Food Science and Technology Research, **27** (5), 725–733, 2021

doi: 10.3136/fstr.27.725

https://jsfst.smoosy.atlas.jp

Original Paper

Protective effects of aqueous extracts of *Rhizopus oryzae* on atopic dermatitis in NC/Nga mice

Takehito SUZUKI*, Yukiko KATO, Arisa KOBAYASHI, and Kurumi SUZUKI

School of Veterinary Medicine, Azabu University, 1-17-71, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

Received December 31, 2020 ; Accepted May 12, 2021

The aqueous extract of *Rhizopus oryzae* U-1 (RU) increases the number of Th1 cells, which defend against *Salmonella* infection and regulate immunity in rats. In this study, we demonstrated the anti-dermatitis effects of RU in a picryl chloride (PiCl)-induced mouse model of atopic dermatitis. RU treatment significantly decreased the time-dependent exacerbation of dermatitis scores and increased the final body weight and food intake. A significant decrease in serum IgE and IL-4 levels confirmed that RU treatment suppressed the exacerbation of atopic dermatitis. Flow cytometric analysis revealed that RU treatment significantly increased $CD4^+$ IFN-γ^+ (Th1) and decreased $CD4^+$ IL-4^+ (IL-4^+ Th) cell numbers. Thus, the oral administration of RU effectively suppressed atopic dermatitis induced by PiCl, and the decrease in the number of $CD4^+$ IL-4^+ cells and serum IL-4 levels may be partially mediated by RU in mice.

Keywords: *Rhizopus oryzae*, atopic dermatitis, helper T cell response, NC/Nga mouse

Introduction

Rhizopus fungi have traditionally been used to produce alcohol in China and tempeh and fermented soy foods in Southeast Asia (Meussen *et al.*, 2012). The aqueous extract of *R. oryzae* U-1 (RU) improves reproductive functions such as fertilization rate, fecundity, and hatchability in quails and chickens (Zhang *et al.*, 1999); prolongs mating season in rats (Higuchi *et al.*, 1979); increases ovarian steroid hormone secretion in rats and rabbits (Higuchi *et al.*, 1979; Horiuchi *et al.*, 1985; Saito, 1980); and improves conception rates in cows (Umezu, 1973). We previously reported that the oral pre-administration of RU effectively suppressed liver injury induced by the oral administration of CCl_4 and that the RU-induced increase in *IGF-I* and *HGF* gene expression may be partially involved in the biological actions of RU in rats (Suzuki *et al.*, 2015). Moreover, oral RU administration protected against *Salmonella* infection by activating peripheral monocytes and improving the Th1/Th2 balance in models of *Salmonella* infection in rats (Suzuki *et al.*, 2007a). The number of viable *Salmonella enteritidis* cells in the liver reduced significantly to approximately 1% of that in the control group; however, the number of viable cells in the spleen and mesenteric lymph nodes was unchanged. Interestingly, oral administration of RU induced a decrease in the number of Th2 cells and activated phagocytes such as macrophages by a relative increase in the number of Th1 cells. For infections caused by intracellular parasites such as *Salmonella*, cell-mediated immunity is important for defense. Cell-mediated immunity involves macrophages and $CD8^+$ cells activated by IFN-α or IL-2 secreted from Th1 cells (Nathan *et al.*, 1983; Pace *et al.*, 1983a; Pace *et al.*, 1983b; Zhang *et al.*, 1998). In cell culture experiments involving human peripheral neutrophils, Zhang *et al.* observed an increased production of superoxide in response to RU treatment. We reported that RU induced apoptosis in the human promyelocytic leukemia cell line HL-60 by activating the caspase cascade, including caspase-3, -8, and -9 (Suzuki *et al.*, 2007b). Thus, the effect of RU in improving adaptive immunity has been established; however, its role in atopic dermatitis (AD) has been examined for the first time in this study.

AD is classified as a type I allergy. An inflammatory response is induced by the release of cytotoxic factors such as reactive oxygen and peroxidase from eosinophils following eosinophilic infiltration into the derma (Carr, 2013; Simon *et*

*To whom correspondence should be addressed. E–mail address: suzuki-t@azabu-u.ac.jp

al., 2004). The hypofunction of the cutaneous barrier caused by the lower water retention ability of the skin and an increase in epidermal transpiration rate exacerbates AD, which is a characteristic of AD. Barrier dysfunction leads to an invasion of environmental antigens such as from ticks or house dust into the epidermis. The engulfment of antigens by antigen-presenting cells causes inflammation. Excessive scratching damages the epidermal cells, which release inflammatory cytokines (Bieber, 2008). At the onset of AD, the type of adaptive immune response is decided by the function of helper T cells. The function of Th2 cells is necessary for AD onset mainly in the acute phase; however, Th1 cells are needed for the chronic phase. Th2 cells produce IL-4, IL-5, and IL-6, which mediate immunoglobulin class switching (Bieber, 2008; Leiferman, 1994). Therefore, when the helper T cell response changes the predominance of Th2 cells, allergies develop as a result of excessive antibody production.

A study on the interaction of intestinal flora and allergy onset or anti-allergic effects of probiotics revealed that the oral administration of *Lactobacillus rhamnosus* GG reduced AD symptoms in pregnant women and newborns (Kalliomäki *et al.*, 2001) and that *Lactobacillus acidophilus* L-92 is effective against AD in adults (Inoue *et al.*, 2014). L-92 induces the production of IL-12 by macrophages and changes the Th1/Th2 balance in favor of Th1. In addition, probiotic administration effectively prevents AD (Lise *et al.*, 1992; Harima-Mizusawa *et al.*, 2015; Kwon *et al.*, 2018;). In addition, fermentation products from fungi such as *Aspergillus* or *Rhizopus* have been reported to reduce symptoms of AD (Matsuda *et al.*, 2012; Kim *et al.*, 2015; Hur *et al.*, 2018). In these reports, AD inhibitory effects were found in extracts with water or low-concentration alcohol from whole fermented products of soybeans and other plants containing fungal organisms. Although RU is a fungal extract, not a fermented extract, it has been found to be effective in promoting Th1 responses (Suzuki *et al.*, 2007a). However, it is unclear whether its RU effect can reduce the symptoms of AD.

For inhibiting or improving the symptoms of AD, the Th2 response, which is induced by the infiltration of immune cells at the site of inflammation, must be regulated. The application of RU locally at the site of dermatitis may effectively inhibit inflammation. We hypothesize the induction of a systemic immunomodulatory effect of helper T cells upon the oral intake of RU, based on our previous findings. To test our hypothesis, we used the Nc/Nga mouse model with picryl chloride-induced AD. The effects of RU administration on AD were analyzed through the immunomodulatory effects of RU on subset analysis of lymphocytes derived from the spleen, such as $CD4^+$ IFN-γ^+ (Th1) and $CD4^+$ IL-4^+ (IL-4^+ Th) cells and serum IFN-γ, IL-4, and IgE levels.

Materials and Methods

Reagents 2-Chloro-1,3,5-trinitrobenzene (picryl chloride; PiCl) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PiCl was recrystallized from 100 % ethanol solution for purification and shielded from light.

Fungal strain and aqueous fungus extracts *R. oryzae* U-1 was isolated from fermentation using ground barley and bran as the culture medium. *R. oryzae* U-1 was cultured for 4 d at 24 °C in malt extract medium (20 g malt extract, 1 g polypeptone, and 20 g glucose in one liter). The fungal cells were collected, suspended in water (10 g/100 mL) at 45 °C, stirred for 30 min, and centrifuged at 3 000 × *g* for 20 min. The supernatant was concentrated in vacuo to produce a dried powder. A working solution of the aqueous extract of RU was produced by dissolving the powder in distilled phosphate-buffered saline (20 mg/mL).

Mice The animals used for this study were 7-week-old, specific pathogen-free, male NC/Nga mice (Charles River Laboratories Japan, Inc., Kanagawa, Japan) weighing 24.2 ± 0.4 g. They were maintained on a commercial diet (CE-2; CLEA Japan Inc., Tokyo, Japan) and tap water ad libitum and kept at a temperature of 22 ± 2 °C, with a relative humidity of 55 % ± 10 % and 12 h light:12 h dark. The 12 animals were divided into two groups; one was sensitized and challenged to develop AD (AD control group; n = 6) and the other was sensitized, challenged to develop AD, and treated with RU (RU group; n = 6).

Model of AD in NC/Nga mice Schematic procedure for the induction of AD-like skin lesions and oral administration of RU in NC/Nga mice is shown in Fig. 1. AD was induced with PiCl in NC/Nga mice according to the instructions provided by Charles River, Japan. For inducing AD-like skin lesions, 150 μL of 5 % (w/v) PiCl in acetone/ethanol (4:1) was applied to the thoracic and abdominal areas and footpads using a micropipette-nose (sensitization). The challenge was performed 3 d the sensitization. A total of 150 μL of 1 % (w/v) PiCl in olive oil was applied to the back and both ears of mice. This procedure was repeated once a week for up to 6 weeks. RU was administered orally at a dose of 0.5 mL/kg (10 mg/kg) using a gastric tube once a day for 10 consecutive days after the fifth challenge. The AD control group was administered an equal volume of phosphate-buffered saline. Body weight and food intake were measured each day from the start of RU administration to the end of the experiment. All procedures were performed under the guidance of the Committee for Animal Experimentation at Azabu University (No. 130410-2).

Scoring of dermatitis The extent of dermatitis was evaluated based on (I) skin flaring and hemorrhage, (II) crust formation and xerosis cutis, (III) edema, and (IV) excoriation and erosion in the pinna and back skin and scored as follows: 0 = no symptoms, 1 = mild, 2 = moderate, and 3 = severe. The

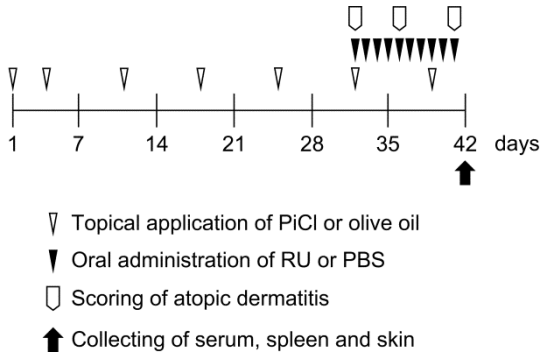

Fig. 1. Experimental design of Rhizopus oryzae U-1 aqueous extract (RU) application in atopic dermatitis (AD) model mice. To induce AD-like dermatitis, sensitization was performed by applying 150 μL of 5 % (w/v) PiCl in acetone/ethanol (4:1 mixture) to the thoracic and abdominal areas and footpads. The challenge was performed after sensitization. A total of 150 μL of 1 % (w/v) PiCl in olive oil was applied to the back and both ears. This procedure was repeated once a week for up to 6 weeks. Mice were either treated with RU or PBS alone for 10 d following the fifth application of PiCl.

total score of pinna and back skin evaluation were considered the dermatitis score.

Determination of serum IgE and cytokine The levels of serum IgE, IL-4, IL-10, IFN-γ, and PGE2 in were determined using commercial ELISA kits (Shibayagi Co., Ltd., Gunma, Japan; Diaclone SAS, Besançon, France; Cayman Chemical Co., Ann Arbor, MI, United States), according to the manufacturer's protocol.

Subset analysis of helper T cells Lymphocytes were purified from pooled mice spleens by density-gradient centrifugation using Percoll (GE Healthcare UK Ltd., Amersham Place, England), seeded at 1×10^6 cells/flask in 25 cm^2 culture flasks, and stimulated with phorbol 12-myristate 13-acetate (final concentration 40 ng/mL) and ionomycin (final concentration 4 μg/mL) for 12 h at 37 °C. Brefeldin A (final concentration 2 μM) was added to the cultures 2 h before cell harvesting. The cells were washed once with PBS and stained with PE-Cy7 conjugated anti-mouse CD4 antibody (BD Bioscience, Flanklin, NJ, United States) for 30 min at 22 °C. The cells were fixed with 4 % formaldehyde for 20 min and permeabilized with 0.5 % saponin PBS solution containing 0.5 % BSA and 0.1 % sodium azide for 10 min at RT. Intracellular cytokines were stained with PreCP-Cy5.5-conjugated anti-mouse IFN-γ antibody and PE conjugated anti-mouse IL4 antibody for 30 min at RT. Antibody staining was analyzed using a EC800 flow cytometer (Sony Imaging Products & Solutions Inc., Tokyo, Japan) on at least 10 000 events, using the software provided.

Statistical analysis Data are expressed as means ± SEM. Differences among groups of mice were assessed using Student's t test and repeated-measures ANOVA (using the Bonferroni multiple comparison). *P*-values < 0.05 were considered statistically significant.

Results

Effect of RU on AD skin lesions in NC/Nga mice The effect of RU on the development of AD-like symptoms by PiCl was evaluated in NC/Nga mice. Typical AD lesions are shown in Fig. 2A. In the RU group, skin lesions (erythema, crust formation, and edema) on the back and pinna in the AD control group were mild. No hemorrhage and tissue defects were observed in the pinna. The dermatitis score was evaluated during 10 d of RU administration. The dermatitis score progressively increased with time. RU prevented this increase, and the difference from the AD control group increased depending on the number of doses (Fig. 2B). The time-dependent change of dermatitis score was significant in both groups, but the increment was significantly smaller in the RU group than in the AD control group ($p < 0.05$).

Effect of RU on growth parameters in NC/Nga mice The effects of RU on growth parameters (initial body weight, final body weight, daily gain, and food intake) are shown in Table 1. Final body weight and food intake in the RU group were significantly higher than those in the AD control group.

Effect of RU on serum IgE and cytokine levels in NC/Nga mice Fig. 3 shows the serum levels of IgE (A) and IL-4 (B) following the end of RU administration. Total serum IgE levels in the AD control and RU groups were approximately 40 936 and 29 698 ng/mL, respectively. IgE levels in the RU group were significantly lower ($p < 0.05$) than those in the AD control group. Serum IL-4 levels in the AD control and RU groups were approximately 42 and 17 ng/mL, respectively. IL-4 levels in the RU group were significantly lower ($p < 0.05$) than that in the AD control group. By contrast, no significant difference in serum IL-10, IFN-γ, and PGE2 levels were observed between mice in both groups.

RU regulates CD4+ T cell proliferation and differentiation Lymphocytes prepared from the spleen were

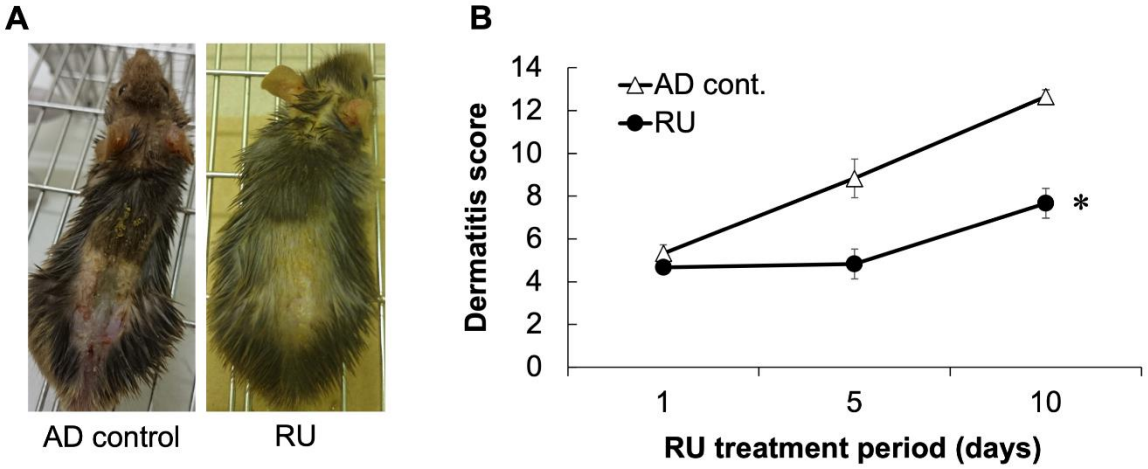

Fig. 2. Effect of Rhizopus oryzae U-1 aqueous extract (RU) treatment on the development of atopic dermatitis (AD)-like skin lesions in NC/Nga mice. (A) Photographs were taken on the last day of the experimental period. (B) The dermatitis scores of mice were evaluated on days 1, 5, and 10 of RU administration. The scores are expressed as the mean ± SEM of six individual animals. Open triangle and closed circle represent the AD control group and RU group, respectively. Data that differ significantly ($p < 0.05$) are indicated by an asterisk on the right side of the line vs. AD control group. Repeated-measures ANOVA (using the Bonferroni multiple comparison) was performed.

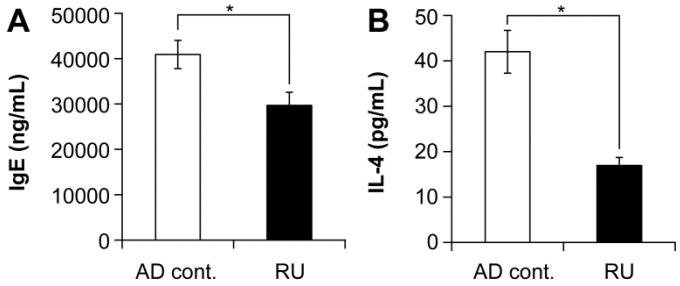

Fig. 3. Suppression of PiCl-induced increased of serum IgE and IL-4 levels by Rhizopus oryzae U-1 aqueous extract (RU). Levels of serum IgE (A) and IL-4 (B) were measured as sensitive markers for atopic dermatitis (AD) after PiCl application in the absence or presence of RU treatment. Data are expressed as means ± SEM of six individual animals. Open and closed bars represent AD control group and RU administration group, respectively. Data that differ significantly ($p < 0.05$) are indicated by an asterisk at the top of the bar.

stained with antibodies specific for CD4, IFN-γ, and IL-4. Fig. 4A shows typical flow cytometric histograms in the absence or presence of RU treatment, and Fig. 4B shows the ratio of helper T cell subsets (Th1: $CD4^+$, IFN-γ^+ or IL-4^+ Th: $CD4^+$, IL-4^+) in 10 000 counts of total lymphocytes. Comparing both histograms, RU treatment reduced IFN-γ-negative and IL-4-positive cells (lower right quadrant), shifting the differentiated subset from IL-4^+ Th cells to Th1. The Th1 cells in the RU group were 2.5 %, which significantly increased compared to 0.3 % in the AD control group ($p < 0.05$). IL-4^+ Th cells were significantly reduced to 9.4 % compared to 41.4 % in the AD control group ($p < 0.05$).

Discussion

Our findings revealed that RU suppresses AD-like dermatitis induced by PiCl application in mice: 1) Treatment (oral administration) of RU significantly suppressed the PiCl-induced increase in dermatitis score (Fig. 2). 2) Final body weight and food intake were significantly increased by RU treatment (Table. 1). 3) The levels of serum markers for AD, such as IgE and IL-4, decreased upon RU administration (Fig. 3). RU treatment improved the helper T cell ratio (significant increase in Th1 cells and significant decrease in Th2 cells) in the spleen (Fig. 4).

In NC/Nga mice, dermatitis symptoms are progressively exacerbated after 6 weeks (Matsuda *et al.*, 1997). In this study,

Table 1. Effects of *Rhizopus oryzae* U-1 aqueous extract on growth parameters in NC/Nga mice.

	AD control	RU
Body weight (g)		
Initial	24.3 ± 0.7	24.2 ± 0.5
Final	24.8 ± 0.7	26.8 ± 0.3*
Daily gain (g)	0.06 ± 0.22	0.19 ± 0.20
Food intake (g/day)	5.14 ± 0.15	5.94 ± 0.23*

Data are expressed as means ± SEM of six individual animals and data that differ significantly ($p < 0.05$) are indicated by an asterisk.

Body weight was measured on the first and last days of the *Rhizopus oryzae* U-1 aqueous extract (RU) treatment.

Daily gain and food intake are shown as per day during RU treatment.

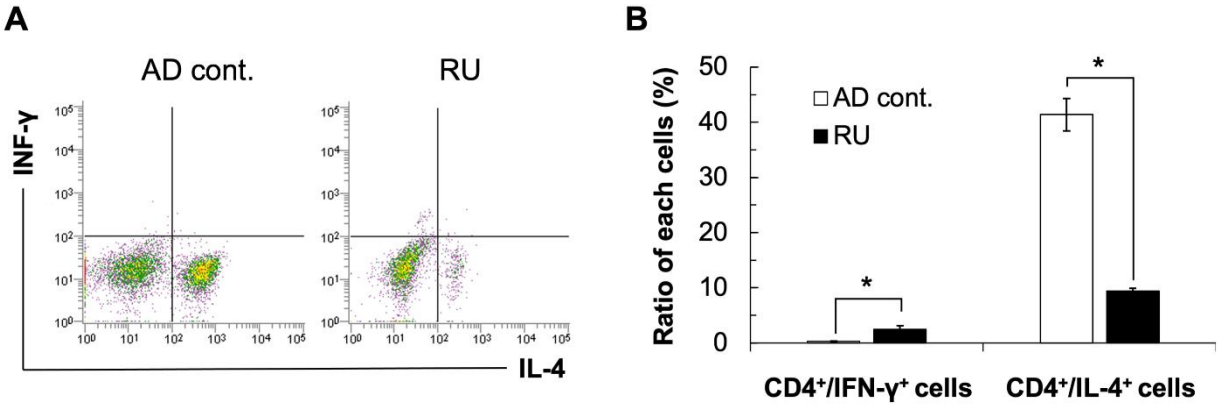

Fig. 4. Change in the population of Th cell subsets upon Rhizopus oryzae U-1 aqueous extract (RU) treatment in NC/Nga mice. (A) Representative flow cytometric histograms indicating $CD4^+$/ IFN-γ^+ (Th1) and $CD4^+$/ IL-4^+ (IL-4^+ Th) cells. The plots were pregated on $CD4^+$ cells and examined by the levels of IFN-γ or IL-4. (B) Ratio of $CD4^+$/ IFN-γ^+ (Th1) cells and $CD4^+$/ IL-4^+ (IL-4^+ Th) cells in total lymphocytes. Data are expressed as means ± SEM of six individual animals. Open and closed bars represent the atopic dermatitis (AD) control and RU administration groups, respectively. Data that differ significantly ($p < 0.05$) are indicated by an asterisk at the top of the bar.

the experiment was completed with the sixth application of PiCl, but AD-like dermatitis appeared in the middle of the exacerbation period, and we expect the symptoms to worsen further if the study period is extended. Therefore, the improvement of dermatitis by RU administration is not due to the disappearance of PiCl-induced AD and spontaneous remission. Notably, the improvement of dermatitis occurred through oral administration and not local application of RU, because RU administration improves AD through changes in immune cell action. Food intake and daily weight gain are suppressed in PiCl-induced AD NC/Nga mice (Seino *et al.*, 2012). Improvements in growth parameters such as high final body weight and food intake in RU-administered mice suggest reduction of stress due to dermatitis. In addition, in PiCl-induced AD NC/Nga mice, the up-regulation of Cdkn1a inhibits the cell cycle and down-regulation of PPARα Acox1, and Cpt2 suppresses β-oxidation of fatty acids and inhibits lipid metabolism to induce the formation of fatty liver (Seino *et al.*, 2012). The promotion of hepatocyte proliferation and suppression of fatty liver by RU (Suzuki *et al*., 2015) may have some effect on AD improvement, but we have not conducted those examinations in this study.

RU was reported to shift the Th1/Th2 balance to Th1 and protect against infection in the *Salmonella* infection model (Suzuki *et al.*, 2007a). The effect of RU on immune cells is expected to be exerted in AD. The number of IL-4 producing (Th2 or Tfh) cells gradually increased in NC/Nga mice as skin inflammation was exacerbated with the induction of AD (Kim *et al.*, 2011) or human allergic disorder (Kamekura *et al.*, 2015). Naive helper T cells differentiated into subclasses, such as Th1 and Th2 cells, upon antigen stimulation and cytokine release but were eliminated by apoptosis if antigen stimulation was not performed for approximately 1 week (Hildeman *et al.*, 2002). In this study, AD-like skin lesions exacerbated with

time because the mice were continuously stimulated with PiCl every week. Therefore, in the AD control group, the symptoms of dermatitis progressively worsened after the fifth application of PiCl (Fig. 2B). However, in the RU group, the dermatitis score at the fifth application of PiCl was maintained for 5 d, and the increase in the dermatitis score was significantly smaller than that in the AD control group. In our previous report, RU induced the differentiation of naive helper T cells into Th1 cells by activating innate immune cells such as monocytes (Suzuki *et al.*, 2007a) and induced apoptosis in leukocytes *in vitro* (Suzuki *et al.*, 2007b). Thus, RU treatment induces the IL-4 producing cells to apoptose and causes the differentiation of naive Th cells into Th1 cells through the components of the innate immune system, such as monocytes and macrophages. Indeed, RU administration significantly reduced the levels of serum IL-4 and IgE, which are produced by activated B cells (Fig. 3A, B). However, no difference in the serum levels of IFN-γ and PGE2, which promote differentiation into Th1 cells through the PI3K pathway, was found between both groups.

Unlike Tfh cells, Th2 cells cannot localize to lymphoid follicles, where B cells differentiate into antibody-producing cells (Fazilleau *et al.*, 2009; Harada *et al.*, 2012; Vijayanand *et al.*, 2012). Although $CD4^+$ $IL\text{-}4^+$ cells are classified as Th2 cells and Tfh cells, they could not be distinguished in this study. However, IL-4 induces a class switch from IgM to IgG1 and IgE in B cells and the differentiation of B cells into plasma cells and memory B cells (Howard *et al.*, 1982). Moreover, peripheral blood Th2 cells were reported to invade the lymphoid follicles and differentiate into Tfh cells (Glatman Zaretsky *et al.*, 2009).

Meanwhile, it has been recently reported that IL-31 produced by Th2 cells is involved in the expression of pruritus in AD (Dillon *et al.*, 2004, Bilsborough *et al.*, 2006), and scratching behavior is reduced in AD model mice in which the IL-31 receptor is knocked out (Dillon *et al.*, 2004, Sakata *et al.*, 2019). In the AD patients, suppression of pruritus has a great effect on improving the QOL. However, in this study, IL-31 gene expression in the local of dermatitis and blood IL-31 concentration were not measured. In the cytokines produced by Th2 cells, IL-31 is an important and essential substance for the expression of pruritus, but then, IL-4 induce class switching of IgE (Delphin *et al.*, 1995) and together with IL-13 damages the skin barrier function by reduce of filaggrin production (Cha *et al.*, 2019, Furue, 2020). Importantly, all of these cytokines are produced by Th2 cells and exerts negative effect on the exacerbation of AD. RU administration significantly reduces the number of IL-4 producing cells (including Th2 cells), blood IL-4 concentration, IgE level, and improves dermatitis score. Therefore, it is considered that RU administration improves AD by regulating the differentiation of helper T cells but not block the pruritus caused by IL-31 in local. This effect of RU may be difficult to improve AD such as immediate pruritus control, but it may be possible to expect a fundamental improvement from a long-term perspective. However, suppression of pruritus is a high priority in the treatment of AD, and it is a very interesting issue whether RU can suppress pruritus in AD.

In addition, there are some very interesting findings regarding Th2 cell differentiation recently. Treg subset that specifically suppresses differentiation into Th2 cells plays an important role in suppressing the Th2 reaction (Curotto *et al.*, 2001, Zheng *et al.*, 2009, Rudra *et al.*, 2012). Meanwhile, it has been reported that Treg cells are reprogrammed into Th2-like cells under inflammatory of Th2 response and promote inflammation by producing IL-4 (Noval *et al.*, 2015). The relationship between these Treg cells and Th2 reaction plays a very important role in the promotion or suppression of AD. In the present study, it has not been possible to determine whether the suppression or promotion mechanism of AD by these functions of Treg cells is involved. Therefore, there is no doubt that the fact that Th2 response is suppressed by RU administration is a major factor in improving AD, but the midstream of the Th2 response suppression mechanism by RU has not been elucidated.

As shown in this study, RU has the effect of alleviating the AD symptoms. RU is a fungal culture extract and is not a purified substance; it is a mixture of various fungus-derived components, but the active ingredients in RU have not been identified. In fermented extracts of *Aspergillus* or *Rhizopus*, which have been reported to have anti-AD effects, the involvement of water-soluble polysaccharides as active ingredients has been suggested (Matsuda *et al.*, 2012; Kim *et al.*, 2015; Hur *et al.*, 2018). Meanwhile, the immune cell activation mechanisms of fungi themselves have been studied in pathogenic fungi, and it has been reported that innate immune responses, mainly phagocytic cells, and adaptive immune responses, mainly helper T cells, are important for infection defense (Bartemes and Kita, 2018). Specific structures such as β-glucan, mannan, and chitin have been reported as water-soluble fungal components recognized by pattern recognition receptors (PRRs) such as Toll-like receptors on antigen presenting cells (Levitz, 2010), and a glycoprotein recognized by the C-type lectin receptor called Dectin-2 has also been newly reported (Ishikawa *et al.*, 2013). Meanwhile, many fungi such as *Aspergillus* and *Candida* are characterized by the presence of β-glucans and chitin as cell wall polysaccharides, while chitin and chitosan are the major polysaccharides of cell wall components in zygomycetes including *Rhizopus*. Chitin is a β1,4-polymer of N-acetylglucosamine in which the hydroxyl group at position 2 of glucose is replaced by an acetylamino group, and chitosan

is a β1,4-polymer of glucosamine in which N-acetylglucosamine is deacetylated. (Younes and Rinaudo. 2015; Gow *et al.*, 2016). N-acetylglucosamine is important as an antigen recognition mechanism in innate immunity by specifically binding to mannan-binding protein (MBP), a component of complement, and activating the complement system. (Kilpatrick, 2003; Nakagawa *et al.*, 2003). MBP is also strongly expressed in the small intestine, suggesting its involvement in mucosal immunity (Uemura *et al.*, 2002; Nakagawa *et al.*, 2003). Administration of N-acetylglucosamine has also been reported to have immunomodulatory effects, activating NK cells and increasing their production of IFN-γ in mice and humans (Matheson *et al.*, 1984; Hulikova *et al.*, 2011).

Since RU is a water-extracted fungal culture, there is a high possibility of containing high levels of polysaccharides derived from the fungal cell wall and monosaccharides derived from it as described above. In some fungal species, such as *Aspergillus*, β-glucans are located inside the mannan layer, which is less permeable and porous in living fungal cell, and may be hardly recognized by PRRs depending on the fungal condition (Brakhage *et al.*, 2010; Gow and Hube, 2012). Since RU does not contain viable fungi, it is likely that these endogenous polysaccharides are exposed during the extraction process and may be involved in the functionality of RU.

In conclusion, RU treatment effectively improve AD in NC/Nga mice, which involved suppression of IgE production from B cells following significant increase of Th1 cells and significant reduction of the number of IL-4^+ Th cells and serum IL-4 levels. However, helper T cells subset that produces IL-4 on which RU acts is unknown.

Acknowledgements The authors would like to thank Mr. Setsuo Ushikoshi and Koichiro Ushikoshi for providing aqueous extracts of *R. oryzae*. We also thank Enago (www.enago.jp) for the English language review. This work was supported by cooperative research expenses from Ushikoshi Research Institute for Physiology Co., Ltd.

Conflict of interest There are no conflicts of interest to declare.

References

Bartemes, K.R. and Kita, H. (2018). Innate and adaptive immune responses to fungi in the airway. *J. Allergy Clin. Immunol.*, **142**, 353–363.

Bieber, T. (2008). Atopic dermatitis. *N. Engl. J. Med*., **358**, 1483–1494.

Bilsborough, J., Leung, D.Y., Maurer, M., Howell, M., Boguniewcz, M., Yao, L., Storey, H., LeCiel, C., Harder, B., and Gross, J.A. (2006). IL-31 is associated with cutaneous lymphocyte antigenpositive skin homing T cells in patients with atopic dermatitis. *J. Allergy Clin. Immunol.*, **117**, 418–425.

Brakhage, A.A., Bruns, S., Thywissen, A., Zipfel, P.F., and Behnsen, J. (2010). Interaction of phagocytes with filamentous fungi. *Curr. Opin. Microbiol.*, **13**, 409–415.

Carr, W.W. (2013). Topical Calcineurin Inhibitors for Atopic Dermatitis: Review and Treatment Recommendations. *Paediatr. Drugs*, **15**, 303–310.

Cha, K.J., Song, C.S., Lee, J.S., Kashif, A., Hong, M.H., Kim, G., and Kim, I.S. (2019). Chaenomeles sinensis Koehne extract suppresses the development of atopic dermatitis-like lesions by regulating cytokine and filaggrin expression in NC/Nga mice. *Int. J. Med. Sci*., **16**, 1604–1613.

Curotto, M.A., Muriglan, S., Sunshine, M.J., Lei, Y., Kutchukhidze, N., Furtado, G.C., Wensky, A.K., Olivares-Villagómez, D., and Lafaille, J.J. (2001). Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. *J. Exp. Med.*, **194**, 1349–1359.

Delphin, S. and Stavnezer, J. (1995). Regulation of antibody class switching to IgE: characterization of an IL-4-responsive region in the immunoglobulin heavy-chain germline epsilon promoter. *Ann. N. Y. Acad. Sci.*, **29**, 123–135.

Dillon, S.R., Sprecher, C., Hammond, A., Bilsborough, J., Rosenfeld-Franklin, M., Presnell, S.R., Haugen, H.S., Maurer, M., Harder, B., Johnston, J., Bort, S., Mudri, S., Kuijper, J.L., Bukowski, T., Shea, P., Dong, D.L., Dasovich, M., Grant, F.J., Lockwood, L., Levin, S.D., LeCiel, C., Waggie, K., Day, H., Topouzis, S., Kramer, J., Kuestner, R., Chen, Z., Foster, D., Parrish-Novak, J., and Gross, J.A. (2004). Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat. Immunol.*, **5**, 752–760.

Fazilleau, N., Mark, L., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2009). Follicular helper T cells: lineage and location. *Immunity*, **30**, 324–335.

Furue, M. (2020). Regulation of Skin Barrier Function via Competition between AHR Axis versus IL-13/IL-4-JAK-STAT6/STAT3 Axis: Pathogenic and Therapeutic Implications in Atopic Dermatitis. *J. Clin. Med.*, **9**, 3741.

Glatman, Zaretsky, A., Taylor, J.J., King, I.L., Marshall, F.A., Mohrs, M., and Pearce, E.J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J. Exp. Med*., **206**, 991–999.

Gow, N.A. and Hube, B. (2012). Importance of the Candida albicans cell wall during commensalism and infection. *Curr. Opin. Microbiol*. **15**, 406–412.

Gow, N.A.R., Latge, J.P., and Munro, C.A. (2017). The Fungal Cell Wall: Structure, Biosynthesis, and Function. *Microbiol Spectr*. **5**.

Harada, Y., Tanaka, S., Motomura, Y., Harada, Y., Ohno, S., Ohno, S., Yanagi, Y., Inoue, H., and Kubo, M. (2012). The 3 ' Enhancer CNS2 Is a Critical Regulator of Interleukin-4-Mediated Humoral Immunity in Follicular Helper T Cells. *Immunity*, **36**, 188–200.

Harima-Mizusawa, N., Kamachi, K., Kano, M., Nozaki, D., Uetake, T., Yokomizo, Y., Nagino, T., Tanaka, A., Miyazaki, K., and Nakamura, S. (2016). Beneficial effects of citrus juice fermented with Lactobacillus plantarum YIT 0132 on atopic dermatitis: results of daily intake by adult patients in two open trials. *Biosci. Microbiota. Food Health*, **35**, 29–39.

Higuchi, T., Seto, K., Hayashi, R., Kojima, H., Saito, H., and Yoshimatsu, K. (1979). Influence of the treatment with gonadotrophin and Rhizopus extract on the LH action in proestrus rats. *Med. Biol*., **99**, 145–147.

Hildeman, D.A., Zhu, Y., Mitchell, T.C., Bouillet, P., Strasser, A., Kappler, J., and Marrack, P. (2002). Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity*, **16**, 759–767.

Horiuchi, C., Tabe, M., Ushikoshi, A., Ushikoshi, S., Ushikoshi, K., Ushikoshi, I., and Seto, K. (1985). The influence of the treatment with gonadotrophin and Rhizopus extracts on LH action in DI rats. *Med. Biol*., **110**, 27–29.

Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T., and Paul, W. E. (1982). Identification of a T cell-derived b cell growth factor distinct from interleukin 2. *J. Exp. Med*., **155**, 914–923.

Hulikova, K., Svoboda, J., Benson, V., Grobarova, V., and Fiserova, A. (2011). N-acetyl-D-glucosamine-coated polyamidoamine dendrimer promotes tumor-specific B cell responses via natural killer cell activation. *Int. Immunopharmacol*., **11**, 955–961.

Hur, S.S., Choi, S.W., Lee, D.R., Park, J.H., and Chung, T.H. (2018). Advanced Effect of Moringa oleifera Bioconversion by Rhizopus oligosporus on the Treatment of Atopic Dermatitis: Preliminary Study. *Evid. Based Complement. Alternat. Med.*, **17**, 7827565.

Inoue, Y., Kambara, T., Murata, N., Komori-Yamaguchi, J., Matsukura, S., Takahashi, Y., Ikezawa, Z., and Aihara, M. (2014). Effects of oral administration of Lactobacillus acidophilus L-92 on the symptoms and serum cytokines of atopic dermatitis in Japanese adults: a double-blind, randomized, clinical trial. *Int. Arch. Allergy Immunol*., **165**, 247–254.

Ishikawa, T., Itoh, F., Yoshida, S., Saijo, S., Matsuzawa, T., Gonoi, T., Saito, T., Okawa, Y., Shibata, N., Miyamoto, T., and Yamasaki, S. (2013). Identification of distinct ligands for the C-type lectin receptors Mincle and Dectin-2 in the pathogenic fungus Malassezia. *Cell Host Microbe*, **13**, 477–488.

Kalliomäki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., and Isolauri, E. (2001). Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet*, **357**, 1076–1079.

Kamekura, R., Shigehara, K., Miyajima, S., Jitsukawa, S., Kawata, K., Yamashita, K., Nagaya, T., Kumagai, A., Sato, A., Matsumiya, H., Ogasawara, N., Seki, N., Takano, K., Kokai, Y., Takahashi, H., Himi, T., and Ichimiya, S. (2015). Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma. *Clin. Immunol*., **158**, 204–211.

Kilpatrick, D.C. (2003). Introduction to mannan-binding lectin. *Biochem. Soc. Trans.*, **31**, 745–747.

Kim, C.H., Cheong, K.A., Park, C.D., and Lee, A.Y. (2011). Glucosamine improved atopic dermatitis-like skin lesions in NC/Nga mice by inhibition of Th2 cell development. *Scand. J. Immunol*., **73**, 536–545.

Kim, O.K., Chang, J.Y., Nam, D.E., Park, Y.K., Jun, W., and Lee, J. (2015). Effect of Canavalia gladiata Extract Fermented with Aspergillus oryzae on the Development of Atopic Dermatitis in NC/Nga Mice. *Int. Arch. Allergy Immunol.* **168**, 79–89.

Kwon, M.S., Lim, S.K., Jang, J.Y., Lee, J., Park, H.K., Kim, N., Yun, M., Shin, M.Y., Jo, H.E., Oh, Y.J., Roh, S.W., and Choi, H.J. (2018). Lactobacillus sakei WIKIM30 Ameliorates Atopic Dermatitis-Like Skin Lesions by Inducing Regulatory T Cells and Altering Gut Microbiota Structure in Mice. *Front. Immunol*., **9**, 1905.

Leiferman, K.M. (1994). Eosinophils in atopic dermatitis. *J. Allergy Clinic. Immunol*., **94**, 1310–1317.

Levitz, S.M. (2010). Innate recognition of fungal cell walls. *PLoS Pathog*., **6**, e1000758.

Lise, M., Mayer, I., and Silveira, M. (1992). Use of probiotics in atopic dermatitis. *Rev. Assoc. Med. Bras.* **64**, 997–1001.

Matheson, D.S., Green, B.J. and Friedman, S.J. (1984). Effect of D-glucosamine on human natural killer activity in vitro. *J. Biol. Response Mod.*, **3**, 445–453.

Matsuda, A., Tanaka, A., Pan, W., Okamoto, N., Oida, K., Kingyo, N., Amagai, Y., Xia, Y., Jang, H., Nishikawa, S., Kajiwara, N., Ahn, G., Ohmori, K., and Matsuda, H. (2012). Supplementation of the fermented soy product ImmuBalance effectively reduces itching behavior of atopic NC/Tnd mice. *J. Dermatol. Sci.*, **67**,130–139.

Matsuda, H., Watanabe, N., Geba, G.P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P.W., and Ra, C. (1997). Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int. Immunol*., **9**, 461–466.

Meussen, B.J., de Graaff, L.H., Sanders, J.P.M., and Weusthuis, R.A. (2012). Metabolic engineering of *Rhizopus*

oryzae for the production of platform chemicals. *Appl. Microbiol. Biotechnol*., **94**, 875–886.

Nakagawa, T., Ma, B.Y., Uemura, K., Oka, S., Kawasaki, N., and Kawasaki, T. (2003). Role of mannan-binding protein, MBP, in innate immunity. *Anticancer Res.*, **23**, 4467–4471.

Nathan, C.F., Murray, H.W., Wiebe, M.E., and Rubin, B.Y. (1983). Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med*., **158**, 670–689.

Noval, R.M., Burton, O.T., Wise, P., Charbonnier, L.M., Georgiev, P., Oettgen, H.C., Rachid, R., and Chatila, T.A. (2015). Regulatory T cell reprogramming toward a Th2-cell-like lineage impairs oral tolerance and promotes food allergy. *Immunity*. **42**, 512–523.

Pace, J.L., Russell, S.W., Schreiber, R.D., Altman, A., and Katz, D.H. (1983a). Macrophage activation: priming activity from a T-cell hybridoma is attributable to interferon-gamma. *PNAS*, **80**, 3782–3786.

Pace, J.L., Russell, S.W., Torres, B.A., Johnson, H. M., and Gray, P.W. (1983b). Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. *J. Immunol*., **130**, 2011–2013.

Rudra, D., deRoos, P., Chaudhry, A., Niec, R.E., Arvey, A., Samstein, R.M., Leslie, C., Shaffer, S.A., Goodlett, D.R., and Rudensky, A.Y. (2012).Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat. Immunol.*, **13**, 1010–1019.

Saito, H. (1980). Influence of the treatment with gonadotrophin and Rhizopus extract on the FSH action in proestrus rats. *Med. Biol.*, **100**, 101–103.

Sakata, D., Uruno, T., Matsubara, K., Andoh, T., Yamamura, K., Magoshi, Y., Kunimura, K., Kamikaseda, Y., Furue, M., and Fukui, Y. (2019). Selective role of neurokinin B in IL-31-induced itch response in mice. *J. Allergy Clin. Immunol.*, **144**, 1130–1133.

Seino, S., Tanaka, Y., Honma, T., Yanaka, M., Sato, K., Shinohara, N., Ito, J., Tsuduki, T., Nakagawa, K., Miyazawa, T., and Ikeda, I. (2012). Atopic dermatitis causes lipid accumulation in the liver of NC/Nga mouse. *J. Clin. Biochem. Nutr*., **50**, 152–157.

Simon, D., Braathen, L.R., and Simon, H.U. (2004). Eosinophils and atopic dermatitis. *Allergy*, **59**, 561–570.

Suzuki, T., Fukuoka, H., Ushikoshi, S. and Morita, H. (2007a). Potency of Water Extracts of Rhizopus oryzae on the Salmonella Infection Rat. *Food Sci. Technol. Res*., **13**, 235–240.

Suzuki, T., Fukuoka, H., Ushikoshi, S., Sato, R., Morita, H., and Takizawa, T. (2015). Protective effect of aqueous extracts from Rhizopus oryzae on liver injury induced by carbon tetrachloride in rats. *Anim. Sci. J*., **86**, 532–540.

Suzuki, T., Ushikoshi, S., and Morita, H. (2007b). Aqueous extracts of Rhizopus oryzae induce apoptosis in human promyelocytic leukemia cell line HL-60. *J. Health Sci*., **6**, 760–765.

Uemura, K., Saka, M., Nakagawa, T., Kawasaki, N., Thiel, S., Jensenius, J.C., and Kawasaki, T.J. (2002). L-MBP is expressed in epithelial cells of mouse small intestine. *Immunol.*, **169**, 6945–6950.

Umezu, M. (1973). Increment of pregnant rats by RV administrations in cow of reproductive disease. *J. Anim. Reprod.*, **19**, 67–76.

Vijayanand, P., Seumois, G., Simpson, L.J., Abdul-Wajid, S., Baumjohann, D., Panduro, M., Huang, X., Interlandi, J., Djuretic, I.M., Brown, D.R., Sharpe, A.H., Rao, A., and Ansel, K.M. (2012). Interleukin-4 production by follicular helper T cells requires the conserved Il4 enhancer hypersensitivity site V. *Immunity*, **36**, 175–187.

Younes, I. and Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Mar, Drugs*. **13**, 1133–74.

Zhang, J., Sugahara, K., Ushikoshi, I., Ushikoshi, S., Seto, K., and Kodama, H. (1999). Effect of water extracts of Rhizopus delemar on the stimulus coupled responses of neutrophils and their modulation by various protein kinase inhibitors. *Clinica. Chimica. Acta*., **282**, 89–100.

Zhang, X., Sun, S., Hwang, I., Tough, D.F., and Sprent, J. (1998). Potent and Selective Stimulation of Memory-Phenotype CD8+ T Cells In Vivo by IL-15. *Immunity*, **8**, 591–599.

Zheng, Y., Chaudhry, A., Kas, A., deRoos, P., Kim, J.M., Chu, T.T., Corcoran, L., Treuting, P., Klein, U., and Rudensky, A.Y. (2009). Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T_H2 responses. *Nature*., **458**, 351–356.