


## ORIGINAL ARTICLE

## Allergens

# Identification of peptides with tolerogenic potential in a hydrolysed whey-based infant formula

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**Summary**

**Background:** Failure to induce oral tolerance may result in food allergy. Hydrolysed cow's milk-based infant formulas are recommended in subjects with a high risk of developing allergic disease. Presentation of T cell epitopes is a prerequisite to generate regulatory T cells that could contribute to oral tolerance.

**Objective:** To investigate whether a specific hydrolysed whey-based infant formula contains peptides that function as T cell epitopes to support the development of oral tolerance to whey.

**Methods:** First, a novel liquid chromatography-mass spectrometry (LC-MS) method was developed to characterize  $\beta$ -lactoglobulin-derived peptides present in a specific infant formula with a focus on region AA#13-48 of  $\beta$ -lactoglobulin, which has previously been described to contain T cell epitopes with tolerogenic potential. Second, the formula was subjected to the ProImmune ProPresent<sup>®</sup> antigen presentation assay and MHC class II binding algorithm to identify relevant HLA-DRB1-restricted peptides. Third, identified peptides were tested on human cow's milk protein-specific T cell lines to determine T cell recognition.

**Results:** Thirteen peptides of minimal 9AAs long that overlap with AA#13-48 of  $\beta$ -lactoglobulin were identified. Six of them were found across all batches analysed. It was further confirmed that these peptides were processed and presented by human dendritic cells. The identified HLA-DRB1-restricted peptides were correlated to AA#11-30 and AA#23-39 of  $\beta$ -lactoglobulin. Importantly, the proliferation assay showed that the synthetic peptides were recognized by cow's milk protein-specific T cell lines and induced T cell proliferation.

**Conclusion and Clinical Relevance:** This study demonstrates that the tested hydrolysed infant formula contains functional HLA-DRB1-restricted T cell epitopes, which can potentially support the development of oral tolerance to whey.

Gouw and Jo are contributed equally.

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## KEYWORDS

food allergy, infant formula, oral tolerance, peptidomics, T cell epitope

## 1 | INTRODUCTION

Oral tolerance is the default response of the immune system to innocuous food proteins and is characterized by suppression of local and systemic immune responses to these proteins.<sup>1</sup> Failure to induce oral tolerance to food proteins may result in food allergy. While extensively hydrolysed and amino acid-based infant formulas are used to manage infants with cow's milk allergy, current international guidelines recommend the use of partially hydrolysed infant formula in infants with increased risk of developing allergic diseases, as a way to prevent the onset of food allergy, when breastfeeding is insufficient or not possible.<sup>2,3</sup>

In a recent clinical trial the preventive effect of a prebiotic supplemented partially hydrolysed whey-based infant formula (pHP) on the development of eczema in infants with increased risk of developing allergic diseases was investigated.<sup>4</sup> In this study, after 6 months of intervention, increased percentages of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>high</sup> regulatory T cells (Tregs) and CD11c<sup>low</sup>CD123w<sup>high</sup> plasmacytoid dendritic cells (DCs) have been detected in infants who received the pHP compared to the ones who received a standard infant formula. Both Tregs and plasmacytoid DCs play a pivotal role in oral tolerance development,<sup>1,5</sup> suggesting that the pHP supplemented with prebiotics might support the development of oral tolerance.

The foundation for all T cell responses, including the induction of Tregs, consists of three interconnected processes; T cell receptor activation, co-stimulation and cytokine signalling.<sup>6</sup> Activation of the T cell receptor on CD4<sup>+</sup> T cells is facilitated by the recognition of specific peptides, called T cell epitopes, presented by MHC class II molecules on antigen-presenting cells. The predominant isotype of MHC class II, HLA-DR, plays a central role in CD4<sup>+</sup> T cell selection and activation. HLA-DR is a heterodimer molecule comprised of HLA-DRA and HLA-DRB chains. While the *HLA-DRA* gene is highly conserved in humans, there are multiple *HLA-DRB* genes. Among those, *HLA-DRB1* is the most polymorphic gene<sup>7</sup> and it is expressed five times higher than its functional paralogs, ie, *HLA-DRB3*, *-DRB4* or *-DRB5*.<sup>8</sup> The presence of T cell epitopes in a pHP is essential for the induction of cow's milk protein-specific Tregs. Previously, we<sup>9</sup> and others<sup>10</sup> have identified T cell epitope-containing domains of  $\beta$ -lactoglobulin (BLG), the most abundant protein in whey and one of the major allergens in cow's milk. Amino acid (AA) #13-48 of mature BLG is of particular interest because we observed that synthetic peptides covering this region were able, in a preventive setting, to reduce the acute allergic skin response in a murine model for cow's milk allergy significantly.<sup>9</sup> Taken together, this region in BLG appears to be important for development of tolerance to whey antigens.

In order to determine the presence of specific sequences in biological samples, mass spectrometry (MS) is the method of choice as opposed to the more traditional techniques that are currently used to characterize protein hydrolysates.<sup>11,12</sup> A recent development in MS, termed peptidomics, allows for the characterization of peptide sequences with great sensitivity and specificity and is rapidly gaining popularity.<sup>13</sup> This technique is capable of closing the gap between understanding the impact of sequence specificity in relation to the biological activity a protein hydrolysate might have.

In this study, we used MS to identify peptides in a pHP similar to that was investigated in the previously published clinical trial<sup>4</sup> aimed to prevent food allergy. Moreover, we showed that the identified peptides are presented by HLA-DRB1 to and recognized by T cells suggesting that they could serve as functional human T cell epitopes and play a role in the development of oral tolerance.

## 2 | MATERIAL AND METHODS

### 2.1 | Hydrolysed infant formula

A mixture of acid whey concentrate and demineralised sweet whey is dissolved in water (purified by reversed osmosis) and afterwards hydrolysed under specific conditions. An established mixture of microbial endopeptidases and exopeptidases is used for hydrolysis. Subsequently, the solution is spray dried. The resulting hydrolysate powder (pHP) is used as sole protein source in infant formulas for children at risk for cow's milk allergy. Ten different batches were analysed. All batches were produced by the same method and taken during a period of 2 months.

### 2.2 | Sample preparation for MS analysis

Samples were essentially prepared as described by Butré et al<sup>14</sup> with the addition of a reduction and alkylation step. All chemicals were obtained from Sigma Aldrich (St. Louis, MO, United States). Briefly, the pHP batches were diluted to 0.5% (v/v) using 50 mmol/L ammonium bicarbonate followed by the reduction of peptides with 4 mmol/L DTT and alkylation with 8 mmol/L iodoacetamide. The mixture was cleared by centrifugation at 20 000 g for 10 minutes and diluted to 0.1% (v/v) using 0.1 mol/L acetic acid.

### 2.3 | Liquid chromatographic-mass spectrometry (LC-MS) analysis

All samples were analysed by nanoflow liquid chromatography using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, United States) coupled online to a LTQ Velos mass spectrometer

(Thermo Fisher Scientific, Waltham, MA, United States). The liquid chromatography part of the system was operated in a setup essentially as described previously.<sup>15</sup> Peptides were trapped at 5  $\mu\text{L}/\text{min}$  in 100% solvent A (0.1 mol/L acetic acid in water) on a 2-cm trap column (100- $\mu\text{m}$  inner diameter, packed in-house using Aqua C18, 5- $\mu\text{m}$  resin [Phenomenex, Torrance, CA, United States]) and eluted to a 20-cm IntegraFrit column (50- $\mu\text{m}$  inner diameter, ReproSil-Pur C18-AQ 3- $\mu\text{m}$ , New Objective, Woburn, MA, United States) at  $\sim 100$  nL/min in a 90-min gradient from 10% to 40% solvent B (0.1 mol/L acetic acid in 8:2 (v/v) acetonitrile/water). The eluent was sprayed via standard coated emitter tips (New Objective) butt connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full scan mass spectra (from  $m/z$  300 to 1,200) were acquired at zoom scan rate after accumulation to a target value of 3,000. The five most intense ions at a threshold above 500 were selected for collision-induced at normalized collision energy of 35% after accumulation to a target value of 10,000.

## 2.4 | MS data analysis

All MS data were processed by Proteome Discoverer (version 2.1, Thermo Scientific). Peak lists were generated using a standard workflow. Peptide identification was performed by searching individual peak lists of CID fragmentation spectra against a database containing selected bovine whey and casein proteins using Mascot (version 2.4.1, Matrix Science Ltd, London, United Kingdom). No enzyme was specified and no missed cleavages were allowed. Precursor ion mass tolerance was set to 0.2 Da and product ion mass tolerance to 0.5 Da. Carbamidomethylation (C) was set as fixed modification. All identifications relevant to this article were validated manually or by correlation to synthetic peptide fragmentation spectra. Synthetic peptides were obtained from JPT technologies. Only peptides containing at least nine AAs were taken into account, as this is the minimal size for binding to MHC class II molecules and subsequent T cell recognition.<sup>16</sup>

## 2.5 | ProlImmune ProPresent<sup>®</sup> antigen presentation assay

Identification of BLG-derived peptides presented by human DCs was performed by ProlImmune, as described earlier.<sup>17</sup> Peripheral blood mononuclear cell samples from 12 HLA-DR-typed healthy adult donors were obtained. The donors were selected based on common 11 HLA-DRB1 alleles (See Supporting Information Table S3). Immature monocyte-derived DCs were generated *in vitro* and matured in the presence of tested pHP. DCs were harvested and lysed in order to obtain HLA-DR complexes using a specific immunoaffinity method. Peptides were eluted from the HLA-DR complexes and subsequently analysed by high-resolution sequencing LC-MS/MS. The presence of six endogenous relevant proteins (ie, ITGAM, ApoB, CLIP, TFRC, FcER2/FcGR2 and LAMP-1/3) was assessed as a control for this assay. Each donor sample had to express a minimum of three

relevant proteins to be qualified for subsequent analysis. Donor P10 failed this screening, therefore was excluded. The resulting data of HLA-DR-restricted peptides were compiled and assessed using sequence analysis software referencing the Swiss-Prot Human Proteome Database with the incorporated test item sequences. The likelihood of peptides to be true positives is described by their expect value  $\leq 0.05$ . The false discovery rate was determined to be  $< 1\%$ .

## 2.6 | MHC Class II binding *in silico* assessment

The identified HLA-DR-restricted peptides were computed into the IEDB MHC Class II Binding Prediction software (<http://tools.iedb.org/mhcii/>) in order to assess binding of discovered peptides to the selected HLA-DRB1 alleles, as described.<sup>18,19</sup> The default IEDB recommended prediction method was selected. For each peptide sequence (15 mers long), a percentile rank was generated by comparing the peptide's score against the scores of five million random 15 mers selected from the Swiss-Prot database. A lower percentile rank indicates a higher affinity of peptide binding to a particular MHC class II allele, in which the IEDB recommends to make a selection based on a consensus percentile rank of the top 10%. In order to be more stringent with the *in silico* assessment, we made an arbitrary selection based on a consensus percentile rank of the top 3%.

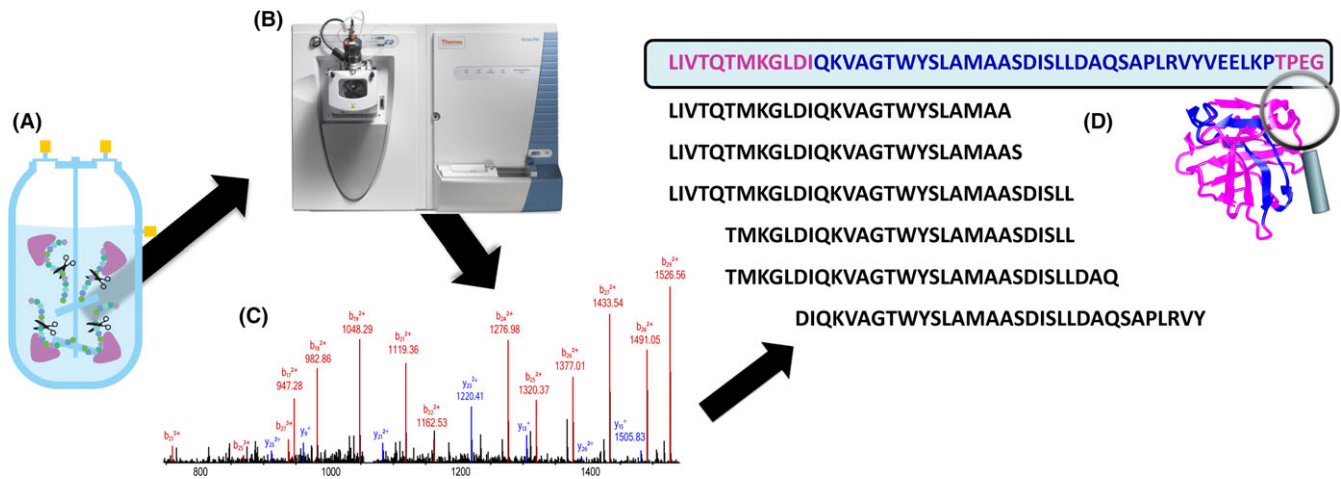
## 2.7 | T cell proliferation assay

Synthetic peptides (JPT Technologies, Berlin, Germany) identical to the peptides identified in pHP were dissolved in dimethylsulfoxide (Sigma Aldrich) at a concentration of 5.3 mmol/L. The peptides were tested on cow's milk protein-specific T cell lines (TCLs) from three infant donors, aged  $< 1$  year, 7.5 months and 6 years old. These TCLs were generated previously and have been shown to recognize epitopes in the region of interest (AA#13-48 of mature BLG).<sup>9</sup> Proliferation was determined as described previously.<sup>20</sup> Stimulation indexes (SIs, ratio between proliferation of allergen/peptide-stimulated and non-stimulated T cells) were calculated and a SI  $\geq 2$  was considered positive.

## 3 | RESULTS

### 3.1 | pHP characterization by MS

To determine the peptide sequences present in the specific pHP, 10 different commercial batches were analysed by LC-MS, which is shown schematically in Figure 1. Among the 314 peptides identified by this approach, 101 could be assigned to BLG leading to a sequence coverage of 90%. Most of the remaining peptides originated from other abundant cow's milk proteins such as  $\alpha$ -lactalbumin and serum albumin. In total, 13 BLG peptides of minimal nine AAs were identified in the region of interest (AA#13-48) (Figure 1 and Supporting Information Table S1). Six of them were identified consistently in all 10 batches. Unambiguous peptide identifications were



**FIGURE 1** Schematic overview of the applied LC-MS methodology. A specific partially hydrolysed infant formula (A) was analysed by mass spectrometry (B) and peptides were identified through database searching (C). In total six peptides were identified consistently in all 10 batches that mapped to BLG (structure is shown in pink and blue fonts) and located to the region of interest (shown in blue font) (D)

obtained by comparing characteristics (retention time, peptide mass and fragmentation spectrum) of the experimental peptide with its stable isotope labelled synthetic equivalent. An example is shown in Supporting Information Figure S1 where the fragmentation spectra of the experimental and synthetic peptide show excellent correlation. In this way, the identity of the six peptides found in all 10 batches (Figure 1) was confirmed.

### 3.2 | In vitro identification of HLA-DR-restricted peptides

The ProPresent<sup>®</sup> antigen presentation assay was performed on 12 healthy donors with known HLA-DRB1 types, in order to identify potential T cell epitopes within the pHP. By focusing on BLG-derived sequences, 15 relevant peptides with an expect value  $\leq 0.05$  were identified within five donor samples (See Supporting Information Table S2). These peptides can be further clustered into two unique sequence groups, ie, DIQ...DIS (AA#11-30) and AMA...APL (AA#23-39) (Table 1). Importantly, both sequence groups overlapped with the region of interest (ie, AA#13-48 of mature BLG). This finding demonstrated that BLG-derived peptides of interest can be presented by HLA-DR molecules on human DCs when incubated with a specific pHP.

Donor ID	DRB1_1	DRB1_2	Unique region (mature BLG)	Number of significant fragments
P2	*01:01	*04:01	AMAASDISLLDAQSAPL	2
P3	*01:01	*07:01	AMAASDISLLDAQSAPL	1
P4	*01:01	*04:05	DIQKVAGTWYSLAMAASDIS	3
P7	*03:01	*09:01	DIQKVAGTWYSLAMAASDIS	7
P12	*01:01	*04:04	AMAASDISLLDAQSAPL	2

**TABLE 1** HLA-DR allele association on unique region of mature BLG with significant fragments were defined by having expect value  $<0.05$

### 3.3 | In silico assessment of BLG-derived peptides' binding to specific HLA-DRB1 alleles

A limitation of the ProPresent<sup>®</sup> antigen presentation assay is the usage of a general anti-HLA-DR antibody, and not a specific antibody against a particular HLA-DRB1 allele (eg, anti-DRB1\*01:01 antibody), to isolate peptide-HLA-DR complexes of interest. Hence, with a donor with heterozygous genotypes of *HLA-DRB1*, eg, \*01:01 and \*04:01, it is uncertain which allele will present the identified peptide. We therefore utilized the MHC class II prediction software to assess whether fragments of the identified two unique sequence groups of BLG would have high affinity to bind to the selected HLA-DRB1 alleles of the characterized donors from the ProPresent<sup>®</sup> assay, ie, HLA-DRB1 \*01:01, \*03:01, \*04:01, \*04:04, \*04:05, \*07:01 and \*09:01.

As shown in Table 2, with an arbitrary threshold of percentile rank set at  $<3\%$  (ie, top 3% high binders), fragments of DIQ...DIS (AA#11-30) were predicted to have high affinity to bind to five HLA-DRB1 alleles, ie, DRB1\*01:01, \*04:01, \*04:04, \*04:05 and \*09:01. In contrast, it was estimated that only one fragment of AMA...APL (AA#23-39) would bind to HLA-DRB1\*07:01 with high affinity. Taken together, these findings suggest that fragments from AA#11-30 had a higher likelihood to be presented as T cell epitopes than the ones from AA#23-39. Moreover, the data suggest that several

**TABLE 2** In silico assessment on potential peptide-HLA-DRB1 complexes of two identified BLG-derived peptides with percentile rank <3%

HLA-DRB1 allele	Predicted 15-mer fragment of DIQVAGTWYSLAMAASDIS	Percentile rank
HLA-DRB1*09:01	AGTWYSLAMAASDIS	0.86
HLA-DRB1*04:05	AGTWYSLAMAASDIS	1.38
HLA-DRB1*04:05	VAGTWYSLAMAASDI	1.40
HLA-DRB1*09:01	VAGTWYSLAMAASDI	1.56
HLA-DRB1*04:05	KVAGTWYSLAMAASD	2.26
HLA-DRB1*01:01	AGTWYSLAMAASDIS	2.37
HLA-DRB1*04:04	AGTWYSLAMAASDIS	2.44
HLA-DRB1*09:01	KVAGTWYSLAMAASD	2.53
HLA-DRB1*04:01	AGTWYSLAMAASDIS	2.84
HLA-DRB1 Allele	Predicted 15-mer fragment of AMAASDISLLDAQSAPL	Percentile rank
HLA-DRB1*07:01	AASDISLLDAQSAPL	2.39

common HLA-DRB1 alleles could present fragments derived from the identified two BLG-derived unique sequence groups.

Of note, these unique sequence groups of BLG (AA#11-30 and #23-39) overlapped for the most part and the consensus sequence (ie, AA#11-39) correlated highly with one consistently identified peptide in the tested pH (AA#11-42; DIQ...RVY; Figure 1). We therefore subjected the sequence of AA#11-42 to the MHC class II prediction software which showed that fragments of this sequence also have high binding affinity (See Supporting Information Table S4). Importantly, the prediction results of AA#11-42 reconfirmed the results of two individual sequences, suggesting that both unique sequences of BLG could be derived from the AA#11-42 peptide that is confirmed to be present in the tested formula. Furthermore, parts of AA#11-42 were predicted to have high affinity to the same sets of identified HLA-DRB1 alleles, ie, DRB1\*01:01, \*04:01, \*04:04, \*04:05, \*07:01 and \*09:01. In conclusion, the in silico assessment confirms our in vitro findings that BLG-derived peptides can bind to common HLA-DRB1 alleles.

### 3.4 | Identified peptides induced proliferation of cow's milk protein-specific T cells

To confirm that the peptides identified in pH were recognized by T cells, synthetic peptides identical to the identified peptides were tested on cow's milk protein-specific human TCLs. As there was a distinct overlap between the identified peptides, we tested five peptides (Supporting Information Table S1, italic sequences) that were identified in all batches and differed by more than 1AA from each other. All tested peptides were able to induce proliferation and showed a concentration-dependent effect (Figure 2). However, each donor showed a different recognition pattern which was confirmed by the in silico prediction data (Supporting Information Table S5). All five peptides induced proliferation of TCL B suggesting that this TCL recognizes either the overlapping part of the peptides (AA#11-27) or

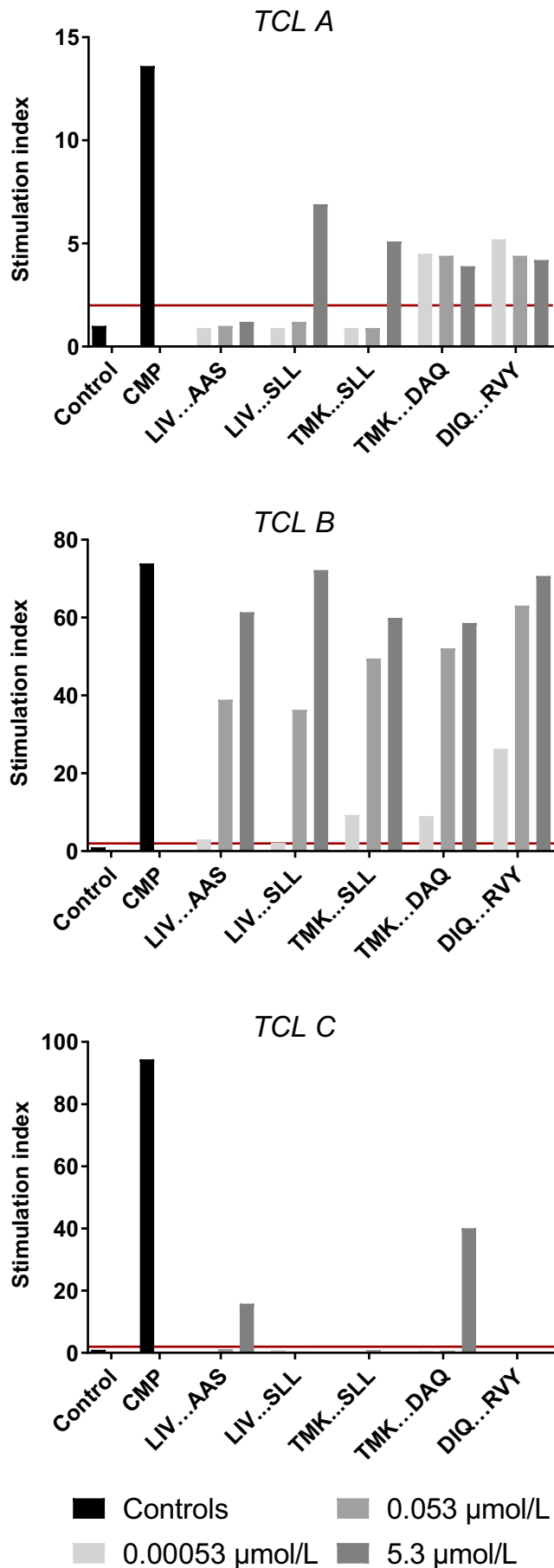
multiple T cell epitopes in this region. TCL A showed a proliferative response after stimulation with four of the five tested peptides. As peptide LIV...AAS (AA#1-27) was not able to induce proliferation, while peptide LIV...SLL (AA#1-32) did, the region containing AA#28-32 (DISLL) was essential for the response of TCL A. Unexpectedly, TCL C recognized peptide LIV...AAS (AA#1-27), while the longer peptide containing the same sequence with five additional AAs, LIV...SLL (AA#1-32), was not able to induce a proliferative response indicating that the structure of the peptide is essential for its ability to stimulate T cell proliferation.

## 4 | DISCUSSION

In this study, we analysed a specific pH to detect the presence of BLG-derived peptides of interest. Moreover, we have investigated the ability of human MHC class II molecules to present these specific BLG-derived peptides and proved that these peptides were functional indeed.

Currently, hydrolysed infant formulas are characterized mainly by methods that provide only information on the degree of hydrolysis and the peptide weight distribution.<sup>11,12</sup> Although important and informative, these characterizations do not provide any information about the sequence and specific activity of the peptides present in the formula. Especially, with the current understanding of sequence specificity in relation to immune modulation, structural information is crucial to understand the overall biological activity of a hydrolysed infant formula. The advantage of the applied methodology is that exact peptide sequences are identified which is important since many factors influence the generation of peptide sequences such as the protein source, used enzymes, hydrolysis time and temperature. However, depending on the applied sample preparation and/or analytical method, physicochemical properties of certain peptides may prohibit straightforward detection. Therefore, some peptides that are present in the sample might not be detected.

Six peptides that originated from a region in BLG known to contain T cell epitopes were identified in ten different production batches of a specific pH. Only two studies investigated the peptide profile of pH previously.<sup>21,22</sup> Although these studies focused on bioactive peptides in general and not specifically on T cell epitopes, none of the peptides we identified were found in these previously tested products. Català-Clariana et al<sup>22</sup> identified a related, but not identical peptide of 9AAs (AA#32-40) in our region of interest (AA#13-48). In addition to the profiles of these pHs, the peptide profile of a non-commercial BLG-based hydrolysate has been determined.<sup>23</sup> Also in this hydrolysate, two peptides (AA#21-40 and AA#25-40) that overlap with our region of interest were identified but none of these peptides had a similar sequence as found in our study. In two of the three previous studies, a hydrolysate from a different manufacturer was investigated.<sup>21,23</sup> The main reason for different peptide sequences found could be the manufacturing processes as indicated above. This is further corroborated by a recent study in which extensively hydrolysed infant formulas from



**FIGURE 2** T cell responses after stimulation with the identified peptides. Cow's milk protein-specific T cell lines (TCLs) of three different donors were stimulated with synthetic equivalents of the identified peptides. Cow's milk protein (CMP, 50  $\mu\text{g/mL}$ ) was taken along as control. A stimulation index  $\geq 2$  was considered significant. The figures are representative examples of two/three experiments

different manufactures were characterized using peptidomics. These authors showed that infant formulas from different manufactures had a distinct signature based on their peptide profile and therefore might have a different effect in clinical trials.<sup>24</sup>

Subsequent to the peptide identification process, we determined whether and which peptides within the tested pHp were presented by HLA-DRB1 molecules. The HLA-DRB1 was prioritized in this study due to the facts that the HLA-DR molecule is the most predominant human MHC class II isotype (>90%)<sup>25</sup> and that *HLA-DRB1* gene locus is polymorphic, while *HLA-DRA1* gene locus is monomorphic (ie, *HLA-DRB1* genotype determines the whole HLA-DR molecule).<sup>26</sup> Furthermore, HLA-DRB1 allele is expressed five times higher than its paralogs (HLA-DRB3, -DRB4 or -DRB5) and is present in all individuals.<sup>8</sup> Two sequence groups (AA#11-30 and AA#23-39) were identified to bind to several HLA-DRB1 molecules. As most donors were heterozygous for HLA-DRB1, the sequences were subjected to the MHC class II binding algorithm to determine which of the two molecules was able to present the peptides with high affinity (top 3%). Importantly, several in silico assessments confirmed the in vitro ProPresent<sup>®</sup> findings on the peptide-HLA-DR complexes, eg, DRB1\*09:01 with AGT...DIS or DRB1\*04:05 with VAG...SDI. In parallel, we demonstrated also that no fragment from the identified two unique BLG sequence groups could bind to HLA-DRB1\*03:01 with high affinity, corroborating the ProPresent<sup>®</sup> result on donor P1. Donor P1 was homozygous for HLA-DRB1\*03:01 and this donor did not present any BLG-derived peptide in the ProPresent<sup>®</sup> assay, suggesting that DRB1\*03:01 did not have enough affinity to bind to the BLG region of interest. Similar arguments could be applied for donors P6 (DRB1\*13:02/\*14:01) and P11 (DRB1\*03:01/\*15:01) as well, in which both donors had HLA-DRB1 alleles that did not bind to BLG-derived peptides with high affinity (data not shown). The remaining results for donors P5 (DRB1\*04:01/\*07:01), P8 (DRB1\*11:01/\*15:01) and P9 (DRB1\*03:01/\*15:01), however, could not be easily explained. These donors had at least one DRB1 allele that in silico could bind to BLG-derived peptides with high affinity, but the prediction was not confirmed by the ProPresent<sup>®</sup> result. The discrepancy could be of methodological origin such as limitations of peptide detection by MS or the tendency of in silico assessments to be overpredictive. Alternatively, aforementioned differences could have a biological cause due to the complexity of MHC class II gene expression.<sup>27</sup> There is a possibility that various HLA-DRB1 alleles might be regulated differently in their expression, resulting in different levels between alleles.<sup>28</sup> Another possibility is the presence of non-classical MHC class II proteins (HLA-DO and HLA-DM) that might modulate the peptide presentation by HLA-DRB1 alleles.<sup>29</sup> Of

note, each tested donor in the ProPresent<sup>®</sup> assay had a unique combination of DRB1 alleles, thus we could not confirm nor refute whether the discrepancy between in silico prediction and ProPresent<sup>®</sup> results (of donor P5-P8-P9) could be generalized to all subjects with the exact same DRB1 allele combination. Therefore, it is prudent to combine both in vitro and in silico approaches in order to identify peptide-MHC class II complexes.

In this study, we predicted that six DRB1 alleles (\*01:01, \*04:01, \*04:04, \*04:05, \*07:01 and \*09:01) could present BLG-derived peptides of interest. As only 11 common HLA-DRB1 alleles were tested, this study was not exhaustive in investigating all possibilities for MHC class II-restricted presentation of BLG-derived peptides. It is possible that the untested HLA-DRB1 alleles, but also HLA-DRB3/4/5, HLA-DP or HLA-DQ molecules, could present BLG-derived peptides of interest. Supporting evidence comes from a study demonstrating that HLA-DQ molecules presented BLG peptides to T cells from a cow's milk allergic patient.<sup>10</sup> Moreover, we only investigated peptides detected in a specific region of interest thus far, whereas previous studies have shown that other regions of BLG also contain T cell epitopes.<sup>9,10</sup> Together this suggests that the MHC class II coverage on presentation of BLG-derived peptides present in the tested pHP might be broader than our current finding.

To confirm that the peptides identified in pHP were recognized by T cells, we tested them on cow's milk protein-specific TCLs and showed that all five peptides tested were indeed able to induce proliferation. It is obvious, however, that not all donors recognized all tested peptides. As each donor expressed a different panel of HLA-DRB1 alleles, these findings support the importance of MHC class II profiles of each individual for presenting peptides of interest to CD4<sup>+</sup> T cells. In silico prediction results confirmed that HLA-DRB1 alleles (\*11:01 & \*04:04) expressed by donor B were able to present fragments of all tested peptides (Supporting Information Table S5). For TCL C, all known HLA-DRB\*03 and \*13 alleles were tested in the algorithm as the genotyping was incomplete and the results indicated that some of the HLA-DRB\*13 alleles were able to present fragments of the tested peptides. However, there are too many uncertainties to make convincing conclusions from these data. TCL A recognizes an overlapping fragment from two tested peptides, ie, TMK...DAQ (AA#6-35) and DIQ...RVY (AA#11-42), which is supported by the algorithm data. In contrast, this TCL was able to recognize two additional tested peptides that were not predicted by the algorithm, namely LIV...SLL and TMK...SLL. However, these proliferative responses were observed upon stimulation with the mentioned peptides at the highest concentration only (Figure 2). One possible explanation is that since our data were primarily based on HLA-DRB1 alleles, other MHC class II molecules (eg, HLA-DRB3/4/5 or HLA-DP or HLA-DQ) could present those tested peptides to T cells and further stimulate them. Alternatively, a lower binding affinity could explain why the peptides were not identified by the algorithm and why stimulation occurs only at the highest concentration. For instance, the prediction results for TCL A show recognition of an overlapping fragment from the two peptides in question, LIV...SLL and TMK...SLL, but at a percentile rank of 4.4% which was just

above the threshold of 3% and as a result was not considered. Even though the affinity of these peptides is lower they could induce stimulation when the concentration of the peptide is high enough. Further research should confirm this.

Several published studies support a notion that dietary antigens (including peptides) within the intestinal lumen can be absorbed and subsequently captured and presented by intestinal antigen-presenting cells, ie, antigen sampling mechanisms, without the need of a disrupted intestinal barrier. There are at least two distinct mechanisms that continuously work to sample dietary antigens at the healthy state, ie, microfold/M cell-mediated transcytosis<sup>30</sup> and goblet cell-associated antigen passage.<sup>31</sup> Both pathways provide dietary antigens to intestinal lamina propria CD103<sup>+</sup> DCs, which in return could imprint gut-homing molecules on T and B cells, could promote differentiation of intestinal IgA-producing plasma cells as well as could generate and activate intestinal Tregs, a key player in the state of hyporesponsiveness to fed antigens known as oral tolerance.<sup>32-34</sup> This suggests that peptides within the tested pHP can be absorbed after oral intake, followed by antigen capture and presentation by intestinal CD103<sup>+</sup> DCs and subsequently culminated in generation of functional Tregs. Previously, we have shown in a murine cow's milk allergy model that administration of the same pHP prior to induction of cow's milk allergy increased the percentage of Tregs in the mesenteric lymph nodes leading to a significantly reduced acute allergic skin response to whey.<sup>35</sup> Moreover, a similar result was seen when synthetic peptides were used for this preventive treatment<sup>9</sup> indicating that the peptides reached the right compartments in the intestine for inducing oral tolerance.

It is imperative that for the development of oral tolerance, T cell epitopes within the tested pHP need to be presented under the right circumstances. As indicated before, in addition to TCR activation, co-stimulation and cytokine signalling play an important role in the generation of Tregs.<sup>6</sup> For example, retinoic acid and TGF- $\beta$  are required for the induction of Tregs. Previously, we have shown that pre- or synbiotics may play an important role in creating the right environment for this predisposition towards Tregs.<sup>9,35-37</sup> The preventive effect of both pHP and synthetic peptides was strengthened in combination with a pre- or synbiotic diet in a murine model for cow's milk allergy. Moreover, clinical evidence for the support of oral tolerance was provided by increased levels of plasmacytoid DCs and Tregs upon nutritional intervention with the pHP supplemented with prebiotics.<sup>4</sup>

In conclusion, this study demonstrated for the first time that a specific pHP contains functional human T cell epitopes. We suggest that this pHP under the right circumstances may stimulate the development of oral tolerance to whey and thereby contribute to allergy prevention.

## CONFLICTS OF INTEREST

JG is head of the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science at the Utrecht University and partly employed by Nutricia Research. LM, SH, JJ, ES, PJ

and LK, as indicated by the affiliations, are part of a strategic alliance between Utrecht University and Nutricia Research and are employed by Nutricia Research. JWJ, EK and AR are employed by Nutricia Research. The other authors declare no further conflicts of interest.

## AUTHOR CONTRIBUTIONS

JWJ, JJ and LM designed and performed experiments, analysed the data and wrote the manuscript. SH performed experiments and analysed the data. EK, ES, PJ and AR were involved in the investigation and revision of the manuscript. AK provided cow's milk protein-specific human T cell lines. JG, LK designed the study and wrote the manuscript. All authors read and approved the manuscript.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Gouw JW, Jo J, Meulenbroek LAPM, et al. Identification of peptides with tolerogenic potential in a hydrolysed whey-based infant formula. *Clin Exp Allergy*. 2018;48:1345-1353. <https://doi.org/10.1111/cea.13223>