



Chapter 2

Use of the *Litomosoides sigmodontis* Infection Model of Filariasis to Study Type 2 Immunity

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Abstract

Helminth parasites infect over 2 billion people worldwide resulting in huge global health and economic burden. Helminths typically stimulate Type 2 immune responses and excel at manipulating or suppressing host-immune responses resulting in chronic infections that can last for years to decades. Alongside the importance for the development of helminth treatments and vaccines, studying helminth immunity has unraveled many fundamental aspects of Type 2 immunity and immune regulation with implications for the treatment of autoimmunity and Type 2-mediated diseases, such as allergies. Here we describe the maintenance and use of *Litomosoides sigmodontis*, a murine model for studying host-parasite interactions, Type 2 immunity, and vaccines to tissue-dwelling filarial nematodes, which in humans cause lymphatic filariasis (e.g., *Brugia malayi*) and onchocerciasis (*Onchocerca volvulus*).

Key words Filariasis, Helminth, Parasite, Infection model, Type 2 immunity

1 Introduction

Infection of resistant (e.g., C57BL/6) and susceptible (e.g., BALB/c) strains of inbred mice with the filarial nematode *Litomosoides sigmodontis* provides a useful laboratory model for studying lymphatic filariasis and onchocerciasis [1]. As with the majority of helminth parasites, *L. sigmodontis* predominantly stimulates a Type 2 immune response, and resistance is dependent upon IL-4 and IL-5 [2, 3]. The model was developed in the laboratory of Dr Odile Bain [4, 5] and is the only filarial parasite that can undergo its full life cycle within inbred laboratory mouse strains. Additionally, *L. sigmodontis* lives within the cavity between the pleural membranes covering the lungs and ribcage, making it very easy to recover parasites and immune cells from the infection site. This has made it an invaluable model for studying immunity to human filarial nematodes, such as *Brugia* spp., *Wuchereria bancrofti*, *Loa loa*, and *Onchocerca volvulus*, which are host specific and do not fully develop in mice. The *L. sigmodontis* model has been

successfully used to study antifilarial vaccines, drug development, infection dynamics, the hygiene hypothesis, and infection-related and fundamental aspects of Type 2 immunity, immune suppression, and immune regulation.

The vector for *L. sigmodontis* is the mite, *Ornithonyssus bacoti*, which transmits the infective third-stage larvae (L3s) to the host during a blood meal. L3s migrate from the skin via the lymphatics arriving in the pleural cavity from day 3 postinfection (pi). Approximately half to two-thirds of larvae die before reaching the pleural cavity, the survivors remaining in the intrapleural space between the pleural membranes for the remainder of the infection. The L3 larvae molt to their fourth larval stage (L4) between 8 and 12 days postinfection (pi), before going through a final molt to the adult stage from days 25 to 30 pi [1]. The timings of the molts can vary between mouse strains. In susceptible strains of mice, male and female parasites develop fully, and the females start releasing the transmission stage microfilaria (Mf) from days 50 to 55 pi onward, which migrates to the blood and circulates in the bloodstream. A fully developed (patent) infection is defined by having detectable levels of Mf in the bloodstream, at which point the host can transmit infection, and is typically assayed at day 60 pi. Although *L. sigmodontis* establishes patent infections in mice, infection is cleared after day 80–100 pi. Long-term chronic infections can be maintained in both gerbils (*Meriones unguiculatus*) and its natural host the cotton rat (*Sigmodon hispidus*) and as a result are used as reservoirs to maintain the life cycle.

Here we describe a protocol for maintaining the *L. sigmodontis* life cycle and performing experimental infections based on the original protocol developed in the laboratory of Dr Odile Bain [4]. In this protocol infections are performed by subcutaneous (s.c.) injection of L3 larvae to allow precise control over the infection dose. However, this has the disadvantage that it bypasses the natural transmission process via mite feeding. Natural infections can be performed by exposing animals to infected mites [6], although it is more difficult to control the infection dose. More recently, a third approach has been described in which gerbils are naturally infected by exposure to *L. sigmodontis*-infected mites, and the L3 parasites are purified from the pleural cavity days 5–6 pi and subsequently used to infect mice [7]. This results in the recovery of large numbers of L3 with higher infectivity. However, it also changes the course of infection with mice developing a patent infection earlier.

2 Materials

2.1 Mite Colony Maintenance

1. 500 mL plastic Erlenmeyer flasks.

2. 20-micron gauze.
3. Fine grade animal bedding.
4. Elastic bands.
5. Climate chamber or room capable of maintaining 27 °C and 70% humidity.
6. Mouse cages and water bottles.
7. Mouse diet.
8. 70% ethanol.
9. Juvenile and/or adult mice.

2.2 *L. sigmodontis* Infections

1. Climate chamber or room capable of maintaining 27 °C and 70% humidity.
2. 500 mL Erlenmeyer flasks.
3. Fine grade animal bedding.
4. 20-micron gauze.
5. Elastic bands.
6. Distilled water.
7. Small paintbrush.
8. Trimmed paintbrush.
9. Metal sieve.
10. 500–1000 mL beakers.
11. Water moat (e.g., large tray or shallow sink).
12. Small trays or beakers for use as platforms.
13. Gerbil cages with bedding, diet, and water bottles.
14. Sealable plastic box with ventilated lid large enough to contain a gerbil cage.
15. Autoclaved glass tubes (approx. 1.5 cm in diameter, 6 cm tall).
16. Filter paper rectangles (1 cm × 2 cm).
17. Nonabsorbent cotton wool.
18. Tissue paper.
19. Cling film.
20. 250 mL glass beaker.
21. Dupont no. 5 forceps.
22. RPMI-1640 media with or without 10% sera (*see Note 15*).
23. Glass petri dish (5 cm).
24. 500 mL beaker of hot water with added detergent.
25. Dissection microscope with dark field (magnification × 6.3–60).
26. 15 cm glass Pasteur pipette and bulb.

27. Syracuse watch glasses.
28. 1 mL disposable syringes.
29. 0.6 × 25 mm (23 gauge (G) × 1 in.) needles.
30. Insect forceps.
31. Bunsen burner.
32. *L. sigmodontis*-infected gerbils or cotton rats.

2.3 Quantitating *L. sigmodontis* Infection Levels and Recovery of Immune Cells

1. Razor or sharp scalpel.
2. 0.5 mm × 16 mm (25G × 5/8 in.) needle.
3. 1.5 mL microcentrifuge tubes.
4. FACS lysing buffer (BD).
5. Hemocytometer.
6. Dissection kit.
7. PBS and/or cell culture media (RPMI-1640, 5% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine).
8. 70% ethanol (<20 dpi).
9. 4% formalin (>20 dpi).
10. 60-micron cell strainers.
11. Dissection microscope with dark field (magnification × 6.3–60).
12. Glass tissue grinder.

3 Methods

3.1 Maintenance of Uninfected *O. bacoti* Mite Colonies

An *O. bacoti* colony must be maintained as a source of uninfected mites for the experimental infections in Subheading 3.2. *O. bacoti* colonies are kept at 27 °C and 70% humidity in a temperature-controlled room or climate chamber (*see* **Note 1**). Water moats are used to contain mites, and all mite work is carried out on raised platforms in a tray of water or dedicated sink. For decontamination, mite-exposed equipment and surfaces are washed with hot water and 70% ETOH (*see* **Note 2**). Bedding containing mites, or excess mites, are kept at –20 °C overnight to kill mites prior to disposal. *O. bacoti* are hematophagous and can be maintained by blood feeding on juvenile (Subheading 3.1.1) or adult (Subheading 3.1.2) mice. Appropriate personal protective equipment including gloves and dedicated laboratory coats should be worn at all time to avoid contact with mites and when animal handling (*see* **Note 3**).

3.1.1 Maintaining Mites in Flasks and Feeding with Juvenile Mice

Maintenance of mite colonies in flasks requires strict management to prevent overcrowding. This ensures effective feeding and minimizes adverse effects to juvenile mice (*see* **Note 4**). One colony

flask started with 200 mites will provide approximately 900 mites after 3 weeks.

1. Start a new colony flask by adding approximately 50 g clean animal bedding to a 500 mL plastic Erlenmeyer flask. Add approximately 200 large female mites to the flask (collected in Subheading 3.2.1). Seal the flask with 20-micron mesh secured with two elastic bands. Keep flasks at 27 °C and 70% humidity (see Note 5).
2. Feed the mites twice weekly for 2 weeks by introducing the appropriate number of juvenile mice to the flask for up to 18 h. After 18 h remove the juveniles, assess their condition, and cull (see Note 6).
3. In the third week, mites are harvested from the flask (Subheading 3.2.1) for *L. sigmodontis* infections or to start new colony flasks. Freeze and then dispose of any excess mites (see Note 7).

3.1.2 Maintaining Mite Colony in Animal Cages and Feeding with Adult Mice

1. Mites are kept in mouse cages containing animal bedding. The cages are positioned on raised platforms surrounded by a water moat to contain the mites (Fig. 1).
2. To feed the mites, introduce one adult mouse to each cage once a week for 48–72 h (see Note 8). Remove and cull the mouse after feeding.
3. Bedding should be refreshed every 3–4 weeks by replacing half with clean bedding. Freeze discarded mite-contaminated bedding prior to disposal (see Note 9).

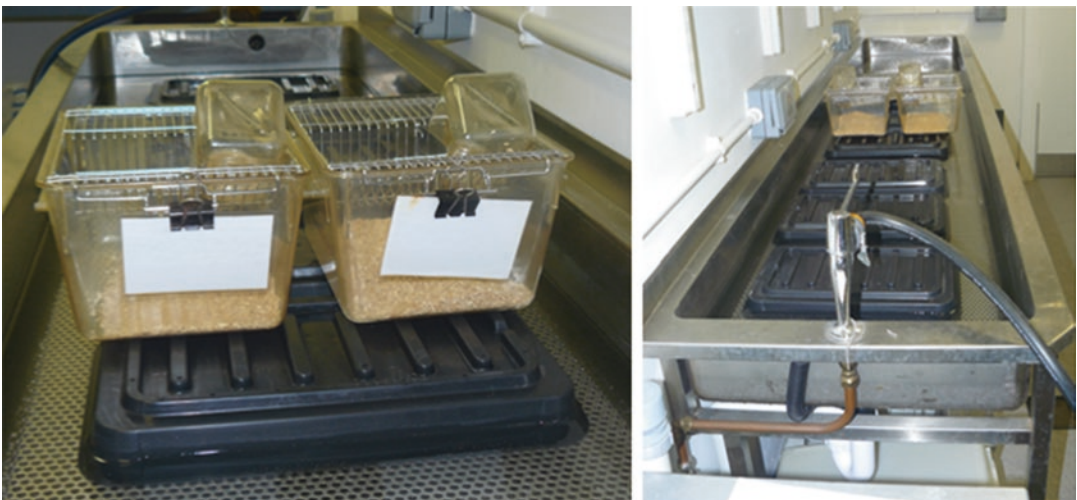


Fig. 1 Large shallow sinks and platforms used to create water moats around animal cages containing *O. bacoti*



Fig. 2 Working area showing moat and raised platforms for collecting uninfected mites from colony flasks

3.2 *L. sigmodontis* Infections

To produce infective *L. sigmodontis* stage 3 larvae (L3s), mites are collected from the uninfected colony (Subheading 3.2.1) and infected with *L. sigmodontis* by allowing them blood feed on *L. sigmodontis*-infected gerbils (Subheading 3.2.2). Mf take 12 days to develop into the infective L3 stage once ingested by the mite, and infective L3 larvae are dissected out of the mite 12–14 days post-ingestion for infections (Subheading 3.2.3). Using one gerbil to infect 450 mites should yield 600–1000 *L. sigmodontis* L3s. This is based on the recovery of 250–350 mites following exposure to infected gerbils. Mites will contain 2–3 L3s each with mite survival at approximately 90%.

3.2.1 Collecting Uninfected Mites

1. Place 5–10 g of animal bedding into the required number of 500 mL plastic Erlenmeyer flasks. For collection of mites from colony flasks, use **steps 2–4**, and for collection of mites from animal cages, go to **step 5**.
2. Set up a working area with a container for collecting mites and waste container surrounded by a water moat (Fig. 2).
3. Sieve the contents of a colony flask (from Subheading 3.1.1) into the collecting container, tapping the sieve on the side of the collecting container so that the bedding is retained in the sieve and mites fall through. Discard the bedding into the waste beaker.
4. Tilt and tap the collecting container so that the mites climb up one side. Using a paintbrush dampened in distilled water, pick 450 large female mites (Fig. 3) from the collecting container into the flask (*see Note 10*).



Fig. 3 Large female mites and smaller protonymphs

5. Pick mites directly from animal cages using a paintbrush dampened in distilled water during the 48–72 h that adult mice are present (*see* **Notes 10** and **11**). Place 450 large female mites (*Fig. 3*) into each flask. Go to **step 6**.
6. Seal the flask with gauze and elastic bands pulling the gauze tight (*see* **Note 5**). Roll the animal bedding around the sides of the flask to remove any water droplets.
7. Keep the collected mites at 27 °C and 70% humidity for 6–9 days without feeding before infecting them with *L. sigmodontis* (Subheading 3.2.2). The starvation period ensures mites feed well when exposed to *L. sigmodontis*-infected gerbils resulting in higher infection levels.

3.2.2 Infection of Mites with *L. sigmodontis*

1. Select a gerbil that has been infected with *L. sigmodontis* for at least 90 days and has detectable microfilaria within the blood (Subheading 3.3.2), preferably greater than 800 Mf/mL. Gerbil weights are taken before and after the procedure to monitor for adverse health effects (*see* **Note 12**).
2. To prevent mite escape, place the gerbil and its cage inside a sealable ventilated box. Add a small amount of bedding from the gerbil cage, and provide food and water (*see* **Note 13**).
3. Pour 450 uninfected mites and their bedding (collected in Subheading 3.2.1) into the gerbil cage and seal the plastic box. Leave the mites to feed on the infected gerbil for 12–18 h.

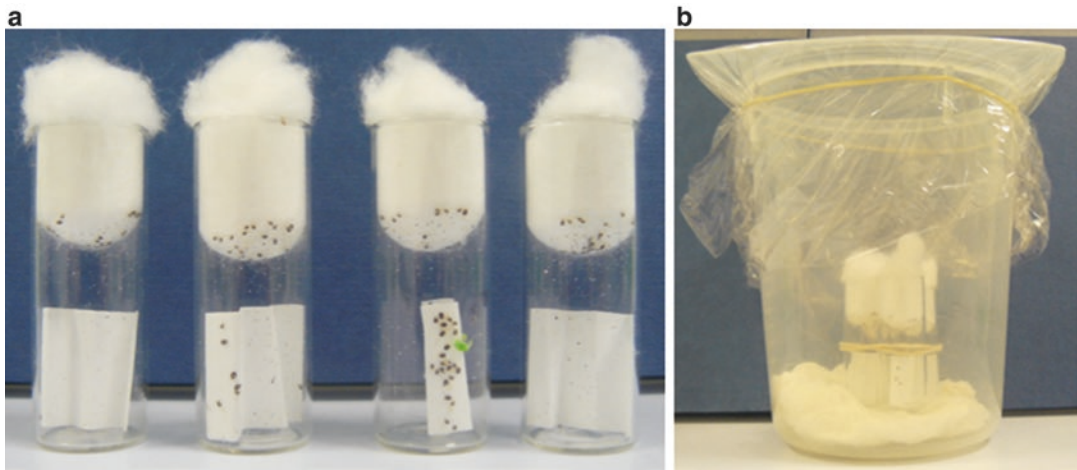


Fig. 4 (a) *O. bacoti* infected with *L. sigmodontis* are collected into glass tubes. (b) The tubes are placed into beaker with damp tissue paper to humidify the local environment

4. Remove the gerbil from the cage, tapping the box on the sides and top prior to opening to dislodge any mites. Tap the lid of the animal cage to dislodge any mites back into the cage. Lift the gerbil cage out of the box, and tap to move the bedding to one side. Sieve the mites from the bedding into a collecting box (as in **step 3** of Subheading 3.2.1) for picking.
5. Collect the infected mites from the ventilated box, and those that were sieved from the bedding, into glass tubes containing a small square of folded filter paper. Use a fine dry paintbrush to collect 50 large well-fed female mites into each tube. Use your gloved thumb to seal the tube to prevent mites escaping while collecting. To seal the tubes, form a plug using nonabsorbent cotton wool. The plug should be dense and fit tightly in the tube (Fig. 4a) (see **Note 14**).
6. Secure the tubes together with an elastic band. Dampen two sheets of tissue paper, and place in bottom of plastic beaker to maintain a local humidified environment. Place tubes in the beaker on top of tissue paper, and secure cling film over the top with an elastic band (Fig. 4b). Ensure tubes remain upright. Keep at 27 °C and 70% humidity for between 12 and 15 days to allow *L. sigmodontis* parasites to develop into the infective L3.
7. Decontaminate mite-exposed equipment, bedding, and surfaces. Equipment and surfaces are washed with hot water and ethanol. Place contaminated bedding at −20 °C overnight prior to disposal.

3.2.3 Infection of Animals with *L. sigmodontis*

1. Set up a working area surrounded by a water moat (Fig. 5a). Working area should include a large beaker full of hot water for decontamination of mite-contaminated items, a glass petri

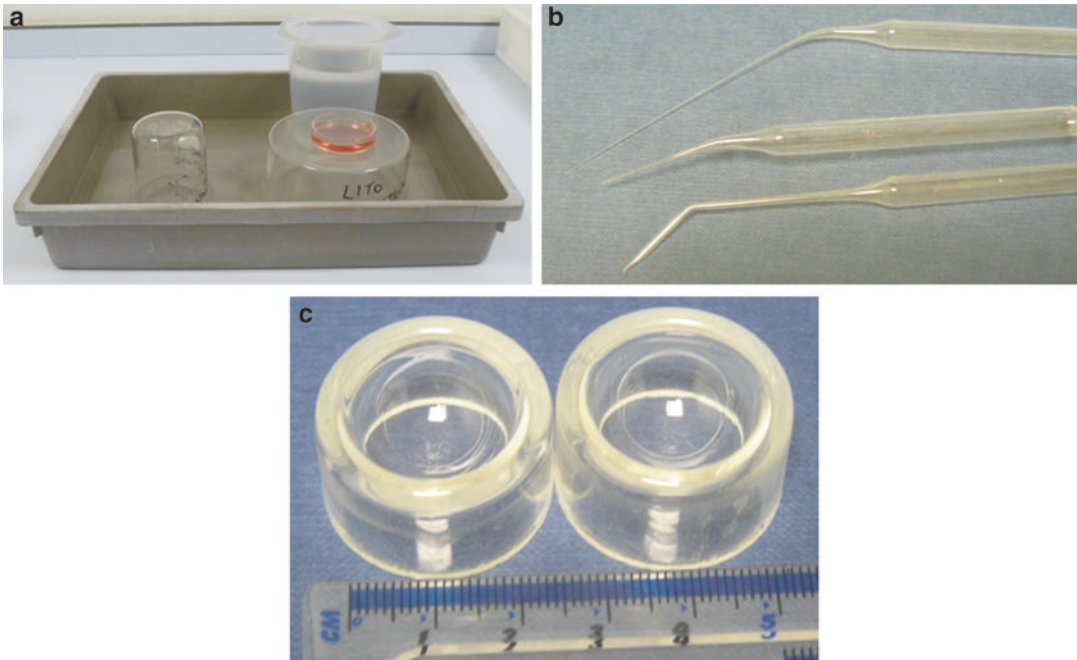


Fig. 5 (a) Setup of water moat and platforms for collection of mite dissection. (b) Drawn-out glass Pasteur pipettes. (c) Syracuse watch glasses

dish containing approximately 5 mL of RPMI media with or without 5–10% sera, and an upside-down 100 mL beaker to act as a small surface on which to collect mites (*see Note 15*).

2. Take a tube containing *L. sigmodontis*-infected mites (Subheading 3.2.2), tap sharply to knock mites down to the bottom, and remove the cotton wool. Place the tube upside down on the upside-down beaker, and using Dumont no. 5 forceps, carefully collect any mites remaining on the cotton wool, and tap mites into the petri dish containing RPMI without putting forceps into media. Discard cotton wool into the beaker of hot water (*see Note 16*).
3. Gently remove the filter paper from the tubes with forceps, pick the mites off with a fine paintbrush (moistened in RPMI), and place into the RPMI-filled petri dish. Place the filter paper into beaker of hot water together with cotton wool to kill any uncollected mites.
4. Keeping the tube upside down on the glass beaker, dislodge the remaining mites by tapping the tube against the beaker. Collect the live large adult mites and transfer to the RPMI-filled petri dish.
5. Repeat for remaining tubes collecting all mites into the same petri dish.

6. Using a dissection microscope, crush the mites gently with fine forceps (Dumont no. 5), checking the abdomen has burst sufficiently to allow L3s to emerge (*see* **Note 17**).
7. Use a Bunsen burner to melt and draw out a glass Pasteur pipette to create a very fine end. Place the neck of the pipette into the Bunsen flame with the tip protruding from the flame and your finger just below the tip. When the tip falls onto your finger, pull the tip to lengthen and reduce the diameter, and simultaneously remove from the flame. Break off the end of the pipette to leave a hole just large enough to collect L3 (Fig. 5b).
8. Infective doses for each animal are counted out and collected individually. Typically, mice are infected with 20–40 *L. sigmodontis* L₃ for experimental purposes, and gerbils are infected with 100 *L. sigmodontis* L3 for life cycle maintenance. Use the Pasteur pipette to collect the required number of L3 for each dose, and transfer them to a Syracuse watch glass (Fig. 5c) (*see* **Note 18**).
9. Let the L3 form a cluster in the bottom of the Syracuse watch glass. The L3s can be recounted using a dissection microscope at this point to double check the accuracy of the dose. Take up the L3 in a 1 mL syringe with 0.6 × 25 mm needle, aiming to collect the dose in 100–200 µL of RPMI media. To prevent the L3 clumping and sticking in the needle, finish by sucking up approximately 100 µL of air.
10. Infect gerbils intraperitoneally for life cycle maintenance and mice subcutaneously for experimental infections using a 0.6 × 25 mm needle (*see* **Note 19**).

3.3 Quantitating *L. sigmodontis* Infection Levels and Recovery of Immune Cells

L. sigmodontis parasites migrate from the skin to the pleural cavity over the first 3–6 days of infection and can be isolated from the pleural cavity by pleural lavage from day 4 pi onward, along with immune cells from the infection site (Subheading 3.3.1). Adult female *L. sigmodontis* parasites start releasing Mf from day 50 pi, which can be measured by blood sampling and pleural lavage (Subheading 3.3.2). Skin-draining LN can be used to sample the immune response for the first 7–12 day pi. Pooled parathymic and posterior mediastinal lymph nodes can be used to sample the infection site (*see* **Note 20**).

3.3.1 Recovering Adult Parasites and Immune Cells from the Pleural Cavity of Mice

1. Once the animal has been culled, open the peritoneal cavity taking care not to damage the diaphragm, or cut into the pleural cavity. Pull organs down away from the diaphragm. Cut off the xiphoid process from the sternum, which should cause a hole to open in the diaphragm next to the sternum. Lavage the peritoneal cavity 1 mL at a time with 10 mL PBS for the col-

lection of parasites. Use culture media if both parasites and cells are to be collected (*see Note 21*).

2. Separate cells and parasites. Prior to day 10 pi, pour lavage fluid into a petri dish under a dissection microscope, and suck up parasites using a P20 pipette. From day 40 pi, parasites can be collected using insect forceps. Between days 10 and 40 of infection, parasites and cells can be separated by passing the lavage fluid through a 60-micron cell strainer. The parasites will remain in the cell strainer, while the cells will pass through. However, at later stages of infection, there is a risk of damaging parasite morphology using a cell strainer. Collect parasites into PBS and retain lavage fluid containing immune cells.
3. Count the number of parasites under a dissection microscope (*see Note 22*).
4. Fixation of parasites (if required). Prior to day 20 pi, fix parasites in 4% formalin. After day 20 pi, fix parasites using warm 70% ethanol (*see Note 23*).

3.3.2 Quantifying Levels of Mf in the Blood and Pleural Cavity

1. Aliquot 95 or 500 μ L BD FACS lysing buffer (prepared as per manufacturer's instructions) into a 1.5 mL Eppendorf tube to assess microfilaria in gerbils or mice, respectively. One tube per animal (*see Note 24*).
2. To quantify microfilaria in the blood, insert a needle into the tail vein, and collect 5 μ L blood from gerbils or 30 μ L blood from mice into the Eppendorf tube containing FACS lysing buffer. Shave the tail of gerbils prior to collection with a razor or scalpel. To quantify the Mf in the pleural cavity of mice, resuspend pleural lavage fluid in 5 mL, mix well, and sample 20 μ L into an Eppendorf tube with FACS lysing buffer.
3. Use a hemocytometer and microscope to count microfilaria in gerbil samples. To count Mf in mouse samples, spin down the sample (17,000 g, 5 min), and remove as much liquid as possible with a pipette. Resuspend in the remaining liquid and smear onto a slide. Count all the Mf in the sample under an inverted microscope.

3.3.3 Preparation of *L. sigmodontis* Antigens

Soluble *L. sigmodontis* antigens can be prepared for in vitro immune cell restimulations and measuring *L. sigmodontis*-specific antibodies via ELISA.

1. On ice, crush *L. sigmodontis* parasites in sterile PBS in a glass tissue grinder. Remove the insoluble fraction by centrifuging at 17000 g for 5 min at 4 °C and collecting soluble fraction. Repeat 2–3 times to remove all insoluble material.

2. Check the protein concentration using standard methods (e.g., Bradford assay), and adjust to the required concentration in sterile PBS. Aliquot and store at -70°C .
3. Coat ELISA plates with *L. sigmodontis* antigen at $5\text{ }\mu\text{g/mL}$ for antigen-specific antibody ELISAs, and use sterile-filtered *L. sigmodontis* antigen at $10\text{ }\mu\text{g/mL}$ for in vitro immune restimulations.

4 Notes

1. Although *O. bacoti* requires high temperature and humidity (approx. 27°C , 70% humidity), it does not have to be as precise as obtained by a climate chamber or specialized room. Colonies can be maintained without climate chambers or specialized facilities so long as the temperature and humidity levels can be raised.
2. Detergent can leave a residue that impairs mite survival. It is better to use non-detergent-based methods for cleaning equipment involved with mite maintenance.
3. *O. bacoti* will bite humans, although it is not able to survive feeding on humans. Precautions should be taken to prevent contact with mites and mite escape. *O. bacoti* tend to climb and will travel up equipment being held. Work quickly, frequently wash off tools (e.g., forceps) with water or place under water, and visually inspect gloves at frequent intervals to check for mites. Wear light-colored gloves and protective personal equipment to allow mites to be seen easily. Double-sided sticky tape can be placed around the cuff of gloves to form a mite barrier.
4. Overcrowding of mites in colony flasks is a major problem. It results in juvenile mice receiving a large number of bites that can cause unnecessary morbidity and may lead to mortality. It also results in mites failing to feed adequately, impairing their development and their survival at later stages in the protocol. The clinical condition of juvenile mice should be assessed immediately on their removal from the flasks, including mobility, anemia, and skin integrity. If any of these are poor, then steps should be taken to reduce the number of mites. Increasing the number of juveniles will not solve the overcrowding problem. The number of mites can also be directly assessed when they are sieved out of the bedding (Subheading 3.2.1). Subheading 3.1.1 should allow feeding of mites with negligible adverse effects for the juvenile mice.
5. Animal bedding should be light colored so that mites stand out against it and is best to be a uniform size for ease of sieving

and collecting mites. We use a fine grade bedding (Gold Chip, LBS Biotechnology). For redundancy use two elastic to prevent the flask unsealing if one breaks. The gauze must be pulled tightly over the top of the flasks; otherwise mites, especially smaller protonymphs, will gather within creases in the gauze on the outside rim making escape more likely. Replace the gauze after each opening, and wash gauzes thoroughly in hot water.

6. Mite feeding can result in trauma to the juveniles, and the severity of trauma relates to the size of the juvenile, strain, and number of mites in the flask. Juvenile mice should be P8–P10 and over 6 g in weight. CD1 juveniles are a recommended strain. Mite feeding efficiency can be reduced if older juveniles are used, while younger or smaller juveniles are more susceptible to adverse health effects. In a well-controlled colony, the required juvenile numbers are predictable, and mite feeding can be performed with minimal adverse effects for the juvenile mice. Typically, two juveniles per flask are used for newly split flasks (feeds 1 and 2 in week 1), increasing to four juveniles per flask as mite numbers increase (feeds 3 and 4 in week 2). If using a less robust strain, such as C57BL/6, then juvenile numbers should be increased, e.g., to three and five, respectively. If mites are not required for *L. sigmodontis* infections, then the four feeds can be given once a week over 4 weeks.
7. Do not keep a colony flask longer than the four feeds as the mites will over expand. Start new flasks to maintain the colony.
8. Exposure to mites can result in stress in adult mice, and adding more than one mouse per cage can result in fighting and resource guarding. The condition of adult mice should be monitored for the period that they are exposed to mites.
9. As the mites live and lay eggs in the animal bedding, it is important not to disturb or change the bedding too frequently. As adult mice are only kept in the cage for 48–72 h, the bedding can be left for 3–4 weeks before partially renewing. The water bottles and cage lids are a favorite egg-laying site and so should only be changed when required. If multiple cages are used, then bridges can be made between cages to allow the migration and mixing of the mite colony. The moats should be cleared and cleaned regularly.
10. It is easier to pick up mites with a damp, rather than dry, paintbrush. However, if the brush is too wet, then this can result in mites trapped in water droplets, and water in the flasks can promote the growth of mold. Use the largest (female) mites for *L. sigmodontis* infections and starting new colony flasks. Transferring too many small protonymphs (Fig. 3) can result

in overcrowding of colony flasks and unnecessarily increase the adverse effects suffered by gerbils.

11. The fed female mites seek egg-laying sites around the top of the cage, under the water bottle, and behind cage card, which are a good location to collect mites.
12. Although more L3s can be produced by using gerbils with higher numbers of Mf/mL, any gerbil with detectable Mf in the blood can be used. A gerbil can be reused to infect different batches of mites, although the number of L3s recovered per mite will decrease with each exposure. Typically, one gerbil can be used to infect four batches of mites before a significant decline in L3 recovery. Gerbils experience a transient weight loss following exposure to mites, and their condition should be monitored to verify that they have fully recovered before reexposure to mites. Resting gerbils for 2 weeks between exposures is recommended although 7 days can be sufficient. A system of rotating gerbils can be used to provide a constant supply of infectious L3.
13. The box vents need to let in air while preventing mite escape. If taking an infected gerbil from a cage containing multiple gerbils, it is important to co-transfer some cage bedding so that the gerbil recognizes their cage mates on reintroduction. Only put a small amount of food directly into the cage, sufficient for 18 h, as the presence of excess food pellets will make it more difficult to collect mites.
14. The paintbrush must be dry as any moisture in the tubes will cause mold growth killing the mites. Use a fine brush and splay the hairs to assist with picking. Avoid introducing bedding and animal dander to the tubes.
15. Once dissected out of the mite, L3 larvae only remain infective for a few hours, and infections should be performed as soon as possible after dissections. L3 collected in RPMI will remain infective for 3–5 h. Adding 10% fetal bovine serum or horse serum to the RPMI increases the length of time they remain infective. If planning to culture immune cells in vitro, then different types of sera should be used for inoculations and in vitro assays; otherwise a recall response may be observed against serum antigens. We prepare L3s in horse serum and perform in vitro restimulations with fetal bovine serum or mouse serum.
16. It is best to use a paintbrush to collect mites as mites will stick to forceps when placed in media and try to climb up the forceps. However, forceps are used for picking mites off the cotton wool. Trimming the paintbrush to a fine point aids the collection of mites.

17. Once the mites are dissected, leave for around 30 min to allow all the L3s to emerge from mites. They will move to the edge of the dish making them easier to collect.
18. Collecting the L3s from around the edges and transferring to a clean petri dish can help separate L3s from mite debris and make collection easier. Approximately two-thirds of injected larvae are killed within 4 days of inoculation. Thus, infecting susceptible BALB/c mice with 20 or 40 L3s will result in the establishment of an average of 7 and 13 L3s, respectively. We recommend injecting 40 L3s for experiments assessing changes in parasite recovery (e.g., vaccination). Injecting 20 L3s is sufficient for experiments which only require the recruitment of immune cells. Using less than 20 L3s increases the risk of single sex infections, which will not result in microfilaria. Infecting with higher L3 numbers increases the magnitude of the immune response but can result in impaired parasite development [5, 8].
19. S.c. infections of *L. sigmodontis* can be performed anywhere on the mouse. We recommend the nape of the neck as it allows a larger volume of liquid to be injected and is drained by the brachial LN, which are relatively large and easy to collect.
20. The precise timings of the *L. sigmodontis* life-cycle shows natural variation over time, and not all mice develop a patent infection or at the same time. In susceptible BALB/c mice, between 50% and 100% of mice can be expected to develop patent infections with female mice being more susceptible than male mice [9]. Although Mf can be detected from day 50 pi, this is not always the case. Typically, day 60 pi is considered the time point by which Mf should be robustly detectable if an infection has fully developed. If blood Mf have not been detected by day 70 pi, it is unlikely that a patent infection will develop.
21. It is recommended to exsanguinate mice prior to lavage to reduce blood leakage into the pleural cavity. Not doing this can result in coagulation and loss of immune cells. The presence of blood will also obscure the LN within the pleural cavity. It is best not to cut a hole directly in the diaphragm as it will enlarge during lavage resulting in the liquid escaping. Cut off as much as the xiphoid process as possible. If a hole does not immediately appear, then it can be opened by brushing up with tweezers just under the cut area. *L. sigmodontis* parasites are fragile and will be damaged if repeatedly aspirated up and down using a Pasteur pipette. Care must be taken if morphological analysis is required.
22. *L. sigmodontis* parasites tend to curl up into ball of complex knots. Placing on ice will slow down their movement while alive allowing them to be teased apart with paintbrushes.

23. The use of formalin to fix large *L. sigmodontis* parasites results in contraction of the parasite making morphological analysis more difficult. Warm ethanol fixes the parasite while better retaining morphology. Ethanol can also be used to fix the earlier parasite stages.
24. Red blood cells must be lysed before fixation; otherwise they will obscure the Mf resulting in inaccurate counts. BD FACS lysing buffer simultaneously lyses red blood cells and fixes Mf for counting at a later date.

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