

T-Cell Proliferation Assay: Determination of Immunodominant T-Cell Epitopes of Food Allergens

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Abstract

Characterization of allergen-specific T cells is critical to understand their contribution to disease pathogenesis. The identification of immunodominant T-cell epitopes is crucial for development of T-cell-based vaccines. Peptide-specific T-cell proliferation studies are usually performed in a library of short synthetic peptides (15mer or 20mer) with 3 or 5 offset spanning the entire length of the allergen. T-cell peptide epitopes lack the primary and tertiary structure of the native protein to cross-link IgE, but retain the ability to stimulate T cells. The peptides sequences can also be obtained either by in silico approaches and in vitro binding assays. The efficacy of T-cell epitope-based peptide immunotherapy has been proven in certain allergies. The present methodology describes T-cell proliferation assays using whole blood sample from allergic subjects.

Key words T cell, T cell proliferation, Peptides, Epitopes, Thymidine incorporation

1 Introduction

Allergen-specific IgE production and activation of mast cells, basophils, and eosinophils are the hallmarks of type I allergic immune response. Both branches of the immune system, innate and adaptive, participate in establishing the TH2 immune response that characterizes the allergic diseases [1]. Dominant TH2 responses to allergens during the sensitization phase lead to immunoglobulin (Ig) class switching to IgE as well as recruitment and activation of pro-inflammatory effector cells such as mast cells, basophils, and eosinophils [2–5]. Allergen-specific T cells are crucial in this inflammatory cascade [5]. Although different types of T lymphocytes (e.g., CD4+, CD8+, and natural killer T cells) have the capacity to respond to allergens, CD4+ T cells are the predominant effector population, which recognize peptides derived from allergens presented by antigen presenting cells (APCs) in the context of major histocompatibility complex (MHC) class II molecules. These cells exert their effects through the production of TH2 cytokines, mainly IL-4, IL-5, and IL-13. IL-4 is critical in

both the development of TH2 cells and antibody isotype switching to IgE [6].

Natural allergens drive diverse T-cell lineages that secrete multiple cytokines. It seems that different allergic phenotypes are linked to various combinations of CD4+ subsets, which include not only the well-established TH2 and TH1 lineages, but also the TH9, TH17, TH22, Treg, TR1, and TFH lineages that have been more recently discovered. Their cytokines have different functional roles in allergy [5]. Regulatory subsets, Treg and TR1, antagonize the activity of TH2 cells. Skewing allergen-specific T-cell responses toward a regulatory phenotype seems to be crucial for the beneficial effects of allergen-specific immunotherapy (ASIT) [7].

The specific immune response to allergens is decisive in the development of clinically healthy or allergic states [8]. In non-allergic subjects, a substantial fraction of allergen-specific T cells have a memory phenotype, but at lower frequencies than in allergic subjects. However, differentiated Th2 cells are present only in allergic subjects and their disappearance correlates with successful immunotherapy and tolerance [7, 8]. However, the T-cell receptor (TCR) of allergen-specific memory T cells from allergic subjects shows higher avidity compared to those of non-allergic subjects; even at a naive stage, suggesting that differences in T-cell repertoire development could exist. Moreover, in non-allergic subjects, the antibody response varies between having no response and the production of allergen-specific IgG₄- or IgG₁ with or without low amounts of allergen-specific IgE. In healthy individuals, during an immune response toward common environmental allergens, antigen-specific TR1 cells have been found to be the dominant subset along with a high concentration of specific IgG₄ and detectable IgE. Similarly, the major components of an immune response after ASIT are IL-10- and TGF- β -producing TR1 cells, IgG₄ antibodies, and suppressed effector cells (i.e., mast cells, basophils, and eosinophils) [7, 8].

Identification of precise T-cell epitopes within allergens is an important step toward elucidating mechanisms of disease pathogenesis and for the design of T-cell vaccines. Peptide-immunotherapy relies on the well-established role of T cells in food and environmental allergies, but with the substantial advantage of surpassing the high rate of adverse reactions caused by traditional immunotherapeutics based on whole proteins or extracts of allergenic sources [9]. Synthetic short peptides lack the primary and tertiary structures of the native protein to cross-link IgE, but retain the ability to stimulate T cells. The efficacy of T-cell epitope-based peptide immunotherapy (IT) has been proven in both bee venom [10] and cat dander allergies [11, 12]. Administration of synthetic peptides that contain the immuno-regulatory epitopes of Fel d 1, the major allergen in cat allergy, has been shown to induce both short-term [12] and long-term [11] beneficial effects (i.e., clinically relevant reduction in rhinoconjunctivitis symptoms).

In food allergy, the T-cell response toward several food allergens has been studied [5, 13]. Multiple T-cell epitopes have been described for a broad range of food allergens, from milk [14], egg [15], celery [16], peanut [17], peach [18–20], beef [21], shrimp [22] to Brazil nuts [5, 13]. The role of T-cell epitopes in cross-reactivity issues, such as the birch pollen associated food allergy, has been demonstrated [16]. Bet v 1₁₄₂₋₁₅₂, the dominant T-cell epitope of birch pollen allergen, is important for the cross-reactivity with Bet v 1 related food allergens [23]. Nevertheless, evidence for a relevant T-cell epitope not cross reactive with homologous pollen allergens has been also demonstrated for the hazelnut allergen Cor a 1.04, a Bet v 1 homologue protein [24]. These findings suggest that T cells specific for food epitopes but nonreactive to pollen do exist which is suggestive of the ineffectiveness of pollen-specific IT to induce tolerance on concomitant food allergy [25].

Several allergenic proteins in peanut have been identified including Ara h 1, Ara h 2, Ara h 3, Ara h 6, Ara h 8, and Ara h 9. Most patients are sensitized to Ara h 1, Ara h 2, or Ara h 3 [26]. One of the major obstacles in identification of T-cell epitopes is the low frequency of allergen peptide-specific CD4⁺ T cells in the circulation, even in sensitized individuals. It has been estimated that the frequency of Ara h 1 specific T cells in a sensitized individual will be around 9 cells per million [27]. Ara h 1 epitopes had been mapped in two previous studies, assaying peptide pools in 12 and 22 patients respectively [27, 28], and using two different approaches: tetramer-guided epitope mapping and CFSE-based proliferation assays using T-cell clones generated from allergic individuals. These methods have high specificity, but low sensitivity, thus requiring external validation with patient samples at the end [29]. The amino acid sequence of Ara h 1 is more than twice that of Ara h 2 and this limits experimental assays with/using overlapping peptides for the entire length of the protein in human blood samples. We have recently utilized an in silico epitope discovery algorithm (NetMHCIIpan [30, 31]) combined with an in vitro HLA binding assay to prospectively preselect Ara h 1 and 2 peptides that can be evaluated by T-cell stimulation assays [32, 33]. This approach has been also successfully used in other food allergens such as shrimp [22].

Here, we provide the T-cell proliferation assay methodology for the determination of immunodominant T-cell epitopes. This assay is based on the principle that soluble short synthetic peptides from allergens will be taken up by the antigen presenting cells, loaded onto MHC class II compartments and presented to CD4⁺ T cells. Only the peptide-specific T cells will be able to interact with the peptide-MHC class II complex leading to proliferation and clonal expansion (Fig. 1). The proliferating T cells can be quantified by tritiated thymidine incorporation followed by DNA harvesting and radioactive scintillation. In this assay, tritiated

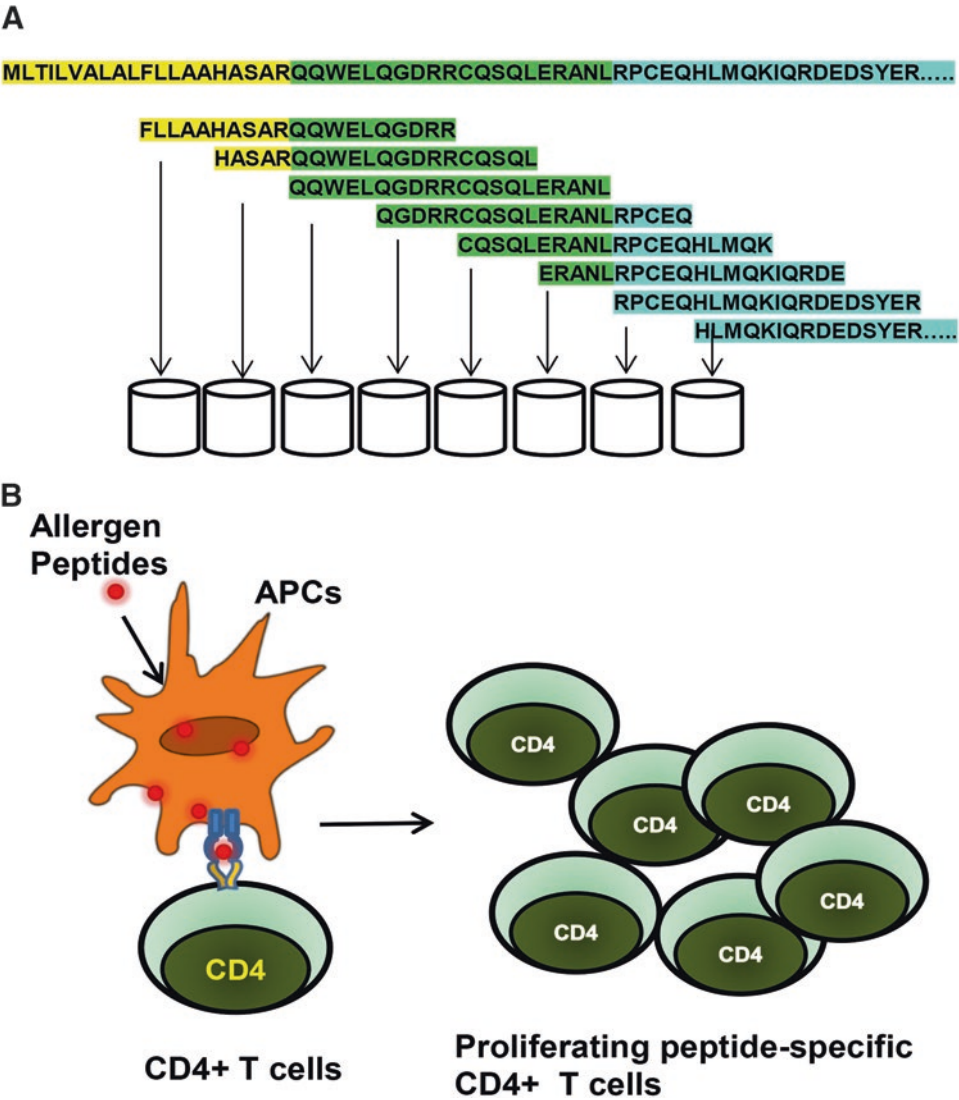


Fig. 1 Schematic representation of T-cell epitope discovery. (a) Overlapping peptides of candidate antigen are generated and plated in multi-well tissue culture plate. (b) Peripheral blood mononuclear cells from allergic subjects were isolated and cultured for 5–7 days. The proliferating T cells were quantified by either tritiated thymidine incorporation or CFSE dilution

thymidine, a radioactive nucleoside, is added during in vitro culture of PBMCs with allergenic peptides. During peptide-specific proliferation of T cells, thymidine is incorporated in the newly synthesized strands of chromosomal DNA. The incorporated radioactivity is measured using a scintillation beta-counter to determine the extent of cell division.

Thymidine incorporation assays have been widely used methodology for the detection of antigen-specific T-cell proliferation. Recent development of flow cytometry-based assays had largely

replaced thymidine incorporation assays due to the versatility of simultaneous detection of multiple markers and other phenotypic characterization of T cells. However, in conditions where the frequency of individual peptide-specific T cells is very low (as in most food allergies) [27], the utilization of flow cytometry (*see Note 1: Alternate methodologies*) is limited because of poor resolution of proliferating T cells. In such cases, a large number of PBMCs are required and must be obtained by leukapheresis [34]. The detection of incorporated radioactive (tritiated) thymidine still proves to be sensitive in this case as it requires relatively less volume of blood compared to flow cytometry.

2 Materials

1. Heparinized whole blood.
2. Ficoll Paque Plus.
3. Serum-free AIM-V medium (store at 4 °C in the dark).
4. Sterile phosphate-buffered saline (PBS).
5. Allergen extracts (Final concentrations: 50 and 100 µg/mL).
6. Allergen peptides (Final concentrations ~20 µg/mL) (*see Note 2*).
7. Anti-CD3-CD28 beads (Dynabeads® Human T-activator CD3/CD28, Life Technologies).
8. 96-U-well plates.
9. Thymidine, [Methyl-3H] (Perkin Elmer) (*see Note 3*).
10. Glass fiber filters.
11. DNA harvestor (Tomtech).
12. Scintillation liquid (Scintisafe 30% cocktail, Fisher Scientific).
13. Scintillation counter (Wallach, Perkin Elmer).
14. Genomic DNA extraction kit (QIAamp DNA Mini Kit—Qiagen).
15. LIFECODES HLA-DRB1*/-DQB/-DPB Typing Kits for use with Luminex (Immuncor Trasplant Diagnostics Inc).
16. Luminex xMAP™ Technology equipment.
17. Thermocycler.

3 Methods

3.1 Experimental Procedure

1. *Blood collection:* Whole blood should be obtained with sodium heparin collection tubes. Preferably, the assay should be performed within 24 h of blood collection as prolonged storage results in low sensitivities.

2. *Isolation of peripheral blood mononuclear cells (PBMC):* Aseptically transfer the blood from each subject into a 50 mL sterile tube. Dilute blood with twice the original volume with sterile PBS. Mix gently. Carefully layer of blood on the top of 15 mL Ficoll in a 50 mL tube. Centrifuge $800 \times g$ for 20 min at 20 °C with no acceleration and with breaks off. Isolate the PBMC layer with a sterile transfer pipette and transfer it to a new 50 mL sterile tube. Resuspend PBMC with sterile PBS (up to 50 mL) and centrifuge $500 \times g$ for 15 min at room temperature, with maximum acceleration and with breaks on. Aspirate the supernatant with a sterile pipette and disturb the pellet by gently tapping the tube. Add 1 mL sterile AIM-V medium. Make sure the pellet is completely resuspended and perform cell count.
3. *Peptide and stimulant plate preparation:* Aliquot peptides in triplicates on a 96-well plate at desired concentration. Leave sufficient empty wells in between to prevent cross-contamination. Include the following control wells: Positive controls: whole allergen extracts (at least two different concentrations); anti-CD3/CD28 beads (T-cell expander) (0.5 μ L/well). Negative controls: Medium-only control (no peptides or allergens); Nonspecific allergen extract control. For peanut whole extract stimulation use a 50 or 100 μ g/mL final concentration.
4. *Peptide-PBMC culture:* Plate 200 μ L of PBMCs (0.5 to 1 million cells) to each well of the peptide and control stimulants. Culture at 37 °C in a humidified 5% CO₂ incubator for 4 days.
5. On day 4 of culture, in the hood, gently resuspend the cells resting at the bottom of the well with multi-channel pipette. Centrifuge the plate at $300 \times g$ for 7 min at room temperature with the centrifuge with maximum acceleration and break on. Aseptically transfer 100 μ L of the supernatant to another 96-round bottom well plate stored at -80 °C for further use (for cytokine analysis). Add 100 μ L of AIM-V to each well and gently resuspend the cell pellet.
6. Add 20 μ L (10 μ Ci) of 3H-Thymidine (diluted working solution) to each well. (Tritiated thymidine working stock: 250 μ L stock 3H-Thymidine (1 mCi/mL) + 4750 μ L AIM-V sterile medium (final volume 5 mL). Culture for another 48 h at 37 °C in a humidified 5% CO₂ incubator.
7. On day 6 of culture, extract DNA from the cells onto a glass fiber filter using DNA harvester. Follow the manufacturer's recommended procedure.
8. Dry the filter paper under infra-red lamp, place it in appropriately sized plastic bag, add scintillation liquid gently to completely wet the filter (do not add excess fluid); remove the extra liquid

by pressing or rolling a cylinder on the surface. Seal all the sides of the filter mat and cut the sides to fit the scintillation cassette.

9. Obtain scintillation counts (counts-per-minute, CPM) on a scintillation counter by following manufacturers' procedures.
10. HLA-Typing: Isolate genomic DNA from venous blood. Perform HLA typing of loci DRB1, DPB1, and DQB1 with the PCR-SSO technique, in which a locus-specific DNA amplification is done using the polymerase chain reaction technique (PCR) and sequence-specific oligonucleotides (SSO) and the automatized system with Luminex® technology following manufacturers' procedure [35].

3.2 Data Analysis

1. The average CPMs for each peptide, whole allergen extracts, and anti-CD3/CD28 beads culture wells are compared with those containing medium alone (unstimulated cells). Calculate within subject variability of CPMs and estimate per-individual coefficient of variation (CV). Calculate stimulation index for each peptide.
2. *Stimulation Index (SI)*: CPM of peptide/antigen-stimulated T cells (test) divided by CPM of unstimulated cells (negative control). If SI exceeds the estimated maximum coefficient of variation [$CV = \text{Standard Deviation } (\sigma) / \text{Mean } (\mu)$] ($>1+CV_{\max}$), the T-cell responses are considered positive. SI that equals or less than estimated maximum CV ($>1 + CV_{\max}$) is considered negligible. Peptides inducing positive proliferative responses ($SI \geq 1.5$) in at least 25% of the recruited subjects were considered significant.
3. *Population Coverage*: One of the major pitfalls in the characterization of immunodominant peptides for food allergens is the diversity of MHC class II molecules (HLA-DR, -DQ, and -DP). Given the extensive polymorphisms of these molecules, a single T-cell epitope may or may not bind a specific MHC class II molecule. In other cases, the peptide can bind more than one MHC molecule. Therefore, for clinical applications, any meaningful characterization of food allergen T-cell epitopes must recognize the MHC diversity and must include appropriate population coverage. This calculation can be done when the HLA genotype frequencies and the existing MHC/peptide recognition patterns are known utilizing tools available at the immuno epitope database (www.icdb.org) [36].

4 Notes

1. *Alternate method to investigate peptide-specific T-cell responses*: Carboxyfluorescein succinimidyl ester (CFSE) dilution assay can be used as an alternative to H^3 -thymidine incorporation

assay (provided the frequency of peptide-specific T cells is high and detectable). T-cell proliferation assay using CFSE dilution followed by flow cytometry [37] has been described for peanut allergy [38]. CFSE (or its analogues such as Oregon Green (OG), SNARF-1, etc.) [39] is a membrane-permeable dye that binds to cytoplasmic proteins. Following each cell division, the intensity of CFSE fluorescence is roughly halved in daughter cells. Thus, CFSE^{low} cells represent cells that are proliferating. The advantage of this methodology is the use of nonradioactive reagents for the experimentation. In addition, it is possible to further characterize the functional subsets of T cells (such as Th2, Treg, etc.) and to follow the changes in T-cell phenotype over a course of time. Other methods to quantify T-cell proliferation include flow cytometric detection of thymidine analog BrdU (5-bromo-2'-deoxyuridine) following its incorporation into newly synthesized DNA [40], or by the expression of proliferation marker Ki-67 [41] in dividing cells.

2. *Peptide solubility and solvent issues*: The success of peptide proliferation assays is very much dependent on the purity and solubility of the peptides tested. Peptides with 90% purity are recommended for proliferation assays. It is advisable to test the solubility of peptides in aqueous solution in physiological pH prior to the assay. Peptide sequences that contain many hydrophobic residues tend to aggregate and are not generally soluble in aqueous solvents. Organic solvents such as dimethylsulfoxide (DMSO), acetonitrile, or dimethylformamide (DMF) are generally used to dissolve peptide sequences containing more than 50% hydrophobic residues. In general for in vitro cellular assays, DMSO is usually well tolerated up to 0.1% final concentration (v/v). Higher concentrations will affect proliferation and ultimately the quality of the data. DMSO concentrations above 1% (v/v) are toxic to cells. For peptides that require organic solvents, control assay conditions using appropriate dilution of solvent (without the peptide) must be incorporated.
3. Working stocks of peptides in 96-well plates can be prepared in advance and stored at -80°C . Long-term storage of aqueous solution of peptides at 4°C is not recommended.
4. The culture conditions need to be standardized and carefully monitored to prevent overgrowth. Excessive number of cells will deplete the nutrients in the medium, and lower pH (medium becomes yellow) resulting in cell death. Scintillation counts should not be taken in overgrown cells. Ideally, the cells should be in the log phase of proliferation to obtain reliable counts.
5. Sterile working stocks of tritiated thymidine can be prepared and stored at 4°C by diluting in culture medium.

6. Cells supernatants for cytokine estimation (if needed) must be collected prior to addition of radioactive thymidine.
7. Adequate precautions must be taken for handling and disposal of radioactive substances following regulations of local laboratories and government agencies.

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