

Therapeutic effects of *Pulsatilla koreana* Nakai extract on ovalbumin-induced allergic rhinitis by inhibition of Th2 cell activation and differentiation via the IL-4/STAT6/GATA3 pathway

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ABSTRACT

Allergic rhinitis (AR), caused by immunoglobulin E (IgE)-mediated inflammation, generally occurs in the upper respiratory tract. T helper type 2 (Th2) cell-mediated cytokines, including interleukin (IL)-4, IL-5, and IL-13, are important factors in AR pathogenesis. Despite various treatment options, the difficulty in alleviating AR and pharmacological side effects necessitate development of new therapies. The root of *Pulsatilla koreana* Nakai (*P. koreana*), a pasque flower, has been used as a herbal medicine. However, its effects on AR remain unclear; therefore, we aimed to explore this subject in the current study. The therapeutic effects of *P. koreana* water extract (PKN) on the pathophysiological functions of the nasal mucosa was examined in ovalbumin (OVA)-induced AR mice. The effect of PKN on Th2 activation and differentiation was evaluated using concanavalin A-induced splenocytes and differentiated Th2 cells from naïve CD4⁺ T cells. We also investigated the effect of changes in JAK/STAT6/GATA3 signaling on IL-4-induced Th2 cells. In OVA-induced AR mice, PKN administration alleviated allergic nasal symptoms and decreased the total number of immune cells, lymphocytes, neutrophils, and eosinophils in nasal lavage fluid; serum levels of OVA-specific IgE, histamine, and IL-13 were also significantly reduced. PKN also ameliorated OVA-induced nasal mucosal tissue thickening by inhibiting inflammation and goblet cell hyperplasia. PKN treatment significantly inhibited Th2 activity and differentiation through the IL-4/STAT6/GATA3 pathway in Th2 cells. PKN is an effective AR treatment with the potential to improve patients' daily lives by regulating the allergic inflammatory response induced by Th2 cells.

1. Introduction

Allergic rhinitis (AR) is known to be caused by immunoglobulin (Ig) E-mediated hypersensitivity reactions following exposure to an allergen and commonly occurs in the nasal airway with symptoms including nasal rubbing, congestion, rhinorrhea, sneezing, and nasal itching [1]. Approximately 10–40 % of the global population experience AR symptoms, and the incidence is expected to increase further [2,3].

Antihistamines, corticosteroids, anticholinergic drugs, and leukotriene inhibitors are among the pharmacological options for AR therapy. However, a complete cure is difficult owing to the side effects of long-term therapy [4,5] and the interaction between immune cells and mediators.

CD4⁺ T helper type 2 (Th2) cells play a critical role in AR by secreting key cytokines such as interleukin (IL)-4, -5, and -13 [6]. IL-4 and IL-13 activate the Janus kinase (JAK)/signal transducer and

Abbreviations: AR, Allergic rhinitis; Con, Control; Con A, Concanavalin A; DEX, Dexamethasone; GATA3, GATA-binding protein 3; H&E, Hematoxylin & Eosin; IFN, Interferon; IgE, Immunoglobulin E; IL, Interleukin; JAK, Janus kinase; NALF, Nasal lavage fluid; OVA, Ovalbumin; PAS, Periodic acid Schiff; *P. koreana*, *Pulsatilla koreana* Nakai; PKN, *P. koreana* water extract; STAT6, Signaling transducer and activator of transcription; Th1, T helper type 1; Th2, T helper type 2; Treg, Regulatory T cell; UPLC-MS/MS, Ultra-high-performance liquid chromatography-tandem mass spectrometry; WT, Wild-type.

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activator of the transcription 6 (STAT6) pathway by binding to the type 1 IL-4 receptor (IL-4R) (complex of IL-4R α and γ -chain (γ c)) and type 2 IL-4R (complex of IL-4R α and IL-13R α 1). This induces the activation and differentiation of Th2 cells by increasing the expression of the GATA-binding protein 3 (GATA3) [7,8]. Allergens inhaled into the nasal cavity activate B cells, which secrete allergen-specific IgE through an allergic immune response. Secreted IgE activates mast cells in the nasal mucosa to release histamine [1,9]. In addition, mucus hypersecretion is promoted by inducing goblet cell metaplasia in the nasal mucosa [9,10]. Eosinophil recruitment at inflammatory sites in the nasal cavity increases the allergic inflammatory response and exacerbates AR symptoms [1]. Therefore, modulation of Th2 cell activity and differentiation in these pathophysiological processes could be a promising therapeutic approach for allergic hyperimmune responses, such as AR.

Traditional herbal medicines are characterized by unique properties and pharmacological activities owing to the structural diversity of their constituents. These multi-target and multifunctional properties can be applied to novel therapeutic strategies. *Pulsatilla koreana* Nakai (*P. koreana*), a pasque flower, is a hairy perennial plant mainly used for ornamental purposes. It grows throughout Korea, except Jeju Island, and is distributed in northeast China and northeast Russia. The root is used primarily as a herbal medicine and has traditionally been used to treat amebic dysentery and malaria [11]. The antitumor, antibiotic, and anti-inflammatory activities of hederoside C and saponin D in the root extract of *P. koreana* have been reported [12,13], but their effects on allergic diseases have not been reported.

Therefore, in this study, we aimed to evaluate the therapeutic effect of water-extracted *P. koreana* (PKN) on ovalbumin (OVA)-induced AR mice, concanavalin A (Con A)-induced splenocytes, and differentiated Th2 cells from naïve CD4⁺ T cells. Further, the effects of changes in JAK/STAT6/GATA3 signaling on IL-4-induced Th2 cells were investigated.

2. Materials and methods

2.1. PKN preparation

P. koreana was purchased from Ungok Herb Co. (Busan, Korea). The voucher specimen of *P. koreana* was deposited at the herbarium of the Korea Institute of Oriental Medicine (KIOM, Daejeon, Korea; voucher number: #JW-158). To prepare the PKN, distilled water (10 L) was added to dried *P. koreana* roots (1 kg) and then refluxed at 100 ± 2 °C. After 3 h refluxing, the extract was passed through a mesh filter (53 μ m), concentrated using a rotary evaporator (N-1200A; Eyela, Tokyo, Japan), and then freeze-dried using a lyophilizer. The PKN was stored at -80 °C until further use.

2.2. Chemical profiling of PKN using ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

The chemical constituents of PKN were characterized as described previously [14–17]. The reference standards, including chicoric acid (Cat. #T6S2391), hederacoside C (Cat. #T3370), pulsatilla saponin D (Cat. #T3909), anemoside A3 (Cat. #T3911), oleanolic acid (Cat. #T2865), and betulonic acid (Cat. #T5S0018), were purchased from Targetmol (Wellesley Hills, MA, USA). UPLC-MS/MS analysis was performed using a Dionex UltiMate 3000 system coupled with a Thermo Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved by an Acquity BEH C18 column (100 $\times$ 2.1 mm, 1.7 μ m) with 0.1 % formic acid in water and acetonitrile. The programed elution condition for UPLC and detailed parameters for MS/MS were employed as previously reported [18]. Data acquisition and processing were performed using Xcalibur and TraceFinder 5.1 softwares (Thermo Fisher Scientific).

2.3. Establishment of an OVA-induced AR mouse model

An OVA-induced AR mouse model was established as described previously [19]. In brief, the Institutional Animal Care and Use Committee (IACUC) of Chonnam National University approved the animal experiments for the OVA-induced AR mouse model (CNU IACUC-YB-2021-104). The AR mice induced using OVA were orally administered PKN every day at 100 or 300 mg/kg doses from days 21 to 27.

2.4. Analysis of histopathological changes in nasal tissues

The nasal tissues of OVA-induced AR mice were paraffin-embedded and sectioned at 5 μ m after fixation and decalcification. The sectioned nasal tissues were stained with a Periodic acid Schiff (PAS) staining kit (Sigma-Aldrich, St. Louis, MO, USA) or a Hematoxylin and Eosin solution (Sigma-Aldrich). Tissue slides were evaluated under a light microscope (Olympus Corporation, Tokyo, Japan) at 400 $\times$ magnification.

2.5. Evaluation of infiltration of immune cells in nasal lavage fluid (NALF)

After sacrificing the OVA-induced AR mice, NALF was harvested with cold phosphate-buffered saline (PBS; 10 mL) from the nasal cavity to evaluate immune cell infiltration. The total number of NALF cells was counted using a cell counter (Thermo Fisher Scientific) and the NALF was centrifuged using Cytospin (Hanil, Seoul, Korea). The number of infiltrated immune cells was investigated using a light microscope (Olympus Corporation) at 400 $\times$ magnification, after staining with Diff-Quik staining reagent (Sysmex, Chuo-ku, Kobe, Japan).

2.6. Analysis of serum parameters

To collect the serum, blood samples were collected from the abdominal vein of OVA-induced AR mice and immediately centrifuged at 1000 g for 15 min. ELISA kits for IL-13 (R&D Systems, Minneapolis, MN, USA), histamine (Enzo Life Science, Farmingdale, NY, USA), and OVA-specific IgE (Cayman Chemical, Ann Arbor, MI, USA) were used to analyze serum parameters.

2.7. Cell culture

Detailed methods of cell culture have been previously described [19]. Briefly, animal experiments for isolation of primary splenocytes and differentiation of Th2 cells were performed by the IACUC of KIOM (approval no.: 2022-002). Primary splenocytes (5×10^6 cells/mL) were cultured in 96-well plates for 24 h after treatment with Con A (Con A; 2.5 μ g/mL; Sigma-Aldrich) and PKN (50, 100, and 200 μ g/mL). Naïve CD4⁺ T cells were differentiated to Th2 cells using a CellXVivo Mouse Th2 Cell Differentiation Kit (R&D Systems), and the cells were cultured with PKN (50, 100, or 200 μ g/mL) during Th2 cell differentiation.

2.8. Analysis of cell viability and Th cytokine secretion

Viability of splenocytes was assessed using an EZ-Cytox Cell Viability Assay Kit (Daeil Lab Service, Seoul, Korea) after treatment with Con A and PKN for 24 h. Cell culture supernatants were collected from primary splenocytes or differentiated Th2 cells, and the secretion levels of Th cytokines (IL-4, IL-5, IL-13, IFN- γ , IL-2, IL-10) were evaluated using a LEGENDplex™ Mouse Th Cytokine Panel (BioLegend, San Diego, CA, USA; Cat. no. 741044).

2.9. Flow cytometry analysis of Th2 cell differentiation

Antibodies for differentiated Th2 cells were obtained from BD Biosciences (Franklin Lakes, NJ, USA): anti-GATA3-BV421 (Cat. no.

563349), Anti-CD4-BB700 (Cat. no. 566407), Anti-CD3-FITC (Cat. no. 561798). Differentiated Th2 cells were resuspended at 1×10^6 cells/test and stained with the indicated antibodies. The cell population was investigated using an LSRFortessa™ X-20 flow cytometer (BD Biosciences) and FlowJo v. 10 software (FlowJo, Ashland, OR, USA).

2.10. Western blot analysis

Naïve CD4⁺ T cells (5×10^6 cells/well) isolated from splenocytes were seeded in a 24-well plate, and Th2 differentiation was induced for 6 days using a CellXVivo Mouse Th2 differentiation kit (R&D Systems) with or without PKN treatment at different concentrations (50, 100, and 200 µg/mL). In addition, differentiated Th2 cells (5×10^6 cells/well) were treated with 20 ng/mL recombinant IL-4 (R&D Systems) and PKN for 5, 10, and 20 min at each concentration. Primary antibodies were as follows: phospho-JAK1 (p-JAK1) (Cell Signaling Technology (Danvers, MA, USA); Cat. no. 74129), JAK1 (Cell Signaling Technology; Cat. no. 3344), phospho-STAT6 (p-STAT6) (Cell Signaling Technology; Cat. no. 56554), STAT6 (Cell Signaling Technology; Cat. no. 5397), GATA3 (Cell Signaling Technology; Cat. no. 5852), IL-4 (R&D Systems; Cat. no. MAB404), and β -actin (Cell Signaling Technology; Cat. no. 4970). Secondary antibodies were as follows: horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology; Cat. no. 7074) and anti-rat IgG (R&D Systems; Cat. no. HAF005). The protein expression levels were detected using the ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA) and the results were quantified using ImageJ version 1.51j8 software (National Institute of Health, Bethesda, MD, USA).

2.11. Statistical analysis

The results of the animal experiments are presented as the mean $\pm$ standard error of the mean, and the results of *in vitro* experiments are presented as the mean $\pm$ standard deviation. Significances were calculated using one-way analysis of variance followed by Tukey's multiple tests using GraphPad Prism version 9 (GraphPad, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Chemical profiling of PKN

In Table 1 and Fig. 1, chicoric acid (1), hederacoside C (2), hederacolchiside E (3), pulsatilla saponin D (4), anemoside A3 (5), oleanolic acid (6), and betulonic acid (7) were identified in PKN, comparing with the fragmentation patterns and retention time of reference standards. The ion fragment pattern of PKN phytochemicals in MS spectra are consistent with previously reports [14–16]. In addition, hederacolchiside E was tentatively identified in accordance with a previous study [17].

3.2. PKN treatment attenuated clinical symptoms of AR in the OVA-induced AR mouse model

In the OVA-induced control mice, the number of rubbing and

sneezing episodes significantly increased compared with that in wild-type (WT) control mice (Fig. 2a and b). The increased number of rubbing episodes was attenuated by treatment with dexamethasone (DEX) or PKN, and a significant difference was observed between DEX ($p < 0.001$) and PKN (300 mg/kg, $p < 0.01$) treatments (Fig. 2a). Similarly, the number of sneezing episodes was significantly decreased following treatment with DEX ($p < 0.001$) or PKN (100 mg/kg, $p < 0.01$; 300 mg/kg, $p < 0.001$) (Fig. 2b). OVA-induced control mice showed severe histopathological changes, such as infiltration of immune cells and increased epithelium thickness and mucus production, compared with WT control mice; these changes were ameliorated by DEX or PKN treatment (Fig. 2c). The epithelium thickness increased in OVA-induced control mice and was reduced following DEX or PKN administration (Fig. 2d). The number of goblet cells increased with OVA treatment but decreased in DEX- or PKN-administered mice (Fig. 2e).

3.3. PKN treatment reduced the infiltration of immune cells in the OVA-induced AR mouse model

The OVA-induced control mice exhibited an increased number of total cells in NALF compared with WT control mice; however, this number was notably reduced following treatment with DEX or PKN (Fig. 3a). Similarly, the infiltration of immune cells, including eosinophils, neutrophils, macrophages, and lymphocytes, markedly increased in the NALF of OVA-induced control mice (Fig. 3b–e). However, after DEX or PKN treatment, immune cell infiltration was significantly reduced in OVA-induced mice (Fig. 3b–e). PKN treatment did not result in a significant difference in macrophage infiltration (Fig. 3d).

3.4. PKN treatment decreased levels of serum parameters in the OVA-induced AR mouse model

Levels of serum parameters, such as OVA-specific IgE (Fig. 4a), histamine (Fig. 4b), and IL-13 (Fig. 4c), in OVA-induced control mice were higher than those in WT control mice; however, DEX or PKN treatment resulted in notably lower levels of these serum components.

3.5. PKN treatment suppressed activation of Th2 cells in splenocytes

Splenocytes isolated from BALB/c mice were treated with PKN after stimulation with Con A to evaluate the suppressive effect of PKN treatment on Th2 cell activation. First, we assessed the cytotoxicity of PKN treatment in splenocytes and found that cell viability did not change upon treatment with concentrations below 300 µg/mL (Fig. 5a). Secretion of Th2 cytokines (IL-4, IL-5, and IL-13) was stimulated by Con A treatment, and PKN treatment significantly inhibited the secretion of Th2 cytokines (Fig. 5b–d). PKN treatment suppressed the secretion of Th1 cytokines, such as IFN- γ (Fig. 5e) but did not change the secretion of IL-2, a well-known Th cytokine related to cell proliferation (Fig. 5f). Secretion of IL-10 (a regulatory T cell [Treg] cytokine) was not affected by PKN treatment (Fig. 5g). These results indicated that PKN treatment could suppress Th2 activation, which was unrelated to the Th1/Th2 balance and Treg regulation.

Table 1

Constituents identified in *Pulsatilla koreana* water extract via ultra-high-performance liquid chromatography-tandem mass spectrometry.

No.	R _t (min)	Calculated (m/z)	Estimated (m/z)	Error (ppm)	Adduct	Chemical formula	MS2 fragments (m/z)	Identification
1	6.48	473.0726	473.0728	0.6246	[M-H] ⁻	C ₂₂ H ₁₈ O ₁₂	179, 149	Chicoric acid*
2	10.45	1265.6172	1265.6173	0.0945	[M+HCO ₂] ⁻	C ₅₉ H ₉₆ O ₂₆	749, 469	Hederacoside C*
3	11.48	1365.6696	1365.6693	0.5800	[M-H] ⁻	C ₆₅ H ₁₀₆ O ₃₀	895	Hederacolchiside E
4	14.42	957.5064	957.5076	1.1680	[M+HCO ₂] ⁻	C ₄₇ H ₇₆ O ₁₇	911	Pulsatilla Saponin D*
5	15.58	751.4627	751.4625	-0.3149	[M+H] ⁺	C ₄₁ H ₆₆ O ₁₂	437, 255, 177, 115, 85	Anemoside A3*
6	20.82	455.3525	455.3537	2.5886	[M-H] ⁻	C ₃₀ H ₄₈ O ₃	-	Oleanolic acid*
7	21.58	453.3374	453.3381	1.4420	[M-H] ⁻	C ₃₀ H ₄₆ O ₃	-	Betulonic acid*

R_t, retention time. *, Compared with retention time (R_t) and mass spectrum of reference standards.

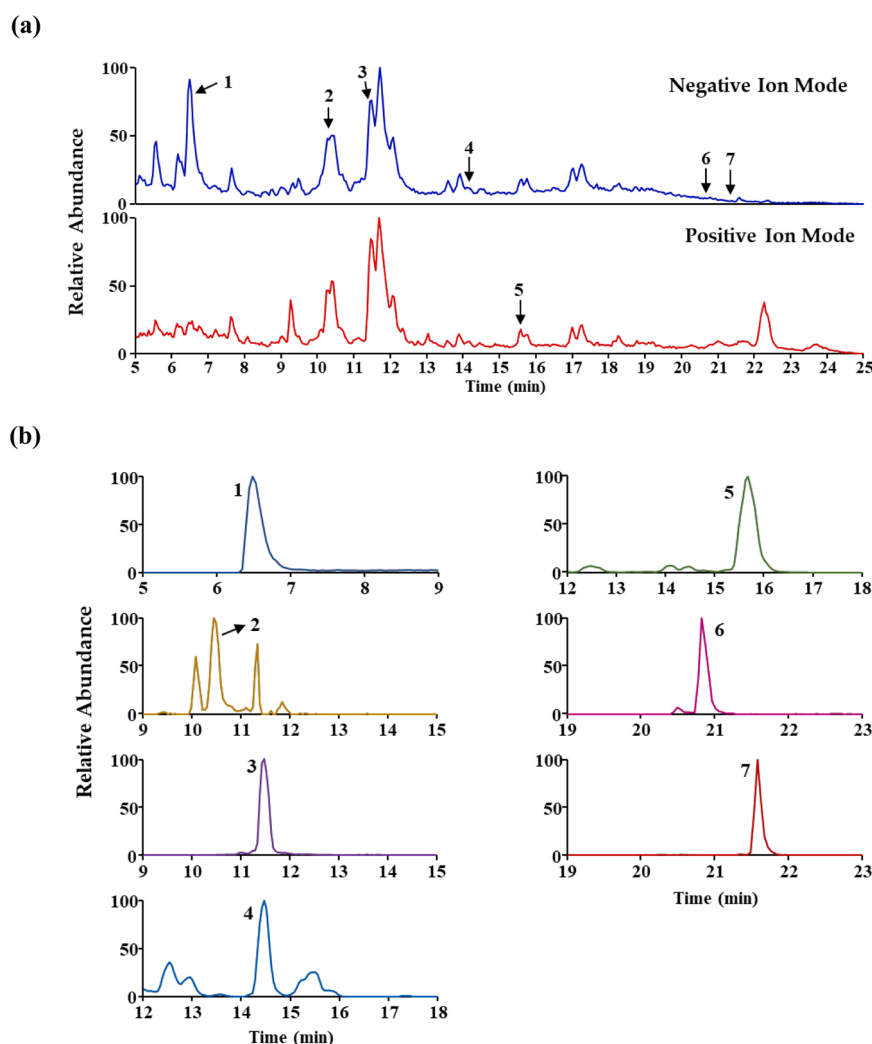


Fig. 1. Ultra-high-performance liquid chromatography-tandem mass spectrometry analysis of *Pulsatilla koreana* water extract (PKN). (a) Total ion chromatogram of PKN in positive and negative ion mode. (b) Extracted ion chromatograms of PKN. 1, chicoric acid; 2, hederacoside C; 3, hederacolchiside E; 4, pulsatilla saponin D; 5, anemoside A3; 6, oleanolic acid; 7, betulonic acid.

3.6. PKN treatment inhibited the differentiation of Th2 cells from naïve CD4⁺ T cells

To further clarify the effect of PKN treatment on Th2 cells, we evaluated the differentiation of Th2 cells from naïve CD4⁺ T cells. As shown in Fig. 6a and b, the population of CD3⁺ CD4⁺ GATA3⁺ cells (differentiated Th2 cells) was 86.57 % ± 0.93 in the control group, and PKN treatment inhibited the cell population in a dose-dependent manner (50 µg/mL, 84.70 % ± 1.43; 100 µg/mL, 83.03 % ± 0.93; 200 µg/mL, 58.57 % ± 2.99, $p < 0.001$). Moreover, the protein expression of IL-4 (Fig. 6c and d) and the secretion levels of IL-5 (Fig. 6e) and IL-13 (Fig. 6f) were significantly decreased by PKN treatment in differentiated Th2 cells.

3.7. PKN treatment suppressed the IL-4/STAT6/GATA3 pathway in differentiated Th2 cells derived from naïve CD4⁺ T cells

To determine whether PKN treatment regulated the IL-4-induced signaling pathway, we analyzed the phosphorylation of JAK1 and STAT6 in differentiated Th2 cells. The phosphorylation of JAK1 and STAT6 was induced by IL-4 treatment in a time-dependent manner (Fig. 7a, left panel), and treatment with PKN did not suppress JAK1 phosphorylation but did suppress STAT6 phosphorylation (Fig. 7a, right panel). STAT6 phosphorylation and GATA3 expression were reduced by

PKN treatment during Th2 differentiation on day 3 in a dose-dependent manner (Fig. 7b). Significant differences in STAT6 phosphorylation (Fig. 7c) and GATA3 expression (Fig. 7d) were observed following treatment with 200 µg/mL PKN.

4. Discussion

In this study, we evaluated whether PKN could alleviate AR by regulating the differentiation and activity of Th2 cells induced by allergens and identified its pharmacological function. Our results show that PKN inhibits Th2 differentiation and activity through inhibition of the IL-4-induced STAT6/GATA3 signaling pathway. The suppression of Th2 cell production by PKN reduces the secretion of Th2-related cytokines such as IL-4, IL-5, and IL-13. In addition, in the OVA-induced AR mouse model, PKN reduced the levels of inflammatory mediators and inflammatory cells and significantly reduced eosinophilic infiltration and goblet cell metaplasia in nasal tissues. Accordingly, we report the potential of PKN as an AR therapeutic agent.

According to the literature, Th2 cells play an important role as central regulators of AR and other allergic diseases [20,21]. External allergens inhaled into the respiratory tract promote differentiation and activation of allergen-specific Th2 cells by antigen-presenting cells, including macrophages and dendritic cells [22–24]. In particular, activated Th2 cells promote increased production of Th2-related cytokines

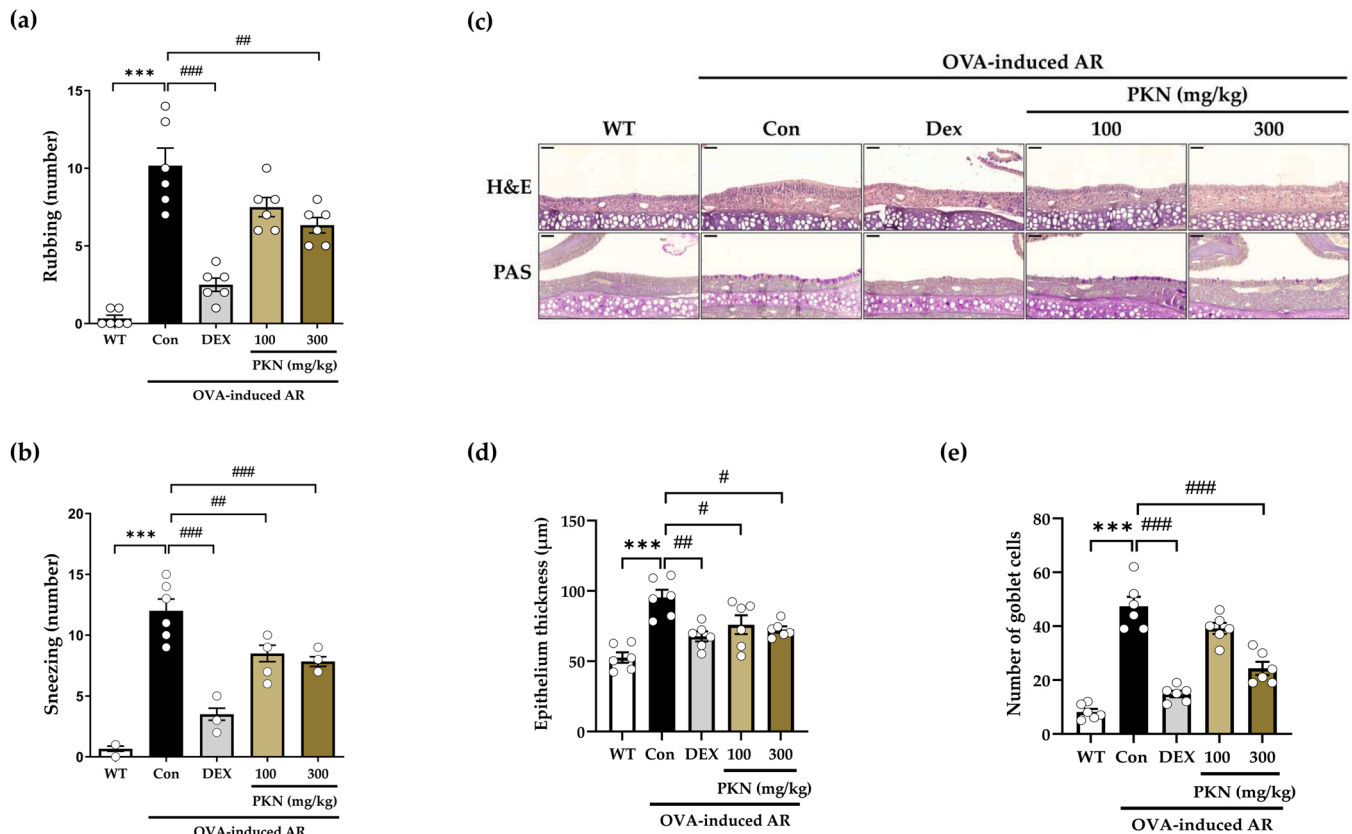


Fig. 2. *Pulsatilla koreana* water extract (PKN) treatment attenuated clinical symptoms of allergic rhinitis (AR) in the ovalbumin (OVA)-induced AR mouse model. The number of rubbing (a) and sneezing (b) episodes was counted for 15 min after the last OVA challenge. (c) The histological changes in nasal tissues were observed after staining with hematoxylin & eosin (H&E) and periodic acid Schiff (PAS) ($\times 400$, scale bar = 50 μm). The epithelium thickness (d) and the number of goblet cells (e) were evaluated using H&E and PAS staining. The data are presented as the mean $\pm$ standard error of mean ($n = 6$ per each group). *** $p < 0.001$ vs. wild-type (WT). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. control (Con). DEX, Dexamethasone.

(IL-4, IL-5, and IL-13) and allergen-specific IgE [1,20]. In addition, IL-4 and IL-13 promote mucus hypersecretion and airway hyper-responsiveness by increasing goblet cell metaplasia in epithelial cells of the nasal mucosa [9,10]. IL-5 induces inflammation by promoting the recruitment of eosinophils in nasal tissues [23,24]. Our findings show that rubbing and sneezing behaviors, characteristic symptoms of AR, were alleviated by PKN in an OVA-induced AR mouse model. In the histological analysis of nasal tissue, a decrease in nasal mucosal epithelial cell thickness and inhibition of goblet cell hyperplasia demonstrate that the cause of AR symptoms is ameliorated by PKN. Moreover, the levels of inflammatory mediators (numbers of eosinophils, neutrophils, and lymphocytes in NALF) and serum parameters (OVA-specific IgE, histamine, and IL-13) were reduced by PKN. These results suggest that the AR therapeutic effect by PKN is closely related to the pathophysiological process of AR mediated by Th2 cytokines.

In this regard, an imbalance of Th1/Th2 cytokines is one of the major causes of AR [25,26]. Tregs also regulate Th2 hypersensitivity through IL-10 cytokines [27]. As shown in the analysis results of cytokine patterns using splenocytes, IL-4, IL-5, and IL-13 (Th2-related cytokines) in splenocytes, which were increased by Con A, were decreased by PKN. IFN- γ (Th1-related cytokine) levels were also decreased by PKN, but IL-2 (Th1/Th2 cytokine) and IL-10 (Treg-related cytokine) levels did not show significant changes. These results suggest that the inhibitory effect of PKN on Th2-related cytokines is not regulated by Th1 or Treg cells but is a direct inhibitory effect on Th2 cells.

Th2 cell differentiation from naïve CD4 $^{+}$ T cells is promoted in a local cytokine milieu by IL-4 and IL-13 [10]. Binding to type 1 and type 2 IL-4R induces dimerization of STAT6 via JAK1/3- or JAK1/2-mediated phosphorylation, respectively. Phospho-STAT6 dimers translocate to

the nucleus and induce the expression of the master regulator GATA3 [28,29]. Studies conducted in GATA3-knockout animal models have confirmed that GATA3 is an essential factor for Th2 cell differentiation and cytokine production [30]. In a Th2 cell differentiation using naïve CD4 $^{+}$ T cells isolated from the spleen, it was confirmed that the decrease in levels of Th2-related cytokines by PKN is closely related to the suppression of GATA3 protein expression during the Th2 cell differentiation process. Furthermore, it was found that inhibition of STAT6 phosphorylation by PKN was the main cause of suppression of GATA3 protein expression.

PKN consisted of various compounds, and triterpenoids saponins are known as the main components. In particular, triterpenoid saponins of lupan- and oleanane-types, which are isolated in PKN, are known as major constituents [31]. Betulinic acid, a typical lupane-type triterpenoid saponin of PKN, improves cellular immunity, humoral immunity, and macrophage activity that can potentially regulate the immune system [32,33]. In addition, it contributes to anti-inflammatory effects through the regulation of cytokine production in Th1/Th2 cells [34]. On the other hand, oleanolic acid, an oleanane-type triterpenoid saponin, suppresses the production of antigen-specific antibodies by inhibiting pro-inflammatory cytokines and inflammation-related factors [35,36]. The mechanism of action for this immune regulation is due to inhibition of NF- κ B and related signaling pathways, STAT and GATA3 signaling pathways [37,38]. Although the therapeutic effect of PKN components on allergic rhinitis was not evaluated in this study, we predict that the active compounds of PKN are due to the effects of oleanolic acid and its glycosides (oleanane-type triterpenoids saponins). Further studies are needed to clarify these hypotheses, and future studies will evaluate the effectiveness of the PKN component as a treatment for allergic rhinitis.

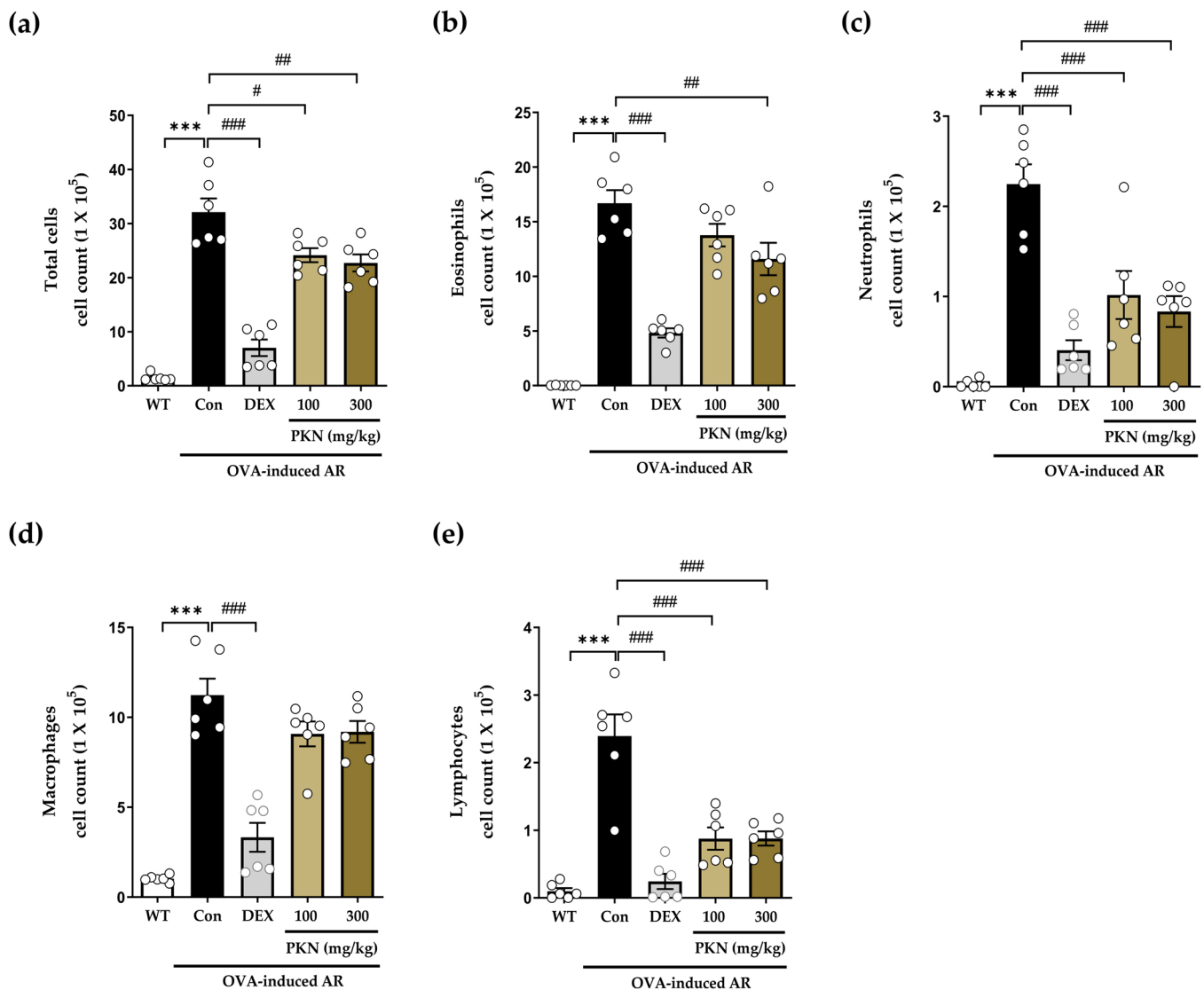


Fig. 3. Pulsatilla koreana water extract (PKN) treatment reduced the infiltration of immune cells in the ovalbumin (OVA)-induced allergic rhinitis (AR) mouse model. The number of infiltrated immune cells, such as total cells (a), eosinophils (b), neutrophils (c), macrophages (d), and lymphocytes (e), was analyzed from the nasal lavage fluid. The data are presented as the mean $\pm$ standard error of mean (n = 6 per each group). ***p < 0.001 vs. wild-type (WT). #p < 0.05, ##p < 0.01, ###p < 0.001, vs. control (Con). DEX, Dexamethasone.

Best of our knowledge, there are no previous study to determine the fatality rate of PKN administration on animal model. However, some groups have been reported for toxicity of Pulsatilla saponins isolated from Pulsatilla chinensis [39]. In this study, PKN did not show the cytotoxicity below 300 μ g/mL concentration in the splenocytes (Fig. 5a), and the morphological changes of splenocytes were not observed in the PKN treatment groups compared to the control group (Supplementary Fig. 1). Moreover, this study performed experiments for serum level of alanine transaminase (ALT) and aspartate aminotransferase (AST) to confirm whether the PKN had a toxicity in the OVA-induced mouse model. The serum levels of ALT and AST were not observed significant differences between the PKN-administered groups and the control group (Supplementary Fig. 2). However, the serum levels of ALT and AST were significantly increased in the DEX administered group compared with the WT and control groups (Supplementary Fig. 2). Thus, it would be considered that the administration doses of PKN used in this study did not have a toxicity. Given that the serum levels of ALT and AST were increased in the DEX-administered mice group, the PKN would be better than DEX on safety and ameliorating effect to allergic rhinitis.

Overall, PKN could serve as a therapeutic agent to ameliorate AR symptoms by inhibiting the allergen-induced IL-4/STAT6/GATA3 signaling pathway, which is important for Th2 cell differentiation.

5. Conclusions

In this study, we demonstrated that PKN ameliorates AR symptoms by reducing cytokine production associated with Th2 cells. Furthermore, we revealed that inhibition of Th2 differentiation by PKN is mediated by inhibiting the IL-4/STAT6/GATA3 signaling pathway. However, further studies on the active components of PKN are needed for the development of AR therapeutics. Despite these study limitations, the current study is the first to report the potential of PKN as a modulator of the immune-inflammatory response in AR.

CRediT authorship contribution statement

D.H.J. and K.-Y.J.: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. A. L., Y. H. H., M.-A.J., B.-J. P., and J.Y.L.: Investigation. T.K.: Conceptualization, Project

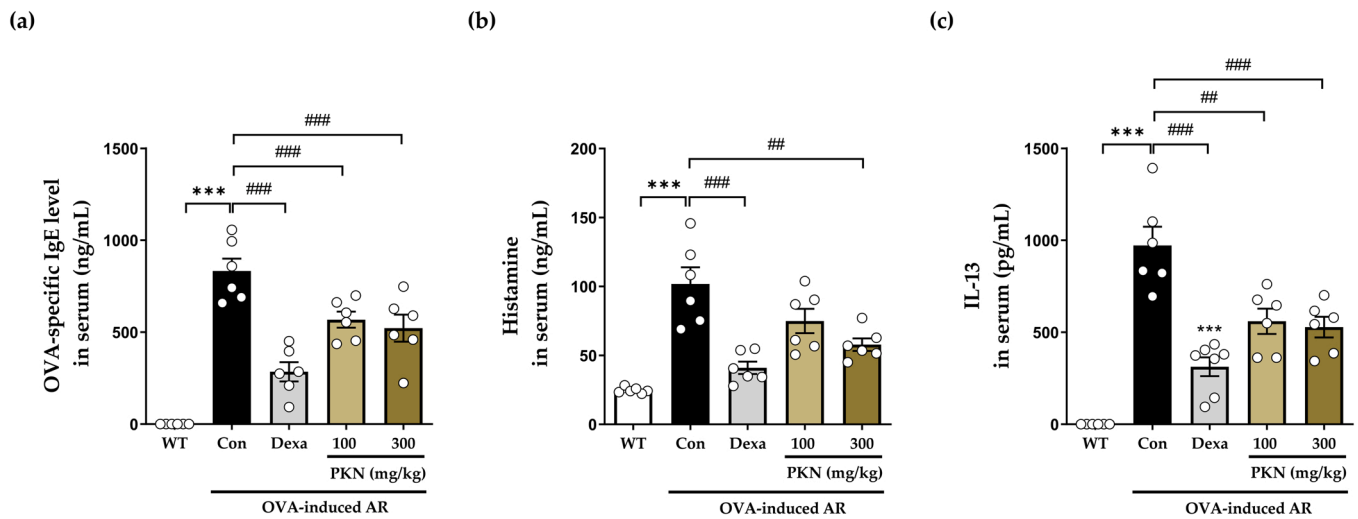


Fig. 4. Levels of serum parameters related to allergic rhinitis (AR) decreased following *Pulsatilla koreana* water extract (PKN) treatment in the ovalbumin (OVA)-induced AR mouse model. The serum levels, including OVA-specific immunoglobulin E (IgE) (a), histamine (b), and interleukin (IL)-13 (c), were analyzed using ELISA. The data are presented as the mean $\pm$ standard error of mean ($n = 6$ per each group). *** $p < 0.001$ vs. wild-type (WT). ## $p < 0.01$, ### $p < 0.001$, vs. control (Con). DEX, Dexamethasone.

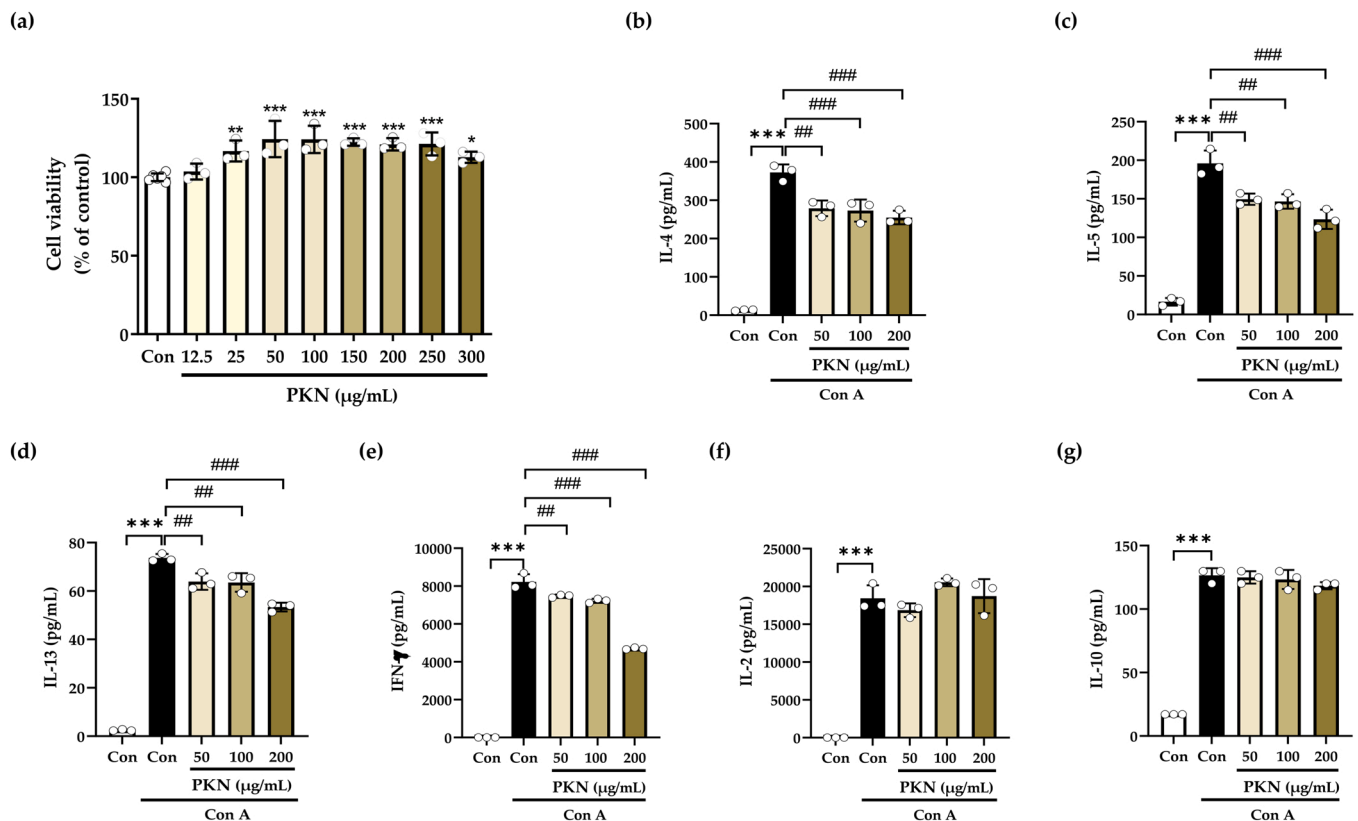


Fig. 5. Activation of Th2 cells was suppressed by *Pulsatilla koreana* water extract (PKN) treatment in splenocytes. (a) The viability of splenocytes was evaluated after treatment with PKN for 24 h, using an EZ-Cytox solution. The secretion levels of Th cytokines, such as interleukin (IL)-4 (b), IL-5 (c), IL-13 (d), interferon (IFN)- γ (e), IL-2 (f), and IL-10 (g), were analyzed by LEGENDplex and flow cytometry. The data are presented as the mean $\pm$ standard deviation ($n = 3$ per each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control (Con). ### $p < 0.01$, #### $p < 0.001$, vs. concanavalin A (Con A) stimulated Con.

administration. K.H.S.: Conceptualization, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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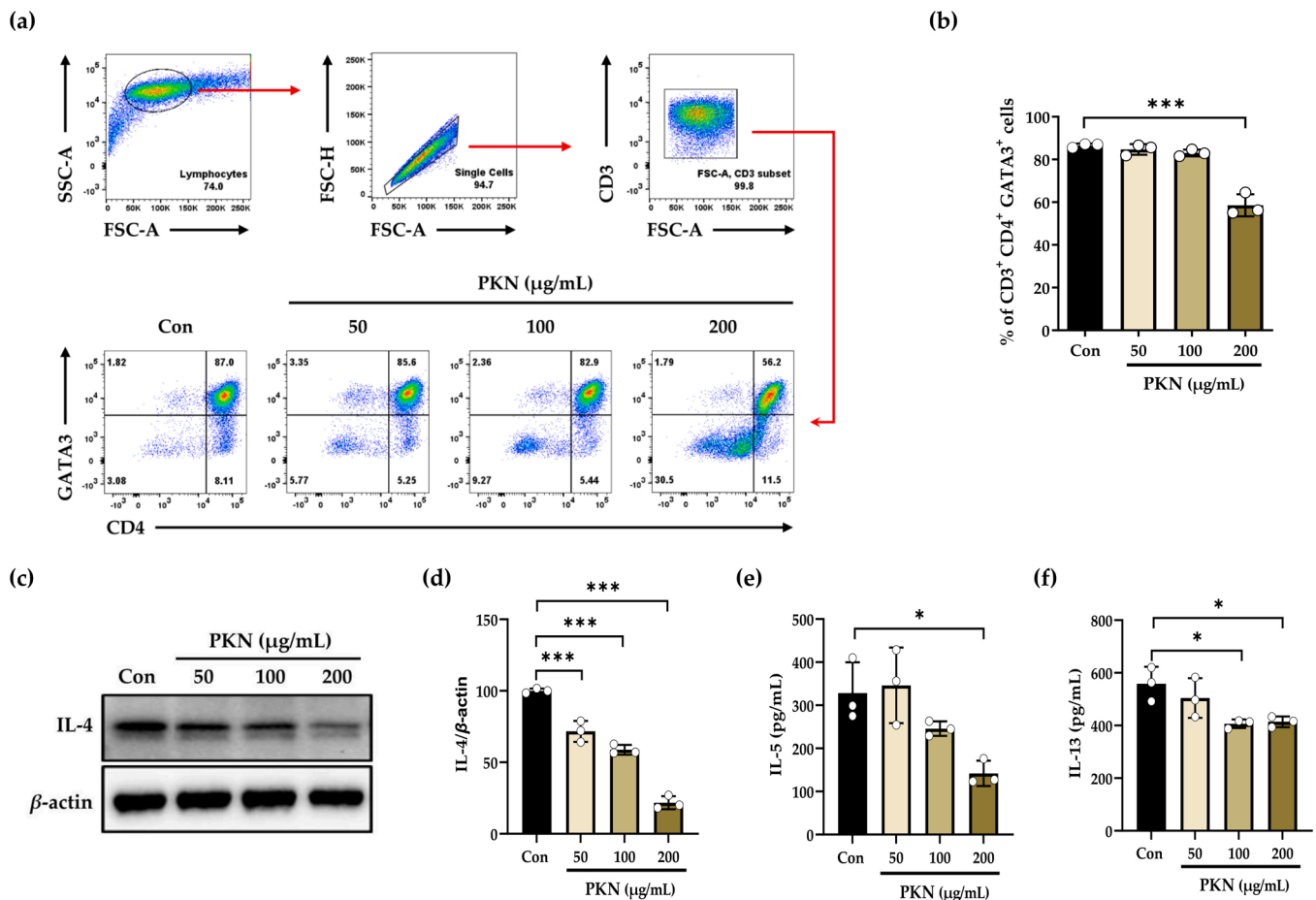


Fig. 6. *Pulsatilla koreana* water extract (PKN) treatment inhibited the differentiation of T helper type 2 (Th2) cells from naïve CD4⁺ T cells. (a and b) The population of differentiated Th2 cells (CD3⁺ CD4⁺ GATA3⁺) was analyzed using flow cytometry. (c and d) The expression of interleukin (IL)-4 was determined by western blot analysis. The levels of secreted IL-5 (e) and IL-13 (f) were evaluated using LEGENDplex and flow cytometry. The data are presented as the mean $\pm$ standard deviation (n = 3 per each group). *p < 0.05, ***p < 0.001 vs. control (Con). GATA3, GATA-binding protein 3.

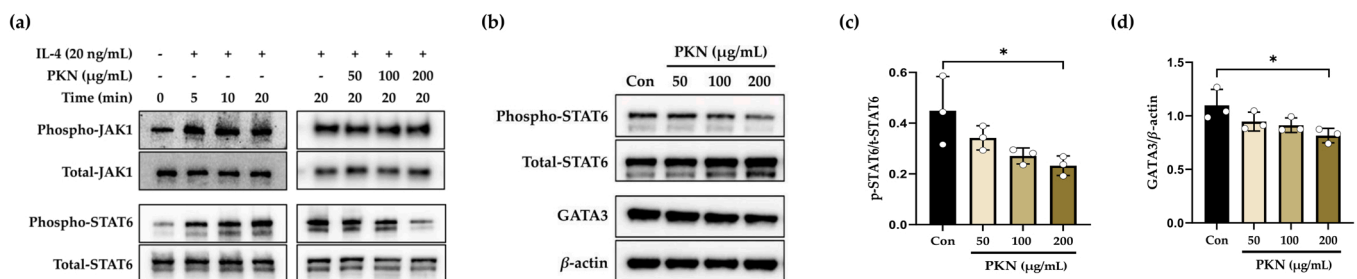


Fig. 7. The interleukin (IL)-4/STAT6/GATA3 pathway was suppressed by *Pulsatilla koreana* water extract (PKN) treatment in the differentiated T helper type 2 (Th2) cells derived from naïve CD4⁺ T cells. (a) The levels of phosphorylation of JAK1 and STAT6 were evaluated using western blot analysis in the differentiated Th2 cells. (b–d) The expression levels of phospho-STAT6 and GATA3 were determined by western blot analysis and quantified using ImageJ software. The data are presented as the mean $\pm$ standard deviation (n = 3 per each group). *p < 0.05, vs. control (Con). GATA3, GATA-binding protein 3; JAK, Janus kinase; STAT6, Signaling transducer and activator of transcription.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2023.114730](https://doi.org/10.1016/j.biopha.2023.114730).

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