BUILDING TOLERANCE

T cell epitopes as a treatment for cow's milk allergy

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HET OPBOUWEN VAN TOLERANTIE
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(met een samenvatting in het Nederlands)

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CONTENTS

CHAPTER 1

General introduction

More than 100 years ago, Wells *et al.* described that continuous feeding of a protein prevented the induction of an immune response to the same protein (1). In addition, Chase *et al.* demonstrated that the immune response to a systemic antigen challenge was inhibited by prior feeding of the antigen (2). This phenomenon is called oral tolerance and is the result of an active regulatory response by the immune system (3, 4). However, in food-allergic patients, oral tolerance to food proteins is disturbed. A combination of multiple factors, such as genetic predisposition, route of exposure, dose of the allergen, and structural characteristic of the allergen, may be responsible for allergy development (3, 5).

Oral tolerance

Several mechanisms have been described for oral tolerance, including anergy or deletion of antigen-specific T cells, and the development of regulatory T cells (Tregs) (3, 4). It is known that high doses of antigen induce T cell anergy/deletion, whereas exposure to low doses induces Tregs. However, it has been suggested that both mechanisms might occur simultaneously (3, 4). T cell anergy/deletion is induced when T cells are activated without co-stimulation (3, 6, 7). In addition, a role for Fas-mediated apoptosis has been described in T cell deletion (7). On the other hand, Tregs are induced after antigen presentation by immature antigen-presenting cells, repeated exposure to antigen and/or exposure to IL-27, IL-10, retinoic acid and/or TGF-β (8-10). There are several inducible Tregs described, namely Foxp3+ Tregs, IL-10-producing Tr1 cells, TGF-β-producing Th3 cells and CD8+ Tregs (3, 4). It has been shown that the induction of Foxp3+ Tregs is favoured by certain subsets of dendritic cells (DCs), such as CD103+ DCs and CD8+ plasmacytoid DCs, which produce TGF-β and retinoic acid (11-13). Inducible Tregs may inhibit immune responses via several mechanisms (9, 14). They may directly kill target cells via granzymes (9, 14) or may disrupt the metabolism of effector T cells by generation of adenosine or by depriving the cells of growth factors (9, 14). Moreover, Tregs may secrete IL-10 and TGF-β that affect multiple immune cells and decrease cytokine production, mediator release, IgE production and antigen presentation (9, 14, 15). In addition, Tregs may inhibit effector and/or antigen-presenting cells via cell-cell contact (9, 14).

The mesenteric lymph nodes (MLN) are an important site for tolerance induction. Worbs *et al.* have shown that removal of MLN almost completely prevented oral tolerance (16). Moreover, they showed that trafficking of DCs from the lamina propria to the mesenteric lymph nodes (MLN) is important for tolerance induction.

Food allergy

If oral tolerance to food proteins is not established or existing tolerance is broken down, this may result in a food allergy (3). Food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly upon exposure to a given food (17). In IgE-mediated food allergy, this immune response consists of two phases, the sensitization phase and the effector phase (18). During the sensitization phase, antigen-presenting cells take up the allergen, process it and present the peptides on MHC class II molecules to naive T cells. These naive T cells differentiate into Th2 cells, which produce pro-inflammatory cytokines,

such as IL-4, IL-5 and IL-13. The cytokines in combination with the food allergen induce a class-switch in allergen-specific B cells. As a result, the B cells produce allergen-specific IgE antibodies that bind to the high-affinity IgE receptor on mast cells and basophils. During the effector phase, the allergen binds to the allergen-specific IgE antibodies on the effector cells and cross-links them, thereby activating these cells. Several mediators, such as histamine, leukotrienes, prostaglandins and cytokines, are released. These mediators act on epithelial, endothelial and smooth muscle cells, and thus induce acute allergic symptoms (19). In addition, the mediators attract and activate other immune cells, such as eosinophils, which may induce late allergic symptoms (18). Although the majority of food allergies and/or food allergy-related adverse reactions are due to allergen-specific IgE antibodies and Th2-mediated immune responses, in some cases food-allergic reactions occur without clear Th2 and IgE involvement (17, 20). Underlying mechanisms of this type of reactions remain to be clarified and might involve, for example, allergen-specific T cells and antigen-specific immunoglobulin free light chains (20, 21).

Cow's milk allergy

The most prevalent food allergy in young children is cow's milk allergy (CMA), affecting 0.3-3.5% of the young children (22, 23). Before their fifth year of life, 60-75% of the children with IgE-mediated CMA spontaneously develop tolerance (24-26). However, they have an increased risk of developing other atopic disorders later in life, such as asthma and rhinoconjuctivitis (24, 26). In adults, the prevalence of CMA is lower and is estimated to be 0.1-0.3% (22, 23). The majority of the adult CMA patients acquired their allergy at adult age (27). Allergic symptoms that occur in CMA patients involve the skin, the gastro-intestinal tract, the respiratory system and the cardiovascular system (28-30). Moreover, cow's milk may induce anaphylaxis in some patients. Previous studies have reported that about 10% of the fatal/near-fatal anaphylactic episodes in children in the UK were due to CMA (29). However, recent data indicate that an increasing part of fatal anaphylaxis is the result of an allergic reaction upon exposure to cow's milk (John Warner, personal communication).

The proteins in cow's milk can be classified in two fractions, the casein and the whey fraction (31, 32). Of these fractions, the casein fraction is most abundant comprising 80% of the total protein content in cow's milk. This fraction contains several proteins, of which αS1, αS2, β and κ caseins are the most important. The major proteins in the whey fraction are α-lactalbumin and β-lactoglobulin. CMA patients may be sensitized to all milk proteins, though αS1 casein, β casein, α-lactalbumin and β-lactoglobulin seem to be most allergenic (32, 33). The frequency of sensitization for the different proteins varies between different studies and depends on the patient population and the methods used to determine IgE levels (32, 33). For example, Shek *et al.* observed that more patients recognized caseins compared to α-lactalbumin and β-lactoglobulin, while Restani *et al.* found that the percentage of patients that recognized these proteins was similar (32, 33).

Current treatment of CMA

To date, there is no curative treatment available for CMA. Therefore, the best strategy to reduce allergic symptoms is to avoid exposure to cow's milk (17, 29, 30). Because cow's milk is found in many foods, this restriction has a great impact on the diet of CMA patients (20). Moreover, accidental exposure to cow's milk occurs frequently and may induce severe symptoms (34). Both the dietary restrictions and the risk of accidental exposure have a great impact on the quality of life of CMA patients and their relatives (35-37).

Hydrolysates

In young children (<2 years old), cow's milk is an important source of nutrition when breast-feeding is not possible (20, 30). Therefore, substitute hypoallergenic formulae have been developed. Hydrolyzed cow's milk proteins are commonly used in these formulae and are produced by enzymatic degradation of casein and/or whey proteins (30). Based on the degree of hydrolysis and the length of the remaining peptides, hydrolyzed proteins are categorized (arbitrary) as partial or extensive hydrolysates (38-40).

Both European and American guidelines indicate that only formulae tested under double-blind placebo-controlled conditions and showing with 95% confidence that they do not induce allergic symptoms in 90% of the CMA patients, may be used as a hypoallergenic formula by CMA patients (41, 42). Because partial hydrolysates contain larger fragments, and thus may still induce allergic symptoms, these formulae are not suitable for treatment purposes (30, 39, 40). Extensive hydrolysates only contain small peptides and are in general well tolerated in CMA patients (30, 40, 43). Nevertheless, allergic symptoms after ingestion of this formula have been observed in severe CMA patients (30). Based on these data, the World Allergy Organization recommends extensive hydrolysates for the treatment of CMA children with a low risk of anaphylactic reactions, whereas for children with a high risk of anaphylaxis formulae containing free amino acids are recommended (20).

While the effect of exposure to allergen on the allergy development is currently under debate (44-46), it has been thought for years that allergen avoidance may prevent the development of allergy (47, 48). Therefore, substitute formulae have also been used in children at risk for developing CMA. Because partial hydrolysates contain larger fragments, which are more immunogenic, it was hypothesized that the use of partial hydrolysate may prevent CMA by inducing tolerance to cow's milk (28, 38). This hypothesis was confirmed in animal studies showing that pre-treatment with partial whey hydrolysates, but not with extensive whey hydrolysates, reduced the sensitization to whey (49-51). Several clinical studies have indicated that both partial as well as extensive hydrolysates may prevent CMA and atopic dermatitis in high-risk children, though the evidence for this effect is limited due to methodological problems and inconsistent findings (40, 43, 52-55). One of the problems is the variation between different hydrolysates caused by differences in their production (40, 56). Another aspect that has to be taken into account is that, although atopic dermatitis is a well-recognized risk factor (17, 57), it might not be the best readout for CMA in trials aimed to validate the preventive capacity of cow's milk hydrolysates.

Oral immunotherapy

Because CMA has a great impact on the quality of life, prevention and therapeutic strategies are highly desirable. A possible way to treat CMA patients may be allergen-specific immunotherapy in which patients are exposed to increasing doses of allergen (3, 17, 44). Whereas this therapy is already used in the clinic for several inhalation allergies, it is not available yet for food-allergic patients (3). The main reason for this are the adverse reactions that are reported (44). The first studies with subcutaneous immunotherapy for food allergy were performed more than 20 years ago in peanut-allergic patients (58, 59). While these studies were successful in inducing tolerance, the treatment induced severe systemic reactions.

In the last 10 years, many studies have investigated the potential of oral immunotherapy for cow's milk (37, 60, 61). However, most of them are of poor quality, i.e. investigating small groups of patients and lacking a control group (37, 60). Because children may spontaneously develop tolerance, a control group, preferably age-matched, is highly desirable to determine whether the tolerance observed is induced by the treatment (44). The studies that have included a control group showed that oral immunotherapy in CMA patients increased the threshold dose for allergic symptoms significantly (37, 60, 62-66). However, in most studies, it is unclear whether the reduced response is the result of tolerance induction or temporarily desensitization (37, 60). In case of tolerance, an individual should be symptom-free after consumption of food even when he/she has not been exposed for weeks (17). So far, only two studies included an allergen-free period (67, 68). Staden *et al.* showed that, directly after treatment, more children were tolerant in the treated group compared to the control group, whereas no difference was found between the groups after a period of allergen avoidance (68). Keet *et al.* observed that almost half of the patients lost desensitization within 6 weeks (67). Moreover, in two patients, desensitization was already reduced after one week of avoidance indicating that patients should strictly adhere to the maintenance dose. In a long-term follow-up of two oral immunotherapy studies, mixed outcomes were observed after 3-5 years. Only 31% of the subjects still tolerated a full serving of cow's milk with minimal or no symptoms (69). It has been suggested that higher doses and longer treatment protocols may be more effective (69).

In addition, also with oral immunotherapy, side effects were frequently reported. While most of the side effects were mild (i.e. minor cutaneous and abdominal reactions), cases of severe respiratory and systemic side effects were described (44, 60). Moreover, more patients needed treatment with epinephrine in the groups treated with immunotherapy compared to the control groups (44, 60). The appearance of side effects was unpredictable. Narisety *et al.* observed systemic reactions in patients at doses that were previously tolerated (70). Those reactions were often correlated with exercise or viral infections.

IgG antibodies in allergy

An important feature of successful conventional immunotherapy is an increase in allergen-specific IgG levels, especially IgG4 (15, 44, 71). IgG4 is considered a blocking antibody. It may reduce allergic symptoms by blocking IgE binding to the allergen or by triggering the inhibitory IgG receptor FcγRIIb on mast cells and/or basophils (15, 72). Moreover, it has been suggested that IgG4 inhibits T cell activation and Th2 skewing by blocking IgE-facilitated antigen

presentation (IgE-FAP) (73-77). In IgE-FAP, allergen-IgE complexes are formed and bind to the low-affinity IgE receptor CD23 on B cells (73, 78, 79). The B cells internalize and process the complexes, and subsequently present the peptides on MHC class II molecules to CD4+ T cells (Figure 1). Previous studies have shown that, in the presence of IgE, B cells are able to activate T cells at lower allergen concentrations (78, 79).

A possible explanation for the increased T cell activation is that the uptake of allergen-IgE complexes by B cells is more efficient than the uptake of allergen via pinocytosis and thus leads to the presentation of more peptides at lower allergen concentrations. IgG4 antibodies may inhibit complex formation and thereby may increase the allergen concentration that is necessary to activate T cells (73-75). Interestingly, CMA and peanut-allergic patients also have high allergen-specific IgG levels but are still allergic (33, 80, 81). The effect of these natural-occurring IgG antibodies on IgE-FAP is unclear.

Peptide immunotherapy

Ideally, both a preventive and curative therapy for CMA should induce tolerance without activating mast cells and basophils. To induce T cell anergy or Tregs, T cells should be activated via their T cell receptor without co-stimulation or in the presence of specific cytokines, such as IL-10 and TGF-β (3, 8, 15). The T cell receptor recognizes peptides of 9-12 amino acids long, which are much smaller than the peptides that are needed to cross-link IgE (minimal 35 amino acids, Figure 2)) (82-85). Therefore, it has been suggested that using peptides that are too small to cross-link IgE but long enough to induce T cell activation may be a safe alternative for conventional immunotherapy.

The potential of peptide immunotherapy has mainly been investigated for inhalation allergies. Preventive and curative treatment with peptides reduced T cell responses, antibody production and/or allergic symptoms in mice. Moreover, curative peptide immunotherapy was effective in cat- and bee venom-allergic patients (86-88). In these studies, a mixture of peptides (10-17 amino

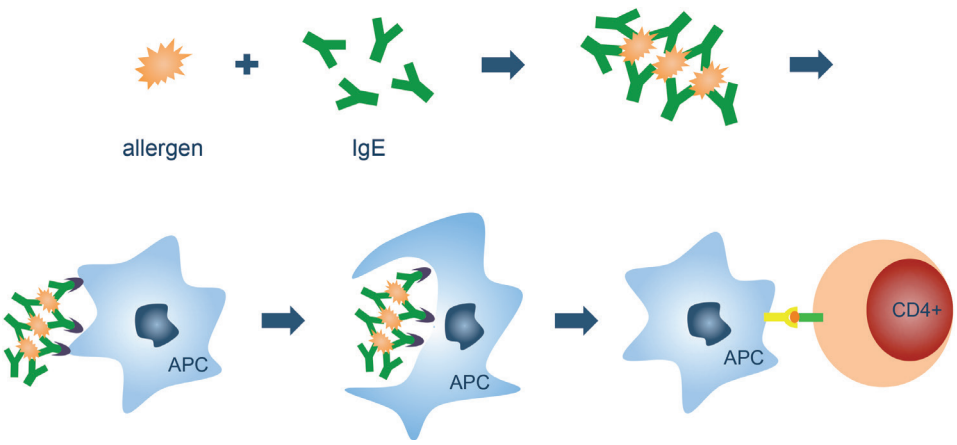

Figure 1 | The principle of IgE-FAP.

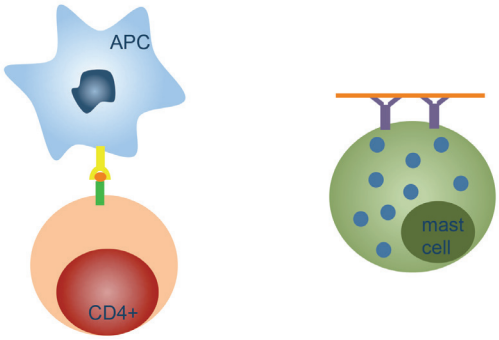

Figure 2 | The difference in peptide size needed for T cell activation compared to basophil/mast cell activation.

acids long) was administered intradermally or subcutaneously. The treatment significantly reduced the allergic symptoms and no acute allergic side effects were observed. In cat-allergic patients, the peptides did induce late allergic symptoms, but these side effects decreased during treatment (89, 90). Interestingly, Patel *et al.* showed that 4 injections of a peptide mixture were already effective and decreased allergic symptoms even 9 months after the therapy was stopped (87).
To date, a limited number of studies have investigated the potential of peptide immunotherapy for food allergy. For example, Rupa *et al.* showed that oral treatment with a peptide of ovomucoid in a curative setting significantly decreased allergic symptoms in a mouse model for egg allergy (91). For cow's milk allergy, only the efficacy of preventive treatment has been investigated. Hirahara *et al.* showed that preventive intradermal treatment with a peptide of αS1 casein reduced T cell and antibody responses to the intact protein in mice (92). Moreover, as described above, previous studies have shown that prophylactic treatment with partial whey hydrolysates reduced allergic symptoms in mouse models for cow's milk allergy (49-51). Interestingly, Knipping *et al.* have indicated that during the hydrolysis of whey proteins there is a certain time point at which the formed peptides are too small to induce basophil activation but long enough to induce T cell activation (93). However, whether these peptides are able to induce tolerance, is unclear.

Immune modulation via dietary components

The efficacy of immunotherapy may be increased by modulating the immune response via dietary components (94). For several dietary components, such as vitamin E, polyunsaturated fatty acids (PUFAs), probiotics and oligosaccharides (prebiotics), it has been shown that they affect food sensitization (94). For example, oligosaccharides found in human breast milk may modulate the gut microbiota and thereby influence the immune system (95). Moreover, it has been shown that addition of a specific mixture of non-digestible short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS) with/without pectin-derived acidic oligosaccharides (pAOS, in a ratio of 9:1:2) to the diet reduced allergic manifestations in both men and mice (96-99). In addition, van Esch *et al.* showed that addition of scGOS/lcFOS/pAOS to the diet increased the tolerance-inducing capacity of whey hydrolysates (van Esch *et al.*, submitted).

OUTLINE OF THIS THESIS

The main focus of this thesis is to investigate the potential of peptide immunotherapy as a preventive and/or curative treatment for CMA. Different technologies have been used, including both *in vitro* as well as *in vivo* research models. In addition, we investigated the effect of the high IgG levels in peanut- and CMA patients on allergen-antibody complex formation and binding to B cells.

In Chapter 2 of this thesis, allergen-antibody complex formation and complex binding to B cells in peanut-allergic and CMA patients was investigated. This was compared to the complex formation and binding in birch pollen-allergic patients, who have much lower allergen-specific IgG levels.

In Chapter 3, the effect of the degree of hydrolysis on the allergenicity and immunogenicity of whey hydrolysates was investigated to determine the time point during the hydrolysis at which hydrolysates are still able to induce T cell proliferation but are unable to cross-link IgE.

Chapter 4 and 5 describe the T cell epitopes of α-lactalbumin and β-lactoglobulin, respectively. Moreover, in Chapter 5, the effect of preventive therapy with synthetic peptides of β-lactoglobulin with/without a diet containing scGOS/lcFOS/pAOS was investigated in a mouse model for CMA (prevention of CMA in mice).

In Chapter 6, the effect of oral immunotherapy (treatment of CMA in mice) with a partial whey hydrolysate or a mixture of synthetic β-lactoglobulin peptides was determined in a mouse model for CMA.

In the final chapter all data are summarized and discussed in a broader sense.

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CHAPTER 2

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IgG antibodies in food allergy influence allergen-antibody complex formation and binding to B cells: A role for complement receptors

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ABSTRACT

Allergen-IgE complexes are more efficiently internalized and presented by B cells than allergens alone. It has been suggested that IgG antibodies induced by immunotherapy inhibit these processes. Food-allergic patients have high allergen-specific IgG levels. However, the role of these antibodies in complex formation and binding to B cells is unknown. To investigate this, we incubated sera of peanut- or cow's milk-allergic patients with their major allergens to form complexes, and added them to EBV-transformed or peripheral blood B cells. Samples of birch pollen-allergic patients were used as control. Complex binding to B cells in presence or absence of blocking antibodies to CD23, CD32, complement receptor 1 (CR1, CD35) and/or CR2 (CD21) was determined by flow cytometry. Furthermore, intact and IgG-depleted sera were compared. These experiments showed that allergen-antibody complexes formed in birch pollen as well as food allergy contained IgE, IgG1 and IgG4 antibodies and bound to B cells. Binding of these complexes to EBV-transformed B cells was completely mediated by CD23, whereas binding to peripheral blood B cells was dependent on both CD23 and CR2. This reflected differential receptor expression. Upon IgG depletion, allergen-antibody complexes bound to peripheral blood B cells exclusively via CD23. These data indicated that IgG antibodies are involved in complex formation. The presence of IgG in allergen-IgE complexes results in binding to B cells via CR2 in addition to CD23. The binding to both CR2 and CD23 may affect antigen processing and presentation and (may) thereby influence the allergic response.

INTRODUCTION

Depending on the antigen/antibody ratio in the circulation, immune complexes may be formed upon exposure to an antigen. These formed complexes play an important role in antigen presentation. They bind to leukocytes via Fc and/or complement receptors, after which they are internalized and processed to facilitate antigen presentation.

Previous research has shown that antigen-antibody complexes formed after vaccination contained complement factors and bound to B cells via complement receptors (CR) 1 (CD35) and CR2 (CD21). These complexes led to more efficient antigen presentation to T cells than complexes without complement (1, 2). In addition, studies with antigen artificially coupled to complement components indicated that these complexes activated T cells at lower antigen concentrations compared to free antigen (3, 4).

Also in allergy, antigen-antibody complex formation has been observed. These allergen-IgE complexes were able to bind to B cells via the low-affinity IgE receptor CD23 (5-7). As with the vaccination studies, complex formation enhanced the antigen uptake and presentation of allergens (5, 6). Furthermore, studies have shown that this IgE-facilitated antigen presentation (IgE-FAP) induced more Th2 skewing (8, 9). As a model system for IgE-FAP, most studies used EBV-transformed B cells (EBV-B cells), which have a high expression of CD23.

So far, the role of IgE-FAP in allergy has mainly been investigated for inhalation allergens, such as birch pollen, grass pollen and house dust mite (6, 7, 10, 11). Only one study has investigated IgE-FAP in food allergy (FA), that is, peanut allergy (PA) (12). In line with other studies, this study revealed that, in presence of specific IgE, peanut-specific T cells are activated at lower allergen concentrations. In PA not only specific IgE levels but also specific IgG levels are elevated (13, 14). This was also demonstrated for cow's milk allergy (CMA) (15). However, the role of these specific IgG antibodies in allergen-antibody complex formation in allergy is unclear.

One study showed that allergen-antibody complexes containing IgE, IgG1 and/or C1q can be formed in the circulation upon a challenge with cow's milk in CMA patients, which suggests that IgG antibodies may be involved in complex formation (16). In contrast, other studies have suggested that IgG antibodies block complex formation and thereby reduce antigen presentation. In patients treated with allergen immunotherapy, specific IgG levels, especially IgG4, were increased, while binding of allergen-IgE complexes to EBV-B cells and subsequent T cell activation were reduced (7, 10, 11, 17, 18). Additional IgG-depletion experiments indicated that IgG antibodies were involved in the inhibition of complex binding and antigen presentation (7, 10, 11).

In this study, the formation of allergen-antibody complexes and their binding to B cells in FA as compared to birch pollen allergy (BPA) was investigated. Complexes were stained for the presence of IgE, IgG1 and IgG4 to determine their composition. Binding to EBV-B cells and to B cells within freshly isolated PBMCs incubated with/without blocking antibodies for several receptor candidates, that is, CD23, the low-affinity IgG receptor FcγRII (CD32), CR1, and CR2, was examined. In addition, IgG antibodies were depleted from the serum to investigate their role in complex formation and binding.

MATERIAL AND METHODS

Patients

Fifteen CMA (age, 26-68 y; median, 39 y), 15 PA (age, 20-37 y; median, 23 y), 15 BPA (age, 18-60 y; median, 39) patients and 4 healthy controls (HCs; age 25-59 y, median 27) were included in this study. The diagnosis of CMA, PA and BPA was based on a suggestive history, a positive double blind placebo-controlled food challenge, positive IgE specific for cow's milk, peanut or birch pollen (determined by CAP system FEIA, Thermo Fisher Scientific, Uppsala, Sweden) and/or a positive skin prick test. After informed consent was obtained, venous blood samples were taken from the patients and the control subjects. Plasma/serum was collected and stored at -20°C until further use. This study was approved by the Ethics Committee of the University Medical Center Utrecht.

Allergens

Cow's milk protein and purified αS1-casein (purity >95%) were obtained from Nizo Food Research (Ede, The Netherlands). Crude peanut extract and purified Ara h 2 (purity >95%) were a kind gift from TNO Innovation for Life (Zeist, The Netherlands). Birch pollen extract was obtained from ALK Abellø (Hørsholm, Denmark), whereas recombinant Bet v 1 (expressed in Escherichia coli, purity >98%) was purchased at Biomay (Vienna, Austria).

Antibodies

HRP-conjugated goat anti-human IgE (1:10,000) was purchased from KPL (Gaithersburg, MD, USA). HRP-conjugated mouse anti-human IgG1 (1:20,000) and -IgG4 (1:30,000) were acquired from Sanquin (Amsterdam, The Netherlands). Unlabeled mouse IgG isotype control (DAK-GO1, 1:2), PE-labeled and unlabeled mouse anti-human CD23 (clone MHM6, 1:10) were obtained from Dako Denmark A/S (Glostrup, Denmark). PE-labeled and unlabeled mouse anti-human CD32 (clone AT10, 1:10) were purchased from AbD Serotec (Martinsried, Germany). PE-labeled mouse IgG1 isotype control (clone MOPC-31C, 1:5), mouse anti-human CR1 (clone E11, 1:10) and CR2 (clone B-ly4, 1:10), streptavidin (1:450), unlabeled mouse anti-human CR2 (clone 1048, 1:5), biotinylated mouse anti-human IgE, IgG1, IgG4 (all 1:250) and FITC-labeled mouse anti-human CD20 (clone 2H7, 1:10) were all acquired from BD Biosciences (San Diego, CA, USA). Unlabeled mouse anti-human CR1 (clone J3D3, 1:50) was bought at Beckman Coulter (Brea, CA, USA).

Allergen-specific IgE, IgG1 and IgG4 ELISA

ELISA to determine allergen-specific IgE, IgG1 and IgG4 levels to cow's milk protein, αS1-casein, crude peanut extract, Ara h 2, birch pollen extract or Bet v 1 was performed as described previously (19). Results are expressed as arbitrary units per milliliter.

Cells

EBV-B cells and IIA1.6 cells (murine cell line expressing human CD32) were cultured in RPMI 1640 + GlutaMAX™-I supplemented with 10% v/v heat-inactivated FBS, 100 IU/ml penicillin and

100 µg/ml streptomycin (all from Invitrogen, Carlsbad, CA, USA). PBMCs were isolated from heparinized venous blood samples from healthy donors by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK).

Allergen-antibody complex binding to B cells

Complex binding to B cells was investigated as described with some adjustments (20). In short, plasma (for 21 subjects) or serum (for 4 subjects) of 25 subjects (n=7 for each allergy group and n=4 for the healthy subjects, see Supplemental Table I) were incubated for 1 hour at 37°C with several concentrations αS1-casein, Ara h 2 or Bet v 1 (range from 0.001-100 µg/ml) in PBS-2% w/v human serum albumin (Sanquin) to form allergen-antibody complexes. Subsequently, $4.5x10^5$ EBV-B cells or freshly isolated PBMCs were added to the samples and incubated for 1 hour at 37°C to allow complexes to bind to the cells. After 1 hour, samples were washed, divided over three tubes, and stained for binding of allergen-antibody complexes with biotinylated mouse-anti-human IgE, IgG1 or IgG4 antibodies for 30 min at 4°C, followed by a PE-labeled streptavidin staining. Simultaneously, peripheral blood B cells (PBBCs) within the PBMCs were stained with FITC-labeled mouse anti-human CD20. Fluorescence was measured with flow cytometry (FACSCanto II, BD Biosciences, Franklin Lakes, NJ, USA).

To evaluate receptors involved in binding of allergen complexes, we preincubated the cells with unlabeled blocking antibodies against CD23, CD32, CR1, CR2 or a mouse IgG isotype control for 30 min at 4°C before they were incubated with the plasma/serum mixtures. Optimal antibody dilutions for receptor blocking were determined by titration. The role of IgG antibodies in the formation and subsequent binding of allergen complexes to the cells was assessed by depleting the antibodies from plasma/serum using ProteoPrep Immunoaffinity Albumin & IgG Depletion kit (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions with a small adjustment. Undiluted serum/plasma samples were used instead of pre-diluted samples. Because samples were diluted twice during IgG-depletion, control samples were diluted to the same extent in these experiments.

CD23, CD32, CR1 and CR2 expression on B cells

To determine receptor expression on EBV-B cells and PBMCs, we incubated $1x10^5$ cells with PE-labeled mouse anti-human CD23, CD32, CR1, CR2 or mouse IgG1 isotype control for 30 min at 4°C. To distinguish PBBCs within the PBMCs, PBBCs were costained with FITC-labeled mouse-anti-human CD20. The fluorescence was measured by flow cytometry.

Binding of heat-aggregated IgG to B cells

To investigate whether IgG complexes are capable of binding to CD32 and to determine the effect of complement activation on this binding, 500 µg/ml heat-aggregated IgG (HA-IgG) in PBS-2% w/v human serum albumin was incubated with/without serum from a healthy control. Subsequently, $4.5x10^5$ EBV-B cells, IIA1.6 cells or freshly isolated PBMCs were added to the samples and incubated for 1 hour at 37°C to allow aggregates to bind to the cells. Bound IgG antibodies were stained as described above. To determine which receptors were involved in the binding, cells were pre-incubated with blocking antibodies to CD23, CD32, CR1 and CR2 as described earlier.

Statistical analyses

All data were analyzed with GraphPad Prism version 5.0d for Macintosh (GraphPad Software, San Diego, CA, USA). The median fluorescence intensities (MFI) of the receptors and of the IgE/IgG/IgG4 binding were corrected for the background staining of the isotype. Negative values were fixed to 1. The MFI of the receptors and the ELISA data were analyzed using the Kruskal-Wallis method followed by a Dunn's post hoc test for selected groups. For blocking and IgG-depletion experiments, the corrected MFIs were log-transformed and analyzed using repeated measures ANOVA with a Bonferroni's multiple comparison posttest for selected groups. The p values <0.05 were considered significant.

RESULTS

High IgG levels in FA

Allergen-specific IgE, IgG1 and IgG4 levels in BPA, PA and CMA patients were measured by ELISA (Figure 1). IgE levels were comparable among the different groups. In contrast, in PA and CMA patients allergen-specific IgG1 levels (median, 53.1 [range, 3.3-1170] and 40.1 [range, 3-678.5] arbitrary units/ml, respectively) were significantly higher than those in BPA patients (median, 4.3; range, 1-50.8). Furthermore, CMA patients had significantly higher levels of allergen-specific IgG4 (median, 106.4; range, 0.2-1440) than PA and BPA patients (median, 10.7 [range, 2.5-200.6] and 3.8 [range, 0.2-16], respectively). Seven patient samples from each group were used for *in vitro* complex formation experiments. In addition, samples of four HCs, which were selected based on high IgG1/IgG4 levels specific for αS1-casein in the absence of IgE, were used for these experiments. The specific antibody levels to the major allergens in the selected patients and in the HCs are shown in Supplemental Table 1.

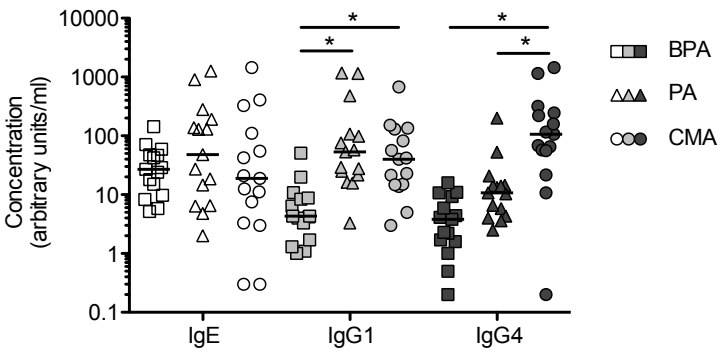

Figure 1 | Allergen-specific IgE, IgG1, and IgG4 levels in BPA, PA and CMA. Birch pollen, peanut or cow's milk-specific IgE, IgG1 and IgG4 levels were determined in BPA, PA, or CMA patients, respectively. Each patient is represented by a square, triangle, or circle in the scatter plot. Lines indicate median values. IgE, IgG1, and IgG4 levels between allergies were compared (n=15, *p < 0.05).

IgG antibodies present in allergen-antibody complexes in BPA and FA

The composition of allergen-antibody complexes binding to EBV-B cells or PBBCs in HCs (Supplemental Figure 1) and BPA, PA, and CMA patients (Figure 2 and Supplemental Figure 2) was investigated using antibodies for IgE, IgG1, and IgG4.

Immune complexes containing all three isotypes were found to bind to EBV-B cells and PBBCs for BPA patients. Also for PA patients, binding of IgE, IgG1, and IgG4 to both cell types was observed. For CMA patients, complex binding to EBV-B cells was observed in six of the seven patients (CMA 1, 3-7), although complex binding was somewhat lower for two patients (CMA 1, 3). In one patient (CMA 2) hardly any complex binding was observed. Binding of complexes to PBBCs as compared to EBV-B cells was more pronounced. For six CMA patients, complexes binding to PBBCs contained IgE, IgG1, and IgG4, whereas in one patient (CMA 2), complexes contained mainly IgG4 and hardly any IgE or IgG1. The optimal allergen concentration for complex binding was 10x higher for CMA (0.01-1 µg/ml) than for PA (0.01-0.1 µg/ml) and BPA patients (0.01-0.1 µg/ml). For the HCs, minimal IgG1 and IgG4 binding to PBBCs was seen for three subjects (HC 2-4). In one of the three subjects (HC 2) also minimal IgE binding was found. Hardly any binding to EBV B cells was observed.

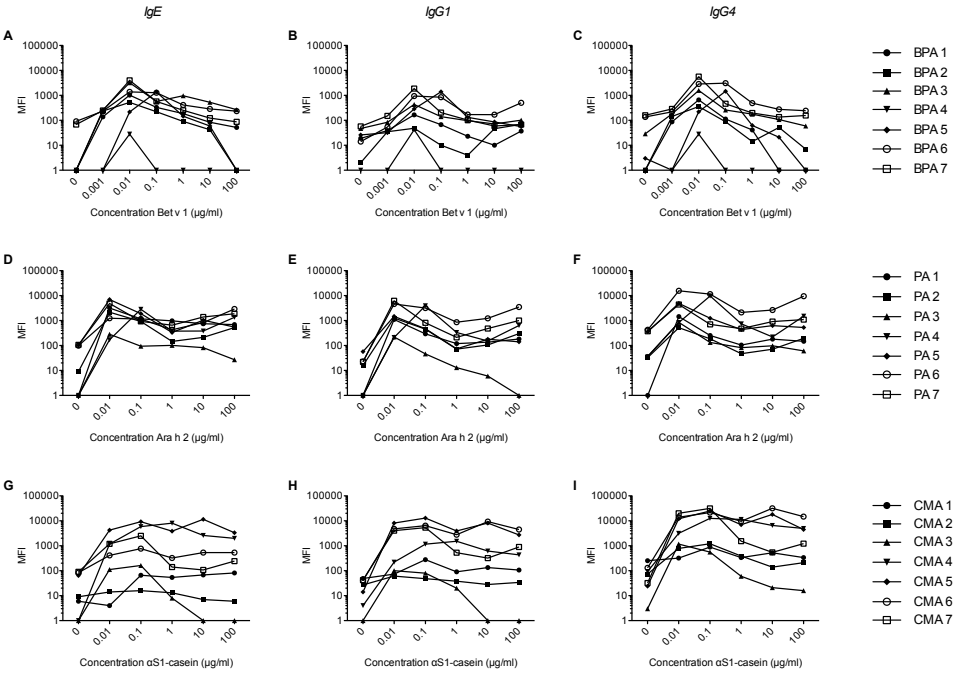

Figure 2 | Allergen-antibody complex binding to PBBCs. MFI of IgE (A, D, G), IgG1 (B, E, H), and IgG4 (C, F, I) present in complexes binding to PBBCs, after incubation of plasma/serum samples of seven BPA (A, B, C), PA (D, E, F) and CMA (G, H, I) patients and different concentrations of the major allergens Bet v 1, Ara h 2, and αS1-casein, respectively.

CR2 involved in complex binding to PBBCs

To determine which receptors were involved in the binding of allergen-antibody complexes, EBV-B cells and PBBCs were pre-incubated with blocking antibodies before incubation with the complexes. For each patient, the optimal allergen concentration for the formation of allergen-antibody complexes was used. For BPA, PA, as well as CMA, binding of complexes to EBV-B cells was mainly mediated by CD23 (Figure 3). In contrast, complex binding to PBBCs was only partially reduced upon blocking this receptor (Figure 4). Blocking CD32 had a minor effect on the complex binding to PBBCs for CMA patients and no effect for PA and BPA patients. However, pre-incubating PBBCs with blocking CR2 antibodies did inhibit the binding of complexes to these cells. Moreover, blocking both CD23 and CR2 virtually abrogated the complex binding for BPA and PA patients, and reduced the binding by almost 90% for CMA patients (significant using a t-test). Blocking CR1 had only a minor effect on the complex binding to PBBCs. Also, in healthy subjects, the complex binding to PBBCs was mediated by CR2 (data not shown).

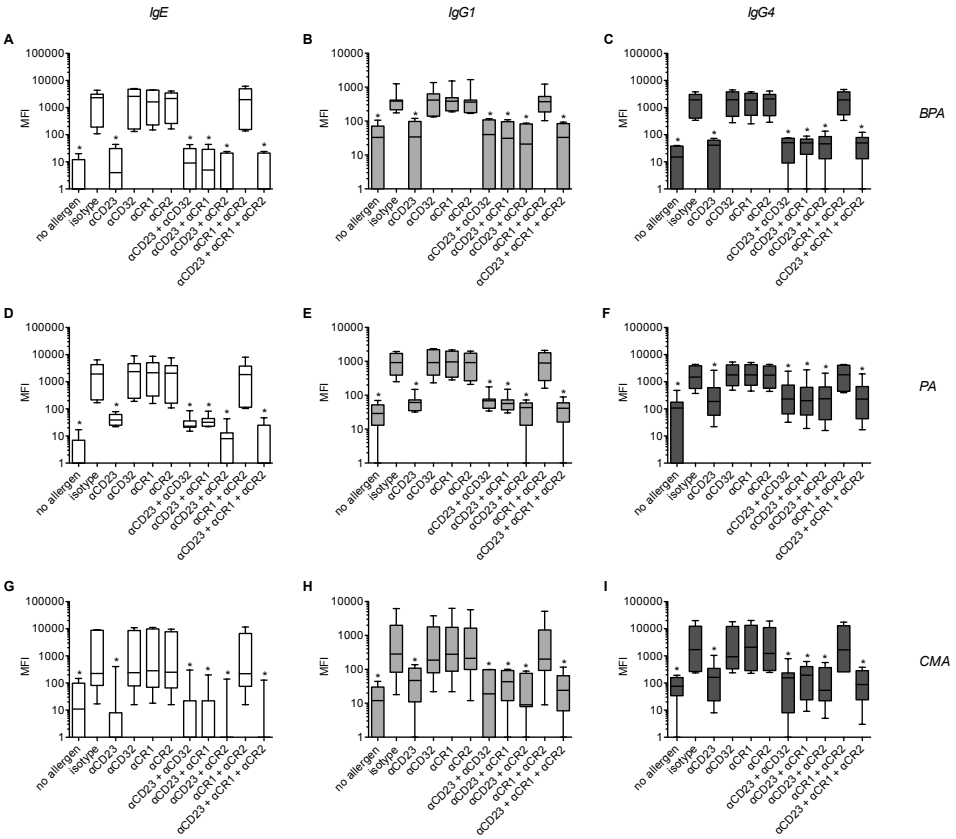

Figure 3 | Allergen-antibody complex binding after blocking several receptor candidates on EBV-B cells. MFI of IgE (A, D, G), IgG1 (B, E, H), and IgG4 (C, F, I) present in complexes binding to EBV-B cells, with and without CD23, CD32, CR1 and/or CR2 blocking in seven BPA (A, B, C), PA (D, E, F), and CMA (G, H, I) patients. All conditions were compared to isotype control with allergen (*p < 0.05).

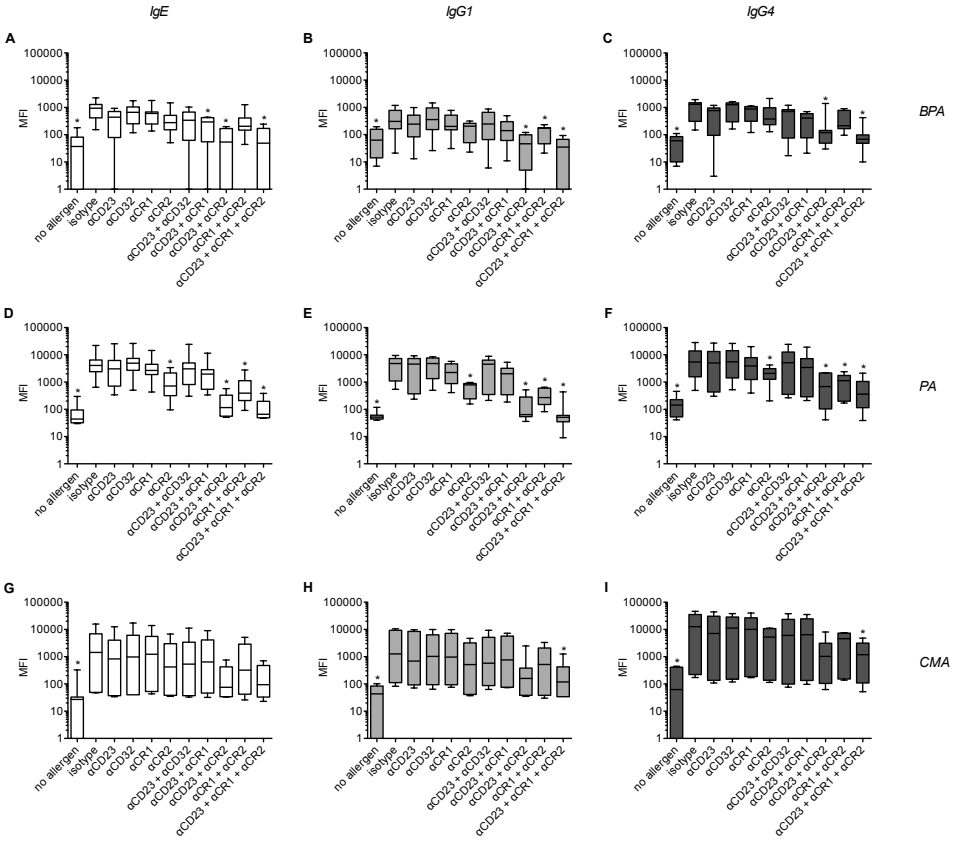

Figure 4 | Allergen-antibody complex binding after blocking several receptor candidates on PBBCs. MFI of IgE (A, D, G), IgG1 (B, E, H), and IgG4 (C, F, I) present in complexes binding to PBBCs, with and without CD23, CD32, CR1, and/or CR2 blocking in seven BPA (A, B, C), PA (D, E, F) and CMA (G, H, I) patients. All conditions were compared to isotype control with allergen (*$p < 0.05$).

Different receptor expression pattern on EBV-B cells compared to PBBCs

To evaluate whether the difference in receptor involvement for EBV-B cells and PBBCs was due to receptor expression, the expression of CD23, CD32, CR1, and CR2 on B cells was determined. Expression of CD23 was much higher on EBV-B cells than PBBCs (Figure 5). In contrast, CR1 and CR2 expression was significantly lower on EBV-B cells compared with PBBCs, whereas CD32 expression was comparable.

Complement deposition on IgG inhibits binding to CD32 and increases binding to CR1/CR2

The effect of complement deposition on the binding of IgG present in antigen-antibody complexes was investigated by comparing the binding of HA-IgG with/without pre-incubation with complement-sufficient serum to EBV-B cells, PBBCs and IIA1.6 cells, of which the last expresses only CD32. Without incubation in serum, binding of HA-IgG to all three cell types was observed and mediated by CD32 (Figure 6).

Upon serum incubation, HA-IgG binding to IIA1.6 cells was strongly reduced, whereas binding to PBBCs slightly increased. Binding to PBBCs and EBV-B cells was now mediated by CR1 and CR2. In both conditions, HA-IgG binding to EBV-B cells was low compared with PBBCs.

IgG antibodies involved in complement activation

To investigate whether IgG antibodies were indeed involved in complement activation, IgG antibodies were depleted from the plasma samples of PA patients before allergen-antibody complex formation. After IgG-depletion, the complexes contained primarily IgE (Figure 7). The binding of these complexes to PBBCs was completely mediated by CD23, whereas binding to CR2 was negligible.

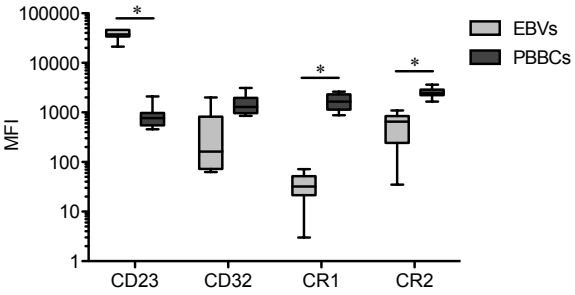

Figure 5 | Expression of CD23, CD32, CR1 and CR2 on EBV-B cells and PBBCs. The MFI of the different receptors on EBV-B cells and PBBCs is depicted. Expression levels between EBV-B cells and PBBCs were compared (n=9, *p < 0.05).

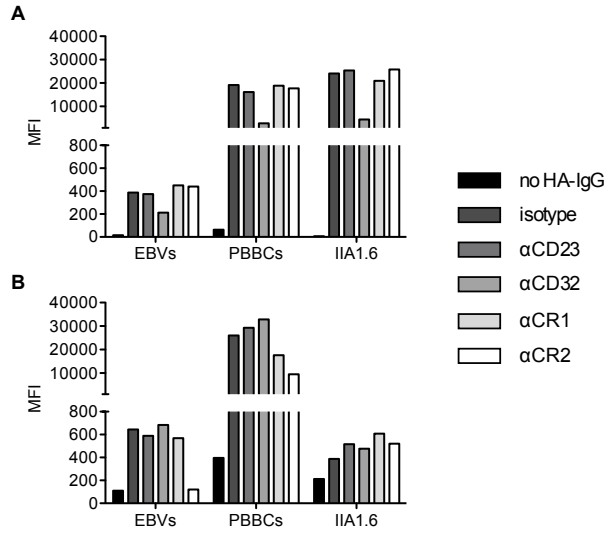

Figure 6 | Binding of HA-IgG with and without serum incubation to several B cells. Binding of HA-IgG without (A) and with (B) serum incubation to EBV-B cells, PBBCs and CD32-expressing IIA1.6 cells pre-incubated with blocking antibodies for CD23, CD32, CR1 or CR2. These figures are representative examples of two experiments.

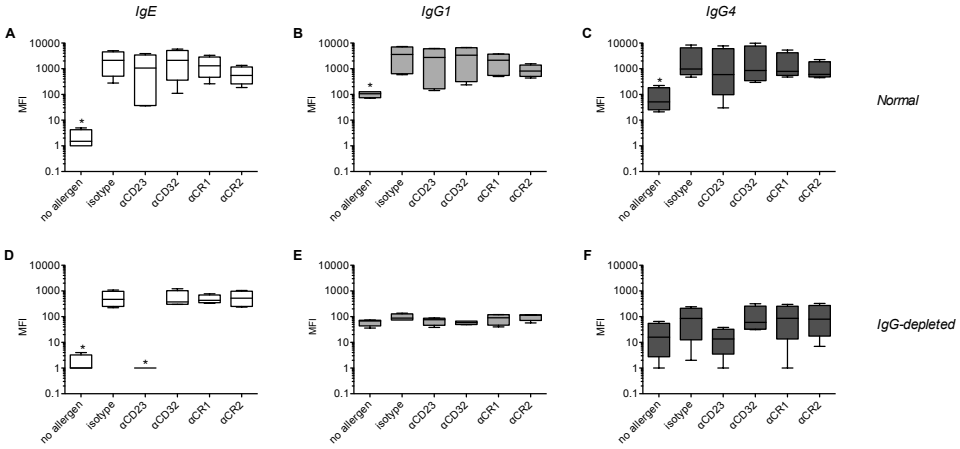

Figure 7 | Effect of IgG depletion on receptor-mediated complex binding to PBBCs. MFI of IgE (A, D), IgG1 (B, E), and IgG4 (C, F) present in complexes binding to PBBCs with and without CD23, CD32, CR1 or CR2 blocking. Complexes were formed by incubating normal or IgG-depleted plasma from four PA patients with Ara h 2. All conditions were compared to isotype control with allergen (*$p < 0.05$).

DISCUSSION

The objective of this study was to investigate allergen-antibody complex formation and binding to B cells in FA in comparison to BPA. Furthermore, the role of specific IgG antibodies in these complexes was examined by determining the antibody composition of the complexes and the receptors involved in complex binding.

Confirming previous studies, IgE binding to EBV-B cells was observed for BPA patients (10, 21). Also for PA patients, binding of complexes to these cells was observed, whereas for CMA patients, binding was less pronounced. Interestingly, not only IgE, but also IgG1 and IgG4 were detected in complexes binding to EBV-B cells.

Although EBV-B cells are commonly used in IgE-FAP studies, complex binding to PBBCs presumably represents the *in vivo* situation better and was therefore also determined. In general, for PA and BPA patients, the binding to PBBCs was comparable to EBV-B cells, whereas for CMA patients complex binding to PBBCs was more pronounced. The optimal allergen concentration to form complexes was different between the allergies. For CMA, this concentration was 10x higher than for PA and BPA. Because it is known that antibody/allergen ratios influence the complex formation, the increase in optimal concentration and the less pronounced complex binding for CMA patients compared to BPA and PA patients may reflect the higher absolute antibody levels, mainly due to significantly higher IgG1 and/or IgG4 levels, against the major allergen αS1-casein in the CMA patients.

Previous studies indicated that also healthy controls have detectable food-specific IgG1 and IgG4 levels (19). To determine whether complex formation and binding also occurs in these subjects, we investigated IgE, IgG1 and IgG4 binding to EBV B cells and PBBCs for four HCs.

As expected, complex binding was less pronounced for the HCs as for the FA patients. Hardly any antibody binding to the EBV B cells was found, whereas some IgG1 and IgG4 binding to PBBCs was observed for three HCs. Because the allergen-specific IgG1 and IgG4 levels in these subjects are comparable with the levels found in the CMA patients (Supplemental Table 1), the less pronounced complex binding probably reflects the lower absolute antibody levels in the HCs because of absence of food-specific IgE levels. Complexes in patients were shown to bind via CD23 and CR2. The data in HCs suggest that absence of IgE in the complexes makes complex binding less efficient. Because of this low complex binding, the functional role of the complexes in healthy controls may be limited.

Although complex binding to EBV-B cells and PBBCs was comparable, the receptors involved in the binding were different. In accordance with previous studies, blocking CD23 on EBV-B cells inhibited the complex binding for BPA patients completely (6, 7, 22). Also in FA, the binding to EBV-B cells was completely reduced upon blocking CD23. Not only was IgE binding to EBV-B cells inhibited by blocking CD23, but also IgG1 and IgG4 binding, which suggest that the formed complexes contained mixed IgE, IgG1 and IgG4 antibodies and that their binding to EBV-B cells was mediated via IgE. Blocking CD23 on PBBCs reduced complex binding only partially. Surprisingly, the remaining binding was not mediated by CD32, but by CR2.

The differences in the receptors involved in complex binding to EBV-B cells and PBBCs were explained by the expression of these receptors on the cells. As mentioned before, EBV-B cells are often used in IgE-FAP as a model system because of their high CD23 expression. However, compared with PBBCs, the expression of CD23 was significantly higher on EBV-B cells, while the expression of CR1 and CR2 was lower. The high expression of CD23 probably leads to preferential binding of allergen-antibody complexes via IgE to this receptor. Apparently, the lower expression of CR2 on EBV-B cells minimized the contribution of this receptor to the complex binding, because blocking CD23 completely abrogated the binding to these cells. This is in line with a previous study in which binding of immune complexes to EBV-B cells was observed only to cells with high CR2 expression (23). The authors suggested that complexes bind to EBV-B cells via CR2 when multivalent attachment of the complexes occurs. This hypothesis may also explain why HA-IgG, which is more potent in binding complement components than complexes containing IgE, IgG1, and IgG4, was able to bind to CR2 on EBV-B cells. However, because of the lower CR2 expression, HA-IgG binding to EBV-B cells was low compared to PBBCs. Together, these data show that EBV-B cells may not be a representative model for allergen-antibody complex binding and IgE-FAP *in vivo*.

Immune complexes that contain IgG1 can activate the classical pathway of the complement system. Activation of this system leads to the fixation of complement component C3b to the complexes. This fragment is subsequently degraded to iC3b and C3dg, which both bind to CR2. In contrast with IgG1, IgG4 may inhibit the activation of the classical pathway, or instead may activate the complement system via the alternative pathway (24, 25). However, this activation requires relatively high antibody concentrations and is weaker than that of the classical pathway by IgG1 (25). Thus, IgG, especially IgG1, present in the mixed allergen-antibody complexes may activate the complement system and results in subsequent binding of the complexes to CR2.

IgG was depleted from serum before complex formation to investigate this. Interestingly, binding of complexes formed in serum depleted for IgG was no longer inhibited by blocking CR2, but was completely dependent on CD23. This indicates that complex binding to CR2 on PBBCs was dependent on the presence of IgG antibodies in the complexes. Furthermore, in BPA patients with low levels of IgG1, complex binding to PBBCs was mediated by CD23 as described previously, whereas in patients with higher IgG1 levels binding occurred via CR2 (data not shown) (11). Also, in PA patients, who all have high IgG1 levels, and in HCs, immune complexes bound to CR2. However, in CMA patients, who have high levels of both IgG1 and IgG4, these effects were less clear. This suggests that, in particular, IgG1 in the complexes was needed for binding to CR2. It would be interesting to deplete IgG1 and IgG4 antibodies separately to further investigate the effects of these antibodies. In addition, the role of IgG2 and IgG3, which both activate the complement system, needs to be investigated.

Interestingly, previous studies have indicated a direct interaction between CD23 and CR2 (26-28). In addition, they showed that the IgE receptor can bind simultaneously to the CR and IgE. Both membrane and soluble CD23 may be involved in the interaction with CR2. Whereas soluble CD23 is not involved in the complex binding observed in this study, the role of the interaction between membrane CD23 and CR2 is unclear. Because blocking of both CD23 and CR2 has a much stronger blocking effect as compared to blocking CD23 or CR2 alone, our data suggest that the receptors act in a cooperative manner to bind the complexes.

Unexpectedly, CD32 was not involved in the binding of immune complexes to B cells. An obvious explanation for this observation is that because of the presence of IgE and IgG4, the allergen-antibody complexes contained too little IgG1 for binding to CD32. Also other studies have shown that immune complexes containing other antibodies in addition to IgG may not bind to CD32 on PBBCs (1, 2, 22). However, the experiments with HA-IgG showed that complexes without serum incubation can bind to B cells via CD32, whereas the same complexes incubated with serum only bind via CRs. This points towards another mechanism, that is, that complement fixation to the complexes apparently abrogated binding via CD32. A similar phenomenon was previously described for the binding of IgG1 to another low-affinity IgG receptor, namely CD16 (29). Moreover, studies investigating the binding sites for Fc receptors and C3 components on IgG have shown that they bind to the same region (30, 31).

The effect of complement activation on the binding and processing of complexes by B cells has already been investigated in several vaccination studies. *In vitro* studies showed that complement deposition on complexes formed using sera after vaccination resulted in more efficient uptake and antigen presentation by antigen-specific and non-specific B cells (1, 2). Moreover, the complement-containing complexes induced an antibody response in antigen-specific B cells, whereas complexes without complement factors did not (32). This is in agreement with *in vivo* mouse studies, which showed reduced antibody responses in CR1/CR2-deficient mice or in mice treated with CR1/CR2 blocking antibodies (33-36). Interestingly, previous research in C3- and CR2-deficient patients has shown that, in particular, IgG4 levels were depressed in these patients, which may indicate that CR2 activation is important for IgG4 production (37, 38).

The effects of allergen-antibody complexes formed in FA patients on antigen presentation by B cells are still unclear. Although previous IgE-FAP studies have shown that IgG antibodies formed after immunotherapy inhibit antigen presentation, these studies were done with EBV-B cells instead of PBBCs (7, 10, 11). Because of differences in receptor expression, complement deposition on complexes has different effects on the binding to receptors on EBV-B cells than PBBCs. Therefore, we investigated the effect of complex formation in FA patients on antigen presentation by both EBV-B cells and PBBCs in a preliminary study. As in previous IgE-FAP studies (7, 10, 11), these preliminary experiments showed that T cell proliferation occurred at a lower concentration when incubating EBV cells with complexes from serum/plasma of BPA and CMA patients, incubated with increasing concentrations of Bet v 1 and αS1 casein, respectively (Supplemental Figure 3). In addition, depleting IgG antibodies from the serum/plasma of CMA patients increased the antigen presentation further. However, when using PBMCs as antigen presenting cells, proliferation was only observed at the highest allergen concentration (100 µg/ml), whereas no effects were seen at the concentrations that showed optimal complex formation (0.01-0.1 µg/ml, Supplemental Figure 3). These data suggest that for PBBCs, the complex formation is not the driving force of the initiation of the T cell response, and that their physiologic relevance for IgE-FAP may be limited. Although this seems in contrast to a vaccination study that showed that complement-containing IgG-influenza complexes can be presented by PBBCs, the data do fit with the data from a recent *in vivo* mouse study (1, 39). This study showed that CD23+ B cells do not present IgE-allergen complexes themselves but transport them to B cell follicles and transfer them there to CD11c+ cells. Depleting these CD11c+ cells abrogated the antigen presentation indicating that these cells were essential for antigen presentation. Interestingly, a role for CR2+ B cells in the transport of immune complexes into follicles also has been described (40, 41).

In conclusion, allergen-antibody complexes formed in FA patients contain IgE, IgG1 and IgG4 antibodies. Their levels influence the composition of the complexes and the subsequent binding to receptors on B cells: mixed IgE/IgG-containing complexes bind not only via CD23, but also via CR2. Preliminary data suggest that PBBCs are not directly involved in IgE-FAP. Moreover, considering the differences in the binding pattern of allergen-antibody complexes and in antigen presentation between EBV-B cells and PBBCs, it is questionable whether EBV-B cells can be used as a representative model for complex binding and IgE-FAP. Therefore, future studies should address the implications of the binding of mixed IgE/IgG-containing complexes to both CD23 and CR2 on antigen presentation and allergic responses *in vivo*.

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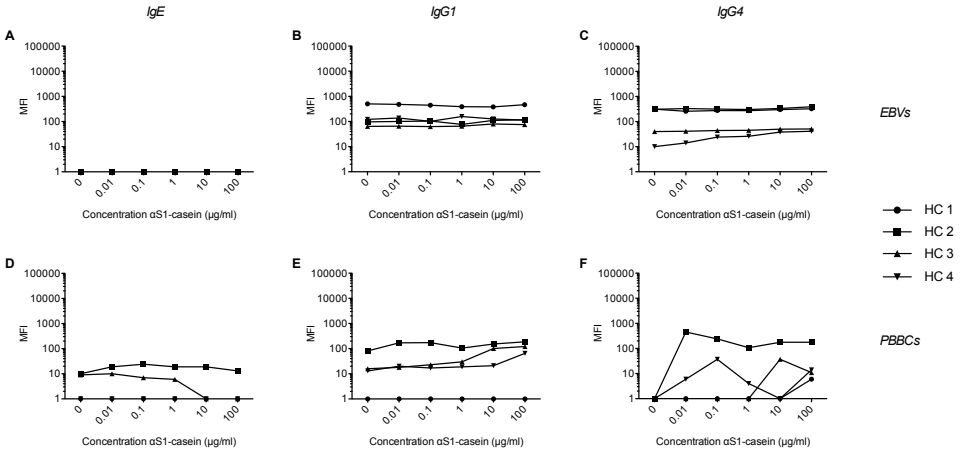
SUPPLEMENTAL DATA

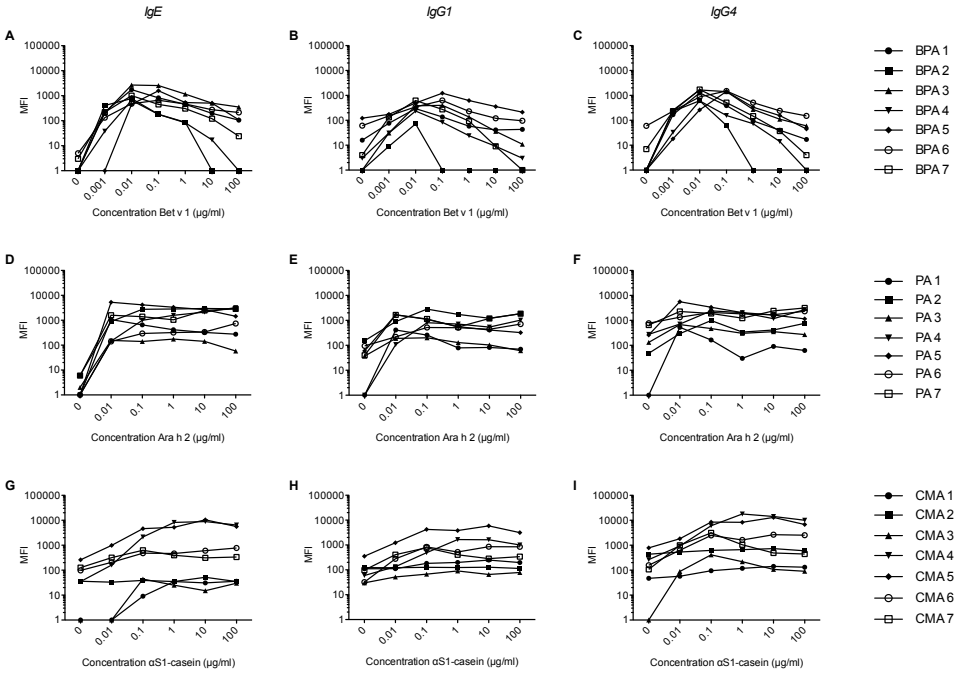
Supplemental Table 1 Specific antibody levels to major allergens in patient samples used for *in vitro* complex formation.

Patients	Major allergen	Samples	IgE (AU)	IgG1 (AU)	IgG4 (AU)
HC 1	αS1-casein	Plasma	nd	703	310
HC 2	αS1-casein	Plasma	nd	632	475
HC 3	αS1-casein	Plasma	nd	233	334
HC 4	αS1-casein	Plasma	nd	407	1137
BPA 1	Bet v 1	Plasma	130	40	7
BPA 2	Bet v 1	Plasma	118	2	3
BPA 3	Bet v 1	Plasma	395	19	20
BPA 4	Bet v 1	Plasma	69	12	20
BPA 5	Bet v 1	Plasma	170	144	27
BPA 6	Bet v 1	Serum	218	59	98
BPA 7	Bet v 1	Serum	271	40	25
PA 1	Ara h 2	Plasma	575	102	15
PA 2	Ara h 2	Plasma	1483	795	66
PA 3	Ara h 2	Plasma	242	340	88
PA 4	Ara h 2	Plasma	922	915	46
PA 5	Ara h 2	Plasma	892	53	11
PA 6	Ara h 2	Plasma	373	383	974
PA 7	Ara h 2	Plasma	292	131	30
CMA 1	αS1-casein	Serum	107	146	344
CMA 2	αS1-casein	Plasma	405	147	22520
CMA 3	αS1-casein	Plasma	458	101	504
CMA 4	αS1-casein	Serum	16877	1598	3801
CMA 5	αS1-casein	Plasma	1547	703	721
CMA 6	αS1-casein	Plasma	302	632	1167
CMA 7	αS1-casein	Plasma	365	233	920

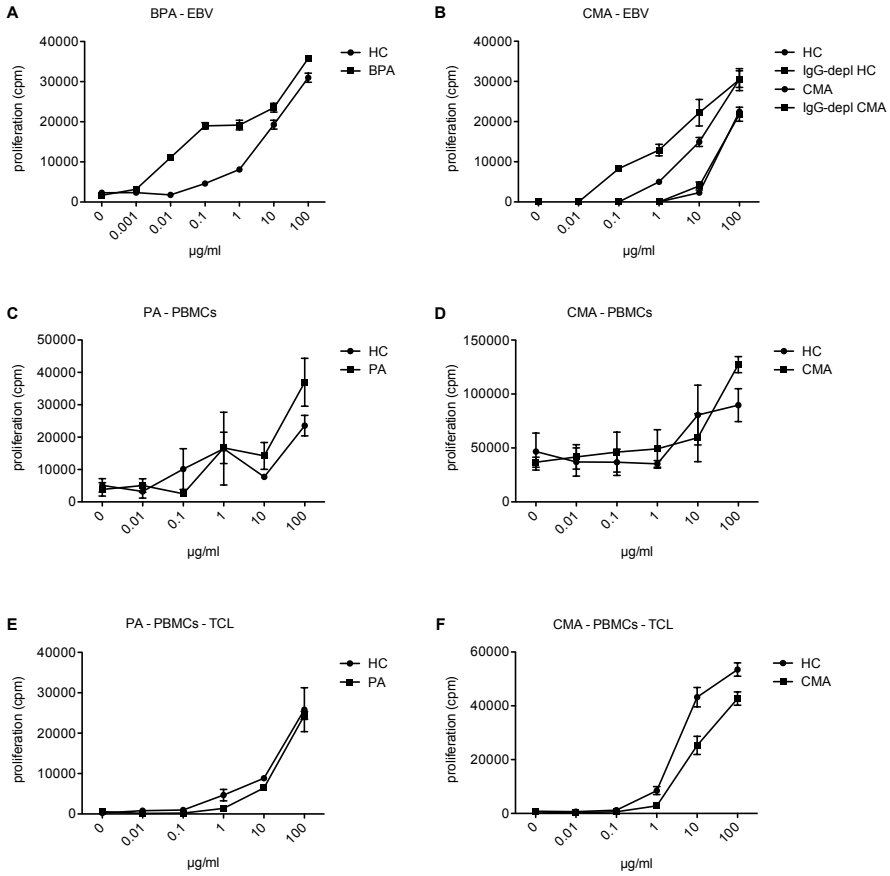
HC, healthy control; BPA, birch pollen allergy; PA, peanut allergy; CMA, cow's milk allergy; AU, arbitrary units; nd, not detectable.



Supplemental Figure 1 | Allergen-antibody complex formation in healthy controls. MFI of IgE (A, D), IgG1 (B, E) and IgG4 (C, F) present in complexes binding to EBV-B cells (A, B, C) or PBBCs (D, E, F), after incubation of plasma samples of four healthy controls with different concentrations of the major allergen αS1-casein.



Supplemental Figure 2 | Allergen-antibody complex binding to EBV-B cells. MFI of IgE (A, D, G), IgG1 (B, E, H) and IgG4 (C, F, I) present in complexes binding to EBV-B cells, after incubation of plasma/serum samples of seven BPA (A, B, C), PA (D, E, F) and CMA (G, H, I) patients with different concentrations of the major allergens Bet v 1, Ara h 2 and αS1-casein, respectively.



Supplemental Figure 3 | Effect of complex binding on antigen presentation to T cells. In general, complexes were formed as described in the article. For the experiments with the T cell clones/lines, the complexes were incubated for one hour with APCs before adding the T cells. Proliferation was determined by measuring [^{3}H]-thymidine incorporation. (A) Proliferation of a BP-specific T cell clone, with its autologous EBV-B cells as APC (kind gift of P. Adler Würtzen, ALK Abelló). Proliferation is 100x more efficient upon complex binding using plasma from a BPA than from a HC subject. (B) Proliferation of a CM-specific T cell clone, with its autologous EBV-B cells as APC. Proliferation is 10x more efficient upon complex binding using plasma from a CMA than from a HC subject. IgG-depletion of plasma has no effect when using HC plasma, but induces proliferation at a 10x lower allergen concentration when using CMA plasma. (C,D) Proliferation of PBMCs from a PA or CMA subject in the presence of PA or CMA plasma, as compared to HC plasma. Only at the highest concentration, proliferation in presence of PA or CMA plasma exceeds proliferation in presence of HC plasma. This concentration is much higher than the optimal concentration for complex binding. (E,F) Proliferation of a P- or CM-specific T cell line from a PA or CMA subject, with its autologous PBMCs as APC. Proliferation is not enhanced upon complex binding using PA or CMA plasma as compared to HC plasma.

CHAPTER 3

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The degree of whey hydrolysis does not uniformly affect *in vitro* basophil and T cell responses of cow's milk-allergic patients

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ABSTRACT

Background

Several studies investigated whether hydrolyzed proteins can induce tolerance to cow's milk in children at risk for developing cow's milk allergy. Due to methodological problems and inconsistent findings, the evidence for a tolerogenic effect is limited. A major problem is that different hydrolysates may give different outcomes due to variations in their production and composition.

Objective

To investigate the effect of the degree of hydrolysis on the allergenicity and immunogenicity of whey hydrolysates.

Methods

The hydrolysis of whey was stopped at different time points between 1 and 60 minutes. In 18 cow's milk-allergic patients, the allergenicity of the hydrolysates was determined by immunoblot and the basophil activation test. To test immunogenicity, cow's milk-specific T cell lines were generated.

Results

In most patients increasing time of hydrolysis decreased IgE recognition and basophil activation. However, in five patients, hydrolyzed proteins induced more basophil activation than non-hydrolyzed proteins. The immunoblot data indicated that these patients recognized either a 25-30 kDa degradation product of casein or a 10 kDa degradation product of whey. Although T cell activation was decreased in all patients over time, half of them still showed a positive response to the proteins after 60 min of hydrolysis.

Conclusion

Increasing the time of hydrolysis reduces both allergenicity and immunogenicity of whey hydrolysates in most but not all patients. This indicates that not the degree of hydrolysis is decisive but the presence and stability of IgE and T cell epitopes in the hydrolysate recognized by individual patients.

INTRODUCTION

Cow's milk allergy (CMA) is defined as an immunological response to one or more cow's milk (CM) proteins. The disease can be characterized by cutaneous, gastro-intestinal, respiratory and/or cardiovascular symptoms that appear after exposure to milk proteins (1-3). Whereas 0.3-3.5% of the young children seems to be affected by CMA, the prevalence in adults is estimated to be only 0.1-0.3% (4, 5). At the moment there is no therapy available for CMA and the only way to reduce the symptoms is avoiding exposure to CM (1, 3).

Because CM is an important nutrient source for infants when breast-feeding is not possible, hypoallergenic formulae were developed for children with CMA or children at risk for developing CMA. These formulae contain either amino acids or hydrolyzed milk proteins (whey, casein or both) (1). Based on the degree of hydrolysis and the length of the remaining peptides, hydrolyzed proteins are categorized as partial or extensive hydrolysates (6-8). The extensive hydrolysates contain only small peptides and are mainly used as a replacement for CM-containing formulae in allergic children (8, 9). In contrast, the partial hydrolysates may contain larger protein fragments and are used in infants at risk for CMA. They were developed with the idea that less hydrolyzed proteins are more immunogenic and therefore may prevent CMA by inducing tolerance to CM (2, 6). However, larger protein fragments also result in increased allergenicity, and therefore these hydrolysates are not suitable for the treatment of CMA children (1, 7, 8).

Several studies investigated the effect of extensive and partially hydrolyzed CM proteins on allergy prevention. Whereas animal studies indicated that only partial hydrolysates induce tolerance (10-12), both partial and extensive hydrolysates seem to prevent CMA and atopic dermatitis in high-risk children (8, 9, 13-16). However, evidence for this beneficial effect in humans is limited due to methodological problems and inconsistent findings (8, 9, 14, 17-19). One of the problems is that the effects may vary between different hydrolysates due to differences in their production, such as differences in protein sources, enzymes and time of hydrolysis (8, 19). Therefore, a better understanding of the influence of these factors on the properties of hydrolyzed proteins is necessary.

In this study, the effect of the degree of hydrolysis on the allergenicity and immunogenicity of hydrolysates was investigated in adult CMA patients. The hydrolysis of whey proteins was stopped on different time points. Allergenicity was determined by immunoblot and the basophil activation test. In addition to high CM-specific IgE levels, CMA patients may also have high CM-specific IgG levels (20). Because IgG antibodies may inhibit basophil activation, activation was tested in the presence and absence of plasma (21). Immunogenicity of the hydrolysates was tested with CM-specific T cell lines (TCLs).

MATERIAL AND METHODS

Patients

Eighteen adult CMA patients were included in this study (Table 1). The diagnosis was based on a suggestive history, positive IgE levels specific for CM and/or a positive double blind placebo-controlled food challenge. Before venous blood samples were taken and plasma samples were collected, an informed consent was obtained from the patients. IgE levels specific for CM, casein, α-lactalbumin (α-LAC) and β-lactoglobulin (β-LG) at the time of inclusion (Table 1) were determined by CAP system FEIA (Thermo Fisher Scientific, Waltham, MA, USA). This study was approved by the Ethics Committee of the University Medical Center Utrecht (approval number: 11-187).

Hydrolysis of whey

Whey (Lactalis, Laval, France) was dissolved at a concentration of 10 mg/ml in demineralized water. The solution was heat-treated and an established mixture of endo- and exopeptidases (enzyme composition confidential by Danone) was added for the hydrolysis. At 16 time points between 0 and 60 min, a sample was obtained. All samples were diluted and heated for 10 min to deactivate the enzymes completely.

Protein pattern of the whey hydrolysates

The protein pattern was analyzed by SDS-PAGE followed with a silver staining and by Liquid Chromatography-Electrospray/Mass Spectrometry (LC-ESI/MS). For SDS-PAGE, the Criterion system with a 10-20% Ready Gel Tris-HCl gel (both Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instructions. The hydrolysates (2 µg per sample) were run on gel under reducing conditions. After protein separation, the proteins were silver-stained as described previously (22). The LC-MS was performed on an Agilent 1100 Series nano-LC System and microTOF-QII MS (Thermo-Quest, San Jose, CA) using a PepSwift Monolithic PS-DVB column (200µm x 5cm, Thermo Fisher Scientific) and a trap column (200µm x 5mm, Thermo Fisher Scientific) filled with the same matrix and directly interfaced with the mass spectrometer. Analysis was performed at a flow rate of 3 µl/min using solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 99.99% LC-MS Chromasolv® aqueous acetonitrile (Sigma Aldrich, St Louis, MO, USA)).

Molecular weight distribution of the whey hydrolysates

The molecular weight distribution was determined by gel permeation chromatography (GPC) using UV absorbency. GPC was performed at a flow of 0.9 ml/min; HPLC-column: Superdex Peptide 10/300 at 30°C (GE Healthcare art. nr. 17-5176-01); UV detector = 200 nm; electronic integrator with GPC mode. Based on the calibration curve, the GPC software calculated the molecular weight of various segments of the chromatogram.

Immunoblot

IgE binding to the major allergens of CM and the whey hydrolysates was determined with immunoblot. Proteins were separated using SDS-PAGE as described above and transferred on

Table 1 Patient characteristics

Code	Müller score[$]	IgE CM (kU_A/l)	IgE Casein (kU_A/l)	IgE α-LAC (kU_A/l)	IgE β-LG (kU_A/l)
01	0	30.4	38.9	10.6	4.5
02	4*	92.5	99	1.58	1.68
03	3	0.41	0.25	0.03	0.59
04	2	87	99.4	0.18	0.2
05	4*	>100	>100	97.8	97.6
06	0	3.98	3.8	0.38	0.96
07	4*	>100	>100	13.9	25.7
08	2/3*	>100	>100	28.5	>100
09	0	4.15	1.08	5.97	0.43
10	0	35	37	0.41	0.88
11	4*	10.9	14.3	0.07	3.79
12	4*	>100	>100	6.57	9.53
13	4*	28.7	28.9	2.86	12
14	4*	32.6	41.6	1.11	0.72
15	1	4.52	5.37	0.04	0.04
16	2/3*	>100	>100	3.7	5.2
17	4*	8.13	8.72	2.14	1.28
18	3	0.15	0.31	0.01	0.2

[$] Müller scores for severity of CMA: 0 = oropharyngeal symptoms, 1 = symptoms of skin and mucous membranes, 2 = gastrointestinal symptoms, 3 = respiratory symptoms, 4 = cardiovascular symptoms. * Müller score based on history (due to the severity of the symptoms, these patients were not challenged), the Müller scores of the other patients were based on the double-blind placebo-controlled food challenge.

a polyvinyldifluoride membrane using the Criterion Blotter system (both Bio-Rad) according to the manufacturer's instruction. The membrane was blocked and incubated with plasma of the CMA patients (diluted 1:25 or more if required in 1% albumin in PBS/Tween). After washing, bound IgE was stained with HRP-conjugated anti-IgE (1:30,000 in 1% albumin in PBS/Tween, KPL, Gaithersburg, MD, USA). The membrane was washed overnight and the bands were visualized using the ECL technique (KPL) according to the manufacturer's instruction.

Direct unwashed and washed basophil activation test

Basophil activation by the major allergens of CM and the whey hydrolysates was analyzed using a flow cytometry-based assay measuring CD63 expression. Heparinized whole blood from the CMA patients was stimulated with equal volumes of allergens diluted in RPMI/IL-3 (2 ng/ml, R&D systems, Minneapolis, MN, USA). The reaction was stopped by adding cold PBS/EDTA (20 mM). Cells were stained using anti-CD63, anti-CD123, and anti-CD203c (all from BioLegend, San Diego, CA, USA). Red cells were lysed by adding FACS Lysing Solution (BD Biosciences, San Jose, CA, USA). Basophil activation was analyzed by flow cytometry using a FACS Canto II (BD Biosciences), and expressed as percentage of CD63-positive cells within CD203c/CD123-

positive cells. The percentages were corrected for spontaneous activation seen with RPMI/IL-3. To determine the general effect of the degree of hydrolysis on basophil activation, the activation by hydrolyzed proteins was expressed as percentage of the activation by non-hydrolyzed proteins.
For part of the experiments, plasma from whole blood was washed away by centrifugation and replaced by RPMI until the original blood volume was reached. Heparin (1 µg/ml, Sigma Aldrich) was added to prevent clotting. Basophil stimulation was performed as described above.

T cell proliferation and cytokine production
To assess T cell activation in response to the whey hydrolysates, CM-specific TCLs were generated and tested as described previously (23). T cell proliferation was determined by measuring [^{3}H]-thymidine incorporation. Stimulation indexes (SIs) were determined by dividing hydrolysate-induced proliferation by background proliferation. A SI $\geq$2 was considered positive. In addition, to determine the general effect of the degree of hydrolysis on T cell proliferation, the proliferation induced by hydrolyzed proteins was corrected for background proliferation and expressed as percentage of the proliferation by non-hydrolyzed proteins. Cytokine levels (i.e. IFNγ, IL-13 and IL-10) in the supernatants were measured by ELISA according to the manufacturer's instructions (Sanquin, Amsterdam, The Netherlands). All values were corrected for background levels.

RESULTS

Protein pattern of the whey hydrolysates
The degradation of CM proteins and the formation of peptides at the different time points of hydrolysis are shown in Figure 1. At 0 min, when the proteins were only heat-treated, 80% of the proteins were larger than 10 kDa. The silver staining of the SDS-PAGE (Figure 1A) and the spectra of the HPLC (Figure 1B, Supplemental Figure 1 and Supplemental Figure 2) show that most of these proteins had an estimated molecular mass of approximately 14 and 18 kDa, which corresponds to the mass of α-LAC and β-LG, respectively. Moreover, both techniques show that adding enzymes for 1 min already partially degraded the proteins. Only small traces of intact protein were visible on the gel after 15 min. However, a degradation product that was formed after 1 min was still visible after 40 min of hydrolysis. The molecular weight distribution indicates that after 60 min of hydrolysis less than 1% of the proteins was larger than 10 kDa and more than 90% was smaller than 3 kDa (Figure 1C).

Protein/peptide recognition by IgE
In most patients (01, 03, 04, 06, 07, 09-11, 14-18), proteins/peptides were recognized when they were hydrolyzed for 11 min or less (Figure 2A). Only in five patients (02, 05, 08, 12, 13), IgE binding to the later time points was observed. Interestingly, one of these patients (02) recognized a band around 25-30 kDa (Figure 2B). This band seemed to appear after hydrolysis of the proteins for 1 min and was still visible after 60 min of hydrolysis. The other four patients

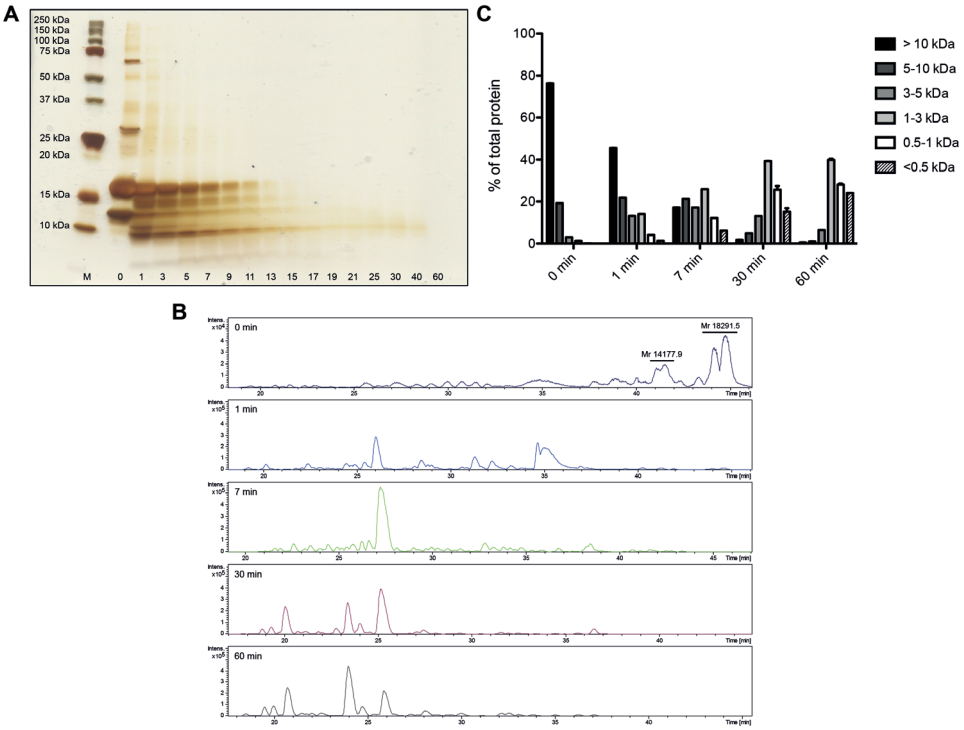

Figure 1 | Protein pattern of the whey hydrolysates. The protein patterns of the hydrolysates on the different time points were visualized with SDS-PAGE followed by a silverstaining (A) and with HPLC spectra of five time points (B). In addition, the molecular weight distribution was determined with GPC (C). The numbers on the bottom of the gel indicate the different time points of the hydrolysates. The marker is designated by an M. The mass of the largest peaks in the HPLC spectra of the non-hydrolyzed proteins was determined by mass spectrometry and is indicated above the peaks.

predominantly recognized a degradation product of approximately 10 kDa (Figure 2C). This band was still visible after 40 min of hydrolysis but no longer at the 60 min time point.

Basophil activation by the hydrolysates

Although IgE levels towards the whey proteins were lower than to casein (Table 1), the median basophil activation in response to the major allergens of cow's milk was similar (Supplemental Figure 3). Only one patient (03) showed no response to any of the major allergens and hydrolysates. Although this patient had positive IgE levels for casein and β-LG (4 kU_A/l and 37 kU_A/l, respectively) in the past, the levels at the time of inclusion were only slightly positive for β-LG (0.59 kU_A/l).

In twelve patients (01, 04, 06, 07, 09-11, 14-18), basophil activation decreased over time when whey was hydrolyzed (Figure 3A). As expected, these were the same patients that showed a decrease in IgE recognition on the immunoblot. While in some patients (01, 06, 10, 15, 18) the basophil activation was lost rapidly (Figure 3C), in others (04, 07, 09, 14, 16, 17) a small percentage

of the cells were still activated when stimulated with the proteins that were hydrolyzed for 60 min (Figure 3E). Of the six patients that still showed a positive response after 60 min of hydrolysis, three patients (04, 07, 16) had high IgE levels against CM. In two other patients (14, 17), the IgE levels were not high but these patients had a history of severe CMA (Müller 4).

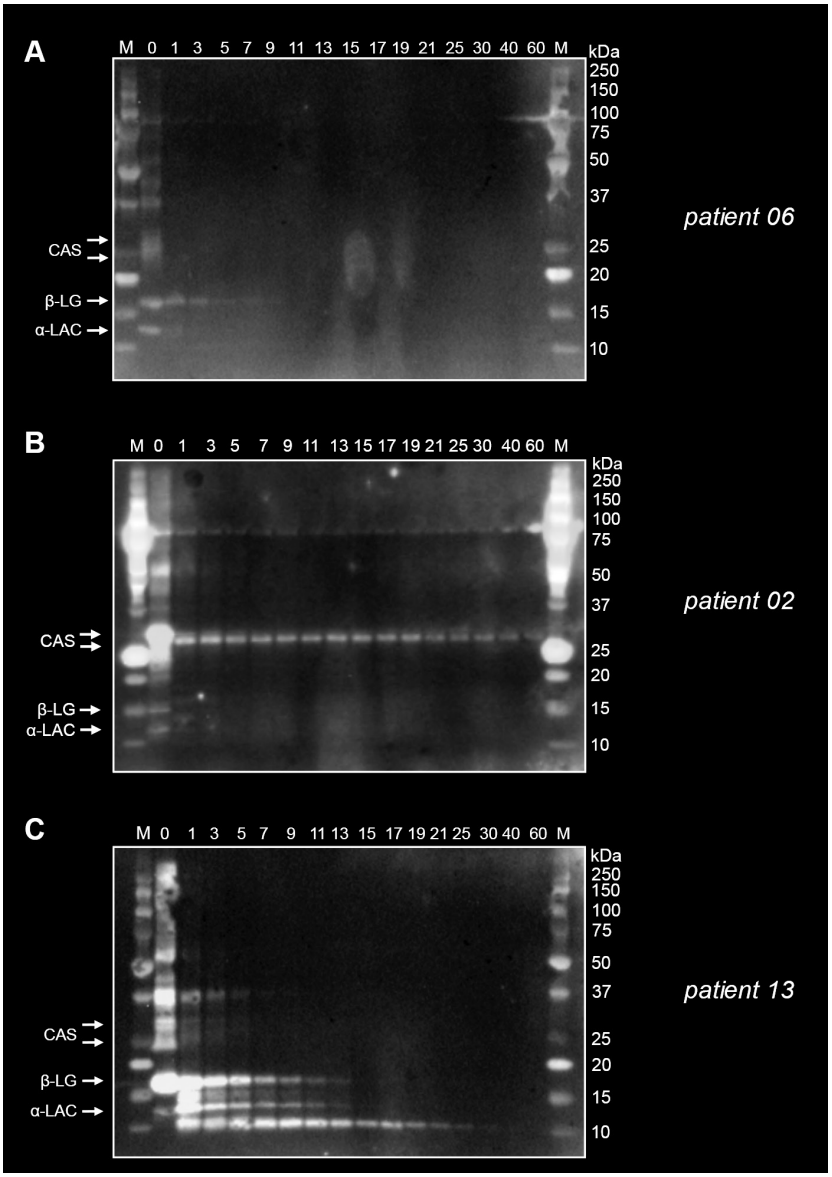

Figure 2 | The recognition of proteins/peptides in the hydrolysates by IgE. The recognition of the hydrolysates by IgE antibodies of one patient (A, 06) who only recognized proteins/peptides when they were hydrolyzed for a short period, and of two patients (B and C, 02 and 13, respectively) who showed IgE binding to the later time points, is depicted. The numbers indicate the different time points of the hydrolysates. The markers are indicated by an M.

In general, the response to the hydrolysates in the twelve patients increased when using washed instead of unwashed basophils (Figure 3B, D and F). This was either indicated by the lower dose that was able to stimulate the basophils or by the fact that proteins hydrolyzed for a longer time were still able to induce basophil activation.
In five patients (02, 05, 08, 12, 13), hydrolysis of whey did not result in decreased basophil activation (Figure 4A). Instead, proteins that were hydrolyzed for only 1 min induced more basophil activation than non-hydrolyzed proteins. This increased activation was still observed after 60 min of hydrolysis. Whereas only four of these patients (02, 05, 08, 12) had high CM-specific IgE levels, the fifth patient (13), who had lower IgE levels, had a history of severe CMA. Only one of the patients (02) showed a dose-response effect of the hydrolysates (Figure 4C). In the other four patients (05, 08, 12, 13), the lowest concentration showed a similar basophil response as the highest concentration, even after 60 min of hydrolysis (Figure 4E). Interestingly, the patient that showed a dose-response effect (02) was the one patient that recognized a different band on the immunoblot.
The effect of using washed instead of unwashed cells was variable for these five patients. In two patients (05 and 08), the background activation of washed basophils was higher than the activation by non-hydrolyzed proteins resulting in negative percentages. Therefore, these patients were excluded from the analysis. In the other three patients (02, 12, 13), the response of the basophils to non-hydrolyzed proteins increased by washing while no differences were observed for the hydrolyzed proteins (Figure 4D and F). When expressing the activation by hydrolyzed proteins as a percentage of the activation by non-hydrolyzed proteins, the percentages for the washed cells were lower while the response of the hydrolyzed proteins was similar (Figure 4B).

T cell activation by the hydrolysates

Unfortunately no specific TCLs could be generated for four patients (01, 04, 07, 10). For the other 14 patients, TCLs showed a decrease in T cell proliferation over time when incubated with the hydrolyzed proteins. The average response decreased with more than 50% after 9 min (range 1-40 min) of hydrolysis (Figure 5A). However, the proteins formed after 60 min of hydrolysis were still able to induce a positive proliferative response (SI >2) in half of the TCLs (patients 02, 05, 09, 11-14, Figure 5B). The cytokine response showed a similar trend as the proliferation (Figure 5C-E). Whereas IL-13 was secreted by all TCLs except patient 15, IFNγ and IL-10 were detectable in the supernatants of part of the TCLs (IFNγ: patients 05, 06, 08, 09, 12-14, 16-18 and IL-10: patients 06, 08, 09, 12, 13, 17, 18).

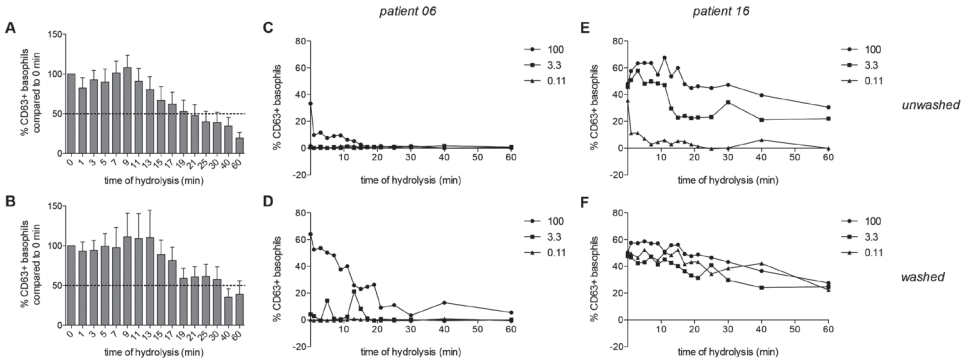

Figure 3 | The activation of unwashed (A, C, E) and washed (B, D, F) basophils in response to the hydrolysates. The average response of twelve patients, who showed a decrease in activation when proteins were hydrolyzed for a longer period, to the highest concentration of the hydrolysates is depicted (A, B). The percentage CD63+ basophils in comparison to the non-hydrolyzed proteins (0 min) was calculated per patient for each time point. The dotted line indicates a response of 50% compared to non-hydrolyzed proteins. The error bars indicate the standard error of the means. An example of the basophil response of a patient (06) that showed a fast decrease (C, D) and a patient (16) that showed a slow decrease (E, F) in response to different concentrations of the hydrolysates is shown. All values were corrected for background activation.

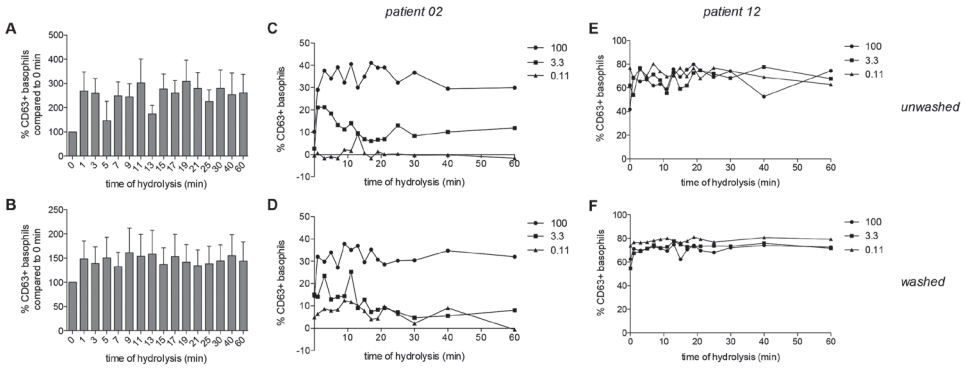

Figure 4 | The activation of unwashed (A, C, E) and washed (B, D, F) basophils in response to the hydrolysates. The average response of five patients, who did not show a decrease in activation when proteins were hydrolyzed, to the highest concentration of the hydrolysates is depicted (A, B). The percentage CD63+ basophils in comparison to the non-hydrolyzed proteins (0 min) was calculated per patient for each time point. The error bars indicate the standard error of the means. An example of the basophil response of a patient (02) that showed a concentration-dependent decrease (C, D) and a patient (12) that showed no decrease (E, F) in response to different concentrations of the hydrolysates is shown. All values were corrected for background activation.

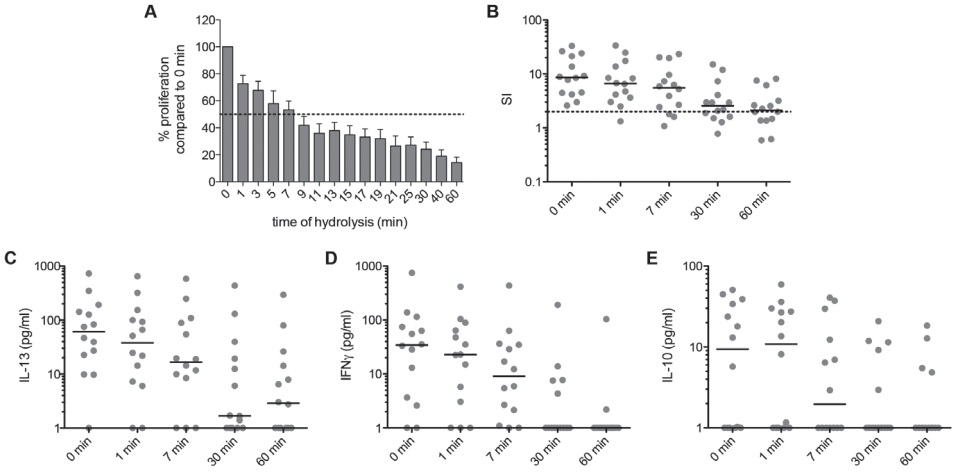

Figure 5 | The activation of TCLs in response to the hydrolysates. The average proliferation (A, n=14), of the TCLs in response to the hydrolysates is depicted. The values were first corrected for background levels, after which the percentage in comparison to the non-hydrolyzed proteins (0 min) was calculated per patient for each time point. The dotted line indicates a response of 50% compared to non-hydrolyzed proteins. The error bars indicate the standard error of the means. In addition, the SIs (B) and the cytokine levels (C, IL-13; D, IFNγ; E, IL-10) at five time points are depicted.

DISCUSSION

The effect of hydrolysates on the primary prevention of CMA may vary between different hydrolysates due to differences in their production. One aspect that may influence the effect of hydrolysates is the degree of hydrolysis. In this study the effect of the degree of hydrolysis on basophil and T cell activation in patients with CMA was investigated.

To determine this effect on both basophils and T cells, a large amount of blood is necessary. Therefore, blood samples from adult CMA patients instead of CMA children were used for this study. Whereas most adult CMA patients recognize casein, α-LAC and β-LG, the highest IgE levels are found for casein. A similar pattern has been described for children with persistent CMA, for which prevention of CMA is most crucial (24). In addition, CM-specific IgE levels in serum are also comparable between these groups, while IgE concentrations in children that achieve tolerance are much lower (24, 25). Although the highest IgE levels are found for casein, whey proteins activated basophils of adult patients to a similar extent as casein, indicating that both casein and whey proteins are important allergens in CMA (Supplemental Figure 3). Together, these data suggest that children with persistent CMA and adult CMA patients have a similar allergic phenotype and that basophils and T cells from adult CMA can be used as a model for children with a persistent allergy.

As expected, the majority of the patients (n=12) showed a decrease in IgE recognition and basophil response when proteins were hydrolyzed for a longer period. Even though less than 1% of the proteins were larger than 10 kDa after 60 min of hydrolysis, this time point could

induce basophil activation in five out of twelve patients. This suggests that this small amount of intact protein is enough to activate basophils and/or that the degradation products formed are able to cross-link IgE. Because a sequential epitope contains 6-12 amino acids and the minimal distance between two IgE molecules on basophils corresponds to 23 amino acids, in theory, a peptide of ~3.8 kDa (35 amino acids) could be able to cross-link IgE (26-28). However, the chance that a peptide of this size contains two epitopes and that the IgE molecules that recognize these epitopes are in such close proximity on the basophil is very small. Therefore, it seems more likely that either larger degradation products or aggregates of small peptides formed after heating of the hydrolyzed proteins induced the basophil response.

In five CMA patients basophil activation did not decrease at all. In contrast, more basophil activation was found in hydrolyzed proteins compared to the non-hydrolyzed proteins. These data suggest that due to the hydrolysis new epitopes became available or were exposed that may cross-link IgE and induce basophil activation. This hypothesis was supported by the immunoblot data. One of the five patients recognized a band around 25-30 kDa. This band appeared after 1 min of hydrolysis. Interestingly, the CAP values and the immunoblot of this patient indicated that this patient mainly recognized casein. These data in combination with the size of the protein suggested that the protein is not a whey protein but a casein protein. Previous studies have already shown that many whey batches are contaminated with casein (29, 30). Also in this study, bands around the molecular size of casein were visible on both the silverstaining and the immunoblots in the lanes loaded with non-hydrolyzed whey.

Four other patients recognized a band around 10 kDa that, like the 25-30 kDa band, appeared after 1 min of hydrolysis. Because this band is still visible after 40 min of hydrolysis on both the silverstaining and the immunoblot but not at the 60 min time point, this fragment may be continuously formed during the hydrolysis until the protein where it originates from is not present anymore. Based on the recognition of major allergens and non-hydrolyzed proteins, this fragment is probably a degradation product of whey.

The activation of T cells was less diverse than the activation of basophils. In all patients, T cell activation decreased when proteins were hydrolyzed for a longer period. Although the proliferative response decreased, proteins that were hydrolyzed for 60 min were still able to induce T cell proliferation in half of the patients. This indicates that not all T cell epitopes were degraded. Both the proliferative and cytokine response showed a similar trend. However, while IL-13 was produced by all TCLs, IFNγ and IL-10 were only released by part of the TCLs, which fits with the Th2-skewed phenotype of allergic responses. Furthermore, the cytokines produced correlated with proliferation. Therefore, TCLs that were strongly activated secreted all cytokines to a higher extent, whereas mildly activated cells secreted only IL-13.

When comparing the T cell response to the basophil response, it seemed that the T cell response is more affected by hydrolysis than the basophil response. While T cell activation is reduced with more than 50% after 9 min of hydrolysis, it takes at least 20 min to reduce the basophil response to the same extent. This is in contrast to a previous study that showed a time point with reduced allergenicity but retained immunogenicity (31). Possible explanations for these contradictive results may be the different techniques and patients groups used in these studies. A major difference between the studies is that Knipping *et al.* determined the allergenicity

of hydrolysates with a serum pool of ten CMA children instead of testing the responses of individual patients as done in the current study. Because the response is highly variable between patients, pooling sera may influence the overall response. In addition, a RBL cell line was used, which may be less sensitive compared to basophils from patients. Furthermore, a large part of our patient population had severe CMA, which is less common in children. It has been shown that RBL cells mainly respond when using serum from more severe CMA patients (32). Another difference is that the T cell lines in the current study were generated with a short protocol in contrast to the long protocol that was used for the T cell lines/clones in the previous study. Because the short-term T cell lines recognize more diverse epitopes, the chance that one of these epitopes is degraded is larger which may lead to a more rapid decrease in the T cell response.

This study indicates that it is difficult to decrease the allergenicity of proteins by hydrolysis without affecting immunogenicity. Whereas previous studies have shown that T cell epitopes are important for tolerance induction, the effect of the presence of IgE epitopes in the hydrolysates on tolerance induction in children who are not sensitized yet is unclear (33-35). Guidelines from European and American organizations indicate that the allergenic activity in formulae intended for prevention should be very low (36-38). According to the European Union, less than 1% of the nitrogen-containing substances in these formulae may be immuno-reactive (37). However, these guidelines are based on studies indicating that avoiding exposure to potential allergens may reduce the risk on developing allergy, whereas recent studies show that exposure may be beneficial for tolerance induction (39-42). As long as the requirements for tolerance induction are unclear, infant formulae should be tested in well-designed clinical trials. The sequences of the remaining peptides may be very different in the different infant formulae even if a similar time of hydrolysis is used for production or if they contain peptides with similar sizes. Therefore, each infant formula should be tested separately.

In conclusion, this study showed that increasing time of hydrolysis in general decreases both the basophil activation and T cell response. However, in some patients, increasing time of hydrolysis had no effect on the basophil response. These patients seemed to recognize stable fragments of casein or whey. Although the T cell response was decreased after 60 min of hydrolysis, there was still partial immunogenicity in most patients. These data indicate that not the hydrolysis grade and the length of the peptides is decisive but the presence of specific IgE and T cell epitopes in the hydrolysate determine the immunological properties of the hydrolysate. Well-designed clinical studies are needed to demonstrate whether limited hydrolysis is a valid approach to support the development of tolerance to CM in infants at risk for developing allergic disease.

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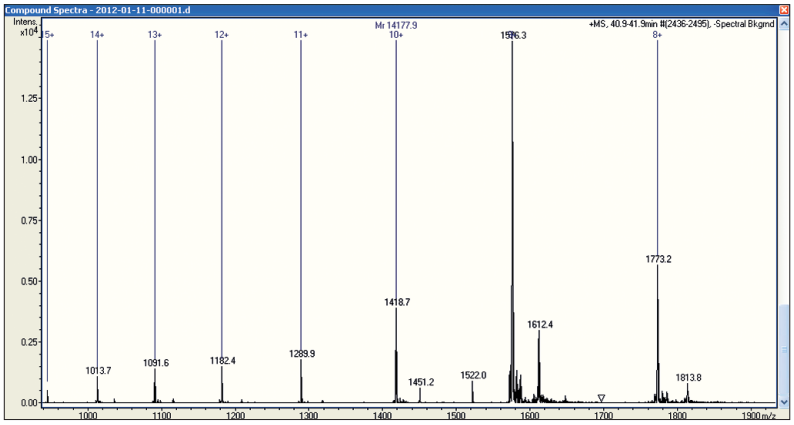
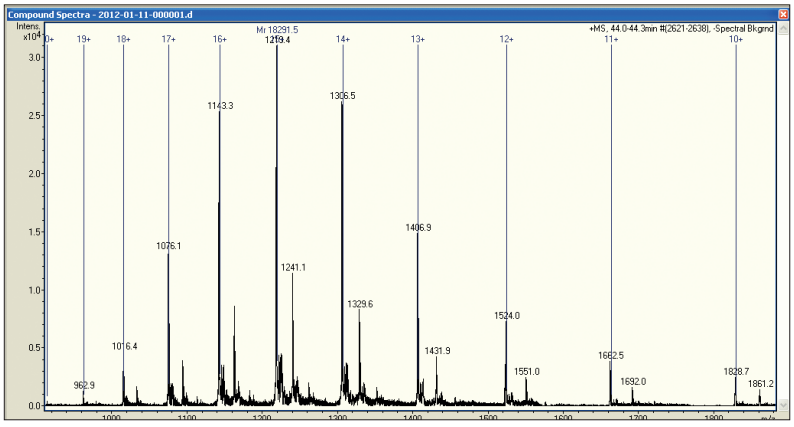
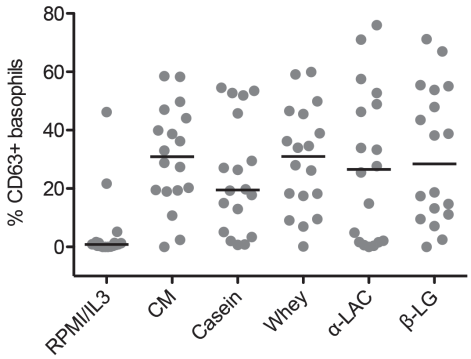
SUPPLEMENTAL DATA



Supplemental Figure 1 | Compound spectrum of the mass spectrometer indicating that the mass of the first large peak is 14 kDa.



Supplemental Figure 2 | Compound spectrum of the mass spectrometer indicating that the mass of the second large peak is 18 kDa.



Supplemental Figure 3 | The activation of unwashed basophils in response to the major allergens of cow's milk. The percentage CD63+ basophils of the total population basophils after stimulation with the highest concentration CM, casein, whey, α-LAC or β-LG is depicted (n=18). RPMI/IL-3 was used as a negative control to determine the spontaneous activation. The black lines indicate the median.

CHAPTER 4

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Characterization of T cell epitopes in bovine α-lactalbumin

Submitted for publication

ABSTRACT

Background

Recent studies have indicated that peptides containing T cell epitopes may be used for immunotherapy. While for several cow's milk allergens the T cell epitopes have been described, the T cell epitopes in the major allergen α-lactalbumin are unknown. Therefore, the aim of this study was to determine the T cell epitopes in α-lactalbumin.

Methods

Nineteen synthetic peptides spanning α-lactalbumin were obtained. Cow's milk-specific T cell lines of 45 subjects were generated and tested for their specificity for α-lactalbumin. The lines responding to α-lactalbumin were subsequently tested to determine their activation in response to the peptides.

Results

More than half of the T cell lines generated did not respond to α-lactalbumin or lost their responsiveness during subsequent experiments indicating that α-lactalbumin has a low immunogenicity. Only eight T cell lines recognized one or more peptides. The recognition of the peptides was diverse and no major epitopes could be defined.

Conclusion

The immunogenicity of α-lactalbumin is very low compared to other major allergens in cow's milk. Moreover, there seems to be no dominant epitope present in the protein. Therefore, it seems unlikely that peptides of this protein can be used for immunotherapy.

INTRODUCTION

Cow's milk allergy (CMA) is a common food allergy, affecting 0.3-3.5% of the young children and 0.1-0.3% of the adults (1, 2). One of the proteins in cow's milk that may induce an allergic response is α-lactalbumin (α-LAC).
α-LAC is a small protein of 123 amino acids (AA) with a molecular weight of 14.2 kDa. It belongs to the whey fraction and is present in relatively high concentrations in milk of all mammalian species (3). The proteins of the different species show a strong homology, for example about 74% of the AA in bovine and human α-LAC are identical (4, 5). α-LAC has an important role in the biosynthesis of lactose by acting as a co-enzyme in the lactose synthase complex. In addition, it is rich in essential AA, such as tryptophan, leucine and lysine, and therefore important in newborn nutrition (3, 6).
Despite the strong homology between human and bovine α-LAC, bovine α-LAC has been described as one of the major allergens in cow's milk (7). However, the percentage of cow's milk-allergic patients that recognizes α-LAC is variable in different study populations. Two studies describe that 75-80% of the patients recognize the protein (5, 7), whereas others observed α-LAC-specific IgE in only 30-35% of the patients (8, 9). According to IgE epitope studies, conformational epitopes play a major role in the IgE recognition of α-LAC (10).
In addition to IgE, also T cells play an important role in food allergy by stimulating B cells to switch to IgE production. On the other hand, they are also important for tolerance induction. Previous studies indicated that immunotherapy with peptides containing T cell epitopes reduced the allergic response to intact protein (11). While for other major allergens in cow's milk T cell epitopes have been described (12, 13), the T cell epitopes in α-LAC are still unknown. In this study, the proliferative and cytokine responses of short-term and long-term T cell lines (TCLs) to synthetic peptides of α-LAC were determined to identify the T cell epitopes.

MATERIAL AND METHODS

Peptides

Nineteen sequential synthetic peptides spanning α-LAC were obtained from JPT Peptide Technologies (Berlin, Germany). The peptides were 18 AA long with a 12-AA overlap (Figure 1). They were dissolved at a concentration of 1 mg/ml in 10% dimethylsulfoxide (Sigma Aldrich, Louis, MO, USA) in PBS, aliquoted and stored at -80°C.

Generation of T cell lines

Cow's milk-specific short-term and long-term TCLs were generated as described previously (14, 15). The short-term TCLs were generated from 12 adult CMA patients. For the long-term TCLs, PBMCs of 15 children with CMA, 11 atopic children without CMA and 7 healthy controls were used. The diagnosis of CMA was based on a suggestive history, positive cow's milk-specific IgE levels, positive skin-prick test and/or a positive double-blind placebo-controlled food challenge. Ethical approval for this study was obtained from the Ethics Committee of the University Medical Center Utrecht.

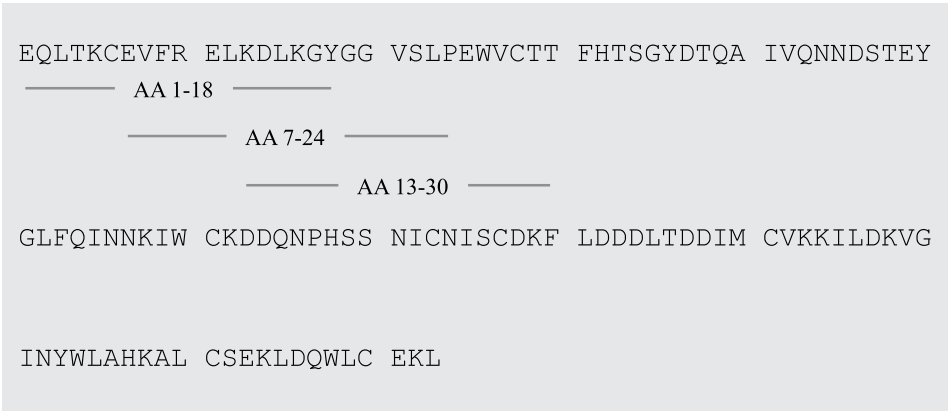

Figure 1 | The amino acid sequence of α-LAC used for the production of the synthetic peptides. The peptides were 18 amino acids long and had a 12 amino acid overlap as indicated in the figure.

T cell activation

T cell activation in response to the major allergens and peptides of α-LAC was determined as described previously (13, 15). For short-term TCLs proliferative and cytokine (IL-13, IFNγ and IL-10) responses were measured, whereas for long-term TCLs only the proliferative response was determined. The major allergens, namely cow's milk protein mixture (a mixture of whey (Lactalis, Laval, France) and casein (FrieslandCampina Domo, Amersfoort, The Netherlands) in a 1:1 ratio), casein, whey, β-lactoglobulin and α-LAC (both from NIZO Food Research BV, Ede, The Netherlands), were tested in a concentration of 50 μg/ml. Due to LPS-contamination, β-lactoglobulin and α-LAC could not be used for the short-term TCLs. The peptides (10 μg/ml) were first tested in mixtures of 2-3 peptides, after which positive peptides were tested separately. The stimulation index (SI), i.e. ratio between the proliferation of allergen/peptide-stimulated cells and non-stimulated cells, was calculated to determine the degree of proliferation. A SI of ≥2 was considered positive.

RESULTS

Of the 33 long-term TCLs that were generated only fifteen TCLs responded to α-LAC. In addition, seven of these fifteen lines lost their response to the protein during the subsequent experiments. Of the remaining α-LAC-specific TCLs, only four, two from children with CMA (TCL1 and 2) and two from atopic children without CMA (TCL3 and 4), responded to the peptides. The fourteen short-term TCLs all responded to whey. However, of these fourteen lines, only four TCLs (5-8) responded to the α-LAC peptides.

The T cell response to the peptides was diverse (Table 1). The eight TCLs recognized ten different peptides, which covered almost the complete protein. Three TCLs (TCL1, 3 and 5) recognized two consecutive peptides suggesting that the overlapping part contains the epitope.

Four peptides (AA 19-36, 25-42, 31-48 and 43-60) were able to induce a proliferative response in two TCLs.
The cytokine response of the short-term TCLs reflected their degree of activation. For example, TCL6 showed a strong proliferative and cytokine response after stimulation with the peptides. Both IL-13 and IFNγ were found in the supernatant of this TCL. In contrast, high IL-13 levels were measured in the supernatant of TCL7 after stimulation with the major allergens but not with the peptides. This correlated with the proliferation of this TCL, which was much stronger in response to the major allergens than to the peptides (SI of 31 vs. 3.5)

DISCUSSION

In this study, short-term and long-term TCLs of in total 45 subjects were generated to investigate the T cell epitopes of α-LAC. The initial specificity tests of the TCLs already indicated that α-LAC has a low immunogenicity. More than half of the long-term TCLs did not respond to α-LAC or lost their responsiveness during the subsequent experiments. In addition, most of the lines that did respond showed a lower SI in response to α-LAC compared to the other major allergens (Table 1, data not shown). A similar effect was seen in a previous study that investigated the primary lymphocyte response of CMA patients (8). A possible explanation for the low immunogenicity of α-LAC may be the high homology between bovine and human α-LAC (4, 5), because the body usually does not respond to 'self' proteins.
Only eight TCLs responded to one or more peptides. The peptides that were recognized were diverse and no discrimination between major and minor epitopes could be made. This may suggest that there is no dominant epitope present in the protein. In two of the short-term TCLs (TCL5 and 7) the proliferative and cytokine response induced by the peptides was relatively low when compared with the response to whey. Because cow's milk protein was used for the generation of the TCLs, it is possible that these TCLs not only recognize α-LAC but also other cow's milk proteins. This hypothesis was supported by the fact that TCL5 and 7 also recognized peptides of β-lactoglobulin, while TCL6 and 8 did not (unpublished data). In addition, by using a mixture of proteins, there is a risk that responses to weakly immunogenic proteins are lost due to the dominance of stronger immunogenic proteins. This may explain why only a small number of TCLs responded to α-LAC.
Several studies in both men and mice have indicated that peptides may be used for immunotherapy (11, 16, 17). Because peptides are too small to cross-link IgE on basophils and mast cells but are capable of activating T cells, they may induce tolerance without provoking side effects as is seen with conventional immunotherapy. However, because of the low immunogenicity of α-LAC, it is questionable whether peptides of this protein are able to induce tolerance.
In conclusion, α-LAC has a low immunogenicity compared to the other major allergens in cow's milk. The TCLs that do respond to α-LAC recognize diverse epitopes suggesting that there is no dominant epitope present in the protein. Therefore, it seems unlikely that peptides of this protein can be used for immunotherapy.

Table 1 Peptide recognition by human T cell lines

TCL	1	2	3	4	5				6				7				8			
	SI	SI	SI	SI	SI	IL-13	IFNγ	IL-10	SI	IL-13	IFNγ	IL-10	SI	IL-13	IFNγ	IL-10	SI	IL-13	IFNγ	IL-10
CMP						14.8	32.7	3.4		108.8	20.8	1.2		193.3	21.8	9.9		8.3	6.0	0
Casein						14.7	32.7	3.8		152.3	57.9	6.3		215.0	32.9	16.4		11.5	12.0	0
Whey						28.1	19.9	0		223.3	34.3	9.2		143.1	16.0	6.3		7.9	14.7	0
α-LAC					ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-LG					ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-18																				
7-24																				
13-30																				
19-36														2.0	3.5	0.7				
25-42																				
31-48																				
37-54																				
43-60																				
49-66						0	2.8	ND												
55-72						0	0	ND												
61-78																				
67-84																				
73-90																				
79-96																				
85-102										93.3	95.0	0								
91-108																				
97-114																		12.1	0.8	17
101-120																				
104-123						0	1.3	0												

The numbers in the left column indicate the amino acids of the peptides. The cytokine concentrations are in pg/ml. α-LAC = α-lactalbumin, β-LG = β-lactoglobulin, CMP = cow's milk protein, ND = not determined, SI = stimulation index, TCL = T cell line

2 < SI < 5
5 < SI < 20
SI > 20

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CHAPTER 5

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

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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, 9 peptides were selected based on human T cell data. Mice were pre-treated orally with 3 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 mixture 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.

INTRODUCTION

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 practise is to consume peanut snacks at an early age, a lower risk of developing peanut allergy was observed compared to 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 galacto- (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 *et al.*, 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 thirty-one 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.

MATERIAL AND METHODS

Peptides

Twenty-five 18-AA-long sequential synthetic peptides with 12-AA overlap spanning the B variant of β-LG (peptides 1-25, Figure 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.

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 (EBV-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). 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-

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.

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 (Figure 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 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 EBV-B cells. A SI of $\geq$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

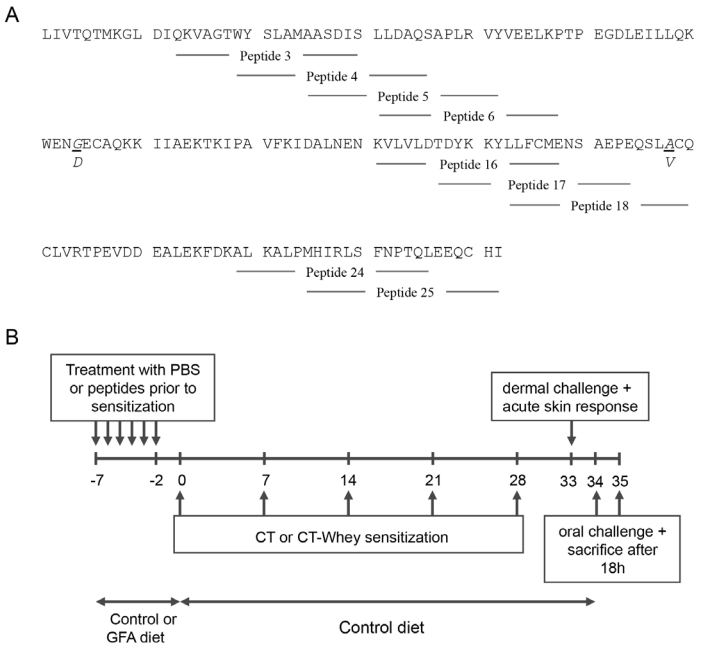

Figure 1 | The amino acid sequence of the B variant of β-LG (A) and a schematic overview of the mouse model for cow's milk allergy (B). The underlined and italicized amino acids (A) are mutated in variant A of β-LG into the amino acids indicated underneath them. For the T cell stimulations, peptides 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.

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 Figure 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 mixture 16-18, while peptides 16 and 17 (AA 91-108 and 97-114) induced a proliferative response in two TCLs.

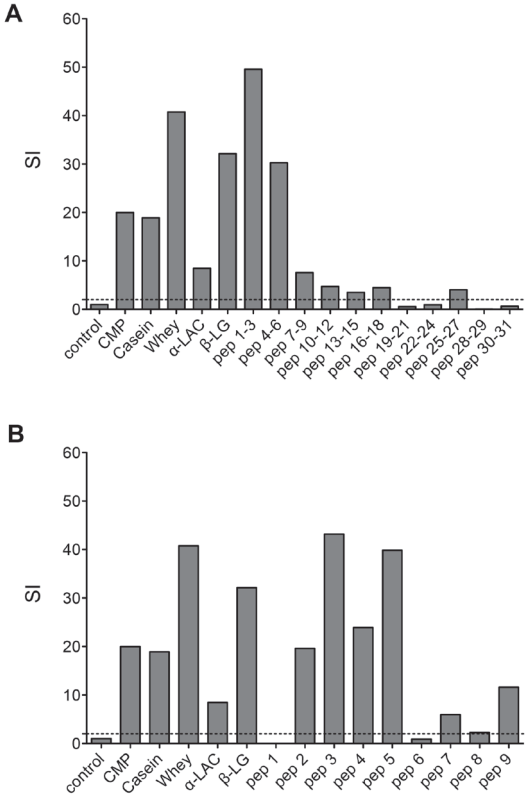

Figure 2 | An example of the proliferation of a TCL in response to the β-LG peptides. SIs of TCL5 in response to the major allergens, peptide mixtures (A) and single peptides (B) are depicted. The dotted line indicates a SI of 2.

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 mixture 1, 2 and 3, Figure 1A).

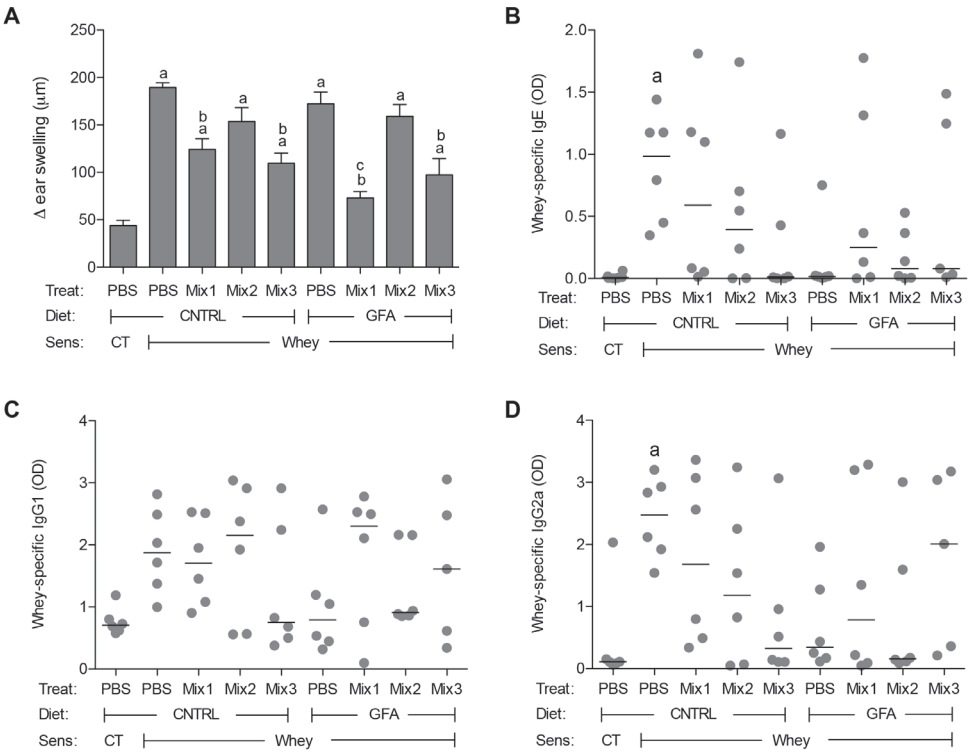

Figure 3 | The acute allergic skin response (A) and whey-specific antibody levels (B-D) in control and whey-sensitized mice pre-treated with PBS or peptide mixtures with/without the scGOS/lcFOS/pAOS (GFA)-containing diet. Antibody levels are expressed as the optical densities (OD) measured by ELISA (n=5/6, 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 1 and 3 reduced the acute allergic skin response in mice

Prior treatment with peptide mixture 1 and 3 significantly reduced the acute allergic skin response to whey, while pre-treatment with mixture 2 showed no effect (Figure 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 mixture 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, i.e. no physical activity after stimulation, were observed in two mice of this group. A similar response was seen in two allergic control mice.

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 (Figure 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).

Peptide 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 (Figure 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 (Figure 4B). These levels tended to be lower than the IgE levels in untreated allergic mice and in mice pre-treated with mixture 1 (both significant using a Mann Whitney test). No reduction in whey-specific antibody levels (Figure 4B-D) was seen with peptide 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 (Figure 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 (Figure 5B) decreased. A similar effect

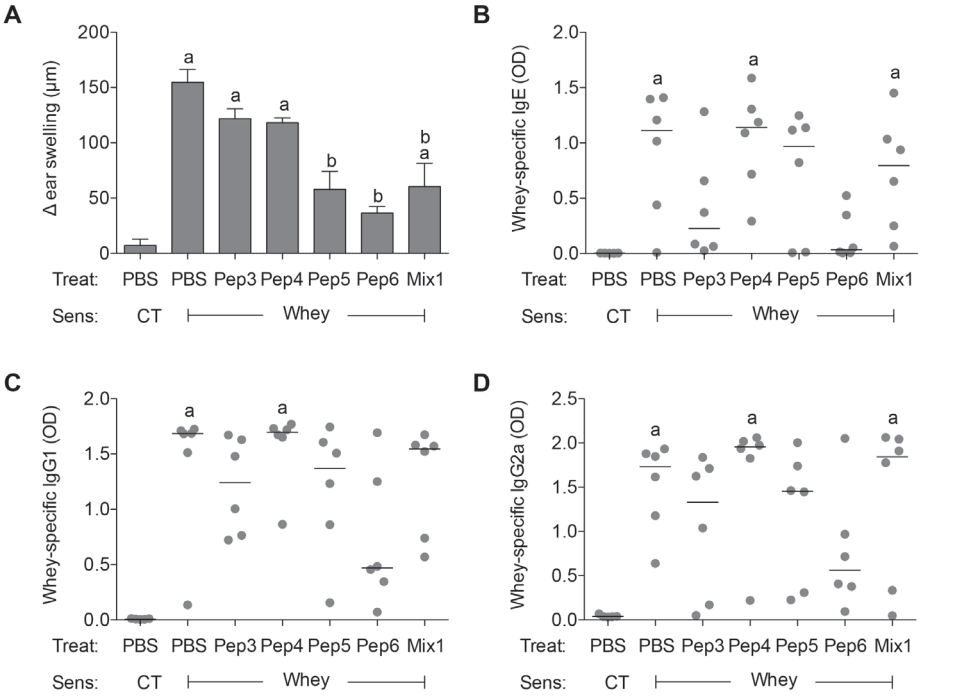

Figure 4 | The acute allergic skin response (A) and whey-specific antibody levels (B-D) in control and whey-sensitized mice pre-treated with PBS, single peptides or a peptide mixture. Antibody levels are expressed as the optical densities (OD) measured by ELISA (n=6, a = $p < 0.05$ compared with control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

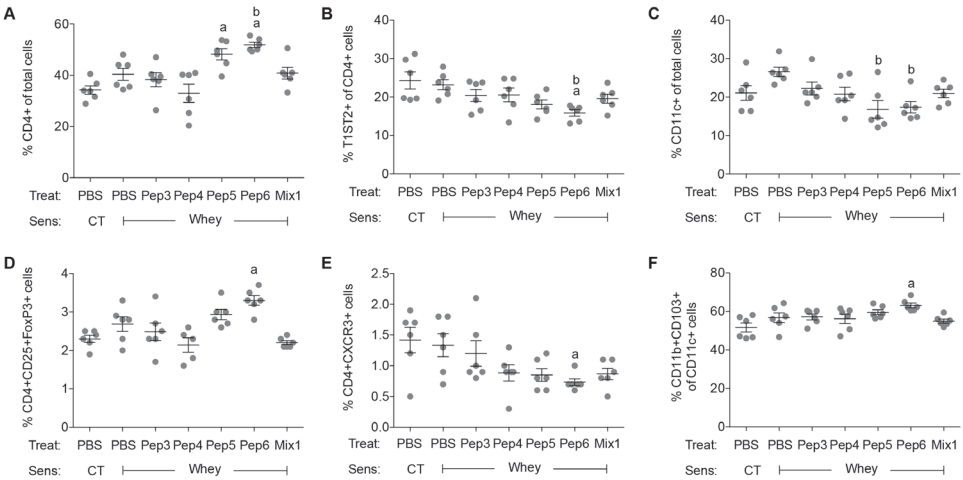

Figure 5 | The percentages of T cell (CD4+ cells, Th2 cells within the CD4+ population, regulatory T cells and Th1 cells (A, B, D, E)) and DC (CD11c+ cells and CD11b+CD103+ cells within the CD11c population (C, F)) subsets in the MLN of control and whey-sensitized mice pre-treated with PBS, single peptides 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).

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 towards increased regulatory T cells and less Th1 cells were found in the treated mice compared with allergic control mice (both significant using a t test, Figure 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 (Figure 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 (Figure 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 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 TCLs. 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 TCLs tested. This may indicated that either, due to the small number of TCLs 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 not only the T cell response 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 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 of 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).

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.

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CHAPTER 6

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Oral immunotherapy with whey peptides reduces allergic symptoms in a mouse model for cow's milk allergy

ABSTRACT

Background

Several studies have investigated the potential of oral immunotherapy for cow's milk allergy. However, due to significant side effects, this therapy is still not used in the clinic. Using peptides instead of proteins may reduce these side effects.

Objective

To investigate the therapeutic effect of oral immunotherapy with a partial whey hydrolysate (pWh) or synthetic β-lactoglobulin peptides in mice sensitized to whey proteins.

Methods

Female C3H/HeOuJ mice were sensitized five times by oral gavage with whey. Five days after the last sensitization, blood was drawn and IgE was measured to determine sensitization. One week later, oral immunotherapy was started. Mice were orally treated 14 times during four weeks with PBS, pWh or a mixture of synthetic β-lactoglobulin peptides. Three days after the last treatment, an intradermal ear and oral challenge were performed. Mice were sacrificed the following day and mesenteric lymph nodes were collected for flow cytometric analysis.

Results

IgE specific for whey was detected in all mice after sensitization. Treatment with pWh and the peptide mixture significantly reduced the acute allergic skin response compared to the PBS-treated mice. No changes were observed in antibody levels between the treatment groups. Whereas the percentage of Foxp3+ T cells decreased, more B cells were observed in the mesenteric lymph nodes directly after peptide treatment. Furthermore, the expression levels of several T cell markers decreased. The observed effect of peptide immunotherapy differed between experiments, which may be due to differences in the extent of sensitization.

Conclusion

Oral immunotherapy with pWh or a mixture of synthetic β-lactoglobulin peptides can reduce the acute allergic symptoms in a mouse model for cow's milk allergy, though the effect is variable and seems to depend on the degree of sensitization. Further studies to optimize the treatment protocol are necessary.

INTRODUCTION

At the moment there is no curative treatment available for cow's milk allergy and the only way to prevent allergic symptoms is to avoid the intake of cow's milk. A possible treatment may be allergen-specific immunotherapy. Immunotherapy for cow's milk allergy has been extensively studied in recent years. However, methods that are relatively safe, such as sublingual immunotherapy, are not effective (1), while approaches that seem to be more effective, like oral immunotherapy, induce side effects (1-4). One way to reduce the side effects of oral immunotherapy is by using peptides instead of intact proteins. Optimally, these peptides should be able to activate T cells, and thereby induce tolerance, without cross-linking IgE, so preventing allergic side effects.

The potential of peptide immunotherapy for the treatment of allergic diseases has been investigated before. For example, intradermal administration of a Fel d 1 peptide reduced allergic symptoms in cat-allergic patients (5, 6). Also in mouse studies, clinical symptoms of cat allergy decreased after intradermal peptide therapy (7). Although no effects were seen on Foxp3+ regulatory T cells in these studies, IL-10 levels increased and blocking this cytokine reduced the beneficial effects. In a recent study, the efficacy of oral peptide immunotherapy for egg allergy was examined (8). Mice that were treated orally with an ovomucoid peptide showed reduced allergic symptoms compared to untreated mice. Also in this study, IL-10 levels increased. However, in contrast to the Fel d 1 study, more Foxp3+ T cells were found in blood of the treated mice.

So far, few studies have investigated the effects of peptide therapy in cow's milk allergy. Beneficial effects were shown in mice after preventive intradermal treatment with a peptide of αS1-casein (9). Additionally, we showed in previous studies that prophylactic oral treatment with a partial hydrolysate of whey (pWh) and with a mixture of synthetic β-lactoglobulin (β-LG) peptides reduced the acute allergic skin response to whey (10, 11). It is unclear whether treatment with peptides is also beneficial in a curative setting.

This study was designed to evaluate the potential of oral immunotherapy using pWh or synthetic peptides of β-LG in a mouse model for cow's milk allergy. Mice were sensitized for whey and subsequently treated with pWh or with a mixture of four β-LG peptides. Efficacy was measured by determining the acute allergic skin response and antibodies levels. To gain more insight into the underlying immune mechanism, effects on several immune cells in the mesenteric lymph nodes (MLN), such as Foxp3+ regulatory T cells and dendritic cell subsets, were determined by flow cytometry. The study was repeated to evaluate the reproducibility of the observed effects.

MATERIALS AND METHODS

Antibodies

Biotin-conjugated rat-anti-mouse IgE, IgG1, IgG2a (all 1:500) and FITC-conjugated rat-anti-mouse CD4 (clone L3T4, 1:100) were purchased from BD Pharmingen (San Diego, CA, USA). FITC-conjugated rat-anti-mouse CD8a (clone 53-6.7, 1:100), CD45R/B220 (clone RA3-6B2, 1:50),

PE-conjugated rat-anti-mouse CXCR3 (clone CXCR3-173, 1:50), CD11b (clone M1/70, 1:50), PerCP-Cy5.5-conjugated rat-anti-mouse CD4, CD25 (clone PC61.5, 1:100), CD11c (N418, 1:50), APC-conjugated rat-anti-mouse CD8a, CD69 (clone H1.2F3, 1:100), CD103 (2E7, 1:100), Foxp3 (FJK-16s, 1:50) and isotype controls were all obtained from eBioscience (San Diego, CA, USA).

Animals

Pathogen-free female C3H/HeOuJ mice (three weeks of age) were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in the animal facility at Utrecht University. They were maintained on cow's milk protein-free standard mouse chow (AIN-93G soy, Special Diets Services, Wijk bij Duurstede, the Netherlands) and treated according to the guidelines of The Dutch Committee of Animal Experiments.

Peptides

pWh was generated as described previously (10) and dissolved in PBS at a concentration of 1 mg/ml. Four 18-amino-acid long peptides of β-LG with 12-amino-acid overlap were synthesized by JPT Peptide Technologies (Berlin, Germany). These peptides were selected based on previous *in vitro* and *in vivo* experiments (Table 1) (11). Prior to the experiment, the peptides were dissolved in PBS and mixed together. The final concentration of each peptide in the mixture was 80 µg/ml. Subsequently, the peptide mixture was aliquoted and stored at -30°C until further use.

Immunotherapy with pWh and synthetic peptides

Mice (n=8/n=16 per group) were sensitized for whey as described previously (12). In short, animals were treated by oral gavage, i.e. intragastrically, with 0.5 ml PBS containing 20 mg whey (DMV International, Veghel, The Netherlands) and 10 µg cholera toxin (CT, Quadratech Diagnostics, Epsom, United Kingdom) at day 0, 7, 14, 21 and 28 (Figure 1). Non-sensitized mice were taken along as negative control and were only treated with CT. At day 33, serum samples were obtained and stored at -70°C until further use.

A week later, immunotherapy started. During four weeks, mice were treated 14 times by oral gavage with 0.5 ml PBS, pWh or peptide mixture: five treatments during the first week and three treatments per week during the subsequent three weeks. Three days after the last treatment, an intradermal ear challenge was performed in one ear pinnae with 10 µg whey in 20 µl PBS. Ear thickness was measured before and 1 hour after the challenge by using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The ear swelling is expressed as delta µm and is an indication of the acute allergic response. In the second experiment, a second intradermal challenge in the other ear pinnae was performed five weeks after the first challenge (Figure 1B). One day after the last intradermal challenge (day 69 in the first experiment, day 104 in the second experiment), mice were challenged orally with 50 mg in 0.5 ml PBS and sacrificed eighteen hours later. Sera, MLN and spleens were collected for further analysis.

Whey-specific IgE, IgG1 and IgG2a ELISA

To determine whey-specific IgE, IgG1 and IgG2a levels in sera, an ELISA was performed as described previously (12).

Table 1 Sequences of the peptides selected for immunotherapy

Peptide sequences
QKVAGTWYSLAMAASDIS
WYSLAMAASDISLLDAQS
AASDISLLDAQSAPLRVY
LLDAQSAPLRVYVEELKP

Flow cytometric analysis of immune cells in MLN

MLN were cut into small pieces and incubated with collagenase IV and DNAse-I (both from Sigma Aldrich) as described by van Esch *et al.* (13). This enzymatic reaction was stopped by adding 0.5 ml FBS. Next, cells were blocked with PBS containing 5% FBS and 1% bovine serum albumin (Boehringer Ingelheim, Ingelheim, Germany). Subsequently, 5 x 10^5 cells were plated and incubated for 30 minutes with anti-CD4 and -CD25 followed by Foxp3 for regulatory T cells, anti-CD4, -T1ST2, -CXCR3 and -CD69 for Th1/Th2 cells, anti-CD11c, -CD11b, -CD8a and -CD103 for dendritic cells or anti-CD11c, -CD11b, -CD45R (B220), -CD8a for plasmacytoid dendritic cells. CD11c-B220+ cells were considered B cells. For the Foxp3 staining, a commercially available Foxp3 staining kit was used according to the manufacturer's protocol (eBioscience). Fluorescence was measured by flow cytometry (FACS Canto™ II, BD Biosciences, Franklin Lakes, NJ, USA).

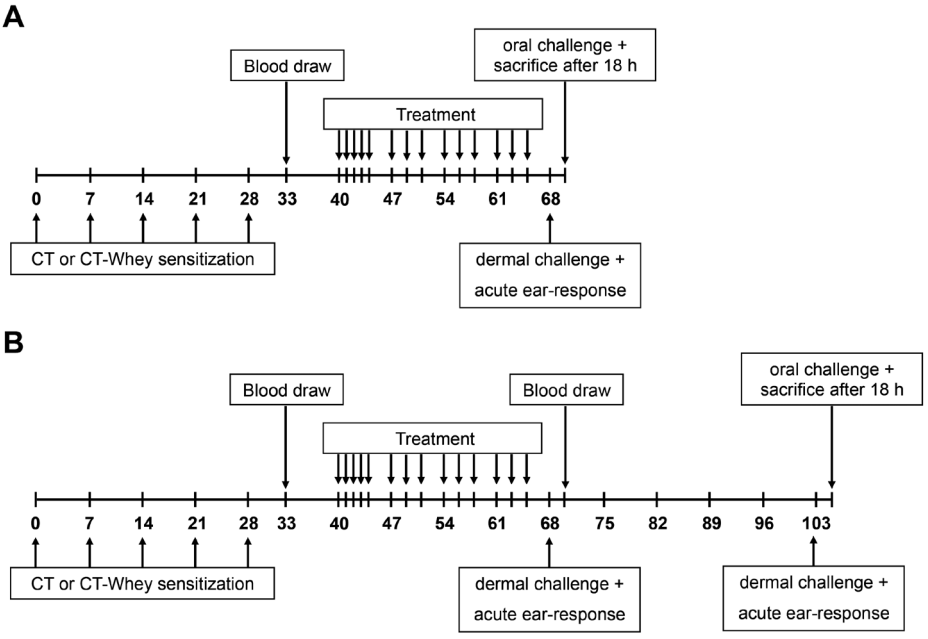

Figure 1 | Schematic overview of the oral immunotherapy mouse model for cow's milk allergy. Mice were sensitized for whey and subsequently treated 14 times with PBS, pWh or peptides. In the first experiment mice were sacrificed 2 days after the intradermal challenge (A). In the second experiment, two intradermal challenges, at day 68 and day 103, were performed and mice were sacrificed two days after the last intradermal challenge (B).

Proliferative and cytokine responses

Single cell suspensions were generated from the spleens. Subsequently, 4 x 10^5 splenocytes were incubated with 1 µg/ml anti-CD3 and 1 µg/ml anti-CD28 (kind gift of Bioceros). After 72h, supernatants were collected and T cell proliferation was determined by measuring [^{3}H]-thymidine incorporation. Cytokine levels (IFNγ, IL-13, IL-4, IL-10 and TGF-β) in the supernatants were determined with ELISA according to the manufacturer's instructions (eBioscience).

Allergenicity of synthetic peptides

The allergenicity of the synthetic peptides was determined both *in vitro* and *in vivo*. To test the allergenicity *in vitro*, the human basophil activation test was used. Heparinized whole blood from nine adult CMA patients (diagnosis based on suggestive history, positive IgE levels specific for cow's milk and/or a double blind placebo-controlled food challenge) was incubated with 100 µg/ml whey or peptide mixture (25 µg/ml per peptide) diluted in RPMI/IL-3 (2 ng/ml, R&D systems, Minneapolis, MN, USA). Cold PBS/EDTA (20mM) was added to stop the reaction and cells were stained using anti-CD63, anti-CD123 and anti-CD203c (all from BioLegend, San Jose, CA, USA). FACS lysing solution was added to lyse the red blood cells. The percentage activated basophils (CD63-positive cells within the CD203c/CD123-positive cells) was determined by flow cytometry (FACS Canto™ II).

To determine the allergenicity of the peptides *in vivo*, mice were sensitized for whey as described above. Five days after the last sensitization, an intradermal ear challenge was performed. Mice were either challenged with whey or a mixture of peptides. Non-sensitized mice challenged with whey were used as negative controls.

Statistical analyses

To analyze the data, GraphPad Prism version 5.0d for Macintosh (GraphPad Software, San Diego, CA, USA) was used. The acute allergic skin response, the flow cytometric data and IgG1, IgG2a and cytokine levels were analyzed with an one-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test for selected groups. The proliferative data were log-transformed and also analyzed with an ANOVA. IgE levels were analyzed with the Kruskal-Wallis test followed by a Dunn's post-hoc test for selected groups.

RESULTS

Immunotherapy with pWh and peptides reduced allergic symptoms

To test whether peptide immunotherapy was able to reduce clinical symptoms of cow's milk allergy, the acute allergic skin response against whey was determined in whey-sensitized mice after treatment with PBS, pWh or a mixture of peptides. The allergic response was compared to the ear swelling in non-sensitized mice, which were used as negative controls. In sensitized mice treated with PBS, a significant increase in ear thickness was observed after challenge with whey compared to non-sensitized mice (Figure 2A). Whey-sensitized mice that were treated with peptides or pWh showed a significantly reduced allergic skin response compared to

sensitized mice treated with PBS. Moreover, the response in pWh- and peptide-treated mice was no longer significantly different from the response in non-sensitized mice. No allergic symptoms were observed after the oral challenge and during treatment.

No differences in whey-specific antibody levels after treatment with pWh and peptides

Next to the acute allergic skin response, whey-specific IgE, IgG1 and IgG2a levels in serum were measured. To investigate whether all mice were sensitized equally to whey before the start of therapy, IgE levels were also determined at day 33. As expected, whey-specific IgE levels were significantly increased in sensitized mice compared to non-sensitized mice before treatment, which was similar in the three treatment groups (Supplementary Figure 1). After treatment, no differences in IgE levels were observed between the treatment groups, including the PBS-treated control group (Figure 2B). In mice treated with the peptides, IgE levels were still significantly higher compared to non-sensitized mice, whereas this was no longer the case in PBS- and pWh-treated mice.

In contrast to the whey-specific IgE levels, whey-specific IgG1 levels (Figure 2C) after therapy were still significantly increased in all treatment groups, including the PBS-treated control group, compared to non-sensitized mice. In addition, whey-specific IgG2a levels (Figure 2D) were significantly higher in the pWh-treated mice compared to non-sensitized mice and a similar trend was shown for the peptide and PBS treatment groups (significant using a student's t test). No differences in whey-specific IgG1 and IgG2a levels were found between the mice treated with PBS, pWh or peptides.

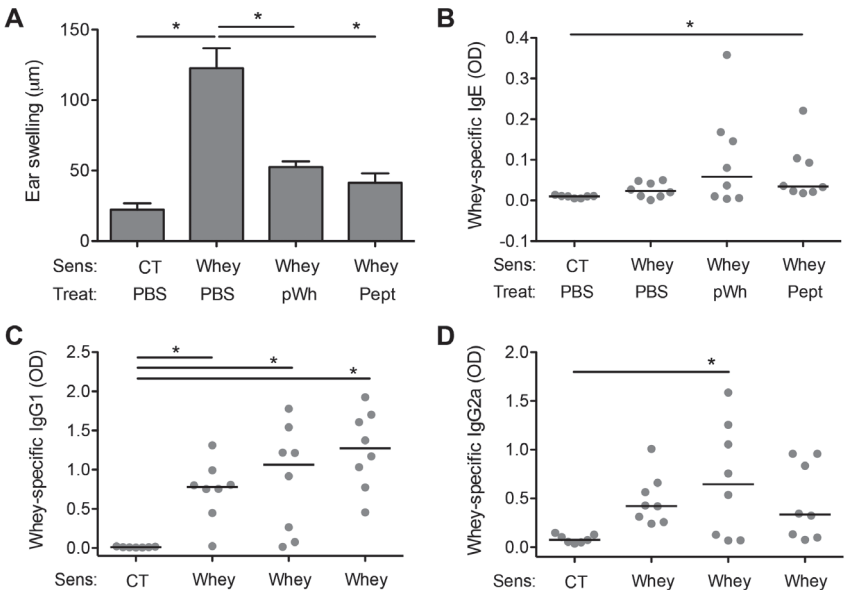

Figure 2 | The acute allergic skin response (A) and whey-specific antibody levels (B-D) in control and whey-sensitized mice treated with PBS, pWh and a mixture of synthetic peptides. Antibody levels are expressed as the optical densities (OD) measured by ELISA (n=7/8, *p < 0.05).

Higher number of B cells in MLNs after peptide immunotherapy

To determine whether immunotherapy influenced the local immune response in the intestine, the effect of therapy with pWh or peptides on the immune cells of the MLN was investigated. The percentage of B cells (CD11c-B220+ cells) increased in peptide-treated mice compared to PBS-treated and non-sensitized mice (Figure 3A). The percentage of regulatory T cells (CD4+CD25+Foxp3+ cells) in the MLN decreased after treatment with pWh and peptides (Figure 3B). This effect was mainly due to a decrease in expression level of CD4 (MFI), as shown in Figure 3D. However, when only gating on CD25+Foxp3+ cells (Figure 3C), the percentages were still significantly decreased compared to PBS-treated and non-sensitized mice. Remarkably, in addition to the expression level of CD4, also the expression of CD8a and CD69 decreased (Figure 3E and F, respectively).

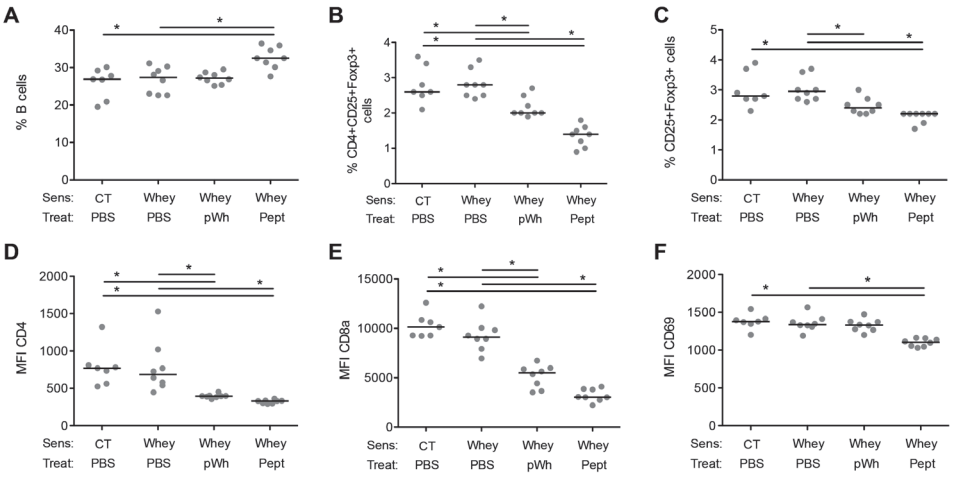

Figure 3 | The percentages of B cells (A) and regulatory T cells (B, C) and the expression levels of CD4 (D), CD8a (E) and CD69 (F) on immune cells in the MLN of control and whey-sensitized mice treated with PBS, pWh and a mixture of synthetic peptides. Expression levels are expressed as the median fluorescence intensity (MFI) measured by flow cytometry (n=7/8, *p < 0.05).

Synthetic peptides are not able to activate basophils/mast cells

Because no allergic symptoms were observed after an oral challenge with whey, the safety of peptide immunotherapy could not be determined. Therefore, a human basophil activation test and a murine intradermal ear challenge were performed to investigate the allergenicity of synthetic peptides. Whereas the basophils of adult cow's milk-allergic patients were activated after incubation with whey, no CD63 expression was observed after incubation with the peptide mixture (Supplementary Figure 2A). Similar results were observed in the *in vivo* mouse model, in which an intradermal challenge with whey induced an acute allergic skin response but no response was observed after a challenge with the peptide mixture (Supplementary Figure 2B).

Variable results with immunotherapy in subsequent experiments

To further investigate the mechanism of peptide immunotherapy, the experiment was repeated. However, no effect of treatment with pWh and the synthetic peptides was seen in the second experiment at day 68 (Figure 4A and B). To determine whether a longer period between treatment and challenge would increase the effect of peptide immunotherapy, a second intradermal challenge (day 103) was performed five weeks after the first challenge (day 68). At the second time point, half of the peptide-treated mice showed a reduced acute allergic skin response, while no effect was seen with pWh. Remarkably, all the responding peptide-treated mice belonged to one cage; therefore this treatment group was split per cage for further analysis (Figure 4C). This analysis indicated that not only the difference between PBS-treated and the responding peptide-treated mice was significant but also the difference between the non-responding and the responding peptide-treated mice. Again, whey-specific IgE, IgG1 and IgG2a levels were similar for all treatment groups on both time points (Figure 5, data not shown).

In contrast to the first experiment, IgE levels after treatment were significantly higher in all sensitized mice compared to the non-sensitized mice. While peptide treatment significantly increased the percentage B cells in the MLN in the first experiment, no effect was seen in the second experiment (data not shown). Also, no differences for other immune cell subsets in the MLN were found, and no effect on CD4, CD8a and CD69 expression levels were observed.

To study the effect of treatment on systemic T cell responses, splenocytes were stimulated with anti-CD3 and anti-CD28. Splenocytes of the mice that responded to the peptide treatment showed a significantly higher proliferative response and higher production of IL-13, IFNγ and IL-10 after stimulation with anti-CD3 and anti-CD28 than splenocytes of non-sensitized and PBS-treated mice (Figure 6).

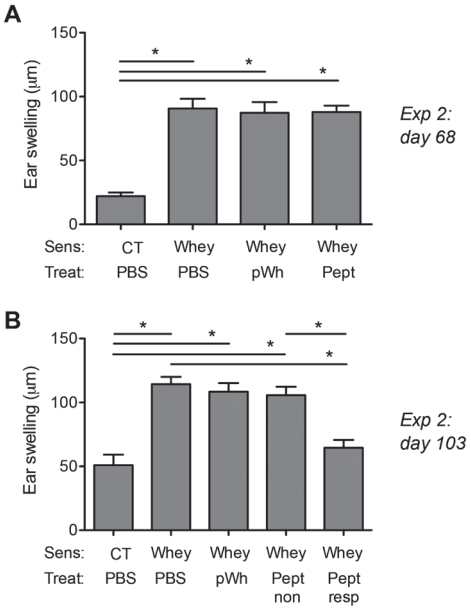

Figure 4 | The acute allergic skin response in control and whey-sensitized mice treated with PBS, pWh and a mixture of synthetic peptides in the second experiment. In this experiment, the intradermal challenge was performed twice, i.e. at day 68 (A) and day 103 (B). At the last time point, a clear difference was observed between the two cages of mice treated with the peptide mixture, therefore this group is split in two groups, a non-responding and a responding group (n=8/15/16, *$p < 0.05$).

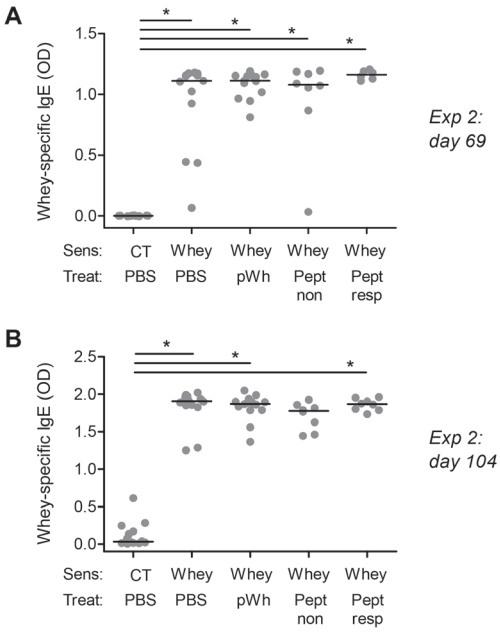

Figure 5 | Whey-specific antibody levels in control and whey-sensitized mice treated with PBS, pWh and a mixture of synthetic peptides on day 69 (A) and day 104 (B) of the second experiment. Antibody levels are expressed as the optical densities (OD) measured by ELISA. At day 104, a clear difference was observed for the acute allergic skin response between the two cages of mice treated with the peptide mixture, therefore this group is split in two groups, a non-responding and a responding group (n=8/15/16, *p < 0.05).

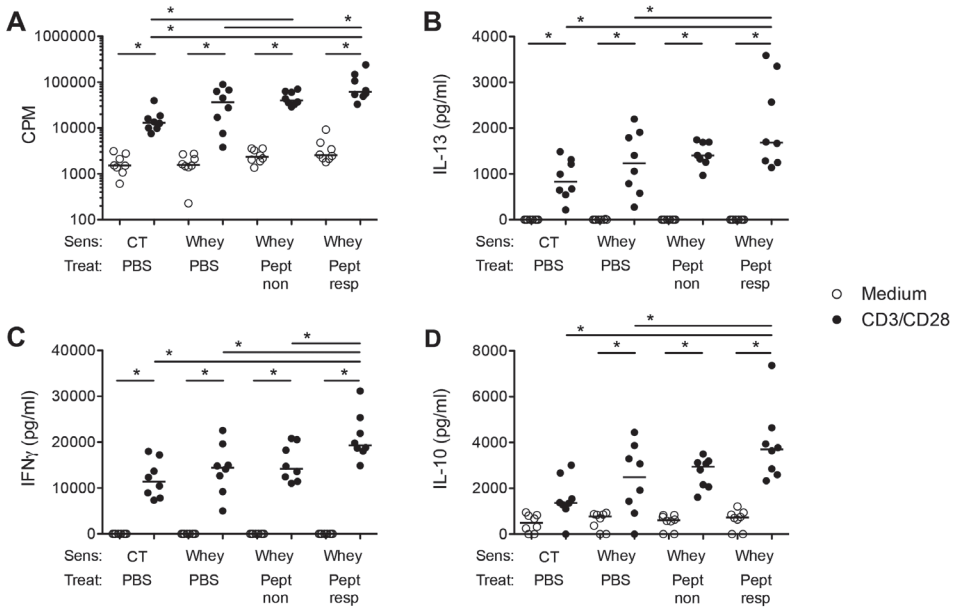

Figure 6 | Proliferative (A) and cytokine responses (B-D) of splenocytes of control and whey-sensitized mice treated with PBS and a mixture of synthetic peptides after incubation for 72h with medium or anti-CD3/CD28. Proliferative responses are expressed as counts per minute (CPM). IL-13 (B), IFNγ (C) and IL-10 (D) levels are expressed in pg/ml (n=8, *p < 0.05).

DISCUSSION

Previous research has shown that preventive oral treatment with pWh or a mixture of β-LG peptides before sensitization to whey significantly reduced allergic symptoms in mice (13). To analyze whether these peptides would also reduce allergic symptoms in a therapeutic setting, the efficacy of peptide immunotherapy as a treatment for cow's milk allergy was investigated in this study.

In line with previous studies (7, 8), this study showed that oral peptide immunotherapy with pWh or a mixture of synthetic peptides significantly reduce the acute allergic symptoms in a mouse model for cow's milk allergy. Interestingly, treating mice with four overlapping peptides of β-LG reduced the allergic response to the entire allergen suggesting a role for linked-epitope suppression as has been described in previous peptide immunotherapy studies (7, 14).

No differences in whey-specific IgE levels were found between the treatment groups, including the PBS-treated control group. The decrease in the acute allergic skin response after treatment is thus not caused by a decrease in IgE. Previous studies investigating peptide immunotherapy in mice showed contradicting results for IgE levels. In two mouse studies no effect was seen (15, 16), while in three other studies IgE levels were reduced upon peptide therapy (7, 8, 17). Similar variable results were also seen with conventional immunotherapy in patients (18, 19).

In addition to IgE, also IgG antibodies can be affected by immunotherapy. Several studies in humans have found increased IgG1 and IgG4 levels after conventional immunotherapy (18). It has been suggested that these antibodies can block allergic responses by either binding to the allergen and thereby preventing the binding of the allergen to and cross-linking of IgE on mast cells and basophils or, in case of IgG1, via the inhibitory IgG receptor FcγRIIB, which after binding of IgG-allergen complexes directly inhibits the activation of effector cells (20). However, the role of IgG in mice is more complicated, because mouse studies have indicated that IgG1, and probably also IgG2a, can both induce and inhibit anaphylaxis (20, 21). In our study, no changes in IgG1 and IgG2a levels were observed after peptide immunotherapy as compared to the PBS-treated group. Whereas two studies have found similar results (15, 22), two other studies have shown that peptide immunotherapy increased IgG levels (8, 16).

Since antibody levels were comparable between PBS-, pWh- and peptide-treated mice, potentially a different mechanism was involved in the reduction of the acute allergic response. It is known that regulatory T cells can actively suppress effector cells and thereby reduce allergic symptoms. However, their role in peptide immunotherapy is still unclear. In the current study, fewer Foxp3+ cells were observed in the MLN of peptide-treated mice. Two previous studies have demonstrated that peptide treatment significantly increased the amount of Foxp3+ regulatory T cells in mice (8, 17), whereas another study has shown comparable levels of these cells in treated and non-treated mice (7). Campbell *et al.* described a role for IL-10-secreting regulatory T cells instead of Foxp3+ regulatory T cells (7). In this study, the percentage of IL-10+ T cells increased significantly in peptide-treated mice and blocking this cytokine inhibited the effects of the therapy completely. In the second experiment of our study, significantly higher IL-10 levels were found in the supernatant of splenocytes of peptide-treated mice compared to splenocytes of non-sensitized and PBS-treated mice after stimulation with anti-CD3/CD28.

However, in contrast to the results of Campbell *et al.*, also IL-13, IL-4 and IFNγ levels were increased (7). They measured the cytokine levels in bronchoalveolar lavage and lung homogenates after an allergen-specific stimulation, which may explain the different results. Unfortunately, *ex vivo* whey-specific stimulation of splenocytes induced no proliferative and cytokine responses in this model, therefore this hypothesis could not be investigated further.
Surprisingly, peptide treatment significantly decreased the expression level of CD4, CD8a and CD69 on T cells. Although this effect has not been described in previous immunotherapy studies, other studies have shown that the expression of CD8 can be diminished during clonal anergy (23, 24). Interestingly, in these studies, cells proliferate vigorously before the down-regulation of CD8, which could explain the significant increase in total number of MLN cells seen in our study (data not shown). However, because also the expression of CD8a on dendritic cells decreased, it seems unlikely that this process is involved. The effect on CD4 and CD69 expression has not been described so far.
Next to T cells, peptide immunotherapy also affected B cells. The percentage of B cells, in our study CD11c-B220+ cells, in the MLN increased significantly in mice treated with peptides compared to the ones treated with PBS. Although B220 is also expressed on thymocytes and a subset of T cells (25, 26), a previous study has shown that the majority of the CD11c-B220+ cells are B cells (19). Therefore, we consider the CD11c-B220+ cells found in our study to be B cells. A previous study has described a B cell subset that can reduce allergic symptoms in mice (27). These regulatory B cells secrete IL-10 and attract regulatory T cells. Moreover, a recent study investigating regulatory B cells in bee venom-allergic patients observed increased numbers of these cells after immunotherapy (28). However, the role of these cells in peptide immunotherapy is unclear.
As has been described for other cow's milk allergy models, oral challenges with whey did not induce clinical symptoms in this model (29). Therefore, the safety of pWh and the peptides could not be tested in this model. A previous study has already shown that, although the allergenicity of pWh is much lower than the allergenicity of whey, pWh can still induce an allergic response in whey-sensitized mice (12). In this study we showed that the peptide mixture was not able to induce activation of mast cells and/or basophils, both in the mouse model as well as on human basophils, suggesting that peptides are too small to cross-link IgE and can be safely used for therapy. Because treatment with both pWh and peptides had a similar clinical effect in our study, the safety of synthetic peptides may be major advantage.
In contrast to the first experiment, no effect of peptide immunotherapy was seen in a second experiment indicating that the results of peptide immunotherapy are variable. A possible explanation for the difference between these experiments may be a difference in the initial sensitization. IgE levels after treatment were much higher in the last experiment when compared to the levels in the first experiment. Interestingly, whereas in the second experiment no effect of peptide therapy on the acute allergic response was seen after the intradermal challenge at day 68, five weeks later half of the peptide-treated mice did respond to the therapy and showed a significant decrease in the acute allergic skin response. These data suggest that in strongly sensitized mice a longer period between the last treatment and the challenge may be necessary to observe a clinical effect. When translating these data to humans, this could imply that strongly

sensitized patients may be more difficult to treat. Indeed, previous oral immunotherapy studies for cow's milk and peanut allergy showed that treatment was less effective and associated with more side effects in patients with higher IgE levels (30, 31). So, a different dosing regimen or longer treatments may be necessary to treat these patients.
In conclusion, this study showed that immunotherapy with pWh and β-lactoglobulin peptides can reduce allergic symptoms in a mouse model for cow's milk allergy, though the effect of therapy is variable and seems to be influenced by the degree of sensitization. Further studies are thus necessary to optimize the therapy. Although the exact mechanism of action of peptide immunotherapy is still unclear and needs further investigation, our data suggest a role for T cells and B cells.

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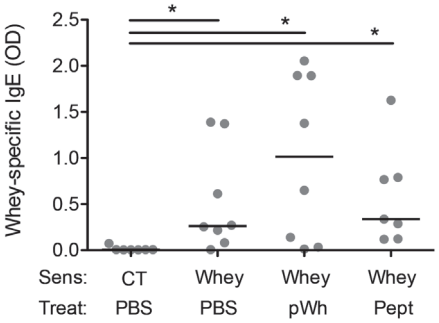
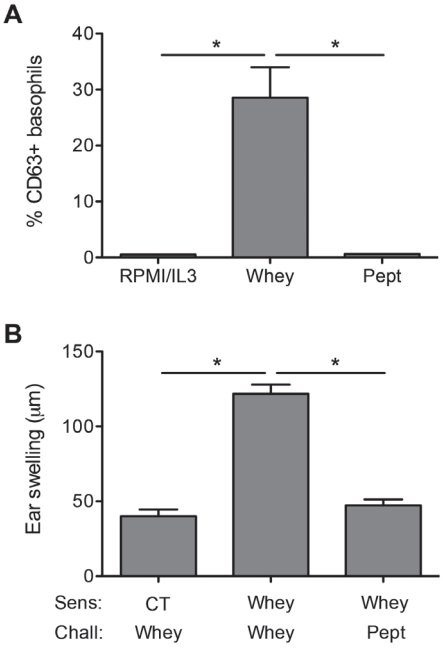
SUPPLEMENTAL DATA



Supplemental Figure 1 | Whey-specific IgE levels in control and whey-sensitized mice before treatment with PBS, pWh and a mixture of synthetic peptides. Antibody levels are expressed as the optical densities (OD) measured by ELISA (n=7/8, *p < 0.05).



Supplemental Figure 2 | (A) Human basophil activation after incubation with whey and a mixture of synthetic peptides. Basophil activation is expressed as the percentage CD63+ basophils within the basophil population measured by flow cytometry (n=9, *p < 0.05). (B) The acute allergic skin response in control and whey-sensitized mice after an intradermal ear challenge with whey or a mixture of synthetic peptides (n=5/6, *p < 0.05).

CHAPTER 7

General discussion

At the moment there is no curative treatment available for food allergy. To prevent allergic symptoms, food-allergic patients are advised to avoid exposure to allergens (1, 2). Because many products contain food allergens, diet choices are limited and there is a risk for accidental exposure (3, 4). Both have a great impact on the quality of life of food-allergic patients (5-7). Therefore, prevention and/or treatment strategies are highly desirable. Several studies have investigated conventional immunotherapy in which increasing doses of allergen were administered subcutaneously, sublingually or orally (7-9). Depending on the route of administration, the therapy was either safe but not effective or was effective but also induced side effects (10-12).

An important feature of successful conventional immunotherapy is the increase in allergen-specific IgG antibodies. It has been suggested that these antibodies block the formation of allergen-IgE complexes and thereby IgE-facilitated antigen presentation (IgE-FAP) (13-17). Interestingly, cow's milk- and peanut-allergic patients already have high allergen-specific IgG levels, and are still allergic (18-20). Therefore, we investigated the influence of these antibodies on the formation of allergen-antibody complexes and their binding to B cells (Chapter 2)

Due to the problems with efficacy and safety, immunotherapy is still not used in the clinic to treat food-allergic patients (4, 7, 8, 21). Previous studies with cat- and bee venom-allergic patients have shown that peptide immunotherapy may be a safe alternative (22-24). Therefore, we investigated in this thesis whether this approach is also effective for cow's milk allergy. First, we tried to use whey hydrolysates by determining the time point at which these hydrolysates are still able to induce T cell proliferation but are unable to cross-link IgE and thus to activate basophils and mast cells (Chapter 3). However, a clearcut time point could not be identified. Because the generation of hydrolysates was difficult and time-consuming, we determined in parallel the T cell epitopes in whey with synthetic peptides (Chapter 4 and 5). Subsequently, peptides containing the T cell epitopes of β-lactoglobulin were used to investigate whether peptides are able to induce tolerance to whey in both a preventive and curative setting (Chapter 5 and 6). Because a previous study has shown that a partial whey hydrolysate is able to prevent allergic symptoms (25), this hydrolysate was also tested in the curative setting (Chapter 6). While both treatments were effective, further optimization of peptide immunotherapy is necessary. There are several factors that are important for peptide immunotherapy, namely the safety of the therapy, the selection of tolerogenic peptides for a large group of patients, the peptide dose, the involved antigen presenting cells and the environment. The role of these factors in both preventive and curative peptide immunotherapy will be discussed here. In addition, the differences between mice and men in context of allergy and immunotherapy will be discussed.

IgG antibodies in allergy/immunotherapy

In Chapter 2 of this thesis, we determined allergen-specific IgE, IgG1 and IgG4 levels in sera of birch pollen-, peanut- and cow's milk-allergic patients. Higher IgG levels were found in food-allergic patients than in birch pollen-allergic patients. Moreover, while IgG1 levels were similar in peanut- and cow's milk-allergic patients, cow's milk-allergic patients had higher IgG4 levels indicating that different types of allergies have a different phenotype. Tay *et al.* compared food-specific IgG levels in healthy subjects with the levels found in food-allergic patients and also

observed different phenotypes in different food allergies (26). Whereas egg-specific IgG levels in both healthy controls and egg-allergic patients were high, lower peanut-specific IgG levels were found in healthy subjects.

Furthermore, we observed that both IgG1 and IgG4 antibodies were present in allergen-antibody complexes. Whereas the binding of these complexes to EBV-transformed B cells (EBV-B cells) was mediated by the low affinity IgE receptor CD23, complex binding to peripheral blood B cells involved both CD23 and complement receptor 2 (CR2). This difference in complex binding was due to differential receptor expression and suggested that EBV-B cells may not be a suitable model to study IgE-FAP. This hypothesis was strengthened by preliminary proliferation data showing that, at optimal allergen concentrations for complex formation, only EBV-B cells were able to induce T cell proliferation and not PBMCs. These data suggest that peripheral blood B cells may not play a direct role in IgE-FAP. Also, Henningsson *et al.* showed in a mouse model that CD23+ B cells were not directly involved in antigen presentation of allergen-IgE complexes (27). Instead, these cells transported and transferred the complexes to CD11c+ cells in B cell follicles. A similar effect has been described for CR2+ B cells. The effect of mixed allergen-antibody complexes that bind to both CD23 and CR2 is unclear and needs to be further investigated.

Recent studies have indicated that IgG antibodies may have different functions depending on the sialylation of their Fc fragment (28). Whereas sialylated IgG antibodies had an immunosuppressive effect, desialylated IgG antibodies induced a proinflammatory response. It can be hypothesized that the antibodies formed during immunotherapy have a different sialylation than the antibodies found in allergic individuals and thus have different effects. Oefner *et al.* have shown that tolerance induction with a T cell-dependent antigen induced sialylated IgG antibodies (28). However, it is unclear whether the IgG antibodies found in food-allergic patients are sialylated. Moreover, the effect of sialylation on complex formation and complex binding to B cells needs to be investigated. In preliminary experiments, we have observed that also allergen-antibody complexes formed using sera from birch pollen-allergic patients treated with immunotherapy contained IgG antibodies and bound to both CD23 and CR2 suggesting that also in these patients IgG antibodies are involved in complex formation and binding (data not shown).

It is known that allergen-IgG complexes may activate the complement system via the classical pathway. Activation of this system leads to the cleavage of C3 in C3a and C3b. C3b binds to the complex and is further degraded to iC3b and C3dg, which both bind to CR2. Our data strongly suggested that the mixed allergen-antibody complexes seen in this study activate the complement system via this pathway. Interestingly, Khodoun *et al.* showed that C3a exacerbated IgE-mediated anaphylactic shock and thus increased allergic symptoms (29). Complex formation and subsequent complement activation may thus aggravate allergy, which needs to be further investigated.

In conclusion, IgG antibodies in food-allergic patients are involved in complex formation and influence the receptors involved in complex binding to B cells, i.e. binding to CR2 and CD23 instead of CD23 alone. The clinical implications of this needs to be further investigated.

Safety

One of the reasons why conventional immunotherapy for food allergy is not used in the clinic yet is its safety profile (21). The intact allergens that are used for immunotherapy may cross-link IgE molecules on basophils/mast cells and thereby induce acute allergic responses. An IgE epitope contains minimal 6 amino acids. Considering that for cross-linking IgE, a peptide needs to contain two IgE epitopes and to cover the distance between two IgE receptors (minimal 23 amino acids) as well, in theory, a molecule of 3.8 kDa (35 amino acids) may be able to activate basophils/mast cells (30-32). However, the chance that a molecule of that size contains two IgE epitopes is very small.

In this thesis, we tested the efficacy of peptide therapy for cow's milk allergy and used both a partial whey hydrolysate and synthetic peptides of 18 amino acids (Chapter 5 and 6). As shown in Chapter 3, after hydrolysing proteins for 60 minutes, more than 90% of the formed peptides are smaller than 3 kDa indicating that most peptides are too small to cross-link IgE. Still, in some patients a strong basophil reaction was observed. This was either due to the small percentage of larger proteins that are still present in the hydrolysate or due to aggregate formation of the smaller peptides. For the immunotherapy study, we used whey proteins that were hydrolyzed for 90 minutes (Chapter 6). A previous study has already shown that this hydrolysate was able to induce tolerance in a preventive setting (33). In Chapter 6, we show that this hydrolysate is also effective in a curative setting. Although no side effects were observed during our study, van Esch *et al.* have shown that this hydrolysate was still able to induce mild acute allergic symptoms after an intradermal challenge in whey-sensitized mice (34). Thus, this hydrolysate was effective and less allergenic that intact proteins but it may still induce side effects.

Considering that minimal 35 amino acids are necessary to induce an acute allergic response, treatment with 18 amino-acid-long synthetic peptides, as used in the studies described in this thesis, should not be able to induce an allergic response. This hypothesis was confirmed for peptide mixture 1 with both *in vitro* and *in vivo* experiments. Incubation with the peptide mixture did not induce CD63 expression, a marker for degranulation, on human basophils (Chapter 6). Moreover, in whey-sensitized mice, the acute allergic skin response after an intradermal challenge with the peptide mixture was comparable to the response seen in mice challenged with PBS (Chapter 6). Also previous studies showed no histamine release after *in vitro* stimulation of human basophils with peptides of the major cat allergen Fel d 1 (35). Moreover, in clinical studies in cat-allergic and bee venom-allergic patients, in which peptides of comparable length (16-18 AA) were administered intradermally or subcutaneously, no acute allergic side effects were observed (23, 24, 35). In contrast, van Hoeyveld *et al*., who fractionated peptides formed after hydrolysis of whey based on size, showed that peptides of 1400-2600 Da (13-23 AA) were able to induce an acute allergic skin response in cow's milk-allergic patients (36). Worm *et al.* has described that peptides can form homo- and/or heterodimers, which may explain why small peptides can still induce an allergic response (35). Therefore, this needs to be taken into consideration when developing a peptide-based therapy.

Although no acute allergic responses were reported in previous clinical studies with cat-allergic patients, some patients in these studies did develop late asthmatic reactions (24, 37, 38). These reactions were caused by direct activation of T cells and were MHC-restricted (37). Because

there was no relation between the ability of the peptides to induce a T cell response *in vitro* and the induction of the late phase reactions, Hasselden *et al.* suggested that the appearance of the late reactions depends on the dose and the threshold for developing these reactions (37). Interestingly, depending on the time between the first and the second treatment, less severe late reactions were observed after the second exposure to the peptides compared to the first exposure indicating that tolerance was induced (24). While peptide therapy induced late asthmatic reactions, no late skin reactions at the site of peptide injection were observed in these patients (37). The late phase reactions can either be induced by the mediators released during mast cell and basophil activation, which attract and activate eosinophils and other immune cells, or by direct activation of T cells, which subsequently produce cytokines that also attract and activate eosinophils and other immune cells. Hasselden *et al.* suggested that for late phase skin symptoms basophil/mast cell activation is necessary, whereas late asthmatic reactions are induced directly by T cells (37). As discussed above peptides are too small to induce basophil/mast cell activation, therefore no late skin responses were observed. Because also cow's milk-allergic patients may experience asthmatic responses (4), further research is necessary to determine whether these responses can be induced by the peptides.

For preventive treatment, not only the induction of allergic symptoms, but also the sensitizing capacity of the peptides is important. One of the criteria for hypoallergenic infant formulas is that the formula is not able to sensitize animals to the protein source they are derived from (39). Because the peptides are able to activate T cells, they may also induce Th2 skewing. Smith *et al.* showed that specific peptides used for the treatment of multiple sclerosis induced IgE antibodies and even anaphylactic symptoms in mice after intravenous administration (40). Because mice normally develop tolerance to orally administered antigens, the sensitizing capacity of the peptides used in our study should be investigated by testing the peptides in combination with the adjuvant cholera toxin, as has been described by van Esch *et al.* (41).

In conclusion, whereas a partial whey hydrolysate is still able to induce mild acute allergic symptoms, 18 amino-acid-long synthetic peptides are not. Further research is necessary to determine their effect on late phase allergic responses and to determine their sensitizing capacity.

Selection of peptides

A second factor that is important for peptide immunotherapy is the selection of the right peptides. To induce tolerance, peptides should contain T cell epitopes. Because CD4+ T cells recognize peptides in a complex with MHC class II molecules, the type of MHC molecules expressed on antigen presenting cells mainly determines which peptides are recognized. Only peptides that are able to bind to the expressed MHC molecule can induce a T cell response. Texier *et al.* showed that each HLA-DR allele expressed a unique binding pattern of Api m1 peptides (a major allergen of bee venom) (42). Individuals that express different MHC molecules on the antigen presenting cells may bind/present different peptides and thus recognize different T cell epitopes. This may explain the diverse T cell response that we observed in our epitope-mapping experiments with whey-derived peptides, which are described in Chapter 4 and 5 of this thesis. None of the T cell epitopes was recognized by all patients.

Due to the large variation in T cell epitopes between patients, it will be difficult to develop a peptide therapy that will work in all patients. Currently, there are three different approaches described, namely (1) using peptides spanning (almost) the whole protein (24), (2) using larger peptides spanning a region that contains multiple epitopes (43, 44), or (3) by selecting T cell epitopes based on the binding to (multiple) MHC molecules (22, 35). When using the first option, there is a minor chance of missing an epitope. However, combining peptides may dampen the effect of tolerogenic peptides, as observed in our preventive study in which a single peptide showed a stronger effect than the mixture of peptides (Chapter 5). A possible explanation for this effect is that the non-tolerogenic peptides may influence the solubility or uptake of the other peptides. Moreover, if the peptides are able to bind to the MHC class II molecules but this peptide-MHC complex is not recognized by T cells, the competition between the peptides for the MHC molecules may affect the tolerogenic response.

An advantage of the second approach, i.e. using larger peptides, is that these peptides still need to be processed and therefore one peptide may generate several T cell epitopes. Counsell *et al.* showed that these peptides may be able to bind to a wide spectrum of MHC class II molecules (45). Moreover, Kämmerer *et al.* showed that larger peptides induce more T cell activation (44). This increased activation may be due to the peptide-flanking regions. Holland *et al.* recently showed that not only the core region but also these peptide-flanking regions are important for binding to MHC class II molecules and may influence the T cell activation (46). Peptides eluted from MHC class II molecules were 12-20 amino acids long. Thus, when using small peptides of 15-18 amino acids, there is a risk that they do not contain the right flanking region. On the other hand, using larger peptides may also increase the risk of side effects. In a study by Fellrath *et al.*, four patients developed mild reactions to treatment with 45-60 amino-acid-long peptides in a clinical trial with bee venom-allergic patients (43).

For the third approach, the binding of peptides to several MHC class II molecules needs to be determined. Worm *et al.* determined the binding affinity of 12 peptides of Fel d 1 to common HLA-DR molecules and verified the binding by investigating the proliferative and cytokine responses of PBMCs of 100 subjects in response to the peptides (35). Based on these data, 7 peptides were selected that bound to a broad range of MHC molecules. Whereas this approach is feasible for small proteins, it is difficult for larger proteins. Previous studies have indicated that, although each HLA allele has a unique binding pattern, there are peptides that can bind to multiple alleles (37, 42, 47). Southwood *et al.* showed that common HLA-DR types have a similar binding pattern (48). Based on this knowledge, they developed an algorithm that can determine peptides with promiscuous binding to the HLA-DR types. A recent study by van Thuijl *et al.* used this algorithm to determine the T cell epitopes of several cow's milk allergens (49). They found one epitope in α-lactalbumin (AA 33-47) and four epitopes in β-lactoglobulin (AA 1-13, 29-43, 68-83, 137-151). Of these epitopes, two epitopes (AA 68-83 and 137-151 of β-lactoglobulin) were not found in our study (Chapter 5) or in other previous human studies (50). On the other hand, peptides from two other regions of β-lactoglobulin (AA 43-60 and AA 91-120) were recognized in our study and in the previous epitope study, but not in the study of Van Thuijl *et al.* These epitopes may be presented by HLA-DP or HLA-DQ molecules with binding patterns that may not be present in the HLA-DR algorithms. This was also observed for

an immunodominant epitope of αS1 casein, as described by Ruiter *et al*., that is presented by HLA-DQ and was not detected by the algorithm used by van Thuijl *et al.* (51). These data indicate that a combination of algorithms is necessary to determine the immunodominant epitopes and that subsequent T cell tests are necessary to verify whether these epitopes are indeed capable of inducing T cell activation.

Are all T cell epitopes able to induce tolerance?

Although it is known that peptides should contain T cell epitopes to induce tolerance, previous studies have indicated that not all T cell epitopes can induce tolerance (52-54). Both Hirahara *et al.* and Hoyne *et al.* showed that subdominant epitopes are not or less effective than dominant epitopes (52, 53). Although the effect of these peptides may be influenced by the dose, Janssen *et al.* suggested that also the affinity of the T cell receptor (TCR) for the peptide is important (54). In their study, a peptide of ovalbumin was not able to induce tolerance in mice, whereas a mutated form of this peptide with a similar affinity for MHC class II did. The mutated form increased proliferation and skewed the T cell response to a Th1 phenotype. Also, other studies investigating the TCR affinity of allergens have indicated that TCR affinity can influence Th1/Th2 skewing (55-58). Juntunen *et al.* suggested that the low TCR affinity of a peptide of Can f 1, a major dog allergen, may contribute to the allergenicity of Can f 1 (57). Furthermore, Daniel *et al.* showed that affinity also plays a role in the induction of regulatory T cells (Tregs) (59). A mutated peptide of insulin with a high affinity for the TCR induced more Foxp3+ T cells and was able to prevent the onset of type I diabetes, while no effect was seen with the wild-type peptide. In addition to TCR affinity, previous studies have also shown that the affinity for MHC molecules may influence the T cell response. By modulating a peptide of myelin basic protein, Kumar *et al.* showed that high affinity for MHC class II leads to a Th1 response, whereas a low affinity resulted in a Th2 response (60).

Several studies for both autoimmune and allergic diseases have generated altered peptide ligands with a high affinity for TCR (61-64). However, altered peptide ligands for multiple sclerosis were successful in animal studies but showed less effect in clinical trials (62, 63, 65). Moreover, Kappos *et al.* showed that the altered peptide ligand induced hypersensitivity symptoms in a phase II trial in patients with multiple sclerosis (63). Further research is necessary to determine the efficacy and safety of these altered peptides.

Is it necessary to determine all T cell epitopes for peptide therapy?

While selecting peptides that contain T cell epitopes and have the right affinity is important for tolerance induction, the question is whether it is necessary to select multiple peptides or whether therapy with one peptide is enough. Previous studies have shown that treatment with one peptide may also affect the response to other T cell epitopes within the same molecule suggesting that it is not necessary to identify all T cell epitopes for tolerance induction (53, 66-68). This phenomenon is called linked-epitope or intramolecular epitope suppression and may explain why both mixture 1 and 3 in our preventive study reduced the acute allergic skin response to intact whey protein with more than 50% (Chapter 5). Whereas Hoyne *et al.* showed that exposure to the intact protein is necessary for linked-epitope suppression (67), in a study

of Anderton *et al.* also effects without exposure to the intact protein were seen (68). Intranasal pre-treatment with peptide MBP[Ac1-9(4Y)] of the myelin binding protein reduced the symptoms of experimental autoimmune encephalomyelitis induced by immunization with another peptide MBP[98-101]. Campbell *et al.* showed that treatment with one peptide could significantly reduce asthmatic symptoms in mice (66). Moreover, they determined the percentage IL-10-producing and peptide-specific T cells, which were both increased after treatment, and showed that the percentage IL-10-producing T cells was more increased than the percentage peptide-specific T cells. These data suggest that also T cells specific for other epitopes switch to a regulatory phenotype. Also in patients, linked-epitope suppression was observed. Campbell *et al.* showed that peptide treatment with 12 peptides not only reduced the proliferative and cytokine responses of PBMCs in response to the treatment peptide but also to peptides not included in the treatment (66).
In addition to linked-epitope suppression, also bystander suppression has been described. During bystander suppression, treatment with peptides of one protein can reduce the allergic response to other proteins (68-70). Previous studies have shown that this only occurs when subjects are exposed to a mixture of proteins (68, 69). It has been suggested that this effect is due to the non-specific effect of inhibitory cytokines, such as IL-10 (71), or due to direct contact between different T cells via, for example, Notch (72). Because cow's milk and other food allergens contain multiple major allergens, treatment with peptides of one protein may influence the response to the mixture and may be enough to decrease allergic symptoms in general.

To summarize, combining non-tolerogenic and tolerogenic peptides may reduce the efficacy of peptide immunotherapy. Moreover, using larger peptides may increase the risk on side effects. In view of safety, selecting 18 amino-acid-long peptides as done in this thesis seems to be a good option for peptide immunotherapy. However, none of the peptides selected in our study were able to induce proliferation in all human subjects tested (Chapter 5). This underlines the importance of determining which MHC molecules present the peptides and to see whether this may explain the diverse response. In addition, it would be interesting to investigate whether peptides of β-lactoglobulin are able to induce tolerance to α-lactalbumin and casein by sensitizing mice with cow's milk instead of whey. For future human studies, using algorithms for HLA-DR, -DP and -DQ in combination with T cell tests, instead of using overlapping peptides, would be a good alternative approach to select T cell epitopes.

Dose

The peptide dose may be an important factor that may influence tolerance induction. It has been suggested that high antigen doses induce T cell deletion/anergy, whereas low antigen doses induce regulatory cells (73). Moreover, Daniel *et al.* showed that there is an optimal concentration for the induction of Foxp3+ regulatory T cells (59). Mice that were subcutaneously exposed to 5-10 μg peptide per day for 14 days via an osmotic pump converted 40-50% of naive peptide-specific T cells into Foxp3+ regulatory T cells, while treatment with 2.5 and 20 μg peptide was less effective in doing so. These observations may explain why in our

experiments oral immunotherapy with 14 administrations of 50 mg partial whey hydrolysate was less effective than therapy with 14x 0.5 mg hydrolysate (Chapter 4, data not shown), although we recognize that caution should be taken by translating results obtained from subcutaneous experiments into oral exposure.

For the synthetic peptides, we orally administered 6x 8-16 mg peptides (mixture of 2, 3 or 4 peptides, 4 mg peptide per administration) in the preventive setting and 14x 160 µg peptides (mixture of 4 peptides, 40 µg peptide per administration) in the curative setting. Two other studies have used oral peptide immunotherapy for allergy in mouse models, of which one in the preventive setting and one in the curative setting (53, 74). Hoyne *et al.* used 3x 3 mg of a Der p1 peptide in a preventive setting. However, this peptide was coupled to glutathione S-transferase, which makes it difficult to compare the concentration (53). Rupa *et al.*, who tested a peptide of ovomucoid in a curative setting, treated the mice orally with 9x 1 mg peptide (74). This concentration is much higher than the concentration we used. In contrast, in other peptide immunotherapy studies, in which the peptides were administered intradermally, a much lower peptide concentration, namely 1 µg, was used (66, 75). However, because in these studies a different route of administration is used, it is difficult to compare these concentrations with the concentrations used in our study. So far, human clinical studies investigated only subcutaneous or intradermal peptide immunotherapy for either allergic or autoimmune diseases. Also, in these studies different peptide concentrations varying from 4x 7.5 µg till 16x 50 mg were used (63, 76).

Only 4 studies investigated several doses (22, 54, 63, 76). In a mouse study by Janssen *et al.*, 0.3 and 3 mg of an ovalbumin peptide was administered intranasally or intradermally to investigate whether peptide immunotherapy may reduce asthmatic symptoms (54). Both dosages were not effective. Two other studies were done in cat-allergic patients (22, 76). Norman *et al.* tested three different peptide doses, i.e. 7.5, 75 and 750 µg, which were administered subcutaneously four times (once per week), and showed that the highest dose was most effective (76). Patel *et al.* showed that treatment with four times 75 µg peptides with an interval of 4 weeks was able to induce a significant effect and that this effect was significantly stronger than when eight times 37.5 µg with an interval of 2 weeks was used (22). Unfortunately, no higher doses were tested in the last study and different peptide mixtures were used in the two studies (two 27-amino-acid-long peptides vs. seven 13/16/17-amino-acid-long peptides), which make it difficult to compare them. In the study by Kappos *et al.*, 5, 20 or 50 mg of NBI 5788, an altered peptide ligand of myelin basic protein, was injected subcutaneously once a week for 4 months in patients with multiple sclerosis (63). Although the study had to be stopped due to hypersensitivity responses, analysis of the data of the patients that finished the whole trial showed that a dose of 5 mg was most effective, indicating again that a higher dose was not more effective in tolerance induction.

In conclusion, these studies clearly indicate that there is an optimal concentration for tolerance induction and that higher and lower doses may be less effective. Although the concentrations used in our experiments were effective, further optimization may increase the efficacy. Therefore, several doses and dose regimens need to be tested to determine the optimal form for peptide therapy.

Antigen presenting cells

The type of antigen-presenting cells that present the peptides influences the efficacy of peptide immunotherapy. Previous studies have shown that antigen presentation by non-activated dendritic cells induce regulatory T cells (77), which are known to play an important role in tolerance induction. Non-activated dendritic cells express basal levels of co-stimulatory molecules and thus will only activate T cells via the TCR/MHC:peptide complex, without additional co-stimulation. Hochwellner *et al.* showed that incubation of dendritic cells with peptides did not up-regulate co-stimulatory molecules on the cells indicating that peptides do not activate dendritic cells and thus may induce tolerance (78).

In addition to non-activated dendritic cells, also specific tolerogenic subsets of dendritic cells have been described (79-81). Both CD103+ dendritic cells and CD8a+ plasmacytoid dendritic cells are present in the intestine, which is indicative for the tolerogenic environment in this organ. Coombes *et al.* and Sun *et al.* showed that CD103+ dendritic cells in the intestine could convert naive T cells into regulatory T cells (79, 80). Furthermore, they indicated that this effect was mediated by TGF-β and retinoic acid, which were both produced by the dendritic cells. In our preventive study, the percentage CD103+ dendritic cells of the total dendritic cell population in the mesenteric lymph nodes was increased after peptide therapy (Chapter 5). It has been shown that these dendritic cells travel from the lamina propria to the mesenteric lymph nodes (79). Therefore, it can be hypothesized that peptide therapy increases the migration of CD103+ dendritic cells to the mesenteric lymph nodes, which may lead to the induction of regulatory T cells and subsequent tolerance induction.

Another dendritic cell subset that has been described as tolerogenic is the CD8a+ plasmacytoid dendritic cell. These cells are present in the lung and in the intestine (81-83). Bilsborough *et al.* showed that this subset has low expression of co-stimulatory molecules and therefore favours tolerogenic responses over immunogenic responses (81). Moreover, naive T cells that are repeatedly stimulated with CD8a+ plasmacytoid dendritic cells differentiate into Tr1-like regulatory T cells. In addition, Lombardi *et al.* showed that these plasmacytoid dendritic cells express RALDH enzymes, which convert retinal into retinoic acid, and thereby induce Foxp3+ regulatory T cells (82). However, no changes in the percentage of CD8a+ plasmacytoid dendritic cells were observed after treatment with synthetic peptides or the partial whey hydrolysate suggesting that these cells were not involved in the tolerance induction seen in our study.

The mucosa of the intestine is well known for its tolerance induction (73). The intestinal immune system is daily exposed to commensal bacteria and food antigens. Because these antigens are harmless, the immune system actively develops tolerance to them. This phenomenon is called oral tolerance. Worbs *et al.* showed that the mesenteric lymph nodes, in which both CD103+ and CD8a+ tolerogenic dendritic cells subsets can be found, are essential for this process (79, 81, 84).

Because subcutaneous conventional immunotherapy for food allergy induced serious side effect, it was hypothesized that due to the tolerogenic environment in the intestine oral immunotherapy may be a safe alternative. However, even with this approach side effects were observed. Therefore, we decided to investigate the efficacy and safety of oral peptide immunotherapy. An advantage of this route of administration in contrast to subcutaneous

administration is that oral administration does not involve needles and is therefore more child-friendly. A disadvantage might be that peptides are exposed to digestive enzymes, which may lead to degradation and can affect the availability of the peptides and thus the peptide dose. Moreover, it is questionable whether the intestinal environment is still tolerogenic in food-allergic children. Previous studies have shown that the intestinal permeability in children with cow's milk allergy is increased (85-87). Although cow's milk-allergic children do not develop an allergic response to every potential food allergen they eat, suggesting that the tolerogenic environment is not compromised, the effect of the increased gut permeability on tolerance induction needs to be investigated.

Whereas most previous peptide immunotherapy studies used intradermal or subcutaneous administration, we showed in this thesis that also oral administration of peptides is effective for inducing tolerance. However, the efficacy and safety of both administration routes should be compared in future studies.

Environmental factors

Environmental factors, such as diet and exposure to microorganisms, may influence tolerance induction. This effect, at least in part, seems to be mediated by the gut microbiota. Sjogren *et al.* described that family size and endotoxin exposure, which are inversely associated with allergy development, influence the diversity of gut microbiota (88). Moreover, they suggested that a low diversity in gut microbiota increases the risk on allergy. Sepp *et al.* showed that in allergic children less bifidobacteria and more clostridia were found in gut microbiota (89). In another study from the same group, the gut microbiota in children from Sweden was compared with the microbiota in Estonian children (90). They observed that the children in Sweden had lower counts of lactobacilli and eubacteria and higher counts of clostridia in their gut microbiota than the children in Estonia indicating that geographic factors may influence the microbiota. Interestingly, in Sweden the prevalence of allergy was much higher than in Estonia.

One dietary factor that affects the gut microbiota is human breast milk (91, 92). The main components in breast milk that are believed to influence microbiota are milk oligosaccharides (91). Previous studies indicated that breast-feeding increased the number of lactobacilli and bifidobacteria (92). A similar effect was seen with a specific mixture of non-digestible oligosaccharides, i.e. short-chain galacto- (scGOS) and long-chain fructo-oligosaccharides (lcFOS, in a ratio 9:1), which was developed to evaluate whether effects of breast milk could be mimicked (93, 94). Moreover, van Esch *et al.* showed that scGOS/lcFOS in combination with pectin-derived acidic oligosaccharides (pAOS, 9:1:2) increased the tolerance-inducing capacity of whey hydrolysates (33). In this thesis, we observed a similar effect for peptide mixture 1 in the preventive study, whereas no effect was observed for peptide mixture 3 (Chapter 5). It can be hypothesized that the peptides have a different mechanism of action and that only some pathways may be affected by exposure to prebiotics. However, further studies are necessary to investigate this. Moreover, the effect of scGOS/lcFOS/pAOS in a curative setting needs to be determined.

Men vs. mice

As described above, many factors can influence tolerance induction. Therefore, it is impossible to study tolerance induction *in vitro*. Additionally, efficacy and safety have to be tested first *in vivo* in animal models before they can be applied to humans. Because much is known about the immune system of mice and a lot of tools are available, we chose to investigate the efficacy of peptide immunotherapy in a mouse model. Although the genetic variation between mice is small, environmental factors may have a great impact on mouse experiments. For example, van Esch *et al.* observed variations in the strength of the allergic response when the mouse model for cow's milk allergy was performed at 4 different locations (95). Similarly, we performed the oral immunotherapy mouse model on two locations and found that this resulted in a large difference in the sensitization response to whey (Chapter 6).

Although studies in mice are still needed, there are important differences between mice and men that need to be taken into account. First of all, antibody isotypes and Fc receptors differ between mice and men (96, 97). Whereas in men IgG1, IgG2, IgG3 and IgG4 can be found, mice express IgG1, IgG2a, IgG2b and IgG3. In addition, human IgG and mouse IgG antibodies can have different effects. For example, in mice both IgE and IgG antibodies are able to induce anaphylaxis, while only IgE antibodies induce anaphylaxis in men (98, 99). Due to the differences in IgG antibodies between mice and men, it is difficult to investigate the exact mechanism of the allergen-antibody complexes described in Chapter 2.

Secondly, also the innate immune system differs between mice and men. Different pattern recognition receptors have been found in both species (100). Garcia Vallerjo *et al.* described in a recent review that, although mice express eight homologues of DC-SIGN, the expression pattern and binding affinities differ from human DC-SIGN and thus the mice ortholog has not yet been found (101). That these receptors may be important for allergy and tolerance induction has been shown by Shreffler *et al.* who described that Ara h 1, a major allergen of peanut, binds to DC-SIGN and thereby activates dendritic cells to induce a Th2 response (102). Thereby, allergens can create an environment via innate signals that may facilitate sensitization to other allergens within the same food.

The above indicates that caution should be taken when extrapolating data obtained in mice models to the human situation. Nevertheless, these models are essential for proof-of-principle studies. From the mouse models used in this thesis we can conclude that oral administration of peptides may induce tolerance to intact proteins, which would not have been possible without these models.

Overall conclusion

This thesis shows that both a partial whey hydrolysate and 18 amino-acid-long synthetic peptides may induce tolerance to whey. While the synthetic peptides seem to be a safe alternative for conventional immunotherapy, further research is necessary to determine their sensitization capacity and their effect on late phase allergic symptoms. The efficacy of peptide immunotherapy is dependent on the selection of peptides. Due to differences in TCR/MHC affinity, some peptides may be more tolerogenic than others. However, the effect of non-tolerogenic peptides can be modulated by changing the peptide dose, the route of

administration and/or the environment, for example by combining peptides with prebiotics. Although effective, partial hydrolysates may still induce allergic side effects and are thus less suitable for immunotherapy. Nevertheless, if the enzymatic process can be more controlled so that the right peptides stay intact, this would be a nice option for the future.

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APPENDICES

SUMMARY

Cow's milk allergy affects 0.3-3.5% of the children and 0.1-0.3% of the adults. As there is no curative treatment, cow's milk-allergic patients are advised to avoid allergen exposure to prevent allergic symptoms. Because many products contain cow's milk proteins, diet choices are limited and there is a risk for accidental exposure. Both have a great impact on the quality of life of cow's milk-allergic patients. Therefore, prevention and/or treatment strategies are highly desirable.
Several studies have investigated allergen-specific immunotherapy, in which patients are exposed to increasing doses of allergens, as a therapy for cow's milk allergy. However, depending on the route of administration, the therapy was either safe but not effective or was effective but also induced side effects. In this thesis we investigated whether preventive/curative treatment with peptides instead of intact proteins may be a safe and effective alternative. In addition, we investigated the effect of naturally-occurring high IgG levels in food-allergic patients on allergen-antibody complex formation and binding to B cells.

Previous studies have indicated that immunotherapy increases allergen-specific IgG levels. Moreover, it has been described that these IgG antibodies inhibit the formation of allergen-IgE complexes and thereby IgE-facilitated antigen presentation by B cells. It has been observed previously that food-allergic patients have naturally high allergen-specific IgG levels but are still allergic. This thesis shows that these IgG antibodies are present in allergen-antibody complexes and influence the complex binding to B cells (Chapter 2). Whereas binding to EBV-transformed B cells was mediated by CD23, binding to peripheral blood B cells involved both CD23 and complement receptor 2 (CR2). However, upon IgG depletion, complex binding to peripheral blood B cells was exclusively mediated by CD23. These data suggest that the complexes containing IgG antibodies induce complement activation and deposition on the complexes, which leads to binding to CR2. This may result in altered antigen presentation or allergen transport to lymph nodes. The difference between EBV-transformed B cells and peripheral blood B cells reflects differential receptor expression and suggests that EBV-transformed B cells are not a representative model for complex binding and IgE-facilitated antigen presentation.

Because the risk/benefit ratio of conventional immunotherapy for food allergy is considered to be too high, this therapy is still not used in the clinic. A possible way to reduce the side effects of immunotherapy is by using peptides, which are too small to cross-link IgE but are still able to activate T cells. Previous studies in cat and bee venom-allergic patients have shown that this therapy may be a safe alternative. Therefore, in this thesis it was investigated whether this approach is also effective for cow's milk allergy. First, whey hydrolysates were investigated by determining the time point at which these hydrolysates are still able to induce T cell proliferation but no mast cell/basophil activation (Chapter 3). Unfortunately, no clearcut time point was found. Whereas in most patients the basophil and T cell responses decreased over time, in some patients more basophil activation was observed after incubation with hydrolyzed proteins compared to non-hydolyzed proteins. Immunoblot data indicated that these patients recognized either a 25-30 kDa degradation product of casein or a 10 kDa degradation product of whey. These data indicate that not the degree of hydrolysis is important for basophil and T cell activation, but the presence and stability of specific IgE and T cell epitopes in the fragments.

In addition, T cell epitopes of the two major whey proteins, α-lactalbumin and β-lactoglobulin, were determined by using 18-amino-acid-long synthetic peptides spanning both proteins and human cow's milk-specific short-term and long-term T cell lines. No major T cell epitopes could be determined for α-lactalbumin (Chapter 4). Moreover, these data showed that immunogenicity of α-lactalbumin is low compared to the other major allergens in cow's milk. More than half of the T cell lines did not respond to this protein or lost their responsiveness during subsequent experiments.

For β-lactoglobulin several T cell epitopes were defined (Chapter 5). Based on these data and previous literature describing T cell epitopes in both men and mice, nine peptides were selected, which were divided over three mixtures, and tested in combination with/without a diet containing a specific mixture of non-digestible short-chain galacto-, long-chain fructo- and pectin-derived acidic oligosaccharides in a mouse model for cow's milk allergy. Prophylactic treatment with two of the three mixtures significantly reduced the acute allergic response to whey (Chapter 5). Moreover, a stronger effect was seen for one of the mixtures in combination with the prebiotic diet. Of the four peptides in this mixture, one peptide, LLDAQSAPLRVYVEELKP, showed the strongest tolerance-inducing effect. This peptide also tended to decrease whey-specific antibody levels and to increase the percentage of regulatory dendritic and T cells in the mesenteric lymph nodes.
Also in a curative setting the peptide mixture was able to reduce the acute allergic response to whey (Chapter 6). However, no effect on antibody responses was seen. Moreover, the percentage regulatory T cells in the mesenteric lymph nodes decreased. The peptide mixture did influence the number of B cells in the lymph nodes. In addition, several T cell markers were affected. Unfortunately, the observed effects differed between experiments, which may have been due to differences in the extent of sensitization. Further research is necessary to optimize the therapy.

In conclusion, this thesis showed that, in contrast to what was expected, IgG antibodies are involved in allergen-antibody complex formation and influence the binding to B cells, which may have an effect on antigen presentation or transport of allergen to lymph nodes. In addition, it was shown that not the degree of hydrolysis of whey proteins is important for basophil and T cell activation, but the presence and stability of specific IgE and T cell epitopes in the fragments. Moreover, this thesis showed that preventive and curative treatment with synthetic peptides containing T cell epitopes of β-lactoglobulin may reduce the acute allergic response to whey, indicating that this therapy may be a safe and effective alternative for conventional immunotherapy.

SAMENVATTING

Koemelkallergie komt voor bij 0.3-3.5 % van de kinderen en 0.1-0.3 % van de volwassen. Aangezien er geen behandeling beschikbaar is, wordt patiënten geadviseerd om koemelkeiwitten in het dieet te vermijden. Echter, omdat veel producten koemelkeiwitten bevatten, zijn de dieetkeuzes beperkt en is er een grote kans op onverwachte blootstelling. Beide hebben een grote impact op de kwaliteit van leven van koemelk-allergisch patiënten. Daarom zijn nieuwe behandelingen om koemelkallergie te voorkomen en/of behandelen nodig.
Eerdere studies hebben onderzocht of allergeen-specifieke immunotherapie, waarbij patiënten blootgesteld worden aan oplopende concentraties allergeen, geschikt is als behandeling van koemelkallergie. Echter, afhankelijk van de toedieningsroute, was de behandeling veilig maar niet effectief of effectief maar traden er bijwerkingen op. In dit proefschrift is onderzocht of preventieve/therapeutische behandeling met peptides in plaats van intact eiwitten een veilig en effectief alternatief is. Verder is het effect van hoge allergeen-specifieke IgG levels, die van nature voorkomen in voedsel-allergische patiënten, op allergeen-antilichaam complex vorming en binding aan B cellen onderzocht.

Eerdere studies hebben laten zien dat immunotherapie de allergeen-specifieke IgG waardes in het serum verhoogd. Bovendien is er beschreven dat deze IgG antilichamen de formatie van allergeen-IgE complexen en daardoor IgE-gefaciliteerde antigeen presentatie door B cellen remt. Ook is aangetoond dat voedsel-allergische patiënten van nature hoge allergeen-specifieke IgG waardes hebben maar nog steeds allergisch zijn. Dit proefschrift laat zien dat de IgG antilichamen betrokken zijn bij de vorming van allergeen-antilichaam complexen en daardoor de binding aan B cellen beïnvloeden (Hoofdstuk 2). Terwijl binding aan EBV-getransformeerde B cellen gemedieerd werd door CD23, zijn zowel CD23 en complement receptor 2 (CR2) betrokken bij de complex binding aan perifere B cellen. Echter, na IgG depletie vindt de binding aan perifere B cellen ook via CD23 plaats. Deze resultaten suggereren dat de aanwezigheid van IgG in allergeen-antilichaam complexen complement activatie en depositie op de complexen induceert, wat leidt tot binding aan CR2. Dit kan een effect hebben op antigeen presentatie en/of op het transport van allergeen naar de lymfeknopen. Het verschil tussen EBV-getransformeerde B cellen en perifere B cellen reflecteert het verschil in receptor expressie op deze cellen en suggereert dat EBV-getransformeerde B cellen geen representatief model zijn voor complex binding en IgE-gefaciliteerde antigeen presentatie.

Omdat het risico op bijwerkingen bij conventionele immunotherapie als behandeling van voedselallergie in het algemeen als te hoog wordt beschouwd, wordt deze behandeling nog niet toegepast in de kliniek. Een mogelijke manier om de bijwerkingen te verminderen is door gebruik te maken van peptides, die te klein zijn om IgE te cross-linken maar nog wel in staat zijn om T cellen te activeren, te gebruiken. Eerdere studies in patiënten met een katten of bijen gif allergie hebben laten zien dat peptide therapie een veilig alternatief kan zijn. In dit proefschrift is onderzocht of deze methode ook effectief is voor koemelkallergie. Eerst is geanalyseerd of wei hydrolysaten gebruikt kunnen worden door het tijdstip te bepalen waarop de hydrolysaten nog wel in staat zijn om T cel proliferatie te induceren maar geen mestcellen/basofielen meer activeren (Hoofdstuk 3). Helaas werd er geen duidelijk tijdstip gevonden. Terwijl in de meeste

patiënten de basofiel en T cel respons in de tijd verminderden, werd er in sommige patiënten meer basofiel activatie geïnduceerd door de gehydrolyseerde eiwitten dan door de niet-gehydrolyseerde eiwitten. Immunoblot data laten zien dat deze patiënten of een 25-30 kDa afbraakproduct van caseïne of een 10 kDa afbraakproduct van wei herkennen. Deze resultaten tonen aan dat niet de mate van hydrolyse belangrijk is voor basofiel en T cel activatie, maar de aan-of afwezigheid van specifieke IgE en T cel epitopen in de fragmenten.
Verder zijn de T cel epitopen in twee belangrijke wei-eiwitten, α-lactalbumine en β-lactoglobuline, bepaald door gebruik te maken van 18-aminozuur-lange synthetische peptides van beide eiwitten en humane koemelk-specifieke korte en lange termijn T cellijnen. Voor α-lactalbumine werden er geen dominante T cel epitopen gevonden (Hoofdstuk 4). Bovendien lieten de resultaten zien dat de immunogeniciteit van α-lactalbumine laag is in vergelijking met andere belangrijke allergenen in koemelk. Meer dan de helft van de T cellijnen reageerde niet op dit eiwit of verloor zijn reactiviteit gedurende de experimenten.

Voor β-lactoglobuline werden er meerdere epitopen gevonden (Hoofdstuk 5). Gebaseerd op deze data en op eerdere artikelen waarin T cel epitopen in zowel mens als muis beschreven zijn, zijn negen peptides geselecteerd, verdeeld over 3 mixen en getest in een muismodel voor koemelkallergie. Dit werd getest al dan niet in combinatie met een prebiotisch dieet, dat een specifieke mix van niet-verteerbare korte keten galacto-, lange keten fructo- en van pectine-afkomstige zure oligosaccharides bevatte. Preventieve behandeling met twee van de drie mixen verminderde de acute allergische symptomen geïnduceerd door wei. Bovendien liet een van mixen een sterker effect zien in combinatie met het prebiotisch dieet. Van de vier peptides in de mix, had één peptide, LLDAQSAPLRVYVEELKP, het sterkste effect. Dit peptide lijkt de wei-specifieke antilichaamwaardes te verminderen en het percentage regulatoire dendritische en T cellen in de mesenteriale lymfeknopen te verhogen.

Ook een therapeutische behandeling met de peptide mix, die het meest effectief was in het preventieve model, was in staat om de acute allergische reactie geïnduceerd door wei te verminderen (Hoofdstuk 6). Echter, er was geen effect op de antilichaam respons te zien en het percentage regulatoire T cellen in de mesenteriale lymfeknopen was verlaagd. Behandeling met de peptides verhoogde wel het aantal B cellen in de lymfeknopen en verschillende T cel markers werden beïnvloed. Helaas was het effect van de therapie wisselend, wat mogelijk te maken heeft met verschillen in de mate van sensibilisatie. Verder onderzoek is nodig om de behandeling te optimaliseren.

Samenvattend heeft dit proefschrift laten zien dat IgG antilichamen, tegen de verwachting in, betrokken zijn bij de vorming van allergeen-antilichaam complexen en daardoor de binding van de complexen aan B cellen beïnvloeden. Dit kan effect hebben op antigeen presentatie en/of transport van allergeen naar de lymfeknopen. Daarnaast is aangetoond dat niet de mate van hydrolyse van wei-allergenen in koemelk belangrijk is voor basofiel en T cel activatie, maar de aan- of afwezigheid van specifieke IgE en T cel epitopen in de fragmenten. Bovendien laat dit proefschrift zien dat preventieve en therapeutische behandeling met synthetische peptides,

die T cel epitopen van β-lactoglobuline bevatten, in staat zijn om de allergische reactie geïnduceerd door wei te verminderen. Deze resultaten suggereren dat peptide immunotherapie een veilig en effectief alternatief is voor conventionele immunotherapie.

DANKWOORD

'Alles komt goed'

Het meest gehoorde en meest gezegde zinnetje van de afgelopen jaren en nu is het dan eindelijk zover: het is goed gekomen!

Hoewel het zeker niet altijd even gemakkelijk was, kijk ik vooral met heel veel plezier terug op de afgelopen tijd. Wat heb ik ontzettend veel leuke mensen ontmoet en heel veel leuke dingen gedaan. Van de borrels, labuitjes en BBQs tot, picknicken in de botanische tuinen, pannekoeken eten in Rhijnauwen, wadlopen, een sledehondentocht maken etc... teveel om allemaal op te noemen. Op de mindere momenten waren er altijd mensen in de buurt met een luisterend oor, een schouder om op te huilen of een helpende hand om te zorgen dat ik toch nog een beetje op tijd naar huis kon. Daarnaast waren er vrienden en familie die interesse toonden in mijn onderzoek, maar vooral voor afleiding zorgden en begrip toonden als ik het op laatste moment toch weer eens afhaakte. Zonder al deze mensen was dit zeker niet gelukt!

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Liefs, Laura

LIST OF PUBLICATIONS

Meulenbroek LA, de Jong RJ, den Hartog Jager CF, Monsuur HN, Wouters D, Nauta AJ, Knippels LM, van Neerven RJ, Ruiter B, Leusen JH, Hack CE, Bruijnzeel-Koomen CA, Knulst AC, Garssen J, van Hoffen E.
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Characterization of T cell epitopes in bovine α-lactalbumin.
Submitted for publication

Van den Elsen LW, **Meulenbroek LA**, van Esch BC, Hofman GA, Boon L, Garssen J, Willemsen LE.
CD25+ Regulatory T-cells transfer n-3 LCPUFA induced tolerance in cow's milk-allergic mice.
Allergy, 2013, in press

CURRICULUM VITAE

Laura Meulenbroek was born on September 27, 1985 in Den Dungen. After graduating from the Sint Janslyceum in 's Hertogenbosch, she started in 2003 with the study Pharmacy at Utrecht University. In 2007, she received her bachelor's degree, after which she continued with the research master Drug Innovation. During this master, she did an internship at the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University under supervision of Dr. Anneke van Houwelingen and Dr. Paul Henricks, in which she investigated the role of collagen fragments in rheumatoid arthritis. Her second internship was performed at the Department Kinetics & Metabolism, Business Unit Biosciences, TNO Quality of Life. During this internship, she investigated the inflammatory response in obesity under supervision of Michiel Balvers, Dr. Heleen Wortelboer and Prof. Dr. Renger Witkamp. In 2008, she received her master's degree and started with her PhD project at the Division of Pharmacology, in which she investigated T cell epitopes as a treatment for cow's milk allergy. Her project was performed in close collaboration with the Department of Dermatology/Allergology, University Medical Center Utrecht and the Department of Immunology, Danone Research Centre for Specialised Nutrition under supervision of Dr. Els van Hoffen, Dr. Léon Knippels, Prof. Dr. Carla Bruijnzeel-Koomen and Prof. Dr. Johan Garssen. During her PhD, she participated in the Utrecht Center for Food Allergy and was trained in immunology in the Infection and Immunity PhD program. In February 2013, she started as a post-doctoral fellow in the group of Jeanette Leusen at the Laboratory for Translational Immunology, University Medical Center Utrecht.