



Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.elsevier.com/jds



Supplementation of the fermented soy product ImmuBalance™ effectively reduces itching behavior of atopic NC/Tnd mice

Akira Matsuda^{a,c}, Akane Tanaka^{b,c,*}, Weijun Pan^{d,e}, Noriko Okamoto^a, Kumiko Oida^c, Nobuyuki Kingyo^e, Yosuke Amagai^c, Yan Xia^c, Hyosun Jang^c, Sho Nishikawa^c, Naoki Kajiwara^c, Ginnae Ahn^a, Keitaro Ohmori^{a,c}, Hiroshi Matsuda^{a,c,*}

^a Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

^b Laboratory of Comparative Animal Medicine, Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

^c Cooperative Major in Advanced Health Science, Graduate School of Bio-Applications and System Engineering, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

^d Beth Israel Deaconess Medical Center at Harvard Medical School, MA 02215, USA

^e Nichimo Co. Ltd., Tokyo, Japan

ARTICLE INFO

Article history:

Received 4 April 2012

Received in revised form 27 May 2012

Accepted 29 May 2012

Keywords:

Atopic dermatitis

NC/Tnd mice

Itch sensation

Probiotics

Fermented soy product

ABSTRACT

Background: Effects of probiotics on the prevention of atopic diseases have been proposed recently. Although we have already reported the suppressive effects of the probiotic, ImmuBalance™, on a mouse model for peanuts allergy, its influence on atopic diseases remains unclear.

Objective: Potential efficacy of ImmuBalance™, which is the fermented soy product, on treatment of atopic dermatitis (AD) was investigated using a mouse model for human AD, NC/Tnd mice.

Methods: For *in vivo* study, ImmuBalance containing chow or a control diet were fed to NC/Tnd mice with moderate dermatitis for 2 weeks. Topical application of FK506 ointment was used as a positive control. Clinical skin severity scores, scratching behaviors, trans-epidermal water loss (TEWL), and histological features were analyzed. For *in vitro* study, suppressive effect of ImmuBalance™ on nerve growth factor (NGF)-activated neurite outgrowth of PC12 cells was examined.

Results: Clinical skin severity scores of the mice fed with ImmuBalance containing chow were gradually reduced as well as the mice treated with FK506. Feeding with ImmuBalance completely inhibited the increase in scratching behavior of NC/Tnd mice. The value of TEWL of NC/Tnd mice fed with ImmuBalance was significantly decreased. In addition, histological examination revealed that application of ImmuBalance decreased the number of PGP9.5-positive neuronal fibers in the lesional skin. When ImmuBalance extract was added to the culture, NGF-activated neurite outgrowth of PC12 cells was diminished through the inhibition of the phosphatidylinositol 3-kinase phosphorylation.

Conclusion: ImmuBalance could exhibit favorable alterations on AD symptoms, particularly through down regulation of the itch sensation.

© 2012 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

1. Introduction

ImmuBalance™ (Nichimo Co. Ltd., Tokyo, Japan) is a proprietary koji fermentation product made with fermenting defatted soybeans with *Aspergillus oryzae*, and lactic acid bacteria (*Pediococcus paewulus* and *Enterococcus faecium*), which is generated by a Japanese fermentation technology. ImmuBalance is one of probiotics;

however, both the probiotic effect by live microorganisms including in ImmuBalance and the indirect biogenic effect by microbial metabolites which are synthesized during fermentation process may be expected [1]. Previously we reported that oral administration of ImmuBalance containing diet chow protected against peanut-induced anaphylaxis in a standard strain of mice [2].

Japanese traditional foods including miso (fermented soybean paste), shoyu (soy sauce), and sake (Japanese liquor) have been generated by Japanese fermentation technology with koji molds [3]. It is controversial whether intake of soy-protein prevents allergic skin disorders, however, some studies showed that intake of fermented soy products or soy sauce prevented allergic rhinitis [4,5]. We have recently reported beneficial effects of ImmuBalance on clinical symptoms of Japanese cedar pollinosis in a pilot study [6].

* Corresponding authors at: Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan. Tel.: +81 42 367 5784; fax: +81 42 367 5916.

E-mail addresses: akane@cc.tuat.ac.jp (A. Tanaka), hiro@cc.tuat.ac.jp (H. Matsuda).

Furthermore, the effect of ImmuBalance on an established murine model of peanut allergy was tested. We clearly demonstrated that ImmuBalance-treated mice exhibited significantly reduced clinical symptoms as compared with control mice against peanut challenge. The evidence allowed us to hypothesize that ImmuBalance might have suppressive properties on skin disorders, including atopic dermatitis (AD).

AD is a chronic and relapsing skin disease characterized by intense pruritus and the development of inflammatory lesions. Strong polarization to Th 2 type responses in AD patients result in the hyperproduction of IgE [7]. Moreover, barrier dysfunction of lesional skins is understood to play a crucial role in the exacerbation of clinical conditions [8]. Recently, several studies have proposed that the early microbial exposure during childhood might decline the risk of AD, which is called hygiene hypothesis [9,10]. Moreover, some human trials suggest that oral probiotics therapy might be useful in the management of AD [11,12]. However, the timing and duration of administration for prevention or treatment of AD is still unclear. The NC/Tnd mouse (formerly known as NC/NgaTnd) is a spontaneous model of human AD that develops chronic lesions when exposed to environmental antigens [13]. Severity of dermatitis in NC/Tnd mice is increased throughout life and clinical features are quite similar to those of human AD including IgE hyperproduction, epidermal hyperplasia, increased trans-epidermal water loss (TEWL), increased numbers of CD4⁺ T cell and mast cells, and elevated serum IL-18 [13–15]. Like human AD, several probiotics treatments have been shown to reduce dermatitis in NC/Tnd mice [16–22]. We reported that administration of heat-treated *Lactobacillus* (L.) *rhamnosus* GG clearly prevented development skin symptoms of AD in NC/Tnd mice, probably through promoted production of IL-10 in intestinal lymphoid organs [16]. Furthermore, our previous studies revealed that oral supplementation of *Lactobacillus johnsonii* NCC533 (La1) or *L. rhamnosus* CGMCC1.3724 prevented the increase of scratching behaviors and reduced the clinical skin severity [17,18]. These studies clarified the prophylactic benefits of probiotics on development of AD in NC/Tnd mice; however, therapeutic effects of those on established AD should be analyzed in more detail.

In the present study, we attempted to examine whether ImmuBalance had therapeutic effects on AD using NC/Tnd mice.

2. Materials and methods

2.1. Animals

For *in vivo* study, NC/Tnd mice were maintained in the air-uncontrolled conventional room. Thirty male NC/Tnd mice with moderate dermatitis were enrolled into the *in vivo* study from 8 weeks of age and were randomly divided into 3 groups. For *in vitro* study, NC/Tnd mice were maintained in the specific-pathogen free (SPF) condition until euthanization for sampling. All mice were housed in the room maintained at 24 ± 1 °C under the cycle of 12/12 h light/dark during the study. All experiments with animals complied with the standards in the guidelines of Tokyo University of Agriculture and Technology.

2.2. Experimental protocol of *in vivo* study

ImmuBalance containing chow was fed to the conventional NC/Tnd mice with moderate dermatitis for 2 weeks. ImmuBalance powders were produced from defatted soybeans fermented with koji mold (a commercially available culture of *A. oryzae*) as previously described [2]. According to the previous data using a mouse model for peanuts allergy, diet chow containing ImmuBalance used in this study were formulated by adding 1.0% of

ImmuBalance to CLEA Rodent Diet CE-2 (CLEA Japan Inc., Tokyo, Japan), which was composed of 1.8×10^8 /g of lactic acid bacteria [2]. An intake of diet chow per mouse is around 6–9 g in one day. CLEA Rodent Diet CE-2 was fed to the other conventional NC/Tnd mice with moderate dermatitis. We applied 100 mg of 0.1% FK506 ointment (0.1% Protopic, Astellas Pharma Inc., Tokyo, Japan) onto the back of conventional NC/Tnd mice daily for 2 weeks as a positive control. For measurement of trans-epidermal water loss (TEWL), the hair in the dorsal skin was clipped before treatment.

2.3. Clinical skin severity scores, scratching analysis, and TEWL

Clinical skin conditions of NC/Tnd mice were macroscopically examined and scored 3 times/week by the person unaware of which mice were in which group, according to a slight modification of the criteria described previously [23]. A total clinical severity score for atopic dermatitis-like lesions was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of five signs and symptoms (itch, erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness). Scratching behavior of mice was quantified by using a SCLABA-Real system (Noveltec Inc., Kobe, Japan) at pre- and post-treatment [24]. Pre- and post-treatment TEWL values were measured on the dorsal skin of NC/Tnd mice under *in vivo* experiments. The mice were anesthetized with isoflurane inhalation and TEWL values were measured with a tewameter TM300 (Courage + Khazaka Electronic GmbH; Cologne, Germany).

2.4. Histological analysis of the skin

Skin samples were collected at the end of the *in vivo* study and fixed with 10% buffered formalin. Using paraffin sections and anti-protein gene product (PGP) 9.5 polyclonal antibody (Millipore Corp., Temecula, CA), immunohistochemistry was performed to visualize extension of nerve fibers in the skin according to the instruction. Paraffin sections were also used for hematoxylin and eosin (H&E) staining for the pathological grading of skin specimens. Researchers who were unaware of treatments in each group evaluated the pathological level according to the four-grade system: 0 (none), 1 (mild), 2 (moderate), and 3 (severe).

2.5. Cells

Spleens were aseptically removed from NC/Tnd mice maintained in the SPF condition and homogenized with glass homogenizers. The cells were passed through a 40- μ m nylon cell strainer (BD Biosciences, San Jose, CA) to prepare a single-cell suspension. For analysis of cytokine expression, spleen cells were resuspended in α -MEM containing 10% fetal bovine serum (FBS; Filtron, Brooklyn, Australia) and antibiotics at 10^6 cells/ml and were cultured with/without pokeweed mitogen (PWM) (3 μ g/ml) and/or ImmuBalance extracts. Bone marrow-derived cultured mast cells (BMCMC) were obtained from bone marrow cells of SPF NC/Tnd mice in α -MEM containing 10% FBS and 10% PWM-stimulated spleen cell-conditioned medium according to the method described previously [25]. For β -hexosaminidase assay and analysis of cytokine expression in BMCMC, they were cultured with/without ImmuBalance extracts. PC 12 cells, a rat pheochromocytoma cell line, was obtained from ATCC (Livermore, CA; CRL1721) and cultured in RPMI 1640 (Invitrogen, Tokyo, Japan) containing 10% FBS and antibiotics.

2.6. Preparation of ImmuBalance extract

For *in vitro* study, ImmuBalance extracts were separated to 2 fractions. The molecular weight of the high fraction was from 3000

Table 1

Characterization of ImmuBalance extracts.

		High fraction	Low fraction
Molecular weight		3000–6000	300–3000
Elemental analysis (w/w, %)	Carbon	38.05	33.66
	Hydrogen	5.23	6.16
	Nitrogen	0.7	6.6
Ninhydrin reaction		Negative	Positive
¹ H NMR analysis		Polysaccharide	Peptide, amino acids
Contents (w/w, %)		3.1	21.5

to 6000 and the molecular weight of the low fraction was from 300 to 3000 (data not shown).

In brief, ImmuBalance powder (1 g) was extracted with distilled water (20 ml) at room temperature for three times. The extract was combined and was filtrated with filter paper and was submitted to preparative gel permeation chromatography.

The preparative gel permeation chromatography was performed on a LC-908 HPLC system using 500 mm × 21.5 mm i.d., 13 μm JAIGEL GS-320 column (Japan Analytical Industry Co. Ltd., Tokyo, Japan) with a RI-5 detector (Japan Analytical Industry Co. Ltd.) and water as an eluting solvent at the flow rate of 3.8 ml/min.

Each fraction was freeze-dried and weighed. Elemental analysis was for carbon, hydrogen, and nitrogen to determining the amount of an element in the fraction. ¹H nuclear magnetic resonance (NMR) analysis was recorded on a spectrophotometer in D₂O at 25 °C. The fractions were detected by ninhydrin reaction after HCl hydrolyzed (Table 1). The high fraction was consisted with polysaccharide that content was 3.1% ImmuBalance, and the low fraction was consisted with peptide and amino acids that content was 21.5% ImmuBalance.

2.7. Effects of ImmuBalance on cytokine expression in spleen cells stimulated with PWM

After 24-h incubation with/without PWM and/or ImmuBalance extract, cells were collected and total RNA was extracted with Fast Pure (Takara bio, Shiga, Japan) according to the manufacturer's instructions. Total RNA obtained from each sample was reverse-transcribed into cDNA with Oligo (dt) (12–18) primers using a PrimeScript 1st strand cDNA synthesis kit (Takara Bio). Real-time PCR was performed with SYBR Premix Ex Taq II (Takara Bio) in the presence of 0.2 μM each of the forward and reverse primers for murine IL-4 (5'-TCTCGAATGTACCAGGAGCCATATC-3' and 5'-AGCACCTTGGAGCCCTACAGA-3'), murine IL-5 (5'-AGCACAGTGGTGAAGAGACCTT-3' and 5'-TCCAATGCATAGCTGGTGATTT-3'), murine IL-10 (5'-GACCAGCTGACAACATACTGCTAA-3' and 5'-GATAAGGCTTGGAACCCAAGTAA-3'), murine TGF-β1 (5'-TGTGGAGCAACATGTGGAACCTCTA-3' and 5'-TTGGTTCAGCCACTGCCGTA-3'), murine IFN-γ (5'-CGGCA-CAGTCATTGAAAGCCTA-3' and 5'-GTTGCTGATGGCCTGATTGTC-3'), murine TNF-α (5'-CATCTTCTCAAATTCGAGTGACAA-3' and 5'-TGGGAGTAGACAAGGTACAACCC-3') or for murine β-actin (5'-TGACAGGATGCAGAAGGAGA-3' and 5'-GCTGGAAGGTGGACAGT-GAG-3') by the following thermal cycling programs: stage 1, 50 °C for 2 min; stage 2, 95 °C for 10 min; stage 3, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence intensity was measured in real time during extension steps by using the ABI Prism 7000 Sequence Detector (Applied Biosystems). Relative expression levels of the target gene were normalized to endogenous reference (β-actin) and calculated by 2^{-ΔΔCT}.

2.8. β-Hexosaminidase assay

BMCMC were generated from bone marrow in NC/Tnd mice as reported [26]. In the presence of various concentrations of

ImmuBalance extracts, IgE-mediated activation of BMCMC was induced by using 2 ng/10⁶ cells of anti-dinitrophenyl (DNP) IgE and 50 ng/ml of DNP-bovine serum albumin (BSA). After 60 min, β-hexosaminidase activity in supernatants and cell lysates were measured by using p-nitrophenyl-N-acetyl-β-D-glucosamine as a substrate and β-hexosaminidase release (%) was calculated as previously described [26].

2.9. Nerve growth factor (NGF)-induced neurite outgrowth of PC12 cells

PC12 cells were plated in six-well plates coated with Cellmatrix Type 1-A (Nitta Gelatin, Osaka, Japan) in the presence of 50 ng/ml 2.5S NGF (Upstate Biologicals Inc., Lake Placid, NY) for a week. The primed PC12 cells were re-incubated with 50 ng/ml 2.5S NGF in the presence of various concentrations of ImmuBalance extracts for 48 h. The neurite-bearing cells were counted under a light microscope as described [26].

2.10. Western blot analysis

PC12 cells were plated in six-well plates coated with Cellmatrix Type 1-A (Nitta Gelatin Inc., Osaka, Japan) in the presence of 50 ng/ml 2.5S NGF for a week. After serum deprivation for 2 h, primed-cells were pretreated for 60 min with or without 50 or 200 ng/ml ImmuBalance extract. The cells were collected at 0, 5, 15, 30, and 60 min after 50 ng/ml NGF stimulation. After washed in PBS, 10⁶ cells were lysed in 50 μl of ice-cold cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, pH 8.0), supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Supernatants were collected by centrifugation, mixed with the same volume of 2× sample buffer (20% glycerol, 10% 2-mercaptoethanol, 4% SDS, 100 mM Tris-HCl, 0.01 Bromophenol Blue, pH 6.8), and boiled for 7 min. Samples were applied to SDS-polyacrylamide gel electrophoresis with the use of 12.5% gels (Bio-Rad Laboratories, Hercules, CA). Separated proteins were transferred onto Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in 5% nonfat dry milk containing Tris-buffered saline (TBS) and was blotted with primary antibodies diluted in a blocking solution. After washed in TBS with 0.05% Tween-20, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Positive reactions were visualized with Immobilon Western (Millipore) as the manufacturer's instruction. Anti-p44/42 mitogen-activated protein kinase (MAPK) antibody, anti-phospho-p44/42 MAPK antibody, anti-phosphatidylinositol 3-kinase (PI3K) p85 antibody, anti-phospho-PI3K p85 antibody, anti-p70 S6K Antibody, anti-phospho-p70 S6K antibody, anti-β-actin antibody, and anti-rabbit IgG, HRP-linked antibody were purchased from Cell Signaling Technology (Beverly, MA).

2.11. Statistical analysis

One- or two-way ANOVA, followed by a Tukey test was used for calculation of *P* values. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Oral application of ImmuBalance improved clinical symptoms in NC/Tnd mice

Using NC/Tnd mice that spontaneously developed AD, we examined whether ImmuBalance had therapeutic relevance on clinical symptoms of AD. In ImmuBalance-applied mice, clinical skin conditions were gradually ameliorated, and at the end of the experiment, clinical scores were significantly reduced as compared

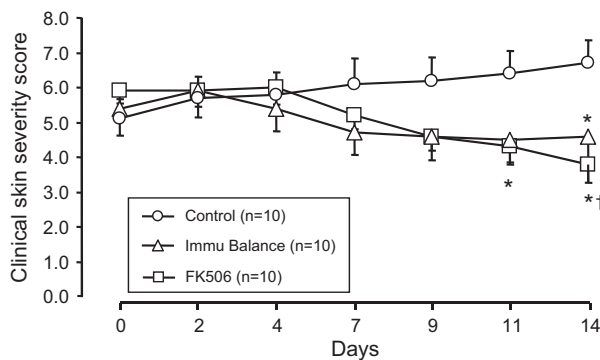


Fig. 1. Clinical skin severity score during ImmuBalance treatment. Clinical conditions of NC/Tnd mice were observed 3 times/week. NC/Tnd mice were divided into 3 groups; Control, mice fed with a regular diet chow; ImmuBalance, mice fed with 2% ImmuBalance containing chow; FK506, mice fed with a regular diet chow and topically applied with 0.1% FK506 ointment on the dorsal skin. Data represent means $\pm$ SE. * $P < 0.05$, when compared with control group. † $P < 0.05$, when compared with day 0.

to a control group (Fig. 1). The clinical scores in the mice which were topically applied 0.1% FK506 ointment as a positive control showed significant decrease (Fig. 1). We also recorded body weight and general condition of each mouse, though no abnormality, such as weight gain or diarrhea, was observed (data not shown).

3.2. Oral application of ImmuBalance prevented the increase in scratching behavior in NC/Tnd mice

Both scratching frequency and duration were significantly increased at the end of treatment in NC/Tnd mice fed with a control

diet; however, scratching behavior was not changed in the mice orally treated with ImmuBalance or topical application of FK506 ointment (Fig. 2A and B). After 2 week-treatment, TEWL values in NC/Tnd mice fed with ImmuBalance or applied with FK506 ointment were significantly lower than those in the control mice (Fig. 2C). Only the mice applied with FK506 showed significant decrease in TEWL. Body weight was not changed through the treatment period in all groups (Fig. 2D).

3.3. Oral application of ImmuBalance decreased the numbers of infiltrated cells and nerve fibers in the lesional skin

Marked edema, spongiosis, acanthosis, epidermal and dermal thickening, and cell infiltration were observed in the skin of NC/Tnd mice fed with a control diet (Fig. 3A). In contrast, such phenomena were weakened and the number of infiltrated cells was significantly decreased in the mice applied with FK506 ointment. In the mice fed with ImmuBalance, the skin condition was improved as well as the mice applied with FK506 ointment; epidermal and dermal thickening was relieved and the number of infiltrated cells was significantly decreased in the mice fed with ImmuBalance (Fig. 3A). Anti-PGP9.5 immunohistochemistry revealed that ImmuBalance application significantly decreased the number of nerve fibers in the skin of NC/Tnd mice (Fig. 3B).

3.4. ImmuBalance directly reduced production of inflammatory cytokines in spleen cells

ImmuBalance contains various constituents generated during fermentation process. Therefore, we extracted active ingredients from ImmuBalance and used them for further *in vitro* experiments.

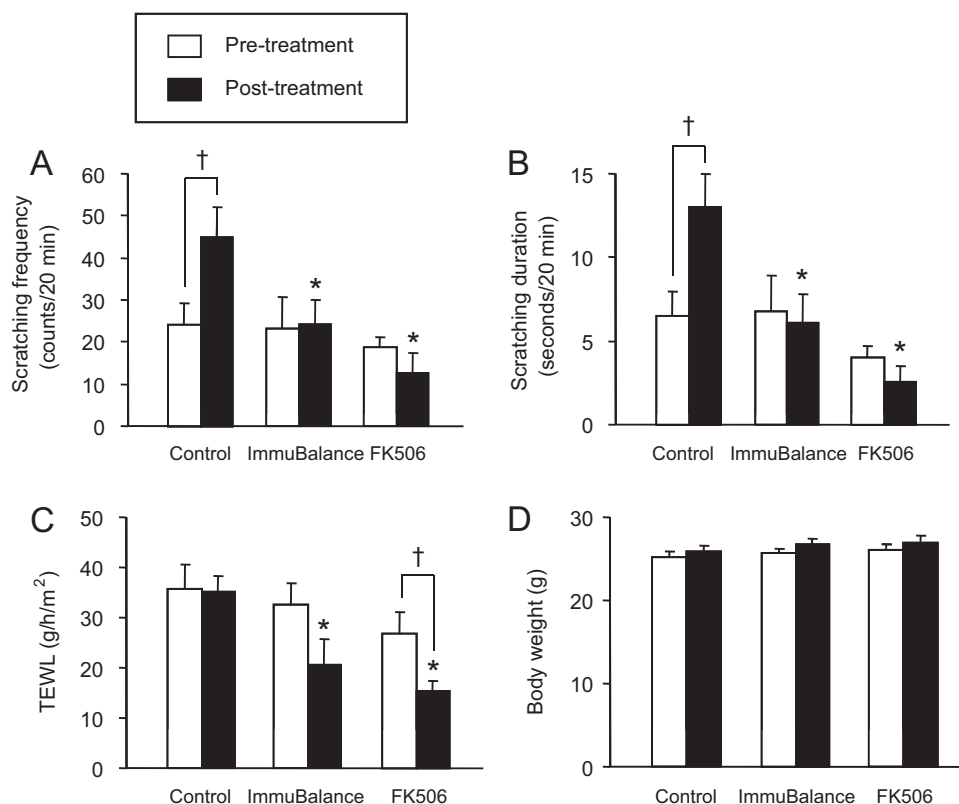


Fig. 2. Effects of oral application of ImmuBalance containing chow on scratching behaviors and TEWL. Scratching behavior of mice was quantified with a SCLABA-Real system (A and B). The mice fed with ImmuBalance containing diet showed reduced scratching frequency and duration compared with control mice. TEWL values were measured with a tewameter TM300 (C). At the end of treatment, TEWL values of ImmuBalance-treated mice were significantly lower than those of control mice. Body weight was measured before and after treatment (D). No significant difference was determined among all groups. Data represent means $\pm$ SE. * $P < 0.05$, when compared with control group. † $P < 0.05$, when compared with pre-treatment.

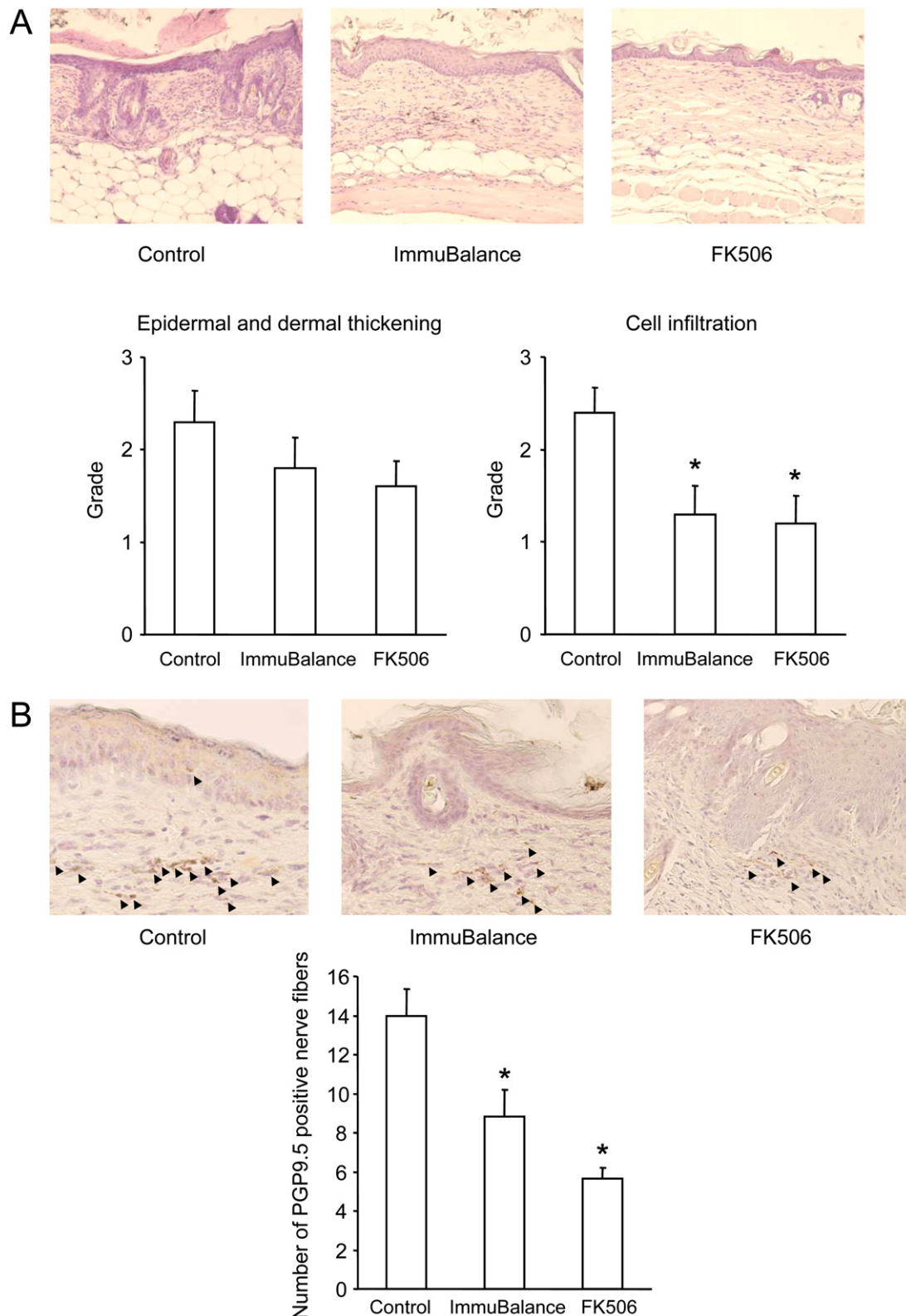


Fig. 3. Histological features of skin lesions of NC/Tnd mice applied with ImmuBalance. Paraffin sections were used for H&E staining (A) and immunohistochemistry (B). Researchers who were unaware of treatments in each group evaluated epidermal and dermal thickening and cell infiltration, according to the four-grade system; 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The number of PGP9.5-positive nerve fibers in five high-power fields from 10 individual skin specimens was counted under a microscope. Data represent means $\pm$ SE. * $P < 0.05$, when compared with control group. Arrow heads show PGP9.5 positive cells.

ImmuBalance extracts were divided into 2 fractions; the high fraction included high molecular weight proteins and the low fraction included low molecular weight proteins. To examine the effect of ImmuBalance on cytokine production of lymphocytes,

mRNA expressions of various cytokines in the spleen cells, which were incubated with PWM and ImmuBalance extracts, were determined. Cytokine expressions of IFN- γ , IL-4, IL-5, and IL-10 in spleen cells stimulated with PWM for 24 h were markedly

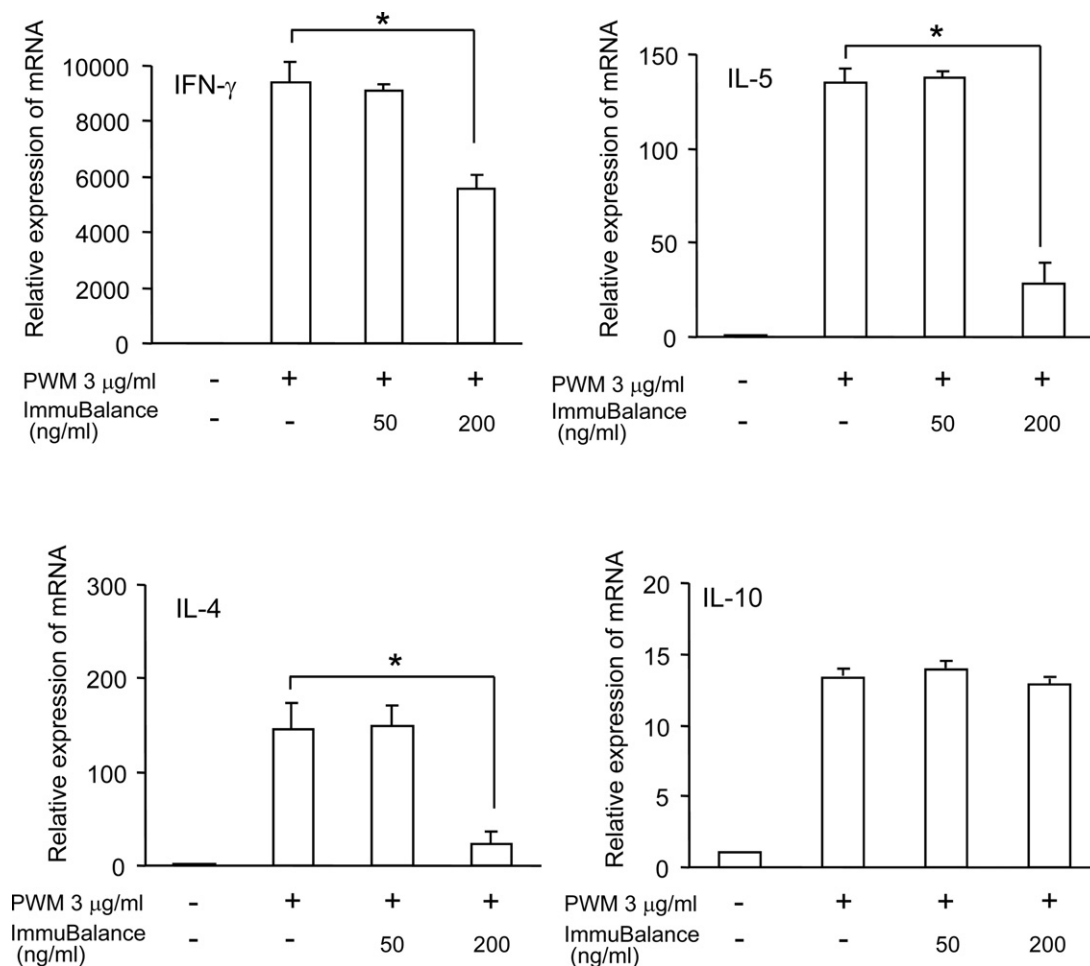


Fig. 4. Effects of the high fraction of ImmuBalance extracts on production of inflammatory cytokines in lymphocytes. Messenger RNA expression in the spleen cells cultured with or without PWM and/or the high fraction of ImmuBalance extracts was measured by real-time PCR. The high fraction of ImmuBalance extract suppressed expression of IFN- γ , IL-4, and IL-5 in spleen cells. Data represent means $\pm$ SE of 3 individual experiments. * $P < 0.05$, when compared with PWM alone.

unregulated. However, the addition of the high fraction of ImmuBalance to culture medium significantly suppressed PWM-induced IFN- γ , IL-4, and IL-5 production (Fig. 4). Production of IL-10 was not changed by the addition of the high fraction of ImmuBalance. The low fraction of ImmuBalance had no effects on *de novo* production of inflammatory cytokines in spleen cells (Fig. S1). Cell viability was evaluated by a trypan blue dye exclusion test, and no cytotoxic effect of ImmuBalance extracts was observed (data not shown).

3.5. ImmuBalance had less effect on degranulation of mast cells

Mast cells play a key role in the inflammatory process, especially in the allergic response. Therefore we examined whether ImmuBalance affected to degranulation of BMCMC. Sensitized BMCMC were degranulated when they were stimulated with antigen DNP-BSA (Fig. 5). ImmuBalance did not accelerate the degranulation of BMCMC. Furthermore, addition of the low fraction or the high fraction of ImmuBalance failed to up-regulate the *de novo* synthesis

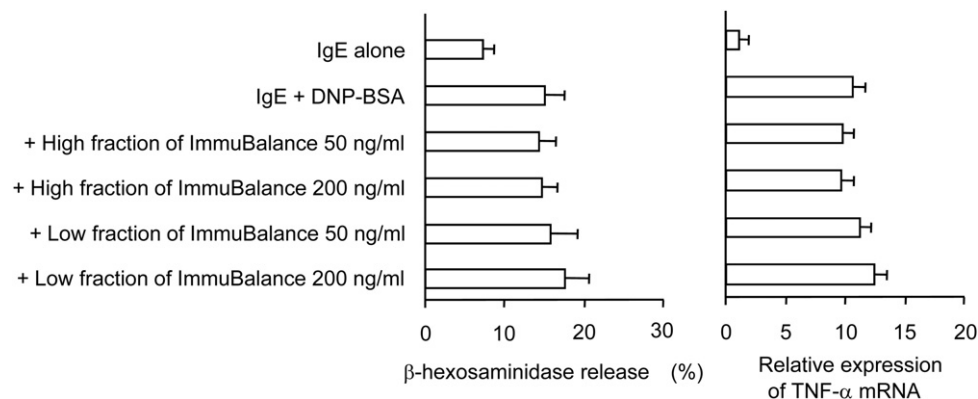


Fig. 5. Effects of ImmuBalance extracts on function of mast cells. BMCMC derived from bone marrow cells separated from SPF NC/Tnd mice were stimulated with anti-DNP IgE and DNP-BSA. The high fraction and the low fraction of ImmuBalance extracts failed to suppress the degranulation of BMCMC. Data represent means $\pm$ SE of 3 individual experiments.

of TNF- α in BMCMC after IgE crosslinkage (Fig. 5). Cell viability was evaluated by a trypan blue dye exclusion test, and no cytotoxic effect of ImmuBalance extracts was observed (data not shown).

3.6. ImmuBalance effectively suppressed neurite outgrowth in PC12 cells

Since the frequency of scratching behavior and the number of PGP9.5-positive nerve fibers in the skin were decreased in NC/Tnd mice fed with ImmuBalance, the suppressive effect of ImmuBalance on the extension and activation of nerve cells was predicted. NGF was reported to stimulate the extension of peripheral sensory neurons and to contribute to skin hypersensitivity in AD [27], therefore the effect of ImmuBalance on neurite outgrowth of PC12

cells was assessed. When primed PC12 cells were stimulated with 50 ng/ml 2.5S NGF in the presence of the high fraction or the low fraction of ImmuBalance extract, neurite outgrowth was significantly suppressed (Fig. 6A and B). Cell viability was evaluated by a trypan blue dye exclusion test, and no cytotoxic effect of ImmuBalance extracts was observed (data not shown).

3.7. ImmuBalance inhibited the phosphorylation of PI3K in PC12 cells

To investigate the effect of ImmuBalance on the activation of intracellular signals activated after NGF stimulation, Western blot analysis was performed. PI3K, S6K, and extracellular signal-regulated kinase (ERK) in PC12 cells were phosphorylated immediately after the stimulation with 50 ng/ml 2.5S NGF (Fig. 7A and B).

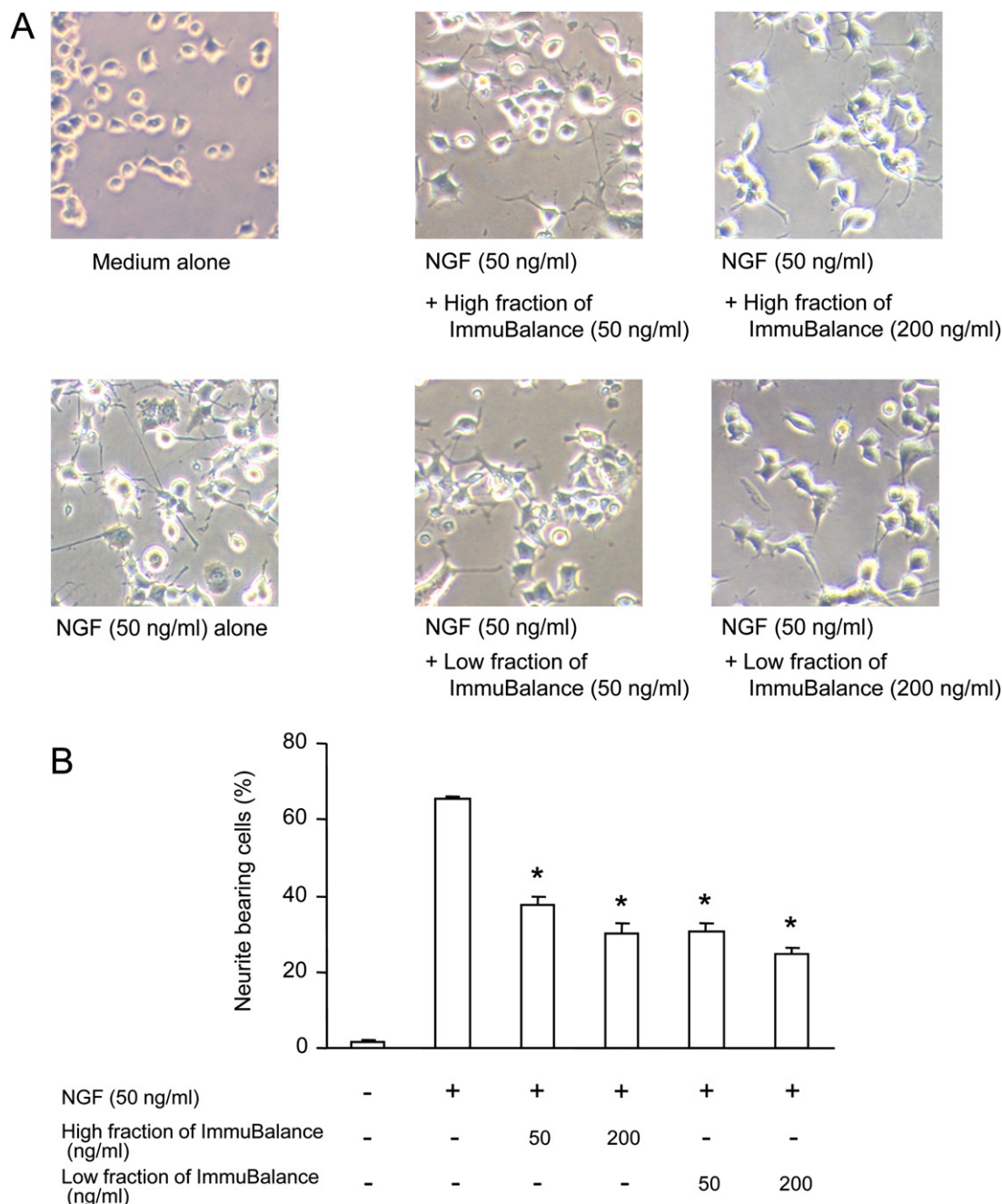


Fig. 6. Suppressive effect of ImmuBalance extracts on neurite outgrowth of nerve cells. The primed PC12 cells were re-incubated with 50 ng/ml 2.5S NGF in the presence of various concentrations of ImmuBalance extracts for 48 h. Typical images (A) and mean percentages (B) of neurite bearing cells were showed. Both the high and low fraction of ImmuBalance extracts reduced neurite outgrowth on PC12 cells. Data represent means $\pm$ SE of 3 individual experiments. * P < 0.05, when compared with NGF alone.

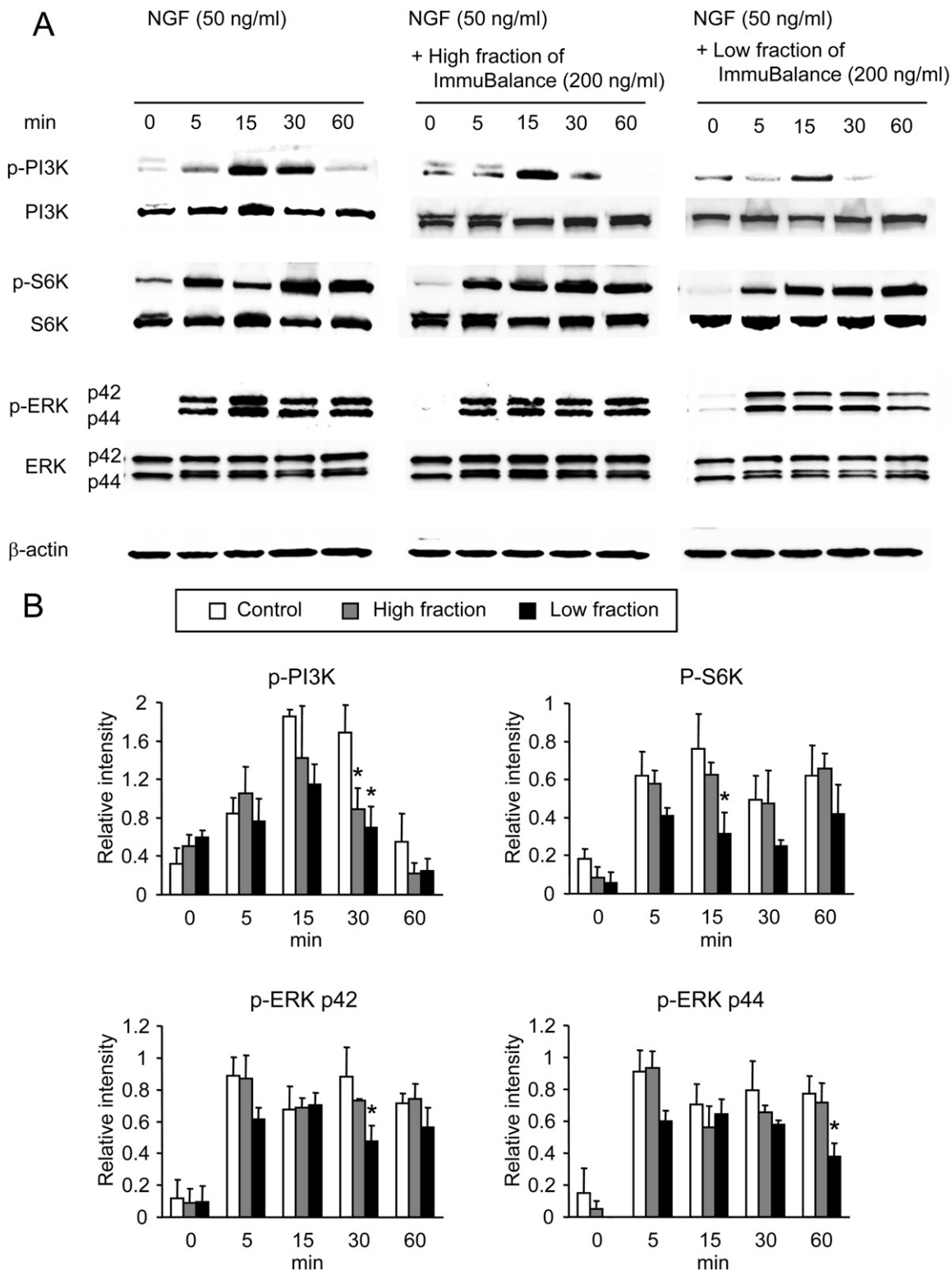


Fig. 7. Inhibitory effect of ImmuBalance extracts on phosphorylation of PI3K and MAPK. The primed PC12 cells were preincubated with or without 200 ng/ml ImmuBalance extracts, and then 50 ng/ml 2.5S NGF was added to the culture. Phosphorylation of PI3K and MAPK were analyzed at 0, 5, 15, 30, and 60 min after stimulation of NGF by Western blot analysis (A). Relative intensity of phosphorylated protein was showed in the graphs (B). β-Actin was used for an endogenous control. Data represent means $\pm$ SE of 3 individual experiments. * $P < 0.05$, when compared with a control.

When PC12 cells were preincubated with the high fraction of ImmuBalance extract, phosphorylation of PI3K was suppressed at 1 h after NGF stimulation. Preincubation with the low fraction of ImmuBalance extract significantly diminished the phosphorylation of PI3K, S6K, and ERK 15–60 min after the stimulation with NGF (Fig. 7A and B).

4. Discussions

In the NC/Tnd mice that were orally administered ImmuBalance containing diet, clinical skin severity score, scratching behavior, and TEWL were all reduced compared to those in control mice. Previous studies have demonstrated the prophylactic benefit of

probiotics on development of AD in NC/Tnd mice [16–18]. In the current study, we provided the first evidence that oral administration of probiotics improved established spontaneous symptoms of AD in NC/Tnd mice. Clinical skin severity scores were gradually decreased with a statistical significance in ImmuBalance-fed group, which was quite similar to those in FK506-treated mice. Oral application of ImmuBalance markedly suppressed the exacerbation of pruritus; thereby, the increase in scratching behavior was restrained. Since TEWL was decreased, skin barrier function might be recovered in mice applied with ImmuBalance. Two-week-treatment of ImmuBalance showed no side effect on NC/Tnd mice.

Whereas the high fraction of ImmuBalance extracts suppressed production of IFN- γ , IL-4, and IL-5 in spleen cells, but not production of IL-10. Some studies reported that the serum concentration or the mRNA expression of IL-10 were elevated in NC/Tnd mice fed with probiotics; on the other hand, those of IFN- γ , IL-4, and IL-5 were down-regulated when compared with control mice [2,16,19]. IL-10 is known as an anti-inflammatory cytokine that inhibits activation and effector functions of T cells, monocytes, and macrophages [28,29]. IL-10 also suppresses the expression of MHC II, CD80, and CD86 on monocytes [30,31], contributing to the anergy to antigens [32]. Therefore, anti-inflammatory effect of ImmuBalance may be caused by suppression of pro-inflammatory cytokines and sustainable production of anti-inflammatory cytokines.

Itch is one of major clinical symptoms in AD [33]. In the epidermis of lesional skins from patients with AD, cutaneous nerve fibers are extended at high densities, reaching to the surface of the skin [34,35]. The observation suggests the hypersensitivity of patients with AD to itch stimulation. NGF is one of the major mediators inducing the nerve fiber expansion [27]. Both fractions of the ImmuBalance extract showed direct suppression on neurite outgrowth of nerve cells promoted by NGF stimulation; and oral application of ImmuBalance decreased infiltration of nerve fibers into skin. These results suggested that ImmuBalance treatment suppressed exacerbation of itch by prevention of the expansion of nerve fibers. Furthermore, analysis of intracellular kinases revealed that both the fractions of ImmuBalance abolished neurite outgrowth of nerve cells through inhibition of the phosphorylation of PI3K signal cascades. The low fraction of ImmuBalance was suggested to have also inhibitory effect on the phosphorylation of MAPK signal cascades. Such additional inhibitory effect of the low fraction suggests that the low fraction may have more strong suppressive effect on neurite outgrowth of nerve cells compared with the high fraction.

The high fraction of ImmuBalance contains polysaccharides. Some polysaccharides have a suppressive effect on PI3K/Akt pathway [36,37]. The low fraction of ImmuBalance may include soy-derived isoflavones. Isoflavones derived from soybeans abolishes the activation of PI3K/Akt pathway and MAPK pathway [38–40]. We found that the high fraction but not the low fraction of ImmuBalance exhibited the inhibitory effects on mitogen-stimulated cytokine production in lymphocytes. In contrast, both fractions suppressed neurite outgrowth of PC12 cells stimulated with NGF by suppressing phosphorylation of the signal molecules. Therefore, these ingredients may be involved in reduction of allergic inflammation and itching behavior, when NC/Tnd mice were orally applied with ImmuBalance. Since ImmuBalance was originated from *A. oryzae*, a possibility to activate immune cells existed. In this study, we confirmed that addition of ImmuBalance extracts into the culture did not induce the activation of mast cells. The results delineate a favorable effect of ImmuBalance on the reduction of allergic inflammation without immuno-active ingredients.

In conclusion, the results in this study indicated that oral supplementation of ImmuBalance reduced the established AD symptoms in NC/Tnd mice as effective as topical treatment with

FK506 ointment. Particularly, the high fraction from ImmuBalance extract exerted anti-inflammatory effects on production of proinflammatory cytokines in lymphocytes. On the other hand, both the high and low fractions of ImmuBalance extract suppressed NGF-induced neurite out growth, explaining the anti-pruritic effect. Taken together, administration of ImmuBalance could be a novel treatment of human AD without serious side effects.

Funding sources

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2012.05.011>.

References

- [1] Gobbetti M, Cagno RD, De Angelis M. Functional microorganisms for functional food quality. *Crit Rev Food Sci Nutr* 2010;50:716–27.
- [2] Zhang T, Pan W, Takebe M, Schofield B, Sampson H, Li XM. Therapeutic effects of a fermented soy product on peanut hypersensitivity is associated with modulation of T-helper type 1 and T-helper type 2 responses. *Clin Exp Allergy* 2008;38:1808–18.
- [3] Manabe M, Tanaka K, Goto T, Matsura S. Producing capability of kojic acid and aflatoxin by koji mould. *Dev Food Sci* 1984;7:4–17.
- [4] Miyake Y, Sasaki S, Ohya Y, Miyamoto S, Matsunaga I, Yoshida T, et al. Soy, isoflavones, and prevalence of allergic rhinitis in Japanese women: the Osaka Maternal and Child Health Study. *J Allergy Clin Immunol* 2005;115:1176–83.
- [5] Kobayashi M. Immunological functions of soy sauce: hypoallergenicity and antiallergic activity of soy sauce. *J Biosci Bioeng* 2005;100:144–51.
- [6] Otsuka Y, Pan W. Effects of the novel symbiotic ImmuBalance as a food supplement in relieving clinical symptoms of Japanese cedar pollinosis: a pilot study. *Clin Exp Pharmacol Physiol* 2007;34:S73–5.
- [7] Beltrani VS. Suggestions regarding a more appropriate understanding of atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2005;5:413–8.
- [8] Leung DY. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol* 2000;105:860–76.
- [9] Yazdanbakhsh M, Kretsinger PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002;296:490–4.
- [10] Farooqi IS, Hopkin JM. Early childhood infection and atopic disorder. *Thorax* 1998;53:927–32.
- [11] Kukkonen K, Savilahti E, Haahela T, Juntunen-Backman K, Korpela R, Poussa T, et al. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 2007;119:192–8.
- [12] Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 2001;357:1076–9.
- [13] 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.
- [14] 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(Suppl. 1):70–5.
- [15] Tanaka T, Tsutsui H, Yoshimoto T, Kotani M, Matsumoto M, Fujita A, et al. Interleukin-18 is elevated in the sera from patients with atopic dermatitis and from atopic dermatitis model mice, NC/Nga. *Int Arch Allergy Immunol* 2001;125:236–40.
- [16] Sawada J, Morita H, Tanaka A, Salminen S, He F, Matsuda H. Ingestion of heat-treated *Lactobacillus rhamnosus* GG prevents development of atopic dermatitis in NC/Nga mice. *Clin Exp Allergy* 2007;37:296–303.
- [17] Tanaka A, Fukushima Y, Benyacoub J, Blum S, Matsuda H. Prophylactic effect of oral administration of *Lactobacillus johnsonii* NCC533 (La1) during the weaning period on atopic dermatitis in NC/NgaTnd mice. *Eur J Dermatol* 2008;18:136–40.
- [18] Tanaka A, Jung K, Benyacoub J, Prioult G, Okamoto N, Ohmori K, et al. Oral supplementation with *Lactobacillus rhamnosus* CGMCC 1.3724 prevents development of atopic dermatitis in NC/NgaTnd mice possibly by modulating local production of IFN- γ . *Exp Dermatol* 2009;18:1022–7.
- [19] Jung BG, Cho SJ, Koh HB, Han DU, Lee BJ. Fermented Maesil (*Prunus mume*) with probiotics inhibits development of atopic dermatitis-like skin lesions in NC/Nga mice. *Vet Dermatol* 2010;21:184–91.
- [20] Wakabayashi H, Nariai C, Takemura F, Nakao W, Fujiwara D. Dietary supplementation with lactic acid bacteria attenuates the development of atopic dermatitis-like skin lesions in NC/Nga mice in a strain-dependent manner. *Int Arch Allergy Immunol* 2008;145:141–51.
- [21] Ogawa T, Hashikawa S, Asai Y, Sakamoto H, Yasuda K, Makimura Y. A new synbiotic, *Lactobacillus casei* subsp. *casei* together with dextran, reduces

- murine and human allergic reaction. *FEMS Immunol Med Microbiol* 2006;46:400–9.
- [22] Watanabe T, Hamada K, Tategaki A, Kishida H, Tanaka H, Kitano M, et al. Oral administration of lactic acid bacteria isolated from traditional South Asian fermented milk 'dahi' inhibits the development of atopic dermatitis in NC/Nga mice. *J Nutr Sci Vitaminol (Tokyo)* 2009;55:271–8.
- [23] Leung DY, Hirsch RL, Schneider L, Moody C, Takaoka R, Li SH, et al. Thymopentin therapy reduces the clinical severity of atopic dermatitis. *J Allergy Clin Immunol* 1990;85:927–33.
- [24] Jung K, Tanaka A, Fujita H, Matsuda A, Oida K, Karasawa K, et al. Peroxisome proliferator-activated receptor gamma-mediated suppression of dendritic cell function prevents the onset of atopic dermatitis in NC/Tnd mice. *J Allergy Clin Immunol* 2011;127:420–9. e1–6.
- [25] Tanaka A, Arai K, Kitamura Y, Matsuda H. Matrix metalloproteinase-9 production, a newly identified function of mast cell progenitors, is downregulated by c-kit receptor activation. *Blood* 1999;94:2390–5.
- [26] Tanaka A, Matsuda H. IgE crosslinkage of Fcepsilon receptor I induces both production and activation of matrix metalloproteinase-9 in mast cells. *Cell Immunol* 2004;228:66–75.
- [27] Pincelli C. Nerve growth factor and keratinocytes: a role in psoriasis. *Eur J Dermatol* 2000;10:85–90.
- [28] Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989;170:2081–95.
- [29] Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683–765.
- [30] de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* 1991;174:915–24.
- [31] Ding L, Linsley PS, Huang LY, Germain RN, Shevach EM. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol* 1993;151:1224–34.
- [32] Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* 1996;184:19–29.
- [33] Wahlgren CF. Itch and atopic dermatitis: clinical and experimental studies. *Acta Derm Venereol Suppl (Stockh)* 1991;165:1–53.
- [34] Tominaga M, Ozawa S, Ogawa H, Takamori K. A hypothetical mechanism of intraepidermal neurite formation in NC/Nga mice with atopic dermatitis. *J Dermatol Sci* 2007;46:199–210.
- [35] Sugiura H, Omoto M, Hirota Y, Danno K, Uehara M. Density and fine structure of peripheral nerves in various skin lesions of atopic dermatitis. *Arch Dermatol Res* 1997;289:125–31.
- [36] Hyun JH, Kim SC, Kang JI, Kim MK, Boo HJ, Kwon JM, et al. Apoptosis inducing activity of fucoidan in HCT-15 colon carcinoma cells. *Biol Pharm Bull* 2009;32:1760–4.
- [37] Kwon MJ, Nam TJ. A polysaccharide of the marine alga *Capsosiphon fulvescens* induces apoptosis in AGS gastric cancer cells via an IGF-IR-mediated PI3K/Akt pathway. *Cell Biol Int* 2007;31:768–75.
- [38] Kim SH, Kim YB, Jeon YT, Lee SC, Song YS. Genistein inhibits cell growth by modulating various mitogen-activated protein kinases and AKT in cervical cancer cells. *Ann N Y Acad Sci* 2009;1171:495–500.
- [39] Park SS, Kim YN, Jeon YK, Kim YA, Kim JE, Kim H, et al. Genistein-induced apoptosis via Akt signaling pathway in anaplastic large-cell lymphoma. *Cancer Chemother Pharmacol* 2005;56:271–8.
- [40] Lee DE, Lee KW, Song NR, Seo SK, Heo YS, Kang NJ, et al. 7,3',4'-Trihydroxyisoflavone inhibits epidermal growth factor-induced proliferation and transformation of JB6 P+ mouse epidermal cells by suppressing cyclin-dependent kinases and phosphatidylinositol 3-kinase. *J Biol Chem* 2010;285:21458–66.

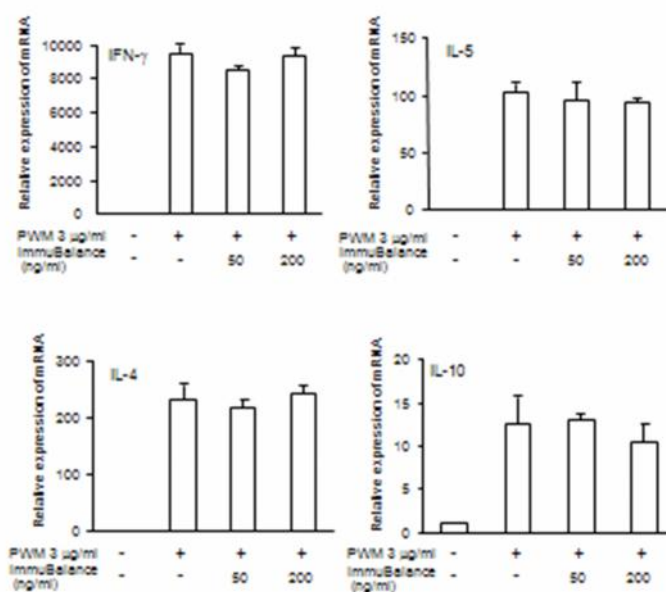


Figure S1 Effects of the low fraction of ImmuBalance extracts on production of inflammatory cytokines in lymphocytes. Messenger RNA expression in the spleen cells cultured with or without PWM and/or the low fraction of ImmuBalance extracts was measured by real-time PCR. The low fraction of ImmuBalance extract had no effects on the production of inflammatory cytokines. Data represent means $\pm$ SE of 3 individual experiments.

Figure S1