GATA binding protein 3 overexpression and suppression significantly contribute to the regulation of allergic skin inflammation

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Abstract. GATA binding protein 3 (GATA3) is a key molecule regulating the balance in the ratio of type 1 helper T (Th1) cells to type 2 helper T (Th2) cells, which is thought to be indicative of the pathogenesis of allergic diseases such as asthma and atopic dermatitis. The aim of this study was to investigate the role of GATA3 in allergic skin inflammation. Transgenic (Tg) mice overexpressing human GATA3 (hGATA3) were produced by the microinjection of *pCMV/hGATA3* constructs into fertilized mouse eggs. The hGATA3 gene was successfully expressed at the protein level in the lymph node and thymus of CMV/hGATA3 transfected cells and Tg mice. CMV/hGATA3 Tg mice showed a significant increase in the allergic skin inflammation response such as ear thickness, draining auricular lymph node (aLN) weight, epidermal thickness, inflammatory cell number and Th2 immunoglobulin (Ig) concentration compared to wild-type (WT) mice after phthalic anhydride (PA) treatment. Furthermore, the secretion of Th2 type cytokines was increased by PA treatment in CMV/hGATA3 Tg mice, while the secretion of Th1 type cytokine was suppressed under the same conditions. However, the increased levels of Th2 type cytokines in CMV/hGATA3 Tg mice were almost recovered by the down-regulation of GATA3 expression with D-pinitol treatment. Therefore, these findings suggest that GATA3 could be considered as a potential target regulating the mechanism responsible for the differences in allergic skin inflammation.

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Introduction

Allergic diseases, such as asthma, rhinitis and atopic dermatitis, are characterized by inflammation driven by type 2 helper T (Th2) cells (1). In allergic individuals, the exposure to allergens results in activation of antigen-specific type 2 helper (Th2) cells and secretion of a variety of cytokines and inflammatory mediators that provoke the inflammatory response (2).

Th2 cytokines are predominantly regulated by the GATA binding protein 3 (GATA3). The zinc-finger transcription factor GATA3 is the master regulator of Th2 cell differentiation and is strongly up-regulated during Th2 differentiation (3). Generally, Th1 and Th2 cells are differentiated from common T precursor cells in the T cell developmental process (4,5). Two polarized subsets can be identified on the basis of the class of cytokines which secreted from each cell. Th1 cells mainly secrete IL-2 and IFN-γ, while Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (6). GATA3 is believed to function by causing chromatin remodeling of the Th2 cytokine locus by binding at multiple promoter sites and thus allowing Th2 cells to express IL-4, IL-5 and IL-13 (7,8). Furthermore, several studies using GATA-3 deletion mutants have suggested that the GATA3 protein plays an important role in vertebrate development and Th2 differentiation (9,10). In addition, transgenic (Tg) mice that overexpress GATA3 spontaneously have asthma-like symptoms. In these mice, the number of GATA3-positive cells is increased in the asthmatic lung and in allergic rhinitis, while these cells are found to further increase after allergen challenge (11,12). In murine asthma models, the Tg mice overexpressing GATA3 in T cells show enhanced airway hyperresponsiveness, increased subepithelial fibrosis, and smooth muscle hyperplasia (13,14). Collectively these studies indicate that GATA3 would be a suitable target for therapeutic down-regulation of Th2 cell function. However, there are no studies regarding the role of GATA3 in allergic

skin inflammation using Tg mice expressing human GATA3 (hGATA3).

Therefore, in this study, in order to investigate the function of GATA3 *in vivo*, we generated Tg mice overexpressing hGATA3. The development of allergic skin inflammation after repeated allergen challenges in the Tg mice was analyzed to clarify the role of GATA3 in the pathogenesis of allergic skin diseases, such as atopic dermatitis and contact dermatitis. These results suggest that GATA3 enhances the allergic skin inflammation response to phthalic anhydride (PA). In addition, this role of GATA3 in allergic skin inflammation was verified by the suppression of GATA3 levels with D-pinitol (DP) treatment.

Materials and methods

Gene construction. In order to construct the CMV/hGATA3 recombinant vector, hGATA3 cDNA (GenBank accession no. 001002295) was first amplified by RT-PCR using cDNA isolated from total-RNA of human Jurkat cells. The following primers were used for amplification: the sense primer, 5'-GGT TTG ACT CAC GGG GAT TT-3' and the antisense primer, 5'-AAA GCA CAT CCA CCT CCT CC-3'. PCR was carried out using 32 cycles of the following program: denaturation at 95˚C for 30 sec, annealing at 62˚C for 30 sec, and extension at 72˚C for 45 sec. The 1,500-bp products of the amplified hGATA3 sequence were cloned into *pcDNA3.1* (Invitrogen Life Technology, CA, USA) in order to express the hGATA3 under the control of the CMV promoter (Fig. 1A).

Luciferase assay. The activity level of luciferase was detected using the Luciferase Reporter assay kit (Promega, WI, USA) according to the manufacturer's instructions. The protein concentration for each lysate was quantified with a BCA protein assay (Thermo Scientific, IL, USA). The relative luciferase activity (RLA) for each sample represents the luciferase counts corrected for the total protein content.

Production and PCR genotyping of Tg mice. The *pCMV/hGATA3* plasmid was digested with *Bgl*II/*Pvu*II in order to remove the prokaryotic sequence, and then microinjected into the male pronucleus of fertilized embryos, which had been obtained by crossing C57BL/6 (female) mice with DBA/2 (male) mice. The injected egg was then transferred into the oviducts of a pseudopregnant ICR recipient female (15). The transgene was identified by PCR analysis of the genomic DNA isolated from the tails of the 3-week-old founder mice. After amplification, the product (416-bp) levels was quantified on 1% agarose gels.

Southern blotting. The presence of the inserted transgene was determined from the genomic DNA extracted from the tail of the 3-week-old founder mice, and the transgenes were detected by Southern blot analysis of the *Hin*dIII-digested tail DNA. After electrophoresis, the DNA was transferred onto nylon membranes in 10X SSC overnight according to the instructions suggested by our laboratory. The membranes were then immersed in 0.4 N NaOH, neutralized with 0.2 M Tris-HCl (pH 7.5) and 2X SSC, and allowed to air dry. Each membrane was pre-hybridized at 42˚C for 2 h in a hybridization buffer. The ^{32}P-labeled hGATA3 probe was added to the membrane and hybridized at 42˚C for 18 h. The membrane was then washed using the following procedure: 1 wash for 15 min using 2X SSC/0.2% SDS at room temperature, 3 washes for 15 min using 1X SSC/0.2% SDS at 65˚C, and 3 washes for 30 min using 0.5X SSC/0.2% SDS at 65˚C. The filters were then exposed to Kodak XAR film (Eastman Kodak Co., NY, USA) at -70˚C. The GATA3-specific probe was prepared by digestion with the *Hin*dIII enzyme of GATA3 cDNA. The fragments were separated by agarose gel electrophoresis and purified by passage through a Jetsorb (Genomed Co., Löhne, Germany).

Western blotting. For Western blotting, 10 μg protein were separated by electrophoresis on a 10% polyacrylamide gel for 3 h and transferred to a nitrocellulose membrane using an electroblot for 2 h. The membrane was incubated with the primary anti-human GATA3 (R&D Systems, MN, USA), and anti-actin (Sigma-Aldrich, MO, USA) antibodies overnight at 4˚C. The membranes were washed with buffer (137 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$ and 0.05% Tween-20) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at a 1:1,000 dilution at room temperature for 2 h. The membrane blots were developed using a Chemiluminescence Reagent Plus kit (ECL, Pfizer and Pharmacia, NY, USA).

Induction of allergic skin inflammation by PA treatment and inhibition by DP administration. PA (Sigma-Aldrich) has been widely used to induce allergic skin inflammation (16). In this study, PA was selected as an appropriate inducer to investigate the GATA3 effect on the allergic skin inflammation. Initially, 6-8-week-old wild-type (WT) and CMV/hGATA3 Tg mice were randomly divided into four subgroups (n=6-8 per group). For the first subgroup (designated as AOO), 50 μl of acetone-olive oil were repeatedly spread on the dorsum of the ear of WT and CMV/hGATA3 Tg mice 3 times a week for 3 weeks. For the second, third and fourth subgroups (designated as 1, 5 and 10% PA, respectively), 50 μl of PA solution in vehicle (4:1 v/v acetone-olive oil) were repeatedly spread on the dorsum of the ear of WT and CMV/hGATA3 Tg mice 3 times a week for 3 weeks. In the case of the DP treatment experiment, CMV/hGATA3 Tg mice were divided into three groups: the first group was treated with acetone-olive oil; the second group was treated only with PA; the third group was co-treated with PA and PD (Sigma-Aldrich). Furthermore, CMV/hGATA3 Tg mice were injected intraperitoneally (i.p.) with 20 mg/kg/day DP in 200 μl from day 18-20.

Measurement of ear thickness. The ear thickness was measured to determine the degree of allergic skin inflammation induced by PA treatment. Briefly, a thickness gauge (Digimatic Indicator, Matstoyo Co., Tokyo, Japan) was used to measure the thickness of the central region of the ear. The values indicating the increases in the ear thickness were then determined by subtracting the measurement of the ear thickness prior to the first treatment.

Enzyme-linked immunosorbent assay (ELISA) for the detection of serum immunoglobulin (Ig) concentration. The concentrations of serum IgE, G1, G2a and G3 were measured using an ELISA kit (IgE from Shibayagi, Gunma, Japan; IgG1,

IgG2a and IgG3 from Immunology Consultant Laboratory, OR, USA) according to the manufacturer's instructions.

Histological analysis. The skins removed from the mouse ears were fixed with 10% formalin, embedded in paraffin wax, and then sectioned into 5 μm slices. The skin sections were then stained with hematoxylin and eosin (H&E) or toluidine blue (Sigma-Aldrich). The stained skin sections were examined using light microscopy for the presence of edema and the accumulation of inflammatory cells.

Measurement of myeloperoxidase (MPO) activity in inflamed tissue. The ear skins were homogenized in 1 ml of T-PER protein extraction reagent (Pierce Biotechnology, IL, USA) containing a protease inhibitor cocktail. The homogenates were then centrifuged at 16,000 x g for 30 min, after which the MPO activity was measured using an enzyme-linked immunosorbent assay kit (Hycult Biotechnology, Uden, The Netherlands) according to the manufacturer's instruction. The protein content of the homogenates was also measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instruction, after which the MPO activity was normalized based on the protein content.

Mouse cytokine array assay. Mouse cytokines were analyzed in the lysate collected from the ear and thymus tissue. The tissues were lysed in 100 μl of protein extraction buffer (Pro-Prep, Intron Biotechnologies, Seoul, Korea), collected and then stored at -70˚C until further analysis. The expression profiles of cytokines derived from the lysate were evaluated using a mouse cytokine array assay kit (RayBiotech, GA, USA) according to the manufacturer's instruction.

Statistical analysis. One-way ANOVA was used to determine if significant differences existed between allergens (PA- and DP-treated) and AOO-treated groups (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL). In addition, differences in the responses of CMV/hGATA3 Tg and WT mice were evaluated using a post-hoc test (SPSS for Windows, Release 10.10, standard version) of the variance and significance levels. All values are reported as the mean ± SD. A P-value of <0.05 was considered significant.

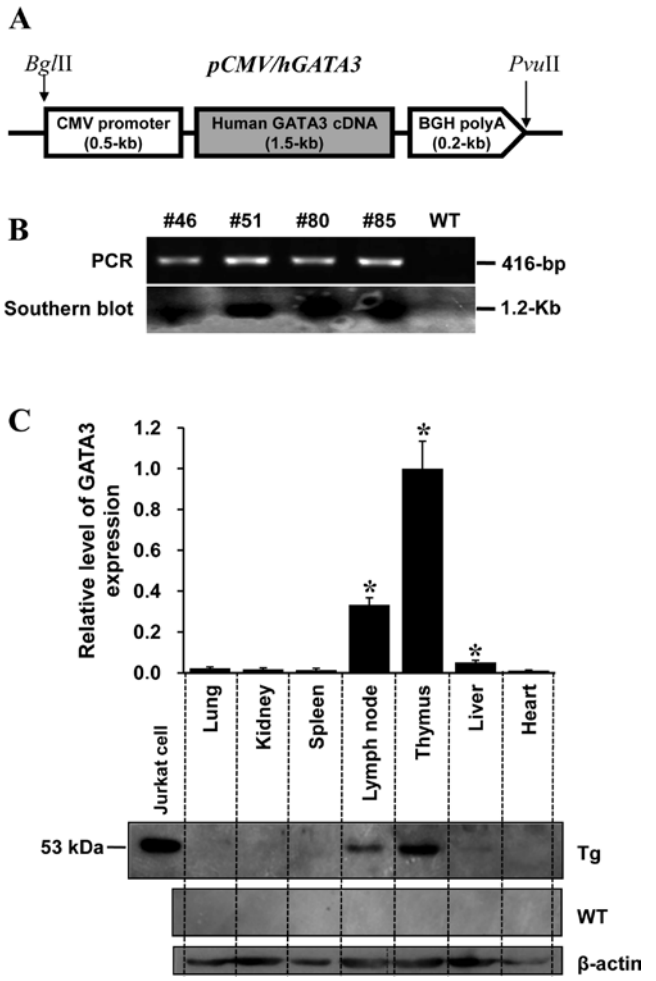

Figure 1. The production of CMV/hGATA3 Tg mice and their expression in various tissues. (A) A *pCMV/hGATA3* transgene was constructed based on the *pcDNA3.1* vector system, which inserted a gene under the control of the CMV promoter. hGATA3 cDNA was amplified from RT-PCR using specific primers and cloned into a *pcDNA3.1* vector. (B) DNA-PCR and Southern blotting were performed on the genomic DNA isolated from the tail of founder mice, and the resulting products (416-bp and 1.2-kb products, respectively) are shown. (C) Expression of hGATA3 protein in the various tissues of CMV/hGATA3 Tg mice was analyzed by Western blotting. The membranes were incubated with antibodies for hGATA3 and β-actin protein. All of these experiments were performed in triplicate. The values are the mean ± SD. *P<0.05 is the significance level compared to the expression levels in lung tissue.

Results

Production of Tg mice and tissue-specific regulation of GATA3 expression. Tg mice were created by injecting eggs with *pCMV/hGATA3*, and transferring them into the oviducts of ICR pseudopregnant mice. The founder mice were then analyzed with regard to the presence of the *hGATA3* gene. Of a total of 53 offspring, four mice (nos. 46, 51, 80 and 85) from the first lineage founders possessed the *CMV/hGATA3* gene as identified by genomic DNA-PCR and Southern blot analysis (Fig. 1B). In order to determine if the regulation of the introduced hGATA3 gene was expressed under the control of the CMV promoter in all tissues, the translational levels of hGATA3 from various tissues including the lung, kidney, spleen, lymph node, thymus, liver and heart of CMV/hGATA3 Tg mice were examined by Western blotting. For tissues of CMV/hGATA3 Tg mice, the highest level of GATA3 proteins (53 kDa) were observed in the thymus, followed by the lymph node and liver of the CMV/hGATA3 Tg mice (Fig. 2C). However, the expression of these proteins was not detected in other organs such as the lung, kidney, spleen or heart of Tg mice.

Role of GATA3 in the induction of allergic skin inflammation. It has been previously reported that PA was a good compound to induce an allergic skin inflammation in various strains of mice (16,17). Therefore, to investigate the role of GATA3 on the allergic skin inflammation response to PA, measurement of the ear thickness, an indicator of allergic skin inflammation, was performed in both WT mice and CMV/hGATA3 Tg mice following topical application of PA solution for 3 weeks. The ear thickness of both WT and CMV/hGATA3 Tg mice gradually increased in the PA treatment groups in a time- and dose-dependent manner, while there were no changes observed in

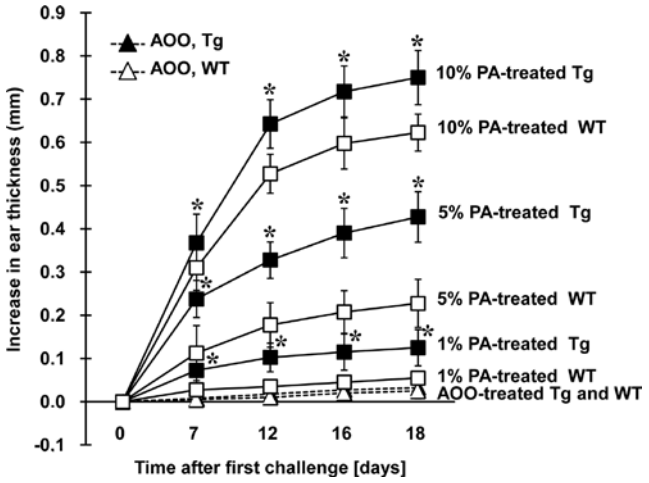

Figure 2. Effect of hGATA3 overexpression on ear thickness. The changes in the allergic skin inflammation between WT mice and CMV/hGATA3 Tg mice were determined on days 0, 7, 12, 16 and 18 post-first challenge based on the ear thickness. The values are the mean ± SD. *P<0.05 is the significance level compared to the WT mice.

the AOO-treatment group (Fig. 2). However, the rate of ear thickness increase was greater in CMV/hGATA3 Tg mice than in WT mice after the PA treatment.

Furthermore, to identify the alterations induced at the tissue level by the allergic skin inflammation, histological analysis was performed using the ear tissue of WT and CMV/hGATA3 Tg mice. In the AOO-treated group, there were no differences in histology, such as migration of inflammatory cells or an increase of the epidermal layer between WT and CMV/hGATA3 Tg mice. For the 5% PA-treated group, edema, epidermal hyperplasia (Fig. 3A) and a large number of inflammatory cells containing mast cells (Fig. 3C) were observed in both WT and CMV/hGATA3 Tg mice compared to AOO-treated mice. Interestingly, the thickness of the epidermal layer was significantly increased in CMV/hGATA3 Tg mice compared to WT mice (Fig. 3B). Also, toluidine blue staining analysis, a method used to specifically identify mast cells, showed a statistically significant increase of mast cell infiltration into the dermis (P<0.05) in CMV/hGATA3 Tg mice compared to WT mice (Fig. 3D).

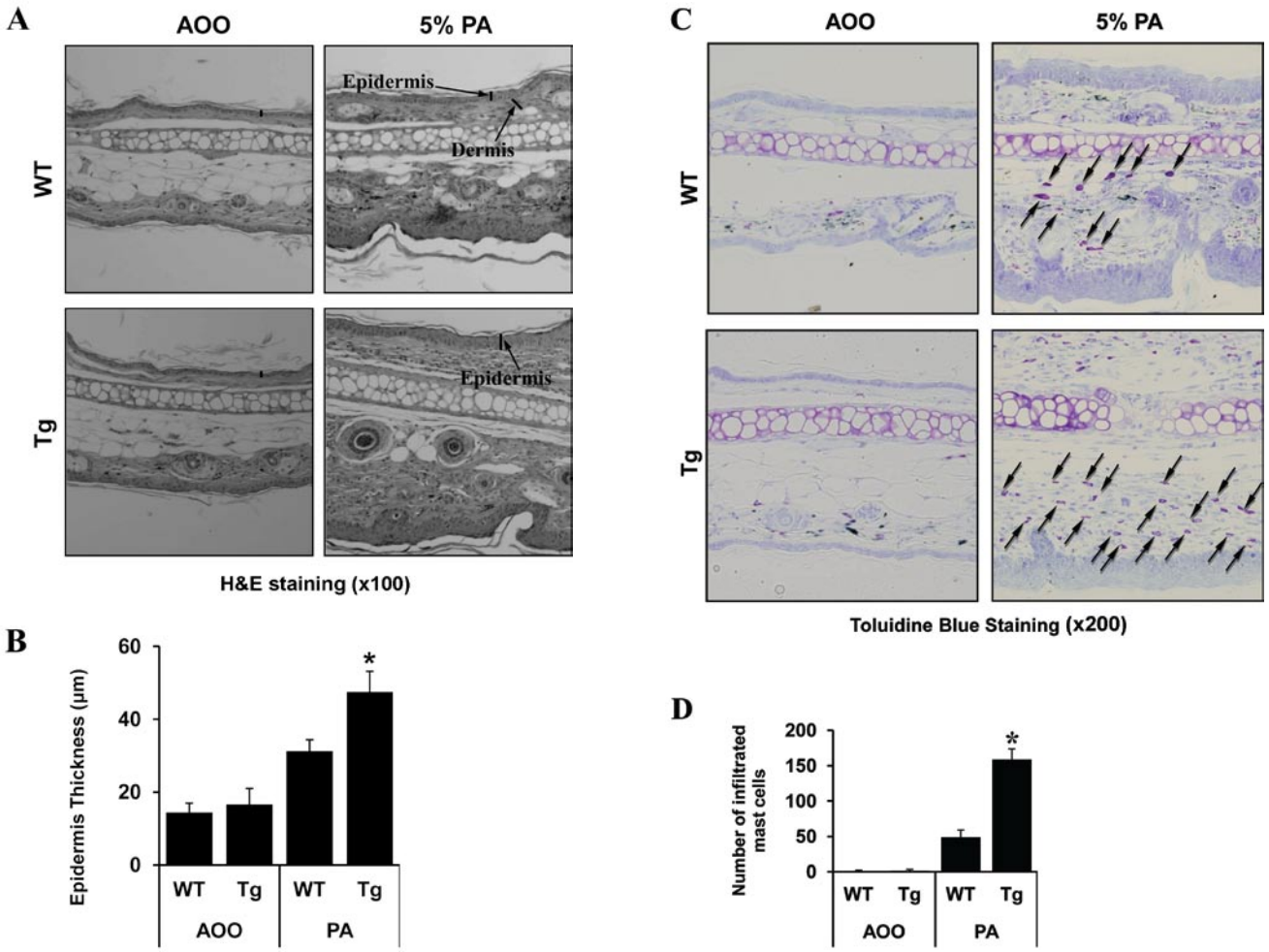

Figure 3. Effect of hGATA3 overexpression on histological structure. (A) A 5% PA solution was repeatedly applied to the dorsum of the ear of WT and CMV/hGATA3 Tg mice. After 3 weeks, the histological change was determined using hematoxylin and eosin (H&E) staining and observed at x100 magnification using light microscopy. (B) Epidermal thickness was calculated by measuring the epidermis width in 3 randomly selected sites of the slide section. The values are the mean ± SD. *P<0.05 is the significance level compared to the WT mice. (C) A 5% PA solution was repeatedly applied to the dorsum of the ear of WT and CMV/hGATA3 Tg mice. After 3 weeks, the alteration of histological structure was assessed. Cells stained with purple color in the dermis of the ear tissue represent mast cells. The slide sections of ear tissue were stained with toluidine blue and observed at x200 magnification. (D) In the lower panel, five fields were randomly chosen and the number of mast cells was counted using light microscopy. The values are the mean ± SD. *P<0.05 is the significance level compared to WT mice.

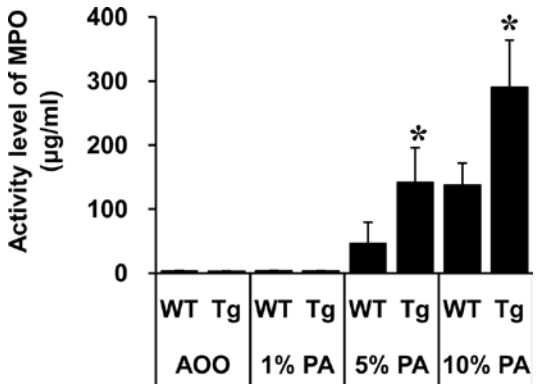

Figure 4. Effect of hGATA3 overexpression on myeloperoxidase (MPO) activity. PA solution was repeatedly applied to the dorsum of the ear of WT and CMV/hGATA3 Tg mice. After 3 weeks, to measure the MPO activity the ear tissues were excised from mice and homogenized. The MPO activity was quantified by an enzyme-linked immunosorbent assay. The values are the mean ± SD. *P<0.05 is the significance level compared to the WT mice.

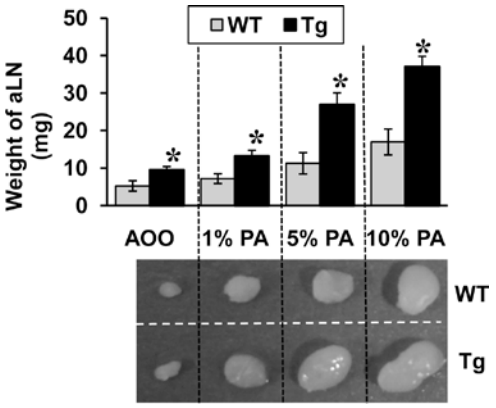

Figure 5. Effect of hGATA3 overexpression on the weight of auricular lymph node (aLN). After PA treatment, mice were sacrificed under anesthesia. aLN were then collected from the neck region of the mice using a microscissor, and their weight was measured with an electronic balance. The values are the mean ± SD. *P<0.05 is the significance level compared to the WT mice.

In addition, neutrophil infiltration, which indicates the presence of the inflammatory response, was detected indirectly by quantifying the neutrophil-specific MPO enzyme activity in the ear tissue, since neutrophils kill engulfed infectious pathogens by using MPO to catalyze the reaction of hydrogen peroxide with chloride ions to produce the microbicidal hypochlorite ion (18-20). The MPO activity increased in the PA treatment group except in the 1% PA-treated group, while it was unchanged in the AOO-treated group as expected (Fig. 4). Particularly, the increased level of MPO activity in the PA-treated group was significantly (P<0.05) higher in CMV/hGATA3 Tg mice than in WT mice. Taken together, these results suggest that Tg mice overexpressing GATA3 were more sensitive to allergic skin inflammation induced by PA treatment. Furthermore, our results show that GATA3 may contribute to the regulation of allergic skin inflammation in CMV/hGATA3 Tg mice.

Effects of GATA3 overexpression on the auricular lymph node (aLN) weight and serum Ig concentration. In order to investigate the effects of GATA3 overexpression on aLN which plays a functional role in the allergic skin inflammation, the weight of aLN collected from WT and CMV/hGATA3 Tg mice was measured. In the AOO-treated groups, aLN weight was slightly higher in CMV/hGATA3 Tg mice than in WT mice (Fig. 5). Also, PA treatment induced the increase of aLN weight in both WT and CMV/hGATA3 Tg mice compared with the AOO-treated group. The rate of their weight increase was dependent on the PA concentration in both groups. In particular, the aLN weight of Tg mice was markedly increased after PA treatment compared with WT mice. Thus GATA3 overexpression may contribute to weight gain of aLN in response to PA. Furthermore, these results showed conclusively that there was a tight link between the increase of aLN weight and the high expression of GATA3.

It is well known that the hyper-production of Th2-type Ig, such as IgE and IgG1, are one of the characteristic features of allergic hypersensitivity (13,21,22) and an indicator of the magnitude of the allergic immune response (23). To investigate whether GATA3 overexpression could induce the change of Th2-type Ig concentration, the concentrations of IgE and IgG1 were measured in serum. The repeated topical application of the PA solution induced an increase in the serum IgE and IgG1 concentrations in both WT and CMV/hGATA3 Tg mice, while AOO application did not induce any change on Ig concentrations (Fig. 6). Interestingly, the CMV/hGATA3 Tg mice were found to have a higher concentration of IgE and IgG1 than the WT mice after PA treatment. However, the concentration of Th1-type Igs, including IgG2a and IgG3 did not show any differences between AOO-treated and PA-treated mice, or between WT and CMV/hGATA3 Tg mice. We could thus deduce that GATA3 overexpression may contribute the regulation of the secretion of only Th2-type Igs.

Effects of GATA3 overexpression on the secretion of cytokines and chemokines. It has been reported that several cytokines and chemokines may regulate the inflammatory response (23,24). Therefore, in order to investigate whether GATA3 overexpression could induce the differences in the secretion of cytokines in allergic skin inflammation, the expression levels of cytokines and chemokines were analyzed in the lysate of thymus and ear tissue in WT mice and CMV/hGATA3 Tg mice using a cytokine array assay. Initially, in this study, one type of Th1 cytokine and three types of Th2 cytokine were selected for analysis of the alteration of cytokine concentrations in the thymus. The concentration of IFN-γ, a representative Th1 cytokine, was higher in WT mice than in CMV/hGATA3 Tg mice treated with AOO (Fig. 7A). However, after PA treatment, their concentrations were significantly decreased in both mice by similar ratios. In the previous study, DP was reported to be an active compound that regulates Th1/Th2 balance via suppressing GATA3 expression (25). To investigate whether the suppression of GATA3 expression could restore the IFN-γ concentration, IFN-γ concentration was measured in mice after DP administration. After administration of DP, the levels of IFN-γ in CMV/hGATA3 Tg mice were restored to those of WT mice. DP treatment induced the rise of IFN-γ concentration in CMV/hGATA3 Tg mice after co-treatment with PA and DP. In terms of the Th2 cytokines, the basal levels of three cytokines including IL-4, IL-5 and IL-13 showed

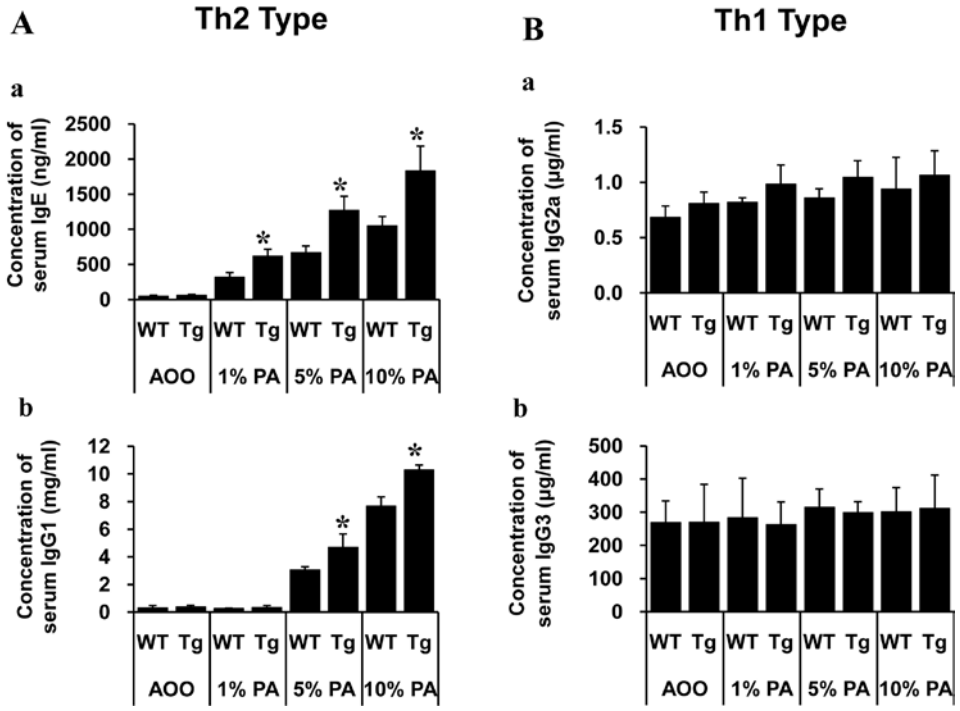

Figure 6. Effect of hGATA3 overexpression on the concentration of serum Ig. The concentrations of Igs from mouse serum blood samples were quantified by an enzyme-linked immunosorbent assay. The values are the mean ± SD. *P<0.05 is the significance level compared to the WT mice.

a different pattern between WT mice and CMV/hGATA3 Tg mice before PA treatment. However, after PA treatment, the concentrations of Th2 cytokines were altered in a similar pattern, with the levels of each Th2 cytokine being significantly higher in CMV/hGATA3 Tg mice than WT mice. Furthermore, DP treatment to suppress the GATA3 expression resulted in a decrease of Th2 cytokines in both groups of mice. The Tg mice in particular showed a high concentration of each cytokine compared with WT mice, although there were differences observed betweem the different experimental conditions. These results suggest that GATA3 overexpression induces the secretion of Th1 type cytokine, but on the other hand, this overexpression suppresses the secretion of Th2 type cytokines. Also, GATA3 suppression restored the Th1 type cytokine suppression induced by GATA3 overexpression and accelerated the Th2 type cytokine suppression induced by PA treatment.

We next assessed the expression levels of other inflammatory cytokines and chemokines, such as RANTES, IL-6, M-CSF and VEGF in the lysate of ear tissue to investigate the effects of GATA3 on the allergic skin inflammation (Fig. 7B). This analysis demonstrated that PA treatment induced the increase of four cytokines in both WT and CMV/hGATA3 Tg mice. Tg mice in the PA-treatment group were observed to have higher levels of RANTES, IL-6, M-CSF and VEGF than WT mice. Interestingly, the administration of DP induced the decrease of RANTES, IL-6, M-CSF and VEGF concentrations in both mice. These results indicate that GATA3 overexpression may induce the high response to PA in the ear tissue. Furthermore, the GATA3 suppression with DP treatment may restore the response induced by PA to almost the no-treatment levels.

Suppression of GATA3 expression by DP treatment. Finally, in order to verify whether GATA3 expression could be regulated by DP treatment in CMV/hGATA3 Tg mice, the expression level of GATA3 was detected in the thymus using Western blotting. The expression level of GATA3 was increased ~3-fold in the thymus of Tg mice after PA treatment (Fig. 8). However, DP treatment induced the suppression of GATA3 expression. Therefore, our data demonstrate that GATA3 expression may be directly regulated by PA and DP treatment.

Discussion

In the present study, we investigated the role of the hGATA3 in the induction process of allergic skin inflammation. We observed that hGATA3 enhanced allergic skin inflammation, whereas assessment of the ear thickness revealed that CMV/hGATA3 Tg mice overexpressing hGATA3 were more sensitive to PA treatment than WT. It is known that allergic skin inflammation is typically characterized by the infiltration of inflammatory cells (26). The results demonstrate that the inflamed skin of CMV/hGATA3 Tg mice overexpressing hGATA3 has a greater mass of infiltrated mast cells than that of WT mice. Furthermore, the increase in MPO activity in the inflamed skin was greater in CMV/hGATA3 Tg mice than that in WT mice. Thus, these results suggest that hGATA3 may induce the accumulation of inflammatory cells, such as mast cells and neutrophils in the inflamed skin. Furthermore, these results were very similar to the response in lung tissue of BALB/c mice which suffered an asthmatic reaction induced by ovalbumin treatment (25).

To evaluate the magnitude of the local immune reaction of mice in response to the topical application of PA, we examined the changes of aLN weight and serum IgE levels. Skin inflammation in response to chemical allergens is generally acknowledged to be dependent on IgE-mediated mechanisms.

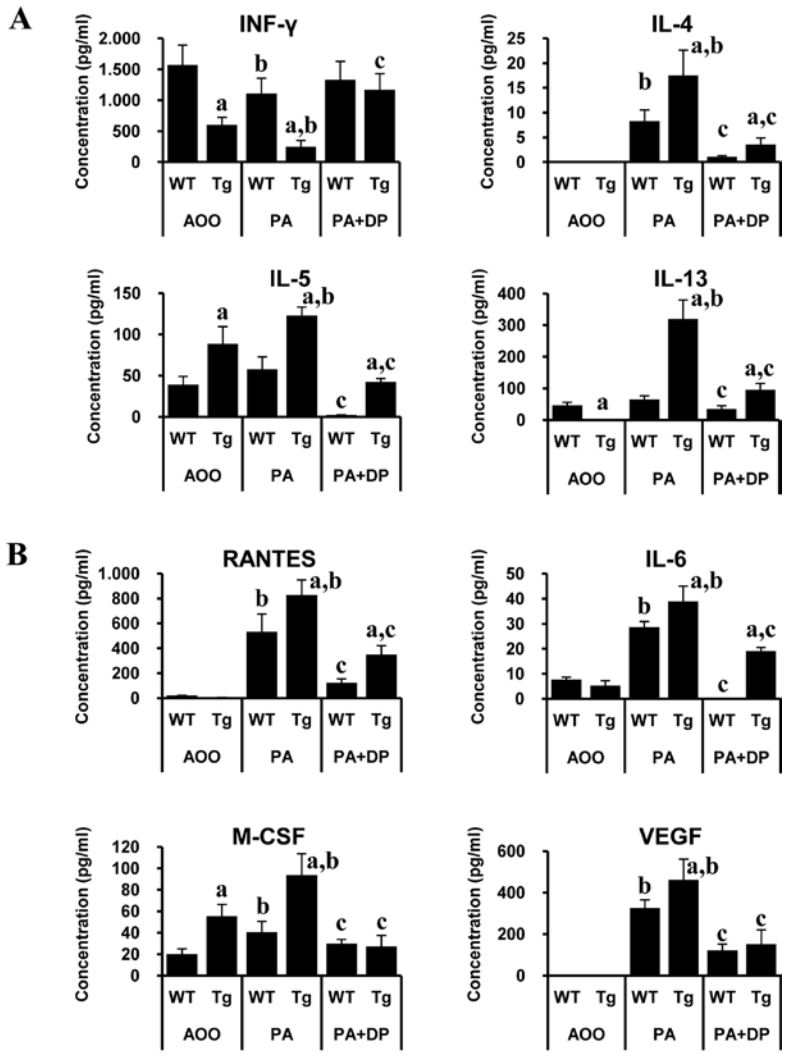

Figure 7. Effect of hGATA3 overexpression and D-pinitol (DP) treatment on the concentration of cytokines and chemokines. PA solution was repeatedly applied to the dorsum of the ear of WT and CMV/hGATA3 Tg mice for 3 weeks. Simultaneously, mice were injected i.p. with 20 mg/kg/day DP in 200 μl from Day 18-20. After 3 weeks, the concentration of cytokines and chemokines in (A) thymus and (B) ear tissues were determined with a cytokine array assay. The values are the mean ± SD. [a]P<0.05, significant difference between WT and CMV/hGATA3 Tg mice treated with the same compound; [b]P<0.05, significant difference between the AOO-treated and PA-treated group/genotype; [c]P<0.05, significant difference between PA-treated group of PA/DP-co-treated group/genotype.

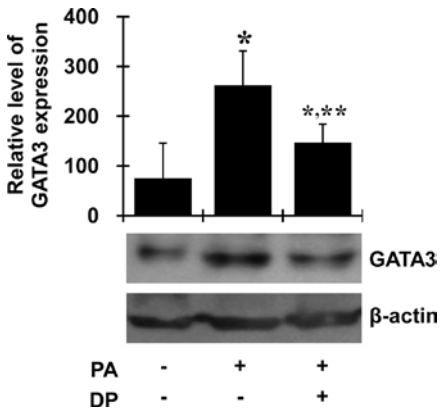

Figure 8. Effect of PA and DP on the overexpression of hGATA3 protein. Expression level of hGATA3 in the thymus of PA and DP-treated CMV/hGATA3 Tg mice was measured by Western blotting. The membranes were incubated with antibodies for GATA3 and β-actin. Three mice/group were assayed by Western blotting. The values are the mean ± SD. *P<0.05 is the significance level compared to the non-treated Tg mice; **P<0.05 is the significance level compared to the PA-treated mice.

Because the aLN weight and serum IgE levels in CMV/hGATA3 Tg mice were higher than those in WT mice following PA treatment, we conclude that GATA3 is involved in the local immune reaction and affects the severity of the allergic skin inflammation. An increase of IgE and IgG1 concentrations has been previously observed in CD2/murine GATA3 Tg mice after ovalbumin treatment (13,22). However, in terms of IgG2 and G3 level, there was a difference observed between CMV/hGATA3 Tg and CD2/mGATA3 Tg mice. In our results, their level did not change by GATA3 overexpression and PA treatment, while it was reported to decrease in CD2/mGATA3 Tg mice (22).

DP, an active component of the traditional anti-diabetic plant, *Bougainvillea spectablilis*, reportedly exerts insulin-like effects (25). DP is also known to exert insulin-like effect, via the driving of creatine and other nutrients into muscle cells (27). In addition, DP has been suggested to possess anti-inflammatory activity. It has been reported that DP regulates Th1/Th2 balance via suppressing Th2 immune response in

ovalbumin-induced asthma (25). Our studies investigated the alterations of key cytokines secreted from Th1 and Th2 cells. Especially, the decrease of IFN-γ concentration was commonly observed in Tg mice overexpressing GATA3 such as the CMV/hGATA3, CD2/mGATA3 (22,28) and BALB/c mice treated with ovalbumin (25). However, their level was maintained only in CD2/mGATA3 Tg mice after ovalbumin treatment (13), while it was enhanced in the CMV/hGATA3 Tg mice after PA treatment. Also, DP treatment successfully restored IFN-γ levels to just over 90% in BALB/c mice that suffered an asthmatic reaction (25). In our study, the reduced level of IFN-γ with hGATA3 overexpression was fully restored by DP treatment.

For the Th2 cytokines, the alterations of three cytokines, including IL-4, IL-5 and IL-13 were detected in our study to investigate the effect of GATA3 in allergic skin inflammation. These three cytokines were significantly increased in CMV/hGATA3 Tg mice, and further enhanced after PA treatment (Fig. 7A). Similar results were observed in CD2/mGATA3 Tg mice after OVA treatment (13). However, in the non-treatment condition, the alteration of the IL-4 concentration was not induced by mGATA3 overexpression (22).

In addition, the expression of inflammatory cytokine and chemokines, such as IL-6, RANTES, M-CSF and VEGF were tightly correlated with inflamed tissues. IL-6 induces the infiltration of mononuclear cells and is believed to play an important role in ongoing chronic skin inflammation (29). RANTES belongs to the CC-chemokine family, which induce eosinophil migration into local tissue (2). VEGF is a potent mediator of angiogenesis that stimulates the migration and proliferation of endothelial cells, increases vascular permeability and stimulates the expression of interacellular adhesion molecules (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on endothelial cells (30-32). Moreover, it has been reported that overexpression of VEGF in the skin of K14/VEGF Tg mice induces an increase in ear thickness, epidermal thickness, angiogenesis and infiltration of T cells (33). In this study, GATA3 overexpression enhanced the levels of these cytokines and chemokines in the PA treatment condition (Fig. 7B). Furthermore, the increased levels of four cytokines and chemokines were restored by GATA3 suppression. On the other hand, a previous study using CD2/mGATA3 Tg mice did not detect the four cytokines and chemokines investigated in our study, but rather detected TGF (13).

In conclusion, our study demonstrated that the enhanced expression of hGATA3 accelerated allergic skin inflammation in allergen-infected tissue. Furthermore, overexpression of hGATA3 induced the Th1/Th2 imbalance in the thymus via suppressing the Th1 cytokine expression and by increasing the Th2 cytokine expression. Furthermore, DP regulated the Th1/Th2 balance by means of suppressing hGATA3 overexpression in the thymus of allergen-induced Tg mice. Therefore, inhibiting the function of GATA3 may be a novel strategy for the treatment of allergic skin inflammation diseases, such as atopic dermatitis.

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