

We thank the editor and reviewers for their positive comments and critiques. We believe we have addressed each comment fully. In several instances, we revised and expanded the text to incorporate points made by reviewers that increase the rigor of the manuscript. In bold below are line-by-line responses to individual editorial comments. We have also provided summarized responses to comments made by Reviewers 1 and 2.

Editor:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread.

2. Please provide email addresses for all authors within the manuscript itself.

E-mail addresses for all authors have been included in the manuscript.

3. Please define all abbreviations before use; e.g., DMEM, FBS, M-CSF.

All abbreviations have been defined upon first use.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Transwell (including in the title), Costar, Thermo Fisher Scientific, Gibco, etc.

Commercial language has been removed from the manuscript, though commercial products are fully referenced in the Table of Material and Reagents.

Protocol:

1. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Additional detail has been included in a number of the protocol steps to address the “how” question.

Specific Protocol steps:

1. 2.1: Where do tumor cells come from? Is this procedure generally done with, e.g., commercially available cell lines?

Additional language stating that work was performed with commercially available cell lines has been included. A comment was also made in the manuscript that the method should be amenable to use with primary tumor cells as well.

2. 2.3: Please express centrifuge speeds as ‘x’g’ instead of rpm.

The substitution of x g has been made.

3. 4.1: How exactly is cell lysate isolated?

The text has been altered to state that cell lysate is isolated per the instructions of the RNA preparation kit vendor.

Figures:

1. Please upload each Figure individually to your Editorial Manager account (i.e., 3 separate files).

The initial combined figures have been separated out for individual loading.

2. Figures 2 and 3: Please explain what the error bars represent in the appropriate figure legend. Please also explain what the stars (*) and crosses (†) represent, including statistical test used, if applicable.

Additional language has been included to describe the indications of statistical significance and the tests used.

Discussion:

1. Please revise the Discussion to explicitly cover the following:

- a) Critical steps within the protocol
- b) Any limitations of the technique
- c) The significance with respect to existing methods

Each point has been addressed within the discussion section. The terms “critical” and “important” have been added to more clearly indicate essential steps. Further commentary on limitations, including a point made by a reviewer, have been incorporated and significance has been stressed, in regard to the ability to use the method to study cancer immune suppression.

References:

1. Please include references within the main text of the manuscript, not as footnotes.

References have been incorporated within the text and as endnotes.

2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

References have been modified to fit the above format.

3. Please do not abbreviate journal titles.

Full journal titles have been substituted for abbreviations.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Materials used have been fully referenced.

Reviewer 1:

We thank Reviewer 1 for their positive comments on our manuscript and for suggestions to improve the scientific rigor of the writing.

Major concerns

Comment #1. Reviewer 1 points out that while the focus of the article is on the macrophage/cancer cell interaction, we spend considerable time discussing the interactions of T cells and cancer cells. We have since revised the introduction to provide a broader overview of the types of tumor/immune signaling interactions and have highlighted relevant tumor/macrophage interactions.

Comment #2. We added additional references and commentary about the use of transwell assays in the context of tumor/macrophage interactions, particularly in the introduction.

Comment #3. Rightfully, the reviewer comments how the M1/M2 paradigm has increasingly become outdated, especially in light of current single-cell sequencing findings. We have since removed mention of M1/M2 polarization.

Minor concerns

Comment #1. We initially mentioned peritoneal macrophages and macrophage cells lines but have since expanded our writing to include bone marrow derived macrophages as well.

Comment #2. Reviewer 2's comments that reagent additions will have consequences on both cell types is absolutely correct. We have since expanded the discussion to address this point, and included references demonstrating potential off-target or unintended effects and some appropriate controls.

Reviewer 2:

We also thank Reviewer 2 for their positive comments.

Major concerns

Comment #1. No major concerns raised.

Minor concerns

Comment #1. Reviewer 2 mentions potential diffusion gradients and the possibility that soluble secreted factors may adhere to cell culture dishes. We have expanded the discussion to address these concerns and highlighted how the added variable may affect overall outcomes.