Allergology International. 2013;62:65-76
DOI: 10.2332/allergolint.12-OA-0473

ORIGINAL ARTICLE

Therapeutic Effects of β1, 4 Mannobiose in a Balb/c Mouse Model of Intranasally-Induced Pollen Allergy

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ABSTRACT

Background: Nutritional prebiotic supplementation represents an attractive approach for interventions of allergy. In this study, the potential therapeutic effect of β-1, 4 mannobiose (MNB) in a murine model of cedar pollinosis was investigated.

Methods: Groups of Balb/c mice were intranasally sensitized to Japanese cedar pollen extract, and subsequently administered with low or high dose MNB. Both intraperitoneal and intranasal challenges were performed to monitor for clinical signs. Frequency of sneezing was recorded. Serum, spleen and Peyer's patches were collected for various biomarker analyses. Anti-allergic activity of MNB using RBL-2H3 cells was also evaluated.

Results: Significant decrease in sneezing frequency, histamine, interleukin (IL)-4 and IL-17A and increase in TGF-β and IL-10 concentration were exhibited by the MNB-treated mice. However, Cry j1 and Cry j 2-specific IgE activity remained unaltered. The high dose MNB treatment increased total IgA activity and IL-10, TGF-β and FoxP3 and decreased IL-4, IL-17A, and RORγT mRNA expression. Inhibition of activation of RBL-2H3 cells was observed via decrease in histamine, intracellular Ca^{2+} concentration, and FcεRI mRNA expression.

Conclusions: We demonstrated the immunomodulatory effects of MNB and conclude that MNB is a potential therapeutic molecular nutritional supplement candidate for treatment of pollen allergy.

KEY WORDS

BALB/c mouse, immunotherapy, pollen allergy, prebiotics, β-1, 4 mannobiose

Abbreviations

APC, Antigen-Presenting Cells; Cj, Cryptomeria Japonica; CT, Cholera Toxin; ELISA, Enzyme-Linked Immunosorbent Assay; FOXP3, Forkhead Box Protein 3; IFN-γ, Interferon γ; Ig, Immunoglobulin; IL, Interleukin; MALT, Mucosa-Associated Lymphoid Tissue; MNB, β-1, 4 mannobiose; PBS, Phosphate Buffered Saline; RORγT, Retinoic Acid-Related Orphan Receptor; RT-PCR, Reverse-Transcription Polymerase Chain Reaction; TGF, Transforming-Growth Factor; Th, T Helper Cell; TLR, Toll Like Receptor; Treg, T Regulatory Cell.

INTRODUCTION

Pollinosis caused by Japanese cedar (*Cryptomeria japonica*, Cj) pollen is a typical type 1 allergy in Japan that is prevalent in 10-20% of the population and poses a major public health problem.[1] Clinical signs of Cj

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Authors' contributions: CY, HK, AN, MI, and YM designed the study, developed the overall research plan, and maintained study oversight; CY conducted the research and CY, YM and PR analyzed the data, performed the statistical analyses, and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest: HK, AN and MI are employed by Fuji Oil Co., Ltd.. The rest of the authors have no conflict of interest.

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Received 13 June 2012. Accepted for publication 4 August 2012.

pollinosis in human subjects are characterized by sneezing, watery rhinorrhea, and conjunctivitis.[2] The proteins Cry j 1 and Cry j 2 have been isolated and have been confirmed as the two dominant allergens associated with Cj pollen allergy.[3] Among 145 patients tested in one study, more than 90% had specific IgE antibodies to both allergens and the remainder had specific IgE to either one or the other[4] and patients had T cell reactivity to both Cry j 1 and Cry j 2 tested in another study.[5] Although many people suffer from Cj pollinosis, an effective and possible therapy to attenuate and cure the disease has not been established yet. Anti-histamine, steroid drug, and anti-IgE antibody therapies have been widely used to suppress the allergic signs, however these treatments exhibit serious side effects.[6]

Several therapeutic approaches for reducing pollinosis using mice as a model have been conducted earlier.[7] Coadministration of cedar pollen allergens and oligonucleotides led to a type-1 shift in immune response and inhibited IgE antibody production, suggesting that co-administration strategy may provide a novel type of immunotherapeutic strategy for cedar pollinosis.[8] It was demonstrated that intramuscular injections with major Cj pollen allergens effectively elicits Cry j 1-specific T helper 1 (Th1)-type immune responses, resulting in inhibition of IgE response to Cry j 1.[9] Also, it was recently demonstrated that Cry j 1-CpG immunization can induce Cry j 1-specific Th1 immune responses, thereby inhibiting IgE response to the pollen allergen.[10] Administration of oligomannose-coated liposomes has been shown to be useful in immunotherapeutic control of allergic reactions to Japanese cedar pollinosis in Balb/c mice.[11] These studies use various intervention approaches, however a safe oral intervention to develop an effective and practical immunotherapeutic approach without any side effects to treat and prevent Cj pollinosis is lacking. Hence, we hypothesize oral prebiotic therapy as one such intervention in the management of pollen allergy.

Dietary interventions with nondigestible prebiotic carbohydrates have been earlier shown to reduce subsequent frequency to allergic reactions.[12-15] The concept intrigued researchers to develop novel prebiotics as a new potential immunotherapeutic approach to treat and prevent allergy. Several major prebiotics, including oligofructose, inulin, galactooligosaccharides, and fructooligosaccharides, have been widely used to stimulate the growth of particular bacterial species and as an attempt to improve host health.[16] It was also recently shown that the oligosaccharide mixture suppressed cow milk allergy in mice via induction of regulatory T (Treg) cells.[14] However, the beneficial effect of prebiotics in suppression of allergic response has not been widely investigated.

The β-1, 4 mannobiose (MNB) is considered as a rare sugar in nature and its biological functions are not revealed. However, its unique structural feature and non-metabolic form are expected to have various immunological functions such as toll like receptor ligand, innate immunity and such small molecular (M.W. 342) can cross via intestinal epithelial cells.[17] We previously observed that chickens fed MNB had increased IgA production and improved *Salmonella enteritidis* clearance,[18] as well as MNB up-regulated local expression of genes related to host defence and innate immunity.[19,20] These results suggest that MNB may serve as a potential immune-modulating agent to exert combined effects on the intestinal immune system. The immune-modulating functions of MNB naturally intrigued us to explore the potential effect of MNB in the suppression of allergic response. Several mouse models of intranasally-induced Japanese cedar pollen allergy have been successfully established to investigate the pathogeneses of pollinosis and evaluate the effects of new therapeutic approaches.[21,22] Also we have recently reported prophylaxis of pollen allergic response using MNB in mice.[23] To explore further, in the present study we have tested the therapeutic effect of MNB in a Balb/c mouse model of intranasally-induced cedar pollinosis and its potential underlying molecular mechanisms. Treatment effects were measured as immune response phenotypes reflecting type-1 and type-2 related immune allergic parameters.

METHODS

ANIMALS, MATERIALS, AND REAGENTS

Female Balb/c mice (6-7 weeks old) were purchased from Charles River Laboratories (Montreal, QC, Canada). Pure MNB (of 99% purity) was prepared from Philippines coconut flour by enzymatic reactions,[19,20] and was kindly provided by Fuji Oil (Osaka, Japan). The structure of MNB was confirmed using C-NMR and H-NMR (Bruker Bio Sp3 Advance III, 400 MHz Spectrometer, Tokyo, Japan) (Data not shown). Japanese cedar pollen crude extract and purified pollen allergens (Cry j 1 and Cry j 2) were purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Cholera toxin (CT) was purchased from List Biologicals Laboratories (Denver, CO, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

ANIMAL SENSITIZATION, MNB ADMINISTRATION, AND CHALLENGE PROTOCOLS

As shown in Figure 1, a total of sixty mice were randomly divided into five groups of twelve mice per group: low dose MNB group; high dose MNB group, positive control group, negative control group, and naïve control group. The protocol for sensitization is well established other researchers.[2,24] All animals were housed in a 12-h lighting cycle and fed a diet (Harlan Teklad global diet, 14% protein and 3.5% fat, Madison, WI, USA). Food and water were available

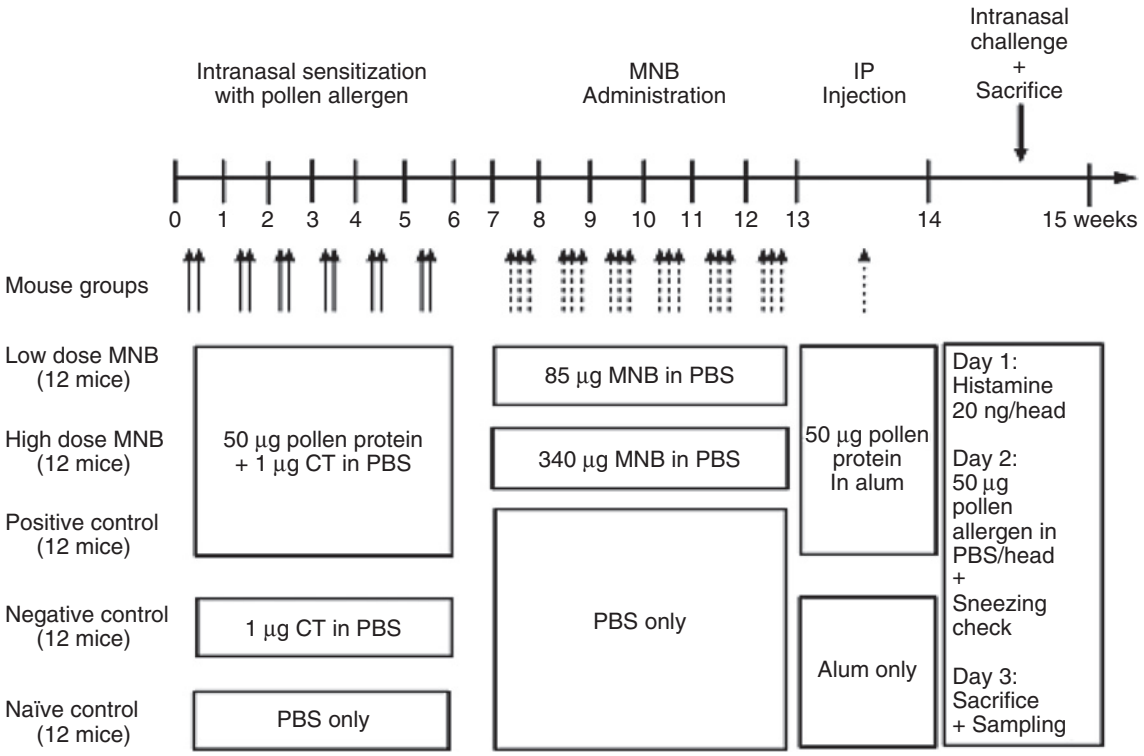

Fig. 1 Experimental protocol: mouse sensitization, MNB oral administration and final nasal challenge. Alum, aluminum hydroxide gel adjuvant; CT, cholera toxin; IP, intraperitoneal; MNB, β-1, 4 mannobiose; PBS, phosphate buffered saline.

ad libitum. All procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care (CCAC) and approved by the Animal Care Committee at the University of Guelph.

Treatment regimen is outlined in Figure 1. Briefly the positive control group and MNB treatment groups were intranasally sensitized with 50 μg of Japanese cedar pollen crude extract, and 1 μg of cholera CT in a solution of phosphate buffered saline (PBS; 25 μL. mouse) twice per week for 6 consecutive weeks. The negative control and naïve control groups received PBS containing 1 μg of CT and PBS alone in a similar way. Mice in the low dose MNB group and high dose MNB group received 85 μg and 340 μg of MNB in 100 μL of PBS solution (oral gavage), respectively, three times per week for 6 consecutive weeks. The other three groups were orally gavaged with 100 μL of PBS alone. Mice in the MNB treated and the positive groups were intraperitoneally injected once with 100 μL of 50 μg Japanese cedar pollen crude extract in aluminum hydroxide gel adjuvant (alum) (2%, Alhydrogel, Westbury, CA, USA). The negative and naïve control groups were given 100 μL of alum only. All mice were intranasally treated with 20 ng of histamine dihydrochloride/mouse. One day post histamine pretreatment, mice were intranasally challenged with 50 μg of Japanese cedar pollen crude extract in 25 μL of PBS. The frequency of sneezing was measured by sight for 5 min in an unrestrained whole body plethysmograph post the last intranasal pollen challenge.[24] After the last intranasal antigen challenge, mice were euthuanized and whole blood and tissue samples were collected.

MEASUREMENT OF HISTAMINE CONCENTRATION

Histamine concentrations in serum and cell culture supernatants were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using a commercial kit (Histamine EIA, LDN Labor Diagnostika Nord, Nordhon, Germany) according to a previously described procedure.[25]

DETERMINATION OF ANTIBODY ACTIVITY

Total immunoglobulin (Ig) E and total IgG levels in the serum were determined by a previously described ELISA procedure.[26] Pollen specific IgE, IgG, IgG1 and IgG2a in the serum were determined by a previously described ELISA procedure with minor modifications such that the Cry j 1 and Cry j 2 both were used for coating.[25,26] The relative levels of allergen-specific IgE, IgG, IgG1, and IgG2a in the serum were expressed as optical density (OD).

ALLERGEN-INDUCED CYTOKINE SECRETION IN SPLEEN CELL CULTURES

Spleen cells were isolated and stimulated by using a previously described procedure.[26,27] Single cell suspensions of splenocytes were seeded in triplicates into 96-well flat-bottomed tissue culture plates (Corning Costar, Fischer Scientific, Pittsburg, USA) (1×10^6 cells/well) and cultured in the presence of 50 μg of Cry j 1 and Cry j 2 allergens. Interleukin (IL)-4 and (interferon) IFN-γ levels were measured by our previously established ELISA procedures.[25] IL-10, transforming-growth factor (TGF)-β and IL17A were measured by ELISA with the ELISA Ready-SET-Go! kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

DETERMINATION OF TOTAL IgA IN FECAL EXTRACTS

Mouse fecal pellets were freshly collected on a weekly basis from each mouse group cage, and were submitted to a previously described extraction procedure.[25] Total IgA levels in fecal extracts were determined by an ELISA procedure.[27]

MAST CELL DEGRANULATION ASSAY

Rat basophilic leukemia cells (RBL-2H3; ATCC CRL-2256) were grown in Eagle's Minimal Essential Medium (EMEM) (Invitrogen Corp., Grand Island, NY, USA) supplemented with 1 mM sodium pyruvate (Invitrogen), 50 U/ml penicillin-streptomycin (Invitrogen), and 10% FBS (HyClone, Logan, UT, USA). Cells were cultured at 37℃ in the presence of 5% CO_2, and medium was replaced every 2-3 days. RBL-2H3 cells were seeded into 48-well plates (Corning Incorporated, Corning, NY, USA) at a density of 1×10^5 cells per well and allowed to adhere for 3 hrs at 37℃ in 5% CO_2. The culture medium was then aspirated and replaced with 0.25 ml of fresh medium containing 0.5 μg/ml of anti-DNP IgE (Clone SPE-7; Sigma-Aldrich) and incubated overnight 37℃ in 5% CO_2. The cells were then rinsed twice with Tyrode's buffer (Sigma-Aldrich) containing 10 mM HEPES and 0.1% BSA, and pre-incubated in Tyrode's buffer for 10 min. The buffer was removed and replaced with 0.25 ml of DNP-HSA (Sigma-Aldrich) at 100 ng/ml in Tyrode's buffer, and cells were incubated for 1 hr 37℃ in 5% CO_2. Degranulation was assessed by intracellular calcium ion assay and histamine ELISA. In order to assess the effect of MNB on mast cell degranulation, RBL-2H3 cells were pre-incubated with MNB at 37℃ for 1 h, following sensitization with anti-DNP IgE before antigenic challenge with DNP-HSA.

MEASUREMENT OF INTRACELLULAR CALCIUM ION CONCENTRATION

Intracellular Ca^{2+} concentration was measured using the fluorescence indicator Fluo-4/AM (Molecular Probes™, Invitrogen, Carlsbad, CA, USA). Briefly, RBL-2H3 cells were seeded into 96 well black opaque culture plates (PerkinElmer, Waltham, MA, USA) at a density of 0.5×10^5 cells per well and allowed to adhere for 3 hrs at 37℃ in 5% CO_2. The culture medium was then aspirated and replaced with 0.125 ml of fresh medium containing 0.5 μg/ml of anti-DNP IgE and incubated overnight 37℃ in 5% CO_2. The cells were then rinsed twice with Tyrode's buffer containing 10 mM HEPES and 0.1% BSA, and pre-incubated with indicated concentrations of MNB in Tyrode's buffer for 1 hr. The buffer was removed and loaded with Fluo-4 NW dye mix (Molecular Probes™, Invitrogen) at 37℃ for 45 min. After the removal of Fluo-4 NW dye mix, cells were challenged for 1 min with 0.125 ml of DNP-HSA at 100 ng/ml in Tyrode's buffer. Intracellular Ca^{2+} concentration was immediately detected at a 485-nm excitation wavelength and a 535-nm emission wavelength at 37℃ with Perkin Elmer Victor^2 TM 1420 Multiple Counter (PerkinElmer).

REAL-TIME RT-PCR ANALYSIS OF GENE EXPRESSION IN PEYER'S PATCHES AND RBL.2H3 BASOPHIL CELLS

Peyer's patches from two mice were pooled within each group (n = 6 samples per group). Total RNA was extracted from Peyer's patches using Aurum total RNA mini kit (Bio-Rad Laboratories Hercules, CA, USA). The real-time RT-PCR reactions were performed with qScript cDNA Synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA) and SYBR Green I Detection kit (Quanta BioSciences) using an iCycler iQ Real Time PCR System (Bio-Rad). The sequences of the primers used for real-time RT-PCR analyses are shown in Supplementary Table 1. The RBL-2H3 cells were analyzed in a similar way for mRNA expression of FcεRI α, β and γ chain for effect of MNB and the sequences of the primers used for real-time RT-PCR analyses are shown in Supplementary Table 2. The supplementary materials are available online.

STATISTICAL ANALYSIS

Statistical calculations were performed using the GraphPad Prism 5.0 package (GraphPad Software, San Diego, CA, USA). All data were expressed as means ± SEM and subjected to ANOVA analyses followed by post hoc multiple comparison using Tukey's test. Comparison of all the endpoint differences with a level of $P < 0.05$ was considered significant.

RESULTS

REDUCTION IN SNEEZING FREQUENCY AND SERUM HISTAMINE RELEASE

Mice that were treated by both dose of MNB had significantly lower sneezing frequency ($P < 0.05$) (Fig. 2a) and serum histamine concentrations ($P < 0.05$) (Fig. 2b) when compared with mice in the posi-

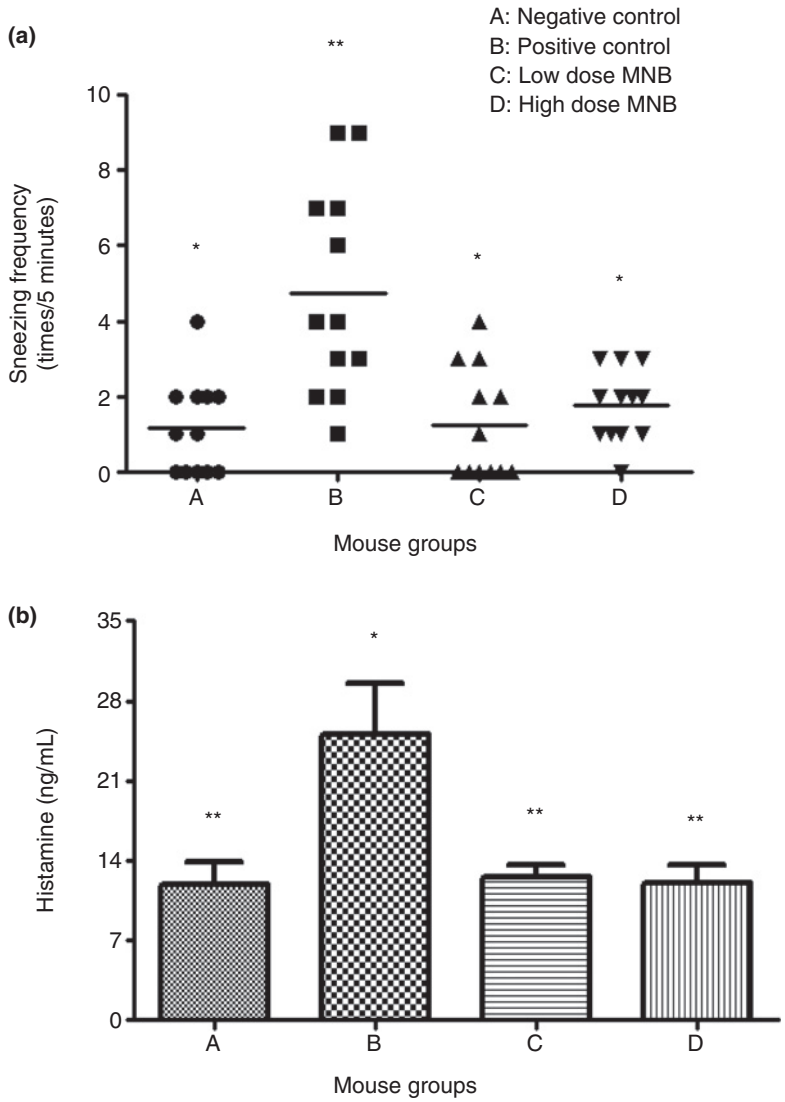

Fig. 2 (**a**) Determination of sneezing frequency in mice during 5 min post challenge by pollen allergen at the end point. Data are represented as mean ± SEM (n = 12 mice per group). (**b**) Histamine concentration in the serum determined at the end point. Serum samples from two mice were pooled in each group and data are represented as mean ± SEM (n = 6). Different asterisks indicate statistically significant differences at $P < 0.05$.

tive control group. However, there were no differences ($P > 0.05$) in the sneezing frequency (Fig. 2a) and the serum histamine concentrations (Fig. 2b) among MNB treated groups, negative control and naïve control groups.

ANTIBODY RESPONSE

As shown in Figure 3a, treatment with low and high dose of MNB decreased total IgE levels in the serum ($P < 0.05$) as compared to those in the positive control group. However, total IgG levels in the serum did not differ among MNB treated mice and mice in the positive control group ($P > 0.05$) (Fig. 3b). There were no differences ($P > 0.05$) in the Cry j 1 and Cry j 2-specific IgE (Fig. 3c), specific IgG (Fig. 3d), specific IgG1 (Fig. 3e) and specific IgG2a (Fig. 3f) activity detected in the serum between the MNB treated groups and the positive control group. Total fecal IgA was only increased in the high dose MNB group as compared to other groups (Fig. 3g).

CYTOKINE PRODUCTION IN SPLEEN CELL CULTURE SUPERNATANT

Culture supernatants stimulated *in vitro* with purified Cry j 1 and Cry j 2 indicated that IL-4 concentration was significantly lower ($P < 0.05$) in the high dose

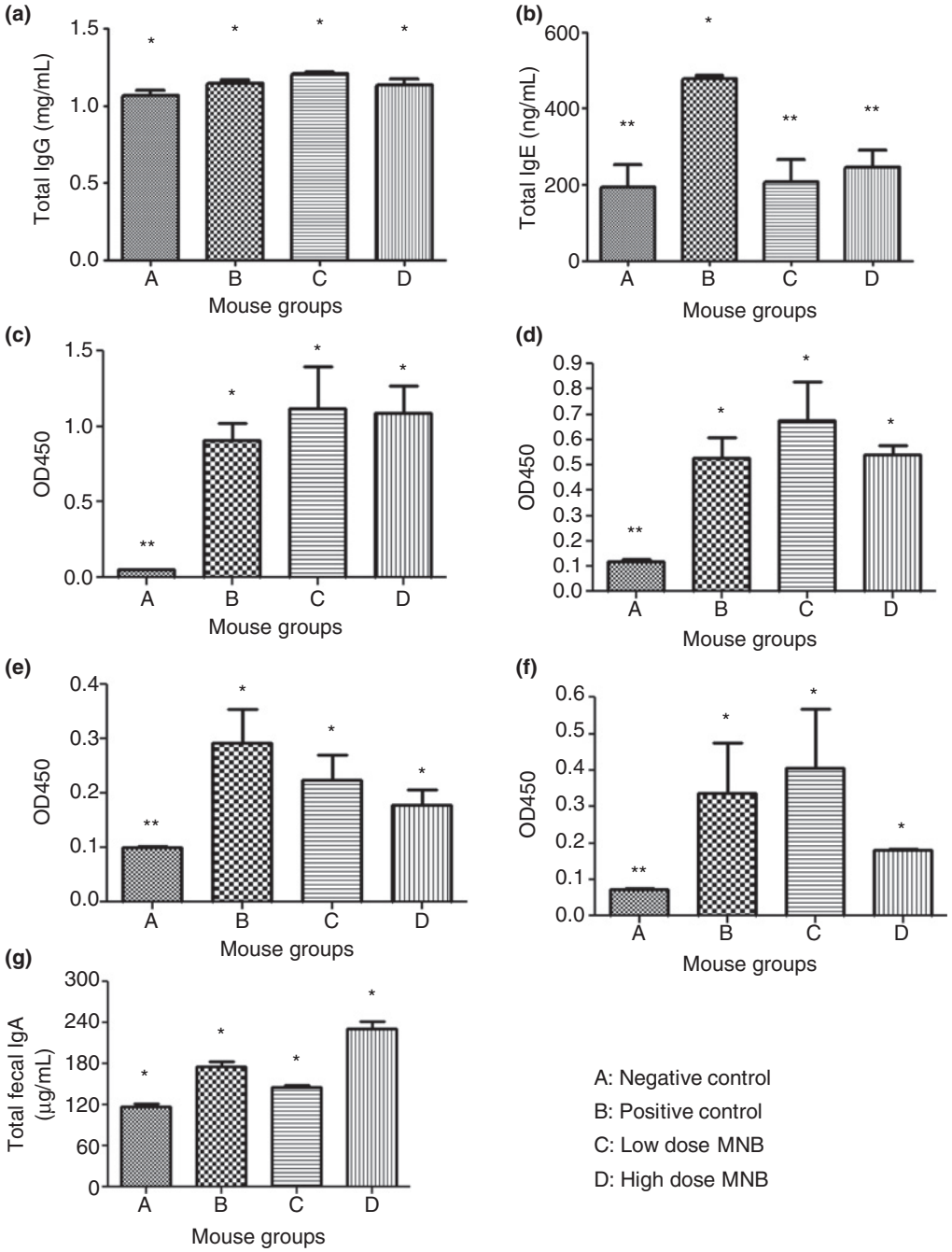

Fig. 3 Total IgE (**a**), total IgG (**b**), pollen allergen (Cry j 1 and Cry j 2)-specific IgG (**c**), IgE (**d**), IgG1 (**e**), and IgG2a (**f**) levels in the serum of pollen allergen-sensitized BALB/c mice after repeated oral administration of MNB. Serum samples from two mice were pooled within each group. Data are represented as mean ± SEM (n = 6). (**g**) Total IgA levels in fecal extracts of mouse at the end point. Data are represented as mean ± SEM (n = 3 replicate tests in fecal extracts). Different asterisks indicate statistically significant differences at $P < 0.05$.

MNB-administered group as compared to mice in the positive control group (Fig. 4a). However, there was no difference ($P > 0.05$) in IFN-γ concentration detected among mice in the MNB-administered groups compared to the positive control group (Fig. 4b). Mice in the low dose MNB-administered group had higher IL-10 concentration ($P < 0.05$) (Fig. 4c) and TGF-β concentration was significantly higher ($P < 0.05$) in both the MNB-administered groups as compared to mice in the positive control group (Fig. 4d).

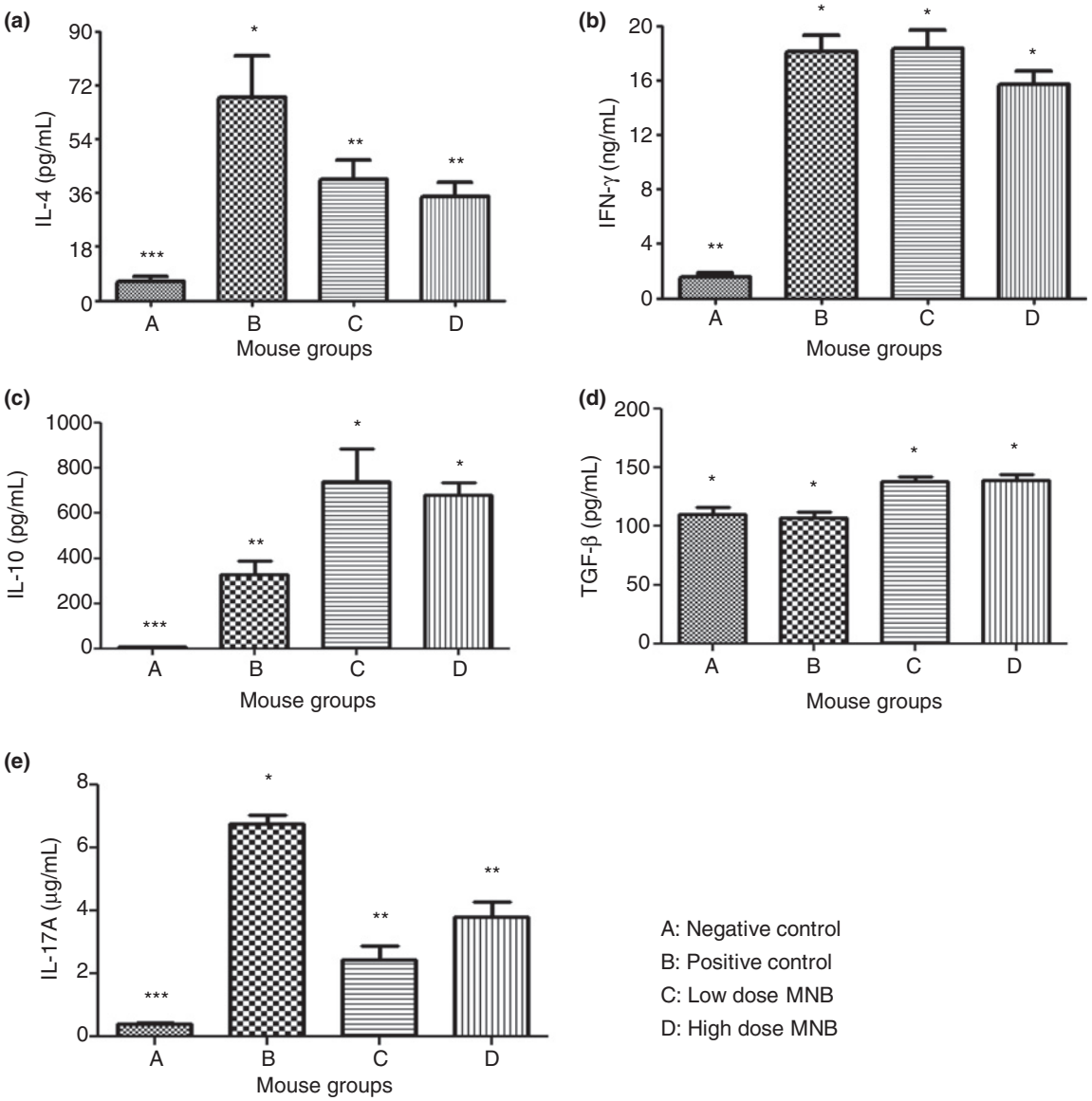

Fig. 4 Cytokine production of IL-4 (**a**), IFN-γ (**b**), IL-10 (**c**), TGF-β (**d**), and IL-17A (**e**) by spleen cells stimulated *in vitro* with pollen allergen (Cry j 1 and Cry j 2). Two spleens from individual mice were pooled within each treatment group (n = 6). Data are represented as mean ± SEM (n = 6). Different asterisks indicate statistically significant differences at $P < 0.05$.

The IL-17A concentration was significantly lower ($P < 0.05$) in both the MNB-administered groups (Fig. 4e).

GENE EXPRESSION IN PEYER'S PATCHES

The IL-4 mRNA expression (Fig. 5a) in Peyer's patches was lower in both the low and the high dose MNB group ($P < 0.05$). The mRNA expression of IL-10 (Fig. 5b), TGF-β (Fig. 5c) and transcriptional factor FoxP3 (Fig. 5e) in Peyer's patches were higher ($P < 0.05$) in the high dose MNB group. The mRNA expression of IL-17A (Fig. 5d) and RORγT (Fig. 5f) in Peyer's patches were lower ($P < 0.05$) in both the low and high dose MNB groups.

EFFECT OF MNB ON *IN VITRO* RBL-2H3 ACTIVATION

As shown in Figure 6a, histamine release was significantly inhibited ($P < 0.05$) at all MNB concentrations when compared to positive control cells (with no MNB). As shown in Figure 6b, relative to positive control cells (with no MNB) intracellular Ca^{2+} level was significantly lower at 50 μg/ml ($P < 0.05$), and 300 μg/ml ($P < 0.05$). The mRNA expressions of FcεRI α chain and γ chain was significantly decreased ($P < 0.05$) by MNB at 50 μg/ml when compared to negative cells with no MNB (Fig. 6c). However, the mRNA expression of FcεRI β chain was affected by

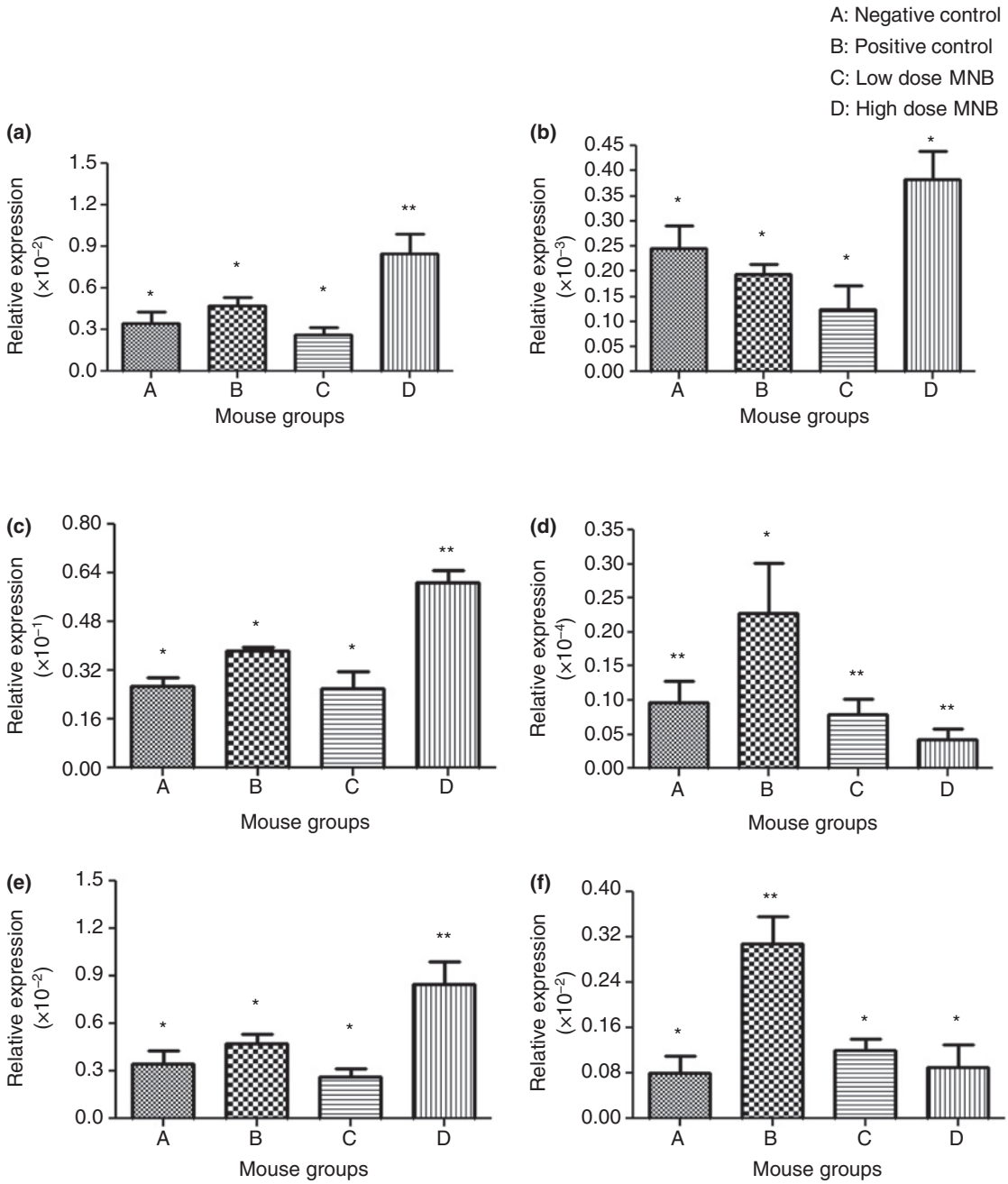

Fig. 5 The mRNA expressions in Peyer's patches determined by real-time RT-PCR analysis for IL-4 (**a**), IL-10 (**b**), TGF-β (**c**), IL-17A (**d**), FoxP3 (**e**), and RORγT (**f**). Data are represented as mean ± SEM ($n = 6$). Different asterisks indicate significant differences ($P < 0.05$).

MNB addition ($P > 0.05$).

DISCUSSION

The major purpose of this study was to test the therapeutic effects of the nutritional supplement molecule MNB against Japanese cedar pollen allergy induced in mice. In this study we have demonstrated alleviation of allergic response in mice via supplementation of MNB as a molecular food nutritional component for uses thereof for a hyposensitization therapy, which may be effective and safe without side effects, different from the conventional hyposensitization therapy.

Sneezing frequency is a major clinical sign of hypersensitivity to assess the pollen allergy response in animal models.[2,24] In the present study, it was demonstrated that MNB significantly decreased the sneezing frequency and the serum histamine concentration

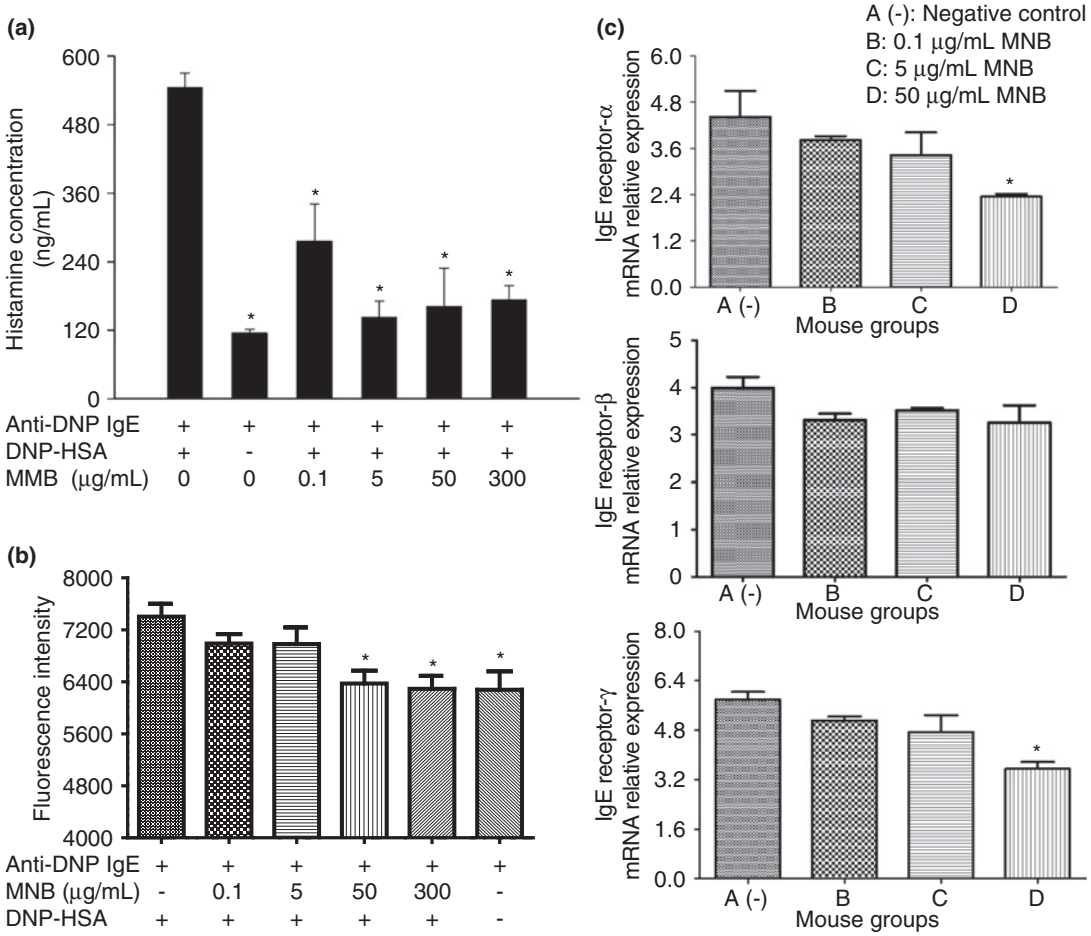

Fig. 6 Effect of β-1, 4 mannobiose (MNB) of RBL-2H3 cell *in vitro* activation. (**a**) Following sensitization with anti-DNP IgE, RBL-2H3 cells were pre-incubated with MNB at the indicated concentrations for 1 hr at 37°C, followed by addition of DNP-HSA. Degranulation was measured by histamine ELISA assay. (**b**) IgE-sensitized RBL-2H3 cells were loaded with Ca^{2+} indicator Fluo-4/AM after treatment with indicated concentrations of MNB and the cells were stimulated with DNP-HAS for 3 min, and fluorescence was measured. (**c**) Total RNA was extracted after incubated with MNB at the indicated concentrations for 24 hr at 37°C. The IgE receptor mRNA expressionS of RBL-2H3 cells were measured by real-time PCR. Values represent mean ± SEM, $n = 3$. *Different from the negative control cells at $P < 0.05$.

and sneezing frequency was positively correlated with the serum histamine levels (R = squared = 0.4128 and *P* value = .002), suggesting that MNB exerts therapeutic effects on Japanese cedar pollen allergy.

Type 1 hypersensitivity reactions including Cj pollinosis are characterized by allergen-specific IgE as a vital biomarker,[28] however, we found that administration of MNB did not affect the serum Cry j 1 and Cry j 2-specific IgE concentration, suggesting that the therapeutic effect of MNB may be due to other regulatory mechanisms inducing immune tolerance to pollen allergens. Similarly, in earlier studies it was observed that specific immunotherapy did not significantly change specific IgE levels post treatment; however, suppression of allergic response was observed.[29,30] Therefore, in this study, measurement of serum IgE was not a better predictor of allergic clinical signs. A possible explanation for this may be that quantification of allergen-specific serum IgE may not reflect allergy-mediating activity of leucocyte FceR1-bound antibody. This type of tolerance may be induced by several other key mechanisms that include clonal deletion and induction of tolerogenic Treg cells that produce IL-10 and/or TGF-β.[31,32] It was great interest that administration of MNB induced a decrease in the total serum IgE activity that may also be attributed to the allergic response. However, the mechanism of decrease in total IgE needs to be further investigated.

To clarify the mechanisms involved in the inhibition of allergy, we examined cytokine production from splenocytes that were stimulated with Cry j 1 and Cry j 2 *in vitro*. We found oral administration of MNB improved type-1/type-2 balance of helper T cells by suppressing IL-4 production (type-1 cytokine) without changing IFN-γ production (type-2 cytokine) and induced the Treg cell responses by increasing TGF-β and IL-10 production, suggesting that MNB may modulate the balance by inducing Treg cell differentiation and alleviating the allergic response. Recently, IL-17A is believed to be involved in the pathogenesis of allergy diseases and is being discussed as a potential therapeutic target for allergy.[33] Our study indicated that Th17 cell responses were suppressed in the spleen by administration of MNB. Although Th17 responses have been reported to be suppressed by Treg cell,[34] molecular mechanisms behind suppressed Th17 response by MNB administration remains to be elucidated. We found high dose MNB increased the mRNA expression of Treg cell-biased cytokines IL-10 and TGF-β, and transcriptional factor FoxP3 in peyer's patches, suggesting that the regulatory $Foxp3^+$ T cells is induced by MNB and responsible for reduction in type-2-cytokine response observed in the spleen and the peyer's patch. Indeed, we also found IL-4 mRNA expression was decreased in Peyer's patches by administration of MNB. In the present study, administration of MNB decreased the mRNA expression of IL17A and the transcriptional regulator of the Th17 pathway (RORγT) in Peyer's patches, suggesting that MNB suppressed Th17 response. Although, cytokine production profiles in the spleen cell culture correlated well with the cytokine mRNA expression in the Peyer's patches, the linkage of systemic immunity and local immunity needs to be further investigated. Also the relationship between nasal-associated lymphoid tissue (NALT) and systemic immune response induced by spleen and Peyer's patches remains to be further explored.

Secretory IgA, the most abundant antibody isotype in mucosal immune response, is produced by B cell and exported by the epithelial polymeric Ig receptor (pIgR)[35] and plays an important role in protecting against infection in the intestinal immune system, and its production was previously shown to be increased by dietary MNB in chickens infected with *Salmonella enteritidis*.[17] Here we found that administration of high dose MNB induced a similar increase in total fecal IgA. Recent research showed that high intestinal IgA played an important role in tolerance and reduced risk of IgE-associated allergic diseases.[36] Moreover, intestinal IgA synthesis is regulated by T-cell dependent and T-cell independent mechanism and are necessary for intestinal immunity homeostasis by selecting and maintaining the appropriate microbial composition.[37] Therefore, the therapeutic effect of MNB on allergy may partially be attributed to intestinal IgA secretions.

Our results also supported that Treg cells played central roles in the MNB-mediated attenuation of pollen allergic responses by improving type-1/type-2 balance, suppressing Th17 response and increasing IgA production. However, whether MNB can interact with mucosa-associated lymphoid tissue (MALT) as well as local/regional mucosa-draining lymph nodes through potential receptors or not, such as TLR4, is still not clear and further investigation is needed. Another potential mechanism behind the anti-pollen allergy effect of MNB is that MNB may modulate gut microbiota which plays a central role in the host innate and adaptive immunity.

In order to further understand the potential anti-allergy activity mechanisms of MNB, in the present study we employed the *in vitro* model of allergic reaction, the rat basophilic leukemia cell line RBL-2H3, characterized as mucosal mast cells[38] to study whether MNB have the ability to influence mast cell functionality *in vitro*. Indeed, we found MNB can inhibit histamine release in the mast cells induced by specific IgE, suggesting that MNB can attenuate the activation of mast cells. The FcεRI receptor is a key molecule in triggering IgE-mediated allergic reaction, and it has been suggested that the downregulation of its expression in mast cells and basophils may lead to the attenuation of IgE-mediated allergic symptoms,[39] therefore FcεRI and IgE sensitization may be an important therapeutic target in the treatment of allergy. Our results indicate that MNB may inhibit mast cell degranulation through reducing FcεRI mRNA expression. Calcium influx is essential for FcεRI-mediated mast cell activation and allergic reaction.[40] The effects of MNB on calcium levels in mast cells were examined. Our results indicated that MNB can significantly reduce intracellular Ca^{2+} concentration. Take together, the role of MNB in attenuation of pollen allergic response in mice could be partially due to the attenuation of activation in the mast cells.

The prophylactic study reported earlier[23] significantly attenuated pollen allergic response using MNB by improving Th1/Th2 balance, increasing regulatory T cell response, decreasing Th17 response, and increasing IgA production. The present therapeutic study provides further evidence for role of MNB in attenuation of pollen allergic response in mice by inducing immune tolerance through several ways that includes a Th2- to a Th1-skewed response, a regulatory response involving the transcription factor FOXP3, induction of regulatory cytokines TGF-β and IL-10, a suppressed Th17 response, enhanced immunoglobulin isotype IgA production, and influencing mast cell functionality. This may suggest that MNB may be a potentially useful candidate both prophylactically and therapeutically for the design of a functional prebiotic food component in targeting management of Cj pollinosis.

ACKNOWLEDGEMENTS

We would like to thank Annette Morrison, Jackie Rombeek, and Linda Groocok from University of Guelph Central Animal Facility, for their skillful technical assistance with the animal studies.

SUPPLEMENTARY MATERIALS

Supplementary Table 1, 2 are available online.

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