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- 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- by innate cells during sublethal infection with *L. monocytogenes* is important for activating macrophages and early control of the pathogen1-3.”

Introduction:

Changed to “Production of IFN- by NK cells is important for macrophage activation and early resistance against the pathogen3; however at high infectious doses, production of IFN- can also be detrimental to pathogen clearance23. NKT cells are also a source of IFN- in the spleen and liver during early 2,24 and this production has been shown to amplify IFN-production by other cell types including NK cells2.”

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- 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- 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 release39. As an alternative to heat-killed listeria, one could also elicit IFN- 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 mice42 and immunodominant MHC Class I epitopes have been described for BALB/c42. 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 mice43,44.”

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 LD50 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 LD50 was 105 CFU for 8 week-old male and 1.5 x 105 CFU for 8 week-old female C57BL6/J mice28. These LD50 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 LD50 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 104 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 105.

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 104 x 2 (dilution factor)=21.5 x 106/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 106/ml=21.5 x 106/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.”