

Induction of Shrimp Tropomyosin-Specific Hypersensitivity in Mice

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Key Words

Seafood allergy · T cell · Cytokines · Shrimp tropomyosin

Abstract

Background: Shellfish hypersensitivity is amongst the most common food allergies. The major shellfish allergen was identified as tropomyosin. Here, we investigated the immediate hypersensitivity responses, IgE and cell-mediated immune response in mice sensitized with recombinant shrimp tropomyosin. **Methods:** Shrimp tropomyosin was cloned and expressed as a His-tagged fusion recombinant protein in *Escherichia coli*. Three- to 4-week-old BALB/c mice were sensitized by intragastric administration of recombinant tropomyosin (0.1 mg) plus cholera toxin (10 µg) on days 0, 12, 19 and 26 and challenged on day 33. Mice fed with phosphate-buffered saline plus cholera toxin were included as controls. Animals were monitored for immediate hypersensitive responses and tropomyosin-specific IgE over time. In addition, shrimp tropomyosin-specific CD4⁺ T cells, interleukin-4 and interferon-γ levels were determined from in vitro splenocyte cultures. A passive cutaneous anaphylaxis assay was also conducted. **Results:** Mice fed with shrimp tropomyosin developed swelling of the snout, increased scratching behavior and shrimp tropomyosin-specific IgE.

Sera from tropomyosin-sensitized mice elicited vascular leakage in naïve mice in the passive cutaneous anaphylaxis assay. Shrimp tropomyosin-specific CD4⁺ T cell proliferations and elevated interleukin-4 over interferon-γ levels were evident in splenocyte cultures of tropomyosin-fed mice upon tropomyosin stimulation. In contrast, shrimp tropomyosin-specific IgE, CD4⁺ T cells and hypersensitive responses were absent in the control mice. **Conclusion:** We have generated a BALB/c model of shrimp allergy. This model provides a useful tool for evaluating the immunopathogenic mechanisms involved in shellfish hypersensitivity.

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Introduction

Seafood allergy is a universal health care issue [1–5] and is one of the most common forms of food allergies [6–10]. In the United States, seafood allergy affects 2.3% of the general population or approximately 6.9 million

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people [10]. This is more than double the 3 million people who confront the day-to-day challenges of peanut or tree nut allergy. Allergic reactions to seafood often develop within minutes after food ingestion. Typical symptoms include nausea, vomiting, emesis, abdominal pain and cramping, respiratory distress, low blood pressure, throat swelling and diarrhea. More severe life-threatening reactions may lead to asphyxia, vascular collapse, irregular heartbeat or myocardial infarction [11].

The US per capita seafood consumption has been increasing steadily from 15.2 lb in 2000 to 16.6 lb in 2004 (<http://www.nmfs.noaa.gov>). Common seafood in the US diet includes fish (cod, salmon and tuna, for example) and shellfish (shrimp, crab, lobster, squid, scallop, clams, mussels and snails). Notably, shellfish allergy is reported by 1 in 50 persons and is 5 times higher than the number of people having fish allergy [10]. Shrimp is the most consumed shellfish, accounting for 15.5% of the total shellfish and 51% of the total crustaceans consumed worldwide. Reports from the US National Marine Fisheries Services showed that annual consumption of shrimp has increased from 3.2 lb in 2000 to 4.2 lb in 2004 (<http://www.nmfs.noaa.gov>). It is believed that the quantity of food required to induce a reaction depends on patient sensitivity, potency of the food allergen and other unknown factors. While some people could outgrow or tolerate certain food allergens, shrimp-hypersensitive individuals are at risk to develop life-threatening anaphylaxis [12, 13].

Shrimp allergy has long been the model for studying shellfish allergies. While the major shrimp allergen has been cloned, sequenced and identified as tropomyosin more than a decade ago and its epitopes mapped [14–17], a detailed understanding of the molecular events in the induction and pathogenesis of shrimp allergy is still lacking. In patients susceptible to shrimp allergies, avoidance is virtually the only way in preventing hypersensitive reactions to shrimp. In the absence of effective treatment modalities in food allergies, appropriate animal models are often needed to investigate the mechanisms of sensitization and immune responses, as well as the possible prophylactic and therapeutic strategies to reduce the allergic responses [17–23]. Since tropomyosin is the major heat-stable shrimp allergen [14, 16] and accounts for 20% of total protein in shrimp [24], we have used the recombinant protein to elicit a focused hypersensitive response to tropomyosin in mice. Amino acid sequence comparison of the tropomyosins in common allergenic seafood showed that they are 96–100% identical in the fast tropo-

myosins, 92–98% identical in the slow tropomyosins and 88–95% between the fast and slow tropomyosins [25]. Furthermore, immunoblotting and inhibition studies demonstrated that the crustacean and mollusk tropomyosins are IgE cross-reactive [26] and thus suggest that the IgE-reactive epitopes of shellfish tropomyosin are very similar if not identical. Therefore, we have taken the shrimp tropomyosin from *Metapenaeus ensis* to study their allergenicity in BALB/c mice.

In this study, humoral, cellular and immediate hypersensitive responses were induced in mice by oral administration of recombinant shrimp tropomyosin rMet e 1. This animal model will provide a valuable tool in understanding the immunophysiological and molecular mechanisms of shellfish allergies.

Material and Methods

Induction of Shrimp Hypersensitivity in BALB/c Mice

Female BALB/c mice, 3–4 weeks of age, were acquired from the Laboratory Animal Services Centre, The Chinese University of Hong Kong (Hong Kong, SAR, China). Mice were maintained on shrimp-free diet under specific pathogen-free conditions. Mice (n = 13; referred to as the 'Trop' group) were sensitized with 0.1 mg of recombinant His-tagged (His-Tag) fusion protein of *M. ensis* tropomyosin (rMet e 1, see below for preparation) in 400 µl of phosphate-buffered saline (PBS) by intragastric gavage together with 10 µg of cholera toxin (CT) per mouse on days 0, 12, 19 and 26. One week after the final sensitization, mice were fasted overnight and challenged with intragastric administration of 1 mg rMet e 1. Mice fed with PBS + CT (PBS group, n = 8) on days 0, 12, 19, 26 and 33 were included as negative control. All mice were sacrificed on day 39. Similarly, BALB/c mice (n = 6) sensitized with a 6× His-Tag recombinant fusion protein of human pyruvate dehydrogenase E2 subunit (PDC-E2) [27] were included as an irrelevant protein control. Animal protocols were approved by the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong.

Chemicals and Reagents

Chemicals were obtained from Sigma Aldrich (St. Louis, Mo., USA) unless stated otherwise.

Preparation of Recombinant Shrimp Tropomyosin

A cDNA coding for the shrimp tropomyosin (Met e 1) [16] was cloned into the 6× His-Tag expression vector pQE30 (Qiagen, Valencia, Calif., USA) and expressed in *Escherichia coli* M15 by isopropyl β-D-thiogalactoside induction. Recombinant fusion protein of His-Tag Met e 1 (rMet e 1) was purified using Ni-NTA agarose beads (Qiagen) under native condition according to the manufacturer's instructions. In brief, frozen cells were subjected to 3 cycles of freezing and thawing and subsequently resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were incubated on ice for 30 min in the presence of lysozyme (1 mg/ml) and then sonicated 6 times for

10 s. After removing cell debris by centrifugation, the supernatant was loaded onto Ni-NTA agarose beads (Qiagen) and placed on a rocking platform overnight at 4°C. Thereafter, nonspecific binding proteins were removed by washing twice with 4 ml wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). The fusion protein was eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluate was then dialyzed against PBS at 4°C. The concentration of the recombinant fusion protein was determined by the BCA assay (Pierce, Rockford, Ill., USA) with bovine serum albumin (BSA) as the standard. Purified proteins were lyophilized, resuspended in PBS, analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis and stored at -20°C until use. The level of endotoxin in the recombinant proteins was analyzed by the Pyrotell Limulus Amebocyte Lysate kit (Associates of Cape Cod Inc., Falmouth, Mass., USA). The detection limit of the kit was 0.03 endotoxin units per milliliter using the gel-clot method. An undetectable level of endotoxin was found in the recombinant tropomyosin.

Assessment of Hypersensitivity Responses

Systemic anaphylaxis responses were evaluated for 30–40 min after challenge, using a scoring system as described [21]: 0 – no symptoms; 1 – scratching and rubbing around the nose and head; 2 – puffiness around the eyes, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate; 3 – wheezing, labored respiration, cyanosis around the mouth and the tail; 4 – no activity after prodding, or tremor and convulsion; and 5 – death.

Determination of Shrimp Tropomyosin-Specific IgE

The levels of shrimp tropomyosin-specific IgE in mice were determined by ELISA. Briefly, 96-well plates (Nunc, Rochester, N.Y., USA) were coated with rMet e 1 (10 µg/ml) in carbonate coating buffer (0.05 M Na₂CO₃, 0.13 M NaHCO₃, 0.003 M NaN₃, pH 9.6). The plates were washed with PBS and blocked with 3% BSA-PBS for 2 h. After washing with PBS/0.05% Tween-20, diluted serum samples (1/10 dilution in 3% BSA-PBS) were added to the plates and incubated overnight at 4°C. Plates were washed with PBS/0.05% Tween-20 and then incubated with 100 µl of 1 µg/ml biotin-conjugated anti-mouse IgE (Pharmingen, San Diego, Calif., USA) for 45 min. After washing, plates were incubated with 100 µl of 1 µg/ml avidin peroxidase (Pierce) for 30 min. The plates were washed and developed with 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) for 30 min, and the optical density at 415 nm was measured using a microplate reader. All incubations were carried out at room temperature, unless otherwise stated. All assays were performed in duplicate. An irrelevant antigen (BSA) was used as control throughout. Data were presented as the difference between IgE reactivity against shrimp tropomyosin and the irrelevant antigen.

Passive Cutaneous Anaphylaxis Test

The backs of 6 naïve mice (in groups of 3) were shaved, followed by intradermal injection of 100 µl undiluted pooled sera from all mice of each group (Trop and PBS), heat-inactivated pooled sera from mice in the Trop group (56°C for 3 h) and 1× PBS as sham control. Twenty-four hours later, mice were injected intravenously with a mixture of 250 µl of 0.5% Evan's blue dye and 250 µg rMet e 1. Thirty minutes after dye-antigen administration,

the mice were killed and the skin of their backs was immediately inverted. Reactions were examined for visible blue color. The diameters of the blue region were measured. A reaction was scored as positive if the blueing of the skin at the site of the injection was larger than 0.5 cm in diameter in any direction.

In vitro Splenocyte Proliferation and Cytokine Profile Determination

Mice were sacrificed 6 days after challenge. Spleens were harvested immediately from each mouse of the 3 different groups, and spleen cells were obtained from the spleen by mincing. Erythrocytes were removed from the suspensions by addition of 0.15 M ammonium chloride solution for 1 min on ice, followed by washing with Hanks' Balanced Salt Solution twice. The spleen cells were then resuspended at 4×10^7 cells/ml in 0.2% BSA/PBS and incubated with 5 µM carboxyfluorescein succinimidyl ester (CFSE) for 8 min at room temperature. After 2 washings with Hanks' Balanced Salt Solution, spleen cells were resuspended in complete culture medium of RPMI-1640 containing 10% fetal bovine serum, 1% penicillin/streptomycin. CFSE-labeled spleen cells (2×10^6 spleen cells/ml/well) were cultured in 24-well plates (Nunc) in the presence or absence of rMet e 1 antigen (100 µg/ml). Cells stimulated with 10 µg/ml phytohemagglutinin and 100 µg/ml BSA were included as positive and negative controls, respectively. The cells were incubated at 37°C in 5% CO₂ for 8 days, harvested and stained with anti-CD4 phycoerythrin. Stained cells were analyzed by 5-color flow cytometry on a FACSVantage (Becton Dickinson, San Jose, Calif., USA) using CellQuest software. The percentage of proliferative CD4+ T cells was calculated following flow cytometric analysis of CFSE expression gated on CD4+ T cells. Supernatants were collected and pooled for cytokine profile determination using the mouse cytokine antibody array kit (Raybio, Norcross, Ga., USA). The levels of 2 cytokines, interleukin (IL)-4 and interferon (IFN)-γ, from an individual supernatant sample were determined by ELISA (ELISA DuoSet, R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions. All the assays were performed minimally in duplicate. The intensity of each spot was semiquantified based on a grey scale using Kodak image analysis software. The intensity was determined by subtracting the mean background grey level from the mean intensity.

Statistics

The data are presented as the mean ± SE. The statistical significance of the data was determined by one-way analysis of variance (ANOVA) or t test using SigmaStat 3.0 (Systat Software Inc., San Jose, Calif., USA). A p value of <0.05 was considered significant.

Results

Induction of Systemic Anaphylaxis Responses after Intragastric Challenge

To develop a mouse model of shrimp tropomyosin hypersensitivity, BALB/c mice were sensitized and challenged with recombinant shrimp tropomyosin. Systemic anaphylactic responses were observed after the challenge

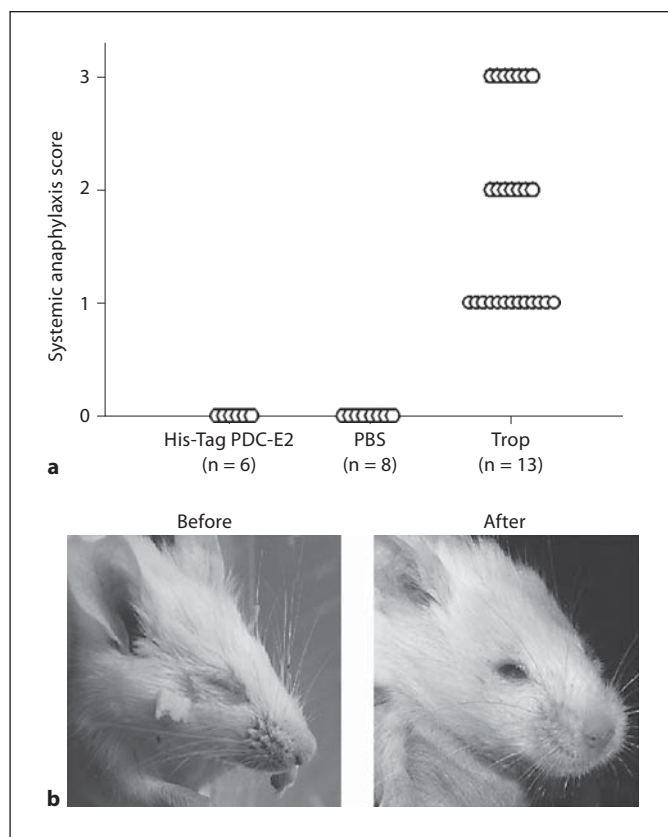


Fig. 1. **a** Systemic anaphylaxis responses after intragastric challenge. Responses of the 3 groups of mice sensitized intragastrically with rMet e 1 + CT (Trop), the 6× His-Tag recombinant fusion protein of PDC-E2 and PBS were evaluated for 30–40 min after challenge based on the scoring system described in Materials and Methods. **b** Representative reaction of rMet e 1 sensitized mice before and after intragastric challenge of tropomyosin. Note the swelling of the snout in the rMet e 1 sensitized mouse.

on day 33. Thirteen out of 13 rMet e 1 sensitized mice had symptoms of scratching around the nose and tail (symptom score 1; fig. 1a). In addition, 7/13 mice also had a cutaneous reaction with puffiness around the eyes (score 2) and cyanosis around the mouth (score 3; fig. 1b), and some of them showed respiratory symptoms such as labored respiration. These symptoms became obvious in the sensitized mice 15–20 min after challenge and peaked at 30–40 min. No animal had symptoms with a score 4 or 5. There were no correlations between the degree of symptoms and IgE level in the corresponding rMet e 1 sensitized mice. None of the mice from the PBS group and the irrelevant protein control group showed any symptoms of anaphylaxis during the course of sensitization and challenge.

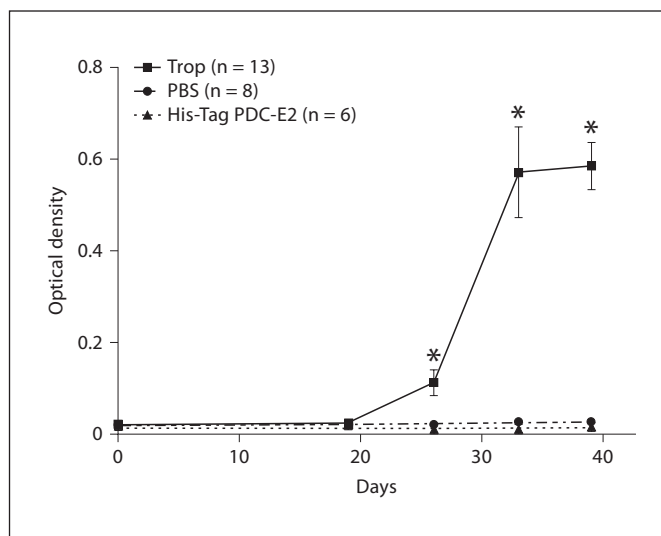


Fig. 2. Induction of serological rMet e 1 specific IgE in rMet e 1 sensitized mice. Sera from different groups of mice were collected on days 0, 19, 26, 33 and 39. The rMet e 1 specific IgE levels were determined by ELISA. Data are expressed as mean $\pm$ SE. * $p < 0.05$, significant differences between rMet e 1 sensitized mice and the PBS control. The rMet e 1 specific IgE level in mice immunized with an irrelevant fusion protein, the 6× His-Tag recombinant PDC-E2, is not significantly different from the PBS control ($p > 0.05$).

Increased Level of rMet e 1 Specific IgE after Intragastric Sensitization and Challenge

Serological IgE specific to rMet e 1 was determined using sera obtained from mice throughout the sensitization (days 0–26) and challenge (day 33) period to post-challenge (day 39). The rMet e 1 specific IgE level increased from day 19 through day 33 in the mice sensitized with rMet e 1 + CT (fig. 2). The rMet e 1 specific IgE levels were significantly higher in the rMet e 1 sensitized group than in the PBS control group, or the group immunized with an irrelevant fusion protein, 6× His-Tag recombinant PDC-E2, on days 26, 33 and 39 ($p < 0.05$, Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method of pairwise multiple comparison). The elevated level of rMet e 1 specific IgE persisted in the Trop group for at least 6 days after the challenge (day 39). In contrast, rMet e 1 specific IgE was undetectable in the PBS control group. Moreover, there were no differences in rMet e 1 specific IgE levels between the PBS control and the group immunized with 6× His-Tag recombinant PDC-E2. Since rMet e 1 specific IgE was absent in both control groups, we only used the PBS group as the control in subsequent analyses.

Table 1. PCA reactions after injection of pooled sera

Donor group	Heat inactivation	Mean diameter $\pm$ SEM, cm	Positive reaction, n/total
Trop	–	0.733 ± 0.102^a	6/6
Trop	+	0.217 ± 0.031^b	0/6
PBS	–	0.217 ± 0.031^b	0/6
Sham	–	0.167 ± 0.033^b	0/6

Pooled sera from all mice of each group were injected intradermally into 6 mice, followed by intravenous challenge of rMet e 1 with Evan's blue. Injection of PBS served as the sham control. Superscripts (a and b) indicate significant difference between groups ($p < 0.05$). Positive reaction refers to the development of a blue spot with a diameter >0.5 cm.

Induction of Passive Cutaneous Anaphylaxis Reaction by rMet e 1 Sensitized Mice Sera

We further used the passive cutaneous anaphylaxis (PCA) test to evaluate the role of IgE in the induction of shrimp hypersensitivity. A significant vascular leakage (blue spots >0.5 cm diameter) was observed when sera from rMet e 1 sensitized mice were passively transferred to naïve mice (table 1). This pattern was similar in all 6 naïve mice tested. In contrast, minor vascular leakages were observed when heat-inactivated sera from the Trop group, sera from the PBS group, or PBS (sham control) were injected. It was believed that the minor leakage was due to the damage of blood capillaries from the insertion of the syringe needle when performing the test. A similar result was also observed in the murine model of IgE-mediated cow's milk hypersensitivity [20]. The diameter of the blue spots which resulted from the injection of sera from rMet e 1 sensitized mice was significantly larger than those in the other treatments including heat-inactivated sera from the Trop group, sera from the PBS group, and the sham control ($p < 0.05$; one-way ANOVA followed by Tukey test), but there were no significant differences among the latter 3 groups ($p < 0.05$; table 1). In summary, the PCA test indicates that IgE is the reagenic antibody in our model.

Proliferation Responses of T Cells under in vitro Stimulation of rMet e 1

To determine the responses of T cells to rMet e 1, splenocytes isolated from the 2 groups of mice were labeled with CFSE and cultured for 8 days in the presence of rMet e 1, irrelevant antigen BSA or medium alone. Proliferation of CD4+ T cells from rMet e 1 sensitized mice was

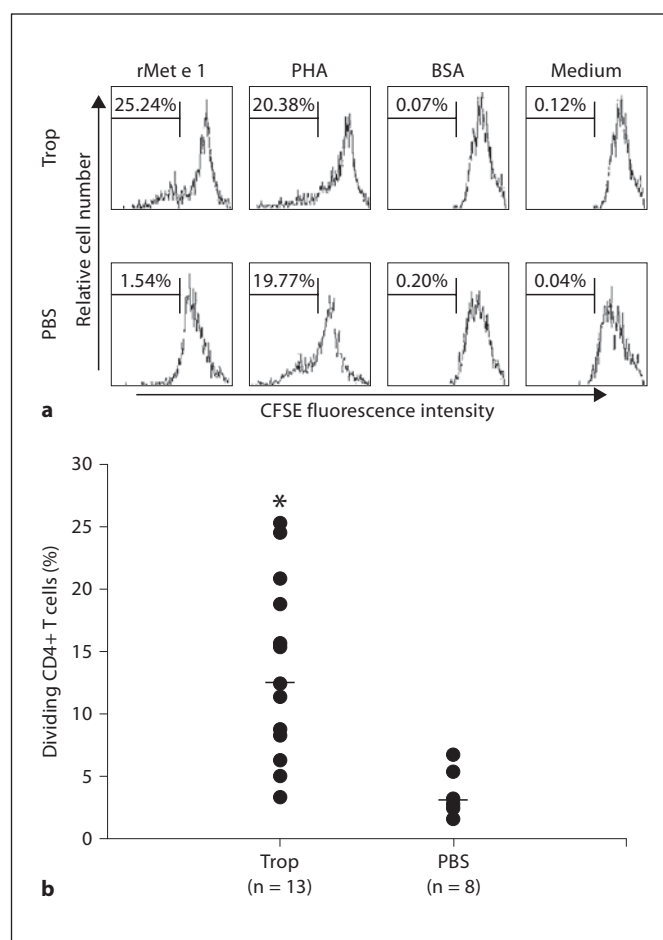


Fig. 3. **a** TROPOMYOSIN-SPECIFIC CD4+ T CELL IN rMet e 1 SENSITIZED MICE. Splenocytes from various groups of mice were first labeled with CFSE and cultured in 100 μ g/ml rMet e 1, 10 μ g/ml phytohemagglutinin (PHA), 100 μ g/ml BSA or medium alone. After culturing for 8 days, the cells were collected, stained with anti-CD4 phycoerythrin and analyzed by FACS. The intensity histograms of CFSE show the number of events gated on CD4+ T cells. Each histogram is from a representative mouse of each group. The y-axis indicates the relative splenocyte number from mice sensitized intragastrically with rMet e 1 + CT (Trop) and PBS. The x-axis represents CFSE fluorescence intensity after culturing. **b** TROPOMYOSIN-SPECIFIC PROLIFERATION OF CD4+ T CELLS FROM DIFFERENT GROUPS OF MICE. Each point represents the percentage of dividing CD4+ T cells determined in 1 mouse, and the horizontal line is the median. * $p < 0.05$, significant difference between the 2 groups.

stimulated by rMet e 1 but not by BSA or when in medium alone (fig. 3a). Furthermore, the frequency of dividing CD4+ T cells from rMet e 1 sensitized mice was significantly higher than that in the PBS control group under in vitro stimulation ($p < 0.05$; fig. 3b).

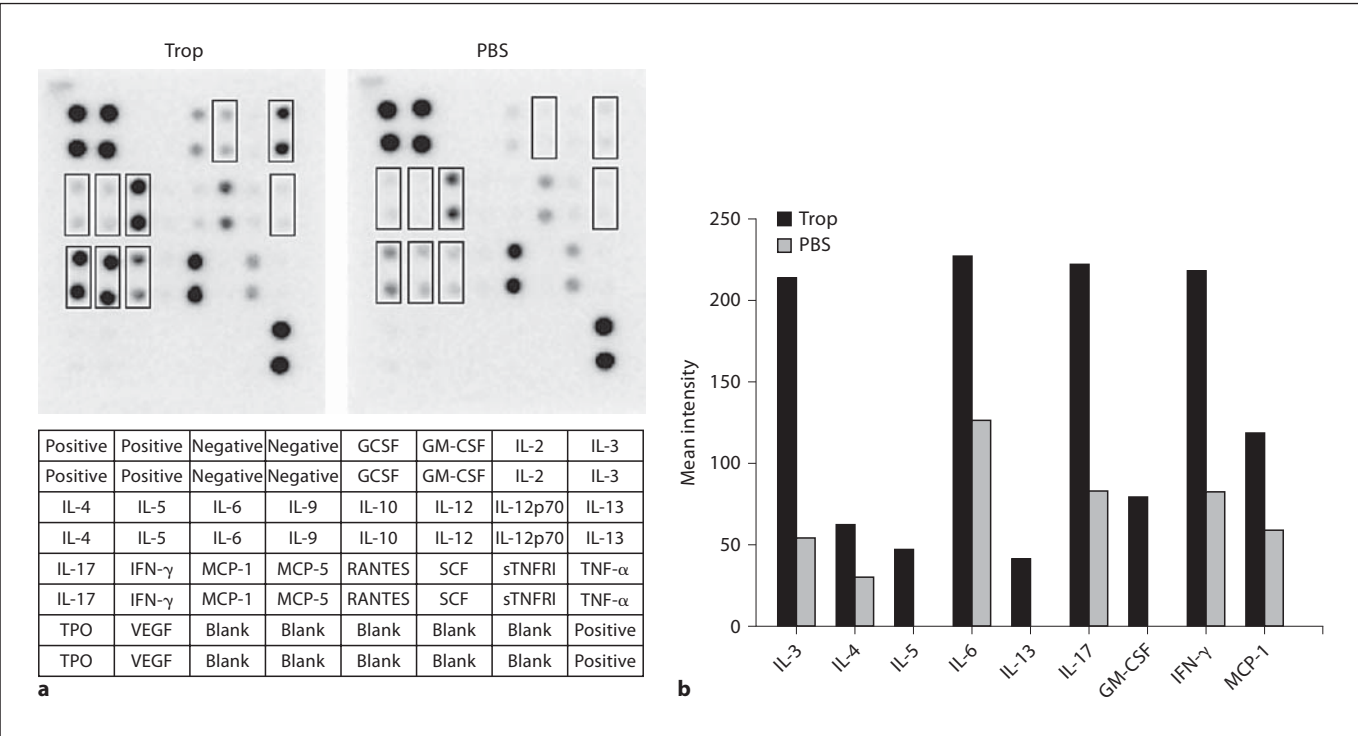


Fig. 4. Cytokine profile of rMet e 1 sensitized and control mice. **a** Culture supernatants from individual spleen cell culture of different groups of mice were collected and pooled for analyzing the expression profile of cytokines by cytokine antibody array. Elevated levels of IL-3, IL-4, IL-5, IL-6, IL-13, IL-17, GM-CSF, IFN- γ and monocyte chemoattractant protein (MCP-1) were observed in rMet e 1 sensitized mice. The boxes represent the types of cytokines showing elevated expression level in rMet e 1 sensitized

mice. GCSF = Granulocyte colony-stimulating factor; SCF = stem cell factor; sTNFRI = soluble tumor necrosis factor receptor I; TNF- α = tumor necrosis factor- α ; RANTES = regulated upon activation normal T cell expressed and secreted; TPO = thrombopoietin; VEGF = vascular endothelial growth factor. **b** Intensities of cytokine spots in rMet e 1 sensitized and PBS control mice. The values represent the average intensities of the spots from 2 independent experiments.

Characterization of the Cytokine Profile of Restimulated Spleen Cells

Splenocyte cytokine profiles from the 2 groups of mice were assayed by cytokine antibody array. In spleen cell cultures of rMet e 1 sensitized mice, levels of IL-3, IL-4, IL-5, IL-6, IL-13, IL-17, IFN- γ , granulocyte macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant protein 1 were elevated when compared with mice in the PBS group (fig. 4).

To further determine the levels of IL-4 and IFN- γ produced in the splenocyte culture supernatant, their levels were measured by ELISA. The level of IL-4 was significantly higher in rMet e 1 sensitized mice than that of PBS-fed mice (18.9 ± 0.2 vs. 0.0 ± 0.2 pg/ml; $p < 0.05$). In addition, the IFN- γ level was also significantly higher in spleen cell cultures of rMet e 1 sensitized mice when compared with the PBS-fed group (683.3 ± 116.5 vs. 88.4 ± 115.1 pg/ml; $p < 0.05$). We also calculated the ratio of IL-

4 to IFN- γ , and the comparison showed that the ratio was significantly higher in the rMet e 1 sensitized mice than in the control group ($p < 0.05$; t test after arcsine transformation). These results suggested a predominance of Th2 response in rMet e 1 sensitized mice.

Discussion

Advances in molecular biology and immunology have revealed the molecular identity of shellfish allergen as tropomyosin [28–30]. Unlike some other food allergies such as egg allergies, shrimp allergy often persists for life and can lead to life-threatening anaphylactic reactions after exposure to minute amounts of shrimp allergen [13]. To further study the mechanism of the hypersensitive response and develop an effective immunotherapeutic regimen for shellfish allergy, appropriate animal models are

eminent. Here, we demonstrated that shrimp tropomyosin-specific hypersensitivity responses, including increase in scratching frequency and changes in snout morphology, can be induced by oral sensitization of BALB/c mice with recombinant tropomyosin. Such hypersensitive responses have also been reported in established animal models of hypersensitivity to peanut [21, 22] and ovalbumin [31]. In addition, tropomyosin-specific IgE was detected in sera of BALB/c mice sensitized with recombinant tropomyosin, in the presence of CT. Significantly higher levels of tropomyosin-specific IgE antibody was detected in mice fed with 0.1 mg tropomyosin plus adjuvant after challenge, as compared with controls. Interestingly, in our preliminary studies, we noted that the level of IgE produced in mice fed with 0.1 mg of rMet e 1 was higher than those found in mice fed with 1 or 2 mg of rMet e 1 which were not significantly different from levels in control mice. Moreover, these mice when treated with 1 or 2 mg rMet e 1 did not develop anaphylaxis (data not shown). A similar observation was reported in a peanut allergy animal model, in which C3H/HeJ mice sensitized with 5 mg of whole peanut developed a higher IgE level and more severe anaphylactic responses than those fed with 25 mg whole peanut [21]. Likewise, oral sensitization of C3H/HeJ and CBA/J mice with a high dose of shrimp extract also had reduced IgE response compared with lower-dose sensitized mice [17]. Thus, there could be a threshold concentration of allergen exposure such that concentrations above the threshold may result in oral tolerance rather than in hypersensitivity. Strobel [32] also demonstrated that the concentration of allergen is a major factor in determining allergenicity or oral tolerance in mice. Our laboratory has demonstrated the presence of rMet e 1 specific IgE in sera from patients with shrimp allergy [16] by immunoblotting. In this study, we showed that rMet e 1 sensitized BALB/c mice also developed rMet e 1 specific IgE by ELISA and immunoblotting (data not shown). Thus, it is evident that both shrimp allergy patients and rMet e 1 sensitized BALB/c mice develop specific IgE reactivities to the same protein.

The specificity of tropomyosin-specific IgE-mediated hypersensitive response was also illustrated in the PCA assay. Similar to the tropomyosin-specific IgE levels, vascular leakage was clearly evident using sera from mice of the Trop group. In contrast, sera from mice of the PBS group as well as heat-inactivated sera from the rMet e 1 sensitized mice only induce a minor vascular leakage in the PCA assay. Our preliminary work showed that sera from mice fed with 1 and 2 mg of rMet e 1 only produced negligible vascular leakage (<0.3 cm) in PCA (data not

shown). This is likely due to the lower level of tropomyosin-specific IgE in these mice.

In addition to the observed humoral immune response, antigen-specific cellular-mediated immune response was also generated in tropomyosin-sensitized mice but not in control mice. Numerous studies have demonstrated that Th2 cytokines play a central role in the pathogenesis of allergic disorders including food allergy [33, 34]. An elevated level of IL-4 was found in the mice fed with 0.1 mg tropomyosin with adjuvant as compared with mice fed with PBS ($p < 0.05$). Furthermore, the ratio of IL-4:IFN- γ in the rMet e 1 immunized mice was also significantly higher than in the control mice, which is consistent with the Th2 response. However, cytokine array data showed that in addition to IL-4, the levels of other cytokines, notably IL-6, IFN- γ and IL-17, were also elevated in splenocyte cultures from mice sensitized with tropomyosin compared with mice fed with PBS. Interestingly, a mixed Th1/Th2 cytokine response has also been reported in a number of oral sensitization models of peanut allergen [35, 36] and buckwheat flour extract [37]. In contrast to the conventional Th1/Th2 model, Th17 cells which secrete IL-17 have been recently shown to be important in various models of immune-mediated tissue injury, including allergic disorder of the lung and skin [38–41]. Moreover, IL-6 along with transforming growth factor- β induces Th17 T cells [42]. Interestingly, GM-CSF, which is a cytokine important in airway inflammation, is also modulated by IL-17. However, there is no previous report regarding IL-17 in food allergy. Clearly, more studies are necessary to further understand the role of IL-17 in food allergy. Future work will be directed to dissecting the role of IL-17 in food allergy using our BALB/c model of shrimp tropomyosin food hypersensitivity. We believe that there is a complexity of cytokine interactions in the development of food allergy. The mechanism is more complicated than the traditional Th1/Th2 dichotomy and likely to involve a complex interplay and fine-tuning of multiple cytokines.

A number of sensitization protocols such as intraperitoneal immunization of food allergen in the presence of adjuvant [43], oral immunization with mucosal adjuvant CT [20, 21], and more recently, epicutaneous sensitization of animals with the allergen without adjuvant [31], have been applied in the development of animal models of food allergy. Clinically, food allergy is also associated with a number of dermatological reactions [44]. Previous work has demonstrated that food allergy can be induced by allergen exposure via the skin [31]. In addition, aerosol

and dermal exposure due to unprotected handling of seafood and its byproducts may account for part of the occupational allergy in fishing and seafood-processing industries [45, 46]. Similar to the cow milk allergy mouse model [20], our model requires multiple sensitizations as opposed to a single sensitization in the peanut allergy model [21]. The specific induction of hypersensitivity via multiple feeding of shrimp tropomyosin shows that shrimp hypersensitivity can be induced in BALB/c mice by the gastrointestinal route. The significance of other routes of allergen exposure in shrimp-induced food hypersensitivity remains to be investigated.

A number of studies have implicated that the selection of mouse strains is important in the development of experimental animal models [17, 47]. Previous work by Reese et al. [17] reported that different mouse strains and different adjuvants have distinct differences with respect to the induction of IgE response. Their data showed that IgE response to shrimp extract could be induced in C3H/HeJ, CBA/J and BALB/c mice by an IgE-mediated hexosaminidase activity assay, with higher response in C3H/HeJ and CBA/J mice than in BALB/c mice. However, data on IgE recognition of sensitized mice sera to shrimp tropomyosin, shrimp tropomyosin-specific T cell response and immediate hypersensitive response in sensitized mice were lacking. In contrast, our preliminary studies using BALB/c and C3H/HeJ mice under the same sensitization and challenge conditions with purified recombinant shrimp tropomyosin showed that although there is no significant difference in the response to IgE production between the 2 strains, only BALB/c showed a significant elevation of IgE level when compared with the PBS control ($p < 0.05$; Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method of pairwise multiple comparison). Thus, it is likely that there are differences, at least in the degree of hypersensitive response, between mouse strains when they are sensitized with shrimp extract as opposed to purified recombinant shrimp tropomyosin. It should be noted that the induction of food allergen-specific IgE, T cell response and Th2

cytokines has been reported in BALB/c mice sensitized orally with cow's milk and peanut allergens [36, 48–50]. In this study, we have successfully induced shrimp tropomyosin-hypersensitive response in BALB/c mice. The specific production of tropomyosin-specific IgE, the elevation of tropomyosin-specific IgE upon challenge and the positive PCA assay with tropomyosin-sensitized mouse sera together with the specific proliferation of CD4⁺ T cells by rMet e 1 and the elevation of IL-4 demonstrate that both humoral and cell-mediated components are involved in this BALB/c mice model of shrimp tropomyosin-induced hypersensitivity.

Future studies will be directed at investigating the mechanism and characteristics of the sensitization and/or the elicitation phase of the allergic reaction as it occurs in atopic humans. These studies are necessary because sensitization studies can obviously not be done in humans and in vitro tests cannot reproduce the complexity of the immune system. Pathogenesis of food allergy in humans is very complex and multifactorial, including variations in susceptibility at different ages, genetic susceptibility, environmental factors, conditions of exposure, allergenicity of antigens and the amount of allergen uptake for an individual. While no animal model can take into account all these factors and produce reliable predictions on the prevalence and severity of allergic reactions, our BALB/c animal model processes most of the critical criteria of an allergen-specific hypersensitive response and will thus allow point by point analysis of the mechanism of food-induced hypersensitive reactions.

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