

ORIGINAL ARTICLE

Food Allergy & Anaphylaxis

Evaluation of the efficiency of hydrolyzed whey formula to prevent cow's milk allergy in the BALB/c mouse model

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Abstract

Background: Partially hydrolyzed milk formulas have been proposed for primary prevention in at-risk infants, but evidence of their efficiency and elucidation of the underlying mechanisms are still lacking. Thanks to a Th2-biased mouse model mimicking at-risk patients, we aimed to assess the potency of a partially hydrolyzed whey formula (pHWF) to induce oral tolerance thus preventing further cow's milk (CM) allergy.

Methods: BALB/c mice were gavaged with pHWF, standard milk formula (SF), or vehicle only (PBS+). All mice were then orally sensitized to CM using cholera toxin and further chronically exposed to CM. Humoral (IgE, IgG1, IgG2a) and cellular (Th2/Th1/Th17 cytokine secretion; frequency of CD4+GATA3+ and CD4+CD25+Foxp3+ T cells in the spleen) responses against β -lactoglobulin (BLG) and whole caseins (CAS) were assessed, as well as a marker of elicitation of allergic reaction (mMCP-1) released after an oral challenge with CM.

Results: All markers of sensitization and of allergic reaction were evidenced in the PBS+ mice and were significantly enhanced upon chronic exposure. Gavage with SF totally and durably prevented sensitization and elicitation of the allergic reaction. Conversely, pre-treatment with pHWF only reduced BLG-specific sensitization (IgE, Th2 cytokines), with no significant effect on sensitization to caseins. However, pHWF pre-treatment significantly reduced mMCP-1 concentration in plasma after CM challenges. CD4+CD25+Foxp3+ Treg cell frequency could not be correlated with tolerance efficiency.

Conclusion: Partially hydrolyzed whey formula only partially prevents the further development of CM allergy in this Th2-biased model. A hydrolysate from both whey and casein fractions may be more efficient.

KEYWORDS

BALB/c mouse, cow's milk allergy, oral tolerance, partially hydrolyzed whey formula, prevention

1 | INTRODUCTION

Oral tolerance (OT) is a suppressive immune response normally induced against food proteins. It guarantees a state of intestinal homeostasis face to the high quantity of food proteins encountered each day. This suppressive activity relies notably on regulatory T cells (Treg cells). Treg cells encompass thymus-derived natural Treg cells, specific to self-antigens and limiting autoimmunity,¹ and Treg cells specific to non-self-antigens generated in the periphery (ie, de novo-generated or de novo-induced Treg cells).² When Treg cells are not functional or not induced, immune-related disorders may occur, such as food allergy (FA) that results from uncontrolled activation of food protein-specific Th2 cells, but also probably Th9 and Th22 cells (recent review in Ref. ³). Actually, a higher number of circulating Treg cells was evidenced in children who had outgrown cow's milk allergy (CMA) compared to children who had an active CMA,⁴ and the development of clinical tolerance to CM was associated with higher number of circulating Treg cells.⁵ This thus suggests that not only the maintenance but also the development of oral tolerance against food antigens requires Treg cells, although other cells may also be involved.³

Cow's milk allergy is one of the most common FAs observed in early childhood, with an estimated incidence ranging between 2% and 3%.⁶ Although recent studies demonstrated an increasing frequency of non-IgE-mediated CMA,⁷ IgE-mediated CMA is still found in more than 50% of the CM-allergic patients. In these latter patients, 75% are sensitized to several cow's milk proteins such as proteins from whey (eg, β -lactoglobulin (BLG), α -lactalbumin, lactoferrin) or from casein (CAS) fraction (ie, α s1-, α s2-, β -, κ -casein).⁸ In high-risk infants, when breast-feeding is not possible or is stopped, primary prevention of CMA is proposed through the use of partially hydrolyzed milk formula in the first 4–6 months of life instead of a standard formula.⁹ These hydrolyzed formulas are derived from either whey or caseins and still contain large peptide fragments. They were shown to reduce the risk of atopic dermatitis¹⁰ and to prevent the development of clinical symptoms of CMA¹¹ in some studies, although not in others.¹²

BALB/c mice are a good model for at-risk individuals, because they show Th2-biased immune responses. They represent a good tool to investigate induction of OT to food proteins or hydrolysates derived thereof.^{13,14} For instance, repeated oral administration of BLG to BALB/c mice induced Treg cells that participate in the inhibition of BLG-specific sensitization and allergic reaction.¹⁵ Large peptides generated from BLG were still efficient to induce tolerance, whereas products derived from extensive proteolysis of BLG, leading to small peptides and probably lacking T cell epitopes, were no more tolerogenic.¹⁴ Accordingly, partially hydrolyzed whey proteins were able to induce OT to intact whey proteins.¹⁶ We recently demonstrated that oral administration of such hydrolysates to BALB/c mice prior to intraperitoneal sensitization with purified BLG led to decreased BLG-specific IgG antibodies compared to non-treated mice.¹⁷ In the present study, we then aim to extend our results in an oral sensitization model to whole CM and to compare the potency of pHWF and non-hydrolyzed standard formula to induce OT.

Key Message

Using a Th2 strain of mice mimicking atopic individuals, the tolerating potency of a partially hydrolyzed milk formula was demonstrated to be lower than that of a cow's milk standard formula. Partial tolerance induced by the whey hydrolysate is restricted to whey proteins, i.e. not efficient on sensitization to caseins. Our model can provide useful information for the development of more effective milk formulas to prevent CMA.

2 | METHODS

2.1 | Animals

Three- to four-week-old female BALB/cByJRj mice (Centre d'Elevage René Janvier [CERJ], Le Genest-Saint-Isle, France) were used and handled according to the European Community rules of animal care (see Appendix S1 for additional information).

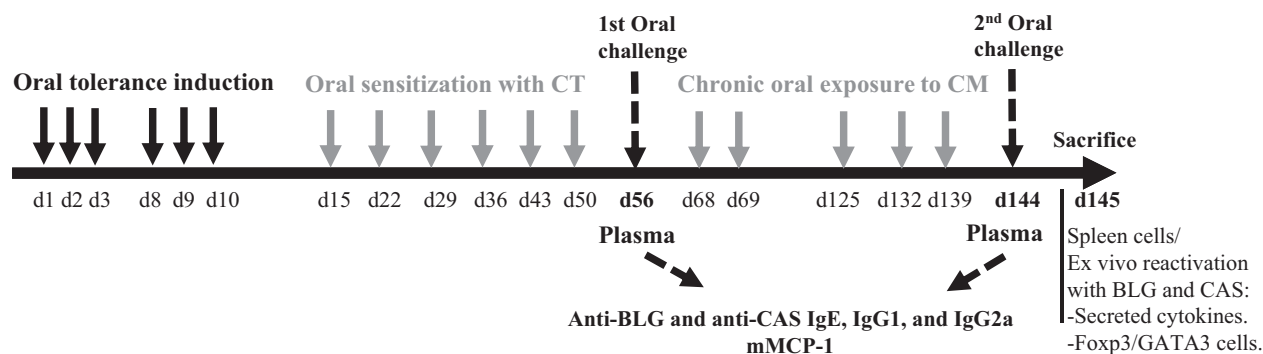
2.2 | Milk formulas, whole cow's milk, and purified allergens

Two milk formulas commercialized in Algeria were used: a standard formula (SF) corresponding to a classical cow's milk-derived formula (Aptamil, 0–6 months; Nutricia, Danone, The Netherlands) or a partially hydrolyzed whey formula (pHWF, Aptamil HA, 0–6 months, Nutricia). Whole CM (UHT sterilized; Monoprix, France) was used for oral sensitization and for oral food challenge (OFC). In the latter case, milk was defatted and then lyophilized to increase the protein concentration. BLG and CAS were purified and characterized as previously described.¹⁸

2.3 | Experimental design: protocols of OT induction and oral sensitization and challenge of mice

The whole experimental protocol, groups, and parameters assessed are summarized in Figure 1. On days 1, 2, 3, 8, 9, and 10, mice received intragastric (ig) gavage with SF or pHWF solubilized in distilled water, using an animal feeding needle (Popper & Sons, New York, NY, USA; 10 mg of proteins/gavage in 200 μ L; $n = 10$ /group).^{14,15} As a positive control of sensitization, one group received only PBS (PBS+). Subsequently, all mice were experimentally sensitized by ig administration of whole CM (6 mg protein/gavage) mixed with cholera toxin (CT, 10 μ g/mice; Sigma-Aldrich, St Louis, MO, USA) on days 15, 22, 29, 36, 43, and 50. A negative control group of sensitization received PBS prior to administration of CT alone (PBS-), and 10 mice were left untreated (N: naïve).

On day 56, an OFC was performed by gavage with 20 mg of CMP. Blood samples were obtained 3 hours after and centrifuged (20 minutes, 2000 $\times$ g, 4°C) to collect plasma. Sodium azide (0.1%) was added as a preservative, and plasma was stored at -20°C until further assays.



Groups	Oral tolerance induction : pre-treatment of mice	Oral sensitization	Chronic oral exposure	Oral challenge
PBS-	PBS	CT in PBS		
PBS+	PBS			
pHWF	Partially hydrolyzed whey formula	Whole CM+ CT	Whole CM	20 mg of CM proteins
SF	Standard formula			
N	None	None	None	

FIGURE 1 Experimental schedule and treatment groups. Ten mice per group were included

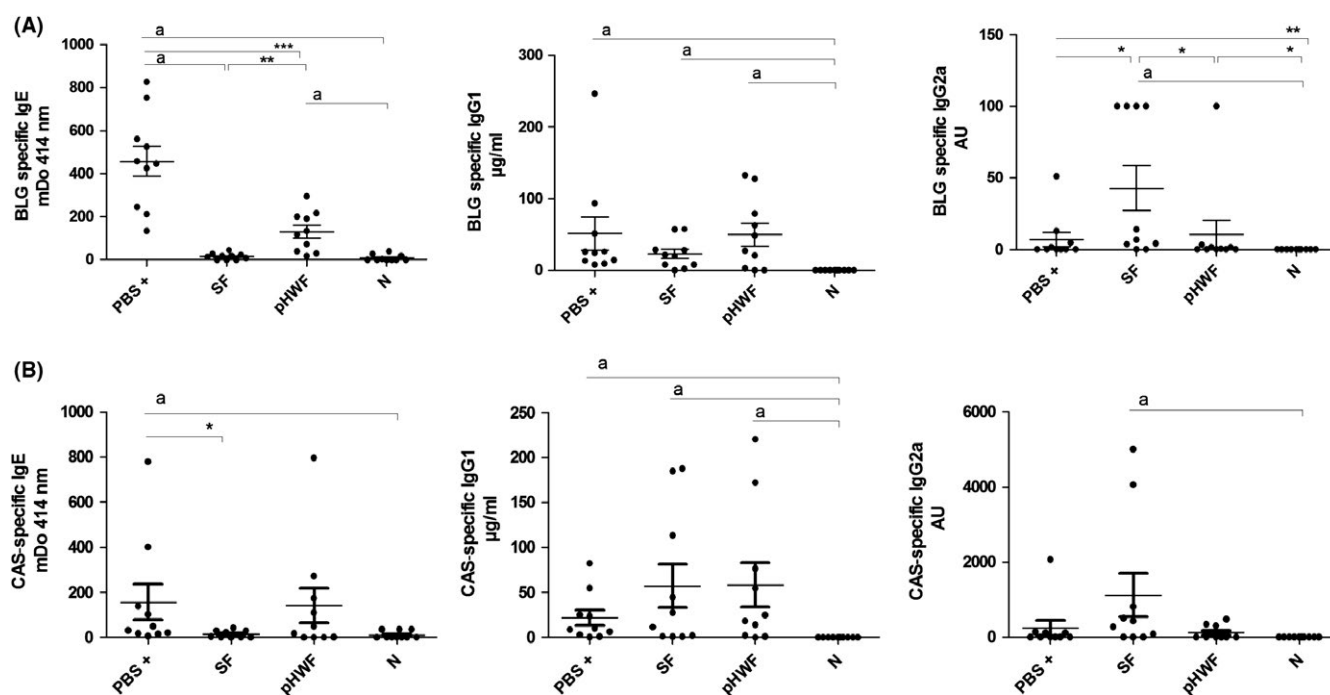


FIGURE 2 Specific IgE, IgG1, and IgG2a antibody responses to β -lactoglobulin (BLG; A) and to casein (CAS; B) at the end of the sensitization phase (day 56) in the different groups of mice. Results obtained on individual plasma from 10 mice/group and the mean for each group are shown. The results are reported as absorbance units at 414 nm (mAU414nm) for IgE, μ g/mL for IgG1, and arbitrary units (AU) for IgG2a. "a" indicates a statistically significant difference between groups using non-parametric Kruskal-Wallis and Dunn's multiple comparison tests comparing all the groups together ($P < 0.05$). Additional non-parametric Mann-Whitney t tests were performed between two specific groups, and corresponding significant P -values are specified (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$)

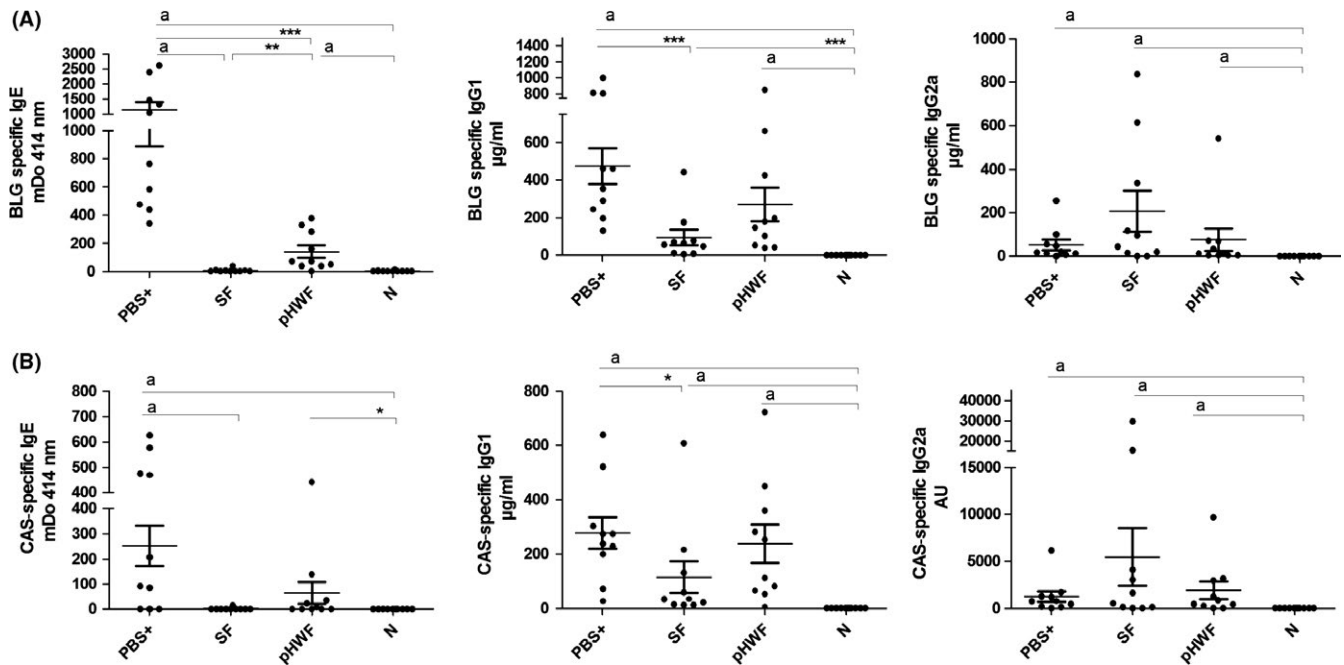


FIGURE 3 Specific IgE, IgG1, and IgG2a antibody responses to β -lactoglobulin (BLG; A) and to casein (CAS; B) after the chronic exposure to CM (day 144; see Figures 1 and 2 for groups, representation of the results, and statistical analysis)

To evaluate the long-term effect of the different treatments, the mice were further chronically exposed to CM by gavage with 200 μ L of whole CM on days 68, 69, 125, 132, and 139. On day 144, an OFC was performed as before. Mice were sacrificed 24 hours after the last challenge, and spleens were removed under sterile conditions for ex vivo reactivation.

2.4 | Assessment of sensitization: assays of BLG- and CAS-specific IgE, IgG1, and IgG2a

Quantitative or relative measurements of BLG- and CAS-specific IgE, IgG1, and IgG2a antibodies were performed on individual plasma, as previously described (Refs¹⁹; see Appendix S1).

2.5 | Assessment of allergic reaction intensity: measurement of serum mMCP-1

The concentration of murine mast cell protease-1 (mMCP-1) was determined in plasma using a commercial kit following the manufacturer's recommendations (mMCP-1 ELISA Ready-SET-Go!TM; eBioscienceTM, Fisher Scientific, Illkirch, France).

2.6 | Ex vivo specific reactivation of spleen for analyses of cytokines, GATA3+ Th2 cells, and Foxp3+ Treg cells

Spleens of two mice from the same experimental group were pooled ($n = 5$ /group) and specifically reactivated ex vivo using

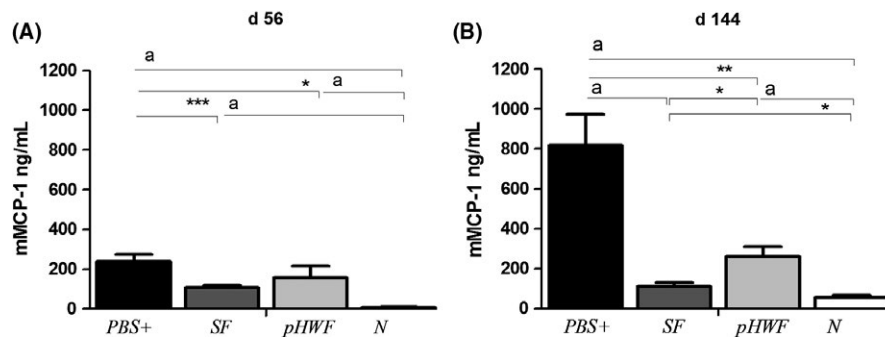


FIGURE 4 Elicitation of the allergic reaction in the different groups of mice. mMCP-1 concentrations (ng/mL) were individually assayed in plasma collected 3 h after an oral food challenge with 20 mg of CMP performed after the initial sensitization phase (day 56; A) and after chronic CM exposure (day 144; B) in mice pre-treated with PBS (black bar), SF (dark gray bar), or pHWF (light gray bar). Untreated, non-sensitized but challenged mice (N) are shown as clear bars. Results are expressed as mean values + SEM of the 10 mice/group. "a" indicates a statistically significant difference between groups using non-parametric Kruskal-Wallis and Dunn's multiple comparison tests comparing all the groups together ($P < 0.05$). Additional non-parametric Mann-Whitney t tests were performed between two groups (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$)

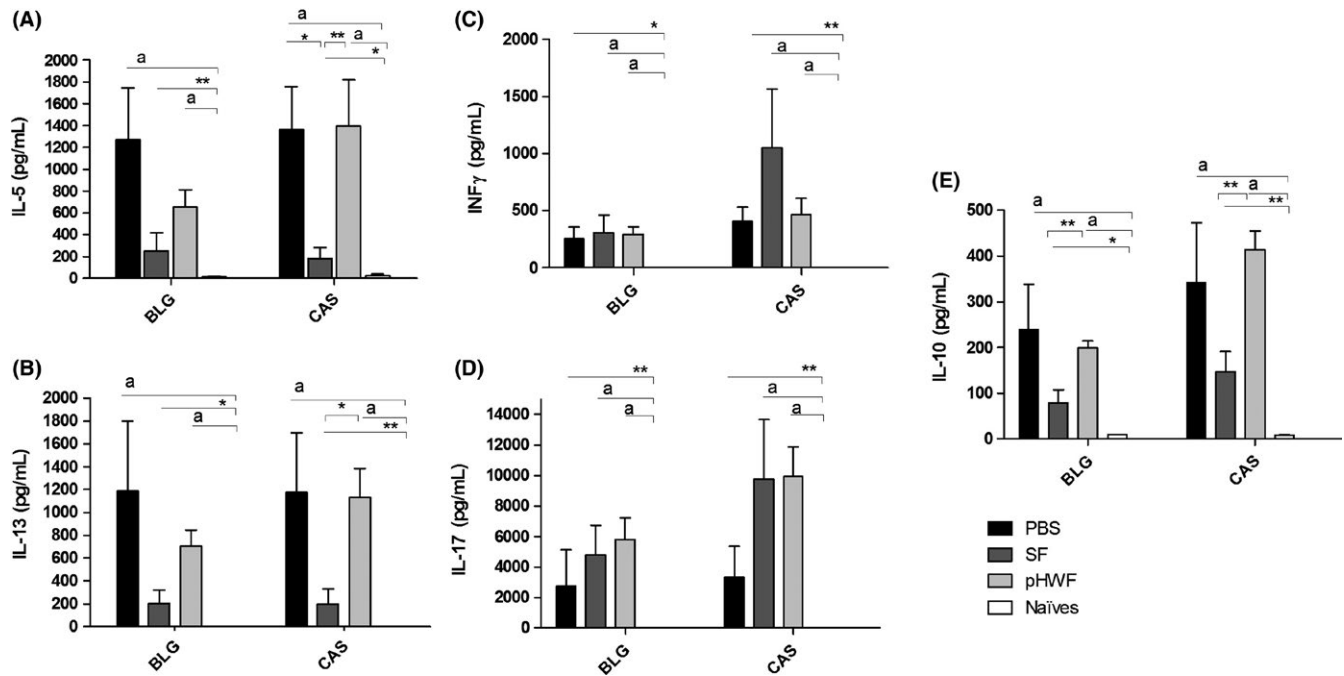


FIGURE 5 Cytokine secretion by splenocytes cultured ex vivo with BLG or CAS. Spleen cells from the different groups of mice were isolated on day 145 (see Figure 1) and reactivated ex vivo with BLG and CAS for 72 h. Mice were pre-treated with PBS (black bar), SF (dark gray bar), or pHWF (light gray bar). Untreated, non-sensitized but challenged mice (N) are shown as clear bars. Controls were performed using media alone (negative control) or mitogenic restimulation with ConA (positive control, not shown). IL-5 (A), IL-13 (B), IFN- γ (C), IL-17 (D), and IL-10 (E) were quantified on supernatants using mouse commercial kits. "a" indicates a statistically significant difference between groups using non-parametric Kruskal-Wallis and Dunn's multiple comparison tests comparing all the groups together ($P < 0.05$). Additional non-parametric Mann-Whitney t tests were performed between two groups (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$)

BLG or CAS, as explained in Appendix S1. After incubation, the plates were centrifuged ($300 \times g$, 10 min, $+4^{\circ}\text{C}$), and supernatants were collected and stored at -80°C until cytokine assay. Cell pellets were collected in PBS, 1% BSA, and 1 mmol/L EDTA for flow cytometry analysis.

2.6.1 | Cytokine analysis

IL-5, IL-13, IL-17, IFN- γ , and IL-10 were assayed in supernatants collected after ex vivo reactivation of splenocytes using Luminex xMAP® technology. Kit and apparatus from Bio-Rad (Hercules, CA, USA) were used, following the manufacturer's recommendations.

2.6.2 | Flow cytometry analysis of GATA3⁺ Th2 cells and Foxp3⁺ Treg cells

After blocking of Fc receptors (anti-CD16/CD32, Clone 2.4G2; BD Pharmingen, Le Pont de Claix, France), T cells were labeled using the following anti-mouse antibodies: anti-CD4 (Clone GK1.5, PE-Cy7; eBioscience), anti-CD25 (Clone PC61, Brilliant Violet 510; Biolegend, San Diego, CA, USA), anti-GATA3 (Clone 16E10A23, Alexa Fluor 647; Biolegend), and anti-Foxp3 (PE; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Intracellular staining of transcription factors was performed after fixation/permeabilization (Foxp3 staining kit; Miltenyi Biotec). Cells were acquired using a NovoCyte flow cytometer (ACEA Biosciences, Inc, San

Diego, CA, USA), and analyses were done using NovoExpress™ software (version 1.0.0; ACEA Biosciences, Inc). Samples were first inspected in all light-scatter patterns and fluorescence channels to confirm quality, and abnormal population were excluded. Unlabeled processed samples and fluorescence-minus-one (FMO) strategies were used for reporting the percentage of positive cells.

2.7 | Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.01 software (San Diego, CA, USA). As the data were not normally distributed, we used the non-parametric Kruskal-Wallis test and Dunn's post-test to compare all the groups together. This test integrates a correction due to multiple testing. For the apparent differences between two specific groups that were not significant using this robust test, we performed additional non-parametric Mann-Whitney tests, then taking into account only the two corresponding groups of interest independently of the others. A P -value < 0.05 was considered significant.

3 | RESULTS

3.1 | Effect of oral administration of SF and pHWF on sensitization to CM

Allergic sensitization to CM was assessed by measuring concentrations of BLG- and CAS-specific antibodies in individual plasma collected

after an OFC performed either after the initial phase of sensitization (day 56; Figure 2) or after a chronic exposure to CM (day 144; Figure 3). No specific IgE, IgG1, or IgG2a were detectable in mice receiving CT alone during the sensitization phase (PBS-, data not shown) or in naïve mice. On the other hand, mice pre-treated with PBS and then exposed to CM and CT (PBS+) demonstrated high levels of BLG- and CAS-specific IgE and IgG1 after the sensitization phase, which were further increased after CM chronic exposure. A concomitant significant production of BLG- and CAS-specific IgG2a antibodies was also observed after the chronic exposure.

Intragastric pre-treatment of mice with standard infant formula totally prevented further induction of BLG- or CAS-specific IgE (SF group; Figure 2), which was sustained even after chronic exposure to CM (Figure 3). A significant decrease in the BLG- and CAS-specific IgG1 production was also observed after the chronic exposure to CM in the SF group compared to the PBS+ group. Conversely, BLG-specific IgG2a increased compared to the PBS+ group on day 56 (Figure 2), but this was no longer evident on day 144 (Figure 3).

BLG-specific IgE were significantly induced in mice pre-treated with pHWF compared to naïve mice, but were significantly reduced compared to the PBS+ mice at both time points analyzed (Figures 2A and 3A). Although the CAS-specific IgE concentration mean value seemed to be lower in the pHWF pre-treatment group compared to the PBS+ group after the chronic exposure, this was not significant (Figure 3B). The pHWF pre-treatment had no effect on the concentrations of BLG- and CAS-specific IgG1 and IgG2a antibodies.

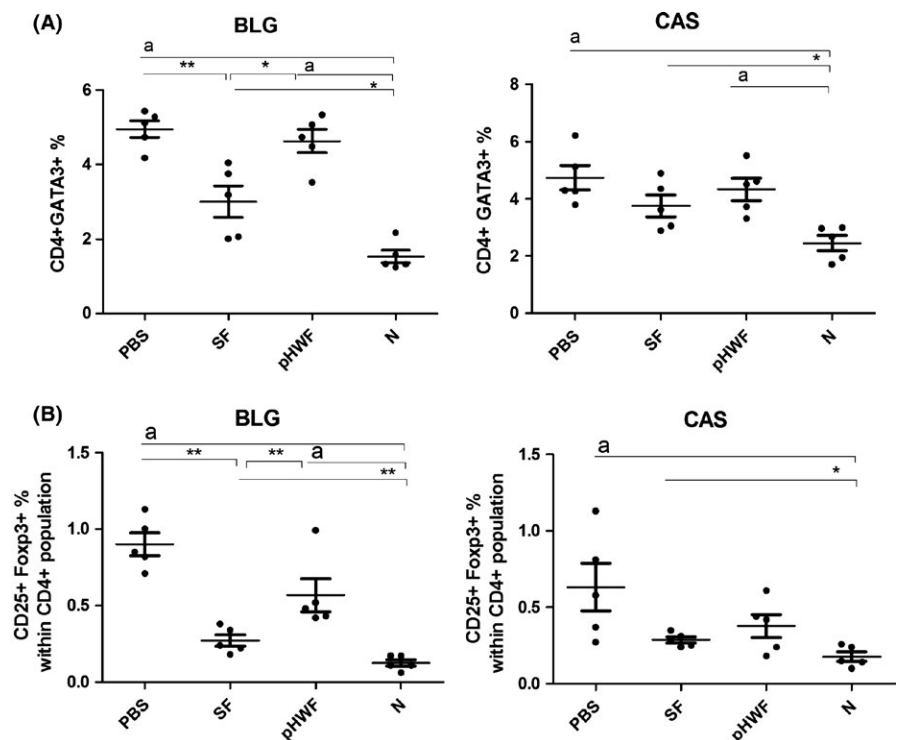
3.2 | Effect of oral administration of SF and pHWF on the allergic reaction

The elicitation of the allergic reaction was assessed by measuring the mMCP-1 concentrations in plasma obtained after the OFC with CM proteins. No mMCP-1 was observed in naïve (Figure 4) nor in PBS- (not shown) challenged mice. Conversely, a significant increase in mMCP-1 concentration was observed in the PBS+ group after the challenge performed after the sensitization phase (day 56; Figure 4A), which was further enhanced when challenge was performed after the chronic exposure to CM (day 144; Figure 4B). This allergic reaction was significantly and highly reduced at both time points in the SF and pHWF mice compared to the PBS+ mice. However, the preventive effect of pHWF was less efficient and persistent than that of SF.

3.3 | Pre-treatment with pHWF reduces the BLG-specific cellular responses in the spleen but not CAS-specific response

No secretion of Th2 (IL-5, IL-13), Th1 (IFN- γ), and Th17 (IL-17) cytokines, nor of IL-10, were observed after culture of splenocytes from naïve (Figure 5) or PBS- mice (not shown) with BLG or CAS. Conversely, splenocytes from the PBS+ mice cultured ex vivo with BLG or CAS produced high levels of IL-5 (Figure 5A) and IL-13 (Figure 5B), associated with IFN- γ (Figure 5C), IL-17 (Figure 5D), and IL-10 (Figure 5E). Th2 cytokine production was significantly and highly reduced in the group of mice pre-treated with SF. A lower mean concentration of IL-5 and IL-13 was noticed in splenocyte

FIGURE 6 GATA3+ and CD25+Foxp3+ regulatory T cell frequency within CD4+ cells in spleen cells obtained after reactivation using BLG or CAS. GATA3+ and CD25+Foxp3+ cells within the CD4+ population were assessed by flow cytometry on spleen cell pellets from the different groups of mice isolated on day 145 (see Figure 1) and reactivated ex vivo with BLG and CAS for 72 h as in Figure 5. "a" indicates a statistically significant difference between groups using non-parametric Kruskal-Wallis and Dunn's multiple comparison tests comparing all the groups together ($P < 0.05$). Additional non-parametric Mann-Whitney t tests were performed between two groups (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$)



supernatants from the pHWF mice after BLG restimulation, but not after CAS reactivation; however, this was not statistically significant (Figure 5A,B). Th1 and Th17 cytokine secretion was comparable between the SF, pHWF, and PBS+ mice. It is worth noting that the production of IL-10 paralleled that of Th2 cytokines (Figure 5E).

3.4 | CD4+GATA3+ and CD4+CD25+Foxp3+ Treg cell frequency in reactivated spleen cell culture

A higher frequency of CD4+GATA3+ cells was observed in spleen culture from the PBS+ mice compared to that from naïve mice, after both BLG and CAS reactivation (Figure 6A). The same was observed in pHWF-treated mice, but not in SF-treated mice, thus suggesting a lower frequency of Th2 memory cells in the SF mice compared to the PBS+ and pHWF mice. CD4+CD25+Foxp3+ Treg cells were more frequent in reactivated spleen cells from the PBS+ and pHWF mice, notably after BLG reactivation (Figure 6B). A lower frequency of CD4+CD25+Foxp3+ Treg cells was noticed in the SF mice, notably after BLG reactivation.

4 | DISCUSSION

A mouse model of experimental sensitization to foods, especially when using a Th2-biased strain mimicking atopic patients and the oral sensitization route, represents a good tool to investigate induction of oral tolerance to food proteins. In the present study, we used such a model to assess the tolerizing capacity of partially hydrolyzed cow's milk proteins from whey compared to that of a standard non-hydrolyzed cow's milk formula.

First, and in line with our previous results,^{15,20} we demonstrated the high sensitization levels to BLG and CAS in BALB/c mice after oral administration of CM with CT that also manifest in a local allergic reaction after an OFC with CMP. Moreover, we now observed that chronic exposure to CM allowed a further significant increase in both the sensitization levels and the intensity of the allergic reaction observed after an OFC, but without inducing severe systemic reactions or chronic symptoms that would present ethical issues.

Using this model, we demonstrated that oral administration of SF is efficient in preventing CMA. Actually, SF pre-administration totally inhibits both sensitization, that is, specific IgE and IgG1 antibody production and Th2 cell activation (as evidenced by reduced Th2 cytokine secretion and lower frequency of CD4+GATA3+ cells in the spleen after specific reactivation), and elicitation of the allergic reaction. A transient increase in the concentration of Th1-associated specific IgG2a antibodies was observed on day 56, but this was not sustained on day 144 nor translated into a significant increase in IFN- γ secretion by reactivated splenocytes. No increase in Th17 cytokine was noticed in these mice. Then, Th2 repression induced by gavage with SF was not related to imbalanced immune response toward Th1/Th17 immune response. On the other hand, no significant increase in CD4+CD25+Foxp3+ Treg cells was evidenced at the end of the protocol after specific reactivation of spleen cells. The results of the present study are therefore not in line with the previous ones,¹⁶ including ours.¹⁵ To preclude the

involvement of Treg cells in the preventive effect observed after SF gavage in the present work, additional markers (eg, LAP or intracellular IL-10, combined Foxp3 and ROR γ t labeling) should have been included.^{21,22} Other time points and sites, notably mucosal site such as small intestine lamina propria, should also be further investigated.

Interestingly, oral administration of partially hydrolyzed whey to mice prior to oral sensitization with whole CM only induced a partial reduction of oral sensitization to BLG and a far less significant one to CAS. Partial OT to BLG can then be induced by large whey fragments present in pHWF, as previously demonstrated in mice using large fragments generated by chemical hydrolysis of BLG,¹⁴ or in a rat model.²³ As previously noticed, the efficiency of the OT seems to correlate with the size of antigenic polypeptides presented to the immune system, with too small peptides as found in extensively hydrolyzed whey formulas sustaining no more tolerogenicity or even immunogenicity.^{14,24} Accordingly, inhibition of IgE and IgG1 responses to three whey allergens (BLG, α -lactalbumin, and BSA) can all be induced after feeding a cow's milk formula for 1 week or a partially hydrolyzed formula for 4 weeks, but not after feeding an extensively hydrolyzed formula.²⁴ Although this may suggest that longer feeding with pHWF would have been more efficient in our model, our protocol allowed to highlight the differences in the tolerating potential of partial hydrolysates vs undigested proteins. Interestingly, the whey hydrolysate does not contain relevant levels of CAS-derived proteins or polypeptides (estimated around 0.8% of total proteins in the pHWF used, as assessed using an in-house competitive ELISA;¹⁸ not shown), and we evidenced that specific immune tolerance cannot develop efficiently against these proteins. These findings thus suggest that whole CM hydrolysates would be more efficient than CAS or whey hydrolysate alone to efficiently prevent CMA. It is worth noting, however, that a significant decrease in the elicitation of the allergic reaction induced by CM proteins was evidenced in pHWF-treated mice, despite the fact that CM contains 80% of caseins and only 20% of whey proteins (in which 50% is BLG).

In conclusion, we evidenced that the pre-treatment with a partially hydrolyzed whey formula has only a partial preventive effect on CMA in a Th2-biased strain mice and this effect is restrained to protein contained in the administered fraction. Our results also suggest that the use of partially hydrolyzed formulas obtained from whole CM would be more efficient than CAS or whey hydrolysate to prevent CMA.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION

KAP, AC, NCP, and HB performed the experiments. KAP and AC designed the study, interpreted the data, and drafted the manuscript.

KAP, KEM, OK, and DS corrected the manuscript. KAP, KEM, and DS revised the manuscript. All authors approved the version to be published.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary file

Materials and Methods

Animals

Mice were housed in filtered cages under normal Specified Pathogen-Free husbandry conditions (autoclaved bedding and sterile water). They acclimated for one week before experiments. They were maintained on a diet deprived of animal proteins and in which BLG was not detected using specific sandwich immunoassays.⁽⁴⁷¹⁸⁾ All animal experiments were performed according to the European Community rules of animal care, with permission 91-368 of the French Veterinary Services and were covered by agreement No. 2009-DSV-074 (October 29, 2009) from the Veterinary Inspection Department of Essonne (France). The protocol was reviewed by our internal animal experimentation ethics committee (Committee C2EA - 44 CETEA – CEA DSV IdF, created and registered on the 6th of June, 2011 by the French Ministry of Higher Education and Research).

Assays of BLG- and CAS-specific IgE, IgG1 and IgG2a

The humoral response was assessed by quantitative or relative measurements of BLG and CAS-specific IgE, IgG1 and IgG2a antibodies on individual sera. For specific IgG1 and IgG2a, anti-BLG or anti-CAS monoclonal antibodies that were produced, characterized and standardized in the lab were used as a reference (ng/ml) when available, or a dilution curve performed using a pool of serum from mice hyper-immunized to CM was used as an internal reference (Arbitrary Unit). For the specific-IgE assay, a reverse format was used, allowing to avoid the inference of specific IgG in the assay. In this case, 96-well microtitre plates (Maxisorp Nunc) were coated with anti-IgE antibody (anti-mouse IgE, clone LO-ME-3, SEROTEC, Clinisciences, France), and purified BLG or CAS, labelled with acetylcholinesterase (AChE), were used as tracers (20).

Ex vivo reactivation of splenocytes

After spleen dilaceration and lysis of red blood cells (180 mM NH₄Cl, 17 mM Na₂EDTA), splenocytes were washed and finally suspended in RPMIc (RPMI medium supplemented with 10% fetal calf serum, 2mM L-glutamin, 100U penicillin, 100 µg/mL streptomycin). Cells were incubated for 72 hours at 37°C (5% CO₂) in 96-well culture plates (106 cells/well) in the presence of BLG (20 µg/mL), CAS (20 µg/mL), Concanavalin A (1 µg/ml, positive control) or PBS (negative control).

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