**Point-by-point Responses to Editorial comments (JoVE52549R1)**

Dear JoVE Editorial Team,

We would like to thank you and our 3 reviewers for the review of our re-submitted, revised manuscript JoVE52549R1 entitled " 'Measuring the Immunosuppressive Activity of Human Mesenchymal Stem Cells in vitro ". We are particularly grateful for the detailed, insightful comments from our reviewers. We greatly appreciate the very helpful advices and have modified the manuscript accordingly. In this revised version, we would like to answer all the questions from the review dated September 25, 2014 on a point-by-point basis.

1. *The length is > 3 pages. Please highlight 2.75 or less for filming.*

**Response: We have highlighted 2.75 pages with yellow color in the revised version.**

1. *Please include the removal of the activating beads at some point. (3.1?)*
   1. **The beads are not removed prior to analysis by flow cytometry because they can easily be distinguished from cells by their distinct size and density.**
2. *Steps 5.4/5.7 could use a reference or more detail.*
   1. **We have added a reference for these isolation techniques, and directed readers to this reference at the beginning of section 5.**
3. *Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.*
4. *Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.*

**Reviewers' comments:**  
  
**Reviewer #1:**

*The invited methods article by M Klinker and coauthors describes the protocol for quantifying the immunosuppressive effect of MSCs in vitro using a proliferative assay or a mixed lymphocyte reaction assay. Overall, the manuscript explicitly and clearly describes the procedure in a way that should be transposable in other laboratories.*  
*I have some minor comments:*

* *Page 4: why the authors use 16.5% of FBS? The percentage of FBS used is generally 10%.* 
  + **We have found that supplementing our MSC culture medium with a higher than normal concentration of FBS speeds the growth and improves the health of MSC used in these experiments. The specific concentration of 16.5% is used mostly for convenience, as adding 100 mL of FBS and 5 mL of Penicillin/Streptomycin to 500 mL of medium yields ~16.5% FBS in the final complete medium.**
* *The authors often use the % of a compound instead of the concentration (for instance: 1% penicillin; 1% glutamine). Even though the supplier and reference of the product are provided, the reviewer suggests to indicate the concentration.*
  + **This is an excellent suggestion, and we have added the final concentrations of media supplements to the manuscript.**
* *Page 4, point 2.2: the FBS used has to be decomplemented: please indicate. In the same paragraph, the authors indicate they use 2x106 cells/ml: please indicate the final quantity of cells used (4x106 cells/2ml should be more appropriate).*
  + **The FBS used in these experiments has been heat inactivated. We have added this information to the manuscript. We have also added the total number of cells and final volume used in each well for this experiment as suggested by the reviewer.**
* *Page 5, point 4.2: incubation of PBMC for 10-14 hours with brefeldin A may be too long. We generally do not incubate further than 10h.*
  + **The reviewer is correct in pointing out that long incubation with brefeldin A can lead to significant toxicity in the cell populations studied. We have therefore altered the protocol to use a shorter 6-10 hour incubation and have noted that longer incubations are toxic to cells.**
* *Page 6, point 4.8: Best results are obtained if analysis is made within 2 days and not longer.*
  + **At the suggestion of the reviewer, we have altered the protocol to suggest that analysis of fixed samples should occur within two days for best results.**

**Reviewer #2:**

*This study addresses the immunosuppressive functions of MSCs and suggests how an appropriate methodological approach can circumvent the differences in immunosuppressive potential by MSCs derived from different donors and species.   
Using several methods for assessing T cell activation that are adaptable to a variety of experimental designs is a key approach successfully demonstrated by the authors.*  
**Reviewer #3:**

*The manuscript presented by Wei. et al has proposed feasible protocols for quantifying immunosuppressive capability of human MSCs in vitro using target cells from both human and murine origin. With protocols lucidly stated and results reasonably illustrated, this work was expected to serve as a reference in testing immunosuppressive effectiveness of MSCs obtained from various sources and culturing techniques. However, with some of the critical assays missed, this system is only partially constructed. Direct evidence demonstrating inhibition of PBMC proliferation and the expression level of effector molecules should be provided by additional tests.   
  
Major Concerns:*

* *The morphological change of PBMCs can provide direct evidence for immunosuppression of MSCs. The results should include photos demonstrating PBMC proliferation is hampered after being co-cultured with MSCs.* 
  + **Thanks for the excellent suggestion. While morphological changes can be indicative of proliferation, they may not unequivocally demonstrate cellular division as the CFSE dilution assay does. Additionally, CFSE dilution allows the investigator to assess proliferation among specific cellular subsets, which is an important consideration when a heterogeneous population such as PBMCs is under investigation. For these reasons, as well as the brevity of the manuscript, we think that CFSE dilution is sufficient for the assessment of proliferation in this manuscript.**
* *It is well acknowledged that IDO is the critical effector molecule in human MSC-mediated immunosuppression. Therefore, the IDO expression level could be a strong indicator to assess the immunosuppressive capability of MSCs. Moreover, the IDO expression level after treatment by various inflammatory cytokines should also be determined to mimic the microenvironment of MSCs after being injected in vivo.* 
  + **We very much appreciate our reviewer’s insightful comment on this point; indeed many reports have observed an important role for IDO activity in MSC-mediated immune suppression. Importantly, however, IDO-independent immune suppression has been observed as well, with various other effector molecules reportedly playing a role. The assays presented in the manuscript are intended to assess the immunosuppressive function of different MSC lots in an unbiased manner without assumptions regarding the mechanism(s) in use. While IDO activity in MSCs has been linked to effective immunosuppression, it is beyond the focus of this manuscript to assess the expression or activity of the various effector molecules attributed to MSCs. Actually we are actively pursuing the molecular mechanisms and pathways involved in the human-MSC mediated immunosuppression in our model system.**
* *In addition to the flow cytometry assay, the production of cytokines by PBMC should also be tested by ELISA or Bio-Plex assay.* 
  + **Thanks for pointing this out, this is an excellent suggestion. Changes in cytokine production identified by the intracellular staining technique included in our manuscript can be confirmed by the methods suggested by the reviewer, and we have added this information to the manuscript.**

*Minor Concerns:*

* *There should be a comma between "individuals" and "they" in line 87.*
  + **A comma has been added to this sentence as suggested by the reviewer.**
* *p values should be added appropriately in all column bar graphs.*
  + **We have indicated where MSCs have significantly inhibited activation in each of the representative results figures.**

We sincerely thank our reviewers for their invaluable expertise and recommendations for the manuscript, which have made this manuscript much clearer and stronger. If there are any other comments or suggestions, we will be very happy to learn and make further changes to the manuscript accordingly.

Thank you and with best regards,

Cheng-Hong Wei, Ph.D

CBER

FDA