

# Reducing the Allergenicity of Shrimp Tropomyosin and Allergy Desensitization Based on Glycation Modification

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**ABSTRACT:** Shrimp is a major allergic food that could trigger severe food allergy, with the most significant and potent allergen of shrimp referred to as tropomyosin (TM). Glycation modification (Maillard reaction) could reportedly weaken the allergenicity of TM and generate hypoallergenic TM, while up to now, there is still a lack of investigations on the hypoallergenic glycosylated tropomyosin (GTM) as a candidate immunotherapy for desensitizing the shrimp TM-induced allergy. This study analyzed the effects of glycation modification on decreasing the allergenicity of TM and generated hypoallergenic GTM and how GTM absorbed to the  $\text{Al}(\text{OH})_3$  function as a candidate immunotherapy for desensitizing allergy. As the results, in comparison to TM, the saccharides of smaller molecular sizes could lead to more advanced glycation end products in GTMs than saccharides of greater molecular sizes, and TM glycosylated by saccharides of different molecular sizes (glucose, maltose, maltotriose, maltopentaose, and maltoheptaose) exhibited lower allergenicity as a hypoallergen upon activating the allergic reactions of the mast cell and mouse model, while TM glycosylated by maltose had insignificant allergenicity changes upon activating the allergic reactions of the mast cell and mouse model. In addition, the hypoallergenic GTM +  $\text{Al}(\text{OH})_3$  was efficient as a candidate immunotherapy; this work intended to offer preclinical data to promote GTM +  $\text{Al}(\text{OH})_3$  as a candidate allergen-specific immunotherapy for desensitizing the allergy reactions for patients allergic to shrimp food.

**KEYWORDS:** tropomyosin, glycation modification, allergenicity, hypoallergen, allergy desensitization, immunotherapy

## INTRODUCTION

Shrimp-induced food allergy is a worldwide food safety issue.<sup>1</sup> The most significant and potent allergen of shrimp has been referred to as tropomyosin (TM), although previous investigations have demonstrated that there are other allergens in several shrimp species;<sup>1–3</sup> TM is still the most commonly investigated allergen in shrimp food.<sup>1–4</sup>

However, currently, except refraining from shrimp food, there is still a lack of desensitization strategies (especially for immunotherapy) for shrimp TM-induced allergic reactions. Previous investigations have demonstrated that hypoallergenic proteins with weaker allergenicity could be used as potential candidates for allergen-specific immunotherapy to desensitize food allergy.<sup>5,6</sup> Glycation (Maillard reaction) is a common chemical reaction that reacts between proteins and reducing saccharides; as reported, glycation could weaken the allergenicity of food allergens.<sup>7–9</sup> Because TM is lysine-rich and susceptible to glycation modification, glycation has been applied to generate hypoallergenic glycosylated tropomyosin (GTM),<sup>9</sup> which could offer hypoallergenic GTM as a candidate to develop immunotherapy to desensitize shrimp TM-induced allergic reactions.

Up to now, allergy desensitization is still the most efficacious strategy for food allergy treatment with extended hyposensitive effects; allergen-specific immunotherapy is a promising therapeutic approach for food allergies that is based on the administration of increasing doses of culprit food allergens.<sup>10</sup> Allergen immunotherapy that consists of hypoallergenic

proteins could relieve food allergy symptoms as a medication strategy. Subcutaneous immunotherapy (SCIT) has been used in food allergy treatment and promoted the tolerance of patients to food allergens.<sup>11–13</sup> Because glycation is a common chemical reaction in foods,<sup>14</sup> glycation modification of TM could be a candidate approach to prepare immunotherapy for desensitizing food allergy symptoms.

In the past years, several investigations have analyzed the hypoallergenic proteins from aquatic products as a desensitization immunotherapy for aquatic-product-induced allergy symptoms (e.g., fish allergy,<sup>6</sup> shrimp allergy,<sup>15</sup> and oyster allergy<sup>16</sup>). Previous researchers have found that the glycation modification of shrimp TM could weaken TM allergenicity and lead to hypoallergenic GTM,<sup>17–19</sup> while currently, there is still a lack of research data on hypoallergenic GTM as a candidate immunotherapy for shrimp-induced food allergy. To investigate whether hypoallergenic GTM could be a candidate immunotherapy, the allergenicity of GTM and the immunotherapeutic efficacy of GTM as an immunotherapy were analyzed in this study via the RBL-2H3 mast cell model and BALB/c mouse model, which could provide a better

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understanding of hypoallergenic GTM as a potential immunotherapy. This study could enhance our understanding on the potential of GTM as a hypoallergen for preclinical immunotherapy, which could provide a novel perspective to develop hypoallergenic GTM as an immunotherapy for patients allergic to shrimp food.

## MATERIALS AND METHODS

**Materials.** 2',7'-Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA), 4-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide (PNAG), acid orange 12 (AO-12),  $\beta$ -mercaptoethanol, cholera toxin (CT), D-glucose (G), maltose (M), maltotriose (MTS), maltopentaose (MPS), maltoheptaose (MHS), and *o*-phthalaldehyde (OPA) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.). The Inject alum adjuvant was purchased from Thermo Scientific, Inc. (Pierce Biotech, Rockford, IL, U.S.A.). The shrimp TM (Lit v 1) was extracted and purified from *Litopenaeus vannamei* according to a previous description.<sup>9</sup>

**GTM Preparation.** The preparation of GTM was according to a previous method.<sup>17</sup> Briefly, TM was dissolved in a buffer solution (1 mmol/L NaHCO<sub>3</sub> and 5 mmol/L  $\beta$ -mercaptoethanol) containing G, M, MTS, MPS, or MHS (10.8%, w/v), respectively, with the protein concentration of 0.2 mg/mL. The mixture of TM with each saccharide was lyophilized and incubated at 60 °C for 48 h to produce different GTMs: TM glycosylated by glucose (TM-G), TM glycosylated by maltose (TM-M), TM glycosylated by maltotriose (TM-MTS), TM glycosylated by maltopentaose (TM-MPS), and TM glycosylated by maltoheptaose (TM-MHS). Afterward, the GTM samples were dialyzed in ultrapure water and then lyophilized. TM incubated at 60 °C for 48 h served as the control.

**Glycation Degree Analysis of GTM. Available Lysine Content Analysis.** The available lysine contents of TM and GTM were investigated referring to a previous method.<sup>18</sup> Briefly, GTM samples (10 mg) were dissolved in 100  $\mu$ L of sodium acetate solution (16.4%, w/v); then 10  $\mu$ L of propionic anhydride was added; and the solution was shaken for 20 min. Afterward, 2 mL of dye solution (0.135% AO-12, 2% oxalic acid dehydrate, 0.34% potassium dihydrogen phosphate, and 6% glacial acetic) was added and shaken for 60 min, forming a protein–dye complex. Then the protein–dye complex was centrifuged at 5000g for 10 min. The samples were diluted 100-fold, and then the absorbance (475 nm) was determined using an ultraviolet–visible (UV–vis) spectrophotometer (UV-2550, Shimadzu, Japan), with the buffer solution (2% oxalic acid dehydrate, 0.34% potassium dihydrogen phosphate, and 6% glacial acetic) as the blank control.

**Free Amino Group Content Analysis.** The free amino group content was determined with the OPA assay. The OPA reagent was prepared by mixing 80 mg of OPA (in 2 mL of ethanol), 5 mmol of sodium tetraborate, 1 g of sodium dodecyl sulfate, and 0.2 mL of  $\beta$ -mercaptoethanol, and the volume was set to 100 mL. Afterward, 200  $\mu$ L of GTM samples was mixed with 4 mL of OPA reagent and then incubated at room temperature for 5 min. The absorbance (340 nm) was determined using an UV–vis spectrophotometer (UV-2550, Shimadzu, Japan). TM served as the control with 100% free amino group contents.

**Advanced Glycation End Product (AGE) Assay of GTM. AGE Assay.** The AGE contents were determined using spectrofluorimetry.<sup>20</sup> The fluorescence intensity of the GTM solution was investigated at maximum emission (440 nm) with excitation at 350 nm using a spectrofluorometer (F-7000, Hitachi, Japan). The AGE contents were normalized to the protein concentration determined by the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, U.S.A.) and presented in astronomical units (AU) per gram of protein.

**N<sup>ε</sup>-(Carboxymethyl)lysine (CML) Determination.** CML is the most common AGE. Basically, CML was investigated by a reversed-phase high-performance liquid chromatography (RP-HPLC) method.<sup>21</sup> First, GTMs were reduced in a buffer (0.1 mol/L sodium tetraborate decahydrate and 0.1 mol/L sodium borate buffer at pH 9.0) at 4 °C overnight. Then, samples were washed with ultrapure

water, concentrated using centrifugal filter units (Amicon Ultra-4, 10 kDa, Meck Millipore, Ireland), and then hydrolyzed in 6 mol/L HCl at 110 °C for 22 h under nitrogen. The hydrolysate (20  $\mu$ L) was diluted with 0.1 mol/L sodium borate buffer (pH 9.0). Afterward, the OPA reagent was added to the sample vial and mixed for derivatization. After reaction for 3 min, the derivatized hydrolysate was injected into a HPLC C<sub>18</sub> column (5  $\mu$ m, 4.6  $\times$  250 mm, Agilent, Santa Clara, CA, U.S.A.) and analyzed using HPLC (Agilent 1260, Santa Clara, CA, U.S.A.) equipped with an Agilent 1260 Infinity detector. The OPA–CML derivatives were determined with excitation and emission wavelengths at 340 and 455 nm, respectively. Quantification was determined using an external CML standard (Cayman Chemical, Ann Arbor, MI, U.S.A.).

**Soluble Receptor for Advanced Glycation End Products (sRAGE) Binding Capacity.** The sRAGE binding assay was determined according to Zhang et al.<sup>18</sup> Briefly, the enzyme-linked immunosorbent assay (ELISA) plates were coated with GTMs dissolved in 1.5 mmol/L sodium carbonate buffer (pH 9.6) and kept overnight at 4 °C. Then, the plate was washed with TBST buffer (0.1 mol/L Tris, 0.15 mol/L NaCl, and 0.05% Tween-20 at pH 7.5) and then blocked with 3% bovine serum albumin (BSA) at 37 °C for 1 h. GTM samples in a series of concentrations (0.25, 2.5, 25, 250, and 2500  $\mu$ g/mL) were pre-incubated at 37 °C with sRAGE (recombinantly produced in *Escherichia coli*, RD172116100, BioVendor, Brno, Czech Republic) in the dilution buffer (1.5% BSA in TBST buffer). After blocking, the coated ELISA plate was washed and the pre-incubation mixture was pipetted followed by incubation at 37 °C for 1 h. The plate was washed, and anti-human RAGE antibody [monoclonal mouse immunoglobulin G2b (IgG2b) human RAGE antibody, MAB11451, R&D Systems, Minneapolis, MN, U.S.A.] was pipetted and incubated for 30 min on the shaker at room temperature. After washing, the detection antibody [horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse immunoglobulins, P0447, Dako, Hamburg, Germany] was pipetted and incubated for 30 min on the shaker at room temperature. After washing, 3,3',5,5'-tetramethylbenzidine (TMB) substrate was pipetted and incubated for 15 min. The coloring was stopped using 2 mol/L H<sub>2</sub>SO<sub>4</sub>. The plate was read using a plate reader (Powerwave XS, BioTek, Winooski, VT, U.S.A.) at 450 nm, with 620 nm as a reference. The percentage of inhibition was calculated using the following formula:

$$\left( \frac{\text{OD}_{\text{sRAGE}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{sRAGE}}} \right) \times 100 (\%)$$

where OD<sub>sRAGE</sub> was the signal obtained for sRAGE incubated without the competition agent (TM or GTMs) in the dilution buffer.

**RBL-2H3 Mast Cell Model Assay. Preparation of the GTM-Immunized Mast Cell.** The RBL-2H3 mast cells were cultured in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), seeded on a 96-well plate (1  $\times$  10<sup>5</sup> cells/well), and incubated at 37 °C for 24 h. Afterward, the mast cells were washed with phosphate-buffered saline (PBS, 0.01 mol/L), and IgE sensitization of mast cells was achieved through pipetting culture medium containing 10% serum from a TM-immunized mouse (100  $\mu$ L/well). After IgE incubation for 24 h, mast cells were washed with PBS. Then, the IgE-sensitized mast cells were challenged using 10  $\mu$ g/mL TM or GTM (100  $\mu$ L/well, incubated at 37 °C for 60 min).

**Histamine Release Assay.** The culture medium supernatants of TM- or GTM-immunized RBL-2H3 mast cells (50  $\mu$ L/well) were harvested to investigate the histamine contents by a histamine ELISA kit (IBL International, Germany).

**Degranulation Degree.** The degranulation degree of RBL-2H3 mast cells was analyzed by determining the  $\beta$ -hexosaminidase enzymatic activity in the culture medium supernatant of RBL-2H3 mast cells. The supernatant and PNAG (1 mmol/L PNAG in 0.1 mol/L citric acid buffer at pH 4.5) were pipetted to the 96-well plate wells. After incubation at 37 °C for 1 h, 0.2 mol/L glycine buffer solution (pH 10.7) was pipetted to each well and the absorbance (OD<sub>450 nm</sub>) was determined. The degranulation rates were presented as the percentage of  $\beta$ -hexosaminidase enzymatic activity in the

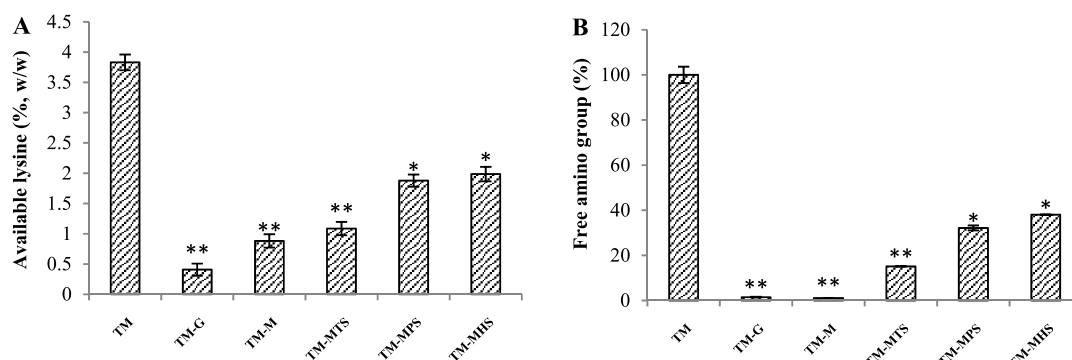


**Figure 1.** (A) Available lysine content and (B) free amino group content of TM and GTMs. TM, tropomyosin; TM-G, TM glycyated by glucose; TM-M, TM glycyated by maltose; TM-MTS, TM glycyated by maltotriose; TM-MPS, TM glycyated by maltopentaose; and TM-MHS, TM glycyated by maltoheptaose. An asterisk showed significant differences compared to TM: (\*)  $p < 0.05$  and (\*\*)  $p < 0.01$ .

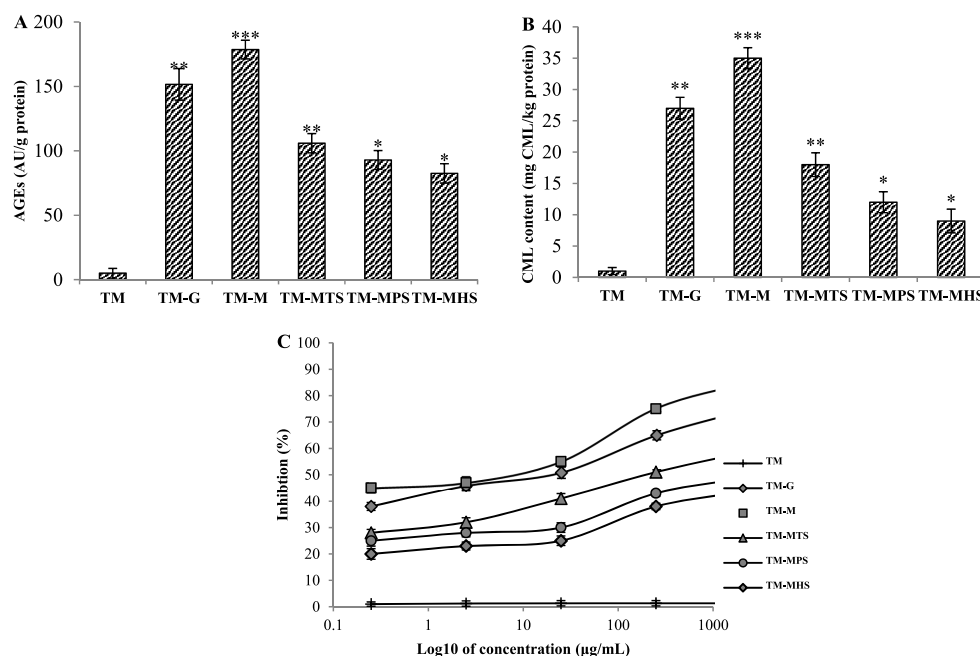


**Figure 2.** (A) AGE content, (B) CML content, and (C) sRAGE binding capacity of TM and GTMs. TM, tropomyosin; TM-G, TM glycyated by glucose; TM-M, TM glycyated by maltose; TM-MTS, TM glycyated by maltotriose; TM-MPS, TM glycyated by maltopentaose; and TM-MHS, TM glycyated by maltoheptaose. An asterisk showed significant differences compared to TM: (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , and (\*\*\*)  $p < 0.001$ .

supernatant compared to the total  $\beta$ -hexosaminidase enzymatic activity in corresponding cell lysates. To determine the total  $\beta$ -hexosaminidase enzymatic activity, the cells were lysed with 0.1% Triton-X 100 before removal of the supernatant. The  $\beta$ -hexosaminidase enzymatic activity of mast cells was analyzed by determining the absorbance ( $OD_{450\text{ nm}}$ ) of hydrolyzed substrates using a plate reader (SpectraMax M5, Molecular Devices, San Jose, CA, U.S.A.).

**GTM Immunotherapy Analysis.** The BALB/c mice (female, 5 weeks old) were housed in the animal care facility of Ocean University of China (Qingdao, China). The subcutaneous immunotherapy (SCIT) experimental protocol was approved by the Ethics Committee of Ocean University of China for the Care and Use of Laboratory Animals. The BALB/c mice were randomly divided into seven equal groups (eight mice per group) before the experiment; the seven groups were named as follows: PBS group, TM group, TM-G group, TM-M group, TM-MTS group, TM-MPS group, and TM-MHS group. Specifically, each mouse of the PBS group was intragastrically (ig) immunized with 0.2 mL of PBS containing 10  $\mu$ g of CT/mouse, and each mouse of other immunized groups was ig immunized with 0.2 mL of PBS containing 6 mg of TM and 10  $\mu$ g of CT on days 0, 1, 2, 7, 14, 21, and 28. After sensitization, each mouse of different groups was subcutaneously (sc) treated in the neck with

0.2 mL of different samples [PBS/TM/GTM +  $\text{Al}(\text{OH})_3$ ] twice per week for 6 weeks since day 42. The PBS +  $\text{Al}(\text{OH})_3$  immunized mice served as the PBS negative control, and all mice of other groups were ig challenged with TM (12 mg/mouse) on day 91 and intraperitoneally (ip) challenged with TM (0.1 mg/mouse) on days 98 and 112. The mouse anaphylactic symptoms were determined in an hour after each sensitization.<sup>22</sup> The body temperature was determined after sc challenge using a rectal thermometry every 10 min for 90 min. The blood of different mouse models was collected for determining immunoglobulin E (IgE), immunoglobulin G1 (IgG1), and immunoglobulin G2a (IgG2a) on days 35, 58, 84, 91, 98, 105, and 115. On day 115, all mice were slaughtered by spinal dislocation, and the spleen tissues were harvested under sterile conditions, obtaining the spleen lymphocyte (SLP) cell suspensions.<sup>18</sup> For cytokine [interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), interferon  $\gamma$  (IFN- $\gamma$ ), and transforming growth factor  $\beta$  (TGF- $\beta$ )] analysis, the SLP cells ( $1 \times 10^6$  cells/well) were cultured in 24-well plates with the presence of TM or GTM at 37 °C for 72 h; the cytokine levels were determined using the corresponding mouse ELISA kits (R&D Systems, Minneapolis, MN, U.S.A.).

**Statistical Analysis.** All experimental data were expressed as the mean  $\pm$  standard deviation (SD). Data were analyzed using one-way

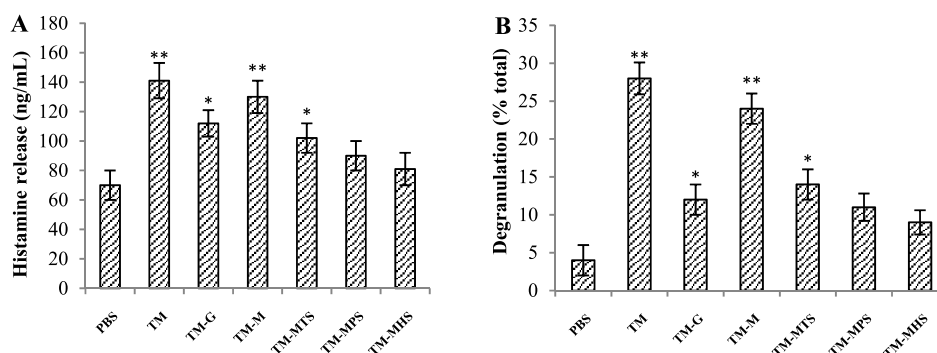


**Figure 3.** (A) Histamine release and (B) degranulation of murine mast cells (RBL-2H3) immunized by TM or GTMs (mean  $\pm$  SD;  $n = 3$ ). TM, tropomyosin; TM-G, TM glycated by glucose; TM-M, TM glycated by maltose; TM-MTS, TM glycated by maltotriose; TM-MPS, TM glycated by maltopentaose; and TM-MHS, TM glycated by maltoheptaose. An asterisk showed significant differences compared to the PBS group: (\*)  $p < 0.05$  and (\*\*)  $p < 0.01$ .

analysis of variance (ANOVA), and the differences between different groups were determined using Duncan's multiple range test by SPSS software (SPSS, version 25, Chicago, IL, U.S.A.). Each experiment was repeated at least 3 times.

## RESULTS

**Glycation Degree of GTM.** Lysine is the most active amino acid during protein glycation; the glycation degree of GTM could be reflected by the available lysine content and free amino group content in GTMs.<sup>14,23</sup> As shown in Figure 1A, in comparison to TM, the available lysine contents of GTMs were reduced in the following order: TM > TM-MHS > TM-MPS > TM-MTS > TM-M > TM-G. With regard to the free amino group contents of GTMs (Figure 1B), the free amino group contents shared similar change tendency with the available lysine contents. Moreover, the available lysine contents had almost the same change tendency as the free amino group contents, which indicated that almost all of the loss of free amino groups belonged to the lysines of TM.

The glycation degree of TM (panels A and B of Figure 1) suggested that TM could be highly glycated by saccharides with smaller molecular sizes, which could be less glycated by saccharides with larger molecular sizes. This might be due to the smaller saccharides having less steric hindrance and, therefore, could highly react with TM, while the greater saccharides had more steric hindrance to react with TM, leading to the reduced glycation degree. Previous investigations had obtained similar findings. Nakamura et al.<sup>23</sup> found that, after glycation by glucose, maltose, and maltotriose, glycated scallop TM exhibited a reduced glycation degree and lower available lysine contents with the promoted saccharide sizes. Chen et al.<sup>24</sup> reported that the greater molecular sizes of saccharides (glucose, maltose, and maltotriose) had a lower glycation degree on  $\beta$ -lactoglobulin. Zhang et al.<sup>25</sup> found that the glycation degree of  $\alpha$ -lactalbumin was reduced with greater saccharide sizes. Zhang et al.<sup>17</sup> found that shrimp TM (Exo m 1) glycated by glucose and maltose exhibited a higher glycation degree than TM glycated by maltotriose.

**AGEs and sRAGE Binding Capacity Analysis.** As reported, the AGEs (e.g., CML) could serve as potent neoepitopes and induce allergic reactions by binding the RAGE on immune cells (e.g., mast cell).<sup>14,26</sup> As shown in Figure 2A, in comparison to non-glycated TM, there were AGEs existing in GTMs, and the AGE contents rose as the following order: TM-M > TM-G > TM-MTS > TM-MPS > TM-MHS > TM, with TM-M exhibited the highest AGE

content. The previous reports suggested that the generation speed and levels of AGEs greatly relied on the different reducing saccharides.<sup>3</sup> The lowest AGE content in TM-MTS, TM-MPS, and TM-MHS in Figure 2A might be due to MTS, MPS, and MHS having greater molecular sizes, which performed a lower possibility to react with TM and generated AGEs.

Previous investigations had proven that CML could serve as a neoepitope, which could enhance the allergenicity.<sup>14,26–30</sup> With regard to the CML contents (Figure 2B), in comparison to non-glycated TM, GTM performed greater CML contents (TM-M > TM-G > TM-MTS > TM-MPS > TM-MHS > TM), which exhibited a similar change tendency to the AGE changes (Figure 2A), suggesting that the saccharides with greater molecular sizes led to less production of AGEs than saccharides with smaller molecular sizes. Moreover, glucose and maltose could induce higher CML production in GTMs than MTS, MPS, and MHS; this might be due to glucose and maltose having smaller molecular sizes and, therefore, having higher accessibility to react with TM and produce more CML. Zhang et al.<sup>31</sup> found that shrimp TM (Exo m 1) glycated by glucose and maltose performed higher AGE and CML contents than TM glycated by maltotriose. Moreover, in comparison to non-glycated TM, TM-M and TM-G exhibited greater RAGE binding capacity, with TM-M > TM-G > TM-MTS > TM-MPS > TM-MHS > TM (Figure 2C), which might be due to the saccharides of smaller sizes exhibiting less steric hindrance and more accessibility to react with TM, which produced more AGEs in TM-M and TM-G, leading to the greater capacity of TM-M and TM-G to bind RAGE.

**GTM Weakened the Allergic Responses of the Mast Cell.** The RBL-2H3 mast cell was used to analyze the allergenicity of TM and GTM (panels A and B of Figure 3). Specifically, as shown in Figure 3A, TM-G, TM-MTS, TM-MPS, and TM-MHS exhibited reduced allergenicity upon activating the IgE-sensitized RBL-2H3 mast cells to secrete histamine, while TM-M insignificantly influenced the secretion of histamine. The  $\beta$ -hexosaminidase enzymatic activity could be applied to reflect the degranulation degree of mast cells.<sup>32,33</sup> In comparison to the PBS-sensitized mast cells, TM could induce greater degranulation (Figure 3B), while TM-G, TM-MTS, TM-MPS, and TM-MHS induced a lower degranulation degree, and TM-M insignificantly affected the degranulation degree of RBL-2H3 mast cells.

**GTM Adsorbed to Al(OH)<sub>3</sub> Served as an Immunotherapy and Relieved Mouse Allergy Reactions.** Allergen

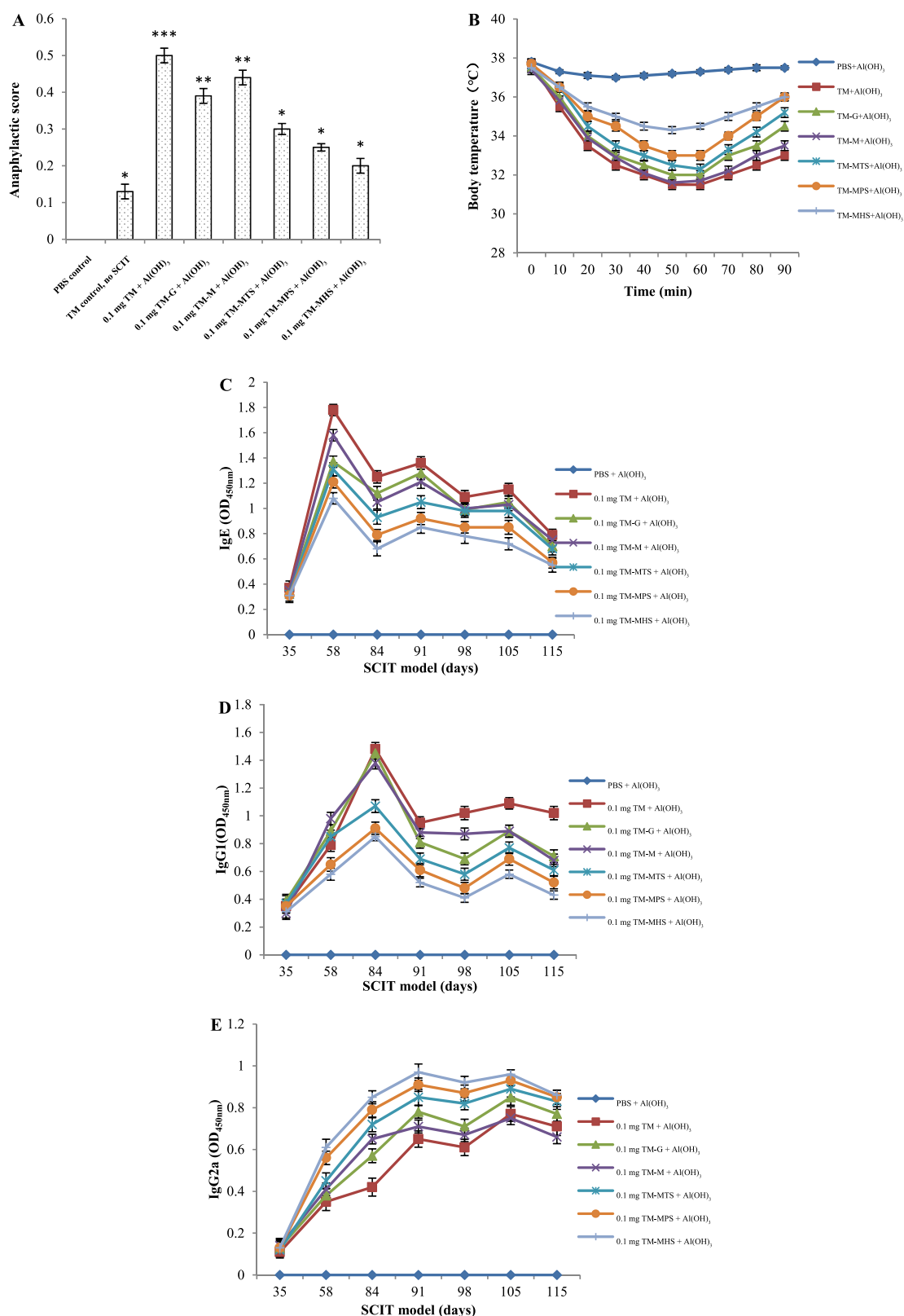


**Figure 4.** Experimental protocol of SCIT for TM or GTMs: (A) anaphylactic score after TM challenge on day 112, (B) body temperature, (C) serum IgE, (D) serum IgG1, and (E) serum IgG2a of mouse models (mean ± SD;  $n = 8$ ). TM, tropomyosin; TM-G, TM glycosylated by glucose; TM-M, TM glycosylated by maltose; TM-MTS, TM glycosylated by maltotriose; TM-MPS, TM glycosylated by maltopentaose; and TM-MHS, TM glycosylated by maltoheptaose. An asterisk showed significant differences compared to the PBS control: (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , and (\*\*\*)  $p < 0.001$ .

immunotherapy has the function to desensitize or tolerize allergic patients. Considering the efficiency of SCIT in allergic disease treatment, SCIT for food allergens has potential to

perform similar allergy desensitization results.<sup>5</sup> As reported, glycation modification could weaken the allergenicity of food allergens.<sup>14</sup> Therefore, glycation could be a candidate strategy

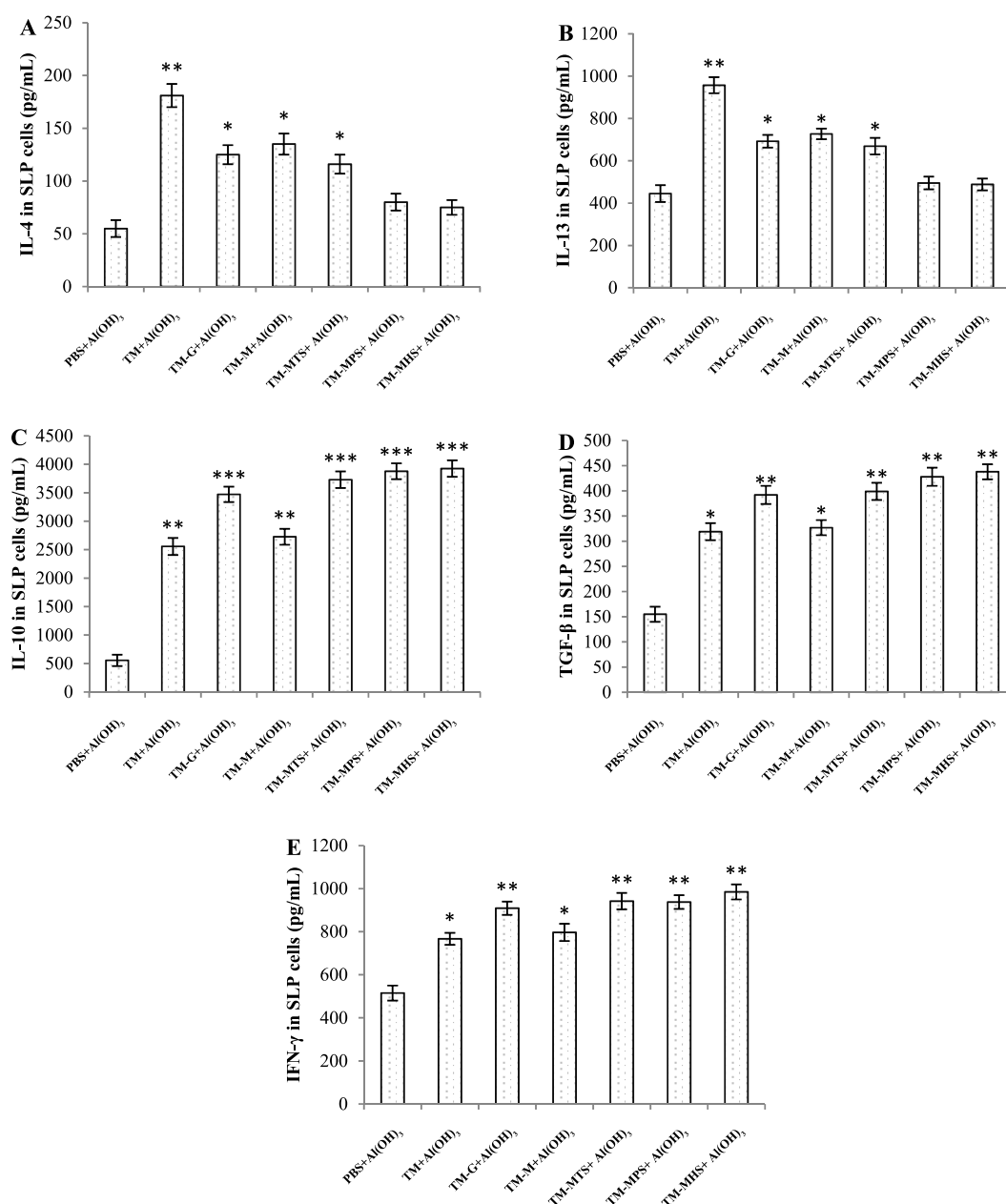


**Figure 5.** (A) IL-4, (B) IL-13, (C) IL-10, (D) TGF- $\beta$ , and (E) IFN- $\gamma$  contents of SLP cells from the mouse treated by TM + Al(OH)<sub>3</sub> or GTMs + Al(OH)<sub>3</sub> immunotherapy (SCIT) on day 115 (mean  $\pm$  SD;  $n = 8$ ). TM, tropomyosin; TM-G, TM glycated by glucose; TM-M, TM glycated by maltose; TM-MTS, TM glycated by maltotriose; TM-MPS, TM glycated by maltopentaose; and TM-MHS, TM glycated by maltoheptaose. An asterisk showed significant differences compared to the PBS + Al(OH)<sub>3</sub> group: (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , and (\*\*\*)  $p < 0.001$ .

to develop SCIT-based immunotherapy for food allergy desensitization. In this study, a mouse model was established to analyze the therapeutic efficacy of GTM + Al(OH)<sub>3</sub> as a candidate immunotherapy. As a result, SCIT of GTM + Al(OH)<sub>3</sub> could weaken the mouse allergic symptoms upon subsequent challenges. As shown in Figure 4A, after SCIT treatment, the mouse models exhibited weaker anaphylactic reactions, which indicated the immunotherapeutic efficacy of GTM + Al(OH)<sub>3</sub> as SCIT for desensitizing allergy responses. In Figure 4B, the PBS + Al(OH)<sub>3</sub> group had a slight body temperature reduction compared to the PBS + Al(OH)<sub>3</sub> group, and the mice of GTM + Al(OH)<sub>3</sub> group exhibited greater reduction of body temperature. For further analysis, the levels of TM-specific antibodies (IgE, IgG1, and IgG2a) secreted in sera were evaluated using the mouse sera of different

immunized groups (panels C–E of Figure 4). IgE and IgG1 belong to Th2-dependent antibodies, and IgG2a is a Th1-dependent antibody.<sup>34</sup> In comparison to the TM-specific IgE in the sera from mice receiving TM sensitization (day 35), the levels of serum TM-specific IgE in the mice treated with GTM + Al(OH)<sub>3</sub> were significantly greater after SCIT desensitization treatment for 3 weeks (day 58), while the levels of TM-specific IgE were significantly reduced after immunotherapy treatment for 6 weeks. As for IgG1, the mouse treated by GTM + Al(OH)<sub>3</sub> immunotherapy performed a steady promotion in TM-specific IgG1 and then performed a decreasing tendency of TM-specific IgG1 in mouse sera. With regard to IgG2a, the mouse treated by GTM + Al(OH)<sub>3</sub> immunotherapy performed a steady development of TM-specific IgG2a in mouse sera. Previous investigations also indicated that the immunothera-

pies with allergens in mouse immunotherapy models could weaken allergen sensitization.<sup>5,35</sup> While the preclinical mouse model of this work could not 100% mimic the human reality as a result of the uncertainties introduced by species differences, this study needs further clinical studies for patients in the future.<sup>7,10,36</sup>

**Immunotherapeutic Efficacy.** The immunotherapeutic efficacy and allergy desensitization mechanism were analyzed by evaluating the allergic cytokines in the spleen. After digestion, the allergenic components and histamine in the lymphocyte cells of intestinal walls could be brought into blood and immune organs and tissues through blood circulation,<sup>37</sup> triggering the secretion of various cytokines in the spleen.

IL-4 and IL-13 are Th2 cytokines; IL-10 and TGF- $\beta$  are Treg cytokines; and IFN- $\gamma$  belongs Th1 cytokine.<sup>26</sup> As shown in panels A–E of Figure 5, in comparison to TM + Al(OH)<sub>3</sub>, TM-G + Al(OH)<sub>3</sub>, TM-MTS + Al(OH)<sub>3</sub>, TM-MPS + Al(OH)<sub>3</sub>, and TM-MHS + Al(OH)<sub>3</sub> exhibited reduced allergenicity on SLP cells by downregulating the Th2 cytokines (IL-4 and IL-13) and upregulating the Treg cytokines (IL-10 and TGF- $\beta$ ) and Th1 cytokine (IFN- $\gamma$ ), while TM-M + Al(OH)<sub>3</sub> inhibited the secretion of Th2 cytokines (IL-4 and IL-13), insignificantly affecting the secretion of Th1 cytokine (IFN- $\gamma$ ) and Treg cytokines (IL-10 and TGF- $\beta$ ). Considering the SCIT data above, the GTM + Al(OH)<sub>3</sub> immunotherapy could desensitize the allergic reactions through downregulating the Th2 cytokines (IL-4 and IL-13) and upregulating the Treg cytokines (IL-10 and TGF- $\beta$ ) and Th1 cytokines (IFN- $\gamma$ ).

## DISCUSSION

This study investigates the allergenicity of GTM and the immunotherapeutic efficacy of GTM as an immunotherapy using the RBL-2H3 mast cell model and BALB/c mouse model. First, as shown in Figure 1, the decreased available lysine contents and free amino group contents in GTMs demonstrated that TM could be highly glycosylated by saccharides with smaller molecular sizes, which could be less glycosylated by the saccharides with larger molecular sizes. This could be due to the saccharides with smaller molecular sizes performing less steric hindrance and being more accessible to react with TM, while the saccharides with larger molecular sizes exhibited greater steric hindrance to react with TM, leading to the reduced glycosylation degree.<sup>17</sup>

Moreover, in comparison to non-glycosylated TM, TM glycosylated by glucose, MTS, MPS, and MHS exhibited weakened activation on the allergic reactions of mast cells (Figure 2), while TM glycosylated by maltose performed unobvious impact on activating the allergic reactions of mast cells. During glycosylation, the TM epitopes with glycosylation sites (e.g., lysine) could be destructed, while there might be production of AGE neoepitopes simultaneously; the altered equilibrium between epitope loss and AGE neoepitope production could lead to either promoted or weakened allergenicity of GTM. With regard to TM-G, TM-MTS, TM-MPS, and TM-MHS, the epitope loss may be greater than the production of AGE neoepitopes, which led to reduced allergenicity. With regard to TM-M, there was greater production of AGE neoepitopes, which could serve as a neoepitope in the induction of immunological reactions induced by AGEs;<sup>38–41</sup> therefore, the production of AGE neoepitopes could partly offset the epitope loss and result in the unobvious influence on TM-M allergenicity.

The RBL-2H3 mast cell model was used to evaluate the allergenicity of TM and GTM (panels A and B of Figure 3). In comparison to the TM-immunized mast cell, the GTM-immunized mast cell performed weakened allergic responses, reflected by the reduced amount of histamine release (Figure 3A) and weaker degranulation degree ( $\beta$ -hexosaminidase; Figure 3B), which suggested that GTM had weaker allergenicity. In a previous investigation by Zhang et al.,<sup>17</sup> the IgE binding capacities of TM and GTM were evaluated by ELISA and western blot; the results indicated that, in comparison to non-glycosylated TM, GTM performed weaker IgE binding capacity.

As reported, IgE is the critical mediator in IgE-mediated food allergy,<sup>42</sup> and the Th2 cytokines (IL-4 and IL-13) could greatly increase the IgE secretion,<sup>43</sup> while the Treg cytokines (IL-10 and TGF- $\beta$ ) and Th1 cytokine (IFN- $\gamma$ ) could reduce the secretion of IgE.<sup>44–47</sup> In this study (Figures 4 and 5), the IgE and Th2 cytokine (IL-4 and IL-13) levels reduced in SLP cells from mice that received GTM + Al(OH)<sub>3</sub> immunotherapy, while the Treg cytokine (IL-10 and TGF- $\beta$ ) and Th1 cytokine (IFN- $\gamma$ ) levels increased in SLP cells, which indicated that the GTM + Al(OH)<sub>3</sub> immunotherapy promoted the Th1 and Treg reactions while suppressing the Th2 reactions. Previous investigations also obtained similar results: The increased levels of Th1 cytokine (IFN- $\gamma$ ) and Treg cytokines (IL-10 and TGF- $\beta$ ) and the reduced levels of Th2 cytokines (IL-4 and IL-13) resulted in food allergy desensitization.<sup>10,48–52</sup> This could be due to the following: (1) the greater saccharide sizes on GTM could reduce the spatial space of the GTM surface, which could mask TM epitopes, and (2) the greater saccharide sizes could serve as a resistance to prevent the binding of TM epitopes to IgE on immune cells (e.g., mast cell).

The preclinical data of mouse models proved the potential of GTM + Al(OH)<sub>3</sub> as immunotherapy to desensitize the shrimp TM-induced allergic reactions. On the basis of the presented data above, it is anticipated that GTM + Al(OH)<sub>3</sub> could reduce the risk of allergic reactions as immunotherapy and offer immunotherapeutic benefits against future food allergen challenges. The preclinical data of this study provide further development of GTM for immunotherapy. Glycosylation between TM and saccharides of larger molecular sizes could produce hypoallergenic GTM with lower allergenicity; after GTM was absorbed to Al(OH)<sub>3</sub>, GTM + Al(OH)<sub>3</sub> could be a potential immunotherapy with high desensitization efficacy. The preclinical data of this work indicated that TM glycosylated by the saccharides of larger molecular sizes could be a potential immunotherapy for desensitizing food allergy.

In summary, this study analyzed the effects of glycosylation modification on decreasing the allergenicity of TM and generated hypoallergenic GTM and how GTM absorbed to Al(OH)<sub>3</sub> functions as a candidate immunotherapy for desensitizing allergy. As a result, in comparison to TM, the saccharides of smaller molecular sizes could lead to more AGEs in GTMs than saccharides of greater molecular sizes and TM glycosylated by saccharides of different molecular sizes exhibited lower allergenicity as a hypoallergen upon activating the allergic reactions of the mast cell and mouse model, while TM glycosylated by maltose (TM-M) had insignificant allergenicity changes upon activating the allergic reactions of the mast cell and mouse model. In addition, hypoallergenic GTM + Al(OH)<sub>3</sub> was efficient as a candidate immunotherapy; this work could offer preclinical research data to promote GTM +

Al(OH)<sub>3</sub> as a candidate immunotherapy for desensitizing patients allergic to shrimp food.

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### Notes

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## ABBREVIATIONS USED

AGE, advanced glycation end product; CT, cholera toxin; GTM, glycated tropomyosin; ig, intragastric; IgE, immunoglobulin E; IgG1, immunoglobulin G1; IgG2a, immunoglobulin G2a; IFN- $\gamma$ , interferon  $\gamma$ ; IL-4, interleukin 4; IL-10, interleukin 10; IL-13, interleukin 13; ip, intraperitoneal; MHS, maltoheptaose; MPS, maltopentaose; MTS, maltotriose; OPA, o-phthalaldehyde; PE, phycoerythrin; PNAG, 4-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide; RAGE, receptor for advanced glycation end products; SCIT, subcutaneous immunotherapy; SLP, spleen lymphocyte; sc, subcutaneously; TGF- $\beta$ , transforming growth factor  $\beta$ ; TM, tropomyosin

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