

T cell epitope immunotherapy ameliorates allergic responses in a murine model of shrimp allergy

C. Y. Y. Wai¹, N. Y. H. Leung¹, P. S. C. Leung² and K. H. Chu¹

¹School of Life Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China and ²Division of Rheumatology/Allergy, School of Medicine, University of California, Davis, CA 95616, USA

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Summary

Background Shellfish allergy is one of the most common food hypersensitivities worldwide but allergen-specific immunotherapy for shellfish allergy is not yet available. We believe that T cell peptide-based immunotherapy holds the potential for modulating allergic responses without IgE cross-linking.

Objective We sought to identify the immunodominant T cell epitopes of tropomyosin, the major shrimp allergen of *Metapenaeus ensis* (Met e 1), and to evaluate their therapeutic effects in a Balb/c mouse model of Met e 1 hypersensitivity.

Methods T cell epitopes of Met e 1 were first identified based on the proliferation and cytokine responses of splenocytes isolated from Met e 1-sensitized Balb/c mice upon stimulation by 18 synthetic peptides that span the full-length Met e 1. The immunodominant T cell peptides identified were then fed orally to Met e 1-sensitized Balb/c mice twice a week for four weeks. Allergic responses, serological antibody levels, intestinal histology and systemic and local cytokine profiles were compared between the treated and the untreated groups.

Results Six major Met e 1 T cell epitopes were identified. Mice treated with the T cell epitope peptide mixture demonstrated an amelioration of systemic allergic symptoms and a significant reduction in Th2-associated antibody and cytokine responses. These benefits were accompanied by a shift to a balanced Th1/Th2 response, induction of IgG_{2a} antibodies possessing *in vitro* and *in vivo* blocking activities and the induction of regulatory T cell responses.

Conclusions and Clinical Relevance T cell epitope-based oral immunotherapy is effective in reducing allergic responses towards shrimp tropomyosin. This is a novel strategy for clinical management of shellfish allergy and is a model for mechanistic studies of oral immunotherapy.

Keywords allergen-specific immunotherapy, Met e 1, peptide immunotherapy, peripheral tolerance, shellfish, T cell epitopes, Th1/Th2 balance

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Correspondence:

Dr P. S. C. Leung, Division of Rheumatology/Allergy, School of Medicine, University of California, Davis, CA 95616, USA.

E-mail: psleung@ucdavis.edu and Dr K. H. Chu, School of Life Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China. Email: kahouchu@cuhk.edu.hk

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Introduction

Allergen-specific immunotherapy (AIT) has been a long-standing goal in the clinical management of allergic disorders [1]. However, adequate methodology is still undergoing development and many clinical studies have been associated with a high risk of developing allergic side-effects and the possibility of new sensitization to the extracts [2–5]. In addition, the duration of treatment, clinical efficacy and risk of anaphylactic reactions remain problematic. Clearly, alternative

approaches employing modalities that alter allergenicity are required to overcome these issues [6]. Indeed, a modulator that reduces allergen-specific IgE production, induces allergen-specific blocking antibodies, restores Th1/Th2 immune balance and induces regulatory T cell responses would be ideal [7–13].

Peptide-based immunotherapy (PIT) has been proposed as a safe and effective therapeutic strategy. Allergen-derived peptides would essentially be small in size but contain the relevant CD4⁺ T cell epitopes, lack secondary or tertiary structure, and would be incapable of cross-link-

ing IgE or activating effector cells [14–16]. Thus far, the development of such peptide immunotherapy is highly encouraging and there have been a number of clinical trials involving cats, house dust mite, bee venom and birch pollen that are suggestive of success [16–20].

Shellfish allergy is the second commonest food sensitivity in the USA and is highly prevalent in the Asia-Pacific region [21, 22]. It is also capable of provoking anaphylaxis [23]. The major allergen in shrimp is tropomyosin, a coiled-coiled muscle protein of 34–38 kDa [24]. Although other proteins in shrimp have been identified as potential allergens [25–29], more than 80% of patients are sensitive to tropomyosin [30] and, interestingly, there is high sequence homology among crustacean and mollusc species [28]. In this study, we took advantage of our recombinant shrimp tropomyosin protein from *Metapenaeus ensis*, coined Met e 1 [30], as well as our murine model of Met e 1-induced anaphylaxis [31], and identified the T cell reactive Met e 1 sequences using a combination of T cell proliferation and cytokine release assays. Furthermore, we evaluated the efficacy of these peptides for oral immunotherapy in modulating tropomyosin-induced anaphylaxis.

Methods

Peptide synthesis

Eighteen overlapping peptides spanning the full length of Met e 1 (Accession number Q25456.1) [30] were synthesized (GenScript, Piscataway, NJ, USA). Each peptide had 20 amino acids (except for peptide 18 which contained 19 amino acids) and with five amino acids overlapping with the adjacent peptides at the N-terminus. Individual peptides were dissolved in distilled water, aliquoted and stored at -20°C until use.

T cell epitope mapping

T cell epitopes were defined based on the proliferation response of T cells from mice intragastrically sensitized with Met e 1 upon peptide stimulation as described [32]. Briefly, immediately upon sacrifice, the spleen of individual Met e 1-sensitized mice ($n = 10$) was aseptically removed and gently crushed in a cell strainer (BD Falcon, San Diego, CA, USA 40 μm Nylon) to remove all connective tissues. Loosened cells were depleted of erythrocytes with 0.85% ammonium chloride/distilled water. After centrifugation, splenocytes were washed in ice-cold DMEM twice and resuspended in DMEM/8% FBS. The cell concentration was determined by trypan blue exclusion method (Sigma-Aldrich, Saint Louis, MO, USA), and the cells were cultured in a 96-well flat-bottom plate (Nunc, Rochester, NY, USA) at a density of 5×10^6 cells/mL at 37°C in a 5% CO_2 incubator. Cells were incubated in

duplicate in the presence of overlapping synthetic peptides (10 $\mu\text{g}/\text{well}$) or 100 μg rMet e 1. Non-stimulated cells were included for background determination. Splenocytes from control mice ($n = 4$) treated with PBS only were included in parallel as a negative control. After 72-h incubation, 10 μL WST-1 tetrazolium salt (Roche, HKSAR, China) was added to each well and further incubated for 2 h at 37°C in a 5% CO_2 incubator prior to measurement of absorbance at 450 nm.

Preparation of recombinant shrimp tropomyosin

cDNA coding for the full-length shrimp tropomyosin Met e 1 was cloned into the His-tag expression vector pET30(a)⁺ (Novagen, Madison, WI, USA) and expressed in *Escherichia coli* BL21 (DE3) (Invitrogen) by culturing in MagicMedia (Invitrogen, Carlsbad, CA, USA) overnight. His-tagged recombinant Met e 1 (rMet e 1) was purified using the HisPur Cobalt Spin Column (Thermo Scientific, Rockford, IL, USA) in accordance with the manufacturer's instructions. The concentration and purity of rMet e 1 produced were determined by BCA assay (Sigma-Aldrich) and SDS-PAGE, respectively.

Mice sensitization and peptide treatment

To induce shrimp allergy, all mice except those in the negative control group were intragastrically sensitized with 0.1 mg rMet e 1 and 10 μg cholera toxin (Sigma-Aldrich) on days 0, 12, 19 and 26, followed by a high dose of 0.5 mg rMet e 1 intragastric challenge on day 33 [31] (Fig. 1). Mice in the T cell peptides treatment group were then treated with a mixture of the six identified T cell epitopes (P2, P4, P6, P10, P15 and P17, each 0.2 mg, total = 1.2 mg) by means of intragastric gavage twice a week for four weeks. Mice fed with six non-dominant T cell epitope peptides of Met e 1 (P1, P5, P8, P12, P14 and P16 that were minimally reactive; these peptides are referred hereafter as non-epitopic peptides) at the same dosage were included as the non-epitopic peptides control group. Mice in the positive control group were sham-treated with PBS. One week after the last treatment (day 70), all animals were administered with 0.5 mg rMet e 1 as second oral challenge. Mice fed with PBS and cholera toxin in the sensitization phase followed by PBS only in the treatment phase were included as negative controls. The experiment was repeated thrice, with seven mice per group in each experiment. As the data from the three experiments were highly consistent, the results from a representative experiment were reported herein. Animal protocols were approved by the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong, in accordance with the Department of Health (Hong Kong) guidelines in Care and Use of Animals. All experiments were performed under licences

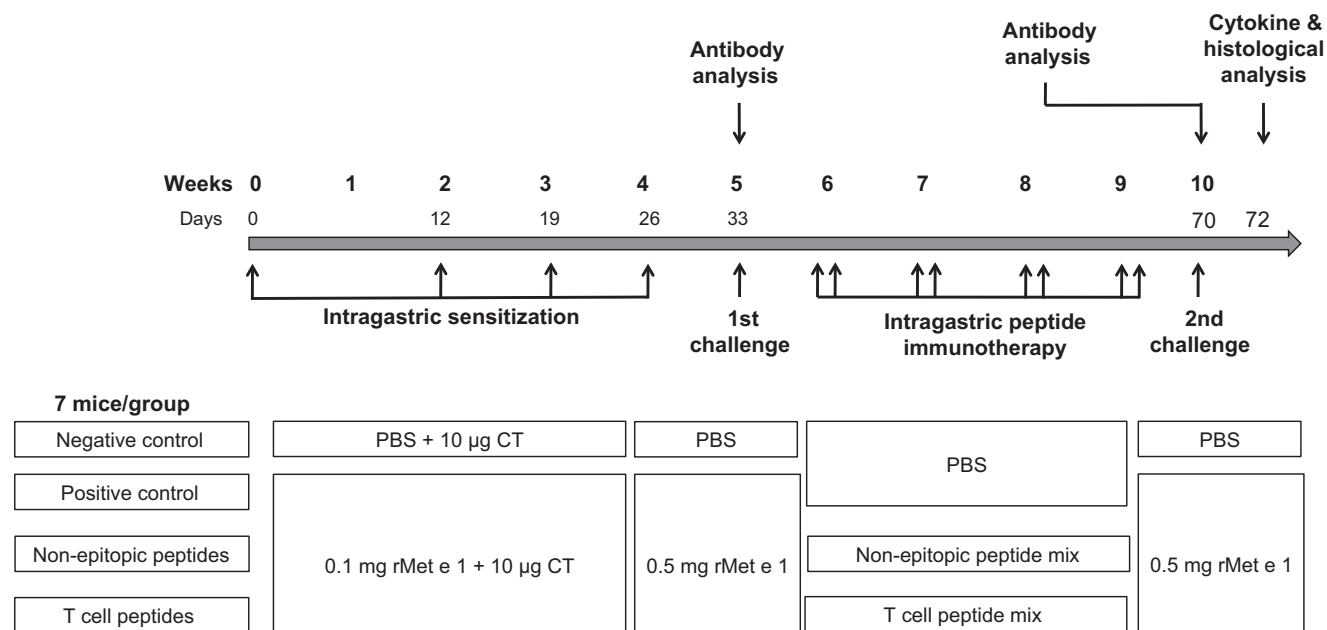


Fig. 1. Treatment regimen design with T cell peptide mixture. Three- to four-week-old Balb/c mice ($n = 7$ per group) were intragastrically sensitized and challenged with Met e 1. T cell peptide mixture was then delivered orally twice a week for four weeks as treatment. Mice were finally given a second Met e 1 challenge for treatment efficacy evaluation. Mice fed with PBS or non-epitopic peptides were included as negative, positive and non-epitopic peptides controls.

granted from the Government of Hong Kong Special Administrative Region, China.

Assessment of hypersensitivity responses

Systemic allergic responses were evaluated in a blinded manner for 30–40 min after challenge, using a scoring system as described [33]: 0 – no symptoms; 1 – scratching and rubbing; 2 – puffiness around eyes or reduced activity; 3 – laboured respiration; 4 – no activity after prodding, tremor and convulsion; 5 – death. Condition of feces was also assessed in a blinded manner during challenge: 0 – hard feces; 1 – soft feces; 2 – liquid feces; and 3 – white, mucus-like feces [34]. Mice showing feces condition of score 2 or above were regarded as displaying diarrhoea.

Levels of serological mMCP-1, IgE and IgG antibodies

Mouse mast cell protease-1 (mMCP-1) levels in blood samples collected 4 h after intragastric challenge were determined using a sandwich ELISA kit (eBioscience, Santa Clara, CA, USA). Met e 1-specific IgE, IgG₁ and IgG_{2a} responses were determined by ELISA at 1 : 10, 1 : 10 000 and 1 : 200 sera dilutions, respectively, as described [35].

Histological analysis

Jejunum sections were prepared as described [36]. Briefly, 4-µm tissue sections were stained with periodic

acid-Schiff (PAS) [37] (Sigma-Aldrich & Leica Microsystems, Wetzlar, Germany) for the detection of goblet cells. Number of PAS-positive cells and total number of villus cells on one side of the villus were counted. Ten randomly selected villi were counted for each tissue sample. The percentage of goblet cells was expressed as the number of PAS-positive cells divided by the total number of villus cells counted [38]. Eosinophils were identified by means of immunohistochemical staining with rat anti-mouse major basic protein monoclonal antibody (MBP) (Mayo Clinic, Scottsdale, AZ, USA) [39]. After colour development by DAB colour developing solution [40] and counterstaining with haematoxylin (Sigma-Aldrich), at least four random sections per tissue sample were analysed. Quantification of eosinophils per square millimetre of villi was performed with the public-domain ImageJ software. Two jejunum sections were stained for each analysis.

Competitive inhibition ELISA

In vitro blocking ability of the IgG antibodies was analysed by competitive inhibition ELISA as described with modifications [35]. Briefly, blood samples were collected after second challenge, and sera (pooled from each group, $n = 7$) were incubated at three dilutions (1 : 10, 1 : 20 and 1 : 40) in rMet e 1-coated plates overnight at 4°C. Thereafter, 100 µL of sera from Met e 1-sensitized mice (pooled, $n = 6$) at 1 : 20 dilution was added and incubated at room temperature for 2 h.

Signals were then developed by incubating with biotinylated anti-mouse IgE antibody (BD Pharmingen, San Diego, CA, USA), avidin D, peroxidase-labelled antibody (vector) and TMB substrate reagent set (BD Biosciences, San Diego, CA, USA). The blocking ability of the sera antibodies was determined using the equation $[(OD_{no\ inhibitor} - OD_{inhibitor}) / OD_{no\ inhibitor}] \times 100\%$ and expressed as percentage inhibition.

Passive cutaneous anaphylaxis

For *in vivo* blocking antibodies analysis, the backs of naïve Balb/c mice were shaved, followed by the intradermal injection of IgE-containing sera from Met e 1-sensitized mice mixed with sera collected after the second Met e 1 challenge (pooled, $n = 7$, undiluted sera in 1 : 1 ratio, with a total volume of 100 μ L) under isoflurane narcosis. Sera collected from the T cell peptides-treated mice were depleted of IgG antibody using the NAb Protein G spin kit (Thermo Scientific). Flow through 1 (FT1) and flow through 2 (FT2) were obtained by passing the PIT sera through the Protein G column once and twice, respectively. The absence of Met e 1-specific IgG, IgG₁ or IgG_{2a} in FT1 and FT2 was confirmed by western blotting and direct ELISA before the PCA experiment. Mice injected with sera containing OVA-specific IgE and PIT sera were included as control to demonstrate the specificity of inhibition. Mice were intradermally injected with 100 μ L sera from negative control mice as control. After 3 h, mice were injected intravenously with a mixture of 100 μ L of 0.5% Evan's blue dye (Sigma-Aldrich) and 0.1 mg rMet e 1. Thirty minutes after dye-rMet e 1 administration, mice were sacrificed and their back skins were immediately inverted for the measurement of the diameters of blue region (i.e. size of dye extravasation). Skin biopsies (0.3 g per mouse) around the injection site were incubated for 24 h at 55°C in 0.5 mL formamide (Sigma-Aldrich) for the extraction of Evan's blue dye. Extravasation of the dye was quantified by absorbance measurements at 610 nm. The experiment was repeated and two absorbance values from two skin biopsies were obtained for data presentation.

Splenic cytokine responses

Splenocytes were prepared as described above and were stimulated with various amounts of synthetic peptides (10, 30, 50 and 100 μ g) or 100 μ g rMet e 1 for 72 h at 37°C in a 5% CO₂ incubator at a density of 5×10^6 cells/mL (1 mL/well) in triplicates. Culture supernatants were collected for the measurement of IL-4, IL-5, IFN- γ and IL-12 using sandwich ELISA kits (BD Biosciences) in accordance with the manufacturer's instructions.

Intestinal gene expression

Ileum sections where the Peyer's patches are exclusively present were collected and immediately stored in ice-cold RNAlater stabilization reagent (Qiagen, Valencia, CA, USA). Total RNA was extracted using the RNeasy mini kit (Qiagen), and cDNA was synthesized from 250 ng/mouse of total RNA using M-MuLV reverse transcriptase (Genesys, Daly City, CA, USA). The expression of the Th1-, Th2- and Treg-associated genes was determined using specific primers (Table S1) with the 7500 fast real-time system (Applied Biosystems, Waltham, MA, USA) using the standard fast PCR protocol. Fast SYBR green PCR master mix (Life Technologies, Carlsbad, CA, USA), 50–125 nM specific primers and 100 ng cDNA were used in all reactions, with the efficiency of all reactions kept between 90% and 110%. The cycle threshold value (C_t) of each gene was normalized to the C_t of the house-keeping gene HPRT-1. The mRNA expression ratios were then determined using the comparative C_t method [41, 42], relative to the negative control group.

Statistical analysis

Data are presented as mean $\pm$ SEM. Data were assessed for normality and statistical comparison was determined by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test using SigmaStat 3.1. The difference was considered statistically significant at p -values < 0.05 . Statistically significant differences in mean values are illustrated with asterisks or different alphabets in the figures and tables.

Results

Identification of six immunodominant T cell epitopes on Met e 1

Splenocytes of Met e 1-sensitized mice had strong proliferation response ($OD_{450\text{ nm}} > 2$) and produced high level of IL-4 (> 400 pg/mL) in response to Met e 1 stimulation while the non-stimulated cultures had undetectable proliferation and cytokine responses (data not shown). Meanwhile, six Met e 1 peptides, viz., P2, P4, P6, P10, P15 and P17, had significantly higher *in vitro* spleen cell proliferation as compared to each of the non-reactive peptides ($p < 0.001$) in ten sensitized mice (Fig. 2a). None of these six peptides induced significant proliferation in the negative control mice. Proliferation intensity was twofold higher in the sensitized than the control mice ($OD_{450\text{ nm}}$ for P2 0.26 ± 0.03 , P4 0.25 ± 0.04 , P6 0.26 ± 0.02 , P10 0.22 ± 0.03 and P15 0.23 ± 0.02) except for P17, which had a threefold higher and the strongest proliferation intensity ($OD_{450\text{ nm}} = 0.34 \pm 0.03$).

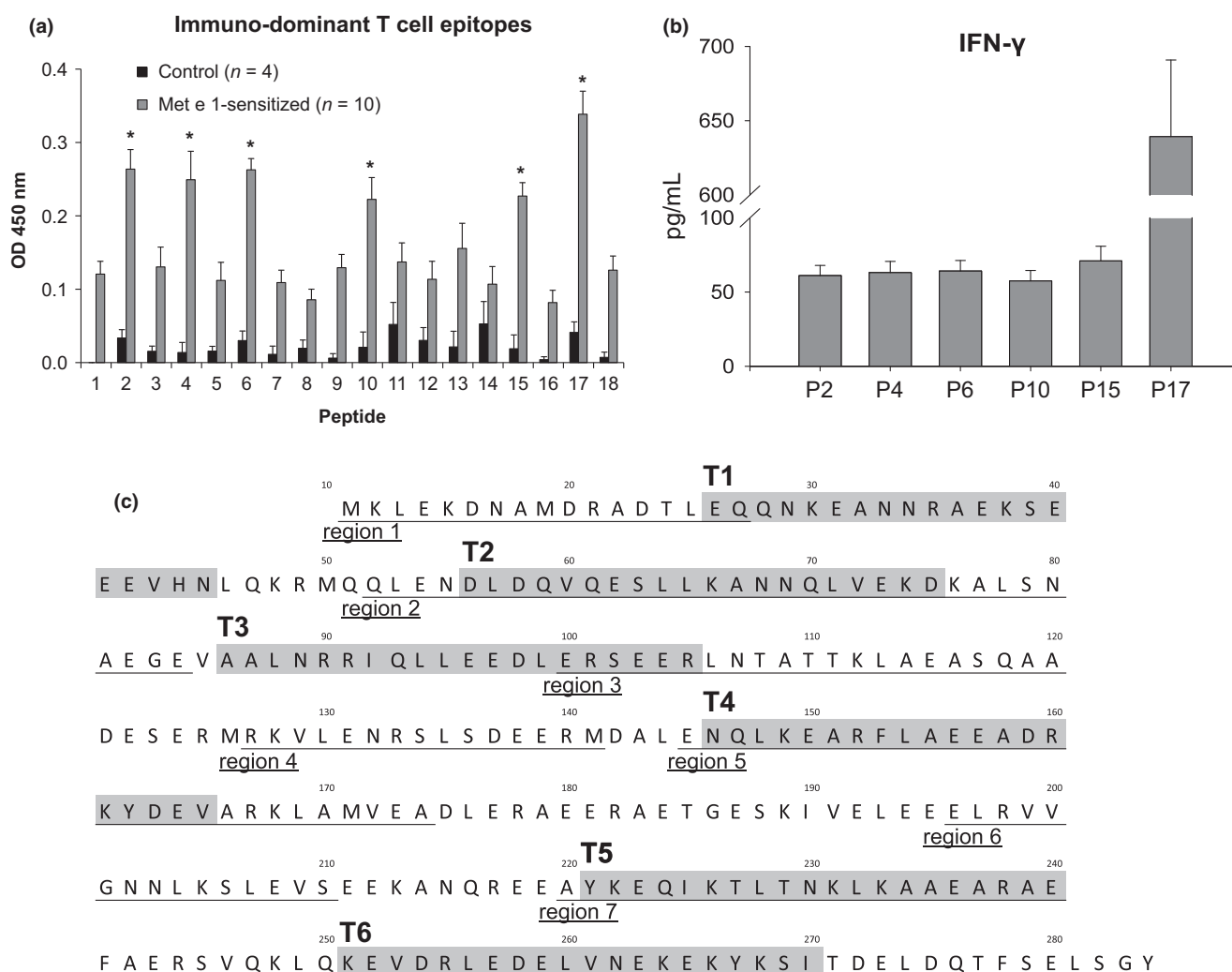


Fig. 2. T cell epitopes of Met e 1. Epitopes were determined by (a) proliferation and (b) cytokine responses of splenocytes from Met e 1-sensitized Balb/c mice. Data are presented as mean $\pm$ SEM ($n = 10$). Asterisks indicate peptides with statistical significant differences compared to each of the non-reactive peptides ($p < 0.001$, one-way ANOVA followed by Student–Newman–Keuls test). (c) Comparison of the T cell reactive regions of Met e 1 (this study, T1–6, shaded) and Pen a 1⁴³ (regions 1–7, underlined).

Furthermore, while none of the positive peptides induced IL-4 (data not shown), high levels of IFN- γ could be detected when the splenocytes were stimulated with 100 μ g of the peptides (Fig. 2b). The amount of IFN- γ detected ranged from 57.4 to 639.2 pg/mL upon stimulation by the six individual positive peptides, as comparing to only 4.5–14.5 pg/mL upon stimulation with the negative peptides. With the ability to induce both T cell proliferation and cytokine production, these six peptides of Met e 1 therefore contained T cell-activating sequences and represented the immunodominant T cell epitopes of Met e 1, namely T1–T6, with positions at Met e 1^{26–45}, Met e 1^{56–75}, Met e 1^{86–105}, Met e 1^{146–165}, Met e 1^{221–240} and Met e 1^{251–270}, respectively (Fig. 2c).

The amino acid sequence of Pen a 1, another common tropomyosin model for the study of shellfish allergy, only differs from Met e 1 by one amino acid position [28]. We therefore compared our identified T cell epitopes with the Pen a 1 epitopes previously identified from shrimp allergy subjects [43] (Fig. 2c). T cell reactive regions 4 and 6 of Pen a 1 were not immunogenic in our study. Despite this discrepancy, epitopes T2, T4 and T5 revealed in our study nest within the T cell reactive regions 2, 5 and 7 of Pen a 1, respectively. Further, epitopes T1, T3 and T6 overlap for two to six amino acid residues with regions 1, 3 and 7 of Pen a 1, respectively. These observations suggest a conserved set of immunogenic sequences between human subjects and Met e 1-sensitized Balb/c mice.

T cell peptide treatment reduces clinical symptoms and Met e 1-specific IgE production

Upon the first Met e 1 challenge (before treatment), mice in all groups (except those in the negative control group) developed hypersensitivity reactions including puffiness around eyes, reduced activity (score 2), laboured respiration (score 3) and convulsion (score 4) (Fig. 3a), and they also showed signs of diarrhoea (score

2 or 3) (Fig. 3b). These symptoms remained in the positive and non-epitopic peptides control groups upon the second Met e 1 challenge (after treatment). On the contrary, allergic responses were markedly reduced after intragastric peptide therapy in the T cell epitopes-treated mice (score 1–2). Specifically, four of the seven treated mice did not develop any allergic symptoms and no mice developed diarrhoea upon challenge after T cell peptide treatment (Fig. 3a and b).

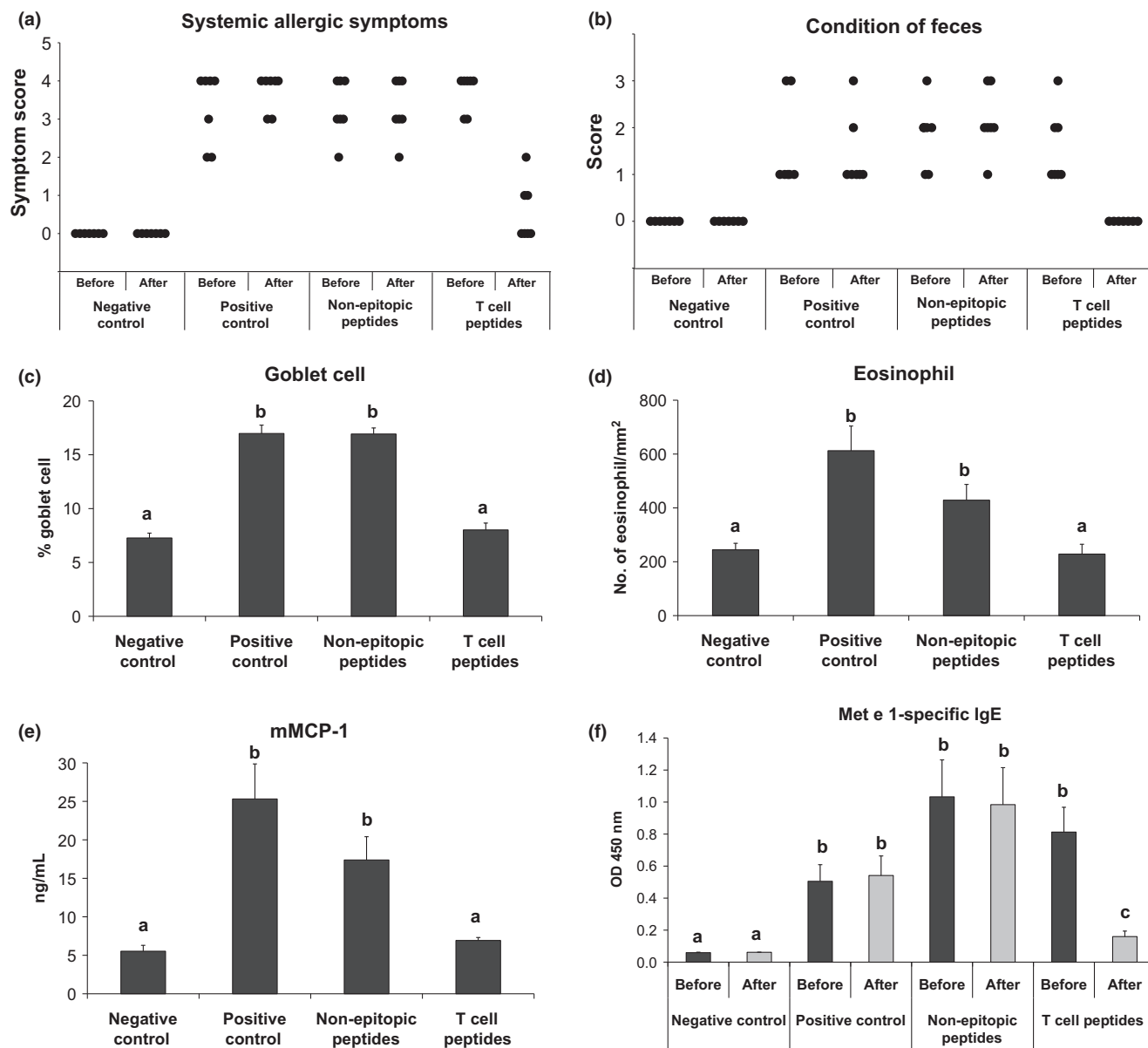


Fig. 3. Amelioration of allergic responses following oral PIT. (a) Systemic allergic symptoms and (b) condition of feces after Met e 1 challenge. (c) Quantification of mucosal goblet cells in the jejunum epithelium expressed as % goblet cells and (d) Eosinophils in jejunum expressed as number of eosinophils/mm². Levels of serological (e) Mast cell protease-1 (mMCP-1) and (f) Met e 1-specific IgE (1 : 10 dilution) determined by ELISA. Data are presented as mean $\pm$ SEM ($n = 7$) and pairwise comparisons among all groups were analysed using one-way ANOVA followed by Student–Newman–Keuls test. Groups denoted by the same alphabet are not significantly different, and groups denoted by different alphabets (a, b or c) are significantly different ($p < 0.05$). Note that T cell peptides-treated mice had significant amelioration in the allergic responses as compared to the positive and non-epitopic peptides controls.

Histologically, mice in the positive and non-epitopic peptides control groups displayed strong goblet cell hyperplasia ($17.0 \pm 0.8\%$ and $16.9 \pm 0.6\%$, respectively) (Figs 3c and S1) and high eosinophil infiltration throughout their lamina propria in the jejunum (612 ± 92 and 429 ± 58 eosinophils/mm², respectively) (Figs 3d and S2). T cell peptides-treated mice were recovered from intestinal inflammation, as indicated by a significantly lower number of goblet cells ($p < 0.001$) in their epithelium ($7.9 \pm 0.6\%$) and a significantly lower number of eosinophils ($p < 0.001$) that were found restricted to the base of villi (229 ± 37 eosinophils/mm²). In addition, the level of serological mMCP-1 was significantly lower in the treatment group when compared with the positive and non-epitopic peptides control groups ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 3e). There were no significant differences in the inflammatory reactions between the negative control and the treatment groups. Moreover, mice in all groups displayed elevated levels of Met e 1-specific IgE when compared to the negative control group upon the first Met e 1 challenge ($p < 0.001$), with no significant differences among these challenged groups (Fig. 3f). Contrary to the sustained level of specific IgE observed in mice from the positive and non-epitopic peptides control groups after the second challenge, IgE level decreased significantly by fivefold in mice after T cell peptides treatment ($p < 0.001$). Level of Met e 1-specific IgE was also significantly lower in these T cell peptides-treated mice as compared to the positive and non-epitopic peptides controls upon the second challenge ($p < 0.001$), while there were no significant differences between the latter two control groups.

T cell peptide treatment induces blocking IgG_{2a} antibody production

The levels of specific IgG₁ antibodies significantly increased ($p < 0.05$) after the second Met e 1 challenge in all of the challenged groups as compared to the levels after the first challenge (Fig. 4a). On the contrary, a high IgG_{2a} level was exclusively detected in the T cell peptides-treated mice, in which the level of IgG_{2a} antibody increased significantly ($p < 0.001$) by almost eightfold after peptide treatment (Fig. 4b). The functionality of these Met e 1-specific IgG_{2a} antibodies was validated by competitive inhibition ELISA. Antibodies from the peptide-treated mice were able to inhibit 79%, 64% and 39% of IgE binding to Met e 1 at 1 : 10, 1 : 20 and 1 : 40 sera dilutions, respectively (Fig. 4c).

PCA assay was also conducted to determine the *in vivo* blocking potential of the IgG antibody. Co-injection of IgE-containing serum and sera from the positive and non-epitopic peptides control groups led to the extravasation of Evan's blue dye into the surround-

ing skin tissue, as indicated by a blue region with diameter > 3 cm at the injection sites, as well as approximately 0.28 and 0.30 absorbance values upon dye extraction, respectively (Fig. 4d and e). On the other hand, the co-injection of IgE-containing serum and sera from T cell peptides-treated mice (PIT sera) did not induce significant Evan's blue extravasation, as indicated by a blue region with a diameter < 1 cm and a lower absorbance value at approximately 0.12. Furthermore, both FT1 and FT2 could not inhibit mast cell degranulation, for which FT1 was depleted of IgG_{2a} but contained IgG₁ antibody while FT2 was deprived of all specific IgG (Fig. S3). These observations indicated the specific role of IgG_{2a} as blocking antibody. Moreover, PIT sera could not inhibit mast cell degranulation induced by OVA-specific IgE. These observations strongly suggest that the IgG_{2a} antibodies present in the sera of the T cell peptides-treated mice could specifically inhibit IgE from binding onto mast cells and the subsequent degranulation process.

Restoration of Th1/Th2 balance and promotion of treg responses upon T cell peptide treatment

Analysis of splenocyte culture revealed high levels of the Th2 cytokines IL-4 and IL-5 and a low level of the Th1 cytokine IFN- γ in the positive and non-epitopic peptides control groups (Table 1), thereby suggesting a typical Th2-oriented response in these mice. Meanwhile, splenocytes from the T cell peptides-treated mice produced significantly lower levels of IL-4 and IL-5 compared to both the positive and non-epitopic peptides control groups ($p < 0.001$). A significantly higher level of IFN- γ was also detected in the cell supernatant of the T cell peptides-treated mice ($p < 0.001$). Interestingly, mice treated with either the non-epitopic peptides or T cell peptides had a high level of IL-12 in their splenocyte cultures.

A clear Th2 response could be detected in the ileum of the positive controls, as seen from the low expressions of IL-12, IL-18 and IFN- γ (Fig. 5a–c) and the overexpressions of IL-4, IL-5 and IL-13 (Fig. 5d–f) as compared to the negative controls ($p < 0.05$). Mixed responses were found in the non-epitopic peptides control group. Although the overexpression of IL-13 and the down-regulated expression of IL-18 could be detected, the expressions of IL-5, IL-12 and IFN- γ of these mice were comparable to the negative controls. Significant overexpression of IL-4 was found only in the positive control group ($p < 0.005$), with no significant difference in its expression among other groups. However, decreased expression of both IL-5 and IL-13 was evident in the T cell peptides-treated group ($p < 0.001$ and $p < 0.05$, respectively), which was accompanied by the normal expression of all Th1

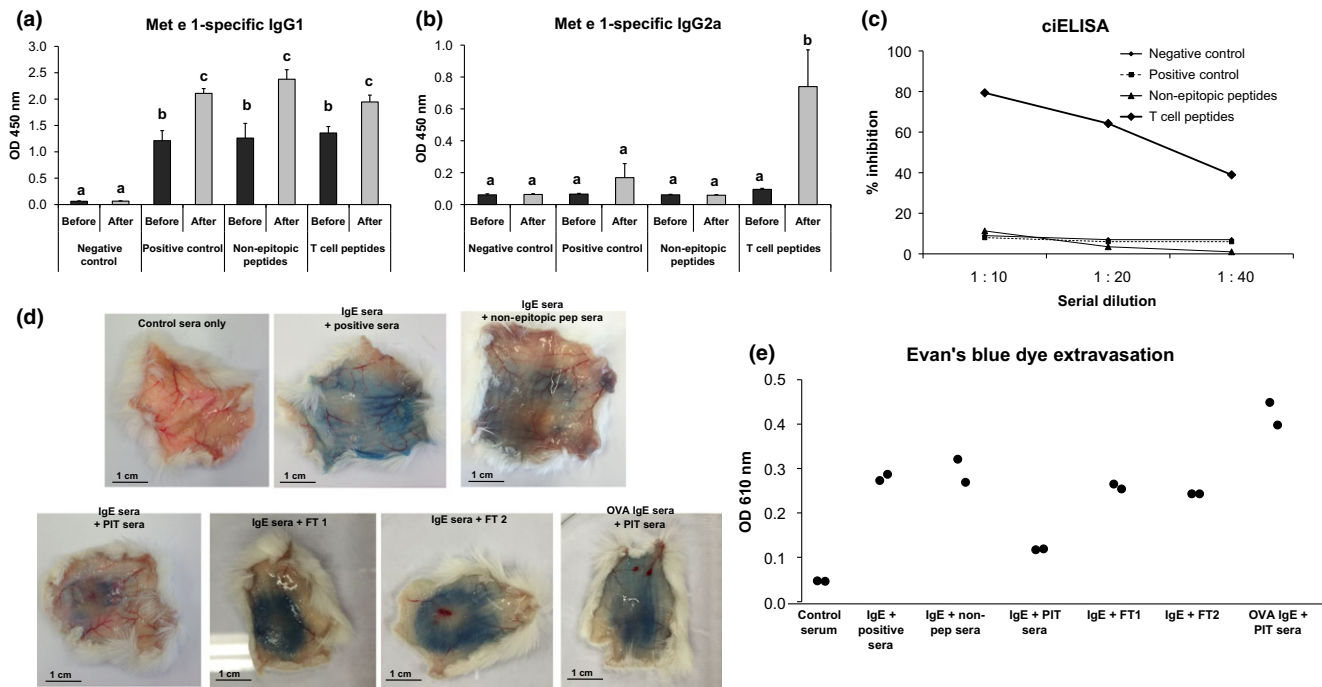


Fig. 4. Induction of IgG antibodies with *in vitro* and *in vivo* blocking potential. Levels of serological Met e 1-specific (a) IgG₁ (1 : 10 000 dilution) and (b) IgG_{2a} (1 : 200 dilution) determined by ELISA. Data are presented as mean $\pm$ SEM ($n = 7$) and pairwise comparisons among all groups were analysed using one-way ANOVA followed by Student–Newman–Keuls test. Groups denoted by the same alphabet are not significantly different, and groups denoted by different alphabets (a, b or c) are significantly different ($p < 0.05$). Note that T cell peptides-treated mice had significantly higher level of IgG_{2a} upon treatment ($p < 0.001$). (c) Pooled sera ($n = 7$) analysed by competitive inhibition ELISA (ciELISA) to determine *in vitro* percentage inhibition. (d) Images of PCA of naïve mice. (e) Quantification of Evan's blue dye amount in the skin tissues after formamide extraction upon PCA assay. PCA assay was repeated to obtain two skin biopsies and each dot represents the absorbance value from each biopsy. Note that PIT sera could inhibit mast cell degranulation but such inhibitory effect was lost when the sera were deprived of IgG_{2a} antibody (IgE + FT1 or FT2).

Table 1. Splenic cytokine levels (pg/mL) upon Met e 1 restimulation*

	Negative control	Positive control	Non-epitopic peptides control	T cell peptides
IL-4	76 $\pm$ 12 ^a	477 $\pm$ 77 ^b	391 $\pm$ 66 ^b	92 $\pm$ 19 ^a
IL-5	25 $\pm$ 0 ^a	355 $\pm$ 40 ^b	352 $\pm$ 48 ^b	27 $\pm$ 1 ^a
IFN- γ	198 $\pm$ 49 ^a	409 $\pm$ 93 ^b	80 $\pm$ 11 ^a	1388 $\pm$ 69 ^c
IL-12	70 $\pm$ 12 ^a	108 $\pm$ 29 ^a	400 $\pm$ 22 ^b	243 $\pm$ 16 ^c

*Individual spleens were collected after the second Met e 1 challenge and isolated splenocytes were restimulated with 0.1 mg rMet e 1. Levels of IL-4, IL-5, IFN- γ and IL-12 in the culture supernatant were determined by ELISA. Data are presented as mean $\pm$ SEM ($n = 7$) and pairwise comparisons among all groups were analysed using one-way ANOVA followed by Student–Newman–Keuls test. For each row, groups denoted by the same alphabet are not significantly different, and groups denoted by different alphabets (a, b or c) are significantly different ($p < 0.05$). Note that T cell peptides-treated mice produced significantly lower levels of the Th2 cytokines IL-4 and IL-5 ($p < 0.001$) and higher level of the Th1 cytokine IFN- γ ($p < 0.001$) as compared to the positive and non-epitopic peptides controls.

cytokines as in the negative controls. In addition, although no significant difference in TGF- β and RUNX3 expressions could be detected (Fig. 5g and h),

expressions of IL-10, Foxp3, CD25 and RUNX1 in the ileum of T cell peptides-treated mice (Fig. 5i–l) were significantly higher than the levels detected in the negative, positive and non-epitopic peptides control groups ($p < 0.001$).

Discussion

Conventional forms of subcutaneous AIT have been reported to be effective in treating airway allergies such as asthma [44], but its applications in food allergies often lead to anaphylactic side-effects and do not lead to significant humoral and cellular immune regulations [45–47]. Oral immunotherapy (OIT), on the other hand, is relatively safe and tightly linked to clinical desensitization in a majority of the recipients and have emerged as novel therapies for food allergies [47]. In this study, we demonstrated that oral administration of T cell peptides of shrimp tropomyosin is capable of alleviating specific allergic responses to shrimp tropomyosin in mice with the restoration of Th1/Th2 balance, the enhancement of Treg responses and the generation of Met e 1-specific blocking antibodies of IgG_{2a} isotype.

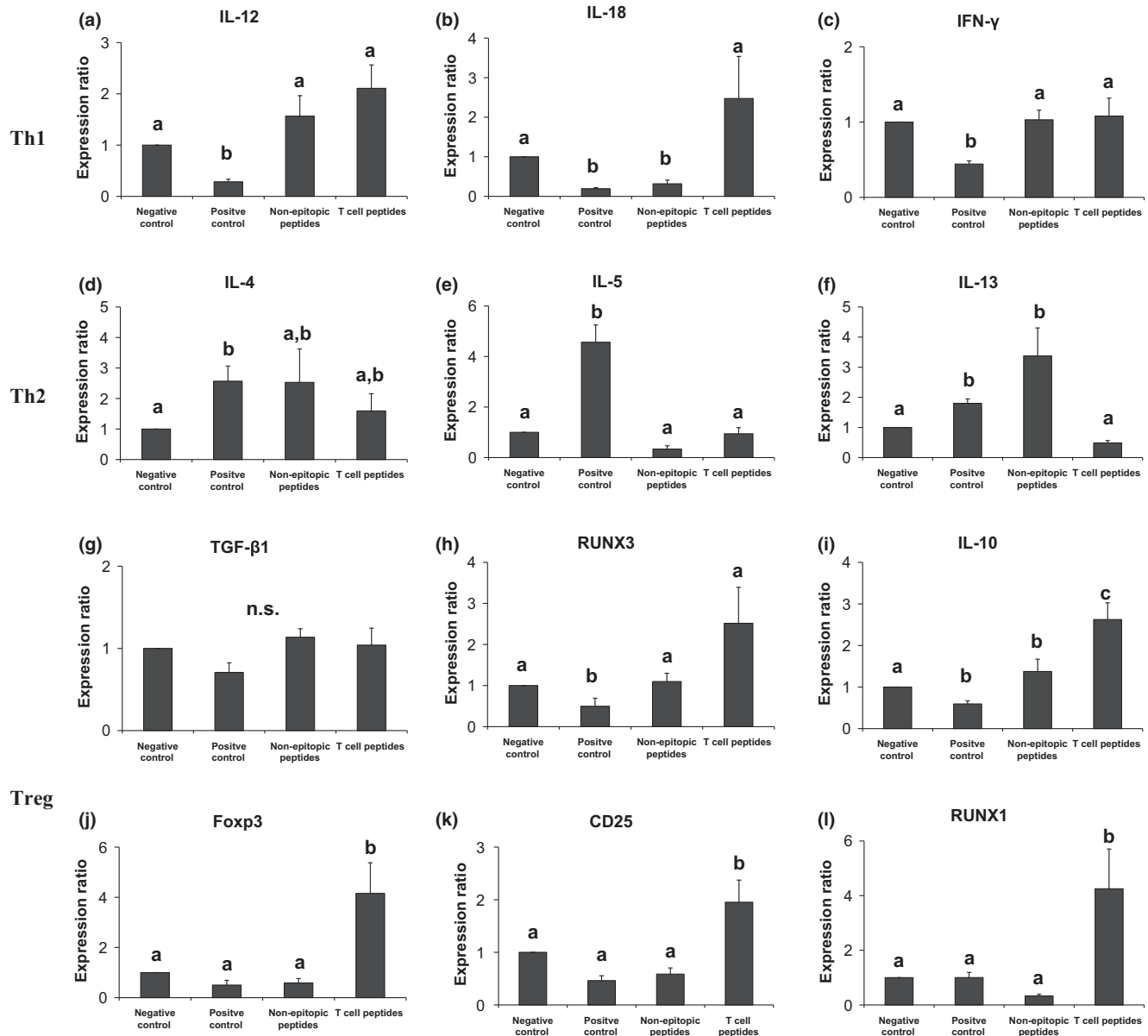


Fig. 5. Intestinal gene expression profile. Expressions of Th1 cytokines (a) IL-12, (b) IL-18 and (c) IFN- γ and Th2 cytokines (d) IL-4, (e) IL-5 and (f) IL-13, as well as Treg cytokines and transcription factors (g) TGF- β 1, (h) RUNX3, (i) IL-10, (j) Foxp3, (k) CD25 and (l) RUNX1 at ileum, were determined by RT-PCR in the negative, positive, non-epitopic peptides controls and T cell peptides treatment groups. Data are presented as mean $\pm$ SEM ($n = 7$), and pairwise comparisons among all groups were analysed using one-way ANOVA followed by Student–Newman–Keuls test. Groups denoted by the same alphabet are not significantly different, and groups denoted by different alphabets (a, b or c) are significantly different ($p < 0.05$). Note that T cell peptides-treated mice had significant down-regulated expression of IL-5 ($p < 0.001$) and IL-13 ($p < 0.05$), but up-regulated expression of IL-10, Foxp3, CD25 and RUNX1 ($p < 0.001$).

Decrease in the levels of allergen-specific IgE and Th2-associated cytokines IL-4, IL-5 and IL-13 was reported in OIT of other food allergies [48–50]. In fact, our data closely mirror those reported in other OIT such that our T cell peptide-based OIT is able to alter the established clinical and immunological allergic responses to shrimp tropomyosin. Firstly, oral T cell peptide treatment markedly reduced the level of Met e 1-specific IgE antibody and was associated with the

reduction in severity of allergic symptoms and the absence of diarrhoea. Secondly, mast cell degranulation (i.e. lower level of mMCP-1), eosinophil infiltration and goblet cells hyperplasia were absent in the treated mice, thereby indicating the alleviation in inflammatory reactions upon our peptide OIT [51–53]. Thirdly, splenic production of IL-4 and IL-5 and the expression of Th1 cytokines (IL-12, IL-18 and IFN- γ) and Th2 cytokines (IL-5 and IL-13) in the gut were restored to normal

levels when compared to the negative controls. These observations of both local and systemic restoration of a balanced Th1/Th2 response in the T cell peptides-treated mice are similar to other OIT and AIT studies [54–56]. The reduction of Th2 cells and the expansion of Th1 cells were also reported in highly allergic children after receiving peanut OIT [57]. Although the indispensability of Th1/Th2 balance restoration upon PIT and AIT remains debatable [9, 58, 59], we suspect that the observed repolarization in the T cell peptides-treated mice might be credited to the IFN- γ -stimulating nature (i.e. the Th1-polarizing nature) of our identified T cell epitopes. Such induction of IFN- γ (but with undetectable level of IL-4) by our major T cell epitopes and the translation of these IFN- γ -stimulating T cell epitopes into PIT to achieve Th1/Th2 immune balance re-orientation are consistent with previous T cell epitope screening and PIT studies [32, 43, 60]. These observations further illustrate the importance of the initial *in vitro* screening of T cell epitopes for the selection of candidate therapeutic peptides.

Previous studies have reported the early increase in allergen-specific IgG₄ antibody level at two months into oral treatment [48, 50]. It is noteworthy that the possession of blocking potential of this IgG₄ antibody remains crucial because the prevention of rapid anaphylaxis depends partly on the fast-acting blocking antibodies [61]. The persistent inhibitory activity of the IgG₄ antibody is also suggested as an explanation of the prolonged efficacy of grass pollen immunotherapy [11]. However, only transient or marginal increases in IgG₄ (clinical studies) or IgG_{2a} (animal studies) levels were reported in intradermal or subcutaneous PIT [60, 62]. Interestingly, our oral T cell peptides treatment is competent in inducing a strong IgG_{2a} antibody response upon re-exposure to Met e 1. Such an exclusive induction of IgG_{2a} antibodies among the T cell peptides-treated mice might be attributed to the high level of IFN- γ detected in the spleen of these mice, in which IFN- γ was found to stimulate IgG_{2a} synthesis by murine B cells [63]. Most importantly, these IgG_{2a} antibodies are validated here with both *in vitro* and *in vivo* blocking ability, in particular when sera deprived of IgG_{2a} but containing IgG₁ antibodies lost their faculty in inhibiting mast cell degranulation (Fig. 4d). It is noteworthy that two Met e 1 challenges were involved in this investigation and blocking IgG_{2a} antibody was synthesized upon allergen re-exposure in the treatment group. This is of utmost clinical importance as this closely reflects the possible clinical improvements when shrimp-allergic subjects are re-exposed to tropomyosin after receiving such oral PIT.

In addition to the resolution of existing allergic pathology and the induction of blocking IgG_{2a} antibodies, our oral PIT is capable of generating regulatory T

cell-associated responses as in other PIT studies [58, 62, 64, 65]. By mRNA expression analysis, the expressions of CD25, Foxp3, IL-10 and RUNX1 were locally up-regulated only in the T cell peptides treatment group. These observations can be recognized in the context of the local recruitment of Treg cells upon oral peptide treatment, which are critical in the clinical improvements of AIT [66, 67]. Specifically, IL-10 is a key Treg cytokine involved in inhibiting IL-4 and IL-5 production, eosinophil survival and IgE synthesis [68]. Its up-regulation might therefore account for the observed reduction of Th2-allergic symptoms in the T cell peptides-treated mice. Although we could not detect a significant increase in TGF- β expression in the T cell peptides-treated mice, a previous study suggests that immuno-suppression could actually be independent of TGF- β [69].

The six non-epitopic peptides were selected for control purpose as they could not induce significant proliferation in Met e 1-primed T cells. Yet such response is still greater than those detected in the negative controls. This is similar to a previous report on the mapping of T cell epitopes of the major peanut allergen Ara h 1 that 65 of 69 peptides could stimulate T cell lines generated from peanut-allergic subjects [70]. Although the mechanism is unclear, it appears that a large number of food allergen-derived peptides could induce T cell responses. We also notice that treatment with these six non-epitopic peptides helped in resuming some local Th1 responses, which might be due to the binding of these peptides to their specific T cells that aid the maturation of dendritic cells and enhancement of Th1 cytokine production [71]. However, this is not sufficient to exert a complete antagonistic effect on the established Th2 response (i.e. a high level of splenic IL-4 and IL-5, as well as an overexpression of IL-13) or to resolve allergic responses in the non-epitopic peptides control mice, suggesting that they are only weak T cell reactive peptides. We therefore believe that the local induction of regulatory T cell responses through the oral delivery of T cell peptides is needed to confer T cell tolerance and achieve significant clinical improvements, although further analysis will be needed to validate the recruitment of regulatory T cells at the intestinal mucosa and this hypothesis.

In our study, 20-mer peptides with five amino acids overlap were used to map the T cell epitopes. We note that T cell epitope recognition is highly dependent on the MHC class II molecules and is a key player in the pathogenesis of shellfish allergy [72]. Although different allelic variants of the MHC class II molecules between mice and humans, as well as the nature of the synthetic peptides used are also factors for consideration in the identification of immunogenic sequences, comparison of the T cell epitopes identified here to that of Pen a 1 mapped from shrimp allergy subjects reveals

great similarity of the immunogenic sequences [43]. In addition, the six T cell peptides did not induce mast cell degranulation in PCA assay (unpublished data from preliminary study) even though these peptides cover IgE-reactive sequences we previously mapped [35], indicating the minimal risk of triggering adverse reactions by these peptides. It is also noteworthy that the peptide dosage used in this study is lower than those used in other similar oral PIT reports [32, 73]. Together, we believe that the epitopes identified here are of great interest and can be translated into clinical use. Yet further validation of these T cell epitopes in shellfish-allergic subjects, as well as using shorter peptides with more overlapping sequences spanning the six T cell epitopes, would help to map the epitopes more precisely for the development of oral PIT for clinical applications.

In summary, our current study presents for the first time a novel immunotherapeutic strategy that effectively ameliorates the allergic responses to shrimp tropomyosin. This peptide-based oral immunotherapy also generates significant immunological improvements that involve alterations in T cell and B cell responses, as well as decrease in the recruitment and activation of effector cells (i.e. mast cells, eosinophils and goblet cells), which are the prime targets of allergen-specific immunotherapy. Because tropomyosin is a highly cross-reactive allergen among shellfish [28], this T cell peptides-based oral therapy might be translated into immunotherapy targeting at shellfish allergy in general. More importantly, to the best of our

knowledge, we are the first to validate the capacity of peptide-based immunotherapy in inducing IgG_{2a} antibodies that possess both *in vitro* and *in vivo* blocking effects. This therapeutic model not only warrants future clinical investigations to advance the clinical management of shellfish allergy disorders, but also helps to unveil mechanistic questions, such as whether the oral delivery of T cell epitopes is a better route as the synthesis of blocking IgG antibodies seems to be exclusively promoted via this approach. The identity, recruitment mechanism and suppressive roles of the mucosal Treg cells induced in oral PIT also warrant further investigations. These will further improve the efficacy and safety of allergen-specific immunotherapy.

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Conflicts of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences used in RT-PCR analysis.

Figure S1. Representative photomicrographs of PAS-stained jejenum sections.

Figure S2. Representative photomicrographs of anti-MBP-stained jejenum sections.

Figure S3. IgG depletion of T cell peptides-treated sera.