

Platycodin D Attenuates Airway Inflammation in a Mouse Model of Allergic Asthma by Regulation NF- κ B Pathway

Tianzhu Zhang,¹ Shihai Yang,² Juan Du,³ Yao Jinfu,^{1,4} and Wang Shumin^{1,4}

Abstract—In this work, the anti-asthma potential of platycodin D (PLD) was studied by investigation of its effect to suppress airway inflammation, a murine model of asthma and the possible mechanisms. A total of 50 mice were randomly assigned to five experimental groups: control, ovalbumin (OVA), OVA+dexamethasone (2 mg/kg) and OVA+PLD (40, 80 mg/kg). Airway resistance (Raw) were measured; airway histological studies were evaluated by the hematoxylin and eosin (HE) staining; interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 in bronchoalveolar lavage fluid (BALF) were evaluated by enzyme-linked immunosorbent assay (ELISA); NF- κ Bp65, p-NF- κ Bp65, p-IKK α , IKK α , p-IKK β , p-IkB α , and IkB α of airway were measured by Western blotting. Our study demonstrated that PLD inhibited OVA-induced increases in Raw and eosinophil count in airway; IL-4, IL-5, and IL-13 were recovered in BALF. Histological studies demonstrated that PLD substantially inhibited OVA-induced eosinophilia in airway tissue. Western blotting studies demonstrated that PLD substantially inhibited NF- κ B pathway. These findings suggest that PLD may effectively ameliorate the progression of asthma and could be used as a therapy for patients with allergic asthma.

KEY WORDS: platycodin D; asthma; NF- κ B pathway.

INTRODUCTION

Allergic diseases of the airway such as asthma or allergic rhinitis are on the rise throughout the world, especially in western countries [1]. One of the most important diseases is bronchial asthma, that is, defined as a chronic inflammation of the bronchial airways and characterized by reversible airway obstruction, increased mucus production, and infiltration of the airway with eosinophils, neutrophils, mast cells, and T-lymphocytes [2]. Interleukin (IL)-4 and IL-13, expressed by T-helper type 2 (Th2) cells, are key cytokines in the pathogenesis of atopy and atopic asthma [3, 4]. Both IL-4 and IL-13 promote acute inflammatory processes and underlying structural changes to the airways [5].

The NF- κ B transcription factor family is the crucial mediator of the inflammatory process and plays an important role in other processes, including development, cell

growth and survival, and proliferation. Cytokines and pathogen-associated molecular patterns (PAMPs) stimulate cell surface receptors including toll-like receptors (TLRs) to initiate a signaling cascade resulting in the activation of NF- κ B, which drives expression of target genes that mediate the release of anti-microbial molecules and cytokines to activate the immune response. Recently, many studies have reported that allergic asthma could be improved through regulating the activation of NF- κ B signaling pathway [6].

Platycodi Radix, the roots of *Platycodon grandiflorum* (Jacq.), has been used traditionally as an expectorant and a remedy for bronchitis, tonsillitis, laryngitis, and suppurative dermatitis in China, Korea, and Japan. In China and Korea, the fresh roots of *P. grandiflorum* have been eaten as pickles for preventing obesity [7]. Platycodin D is a major pharmacological constituent of *Platycodi Radix*, and it has been shown the antidiabetic [8], anti-inflammatory [9], anticancer [10], antinociceptive [11], and immunomodulatory [12] activities. Despite the several known effects of platycodin D (PLD), no work on ALI prevention promoted by this compound has been reported to date. The present study was designed to examine the effect of PLD on ovalbumin-

¹ Changchun University of Chinese Medicine, Changchun, 130117, China

² Jilin Agricultural University, Changchun, 130118, China

³ School of Life Science, Peking University, Beijing, 100871, China

⁴ To whom correspondence should be addressed at Changchun University of Chinese Medicine, Changchun, 130117, China. E-mails: yaojinfu126@126.com; Wangshuminch@126.com

induced airway inflammation in asthma and its possible mechanism.

MATERIALS AND METHODS

Materials

PLD powder (pure: 98.7 %) was purchased from National Institutes for Food and Drug Control (Beijing, China), and Mat solution (4 mg/mL) was prepared in distilled water. Dexamethasone was purchased from Xiansheng drug Store (Nanjing, China). Ovalbumin (OVA) (Sigma Chemical Co., St Louis, MO), aluminum hydroxide (Pierce Biotechnology, Rockford, USA), Wright–Giemsa staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). ELISA kits (R&D, Minneapolis, MN, USA). Antibodies of NF- κ B p65, phospho-NF- κ B p65 (Ser536), IKK α , IKK β , phospho-IKK α / β (Ser176/180), I κ B α , and phospho-I κ B α (Ser32) were purchased from Cell Signaling Technology.

Animals

Specific pathogen-free female BALB/c mice, aged 6–8 weeks, which were routinely screened serologically for relevant respiratory pathogens, were purchased from Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Animal experiment was carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University China Pharmaceutical University. A total of 60 female BALB/c mice were obtained from Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments and provided with water and standard chow *ad libitum*. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of China.

Sensitization, Airway Challenge, and Treatment

Mice were divided into five groups (each of 10 animals): (1) normal control, (2) asthma-model control, (3) asthma treated with dexamethasone (2 mg/kg, administered by gavage), (4, 5) asthma treated with PLD (40, 80 mg/kg, administered by gavage). Allergic asthma was induced by ovalbumin (Grade V) in five of the groups, using the method described by Oh *et al.* [13]. Mice were

immunized *via* intraperitoneal (i.p.) injection with 10 μ g chicken OVA and 2 mg aluminum hydroxide in 200 μ L phosphate buffered saline (PBS) (pH 7.4), on days 0 and 14. Mice were exposed to a 1 % (w/v) OVA solution in PBS, for 20 min, using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 28, 29, 30 after initial sensitization. Animals were sacrificed 48 h after the last challenge (thus on day 32) to characterize the suppressive effects of PLD. A schematic diagram of the treatment schedule is shown in Fig. 1.

Measurement of Airway Resistance (Raw)

The forced oscillation technique was used to measure respiratory mechanics. Regular ventilation was interrupted, and a computer generated volume signal that consisted of waveforms of mutually primed frequencies was delivered to the airway opening. Piston displacement and cylinder pressure were measured. Impedance values were obtained before and after the delivery of increasing concentrations of methacholine aerosols. Prior to the start of the methacholine concentration response curve, two total lung capacity breaths were delivered. Methacholine (Sigma) dissolved in saline was given *via* an ultrasonic nebuliser (Hudson RCI, Teleflex Medical) in increasing concentrations (0.125, 0.25, 0.5, and 1 mg mL⁻¹). After delivery of each aerosol, forced oscillations were delivered every 15 s, over a 5-min duration. In between impedance measurements, regular ventilation was resumed. The peak response for each variable was determined.

Collection of Bronchoalveolar Lavage Fluid

Mice were sacrificed using an overdose of 50 mg/kg of pentobarbital 48 h after the last challenge, and tracheotomy was performed. After instilling ice-cold PBS (0.5 mL) into a lung, bronchoalveolar lavage (BAL) fluid was obtained by three successive aspirations (total volume 1.5 mL) *via* tracheal cannulation [14]. BAL fluid (BALF) samples were centrifuged at 1500 rpm for 10 min at 4 °C; the supernatants were stored in –80 °C for analysis

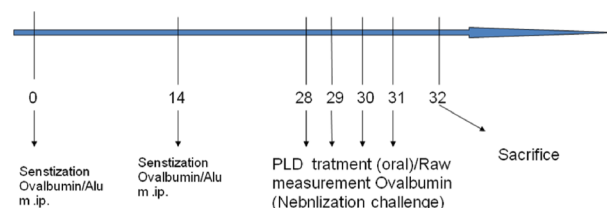


Fig. 1. Sensitization, challenge treatment protocols for the different groups in this study.

of cytokine concentrations, and the pellet was resuspended in 100 μ L of saline, centrifuged onto slides, and stained for 8 min with Wright–Giemsa staining. The slides were quantified for differential cell count by counting a total of 200 cells/slide at 40 magnification.

Histological Assessment

Airway tissues were detached from the mice and fixed with Carnoy's solution overnight at 4 °C. The fixed tissues were embedded in paraffin and cut into 4- μ m sections with a microtome (Leica, Nussloch, Germany). The sections were placed on slide glasses, deparaffinized, and stained with hematoxylin and eosin (Sigma, Korea) in order to examine the cells that had infiltrated into the airway tissues. Airway cells count based on a five-point scoring system was performed as previously described to estimate the severity of leukocyte infiltration [15, 16]. The scoring system was 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cell layers deep; and 4, a ring of cells more than 4 cell layers deep.

Enzyme-Linked Immunosorbent Assay Detection of BALF Cytokines IL-4, IL-5, and IL-13

BALF levels of IL-4, IL-5, and IL-13 were measured by ELISA according to the manufacturer's instructions (R&D, Minneapolis, MN, USA). All measurements were performed in duplicate. Briefly, the BALF samples were added in duplicate to 96-well plates with 100 μ L per well. The appropriate biotin-conjugated antibodies were added to each well. The samples were incubated at room temperature for 2 h. The wells were then aspirated, and each well was washed 5 times. The substrate solutions were added to each well and were incubated for 30 min at room temperature in the dark. The optical density (O.D.) of each well was determined using a microplate reader (Bio-Rad Model 680, USA) that was set to 450 nm. A standard curve was created of the average of the O.D. duplicate readings. The results were calculated using Excel 2007 (Microsoft office, USA).

Western Blotting of NF- κ B Pathway

The airway tissues were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of lung proteins. The samples were loaded to 10 % SDS-PAGE gels and were electrotransferred to nitrocellulose. The blots were incubated with the appropriate concentration of specific antibody. After washing, the blots were

incubated with horseradish peroxidase-conjugated second antibody. The membranes were stripped and reblotted with anti-GAPDH antibody (Sigma) to verify the equal loading of protein in each lane. Quantification of protein expression was normalized to GAPDH using a densitometer (Imaging System).

Statistical Analysis

All values were expressed as the mean $\pm$ SD and analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS version 13.0 software; a *P* value of less than 0.05 was considered significant, and *P* < 0.01 was considered to be statistically very significant.

RESULTS

Effect of PLD on Airway Resistance (Raw)

Measurements of airway resistance (Raw) during methacholine-induced constriction are shown in Fig. 2. The Raw value of the OVA-challenged group was significantly higher than that of the control group (*P* < 0.05). The treatment groups showed a significant decrease with acetylcholine 0.5 and 1 mg mL⁻¹ (*P* < 0.05 and 0.01, compared with model group, respectively).

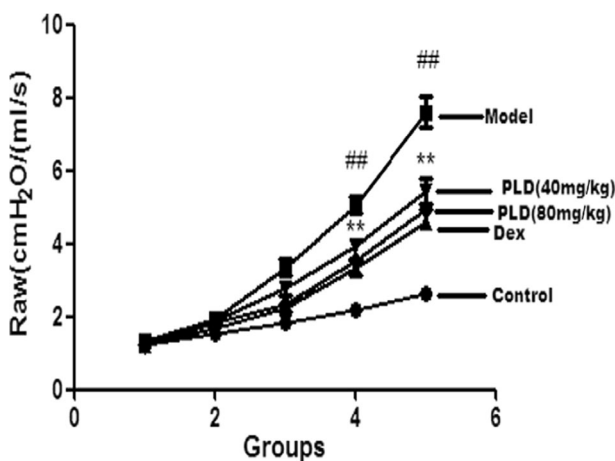


Fig. 2. Effect of PLD on airway resistance (Raw). Values are expressed as mean $\pm$ SDs. Compared with control: #*P* < 0.05; ##*P* < 0.01. Compared with model: **P* < 0.05, ***P* < 0.01.

Effect of PLD on OVA-Induced Eosinophilia and BAL Fluid Composition

We examined changes in total cell levels in the BAL fluid to determine the effects of PLD on asthma. Suppression of eosinophilia by Mat in OVA-challenged mice was estimated by counting of cells recruited to the BAL fluid 24 h after the last challenge. As shown in Table 1, OVA induced a marked influx of leukocytes into BAL fluid in the control group. Eosinophil levels markedly increased in the BAL fluid of OVA-challenged mice. OVA-sensitized and challenged mice treated with GA displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction.

Effect of PLD on OVA-Induced Eosinophilia in Airway Tissue

Because PLD inhibits inflammatory cell recruitment into the airway tissue, we next examined the anti-asthmatic effects of the PLD. Airways were collected 24 h after the final OVA challenge. In OVA-induced asthmatic airway tissue, we observed marked infiltration of inflammatory cells into airway tissues, compared with the normal tissue. Moreover, most leukocytes were eosinophils. The control group displayed no change in the extent of inflammatory cell infiltration, similar to what was seen in dexamethasone-treated positive control mice. Eosinophilia in OVA-sensitized and challenged mice treated with PLD was significantly attenuated compared with the level seen in OVA-challenged mice as shown in Fig. 3.

Effect of PLD on Cytokine Levels in BAL Fluid

To determine whether PLD influenced cytokine secretion into BAL fluid, IL-4, IL-5, and IL-13 in the BAL fluid were measured by ELISA 24 h after the final challenge. The results are shown in (Fig. 4), as for BALF levels

of IL-4, IL-5, and IL-13, compared with control group, levels of IL-4, IL-5, and IL-13, in model group was markedly increased ($P<0.05$), while compared with model group, the treatment groups can markedly decreased levels of IL-4, IL-5, and IL-13.

Effect of PLD on NF- κ Bp65 Pathway

The expressions of inflammation-related proteins NF- κ Bp65 signaling were changed by OVA in lung. As shown in Fig. 5. NF- κ B pathway was significantly activated in OVA-induced mice. The increased phosphorylation of NF- κ B(p-NF- κ Bp65/NF- κ Bp65) was detected with activation of IKK (p-IKK α /IKK α ;p-IKK β /IKK β) and p-I κ B α /I κ B α at protein levels in OVA-induced mice. These data demonstrated airway inflammatory in this model. In PLD, the protein levels of NF- κ Bp65 signaling were significantly decreased in a dose-dependent manner compared to the model group, respectively.

DISCUSSION

Asthma, a chronic inflammatory disease of the airways, affects many individuals worldwide. The disease may cause severe morbidity and even mortality if exacerbated. To date, a variety of drugs have been developed to treat asthma; these include steroids, leukotriene inhibitors, mast cell stabilizers, and β 2 adrenergic agonists. Many herbal medicines, including PLD, were commonly used to treat asthma, but the therapeutic efficacies and modes of action of such medicines are currently unclear. Here, using an OVA-induced mouse model of asthma, we show for the first time that PLD can be effective as a therapeutic drug for the treatment of allergic asthma.

Asthma is defined as a variable level of airway obstruction usually accompanied by aryl hydrocarbon

Table 1. Effect of PLD on OVA-Induced Eosinophilia and BAL Fluid Composition

Groups	Dose/mg.kg ⁻¹	White blood cells /10 ⁶ /L	Lymphocytes	Neutrophil	Monocytes	Eosinophil
Control	—	18.63±5.58	2.47±1.60	2.59±1.51	14.531±4.18	0.08±0.07
Model	—	28.88±3.68 ^{###}	7.8±4.49 ^{###}	4.81±1.63 [#]	13.88±3.87	5.98±1.97 ^{###}
Dex	2	10.50±5.90 ^{**}	2.29±2.08 ^{**}	2.09±1.59 ^{**}	5.49±2.21 ^{##}	0.93±0.62 ^{**}
PLD	40	19.±12.01 ^{**}	4.13±2.35 [*]	3.61±2.93 [*]	9.93±6.66 [*]	1.83±1.52 [*]
PLD	80	24.13±5.38 ^{**}	3.53±2.53 [*]	3.14±2.53 [*]	7.77±3.35 ^{**}	1.79±1.05 ^{**}

Values are expressed as means±SDs

Compared with control: [#] $P<0.05$; ^{###} $P<0.01$. Compared with model: ^{*} $P<0.05$; ^{**} $P<0.01$

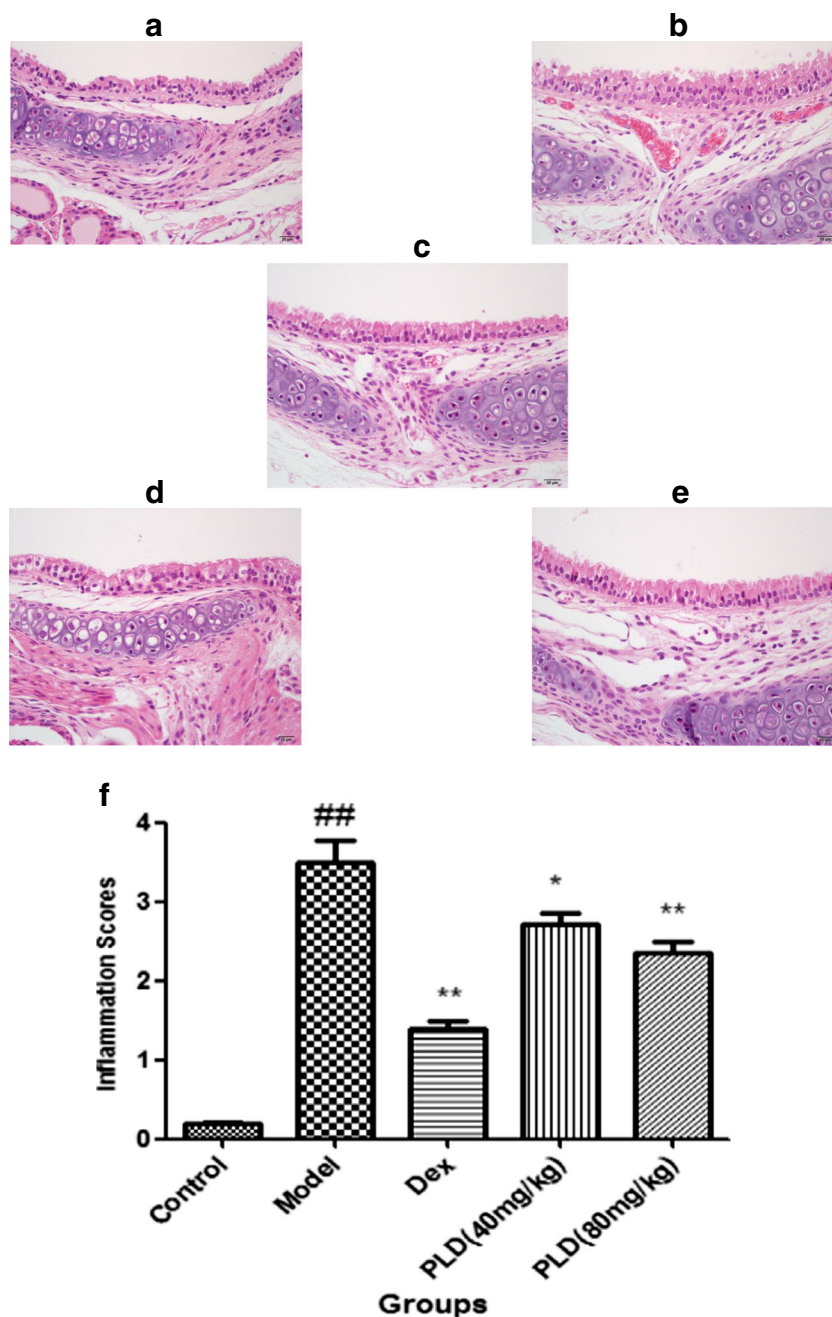


Fig. 3. Effect of PLD on cytokine levels in BAL fluid. Values are expressed as means $\pm$ SDs. Compared with control: $^{\#}P<0.05$; $^{##}P<0.01$. Compared with model: $^{*}P<0.05$; $^{**}P<0.01$.

receptor airway hyper responsiveness (AHR). Bronchoconstriction attributable to contraction or hypertrophy of airway smooth muscle (ASM), and inflammation within the airway, leads to decreased lung function [17]. AHR is a measure of the bronchial constriction commonly observed in individuals with asthma. We monitored the

effects of PLD on AHR by measuring Raw. These experiments disclosed that Mat inhibited the OVA-induced AHR in response to inhaled methacholine.

The mechanisms of recruitment of inflammatory cells associated with and presumably causing AHR have been well studied. Migration of inflammatory cells, specifically

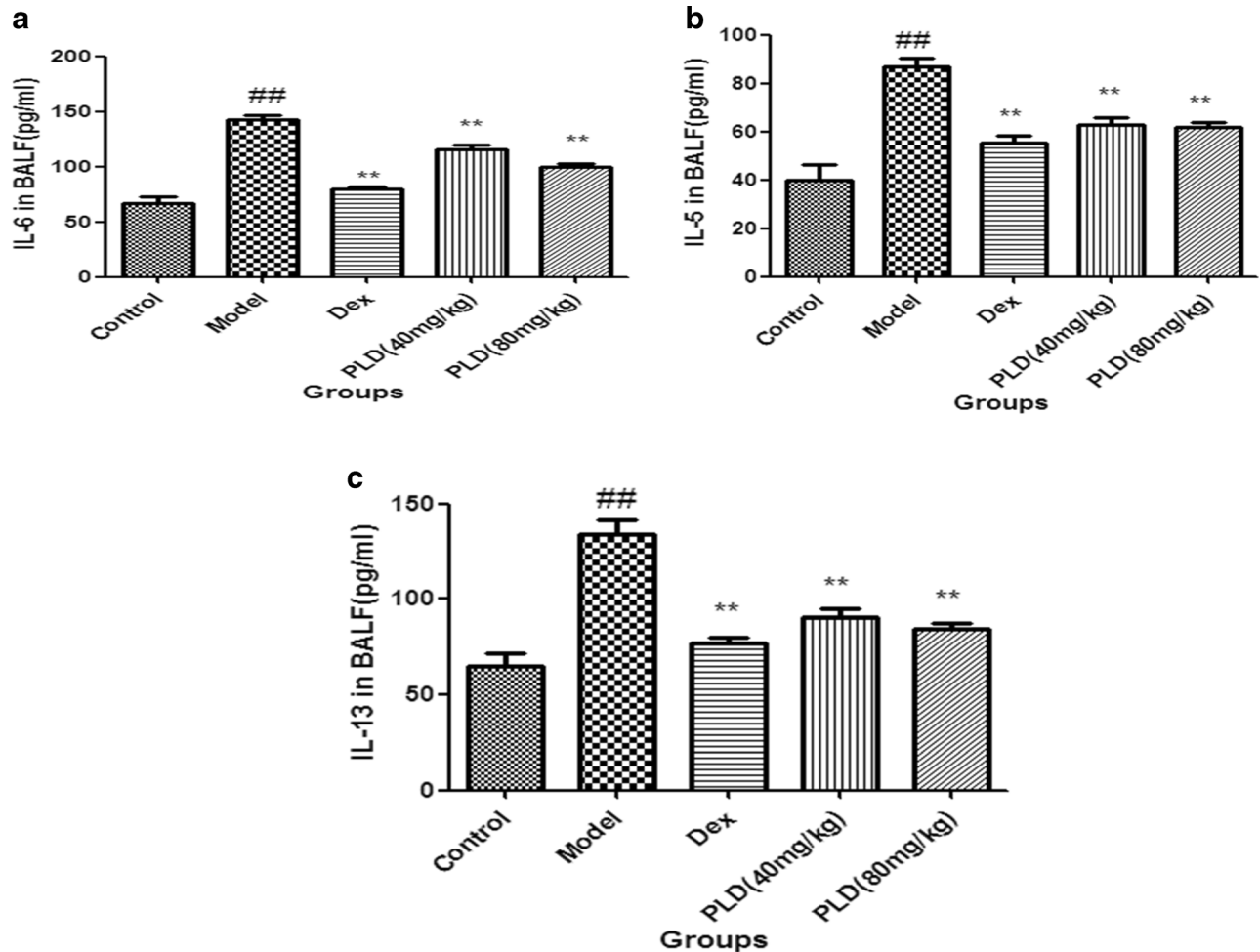


Fig. 4. Pathological changes of lung tissues observed by HE staining (Light microscopy, $\times 200$): **a** control, **b** model, **c** dexamethasone (2 mg/kg), **d** PLD (40 mg/kg), **e** PLD (80 mg/kg). Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections. Values are expressed as means $\pm$ SDs. Compared with control: $^{\#}P < 0.05$; $^{##}P < 0.01$. Compared with model: $^{*}P < 0.05$; $^{**}P < 0.01$.

eosinophils and lymphocytes, into the lung is a major contributor to the development of airway inflammation [18]. A rise in the number of eosinophils in the BAL fluid is a characteristic of asthma. The present results clearly demonstrate that Mat significantly reduces eosinophil numbers in the airway tissue.

Several lines of evidence indicate enhanced NF- κ B pathway activation in asthmatic tissues. Small animal models of allergic asthma using gene deficient mice highlight the importance of NF- κ B in disease pathogenesis. While a wealth of small animal studies have demonstrated the role of NF- κ B in inflammation following respiratory viral or bacterial infections [19]. NF- κ B also appears to be important in the allergic response. In the ovalbumin (OVA)

sensitisation and challenge model, total lung extracts from Brown-Norway rats exhibit enhanced NF- κ B activity [20]. In a murine model, bronchial epithelium exhibits robust and rapid NF- κ B p65 nuclear translocation and IKK α/β activity, compared to controls. Mice that lack NF- κ B p50 have reduced eosinophilic responses to aerosolised allergen. This effect was shown to be due to a lack of T cell production of Th2 cytokines, IL-13, IL-4, and the eosinophil growth factor IL-5 (Das *et al.*), and also the chemokine eotaxin [21]. The present results clearly demonstrate that PLD significantly inhibited NF- κ Bp65 signaling. The results support that the use of PLD is beneficial in the treatment of asthma and the exact mechanism and clinical application needs further investigation

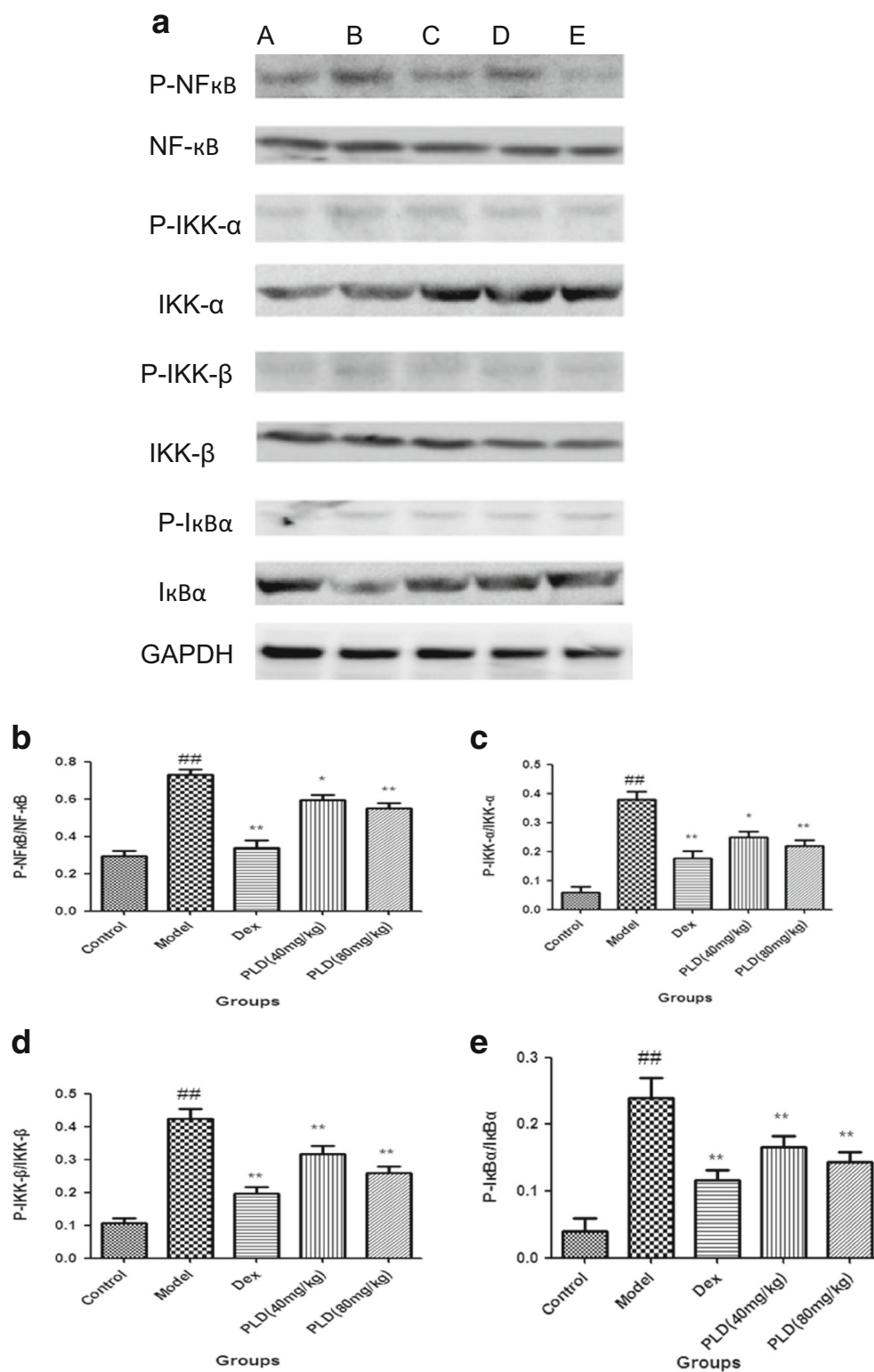


Fig. 5. Effect of PLD on NF- κ B. **a** control, **b** model, **c** dexamethasone (2 mg/kg), **d** PLD (40 mg/kg), **e** PLD (80 mg/kg). Values are expressed as means $\pm$ SDs. Compared with control: [#] P <0.05; ^{##} P <0.01. Compared with model: ^{*} P <0.05; ^{**} P <0.01.

REFERENCES

1. Beasley, R., J. Crane, C.K. Lai, and N. Pearce. 2000. Prevalence and etiology of asthma. *Journal of Allergy and Clinical Immunology* 105: 466–72.
2. Busse, W.W., and R.F. Lemanske Jr. 2001. Asthma. *New England Journal of Medicine* 344: 350–62.
3. Brightling, C.E., F.A. Symon, S.S. Birring, et al. 2002. TH2 cytokine expression in bronchoalveolar lavage fluid T lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis. *Journal of Allergy and Clinical Immunology* 110: 899–905.
4. Robinson, D.S., Q. Hamid, S. Ying, et al. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *New England Journal of Medicine* 326: 298–304.
5. Hershey, G.K. 2003. IL-13 receptors and signaling pathways: an evolving web. *Journal of Allergy and Clinical Immunology* 111: 677–690.
6. Ci, X., X. Chu, X. Xu, H. Li, and X. Deng. 2012. Short-term roxithromycin treatment attenuates airway inflammation via MAPK/NF-kappaB activation in a mouse model of allergic asthma. *Inflammation Research* 61: 749–58.
7. Han, B., J. Xu, and Y. Kimura. 2000. Platycodi radix affects lipid metabolism in mice with high fat diet-induced obesity. *Journal of Nutrition* 130(11): 2760–2764.
8. Han, L.-K., Y.-N. Zheng, B.-J. Xu, H. Okuda, and Y. Kimura. 2002. Saponins from Platycodi radix ameliorate high fat diet-induced obesity in mice. *Journal of Nutrition* 132(8): 2241–2245.
9. Kim, Y.P., E.B. Lee, and S.Y. Kim. 2001. Inhibition of prostaglandin E2 production by platycodin D isolated from the root of *Platycodon grandiflorum*. *Planta Medica* 67(4): 362–364.
10. Kim, M.-O., D.-O. Moon, H.C. Yung, J.-D. Lee, D.K. Nam, and G.-Y. Kim. 2008. Platycodin D induces mitotic arrest *in vitro*, leading to endoreduplication, inhibition of proliferation and apoptosis in leukemia cells. *International Journal of Cancer* 122(12): 2674–2681.
11. Choi, S.-S., E.-J. Han, T.-H. Lee, et al. 2002. Antinociceptive mechanisms of platycodin D administered intracerebroventricularly in the mouse. *Planta Medica* 68(9): 794–798.
12. Xie, Y., H.-X. Sun, and D. Li. 2009. Platycodin D is a potent adjuvant of specific cellular and humoral immune responses against recombinant hepatitis B antigen. *Vaccine* 27(5): 757–764.
13. Oh, S.R., M.Y. Lee, K. Ahn, B.Y. Park, O.K. Kwon, H. Joung, J. Lee, D.Y. Kim, S. Lee, J.H. Kim, and H.K. Lee. 2006. Suppressive effect of verproside isolated from *Pseudolysimachion longifolium* on airway inflammation in a mouse model of allergic asthma. *International Immunopharmacology* 6: 978–86.
14. Djukanović, R., W.R. Roche, J.W. Wilson, C.R. Beasley, O.P. Twentyman, R.H. Howarth, and S.T. Holgate. 1990. Mucosal inflammation in asthma. *American Journal of Respiratory and Critical Care Medicine* 142: 434–57.
15. Duan, W., J.H. Chan, C.H. Wong, B.P. Leung, and W.S. Wong. 2004. Anti inflammatory effects of mitogen-activated protein kinase inhibitor U0126 in an asthma mouse model. *The Journal of Immunology* 172: 7053–7059.
16. Wenzel, S.E. 2006. Asthma: defining of the persistent adult phenotypes. *Lancet* 368: 804–13.
17. Elsner, J., and A. Kapp. 1999. Regulation and modulation of eosinophil effector functions. *Allergy* 54: 15–22.
18. Haddad, J.J. 2002. Nuclear factor (NF)-kappa B blockade attenuates but does not abrogate LPS-mediated interleukin (IL)-1 beta biosynthesis in alveolar epithelial cells. *Biochemical and Biophysical Research Communications* 293: 252–257.
19. Haeberle, H.A., R. Takizawa, A. Casola, A.R. Brasier, H.J. Dieterich, N. Van Rooijen, et al. 2002. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. *Journal of Infectious Diseases* 186: 1199–1206.
20. Poynter, M.E., C.G. Irvin, and Y.M. Janssen-Heininger. 2002. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *American Journal of Pathology* 160: 1325–1334.
21. Yang, L., L. Cohn, D.H. Zhang, R. Homer, A. Ray, and P. Ray. 1998. Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *Journal of Experimental Medicine* 188: 1739–1750.