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천식 모델 생쥐에서 필발이 CD25+T 세포수, IgE, Histamine 생성량과 *in vitro*에서 Th1/Th2 Cytokine Balance에 미치는 영향

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Effects of Piperis Longi Fructus on Regulatory T Cells Number, IgE, Histamine Production in Asthma Model Mice and Th1/Th2 Cytokine Balance *in vitro*

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Abstract

Objectives : It has been recently shown that Piperis Longi Fructus (PLF) is involved in the reduction of eosinophil recruitment and production of Th2 cytokines *in vivo*. However, the main therapeutic mechanisms of PLF remains a matter of considerable debate. To investigate the therapeutic mechanisms of PLF, we examined the influence of PLF on regulatory T cells number, IgE, histamine production *in vivo* and Th1/Th2 cytokine balance *in vitro*.

Methods : All mice were immunized on two different days (21 days and 7 days before inhalational exposure) by i.p. injections of 0.2 ㎖ alum-precipitated Ag containing 100 ㎍ of OVA bound to 4 mg of aluminum hydroxide in PBS. Seven days after the second sensitization, mice were exposed to aerosolized ovalbumin for 30 min/day on 3 days/week for 12 weeks(at a flow rate of 250 L/min, 2.5% ovalbumin in normal saline) and PLF (150 mg/kg) were orally administered 3 times a week for 8 weeks. Splenocytes from C57BL/6 mice at 8 weeks of age were stimulated with anti-CD3 (1 mg/ml) plus anti-CD28 (1 mg/ml) antibody for 48hrs. IL-4 and IFN-γ in the culture supernatants were measured by ELISA

Results : The suppressive effects of PLF on asthma model were demonstrated by the increase the number of regulatory T cells and by reducing IgE, histamine production *in vivo* and modulation of Th1/Th2 cytokine balance.

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Conclusions : These results indicate that PLF has a deep inhibitory effects on asthma model mice by increase the number of regulatory T cells, and by reducing IgE, histamine production.

Key words : Piperis Longi Fructus (PLF), asthma, regulatory T cell, IgE, histamine

Introduction

Allergic asthma is characterized by airway hyperresponsiveness and chronic mucosal inflammation mediated by CD4+ Th2 lymphocytes[1]. CD4+CD25+ T cells can suppress Th2 maturation[2], possibly by inhibiting IL-4 production[3]. CD4+CD25+ T cells are important components of the homeostasis of the immune system, as impaired CD4+CD25+ T cell activity can cause autoimmune diseases, allergy and asthma.

CD4+CD25+ T cells can suppress the activation and proliferation of other CD4+ and CD8+ T cells in an antigen-nonspecific manner[4,5]. Increased numbers of CD4+ T lymphocytes have been found in asthmatic airways that show signs of activation[6,7]. It had been reported that depletion of CD4+ cells prevented bronchial eosinophilia and AHR in a murine asthma model[8]. Regarding the cytokine expression of CD4+ cells, it has been well established over recent years that asthmatic airway inflammation is characterized by an increased expression of the Th2-type cytokines IL-4, IL-5 and IL-13[9,10]. These cytokines are of major importance because IL-4 and IL-13 induce the production of IgE by B cells and IL-5 regulates the growth, differentiation, and activation of eosinophils[11].

Our previous studies have shown that treatment with Piperis Longi Fructus (PLF) reduced eosinophil recruitment and production of cytokines (IL-4, IL-5, and IL-13)[12,13]. A crude methanol extract of PLF was found to be active against the larvae, and the hexane fraction of the methanol extract showed a strong larvicidal activity of 100% mortality[14]. Other reports have shown that PLF has weak opioid but potent NSAID(nonsteroidal anti-inflammatory drug) type of analgesic activity[15]. Piperine was the first amide isolated from piper species and was reported to display central nervous system depression, antipyretic, anti-tumor and anti-inflammatory activity[16], and crude methanol extracts of PLF is effective Caecal amoebiasis in mice[17]. Especially, constituents of piper species have inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro*[18]. Dehydropipernonaline that has coronary vasorelaxant activity was isolated from the fruit of PLF[19]. Some people have reported the alcoholic extract of the fruits of the plant Piper longum and its component piperine have immunomodulatory and antitumor activity[20].

It has been recently shown that PLF is involved in the reduction of eosinophil recruitment and production of Th2 cytokines *in vivo*. However, the main therapeutic mechanisms of PLF remains a matter of considerable debate. To investigate the therapeutic mechanisms of PLF, we examined the influence of PLF on regulatory T cells number, IgE, histamine production *in vivo* and Th1/Th2 cytokine balance *in vitro*. In this study, we focused on the immunoregulatory effects of PLF on the regulatory T cells and Th1/Th2 balance in ovalbumin (OVA)-induced asthma model mice.

Materials and methods

1. Plant material and preparation of extracts

PLF was purchased from Oriental Medical Hospital (Daejeon, Korea) in August, 2003. The voucher specimens (PLF) are deposited in our laboratory (Department of Herbology, College of Oriental Medicine, Sanji University Wonju 220-702, Republic of Korea). Plant material (200 g) was extracted three times with H_2O. Then, the extract was filtered and evaporated on a rotatory evaporator (Rotary evaporator, BUCHI B-480, Switzerland) and

finally dried by a freeze drier (Freeze dryer, EYELA FDU-540, Japan) to yield the extracts PLF (30 g).

2. Animals

Seven to eight-week-old male C57BL/6 mice were obtained at Daehan Biolink Co. LTD. (Eumsung, Republic of Korea). The animals were housed in plastic cages in an air-conditioned room at 23±2℃ with a relative humidity of 55±10% under a 12-h light-dark cycle, fed a standard laboratory diet and given water ad libitum. Our study was approved by the committee for animal welfare at the institution (Daejeon University). All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

3. Digestion of pulmonary tissue and cell preparations

Single cell suspensions from lung tissues and BALF were isolated by mechanical disruption in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ㎍/ml streptomycin, 50 ㎛M 2-mercaptoethanol, 20 mM HEPES, and 2% heat-inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY). Briefly, Lungs were subsequently removed from thoracic cavity. After mincing using sterile scalpels, tissue was incubated in PBS containing 1 mg/ml Collgenase IV and 2 mg/ml DispaseⅡ for 40 min at 37°C in a sterile polypropylene tube. After incubation, lung tissue was vigorously pipetted up and down to further dissolve remaining tissue clumps and then filtered using 70 ㎛ cell-strainer (Falcon, Le Pont de Claix, France). Total cells of each samples were counted.

4. Ovalbumine sensitization and inhalation

OVA (500 ㎍/㎖) in PBS was mixed with equal volumes of 10% (w/v) aluminum potassium sulfate (alum; Sigma) in distilled water. Then incubated for 60 min at RT after adjustment to pH 6.5 using 10 N NaOH, and centrifuged at 750×g for 5 minutes. OVA/alum pellet was resuspened to the original volume in distilled water. All mice were immunized on two different days (21 days and 7 days before inhalational exposure) by i.p. injections of 0.2 ㎖ alum-precipitated Ag containing 100 ㎍ of OVA (Sigma-Aldrich Korea , Korea) bound to 4 mg of aluminum hydroxide (Sigma-Aldrich Korea. Korea) in PBS. Seven days after the second sensitization, mice were exposed to aerosolized ovalbumin for 30 min/day on 3 days/week for 12 weeks (at a flow rate of 250 L/min, 2.5% ovalbumin in normal saline) and intratracheally injected 250 ㎍ of OVA (on day 8) on the back of the tongue. PLF (150 mg/kg) were orally administered 3 times a week for 12 weeks. One day after the last of the OVA exposures, samples(bronchoalveolar lavage (BAL) fluid, lung cells, and blood) were collected[21-24].

5. Bronchoalveolar lavage fluid (BALF)

To evaluate airway inflammation, we experimented the accumulation of eosinophils in BALF. Mice were sacrificed with an intraperitoneal injection of sodium pentoparbitone (100 ㎎/㎏). The trachea was cannulated and the left bronchi were tied for histological experiment.

Immediately after sacrifice, cells in the lungs were recovered by flushing 1 ml of BAL fluid (1 mM EDTA, 10% FBS, PBS) into the lungs via the trachea. Total cell counts were determined and 100 ㎕ of fluid were cytospun onto glass slides using a Cytospin centrifuge (Cellspin, Hanil, Korea) (400 g for 4 minutes). Differential cell counts were performed after staining with Diff-Quik Stain Set (Baxter Healthcare Corp., Miami, Florida, USA). The supernatant of BALF was stored at -25℃ for determination of cytokines[23,24].

6. Antibodies and flow cytometric analysis

All antibodies for flow cytometric analysis were

purchased from Becton Dickinson (BD) PharMingen (San Diego, CA). Cells from lung tissues and BALF were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% NaN3) for 10 min on ice, and analyzed by two color flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences, Mountain View, CA).

7. Enzyme-Linked Immunosorbent Assay (ELISA)

IgE and histamine production from BALF and serum of the indicated mice (n=5) was measured by ELISA according to the manufacturer's instruction on a monoclonal antibody-based mouse interleukin ELISA kit (R&D system). Splenocytes from C57BL/6 mice at 8 weeks of age were stimulated with anti-CD3 (1 mg/ml) plus anti-CD28 (1 mg/ml) antibody for 48hrs. IL-4 and IFN-γ in the culture supernatants were measured by ELISA. All data represent the standard deviation of at least three different determinants and were compared using Student's unpaired t-test.

8. Hematoxylin & eosin (H&E) and Masson trichrome staining in murine OVA-indued asthma lung cells

C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of PLF for the later 8 weeks (3 times/week). At the end of the experiment, the mice lungs were removed and analyzed histology. Briefly, after removal of blood, lungs were slowly perfused with 10 ml PBS via the right ventricle, and then perfused with 4% paraformaldehyde and immersed in fixative solution overnight. Lungs were embedded in paraffin, and 5 ㎛ sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson Trichrome (MT). The slides were evaluated by microscopy.

9. Statistical Analysis

For statistical analysis of data, P-values were analyzed using a unpaired Student t-test software program (Startview 5.1; Abacus Concepts, Berkeley, CA). Results were considered statistically significant if p values were < 0.05 (*), < 0.01 (**), or < 0.001 (***).

Results

1. Effects of PLF on CD4+CD25+ regulatory T cells (%) population in murine OVA-induced asthma lung cells

Comparison with OVA control revealed, that population of CD4+ T helper cells and CD69+/CD3+ double positive cells in PLF treated group were significantly reduced (27.8% and 24.3% versus 19.5% and 15.8% respectively; $p < 0,05$) (Fig. 1).

Effects of PLF on CD4+CD25+ regulatory T cells in lung, there were marked change in numbers of CD4+CD25+ T cells(regulatory Th cells) in lung compared to control group. PLF treated group with OVA resulted in further significant increase in CD4+CD25+ T cells (Fig. 1, ** : $p < 0.01$).

2. Measurement of IgE and histamine levels in BAL fluid and serum

To investigate the effects of PLF on IgE synthesis and histamine release, PLF (150 mg/kg) were orally administered 3 times a week for 8 weeks. PLF prevents IgE production in both BALF and serum, but not histamine release. Because IgE levels in serum are dependent upon IL-4 and may be considered an additional index of Th2-cytokine secretion, we measured IgE in BALF and serum from mice in all groups. We found that IgE levels in serum from OVA-induced murine model of asthma were significantly increased compared with normal groups (PBS only). PLF treated mice significantly was inhibited the production of IgE. These results support the conclusion that PLF

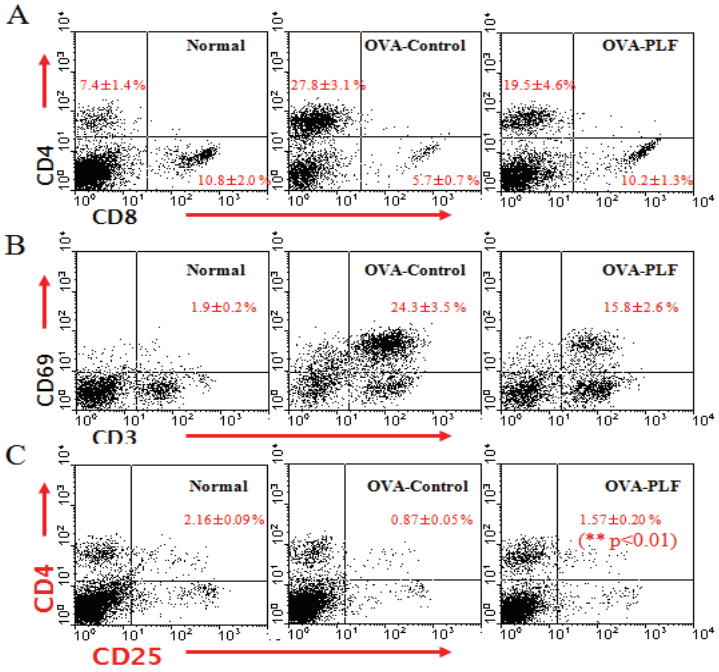

Fig. 1. Effects of PLF on the number of regulatory T cells (CD4+CD25+), CD4, CD8 T lymphocyte subsets and CD3+CD69+ (early activated T cells) cells in OVA-induced murine model of asthma
Normal : Normal C57BL/6 mice. OVA-Control : Ovalbumin inhalation + vehicle. OVA-PLF : OVA + PLF (150 ㎎/㎏).

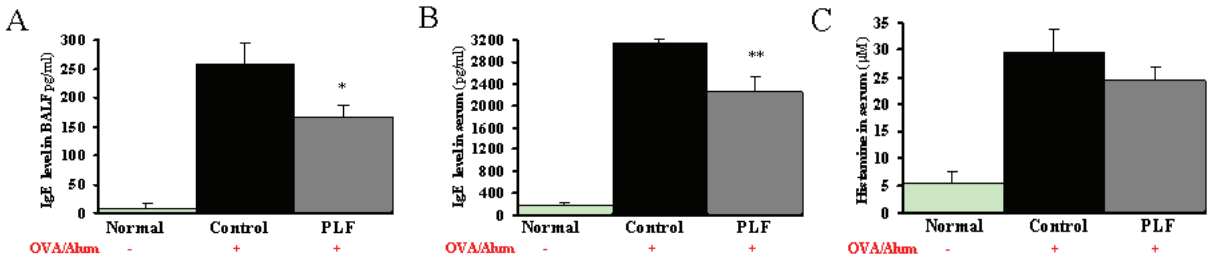

Fig. 2. Effects of PLF on immunoglobulin E level in BALF and serum in OVA-induced murine model of asthma
C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of PLF for the later 8 weeks. At the end of the experiment, BALF and serum were collected(materials and method) and anaylzed by ELISA.
Normal : Normal C57BL/6 mice.
OVA-control : Ovalbumin inhalation (control).
PLF : OVA + PLF (150 mg/kg).
The results are expressed the mean±S.E (N=5). Statisticallysignificant value compared with control group data by T test (* : $p < 0.05$, ** : $p < 0.01$).

suppressed the generation of a Th2-type immune response in this animal model of asthma.

3. Immunomodulation of OVA-specific Th1/Th2 cytokines production in spleen cells

Spleen cells were isolated from experimental murine model of asthma (in materials and methods) and stimulated in the absence PLF.

To study whether PLF were related to Th1/Th2 cytokine balance,after 48 hrs of culture, supernatant from the splenocytes of PLF treated group were analyzed the levels of IL-4 and IFN-γ in each samples.

As shown in Fig. 3, IL-4 productions in spleen were significantly suppressed by PLF. On the contrary, PLF enhanced the secretion of IFN-γ levels.

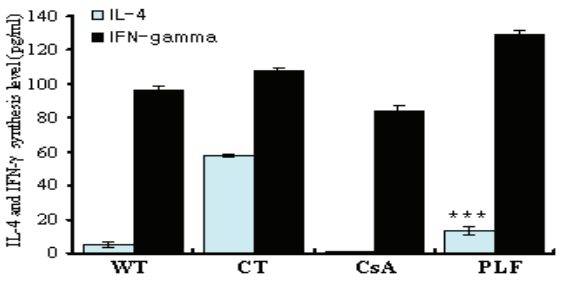

Fig. 3. Immunomodulatory effects of PLF on OVA-specific Th1/Th2 cytokines production in spleen cells

Splenocytes from C57BL/6 mice at 8 weeks of age were stimulated with anti-CD3 (1 mg/ml) plus anti-CD28 (1 mg/ml) antibody for 48hrs. IL-4 and IFN-γ in the culture supernatants was measured by ELISA.
WT : Normal C57BL/6 mice.
CT : anti-CD3 (1 mg/ml) plus anti-CD28 (1 mg/ml) antibody coated plate.
CsA : anti-CD3 plus anti-CD28 plus cyclosporin A (10 mg/ml).
PLF : anti-CD3 plus anti-CD28 plus PLF (100 mg/ml)
The results are expressed the mean±S.E (N=5). Statistically significant value compared with control group data by T-test (*** : $p < 0.001$).

4. Histological analyses of lung sections from OVA-induced asthma model mice after final antigen challenge

To clarify the efficacy of PLF on lung cells of murine asthma model, the left lungs were histologically examined 24h after the final antigen challenge. Histological analyses of lungs from PBS-exposed sensitized mice showed normal lung histology(Fig. 4). In contrast, histological sections of lung tissue from OVA-exposed mice exhibited airway inflammation. In a quadrangle area, infiltrating eosinophils were chiefly observed in the peribronchial regions of the lung. While on the other hand, exhibition of airway infammation was decreased in histological sections of lung tissue from PLF treated mice (Fig. 4).

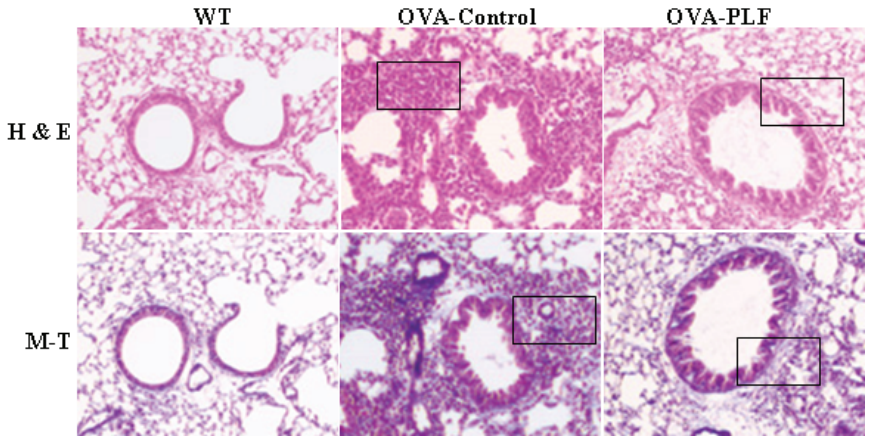

Fig. 4. Effects of PLF on histology of lung tissue (H&E and Masson trichrome staining) in lung cells of OVA-indued murine model of asthma

C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of PLF for the later 8 weeks (three times a week). At the end of the experiment, the mice lungs were removed and analyzed histology.
H&E : hematoxyline-eosin stain.
WT : Normal C57BL/6 mice.
OVA-control : Ovalbumin inhalation.
OVA-PLF : OVA + PLF (150 mg/kg).

Discussion

PLF is one of the well known herb used in oriental medicine for treatment anti-inflammatory and many allergic diseases. Therapeutic mechanisms of PLF in the development of OVA-induced eosinophilia and hyperresponsiveness in murine model of asthma have not been fully investigated *in vivo* and *in vitro*.

CD4+ T lymphocytes are the responsible cells accounting for the pathologies associated with allergic airway disease. From numerous animal

models, the initiation of pulmonary inflammatory responses is absolutely dependent on CD4+ T lymphocytes and to this can be added an essential role for IL-4; genetic elimination or IL-4 during the initiation or sensitization phase is associated with a lack of AHR, allergen-specific IgE, and airway eosinophilia development[8].

CD8+ T cells play a important role in both the regulation and progression of allergic diseases, including asthma. Despite a number of articles describing CD8+ T cells as suppressors of AHR and airway inflammation[25,26], Erwin et al have shown that CD8+ T cells are important contributors to the development of allergic responses in the lung[27]. However, the main role of CD8+ T cells remains a matter of considerable debate. In our results, PLF also played a role as a suppressor of chronic asthma model.

Above of all, regulatory T cells can regulate airway function by suppressing Th2 maturation and Th2 cytokine production. Regulatory T cells can also be generated in the periphery from either CD4+ or CD8+ T cells under specific conditions. CD4+CD25+ T cells can suppress the activation and proliferation of other CD4+ and CD8+ T cells in an antigen-nonspecific manner[28,29]. Furthermore, CD4+CD25+ T cells play a key role in regulating airway eosinophilic inflammation.

Regulatory T cells (Tregs) appear to play important roles in regulation of B cell Ig response. In several autoimmune diseases with aberrant Ab production, the function or number of Tregs is decreased[30].

It was recently suggested that a transient activation-induced CD69 surface expression may be important for regulating T cell trafficking[31]. Our results showed that PLF down-regulate CD4+ T lymphocyte subsets and CD3+CD69+ (early activated T cells) cells(Fig. 1).

IgE production is considered to be due to the development and activation of Th2 cells and B cells. This specific Th2 cell produces predominantly IL-4 and IL-5. IL-4 plays a crucial role in inducing class switching of the IgE isotype and its production. Moreover, excessive IL-4 production by Th2 cells has been associated with an elevation of IgE levels and allergic reaction.

We showed that PLF administration to mice suppressed asthma induced by OVA stimulation and that the increase in serum histamine of PLF treated group was lower as compared with that of the control group. Also, secretion of Th2-driven BALF and serum IgE were markedly inhibited by oral administration of PLF(Fig. 2).

It is now well established that asthma is characterized by the production of large quantities of IgE antibodies by B cells and by a decrease of the IFN-γ /IL-4 (Th1/Th2) ratio. Herbal medicines to modulate the Th1/Th2 cytokine balance in the prevention of asthma remains attractive. Aside from the use of them to alter the Th1/Th2 balance, several other strategies are under current investigation. One such strategy is the invention of novel anti-allergic agents to modulate the Th1/Th2 balance.

It has been demonstrated that the Th1/Th2 cell response is shifted to a predominantly Th1 cell response during autoimmune diseases, while an overwhelming Th2 response elicits allergic disorders[32]. Investigations on the balance of Th1/Th2 cytokines production should be helpful to understand the outcomes of different immune responses, and are clinically useful in treating immunologically dysregulated

states[33]. Thus, modulation of the Th1/Th2-balanced immune response is one of the most strategic immunotherapies for allergic diseases. Since Th1 and Th2 types of reactions appear to be reciprocally regulated *in vivo*, modulation of the Th1/Th2 balance should become a common strategy for asthma therapy[34].

Spleen cells obtained from the mouse produced more interleukin IL-4 but less interferon IFN-γ than T cells from nonsensitized control animals (Fig.3). However, PLF reduced the production of IL-4 and the production of IFN-γ returned to the control level. Moreover, in our previous results, the IL-4 level was increased in the BAL fluid of the

OVA-sensitized animals compared to the nonsensitized control, while the IFN-γ levels decreased. PLF reduced the IL-4 levels in the BAL fluids and returned the IFN-γ level to control levels. Exhibition of airway infammation was decreased in histological sections of lung tissue from PLF treated mice(Fig. 4)

The present study indicates that PLF can suppress allergen-specific IgE-mediated reactivity in a murine model of asthma, which can be resulted from shifting from a Th2-dominated to a Th1-dominated immune response.

In conclusion, our results strongly indicate that PLF reduces allergic airway inflammation and hyperresponsiveness due to the alteration of Th1/Th2 polarization via the suppression of CD4+, CD69+ T cells and increase of CD8+ T cells and CD25+ regulatory T cells. Therefore, our data suggest that PLF might offer a new therapeutic approach to allergic airway diseases.

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