

Prophylaxis of Intranasally Induced Pollen Allergy in a BALB/C Mouse Model Using a Potential Prebiotic β -1, 4 Mannobiose

Chengbo Yang¹, Prithy Rupa¹, Hiroyuki Kanatani², Akihiro Nakamura²,
Masahisa Ibuki³ and Yoshinori Mine¹

ABSTRACT

Background: Dietary supplementation with unique prebiotic nondigestible carbohydrates has been shown to suppress allergy. In the present study, the prophylactic efficacy of a disaccharide β -1, 4 mannobiose (MNB) in a BALB/C mouse model of intranasally-induced pollen allergy was characterized.

Methods: Balb/c mice were pretreated with MNB orally and sensitized with pollen extract intraperitoneally and intranasally and challenged with histamine and crude pollen extract. Outcomes were measured as clinical signs, antibody isotypes, cytokine gene and protein expression patterns.

Results: The MNB-treated mice had lower sneezing frequency as compared to the positive control mice ($P < 0.05$). The low dose MNB-treated mice had less histamine ($P < 0.05$). However, the Cry j1 and Cry j 2-specific IgE, IgG, IgG1 and IgG2a antibody activity did not differ between groups ($P > 0.05$). The MNB-treated mice had increased IFN- γ ($P < 0.05$), and decreased IL-4 ($P < 0.05$). Mice in the high dose group had increased IL-10 ($P < 0.05$). However, TGF- β and IL-17 concentration did not differ between groups ($P > 0.05$). Both total and Cry j1 and Cry j 2-specific IgA were increased in the high dose group. Real-time RT-PCR analysis indicated that IL-4 and IL-17 mRNA expression were lower in MNB-treated mice ($P < 0.05$).

Conclusions: This work provides insights into using MNB as a potential prebiotic immunomodulator via decreased clinical signs, improved type1/type 2 balance, and IgA production, thus validating the potential use of MNB as a prophylactic prebiotic candidate to attenuate allergic response.

KEY WORDS

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

ABBREVIATIONS

APC, Antigen-Presenting Cells; Cj, Cryptomeria Japonica; CT, Cholera Toxin; ELISA, Enzyme-Linked Immuno-Sorbent Assay; FOXP3, Forkhead Box Protein 3; IFN- γ , Interferon γ ; Ig, Immunoglobulins; IL, Interleukin; MALT, Mucosa-Associated Lymphoid Tissue; MNB, β -1, 4 mannobios; PBS, Phosphate Buffered Saline; ROR γ T, 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 serious disease of concern that

is prevalent in 10-20% of the population and poses a major public health problem.¹ The clinical signs of Cj pollinosis in humans are characterized by sneezing, watery rhinorrhea and conjunctivitis.² Allergens Cry j

¹Department of Food Science, University of Guelph, Ontario, Canada, ²Tsukuba R&D Centre, Fuji Oil Co., Ltd., Ibaraki and ³Soy Protein Processed Food, Overseas Sales Department, Fuji Oil Co., Ltd., Tokyo, Japan.
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Correspondence: Professor Yoshinori Mine, PhD, Department of Food Science, University of Guelph, Guelph, Ontario N1G2W1, Canada.
Email: ymine@uoguelph.ca
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1 and Cry j2 have been isolated earlier and are reported as the two dominant allergens responsible for causing Cj pollen allergy.³ Although many people suffer from Cj pollinosis, an effective therapy to attenuate and cure the clinical signs has not been established yet. Anti-histamine and steroid drug remedies have been widely used to suppress the allergic signs, however these have been shown to have various side effects and cause inconvenience.⁴ Therefore, it is important to develop an effective prophylactic approach without any side effects to prevent Cj pollinosis.

Dietary interventions with prebiotic nondigestible carbohydrates have been shown to reduce the development of allergic disease.⁵⁻⁸ There are several major prebiotics including oligofructose, inulin, galactooligosaccharides, and fructooligosaccharides, which have been widely used to stimulate the growth of particular bacterial species and improve host health.⁹ Studies in infants with allergy, fed such prebiotic formulae demonstrated a reduced risk of expression of allergy.¹⁰ Similar antiallergic effects induced by oligosaccharides have also been demonstrated in animals sensitized with ovalbumin⁶ or cow milk proteins.¹¹ Much recent research showed that oligosaccharides suppressed cow milk allergy in mice by induction of regulatory T (Treg) cells,⁷ indicating that the inhibition of allergic signs were not solely due to decrease in type-2 and increase in type-1 stimulation. However, the beneficial effect of prebiotics, in particular using short chain disaccharides for suppression of pollen allergic response has not been widely investigated in detail, which intrigued us to carry out this study.

The β -1, 4 mannobiose is a disaccharide derived from a coconut meal enzymatic hydrolysate, which contained approximately 13% β -1, 4 mannobiose and only residual mannose (<3% mannose, Fuji Oil, Osaka, Japan). Mannobiose is more water soluble and more homogenous than the large mannose-containing molecules like mannoooligosaccharides. It was reported earlier that MNB may be potential ligands for TLR2/4.¹² We previously observed that supplementation of mannobiose during the early stage of broiler production was able to effectively reduce gut *Salmonella* and chickens fed MNB had increased IgA production and improved *Salmonella enteritidis* clearance,¹³ as well as MNB was able to up-regulate the local expression of genes related to host defence and enhances innate immunity thereby acting as an immuno-modulator.^{14,15} These results suggest that MNB may act as a potential immune-modulating agent in suppression of pollen allergic response. In the present study, we investigated the prophylactic effect of β -1, 4 mannobiose in a BALB/C mouse model of intranasally-induced pollen allergy and examined candidate immune response polarizers involved in immune modulation.

METHODS

PREPARATION OF β -1, 4 MANNOBIOSE

Pure β -1, 4 mannobiose (about 99% purity) was prepared from Philippine coconut flour by enzymatic reactions and was provided by Fuji Oil.

PRETREATMENT WITH MNB, ANIMAL SENSITIZATION AND CHALLENGE

A total of forty-eight BALB/C female mice (6-7 weeks old) were purchased from Charles River laboratories (Montreal, QC, Canada) and randomly divided into 4 groups of twelve mice in two cages as described in Figure 1 and as follows: Negative control; Positive control; MNB low dose treated group and MNB high dose treated group. All animals were housed in the campus animal facility at the University of Guelph 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 *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.

Following a 1-week adaptation period, mice in the MNB low dose group and MNB high dose group were given 5.0 and 20 mg/kg bodyweight of MNB in a 100 μ L of PBS solution by oral gavage three times a week throughout the experimental period, respectively. All other groups were orally gavaged with 100 μ L of PBS placebo solution. From the 7th week onwards, mice in the positive control group, MNB low dose group, and MNB high dose group were intraperitoneally injected with 50 μ g pollen extract in 100 μ L of aluminum hydroxide gel adjuvant (alum) (2%, Alhydrogel, Westbury, CA, USA) once a week for 4 weeks. Mice in the negative control were intraperitoneally (IP) injected with 100 μ L of aluminum hydroxide gel adjuvant (alum) (2%, Alhydrogel) once a week for 4 weeks. Following the 4-week IP injection period, mice in the positive control group, MNB low dose group, and MNB high dose group were intranasally sensitized with 25 μ L/mouse of a PBS solution containing 50 μ g of Japanese Cedar Pollen crude extract, and 1 μ g of cholera toxin (CT) (List Biologicals Laboratories, Denver, CO, USA), twice a week for 2 weeks. The negative control group was orally gavaged according to an identical schedule, with 25 μ L/mouse of a PBS solution containing 1 μ g of CT. The CT was used as an immunological adjuvant given to mice by the intranasal route to enhance the immune response.

In the 13th experimental week, all mice were intranasally given 20 ng of histamine dihydrochloride/mouse once and intranasally challenged with 50 μ g of Japanese Cedar Pollen crude extract in 25 μ L of PBS a day later. The frequency of sneezing was measured for 5 min using an unrestrained whole body plethys-

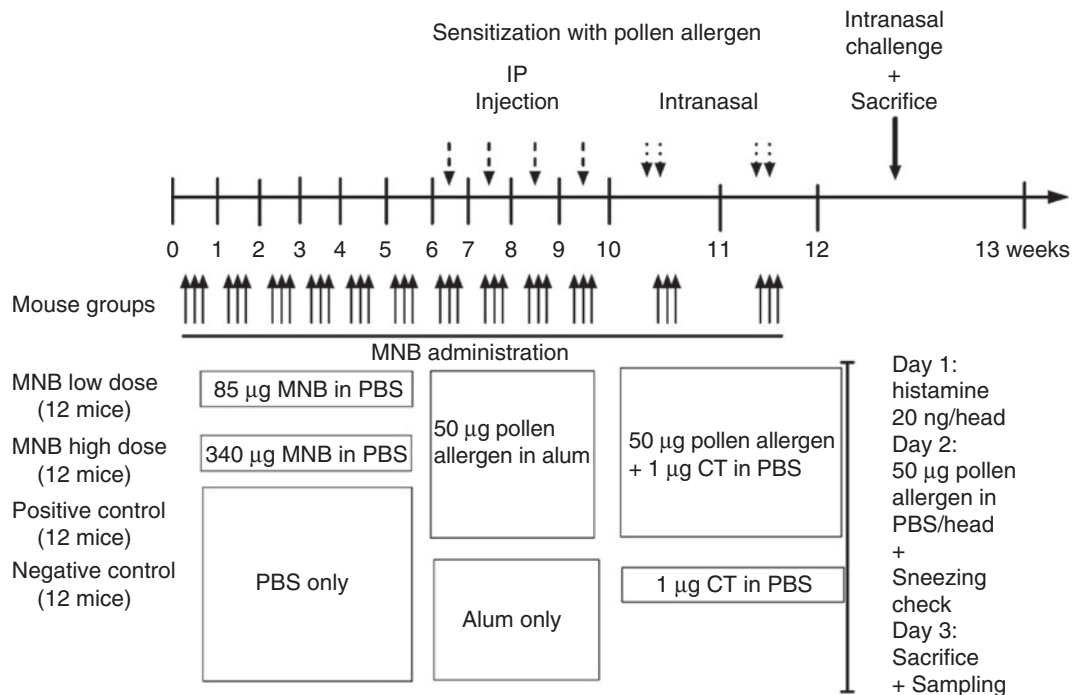


Fig. 1 Experimental timeline: MNB oral administration, mice sensitization, and final nasal challenge. Alum, aluminum hydroxide gel adjuvant; CT, cholera toxin; IP, intraperitoneal; MNB, β -1, 4 mannobiose; PBS, phosphate buffered saline.

mograph post intranasal pollen challenge.¹⁶ Twenty-four hours post the intranasal antigen challenge, all mice were killed and whole blood and tissue samples were collected.

TISSUE SAMPLING

Whole blood was collected by cardiac puncture and kept at 4°C overnight. Blood samples were then centrifuged with 10,000 rpm for 10 min at 4°C and sera from each two mice were pooled in equal volumes within each group, due to the limitation in volume. Serum samples were frozen at -80°C until further use. Spleen was aseptically removed and stored in a 50-ml conical tube with 10 ml of ice cold RPMI 1640 medium (Gibco Invitrogen, Grand Island, NY, USA), containing sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 units/mL), and streptomycin (50 µg/mL), and splenocytes were isolated. Peyer's patches in the intestine were collected into a 2-ml tube containing 1 ml of RNAlater solution and stored at -80°C for RNA isolation.

MEASUREMENT OF SERUM HISTAMINE CONCENTRATION AND mMCPT-1 MEASUREMENTS

At the end point of experiment, mice were killed and whole blood samples were collected by cardiac puncture, and sera from each two mice were pooled in equal volumes within each group. Histamine concentrations were determined by ELISA using a commer-

cial kit (Histamine EIA, LDN Labor Diagnostika Nord, Nordhonn, Germany). Serum samples were diluted (1 : 10) with diluent buffer (provided in the kit) prior to application onto the plate and were run in duplicate wells along with the histamine standards. The mouse mast cell protease (mMCPT-1) concentration was quantified by ELISA according to the manufacturer's instructions (eBiosciences, San Diego, CA, USA), and all the samples were diluted 1 : 10.

DETERMINATION OF SERUM TOTAL IgE AND TOTAL IgG

Total IgE and total IgG antibody activity in serum were determined by ELISA. Briefly, flat-bottom 96-well ELISA plates (Corning, Corning, NY, USA) were coated with 100 µl of purified rat anti-mouse IgE monoclonal antibody (Caltag Laboratories, Burlingame, CA, USA; RMGE00) or purified goat anti-mouse IgG (EMD Biosciences, CA, USA) diluted 1 : 1000 in 50 mM sodium bicarbonate buffer (pH 8.5) at 4°C overnight. Plates were washed four times with 200 µl of 0.05% Tween 20 in phosphate buffered saline (PBST) and then blocked with 200 µl of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2 h at 37°C. The plates were washed four times with 200 µl of PBST and serum samples were diluted (1 : 20 for total IgE and 1 : 25,000 for total IgG) in 1% BSA in PBST, and 100 µl of diluted samples and a duplicate dilution series of recombinant mouse IgE (BD Pharmingen, San Diego, CA, USA; 553413) as stan-

dards were added to the wells and incubated for 1 h at 37°C. The plates were washed four times with 200 µl of PBST. For determination of total IgE, monoclonal anti-mouse IgE conjugated to biotin (Caltag; RMGE15) was used in combination with avidin-horse radish peroxidase (Av-HRP) conjugate (BD Pharmingen), and color development was revealed by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic substrate (Sigma-Aldrich, St. Louis, MO, USA). For determination of total IgG, polyclonal goat anti-mouse IgG (Fab specific) conjugated to alkaline phosphatase (Sigma-Aldrich) was employed followed by the addition of *p*-nitrophenyl phosphate (*p*NPP, Sigma-Aldrich). The reaction was terminated after 30 minutes by addition of 50 µl/well of 2N H₂SO₄, and absorbance values were determined at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

DETERMINATION OF POLLEN ALLERGEN CRY J 1 AND CRY J 2 SPECIFIC IgE, IgG, IgG1 AND IgG2a IN SERUM

Pollen specific IgE, IgG, IgG1 and IgG2a antibody activity in serum samples were determined by sandwich ELISAs. Briefly, flat-bottom 96-well ELISA plates (Corning) were incubated with 100 µl/well of 2.7 µg/mL of purified pollen allergen (Cry j 1 and Cry j 2, Hayashibara Biochemical Laboratories, Okayama, Japan) in 100 mM of sodium bicarbonate buffer (pH 8.5) at 4°C overnight. The plates were washed four times with 200 µl/well of PBST. Mouse serum were diluted in 1% BSA in PBST (1 : 5 for p pollen Cry j 1 and Cry j 2 specific IgE, 1 : 5000 for pollen Cry j 1 and Cry j 2 specific IgG and IgG1, 1 : 2000 for pollen Cry j 1 and Cry j 2 specific IgG2a) and assays were duplicated. For determination of pollen Cry j 1 and Cry j 2 specific IgE, monoclonal anti-mouse IgE conjugated to biotin (Caltag) was used in combination with avidin-horse radish peroxidase (Av-HRP) conjugate (BD Pharmingen), and color development was revealed by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic substrate (Sigma-Aldrich). For determination of pollen Cry j 1 and Cry j 2 specific IgG, polyclonal goat anti-mouse IgG (Fab specific) conjugated to alkaline phosphatase (Sigma-Aldrich) was employed followed by the addition of *p*-nitrophenyl phosphate (*p*NPP, Sigma-Aldrich). For determination of pollen Cry j 1 and Cry j 2 specific IgG1 and IgG2a, monoclonal rat anti-mouse IgG1 antibody conjugated to biotin (1 : 2000) or anti-mouse IgG2a conjugated to biotin (1 : 2000) (BD Pharmingen) was added to the plates followed by incubation with avidin-horse radish peroxidase (Av-HRP) conjugate (BD Pharmingen). Color development was revealed by the addition of TMB substrate (Sigma-Aldrich). The reaction was terminated after 30 minutes or bright yellow solution showing up by addition of 50 µl/well of 2N H₂SO₄, and absorbance values were de-

termined at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories). The relative levels of allergen-specific IgE, IgG, IgG1, and IgG2a in serum were expressed as OD.

ALLERGEN-INDUCED CYTOKINE SECRETION IN SPLEEN CELL CULTURES

Single cell suspensions were prepared by gently grinding spleens using a syringe plunger and passing the cells through a 100 µm nylon membrane cell strainer (BD Biosciences, San Jose, CA, USA). The spleen cell suspension was transferred onto a 15 mL tube and subjected to centrifugation for 10 min at 100 × *g*. The supernatant was discarded, erythrocytes were depleted by the addition of 1 mL of red blood cell lysing buffer (Sigma-Aldrich), and the reaction was stopped by adding 10 mL of sterile RPMI 1640 medium. After resuspension, the sample was centrifuged for another 10 min at 100 × *g* and washed twice with 10 mL of RPMI 1640 medium. The splenocytes were resuspended in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS) (Hyclone, Fisher Scientific, Ottawa, ON, Canada), and cell viability was assessed by trypan blue exclusion. Cells were cultured in 24-well plates (Corning) at a density of 2.5×10^6 /mL in the absence (negative control wells) or presence of pollen antigen (100 µg/mL).

Supernatants were collected after 72 h of culture in a 5% CO₂ humidified incubator and assayed for the presence of cytokines IFN-γ (Th1-biased), IL-4 (Th2-biased), TGF-β and IL-10 (regulatory cytokines) and IL-17 (Th17). The IFN-γ and IL-4 levels were measured by our previously established ELISA procedure.¹⁷ All antibodies were purchased from BD Pharmingen and the standard ranges of IFN-γ and IL-4 are 1000-31,250 pg/mL and 500-7.8 pg/mL, respectively. Regulatory cytokines TGF-β and IL-10 and Th17 cytokine IL-17 were measured by ELISA with the ELISA Ready-SET-Go! kits (eBioscience) according to the manufacturer's protocol. Each sample was analyzed in duplicate. Cell proliferation was determined by WST-1 assay (Roche Diagnostics, Mannheim, Germany) following *in vitro* stimulation of spleen cell cultures with pollen allergen (Cry j 1 and Cry j 2). Briefly, 290 µl of RPMI 1640 medium with 8% FBS and 10 µl of WST-1 solution were added into each well after collection of supernatant and then the 24-well plate was incubated for 30 min at 37°C. The supernatant was transferred into flat-bottom 96-well ELISA plates (Corning) and absorbance was measured at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories).

DETERMINATION OF POLLEN-SPECIFIC IgA AND TOTAL IgA IN FECAL PELLETS

Mouse fecal pellets were freshly collected on a weekly basis from each mouse group cage and fecal pellets from two cages in the same group were

pooled and freeze-dried. A mass of roughly 1.0 g of fecal pellets were added to 7 ml of PBS buffer in a 15-mL conical tube and vortexed for 2×30 seconds. Samples were subsequently centrifuged at $1600 \times g$ for 15 min at 4°C to remove large fibrous particles and supernatants were carefully collected in new tubes (2 ml) and centrifuged at $7200 \times g$ for 10 min at 4°C . The supernatant was collected for ELISA immediately or stored at -80°C for further experiments.

Concentrations of pollen-specific IgA were determined using an indirect ELISA. Flat-bottom 96-well ELISA plates (Corning) were incubated with 100 μL /well of 2.7 $\mu\text{g}/\text{mL}$ purified pollen allergen (Cry j 1 and Cry j 2, Hayashibara Biochemical Laboratories) in 100 mM of sodium bicarbonate buffer (pH 9.5) and incubated overnight at 4°C . Plates were subsequently washed four times with 200 μL /well of PBST, and blocked with 200 μL /well of 1% BSA in PBS and incubated for 2 h at 37°C . An additional four washes were performed (200 μL /well of PBST) and 100 μL /well of fecal extracts diluted in 1% BSA in PBST (1 : 2) were added onto the plate in triplicate wells for a 2 h incubation at 37°C . The plates were washed four times with PBST and incubated with 100 μL /well of biotinylated-monoclonal anti-mouse IgA (1 : 500; BD Pharmingen) diluted in 1% BSA in PBST for 1 h at 37°C . The wells were further washed four times with 200 μL /well of PBST, and 100 μL /well of avidin-horse radish peroxidase (HRP) conjugate (1 : 2000; BD Pharmingen) were applied onto the plate for 30 min incubation at 37°C . After a final four-wash cycle with PBST, pollen-specific IgA binding activity was revealed by addition of 100 μL /well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). The reaction was terminated after 30 min by addition of 50 μL /well of 2N H_2SO_4 , and absorbance values were determined at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories).

Total IgA levels in fecal sample were determined by ELISA procedure. Flat-bottom 96-well ELISA plates (Corning) were incubated with 100 μL /well of diluted purified anti-mouse IgA antibody (1 : 1000; BD Pharmingen; #556969) at 4°C overnight. The plates were washed four times with 200 μL /well of PBST, and blocked with 200 μL /well of 1% BSA in PBS and incubated for 2 h at 37°C . An additional four washes were performed (200 μL /well of PBST) and 100 μL /well of fecal extracts diluted in 1% BSA in PBST (1 : 2000) were added onto the plate in triplicate wells for a 2 h incubation at 37°C . Purified mouse IgA (BD Pharmingen, #553476) as standards were added to the plate for generation of a standard curve to calculate concentration of unknown samples. The plates were washed four times with PBST and incubated with 100 μL /well of biotinylated-monoclonal anti-mouse IgA (1 : 500; BD Pharmingen; #556978) diluted in 1% BSA in PBST for 1 h at 37°C . The wells were further washed four times with 200 μL /well of

PBST, and 100 μL /well of avidin-horse radish peroxidase (HRP) conjugate (1 : 2000; BD Pharmingen) were applied onto the plate for 30 min incubation at 37°C . After a final four-wash cycle with PBST, total IgA binding activity was revealed by addition of 100 μL /well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). The reaction was terminated after 30 min by addition of 50 μL /well of 2N H_2SO_4 , and absorbance values were determined at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories).

REAL-TIME RT-PCR ANALYSIS OF GENE EXPRESSION IN PEYER'S PATCHES

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). The total RNA integrity was assessed on 1% agarose gels, and respective concentrations and purity were determined with Nanodrop 8000 (NanoDrop Products, Wilmington, DE, USA). The cDNA was synthesized from 1.0 μg of total RNA using qScript cDNA Synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA) following the manufacture's instruction. The real-time PCR reactions were performed with SYBR Green I Detection kit (Quanta BioSciences) using an iCycler iQ Real Time PCR System (Bio-Rad Laboratories). The temperature profile was 95°C for 15 min, then 15 s at 95°C , 56°C for 15 s and 72°C for 30 s, repeated for 40-50 cycles. Melt curve analysis was conducted to confirm the specificity of each product. The target gene expression was normalized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and relative gene expression was determined using $R = 2^{(\text{Ct}(\text{reference}) - \text{Ct}(\text{test}))}$.¹⁸ Threshold cycle (Ct) values were obtained at the cycle number at which the gene is amplified beyond the threshold of 30 fluorescence units. Real-time PCR efficiencies were acquired by amplification of dilution series of cDNA according to the equation $10^{(-1/\text{slope})}$.¹⁹ The efficiencies of all primers used in this study were between 96-105%. Each sample was analyzed in duplicate for each gene. The sequences of the primers used for real-time RT-PCR analyses are shown in Table 1.

STATISTICAL ANALYSIS

All data were expressed as means $\pm$ SEM and subjected to ANOVA analysis 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. Statistical calculations were performed using the GraphPad Prism 5.0 package (GraphPad Software Inc., San Diego, CA, USA).

Table 1 Sequences of primers used in RT-PCR analyses in this study

Mouse Gene	Sequence (5'-3')	T _m value	PCR product size	Accession number
IL-4	FP: CCTCACAGCAACGAAGAACA	60.02	155 bp	NM_021283
	RP: ATCGAAAAGCCCGAAAGAGT	60.21		
IL-17A	FP: CCAGGGAGAGCTTCATCTGT	59.4	125 bp	NM_010552
	RP: AGGAAGTCCTTGGCCTCAGT	60.25		
GAPDH	FP: AACTTTGGCATTGTGGAAGG	59.97	132 bp	NM_008084
	RP: GGATGCAGGGATGATGTTCT	59.89		

FP, forward primer; RP, reverse primer; T_m, melting temperature; IL4, interleukin-4; IL17A, interleukin 17A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

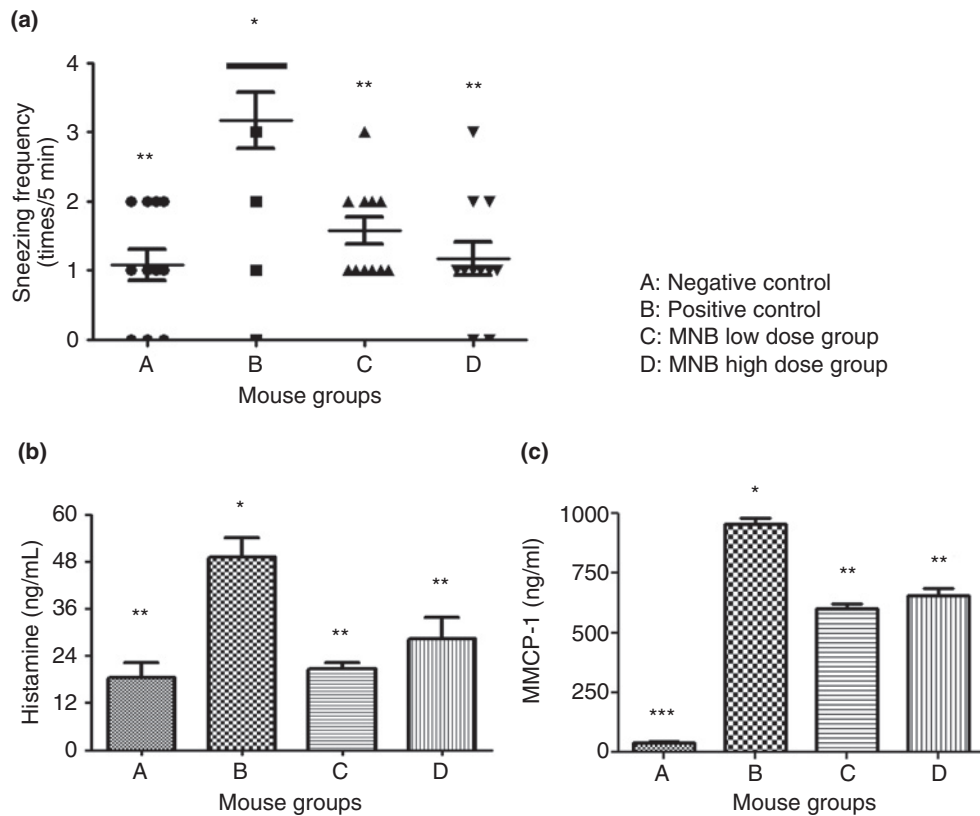


Fig. 2 (a) Determination of sneezing frequency in mice during 5 min post challenge by crude pollen allergen at the end point. Data are represented as mean $\pm$ SEM ($n = 12$ mice per group). (b) Histamine concentration in mouse serum determined by ELISA at the end point. Two serum samples were pooled in each group and data are represented as mean $\pm$ SEM ($n = 6$). (c) Mouse mast cell protease concentration determined in mouse serum by ELISA at end point. Different asterisks indicate statistically significant differences at $P < 0.05$.

RESULTS

REDUCTION IN FREQUENCY OF CLINICAL SIGNS AND HISTAMINE CONCENTRATION

Mice in both low and high dose MNB groups had significantly reduced ($P < 0.05$) sneezing frequency as compared to mice in the positive control group (Fig. 2a). However, there was no difference ($P > 0.05$) in

sneezing frequency among MNB treatment groups and negative control group. Mice in MNB treated groups and negative control groups also had lower ($P < 0.05$) serum histamine concentration (Fig. 2b) and less mMCP-1 concentration ($P < 0.05$) as compared to the positive control group (Fig. 2c).

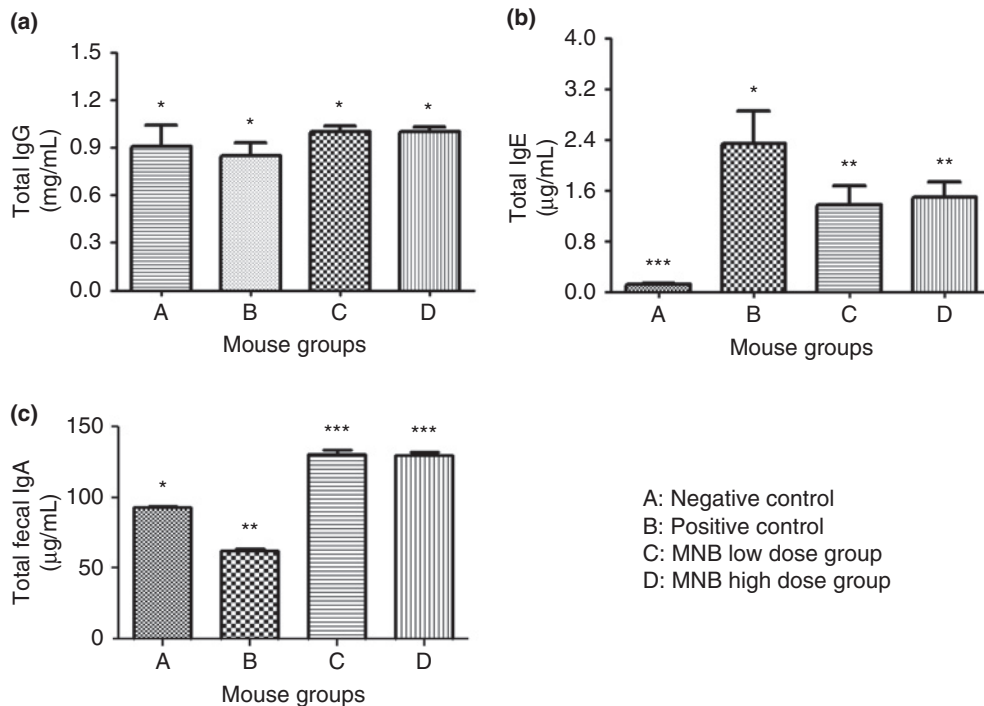


Fig. 3 Serum total IgE (a) and IgG (b) concentration post challenge by pollen allergen at the end point. Two serum samples were pooled in each group and concentration of serum total IgE and IgG were measured by ELSA. Data are represented as mean $\pm$ SEM ($n = 6$). (c) Total IgA concentration in the mice fecal extracts at the endpoint of experiment. Data are represented as mean $\pm$ SEM ($n = 3$ replicate tests in fecal extracts). Different asterisks indicate statistically significant differences at $P < 0.05$.

EFFECTS OF ANTIBODY ACTIVITY ON MNB PRETREATMENT

There was no significant difference in total and specific IgG and IgE antibody activity ($P > 0.05$) in the MNB treated mice as compared to those of the positive control (Fig. 3a, b). However, total fecal IgA levels were higher in MNB-treated mice as compared with those of the positive control mice (Fig. 3c).

There were no significant differences ($P > 0.05$) in Cry j 1 and Cry j 2-specific IgE (Fig. 4a), specific IgG (Fig. 4b), specific IgG1 (Fig. 4c) and specific IgG2a (Fig. 4d) antibody activity in serum of MNB treated mice as compared to the positive control group. However, Cry j 1 and Cry j 2 specific fecal IgA antibody activity was higher in the MNB-treated mice as compared with those of the positive control mice (Fig. 4e).

CYTOKINE CONCENTRATION IN SPLENOCYTES

Analyses of culture supernatants stimulated *in vitro* with purified Cry j 1 and Cry j 2 indicated that IL-4 (Th2-biased cytokine) concentrations were significantly lower ($P < 0.05$) in both MNB-administered groups as compared to mice in positive control group (Fig. 5a). IFN- γ (Th1-biased cytokine) concentrations

were significantly higher ($P < 0.05$) in both MNB-administered groups, compared with mice in positive control group (Fig. 5b). Also, mice in the high dose MNB-administered group had higher IL-10 (regulatory T-cell biased cytokine) concentration as compared to animals in positive control group ($P < 0.05$) (Fig. 5c). However, there was no significant difference ($P > 0.05$) in TGF- β (regulatory T cell-based cytokine) concentration (Fig. 5d) and IL-17 concentration (Fig. 5e) between groups.

CYTOKINE GENE EXPRESSION IN PEYER'S PATCHES

The mRNA gene expression of IL-4 gene was decreased in the Peyer's patches collected from the low dose MNB mice as compared to the positive control and high dose mice ($P < 0.05$; Fig. 6a). In addition, the mRNA level of IL-17 was also decreased in the Peyer's patches collected from both the high and low dose group of mice as compared to the positive control ($P < 0.05$; Fig. 6b). The mRNA levels of other genes IL-10, TGF-beta, IFN-gamma, T-bet, foxp3, GATA-3 and RORyt remained unchanged in the Peyer's patches of all treated groups (data not shown). No significant changes were observed in cytokine expression in the Peyer's patches collected

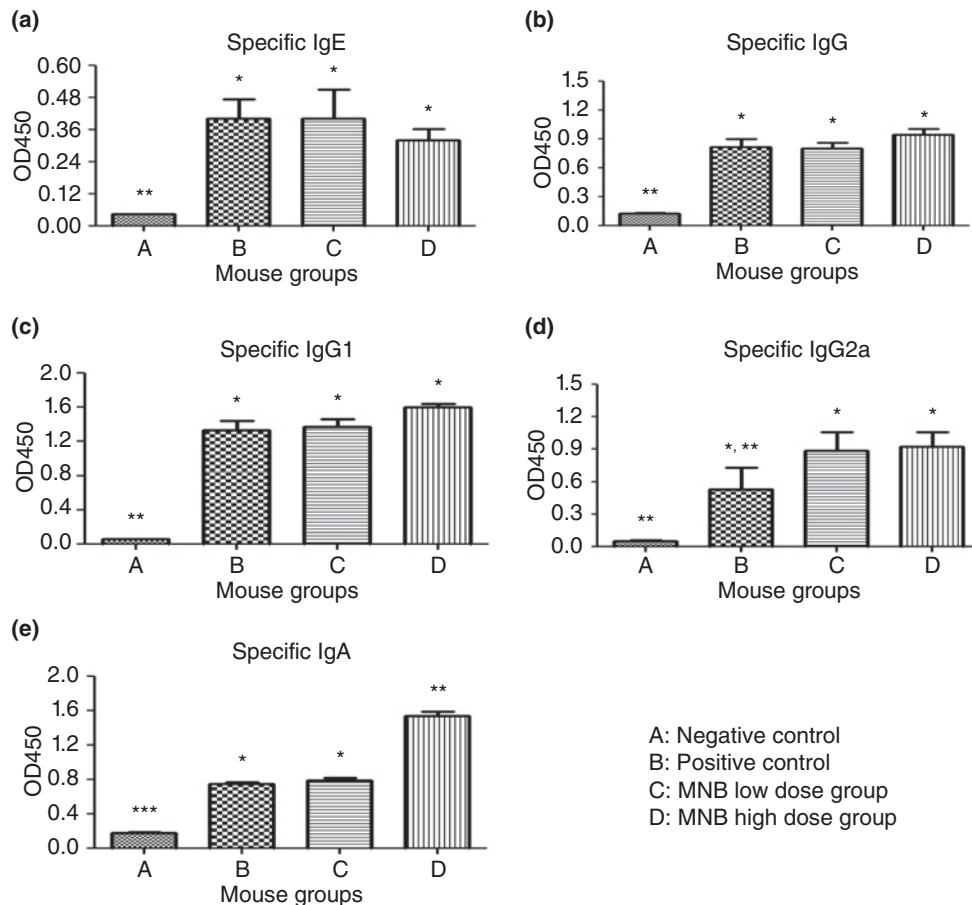


Fig. 4 Serum pollen allergen (Cry j 1 and Cry j 2)-specific IgE (a), IgG (b), IgG1 (c), and IgG2a (d) levels post challenge by pollen allergen at the end point. Every two serum samples were pooled in each group and levels of serum specific IgE, IgG, IgG1, and IgG2a were measured by ELSA. Data are represented as mean $\pm$ SEM ($n = 6$). (e) Specific IgA levels in the mouse fecal extracts at the endpoint of experiment. Data are represented as mean $\pm$ SEM ($n = 3$ replicate tests in fecal extracts). Different asterisks indicate statistically significant differences at $P < 0.05$.

from the negative control group ($P > 0.05$).

DISCUSSION

The present study was undertaken to provide a novel platform on use of prebiotic disaccharides to attenuate allergic reactions. The research addresses the contribution of a novel disaccharide, the β -1,4 manno-bose in lieu of being an immune-modulator in alleviating clinical signs of allergy in a mouse model of pollen allergy and to gain further insights into the underlying mechanisms by which the disaccharide MNB exerts an effect. Mice pre-fed with MNB diet before sensitisation with pollen extract showed a strong reduction in the allergic effector response when compared with mice fed PBS. Sneezing frequency is a major clinical sign to assess the pollen allergic response in a given animal model.²⁰ In the present study, MNB pretreatment significantly decreased the sneezing frequency serum histamine concentration and mast cell

protease enzyme activity in mice, suggesting that MNB exerts prophylactic effects of Japanese cedar pollen allergy.

Type 1 allergic diseases including Cj pollinosis are characterized by allergen-specific immunoglobulin E (IgE) activity.²¹ Here, we found that administration of MNB did not affect the serum Cry j 1 and Cry j 2-specific IgE, suggesting that the therapeutic effect MNB on allergy response may be due to induction of tolerance via other local regulatory mechanisms, indicating immune regulation. Also, measurements of serum Cry j 1 and Cry j 2-specific IgG, and sub-isotypes IgG1 and IgG2a, did not differ among treated groups, which concludes that MNB pretreatment did not affect antibody isotypes. Therefore, it is most likely that allergic suppression induced by MNB diet protects against the development of allergic signs, by a non-antibody isotype-mediated mechanism. It remains elusive how MNB may exert its effect on the allergic

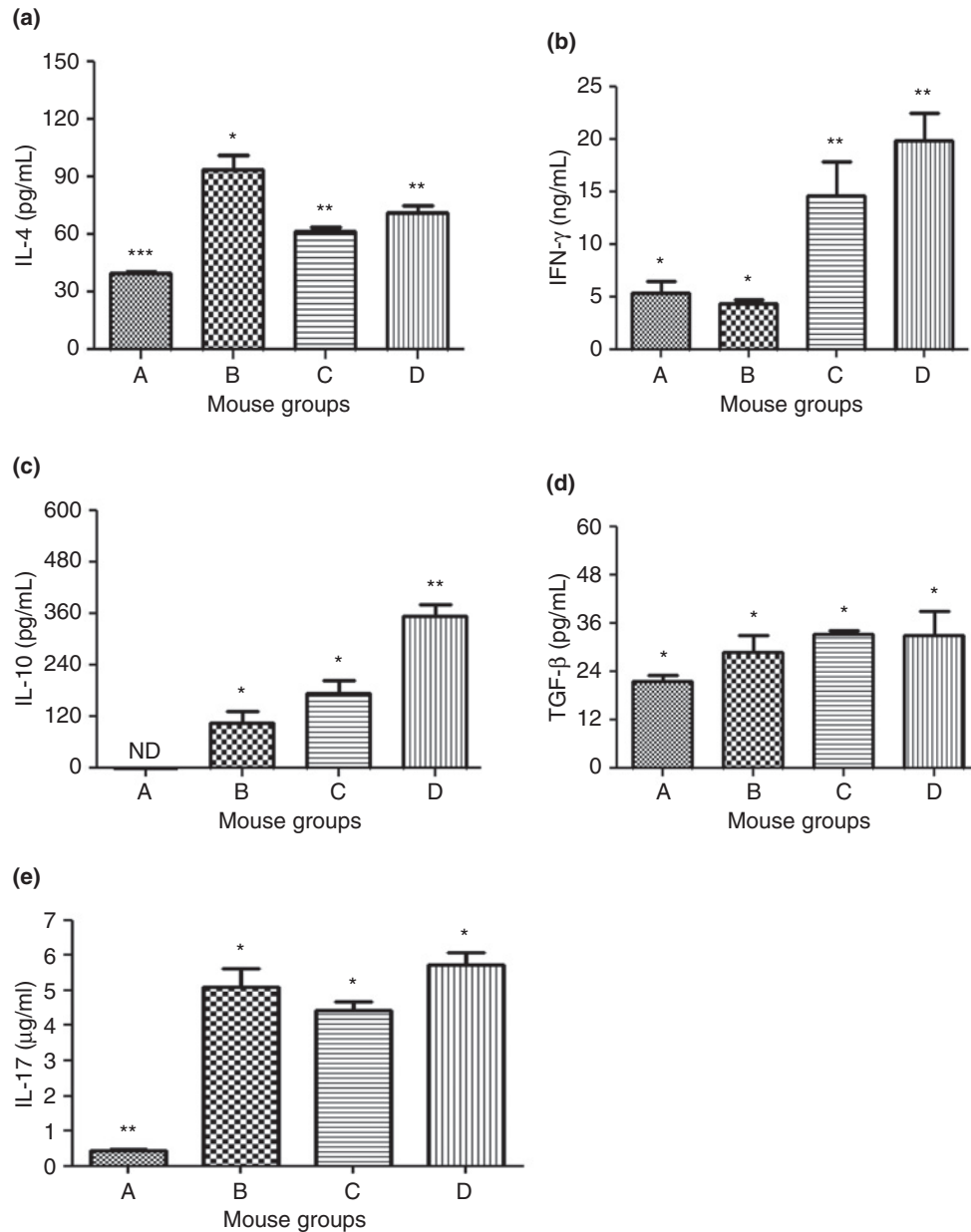


Fig. 5 Cytokine production of IL-4 (a), IFN- γ (b), IL-10 (c), TGF- β (d) and IL-17 (e) by spleen cells stimulated *in vitro* with pollen allergen (Cry j 1 and Cry j 2). Cytokine levels in the culture supernatants were determined by ELISA. Every two spleens were pooled for cell isolation in each group. Data are represented as mean $\pm$ SEM ($n = 6$). ND, non-detectable. Different asterisks indicate statistically significant differences at $P < 0.05$.

effector response since IgG and IgE antibody isotypes remain unaltered. It has been earlier reported by El-Naggar *et al.*,²² that immunotherapy with OVA-pullulan significantly decreased nasal airway resistance, dye leakage and histamine content in nasal irrigation after allergen challenge, but no significant difference was found in IL-4 and IL-5 mRNA expression or in specific IgE level among the groups. Also Nakamoto *et al.*²³ and several others have investigated the effects of allergen immunotherapy on the

nasal responses in guinea pigs with allergic rhinitis and found no significant differences in the number of allergen-specific IgE and IgG antibodies. This was consistent in our case as well in which antibody isotype for specific IgE did not differ among treatment groups. While specific IgE may be used as a useful marker of allergic responses, it is required however isn't mandatory to reflect the actual allergic status, since more IgE is also observed in the state of clinical tolerance. In the present study the total IgE differed

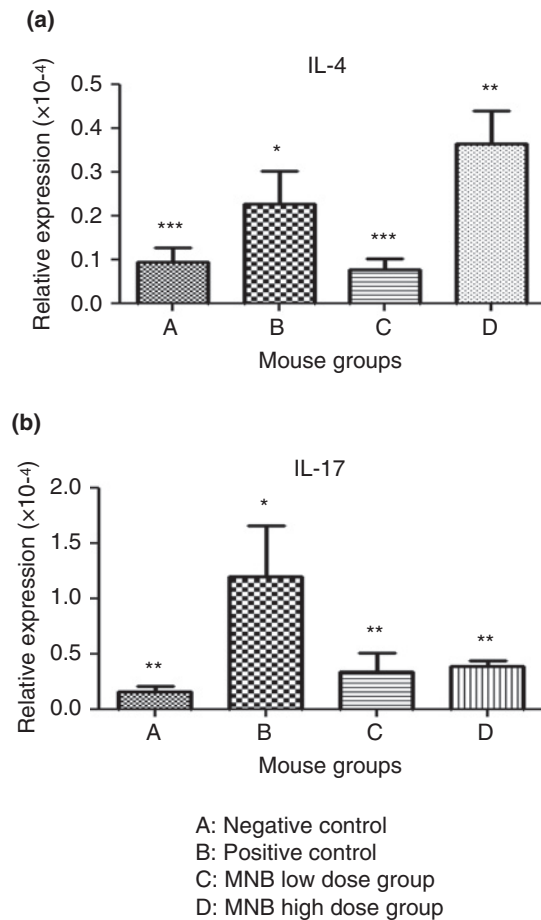


Fig. 6 Messenger RNA expression in Peyer's patches as determined by real-time RT-PCR analyses for IL-4 and IL-17A. Data are represented as mean $\pm$ SEM ($n = 6$). Different asterisks indicate significant differences ($P < 0.05$), between groups of mice.

among treatment groups, however; other than IgE, there may be other immuno-regulatory markers responsible for reduction of sneezing frequency.

There was an increase in both the total and specific IgA in the high dose treated group and total IgA increase was observed in the low dose treated group as well. Being the most abundant antibody isotype in mucosal secretions, IgA is produced by B cell and exported by the epithelial polymeric Ig receptor (pIgR)²⁴ 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*,¹³ which is consistent with the results observed here. Both the low and high MNB treated groups had reduced sneezing frequency, however with regards to fecal IgA content the specific IgA was only increased in the high dose group, whereas the total IgA was increased in both treated groups. It remains to be explored in this case the rea-

son for the difference in response between the two groups. Recent research showed that high intestinal IgA play an important role in induction of tolerance and reduced risk of IgE-associated allergic diseases.^{25,26} Moreover, intestinal IgA synthesis is regulated by T-cell dependent and T-cell independent mechanism and is necessary for intestinal immunity homeostasis by selecting and maintaining the appropriate microbial composition.²⁷ Therefore, the prophylactic effect of high dose MNB on allergy may be partially attributed to increased intestinal IgA secretions, indicating an active regulation of the mucosal immune response occurring at the gut level. It may be postulated that the modulating effects of these disaccharides are induced by change in gut microflora or in a microbiota independent mechanism of direct interaction on cells of the immune system, however in this study it seems that it may be attributed to the second mechanism.

The mechanism of pathogenesis of type I allergy is initiated by phagocytosis of allergens by antigen-presenting cells (APC), which present antigen to naïve T cells. Conventional CD4⁺ T cells can be subdivided into four distinct populations, the T helper type 1 (Th1), T helper type 2 (Th2), T helper 17 (Th17), and regulatory T (Treg) cells.²⁸ The Th2 cells predominantly produce interleukin (IL)-4 and IL-5, and promote IgE synthesis. In contrast, the Th1 cells mainly secrete cytokines such as IL-2 and interferon (IFN)- γ , and prevent IgE production.²⁹ The balance between Th1- and Th2- is thought to be important for the development of allergy disease and in immune homeostasis. The Th17 cells mainly secrete IL17A and Treg cells mainly secrete TGF- β and IL-10.³⁰ Regulatory T cells can regulate the Th1/Th2 balance and suppress the allergic response.¹⁷ To clarify the mechanisms involved in the inhibition of allergy, we examined cytokine production from spleen cells that were stimulated with Cry j1 and Cry j 2 *in vitro*. We found oral administration of MNB (both low and high dose) improves Th1/Th2 balance by decreasing Th2-biased cytokine IL-4, and increasing Th1-biased cytokine IFN- γ . The high dose also induced the Treg cytokine response by increasing IL-10 production, suggesting that MNB may modulate the Th1/Th2 balance by inducing Treg cell differentiation thereby attenuating the allergy response. However staining cells for CD25⁺ Foxp3⁺ was not done here which are part of ongoing investigations elucidating further insights into mechanistical evidence of MNB abrogating allergic suppression, to test if T-regs are directly involved in suppression of allergic response. Also the cytokine TGF β did not differ among groups. This is not surprising since Bohle *et al.* earlier reported that suppression of allergen-specific T cell proliferation was observed at 4 weeks of birch pollen immunotherapy mediated by IL-10 but not TGF-beta.³¹ Similar observation was made in our study in which the high

dose group had more IL-10 production, but no difference was observed in TGF- β production among groups. It could be that IL-10-producing CD4⁺ T cells seen in our study may be a result of expansion of bystander cells as described earlier in murine disease models.³²

The Peyer's patches are the primary site for antigen uptake in the intestine and play an important role in immune homeostasis.³³ The genes T-bet, GATA3, ROR γ T, and FoxP3 have emerged as the key transcriptional factors responsible for controlling the fate of both innate and adaptive immune cells, and its expression in Th1, Th2, Th17, and Treg cells, respectively.^{30,34} To clarify the mechanisms involved in the inhibition of allergy, we also examined the mRNA expression of cytokines and transcriptional factors in Peyer's patches. We found IL-4 mRNA expression was decreased upon administration of low dose MNB, suggesting MNB can suppress Th2 response in the Peyer's patches in that group. However the high dose group had increased IL-4 expression. Since the IL-4 protein concentration was low with both the treatments, it could be argued that validating IL-4 concentrations at the protein level would seem more appropriate as compared to the mRNA expression data. In the present study, administration of MNB decreased IL17A mRNA expression suggesting that MNB suppresses the Th17A response. Recently, IL17A has been believed to be involved in the pathogenesis of allergic diseases and is being targeted as a potential therapeutic candidate for allergy³⁵ and in that context the reduction of IL-17 expression in both treatment groups is of interest.

Both the low and high dose of MNB alleviated clinical signs of pollen allergy using various mechanisms. The low dose group had less histamine, less mMCPT-1 concentration, less total IgE, more total IgA, less IL-4, more IFN- γ and less IL-4 and IL-17 mRNA expression. On the other hand the high dose group showed less histamine, less mMCPT-1 concentration, less total IgE, more total and specific IgA, less IL-4 and more IFN- γ and IL-10 and less IL-17 mRNA expression. Overall, in conclusion, dietary intervention with MNB significantly attenuated pollen allergic response by improving Th1/Th2 balance, increasing regulatory T cell response, decreasing Th17 response, and increasing IgA production. However, how MNB interacts with mucosa-associated lymphoid tissue (MALT) as well as local/regional mucosa-draining lymph nodes (LNs) by potential receptors, such as TLR4, is still not clear and further investigation is needed to be conducted. This study provides insight into using MNB as a prophylactic immune modulator candidate for suppression of pollen allergy.

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