

Low Doses of Allergen and Probiotic Supplementation Separately or in Combination Alleviate Allergic Reactions to Cow β -Lactoglobulin in Mice^{1,2}

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Abstract

Probiotic supplementation and oral tolerance induction can reduce certain types of food allergy. The objectives of this study were to investigate the allergy-reducing effects of probiotics (VSL#3) and/or oral tolerance induction via low doses of an allergen supplementation in β -lactoglobulin (BLG)-sensitized mice. Three-week-old, male BALB/c mice were divided into 6 groups ($n = 8$ /group): sham-sensitized negative control (CTL−), BLG-sensitized positive control (CTL+), oral tolerance-induced and BLG-sensitized group (OT), probiotic-supplemented OT group (OTP), probiotic-supplemented CTL− (PRO), and probiotic-supplemented and BLG-sensitized (PROC) groups. Mice were i.p. sensitized with BLG and alum and then orally challenged with BLG. Immunological responses were assessed by monitoring hypersensitivity scores and measuring levels of BLG-specific serum IgS, total serum IgE and fecal IgA, and cytokines from serum and spleen lysates. Hypersensitivity scores were significantly lower in the PROC (2.00 ± 0.53), OT (0.75 ± 0.46), and OTP mice (1.00 ± 0.53) than in the CTL+ mice (2.63 ± 0.52) as were BLG-specific serum IgE concentrations (34.3 ± 10 , 0.442 ± 0.36 , 3.54 ± 3.5 , and $78.5 \pm 8.7 \mu\text{g/L}$ for PROC, OT, OTP, and CTL+, respectively). Our results suggest that supplementation of VSL#3 suppressed the allergic reaction mainly through increased intestinal secretory IgA (sIgA) in PROC mice, and oral tolerance offered allergen-specific protective effects to BLG-induced allergy, probably through CD4+CD25+ regulatory T cell-mediated active suppression. In OTP mice, probiotics did not induce a further reduction of hypersensitivity score compared with OT mice but may provide additional protection to unforeseen nonspecific challenges through increased intestinal sIgA. J. Nutr. doi: 10.3945/jn.112.169466.

Introduction

Food allergy is a complex disease that poses a growing threat to public health and the underlying immunological mechanisms are still not fully understood. Cow milk protein allergy (CMA)⁵ is the most common food allergy in infants and children from developed countries, with an estimated incidence rate of 2.2% (1). Although CMA patients may be allergic to several milk proteins, caseins and β -lactoglobulin (BLG) are considered the most allergic cow milk proteins (2). Depending on the nature of immunological response, CMA may be divided into IgE-

mediated (immediate reactions) and non-IgE-mediated (delayed) reactions. Clinical manifestations of CMA include skin reactions (e.g., urticaria, angioedema, and contact dermatitis), respiratory reactions (e.g., wheezing, rhinitis, dry cough, laryngeal edema, and asthma with severe respiratory distress), and gastrointestinal reactions (e.g., diarrhea and vomiting, bloody stools, constipation, severe irritability colic). IgE-mediated allergic reactions are exhibited within minutes to an hour after an allergen exposure (typically within 10–20 min) with a risk of anaphylaxis, whereas non-IgE-mediated reactions are displayed within an hour to several days after ingestion of an allergen (3,4). In general, healthy individuals with mature immune systems have tolerance to most ingested food proteins and commensal bacteria (5). Food allergy may develop when specific oral tolerance against ingested food is impaired or failed to develop. Results from mouse models have shown promising protective effects of oral tolerance induction on food allergy (6,7). On the other hand, the emergence of the hygiene hypothesis and the findings from a germ-free mouse study revealed a protective role of gut microbiota in food allergy

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⁵ Abbreviations used: BLG, β -lactoglobulin; CMA, cow milk protein allergy; CTL−, sham-sensitized negative control group; CTL+, β -lactoglobulin-sensitized positive control group; OT, oral tolerance-induced and β -lactoglobulin-sensitized group; OTP, oral tolerance-induced and probiotic-supplemented group; PRO, control group for probiotics (probiotic-supplemented sham-sensitized mice); PROC, probiotic-supplemented and β -lactoglobulin-sensitized group; sIgA, secretory IgA; Th, T helper cell; Treg, regulatory T cell.

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development (8). Recently, a mouse model study of CMA reaffirmed that germ-free mice were more susceptible to CMA than conventional mice (9). Another recent finding demonstrated that continuous microbial exposure at the early stage of life was necessary in the establishment of normal gut microbiota, and this process was compromised under excessive hygienic conditions (10). Gut microbiota are essential for the initiation of an infant's own IgA production and influence development of the entire IgA system (11). We hypothesized that mice with prior supplementation of low doses of an oral allergen and probiotics individually or concurrently before the CMA challenge would alleviate CMA attack. Therefore, the purpose of this study was to investigate the effects of oral tolerance and/or probiotics on allergic responses to bovine BLG.

Materials and Methods

Mice and experimental design. Three-week-old, male BALB/c mice (Charles River Laboratories) were fed a diet free from animal proteins (no. 2918 Teklad Harlan Global Diet) (12) and raised as previously described (13). Our choice of just-weaned, 3-wk-old mice was intended to better reflect human infant food allergy. Mice were used in the experiment after a 1-wk acclimation period. Animal care use and protocols were approved by the McGill University Animal Care Committee.

A food allergy sensitization method recently developed in our laboratory was used in this study with minor adjustments (13). Systemic sensitization through i.p. injections of allergens with alum was used in this study, because alum has been increasingly applied in mouse models for oral tolerance and food allergy studies (6,14) due to its relative safety and strong T helper cell (Th2)-favored adjuvant nature.

Mice were divided into 6 groups: oral tolerance-induced mice (OT), probiotic-supplemented mice (PROC), oral tolerance-induced and probiotic-supplemented mice (OTP), control mice for probiotic supplementation that received probiotics but were sham-sensitized with PBS (PRO), BLG-sensitized positive control mice (CTL+), and sham-sensitized negative control (CTL-), with 8 mice/group. The oral tolerance induction, BLG sensitization, and probiotic supplementation schedules are detailed in Figure 1. Briefly, starting at 28 d of age, oral tolerance induction to OT and OTP mice was performed by i.g. administration of 2 mg of BLG (Sigma Aldrich) in 200 μ L of PBS for 5 consecutive days. VSL#3 probiotics were supplemented daily via gavage to OTP, PROC, and PRO mice (1.5×10^9 CFU in 250 μ L of PBS) from wk 4 to 9 of age. VSL#3 is a well-recognized mixture of probiotics that contains 8 species of freeze-dried, live probiotics: *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *Bulgaricus*, *Bifidobacteria breve*, *Bifidobacteria infantis*, *Bifidobacteria longum*, and *Streptococcus salivarius* subsp. *thermophilus*. VSL#3 has been shown to promote gut health and prevent

chronic intestinal inflammation through stimulation of epithelial innate immunity (15) and also attenuate liver fibrosis (16). The dosages of BLG and VSL#3 were determined after careful consideration of dosages used in previously published papers (7,14,15,17). OT, OTP, PROC, and CTL+ mice were i.p. sensitized with 3 weekly (at wk 6, 7, and 8) doses of 250 μ L PBS-based solution that contained BLG (50 μ g) and 2 mg alum (Pierce Chemical). One week after the last sensitization, mice were i.g. challenged with 2 doses of 30 mg BLG in 250- μ L PBS solutions 30 min apart. In every step of the treatment and sensitization, negative control mice were sham-administered through the same route with the same volume of PBS solution devoid of BLG or probiotics.

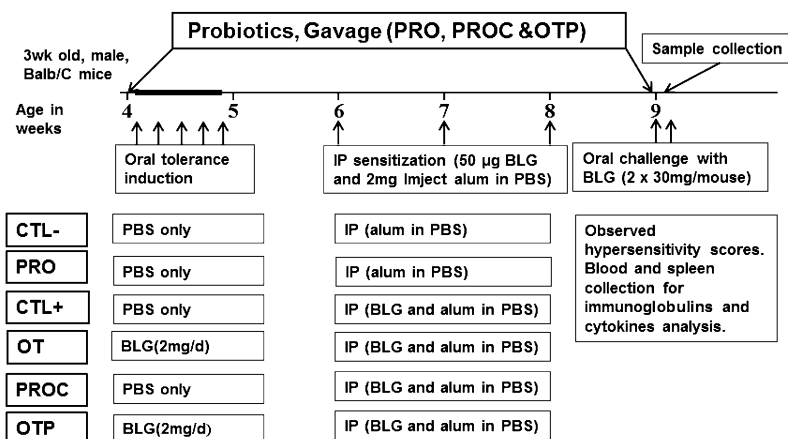
Reagents. HRP conjugated anti-mouse antibodies were obtained from Bethyl Laboratories. Purified anti-mouse Igs were obtained from BD Pharmingen and ABTS peroxidase substrate was purchased from Mandel Scientific. Bio-Plex Pro Mouse Cytokine Assay kits (Bio-Rad) were used to detect spleen lysate cytokines. VSL#3 was obtained from VSL pharmaceuticals. All other chemicals were purchased from either Sigma or Fisher Scientific.

Evaluation of hypersensitivity symptoms. Within 1 h following the final oral BLG challenge, hypersensitivity symptoms were scored by a person unaware of the study treatments by using the score system as described by Schouten et al. (18). The scores were as follows: 0 = no symptom; 1 = scratching and rubbing around the nose and head; 2 = reduced activity; 3 = activity after prodding and puffiness around the eyes and mouth; 4 = no activity after prodding, labored respiration, and cyanosis around the mouth and the tail; and 5 = death.

Fecal, serum, and spleen sample collections. Fresh fecal pellets were collected at d 51 of age by transferring mice into new cages that were lined with moist paper towels. Fecal pellets were immediately stored in -80°C until further analysis. Three hours after the final oral BLG challenge, mice were killed by CO_2 inhalation and blood samples were collected by intra-cardiac puncture. Blood samples were collected into serum separator tubes (Sarstedt). The serum portion was separated by centrifugation at $10,000 \times g$ for 5 min at 20°C . Serum samples were then aliquoted into Eppendorf tubes and stored at -20°C until analysis. Spleens were also aseptically collected, snap frozen in liquid nitrogen, and stored at -80°C until further analysis.

Determination of serum Igs and fecal IgA. BLG-specific serum IgG1, IgG2a, and IgA as well as total IgE and total fecal IgA were measured by indirect ELISAs by adopting a previously described procedure with minor modifications (13). Briefly, 96-well plates (Dynatech Laboratories) were coated with 50 mg/L BLG in 0.1 mol/L Na-bicarbonate/carbonate coating buffer (pH 9.6). Serum samples were diluted 1:100 for BLG-specific serum IgE, 1:200 for total IgE, and 1:1000 for others before being used in the assays. All other procedures and conditions, such as washing, blocking, incubation, volume, and diluent used, were exactly the same as the procedure previously described (13).

FIGURE 1 Schematic representation of experimental protocol. Corresponding treatments ($n = 8/\text{treatment}$) were given to the mice as stated in the above diagram. Three hours after the final oral BLG challenge, mice were killed by CO_2 inhalation and samples were collected. Alum, aqueous solution of aluminum and magnesium hydroxide adjuvant; BLG, β -lactoglobulin; CTL-, sham-sensitized negative control group; CTL+, β -lactoglobulin-sensitized positive control group; OT, oral tolerance-induced and β -lactoglobulin-sensitized group; OTP, oral tolerance-induced and probiotic-supplemented group; PRO, control group for probiotics (probiotic-supplemented sham-sensitized mice); PROC, probiotic-supplemented and β -lactoglobulin-sensitized group.



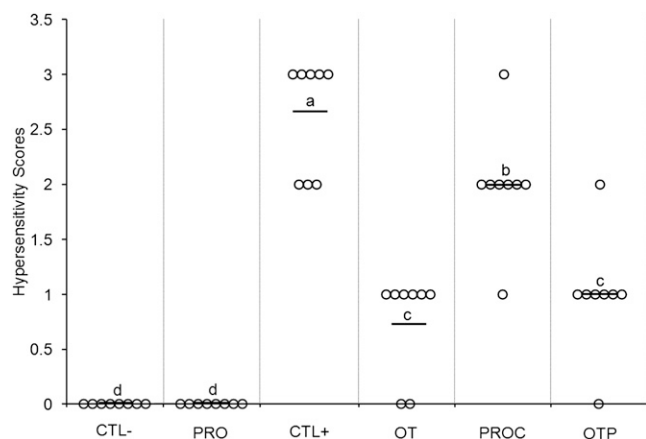


FIGURE 2 Hypersensitivity scores for individual mice recorded within 1 h after the final oral BLG challenge. Each point represents an individual mouse. Mean scores ($n = 8$) are shown by horizontal lines. Means without a common letter differ, $P < 0.05$. BLG, β -lactoglobulin; CTL-, sham-sensitized negative control group; CTL+, β -lactoglobulin-sensitized positive control group; OT, oral tolerance-induced and β -lactoglobulin-sensitized group; OTP, oral tolerance-induced and probiotic-supplemented group; PRO, control group for probiotics (probiotic-supplemented sham-sensitized mice); PROC, probiotic-supplemented and β -lactoglobulin-sensitized group.

For detection of total and BLG-specific serum IgE, a Signal Boost Immunoreaction Enhancer Kit (EMD Millipore) was used to dilute serum and HRP-conjugated anti-mouse IgE. For fecal IgA concentration, fecal supernatants were prepared from fecal samples as described by Adel-Patient et al. (19). Fecal pellets (100 mg) were placed in 1 mL of PBS containing 0.01% (v:v) of the protease inhibitor cocktail (Sigma-Aldrich). The mixture was vortexed and centrifuged at $16,000 \times g$ for 10 min. Supernatants were collected. Undiluted supernatants were used to determine fecal secretory IgA (sIgA) by ELISA. All analyses were performed in duplicates or triplicates and the mean values were used. Relative Ig concentrations were determined by extrapolation from standard curves with purified mouse Ig standards. The limit of detection for the BLG-specific IgE, IgA, and IgG subclasses was $3.7 \mu\text{g/L}$.

Cytokine analyses of serum samples and spleen lysates. Spleen lysates were prepared as previously described (13,20). Briefly, individual spleens were placed in Eppendorf tubes in 0.5 mL PBS containing 0.01% (v:v) of the protease inhibitor cocktail. Cytokines from spleen lysates and serum samples were analyzed by Mouse Cytokine 23-plex Assay kits. After following the manufacturer's protocol, beads were read with a Bio-

Plex Reader and data were analyzed with Bio-Plex Manager 2.0 software (Bio-Rad Laboratories).

Statistical analyses. Data were analyzed by using PROC MIXED of SAS 9.3 (SAS Institute, 2003) except for the anaphylaxis data, which were analyzed using the Kruskal-Wallis test and SigmaStat software (Systat Software). Differences among treatment means were tested using the Scheffé multiple comparison test. Data are expressed as means $\pm$ SE and a $P < 0.05$ was considered significantly different.

Results

Hypersensitivity scores. Hypersensitivity scores were recorded within 1 h after the final challenge with BLG. Hypersensitivity scores were significantly lower in the OT, OTP, and PROC groups than that in the CTL+ group (Fig. 2).

BLG-specific and total Igs from serum. As shown in Table 1, serum BLG-specific IgE and total IgE concentrations were higher in CTL+ mice than in all other groups ($P < 0.05$). Serum BLG-specific IgE and total IgE concentrations in the PROC group were lower than those in the CTL+ mice, but were higher than those in the CTL-, OT, and OTP groups ($P < 0.05$). Serum BLG-specific IgE and total IgE concentrations did not differ among the CTL-, PRO, OT, and OTP groups. In contrast to their serum BLG-specific IgE and total IgE concentrations, the BLG-specific serum IgG1 concentration in the PROC group was higher than that in the CTL+ group ($P < 0.05$) (Table 1). BLG-specific serum IgG2a concentrations in the CTL+ and PROC groups were similar and higher than those in the CTL-, OT, and OTP groups ($P < 0.05$) (Table 1).

BLG-specific serum and total fecal IgA. Both the OT and OTP groups had significantly lower BLG-specific serum IgA concentrations compared with the PROC group (Table 1). In fecal samples, nonspecific sIgA concentrations were detectable from all treatment groups. However, probiotic-supplemented mice in the PRO, PROC, and OTP groups had higher total fecal sIgA concentrations than groups of mice that did not receive probiotic (i.e., CTL-, CTL+, and OT; $P < 0.05$) (Table 1).

Cytokine concentrations in serum and spleen lysates. The serum and spleen lysate cytokine concentrations of PRO mice were lower than those of other groups (Tables 2 and 3). The IL-4 concentration in spleen lysates was higher in the CTL+ group

TABLE 1 Effect of low doses of allergen and probiotic supplementation on serum and fecal antibodies in mice¹

	CTL-	PRO	CTL+	OT	PROC	OTP
BLG-specific						
serum antibody						
IgE, $\mu\text{g/L}$	ND	ND	78.5 ± 8.7^a	0.442 ± 0.36^c	34.3 ± 10^b	3.54 ± 3.5^c
IgG1, mg/L	ND	ND	40.5 ± 4.1^b	11.2 ± 2.1^c	58.0 ± 3.8^a	11.0 ± 1.7^c
IgG2a, mg/L	ND	ND	10.1 ± 2.3^a	0.334 ± 0.29^b	9.32 ± 1.5^a	0.924 ± 0.68^b
IgA, mg/L	ND	ND	4.83 ± 0.93^a	0.334 ± 0.13^b	4.21 ± 0.90^a	0.541 ± 0.19^b
Total antibody						
Serum IgE, $\mu\text{g/L}$	32.1 ± 3.9^c	23.7 ± 1.7^c	101 ± 7.5^a	26.4 ± 2.3^c	62.4 ± 6.2^b	30.2 ± 3.8^c
Fecal IgA, $\mu\text{g/L}$	14.3 ± 0.80^b	25.6 ± 2.3^a	14.0 ± 1.3^b	15.3 ± 1.3^b	22.9 ± 1.8^a	22.2 ± 1.0^a

¹ Values are mean $\pm$ SE, $n = 8$. Means without a common letter differ, $P < 0.05$. BLG, β -lactoglobulin; CTL-, sham-sensitized negative control group; CTL+, β -lactoglobulin-sensitized positive control group; ND, not detectable; OT, oral tolerance-induced and β -lactoglobulin-sensitized group; OTP, oral tolerance-induced and probiotic-supplemented group; PRO, control group for probiotics (probiotic-supplemented sham-sensitized mice); PROC, probiotic-supplemented and β -lactoglobulin-sensitized group.

TABLE 2 Effect of low doses of allergen and probiotic supplementation on spleen lysate cytokines in mice¹

Cytokine	CTL−	PRO	CTL+	OT	PROC	OTP
	ng/L					
IL-4	206 ± 4.8 ^c	232 ± 10 ^{bc}	342 ± 51 ^a	278 ± 12 ^{a-c}	316 ± 9.9 ^{ab}	265 ± 18 ^{a-c}
IFN γ	89.0 ± 24 ^b	106 ± 27 ^b	192 ± 18 ^{ab}	234 ± 18 ^a	192 ± 24 ^{ab}	179 ± 18 ^{ab}
IL-10	52.1 ± 7.8 ^c	80.2 ± 9.8 ^{bc}	112 ± 7.1 ^{ab}	149 ± 7.6 ^a	134 ± 11 ^a	118 ± 8.7 ^{ab}
IL-12	633 ± 29 ^b	787 ± 50 ^{ab}	829 ± 20 ^{ab}	971 ± 51 ^a	937 ± 42 ^a	977 ± 63 ^a

¹ Values are mean ± SE, $n = 8$. Means without a common letter differ, $P < 0.05$. CTL−, sham-sensitized negative control group; CTL+, β -lactoglobulin-sensitized positive control group; OT, oral tolerance-induced and β -lactoglobulin-sensitized group; OTP, oral tolerance-induced and probiotic-supplemented group; PRO, control group for probiotics (probiotic-supplemented sham-sensitized mice); PROC, probiotic-supplemented and β -lactoglobulin-sensitized group.

compared with that in the CTL− group ($P < 0.05$); the concentrations of the other groups were between those of the CTL+ and CTL− groups and did not differ from one another (Table 2). Spleen lysate IFN γ concentrations in the OT group were significantly higher compared with the CTL− and PRO groups but did not differ from the CTL+, PROC, and OTP groups (Table 2). Spleen lysate IL-10 concentrations were significantly higher in the BLG-sensitized groups (i.e., CTL+, OT, PROC, and OTP) compared with groups without BLG sensitization (PRO and CTL−) (Table 2). The OT and OTP groups had significantly higher concentrations of serum IL-12 (p40) compared with CTL+ group, but no differences were observed among the PROC, PRO, CTL+, and CTL− groups or between the OT and OTP groups (Table 3). Serum cytokine concentrations (both Th1- and Th2-mediated profile) were similar in PROC and CTL+ mice (Table 3).

Mouse MCP-1 from serum and spleen lysates. MCP-1 concentrations in both serum and spleen lysates were significantly higher in the OT group compared with the other groups. Serum MCP-1 concentrations were significantly lower in the PRO group and spleen lysates MCP-1 concentrations were also significantly lower in the CTL− and PRO groups compared with other groups (Table 4).

Discussion

Probiotic supplementation and oral tolerance induction, individually or concurrently, reduced bovine BLG-induced allergy in this study as indicated by significantly lower hypersensitivity scores and BLG-specific serum IgE concentrations in OT, OTP,

and PROC mice compared with CTL+. An examination of immunological variables suggested that oral tolerance induction and/or probiotic supplementation may have distinct mechanisms to reduce allergy.

Two widely acknowledged mechanisms for oral tolerance induction are active suppression through CD4+CD25+ regulatory T cells (Tregs) (21) and clonal anergy or deletion through apoptosis (22). Active suppression could be induced by low doses of allergen supplementation and clonal anergy could be induced by one time supplementation of a high-dose allergen (23). However, researchers now have focused mainly on low doses of allergens to induce oral tolerance, because high-dose allergen supplementation is often associated with anaphylactic shock. Thus, we administered low doses of BLG (2 mg BLG for 5 d) to induce active suppression in mice. Elevated food allergy markers, such as higher hypersensitivity scores along with higher BLG-specific serum IgE, IgG1, and spleen-derived IL-4, in CTL+ mice (Tables 1 and 2) confirmed the validity of our model.

OT mice were capable of suppressing both an allergen-specific Th1 response, indicated by reduced BLG-specific serum IgG2a and elevated IL-12(p40), and a Th2 response, as indicated by lower BLG-specific serum IgE and IgG. Our results were similar to those from a mouse peanut allergy model study (24). In addition, our results were in full agreement with the observation from Adel-Patient et al. (6), who reported that oral tolerance and Tregs were induced in BALB/c mice after gavage with bovine BLG. Furthermore, recent studies have demonstrated that allergy suppression induced by oral tolerance was mediated by CD4+CD25+ Tregs that express the transcription factor forkhead p3 (Foxp3) (6,25,26). All of these reports clearly support the notion that the major pathway for OT-induced

TABLE 3 Effect of low doses of allergen and probiotic supplementation on serum cytokines in mice¹

Cytokine	CTL−	PRO	CTL+	OT	PROC	OTP
	ng/L					
Th1 cytokine						
IL-12 (p40)	130 ± 3.3 ^b	109 ± 6.9 ^b	139 ± 4.7 ^b	191 ± 14 ^a	133 ± 9.2 ^b	190 ± 11 ^a
IFN γ	154 ± 14 ^a	64.0 ± 10 ^b	127 ± 22 ^{ab}	119 ± 14 ^{ab}	90.0 ± 12 ^{ab}	115 ± 9.4 ^{ab}
Th2 cytokine						
IL-5	16.0 ± 1.4 ^a	5.50 ± 1.4 ^b	17.1 ± 1.9 ^a	18.6 ± 1.4 ^a	12.6 ± 1.9 ^a	14.2 ± 1.0 ^a
IL-9	211 ± 10 ^{ab}	160 ± 6.8 ^b	207 ± 14 ^{ab}	217 ± 7.9 ^a	158 ± 13 ^b	200 ± 14 ^{ab}
IL-13	161 ± 12 ^a	85.0 ± 10 ^b	157 ± 23 ^a	141 ± 9.0 ^{ab}	109 ± 17 ^{ab}	145 ± 9.0 ^{ab}

¹ Values are mean ± SE, $n = 8$. Means without a common letter differ, $P < 0.05$. CTL−, sham-sensitized negative control group; CTL+, β -lactoglobulin-sensitized positive control group; OT, oral tolerance-induced and β -lactoglobulin-sensitized group; OTP, oral tolerance-induced and probiotic-supplemented group; PRO, control group for probiotics (probiotic-supplemented sham-sensitized mice); PROC, probiotic-supplemented and β -lactoglobulin-sensitized group.

TABLE 4 Effect of low doses of allergen and probiotic supplementation on serum and spleen lysate MCP-1 in mice¹

MCP-1	CTL–	PRO	CTL+	OT	PROC	OTP
	ng/L					
Serum MCP-1	303 ± 12 ^b	175 ± 17 ^c	267 ± 28 ^{bc}	436 ± 32 ^a	232 ± 17 ^{bc}	288 ± 16 ^b
Spleen MCP-1	466.0 ± 119 ^c	520.2 ± 140 ^c	1787 ± 170 ^b	2940 ± 230 ^a	1473 ± 203 ^b	2008 ± 218 ^b

¹ Values are mean ± SE, *n* = 8. Means without a common letter differ, *P* < 0.05. CTL–, sham-sensitized negative control group; CTL+, β -lactoglobulin-sensitized positive control group; OT, oral tolerance-induced and β -lactoglobulin-sensitized group; OTP, oral tolerance-induced and probiotic-supplemented group; PRO, control group for probiotics (probiotic-supplemented sham-sensitized mice); PROC, probiotic-supplemented and β -lactoglobulin-sensitized group.

tolerance in our study was associated with Foxp3⁺Treg-mediated active suppression.

Intestinal sIgAs may not be involved in OT-induced tolerance, because total fecal IgA and BLG-specific serum IgA concentrations in OT mice were lower than those in CTL+ mice (Table 1). Our finding of lower allergen-specific serum IgA and fecal IgA in OT mice was also in full agreement with the result of a recently published paper (7), which stated that ovalbumin-specific serum IgA was suppressed at the basal level in oral tolerance-induced mice. In addition, we chose MCP-1 as a complementary marker for histamine release by basophils and mast cells, because MCP-1 causes mediator release from these cells in a dose-dependent manner (27). Interestingly, both serum and spleen MCP-1 concentrations in OT mice were higher than those in the CTL+ group (Table 4) and the OTP group had a similar MCP-1 concentration to the CTL+ group. This suggests that oral tolerance may induce higher concentrations of MCP-1 and probiotics may prevent an oral tolerance-induced increase in MCP-1 production. Consistent with our results from the OT group, a recent CMA desensitization study revealed higher concentrations of serum MCP-1 as well as lower CMP-specific IgE in CMA subsided children (28).

Probiotics have also been used to suppress food allergy. Our results from PRO mice indicated that under normal, unchallenged conditions, probiotics have a tendency to suppress both Th1- (lower serum IFN γ) and Th2- (lower serum IL-5, IL-9, and IL-13) associated immune responses (Table 3). However, this probiotic-induced, basal, immunological setting changed when the mice were sensitized and challenged with BLG. An examination of immunological responses in PROC mice revealed that probiotic supplementation may alleviate allergic reactions mainly through producing sIgAs.

Higher fecal IgA concentrations in the PROC group than in the CTL+ group support the notion that sIgA was responsible for the allergy-reducing effect of probiotics in this study. In line with our findings, a study conducted in a cohort of allergy-prone infants revealed that probiotic supplementation tended to increase fecal IgA and it is associated with reduced risk for IgE-mediated allergic diseases (29). The immunological response pattern of VSL#3-supplemented mice in our study was also in complete agreement with the findings of a recent study (30) that reported the allergy suppression effects of VSL#3 through elevated fecal IgA in shrimp tropomyosin protein-sensitized mice. Probiotics have been reported to increase total and antigen-/allergen-specific sIgAs (31). Large amounts of noninflammatory IgA can be generated in the gut with or without T cell involvement. T cell-dependent intestinal IgA production is initiated by Ig class switching of IgM⁺ B cells to IgA⁺ B cells in Payer's patches that requires IgA class-switching factors, such as B cell-activating factor of the TNF family and a proliferation-inducing ligand released by dendritic cells and other cell types

(32–34). On the other hand, T cell-independent sIgAs are generated in lamina propria without T cell involvement. It was estimated that almost one-half of the intestinal sIgAs were T cell-independent (35). Taken together, these findings strongly suggest that CMA reduction in our PROC mice was achieved at least partly through high- and low-affinity IgA produced by activated B cells.

PROC mice had lower concentrations of BLG-IgE and higher concentrations of BLG-IgG1 compared with CTL+ mice (Table 1). However, similar concentrations of Th1-favored IgG2a and IL-12 and Th2 cytokines IL-5 and IL-9 were observed between the 2 groups (Table 3). Furthermore, high concentrations of BLG-IgG1 were observed in PROC mice (Table 1). These contradictory results did not strongly support the Th-shifting from Th2 to Th1. Although lower allergen-specific IgE and IgG1 are commonly accepted indicators of allergy relief, high IgG1 is not always associated with strong allergy reactions. Allergen-specific IgG1 can be produced by IL-4-dependent and IL-4-independent (via IL-12 or IFN γ stimulation) manners (36,37). IgG1 synthesized by the later manner causes no allergic reactions (37). Considering the fact that the IL-4 concentration in PROC was not higher than that in CTL+ (Table 2), the increased concentrations of IgG1 in the PROC group may have been generated through an IL-4-independent manner.

To our knowledge, there are no published studies investigating the combined effects of oral tolerance and probiotics on CMA or food allergy. In OTP mice, probiotic supplementation did not further reduce hypersensitive scores but increased intestinal sIgAs compared with the OT group (Table 1). Similar to the OT group, the OTP group was capable of suppressing both the allergen-specific Th1 response and Th2 response. The higher intestinal sIgA may contribute an immunoregulatory role against other antigens/allergens in a nonspecific fashion.

Under the current experimental conditions, oral supplementation of low doses of allergen would induce allergen-specific oral tolerance and suppress the development of allergic reaction upon subsequent exposure to that same allergen. VSL#3 supplementation could reduce allergic reaction to BLG mainly through generation of low- and high-affinity sIgAs. Our findings also suggest that concurrent application of low doses of oral allergen and VSL#3 supplementation might offer both allergen-specific protection, through active suppression, and allergen-nonspecific protection, through production of low-affinity intestinal sIgA.

Acknowledgments

C.L.T. designed and conducted the study, acquired the data, and drafted the manuscript; J.I.B. was involved in planning the study and revising the manuscript; and X.Z. designed the study, interpreted the data, and revised the manuscript critically. All authors read and approved the final manuscript.

Literature Cited

- Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, Plaut M, Cooper SF, Fenton MJ, Arshad SH, et al. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. *J Allergy Clin Immunol*. 2010;126:S1–S8.
- Wal JM. Cow's milk proteins/allergens. *Ann Allergy Asthma Immunol*. 2002;89:3–10.
- Fiocchi A, Brozek J, Schunemann H, Bahna SL, von Berg A, Beyer K, Bozzola M, Bradsher J, Compalati E, Ebisawa M, et al. World Allergy Organization (WAO) diagnosis and rationale for action against Cow's Milk Allergy (DRACMA) Guidelines. *Pediatr Allergy Immunol*. 2010;21 Suppl 21:1–125.
- Solinas C, Corpino M, Maccioni R, Pelosi U. Cow's milk protein allergy. *J Matern Fetal Neonatal Med*. 2010;23 Suppl 3:76–9.
- Burks AW, Laubach S, Jones SM. Oral tolerance, food allergy, and immunotherapy: implications for future treatment. *J Allergy Clin Immunol*. 2008;121:1344–50.
- Adel-Patient K, Wavrin S, Bernard H, Meziti N, Ah-Leung S, Wal JM. Oral tolerance and Treg cells are induced in BALB/c mice after gavage with bovine beta-lactoglobulin. *Allergy*. 2011;66:1312–21.
- Yamashita H, Takahashi K, Tanaka H, Nagai H, Inagaki N. Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. *Allergy*. 2012;67:201–9.
- Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol*. 1997;159:1739–45.
- Rodriguez B, Prioult K, Bibiloni R, Nicolis I, Mercenier A, Butel MJ, Waligora-Dupriet AJ. Germ-free status and altered caecal subdominant microbiota are associated with a high susceptibility to cow's milk allergy in mice. *FEMS Microbiol Ecol*. 2011;76:133–44.
- Schmidt B, Mulder IE, Musk CC, Aminov RI, Lewis M, Stokes CR, Bailey M, Prosser JJ, Gill BP, Pluske JR, et al. Establishment of normal gut microbiota is compromised under excessive hygiene conditions. *PLoS ONE*. 2011;6:e28284.
- Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science*. 2000;288:2222–6.
- Theil EC, Chen H, Miranda C, Janser H, Elsenhans B, Nunez MT, Pizarro F, Schumann K. Absorption of iron from ferritin is independent of heme iron and ferrous salts in women and rat intestinal segments. *J Nutr*. 2012;142:478–83.
- Thang CL, Baurhoo B, Boye JJ, Simpson BK, Zhao X. Effects of *Lactobacillus rhamnosus* GG supplementation on cow's milk allergy in a mouse model. *Allergy, Asthma Clin Immunol*. 2011;7:20.
- Shin JH, Kang JM, Kim SW, Cho JH, Park YJ. Effect of oral tolerance in a mouse model of allergic rhinitis. *Otolaryngol Head Neck Surg*. 2010;142:370–5.
- Pagnini C, Saeed R, Bamias G, Arseneau KO, Pizarro TT, Cominelli F. Probiotics promote gut health through stimulation of epithelial innate immunity. *Proc Natl Acad Sci USA*. 2010;107:454–9.
- Velayudham A, Dolganiuc A, Ellis M, Petrusek J, Kodys K, Mandrekar P, Szabo G. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology*. 2009;49:989–97.
- Wang X, O'Gorman MR, Bu HF, Koti V, Zuo XL, Tan XD. Probiotic preparation VSL#3 alters the distribution and phenotypes of dendritic cells within the intestinal mucosa in C57BL/10J mice. *J Nutr*. 2009;139:1595–602.
- Schouten B, van Esch BCAM, Hofman GA, van Doorn SACM, Knol J, Nauta AJ, Garssen J, Willemsen LEM, Knippels LMJ. Cow milk allergy symptoms are reduced in mice fed dietary synbiotics during oral sensitization with whey. *J Nutr*. 2009;139:1398–403.
- Adel-Patient K, Ah-Leung S, Creminon C, Nouaille S, Chatel JM, Langella P, Wal JM. Oral administration of recombinant *Lactococcus lactis* expressing bovine β -lactoglobulin partially prevents mice from sensitization. *Clin Exp Allergy*. 2005;35:539–46.
- Alkan SS, Akdis AC, Feuerlein D, Gruninger M. Direct measurement of cytokines (IFN- γ , IL-4, -5, and -6) from organs after antigenic challenge. *Ann N Y Acad Sci*. 1996;796:82–90.
- Dubois B, Chapat L, Goubier A, Papiernik M, Nicolas JF, Kaiserlian D. Innate CD4+CD25+ regulatory T cells are required for oral tolerance and inhibition of CD8+ T cells mediating skin inflammation. *Blood*. 2003;102:3295–301.
- Friedman A, Weiner HL. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci USA*. 1994;91:6688–92.
- Faria AM, Weiner HL. Oral tolerance. *Immunol Rev*. 2005;206:232–59.
- Strid J, Thomson M, Hourihane J, Kimber I, Strobel S. A novel model of sensitization and oral tolerance to peanut protein. *Immunology*. 2004;113:293–303.
- Dubois B, Joubert G, Gomez de Agüero M, Gouanvic M, Goubier A, Kaiserlian D. Sequential role of plasmacytoid dendritic cells and regulatory T cells in oral tolerance. *Gastroenterology*. 2009;137:1019–28.
- Rupa P, Mine Y. Oral immunotherapy with immunodominant T-cell epitope peptides alleviates allergic reactions in a Balb/c mouse model of egg allergy. *Allergy*. 2012;67:74–82.
- Conti P, Boucher W, Letourneau R, Feliciani C, Reale M, Barbacane RC, Vlagopoulos P, Bruneau G, Thibault J, Theoharides TC. Monocyte chemoattractant protein-1 provokes mast cell aggregation and [3H]5HT release. *Immunology*. 1995;86:434–40.
- Glez PP, Franco YB, Matheu V. MIP-1 α , MCP-1, and desensitization in anaphylaxis from cow's milk. *N Engl J Med*. 2012;367:282–4.
- Kukkonen K, Kuitunen M, Haahtela T, Korpela R, Poussa T, Savilahti E. High intestinal IgA associates with reduced risk of IgE-associated allergic diseases. *Pediatr Allergy Immunol*. 2010;21:67–73.
- Schiavi E, Barletta B, Buteroni C, Corinti S, Boirivant M, Di Felice G. Oral therapeutic administration of a probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy. *Allergy*. 2011;66:499–508.
- Galdeano CM, Perdigon G. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clin Vaccine Immunol*. 2006;13:219–26.
- Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, Cerutti A. DCs induce CD40-independent immunoglobulin class switching through BLYS and APRIL. *Nat Immunol*. 2002;3:822–9.
- He B, Xu W, Santini PA, Polydorides AD, Chiu A, Estrella J, Shan M, Chadburn A, Villanacci V, Plebani A, et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity*. 2007;26:812–26.
- Xu W, He B, Chiu A, Chadburn A, Shan M, Buldys M, Ding A, Knowles DM, Santini PA, Cerutti A. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. *Nat Immunol*. 2007;8:294–303.
- Kroese FG, Butcher EC, Stall AM, Lalor PA, Adams S, Herzenberg LA. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. *Int Immunol*. 1989;1:75–84.
- Faquim-Mauro EL, Coffman RL, Abrahamson IA, Macedo MS. Cutting edge: mouse IgG1 antibodies comprise two functionally distinct types that are differentially regulated by IL-4 and IL-12. *J Immunol*. 1999;163:3572–6.
- Dearman RJ, Kimber I. Animal models of protein allergenicity: potential benefits, pitfalls and challenges. *Clin Exp Allergy*. 2009;39:458–68.