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Water soluble extracts from *Actinidia arguta*, PG102, attenuates house dust mite-induced murine atopic dermatitis by inhibiting the mTOR pathway with Treg generation

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ABSTRACT

Ethnopharmacological relevance: *Actinidia arguta* is widespread in northeastern Asia, being found in Siberia, Korea, Japan, and northern China. These fruits have been documented to regulate the uncontrolled heat of body resulting in various allergic diseases in the Korean traditional medicine. PG102, a water-soluble extract from an edible fruit, *A. arguta*, has been previously shown to control various factors involved in allergic pathogenesis.
Aim of the study: In this study, we investigated whether PG102 prevents chronic allergic reactions via the generation of Tregs, which play a preventive role in the pathogenesis of allergic disease.
Methods and results: In dust mite extract-induced chronic atopic dermatitis, orally administered PG102 inhibited symptoms of dermatitis, including ear swelling and erythema, and decreased lymphocyte infiltration into the inflamed region. Moreover, PG102 reduced inflammatory T cell responses and increased the expression levels of *Foxp3* and other Treg-related genes. PG102 treatment enhanced the induction of CD4+Foxp3+ Tregs from naive CD4+CD62L+ T cells, probably via the inhibition of mTOR activation and the phosphorylation of STAT5 rather than using the TGF-β signaling pathway.
Conclusion: PG102 may have potential as an orally active immunosuppressor for preventing chronic inflammatory diseases.

1. Introduction

Atopic dermatitis (AD) is a multifactorial chronic inflammatory skin disease characterized by pruritic and eczematous skin lesions; the prevalence of AD in childhood is 15–30% worldwide (Bieber, 2008; Boguniewicz and Leung, 2011). AD is caused by complex pathogenic factors, including genetic susceptibility, skin barrier defects, microbial colonization, immune dysregulation and environmental triggers, such as house dust mites and pollen (Leung et al., 2004). In particular, *Dermatophagoides farinae* extracts (*Df*E) have been considered to be the most common among house dust mites (Matsuoka et al., 2003).

AD is caused by a complex immune reaction mediated by various immune responses. T helper type 2 (Th2) cytokines play an important role in the development of AD (Homey et al., 2006), while the T helper type 1 (Th1) cytokine IFN-γ is also involved in the maintenance of the chronic stage of AD (Rebane et al., 2012). In many recent studies, a possible role of IL-17-producing Th17 cells in AD has been discussed (Gittler et al., 2012; Nakajima et al., 2014). There have been several pieces of evidence suggesting a curative role of regulatory T cells (Tregs) in the pathogenesis of AD. It has been shown that the percentage of Th17 cells was higher in the peripheral blood of AD patients compared with healthy individuals (Koga et al., 2008; Rabin and Levinson, 2008), whereas the severity of AD was negatively correlated with the percentage of Tregs (Ma et al., 2014). Fyhrquist et al. (2012) reported that forkhead box P3 (Foxp3)$^+$ Tregs were essential suppressors of AD-

Abbreviations: AD, atopic dermatitis; APCs, antigen presenting cells; Dex, dexamethasone; *Df*E, *Dermatophagoides farinae* extracts; dLN, draining lymph node; DNCB, dinitrochlorobenzene; Foxp3, that forkhead box P3; H&E, hematoxylin and eosin; PTEN, phosphatase and tensin homologue deleted on chromosome 10; mLN, mesenteric lymph node; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RORγT, Retinoic acid-related orphan receptor γT; TCR, T cell receptor; Teff cells, effector T cells; TGF-β, transforming growth factor-β; Th, T-helper; Th17 cells, IL-17-producing Th cells; Tregs, regulatory T cells

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like skin inflammation. Based on these findings, numerous efforts have been made to treat AD by enhancing Tregs.

The generation of Foxp3$^+$ Tregs is driven by transforming growth factor-β (TGF-β) and IL-2 (Gabrysova et al., 2011). There is also a TGF-β-independent pathway, which involves the serine/threonine protein kinase mammalian target of rapamycin (mTOR) pathway (Delgoffe et al., 2009; Thomson et al., 2009). mTOR exists in at least two complexes: mTOR complex (mTORC) 1 and mTORC2 (Loewith et al., 2002). mTORC1 tends to promote Th1 differentiation (Delgoffe et al., 2009; Lee et al., 2010), whereas mTORC2 may positively regulate Th2 response via AKT (Lee et al., 2010). The inhibition of both complexes is required for optimal induction of Foxp3$^+$ Treg (Cobbold, 2013). The expression of *Foxp3* is prevented through the activation of the PI3K-AKT-mTOR signaling pathway. However, the expression of *Foxp3* is likely induced by the activation of STAT5 during the initial stimulation (Thomson et al., 2009). The binding of STAT5 to the *Foxp3* promoter has been shown to facilitate the generation of induced Tregs in the periphery (Yao et al., 2007).

According to the theory of Korean traditional medicine, it is known that allergy results from the unregulated overflow of energy in the body of affected individuals. The fruits of *Actinidia arguta (Siebold & Zucc.) Planch. ex Miq.* (*A. argute*; hardy kiwifruit; 软枣猕猴桃; 다래) which corresponds to the lastest revision in "The plant List" (www.theplantlist.org), may govern such an uncontrolled high level energy, leading to the improvement of allergic responses. Indeed, various extracts and compounds from several *Actinidia* species have been reported to be effective for the treatment of several inflammatory diseases (Nishiyama et al., 2004; Takano et al., 2004; Whang et al., 2000) in China and Japan. PG102 is a water-soluble extract prepared from the fruit part of hardy kiwifruit, *A. arguta*, which is rich in vitamins and flavonoids, such as vitamin C and quercitrin, respectively (Park et al., 2007). In previous studies, we demonstrated that PG102 effectively improved the Th2 cell-mediated symptoms associated with bronchial asthma (Kim et al., 2009a), spontaneous dermatitis (Park et al., 2007) and allergic diarrhea (Kim et al., 2009b) in respective mouse models. Furthermore, in an exploratory human trial i involving 156 asymptomatic subjects with atopy, PG102 has been reported to decrease the serum IgE levels and inflammatory markers associated with allergic diseases, indicating its potential as a preventive or therapeutic agent (Kim et al., 2011). However, the molecular and cellular mechanisms of the preventive effects of PG102 on atopic dermatitis have not yet been clearly elucidated.

The aim of this study was to determine whether orally administered PG102 could relieve the allergic symptoms of AD induced by the common allergen *Df*E, as well as to investigate whether PG102 has any effect on the Treg generation. PG102 decreased the clinical score, the severity of ear swelling and the number of immune cells, resulting in the prevention of inflammatory cytokine infiltration into the ear. Histological analysis indicated that PG102 also inhibited the infiltration of inflammatory cells in the dermis. Furthermore, oral administration of PG102 induced the generation of CD4$^+$Foxp3$^+$ Tregs and suppressed the activation of Th1, Th2 and Th17 cells by *Df*E in the inflammatory region. These in vitro and in vivo data clearly indicate that PG102 might act as an effective immunosuppressor for AD.

2. Materials and methods

2.1. Sample preparation

The *A. arguta* used in this study was identified by KMD. D.S. Lee, ViroMed Co., Ltd. (Seoul, Korea). The specimen (PG102 batch #090910) has been kept in ViroMed Co., Ltd. (Seoul, Korea). PG102 was prepared from *A. arguta* as previously described (Kim et al., 2009a, 2009b, 2011, 2013; Park et al., 2007). Briefly, *A. arguta* had the moisture content of $<10\%$ by weight through air-drying. The dried fruit (10 g) was extracted three times by heating in distilled water (DW) to prepare PG102. The extract was filtered by filter paper (No. 2; 110 mm, Whatman International Ltd., Kent, England) concentrated by rotary evaporator, followed by freeze-drying process and proximate analyses were performed to determine components of PG102. PG102 were dissolved in DW at concentration 2.5–25 mg/mL for in vivo experiment or in 1% DMSO at concentration 200 mg/mL for in vitro experiments. Based on weight, less than 4% of PG102 is insoluble in DW. The peak pattern between PG102 in 1% DMSO and PG102 in TDW by HPLC analysis was very similar, and no significant difference between two samples was observed in its biological activities when measured for IL-4 production in A23187-induced RBL-2H3 as described by Kim et al. (2009b). The quality of PG102 was controlled using bioassays, as previously described (Kim et al., 2009a; Park et al., 2007). PG102 extracts were dissolved in distilled water (DW) or $\leq 0.1\%$ DMSO (Sigma-Aldrich, St. Louis, MO) before use.

2.2. Antibodies

The following antibodies were used: anti-CD3 (17A2), anti-CD28 (37.51), allophycocyanin (APC)-labeled anti-mouse CD8a (53-6.7), APC-labeled anti-mouse CD11c (N418), phycoerythrin (PE)-labeled anti-CD4 (H129.19), APC-labeled anti-Armenian Hamster IgG (all from BioLegend, San Diego, CA), APC-labeled anti-Foxp3 (FJK-16s), PE-labeled anti-rat IgG1κ, APC-labeled anti-rat IgG2aκ (all from eBioscience, San Diego, CA), FITC-labeled rat anti-mouse CD11b (M1/70), FITC-labeled anti-rat IgG2bκ (both from BD PharMingen, San Diego, CA), and neutralizing antibody to TGF-β (1D11; R&D Systems, Minneapolis, MN, USA).

2.3. Animals

Four-week-old female BALB/c mice (18–20 g) were purchased from OrientBio Inc. (Kyeonggi, Korea) and housed at 23 ± 2 °C with a 12-h light/dark cycle and free access to food and water. All experimental procedures were performed in compliance with the guidelines set by the University Animal Care and Use Committee at Seoul National University with special attention to minimizing animal pain. The study protocol was approved by the Seoul National University Institutional Animal Care and Use committee (Approval Number: SNU-130913-1). All animals were not sacrificed or died as a result of the AD induction experiment.

2.4. Induction of atopic dermatitis in the mouse ear

Mice were divided into naïve (n=10), sham (n=10), PG102 25 mg/kg (n=10), PG102 250 mg/kg (n=10) and dexamethasone (n=10) groups. For the induction of experimental AD in BALB/c mice, the ear surface was stripped three times with surgical tape (Daesung Pharm, Korea). After stripping, 20 μL of 1% dinitrochlorobenzene (DNCB, Sigma Aldrich, St Louis, MO, USA) dissolved in an acetone/olive oil solution (1:3 acetone/olive oil ratio) was painted on each ear surface. Three days after DNCB painting, the ears were subjected to the tape stripping procedure again and painted with 20 μL of 10 mg/mL mite extracts (*Dermatophagoides farinae*, GREER source materials, Lenoir, NC, USA). Alternating painting with DNCB or mite antigen was performed. Tape stripping and DNCB/mite extract painting were continued for 5 weeks. PG102, dexmethasone (Dex) or distilled water (DW) was orally administered to respective groups every day for 3 weeks before AD was induced by house dust mite and DNCB (Fig. 1a). The ear

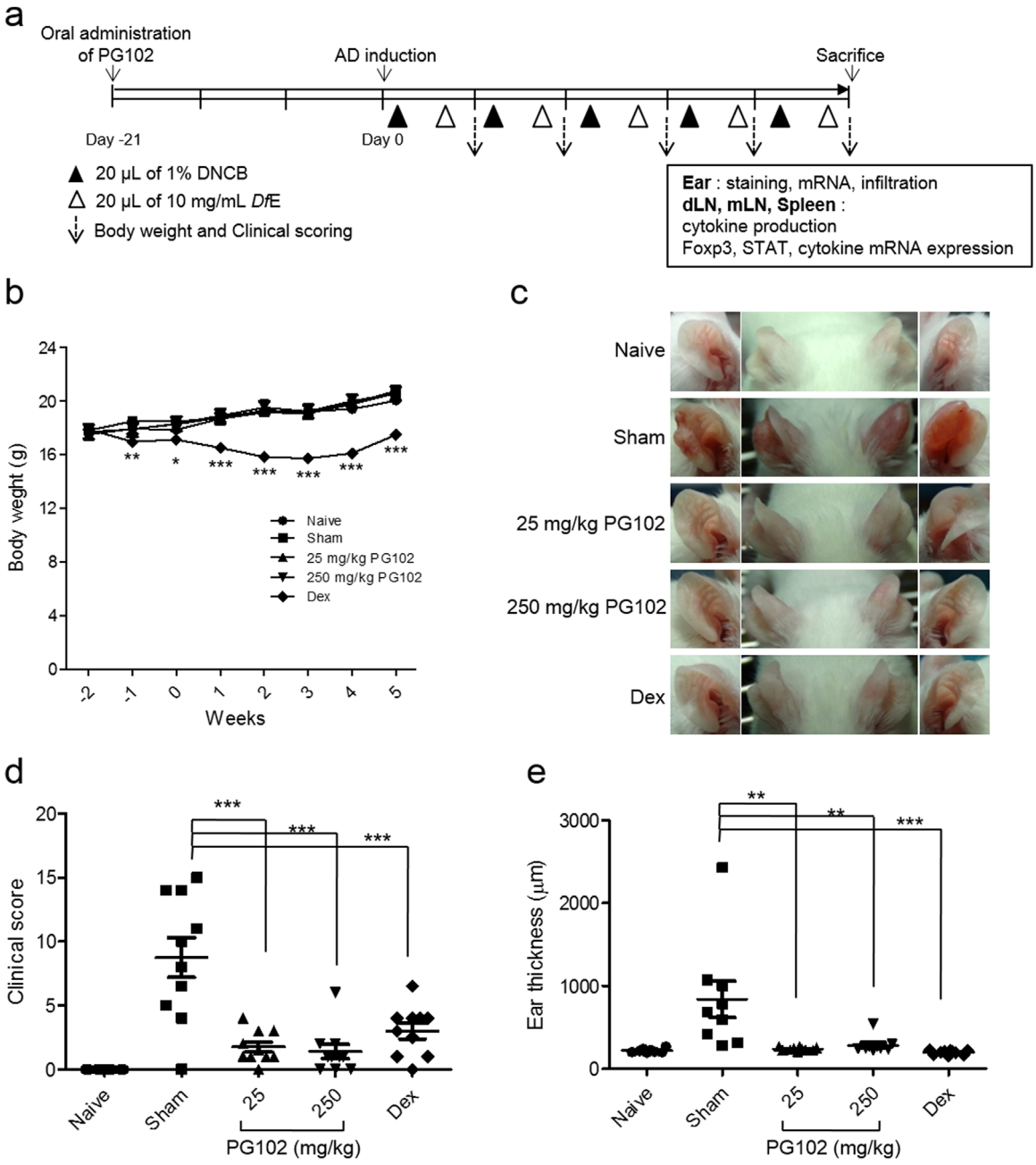

Fig. 1. Preventive effect of PG102 on symptoms of experimental atopic dermatitis. (a) Scheme of atopic dermatitis (AD) induction and dosing. (b) Body weights of each treatment group are shown. (c) Representative mice of each treatment group at 5 weeks induction are shown. Five weeks after the AD induction, clinical score (d) and ear thickness (e) were measured at 24 h after the final mite extract application. Data are presented as the mean ± standard deviation (SD) of n = 10 and were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. *P < 0.05, **P < 0.005 and ***P < 0.001 vs. control, respectively. Figures shown are representative of 3 independent experiments.

thickness and clinical score were measured 24 h after mite application. Only tape stripping and painting with vehicle were performed on the control mice.

2.5. Measurement of ear swelling and clinical score

Ear thickness was measured 24 h after the application of DNCB or mite extract with a spline micrometer (Mitutoyo Corp., Japan). A mouse from each group with a representative clinical score was photographed to show the clinical symptoms. To evaluate the clinical symptoms of each mouse, erythema, edema, excoriation and dryness on the ear surface were scored as 0 (not visible), 1 (mild), 2 (moderate), or 3 (severe), as previously described (Ohmen et al., 1995). Scoring was performed by three independent observers, and an average for each group was taken as the final score.

2.6. Tissue preparation for histological analysis

The excised ears of each group were fixed in 10% paraformaldehyde and embedded in paraffin. Then, 6 μm sections were stained with hematoxylin and eosin (H&E) and toluidine blue (TB) at Reference Biolabs (Seoul, South Korea). Lymphocyte infiltration, thickening of the epidermis and fibrosis in the dermis were observed by microscopy. Immunohistochemistry for analysis of *Foxp3* expression was performed by a commercial company (Reference

Biolabs, Seoul, South Korea) using anti-Foxp3 (ab54501, Abcam, Cambridge, MA, USA).

2.7. Ear cell preparation and FACS staining

Mouse ear cells were prepared with 1.0 mM EDTA in 5% FBS in a small fleaker and stirred using a stir bar for 20 min at room temperature. A buffer containing 0.5 mg/mL collagenase IV in RPMI in a 50 mL Falcon tube was used to obtain cell suspensions for 1 h at 37 °C. The population of infiltrated immune cells in total ear cells was detected by FACS. After Fc receptors were blocked for 15 min, cells were stained with APC-labeled anti-mouse CD8a (53-6.7), APC-labeled anti-mouse CD11c (N418), PE-labeled anti-CD4 (H129.19) and FITC-labeled rat anti-mouse CD11b (M1/70) antibodies in FACS buffer at 4 °C for 30 min. Data were acquired by flow cytometry using a BD FACS Canto (BD Biosciences) and analyzed using FlowJo software (Treestar, San Carlos, CA).

2.8. Cell purification and functional differentiation of naïve CD4+ T cells

The spleen and mesenteric lymph node (mLN) were aseptically removed from each mouse. The spleens and mLNs were then homogenized into single-cell suspensions, 5×10^6 cells/mL were added to RPMI medium, and cell viability was determined using trypan blue dye exclusion. Naïve $CD4^+$ T cells from the spleens and mLNs of BALB/c mice were isolated using a magnetic-activated cell sorting (MACS) $CD4^+CD62L^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany), following the manufacturer's instructions. The purity of the isolated cells was confirmed using FACS. Naïve BALB/c $CD4^+$ T cells (1×10^6 cells/well) were cultured in 96-well plates in the presence of 0 − 100 μg/mL PG102 extract in 0.1% DMSO with or without T cell receptor (TCR) stimulation at 37 °C for 4–72 h in a 5% CO_2 humidified incubator. TCR stimulation was performed using 2 μg/mL plate-bound anti-CD3 and 1 μg/mL soluble anti-CD28 antibodies. The population of $CD4^+Foxp3^+$ T cells was then analyzed using FACS. Collected culture supernatants and cells were used for the analysis of inflammatory mediators.

2.9. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

RNA from the spleens and mLNs was extracted and purified by using TRIzol Reagent (GIBCO BRL, Carlsbad, CA), and cDNA was synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Takara Bio., Japan) according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR green (Takara Bio), using the Thermal Cycler Dice Real Time System (Takara) with the following protocol: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The synthesized PCR primer sets (Bioneer Co. Ltd., Seoul, Korea) are as follows: *β-actin* (5′-CCCTAAGGCCAACCGTGAAA-3′; 5′-TGGCGTGAGGGAGAGCATAG-3′; housekeeping gene), *GAPDH* (5′-CTGGAAAGCTGTGGCGTGAT-3′; 5′-CCAGGCGGCACGTCAGATCC-3′), *TNF-α* (5′-ACTGAACTTCGGGGTGATCGGTCC-3′; 5′-GTGGGTGAGGAGCACGTAGTCG-3′), *IL-1β* (5′-ATCTTTTGGGGTCCGTCAACT-3′; 5′-GCAACTGTTCCTGAACTCAACT-3′), *IFN-γ* (5′-GAACTGGCAAAAGGATGGTGA-3′; 5′-TGTGGGTTGTTGACCTCAAAC-3′), *T-bet* (5′-TGCCTGCAGTGCTTCTAACA-3′; 5′-TGCCCCGCTTCCTCTCCAACCAA-3′), *IL-4* (5′-GTATCCACGGATGTAACGACAGC-3′; 5′-AGGACTGCAAGTATTTCCCTCGTA-3′), *GATA-3* (5′-GAAGGCATCCAGACCCGAAAC-3′; 5′-ACCCATGGCGGTGACCATGC-3′), *IL-17F* (5′-TGCTACTGTTGATGTTGGGAC-3′; 5′-AATGCCCTGGTTTTGGTTGAA-3′), *RORγt* (5′-CCGCTGAGAGGGCTTCAC-3′; 5′-TGCAGGAGTAGGCCACATTACA-3′), *TGF-β* (5′-GAAGGCAGAGTTCAGGGTCTT-3′; 5′-GGTTCCTGTCTTTGTGGTGAA-3′), *CTLA-4* (5′-AGAACCATGCCCGGATTCTG-3′; 5′-CATCTTGCTCAAAGAAACAGCAG-3′), *PD-1* (5′-AAGCTTATGTGGGTCCGGC-3′; 5′-GGATCCTCAAAGAGGCC-3′), *AhR* (5′-TGCACAAGGAGTGGACGA-3′; 5′-AGGAAGCTGGTCTGGGGTAT-3′), *IL-10* (5′-CAGAGCCACATGCTCCTA-3′; 5′-GGAGTCGGTTAGCAGTATG-3′) and *Granzyme B* (GranB, 5′-CTCCTAAAGCTGAAGAGTAAG G-3′; 5′-TTTAAAGTAGGACTCACACTCCC-3′). A housekeeping gene (*β-actin* or *GAPDH)* was used to normalize mRNA expression levels. The data are shown as relative delta CT (ΔΔCT) values, and the fold-induction of each gene was calculated as follows: Δ Threshold cycle (ΔCt)=(Ct of target mRNA)−(Ct of housekeeping gene); ΔΔCt=(ΔCt of mRNA in target gene)−(ΔCt of mRNA in control gene); fold-induction$=2^{-\Delta\Delta Ct}$.

2.10. Western blot analysis

For general Western blot analysis, total cell lysates were prepared 48 h after cell culture using Phosphosafe lysis buffer (Novagen, San Diego, CA, USA). Protein samples were subjected to SDS-PAGE on a 5–10% polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated with antibodies specific for phospho-STAT1, 3 and 5 (1:1000; Cell Signaling, Danvers, MA, USA), phospho-STAT6 (1:1000; Abcam, Cambridge, UK), phospsho-AKT-Ser473, phospho-P70S6K-Y389, PTEN, phospho-AMPKα (1:1000; Cell Signaling) and β-actin (1:5000; Sigma) overnight at 4 °C. Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:100,000; Sigma), and bands were visualized with ECL (Millipore, Billerica, MA, USA).

2.11. Cytokine assays

Cells were plated at 1×10^6 cells/well at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 72 h, and then the culture supernatants were taken. The levels of IFN-γ, IL-4, IL-17A/F (BioLegend) and TGF-β (R&D systems, Minneapolis, MN, USA; eBioscience Inc., San Diego, CA, USA) in the supernatants were measured by ELISA, according to the manufacturer's instructions.

2.12. Statistical analysis

Data are presented as the mean ± standard deviation (SD) of triplicate measurements. Comparisons between two groups were analyzed using Student's *t*-test. Differences between the other experimental data were analyzed by either one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test or ANOVA followed by Student's *t* test to compare each group. ND=not detected. Significant differences are presented as $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Theory/calculation

3.1. Theory

We have previously reported that PG102 could effectively improve the Th2 cell-mediated symptoms of bronchial asthma (Kim et al., 2009a), spontaneous dermatitis (Park, et al., 2007) and allergic diarrhea (Kim et al., 2009b) in the respective mouse models and also in an exploratory human trial involving asymptomatic subjects with an abnormally high level of IgE (Kim, et al., 2011). Recent studies showed that Th1 and Th17 cells also play important roles in maintaining the chronic stage of AD (Rebane et al., 2012). The aim of this study was to determine the roles of Tregs in preventive or therapeutic effects of PG102.

3.2. Calculation

Effects of PG102 were tested on the generation of $CD4^+$ $Foxp3^+$ Treg cells. First, it was investigated whether PG102 exerted any preventive effect in the mouse model simulating house dust mite-induced AD in human and if so, whether regulatory T cells were involved. After positive effects of PG102 and the involvement of Tregs were observed, it was examined how PG102 up-regulated the *Foxp3* expression, one of the most important markers for Tregs, in $CD62L^+CD4^+$ naïve T cells. Having obtained the evidence for PG102-mediated enhancement of peripheral Tregs, the possible involvement of the PI3K-AKT-mTOR axis was

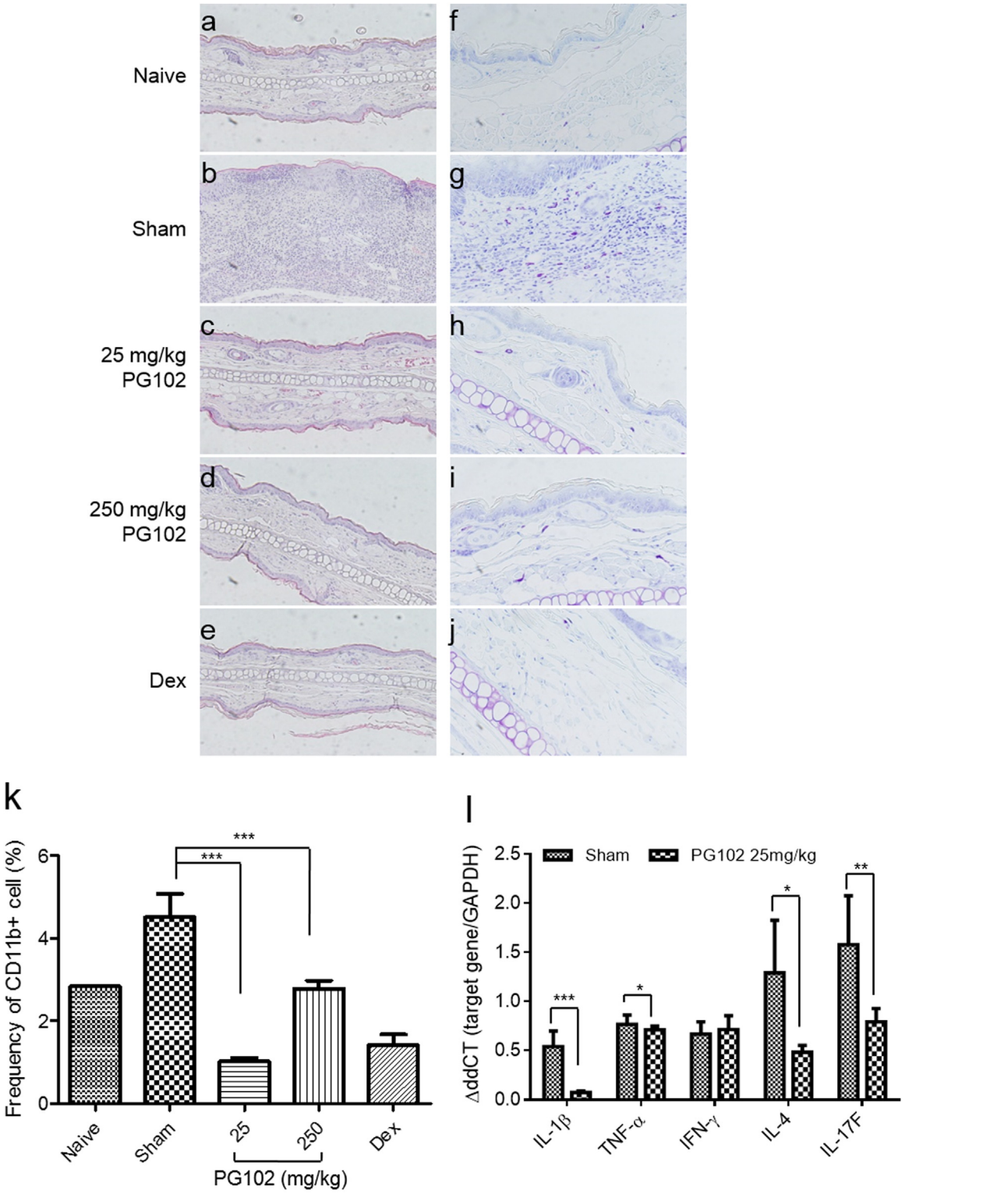

Fig. 2. Inhibitory effect of PG102 on the infiltration of inflammatory cells and effector T cells in the ear. Infiltrated lymphocytes in the ears were observed by H&E staining (a-e) and TB staining (f-j). (k) The population of $CD11b^+$ macrophage cells in total ear tissue was detected by FACS. (l) mRNA expression levels of cytokines were determined by quantitative RT-PCR. Data are presented as the mean ± standard deviation (SD) of n=10 and were analyzed using analysis of variance (ANOVA) followed by Student's *t* test. $^*P < 0.05$,$^{**}P < 0.005$ and $^{***}P < 0.001$ vs. control, respectively. Figures shown are representative of 3 independent experiments.

analyzed. Our approach was designed to confirm anti-allergic activities of PG102 in the mouse model relevant to human AD and to unravel the molecular and cell biological mechanism(s), which could possibly lead to the development of an innovative functional food for atopic dermatitis.

4. Results

4.1. Oral administration of PG102 alleviated symptoms associated with dust mite-induced AD

To investigate the effect of PG102 on AD-like symptoms, 25 or 250 mg/kg/day of PG102 was orally administered on a daily basis to BALB/c mice for 3 weeks before the induction of the disease. Experimental AD was induced on both ears of mice by alternative painting of DNCB and mite extract with an additive oral administration of PG102 for 5 weeks. The symptoms of dust mite-induced AD were evaluated and scored as described by (Ohmen et al., 1995). Dexamethasone (Dex) (2.5 mg/kg/day) was employed as a positive control. No gross signs of toxicity (i.e., changes in body weight) were observed in mice receiving the treatment regimens (Fig. 1b). Severe dust mite-induced AD symptoms were observed in the sham group, including erythema, edema, excoriation and dryness on the ear surface (Clinical score: 8.75 points; Ear swelling: 841 μm; Fig. 1c). In contrast, oral treatment with 25 and 250 mg/kg/day PG102 significantly decreased the clinical score and the level of ear swelling at 5 weeks after DNCB application (Fig. 1d and e).

4.2. Oral administration of PG102 abrogated the infiltration of inflammatory cell and the responsiveness of Teff cells in AD mice

Eczematous skin samples from mice with dust mite-induced AD were subjected to histological examination with H&E (Fig. 2a-e) and TB (Fig. 2f-j) stainings and were then compared with skin from mice receiving PG102. By the fifth week after dust mite treatment, the lesional skin from AD mice showed all of the characteristic symptoms of eczematous inflammation observed in human AD, including thickening of the epidermis and fibrosis and mononuclear cell infiltration in the dermis. These features were not present in mice receiving either PG102 or Dex. When inflammatory cells were examined by FACS, the infiltration of macrophages into the ear was suppressed in PG102-treated mice (Fig. 2k). Cytokines produced from pathogenic effector T (Teff) cells, such as Th1, Th2 and Th17 cells, are crucial factors in AD pathogenesis (Wierenga et al., 1991). T cells reactive to house dust mite indeed produced cytokines, such as IFN-γ, IL-4, and IL-17A/F (Schmitt and Williams, 2013). To elucidate the mechanism underlying the effects of PG102, mRNA expression levels of AD-related pathogenic cytokines were measured from ear tissues (Fig. 2l). In this ear area, oral application of 25 mg/kg PG102 significantly reduced the mRNA levels of not only inflammatory proteins (*TNF-α* and *IL-1β*) but also of Th2 (*IL-4*) and Th17 (*IL-17F*) cytokine genes. This suggested that the oral administration of PG102 attenuated AD symptoms, presumably by inhibiting the infiltration of immune cells and expression of cytokines in the ear.

4.3. PG102 enriched $CD4^+Foxp3^+$ Treg cells at sites of inflammation

$CD4^+Foxp3^+$ Tregs control Teff cells through cell–cell contact, metabolic disruption, the secretion of inhibitory cytokines or focal increment at the inflamed sites (Zou et al., 2012). Therefore, *Foxp3* expression was examined at the inflammatory sites, first using the histological data. The ears of AD mice treated with 25 mg/kg/day or 250 mg/kg/day of PG102 showed a significant increase in the *Foxp3* expression compared with those of the sham groups (Fig. 3a). In addition, the number of $CD4^+Foxp3^+$ Tregs present at the superficial cervical nodes (the lymph nodes proximate to the ear) was also measured by FACS. The PG102 group showed a significantly increased number of $CD4^+Foxp3^+$ T cells compared with the sham group (7.06% in the 25 mg/kg/day group vs. 2.28% in the sham group; Fig. 3b and c). These Tregs in dLN appear to have come from mLN as the population of $Foxp3^+$ cells was also highly increased in intestinal lymph node (Fig. 3d). In PG102-treated mice, the expression levels of Treg-related genes, such as *PD-1, CTLA-4, AhR* and *IL-10,* were increased (Fig. 3e), whereas those of inflammatory cytokines, such as IFN-γ, IL-4 and IL-17A/F, were decreased in dLNs, when compared with those in the control group (Fig. 3f). These results suggested that PG102-mediated enrichment of Tregs in the inflammatory site might lead to alleviation of the pathogenic responses.

To be certain, the effect of PG102 was also tested on the population of $CD4^+Foxp3^+$ Tregs in normal healthy mice. The *Foxp3* levels in mLNs and dLNs from mice treated with DW or 25 mg/kg/day PG102 for 8 weeks were analyzed by FACS. The frequency of $Foxp3^+$ cells in $CD4^+$ gated T cell increased significantly in mLN of PG102-fed normal mice but not in dLNs (Fig. 3g). Taken together, PG102 administration could induce the generation of $CD4^+Foxp3^+$ Tregs in gut-associated lymphoid tissue, which might subsequently migrate into inflammatory sites only under pathological conditions.

4.4. PG102 promoted the generation of $CD4^+Foxp3^+$ T cells from $CD62L^+CD4^+$ naïve T cells

In an effort to understand the immunosuppressive mechanism of PG102 in this AD mouse model, it was investigated whether PG102 could specifically increase the level of *Foxp3* expression from $CD62L^+CD4^+$ naïve T cells. $CD62L^+CD4^+$ naïve T cells isolated from the mLNs and splenocytes were co-treated with anti-CD3/-CD28 antibodies and 10 μg/mL PG102. The number of $CD4^+Foxp3^+$ cells was measured using FACS. Compared with the control, PG102 significantly increased the $CD4^+Foxp3^+$ cells population (Fig. 4a and b). Q-RT PCR was then used to determine whether the expression of other Treg-related genes could be enhanced during PG102-mediated induction of Tregs from $CD62L^+CD4^+$ naïve T cells. The RNA levels of *CTLA-4, AhR* and *GranB* were increased in the PG102-treated group compared with the control group (Fig. 4c). However, the levels of *T-bet* (a Th1-specific T box transcription factor), *GATA-3* (a Th2-polarized transcription factor), and *RORγT* (Retinoic acid-related orphan receptor γT; a master-regulator transcription factor of Th17 cells) were not affected. Therefore, these results showed that PG102 enhanced the induction of $CD4^+Foxp3^+$ Tregs from $CD62L^+CD4^+$ naïve T cells.

4.5. PG102 regulated Foxp3 expression independently of TGF-β signal pathway

Foxp3 expression in $CD62L^+CD4^+$ naive T cells has been reported to be regulated by the mTOR pathway, either dependent on or independent of TGF-β (Horwitz et al., 2008). To test the role of TGF-β in PG102-mediated induction of $CD4^+Foxp3^+$T cell, $CD4^+CD62L^+$ naïve T cell from splenocytes and mLN cells were stimulated with anti-CD3/anti-CD28 antibodies in the presence of 10 μg/mL PG102. PG102 treatment did not increase the mRNA and protein levels of TGF-β in these naïve T cells (Fig. 5a and b). The use of neutralizing antibody to TGF-β could not block the PG102-mediated appearance of Foxp3 expression (Fig. 5c). This is consistent with the above data showing that PG102 did not affect the level of TGF-β in dLN having the increased level of *Foxp3* (Fig. 3h and l).

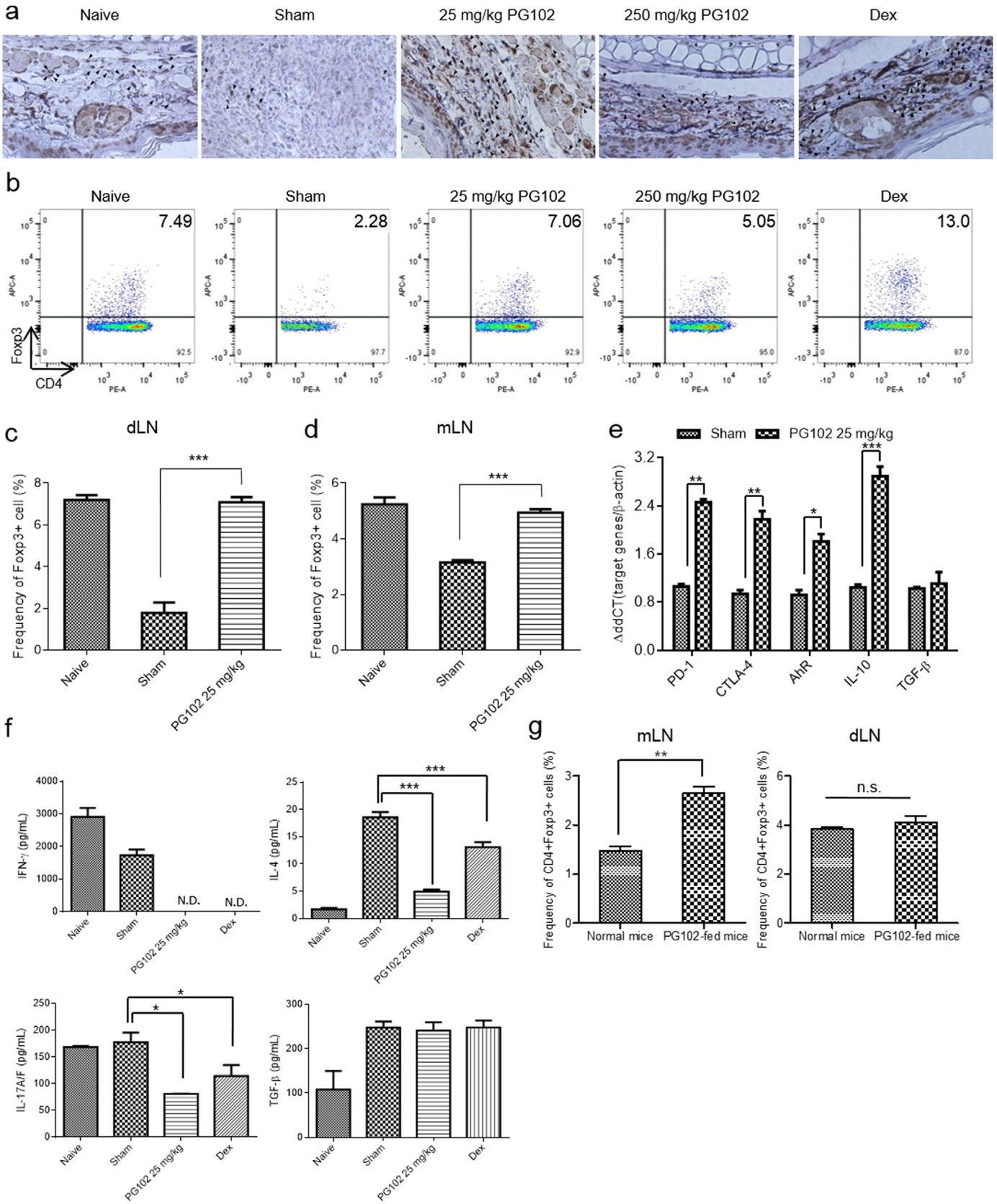

Fig. 3. Effect of PG102 on the induction of CD4$^+$Foxp3$^+$Tregs in the ear and LNs of house dust mite-induced atopic dermatitis mice. (a) Immunohistochemical staining for *Foxp3* in middle center of ear tissue from mice fed either DW or PG102 for 8 weeks. Magnification, × 400. Foxp3 levels were analyzed by FACS in the dLNs (b and c) or mLNs (d) from AD mice fed either DW or PG102 for 8 weeks. (e) Expression levels of Treg-associated molecules were compared in the dLNs from each group by quantitative RT-PCR. (f) The dLNs in each treatment group were stimulated with PMA (0.5 μg/mL) and ionomycin (1 μM) for 72 h, then cytokine production levels of IFN-γ, IL-4, IL-17A/F and TGF-β were determined by ELISA. Foxp3 levels were analyzed by FACS in the LNs from normal mice (g) fed either DW or PG102 for 8 weeks. Data were analyzed and described for Fig. 1. Figures shown are representative of 3 independent experiments.

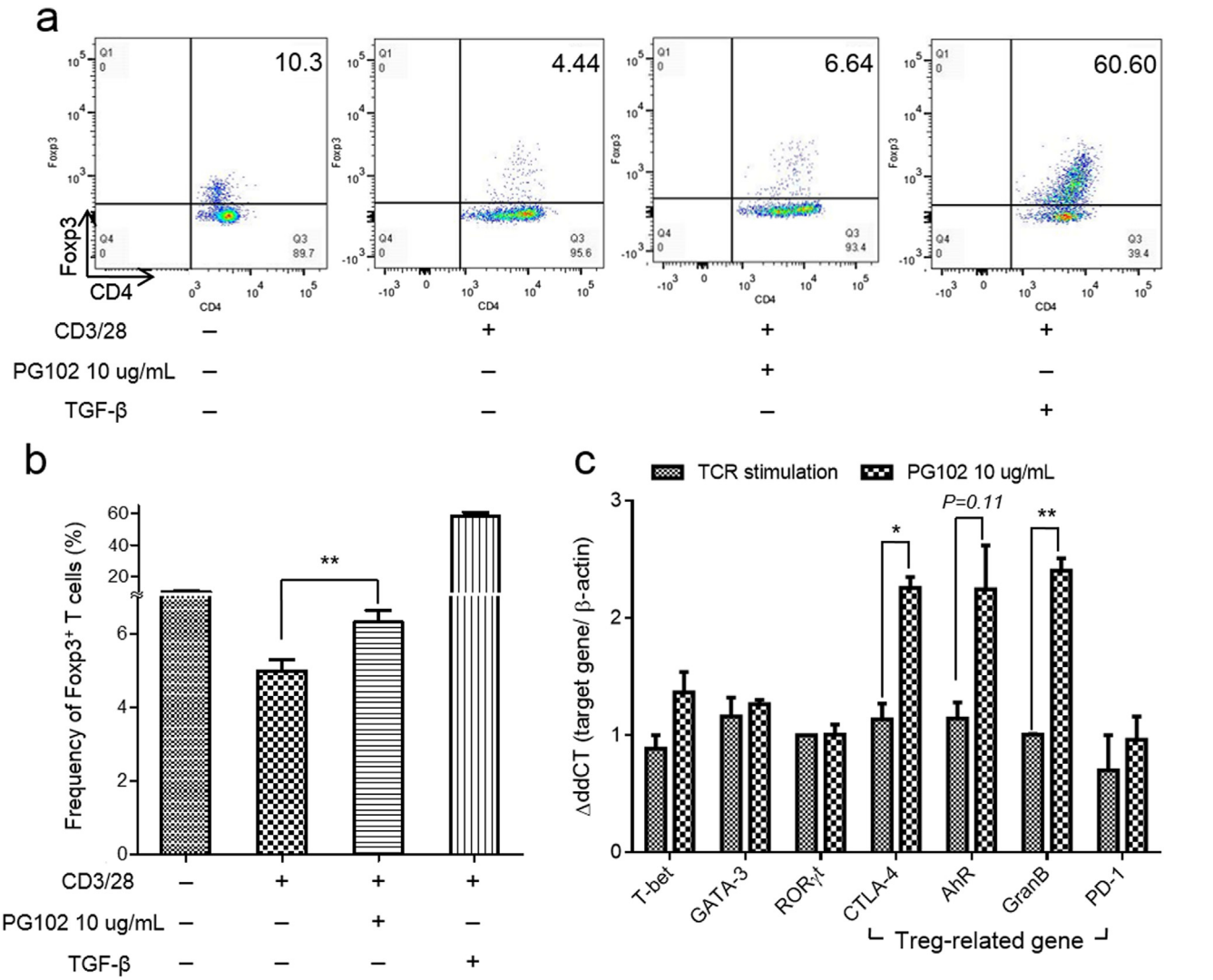

Fig. 4. Inductive effect of PG102 on the generation of Foxp3$^+$Tregs from CD62L$^+$CD4$^+$T cells in vitro. CD4$^+$CD62L$^+$ naïve T cells from splenocytes and mLN cells from BALB/c mice were cultured with TCR stimulation in the presence of 10 μg/mL PG102. (a, b) After 3 days, the expression levels of Foxp3 in gated CD4$^+$ cells were analyzed by flow cytometry. A plot from one representative experiment indicating the frequency of CD4$^+$Foxp3$^+$ T cells is shown. (c) The RNA levels of T cell differentiation-related genes were determined by quantitative RT-PCR after 24 h of culture. A plot from one representative experiment is shown. Data are presented as the mean ± standard deviation (SD) of triplicate determinations and were analyzed using analysis of variance (ANOVA) followed by Student's *t* test. *$P < 0.05$ vs. control; **$P < 0.01$. Figures shown are representative of 3 independent experiments.

4.6. PG102 expressed Foxp3 via the inhibition of mTOR pathway

It was recently reported that the inhibition of the PI3K-AKT-mTOR axis enhances the development of peripheral Tregs independently of TGF-β (Han et al., 2012; Sauer et al., 2008). To test the possibility of PG102 using this pathway, the level of AKT phosphorylation at Ser473 was determined (Sarbassov et al., 2005). As shown in Fig. 6a, PG102 treatment diminished the level of Ser 473 phosphorylation of AKT, which has been known to be regulated by mTORC2. In addition, it was found that the phosphorylation of P70S6K, a direct target of mTORC1-regulated p70 S6 kinase S6K, was decreased with PG102 treatment (Han et al., 2012). However, the levels of two major negative regulators in the PI3K/AKT pathway remain unchanged, AMPK (an enzyme that antagonizes mTOR activation (Sarbassov et al., 2005)) and PTEN (phosphatase and tensin homologue deleted on chromosome 10 (Hagenbeek et al., 2004)). Therefore, PG102 appears to enhance *Foxp3* expression by directly inhibiting the mTOR signaling pathway in CD62L$^+$CD4$^+$ naive T cells.

The STAT5 pathway is activated by the restriction of the PI3K-AKT-mTOR signaling axis and stimulation with endogenous cytokines, such as IL-2, leading to the control of *Foxp3* expression in peripheral T cells (Battaglia et al., 2005; Thomson et al., 2009). The role of STAT5 in the PG102-mediated induction of Tregs was investigated by stimulating CD62L$^+$CD4$^+$ naïve T cells with anti-CD3/-CD28 antibodies in the presence of PG102 and measuring the level of four STAT proteins by western blot analysis. PG102 treatment highly increased the levels of pSTAT5 and lowered the levels of pSTAT1, 3 and 6, thereby suppressing Teff cells differentiation (Fig. 6b).

To confirm the above data, the effects of PG102 on these protein factors were studied. When animals were administered with 25 mg/kg/day PG102, the level of phosphorylated forms of P70S6K and AKT was decreased, while the amount of pSTAT5 was increased when compared to untreated AD mice (Fig. 6c). These data indicated that PG102 attenuated AD by suppressing the mTOR pathway with Treg generation.

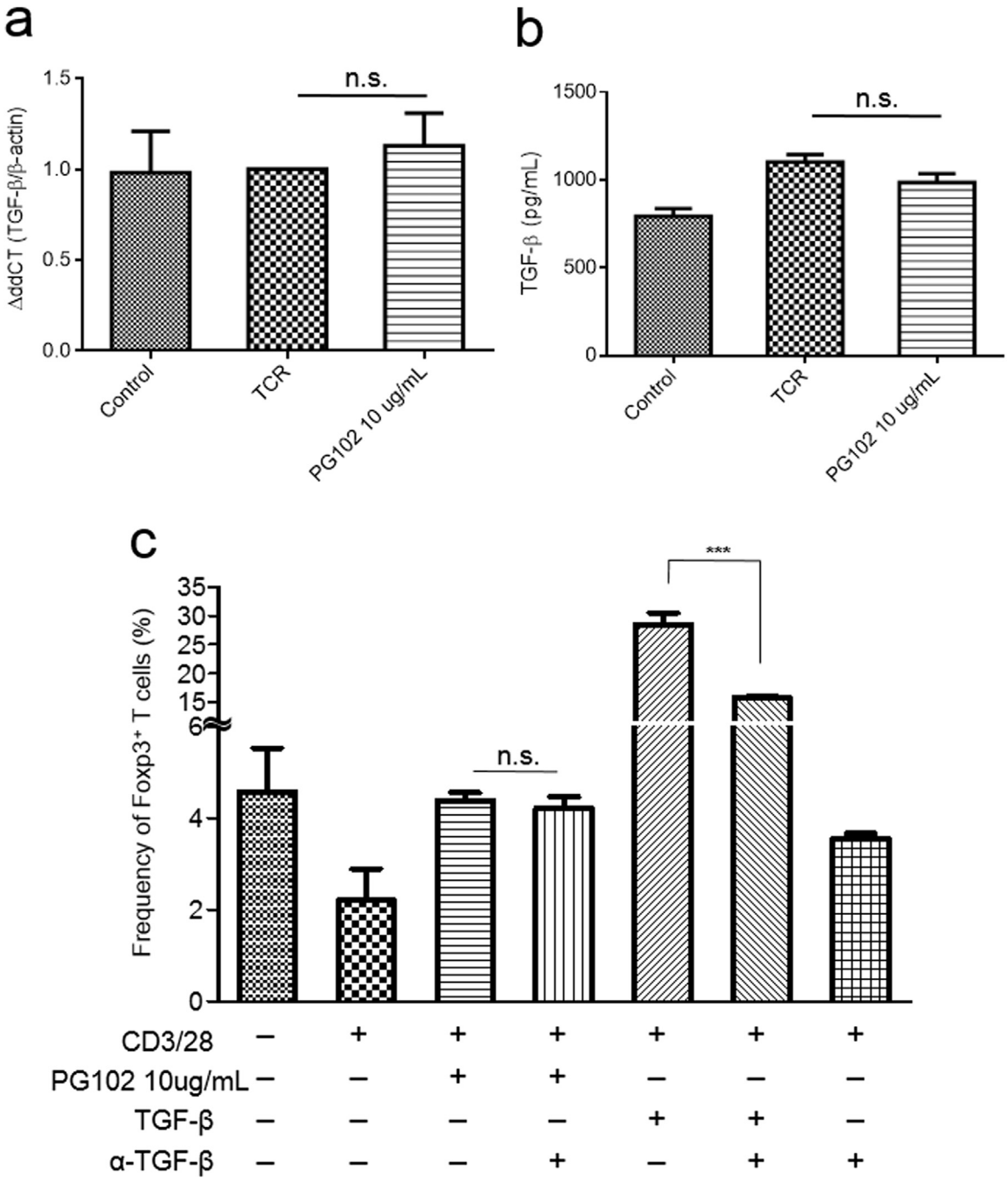

Fig. 5. The Foxp3$^+$Treg generation by PG102 independently of TGF-β pathway. CD4$^+$CD62L$^+$ naïve T cell from splenocytes and mLN cells from BALB/c mice were cultured with TCR stimulation in the presence of 10 μg/mL PG102. (a) After 24 h, the RNA levels of TGF-β were analyzed by quantitative RT-PCR. (b) After 3 days, the protein level of TGF-β was analyzed by ELISA. (c) After 3 days of culturing with neutralizing antibody to TGF-β (α-TGF-β; 12 μg/mL), the expression levels of Foxp3 in gated CD4$^+$ cells were analyzed by flow cytometry. Data are presented as the mean ± standard deviation (SD) of triplicate determinations and were analyzed by one way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test.: $^*P < 0.05$,$^{**}P < 0.005$ and $^{***}P < 0.001$ vs. control, respectively. Figures shown are representative of 3 independent experiments. TCR: TCR stimulation.

5. Discussion

In house dust mite-induced experimental atopic dermatitis, we confirmed the role of PG102 as a potent oral immuno-regulator and unraveled the underlying mechanism of PG102. PG102 induced *Foxp3* expression in CD62L$^+$CD4$^+$ naive T cells by inhibiting mTOR activation independently of TGF-β. The enrichment of CD4$^+$Foxp3$^+$ Tregs at the sites of inflammation was associated with the inhibition of AD pathogenesis.

It is not yet clear how PG102 promotes the generation of CD4$^+$Foxp3$^+$ cells. One possibility is that PG102 may directly control *Foxp3* expression in this particular type of naive T cell to induce immunological tolerance. PG102 alone could enhance the population of CD4$^+$Foxp3$^+$ Tregs when stimulated with anti-CD3/CD28 antibodies for 3 days, namely without antigen presenting cells (APCs). In an ovalbumin-induced mouse model where hyperactive Th2 cells play key pathologic roles, the therapeutic effect of PG102 administration was thought to have resulted from the inhibition of serum IgE levels, resulting in the suppression of the infiltration of mast cell (Kim et al., 2009a, 2009b). However, 20–30% of AD cases are reported to be triggered by non-IgE-mediated sensitization known as the intrinsic form, in which immunologic

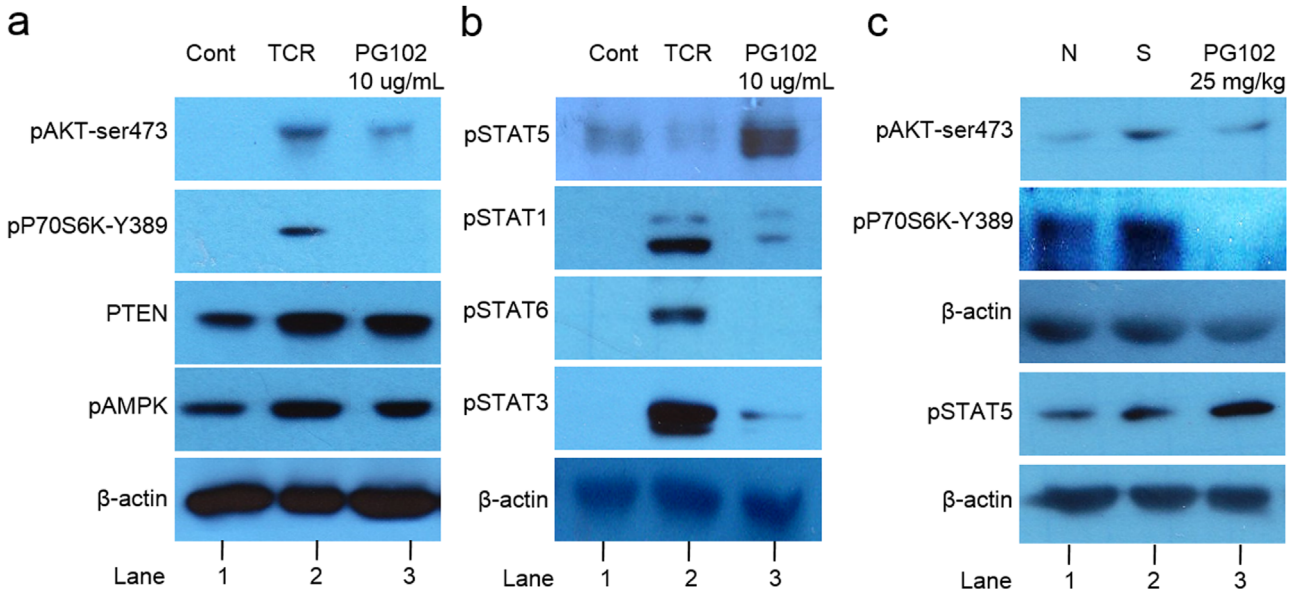

Fig. 6. Inductive effect of PG102 on the generation of Foxp3$^+$Tregs via the inhibition of the mTOR signaling pathway. CD4$^+$CD62L$^+$ naïve T cell from splenocytes and mLN cells from BALB/c mice were cultured with TCR stimulation in the presence of 10 μg/mL PG102. (a) After 48 h of culture, total cell lysates were immunoblotted for the indicated proteins. PTEN-AKT-mTOR signaling was quantified and normalized relative to β-actin. (b) P-STAT5, 1, 6 and 3 signaling were quantified and normalized relative to β-actin. (c) The mLNs from ad AD mice fed either DW or PG102 for 8 weeks were stimulated with PMA (0.5 μg/mL) and ionomycin (1 μM) for 48 h. Then, the total cell lysates were immunoblotted for the protein of mTOR signaling and P-STAT5 was quantified and normalized relative to β-actin. Figures shown are representative of 3 independent experiments. Cont: control, TCR: TCR stimulation, N: Naive, S: Sham.

mechanisms other than the IgE-mediated pathway are thought to be involved (Schmid-Grendelmeier et al., 2001). In this study, we clearly showed that PG102 could generate immunosuppressive CD4$^+$Foxp3$^+$ Tregs in vitro and in vivo. PG102 treatment inhibited the phosphorylation of both AKT-Ser473 and P70S6K-Y389 without affecting TGF-β levels. The decreased level of mTORC2-mediated AKT phosphorylation at Ser473 promotes the inclusion and activation of FOXO transcription factors in the nucleus (Huang and Manning, 2009; Sarbassov et al., 2005). Such AKT kinase inhibition also leads to the suppression of mTORC1, resulting in activation of P70S6K (Maul et al., 2005). However, PG102 did not enhance PTEN, which has previously been known to inhibit the activation of AKT and AMPK to suppress mTORC1. Taken together, our data suggested that PG102 might directly suppress mTOR activation to induce the *Foxp3*-driven differentiation of Tregs.

How does the generation of CD4$^+$Foxp3$^+$ Tregs induced by oral administration of PG102 alleviate the AD symptoms in the ear? CD4$^+$Foxp3$^+$ Tregs are able to circulate through the lymphoid tissues and enter inflamed regions (Matsuoka et al., 1995). Particularly, the circulation of Treg cells between skin and lymphoid organ during cutaneous immune responses could inhibit the activation of effector T cells (Matsuoka et al., 1995; Tomura et al., 2010). A decreased number of Tregs in inflammatory regions is associated with inflammatory diseases, such as AD (Leung et al., 2004). In our study, mice having dust mite-induced AD also showed reduced *Foxp3* expression in both inflamed site and dLN compared with DW-treated mice, but oral administration of PG102 increased its expression in the inflammation sites. In addition, PG102 administration significantly increased the CD4$^+$Foxp3$^+$ Treg population in the mLN and inhibited the mTOR signaling pathway. Under the normal condition, PG102 seems to still induce the generation of CD4$^+$Foxp3$^+$ Treg cells in mLNs, but these cell were confined to intestinal LNs, rather than moving to dLN. Taken together, it appears that Tregs are generated by PG102 in the mLN, and then migrate to the inflammatory sites, only under the pathological condition, to suppress dust mite-induced pathogenic Teff cells, eventually generating protective effects in dust mite-induced AD.

Immunosuppressors, such as glucocorticoids and leukotriene receptor antagonists, have been used for the treatment of AD. However, these pharmacological agents produced not-so-trivial side-effects and did not satisfy a significant number of patients due to the lack of preventive and curative therapies. Therefore, there has been a strong need for the development of safer, more cost-effective, and more efficacious agents for the treatment of AD. PG102 is a water-soluble extract prepared from an edible fruit, *A. arguta* which has been widely used in northeastern Asia such as Korea, Japan, and northern China. Although allergenic proteins, such as actinidin and kiwellin, are known to be present in kiwifruits, heat-processed hardy kiwifruit, namely PG102, has been shown to modify the allergenic protein structure and to dramatically reduce the risk of eliciting allergic symptoms (Chen et al., 2006). Our previous toxicity study indeed showed that PG102 is a very safe agent (Park et al., 2005). Data shown in this report indicate that the oral administration of PG102 may provide a safe and effective means of controlling AD. Given its highly interesting biological activities, as well as a high degree of safety, further scientific and clinical investigations are warranted.

6. Conclusion

PG102 exerts potent immunosuppressive effects in the murine atopic dermatitis model induced by house dust mite by generating CD4+Foxp3+ Tregs via the inhibition of the mTOR pathway but not via the better known TGF-β signaling pathway. PG102 administration may provide a safe and effective means of treating AD.

Author contributions

M. Bae (mjbae1231@snu.ac.kr), and S. Kim (sunyoung@snu.ac.kr) planed conception and designed research. M. Bae, D. Lee (muzuck223@hanmail.net), S. Lim (kindman10@snu.ac.kr), K. Ko (kyeong0323@naver.com) and W. Lee (summersnow97@snu.ac.kr)

performed experiments and analyzed data. M. Bae prepared figures and drafted manuscript. M. Bae and S. Kim discussed and revised manuscript. All authors approved final version of manuscript.

Conflict of interest

S. Kim and D.S. Lee are the employee of ViroMed Co. Ltd. S. Kim owns stocks of this company. Other authors have no conflict of interest.

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