



Experimental Mouse Models of Ragweed- and Papain-Induced Allergic Conjunctivitis

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Abstract

Mouse models of allergic conjunctivitis mimic various aspects of human allergic conjunctivitis. They are useful as acute models of allergic conjunctivitis to study immunological aspects of this condition. In this chapter, we will describe ragweed-pollen-induced experimental allergic conjunctivitis (mostly driven by adaptive immunity), and papain-soaked contact lens-induced experimental allergic conjunctivitis (mostly driven by innate immunity). Giemsa staining of histological sections is used for quantification of the number of infiltrating eosinophils, which is useful to evaluate the severity of the allergic inflammation. Immunohistochemical staining and quantitative PCR are used to clarify spatiotemporal expression of proinflammatory molecules in the conjunctival tissue. Flow cytometric analysis of conjunctival tissue is used for the detection of innate lymphoid cell type 2 (ILC2) in the ocular surface tissues.

Key words Mouse conjunctivitis model, Allergy, Ragweed pollen, Papain, Immunohistochemistry, Quantitative PCR, IgE, Innate lymphoid cell type 2 (ILC2)

1 Introduction

Allergic conjunctivitis is a common disorder in developed countries. There are four subtypes of allergic conjunctivitis: seasonal/perennial allergic conjunctivitis (SAC/PAC), vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and giant papillary keratoconjunctivitis (GPC). In this chapter, we describe methods for the induction and analysis of a ragweed-induced experimental allergic conjunctivitis model (RW-EAC) and a papain-soaked contact lens-induced allergic conjunctivitis (papain-CL conjunctivitis) model, which mimic human SAC [1] and GPC [2], respectively. The RW-EAC model is IgE-dependent, whereas the papain-CL conjunctivitis model is IgE-independent [2, 3]. Mouse models are superior to other allergic conjunctivitis models using guinea pigs or rats because of the availability of a wide range of antibodies for functional analysis, genetic information, and genetically modified animals.

2 Materials

2.1 RW EAC Mouse Model

1. Ragweed pollen (RW): 19–20- μ m sized ragweed pollen collected from *Ambrosia artemisiifolia*.
2. 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 of Na₂HPO₄, 8.0 g of NaCl, 0.24 g of KH₂PO₄, and 0.2 g of KCl in distilled water and adjust the pH to 7.4. Bring the final volume to 1 L with distilled water.
3. Imject™ Alum Adjuvant: a commercially available formulation containing 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide.
4. Female BALB/c mice: 8–12 weeks old (*see Note 1*).
5. 1-mL Syringe.
6. 1.5-mL Microfuge tubes for eye and blood collection.
7. Micropipettes and tips.

2.2 Papain-CL Conjunctivitis Mouse Model

1. Soft contact lens: 2 mm in diameter, negatively charged, disposable. Trim commercially available contact lenses using a 2 mm diameter biopsy punch.
2. Papain solution: 25 mg/mL in PBS, pH 7.4. Prepare a fresh solution just before use (no need to be sterilized). Heat-inactivated papain solution is made by heating the papain solution at 95 °C for 30 min.
3. 96-Well cell culture plates: Used to soak contact lenses in the papain solution.
4. 8-0 nylon surgical suture.
5. Female C57BL/6 mice: 8–12 weeks old (*see Note 1*).

2.3 Tissue Collection

1. 1-mL Syringes.
2. Pentobarbital: 6.5 mg/mL in saline solution for general anesthesia.
3. 0.4% (w/v) Oxybuprocaine hydrochloride: for topical anesthesia of the eye.
4. Microscissors.
5. Dissection microscope.

2.4 Quantitative PCR (qPCR) of the Mouse Conjunctival Tissue

1. RNA stabilization solution: RNeasyLysis™ or equivalent.
2. Nuclease-free 1.5-mL microfuge tubes.
3. RNA isolation kit: NucleoSpin II or equivalent.
4. Reverse transcriptase kit for the preparation of cDNA.
5. RNase inhibitor.

Table 1
Primers used for quantitative PCR

Gene	Forward primer	Reverse primer
<i>il4</i>	5'-TCCAAGGTGCTTCGCATATTTT-3'	5'-CAGCTTATCGATGAATCCAGGC-3'
<i>il5</i>	5'-TCCAAGGTGCTTCGCATATTTT-3'	5'-TCCAATGCATAGCTGGTGATT-3'
<i>il13</i>	5'-GGCAGCAGCTTGAGCACATT-3'	5'-GGCATAGGCAGCAAACCATG-3'
<i>ccl5</i>	5'-ACTATGGCTCGGACACCA-3'	5'-ACACACTTGGCGGTTCT-3'
<i>ccl11</i>	5'-AGATGCACCCTGAAAG-3'	5'-GCATCCTGGACCCACT-3'
<i>il33</i>	5'-TCCAACTCCAAGATTTCCTCCG-3'	5'-CATGCAGTAGACATGGCAGAA-3'
<i>gapdh</i>	5'-AAGGGCTCATGACCACAGTC-3'	5'-GGATGACCTTGCCCACAG-3'

6. NanoDrop spectrophotometer or equivalent for quantitation of RNA.
7. PCR tubes with lids.
8. Forward and reverse primers: *See* Table 1.
9. Universal quantitative PCR reaction master mix: KAPA SYBR[®] FAST or equivalent.
10. Real-time qPCR thermal cycler.

2.5 Histological Analysis of Mouse Conjunctival Tissue

1. 10× Phosphate-buffered saline stock solution (10× PBS): 100 mM Na₂HPO₄, 18 mM KH₂PO₄, 1.37 M NaCl, 27 mM KCl, pH 7.4. Dissolve 14.4 g of Na₂HPO₄, 80 g of NaCl, 2.4 g of KH₂PO₄, and 2 g of KCl in distilled water and adjust the pH to 7.4. Bring the final volume to 1 L with distilled water.
2. 12% (w/v) Paraformaldehyde (PFA) stock solution: Dissolve 120 g PFA in 800 mL of distilled water on a hot plate (50–50 °C) with a magnetic stirrer. Add a few drops of 1 M NaOH until the solution becomes translucent. Adjust the final volume to 1 L with distilled water. Store at 4 °C.
3. 4% (w/v) PFA in PBS: Just before use, prepare 100 mL of 4% PFA solution by mixing 25 mL of 12% PFA stock solution, 10 mL of 10× PBS, and 65 mL of distilled water.
4. Ethanol solutions for dehydration and rehydration: 100% and 70% ethanol solutions for rehydration; 50%, 70%, 80%, 90%, 95%, and 100% ethanol solutions for dehydration.
5. Xylene.
6. Paraffin wax: histological grade.
7. Plastic molds: for embedding tissue in paraffin blocks.
8. Microtome for paraffin block sectioning.

9. Glass slides: histological grade, adhesive coated.
10. Giemsa staining solution: commercially available.
11. Mounting medium.
12. Coverslips.
13. 30% (w/v) Sucrose in PBS: Dissolve 300 g of sucrose in 1 L of PBS. Prepare just before use.
14. Plastic tissue-embedding mold: 15 mm × 15 mm × 5 mm.
15. Tissue-embedding compound: O.C.T. compound or equivalent.
16. Cryostat.
17. Blocking buffer: 10% normal donkey serum and 1% bovine serum albumin in PBS.
18. Hydrophobic pen.
19. Primary antibodies for immunohistochemical staining:
 - (a) Goat anti-mouse IL-33 polyclonal antibody.
 - (b) Rat anti-mouse major basic protein (MBP) antibody.
20. Secondary antibodies for immunohistochemical staining:
 - (a) Donkey Alexa 488-conjugated anti-rat IgG antibody.
 - (b) Donkey Alexa 594-conjugated anti-goat IgG antibody.
21. Anti-fade and nuclear staining mounting medium: 0.3 µg/mL DAPI, 3% DABCO, and 50% glycerol in distilled water.
22. Confocal laser scanning microscope.

2.6 Mouse Serum IgE Quantification

1. 96-Well ELISA plates and sealers.
2. Multichannel pipettors and tips.
3. ELISA kit for mouse IgE: commercially available.
4. Coating buffer: 100 mM NaHCO₃, 33.6 mM Na₂CO₃, pH 9.5. Dissolve 8.4 g NaHCO₃ and 3.56 g Na₂CO₃ in 800 mL of distilled water. Adjust the pH and bring the volume to 1 L.
5. Wash buffer: 0.05% (v/v) Tween 20 in PBS, pH 7.4.
6. Assay diluent: 1% bovine serum albumin in PBS.
7. Orbital shaker.
8. TMB substrate reagents: commercially available ELISA reagent set with 0.005% (w/v) 3,3',5,5'-Tetramethylbenzidine solution and hydrogen peroxide.
9. Stop solution: 2 N H₂SO₄.
10. Spectrophotometric microplate reader.

**2.7 Flow Cytometric
Analysis of Mouse
Conjunctival Tissue
for Innate Lymphoid
Cell Type 2 (ILC2)**

1. Dissection tools.
2. Plungers from 10-mL syringes.
3. Hank's balanced salt solution (HBSS): Dissolve 400 mg KCl, 60 mg KH_2PO_4 , 350 mg NaHCO_3 , 8 g NaCl, 48 mg Na_2HPO_4 , and 1 g D-glucose, in 800 mL of distilled water. Adjust the final volume to 1 L with distilled water.
4. FACS buffer: 2% fetal calf serum (FCS) in HBSS. Add 2 mL of FCS in 98 mL HBSS.
5. Cell strainers: 70 μm , sterile.
6. 50-mL Conical tubes.
7. Digestion buffer: HBSS containing 1 mg/mL collagenase type IV and 0.5 mg/mL DNase I.
8. 3-mL Syringes.
9. Red blood cell (RBC) lysis buffer: 155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 8.0. Mix 8.29 g NH_4Cl , 1 g KHCO_3 , and 0.2 mL of 0.5 M EDTA in 1 L of distilled water.
10. Fc receptor blocking reagent: rat anti-mouse CD16/CD32 antibody, clone FCR4G8.
11. Fluorochrome-conjugated monoclonal antibody cocktails: Mix 0.5 μg each of the following monoclonal antibodies per 1×10^6 cells in FACS buffer.
 - (a) Brilliant Violet 421-anti-mouse-CD45 (30-F11).
 - (b) PE-conjugated anti-mouse lineage marker cocktail: CD3 (145-2C11), CD19. (6D5), NK1.1 (PK136), CD11b (M1/70), Gr1 (RB-8C5), and Ter119 (Ter-119).
 - (c) FITC-conjugated anti-mouse ST2L (DJ8).
 - (d) Cy7-conjugated anti-mouse CD127 (A7R34).
 - (e) APC-conjugated anti-mouse CD25 (PC61).
 - (f) APC-Cy7-conjugated anti-mouse CD90.1 (30-H12).
12. FACS tubes with 70- μm nylon mesh tops.
13. 7-AAD (7-amino-actinomycin D) nuclear staining solution: commercially prepared 50 $\mu\text{g}/\text{mL}$ 7-AAD solution in PBS with 0.09% sodium azide, pH 7.2. Store in the dark at 2–8 °C. Use at the final concentration of 2 $\mu\text{g}/\text{mL}$ in FACS buffer.
14. MAQS Quant[®] flow cytometer or equivalent.

3 Methods

3.1 RW EAC Mouse Model

3.1.1 Sensitization

1. Mix 1 mg of RW with 1 mL of Imject™ Alum and stir overnight at room temperature to make RW emulsion (*see Note 2*).
2. For the first sensitization, inject 50 μ L of the emulsified RW into the left hind foot and root of the tail subcutaneously on Day 0.
3. Collect the serum from the tail vein blood for quantitation of presensitization IgE levels.
4. For the second sensitization, inject the emulsified RW into the right footpad subcutaneously on Day 14.

3.1.2 Allergen Challenge with RW Eye Drop Solution

1. Make an eye drop solution by mixing RW with PBS (2 mg in 10 μ L/eye) and vortex (*see Note 2*).
2. Using a micropipette, instill 10 μ L of the RW eye drop solution per eye from Days 26 to 29, once per day for 4 days (*see Note 3*).
3. Twenty minutes after the last eye drop challenge, Evaluate EAC using the following 12-point scoring system based on the criteria described by Magone et al. [4]. (Fig. 1; *see Note 4*).
 - (a) Chemosis: 0 = absent; 1 = mild; 2 = obvious; 3 = severe.
 - (b) Redness: 0 = absent; 1 = mild; 2 = moderate; 3 = severe.
 - (c) Lid edema: 0 = absent; 1 = mild; 2 = moderate; 3 = severe, unable to open eye.
 - (d) Tearing and discharge: 0 = absent; 1 = mild; 2 = obvious; 3 = severe.

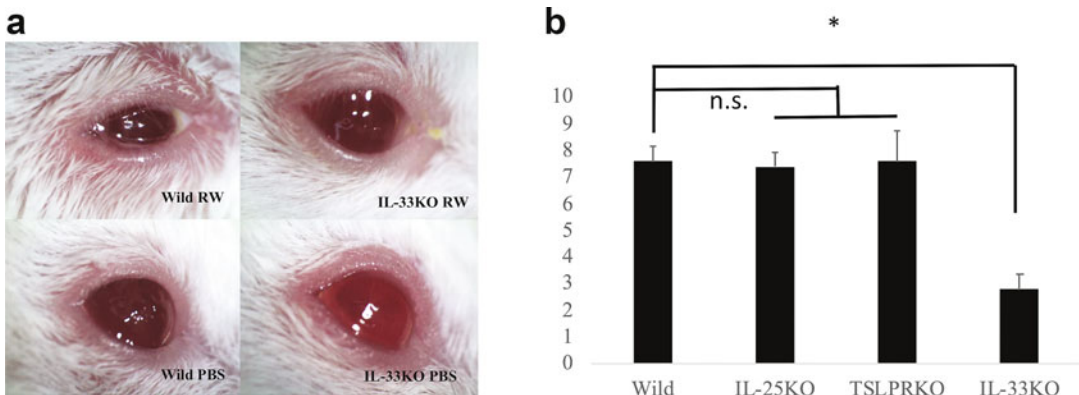


Fig. 1 Representative photographs of RW-EAC models, using wild-type mice and IL-33 knockout (KO) mice challenged either with RW-PBS (upper row) or with PBS alone (lower row) taken 20 min after the last eye drop challenge (**a**). The clinical scores of the RW-challenged EAC models are shown (**b**). * $P < 0.05$, Mann–Whitney U-test. *Wild* wild-type, *KO* IL-33 KO mice. Reproduced from Invest Ophthalmol Vis Sci. 56: 5194–5202, 2015 [1]

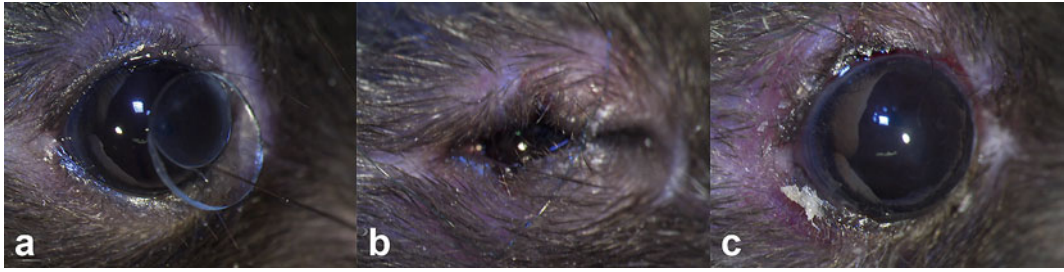


Fig. 2 Papain-contact lens (papain-CL)-induced inflammation in the mouse eye. On Day 0, a papain-CL is inserted into the conjunctival-sac of the right eye (a), and the eyelid is sutured with 8-0 nylon (b). The papain-CL is exchanged once on Day 2. The second papain-CL is removed, and the eye is sampled for further analysis on Day 5 (c). Reproduced from *Immun Inflamm Dis.* 5: 515–525, 2017 [2]

4. At 24 h after the last eye drop challenge, euthanize the mice by a method approved by your institution. Collect the eyeballs (with lids and conjunctival tissue) for histological and gene expression analysis, and blood for postantigen challenge IgE levels in the serum.

3.2 Papain-CL Conjunctivitis Mouse Model

1. Soak negatively charged soft contact lenses (CLs) in 25-mg/mL papain solution for 24 h to prepare papain-CLs (*see Note 5*). For this purpose, dispense 200 μ L of the papain solution to 96-well cell culture plates and immerse a 2-mm diameter soft contact lens in each well.
2. Anesthetize the animals by administering an intraperitoneal injection of pentobarbital at 50 mg/kg body weight. Instill oxybuprocaine hydrochloride topical anesthesia eye drops into the eyes (*see Note 6*).
3. After general anesthesia, place a papain-CL in the conjunctival sac of the right eye (Day 0). Suture the eyelid with an 8-0 nylon suture (Fig. 2). Use either heat-inactivated papain-CLs or PBS-soaked CL as a negative control.
4. Two days after the initial surgery (Day 2), repeat **steps 1–3** above to replace the first papain-CL with a newly prepared papain-CL in the same conjunctival sac. Resuture the eyelids.
5. Three days after the second surgery (Day 5), euthanize the mice and remove the second papain-CL. Collect the eyelid and conjunctival tissue for gene expression analysis, histological analysis, and/or fluorescence-activated cell sorting (FACS) analysis.
6. For gene expression analysis, immerse the enucleated eyes in RNeasy lysis solution. Collect conjunctival tissue under a dissecting microscope using microscissors and forceps (Fig. 3). Store the tissue RNeasy lysis solution at -20°C until analysis.

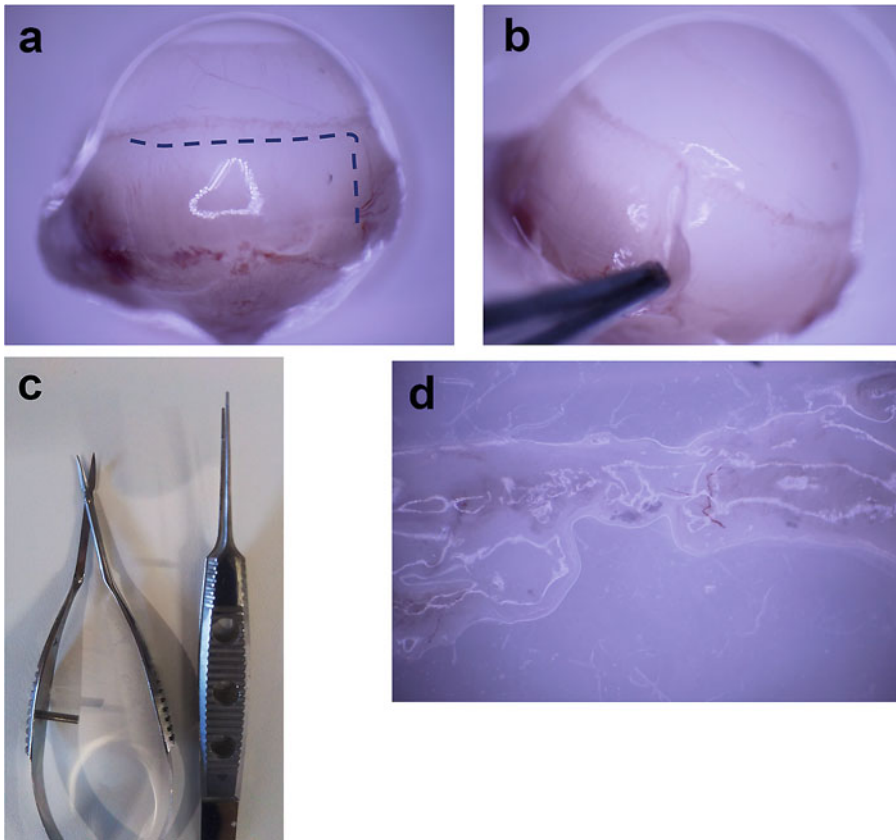


Fig. 3 Conjunctival tissue separation from an eyeball. Cut (a: as dotted line) and peel off (b) conjunctival tissue from the eyeball using microscissors and forceps (c). Isolated conjunctival tissue is shown as a sheet (d)

7. For histological analysis, enucleate the eye with the eyelid using microscissors and fix the tissue immediately with 4% PFA.
8. For FACS analysis, keep the enucleated eyes and microdissected conjunctival tissue in FACS buffer.

3.3 Quantitative PCR (qPCR) for Gene Expression Analysis of Th2 Cytokines

1. Isolate conjunctival tissues from mouse eyes using microscissors and forceps under a dissecting microscope (Fig. 3). Immerse the isolated tissues immediately in RNAlater solution and keep at -20°C until RNA extraction.
2. Extract total RNA from the conjunctival tissues using a NucleoSpin II RNA Isolation Kit or equivalent according to the manufacturer's instruction (see Note 7). Quantify the amount of RNA in each sample using a NanoDrop spectrophotometer.
3. Prepare cDNA from the conjunctival RNA extracts using a reverse transcriptase kit with random primers and RNase inhibitors according to the manufacturer's protocol.

4. Quantify the relative expression of the target genes by qPCR using a real-time thermal cycler using the primers listed in Table 1 (*see* **Note 8**). The conditions of qPCR depend on the machine and reagents used for qPCR analysis. If KAPA SYBR[®] FAST qPCR master mix is used with a Light Cycler (Roche Diagnostics), use the following condition: 3-min preincubation at 95 °C; 40 cycles of 3-step amplifications (95 °C for 10 s, 60 °C for 20 s, 72 °C for 1 s).

3.4 Histological Analysis of the Mouse Conjunctiva

3.4.1 Giemsa Staining for Eosinophil Counting

1. Dissect mouse eyes with conjunctival tissues and the eyelids intact and fix in 4% PFA for 3 h at 4 °C.
2. Dehydrate the tissue through ethanol gradients by incubating in 50%, 70%, 80%, 90%, 95%, and 100% ethanol solutions. Transfer the tissue in xylene to clear.
3. Incubate the tissue in melted paraffin at 58–60 °C. Place the tissue in a plastic mold and embed it in a paraffin block.
4. Make vertical 2- μ m-thick sections on a microtome and mount them on glass slides (*see* **Note 9**).
5. Deparaffinize, rehydrate, and stain the sections with Giemsa solution according to the supplier's instructions.
6. Dehydrate the sections as described in **step 2**. Coverslip to observe under the microscope (Fig. 4).
7. Count the infiltrating eosinophils in the lamina propria of the tarsal and bulbar conjunctiva throughout each section in the central portion of the eye.

3.4.2 Immuno-histochemical Staining of the Conjunctival Tissue

1. Trim the eye and eyelids under a dissecting microscope after 30 min fixation in 4% PFA.
2. Remove the lens and make toroidal tissue including the eyelid, cornea, conjunctiva, iris, and retina (Fig. 5).
3. After dissection, fix the eye tissue again in 4% PFA for another 30 min.
4. Immerse the tissue in 30% sucrose-PBS for cryoprotection for 30 min.
5. Take the tissue out of the sucrose-PBS solution and remove the excess solution using filter paper (*see* **Note 10**).
6. Transfer the tissue in a plastic mold to embed in O.C.T. compound and freeze on a liquid nitrogen-cooled metal plate. Do not freeze the tissue directly in liquid nitrogen to prevent cracking of the tissue.
7. Make 5- μ m-thick frozen sections using a cryostat and air-dry the tissue sections on glass slides.
8. Rinse the slides in PBS and incubate in the blocking buffer for 30 min at room temperature.

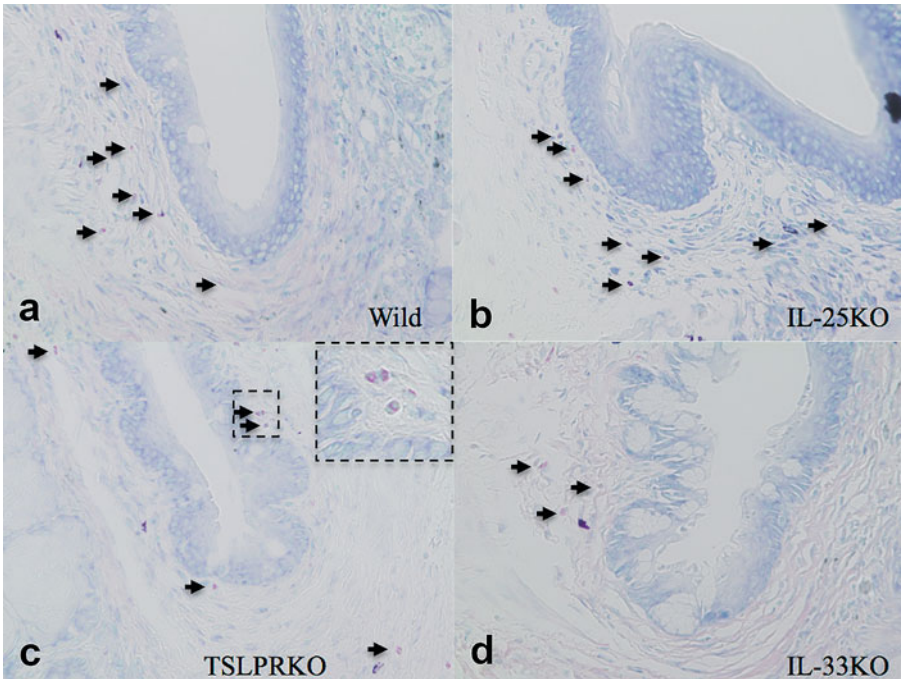


Fig. 4 Infiltrating eosinophils are identified by Giemsa staining. Compared to wild-type (a) and IL-25 KO (b) mice, significant reduction in eosinophil numbers is observed in the models using TSLP receptor (c: TSLPRKO) and IL-33(d: IL-33KO) knockout mice. Infiltrating eosinophils are shown at a higher magnification in the inset (dotted box in c). *Wild* wild-type, *KO* knockout mice. Reproduced from *Immun Inflamm Dis.* 5: 515–525, 2017 [2]

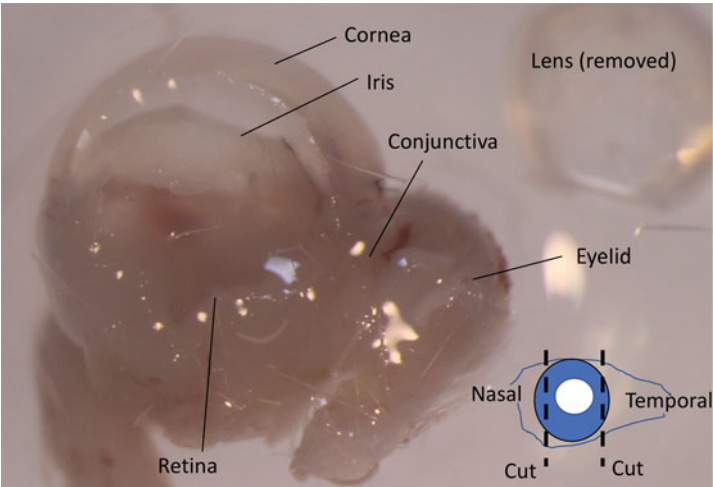


Fig. 5 Enucleated eye and eyelids are dissected using microscissors. Parts of corneal and scleral tissues are removed from both the nasal and temporal sides. The lens is also removed. The toroidal-shaped eye tissue can be embedded in O.C.T. compounds using a plastic mold

9. Use a hydrophobic pen to draw circles around the tissue sections on the slides to minimize the amount of antibody solution.
10. To perform double immunostaining, apply anti-IL-33 (1:100 dilution using donkey blocking buffer) and anti-MBP antibody solutions (1:500 dilution with the blocking buffer) within the circles on the slides, covering the eye sections completely. As negative controls, incubate additional specimens to be incubated with control goat IgG or rat IgG at the same concentrations as the IL-33 and MBP primary antibodies, respectively.
11. Incubate the slides in the antibodies for 2 h at room temperature or overnight at 4 °C.
12. Remove the antibody solution and wash the tissue sections in PBS for 5 min, 3 times.
13. Apply two secondary antibodies (Alexa-488-anti-rat IgG and Alexa-594-anti-goat IgG antibodies, dilution 1:1000 each) to the slides for 30 min at room temperature.
14. Remove the antibody solution and wash the tissue sections in PBS for 5 min, 3 times.
15. Coverslip using an anti-fade mounting medium. Use a confocal laser scanning microscope to observe MBP-positive cytoplasmic staining in green (a marker for eosinophils) and IL-33 positive nuclear staining in red (Fig. 6).

3.5 Mouse Serum IgE Quantification

1. Collect blood samples from mice at presensitization (Day 0) and postchallenge (Day 30) to determine the total serum IgE levels.
2. Leave the collected blood samples in 1.5-mL microfuge tubes for 20–30 min at room temperature to coagulate the blood.
3. After coagulation, centrifuge the tubes for 5 min at $20,000 \times g$ and transfer the supernatant (serum) in new microfuge tubes. Store at -80°C until analysis.
4. Select a commercial ELISA kit for total IgE detection. One day before ELISA analysis, coat 96-well microwell ELISA plates with mouse IgE capture antibody diluted in the coating buffer. Seal the plate and incubate overnight at 4 °C.
5. Remove the captured antibody and rinse the wells with the wash buffer. Block the wells with the assay diluent for 2 h at room temperature.
6. If frozen serum samples are used, thaw the sample completely and keep in the ice until dilution. Make 1:10 dilution of Day 0 (preimmune) sera and 1:100–1:1000 dilution of Day 30 (postchallenge) sera using the assay diluent of the selected ELISA system (*see Note 11*).

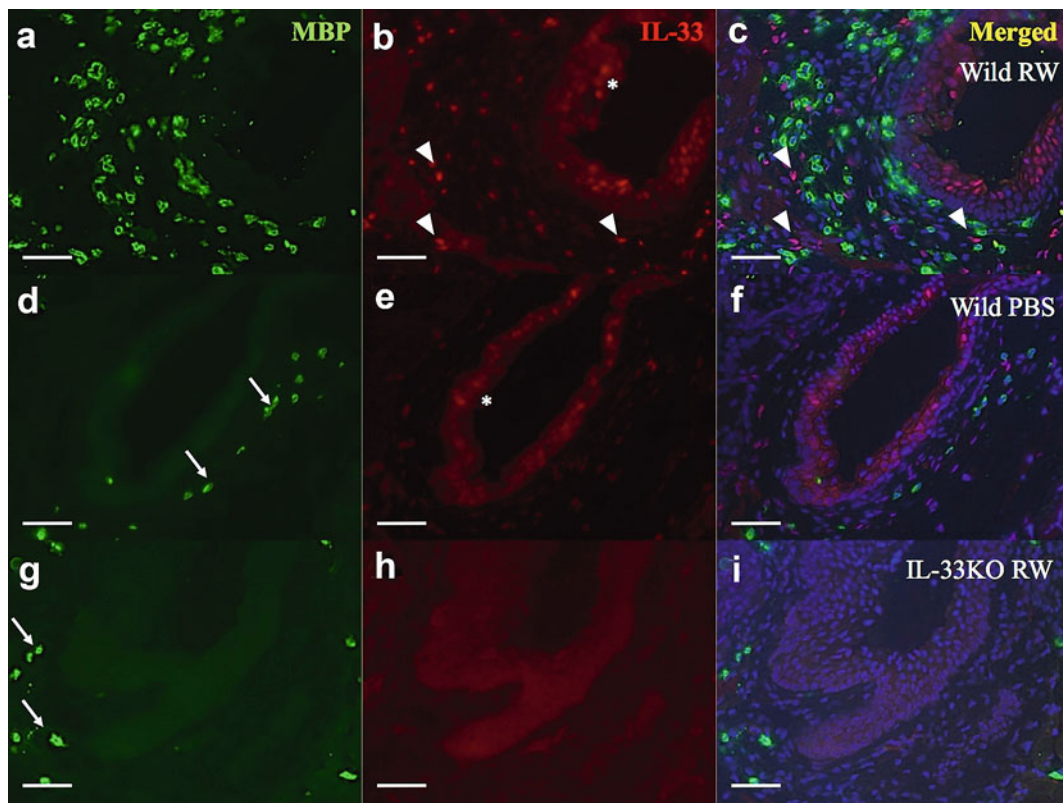


Fig. 6 MBP (a, d, g) and IL-33 (b, e, h) immunostaining of the conjunctivae obtained from RW-EAC models is shown. Massive infiltration of MBP-positive eosinophils (green) is observed in the substantia propria of RW-challenged EAC in wild-type mice (a). On the other hand, sparse infiltration of eosinophils is observed in PBS-challenged wild-type mice (d) and RW-challenged IL-33 KO mice (g). IL-33 protein expression (asterisks) can be observed in the conjunctival epithelial cells of the wild-type mice (b, c, e, f). The cells infiltrating the substantia propria in the vicinity of MBP-positive eosinophils (arrowheads) are also immunopositive for IL-33 (b, c). No IL-33 positive cells are observed in the conjunctiva of IL-33 KO mice (h, i). *Wild* wild-type, *KO* knockout mice. Scale bar = 50 μ m. Reproduced from Invest Ophthalmol Vis Sci. 56: 5194–5202, 2015 [1]

7. After blocking the plate, remove the assay diluent and add 100 μ L of the IgE standard and serum samples to the wells and incubate for 2 h with gentle shaking.
8. Wash the plate with the wash buffer 4 times. Add 100 μ L of the detection antibody and incubate for 1 h at room temperature.
9. Remove the detection antibody and wash it again 4 times with the wash buffer.
10. Add 100 μ L of TMB substrate solution and incubate in the dark for 20 min.
11. Add 100 μ L of stop solution to terminate the color development and read the absorbance at 450 nm using a microplate reader.

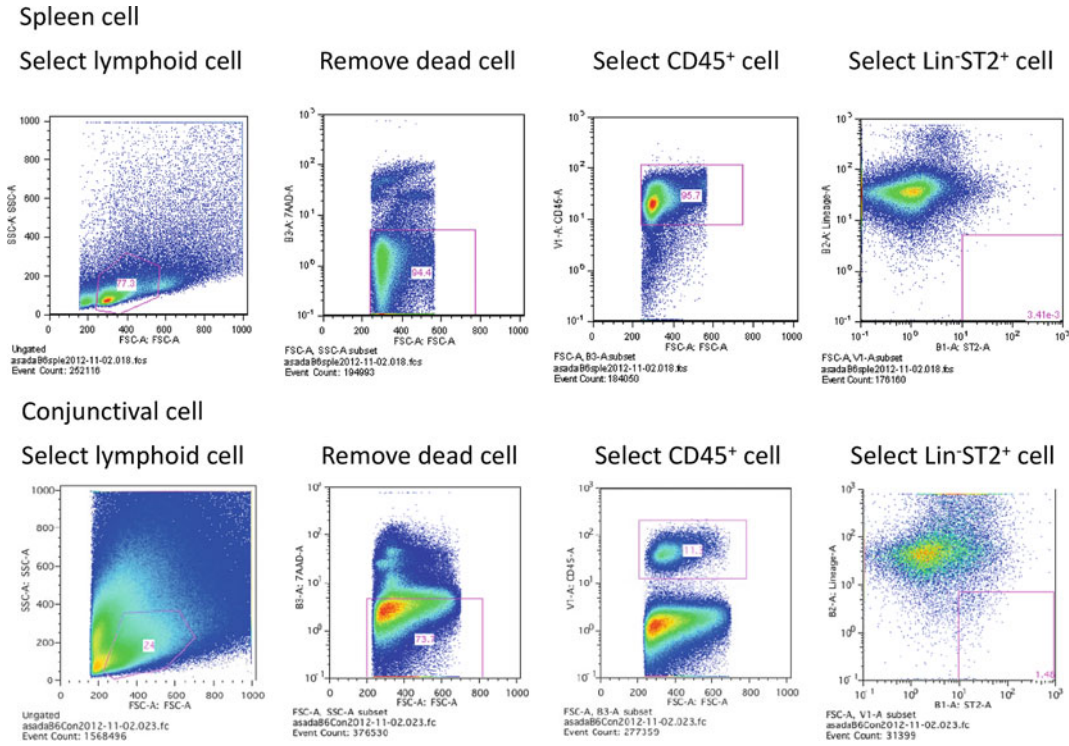
3.6 FACS Analysis of Mouse Conjunctival Tissue

3.6.1 Cell Preparation

1. For spleen cells, harvest the spleen from each mouse and use one half of the spleen to prepare spleen cell suspension. Triturate the spleen tissue with the flat end of the plunger of a 10-mL disposable syringe.
2. For conjunctival cells, collect the conjunctival tissue around the sensitized eye from each mouse (*see Note 12*). Pool conjunctival tissues from 10 mice (=10 eyes) and mince the tissues using scissors. Incubate the tissue fragments in the digestion buffer for 15 min at 37 °C, and then triturate the tissue fragments using the end of a 10-mL syringe plunger.
3. Set a 70- μ m cell strainer on top of a 50-mL centrifuge tube and pass the cell suspension prepared above through the cell strainer. Fill the tube with 10–15 mL of HBSS for washing.
4. Centrifuge the samples at $400 \times g$ for 5 min at 4 °C and discard the supernatant.
5. Resuspend the cells with 1 mL of RBC lysis buffer and incubate for 5 min at room temperature.
6. Fill the tube with 10–15 mL of HBSS for washing.
7. If cell debris is seen, repeat **step 3** and strain the debris.
8. Centrifuge the samples at $400 \times g$ or 5 min at 4 °C and discard the supernatant.
9. Resuspend the cells in 500 μ L of FACS buffer and transfer to 1.5-mL microfuge tubes. Usually, $1\text{--}5 \times 10^6$ cells are obtained from conjunctival tissues pooled from 10 mice, and $1\text{--}5 \times 10^8$ cells are obtained from one-half of spleen tissue.

3.6.2 Cell Staining

1. To the cell suspensions from Subheading 3.6.1, **step 9** above ($2\text{--}5 \times 10^6$ conjunctival cells and 1×10^8 spleen cells), add 1 μ g of Fc receptor blocking reagent. Incubate for 15 min at 4 °C.
2. Add 100 μ L of the fluorochrome-conjugated primary antibody cocktail or fluorochrome-conjugated isotype-matched control antibodies in FACS buffer. Mix gently by pipetting and incubate for 30 min at 4 °C in the dark.
3. Wash the cells 3 times with 1 mL of FACS buffer and centrifuging at $800 \times g$ for 1 min at 4 °C. Discard the supernatants and resuspend the cells with 200 μ L of FACS buffer.
4. Pass the cell suspensions through a 70- μ m nylon mesh into FACS tubes.
5. Add 200 μ L of 7-AAD nuclear staining solution and incubate for 5 min at room temperature.
6. Analyze the cell proportion in the cell suspension by flow cytometry as below.



3.6.3 Flow Cytometric Analysis

1. Select the population of lymphocytes by gating using the scatter plot of FSC and SSC (Fig. 7: Select lymphoid cells).
2. Select the population of live cells by gating the 7-AAD-negative region (Fig. 7: Remove dead cells).
3. Select the population of cells by gating the CD45-positive region in live cells (Fig. 7: Select CD45⁺ cells). Most of the spleen cells are CD45-positive lymphoid cells.
4. Select the population of cells by gating the lineage marker-negative and ST2-positive region (Fig. 7: Select Lin⁻ST2⁺ cells).
5. Further confirm the identity of the cells as ILC2 [5] by assessing the cell surface expression of CD25, CD127, and CD90.1 (Fig. 8). Most of the Lin⁻/CD45⁺/ST2⁺ cells are ILC2.

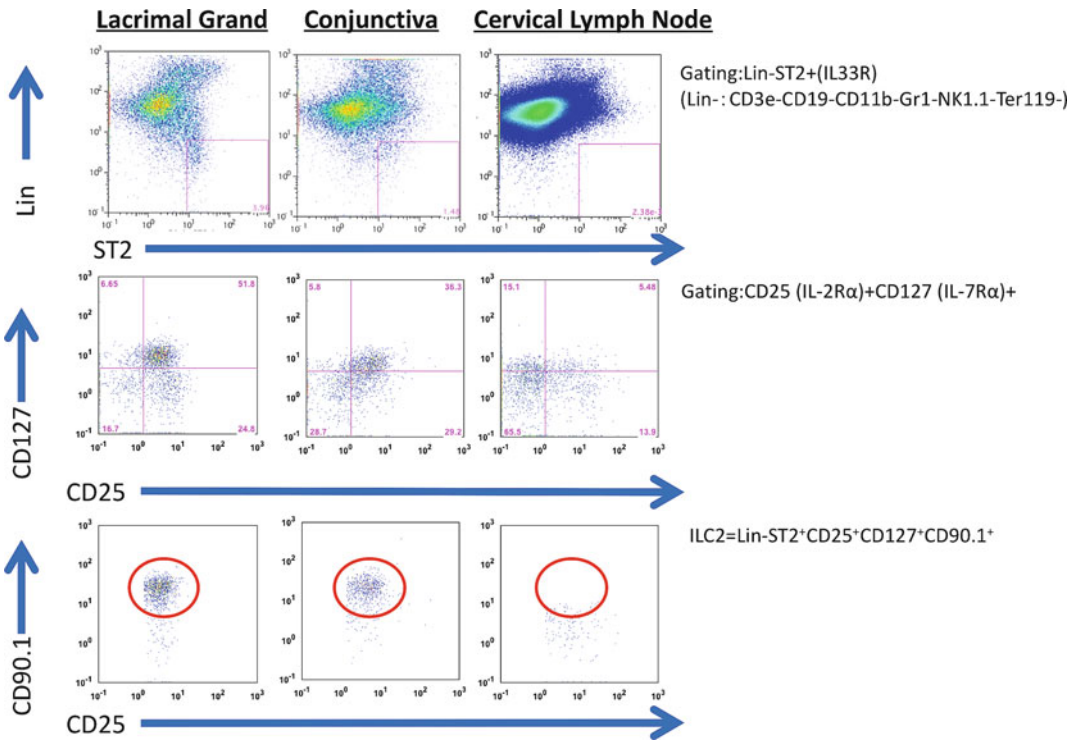


Fig. 8 Detection of ILC2 in the mouse conjunctival tissue by flow cytometry. ILC2s (Lin⁻ST2⁺CD25⁺CD127⁺CD90.1⁺) are identified in the conjunctival tissue (bottom left) and lacrimal gland tissue (bottom center) from the papain-CL model. On the other hand, only a few ILC2s are detected in the cervical lymph node (bottom right). Reproduced from Immun Inflamm Dis. 5: 515–525, 2017 [2]

4 Notes

1. The BALB/c strain is preferred for the RW-EAC model because the mouse has a Th2-prone nature. We use the C57BL/6 strain for the papain-CL conjunctivitis model because of the availability of genetically modified mice.
2. It is essential to stir overnight to make the alum-RW emulsion. Mix RW-PBS eye drops well before instillation into the eye. We usually prepare 300 μ L of solution for 10 mice.
3. After the instillation of the RW eye drops, the mouse should be kept on a rough mesh on top of the mouse cage by gently holding the tail to keep the mouse from escaping. Allow the mouse to blink and scratch for 3 min. This procedure facilitates RW particle retention within the conjunctival sac and eyelids.
4. For clinical grading, we recommend video recording of the mouse eye under a dissecting microscope using a constant magnification. Clinical evaluation can be carried out by

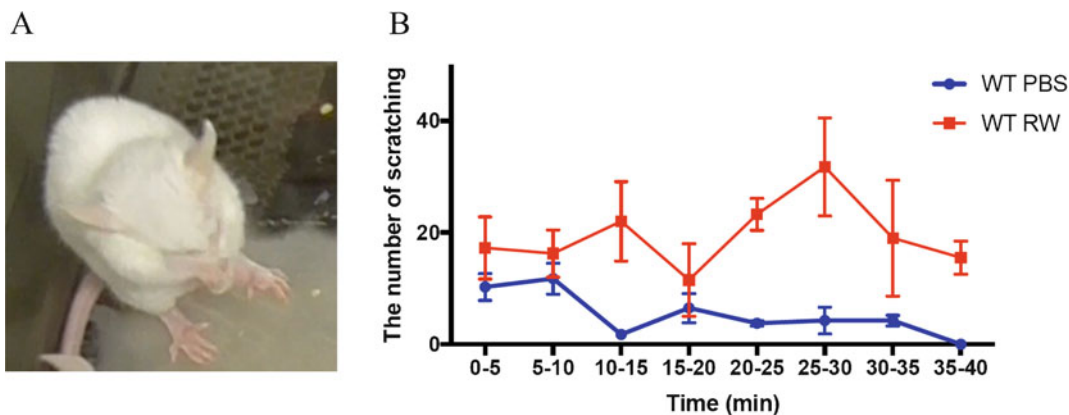


Fig. 9 A mouse challenged with RW eye drop is showing scratching behavior (a). The number of scratching behaviors is counted (b). Time (min) indicates the time after RW eye drop instillation. *WT PBS* wild-type mouse challenged with PBS eye drops. *WT RW* wild-type mouse challenged with RW eye drops. Typically, the number of scratching behaviors peaks at 20–30 min after RW eye drop instillation

examiners familiar with ophthalmological findings. Counting the number of scratching behaviors is one of the indicators of the itching sensation [6] (Fig. 9).

5. It is essential to use negatively charged contact lenses to retain papain molecules on the surface of the contact lens because papain is positively charged. Negatively charged disposable soft contact lenses can be obtained from commercial sources; we use 1-Day Acuvue Moist and trepan the lenses using a 2-mm diameter biopsy punch.
6. General anesthesia of mice may be performed in various ways. We also carry out general anesthesia using sevoflurane with a vaporization machine. Methods of anesthesia should adhere to the regulations of the animal research committee at your institution.
7. Typically, conjunctival tissue from one eye yields 500–1000 ng of RNA. The RNA concentration and quality should be estimated using the NanoDrop spectrophotometer (Thermo Fisher) or equivalent. RNA yield and quality may be affected by the method of conjunctival tissue disruption. We perform tissue disruption in the lysis buffer of the RNA isolation kit using microscissors. Tissue Ruptor (Qiagen) is also useful.
8. Primer pairs for SYBR Green-based qPCR analysis can be designed using Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) software. The relative gene expression is quantified by comparative Ct methods using mouse *gapdh* expression in the same cDNA as the controls.

9. Care should be taken to make vertical (not oblique) sections and not to include the nictitating membrane. It is important to fix the eye with eyelid tissue in the wide-open position. If the eyelids are closed, it is difficult to maintain the vertical plane of the eyeball at the time of paraffin embedding.
10. This step prevents curling of the tissue during the cryosection procedure.
11. The optimal dilutions of serum samples need to be determined by individual laboratories. The total serum IgE concentration is affected by the environment of the experimental mouse. For example, total IgE tends to be lower in the specific-pathogen-free condition compared to the conventional condition.
12. For the papain conjunctivitis model, only one eye may be sensitized to establish conjunctivitis in a mouse. Therefore, conjunctival tissues from one eye are harvested from each mouse.

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