

ORIGINAL ARTICLE

Multiple T cell epitope peptides suppress allergic responses in an egg allergy mouse model by the elicitation of forkhead box transcription factor 3- and transforming growth factor- β -associated mechanisms

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Summary

Background Peptide-based immunotherapy (PIT) represents an attractive approach for targeted interventions in immunological disorders, but has not been widely explored in the context of food allergy.

Objective In this study, we built on the information obtained from the recent identification of three immunodominant T cell epitopes of hen ovalbumin (OVA), a major egg allergen, to assess the therapeutic potential of PIT for food allergy, using the BALB/c mouse model.

Methods Groups of mice were sensitized to OVA by repeated oral gavages, and subsequently administered with single or multiple synthetic peptides containing OVA T cell epitopes.

Following the peptide administration period, all mice were orally challenged with high doses of OVA to elicit active anaphylaxis. Serum, spleen, and intestinal tissues were collected for the determination of immunoglobulin levels, cytokine secretions, and intestinal gene expression.

Results Significantly lower anaphylactic scores were exhibited by mice that received multiple epitope-containing peptides, accompanied by lower serum histamine and OVA-specific IgE levels, compared with placebo-treated mice. Mechanistically, the quantification of cytokine secretions in splenocyte cultures revealed a T helper type 1-biased response (IFN- γ) in all peptide-treated mice to the detriment of a T helper type 2-response (IL-4). Interestingly, a similar cytokine expression profile was determined in intestinal tissues, accompanied by a pronounced mRNA expression of regulatory molecules TGF- β and forkhead box transcription factor 3 (FOXP3). These data suggest the activation of local repressive mechanisms mediated by subsets of regulatory T cells.

Conclusion We demonstrated the therapeutic potential of PIT in a mouse model of food allergy model and provided evidence that mechanistic pathways entailing regulatory molecules TGF- β and FOXP3, stand as promising trails for the further understanding of peptide-based strategies for food allergy.

Keywords BALB/c mouse, food allergy, FOXP3, ovalbumin, peptide-immunotherapy, T cell epitope, TGF- β

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Introduction

Conventional immunotherapy using whole allergens is burdened with the risks of inducing severe side-effects related to the presence of multivalent B cell epitopes and the cross-linking of IgE at the surface of mast cells and basophils. Approaches that alter or bypass the IgE cross-linking activity provide, therefore, a scope for the development of novel strategies; peptide-based immun-

otherapy (PIT) represents one of them. The strategy presents several advantages based on the criteria that the production of synthetic peptides has today become a low-cost routine exercise [1] and that it offers an alternative to the diligent task of producing chemically or genetically engineered proteins, while maintaining the precision and specificity of treatment [2, 3]. To date, several inhalant allergens have been the object of PIT investigations: the major cat allergen Fel d 1, the bee venom allergen Api m 1

[2], and more recently the ragweed allergen Amb a 1 [4] and the dog allergen Can f 1 [5]. The most promising results were obtained with cat allergens, and have been recently reviewed [4, 6]. In the context of food allergy, even though the approach has been praised as a promising therapeutic strategy in a number of reviews [7–10], there is virtually no supportive information on the curative potential of PIT in dietary allergies. Almost a decade ago, one *in vitro* study showed that peptides derived from the peptic digestion of peanut allergens, which contained T cell epitopes but no IgE-binding epitopes, could preferentially induce the production of IFN- γ , a T helper (Th) type 1-biased cytokine, in a concentration-dependent manner [11]. A recent review reported unpublished results describing that the repeated subcutaneous administration of overlapping peptides, covering the entire sequence of the major peanut allergen Ara h 2, led to a significant reduction of specific IgE and histamine levels in peanut-sensitized C3H/HeJ mice, compared with placebo-treated mice. These changes were accompanied by lower anaphylaxis scores and an increased production of IFN- γ by splenocytes stimulated *in vitro* [8]. However, to the best of our knowledge, no further investigations were reported.

In recent years, the existence of a population of regulatory T cells (T_{reg}) has been increasingly recognized and has shifted focuses in allergy research toward the exploration of immunotherapies inducing active regulation [12]. It has in fact been suggested that the administration of T cell epitope-containing peptides could elicit such regulatory effects [4]; however, the nature of the underlying mechanisms remains yet to be elucidated. The present study aimed at exploiting the recently reported T cell epitope map of ovalbumin (OVA) [13] for the assessment of PIT in a mouse model of food allergy using the commonly used BALB/c strain. More specifically, synthetic peptides comprising immunodominant T cell determinants of OVA, a major egg allergen, were administered to orally sensitized BALB/c mice via subcutaneous injections. Measurements of anaphylactic scores, serum and fecal immunoglobulin levels, cytokine secretions and intestinal gene expression ratios were conducted to shed

light on the immunomodulatory and/or the allergy-suppressive effects induced.

Materials and methods

Design of epitope-containing synthetic peptides

We recently reported the identification of novel T cell epitopes of OVA in the BALB/c mouse [13]. In that study, three peptide sequences were documented as immunodominant T cell determinants i.e. peptide fragments A39R50, S147R148 and A329E340, and were selected for the development of a PIT mouse model for food allergy. Fifteen amino acid synthetic peptides, respectively, labelled A39I53 (AI-15), S147L161 (SL-15) and A329G343 (AG-15) were designed to contain one immunodominant T cell epitope of OVA (Table 1). The 15-mer peptides were purchased from a manufacturer (GL Biochem, Shanghai, China) with a purity >85% and their sequences were confirmed by mass spectrometry (MALDI-TOF) analyses (quality control). The optimal peptide length was determined primarily based on the binding characteristics defined for BALB/c major histocompatibility complex (MHC) (H2^d) class II molecules [14] and based on previous studies, which have documented that short linear synthetic peptides with sizes of 16–17 residues promoted a presentation to T cells in a non-immunogenic manner (e.g. absence of antigen-processing or costimulatory signals) and exhibited negligible IgE cross-linking activity [3, 15, 16]. In clinical studies, the use of peptides longer than 20 amino acids has, in fact, been associated with adverse effects in the patients [2].

Allergen sensitization and synthetic peptide administration

Female BALB/c mice were purchased from Charles River Laboratories (Montreal, QC, Canada) at 6–8 weeks of age and randomly divided into six groups (six mice per group). All animals were housed in the campus animal facility at the University of Guelph under an egg-free diet

Table 1. Design of OVA synthetic peptides for development of PIT for food allergy in the BALB/c mouse model

OVA immunodominant T-cell determinants			Synthetic peptide constructs for PIT design		
Name	Primary sequence	OVA region	Name	Primary sequence	OVA region
AR-12	AMVYL GAKD STR	39–50	AI-15	AMVYL GAKD STR TQI	39–53
SR-12	SWVESQTNGI IR	147–158	SL-15	SWVESQTNGI IR NVL	147–161
AE-12	AAHAEINEAG RE	329–340	AG-15	AAHAEINEAG RE VVG	329–343

Amino acid sequences of three OVA T-cell determinants (AR-12, SR-12 and AE-12), identified as immunodominant, are presented in the left column. Each of the T-cell epitope sequences was elongated by an additional three amino acids (indicated in bold) corresponding to the three residues adjacent to their last C-terminal residue as found in the OVA native sequence, and are detailed in the right column. The resulting 15-mer peptide sequences AI-15, SL-15 and AG-15 were ordered with purity greater than 85%, and administered to BALB/c mice orally sensitized to OVA. OVA, ovalbumin; PIT, peptide-based immunotherapy.

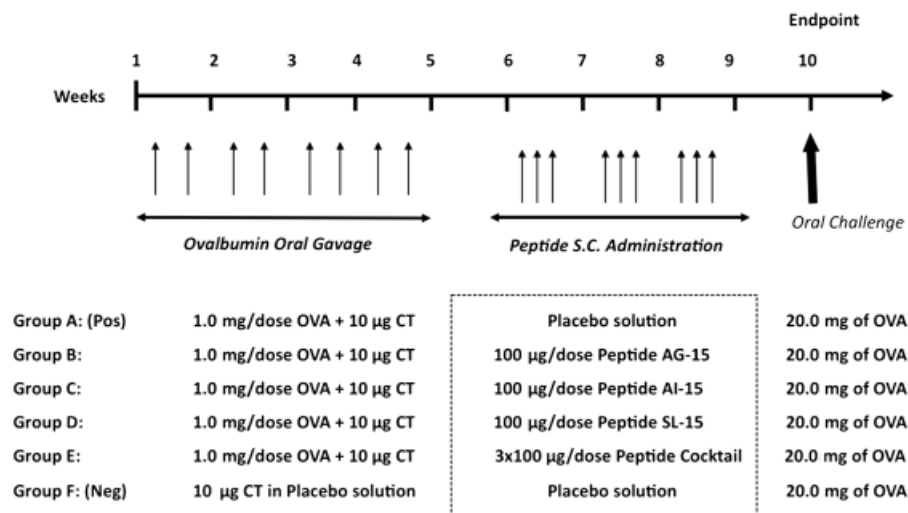


Fig. 1. Mouse experimental protocol. CT, cholera toxin; OVA, ovalbumin; PBS, phosphate-buffered saline; SC, subcutaneous.

[Teklad global diet, 14% protein (wheat and corn) and 3.5% fat] in a 12 h lighting cycle. Food and water were available *ad libitum*. All procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care (CCAC) and approved by the Animal Care Committee at the University of Guelph.

Following a 1-week acclimatization period, mouse groups A, B, C, D and E were sensitized by oral gavage at a dose of 1.0 mg of HPLC-purified OVA and 10 µg of cholera toxin (List Biologicals, Denver, CO, USA), twice per week for a duration of 4 weeks. Groups B, C and D were subsequently administered 100 µg/mouse of a single synthetic peptide in sterile phosphate-buffered saline (PBS), three times weekly for a period of 3 weeks by subcutaneous injection i.e. peptides AG-15, AI-15 and SL-15, respectively. On the other hand, group E received a mixture of the three peptides (or peptide cocktail) following an identical injection schedule and each dose of peptide cocktail contained a mixture of peptides AI-15, SL-15 and AG-15 at an equal amount of 100 µg each. Mice comprised in group A served as positive controls, and following the sensitization period with OVA, the animals were placebo-treated with a PBS solution devoid of peptides. Mice comprised in group F served as negative controls and were both sham-sensitized and placebo-treated (Fig. 1).

Assessment of systemic anaphylactic signs

To elicit active anaphylaxis, all mice were orally challenged with a high dose of OVA following the peptide administration period i.e. 20.0 mg/mouse in a sterile saline solution. Anaphylactic responses were evaluated for approximately 30 min after the challenge by three independent and blind observers, according to a scoring system described previously for the determination of IgE-

mediated responses in mouse models of food allergy [17]. Briefly, score 0 was assigned if no symptoms were observed; score 1 represented scratching and rubbing around the snout and head; score 2 for puffiness around the eyes and snout, diarrhoea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate; score 3 for wheezing, laboured respiration, cyanosis around the mouth and the tail; score 4 for no activity after prodding, or tremor and convulsions; and at last, score 5 was assigned when death of the animal occurred.

Serum histamine and immunoglobulins analyses

Following the final oral challenge, whole blood was collected by cardiac puncture and two serum samples were pooled in equal volumes within each group, a total of three pooled serum samples per group due to limited volume in individual mouse serum for the following ELISA assays. Histamine concentrations were assayed using a commercial ELISA kit (Labor Diagnostika Nord, Nordhon, Germany) following the manufacturer's instructions. Specific IgE and IgG were determined according to an ELISA procedure described previously [18, 19].

Real-time reverse-transcription polymerase chain reaction analyses

Following the final oral challenge, mouse ileums from preselected groups (A, C, E and F) were freshly and individually isolated ($n = 6$ samples per group), and immediately placed in ice-cold RNA later[®] solution (Sigma-Aldrich, St. Louis, MO, USA) followed by storage at -30°C . Total RNA was extracted from individual mice (30 mg of tissue/mouse) using the spin column procedure from RNeasy kits (Qiagen Inc., Valencia, CA, USA). Total RNA integrity was assessed on 1% agarose gels, and the

respective concentration and purity were determined by measuring the absorbance ratios A260/A280. Complementary DNA (cDNA) was synthesized from 1.0 µg/mouse of total RNA using Quantitect Rev kit (Qiagen Inc.), following the manufacturer's instructions. Real-time fluorescence-monitored PCR reactions were performed using an iCycler (Bio-rad Laboratories Inc., Hercules, CA, USA) detection system. The temperature profile was 95 °C for 15 min, then 15 s at 95 °C (denaturation), 56 °C for 15 s (annealing) and 72 °C for 30 s (extension), repeated for 45–50 cycles. The efficiency of all qPCR reactions (primer pairs) was between 90% and 110% as per standard PCR primer design parameters. All data were normalized to the house-keeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the relative mRNA expression ratios were determined using the $2^{-\Delta C_t}$ (threshold cycle) method [20]. The sequences of the primers used for real-time RT-PCR analyses are presented in Table 2.

Mouse spleen cell cultures and determination of cytokine secretion

At the experimental end-point, following the final oral challenge, spleens from individual mouse were aseptically removed into ice-cold RPMI-1640 medium (Gibco Invitrogen, New York, NY, USA), containing sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL), and two whole spleens were pooled

within each group and made samples per group. Single cell suspensions were prepared as described previously [21] and cell viability was assessed by trypan blue exclusion. Cells were cultured in 24-well plates (Corning Inc., Corning, NY, USA) at a density of 2.5×10^6 /mL in the absence (negative control wells) or presence of purified OVA (100 µg/mL). Supernatants were collected after 72 h of culture in a 5% CO₂ humidified incubator, and assayed for the presence of cytokines IFN-γ (Th1-biased) and IL-4 (Th2-biased), in triplicate wells, using antibody reagents purchased from commercial kits (BD Pharmingen, Mississauga, Canada) following the manufacturer's instructions.

Determination of ovalbumin-specific immunoglobulin A in mouse fecal pellets

Mouse fecal pellets were freshly collected on a weekly basis from each mouse group cage, and were submitted to the following extraction procedure. In brief, a mass of 1.0 g of fecal pellets were added to 7.5 mL of PBS buffer pH 7.4 and homogenized for 2 × 30 s using a rotor stator homogenizer (Polytron® model PT-2000; Kinematica, Luzern, Switzerland). Samples were subsequently centrifuged at 500 g for 20 min and the supernatants were carefully passed through a 0.45 µm diameter syringe filter. Concentrations of OVA-specific IgA were determined using an indirect ELISA procedure. Flat-bottom 96-well ELISA plates (Corning Inc.) were incubated with 100 µL/well of purified OVA (5 µg/well) in 100 mM of sodium bicarbonate buffer (pH 9.5) and stored overnight at 4 °C.

Table 2. Sequences of primers used for real-time RT-PCR analyses

Mouse gene	Sequence (5'–3')	T_m value	PCR product size (bp)	Accession number
IL-4	FP: CCTCACAGCAACGAAGAACA	60.02	155	NM_021283
	RP: ATCGAAAAGCCCGAAAGAGT	60.21		
IL-5	FP: GAAGTGTGGCGAGGAGAGAC	59.99	177	NM_010558
	RP: GCACAGTTTGTGGGGTTTT	59.88		
IL-13	FP: CAAGACCAGACTCCCTGTG	60.70	151	NM_008355
	RP: GGTACAGAGGCCATGCAAT	59.96		
IL-12p40	FP: TCAGGGACATCATCAAACCA	59.96	160	NM_008352
	RP: TTTTCTTTCTTGCCTGGAT	59.99		
IL-10	FP: GCCTTATCGGAAATGATCCA	59.86	151	NM_010548
	RP: AGGGGAGAAATCGATGACAG	59.09		
IL-18	FP: TTGCTTGCCAAAAGGAAGAT	59.82	121	NM_008360
	RP: CAAACCCTCCCACTAACT	60.22		
IFN-γ	FP: GCTCTTCCTCATGGCTGTTT	59.43	154	NM_008337
	RP: GTCAACATCCTTTGCCAGT	59.97		
TGF-β1	FP: TTGCTTCAGCTCCACAGAGA	59.86	156	NM_011577
	RP: TACTGTGTGTCAGGCTCCA	60.31		
FOXP3	FP: TCCTTCCAGAGTTCTTCCA	59.77	157	NM_054039
	RP: CGAACATGCGAGTAAACCAA	59.73		
GAPDH	FP: AACCTTGGCATTGTGGAAGG	59.97	132	NM_008084
	RP: GGATGCAGGGATGATGTTCT	59.89		

FP, forward primer; RP, reverse primer; FOXP3, forkhead box transcription factor 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T_m , melting temperature.

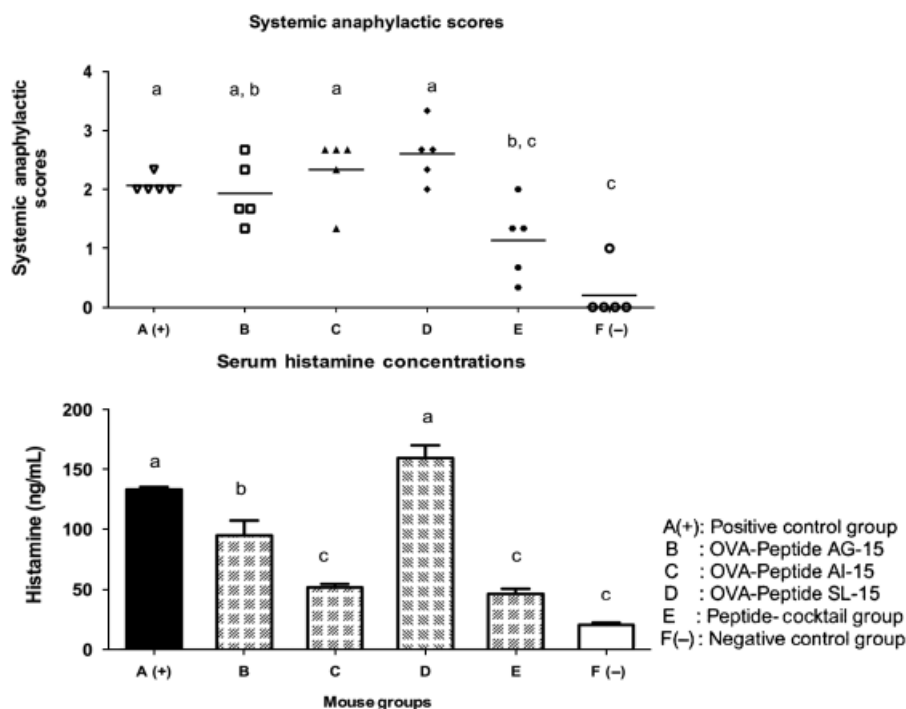


Fig. 2. Systemic anaphylactic scores (a) and serum histamine levels (b). Histamine: data are represented as mean $\pm$ standard deviation ($n = 3$ pooled sera). Anaphylactic scores: each triangle stands for an individual mouse, and represents the average of scores assigned by three independent and blind observers. Different letters indicate statistically significant differences ($P < 0.05$), between groups of mice.

Plates were subsequently washed four times with 200 μ L/well of PBST, and blocked with 200 μ L/well of 1% bovine serum albumin (BSA) in PBS and incubated for 2 h at 37 $^{\circ}$ C. An additional four washes were performed (200 μ L/well of PBST), and 100 μ L/well of filtered fecal extracts diluted in 1% BSA in PBST (1 : 5) were added to the plate in triplicate wells for 2 h incubation at 37 $^{\circ}$ C. The plates were washed four times with PBST and incubated with 100 μ L/well of biotinylated monoclonal anti-mouse IgA (1 : 500; BD Pharmingen) diluted in 1% BSA in PBST for 1 h at 37 $^{\circ}$ C. The wells were further washed four times with 200 μ L/well of PBST, and 100 μ L/well of avidin-horse radish peroxidase (HRP) conjugate (1 : 2000; BD Pharmingen) was applied to the plate for 30 min incubation at 37 $^{\circ}$ C. After a final four-wash cycle with PBST, OVA-specific IgA binding activity was revealed by the addition of 100 μ L/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (ready-to-use solution; Sigma-Aldrich). The reaction was terminated after 20 min by the addition of 50 μ L/well of 2 N H_2SO_4 , and the absorbance values were determined at 450 nm using an ELISA microplate reader (BioRad Laboratories).

Statistical analysis

Histamine, immunoglobulin and cytokine concentrations measured by ELISA assays were subjected to ANOVA analyses followed by *post hoc* multiple comparison using

Tukey's test. Statistical differences in the expression ratios determined by real-time RT-PCR analyses were assessed using the Kruskal-Wallis test, followed by Dunn's *post hoc* test. In all cases, P -values < 0.05 were considered statistically significant. Statistical calculations were performed using the GraphPad Prism[®] 5.0 package (GraphPad Software Inc., San Diego, CA, USA).

Results

Decreases in hypersensitivity reactions and serum histamine release

We asked whether the severity of anaphylactic responses was positively correlated with serum histamine levels, as suggested by previous reports [18, 22]. Compared with placebo-treated mice (group A), animals administered with a mixture of epitope-containing peptides, developed significantly lower hypersensitivity reactions, and the scores were in fact statistically comparable with sham-sensitized mice (group F). Interestingly, serum histamine levels in group E were indeed significantly inferior (two- to three-fold difference) compared with placebo-treated mice (Fig. 2). On the other hand, mice comprised in groups B, C and D, treated with a single peptide, exhibited severe anaphylactic signs (reaching up to level 4). Serum histamine levels were divergent in these latter groups; while group B (peptide AG-15) and group C (peptide AI-15)

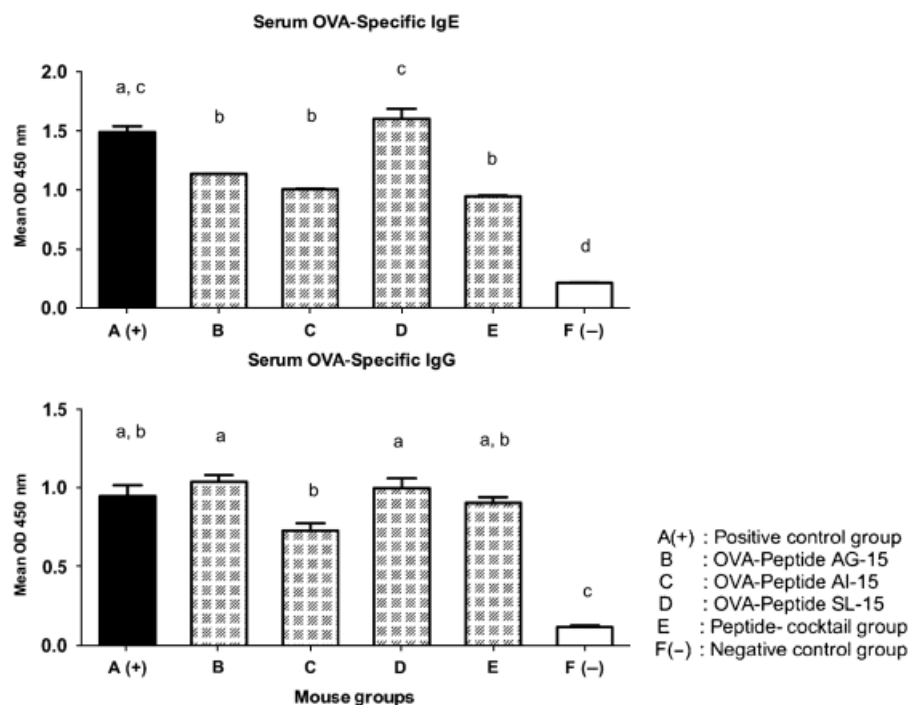


Fig. 3. Ovalbumin (OVA)-specific IgE (a) and OVA-specific IgG (b) levels in OVA-sensitized BALB/c mice after repeated subcutaneous administration of T cell determinant containing-peptides. Data are represented as mean $\pm$ standard deviation ($n = 3$ pooled sera). Different letters indicate statistically significant differences ($P < 0.05$), between groups of mice.

showed declined serum histamine levels, higher histamine concentration was determined in sera obtained from group D (peptide SL-15). Variations in group D remain, however, in line with the observation that this group was assigned the highest anaphylactic scores (Fig. 2).

Significant reduction of specific immunoglobulin E; absence of variation in specific immunoglobulin G levels

Between-group variations obtained for OVA-specific IgE levels (Fig. 3a) resemble the variation pattern described above with serum histamine concentrations (Fig. 2b). More specifically, groups B, C and E showed lower titres in both OVA-specific IgE and serum histamine. On the other hand, group D tended to show greater specific IgE titres, which corroborates with the elevated titres of serum histamine and the anaphylactic scores described earlier (Fig. 2). This brings support to the fact that in the BALB/c mouse model, there may be a positive correlation between the levels of specific IgE and the severity or risk of developing an anaphylactic response.

Compared with placebo-treated mice, no significant variation of OVA-specific IgG was observed in any peptide-treated group (Fig. 3b). These results tend to rule out the possibility that the production of the so-called 'blocking IgG' [23] may be involved in the immunomodulatory mechanisms induced by our PIT design. In sham-sensi-

tized mice (group F), none or negligible OVA-specific IgE or IgG titres were detected.

Augmentation of fecal-specific immunoglobulin A upon oral administration of T cell determinants

Our interest in the determination of OVA-specific IgA is based on reports showing that low or decreased levels of allergen-specific IgA were associated with the development of food allergies in humans [24, 25], as well as in experimental mouse models [26]. Our results indicate that the peptide injection period (starting at week 6) coincided with a gradual but robust increase in fecal OVA-specific IgA, particularly prominent in group C (peptide AI-15), as well as in group E (Cocktail) (Fig. 4). A significant augmentation was detected as early as seven days after starting the administration of peptides in group C; whereas in group E, levels of specific-IgA started to peak at 15 days post-peptide administration. These elevations in specific IgA levels may be associated with earlier observations that groups C and E were the only two groups showing distinct reduction in histamine and specific IgE levels (Figs 2 and 3). Interestingly, unlike single peptide-treated groups, a substantial rise in specific IgA was observed in group E (cocktail) during the sensitization period. This latter observation suggests that a higher *a priori* production of specific IgA at the mucosal level might have contributed to the allergy-suppressive effect

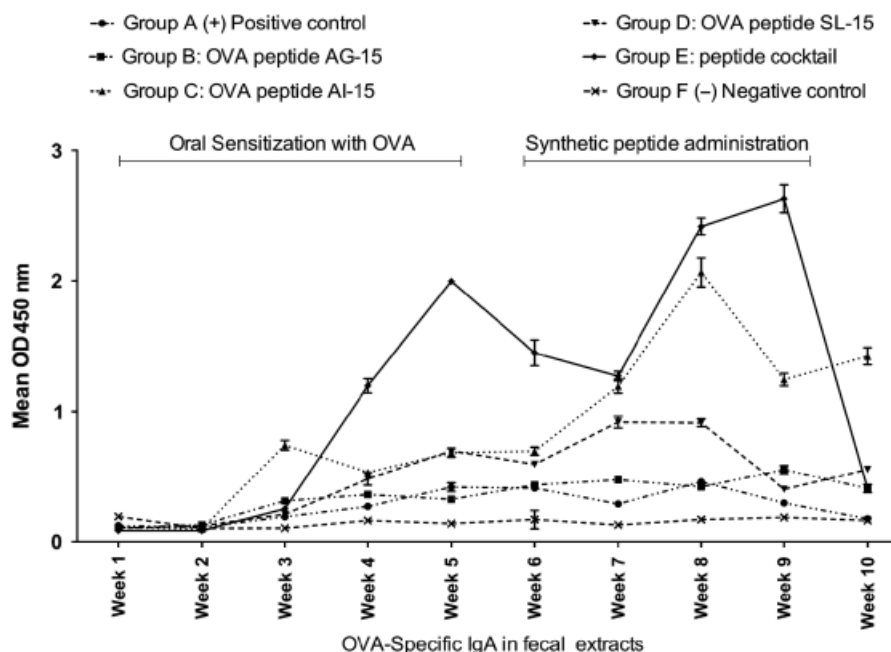


Fig. 4. Time-course monitoring of ovalbumin (OVA)-specific IgA levels in mouse fecal extracts before and during repeated administration of T cell epitope-containing peptides in groups A–E. Data are represented as mean $\pm$ SD ($n = 3$).

of our PIT design, reflected in particular by a significant reduction in the hypersensitivity reactions elicited in group E (Fig. 2a).

Promotion of a T helper type 1-biased response upon administration of epitope-containing peptides

In mouse models, CD4⁺ T cells are classically divided into two subsets, i.e. Th1 and Th2, particularly well known for their ability to counter-regulate one another. The concept of the Th1/Th2 balance model has thus provided valuable insight into understanding the basic mechanisms underlying allergic diseases. In the present study, analyses of culture supernatants stimulated *in vitro* with purified OVA indicate that IFN- γ (Th1-type cardinal cytokine) concentrations were significantly higher in all peptide-administered groups, compared with placebo-treated mice (Fig. 5a). In line with the Th1/Th2 balance, a significant decline in IL-4 concentrations was measured in all mice groups treated with epitope-containing peptides (Fig. 5b).

The combined results obtained from anaphylactic scores, serum and fecal sample analyses (Figs 2–4) indicate that the peptide-treated group C (peptide AI-15) and group E (cocktail) presented overall the most significant improvements in their OVA-specific allergic responses, in comparison with the remaining peptide-treated groups. Tissues collected from groups C and E were, therefore, selected for further analysis by real-time RT-PCR analyses, which also included positive and negative control mice i.e. groups A and F. Unlike placebo-treated mice (Pos), sham-sensitized mice (Neg) showed

very low expression levels of all parameters analysed – except for mRNA expressions of IL-4 and IL-10; however, the variations were not statistically significant (Figs 6b and c). These results are in line with recent reports investigating murine models of food allergy [27].

Mixed results were obtained in group C, in which mice were administered with the single T cell determinant AI-15. Results tend to show a reduction in their intestinal expression of both Th1- and Th2-biased cytokines, illustrated by a decreasing trend in IFN- γ and IL-12 (type-1 cytokines), as well as in IL-4, IL-5 and IL-13 (type-2 cytokines). Nevertheless, down-regulation of both type-1 and type-2 responses has previously been reported in a number of studies exploring immunotherapeutic treatments [4] and such variations are, therefore, not uncommon. Matters were further complicated with a marked elevation in the expression ratio of IL-18 (deemed type-1 cytokine), along with lower expression ratios for TGF- β , IL-10 and forkhead box transcription factor 3 (FOXP3), compared with placebo-treated mice (Pos). The mixed results obtained in group C remain to be clarified, but suggest that mechanisms more complex than the mutual antagonism Th1/Th2 are involved.

On the other hand, the administration of the peptide-cocktail (group E) provided a more defined picture. Indeed, in comparison with placebo-treated mice, a dominant Th1-biased profile (IFN- γ , IL-12 and IL-18, Fig. 6a) is manifest in this group. Furthermore, unlike group C, which was administered with single T cell determinant AI-15, mice comprised in group E showed a several-fold augmentation in both TGF- β and FOXP3 expression ratios

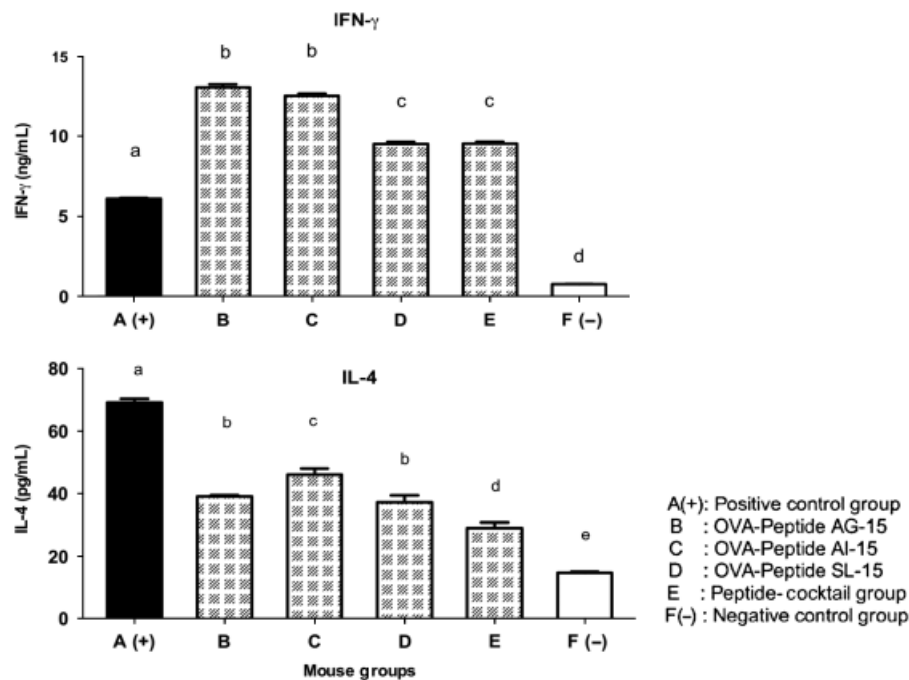


Fig. 5. Determination of cytokine concentrations of IFN- γ (a) and IL-4 (b) following *in vitro* restimulation of spleen cell cultures with ovalbumin. Data are represented as mean $\pm$ standard deviation ($n = 3$ pooled spleens). Different letters indicate statistically significant differences ($P < 0.05$), between groups of mice.

(Fig. 6c), significantly above the levels detected in placebo-treated mice. Altogether, these data strongly suggest that the administration of epitope-containing peptides promoted a systemic (spleen) and local (intestinal) Th1-biased cytokine response, with a concomitant up-regulation of T_{reg}-associated responses.

Discussion

PIT stands as an appealing alternative to other forms of immunotherapies that are based on the use of whole allergens. Surprisingly, the therapeutic utilization of PIT in food allergy, as well its underlying mechanisms remain unexplored. In the present study, we document the curative potential of PIT using the BALB/c mouse, a commonly used experimental model for food allergy [28].

Here are the main findings brought by our study: first, we showed that in comparison with placebo-treated mice, the administration of multiple T cell epitope-containing peptides promoted a robust reduction in hypersensitivity reactions, serum histamine and OVA-specific IgE levels, variations which were not observed in single peptide-treated mice (groups B–D) (Figs 2 and 3). Previous *in vitro* studies were described in which the use of peptides containing multiple rather than single T cell epitopes could exert suppressive effects on allergen-specific responses in a synergistic fashion [29]. Mixed responses were obtained with single peptide-treated mice: when administered individually, peptide SL-15 (group D) was

shown to exacerbate the allergic response to OVA; in counterpart, the co-administration of peptide AI-15 and AG-15 may have counterbalanced its effects and favoured inhibition of the specific allergic response. The question arises, what distinguishes peptide SL-15 from peptides AI-15 or AG-15. Previous investigations reported that immune reactivity to individual epitope regions could vary considerably [30–33] and differential MHC-binding affinity was suggested as one possible explanation. For example, using the OVA epitope model I323R339, mouse studies reported that analogue peptides with lower binding affinity to MHC class II molecules favoured the development of Th2-biased responses, whereas peptide analogues with higher binding affinity promoted a Th1-response [34, 35]. We believe that such a phenomenon is applicable to our study; but would need to be proven experimentally. Secondly, *in vitro* stimulation of splenocytes prepared from peptide-treated mice vs. placebo-treated mice suggested that a shift from a Th1-biased (IFN- γ) to a Th2-biased (IL-4) response might have occurred (Fig. 5). Interestingly, the same cytokine pattern was found *in vivo* upon analysis of intestinal gene expression as evidenced by higher expression trends of Th1-type cytokines, to the detriment of Th2-type cytokines (Figs 6a and b). Mechanistically, the most noteworthy finding may be that co-administration of peptides containing three OVA T cell determinants yielded significantly higher mRNA expression of FOXP3 and TGF- β in intestinal tissues, compared with placebo-treated mice.

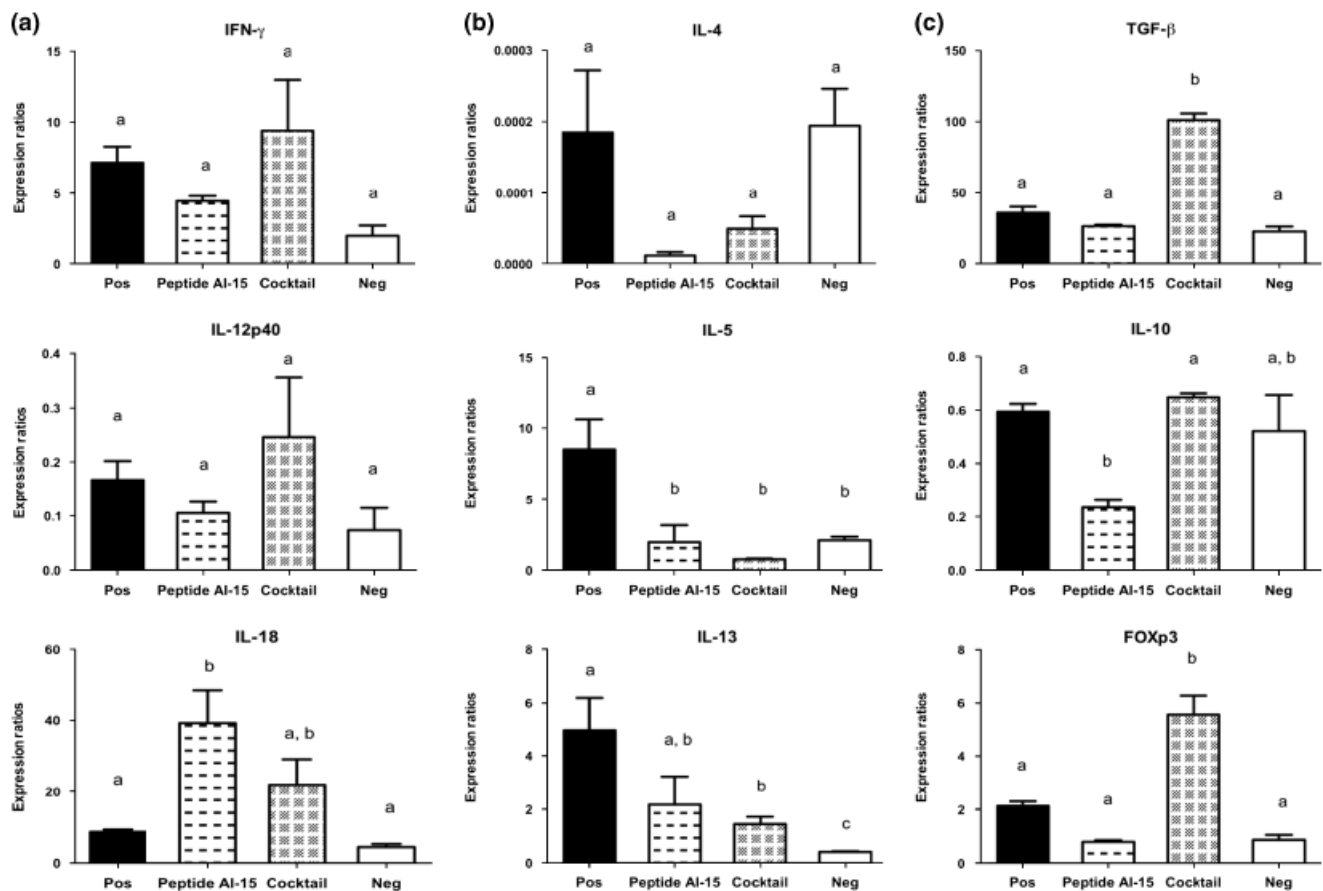


Fig. 6. Modulation of intestinal gene expression patterns in peptide-treated mice. Expression ratios determined by real-time RT-PCR analyses for Th1-biased cytokines IFN- γ , IL-12p40 and IL-18 (a), Th2-biased cytokines IL-4, IL-5 and IL-13 (b) and T_{reg}-associated molecules TGF- β , IL-10 and FOXP3 (c). Data are represented as mean $\pm$ SEM ($n = 6$ individual mice). Different letters indicate significant differences ($P < 0.05$), between groups of mice. Pos, positive control, i.e. group A; Neg, negative control, i.e. group F.

Again, such variations were not observed in single peptide-treated mice (Fig. 6c). The synergistic effect mentioned earlier, may be responsible for the up-regulation of gene expression for these two regulatory molecules. Here, we suggest that the variations observed in TGF- β and FOXP3 expression are major contributors to the allergy-suppressive effect elicited in mice that received the peptide cocktail. Indeed, TGF- β was shown to directly inhibit the proliferation and differentiation of effector T cells [36] and FOXP3-expressing T cells were well documented for their inhibitory effects on Th2-allergic responses [37]. Furthermore, as the enhanced ratios of FOXP3 and TGF- β were observed concomitantly, we further propose that the two events may be causally linked and more specifically, that in our experimental conditions, FOXP3 expression might be mainly induced by the action of TGF β . In line with this argument, cytokine TGF- β was highly documented as a key regulator in the induction of FOXP3 expression in regulatory T cells [36, 38, 39] and in mice, studies involving transgenic models reported that TGF- β -producing Th3 cells were essential to the induction and main-

tenance of tolerance by their contribution to the differentiation of FOXP3-expressing regulatory cells in the periphery [40]. Supplementary analyses aimed at verifying the expression of TGF- β and FOXP3 as functional proteins would be helpful additions to confirm our findings.

In mice, the expression of transcription factor FOXP3 was reported to be highly exclusive of T_{reg} cell lineage [41, 42] and the induction of FOXP3 in mice was shown to confer a suppressive phenotype in a consistent manner [43]. Transient expression of FOXP3 has been reported to occur in human effector T cells as an early marker of T cell activation, concomitantly with other activation markers such as cytotoxic T-lymphocyte antigen-4 and glucocorticoid-induced tumour necrosis factor receptor [44, 45]. One study was found in which the transient expression of FOXP3 was reported in mouse effector T cells; however, the study was based on the stimulation of T cells by dendritic cells presenting foreign antigen and leading to a highly activated state [46]. We can, therefore, propose with confidence that local regulatory mechanisms

associated with the expression of FOXP3 were induced upon administration of multiple peptides containing immunodominant T cell epitopes.

Third, besides cell-mediated responses, we also analysed the effect of systemic administration of T cell determinant containing-peptides on antibody responses. While it has now been accepted that allergic conditions are commonly associated with elevated levels of allergen-specific IgE resulting from IL-4-driven mechanisms, the role and contribution of other major isotypes such as IgG and IgA remain the matters of conflicting investigations [47]. In our study, the ELISA analysis of sera obtained from peptide-treated mice failed to highlight any significant variation in the production of OVA-specific IgG. Similar results have been documented in several reports [48, 49], but differ however from previous mouse studies completed in our laboratory using recombinant allergens [18]. This could signify that the mechanisms underlying PIT differ from those underlying immunotherapy using recombinant molecules. This was also suggested in recent reviews [2, 50]. On the other hand, we described a marked elevation of specific IgA levels, which occurred concomitantly with the administration of OVA-T cell epitope peptides (Fig. 4). This augmentation could signify either (1) that mucosally derived IgA contributed to the allergy-suppressive effects of our PIT design through mechanisms that merit further investigations or (2) that specific IgA levels may be used as a monitoring tool to predict the efficacy of a PIT-based treatment. Earlier studies also reported increases in local allergen-specific IgA following immunotherapeutic treatment, but using inhalant allergen extracts [51, 52]. Coincidentally, the cytokine TGF- β is particularly well known as the 'switch factor', governing the switch of B cell to IgA synthesis [53]. Based on this information, we currently explore how an enhanced intestinal expression of TGF- β and its functional gene product may elicit the production and secretion of allergen-specific IgA at intestinal surfaces.

In conclusion, the present study established that the repeated administration of short synthetic peptides containing multiple T cell determinants, promoted *in vivo* allergy-suppressive effects in a food allergy mouse model. Thus, we successfully developed a PIT mouse model that may serve as a valuable tool for further investigations into the mechanisms underlying the peptide-based approaches in food allergy. Mechanistically, we showed that beyond the shift from a Th2- to a Th1-skewed response, regulatory pathways involving the transcription factor FOXP3, the regulatory cytokine TGF- β and immunoglobulin isotype IgA are major contributors to the induction of immune tolerance.

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