



Two allergens from *Scylla paramamosain* share common epitopes showed different allergenic potential in Balb/c mice

Yang Yang^{a,b}, Xin-Rong He^a, Shao-Gui He^b, Meng Liu^a, Yong-Xia Zhang^a, Fei Xia^a, Min-Jie Cao^a, Wen-Jin Su^a, Guang-Ming Liu^{a,*}

^a College of Ocean Food and Biological Engineering, Jimei University, 43 Yindou Road, Xiamen, Fujian 361021, China

^b College of Environment and Public Health, Xiamen Huaxia University, 288 Tianma Road, Xiamen, Fujian 361024, China

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ABSTRACT

Filamin C (FLN c) and triosephosphate isomerase (TIM) are novel allergens of crab (*Scylla paramamosain*) which are sharing common epitopes. This work aimed to assess their contributions to the induction and elicitation of allergenic responses. Balb/c mice were sensitized by intraperitoneal injections and challenged by intragastric gavage with purified proteins. Upon oral challenge, FLN c triggered more severe anaphylactic symptoms, higher levels of specific antibodies and histamine in serum than TIM, while TIM was a more active promotor of early specific antibody production and stimulated stronger Th2-biased responses. Combined with the results of *in vitro* assays, the data demonstrated that though with common epitopes, the two allergens showed a different allergenicity, TIM favored Th2 polarization in sensitization stage, while FLN c had a better ability to stimulate B cells and is highly immunogenic in oral challenge stage. The findings can help with the better understanding of allergenicity of crab allergens.

1. Introduction

Food allergy is a pathological reaction of the immune system triggered by the ingestion of a food allergen (Yu, Freeland, & Nadeau, 2016). Exposure to very small amounts of allergenic foods can trigger clinical symptoms such as gastrointestinal disorders, urticaria and airway inflammation, ranging in severity from mild to life-threatening (Yu et al., 2016). Food allergy has become a major issue related to food safety, there are extensive data to suggest that food allergies are common (up to 10% of population affected), and that a rather short list of foods account for most of the more serious disease burden, namely peanuts, tree nuts, fish, shellfish, eggs, milk, wheat, sesame, and seeds (FAO/WHO, 2021). The hypersensitivity reaction is generally influenced by regional factors, genetics, dietary habits and traditions, e.g., tree nut allergy is common in the USA, while shellfish allergy is fairly prevalent in Asia (Smeekens, Bagley, & Kulis, 2018; Moonesinghe, et al., 2016).

Allergy to crabs, a prominent form of shellfish allergy, belongs to type I hypersensitivity reaction, which is mediated by the binding of allergens to specific immunoglobulin (Ig) E (Khora, 2016). The characterization of food allergens, including the physicochemical feature and allergenicity, is an important issue of food chemistry. Over the past decades, a variety of crab allergens have been identified and analyzed. Tropomyosin and arginine kinase are major allergens found in crab with the ability to trigger severe anaphylactic reaction *in vivo* (Faber et al., 2017; Lv et al., 2019; Ruethers et al., 2018). In addition, several other allergenic components have been identified in crab. Sarcoplasmic calcium-binding protein and myosin light chain are novel allergens with various epitopes that have been identified and their antigenicity has been assessed (Yang et al., 2019; Hu et al., 2017; Li et al., 2019). In our recent study, filamin C (FLN c) and triosephosphate isomerase (TIM) are novel allergens of crab and crayfish (*Procambarus clarkii*), which share common epitopes (Yang et al., 2017a; Yang et al., 2019; He et al., 2020).

TIM has been identified by the International Union of Immunological

Abbreviations: AA, amino acid; APC, allophycocyanin; BSA, bovine serum albumin; CFSE, carboxyfluorescein diacetate succinimidyl ester; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FLN c, filamin C; GM-CSF, granulocyte-macrophage colony-stimulating factor; HRP, horse radish peroxidase; mIL-4, mouse interleukin-4; MLN, mesenteric lymph nodes; NTA, Ni²⁺-Nitrilotriacetic acid; PE, phycoerythrin; Percp, peridinin chlorophyll protein; TBST, Tris-HCl buffer solution-Tween; Th2, T helper cell 2; TIM, triosephosphate isomerase; TMB, 3,3',5,5'-tetramethylbenzidine.

* Corresponding author at: College of Ocean Food and Biological Engineering, Jimei University, Xiamen 361021, Fujian, China.

E-mail address: gmlu@jmu.edu.cn (G.-M. Liu).

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Societies as a food allergen in seafood (Lopata, Kleine-Tebbe, & Kamath, 2016). In our previous research, TIM was identified as an allergen in crayfish and octopus (*Octopus fangsiao*) (Yang et al., 2017a; Yang et al., 2017b). During our investigation on crayfish TIM, FLN c attracted our attention for its IgG and IgE cross-reactivity with TIM. The cross-reactivity was then proved to be result from their common epitopes in subsequent research (Yang et al., 2017a). Following the first time that FLN c has been recognized as an allergen in crayfish, FLN c and TIM showed common epitopes were identified in crab (*Scylla paramamosain*) (Yang et al., 2019). Despite sharing common amino acids in the epitopes, FLN c induced much higher IgE cross-linking and higher degree of basophil activation than TIM in an *in vitro* basophil activation test. Moreover, patients who also recognized FLN c suffered more severe clinical symptoms (Yang et al., 2019). To clarify the discrepancy between these two allergens, further investigation on the mechanism by which FLN c and TIM act during anaphylaxis should be investigated.

The induction of sensitization and triggering of anaphylaxis to food proteins is a poorly understood and complex process. Numerous innate immune cells, including dendritic cells, basophils, innate lymphoid cells and others all interact to elicit ultimately T helper cell 2 (Th2) immune responses leading to IgE induction (Sampson, O'Mahony, Burks, Plaut, Lack, & Akdis, 2018). Not only host factors are involved in this process, but quantity, frequency and site of allergen contact are also important (Ekezie, Sun, & Cheng, 2019). The properties and epitopes of FLN c and TIM are well-known, however, compared with other crab allergens, investigation on the abilities of FLN c and TIM to promote sensitization and elicitation of allergic reactions are lacking. No research on the relationship between the allergen properties, epitopes, and the capacity of triggering anaphylaxis has been reported. As a consequence, there is a clear need to use models that take the multifactorial aspects of the sensitization and elicitation phase into account.

Balb/c mice is the most common mouse strain used for developing a murine model of food allergy as it is a good developer of Th2 and elicit IgE mediated immunological response for antigen challenges (Arámburo-Gálvez et al., 2018). Balb/c mice were used to compare the ability of FLN c and TIM to promote sensitization and elicitation of allergic reactions. The effects of FLN c and TIM on the serum antibody level, cytokine responses, lymphocyte proliferation, and the development of clinical signs upon challenge were assessed. Thus, to address the capacity of the two allergens to sensitize and trigger the manifestations, and to further clarify the relationship between their epitopes and allergenicity. The allergic potential of two cross-reactive allergens investigated in the present study might provide valuable insights for further research into different allergens in various other types of allergenic foods.

2. Materials and methods

2.1. Chemicals

Inject Alum was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The horse radish peroxidase (HRP)-conjugated goat anti-mouse IgE, IgG1, and IgG2a antibodies were obtained from Abcam (Cambridge, UK). 3,3',5,5'-tetramethylbenzidine (TMB) was from TIAN-GEN (Beijing, China). ELISA kits of interleukin (IL)-4, IL-13, IL-10, IL-17, interferon (IFN)- γ , and histamine were purchased from R&D Systems (Minneapolis, MN, USA.) and IBL (Hamburg, Germany), respectively. RPMI 1640, fetal calf serum (FCS), and fluorescein isothiocyanate (FITC) were purchased from Invitrogen (New York, NY, USA). Recombinant proteins of the mouse IL (mIL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech (Rocky Hill, NJ, USA). The carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Invitrogen (Oslo, Norway). All antibodies for flow cytometry analysis were purchased from Biolegend (San Diego, CA, USA).

2.2. Animals

Live crab (*S. paramamosain*) was purchased from a local aquatic products market in Xiamen, China in June 2019. The obtained samples were handled with care by placing them in polystyrene boxes, which were filled with water, twice that of the sample weight. The crabs were immediately transported to Jimei University within approximately 30 min. On arrival to the laboratory, crabs were subjected to washing using water ($\sim 20^{\circ}\text{C}$) to remove extraneous matter.

Specific pathogen-free female Balb/c mice, 4–6 weeks of age, were obtained from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). Mice were housed in a specific pathogen-free conditions and maintained at $22 \pm 1^{\circ}\text{C}$ with a relative humidity of $55 \pm 10\%$. No crab protein was present in the diet. All protocols involving animals was performed in strict accordance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85–23 Rev. 1985). Protocols were approved by the Institutional Animal Care and Use Committee of Animal Laboratory Center of Jimei University (Xiamen, China, No.).

2.3. Purification of allergens

The isolation and purification of native FLN c from crab myosinogen was performed as described previously (Yang et al., 2017a). Recombinant TIM from crab was expressed in *Escherichia coli* and purified by nickel-affinity chromatography (Xia et al., 2019). The identity of the purified protein was confirmed by western blot using rabbit anti-crab FLN c polyclonal IgG antibody and rabbit anti-crayfish TIM polyclonal IgG antibody as the primary antibodies and the bovine serum albumin (BSA) as negative control (He et al., 2020; Xia et al., 2019). The purified allergens were further analyzed by MALDI-TOF/TOF (Winnvate Bio, Shenzhen, China).

2.4. Sensitization and challenge protocols

The mice were sensitized and challenged by purified allergens as previously reported (Liu et al., 2018). For sensitization, mice were exposed to 150 μL of purified FLN c or TIM (200 $\mu\text{g}/\text{mouse}$, $n = 9$) with alum adjuvant by intraperitoneal (i.p.) injection on days 0 and 14. Blood was collected on day 15, and 3 mice from each group were sacrificed for the collection of splenic lymphocytes. From day 28, mice were orally challenged with 10 mg purified FLN c or TIM in saline every 3 days, for a total challenge of 5 times. All mice received the challenge of FLN c or TIM intragastrically were sacrificed 1 day later to collect the blood and mesenteric lymph nodes (MLN) lymphocytes (Fig. 2A). Mice which received phosphate-buffered saline served as controls.

2.5. Measurement of specific antibodies and histamine

The allergen-specific IgE, IgG1 and IgG2a levels in sera were determined by indirect ELISA (Zhang, Xiao, & Zhou, 2019). Briefly, the wells of a microtiter plate (Nunc Maxisorb, Copenhagen, Denmark) were coated overnight at 4°C with 150 $\mu\text{g}/\text{well}$ purified allergens and blocked with 5% skimmed milk in Tris-HCl buffer solution-Tween (TBST, 20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0) at 37°C for 2 h. The wells were then incubated with 100 μL diluted sera (dilution 1:5) at 37°C for 2 h. The specific antibodies were detected with 100 μL of HRP-conjugated goat anti-mouse IgE (dilution 1:2000), HRP-conjugated goat anti-mouse IgG1 (dilution 1:5000), or HRP-conjugated goat anti-mouse IgG2a (dilution 1:5000). The reactions were developed with 100 μL of TMB substrate solution and terminated with 2 M H_2SO_4 . The absorbance at 450 nm was measured on an automatic microplate reader (Infinite M200 PRO, Tecan, Austria). The plates were washed 5 times with TBST between each step. The 1% skimmed milk in TBST was used as diluent for the sera and HRP-conjugated antibodies. For the measurement of histamine, sera were collected 1 h after the last challenge, and the

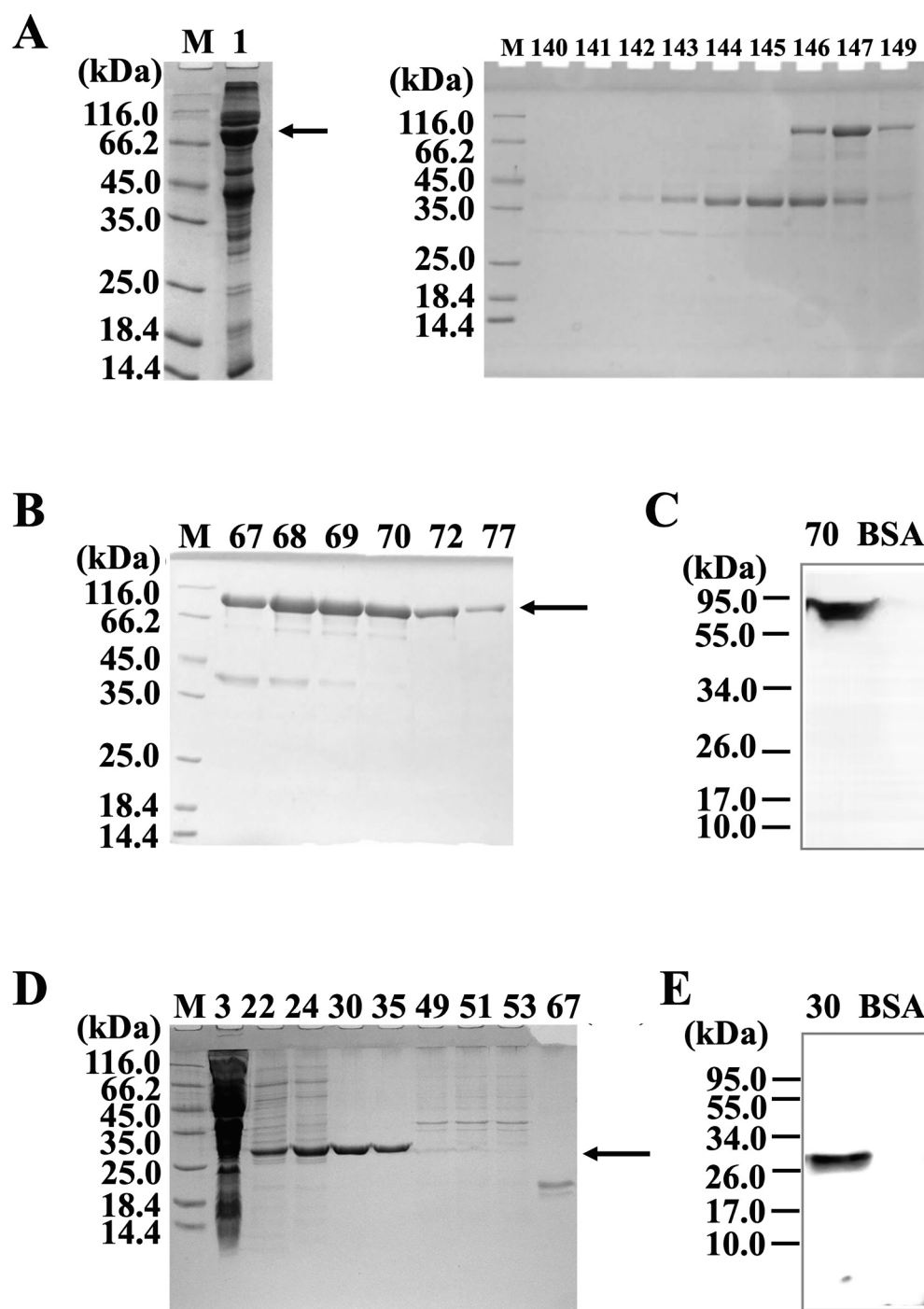


Fig. 1. Purification and identification of *S. paramamosain* FLN c and TIM. A: SDS-PAGE analysis of the crab muscle protein after the first Q-Sepharose chromatography. Lane M, protein marker; lane 1, the crab muscle protein after ammonium sulfate precipitation; Lane 140 ~ 149, the elution fraction number by the Q-Sepharose chromatographic column. B-C: SDS-PAGE (B) and western blot (C) analysis of the protein after the second Q-Sepharose chromatography. Lane M, protein marker; lane 67, 68, 69, 70, 72, 77, the elution fraction number by the Q-Sepharose chromatographic column. D-E: SDS-PAGE (D) and western blot (E) analysis of expressed recombinant protein in *E. coli* after Ni^{2+} -NTA chromatography. Lane M, protein marker; lane 3, 22, 24, 30, 35, 49, 51, 53, 67, the elution fraction number by the Ni^{2+} -NTA chromatographic column.

concentration of histamine in sera were determined using commercially available ELISA kits following the manufacturer's instructions.

2.6. Subpopulation assay and cytokine measurements of immune cells

Splenic lymphocytes from each group were prepared by aseptic removal on day 15 as previously reported (Pablos-Tanarro, Lozano-Ojalvo, Molina, & López-Fandiño, 2018a). To detect the effects of allergens on the cell population of T/B lymphocytes, the isolated splenic lymphocytes were stained with allophycocyanin (APC) anti-mouse CD3, phycoerythrin (PE) anti-mouse CD4 and peridinin chlorophyll protein (Percp)/cy5.5 anti-mouse CD19 and analyzed by flow cytometry (Focke-Tejkl et al., 2014).

Splenic lymphocytes from individual mice pooled from each group were cultured in 24-well plates (2×10^6 cells/well) and stimulated with FLN c or TIM (100 $\mu\text{g}/\text{mL}$), and RPMI-1640 medium as a negative control. Supernatants were collected after 72 h at 37°C in 5% CO_2 to measure the release of the cytokines IL-4, IL-13, IL-17, and IFN- γ , using ELISA kits following the manufacturer's instructions.

For the measurement of lymphocyte proliferation, MLN lymphocytes were collected and processed under sterile conditions immediately after the mice were sacrificed on day 37 (Pablos-Tanarro et al., 2018a). Cells were labeled with CFSE according to the method of Focke-Tejkl et al. (2014) and then cultured with purified allergens (10 $\mu\text{g}/\text{well}$), or medium alone (negative control), for 7 days at 37°C in 5% CO_2 . Cells were then stained with APC anti-mouse CD3, PE anti-mouse CD4 and Percp/

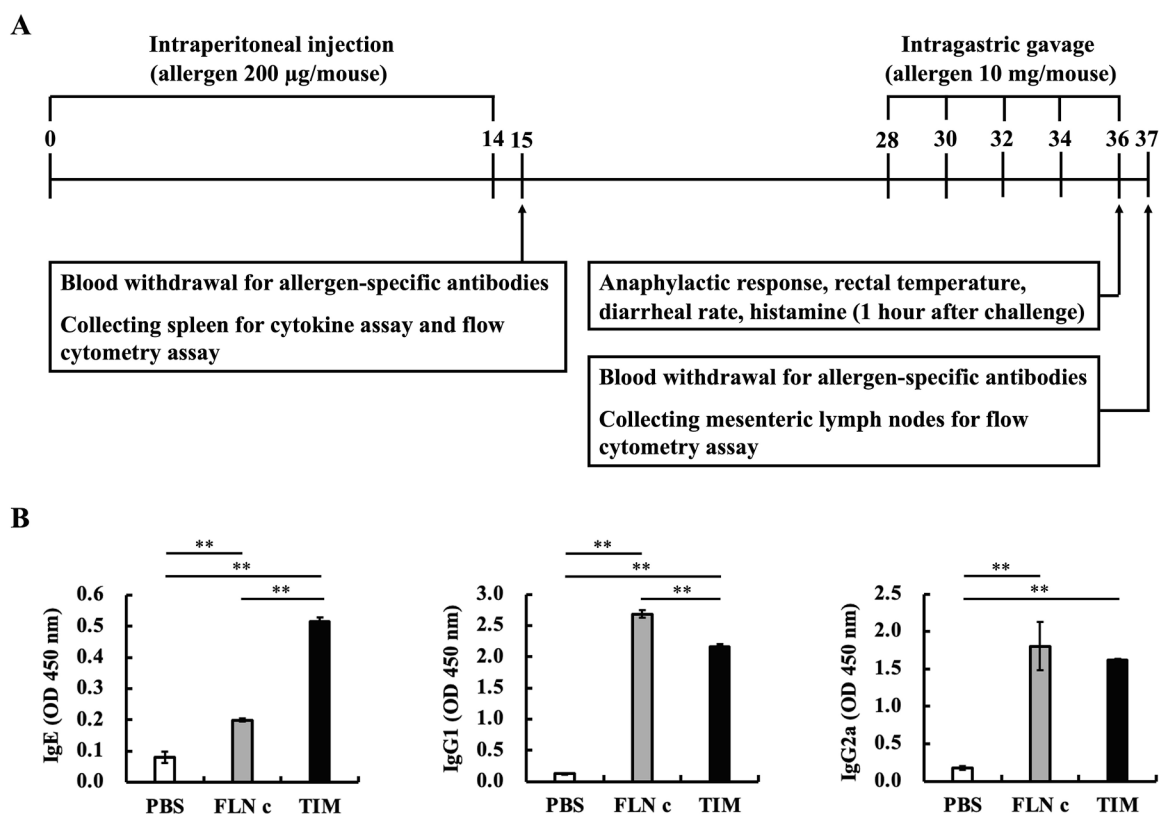


Fig. 2. Design of animal experiments and effects of allergens on specific antibodies in sensitized mice. **A:** Protocol for the food allergy model. Groups of mice ($n = 9$ mice per group) were sensitized twice by intraperitoneal injection of purified allergen (200 µg/mouse) and orally challenged with 10 mg of purified allergen five times every 3 days. **B:** Effect of allergens on specific-IgE, IgG1, and IgG2a in the sensitization stage. The sera from eyeball blood at day 15 were collected for ELISA. All experiments were repeated three times, the data represent the mean $\pm$ SD. Statistical differences are indicated by p values (one-way ANOVA), $*p < 0.05$, $**p < 0.01$. The specific antibody level of sham-sensitized mice, FLN c-sensitized mice, and TIM sensitized mice were showed by white bars, grey bars, and black bars, respectively.

cy5.5 anti-mouse CD19 and analyzed by flow cytometry.

2.7. Measurement of anaphylactic symptoms, diarrhea rates, and rectal temperatures

Anaphylactic symptoms and diarrhea rates were evaluated 1 h after the last challenge. Clinical signs were graded with the following scale: 0 = no signs; 1 = scratching nose and mouth less than ten times in 15 min; 2 = puffiness around eyes and mouth, scratching nose and mouth more than ten times in 15 min; 3 = wheezing and labored respiration, cyanosis around the mouth and tail, difficulty in walking normally; 4 = no activity after prodding; and 5 = death (Pablos-Tanarro, Lozano-Ojalvo, Martínez-Blanco, Molina, & López-Fandiño, 2018b). Rectal temperatures were measured for 0–1 h after the last challenge using a rectal electronic thermometer (Prosper, Shanghai, China).

2.8. In silico prediction of T- and B-cell epitopes

The sequences of FLN c and TIM were computed into the NetMHCII 2.3 server (<http://www.cbs.dtu.dk/services/NetMHCII/>) and IEDB MHC Class II Binding Prediction software (<http://tools.iedb.org/mhcii/>) in order to predict the T cell epitopes, the default IEDB recommended, NetMHCII pan 3.2, NN align, and NetMHCII 2.3 server prediction methods were selected (Jensen et al., 2018). For B cell epitopes, the DNASTAR Protein (<https://www.dnastar.com>), BepiPred 1.0 server (<http://www.cbs.dtu.dk/services/BepiPred-1.0/>), Immunomedicine Group (<http://imed.med.ucm.es/Tools/antigenic.pl>), and IEDB Antigen Sequence Properties (<http://tools.iedb.org/bcell/>) were used (Soria-Guerra, Nieto-Gomez, Govea-Alonso, & Rosales-Mendoza, 2015). The

regions recognized frequently by three or more than three tools were selected.

2.9. Statistical analysis

All experiments were repeated at least three times, the results were presented as the means $\pm$ SD. Data were analyzed by the General Linear Model and ANOVA of Duncan's test. The threshold p value for significance was set at 0.05.

3. Results and discussion

3.1. Purification and identification of FLN c and TIM

According to our previously reported method (Yang et al., 2017a), the fractions eluted from the first Q-Sepharose column that contained the 90-kDa protein were pooled (Fig. 1A) and separated on the second Q-Sepharose column (Fig. 1B), one major band with a molecular mass of approximately 90 kDa was eluted and recognized by the rabbit anti-FLN c IgG polyclonal antibody (Fig. 1C). The amino acid sequence of the protein was further analyzed (Supplementary Information, Fig. S1), a total of 3 peptides had 100% identity with the amino acid sequences of FLN c from *S. paramamosain* (gi|338224387). The recombinant TIM was purified using Ni^{2+} -Nitrilotriacetic acid (NTA) chromatography. The eluted 28-kDa protein (Fig. 1D) that can be recognized by the rabbit anti-TIM IgG polyclonal antibody (Fig. 1E) was subjected to mass spectrometry, six peptides were 100% identical to the sequences of TIM of *S. paramamosain* (gi|1122816254) (Supplementary Information, Fig. S2).

3.2. Sensitizing capacity of the allergen

An animal model of allergy was used to test the immunogenicity of allergens *in vivo* following i.p. injection using the protocol shown in Fig. 2A. As has been described, numerous IgE-binding epitopes evenly distributed along the structure of the two allergens (Yang et al., 2019), the same amount of each protein was used for sensitization and challenge, to provide mice with equivalent epitope loads, following an approach of another comparative study (Liu et al., 2017). The immune system produces an allergic response by the release of specific antibodies upon exposure to an allergen. Thus, on the 15th day, the levels of allergen-specific IgE, IgG1 and IgG2a in sera were measured. It can be observed that mice were sensitive to both allergens. FLN c-sensitized and TIM-sensitized mice had significantly higher levels of specific IgE, specific IgG1, and specific IgG2a than the PBS-treated control group

(Fig. 2B). In addition, the specific IgE level in TIM group was two-fold higher than that in the FLN c group (Fig. 2B), indicating that the *in vivo* immunogenic capacity of TIM is higher than FLN c. While the levels of IgG1 and IgG2a in FLN c group were higher than that in TIM group (Fig. 2B).

3.3. Effects of the allergens on the splenic lymphocytes

To examine the change in subpopulation of splenic lymphocytes in the three groups of mice, flow cytometry experiments were performed (Fig. 3A). The percentage of CD19⁺ B cells in splenocytes increased from $13.52 \pm 0.42\%$ (sham-sensitized mice) to $15.85 \pm 1.43\%$ (FLN c-sensitized mice) and $40.66 \pm 2.00\%$ (TIM-sensitized mice), respectively. The percentage of CD19⁺ B cells in splenocytes from TIM-sensitized mice was significantly higher than that of FLN c-sensitized mice and sham-

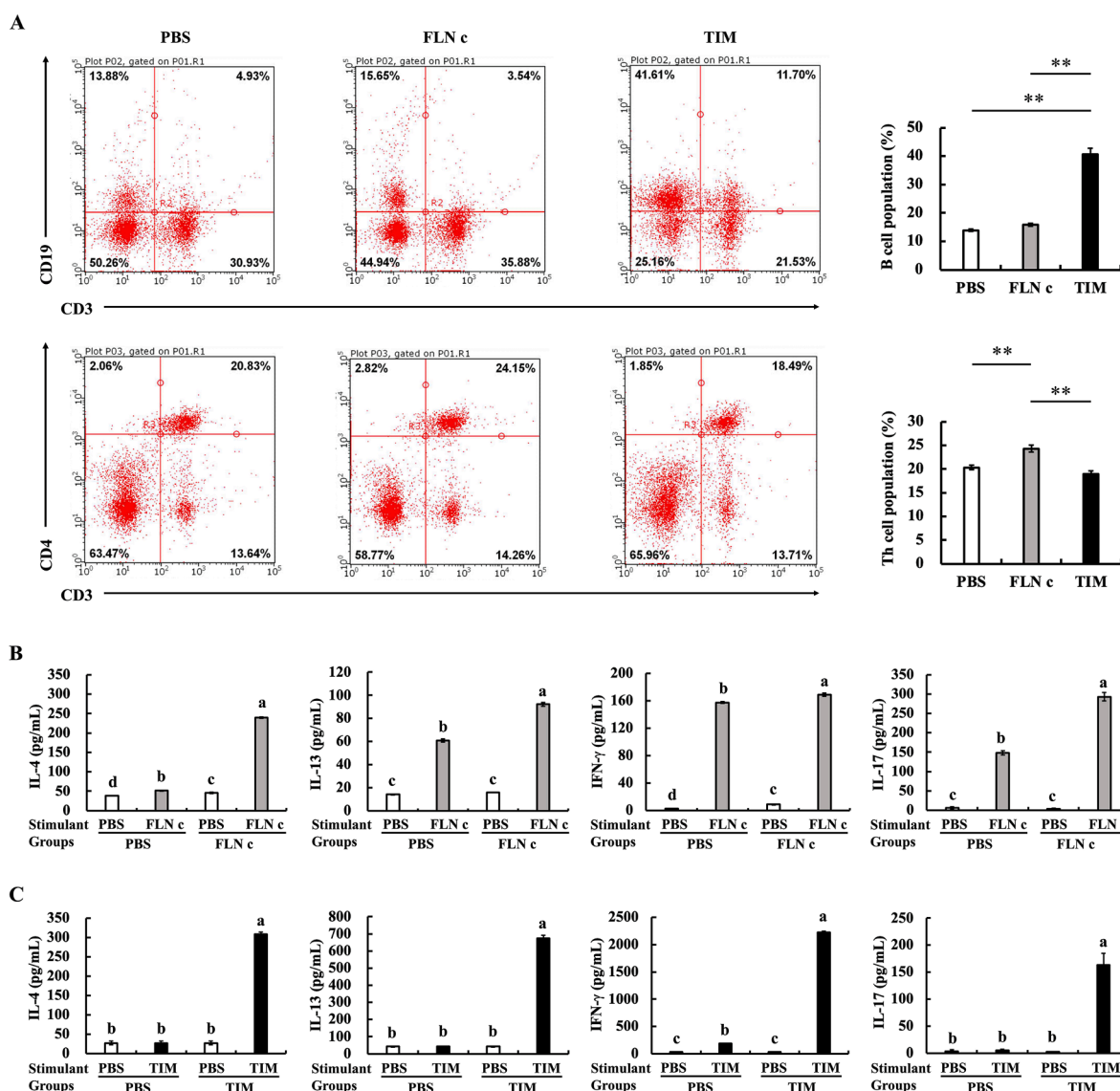


Fig. 3. The effects of allergens on the subpopulation and cytokine release of splenic lymphocytes in sensitized mice. A: Effects of allergens on the cell population of T/B lymphocytes. Splenic lymphocytes were isolated from each group of mice at day 15 and labeled with anti-CD3, anti-CD4, and anti-CD19 antibodies. All cells were gated in cell population from FSC/SSC dot plot. Three repeated mice experiments with similar results were carried out, and the exhibitory results that were displayed as the mean $\pm$ SD from mice in each group were from one of the mice experiments. Statistical differences are indicated by *p* values (one-way ANOVA), $*p < 0.05$, $**p < 0.01$. The exhibitory results of cell population of sham-sensitized mice, FLN c-sensitized mice, and TIM sensitized mice were showed by white bars, grey bars, and black bars, respectively. B – C: Effect of FLN c (B) and TIM (C) on cytokine release in splenocyte cultures. Splenic lymphocytes were isolated from each group of mice at day 15 and stimulated with allergens (100 μ g/mL) for 72 h. The amount of cytokine released into the culture supernatant were measured by ELISA. Each bar shows three independent experiments. One-way ANOVA was performed, and significance ($p < 0.05$) is precised as different letters. The cytokine release in splenocyte cultures that stimulated by PBS, FLN c, and TIM were showed by white bars, grey bars, and black bars, respectively.

sensitized mice. While there was also a significant increase in CD3⁺CD4⁺ T cell percentages in splenocytes from the FLN c-sensitized mice ($24.31 \pm 0.75\%$) compared with sham-sensitized mice ($20.30 \pm 0.48\%$) and TIM-sensitized mice ($18.95 \pm 0.61\%$), but no statistical difference was observed between the sham-sensitized and TIM-sensitized groups.

To further examine the change in CD3⁺CD4⁺ T cells in the three groups of mice, splenic lymphocytes from the sensitized mice were stimulated with the proteins used for sensitization. Increased production of the Th2 cytokines, IL-4, and IL-13, as well as the Th1 cytokines, IFN- γ , were detected in the splenic lymphocytes from mice belonging to the FLN c-sensitized group following stimulation with this protein (Fig. 3B). Beyond that, stimulation of the splenic lymphocytes from sham-sensitized mice with FLN c also significantly increase the production of IL-13, IFN- γ , and IL-17 (Fig. 3B). Similarly, compared to the splenic lymphocytes from sham-sensitized mice, splenic lymphocytes from TIM-sensitized mice showed significantly higher production of IL-4, IL-13, and IFN- γ by stimulated with TIM (Fig. 3C). In addition, FLN c as well as TIM caused a more abundant release of IL-17 by the splenic lymphocyte from sensitized mice (Fig. 3B, C).

The high level of specific IgE in the TIM-sensitized mice can be explained by the high secretion of Th2 type cytokines in the lymphocytes. In the sensitization stage, allergens are captured and degraded by dendritic cells, and the degraded peptide fragments are presented by dendritic cells and recognized by naïve T cells (Schraml et al., 2015). These naïve T cells differentiate into Th2 cells and secrete IL-4 and IL-13, which are able to induce B cell activation and antibody class switching to IgE (De Martinis, Sirufo, Suppa, & Ginaldi, 2020). High levels of Th2 type cytokines secreted by splenocytes were observed in the TIM stimulated groups, and high secretion of IL-17, which is able to induce Th2 type cytokine production (Moschen, Tilg, & Raine, 2019), was also detected in the supernatant of TIM-stimulated splenocytes. As to the

antibodies, high concentration of IgE was detected in TIM-sensitized group. These results indicated that in primary exposure, TIM could induce high levels of Th2 type cytokines in activated Th cells, which, in turn, activate the B cells to produce high level of specific IgE.

3.4. Eliciting capacity of the allergens

At two weeks after the last sensitization dose, mice sensitized to FLN c and TIM were orally challenged with the respective allergen. The sham-sensitized mice receive phosphate-buffered saline served as controls. Anaphylaxis in mice was assessed by anaphylactic score, rectal temperature and diarrhea rate (Fig. 4A-C). All of the allergen sensitized mice experienced significant clinical signs following oral challenge. The most severe reactions were observed in mice sensitized and challenged with FLN c (Fig. 4A). Significant decrease in the rectal temperature and increase in diarrhea rate, with respect to control mice, were detected in both FLN c and TIM challenged mice (Fig. 4B, C). The levels of serum antibodies as well as the degrees of serum histamine were the highest in the FLN c-sensitized group, followed by the TIM-sensitized and control groups after oral challenge (Fig. 4D, E), despite, before challenge, the concentration of FLN c-specific IgE being lower than that of TIM-specific IgE.

The results were in accordance with our previous finding that the level of specific IgE in TIM-allergic patients are higher than that in FLN c-allergic patients, while FLN c induces a significantly higher basophil activation (Yang et al., 2019). In addition, more severe clinical symptoms were observed in FLN c-allergic patients in the previously research (Yang et al., 2019). The circumstance is not rare, in a previous investigation on egg allergens by Pablos-Tanarro et al. (2018a), lysozyme promoted an early production of specific IgE antibodies, which subsequently declined in the orally challenged stage, and ovomucoid showed the opposite trend. The discrepancy was then found to be a result of the

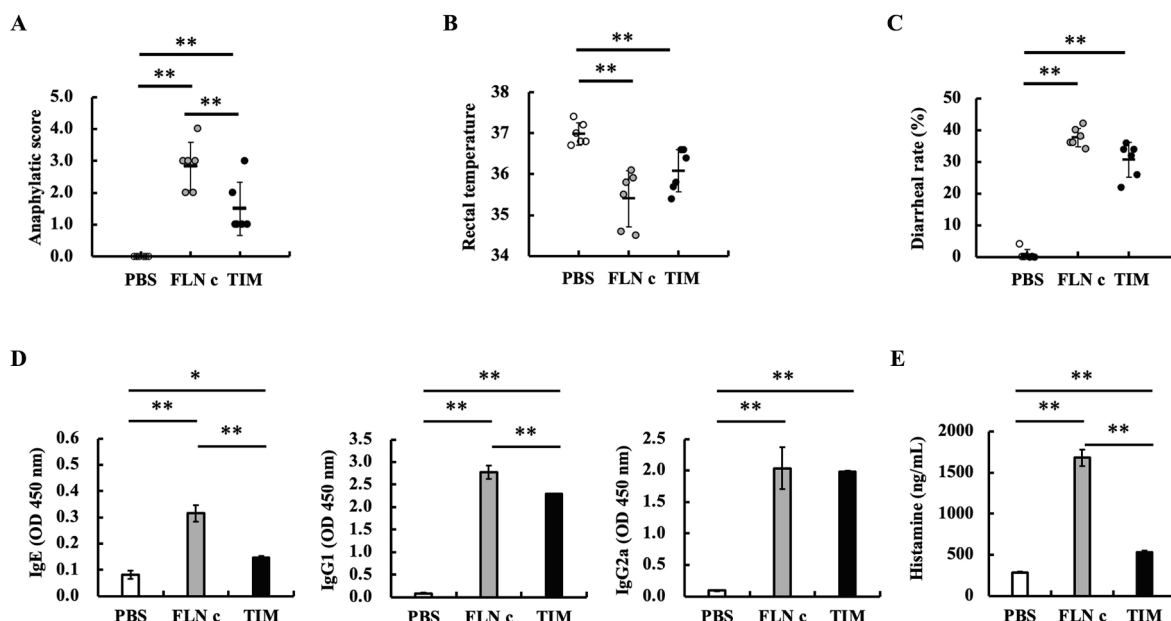


Fig. 4. The effects of allergens on the allergy symptoms, specific antibodies, and histamine in mice received oral challenge. A – C: Anaphylactic scores (A), rectal temperature (B), and rates of diarrhea (C) of mice for 1 h after the fifth challenge. Three repeated mice experiments with similar results were carried out, and the exhibitory results that were displayed as an average value from 6 mice in each group were from one of the mice experiments. Statistical differences are indicated by *p* values (one-way ANOVA). **p* < 0.05, ***p* < 0.01. Allergy symptoms of mice from control group, FLN c group, and TIM group were showed by white dots, grey dots, and black dots, respectively. D: Effect of allergens on specific-IgE, IgG1, and IgG2a in oral challenge stage. The sera from eyeball blood at day 37 were collected for ELISA. All experiments were repeated three times, the data represent the mean $\pm$ SD. Statistical differences are indicated by *p* values (one-way ANOVA), **p* < 0.05, ***p* < 0.01. The specific antibody level of mice from control group, FLN c group, and TIM group were showed by white bars, grey bars, and black bars, respectively. E: Effect of allergens on histamine level in oral challenge stage. Sera from the tail veins of mice were obtained 1 h after the last challenge. All experiments were repeated three times, the data represent the mean $\pm$ SD. Statistical differences are indicated by *p* values (one-way ANOVA), **p* < 0.05, ***p* < 0.01. The histamine level of mice from control group, FLN c group, and TIM group were showed by white bars, grey bars, and black bars, respectively.

different immunogenic capacity and capability of provoking IgE mediated hypersensitivity response of each allergen. These results indicated that TIM was highly immunogenic while FLN c had a better ability in eliciting anaphylactic reactions upon oral challenge.

Inoue et al. (Inoue, Moran, Shinnakasu, Phan, & Kurosaki, 2018) explained that this discrepancy is result from the differentiation of an

activated B-cell into plasma cells or memory B cells. In sensitization stage levels of specific IgG1 and IgG2a in FLN c-sensitized mice were significantly increased, indicating that the allergen-specific IgG response precedes IgE response. This phenomenon has also been reported in a recent study by Jiménez-Saiz et al. (2019), which demonstrated that the expansion of allergen-specific IgG1-producing memory B cells was

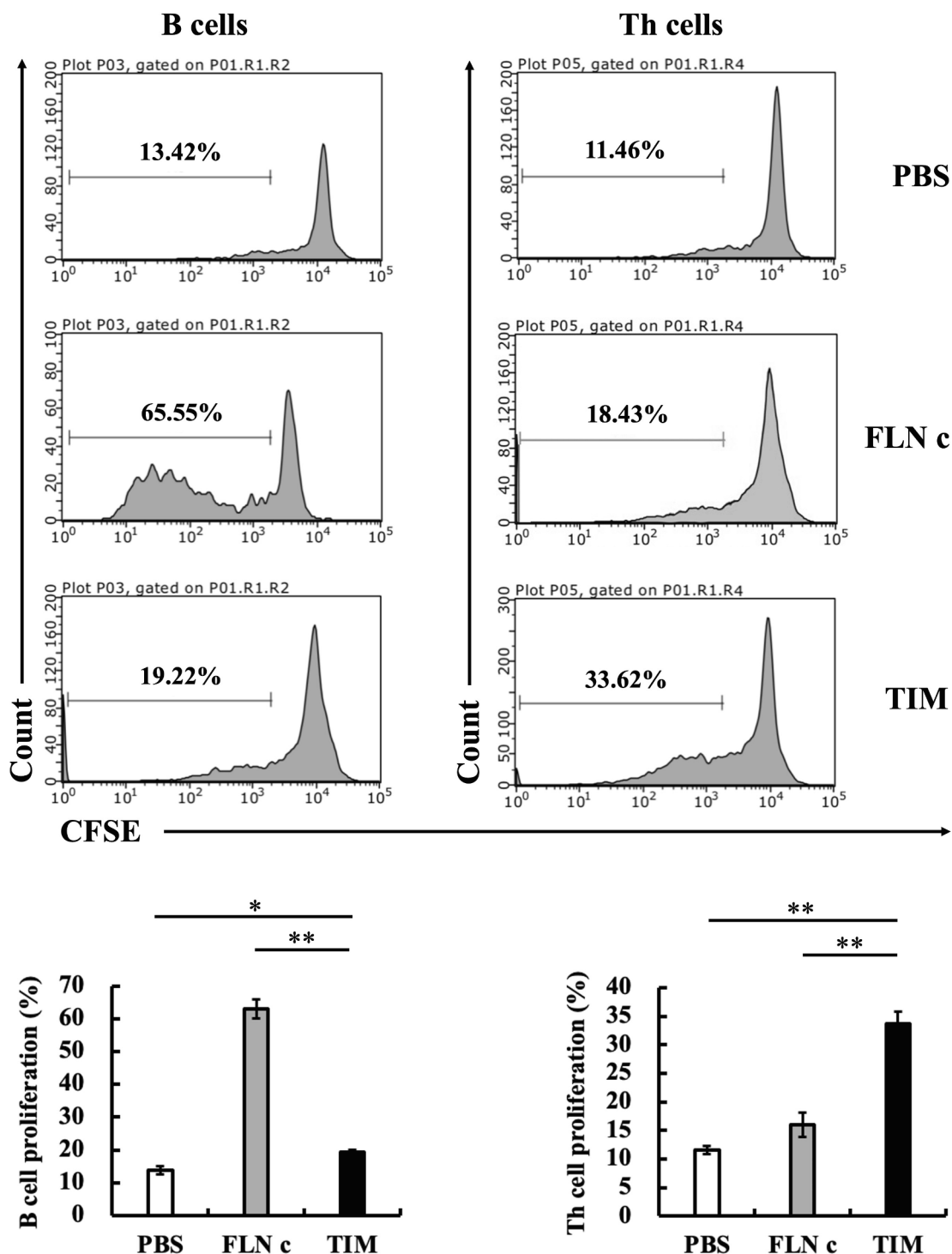


Fig 5. Effects of allergens on the cell proliferation of T/B lymphocytes in MLNs of mice received oral challenge. MLNs were isolated from each group of mice at day 37, labeled with CFSE, and stimulated with allergens (100 µg/mL) for 7 days to examine the extent of CD3⁺CD4⁺ lymphocytes and CD19⁺ lymphocytes proliferation based on CFSE dilution. Three repeated experiments with similar results were carried out, and the exhibitory results that were displayed as an average value from 3 samples in each group were from one of the experiments. Statistical differences are indicated by *p* values (one-way ANOVA). **p* < 0.05, ***p* < 0.01. The cell proliferation of T/B lymphocytes in MLNs of mice from control group, FLN c group, and TIM group were showed by white bars, grey bars, and black bars, respectively.

accompanied by bone marrow reconstitution with IgE-producing plasmablasts in mice rechallenged with allergen after sensitization. According to another study by He et al. (2017), memory B cells that produce IgG could induce antigen-specific IgE memory responses. Thus, it is speculated that in FLN c-sensitized mice, the activated B cells mainly differentiated into IgG-producing memory B cells, and these memory B cells boosted the proliferation of IgE-producing plasmablasts in the rechallenged stage, which, then resulted in a high level of specific IgE secretion. In contrary, it seems that the activated B cells in TIM-sensitized mice mainly differentiated into plasma cells. Long-lived plasma cells are not likely to be found in blood (Hoof et al., 2020), which gives an explanation to the drop of specific IgE in TIM-sensitized mice upon rechallenge.

3.5. Immunostimulating capacity of the allergens in MLN lymphocytes

During oral challenge, the inductive sites for immune responses in the gut are Peyer's patches, isolated lymph follicles and MLNs. MLNs are the largest lymph nodes in the body which serve as a crossroad between the peripheral and mucosal recirculation pathways (Velickovic, & Gavrovic-Jankulovic, 2014). To further characterize the nature of the anaphylaxis responses, the proliferation of MLN lymphocytes induced by the allergens was measured by CFSE staining and gated on CD19⁺ B cells and CD3⁺CD4⁺ T cells. The fluorescence intensity of CFSE-labeled cells decreased by one half after each cell division, thus, cells with low levels of fluorescence (CFSE-low) represent the cells that underwent one or more divisions. As it can be observed in Fig. 5, the highest proliferation rate of CD19⁺ B cells can be found in the FLN c-stimulated cultures, which represented $62.97 \pm 3.03\%$ CFSE-low CD19⁺ lymphocytes of the cells in culture, higher than the CFSE-low CD19⁺ in TIM-stimulated cultures ($19.33 \pm 0.76\%$) and PBS-stimulated cultures ($13.91 \pm 1.25\%$) with statistic difference. However, the CFSE-low CD3⁺CD4⁺ lymphocytes showed a relatively low level in FLN c-stimulated cultures ($16.00 \pm 2.16\%$), with no statistic difference with that of PBS-stimulated cultures ($11.59 \pm 0.73\%$). In contrast, $33.62 \pm 2.16\%$ of the CD3⁺CD4⁺ lymphocytes underwent division during TIM restimulation, which is significantly higher than that of PBS- and FLN c-stimulated cultures.

Activated B cells have the ability to proliferate, present antigen, and secrete antibody, and activated T cells have the ability to proliferate and differentiate into effector cells, with killing effect or production of

cytokines (Mandl, Torabi-Parizi, & Germain, 2014). These results suggested that in oral challenge stage, FLN c could immediately be recognized by the B cells, while TIM acted on the T cells. In other word, sensitization to TIM mainly caused a distinct Th2 biasing effect, while FLN c had a better ability to immediately stimulate B cells and then trigger severe symptoms in oral challenge stage. The finding, in addition, gave evidence to the speculation that more memory B cells were formed in FLN c-sensitized mice.

3.6. In silico analysis of T- and B-cell epitopes of the allergens

Though FLN c and TIM were reported to share common epitopes, which resulted in their cross-reaction (Yang et al., 2017a; Yang et al., 2019), major differences in their allergenic potential were observed. Both the B cell epitope and T cell epitope are capable to boost allergen-specific IgE via different pathways (Narayanan et al., 2017). For further verifying if this discrepancy is caused by the epitope type of the two allergens, four publicly available tools on different datasets were employed for determination of H-2-I binders. Four T cell epitope regions of FLN c: amino acid (AA) 224–236, AA 360–372, AA519–530 and AA779–790, and 3 T cell epitope regions of TIM: AA 140–155, AA 159–172, and AA 201–215 were identified as promiscuous binders of MHC II by taking consensus from the prediction data (Table 1, Supplementary Information, Fig. S3). In addition, the B cell epitopes of FLN c and TIM were also predicted by 4 sequence based methods. By taking a consensus among the commonly predicted B cell epitopes by various prediction tools used in the study, 18 regions in FLN c and 4 regions in TIM were predicted as B cell epitopes (Table 1, Supplementary Information, Fig. S4).

From the aspect of quantity, the predominant epitope type is B cell epitope in FLN c while T cell epitope in TIM. In the present study, TIM induced remarkable T cell response, including proliferation of the subpopulation of Th cells and the increasing in cytokines. This might be resulted from the T cell epitopes, which are presented after processing by antigen-presenting cells to T lymphocytes (Sampson et al., 2018). Narayanan et al. (2017) stated that B cell epitopes might have the ability of boosting allergen-specific hypersensitivity responses without allergen-specific T cell help, which provides explanation for the eliciting capacity of FLN c during oral challenge. While the indepth pathway of the two allergens to promote sensitization and elicitation of allergic reactions deserve further investigation.

Table 1

In silico assessment on T- and B-cell epitopes of FLN c and TIM.

	No.	FLN c	TIM
T cell epitopes	1	M224G225N226H227I228A229N230S231P232 F233K234 I235N236	E140E141V142V143F144A145Q146M147 K148A149L150V151P152N153I154S155 R159V160V161I162A163Y164E165P166 V167W168A169I170G171T172 A201E202S203T204R205I206I207Y208G209 G210S211V212S213A214G215
	2	C360R361L362T363F364K365L366P367G368 I369S370P371F372	
	3	S519P520Y521K522V523Y524I525T526P527 S528L529G530	
	4	T779V780N781A782S783G784A785G786N787N788I789 L790	
B cell epitopes	1	P25R26E27E28G29	G103E104O105D106Q107L108I109
	2	E40H41V42Q43G44	K130L131E132E133R134E135S136N137 R138T139
	3	Y58V59T60A61Y62G63P64	K174T175A176T177O178E179Q180A181
	4	I140R141G142S143	V196S197P198Q199V200A201E202
	5	E152G153R154K155R156N157	
	6	L255S256E257G258Q259T260H261K262E263	
	7	D272A273G274Y275G276	
	8	K293D294N295E296D297G298	
	9	T307E308P309G310Y311Y312	
	10	N321H322V323P324G325S326	
	11	E335G336T337N338R339Q339T340E341R342 I343K344R345Q346R347E348A349V350P351 L352T353 E354V355	
	12	H420I421P422G423S424P425	
	13	G437A438H439R440V441H442A443G444	
	14	E449R450G451E452Q453G454M455	
	15	K486D487R488K489D490	
	16	D513K514H515I516P517D518S519	
	17	L540P541E542Q543G544S545M546P547N548 K549	
	18	V735E736K737T738K739S740G741D742K743 G744H745	

In addition to the ability of stimulating B cells, the characteristics of IgE epitopes might also be used to explain the anaphylactic responses. Among the predicted epitopes of FLN c, two B cell epitopes (AA 255–263 and AA 420–425) were consistent with the reported linear IgE epitopes; one T cell (AA 224–236) and 5B cell epitopes (AA 255–263, AA 293–298, AA 449–455, AA 540–545, and AA 735–745) distributed in the reported conformational IgE epitope region (Yang et al., 2019). For TIM, the predicted T cell epitope AA 201–215 and predicted B cell epitope AA 103–109 were in the same location of the reported linear epitopes; In addition, the predicted T cell epitope AA 159–172 and 2 predicted B cell epitopes (AA 130–139 and AA 174–181) distributed in the reported conformational IgE epitopes (Yang et al., 2019). However, none of these predicted continuous epitope sequences located in the previously identified common IgE epitopes of TIM and FLN c (data not shown), for majority of the common epitopes were conformational (Yang et al., 2017a; Yang et al., 2019).

Evidence suggests that some determinants of IgE recognition define the strength of a clinical response: abundance, epitope diversity, and epitope proximity (Dang et al., 2018). It has been reported that IgE responses to a higher number of epitopes were linked to the severity of milk allergy (Caubet et al., 2017). Gieras et al. (2016) demonstrated that closer proximity of epitopes results in a stronger IgE-dependent mediator release. FLN c contains higher number and closer proximity epitopes than TIM, and it showed better basophil activation capacity *in vitro* (Yang et al., 2019). The present results demonstrated the better eliciting capacity of FLN c during oral challenge, which is in line with the previous observation. Furthermore, though common IgE epitopes of FLN c and TIM were well documented, it seemed that these common epitopes are not predominant on their abilities of inducing and eliciting allergic responses. Nevertheless, the common epitopes might be meaningful in food allergen detection and clinical diagnosis.

4. Conclusions

It is of interest to note that although FLN c and TIM share common epitopes, they showed allergenic potential discrepancy *in vivo*. In conclusion, the present work showed that TIM was an active promotor of sensitization, and FLN c had a better elicitation capacity of the allergic reaction. This suggests that multiple mechanisms may act synergistically in the development of allergy, the differentiation phenotypes of active B cells, the predominant type of epitopes, as well as abundance, and proximity of epitopes are involved in the triggering of anaphylactic reaction. This knowledge is important for a better understanding of the biochemical features of food allergens and the relationship between epitope and allergenic potential of allergens. Future experiments are planned to investigate the potential of *in vivo* cross-reactivity depended upon the common epitopes of FLN c and TIM.

CCRediT authorship contribution statement

Yang Yang: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Visualization. **Xin-Rong He:** Investigation, Data curation. **Shao-Gui He:** Conceptualization, Methodology. **Meng Liu:** Methodology. **Yong-Xia Zhang:** . **Fei Xia:** Methodology. **Min-Jie Cao:** Supervision, Writing - review & editing. **Wen-Jin Su:** Writing - review & editing. **Guang-Ming Liu:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.131132>.

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1**Two allergens from *Scylla paramamosain* share common epitopes**

2**showed different allergenic potential in Balb/c mice**

3**Running title:** Assessment of allergenic potential of two crab allergens

4Yang Yang ^{1,2}, Xin-Rong He ¹, Shao-Gui He ², Meng Liu ¹, Yong-Xia Zhang ¹, Fei Xia

5¹, Min-Jie Cao ¹, Wen-Jin Su ¹, Guang-Ming Liu ^{1,*}

6

7¹ College of Ocean Food and Biological Engineering, Jimei University, 43 Yindou Road, Xiamen,

8Fujian, 361021, China.

9² College of Environment and Public Health, Xiamen Huaxia University, 288 Tianma Road,

10Xiamen, Fujian, 361024, China.

11

12***Corresponding authors**

13Guang-Ming Liu, College of Ocean Food and Biological Engineering, Jimei

14University

15Xiamen 361021, Fujian, China

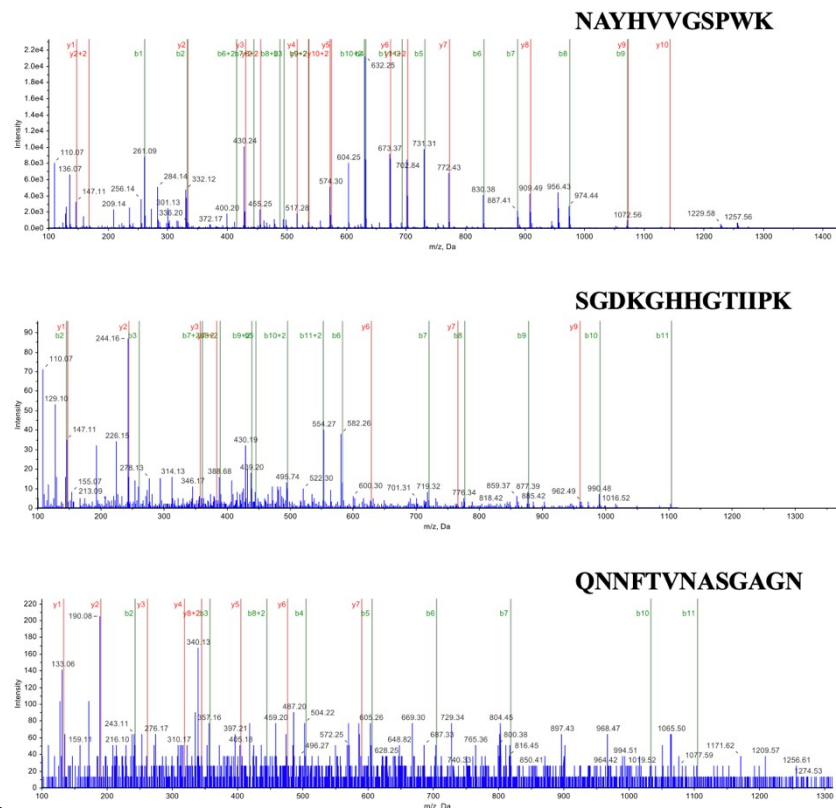
16Tel: +86-592-6183383

17Fax: +86-592-6180470

18Email: gmliu@jmu.edu.cn

19Fig. S1. Summary of mass spectrometry of purified 90-kDa protein

20A



21

22B

Sequence of peptide	Calculated mass	Observed mass	ΔMass	Assession
NAYHVVGSPWK	1402.66626	1402.688118	0.02191930	gi 338224387
SGDKGHHGTIIPK	1345.70911	1345.710205	0.00117212	gi 338224387
QNNFTVNASGAGN	1292.57312	1292.574585	0.00154602	gi 338224387

<i>Scylla paramamosain</i>	NAYHVVGSPWKVRCCTGEAHAERGTIQESSVVVETVEKTKSGDKGHHGTIIPKFEHSDANK	60
Purified protein	NAYHVVGSPWK.....SGDKGHHGTIIPK.....	23
Consensus	nayhvvgsppwksgdkghhgtiipk	
<i>Scylla paramamosain</i>	VTSKGLGLKKAHINPQNNFTVNASGAGNNML	61
Purified protein	QNNFTVNASGAGN...	37
Consensus	qnnftvnasgagn	

23

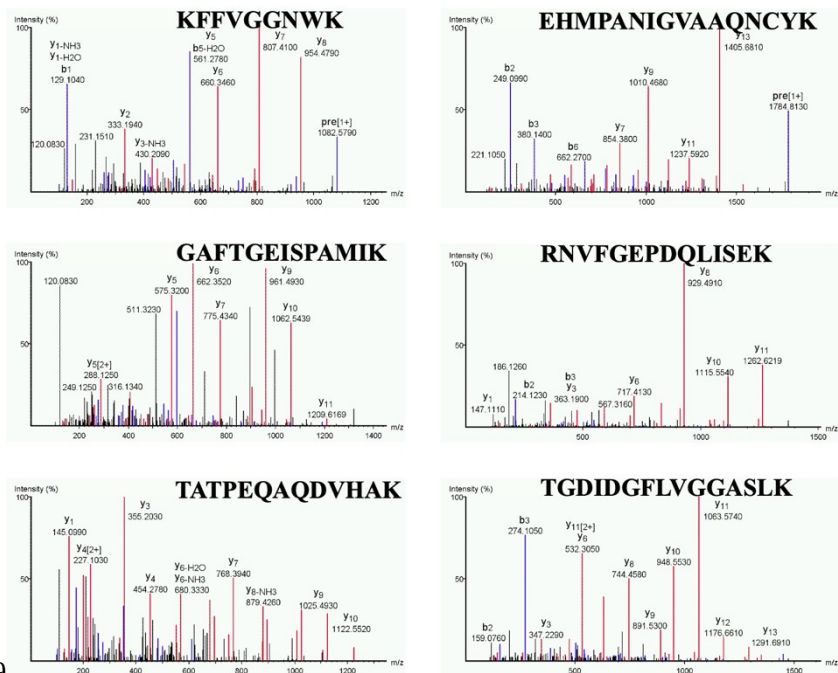
24A: Maps of MALDI-TOF/TOF of the purified 90-kDa protein.

25B: Sequence alignment results of the mass spectrometry peptides and matching protein (*S.*

26*paramamosain* FLN c).

27Fig. S2. Summary of mass spectrometry of purified recombinant 28-kDa protein

28A



29

30B

Sequence of peptide	Calculated mass	Observed mass	Δ Mass	Assession
KFFVGGNWK	1081.563632	1081.570863	0.007231	gi 1122816254
EHMPANIGVAAQNCYK	1802.792578	1802.808097	0.015519	gi 1122816254
GAFTGEISPAMIK	1320.672709	1320.674761	0.002052	gi 1122816254
RNVFGEPDQLISEK	1630.829037	1630.831456	0.002419	gi 1122816254
TATPEQAQDVHAK	1394.678943	1394.679073	0.000130	gi 1122816254
TGDIDGFLVGGASLK	1448.749038	1448.751139	0.002101	gi 1122816254

<i>Scylla paramamosain</i>	MANQRKFFVGGNWKMNMGDKAAIDGIIISFMKGFLNADTEVVVGCPQCXYLMYTR	EHMPANIG	60
Purified protein	KFFVGGNWK.....	EHMPANIG	17
Consensus	kffvgggnwk	ehmpanig	
<i>Scylla paramamosain</i>	VAAQNCYKT.ARGGAFTGEISPAMIKDCGCEWVILGHSERRNVFGEPDQLISEKVGHALEA		120
Purified protein	VAAQNCYK....GAFTGEISPAMIK.....	RNVFGEPDQLISEK	52
Consensus	vaaqncyk gaftgeispamik	rnvfgepdqlisek	
<i>Scylla paramamosain</i>	GLKVIPICIGEKLEERESNRTEEVVFAQMKAIVPNISDWSRVVIAEYFPVWAIGTGTATPE		180
Purified protein	TATPE		57
Consensus	tatpe		
<i>Scylla paramamosain</i>	QAQDVHAKLRQWLKRDNVSPQVAESTRIIYGGSVSAGNCKELAKTGDIDGFLVGGASLKPD		240
Purified protein	QAQDVHAK.....TGDIDGFLVGGASLK..		80
Consensus	qaqdvhak	tgdidgflvvggask	
<i>Scylla paramamosain</i>	FVTIINAR		247
Purified protein			80

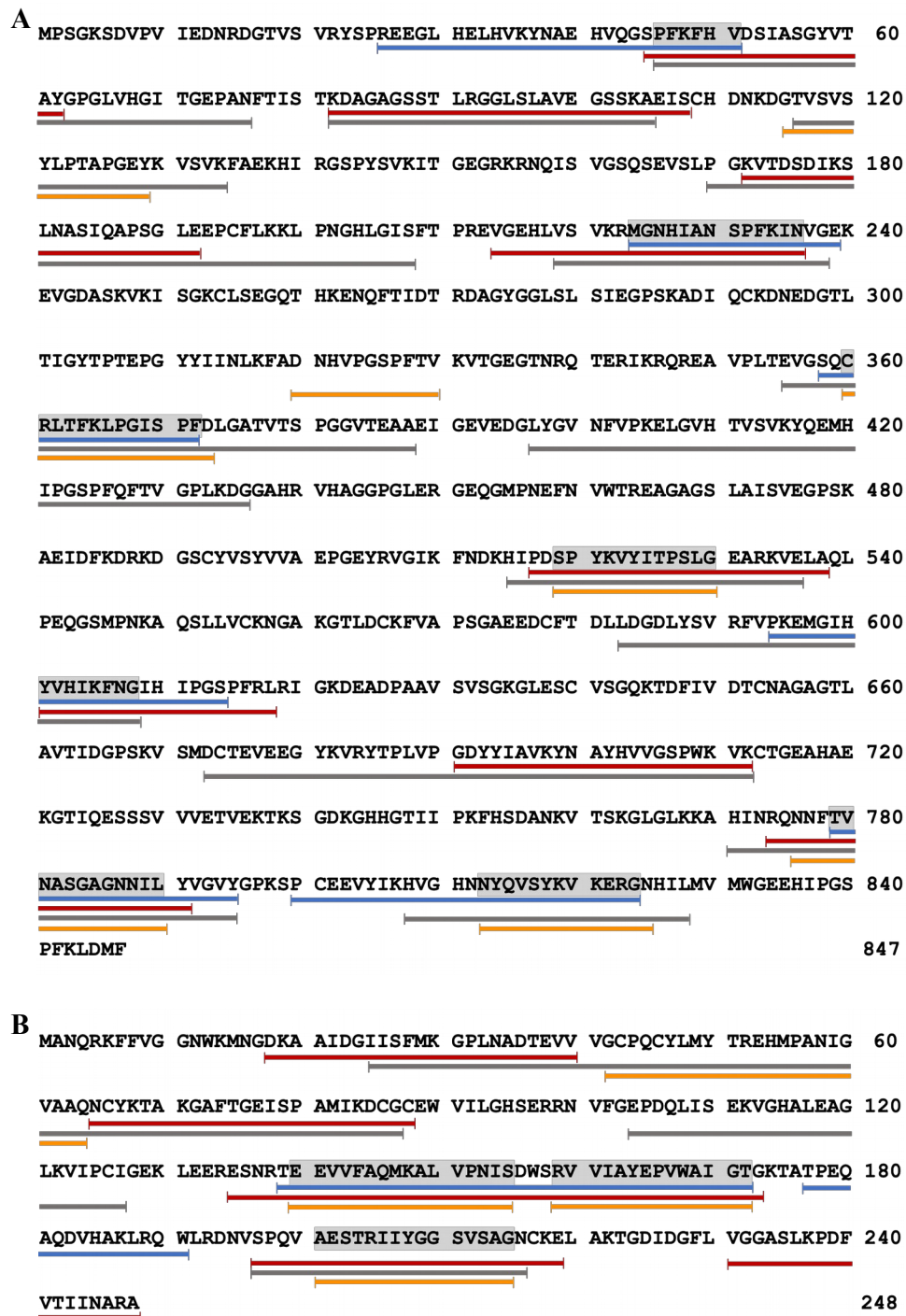
31

32A: Maps of MALDI-TOF/TOF of the purified 28-kDa protein.

33B: Sequence alignment results of the mass spectrometry peptides and matching protein (*S.*

34*paramamosain* TIM).

35Fig. S3. *In silico* prediction of T-cell epitopes



36
37A: T-cell epitope prediction of FLN c.

38B: T-cell epitope prediction of TIM.

39The predicted T-cell epitopes were underlined by red lines (NetMHCII 2.3 server), grey lines

40(NetMHCII pan 3.2), orange lines (NN align), and blue lines (IEBD recommended), respectively.

41The regions recognized frequently by three or more than three tools were selected and marked

42with grey.

43Fig. S4. *In silico* prediction of B-cell epitopes



44

45A: B-cell epitope prediction of FLN c.

46B: B-cell epitope prediction of TIM.

47The predicted T-cell epitopes were underlined by light blue lines (DNASTAR Protean), yellow lines

48(BepiPred 1.0 server), purple lines (Immunomedicine Group), and green lines (IEDB Antigen

49Sequence Properties), respectively. The regions recognized frequently by three or more than three
50tools were selected and marked with black.