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An inactivated *Pseudomonas aeruginosa* medicament inhibits airway allergic inflammation and improves epithelial functions

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Abstract The features of asthma are airway hyper-responsiveness (AHR), excess production of Th2 cytokines, and eosinophil accumulation in the lungs. To investigate the antiasthmatic potential of an inactivated *Pseudomonas aeruginosa* medicament (PPA), as well as the underlying mechanism involved, we studied the effects of PPA on airway and epithelial functions. Airway resistance, cell enumeration, and IL-4, IFN-γ, and IL-17 secretion in bronchoalveolar lavage fluid were assayed on an OVA-sensitized AHR animal model. Flow cytometry was used to observe the effects of PPA on cell proliferation, real-time PCR was used to test the expressions of toll like receptor 4 and 5, and Th17 signal molecules Act1, NF-kB negative regulator A20, and western blot were used to detect NF-kB expression on cultured human bronchial epithelial cells (BECs). PPA-treated animals had suppressed airway resistance, eosinophil and lymphocytes infiltration, and IL-4 and IL-17 secretion. PPA can stimulate toll-like receptor-4 and 5 expressions, promote cell proliferation in normal and OVA-treated BECs, significantly decrease Act1 and NF-kB, and increase A20 expression in BECs treated by OVA. Our data suggest the therapeutic mechanism by which PPA effectively treats allergic inflammation on reductions of airway responsiveness, eosinophil infiltration, IL-4 and IL-17 secretion, and improvements of epithelial functions.

Introduction

The incidence of diseases with airway hyperresponsiveness (AHR) such as chronic bronchitis, asthma, and emphysema is increasing year by year, consuming a large amount of medical and economic resources and becoming a global public health problem. Asthma is a complex inflammatory disease characterized by AHR, imbalance of airway innate and adaptive immunity, and airway chronic inflammation. The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall by mast cells, lymphocytes, and eosinophils. The CD4+ effector cells are initially separated into Th1 and Th2 cells. Excess production of Th2 cytokines (IL-4, IL-5) relative to the Th1 cytokine IFN-γ has a crucial role in the pathogenesis of asthma. Another lineage of T cells that produce IL-17 and Th17 cells has been recently identified that is highly proinflammatory and also plays a very important role in asthma.

Bronchial epithelial cells (BECs) have their own repertoire of innate immune functions, expressing chemokines and cytokines to recruit and activate phagocytic cells in response to pathogens. BECs are readily activated by superficial exposure to bacterial ligands and toll-like receptors (TLRs) mediate such responses. By enhancing the function of TLRs can promote the recognition and removal of pathogens. Several studies have shown that TLR-4 agonists can decrease allergic airway inflammation [1], mediate airway epithelial cells to identify and eliminate diesel particles [2], and recognize endogenous harmful substances such as heat shock proteins, hyaluronic acid,

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degradation products, and saturated and unsaturated fatty acids [3]. In addition, TLR-4 plays an important role in chronic inflammatory processes. TLR-4 mainly expresses in macrophages and epithelia [4]. Defective expression or poor response of TLR-4 can lead to chronic infection in the body. TLR-5 signaling can promote epithelial wound repair, proliferation, and survival [5]. Typically, BECs present a certain amount of TLR-4 and TLR-5, but they decrease with age and many kinds of inflammatory diseases [6, 7]. In addition, epithelial shedding is a common feature of asthma, so the promotion of epithelial repair will effectively alleviate the diseases with AHR.

Pseudomonas aeruginosa is a common normal flora of the human body and a common pathogen of chronic infection of the respiratory tract. Among pathogens in respiratory tract infection, Gram-negative bacteria account for 77.3 %, of which 57.7 % are *Pseudomonas aeruginosa*. The flagellar antigen of *Pseudomonas aeruginosa* is a natural TLR-5 ligand. The adhesion portion of type 1 fimbria FimH from avian pathogenic *E. coli* strain can effectively activate TLR-4 [8, 9]. So, in this study, we use an inactivated *Pseudomonas aeruginosa* medicament transfected with FimH (PPA) to observe its effect in allergic airway inflammation, so exploring the effects of the *Pseudomonas aeruginosa* medicament in airway allergic diseases.

Materials and methods

Reagents

The inactivated *Pseudomonas aeruginosa* medicament was obtained from Beijing Wanteer Biological Pharmaceutical. The medicament is transfected with type 1 fimbriae which can effectively activate TLR-4. Now, it is mainly used in clinical treatments of Gram-negative bacterial infections. Interleukin IFN-γ, IL-4 and IL-17 (rat) and anti-rat oval-bumin (OVA) IgE ELISA kits were purchased from Boster, Wuhan. All chemicals were purchased from Sigma unless otherwise stated.

Animals and treatments

SD rats, each weighing 210 ± 20 g, were obtained from the experimental animal center, Xiangya School of Medicine, Central South University, and maintained in a path-ogen-free environment. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee. Animals were divided into four groups including normal control group, OVA group, control plus PPA group, and OVA plus PPA group, with 6 animals in each group. Animals in the OVA group were intraperitoneally injected with 1 mL of 10 % OVA plus 10 % Al (OH)$_3$ antigen on Day 1 and inhaled 1 % OVA for 30 min for the following 14 days. In the control group, animals were caged in the same conditions, but injected with 1 mL of NS on Day 1 and inhaled NS for 30 min for the following 14 days. Animals in the OVA plus PPA group were intraperitoneally injected with 1 mL of 10 % OVA plus 10 % Al (OH)$_3$ antigen at Day 1. Then, 4 h after animals inhaled 1 % OVA for 30 min, they were intranasally administered with 0.2 mL PPA (1×10^7) for the following 14 days. Animals in the PPA group were injected with 1 mL of NS on Day 1, inhaled NS for 30 min and were intranasally administered with 0.2 mL PPA (1×10^7) for the following 14 days.

In vivo assessment of airway function

Airway function was assessed in vivo in anesthetized, mechanically-aspirated rats by measuring changes in lung resistance in response to increasing doses of inhaled hydrochloric acid histamine, as previously described [10]. The animals were intubated with a 6-mm tracheal cannula. The other end of the catheter was connected to a pressure transducer and the collateral arm to an airflow transducer, respectively. Thus, the intrapulmonary pressure and airflow signal could be recorded synchronically. Baseline values were recorded from data obtained after challenge with aerosolized PBS. Lung resistance (R_L) could then be calculated according to the formula: $R_L = $ (intrapulmonary pressure $-$ atmospheric pressure)/airflow (cmH$_2$O/mL s).

Cell enumeration and ELISA assay of bronchoalveolar lavage fluid

Immediately following assessment of AHR, mice were sacrificed with an i.p injection of sodium pentobarbitone (100 mg kg^{-1}). The trachea was cannulated and broncho-alveolar lavage fluid (BALF) was obtained by washing the airway lumina. Briefly, cells in the lungs were recovered by flushing 1 mL of BALF [1 mM EDTA, 10 % fetal bovine serum (FBS), PBS] into the lungs via the trachea. BALF was centrifuged and the cell pellet was suspended in 200 μL of PBS and counted using a hemocytometer. The cell suspensions were then centrifuged onto glass slides using a cytospin centrifuge at 1,000g for 5 min at room temperature. Cytocentrifuged cells were air-dried and stained with a Wright stain or immunofluorescence which allows differential counting of various cells. At least 300 cells per sample were counted by direct microscopic observation. The supernatant of BALF was used for determination of cytokine levels. Interleukin IFN-γ, IL-4, and IL-17 production in BALF and anti-OVA IgE in serum were measured by ELISA according to the manufacturer's instructions.

Cell culture and treatments

Human BECs (derived from normal humans) were incubated in DMEM:F12 (1:1) containing 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10 % heat inactivated FBS at 37 °C in an atmosphere containing 5 % CO_2. After the cells were grown in 6-well round-bottom plates with 70 % confluence, BECs or BECs pretreated with OVA for 30 min were added with 0.2 mL PPA (1×10^7) PPA at 37 °C for additional 24 h.

Real-time PCR assay

RNA was isolated from rat lung tissues or human BECs using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. Each sample was reverse transcribed into cDNA and analyzed by quantitative real-time PCR with SYBR Green I. The primers (Invitrogen, China) for TLR4 were 5′ GATGCTTCTTGCTGGCTGC 3′ and 5′ ACCTTCATGGATGATGTTGGC 3′ (205 bp); for TLR5 were 5′ TTGCTCAAACACACCTGGACAC 3, and 5′ CTGCTCACAAAGACAAACGAT 3 (148 bp); for Act1 were 5′ AGTCAGCGAGCCTGCGTC 3′ and 5′ CCATCT CCTGGCTACCGC 3′ (197 bp); for A20 were 5′ GGCAG GAAAACAGCGAGC 3′ and 5′ GGCAGGAAAACAGCG AGC 3′ (168 bp). Briefly, 2 μL (out of 20 μL) of the reverse-transcribed reaction mix was added to 50 μL of PCR mixture for 35 cycles. Each cycle included 94 °C for 20 s, 60 °C for 30 s and 72°C for 30 s. Raw data were normalized to β-actin (5′ TGACGTGGACATCCGCAA AG 3′ and 5′ CTGGAAGGTGGACAGCGAGG 3′).

NF-kappa B/p65 assay by western blot

BECs were washed by ice-cold PBS twice, and resolved in RIPA buffer containing 1 mM PMSF, 5 mM β-glycerophosphate, and 1 % of a standard protease inhibitor cocktail (Sigma) on ice for 30 min. Insoluble materials were removed by centrifugation for 20 s at 12,000g at 4 °C. The supernatant was collected and the protein concentration was measured by the Bradford method to be adjusted to a final concentration of 10 mg/mL. The supernatant were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane were blocked with 3 % BSA in PBS for 2 h and then incubated with polyclone rabbit anti-NF-kappa B antibody and appropriate horseradish peroxidase-conjugated secondary antibody. Detection was made using the enhanced chemiluminescence system.

Measurement of cell cycle by flow cytometry

After being cultured in 6-well plates, cells were harvested at a density of 1×10^6 cells/mL, fixed in cold 70 %

ethanol, and stored at −20 °C overnight. The fixed cells were washed twice with phosphate-buffered saline, stained in a propidium iodide solution (50 μg/mL) for 1 h, and treated with a ribonuclease A solution (20 μg/mL) for 30 min. Flow cytometry was then using to examine the cell cycle. Experiments were repeated 4 times.

Statistical analysis

Calculations were performed with SPSS software. All values were expressed as mean ± SE. Measurements of lung mechanics were analyzed by two-factor ANOVA. Other data were compared by use of the Student's t test. A P value of <0.05 was considered significant.

Results

Influences of PPA on airway functions of an OVA-induced airway hyperresponsiveness model

After the animals were challenged by different concentrations of hydrochloric acid histamine (0.02, 0.04, 0.08, 0.16, and 0.32 mg mL^{-1}), the R_L of animals increased in a dose-dependent manner. The R_L of OVA-treated animals, which were challenged by 0.16 and 0.32 mg mL^{-1} of hydrochloric acid histamine, increased significantly compared with those of the control animals; while the R_L of OVA-treated animals significantly decreased after treatment with PPA (**$P < 0.01$; Fig. 1).

The lungs of animals treated with OVA showed a significant increase in the number of total cellular score and eosinophils. PPA decreased the total cells and eosinophils in BALF induced by OVA (Table 1). The numbers of
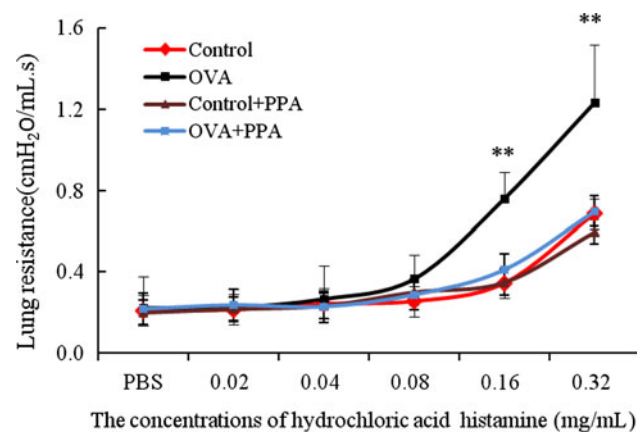

Fig. 1 Lung resistance (R_L) assay induced by different concentrations of hydrochloric acid histamine. OVA resulted in increased R_L to hydrochloric acid histamine (at the concentrations of 0.16 and 0.32 mg mL^{-1}) and PPA abolished the increased R_L induced by hydrochloric acid histamine ($n = 6$, *$P < 0.01$ vs. OVA plus PPA)

Table 1 Total and differential cells counts in BALF

Group	Total cellular score	Macrophage	Eosinophils	Neutrophils	Lymphocytes		
					CD3+	CD4+	CD8+
Control	2.36 ± 0.37	0.87 ± 0.48	0.29 ± 0.11	0.44 ± 0.17	0.67 ± 0.18	0.48 ± 0.14	0.17 ± 0.16
OVA	4.53 ± 1.21**	0.95 ± 0.53	0.76 ± 0.41**	0.63 ± 0.44	1.83 ± 0.47**	1.21 ± 0.29**	0.52 ± 0.21**
Control + PPA	1.94 ± 0.87	0.59 ± 0.33	0.28 ± 0.25	0.49 ± 0.36	0.76 ± 0.25	0.41 ± 0.17	0.25 ± 0.22
OVA + PPA	2.05 ± 1.33##	0.83 ± 0.32	0.34 ± 0.18##	0.56 ± 0.35	0.84 ± 0.38##	0.47 ± 0.25##	0.29 ± 0.18#

Data are presented as (mean ± SE) × 10^5 cells/mL

** $P < 0.01$, significantly different from control group ($n = 6$); ## $P < 0.01$, significantly different from OVA group ($n = 6$); # $P < 0.05$, significantly different from OVA group ($n = 6$)
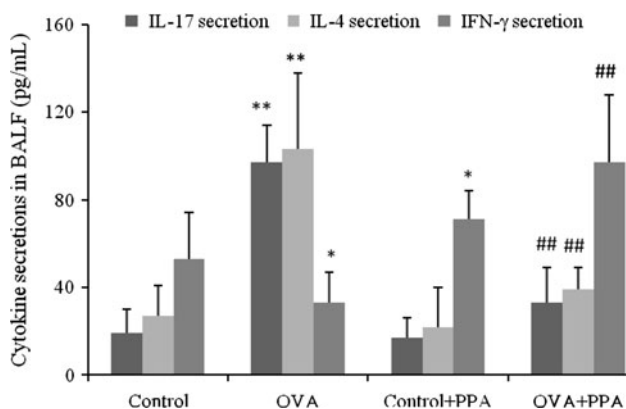

Fig. 2 Effects of PPA on IL-4, IL-17 and IFN-γ production in BALF. OVA promoted the production of IL-4 and IL-17 and decreased the production of IFN-γ. PPA decreased OVA-induced IL-4 and IL-17 secretion and increased IFN-γ secretion (** $P < 0.01$,* $P < 0.05$ vs. normal BECs group; ## $P < 0.01$ vs. OVA group, $n = 6$)

CD3-, CD4-, and CD8-positive cells in the lungs of OVA-challenged animals were increased compared to the control group (Table 1). PPA administration similarly resulted in significant reduction in T cell subtypes.

OVA-specific IgE in serum can be observed in all OVA-treated animals by using a qualitative ELISA Kit. PPA decreased OVA-specific IgE (data not shown). The lungs of animals treated with OVA showed a significant increase in the production of IL-4 and IL-17 and a decrease in the production of IFN-γ. PPA decreased OVA-induced IL-4 and IL-17 secretion and increased IFN-γ secretion (Fig. 2).

Influences of PPA on TLR-4 and TLR-5 expressions of BECs

It was shown that PPA stimulated TLR-4 and TLR-5 expression in normal BECs in a dose-dependent manner (Fig. 3a). According to this result, 2×10^6 PPA was chosen to do subsequent experiments. It was shown that TLR-4 and TLR-5 expression substantially reduced after OVA treatment and PPA induced TLR-4 and TLR-5 mRNA expression in control and OVA-treated BECs (Fig. 3b).

Influences of PPA on Act I or NF-kappa B/A20 expression of BECs

By using real-time PCR, we observed that OVA stimulated the expression of Act1 and PPA significantly decreased its expression in BECs treated by OVA ($P < 0.01$; Fig. 4a); OVA also stimulated NF-kappa B expression and decreased A20 expression in BECs. PPA inhibited NF-kappa B expression and increased A20 mRNA expression in normal or OVA-treated BECs ($P < 0.01$; Fig. 4b).

Influences of PPA on cell cycle of BECs

By using flow cytometry, we observed that the ratio of G1-phase in cells treated with OVA significantly increased, while S-phase significantly decreased when compared to normal BECs. PPA significantly increased the numbers of S-phase cells in normal BECs and BECs plus OVA groups ($P < 0.01$) (Fig. 5).

Discussion

Allergic asthma is characterized by airway hyperresponsiveness, recruitment of eosinophils to the airways, and immune abnormalities in a wide variety of cell populations. The experiments showed that an allergic animal model was successfully built by OVA challenge. The AR of OVA-treated animals increased significantly compared with that of the control animals. The cellular population in BAL fluid was altered. Especially significant are the increases in eosinophils and lymphocytes. PPA prevented the development of AHR, airway eosinophilia, decreased IL-4 and IL-17, and increased IFN-γ productions in BALF. PPA alone could stimulate IFN-γ production. These results demonstrate that PPA has profound regulatory effects on the development of lung allergic responses in the OVA-induced asthma model.

BECs are not only an important barrier against the environmental injury factors but also an active participant
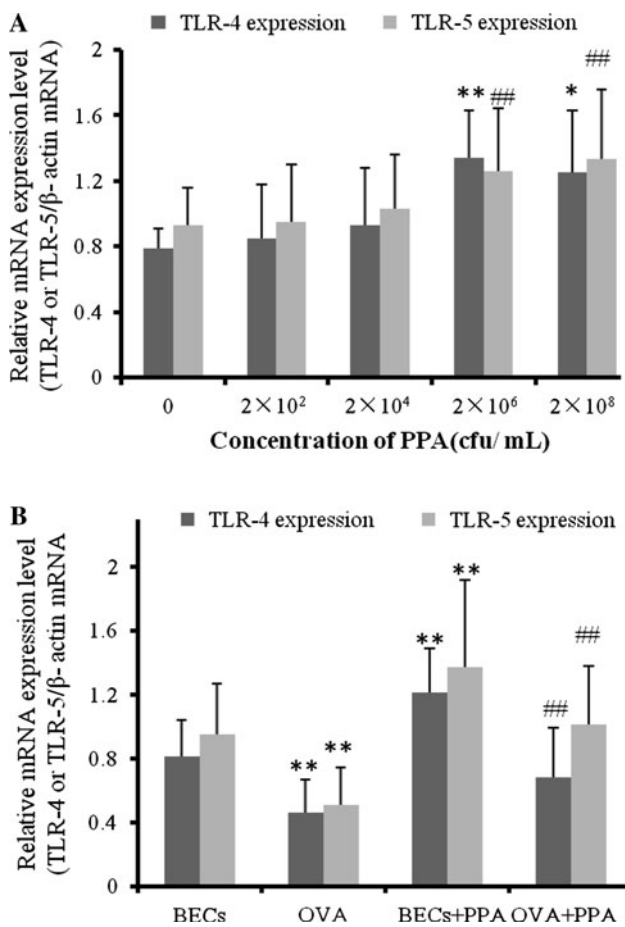
Fig. 3 Human TLR-4 or TLR-5 expression in BECs was determined by real time PCR. **a** Human TLR-4 or TLR-5 mRNA expression in normal BECs significantly induced by PPA in a dose-independent manner. **b** The TLR-4 or TLR-5 expression substantially reduced after OVA treatment and PPA induced TLR-4 or TLR-5 mRNA expression in normal and OVA-treated BECs. Data are means ± SE of 4 experiments (**$P < 0.01$ vs. normal BECs group; ##$P < 0.01$ vs. OVA group)

Fig. 4 a Human Act1 expression in BECs was determined by real-time PCR. The Act1 expression substantially increased after OVA treatment and PPA significantly decreased Act1 expression in BECs treated by OVA. **b** Human NF-kappa B (western blot) and A20 expression (real-time PCR) assay. Data are means ± SE of 4 experiments (**$P < 0.01$ vs. normal BECs group; ##$P < 0.01$ vs. OVA group)

in a variety of acute and chronic inflammatory reactions in the airway. Through making use of TLRs that recognize conserved microbial structures, epithelial cells are able to sense microbes, which lead to the inducible secretion of further mediators and corresponding T cell responses. Because PPA has a cloudy distribution of flagellar (TLR-5 natural ligand) and type 1 fimbriae (TLR-4 natural ligand) around the whole body, which are both strong suppressors of allergic responses [11], we selectively investigated the expression of TLR-4 and TLR-5 on BECs. The results showed that the TLR-4 and TLR-5 mRNA expressions substantially reduced after OVA treatment and PPA induced TLR-4 and TLR-5 mRNA expressions in normal and OVA-treated BECs, indicating that PPA can enhance the functions of TLR-4 and TLR-5, so promoting the recognition and removal of pathogens.
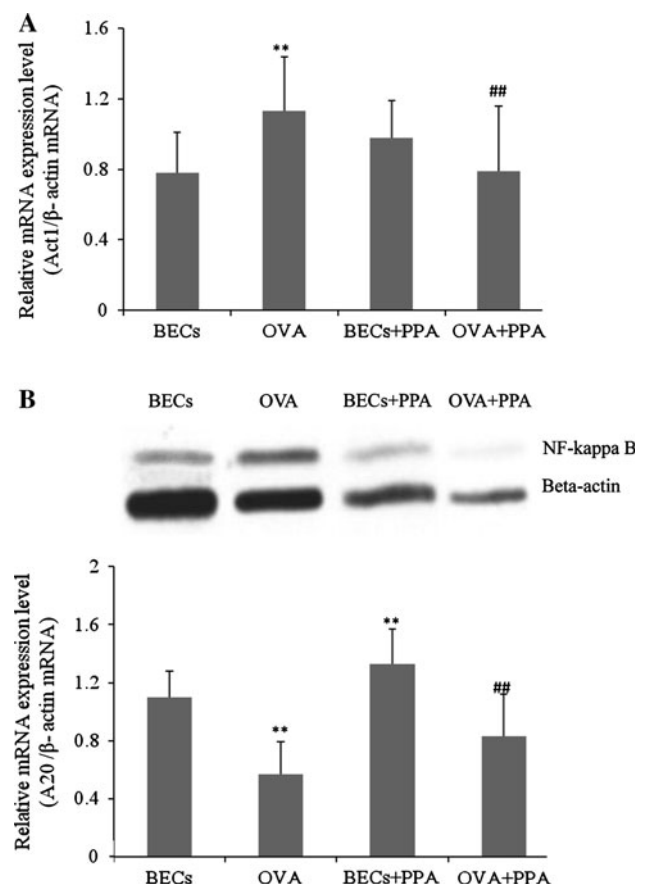
BECs-derived signal molecules are also very important in modulation of T cell responses. Act1 gene was first cloned as an NF-kB activator. Later studies have shown that Act1 has two important functions. On the one hand, Act1 is a key component in IL-17A signaling and induces Th2 differentiation [12], while on the other hand, it was recruited to CD40 and the TNF family member B cell-activating factor receptor in B cells through its interaction with TNFR-associated factor, negatively regulates B cell survival [13]. Swaidani's [14] research showed that epithelium-derived Act1 has the essential role in allergic pulmonary inflammation through the distinct impact of the IL-17R-Act1 and IL-25R-Act1 axes and acts as a potential therapeutic target for allergic pulmonary inflammation. PPA can significantly decrease Act1 mRNA expression on OVA-treated BECs, indicating that PPA prevented Th17 differentiation.

A20, which is viewed as a potential therapeutic target for inflammatory disease, is an NF-kB-inducible protein
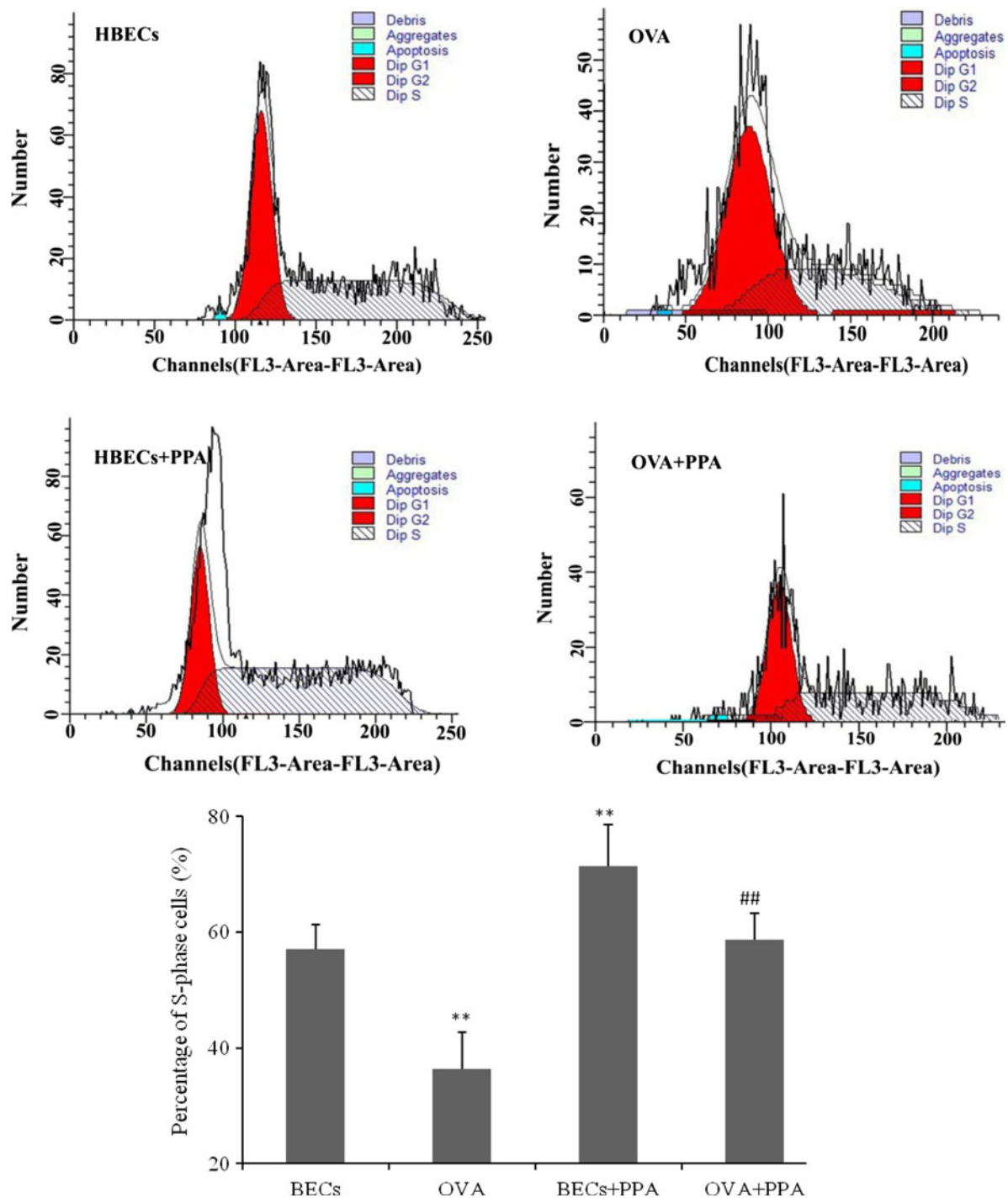
Fig. 5 Effects of PPA on the cell cycle of BECs were assayed by flow cytometry. Results indicated that OVA inhibited proliferation of BECs and PPA stimulated proliferation of normal and OVA-induced BECs. Data are means ± SE of 6 experiments. **$P < 0.01$ vs. BECs group; ##$P < 0.01$ vs. OVA group

and negatively regulates inflammatory signaling pathways [15]. PPA can significantly decrease NF-kB expression and increase A20 expression in normal or OVA-treated BECs, indicating that PPA is a hopeful therapeutic for airway inflammation.

Proliferation and wound repair of BECs are recognized as effective improvements of airway functions. In the present study, we observed that PPA can stimulate cell proliferation of resting or OVA-induced BECs. *Pseudomonas aeruginosa* may also have other ways to promote

epithelial proliferation including the role of shielding to protect and promote the release of epidermal growth factor [7, 16].

Taken together, PPA can decrease airway inflammation, improve epithelial functions, and stimulate recovery from abnormal airway microenvironment. These results might provide an opportunity for the development of novel therapeutics to treat asthma. Our result is not sufficient for the explanation of the precise mechanism, and the mechanism is more complicated. However, it is a possible mechanism that PPA shifts immunity from a Th2 to a Th1 bias in a rat model of asthma. It would be interesting to precisely identify the complicated mechanisms of our results concerning Th1/Th2 transcription factors in future studies. Moreover, additional studies are needed to characterize the precise mechanism of therapeutic action of PPA for treatment of asthma.

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