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A Mouse Infection Model with a Wildtype *Salmonella enterica* Serovar Typhimurium Strain for the Analysis of Inflammatory Innate Immune Cells

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[Abstract] *Salmonella enterica* serovar Typhimurium *(S.* Typhimurium*)* is a Gram-negative, facultative intracellular bacterium, which causes gastrointestinal disorders in humans, and systemic, typhoid fever-like infections in mice. Our current knowledge regarding the involvement of cellular and humoral immunity in the defense from *S.* Typhimurium infections is largely based on animal models with attenuated strains. Cells of the innate immune system act as one of the first barriers in the defense from bacteria. We established a robust experimental model for the characterization of these cell types and their response during host-pathogen interactions. Therefore, this protocol focuses on the characterization of macrophages, monocytes, and neutrophils in the spleens of infected animals by employing multi-color flow cytometry.

Keywords: Macrophages, Intracellular bacteria, Gram-negative bacteria, Infection control

[Background] *Salmonella enterica* is a highly iron-dependent siderophilic intracellular Gram-negative pathogen, which can cause local intestinal disease, or severe systemic infection and septicemia. Hence, it was included in the WHO list of the most serious infectious disease threats to human health (Keestra-Gounder *et al.*, 2015; Bumann and Schothorst, 2017). *Salmonella* invades and multiplies within mononuclear phagocytic cells in the liver, spleen, lymph nodes, and Peyer's patches, in so-called *Salmonella-containing* vacuoles (SCV) (Steele-Mortimer, 2008). However, *Salmonella* exhibits different mechanisms to evade antimicrobial activities, for example by inhibiting phagolysosomal fusion (Baumler and Fang, 2013; Bhutta *et al.*, 2018).

Several immune cells are important in the defense against bacterial infections. While some act as the first barrier, by taking up and killing bacteria, others are responsible for long-term immunity or for coordinating efficient anti-microbial immune responses. Macrophages, dendritic cells (DCs), and neutrophils represent one of the first lines of defense against invading pathogens like *Salmonella,* by recognizing specific pathogen-associated molecular patterns (PAMPs), and danger-associated-molecular patterns (DAMPs) (de Jong *et al.*, 2012). Recognition of Gram-negative bacteria in macrophages involves the binding of LPS to a receptor complex, leading to the secretion of pro- and anti-inflammatory cytokines (Nagai *et al.*, 2002; Bode *et al.*, 2012). Macrophages can polarize dependending on local factors within the microenvironment, microbial stimuli, and cytokines secreted by other cells. Pro-inflammatory macrophages are essential components of anti-microbial host defense mechanisms, and can be characterized by the synthesis of inducible nitric oxide synthase (iNOS). Anti-

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

inflammatory macrophages dampen the inflammatory state, by producing anti-inflammatory cytokines and Arginase-1 (ARG1). ARG1 cleaves L-arginine, the substrate of iNOS, and thereby impairs the control of various intracellular pathogens (Mosser and Edwards, 2008; Murray, 2017).

Several studies have shown that *Salmonella* is also phagocytosed by dendritic cells, which serve as an important link between innate and adaptive immunity (Steinman and Hemmi, 2006). Upon phagocytic internalization of bacteria, DCs mature and migrate to defined lymphoid tissues. Thereby, they increase the expression of the major histocompatibility complex II (MHCII) to present specific antigens to T cells, initiating adaptive immune response.

Due to the widespread development of multidrug resistances against antibiotics, current antibiotic treatments of invasive salmonellosis is often not successful. In addition, it is uncertain whether standard antibiotics can penetrate into the *Salmonella*-containing vacuole. Thus, it is necessary to better understand the host-pathogen interplay in salmonellosis, to develop novel effective antimicrobial therapies targeting intracellular pathogens (Lahiri *et al.*, 2010; Navarre *et al.*, 2010; Mastroeni and Grant, 2011).

Materials and Reagents

1. 10 cm bacteriological Petri dish (Falcon, catalog number: 351029)
2. Cell strainer 100 µm (Falcon, catalog number: 352360)
3. 5 ml syringe (BD, Discardit™ II 309050)
4. Sterile wedge shaped spreader (Microspec, catalog number: 211738)
5. Syringes for intraperitoneal injection (30G × ½" 0.3 mm × 12 mm, Braun, Omnican F 9161502S)
6. 1.5 mL Eppendorf Tubes (Eppendorf, catalog number: T9661)
7. 96 well round-bottom plates (BRAND, catalog number: 781600)
8. Cryovials 2 mL round bottom (Simport, catalog number: T311-3)
9. *Salmonella enterica* serovar Typhimurium ATCC14028 (ATCC)
10. LB Broth Lennox (Roth, catalog number: X964.2)
11. Phosphate buffer saline (PBS) (Lonza, catalog number: 17-515 F)
12. Agar-Agar Kobe I (Roth, catalog number: 5210.3)
13. Paraformaldehyde (Sigma-Aldrich, Formalin-solution, neutral buffered 10%, HT501128)
14. APC anti-mouse CD11b (BioLegend, catalog number: 101212)
15. FITC anti-mouse CD45 (BioLegend, catalog number: 103108)
16. BV421 rat anti-mouse F4/80 (BD Biosciences, catalog number: 565411)
17. BV510 anti-mouse Ly6C (BioLegend, catalog number: 128033)
18. PerCP-eFluor™ 710 anti-mouse Ly6G (Invitrogen, catalog number: 46-9668-82)
19. PerCP/Cyanine5.5 anti-mouse MHCII (BioLegend, catalog number: 116416)
20. BV421 anti-mouse CD11c (BioLegend, catalog number: 117330)
21. PE-Cyanine7 anti-mouse iNOS (Invitrogen, catalog number: 25-5920-80)
22. anti-mouse ARG1 (Invitrogen, catalog number: 17-3697-82)

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

23. Glycerol (Sigma-Aldrich, catalog number: G5516)
24. Fetal Bovine Serum heat inactivated (FBS) (PAN Biotech, catalog number: P30-3031)
25. Ethylenedinitrilotetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
26. Ammonium chloride (NH_4Cl) (Sigma-Aldrich, catalog number: 254134)
27. Potassium hydrogen carbonate ($KHCO_3$) (Sigma-Aldrich, catalog number: 237205)
28. Ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA) (Sigma-Aldrich, catalog number: E5134)
29. Triton X-100 (Roth, catalog number: 3051.3)
30. LB-Agar plates (see Recipes)
31. ACK lysis buffer (see Recipes)
32. FACS buffer (see Recipes)
33. Triton buffer (see Recipes)
34. LB agar plates (see Recipes)
35. LB medium (see Recipes)
36. 4% PFA (see Recipes)
37. LB-medium with 30% glycerol (see Recipes)

Equipment

1. Incubator 37°C (ELOS Breed, catalog number: B110N)
2. Shaking incubator (VWR, GFL, catalog number: 3031)
3. Photometer (Eppendorf, BioPhotometer D30, catalog number: 6133000001)
4. Centrifuge (Hettich Micro 200R, catalog number: Z652113)
5. CytoFLEX S V4-B4-R2-I2 Flow Cytometer (13 detectors, 4 lasers, Beckman Coulter, catalog number: C01161)
6. CASY TT counting system (OMNI Life Science, catalog number: TT-20A-2571)

Software

1. FlowJo v10.7.0 (BD Biosciences, https://www.flowjo.com/solutions/flowjo)
2. GraphPad Prism V8.4.1 (https://www.graphpad.com/scientific-software/prism)

Procedure

A. Grow *Salmonella enterica* serovar Typhimurium ATCC14028 in an Erlenmeyer flask, until they reach the logarithmic growth phase
   1. Open the original vial and rehydrate the entire pellet in 1 mL of LB-medium.
   2. Mix well by pipetting up and down.
   3. Pipette 10 µL in 10 mL of LB-medium as pre-culture.
      Note: Centrifuge the remaining 950 µL at 1920 × g at room temperature (RT) for 5 min,

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

resuspend the pellet in 1 mL of LB-medium containing 30% Glycerol, aliquot as 10 × 100 µL in cryovials, and freeze the aliquots at -80°C. This will be the stock for the pre-culture in further experiments.

4. Incubate the pre-culture in a rotating incubator at 200 rpm at 37°C overnight.
5. Next day, pipette 50 µL of this overnight pre-culture in 10 mL of LB-medium in an Erlenmeyer flask.
6. Incubate in an orbital shaker at 200 rpm at 37°C for 1–2 h, until an OD_{600} of 0.5 (measured in a photometer using LB-medium as blank).

B. Counting of viable *Salmonella enterica serovar* Typhimurium using for example a CASY TT counting system

1. Use the 45 µm capillary.
2. Measure the background, by placing a new Casy cup with fresh Casy ton buffer under the measuring unit.
3. Select program for background measurement (Table 1).
4. Measure background. This should be below 30 counts and 1 µm size. Otherwise, wash the system.
5. Prepare a new Casy cup with 10 mL of Casy ton buffer, and add 5 µL of *S. typhimurium* solution.
6. Shake gently.
7. Place sample under the measuring unit.
8. Select program for measuring between 1–3 µm (Table 1).
9. Measure.
10. Click next to get the number of viable counts per milliliter = viable *S.tm*/mL.
11. After the measurement is completed, remove the sample cup, and add a fresh Casy cup with 10 mL Casy ton buffer.
12. Perform Casy Clean up to five times.
13. Select Program for Washing (Table 1).
14. After washing is completed, check the background.
15. If the background is below 30, the Casy counting system can be turned off; otherwise, continue washing.

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

Table 1. Programs CASY TT Counting system

	Background Measurement	**Measurement of *S. Typhimurium***	**Washing Program**
Capillary	45 µm X-Axis: 5 µm	45 µm X-Axis: 3 µm	45 µm X-Axis: 5 µm
Sample Volume	200 µL Cycles: 1	200 µL Cycles: 3	200 µL Cycles: 10
Dilution	1.00×10^0	2.00×10^3	1.00×10^0
Y-Axis	Auto	Auto	Auto
Eval.Cursor	1.00–4.89 µm	0.75–2.93 µm	0.00–5.00 µm
Norm. Cursor	0.5–4.89 µm	0.3–2.93 µm	0.00–5.00 µm
%Calculation	%ViaDebris: On	%ViaDebris: On	%ViaDebris: On
Aggregation Correction	Auto	Auto	Auto
Interface	ParP.Feed: On	ParP.Feed: On	ParP.Feed: On
Print Mode	Manual Graphic: On	Manual Graphic: On	Manual Graphic: On

C. Intraperitoneal injection of mice

Using Omnican F syringes (30G × ½" (0.3 mm × 12 mm), intraperitoneally (i.p.) inject mice with 1000 bacteria in 200 µL of PBS, according to the measurement in the Casy counting system.

Negative control mice are injected with 200 µL of PBS.

A video demonstrating the procedure can be found at: Intraperitonelal Injection in the Mouse: https://researchanimaltraining.com/articles/intraperitoneal-injection-in-the-mouse/.

Notes:

1. *The injection of mice is performed in a bio-safety level 2 animal facility, where mice are housed in individually ventilated cages (IVC). After infection, all cages, litter, and used material must be autoclaved.*
2. *Weight and body surface temperature are important markers for monitoring the mice during the infection. Therefore, mice should be familiarized to daily weighing and temperature measurements 3–5 days prior to the experiment. This will avoid stress-induced weight loss and changes in body temperature.*
3. *All substances used should be warmed to RT. The injection of cold substances influences the wellbeing of the mice, and can lead to a sudden drop in body temperature.*

D. Monitoring of mice during the infection

Note: Mice on a C57BL/6 background express a non-functional NRAMP1 (natural resistance associated macrophage protein 1) protein. NRAMP is a phagolysosomal membrane transporter for iron, protons, and divalent cations, and is associated with host resistance to various intracellular pathogens. Non-functional NRAMP leads to intracellular persistence of S. typhimurium within macrophages, and early death after 5 days at the latest. Therefore, it is crucial to monitor the mice in the most careful way.

1. Body weight:

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

The body weight should be measured at the same time each day, to avoid behavioral changes due to food intake. It is important to use a lockable box and a precision scale, which can be disinfected.

2. Body temperature:
   The body temperature is measured with an infrared thermometer at the same time each day. Pick the mouse up by the middle of the tail and expose its abdomen. Allow the mouse to hold on to the grid of a cage with its forepaws, which allows the mouse to stretch its upper body and expose the abdomen. Hold the trigger to measure the temperature, taking care to aways measure at the same location of the abdomen.
3. General appearance of the mice:
   The general appearance of the mice should be documented twice a day, for example according to the M-CASS scoring sheet in Lilley *et al*. (2015), and your experimental animal license. Severe pain, suffering, and distress must be prevented.

E. Isolation of splenocytes

1. After 72 h of infection, isolate the spleens, and press the organs through a 100 µm cell strainer with the plunger of a 5 mL syringe, onto a Petri dish with 5 mL of cold PBS.
2. Centrifuge at 300 × *g* and 4°C for 5 min, and wash once with 5 mL of PBS (300 × *g* at 4°C for 5 min), discard the supernatant.
3. Subject the cell pellet to erythrocyte lysis, by resuspending in 2 mL of ACK lysis buffer.
4. Incubate at RT for 2 min.
5. Wash once with 5 mL of FACS buffer (300 × *g* at 4°C for 5 min).

F. Flow Cytometry stain of innate immune cells

All procedures are performed in 1.5 mL Eppendorf tubes.

1. Extracellular stain (Table 2)
   a. Prepare an appropriate mix of antibodies (1:200) in 100 µL of FACS buffer per sample (Table 2).

Table 2. Antibodies for flow cytometry – extracellular stain

Antibody	Clone	Company	Catalog number	expressed on
CD11b APC	M1/70	BioLegend	101212	neutrophils, monocytes
CD45 FITC	30-F11	BioLegend	103108	leukocytes
F4/80 BV421	T45-2342	BD Biosciences	565411	macrophages
Ly6C BV510	HK1.4	BioLegend	128033	monocytes
Ly6G PerCP-eFluor™710	1A8-Ly6g	Invitrogen	46-9668-82	neutrophils
MHCII PerCP/Cyanine5.5	AF6-120.1	BioLegend	116416	dendritic cells
CD11c BV421	N418	BioLegend	117330	dendritic cells

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

a. Resuspend the cell pellet in this mix.
b. Incubate in the dark at 4°C for 15 min.
c. Wash once with 1,000 µL of FACS buffer (300 × *g* at 4°C for 5 min).
d. Resuspend the pellets in 4% paraformaldehyde (PFA) to fix the cells for intracellular staining.
e. Transfer into 96-well round-bottom plates.
f. Analyze directly in a flow cytometer.

2. Intracellular stain (Table 3)
   a. Prepare extracellular stain as described in steps 1a–d.
   b. Resuspend the pellet in 4% PFA.
   c. Incubate at 4°C for 15 min in the dark.
   d. Centrifuge and resolve the pellet in Triton buffer.
   e. Mix well and incubate at room temperature for 15 min in the dark.
   f. Prepare appropriate mix of antibodies (1:100) in 50 µL of Triton buffer per sample (Table 3).

Table 3. Antibodies for flow cytometry – intracellular stain

Antibody	Clone	Company	Catalog number	expressed on
iNOS PE-Cyanine7	CXNFT	Invitrogen	25-5920-80	pro-inflammatory macrophages
ARG1 APC	A1exF5	Invitrogen	17-3697-82	anti-inflammatory macrophages

   g. Resuspend the cell pellet in this mix.
   h. Incubate at 4°C for 45 min.
   i. Wash once with 1,000 µL Triton buffer (300 × *g* at 4°C for 5 min).
   j. Resuspend the pellets in 4% PFA.
   k. Transfer into 96-well round-bottom plates.
   l. Analyze directly in a flow cytometer.

Data analysis

FlowJo software can be used to analyze data. Plot a linear FSC (forward scatter) versus SSC (side scatter) dot plot, and create a gate to select all cells. Using these cells, select $CD45^+$ cells (pan-leucocytes), and follow the gating strategy described in Figure 1.

Statistical analysis and graphs are performed and generated in GraphPad Prism. When data are normally distributed (assess by using histograms and statistical tools, for example the Shapiro-Wilk test), analyze by the unpaired Student's t-test for samples with equal variances, or by two-way ANOVA (*F* test). When data are not normally distributed, the Mann-Whitney U, or an ANOVA on ranks tests can be performed.

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

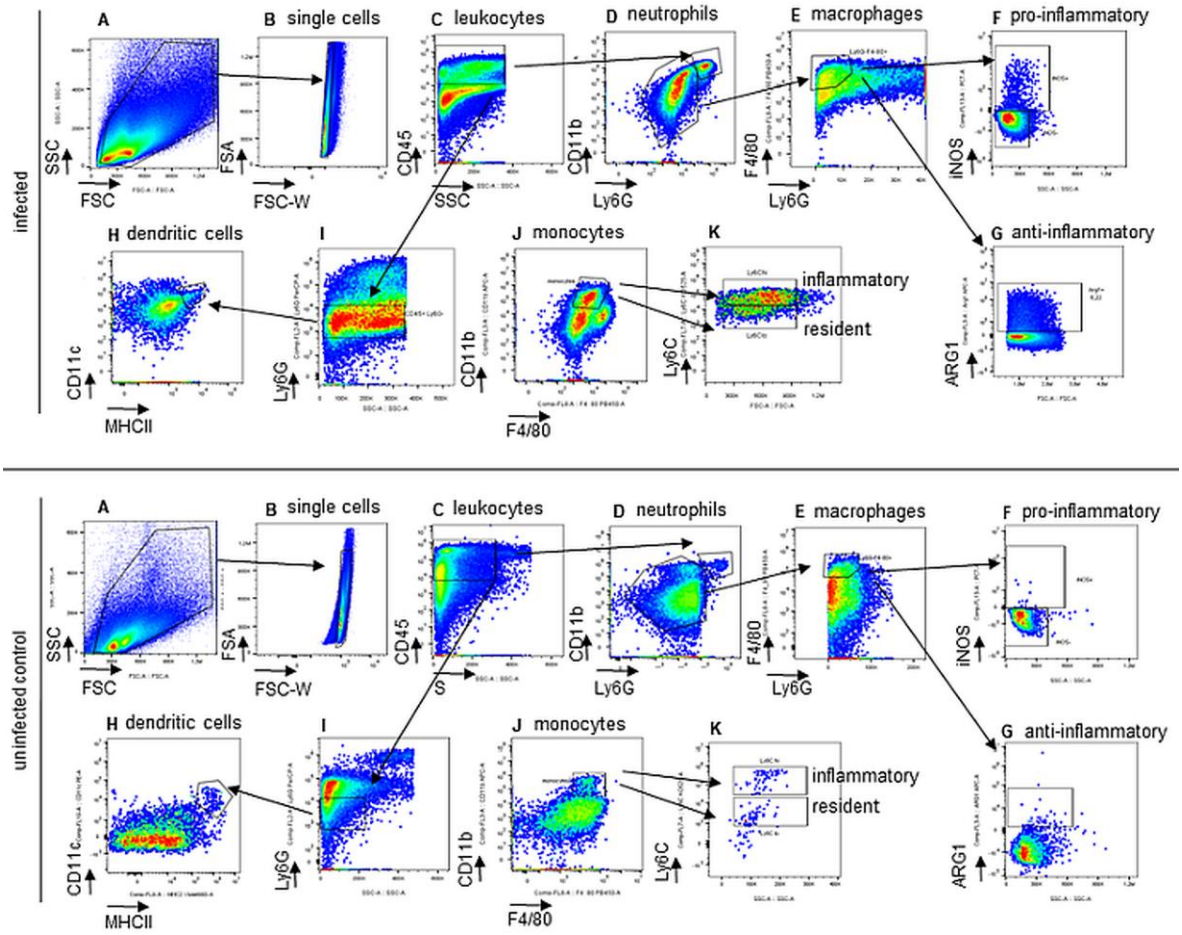

Figure 1. Gating strategy for the analysis of innate immune cells (infected mouse - upper panel, and uninfected mouse – lower panel). (A) forward scatter (FSC) against side scatter (SSC), (B) exclusion of doublets, (C) leukocytes: $CD45^+$, (D) neutrophils: $CD45^+Ly6G^+CD11b^+$, (E) macrophages: $CD45^+CD11b^-Ly6G^-F4/80^+$, (F) proinflammatory macrophages express iNOS, (G) antiinflammatory macrophages express Arginase, (H) dendritic cells: $CD45^+Ly6G^-CD11c^+MHCII^+$, (I) + (J) monocytes: $CD45^+Ly6G^-F4/80^+CD11b^+$, (K) inflammatory and resident monocytes: $Ly6C^{high}$ or $Ly6C^{low}$, respectively.

Recipes

1. FACS buffer
   PBS with 0.5% heat-inactivated FBS
   2 mM EDTA
2. ACK (Ammonium-Chloride-Potassium) lysis buffer
   150 mM NH_4Cl
   10 mM $KHCO_3$
   0.1 mM Na_2EDTA
   pH 7.2–7.4
3. Triton buffer

Bio-protocol 12(07): e4378.
DOI:10.21769/BioProtoc.4378

PBS with 0.05% Triton X-100

4. LB agar plates
   2% LB-Broth
   1.5% Agar-Agar
   Autoclave (121°C for 20 min), cool down, and pipette 15 mL in 10 cm bacteriological Petri dishes. Store at 4°C.
5. LB medium
   2% LB-Broth
   Autoclave (121°C for 20 min).
6. 4% PFA
   Add 40 µL of PFA to 960 µL of PBS.
7. LB-medium with 30% glycerol
   Add 300 µL of glycerol to 700 µL of LB-medium.

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Ethics

Animal experiments were approved by the Austrian Federal Ministry of Science and Research according to the directive 2010/63/EU.

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