Rebuttal Letter for Reviewers' comments

**Reviewers' comments:**  
**Reviewer #1:**  
Major Concerns:  
1. Some of the protocols lack detail. More detail should be added to protocol 1 especially, as this is a key first step and a person who is unfamiliar could not complete this protocol by your instructions alone. It would be helpful to give more details on this protocol. For example, what result are you looking for with the gel electrophoresis? In protocol 2, peritoneal macrophage isolation is not a trivial technique, especially for people who have not done it before. I would recommend adding helpful statements such as inserting the needle with the bevel up, to avoid internal organs and tissues in step 2.5.

Reply: Revised. More details were added into section 1 and 2.  
2. The video does not replicate the steps exactly. For example, 5:00 of the video, protocol 2, the video did not show trimming the fur to expose the abdominal cavity or massaging the abdomen when isolating macrophages. This is not a preferred method for isolating peritoneal macrophages, especially for people who are just learning. It is very difficult to avoid puncturing organs, when you cannot see them. Massaging the cavity is an important step that can help increase cell yield.

Reply: Revised. The clip of massage the mouse cavity had been added in the video.  
3. Using thioglycolate to elicit macrophages may complicate phagocytosis experiments, as macrophages can phagocytose the agar. Also, there is concern that LPS may be a contaminant in thioglycolate. See the methods article "Activation of murine macrophages" by Zhang and Mosser 2008. This could have implications on the results of these experiments. This is worth mentioning in the text, as well as describing that resident peritoneal macrophages can be isolated without thioglycolate, with lower macrophage yields.

Reply: Thanks for the advice. We've added some explains in the text.

Studies have shown that Brewer’s thioglycollate recruits numerous macrophages, but does not activate them[1]. On the other hand, Brewer’s thioglycollate elicited macrophages showed an increase in lysosomal enzyme but a decrease in killing ingested microorganisms. However, the phagocytic capacity was not affected when compared with non-elicited macrophages[2].

[1]. Leijh, P. C., van Zwet, T. L., ter Kuile, M. N., van Furth, R. Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. Infect Immun. 46, (2), 448-452 (1984).

[2]. Layoun, A., Samba, M., Santos, M.M. Isolation of Murine Peritoneal Macrophages to Carry Out Gene Expression Analysis Upon Toll-like Receptors Stimulation. J. Vis. Exp. (98), e52749, doi:10.3791/52749 (2015).  
4. The authors did not count macrophage numbers in these methods, only total numbers of peritoneal cells. There is an issue with the comparison of phagocytic ability of peritoneal macrophages from young vs aged mice, when the numbers of macrophages plated have not be equalized between the two groups. Different numbers of macrophages plated in each group could potentially skew the results, as there may be a different ratio of bacteria per macrophage in the groups. This can also affect reproducibility between experiments. Authors should either count and equalize plating of macrophages first (they are larger and have a distinct appearance from other peritoneal cells) or dissociate and count macrophages after adherence of peritoneal cells.

Reply: In fact, we do count the cell numbers at first. Because most of the cells were lymphocytes, it is hard to determine the exact number of macrophages at the beginning. However, we may count the nuclei which stain by DAPI under the fluorescence microscope to normalized the cell density. Besides, the flow cytometry results can also help to verify the results.  
5. I don't recall the authors including statements indicating exactly how many bacteria to add to the macrophages. I think that an exact number should be included, not an approximation, as this is also very important for reproducibility between experiments and for comparison between two experimental mouse groups.

Reply: The number was given in the tables.  
6. Were experiments on young and aged mice done in parallel? I think that this is important and should be specified in the video and manuscript. (especially if approximate numbers of bacteria are added to cells and macrophages were not counted)

Reply: Yes, the young and aged mice were done in parallel.   
7. The number of internalized bacteria per cell would be a helpful assessment of phagocytic ability in addition to the % EGFP F4/80 co-positive cells. If possible, the authors should include quantifications of internalized bacteria per cell from the microscopy data, since they have stated that there are differences in phagocytic ability between aged and young mice.

Reply: That's a good quesiton and it's possible to calculate the number of internalized bacteria per cell. However, the calculation may be complicated. We may get that data from the flow cytometry data. Because the more bacteria a macrophage internalized, the stronger EGFP signal it was. We can divided the EGFP positive cells into two subgroups: strong positive and weak positive. It can be observed on Figure 2, the proportion of strong positive cells in young group was much higher than the aged group. These data may have similar significance to calculation the number of internalized bacteria per cell.

8. Please include the tables in the manuscript, they are missing from submitted files. It is very hard to follow only having Table 2 in the video. Also, table 1 was not in the video or the manuscript.

Reply: Revised.  
9. In the video, it would be helpful to include captions throughout with important details of the step required (especially for protocol 1), similar to 3:30 and 4:31 of protocol 2 in the video.

Reply: Thanks for the advice. Revised.  
  
Minor Concerns:  
Manuscript  
1. In the introduction line 51, reference 2 and 3 indicate that innate immune function is not impaired in the aged. I would specify that you are demonstrating in this paper that there may be decreased phagocytic ability in the aged, otherwise it sounds as like this is said in these papers.

Reply: Thanks for the advice. Revised.  
2. Line 51 again, it would be helpful to mention here, at least briefly, what the other methods are. You have described them in the discussion, but it would be helpful for readers to have a comparison in the introduction when you say that this method is an improvement.

Reply: Revised.  
3. Line 70 of the introduction, the authors say they have highlighted critical steps that the researcher may modify to meet the needs of their experiment. I do not think the authors did this throughout the protocols. Please add statements that indicate steps that can be modified. For example, it may be helpful to indicate specifically in protocol 2 that young vs aged mice or diseased vs healthy mice could be used with this protocol to test different experimental questions. Also, can other types of bacteria be used in this protocol?

Reply: Thanks for the advice. Revised. To the question " can other types of bacteria be used in this protocol?", the answer is yes. Some researchers use Staphylococcus aureus, S. enteritidis or K. pneumoniae according to their purpose. However, the pET-SUMO-EGFP plasmid is more suitable transferred into BL21(DE) than other E. coli strain.  
4. In step 2.1, do current institutional animal ethics allow 1 mL of volume for intraperitoneal injections? Some ethics do not allow this anymore.

Reply: The needle of 1 mL syringe is 25 gauge, and 1 mL is a safe volume for i.p., thus, currently, it is still approved by the Animal Care and Use Committee.   
5. In step 2.1, what is the reasoning behind caging mice individually? Animal ethics tend to discourage this, unless necessary. Please justify in text if this is necessary.

Reply: Revised.  
6. In step 2.1, if thioglycolate is needed for this protocol, please specify in the text what the purpose of thioglycolate is, for someone who is unfamiliar.

Reply: Revised.  
7. In step 2.2, how long does a person need to wait and what level of anesthesia is needed before cervical dislocation can be performed?

Reply: Revised. Sevoflurane take effect in just 30-60 seconds.   
8. In step 2.4, the authors should indicate that if the bowel (or any other organ) is punctured, that the mouse and cells should no longer be used for experiments, as this may activate cells and skew the results.

Reply: Revised.  
9. In step 2.6, please add a statement that indicates cells should be counted. The number of macrophages can also specifically be counted at this step. Counting the cells is a very important step, and there can be huge differences in cell yield between experimenters and between mice.

Reply: Revised.  
10. In step 2.7, be specific that the adherent cells are the macrophages and that they adhere well to tissue culture treated plastic.

Reply: Revised.  
11. In step 3.1, again, cell and bacteria numbers should not be estimated they should be counted and known for each experiment, especially when there are comparisons between different mice.

Reply: Revised.   
12. For protocol 3, step 3.3, should the authors quench florescence with crystal violet for the florescence, as with the flow cytometry protocol, to avoid detecting adherent but not phagocytosed bacteria? If not, please explain in text why it is not required for this protocol.

Reply: For the fluorescence microscopy, there is no need to use crystal violet. Because the use of phalloidin to stain the F-actin, it can be easily distinguish if there were EGFP-expressing E. coli adherent to the cell surface.  
13. In step 3.6, please indicate why F-actin is being stained in the manuscript text.

Reply: By staining the F-actin, we can outline the cytoplasm to distinguish the internalized bacteria.  
14. In step 3.8, please indicate what concentration the DAPI working solution is.

Reply: Revised.  
15. In step 4.1, after the cells are detached macrophages can be counted as they are the adherent cells.

Reply: Yes. But this step may cause some cells dead, thus, a number which include both dead and live cells was not accurate.  
16. For section 4.3, (9:00 of video) crystal violet was used to quench florescence for the 37°C incubated cells, but not the control cells on ice. Shouldn't the fluorescence be quenched as well for this control? Please explain why not. Even if they won't bind to the cells, it is important to treat all controls with the same experimental conditions.

Reply: The group on ice was intend to exclude the non-specific EGFP signal, so it is no need to quench. The F4/80 positive cells in this group (ice) will be EGFP negative, thus, exclude the non-specific EGFP signal.  
17. For step 4.7, typically more than one marker should be used to identify macrophages for flow cytometry, as well as a live/dead stain (example, detect % live F4/80/CD11b/EGFP positive, CD11c negative cells). Were Fcγ receptors blocked (for example with 2.4G2 antibody) prior to staining for flow cytometry, to prevent non-specific binding?

Reply: Yes. Usually for blood cells, typically using two or more markers to identify macrophages. As for the in *vitro* primary cells, especially in this scenario, F4/80 is sufficient to gate the macrophage. Also, FC blocks were not essential in this case, because only one antibody used. In addition, we examined the proportions of EGFP+ F4/80+ cells with or without FC blocks, the results were the same.   
18. The microscopy images in Figure 1 are of poor quality. They are not clear enough to see the punctate internalized E. coli. The pictures in the video at 7:35 and 10:18 should be used instead, if possible, as they provide a much clearer image.

Reply: Revised.  
19. In line 205 and 209, it should be called a scale bar, rather than white bar.

Reply: Revised.  
20. The results should not be explained in the figure legends, this should be in the representative results. The figure legends should include a title that indicates the main result and a short description of the methods of the experiment. The authors should also include the number of samples and number of experiments preformed, whether they were independently performed, and the statistical test used.

Reply: Revised.  
21. In figure legend 2, line 218-219, you do not need to indicate that it isn't shown in the histogram if you show the flow plots.

Reply: Revised.  
22. In line 235 of the discussion, the phrase "seemed not to work well" isn't formal enough of a phrase for a scientific paper. Instead, you can say "was not effective". Also please reference the literature that you are referring to.

Reply: Revised.  
23. In line 238, you have said cell density, but you are referring to cell numbers from mice. Also, it might be helpful to indicate within protocol 2 the typical yield of macrophages per mouse and that macrophages from multiple mice can be pooled for experiments.

Reply: Revised.  
24. In line 250, the examples you gave are used for phagocytosis, but are not markers of phagocytosis.

Reply: Revised.  
25. In line 265, the authors should say instead "the kit may be cost prohibitive" instead of "the cost of this assay kit was not friendly enough", as that judgement may depend on the laboratory.

Reply: Revised.  
  
Video  
1. Mice should be held at a slight angle, with the head lower than the hind region during an intraperitoneal injection to avoid puncturing organs. The animal should be angled in 4:02 of video. This could be specified in the manuscript, along with placing the needle bevel up at a 30-40° angle.

Reply: The text were added.  
2. For Table 2 and 7:44 in the video, it would be helpful to describe the rationale behind each control included during each step of the video and in the manuscript, for someone who has not done this before. For example, why is there a control group on ice?  
Reply: Revised.  
  
**Reviewer #2:**  
Major Concerns:  
What would greatly strengthen trust in the assay is if it were demonstrated that the internalized fluorescence is specific to the phagocytosis pathway. For example, does the presence of latrunculin A (which interferes with actin dynamics and phagocytosis) prevent fluorescent signal acquisition by macrophages (as measured by fluorescence microscopy or flow cytometry)? A control along these lines is critical to demonstrate specificity.

Reply: Thanks for the advice. To ensure the internalized fluorescence is specific to the phagocytosis, we incubated one control group with both EGFP E. coli. and F4/80 on ice. The phagocytosis is stopped on ice, which is similar to the effect of the presence of latrunculin A.  
The images in figure 1 are of low quality and the magnification is too low to properly assess if E.coli are internalized. Revised figure should also include brightfield images.  
Reply: We revised the figure 1 to make it clear as possible as we can. The red fluorescence by F-actin can help to assess whether E. coli are internalized.