

Research article

**Partially hydrolyzed whey proteins prevent clinical symptoms in a cow's milk allergy mouse model and enhance regulatory T and B cell frequencies**

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**Abstract**

**Scope:** Partially hydrolyzed cow's milk proteins are used to prevent cow's milk allergy in children. Here we studied the immunomodulatory mechanisms of partial cow's milk hydrolysates *in vivo*.

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**Methods and results:** Mice were sensitized with whey or partially hydrolyzed whey using cholera toxin. Whey-specific IgE levels were measured to determine sensitization and immune cell populations from spleen, mesenteric lymph nodes and Peyer's patches after oral whey administration were measured by flowcytometry. Whey-specific IgE and IgG1 levels in partial whey hydrolysate sensitized animals were enhanced, but challenge did not induce clinical symptoms. This immunomodulatory effect of partial whey hydrolysate was associated with increased regulatory B and T cells in the spleen, together with a prevention of IgM-IgA class switching in the mesenteric lymph nodes and an increased Th1 and activated Th17 in the Peyer's patches.

**Conclusion:** Partial hydrolysate sensitization did not induce whey induced clinical symptoms, even though sensitization was established. Increased regulatory cell populations in the systemic immune system and a prevention of increased total Th1 and activated Th17 in the intestinal immune organs could contribute to the suppression of allergic symptoms. This knowledge is important for a better understanding of the beneficial effects of hydrolysates.

**Keywords:** Breg, cow's milk allergy, infant formula, partial whey hydrolysate, sensitization study.

**Abbreviations:**

**Treg**, regulatory T cells, **PP**, Peyer's patches, **MLN**, mesenteric lymph nodes, **CT**, cholera toxin, **FCS**, Fetal calf serum, **RT**, room temperature, **Breg**, regulatory B cells.

**Introduction**

In a conventional homeostatic situation, the intestinal immune system maintains oral tolerance. In this state, no immune response against harmless food derived molecules is

induced, while the immune system is still able to evoke a protective immune response against harmful pathogens [1]. When this homeostasis is disturbed, food allergy can develop. Cow's milk allergy is one of the major food allergies in children, with a prevalence of 2-3% [2]. Although the awareness of cow's milk allergy as a serious health issue is growing [3], the only therapy available is allergen avoidance [4].

Avoidance of allergens can be accomplished by allergen destruction. Enzymatic hydrolysis destroys linear and structural epitopes and is considered the most effective mode to reduce allergenicity. Depending on the hydrolysis degree, hydrolyzed proteins are either used to treat existing cow's milk allergy (extensively hydrolyzed) or prevent allergic symptom development (partially hydrolyzed). The efficacy of partially hydrolyzed cow's milk protein based hypoallergenic infant formulas in reducing the development of cow's milk allergy in at-risk infants was demonstrated in multiple clinical trials [5,6]. Recently it had been recognized that postponing the contact with intact protein by destruction of epitopes might not be the only explanation for the hypoallergenic properties of partially hydrolyzed cow's milk proteins. It was demonstrated that especially partial hydrolysates can actively modulate immune responses [7].

During hydrolysis, smaller peptides are released from the intact protein. These peptides were found to possess a range of immunomodulatory capacities [8-11]. This suggests that specific peptides might contribute to hypoallergenic effects of hydrolysates. This has been confirmed in mouse models in which pre-treatment with partially hydrolyzed cow's milk proteins before sensitization induced oral tolerance and reduced allergic responses to whey protein [12,13]. This effect is associated with an increased percentage of regulatory T cells (Treg) in the mesenteric lymph nodes [13]. Transferring immune cells including Treg from tolerized mice protected recipients from developing an acute skin

response after a challenge with whey [12,14]. No oral tolerance was induced when pre-treatment was performed with proteins that were more extensively hydrolyzed [12,13].

Previously, we used an *in vitro* technology platform to screen immunomodulatory effects of a range of cow's milk proteins in a systematic way [15]. Our data showed that partially hydrolyzed whey induced a higher anti-inflammatory/pro-inflammatory cytokine ratio than intact whey or extensively hydrolyzed whey. Since higher levels of anti-inflammatory cytokines have a regulatory function in allergy [16,17], we hypothesized that partially hydrolyzed whey may also have *in vivo* immunomodulatory effects in a cow's milk allergy setting. Therefore, we investigated the effect of the most potent partial hydrolysate with immunomodulatory capacities in a cow's milk allergy mouse model. This mouse model has been developed and validated to be used in the pre-clinical safety evaluation of hypoallergenic formulas [18]. The IgE-mediated allergic response in these animals, together with the orally induced sensitization, can be closely followed. In the present study, mice were sensitized with either whey or its partial hydrolysate by using cholera toxin. To study whether sensitization with the partially hydrolyzed proteins can induce clinical allergic symptoms, we investigated acute skin response, body temperature, and anaphylactic reactions following intradermal whey administration. Whey-specific IgE and IgG1 were measured in serum. To assess the role of possible immunomodulatory effects of the hydrolysate, we studied immune cells populations in the spleen, Peyer's patches (PP) and mesenteric lymph nodes (MLN) following an oral whey challenge.

## Materials and methods

## **Cow's milk proteins and hydrolysates**

Intact whey (WPC80) and whey protein hydrolysate were provided by FrieslandCampina (Amersfoort, the Netherlands). The whey protein was partially hydrolyzed by two-step digestion to produce the hydrolysate with a hydrolysis degree of 9.2%. Peptide and amino acid patterns were obtained with RP-UHPLC method. Separations were performed on a Hypersil GOLD analytical column with Spherical silica packing (1.9  $\mu\text{m}$ , 175Å, 2.1 x 100 mm). The gradient elution was carried out with a mixture of 0.1% trifluoroacetic acid (TFA) and 1% acetonitrile (ACN) in water and 01% TFA and 90% ACN in water.

## **Mice**

Three- to four week old pathogen-free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France). Mice were housed in the animal facility of the University Utrecht and maintained on semi-purified cow's milk protein-free mouse chow (Research Diet Services, Wijk bij Duurstede, The Netherlands). Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

## **Experimental set-up**

Two groups of mice (both n=8) were sensitized by oral administration on day 0, 7, 14, 21, and 28 of 20 mg whey or whey hydrolysate, in 0.5 ml PBS containing 10  $\mu\text{g}$  cholera toxin (CT) (Quadrant Diagnostics, Epsom, UK) to break tolerance (figure 1). Non-sensitized mice (n=8) received only cholera toxin in PBS (CT group). To determine clinical allergic symptoms, animals were challenged with intact whey. On day 33, mice were intradermally

challenged in the ear with 10  $\mu\text{g}$  whey after which acute allergic skin responses, anaphylactic reactions and body temperature were determined. After 6 hours, mice were orally challenged with 50 mg whey. Eighteen hours later, animals were terminated and blood, spleen, MLN and PP of 6 out of 8 mice were collected for each group. The organs were kept in RPMI containing de complemented fetal calf serum (FCS) on ice until processing for flow cytometry. Blood samples were centrifuged for 15 min at 13.500 rpm and stored at  $-20^{\circ}\text{C}$ .

### **Acute allergic skin response**

In all animals, the acute allergic skin response was determined 1 hour after the intradermal challenge with 10  $\mu\text{g}$  whey in the ear pinnae. 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 ear thickness with the basal ear thickness. The delta ear swelling is expressed as  $\mu\text{m}$ .

### **Anaphylactic shock score and body temperature**

To evaluate the sensitization capacity of the samples, the anaphylactic reaction and body temperature were determined after the intradermal whey challenge. To establish the shock severity, a validated anaphylactic scoring table (0: no symptoms; 1: scratching nose and mouth; 2: swelling around the eyes and mouth, pillar erecti, reduced activity, higher breathing rate; 3: shortness of breath, blue rash around the mouth and tail, higher breathing rate; 4: no activity after stimulation, shivering and muscle contractions; 5: death by shock) was used, as adapted from Li et al.[19]. To measure changes in body temperature, all mice were given an implantable electronic ID transponder (Bio Medic Data Systems, Delaware, USA) on day 16.

### Measurement of whey-specific serum IgE and IgG1

Concentrations of serum whey-specific IgE and IgG1 were determined by ELISA as described previously [12]. In short, Microton plates (Greiner Bio-one, Monroe, USA) were coated with whey protein for 18 hours at 4°C. Plates were washed after each incubation step. Serum samples were applied and incubated for 2 hours at room temperature (RT), followed by incubation with biotin-labeled IgE or IgG1 for 90 minutes. The plates were incubated with streptavidin-horseradish peroxidase for 1 hour at RT and developed with *o*-phenyldiamine (Sigma-Aldrich). The reaction was stopped with 4 M H<sub>2</sub>SO<sub>4</sub> and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). In the IgG1 ELISA, serum samples were diluted 50 times.

### Flow cytometer analysis of T and B cell subsets in spleen, MLN, and PP

T and B cell (sub)populations were investigated after the oral challenge using flow cytometry. Cells were isolated from the spleen, MLN and PP. The spleen is considered to be representative for the systemic immune response [20]. The MLN are the draining lymph nodes from the gut [21], and the PP represent the local intestinal immune response [22].

All procedures were performed on ice. In order to isolate cells, organs were crushed between microscope slides in 2 ml of medium (RPMI with 10% decompemented FCS). To lyse erythrocytes, the spleen cell suspensions were incubated for 10 min with 4 ml ammonium chloride solution. Spleen cell suspensions were centrifuged at 1800 rpm for 5 min at 4°C and washed with 4 ml FACS buffer 2% (PBS supplemented with 2% decompemented FCS). Cell suspensions from all organs were filtered using a cell suspension filter (Greiner,

Alphen aan de Rijn, The Netherlands), and counted using a coulter counter (Beckton Dickinson BV, Breda, the Netherlands).

To stain the T and B cell subsets, cells ( $1 \times 10^6$ /well) were transferred to a roundbottom 96-wells plate and centrifuged at 1800 rpm for 5 min at 4°C. Cells were resuspended in 25  $\mu$ l extracellular blocking buffer (20% normal rat serum, 2% FC (CD16/CD32) block, in FACS buffer 10%) for 15 min. After centrifuging, cells were resuspended in 25  $\mu$ l extracellular antibody mix (antibodies as listed in supplementary file 1 or isotype controls, in FACS buffer 10% containing 5% normal rat serum) and incubated for 30 min in the dark. After washing and fixation for 30 min in fix-lysing buffer (Beckton Dickinson BV, Breda, the Netherlands) and washing again, cells were incubated with 200  $\mu$ l permeabilization buffer (eBioscience, Vienna, Austria) for 10 minutes. Cells were resuspended in 50  $\mu$ l intracellular blocking buffer (20% normal rat serum in permeabilization buffer) and incubated for 15 min. After centrifuging, cells were resuspended in 50  $\mu$ l intracellular antibody mix (antibodies as listed in supplementary file 1 or isotype controls, and 5% normal rat serum in permeabilization buffer) for 30 min in the dark. Cells were washed in permeabilization buffer 3 times, and resuspended in 100  $\mu$ l FACS buffer. Cells were analyzed on a FACSVerse flow cytometer. UltraComp eBeads were used to set compensation values for the antibody panels. The corresponding isotype control antibodies were used to set positive gates, using a margins of 1%. FlowJo VX software (FlowJo, Oregon, USA) was used to analyze lymphocyte subsets.

Flow cytometry gating strategies are described in the supplementary files 2, 3 and 4. Single cells were selected by gating FSC-W vs FSC-H. CD3 was used as a marker for T cells, and within this T cell population we distinguished Thelper cells ( $CD4^+$ ) and cytotoxic T cells ( $CD8^+$ ). Within the Thelper cell population we identified Th1 cells ( $Tbet^+$ ), Th2 cells

(Gata3<sup>+</sup>), Treg (FoxP3<sup>+</sup>) and Th17 cells (RoRγT<sup>+</sup>). B220 was used as a B cell marker. Within this B cell population, regulatory B cells (Breg) were distinguished (CD5<sup>+</sup>). Also within B220<sup>+</sup> cells, IgM<sup>+</sup> and IgA<sup>+</sup> cells were measured to indicate class-switching. CD138<sup>+</sup> cells were considered to be plasma cells. Within all populations, CD69<sup>+</sup> cells were considered to be activated cells [23].

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, CA, USA). For all data, normality was tested using a Kolmogorov-Smirnov test. The ear swelling, body temperature and anaphylactic shock score data were normally distributed and shown as mean ± standard deviation (SD). Significant differences were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The antibodies and flow cytometry data were not normally distributed, and therefore the data were shown as median ± range. Significant differences were tested using a Kruskal Wallis test followed by Dunn's multiple comparisons test. A *p*-value of <0.05 was considered to indicate a significant difference.

## Results

### Chemical characterization of partial whey hydrolysate

The whey protein hydrolysate was obtained by a two-step hydrolysis of whey protein (WPC80). The degree of hydrolysis of the obtained hydrolysate was 9.2%. Peptide pattern (reversed phased chromatography) and molecular weight distribution are shown in figure 2.

As shown in figure 2B, the hydrolysate contained mainly peptides smaller than 5 kD, and is therefore considered partially hydrolyzed.

### **Whey hydrolysate sensitized mice showed an IgE and IgG1 response but no clinical signs of anaphylactic shock and acute skin response after whey administration**

Whey sensitized mice showed increased levels of IgE compared to the non-sensitized control group that only received cholera toxin ( $p<0.01$ ) (figure 3A). Partial hydrolysis of whey could not prevent whey-specific IgE enhancement as IgE levels were also significantly elevated in whey hydrolysate sensitized mice compared to the CT group ( $p<0.05$ ). Whey-specific IgG1 levels were also elevated in both the whey and hydrolysate group compared to the control group ( $p<0.01$  and  $p<0.05$ ) (figure 3B). To study the allergic response against whey, the acute skin response was measured in mice after an intradermal whey challenge. As expected, an increased acute skin response was detected in intact whey protein sensitized animals ( $p<0.0001$ ) compared to the CT group (figure 3C). Interestingly, sensitization with a hydrolyzed whey did not lead to an acute skin response upon a whey challenge (figure 3C). Two other clinical parameters were measured after the acute skin response, i.e. the presence of an anaphylactic shock and body temperature. Mice sensitized with intact whey protein showed low to severe anaphylactic symptoms ( $p<0.0001$ ), while whey hydrolysate sensitized animals did not show anaphylactic symptoms (figure 3D). A drop in body temperature was clearly present in the whey sensitized animal group ( $p<0.01$ ) but absent in the whey hydrolysate sensitized animals (figure 3E).

## **Sensitization with whey hydrolysate induced systemic changes in T cell percentage, subtypes, and activation after oral whey administration.**

Since we observed that food allergy related clinical symptoms were absent in hydrolysate sensitized mice despite the presence of whey-specific IgE, we questioned whether whey hydrolysate could have an immunomodulating effect. Therefore, we studied the differences in immune responses between intact whey and hydrolysate treated animals and CT controls. We first investigated the systemic immune system after the oral challenge. As shown in figure 4 the total percentage of lymphocytes did not differ between groups, but the percentage of T cells in the spleens of mice sensitized with whey hydrolysate was significantly decreased compared to the CT group ( $p<0.001$ ), while there were no differences between the whey group and the CT group. The hydrolysate sensitized animals showed significant differences compared to the whey group and the CT group for several T cell subsets. They had a higher percentage of Thelper cells ( $p<0.05$ ) compared to the whey group and a decreased percentage of cytotoxic T cells compared to the CT group ( $p<0.01$ ) and whey group ( $p<0.05$ ). The whey group showed no differences in these T cell subtypes compared to the CT group. Also, hydrolysate sensitized animals showed a higher percentage of activated Thelper cells ( $p<0.05$ ) compared to the CT group. Activated cytotoxic T cell percentages did not differ between any group.

## **The percentage of activated Th2 and Treg increased in the spleen of whey hydrolysate sensitized mice after an oral whey challenge**

As food allergy is considered to be a Th2 type immune response and balanced by Treg cells, we decided to further explore the different subsets of splenic Thelper cells. While the total

percentages of Th1, Th2, Treg and Th17 cells did not differ among groups (supplementary file 5), differences were found when analyzing the activated T-cells, i.e. CD69 positive cells, within the subsets (figure 4). No effects on the percentage of activated Th1 cells were observed, while the percentage of activated Th2 cells was increased in the hydrolysate group compared to the CT group ( $p<0.05$ ), but not in the whey group. However, the higher percentage of activated Th2 cells was accompanied by a significantly increased percentage of activated Treg cells in the hydrolysate group compared to the CT group ( $p<0.01$ ). No effects were observed in the whey group compared to the CT group. The percentages of activated Th17 cells in the spleen did not differ between any group.

#### **The percentage of CD5+ Breg was increased in the spleen of whey hydrolysate sensitized mice after an oral challenge**

Helper cells in the spleen are found to be important in the activation and class-switching of B cells, which plays a crucial role in the development of food allergy [24]. Therefore, it was investigated whether sensitization with a whey hydrolysate affected B cell subsets after an oral whey challenge. As shown in figure 5, the total percentage of B cells was decreased in the hydrolysate group compared to the CT group ( $p<0.05$ ), but the percentage of B cells of the whey group did not differ from the CT group. The percentage of activated B cells did not differ among groups. Furthermore, a significant increase in the percentage of Breg was observed in the spleens of whey hydrolysate sensitized mice compared to the whey group ( $p<0.05$ ). The whey group did not differ significantly from the CT group. No differences in percentage of plasma cells, IgM+IgA- and IgM-IgA+ B cells were observed between groups.

## **In the MLN, sensitization with a whey hydrolysate decreased activated Th1 cells and prevents the class-switching to IgA**

Since we observed multiple hydrolysate-specific effects in the systemic immune system, we investigated possible differences in the local intestinal immune organs, since the mucosal immune system is the primary site where the induction of an allergic reaction against orally administered antigens occurs [25]. Effects in the intestine might affect the systemic immune response [26]. We first studied differences in the MLN, since the MLN are assumed to play a pivotal role in food allergy and maintaining oral tolerance [27]. We found a decrease of the total percentage of lymphocytes in the whey hydrolysate sensitized animals compared to the CT group ( $p<0.05$ ), but there was no difference between the whey group and CT group (figure 6). The total percentage of T cells was increased in the whey group compared to the hydrolysate group ( $p<0.05$ ). Activated cytotoxic T cells were slightly increased in the whey hydrolysate group compared to the whey group ( $p<0.05$ ). Also, when analyzing the activated Thelper subsets, sensitization with whey hydrolysate was found to result in a decreased percentage of activated Th1 cells compared to the CT group ( $p<0.01$ ) (figure 6). This effect was not seen in the whey group. We found no differences when analyzing total percentage of Thelper cells and cytotoxic T cells, activated Thelper cells (figure 6), total Th1, Th2, Treg and Th17 cells (supplementary file 6), and activated Th2, Treg and Th17 cells (figure 6).

For the B cells, we found no differences among groups for the total percentage of B cells, activated B cells, Breg, and plasma cells (figure 7). However, when focusing on antibody producing cells, we found that class-switching in B cells was different among groups. In the group of mice sensitized with whey the percentage of IgM+IgA- B cells decreased compared to the hydrolysate group ( $p<0.05$ ), while the percentage of IgM-IgA+ B cells increased compared the hydrolysate group ( $p<0.05$ ). This suggests that class-switching

to IgA happened after sensitization with intact whey. This did not occur in mice sensitized with whey hydrolysate.

**The increase of Th1, and activated Th17 and Treg cells and decrease of Breg was prevented in the PP of whey hydrolysate sensitized mice**

The first immune organs to come into contact with antigen after an oral challenge are the PP, located in the lamina propria of the intestine. They are important antigen sampling sites [22], and were found to affect the systemic immune system [26]. No differences were found for the total percentage of lymphocytes, T cells, Thelper cells or cytotoxic T cells, activated Thelper cells and cytotoxic T cells, nor for activated Th1 and Th2 cells among groups in the PP (figure 8). However, mice sensitized with intact whey showed increased activated Treg ( $p<0.05$ ) and Th17 ( $p<0.05$ ) percentages compared to the CT group (figure 8), while hydrolysate sensitized mice did not show these effects. Here, we did not only observe differences in activated Thelper subsets, but also the total percentages of the Th1 and Treg were increased after sensitization with whey compared to the CT group (both  $p<0.05$ ), while sensitization with whey hydrolysate did not have such effects (supplementary file 7).

Sensitization with whey or whey hydrolysate did not have an effect on the total percentage of B cells, activated B cells, plasma cells, and IgM and IgA expression of B cells (figure 9). When mice were sensitized with whey, a significant decrease in Breg was seen in the PP compared to the CT group ( $p<0.05$ ). This decrease was not observed in animals sensitized with a whey hydrolysate.

## Discussion

Recent findings suggest that besides the destruction of epitopes [28], immunomodulatory effects of hydrolysates can be an underlying mechanism contributing to the hypoallergenic properties of partially hydrolyzed whey proteins [29]. However, most animal studies showing beneficial effects of hydrolysates in allergy mainly focus on immunoglobulin levels as a measure for sensitization [12,13,30-32], while only a few studies investigated effects of whey hydrolysates on immune populations involved in the allergic response [12,14]. In this study, we investigated the effects of sensitization with a partially whey hydrolysate on allergy parameters and serum whey-specific IgE and IgG1 levels after an intradermal whey challenge. To investigate immune effects, a range of immune populations of both systemic and intestinal immune organs was also measured after the oral whey challenge. We found that anaphylactic shock was not induced in whey hydrolysate sensitized mice, even though increased serum whey-specific IgE and IgG1 levels were detected. In the spleen, sensitization with the whey hydrolysate resulted in an increase of Breg and activated Treg. In the local immune organs hydrolysis of whey proteins prevented both IgM-IgA class switching (MLN) and the increase of the percentage of Th1, activated Th17 and activated Treg cells (PP) (figure 10).

We found that after sensitization with the whey hydrolysate, the intradermal challenge did not induce allergic symptoms like an anaphylactic shock, drop in body temperature and an acute skin response. This finding corresponds with previous experiments in the same and other mouse models using partially hydrolyzed whey proteins [33,34,12]. Interestingly, despite the absence of clinical symptoms, we found a significantly increased whey-specific IgE response in whey hydrolysate sensitized animals. This also corroborates the findings of other groups showing no clinical symptoms despite a high specific IgE response in sensitized

mice [35-37]. It should be noted that variability between individual mice in the whey hydrolysate group was high. Four animals in the group showed a high IgE response, while 2 animals showed a low response and IgE levels were not increased in 2 animals. However, high variability between individual animals is a known phenomenon in sensitization studies [38]. None of these animals developed clinical symptoms.

As we observed IgE induction, our data suggest that the partial whey hydrolysate still contains whey epitopes which are recognized by the immune system. After hydrolysis 10% of the total amount of proteins was still larger than 10.000 Da (figure 2B). It is likely that intact  $\alpha$ -lactalbumin (14 kD) and  $\beta$ -lactoglobulin (18 kD), which are the main allergens in whey, are still present in this 10.000 Da fraction [39]. Lower doses of antigen were described before to be to induce a stronger Th2 response than higher doses [40,41]. This could explain why the IgE levels in the hydrolysate group are higher compared to the IgE levels in the whey group.

Despite the increased whey-specific IgE levels, the hydrolysate does not evoke an allergic reaction after an intradermal challenge. We hypothesized that this could be due to an immunomodulatory effect of the hydrolysate. To study the immunomodulatory effects of whey hydrolysates, we studied the immune populations in the spleen, MLN and PP after an oral whey challenge. In this challenge model, antigens will first encounter the PP and MLN, which may affect the peripheral immune response. We found hydrolysate-induced effects in the spleen, i.e. the peripheral immune response. Some of these effects are associated with (food) allergy and suggest that the whey hydrolysate does sensitize against whey, which is in line with the detected increased IgE levels. This includes an increased percentage of (activated) Thelper cells and a decreased percentage of cytotoxic T cells [42,43], together with an increase in activated Th2 cells [44]. The fact that despite this established sensitization no clinical symptoms were observed, suggests a stimulated immune regulatory effect of the

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whey hydrolysate. Such an immunomodulatory effect could be induced by Treg and Breg, since we observed an increased percentage of these cells in the spleen. The increased percentage of Treg in the spleen may suppress mast cells and basophils, thereby suppressing the effector phase of the allergic reaction [37]. Breg have been recognized as an important suppressor cell in multiple diseases, including cow's milk allergy [45]. It was shown that an adoptive transfer of CD5+ Breg from the MLN could reduce the allergic responses in sensitized recipients [46]. The induction of Treg and Breg by partially hydrolyzed whey could therefore be a mechanism involved in the dampening of the clinical allergic symptoms in the whey hydrolysate sensitized mice.

During oral sensitization and challenge, the intestinal immune cells are the first to encounter the administered antigen. Food allergy might already be initiated at this intestinal mucosal level as both the removal of the Peyer's patches or the MLN results in a decrease in allergic responses [26]. The role of the MLN in oral tolerance and regulating the allergic reaction has been studied most. However, we only found differences in the B cell subset in the MLN. Whey sensitization increased the percentage of IgA+IgM- B cells and decreased the percentage of IgA-IgM+ cells, which indicates class switching from IgM to IgA. However, this effect was not observed in the hydrolysate sensitized group. B cells from the MLN are known to migrate to the intestine to regulate local protection by producing immunoglobulins [47]. In allergy, coating of antigens by IgA could be an important protection mechanism [48].

Although the Peyer's patches play a crucial role in the development of an allergic reaction in food allergy [26], it is unknown which cell populations are involved. In our study, after the oral challenge, multiple effects were found in the Peyer's patches of whey sensitized animals which were lacking in hydrolysate sensitized animals. These differences might be

associated with immune dysregulation and development of an allergic reaction. Whey sensitized animals showed an increased percentage of total Th1, activated Th17 and activated Treg cells; these increases were not observed in animals sensitized with the whey hydrolysate. An increase of Th1 and Th17 cells might indicate intestinal dysregulation and the start of proinflammatory processes [49,50], which is compensated by the increased numbers of Tregs, as suggested previously [51]. These findings seem to be in line with the finding of a decreased percentage of Breg in the PP of whey sensitized animals but not in the whey hydrolysate sensitized mice. A lowering of these cells was reported before to precede intestinal inflammation and dysregulation [52,53]. These dysregulated inflammatory processes and differences between intact whey and hydrolyzed whey treated animals might be due to intestinal differences initiated during the systemic allergy development. Since we focused in this study on the effects 18 hours after the last challenge, we do not have full insight in the changes over time.

In summary, we showed that administration of a partially whey hydrolysate can induce sensitization against intact whey as expressed by increased whey-specific IgE levels, but that the occurrence of clinical symptoms induced by a challenge with intact whey was not observed. We suggest that the increase of Breg and Treg in the spleen and a prevention of increased Th1, Th17 and IgM-IgA class switching in the mucosal immune organs in partial hydrolysate sensitized animals can contribute to a suppression of clinical symptoms. We believe that these data are a first stepwise analysis of immune cell populations in different immune organs of whey and partially hydrolyzed whey sensitized animals. This knowledge is important for a better understanding of the beneficial effects of hydrolysates.

**Author contributions**

BCAMVE, MBGK, PDV and MMF conceived and designed the experiments. BCAMVE performed the animal experiment and MBGK performed the flow cytometry experiment and analyzed the data. MBGK, JG, BCAMVE, MMF and PDV wrote the paper.

**Conflict of interest statement**

MBGK, MMF and PDV declared no conflict of interest. The project was funded by the University Medical Center Groningen. BCAMVE and JG are partly employed by Nutricia Research.

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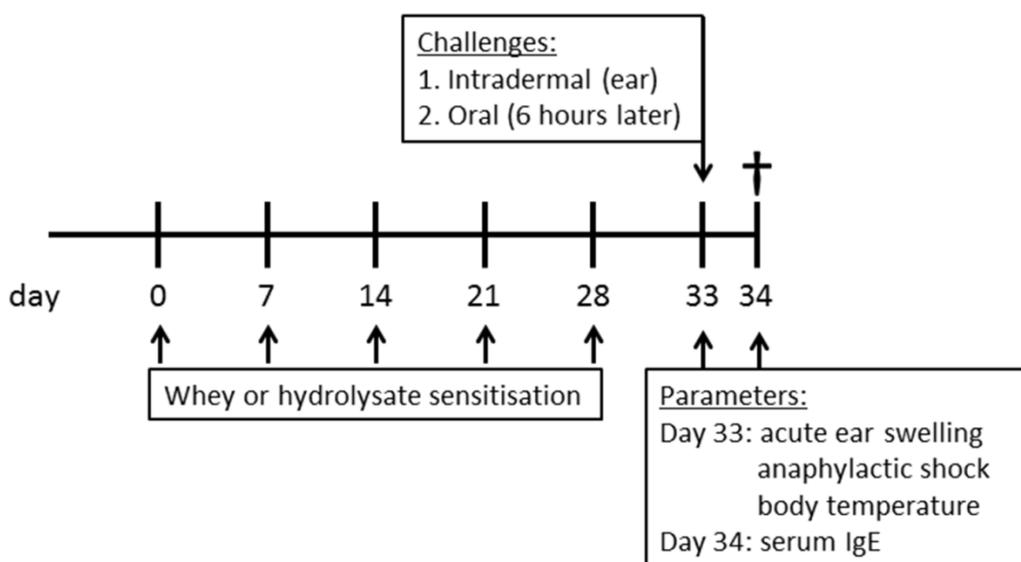
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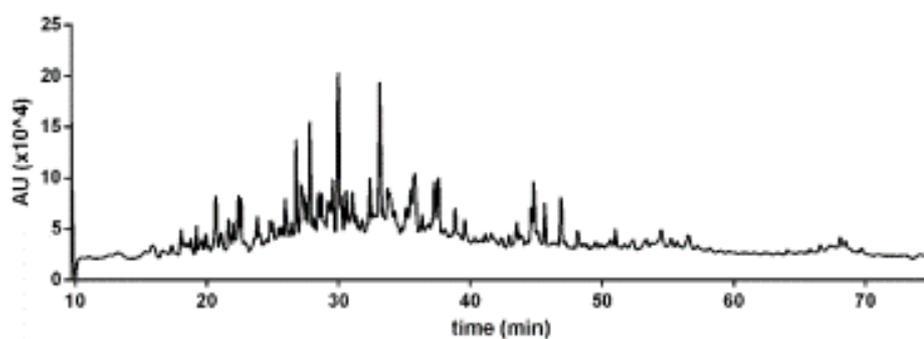
## Figure legends

**Figure 1. Experimental set-up.** In order to induce sensitization the mice received 20 mg intact whey or whey hydrolysate homogenized in PBS and mixed with 10  $\mu$ g cholera toxin on day 0, 7, 14, 21 and 28 via the oral route. The control group only received cholera toxin on those days. On day 33, mice were first intradermally challenged in the ear with 10  $\mu$ g intact whey after which the acute skin response, anaphylactic reaction and body temperature were measured. After 6 hours, mice were orally challenged with 50 mg intact whey. On day 34 (18 hours after the oral challenge) the animals were terminated and serum and organs were collected.



**Figure 2. Characterization of partial whey hydrolysate.** Peptide and amino acid patterns were obtained with a RP-UHPLC method (A). This was used to calculate the molecular weight distribution (B).

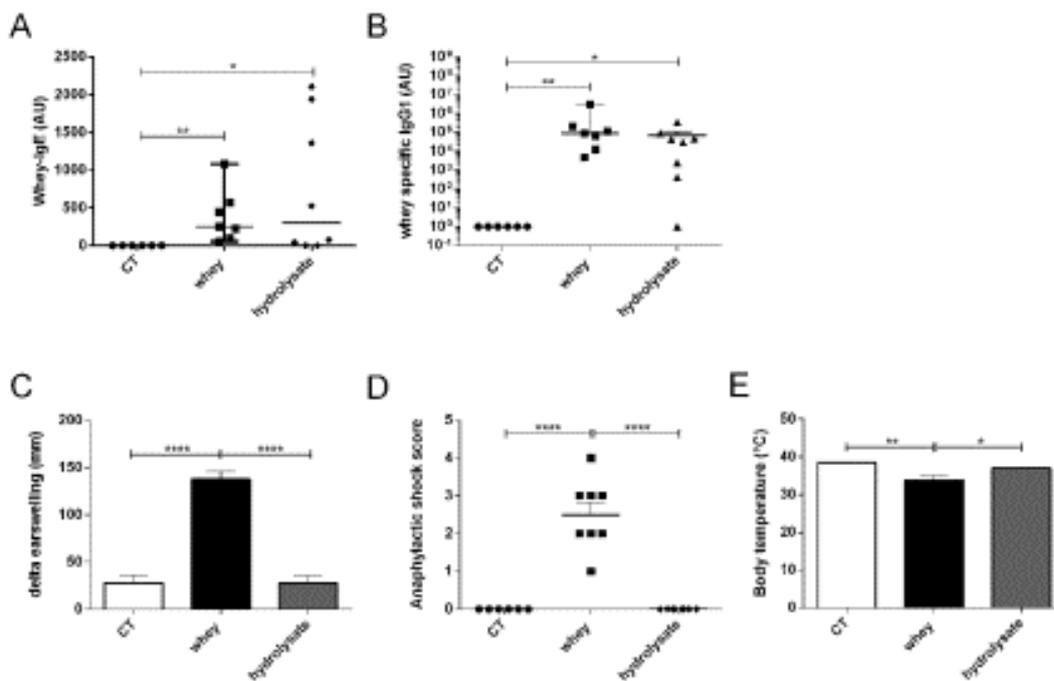
A



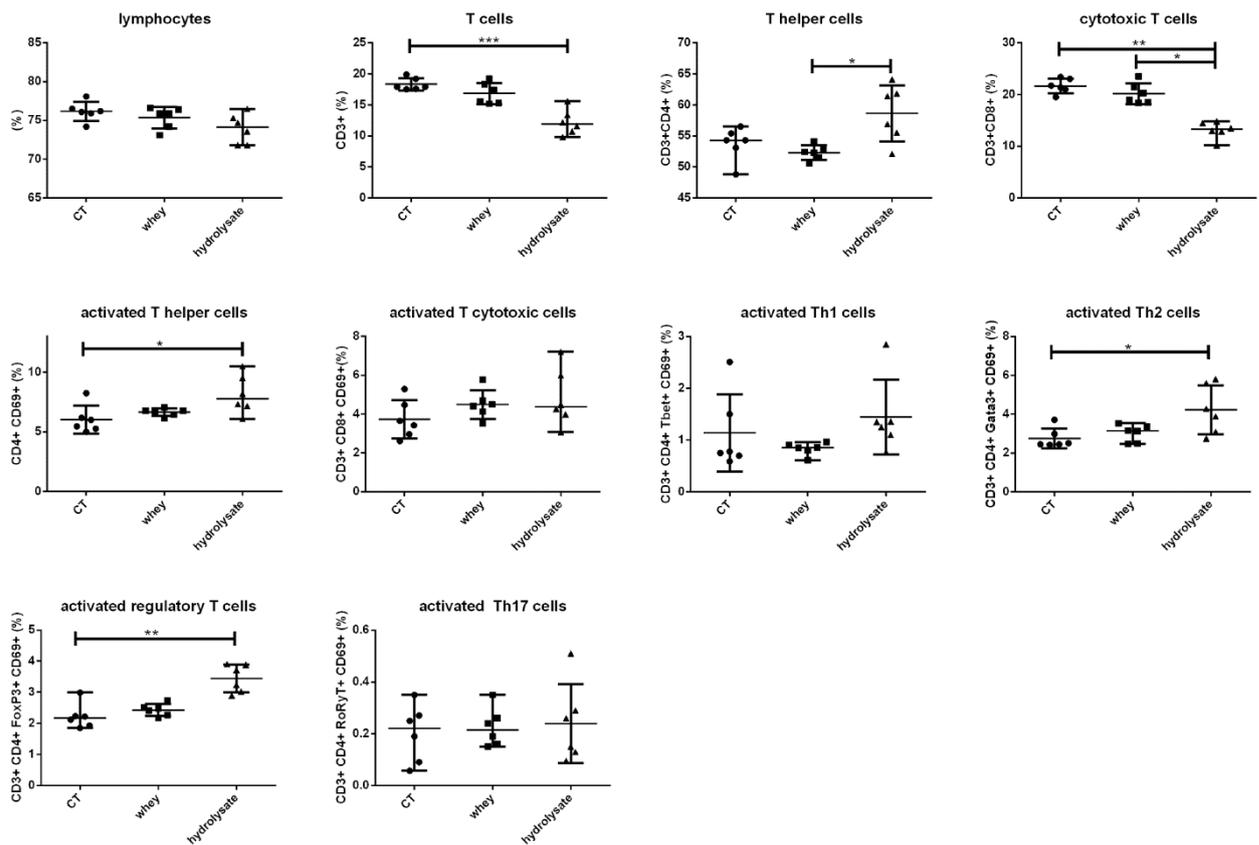
B

Weight (Da)	percentage
>10,000	10%
10,000 - 5,000	6%
5,000 - 2,000	13%
2,000 - 1,000	13%
1,000 - 500	16%
< 500	43%

**Figure 3. Whey-specific IgE and IgG1 response and clinical symptoms in mice after the challenge with whey.** IgE and IgG1 levels were significantly increased in intact whey and whey hydrolysate sensitized animals compared to the CT group (A,B). Clinical symptoms being acute skin response, symptoms of anaphylactic shock and a drop in body temperature were all increased in the whey sensitized animals, but not in the hydrolysate sensitized mice (C, D, E). Significant differences were indicated by \*.

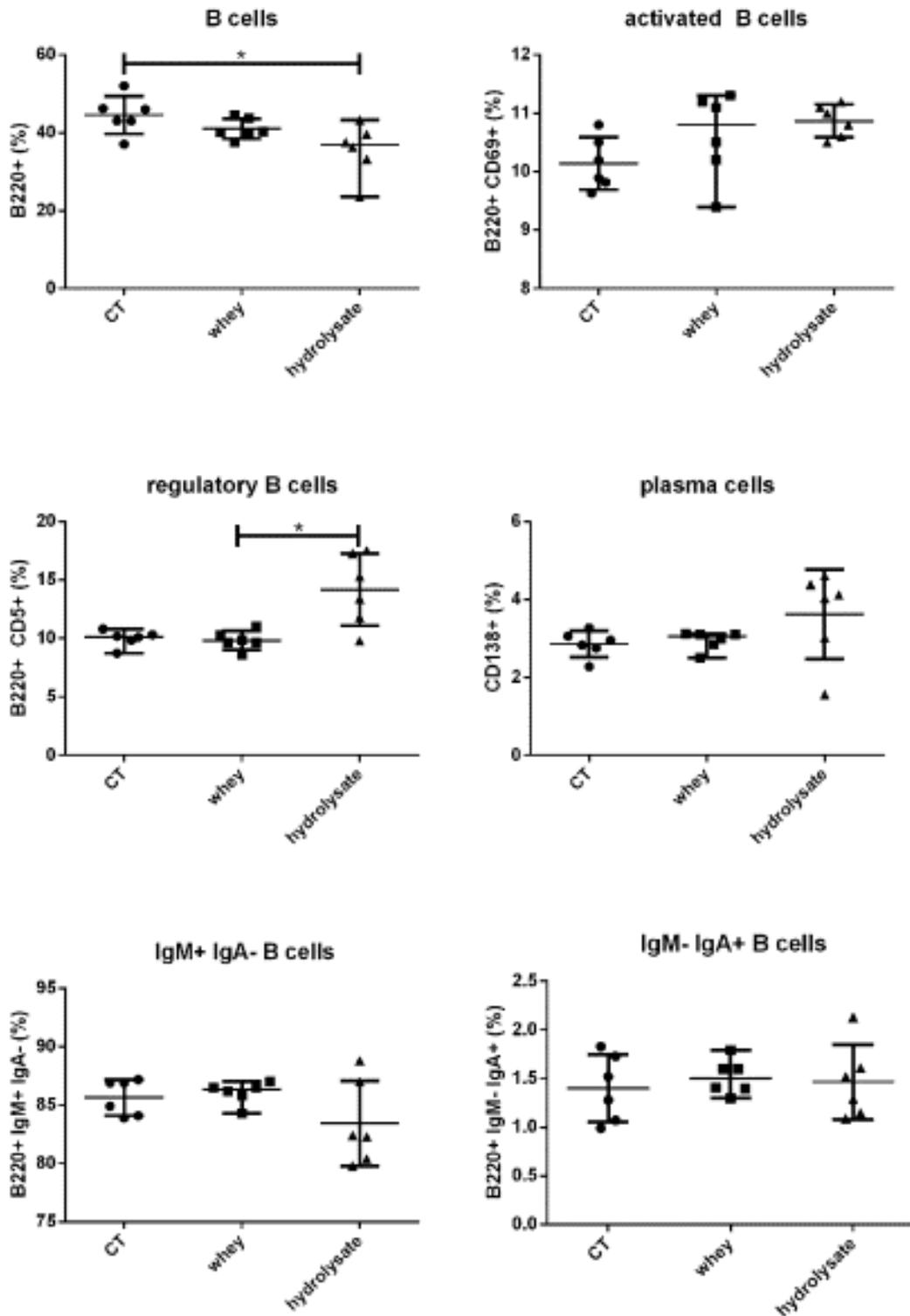


**Figure 4. Percentages of lymphocytes, T cells and T(helper) cell subsets in the spleen. A** decrease in total percentage of T cells and cytotoxic T cells was observed in the hydrolysate group compared to the CT group, while the percentage of Thelper cells and activated T cells increased. Activated Th2 and Tregs were increased in hydrolysate sensitized animals, but not in whey sensitized mice. Statistical differences were indicated by \*.

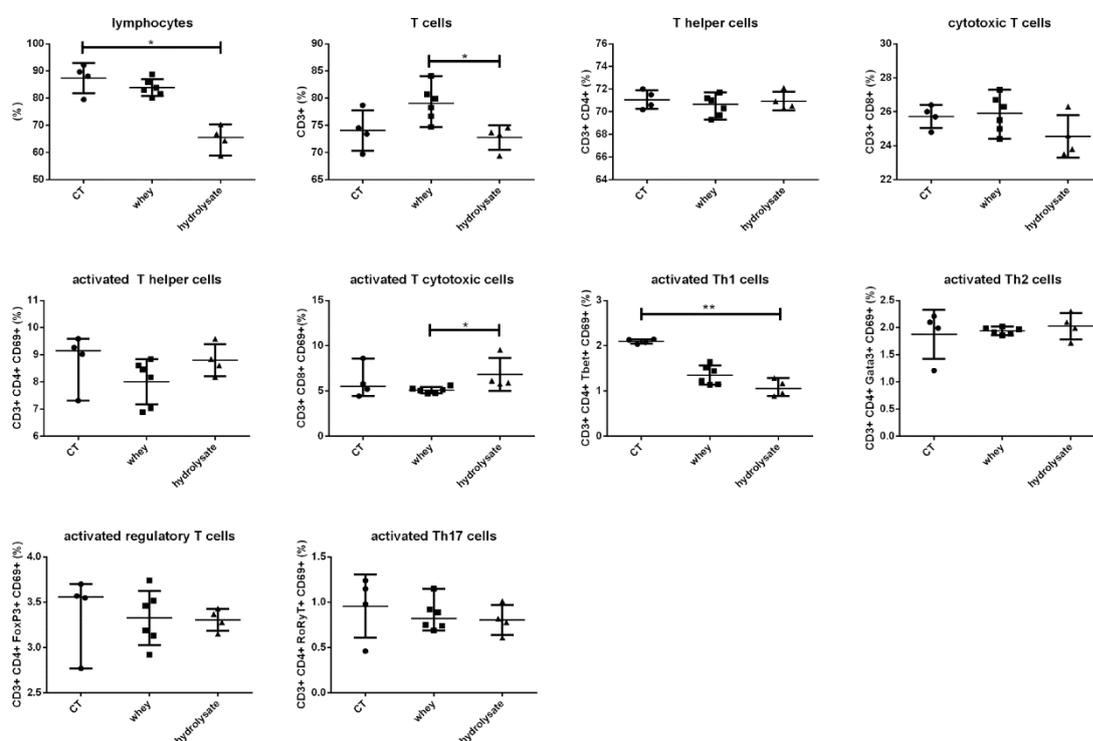


**Figure 5. Percentages of B cells and B cell subsets in the spleen.** The total percentage of B cells was decreased in hydrolysate sensitized mice compared to the CT group. The percentages of Breg were increased in mice sensitized with hydrolysate. Statistical differences were indicated by \*.

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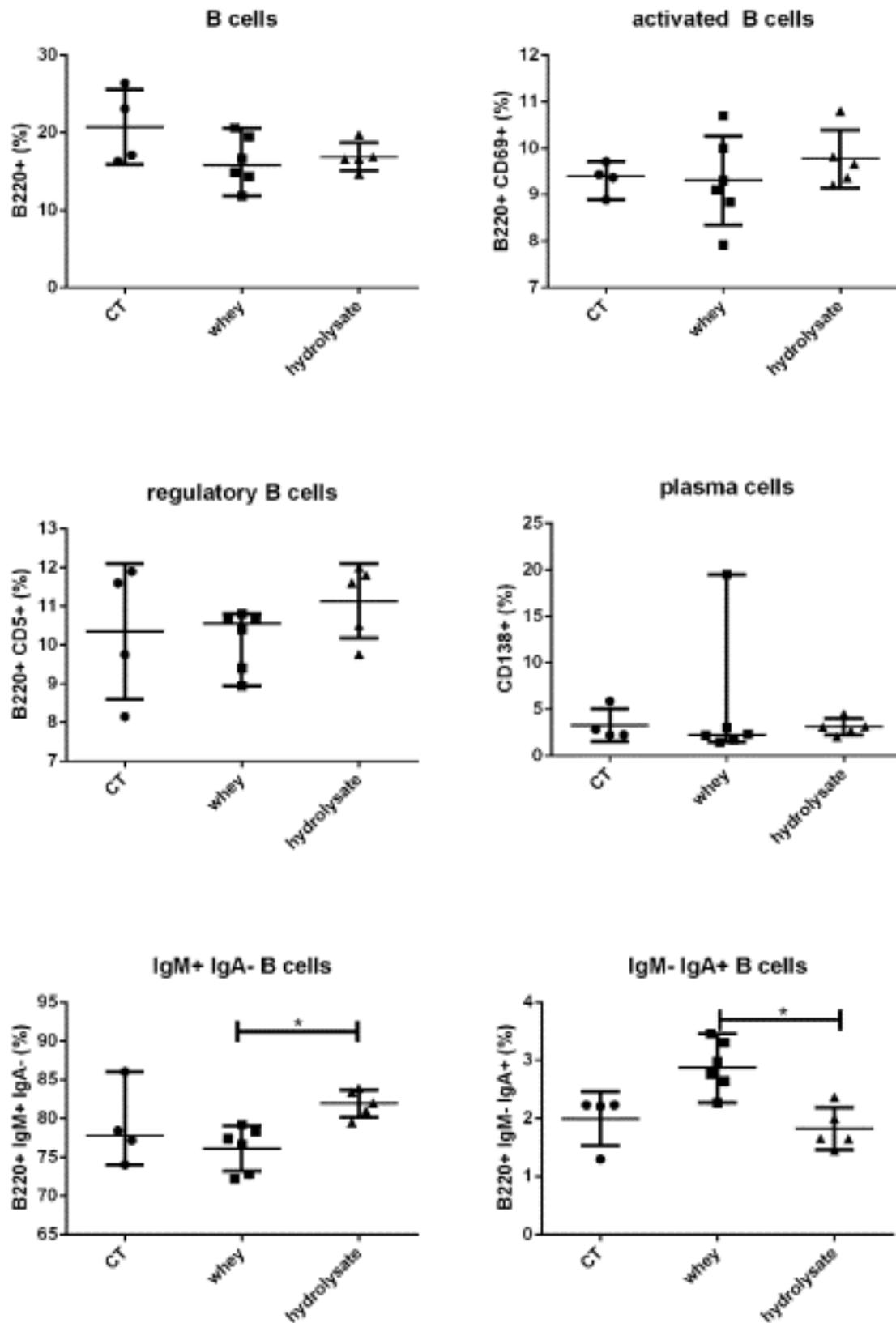


**Figure 6. Percentages of lymphocytes, T cells and T(helper) cell subsets in the MLN.** The total percentage of lymphocytes was decreased in hydrolysate sensitized mice, but not in whey sensitized mice. The percentages of T cell increased in the whey group compared to the hydrolysate group. The percentage of activated Th1 cells was decreased in animals sensitized with whey hydrolysate compared to the CT group. Activated cytotoxic T cells slightly increased in the hydrolysate group compared to the whey group. Statistical differences were indicated by \*.

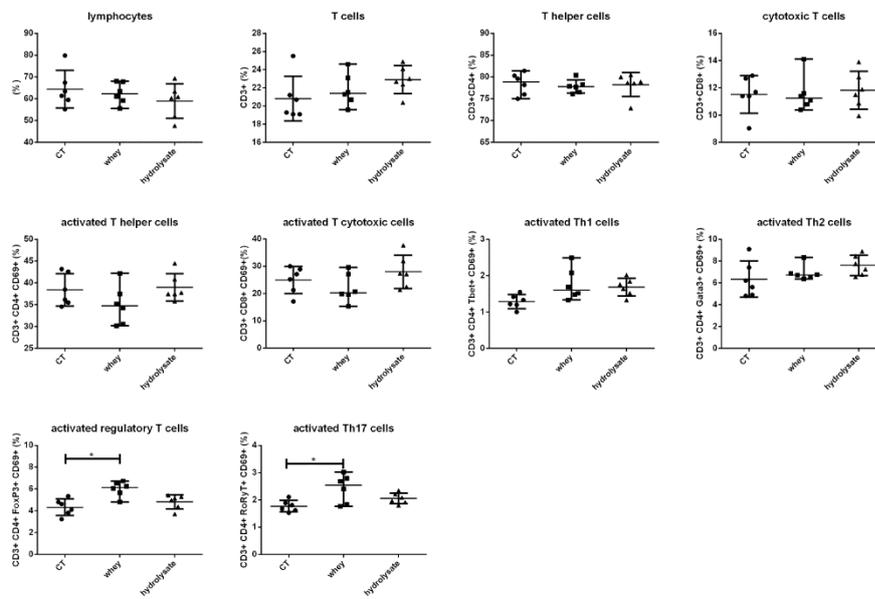


**Figure 7. Percentages of B cells and B cell subsets in the MLN.** The percentage of IgM-IgA+ B cells was increased in whey sensitized mice compared to the hydrolysate sensitized animals. Statistical differences were indicated by \*.

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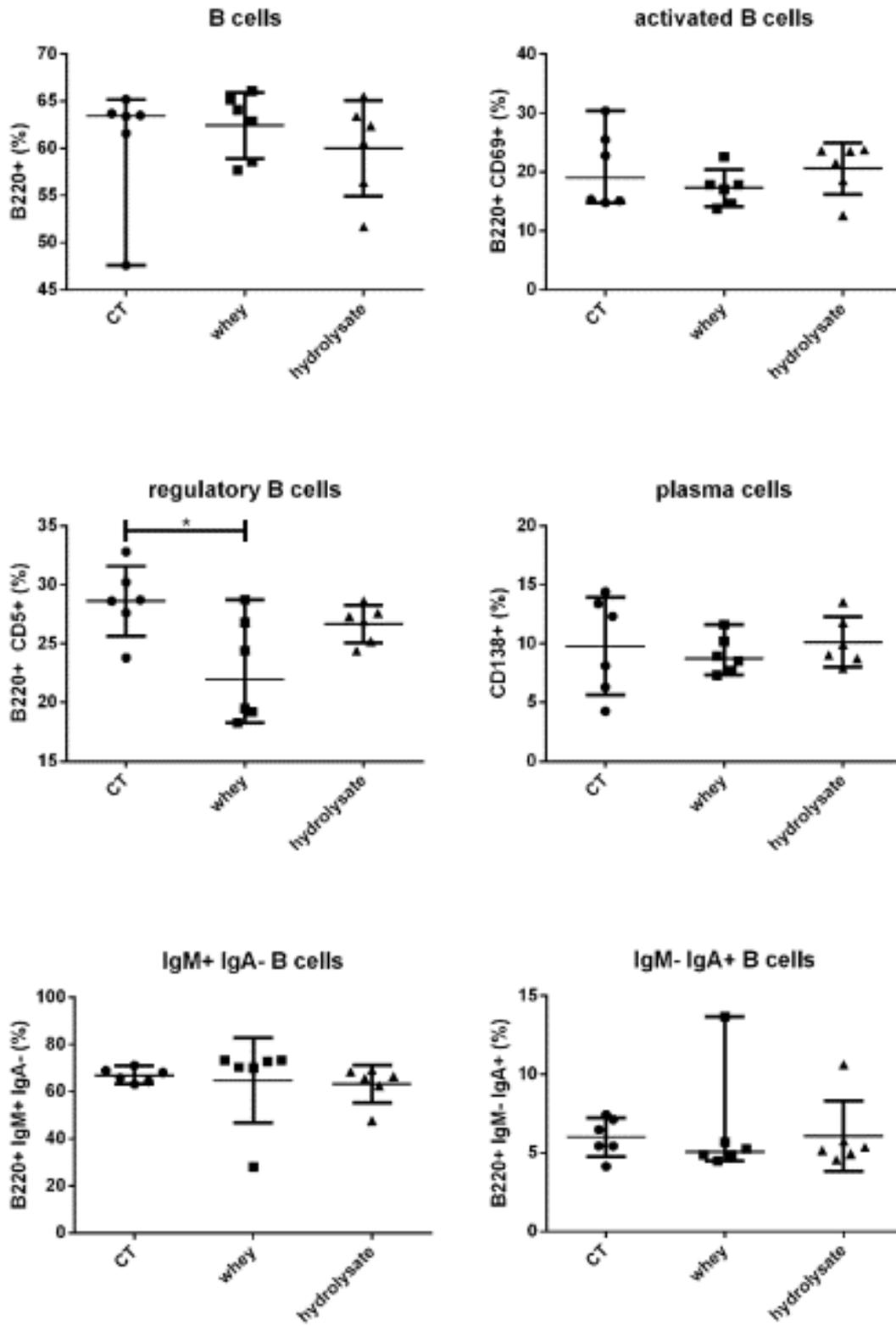


**Figure 8. Percentages of lymphocytes, T cells and T(helper) cell subsets in the PP.** No differences between groups in percentages of total lymphocytes, T cells and T cells subsets were observed. Activated Th17 and Treg cells were increased in the whey group compared to the CT group. Statistical differences were indicated by \*.

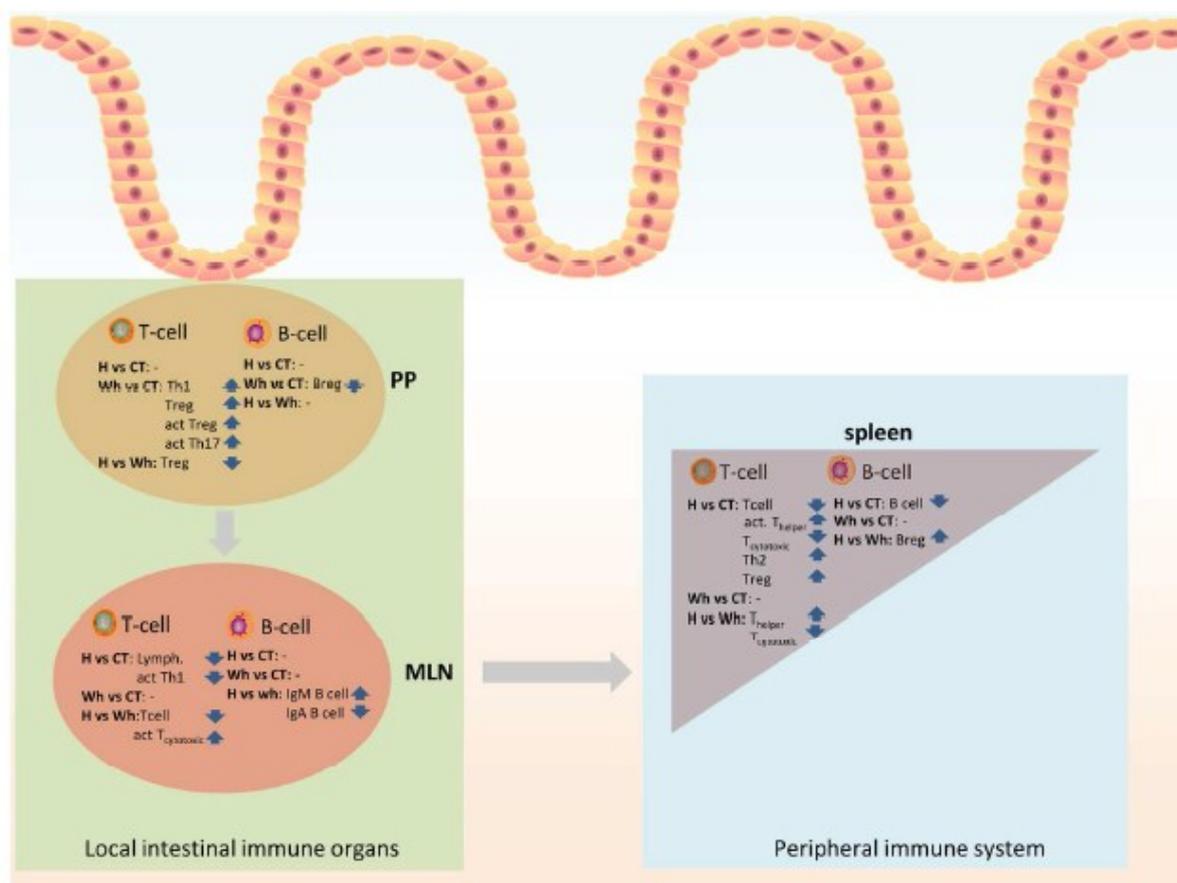


**Figure 9. Percentages of B cells and B cell subsets in the PP.** The percentage of Breg was decreased in mice sensitized with whey compared to the CT group, while no effect was observed in hydrolysate sensitized animals. Statistical differences were indicated by \*.

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**Figure 10. Overview of effects in immune cell populations observed in the study.** Many differences have been observed in T and B cell populations between hydrolysate sensitized animals (H), intact whey sensitized animals (Wh) and non-sensitized animals (CT). In the spleen of whey hydrolysate sensitized mice an increase of Breg and activated Treg was observed. In the local immune organs hydrolyzation of whey proteins lead to prevention of IgM-IgA class switching (MLN) and increased percentages of Th1, and activated Th17 and Treg cells.



## Text graphical abstract

We studied the immunomodulatory effects of partially hydrolyzed whey proteins in a mouse model for cow's milk allergy. It was shown that administration of a partially whey hydrolysate can induce sensitization against intact whey as expressed by increased whey-specific IgE levels, but that no clinical symptoms were present in these mice after a challenge with intact whey. Increased regulatory T and B cells in the systemic immune system and the prevention of increased Th1 and activated Th17 in the intestinal immune organs could contribute to the suppression of allergic symptoms.

