



Effects of enzymatic hydrolysis on the allergenicity of natural cow milk based on a BALB/c mouse model

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ABSTRACT

Cow milk allergy is one of the most prevalent food allergies worldwide, particularly in infants and children. To the best of our knowledge, minimal research exists concerning the antigenicity of cow milk (CM). This study was performed to evaluate the allergenicity of enzymatically hydrolyzed cow milk (HM) in a BALB/c mouse model. The mice were randomly divided into 5 groups ($n = 12/\text{group}$), which were sensitized with phosphate-buffered saline, CM, and HM (Alcalase-, or Protamex-, or Flavorzyme-treated cow milk; Novo Nordisk; AT, PT, FT, respectively), respectively, using cholera toxin as adjuvant on d 0, 7, 14, 21. On d 28, the test mice were orally challenged with phosphate-buffered saline, CM, and HM (AT, PT, or FT) alone. Anaphylactic symptoms were monitored in the mice. Antibody, cytokine, histamine, and mouse mast cell protease-1 (mMCP-1) levels were measured using enzyme-linked immunosorbent assays. In addition, the numbers of T helper (Th)1 and Th2 cells, as well as the proportions of $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+\text{Tregs}$ cells, in mouse spleens were detected using flow cytometry. Statistical significance was determined by one-way ANOVA. The results revealed significant differences between CM- and HM-challenged mice. Among these, the clinical scores of HM-challenged mice (AT, 1.50; PT, 2.00; FT, 1.92) were lower than those of CM-challenged mice (positive control, 2.83), but body weight and temperature of HM-challenged mice were higher than those of CM-challenged mice. In addition, significant reductions of allergen-specific IgE, IgG, histamine, and mMCP-1 were showed in HM-challenged mice, especially for histamine, ranging from 171.42 ng/mL to 214.94 ng/mL. Remarkable reductions of IL-4, IL-5, and IL-13 levels, as well as elevations of interferon- γ and IL-10 levels in the spleens of HM-challenged mice were also detected.

Moreover, the number of Th2 cells decreased in the HM-challenged mice, to 2.36% (AT), 1.79% (PT), and 4.03% (FT), respectively, whereas the numbers of Th1 cells (AT, 6.30%; PT, 6.70%; FT, 6.56%) and the proportions of $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+\text{Tregs}$ (AT, 8.86%; PT, 9.21%; FT, 9.16%) increased significantly. Our findings indicate that exposure to HM was sufficient to induce a shift toward a Th1 response, thereby reducing potential allergenicity. Importantly, these results will lay a theoretical foundation for the development of hypoallergenic CM products.

Key words: allergenicity, cow milk, enzymatic hydrolysis, mouse model

INTRODUCTION

Food allergy is an immediate hypersensitivity response to otherwise harmless food or food components, which affects approximately 1 to 3% of adults and 2 to 6% of infants (Sampson, 2005). The prevalence and severity of food allergy is increasing worldwide (Sampson, 2016). The major sources are cow milk (CM), eggs, soy, peanuts, tree nuts, fish, shellfish, and wheat (Fernández-Rivas et al., 2006). Cow milk allergy (CMA), which affects approximately 2 to 3% of infants and children (Sicherer, 2011), can induce severe or fatal allergic reactions, such as pruritus, atopic dermatitis, allergic rhinitis, and more (Sampson, 2002). Cow milk allergy is associated with protein constituents, the most prominent allergens being caseins and whey proteins (β -lactoglobulin and α -lactalbumin; Wal, 1998), as well as some low-content proteins (e.g., bovine serum, lactoferrin). There is no cure for CMA, and the avoidance of CM in any form is an effective method for managing CMA, but this can affect growth and development of infants and children (Christie et al., 2002). Several processing methods have been used to reduce or eliminate the allergenicity of CM, including heat treatment, γ -irradiation, and enzymatic hydrolysis.

Heat treatment is a common method during milk processing. Elagamy (2000) has reported that IgG activities in cow, buffalo, and camel milk were significantly

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affected after heat treatment at 65°C for 30 min, with activity entirely lost in cow and buffalo milk after heat treatment at 75°C for 30 min, and 68.7% lost in camel milk. Shi et al. (2014) demonstrated that the IgE-binding ability of CM proteins with *Lactobacillus casei* were significantly reduced by 15 to 90%, compared with raw milk, whereas heat treatment would affect the quality and nutritional properties of CM. Additionally, several studies have suggested that the allergenicity of food proteins could be reduced by γ -irradiation (Meng et al., 2016), but this is not widely accepted by consumers who are unfamiliar with the technology (Parlato et al., 2014). Calvo and Gómez (2002) found that enzymatic hydrolysis and heat treatment of whey proteins could reduce the content of proteins, and significantly lowered the antigenicity of CM proteins. Similarly, Wróblewska and Troszyńska (2005) demonstrated that enzymatic hydrolysis with Alcalase could reduce the antigenicity of whey protein concentrate.

Due to the complexity of the food allergy mechanism, animal models could be used as a direct method to evaluate the potential immunoreactivity of food. The BALB/c mouse is widely used (Fritsché, 2003), as these inbred strains have the advantage of favoring the development of T helper 2-type immune responses and production of IgE antibodies (Niggemann et al., 2001). Moreover, BALB/c mice can be administered CM proteins to simulate different parameters of human allergic responses (Hsieh et al., 2003). Gizzarelli and colleagues established a soybean protein-sensitized BALB/c mouse model by using cholera toxin (CT) as an adjuvant (Gizzarelli et al., 2006), and van Esch et al. (2011) developed a BALB/c mouse model to evaluate the allergenicity of hydrolyzed whey proteins. To the best of our knowledge, previous studies have focused only on the allergenicity of specific proteins in CM; few studies have investigated the antigenicity of CM. In this study, the BALB/c mouse model was used to evaluate the effects of enzymatic hydrolysis on the potential allergenicity of CM. Specific antibodies, cytokines, histamine, mouse mast cell protease-1 (mMCP-1), the numbers of T helper (Th)1 and Th2 cells, and the proportions of CD4⁺CD25⁺Foxp3⁺Tregs cells were measured. The results will provide a theoretical foundation for the development of high-quality hypoallergenic dairy products.

MATERIALS AND METHODS

Materials

Fresh CM was obtained from a local farm in Shenyang, China. Alcalase (EC 3.4.21.62), Protamex

(EC 3.4.24.28), and Flavorzyme (EC 3.4.11.1) were purchased from Novozymes (Novo Nordisk). Cholera toxin was purchased from Sigma-Aldrich Chemical Co. Mouse IgE and IgG ELISA kits, as well as mouse IFN- γ , IL-4, IL-5, IL-13, IL-10, and mMCP-1, were obtained from eBioscience. Mouse histamine ELISA kits were acquired from Demeditec Company. Peridinin chlorophyll protein complex (PerCP) anti-mouse CD3e, fluorescein isothiocyanate (FITC) anti-mouse CD4, phycoerythrin (PE) anti-mouse CD25, allophycocyanin (APC) anti-mouse Foxp3, PE anti-mouse IL-4, and PerCP anti-mouse IFN- γ antibodies were purchased from BD Pharmingen. All chemicals used were of analytical grade.

Preparation of Samples

Fresh CM samples, including mature milk (1–6 mo postpartum), were collected from 120 Holstein cows (2–4 yr of age and fed only on grass) on a local farm in Shenyang, China. After collection, CM samples were placed in ice-boxes and transported to the laboratory, frozen at –80°C, and kept at this temperature until further analysis. The somatic cell count of CM samples was $<2.5 \times 10^5$ /mL, the total number of colonies was $<2.0 \times 10^6$ cfu/mL, and the contents of protein, fat, ash, moisture, lactose, and dry matter were 3.05 g/100 mL, 3.54 g/100 mL, 1.12 g/100 mL, 87.80 g/100 mL, 4.98 g/100 mL, and 12.35 g/100 mL, respectively. Skimmed CM was prepared by removing fat with high-speed centrifugation at $15,000 \times g$ for 30 min at 4°C. Solutions of Alcalase, Protamex, and Flavorzyme were prepared with distilled water. Enzyme solutions and CM were preheated for 20 min at their respective optimal temperatures (Alcalase: $55 \pm 5^\circ\text{C}$; Flavorzyme: $50 \pm 5^\circ\text{C}$; Protamex: $50 \pm 5^\circ\text{C}$). Subsequently, the enzyme solutions were added to CM (150 mL), in accordance with the enzyme activity-to-substrate ratio (Alcalase, 10,000 U/g; Protamex, 10,000 U/g; Flavorzyme, 6,000 U/g). These mixtures were incubated for 60 min (Alcalase), 120 min (Protamex), and 60 min (Flavorzyme), respectively, based on the results of our earlier work (Liang et al., 2021). Each enzyme solution was inactivated by heating at 90°C for 10 min and then immediately cooled in an ice bath. The resulting CM hydrolysates were centrifuged at $5,000 \times g$ for 10 min, freeze-dried, and stored at –80°C for use in animal experiments.

Animals

Six-week-old female BALB/c mice (specific-pathogen-free grade; weight, 20 ± 2 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd.

Table 1. Anaphylactic symptom scoring

Score	Symptom
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erection; reduced activity; high breathing rate
3	Shortness of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation; shivering and muscle contractions
5	Death by shock

All mice were housed at the Animal Center of Shenyang Agricultural University (Shenyang, China) and handled in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85-23, 1996). All experimental animal procedures were approved by the Ethical Committee for the Experimental Use of Animals at Shenyang Agricultural University (license no. SYXK < Liao > 2011-0001). The mice were maintained on a CM-free standard diet and continuously propagated for 3 generations. The female mice in the third generation were used as experimental animals. The mice were provided tap water and food ad libitum; they were housed in an animal research facility at a temperature of 19 to 25°C and relative humidity of 55 to 65%, with light exposure for 10 to 12 h/d.

Mouse Sensitization and Oral Challenge

Mouse sensitization was performed in accordance with a modified version of the method described previously (Yang et al., 2015). As illustrated in Figure 1, following a 1-wk acclimatization period, the mice were randomly divided into 5 groups (n = 12 per group): negative control (NC), positive control (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), and Flavorzyme-treated cow milk (FT). All

mice were sensitized by oral challenge on d 0, 7, 14, and 21. Specifically, mice in the NC group were sensitized with 10 µg of CT in 300 µL of PBS, and mice in the PC group were sensitized with 5 mg of CM and 10 µg of CT in 300 µL of PBS. The other groups were sensitized with 5 mg of the corresponding samples (AT, PT, or FT) and 10 µg of CT in 300 µL of PBS. On d 28, the NC group was orally challenged with PBS alone, and the PC group was orally challenged with 20 mg of CM in PBS; the other groups were orally challenged with 20 mg of the corresponding samples (AT, PT, or FT) in PBS. At 30 min after the final challenge, anaphylactic symptoms were assessed using a validated anaphylactic scoring index (Table 1; Rupa and Mine, 2012); the body temperature and weight of the mice were also measured. Thereafter, the mice were humanely killed by dislocation, and blood samples were collected for biomarker assays, as well as analysis of histamine, mMCP-1, and specific Ig levels. Finally, mouse spleens were collected for flow cytometry analysis.

Measurement of Specific Antibody and mMCP-1 Levels

The levels of specific IgE and IgG antibodies in serum were determined by ELISA, as previously described (Ren et al., 2014). A 96-well microplate was

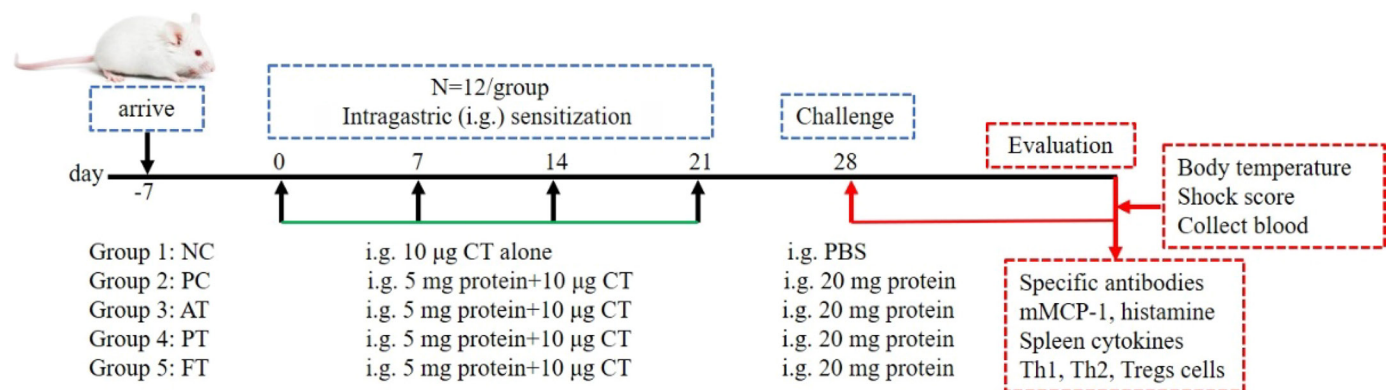


Figure 1. Oral immunization experimental scheme of BALB/c mice with negative group (NC), positive group (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), Flavorzyme-treated cow milk (FT; all from Novo Nordisk). CT = cholera toxin; Th1, Th2 = T helper 1 and 2 cells; mMCP-1 = mouse mast cell protease-1.

coated with 5 µg/mL antigenic proteins (100 µL/well) in 0.05 M sodium carbonate buffer (pH 9.6), and the microplate was incubated overnight at 4°C. The next day, the microplate was washed thrice with PBS-Tween (PBST). Then, gelatin (250 µL/well) was added for blocking and incubated for 1 h at 37°C for. Again, the microplate was washed thrice with PBST. Each diluted serum was added and incubated at 37°C for 1 h. Then, horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and IgE antibodies (1:2,000, 100 µL/well) were added and incubated at 37°C for 1 h. The microplate was washed thrice with PBST, and *o*-phenylenediamine (OPD, 100 µL/well) was added and incubated in the dark at 37°C for 15 min. The reaction was terminated with 2 M H₂SO₄. The optical density was determined at 490 nm with a microplate reader (BioRad). The mMCP-1 levels in serum were quantified in accordance with the manufacturer's instructions using an ELISA kit (eBioscience).

Measurement of Histamine Level in Plasma

Mouse blood samples (n = 12 per group) were collected in anticoagulant tubes containing heparin sodium. The blood sample supernatants were centrifuged at $4,000 \times g$ for 10 min at 4°C and stored at -80°C until further analysis. The levels of plasma histamine were determined by using a commercial ELISA kit in accordance with the manufacturer's protocol (Demeditec).

Detection of Cytokines in Splenocyte Cell Cultures

Spleen samples (n = 12 per group) were aseptically collected from individual mice and gently ground with a syringe plunger in ice-cold RPMI-1640 medium (Gibco). The resulting tissue fragments were passed through a 100-µm nylon membrane cell strainer, and single-cell suspensions were prepared. The suspensions were transferred to 10-mL tubes, and supernatants were discarded by centrifugation at $1,000 \times g$ for 5 min at 4°C. Erythrocyte lysate was then added and incubated at room temperature for 5 min; the resulting cell suspension was mixed with RPMI-1640 medium containing 10% fetal bovine serum (HyClone). Cell viability was evaluated by means of trypan blue staining. Cell density was adjusted to 4×10^6 cells/mL; the isolated splenocytes were cultured in 24-well plates in triplicate wells. The cells were stimulated with samples (CM, AT, PT, or FT) and cultured for 72 h at 37°C in an atmosphere of 5% CO₂. The supernatants were collected and used for measurement of cytokine levels (i.e., IFN-γ, IL-4, IL-5, IL-10, and IL-13) with ELISA kits (eBioscience).

Assessment of Th1 and Th2 Cell Numbers Among Mouse Splenocytes

The numbers of Th1 and Th2 cells were determined by assessing the presence of intracellular IFN-γ and IL-4. We stained 100-µL single-cell splenocyte suspensions (4×10^6 cells/mL) with FITC-labeled anti-mouse CD4 (BD Pharmingen), and the mixtures were incubated at 4°C in darkness for 30 min. Fixative and rupture agents were then added to the cell mixtures; the supernatants were discarded by centrifugation ($1,000 \times g$ for 5 min at 4°C). Subsequently, PE anti-mouse IL-4 and PerCP anti-mouse IFN-γ antibodies were added, and the mixtures were incubated at 4°C in darkness for 30 min. Single cells were resuspended in flow buffer (300 µL); Th1 and Th2 cells were then identified using a BD FACS-Calibur Flow Cytometer (Becton Dickinson). Data were analyzed by using Accuri Express software (V10; BD Biosciences).

Detection of Tregs Among Mouse Splenocytes

Single-cell suspensions of splenocytes were mixed with PerCP anti-mouse CD3e, FITC anti-mouse CD4, and FE anti-mouse CD25 antibodies; the mixtures were incubated at 4°C in darkness for 30 min. Fixation and permeabilization reagents were added, and the mixtures were incubated at 4°C in darkness for an additional 30 min. Subsequently, wash and permeabilization buffer was added, and supernatant was discarded. Then, APC anti-mouse Foxp3⁺ antibody was added, and the mixture was incubated at 4°C in darkness for 30 min. Single cells were resuspended in flow buffer (300 µL); Treg cells were identified using a BD FACS-Calibur Flow Cytometer. Data were analyzed by using Accuri Express software (BD Biosciences).

Statistical Analysis

All tests were performed in triplicate. Statistical analysis of the experimental data was performed using Graphpad Prism 7.0 (GraphPad Software). Results are expressed as mean values ± standard errors of the means. Statistical significances between groups were determined by one-way ANOVA (SPSS version 19.0 for Windows; IBM Corp.) followed by Tukey's test with α-level of 0.05.

RESULTS

Assessments of Allergy Symptoms, Body Temperatures, and Body Weights

The allergenicity of enzymatically hydrolyzed cow milk (HM) in vivo was evaluated in a BALB/c mouse

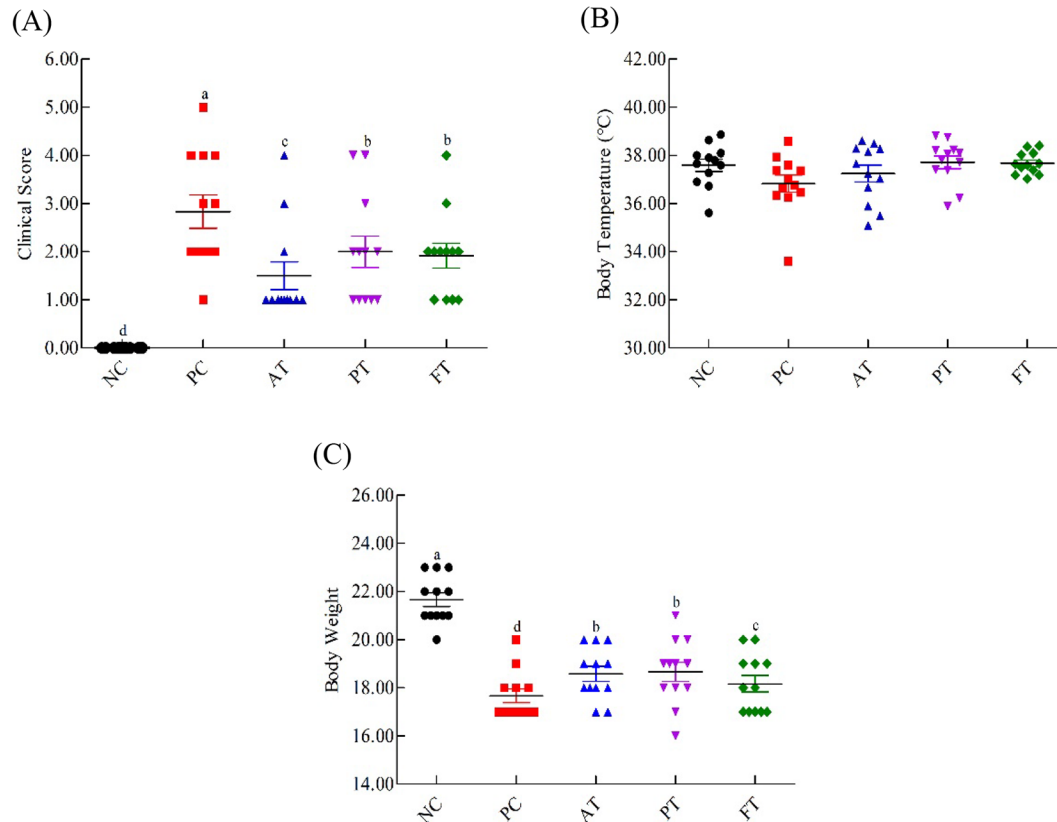


Figure 2. Clinical scores of hypersensitivity symptoms (A), body temperature (B), and body weight (C). Groups included negative group (NC), positive group (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), Flavorzyme-treated cow milk (FT; all from Novo Nordisk). Different letters (a–d) indicate significant differences ($P < 0.05$); error bars represent SEM.

model (Figure 1). The allergy scores of the mice after oral challenge are shown in Figure 2A. The mice in these groups exhibited different sensitization statuses. No signs of anaphylaxis were observed in the NC group. However, mild anaphylactic reactions were identified in the AT, PT, and FT groups; these significantly differed from the reactions in the NC group ($P < 0.01$). In addition, all mice in the PC group developed severe anaphylactic reactions (mean value, 2.73); 1 mouse in the PC group died of shock (Figure 2A). Changes in mouse body temperature and weight are shown in Figure 2B and C. The average body temperature of the mice in the NC group was 37.59°C, and body temperatures were lower among mice in the PC group than among mice in the NC group; however, body temperatures did not significantly differ between the PC and NC groups ($P > 0.05$). Body temperatures tended to be higher in the AT, PT, and FT groups than in the PC group (Figure 2B); however, these differences were not statistically significant ($P > 0.05$). Mouse body weights were measured after the final challenge (Figure 2C). Mice in the NC group had the highest weight (21.43 g), and mice in the PC group had the lowest weight (17.86 g).

Mice in the AT, PT, and FT groups had higher weights than mice in the PC group but lower weights than mice in the NC group (AT, PT, and FT groups: $P < 0.001$, compared with the NC group).

Assessments of Specific IgE and IgG Levels

The serum-specific IgE and IgG levels were compared among the groups of mice in this study. As shown in Figure 3, significant elevations of IgE and IgG antibodies were detected in the PC group, compared with the NC group ($P < 0.001$). Furthermore, the levels of IgE and IgG antibodies in the AT, PT, and FT groups were significantly lower than the levels in the PC group (all $P < 0.001$), whereas they were significantly higher than the levels in the NC group (all $P < 0.001$).

Levels of the Plasma Histamine and Serum mMCP-1

Figure 4 shows the comparison of histamine and mMCP-1 levels among mice in this study. Notably, enzymatic hydrolysis treatment significantly reduced mMCP-1 and histamine levels (all $P < 0.001$). Hista-

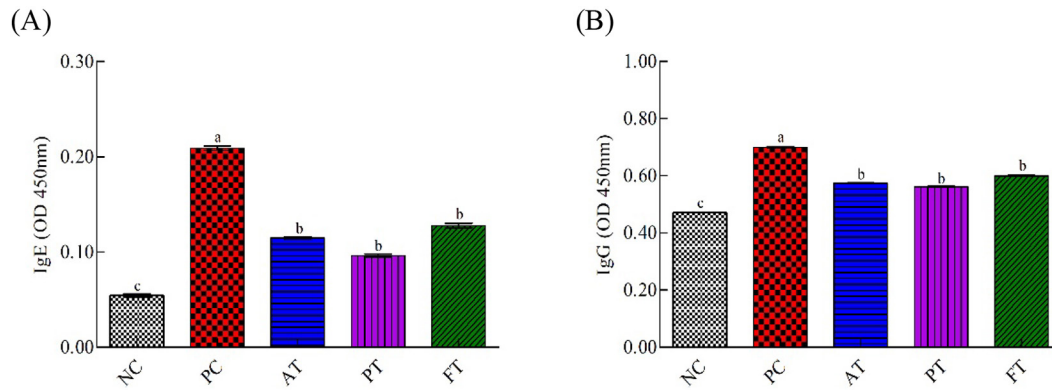


Figure 3. Specific IgE (A) and IgG (B) levels in serum of the mice; optical density (OD) at 450 nm. Negative group (NC), positive group (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), Flavorzyme-treated cow milk (FT; all from Novo Nordisk). Different letters (a–c) indicate significant differences ($P < 0.05$); error bars represent SEM.

mine content was lowest in the NC group (96.22 ng/mL), whereas it was highest in the PC group ($P < 0.001$). The AT, PT, and FT groups showed lower levels of histamine compared with the PC group (all $P < 0.001$). In addition, the mMCP-1 levels were significantly greater in the PC group than in the NC group (15.13 ng/mL vs. 4.67 ng/mL; $P < 0.001$). The mMCP-1 levels in the AT (9.58 ng/mL), PT (8.44 ng/mL), and FT groups (8.79 ng/mL) were significantly lower than the level in the PC group (all $P < 0.001$); they were also significantly higher than the level in the NC group (all $P < 0.001$).

Cytokine Levels in Splenocyte Supernatants

The levels of Th1-associated IFN- γ ; Th2-associated IL-4, IL-5, and IL-13; and Treg-associated IL-10 were detected in splenocyte supernatants (Figure 5). We observed that IL-4, IL-5, and IL-13 were significantly

upregulated in the PC group, compared with the NC group (all $P < 0.001$). The levels of these cytokines were significantly lower in the AT, PT, and FT groups, compared with the levels in the PC group (all $P < 0.01$); they were also significantly higher than the levels in the NC group (all $P < 0.01$). Furthermore, the levels of IFN- γ and IL-10 were significantly lower in the PC group than in the NC group (both $P < 0.001$). The levels of IFN- γ and IL-10 significantly increased in the AT, PT, and FT groups, compared with the levels in the PC group (all $P < 0.01$).

Assessment of Differentiation and Regulation of Th1/Th2 Balance

The numbers of Th1 and Th2 cells were compared among mouse splenocytes in this study (Figure 6). The number of Th1 cells was significantly lower in the PC group than in the NC group (4.18% vs. 8.08%; $P <$

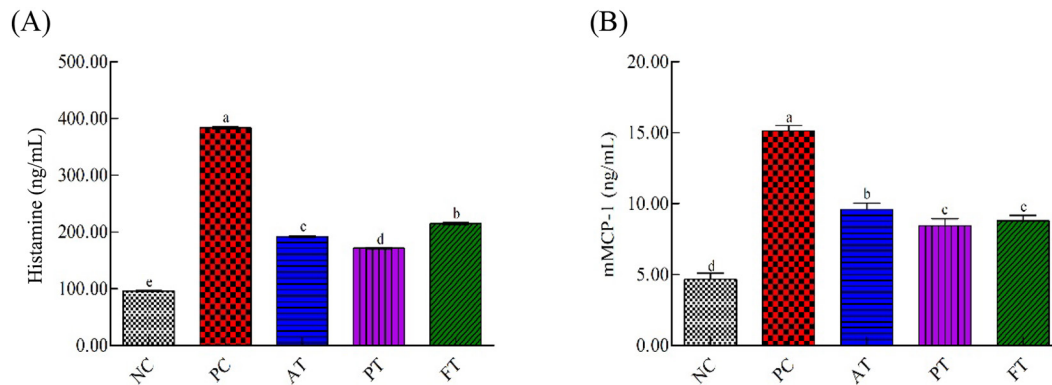


Figure 4. Plasma histamine levels (A) and mouse mast cell protease-1 (mMCP-1) levels in serum (B) of experimental mice. Groups included negative group (NC), positive group (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), Flavorzyme-treated cow milk (FT; all from Novo Nordisk). Different letters (a–e) indicate significant differences ($P < 0.05$); error bars represent SEM.

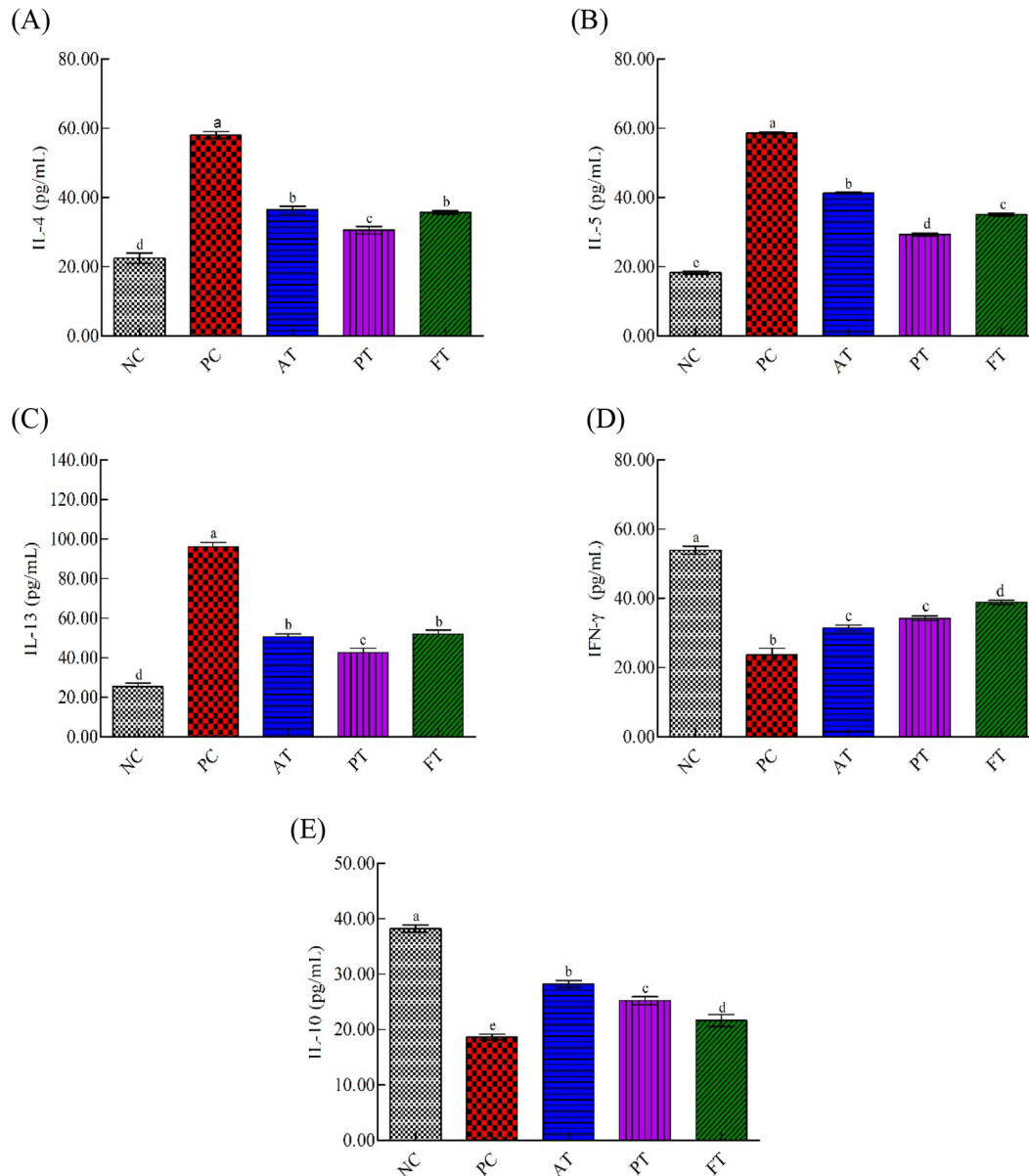


Figure 5. Levels of cytokines secreted by mouse spleen cells: IL-4 (A), IL-5 (B), IL-13 (C), IFN- γ (D), and IL-10 (E). Groups included negative group (NC), positive group (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), Flavorzyme-treated cow milk (FT; all from Novo Nordisk). Different letters (a-e) indicate significant differences ($P < 0.05$); error bars represent SEM.

0.001). Notably, the numbers of Th1 cells in the AT, PT, and FT groups significantly increased, compared with the level in the PC group (all $P < 0.001$). The numbers of Th2 cells followed a pattern that contrasted with the changes in Th1 cells. Specifically, the numbers of Th1 cells significantly increased in the AT, PT, and FT groups (all $P < 0.001$), whereas the numbers of Th2 cells decreased in these groups (AT and PT compared with PC group, $P < 0.001$; FT compared with PC group, $P > 0.05$). These results were consistent with the changes in cytokine levels.

Assessment of $CD4^+CD25^+Foxp3^+$ T Cells

The levels of $CD4^+CD25^+Foxp3^+$ T cells in mouse spleens were determined using flow cytometry. Figure 7 shows the proportions of $CD4^+CD25^+Foxp3^+$ T cells among mouse splenocytes in this study. The proportion of $CD4^+CD25^+Foxp3^+$ T cells was highest in the NC group and lowest in the PC group (10.66% vs. 6.22%; $P < 0.001$). Notably, the proportions of $CD4^+CD25^+Foxp3^+$ T cells gradually increased in the AT, PT, and FT groups; these proportions were significantly dif-

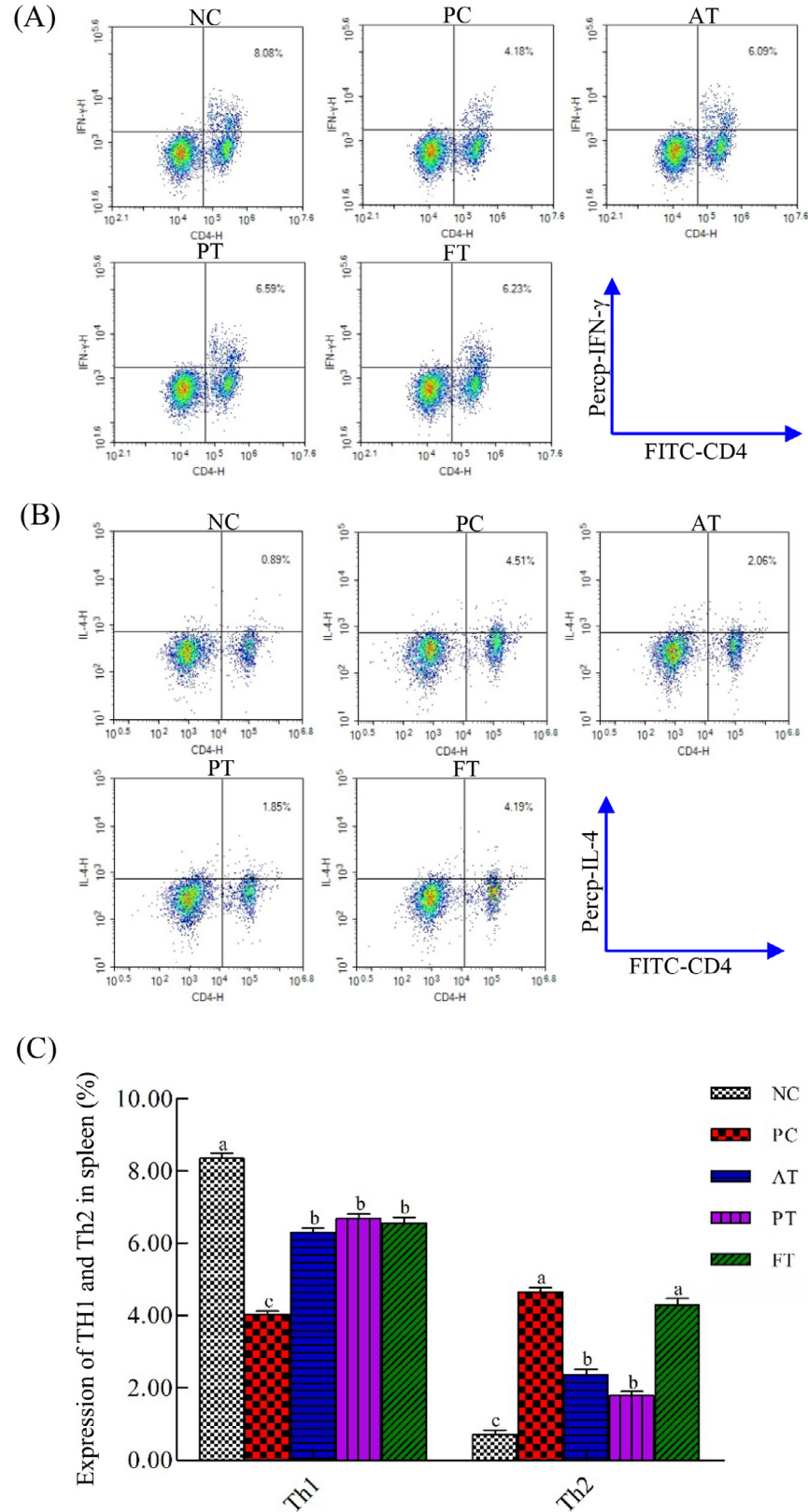


Figure 6. Differentiation balance of T helper (Th)1 and 2 cells in mouse spleens. Expression of Th1 (A), expression of Th2 (B), the percentage of Th1/Th2 expression (C). Groups included negative group (NC), positive group (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), Flavorzyme-treated cow milk (FT; all from Novo Nordisk). Different letters (a-c) indicate significant differences ($P < 0.05$).

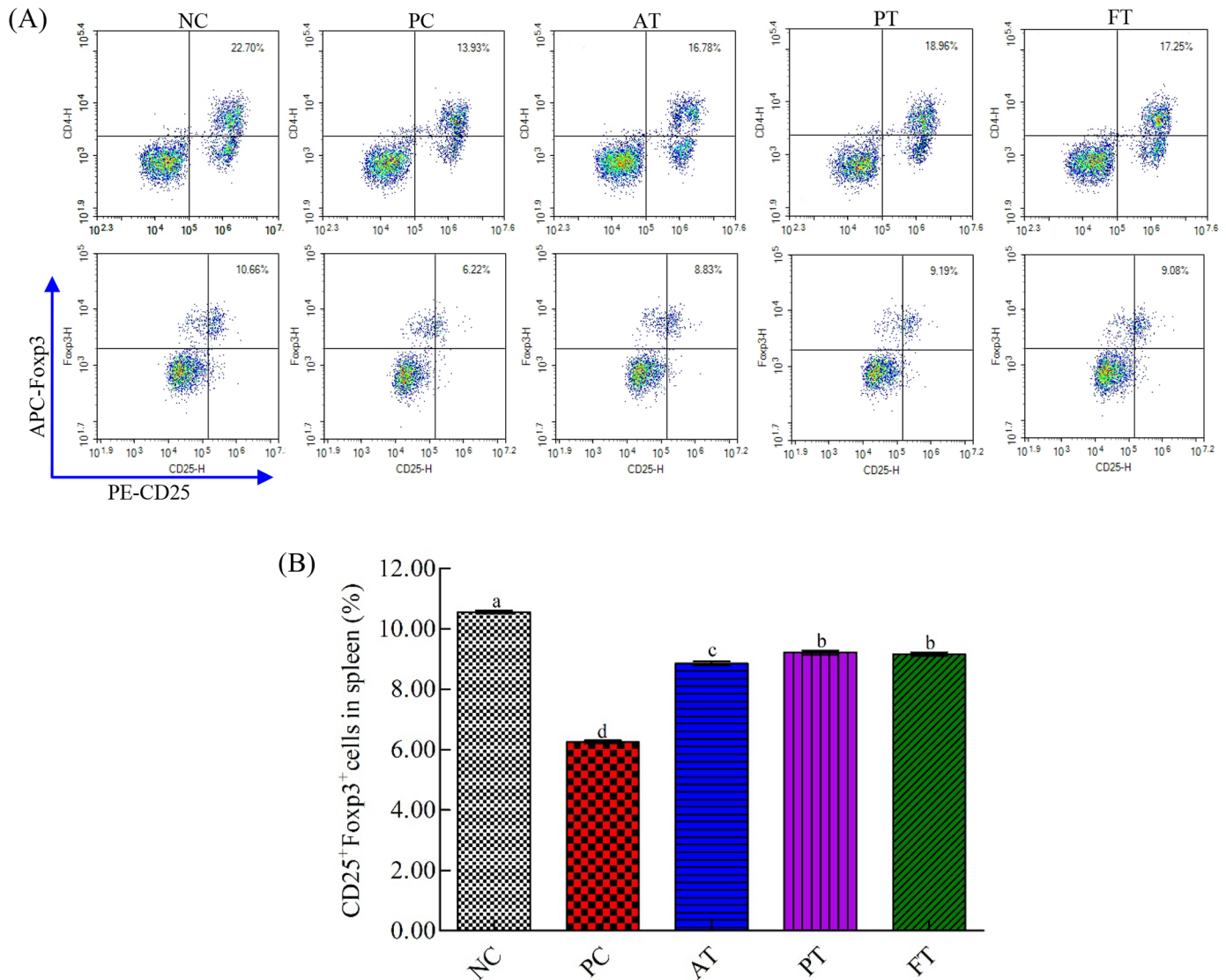


Figure 7. Identification of the Treg cells in the spleen of the mice. CD3⁺CD4⁺CD25⁺Foxp3⁺ expression of Tregs in mouse spleens (A); percentage of CD25⁺Foxp3⁺ in CD4⁺ cell population (B). Groups included negative group (NC), positive group (PC), Alcalase-treated cow milk (AT), Protamex-treated cow milk (PT), Flavorzyme-treated cow milk (FT; all from Novo Nordisk). Different letters (a–d) indicate significant differences ($P < 0.05$).

ferent from the proportion in the PC group (all $P < 0.001$).

DISCUSSION

Animal models can imitate food allergies mediated by specific IgE in humans; this approach is a common and direct method for evaluating the food allergens in vivo. This study was performed in female BALB/c mice, which are known to have augmented Th2-type immune reactions and robust generation of IgE antibodies (Rizzo et al., 1991); thus, these mice are particularly sensitive to Th2-driven allergic reactions. Additionally,

the immune system in BALB/c mice is similar to the human immune system in terms of overall structure. Accordingly, BALB/c mice have been widely used to evaluate the potential sensitization of food proteins such as eggs and tree nuts. Saldanha et al. (2004) developed a BALB/c mouse model to investigate the intestinal and systemic consequences of egg white solution ingestion. Kumar et al. (2018) used BALB/c mice to assess the allergenicity of a *Buchanania lanzan* protein extract. In the present study, the allergenicity of HM was systematically evaluated by assessment of body temperature, weight, and anaphylactic symptoms; levels of mMCP-1, histamine, and cytokines; numbers of

Th1 and Th2 cells; and proportions of Treg cells, using a BALB/c mouse model.

In this work, CM and HM induced different levels of allergic symptoms in BALB/c mice. In particular, sensitization with HM led to lower anaphylactic scores, relative to sensitization with CM. These results implied that HM had lower allergenicity, compared with CM. Notably, allergic reactions in mice may be caused by multiple mechanisms. Cow milk allergy is a type I allergic reaction, characterized by an elevated level of allergen-specific IgE antibody. Duan et al. (2014) showed that allergic reactions in mice are triggered by various mechanisms; among these, allergen-specific IgE antibody plays a key role in mediating the type I allergic reaction during CMA. Our findings revealed that the levels of IgE and IgG antibodies were significantly lower in mice sensitized with HM (i.e., mice in the AT, PT, and FT groups) than in mice in the PC group (Figure 3). Therefore, mice in the AT, PT, and FT groups were presumed to have less hypersensitivity than mice in the PC group. Similarly, Gomes-Santos et al. (2015) found an elevated level of allergen-specific IgE antibody in BALB/c mice with CMA. Furthermore, soybean protein has been shown to induce high levels of antigen-specific IgE and IgG₁ antibodies in a BALB/c mouse model with intragastric exposure (Gizzarelli et al., 2006).

Mast cells are important effector cells that secrete a variety of cytokines and participate in immune regulation. Activated mast cells undergo degranulation and release various inflammatory mediators (e.g., histamine, prostaglandin D₂, leukotriene, cysteine, and CysL) that contribute to the development of allergic reactions (Levy et al., 2012). Notably, mMCP-1 is mainly expressed in mast cells of the intestinal mucosa, where it exerts important effects on the intestinal epithelial barrier. Elevated mMCP-1 content is presumed to enhance intestinal permeability, promote Th2 inflammatory responses, and cause allergic reactions (Tong et al., 2018). Bailón et al. (2012) found a high level of histamine in CM protein-stimulated BALB/c mice. The levels of histamine and mMCP-1 significantly increased in the PC group, whereas the AT, PT, and FT groups showed a significant decreasing trend; these findings were consistent with the differences in anaphylactic shock symptoms among groups and implied that HM sensitization could effectively reduce allergic reactions in the mice.

In the context of food allergies, various cytokines constitute important bioactive mediators in conjunction with histamine and mMCP-1. The Th2-associated cytokines IL-4, IL-5, and IL-13 are important for promoting the production of allergen-specific IgE antibody by B cells (Mathias et al., 2011). In contrast, IFN- γ is a

Th1-type cytokine that can suppress the production of IgE antibody and inhibit allergic responses by causing Th2 cell inactivation (Adel-Patient et al., 2012). To elucidate the molecular mechanism involved in the HM sensitization effect observed in our study, we examined the levels of cytokines in supernatants of splenocytes that had been stimulated with CM, AT, PT, and FT in vitro. We found that the AT, PT, and FT groups exhibited significantly higher levels of IFN- γ and IL-10, as well as lower levels of IL-4, IL-5, and IL-13, compared with the PC group (Figure 5). A similar result was demonstrated by Matsubara et al. (2018), who suggested that whey protein hydrolysate significantly reduced the levels of IgE, IL-4, and IL-5, while increasing IFN- γ levels, in challenged mice. Therefore, our findings imply that HM could contribute to amelioration of allergic responses by modulating the Th1/Th2 balance.

The CD4⁺ T cells comprise the most complex immune cell population involved in cellular immune responses. Naïve CD4⁺ T cells can differentiate into different types of Th cells when stimulated by antigens, each of which performs distinct biological functions. T helper cell precursors first differentiate into intermediate Th0 cells, which selectively migrate and differentiate into Th1 or Th2 cells. IL-12 and IFN- γ induce Th0 cells to differentiate into Th1 cells; IL-4 induce Th0 cells to differentiate into Th2 cells; and IL-10 and transforming growth factor- β induce Th0 cells to differentiate into Tregs. Under normal conditions, Th1 and Th2 cells are in a relatively balanced state in the body. When foreign antigens are encountered, the dynamic equilibrium of Th1/Th2 is disrupted, which leads to changes in the numbers of Th1 and Th2 cells (Talaat et al., 2015; Vargas-Rojas et al., 2016). Recent studies have shown that Th1/Th2 imbalance is closely related to autoimmune diseases (Shin et al., 2015). Perez-Mazliah and Langhorne (2015) demonstrated that the Th1/Th2 balance is closely related to immunity and disease, in that Th2 cells stimulated humoral immunity through IL-4 secretion to promote B cell proliferation, antibody production, and allergic reaction onset. In this study, we examined the numbers of Th1 and Th2 cells. Our results suggest that HM sensitization significantly enhanced the number of Th1 cells and reduced the number of Th2 cells, which mitigated Th1/Th2 imbalance (Figure 6). Presumably, enzymatic hydrolysis served to attenuate CMA symptoms through inhibition of Th2 and enhancement of Th1 functions. These results were confirmed by analyses of cytokine and antibody levels.

Analysis of Th1/Th2 balance is insufficient for comprehensively and effectively identifying the mechanism of immune system dysregulation in the context of food allergy; Tregs could maintain the body in a normal state by regulating the balance of Th1/Th2 expression,

which implies that the role of Tregs should also be considered (Kanjrawi et al., 2011). $CD4^+CD25^+Foxp3^+$ T cells are important in the maintenance of immune tolerance and responses, such that deficiency and dysfunction involving these cells contributes to allergic diseases (Qamar et al., 2015). Therefore, we investigated the proportions of $CD4^+CD25^+Foxp3^+$ T cells in this study. We found that the AT, PT, and FT groups had significantly greater proportions of $CD4^+CD25^+Foxp3^+$ T cells; these changes were consistent with the findings concerning Th1 and Th2 cells. The allergic reactions of the mice sensitized with HM were significantly weakened and tended to suggest a tolerant state. These results implied that HM sensitization could regulate differentiation of Tregs and shift the Th1/Th2 balance toward a Th1-type response, thus reducing allergic reactions. Noval Rivas and Chatila (2016) demonstrated that healthy individuals have a higher tolerance to allergens than individuals with allergy, mainly due to elevated Treg levels in the body, which induces immune tolerance to foreign antigens and thus suppresses the allergic response. In addition, Zhou et al. (2016) suggested that during enzymatic hydrolysis, some peptides or disulfide bonds were broken, resulting in the collapse of conformational or sequential epitopes; consequently, the allergenicity of food proteins was reduced or eliminated. Thus, we suspect that enzymatic hydrolysis might alter the spatial structure of conformational or sequential epitopes of CM proteins that elicit an allergic reaction.

In conclusion, our results indicated that HM exhibited low potential allergenicity in vivo, such that it suppressed the levels of IgE, IgG, mMCP-1, and histamine. Moreover, HM sensitization contributed to a shift in immune response type (from Th2 to Th1) and maintenance of the Th1/Th2 immune balance. These findings imply that enzymatic hydrolysis treatment can effectively reduce CM sensitization, providing an effective method for production of hypoallergenic CM products.

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