



## A Probiotic Preparation Alleviates Atopic Dermatitis-Like Skin Lesions in Murine Models

Min-Soo Kim<sup>†</sup>, Jin-Eung Kim<sup>†</sup>, Yeo-Sang Yoon, Jae-Gu Seo, Myung-Jun Chung and Do-Young Yum

R&D Center, Cell Biotech Co., Ltd., Gimpo, Korea

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Atopic dermatitis (AD) is a chronic inflammatory skin disease with a complex etiology that encompasses immunologic responses. AD is frequently associated with elevated immunoglobulin (Ig) E levels, and common environmental factors contribute to its pathogenesis. Several recent studies have documented the role of specific lactic acid bacteria in the treatment and prevention of AD in humans and mice. In this study, the efficacy of Duolac ATP, a probiotic preparation, was determined in a mouse model with AD-like skin lesions. Alterations in the cytokine levels and histological staining suggested the alleviation of AD. The *in vivo* test showed that T helper (Th)2 cytokines, IgE, interleukin (IL)-4, and IL-5, were significantly downregulated, whereas Th1 cytokines, IL-12p40 and interferon (IFN)- $\gamma$ , were upregulated in all groups of mice treated with Duolac ATP compared to that observed in the group of mice treated with 1-chloro-2,4-dinitrobenzene (DNCB) alone. Moreover, the scratch score decreased in all mice treated with Duolac ATP. Staining of the dorsal area of the mice in each group with hematoxylin and eosin and toluidine blue further confirmed the alleviation of AD in mice orally treated with Duolac ATP. These results suggest that Duolac ATP inhibits the development of AD-like skin lesions in NC/Nga mice by suppressing the Th2 cell response and increasing the Th1 cell response. Thus, Duolac ATP is beneficial and effective for the treatment of AD-like skin lesions.

**Key words:** Atopic dermatitis, Duolac ATP, Immunoglobulin E, NC/Nga mouse

### INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease, characterized by pruritic and eczematous skin lesions (1). It is a multifactorial condition that involves genetic and environmental components such as infectious agents, food allergens, and aeroallergens (2). The early onset of AD in infancy is recognized as the most prevalent chronic skin disorder in childhood, which may persist or aggravate throughout the life of the affected individual, causing serious problems in adulthood (1). Recently, the prevalence of AD in infants and young children is increas-

ing rapidly worldwide (3); it affects up to 20% of children and 1~3% of adults globally (4). The majority of patients with AD show elevated serum immunoglobulin E (IgE) levels against specific environmental allergens or microbial proteins (5). Pruritus, a sensation that urges the affected individuals to scratch, is an important clinical feature of AD; it destroys the skin barrier and aggravates dermatitis, resulting in typical skin lesions (5). However, the exact pathophysiology of AD has not yet been determined. Till date, AD has been known to be a multifactorial disease associated with genetic susceptibility; immune response to increased antigens, as indicated by the impaired skin and the gut barrier function; and a certain lifestyle (e.g., dietary habits) and environment (6). It is difficult to treat AD, and it is necessary to manage some of its modifiable risk factors and modulate the immune reactions elicited by it.

Allergic diseases such as rhinitis, asthma, and atopic eczema produce an immune response that is dominated by the hypersensitivity of T helper (Th) 2 cells, leading to production of IgE and accumulation of eosinophils (7). A breakdown in the balance between Th1 and Th2 cytokines has been reported to be central to the pathology of AD (8). Therefore, most patients with AD have increased circulat-

Correspondence to: Do-Young Yum, R&D Center, Cell Biotech Co., Ltd., 134 Gaegok-ri, Wolgot-myeon, Gimpo-si, Gyeonggi-do 415-872, Korea  
E-mail: [dyyum@cellbiotech.com](mailto:dyyum@cellbiotech.com)

<sup>†</sup>These authors contributed equally to this study.

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ing eosinophils and IgE levels due to the elevated levels of interleukin (IL)-4, IL-5, and IL-13 produced by Th2 cells (9). Th2 immune responses are known to play an important role in the pathogenic mechanism of AD. It is believed that the immune response in AD is skewed toward a Th2 response, thus resembling a Th1 deficiency. Studies have shown that Th2 cytokines such as IL-4, IL-5, and IL-13 are overproduced, while the Th1 cytokines interferon-gamma (IFN- $\gamma$ ) and IL-12 are underproduced in patients with AD as compared to non-AD control subjects (10). Thus, the imbalance between Th1 and Th2 immune responses plays an important role in the development of AD (11).

Until recently, steroid therapy was widely used in the treatment of AD. However, this treatment was associated with severe side effects, including atrophy, acne, cataracts, growth retardation, and red burning skin. Thus, the long-term use of this therapy is prohibited (6,12,13). More importantly, steroid treatment has an overall immunosuppressive effect, which is mediated by a reduction in cytokine production by both Th2 and Th1 cells (14). Despite their severe side effects, steroid-based drugs are used to treat dermatological disorders in over 80% of the world's population (15). To circumvent the toxicity of steroid-based drugs, several researchers have conducted clinical trials of complementary or alternative medicines. However, the efficacy and safety as well as the therapeutic mechanisms of these medicines remain unclear (16,17).

Lactic acid bacteria (LAB) are non-invasive, non-pathogenic, gram-positive commensal bacteria, which are recognized for their health-promoting effects in humans (11). *Lactobacillus* and *Bifidobacterium* strains can be found in many dairy products and have probiotic properties, which confer a number of health benefits by regulating the immune system of the host (18). Recent reports have documented the role of strain-specific LAB in the prevention of allergic disorders in mice and humans. Moreover, some LAB strains such as *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Bifidobacterium lactis* are effective in the treatment of AD in mice and humans (14,17). However, to date, these results have not been obtained consistently (16). It is also reported that the effectiveness of this treatment may vary with the type of LAB strain used (6).

NC/Nga mice originated from Japanese fancy mice at Nagoya University, Japan in 1957. These mice develop AD-like skin lesions and show overproduction of IgE under conventional conditions with itching, erythema, and hemorrhage, followed by edematous superficial erosion, deep excoriation, scaling, dryness of the skin, and retarded growth (19). These pathophysiological observations of AD in NC/Nga mice highly resemble those observed in AD in humans. This mouse strain is therefore considered a useful animal model for the evaluation of pathologic mechanisms of AD in humans (20).

In this study, we evaluated the effect of Duolac ATP, a

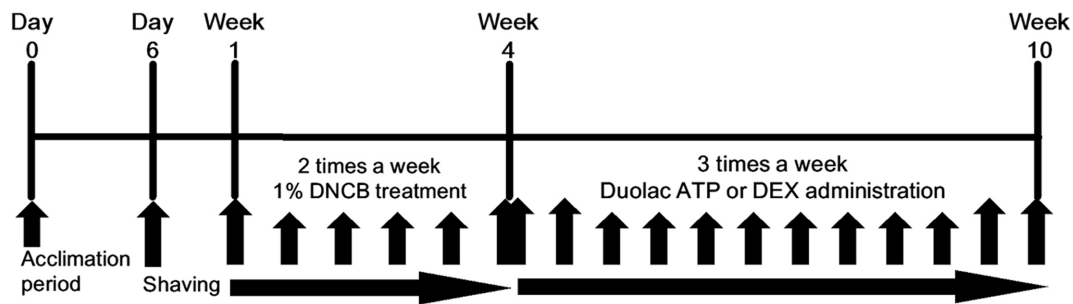
probiotic preparation containing four LAB strains, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Bifidobacterium lactis*, on AD-like skin lesions in NC/Nga mice. Duolac ATP has been previously evaluated for anti-inflammatory activity *in vitro* and *in vivo*, where treatment with the formulation significantly reduced the lipopolysaccharide (LPS)-induced NO production from RAW 264.7 cells and suppressed inflammatory responses in a trinitrobenzene sulfonic (TNBS)-induced colitis model (21). We observed that Duolac ATP selectively upregulated the levels of Th1 cytokines, IL-12p40 and IFN- $\gamma$ . In contrast, the levels of IgE and Th2 cytokines, IL-4 and IL-5, were reduced by orally treatment with Duolac ATP *in vivo*. Furthermore, the histological examination of the dorsal area of the mice treated with Duolac ATP revealed an improvement in AD. These results suggest that Duolac ATP possesses preventive potential against AD and might serve as an effective immunomodulatory agent for patients with AD.

## MATERIALS AND METHODS

**Preparation of LAB.** Duolac ATP is a probiotic formulation that includes *Lactobacillus casei* CBT LC5 (KCTC 12398BP), *Lactobacillus plantarum* CBT LP3 (KCTC 10782BP), *Lactobacillus rhamnosus* CBT LR5 (KCTC 12202BP), and *Bifidobacterium lactis* CBT BL3 (KCTC 11904BP). Duolac ATP was supplied by Cell Biotech Co., Ltd., Gimpo, Korea. LABs were cultured in MRS broth (Difco, NJ, USA) at 37°C for 18~24 hr.

**Animals.** Six-week-old male NC/Nga mice were purchased from the Shizuoka Laboratory Animal Center, Tokyo, Japan. The mice were randomized and housed in stainless steel cages in a controlled environment with a temperature of  $22 \pm 2^\circ\text{C}$  and humidity of 40~60% under a 12 : 12-hr light-dark cycle. They were fed standard laboratory food and water prior to the experiments. All the experimental procedures were carried out in accordance with The Animal Use and Care Protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Cell Biotech Co., Ltd., Gimpo, Korea.

**Murine AD model.** Six-week-old NC/Nga mice were maintained in an air-conditioned room and allowed to adapt to the environment for at least 1 week prior to use. To induce AD-like symptoms in the mice, 1-chloro-2,4-dinitrobenzene (DNCB) (Sigma-Aldrich, MO, USA) was used (22). Briefly, the dorsal hair of the mice was removed using an electronic clipper 2 days before DNCB treatment. DNCB was prepared at a concentration of 1% in acetone-olive oil (3 : 1), after which it was applied to the dorsal skin at the indicated times over a course of 4 weeks (Fig. 1). The mice were divided into the following six treatment groups ( $n = 8$  per group):  $1 \times 10^7$  CFU/day of Duolac ATP,  $1 \times 10^8$  CFU/



**Fig. 1.** DNCB-induced mouse model of atopic dermatitis (AD)-like skin lesions and oral administration of Duolac ATP in NC/Nga mice. The dorsal skin of mice was treated with 1% DNCB six times over 3 weeks to induce AD-like skin lesions. Duolac ATP was administered following the last treatment with DNCB at week 4. The experimental groups were as follows: Duolac ATP-L,  $1 \times 10^7$  CFU/day; Duolac ATP-M,  $1 \times 10^8$  CFU/day; Duolac ATP-H,  $1 \times 10^9$  CFU/day; 2.5 mg/kg/day of dexamethasone (DEX); and 200  $\mu$ L/day of DW. At week 10, the mice were sacrificed and samples were collected for immunological analyses and dorsal skin histological analysis.

day of Duolac ATP,  $1 \times 10^9$  CFU/day of Duolac ATP, 2.5 mg/kg/day of dexamethasone (DEX), and 200 mL/day of distilled water (DW) once a day, orally administered from 4 to 10 weeks of age (Fig. 1). As a negative control (NC) for AD, NC/Nga mice not treated with DNCB were maintained under specific-pathogen-free conditions and then sacrificed for examination.

#### **Evaluation of the severity of the AD-like skin lesions.**

The severity of dermatitis in each mouse at 10 weeks was assessed by macroscopic observation before each blood sampling, and scored according to the severity grade (23). The severity scores were expressed as a sum of the individual score and graded on a scale of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of the following five signs and symptoms: itching, erythema/hemorrhage, oedema, excoriation/erosion, and scaling/dryness.

#### **Evaluation of scratching behavior in mice with AD-like skin lesions.**

Individual mice were placed in clear plastic cages and allowed to acclimatize for 15 min. Their behavior for the next 10 min was then observed. Scratching of the nose, ears, and dorsal skin with hind paws was recorded, while licking of the belly and dorsal skin during grooming was not recorded. Scratching behavior within the treatment area was evaluated according to the scratching score. All evaluations were performed in a blinded manner. Each incidence of scratching of the head, neck, dorsal skin, ears, and nose was recorded and assigned a score to obtain the maximum score.

**Measurement of cytokine levels in serum.** The immunological response of the mice following DNCB-induced AD was monitored by measuring the serum levels of IFN- $\gamma$ , IgE, IL-4, IL-5, and IL-12 (BD Biosciences, CA, USA). Whole blood was collected from 16-week-old mice from each of the treatment groups. The serum was then collected by centrifugation for 20 min at 12,000 rpm and stored at

$-80^\circ\text{C}$ , until further use. The cytokine levels were determined by ELISA by using detection kits for mouse cytokines. Briefly, monoclonal anti-mouse antibodies diluted in phosphate buffered saline (PBS; 1 : 250) were placed in an Immunoplate (Nunc, Roskilde, Denmark) and incubated at  $4^\circ\text{C}$  overnight. After washing three times with washing buffer (PBS containing 0.05% Tween-20), 200  $\mu$ L of PBS containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, UT, USA) was added to each well. The samples were then incubated for 1 hr at room temperature (RT), following which the plates were washed three times with the washing buffer. Serum samples (100  $\mu$ L) were then added to the wells, and the plates were incubated for 2 hr at RT. The samples were first diluted with PBS containing 10% FBS for each type of cytokine. The plates were then washed three times, and 100  $\mu$ L of streptavidin-horseradish peroxidase-conjugated detection antibody (SAv-HRP, BD Biosciences, CA, USA) was added. The plates were then incubated for 1 hr at RT and washed five times, and the enzymatic reaction was initiated by the addition of 100  $\mu$ L of substrate solution (0.1 M citric acid, 0.2 M  $\text{Na}_2\text{HPO}_4$ , *o*-phenylenediamine, and  $\text{H}_2\text{O}_2$ ; BD Biosciences). The reactions were allowed to proceed for 20 min at RT and then terminated by the addition of a stop solution (50  $\mu$ L of 2 N  $\text{H}_2\text{SO}_4$ ; BD Biosciences). The absorbance of each well was then immediately measured at 450 nm by using an ELISA reader (Bio-Rad Laboratories, CA, USA).

**Histological analysis.** DNCB was repeatedly applied to the backs of the mice and removed on the final day of the experiment (week 10). The dorsal skin was removed and fixed in 4% paraformaldehyde (Sigma-Aldrich). The paraffin-embedded skin sections were heat immobilized, deparaffinized by immersing in xylene (Sigma-Aldrich), rehydrated using a graded series of ethanol, and washed with DW. The dorsal skin samples were then cut into 4- $\mu$ m sections and subjected to either hematoxylin and eosin (H&E) (Sigma-Aldrich) or toluidine blue (Sigma-Aldrich) staining; they

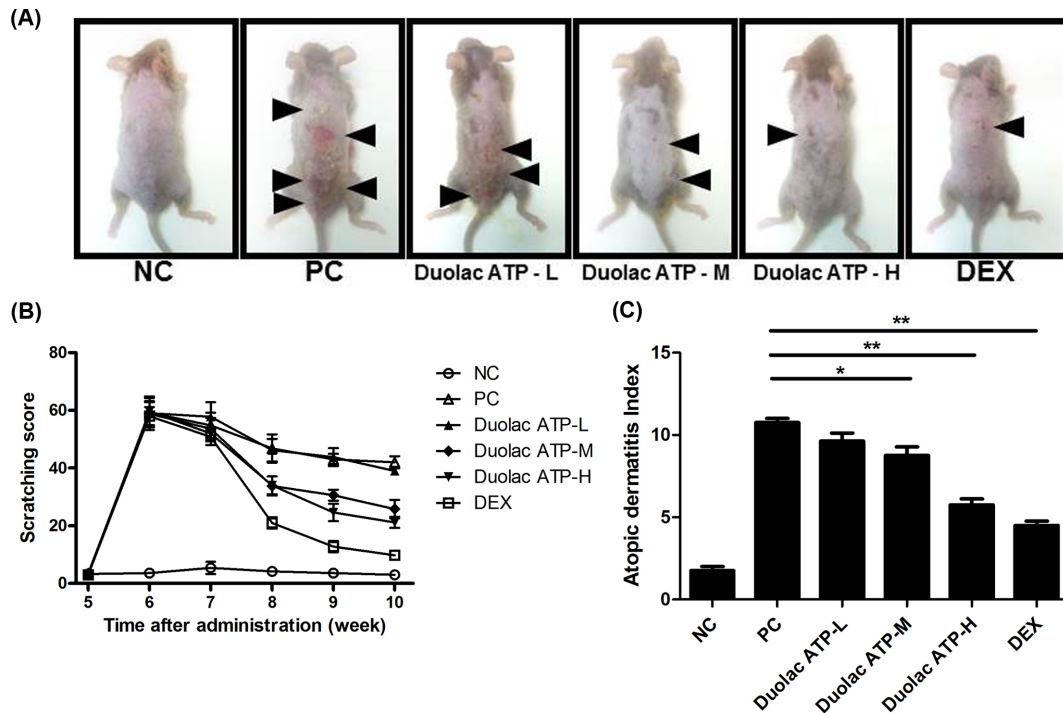
were then examined by light microscopy (Leica Microsystems, Wetzlar, Germany) for histological evaluation. Mast cells in each mouse were counted in three different microscopic fields, and the number of cells per dorsal skin surface area ( $\text{n/mm}^2$ ) was calculated.

**Statistical analysis.** The data were processed using (GraphPad Software, CA, USA), and the statistical parameters, mean value, and standard deviation were calculated and compared among the groups. Statistical significance was determined using ANOVA ( $P < 0.05$ ).

## RESULTS

**Alleviation of DNCB-induced AD-like skin lesions in NC/Nga mice treated with Duolac ATP.** To assess the therapeutic potential of Duolac ATP *in vivo*, we established a mouse model for AD-like skin lesions by using NC/Nga mice. Briefly, the mice were sensitized with 1% DNCB six times over 3 weeks and simultaneously administered DW (200  $\mu\text{L/day}$ ), Duolac ATP ( $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/day, and  $1 \times 10^9$  CFU/day), or DEX (2.5 mg/kg/day) orally,

as shown in Fig. 1. After 4 weeks, DNCB treatment was stopped and administration of Duolac ATP was initiated. After the cessation of DNCB treatment, the AD-like skin lesions were observed to be markedly different in the positive control (PC, only DNCB) and Duolac ATP-treated mice. There were obvious signs of AD-like skin lesions in PC mice treated with DW, including bleeding, severe itching, and rashes. However, the AD-like skin lesions in mice treated with Duolac ATP were milder than those in the PC mice. There were no significant differences in the AD-like skin lesions among mice treated with different doses of Duolac ATP ( $1 \times 10^7$ ,  $1 \times 10^8$ , and  $1 \times 10^9$  CFU/day). The severity of the skin lesions in the control mice increased gradually with increase in the number of DNCB challenges. Oral administration of Duolac ATP for 6 weeks suppressed the development of AD-like skin lesions. The severity of the skin lesions in each group of mice at week 10 is shown in Fig. 2A. The severity in each group was more or less similar up to 4 weeks from the day of sensitization. However, after 4 weeks, a radical change was observed in the skin lesions in PC mice compared with those in the Duolac ATP-treated mice.



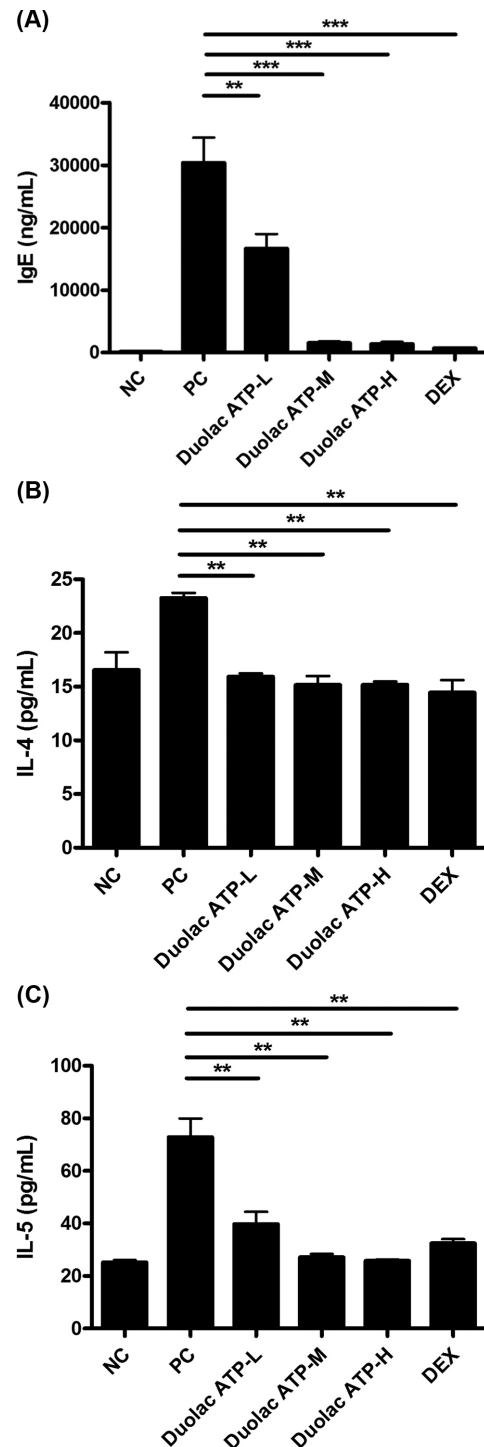
**Fig. 2.** The effect of Duolac ATP on AD. (A) DNCB induced AD-like skin lesions in NC/Nga mice. Duolac ATP demonstrated an inhibitory effect on the pathogenesis of AD-like skin lesions, when compared with that observed in the positive control (PC, only DNCB) mice. Three different doses of Duolac ATP ( $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/day, and  $1 \times 10^9$  CFU/day) and 2.5 mg/kg/day of DEX were administered after DNCB sensitization. The photographs were obtained at week 10 before the mice were sacrificed. Arrow heads indicate macroscopic features of atopic dermatitis-like skin lesions on the dorsal skin. (B) The scratching behavior of each mouse was observed for 10 min after sensitization. The graph shows the mean scratching behavior of mice treated with Duolac ATP or DEX for 6 weeks. (C) Skin severity score. The skin severity of the mice was assessed macroscopically. The dermatitis index was evaluated on week 10. The symptoms considered were itching, erythema/hemorrhage, oedema, excoriation/erosion, and scaling/dryness.

The chronological profile of the scratching behaviors observed in NC, PC, Duolac ATP-treated, and DEX-treated NC/Nga mice is shown in Fig. 2B. The number of spontaneous scratching episodes remained almost constant from week 4 to week 10 in NC mice and significantly increased in PC ( $42.0 \pm 2.1$ ) and Duolac ATP-treated mice. However, the scratching score was lower when the Duolac ATP dose was  $1 \times 10^8$  CFU/day and  $1 \times 10^9$  CFU/day ( $25.8 \pm 3.2$  and  $21.2 \pm 1.9$ , respectively) compared to that when the dose was  $1 \times 10^7$  CFU/day ( $39.0 \pm 1.0$ ). The scratching behavior in mice treated with DEX ( $9.8 \pm 1.5$ ) was almost completely inhibited during this period (Fig. 2B).

The severity of skin lesions in each group was more or less similar up to 6 weeks from the day of sensitization. However, after 6 weeks, there was radical change in the skin lesions of the PC group compared with the ATP administration groups. The skin severity score of each group is shown in Fig. 2C. To compare the skin lesions from NC mice, we utilized the clinical severity scores proposed by Leung for human AD (24). Fig. 2C shows changes in the atopic dermatitis index. In NC mice, the dermatitis score was zero from 10 weeks, but in PC mice, dermatitis appeared at 6 weeks and deteriorated with time. This can be inferred from scratching score (Fig. 2B). In ATP mice, the development of dermatitis was inhibited significantly when compared with PC mice. The effects of ATP-L, -M, -H for each dosage AD index is  $9.6 \pm 1.7$ ,  $8.8 \pm 1.5$ , and  $5.8 \pm 1.0$ , respectively. DEX showed the lowest  $4.5 \pm 0.8$ .

**Changes in serum levels of IgE and Th2 cytokines in NC/Nga mice treated with Duolac ATP.** In addition to the clinical features, IgE, IL-4, and IL-5 levels in the sera of NC/Nga mice were assessed to characterize the immunological response during disease progression because an elevated IgE level is a common indicator of AD. At week 10, the concentration of soluble serum IgE was significantly reduced in Duolac ATP-treated mice. IgE levels in mice treated with  $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/day, and  $1 \times 10^9$  CFU/day of Duolac ATP were  $16,652 \pm 430$ ,  $1,565 \pm 247$ , and  $1,393 \pm 125$  ng/mL, respectively, whereas the IgE level in PC mice was  $30,382 \pm 1,731$  ng/mL. The NC group of NC/Nga mice without AD produced much lower serum IgE at  $155 \pm 38$  ng/mL (Fig. 3A). Taken together, these results show that the increase in serum IgE correlates with the onset of AD-like skin lesions. Treatment with Duolac ATP reduces the production of IgE, which in turn inhibits the symptoms of AD-like skin lesions. Thus, Duolac ATP alleviates AD-like symptoms through the concomitant down-regulation of IgE.

As Duolac ATP treatment differentially affected the decrease of IL-4 and IL-5 in relation to Th2 responses, we examined the effect of this probiotic preparation on Th2 cytokine responses. We measured the levels of IL-4 and IL-5, which represented Th2 immune responses in sera iso-

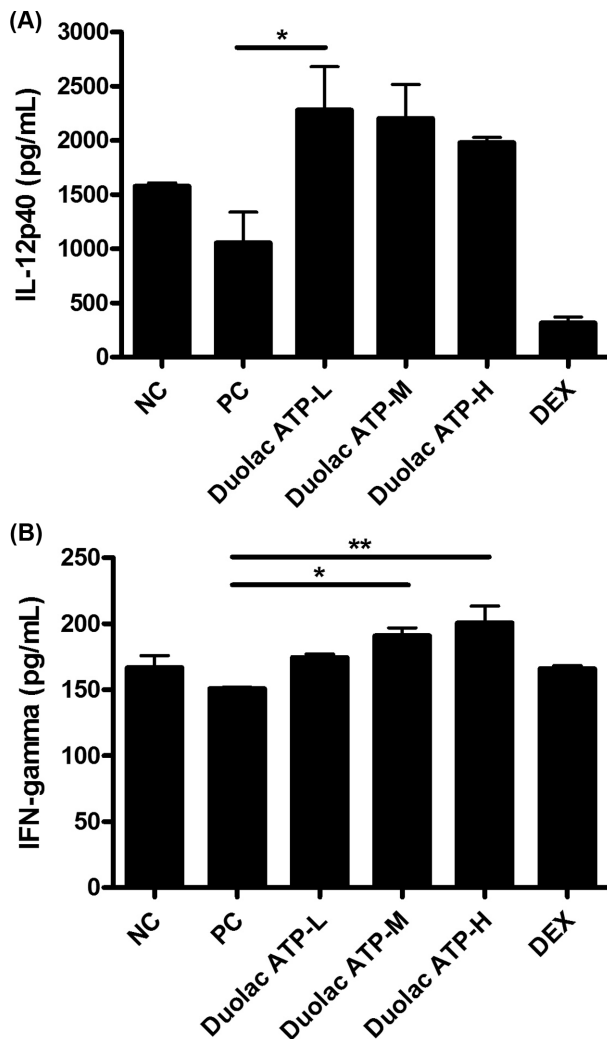


**Fig. 3.** Serum IgE and Th2 cytokine levels in DNCB-sensitized NC/Nga mice. Blood was collected from NC/Nga mice treated with Duolac ATP or DEX at week 10 for which the (A) serum IgE levels, (B) IL-4 levels, and (C) IL-5 levels were determined using ELISA. The experimental groups were as follows: Duolac ATP-L,  $1 \times 10^7$  CFU/day; Duolac ATP-M,  $1 \times 10^8$  CFU/day; Duolac ATP-H,  $1 \times 10^9$  CFU/day; and 2.5 mg/kg/day of DEX. Data represent mean  $\pm$  SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with the PC mice (ANOVA).

lated from week 10 NC/Nga mice. In the PC mice, the serum IL-4 levels of  $23.8 \pm 0.8$  pg/mL decreased to  $15.9 \pm 0.8$ ,  $15.2 \pm 2.0$ , and  $15.2 \pm 0.5$  pg/mL when treated with  $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/day, and  $1 \times 10^9$  CFU/day of Duolac ATP, respectively. These levels were similar to those observed in NC mice ( $16.6 \pm 2.3$  pg/mL) (Fig. 3B). These findings show that treatment with Duolac ATP decreased both serum IgE and IL-4 levels.

Moreover, the serum IL-5 levels of  $72.9 \pm 14.1$  pg/mL in the PC mice decreased to  $39.8 \pm 10.4$ ,  $27.1 \pm 2.8$ , and  $25.8 \pm 0.9$  pg/mL when treated with  $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/

day, and  $1 \times 10^9$  CFU/day of Duolac ATP, respectively (Fig. 3C). These results show that treatment with Duolac ATP decreased both serum IgE and IL-4 levels (Fig. 3A, 3B). Overall, the *in vivo* results demonstrated that Duolac ATP doses of  $1 \times 10^8$  CFU/day and  $1 \times 10^9$  CFU/day rather than that of  $1 \times 10^7$  CFU/day are more effective in reducing AD-like lesions. Treatment with DEX induced a pattern of cytokine production similar to that observed with treatment with Duolac ATP (Fig. 3), with the IL-4, IL-5, and IgE levels being  $14.4 \pm 2.3$  pg/mL,  $32.5 \pm 2.7$  pg/mL, and  $698 \pm 89$  ng/mL, respectively.



**Fig. 4.** Serum IL-12p40 and IFN- $\gamma$  cytokine levels in DNCB-sensitized NC/Nga mice. Blood was collected from NC/Nga mice treated with Duolac ATP or DEX at week 10 for which the (A) serum IL-12p40 levels and (B) IFN- $\gamma$  levels were determined using ELISA. The experimental groups were as follows: Duolac ATP-L,  $1 \times 10^7$  CFU/day; Duolac ATP-M,  $1 \times 10^8$  CFU/day; Duolac ATP-H,  $1 \times 10^9$  CFU/day; and 2.5 mg/kg/day of DEX. Data represent the mean  $\pm$  SD. \* $P < 0.05$  and \*\* $P < 0.01$  compared with the PC mice (ANOVA).

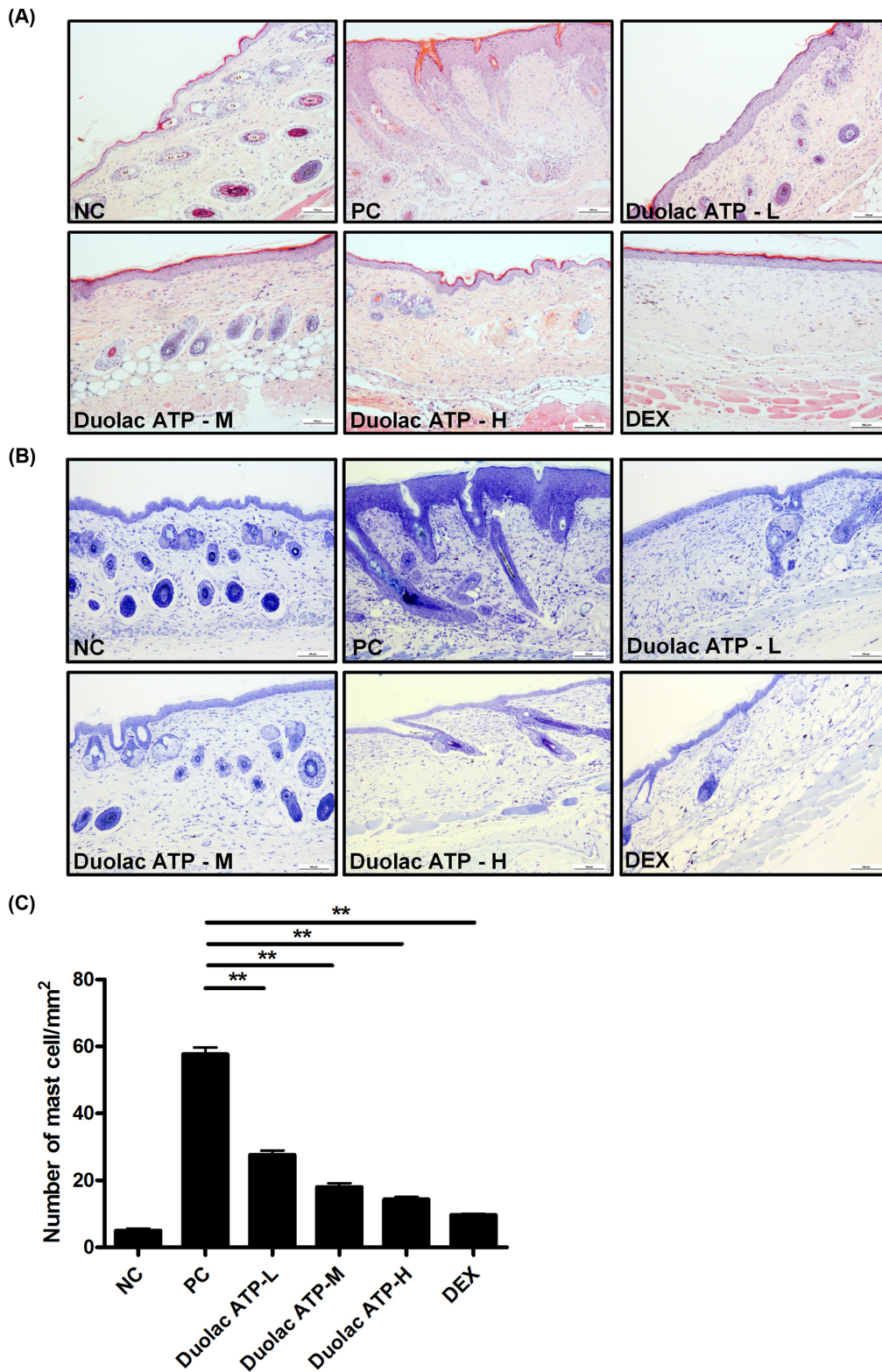
#### **Duolac ATP upregulates IFN- $\gamma$ and IL-12p40 cytokines in sera of NC/Nga mice.**

As treatment with Duolac ATP differentially affected the increase in IL-12p40 and IFN- $\gamma$  levels in relation to Th1 responses, we examined the effect of Duolac ATP on Th1 cytokine responses. For this, we measured the levels of IL-12p40 and IFN- $\gamma$ , which represented Th1 immune responses in sera isolated from week 10 NC/Nga mice. Serum IL-12p40 level of  $1,056 \pm 489$  pg/mL in the PC mice increased to  $2,281 \pm 566$ ,  $2,205 \pm 441$ , and  $1,981 \pm 66$  pg/mL when treated with  $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/day, and  $1 \times 10^9$  CFU/day of Duolac ATP, respectively (Fig. 4A). In contrast, the IL-12p40 levels in mice treated with DEX decreased to  $264 \pm 94$  pg/mL, which clearly differed from the levels observed in PC mice. Moreover, IFN- $\gamma$  level of  $153 \pm 4$  pg/mL in PC mice increased to  $175 \pm 5$ ,  $191 \pm 12$ , and  $201 \pm 22$  pg/mL when treated with  $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/day, and  $1 \times 10^9$  CFU/day of Duolac ATP, respectively (Fig. 4B). These data showed that treatment with Duolac ATP increased both serum IL-12p40 and IFN- $\gamma$  levels. The results demonstrated that  $1 \times 10^7$  CFU/day,  $1 \times 10^8$  CFU/day, and  $1 \times 10^9$  CFU/day of Duolac ATP is efficient in decreasing AD-like lesions as well. Treatment with DEX induced a pattern of cytokine production similar to that observed with treatment with Duolac ATP low dosage (Fig. 4B), with IFN- $\gamma$  level being  $168 \pm 4$  pg/mL.

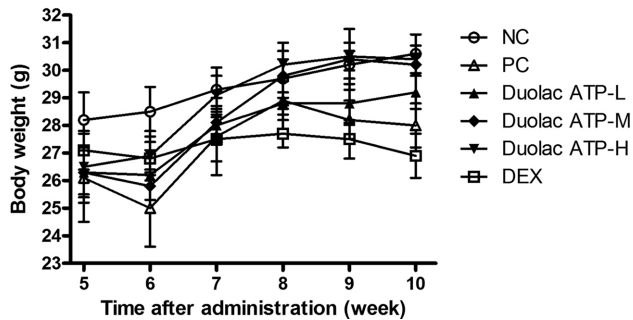
#### **Improvements in epidermal skin lesions in Duolac ATP-treated NC/Nga mice.**

PC NC/Nga mice with fully developed AD-like skin lesions showed hypertrophy and hyperkeratosis, which are typical features exhibited in the epidermis of patients with AD (Fig. 5A). To analyze the effect of Duolac ATP on skin hypertrophy infiltration in DNCB-induced NC/Nga mice, the Duolac ATP-treated skin was stained with H & E and examined using an optical microscope. As shown in Fig. 5A, acanthosis was clearly suppressed in NC/Nga mice orally administrated with Duolac ATP when compared to PC mice. These results indicated that Duolac ATP alters skin hypertrophy in DNCB-induced NC/Nga mice.

Staining of the dorsal skin sections with toluidine blue revealed more infiltration and degranulation of mast cells in



**Fig. 5.** Comparison of the histopathological analysis of dorsal skin lesions in DNCB-treated NC/Nga mice following repeated oral administration of Duolac ATP. (A) and (B) Images are representative examples from each group. Hematoxylin and eosin (H&E)- or toluidine blue-stained sections of PC, Duolac ATP-, and DEX-treated NC/Nga mice (original magnification,  $\times 100$ ). (C) Comparison between the number of mast cells in dorsal skin lesions in PC, Duolac ATP-, and DEX-treated mice. Data represent the mean  $\pm$  SD.  $**P < 0.01$  compared with the PC mice (ANOVA).



**Fig. 6.** Duolac ATP increased the body weight of mice treated with DNCB. Effects of Duolac ATP on body weight in the AD mouse model. AD was induced in the NC/Nga mice by treatment with 1% DNCB. Duolac ATP and DEX were orally administered three times per week for 6 weeks. Data represent the mean  $\pm$  SD. There were no significant differences between groups (ANOVA).

the upper dermis of PC mice than in the upper dermis of Duolac ATP-treated mice, as shown in Fig. 5B. Fig. 5C shows the number of mast cells in the lesional skin. PC mice and those treated with Duolac ATP-L, Duolac ATP-M, Duolac ATP-H, and DEX showed  $57.7 \pm 3.5$ ,  $27.7 \pm 2.1$ ,  $18.0 \pm 2.0$ ,  $14.3 \pm 1.2$  and  $9.7 \pm 0.6$  mast cells per millimeter square, respectively. After week 10, the number of mast cells significantly decreased by 52.0%, 68.8%, 75.1%, and 83.2% in the Duolac ATP- and DEX-treated mice as compared to PC mice. These results indicate that orally administered Duolac ATP suppressed DNCB-induced AD-like skin lesions in NC/Nga mice by reducing the infiltration of mast cells.

## DISCUSSION

AD is a major allergic disease that results from dermal inflammation. Although the etiology and pathology of AD is not fully understood, several studies have suggested that the typical symptoms of this disease involve increased levels of Th2-mediated cytokines and a deficiency in Th1-derived cytokines (1). In such a model, the key element is the sequential activation of the Th2-cell subsets during the initiation phase followed by that of the Th1-cell subset, to account for the persistence of the inflammatory response (25). Therefore, enhancement of Th1 is not necessarily beneficial for the treatment of AD in humans. However, despite a controversy over the pathogenesis of AD, a common goal for AD therapy is the manipulation of the cytokine network to selectively promote the Th1 response or preferentially inhibit the Th2 response. Several studies have suggested that the Th1 and Th2 reaction types can reciprocally regulate one another. Thus, given the immunologic imbalance in AD, one of the goals of AD therapy is to manipulate the cytokine network to inhibit the Th2 response preferentially.

In addition, the NC/Nga mouse strain was the first animal model for AD, reported by Matsuda *et al.* (23). Th2 responses are key elements underlying the pathogenesis of atopic disorders. NC/Nga mice were the first models of AD (23). However, mice treated with DNFB, DNCB, or picryl chloride have also been used as animal models of AD (19). Elevated levels of serum total IgE have been reported to correlate with the appearance of AD-like lesions in NC/Nga mice, with massive infiltration of IL-4- and IL-13-producing Th2 cells and degranulation of mast cells and eosinophils (26). Mast cells are the key effector cells in IgE-mediated allergic disorders and are activated by cross-linking of a high-affinity IgE receptor (9). Upon activation, mast cells undergo degranulation and release a variety of biologically active substances that play an important role in host defense and allergic reactions, including AD. The infiltration of mast cells into the dermis is a necessary characteristic for defining an appropriate animal model of AD (18). Skin changes develop spontaneously in NC/Nga mice, in response to their exposure to various environmental aeroallergens that closely mimic human AD. NC/Nga mice display mutations on chromosome 9, which are linked to increased IgE production and Th2 responses (27). Together with the skin changes, NC/Nga mice exhibit preferential Th differentiation toward Th2 cells, dense accumulation of eosinophils, mast cells in skin lesions, and an increase in serum IgE levels (19). Therefore, Duolac ATP consists of 4 probiotic species. Each bacterial strain reported to increase the Th1 cytokines. Because using the probiotic to increase the Th1 cytokines, we expected that will regulate the collapse of the balance of Th1/Th2 by the AD. By increasing the Th1 cytokines by Duolac ATP, it was confirmed the effect of treatment of AD-like in NC/Nga mice (28). Although the treatment of AD with steroids is effective, it could lead to several undesirable and adverse side effects. Therefore, steroids should only be used in an emergency. This has resulted in the use of Duolac ATP as a viable alternative. Several studies investigating the use of probiotics for the treatment of AD have been conducted in both humans and animals, with most studies employing *Lactobacillus* and *Bifidobacterium* species (4,6,22). It is interesting to note that the skin lesions of mice treated with DEX were cleaner than those of mice treated with Duolac ATP. The effect of DEX was confirmed with the scratching score, the levels of IgE and Th2 cytokines, and histological analysis. However, despite the treatment of AD, the body weights of the mice were not restored to the near-normal state (Fig. 6). Thus, probiotics stabilize the imbalance between Th1 and Th2 cytokines without any side effects, thereby reinforcing the immune function, as previously reported. Mice treated with Duolac ATP also exhibited no such visible adverse effects. These *in vivo* results demonstrated the safety of Duolac ATP.

In the current study, Duolac ATP treatment increased the



levels of Th1-mediated IL-12p40 and IFN- $\gamma$  in the sera of DNCB-sensitized NC/Nga mice (Fig. 4). These results confirm that Duolac ATP can restore the impaired balance between Th1 and Th2 responses by enhancing the secretion of Th1 cytokines. In contrast to its effects on Th1 cytokines, Duolac ATP induced a significant decrease in IgE and Th2-mediated cytokines (IL-4 and IL-5) in the sera of NC/Nga mice (Fig. 3). IL-4 is an important factor for IgE class switching and directly induces the release of IgE (29). Although a few studies report that IgE plays a major role in the pathogenesis of AD, it has been reported that abnormally high serum IgE levels correlate with the severity of AD (30). It has also been reported that another Th2 cytokine, IL-5, is involved in eosinophilia (31). IL-5 has eosinophil chemotactic activity and has been shown to prolong eosinophil survival by delaying apoptotic death, increasing the adhesion of eosinophils to endothelial cells, and enhancing eosinophil effector function (24). However, although IL-5 signaling is critical to the development of eosinophilia (24), IL-5 null mice have residual eosinophils; this underscores the importance of other pathways in the generation of eosinophilia (32). The results of the present study indicate that oral administration of Duolac ATP in NC/Nga mice treated with DNCB suppressed the development of AD-like skin lesions. Macroscopic analysis showed severe hemorrhage, acanthosis, and excoriation in the control mice, whereas Duolac ATP treatment prevented these skin changes. Histologically, Duolac ATP administration decreased hypertrophy, hyperkeratosis, and infiltration of inflammatory cells in the skin (Fig. 2A). Together with the biological activities of Duolac ATP, these results indicate that Duolac ATP could serve as a natural and safe therapeutic agent for the treatment of various allergic disorders with a minimal risk of adverse effects.

Also, important questions remain in establishing the clinical applications for probiotics, such as the optimal duration of probiotic administration as well as preferred microbial dosage and species. There are many treatment cases of various disease including atopic dermatitis using probiotics; the normal dosage for the treatment cases is  $10^9$ – $10^{11}$  CFU/day (9). On the other hand, the dosage of  $10^7$ – $10^9$  CFU/day, used for the animal study, is determined by referring to the theories (9). In other words, there are at least more than 100 fold differences between the body weight of the mice and human.

In conclusion, Duolac ATP downregulated the levels of IgE in AD-like skin lesions in mice through the modulation of Th1 and Th2 immune responses. It also caused an increase in Th1-mediated cytokines and a decrease in Th2-mediated cytokines. The optimal doses of Duolac ATP for maximum inhibition of AD symptoms were  $1 \times 10^8$  CFU/day and  $1 \times 10^9$  CFU/day. These results suggest that Duolac ATP has beneficial therapeutic effects on AD symptoms and represents an effective drug candidate for the treatment of AD without severe adverse effects.

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