

Identification of the Dominant T-Cell Epitopes of Lit v 1 Shrimp Major Allergen and Their Functional Overlap with Known B-Cell Epitopes

Li Li Xu, María Gasset, Hong Lin, Chuang Yu, Jin Long Zhao, Xue Wen Dang, and Zhen Xing Li*



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ABSTRACT: Development of efficient peptide-based immunotherapy for shrimp allergy relies on the identification of the dominant T-cell epitopes of its major allergen, tropomyosin. In this study, immunoinformatic tools, T-cell proliferation, cytokine release, IgG/IgE binding, and degranulation assays were used to identify and characterize the T-cell epitopes in Lit v 1 in comparison with previously validated B-cell epitopes. The results showed that of the six in silico predicted T-cell epitopes only one (T2: VQESLLKANIQLVEK, 60–74) promoted T-cell proliferation, the release of IL-2, and upregulated secretion of Th2-associated cytokines in the absence of IgG/IgE binding and degranulation activities. These findings support T2 as a candidate for the development of an efficient peptide-based vaccine for the immunotherapy for shrimp-allergic patients.

KEYWORDS: shrimp allergy, tropomyosin, Lit v 1, T-cell epitope, B-cell epitope

INTRODUCTION

Shellfish are widely consumed as food all over the world because of their delicate flavor and high nutritive value.¹ However, shrimps account for one of the most prevalent events of long-lasting IgE-mediated anaphylactic reactions particularly in the USA and Asia-Pacific regions.^{2,3} Peptide-based immunotherapy has been recently deemed as a safe and effective therapeutic strategy.⁴ Thus far, epitope-based vaccines are highly encouraging and the numbers of clinical trials involving pollen and house dust mite are suggestive of success.^{4–9} The used peptides are designed to comprise allergen T-cell epitopes lacking both the IgE binding and the stimulatory capacity of inflammatory cells. A large number of clinical trials using this approach for house dust mite and birch pollen allergies have provided encouraging results.^{10,11} However, the use of this approach in shrimp allergy such as the one induced by *Litopenaeus vannamei* remains unexploited.

IgE-mediated hypersensitivities mainly include two sequential phases known as the sensitization phase and the effector phase. In the sensitizing phase, antigens are picked up by the antigen-presenting cells (APC), mainly the dendritic cell. The allergens are degraded and processed into fragments. Some of these fragments combined with major histocompatibility molecules can be recognized by T-lymphocytes and induce their proliferation which promote their differentiation into Th2 type that leads to B-cell activation and IgE production.¹² The resulting allergen-specific IgEs are then recognized by the FcεRI receptor on the surface of mast cells for the acquisition of the sensitized state. The peptides activating the T-lymphocytes are referred to as T-cell epitopes.^{13,14} In the effector phase, the allergen to which the individual is sensitized binds to and cross-links the IgE molecules at the mast/basophile cell surface triggering the release allergic mediators

into the bloodstream and tissues.¹² The allergen regions recognized by the IgEs are referred to as B-cell epitopes.

The major allergen in shrimp is tropomyosin (TM), a salt-soluble coiled-coiled protein homodimer of 34–38 kDa.^{15,16} IgE binding studies have provided a variety of linear epitopes of TM from different shrimp species. These B-cell epitopes include five regions in Pen a 1 (sequences 43–57, 85–105, 133–148, 187–202, and 247–284), 10 regions in Lit v 1 (sequences 12–25, 28–41, 47–61, 97–108, 117–132, 149–166, 178–191, 209–224, 244–257, and 261–274), 9 regions in Met e 1 (sequences 25–30, 43–60, 87–103, 146–154, 161–165, 191–211, 236–241, 247–255, and 269–281), and 10 regions in Fen c 1 (12–25, 47–60, 87–105, 112–128, 131–147, 155–167, 187–203, 221–239, 243–259, and 263–280).^{1,17–23} On the other hand, the search for T-cell epitopes in shrimp tropomyosin have provided six regions in Met e 1 (regions 26–45, 56–75, 86–105, 146–165, 221–240, and 251–270) and five regions in Pen a 1 (regions 25–39, 58–75, 193–207, 223–264, and 265–284).^{2,22} Notwithstanding, some of the reported T-cell epitopes overlap sequences described as B-cell epitopes and may display undesired activities.^{2,24}

Thus, taking Lit v 1 as the shrimp tropomyosin model, this study aims to identify the dominant T-cell epitopes using a 15-mer peptide length and analyze their functional relation with previously validated B-cell epitopes.

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MATERIALS AND METHODS

Materials. Shrimps (*Litopenaeus vannamei*) were obtained from a local supermarket (Liquan supermarket, Qingdao, Shandong, China). Lit v 1 was purified from raw shrimp muscles as described.²⁵ The anti-Lit v 1 IgG was obtained from Lit v 1-immunized mouse sera and purified by a Protein A Sepharose Column.²⁶ Goat anti-mouse IgG, goat anti-mouse IgE, and goat anti-human IgE antibodies were purchased from Zhongshan Jinqiao Co. (Beijing, China). The RBL-2H3 (Rat Basophilic Leukemia cells, ATCC CRL-2256) cell line was acquired from the Chinese Academy of Sciences (Shanghai, China). The enhanced chemiluminescent kit (ECL) and alum adjuvant were purchased from Thermo Fisher (Thermo Fisher Scientific, MA, USA). The ELISA kits for histamine, IL-2, IL-4, IL-10, IL-12, IL-13, and interferon (IFN)- γ determinations were acquired from R&D systems (Minneapolis, MN, USA). All the other reagents used were of analytical grades.

Shrimp-Allergic Patient Sera. The sera from 14 shrimp-allergic patients with a documented clinical history and two from healthy controls were obtained from The Affiliated Hospital of Qingdao University (Qingdao, Shandong, China) (Table 1). The study

Table 1. Clinical and Serological Features of Shrimp-Allergic and Nonallergic patients^a

no.	age (years)	sex	symptoms	specific shrimp IgE (kUA/L)
S1	34	F	U, P	1.96
S2	21	M	Dr	2.31
S3	7	M	U, P	4.77
S4	13	M	Ds	8.58
S5	4	F	Dr, P	2.56
S6	37	F	U	6.94
S7	62	M	P, C	2.70
S8	52	F	Dr	0.83
S9	56	M	U, P	1.57
S10	24	F	A, P	0.98
S11	4	M	U, P	2.52
S12	30	M	Dr	0.95
S13	7	F	A, C	4.41
S14	5	M	Ds	0.61
S15	21	F	/	/
S16	18	M	/	/

^aThe abbreviation used are: F, female; M, male; A, asthma; AR, anaphylactic rhinitis; C, cheilitis; Dr, dermatitis; Ds, dyspnea; P, pruritus; and U, urticaria. A serum with specific IgE >0.35 (kUA/L) is defined as positive. S15 and S16 were used as the negative control.

protocol and the methods used were approved by the medical ethics committee (File No. QD-PF-20200519685). All patients and controls signed the institutionally approved clinical consent files.

T-Cell Epitope Prediction and Synthesis. In silico analysis of T-cell epitopes in the Lit v 1 sequence (UniprotKB B4YAH6 entry) was performed using a 15-mer peptide window and MHCpred 2.0 (<http://www.ddg-pharmfac.net/mhcpred/>), IEDB (<http://tools.immuneepitope.org/>), ProPred (<http://crdd.osdd.net/raghava/propred/>), NetMHCII 2.3 (<http://www.cbs.dtu.dk/services/NetMHCII/>), Immunomedicine Group (<http://imed.med.ucm.es/>), and SYFPEITHI (<http://www.syfpeithi.de/>) tools.^{27,28} The predicted peptides and three previously validated Lit v 1 B-cell epitopes²³ were synthesized by Sango Biotech (Shanghai) Co., Ltd. (Shanghai, China) with free N- and C-terminal ends and with purities higher than 95% (Table 2).

Lit v 1-Mice Sensitization. All experiments were performed according to the guidelines of the Ethical Committee of Experimental Animal Care at the Ocean University of China (SPXY20200521001), using protocols previously described.^{29,30} Briefly, BALB/c female mice at the age of six-week-old were obtained from Jinan Laboratory at the Animal Center (Jinan, China), kept in a pathogen-free environment,

and fed food devoid of fish and shrimp proteins (LAD3001M, Trophic Animal Feed High-tech Co., Ltd., Jiangsu, China). After a week, the mice were boosted with 50 μ g Lit v 1 dissolved in an alum adjuvant by intraperitoneal injection at days 1, 7, 13, 19, 25, and 31 followed by a stimulation with 100 μ g Lit v 1 in phosphate buffered saline (PBS) on days 34, 37, 40, and 43. At day 50, mice were injected with 1 mg Lit v 1 dissolved in PBS. After overnight fasting, the mice were anesthetized and killed by cervical dislocation, and the blood and spleen were collected under sterile conditions for further processing.

T-Cell Proliferation Assay. CD4⁺ T-cells were isolated from spleens using Easysep CD4 + T Kit (Stemcell Technologies, Vancouver, Canada) according to the manufacture's protocol.^{2,24} The isolated lymphocytes (5–10 $\times$ 10⁶ cells/mL) in PBS were stained with 5 μ M carboxyfluorescein succinimidyl ester (CFSE, BD Biosciences, San Jose, USA) final concentration for 15 min at 37 °C in a water bath with protection from light and with two gentle shaking. Labeling was quenched by dilution with 5 volumes of RPMI-1640 medium containing 10% fetal bovine serum (FBS, Biological Industries, Israel) and a 5 min incubation in ice. After two washes with RPMI-1640 medium containing 10% FBS, 1% nonessential amino acids (NEAA, Solarbio Life Sciences, USA), 1% HEPES, 1% sodium pyruvate, and 1% β -mercaptoethanol, the cells were seeded in a 24-well plate (2 $\times$ 10⁶ cells/mL) and stimulated in the absence or presence of 200 μ g/mL peptides (T1–T6, B1–B3), 20 μ g/mL Lit v 1, or 20 μ g/mL Conavalin A (Con A, Solarbio Life Sciences, USA) final concentration. After 72 h at 37 °C in a humidified incubator with 5% CO₂, the cell solutions were centrifuged (400 g, 10 min) and the supernatant was collected for IL-2, IL-4, IL-10, IL-12, IL-13, and IFN- γ determination using the corresponding commercial ELISA kits. The pellets containing the cells were washed twice with PBS, stained with anti-mouse CD4 APC (BD Biosciences, San Jose, USA) in 5% FBS-supplemented PBS, and analyzed on a flow cytometer (BD FACS Verse, Biosciences, San Jose, USA).

Dot-Blot Assay. Synthetic peptides (10 μ g) comprising the predicted T-cell and validated B-cell epitopes dissolved in PBS (pH 7.4) were spotted on prehydrated nitrocellulose membranes (0.45 μ m pore size) and allowed to air-dry.^{30,31} Membranes were first incubated with a blocking buffer (PBS containing 0.1% Tween-20, 5% skim milk, and 1% bovine albumin) for 2 h at 37 °C and then for 1.5 h at 37 °C with sera (1:5 dilutions in blocking buffer). After several washes with PBS containing 0.1% Tween-20 (PBST), the membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-human IgE (1:2000 dilution in PBST) for 1.5 h at 37 °C. After three washes with PBST, the membranes were developed with the ECL kit and the signal recorded in a ChemiDoc XRS+ equipment with 3 min exposure time (Bio-Rad Laboratories, Inc. Hercules, CA, USA). Based on the intensity, IgE binding was classified into four groups: negative intensity (score: 0, white dot, no IgE binding), weak intensity (score: 1, light gray dot, weak IgE binding), medium intensity (score: 2, dark gray dot, medium IgE binding), and strong intensity (score: 3, black dot, strong IgE binding).

Indirect ELISA Assay. ELISA assay was carried out as described.²⁶ In brief, plate wells were coated with either 30 μ g of synthetic peptides, 3 μ g Lit v 1 (positive control), or 3 μ g bovine serum albumin (BSA, negative control) in PBS (pH 7.4) by an overnight incubation at 4 °C followed by several washes with PBST. Wells were blocked for 2 h at 37 °C with blocking buffer and then incubated for 2 h at 37 °C with the mouse anti-Lit v 1 polyclonal antibody (1:5000 dilution in PBST), Lit v 1-immunized mouse serum (1:10 dilution in PBST), or pool made of equal volumes the sera S1, S5, S7, and S10 of shrimp-allergic patients (1:4 dilution in PBST). After several washes with PBST, the PBST solutions of HRP-conjugated goat anti-mouse IgG (1:5000 dilution), HRP-conjugated goat anti-mouse IgE (1:5000 dilution), or HRP-conjugated goat anti-human IgE (1:2000 dilution) were added to the corresponding wells for 1 h incubation at 37 °C. After washing, the plate was developed with a 1-Step TMB Turbo (Thermo Fisher Scientific, MA, USA) ELISA substrate and the absorbance at 450 nm was recorded in a microplate reader (CMax Plus, Molecular Devices, LLC., San Jose, CA, USA).

Table 2. Prediction of T-Cell Epitopes in Lit v 1

no	sequence	position	allele	ProPred MHC class II binding score	NetMHC II IC% rank	NetMHCII pan IC ₅₀ nM binding score	NN-align IC ₅₀ nM binding score	SMM-align IC ₅₀ nM binding score	RANKPEP optimal score (%)	SYFPEITI binding score	MHC Pred IC ₅₀ binding score
T1	KMQAMKLEKDNAMDR	7–21	DRB1–0101			373.07		473	30.01	19	6.67
			DRB1–0301	4.0	4.50	340.41	472.6	158		8	
			DRB1–0401	1.70	4.50		314.5	321	18.21	12	
			DRB1–0701						28.69	4	
			DRB1–1101							20	
			DRB1–1501							4	
T2	VQESLLKANIQLVEK	60–74	DRB1–0101		7.50	47.14	27.5	65		9	10.19
			DRB1–0301			469.7	200.7			17	
			DRB1–0401							7	
			DRB1–0701			211.59	105.2	312	31.15	14	
			DRB1–1101	1.40						0	
			DRB1–1501			395.4				14	
T3	AEGEVAALNRRQLL	81–95	DRB1–0101			183.04	47.2	297		10	
			DRB1–0301			426.91				8	
			DRB1–0401							18	
			DRB1–0701			347.53		342		20	59.70
			DRB1–1101	1.20	3.00	129.21	15.2	30		3	
			DRB1–1501							8	
T4	EERLNTATTKLAEAS	103–117	DRB1–0101			64.94	48	108		24	10.96
			DRB1–0301							11	
			DRB1–0401		9.00	159.67	302.2	304		26	
			DRB1–0701			197	160.5	232		26	
			DRB1–1101			393.83	478.5			13	
			DRB1–1501							18	
T5	EELRVVGNNLKSLEV	195–209	DRB1–0101		8.00	68.93	135.9	88		10	
			DRB1–0301			283.48	137.6			9	
			DRB1–0401	2.40	3.50	350.84	347.9		13.99	12	
			DRB1–0701			181.44	143.9	388		6	
			DRB1–1101			205.42	397.1			8	
			DRB1–1501		5.50	274.64	229.4	265	25.54	14	
T6	YKEQIKTLTNKLKAA	221–235	DRB1–0101		6.50	30.9	35.9	81		17	
			DRB1–0301			381.91				12	
			DRB1–0401	1.60	5.50	150.05	96.8	202	28.29	12	

Table 2. continued

no	sequence	position	allele	ProPred MHC class II binding score	NetMHC II IC ₅₀ rank	NetMHCII pan IC ₅₀ nM binding score	NN-align IC ₅₀ nM binding score	SMM-align IC ₅₀ nM binding score	RANKPEP optimal score (%)	SYFPEITHI binding score	MHC Pred IC ₅₀ binding score
			DRB1-0701			80.71	244.2	259		24	
			DRB1-1101		3.00	21.74	29.8	81		1	
			DRB1-1501		7.00	110.58	166.8	322		8	

Mediator Release Assay. The RBL-2H3 cells were cultured in DMEM medium with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified incubator with 5% CO₂ as described.^{26,32} The cells were seeded in a 96-well plate (1 × 10⁶ cells/mL) and passively primed with Lit v 1-sensitized mouse sera IgE (1:15 dilution in culture medium). After 24 h, the medium was replaced by Tyrode's buffer containing each of the synthetic peptides (200 μg/mL), Lit v 1 (20 μg/mL, positive control), Compound 48/80 (20 μg/mL, positive control), or none (negative control). After 8 h of incubation, the media were collected for analysis. Histamine levels were determined using ELISA kits according to the manufacturer's instructions. The β-hexosaminidase release was measured using the colorimetric assay based on *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide hydrolysis previously described.³³

Statistical Analysis. Data were presented as mean ± standard deviation (*n* = 3) and all the statistical analysis and the figures were performed using SPSS 22.0 and Origin 8 software. The difference in significance was determined using a one-way analysis of variance of the Duncan test with *p*-value <0.05.

RESULTS AND DISCUSSION

In Silico Prediction of T-Cell Epitopes of Lit v 1. To identify the potential T-cell epitopes, regarded as the peptides binding to multiple HLA-class II molecules (Figure 1A), the

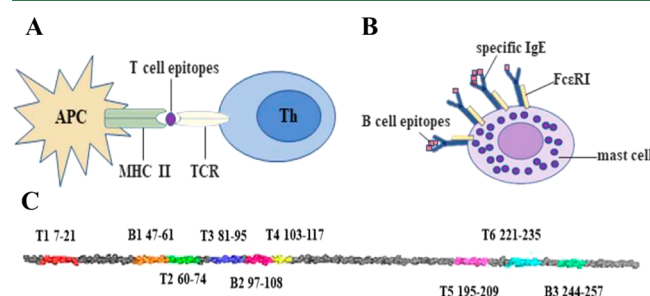


Figure 1. Location and structure of T-cell epitopes and B-cell epitopes in Lit v 1 tropomyosin. Schematic representation of the basic differential interactions of (A) T-cell epitopes and (B) B-cell epitopes. (C) Relative location of the predicted T-cell epitopes (T1–T6) and validated B-cell epitopes (B1–B3) in Lit v 1. The Lit v 1 model was performed using PyMOL tools. T1–T6 sequences are described in Table 2. The sequences of B-cell epitopes are: B1, QKRMQQLENDLDQVQ; B2, EDLERSEERLNT; and B3, RSVQKLQKEVDRL.

Lit v 1 sequence was analyzed using MHCpred 2.0, IEDB, ProPred, NetMHCII 2.3, Immunomedicine Group, and SYFPEITHI algorithms. Of the different HLA-class II molecules, only DRB1–0101, DRB1–0301, DRB1–0401, DRB1–0701, DRB1–1101, and DRB1–1501 alleles were common to all algorithms and their use as templates was successful in previous studies.^{34,35} Table 2 lists the peptides with positive hits in a least one of the algorithms and their

interaction parameters. The predicted T-cell epitopes comprise T1 (MQAMKLEKDNAMDR, 7–21), T2 (VQESLLKANIQLVEK, 60–74), T3 (AEGEVAALNRRIQLL, 81–95), T4 (EERLNTATTKLAEAS, 103–117), T5 (EELRVVGNNLKSLEV, 195–209), and T6 (YKEQIKTLTNKLKAA, 221–235). Of them, only T2–T3–T4–T6 and T2–T5–T6 partially overlap the T-cell epitopes found in Met e 1 and Pen a 1, respectively.^{2,24} The predicted 15-mer sequence regions were synthesized as independent peptides using solid phase methods with a purity higher than 95% for further use (Table 2). Also, the synthetic peptides B1–B3 comprising the regions of Lit v 1 with IgE binding capacity (Figure 1B) and consequently regarded as B-cell epitopes validated as IgE binders were included in the study.²³ The relative location of all these regions in the 3D structure is displayed in Figure 1C.

T-Cell Proliferation and IL-2 Release. T-cell epitopes could bind to HLA-class II molecules and stimulate T-cell proliferation, and promote IL-2 release. To test the predictions, the behavior of the distinct peptides was analyzed using carboxyfluorescein succinimidyl ester (CFSE)-based lymphocyte proliferation. Splenic lymphocytes isolated from Lit v 1-sensitized mice showed a significant lymphocyte proliferation on stimulation with the positive controls Lit v 1 and Con A. Of the distinct epitopes, only the predicted T2 and the validated B1 yielded significantly higher proliferation rates (*p* < 0.01). Other peptides such as T1, T3–T6, B2, and B3 exhibited values similar to the negative control (Figure 2A).

On the other hand, the analysis of the IL-2 levels released upon stimulation showed a similar trend to the lymphocyte proliferation assay (Figure 2B). In this assay, the controls Lit v 1 and Con A produced the highest levels of IL-2 in cell supernatants and the peptides T2 and B2 yielded IL-2 levels significantly higher (*p* < 0.01) than those of the negative control. The peptides T1, T3–T6, B2, and B3 released IL-2 levels lacking significant difference compared with the negative control.

Thus, based on the high rate of T-cell proliferation and IL-2 level, the peptide T2 (VQESLLKANIQLVEK, 60–74) could be deemed as T-cell epitopes of Lit v 1. This sequence is a shorter form of one of the T-cell epitopes found in Pen 1 and of Met e 1.^{2,24} It merits comment that the peptides T1 and T6, predicted as T-cell epitopes but lacking both IL-2 releasing and T-cell proliferating activities, displayed partial overlap with the B-cell epitopes found in Lit v 1 (VSEKANQREEAYKEQ, 209–224), Pen m 1 (SEKANQREEAYKEQ, 210–224), and Fen c 1 (YKEQIKTLTNKLKAAEARA, 221–239).^{1,17–22} Importantly, the B-cell epitope B1, N-terminally located with respect T2 in the Lit v 1 sequence, also displayed T-cell proliferation and IL-2 release activities.

Cytokine Profiles of Lit v 1 Peptides. To further characterize the responses triggered by these peptides, the

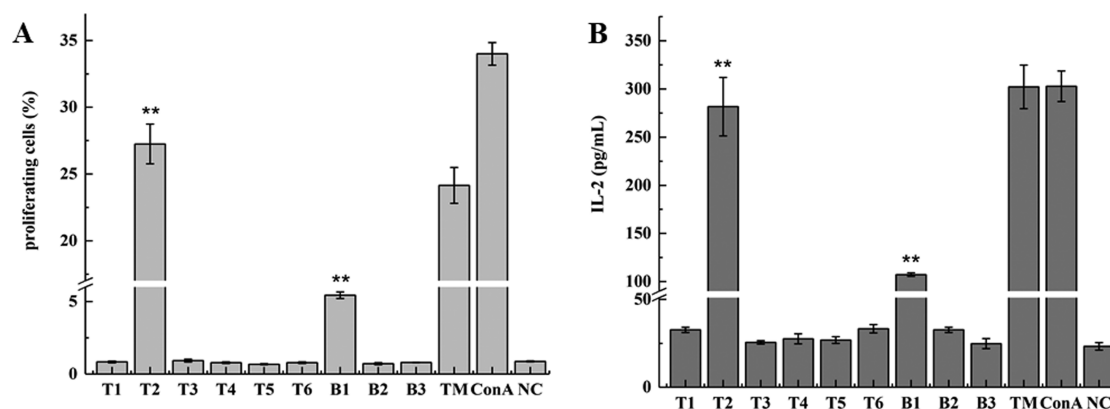


Figure 2. T-cell epitope activities of Lit v 1 peptides. (A) T-cell proliferation measured using CFSE-based assay. (B) IL-2 concentration released in the supernatant of stimulated cells determined by ELISA. The abbreviations used are: T1–T6, predicted T-cell epitopes; B1–B3, validated B-cell epitopes. Lit v 1 and Con A were used as the positive control, and RPMI-1640 medium was used as the negative control. The data are displayed as mean $\pm$ SD ($n = 3$), and the marker “*” stands for significant difference ($p < 0.05$) and “**” stands for significant difference ($p < 0.01$) as compared to the negative control.

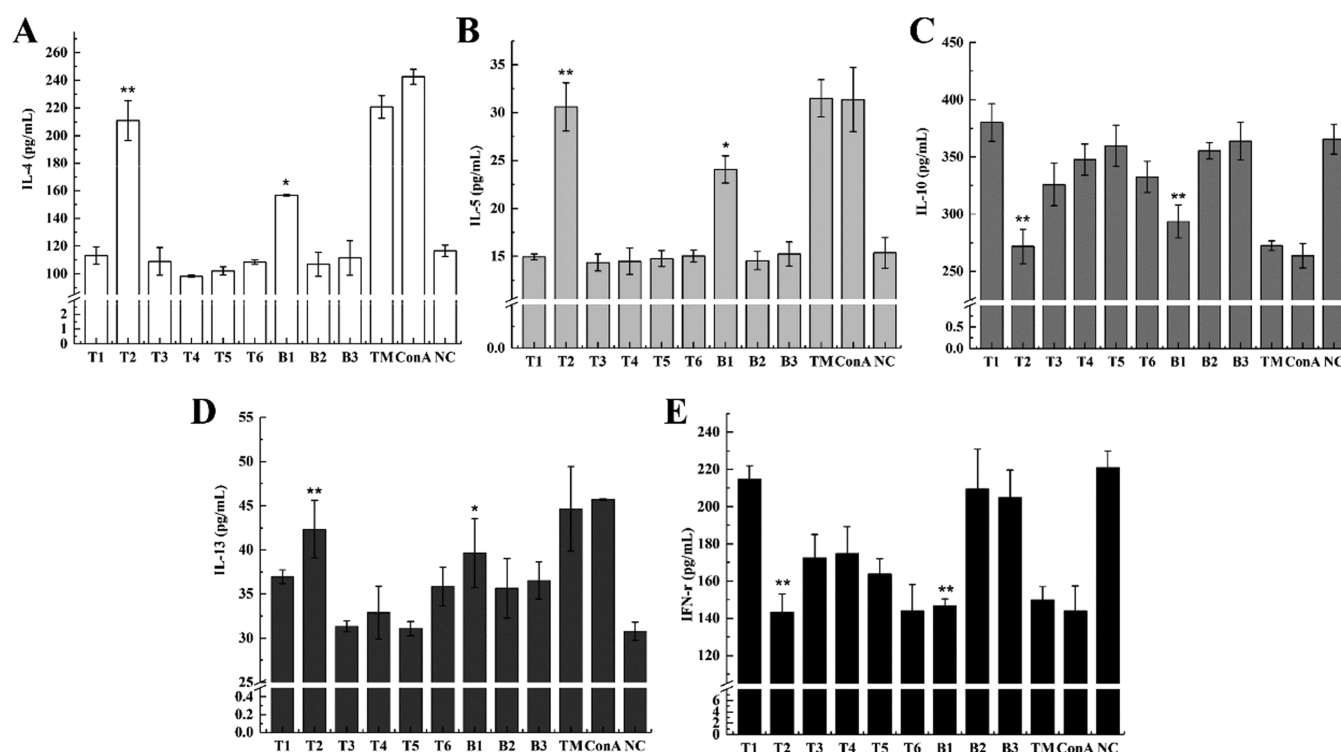


Figure 3. Cytokine response by spleen cells. Concentration in the supernatants of peptide-stimulated splenic lymphocytes of Lit v 1-sensitized mice of (A) IL-10, (B) IFN- γ , (C) IL-4, (D) IL-5, and (E) IL-13. Cytokine levels were determined using commercial ELISA kits. The data are displayed as mean $\pm$ SD ($n = 3$) and the marker “*” stands for significant difference ($p < 0.05$) and “**” stands for significant difference ($p < 0.01$) as compared to the negative control.

Th1- (IL-10 and IFN- γ) and Th2 (IL-4, IL-5 and IL-13)-response-related cytokines were measured in cell culture supernatants of the splenic lymphocytes isolated from shrimp-sensitized mice after stimulation (Figure 3). Figure 3A,B shows that the stimulation with T2 and B1 significantly downregulated the secretion of the Th1 IL-10 and IFN- γ cytokines to levels similar to the positive controls. The stimulation with T3, T4, T5, and T6 provoked a mild reduction, whereas the use of B2 and B3 yielded values similar to the negative control. On the other hand, although proliferative responses to T2, B1, Lit v 1, and Con A could be associated with greater cytokine production, all these

peptides significantly upregulated ($p < 0.01$) the IL-4 (Figure 3C), IL-5 (Figure 3D), and IL-13 levels (Figure 3E). Interestingly, the stimulation with peptides T1, T6, B2, and B3 only displayed minor effects on IL-13 production (Figure 3C–E). Taken together, these results indicate that only T2 and B1 alter the Th1/Th2 balance by decreasing Th1 and increasing Th2, as described for allergic reactions.^{13,36}

IgG Binding and Specific IgE Binding of Lit v 1 Peptides. The recognition the synthesized peptides by the anti-Lit v 1 IgG produced in Lit v 1-immunized mice were determined by ELISA. As shown in Figure 4A, comparing with the negative control, three B-cell epitopes (B1–B3) showed

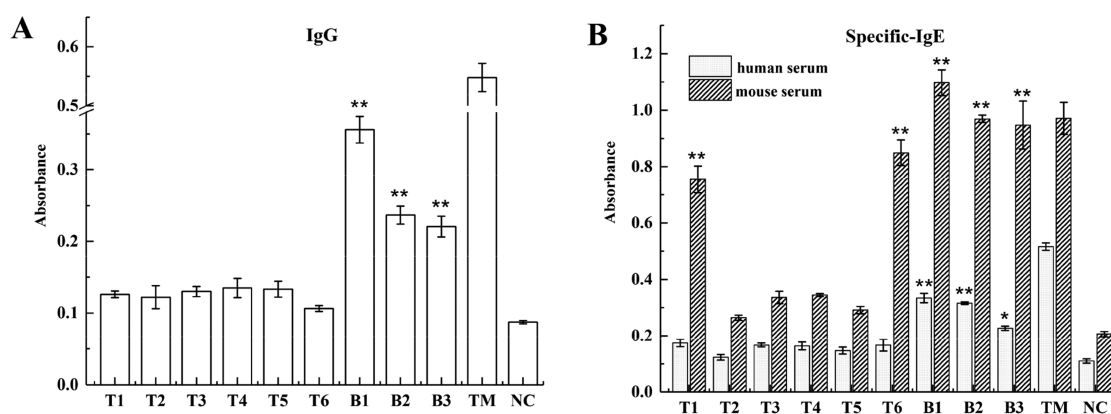


Figure 4. IgG/IgE binding of Lit v 1-peptides. (A) Binding of the IgG contained in the sera of Lit v 1-immunized mice probed by ELISA. (B) Binding of the IgE contained in the pools of the sera of shrimp-allergic patients and of Lit v 1-immunized mice. The abbreviations used are: T1–T6, predicted T-cell epitopes; B1–B3, validated B-cell epitopes. Lit v 1 was used as the positive control, and BSA was used as the negative control. The data are displayed as mean $\pm$ SD ($n = 3$) and the marker “*” stands for significant difference ($p < 0.05$) and “**” stands for significant difference ($p < 0.01$) as compared to the negative control.

Table 3. Peptide Recognition by the IgE Contained in the Sera of Shrimp-Allergic and Nonallergic Patients

Sera	epitopes									Positive No.
No.	T1	T2	T3	T4	T5	T6	B1	B2	B3	
Control 1										0
Control 2										0
S1										5
S2										4
S3										5
S4										4
S5										5
S6										5
S7										4
S8										4
S9										5
S10										2
S11										4
S12										2
S13										5
S14										4
Positive individuals, n	6	3	2	3	2	5	13	12	12	
Intensity $\geq 50\%$ individuals	10	3	3	4	2	7	33	31	27	
> mean intensity $\pm$ SD							+	+	+	

^aDots of the dot-blot membranes displayed in Supporting Information Figure S1 were transformed using the following white/black scale: white-negative (0 points); light gray-weak binding (1 points); dark gray-medium binding (2 points), and black-strong binding (3 points).

significantly higher IgG binding ($p < 0.01$), whereas none of peptides representing the predicted T-cell epitopes (T1–T6) showed differences compared to the negative control. This differential IgG recognition supports their B-cell and T-cell epitope features.

On the other hand, IgE binding to Lit v 1-peptides was assessed by both dot-blot and ELISA using the independent or pooled sera of allergic patients, the sera of two healthy donors and the sera of Lit v 1-sensitized mice (Table 3, Figure 4B). Dot-blot assay confirmed that peptides representing all previously validated B-cell epitopes (B1, B2, and B3) were recognized by the IgE contained in ≥ 12 sera of allergic patients (Table 3, Supporting Information Figure S1). These binding

trends exceeded 50% of sera samples with the intensity scores ≥ 27 . Importantly, under identical conditions, the sera of healthy donors tested negative for the binding of all peptides (T and B). On the contrary, T1–T6 peptides representing the predicted T-cell epitopes were recognized by 6 (42.86%), 3 (21.43%), 2 (14.29%), 3 (21.43%), 2 (14.29%), and 5 (35.71%) sera of allergic patients with intensity scores of 10, 3, 3, 4, 2, and 7, respectively. To confirm these results, the IgE binding of these peptides was probed by ELISA using a sera pool of shrimp-allergic patients and of Lit v 1-sensitized mice. Figure 4B shows that B1, B2, and B3 are recognized by the IgE present in the sera pools of both shrimp-allergic patients and Lit v 1-immunized mice sera. However, of the six peptides

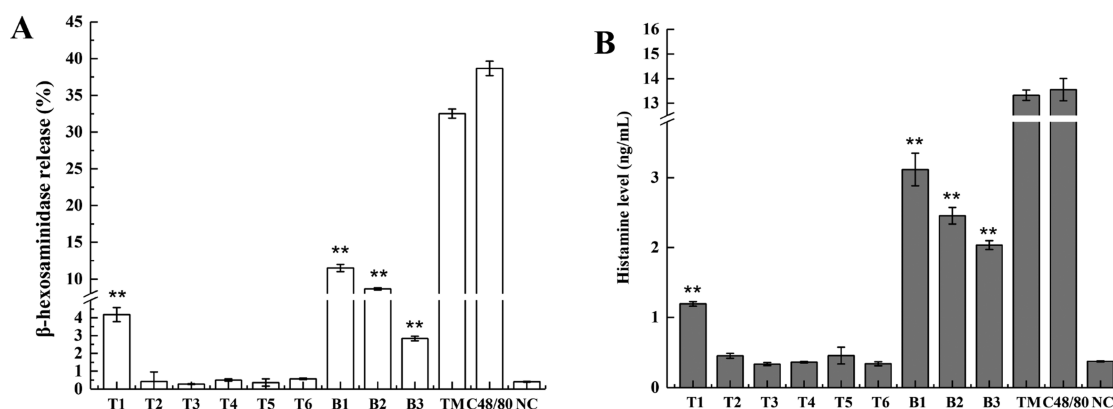


Figure 5. Peptide-induced mediator release from sera-primed RBL-2H3 cells. (A) Percentage of β -hexosaminidase release and (B) histamine concentrations in the supernatant were assessed by ELISA. The abbreviations used are: T1–T6, predicted T-cell epitopes; B1–B3, validated B-cell epitopes. Lit v 1 and C48/80 were used as the positive control, and Tyrode's buffer was used as the negative control. The data are displayed as mean $\pm$ SD ($n = 3$) and the marker “*” stands for significant difference ($p < 0.05$) and “***” stands for significant difference ($p < 0.01$) as compared to the negative control.

representing the predicted T-cell epitopes, only T6 was significantly ($p < 0.05$) recognized by the IgE of the human and mouse sera pools as compared to the negative control, and T1 showed a significantly higher ($p < 0.01$) IgE binding when probed with the sera of immunized mice. Then, the absence of significant IgE and IgG binding together with the T-cell proliferation and cytokine secretion profiles support T2 as a T-cell epitope suitable for its use in peptide vaccines.

Degranulation Capacity of Lit v 1-Peptides. In addition to the essential functional properties of T2 as a T-cell epitope, its safe exploitation relies on its incapacity to induce the degranulation of effector cells.^{23,28} Figure 5 shows that the addition of IgE binding peptides such as B1, B2, B3, and T1 to RBL-2H3 cells primed with Lit v 1-sensitized mouse sera significantly increased the release ($p < 0.05$) of β -hexosaminidase and histamine compared to the negative control. Importantly, these results underline that peptide lengths of 12–15 residues suffice the cross-linking capacity of the cell membrane-bound IgE required for degranulation.^{1,23} Notwithstanding, it must be stressed that such peptide lengths allow the formation of α -helix and β -sheet-based oligomers that ensure polyvalent states.³⁷ Contrary to the previous peptides assayed, T2 as well as T3, T4, T5, and T6 yielded β -hexosaminidase and histamine levels in cell supernatants similar to that of the negative control. Therefore, T2 suffices the properties of a T-cell epitope for safe use in peptide-based immunotherapy (PIT).

However, single T-cell epitopes fulfilling the criteria such as inducing T-cell proliferation, IL2 and Th2-cytokine releases, and lacking IgE/IgG binding and primed effector cell degranulation may display a variable interaction with the distinct alleles of HLA-class II. To strengthen its validity for a global use, the initial *in silico* analysis was extended including the reference set of HLA-class II alleles covering 99% of the population.³⁸ Table 4 shows that NetMHCII predicts T2 strongly or weakly binding to 12 of the 17 DRB alleles considered, 3 of the 6 DPA/B, and only 1 of the 6 DQA/B included, whereas the NN-align predicts roughly half of the bindings. Therefore, T2 may lack the universal HLA-class II recognition required for its singly global use. In fact, since the T2 sequence is not conserved along all shrimp allergenic tropomyosins, a peptide-based shrimp allergy could contain multiple peptides T2 being one of the components. In this

Table 4. Neural Network-Predicted Binding Scores of T2 with the Most Frequent HLA-Class II Alleles

allele	NetMHCII pan IC ₅₀ nM	NN-align IC ₅₀ nM
DRB1–0101	39.43	21.00
DRB1–0301	850.27	430.70
DRB1–0401	369.32	1142.40
DRB1–0405	417.33	312.20
DRB1–0701	114.18	105.20
DRB1–0802	440.6	2522.00
DRB1–0901	150.66	1007.70
DRB1–1101	589.23	1926.70
DRB1–1201	362.03	
DRB1–1202	750.66	
DRB1–1302	44.48	66.90
DRB1–1501	241.94	837.20
DRB1–1502	351.46	
DRB3–0101	1389.08	1198.00
DRB3–0202	336.12	663.40
DRB4–0101	150.76	152.80
DRB5–0101	533.24	906.90
DPA1*01:03/DPB1*02:01	316.94	835.00
DPA1*01:03/DPB1*04:01	632.79	968.10
DPA1*02:01/DPB1*01:01	448.45	350.60
DPA1*02:01/DPB1*05:01	1105.58	1522.00
DPA1*02:01/DPB1*14:01	1837.11	2612.80
DPA1*03:01/DPB1*04:02	254.62	128.00
DQA1*01:01/DQB1*05:01	2043.38	2555.30
DQA1*01:02/DQB1*06:02	436.55	616.60
DQA1*03:01/DQB1*03:02	3187.11	2819.30
DQA1*04:01/DQB1*04:02	2975.91	1268.20
DQA1*05:01/DQB1*02:01	1958	3809.10
DQA1*05:01/DQB1*03:01	992.31	982.90

sense, a peptide mixture comprising a larger form of T2 of T-cell epitopes supplied intragastrically conferred partial protection against shrimp allergy in a murine model.²⁴ In addition to a peptide mixture, recombinant constructs with T-cell epitope tandem arrays have also yielded promising results.³⁹

On the other hand, the use of peptides previously validated as B-cell epitopes by virtue of their IgG/IgE binding and degranulation capacity has provided insights into their activity

and plasticity as epitopes. For instance, the B-cell epitope of B1 sums the capacity to induce the proliferation of T-cells and the release of IL-2. This duality suggests that B1 could comprise shorter cores in which the T-cell and B-cell epitope features are separated. This overlap was previously found in Anis 1 in which the T-cell epitope NRFTTIKNCEQH (region 49–69) shares with the IgE and IgG4 epitopes the core NRFTTIKN.⁴⁰ Then, work addressing the identification of the minimal cores and their structural properties may provide key knowledge for improving the distinction between T-cell epitopes and B-cell epitopes.

In conclusion, these findings provide the peptide VQESLL-KANIQLVEK as a candidate peptide member for the design of vaccines for Lit v 1 allergen.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c02231>.

Recognition of T and B peptides by the IgE of the sera of shrimp-allergic and nonallergic patients; dot composition used for the quantification and analysis displayed in Table 3; original dot-blot membrane; loading was performed on a graphite-pencil grided membrane to allow independent performance of independent reactions (Figure S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Zhen Xing Li – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China; orcid.org/0000-0002-7866-681X; Phone: +86-532-82032389; Email: lizhenxing@ouc.edu.cn; Fax: +86-532-82032389

Authors

Li Li Xu – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China; orcid.org/0000-0001-8759-1568

María Gasset – Institute of Physical Chemistry Rocasolano (IQFR), Spanish National Research Council (CSIC), 28006 Madrid, Spain

Hong Lin – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Chuang Yu – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Jin Long Zhao – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Xue Wen Dang – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.jafc.1c02231>

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Notes

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Figure S1. Recognition of T and B peptides by the IgE of the sera of shrimp allergic and non allergic patients. (A) Dot composition used for the quantification and analysis displayed in Table 3. (B) Original dot-blot membrane. Loading was performed on a graphite-pencil grided membrane, to allow independent the performance of independent reactions.

