



A Mouse Model of Oral Sensitization to Hen's Egg White

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Abstract

Egg allergy is one of the most common food allergies in children, being the most important allergenic proteins found in the egg white (EW). Allergy to EW shows a complex phenotype that involves a multifaceted reaction that can only be assessed *in vivo*. Although other routes of sensitization have been described, oral exposure to food antigens is one of the most suitable in humans. In mice, oral administration of allergenic proteins results in the development of tolerance, and the use of adjuvants, such as cholera toxin (CT), is required to promote Th2-biased immune responses over tolerogenic responses. In this regard, among the mouse strains that readily display Th2 responses, Balb/c has been widely used. Here, we describe a frequently used protocol of oral EW sensitization by using CT as an adjuvant and we explain in detail the methods that we have developed to analyze the sensitizing and eliciting capacity of EW proteins including evaluation of signs, measurement of serum levels of specific immunoglobulins, mast cell degranulation, cytokine secretion profile of allergen-reactive T cells, phenotyping of mesenteric lymph node- and spleen-derived dendritic and T cells by flow cytometry, and quantification of intestinal gene expression.

Key words Food allergy, Egg white proteins, Intestinal gene expression, Th2 response, Balb/c model, T cells, Dendritic cells

1 Introduction

Hen's egg is the second most frequent source of allergic reactions in children [1]. The described overall prevalence of self-reported egg allergy reaches 2.5%, being higher in younger age groups (5.7% in children 2–5 years old) compared to older ones (2.0% people >18 years old) [1].

Egg allergy is an IgE antibody-mediated reaction defined as an adverse immune response triggered by the ingestion of egg proteins in sensitized individuals [2]. The main allergenic proteins of hen's egg are found in egg white (EW), ovomucoid and ovalbumin being the two most important proteins that can generate allergic reactions [2]. In most cases (25–50%), symptoms occur within 2 h after ingestion, including abdominal pain, bloating, vomiting, diarrhea (gastrointestinal symptoms), itching, urticaria, angioedema

(cutaneous symptoms), asthma, and/or rhinitis (respiratory symptoms) [3].

Antigenicity of food proteins has been tested by in vitro methods including IgE antibody quantification and cell-based reactivity, mainly using mast cell or basophil activation assays [4, 5]. However, egg allergy shows a complex pattern that involves multifaceted reactions that can only be assessed in vivo. In this regard, murine models offer the opportunity to evaluate food-induced allergic responses, which would otherwise not be possible to estimate in human allergic patients.

Traditionally, in the field of food allergies, experimental models have been specially directed to certain mouse strains that readily display allergenic responses, Balb/c being one of the most widely used. As in humans, oral administration of food antigens to mice results in oral tolerance, with a little or no immune response or disease association [6]. To overcome the physiological tolerance to ingested antigens in experimental studies, immune adjuvants are often coapplied with food allergens in order to induce sensitization and to promote Th2-biased responses over Th1 [7]. The cholera toxin (CT), an endotoxin produced by *Vibrio cholera*, is one of the best-known mucosal adjuvants. Their nontoxic subunit B links to the allergen of interest, which acquires the capacity to bind to the ganglioside receptor GM-1 on intestinal epithelial cells [8]. In addition, CT induces migration and activation of dendritic cells (DCs) from the lamina propria to the mesenteric lymph nodes (MLNs) and promotes a Th2-associated DC phenotype, by the upregulation of the surface costimulatory molecule, OX40L [9].

The oral coadministration of CT together with egg proteins has been used by our group in order to evaluate the effect of food processing and matrix on their capacity to induce sensitization or elicit allergic responses [10], to compare the sensitizing capacity of different egg components (egg white and yolk) [11], and as a pre-clinical approach to study the efficacy of new preventive and therapeutic strategies [12].

In this experimental protocol, we explain how to establish EW-induced allergy in mice and how to evaluate their allergic response assessing their clinical symptoms, measuring their serum levels of antigen-specific immunoglobulins and mouse mast cell protease-1 (mMCP-1), and analyzing the phenotype of dendritic and T cells from MLNs and spleens and the intestinal gene expression. Moreover, we describe how to restimulate the T cells from MLNs and spleens with EW to study their capacity to secrete Th2 cytokines.

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Prepare and store all reagents at room temperature, unless indicated otherwise. Diligently follow all waste disposal regulations when placing waste materials.

2.1 Egg White Sensitization (See Note 1)

1. Phosphate-buffered saline (PBS): pH 7.4. Commercially available formulation without Ca^{2+} and Mg^{2+} . Suitable for cell culture.
2. Sensitizing solution for the experimental group: Dissolve 5 mg of EW lyophilized powder (*see Note 2*) and 10 μg of CT in 200 μL of PBS per mouse (*see Note 3*).
3. Sensitizing solution for the control group: Dissolve 10 μg of CT in 200 μL of PBS per mouse.
4. Five-week-old BALB/c female mice, ideally specified pathogen-free (*see Note 4*).
5. Freeze dryer.
6. Analytical balance.
7. 50-mL Centrifuge tubes.
8. Centrifuge.
9. 1-mL Syringes.
10. Feeding tubes: 30 mm.

2.2 Egg White Challenge

1. PBS: pH 7.4. Commercially available formulation without Ca^{2+} and Mg^{2+} . Suitable for cell culture.
2. Allergen challenge solution (without CT): Dissolve 50 mg of EW (*see Note 2*) in 200 μL of PBS per mouse (*see Note 3*).
3. 1-mL syringes.
4. Feeding tubes: 30 mm.
5. A rectal thermometer.

2.3 Fecal and Organ Sample Collection

1. CO_2 for euthanasia.
2. Dissection instruments: scissors and forceps.
3. Sample tubes: 50 and 1.5 mL.
4. Collection buffer for MLNs: 25 mM Hepes; 238.3 g/mol) and 0.1% glucose in PBS. Weigh 3 g of Hepes and 0.5 g of glucose. Dissolve solutes in 500 mL of PBS. Mix and filter through a pore size membrane of 0.2 μm . Store at 4 °C.
5. Collection buffer for spleens and intestines: 0.1% glucose in PBS. Weigh 0.5 g of glucose and dissolve in 500 mL of PBS. Mix and filter through a pore size membrane of 0.2 μm . Store at 4 °C.

6. RNA preservation buffer (lysis buffer for lysing cells and tissues recommended by commercial RNA isolation kits).
7. Tissue homogenizer: Ultra-Turrax T8 or equivalent.

2.4 Splenocyte and MLN Cell Isolation

1. 70- μ m Cell strainers.
2. PBS: *See* Subheading 2.2.
3. Completed RPMI: Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μ g/mL streptomycin.
4. FACS buffer: 1 mM ethylenediaminetetraacetic acid (EDTA) and 2% bovine serum albumin (BSA) in PBS. Weigh 0.29 g of EDTA and 20 g of BSA. Dissolve solutes in 1 L of PBS. Store at 4 °C.
5. Red blood cell (RBC) lysis buffer: Commercially available.

2.5 Splenocyte and MLN Cell Culture

1. Sterile 48-well culture plates.
2. Negative control stimulating solution: completed RPMI (*see* Subheading 2.4).
3. Positive control stimulating solution: 25 μ g/mL Concanavalin A (ConA) dissolved in completed RPMI (*see* Subheading 2.4).
4. Egg white stimulating solution: 2 mg/mL EW lyophilized powder (*see* Note 2) dissolved in completed RPMI (*see* Subheading 2.4).

2.6 Measurement of EW-Specific Immunoglobulins, mMCP-1, and Cytokines by ELISA

1. Clear flat-bottom polystyrene high-bind 96-well plates.
2. Egg white lyophilized powder (*see* Note 2).
3. 10 $\times$ PBS stock solution: 80.9 mM Na₂HPO₄·12H₂O, 14.7 mM KH₂PO₄, 1370 mM NaCl, and 26.8 mM KCl. Weigh 29 g of Na₂HPO₄·12H₂O, 2 g of KH₂PO₄, 80 g of NaCl, and 2 g of KCl. Dissolve solutes in 800 mL of distilled water. Adjust the pH to 7.4 and make up to 1 L with distilled water.
4. PBS: Mix 100 mL of PBS 10 $\times$ with 900 mL of distilled water.
5. PBS-BSA: 1% BSA in PBS (w/v). Weigh 1 g of BSA and dissolve solute in 100 mL of PBS. Store at 4 °C.
6. 0.1 M Sodium bicarbonate (NaHCO₃) buffer: pH 9.6. Weigh 0.84 g of NaHCO₃ and dissolve solute in 90 mL of PBS. Adjust pH to 9.6 and make up to 100 mL with distilled water. Store at 4 °C.
7. PBS-Tween (PBS-T): 2.5% Tween 20 in PBS (v/v). Dissolve 5 mL of Tween 20 in 200 mL of PBS. Store at 4 °C.

Table 1
Overview of immunoglobulin ELISA

ELISA	Layer	Component	Dilution (dil.) or concentration	Diluent
sIgE	Capture	EW ^a ; rat anti-mouse IgE	5 µg/mL; 2 µg/mL	PBS
	Block	Tween	2.5%	PBS
	Samples	Serum samples; mouse-IgE	1:20 dil.; 7–500 ng/mL ^b	PBS-T
	Detection	Biotinylated rat anti-mouse IgE	2 µg/mL	PBS-T
	Developing	Avidin–horseradish peroxidase	1:1000 dil.	PBS-T
sIgG1	Capture	EW; rat anti-mouse IgG1	2 µg/mL; 2 µg/mL	PBS
	Block	BSA	1%	PBS
	Samples	Serum samples; mouse-IgG1	1:1000 dil.; 2–150 ng/mL ^b	PBS–BSA
	Detection	Biotinylated rat anti-mouse IgG1	0.1 µg/mL	PBS–BSA
	Developing	Avidin–horseradish peroxidase	1:4000 dil.	PBS–BSA
sIgG2a	Capture	EW; rat anti-mouse IgG2a	2 µg/mL; 2 µg/mL	PBS
	Block	BSA	1%	PBS
	Samples	Serum samples; mouse IgG2a	1:100 dil.; 4–1000 ng/mL ^b	PBS–BSA
	Detection	Biotinylated rat anti-mouse IgG2a	0.1 µg/mL	PBS–BSA
	Developing	Avidin–horseradish peroxidase	1:4000 dil.	PBS–BSA
sIgA	Capture	EW; rat anti-mouse IgA	1 µg/mL; 2 µg/mL	0.1 M NaHCO ₃
	Block	Tween	2.5%	PBS
	Samples	lyophilized feces; mouse IgA	1:10 dil.; 3–50 ng/mL ^b	PBS
	Detection	Biotinylated rat anti-mouse IgA	1 µg/mL	PBS–BSA
	Developing	Avidin–horseradish peroxidase	1:2000	PBS–BSA

^aSeparated from whole hen's egg and lyophilized (*see* Subheading 2.5)

^bIndicates the range of mouse immunoglobulin concentrations used for the standard curve

8. Wash buffer: 0.05% (v/v) Tween 20 in PBS. Dissolve 500 µL of Tween 20 in 200 mL of PBS. Store at 4 °C.
9. Specific antibodies against IgE, IgG1, IgG2a, and IgA and mouse IgE, IgG1, IgG2a, and IgA. For an overview of all antibodies and reagents, *see* Table 1.
10. ELISA kits for mMCP-1 and cytokines of interest, such as IL-4, IL-5, IL-10, IL-13, and IFN γ .
11. 3,3',5,5'-Tetramethylbenzidine (TMB).
12. Stop Solution: 0.5 M H₂SO₄. Add 2.8 mL of concentrated H₂SO₄ (*see* **Note 5**) to 50 mL of distilled water and make up to 100 mL with distilled water.
13. Plate shaker.
14. ELISA plate washer (optional).
15. Absorbance microplate reader.

2.7 Phenotypic Analysis of Dendritic and T Cells From MLNs and Spleens

1. Clear V-bottom polystyrene high-bind 96-well plates.
2. PBS: *See* Subheading 2.2.
3. FACS buffer: *See* Subheading 2.3.
4. Specific antibodies against DC and T-cell markers. For an overview of all antibodies and reagents, *see* Table 2.
5. Viability staining solution: Dilute 0.1 μL of LIVE/DEAD™ Fixable Cell Stain in 200 μL of PBS per sample (*see* Note 3).
6. Fc-blocking solution: Dilute 0.25 μL of anti-CD16/CD32 in 25 μL of FACS buffer per sample (*see* Note 3).
7. Antibody cocktail for detection of MLNs (*see* Note 6): Dilute 0.3 μL of Alexa Fluor 700-anti-CD4, 0.4 μL of APC-anti-CXCR3, 0.4 μL of PerCP Cy5.5-anti-CD69, 0.7 μL of PE-anti-ST2, 2.5 μL of PE Cy7-anti-CD39, and 2 μL of FITC-anti-CD25 in 45.5 μL of FACS buffer per sample (*see* Note 3).
8. Antibody cocktail for detection of DCs (*see* Note 6): Dilute 1 μL of PE Cy7-anti-CD11c, 0.2 μL of Alexa Fluor 700-anti-CD11b, 2 μL of PE-anti-CD103, 1.5 μL of APC-anti-CD80, 1.5 μL FITC-anti-CD86, and 1 μL PerCP Cy5.5-anti-CD8 α in 42.8 μL of FACS buffer per sample (*see* Note 3).
9. Flow cytometer.
10. 5-mL Flow cytometry tubes.
11. Flow cytometry data analysis software.

Table 2
Antibodies used for FACS analysis of DCs and T cells

Specificity	Clone	Isotype	Target	Supplier
Anti-CD16/CD32	2.4G2	Rat IgG2b, κ	DCs, T cells	BD Biosciences
PE Cy7-anti-CD11c	N418	Armenian Hamster IgG	DCs	BioLegend
Alexa Fluor 700-anti-CD11b	M1/70	Rat IgG2b, κ	DCs	BioLegend
PerCP Cy5.5-anti-CD8 α	53-6.7	Rat IgG2a, κ	DCs	eBiosciences
PE-anti-CD103	2E7	Armenian Hamster IgG	DCs	BioLegend
APC-anti-CD80	16-10A1	Armenian Hamster IgG	DCs	eBiosciences
FITC-anti-CD86	GL1	Rat IgG2a, κ	DCs	eBiosciences
Alexa Fluor 700-anti-CD4	GK1.5	Rat IgG2b, κ	T cells	eBiosciences
PerCP Cy5.5-anti-CD69	H1.2F3	Armenian Hamster IgG	T cells	eBiosciences
FITC-anti-CD25	3C7	Rat IgG2b, κ	T cells	BioLegend
PE-anti-ST2	RMST2-2	Rat IgG2a, κ	T cells	eBiosciences
APC-anti-CXCR3	CXCR3-173	Armenian Hamster IgG	T cells	eBiosciences
PE Cy7-anti-CD39	Duha59	Rat IgG2a, κ	T cells	BioLegend

2.8 Intestinal Gene Expression

1. Tissue homogenizer.
2. NucleoSpin RNA Isolation Kit or equivalent (*see Note 7*).
3. Agilent 2100 Bioanalyzer (*see Note 8*).
4. PCR tubes or plates.
5. cDNA synthesis kit: PrimeScript RT Reagent Kit or equivalent.
6. Real-time PCR system: SYBR Premix Ex Taq II (Tli RNase H Plus) or equivalent.
7. Primer pairs: The primer sequences used in the analyses of intestinal gene expression by reverse transcriptase quantitative PCR (RT-qPCR) are shown in Table 3.
8. Real-time PCR thermocycler.

3 Methods

3.1 Egg White Sensitization and Blood Sample Collection

1. Administer 200 μ L per mouse of the sensitizing solutions by oral gavage during three consecutive days on the first week and once per week during the following 5 weeks (*see Note 9* and Fig. 1).
2. Extract blood samples by cheek puncture 3 days after the last sensitization dose to measure EW-specific immunoglobulin levels. Keep them on ice until continuing with the serum isolation step described below.
 - (a) Centrifuge blood samples at $2000 \times g$ for 15 min at 4 °C.
 - (b) Take sera from supernatants and transfer to a set of new tubes.
 - (c) Freeze serum samples and store at -20 °C until analysis.

3.2 Egg White Challenge and Anaphylactic Response Evaluation

1. One week after the last sensitization dose (*see Fig. 1*), administer 200 μ L of the allergen challenge solution by oral gavage.
2. Thirty minutes after oral challenge, evaluate the severity of anaphylaxis by measuring the body temperature with a rectal thermometer. Score the severity of the clinical signs according to the graded score scale from Table 4.
3. Extract blood samples by cheek puncture 30 min after oral challenge to analyze mMCP-1 serum levels. Keep them on ice until continuing with the isolation step described in Subheading 3.1, step 2.

3.3 Fecal Sample Collection

1. Collect feces from the cage in a 5-mL tube once a week (*see Note 10*).
2. Lyophilize and weigh the fecal samples. Dissolve one part of each fecal sample in six parts of PBS (w/v).

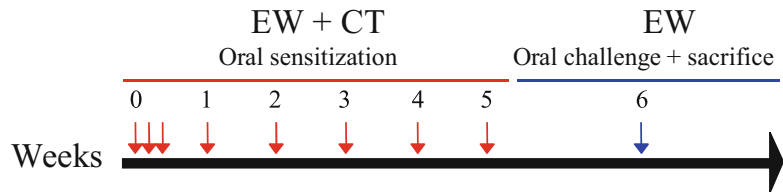
Table 3**Primer sequences used for RT-qPCR analyses of intestinal relative gene expression**

Gene	Primer pair	Extension temperatures
<i>Actb</i> ^a	<i>fw</i> 5' AGCTGCGTTTACACCCTTT 3' <i>rv</i> 5' AAGCCATGCCAATGTTGTCT 3'	60 s at 60 °C
<i>Aldh1 a1</i>	<i>fw</i> 5' CTCTGTTCCCCAGGTGTTGT 3' <i>rv</i> 5' TCATGCAAGGGTGCCTTTAT 3'	
<i>Foxp3</i>	<i>fw</i> 5' ACAACCTGAGCCTGCACAAGT 3' <i>rv</i> 5' GCCCACCTTTTCTTGTTTTG 3'	
<i>Gata3</i>	<i>fw</i> 5' CCTTAAACTCTTGCGCTCC 3' <i>rv</i> 5' AGACACATGTCATCCCTGAG 3'	
<i>Il12p40</i>	<i>fw</i> 5' AGGTGCGTTCCTCGTAGAGA 3' <i>rv</i> 5' AAAGCCAACCAAGCAGAAGA 3'	
<i>Il17a</i>	<i>fw</i> 5' TGCCTGTGGCACTGAAGTAG 3' <i>rv</i> 5' TTCATGGCTGCAGTGAAAAG 3'	
<i>Il22ra2</i>	<i>fw</i> 5' TCAGCAGCAAAGACAGAAGAAAC 3' <i>rv</i> 5' GTGTCTCCAGCCCAACTCTCA 3'	
<i>IL-25</i>	<i>fw</i> 5' ACAGGGACTTGAATCGGGTC 3' <i>rv</i> 5' TGGTAAAGTGGGACGGAGTTG 3'	
<i>IL-33</i>	<i>fw</i> 5' ATTTCCCCGGCAAAGTTCAG 3' <i>rv</i> 5' AACGGAGTCTCATGCAGTAGA 3'	
<i>Irf4</i>	<i>fw</i> 5' TCCTCGTCCCTTGCTGAAAC 3' <i>rv</i> 5' GGGCTTTGGGGCTTCTAGTT 3'	
<i>Irf8</i>	<i>fw</i> 5' ACCGGCGGCAGGATGT 3' <i>rv</i> 5' ACAGCGTAACCTCGTCTTCC 3'	
<i>Jag2</i>	<i>fw</i> 5' GGCAAAGAATGCAAAGAAGC 3' <i>rv</i> 5' GCTCAGCATTGATGCAGGTA 3'	
<i>Muc2</i>	<i>fw</i> 5' GCTGACGAGTGGTTGGTGAATG 3' <i>rv</i> 5' ATGAGGTGGCAGACAGGAGAC 3'	
<i>Rorc</i>	<i>fw</i> 5' TCACCTGTGAGGGGTGCAAG 3' <i>rv</i> 5' GTTCGGTCAATGGGGCAGTT 3'	
T-bet	<i>fw</i> 5' GTATCCTGTTCCCAGCCGTTTC 3' <i>rv</i> 5' ACTGTGTTCCCAGGTGTCC 3'	
<i>Tslp</i>	<i>fw</i> 5' AGGCTACCCTGAAACTGAGA 3' <i>rv</i> 5' GGAGATTGCATGAAGGAATAC 3'	

(continued)

Table 3
(continued)

Gene	Primer pair	Extension temperatures
<i>Actb</i> ^a	<i>fw</i> 5' AGCTGCGTTTTACACCCTTT 3' <i>rv</i> 5' AAGCCATGCCAATGTTGTCT 3'	45 s at 58 °C 15 s at 60 °C
<i>Aldh1 α2</i>	<i>fw</i> 5' ACCGTGTTCTCCAACGTCACGTGAT 3' <i>rv</i> 5' TGCATTGCGGAGGATACCATGAGA 3'	
<i>Cldn2</i>	<i>fw</i> 5' GTCATCGCCCATCAGAAGAT 3' <i>rv</i> 5' ACTGTTG GACAGGGAACCAG 3'	
<i>Il6</i>	<i>fw</i> 5' TTCCATCCAGTTGCCTTCTTG 3' <i>rv</i> 5' GGGAGTGGTATCCTCTGTGAAGTC 3'	
<i>Il10</i>	<i>fw</i> 5' GCCTTATCGGAAATGATCCA 3' <i>rv</i> 5' AGGGGAGAAATCGATGACAG 3'	
<i>Il22</i>	<i>fw</i> 5' CATGCAGGAGGTGGTACCTT 3' <i>rv</i> 5' CAGACGCAAGCATTCTCAG 3'	
<i>Il27</i>	<i>fw</i> 5' CTCTGCTTCCTCGCTACCAC 3' <i>rv</i> 5' GGGGCAGCTTCTTTCTTCT 3'	
<i>Tfjb1</i>	<i>fw</i> 5' TTGCTTCAGCTCCACAGAGA 3' <i>rv</i> 5' TACTGTGTGTCCAGGCTCCA 3'	
<i>Tjp1</i>	<i>fw</i> 5' TACCTCTTGAGCCTTGAACCTT 3' <i>rv</i> 5' ACAGAAATCGTGCTGATGTGC 3'	

fw forward primer, *rv* reverse primer^a*Actb* may be used as a reference gene**Fig. 1** Experimental protocol for oral sensitization of Balb/c mice with egg white (EW) plus cholera toxin (CT)

- Homogenize the samples in PBS using an electric tissue homogenizer.
- Centrifuge the samples at $2000 \times g$ for 15 min at 4 °C.
- Collect the supernatants, transfer to new tubes, and store them at -80 °C until use.

3.4 Organ Collection

- Thirty minutes after challenge, sacrifice the mice by CO₂ inhalation and individually collect the whole spleen and the MLNs in 1 mL of cold, tissue-specific collection buffer. Keep samples on ice until cell isolation.

Table 4
Clinical severity score

Score	Clinical signs
0	No signs
1	Scratching nose and mouth less than ten times in 15 min
2	Puffiness around eyes and mouth, scratching nose and mouth more than ten times in 15 min
3	Wheezing and labored respiration, cyanosis around the mouth and tail, diarrhea and difficulty in walking normally
4	No activity after prodding
5	Death

2. Collect the whole gut in 5 mL of cold collection buffer specific for intestines. Flush the intestinal contents with the collection buffer, three times using a 5-mL syringe.
3. Place a 2-cm segment portion from each of the cleaned duodenum, jejunum, ileum, caecum, or colon in 350 µL of RNA preservation buffer. Store at −80 °C until its use.

3.5 Splenocyte and MLN Cell Isolation

1. Transfer the whole spleen or the MLNs to a 70-µm cell strainer held on a 50-mL conical tube and mechanically disrupt the organ using the plunger of a 1-mL syringe.
2. Rinse the filter five times using 5 mL of PBS.
3. Centrifuge the tubes at $450 \times g$ for 10 min at 4 °C, and discard the supernatant.
4. Add 2.5 mL of RBC lysis buffer to the tube. Homogenize the cell suspension by pipetting and incubate for 3 min at room temperature.
5. Add 25 mL of PBS and divide the cell suspension into two tubes.
6. Centrifuge the tubes at $450 \times g$ for 10 min at 4 °C, discard the supernatants, and resuspend the cells in the appropriate volume of completed RPMI (for cell culture) or FACS buffer (for flow cytometry staining) to obtain a final density of 4×10^6 cell/mL.

3.6 Splenocyte and MLN Cell Culture

1. Seed 1 mL of the splenocytes and MLN cells resuspended in completed RPMI in a 48-well plate.
2. Add 100 µL of stimulating solution to appropriate wells:
 - (a) Negative control: completed RPMI.
 - (b) Positive control: 2.5 µg/mL ConA.
 - (c) Egg white stimulation: 200 µg/mL EW.

3. Incubate the cell culture for 72 h in a 5% CO₂ incubator at 37 °C.
4. Centrifuge the plate at 450 × *g* for 10 min at room temperature.
5. Collect the supernatants and store them at −80 °C for cytokine secretion analysis. Collect the remaining cells in RNA preservation buffer (such as RNeasy Lysis Buffer™ or TRIzol™) and store them at −80 °C until gene expression analysis.

3.7 Quantification of EW-Specific Murine IgE, IgG1 e IgG2a in Sera and IgA in Feces

1. Make coating solutions for EW-specific immunoglobulin ELISAs with the lyophilized EW. Determine the protein concentration of lyophilized EW powder by the Kjeldahl method (*see Note 2*) and prepare the following EW solutions with the specified concentrations in PBS.
 - (a) For coating IgE ELISA plate: 5 µg/mL EW.
 - (b) For coating IgG1 or IgG2a ELISA plate: 2 µg/mL EW.
 - (c) For coating IgA ELISA plate: 1 µg/mL EW.
2. Coat 96-well plates with 100 µL/well of appropriate EW solutions from **step 1**.

For the reference curves, coat the designated wells with 100 µL of rat anti-mouse IgE, IgG1, and IgG2a in PBS or rat anti-mouse IgA in 0.1 M NaHCO₃, pH 9.6, each at 2 µg/mL (Table 1). Incubate the plates overnight at 4 °C.
3. Aspirate the wells and wash four times with 250 µL/well of the wash buffer (*see Note 11*).
4. Block the wells with 250 µL/well of PBS-T. Incubate at room temperature for 2 h.
5. Prepare a 1:20 dilution of serum samples in PBS-T for IgE ELISA, a 1:1000 dilution in PBS-BSA for IgG1 ELISA, and a 1:100 dilution in PBS-BSA for IgG2a ELISA. For IgA ELISA, prepare a 1:10 dilution of fecal samples in PBS. For the reference curves of mouse IgE, IgG1, IgG2a, and IgA, prepare serial dilutions using the ranges indicated in Table 1.
6. Repeat **step 3** and add 100 µL/well of diluted samples. Seal the plate and incubate at room temperature for 2 h (*see Note 12*).
7. Repeat **step 3** and add 100 µL/well of biotinylated rat anti-mouse IgE, IgG1, IgG2a, and IgA (2 µg/mL for IgE, 0.1 µg/mL for IgG1, and IgG2a, and 1 µg/mL for IgA ELISA). Buffers used for biotinylated antibody dilutions are PBS-T for IgE and PBS-BSA for IgG1, IgG2a, and IgA ELISA. Seal the plate and incubate at room temperature for 1 h.
8. Repeat **step 3** and add 100 µL/well of avidin-horseradish peroxidase (1:1000 dilution for IgE, 1:4000 dilution for IgG1 and IgG2a, and 1:2000 for IgA ELISA). Buffers used for biotinylated antibody dilutions are PBS-T for IgE and PBS-

BSA for IgG1, IgG2a, and IgA ELISA. Seal the plate and incubate at room temperature for 30 min.

9. Repeat **step 2** and add 100 μL /well of TMB. Incubate at room temperature for 15 min in the dark.
10. Add 50 μL /well of the stop solution.
11. Read plate at 450 nm on an absorbance microplate reader.

3.8 Quantification of mMCP-1 in Sera and Cytokines Levels in Supernatants of Cultured Splenocytes and MLNs

1. Determine the levels of mMCP-1 in serum samples by using a commercial ELISA kit, according to the manufacturer's protocol.
2. Determine cytokines released during cell culture (IL-4, IL-5, IL-10, IL-13, IFN- γ , and TNF- α) by using commercial ELISA kits or Luminex immunoassay kits, according to the manufacturer's protocol.
3. Collect Luminex data in a Luminex Instrument 100 IS (Luminex Corporation, Texas, US).
4. Analyze data using ProcataPlex Analyst 1.0 software (Fig. 2).

3.9 Phenotypic Analysis of Dendritic Cells and T Cells From MLNs and Spleens

3.9.1 Staining for Flow-Cytometric Analysis

1. Distribute the cells isolated from the MLNs and spleens (*see* Subheading 3.3) in two 96-well plates each (1×10^5 cells per well) (*see* **Note 13**). Prepare a pool of cells with leftover cell suspensions of MLNs and spleens for unstained, viability, Fluorescence Minus One (FMO), and single staining controls, and distribute 1×10^5 cells per well (one per control) on each plate. Centrifuge cells at $500 \times g$ for 5 min at 4°C .
2. Resuspend the cell pellets in 200 μL of LIVE/DEADTM viability staining solution and incubate for 30 min at 4°C on ice, protected from light (*see* **Note 14**).
3. Add 100 μL of PBS and centrifuge for 5 min at $500 \times g$, 4°C .
4. Add 100 μL of FACS buffer and centrifuge for 5 min at $500 \times g$, 4°C .
5. Resuspend the cell pellets in 25 μL of Fc-blocking solution and incubate for 15 min at 4°C on ice, protected from light (*see* **Note 15**).

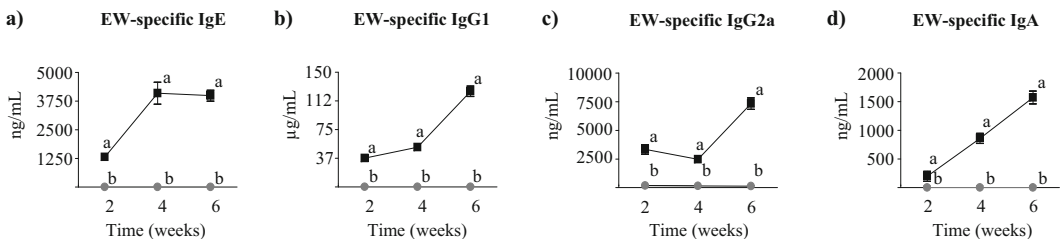


Fig. 2 Egg white (EW)-specific IgE (a), IgG1 (b), IgG2a (c), and IgA (d) antibodies from mice after administration of EW plus cholera toxin (CT) or CT alone (Adapted with permission from Lozano-Ojalvo et al. [13])

6. Add 150 μL of FACS buffer and centrifuge at $500 \times g$ for 5 min at 4°C .
7. Resuspend the cell pellets in 200 μL of FACS buffer and centrifuge for 5 min at $500 \times g$ at 4°C .
8. Resuspend the cell pellets in 50 μL of appropriate antibody solutions and incubate for 30 min at 4°C on ice, protected from light (*see Note 16*).
9. Add 150 μL of FACS buffer and centrifuge for 5 min at $500 \times g$ at 4°C .
10. Add 200 μL of FACS buffer and centrifuge for 5 min at $500 \times g$ at 4°C .
11. Resuspend the cell pellets in 200 μL of FACS buffer and centrifuge for 5 min at $500 \times g$ at 4°C .
12. Resuspend the cell pellets in 200 μL of FACS buffer and keep at 4°C until data acquisition on the flow cytometer (*see Note 17*).

3.9.2 Flow-Cytometric Gating Strategy for DCs

1. Gate cells on the basis of forward scatter-integral (FS-I), live cells, and CD11c population (*see Fig. 3*).
2. Further gate on CD11b and CD103, which are markers used for identifying different DC populations. CD103+CD11b+ and CD103+CD11b– populations correspond to lamina-

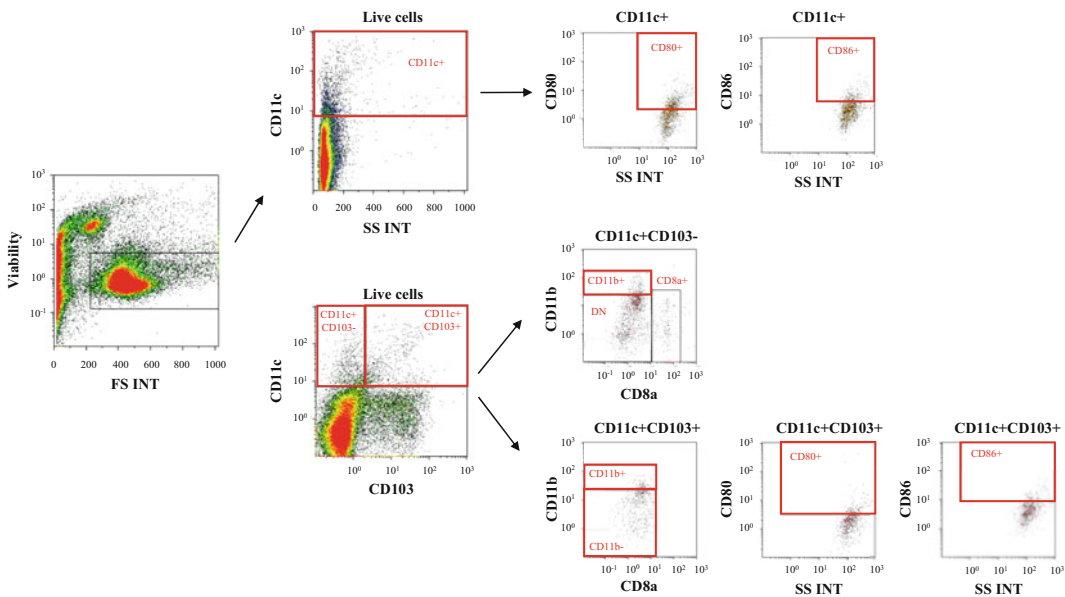


Fig. 3 Gating strategy for identification of dendritic cell (DC) subsets in the mesenteric lymph nodes (MLNs) of mice sensitized to egg white (EW). Cells are gated based on the viability and CD11c staining and subsequently analyzed based on CD80, CD86, CD11b, CD8 α , or CD103 expression. Pictures show representative dot plots of DC analysis in MLNs (Adapted with permission from Pablos-Tanarro et al. [11])

propria-derived DCs that bear the integrin α -chain CD103 [13] with the capacity to present antigens to naïve T cells and induce a Th2-skewed response in the MLNs. CD103⁺ DCs (either CD11b⁺CD8 α ⁺, CD11b⁺CD8 α ⁺ or CD11b⁺CD8 α ⁺) correspond to resident MLN-DCs [14].

- 3. Further gate on CD80 and CD86 to identify the upregulation of these costimulatory molecules.

3.9.3 Flow-Cytometric
Gating Strategy for T-Cells
Subsets

- 1. Gate cells on the basis of FS-I and live cells (*see* Fig. 4).
- 2. Further gate CD4⁺ cells.
- 3. To detect different subsets of activated T cells, gate on CD69⁺CXCR3⁺ for Th1 cells, CD69⁺T1/ST2⁺ for Th2 cells, and CD25⁺CD39⁺ for regulatory T cells (Treg).

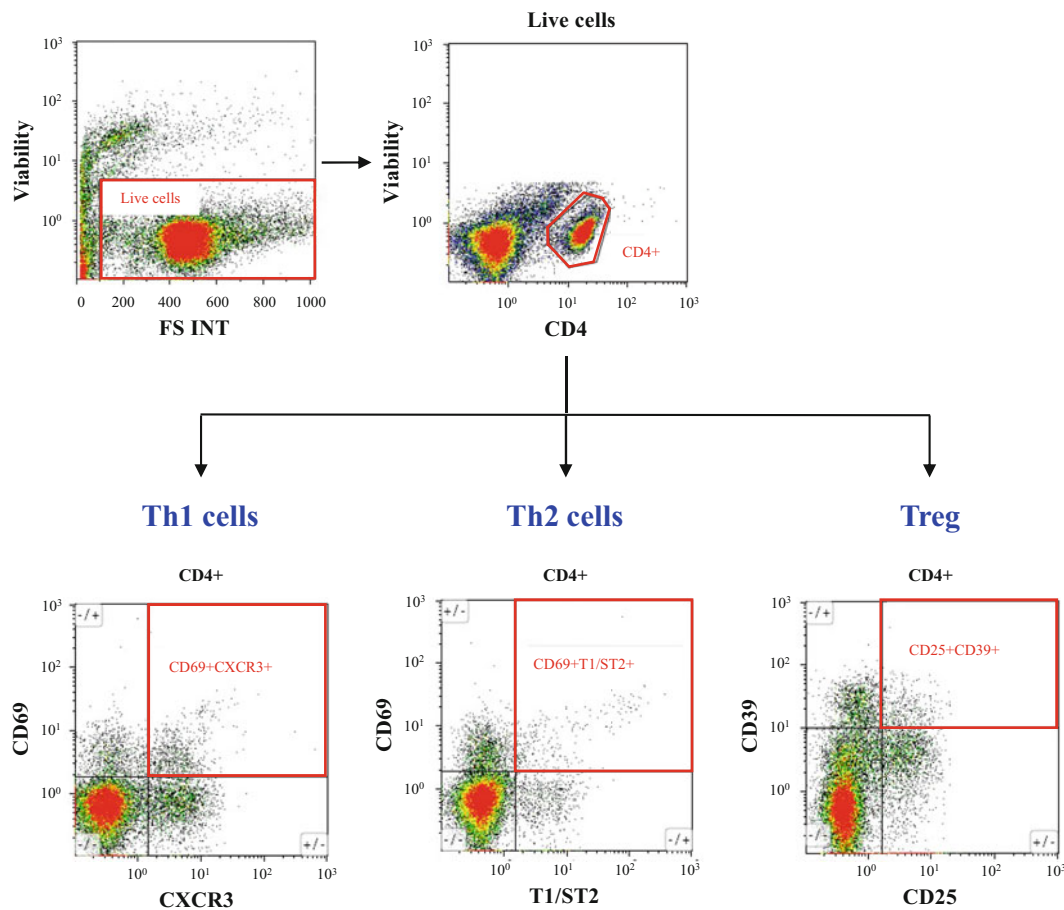


Fig. 4 Gating strategy for identification of T-cell subsets in the mesenteric lymph nodes (MLNs) and spleens of mice sensitized to egg white (EW). Cells are gated based on the viability and CD4 staining, and subsequently analyzed based on their CD69, CXCR1, T1/ST2, CD25, and CD39 expression. Pictures show representative dot plots of T-cell analysis in the spleen

3.10 Intestinal Gene Expression

1. Disaggregate and homogenize the thawed intestinal samples using an electronic tissue homogenizer.
2. Perform RNA isolation following the specific instructions given by the RNA isolation kit manufacturer. Elute the RNA with 60 μL of RNase-free water to obtain approximately 1500 ng/mL of RNA.
3. Determine the integrity, purity, and concentration of the isolated RNA using an Agilent 2100 Bioanalyzer (*see Note 8*).
4. Use 2 μg of the RNA samples to synthesize cDNA following the specific instructions given by a selected cDNA synthesis reagent kit.
5. Prepare a master mix following the instructions provided by a selected real-time PCR reagent system. Use less than 100 ng of the synthesized cDNA as the template for qPCR reactions.
6. Reconstitute lyophilized primers (store at 10 μM and -80°C) in RNase-free water and use them at a final concentration of 100 nM in the reaction (*see Note 18*).
7. Perform the qPCR in a real-time thermocycler. The thermal cycling conditions include an initial denaturation step of 30 s at 95°C , followed by 40 cycles consisting of a denaturation step of 15 s at 95°C and a polymerase extension step. For the polymerase extension step appropriate for each primer set, follow the thermal conditions indicated in the Table 3. After qPCR cycling, perform a melting curve analysis to confirm the specific product formation.
8. For the analysis of the results, determine the relative gene expression by normalizing the data to the expression of a reference gene (e.g., *Actb*, *see Table 3*), as described by Livak and Schmittgen [15].

4 Notes

1. All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee and must follow officially approved procedures for the care and use of laboratory animals.
2. Separate the egg white from whole fresh hen's eggs and lyophilize it to concentrate EW. Its endotoxin content may be quantified using a commercial endotoxin detection kit, and protein composition (based on total nitrogen content) may be determined by the Kjeldahl's method (The procedure and performance characteristics of the method for determination of total nitrogen content of egg are described in both AOAC Method 991.20 [16]). Egg white solutions in sterile PBS can be made beforehand and stored in the -20°C freezer for 2 months.

3. Prepare an excess amount of each solution enough for three additional numbers of mice (or samples).
4. Five to six mice per group are usually enough to obtain statistically significant results.
5. Handle in a fume hood, with gloves, lab coat, and safety glasses.
6. Several panel designs targeting the same cell surface markers are possible. If another panel is designed, or if antibodies are bought from different suppliers, we recommend choosing the same antibody clones. New antibodies should first be validated, their optimal dilution determined, and the staining conditions decided before using them in the complete staining mixture.
7. This isolation kit is used as an example in this chapter, but any RNA isolation kit commercially available from different brands can be chosen depending on the experimental design.
8. The integrity of the isolated RNA can also be determined by agarose gel electrophoresis, and its purity and concentration can be determined by a NanoDrop spectrophotometer. A 260/280 ratio of ~2.0 is generally accepted for pure RNA and 260/230 values are commonly in the range of 2.0–2.2.
9. Pay attention and do not put the feeding tubes into the trachea.
10. Animals are individually isolated in an empty box for 5–10 min until 2–3 fecal pellets are obtained per animal.
11. Blot the plate on absorbent paper to remove any residual buffer.
12. For maximal sensitivity, incubate overnight at 4 °C.
13. One plate will be used for T-cell staining and a different one for DC staining.
14. Add 200 μ L of PBS to tubes containing the unstained and single staining controls instead of the LIVE/DEAD™ viability staining solution.
15. Resuspend the cells for unstained and viability controls with 25 μ L of FACS buffer instead of Fc-blocking solution.
16. For FMO controls, add all fluorophore-conjugated antibodies in the antibody mixture, *except* one. Use FACS buffer in the place of the excluded antibody. For single staining controls, resuspend the cell pellets in 50 μ L of FACS buffer containing only one fluorophore-conjugated antibody.
17. Acquire cells immediately or keep them in a fridge, covered in parafilm and foil for a maximum of 24 h. For longer storage periods (no more than 3 days), fix the cells after staining with 4% paraformaldehyde at room temperature for 5–10 min.
18. To determine the appropriate template amount, make serial dilutions of the synthesized cDNA. The optimal quantity varies depending on the number of target copies present in the template solution.

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