



Chapter 21

T-Cell Epitope Immunotherapy in Mouse Models of Food Allergy

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Abstract

Food allergy has been rising in prevalence over the last two decades, affecting more than 10% of the world population. Current management of IgE-mediated food allergy relies on avoidance and rescue medications; research into treatments that are safer and providing guaranteed and durable curative effects is, therefore, essential. T-cell epitope-based immunotherapy holds the potential for modulating food allergic responses without IgE cross-linking. In this chapter, we describe the methods in evaluating the therapeutic capacities of immunodominant T-cell epitopes in animal models of food allergy. Moreover, we explain in detail the methods to measure the allergen-specific antibody levels, prepare single-cell suspension from spleen, and prepare small intestine for immunohistochemical analysis of eosinophils and Foxp3⁺ cells.

Key words Shellfish allergy, Egg allergy, Cow's milk allergy, Oral immunotherapy (OIT), Mouse model, ELISA, Immunohistochemical staining (IHC), Swiss roll, Eosinophils, Regulatory T cell (Treg)

1 Introduction

Food allergy is an immune-mediated hypersensitivity resulted from the failure to develop or the abrogation of oral tolerance against food proteins that are harmless to most of the population. Most cases of food allergy are IgE-mediated, by which type 2 immune response activated by the culprit allergen triggers the release of allergen-specific IgE that cross-link the allergenic proteins on mast cells or basophils, leading to the release of inflammatory mediators such as histamine, prostaglandins, and leukotrienes. These events result in immediate hypersensitivity symptoms involving a single or multiple organs, and they vary from mild reactions at skin, respiratory, and gastrointestinal tracts to life-threatening anaphylaxis such as hypovolemic shock and deterioration in respiratory functions.

Food allergy poses substantial burden on global health affecting approximately 8–10% of children and adults, respectively, based

on self-reports and approximately 3% adults and children when diagnosed by oral food challenges [1, 2]. The prevalence of food allergy has doubled over the past two decades and has loomed as a “second wave” of allergy epidemic after the rise in prevalence of asthma and allergic rhinitis [3]. Although the prevalent allergens vary with cultural and geographical factors, shellfish, peanut, cow’s milk, and egg often top the lists worldwide [4, 5].

Despite the high impact, treatment for food allergy has remained unchanged for decades; strict avoidance and rescue medication with antihistamines and epinephrine autoinjector are the mainstay “treatments.” Allergen-specific immunotherapy (AIT) deploying standardized food extracts or unmodified allergens is by far the only disease-modifying option. AIT is highly effective against aeroallergens, but frequent adverse events, low patient adherence, and lack of evidence in developing durable immunological and clinical tolerance remain the major obstacles in food allergy AIT [6]. In this regard, immunotherapy using allergen-specific peptides has been shown to be safe and effective. These peptides are essentially small in size without secondary or tertiary structure, making them incapable of cross-linking IgE on the effector cells. Thus far, immunodominant T-cell epitopes of major food allergens such as peanut Ara h 1 and 2, ovalbumin (OVA) and ovomucoid (Ovm), β -lactoglobulin (BLG), and shrimp tropomyosin have been identified [7–14]. Administration of the T-cell epitopes has been shown to suppress anaphylactic and/or diarrhea scores, decrease allergen-specific IgE levels, increase the inhibitory IgG_{2a} antibodies, reduce Th2 cytokine responses, and increase Th1 and regulatory T-cell responses such as the expression of intestinal *FOXP3* and *TGF- β* in animal models of food allergy [13, 15, 16]. Whereas the therapeutic efficacy of oral peptide immunotherapy was superior to subcutaneous treatment [17], peptide-based treatment for food allergy still warrants further optimization and in-depth characterization of the mechanisms of desensitization involved.

In this chapter, we detail how the therapeutic efficacies of T-cell epitope immunotherapy can be evaluated in mouse models of different food allergies, including shellfish, cow’s milk, and egg allergies. Based on our laboratory experiences, we also provide protocols of the most important efficacy assessments for translational studies. These include methods of immediate allergic responses assessment, blood sampling, serum allergen-specific IgE and IgG analyses, and preparation of mouse splenocytes for flow cytometry and cytokine level measurements, as well as detailed methodologies in preparing the small intestine for immunohistochemical (IHC) staining of eosinophils and Foxp3+ T cells.

2 Materials

2.1 Allergen Sensitization and T-Cell Epitope Immunotherapy

1. Female BALB/c mice: 3–5 weeks old.
2. Culprit allergen: recombinant shrimp tropomyosin (purified rMet c 1), OVA, Ovm, or BLG.
3. Cholera toxin: from *Vibrio cholera*, azide-free.
4. Aluminum hydroxide: Al(OH)₃, Imject[®] Alum. An aqueous solution of 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide plus inactive stabilizers.
5. T-cell epitope peptides: 15–22 mer, >95% purity. Purified by high-performance liquid chromatography (HPLC) and validated by mass spectrometry. Reconstitute individual peptides in ultrapure water at 1 µg/µL concentration, aliquot, and store at –20 °C until use. Avoid repeated freeze-thaw cycles (*see Note 1*).
6. Sterile phosphate-buffered saline (PBS): 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4. Dissolve 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, 8.0 g NaCl, and 0.2 g KCl in 800–900 mL of ultrapure water. Adjust the pH to 7.4 with HCl and add water to make the final volume of 1 L. Sterilize by using a 0.25-µm filter unit or autoclaving.
7. 1-mL Syringe with a 22-gauge stainless gavage needle.
8. 1.5-mL Microfuge tubes.
9. 1-mL Syringe with a 23-gauge needle.

2.2 Allergen Challenge

1. 1-mL Syringe without needle.
2. 22-Gauge stainless steel gavage needle.
3. Microprobe thermometer with a RET-3 mouse-specific rectal probe.
4. Restrainer.
5. Warming chamber.
6. 23-Gauge syringe needle.
7. Capillary tube.
8. 1.5-mL Microfuge tubes.

2.3 ELISA for Allergen-Specific Antibodies

1. 96-Well flat-bottom enzyme-linked immunosorbent assay (ELISA) plates.
2. Coating buffer: 50 mM Na₂CO₃, 130 mM NaHCO₃, 3 mM NaH₃. Dissolve 5.3 g Na₂CO₃, 10.9 g NaHCO₃, and 1.953 g NaH₃ in 1 L of ultrapure water. Store at 4 °C.
3. PBS-T: 0.05% (v/v) Tween-20 in PBS. Add 0.5 mL Tween-20 in 1 L PBS, pH 7.4.

4. Blocking buffer: 5% fetal bovine serum (FBS) in PBS. Add 2.5 mL of FBS in 47.5 mL of PBS.
5. Mouse serum: Dilute 1:10 in the blocking buffer.
6. Secondary antibody: biotin-labeled goat anti-mouse IgE, IgG₁, and IgG_{2a} antibodies. Dilute 1:1000 in the blocking buffer.
7. Horseradish peroxidase-labeled avidin D (Av-HRP): Dilute 1:1000 in the blocking buffer.
8. TMB substrate solution set: 3,3',5,5'-Tetramethylbenzidine (TMB). Commercially available.
9. Stopping solution: 2 N H₂SO₄. Dissolve 55.6 mL of 18 M H₂SO₄ in 944.4 mL of ultrapure water.
10. Plate reader: for colorimetric quantitation.

2.4 Mouse Splenocytes Preparation

1. 70% Ethanol: use for sterilizing the tools and mouse fur during dissection.
2. Dulbecco's Modified Eagle Medium (DMEM), high glucose.
3. Complete culture medium: 10% FBS in DMEM. Add 50 mL of FBS and 500 µL of penicillin–streptomycin (10,000 U/mL) in 500 mL of DMEM, high glucose.
4. Red blood cell depletion reagent: 0.85% NH₄Cl. Dissolve 0.85 g NH₄Cl in 100 mL of ultrapure water.
5. 0.4% (w/v) Trypan blue solution: commercially available as a ready-to-use solution.
6. Dissection board.
7. Sterile forceps and scissors: autoclaved. Keep in a sterile beaker with 70% ethanol.
8. 5-mL Syringe plunger.
9. 40-µm Nylon cell strainer.
10. 60-mm Tissue culture dishes.
11. 24-Well tissue culture plates.
12. 50-mL Centrifuge tubes.
13. 1.5-mL Microfuge tubes.

2.5 Intestinal Preparation for Histological Analysis

1. 70% Ethanol.
2. PBS: pH 7.4, ice-cold.
3. Fixative: 4% paraformaldehyde (PFA). Dissolve 4 g of paraformaldehyde in 100 mL of PBS, pH 7.4. Heat at 56 °C to dissolve completely. Sterile filter through 0.4 µm polyethersulfone (PES) membrane (*see Note 2*).
4. Dissection board.

5. Sterile forceps and scissors: autoclaved. Keep in a sterile beaker with 70% ethanol.
6. Petri dish.
7. 10-mL Syringe.
8. 20-Gauge stainless steel gavage needle.
9. 15-mL Centrifuge tubes.
10. Cardboard.
11. Toothpicks.
12. Embedding cassettes.
13. Adhesive microscope slides: positively charged.

2.6 Immuno-histochemical Staining for Eosinophils and Foxp3+ Cells

1. Xylene: Prepare two baths of xylene for deparaffinization of tissue sections.
2. Ethanol: Prepare 100%, 95%, 70%, 50%, and 30% ethanol solutions for rehydration of tissue sections.
3. Tris-EDTA antigen retrieval buffer: 5% Tris, 1.5% 2-butoxyethanol, and 5% sodium ethylenediaminetetraacetic acid (EDTA), pH 9.0.
4. 1-L Glass beaker.
5. Microwave oven and/or hotplate.
6. Hydrophobic barrier pen for histology.
7. Hydrogen peroxide solution for eosinophil staining: 3% hydrogen peroxide in PBS.
8. Hydrogen peroxide solution for Foxp3+ cell staining: 0.3% hydrogen peroxide in absolute methanol.
9. Pepsin solution: 0.25% pepsin with 0.1% sodium azide as preservative in Tris buffer, pH 2.0. Commercially available.
10. PBS: pH 7.4.
11. Blocking buffer: 10% (v/v) goat serum in PBS, pH 7.4. Add 1 mL of goat serum in 9 mL of PBS. Store at 4 °C.
12. 0.1% (v/v) Triton-X 100: Prepare in PBS, pH 7.4. Dissolve 100 µL of Triton-X 100 in 100 mL of PBS. Store at room temperature.
13. Rat anti-mouse major basic protein (MBP) antibody. Dilute 1:500 to 1:15,000 in the blocking buffer for eosinophil staining.
14. Rat anti-mouse Foxp3 antibody. Dilute 1:100 in PBS with 0.1% Triton-X 100.
15. HRP-conjugated anti-rat IgG antibody.
16. Diaminobenzidine (DAB) chromogen substrate kit: commercially available.

17. Hematoxylin Gill #3: commercially available.
18. Mounting medium: Permount or equivalent.
19. Coplin jars.
20. Moisture chamber/slide tray: used for preventing evaporation of antibody solutions during incubation.
21. Glass coverslips.

3 Methods

3.1 Allergen Sensitization

1. Prepare the sensitization allergen and adjuvant. For intragastric sensitization, mix 100 μg (recombinant shrimp tropomyosin) or 1 mg (OVA and Ovm) allergen with 10 μg cholera toxin in 400 μL of sterile PBS per mouse (*see Note 3*).
2. For intraperitoneal sensitization, prepare 50 μg (BLG) or 100 μg (recombinant shrimp tropomyosin) allergen per mouse adsorbed in 1–2 mg of $\text{Al}(\text{OH})_3$ by adding thoroughly vortexed adjuvant solution into the allergen solution drop by drop. The final total volume should be 200–300 μL per mouse (*see Note 3*).
3. Randomly house the mice in cages (*see Note 4*). Using a 1-mL syringe and 22-gauge stainless steel gavage needle, sensitize the animals by orally administering the allergen solution prepared in **step 1** above at weekly intervals for 4 weeks as illustrated in Fig. 1.
4. Alternatively, use a 1-mL syringe with a 23-gauge needle to sensitize the animals by intraperitoneally administering the allergen solution prepared in **step 2** above at weekly intervals for 3 weeks as illustrated in Fig. 1.

3.2 T-Cell Epitope Treatment

1. Randomly assign the animals sensitized in Subheading 3.1 into the positive control and treatment groups with 6–8 animals per experimental group. Validating allergen-specific IgE levels prior to immunotherapy is recommended (*see Note 5*).
2. For single peptide immunotherapy, administer 1 mg of single peptide per mouse. For cocktail peptide immunotherapy, first prepare a mixture of peptides (3–6 peptides, each at 0.2–1 mg) before administering to mice.
3. Treat mice with the peptide(s) 2–3 times a week for 3–4 weeks intragastrically or subcutaneously as described below (also *see Fig. 1*).
 - (a) For shrimp allergy: administer 1.2 mg cocktail peptides via intragastric gavage.
 - (b) For egg white allergy (Ovm): administer 1 mg single peptide or 3 mg cocktail peptides via intragastric gavage.

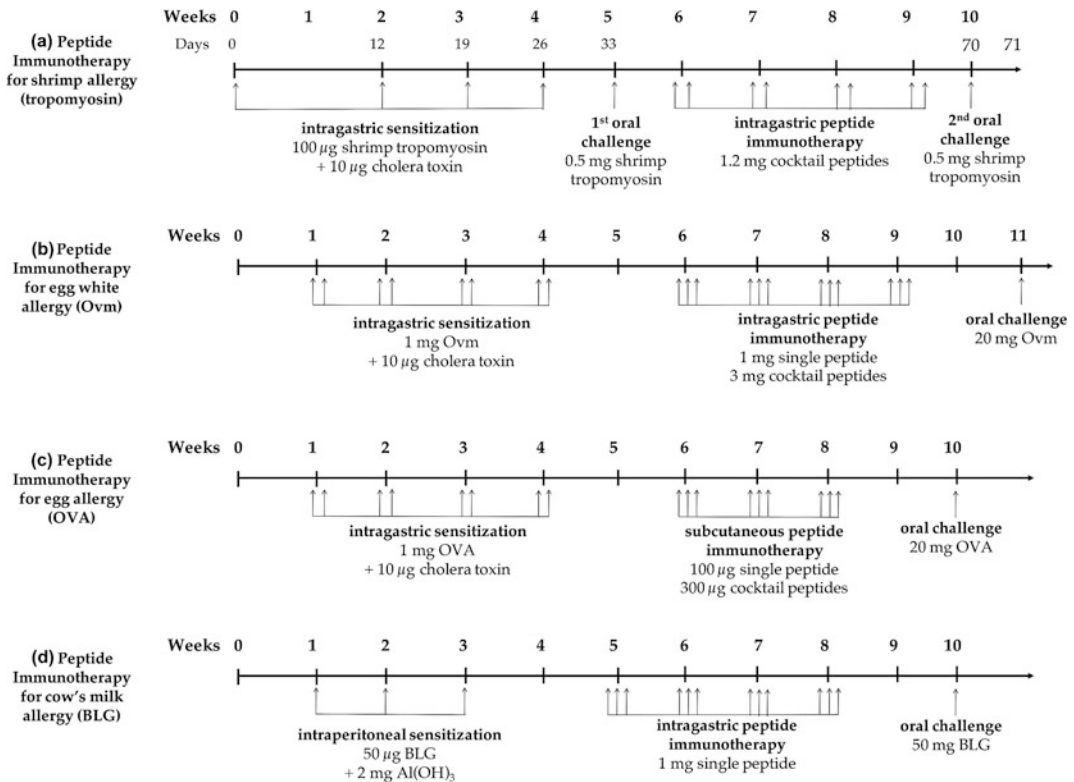


Fig. 1 Regimen designs for T-cell peptide immunotherapy of food allergy. **(a)** Peptide therapy for shrimp tropomyosin allergy [13]. BALB/c mice are sensitized with 100 μ g recombinant shrimp tropomyosin with 10 μ g cholera toxin (CT) on Days 0, 12, 19, and 26 followed by an oral challenge with 0.5 mg tropomyosin on Day 33. Mice are then intragastrically treated with a mix of six T-cell peptides, each at 0.2 mg (total = 1.2 mg), twice a week for 4 weeks. A second 0.5 mg tropomyosin oral challenge is given on Day 70 (1 week after last treatment). **(b)** Peptide treatment for egg white allergy [16]. BALB/c mice are sensitized with 1 mg ovomucoid (Ovm) and 10 μ g CT twice a week for 4 weeks. One week after the last sensitization, mice are intragastrically treated with either 1 mg single peptide or 3 mg cocktail peptides three times a week for 4 weeks. An oral challenge with 20 mg Ovm is given 2 weeks after the last treatment. **(c)** Peptide treatment for egg allergy [15]. BALB/c mice are sensitized with 1 mg ovalbumin (OVA) and 10 μ g CT twice a week for 4 weeks. One week after the last sensitization, mice are subcutaneously treated with 100 μ g single peptide or 300 μ g cocktail peptides three times a week for 3 weeks. An oral challenge with 20 mg OVA is given 2 weeks after the last treatment. **(d)** Peptide treatment for cow's milk allergy [17]. BALB/c mice are intraperitoneally sensitized with 3 weekly doses of 50 μ g β -lactoglobulin (BLG) and 2 mg Al(OH)₃. Single peptide at 1 mg is then given intragastrically three times a week for 4 weeks. A 50 mg BLG oral challenge is finally given 2 weeks after the last treatment

- (c) For egg white allergy (OVA): administer 100 μ g single peptide or 300 μ g cocktail peptides via subcutaneous injection.
- (d) For cow's milk allergy (BLG): administer 1 mg single peptide via intragastric gavage.

3.3 Allergen Challenges

Allergen challenge can be introduced to sensitized mice 1 week after the last sensitization and 1–2 weeks after the last peptide treatment (*see* **Note 6**). General protocols only involve challenging the animals after the peptide treatment and comparing the allergic parameters among different experimental groups to assess the therapeutic effects of the T-cell epitopes (*see* **Note 7**).

3.3.1 Preparation and Administration of Culprit Allergens

1. Fast the animals overnight for 16–18 h before allergen challenge. Water is allowed.
2. Prepare the challenge solution by reconstituting the appropriate culprit allergen in PBS. For each mouse, use 500 µg recombinant shrimp tropomyosin, 20 mg OVA, 20 mg Ovm, or 50 mg BLG in sterile PBS. The total volume should not exceed 600 µL per mouse (*see* **Note 3**).
3. On the day of challenge, record the rectal temperature using a microprobe thermometer before allergen challenge, and then intragastrically deliver the culprit allergen with a 1-mL syringe and a 22-gauge stainless steel gavage needle.
4. Return the mice to their home cage for observation and proceed to Subheading 3.3.2.

3.3.2 Assessment of Immediate Allergic Responses

1. Record the rectal temperature at 15, 30, and 45 min postchallenge (*see* **Note 8**).
2. Observe and record the immediate hypersensitivity symptoms within 40 min postchallenge using a scoring system listed in Table 1 [18]. Scoring should be performed by an independent investigator in a blinded manner (*see* **Note 9**).
3. Observe and record the condition of feces for signs of diarrhea using a scoring system listed in Table 2 [19]. Scoring should be performed by an independent investigator in a blinded manner (*see* **Note 9**).

3.3.3 Tail Vein Blood Sampling

Blood sampling is recommended at 24 h postchallenge for maximum allergen-specific IgE titer.

1. Keep the mouse inside a restrainer and apply local anesthetic cream on the tail surface 30 min before blood sampling.
2. Warm the animal in a warming chamber until the tail vein becomes visible.
3. Insert a 23-gauge needle into the blood vessel and use a capillary tube to collect blood into a 1.5-mL microfuge tube.
4. Put gentle pressure on the bleeding spot upon completion of blood collection.
5. Centrifuge the whole blood samples at $10,000 \times g$ for 20 min at room temperature to separate the serum portion. Store serum samples at -20°C until analyses.

Table 1
Scoring system of systemic allergic responses

Score	Symptoms
0	No symptom
1	Scratching and rubbing around the nose, head, and tail
2	Puffiness around eyes and/or mouth; reduced activity
3	Labor respiration
4	No activity after prodding; tremor; convulsion
5	Death

Table 2
Scoring system of fecal condition

Score	Fecal condition
0	Hard feces
1	Soft feces
2	Liquid feces
3	White, mucus-like feces

3.4 ELISA for Allergen-Specific Antibodies

1. Prepare a culprit allergen in the coating buffer at 5 µg/mL concentration.
2. Coat a 96-well plate with 100 µL/well of the diluted allergen overnight at 4 °C (*see Note 10*).
3. Wash plate with PBS-T twice (*see Note 11*).
4. Block the plate with 200 µL/well of the blocking buffer for 2 h at room temperature.
5. Wash plate with PBS-T once.
6. Prepare serum samples in duplicate and add 100 µL of diluted serum samples to each well. For measuring the levels of IgE, IgG₁, and IgG_{2a} antibodies, prepare dilutions at 1:10, 1:10,000, and 1:200 with the blocking buffer, respectively (*see Note 12*). Incubate the plate overnight at 4 °C.
7. Wash plate with PBS-T for three times (*see Note 11*).
8. Incubate the plate with 100 µL/well of the diluted secondary antibody for 1 h at room temperature (*see Note 12*).
9. Wash plate with PBS-T for four times (*see Note 11*).
10. Incubate the plate with 100 µL/well of the diluted Av-HRP for 30 min at room temperature in the dark.

11. Wash plate with PBS-T for five times and PBS once (*see Note 11*).
12. Allow TMB substrate to reach room temperature before use and add 100 μL /well of TMB substrate for signal development; protect the plate from light.
13. After sufficient color development, stop the signal reaction by adding 50 μL /well of 2 N H_2SO_4 (*see Note 13*).
14. Measure the absorbance of each well (Optical Density at 450 nm) with a plate reader (*see Note 14*).

3.5 Mouse Splenocytes Preparation

1. At the end of the experiment, euthanize mice according to a method approved by the Institutional Animal Care and Use Committee of your institution.
2. Place a sacrificed mouse on a clean dissection board and rinse the mouse with 70% ethanol to sanitize the fur (*see Note 15*). Dissect and remove spleen (*see Note 16*). Place the spleen in a 60-mm tissue culture dish with 3 mL of the complete culture medium.
3. Place a 40- μm nylon cell strainer in a new 60-mm tissue culture dish with 5 mL of DMEM. Place the spleen into the cell strainer and press the spleen gently using the seal of a sterile 5-mL syringe plunger until only the connective tissues are left behind (*see Note 17*).
4. Transfer the spleen cells with all medium to a 50-mL centrifuge tube.
5. Add 10 mL of DMEM (without any supplements) to the same culture dish. Pipette up and down to remove any residual spleen cells (*see Note 18*). Transfer the medium to the same 50-mL centrifuge tube.
6. Centrifuge the spleen cells at $250 \times g$ for 5 min at room temperature. Discard supernatant using pipettes.
7. Add 5 mL of the red blood cell depletion reagent to the spleen cells. Pipette up and down to resuspend the cells (*see Note 18*). Slowly add the resuspended spleen cells to 1 mL of FBS.
8. Centrifuge at $250 \times g$ for 5 min at room temperature. Discard supernatant using pipettes.
9. Wash spleen cells by resuspending in 10 mL of unsupplemented DMEM (*see Note 18*). Centrifuge at $250 \times g$ for 5 min at room temperature. Discard supernatant using pipettes. Repeat this step twice.
10. Resuspend the spleen cells in 5 mL of the complete culture medium. Take 200 μL of cells into a new microfuge tube and add 300 μL DMEM and 500 μL 0.4% trypan blue solution (dilution factor = 5). Apply the mixture to a hemocytometer for counting the number of viable cells (*see Note 19*).

11. For flow cytometric analysis, resuspend the cells at a density of 1×10^6 to 1×10^8 cells/mL in an appropriate staining buffer and proceed to cell staining immediately.
12. For cytokine analysis, resuspend the cells at a density of 5×10^6 cells/mL in the complete culture medium. Place 1 mL of the cell suspension in each well and culture the cells in a 24-well tissue culture plate with 50–100 μ g of the culprit allergen.
13. After culturing for 72 h at 37 °C in a 5% CO₂ incubator, harvest the cells in microfuge tubes. Centrifuge at $250 \times g$ for 5 min at room temperature. Collect the supernatant in microfuge tubes and store at –20 °C until analysis.
14. Quantify the levels of target cytokine(s) using commercial mouse cytokine ELISA kits using the harvested cell supernatants.

3.6 Intestinal Preparation for Histological Analysis

1. Place the sacrificed mouse on a clean dissection board and rinse the mouse with 70% ethanol. Dissect and identify the stomach to trace its junction with the small intestine.
2. Carefully make an incision at the junction and gently isolate the small intestine from the mesenteric membrane. Make another incision at junction with the cecum and transfer the entire small intestine from the abdomen to a clean Petri dish containing ice-cold PBS.
3. Divide the small intestine into three segments by locating the duodenum, jejunum, and ileum.
4. Fill a 30-mL syringe with ice-cold PBS and attach a gavage needle to the syringe. Flush the contents of each intestinal segment with ice-cold PBS.
5. Fill a new 10-mL syringe with ice-cold 4% PFA and attach a gavage needle to it. Gently fill each intestinal segment for immediate fixation (*see Note 20*).
6. Transfer each segment into a 15-mL centrifuge tube. Fill each tube full of 4% PFA. Allow fixation for 4 h at 4 °C (*see Note 21*).
7. Transfer the intestinal tissue on a cardboard and orient each intestinal segment such that the proximal (stomach) end is closest to the operator. Then, use a pair of forceps to hold one end of the small intestine with gentle tension, and with another, use scissors to cut open the intestinal segment longitudinally in a straight line along the mesenteric line (*see Note 22*).
8. Keep the luminal side of the intestinal segment facing upward and the segment flat open on the cardboard.

9. Hold the distal (cecum) end with a pair of forceps and wrap the edge of the distal end around a toothpick held with another hand. Gently roll the toothpick with fingers to wrap the intestinal segment around the toothpick to form a “Swiss roll” (*see Note 23*). Be sure to overlay each successive rolled layer so that the edges are flush. Avoid rolling the intestinal tissues too tightly as this will lead to compression of the villi and degrade the morphological appearance.
10. Upon rolling up the entire intestinal segment, slide the Swiss roll off the toothpick gently with a pair of forceps (*see Note 24*). Place the Swiss roll in the original 15-mL centrifuge tube filled with 4% PFA overnight at 4 °C for fixation (*see Note 25*).
11. On the next day, transfer the fixed tissue to a clean Petri dish and rinse the tissue with ice-cold PBS until the fixative is completely removed. Place the cleaned tissue in an embedding cassette.
12. Proceed with processing the PFA-fixed tissues within 2 weeks to avoid potential tissue damage. Embed the processed tissue in paraffin with the tissue oriented on its side such that the entire length of each intestinal segment is exposed.
13. Cut 4- μ m-thick sections with a microtome and collect the sections on positively charged adhesive slides (*see Note 26*). Bake the slides in a 55 °C oven for 2 h. Either let cool to room temperature or keep in 4 °C until using them for staining.

3.7 Immuno-histochemical Staining for Eosinophils and Foxp3+ Cells

1. Deparaffinize tissue sections of slides by immersing the slides sequentially in xylene and ethanol solutions in the following order: two baths of xylene for 5 min each; one bath each of 100%, 95%, 70%, 50%, and 30% ethanol for 1 min each. Place the slides in a water bath and rinse gently in running water for 1 min (*see Note 27*).
2. For Foxp3 staining only, perform heat-induced antigen retrieval. Boil 500 mL Tris-EDTA buffer in a 1-L glass beaker with a microwave. Place the beaker with the boiled buffer on a hotplate with a high-heat setting. Transfer the deparaffinized slides from tap water to the boiled buffer and heat the slides for 10 min.
3. Remove the beaker from the hotplate with the slides still in it and allow the slides and Tris-EDTA buffer to cool to room temperature.
4. Circumscribe tissue sections on the slides with a hydrophobic barrier pen.
5. Block endogenous peroxidases with a hydrogen peroxide solution at the following concentrations in PBS:

- (a) For eosinophil staining: immerse slides in Coplin jar filled with 3% hydrogen peroxide in PBS for 8 min at room temperature.
 - (b) For Foxp3+ cell staining: immerse slides in Coplin jar filled with 0.3% hydrogen peroxide in absolute methanol for 30 min at 4 °C.
6. Wash slides once with PBS for 5 min on a rocker.
7. For eosinophil staining only, incubate each tissue section with 200 µL of the pepsin solution for 5 min at room temperature. Wash slides thrice with PBS, each for 5 min on a rocker.
8. Block each tissue section with 200 µL of the blocking buffer for 1 h at room temperature. Wash slides once with PBS for 5 min on a rocker.
9. Add 100 µL of diluted primary antibody (*see Note 28*) to tissue section slides that have been appropriately pretreated for the particular antibody (*see steps 2, 5, and 7* above).
 - (a) For eosinophil staining: dilute rat anti-mouse MBP antibody at 1:500–1:15,000 in the blocking buffer.
 - (b) For Foxp3+ cell staining: dilute rat anti-mouse Foxp3 antibody at 1:100 in 0.1% Triton-X 100 in PBS.

Incubate the slides in a moisture chamber/slide tray overnight at 4 °C to prevent drying of the slides that may result in inconsistent staining (*see Note 29*).
10. Rinse slides with PBS once quickly, then wash the slides with PBS thrice, each for 5 min on a rocker. Apply 100 µL of HRP-conjugated anti-rat IgG antibody to each tissue section for 30 min at room temperature.
 - (a) For eosinophil staining: 1:250 dilution in the blocking buffer.
 - (b) For Foxp3+ cell staining: 1:100 dilution in 0.1% Triton-X 100 in PBS.
11. Rinse slides with PBS once quickly, then wash the slides with PBS thrice, each for 5 min on a rocker. Bring the DAB substrate solution to room temperature before use and incubate each tissue section slide with 100 µL of DAB color development substrate until a brownish color is developed.
12. Discard the DAB substrate, and then rinse slides with PBS once followed by washing the slides in distilled water thrice, each for 5 min on a rocker (*see Note 30*).
13. Subject slides to hematoxylin counter stain, and then rinse the slides under running tap water for 5 min. Dehydrate the slides by sequentially immersing the slides as follows: rinse in 70% and 95% ethanol; two baths of 100% ethanol for 2 min each;

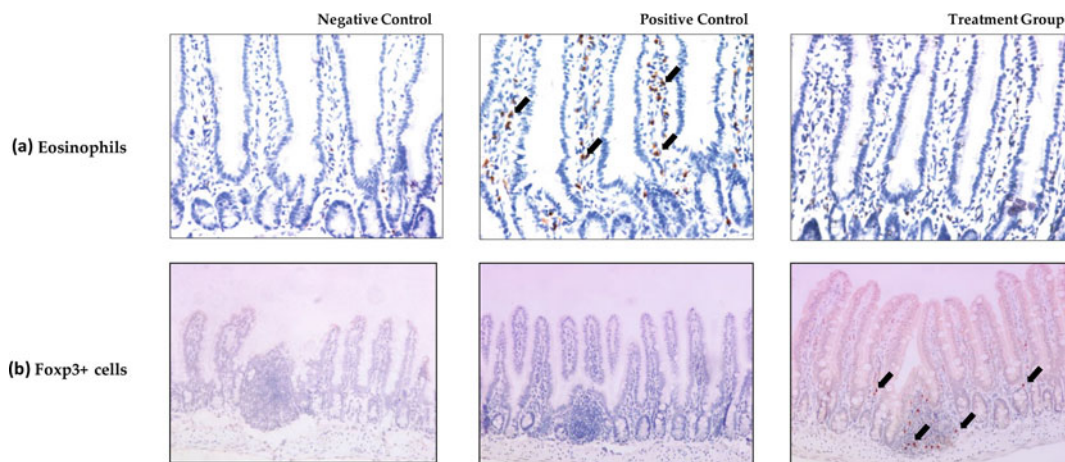


Fig. 2 Immunohistochemical staining of eosinophils and Foxp3+ cells. Representative photomicrographs of (a) eosinophils ($\times 400$ magnification) and (b) Foxp3+ cells ($\times 200$ magnification). Black arrows indicate eosinophils and Foxp3+ cells stained brown in IHC

one bath of 50% xylene/absolute ethanol for 2 min; and two baths of xylene for 2 min each.

14. Allow the slides to air-dry. Mount glass coverslips onto the slides with Permount.
15. Select random fields on the slide and capture images at $200\times$ (Foxp3) and $400\times$ (eosinophils) magnifications by an investigator unfamiliar with the key codes on the slides.
16. Count the number of cells stained brown (Fig. 2) using the ImageJ software, and express the cell count as number of cells/ mm^2 of villi or crypt area.

4 Notes

1. All peptides should first be dissolved in ultrapure water. If the peptide cannot be completely dissolved, add 10–30% acetic acid solution or trifluoroacetic acid (TFA; $<50\ \mu\text{L}$) to solubilize if the overall charge of the peptide is positive. For peptide that has a negative overall charge, add NH_4OH ($<50\ \mu\text{L}$). Add organic solvents like acetonitrile, methanol, or isopropanol to solubilize peptide that have an overall charge of zero. Add a small amount of dimethylsulfoxide (DMSO) for insoluble and highly hydrophobic peptides. 6 M guanidine-HCl or 8 M urea can be added for peptides that tend to aggregate.
2. Use autoclaved PBS to prepare the 4% PFA fresh and use within 2 days. Paraformaldehyde is recommended for fixation; the use of standard fixatives such as 10% buffered formalin yields intestinal tissue that is stiff and increases difficulty in bundling the

intestinal tissue and subsequent immunohistochemical staining.

3. The minimum recommended volume of allergens and peptides for sensitization, treatment, and challenge is 350 μL . While the maximum volume should not exceed 600 μL , dead volume within the syringe and gavage needle need to be considered when calculating the total amount of sensitization solution.
4. A 1-week acclimatization period is recommended before commencing the sensitization procedures. Mice should be provided with a diet free of the allergen used for sensitization.
5. In our general experience, sensitization success rate is approximately 70–90% with the intragastric route and depending on the source of cholera toxin used. Blood sampling 1 week after the last sensitization or 24 h after the first allergen challenge for measuring allergen-specific IgE levels with ELISA (*see* Subheading 3.2) is recommended prior to choosing candidates for the immunotherapy stage. Mice with allergen-specific IgE ELISA reading of equal to or greater than 0.3 OD (optical density) at 450 nm should be selected.
6. Introduction of allergen challenge 1–2 weeks posttreatment only evaluates the short-term effects of the peptide immunotherapy. An allergen challenge at 1–3 months off-treatment is recommended to examine the long-term effects of the peptide immunotherapy.
7. We also recommend introducing an allergen challenge after sensitization but before peptide treatment to compare the changes in antibody levels and immediate allergic symptoms within the same animal.
8. The changes in rectal temperature in challenged mice showing hypersensitivity reaction can be up to -8°C .
9. Most common allergic symptoms in challenged mice include continuous scratching and rubbing around the nose, head, and tail for over 15 s, puffiness around mouth, and reduced activity. Convulsion is also common in mice sensitized through the intraperitoneal route using $\text{Al}(\text{OH})_3$ as adjuvant. Postchallenge death in mice is not common except for peanut allergy.
10. Positive and negative controls should always be included along with the samples to ensure proper interpretation of results. Include “blank controls” by coating dedicated microwells with the culprit allergen and blocking with the blocking buffer. No serum samples or detector antibodies are added to the blank control wells, but only blocking buffer is added in place. “Nonspecific binding controls” should also be added to assess the contribution of the labeled detector antibody. The nonspecific binding control microwells are coated with

the culprit allergen and blocked with the blocking buffer. No serum sample is added, but the addition of detector antibodies is included as usual.

11. Improper plate-washing can lead to inconsistent results, low signals, or high background across replicate samples and control wells. For all washing steps, use approximately 400 μL /well of PBS-T or PBS with thorough aspiration of microwell contents. Allow the wash buffer to sit in the wells for 15–30 s before aspiration. Blot-dry on tissue papers between each wash. To minimize cross-well contamination, avoid over-flooding of microwells during the wash steps. If alkaline phosphatase-conjugated antibodies are used for colorimetric reactions, use Tris-buffered saline instead of PBS.
12. Suboptimal dilutions of serum and detection antibodies can also lead to inconsistent results. Optimization of serum and detection antibody dilutions can be accomplished by serial dilution of the serum and/or detection antibody across the plate [20].
13. Incubate plate at room temperature until the desired color develops or stop the reaction before any well displays a green product. Color development usually takes 15–30 min at room temperature.
14. The expected OD signal for blank control wells is as low as zero. High ODs in blank control wells may indicate a plate-washing problem. The desired OD signal for nonspecific binding control wells is slightly over the blank control wells but not over the sample wells. High ODs in nonspecific binding control wells may indicate improper blocking procedures and/or concentrations of the detection antibodies. Increasing the concentration of FBS up to 8% or replacing FBS by Bovine Serum Albumin (BSA) may reduce nonspecific binding signals. Optimize dilutions of detection antibody as described in **Note 11**.
15. All procedures should be performed under sterile conditions in a biological safety cabinet to prevent contamination.
16. Immediate processing of the spleen into single-cell suspensions is recommended. If immediate processing is not possible, keep the spleen in 5 mL of the complete culture medium in a clean 15-mL centrifuge tube at 4 °C to maintain cell viability. However, avoid temperature shock from ice and warming up to room temperature.
17. Lift the cell strainer off the bottom of the Petri dish regularly during the straining process to allow the cells to pass through.
18. Gentle pipetting is recommended to avoid damaging the cells when resuspending the cells during each washing step.

19. The anticipated recovery of live lymphocytes from the spleen per mouse is $5\text{--}15 \times 10^7$ cells.
20. Delay in fixation would lead to irreparable damage to the small intestine tissue. Immediately place the intestinal segments into iced-cold PBS and instill 4% PFA into the gut lumen within 10 min to reduce potential tissue damage.
21. Before cutting intact intestinal segments open, fixation in 4% PFA for 3–4 h is recommended to minimize distortion of the villi and to prevent unrolling of the intestinal tissues. Avoid overnight fixation of intact intestinal segments in 4% PFA as overnight fixation yields stiff intestinal tissues that are too hard to roll. Flexibility of the tissue is critical in the Swiss roll technique.
22. When cutting open the intestines, uneven edges make it more difficult to keep the intestine flat and result in poorer bundling of the tissue. Keep a steady but gentle tension on the intestine with forceps and cut smoothly and steadily to give a straight edge.
23. Bundling all intestinal segments from distal (cecum) to proximal (stomach) ends is recommended to minimize compression of the villi and for estimating the anatomical location in histological analysis.
24. Unrolling of the bundled intestinal tissues represents a common problem; however, pinning of the intestinal roll is not recommended, as this would degrade the fixation quality.
25. Avoid overfixation as this leads to masking of epitopes and failure of immunohistochemical staining. A maximum of 24-h fixation in 4% PFA is recommended.
26. Adhesive slides are recommended over uncoated slides to reduce the tendency of tissue detachment especially when heat-induced epitope retrieval is required prior to immunohistochemical staining.
27. Use fresh xylene in deparaffinization to prevent high background staining. Warm the slides in a 55 °C oven for 10–15 min to soften paraffin if the slides were stored at 4 °C prior to use.
28. Optimization of the dilution of the primary antibody for IHC is required. The recommended dilution for rat anti-mouse MBP antibody for eosinophil staining is between 1:500 and 1:15,000, whereas that for anti-mouse Foxp3 antibody is at 1:100. The dilution adopted in our experiment for eosinophil staining is 1:15,000 with overnight incubation for maximum signal.
29. An isotype control antibody should be included as a control to rule out nonspecific antibody staining.

30. Optimization of the incubation time with DAB substrate is recommended to maximize signal while minimizing background noises. Recommended incubation time for eosinophil staining is 1 min 30 s and 4 min for Foxp3+ cell staining. Handle DAB with great caution for it is potentially carcinogenic. Discard used DAB substrate solution into a designated container and dispose of it according to the guidelines of your institution.

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