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Experimental Infection with Listeria Monocytogenes as a Model for Studying Host Interferon- γ responses

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Abstract:	L. monocytogenes is a gram-positive bacterium that is a cause of food borne disease in humans. Experimental infection of mice with this pathogen has been highly informative of the role of innate and adaptive immune cells and specific cytokines in host immunity against intracellular pathogens. Production of IFN-γ by innate cells during sublethal infection with L. monocytogenes is important for activating macrophages and early control of the pathogen1-3. In addition, IFN-γ production by memory adaptive lymphocytes is important for priming the activation of innate cells upon reinfection4. The L. monocytogenes infection model thus serves as a great tool for investigating whether new therapies that are designed to increase IFN-γ production have an impact on IFN-γ responses in vivo and have productive biological effects such as increasing bacterial clearance or improving mouse survival from infection. Described here is a basic protocol for how to conduct intraperitoneal infections of C57BL6/J mice with the EGD strain of L. monocytogenes and to measure IFN-γ production by NK cells, NKT cells, and adaptive lymphocytes by flow cytometry. In addition, procedures are described to: (1) grow and prepare the bacteria for inoculation, (2) measure bacterial load in the spleen and liver, and (3) measure animal survival to endpoints. Representative data are also provided to illustrate how this infection model can be used to test the effect of specific agents on IFN-γ responses to L. monocytogenes and survival of mice from this infection.
Author Comments:	In terms of filming. I think it would best to film how: (1) the bacteria are grown for inoculation and injected, (2) how the liver and spleen are dissected, (3) how bacterial load is measured in the spleen and liver, and (3) how the spleen is processed in

	preparation for measuring IFN-gamma responses by flow cytometry. I am not interested in filming the sick mice with infection or mice handled within the animal facility.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

TITLE:

Experimental Infection with *Listeria Monocytogenes* as a Model for Studying Host Interferon-γ responses

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KEYWORDS:
Listeria monocytogenes; bacteria; infection model; mice; flow cytometry

SHORT ABSTRACT:
This protocol describes how to inoculate C57BL6/J mice with the EGD strain of *Listeria monocytogenes (L. monocytogenes)* and to measure interferon-γ (IFN-γ) responses by natural killer (NK) cells, natural killer T (NKT) cells, and adaptive T lymphocytes post-infection. This protocol also describes how to conduct survival studies in mice after infection with a modified LD_{50} dose of the pathogen.

LONG ABSTRACT
L. monocytogenes is a gram-positive bacterium that is a cause of food borne disease in humans. Experimental infection of mice with this pathogen has been highly informative of the role of innate and adaptive immune cells and specific cytokines in host immunity against intracellular pathogens. Production of IFN-γ by innate cells during sublethal infection with *L. monocytogenes* is important for activating macrophages and early control of the pathogen[1-3]. In addition, IFN-γ production by memory adaptive lymphocytes is important for priming the activation of innate cells upon reinfection[4]. The *L. monocytogenes* infection model thus serves as a great tool for investigating whether new therapies that are designed to increase IFN-γ production have an impact on IFN-γ responses *in vivo* and have productive biological effects such as increasing bacterial clearance or improving mouse survival from infection. Described here is a basic protocol for how to conduct intraperitoneal infections of C57BL6/J mice with the EGD strain of *L. monocytogenes* and to measure IFN-γ production by NK cells, NKT cells, and adaptive lymphocytes by flow cytometry. In addition, procedures are described to: (1) grow and prepare the bacteria for inoculation, (2) measure bacterial load in the spleen and liver, and (3) measure animal survival to endpoints. Representative data are also provided to illustrate how this infection model can be used to test the effect of specific agents on IFN-γ responses to *L. monocytogenes* and survival of mice from this infection.

INTRODUCTION:
IFN-γ is a cytokine that is crucial for mediating immunity against intracellular pathogens and for controlling tumor growth[5]. The importance of this cytokine in bacterial resistance is evident in the observation that humans with mutations in the IFN-γ signaling pathway are highly susceptible to infection with mycobacteria and *Salmonella*[6]. Similarly, mice deficient in either IFN-γ or the IFN-γ receptor exhibit defects in resistance to mycobacterium[7-9] and other intracellular pathogens including *L. monocytogenes*[10,11], *Leishmania major*[12], *Salmonella typhimurium*[13], and certain viruses[11]. In addition to combatting pathogens, IFN-γ plays a crucial role in host-defense against tumors[14]. Though higher production of IFN-γ is beneficial in the context of infection or cancer, prolonged production of this cytokine has been linked to the development of systemic autoimmunity[15-17] and the acceleration of type I diabetes in the non-obese diabetic mouse model[18].

The major sources of IFN-γ include NK cells, NKT cells, γδ T cells, T helper 1 (Th1) cells, and cytotoxic T lymphocytes (CTL)[5,19,20]. IFN-γ enhances both innate and adaptive immunity by: (1) up-regulating major histocompatibility complex (MHC) class I and II expression, (2) increasing the expression of co-stimulatory molecules on antigen presenting cells, (3) enhancing macrophage phagocytosis and the production of pro-inflammatory cytokines and microbicidal factors (*e.g.*, nitric oxide and reactive oxygen species), (4) promoting the differentiation of naïve $CD4^+$ T cells into Th1 effector cells, (5) promoting antibody class switching to immunoglobulin (Ig)2a and IgG3 (in mouse), (6) inducing the production of chemokines to recruit immune cells to sites of infection, and (7) enhancing NK cell and CTL responses[5,19]. Given the crucial importance of IFN-γ in the host response to pathogens and tumors, recombinant IFN-γ has been tested as a treatment for various infections and malignancies (reviewed in[19]). However, because systemic administration of IFN-γ or the Th1 promoting cytokine interleukin-12 (IL-12) is associated with side effects and dose-related toxicity[19,21], there is interest in developing alternative strategies to increase IFN-γ production by immune cells. Development of new biologics and small molecules requires *in vivo* screening tools to test whether such agents increase IFN-γ production during an immune response and whether this translates into meaningful biological effects such as increases in animal survival.

Experimental infection of mice with the gram-positive bacterium *L. monocytogenes* has been an instrumental model for deciphering the role of IFN-γ in host-immunity against intracellular pathogens[1,22]. Infection of mice with the pathogen intravenously or intraperitoneally (i.p.) leads to the rapid dissemination of the bacteria to the spleen and liver, where they become internalized by resident macrophages and hepatocytes with peak bacterial loads in the spleen occurring between 3 and 4 days post-infection[1,3,22]. Production of IFN-γ by NK cells is important for macrophage activation and early resistance against the pathogen[3]; however at high infectious doses, production of IFN-γ can also be detrimental to pathogen clearance[23]. NKT cells are also a source of IFN-γ in the spleen and liver during early [2,24] and this production has been shown to amplify IFN-γ production by other cell types including NK cells[2]. On the other hand, later-acting adaptive T lymphocytes, $CD8^+$ T cells in particular, are important for mediating the clearance of the pathogen and providing protection against re-infection[1,4,22].

This infection model has been attractive to researchers for a number of reasons (reviewed in[1]). First, infection with the pathogen is highly reproducible and induces a strong Th1 and cellular immune response. Secondly, during sublethal infection, bacterial load is concentrated in the liver and spleen where it can be easily measured. Thirdly, the pathogen can be safely handled under Biosafety Level 2 (BSL2) conditions. Fourthly, the organism and the immune response that it generates have been extensively characterized. Finally, a variety of mutant and genetically-modified strains have been developed that are available for use.

Described here is a basic protocol for inoculation of C57BL6/J mice with the EGD strain of *L. monocytogenes*[25] and for measuring IFN-γ responses by NK, NKT, and adaptive lymphocytes post-infection. Also described is how to measure bacterial load in the spleen and liver after sublethal infection and to carry out survival studies after infection with a modified LD_{50} dose of the pathogen. Finally, representative data are shown of how this protocol can be used to

screen the effect of new treatments on IFN-γ responses and mouse survival from *L. monocytogenes* infection.

PROTOCOL:

Safety Statement.

This protocol describes infection of mice with live *L. monocytogenes*. The pathogen is handled safely under BSL2 conditions by trained personnel who are not immunocompromised. Immunocompromised people include pregnant women, the elderly, individuals who are HIV-infected or have chronic conditions that require treatment with immunosuppressive therapy. Personnel should don a protective lab coat or gown, gloves, mask, and eye protection while handling infected samples. The work described herein was performed under BSL2 conditions under a certificate (#32876) that was issued by the University Health Network (UHN) Biosafety office. Carcasses from infected mice or any unused tissues were double-bagged and disposed of in biohazard waste. Cages from infected mice were also decontaminated by autoclaving.

Ethics Statement.

Mice were maintained and infected in a quarantine room within UHN animal facilities and were cared for in accordance with the guidelines set by the Canadian Council on Animal Care. All procedures on mice were carried out under animal use protocol #3214 that was approved by the UHN animal care committee. Due to ethical considerations, death was not used as an endpoint for survival studies. The modified LD_{50} dose reported here for *L. monocytogenes* infection was determined to be the dose at which 50% of the mice reached specific endpoints, which consisted of a 20% loss in body weight or showing at least two of the following clinical signs: lethargy, ruffled fur, hunched posture, labored breathing, dull or sunken eyes. Mice were euthanized when they reached endpoints via exposure to carbon dioxide (CO_2) according to UHN facility guidelines.

1) Preparation of glycerol stocks for long-term storage.

NOTE: This procedure describes how glycerol stocks of the EGD strain of *L. monocytogenes* are prepared from an original glycerol stock. Steps that have the potential to generate aerosols should be performed within a certified biosafety cabinet (BSC).

1.1) Prepare brain heart infusion (BHI) agar plates for bacterial growth. For this add 3.8% (w/v) BHI broth and 1.5% (w/v) agar to double distilled H_2O (ddH_20). Autoclave liquid. Once the agar cools to 50 °C, dispense liquid into bacterial petri dishes (25 ml/dish) and let plates dry (uncovered) in the BSC for 1 hr.

NOTE: Transfer BHI agar into a 50 °C water bath after autoclaving to avoid solidification prior to pouring plates. Store BHI plates at 4 °C upside down (with media side on top) until ready for use.

1.2) Prepare liquid BHI media. For this, mix 3.8% (w/v) BHI broth in ddH_20. Autoclave.

1.3) Remove frozen glycerol stock of the *L. monocytogenes* EGD strain from the -80 °C freezer and thaw to room temperature.

1.4) Dip a sterile pipette tip in the thawed glycerol stock and immediately streak the tip back and forth across a section of a BHI plate. This is the primary streak.

1.5) Turn the plate by 90 °C and using a fresh pipette tip, drag through the first streak and spread it to the next ¼ of the plate (this is the secondary streak). Repeat once more to make the tertiary streak.

1.6) Turn plate upside down and incubate at 37 °C overnight. Single uniform colonies should be obtained in the last set of streaks and visible between 16 and 24 hr.

1.7) Dispense 10 ml of sterile BHI broth into a sterile vented 50 ml tube. Pick one colony of *L. monocytogenes* from the plate using a sterile pipette tip and inoculate the broth. Incubate the culture in a 37 °C orbital shaking incubator overnight or until OD_{600}=1.0 with settings at 225 rotations per min (rpm).

NOTE: Glass or disposable plastic Erlenmeyer flasks can also be used to culture bacteria. Regardless of the type of container used, make sure that it is sterile, vented and that the volume of culture does not exceed 20% of the total volume of the container to ensure appropriate aeration of the bacteria. If using a culture tube, keep it tilted at a 45 ° angle during culture.

1.7) Prepare glycerol stocks by mixing sterile 100% glycerol with overnight bacterial liquid culture at a 1:1 ratio. Distribute the bacterial/glycerol mixture into 2 ml cryogenic vials (500 µl/vial) and transfer vials to -80 °C freezer for storage.

NOTE: Bead stock methods can also be used in place of glycerol stocks to store bacteria. By this method, porous microbeads are inoculated with a pure culture of *L. monocytogenes* and are stored at -80 °C. Each bead can be used to inoculate a fresh culture as needed. See materials list for further information.

2) Determination of growth curve of *L. monocytogenes* in day culture.

NOTE: This procedure describes how to generate the growth curve for *L. monocytogenes* that is used to estimate the colony forming units (CFU) for infection studies. All steps that have the potential to generate aerosols should be performed within a certified BSC.

2.1) Take 100 µl of overnight culture generated in Step 1.7 to 10 ml BHI media in a vented 50 ml tube and grow at 37 °C in a shaking incubator (225 rpm, tilted at 45 degree angle). Use a non-inoculated tube as a control.

2.2) Take 0.5 ml samples of the culture at hourly intervals (1, 2, 3, 4, 5, 6 hr, etc.). Dilute each aliquot 1:1 (v/v) with BHI media in a plastic cuvette. Pipette up and down to mix. Measure the optical density (OD) at 600 nm (OD_{600}) using a spectrometer. Continue culturing bacteria until OD_{600}=1.

2.3) At the same time, take a 100 µl sample of the culture and dilute with 900 µl BHI media in a sterile 1.5 ml microcentrifuge tube (this is the 10^{-1} dilution). Centrifuge the bacteria at 6000 x g for 5 min, and aspirate the supernatant.

2.4) Wash bacteria twice by resuspending the pellet in 1 mL of BHI media, centrifuging for 5 min at 6000 x g, and then aspirating the supernatant. Resuspend the pellet in 1 ml BHI media. Prepare a 10-fold dilution series of this sample in BHI media (10^{-2} to 10^{-9}). Spread 100 µl of each diluent onto separate BHI agar plates. Incubate plates overnight at 37 °C in an incubator.

2.5) The next day, pick plates that have between 30-300 colonies. Discard the rest. Count the colonies on these plates. Table 1 shows an example of counts obtained in an aliquot that was taken when OD_{600}=0.84. In this example, one of the plates (i.e., 10^-6 dilution) had colony counts between 30-300 and was used for the CFU/ml calculation.

NOTE: Plates with greater than 300 colonies are not used, since overcrowding can hinder bacterial growth and also makes it difficult to discern and enumerate individual colonies. Plates with counts < 30 are also not used because small errors in dilution technique or the presence of contaminants can have a large impact on the precision of counts at the lower end of the range.

2.6) Divide the number of colonies by the volume plated and then multiply by the dilution factor to obtain the CFU/ml value for a particular dilution. In the example in Table 1, the count at the 10^{-6} dilution was 70. Divide this value by 0.1 ml to get the CFU/ml value for the diluted culture. Then multiply this value by the dilution factor (10^6) to obtain the CFU/ml value of the undiluted culture (7.0×10^8).

2.7) Plot the OD_{600} (y-axis) versus time in h (x-axis) to identify the logarithmic phase of growth[26].

NOTE: Choose a OD_{600} reading that is in the logarithmic phase of growth that can be used as a target OD_{600} for growing day cultures. This growth curve provides an estimate of the CFU/ml of the day culture when grown to a certain OD reading. These data now can be used to estimate the CFU in a culture for preparation of inoculum (Procedure 3).

3) Preparation of the inoculum for experimental infection with *L. monocytogenes*.

NOTE: This procedure describes the preparation of the infectious inoculum from a day culture that was started from an overnight culture (prepared in Procedure 2). All of these steps are performed in the BSC unless otherwise indicated.

3.1) Calculate the number of CFU required for infection based on the number of mice and experimental design of the study. Add an appropriate volume of BHI media to a sterile vented Erlenmeyer flask or culture tube.

NOTE: The CFU of bacteria prepared will be dependent on the type of experiment performed. For studying NK and NKT cell responses during infection, each mouse is inoculated with 10^5 CFU of bacteria (section 6). If studying adaptive T cell responses to infection or measuring bacterial load, each mouse is inoculated with 2×10^4 CFU of bacteria (Procedure 8). If studying survival to endpoints, each mouse is inoculated with the LD_{50} dose of the pathogen (which is 10^5 CFU for males and 1.5×10^5 CFU for females, see section 9).

3.2) Inoculate the tube containing BHI media with 100 µl of overnight culture. Incubate the culture in a 37 °C orbital shaking incubator (225 rpm) until target OD_{600} is reached. Transfer culture contents into a sterile centrifuge tube.

3.3) Centrifuge bacteria into a pellet for 5 min at 6000 x g using a centrifuge. Aspirate the supernatant using a vacuum attached to a trap flask containing bleach.

3.4) Wash pellet twice with 1 x phosphate buffered saline (PBS), centrifuging (5 min at 6000 x g) in between.

3.5) Aspirate the second wash and re-suspend bacteria in a volume of 1 x PBS such that the desired CFU (i.e., 10^5 CFU for NK cell experiment) will be delivered to each mouse in a 200 µl volume.

NOTE: It is best to use a commercial source of sterile 1 x PBS for washing bacteria and for preparation of the inoculum, since lab glassware can introduce immunological contaminants such as lipopolysaccharide.

4) Experimental infection of mice with *L. monocytogenes*

NOTE: This procedure describes how to infect mice with the inoculum prepared in Procedure 3 and how to verify the CFU delivered in the inoculum. Handling of mice and injections are performed in a BSC.

4.1) Order a sufficient number of male or female C57BL6/J mice for your experiment. Also order mice to serve as uninfected controls.

4.2) Allow mice to acclimatize for 1 week prior to bacterial inoculation.

NOTE: This is because the stress associated with transport of the animals can trigger a transient increase in stress hormone production and lymphopenia[27,28].

4.3) On the day of inoculation, obtain a baseline body weight for each mouse and record it in the lab notebook.

4.4) In the BSC, mix the bacterial suspension up and down using a sterile pipette to ensure that the bacteria are evenly distributed and then take up 200 µl of the inoculum into a 1 mL safety engineered syringe fitted with a 25 G needle.

4.5) Inject a mouse i.p. with 200 µl of prepared inoculum (e.g. 10^5 CFU for NK cell infection, section 6). For this procedure, scruff mice with the less dominant hand by grabbing the loose skin around the mouse's shoulders. After ensuring that the mouse is well-restrained, inject the mouse in the lower quadrant of the abdomen, just lateral to the midline to avoid the bladder.

4.6) Dispose of the needle and syringe in a biohazard sharps container.

4.7) Repeat steps 4.3-4.6 until all mice are injected. Conduct similar steps with 1 x PBS injected mice (non-infected controls).

NOTE: Since the CFU is an estimate based on the growth curve, it is also good practice to check the actual CFU in the inoculum. For this, prepare 3-4 different dilutions of the prepared inoculum (using 10-fold dilution series) that you expect will result in countable colonies. Spread 100 µl of each diluent onto a BHI agar plate and incubate overnight at 37 °C. Count the colonies and calculate the actual CFU/ml as described in Procedure 2.

5) Preparing heat-killed *L. monocytogenes* for immune studies.

NOTE: All steps that have the potential to generate aerosols are performed within the BSC.

5.1) Grow day culture until OD_{600} values are reached that are within the logarithmic phase. Dispense culture into 1.5 ml microcentrifuge tubes.

5.2) Incubate tubes in a 70 °C in a water bath for 1 hr to kill bacteria.

5.3) Wash bacteria twice with 1 x PBS as in Steps 3.3 and 3.4. Resuspend in sterile complete RPMI media containing fetal calf serum (FCS) (see Supplemental File 1 for recipe) at a concentration of 4 x 10^6/ml. Aliquot killed bacteria into 2 ml sterile cryogenic vials and store at -80 °C.

5.4) Confirm the death of the bacteria by spreading 100 µl of heat-killed bacteria preparation onto BHI agar plates and incubating overnight at 37 °C.

NOTE: This heat-killed bacteria should be ready for stimulating lymphocytes in culture in Procedure 8. If there are any colonies growing on the BHI agar plate, repeat heat-killing procedure.

6) Measurement of IFN-γ responses by NK and NKT cells during infection.

NOTE: This procedure describes how to measure the IFN-γ responses by NK and NKT cells in mice at 24 hr after infection with 10^5 CFU of the *L. monocytogenes*. This dose is used because it induces robust IFN-γ responses by NK and NKT cells in the spleen[24]. Conduct all steps in the BSC. To help maintain cell viability, keep cells on ice whenever possible and use ice-cold buffers.

6.1) Inoculate mice as described in Procedure 4 with 10^5 CFU of the *L. monocytogenes*. At the same time, inject non-infected control mice i.p. with an equal volume of 1 x PBS.

6.2) Euthanize mice at 24 hr post-inoculation by CO_2 inhalation according to institutional guidelines.

6.3) Lie each mouse on its right side and wet down the skin with 70% ethanol using a squeeze bottle.

6.4) Using aseptic or sterile forceps and tough-cut scissors, incise the skin just below the bottom of the rib cage.

6.5) Spray down the exposed muscle layer with 70% ethanol. The spleen should be visible underneath the muscle layer (open arrow head in **Figure 1**).

6.6) Using aseptic or sterile forceps and fine scissors, incise the muscle layer to reveal the spleen. Gently grab the spleen with the forceps and use fine scissors to cut the spleen away from surrounding connective tissue.

6.7) Place the spleen in a 15 ml conical tube containing sterile 1 x PBS.

6.8) Half-fill sterile petri dishes with sterile 1 x PBS. Dissociate the spleen through a 70 µm nylon cell strainer into the petri dish using the flat end of a sterile 3 ml syringe.

6.9) Transfer the splenocyte suspension into a clean sterile 15 ml conical tube using a sterile 10 ml serological pipette.

6.10) Centrifuge samples at 335 x g for 10 min at 4 °C.

6.11) Aspirate the supernatant into a trap flask containing bleach. Loosen the cell pellet by flicking the tube with a finger or by dragging the bottom tube back and forth along a corrugated surface (e.g. air flow vent in the BSC).

6.12) Lyse red blood cells by adding 1.5 ml of Ammonium-Chloride-Potassium (ACK) lysis buffer (see Supplemental File 1 for recipe) to each spleen. After exactly 1 min and 15 seconds, fill the tube with 1 x PBS to stop the cell lysis.

6.13) Centrifuge cells as described in step 6.10. Aspirate the supernatant and resuspend the cell pellet in 10 ml of fluorescence-activated cell sorting (FACS) Buffer (sterile 1 x PBS containing 2% FCS).

6.14) Count the cells using a hemocytometer. For this, take two aliquots of cell suspension for counting. Add 15 µl of each cell suspension to an equal volume of Trypan blue (0.04%, made by diluting 0.4% trypan blue solution in ddH_20). Load 15 µl of each cell/Trypan blue suspension into the chamber of the hemocytometer (i.e., one in the top chamber, one in the bottom chamber).

6.15) Using a microscope, count all non-blue cells in five large squares of the central grid in each chamber (**Figure 2**). Take this count and divide it by 10 to obtain the number of cells in 10^6/ml. In the example in Figure 2, the count is 215 cells in the 5 squares; therefore, the cell concentration is 21.5 x 10^6/ml. Average the cell concentrations obtained from the two samples.

6.16) Seed 1 x 10^6 cells per well/stain in a 96-well round-bottom plate for flow cytometry staining. Make sure to also seed cells for unstained and fluorescence minus one (FMO) controls. See recommended flow cytometry staining panel in **Table 2**.

NOTE: For the following steps, it is recommended to keep cells on ice or at 4 °C and to protect cells from light with foil when fluorochromes are present. A multichannel pipette can be used to dispense liquids into 96-well staining plates to speed up processing. Be careful not to disturb the cell pellet when aspirating the supernatant from the centrifuged plate. Staining can also be done in FACS tubes if the centrifuge is not fitted with plate adapters.

NOTE: All centrifuge steps from this point on are done at 456 x g for 5 min at 4 °C.

6.17) Centrifuge the plate and then wash cells twice with FACS buffer. One wash is done by adding 200 µl of FACS buffer to each well, centrifuging the plate, and then aspirating the supernatant.

6.18) Perform blocking step by adding 50 µl/well of FACS buffer containing anti-mouse CD16/CD32 (purified Fc block) (0.5 µg). Incubate cells at 4 °C for 15 min. Wash cells once in 1 x PBS as described above.

6.19) Add 100 µl viability dye (fixable viability dye diluted 1:1000 in 1 x PBS) to cells. Stain cells at 4 °C in the dark (in refrigerator) for 30 min. Wash cells twice in FACS buffer as described above.

6.20) After a second wash, add 100 µl of cell surface antibodies or tetramers to respective wells according to the staining panel described in **Table 2**. Stain cells at 4 °C in the dark (in refrigerator) for 30 min. At this time, also add antibodies for staining single positive and FMO controls.

NOTE: Regarding single positive controls, it is recommended to either use splenocytes that are stained with various fluorochrome versions of the CD4 antibody or commercial compensation beads that are stained with the antibodies used in the panel. Prior to conducting this staining procedure, all FACS antibodies should be titrated in test studies to determine optimal concentrations for staining.

6.21) Wash cells twice in FACS buffer as described above and then fix cells by resuspending them in 50 µl of 4% paraformaldehyde (16% paraformaldehyde stock diluted in ddH_2O) and incubating for 10 min at room temperature. **CAUTION:** The paraformaldehyde is toxic and should only be handled in the fume hood.

6.22) Wash cells twice in FACS buffer, centrifuging in between. Resuspend cells in FACS buffer. Continue to next step or store cells in the refrigerator protected from light for up to three days.

6.23) Centrifuge cells, remove the supernatant, and wash cells twice with 150 µl of 1 x Permeabilization/Wash Buffer (Perm/Wash buffer), centrifuging in between. The Perm/Wash Buffer is prepared from a 10 x stock by diluting 1:9 (v/v) in ddH_20.

6.24) After the second wash, resuspend the cells in 150 µl of Perm/Wash Buffer and incubate for 15 min at 4 °C in the dark.

6.25) Centrifuge cells again and then aspirate the supernatant. Resuspend cells in 50 µl of 1 x Perm/Wash buffer containing anti-IFN-γ and incubate for 1 hr at 4 °C in the dark.

6.26) Wash cells twice with 1 x Perm/Wash buffer, centrifuging in between. Resuspend cells in 250 µl of FACS buffer and then transfer cells to FACS tubes.

6.27) Proceed to flow cytometry acquisition using a flow cytometer that has an appropriate laser configuration and filter set to discriminate fluorochromes used in the staining panel described in Table 2[29]. Collect at least 200,000 events per sample and 10,000 events for compensation controls.

6.28) Apply compensation matrix and analyze data using flow cytometric analysis software[30].

7) Measurement of bacterial load in the spleen and liver at the time of peak infection.
NOTE: All steps are performed within a BSC unless otherwise noted.

7.1) Inoculate mice i.p. with 2 x 10^4 CFU of the pathogen using procedures described in Procedure 4.

7.2) On day 3 post-infection, prepare sterile 1.5 ml microcentrifuge tubes, each containing 500 µl of ice-cold sterile 0.1% Triton X-100 in 1 x PBS and 0.2-0.3 g of 1.5-2 mm acid-washed sterile glass beads. Weigh each tube.

NOTE: Glass beads are acid washed by incubating in 10% acetic acid in a beaker on a magnetic stirrer for 1 hr. These beads are then extensively washed with ddH_20 to remove acid, are air-dried, and then autoclaved prior to use.

7.3) Euthanize mice by CO_2 exposure.

7.4) For dissection of organs, lay the animal on its back on a dissecting board and pin the limbs of the mouse to the board using 25 G needles. Disinfect skin by wetting with 70 % ethanol.

7.5) Using sterile tough cut scissors, make a midline incision in the skin from the groin to the mid chest and then from the mid groin towards each knee and from the mid chest towards each elbow. Blunt dissect and reflect back the skin, pinning it open using 25 G needles.

7.6) Disinfect muscle layer by wetting it with 70 % ethanol and then using sterile fine scissors make a midline incision in the peritoneal wall. Grab the xiphoid process with forceps and then, using the same fine scissors, make cuts in the peritoneal wall from the xiphoid process laterally on each side, following the rib cage, just below the diaphragm.

7.7) Using forceps, reflect back the peritoneal wall to reveal the liver. Cut out a ~100 mg piece of the liver (use the same lobe for all mice) using sterile scissors and place it in a pre-weighed 1.5 ml microcentrifuge tube.

7.8) Use forceps to gently push aside the organs on the left side of the peritoneal cavity to visualize the spleen. Gently grab the spleen with a pair of forceps and release it from the peritoneal cavity by cutting away the surrounding connective tissue.

7.9) Place the spleen in the pre-weighed 1.5 ml microcentrifuge tube containing beads. Transport tissues to the laboratory in a leak-proof container containing ice. Re-weigh the tubes containing the organs to determine the tissue weights in mg.

7.10) Homogenize the tissues by shaking the tubes using a bead mill homogenizer for 3 min at frequency of 30 hertz.

NOTE: The bead mill method is preferred for homogenization as it is amenable for processing a large number of samples and creates less mess and potential exposure to the pathogen. However, automatic homogenizers or autoclaved 2 ml manual glass tissue homogenizers could be used as an alternative.

7.11) Prepare a 10-fold dilution series of the homogenates in 0.1% Triton-X-100 in 1 x PBS with 250 µl volume per dilution (ranging from undiluted to 10^{-7}).

7.12) Spread 50 µl of each diluted homogenate onto half of a BHI agar plate (in duplicate) using a sterile spreader. Transfer plates to a 37 °C incubator and incubate overnight.

7.13) Keep the plates that contain up to 300 colonies/plate, discard the rest. Count colonies in each half of the plate and determine the mean number of colonies for duplicate spreads.

7.14) Calculate the CFU/mg according the following equation:

CFU/mg = CFU/ml in the homogenate, multiplied by the ml of homogenate prepared, divided by the mg weight of the tissue homogenized.

NOTE: For example, if a mean of 30 colonies were counted after plating 50 µl of 10^{-2} diluted homogenate prepared from a 120 mg piece of liver that was homogenized in 0.5 ml, the calculations would be as follows:

CFU/ml=30 colonies x 100 (dilution factor)/0.05 ml (volume spread)=60000 CFU/mL.

CFU/mg=60000 CFU/ml x 0.5 ml homogenate / 120 mg tissue= 250 CFU/mg.

8) Effects of *L. monocytogenes* on IFN-γ responses by $CD4^+$ and $CD8^+$ cells

NOTE: This procedure describes how to measure IFN-γ production by splenic $CD4^+$ and $CD8^+$ T effector cells harvested at the time of the peak of the adaptive immune response (~ 7 d post-infection) using two methods: (1) flow cytometry to measure IFN-γ by $CD4^+$ and $CD8^+$

cells by intracellular cytokine staining, and (2) ELISA to measure total IFN-γ levels produced by splenocytes (includes all T cells). Procedures are performed within the BSC.

8.1) Infect mice by injecting i.p. with 2 x 10^4 CFU of the pathogen using procedures described in Procedure 4.

8.2) On day 7 post-infection, euthanize mice by CO_2 inhalation according to institutional guidelines.

8.3) Dissect the spleen (as described above) and place in a 15 ml conical tube containing sterile 1 x PBS. Transport the tubes to the laboratory in a leak-proof container containing ice.

8.4) Process the spleens into a single cell suspension, lyse red blood cells as described in section 6, and then resuspend cells in complete RPMI media containing 10% FCS. Count cells using a hemocytometer.

8.5) Set up cultures for measurement of IFN-γ responses. For this dispense cells (4 x 10^6 in 1 ml/well) into 24-well plates together with and equal number (4 x 10^6 or 1 ml/well) of thawed heat-killed *L. monocytogenes* (prepared in Procedure 5). Transfer cells to a 37 °C incubator.

8.6) After 20 h of incubation, add 0.66 µl/ml of protein transport inhibitor to wells and continue incubations.

8.7) Four hr later, transfer plate to BSC and collect 500 µl of culture supernatant and freeze (at -80 °C) for the later measurement of IFN-γ levels using a commercial enzyme-linked immunosorbent assay (ELISA) kit[31]. Then collect cells into a sterile 15 ml tube. Wash wells with 1 x PBS and pool this wash together with collected cells.

8.8) Conduct cell-surface staining and intracellular staining for IFN-γ on $CD4^+$ and $CD8^+$ cells as described in Procedure 6 except use the staining panel described in **Table 3**. Proceed to flow cytometer acquisition (collecting 200,000 events/sample) and analyze data[29] using flow cytometry analysis software[30].

9) Measuring mouse survival to endpoints after *L. monocytogenes* infection.

NOTE: This procedure describes the effect of an agent on mouse survival to endpoints post-infection with the modified LD_{50} dose of the pathogen. All these procedures are conducted in the BSC in the animal facility.

9.1) Inject mice i.p. with the modified LD_{50} dose of *L. monocytogenes* as described in Procedure 4. This was determined to be 10^5 CFU (for male) or 1.5 x 10^5 CFU (for female)[31].

9.2) Follow mice twice daily for clinical signs and record these signs and animal body weights in a lab notebook. Euthanize mice if they show a 20% loss in body weight or two clinical signs of listeriosis (lethargy, ruffled fur, hunched posture, labored breathing, dull or sunken eyes).

9.3) After 14 days, euthanize surviving mice via CO_2 inhalation.

9.4) Prepare Kaplan-Meier plots of the data by plotting the percent survival of each group against time[32].

NOTE: If mice succumb to infection (meet endpoints described in Ethics Statement), this usually occurs by day 5 post-infection (**Figure 7**).

REPRESENTATIVE RESULTS:

Figure 3 presents some typical flow cytometry staining of IFN-γ in splenic NK and NKT cells at 24 hr post-infection with 10^5 CFU of the pathogen. This figure also illustrates the gating strategy for the staining panel described in **Table 2**. **Figure 4** shows some representative data that were obtained in one experiment where male mice were treated with the PPARα antagonist IS001 or vehicle control, infected with 10^5 CFU *L. monocytogenes,* and then analyzed for IFN-γ in NK and NKT cells after 24 hr. This figure shows that treatment with IS001 boosted IFN-γ responses by NKT cells, but not NK cells after infection with the pathogen. **Figure 5** shows representative staining for IFN-γ in splenic $CD4^+$ and $CD8^+$ T cells at 7 days post-infection after re-stimulation *ex vivo* with heat-killed pathogen. This figure also shows the gating strategy for the staining panel described in **Table 3**. **Figure 6** shows representative data that were obtained in one experiment where male mice were treated daily with the PPARα antagonist IS001 or vehicle control, infected with a sublethal dose of *L. monocytogenes,* and analyzed at 7 days post-infection. This experiment shows that treatment with IS001 enhanced IFN-γ responses by both $CD4^+$ and $CD8^+$ lymphocytes. **Figure 7** shows representative data from a study that investigated the effect of the PPARα antagonist IS001 on mouse survival to endpoints after infection with the modified LD_{50} dose of the pathogen. Plotted is the percent survival of mice against time post-infection. This figure shows that treatment with IS001 increased the survival of male mice to endpoints. Together these data illustrate how this model can be applied to investigate the effects of new drugs or treatments on IFN-γ responses *in vivo* and to explore how these immune changes impact animal survival from infection.

FIGURE LEGENDS:

Figure 1. Dissecting the spleens from infected mice.

This series of photos shows how to dissect the spleen from a dead mouse. (a) Lie the mouse on its right side and spray down the skin with 70% ethanol. (b) Using aseptic or sterile forceps and tough-cut scissors, incise the skin just below the bottom of the rib cage. (c) Spray down the exposed muscle layer with 70% ethanol. The spleen should be visible underneath the muscle layer (open arrow head). (d) Using aseptic or sterile forceps and fine scissors, incise the muscle layer to reveal the spleen. (e) Gently grab the spleen with the forceps and use fine scissors to cut the spleen away from surrounding the connective tissue. (f) Place the spleen in a 15 ml conical tube containing sterile 1 x PBS.

Figure 2. Counting splenocytes using a hemocytometer.

(a) shows the central grid of the hemocytometer. (b) shows an enlarged view of the central grid that contains 25 large squares (that each contain 16 smaller squares). The five large squares used for counting are highlighted in grey (4 corner squares plus the center square in

the central grid). (c) shows an enlarged view of one of the large grey squares. To determine the cell volume in 10^6/ml, first count all the viable cells within the five large grey squares. In the example shown, this count is 215. When counting, make sure to only count all of the clear (non-blue) cells, including those that are touching the double lines on the right and bottom of the grid. Do not count the cells touching the double lines on the left and top of the grid. Take the total five square count and divide it by 10 to obtain the number of cells in 10^6/ml. In the example, 215 divided by 10 is 21.5 x 10^6 cells/ml. Note that these calculations only work if you are counting 5 of the large squares as highlighted and dilute your cells 1:1 in trypan blue.

Figure 3. Gating strategy for detection of IFN-γ production in NK and NKT cells.
First gate on lymphocytes on FSC-A by SSC-A plot. Then gate on those events that are on the diagonal on the FSC-H/FSC-A plot. These are the singlets. Then gate on live (AmCyan$^-$) and CD8$^-$ cells. Then plot the tetramer staining against TCRβ. The NKT cells are within the double positive population and the NK cells are within the double negative population. Gate on the double positive cells, and plot NKp46 versus FSC. Gate on the NKp46 negative population, which are the NKT cells (this gate can be set by finding the point of division in the two populations from the NK cell plot). The NK cells are the tetramer$^-$ TCRβ$^-$NKp46$^+$ population. Within NK and NKT cell gates, the IFN-γ$^+$ cells in the PE channel are identified after setting a gate based on the FMO control.

Figure 4. **Representative data obtained for the frequencies of IFN-γ$^+$ NK and NKT cells at 24 h post-infection.**
In this experiment, male C57BL6/J mice (N=3-4/group) were infected i.p. with 10^5 CFU of *L. monocytogenes* or were left un-infected. Mice were also administered the drug IS001 or vehicle (0.5% carboxymethyl cellulose) at the same time of inoculation and 12 h later. Twenty-four hours after inoculation, mice were euthanized and the spleens were removed and were processed individually and stained for flow cytometry. Shown are the mean +/- SEM frequency of IFN-γ$^+$ cells in the NK (a) or NKT cell gates (b) in uninfected or infected mice after treatment with a vehicle or the drug IS001. *Indicates a difference (P<0.05) from vehicle control by two-tailed T-test. Data are re-printed from[31] with permission from the *Journal of Immunology* (volume 195, pp. 5189-5202, 2015). Copyright 2015. The American Association of Immunologists, Inc.

Figure 5. Gating strategy for detection of IFN-γ production in CD4 and CD8 cells.
First gate on lymphocytes on FSC-A by SSC-A plots. Then gate on those events that are on the diagonal on the FSC-H/FSC-A plot. These are the singlets. Within this gate, gate on live (AmCyan-) CD45$^+$ cells. Then gate on either CD8$^+$ or CD4$^+$ populations. Within each gate, the IFN-γ$^+$ cells in the PE channel are identified by comparing the staining to the FMO control.

Figure 6. Representative data obtained for the frequencies of IFN-γ$^+$ CD4$^+$ and CD8$^+$ T cells at 7 days post-infection with *L. monocytogenes* (EGD strain).
In this experiment, male C57BL6/J mice were infected i.p. with 2 x 10^4 CFU *L. monocytogenes* (N=7/group) or were left uninfected (N=3/group). Mice were also administered the drug IS001 or vehicle (0.5% carboxymethyl cellulose) twice daily starting on the day of inoculation. Seven days later, mice were euthanized and the spleens were removed and were processed individually for cell culture. Splenocyte mononuclear cells

were stimulated for 24 hr with heat-killed *L. monocytogenes* with protein transport inhibitor added for the final 4 hr of culture. Cells were then stained for flow cytometry. Shown are the mean +/- SEM frequency of IFN-γ^+ cells in the $CD4^+$ (a) or $CD8^+$ cell gates (b) in uninfected or infected mice after treatment with a vehicle (0.5% carboxymethyl cellulose) or the drug IS001. * indicates a difference ($P<0.05$) from the vehicle control counterpart as determined by two-tailed T test. Data are re-printed from[31] with permission from the *Journal of Immunology* (volume 195, pp. 5189-5202, 2015). Copyright 2015. The American Association of Immunologists, Inc.

Figure 7. Representative data obtained during an experiment that compared the effect of a PPARα antagonist 1S001 on mouse survival to endpoints after infection of male C57BL6/J mice with *L. monocytogenes* (EGD strain)

In this experiment, male C57BL6/J mice (N=10 mice/group) were infected i.p. with the modified LD_{50} dose of the pathogen (10^5 CFU) of *L. monocytogenes*. Mice were also administered the drug IS001 or vehicle (0.5% carboxymethyl cellulose) twice daily starting on the day of inoculation. Mice were followed daily for clinical signs and were euthanized if humane endpoints were met. Shown is the percent survival of mice to endpoints over time * indicates a difference in the survival between groups as determined by log-rank test ($P<0.05$). Data are re-printed from[31] with permission from the *Journal of Immunology* (volume 195, pp. 5189-5202, 2015). Copyright 2015. The American Association of Immunologists, Inc.

Table 1: Shows some representative calculations for determining CFU in an aliquot of day culture.

[insert Table 1 here]

In this example, an aliquot of day culture was taken and was diluted 1:1 with BHI media. The OD_{600} of this diluted sample was determined to be 0.84. In addition, a 100 µl aliquot was taken for CFU determination. This sample was diluted with 900 µl of BHI media (10^{-1}) and was washed and resuspended in 1 ml BHI. A 10-fold dilution series of this sample was prepared (10^{-2} to 10^{-9}) diluted samples were plated on BHI agar plates (only values for 10^{-4} to 10^{-9} are shown). The next day colonies were counted. Only those plates that had colony numbers between 30-300 were considered for the calculation (i.e., 10^-6 plate, highlighted in yellow). The number of colonies on this plate (70) was then divided by 0.1 (volume in ml plated) to get the CFU/ml of the diluted sample. This value was then multiplied by the dilution factor (10^6) to obtain the CFU/ml reading of the undiluted culture. TMTC=too many to count.

Table 2: Staining panel for detection of IFN-γ in NK and NKT cells

[insert Table 2 here]

Note that either compensation beads stained with the flow antibodies used in the panel or splenocytes stained with various fluorochrome versions of CD4 antibody clone GK1.1 can be used as single positive controls.

Table 3: Staining panel for detection of IFN-γ in $CD4^+$ and $CD8^+$ cells

[insert Table 3 here]

Note that either compensation beads stained with the flow antibodies used in the panel or splenocytes stained with various fluorochrome versions of CD4 antibody clone GK1.1 can be used as single positive controls.

DISCUSSION:

Here we describe a protocol of how to carry out a basic experimental infection with the EGD strain of *L. monocytogenes*[25] in male or female C57BL6/J mice. This protocol was set up for the purpose of studying the effect of a novel small molecule IS001 on IFN-γ production by innate and adaptive lymphocytes *in vivo*[31]. By monitoring bacterial clearance and survival post-infection, insights were gained into how these changes in IFN-γ impacted the host's ability to control the infection.

Critical considerations in the protocol.

An important consideration in the design of this type of study is that each experiment be adequately powered and appropriately controlled. Due to biological variation in the immune response to infection (see **Figures 4 and 6**), it is recommended that N=4-5 mice per group be used for the initial immune studies. If after these studies there is a trend in the data, but no significant difference apparent between groups, a power calculation could be done to determine the least number of animals required in subsequent studies to achieve statistical significance. Regarding controls, it is important to include uninfected controls for determination of baseline IFN-γ responses for immune studies and vehicle controls to help distinguish the effect of the treatment from the stress associated with administering the treatment. Another important consideration is the timing of treatment. Since the innate response to *L. monocytogenes* is very rapid, it is recommended that the first treatment be administered on the day prior to, or at the same time as, inoculation in order to ensure that therapeutic levels of the reagent be achieved prior to the initiation of the innate immune response.

Yet another important consideration is the dose of the pathogen to be used for infection. A sublethal dose is recommended for measurement of bacterial load, since it increases the chance that the pathogen will be concentrated within the spleen and liver, allowing for the more accurate enumeration of the bacteria. A sublethal dose is also recommended for enumerating IFN-γ responses by adaptive lymphocytes to ensure that animals do not succumb to listeriosis prior to the time of peak T cell expansion. In contrast, it is recommended that a higher infectious dose be used for measurement of the early NK and NKT cell response at 24 hr in order to maximize the IFN-γ production by these cells.

The classical LD_{50} is the dose of pathogen that results in 50% lethality of mice. Since death was not an acceptable endpoint at our institution and since many symptoms of listeriosis can predict whether an animal is likely to succumb to an infection, we used a defined list of clinical signs instead of death as an endpoint in our studies. Using this method, it was determined that the modified LD_{50} was 10^5 CFU for 8 week-old male and 1.5×10^5 CFU for 8 week-old female C57BL6/J mice[31]. These LD_{50} doses were determined by measuring the

percent survival of mice to endpoints in step-wise dose-escalation studies (N=5 studies in total) that each contained N=8 mice per group (e.g., mice were infected first with 10000 CFU, then a second batch with 20000 CFU, etc.). The LD_{50} calculation was determined from a regression plot of the log (CFU) (x-axis) versus the probit of the percent survival values (y-axis) (website: userwww.sfsu.edu/efc/classes/biol710/probit/ProbitAnalysis.pdf).

Note that the modified LD_{50} dose determined in our lab may differ from that in another lab even when infecting mice with the same strain of *L. monocytogenes*. Part of this variability may relate to the subjective nature of monitoring clinical signs of listeriosis compared to the more absolute endpoint of death. Additional variability can result from differences in environmental factors such as mouse diet or the microbiota or differences in the preparation of inoculum between labs. Thus, it is recommended that prior to embarking on any survival studies, a pilot study be performed where female mice (N=8 mice/group) are infected with 1.5×10^5 CFU of the same strain of *L. monocytogenes* as used in this study and symptoms monitored to determine if this dose indeed results in 50% survival to endpoints. If survival is lower or higher than 50% at this CFU, step-wise dose escalation or dose de-escalation studies could be performed to quickly narrow in on the LD_{50} dose.

Another important consideration is the strain or substrain of mice used for infection studies. This protocol describes infection of the commonly-used inbred mouse strain C57BL6/J. This strain is well-suited for measurement of IFN-γ responses since this mouse is considered to be a Th1-prone strain[33] and as a result, is relatively resistant to *L. monocytogenes* infection (compared to Th2-prone mouse strains such as BALB/c)[34,35]. Adapting this protocol to other mouse strains will require knowledge of the infectious dose of the pathogen for the particular strain. It is also recommended to use mice of the same age, sex and vendor as outlined in this protocol in order to reduce the amount of trouble-shooting involved in setting up the model. For example, C57BL6 mice ordered from one vendor (e.g., C57BL6/J) can exhibit genetic differences than C67BL6 mice ordered from another vendor (e.g., C57BL6/NTac)[36]. In addition, the intestinal microbiota differs between C57BL6 substrains obtained from different vendors, which can influence the balance of Th1 and Th17 responses in the mouse[37].

Potential modifications to technique.
Mice are most commonly inoculated i.p. or intravenously as opposed to the natural route of infection in humans, which is through the gastrointestinal tract. Oral infections are less common because standard strains of *L. monocytogenes* inefficiently infect the intestinal epithelium of mice[38]. This because there is a single amino acid change in the sequence of mouse E-cadherin from human E-cadherin that results in loss of recognition of E-cadherin by the listerial invasion protein, internalin A (InIA)[39]. To overcome this barrier, researchers use mice that are transgenic for human E-cadherin protein or use listeria that have been engineered to express a mutated sequence of InIA ($InIA^{mut}$) that binds to mouse E-cadherin with the same affinity as WT EGD for human E-cadherin[40]. Thus, one potential modification of this technique is to infect mice via the oral route. The reader is referred to another JoVE publication that describes oral inoculation methods[38]. Note that altering the mode of infection will affect the infectious dose as well as the kinetics of dissemination of the pathogen.

This protocol describes using heat-killed listeria to elicit IFN-γ production by $CD4^+$ and $CD8^+$ T cells. Heat-killed *L. monocytogenes* was chosen as a stimulus in our studies, because this antigen is inexpensive and because our lab was primarily interested in $CD4^+$ T cell responses to the pathogen. One limitation is that heat-killed bacteria does not efficiently prime $CD8^+$ T cell responses either *in vitro*[41] or *in vivo*[42,43] infection. Thus, the $CD8^+$ T cell IFN-γ production that we observed by splenocytes harvested at the peak of infection (i.e., **Figure 6**) likely is in response to the residual live bacteria present in the splenocyte cultures or was elicited as a result of cytokine-induced cytokine release[41]. As an alternative to heat-killed listeria, one could also elicit IFN-γ responses *ex vivo* by exposing T cells to peptides encoding epitopes on listerial proteins. Indeed, immunodominant MHC Class II-restricted epitopes for listeriolysin O and the p60 hydrolase and have been described for C57BL6 and BALB/C mice and immunodominant MHC Class I epitopes have been described for BALB/c[44]. Yet another approach is to infect mice with strains of *L. monocytogenes* that have been engineered to express model antigens such as ovalbumin or viral antigens in order to take advantage of existing MHC Class I- and MHC Class II-tetramer reagents to enumerate antigen-specific T cells in infected mice[45,46].

Other limitations of the protocol.
Another limitation of this model is that it only measures IFN-γ production by immune cells in the spleen. In addition to the use of tetramers to enumerate antigen specific T cells (of ovalbumin-expressing variants of *L. monocytogenes*), flow cytometry staining panels described here could be easily modified to measure the production of other cytokines such as TNF or IL-2 or effector molecules that participate in CD8 T cell or NK-mediated killing of the pathogen such as perforin or granzyme B. In addition, this protocol could also be adapted to examine IFN-γ produced by immune cells in the liver.

Future applications.
Once this protocol is mastered, it can serve as a simple *in vivo* model to screen the effects of various agents or genes on Th1 and cellular immunity.

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No conflicts of interest to disclose.

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Fig. 1.

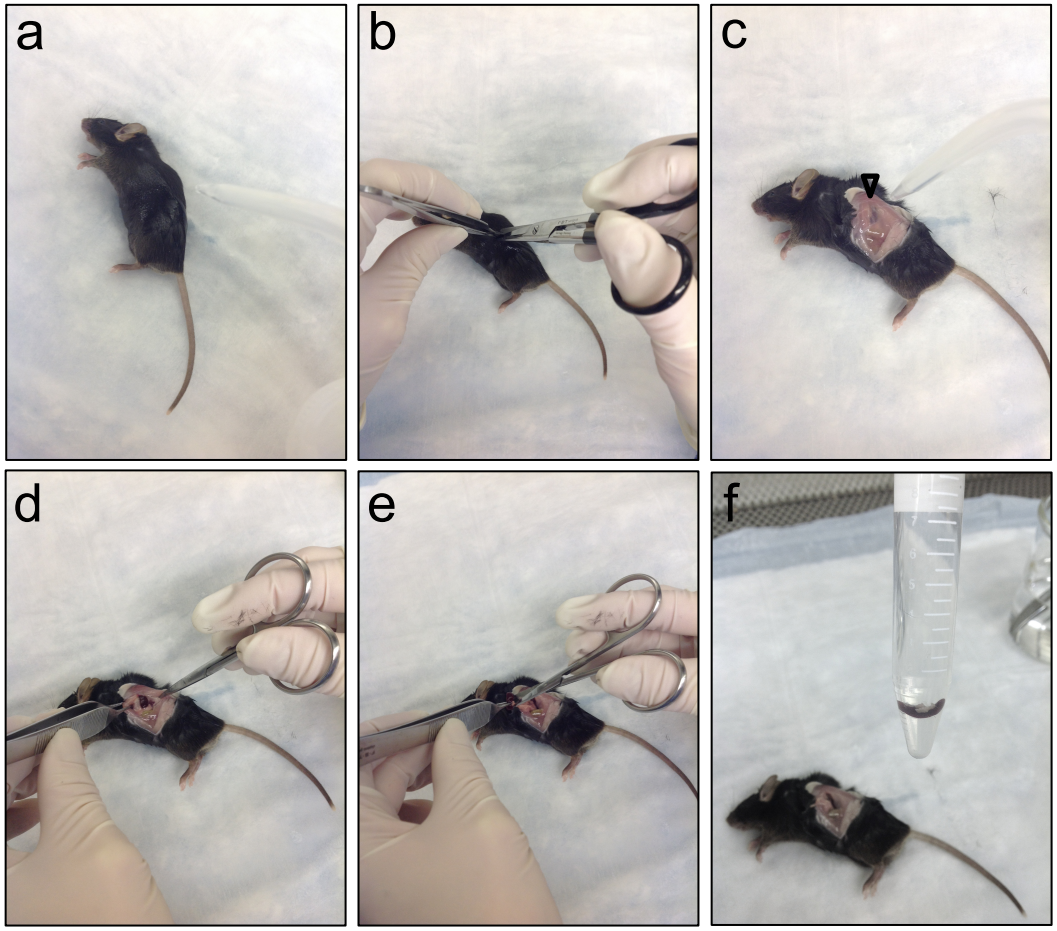

Fig. 2.

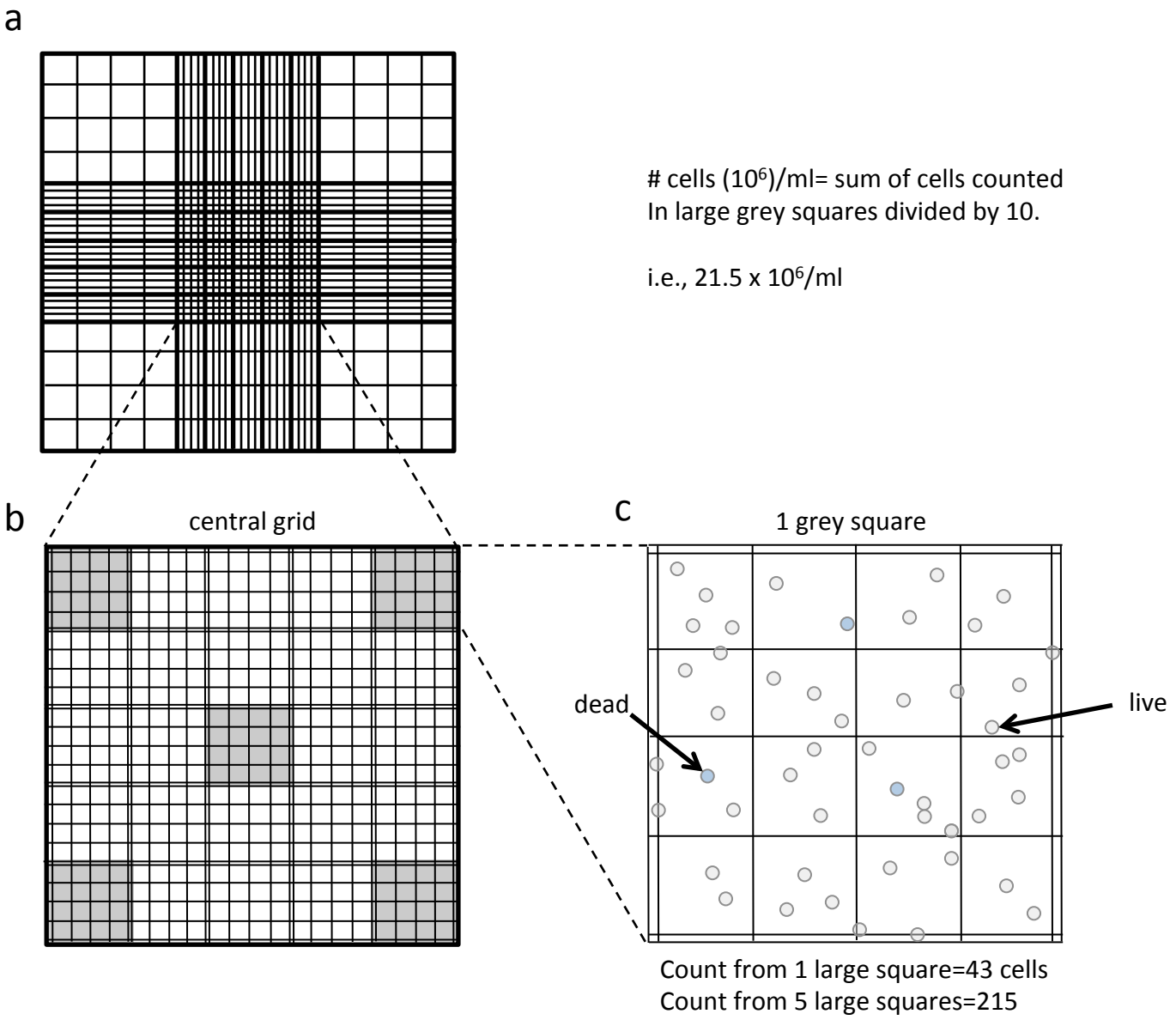

Fig. 3

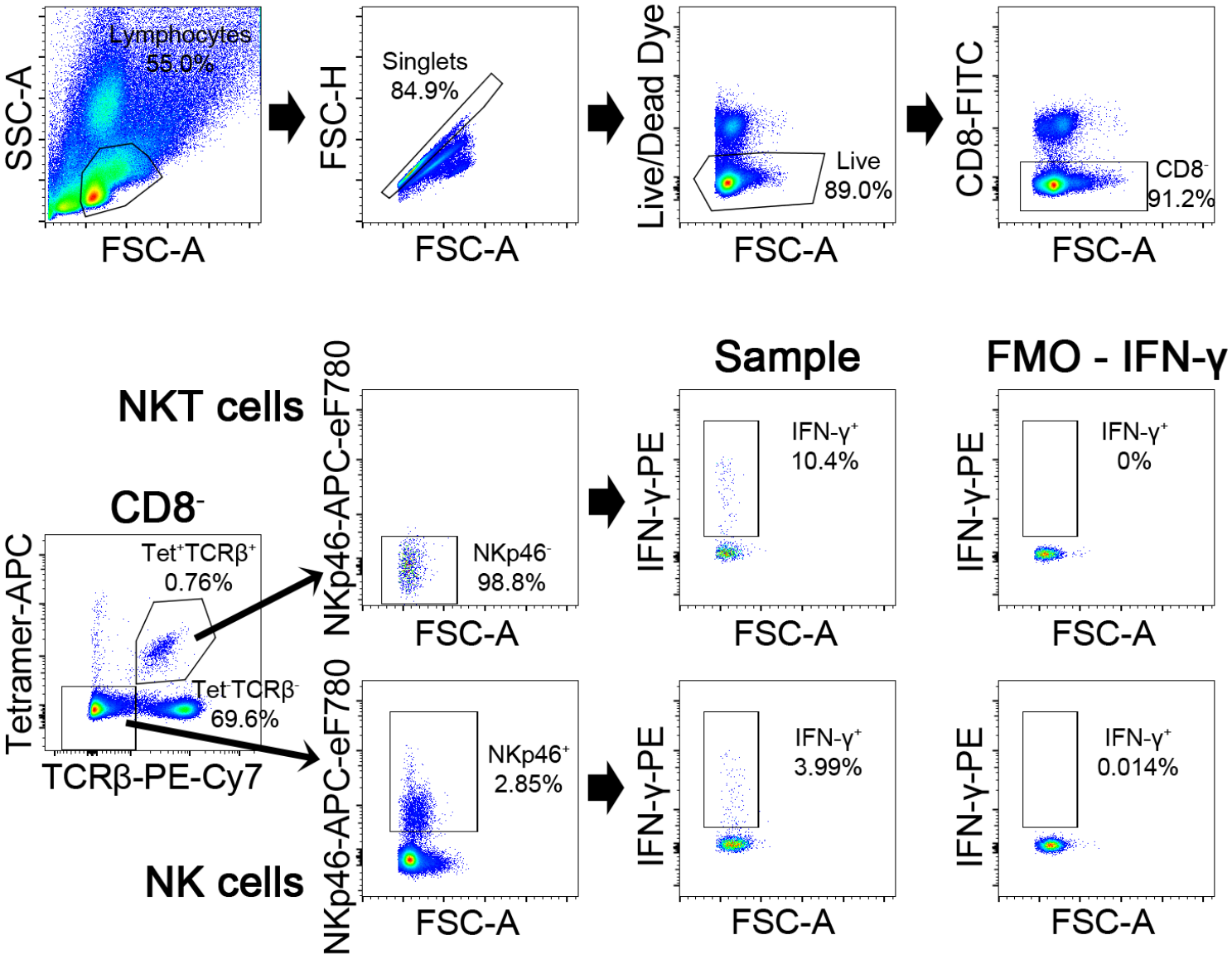

Fig. 4

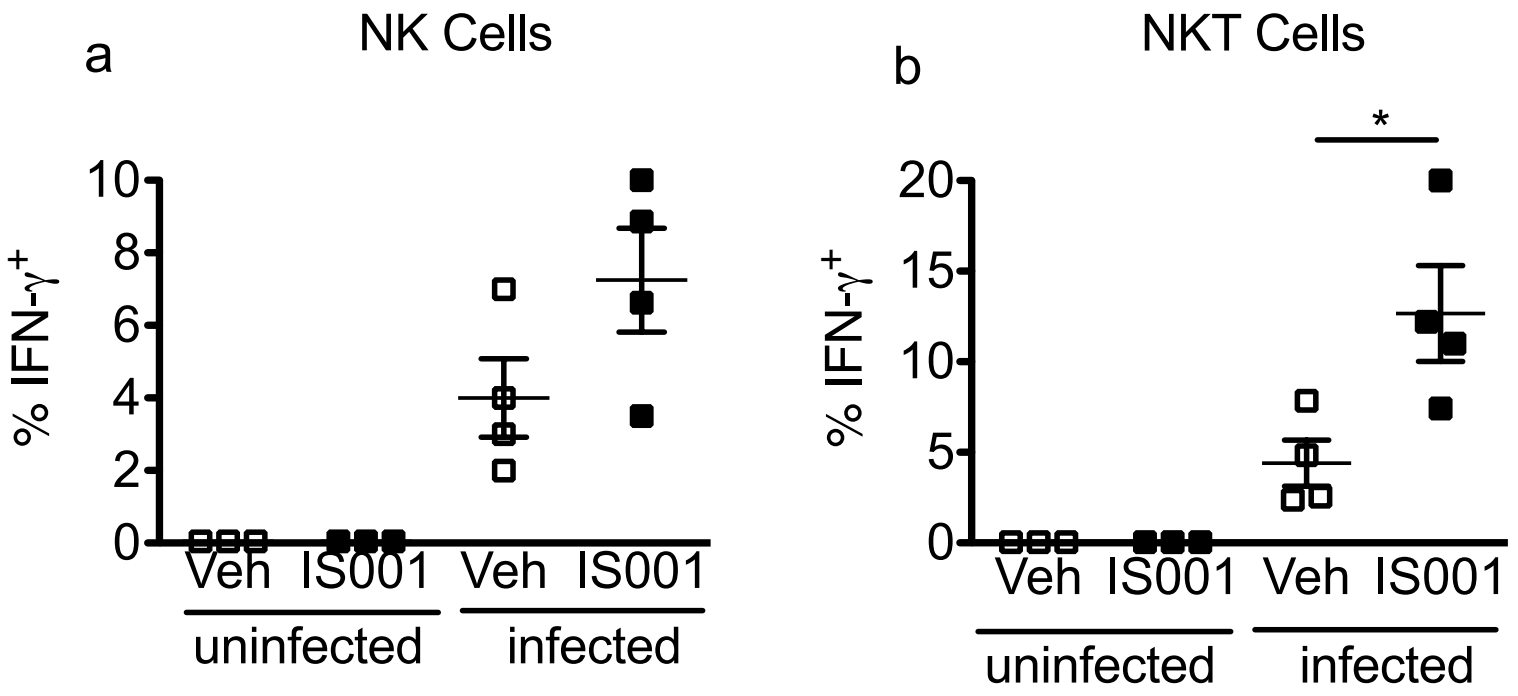

Fig. 5

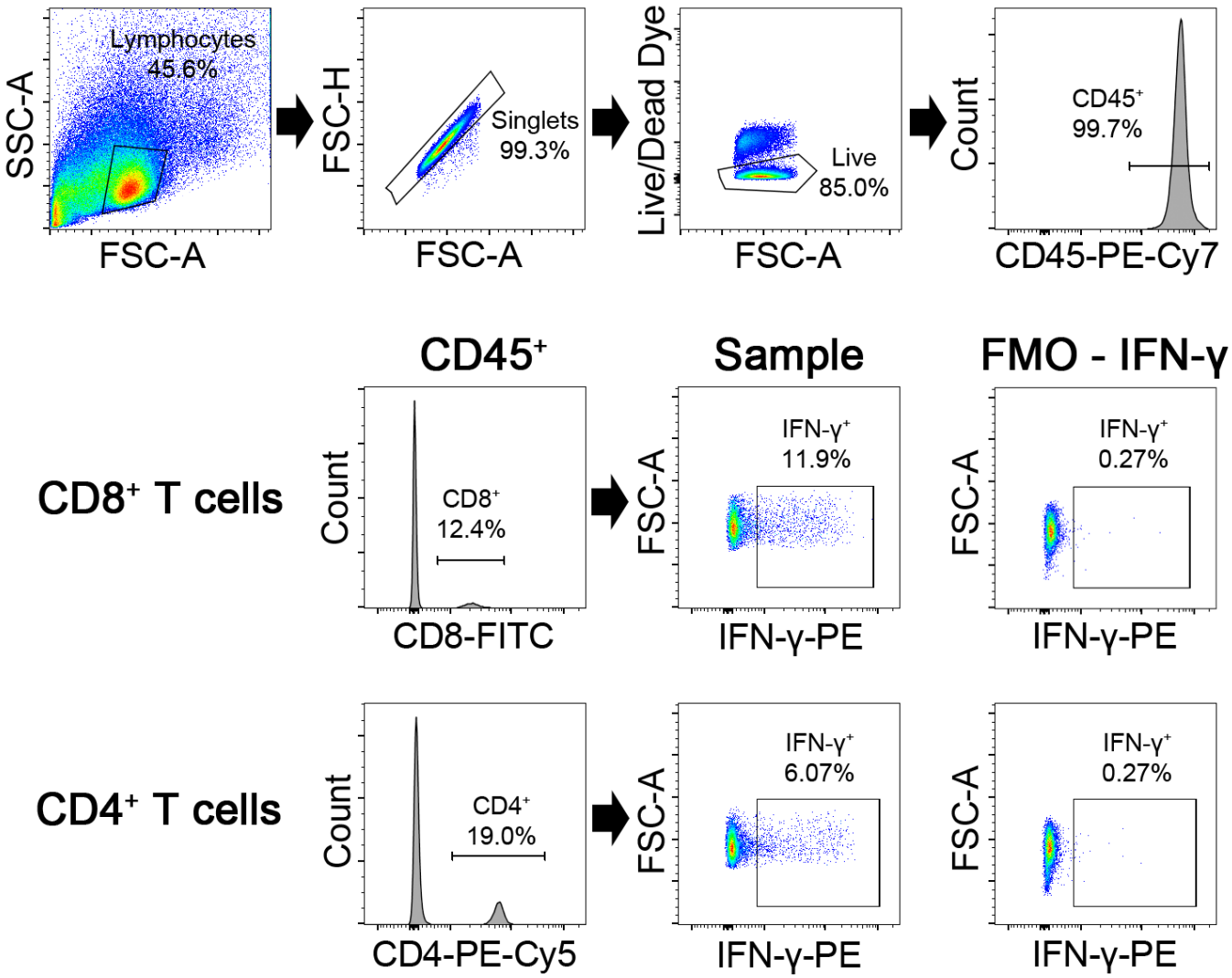

Fig. 6

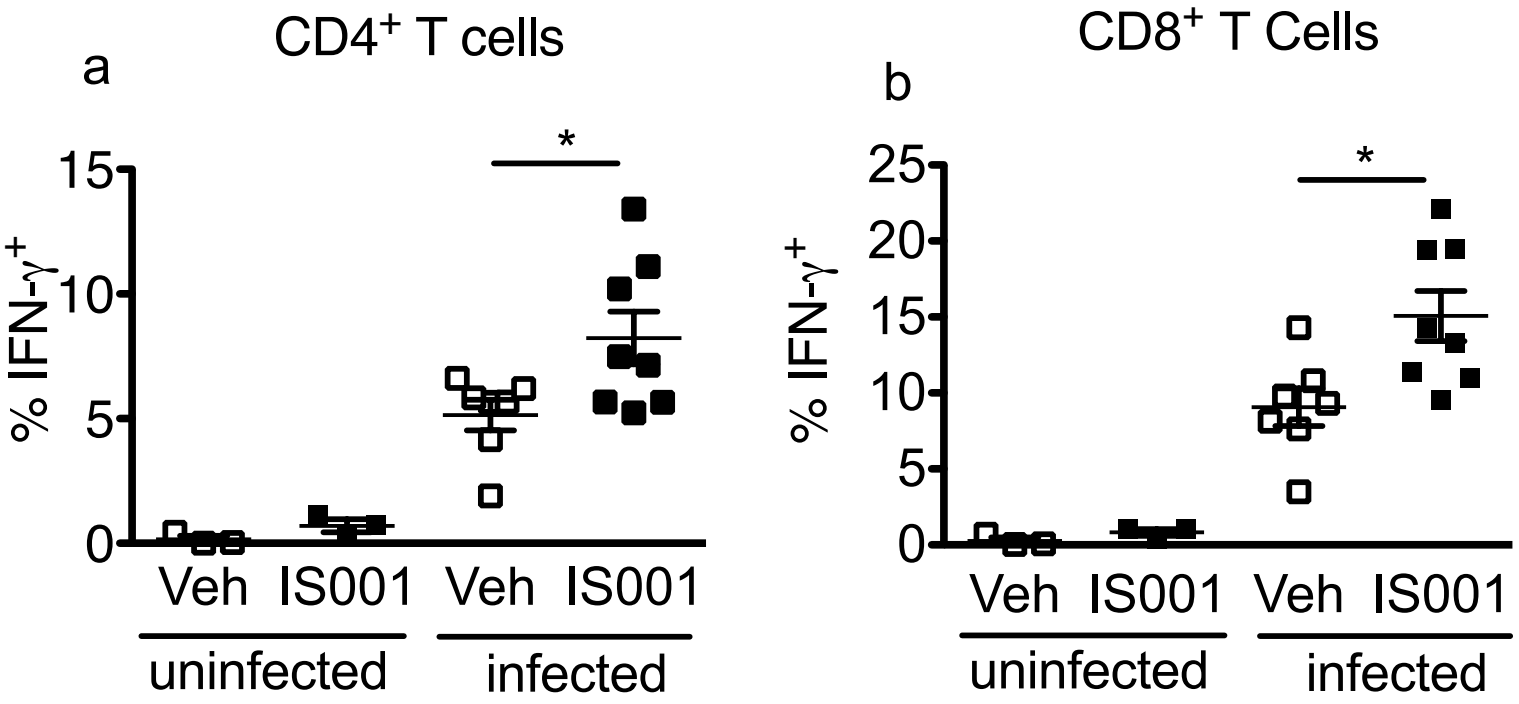

Fig. 7

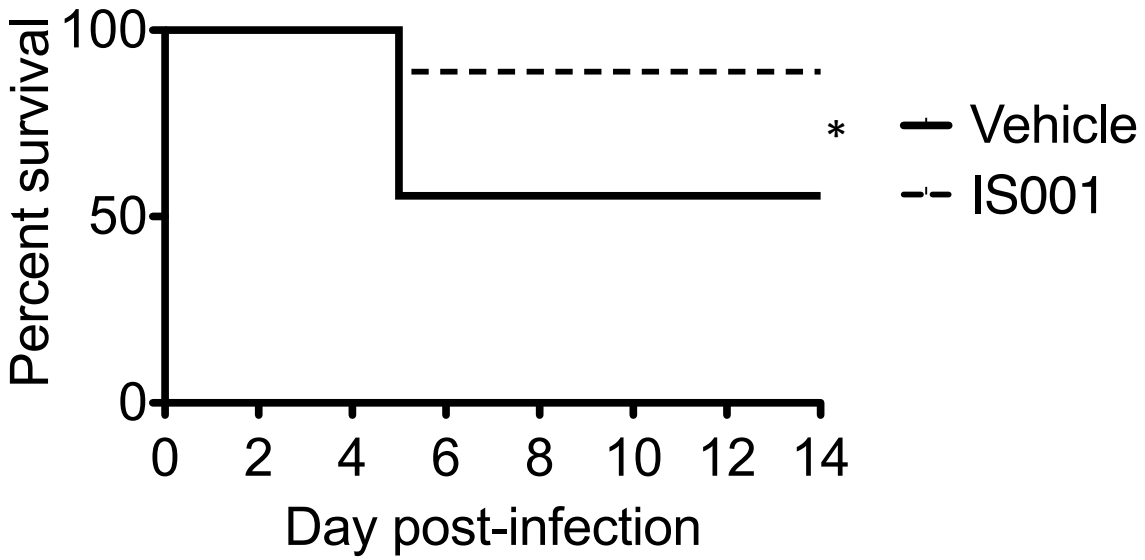

Table 1: Calculation of CFU from a sample taken from L. monocytogenes culture (OD600=0.84)

Dilution	Colony Count	CFU/ml in diluted sample	CFU/ml in undiluted sample
10^-4	TMTC		
10^-5	660		
10^-6	70	700	7 x 10^8
10^-7	9		
10^-8	none detected		
10^-9	none detected		
CFU/ml			**7 x 10^8**

Table 2: Staining panel for detection of IFN-γ in NK and NKT cells

Stain	Purpose
Viability Dye eFluor 506, CD8-FITC, TCRβ-PE-Cy7, NKp46-APC-eFluor780, PBS57/mCD1d tetramer-APC, IFNγ-PE	Core staining panel to identify live IFN-γ producing cells in the NK and NKT cell gates.
FITC-labeled beads or CD4-FITC-labeled splenocytes	Single positive control for compensation
PE-Cy7-labeled beads or PE-Cy7-CD4-labeled splenocytes	Single positive control for compensation
APC-eFluor780-labeled beads or APC-eFluor780-CD4-labeled splenocytes	Single positive control for compensation
APC-labeled beads or APC-CD4-labeled splenocytes	Single positive control for compensation
Viability Dye-stained splenocytes (unstained splenocytes mixed 1:1 with heat-killed splenocytes)	Control for compensation
Unstained cells or unlabeled beads	Control for compensation
Viability Dye eFluor 506, CD8-FITC, TCRβ-PE-Cy7, NKp46-APC-eFluor780, PBS57/mCD1d tetramer-APC	FMO control #1: Used to set gate for IFNγ$^+$ cells.

Table 3: Staining panel for detection of IFN-γ by $CD4^+$ and $CD8^+$ T cells

Stain	Purpose
Viability Dye eFluor 506, CD45-PE-Cy7, CD8-FITC, CD4-PE-Cy5, IFNγ-PE	Core staining panel to identify live IFN-γ producing cells in the CD4 and CD8 cell gates.
FITC-labeled beads or FITC-CD4-labled splenocytes	Single positive control for compensation
PE-Cy7-labeled beads or PE-Cy7-CD4-labeled splenocytes	Single positive control for compensation
PE-Cy5-labeled beads or PE-Cy5-CD4-labeled splenocytes	Single positive control for compensation
Viability Dye-labeled splenocytes (unstained splenocytes mixed 1:1 with heat-killed splenocytes)	Single positive control for compensation
Unstained splenocytes or beads	Control for compensation
Viability Dye eFluor 506, CD45-PE-Cy7, CD8-FITC, CD4-PE-Cy5	FMO control #1: Used to set gate for $IFN\gamma^+$ cells.

Name of Material/ Equipment	Company	Catalog Number
Brain Heart Infusion Broth, Modified	BD	299070
Agar	BD	214010
Triton X-100	Sigma-Aldrich	X100
1xPBS	Sigma	D8537
TissueLyser II	Qiagen	85300
Ammonium Chloride (NH3Cl)		
KHCO3		
Na2EDTA		
RPMI 1640	Gibco	22400089
Fetal Bovine Serum	Gibco	12483
L-glutamine	Gibco	25030
Non-essential amino acids	Gibco	11140
Penicillin/Streptomycin	Gibco	15140
GolgiStop Protein Transport Inhibitor (containing Monensin)	BD	554724
16% Paraformaledehye	Electron Microscopy Sciences	15710
10 x Perm/Wash buffer	BD	554723
Fc block, Anti-Mouse CD16/CD32 Purified	eBioscience	14-0161
Fixable Viability Dye eFluor 506	eBioscience	65-0866
anti-Mouse CD4-PE-Cy5 (GK1.5)	eBioscience	15-0041
anti-Mouse CD8-FITC (53-6.7)	eBioscience	11-0081
PBS57/mCD1d tetramer-APC	NIH Tetramer Core Facility	N/A
anti-Mouse TCRβ-PE-Cy7 (H56-597)	eBioscience	25-5961
anti-Mouse NKp46-APC-eFluor780 (29A1.4)	eBioscience	47-3351
anti-Mouse CD45 PE-Cyanine7 (30-F11)	eBioscience	25-0451
anti-Mouse IFN gamma-PE (XMG1.2)	eBioscience	12-7311
OneComp eBeads	eBioscience	01-1111
Mouse IFN gamma ELISA kit	eBioscience	88-7314
50 mL vented tubes for culture		

1.5 ml microcentrifuge tubes		
bacterial petri dishes		
2 ml cyrovials		
UV spectrometer		
safety engineered needles		
C57BL6/J	Jackson laboratories	Stock#000664
Bleach		
70% Ethanol		
Glass beads		
Centrifuge		
Microcentrifuge		
Sterile Glycerol		
Pipette Tips		
Pipette		
Surgical instruments		
70 micron strainers		
3 ml syringe		
Pipette gun		
Filtration Units		
Trypan Blue		
Hemocytometer		
Round bottomed plates		
FACs tubes	BD	
BD LSR II	BD	
Flowjo software	Treestar	
Multichannel pipettor (0-300 µl)	Eppendorf	
Acetic Acid		
Microbank Bacterial Preservation System	Pro-lab Diagnositics	

Comments/Description

any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
Before use, heat-inactivate at 56 °C for 30 min
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate

Use 4 µl in 6 ml cell culture

Dilute to 4% PFA in ddH_20 or 1xPBS

Dilute 10x in ddH_20

Dilute 1:50
Dilute 1:1000 (we have also used viability dyes from Molecular Probes)
Manufacturer recommends a certain test size; however this should be titrated before use.
Manufacturer recommends a certain test size; however this should be titrated before use.
Obtained as a gift from the facility
Manufacturer recommends a certain test size; however this should be titrated before use.
Manufacturer recommends a certain test size; however this should be titrated before use.
Manufacturer recommends a certain test size; however this should be titrated before use.
Manufacturer recommends a certain test size; however this should be titrated before use.
Manufacturer recommends a certain test size; however this should be titrated before use.
Used for measuring the interferon gamma in the culture supernatant
Used for culturing the bacteria, any brand should be appropriate

any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
Order for arrival at 7 wks
For decontamination
For decontamination
any brand should be appropriate
rotor, buckets, bucket covers.
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
any brand should be appropriate
Dilute 1 to 9 in ddH20, any brand should be appropriate
any brand should be appropriate
any brand should be appropriate

Any flow cytometer could be used for acquisition that has an appropriate laser configuration and filter set to discriminate the fluorochor
Used for data analysis. Other types of data analysis software will also be appropriate
Used for washing cells and adding antibodies during flow cytometry staining
Used for washing glass beads, any brand should be appropriate
Used as an alternative to glycerol stocks for long-term storage of bacteria

mes

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CORRESPONDING AUTHOR:

Name: Shannon Dunn

Department: Toronto General Research Institute

Institution: University Health Network

Article Title: Experimental infection with Listeria monocytogenes (L. monocytogenes) as a model for studying host TRNS response

Signature: Shannon Dunn Date: 1/31/16

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Dear Jaydev,

Thanks for conducting a thorough review of our protocol. The reviewers raised some great points, which we addressed both in this letter and in the manuscript text. Though Reviewer#1 was negative, he/she still had some very good comments and suggestions. The other three reviewers were more supportive and also provided helpful comments. We feel that the protocol has improved significantly from this thorough review. Part of these recommendations was to discuss in greater detail how we used a modified LD50 dose as this is a refinement in terms of animal welfare. We have therefore discussed this point in greater detail. In addition, in response to the comments of Reviewer 3, we have used alternative data for the listeria growth curve in Table 1.

Sincerely,
Shannon Dunn

The following is a point-by-point response to both the Editorial comments and the Reviewer comments:

Response to Editor:
1. Please remove abbreviations from title.
This has been done.

2. Please define all abbreviations at first occurrence.
We re-read the paper and have done this.

3. PFA is toxic and requires a caution statement.
We have added a caution statement at step 6.17 where we use this reagent.

4. Please remove underlining from the protocol (i.e., step 4.6).
This has been done.

5. Please use “hr” as the abbreviation for hour(s).
This has been changed throughout.

6. Please use “refrigerator” rather than the informal “fridge”.
This has been changed throughout.

7. Please delete the note under step 8.10 as this material appears in 8.6.
This has been done.

8. Suggestions of where to place the figures should not appear between the figure title and the legend.
We removed this.

Additional Detail Required:
9. 4.4 How many bacteria are injected per mouse?

Reply: We inserted the following note after step 3.1.

The CFU of bacteria injected will be dependent on the type of experiment performed. Refer to procedures below for specific recommendations for each experiment.

10. 6.2. Please include a citation or describe using text. The actions are difficult to see in the figure.

Reply. The actions are outlined in detail in the figure legend. I have added a statement to refer the reader to the legend. We also to film a spleen removal in the video.

11. 6.24. Please provide a citation.
Reply: To cite a flow cytometer manual is not appropriate as we are not allowed to specify what machine to use and many would be appropriate for this type of analysis. We therefore inserted the following to this statement, "Collect at least 200,000 events per sample and 10,000 events for compensation controls **using operating procedures that are appropriate for the instrument used**".

12. How are the piece of liver and spleen removed? What cuts are made, is the animal disinfected first?. Stepwise detail is required to film removal.

Reply: This dissection was to be included as part of the filming (this step was highlighted in yellow). Nonetheless, we have expanded this section to describe the dissection procedures (steps 7.4-7.8).

13. Branding should be removed. Jackson Laboratory, stock number should appear in the materials table rather than in the text protocol.
This has been done.

14. Remove Golgistop from legend.
This has been done.

15. Results:
Please describe what the data in each figure mean in the results section. For instance, what did the data in Figure 4 show? We have added a line after Figure 4, 6, and 7 to explain what the figures show.

This has been done.

16. Please define the error bars in Figure 4 and 6.
This has been done.

Reviewer 1
Reviewer 1 was generally negative and found that the usefulness and impact of the manuscript are quite low, given that we are presenting standard protocols that have been used for decades. The reviewer also said that none of the techniques are complicated enough to require a visual aid to perform correctly.

Nonetheless, reviewer 1 provided valuable comments that have improved the protocol.

Reply: We agree with the reviewer the protocols are standard and not necessarily novel. I did originally raise this issue with the editor who solicited the manuscript (Teena Mehta) and she insisted that she thought that it would be a useful protocol for the readership given that similar protocols were highly accessed. Our group feels that we certainly would have benefited from such a protocol if it had been available at JOVE when we established these procedures in our laboratory. Regarding the comment that none of the techniques are complicated enough to require a visual aid. I disagree with this statement. There are many who may be unfamiliar with the method or do not have the appropriate expertise and therefore would benefit from illustrated techniques.

Specific comments:
1. Lines 65-67 and lines 117-119.

The reviewer flagged the following statement in the Long abstract and the introduction "Early production of IFN-γ by NK cells and NKT cells has been shown to be crucial for macrophage activation and the early control of pathogen". He/she said that this statement oversimplifies current paradigms in the published literature and is somewhat misleading. This reviewer also mentioned that the role of NK cells during Listeria infection is controversial with some studies suggesting that a lack of NK cells actually promotes clearance.

Reply: We thank the reviewer for raising this point. The reviewer is correct that during lethal infection, an over production of IFNgamma and other cytokines can be detrimental to pathogen clearance and survival as it may block migration of granulocytes in the spleen. We have now added this information and an appropriate citation (Viegas et al.) and have re-worded the following passages to be more accurate.

Long abstract:
Changed to "Production of IFN-γ by innate cells during sublethal infection with *L. monocytogenes* is important for activating macrophages and early control of the pathogen[1-3]."

Introduction:
Changed to "Production of IFN-γ by NK cells is important for macrophage activation and early resistance against the pathogen[3]; however at high infectious doses, production of IFN-γ can also be detrimental to pathogen clearance[23]. NKT cells are also a source of IFN-γ in the spleen and liver during early □□□□□□□□□□[2,24] and this production has been shown to amplify IFN-γ□production by other cell types including NK cells[2].□"

2. Line 170 – Why are you recommending that the BHI agar be used within 2 weeks? It will support Listeria growth after 2 weeks.

Reply: We had been provided this advice by the colleague who helped us set up the model. Since, we have not specifically tested how long the BHI agar plates will support growth of bacteria, we have removed this line.

3. Line 185 – Colonies with a single, uniform morphology should be obtained.
Reply. We have re-worded this sentence as per the reviewer's suggestion in Step 1.5.

4. Line 206—you have instructed to remove 1 ml samples at different time points but started with only 3 ml of culture. Using up the whole sample will cause the culture to have a significantly different degree of aeration in the latter half of the incubation period than during the first half, which could affect the growth rate.

Reply. We thank the reviewer for catching this error. We increased both the volume of the culture (10 mL) and size of the tube (50 ml). This way it will be less likely the sampling will impact the aeration and growth rate of the bacteria. We also inserted the following note after step 1.6. "Regardless of the type of container used, make sure that it is sterile, vented and that the volume of culture does not exceed 20% of the total volume of the container to ensure appropriate aeration of the bacteria. If using a culture tube, keep it tilted at a 45 ° angle during culture."

5. Line 212: You don't state the diluent.
Reply: It is BHI media and we have changed this accordingly.

6. The calculation of CFU shown in Table 1 should explain why the count for the 10^5 plate is not used in determining the average. The only explanation is (up to 300) on line 216 in the text. This is standard microbiology practice; anyone who would need to read this protocol would need a better explanation than that (overcrowding reduces growth; difficult to discern individual colonies, etc.)
Reply: We agree with the reviewer and have added a statement outlining why we don't use plates with greater than 300 or less than 30 colonies. This note has been inserted after step 2.5.
NOTE: Plates with greater than 300 colonies are not used, since overcrowding can hinder bacterial growth and also makes it difficult to discern and enumerate individual colonies. Plates with counts < 30 are also not used because small errors in dilution technique or the presence of contaminants can have a large impact on the precision of measurements at the lower end of the range.

7. Lines 240-241. This is not a good way to prepare the Listeria. Although you are using identical tubes and theoretically inoculating each with the same number of bacteria from the glycerol stock, there are numerous reasons why the growth could be slightly different in each tube (hot spots in the incubator, caps tighter or loser, etc). For this work to inject each mouse with a uniform inoculum, you would need to pool the growth from each tube together first and then wash and prep the pooled cultures. But this causes the investigator to measure the OD from multiple tubes. Why would you not just grow a larger volume say 10-15 ml

in a 125 ml culture flask and prepare the growth curve and the cultures for injecting that way every time?

Reply: I totally agree. The procedure that my student was using is not optimal. We appreciate the suggestion and have changed the protocol to grow a larger volume of culture (10 ml) in a larger tube or flask.

8. Line 251—Wash pellet twice with sterile 1 x PBS.
Reply: The typo has been changed (was changed to wash).

9. The name of the strain and origin of the Listeria monocytogenes strain used to generate the representative data should be given.
Reply: The strain is EGD was obtained from Pascale Cossart and we have provided an appropriate reference for the strain (Becavin et al., 2014).

10. Line 342-I don't think that "finker flicking" is clear.
Reply: Although finger flicking is a term often used to describe this technique, we have re-worded this sentence for clarity.
"Loosen the cell pellet by flicking the tube with a finger or by dragging the bottom tube back and forth along a corrugated surface (e.g. air flow vent in the BSC)."

11. Line 360-2-3 x 106 cells is a lot to use per sample for flow analysis-far more than is needed. You should indicate the range of cells that can be used if the investigator would prefer to be frugal using up their precious antibodies.

Reply: I agree that this number of cells is excessive for the amount of cells that need to be collected (200000). 1 million should be sufficient and we have changed this accordingly.

12. Line 406, and throughout. "Aspirate" would be a better verb to use than "vaccum"

Reply: We agree and have changed this throughout.

13. Line 466. The term "recall" here is misleading—it sounds like you are measuring a secondar/recall memory response, when in fact you are harvesting effector T cells at the peak of the primary response and exposing them to infected cells, so they are displaying their effector functions.

Reply: We agree that the terminology is misleading and have changed this to the following "This procedure describes how to measure IFN-γ production by $CD4^+$ and $CD8^+$ **T effector cells** at the time of the peak of the adaptive immune response (~ 7 d post-infection) using two methods:…"

14. Line 469- by using heat-killed Listeria as the in vitro stimulus, you are measuring IFNg produced by CD4, NK, and NKT cells, and innate (cytokine-induced) production by CD8 T cells, but you are probably NOT measuring antigen-specific IFNg production from CD8 T cells because presentation of CD8

antigens on MHC Class I requires infection with live replicating Listeria that can access the host cell cytosol.

Reply: We thank the reviewer for raising this point. We have now modified the discussion accordingly to raise this limitation. "One limitation is that heat-killed bacteria does not efficiently prime $CD8^+$ T cell responses either *in vitro*[39] or *in vivo*[40,41] infection. Thus, the $CD8^+$ T cell IFN-γ production that we observed by splenocytes harvested at the peak of infection (i.e., **Figure 6**) is likely in response to the residual live bacteria present in the splenocyte cultures or was elicited as a result of cytokine-induced cytokine release[39]. As an alternative to heat-killed listeria, one could also elicit IFN-γ responses *ex vivo* by exposing T cells to peptides encoding epitopes on listerial proteins. Indeed, immunodominant MHC Class II-restricted epitopes for listeriolysin O and the p60 hydrolase and have been described for C57BL6 and BALB/C mice[42] and immunodominant MHC Class I epitopes have been described for BALB/c[42]. Yet another approach is to infect mice with strains of *L. monocytogenes* that have been engineered to express model antigens such as ovalbumin or viral antigens in order to take advantage of existing MHC Class I- and MHC Class II-tetramer reagents to enumerate antigen-specific T cells in infected mice[43,44]."

15. Figure 7 – please add more tick marks to the X-axis so it is clear to determine which day post-infection the mice die/were euthanized.

Reply: We have fixed this figure and added more tick marks to the x-axis.

16. Line 719 – avoid the slang term "Black 6" mice –say C57BL6/J.

Reply: We have now changed the model name at Taconic (Black 6) to the appropriate strain nomenclature (C57BL6/NTac).

17. Line 751 – the technique described here is a basic method – it is not a novel technique, so there is nothing to compare to "existing methods".

Reply: We agree with the reviewer and only added this section at the request of the editor. We will remove it as we agree that this section is not required as this technique is a basic method.

Reviewer # 2.
Manuscript summary.

Overall, this manuscript is a good review of the topic and very applicable to many different types of related research.

Major concerns: N/A

Minor concerns:
1. Section 1.4.1. – 90 °C should be 90°C.

The JOVE journal requires that a space be inserted between the degree symbol and the C.

2. Section 3 – It may be helpful to mention that when using PBS you should ensure a lack of contaminants (e.g. use of commercially bought PBS). Using lab glassware to make PBS can introduce immunogenic contaminants (i.e., LPS) regardless of autoclaving.

Reply: We have added the following note after step 3.5. "Note: We used a commercial source of sterile 1 x PBS to ensure a lack of contaminants, since lab glassware can introduce immunological contaminants such as lipopolysaccharide."

3. Section 4.1/Discussion – You give specific numbers people should use for mice given a specific study (e.g. at least 5 mice per arm for immune studies) in section 4.1 and give further recommendations in the Discussion section. You should have some references for these recommendations, provide more discussion on this topic, or not use such prescriptive language. I am not aware of a specific group size for survival studies either. It seems that for all studies the smallest number of animals possible should be used in order to gain the needed information, unless there is a requirement otherwise set-forth by the funding agency, etc.

Reply: We removed many of the specific recommendations for animal numbers from the body of the protocol. We still made some recommendation in our discussion, based on the variability that we observed in our own data.

" Due to biological variation in the immune response to infection (see **Figures 4 and 6**), it is recommended that N=4-5 mice per group be used for the initial immune studies. If after these studies there is a trend in the data, but no significant difference apparent between groups, a power calculation could be done to determine the least number of animals required in subsequent studies to achieve statistical significance."

4. Section 4.2 – Acclimatization periods may differ between institutions. 2 wks is a long period.

Reply: We thank the reviewer for this comment and we have revisited the literature. It is known that animal transport causes transient increases in stress hormone levels, lymphopenia, and decreases in NK cell activity, but that these parameters return to baseline within 1-7 days of acclimatization (reviewed in Conour et al. ILAR J (2006) 47 (4): 283-293; Obernier and Baldwin, ILAR 2006; 47 (4): 364-369). Based on these data, we changed the recommendation from 1-2 weeks to 1 week.

5. Section 4.4 – Add more details on mouse injections. What anatomical landmarks do you look for when doing IP injections? Are you pulling back on the syringe to ensure that you have a vacuum? Are you restraining the mouse with your less-dominant hand? Are you agitating the inoculum in the syringe prior to injection.

Reply: Since this step was going to be filmed in the video, we had not included details; however these have now been added some details of the injection in Procedure 4.

6. Section 4.5. Add ")" at the end of sentence.

Reply: We have changed this.

7. Section 9/Discussion section – These sections should be expanded to include the use of modified LD50 studies. In many cases, humane endpoints (in which the animal is likely to die) can replace actual death of the animal. These studies are a major refinement in animal welfare and need to be discussed in this section. You have certain endpoints discussed in section 4 that work nicely if used in a modified LD50 study.

Reply: We had used a modified LD50 dose for our studies and now clarify this in the ethics statement. We agree that this modified LD50 is a refinement in animal welfare and have expanded upon this in the discussion. We also referred to instructions on how to calculate the LD50 dose.

"The classical LD_{50} is the dose of pathogen that results in 50% lethality of mice. Since death was not an acceptable endpoint at our institution and since many symptoms of listeriosis can predict whether an animal is likely to succumb to an infection, we used a defined list of clinical signs instead of death as an endpoint in our studies. Using this method, it was determined that the modified LD_{50} was 10^5 CFU for 8 week-old male and 1.5 x 10^5 CFU for 8 week-old female C57BL6/J mice[28]. These LD_{50} doses were determined by measuring the percent survival of mice to endpoints in six step-wise dose-escalation studies that each contained N=8 mice per group (mice infected with 10000, 20000, 50000, 100000, 150000, or 200000 CFU). The LD_{50} calculation was determined from a regression plot of the log (CFU) (x-axis) versus the probit of the percent survival values (y-axis) (website: userwww.sfsu.edu/efc/classes/biol710/probit/ProbitAnalysis.pdf)."

8. Discussion – Line 688 –Remove or rephrase the comment "as opposed to going systemic." You seem to be referring to sepsis. An IP injection will be systemic.

Reply: We agree and have removed this line.

9. Discussion – Paragraph starting at line 712 – Should expand the discussion to include genetic differences between vendors (i.e., creation of sub-strains), not just microbiota.

Reply: We have added this and have referenced a paper that explored the genetic differences between different C57BL6 vendors by SNP analysis.

10. Table 1 – Expand description of this table and better explain how you found your 1 x 10^9 CFU/ml. You talk about it in section 2, but it would be beneficial in the table description as well.

Reply: At the request of reviewer # 3, we have provided alternative data for Table 1. We now provide a better explanation for how we calculated the CFU/ml both in the protocol text and in the new legend of this table.

Reviewer # 3.

Manuscript summary: The manuscript describes intraperitoneal injection of C57BL6/J mice with the EGD strain of Listeria monocytogenes to measure IFN-gamma production in splenic cells.

Major concerns:
1) 4.1 Groups of mice have to be males or females since host-response to *L. Monocytogene*s is sex-dependent.

Reply, we have changed the text to state, "order male **or** female mice".
We also more clearly noted the differences in modified LD50 of the pathogen for male and female mice at various places throughout the text.

2) 7.9. As far as I understand the experimental setting, the calculation is wrong. It is actually CFU/mg-CFU/ml x ml homogenate/mg weight of tissue.

We thank the reviewer for catching this error and have changed the text accordingly.

3) 9.1. This is not clear if you kill the mice when they meet humane endpoints, which is accurate for a Listeria infection, or if you let them die when you calculate the LD50 (this being ethically questionable for the well-studied Listeria infection).

Reply: We apologize if this wasn't clear and always euthanize mice at humane endpoints. Our LD50 dose is a modified LD50 dose; the median dose at which half of the mice succumb to the endpoints (not death). We did not use death as an endpoint due to ethical guidelines at our institution. We have made this more clear in the text (starting in the Ethics Statement where we describe these endpoints in detail). At the request of reviewer 2, we have also expanded on this point in the discussion.

4) In the discussion, you can cite the work of Becavin (MBio 2014) for the Listeria reference strain to be used. Indeed, EGD is a PrfAstair strain which differs from EGD-e. Both strains are sometimes mixed and it is of importance to know which strain is actually used to compare experimental studies.

Reply: We agree and now cite the Becavin et al paper as a source of the strain that we are working with.

5) 3.3. Listeria can be recovered after a 6000 x g centrifugation in a 1.5 ml or 15 ml tube (not all centrifuges can reach 17949 g).

Reply. We thank the reviewer for making this point as it will certainly streamline this step. We have changed the protocol accordingly.

6) 6.17. Cells can be stained on ice using an aluminum sheet.

Reply. That also makes sense, however the lower temperature may slightly modify antibody binding. All of our stains have been optimized for 4 degrees celsius incubations so we do not wish to change this.

7) 100 ul on a ¼ plate is a lot. I prefer spreading 50 µl on a ¼ plate or 100 ul on a ½ plate.

Reply: We agree and changed this.

8) Table 1: when we use EGD, we have frequently 8 x 10^8 CFU/ml for a OD of 0.8. In the Table 1, the quantification at 10^6 seems high compared to 10^5 and 10^7. Can you give another example where your dilutions are more close to what you expect (10 fold between dilutions)?

Reply: We thank the reviewer for pointing this out. After finding the source of the original data, we found it to have been from a growth curve for another strain of EGD. We now present alterative data in Table 1 that is from a growth curve for EGD.

9) Line 732: the reference should be Lecuit et al. J. Embo 1999.

Reply: This has been changed.

Reviewer # 4.

Manuscript Summary.

The manuscript is well compiled and the revisions that were provided on line have corrected many issues. It is a well thought out and thorough description of the procedures.

No major concerns.

Minor concerns.

1. Some typos: p5. Line 180 C after degree symbol for rotation.

Reply: This has been changed.

2. For cell counting. Usually count the average number of cells in both centre grids of a hemacytometer and then multiply it by 10^4 and then times the dilution factor. In the example in figure 2, cell number of 43 would be 430,000 x dilution factor of 2= 860,000 or 8.6 x 10^5.

Reply: Yes, the reviewer is correct about this calculation is a common one used if the cells are dilute. However, we typically use more concentrated cells. Also the number 43 refers to the count obtained in one of the 25 squares that sit in the central grid, not the center grid itself. If we were to use the reviewer's calculations with our example, we would come up with the same answer as our calculation.

Reviewer's count: 43 cell count (one square in central grid) x 25 squares=1075 cells in central grid. 1075 x 10^4 x 2 (dilution factor)=21.5 x 10^6/ml.

The way we calculate it is that we count 5 squares within the large central grid (each containing 16 small squares) within the central grid. According to our example, this count would be ~ 215 (43 x 5). We would then divide this by 10 to get the answer in x 10^6/ml=21.5 x 10^6/ml.

Nonetheless, after re-examining Figure 2 and re-reading the legend for this figure, we agree that it could be made more clear and we have altered the figure and legend accordingly.

3. Consider mentioning bead stock methods, such as Microbank which can be very convenient to use especially if shipping strains at room temperature.

Reply: We thank the reviewer for mentioning these methods. The Microbank method does appear to be an improvement over our glycerol stock method, particularly because it avoids repeated subculture. Since we do not have experience with these methods, we will not describe them; however, we have incorporated the following note at the end of Procedure 1. Since we are not permitted to use any branding, we have also listed Microbank as a potential source of these beads in the materials list.

"NOTE: Bead stock methods can also be used in place of glycerol stocks to store bacteria. By this method, porous microbeads are inoculated with a pure culture of *L. monocytogenes* and are stored at -80 °C. Each bead can be used to inoculate a fresh culture as needed. See materials list for further information."

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Date: March 4, 2016

Jaydev Upponi, Ph.D.
Science Editor
JoVE
1 Alewife Center, Suite 200, Cambridge, MA 02140

Subject: Revision of manuscript

Dear Dr. Upponi:

We have revised our manuscript according you your suggestions. We have also edited it to increase clarity and have removed Figure 1 since our clinical veterinarians thought it was fine for us to video a healthy mouse being injected. We have also received the necessary permissions from the *Journal of Immunology* to re-use 3 of the figures and have altered the figure legends accordingly to satisfy your guidelines and the journal guidelines. I have also included another author (Dr. Thierry Mallevaey) who helped to critically read the manuscript.

Here is a point-by-point response to the editorial comments:

1. 1. Please adjust the numbering of your protocol section to follow JoVE instructions for authors, 1. should be followed by 1.1) and then 1.1.1) if necessary and all steps should be lined up at the left margin with no indentations.

The numbering has been adjusted.

2. The Safety statement of the protocol section is too long. Please break the statements down intro 2-3 sentences and place them as NOTES where applicable in the protocol.

I reduced the length of the safety statement, but felt it was important to stress that immunocompromised individuals should not work with the pathogen and to define whom these people are. If an immunocompromised person does become infected with the pathogen, they have a high chance of dying. This is a pretty serious issue; hence I felt that it was important to stress this point at the outset. I also included brief statements at the beginning of each procedure about BSC use. The biosafety people at our institution thought that it was important to stress this upfront rather than having it buried in the notes.

3. Due to the nature of being a video-based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in your protocol, please only add the following information to your text where applicable:

a) Please include an ethics statement before your numbered protocol steps indicating your protocol follows the guidelines of your institutions animal research ethics committee.

This was already done and has not been changed.

b) Please specify the euthanasia method

We had specified the euthanasia method in the protocol itself, but have now included this information in the ethics statement before the start of the protocol.

c) Please mention how animals are anesthetized and how proper anesthetization is confirmed.

This protocol does not involve animal anesthesia.

d) Use of vet ointment on eyes to prevent dryness while under anesthesia.

This protocol does not involve animal anesthesia.

e) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

This protocol does not involve animal surgery.

f) Do not leave an animals unattended until it has regained sufficient consciousness to maintain sternal recumbency.

This protocol does not involve animal anesthesia or surgery.

g) Do not return an animal that has undergone surgery to the company of other animals until fully recovered.

This protocol does not involve animal anesthesia or surgery.

h) Discuss maintenance of sterile conditions during survival surgery.

This protocol does not involve animal anesthesia.

4. Please add a one line space between each step and sub-steps of your protocol section.

This has been done.

5. The (mu) of the micro liter should be greek “mu” symbol. Please change this throughout the manuscript.

This has been done. We also changed the gamma symbol to the greek gamma symbol.

6. Please provide the dilutions for the antibody used throughout the protocol, for example, see section 6

It is not appropriate to specify the dilutions for the antibodies used. This is because there is a lot to lot variation for antibodies. Every time, we buy a new lot of antibody (same catalog number), we have to re-titrate the antibody. Thus it is not appropriate to list antibody dilutions as these will fluctuate over time with different lots. This is a point that we stressed in the notes sections of Procedures 6 and 8.

7. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript and replace it with a more generic term as much as possible throughout the entire manuscript. All commercial products should be sufficiently referenced in the table of materials/reagents. Examples of commercial sounding language in your manuscript are TissueLyser II, falcon, etc.

We have removed any commercial sounding language from the manuscript.

8. Please un-highlight the written paragraphs under the sub-headings as this may constitute to the overall length.

This has been done.

9. Please present Recipes as supplemental file or as tables.

The recipes are now presented in Supplemental File 1.

10. After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10 page limit for the protocol text, but there is a 3 pages limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages (or less) of text to identify which portions of the protocol are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Our protocol is still within the 10 page limit and we have highlighted the 3 pages of text that we wish to have videod.

11. Please revise the manuscript text to avoid the use of any pronouns (i.e. "we", "you", "your", “our” etc.). If you feel it is very important to give a personal example, you may use the royal "we" sparingly and only as a "NOTE:" after the relevant protocol step. Please use the Ctrl+F function to find and replace the pronouns.
We have removed the majority of we’s, you’s, and ours’ as suggested. A few ‘we’s” remain in the

discussion, but we felt that it was best to keep them as we were discussing how we trouble shooted something.

12. “Representative results section” and “Figure legends” are different sections.
We had carefully followed the format of the provided example. Since this was not clear, we have not included a separate heading for the Figure legends. We have also re-written the representative results section.

13. Please add at least one paragraph of results text that explains your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.

We had followed the provided template for this. Since this was not clear, we have re-written this paragraph to discuss only the representative results and not the figures that referred to experimental steps.

14. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.
We have received re-print permission from Journal of Immunology and have included these comments in the figure legends. We will also upload a copy of the re-print permission.

15. Please make sure that the “Discussion” is written under the following sections.
a) Critical steps within the protocol.
b) Modifications and troubleshooting.
c) Limitations of the technique.
d) Significance of the technique with respect to existing/alternative methods.
e) Future applications or directions after mastering this technique.
We have re-organized our discussion using slightly modified subheadings. We do agree that this improves the discussion organization. I did not discuss about troubleshooting, as there are so many areas in this protocol that should be troubleshooted and the discussion is already very long. I only discussed the critical parameter to be troubleshooted, which is the infectious dose. This was discussed under the critical steps section.

16. NOTE: Please copyedit the entire manuscript for any grammatical errors you may find. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol. Please thoroughly review the language and grammar of your article text prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

I have read through the manuscript and am a competent native English speaker. I have had a colleague

also critique and view the manuscript and because of his intellectual input have included him as a co-author.

Sincerely,

Shannon Dunn, Ph.D.

Supplemental code file (if applicable)