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1 # Allergenicity and Oral Tolerance of Enzymatic Cross-linked
2 # Tropomyosin Evaluated Using Cell and Mouse Models

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15 Running title: Allergenicity analysis of enzymatic cross-linked tropomyosin

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Abstract

The enzymatic cross-linking of proteins to form high-molecular-weight compounds may alter their sensitization potential. The IgG-/IgE-binding activity, digestibility, allergenicity and oral tolerance of cross-linked tropomyosin with tyrosinase (CTC) or horseradish peroxidase (CHP) were investigated. ELISA results demonstrated CTC or CHP reduced its IgE-binding activity by 34.5% ±1.8 and 63.5% ±0.6, respectively. Compared with native tropomyosin or CTC, CHP was easily digested into small fragments; CHP decreased the degranulation of RBL-2H3 cells, and increased endocytosis by dendritic cells. CHP can induce oral tolerance and reduce allergenicity in mice by decreased serum levels of IgE and IgG1, the production of T-cell cytokines, and the percentage composition of dendritic cells. These findings demonstrate CHP has more potential of reducing the allergenicity than CTC via influencing the morphology of protein, changing the original method of antigen-presenting, modulating the Th1/Th2 immunobalance, and induce the oral tolerance of the allergen tropomyosin.

Keywords: allergenicity reducing; tropomyosin; enzymatic cross-linking; horseradish peroxidase; oral tolerance potency

44 **1. Introduction**

45 Food allergy is becoming a worldwide problem and it affected about an estimated
46 6%–8% of children. The perceived prevalence of food allergy is even higher with an
47 estimated 20% of children adhering to some form of elimination diet.[1] Shellfish
48 allergy is the most frequent cause of food allergy and it is responsible for the majority
49 of emergency department visits related to severe food allergy.[2] Shellfish allergy in the
50 Asia-Pacific is fairly prevalent and ranks as one of the most common foods causing
51 allergy.[3] In China, 16.7% of the rural population is sensitized to shellfish.[4]

52 Shellfish allergy is a type I hypersensitivity reaction, which is mediated by the
53 binding of allergens and specific immunoglobulin E (IgE).[5] Different methods of food
54 processing can influence the allergenicity and immunogenicity of allergens. It may
55 involve both the sensitivity and effector phases of food allergy by influencing allergen
56 stability, conformation and digestibility.[6] Different processing technologies such as
57 heating, pasteurization and enzymatic cross-linking have different effects on allergens
58 in the complex foods.[7] Cross-linking of dietary proteins can increase the molecular
59 weight of proteins and change the secondary structure.[8] Tyrosinase is usually applied
60 to proteins and peptides by catalyzing the oxidation of tyrosine and results in
61 oxidative cross-linking of tyrosine side-chains. It has also been shown that tyrosinase
62 can polymerize peptides containing tyrosine.[9] Cross-linked β-casein by tyrosinase
63 contributed to a decrease in allergenicity.[10] Peroxidases are a diverse group of
64 oxidoreductases that use H_2O_2 as an electron acceptor to oxidize a variety of organic
65 and inorganic substrates such as phenols, aromatic phenols, phenolic acids and amino

66 acids.[11] The best-characterized plant peroxidase is from horseradish (HRP). It has
67 been hypothesized that peroxidases affect the gluten network by the cross-linking of
68 gluten proteins or by attaching arabinoxylans to gluten proteins via ferulic acid
69 moieties and lysine, tyrosine, or cysteine residues.[12] Therefore, enzymatic
70 cross-linking is an effective approach in food processing.

71 Several independent biochemical and immunological studies have identified the
72 major shellfish allergen as tropomyosin (TM).[13,14] It has been reported that the
73 molecular weight of TM is 38 kDa in crab and shrimp.[3,15] TM is a highly conservative
74 allergic protein with a high degree of amino acid sequence identity among different
75 species.[2-4,13] Currently, the most sufficient amino acids in TM epitopes, which have
76 been identified, are tyrosine, arginine, glutamic acid, serine, and phenylalanine.[16]
77 These amino acids are extremely sensitive to oxidative stress.[17] A previous study
78 found that enzymatic cross-linking with crab arginine kinase (AK) by tyrosinase had
79 reduced IgE-binding activity and allergenicity.[7] However, TM exhibits strong
80 resistance to digestive fluids, and retains its IgE-binding ability even after prolonged
81 heating due to the exceptionally stable alpha helical coiled-coil secondary
82 structure.[18,19] Therefore, it is necessary to assess whether TM can be cross-linked by
83 enzymes as enzymatic cross-linking can be used to influence TM stability,
84 conformation and digestibility, and reduce allergenicity and immunogenicity.

85 Mouse and cellular models are usually used to evaluate allergenicity and
86 immunogenicity in vivo and in vitro. Following exposure of allergens in vivo,
87 inflammation can occur, which is induced by cellular components and mediated by

88 dendritic cells (DCs), T cells and basophils.[20] DCs are key effector cells in allergy,
89 and once activated they can take in, process and present antigens to T cells and release
90 cytokines.[21] In addition, T regulatory (Treg) cells play an important role in the
91 inhibition of immune responses.[22] Therefore, T cells, DCs, basophils and Treg cells
92 are important indicators in assessing allergic reactions, but the effect of cross-linked
93 TM is unknown.

94 In the present study, the effect of crab TM cross-linking with horseradish
95 peroxidase or tyrosinase on the digestibility and IgG-/IgE-binding activity was
96 investigated with the simulated digestion and ELISA in vitro. The allergenicity and
97 cellular uptake of cross-linked TM were investigated by using cell models. The mouse
98 model was to investigate the potential of allergenicity and oral tolerance. The aim of
99 this study was to investigate the potential of enzymatic processing and provide
100 valuable references for further research into the different allergens in a complex food
101 system.

102

103 **2. Materials and methods**

104 **2.1 Chemicals**

105 The horseradish peroxidase (HRP) and tyrosinase from mushroom were purchased
106 from Sigma-Aldrich (Seelze, Germany). The goat anti-rabbit IgG antibody or goat
107 anti-human IgE antibody was from Kirkegaard and Perry Laboratories (Gaithersburg,
108 MD, USA). The goat anti mouse IgE, IgG1, IgG2a antibodies were purchased from
109 Abcam (Cambridge, UK). ELISA kit of histamine was purchased from IBL (Hamburg,

110 Germany). The ELISA kit of IL-4, IL-13, interferon (IFN)-γ and mouse mast-cell
111 protease-1 (mMCP-1) was purchased from R&D Systems (Minneapoils, MN, USA).

112 **2.2 Mice**

113 Five-week-old female BALB/c mice were purchased from Shanghai Laboratory
114 Animal Center of Chinese Academy of Sciences (Shanghai, China). All mice were
115 housed under specific pathogen-free conditions. No crab protein was present in the
116 diet. Experiments were approved by the Fisheries College of Jimei University (SCXK
117 2012-0005, Xiamen, China).

118 **2.3 Human sera**

119 Human sera from crab-allergic and shrimp-allergic patients (No. 7543, 4768, 2389,
120 1426, 8734, 5396 and 4658) were provided by the Xiamen Second Hospital (the
121 human ethical approval number is XSH2012-EAN019, Xiamen, China). The specific
122 IgE levels to crab and shrimp were assessed in vitro using an ImmunoCAP (Phadia
123 AB, Uppsala, Sweden). Table 1 shows the data of patients who were allergic to crab
124 and shrimp. All sera were stored in -30 ℃ until used.

125 **2.4 Protein purification and enzymatic cross-linking reaction**

126 TM was purified from *Scylla paramamosain* according to the method of Liang et
127 al.[23] The activity of HRP (670 nKat/mL) and the tyrosinase (1900 nKat/mL) was
128 assessed as described previously.[8,24] Cross-linking of TM was preliminarily produced
129 a higher molecular weight protein complex.[7] The optimal conditions for the
130 cross-linking of TM were as follows: HRP (800 nKat/g), and tyrosinase (1000 nKat/g)
131 at 37 ℃ for 1 h, and the protein complex were named CHP and CTC, respectively. In

addition, CTC and CHP will be heated at 95 ℃ for 10 min to inactivate enzymes. In the case of CTC, 0.25 mM caffeic acid was added as a mediator.

2.5 Electrophoretic and scanning electron microscopy analysis

The target protein was collected and detected on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.[7] The protein concentrations in the eluted solutions were estimated by measuring the absorbance at 280 nm.

Cross-linked TM and TM were coated with gold, and their morphology observed using a scanning electron microscope (SEM) (HITACHI S-4800, Zeiss, Germany). [7] The SEM analysis was detected under the following conditions: Mag=x1.00 k, Vacc=3.0 kV, WD=7.2 mm and SignalName=SE(M)

2.6 ELISA analysis

The IgG-/IgE-binding activity of TM was measured by ELISA, which was performed as previously described, with some modifications.[25] An ELISA plate (P1) was coated with native purified TM (100 μg/mL) and incubated overnight at 4 ℃. The plate was washed and then blocked with 5% skimmed milk at 37 ℃ for 1.5 h. In another ELISA plate (P2), cross-linked TM was incubated with a rabbit anti-crab (*Scylla paramamosain*) TM poly-clonal antibody or crab allergic patients' sera at 37 ℃ for 2 h. After washing P1, P2 solution was added to P1 at 37 ℃ for 2 h. Then washing P1, it was incubated with goat anti-rabbit IgG antibody or goat anti-human IgE antibody (diluted 1:2000) at 37 ℃ for 1.5 h. After a final wash, tetramethylbenzidine was added and P1 was incubated at 37 ℃ for 20 min. The

153 reaction was terminated by sulfuric acid and it was measured at 450 nm using an
154 automatic microplate reader (Infinite ® M200 PRO, Tecan, Austria).

155 **2.7 Secondary structure and surface hydrophobicity analysis**

156 Far-UV CD spectra were measured with a Chirascan circular dichroism
157 spectrometer (Applied Photophysics Ltd., Surrey, UK), according to a method
158 described previously.[26] Each spectrum represents the scan at 25 ℃ with TM
159 concentration adjusted to 0.5 mg/mL. The operating parameters were as follows: scan
160 rate, interval and bandwidth were set to 100 nm/min, 0.25 s and 1.0 nm, respectively.
161 The final results are shown as mdeg (deg cm^2 $dmol^{-1}$).

162 The surface hydrophobicity of cross-linked TM was determined using
163 8-anilino-1-naphthalenesulfonic acid as the fluorescence probe, according to a method
164 described previously.[27]

165 **2.8 In vitro digestion assay**

166 The simulated gastric fluids (pepsin) were prepared as described previously.[28] The
167 final concentration of TM and cross-linked TM was adjusted to 0.5 mg/mL.
168 SDS-PAGE and western blot analysis (using the goat anti-rabbit IgG antibody) were
169 to determine the digestibility and IgG-binding activity.

170 **2.9 RBL-2H3 and dendritic cells assay**

171 The rat mast cell line RBL-2H3 was obtained from the American Type Culture
172 Collection (Bethesda, MD, USA). The cells were cultured as described previously.[29]
173 The release of β-hexosaminidase and histamine from RBL-2H3 cells was measured as
174 a model of IgE-mediated mast cell allergic reaction,[29] using the sensitization of mouse

sera and TM or cross-linked TM (500 ng/mL) for 6 h and 15 min at 37 ℃, respectively. The cell supernatant was measured the release levels of β-hexosaminidase and histamine using an ELISA kit (IBL, Hamburg, Germany).

Bone marrow cells from mice were cultured at 10^6 cells/mL in complete RPMI 1640 (Hyclone, Logan, USA) with 10 ng/mL of granulocyte-macrophage colony stimulating factor and 5 ng/mL of interleukin (IL)-4. At day 6, approximately 90% of cells expressed medium–high levels of CD11c and major histocompatibility complex (MHC) class II. To assess protein endocytosis, pHrodo Green (Invitrogen, Life Technologies, Breda, Netherlands)-labeled TM or cross-linked TM was incubated with 10^6 cells for 0, 15, and 30 min. The pHrodo Green-positive DCs were measured by using an automatic microplate reader.

2.10 In vivo studies

2.10.1 Oral sensitization to TM

The oral sensitization testing was used according to the method of Radosavljevic et al.[8] Mice were exposed to 3 mg TM (n=6) or cross-linked TM (n=6) with 7.5 μg cholera toxin by intragastric gavage for 3 consecutive days, and this was repeated once a week for 4 weeks. The control group (n=6) received phosphate buffer saline. All mice received a challenge of 6 mg TM or cross-linked TM intragastrically on day 28, and were sacrificed 1 day later to measure the levels of anti-TM IgG2a, IgE, and IgG1. Blood sample used to measure the levels of mMCP-1 and histamine was collected at 30 min after oral challenge on day 28.

196 Splenocytes and mesenteric lymph nodes (MLN) lymphocytes were prepared by
197 aseptic removal on day 29 and cell supernatant were measured the levels of the IL-4,
198 IL-13 and IFN-γ after 3 days.

199 The spleen and MLNs cells (2×10^5 cells/well) were isolated from mice on day 29 to
200 detect changes in the number of Treg cells. Cells were stained with anti-CD4-PerCP
201 Cy5.5 and anti-Foxp3-Alexa 647 (BD Pharmingen, San Diego, CA, USA)
202 fixation/permeabilization and analyzed by flow cytometry. All flow cytometry
203 experiments were performed with the Guava easyCyte 6−2L system and GuavaSoft
204 3.1.1 software (Millipore, MA, USA).

205 **2.10.2 Induction of oral tolerance to TM**

206 Mice (n=6) received 1 mg TM or cross-linked TM or phosphate buffer saline via
207 intragastric gavage for 3 consecutive days. This was followed by intraperitoneal
208 immunization with 200 μg TM/alum adjuvant (Pierce, Rockford, IL, USA) 14 and 21
209 days after the last exposure. Blood was obtained on day 34 and the animals were
210 subsequently sacrificed.

211 The blood was collected on day 34 to measure the level of specific antibodies.
212 Spleen and MLN single-cell suspensions (1×10^5 cells in 200 μL of complete RPMI
213 1640) were incubated for 3 days. IL-4, IL-13 and IFN-γ levels in the culture
214 supernatants of splenocytes and MLN were determined by ELISA kits.

215 Splenocyte and MLN cell suspensions were evaluated to determine changes in the
216 numbers of DCs. Cells were stained with anti-CD11c-APC and anti-MHC-Ⅱ-FITC

217 (BD Pharmingen) and analyzed by flow cytometry. All flow cytometry experiments
218 were performed with the Guava easyCyte 6−2L system and GuavaSoft 3.1.1 software.

219 **2.11 Statistical analysis**

220 Data from the in vivo and in vitro studies were presented as mean ± SD. Data were
221 analyzed by the General Linear Model and ANOVA of Duncan test. Differences
222 between groups were considered significant when p-values were <0.05. Each
223 experiment was repeated at least 3 times.

224

225 ## 3. Results

226 ### 3.1 Electrophoretic and scanning electron microscopy analysis

227 SDS-PAGE analysis showed that enzymatic cross-linked caused the polymerization
228 of TM. Compared to that of TM, both CTC and CHP produced higher molecular
229 weight (MW) proteins (Figure 1A). CTC produced the MW proteins over 66 kDa.
230 CHP produced more complicated protein complex, containing proteins of MW over
231 45 kDa and others greater than 116kDa. In addition, SEM results showed (Figure 1B)
232 that both CTC and CHP were built from rod proteins, which connected to form a
233 network through the enzymatic cross-linked reaction. The extent of CTC was tighter
234 than that with CHP, while the extent of CHP resulted in a loose network.

235 ### 3.2 Analysis of the IgG-/IgE-binding activity of cross-linked TM

236 The IgG-/IgE-binding activity of CTC and CHP was investigated to the
237 allergenicity (Figure 1C). ELISA analysis revealed that the IgG-binding activity of

238 CTC and CHP decreased by 16.9% and 70.8%, and the IgE-binding activity of CTC

239 and CHP reduced by 34.5% ±1.8 and 63.5% ±0.6, respectively.

240 **3.3 Secondary structure and surface hydrophobicity of cross-linked TM**

241 Far-ultraviolet CD data were used to detect the secondary structure of cross-linked

242 TM (Figure 1D). After HRP and tryosinase treatment, the positive and negative molar

243 residue ellipticity peaks at near 208 nm and 220 nm of the samples decreased, which

244 indicated that the content of α-helix decreased. Further analyses performed with

245 CDNN software showed that the amount of α-helix in CTC was reduced to 78.5% and

246 in CHP was reduced to 86.8%. The percentage of β-sheet, at around 198 nm,

247 increased to 2.3% in CTC and increased to 1.5% in CHP. In addition, the percentage

248 of β-turn in CTC was up to 10.2% and in CHP was up to 8.6%. The amount of

249 random coil, at near 220 nm and 230 nm, increased to 7.9% in CTC and increased to

250 5.0% in CHP.

251 Based on the results of surface hydrophobicity (Figure 1E), CTC had high

252 hydrophobicity compared with TM and the fluorescence intensity peak showed a red

253 shift, while CHP had low hydrophobicity compared with TM, and the fluorescence

254 intensity peak decreased.

255 **3.4 The stability of cross-linked TM in a simulated digestion in vitro**

256 TM was digested into 32 kDa fragment by pepsin within 10 min and 32 kDa

257 fragment also failed to degrade until 60 min (Figure 2A). CTC remained as a

258 macromolecular band and 32kDa fragment were seen up to 60 min (Figure 2B), while

259 CHP was easily digested into small fragments within 10 min (Figure 2C). CTC was

260 more difficult to digest than TM, while the CHP exhibited easier degradation than TM
261 or CTC. As shown in Figure 2D–F, western blotting showed that the IgG-binding
262 activity of CTC digested fragments should be in Figure 2E looks increasing intensity,
263 but CHP and its digested fragments had no IgG-binding activity. In addition, there
264 was little change in IgG-binding activity of the TM fragments.

265 **3.5 The impact of cross-linked TM in IgE-mediated RBL-2H3 cells and uptake**
266 **by DCs**

267 RBL-2H3 cells are the IgE-mediated mast cell model used to release
268 β-hexosaminidase and histamine.[29] TM induced significant degranulation in
269 RBL-2H3 cells, while CTC and CHP induced a lower level of degranulation than that
270 of TM (Figure 3A-B). The release of β-hexosaminidase in the CTC group reduced
271 11.4% and it in CHP group reduced 21.3%. In addition, the release of histamine in the
272 CTC group decreased 51.5% and it in CHP group decreased 60.2%.

273 The pHrodo Green dye was used to label proteins as it fluoresces brightly at acidic
274 pH with almost no fluorescence at neutral pH, which makes this dye a good indicator
275 of the localization of TM or cross-linked TM in the endolysosomal compartments.
276 Endocytosis of pHrodo-labeled TM was internalized at a much slower rate, and at 30
277 min there was no obvious increase. pHrodo-labeled CTC was slightly increased at 30
278 min, and pHrodo-labeled CHP reached saturation at 30 min and was obviously
279 increased compared to TM and CTC (Figure 3C).

280 **3.6 Allergic responses following intragastric exposure to cross-linked TM**

281 An animal model of allergy was used to test the allergenicity of cross-linked TM in
282 vivo (Figure 4A). First, CTC and CHP decreased in the levels of the TM-specific IgE
283 and IgG1 in mouse serum; however, CHP led to an obvious increase in the level of the
284 TM-specific IgG2a in serum (Figure 4B). Second, the level of histamine in sera in the
285 CTC and CHP group (Figure 4C) was significantly decreased compared with the TM
286 group. In addition, it should be noted that the changes in mMCP-1 between TM and
287 cross-linked TM (Figure 4D) showed that TM, CTC and CHP reached 28 ng/mL, 17
288 ng/mL and 16 ng/mL, respectively. Third, the levels of IL-4 and IL-13 as
289 representative Th2-related cytokines (Figure 4E–F) in spleen cells were decreased.
290 IFN-γ, a Th1-related cytokine in spleen cells, was increased (Figure 4G). However,
291 the levels of IL-4 and IL-13 (Figure 4H–I) in MLN lymphocytes was increased. In the
292 MLN lymphocyte, compared to the production of IFN-γ in TM, that of CTC was
293 slightly increased and that of CHP was unchanged (Figure 4J).
294 To examine the change in $CD4^+$ fork head box P3 (Foxp3) T cells in the four groups
295 of mice, flow cytometry experiments were performed (Figure 4K). In splenocytes, the
296 percentage of $CD4^+Foxp3^+$ Treg cells increased from 1.68% (TM group) to 1.91%
297 (CTC group) and 2.42% (CHP group). No significant change in MLN lymphocytes
298 (CTC group) was observed, while CHP group slightly increased.

299 **3.7 Oral tolerance induced by cross-linked TM**

300 The ability of cross-linked TM to induce gut and systemic immune tolerance was
301 investigated in the oral tolerance testing (Figure 5A). Compared with mice in the
302 TM-TM group, the CTC-TM and CHP-TM groups showed reduced levels of the

303 TM-specific IgE, IgG1, but not IgG2a (Figure 5B). The levels of IL-4, IL-13 and

304 IFN-γ were measured in spleen cell and MLN lymphocyte (Figure 5C–H). Compared

305 with TM-TM, CTC-TM and CHP-TM decreased the production of IL-4 and IL-13,

306 and increased the production of IFN-γ in spleen cells. However, in the CTC-TM and

307 CHP-TM groups, the production of IL-13 was increased, IFN-y in MLN lymphocytes

308 in CTC-TM was slightly increased while CHP-TM slightly decreased, and the

309 production of IL-4 in MLN lymphocytes was unchanged. To examine changes in the

310 numbers of DCs in the four groups of mice, flow cytometry experiments were

311 performed (Figure 5I). In splenocytes, the percentage of CD11c + MHC-Ⅱ+ DCs

312 decreased from 7.03% (TM-TM group) to 6.42% (CTC-TM group) and 6.02%

313 (CHP-TM group). However, the percentage of CD11c + MHC-II+ DCs increased

314 from 1.54% (TM-TM group) to 2.25% (CTC-TM group) and 2.92% (CHP-TM group)

315 in MLN lymphocytes.

316

317 **4. Discussion**

318 The present study shows that cross-linked TM with tyrosinase or HRP possesses

319 allergenic and immunologic properties. The results that cross-linked proteins CTC and

320 CHP were formed complicated protein complex and it was consistent with previous

321 studies.[7,30,31] Moreover, many studies have shown that cross-linked dietary products

322 further influenced their allergenicity.[7,30-32] Tyrosinase catalyzes the oxidation of

323 tyrosine and results in oxidative cross-linking of tyrosine side-chains, so cross-linking

324 of TM by tyrosinase was catalyzed the oxidation of the amino acid residues of Y_{162},

325 Y_{221} and Y_{267} in TM linear epitopes. Peroxidases are a diverse group of
326 oxidoreductases that use H_2O_2 as an electron acceptor to oxidize a variety of organic
327 and inorganic substrates such as phenols, aromatic phenols, phenolic acids and amino
328 acids, therefore cross-linking of TM by HRP was catalyzed the oxidation of the amino
329 acid residues of Y_{162}, Y_{221}, Y_{267} and F_{153} in TM linear epitopes. Therefore, TM linear
330 epitopes were covered or destroyed to reduce the IgE-binding activity.
331 Due to the effect of enzymatic cross-linked reaction, the secondary structure of
332 cross-linked TM has changed. The polymerization of TM produced the more complex
333 structures, so the percentage of α-helix in cross-linked TM reduced and the amount
334 of β-turn, random coil increased. In addition, from the results of SEM, the
335 cross-linked TM generated the complex change. Enzymatic cross-linking also caused
336 the hydrophobicity. The cross-linking of TM by tyrosinase makes the hydrophilic
337 amino acids covered to increase the hydrophobicity, while cross-linking of TM by
338 HRP makes the hydrophilic amino acids exposed to decrease the hydrophobicity. In
339 addition, the correlation was found between hydrophobicity and digestion in two
340 recent studies.[31,33] CTC had high hydrophobicity compared with TM or CHP, which
341 also explained its digestibility, as it was difficult for pepsin to find cleavage sites.
342 Interestingly, CHP, which forms a loose network, increased digestibility by reducing
343 its hydrophobicity. In addition, CHP resulted in more digestion of the cleavage sites,
344 and the new small fragments did not have IgG-/IgE-binding activity. Hence, this may
345 explain why these two treatments behaved differently in simulated digestion and
346 surface hydrophobicity. This eventually elicited a different immunological response

347 compared to the native TM. From the results of endocytosis of pHrodo-labeled TM or
348 cross-linked TM, it is associated with digestion. pHrodo-labeled CHP was easily
349 digested within 10 min, hence it can be faster accumulation and processing in DCs
350 and it fluoresces brightly at acidic pH in the lysosome of DCs. Therefore, the
351 fluorescence intensity of CHP is stronger than CTC or TM.

352 Cross-linked TM influenced allergenicity and the degranulation in RBL-2H3 cells.
353 This was in agreement with the previous study where the treatment of AK with
354 tyrosinase/caffeic acid decreased degranulation.[7] The response of RBL-2H3 cells may
355 be explained by the aggregation of cross-linked TM which covered the IgE epitopes,
356 thus successfully decreasing the degranulation of basophils.

357 Treg cells inhibited $CD4^+$ T cells, thus reducing the secretion of IL-4.[7] As a result,
358 the percentage of $CD4^+Foxp3^+$ Treg cells (cross-linked TM group) increased in
359 splenocytes compared to the TM group and this was consistent with the production of
360 cytokines. In addition, cross-linked TM relieved allergy symptoms in mice compared
361 to TM, as shown by significantly lower levels of IgG1 and IgE and cross-linked TM
362 reduced the levels of mMCP-1 and the release of histamine.

363 The influence between spleen and MLN cells has been reported.[34,35] Our results
364 show that the percentage of $CD4^+Foxp3^+$ Treg cells in MLN cells was not
365 significantly changed and the production of cytokines was increased. The
366 concentration of antigen reaching the circulation may change by the nature and dose
367 of antigen.[36] Cross-linked TM after gastrointestinal digestion will produce some
368 allergic degradation segments, but this was gradually degraded into smaller

non-allergenic molecular fragments while the time increased. The low concentration of allergenic degradation segments only caused local gastrointestinal lymphocyte immune responses and smaller molecular fragments did not cause an immune response in the spleen. Thus, cross-linked TM changed the antigens presented from MLN to the spleen.

DCs as antigen-presenting cells play a central role in oral tolerance.[37,38] In addition, DCs carry antigens from MLN to the spleen.[39,40] The number of DCs in the spleen in the CTC-TM and CHP-TM groups decreased compared with the TM-TM group. Hence, the cross-linked TM altered the method of antigen digestion and not only affected antigen presentation, but also induced oral tolerance in mouse spleen cells.

Oral tolerance alleviates the immune responses and it plays an important role in immune homeostasis. The intestinal immune system includes inductive and effector sites. Inductive sites contain the gut-associated lymphoid tissues; the main effector sites contain the lamina propria (LP) and epithelium, harboring large populations of activated T cells and antibody-secreting plasma cells. The LP may also contribute to the induction of tolerance, as a site of antigen uptake and loading of migratory DCs that encounter naive T cells in the MLN.[37] $CD103^+$ DCs from the LP and MLN induce the expression of gut-homing molecules on T cells to cause the release of cytokines on T cells in the MLN. Combined with the results of endocytosis by DCs in vitro, CHP and CTC can easily be taken up by DCs; therefore, this influenced the number of DCs in the MLN of mice. Orally administered antigens can inhibit following immune responses in the gut and the systemic immune system; however,

391 cross-linked TM only suppresses immune responses in the systemic immune system.
392 Low molecular weight protein may pass directly across the epithelium by paracellular
393 diffusion, while larger molecular proteins can be taken across enterocytes. These
394 findings showed that cross-linked TM was different from TM in the immunological
395 response. In addition, the variation in antigen-presenting from the MLN to the spleen
396 was unknown. A further in-depth study of these issues is planned for the future.

397 In our previous study,[7] enzymatic cross-linking was an effective way of reducing
398 crab allergens, which AK is the main allergens. Compared with cross-linked AK, CTC
399 had a shorter cross-linking time and did not require heating of TM in advance. In
400 addition, we also found that CHP reduced IgG-/IgE-binding activity more than CTC
401 and TM, and decreased the stability of pepsin digestion. Furthermore, some of the
402 crab allergens achieved the same effect of reducing of allergenicity due to enzymatic
403 cross-linking. Therefore the hypoallergenic crab meat was developed and applied for
404 a Chinese patent (No. 2015108074188) based on the enzymatic cross-linking process
405 technique. Clinical trials are required to confirm that tyrosinase and HRP cross-linked
406 crab allergens may reduce the crab allergy.

407 In conclusion, enzymatic treatment of TM with tyrosine or HRP yielded high
408 molecular weight compounds. CHP, which forms a loose network, decreased
409 digestibility by reducing its hydrophobicity. It also has reduced IgG-/IgE-binding
410 activity and allergenicity. The digestible CHP changed the original method of
411 antigen-presenting and induced oral tolerance in the systemic immune system. In

future investigations, it is important to examine the effects of cross-linked reaction on the different allergens in a complex food system.

Conflict of interest

The authors declare that there is no conflict of interests.

Abbreviations

CHP: cross-linked tropomyosin with horseradish peroxidase

CTC: cross-linked tropomyosin with tyrosinase/caffeic acid

DCs： dendritic cells

ELISA: enzyme linked immunosorbent assay

HRP: horseradish peroxidase

mMCP-1: mouse mast-cell protease-1

MLN： mesenteric lymph nodes

TM: tropomyosin

Treg： regulatory T

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435 **References**

436 (1) Noimark, L., Cox, H. E. Nutritional problems related to food allergy in childhood. *Pediatr.*
437 *Allergy Immunol*. **2008**, *19*, 188-195.
438 (2) Sicherer, S. H., Muñoz-Furlong, A., Sampson, H. A. Prevalence of seafood allergy in the
439 United States determined by a random telephone survey. *J. Allergy Clin. Immunol*. **2004**, *114*,
440 159-165.
441 (3) Lee, A. J., Gerez, I., Shek, L. P. C., Lee, B. W. Shellfish allergy-an Asia-Pacific
442 perspective. *Asian Pac. J. Allergy Immunol.* **2012**, *30,* 3−10.
443 (4) Kim, J. S., Ouyang, F., Pongracic, J. A., Fang, Y., et al. Dissociation between the prevalence of
444 atopy and allergic disease in rural China among children and adults. *J. Allergy Clin.*
445 *Immunol*. **2008**, *122*, 929-935.
446 (5) Martos, G., Lopez-Exposito, I., Bencharitiwong, R., Berin, M. C., et al. A. Mechanisms
447 underlying differential food allergy response to heated egg. *J. Allergy Clin. Immunol*. **2011**, *127*,
448 990-997.
449 (6) Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., et al. Crosslinking food proteins for
450 improved functionality. *Ann. Rev. Food Sci. Technol*. **2010**, *1*, 113-138.
451 (7) Fei, D. X., Liu, Q. M., Chen, F., Yang, Y., et al. Assessment of the sensitizing capacity and
452 allergenicity of enzymatic cross‐linked arginine kinase, the crab allergen. *Mol. Nutr. Food Res*.
453 **2016**, *60*, 1707–1718.
454 (8) Radosavljevic, J., Nordlund, E., Mihajlovic, L., Krstic, M., et al. Sensitizing potential of
455 enzymatic cross‐linked peanut proteins in a mouse model of peanut allergy. *Mol. Nutr. Food*
456 *Res*. **2014**, *58*, 635-646.

457 (9) Mattinen, M. L., Lantto, R., Selinheimo, E., Kruus, K., et al. Oxidation of peptides and
458 proteins by Trichoderma reesei and Agaricus bisporus tyrosinases. *J Biotechnol.* **2008**, *133*,
459 395-402.
460 (10) Stanic, D., Monogioudi, E., Dilek, E., Radosavljevic, J., et al. Digestibility and allergenicity
461 assessment of enzymatic crosslinked β‐casein. *Mol. Nutr. Food Res*. **2010**, *54*, 1273-1284.
462 (11) Malencik, D. A., Anderson, S. R. Dityrosine formation in calmodulin: cross-linking and
463 polymerization catalyzed by Arthromyces peroxidase. *Biochemistry*. **1996**, *35*, 4375-4386.
464 (12) Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., Monogioudi, E., Faccio, G., ... & Lantto,
465 R. Crosslinking food proteins for improved functionality. *Ann. Rev. Food Sci. Technol*. **2010**, *1*,
466 113-138.
467 (13) Lopata, A. L., O'hehir, R. E., Lehrer, S. B. Shellfish allergy. *Clin Exp Allergy*. **2010**, *40*,
468 850-858.
469 (14) Lopata, A. L., Jeebhay, M. F. Airborne seafood allergens as a cause of occupational allergy
470 and asthma. *Curr. Allergy Asthm. R*. **2013**, *13*, 288-297.
471 (15) Gill, B. V., Rice, T. R., Cartier, A., Gautrin, D., et al. Identification of crab proteins that elicit
472 IgE reactivity in snow crab–processing workers. *J. Allergy Clin. Immunol*. **2009**, *124*, 1055-1061.
473 (16) Zheng, L. N., Lin, H., Pawar, R., Li, Z. X., et al. Mapping IgE binding epitopes of major
474 shrimp (*Penaeus monodon*) allergen with immunoinformatics tools. *Food Chem. Toxicol*. **2011**, *49*,
475 2954-2960.
476 (17) Chondrogianni, N., Petropoulos, I., Grimm, S., Georgila, K., et al. Protein damage, repair and
477 proteolysis. *Mol. Aspects Med.* **2014**, *35*, 1-71.
478 (18) Kamath, S. D., Rahman, A. M. A., Komoda, T., Lopata, A. L. Impact of heat processing on

479 the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using
480 specific monoclonal antibodies. *Food Chem*. **2013**, *141*, 4031-4039.
481 (19) Yadzir, Z. H. M., Misnan, R., Bakhtiar, F., Abdullah, N., et al. Tropomyosin, the major
482 tropical oyster *Crassostrea belcheri* allergen and effect of cooking on its allergenicity. *Allergy,*
483 *Asthma Clin. Immunol.* **2015**, *11*, 1.
484 (20) Longo, G., Berti, I., Burks, A. W., Krauss, B., et al. IgE-mediated food allergy in
485 children. *The Lancet*, **2013**, *382*, 1656-1664.
486 (21) Pouniotis, D., Tang, C. K., Apostolopoulos, V., & Pietersz, G. Vaccine delivery by penetratin:
487 mechanism of antigen presentation by dendritic cells. *Immunol. Res.* **2016**, 1-14.
488 (22) Brandenburg, S., Takahashi, T., de la Rosa, M., Janke, M., et al. IL‐2 induces in vivo
489 suppression by $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells. *Eur. J. Immunol.* **2008**, *38*, 1643-1653.
490 (23) Liang, Y. L., Cao, M. J., Su, W. J., Zhang, L. et al. Identification and characterisation of the
491 major allergen of Chinese mitten crab (*Eriocheir sinensis*). *Food Chem*. **2008**, *111*, 998-1003.
492 (24) Srinroch, C., Srisomsap, C., Chokchaichamnankit, D., Punyarit, P., et al. Identification of
493 novel allergen in edible insect, *Gryllus bimaculatus* and its cross-reactivity with *Macrobrachium*
494 spp. allergens. *Food Chem*. **2015**, *184*, 160-166.
495 (25) Mao, H. Y., Cao, M. J., Maleki, S. J., Cai, Q. F., et al. Structural characterization and IgE
496 epitope analysis of arginine kinase from Scylla paramamosain. *Mol. Immunol.* **2013**, *56*, 463-470.
497 (26) Zhang, Y. X., Chen, H. L., Maleki, S. J., Cao, M. J., Zhang, L. J., Su, W. J., & Liu, G. M.
498 Purification, characterization, and analysis of the allergenic properties of myosin light chain in
499 *Procambarus clarkii*. *J. Agric. Food Chem.* **2015**, *63*, 6271-6282.
500 (27) Puppo, C., Chapleau, N., Speroni, F., de Lamballerie-Anton, M., et al. Physicochemical

501 modifications of high-pressure-treated soybean protein isolates. *J. Agric. Food Chem.* **2004**, *52*,
502 1564-1571.
503 (28) Chen, H. L., Mao, H. Y., Cao, M. J., Cai, Q. F., et al. Purification, physicochemical and
504 immunological characterization of arginine kinase, an allergen of crayfish (*Procambarus*
505 *clarkii*). *Food Chem. Toxicol*, **2013**, *62*, 475-484.
506 (29) Liu, Q. M., Yang, Y., Maleki, S. J., Alcocer, M., et al. Anti-Food Allergic Activity of Sulfated
507 Polysaccharide from Gracilaria lemaneiformis is Dependent on Immunosuppression and Inhibition
508 of p38 MAPK. *J. Agric. Food Chem.* **2016**, *64*, 4536-4544.
509 (30) Bowman, C. C., Selgrade, M. K. Failure to induce oral tolerance in mice is predictive of
510 dietary allergenic potency among foods with sensitizing capacity. *Toxicol. Sci.* **2008**, *106*,
511 435-443.
512 (31) Xie, N., Wang, B., Jiang, L., Liu, C., et al. Hydrophobicity exerts different effects on
513 bioavailability and stability of antioxidant peptide fractions from casein during simulated
514 gastrointestinal digestion and Caco-2 cell absorption. *Food Res. Internat.* **2015**, *76*, 518-526.
515 (32) Monogioudi, E., Faccio, G., Lille, M., Poutanen, et al. Effect of enzymatic cross-linking of
516 β-casein on proteolysis by pepsin. *Food Hydrocoll.* **2011**, *25*, 71-81.
517 (33) Zhou, F., Zhao, M., Cui, C., Sun, W. Influence of linoleic acid-induced oxidative
518 modifications on physicochemical changes and in vitro digestibility of porcine myofibrillar
519 proteins. *LWT - Food Sci. Technol.* **2015**, *61*, 414-421.
520 (34) Roth‐Walter, F., Berin, M. C., Arnaboldi, P., Escalante, C. R., et al. Pasteurization of milk
521 proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy.*
522 **2008**, *63*, 882-890.

523 (35) Karikalan, M., Rajukumar, K., Mishra, N., Kumar, M., et al. Distribution pattern of bovine
524 viral diarrhoea virus type 1 genome in lymphoid tissues of experimentally infected sheep. *Vet. Res.*
525 *Commun*. **2016**, *40*(2), 55-61.
526 (36) Luo, X., Zheng, Y., Wen, R., Deng, X., et al. Effects of ceftriaxone induced intestinal
527 dysbacteriosis on lymphocytes in different tissues in mice. *Immunobiol.* **2016***, 221,* 994-1000.
528 (37) Pabst, O., & Mowat, A. M. Oral tolerance to food protein. *Mucosal Immunol.* **2012**, *5*,
529 232-239.
530 (38) Stojadinovic, M., Pieters, R., Smit, J., Velickovic, T. C. Cross-linking of β-lactoglobulin
531 enhances allergic sensitization through changes in cellular uptake and processing. *Toxicol.*
532 *Sci.* **2014**, *140*, 224-235.
533 (39) Pouniotis, D., Tang, C. K., Apostolopoulos, V., Pietersz, G. Vaccine delivery by penetratin:
534 mechanism of antigen presentation by dendritic cells. *Immunol. Res.* **2016**, 1-14.
535 (40) Wen, Q., Zhou, C., Xiong, W., Su, J., et al. MiR-381-3p regulates the antigen-presenting
536 capability of dendritic cells and represses antituberculosis cellular immune responses by targeting
537 CD1c. *J. Immunol.* **2016**, *197*, 580-589.
538

539 **Figure legends**

540 **Figure 1. The polymerization and IgG-/IgE-binding activity of enzymatic cross-linked TM**

541 **compared with TM.**

542 A, Cross-linked tropomyosin with tyrosinase/caffeic acid (CTC), cross-linked tropomyosin with

543 horseradish peroxidase (CHP) resolved in 12% SDS-PAGE;

544 B, SEM of TM, CTC, and CHP. The scale of the photo has been added;

545 C, ELISA of TM, CTC and CHP, rabbit anti-TM IgG or patient sera were used as the antibody;

546 D, Secondary structure analysis of cross-linked TM;

547 E, Surface hydrophobicity analysis of cross-linked TM. The data represent the mean ± SD from

548 triplicate determinations.

549

550 **Figure 2. The stability of cross-linked TM by simulating pepsin digestion.**

551 A-C, SDS-PAGE analysis of TM, CTC and CHP stability to pepsin digestion;

552 D-F, Western blot analysis of TM, CTC and CHP treated with pepsin.

553

554 **Figure 3. The impact of cross-linked TM in IgE-mediated RBL-2H3 cells and uptake by**

555 **DCs.**

556 A, The release of β-hexosaminidase;

557 B, The level of histamine;

558 C, Uptake of pHrodo Green-labeled cross-linked TM and TM by DCs. The data represent the

559 mean ± SD from triplicate determinations.

560

561 **Figure 4. The sensitization experiment. Cells were isolated from spleens and MLNs and**

562 **stained for CD4 and Foxp3.**

563 A, Sensitization protocol;

564 B, The levels of IgE, IgG1, and IgG2a measured on day 29;

565 C-D, The release of histamine/mMCP-1 measured in serum;

566 E-G, The production of IL-4, IL-13 and IFN-γ was measured in the spleen cell culture;

567 H-J, The production of IL-4, IL-13 and IFN-γ was measured in the MLN cell culture;

568 K, The spleen and MLNs cells were isolated from the mouse models of food allergy on day 29.

569 Cells were cultured in the presence of 25 μg/mL of anti-CD4-PerCP Cy5.5 and 25 μg/mL of

570 anti-Foxp3-Alexa 647 for 30 min. $CD4^{+}Foxp3^{+}T$ cells were analyzed by flow cytometry. *$p <$

571 0.05, **$p < 0.01$. The data represent the mean ± SD from triplicate determinations.

572

573 **Figure 5. The sensitizing potential of cross-linked TM in the tolerance induction experiment.**

574 **Cells were isolated from spleens and MLNs and stained for CD11c and MHC-Ⅱ.**

575 A, Oral tolerance induction protocol;

576 B, The levels of IgE, IgG1, and IgG2a measured in serum;

577 C-E, The production of IL-4, IL-13 and IFN-γ was measured in the spleen cell culture;

578 F-H, The production of IL-4, IL-13 and IFN-γ was measured in the MLN cell culture;

579 I, The spleen and MLNs cells were isolated from the mouse models of food allergy on day 34.

580 Cells were cultured in the presence of 25 μg/mL of anti-CD11c-APC and 25 μg/mL of

581 anti-MHC-II-FITC for 30 min. CD11c+ MHC-Ⅱ+DCs were analyzed by flow cytometry. *$p <$

582 0.05, **$p < 0.01$. The data represent the mean ± SD from triplicate determinations.

Table 1. Specific IgE levels in patients in relation to the crab and shrimp.

ID	Age	Sex	Specific IgE (kU/L)
			Crab or shrimp
7543	3	F	0.93
4768	5	M	1.93
2389	2	M	2.93
1426	29	M	3.93
8734	45	F	4.93
5396	23	M	5.93
4658	3	F	6.93

A serum with the specific IgE > 0.35 (KU/L) is defined as positive. M, male; F, female

Table 2. Experimental groups of tolerance induction.

Group Name	Sensitization	Challenge
PBS-TM	PBS	TM
TM-TM	TM	TM
CTC-TM	CTC	TM
CHP-TM	CHP	TM

Figure 1

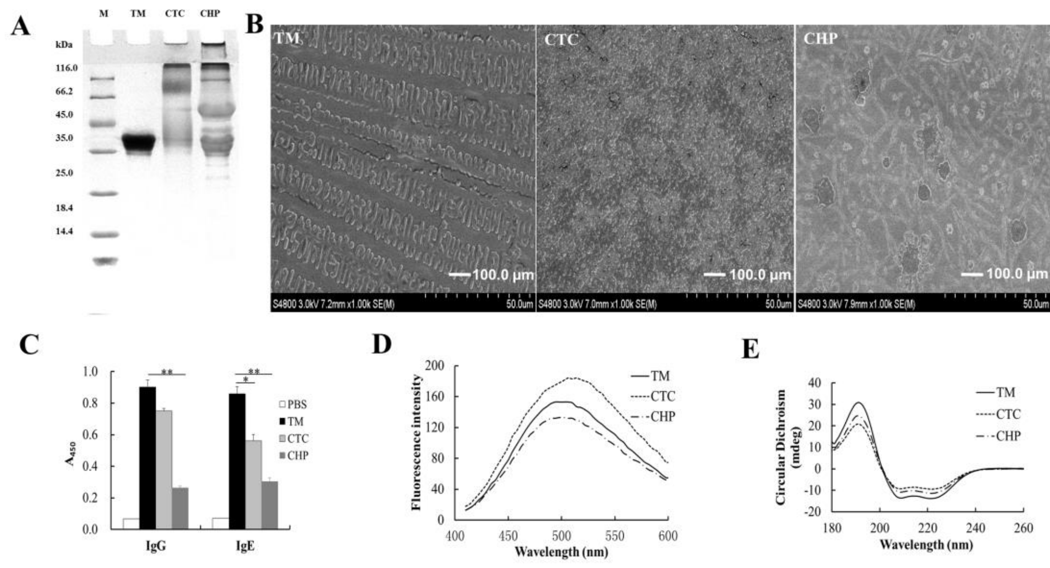
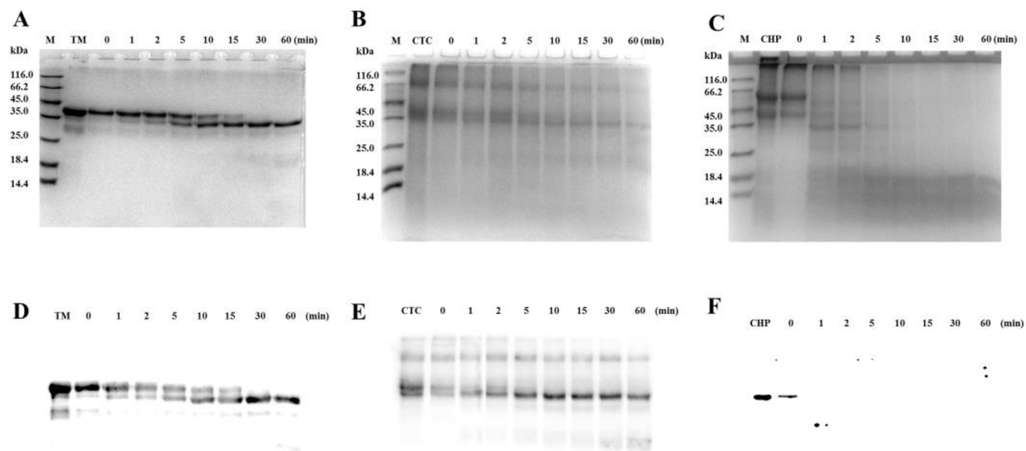


Figure 2



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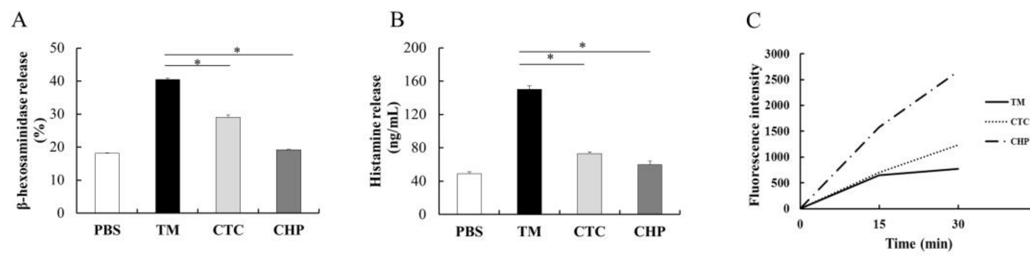


Figure 4

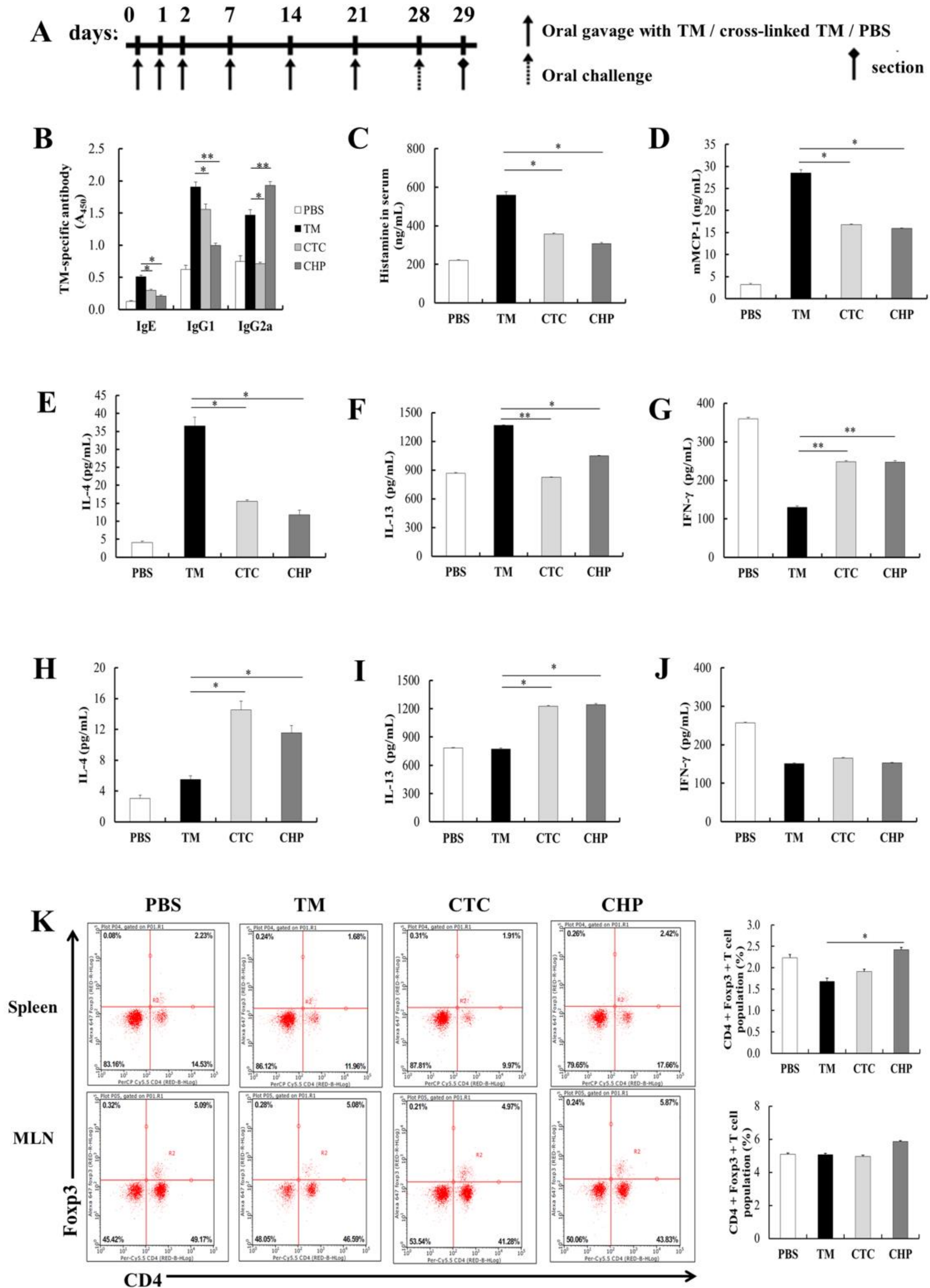
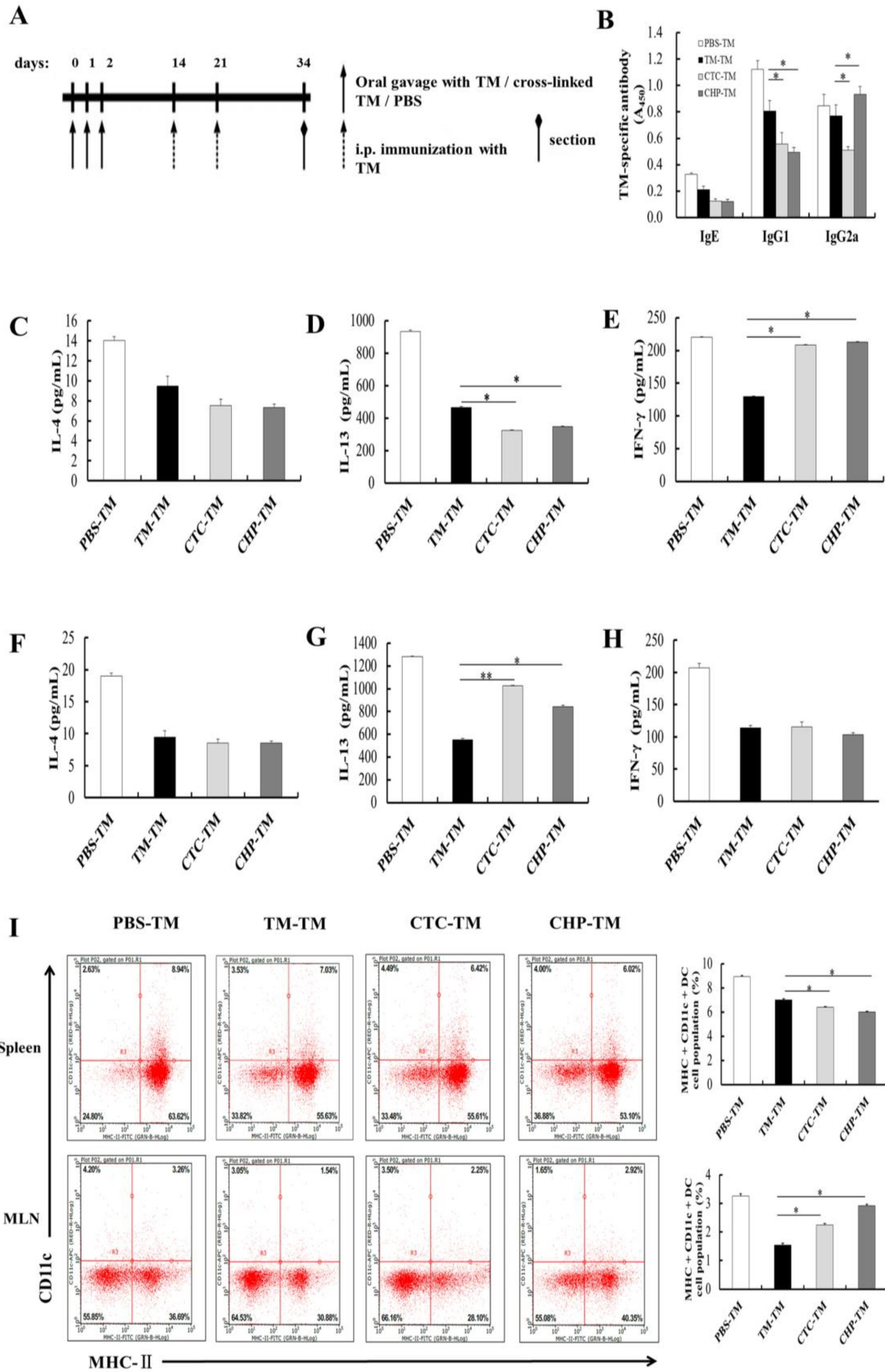


Figure 5



Graphic for table of contents