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Identification and validation of shrimp-tropomyosin specific CD4 T cell epitopes.

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Abbreviations: MFI, Mean Fluorescence Intensity.

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Abstract

Background: Shellfish allergy is an immune-mediated adverse reaction to allergenic shellfish and is responsible for significant morbidity and mortality. CD4 T cell responses play an important role in the pathophysiological mechanisms of sensitization and in production of IgE.

Objective: We sought to identify and validate CD4 T cell shrimp tropomyosin-derived epitopes and characterize CD4 T cell responses in subjects with a clinical history of shellfish allergy.

Method: Using an *in vitro* MHC-peptide binding assay, we screened 91 overlapping peptides and identified 28 epitopes with moderate and strong binding capacities; 3 additional peptides were included based on MHC binding prediction score. These peptides were then examined in proliferation and cytokine release assays with T cells from allergic subjects.

Result: 17 epitopes restricted to DRB*01:01, DRB1*03:01, DRB1*04:01, DRB1*09:01, DQB1*02:01, DQB1*03:02 and DQB1*05:01 alleles were identified and validated by both the MHC binding and the functional assays. Two peptides showed specificities to more than one MHC class II allele. We demonstrated that these peptides exert functional responses in an epitope specific manner, eliciting predominantly IL-6 and IL-13.

Conclusion: The identified epitopes are specific to common MHC class II alleles in the general population. Our study provides important data for the design of peptide-based immunotherapy of shrimp-allergic patients.

1. Introduction

Shellfish allergy is an immune-mediated adverse reaction to substances derived from shellfish. It is the most frequent cause of food allergy, affecting more than 2% of the US adult population and is responsible for the majority of emergency department visits related to severe food allergy [1-4]. The prevalence of shellfish allergy appears to be increasing more recently worldwide because of a substantial increase of sea food consumption over the last decade [1-4]. The mild adverse reactions, which might persist throughout life, include hives, vomiting, abdominal pain and diarrhea. The severe and life threatening systemic anaphylactic reactions include a dramatic fall in blood pressure, wheezing, severe upper airway obstruction and even death. Currently, the only treatments for shellfish allergy are food avoidance and prompt effective management of severe reactions caused by allergen exposure [1-4].

Several independent biochemical and immunological studies have reported the identification of the major shellfish allergen as tropomyosin [2, 3, 5]. Tropomyosin belongs to a family of highly conserved proteins with multiple isoforms found in both muscle and non-muscle cells of vertebrates and invertebrates, with a high degree of amino acid sequence identity among different species [4-6]. At least 80% of shrimp-allergic subjects react to tropomyosin [5]. Extracts of tropomyosin bind approximately 75% of the shrimp-specific IgE from shrimp-allergic subjects. Allergic reactions to crustaceans and mollusks are often cross-reactive, which is explained by the highly conserved amino acid sequences of tropomyosins among these species [2].

Shellfish allergy is an immediate-type hypersensitivity reaction that is mediated by IgE. Naïve CD4⁺ T cell recognition of antigenic tropomyosin peptides presented in the context of HLA class II molecules are thought to be a key component in the pathophysiological mechanism of sensitization and production of IgE. Specific subsets of activated CD4⁺ T cells, particularly T_H2 cells, favor the production of IgE and disease development [9-11]. Little is known about the specific CD4⁺ T cell tropomyosin-derived epitopes and mechanisms of antigen presentation that selectively evoke T_H2 cells in patients with shellfish allergy.

Clinical studies of other types of allergies have shown promising results in the application of CD4 T-cell immunotherapy in allergic patients by administering the whole allergen to achieve desensitization [12-16]. The risk of potential adverse reactions, however, still remains as the allergen can bind specific IgE antibodies and cause severe symptoms. In this regard, peptide-based immunotherapy has an advantage because peptides do not bind IgE but may cause more effective and targeted immune responses [17, 18].

Previously, we have shown proliferation of peripheral mononuclear cells and T-cell lines derived from shrimp-allergic subjects after stimulation with native tropomyosin protein [19]. The purpose of this study was to identify and validate CD4 T cell tropomyosin-derived epitopes and to characterize CD4 T cell responses in subjects with clinical history of shellfish allergy. Using an *in vitro* MHC-peptide binding assay as well as *ex vivo* proliferation and cytokine release assays, we have identified and validated 17 epitopes restricted to multiple MHC class II alleles. We also demonstrate that these peptides exert functional responses in an epitope specific manner, capable of inducing CD4 T cell proliferation and eliciting predominant production of IL-6 and

- 84 IL-13 cytokines. Our study provides important data for the design of a peptide-based
85 immunotherapy of shrimp-allergic patients.

2. Materials and Methods

2.1. Study subjects

Study subjects with a clinical history (subjects 1-11) of allergy to shrimp were recruited based on history that included the time between exposure and the type and severity of reaction. Reactions included urticaria, erythema, swelling, vomiting, diarrhea, rhinitis, coughing, and dyspnea. Individuals were skin prick tested with extracts of shrimp as described before [19]. All blood draws were carried out according to the approved guideline procedures established by ARUP laboratories and the University of Utah Institutional Review Board. Serum samples were prepared and tested for IgE specific to tropomyosin from brown shrimp (Phadia ImmunoCAP® System, ThermoFisher). Control group (subjects 12-17) consisted of subjects without clinical history of shrimp allergy, who tested negative for the specific IgE levels (<0.35 kU/l). All subjects were genotyped for HLA-DRB1, HLA-DQA1 and HLA-DQB1 alleles.

2.2. MHC-peptide binding assay and epitope prediction

A total of 91 overlapping peptides (15-mers with offset by 3 amino acids), corresponding to tropomyosin of brown shrimp (Swiss-Prot: Q3Y8M6), were synthesized and tested by REVEAL™ MHC-peptide binding assay (Proimmune Ltd., Bradenton, FL) against the following MHC class II alleles: HLA- DRB1*01:01, DRB1*15:01, DRB1*03:01, DRB1*04:01, DRB1*11:01, DRB1*13:01, DRB1*07:01, DRB1*09:01, DRB1*10:01 and DQA1*01:01; DQB1*05:01, DQA1*05:01; DQB1*02:01, DQA1*01:02; DQB1*06:02 and DQA1*03:01; DQB1*03:02 (Supplement 1). This binding assay determines the ability of each candidate peptide to bind one or more MHC class II alleles and stabilize the MHC-peptide complex [20].

Detection is based on the presence or absence of the native conformation of the MHC-peptide complex via a labeled antibody. Each peptide is given a score relative to the positive control peptide, which is a known T-cell epitope for each MHC class II allele tested. The binding score for each peptide-MHC complex is calculated at 0 and 24 hours by comparison to the binding of the relevant positive control. The score of the test peptide is reported quantitatively as a percentage of the signal generated by the positive control peptide. Assay performance was confirmed by including an intermediate control peptide that is known to bind with weaker affinity to the allele under investigation. Using this screening method, peptides with scores $\geq 10\%$ of the positive control were considered as good binders and further investigated. MHC class II peptide binding prediction was carried out using the consensus prediction method available at the Immune Epitope Database (IEDB) analysis resource (www.iedb.org) [21].

2.3. Tritiated thymidine proliferation assay

The assay was carried out as described previously [22]. Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation over Ficoll-Paque medium (GE Healthcare, Pittsburg, PA). The cells were cultured in RPMI1640 media containing 10 % of human AB serum (Sigma, Saint Louis, MO), penicillin (10 IU/ml), streptomycin (100 mg/ml) and glutamine (1 mM) (Sigma, Saint Louis, MO). 2×10^5 of cell were dispensed into each well in 200 μ l of complete media. The peptides were at the concentration of 50 μ g per ml. Tetanus Toxoid was used as positive control (0.3 μ g/well). Negative control was media collected from unstimulated cell cultures. After 5 days of incubation, tritiated thymidine (1 μ Ci per well) was added for another 18 hours of incubation. The stimulation index (SI) was calculated and used as

a measurement of proliferation (SI = c.p.m of stimulated cells/c.p.m of negative control). The experiment was carried in triplicate. The mean of the triplicate assays was used to calculate the SI. The SI equal or higher than 2 was considered as a positive response [22]

2.4. CFSE CD4 T cell proliferation assay

PBMC were washed twice in cold PBS prior labeling with Carboxyfluorescein succinimidyl ester (CFSE), (Invitrogen, Carlsbad, CA). The cell density was adjusted to 1×10^7 cells per ml in pre-warmed RPMI1640 media without human AB serum. To avoid the carry-over of unlabeled cells, the samples were transferred into fresh tubes prior the labeling with CFSE. CFSE labeling was carried out at 37°C for 8 minutes with 1.25 mM CFSE. The reaction was quenched with cold, complete RPMI 1640 media with 10% of human AB serum and then washed twice. The cell density and stimulation conditions were the same as described in the tritiated thymidine proliferation assay. 2×10^5 of cell were dispensed into each well in 200 μ l of complete media. The peptides were at the concentration of 50 μ g per ml. The experiments were carried out in triplicate. Cells were cultured for 5 days, after which they were stained with CD3-APC and CD4-PE (BD Pharmingen) antibodies and analyzed by flow cytometry. Cells that had undergone proliferation were distinguished from undivided CD4 T cells by their low CFSE and higher CD4-PE fluorescence intensities (Supplement 2). The mean of the triplicate assays was used to calculate frequency of proliferating CD4 T cells per 100,000 of the total CD4 T cell population.

2.5. Cytokine release assay

Triplicate cell culture media samples from the CFSE CD4 T cell proliferation assays were harvested on day 5 after stimulation, and tested for the released cytokines by the in-house developed multiplexed fluorescent microsphere immunoassay as described previously [23]. The following cytokines were measured: TNF- α , IL-1 β , IL-4 and IL-5, IL-6, IL-12, IL-13 and IFN- γ . The cytokine production above the average of unstimulated samples plus 2 standard deviations (SD) of the triplicate unstimulated cultures, calculated separately for each particular cytokine on the panel, was considered positive.

2.6. Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). The Mann–Whitney U test was used to determine statistical significance of the results, with p-values equal of 0.05 or less.

3. Results

3.1. Identification of CD4 T cell epitopes based on peptide/MHC binding properties

The initial screening of potential tropomyosin-derived CD4⁺ T cell epitopes was carried out using the REVEAL™ MHC-peptide binding assay [20]. Of 91 tropomyosin peptides tested, 28 peptides were confirmed to have relative binding affinities $\geq 10\%$ of the positive control. All 91 peptides were also evaluated by the MHC class II peptide binding prediction tool available at the IEDB (www.iedb.org). Peptides 18, 43 and 80 with binding scores of $\leq 10\%$, using the REVEAL™ MHC-peptide binding assay, were found to be potential MHC class II epitopes by the consensus prediction method (data not shown) [21]. Thus, a total of 31 peptides were selected for further investigation. The peptides amino acid sequences, HLA restriction and binding scores are shown in Table 1. Using this method, we also found additional MHC alleles with potential specificities for peptides 21 and 54 (Table 1).

3.2. Proliferative T cell responses measured by tritiated-thymidine incorporation

To determine whether the selected peptides are immunologically significant, we measured proliferative T cell responses in subjects with a clinical history of shrimp allergy. Subject demographics and HLA restriction are shown in Table 2. All the subjects tested positive by skin prick test, but only 6 of 11 of them (54.4%) have elevated tropomyosin-specific IgE levels. The majority of subjects carried HLA-DRB1 and HLA-DQB1 alleles matching to one or more of the selected peptides. To avoid ambiguity, we choose those subjects that have only one HLA-matched allele. Peptides 15, 53 and 58 were excluded from the investigation because no

HLA-matched subjects were available. T cells from all of the subjects, except subject # 2, showed proliferation by tritiated-thymidine incorporation to at least one of the selected peptides (Supplement 3). We found that 18 out of 28 tested peptides elicited T cell proliferation ($SI \geq 2$), although not all of the subjects responded positively (Supplement 3). This is likely due to the limited sensitivity of the assay in which a cell population with a very low frequency is under investigation. Figure 1 shows the data of positively responded T cells and peptides used in the experiment. The SI was in range from 2.12 to 5.91. Of these 18 peptides, 10 peptides (1, 4, 5, 24, 36, 43, 54, 66, 80 and 81) elicited positive responses in 2 or more subjects. Stimulation with Tetanus Toxoid showed much higher proliferative responses in T cells from all of the subjects, indicating the subject's immunocompetence (Supplement 3).

3.3. Proliferation of CFSE-labeled CD4 T cells

To confirm that the proliferative responses measured by tritiated-thymidine incorporation were specific to CD4 T cell population, we examined dilution of CFSE after peptide stimulation in CD4 T cells from the same subjects, performed in parallel on the same samples. All of the subjects showed proliferation of CFSE-labeled CD4 T cells to at least one of the selected peptides (Figure 2 and Supplement 4). Figure 3 shows examples of the results. Specifically, we found that 23 out of 28 tested peptides elicited proliferation of CFSE-labeled CD4 T cells (Figure 2, Supplement 4). The number of cells that had undergone cell division upon peptide stimulation in subjects with history of shrimp allergy varied from 5 to 164 per 100,000 cells of the total CD4 T cell population. In HLA-matched control subjects without history of shrimp allergy, peptides did not elicit proliferation of CSFE-labeled CD4 T cells (Figure 4). All the

subjects tested showed much greater level of CD4 T cell proliferation to Tetanus Toxoid stimulation, but no proliferation was observed when stimulated with media alone (Figure 3, 4). MFI of CD4 fluorescence of both peptide and Tetanus toxin stimulated cells was consistently 2-fold higher compared to unstimulated cells (Figure E1). This qualitative difference indicates that even those with very low numbers of positive CD4 T cells are truly proliferated cells and are not the result of instrument noise.

3.4. Cytokine release upon peptide stimulation

We next examined cytokine release in the culture media collected from samples that were investigated by the CFSE proliferation assay. Very low levels of TNF- α , IL-1 β , IL-4 and IL-5 cytokines were observed, with no discernible differences (data not shown). IL-6 and IL-13 were found to be the most responsive cytokines in the panel (Figure 5). IL-6 secretion was detected in 42 peptide stimulation cultures, representing 24 different peptides. IL-13 secretion was observed in 25 peptide stimulation cultures, representing 18 different epitopes. Eighteen peptide stimulation cultures secreted both IL-13 and IL-6. Secretion of IL-12 and IFN- γ was detected in 10 and 14 peptide stimulation cultures, each representing 8 different epitopes. Cosecretion of these cytokines was observed only in 5 cultures. Combined, IL-6 and IL-13 secretion were detected in 42 out of 136 peptide stimulation cultures, whereas IL-12 and IFN- γ secretion were detected in only 18 cultures. Taken together, the results indicate predominance of IL-6 and IL-13 secretion after *in vitro* stimulation with tropomyosin-derived peptide in patients with history of shrimp allergy.

230 3.5. *Compilation of shrimp-tropomyosin CD4⁺ T cell epitopes*

231 In this study we use the following criteria for validation of shrimp-tropomyosin specific
232 CD4 T cell epitopes. First, the peptides that were initially identified by MHC binding assay and
233 prediction score must evoke proliferation of CD4 T cell as determined by CFSE dilution assay.
234 Then, the positive epitopes must be confirmed by either or both the tritiated-thymidine
235 incorporation and the cytokine release assays. Based on these criteria, we compiled a list of 17
236 tropomyosin-specific CD4 T cell epitopes restricted to multiple HLA alleles. Table 3 shows their
237 amino acid sequences, position within tropomyosin, HLA allele restriction and the functional
238 assays they tested positive. Peptides 4 and 5 overlap by 12 amino acids, are restricted to
239 DRB1*03:01 and likely represent the same epitope.

4. Discussion

This study was focused on identification and validation of CD4 T cell epitopes localized to tropomyosin from brown shrimp, an immunodominant shrimp allergen [5]. Our approach consisted of the initial screening of 91 overlapping peptides by a MHC-peptide binding assay and the subsequent validation of the selected epitopes using two types of proliferation assays and by the assessment of cytokine release. In order to determine MHC binding properties, we used the REVEALTM (Rapid Epitope Discovery System) binding assay [20]. The assay determines the ability of each overlapping peptide to bind to one or more MHC class II alleles in a stabilized MHC-peptide complex, which then is compared to the affinity of known T-cell epitopes. This allows rapid identification of the most likely immunogenic peptides and determines the epitope amino acid sequences. The screening is based on whether or not the native conformation of the MHC-peptide complex is present. The other advantage of this system is that it allows rapid identification of MHC allele restriction. The screening, along with MHC class II epitope prediction, identified 31 potential epitopes that are restricted to HLA-DRB1 and HLA-DQB1 MHC class II alleles. There are some limitations to this assay in terms of epitope identification. MHC-peptide binding *in vitro* is not always indicative that those identified are truly functional epitopes, which is critical in terms of the design of peptide-based immunotherapy of shrimp-allergic patients. There are a number of examples where a peptide with good MHC binding properties, determined *in vitro*, is unable to elicit *ex vivo* or *in vivo* functional responses and vice versa[24]. That being said, neither the MHC-peptide binding nor the MHC class II epitope prediction are perfect methods for epitope identification, but their combination may provide a better approach for the initial identification of epitope candidates.

In order to confirm that an epitope is biologically significant, it has to be examined on whether it can trigger functional responses, such as the ability to cause proliferation and/or produce cytokines upon stimulation of CD4 T cells. In this study, we employed two well established proliferation assays to validate the initially selected tropomyosin-specific MHC binding peptides. The tritiated-thymidine incorporation assay is the most utilized in laboratories for many decades because of its simplicity and interpretation of the results. After examining all of the initially identified epitopes, slightly more than half of them were confirmed by this assay. This indicates that some of the peptides might be functionally inactive with respect to eliciting proliferation. Alternatively, the sensitivity of this assay is likely to be limited due to a very low frequency of the cell population under investigation, which is typical for memory CD4 T cells. Despite its ease of use, the tritiated-thymidine incorporation assay is limited in terms of differentiating the subset of T cells that has undergone proliferation, unless these cells can be isolated and reconstituted with antigen-presenting cells. In our study, this was not an option as we had to screen many peptides and cell samples from many subjects. In parallel, we also examined CD4 T cell proliferation measuring CFSE dilution under the same stimulation conditions. After comparing results of both assays, we have found that the CFSE dilution assay revealed more positive responders compared to what was revealed with the tritiated-thymidine incorporation (Figure 1 and 2). This difference is likely due to the better sensitivity of the CFSE assay, as it provides both quantitative and qualitative measurements, which is important in examining rare subsets of cells. In particular, the high levels of CD4 fluorescence turned out to be very useful in proving that those with very low numbers of positive CD4 T cells are indeed proliferated cells. As little as 5 proliferated cells per 100,000 of the total CD4 T cell population were distinguished based on that qualitative characteristic. High levels of CD4 on the surface of

T cells specific for recent antigen exposure have been well demonstrated [25]. The other explanation of the difference between assays is likely due to the low frequencies of tropomyosin-specific CD4 T cells present in the patients, which has been described in patients with other types of allergies. For example, DeLong and colleagues estimated frequencies of Ara h1-specific CD4 T cells in patients with peanut allergy to be as low as 9 cells per million [26]. The lower their numbers, the greater coefficient variability both within and between assays would be determined. This has been documented in other functional cell-based assays [27, 28]. Both explanations are not mutually exclusive. One of the solutions to overcome this problem – that is related to both assays -- is to significantly increase the cell numbers. However, as mentioned above, it is constrained by the number of peptides and samples that were examined in this study. With respect to the tritiated-thymidine incorporation assay, one might question whether SI is too stringent for this type of the experiments. This concern might be valid and should be addressed in future studies. Clearly, the lowered SI would enable identification of more positively responding cells. In this investigation, we used a previously published protocol from a study that investigated similar questions [22].

Finally, we examined cytokine release into the culture media after 5 days of peptide stimulation, employing a multiplexed fluorescent microsphere immunoassay system. The assay is very sensitive compared to the tritiated-thymidine incorporation and CFSE dilution assays, as it enables one to detect picograms of the released cytokines. The other advantage of this system is the ability to measure multiple cytokines in a single sample, which is useful in comparing T_H1 and T_H2 cytokine profiles. Although the latter was not the main focus of this study, we were interested in examining secretion of these molecules since many recent studies of other types of allergies have implicated T_H2 cytokines in the pathophysiological mechanisms of allergic

inflammatory disease [10, 11, 29]. In our study, we were unable to detect levels of IL-4 and IL-5, which are specific markers of T_H2 responses. This might be due different kinetics and stabilities of these cytokines in cell cultures. An ideal approach to address the question of T_H1 versus T_H2 would be isolation and enrichment of the antigen-specific CD4 T cells and setting up a short-time (5-6 hours) stimulation assay as it was recently described [11]. Interestingly, the secretion of IL-6 and IL-13 dominated over IFN- and IL-12 in peptide stimulation cultures when examined separately or together in the cultures. In addition, their production was observed in response to more than twice the number of peptides compared to those that evoke secretion of IFN- and IL-12. Both IL-6 and IL-13 have been implicated in pathophysiology of allergic disorders. IL-13 is considered as a major regulator of allergy [30-32]. Studies of murine models have demonstrated that IL-13 alone is sufficient to trigger allergic responses. It induces a variety of signaling pathways in patients with allergic asthma [33, 34]. IL-6 functions in the maturation of B cells by promoting T_H2 while inhibiting T_H1 differentiation [35]. Recent studies have shown that it can also promote T_H17 differentiation, a subset of T cells that is implicated in T_H2 -mediated allergic disorders. IL-6 also regulates the intensity of immune responses by inhibiting Treg cell differentiation. Interestingly, elevated levels of IL-6 correlate with IL-13 levels in allergic asthma patients, but not with the proinflammatory cytokines such as IL-1 and TNF [36]. It is important to note that IL-6 is primarily produced by antigen presenting cells. Future studies examining intracellular cytokine expression in peptide-stimulated cultures will be necessary to confirm the source of IL-6 in these cell cultures.

After compiling data from all the three assays, we have identified 17 epitopes restricted to 4 HLA-DRB1 and 4 HLA-DQB1 MHC class II alleles (Table 3). Two epitopes show restriction to multiple alleles: peptide 66 is specific to DQB1*02:01 and DQB1*04:01, while

peptide 74 has restriction to DRB1*01:01, DQB1*04:01 and DQB1*05:01. The rest of the epitopes show restriction to a single MHC class II allele. Four epitopes have a significant overlap (12 amino acids) between each other; yet show specificities to different alleles. Peptides 3 and 4 are specific to DRB1*0401 and DRB1*0301 correspondingly, while peptides 80 and 81 are restricted to DQB1*0501 and DRB1*0101. There are two peptides (4 and 5) that also have 12 amino acid overlap but share the same allele specificity and therefore likely represent the same epitope. It is important to note that the number of tropomyosin-derived epitopes is likely to be greater than what have been found in this study. Some of the 91 synthesized peptides that had failed by MHC-peptide binding assay might have specificities to other MHC alleles that were not examined during the initial screening. In addition, some of these with the score below 10 might elicit CD4 T cell functional responses and, therefore, be considered as valid epitopes. It is important to note that in this study only DR and DQ specificities were examined. Thus, it is possible that the identified epitopes might also show restriction to DP or DRB-associated MHC alleles, which were not examined in this study.

Identification of these tropomyosin-specific immunodominant epitopes is important in terms of developing peptide-based immunotherapy of shrimp and other shellfish-allergic patients. Significant progress has been made in developing peptide-based immunotherapies in studies with other types of allergies [39, 40]. Vaccination with soluble peptides has several advantages over vaccination with the whole antigens. Peptides can be chosen based on their length, solubility and their restriction to multiple alleles in order to cover a wider population range. Administration with peptides improves safety and reduces the time of immunotherapy. Worm and colleagues have recently shown that unlike vaccination with the whole cat allergen, vaccination with peptides does not induce histamine release. A single administration was safe

and efficacious [41]. However, implementation of these studies would require a significantly larger group of individuals. The results of our study give a good start to initiate this type of studies. The other important aspect of our study is the development of reagents that could be utilized in clinical trials of patients undergoing immunotherapy, with the focus on understanding CD4 T cell biology and the interplay between T_H1 , T_H2 and T_H17 responses in allergic disorders. The utility of MHC class II multimers is proving to be useful tool for enumeration and characterization of antigen-specific CD4 T cells [37, 38]. This study provides data that can be used for developing these reagents that would be exceptionally useful in studying the CD4 T cell phenotypes and their functional responses measured on a single cell level.

364

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480

481 **Tables**

482 Table 1. MHC-peptide binding score of tropomyosin-derived peptides selected for validation.

483 Table 2. Demographics and HLA genotyping of shrimp allergic subjects.

484 Table 3. Validated tropomyosin-specific CD4 T cell epitopes

485

486

487 **Figure legends**

488 Figure 1. Results of T-cell proliferation responses by tritiated thymidine incorporation assay in
489 subjects with history of shrimp allergy. The graph shows responders with SI equal or above 2.

490 The complete data is presented in Supplement 2.

491 Figure 2. Results of proliferation of CFSE-labeled CD4 T cells after 5 days of peptide
492 stimulation in vitro culture in subjects with history of shrimp allergy. CD4 T cell frequency is
493 shown per 100,000 CD4 T cells. The graph shows positive responders. The complete data is
494 presented in Supplement 4.

495 Figure 3. Examples of results of proliferation of CFSE-labeled CD4 T cells after peptide
496 stimulation in subjects with history of shrimp allergy. Positive response corresponds to CFSEdim
497 (x axis) and CD4⁺ high (y axis) cell populations.

498 Figure 4. Examples of results of proliferation of CFSE-labeled CD4 T cells after peptide
499 stimulation in control subjects showing absence of proliferation of CD4 T cells. X axis
500 corresponds to CFSE, and y axis shows CD4 fluorescence intensities.

501 Figure 5. Levels of cytokines secreted by T cells in subjects with history of shrimp allergy after 5
502 days of peptide stimulation. Only those responses that were above the average of unstimulated
503 samples plus 2 of their standard deviation are presented.

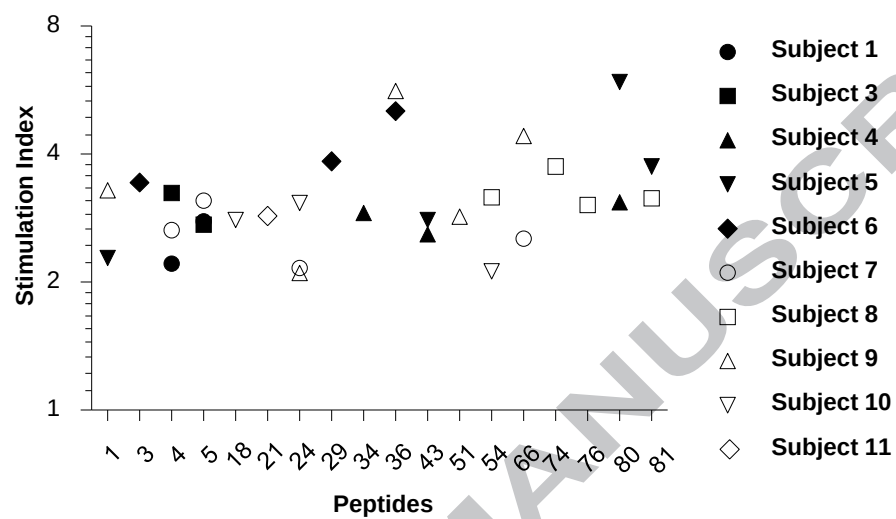
Supplement material text

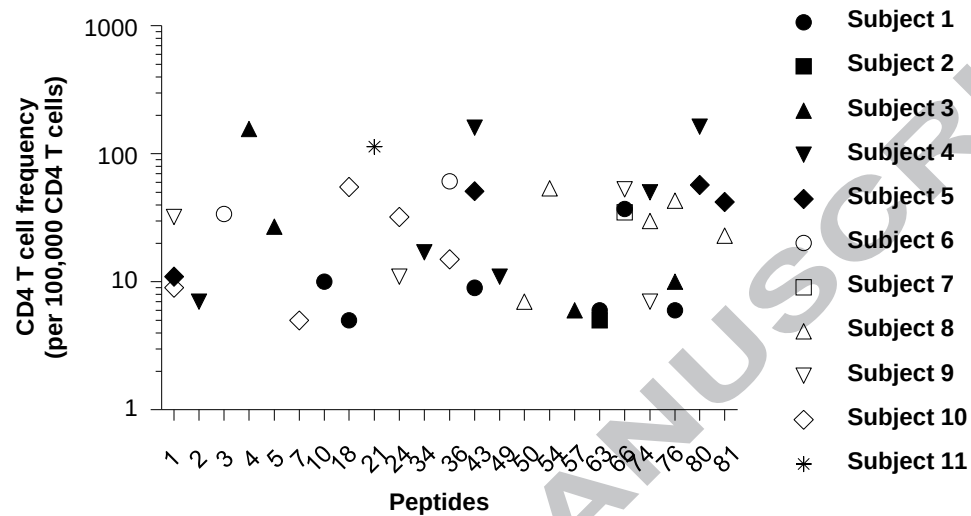
Supplement 1. List of tropomyosin-derived peptides tested in the study.

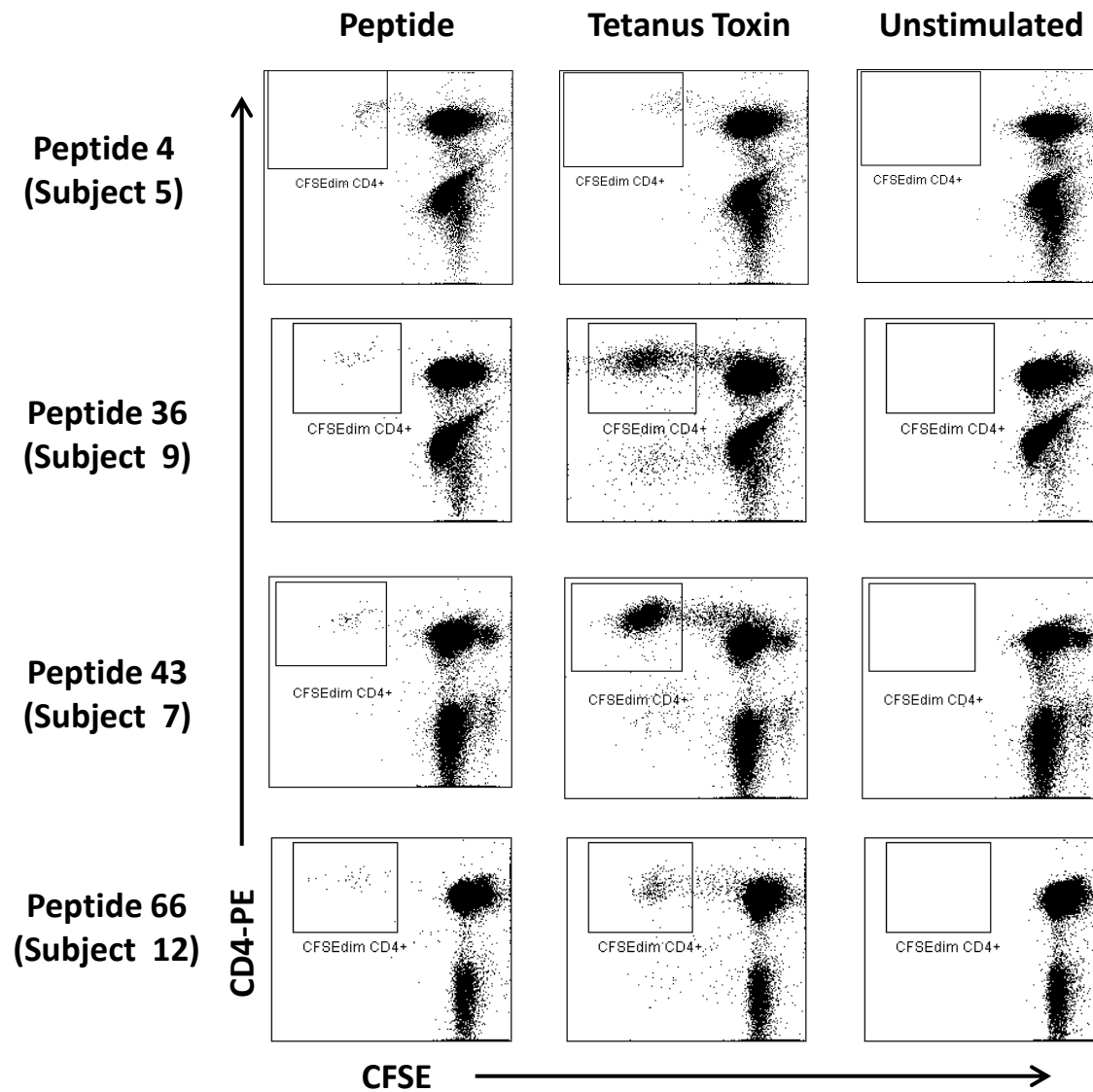
Supplement 2. CD4 fluorescence levels on stimulated and unstimulated CD4 T cells. Proliferated CD4 T cells (left quadrant) show two fold increase in MFI compared to unstimulated cells (right quadrant).

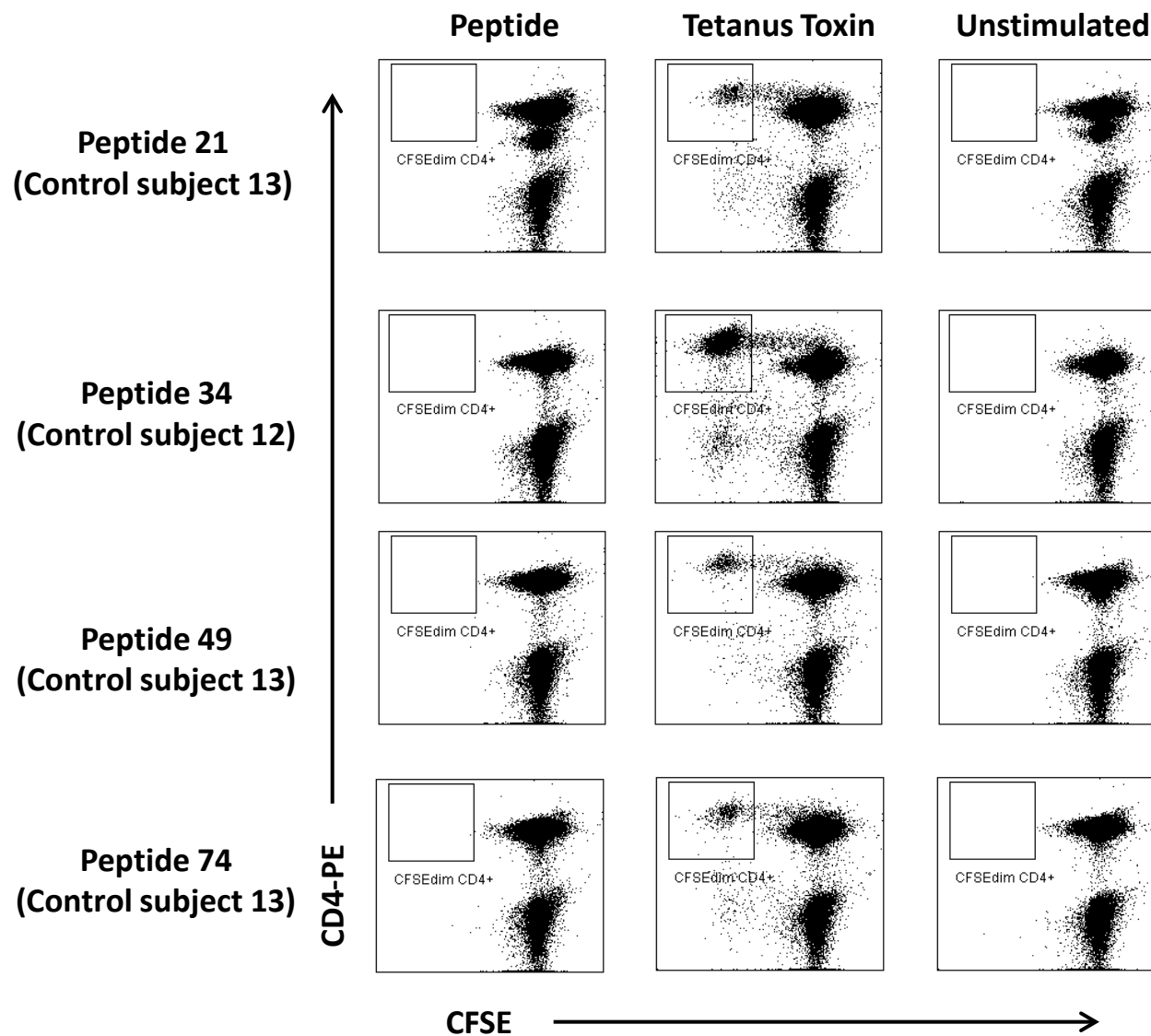
Supplement 3. Results of tritiated thymidine proliferation assays in shrimp-allergic patients. Results are shown in stimulation index (SI) units. SI results equal or higher than 2 were considered as a positive response.

Supplement 4. Results of CFSE proliferation assays in shrimp-allergic patients after 5 days of peptide stimulation in vitro culture. Results are shown per 100,000 CD4 T cells. Positive responders are indicated in bold.

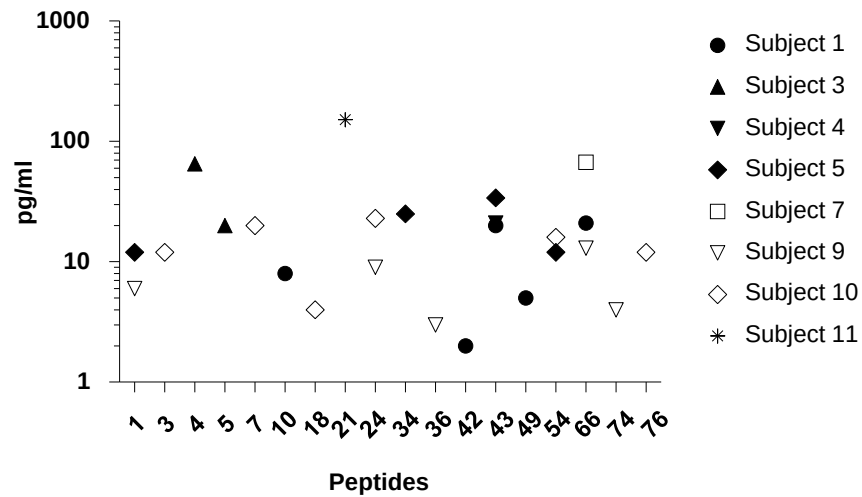




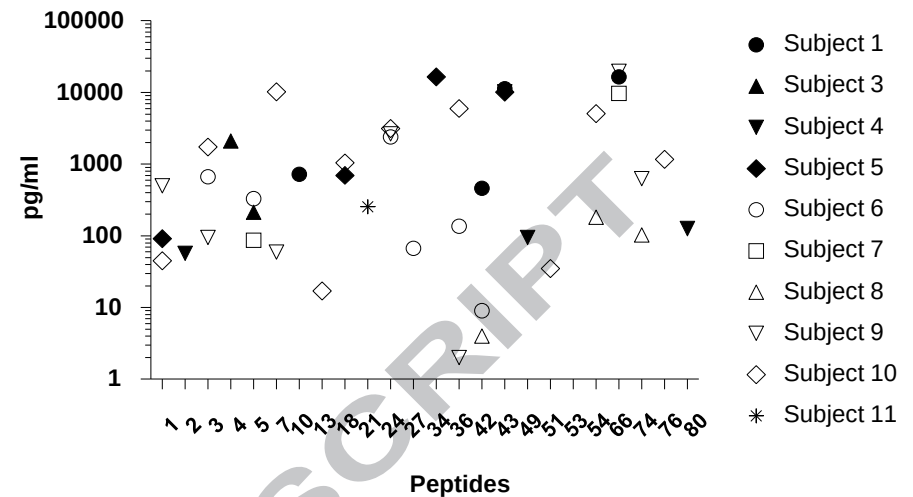




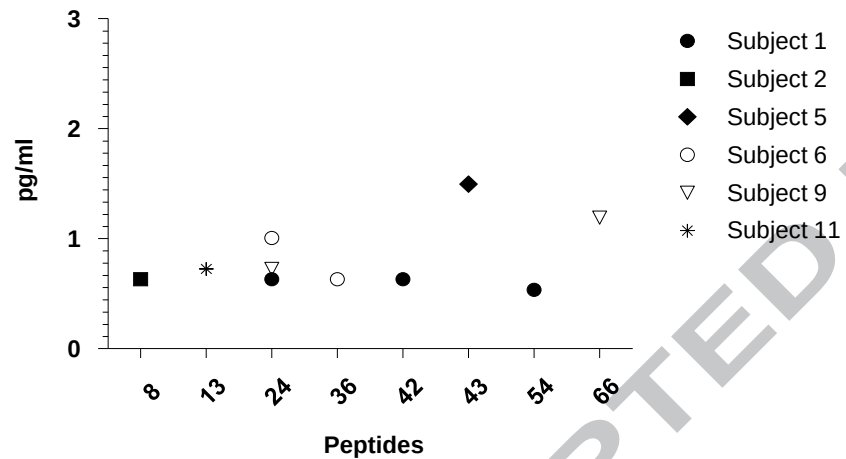
IL-13



IL-6



IL-12



IFN- γ

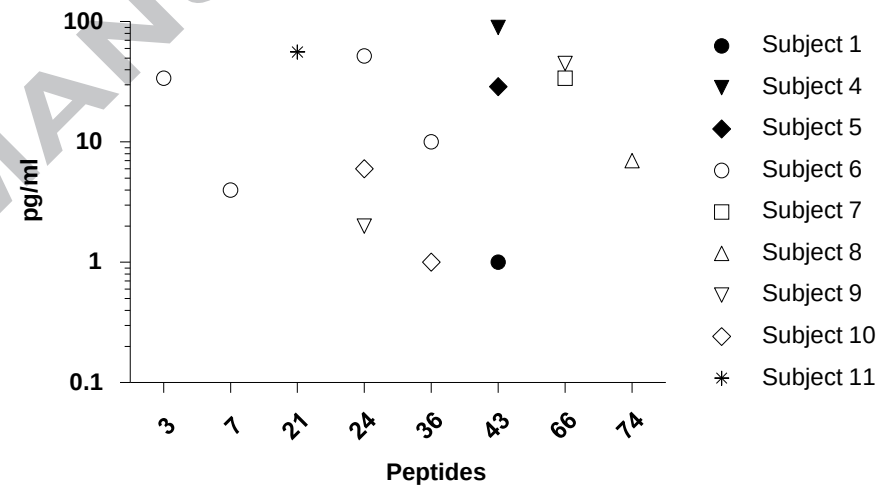


Table 1. MHC-peptide binding score of tropomyosin-derived peptides selected for validation.

Peptide ID	Peptide sequence	Starting Amino Acid #	HLA alleles	Binding score
1	MDAIKKKMQAMKLEK	1	DRB1*15:01	14.02
			DRB1*11:01	18.74
			DQB1*03:02	35.20
2	IKKKMQAMKLEKDNA	4	DRB1*01:01	63.69
			DRB1*13:01	26.06
			DQB1*05:01	13.94
3	KMQAMKLEKDNAMDR	7	DRB1*04:01	23.52
4	AMKLEKDNAMDRADT	10	DRB1*03:01	74.69
5	LEKDNAMDRADTLEQ	13	DRB1*03:01	66.31
7	MDRADTLEQQNKEAN	19	DRB1*04:01	22.16
8	ADTLEQQNKEANNRA	22	DQB1*02:01	20.74
10	QNKEANNRAEKSEEE	28	DQB1*02:01	25.17
13	EKSEEEVHNLQKRMQ	37	DRB1*07:01	26.79
15	VHNLQKRMQQLENDL	43	DRB1*11:01	11.01
18	QLENDLDQVQESLLK	52	DRB1*04:01	9.10*
21	QESLLKANIQLVEKD	61	DRB1*01:01	30.10
			DRB1*09:01	8.32*
24	QLVEKDALSNAEGE	70	DRB1*03:01	45.59
			DRB1*04:01	20.62
29	VAALNRRRIQLLEEDL	85	DRB1*01:01	40.42
			DRB1*11:01	22.35
			DRB1*13:01	19.31
34	ERSEERLNTATTCLA	100	DQB1*05:01	22.67
36	LNTATTKLAEASQAA	106	DRB1*04:01	43.87
42	ERMRKVLENRSLSDE	124	DRB1*01:01	21.57
43	RKVLENRSLSDEERM	127	DQB1*05:01	7.11*
49	ENQLKEARFLAEEAD	145	DQB1*05:01	19.65
50	LKEARFLAEEADRKY	148	DRB1*01:01	25.28
51	ARFLAEEADRKYDEV	151	DRB1*03:01,	15.03,
			DRB1*04:01	11.06
53	EADRKYDEVARKLAM	157	DRB1*11:01	13.67
54	RKYDEVARKLAMVEA	160	DRB1*01:01	11.62
			DQB1*03:02	7.98*
57	LAMVEADLERAEERA	169	DRB1*03:01	19.46
58	VEADLERAEERAETG	172	DRB1*10:01	39.12
63	ESKIVELEELRVVG	187	DRB1*03:01	29.06
66	ELRVVGNNLSLEVS	196	DRB1*04:01	25.05
			DQB1*02:01	24.15
74	AYKEQIKTLTNKLKA	220	DRB1*01:01	90.89
			DRB1*04:01	41.94
			DRB1*11:01	10.94
			DRB1*07:01	74.20
			DQB1*05:01	14.73
			DQB1*03:02	19.28
76	KTLTNKLKAAEARAE	226	DRB1*01:01	100
			DRB1*09:01	89.92
			DRB1*10:01	37.57
80	RAEFAERSVQKLQKE	238	DQB1*05:01	9.26*
81	FAERSVQKLQKEVDR	241	DRB1*01:01	17.78

*Peptide and MHC allele selected for further validation based on the consensus prediction method.

Table 2. Demographics and HLA genotyping of the study subjects

Subjects no.	Gender	Age	DQB1 allele	DRB1 allele	Skin test	Tropomyosin IgE (kU/L)	Shrimp allergy history
1	F	28	DQB1*02:01/DQB1*05:01	DRB1*01:01/DRB1*03:01	+	70.9	Yes
2	M	34	DQB1*02:01/DQB1*06:03	DRB1*03:01/DRB1*13:01	+	33.6	Yes
3	M	33	DQB1*02:01/DQB1*03:02	DRB1*03:01/DRB1*04:07	+	25.2	Yes
4	F	29	DQB1*05:01/DQB1*06:04	DRB1*01:03/DRB1*13:02	+	<0.10	Yes
5	M	39	DQB1*03:02/DQB1*05:01	DRB1*01:01/DRB1*04:04	+	0.85	Yes
6	F	29	DQB1*03:02/DQB1*05:01	DRB1*01:01/DRB1*04:01	+	<0.10	Yes
7	F	52	DQB1*02:01/DQB1*02:01	DRB1*03:01/DRB1*03:01	+	<0.10	Yes
8	M	45	DQB1*05:04/DQB1*06:03	DRB1*01:01/DRB1*13:01	+	2.07	Yes
9	F	28	DQB1*03:02/DQB1*06:03	DRB1*04:01/DRB1*13:01	+	<0.10	Yes
10	M	23	DQB1*03:02/DQB1*03:03	DRB1*04:01/DRB1*07:01	+	<0.10	Yes
11	M	26	DQB1*03:03/DQB1*03:03	DRB1*09:01/DRB1*09:01	+	0.74	Yes
12	F	25	DQB1*05:01/DQB1*05:01	DRB1*01:01/DRB1*01:01	-	<0.10	No
13	F	27	DQB1*03:02/DQB1*05:01	DRB1*01:01/DRB1*09:01	-	<0.10	No
14	F	41	DQB1*03:01/DQB1*03:01	DRB1*04:01/DRB1*11:01	-	<0.10	No
15	M	32	DQB1*02:01/DQB1*03:01	DRB1*03:01/DRB1*11:01	-	<0.10	No
16	M	49	DQB1*02:01/DQB1*02:02	DRB1*03:01/DRB1*07:01	-	<0.10	No
17	M	53	DQB1*03:03/DQB1*06:01	DRB1*09:01/DRB1*15:02	-	<0.10	No

Table 3. Validated tropomyosin-specific CD4 T cell epitopes

Peptide ID	Peptide sequence	Starting Amino Acid #	HLA alleles	Positivity by functional assay		
				CFSE dilution	tritiated-thymidine incorporation	Cytokine release*
1	MDAIKKKMQAMKLEK	1	DQB1*03:02	+	+	+
3	KMQAMKLEKDNAMDR	7	DRB1*04:01	+	+	+
4	AMKLEKDNAMDRADT	10	DRB1*03:01	+	+	+
5	LEKDNAMDRADTLEQ	13	DRB1*03:01	+	+	+
18	QLENDLDQVQESLLK	52	DRB1*04:01	+	+	+
21	QESLLKANIQLVEKD	61	DRB1*09:01	+	+	+
24	QLVEKDKALSNAEGE	70	DRB1*04:01	+	+	+
34	ERSEERLNTATTKLA	100	DQB1*05:01	+	+	+
36	LNTATTKLAEEASQAA	106	DRB1*04:01	+	+	+
43	RKVLENRSLSDDEERM	127	DQB1*05:01	+	+	+
49	ENQLKEARFLAEEAD	145	DQB1*05:01	+	-	+
54	RKYDEVARKLAMVEA	160	DRB1*01:01	+	+	+
66	ELRVVGNNLKSLEVS	196	DQB1*04:01, DQB1*02:01	++	++	++
74	AYKEQIKTLTNKLKA	220	DRB1*01:01, DRB1*04:01	++	+-	++
			DQB1*05:01	+	+	-
76	KTLTNKLKAAEARAE	226	DRB1*01:01	+	+	+
80	RAEFAERSVQKLQKE	238	DQB1*05:01	+	+	-
81	FAERSVQKLQKEVDR	241	DRB1*01:01	+	+	-

* Positive response detected for at least one of the cytokines in the panel