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***Salmonella* Typhimurium Infection of Human Monocyte-Derived Macrophages**

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Abstract

The successful infection of macrophages by non-typhoidal serovars of *Salmonella enterica* is likely essential to the establishment of the systemic disease they sometimes cause in susceptible human populations. However, the interactions between *Salmonella* and human macrophages are not widely studied, with mouse macrophages being a much more common model system. Fundamental differences between mouse and human macrophages make this less than ideal. Additionally, the inability of human macrophage-like cell lines to replicate some properties of primary macrophages makes the use of primary cells desirable. Here we present protocols to study the infection of human monocyte-derived macrophages with *Salmonella* Typhimurium. These include a method for differentiating monocyte-derived macrophages *in vitro* and protocols for infecting them with *Salmonella* Typhimurium, as well as assays to measure the extent of infection, replication, and death. These protocols are useful for the investigation of both bacterial and host factors that determine the outcome of infection.

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DISCLOSURES:

The authors have nothing to disclose.

Keywords

Macrophages; *Salmonella*; bacteria; infection; monocyte-derived macrophage; intracellular pathogen

INTRODUCTION

In humans, infection with *Salmonella enterica* spp. *enterica* serovars cause two major types of disease. The human-specific serovars Typhi and Paratyphi cause Typhoid fever, a systemic bacterial infection, while non-typhoidal serovars primarily cause gastroenteritis, and represent a major cause of foodborne illness. In susceptible strains of mice, the non-typhoidal serovar Typhimurium causes a systemic disease that more resembles Typhoid fever in humans, and is traditionally used as a model for the study of this disease.¹ These studies have shown that the development of Typhoid-like systemic disease in mice depends upon the successful infection of and replication within macrophages².

While human infections with non-typhoidal serovars such as Typhimurium are most often self-resolving, these serovars can also cause a serious, often fatal systemic infection, particularly in certain immunocompromised patient populations³. The largest populations that are susceptible to these infections are patients with HIV AIDS, and malnourished or malaria-infected children. In sub-Saharan Africa, non-typhoidal *Salmonella* are one of the most commonly isolated bacteria from the blood, and associated with a case fatality rate of 20–25%⁴.

Systemic infection with non-typhoidal *Salmonella* in humans likely also depends upon macrophage infection, but few studies of the interactions of non-typhoidal *Salmonella* with human macrophages have been published. This may be in part because *Salmonella* Typhimurium reportedly does not replicate well in human macrophages, unlike what is seen in the macrophages of susceptible mouse strains⁵, and this is thought to be one reason that most human infections are confined to the intestines. A better understanding of the way the bacteria interact with human macrophages, and the conditions under which they survive and replicate in these cells, is important to better understand these deadly systemic infections. Important differences between mouse and human macrophages^{5–7}, and the inability of macrophage-like cell lines to recapitulate some properties of primary cells⁸, make human monocyte-derived macrophages ideal for these studies.

The way in which these macrophages are differentiated *in vitro* plays a large part in determining whether the bacteria will be killed once they are taken up, or go on to survive and replicate.⁹ Protocols in which monocytes are matured in the presence of activating agents result in the development of “M1” macrophages, cells with bacteriocidal properties that efficiently control the bacteria. This is likely the basis of at least some of the reports describing that *Salmonella* Typhimurium is unable to survive and replicate in human macrophages. The differentiation of monocyte-derived macrophages as described in Basic Protocol 1 results in a macrophage phenotype that is more permissive for bacterial survival and replication. This is more likely to represent what may happen during infection of resident, non-inflammatory macrophages, commonly described as “M2”, *in vivo*.

The expression of the type III secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (referred to as the SPI1 T3SS) is essential for the invasion of non-phagocytic cells, such as the epithelial cells of the intestine. However, recognition of this T3SS by macrophages induces activation of the inflammasome, resulting in a caspase-1-mediated inflammatory cell death termed pyroptosis.^{10,11} While the exact conditions under which the SPI1 genes are expressed *in vivo* are unknown, during broth culture these genes are transiently upregulated in the late log phase of growth^{12,13}. Stationary phase cultures, in which the bacteria do not express appreciable levels of the SPI1 genes, are commonly used for studies of macrophage infection to avoid triggering pyroptosis. However, we have previously shown that the induction of widespread pyroptotic death during infection with SPI1 T3SS-expressing bacteria can be avoided through the use of a low multiplicity of infection (MOI) and short infection time⁹. Therefore, adherence to the infection conditions described in Basic Protocol 2 will allow for the study of bacteria expressing these important virulence factors during macrophage infection. If the study of infection with bacteria not expressing the SPI1 T3SS is desired, Alternative Protocol 1 describes infection conditions using bacteria grown to stationary phase instead.

Also addressed in this unit are ways to assess the outcome of infection. The accurate assessment of bacterial survival and replication requires careful analysis. Quantification of the number of live intracellular bacteria at desired times post-infection is most easily and efficiently done through the gentamicin protection assay described in Basic Protocol 3. However, microscopic analysis, as described in Basic Protocol 4, is also essential for confirming these results, as early macrophage death due to bacterial replication can lead an underestimate of bacterial numbers. Additionally, microscopy yields valuable information about the number of bacteria per cell, the overall percentage of infected cells, and heterogeneity in the outcome of infection between individual cells. Together, these techniques allow for careful study of the infection, survival, and replication of *Salmonella* Typhimurium within human macrophages (see Figure 1). These protocols can also be used to investigate the relative pathogenicity of different strains of interest, including clinical isolates, or mutants to determine the role of particular genes in human macrophage infection. In addition, this macrophage differentiation protocol can be adapted to produce macrophages of various phenotypes, in order to investigate their mechanisms for controlling infection.

CAUTION: *Salmonella enterica* ser. Typhimurium is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

BASIC PROTOCOL 1. CULTURE OF MACROPHAGES FROM ISOLATED MONOCYTES

Below, monocytes are differentiated into macrophages over a 7-day period by culture in a medium that includes human serum and human macrophage colony stimulating factor (M-CSF). This protocol yields cells that are “M2-like” (see Figure 2). In order to prevent unwanted activation of the monocytes, we recommend using monocytes enriched through a negative selection protocol. We routinely use the Dynabeads Untouched Human Monocytes Kit (ThermoFisher Scientific), following the product protocol closely. We begin the enrichment with 1×10^8 human peripheral blood mononuclear cells (PBMC) obtained through gradient centrifugation of peripheral blood that has been enriched for monocytes by apheresis.⁹ Other sources and numbers of PBMC also may be used, by scaling the volumes according to cell number. Either fresh or frozen cells may be used. For convenience, PBMC may be stored prior to enrichment at 1×10^8 cells/ml in a freezing medium of 90% fetal bovine serum/10% DMSO for at least 1 year in liquid N₂.

The authors use human blood collected from randomly selected and anonymized donors by the Department for Transfusion Medicine, National Institutes of Health. Signed informed consent is obtained from each donor, acknowledging that his or her donation will be used for research purposes by intramural investigators throughout the National Institutes of Health.

The use of medium containing human serum (rather than fetal bovine serum) for macrophage culture is critical to the development of an M2 phenotype. We have compared the development of cells in each medium and found that those developed in the presence of fetal bovine serum have a phenotype that is less permissive for bacterial replication upon infection⁹.

Materials

Human monocytes isolated by negative selection

Complete medium (see recipe in Reagents and Solutions section)

Human recombinant macrophage colony stimulating factor (M-CSF) (PeproTech) stock at 100 µg/ml reconstituted as directed and diluted to 100 µg/ml in culture medium. Aliquoted and frozen at –80°C until use.

Desired sterile tissue-cultured treated plates (see Table 1)

1. Determine the number of cells needed for the plate(s) or dish(es) being used (see Table 1). Selection of the dishes depends upon the desired assay(s). Note that triplicate wells should be used for the gentamicin protection assay described in Basic Protocol 3, and duplicate wells should be used for the microscopic analysis described in Basic Protocol 4. Transfer the volume of cell suspension required for this number of cells into a fresh 15 ml or 50 ml tube (depending upon the target volume).
2. Centrifuge at 4°C, $200 \times g$ for 5 min to pellet the cells. Resuspend the cells in the appropriate volume (see Table 1, Culture Volume) of complete medium

containing 100 ng/ml of human recombinant macrophage colony stimulating factor (M-CSF). Add the appropriate volume of cell suspension to the plates or dishes. The day of plating is counted as day 0. Incubate cells at 37°C, 5% CO₂.

3. On day 3, remove half the volume of media from the top of the well, and replace with an equal volume of fresh complete macrophage media containing 200 ng/ml of human recombinant M-CSF, prewarmed to 37°C. Avoid disturbing the cells on the bottom of the dish. Replace in incubator and repeat on day 5.
4. On day 7, macrophages are considered mature and are ready to use. If used after day 7, replace the media as described in step 3 on day 7 and then every other day. On day 7, we find the number of live macrophages to be approximately equal to the number of monocytes originally plated on day 0. Thereafter, cell density slowly increases.

BASIC PROTOCOL 2. Infection of macrophages with SPI1-induced late log phase WT (SL1344)

This protocol and Alternative Protocol 1, below, are specific to *Salmonella* Typhimurium strain SL1344. Other strains may be used, but the optimal conditions for SPI1-induction may vary and will need to be determined (See Critical Parameters and Troubleshooting). The addition of L-histidine is specific to strain SL1344, a histidine auxotroph, as is the use of streptomycin sulfate in the growth medium, since it is resistant to this antibiotic.

If using a mutant strain unable to express the SPI1-encoded T3SS, infection efficiency will be much lower. If equal numbers of bacteria per cell is desired between such a mutant and one expressing the T3SS, a higher MOI will be needed to improve the infection efficiency of the SPI1-deficient strain. 3×–5× more bacteria than used for the WT SL1344 strain is a good starting point. Even with using higher number of bacteria, however, note that it may be difficult to achieve an infection percentage equal to that of the WT strain.

The use of centrifugation during the infection process is an additional way to improve infection efficiency of strains that do not express the SPI1 T3SS. Centrifuge the cells 5 min at 500 × *g*, 37°C, immediately after addition of the bacteria. Note that the total infection time may need to be extended by a few minutes. This procedure will also increase the infection efficiency of bacteria expressing the SPI1 T3SS. Therefore, to avoid an increase in the number of bacteria per cell, a reduction in the MOI is required. For example, when centrifuging cultures, we use approximately 60% of the amount of bacteria used in the absence of centrifugation.

In all of these infections, our goal is to infect approximately 30% of total cells, with 1–5 bacteria per cell, in order to minimize pyroptosis. Infection percentages and bacteria per cell were measured microscopically using the technique described in Protocol 4. Adjustments in bacterial numbers may need to be made until your goals are achieved.

Materials

Salmonella enterica ser. Typhimurium strain SL1344 (and any mutants of interest)

LB-Lennox agar plates containing 100 µg/ml streptomycin sulfate (or other appropriate antibiotics as needed for mutant strains)

LB-Miller culture broth (we recommend US Biologicals cat# L1520)

Streptomycin sulfate solution at 100 mg/ml in H₂O, sterile.

14 ml round bottom sterile culture tubes

125 ml sterile Erlenmeyer flasks

Sterile 1.6 ml microfuge tubes

Hank's Balanced Salt Solution (HBSS), no calcium or magnesium (Gibco #14175 or equivalent)

Complete medium (see recipe in Reagents and Solutions section)

Gentamicin sulfate (Sigma-Aldrich G1264) stock solution at 50 mg/ml in H₂O, sterile.

L-histidine (Sigma-Aldrich H8000) stock solution at 30 mg/ml in H₂O, sterile.

Differentiated macrophages (day 7 or later)

Count-up timer

1. Streak bacteria from frozen glycerol stock on an LB-Lennox agar plate containing 100 µg/ml streptomycin sulfate (or other antibiotic as appropriate) 2–6 days prior to the start of the infection to achieve single colonies. Grow at 37°C until colonies of 1–2 mm in diameter are present. Store for up to 1 week at 4°C.
2. 19.5–21.5 h prior to beginning of infection, inoculate 2 ml LB-Miller broth containing 100 µg/ml streptomycin sulfate (for WT SL1344), or other antibiotics as appropriate for the strain in a 14 ml round bottom culture tube with a single colony of bacteria. Shake at 225 rpm, 37°C for 16 – 18 h.
3. Subculture each strain of bacteria by transferring 300 µl of overnight culture into 10 ml of LB-Miller broth (without antibiotic) in a sterile 125 ml Erlenmeyer flask, and shake at 225 rpm, 37°C for 3.5 h.
4. At least 1 h prior to the start of the infection prepare all tubes of media that will be needed during the infection process and warm in a 37°C water bath or incubator. (Volumes will depend upon the number of type of wells being infected.)
5. At 3.5 h after subculture start, transfer 1 ml of each subculture into a sterile microfuge tube and centrifuge 2 min at 8,000 × *g*, RT to pellet the bacteria.
6. Optional: While bacteria are pelleting, use a spectrophotometer to check the OD₆₀₀ of the culture, to verify it has grown as expected. The growth of the bacteria in the culture is tightly linked to SPI1 induction, making this a convenient way to verify that the bacteria are expressing these genes. In our laboratory, we expect to see an O.D.₆₀₀ of about 4.0 – 4.3. (A 1:5 dilution of the culture in LB is used to obtain a reading within the linear range).

Note: For steps 7 – 11, work quickly!

7. Remove 900 µl of supernatant from the pelleted bacteria, carefully avoiding the pellet, then add 900 µl of prewarmed Hank's balanced salt solution (HBSS) and resuspend the pellet gently but thoroughly using a 1,000 µl micropipettor. Vortexing should be avoided, as it can lead to shearing of flagella.
8. Make a 1:20 dilution of the bacterial suspension by adding 50 µl bacteria to 950 µl prewarmed (37°C) HBSS, and mix thoroughly with a 1000 µl micropipettor. Set aside the original tube of resuspended bacteria for plating to calculate the inoculum amount (see step 12).
9. Use the diluted bacterial suspension to make an "infection mix" of prewarmed (37°C) complete medium + bacteria. **The volume of bacterial suspension to add to the media will have to be determined experimentally, with a final goal of adding approximately 10 bacteria per cell.** Refer to Table 1 for the infection media volumes to use for each type of plate or dish. The number of macrophages per well on day 7 is approximately the original number that was plated. As a starting point, when infecting the Ibidi 8-well chambered coverslips our laboratory adds 15 µl of the diluted bacterial suspension per 1 ml of medium to achieve an approximate MOI of 10 bacteria per cell. Therefore, for each 1 ml of mix needed, we add 15 µl diluted bacteria to 985 µl complete medium.
10. Infection: Start a timer counting up as you begin to remove the media from the macrophages gently, using a pipettor. Remove media from no more than 8 wells at a time to prevent drying, then immediately replace with the infection mix in the same order. Keep track of the timing and order as you remove and replace the media; this should be achieved as quickly as possible. Note: A multichannel pipette and sterile basin may be used to more efficiently infect 96-well plates. All subsequent washing steps will then require the use of the multichannel pipette and additional sterile basins for each medium, in order to keep the timing consistent.
11. Return the plate(s) to 37°C, 5% CO₂ as soon as all infections are complete. Infections will be complete by 10 minutes.
12. Inoculum plating: During incubation, make dilutions of the original bacterial suspension for plating, in order to back-calculate the number of bacteria that were added to the cells. When time is short, the first dilution (step 12a) is made and stored at 4°C until the further dilutions and plating can be completed at the first opportunity.
 - a. Make serial 1:10 dilutions in PBS (add 100 µl bacteria to 900 µl PBS in a microfuge tube, vortex, then add 100 µl of this suspension to 900 µl PBS, etc).
 - b. Plate the 10⁻⁶ and 10⁻⁷ dilutions by spotting five 10 µl spots of each of these dilutions onto LB-Lennox agar plates.

- c. Incubate these plates for approximately 10–11 h at 37°C, 14–15 h at 30°C, or 24 h at RT, until colonies are countable. Take care not to allow overgrowth, which can make it difficult to distinguish individual colonies.
- d. Count the colony forming units (CFU) of the five spots from whichever dilution yields counts within the range of 3 to 30 colonies per spot. Determine the original starting bacterial culture concentration as follows:

$$B = m \times DF \times 100 \quad (\text{Equation 1})$$

B = bacteria count per ml

m = mean of the counts for 5 spots

DF = dilution factor of spots counted (1, 10 or 100)

Mean of the 5 CFU counts $\times$ dilution factor $\times$ 100 = bacteria/ml.

- 13. Wash: Remove the plate(s) from the incubator 30 – 60 sec prior to the 10 min mark, so that they are ready at exactly 10 min post-infection (p.i.). When the timer reaches 10 min, begin removing the infection media and replacing with prewarmed HBSS of the same volume. Keep the timing and order consistent with that of step 10, so that all cells have infection media for about 10 minutes.
- 14. “Chase”: Beginning immediately after step 13, and following the same order and timing, remove the HBSS and replace with the same volume of prewarmed complete media (without M-CSF). Return to the incubator until 30 min p.i. This step allows attached bacteria to complete internalization.
- 15. “High” gentamicin treatment: At 30 min p.i., replace the media with prewarmed complete media containing 50 μ g/ml gentamicin, following the same order and timing as before. Return to the incubator.
- 16. Switch to “Low” gentamicin medium: At 45 min p.i., replace the media with prewarmed complete media containing 10 μ g/ml gentamicin and 500 μ g/ml L-histidine, again following the same order and timing. Return to the incubator until it is time to perform your desired assay(s).

ALTERNATE PROTOCOL 1. Infection of Macrophages with Stationary Phase Bacteria

For experiments in which it is desirable to use bacteria NOT expressing the SPI1 T3SS, the infection process is slightly different. The bacteria are opsonized prior to infection to improve their uptake by the macrophages. Also, a much higher MOI of 100 bacteria/cell is used, to further improve infection efficiency. As these bacteria do not express the SPI1 T3SS, there is no pyroptosis of the macrophages even at a high MOI. If desired, bacteria may be centrifuged onto the macrophages (5 min at 500 $\times$ g, 37°C) to further improve

uptake. We have found that when using a high MOI and opsonization this is generally unnecessary for our studies.

Materials

Salmonella enterica ser. Typhimurium strain SL1344 (and any mutants of interest)

LB-Lennox agar plates containing 100 µg/ml streptomycin sulfate (or other appropriate antibiotics as needed for mutant strains)

LB-Miller culture broth (we recommend US Biologicals cat# L1520)

Streptomycin sulfate solution at 100 mg/ml in H₂O, sterile.

125 ml sterile Erlenmeyer flasks

Sterile 1.6 ml microfuge tubes

Hank's Balanced Salt Solution (HBSS), no calcium or magnesium (Gibco #14175 or equivalent)

Complete medium (see recipe in Reagents and Solutions section)

Human male AB serum, NOT heat-inactivated

Gentamicin sulfate (Sigma-Aldrich G1264) stock solution at 50 mg/ml in H₂O, sterile.

L-histidine (Sigma-Aldrich H8000) stock solution at 30 mg/ml in H₂O, sterile.

Differentiated macrophages (day 7 or later)

Count-up timer

1. Streak bacteria from a frozen glycerol stock on an LB-Lennox agar plate containing 100 µg/ml streptomycin sulfate (or other antibiotic as appropriate) 2–6 days prior to the start of the infection to achieve single colonies. Grow at 37°C until colonies appear. Store for up to 1 week at 4°C.
2. Approximately 18.5 h prior to the desired infection time, inoculate 10 ml of LB-Miller in a 125 ml Erlenmeyer flask with a single colony of bacteria.
3. Shake for 18 h at 225 rpm, 37°C.
4. At least 1 h prior to the start of the infection prepare all tubes of media and warm in a 37°C water bath or incubator. (Volumes needed will depend upon the number and type of wells being infected.) Also prewarm human male AB serum in a microfuge tube; you will need 50 µl of serum for each strain being used.
5. Collect 1 ml of bacteria into a microfuge tube and pellet by centrifugation 2 min at 8,000 × g, RT.
6. Optional: While centrifuging the bacteria, measure the OD₆₀₀ of a 1:5 or 1:6 dilution of bacterial culture, to confirm that the culture grew as expected.

7. Remove 900 µl of supernatant from pelleted bacteria, carefully avoiding the pellet, and resuspend gently but thoroughly with 900 µl of prewarmed HBSS using a 1,000 µl micropipettor.
8. Opsonization: Add 50 µl of the bacterial suspension to the tube containing 50 µl prewarmed human male AB serum. Incubate 30 min at 37°C.
9. Inoculum plating: During incubation, make dilutions of the original bacterial suspension for plating, in order to back-calculate the number of bacteria that were added to the cells.
 - a. Make serial 1:10 dilutions in PBS (add 100 µl bacteria to 900 µl PBS in a microfuge tube, vortex, then add 100 µl of this suspension to 900 µl PBS, etc).
 - b. Plate the 10^{-6} and 10^{-7} dilutions by spotting five 10 µl spots of each of these dilutions onto LB-Lennox agar plates.
 - c. Incubate these plates for approximately 10–11 h at 37°C, 14–15 h at 30°C, or 24 h at RT, until colonies are countable. Take care not to allow overgrowth, which can make it difficult to distinguish individual colonies.
 - d. Count the colony forming units (CFU) of the five spots from whichever dilution yields counts within the range of 3 to 30 colonies per spot. Determine the original starting bacterial culture concentration as follows:

$$B = m \times DF \times 100 \quad (\text{Equation 1})$$

B = bacteria count per ml

m = mean of the counts for 5 spots

DF = dilution factor of spots counted (1, 10 or 100)

10. Dilute the opsonized bacteria/serum mixture 1:10 by adding 900 µl of prewarmed HBSS and mixing well.
11. Add the appropriate amount of diluted opsonized bacteria to prewarmed complete medium to achieve an MOI of approximately 100 bacteria/cell. **The volume of bacteria to achieve an MOI of approximately 100 will need to be experimentally determined in advance.** Refer to Table 1 for the infection media volumes to use with each type of cell culture dish. As a starting point, our laboratory uses 10 µl of opsonized bacteria suspension per well of an 8-well Ibidi chambered coverslip. Therefore, we add 50 µl of opsonized bacteria for each 950 µl of medium.
12. Infection: Remove media from the cells with a pipette, hitting “start” on a count-up timer as you begin. Immediately add the bacteria/medium mixture to the cells. Remove and replace medium with bacterial mixture no more than 8 wells at

time, to prevent drying. Keep track of the timing and order as you remove and replace the media; this should be achieved as quickly as possible. Return cells to 37°C, 5% CO₂ as quickly as possible.

13. Wash: Remove the plate(s) from the incubator 30 – 60 sec prior to the 10 min mark, so that they are ready at exactly 10 min post-infection (p.i.). When the timer reaches 10 min, begin removing the infection media and replacing with prewarmed HBSS of the same volume. Keep the timing and order consistent with that of step 10, so that all cells have infection media for about 10 minutes.
14. “Chase”: Beginning immediately after step 13, and following the same order and timing, remove the HBSS and replace with the same volume of prewarmed complete media (without M-CSF). Return to the incubator until 30 min p.i. This step allows attached bacteria to complete internalization.
15. “High” gentamicin treatment: At 30 min p.i., replace the media with prewarmed complete media containing 50 µg/ml gentamicin, following the same order and timing as before. Return to the incubator.
16. Switch to “Low” gentamicin medium: At 45 min p.i., replace the media with prewarmed complete media containing 10 µg/ml gentamicin and 500 µg/ml L-histidine (for strain SL1344), again following the same order and timing. Return to the incubator until it is time to perform your desired assay(s).

BASIC PROTOCOL 3. ENUMERATION OF INTRACELLULAR RECOVERABLE SALMONELLA

This gentamicin-protection CFU assay provides a convenient measure of the recoverable bacterial load within the macrophages. The protocol below is for an infection done in triplicate in a 96-well plate. For experiments with a large number of wells, dilutions can be made in deep well 96-well plates using a multi-channel pipette for convenience (see Option 2). This protocol can also be adapted for infections done in dishes or plates with larger surface area by using a larger volume of 0.2% DOC (enough to cover the bottom of the vessel), and diluting into a larger volume of PBS in appropriately sized tubes. For example, if wanting to determine the number of intracellular bacteria in cells grown and infected in a well of the *ibidi* dishes recommended for microscopic analysis, lysis in 100 µl of DOC and subsequent dilution into 900 µl of PBS in a microfuge tube is recommended for step 3. Be sure to vortex the tubes gently but thoroughly before making additional dilutions through the transfer of 100 µl into 900 µl of PBS.

Materials

Stock solution of 10% (w/v) sodium deoxycholate (DOC) in water (see recipe in Reagents and Solutions)

Sterile phosphate buffered saline (PBS)

LB-Lennox agar plates (without antibiotics)

Option 1: microfuge tubes

Option 2: sterile deep (1 ml) 96-well plate

- 1** Make a solution of 0.2% DOC in PBS from the 10% stock solution, by adding 20 µl of the stock solution for each 1 ml of PBS (1:50 dilution). This is best done shortly before the time point, and made fresh for each time point.
- 2** At the desired time post-infection, remove 150 µl of the medium from each well. This leaves 50 µl of liquid per well.
- 3** For each set of triplicate wells, remove the remaining 50 µl of medium from the three wells, and replace with 50 µl per well of 0.2% DOC. The cells should lyse almost instantly. Using the P200 pipette, scrape the well, collect the lysate, and transfer into PBS by pipetting up and down several times. Complete the lysis and transfer of all three samples in the triplicate set before moving onto the next, so that none remain in undiluted 0.2% DOC for a long period of time. Work quickly.

Option 1: dilution in microfuge tubes

- 4a** Transfer the 50 µl of lysate into a 1.5 ml microfuge tube containing 450 µl of sterile PBS. Vortex well. Consider this the “undiluted” sample.
- 5a** Make a 1:10 dilution by transferring 100 µl of the undiluted sample into a tube with 900 µl sterile PBS and vortex well.
- 6a** Make a 1:100 dilution by transferring 100 µl of the 1:10 dilution into 900 µl PBS and vortex well.
- 7a** Plate five 10 µl spots from each dilution onto a LB-Lennox agar plate.

Note: If there is any condensation in the plates it is recommended to dry them prior to plating. This can be done by leaving them unwrapped (with lids on) in an unhumidified 37°C incubator or on the benchtop for several hours to overnight, or with the lids off in a laminar flow hood for about 1 h. Moisture can cause the spots to run together and make it difficult to accurately count colonies. All dilutions from a single sample may be plated on one plate, if desired, by dividing it into 3 sections.

Option 2: dilution using a deep 96-well plate (1 ml volume per well). This is a way to make dilutions more quickly for a larger number of samples (several different strains or conditions)

- 4b** Prepare a sterile, deep 96-well plate by positioning it vertically, so that there are 8 wells across the top. Each column will contain the triplicates from one strain or condition. Mark a horizontal line between rows 4 and 5, and rows 8 and 9. Put 50 µl of sterile PBS in the top row, 5th row, and 9th row, for as many columns as you have sets of triplicates. (More than 8 sets of triplicate samples will require an additional plate).

- 5b** Transfer the 50 µl of lysate from each well into a well containing 50 µl of PBS. The first sample of the triplicate will be transferred into the top row, the 2nd sample of the triplicate into the 5th row, and the 3rd into the 9th row.
- 6b** Repeat with the remaining samples, one set of triplicates at a time.
- 7b** When all samples have been transferred into the plate, use a multichannel pipette to deliver 400 µl of sterile PBS to each row containing lysates (rows 1, 5 and 9), carefully pipetting 10 times to mix, avoiding bubbles. Consider these rows to be the undiluted (10⁰) samples.
- 8b** Use a multichannel pipette to transfer 50 µl of the 10⁰ samples to the row below (rows 2, 6, and 10). Create the 10⁻¹ dilutions by adding 450 µl with a multichannel pipette, and mixing by pipetting 10 times.
- 9b** Create the 10⁻² dilutions by repeating step 8b: Transfer 50 µl to the row below (rows 3, 7, 11) and add 450 µl PBS.
- Note: there is an additional blank row, allowing for a 10⁻³ dilution to be made if desired.
- 10b** Plate five 10 µl samples from each dilution onto LB-Lennox agar plates. When using the 96-well dilution method, it is possible to plate more quickly using a 2–20 µl multi-channel pipette, if desired, by plating a sample of all three dilutions at the same time. Dry plates are particularly important when doing this; see note in step 8a for suggestions regarding plate preparation.

Either option

- 11** Let the spots dry before moving the plates, and then incubate them, upside down, for approximately 10–11 h at 37°C, or 14–15 h at 30°C. They can also be left at RT for approximately 24–26 h. Take care not to overgrow, as this will make it difficult to resolve single colonies.
- 12** Count the colonies of the five spots from whichever dilution results in 3 to 30 colonies per spot.
- 13** Calculate the CFU per well using the following calculation:

$$B = m \times DF \times PF \quad (\text{Equation 2})$$

where B = total number of intracellular bacteria in a well

m = mean of the counts from the 5 replicate spots

DF = dilution factor of the spots counted (1, 10 or 100)

PF = plating factor = volume plated (10 µl)/10⁰ sample's volume (500 µl)

Note: this protocol dilutes the lysate in a total of 500 µl, so the PF = 50. If lysing cells from a larger well this may be altered. For example, with a larger well that is lysed with 100 µl of

DOC, dilution in a total of 1 ml is recommended. This will result in 10 µl/1000 µl, for a PF of 100.

BASIC PROTOCOL 4. IMMUNOFLUORESCENCE STAINING OF CELLS

Immunofluorescence microscopy analysis is a powerful method for probing host-pathogen interactions because it provides spatial information at the single cell level. We suggest a protocol that labels the bacteria and a host vesicle marker, LAMP1. To avoid differences in macrophage phenotype that may occur due to growth on a glass versus plastic surface, we suggest growing and infecting cells in Ibidi brand 8-well ibidiTreat chambered coverslips. These dishes have an optically clear, tissue culture-treated plastic surface that closely mimics the surface of a traditional plastic tissue-culture treated plate or dish.

Materials

Infected cells in tissue culture treated 8-well chambered coverslips (e.g. ibiTreat, Ibidi USA Inc.)

Mowiol mounting medium (see recipe in Reagents and Solutions section)

Stock solution of 25% paraformaldehyde (PFA) (see recipe in Reagents and Solutions section)

10× phosphate buffered saline (PBS).

SS-PBS (see recipe in Reagents and Solutions section).

Normal Donkey Serum

Goat anti-Salmonella common structural antigen (CSA-1) antibody (KPL cat# 01-91-99), reconstituted at 1mg/ml in 1:1 (v/v) glycerol:water. Store for years at −20°C.

Mouse anti-LAMP1 antibody (clone H4A3, Dev. Studies Hybridoma Bank, U of Iowa), glycerol added to 1:1 (v/v). Store for years at −20°C.

Donkey anti-goat-Alexa488 (Molecular Probes/Thermo-Fisher cat#A-11055), glycerol added 1:1 (v/v). Store for years at −20°C.

Donkey anti-mouse-Alexa568 (Molecular Probes/Thermo-Fisher cat#A-10037), glycerol added 1:1 (v/v). Store for years at −20°C.

Optional: solution of DAPI (stock of 300µM or greater).

1. Prepare Mowiol mounting medium in advance of the experiment.
2. On the day of the experiment, freshly prepare 2.5% PFA in PBS. Thaw an aliquot of the 25% stock by heating at 70°C with gentle/occasional shaking until no precipitate is visible. To 1 part 25% PFA, add 1 part 10× PBS, and 8 parts ddH₂O. Store for up to 24 h at RT.
3. At each desired time point, carefully remove the media from the wells of the dish and add 200 µl of 2.5% PFA/PBS to each well to fix. Incubate 20 min at 37°C.

4. Remove the PFA and replace with 200 μ l of PBS to wash. Repeat the wash. Store samples at 4°C in PBS until all dishes are ready for staining. Stain within 24 h if possible, although they may be stored longer. As paraformaldehyde fixation is reversible, we recommend doing a second fixation step of 10 min just prior to staining if cells have been stored for more than a few hours.
5. Permeabilization, blocking, and antibody dilutions are diluted in SS-PBS. In order to calculate the volume of SS-PBS needed, multiply the # of wells $\times$ 0.2 ml $\times$ 6 ml. Round up to the nearest ml. For a full 8-well coverslip: $8 \times 0.2 \times 6 = 9.6 \rightarrow 10$ ml.
6. Remove PBS from dishes and replace with 200 μ l SS-PBS to block/permeabilize for 30 min at RT.
7. Prepare the primary antibody mix: goat anti-CSA at 1:300, mouse anti-LAMP1 at 1:750 in SS-PBS.
8. Remove SS-PBS and add 200 μ l of primary antibody mix per well. Incubate 45 min at RT.
9. Remove antibody mix, and add 200 μ l SS-PBS per well to wash. Remove and wash two times with PBS. If needed, dishes can be stored in PBS for a few hours at RT, or overnight at 4°C.
10. Remove PBS and add 200 μ l SS-PBS per well. Incubate 15 min at RT.
11. Prepare the secondary antibody mix: donkey anti-goat Alexa488, donkey anti-mouse Alexa568, each at 1:400 in SS-PBS. It is possible to use a different combination of fluorophores, keeping in mind that the LAMP1 staining in macrophages will generally be brighter than the anti-CSA staining.
12. Remove SS-PBS from wells and replace with secondary antibody mix. Incubate 45 min at RT, protected from light.
13. Remove antibody mix and add 200 μ l SS-PBS to wash. Remove and wash two additional times with PBS. While we recommend imaging cells as soon as possible after staining, if necessary they can then be stored in PBS, in the dark, for at least 2 days at 4°C.
14. Optional: Stain DNA, to detect the nucleus, using 0.3 μ M DAPI in ddH₂O. Incubate 15 min at RT. Wash once with PBS.
15. Preserve stained cells by removing the PBS and adding 200 μ l of RT Mowiol mounting media per well. Pipette slowly to avoid bubbles. Remove PBS from only 1 – 2 wells at a time, to prevent drying. Store dishes (with lid on) protected from light at 4°C. Mowiol will not completely harden, but will provide protection from drying.
16. Use a fluorescence microscope, preferably with a 60 $\times$ oil immersion lens, to count bacteria per cell and total cells per field. It is best to analyze within 1 week of staining. Count approximately 100 cells per well, in randomly chosen fields,

with duplicate wells. Cell density varies across the well in these dishes, so it is best to choose fields from all parts of the dish.

REAGENTS AND SOLUTIONS

Complete Medium

To make 100 ml of media:

- 91 ml RPMI 1640 w/o L-glutamine (such as Gibco cat#21870)
- 1 ml MEM non-essential amino acids solution (100×) (such as Gibco cat# 11140050)
- 1 ml 1 M HEPES
- 1 ml 200 mM L-glutamine
- 1 ml 100 mM sodium pyruvate
- 5 ml human male AB serum (heat inactivated @56°C for 1 h, store for years at -20°C).

10% (w/v) sodium deoxycholate (DOC) in water

At least one day in advance, measure out the DOC, add diH₂O, and dissolve by VERY slow rocking or stirring overnight at RT. When it has dissolved, filter sterilize and store tightly closed and protected from light. Store for several months at RT.

Mowiol mounting medium

Mowiol® 4-88, a solution of polyvinyl alcohol, is an anti-fade medium that is used for immunofluorescence. When used to mount coverslips onto glass slides it will harden but when used as described here in open wells it will not harden.

1. To a 150 ml glass beaker, add:
 - 4.8 g of Mowiol® 4-88,
 - 1.25 g DABCO,
 - 12.0 ml diH₂O,
 - 24 ml of 0.2M Tris (pH 8.5).
2. Mix well using a stir bar.
3. Slowly add 9.6 ml glycerol. Cover and allow to stir overnight.
4. In the morning, set the heat on the stir plate to 50°C and continue to stir for 5–6 h.
5. Clarify by centrifugation: Most of the Mowiol should have gone into solution during the stir time, but there will likely be some crystalline material that needs to be removed by centrifugation. Transfer the solution to a 50 ml centrifuge tube capable of withstanding centrifugation at 5,000 × g. Centrifuge 15–20 min at 5,000 × g.

6. Use a pipette to carefully transfer the supernatant into a clean beaker or tube, avoiding the clear, thick “pellet”. Store 1 ml aliquots in screw cap microcentrifuge tubes at -20°C . At least 30 min before use, remove tube(s) from the freezer to allow them to warm to RT. Opened tubes may be stored at 4°C for approximately 1 month. Discard if any crystalline material is seen.

25% (w/v) Paraformaldehyde (PFA)

Caution: PFA is an irritant, weigh in a fume hood and use a mask and gloves. Dispose of properly.

1. Add 30 ml ddH₂O to 10 g PFA in a small beaker. Cover and heat to 75°C , stirring, on a hot plate.
2. Slowly add 1 N NaOH dropwise until the PFA fully dissolves, 5 – 10 drops. Add ddH₂O to a final volume of 40 ml.
3. Make a small amount of working stock (2.5%) by diluting 1:10 in PBS, and verify that the pH is 7.2 – 7.4 using a pH strip. If so, aliquot the stock solution, 500 μl per 1 ml microfuge tube. Store for years at -20°C .
4. To thaw for use: place tubes on a shaking heat block at 70°C for 5–10 min.

SS-PBS

0.1% saponin (w/v) and 10% (v/v) normal donkey serum in PBS.

For 10 ml of SS-PBS combine:

1 ml normal donkey serum
200 μl 10% saponin
1 ml 10 $\times$ PBS
7.8 ml ddH₂O

(It is recommended to create a 10% (w/v) stock of saponin in ddH₂O. Store single use aliquots for years at -20°C)

COMMENTARY

Background Information

Macrophage differentiation—Many protocols exist for the development of monocyte-derived macrophages. Different protocols can produce macrophages with very different phenotypes, which greatly affects their response to pathogen infection. Few previous studies of *Salmonella* infection in human macrophages have reported bacterial replication, and some researchers simply report that human macrophages kill the bacteria. However, when we infect macrophages differentiated according to this protocol with late log phase bacteria, we consistently observe bacterial survival and replication. While infection of these cells with bacteria at stationary phase results in little replication, the bacteria generally survive well up to at least 24 h. Therefore, differences in macrophage differentiation conditions between

different studies are a likely reason for the observed discrepancies. Additionally, the use of M-CSF versus GM-CSF as a growth factor results in vastly different cell phenotypes¹⁴.

The differentiation of monocyte-derived macrophages in media containing M-CSF yields macrophages with an “M2” phenotype, by common designations¹⁵. However, the phenotype can vary widely due to both the monocyte isolation protocol used (negative selection, positive selection, or adherence enrichment) and the composition of the growth medium. For example, we have found that the use of fetal bovine serum versus human serum in the macrophage growth medium results in macrophages which differ in their surface molecule expression, phagocytic ability, and ability to control *Salmonella* replication⁹. We have also observed that the surface on which the cells are grown can impact the phenotype as assessed by expression of cell surface markers (not shown). Phase-contrast microscopy images of cells grown according to our protocol, including 5% human serum, show a morphology that varies somewhat between donors, but can be described as a mixture of “spindly” and “fried egg” morphologies, with the fried egg morphology dominating (Figure 2A). Analysis of cells grown according to this protocol by flow cytometry shows that, regardless of donor, most cells express MHC class II (HLA-DR), but not the costimulatory molecule CD80 (Figure 2B). These cells also express a prototypical M2 marker, the macrophage mannose receptor CD206, although very few cells express another M2-associated marker, the scavenger receptor CD163. Therefore, these cells likely fall within the wide spectrum of “M2” macrophages, which is known to comprise many subsets of cells¹⁴. Although we chose to use human serum in our cultures because it is physiologically more appropriate, we have compared the use of fetal bovine serum (FBS) instead of human serum, and shown that this results in cells with a more spindly morphology, and uniformly higher levels of CD14, CD16, CD80 and CD163⁹. Cells grown in FBS were also more phagocytic, and controlled the replication of *Salmonella* more effectively, than those grown in human serum⁹.

Some other studies of macrophage infection use human monocytic cell lines such as THP-1 as a model. We have confirmed, in agreement with the findings of others, that very little to no replication occurs in THP-1 cells (not shown). THP-1 cells require stimulation (commonly via the addition of phorbol 12-myristate 13-acetate) to acquire essential macrophage-like properties such as adherence, and this activation may cause them to acquire properties more similar to a bactericidal “M1” macrophage. Additionally, we have confirmed that monocyte-derived macrophages skewed to an M1 phenotype through the addition of LPS and IFN γ also efficiently kill *Salmonella*⁹. Accordingly, this infection protocol can easily be modified to study the ability of macrophages with other phenotypes to control bacterial growth, if desired.

Bacterial culture and infection conditions—In addition to differences in macrophage phenotype, we have shown that *Salmonella* grown to late log phase before infection have increased intracellular replication versus those in stationary phase. Therefore, differences in bacterial growth conditions have a profound influence on the outcome of infection. Most investigations of macrophage infection have been done with stationary phase bacteria, to avoid macrophage pyroptosis triggered by recognition of the SPI1 T3SS. However, in our hands, late log phase (SPI1-induced) bacteria can be internalized without inducing significant pyroptosis as long as the majority of cells are not infected with more than 1–3

bacteria. To optimize the infection, we suggest carefully titrating MOIs when first establishing the experimental conditions. While these infection conditions result in a relatively low percentage of infected cells and a low number of bacteria per cell compared to some others, this may actually be more representative of the level of infection *in vivo*.

It is also worth mentioning that the level of gentamicin used in Basic Protocol 2 is considerably less than that often used in other similar protocols. Macrophages will take up gentamicin from the extracellular medium by pinocytosis, potentially exposing the intracellular bacteria to the antibiotic when high levels are used over an extended period of time¹⁶. We use the moderately high concentration of 50 µg/ml only very early in the infection protocol, to kill extracellular bacteria, and then substitute with a lower “maintenance” level of 10 µg/ml, which is sufficient to control extracellular bacterial proliferation while still minimizing the exposure of intracellular bacteria to gentamicin.

Critical Parameters and Troubleshooting

Bacterial Cultures—Growing *Salmonella* Typhimurium SL1344 to late log phase, as detailed in this protocol, causes a significant portion of the bacteria to express the SPI1 T3SS¹³. The expression of the SPI1 genes during subculture can be followed by using a strain that contains a plasmid-encoded SPI1 gene promoter fused to GFP as a reporter, such as *PprgH-gfp*[LVA]¹³. Under the culture conditions detailed in Basic Protocol 2, we found that the maximum expression of GFP occurred after 3.5 h of subculture (Figure 3). Although this experiment indicates that approximately 20% of bacteria are SPI1-induced, staining of bacteria with an antibody to GFP, to amplify the signal, reveals that the actual number is much higher (not shown). However, variations in LB formulations, such as salt content and nutrient source, will influence the growth rate and upregulation of SPI1 genes in broth culture. LB from different suppliers can also vary considerably. Therefore, it is critical to experimentally validate the concentration of bacteria typically achieved under the conditions in your laboratory prior to infection. A few “practice” cultures should be done, followed by dilution and plating of the culture to determine the typical bacterial density, in order to determine the proper dilution of bacteria needed to achieve the desired MOI. As the growth rate will also influence the timing of the upregulation of SPI1 genes, different conditions may result in different optimal subculture times. Also note that other strains of *Salmonella* may exhibit different rates of growth. For example, we have observed that the commonly used strain ATCC 14028 grows more quickly than SL1344, and reaches maximum SPI1 expression sooner, approximately 3 h after the subculture initiation (not shown). Therefore, it is important to determine the subculture conditions that result in the peak expression of SPI1, either using a reporter strain such as the one described above, or by measuring the efficiency of SPI1-dependent invasion of a non-phagocytic cell line (such as HeLa cells) by bacteria subcultured for varying lengths of time.

Infection Conditions—Although macrophages are phagocytic and capable of taking up bacteria in a T3SS1-independent manner, expression of the SPI1 regulon allows the bacteria to actively invade and the proportion of bacteria expressing SPI1 will directly influence the number of bacteria internalized. The protocol detailed here uses a short infection time of ten minutes, and adds a fairly low number of bacteria (an MOI of about 10 bacteria per cell)

with the goal of achieving a synchronized infection with a small number of bacteria per cell. In our hands, this protocol generally results in approximately 20–30% of cells being infected, with the vast majority containing less than 5 bacteria each (Figure 4). However, this can vary considerably due to bacterial culture conditions and donor-related differences in the uptake efficiency of the cells. As the percentage of infected macrophages increases, there is a directly proportional increase in the average number of intracellular bacteria per cell (Figure 4). We have also observed that, although an increase in the number of bacteria added results in infection of a greater percentage of cells, it also results in an increased percentage of the macrophages dying from caspase-1 mediated pyroptosis shortly after infection⁹. Therefore, the goal of the experiment must be kept in mind when choosing the MOI and the infection conditions require careful optimization if one wishes to avoid early macrophage death. The included protocol for assessment of bacteria by immunofluorescence microscopy can be used to determine the percentage of cells infected and the typical number of bacteria per cell. For a simple assessment of pyroptosis at 1 – 2 h p.i. a cell viability stain such as propidium iodide can be used.

Selection of Assay Time Points—The commonly used gentamicin protection assay (Basic Protocol 4) provides a convenient way to measure the change in intracellular bacterial numbers, but this actually reflects the balance between bacterial replication and death. Therefore, these data have potential to be misleading. For example, if a strain or mutant of interest replicates more quickly than anticipated, it may cause the death of the macrophages before the selected terminal time point of the assay. This exposes the bacteria to the gentamicin in the culture medium, resulting in the killing of the bacteria and leading to an underestimation of the extent of bacterial proliferation. Therefore, the recovered CFU of that strain would be lower at the terminal time point, and the researcher may incorrectly conclude that this strain has a replication defect. By doing a microscopic analysis of the cells at carefully chosen time points, one can be certain to select time points that will accurately assess the change in intracellular bacterial populations. Therefore, it is recommended that microscopy be used to verify results, especially when investigating new strains or altering infection conditions.

Microscopic analysis—The largest variable of success in this protocol lies with the selection of antibodies. While antibodies other than those listed can certainly be used, each will require trials to confirm their ability to bind to the cells after fixation, and to determine the optimal dilution to use while staining. It is important to use high quality secondary antibodies, which have been cross-adsorbed to limit non-specific staining, with the brightest and most stable fluorophores. Of course, the fluorophore choice is determined by the imaging system. For example, if using a confocal microscope the fluorophores must be matched to wavelength of the lasers. To minimize photobleaching and oxidation, which will cause loss of fluorescence signal over time, it is also essential to use a suitable mounting medium. The mowiol mounting media that we use contains the additive 1,4-diazabicyclo[2.2.2]octane (DABCO) that considerably reduces fading by acting as a free-radical scavenger. Commercially available mounting media can also be used, although they are a lot more expensive. Fluorescently labeled samples should always be stored in the dark. Also, be aware that lower-affinity antibodies may require analysis within 24 h of staining,

rather than being stable for a week or more as those described in Protocol 4. Alternatively, the samples may be fixed a second time with PFA after antibody staining.

Statistical Analyses

Primary cells from different donors differ in their susceptibility to *Salmonella* infection and we have observed considerable variation in the extent of replication (See Figure 5B as an example). This can have a huge impact on experiments, for example, if you are looking for replication defects, donor cells that support a higher level of WT replication are more likely to reveal a defect. For each experiment, we strongly recommend using cells from at least 3 independent donors and at least 3 independent experiments. In addition, when performing the gentamicin protection assay, the use of triplicate samples to obtain three CFU values which will then be averaged is essential to obtaining consistent results. For microscopic analyses we use duplicate wells, averaging the data obtained from each. When comparing different bacterial strains or mutants it is often necessary to normalize the replication of each strain to that of WT. Comparisons can then be made between data obtained with cells from different donors, as long as the baseline level of WT replication is high enough.

Comparisons between the extent of replication of two strains (generally WT and a mutant) can be done using the Student's T test. When more than two strains or conditions are being compared, Analysis of Variance (ANOVA) must be used. One-way ANOVA is used if there is only one independent variable whereas two-way ANOVA is required if there are two independent variables. If this shows an overall statistically significant difference in group means (i.e., a statistically significant ANOVA result) a post hoc test is used to identify which groups are statistically different. If multiple pairs of experimental groups and controls are compared then a "multiple comparison analysis" test must be used. Some of the commonly used ones include Tukey, Bonferroni and Dunnett. Each of these statistical tools have specific uses, advantages and disadvantages depending on the type of data. We use GraphPad Prism software for statistical analysis.

Understanding Results

Gentamicin Protection Assay (Basic Protocol 3)—The gentamicin protection assay provides a convenient way to enumerate the number of intracellular bacteria at any given time point. A comparison of the recoverable colony forming units (CFU) over multiple time points can be used to measure the net change in the number of intracellular bacteria over the course of the infection. As shown by the example of Figure 5A, the data from independent experiments using cells from different donors can vary considerably. To simplify comparisons between different experiments using different donors, the data can be represented as the fold-change in CFU as compared to an initial time point, such as the 2 h p.i. time point in these experiments (Figure 5A, right panel). The extent of donor-to-donor variability of the net replication of the bacteria is apparent when comparing the fold change in recoverable CFU in macrophages between 2 and 18 h p.i. from 15 different donors (Figure 5B). Selection of the terminal time point in these experiments is critical, since death of macrophages at late time points will expose bacteria to gentamicin in the media, resulting in an underestimate of replication. In these experiments, 18 h was selected because immunofluorescence microscopy and live cell imaging showed that death of the most

highly-infected macrophages begins to occur at time points just after 18 h. As a result, in these same experiments assays done at 24 h p.i. often showed lower CFU values than at 18 h p.i. (not shown).

The gentamicin protection assay is an efficient way to compare the ability of different strains to survive and/or replicate in the macrophages. Figure 5C shows data comparing the fold change in CFU over time in three independent experiments comparing WT and bacteria lacking *phoP*. As this strain has a known replication defect in macrophages^{9,17}, this data confirmed its similar importance to replication in human monocyte-derived macrophages.

Immunofluorescence microscopy (Basic Protocol 4)—Immunofluorescence (IF) microscopy analysis provides valuable information on the percentage of cells infected and the number of bacteria per cell at each time point evaluated. Figure 6A shows an example of IF images of infected cells obtained with a confocal microscope. These macrophages show abundant LAMP1+ve vesicles, and therefore staining for LAMP1 allows for visualization of the cell as well as the *Salmonella*-containing vacuoles. Bacteria can be visualized using an LPS-reactive antibody, as in this protocol, or by the use of a strain constitutively expressing a fluorescent molecule. Some care must be exercised when choosing fluorescent strains, as some show compromised growth and survival, likely due to excessive production of the plasmid and/or the fluorescent molecule it encodes¹⁸.

One effective way to present IF microscopy data is by categorizing, or “binning”, the infected cells according to the number of intracellular bacteria they contain, as shown in Figure 6B. In this experiment, macrophages on day 7 were infected with WT SL1344 bacteria, and cells were fixed at 2, 8 and 18 h p.i. Infected cells were stained as described in Basic Protocol 4, and then determined to contain less than five, five to nine, or 10 or more bacteria using a widefield fluorescence microscope. Easily observable is the increase in the proportion of infected cells containing five to nine or 10 or more bacteria (shown here in gray and black) over time. The number and parameters of “bins” can be selected in order to most effectively convey the data. Shown in Figure 6C are data from a set of three independent experiments comparing infection with WT to the isogenic *phoP* mutant strain (these are the same experiments shown in Figure 5C). For this figure, only one bin is shown: cells containing ten or more bacteria. The replication defect of the mutant is clearly demonstrated by these data. By also considering results showing a slight decrease in the total percentage of cells infected with the mutant strain over time (not shown here), we can conclude that *phoP* contributes to both replication and survival in these cells.

Infections with stationary phase bacteria (Alternative Protocol 1)—Although the above data were obtained from infections using late log phase bacteria, most published studies of *Salmonella* Typhimurium infection of macrophages use bacteria grown to stationary phase. Macrophages grown according to Basic Protocol 1 will internalize stationary phase bacteria, but much less efficiently due to the fact that bacteria in a stationary phase culture generally do not express the SPI1-encoded T3SS1 (Figure 7A)^{13,19}. Without this T3SS, these bacteria will not induce early pyroptotic death of the macrophages, even when multiple bacteria are taken up per cell. The uptake of these bacteria by macrophages can be enhanced by opsonization in serum (as described in this protocol), and/or purified

complement components in order to enhance phagocytosis through engagement of complement receptors on the macrophage. Using a high MOI of bacteria opsonized with human serum as described in this protocol, we achieved an average of about 70% of cells infected, as assessed by IF microscopy at 2 h p.i. (Figure 7B). This induced no measureable cell death by 1.5–2 h p.i. (not shown). Even with opsonization, the uptake is inefficient, with only about 0.1–0.2% of the total bacteria added to the cells during the infection being found intracellularly at 2 h p.i., as measured by a gentamicin protection assay (Figure 7C). However, uptake can be increased by using longer infection times and/or centrifugation of the bacteria onto the cells, if these manipulations are compatible with the experimental plan. As we have previously reported⁹, we see much lower levels of bacterial replication when infecting with bacteria grown to stationary phase versus what we usually observe with late-log phase bacteria, both by gentamicin protection assay (Figure 7D) and IF microscopy (Figure 7E). There were slight, but not statistically significant, increases in the recoverable CFU and the number of bacteria per cell over the course of the shown 18 h experiment.

Time Considerations

Basic Protocol 1 takes approximately 15 min, then 7 days of differentiation time. During this time, a media change is done on days 3, 5, and 7 (if needed), with each media change requiring approximately 30 mins to prewarm media, and about 15 min to complete the media change, depending on the number of plates being cultured.

Basic Protocol 2 requires an overnight culture of 16–18 h, followed by a subculture of 3.5 h. The infection process itself takes approximately 1 h.

Alternative Protocol 1 requires an 18 h overnight culture, followed by about 45 min to collect and opsonize the bacteria. The infection process itself takes approximately 1 h.

Basic Protocol 3 requires about 30 min at each selected time point, followed by growing of plates for 12–24 h (depending upon selected method), and then about 5 min per plate to count colonies.

Basic Protocol 4 requires about 20 min at each selected time point to fix the cells. The staining protocol takes about 2.5 – 3 h.

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References

1. Tsolis RM, Xavier MN, Santos RL, Baumler AJ. How to become a top model: impact of animal experimentation on human *Salmonella* disease research. *Infect Immun*. 2011; 79:1806–1814. DOI: 10.1128/IAI.01369-10 [PubMed: 21343352]

2. Fields PI, Swanson RV, Haidaris CG, Heffron F. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A*. 1986; 83:5189–5193. [PubMed: 3523484]
3. Gordon MA. *Salmonella* infections in immunocompromised adults. *The Journal of infection*. 2008; 56:413–422. DOI: 10.1016/j.jinf.2008.03.012 [PubMed: 18474400]
4. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal *salmonella* disease: an emerging and neglected tropical disease in Africa. *Lancet*. 2012; 379:2489–2499. DOI: 10.1016/S0140-6736(11)61752-2 [PubMed: 22587967]
5. Schwan WR, Huang XZ, Hu L, Kopecko DJ. Differential bacterial survival, replication, and apoptosis-inducing ability of *Salmonella* serovars within human and murine macrophages. *Infect Immun*. 2000; 68:1005–1013. [PubMed: 10678900]
6. Gross TJ, et al. Epigenetic silencing of the human NOS2 gene: rethinking the role of nitric oxide in human macrophage inflammatory responses. *J Immunol*. 2014; 192:2326–2338. DOI: 10.4049/jimmunol.1301758 [PubMed: 24477906]
7. Schneemann M, Schoeden G. Macrophage biology and immunology: man is not a mouse. *J Leukoc Biol*. 2007; 81:579. discussion 580.
8. Kortmann J, Brubaker SW, Monack DM. Cutting Edge: Inflammasome Activation in Primary Human Macrophages Is Dependent on Flagellin. *Journal of immunology*. 2015; 195:815–819. DOI: 10.4049/jimmunol.1403100
9. Lathrop SK, et al. Replication of *Salmonella enterica* Serovar Typhimurium in Human Monocyte-Derived Macrophages. *Infect Immun*. 2015; 83:2661–2671. DOI: 10.1128/IAI.00033-15 [PubMed: 25895967]
10. Brennan MA, Cookson BT. *Salmonella* induces macrophage death by caspase-1-dependent necrosis. *Molecular microbiology*. 2000; 38:31–40. [PubMed: 11029688]
11. Monack DM, Navarre WW, Falkow S. *Salmonella*-induced macrophage death: the role of caspase-1 in death and inflammation. *Microbes Infect*. 2001; 3:1201–1212. [PubMed: 11755408]
12. Lundberg U, Vinatzer U, Berdnik D, von Gabain A, Baccarini M. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. *Journal of bacteriology*. 1999; 181:3433–3437. [PubMed: 10348855]
13. Ibarra JA, et al. Induction of *Salmonella* pathogenicity island 1 under different growth conditions can affect *Salmonella*-host cell interactions in vitro. *Microbiology*. 2010; 156:1120–1133. DOI: 10.1099/mic.0.032896-0 [PubMed: 20035008]
14. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports*. 2014; 6:13. [PubMed: 24669294]
15. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol*. 2006; 177:7303–7311. 177/10/7303 [pii]. [PubMed: 17082649]
16. Flannagan RS, Heit B, Heinrichs DE. Intracellular replication of *Staphylococcus aureus* in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination. *Cellular microbiology*. 2016; 18:514–535. DOI: 10.1111/cmi.12527 [PubMed: 26408990]
17. Miller SI, Kukral AM, Mekalanos JJ. A two-component regulatory system (phoP phoQ) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci U S A*. 1989; 86:5054–5058. [PubMed: 2544889]
18. Knodler LA, et al. Cloning vectors and fluorescent proteins can significantly inhibit *Salmonella enterica* virulence in both epithelial cells and macrophages: implications for bacterial pathogenesis studies. *Infect Immun*. 2005; 73:7027–7031. DOI: 10.1128/IAI.73.10.7027-7031.2005 [PubMed: 16177386]
19. Steele-Mortimer O, Meresse S, Gorvel JP, Toh BH, Finlay BB. Biogenesis of *Salmonella typhimurium*-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cellular microbiology*. 1999; 1:33–49. [PubMed: 11207539]

SIGNIFICANCE STATEMENT

Salmonella enterica serovar Typhimurium (*Salmonella* Typhimurium) primarily causes gastrointestinal disease in healthy humans. However, serious, often fatal systemic infections with non-typhoidal *Salmonella* strains such as Typhimurium can also occur, particularly in immunocompromised populations, and these are likely dependent upon infection of macrophages. Therefore, the interactions between *Salmonella* and human macrophages are of great interest, but not widely studied, perhaps due to reports that human macrophages can readily kill *Salmonella* Typhimurium. Here we provide protocols for the infection of primary monocyte-derived macrophages, a model in which the survival and replication of *Salmonella* Typhimurium can be observed and studied.

Figure 1. Schematic overview of the protocols
Numbers refer to the protocol describing each procedure.

Figure 2. Phenotype of mature macrophages at day 7

A) Phase-contrast photos of macrophages after 7 days of differentiation as described in the protocol. Cells from 3 different donors show somewhat variable morphology. Bar = 100 μ m

B) Representative flow cytometry data of day 7 macrophages showing the expression of macrophage cell surface markers. Cells were removed from 6-well plates and stained as previously described⁹. Cells were gated on CD33 and CD11b expression. The background autofluorescence level of unstained cells is shown in gray.

Figure 3. SPI1-expression of bacteria cultured in LB-Miller with aeration as described in Basic Protocol 3

Bacteria containing a plasmid-encoded fluorescent reporter for a SPI1 gene promoter, *PprgH-gfp[LVA]*¹³, were subcultured as described in the protocol, and samples removed at the indicated times. Bacteria were fixed for 15 min at R.T. with 2.5% PFA, and stained with the nucleic acid stain Syto 41 (ThermoFisher Scientific). The GFP fluorescence intensity of Syto 41⁺ events was detected by flow cytometry, and the percentage of bacteria expressing GFP was determined using FlowJo analysis software. Shown is the average percentage of GFP+ve bacteria (left axis, green circles) and the corresponding O.D. at 600 nm (right axis, black triangles) at each time point in two experiments, +/- the range.

Figure 4. The total percentage of cells infected directly correlates with the number of bacteria per cell

Data from 10 independent experiments/donors show that as the percentage of infected cells increases, so does the proportion of cells with 5 or more bacteria. The linear regression line is shown, $r^2 = 0.96$.

Figure 5. Assessment of intracellular bacteria as recoverable colony-forming units (CFU) by gentamicin protection assay

(A) Macrophages grown as described in Basic Protocol 2 were infected with late log phase strain SL1344, and the recoverable CFU at 2, 8 and 18 h p.i. were determined as described in the protocol. Each symbol represents data from one independent experiment. Different donors were used in each experiment. *Left*, recoverable CFU per well. *Right*, the CFU values normalized to 2 h p.i., to facilitate comparisons between experiments. (B) Shown is the fold change in recoverable CFU between 2 and 18 h p.i. for cells infected with late log phase SL1344 in 15 independent experiments, using different donors. Note the wide range of replication between different experiments and donors. (C) Cells were infected with WT SL1344 or an isogenic strain lacking *phoP*. Recoverable CFU were determined at 2, 4, 8 and 18 h p.i., Each of 3 independent experiments using different donors is represented by a unique set of lines and symbols (circles, WT; squares, *phoP*). Data are normalized to the 2 h value.

Figure 6. Immunofluorescence microscopy analysis showing increased number of intracellular bacteria per cell over time

Cells were infected with SPI1-induced SL1344 bacteria according to the protocol, fixed at 2, 8 and 18 h p.i., and stained for IF microscopy. (A) Representative images showing infected cells at 2 h and 18 h p.i. Red = LAMP1, Green = *Salmonella* (LPS), Blue = DAPI (DNA). Bar = 10 μ m. (B) The proportion of infected cells having 1 – 4, 5 – 9, and 10 bacteria is shown at the indicated time p.i. for a representative experiment. (C) Cells were infected with either WT SL1344 or an isogenic strain lacking *phoP*, and the percentage of infected cells containing ≥ 10 bacteria at 2, 8 and 18 h p.i. is shown. Data are the average of 3 independent experiments using cells from different donors. Copyright © American Society for Microbiology, *Infection & Immunity*, 2015, 83:2661–2671, DOI: [10.1128/IAI.00033-15](https://doi.org/10.1128/IAI.00033-15)

Figure 7. Infection of macrophages with stationary phase bacteria results in less replication than seen by late log phase bacteria

A) Bacteria containing the SPI1 reporter *PprgH-gfp*[LVA] were grown either to stationary phase (left) or late log phase (middle), as described in the protocols. The percentage of GFP +ve bacteria then detected by flow cytometry is shown. For determination of background fluorescence, WT bacteria lacking the *PprgH-gfp*[LVA] reporter are shown in the panel on the right. (B–E) Macrophages were infected with stationary phase bacteria (Alternative Protocol 1), followed by assessment of intracellular bacteria by gentamicin protection assay (Basic Protocol 4) and immunofluorescence microscopy (Basic Protocol 5) at 2 and 18 h p.i. Three independent experiments were done with different donors. All data are the mean $\pm$ SE.

SD. (B) The percentage of total cells containing bacteria at 2 and 18 h p.i. was assessed by IF microscopy. (C) The efficiency of bacterial internalization was calculated by dividing the number of intracellular bacteria at 2 h p.i. by the number of live bacteria that was added to the cells during the infection. (D) The recoverable CFU at 2 h and 18 h p.i. E) The number of bacteria per infected cell as assessed by IF microscopy is represented by binning of the infected cells into 3 categories according to the number of bacteria they contain.

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Table 1
Appropriate cell numbers and volumes for plating of monocytes in different dishes and plates.

Vessel	Cells/well	Culture Volume	Infection Volume	Potential applications	Notes
96-well plate (flat bottom)	4.0×10^4	200 μ l	50 μ l	Gentamicin protection assay	Plate triplicate wells
<i>ibidi</i> * 8-well chambered coverslip	8.0×10^4	200 μ l	100 μ l	Immunofluorescence or live cell microscopy	Plate duplicate wells
12-well plate	4.0×10^5	1 ml	500 μ l	Supernatant collection, RNA analysis of host cells	Plate triplicate wells
6-well plate	8.0×10^5	2 ml	1 ml	Flow cytometry, Western blot	Single or duplicate wells

* the use of *ibidi*Treat 8-well chambered plastic coverslips is recommended (<https://ibidi.com>)