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Bacterial infection allows for functional examination of adoptively transferred innate lymphoid cell subsets

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Abstract

Innate lymphoid cells (ILCs) are important regulators of the early responses to infection at mucosal barriers, including the intestine. Recently, we have shown that specific ILC3 subsets protect against enteric bacterial pathogens. Here, we describe a mouse model of oral infection by *Yersinia enterocolitica (Y. enterocolitica)* and several different methodologies to assess the severity of the infection. We also detail how ILC3 subsets can be isolated from the mouse small intestine and transferred into recipient immune deficient mice to study the function of these ILCs in the small intestine.

Keywords

Adoptive transfer; group 3 innate lymphoid cells; innate immunity; small intestine lamina propria; *Yersinia enterocolitica*

1 Introduction

ILCs are a heterogeneous lymphoid population that do not express rearranged antigen receptors, and they respond to infection and other insults promptly by secreting effector cytokines. Therefore, ILCs contribute to barrier integrity, homeostasis, and host defense of mucosal surfaces. (1). ILC subsets have been defined by patterns of expression of effector cytokines, transcription factors and cell surface markers. These subsets can be broadly defined as natural killer (NK) cells, as well as ILC1, ILC2 and ILC3. While natural killer (NK) cells share features with $CD8^+$ cytotoxic T cells (1), ILC1, ILC2 and ILC3 subsets share features with $CD4^+$ T helper (Th)1, Th2 and Th17 subsets, respectively. Shared features include expression of key transcription factors and cytokine production. In mice, ILC3s are further divided into subsets distinguished by CCR6 and NKp46 expression (2–5). $CCR6^+$ ILC3, which are lymphoid-tissues-inducer cells (LTi) cells, produce IL-22 and IL-17 (6). $CCR6^{neg}$ ILC3, which include $NKp46^+$ and $NKp46^-$ cells, can express T-bet and produce IFN-γ in addition to IL-22 (2). ILC3s are abundant at some mucosal sites and involved in the innate immune response to bacterial infection. ILC3 are important for host

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defense against bacterial infection of the large intestine, including *Citrobacter rodentium* (4, 7, 8), *Helicobacter hepaticus* (9) and *Salmonella typhimurium* (2, 10). Interestingly, however, ILC3 are more abundant in the small intestine than in the large intestine (11).

Y. enterocolitica is a facultative intracellular bacterium that targets the small intestine and causes food borne illness. After oral uptake, *Y. enterocolitica* replicates in the small intestine, invades Peyer's patches of the distal ileum and disseminates to the spleen and liver (12). Frequently the infection is cleared in 1 to 2 weeks (13, 14), suggesting that innate immune cells play important roles in protection from *Y. enterocolitica* infection. Our studies recently demonstrated that ILC populations, in particular CCR6^{neg} ILC3s, are important for resistance to *Y. enterocolitica* infection in the small intestine by secreting IFN-γ (15).

In this chapter, we provide a strategy to isolate ILC3 subsets from the small intestine, based on cell surface marker expression, for the purpose of adoptive transfer. Following transfer, we describe the establishment and monitoring of *Y. enterocolitica* infection model and using analysis of infection parameters for study of ILCs.

2 Materials

2.1 Isolation of ILC3 subsets and Adoptive Transfer of ILC3 subsets

1. Donor *Rag1*$^{-/-}$ mice
2. Recipient *Rag2*$^{-/-}$*gc*$^{-/-}$ mice, age 8-10 weeks
3. *RPMI Wash Buffer 1*: 1000mL RPMI + 25mL of 1M HEPES (final 25mM) + 10mL of 100X P/S/G (Penicillin-Streptomycin-Glutamine) + 100mL of FBS (final 5%)
4. *RPMI Wash Buffer 2*: 1000mL RPMI + 25mL of 1M HEPES (final 25mM) + 10mL of 100X P/S/G + 200mL of FBS (final 10%)
5. *EDTA Buffer*: 500mL HBSS + 12.5 mL of 1M HEPES (final 25mM) + 5ml of 0.5M EDTA (final 5mM)
6. *Collagenase Buffer*: 100mL of *RPMI Wash Buffer 2* + 1mL of Collagenase Type-8 (1:100 dilution; Sigma # C2139-1g, Stock=50mg/mL; Final=0.5mg/mL) + 50μL DNaseI (1:2000 dilution; Sigma, Stock=100mg/mL; Final=0.05mg/mL)
7. *100% Percoll*: 45mL of *Percoll* + 5mL of 10x HBSS
8. *40 % Percoll*: 30mL of *RPMI Wash Buffer* 1 + 20mL of *100% Percoll* (final 40%)
9. *80 % Percoll*: 10mL of *RPMI Wash Buffer* 1 + 40mL of *100% Percoll* (final 80%)
10. Fluorescence-activated cell sorting (FACS) staining buffer: 49mL of 1x Phosphate buffered saline (PBS) + 1mL of FBS (final 2%)
11. Dissecting scissors
12. Forceps

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13. Petri dishes
14. 70 μm cell strainers
15. 37°C incubating orbital shaker
16. Centrifuge
17. 50mL conical tubes, FACS tubes

2.2 *Y. enterocolitica* Infection Model: Infection

1. Mice, age 8-10 weeks, *Ifng*$^{-/-}$ mice (*see* Note 1)
2. *Y. enterocolitica* strain WA-C (pYV::CM) (*see* Note 2) (16)
3. Luria broth (LB) broth media; LB agar plates supplemented with chloramphenicol (final 30 μg/mL)
4. 30°C incubating orbital shaker, 30°C microbiological incubator
5. Sterile bacteria culture tubes
6. Spectrophotometer
7. Oral gavage needle with bulbous tip (Curved Feeding Needles)
8. 1mL syringe

2.3 *Y. enterocolitica* Infection Model: Monitoring and quantifying infection

1. zinc formalin (Medical Chemical Corporation)
2. 70% isopropanol
3. homogenizer
4. Chloramphenicol (final 30 μg/mL)-containing LB agar plates

3. Methods

Fig. 1 outlines the entire procedure for measuring ILC function against *Yersinia* infection. To determine if there is a role for ILC3 subsets during *Y. enterocolitica* infection, isolated ILC3 subsets from the small intestinal lamina propria lymphocytes (SI-LPLs) of *Rag1*$^{-/-}$ mice, which lack B and T cells, are transferred into recipient *Rag2*$^{-/-}$*gc*$^{-/-}$ mice, which lack all ILC subsets, as well as B and T cells. Recipient mice are then monitored to assess the infection. The following description of infection of mice with *Y. enterocolitica* and subsequent monitoring has been approved by the La Jolla Institute for Immunology Institutional Animal Care and Use Committee (IACUC). All procedures must be approved by a relevant institutional animal ethics committee.

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1. If possible, use *Ifng*$^{-/-}$ mice as a positive control of *Y. enterocolitica* infection to check the virulence of *Y. enterocolitica* at each experiment. *Ifng*$^{-/-}$ mice start losing their body weight and survival diminishes beginning around day 4.
2. This strain is resistant to chloramphenicol and was obtained from Dr. J. Heesemann (Ludwig Maximilian University of Munich, Germany) (16).

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3.1 Isolation of ILC3 subsets and Adoptive Transfer of ILC3 subsets

3.1.1 Lamina propria lymphocyte from small intestine (*see* Note 3)

1. Cut the entire small intestine from *Rag1*$^{-/-}$ mice and open longitudinally (*see* Note 4).
2. Wash briefly with *RPMI Wash Buffer 1* and cut into 1.5 cm pieces.
3. Transfer the tissue into a 50mL conical tube containing 25mL of *EDTA Buffer*. Incubate the tissue pieces in a 37°C incubating orbital shaker at 225 rpm for 10-15 minutes.
4. After incubation, pour off tissue pieces and liquid into a petri dish, collect all tissue pieces, put them back in a 50mL conical tube, and then add 25mL of pre-warmed (*see* Note 5) *EDTA Buffer* into a 50mL conical tube. Incubate the tissue pieces in the 37°C incubating orbital shaker at 225 rpm for 10-15 minutes. Repeat steps 3-4 several times until the epithelial layer is removed (*see* Note 6).
5. Pour off tissue pieces and liquid in the petri dish, collect all tissue pieces, put them back in a 50mL conical tube. Add 20mL of *RPMI Wash Buffer 2* into a 50mL conical tube and wash tissue pieces by shaking by hand 20 times.
6. Pour off tissue pieces and liquid in the petri dish, collect all tissue pieces, and transfer tissues to a 50mL conical tube containing 25 ml of pre-warmed *Collagenase Buffer*. Incubate the tissue pieces in the 37°C incubating orbital shaker at 225 rpm for 20-25 minutes.
7. Separate lamina propria lymphocytes from digested tissues by passing cells through a 70 μm cell strainer and add 20mL of *RPMI Wash Buffer 2*.
8. Centrifuge the flow-through cell suspension at 1,500 rpm at 4°C for 5 minutes.
9. Pour off the supernatant. Resuspend pellet well in 2 mL of *40% Percoll* and gently load this cell containing fraction over 2 mL of *80% Percoll* in a FACS tube. (*see* Note 7).
10. Centrifuge the gradient at 2,000 rpm at room temperature for 10 minutes (*see* Note 8). This step must be completed at room temperature.
11. Remove the upper phase containing fat and cell debris with a suction pump. Collect the ring interphase between the *80% Percoll* and *40% Percoll*, transfer it into a 50mL conical tube, add 30-40mL of *RPMI Wash Buffer 2*, and invert the tube several times. (*see* Note 9).

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[3]. Based on experience, one male *Rag1*$^{-/-}$ donor mouse (6-8 weeks old) should yield approximately 0.3-0.6×10^5, 0.5-1.2×10^5 and 0.5-1.2×10^5 cells in the CCR6$^+$ ILC3s, CCR6$^-$NKp46$^+$ ILC3s and CCR6$^-$NKp46$^-$ ILC3s, respectively.
[4]. Peyer's patches do not develop in *Rag1*$^{-/-}$ mice. Therefore, there is no need to remove Peyer's patches.
[5]. Before using the buffers, pre-warm at 37°C.
[6]. While it depends on the condition of the intestine, these steps usually need repeating 5-7 times.
[7]. *Percoll* must be kept at room temperature before use, because temperature differences will affect the gradient.
[8]. Even though you centrifuge the cells with a brake, it does not affect the gradient.
[9]. Before centrifuging, be sure to mix the tube well and completely disrupt the remaining *Percoll* layer.

12. Centrifuge the cell suspension at 1,500 rpm at 4°C for 5 minutes. Pour off the supernatant and resuspend cells in the FACS staining buffer.

3.1.2 Staining, Sorting of ILC3 subsets by Flow Cytometry for Adoptive Transfer—

ILC3s are divided into three subsets distinguished by CCR6 and NKp46 expression in mice. These include the CCR6^{+} ILC3 LTi subset and the CCR6^{neg} populations, which include NKp46^{+} and NKp46^{-} subsets. Previously, Guo, *et al.* showed that intestinal ILC3 from mice are identified by their differential surface expression of CD45 and CD90 (17). Here, we further show how to sort the individual ILC3 subsets from the SI-LPLs by flow cytometry.

1. When lamina propria lymphocytes are ready, resuspend the cells with FACS staining buffer containing anti-mouse CD16/CD32 (Mouse Fc Block) and incubate for 15 minutes at 4°C.
2. Prepare antibody mixture for ILC staining and sorting of ILC3 subsets.
3. Add antibody mixture to the cells and incubate for 30 minutes at 4°C in the dark.
4. Wash the cells with FACS staining buffer, centrifuge the cell suspension at 1,500 rpm at 4°C for 5 minutes and pour off the supernatant (*see* Note 11).
5. Resuspend cells with 1X PBS containing LIVE/DEAD Fixable Dead Cell Stain and incubate the cells for 30 minutes at 4°C. Wash the cells with FACS staining buffer, centrifuge the cell suspension at 1,500 rpm at 4°C for 5 minutes and pour off the supernatant (*see* Note 11).
6. Resuspend the cells with FACS staining buffer up to 100×10^{6} cells/mL (*see* Note 12) and filter the cells using a 70 μm cell strainer to remove cell debris.
7. The stained cells are ready for sorting. Proceed to the cell sorter. ILC3 subsets should be sorted according to the strategy detailed in Fig. 2.
8. Wash the sorted ILC3 subsets with 1x PBS and resuspend the cells in PBS at $1.5\text{~}5 \times 10^{5}$/200μL.
9. Transfer 200μL of sorted cells ($1.5\text{~}5 \times 10^{5}$/mouse) into *Rag2*$^{-/-}$*gc*$^{-/-}$ recipients at day −1 by retro-orbital injection. After the injection, monitor the mouse to ensure it is breathing and behaving normally.

	Antigen	Clone	Final Dilution
Lineage	CD19	ID3	1:400
	B220	RA3-6B2	1:400
	Gr1	RB6-8C5	1:400
	CD11c	N418	1:400

11. Wash the cells twice to remove the unbound antibody or LIVE/DEAD Fixable Dead Cell Stain from the cells.
12. Adjust the cell concentration as necessary to achieve a maximum sort rate.

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	Antigen	Clone	Final Dilution
	CD11b (*see* Note 10)	M1/70	1:400
	CD3	145-2C11	1:400
	CD45	30-F11	1:400
	Thy1.2	53-2.1	1:400
	NK1.1	PK136	1:400
	NKp46	29A1.4	1:400
	CCR6	140706	1:20

3.2 *Y. enterocolitica* Infection Model: Infection

1. To recover *Y. enterocolitica* from the glycerol stock (*see* Note 13), open the tube and use a sterile loop, or pipette tip to scrape some of the frozen bacteria off of the top. Inoculate *Y. enterocolitica* to 4mL of sterile LB media (*see* Note 14) in the culture tube. Incubate the culture tube in a 30°C incubating orbital shaker overnight at 200rpm (*see* Note 15).

2. For 2nd culture of *Y. enterocolitica*, add 500 μL of the overnight culture to 50mL of sterile LB media in a new 200mL culture flask and place in a 30°C incubating orbital shaker at 200rpm for 5-6 hours.

3. Measure the optical density of the 2nd culture with a spectrophotometer at 600nm to ensure that the correct concentration has been attained prior to oral gavage. The OD600 should be approximately 0.6 which is in the log or exponential phase of bacteria growth and is the most reproductive portion of the growth curve. If the OD600 is less 0.6 after 2nd culture, incubate them until OD600 reach 0.6. In our hands, OD600≈0.6 is about 5.0-10.0 × 10^8 CFU/mL.

4. To confirm the accuracy of the inoculum preparation colony density, serially dilute 100μL of the 2nd culture tenfold until a 10^8-fold dilution of the starting material has been reached. Then plate 30μL of serial dilutions of 10^6 - 10^8 in duplicate or triplicate onto chloramphenicol-containing LB agar plate to determine the bacterial density of the inoculate. Incubate the plates at 30°C incubator for 2 days, and then count the number of the colonies on the 10^6 - 10^8 in plates and calculate the CFU/mL.

5. Proceeding from step 3, prepare a 1mL syringe for oral gavage. Swirl the bacterial culture to ensure a homogenous mixture before loading the syringe. Firmly attach the oral gavage needle onto the syringe and load the bacterial liquid (*see* Note 16).

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[10]. If you want to sort Group 1 ILCs, do not add CD11b for a lineage marker because intestinal NK cells can express CD11b.

[13]. For long term storage of *Y. enterocolitica*, make a glycerol stock. After you have 2nd culture, add 700 μL of the *Y. enterocolitica* culture to 300 μL of 50% glycerol in a 2 mL screw top tube or cryovial and gently mix by pipetting. Freeze the glycerol stock tube at −80°C. Do not let the glycerol stock unthaw.

[14]. Be sure that LB media looks clear at inoculation to confirm LB media is not contaminated.

[15]. Ensure that the cap of the culture tube is loose letting oxygen enter and adjust the shaking speed as necessary for full aeration.

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6. Weigh the mouse, and then slowly inject approximately 100-200μL of culture into the esophagus and remove the gavage needle at the same angle. In our hands, 100-200μL of 2nd culture is about 1×10^8 to 2×10^8 CFU of *Y. enterocolitica*. After the oral gavage, monitor the mouse to ensure it is breathing and behaving normally.

3.3 *Y. enterocolitica* Infection Model: Monitoring and quantifying infection

1. Survival rate and monitoring for other qualitative signs of infection daily: After *Y. enterocolitica* infection, check the mouse survival rate daily and monitor mice including appearance (scruffy, skin tenting as evidence of dehydration etc.) and behavior and activity levels (reduced activity, hunched etc.) daily, until day 5-7. Hunched appearance and reduced activity are a clear sign of severe infection, so these mice should be monitored closely and euthanized if severely ill according to standards set by the IACUC.
2. Body weight: After *Y. enterocolitica* infection, weigh the mice every day until day 5-7 as measure of overall health. Weight loss is also a clear sign of severe infection, so mice experiencing weight loss should be monitored closely as above.
3. Bacterial translocation: When mice are euthanized, approximately at day 5, collect ileum contents, spleen and liver for quantification of the bacterial burdens (CFU/g).
   a. Ileum contents: Collect ileum contents by longitudinally cutting open the ileum and scraping out contents using the rounded back of forceps. Place contents into pre-weighed 1.5mL tube. Weigh and record weight of ileum contents and resuspend in 1mL 1x PBS. Serially dilute the contents, according to diagram in Fig. 3a, until a dilution of 1:20,000 has been reached. Next, plate 30μL of the 1:200, 1:2,000 and 1:20,000 dilutions in triplicate onto chloramphenicol-containing LB agar plate to determine the bacterial colonies. Incubate the plates in a 30°C incubator for 2 days, and then count the number of the colonies in plates and calculate as follow; CFU/g = (no. of colonies in 1mL of PBS x dilution factor) / (grams of tissue) (Fig. 3a).
   b. Spleen and liver: Collect approximately half of the spleen (reserving the rest for histology) and approximately 100-200 mg of the liver, from the left lateral lobe, into pre-weighed 1.5mL tubes and weigh the tissues. Transfer them into a FACS tube (*see* Note 17), add 1mL of PBS into the tube and homogenize the tissue on ice with the homogenizer. Serially dilute the homogenized tissue, according to diagram Fig. 3a, until dilutions of 1:200 have been reached. Next, plate 30μL of the 1:20 and 1:200 dilutions in triplicate onto chloramphenicol-containing LB agar

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16. Ensure removal of air bubbles by flicking the syringe.

17. Do not fix the tissues with zinc formalin for a long time. After 24 hours, samples should be placed in 70% isopropanol for long term storage.

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plate to enumerate bacterial colonies. Incubate the plates in a 30°C incubator for 2 days, and then count the number of the colonies in plates and calculate CFU (Fig. 3a).

4. Histopathology: A systemic infection of *Y. enterocolitica* leads to focal abscess formation with necrosis containing *Y. enterocolitica* colonies in the liver and spleen. To assess a systemic infection by *Y. enterocolitica*, prepare samples for hematoxylin and eosin (H&E) stain or Warthin-Starry silver stain to allow for assessment of necrosis in the tissues and *Yersinia* colonies, respectively (Fig. 3b).

   a. Collect the tissues and fix them with zinc formalin overnight and the next day change out the fixation buffer for 70% isopropanol (*see* Note 18).

   b. Complete the H&E stain or Warthin-Starry Silver stain according to standard protocols.

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[18] A 1.5mL tube is not suitable for homogenization of spleen and liver. Thus, a bigger tube, such as a FACS tube, should be used.

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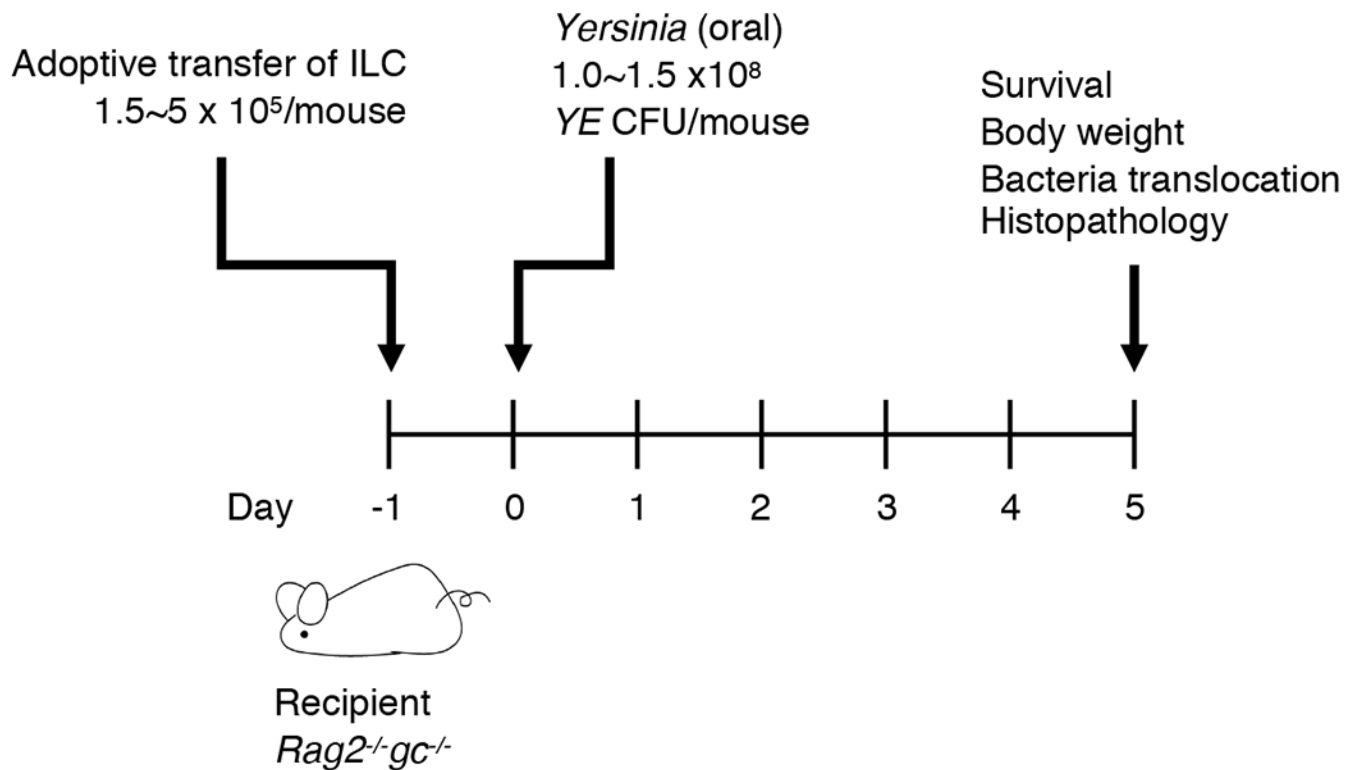

Fig. 1. Measuring ILC function against *Yersinia* infection.

Schematic outline of the entire procedure to measure ILC function against *Yersinia* infection. Isolated SI-LPLs from uninfected *Rag1*$^{-/-}$ are stained with fluorophore-conjugated antibodies. ILC3 subsets, such as NKp46^{-}ILC3, NKp46^{+}ILC3, and CCR6^{+}ILC3 cells, are sorted by flow cytometry (BD FACSAria Fusion). Next, ILC3 subsets are transferred by retro-orbital injection into *Rag2*$^{-/-}$*gc*$^{-/-}$ recipients at day −1, and the mice are infected with *Y. enterocolitica* the following day (Day 0). Control mice are injected with PBS. After *Y. enterocolitica* infection, mice are monitored by body weight and survival rate daily until day 5-7. At which point, the mice are euthanized and tissues are collected to assess the infection.

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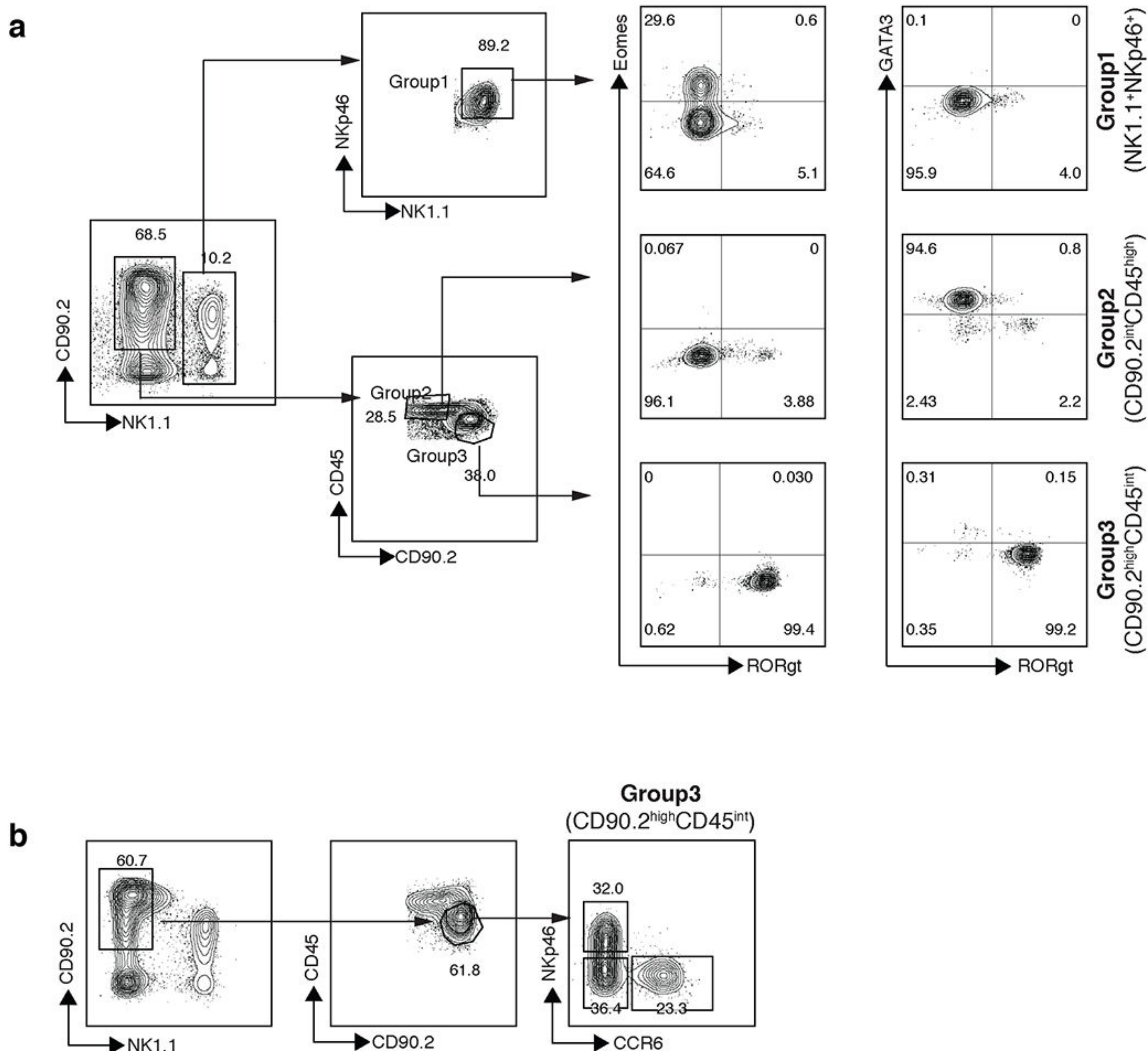

Fig. 2. Gating strategy for isolation of intestinal ILC3 subsets from the small intestine of *Rag1*$^{-/-}$ mice by flow cytometry.

(a) Strategy for staining of RORγt^{+}ILC3s from the small intestine of *Rag1*$^{-/-}$ mice by their differential surface expression of CD45 and CD90. Gating is performed on lymphocytes and doublets are excluded. Gating is performed on live CD45^{+} Lineage (Lin)$^{-}$CD3^{-} cells. Lineage markers are CD19, B220, CD11c and Gr1. Data show initial flow cytometry plot gated on live CD45^{+}Lin^{-}CD3^{-}NK1.1^{+}cells and CD45^{+}Lin^{-}CD3^{-}CD90.2^{+}NK1.1^{-}cells. Cells are identified as Group 1, Group 2 and Group 3 by their differential surface expression of CD45, CD90, and NK1.1. Group 1 (CD45^{+}Lin^{-}CD3^{-}NK1.1^{+}NKp46^{+} cells) include NK cells, ILC1 and ILC3. Group 2 (CD45^{+}Lin^{-}CD3^{-} CD90^{int}CD45^{high} cells) are mainly GATA3^{+} ILC2, but also contain a few RORγt^{+}ILC3. Group 3 (CD45^{+}Lin^{-}CD3^{-}

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$CD90^{high}CD45^{int}$ cells) are 99% $ROR\gamma t^{+}ILC3$. (b) Gating strategy for isolation of intestinal ILC3 subsets from *Rag1*$^{-/-}$ mice. Gating is performed on live $CD45^{+}Lin^{-}CD3^{-}CD90.2^{+}NK1.1^{-}$cells. Then, $CD90^{high}CD45^{int}$ cells are selected, and divided further based on NKp46 and CCR6 expression as follows: $NKp46^{-}ILC3$ ($CD45^{+}Lin^{-}CD3^{-}NK1.1^{-}CD90.2^{high}CD45^{int}CCR6^{-}NKp46^{-}$), $NKp46^{+}ILC3$ ($CD45^{+}Lin^{-}CD3^{-}NK1.1^{-}CD90.2^{high}CD45^{int}$ $CCR6^{-}NKp46^{+}$), and $CCR6^{+}ILC3$ ($CD45^{+}Lin^{-}CD3^{-}NK1.1^{-}CD90.2^{high}CD45^{int}CCR6^{+}NKp46^{-}$).

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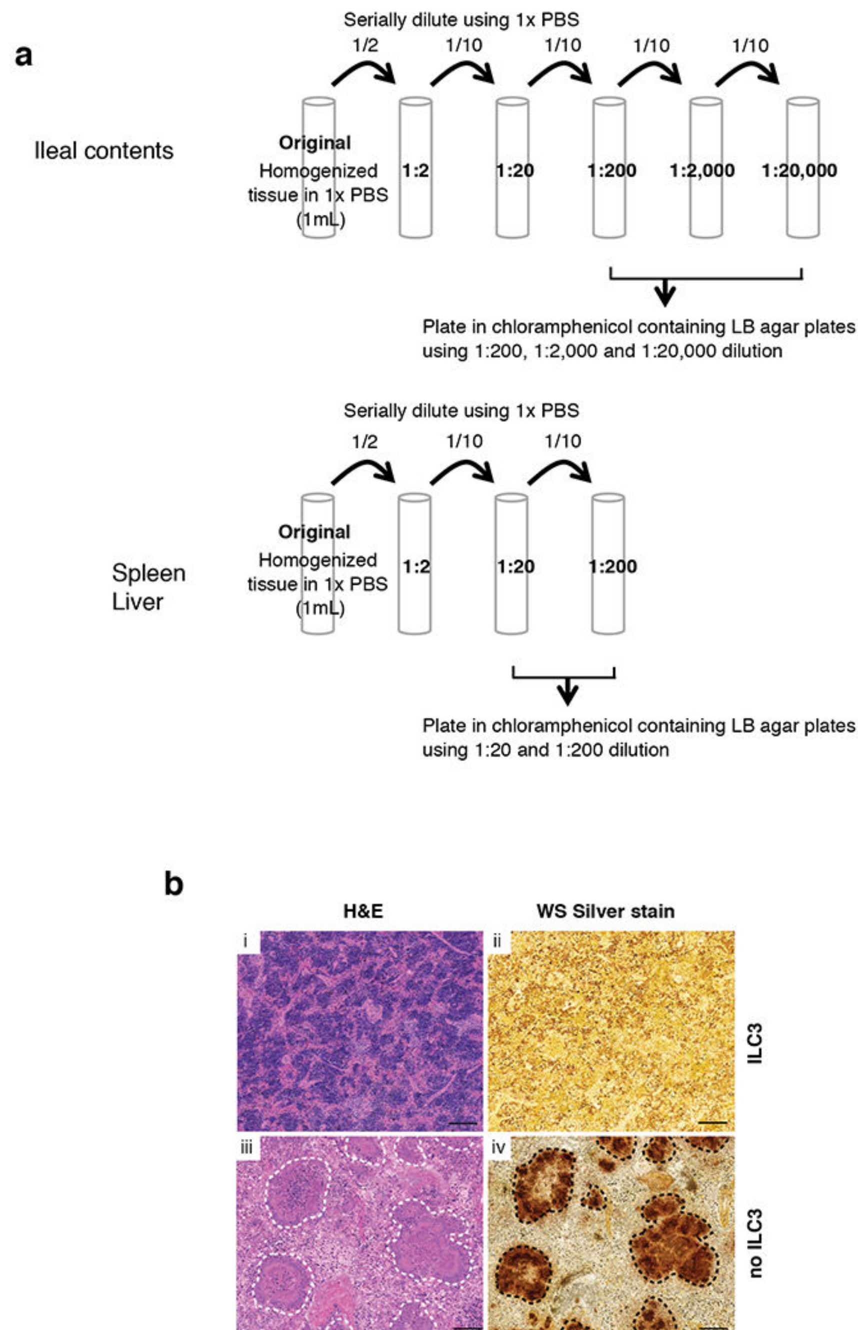

Fig.3. Quantifying *Yersinia enterocolitica* Infection

(a) Serial dilution of bacterial cultures. The CFU/g can be calculated using the formula: CFU/g = (no. of colonies in 1mL of PBS x dilution factor) / (grams of tissues). (b) Representative H&E-stained (i,iii) and Warthin-Starry silver stained (ii,vi) splenic sections of *Rag2*$^{-/-}$*gc*$^{-/-}$ recipients with/without adoptive transfer of ILC3 after *Y. enterocolitica* infection at day 5 p.i. Scale bars, 100μm. *Rag2*$^{-/-}$*gc*$^{-/-}$ mice without adoptive transfer of ILC3 infected with *Y. enterocolitica* exhibit large areas of splenic necrosis (white dotted lines, Fig. 3b-ii) that contain *Y. enterocolitica* colonies (black dotted lines, Fig. 3b-vi) in the

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spleen. However, *Rag2*$^{-/-}$*gc*$^{-/-}$ recipients infected with *Y. enterocolitica* are rescued from large areas of splenic necrosis that contain *Y. enterocolitica* colonies by transfer of purified ILC3 (Fig. 3b-i,iii).