

ORIGINAL ARTICLE

Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3⁺ regulatory T-cells in the mesenteric lymph nodes

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Keywords

oral tolerance; hypoallergenic formulas; cow's milk allergy; partial whey hydrolyzates; extensive whey hydrolyzates; food allergy; avoidance, prevention; Foxp3⁺ regulatory T-cells; transfer.

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Abstract

Background: Hypoallergenic formulas are considered a good option for infants at risk for cow's milk allergy. The aim of this animal study was to investigate whether whey hydrolyzates (WH) have the capacity to induce oral tolerance to whey.

Methods: Whey, partial or extensive WH was given via gavages to naïve mice prior to oral whey sensitization using cholera toxin as an adjuvant. The acute allergic skin response, mouse mast cell protease-1 (mMCP-1), whey-specific IgE, IgG₁ and effector Th2-cells, Th1-cells, and Foxp3⁺ regulatory T-cells were determined in the mesenteric lymph nodes (MLN). MLN cells from tolerized mice were adoptively transferred to naïve recipient mice prior to whey sensitization.

Results: In contrast to the extensive WH, pre-treatment of naïve mice with whey or partial WH reduced the acute allergic skin response and mast cell degranulation after whey challenge. However, only treatment with whey prevented the generation of serum-specific IgE/IgG₁. In partial WH tolerized mice, Foxp3⁺ regulatory T-cell numbers in the MLN were increased compared to whey-sensitized mice. Both whey and partial WH treatment showed a tendency toward a decreased number of effector Th2-cells. Transfer of MLN cells from tolerized mice protected recipient mice from developing an acute allergic skin response.

Conclusion: These results show that partial WH with limited sensitizing properties reduced the effector response upon whey challenge. This effect is transferable using MLN cells and was associated with enhanced Foxp3⁺ regulatory T-cell numbers in the MLN. Partial WH retained the capacity to induce active immune suppression in mice which may be relevant for allergy prevention.

Prevention of food allergy deserves high priority either by avoidance of the allergen (e.g., epitope exclusion) or the induction of oral tolerance. Oral tolerance enables the mucosal immune system to remain quiescent when harmless proteins such as food allergens enter the intestine while a protective inflammatory response is generated against potential pathogens. Oral tolerance induction most likely results from the deletion of antigen-specific Th2-cells as well as active suppression by regulatory T-cells that encompass natural or the novo-generated CD4⁺CD25⁺Foxp3⁺ and TGF- β - and IL-10-producing regulatory T-cells (1–4). Human data showed that regulatory T-cells were associated with tolerance

induction (5, 6). Moreover, children born with dysfunctional Foxp3, a key gene for development of CD25⁺ regulatory T-cells, develop severe dermatitis with high levels of IgE and occasionally eosinophilia which suggest a suppressive role for Foxp3⁺ regulatory T-cells in allergic immune reactions in humans. Hypoallergenic formulas are commonly used to manage cow's milk allergy and are generally categorized into partial and extensive hypoallergenic formulas based on the degree of hydrolysis and consequently the length of the remaining peptides. Several clinical trials show the effectiveness of partial whey hypoallergenic formulas in reducing clinical symptoms of allergy in high-risk children (7–11).

However, these studies are limited in answering whether the beneficial effects are owing to avoidance of the allergic epitope or a result of oral tolerance induction. Animal models using oral sensitization represent a good tool to investigate oral tolerance to food proteins (12–15). However, the tolerizing capacity of whey hydrolyzates (WH) and its underlying mechanism has not been investigated in animal models using oral sensitization so far. In the current study, the tolerizing capacity of a partial WH and extensive WH was assessed in mice using a mouse model of orally induced cow's milk allergy. Moreover, the number of Foxp3⁺ regulatory T-cells was determined and the contribution of the mesenteric lymph nodes (MLN) cells in the transfer of tolerance was investigated. This study implies that the partial and not the extensive WH possess the putative capacity to induce clinical tolerance to whey in mice and that Foxp3⁺ regulatory T-cells from the MLN may contribute to this effect.

Material and methods

Cow's milk proteins and hydrolyzates

Whey was obtained from DMV International, Veghel, The Netherlands. A partial whey hydrolyzate (WH) was manufactured at Danone Research Centre for Specialised Nutrition by enzymatic hydrolysis under specified conditions. The enzymatic process was stopped by fast cooling. The partial WH was further characterized by analysis of the peptide size (85% <1 kD, 8% <2 kD, 4% <5 kD, 1% <10 kD, 0.6% <20 kD, and 1.4% >20 kD) by means of high-pressure liquid chromatography. Subsequently, filtering of the partial WH using a filter with a cutoff of 5 kD resulted in an extensive WH. These experimental WH were used in all animal studies as mentioned later.

Reagents and antibodies

Cholera toxin is purchased from Quadrantech Diagnostics, Epsom, UK. Biotin-labeled rat anti-mouse IgE, IgG₁ and perCp-conjugated anti-CD4, FITC-conjugated-CD4 (L3T4), PE-conjugated CD25, and isotype control mAb were from Pharmingen, Alphen a/d Rijn, The Netherlands. APC-conjugated Foxp3, PE-conjugated CD69, FITC-conjugated T1St2, FITC-conjugated CD69, PE-conjugated CXCR3, and isotype controls were obtained from eBioscience, San Diego, CA, USA. All other chemicals were obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands.

Mice

Three- to 4-week-old pathogen-free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maastricht, The Netherlands), maintained on cow's milk protein free standard mouse chow (AIN-93G soja; Special Diets Services, Wijk bij Duurstede, The Netherlands) and housed in the animal facility at the Utrecht University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

Oral tolerance induction, oral sensitization, and challenge of mice

Prior to whey sensitization, mice were treated orally (daily; day 7 until day 2) with 50 mg of partial or extensive WH or whey (positive control for oral tolerance induction) or PBS (sham), using a blunt needle. Subsequently, mice were sensitized orally on day 0, 7, 14, 21, and 28 with 20 mg of whey per mouse homogenized in PBS (0.5 ml; Cambrex Bio Science, Verviers, Belgium) mixed with 10 µg cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin in PBS only. One week after the last sensitization, the acute allergic skin response (ear swelling at 1 h) after intra dermal whey challenge was measured. Mice were challenged orally with 0.5 ml of whey (200 mg/ml PBS), and 18 h later, blood samples were collected, centrifuged for 15 min at 20,000 g and stored at -70°C (see Table 1 and flow chart in Fig. 1).

Acute allergic skin response

An acute allergen-specific ear swelling in whey-sensitized mice was determined at 1 h after intra dermal challenge with 10 µg total whey protein in the ear pinnae. As a negative control, non-sensitized mice were challenged in the ear with similar whey protein. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling owing to local injection in the non-sensitized mice. The ear swelling is expressed as delta micrometer.

Measurement of serum-specific antibodies and mMCP-1

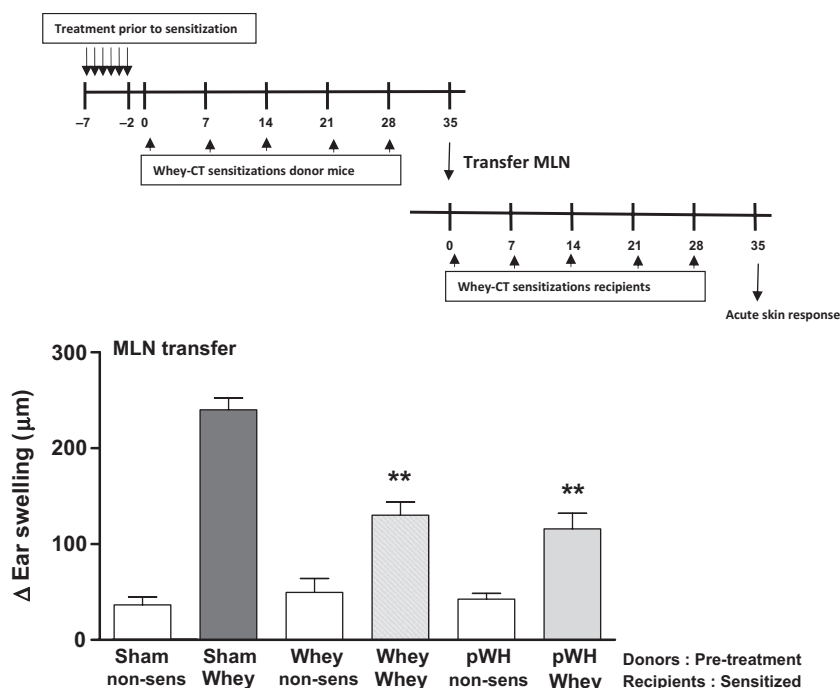
Whey-specific IgE and IgG₁ levels were measured in serum by means of ELISA. Microton plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 20 µg of whey in coating buffer (Sigma) for 18 h at 4°C. Plates were washed and blocked for 1 h with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl/0.05% Tween, and 0.5% BSA (Sigma-Aldrich, Zwijndrecht, The Netherlands). Serum samples were incubated for 2 h at room temperature. Plates were washed and incubated with 1 µg biotin-labeled rat anti-mouse IgE or IgG₁ (Pharmingen) for 1 h at room temperature. After washing, the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, The Netherlands) for 1 h, washed and developed with o-phenyldiamine (Sigma). The reaction was stopped after 10 min with 4 M

Table 1 Treatment groups

Group	Pre-treatment	Sensitization	Challenge
Non-sens	PBS	Cholera toxin in PBS	Whey
CNTR	PBS	Whey-cholera toxin	Whey
Whey	Whey	Whey-cholera toxin	Whey
Partial WH	Partial WH	Whey-cholera toxin	Whey
Extensive WH	Extensive WH	Whey-cholera toxin	Whey

WH, whey hydrolyzates.

Figure 1 Adoptive transfer of mesenteric lymph nodes (MLN) cells of both whey or partial (p) WH pre-treated sensitized donor mice significantly reduced the acute allergic skin response in whey-sensitized recipient mice when compared to recipient mice receiving sham-treated MLN cells ($p < 0.01$). Donor mice were pre-treated with PBS (sham), whey, or pWH prior to whey sensitization using cholera toxin as an adjuvant. MLN cells from donor mice were injected in recipient mice and subsequently sensitized to whey using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only. WH, whey hydrolyzates.



H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, The Netherlands), and results were expressed as arbitrary units (AU). Serum concentrations of mouse mast cell protease-1 (mMCP-1) were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific Ltd., Midlothian, UK).

Flow cytometer analysis of regulatory T-cells and effector Th1- and Th2-cells

Mesenteric lymph nodes were removed and cut into small pieces and incubated with 0.2% collagenase IV and 2 kU/ml DNase 1 (Roche Diagnostics, Almere, The Netherlands) for 30 min at 37°C, resuspended, and incubated for another 30 min. The enzymatic reaction was stopped by adding 50 μl FCS. Single-cell suspensions (5×10^5) of MLN were blocked with PBS containing 5% FCS, 1% BSA, and subsequently incubated for 20 min with either mAb against CD4 and CD25 for regulatory T-cells, mAb against the activation marker CD69 and T1ST2 for the detection of effector Th1-cells, mAb against CD69 and CXCR3 for the detection of effector Th1-cells or isotype controls (Pharmingen). For detection of Foxp3 (FJK-16), cells were permeabilized and incubated with anti-Foxp3 according to the manufacturer's protocol (eBioscience). Stained cells were analyzed using a BD FACSCalibur flow cytometer.

Adoptive transfer experiments

Oral treatment with partial WH or total non-hydrolyzed whey protected the mice from developing an allergic effector response. Regulatory cells generated in the MLN may be responsible for the transfer from local (gut associated)

toward systemic tolerance. To test whether tolerance could be transferred using MLN cells, an adoptive transfer experiment was performed. MLN were obtained from donor mice that were whey, partial WH or sham treated prior to sensitization. Single-cell MLN suspensions were prepared using cell strainers and three different groups (sham, whey, and partial WH) were pooled ($n = 12$ per group). Single-cell suspensions were injected intravenously (100 μl , 4×10^6 cells per mouse) in six different groups of recipient mice ($n = 6$ per group). The recipient mice were non-sensitized or whey sensitized as described earlier without prior pre-exposure to whey or partial WH. The acute allergic skin reaction was measured to determine allergic sensitization (see Fig. 1).

Statistics

All data were analyzed using one-way ANOVA and *post hoc* Dunnett's test. Whey-specific antibodies were analyzed using Kruskal–Wallis as variances differed between groups. A probability value $p < 0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism software. Data are represented as mean $\pm$ s.e.m. of 12 mice per group. Data except for activated T-cell numbers $n = 6$ were shown as mean $\pm$ s.e.m. from $n = 2$ independent experiments.

Results

Reduced whey-specific antibodies in mice orally exposed to whey prior to whey sensitization

In whey-sensitized mice (CNTR), whey-specific IgE and IgG₁ levels were augmented in serum when compared to non-sensitized mice (Fig. 2, $p < 0.05$). No inhibitory effect on IgE or

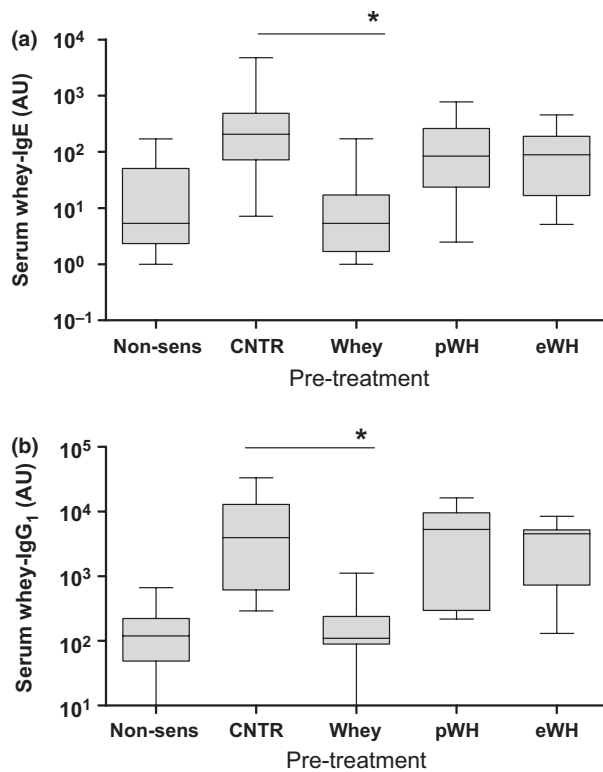


Figure 2 Reduced whey-specific serum IgE (a) and IgG₁ (b) concentrations in whey pre-treated sensitized mice compared to control (CNTR)-sensitized mice ($p < 0.050$). No effect of the partial (p)WH or extensive (e)WH on serum IgE/IgG₁ concentrations was observed. Mice were treated with PBS (CNTR) or 50 mg whey, pWH, or eWH prior to whey sensitization using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only. Data were shown as mean $\pm$ s.e.m. from $n = 2$ independent experiments. WH, whey hydrolyzates.

IgG₁ levels was observed when mice were pre-treated with the partial WH before sensitization indicating that sensitization to whey was not completely prevented, the extensive WH showed similar results (Fig. 2). As expected, pre-treatment of mice with whey prior to whey sensitization prevented the induction of whey-specific IgE ($p < 0.05$) and IgG₁ ($p < 0.01$).

Reduced acute skin response in whey and partial WH-treated mice

To study whether oral administration of whey, partial, or extensive WH prior to sensitization induced systemic tolerance at the effector level, the acute allergen-specific skin response was measured in mice after intra dermal whey challenge. In whey-sensitized control mice, ear challenge with whey induced a significant ear swelling at 1 h compared to non-sensitized mice (Fig. 3; 120.9 ± 12.4 vs. 25.4 ± 6.8 μ m; $p < 0.01$). Interestingly, administration of the partial WH prior to sensitization reduced the acute allergic skin response

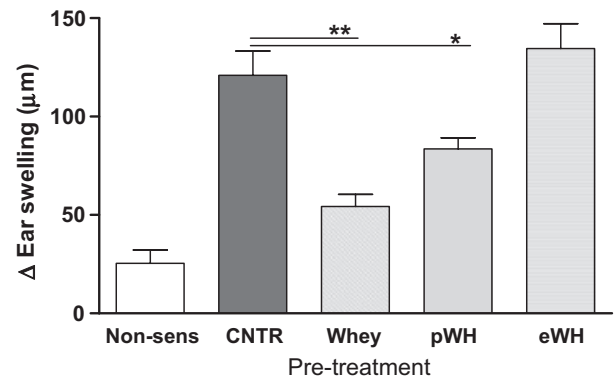


Figure 3 Reduced acute allergic skin responses in whey ($p < 0.01$) or partial (p)WH ($p < 0.050$) pre-treated sensitized mice compared to control (CNTR)-sensitized mice. No effect of the extensive (e)WH on the acute allergic skin response was observed. Mice were treated with PBS (CNTR) or 50 mg whey, pWH, or eWH prior to whey sensitization using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only. Data were shown as mean $\pm$ s.e.m. from $n = 2$ independent experiments. WH, whey hydrolyzates.

(83.6 ± 5.6 μ m; $p < 0.05$) as well although the induction of whey-specific IgE was not significantly reduced. Administration of an extensive WH prior to sensitization did not affect the acute ear swelling response (134.6 ± 12.55 μ m) compared to control whey-sensitized mice (Fig. 3). As expected by the absence of detectable serum levels of whey-specific IgE or IgG₁, administration of whey prior to oral sensitization showed a significant reduction in the acute ear swelling compared to whey-sensitized control mice (54.3 ± 6.1 μ m; $p < 0.01$).

Reduced serum mMCP-1 after oral challenge in whey and partial WH-treated mice

To assess whether mucosal mast cell degranulation upon oral challenge was affected, mMCP-1 serum concentrations were determined. Administration of whey or partial WH prior to sensitization significantly reduced mMCP-1 concentrations (24.7 ± 5.6 and 35.1 ± 6.2 ng/ml respectively) compared to whey-sensitized control mice (69.8 ± 13.4 ng/ml) ($p < 0.05$, Fig. 4). Pre-treatment of mice with the extensive WH did not affect the serum mMCP-1 concentration (67.6 ± 16.3 ng/ml) compared to control-treated, whey-sensitized mice.

Number of regulatory T-cell, effector Th2- and Th1-cells in the MLN

To investigate whether active Th2-cell suppression might underlie the observed protective effect of whey and the partial WH, the number of Foxp3⁺ regulatory T-cells and effector Th2-cells were determined in the MLN. Oral administration of the partial WH prior to sensitization increased the relative numbers of CD4⁺CD25⁺Foxp3⁺ regulatory T-cells compared to whey-sensitized control mice

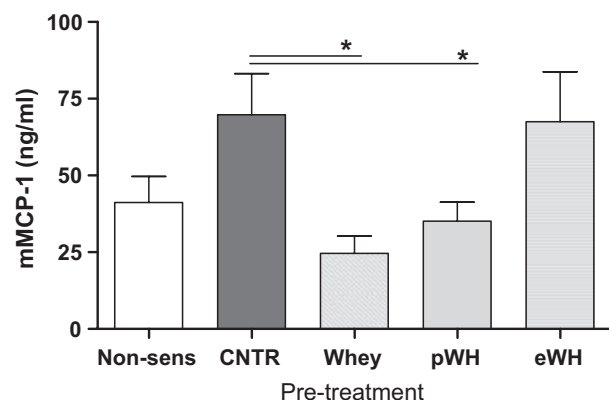


Figure 4 Reduced serum mMCP-1 serum concentration was observed in whey or partial (p)WH pre-treated sensitized mice compared to control (CNTR)-sensitized mice ($p < 0.050$). No effect of extensive WH (eWH) on mMCP-1 was observed. Mice were treated with PBS (CNTR) or 50 mg whey, pWH, or eWH prior to whey sensitization using cholera toxin as an adjuvant. Non-sensitized mice received cholera toxin only. Data were shown as mean $\pm$ s.e.m. from $n = 2$ independent experiments. WH, whey hydrolyzates.

(Fig. 5a; 4.2 ± 1.1 vs. 3.1 ± 0.5 ; $p < 0.05$). The numbers of regulatory T-cells was not increased in whey pre-treated mice. However, a tendency toward increased numbers of effector Th1-cells compared to whey-sensitized control mice was observed in the latter group indicating that these cells might contribute to the observed protective effects of whey (Fig. 5b; $p = 0.10$). Both whey and pWH treatment prior to whey sensitization showed a trend in reducing the number of activated Th2-cell after oral whey challenge (Fig. 5c; $p = 0.092$ for both). No effect on T-cells numbers was observed in extensive WH pre-treated mice (Fig. 5).

Adoptive transfer of MLN

Mesenteric lymph nodes single-cell suspensions from whey or partial whey tolerized (donor) mice were injected into recipient mice prior to PBS (non-sensitized) or whey sensitization (see scheme Fig. 1). As expected, an acute allergic skin response was measured in recipient mice upon whey challenge when mice received MLN cells from control donor mice (Fig. 1, $p < 0.01$). Recipient mice transferred with MLN cells of donor mice tolerized with whey or partial WH prior to whey sensitization developed a reduced acute allergic skin response compared to recipients transferred with control MLN cells (Fig. 1, $p < 0.01$). These results indicate that regulatory mechanisms generated in the MLN contribute to the protective effects of whey and partial WH.

Discussion

Establishment of oral tolerance to harmless food proteins might be of importance to prevent the development of food allergies and other allergies later in life. In the current study,

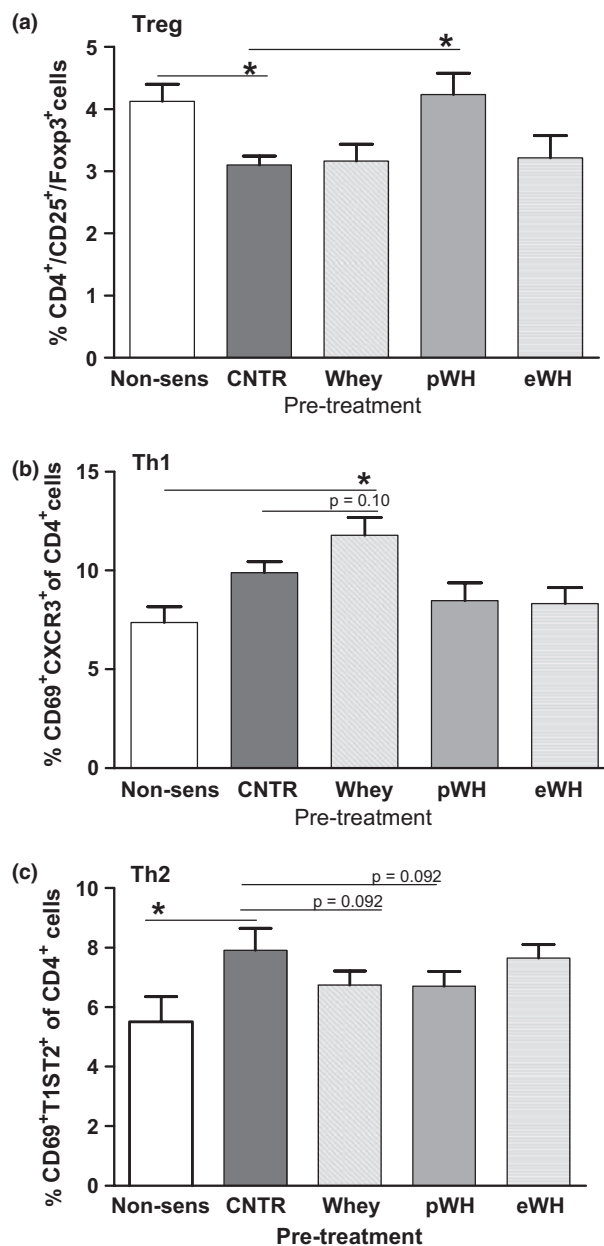


Figure 5 Increased numbers of Foxp3⁺ regulatory T-cell in mesenteric lymph nodes were observed in partial (p)WH pre-treated sensitized mice compared to control (CNTR)-sensitized mice (a; $p < 0.050$). Data were shown from $n = 2$ independent experiments. Whey pre-treatment tended to increase the number of activated Th1-cells compared to whey-sensitized mice while this was significant compared to sham-sensitized mice (b; $p = 0.10$, $p < 0.05$). Administration of whey and pWH prior to sensitization tended to reduce the number of recently activated Th2 cells (c; $p = 0.092$). No effect was observed after pre-treatment with an extensive (e)WH. Mice were treated with PBS (CNTR) or 50 mg whey, pWH, eWH prior to whey sensitization using cholera toxin as an adjuvant. WH, whey hydrolyzates.

it was demonstrated that partial and not extensive WH retained the putative capacity to prevent allergic symptoms when given orally prior to sensitization, in a mouse model of orally induced cow's milk allergy. The protective effects coincided with elevated numbers of regulatory T-cells in the intestine and could be adoptively transferred using MLN cells.

In a recent study of our group, a partial WH was found to have limited sensitizing capacities in a mouse model of cow's milk allergy using oral sensitization (16, 17). In the current study, it was investigated whether this partial WH still was able to protect against sensitization to whey in relation to intestinal Foxp3⁺ regulatory T-cells. Our results show that treatment of naïve mice with the partial WH prior to oral whey sensitization reduced the acute allergic skin response and mast cell degranulation upon whey challenge. Surprisingly, partial WH treatment of naïve mice prior to oral sensitization decreased the acute allergic skin response and mast cell degranulation without affecting whey-specific IgE/IgG1 serum levels. This implies that feeding a partial WH did not prevent sensitization to whey but protects against the development of an allergic effector response (e.g., clinical symptoms). One of the possible explanations of a reduced effector response in the presence of whey-specific IgE/IgG1 may be that IgE receptor signaling of mast cells is diminished by active suppression involving regulatory T-cells which can be mediated via cell-cell contact or mediator release (18, 19). No protective effect of the extensive WH was observed. As expected, whey treatment (as a positive control) prior to sensitization prevented the induction of whey-specific IgE and IgG₁ and as a result the allergic effector response upon whey challenge.

Oral tolerance induction by partial WH in mice was widely studied in the past more than 10 years ago using systemic sensitization rather than oral sensitization. In contrast to the current study, these animal studies showed that partial hydrolyzed formulae prevented the generation of whey-specific IgE antibodies for β -lactoglobulin in mice (20–22). Children suffering from cow's milk allergy are most likely sensitized via the oral route; therefore, animal models using oral sensitization instead of systemic sensitization might give a more accurate reflection of the sensitization route in children. In mouse models using oral sensitization with cholera toxin as an adjuvant, it was shown that orally administered food proteins prior to oral sensitization prevented the generation of serum-specific antibodies (12–15) and decreased local Th2-cell responses to beta-lactoglobulin (12, 15).

In mice, Foxp3 is exclusively expressed by naturally occurring and locally induced CD4⁺CD25⁺ regulatory T-cells (5). Support for a role for regulatory T-cells in mediating oral tolerance has been obtained from feeding experiments in mice (23–25) and human studies (5). The tolerizing capacity of WH in relation to effects on intestinal Foxp3⁺ regulatory T-cells has not been investigated in a mouse model using oral sensitization so far.

The number of CD25⁺Foxp3⁺ regulatory T-cells in the MLN was decreased in whey-sensitized mice compared to

non-sensitized mice indicating that regulatory T-cells contribute to the development of the allergic response. The tolerogenic features of the partial WH were associated with enhanced local numbers of CD25⁺Foxp3⁺ regulatory T-cell in the MLN. Concomitant with the increase in intestinal regulatory T-cells, a tendency toward decrease in intestinal activated Th2-cells was observed indicating a possible mechanism for the protective effects as induced by the partial whey hydrolyzate. In whey tolerized mice, regulatory T-cell numbers remained low when naïve mice were fed whey prior to sensitization. A concomitant Th1 response may be involved in redirecting the Th2-type allergic response, contributing to the protective effects of whey feeding prior to sensitization. However, further investigation is needed to examine whether effector Th1-cells contribute to the observed protective effects of non-hydrolyzed whey tolerization.

Studies in mice and humans have demonstrated that Foxp3⁺ regulatory T-cells convert local generated tolerance to systemic tolerance (26). The MLN connects the local and the systemic immune system creating one of the possibilities to convert the gut-induced regulatory responses to systemic tolerance. To confirm whether the inhibitory effects of the partial WH on the acute allergic effector response (e.g., skin response and mMCP-1 levels) could be converted at the level of MLN, an adoptive transfer using MLN derived cells was conducted. Indeed, adoptive transfer of MLN cells of tolerized donor mice reduced the acute allergic skin response of whey-sensitized recipient mice. Hence, MLN cells from partial WH as well as whey tolerized mice could transfer tolerance to recipient mice. The protective effect of MLN cells of the partial WH tolerized donor mice may relate to the increased number of Foxp3⁺ regulatory T-cells found in the MLN of these mice.

In this study, it was shown that partial WH and not extensive WH treatment of naïve mice prior to whey sensitization reduced the induction of clinical symptoms to whey. The tolerizing capacity of partial WH coincided with elevated numbers of Foxp3⁺ regulatory T-cells in the MLN, and the protective effects could be transferred using MLN cells. These results indicate that Foxp3⁺ regulatory T-cells might contribute to a reduced effector response (e.g., clinical tolerance) induced by partial WH in mice. This might be of relevance in the prevention of cow's milk allergy in children.

Conflict of interest

First author as indicated by the second affiliation is part of a strategic alliance between Utrecht University and Danone Research Centre for Specialised Nutrition. Prof. Dr. Johan Garssen is head of the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science at the Utrecht University. Independent research for Danone and other (food) industries is performed at this setting and we would like to declare that there is no conflict of interest in the paper.

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