



Mechanism underlying the effect of combined therapy using glucosamine and low-dose cyclosporine A on the development of atopic dermatitis-like skin lesions in NC/Nga mice

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ARTICLE INFO

Article history:

Received 3 September 2012

Received in revised form 8 December 2012

Accepted 7 January 2013

Available online 22 January 2013

Keywords:

Glucosamine

Cyclosporine A

Combination therapy

Atopic dermatitis

NC/Nga mice

ABSTRACT

Combination therapy is often used in the treatment of atopic dermatitis (AD) to improve clinical efficacy or to spare the dose of each drug. Cyclosporine A (CsA) is a calcineurin inhibitor that was developed for the treatment of AD. Glucosamine (Glu) is a potent immunosuppressant that inhibits Th2-mediated immunity. We previously reported that Glu has an ameliorative effect on the development of the pathology in NC/Nga mice. The aims of our study were to investigate the therapeutic efficacy of combination of Glu and low-dose CsA in dermatophagoides farinae (Df)-induced AD-like skin lesions in NC/Nga mice and to determine the underlying therapeutic mechanisms. The Df-induced NC/Nga mice with a clinical score of 7 were used for treatment with Glu (500 mg/kg) alone, low-dose CsA (2, 5, and 10 mg/kg) or in combination. The clinical scores were reduced significantly by the combination treatment with Glu and low-dose CsA. The suppression of dermatitis by combined therapy was accompanied by decrease in the plasma level of IgE and in the splenic level of IL-4, IL-5, IL-13, TARC and eotaxin. Histological analysis of the skin also revealed that combination treatment significantly reduced the inflammatory cellular infiltrate, including mast cells and eosinophils. Particularly, immunological evaluation reveals an increase of CD4⁺CD25⁺ Treg cells in the combined treatment. The induction of TSLP, which leads to systemic Th2 response, was reduced in the skin on combination treatment. The protein expression of filaggrin and involucrin was recovered by combination treatment in the skin lesions, whereas the protein expression of keratin-10 and keratin-14 decreased in the combination treatment. Collectively, our findings suggest that combination treatment of Glu and low-dose CsA leads to the therapeutic effects in Df-induced AD-like skin lesion in NC/Nga mice through inhibition of IgE, inflammatory cellular infiltrate, and recovery of skin barrier function via a mechanism that may inhibition of Th2-mediated immune responses, in part, increment of CD4⁺CD25⁺ Treg cells. These results suggest that this combined immunosuppressive treatment may provide important implications for the design of therapeutic strategies aimed at AD treatment.

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1. Introduction

Atopic dermatitis (AD) is a chronically relapsing, non-contagious, and pruritic skin disorder with allergic inflammation [1,2]. Although the pathogenesis of AD is unclear, it thought that dysregulated T-helper 1 (Th1) and T-helper 2 (Th2) immune responses, allergen-specific IgE predominance, and disrupted epidermal barrier function is an important pathogenic mechanism of AD [3–5]. In addition, the presence of inflammatory cells such as CD4⁺ T cells, eosinophils, and mast cells in the majority of patients with AD supports Th2 dominance in AD [6,7]. Some studies have demonstrated that regulatory T (Treg) cells suppresses

the production IgE by B cells, the production of Th2 cytokine from Th2 cells, and regulate various allergic diseases [8,9]. Therefore, it is important to consider the balance of Th1/Th2 cytokine and Treg in patients with immune diseases in allergic diseases such as AD.

Glucosamine (Glu) suppresses proliferation and differentiation of unprimed CD4⁺ T cells and is inhibitor toward the development of Th2-mediated immune responses than Th1-mediated immune responses [10]. Thus, Glu has immunosuppressive properties. We recently reported that prophylactic treatment with Glu improved clinical symptoms in *Dermatophagoides farinae* (Df)-induced NC/Nga mice, with no apparent side effects [11]. These results suggest that inhibitory dermatitis effect of Glu on Df-induced NC/Nga mice might be mediated by selectively suppressing Th2-mediated immune responses and decreasing the level of IgE.

Cyclosporine A (CsA) has been shown to be effective in the treatment of AD and is approved for the treatment of moderate to severe AD [12]. However, the possibility of side effects from CsA frequently

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limits their long-term clinical treatment or do relapse after CsA treatment has stopped [13]. The main side effects are renal dysfunction and hypertension. To improve clinical efficacy, to spare the dose of each drug, or reduce the risk of adverse effects, low-dose CsA has been administered with other immunosuppressant therapy [14,15]. These immunosuppressive drug combination therapies may prove superior to monotherapy with either of the agents in improving overall dermatological scores.

The present study investigated the therapeutic potential of Glu and low-dose CsA in combination on AD by an *in vivo* experiment performed using Df-induced dermatitis in NC/Nga mice, which is histologically and clinically similar to AD in humans [16], and determined its underlying mechanisms of this combination therapy.

2. Materials and methods

2.1. Animals

Eight-week-old male NC/Nga mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) for this study. The mice were maintained in uncontrolled conventional Laboratory Animal Facilities at the Dongguk University School of Medicine. The animal care and use committee of the research institute at Dongguk University Hospital approved all described studies.

2.2. Drugs and reagents

Glu and CsA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and ChongKunDang Pharmaceutical Co. (Seoul, Korea), respectively. Df body ointment was prepared by Biostir Inc. (Kobe, Japan). 1 g of Df body ointment contained 136.4 mg protein, 234 µg Der f 1, and 7 µg Der f 2.

2.3. Induction of AD in the NC/Nga mice

Induction of AD using Df body ointment was performed as described previously [11]. The hair on the backs of the NC/Nga mice was shaved using an electric shaver, followed by treatment with a skin-hair-remover (Niclean, Ildong, Korea). Barrier disruption was achieved by 4% sodium dodecyl sulfate treatment on the shaved dorsal skin and both surfaces of each ear 3 h before Df body ointment (100 mg/mouse) application. These procedures were repeated twice a week for four weeks.

2.4. The administration of drugs on Df-induced NC/Nga mice

The mice with a clinical score of 7 were used for treatment with drug alone or in combination. We investigated a dose response (250, 500, and 1000 mg/kg) for Glu in the therapeutic experiment. However, there was not any therapeutic effect in these dose-responses [data not shown]. We recently reported that Glu (500 mg/kg) combined with tacrolimus (FK-506) significantly showed therapeutic effects of atopic dermatitis in NC/Nga mice [17]. Therefore, we administered 500 mg/kg of Glu in further study. The mice were divided into three groups according to the dose of CsA: 2 mg, 5 mg, and 10 mg/kg. For the control group, PBS was administered instead of each drug. Glu was administered to the mice orally once a day for 4 weeks by gavage (oral zoned needle) in water, either alone or in combination with CsA. Each group consisted of five mice.

2.5. Scoring of skin lesions

The extent of: (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total skin score was defined as the sum of the individual scores [18].

2.6. Assay of serum IgE concentration

Mice serum was collected at one week after the final administration. The concentration of total IgE in mouse serum was measured using ELISA kit (Yamasa, Tokyo, Japan). The ELISA was performed in accordance with the manufacturer's instructions.

2.7. Assay of cytokines, chemokines production

One week after the final administration, single-cell suspension were prepared from the spleen and incubated with 20 ng/mL of Phorbol 12-Myristate 13-Acetate (PMA, sigma) and 1 µM of ionomycin (Calbiochem, La Jolla, CA, USA) at 37 °C in 5% CO₂ for 24 h. After the incubation period, the culture supernatant was collected. The concentrations of IL-4, IL-5, IL-13, IFN-γ, CCL17/TARC, and eotaxin were determined using the ELISA kit (R&D System, Minneapolis, Minnesota, USA). For measuring thymic stromal lymphopoietin (TSLP) level, the dorsal skin of mice were removed and stored at −80 °C. Later, the skins were homogenized using the Bullet Blender™ Blue (Next Advance, Averill Park, NY) at 4 °C. The homogenates were then centrifuged by 10,000 rpm for 10 min, and then supernatants were stored at −80 °C.

The concentrations of TSLP were determined using the ELISA kit (R&D System, Minneapolis, Minnesota, USA).

2.8. Flow cytometry of regulatory T cells

Single-cell suspension prepared from the spleen were stained with FITC-anti-CD4 (GK1.5) and PE-anti-CD25 (280406) (R&D System, Minneapolis, Minnesota, USA). Then they were incubated in the dark for 30 min at 4 °C in PBS, 2% FBS. These samples washed twice and analyzed using CellQuest software (Becton Dickinson).

2.9. Histological analyses

Paraformaldehyde-fixed and paraffin-embedded back skin samples from the mice were sliced and then stained with toluidine blue and Congo red to count the mast cells and eosinophils, respectively. The cell density was expressed as the number of cells per five high-power fields (×400) for each section.

2.10. Western blot analysis

Skin was harvested from the back of mice and stored at −80 °C. The samples were homogenized by mortar and pestle and lysed in Lysis buffer (10 mM Tris-HCl containing 50 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM leupeptin, and 10 µg/ml aprotinin, pH 7.5). Equal amounts of proteins (20 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a NC membrane. After blocking, the membrane was incubated with 1:1000 diluted antibodies against Filaggrin, Involucrin, β-actin, and keratin-14 (Santa Cruz, CA, USA) or keratin-10 (Sigma-Aldrich Co. (St. Louis, MO, USA)). After incubation with appropriate HRP-conjugated secondary antibodies (diluted 1:3000), the blots were visualized with ECL Plus™ Western Blotting Detection Reagents (Amersham, Piscataway, NJ, USA) and the signals were captured on an Image Reader (LAS-3000; Fuji Photo Film, Tokyo, Japan).

2.11. Statistical analysis

The statistical analysis of the experimental data was performed using the Student's *t*-test. The results are expressed as the mean ± S.D. A *p*-value less than 0.05 were considered significant.

3. Results

3.1. Effect of CsA on Df-induced AD-like skin lesions in NC/Nga mice

To investigate the effect of CsA on AD, Df-induced NC/Nga mice with a clinical score of 7 were used for treatment with a single oral dose (from 2 to 100 mg/kg) of CsA once a day for 4 weeks. The clinical skin score was calculated by the sum of the individual scores based on the symptoms of erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion. The symptoms severity scores in the high-dose (45 and 100 mg/kg) of CsA were significantly lower than those in the control group (Df alone) (Fig. 1). However, there was no significant difference between the low-dose (2, 5, and 10 mg/kg) of CsA and the control group (Df alone) (Fig. 1). Therefore, we used CsA at a low-dose (2, 5, and 10 mg/kg) in the following experiments.

3.2. Improvement of Df-induced AD-like skin lesions in NC/Nga mice by combined treatment with Glu and low-dose CsA

To evaluate the therapeutic efficacy of combination treatment on Df-induced AD-like skin lesions in the NC/Nga mice, the mice with a clinical score of 7 were used for treatment with Glu (500 mg/kg) and/or CsA (2, 5, and 10 mg/kg) once a day for 4 weeks. The clinical skin score was calculated by the sum of the individual scores based on the symptoms of erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion. These symptoms severity scores in the combination groups of Glu and low-dose CsA were significantly ameliorated or resolved than those in the group of Glu alone or low-dose CsA alone (Fig. 2A). There was no significant difference between the Glu alone or low-dose CsA alone and the control group (Df alone) (Fig. 2A). Representative clinical features of NC/Nga mice are shown in Fig. 2B.

3.3. Inhibition of Df-induced serum IgE level in NC/Nga mice by combined treatment with Glu and low-dose CsA

Serum levels of IgE were examined in the Df-induced NC/Nga mice after treatment with drug alone or in combination. As shown in Fig. 3, the total serum IgE levels were significantly decreased in the combination

groups of Glu and low-dose CsA compared to the Glu alone or CsA alone. There was no significant difference between the Glu alone or low-dose CsA alone and the control group (Df alone) (Fig. 3).

3.4. Changes of immune responses by combined treatment with Glu and low-dose CsA

We examine the effects of combined treatment by Glu and low-dose CsA on Th2 cytokine and chemokines production in Df-induced NC/Nga mice because inhibition of IgE production by combination treatment at the time (Fig. 3). As shown in Table 1, the production of IL-4, IL-5, IL-13, TARC, and eotaxin (Th2 cytokines) showed marked inhibition in the combination groups of Glu and low-dose CsA, compared to the Glu alone or low-dose CsA alone group and the control group (Df alone). Furthermore, it was also observed that the production of TSLP in the skin tissue of Df-induced NC/Nga mice were significantly inhibited by combined treatment. No difference, however, was observed in IFN- γ production among all groups (data not shown).

3.5. Effect of combined treatment with Glu and low-dose CsA on regulatory T (Treg) cells

We investigated whether this combination treatment influenced the number of CD4⁺CD25⁺ Treg cells. We found that the production of CD4⁺CD25⁺ Treg cells in the spleen cells of Df-induced NC/Nga mice were significantly increased by combined treatment (Fig. 4). On the other hand, there was no significant difference in CD4⁺CD25⁺ Treg cells population between the Glu alone or low-dose CsA alone and the control group (Df alone).

3.6. Improvement of mast cells and eosinophils infiltration by combined treatment with Glu and low-dose CsA

In order to investigate whether combination treatment decreased infiltration of these inflammatory cells into the skin, in the Df-induced NC/Nga mice, tissue sections were stained with toluidine blue or Congo red. As shown in Fig. 5A and B, the number of infiltrated cells, both mast cells and eosinophils, was significantly reduced in the combination groups of Glu and low-dose CsA, compared to the Glu alone ($P=0.00035$ and $P=0.0002$, respectively) or CsA alone group ($P=0.0006$ and $P=0.009$, respectively) and the control group (Df alone) ($P=0.00024$ and $P=0.0016$, respectively).

3.7. Recovery of skin barrier function by combined treatment with Glu and low-dose CsA

The recurrence of skin barrier defect is an important target of AD treatment. We investigated whether combination treatment change the expression of protein associated with skin barrier defect in the Df-induced NC/Nga mice. As shown in Fig. 6A, filaggrin and involucrin, keratinocytes differentiation marker, demonstrated a significant increase in the combination groups of Glu and low-dose CsA compared with the control group (Df alone) ($P<0.05$). It was more increased in the combination groups compared with the Glu alone or CsA alone group, although the differences had no statistical significance. In contrast, the expressions of keratin-10 and keratin-14 were significantly decreased in the combination groups of Glu and low-dose CsA, compared to the Glu alone or CsA alone group and the control group (Df alone) ($P<0.05$; Fig. 6B).

4. Discussion

AD is associated with genetic, environmental and psychological factors, immune system dysregulation, and epidermal barrier defects [19]. Although topical steroids (corticosteroids) or calcineurin inhibitor

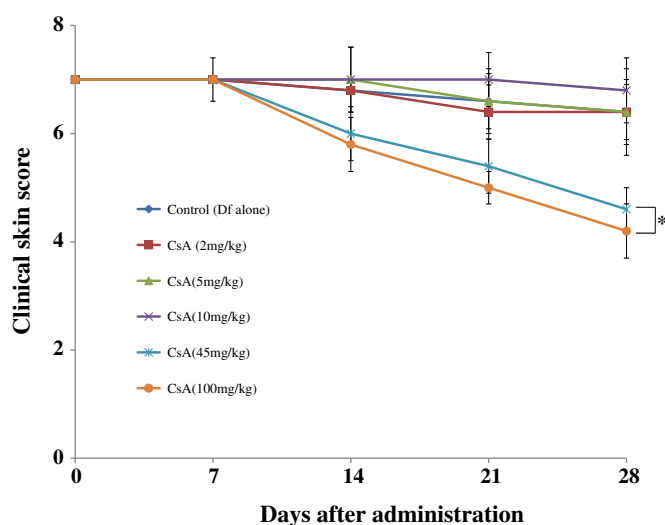


Fig. 1. Effect of CsA on the Df-induced AD-like skin lesions in NC/Nga mice: CsA was administrated to Df-induced NC/Nga mice orally once a day for 4 weeks at various doses (from 2 to 100 mg/kg). The clinical skin score is the sum of individual scores judged by the symptoms of erythema/hemorrhage, scarring/dryness, edema, excoriation/erosion. Each data represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P<0.05$ using a Student's *t*-test compared with all other groups. The data shown are representative of two independent experiments.

A

Groups	Dermatitis score (Day 0)	Dermatitis score (Day 28)
Control (Df alone)	7.0± 0.0	6.8± 0.4
CsA (2mg/kg)	7.0± 0.0	6.4± 0.3
CsA (5mg/kg)	7.0± 0.0	6.2± 0.5
CsA(10mg/kg)	7.0± 0.0	6.4± 0.3
Glu(500mg/kg)	7.0± 0.0	6.4± 0.4
CsA(2mg/kg)+ Glu(500mg/kg)	7.0± 0.0	5.0± 0.4 [*]
CsA(5mg/kg)+ Glu(500mg/kg)	7.0± 0.0	5.0± 0.6 [*]
CsA(10mg/kg)+ Glu(500mg/kg)	7.0± 0.0	4.8± 0.5 [*]

B

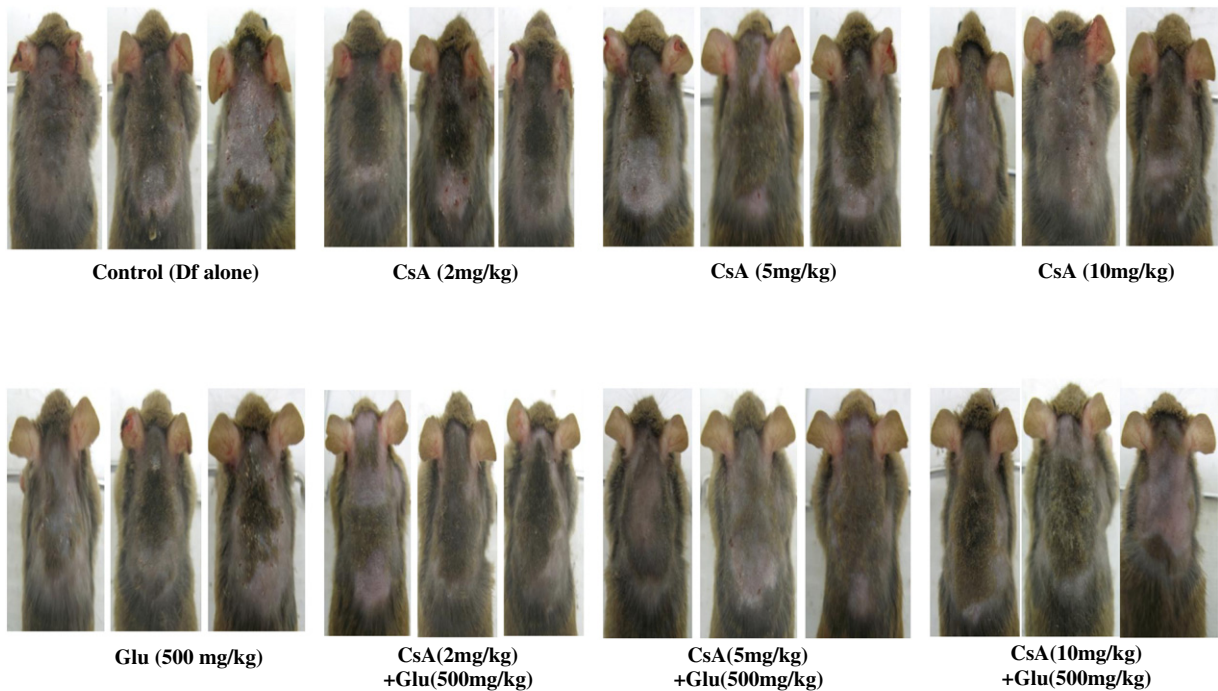


Fig. 2. Therapeutic effect of combination therapy on the Df-induced AD-like skin lesions in NC/Nga mice: (A) after the removal of hair, Df body ointment (100 mg/mouse) was swabbed onto the dorsal skin and both surfaces of each ear twice a week for 4 weeks. The clinical skin score is the sum of individual scores judged by the symptoms of erythema/hemorrhage, scarring/dryness, edema, excoriation/erosion. Df-induced AD-like skin lesions in NC/Nga mice were untreated (Df alone), or treated with Glu (500 mg/kg), CsA (2, 5, and 10 mg/kg), or Glu plus CsA orally once a day for 4 weeks. Each data represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P < 0.05$ using a Student's *t*-test compared with all other groups. (B) representative clinical features of Df-induced NC/Nga mice in the untreated (Df alone), or treated with Glu (500 mg/kg), CsA (2, 5, and 10 mg/kg), or Glu plus CsA. The data shown are representative of two independent experiments.

(tacrolimus and pimecrolimus) are the current standard treatment for severe AD, use may be limited by potentially serious adverse effects [20]. Therefore, identified combinations of immunosuppressive agents are often necessary to improve clinical efficacy, to facilitate drug dosage reduction, or to reduce the risk of adverse effects for AD treatment. It has been reported that use of combinations of

immunosuppressive agents may be more effective than single-modality treatment with either agent [21,22]. Recently, we previously showed that combination therapy of Glu plus FK-506 was more synergistic efficacy than single modality treatment with either alone to improve the development of established dermatitis in NC/Nga mice model [17].

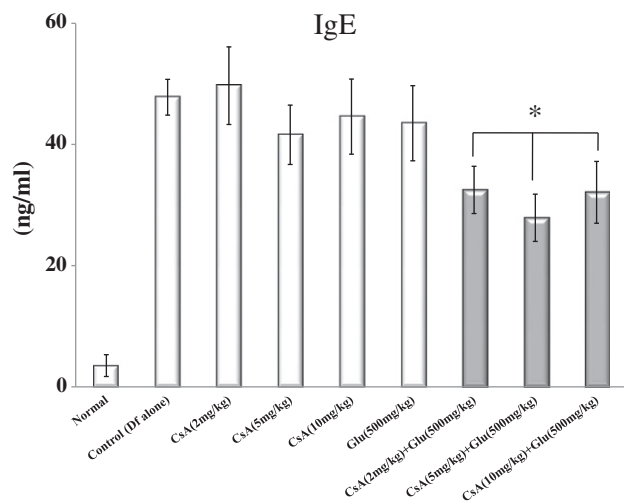


Fig. 3. Serum level of IgE by combination therapy on the Df-induced AD-like skin lesions in NC/Nga mice: the concentration of IgE in the serum was measured at one week after the final administration. Each data represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P < 0.05$ using a Student's *t*-test compared with all other groups. The data shown are representative of two independent experiments.

In this study, single administration of high-dose CsA (45 and 100 mg/kg) to NC/Nga suppressed the development of AD-like skin lesions, but little effects in NC/Nga mice administrated with low-dose CsA (2, 5, and 10 mg/kg). To look for potential effects of low-dose of CsA, CsA was administrated with Glu. We found that combination therapy with Glu and low-dose CsA of immunosuppressive agent has a potential effect on Df-induced AD-like skin lesions in NC/Nga mice. For instance, combination treatment with Glu and low-dose CsA improved the severity of the dermatitis with reduction of IgE and Th2 cytokine (IL-4, IL-5, and IL-13). A majority of the symptoms associated with AD manifest due to strong polarization of Th2 immune responses, resulting in the hyperproduction of IgE. Since IL-4 is important in the development of Th2 cells and in changes to the IgE isotype [23], the reduction of IgE levels might result from suppression of the Th2 response. In fact, the correlation between the elevation of serum levels of total IgE and the production of Th2 cytokines has been previously reported [24]. These results suggest that the improvement of clinical symptoms by combination treatment of Glu and low-dose CsA against therapeutic effects of Df-induced NC/Nga mice might be mediated, at least in part, by its inhibitory effect on Th2-mediated cytokines and IgE. Moreover, the present study of immune responses clearly also shows that Th2 chemokines (TARC and eotaxin) and TSLP in combination treatment with Glu and low-dose CsA were significantly lower than in the single

modality treatment with either alone and the control mice (Df alone). Eotaxin, together with IL-5, is known to be a potent chemoattractant for eosinophils, whereas TARC produced by Th2 cells, and induced the pathological responses typically associated with AD [25,26]. Additionally, thymic stromal lymphopoietin (TSLP) plays a critical role in initiating systemic Th2 immunity favorable for the development of AD [27]. TSLP is associated with the induction of Th2 immune response, TARC chemokine, and IgE [28]. Therefore, these results imply that combination treatment of Glu and low-dose CsA inhibited the production of TARC, eotaxin, and TSLP, resulting in the inhibition of Th2-mediated cytokines.

Although the role of Treg cells in AD remains controversial, $CD4^+CD25^+$ Treg cells have the capacity to suppress Th2 responses to allergen and the therapeutic potential for Th2-mediated diseases such as AD and asthma [29,30]. It has been reported that the population of Treg cells in AD patients treated with low-dose CsA, but not high-dose CsA, was increased [31]. Thus, we investigated whether combination treatment of glucosamine and low-dose CsA influenced the number of $CD4^+CD25^+$ Treg cells. We showed that combination treatment led to a higher increase in the $CD4^+CD25^+$ Treg cell numbers compared to the control groups. These results indicated that combination treatment inhibited Th2-mediated cytokine, which may contribute to induce $CD4^+CD25^+$ Treg.

AD skin is characterized by the restoration of infiltration of inflammatory cell and skin barrier defect [32,33]. Moreover, mast cells are recognized as key effector cells in IgE-associated Th2 type immune responses, because they are activated by cross-linking of FcεRI. Th2 cytokines can down-regulate filaggrin and involucrin [34,35]. These filaggrin and involucrin demonstrated a significant decrease in lesional AD skin compared to non-lesional skin. In addition, keratin-10 and keratin-14 as a marker for differentiation and proliferation, respectively, increased in AD compared with normal epidermis [36–38].

Therefore, recruitment of the inflammatory cell and the epidermal barrier is an important objective for the management of AD. Several previous studies demonstrated that calcineurin inhibitors can in part recovery the skin barrier function in AD [39,40]. We found that combination treatment of Glu and low-dose CsA reduced infiltration of inflammatory cells and recovered barrier function in the skin. These results suggest that combination treatment might suppress the development of dermatitis by inhibition of mast cells and eosinophils and by up-regulation of filaggrin and involucrin or down-regulation of keratin-10 and keratin-14, presumably resulting in the inhibition of Th2-mediated immune responses.

In conclusion, the results of this study demonstrated that this combination treatment improved Df-induced AD-like skin lesions in an NC/Nga mouse model by dominantly reducing IgE, suppressing Th2-mediated cytokine levels and Th2 chemokines expression, increasing Treg cells, inhibiting inflammatory cells, as well as improving skin barrier function, and these findings correlated with the observed

Table 1

Effects of combination therapy on the level of Th2 cytokines and Th2 chemokines in Df-induced NC/Nga mice.

Groups	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	TSLP (pg/ml)	Eotaxin (pg/ml)	TARC (pg/ml)
Df alone	122.1 $\pm$ 25	76.4 $\pm$ 12.9	344.5 $\pm$ 61.8	23.1 $\pm$ 1.8	276.7 $\pm$ 11.5	132.6 $\pm$ 10.6
CsA (2 mg/kg)	98.5 $\pm$ 4.1	74.6 $\pm$ 5.1	353.8 $\pm$ 50.9	20.2 $\pm$ 1.5	291.2 $\pm$ 12.7	113.2 $\pm$ 10.9
CsA (5 mg/kg)	103.3 $\pm$ 8.8	79.5 $\pm$ 3.9	392.1 $\pm$ 17.5	21.3 $\pm$ 1.1	285 $\pm$ 6.9	125.8 $\pm$ 6.9
CsA (10 mg/kg)	105.8 $\pm$ 9.6	83.6 $\pm$ 7.8	345.9 $\pm$ 17.5	19.9 $\pm$ 0.6	261.1 $\pm$ 9.4	115.9 $\pm$ 15.1
Glu (500 mg/kg)	115.5 $\pm$ 7.3	78.5 $\pm$ 3.1	385.9 $\pm$ 23.3	19.2 $\pm$ 3.4	266.6 $\pm$ 17.4	110.7 $\pm$ 17.1
CsA (2 mg/kg) + Glu (500 mg/kg)	64.2 $\pm$ 2.3**	42.2 $\pm$ 8.2**	277.2 $\pm$ 11.7**	13.5 $\pm$ 2.6	214.7 $\pm$ 17.4*	84.2 $\pm$ 8.8*
CsA (5 mg/kg) + Glu (500 mg/kg)	57.9 $\pm$ 2.6**	47.1 $\pm$ 2.9**	203.6 $\pm$ 12.6**	14.7 $\pm$ 1.6*	219.2 $\pm$ 16.7*	83.1 $\pm$ 5.5**
CsA (10 mg/kg) + Glu (500 mg/kg)	54.6 $\pm$ 2.8**	40.3 $\pm$ 1.4**	198.2 $\pm$ 11.9	13.2 $\pm$ 1.1*	288.1 $\pm$ 15.1*	90.6 $\pm$ 6.4*

IL-4, IL-5, IL-13, eotaxin, TARC, and TSLP were measured on day 7 after the final administration. Data are mean $\pm$ S.D. of five mice.

* $p < 0.01$ as compared with untreated control (Df alone) mice (Student's *t* test).

** $p < 0.05$ as compared with untreated control (Df alone) mice (Student's *t* test).

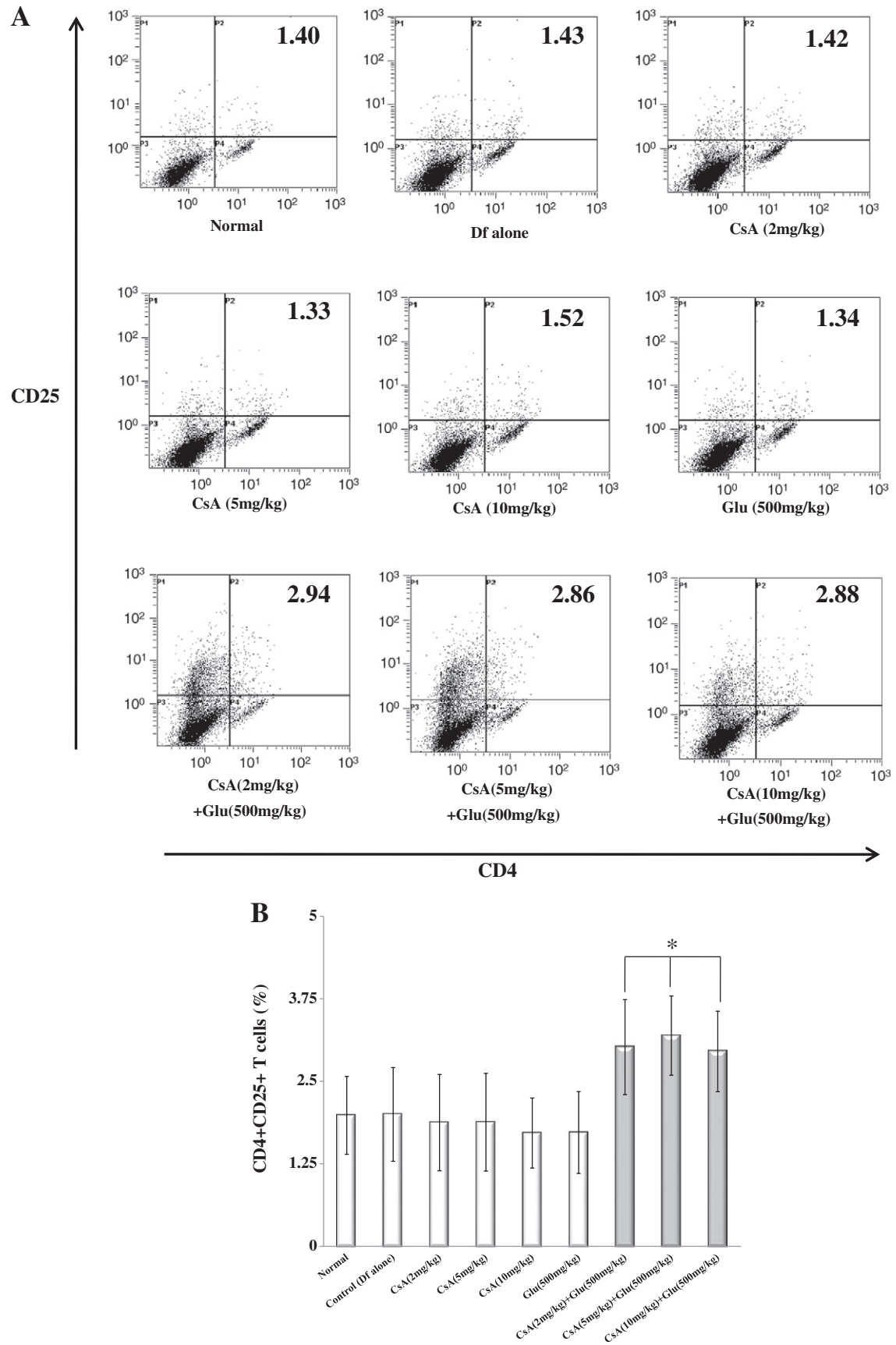


Fig. 4. Effect of combination therapy on CD4⁺CD25⁺ Treg cells: one week after the final administration, single-cell suspensions prepared from the spleen were stained with FITC-anti-CD4 and PE-anti-CD25. These samples washed twice and analyzed using CellQuest software. (A) representative FACS dot plots demonstrated the percentage of CD4⁺CD25⁺ Treg cells in splenocytes. (B) bar graph shows the mean results. Each data represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P < 0.05$ using a Student's *t*-test compared with all other groups. The data shown are representative of two independent experiments.

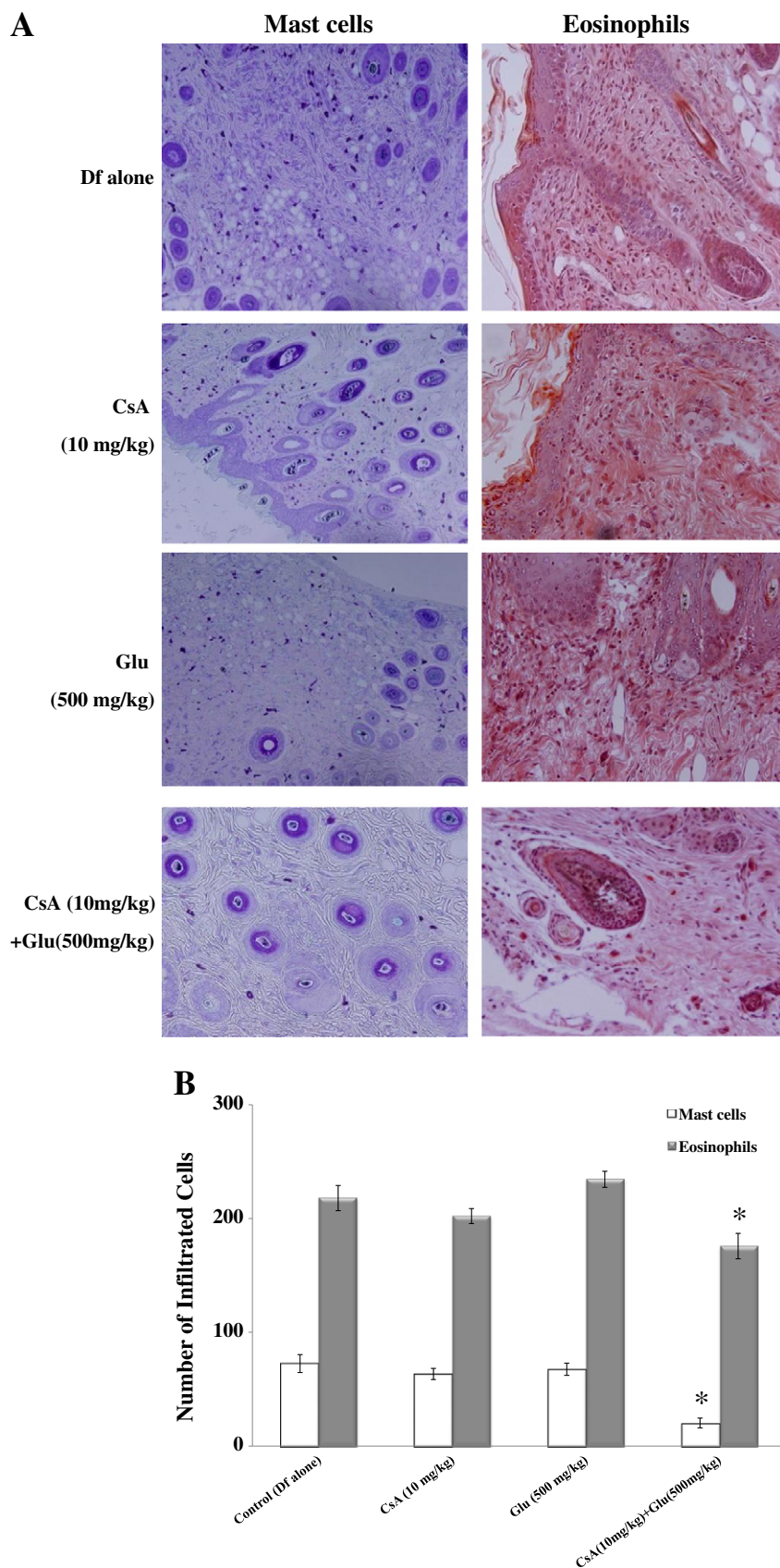


Fig. 5. Histological analysis of the skin lesions: skin samples from the back of the mice were studied at one week after the final administration. The sections were then stained with toluidine blue and Congo red for mast cells and eosinophils, respectively; (A) representative histological features in the Df-induced NC/Nga mice from the untreated (Df alone), or treated with Glu (500 mg/kg), CsA (10 mg/kg), or Glu plus CsA; (B) the cell density was expressed as the number of mast cells and eosinophils in five high-power fields ($\times 400$ for count) for each section. The data represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P < 0.05$ using a Student's *t*-test compared with all other groups. The data shown are representative of two independent experiments.

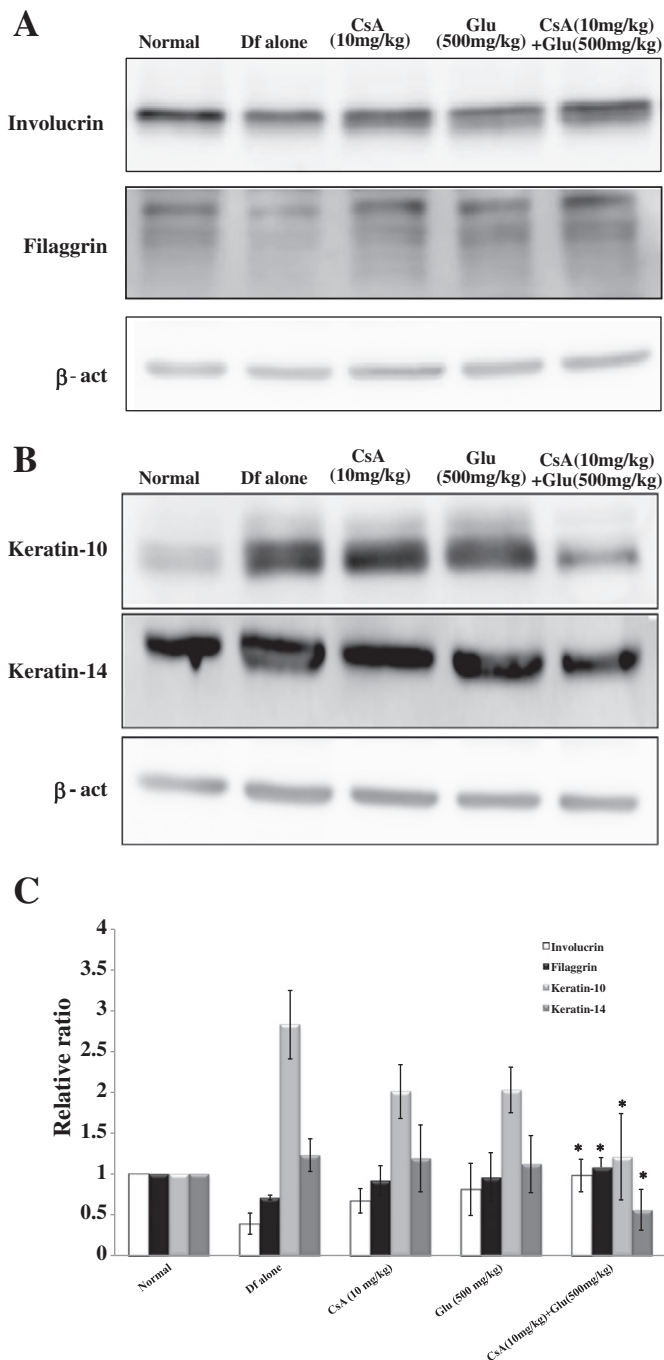


Fig. 6. Recovery of skin barrier functions by combination therapy on the Df-induced AD-like skin lesions in NC/Nga mice: the protein was prepared from the back skin of mice treated with untreated (Df alone), or Glu (500 mg/kg), CsA (10 mg/kg), or Glu plus CsA and western blotting was performed. The protein expression of filaggrin and involucrin (A), keratin-10 and keratin-14 (B) were determined. The relative ratio of each protein was determined by calculating the ratio of each protein relative to the optical density of a normal control (C). * $p < 0.005$ when compared with control mice (Df alone) (Student's *t* test). The data shown are representative of two independent experiments.

clinical symptoms. These findings have important implications for the design of therapeutic strategies aimed at AD treatment.

Conflicts of interest

None declared.

Acknowledgments

This work was supported by a grant of the Korea Healthcare technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant No.: A103017). This work was supported by the National Research Foundation of Korea [NRF] grant funded by the Korea government [MEST] (No. 2011-0028962).

References

- [1] Hoare C, Li Wan Po A, Williams H. Systematic review of treatments for atopic eczema. *Health Technol Assess* 2000;4:1–191.
- [2] Leung DYM, Bieber T. Atopic dermatitis. *Lancet* 2003;361:151–60.
- [3] Spergel JM, Mizoguchi E, Oettgen H, Bhan AK, Geha RS. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest* 1999;103:1103–11.
- [4] Miraglia del Giudice M, Decimo F, Leonardi S, Maiello N, Amelio R, Capasso A, et al. Immune dysregulation in atopic dermatitis. *Allergy Asthma Proc* 2006;27:451–5.
- [5] Wolf R, Wolf D. Abnormal epidermal barrier in the pathogenesis of atopic dermatitis. *Clin Dermatol* 2012;30:329–34.
- [6] Uehara M, Izukura R, Sawai T. Blood eosinophilia in atopic dermatitis. *Clin Exp Dermatol* 1990;15:264–6.
- [7] James EA, Kwok WW. Autoreactive CD4(+) T cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 2011;128:100–1.
- [8] Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* 2005;116:961–8.
- [9] Inoue J, Aramaki Y. Suppression of skin lesions by transdermal application of CpG-oligodeoxynucleotides in NC/Nga mice, a model of human atopic dermatitis. *J Immunol* 2007;178:584–91.
- [10] Forchhammer L, Thorn M, Met O, Gad M, Weidner MS, Claesson MH. Immunobiological effects of glucosamine in vitro. *Scand J Immunol* 2003;58:404–11.
- [11] Kim CH, Cheong KA, Park CD, Lee AY. Glucosamine improved atopic dermatitis-like skin lesions in NC/Nga mice by inhibition of Th2 cell development. *Scand J Immunol* 2011;73:536–45.
- [12] Guglielmetti S, Dart JK, Calder V. Atopic keratoconjunctivitis and atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2010;10:478–85.
- [13] Akdis CA, Akdis M, Bieber T, Bindslev-Jensen C, Boguniewicz M, Eigenmann P, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *J Allergy Clin Immunol* 2006;118:152–69.
- [14] Campione E, Paternò EJ, Diluvio L, Bianchi L, Giorgetti G, Chimenti S. Combination of low-dosage cyclosporine and topical pimecrolimus in severe atopic dermatitis with chronic hepatitis B. *Acta Derm Venereol* 2008;88:74–5.
- [15] Nahm DH, Kim ME. Treatment of severe atopic dermatitis with a combination of subcutaneous allergen immunotherapy and cyclosporin. *Yonsei Med J* 2012;53:158–63.
- [16] Jin H, He R, Oyoshi M, Geha RS. Animal models of atopic dermatitis. *J Invest Dermatol* 2009;129:31–40.
- [17] Kim CH, Cheong KA, Park CD, Lee AY. Therapeutic effects of combination using glucosamine plus tacrolimus (FK-506) on the development of atopic dermatitis-like skin lesions in NC/Nga mice. *Scand J Immunol* 2012;75:471–8.
- [18] Suto H, Matsuda H, Mitsuishi K, Hira K, Uchida T, Unno T, et al. NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol* 1999;120:70–5.
- [19] Fonacier LS, Dreskin SC, Leung DY. Allergic skin diseases. *J Allergy Clin Immunol* 2010;125(2 Suppl. 2):S138–49.
- [20] Gelbard CM, Hebert AA. New and emerging trends in the treatment of atopic dermatitis. *Patient Prefer Adherence* 2008;2:387–92.
- [21] Kubota Y, Yoneda K, Nakai K, Katsuura J, Moriue T, Matsuoka Y, et al. Effect of sequential applications of topical tacrolimus and topical corticosteroids in the treatment of pediatric atopic dermatitis: an open-label pilot study. *J Am Acad Dermatol* 2009;60:212–7.
- [22] Nakahara T, Koga T, Fukagawa S, Uchi H, Furue M. Intermittent topical corticosteroid/tacrolimus sequential therapy improves lichenification and chronic papules more efficiently than intermittent topical corticosteroid/emollient sequential therapy in patients with atopic dermatitis. *J Dermatol* 2004;31:524–8.
- [23] Vercelli D, Geha RS. Regulation of isotype switching. *Curr Opin Immunol* 1992;4:794–7.
- [24] Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, et al. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997;9:461–6.
- [25] Nakatani T, Kaburagi Y, Shimada Y, Inaoki M, Takehara K, Mukaida N, et al. CCR4 memory CD4+ T lymphocytes are increased in peripheral blood and lesional skin from patients with atopic dermatitis. *J Allergy Clin Immunol* 2001;107:353–8.
- [26] Ma W, Bryce PJ, Humbles AA, Laouini D, Yalcindag A, Alenius H, et al. CCR3 is essential for skin eosinophilia and airway hyperresponsiveness in a murine model of allergic skin inflammation. *J Clin Invest* 2002;109:621–8.
- [27] Liu YJ. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med* 2006;203:269–73.
- [28] Liu YJ. Thymic stromal lymphopoietin and OX40 ligand pathway in the initiation of dendritic cell-mediated allergic inflammation. *J Allergy Clin Immunol* 2007;120:238–44.

- [29] Stassen M, Jonuleit H, Müller C, Klein M, Richter C, Bopp T, et al. Differential regulatory capacity of CD25 + T regulatory cells and preactivated CD25 + T regulatory cells on development, functional activation, and proliferation of Th2 cells. *J Immunol* 2004;173:267–74.
- [30] Larché M. Regulatory T, cells in allergy and asthma. *Chest* 2007;132:1007–14.
- [31] Brandt C, Pavlovic V, Radbruch A, Worm M, Baumgrass R. Low-dose cyclosporine A therapy increases the regulatory T cell population in patients with atopic dermatitis. *Allergy* 2009;64:1588–96.
- [32] Kubo A, Nagao K, Amagai M. Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. *J Clin Invest* 2012;122:440–7.
- [33] Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005;6:135–42.
- [34] Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, DeBenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 2009;124(3 Suppl. 2):R7–12.
- [35] Kim BE, Leung DY, Boguniewicz M, Howell MD. Loricrin and involucrin expression is down-regulated by Th2 cytokines through STAT-6. *Clin Immunol* 2008;126:332–7.
- [36] Wakita H, Yamamoto Y, Furukawa F. Aberrant suprabasal P-cadherin expression in acanthotic but not psoriatic thickened epidermis. *Arch Dermatol Res* 2003;295(Suppl. 1):S71–4.
- [37] Theerawatanasirikul S, Sailasuta A, Thanawongnuwech R, Suriyaphol G. Alterations of keratins, involucrin and filaggrin gene expression in canine atopic dermatitis. *Res Vet Sci* 2012;93:1287–92.
- [38] Mantel A, Carpenter-Mendini AB, Vanbuskirk JB, De Benedetto A, Beck LA, Pentland AP. Aldo-keto reductase 1C3 is expressed in differentiated human epidermis, affects keratinocyte differentiation, and is upregulated in atopic dermatitis. *J Invest Dermatol* 2012;132:1103–10.
- [39] Draelos ZD. Use of topical corticosteroids and topical calcineurin inhibitors for the treatment of atopic dermatitis in thin and sensitive skin areas. *Curr Med Res Opin* 2008;24:985–94.
- [40] Jensen JM, Pfeiffer S, Witt M, Bräutigam M, Neumann C, Weichenthal M, et al. Different effects of pimecrolimus and betamethasone on the skin barrier in patients with atopic dermatitis. *J Allergy Clin Immunol* 2009;123:1124–33.