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Abstract	Th2 responses such as peripheral and tissue eosinophilia are characteristic features in the host animals infected with *Strongyloides venezuelensis* and *Trichinella spiralis*. Th2 responses are characterized by a specific profile of cytokines and chemokines induced during the course of infection. In this chapter, we describe the methodology that is utilized in our laboratories to study the production of cytokine, chemokine, and antibodies related to the eosinophilia seen in mice infected with the parasites. Furthermore, protocols are described for the different methods used to study eosinophil functions in the blood and tissues of these experimental models of parasitic infections.	
Keywords (separated by "-")	*Strongyloides venezuelensis* - *Trichinella spiralis* - Eosinophil - CCL11/eotaxin-1	

Chapter 18

Qualitative and Quantitative Studies of Eosinophils in Parasitic Infections

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Abstract

Th2 responses such as peripheral and tissue eosinophilia are characteristic features in the host animals infected with *Strongyloides venezuelensis* and *Trichinella spiralis*. Th2 responses are characterized by a specific profile of cytokines and chemokines induced during the course of infection. In this chapter, we describe the methodology that is utilized in our laboratories to study the production of cytokine, chemokine, and antibodies related to the eosinophilia seen in mice infected with the parasites. Furthermore, protocols are described for the different methods used to study eosinophil functions in the blood and tissues of these experimental models of parasitic infections.

Key words *Strongyloides venezuelensis*, *Trichinella spiralis*, Eosinophil, CCL11/eotaxin-1

1 Introduction

Chemokines are derived from heterogeneous sources and serve to direct leukocytes to sites of inflammation. Eosinophilia is strongly linked with parasitic infections. Eotaxin is a fundamental regulator of eosinophil trafficking during both healthy status and inflammation. Expression of CCL11/eotaxin-1 and CCL24/eotaxin-2 in various tissues correlates with the number of eosinophils infiltrating inflammatory tissues. During infection with *T. spiralis*, CCL11/eotaxin-1 is important in intestinal tissue eosinophilia but not in peripheral eosinophilia, while CCL24/eotaxin-2 is only induced in the infected intestine [1]. Increased levels of CCL11/eotaxin-1 have been observed in the sera of patients with *Strongyloides stercoralis* when compared to healthy donors [2]. Eosinophils have been suggested as effector cells for innate immunity to *S. stercoralis* larvae [3] and for adaptive immunity to *S. venezuelensis* [4] and *S. ratti* [5]. However, the precise role and function of eosinophils still remain uncertain during the course of parasitic helminth infections [6, 7]. We describe here parasitological and immunological methodologies for studying the role of eosinophils in parasitic infections.

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2 Materials

2.1 Serial Passage and Experimental Infection of Parasites and Antigen Preparation

1. Filter paper (30 × 30 cm, No. 50).
2. 250 μL Glass syringes with a 27G needle (Ishizawa Manufacturing Co., Ltd., Ibaraki, Japan).
3. Penicillin G potassium (10^5 units/mL) and streptomycin sulfate (100 mg/mL, were diluted in sterile saline, aliquoted, and stored at −20 °C.
4. Phosphate-buffered saline (PBS): 9.6 g of the powder containing 8,000 mg sodium chloride, 200 mg potassium chloride, 1,150 mg disodium phosphate (anhydrous), and 200 mg monopotassium phosphate (anhydrous) dissolved in 1 L distilled water; sterilize by autoclaving.
5. A testing sieve (JIS Z8801, aperture 75 μm, wire diameter 52 μm, Iida Manufacturing Co., Ltd. Osaka, Japan).

2.2 Collection of Samples

1. Teflon feeding tube (1.2 × 50 mm), Fuchigami Kiki, Kyoto, Japan.
2. Homogenizer, sonicator, standard centrifuge.
3. TRIzole.
4. Gilconium ball (5 mm in diameter, Asone Corporation, Osaka, Japan). [AU2]
5. Carnoy's fixative: Ethanol (6 vol.), chloroform (3 vol.), acetic acid (1 vol.).

2.3 Cytospin, Cell Culture, ELISPOT, ELISA, PCR, and Histology

1. Diff-Quik.
2. Hinkelman's solution: 0.5 % w/v Eosin Y, 0.5 % w/v phenol, and 0.185 % v/v formaldehyde in distilled water.
3. Cytocentrifuge.
4. Cell strainer (40 μm Nylon): 48- or 96-well cell culture plate.
5. Cell culture medium: RPMI-1640 medium supplemented with 2 mM L-glutamine, 5 % heat-inactivated fetal bovine serum (FBS), 0.1 mM sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL streptomycin, 15 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid (HEPES), and 0.05 mM 2-mercaptoethanol.
6. RPMI-1640 containing 2 % FBS and 30 mg/L DNase I.
7. Tris (2.05 g/L)-buffered ammonium chloride (7.5 g/L).
8. ELISPOT assay multiscreen HA nitrocellulose filter-based 96-well plate (Merck Millipore, Billerica, MA, USA). Capture antibody: 10 μg/mL anti-mouse CCL11/eotaxin (affinity-purified goat IgG). Detection antibody: dilute appropriately anti-mouse CCL11/eotaxin (biotinylated affinity-purified goat IgG).

9. ELISPOT Color-development reagent: Dissolve 8 mg of 4-chloro-1-naphthol in 1 mL of ethanol. Add 20 mL of 50 mM Tris/HCl (pH 7.5). Filter the solution to remove debris. Add 7 μL of 30 % H_2O_2 just before use.
10. Takara PrimerScript RT regent kit (Takara Bio Inc., Shiga, Japan).
11. SYBER Green (FastStart Universal Probe Master (ROX), Roche-Diagnostics, Tokyo, Japan).
12. MixerMill, thermal cycler (PCR system 9700) and Genetic analyzer.
13. The primer set of CCL11/eotaxin-1 and β-actin: CCL11/eotaxin-1: Upper primer; 5′ GGC TTC ATG TAG TTC CAG AT 3′, lower primer; 5′ TTC CTC AAT AAT CCC ACA TC 3′. β-Actin: Upper primer; 5′ AGC ACC ATG AAG ATC AAG 3′, lower primer; 5′ GTA AAA CGC AGC TCA GTA A 3′.
14. ELISA plate: 96-Well plates.
15. PBS/T: PBS containing 0.05 % Tween 20.
16. IgG1 conjugated to peroxidase.
17. TMB: 3,3′, 5,5′-Tetramethylbenzidine liquid substrate system.
18. PD-10 column (Amersham Biosciences Europe, Freiburg, Germany).
19. Water-soluble biotin *N*-hydroxysuccinimide ester.
20. Murine total IgE ELISA (Alpha Diagnostic, San Antonio, TX).
21. Avidin-peroxidase.
22. Orthophenylenediamine.
23. H_2O_2 (Sigma Aldrich).
24. 0.5 % Alcoholic Congo red solution of Highman.
25. Wright–Giemsa stain.

3 Methods

3.1 Faecal Culture and Inoculation of S. venezuelensis

1. A strain of *S. venezuelensis* has been maintained by serial passage in 7–19-week-old male Wistar rats infected with 40,000 filariform larvae (L3) every 2 weeks. C57BL/6 or BALB/c male mice were infected subcutaneously with 1,000 L3 4 weeks before a challenge infection with the same dose.
2. Collect faeces from cages of rats which were infected with the parasites 6–8 days ago.
3. Spread paste of rat faeces on a sheet of filter paper (7.5 × 30 cm). Fold the sheet of filter paper and place in a 500 mL beaker containing 150 mL of tap water (*see* **Note 1**). Cover the top of beaker with aluminum foil (Fig. 1).

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Fig. 1 Faecal culture of *Strongyloides venezuelensis* using a filter paper

4. Incubate the faecal culture at 26–27 °C for 5–7 days.
5. Discard the filter paper, and collect the tap water containing L3. Filtrate debris through double-gauze clothes.
6. Decant and wash with saline. Repeat several times.
7. After washings, add 0.5 mL of conc. penicillin and streptomycin into the decanter. Keep it for at least 60 min and more.
8. Decant and wash with sterile saline.
9. Count the number of L3 in 10 μL of the suspension. Adjust the suspension to 1,000 L3/200 μL.
10. Anesthetize mice with diethyl ether and/or Nembutal, if necessary; shave skin on the trunk region of the mice 1 day before an inoculation. Subcutaneously inoculate 200 μL of the suspension using a glass syringe attached with a needle.

3.2 Experimental Infection with *T. spiralis*

1. A strain of *T. spiralis* has been maintained by serial passage in 7-week-old ddY or CD1 (outbred) mice. Mice are infected orally with 400 muscle larvae.
2. Sacrifice mice with diethyl ether.
3. Eviscerate and cut mouse carcasses into small pieces.
4. Digest them for 1 h for infection or overnight for counting muscle worm burden at 37 °C in a pepsin–HCl digestion fluid (*see* **Note 2**).
5. Pour and pass digested carcasses through double-gauze clothes into a 500 mL beaker.
6. Pour the fluid through the testing sieve.
7. Wash the muscle larvae on the sieve with running tap water.
8. Pour the muscle larvae into a funnel on a 50 mL graduated cylinder, and then fill with saline to the top of the cylinder.

9. Decant.
10. Wash and decant with saline.
11. Transfer suspension of muscle larvae to a 20 mL glass vial.
12. Count and adjust the number of larvae.
13. Make aliquots containing 400 muscle larvae per 200 μL of saline in Eppendorf tube (*see* **Note 3**).
14. Inoculate the larvae to mice orally using a feeding tube attached with a tuberculin syringe (*see* **Note 4**).

3.3 Parasite Somatic Crude Antigens

1. Homogenize *S. venezuelensis* L3 or *T. spiralis* muscle larvae using a high-speed homogenizer.
2. Sonicate the homogenate on ice.
3. Centrifuge the suspension at 435,000×*g* for 1[illegible] min by ultracentrifuge.
4. Measure the protein concentration of the supernatant.
5. Make aliquots, and store them at −30 °C until use.

3.4 Collection of Samples

1. At given days, sacrifice mice to collect tissues and organs.
2. Anesthetize mice with diethyl ether, cut a tip of the mouse tail, take each 5 μL of blood to put into 20 μL of Hinkelman's solution, and count the number of eosinophils in peripheral blood using an improved Neubauer hemocytometer.
3. To collect sera, make an incision on the inguinal skin to cut the femoral artery and vein to collect blood into Eppendorf tubes. Collect sera after clotting.
4. To collect bronchus alveolar lavage fluid (BALF), make an incision on the neck to expose the trachea. Put a cotton ligature under the trachea, and make a 0.5–1 mm long incision vertically on the trachea. Insert Teflon feeding tube (*see* **Note 5**) attached with a tuberculin syringe filled with 1 mL of cold PBS. If necessary, use PBS containing 1 % bovine serum albumin (BSA) or normal mouse serum. Tighten the ligature beneath the incision to secure the tubing. Slowly push and pull the piston of the syringe, making sure that both lungs are filled with PBS. BALF should be kept on ice.
5. For RT-PCR, collect a piece of tissues of the lungs, small intestine, spleen, lymph nodes, and skin into a 2 mL screw-capped tube containing cold 0.5–1 mL of TRIzole with a gilconium ball.
6. For pathology, keep the tissues in 5 % buffered formalin until use. When mucosal mast cells are to be examined, tissues are fixed with Carnoy's fixative overnight and processed.
7. To remove the femoral bone, and flush out the bone marrows into an Eppendorf tube using a tuberculin syringe with 25G needle containing 1 mL of cold PBS.

3.5 Cytospin

1. Count the number of cells in cell suspension from BALF, bone marrow washings, etc.
2. Centrifuge cell suspension at 400×*g* for 10 min. Keep the supernatant at −80 °C.
3. Adjust cell suspension at 1×10^6 cells/mL with 30 % normal mouse serum (*see* **Note 6**) in PBS.
4. Apply 100 μL of the suspension to a cytospin apparatus attaching slide glass.
5. Centrifuge at 650 rpm for 3 min.
6. Fix and stain cytospin specimens with Diff-Quik.

3.6 Cell Culture

1. Remove aseptically the spleen, mesenteric lymph nodes, etc. from mice.
2. Squeeze the tissues through a cell strainer with the plunger of a plastic syringe.
3. Rinse with 15 mL of RPMI-1640 containing 2 % FBS and 30 mg/L DNase I.
4. Allow the debris to settle.
5. Transfer cell suspension to a 15 mL tube.
6. Centrifuge 400×*g* for 10 min.
7. Resuspend in 2 mL of Tris/ammonium chloride for 2 min on ice to lyse erythrocytes.
8. Wash twice.
9. Resuspend in culture medium.
10. Count the number of cells using 0.1 % trypan blue solution.
11. Adjust cell suspension to 5×10^6 cells/mL (depending on the desired experiment).
12. Apply cells to 96- or 24-well flat-bottom culture plate.
13. Add antigen or supplements (depending on the desired experiment).
14. Incubate the culture plate for 48 h at 37 °C in a CO_2 incubator.
15. Centrifuge the plate at 400×*g* for 10 min to obtain supernatant.
16. Store the supernatant at −80 °C until use.

3.7 ELISPOT Assay

1. Coat each well of a multiscreen HA nitrocellulose filter-based 96-well plate with 50 μL of capture antibody and incubate at 4 °C overnight.
2. Wash three times with PBS.
3. Block each well with 250 μL RPMI-1640 containing 1% BSA at room temperature for 3 h or at 37 °C for 1 h.

4. Wash with RPMI-1640.
5. Seed cells at concentrations of 10^3, 10^4, and 10^5 cells/well in 100 μL of culture medium.
6. Culture at 37 °C for 2–6 h in a CO_2 incubator.
7. Wash three times with PBS, and wash once with PBS-T.
8. Apply 50 μL/well of detection antibody at 37 °C for 1 h.
9. Wash three times with PBS-T.
10. Apply 50 μL/well of HRP–streptavidin.
11. Wash three times with PBS-T.
12. Apply 150 μL/well of color-development reagent, and incubate at room temperature for 10–60 min in the dark.
13. Wash three times with DW.
14. Dry completely.
15. Count the spots using a dissecting microscope.

3.8 RNA Extraction, cDNA Synthesis, and Real-Time Quantitative PCR

1. Extract the total RNA from the lung or any tissues using TRIzole reagent.
2. Put a small piece (less than 50 mg) of the lung tissue into 0.5 mL TRIzole in a 2 mL tube containing a gilconium ball.
3. Shake vigorously the tube using MixerMill (30 cycles/s for 2 min).
4. After cell destruction, store the samples in a deep freezer (−80 °C) until use.
5. Extract RNA followed by the manufacturer's protocol (Invitrogen™).
6. Reverse-transcribe RNA samples (500 ng) to cDNA using Takara PrimerScript RT regent kit. Amplification conditions: 37 °C for 15 min, 85 °C for 5 s, and then 4 °C using a thermal cycler.
7. Perform real-time quantitative PCR (qPCR) in a 25 μL volume using SYBER Green. Amplification conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 63 °C for 1 min using a genetic analyzer.
8. Calculate the fold changes in mRNA expressions for targeted genes which are relative to the respective vehicle groups of mice after normalization to β-actin using ΔΔ Ct method.

3.9 ELISA for Parasite-Specific IgG

1. To detect parasite-specific IgG1 antibody, coat each well of a 96-well plate with 100 μL of the parasite antigen solution (*see* **Note 7**) at 4 °C overnight.
2. Wash four times with PBS/Tween 20.
3. Block with 100 μL/well of 1 % BSA in PBS at 37 °C for 1 h.
4. Wash with PBS/Tween 20.

5. Apply serum samples at 1:10 dilution.
6. Incubate at 37 °C for 1 h.
7. Wash four times with PBS/Tween 20.
8. Apply 100 µL/well of goat anti-mouse IgG1 conjugated to peroxidase at a 1:4,000 (v/v) dilution (*see* **Note 8**).
9. Incubate at 37 °C for 1 h.
10. Wash four times with PBS/Tween 20.
11. Apply 100 µL/well of TMB to the wells. The plate should be kept in the dark during the reaction.
12. Add 100 µL/well of 0.5 M H_2SO_4 to stop the reaction.
13. Read absorbance values using a spectrophotometer at 450 nm.

3.10 ELISA for Parasite-Specific IgE and Total IgE

Trichinella spiralis excretory/secretory (Ts E/S) antigen preparation and biotin labeling are performed according to Del Prete et al. [8], following the following steps:

1. Culture *T. spiralis* muscle larvae, obtained as described above, in RPMI-1640 medium containing streptomycin (500 µg/mL) at 37 °C in a 5 % CO_2 atmosphere.
2. After 18 h, collect the supernatant and desalt it into the appropriate buffer with a PD-10 column.
3. The TsE/S antigen protein concentration is estimated by means of absorbance at 280 nm using a Varian Cary Bio 50 spectrophotometer.
4. Incubate TsE/S antigen (4 mg/mL) in sodium bicarbonate buffer, pH 8.5, with water-soluble biotin *N*-hydroxysuccinimide ester for 2 h at room temperature.
5. Add glycine (10 mg) to stop the reaction, and dialyze extensively biotinylated TsE/S antigen against PBS.

3.11 Measurement of Total and Ts E/S-Specific IgE in Plasma

Total mouse IgE levels in the plasma are assessed by using a specific ELISA according to the manufacturer's instruction. Ts E/S-specific IgE in plasma samples is determined by means of a modification of the ELISA assay for total IgE:

1. Seed plasma samples diluted 1:2 in PBS in the microplates coated with the anti-mouse IgE derived from the commercial kit for total IgE assay (0.1 mL/well), and incubate it for 4 h at room temperature.
2. Wash microplates with PBS–0.05 % Tween 20.
3. Add biotinylated Ts E/S antigen (10 µg/mL).
4. Keep plates for 4 h at room temperature.
5. Wash microplates with PBS–0.05 % Tween 20.
6. Add avidin-peroxidase to each well.

7. Incubate with orthophenylenediamine for 30 min with H_2O_2; the plate should be kept in the dark during the reaction.
8. Stop the reaction with H_2SO_4.
9. Read OD at 490 nm by a microplate spectrophotometer.

3.12 Histochemical Identification of Eosinophils

Appropriate sections are stained with hematoxylin/eosin/azure II which stains eosinophils pink [9] or with 0.5 % alcoholic Congo red solution of Highman or modified Luna's methodology which stains eosinophils orange [10–12]. Wright–Giemsa stain can also be used in some tissue sections [13] to identify all granulocytes (Fig. 2).

1. Collect a sample of tongue at 42 days postinfection with *T. spiralis* from each sacrificed animal, and process them for routine histology of formalin-fixed paraffin-embedded tissues.
2. From each animal cut 5 μm sections of the tongue at different depths.
3. Mount the specimens on glass slides, and then after routine processing, stain with hematoxylin and eosin to evaluate total inflammation or with Congo red which is suitable for eosinophil counts.
4. Observe the slides by a microscope and acquire by a video camera. Image analysis system is accomplished using an appropriate software program (Adobe® Photoshop® CS3). Through software tools, the inflammatory infiltrate around the nurse cell–parasite complex is measured in pixels calculating the difference between the whole area of nurse cell–parasite complex plus surrounding inflammation and the area delimited by the collagen capsule. The inflammatory pixel value, analyzed for more than 300 larvae per experimental group on different sections, is then converted in μm^2 (50 μm^2 = 78,478.8 pixel) [14].

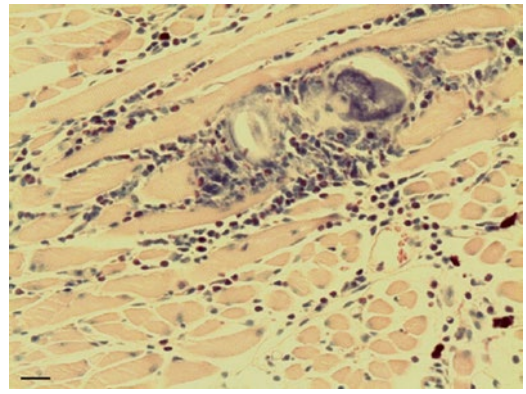

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Fig. 2 Tongue tissue of mouse experimentally infected with *Trichinella spiralis* on day 21. Giemsa stain. Bar = 50 μm

3.13 Other Techniques for Blood Eosinophil Count in Rodents

1. Collect peripheral blood in Na-heparin tubes from the retro-orbital venous plexus before infection and at different time points after infection.
2. Obtain blood smears and stain with May-Grünwald method to establish the percentage of eosinophils, and count total leukocytes in parallel by means of a Thoma's chamber or a cell counter after lysis of the erythrocytes with a hypotonic solution of NaCl [9]. Alternatively, dilute blood 1/2 with PBS containing 100 U/mL heparin.
3. Dilute the mixture 1/10 in Discombe's fluid (0.05 % eosin Y), and count eosinophils directly in a hemocytometer [15, 16].

3.14 Eosinophil Apoptosis Evaluation

1. Seed cells at a concentration of 1×10^6/mL in 48-well flat-bottom culture plates.
2. Incubate at 37 °C in a humidified atmosphere with 5 % CO_2.
3. Stain the smears obtained from these cells with May-Grünwald–Giemsa.
4. Count a number of apoptotic eosinophils in a total of 200 cells/slide under a light microscope at 1,000× magnification.
5. Use the following criteria to distinguish apoptotic from non-apoptotic cells: in both cases the cytoplasm granules are present. Normal eosinophils have ringlike nuclei, whereas those in apoptosis are smaller than normal and the nuclei are condensed basophilic with a round shape ([17] and Chapter 17).

4 Notes

1. Do not use deionized water.
2. Two hundred mL of 1 % pepsin in 1 % HCl solution per mouse: The solution can be stored in a freezer.
3. Use a yellow tip from which the tip end is cut.
4. Apply again 200 μL saline into the same syringe, and inoculate again. Sometimes the larvae remain inside the syringe.
5. The ball-ended tip of the tubing should be cut to an acute angle.
6. 5–10 % BSA can be used instead of serum to adhere cells to a glass slide.
7. Antigen solution is adjusted to 10 μg/mL in PBS containing 0.1 % normal goat IgG or BSA.
8. Checkerboard titration is necessary for every lot of antibody for appropriate dilution of sera and detection antibody.

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