

ORIGINAL ARTICLE

Oral treatment with β -lactoglobulin peptides prevents clinical symptoms in a mouse model for cow's milk allergy

Laura A. P. M. Meulenbroek^{1,2}, Betty C. A. M. van Esch^{1,3}, Gerard A. Hofman¹, Constance F. den Hartog Jager², Alma J. Nauta^{1,4}, Linette E. M. Willemsen¹, Carla A. F. M. Bruijnzeel-Koomen², Johan Garssen^{1,3}, Els van Hoffen² & Léon M. J. Knippels^{1,3}

¹Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands;

²Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht, The Netherlands; ³Department of Immunology, Danone Research Centre for Specialised Nutrition, Wageningen, The Netherlands; ⁴Department of Immunology, Danone Research Centre for Specialised Nutrition, Singapore

To cite this article: Meulenbroek LAPM, van Esch BCAM, Hofman GA, den Hartog Jager CF, Nauta AJ, Willemsen LEM, Bruijnzeel-Koomen CAFM, Garssen J, Van Hoffen E, Knippels LMJ. Oral treatment with β -lactoglobulin peptides prevents clinical symptoms in a mouse model for cow's milk allergy. *Pediatr Allergy Immunol* 2013; **00**.

Keywords

animal model; β -lactoglobulin; CD103+ dendritic cells; cow's milk allergy; non-digestible oligosaccharides; oral tolerance; peptides; prophylaxis; regulatory T cells; T cell epitopes

Correspondence

Léon Knippels, Department of Immunology, Danone Research Centre for Specialised Nutrition, PO Box 7005, Wageningen 6700 CA, The Netherlands.
Tel.: +31 30 2095 0000
Fax: +31 6 5267 9187
E-mail: Leon.Knippels@danone.com

Present address

Els van Hoffen, NIZO Food Research BV, Ede, The Netherlands.

Accepted for publication 18 July 2013

DOI:10.1111/pai.12120

Abstract

Background: Prior exposure to partial whey hydrolysates has been shown to reduce the allergic response to whey in mice. This effect was more pronounced in combination with a diet containing non-digestible oligosaccharides (scGOS/lcFOS/pAOS). It is unknown which fractions/epitopes are responsible for this effect. Therefore, the prophylactic ability of synthetic peptides of β -lactoglobulin with/without a scGOS/lcFOS/pAOS-containing diet to reduce the allergic response in a mouse model for cow's milk allergy was investigated.

Methods: Of 31 peptides, nine peptides were selected based on human T cell data. Mice were pre-treated orally with three peptide mixtures or single peptides for six consecutive days. During this period, they received a control or scGOS/lcFOS/pAOS-containing diet. Subsequently, mice were orally sensitized to whey and received an intradermal and oral challenge. After sacrifice, serum and mesenteric lymph nodes (MLN) were collected for further analysis.

Results: Prior exposure to peptide mixtures 1 and 3 significantly reduced the acute allergic skin response to whey. Mixture 2 showed no effect. An additive effect of the scGOS/lcFOS/pAOS-containing diet was only observed for mixture 1. Of the peptides in mixture 1, one peptide (LLDAQSAPLRVYVEELKP) showed the strongest effect on the acute allergic skin response. This peptide also tended to decrease whey-specific antibody levels and to increase the percentages of CD11b+CD103+ dendritic cells and CD25+Foxp3+ T cells in the MLN.

Conclusions: Prior exposure to specific peptides of β -lactoglobulin reduces the allergic response to whey, which may involve regulatory dendritic and T cells. Combining peptides with a sGOS/lcFOS/pAOS-containing diet enhances this effect.

Cow's milk allergy (CMA) is a general health problem, affecting 0.3–3.5% of the young children (1, 2). Although 60–75% of the children with an IgE-mediated CMA spontaneously develop tolerance before their fifth year of life, the risk of developing other atopic disorders later in life, such as asthma and rhinoconjunctivitis, is increased (3–5).

In food-allergic patients, oral tolerance to food proteins is disrupted. For years, it was thought that decreasing the exposure to allergens would reduce the development of allergy (6, 7). Therefore, children at high risk of developing food

allergy have been advised to eliminate allergens from their diet. However, recent studies have indicated that exposure to allergens may be beneficial (8, 9). In Jewish children in Israel, in which the common practice is to consume peanut snacks at an early age, a lower risk of developing peanut allergy was observed compared with Jewish children in the UK who were not exposed to peanut (8).

Interestingly, not only entire proteins but also protein fragments may be used to induce oral tolerance. Previous research in mice has demonstrated that preventive oral

treatment with peptides obtained by tryptic hydrolysis of β -lactoglobulin (β -LG), the major allergen in the whey fraction of cow's milk, reduced the allergic responses to intact β -LG (10). Recently, we have shown that preventive oral treatment with partial whey hydrolysates diminished the allergic symptoms in a mouse model for CMA (11). However, treatment with extensive whey hydrolysates, which contained only small protein fragments (<5 kDa), had no effect. Similar results were seen in a study with rats (12). Although tolerance could be induced using partial whey hydrolysates, it remains to be elucidated which exact fragments are responsible for the observed effects.

Not only exposure to proteins but also other factors may influence tolerance induction. Previous studies have indicated that a specific mixture of non-digestible short-chain galactose (scGOS) and long-chain fructo-oligosaccharides (lcFOS, in a ratio of 9:1) can influence the intestinal microbiota (13, 14). This diet stimulated the growth of *Bifidobacteria* in formula-fed infants to similar counts as in breast-fed infants (14, 15). Moreover, *in vivo* studies in both human and mice revealed that the scGOS/lcFOS diet with/without pectin-derived acidic oligosaccharides (pAOS, in a ratio of 9:1:2) reduced allergic manifestations (16–19). Mouse studies indicated that this effect was mediated through the induction of Th1 cells or regulatory T cells (18, 19). Also, the effect of oligosaccharides on tolerance induction has been investigated before. The tolerance-inducing capacity of whey hydrolysates in a mouse model for CMA was increased by the addition of scGOS/lcFOS/pAOS to the diet (van Esch BCAM, de Kivit S, Hofman GA, Garssen J, Willemsen LEM, Knippels LMJ, submitted).

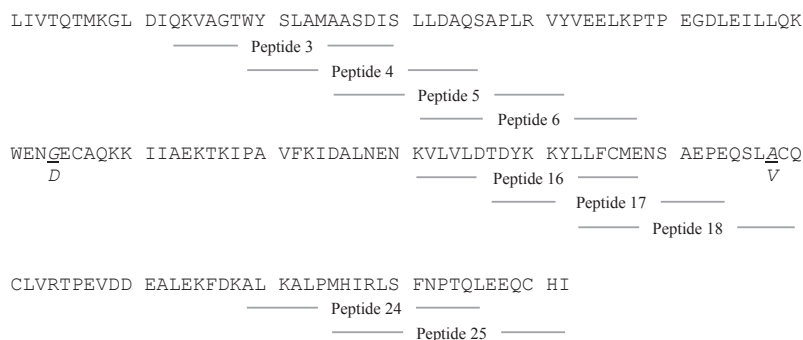
This study was designed to evaluate whether prior oral exposure to specific synthetic peptides of β -LG was able to induce oral tolerance to whey in a mouse model for CMA. The immunogenicity of 31 peptides spanning β -LG was determined using cow's milk-specific human T cell lines. Based on these data, nine β -LG peptides were selected to study their tolerogenic properties in a mouse model for CMA and to examine the effect of a scGOS/lcFOS/pAOS-containing diet on the treatment.

Methods

Peptides

Twenty-five 18-AA-long sequential synthetic peptides with 12-AA overlap spanning the B variant of β -LG (peptides 1–25, Fig. 1a) and six additional synthetic peptides with the mutations of the A variant (peptide 26–28 corresponding to peptides 9–11, and peptides 29–31 corresponding to peptides 18–20, respectively) were obtained from JPT Peptide Technologies (Berlin, Germany). Peptides were dissolved in 10% dimethylsulfoxide (DMSO, Sigma Aldrich, St Louis, MO, USA) in PBS at a concentration of 1 mg/ml. For the animal studies, nine peptides were dissolved in PBS shortly prior to the experiment and combined in three mixtures. The final concentration of each peptide in the mixture was 8 mg/ml. In addition, single peptides with the same concentration were tested.

(a)



(b)

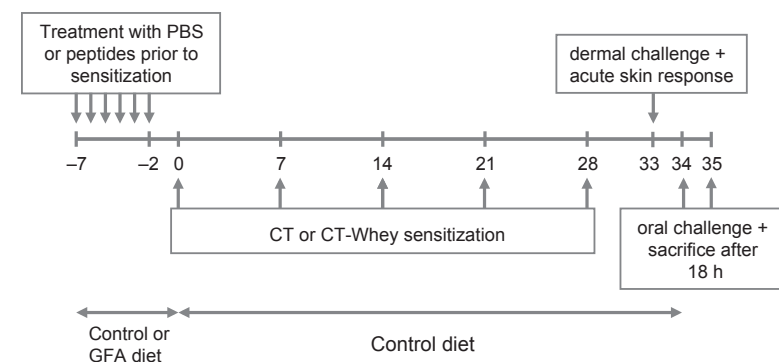


Figure 1 The amino acid sequence of the B variant of β -LG (a) and a schematic overview of the mouse model for cow's milk allergy (b). The underlined and italicized amino acids (a) are mutated in variant A of β -LG into the amino acids indicated underneath them. For the T cell stimulations, peptides covering both the A and B variant of β -LG were used. The nine peptides selected for the *in vivo* experiments derived from the sequence of the B variant. The synthetic peptides are 18 AA long and have a 12-AA overlap as shown for the peptides used for the *in vivo* experiments.

T cell proliferation

T cell proliferation in response to β -LG peptides was tested on cow's milk-specific long-term and short-term T cell lines (TCLs). Long-term TCLs can be generated from small blood samples as were obtained from children. These long-term TCLs can be maintained and expanded in culture and can therefore be tested multiple times, but they may have undergone stronger selection of immunodominant T cells. Short-term TCLs are more polyclonal due to a shorter selection protocol, but require a large blood sample, which was only available in adult patients.

The lines were generated as described previously (20, 21). The long-term TCLs were generated from peripheral blood mononuclear cells (PBMCs) of three non-atopic children, four atopic children without CMA, and three children with CMA (Table 1). For the short-term TCLs, PBMCs of three adult CMA patients were used. TCLs, PBMCs, and/or EBV-transformed B cells were frozen in 10% DMSO/90% fetal calf serum (FCS) and stored in liquid nitrogen. CMA diagnosis was based on a suggestive history, positive cow's milk-specific IgE levels, or a positive skin-prick test and confirmed by a positive double-blind placebo-controlled food challenge. The study was approved by the Ethics Committee of the University Medical Center Utrecht.

The proliferative response of the long-term TCLs to the major allergens, that is, cow's milk protein mixture (CMP, a mixture of whey [Lactalis, Laval, France] and casein [FrieslandCampina Domo, Amersfoort, the Netherlands] in a 1:1 ratio), whey, casein, β -LG, and α -lactalbumin (α -LAC, both from NIZO Food Research BV, Ede, the Netherlands), and to the β -LG peptides was determined as described previously (22). The short-term TCLs were tested as described by Flinterman et al. (21).

Table 1 The characteristics of and the peptide recognition by the human T cell lines

TCL	Long-term/ short-term	Atopic status	Recognized peptide mixtures	Recognized peptides
TCL1	Short-term	CMA	1–3	1
TCL2	Short-term	CMA	13–15, 16–18, 25–27	13, 14, 15, 16, 17, 18 and 25
TCL3	Short-term	CMA	4–6, 16–18	16, 17 and 18
TCL4	Long-term	CMA	16–18	ND
TCL5	Long-term	CMA	1–3, 4–6, 7–9	2, 3, 4, 5, 7, 8, and 9
TCL6	Long-term	CMA	ND	18, 19, 21, 26, 29 and 30
TCL7	Long-term	A	1–3, 4–6, 7–9	3, 4, 5 and 9
TCL8	Long-term	A	4–6	4 and 5
TCL9	Long-term	A	ND	3, 4 and 5
TCL10	Long-term	A	ND	8, 9 and 26
TCL11	Long-term	NA	4–6	5
TCL12	Long-term	NA	1–3, 13–15	ND
TCL13	Long-term	NA	25–27	26 and 27

TCL, T cell line; CMA, cow's milk allergic; A, atopic; NA, non-atopic; ND, not determined.

The peptides were first tested in mixtures of 2 or 3 peptides, after which the peptides of the positive mixtures were tested separately. In case multiple mixtures were positive, the peptides of the three mixtures giving the highest proliferative responses were tested again. For some TCLs, the response to either the mixtures or the single peptides was determined due to low cell numbers.

Animals

Three-week-old pathogen-free female C3H/HeOuJ mice (Charles River Laboratories, Wilmington, MA, USA) were maintained on cow's milk protein-free standard mouse chow (AIN-93G soy; Research Diet Services, Wijk bij Duurstede, the Netherlands) with/without a 1%, w/w, specific mixture of non-digestible scGOS (Vivinal GOS, FrieslandCampina Domo), lcFOS (Raftiline HP; Beneo-Orafti, Tienen, Belgium), and pAOS (Südzucker AG, Mannheim, Germany) in a 9:1:2 ratio. Mice were housed in the animal facility at the Utrecht University and treated according to the guidelines of the Dutch Committee of Animal Experiments.

Oral tolerance induction with synthetic peptides

To investigate whether preventive treatment with peptides of β -LG induced oral tolerance to whey, the mouse model as described by van Esch et al. (11) was used (Fig. 1b). Mice were treated by oral gavage, that is, intragastrically, with 0.5 ml of the peptide mixtures, single peptides, or PBS in the week prior to sensitization (daily, from day -7 until day -2). During this week, mice were fed either standard AIN-93G (control) diet or the scGOS/lcFOS/pAOS-containing diet. From day 0, all mice were maintained on the control diet. Oral tolerance was defined as a reduced acute allergic skin response.

Whey-specific IgE, IgG1, and IgG2a ELISA

Whey-specific IgE, IgG1, and IgG2a levels in sera were determined by ELISA as described previously (11).

Flow cytometry analysis of T cell and dendritic cell subsets

Immune cells were isolated from the mesenteric lymph nodes (MLN) as described by van Esch et al. (11). In short, MLN were cut into small pieces and treated with DNase I and collagenase IV (Sigma Aldrich). The enzyme reaction was stopped by adding FCS. To determine which immune cells were present in the MLN, Th1/Th2 cells were stained for the selection markers CD4, T1ST2, CXCR3, and the activation marker CD69. For dendritic cells (DCs), the selection markers CD11c, CD11b, CD8a, and CD103 and for plasmacytoid DCs, the selection markers CD11c, CD11b, CD45R (B220), and CD8a were used. Regulatory T cells were first stained for the selection markers CD4 and CD25 after which an intracellular Foxp3 staining was performed according to the manufacturer's protocol. All antibodies were obtained from eBioscience (San Diego, CA, USA) except FITC-conjugated anti-CD4, which was purchased at BD Pharmingen (San Diego, CA, USA).

Fluorescence was measured by flow cytometry (FACS Canto™ II; BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analyses

For the T cell proliferation, stimulation indexes (SIs) were calculated as a ratio between the proliferation of allergen-/peptide-stimulated cells and non-stimulated T cells. The proliferation of the long-term TCLs was corrected for the background proliferation levels of Epstein Barr Virus-transformed B cells (EBV-B cells). An SI of ≥ 2 was considered positive (21, 23). Ear swelling was determined by subtracting the basal ear thickness from the ear thickness measured one hour after the intradermal challenge. Differences among groups were analyzed by one-way ANOVA followed by a Bonferroni's multiple comparison post-test for selected groups. Antibody levels were analyzed using the Kruskal–Wallis method followed by a Dunn's *post hoc* test for selected groups. The flow cytometry data were analyzed with BD FACSDiva software version 6.1.3 (BD Biosciences). All statistical analyses were performed with GraphPad Prism version 5.0d for Macintosh (GraphPad Software, San Diego, CA, USA).

Results

Three regions of β -LG selected based on human T cells

To determine which β -LG peptides were recognized by human T cells, the proliferative response of long-term and short-term TCLs to these peptides was investigated. An example of this response is shown in Fig. 2.

Five of the thirteen TCLs responded to a mixture of peptides 4–6. In addition, peptide mixtures 1–3 and 16–18 activated four and three TCLs, respectively. Other mixtures that induced a proliferative response are depicted in Table 1. Of the peptides in mixtures 1–3 and 4–6, mainly peptides 3, 4, and 5 (AA 13–30, 19–36, and 25–42, respectively) were recognized. Peptide 18 (AA 103–120) was able to activate all three TCLs that recognized mixtures 16–18, while peptides 16 and 17 (AA 91–108 and 97–114) induced a proliferative response in two TCLs.

A previous study showed that a fragment containing AA 30–47 and a fragment stretching the end of the β -LG molecule (AA 142–162) were each able to activate a TCL of one of the four CMA patients tested (24). These fragments correspond to peptide 6 (AA 31–48) and peptides 24 and 25 (AA 139–156 and 145–162, respectively) used in our study. Peptide 6 was not recognized by our TCLs. However, peptide 5, which has a 12-AA overlap with peptide 6 and a 13-AA overlap with the fragment, was able to activate several TCLs.

Peptide 25 was recognized by one of the TCLs. Furthermore, previous *ex vivo* mice experiments have shown that peptides in this region (AA 129–153) were able to activate T cells of C3H/He(N) mice immunized with β -LG (25, 26).

Based on the TCL data and on the previous literature, nine peptides, that is, peptides 3–6, 16–18, and 24–25 were selected and combined in three mixtures to test their tolerance-inducing

capacity in mice (further referred to as mixtures 1, 2, and 3, Fig. 1a).

Pre-treatment with mixtures 1 and 3 reduced the acute allergic skin response in mice

Prior treatment with peptide mixtures 1 and 3 significantly reduced the acute allergic skin response to whey, while pre-treatment with mixture 2 showed no effect (Fig. 3a). In mice that were provided with the scGOS/lcFOS/pAOS-containing diet in combination with peptide mixture 1, the acute allergic skin response was significantly lower. The diet had no additional effects on mixtures 2 and 3. Although the combination of mixture 1 and the prebiotic diet showed the largest reduction in the acute allergic skin response, half an hour after the intradermal challenge, severe shock symptoms, that is, no physical activity after stimulation, were observed in two mice of this group. A similar response was seen in two allergic control mice.

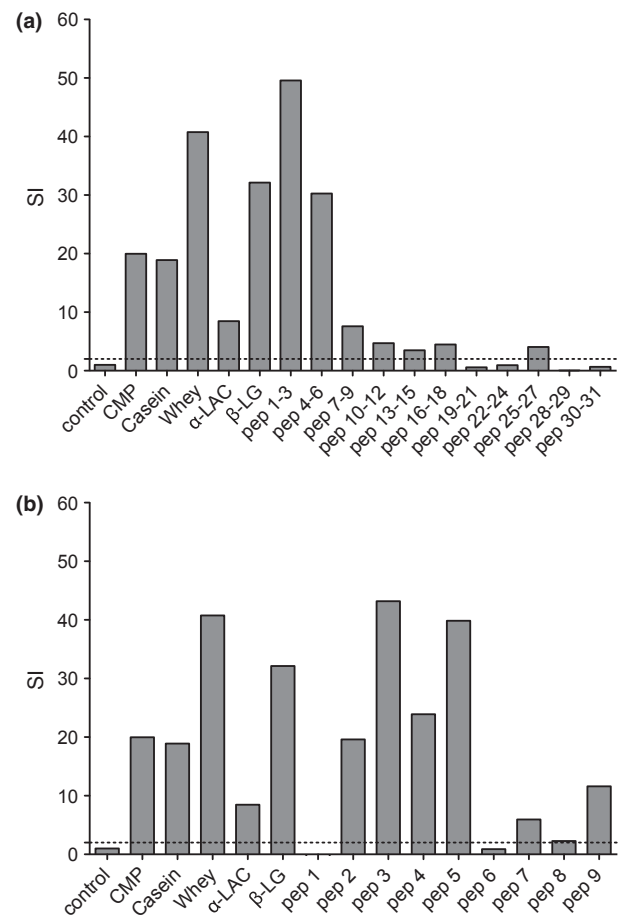


Figure 2 An example of the proliferation of a TCL in response to the β -LG peptides. SIs of TCL5 in response to the major allergens, peptide mixtures (a), and single peptides (b) are depicted. The dotted line indicates an SI of 2.

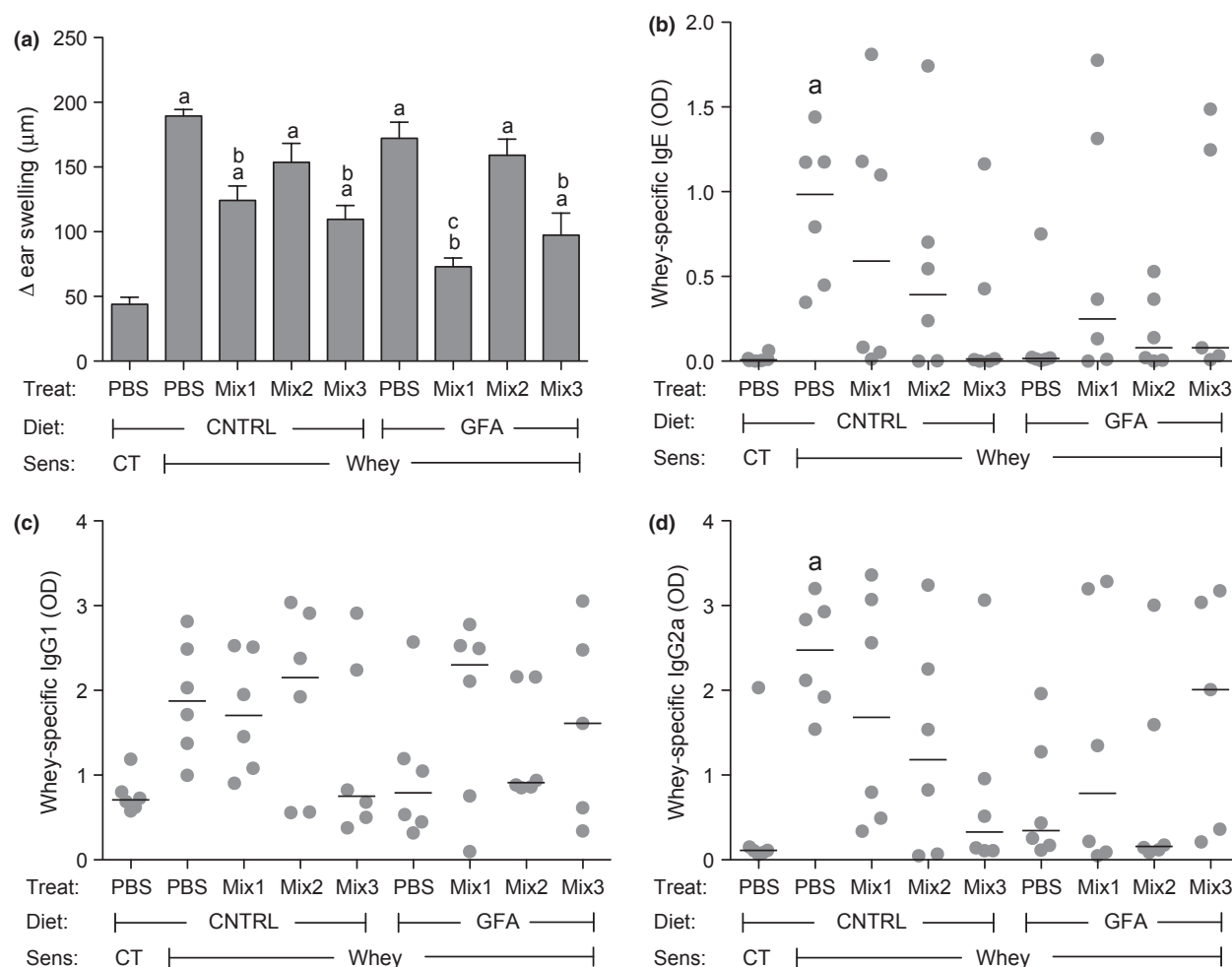


Figure 3 The acute allergic skin response (a) and whey-specific antibody levels (b–d) in control and whey-sensitized mice pre-treated with PBS or peptide mixtures with/without the scGOS/lcFOS/pAOS (GFA)-containing diet. Antibody levels are expressed as the optical densities (OD) measured by ELISA ($n = 5/6$, $a = p < 0.05$ compared with control mice, $b = p < 0.05$ compared with PBS-treated whey-sensitized mice, $c = p < 0.05$ compared with treatment without the GFA-containing diet).

Pre-treatment with mixture 3 tends to reduce the antibody response to whey

Although pre-treatment with mixture 1 reduced the acute allergic skin response, its effect on the antibody levels was highly variable (Fig. 3b–d). Half of the mice showed a reduced IgE and IgG2a response, while in the other half, the levels were similar to the allergic control group. No effect on IgG1 levels was seen. The effect of mixture 2 was similar to mixture 1. In line with the acute allergic skin response, mixture 3 had the strongest effect on IgE and IgG2a. This effect was significant when compared with the untreated allergic mice using a Mann–Whitney test. However, after correcting for multiple comparisons, significance was no longer observed, due to the large variation in the antibody levels. Peptide treatment in combination with the scGOS/lcFOS/pAOS-containing diet had no additional effect on the antibody levels, although the diet alone tended to reduce the whey-specific IgE and IgG2a response in allergic mice (significant using a Mann–Whitney test).

Peptides 5 and 6 responsible for effect mixture 1 on acute allergic skin response

Next, the single peptides of mixture 1 were tested separately. The peptides of this mixture were selected based on the strongest reduction in the acute allergic skin response and the observation that the peptides in this mixture were mainly recognized by human TCLs. Peptides 5 and 6 significantly reduced the acute allergic skin response (Fig. 4a). Pre-treatment with peptides 3 and 4 had no effect on the allergic response.

Antibody response to whey tends to be reduced by pre-treatment with peptide 6

Compared with mixture 1, pre-treatment with peptide 6 showed a more predominant reduction in whey-specific IgE levels (Fig. 4b). These levels tended to be lower than the IgE levels in untreated allergic mice and in mice pre-treated with

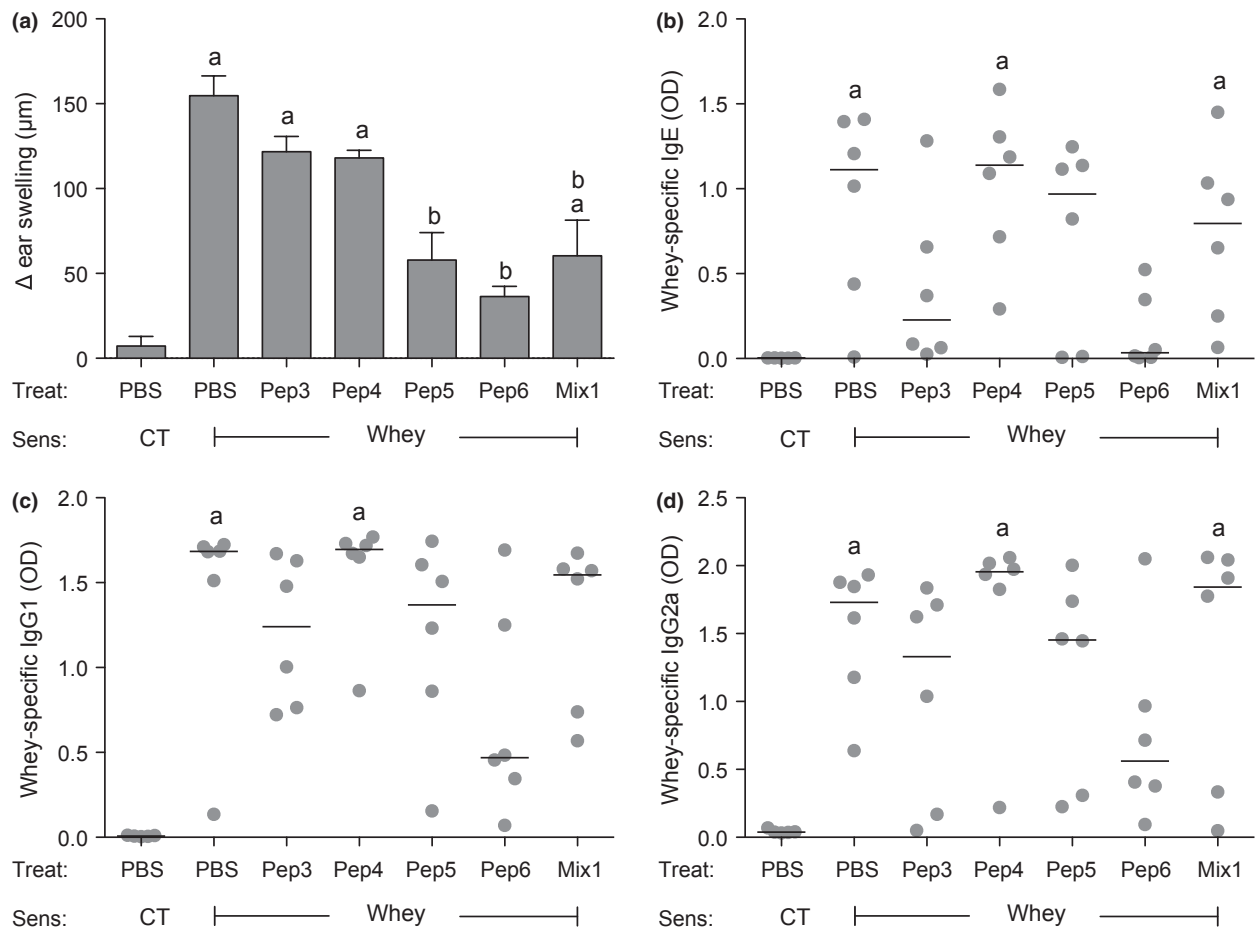


Figure 4 The acute allergic skin response (a) and whey-specific antibody levels (b–d) in control and whey-sensitized mice pre-treated with PBS, single peptides or a peptide mixture. Antibody levels are expressed as the optical densities (OD) measured by ELISA ($n = 6$, $a = p < 0.05$ compared with control mice, $b = p < 0.05$ compared with PBS-treated whey-sensitized mice).

mixture 1 (both significant using a Mann–Whitney test). No reduction in whey-specific antibody levels (Fig. 4b–d) was seen with peptides 3, 4, and 5.

Pre-treatment with peptide 6 altered T cell and DC subsets in MLN

Prior exposure to peptide 6 significantly increased the percentage of CD4⁺ cells in the MLN (Fig. 5a). Within the CD4⁺ population, the percentage CD25⁺ Foxp3⁺ cells was similar in the mice treated with peptide 6 compared with the allergic control mice (data not shown). However, the percentage of Th1 (data not shown) and Th2 cells (Fig. 5b) decreased. A similar effect but less pronounced was seen on activated CD69⁺ Th1 and Th2 cells (data not shown). When determining the percentage of the total MLN cells, a trend toward increased regulatory T cells and less Th1 cells was found in the treated mice compared with allergic control mice (both significant using a *t*-test, Fig. 5d and e, respectively). No differences were observed for the Th2 cells (data not shown).

In contrast to the percentage CD4⁺ cells, the percentage CD11c⁺ cells in the MLN decreased after pre-treatment with peptide 6 (Fig. 5c). However, the percentage of CD11b⁺CD103⁺ cells within the DC population increased significantly compared with the non-sensitized mice and tended to be increased compared with the allergic control mice (Fig. 5f). Peptide 5 showed similar effects as peptide 6 on both T cells and DCs but less pronounced.

Discussion

This study showed that prior exposure to specific β -LG peptides induces oral tolerance to whey. Oral tolerance was defined as a reduced acute allergic skin response. Combining the treatment with a scGOS/lcFOS/pAOS-containing diet enhanced the effect of some peptides.

The nine peptides that were tested in the mouse model were selected based on human T cell data and previous literature. Whereas previous *ex vivo* studies showed that the peptides of mixture 1 were not recognized by T cells of β -LG-immunized

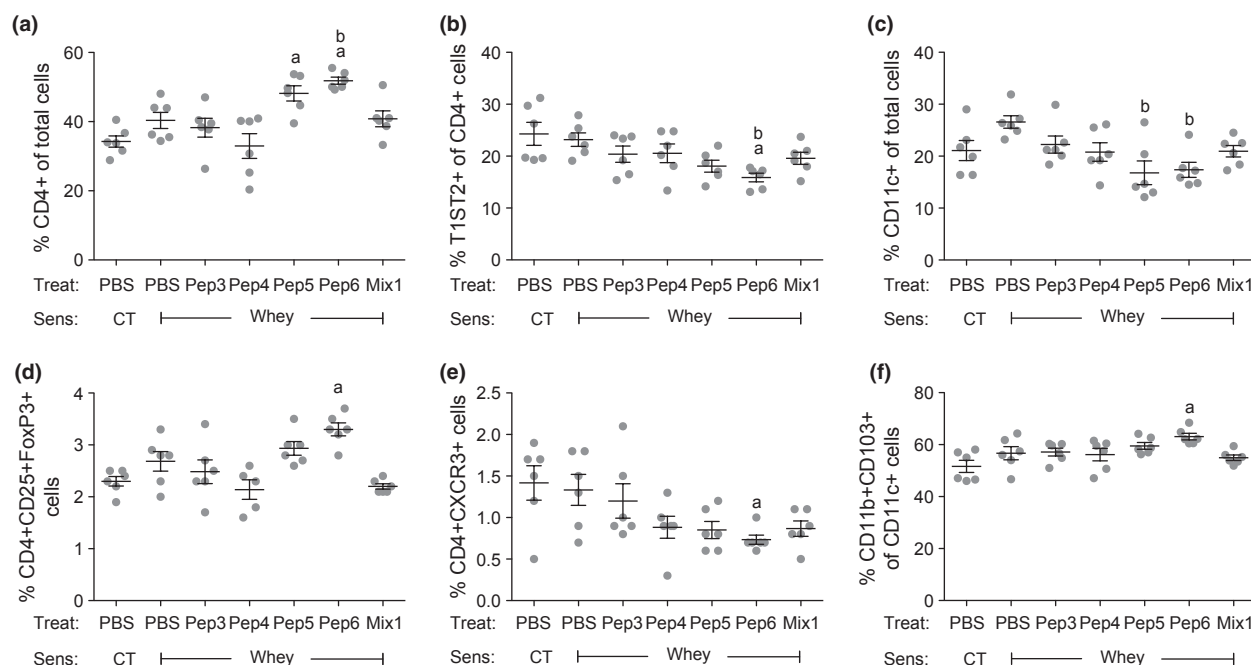


Figure 5 The percentages of T cell (CD4+ cells, Th2 cells within the CD4+ population, regulatory T cells, and Th1 cells [a,b,d,e]) and DC (CD11c+ cells and CD11b+CD103+ cells within the CD11c population [c,f]) subsets in the mesenteric lymph nodes (MLN) of control and whey-sensitized mice pre-treated with PBS, single peptides, or a peptide mixture ($n = 6$, $a = p < 0.05$ compared with control mice, $b = p < 0.05$ compared with PBS-treated whey-sensitized mice).

C3H/He(N) mice, these peptides were able to induce tolerance in the CMA model with C3H/HeOuJ mice in our study (25, 26). On the other hand, the peptides of mixture 2 induced T cell activation in previous *ex vivo* mice experiments but showed no effects *in vivo* in our study. Although C3H/He(N) and C3H/HeOuJ mice have a similar genetic background, differences in T cell recognition by these mice strains may explain the conflicting results. The lack of tolerance induction by mixture 2 may also be explained by the fact that not all T cell epitopes are able to induce tolerance as shown in previous studies (27, 28). Again, this may depend on the T cell repertoire present in specific mouse strains, because a fragment (AA 92–99/100) of β -LG comparable to peptide 16 (AA 91–108), which is present in mixture 2, has been described as tolerogenic in a study that used a different mouse strain (10).

Of the peptides in mixture 1, peptides 5 and 6 (AA 25–42 and AA 31–48, respectively) showed the strongest effect on the acute allergic skin response. This indicates that the tolerogenic T cell epitope for mice is probably present in the overlapping region (LLDAQSAPLRVY) of these peptides. In contrast to the mouse data, peptide 6 was not able to activate human T cells. A possible explanation for the difference between the *in vitro* human experiments and the *in vivo* mice experiments may be that men and mice present and recognize different epitopes. Interestingly, a previous study has shown that a fragment (AA 30–47) comparable to peptide 6 induced T cell proliferation in one of four human T cells tested. This may indicate that either, due to the small number of T cells tested in both studies, epitopes are missed or that AA 30, which is

present in the fragment and in peptide 5 but not in peptide 6, is important for T cell activation in humans.

Interestingly, the effects of peptide 6 were stronger than the effects of mixture 1, which contained a similar concentration of the peptide. These data suggest that combining tolerogenic and non-tolerogenic peptides may lead to reduced oral tolerance induction. A possible explanation may be a difference in the solubility or uptake of the peptides. Alternatively, peptides that have a tolerogenic effect and peptides that do not may compete for binding to MHC class II. If fewer tolerogenic peptides are presented by antigen presenting cells, their effects on T cells may be less pronounced. A previous study investigating peptide immunotherapy has indicated that there is an optimal dose for tolerance induction. Both higher and lower doses showed less effect (29).

As shown in previous studies, single peptides may induce linked epitope suppression. In linked epitope suppression, the T cell response not only to the epitope used for treatment but also to other epitopes within the same molecule is reduced (30, 31). In this study, treating mice with one epitope of β -LG reduced the allergic response to the intact protein that contains multiple T cell epitopes. This suggests that the response to other T cell epitopes within β -LG is also affected. However, *ex vivo* T cell experiments are necessary to confirm this.

Although pre-treatment with mixture 1 significantly reduced the acute allergic skin response in mice, the effects on whey-specific antibody levels were less clear. A similar effect was seen for peptide 5, whereas peptide 6 tended to reduce IgE and

IgG2a levels. Reduction in allergic symptoms while IgE levels remained high has been observed before, both in humans and in mice (10, 30, 32). Possible explanations may be active suppression of mast cell and basophil activation by regulatory T cells, a difference in affinity of the induced antibodies, which leads to less allergen binding and cross-linking, a decreased reactivity of mast cells and basophils, or the formation of blocking antibodies. Because the peptides were administered before sensitization and they are too small to cross-link IgE, it seems unlikely that they directly activated the effector cells and thereby induced unresponsiveness of mast cells and basophils as is seen with conventional immunotherapy. It is most likely that allergen-specific regulatory T cells that were induced or modulated by exposure to the peptides before sensitization played a role in the reduction in the effector response.

Previous research has indicated that the MLN play an important role in tolerance induction (33). Prior exposure to peptide 6 tended to increase the percentage of CD25+Foxp3+ regulatory T cells in the MLN. This is in line with a previous study that indicates that oral tolerance most likely involves the induction of regulatory T cells (34). The induction of a regulatory response by peptide 6 was further supported by the DC data. Whereas the percentage CD11c+ cells in the MLNs decreased, the percentage CD11b+CD103+ cells within this population increased. These cells are known for their ability to induce regulatory T cells and thus may play an important role in oral tolerance induction (35, 36).

The addition of scGOS/lcFOS/pAOS to the diet had a beneficial effect on the preventive treatment with some peptides. Previous studies have shown that a prebiotic diet can skew the immune response to a Th1 response (18, 37, 38). However, in the current study, no effects were seen on this cell

type in the MLN (data not shown). Other studies indicated a role for regulatory T cells (CD4+CD25+Foxp3+), although this effect was only seen after depletion of these cells (19, 38). Also in the current study, no effect was seen on the percentage of regulatory T cells in the MLN (data not shown). Further investigation is necessary to determine whether this cell type is involved.

In conclusion, preventive treatment with synthetic β -LG peptides can reduce the acute allergic skin response to whey. This effect seems to be mediated by regulatory DCs and T cells. Combining the peptides with a scGOS/lcFOS/pAOS-containing diet may increase the tolerance-inducing capacity of the peptides, indicating that not only the exposure to peptides but also the environment in the intestine during exposure is important. This knowledge may be used to optimize both preventive and curative therapies for CMA.

Funding

This study was financially supported by the Utrecht Institute for Pharmaceutical Sciences and Danone Research Centre for Specialised Nutrition.

Conflict of interest

B.C.A.M. van Esch, A.J. Nauta, J. Garssen, and L.M.J. Knippels are employees of Danone Research Centre for Specialised Nutrition. L.A.P.M. Meulenbroek and E. van Hoffen received funding from Danone Research Centre for Specialised Nutrition. L.E.M. Willemsen, G.A. Hofman, C.F. den Hartog Jager, and C.A.F.M. Bruijnzeel-Koomen have no conflict of interest.

References

- Venter C, Arshad SH. Epidemiology of food allergy. *Pediatr Clin North Am* 2011; **58**: 327–49.
- Rona RJ, Keil T, Summers C, et al. The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 2007; **120**: 638–46.
- Saarienen KM, Pelkonen AS, Makela MJ, Savilahti E. Clinical course and prognosis of cow's milk allergy are dependent on milk-specific IgE status. *J Allergy Clin Immunol* 2005; **116**: 869–75.
- Vanto T, Helppila S, Juntunen-Backman K, et al. Prediction of the development of tolerance to milk in children with cow's milk hypersensitivity. *J Pediatr* 2004; **144**: 218–22.
- Host A, Halken S, Jacobsen HP, Christensen AE, Herskind AM, Plesner K. Clinical course of cow's milk protein allergy/intolerance and atopic diseases in childhood. *Pediatr Allergy Immunol* 2002; **13**(Suppl. 15): 23–8.
- Zeiger RS, Heller S, Mellon MH, et al. Effect of combined maternal and infant food-allergen avoidance on development of atopy in early infancy: a randomized study. *J Allergy Clin Immunol* 1989; **84**: 72–89.
- Arshad SH, Matthews S, Gant C, Hide DW. Effect of allergen avoidance on development of allergic disorders in infancy. *Lancet* 1992; **339**: 1493–7.
- Du Toit G, Katz Y, Sasieni P, et al. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *J Allergy Clin Immunol* 2008; **122**: 984–91.
- Poole JA, Barriga K, Leung DY, et al. Timing of initial exposure to cereal grains and the risk of wheat allergy. *Pediatrics* 2006; **117**: 2175–82.
- Pecquet S, Bovetto L, Maynard F, Fritsche R. Peptides obtained by tryptic hydrolysis of bovine beta-lactoglobulin induce specific oral tolerance in mice. *J Allergy Clin Immunol* 2000; **105**: 514–21.
- van Esch BC, Schouten B, de Kivit S, et al. 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. *Pediatr Allergy Immunol* 2011; **22**: 820–6.
- Fritsche R, Pahud JJ, Pecquet S, Pfeifer A. Induction of systemic immunologic tolerance to beta-lactoglobulin by oral administration of a whey protein hydrolysate. *J Allergy Clin Immunol* 1997; **100**: 266–73.
- Agostoni C, Axelsson I, Goulet O, et al. Prebiotic oligosaccharides in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr* 2004; **39**: 465–73.
- Boehm G, Stahl B. Oligosaccharides from milk. *J Nutr* 2007; **137**: 847S–9S.
- Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch Dis Child* 2006; **91**: 814–9.
- Gruber C, van Stuijvenberg M, Mosca F, et al. Reduced occurrence of early atopic dermatitis because of immunoactive prebiotics among low-atopy-risk infants. *J Allergy Clin Immunol* 2010; **126**: 791–7.

17. Arslanoglu S, Moro GE, Schmitt J, Tandoi L, Rizzardi S, Boehm G. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. *J Nutr* 2008; **138**: 1091–5.
18. Vos AP, van Esch BC, Stahl B, et al. Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. *Int Immunopharmacol* 2007; **7**: 1582–7.
19. Schouten B, van Esch BC, Hofman GA, et al. Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. *J Nutr* 2010; **140**: 835–41.
20. Schade RP, Van Ieperen-Van Dijk AG, Van Reijssen FC, et al. Differences in antigen-specific T-cell responses between infants with atopic dermatitis with and without cow's milk allergy: relevance of TH2 cytokines. *J Allergy Clin Immunol* 2000; **106**: 1155–62.
21. Flinterman AE, Pasmans SG, den Hartog Jager CF, et al. T cell responses to major peanut allergens in children with and without peanut allergy. *Clin Exp Allergy* 2010; **40**: 590–7.
22. Ruiter B, Tregoeat V, M'rabet L, et al. Characterization of T cell epitopes in alpha s1-casein in cow's milk allergic, atopic and non-atopic children. *Clin Exp Allergy* 2006; **36**: 303–10.
23. Schulten V, Nagl B, Scala E, et al. Pru p 3, the nonspecific lipid transfer protein from peach, dominates the immune response to its homolog in hazelnut. *Allergy* 2011; **66**: 1005–13.
24. Inoue R, Matsushita S, Kaneko H, et al. Identification of beta-lactoglobulin-derived peptides and class II HLA molecules recognized by T cells from patients with milk allergy. *Clin Exp Allergy* 2001; **31**: 1126–34.
25. Tsuji NM, Kurisaki J, Mizumachi K, Kaminogawa S. Localization of T-cell determinants on bovine beta-lactoglobulin. *Immunol Lett* 1993; **37**: 215–21.
26. Totsuka M, Ametani A, Kaminogawa S. Fine mapping of T-cell determinants of bovine beta-lactoglobulin. *Cytotechnology* 1997; **25**: 101–13.
27. Hirahara K, Hisatsune T, Choi CY, Kaminogawa S. Profound immunological tolerance in the antibody response against bovine alpha s1-casein induced by intradermal administration of a dominant T cell determinant. *Clin Immunol Immunopathol* 1995; **76**: 12–8.
28. Hoyne GF, Callow MG, Kuo MC, Thomas WR. Inhibition of T-cell responses by feeding peptides containing major and cryptic epitopes: studies with the Der p I allergen. *Immunology* 1994; **83**: 190–5.
29. Daniel C, Weigmann B, Bronson R, von Boehmer H. Prevention of type 1 diabetes in mice by tolerogenic vaccination with a strong agonist insulin mimotope. *J Exp Med* 2011; **208**: 1501–10.
30. Hoyne GF, Jarnicki AG, Thomas WR, Lamb JR. Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. *Int Immunol* 1997; **9**: 1165–73.
31. Campbell JD, Buckland KF, McMillan SJ, et al. Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. *J Exp Med* 2009; **206**: 1535–47.
32. Muller U, Akdis CA, Fricker M, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998; **101**: 747–54.
33. Worbs T, Bode U, Yan S, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med* 2006; **203**: 519–27.
34. Adel-Patient K, Wavrin S, Bernard H, Meziti N, Ah-Leung S, Wal JM. Oral tolerance and Treg cells are induced in BALB/c mice after gavage with bovine beta-lactoglobulin. *Allergy* 2011; **66**: 1312–21.
35. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103 + DCs induces Foxp3 + regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 2007; **204**: 1757–64.
36. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 2007; **204**: 1775–85.
37. van't Land B, Schijf M, van Esch BC, et al. Regulatory T-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides. *Vaccine* 2010; **28**: 5711–7.
38. Schijf MA, Kruijsen D, Bastiaans J, et al. Specific dietary oligosaccharides increase Th1 responses in a mouse respiratory syncytial virus infection model. *J Virol* 2012; **86**: 11472–82.