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Comparison of allergenicity among cow, goat, and horse milks using a murine model of atopy

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Due to the prevalence and severity of cow milk (CM) allergy (CMA), an ideal substitute is urgently needed to develop hypoallergenic infant formula for infants who experience anaphylaxis to typical whey-based CM formula. Goat milk (GM) and horse milk (HM) are considered appropriate substitutes; however, whether GM and HM are less allergenic than CM is unclear. In the present study, the difference in allergenicity among CM, GM, and HM was investigated using the Balb/c mouse model. The number of mice with severe respiratory symptoms was significantly lower in the GM- and HM-sensitised groups than in the CM-sensitised group. Furthermore, histologic examination of intestinal and lung tissues revealed a thinner lamina propria of the small intestine and obvious inflammation and congestion in lungs in the CM-sensitised group than in the GM- and HM-sensitised groups. CM-specific immunoglobulin (Ig) E, serum IgG1, and plasma histamine levels were also higher in CM-sensitised mice than in GM- or HM-sensitised mice. In addition, higher interleukin (IL) 4 and IL-17A levels and lower interferon- γ (IFN- γ) and IL-10 levels were observed in CM-sensitised mice compared with GM- and HM-sensitised mice, according to qPCR, indicating Th1/Th2 and Treg/Th17 imbalances. The CM-sensitised group had a higher proportion of IL-4- and IL-17A-producing CD³⁺ T cells but a lower proportion of IFN- γ - and IL-10-producing CD³⁺ T cells compared with the GM- and HM-sensitised groups, confirming the Th1/Th2 and Treg/Th17 imbalances. In conclusion, GM and HM were less allergenic than CM in mice as a result of a shift in the Th1/Th2 and Treg/Th17 imbalances; however, HM was less allergenic than GM and can be used as an alternative milk to develop infant formulas for children with CMA.

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Introduction

Cow milk (CM) has always been used as an important raw material in infant formula; however, CM allergy (CMA) has a high incidence (approximately 2–7.5%) in infants and young children.¹ In addition, the incidence of CMA in young children is steadily increasing. Human milk includes a large number of protective factors against atopic disorders.^{2,3} However, breastfeeding is not always possible due to insufficient breast milk production, and in most cases, children receive breast milk for less than 6 months because mothers have to return to work.

Therefore, children are usually given a CM-based infant formula powder at the end of the breastfeeding period, causing them to become sensitised to CM and develop immediate symptoms after ingestion.⁴ For these children, a milk substitute must be provided,⁵ and the management of CMA is a major challenge in the 21st century.

Complementary and alternative approaches have been implemented to reduce the allergenicity of CM proteins, including heat treatment,⁶ glycation,^{7,8} and enzymatic hydrolysis.⁹ However, these treatments have disadvantages. For example, heat treatment can destroy bioactive substances in CM, and enzymatic hydrolysis can generate several bitter compounds that affect the taste of hydrolysates. Furthermore, some children reportedly experienced strong anaphylactic reactions to both whey¹⁰ and casein hydrolysates.^{11,12} During hydrolysis, some molecular structures that cause allergenic reactions are potentially destroyed, however, new binding sites that can cause allergenicity are exposed. Theoretically, controlling the degree of CM protein hydrolysis to completely eliminate allergenicity is difficult. In addition, the hydrolysed (partially or extensively) milk is lacking the bioactive peptides

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present in untreated CM, which play important roles in immunomodulation. Therefore, discovering novel raw materials with naturally low allergenicity without the need for any treatment is important.

A more suitable protein source is needed for children with CMA. Camel milk has been proven safer than goat milk (GM) for children with CMA because it lacks β -lactoglobulin, which is the main component that causes the allergy;¹³ however, camel milk production is low and cannot satisfy the needs of children with CMA. GM and horse milk (HM) have also been suggested as a substitute for CM in children who are allergic to CM but tolerate GM or HM derivatives.¹⁴ However, in several studies, children with CMA were also sensitive to GM or HM protein and showed cross-reactivity between CM and GM proteins.^{15–17}

Nevertheless, sufficient evidence suggests that GM is less allergenic than CM.¹⁸ Casein and β -lactoglobulin are the main allergens causing CMA.^{19,20} Although five principal proteins in goat milk (α_{s1} -casein, α_{s2} -casein, β -casein, β -lactoglobulin and α -lactoferrin) are reported to closely resemble their homologs in cow milk,¹⁸ α_{s1} -casein is found to account for 43% of the total protein in CM²¹ and only 1–3% of that in GM.²² In addition, the β -lactoglobulin content is lower in GM than CM. Thus, GM could be less immunogenic when introduced into the diet of non-sensitised children. In a previous study, GM-based formula was shown to provide similar and even better nutritional support to infants compared with CM-based formula. Specifically, research on GM oligosaccharides showed decreased intestinal inflammation in rats with colitis and improved barrier function in epithelial cell co-cultures.²³ Furthermore, due to specific nutritional and functional properties, GM is a valuable and healthier alternative to CM.

HM has also received increased interest for its biophysical and biochemical properties, which are closer to human milk than CM. The casein content in mare milk is similar to that of breast milk but lower than CM and the homologies between α_{s1} -casein and β -casein from HM and CM are 39% and 56%, respectively.²⁴ The cross-reactivity between CM proteins and the milk proteins of other mammals has been examined in previous studies, and the results indicated that IgE from the sera of children allergic to CM recognised defined epitopes of milk proteins from other mammals, including goat and buffalo.²⁵ However, the cross-reactivity between HM and CM proteins was weak,²⁶ probably due to differences between the amino acid sequences.²⁷ Furthermore, HM has an optimal casein/whey protein ratio, similar to that of breast milk,²⁸ supporting its potential value as a breast milk substitute.

Female Balb/c mice are generally recognised as the most suitable model for detecting the sensitisation of proteins because they induce Th2-biased immune responses. Systematic and comprehensive studies in which the differences in allergenicity were investigated among CM, GM, and HM are lacking. In the present study, the differences in sensitisation among CM, GM, and HM in terms of mucosal and cellular immunities were evaluated using a mouse model of orally induced atopy.

Materials and methods

Experimental reagents and milk proteins

Cholera toxin (CT) and Concanavalin A (ConA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies for enzyme-linked immunosorbent assay (ELISA) were purchased from Bio-Rad Laboratories, Inc. (formerly AbD Serotec) (Hercules, California, USA), including goat anti mouse IgE (polyclonal antibody) conjugated to horseradish peroxidase (HRP), goat anti-mouse IgG2a (polyclonal antibody) conjugated to HRP, or goat anti-mouse IgG1 (polyclonal antibody) conjugated to HRP. All other products were of the best grade available and were purchased from Sigma.

CM, GM and HM were purchased from a farm in Changchun (Chang Chun, Jilin, China). CM, GM and HM were centrifuged at 1000g for 10 min at 4 °C, and the upper layer of fat was removed to obtain a skimmed milk. Both milks were freeze-dried.

Mice and diets

Female Balb/c mice aged 3 weeks (immediately after weaning) were obtained from the Laboratory Animal Center of Tumour Hospital of Harbin Medical University (Harbin, Heilongjiang, China). The mice were housed in a controlled environment with a 12 h light/12 h dark cycle and room temperature (approximately 22 °C) with free access to milk-free mouse chow diet and water.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Northeast Agricultural University and approved by the Animal Ethics Committee of Northeast Agricultural University.

Sensitization and challenge by oral administration of antigen

Mice ($n = 8$ per group) were sensitised intragastrically with CM, GM or HM plus CT as an adjuvant and boosted five times at weekly intervals. Intragastric feeding was administered using a stainless-steel blunt feeding needle (Changchun BAIAO biological instruments Co., Ltd, Changchun, Jilin, China). Mice received CM, GM or HM (1 mg per g body weight) together with CT (0.3 μ g per g body weight). To control for the effect of the adjuvant, one group of mice received CT in PBS (CT group), and another group received PBS only (control group). Six weeks after the first oral gavage, mice were fasted overnight and challenged intragastrically. The CM, control and CT groups received two doses of CM protein (30 mg per mouse) administered 30 min apart. The GM and HM groups received two doses of GM or HM (30 mg per mouse), respectively, administered 30 min apart. Our previous experiments showed identical results in the control group challenged with CM, GM or HM (data not shown).

Evaluation of hypersensitivity responses

Immediately after challenge, hypersensitivity responses were evaluated using a scoring system modified slightly from a previous report⁵ as follows: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the

eyes and mouth and pilar erecti; 3 = decreased activity with increased respiratory rate; 4 = wheezing and laboured respiration accompanied with diarrhea; 5 = cyanosis around the mouth and the tail; and 6 = death.

Determination of serum CM- or GM-specific IgE, IgG1 and IgG2a levels

Blood was collected by removing the eyeball. Serum was obtained by centrifugation (1000g for 10 min, 4 °C), collected, and frozen at −80 °C. The antigen-specific IgE level was determined by ELISA. Briefly, 96-well plates were coated with 10 µg mL^{−1} CM, GM, or HM in coating buffer (0.05 M sodium carbonate/bicarbonate buffer, pH = 9.6 ± 0.2). After an overnight incubation at 4 °C, the plates were washed three times with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20), and the reactions were blocked with blocking solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin) for 1 h and then washed three times. Next, serum samples (1:10 for IgE, 1:1000 for IgG1, and 1:100 for IgG2a) were added to the plates and incubated for 2 h at 37 °C. The plates were washed four times, and 100 µL goat anti-mouse IgE antibody conjugated to HRP, goat anti-mouse IgG2a conjugated to HRP, or goat anti-mouse IgG1 conjugated to HRP was added for 1 h at 37 °C; then, the plates were washed four times. Staining was performed using 3,3',5,5'-tetramethyl-benzidine (Sigma) for 30 min at 25 °C in the dark, and the reaction was stopped using 1 M H₂SO₄. The plates were read using an enzyme microplate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 414 nm. Values are expressed in arbitrary units, deduced from the optical densities of the reference serum curve with a high level of antibodies after subtracting the blanks. Serial dilution of a pool of hyperimmune serum samples was used as a standard for IgE, IgG1, or IgG2a on each assay plate. The results are expressed in arbitrary units. All analyses were performed in triplicate.

Determination of the plasma histamine level

Plasma was separated into chilled tubes containing heparin sodium as an anticoagulant. After centrifugation (1713g) for 10 min at 4 °C, plasma aliquots were collected and frozen at −80 °C. Histamine levels were determined using an enzyme immunoassay kit (Usin Life Science & Technology Co., Ltd, Wuhan, Hubei, China) according to the manufacturer's instructions.

Assessment of pathologic abnormalities

For assessment of pathologic alterations, jejunum and lung samples were fixed in neutral-buffered formaldehyde and embedded in paraffin. Three-micrometer sections were stained with hematoxylin and eosin (H and E) and periodic acid-Schiff (PAS) reagent.

Culture of spleen cells

Spleen cells were collected and homogenised in complete RPMI-1640 culture medium (HyClone Laboratories, Inc., Omaha, NE, USA), supplemented with 100 U mL^{−1} penicillin,

100 µg mL^{−1} streptomycin, and 10% foetal bovine serum (HyClone). After centrifugation at 1000g for 5 min, erythrocytes were lysed in lysis buffer (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) for 1 min at 4 °C. Resting cells were counted using a haemocytometer and cultured for stimulation assays, real-time polymerase chain reaction (qPCR), or flow cytometry. Cells were incubated at 37 °C and humidified 5% CO₂.

Determination of cytokine production

The spleen-derived lymphocytes were cultured in six-well plates (1 × 10⁷ per well) in 5 mL medium and incubated for 24 h in the presence of ConA (5 µg mL^{−1}), CM protein, GM protein, or HM protein (100 µg mL^{−1}). The supernatants were collected after 48 h of incubation. Interleukin (IL)-4, IL-10, IL-17A, and interferon (IFN)-γ levels were determined by ELISA according to the manufacturer's instructions (BioLegend). The serum IL-4, IFN-γ, IL-10, and IL-17A levels were also detected using the same method described above.

Analysis of cytokine mRNA expression by quantitative real time polymerase chain reaction (qPCR)

Total RNA was isolated from cultured cells stimulated by CM, GM, or HM in the presence of ConA or from cells stimulated with PBS in the presence of ConA using TRIPURE reagent according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The total RNA (2 mg) was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) at 42 °C for 60 min in a 25 mL reaction volume. For qPCR, cDNA was amplified using FS Universal SYBR Green Real Master (Roche) on the Bio-Rad CFX96TM Real Time Detection System. The conditions used for qPCR were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate. The results were analysed using CFX Manager Software. Data were analysed using the 2^{−ΔΔCt} method, with normalisation to GAPDH expression. The primer sequences used for qPCR are shown in Table 1.

Flow cytometry

The proportion of CD³⁺CD⁴⁺ and CD³⁺CD⁸⁺ T cells was detected by flow cytometry using PerCP/cyanine 5.5 anti-mouse CD3 (clone 145-2C11), APC anti-mouse CD4 (clone GK1.5), and PE anti-mouse CD8a (clone 53-6.7) antibodies. CD³⁺ specific splenocyte intracellular cytokines, IL-4, IFN-γ, IL-10, and IL-17A, were stained with PE anti-mouse IL-4 (clone 11B11), FITC anti-mouse IFN-γ (clone XMG1.2), FITC anti-mouse IL-10 (clone JES5-16E3), and FITC anti-mouse IL-17A (clone TC11-18H10.1) antibodies, respectively. All antibodies were purchased from BioLegend.

During the final 4 h of incubation, 1 µM brefeldin A was added to block cytokine secretion. Intracellular IL-4, IFN-γ, IL-10, and IL-17A were analysed after fixation/permeabilisation. Lysing solution was added to lysed erythrocytes. The stained samples were washed twice, and supernatants were discarded before acquisition on FACSaria II flow cytometry (BD

Table 1 Primer sequences for real-time PCR

Gene	Primer sequence (5'-3')	Length of amplified fragment (bp)
IL-4	ACAGGAGAAGGGACGCCAT (forward) GAAGCCCTACAGACGAGCTCA (reverse)	95
GATA-3	GAAGGCATCCAGACCCGAAAC (forward) ACCCATGGCGGTGACCATGC (reverse)	255
IFN- γ	TCAAGTGGCATAGATGTGGAA (forward) TGGCTCTGCAGATTTTCATG (reverse)	92
T-bet	GCCAGGGAACCGCTTATATG (forward) GACGATCATCTGGGTACATT (reverse)	137
IL-17A	GCGCTGTGTCAATGCGGAGG (forward) CTGGCGGACAATCGAGGCCA (reverse)	169
ROR γ t	TACGGCAGCGCACCAACCTC (forward) AGCCGCTTGGCAAACTCCACC (reverse)	136
IL-10	TGAGGATCAGCAGGGGCCAGT (forward) CTGGCTGAAGGCAGTCCGCA (reverse)	104
Foxp3	GGAAGCCCCGGAGAGGCAGA (forward) TGCAGGCTCAGGTTGTGGCG (reverse)	123
Gapdh	TTCACCACCATGGAGAAGGC (forward) GGCATGGACTGTGGTCATGA (reverse)	237

Biosciences, San Jose, CA, USA). Non-stimulated control cells were used to determine the threshold quadrants of the background response.

Statistical analysis

All the experiments were independently repeated at least three times. The results are expressed as means $\pm$ standard error of the mean. Statistical significance ($p < 0.05$) was determined by one-way ANOVA using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). The figures were generated using GraphPad Prism 5 software (GraphPad Prism Software, Inc, San Diego, CA, USA).

Results

Evaluation of body weights and symptoms

The mice in each group were weighed on days 0, 7, 14, 21, 28, and 35 of the experimental period. The body weight of mice increased in each group. Although the degree of the increase differed among the groups, significant differences were not observed (Fig. 1A).

Mice were challenged intragastrically with CM, GM, or HM (30 mg per mouse) after 6 weeks of sensitisation. The symptoms were evaluated within 15–30 min. The severity of the reaction was scored based on the standard mentioned in the Materials and Methods section. The most severe reactions were observed in CM-sensitised mice (Fig. 1B) compared with the GM- and HM-sensitised mice. In addition, the GM-sensitised mice showed weaker symptoms compared with the HM-sensitised mice. The control mice showed no symptoms of hypersensitivity after CM challenge, and only one mouse in the CT group showed slight symptoms of scratching and rubbing around the nose and head. The average severity score was significantly higher ($p < 0.01$) in the GM group than in the control group but without statistical significance. The results indicated less severe sensitisation in the GM group.

Histamine level in plasma after challenge of sensitised mice

In food allergy studies, histamine released by mast cells was found to be effective in IgE-mediated hypersensitivity reactions.^{29,30} Therefore, the plasma histamine level was examined in the mice. The plasma histamine level was significantly higher in CM-sensitised mice than in GM- and HM-sensitised mice (Fig. 1C), suggesting that CM stimulated the mast cells most strongly. However, the plasma histamine level was similar between GM- and HM-sensitised mice, indicating that GM and HM equally stimulated the mast cells to release histamine. Notably, mice with the highest symptom score also had the highest histamine level, indicating that the higher histamine level was responsible for the allergy symptoms.

Effects of CM, GM and HM on specific humoral responses in serum and intestinal juice

CM-, GM-, and HM-specific IgE levels in serum and intestinal juice were evaluated. As shown in Fig. 1D–I, CM-specific IgE and IgG1 levels in the serum and intestinal juice of mice intragastrically challenged with CM were markedly increased compared with the CT-sensitised mice ($p < 0.01$), indicating a significantly higher level of antibody response. GM- and HM-sensitised mice showed the same trend as that of the CM-sensitised mice. However, a significant difference was not observed between GM- and HM-sensitised mice in either the antigen-specific IgE or IgG1 level in serum. However, a significant difference was observed in the antigen-specific IgG1 level in intestinal juice between GM- and HM-sensitised mice ($p < 0.05$).

Conversely, the IgG2a level was significantly higher in GM- and HM-sensitised than in CT- and CM-sensitised mice. No significant difference was observed between GM- and HM-specific IgG2a levels in serum; however, the IgG2a level in the intestinal juice was significantly higher in GM- than HM-sensitised mice ($p < 0.05$). Furthermore, CM-specific IgG2a levels in intestinal juice were markedly lower ($p < 0.01$) in CM- than CT-

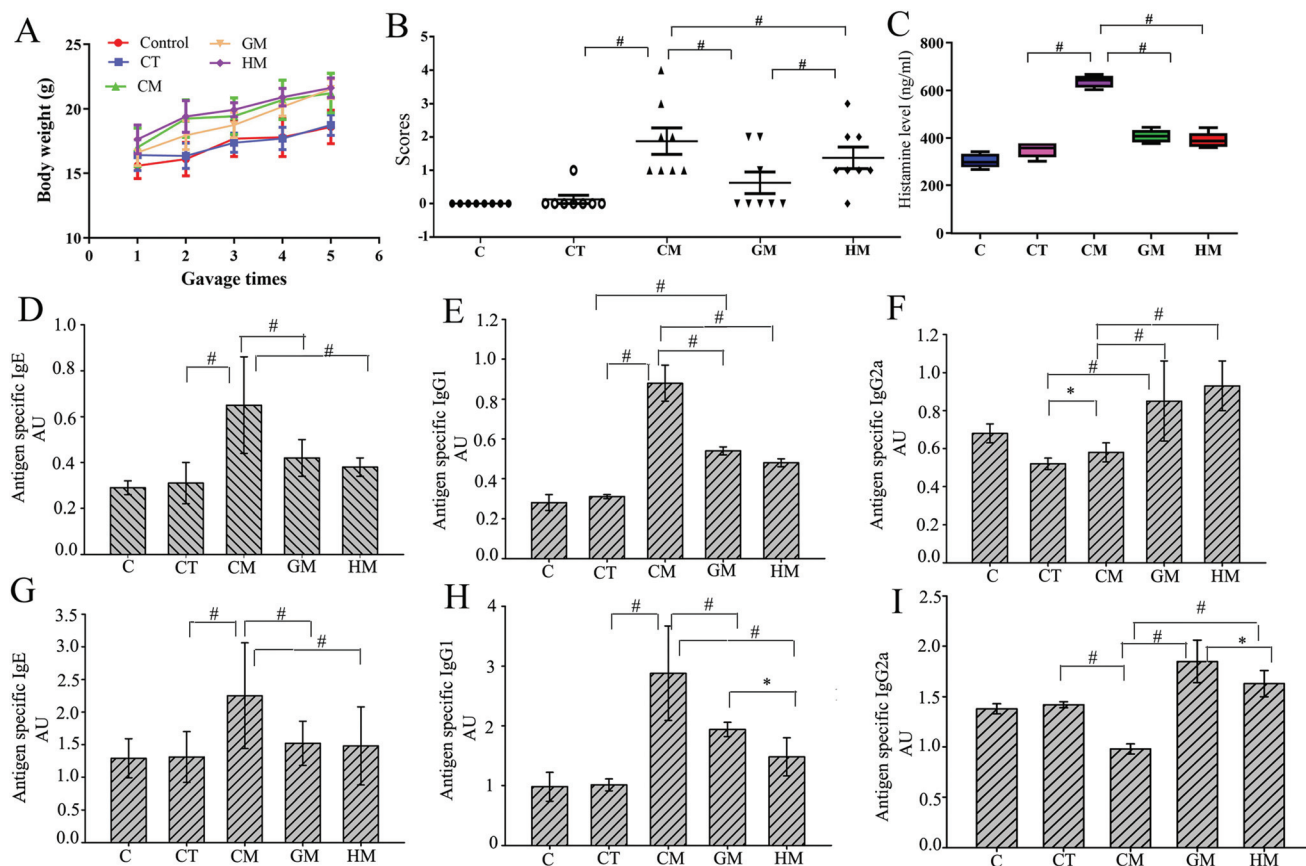


Fig. 1 Effects of CM, GM and HM on body weight, hypersensitivity symptoms, histamine levels AND specific humoral responses in serum and intestinal juice. Evaluations of (A) body weight, (B) symptom scores, and (C) plasma histamine levels in sensitised mice, antigen-specific IgE, IgG1, and IgG2a levels in serum (D, E, F) and intestinal juice (G, H, I). Mice ($n = 8$ per group) were challenged with cow milk (CM), goat milk (GM), or horse milk (HM) by oral administration. One mouse group was administered cholera toxin (CT group), and the control group (C group) was administered PBS. Body weight was measured weekly. The hypersensitivity symptoms were scored (blinded) on a scale from 0 (no symptoms) to 6 (death), as described in the Materials and Methods section. The plasma histamine level (ng mL^{-1}) was determined using ELISA and expressed as the mean $\pm$ standard error of the mean (SEM). ANOVA was used to determine statistical significance ($p < 0.05$ or $p < 0.01$). Specific IgE, IgG1, and IgG2a levels in sera were determined using ELISA. The results are expressed as means $\pm$ SEM. ANOVA was used to determine the statistical significance ($p < 0.05$ or $p < 0.01$). * denotes significance ($p < 0.05$), # denotes significance ($p < 0.01$).

sensitised mice, but significantly higher ($p < 0.05$) in CM- than CT-sensitised mice in serum.

Characterization of intestinal reactions and pulmonary reactions

Histological analysis indicated that both CM and GM sensitisation resulted in an increase in the number of Paneth cells, a type of secretory cell located at the bottom of the enteraden. An increased number of Paneth cells causes an increase in the number of eosinophils in Paneth cells. The HM- and CT-sensitised mice did not show obvious changes in the number of Paneth cells (Fig. 2). The lamina propria in the small intestine was thinner in CM-sensitised mice than in GM- and HM-sensitised mice, probably due to complex inflammatory responses stimulated by CM.

In the present study, the CM-induced immediate reactions were frequently accompanied by respiratory symptoms, including laboured respiration and wheezing. The lungs from CM-

sensitised mice showed obvious inflammation and contained a large number of congestion points compared with mice in the other groups. Furthermore, several areas of the bronchial lumen appeared to be filled with mucus. The GM-sensitised mice had more small congestion points compared with HM-sensitised mice.

Effects of CM, GM or HM on cytokine levels in serum and splenocyte supernatants

Cytokines play an important role in immunoregulation. In the present study, IL-4, IFN- γ , IL-10, and IL-17A levels in splenocyte supernatants were detected using an ELISA kit according to the manufacturer's instructions. After CM stimulation, the levels of secreted IL-4 and IL-17A were significantly increased, and those of IFN- γ and IL-10 significantly decreased, compared with mice in the other groups (especially in the GM- and HM-sensitised groups), indicating Th1/Th2 and Treg/Th17 imbalances (Fig. 3A). Significant differences in the IL-4, IFN- γ ,

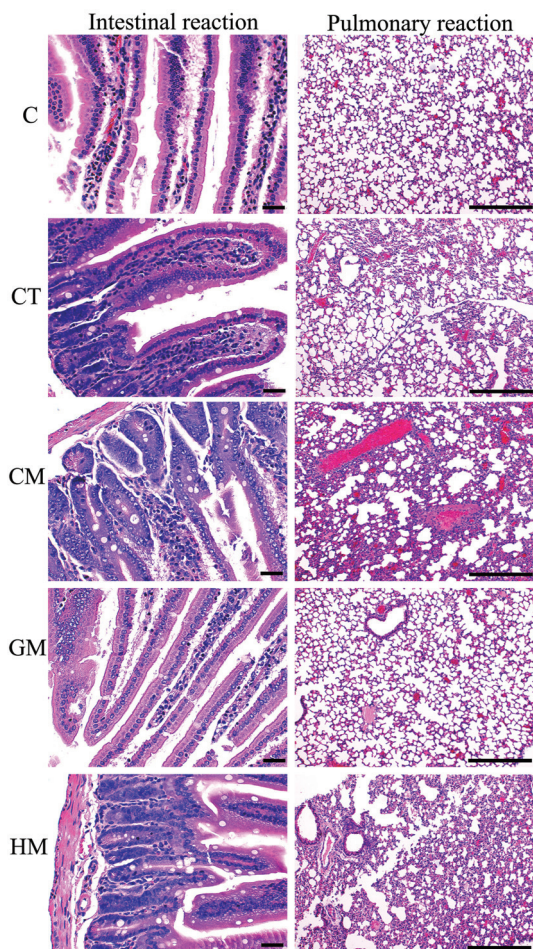


Fig. 2 Characterisation of intestinal and pulmonary reactions. The pathologic changes in the jejunum and lung were observed under a microscope after haematoxylin and eosin staining. Both CM and GM sensitisation resulted in an increased number of Paneth cells, indicating an increase in the number of eosinophils. In addition, the lamina propria of the small intestine was thinner in CM-sensitised mice than in GM- and HM-sensitised mice. The lungs of CM-sensitised mice showed obvious inflammation and a large number of congestion points compared with mice in the other groups. Bar = 100 μ m. C: PBS-sensitised group; CT: CT-sensitised group; CM: CM-sensitised group; GM: GM-sensitised group; HM: HM-sensitised group.

and IL-17A levels in the supernatants were not observed between GM- and HM-sensitised mice (Fig. 3B). In addition, the levels of IL-4, IFN- γ , IL-10, and IL-17A in serum were measured and showed similar trends as those in the splenocyte supernatants.

Effects of CM, GM or HM on the expression of cytokines and transcription factors

To further identify changes in the levels of cytokines and their transcription factors, the mRNA expression of IL-4, GATA-3, IFN- γ , T-bet, IL-10, Foxp3, IL-17A, and ROR γ t in both splenocytes and spleen was examined using qPCR. The IFN- γ , T-bet, IL-10, and Foxp3 mRNA levels were significantly lower, but the IL-4, GATA-3, IL-17A, and ROR γ t mRNA levels significantly

higher, in the CM-sensitised group compared with the GM- and HM-sensitised groups (Fig. 4A and B). Notably, the changes in cytokine expression confirmed the results observed in the splenocyte supernatants and serum, confirming Th1/Th2 and Treg/Th17 imbalances.

Significant and non-significant differences in the expression of the cytokines and transcription factors evaluated were observed between the GM- and HM-sensitised groups. Overall, the GM-sensitised mice expressed higher cytokine and transcription factor levels compared with the HM-sensitised mice, indicating stronger sensitisation induced by GM than HM.

Flow cytometric analysis

Compared with the ConA-sensitised group, the proportion of CD $^{3+}$ CD $^{4+}$ and CD $^{3+}$ CD $^{8+}$ T cells was not significantly affected by *ex vivo* stimulation with CM, GM, or HM (Fig. 5A–E). The proportion was moderately decreased in the CM-sensitised group but not significantly (Fig. 5F).

As shown in Fig. 6, the proportion of IL-4-producing CD $^{3+}$ T cells was significantly higher in the CM-sensitised group compared with the ConA- and HM-sensitised groups. A mild decrease in the proportion was observed in the GM-sensitised group but without statistical significance ($p > 0.05$); however, the proportion of IL-4-producing CD $^{3+}$ T cells was significantly lower in the HM- than GM-sensitised group. Notably, the change in the proportion of IFN- γ -producing CD $^{3+}$ T cells was the opposite of that of the IL-4-producing CD $^{3+}$ T cells, with the HM-sensitised cells showing the highest proportion ($p < 0.01$) compared with the other groups. No significant difference in the proportion of IFN- γ -producing CD $^{3+}$ T cells was observed between the CM- and GM-sensitised groups; however, both groups had a remarkably higher proportion compared with the ConA-sensitised group. Furthermore, although the proportion of IL-10-producing CD $^{3+}$ T cells differed between the GM- and HM-sensitised groups, it was significantly lower in the CM-sensitised group compared with all groups except for the control. Finally, the proportion of IL-17A-producing CD $^{3+}$ T cells was also increased in CM-sensitised group compared with the GM- and HM-sensitised groups. The HM-sensitised group also had a higher IFN- γ level and proportion of IL-10-producing CD $^{3+}$ T cells but a lower proportion of IL-4 and IL-17A-producing CD $^{3+}$ T cells, which implied weaker splenocyte stimulation and confirmed the presence of the Th1/Th2 and Treg/Th17 imbalances.

Discussion

In the present study, the Balb/c mouse was used as an animal model to investigate the difference in the allergenicities of CM, GM, and HM. The question of whether GM or HM is less allergenic than CM was investigated to identify a potential hypoallergenic milk source for infants who have CMA. CT, which has been shown to stimulate the production of IgE, Th2

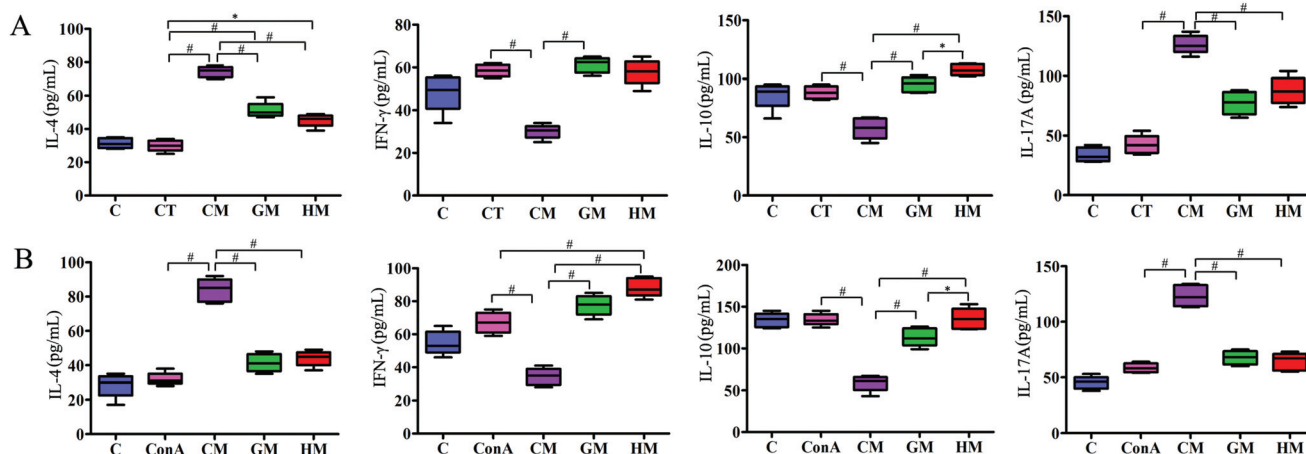


Fig. 3 Changes in cytokine levels in the serum (A) and splenocyte supernatant (B). Sera were collected by removing the eyeball and centrifuged (1000g, 10 min, 4 °C). The splenocytes were cultured in six-well plates (1×10^7 per well) in the presence of ConA ($5 \mu\text{g mL}^{-1}$), CM protein, GM protein, or HM protein ($100 \mu\text{g mL}^{-1}$). The supernatants were collected after 48 h of incubation. IL-4, IFN- γ , IL-10, and IL-17A levels in splenocyte supernatants or serum were detected using ELISA. CT: CT-sensitized group; CM: CM-sensitized group; GM: GM-sensitized group; HM: HM-sensitized group. The results are expressed as means $\pm$ SEM. ANOVA was used to determine the statistical significance ($p < 0.05$ or $p < 0.01$). * denotes significance ($p < 0.05$), # denotes significance ($p < 0.01$).

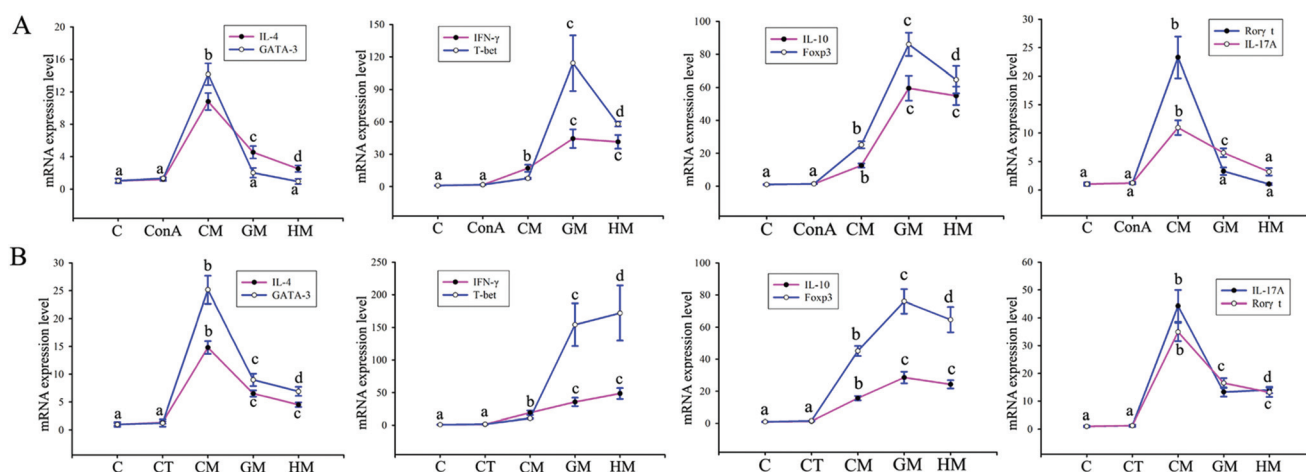


Fig. 4 Effects of CM, GM, or HM on the expression of cytokines and transcription factors in splenocytes (A) or the spleen (B). The splenocytes were cultured in six-well plates (1×10^7 per well) in the presence of ConA ($5 \mu\text{g mL}^{-1}$), CM protein, GM protein, or HM protein ($100 \mu\text{g mL}^{-1}$). Then, the cells were collected after 48 h of incubation, and the mRNA expression of cytokines and transcription factors in splenocytes was measured using qPCR. The mouse spleens were collected after the last challenge, and the mRNA expression of cytokines and transcription factors was detected using qPCR. The expression of all splenocyte genes was normalised to GAPDH expression. C: PBS-sensitized group; ConA: ConA-sensitized group; CT: CT-sensitized group; CM: CM-sensitized group; GM: GM-sensitized group; HM: HM-sensitized group. The results are expressed as means $\pm$ SEM. ANOVA was used to determine the statistical significance. Different letters on the bars represent significant differences ($p < 0.01$).

cells, and IL-4,^{31,32} was used to prevent the development of oral tolerance.

The CM-sensitized group of Balb/c mice had the most severe hypersensitivity symptoms, highest plasma histamine level, highest antigen-specific IgE and IgG1 levels in serum or intestinal juice, and lowest serum antigen-specific IgG2a level, compared with the GM- and HM-sensitized groups. Mice in all groups had an IgE-mediated allergic reaction. Mouse IgE antibodies are thought to bind to high-affinity FcεRI receptors on the surface of mast cells to induce their release of histamine;

thus, IgE appears to have been responsible for the elevated plasma histamine level in the present study. This result concurs with previous studies in which IgE induced type I hypersensitivity in mice.^{33,34} A significantly increased plasma histamine level was the probable cause for the hypersensitivity symptoms observed in the present study. In addition, HM-sensitized mice had lower histamine, specific IgE, and IgG1 levels but a higher IgG2a level compared with the GM- and CM-sensitized mice, indicating that HM produced a milder allergic reaction in mice.

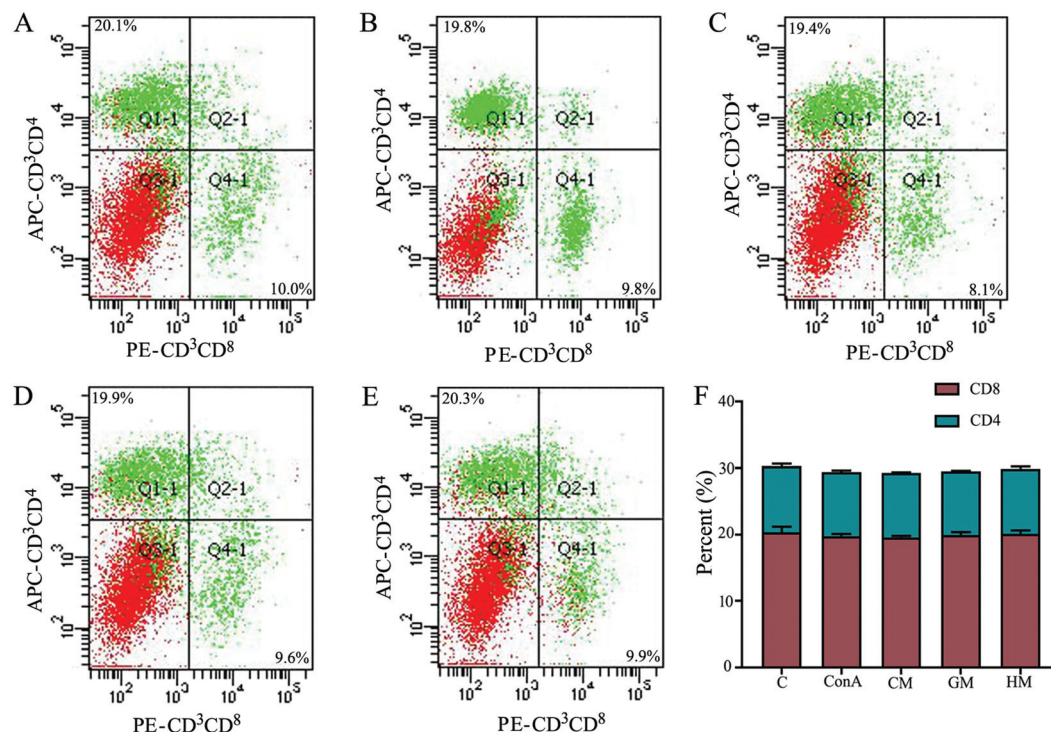


Fig. 5 The proportion of CD³⁺CD⁴⁺ and CD³⁺CD⁸⁺ cells among splenocytes. A: PBS-sensitised group; B: ConA-sensitised group; C: CT-sensitised group; D: CM-sensitised group; E: GM-sensitised group; F: HM-sensitised group. CD³⁺ cells were labelled with percp-cy5.5, CD⁴⁺ cells were labelled with APC, and CD⁸⁺ cells were labelled with PE. Non-stimulated control samples were used to establish the threshold quadrants of the background response.

Furthermore, CM provoked a strong IgE response after intragastric challenge, whereas GM and HM failed to induce a similar response. Significant vascular congestion and oedema of the lamina propria were observed on histologic examination of the small intestine, showing that intestinal anaphylaxis occurred after intragastric challenge with CM. The results support the hypothesis that systemic anaphylaxis is a consequence of intestinal anaphylactic events, which allows the CM antigen to rapidly pass into the systemic circulation and induce mediator release from mast cells and other cells at extra-intestinal sites.³⁵ Furthermore, respiratory symptoms were observed in this animal model. Histologic examination of animal lungs revealed significant inflammation and increased bronchial mucus. This result is consistent with a previous study in which intragastrically administered antigen induced pulmonary reactions.³⁵ In another study, hypersensitivity reactions to food, primarily IgE-mediated hypersensitivity reactions, were shown to trigger asthmatic episodes in humans.³⁶ In the present study, ingested CM proteins penetrated the intestinal mucosa and circulated to the lungs and may have contributed to the pulmonary inflammatory response. However, in other relevant studies, antigen-specific intestinal lymphocytes migrated to the lungs and induced pulmonary symptoms because the activated lymphocytes could migrate from one mucosal site to another.^{37,38} In the GM- or HM-sensitized groups, significant respiratory and intestinal tract symp-

toms were not observed, indicating that GM and HM produced a weaker allergic reaction than did CM.

The Th1/Th2 balance is a critical factor in the pathogenesis of allergic diseases. CMA generally corresponds to an inappropriate immune response characterised by a disruption of the Th1/Th2 balance toward a Th2 profile.³⁹ Our results showed that CM stimulation was associated with a significantly higher IL-4 level and significantly lower IFN- γ level in serum and splenocyte supernatants, indicating that CM stimulation might induce differentiation of Th cells towards the Th2 profile; the significant elevation in IL-4 and GATA-3 mRNA expression confirmed this hypothesis. The identification of Th17 and Tregs introduced a new multifactorial balance concept in the mechanism of allergic diseases.⁴⁰ CMA was previously considered a Th2-dominant disease; however, roles of Th17 and Tregs in allergic diseases have since been identified.^{41,42} In the present study, a Treg/Th17 imbalance was also involved in CMA. IL-10 is a strong anti-inflammatory cytokine that shows antigen-specific and non-specific tolerance induction *via* local production of IL-10,^{43–45} and IL-17A has been implicated in host defences against a series of extracellular pathogens, such as *Klebsiella pneumoniae* and *Candida albicans*.^{46,47} In the present study, after stimulation with CM, the levels of secreted IL-17A and ROR γ t mRNA were significantly elevated. Conversely, the levels of secreted IL-10 and Foxp3 mRNA were significantly lower in the CM- than GM- and

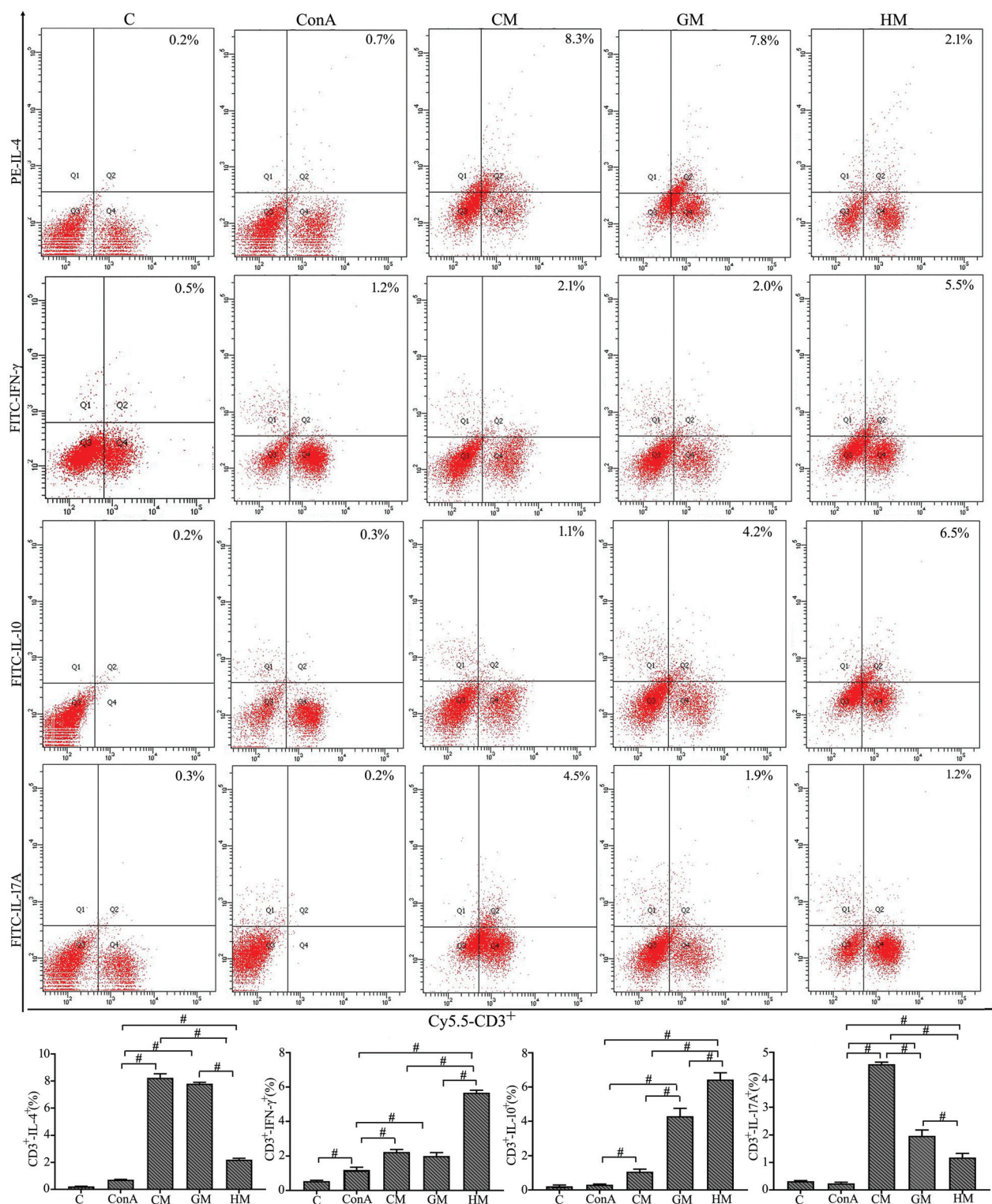


Fig. 6 Flow cytometric detection of antigen-specific IL-4 (PE-labelled), IFN- γ (FITC-labelled), IL-10 (FITC-labelled), and IL-17A (FITC-labelled)-producing CD3⁺ T cells. C: PBS-sensitized group; ConA: ConA-sensitized group; CM: CM-sensitized group; GM: GM-sensitized group; HM: HM-sensitized group. * denotes significance ($p < 0.05$), # denotes significance ($p < 0.01$). Non-stimulated control samples were used to establish the threshold quadrants of the background response.

HM-sensitised groups. Therefore, the inflammatory effects were enhanced by the lower IL-10 and higher IL-17A levels, which confirmed the Treg/Th17 imbalance. In addition, IFN- γ was shown to inhibit Th17 cell development;⁴⁸ thus, it could be inferred that enhanced IL-17A secretion will be accompanied by decreased IFN- γ levels, which was verified by our results. Furthermore, after stimulation with GM or HM, neither the IL-4 nor IL-17A level was significantly increased, and IL-10 and IFN- γ levels were significantly higher, compared with after stimulation with CM, indicating that GM and HM were less allergenic. This was consistent with the antigen-specific IgE, IgG1, and IgG2a results.

To further confirm the Th1/Th2 and Treg/Th17 imbalance after CM sensitisation, the proportion of CD³⁺CD⁴⁺ and CD³⁺CD⁸⁺ cells was evaluated. However, no significant difference was observed among the CM-, GM- and HM-sensitised groups; there was only a moderate decrease in the CM-sensitised group. Notably, the CM-sensitised group had a higher proportion of IL-4- and IL-17A-producing CD³⁺ T cells but a lower proportion of IFN- γ - and IL-10-producing CD³⁺ T cells, confirming the Th1/Th2 and Treg/Th17 imbalance. Furthermore, the significantly lower proportion of IL-4 and IL-17A-producing CD³⁺ T cells in the HM-sensitised group compared with the GM-sensitised group implied weaker splenocyte stimulation. This result was in agreement with the changes in cytokine and transcription factor expression.

Conclusions

In conclusion, the allergenicities of CM, GM, and HM were compared using a murine model. The results indicated that GM and HM were less immunogenic than CM by shifting the Th1/Th2 and Treg/Th17 imbalances, and that HM produces weaker sensitisation than GM. Both GM and HM were with the potential to be used as suitable substitutes for CM in infant formulas for children with CMA, and HM was superior to GM on account of lower allergenicity.

Author contributions

Cuicui Duan completed most of the experiments, wrote the manuscript and collected the data. Lin Ma, Lin Cai and Xiaolei Li completed a part of experiments, collected the data. Fumin Ma and Juliang Chen completed a part of experiments and analysed data using software. Guicheng Huo and Dan Li designed the experiment and provided good idea. All the authors agree to be accountable for the contents of the work.

Conflicts of interest

The authors declare no completing financial interest.

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