza virus could suppress immune response and decrease survival.[22] Another study by Bhat *et al*[12,23] suggested that smoke exposure reduces the adaptive immune response to *Haemophilus influenzae*. Second, "T cell depletion" was recently proposed as the exhaustion of T cells gradually after long-term exposure to persistent antigens and inflammation.[24-26] Bhat *et al* also observed that the mouse model of COPD combined with *Haemophilus influenzae* infection has increased number of inflammatory cells in the lung tissue, but decreased amounts of lymphocytes, especially T lymphocytes, as well as reduced IFN-γ and IL-4 in the lung.[23] Moreover, the effect of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) blockade on T cell responses in COPD may represent a

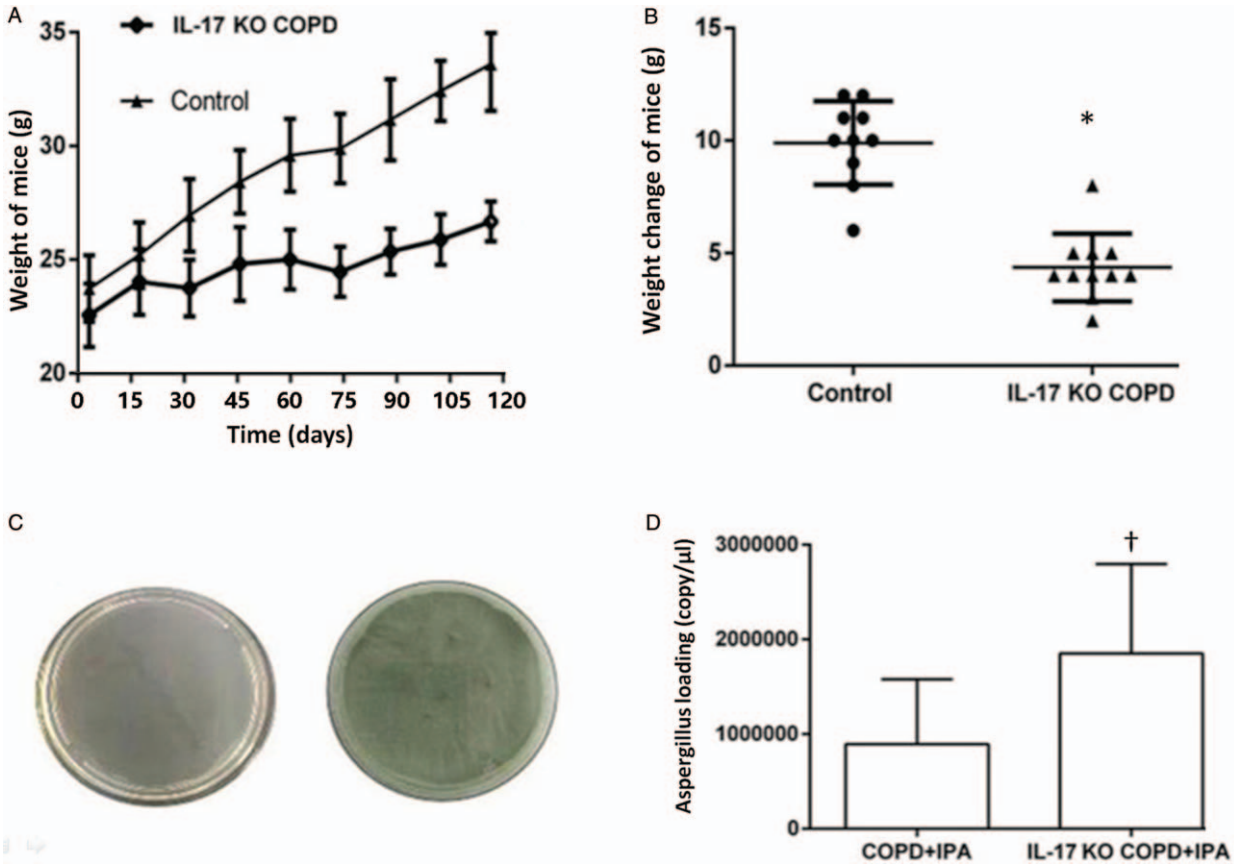

Figure 6: COPD model in IL-17 KO mice and *Aspergillus* load in IL-17 KO COPD+IPA and COPD+IPA mice. (A) Body weights in both groups increased with time. (B) Overall weight gain in the IL-17KO COPD+IPA group was reduced compared with the control value. (C) Lung homogenates were cultured on potato dextrose agar. The control group showed no fungal growth, while the IL-17 KO COPD+IPA group had *Aspergillus* colonies (blue-green) covering the whole plate. (D) *Aspergillus* load as analyzed by RT-PCR in the IL-17 KO COPD +IPA group and the COPD +IPA group. $^{*}P < 0.05$ *vs.* control; $^{\dagger}P < 0.05$ *vs.* COPD+IPA group. COPD: Chronic obstructive pulmonary disease; IL-17: Interleukin-17; KO: Knockout; IPA: Invasive pulmonary aspergillosis.

source for immunosuppression of antibacterial immunity.[12,23] On the other hand, blocking of immune checkpoint receptors CTLA-4 and PD-1 resulted in increased T cell proliferation and IFN-γ production, which in turn strengthens the over expression of these molecules on COPD T cells.[27] Therefore, our findings suggested that the Th17 cell-associated immune pathway may be involved in cytokine production/secretion in the pathogenesis of COPD combined with IPA. Meanwhile, Th17 cells did not effectively react to *Aspergillus* infection in the lung in the context of COPD.

In this study, compared with the control group, the COPD group only showed a trend of higher amounts of blood Th17 cells, serum levels of IL-17 and IL-23. This result is similar to that reported in several previous studies. Paats *et al*[28] found that CD4+Th17 cells/IL-17 level in blood were similar in COPD patients and non-smokers in control group. IL-17 level in sputum sample and Th17 cells in lung tissue were reported similar in COPD patients and non-smokers.[22,29,30] However, elevated levels of Th17 cells, IL-17 and IL-23 in blood and lung of COPD patients were reported in other studies.[31,32] This elevation may correlate with IL-18 induced endothelial activation and enhanced neutrophil recruitment to the sites of inflammation.[33] Furthermore, these differences may be caused by the different methods used in evaluation of Th17 and IL-17 levels.[22] A study suggested that Th17 and IL-17 levels were prone to be similar between COPD and non-COPD groups when evaluated with immunohistochemical analysis.[34]

Finally, to assess whether the Th17 cell pathway is totally ineffective in COPD mice infected by *Aspergillus*, and whether IL-17 has a protective role against *Aspergillus* infection, we established a COPA+IPA animal model in IL-17 KO mice. As shown above, *Aspergillus* load in IL-17 KO COPD+IPA mice was almost two times that of COPD +IPA mice. This finding indicates that in IL-17 KO mice, recruitment, migration and activation of granulocytes may be reduced, leading to increased *Aspergillus* load and more severe infection. This study demonstrated that IL-17 has an important protective immune function in the context of COPD complicated with *Aspergillus* infection, although Th17 cells were partially depleted in the lung of COPD mice. This is consistent with findings reported by previous studies showing that IL-17-deficient mice have increased fungal load after invasive fungal infection, with the mechanism involving altered neutrophil recruitment and cytokine expression.[35,36]

Our findings suggested that in COPD murine model, Th17/IL-17 induces a protective immune response in COPD +IPA. However, the chronic pulmonary inflammation of

COPD is accompanied by the induction of defective immune responses with the chronic depletion of T cells, which worsens the inflammatory lung microenvironment and leads to a damaged antifungal immunity, and a high mortality of IPA in COPD.

In summary, this study provided evidence that the Th17 cell-associated immune pathway may be involved but inhibited in the immunopathological process of COPD combined with IPA. We also demonstrated the infection-protection role of IL-17 in IL-17 KO COPD combined with IPA animal model. However, further studies, including clinical trials, are required to validate these findings.

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Conflicts of interest

None.

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