

Therapeutic Effects of Combination Using Glucosamine Plus Tacrolimus (FK-506) on The Development of Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

C.-H. Kim^{*1}, K. A. Cheong^{†1}, C. D. Park[‡] & A.-Y. Lee[†]

^{*}Dongguk University Research Institute of Biotechnology, Medical Science Research Center, Goyang, Korea; [†]Department of Dermatology, Dongguk University School of Medicine, Goyang, Korea; and [‡]Research Institute of Hurim Biocell, Gayang-Dong, Gangseo-Gu, Korea

Received 4 May 2011; Accepted in revised form 21 August 2011

Correspondence to: A.-Y. Lee, Department of Dermatology, Dongguk University Ilsan Hospital, 814 Siksa-dong, Ilsandong-Gu, Goyang-Si, Gyeonggi-Do 410-773, South Korea. E-mail: leeay@duh.org

¹These authors contributed equally to this study.

Abstract

Tacrolimus (FK-506) has been found to exhibit potent inhibitory effects on spontaneously developed dermatitis. We previously showed that glucosamine prevents the development of Atopic dermatitis (AD)-like skin lesions in NC/Nga mice. The aims of our study were to investigate the synergistic therapeutic efficacy of combination of glucosamine plus FK-506 in dermatophagoides farina (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 8 were used for treatment with glucosamine (500 mg/kg) alone, FK-506 (1.0 mg/kg) or in combination. The synergistic effects of combination therapy were evaluated by dermatitis scores, skin histology and immunological parameters such as IgE, Th2-mediated cytokines and chemokines, CD3⁺ T cells and CLA⁺ T cells. Combined therapy using glucosamine plus FK-506 improved the development of AD-like skin lesions as exemplified by a significant decrease in total skin symptom severity scores. The suppression of dermatitis by combined therapy was accompanied by a decrease in the plasma level of IgE and in the splenic level of IL-5, IL-13, TARC and eotaxin. Histological finding indicated that the dermal infiltration of inflammatory cells including mast cells and eosinophils was greatly reduced. Particularly, immunohistological evaluation reveals a reduction in CD3⁺ T cells and CLA⁺ cells in the combined therapy. Our findings suggest that combination therapy of glucosamine 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. This combined immunosuppressive therapy may provide an effective therapeutic strategy for the treatment of AD.

Introduction

Atopic dermatitis (AD), or atopic eczema, is a common, chronic, inflammatory skin disease [1, 2]. The worldwide lifetime prevalence of AD in children is 10–20%, and in adults it is 1–3% [3]. Several lines of evidence suggest the contribution of immunological mechanisms in the pathogenesis of AD. Several immunology reports have suggested T-helper 1 (Th1)/T-helper 2 (Th2) imbalance in AD [4, 5]. This imbalance favours Th2, and high serum immunoglobulin (Ig) E levels as well as infiltration with immune cells such as eosinophils, mast cells

and cutaneous lymphocyte antigen (CLA) T cells [6–8], which are all characteristics of AD, are provoked by Th2 cytokines, interleukin-4 (IL-4), IL-5 and IL-13 [9]. Patients with AD show elevated plasma IgE levels in response to many kinds of allergens, while keratinocytes of patients with AD exhibit the propensity to produce exaggerated amounts of cytokines, a phenomenon that can play a major role in the promotion and maintenance of inflammation [10, 11].

Glucosamine is a common constituent of the glycosaminoglycans in the cartilage matrix and synovial fluid. Use of glucosamine is common in patients with

osteoarthritis, because of its pharmacological effects on articular cartilage and joint tissue [12, 13]. In fact, its anti-inflammatory activity may allow for reduced doses of non-steroidal anti-inflammatory agents. The suppression of inflammatory activity may result from the potential immunoregulatory capability of glucosamine. It has been reported that glucosamine suppresses proliferation and differentiation of unprimed CD4⁺ T cells and is more inhibitory towards the development of Th2-mediated immune responses than Th1-mediated immune responses [14]. Thus, glucosamine has immunosuppressive properties also [15]. We recently reported that prophylactic treatment with glucosamine improved clinical symptoms in *Dermatophagoides farinae* (Df)-induced NC/Nga mice, with reduced infiltration of mast cells and eosinophils into skin, and that it selectively suppressed Th2-mediated immune responses [16]. These results suggest that the *in vivo* potentially prevent dermatitis effect of glucosamine on Df-induced NC/Nga mice and provides evidence that glucosamine selectively suppresses Th2-mediated immune responses.

Tacrolimus (FK-506) is a calcineurin inhibitor that was developed especially for the treatment of AD [17]. The immunosuppressive action of tacrolimus was found to be T cell specific, because it did not inhibit B cells, natural killer cells or various bone marrow-derived cell lines [18]. It also inhibits the production of several proinflammatory cytokines such as IL-3, IL-4, IL-5, IFN- γ , tumour necrosis factor- α and granulocyte/macrophage colony-stimulating factor [19]. In NC/Nga mice, tacrolimus inhibited the spontaneous dermatitis and was effective against established dermatitis by suppressing T cells, eosinophils, mast cells, IL-4, IL-5 and IgE [20, 21]. In a study on patients with AD, concomitant treatment with tacrolimus and another immunosuppressive agent was proven superior to monotherapy with either of the agents in improving overall dermatological scores [22].

Current treatments for severe AD are not always effective, and therefore, alternative therapies that are more effective need to be identified. The present study investigated the therapeutic potential of glucosamine and tacrolimus 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 [23], and determined its underlying therapeutic mechanisms.

Materials and methods

Animals. Eight-week-old male NC/Nga mice purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) were included in the study. The mice were maintained under uncontrolled conventional air conditions in the Laboratory Animal Facilities at the Dongguk University School of Medicine. The animal care and use com-

mittee of the research institute at Dongguk University Hospital approved all described studies.

Drugs and reagents. Glucosamine was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Tacrolimus (FK-506) was kindly provided by Chong Kun Dang pharma Inc. (Seoul, Korea). Df body ointment was prepared by Biostir Inc. (Kobe, Japan), and 1 g of Df body ointment contained 136.4 mg protein, 234 μ g Der f 1 and 7 μ g Der f 2.

Induction of AD in NC/Nga mice. Induction of AD using Df body ointment was performed as described previously [24]. The hair on the back 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 sulphate treatment on the shaved dorsal skin and both surfaces of each ear 3 h before the Df body ointment (100 mg/mouse) application. These procedures were repeated twice a week for 4 weeks.

Scoring of skin lesion. The extent of (1) erythema/haemorrhage, (2) scarring/dryness, (3) oedema and (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 individual scores [25].

The administration of drugs on Df-induced NC/Nga mice. Mice with a clinical score of 8 were used for treatment with drug alone or in combination. 'The dosages of glucosamine used in this study are the minimal concentration having significant effect in the previous dose-response prophylactic experiment' [16]. Also, it has been reported that tacrolimus at a daily dose of 1.0 mg/kg significantly showed prophylactic effects of atopic dermatitis in NC mice [26]. Accordingly, glucosamine and tacrolimus were selected for further study in inhibitory effects of combination therapy on AD by *in vivo* experiment using Df-induced dermatitis in NC/Nga mice. Glucosamine was administered to the mice orally once a day for 3 weeks by gavage (oral zoned needle) in water, either alone or in combination with tacrolimus. There was a sham gavage in the control group. Each group consisted of five mice.

Assay of serum IgE concentration. Mice serum was collected at 1 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.

Assay of cytokine and chemokine production. One week after the final administration, single-cell suspension was prepared from the spleen and incubated with 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St Louis, MO, USA) and 1 μ M 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-5, IL-13,

IFN- γ , CCL17/TARC and eotaxin were determined using the ELISA kit (R&D System, Minneapolis, MN, USA).

Histological analyses. Formalin-fixed and paraffin-embedded back skin samples from mice were sliced and then stained with toluidine blue and Congo red for counting mast cells and eosinophils, respectively. Cell density was expressed as the number of the cells in five high-power fields ($\times 400$) for each section.

Immunohistochemistry. For immunofluorescence staining (IHC) of CD3⁺ T cells or CLA, the skin samples from each mouse groups were embedded in paraffin wax and 5- μ m sections were obtained. After deparaffinization and rehydration, the sections were boiled in a 100 mM citrate buffer (pH 6.0) for 5 min on a hot plate. The sections were preincubated with 3% bovine serum albumin for 1 h at room temperature and then reacted sequentially with 1:100 anti-CD3 antibodies (rabbit polyclonal; Abcam, Cambridge, UK) or anti-CLA antibodies (rat monoclonal; Novus Biologicals, Littleton, CO, USA) for overnight at 4 °C and Alexa Fluor-labelled goat anti-rabbit IgG (594; Invitrogen, Eugene, OR, USA) for CD3 staining or Alexa Fluor-labelled goat anti-rat IgG and IgM (488; Invitrogen) for CLA staining for 1 h at room temperature. The nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). The stained specimens were evaluated using an image analysis system (Dp Manager 2.1; Olympus Optical Co., Tokyo, Japan).

Statistical analysis. The statistical analysis of the experimental data was performed by the Student's *t*-test. The results are expressed as mean $\pm$ SD. The *P*-value < 0.05 was considered significant.

Results

Therapeutic effect of combination therapy in Df-induced AD-like skin lesions in the NC/Nga mice

To examine whether the combination therapy with glucosamine plus tacrolimus (FK-506) on Df-induced AD-like skin lesions in the NC/Nga mice has synergistic therapeutic effects, mice with a clinical score of 8 were used for treatment with glucosamine (500 mg/kg) and/or tacrolimus (FK-506; 1.0 mg/kg) once a day for 3 weeks. The clinical skin score was calculated by the sum of the individual scores based on the symptoms of erythema/haemorrhage, scarring/dryness, oedema and excoriation/erosion. These symptom severity scores in the combination groups of glucosamine plus tacrolimus (FK-506) were significantly ameliorated or resolved than those in the group of glucosamine alone or tacrolimus (FK-506) alone (Fig. 1A). There was no significant difference between the glucosamine alone or tacrolimus (FK-506) alone and the control group (Fig. 1A). Representative clinical features of NC/Nga mice are shown in Fig. 1B.

Therapeutic effect of combination therapy on skin infiltration of mast cells and eosinophils

The Th2 cytokine induces proliferation and activation of mast cells and eosinophils with skin inflammation [5]. To investigate whether combination therapy 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. 2A,B, the number of infiltrated cells, both mast cells and eosinophils, was significantly reduced in the combination groups of glucosamine plus tacrolimus (FK-506), compared to the glucosamine alone or tacrolimus (FK-506) alone group (*P* = 0.003 and *P* = 0.002, respectively) and the control mice (*P* = 0.001). In addition, there was no significant difference between the combination group and normal (no dermatitis) group.

Inhibitory effect of combination therapy on Df-induced serum IgE levels in NC/Nga mice

A majority of the symptoms associated with AD manifest because of strong polarization of Th2 immune responses [5], resulting in the hyperproduction of IgE. Therefore, 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 glucosamine plus tacrolimus (FK-506) compared to the glucosamine alone or tacrolimus (FK-506) alone (*P* = 0.002 and *P* = 0.003, respectively). There was no significant difference between the glucosamine alone or tacrolimus (FK-506) alone and the control group (Fig. 3).

Inhibitory effect of combination therapy on the production of Th2-mediated cytokine and chemokines

To examine the effects of combination therapy using glucosamine plus tacrolimus (FK-506) on Th2 cytokine and chemokine production in Df-induced NC/Nga mice, ELISA targeting of IL-5, IL-13, eotaxin and TARC was performed using spleen cells. As shown in Fig. 3, the expression levels of IL-5 (Fig. 4A), IL-13 (Fig. 4B), TARC (Fig. 4C) and eotaxin (Fig. 4D) showed marked inhibition in the combination groups of glucosamine plus tacrolimus (FK-506), compared to the glucosamine alone or tacrolimus (FK-506) alone group (*P* = 0.008 and *P* = 0.011, respectively) and the control group (*P* = 0.001). No difference, however, was observed in IFN- γ production among all four groups (data not shown).

Immunohistological staining of CLA⁺ cells and CD3⁺ T cells

Cutaneous lymphocyte antigen is highly expressed on skin-infiltrating T cells in inflammatory skin diseases,

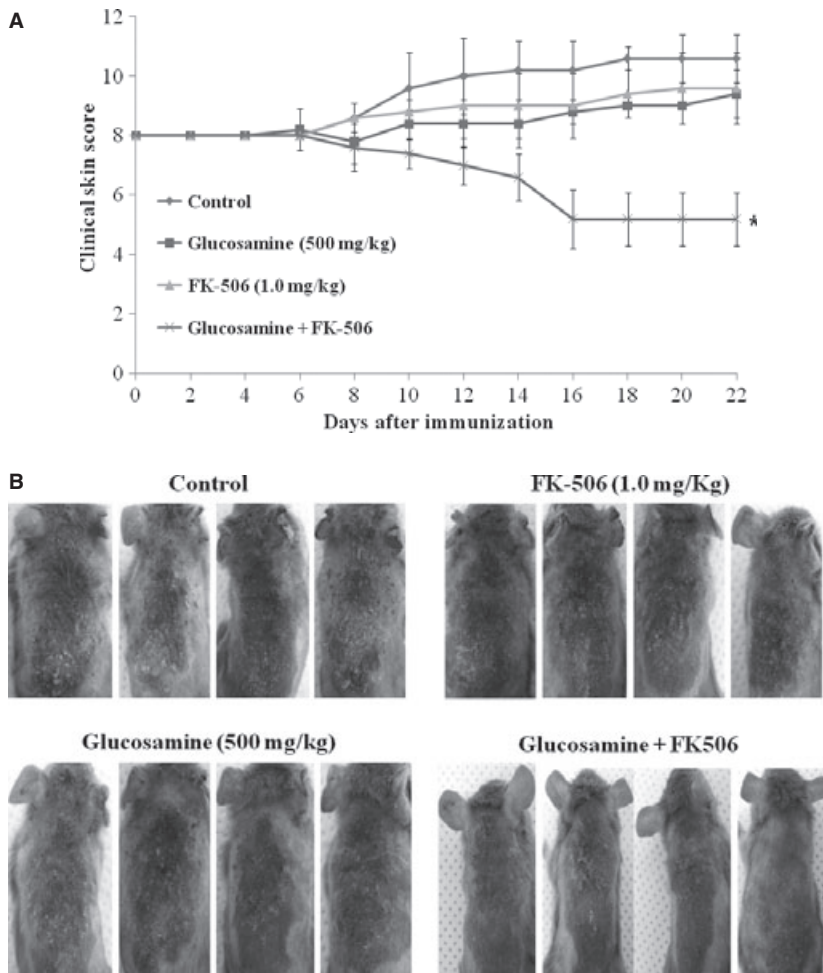


Figure 1 Therapeutic effect of combination therapy on Df-induced Atopic dermatitis (AD)-like skin lesions in NC/Nga mice. (A) After the removal of hair, Df body ointment (100 mg/mouse) was swabbed onto the dorsal skin and both surfaces of each ear twice a week for 4 weeks. The dermatitis scores of NC/Nga mice increased by the repeated application of Df body ointment and became remarkable (approximately 1–2) 2 weeks after the first elicitation. The clinical skin score is the sum of individual scores judged by the symptoms of erythema/haemorrhage, scarring/dryness, oedema and excoriation/erosion. Df-induced AD-like skin lesions in NC/Nga mice were untreated (none) or treated with glucosamine (500 mg/kg), tacrolimus (FK-506; 1.0 mg/kg), or glucosamine plus tacrolimus (FK-506) orally once a day for 3 weeks. Each datum represents the mean $\pm$ SD of five 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 (none) or treated with glucosamine (500 mg/kg), tacrolimus (FK-506; 1.0 mg/kg), or glucosamine plus tacrolimus (FK-506). The data shown are representative of two independent experiments.

including allergic contact dermatitis and atopic dermatitis [27]. The expressions of peripheral blood CD3⁺ CLA⁺ T cells were significantly increased in children with AD compared with those in control subjects [28]. We found that the infiltration of Df-induced CLA⁺ and CD3⁺ T cells (coloured green with cell surface) in NC/Nga mice was inhibited by combination therapy of glucosamine plus tacrolimus (FK-506) (Fig. 5A,B). In addition, there was no significant difference between the combination group and normal (no dermatitis) group.

Discussion

Atopic dermatitis has been treated by the regular use of corticosteroids, which is not a perfect treatment because sufficient results cannot be provided in a number of cases as a result of adverse events such as steroid-induced skin atrophy. Therefore, identified combinations of immunosuppressive agents are expected to be among the important future strategies for improved treatment of AD. It has been reported that the use of combinations of immunosuppressive agents may be more effective than single-

modality treatment with either agent. In this study, we found that combination therapy with immunosuppressive agent glucosamine plus tacrolimus (FK-506) has a synergistic effect on Df-induced atopic dermatitis-like skin lesions in NC/Nga mice. For instance, combination treatment with glucosamine plus tacrolimus (FK-506) improved the severity of the dermatitis with reduction in inflammatory cellular infiltrate, such as mast cells and eosinophils. For each parameter, we have repeated the experiment once using the same number of animals per group and found a similar type of profile, indicating that the results are reproducible (data not shown). These results indicated that combination therapy suppressed the development of Df-induced dermatitis, probably by controlling various inflammatory cells including mast cells and eosinophils. Because Th2 cytokines induce proliferation and activation of mast cells as well as eosinophils in the skin, massive infiltration of mast cells and eosinophils would be expected in Df-induced NC/Nga mice, as previously reported [24].

Th2 cytokines are considered to play a major role in the pathogenesis of AD [5]. In fact, Th2 immune

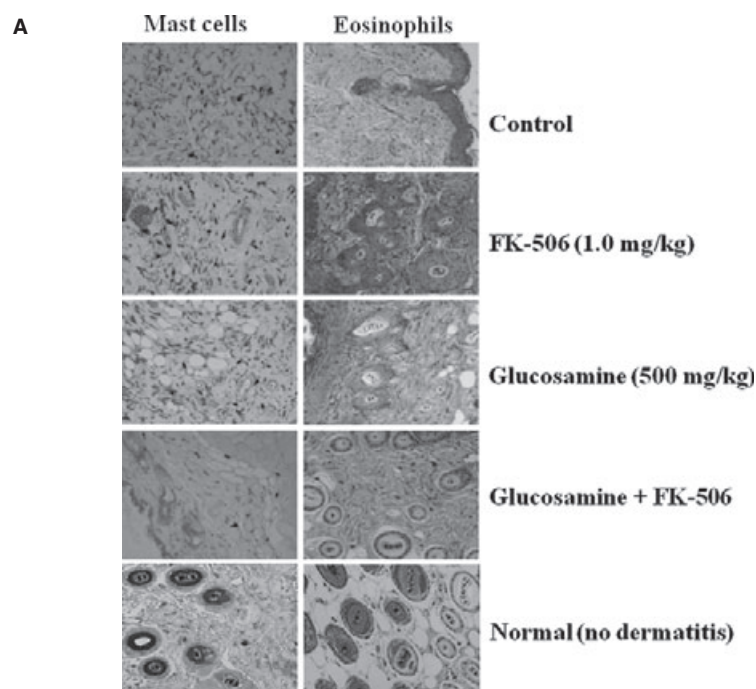


Figure 2 Histological analysis of the skin lesions. Skin samples from the back of the mice were taken at the end of the experiment (1 week after the final administration). The sections were then stained with toluidine blue and Congo red for mast cells and eosinophils, respectively. (A) Representatives of histological features in the Df-induced NC/Nga mice from the untreated (none) or treated with glucosamine (500 mg/kg), tacrolimus (FK-506; 1.0 mg/kg), or glucosamine plus tacrolimus (FK-506). (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 $\pm$ SD of five mice. * $P < 0.005$ when compared with untreated control mice (Student's *t*-test). The data shown are representative of two independent experiments.

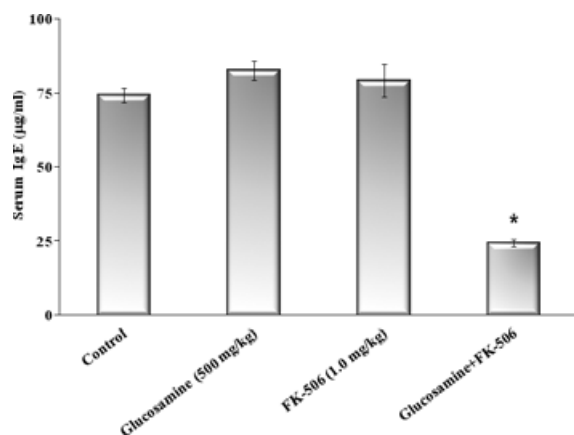
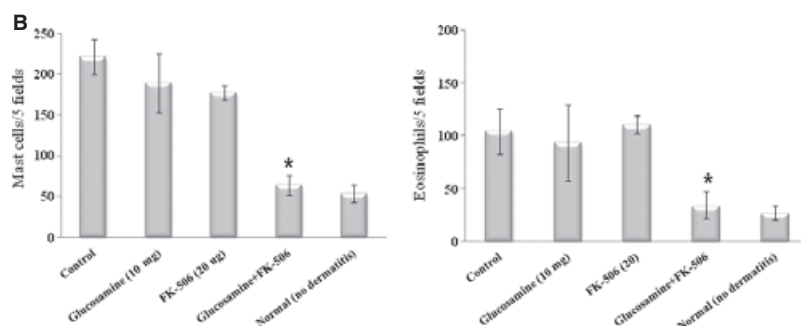


Figure 3 Serum level of IgE by combination therapy on the Df-induced Atopic dermatitis-like skin lesions in NC/Nga mice. The concentration of IgE in the serum was measured at 1 week after the final administration. Each datum represents the mean $\pm$ SD of five mice. *Statistically significant at $P < 0.05$ using a Student's *t*-test compared with all other groups. The data shown are representative of two independent experiments.

responses mediated by IL-4, IL-5 and IL-13 are critical in the pathogenesis of AD [9], because the upregulation of IgE production, one of the major causes of atopic inflammation, has been extensively studied with Th2 cytokines, IL-5 and IL-13. Moreover, Th2 cell numbers are increased in lesional tissue of patients who suffer from patients with AD frequently show elevated IgE levels in response to many kinds of allergens, including mite antigen [29]. The NC/Nga mouse model also displays Th2-dominant responses, with overproduction of Th2 cytokines (IL-4, IL-5 and IL-13) and IgE antibody [23, 30]. We have already shown that glucosamine downregulates the overproduction of IgE and Th2 cytokines in an NC/Nga mice model of Df-induced AD, a major Th2-dominant disease [16]. In addition, Th2-specific chemokines, TARC and eotaxin, have been reported to be highly expressed in the NC/Nga mice [30]. A previous report showed that tacrolimus (FK-506) markedly inhibited Df-induced expression of TARC and eotaxin [31]. The present study of immune responses clearly shows that IgE, Th2 cytokine (IL-5 and IL-13) and Th2

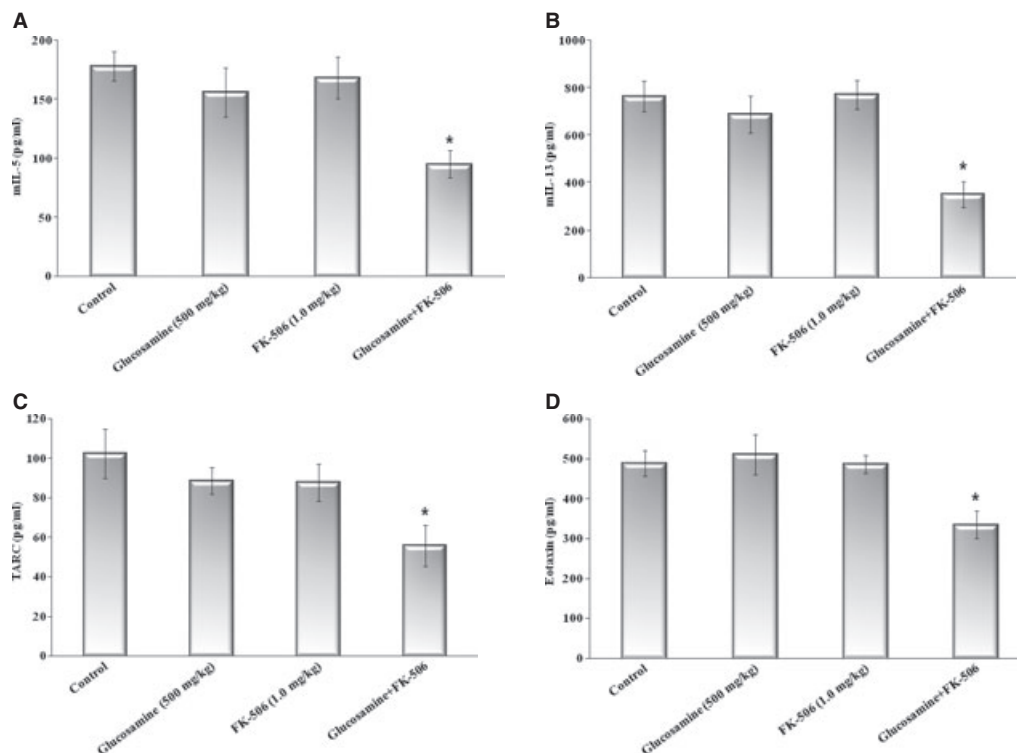


Figure 4 Cytokine and chemokine production by combination therapy on Df-induced Atopic dermatitis-like skin lesions in NC/Nga mice. Spleen cells were obtained from mice that had been left untreated (none) or treated with glucosamine (500 mg/kg), tacrolimus (FK-506; 1.0 mg/kg), or glucosamine plus tacrolimus (FK-506). These cells were stimulated with PMA and ionomycin. ELISA targeting IL-5 (A), IL-13 (B), TARC (C) and eotaxin (D) was performed using supernatants obtained 24 h after the stimulation. Each datum represents the mean $\pm$ SD of five 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.

chemokines (TARC and eotaxin) in combination treatment with glucosamine plus tacrolimus (FK-506) were significantly lower than in the single-modality treatment with either alone. These results suggest that the improvement in clinical symptoms by combination treatment of glucosamine plus tacrolimus (FK-506) against therapeutic effects of Df-induced NC/Nga mice might be mediated, at least in part, by its inhibitory effect on IgE, Th2-mediated cytokine and chemokines. In fact, the correlation between the elevation of serum levels of total IgE, the production of Th2 cytokine and chemokines has been reported [30, 32].

In this study, immunohistochemical analysis showed that treatment with glucosamine plus tacrolimus (FK-506) led to a higher decrease in the CD3⁺ T and CLA⁺ cell numbers compared to controls. Skin-homing T cells expressing CLA are important in the pathogenesis of AD [27]. In patients with AD, there is a significant increase in the number of circulating CLA⁺ cells, which have an augmented capability to produce IL-4 and IL-13 compared to the cells from non-affected individuals [28]. It has been reported that cyclosporine treatment significantly reduced the percentages of CD3⁺ T cells and CLA⁺ cells in children with severe AD [33]. These results imply that CD3⁺ T cells and CLA⁺ cells may be impor-

tant in the pathogenesis of AD and in the mechanism of action of this combination treatment.

Current studies suggest that a single type of immunosuppressive therapy may be able to deal with all facets in the treatment of AD. However, a rational combination of synergistic therapy could provide a successful clinical approach to AD. An important finding in this study showed synergistic efficacy of combination therapy with glucosamine plus tacrolimus (FK-506) in Df-induced NC/Nga mice.

In conclusion, our findings indicated that this combined immunosuppressive therapy was more efficacious than monotherapy in reducing IgE, Th2 cytokine levels and Th2 chemokine expression and in inhibiting inflammatory cells and CLA⁺ cell infiltration, and these findings correlated with the observed clinical symptoms. These findings have important implications for the design of therapeutic strategies aimed at AD treatment.

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0004049). This study was

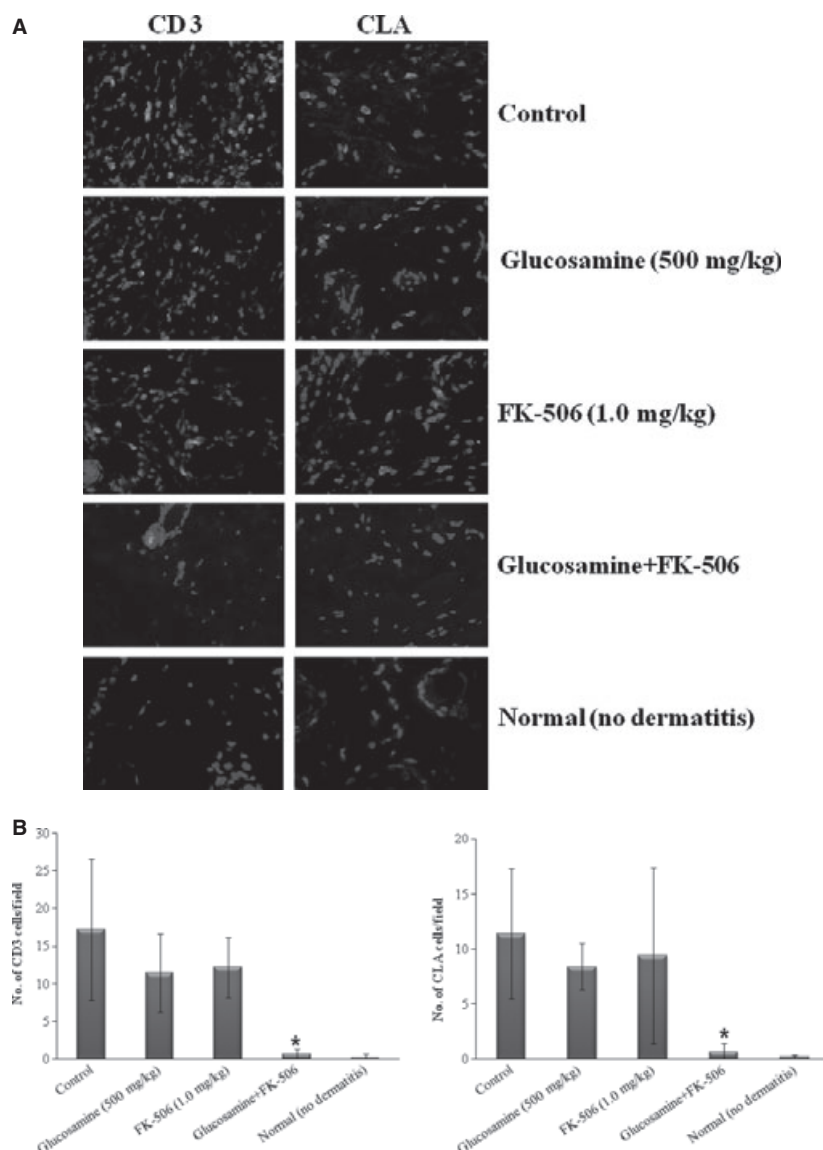


Figure 5 Immunohistological staining of CD3⁺ T cells and CLA⁺ cells. Skin samples from the back of the mice were taken at the end of the experiment (1 week after the final administration). The sections were then stained with anti-CD3 and anti-CLA as described in Materials and methods. (A) Representatives of histological features in the Df-induced NC/Nga mice from the untreated (none) or treated with glucosamine (500 mg/kg), tacrolimus (FK-506; 1.0 mg/kg), or glucosamine plus tacrolimus (FK-506). (B) The cell density was expressed as the number of CD3⁺ T cells and CLA⁺ cells (coloured green with cell surface) in five high-power fields (×400 for count) for each section. The data represents the mean ± SD of five mice. **P* < 0.05 when compared with untreated control mice (Student's *t*-test).

supported by a grant from the Korea Health 21 R&D Project by Ministry of Health and Welfare (A010251).

Conflicts of interest

None declared.

References

- Hoare C, Li Wan Po A, Williams H. Systematic review of treatments for atopic eczema. *Health Technol Assess* 2000;4:1–191.
- Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003;361:151–60.
- Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. *J Clin Invest* 2004;113:651–7.
- Wollenberg A, Kraft S, Oppel T, Bieber T. Atopic dermatitis: pathogenetic mechanisms. *Clin Exp Dermatol* 2000;25:530–4.
- Grewe M, Bruijnzeel-Koomen CA, Schöpf E. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 1998;19:359–61.
- Uehara M, Izukura R, Sawai T. Blood eosinophilia in atopic dermatitis. *Clin Exp Dermatol* 1990;15:264–6.
- Cooper KD. Atopic dermatitis: recent trends in pathogenesis and therapy. *J Invest Dermatol* 1994;102:128–37.
- Beltrani VS. Suggestions regarding a more appropriate understanding of atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2005;5:413–8.
- Leung DY, Soter NA. Cellular and immunologic mechanisms in atopic dermatitis. *J Am Acad Dermatol* 2001;44:S1–12.
- Sasakawa T, Higashi Y, Sakuma S *et al.* Atopic dermatitis-like skin lesions induced by topical application of mite antigens in NC/Nga mice. *Int Arch Allergy Immunol* 2001;126:239–47.
- Esche C, de Benedetto A, Beck LA. Keratinocytes in atopic dermatitis: inflammatory signals. *Curr Allergy Asthma Rep* 2004;4:276–84.
- Towheed TE, Anastassiades T. Glucosamine therapy for osteoarthritis: an update. *J Rheumatol* 2007;34:1787–90.
- Reginster JY, Deroisy R, Rovati LC *et al.* Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. *Lancet* 2001;357:251–6.

- 14 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.
- 15 Ma L, Rudert WA, Harnaha J *et al.* Immunosuppressive effects of glucosamine. *J Biol Chem* 2002;277:39343–9.
- 16 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.
- 17 Rustin MH. The safety of tacrolimus ointment for the treatment of atopic dermatitis: a review. *Br J Dermatol* 2007;157:861–73.
- 18 Gummert JF, Ikonen T, Morris RE. Newer immunosuppressive drugs: a review. *J Am Soc Nephrol* 1999;10:1366–80.
- 19 Wollenberg A, Sharma S, von Bubnoff D, Geiger E, Haberstok J, Bieber T. Topical tacrolimus (FK506) leads to profound phenotypic and functional alterations of epidermal antigen-presenting dendritic cells in atopic dermatitis. *J Allergy Clin Immunol* 2001;107:519–25.
- 20 Hiroi J, Sengoku T, Morita K *et al.* Effect of tacrolimus hydrate (FK506) ointment on spontaneous dermatitis in NC/Nga mice. *Jpn J Pharmacol* 1998;76:175–83.
- 21 Sasakawa T, Higashi Y, Sakuma S *et al.* Topical application of FK506 (tacrolimus) ointment inhibits mite antigen-induced dermatitis by local action in NC/Nga mice. *Int Arch Allergy Immunol* 2004;133:55–63.
- 22 Torok HM, Maas-Irslinger R, Slayton RM. Clocortolone pivalate cream 0.1% used concomitantly with tacrolimus ointment 0.1% in atopic dermatitis. *Cutis* 2003;72:161–6.
- 23 Jin H, He R, Oyoshi M, Geha RS. Animal models of atopic dermatitis. *J Invest Dermatol* 2009;129:31–40.
- 24 Yamamoto M, Haruna T, Yasui K *et al.* A novel atopic dermatitis model induced by topical application with dermatophagoides farinae extract in NC/Nga mice. *Allergol Int* 2007;56:139–48.
- 25 Suto H, Matsuda H, Mitsuishi K *et al.* NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol* 1999;120:70–5.
- 26 Maekawa T, Nojima H, Kuraishi Y, Aisaka K. The cannabinoid CB2 receptor inverse agonist JTE-907 suppresses spontaneous itch-associated responses of NC mice, a model of atopic dermatitis. *Eur J Pharmacol* 2006;542:179–83.
- 27 Santamaria-Babi LF. Skin-homing T cells in cutaneous allergic inflammation. *Chem Immunol Allergy* 2006;91:87–97.
- 28 Teraki Y, Hotta T, Shiohara T. Increased circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)+ type 2 cytokine-producing cells, and decreased CLA+ type 1 cytokine-producing cells in atopic dermatitis. *Br J Dermatol* 2000;143:373–8.
- 29 Van Bever HP. Recent advances in the pathogenesis of atopic dermatitis. *Eur J Pediatr* 1992;151:870–3.
- 30 Vestergaard C, Yoneyama H, Murai M *et al.* Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Invest* 1999;104:1097–105.
- 31 Oshio T, Sasaki Y, Funakoshi-Tago M *et al.* Dermatophagoides farinae extract induces severe atopic dermatitis in NC/Nga mice, which is effectively suppressed by the administration of tacrolimus ointment. *Int Immunopharmacol* 2009;9:403–11.
- 32 Matsuda H, Watanabe N, Geba GP *et al.* Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997;9:461–6.
- 33 Lee SY, Shim JY, Kim JH *et al.* Cyclosporine treatment decreases the percentage of cutaneous lymphocyte antigen (CLA)(+) CD4(+) T cells in children with severe atopic dermatitis. *Allergy* 2004;59:1129–30.