ORIGINAL ARTICLE-EXPERIMENTAL
MODELS OF ALLERGIC DISEASE

Inhibition of cow's milk allergy development in mice by oral delivery of β-lactoglobulin-derived peptides loaded PLGA nanoparticles is associated with systemic whey-specific immune silencing

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Abstract

Background: Two to four percentage of infants are affected by cow's milk allergy (CMA), which persists in 20% of cases. Intervention approaches using early oral exposure to cow's milk protein or hydrolysed cow's milk formula are being studied for CMA prevention. Yet, concerns regarding safety and/or efficacy remain to be tackled in particular for high-risk non-exclusively breastfed infants. Therefore, safe and effective strategies to improve early life oral tolerance induction may be considered.

Objective: We aim to investigate the efficacy of CMA prevention using oral pre-exposure of two selected 18-AA β-lactoglobulin-derived peptides loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) in a whey-protein induced CMA murine model.

Methods: The peptides were loaded in PLGA NPs via a double emulsion solvent evaporation technique. In vivo, 3-week-old female C3H/HeOuJ mice received 6 daily gavages with PBS, whey, Peptide-mix, a high- or low-dose Peptide-NPs or empty-NP plus Peptide-mix, prior to 5 weekly oral sensitizations with cholera toxin plus whey or PBS (sham). One week after the last sensitization, the challenge induced acute allergic skin response, anaphylactic shock score, allergen-specific serum immunoglobulins and ex vivo whey-stimulated cytokine release by splenocytes was measured.

Results: Mice pre-treated with high-dose Peptide-NPs but not low-dose or empty-NP plus Peptide-mix, were protected from anaphylaxis and showed a significantly lower acute allergic skin response upon intradermal whey challenge compared to whey-sensitized mice. Compared with the Peptide-mix or empty-NP plus Peptide-mix pre-treatment, the high-dose Peptide-NPs-pre-treatment inhibited ex vivo whey-stimulated pro-inflammatory cytokine TNF-α release by splenocytes.

Conclusion & Clinical relevance: Oral pre-exposure of mice to two β-lactoglobulin-derived peptides loaded PLGA NPs induced a dose-related partial prevention of CMA

symptoms upon challenge to whole whey protein and silenced whey-specific systemic immune response. These findings encourage further development of the concept of peptide-loaded PLGA NPs for CMA prevention towards clinical application.
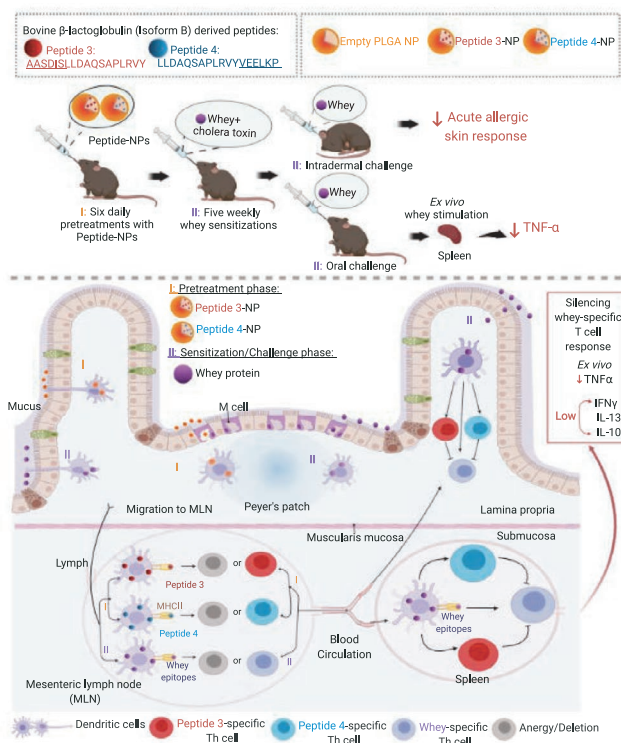
GRAPHICAL ABSTRACT

Oral pre-exposure to PLGA nanoparticles encapsulated with two selected 18-AA β-lactoglobulin-derived peptides for 6 days, prior to 5 weekly oral sensitizations to whole whey protein, significantly reduced the acute allergic skin response of 3 week-old female C3H/HeOuJ mice upon intradermal whey challenge dose dependently. Ex vivo whey stimulation recalled low IL-13, IFN-γ and IL-10 and significantly lowered TNF-α release by splenocytes in the 160 μg PLGA-encapsulated peptides recipients, but not empty PLGA nanoparticles plus peptides recipients, indicating a systemic whey silencing effect.

1 | INTRODUCTION

Cow's milk allergy(CMA) is one of the most prevalent food allergies in newborns, afflicting an estimate of 1.8–7.5% of infants in the first year of life.[1] CMA infants show symptoms including atopic dermatitis, rhinitis, diarrhoea and even anaphylactic shock upon ingestion of cow's milk-contaminated food. Currently, dietary avoidance remains as a major approach for CMA management.[2] However, children with CMA tend to develop allergy to other mammalian milk proteins, due to their structural similarity with cow's milk proteins and/or their atopic constitution.[3] Without appropriate substitutes for cow's milk, inappropriate feeding can lead to nutritional deficiencies of CMA patients. Despite most children outgrow CMA by the age of 3–5 years, they are at a higher risk of developing other atopic diseases such as asthma and rhino-conjunctivitis.[4] Thus, early intervention to prevent CMA development is imperative for augmenting CMA patients and caregivers' quality of life via alleviation of symptoms or acceleration of outgrowth of CMA.

KEY MESSAGES

- Oral pre-treatments with β-lactoglobulin-derived peptides loaded PLGA nanoparticles (BLG-peptide-NPs) prior to whey-sensitization protected mice from developing cow's milk allergy.
- BLG-peptide-NPs largely prevented the whey challenge induced acute allergic skin response in a dose-dependent manner.
- Oral tolerance induction by BLG-peptide-NPs was associated with systemic whey-specific immune-silencing and reduced TNF-α release.

The learning early about peanuts (LEAP) study showed the early introduction of peanut to high-risk infants with eczema and/or egg allergy, starting from 4 to 11 months of age, predominantly

induced allergen-specific oral tolerance and thus reduced the prevalence of peanut allergy.[5–7] These prevention effects were not found for CMA in the enquiring about tolerance (EAT) study.[8] Yet, an observational study reported a protective effect of introducing cow's milk protein to infants within 14 days after birth.[9] Nonetheless, the potential risk of adverse reactions to cow's milk protein presents as a challenge for the prevention of CMA, in particular in children at high inherited risk of developing allergies. Nowadays, partially (or extensively) hydrolysed cow's milk formulas are recommended for high-risk infants when breastfeeding is not possible. The partially hydrolysed cow's milk formulas may benefit primary prevention, however, these effects are inconsistent.[2,10,11] In previous studies, a mixture of 18-AA peptides derived from the cow's milk allergenic protein β-lactoglobulin (BLG), which may function as T cell epitopes, were shown to induce oral tolerance to whole whey protein in mice.[12,13] These peptides may be added to enrich hydrolysed infant milk formulas for non-exclusively breast-fed infants at risk, aiming to enhance oral tolerance induction for cow's milk for the purpose of primary prevention. In this study, we selected only two of these small peptides, which are too small to provoke symptoms, since they are unable to bridge two effector cell bound IgE molecules, hence preventing dimerization and mast cell degranulation.[14] However, they can still be presented in the MHCII pocket of antigen-presenting cells which allows them to provoke allergen-specific T cell responses. The T cell receptor recognition of mixtures of nine 18-AA synthetic peptide sequences derived from BLG, was tested via their capability to induce human cow's milk-specific T cell activation.[12] Oral pre-exposure using four of the nine peptides prior to whole whey protein sensitization rendered oral tolerance to whey in a CMA mouse model.[12] However, due to proteolytic degradation in the gastrointestinal tract, oral pre-exposure to these peptides would require a large dose, which is associated with an instable protective effect. To tackle this issue, Kostadinova et al. [13] encapsulated two peptides derived from BLG in poly(lactic-co-glycolic acid) (PLGA) nanoparticles PLGA (NPs) using four BLG peptides. The US Food and Drug Administration (FDA) approved PLGA as generally graded as safe (GRAS) polymer, which would allow further development of the approach for future human oral application. It is likely that the PLGA NP increase not only the survival of the peptides in the gastro-intestinal tract but also the uptake by antigen-presenting cells in the intestinal mucosa, while skewing the immune response from allergy prone T helper 2 (Th2) towards Th1 and regulatory T cell responses.[15,16]

In this study, we aim to identify the tolerance induction efficacy of only two BLG-derived peptides encapsulated PLGA NPs in CMA murine model. To this end, NP size and encapsulation efficiency (EE) of these two selected BLG-derived peptides loaded PLGA NPs were further optimized. In this study, we investigated oral tolerance induction efficacy and the underlying immunomodulatory mechanism of only two BLG-derived peptides encapsulated in PLGA NP using two different dosages in a CMA murine model.
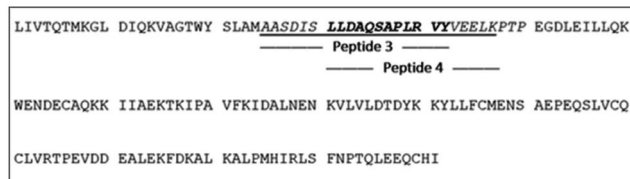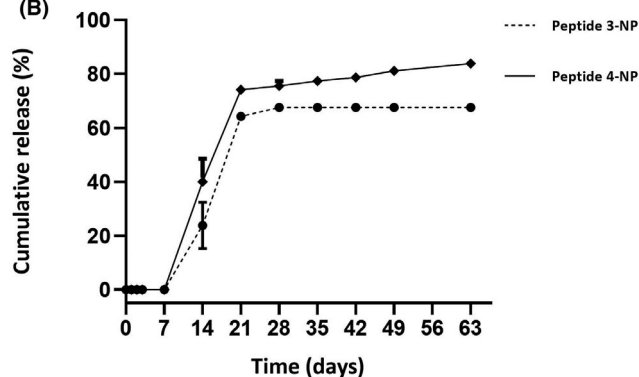

FIGURE 1 Amino acids sequence of B variant of β-lactoglobulin (A). The two selected sequential peptides (Peptide 3 and Peptide 4) used in the study were underlined and italicized. The bold amino acids sequence is the overlapping 12 amino acids. In vitro release of Peptide 3 and Peptide 4 from PLGA NPs in PBS at 37°C was conducted using cumulative method (B). Data are presented as mean ± SEM, n = 3 per formulation

2 | METHODS

2.1 | Peptides

Two 18-AA-long sequential synthetic peptides, derived from chain B of BLG, were purchased from JPT Peptide Technologies (Berlin, Germany). These two 12-AA overlapping BLG-derived peptides are indicated as Peptide 3 and Peptide 4, spanning AAs 25–47 in B variant of BLG (Figure 1A). The peptides were encapsulated in PLGA with double emulsion solvent evaporation method as published previously,[13] with applied adaptations to optimize NP size and increase EE of the peptide cargos. The detailed methods are described in the supplemental Materials and Methods section.

2.2 | In vitro studies

2.2.1 | Culture of human monocytes-derived dendritic cells (moDC)

Freshly donated and condensed blood (buffy coat) (time between donation and experiments was 24 h at most) was obtained from Sanquin Blood Supply (Amsterdam, the Netherlands). Human monocytes were isolated and differentiated into human monocytes-derived dendritic cells (moDC) using the method described in the study by Ayechu Muruzabal et al[17] (see supplemental materials and methods for details).

2.2.2 | Stimulation of human moDC and subsequent cytokine production

On day 8 immature moDC were used for co-incubation with peptide mix, Peptide-NPs or empty-NP plus Peptide mix as described in the following sections respectively, LPS was used as a positive control for moDC maturation. 5×10^5 cells in 500 μL RPMI 1640 medium (Sigma-Aldrich)/10% FBS+1% penicillin /Streptomycin (Gibco) were pulsed with 100 ng/ml Lipopolysaccharide (LPS-EB Ultrapure, from E. coli 0111:B4 strain, TLR4-based adjuvant, Invivogen), 0.4 mg/ml empty-NP, Peptide mix (containing 1.6 μg/ml Peptide 3 and 1.9 μg/ml Peptide 4), Empty-NP plus Peptide mix (containing 1.6 μg/ml Peptide 3, 1.9 μg/ml Peptide 4 and 0.2 mg/ml empty-NP), Peptide-NPs (containing 0.2 mg/ml Peptide 3-NP and 0.2 mg/ml Peptide 4-NP) for 24 h at 37°C and 5% CO_2 respectively. Subsequently, supernatants were collected for cytokine analysis. IL-10, IL-12p70, TNF-α and IL-6 were measured with human ELISA Kit (Invitrogen, Fisher Scientific) in accordance with the manufacturer's protocols. Human moDC were washed to remove free NPs and analysed with flow cytometry.

2.3 | Flow cytometry

First, human moDC were stained with fixable viability dye eFluor 780 (ThermoFisher Scientific) to exclude dead cells from analysis. No-specific staining was excluded by incubating the cells with anti-human Fcγ Receptors (Human BD Fc Block™, BD Pharmingen™) in PBS/1% BSA/2% FBS for 10 min in the dark at 4°C. For analysing the percentage of moDC, cells were stained with CD11c-PerCP/eFluor 710 and HLA-DR-PE. To analyse the expression of

costimulatory markers, cells were stained with CD80-FITC and CD86-PE/Cy7 (All from Invitrogen, Thermo Fisher Scientific). The results were obtained with BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes) and processed with FlowLogic software (Inivai Technologies).

2.4 | In vivo study

2.4.1 | Animals

Three-week-old pathogen-free female C3H/HeOuJ mice were ordered from Charles River Laboratories (Sulzfeld, Germany) and housed under standard conditions in the animal facility of Utrecht University. The work protocol was approved by the Animal Ethics committee of Utrecht University and the Center Commission for Animal use (CCD) with an approval number of AVD108002015262. Animal care and use in this study follows the guidelines of the Animal Ethics committee of Utrecht University.

2.4.2 | Oral tolerance induction, sensitization and challenge

The CMA prevention murine model has been established previously by van Esch et al.[18] To evaluate oral tolerance induction by Peptide-NPs, mice were randomly allocated into seven groups (Figure 2) and received six consecutive daily oral gavage with 0.5 ml PBS (Sigma-Aldrich) for Group 1 (sham) and 2 (whey-sensitized), 50 mg whey protein (DMV International, Veghel, the Netherlands) for Group 3 (whey tolerant), 80 μg per Peptide 3 and 4 for Group 4 (Peptide
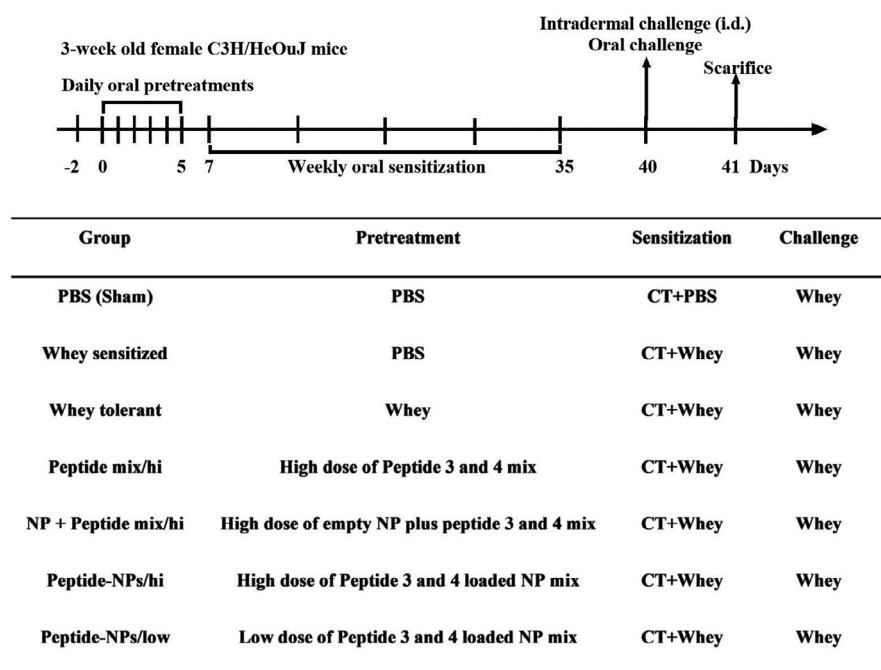

Group	Pretreatment	Sensitization	Challenge
PBS (Sham)	PBS	CT+PBS	Whey
Whey sensitized	PBS	CT+Whey	Whey
Whey tolerant	Whey	CT+Whey	Whey
Peptide mix/hi	High dose of Peptide 3 and 4 mix	CT+Whey	Whey
NP + Peptide mix/hi	High dose of empty NP plus peptide 3 and 4 mix	CT+Whey	Whey
Peptide-NPs/hi	High dose of Peptide 3 and 4 loaded NP mix	CT+Whey	Whey
Peptide-NPs/low	Low dose of Peptide 3 and 4 loaded NP mix	CT+Whey	Whey

FIGURE 2 Schematic overview of the animal model for CMA prevention and different treatments per group

mix), 18.7 mg empty-NP plus 80 µg per Peptide 3 and 4 for Group 5 (NP + Peptide mix/hi), 18.9 mg Peptide-NPs mixture (containing 80 µg per encapsulated Peptide 3 and 4) for Group 6 (Peptide-NPs/hi) and 8.5 mg Peptide-NPs mixture (containing 40 µg encapsulated Peptide 3 and 4) for Group 7 (Peptide-NPs/low) (Figure 2). For oral gavage, the peptides and NPs were dissolved or dispersed respectively in 0.5 ml PBS per mouse. Two days after the last oral administration with the indicated pre-treatment, mice received five consecutive weekly oral sensitizations with 20 mg whey plus 10 µg cholera toxin (CT; List Biological Laboratories, Inc.) as adjuvant in 0.5 ml PBS for Group 2–7 to break oral tolerance for whey, and 10 µg cholera toxin in 0.5 ml PBS for the non-sensitized mice in Group 1. Five days after the last oral sensitization, mice were intradermally and orally challenged with 10 µg or 20 mg whey respectively. The allergic response including acute skin response (Δ ear swelling thickness), body temperature and anaphylactic shock core within 1 h upon intradermal challenge were assessed. Eighteen hours after oral challenge, mice were anaesthetized with isoflurane and euthanized.

2.5 | Evaluation of acute allergic response

To evaluate the efficacy of the allergy prevention of the different pre-treatments, the acute allergic skin response upon intradermal challenge with 10 µg whey protein in 20 µL PBS was recorded as the primary outcome. The ear pinnae thickness of the mice was measured twice with a digital micrometer (Mitutoyo) before and 1 h after intradermal ear challenge with whey. Mice were under anaesthesia by inhalation of isoflurane prior to measurement of the ear pinnae and injection of whey. Δ Ear pinnae thickness is calculated by subtraction of the basal ear pinnae thickness as indicated in the following formula. Simultaneously, the anaphylaxis symptoms score was determined at 15 min and 1 h after intradermal challenge with whey according to Schouten et al.[19] The evaluation of the acute allergic response was performed with the cage number concealed to avoid bias

$$\Delta \text{Ear pinnae thickness} = \text{Average thickness}_{\text{1h after id challenge}} - \text{Average thickness}_{\text{before id challenge}}$$

2.6 | ELISA detection of serum Whey- and BLG-specific immunoglobulins

Whey-specific and BLG-specific immunoglobulins were measured in the sera collected 18 h after oral challenge with whey using a previously reported method.[20]

2.7 | Cells isolation from spleen

Splenocytes were isolated by sieving the spleens through 70 µm sterile cell strainers (Nylon, Falcon) and rinsed with 10 ml RPMI 1640 medium. To lyse red blood cells, 1 ml lysis buffer (155 mmol

NH_4Cl, 10 mmol $KHCO_3$ and 0.127 µmol EDTA, filtered with 0.22 µm filter (Cellulose Acetate, Whatman™, GE Healthcare Life Sciences), was added into the splenocyte suspension and incubated on ice for 4 min. The lysis of red blood cells was stopped by the addition of RPMI 1640 medium (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% FBS, 1% Penicillin/Streptomycin. The isolated splenocytes were resuspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 1% Penicillin/Streptomycin on ice until use.

2.8 | Ex vivo re-stimulation of splenocytes and cytokine measurements

6×10^5 splenocytes per well and suspended in 200 µL RPMI 1640 medium supplemented with 10% FBS +1% Penicillin/Streptomycin were cultured in a round bottom culture plate, and the cells were stimulated with either 500 µg/ml whey protein (DMV International), or medium as control and cultured at 37°C, 5% CO_2. After 5 days stimulation with whey protein and medium, the supernatants were collected and stored at −20°C until further analysis. The cytokine concentrations in the collected supernatants were measured with mouse IFN-γ, IL-13, IL-5, IL-17A, IL10 or TNF-α ELISA kits (ThermoFisher), conforming to the ThermoFisher protocols. Absorbance was measured at 450 nm by a Benchmark plate reader (Bio-Rad). In addition, cytokine production was measured in the supernatant after 2 days ex vivo stimulation of splenocytes with 1 µg/ml anti-mouse CD3 antibody (eBioscience, ThermoFisher Scientific) or medium as control and cultured at 37°C, 5% CO_2.

2.9 | Statistical analysis

The obtained *in vivo* data were analysed with GraphPad Prism 8.0.1 software (GraphPad Software). For each statistical analysis, multiple comparisons were conducted for the selected pairs: the whey-sensitized group was compared to all the other groups and the Peptide-NPs/hi group was compared to all groups except the non-sensitized group. For data sets that were not normal distributed as indicated by the Kolmogorov–Smirnov test or the Shapiro–Wilk test, square root transformation was performed. One-way ANOVA followed by Bonferroni's *post hoc* test for selected pairs was performed. Otherwise, Kruskal–Wallis' non-parametric test followed by Dunn's *post hoc* test for selected pairs was applied. Data are presented as mean ± SEM for *n* = 9–10 per group except for the non-sensitized group (*n* = 3) and the whey-tolerant group (*n* = 6). For correlation analysis of acute allergic skin response and sera immunoglobulins, non-parametric Spearman correlation coefficient test was conducted using GraphPad Prism 8.0.1 software.

TABLE 1 Characteristics of Peptide 3-NP, Peptide 4-NP and empty-NP (mean ± SD)

Nanoparticles (NP)	Z-Ave (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Loading capacity (%)
Empty-NP (*n* = 6)	280 ± 28	0.13 ± 0.07	−1.3 ± 0.2	NA	NA
Peptide 3-NP (*n* = 4)	287 ± 40	0.13 ± 0.10	−1.1 ± 0.1	78 ± 10	0.8 ± 0.1
Peptide 4-NP (*n* = 4)	271 ± 30	0.11 ± 0.01	−1.1 ± 0.1	84 ± 7	0.9 ± 0.1

3 | RESULTS

3.1 | Characteristics of empty and BLG-derived peptides loaded PLGA NPs

Placebo and PLGA NPs loaded with BLG-derived Peptide 3 or 4 were prepared using a double emulsion solvent evaporation method. Empty-NP, Peptide 3-NP and Peptide 4-NP had a close to neutral zeta potential and similar size of 280 ± 28, 287 ± 40 and 271 ± 30 nm respectively (Table 1). 18 Amino acids sequence of Peptides 3 and 4 were selected from the B variant of BLG protein (adapted from Meulenbroek et al[12]) (Figure 1A). Both Peptide 3-NP and Peptide 4-NP were encapsulated with a high EE of 78 ± 10 and 84 ± 7% respectively. Peptide 3-NP and Peptide 4-NP have a loading capacity of 0.8 ± 0.1 and 0.9 ± 0.1% respectively. Peptide 3-NP and Peptide 4-NP showed no burst release in the in vitro release study (Figure 1B). Sustained-release profiles of Peptide 3 and 4 from PLGA NP matrices were observed from 1 to 3 weeks, showing a cumulative release of 68% and 85% respectively (Figure 1B).

3.2 | Effect of BLG-derived peptides loaded NPs (Peptide-NPs) on human moDC activation in vitro

Figure 3A shows incubation of medium, LPS, Peptide-NPs, Peptide mix or empty-NP plus Peptide mix for 24 h did not affect cell viability. Figure 3B shows that incubation of immature human moDC with Peptide-NPs, Peptide mix or empty-NP plus Peptide mix did not influence HLA-DR expression on. As for human moDC maturation, pulsing immature human moDC with Peptide-NPs, Peptide mix or empty-NP plus Peptide mix did not induce an increase in surface expression of costimulatory molecules CD80 or CD86 compared to the medium control, whereas positive control LPS induced a significant higher surface expression of costimulatory molecules CD80 and CD86 on moDC than the other groups (Figure 3C,D). moDC incubation with LPS elicited a significant increase in production of IL-12p70, IL-10, TNF-α and IL-6 compared to medium controls (Figure 3E,H). Compared to the medium-treated group, moDC co-incubated with empty PLGA-NP also showed a higher cytokine release of IL-12p70, IL-10 and TNF-α. However, this effect was lost when the moDC were incubated with empty PLGA-NP plus free peptides or the peptide-encapsulated NPs.

The free peptide mixture did not provoke cytokine release by moDC (Figure 3E,H).

3.3 | Oral pre-exposure to BLG peptides loaded PLGA NPs dose dependently induces allergen-specific oral tolerance for whole whey protein

The acute allergic skin response of mice from all groups was measured prior to and 1 h upon i.d. challenge in the ear pinnae with whole whey protein (Figure 4). Notably, the PBS-pre-treated whey-sensitized group showed a significantly increased acute skin response when compared to the non-sensitized group (***p < .001), while whole whey-pre-treatment largely prevented allergic symptoms indicating oral tolerance induction in this whey-tolerant group. A significant alleviated acute allergic skin response was observed in the high-dose Peptide-NPs pre-treated group (*p < .05) compared to the whey-allergic mice, the high-dose free Peptide mix group and also the low dose of Peptide-NPs group. The latter two interventions were unable to prevent allergy development. However, also the pre-treatment with empty-NP plus high-dose free Peptide mix tended to reduce the acute allergic skin response in comparison to the whey allergic group, albeit this effect was not significant.

Anaphylactic shock scores of the mice were recorded 15 min and 60 min upon intradermal challenge with whole whey protein. As a result, the whey-sensitized mice showed a significant higher shock score than the other groups 15 min after the challenge. After 60 min, all the non-sensitized mice and whey-tolerant mice scored 0 for anaphylaxis, while the observed mice with the highest shock score 3 were from the whey-allergic and the empty-NP plus high-dose Peptide mix pre-treated group. The maximum shock score in the high-dose Peptide-NPs group was 1.

3.4 | Whey-specific and BLG-specific serum immunoglobulins

To investigate the influence of the pre-treatments on the humoral immune response to whole whey protein or BLG, whey-specific and BLG-specific-immunoglobulins were measured in the sera collected 18 h after oral challenge with whole whey protein. The whey-sensitized group showed a significant higher level of whey-specific IgG1 and BLG-specific IgG1 in comparison to that of the non-sensitized group (Figure 5B,E). Even though the different pre-treatments modified the pattern of immunoglobulin release similar to the effects shown on the ear swelling response, no significant results were obtained.

FIGURE 3 Effect of Peptide-NPs, Peptide mix, empty-NP and Peptide mix plus empty-NP on human moDC activation in vitro. After incubation of human moDC with medium, LPS, Peptide mix, empty-NP, Peptide mix plus empty-NP or Peptide-NPs for 24 h, cell viability (A), % MHC class II expression by human moDC (B) and surface expression of co-stimulatory molecules in HLA-DR-positive moDC (C,D) were evaluated by flow cytometry. IL12p70 (E), IL-10 (F) and pro-inflammatory IL-6 and TNF-α (G,H) concentrations were measured in the supernatant. Data are presented as mean ± SEM for n = 3 independent experiments per group. Comparison between medium and the other groups were analysed with one-way ANOVA followed by Bonferroni's post hoc test for selected pairs for (A–D), after square root transformation for (E), (G) and (H) and after log transformation for (F).*p < .05, **p < .01, ***p < .001, ****p < .0001
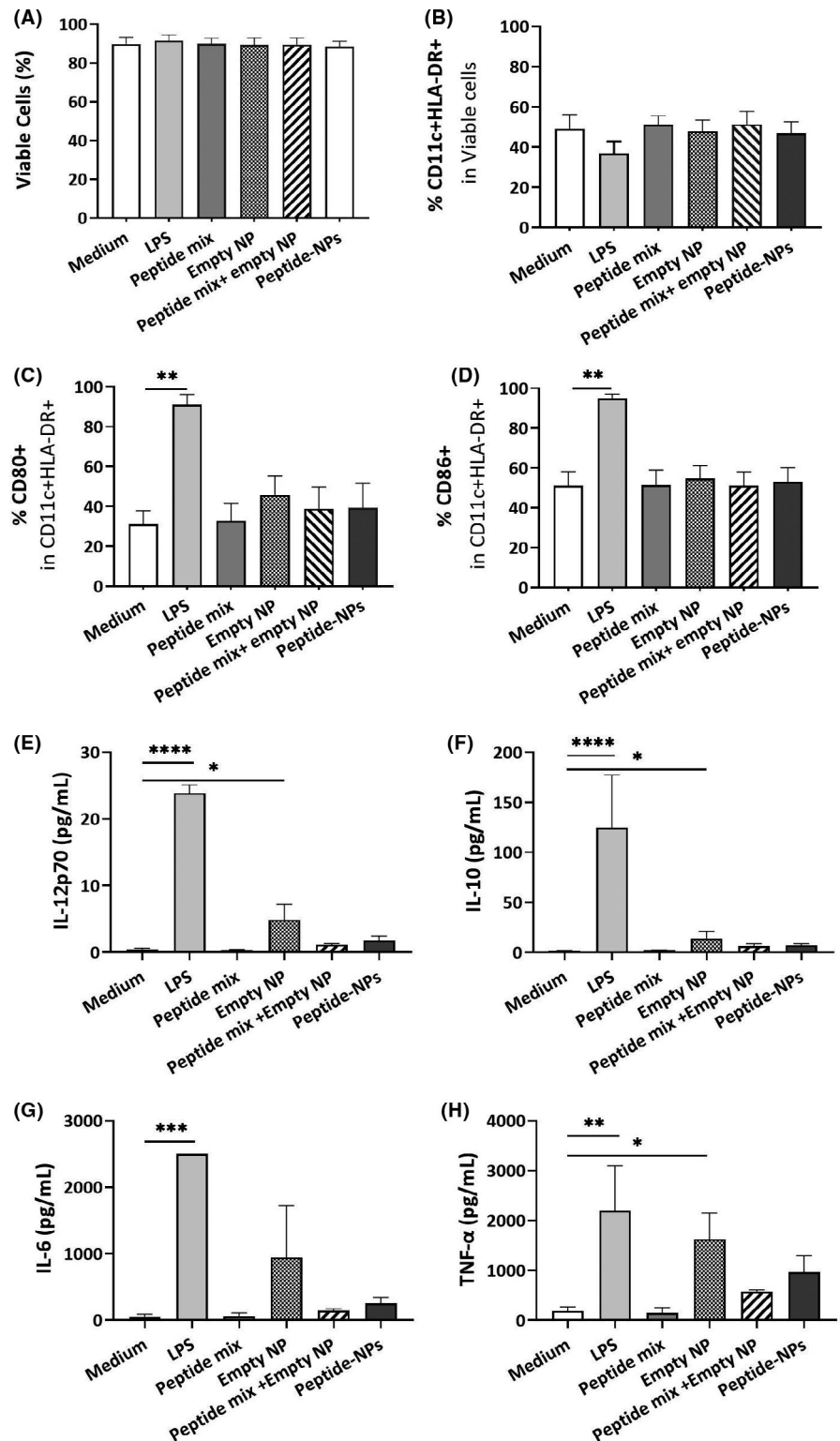
3.5 | Cytokine production by splenocytes upon ex vivo re-stimulation with whey

Upon ex vivo whey re-stimulation, the general pattern of the cytokine secretion levels of IFN-γ, IL-13, IL-10 and TNF-α was not significantly different between the non-sensitized, whey-sensitized and whey-tolerant group (Figure 6A–D) (for full data set see Figure S3). However, the high-dose Peptide-NPs pre-treated group

showed low levels of IL-13, IL-10 and TNF-α release. Similar to the anti-CD3 stimulated groups (Figure S4A–D), the high-dose Peptide-NPs pre-treated group showed significantly lower whey-stimulated TNF-α release than the whey-sensitized mice (**p < .01) as well as the high-dose free Peptide mix (***p < .001), and the empty-NP plus high-dose Peptide mix (**p < .01). In addition, whey and anti-CD3 induced secretion of IL-5 and IL-17A by splenocytes were measured (Figure S5A–D).
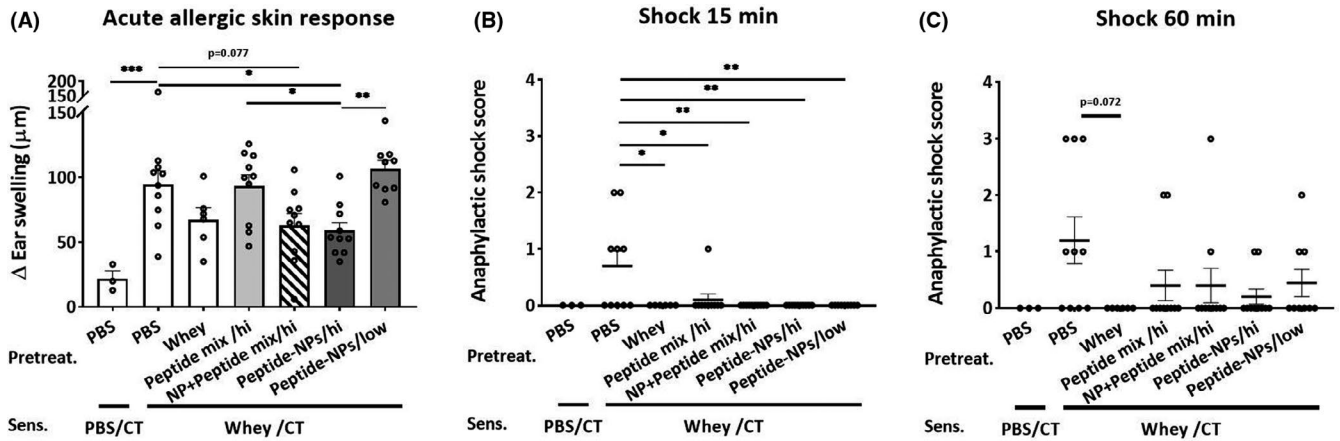
FIGURE 4 Acute allergic skin response and anaphylaxis score. Five days after last sensitization, mice were i.d. challenged in the ear pinnae with 10 µg whey followed by oral challenge. The acute allergic skin response was measured 60 min afterwards (A), and signs of anaphylaxis were scored after 15 min (B) and 60 min (C). Data are presented as mean ± SEM for n = 9–10 per group except for the non-sensitized group, n = 3 and whey-tolerant group, n = 6. (A) is analysed by one-way ANOVA, followed by Bonferroni's *post hoc* test for selected pairs; (B) and (C) are analysed with Kruskal–Wallis' non-parametric test, followed by Dunn's *post hoc* test for selected pairs. *p < .05, **p < .01, ***p < .001. CT, Cholera toxin
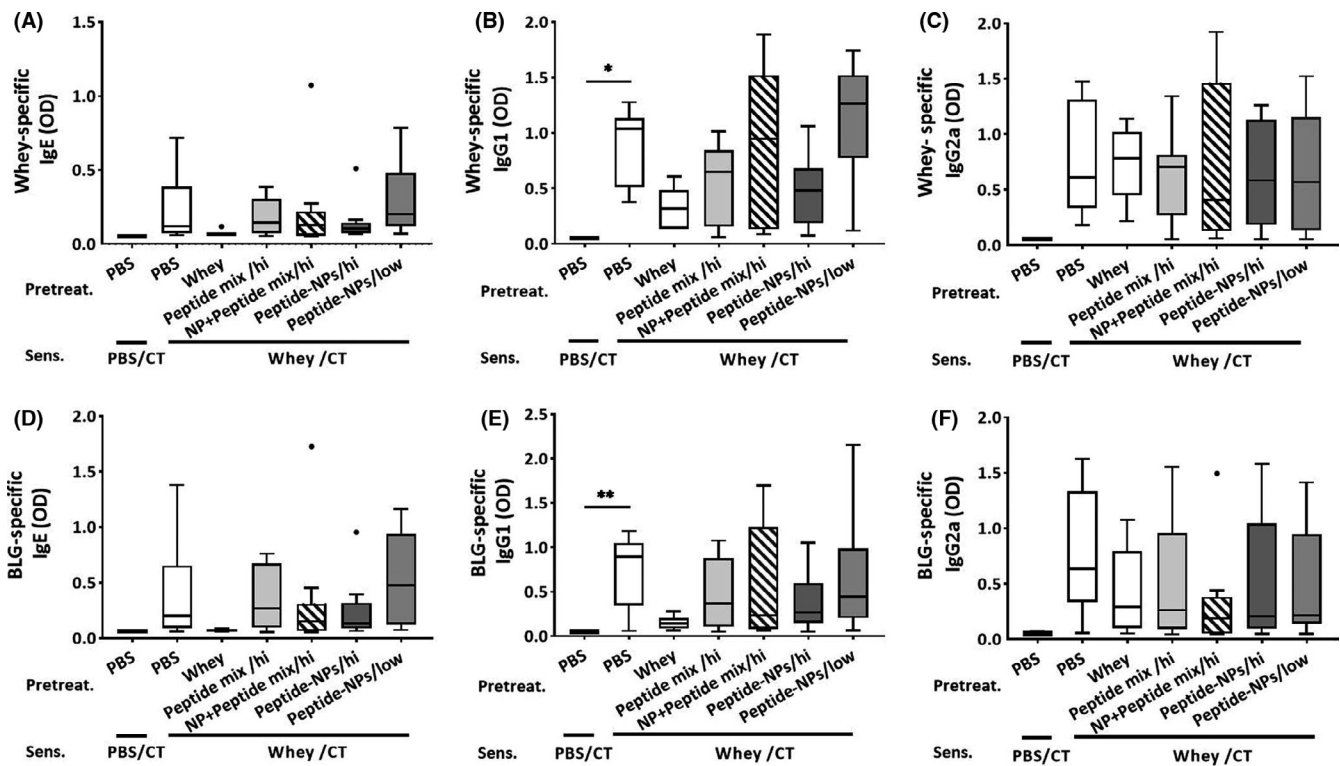

FIGURE 5 Whey- and BLG-specific serum immunoglobulins levels. Whey- (A–C) and BLG-specific (D,F) IgE, IgG1 and IgG2a levels are measured in sera, which were collected 18 h after last oral challenge with whey in mice from all groups. Data are presented as Tukey box-and-whisker plots for n = 9–10 per group except for the non-sensitized group, n = 3 and whey-tolerant group, n = 6. (A–F) are analysed with the Kruskal–Wallis non-parametric test, followed by Dunn's *post hoc* test for selected pairs; *p < .05, **p < .01; BLG, β-lactoglobulin; CT, Cholera toxin

4 | DISCUSSION

CMA is one of the first allergies to develop early in life and disrupts the quality of life of newborns and their caretakers. Gut-associated lymphoid tissues (GALT) have been regarded as an ideal site for

oral tolerance induction, as intestinal dendritic cells in the GALT can take up the oral administered antigens and present to T cells in the Peyer's patch and mesenteric lymph nodes to generate systemic immune tolerance.[14,21–25] Therefore, our study investigated and confirmed the early life allergen-specific tolerance induction

FIGURE 6 Cytokine production after *ex vivo* re-stimulation of splenocytes with whole whey protein. Splenocytes were *ex vivo* re-stimulated either with whole whey protein for 5 days, Th1- (A), Th2- (B), regulatory (C) and proinflammatory (D) associated cytokines were measured in supernatants; Data are presented as mean ± SEM for n = 9–10 per group except for the non-sensitized group, n = 3 and whey-tolerant group, n = 6. (A)–(D) are analysed with one-way ANOVA followed by Bonferroni's *post hoc* test for selected pairs, after log transformation for (A–C) and after square root transformation for (D); *p < .05, **p < .01, ***p < .001; CT, Cholera toxin
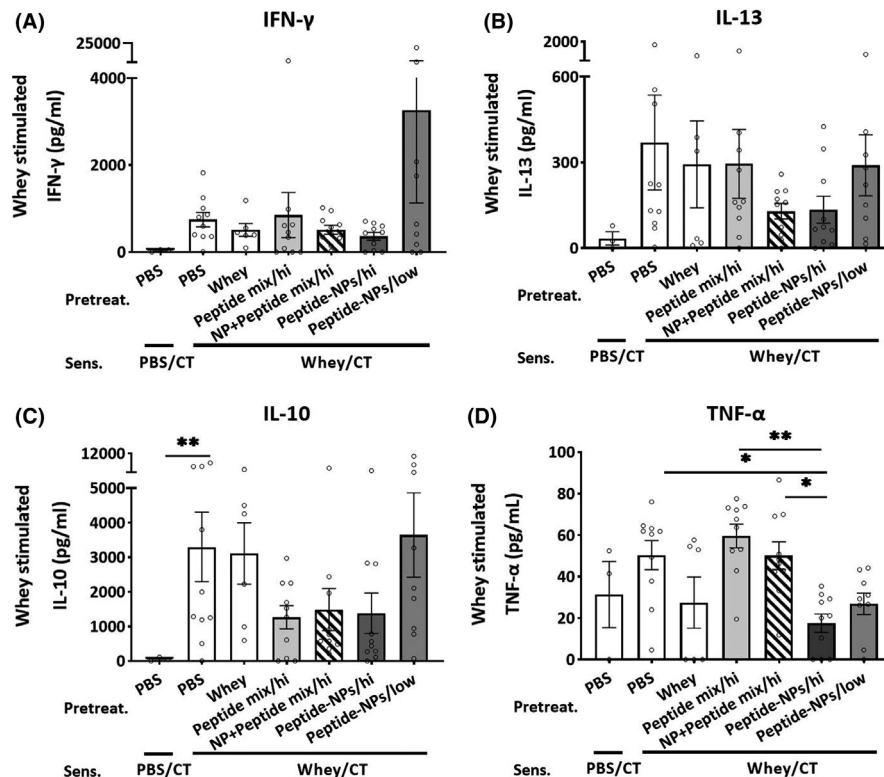
effect in a murine CMA prophylatic model via oral pre-exposure to only 160 µg encapsulated two selected 18-AA BLG peptides in PLGA NPs.

Previously, Kostadinova et al.[13] reported oral delivery of four selected 18-AA BLG-derived peptides (40 µg per Peptide), namely Peptide 1 and 2 mix plus PLGA NPs encapsulated Peptide 3 and 4, effective in modulating mucosal immunity by increase in *ex vivo* whey stimulated TGF-β release by intestinal lamina propria cells. This study only made use of the two selected Peptide 3 and 4 loaded PLGA NPs, which were further optimized in terms of NP size, EE and *in vitro* release profile. The NP size of ~280 nm (Table 1) is smaller than the previous mean NP size of 320 nm,[13] facilitating penetration through the mucus and cellular uptake by intestinal M cells or enterocytes.[26,27] In addition, the EE of Peptide 3 in PLGA NPs has now been improved from 30% in the previous study[13] to 78% in this study. In vitro release study of Peptide 3-NP and Peptide 4-NP in PBS showed no burst release and a lag phase of 7 days, followed by a sustained release from week 1 to 3, which is likely resulting from hydrolysis of the PLGA polymeric matrix.[28] This observed absence of burst release of Peptide 3-NP is likely to be attributed to sufficient washing procedures and/or absence of porosity in the lyophilized Peptide-NPs, confirming the successful and improved encapsulation of these two peptides.

In vitro cellular uptake of empty PLGA NP by murine bone marrow-derived dendritic cells and human moDC was reported previously.[13,29] In vitro, we found human moDC remained immature phenotype upon stimulation with Peptides-NPs, showing no up-regulation of surface expression of co-stimulatory molecules CD80/

CD86. This immature human moDC is often regarded as a prerequisite for tolerance induction. As reported, the CD80^{low}/CD86^{low} human immature dendritic cells have the potential to induce tolerance by promoting differentiation of naïve T cells into Treg cells after repetitive stimulation under steady conditions.[30–32] Furthermore, empty-NP induced a small increase in Th1, pro-inflammatory and regulatory associated cytokines, namely IL-12p70, TNF-α and IL-10 release by human moDC. Remarkably, this phenomenon was abolished when the moDC were pulsed with co-administration of the two BLG peptides plus PLGA NPs. This neutralization effect for Th1 associated cytokine IL-12p70 release, but not TNF-α release, was also found when pulsing BMDC with Peptide 1, 3 and 4 loaded PLGA NP in a previous study.[13] It is not unlikely that the previously reported in vivo Th1 and Th2 immune silencing effect of Peptide 4, neutralized the Th1 skewing effect of the PLGA NPs.[12] Yet, future studies are warranted to confirm this hypothesis.

In this study, the oral pre-exposure to a relatively low required dose (160 µg) of Peptide 3 and 4 loaded PLGA NPs (160 µg) and short duration (6 days) prior to whey sensitizations, significantly reduced the acute allergic skin response upon intradermal whole whey protein challenge. The dose of peptides used in this study is very small as compared to the 4 mg per Peptide 3 or 4 in the previous murine prophylatic CMA study by Meulenbroek et al.[12]

Previous studies showed PLGA NPs exert a Th1 adjuvant effect, but PLGA NP alone did not protect against allergy development.[13,33] However, the simultaneous presence of PLGA NPs and antigen may have facilitated allergen-specific tolerance concomitantly. As reported previously,[34–36] NPs of approximately 200 nm or less can

efficiently diffuse into the lymphatic system and be internalized by dendritic cells, which may account for the observed improved tolerance induction effect in our study.

Consistent with a previous study by Kim et al.,[22] this observed preventive effect is dose dependent, since the dosage of 40 μg per encapsulated peptide in PLGA NP did not protect against allergy development. Although oral pre-exposure to a high dose of Peptide-NPs, prior to whey sensitization, did not lower whey- and BLG-specific IgE, IgG1 and IgG2a compared to the whey allergic group. A similar declining pattern in immunoglobulin levels comparable to the skin response was observed. In accordance with the previous study,[19] the acute allergic skin response and whey- and BLG-specific IgE, IgG1 and IgG2a levels were positively correlated (Figure S1), connecting the intensity of allergic sensitization and the outcome of the effector response.

In contrast to previous findings of regulatory T cells' development induced by PLGA NP,[16] the frequency of the systemic Treg, as well as CD4+ cells, Th1 and Th2 subsets in splenocytes remained unaffected in this study (Figure S2). However, oral pre-exposure to PLGA NP, with or without peptides incorporation, attributed to an increasing pattern of mRNA expression of regulatory cytokine IL-10 in middle intestines after oral whey challenge (Figure S6). Regulatory cytokines, such as IL-10, and its increased intestinal expression were found associated with allergy protection.

Despite no significant decrease observed, the high-dose Peptide-NPs pre-treatment maintained a relative low ex vivo whey stimulated IL-13 and IL-10 release by splenocytes compared to the whey-sensitized group. Furthermore, the high-dose Peptide-NPs pre-treatment, but not the low-dose counterpart, significantly reduced the whey-stimulated TNF-α release, indicating allergen-specific immune silencing was established in a dose-dependent manner. TNF-α is principally associated with Th1-mediated inflammation,[37] but also plays a key role in enhancing Th2-polarizing cytokines release and antigen-specific IgE production.[38,39] Indeed, the silencing of ex vivo whey-stimulated TNF-α release by splenocytes suggests the systemic immunomodulatory effect of oral pre-exposure to Peptide-NPs, in association with the observed ameliorated acute allergic skin response.

Only the high-dose Peptide-NPs, but not empty-NP plus equal amount of free Peptide mix, showed high efficacy in immune silencing and significant antigen-specific suppression of TNF-α as compared to the whey-sensitized group. This further underlines the relevance and importance of the finding that the high dose of Peptide-NPs (80 μg per peptide) is capable of largely protecting against whole whey sensitization. Furthermore, it has highlighted the superiority of PLGA NP-encapsulated peptides over the treatment with peptides that were co-delivered with PLGA NP, which may be attributed a more potent immunomodulatory effect by the antigen-encapsulated PLGA NPs as was also reported by Gu et al.[40] Indeed, previous studies have shown peptide 3 to be able to activate human T cells and it also was predicted in silico to be presented by human MHCII (HLA-DRB1).[41] Future studies are needed to confirm in vivo presentation

of the PLGA NPs delivered peptides in the MHCII cleft of the DC and the generation or deletion of T cells recognizing peptides 3 or 4.

Our findings in the dose-dependent tolerance induction effect of the peptide-encapsulated PLGA NPs are consistent with the previous studies,[42,43] suggesting different mechanisms of oral tolerance involved for low and high dose of oral administered antigens. As reported, deletion of antigen reactive Th1 and Th2 cells induced by high dose of oral administered antigens, is abolished when using a lower antigen dosage. Instead, a lower dosage of oral administered antigens showed an active suppression by increasing antigen-stimulated IL-10 release.[43] Nevertheless, the high-dose Peptide-NPs group showed both lower ex vivo generic T cell receptor anti-CD3 stimulated regulatory and inflammatory associated cytokine production, IL-10 and TNF-α, than the whey-sensitized group (Figure S4) and lowered whey-stimulated TNF-α release, while maintaining low levels of whey-specific IL-13, IFN-γ and IL-10. We hypothesize that the underlying mechanism by which Peptide-NPs mediated this immune silencing differs from the mechanism by which whole whey protein facilitated tolerance induction. In the whey-tolerant group, the regulatory IL-10 secretion remained unaffectedly high, whereas TNF-α secretion was low, hence shifting the immune balance in favour of the regulatory response which may have dampened allergic sensitization and clinical symptoms. Similarly, the low-dose Peptide-NPs also showed this pattern, but in this group the IFN-γ and IL-13 release tended to increase. In contrast to whey pre-treated mice, the low-dose Peptide-NPs pre-treatment did not tend to lower the whey-immunoglobulin levels. Therefore, the T cell modulatory effect in the low-dose Peptide-NPs may have been insufficient to affect allergic sensitization and whey-induced symptoms. Collectively, the high-dose Peptide-NPs (160 μg) or 50 mg whey protein showed similar reduction in acute allergic skin response but disparate whey stimulated cytokine release pattern, indicating a different tolerance induction mechanism.

Ultimately, this study indicates full tolerance induction/ immune silencing for whole whey protein at the level of the T cells, while using only 160 μg two small peptides derived from BLG. This may be accomplished if these peptide 3 or 4 sequences consist of immunodominant epitopes of whey proteins, when processed and expressed in MHCII by DC and presented to the T cells. The small required dose of these BLG peptides may be attributed to the prolonged retention time of the PLGA NP-encapsulated peptides in dendritic cells as reported in previous studies.[33,44,45] Furthermore, the BLG peptides as well as PLGA nanocarriers have been shown to instruct regulatory T cell development in vivo and in the in vitro studies.[12,16] We hypothesize that the two PLGA NPs encapsulated BLG peptides may instruct a tolerogenic DC phenotype, presenting the antigen via their MHC class II, which contributes to the immune silencing effect at the T cell level even for whole whey protein either in an epitope-specific fashion or via bystander suppression.

Silencing of the T cell response may consequently suppress the generation of whey- and BLG-specific IgE/IgG1, but here no statistical significance was observed. Allergen-specific B cell selection

occurs via direct binding of the allergen to the B cell receptor. B cells may therefore be responsive to a broader/different spectrum of epitopes (B cell epitopes) as compared to T cells. Hence, to further improve tolerance induction also at the level of the B cells, supplemental addition of other whey/BLG epitopes may help which needs to be addressed in future studies.

In summary, this study has proven that oral delivery of only two BLG peptides encapsulated in PLGA NP is effective in partial oral tolerance induction for whole whey protein in a dose-dependent manner. Our findings substantiate PLGA NP encapsulation of the peptide cargo as a relevant strategy for optimal antigen-specific oral tolerance induction. Oral pre-treatment with BLG peptides loaded PLGA NP might be a promising CMA prevention strategy, circumventing the potential adverse effect for children at high risk of CMA while enhancing the chance to develop oral tolerance, that warrants further clinical validation. For future study, incorporation of additional BLG epitopes and a Th1 and Treg skewing adjuvant may further optimize the tolerance induction effect of the current preventive approach or for development of therapeutic purposes.

AUTHORS CONTRIBUTIONS

L.E.M. Willemsen, M. Liu and S. Thijssen designed the experiments; M. Liu and S. Thijssen performed the experimental procedures. M. Liu performed data collection and analyses and drafted the manuscript. L.E.M. Willemsen, C.F. van Nostrum, W.E. Hennink and J. Garssen contributed to data interpretation and critically revised the manuscript.

DATA AVAILABILITY STATEMENT

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.