Journal of Visualized Experiments

# Measuring the Immunosuppressive Activity of Human Mesenchymal Stem Cells in vitro

--Manuscript Draft--

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| **Manuscript Number:** | JoVE52549R2 |
| **Full Title:** | Measuring the Immunosuppressive Activity of Human Mesenchymal Stem Cells in vitro |
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Keywords:** | Mesenchymal Stem Cells<br>Immunosuppression<br>Immune Regulation<br>T Cells<br>Flow Cytometry<br>T cell activation<br>Immunology |
| **Manuscript Classifications:** | 95.51.10: biology (general); 95.51.22: immunology; 95.51.23: immunosuppression |
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| **Abstract:** | Mesenchymal stem cells (MSCs) can be isolated from a wide variety of accessible human tissues and exhibit multipotency and immunoregulatory properties. Because these cells are easily expanded in vitro and do not induce allogeneic immune responses upon transfer to unmatched individuals, they have received much attention as a practical source for cell-based therapies in regenerative medicine and for the treatment of immune-mediated disorders. Several clinical trials testing the efficacy of MSC-based treatment for autoimmune disease have shown mixed results, with some showing effective immunosuppression and others little or no effect. One possible explanation for these discrepancies is intrinsic variability of immunosuppressive functions between MSC lots arising from different human donors or as a consequence of differing manufacturing protocols. The experiments presented here use in vitro methods to compare the immunosuppressive activity of MSC lots using both human and murine target cells. The expansion and harvest of MSC cultures is described, as well as methods for co-culture of MSCs with targets cells derived from human peripheral blood cells and murine splenocytes and lymphocytes. Several methods for assessing activation of T cells are described as well, using flow cytometry to measure expression of activation-induced cell surface markers, proliferation, and production of inflammatory cytokines in T cells. These methods are adaptable to a variety of |

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| | experimental designs, such as comparing the immunosuppressive activity of MSCs obtained from different donors or expansion techniques, and investigating potential immunosuppressive mechanisms used by MSCs against both human and murine target cells. |
| **Author Comments:** | Dear JoVE editors,<br>Our lab has just moved from NIH campus to White Oak campus on 8/8/2014, it will take some time (probably 6-8 weeks) before we can settle down and start working in the labs again. So we may need to touch base again concerning the date of the filming down the road of reviewing and revision.<br>Thanks,<br>Cheng-Hong Wei |
| **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. | Nov 30, 2014 |
| If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter. | |

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Re: JoVE Cover Letter

Dear Editors,

We are submitting the manuscript titled "**Measuring the Immunosuppressive Activity of Human Mesenchymal Stem Cells in vitro**" for consideration as a research article in the Journal of Visualized Experiments (JoVE).

Duto to its unique differentiation multipotency and immunomodulatory capacity, multipotent Stromal cells (MSCs, also termed mesenchymal stem cells), have been extensively used in investigational studies to treat a variety of tissue injuries and immune diseases both in experimental and clinical settings.

Currently, there is increased need to establish novel quantitative bioassays suitable for measuring differences in immune-inhibitory activity of MSCs from different donors or at different passages in tissue culture. There is a related scientific need to identify the molecular mechanisms underlying MSC-mediated immunosuppression, which also requires accurate assays to measure the immunosuppressive activity of MSCs. Such methods could potentially also be used to predict MSC behavior after transplantation.

To address this need, the manuscript submitted here describes a novel method to characterize the immunosuppressive acitivity of human MSCs. We developed novel immune inhibition assays using clonal murine T cell populations responding to known peptide antigens, and MSCs derived from human donors. MSCs are known to be immunosuppressive across xenogeneic barriers, allowing us to assess the use of easily obtained clonal murine T-cells as a method to reduce variability in T-cell based in vitro immune suppression assays. In our system, hMSC clearly show inhibitory properties, altering multiple aspects of murine T cell activation, including reduced proliferation, inhibited or stimulated cell surface marker expression, and decreased cytokine expression. Therefore, clonal murine T cells can be used to characterize and quantify the immunosuppressive activity of human MSCs, representing a promising approach to improve bioassays and to elucidate the mechanisms for MSC-mediated immunosuppression.

We believe that this manuscript is very relevant in light of the wide potential application of MSCs in treating GVHD and various autoimmune diseases (such as Crohn's disease, type 1 diabetes, multiple sclerosis etc.). The findings reported in this study would be of general interest to immunologists, stem cell biologists, transplantation biologists and scientists in the cellular therapy field. It would be of special interest to the autoimmune disease and immunoregulation community. This manuscript is not under consideration elsewhere, and all authors have approved its submission.

We welcome your comments and thank you for your consideration.

Sincerely,

Cheng-Hong Wei, PhD.

**TITLE:**
Measuring the Immunosuppressive Activity of Human Mesenchymal Stem Cells *in vitro*

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**KEYWORDS:**
Mesenchymal Stem Cells, Immunosuppression, Immune Regulation, T Cells, Flow Cytometry, T cell activation, Immunology

**SHORT ABSTRACT:**
Mesenchymal stem cells (MSCs) are a readily-accessible source of immunoregulatory cells that can potently inhibit pathogenic immune responses, but significant variability exists between MSC lots. Here, we present protocols for quantifying and comparing the immunosuppressive effectiveness of MSCs in vitro using both human and murine target cells.

**LONG ABSTRACT:**
Mesenchymal stem cells (MSCs) can be isolated from a wide variety of accessible human tissues and exhibit multipotency and immunoregulatory properties. Because these cells are easily expanded in vitro and do not induce allogeneic immune responses upon transfer to unmatched individuals, they have received much attention as a practical source for cell-based therapies in regenerative medicine and for the treatment of immune-mediated disorders. Several clinical trials testing the efficacy of MSC-based treatment for autoimmune disease have shown mixed results, with some showing effective immunosuppression and others little or no effect. One possible explanation for these discrepancies is intrinsic variability of immunosuppressive functions between

MSC lots arising from different human donors or as a consequence of differing manufacturing protocols. The experiments presented here use in vitro methods to compare the immunosuppressive activity of MSC lots using both human and murine target cells. The expansion and harvest of MSC cultures is described, as well as methods for co-culture of MSCs with targets cells derived from human peripheral blood cells and murine splenocytes and lymphocytes. Several methods for assessing activation of T cells are described as well, using flow cytometry to measure expression of activation-induced cell surface markers, proliferation, and production of inflammatory cytokines in T cells. These methods are adaptable to a variety of experimental designs, such as comparing the immunosuppressive activity of MSCs obtained from different donors or expansion techniques, and investigating potential immunosuppressive mechanisms used by MSCs against both human and murine target cells.

## INTRODUCTION

Mesenchymal stem cells (MSC, also known as multipotent stromal cells or mesenchymal stromal cells) are a heterogeneous cell population with the capacity to differentiate into several types of cells under appropriate conditions *in vitro*, including osteocytes, chondrocytes, and adipocytes[1]. They are present in a wide variety of clinically-accessible tissues including bone marrow, umbilical cord blood, adipose tissue, and dental pulp, and are easily isolated and expanded *in vitro*[2]. Importantly, MSCs do not elicit an allogeneic response upon transfer into an unmatched recipient due to their low expression of MHC class I and class II molecules and their use of multiple active immunoregulatory mechanisms[3,4]. These combined qualities have led to intense interest in MSCs for their potential as a cell-based therapy for a wide variety of diseases.

Several clinical trials testing the efficacy of MSCs in treating autoimmune disorders have completed or are currently underway[5]. These studies have yielded mixed results, with the effectiveness of MSCs in ameliorating autoimmune symptoms varying between studies[6]. One possible explanation for these discrepant results may be variability between MSC preparations derived from different donors or expanded by different commercial production methods. A standardized and repeatable bioassay for comparing the immunosuppressive activity of MSC preparations could help to identify MSC lots most likely to be effective clinically. Additionally, such a bioassay would aid in the identification of biomarkers correlated with immunosuppression in MSCs, or better characterize molecular mechanisms important for their *in vivo* function.

We present here protocols for assessing the immunosuppressive activity of human MSCs using both human and murine target cells. Using peripheral blood mononuclear cells (PBMC) as target cells allows for the assessment of the full complement of immunosuppressive mechanisms used by MSCs, but requires a less biologically-relevant stimulus to elicit T cell stimulation (in this case, activation by beads coated with anti-CD3 and anti-CD28). Additionally, PBMCs from distinct donors may have varying susceptibility to MSC-mediated immunosuppression, which makes comparisons between experiments more difficult to interpret. In contrast, using murine target cells allows for a more biologically-relevant, repeatable, and reliable form of T cell stimulation (antigenic stimulation of transgenic T cells), but has the disadvantage that although

cross-species effects of human MSCs have been reported, some mechanisms employed by MSCs might not be cross reactive between species[7,8].

**PROTOCOL**

All procedures performed on animals were approved by the Institutional Animal Care and Use Committees at the Center for Biologics Evaluation and Research and performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. For more detailed protocols for isolation immune cells from spleens and lymph nodes see Kruisbeek, 2001[9]. Perform all steps in a sterile laminar flow hood.

**1) Expanding MSCs in Culture**

1.1) Purchase MSCs from a commercial vendor at passage 1, or isolate from human tissues according to established protocol[10].

1.2) Prepare MSC culture medium using α-MEM supplemented with 16.5% fetal bovine serum, 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine.

1.3) Culture MSCs in T175 flasks at an initial concentration of 60 cells/$cm^2$ in MSC culture medium.

1.4) When MSC cultures attain 80% confluence, remove media and detach the adherent MSCs by incubating with 0.25% Trypsin/EDTA and for 5 minutes at 37$^oC$. Collect cells and wash with HBSS. Return MSCs to culture in T175 flasks at 60 cells/$cm^2$.

1.5) For cryopreservation, harvest MSCs after passage 3 or greater as in step 1.4 and wash with HBSS. Re-suspend MSCs in freezing media containing 5% DMSO, 30% FBS (heat inactivated), and 100 U/mL penicillin/streptomycin at $10^6$ cells/ml.

1.6) Aliquot the cell suspension into cryoprotective vials and place into a passive freezing device. Place the passive freezing device in a -80$^oC$ freezer overnight, then transfer MSC aliquots to a liquid nitrogen storage vessel until needed for experiments.

1.7) To use cryopreserved MSCs in subsequent experiments, culture a thawed aliquot of MSCs as in step 1.3 until 80% confluent, then harvest and wash as in step 1.4.

**2) Co-culture of MSCs with human peripheral blood mononuclear cells (PBMCs)**

2.1) Obtain cryopreserved PBMCs from a commercial vendor or isolate from human blood according to established protocol [11].

2.2) On the day before cultured MSCs will be ready for experiments, thaw an aliquot of cryopreserved PBMCs and culture overnight in standard RPMI medium (RPMI 1640 +

L-glutamine medium supplemented with 10% FBS (heat inactivated) and 100 U/mL penicillin/streptomycin) at $10^7$ cells/5 ml in 6-well plates.

2.3) Harvest PBMCs from culture plates, wash with HBSS, and count using a hemocytometer. Re-suspend PBMC at 20 x $10^6$/ml in pre-warmed HBSS containing 0.5 µM CFSE (carboxyfluoroscein diacetate, succinimidyl ester). Incubate cells for 20 minutes at 37$^o$C. Wash cells with HBSS and re-suspend in an appropriate volume of standard RPMI medium.

2.4) Harvest MSCs from culture flasks as described in step 1.4. Wash with HBSS, count, and re-suspend in an appropriate volume of standard RPMI medium.

2.5) Wash T cell activating beads by diluting with HBSS and placing the vial in a magnet. Pour off HBSS while the magnet retains the beads in the vial. Re-suspend beads in an appropriate volume of standard RPMI medium.

2.6) Set up PBMC/MSC co-cultures in flat-bottomed 96-well plates. Add PBMCs (0.1 x $10^6$ – 0.2 x $10^6$ cells/well), MSCs (5,000-20,000 cells/well), and T cell activating beads (at a 1:1 bead to PBMC ratio) to each well with a final volume of 200 µl/well.

**3) Assessing activation-associated surface markers and proliferation of human T cells**

3.1) After 3-4 days in culture, remove PBMCs from a flat-bottomed plate and move to a round-bottomed plate. Wash cells twice with staining buffer (1X HBSS, 2% heat-inactivated FBS).

**NOTE:** Activating beads are easily identified and excluded from flow cytometry analysis by their distinctive size and density, and therefore there is no need to separate cells from beads.

3.2) Prepare an antibody cocktail with fluorochrome-labeled antibodies against surface antigens in staining buffer according to manufacturer's specifications.

**NOTE:** Useful antibodies for comparing the activation status of T cells include anti-CD4, anti-CD8, anti-CD25, anti-CD69, and anti-CD44.

3.3) Spin cells down at 300 x g for 10 minutes and remove excess staining buffer after final wash. Re-suspend with a vortex and add 50 µl of the antibody cocktail. Incubate cells at room temperature for 30 minutes while protecting them from light.

3.4) Wash cells twice with 200 µl staining buffer, then re-suspend in 200 µl of staining buffer and read immediately on an appropriate flow cytometer. Alternatively, washed and stained cells can be fixed overnight in 2% formalin and analyzed by a flow cytometer within 2 days of fixation.

**4) Quantifying cytokine-producing human T cells**

4.1) Set up PBMC/MSC co-culture plates as described in step 2.1 through 2.6.

4.2) After 2-5 days in culture, remove and reserve 100 µl of supernatant and replace with 100 µl of standard media containing a secretion inhibitor such as brefeldin A. Return plates to the incubator and allow cytokines to build up in PBMCs for 6-10 hours.

**NOTE:** Avoid incubation periods longer than 10 hours, as brefeldin A is toxic to cells and can lead to significant cell death.

4.3) Remove PBMCs from a flat-bottomed plate and move to a round-bottomed plate. Wash cells twice with staining buffer.

4.4) Prepare an antibody cocktail with fluorochrome-labeled antibodies against CD4 and CD8 in staining buffer. Stain cells as described in step 3.3.

4.5) After staining, wash cells once in staining buffer and fix by adding 100 µl of fixation/permeabilization buffer. Incubate for 20 minutes at 4$^{o}$C. Wash twice with 150 µl of BD permeabilization buffer to permeabilize the cells.

4.6) Prepare an antibody cocktail with fluorochrome-labeled antibodies against cytokine antigens in permeabilization buffer according to manufacturer's specifications. Useful antibodies for comparing the activation status of T cells include anti-TNFα and anti-IFNγ.

4.7) Incubate cells in with antibody cocktail for 30 minutes at 4$^{o}$C while protecting from light.

4.8) Wash twice with 200 µl of permeabilization buffer, and once with 200 µl of staining buffer. Re-suspend cells in 200 µl of staining buffer and store in the dark at 4$^{o}$C until ready for analysis on a flow cytometer. For best results cells analyze within two days of staining. To confirm results obtained by flow cytometry, measure the concentration of cytokines in the supernatant reserved in step 4.2 by enzyme linked immunosorbent assay (ELISA) or a multiplex cytokine quantification method.

**5) Co-culture of human MSCs with murine transgenic T cells**

5.1) Obtain T cell transgenic mice such as the NOD 8.3 TCR transgenic strain from a commercial source. CD8$^{+}$ T cells from NOD 8.3 mice express a MHC class I-restricted TCR that recognizes an epitope derived from IGRP, an islet-associated antigen.

5.2) Obtain wild type control mice from a commercial vendor. For the NOD 8.3 mice, the appropriate control strain is NOD/ShiLtj.

**NOTE:** These mice will be used as a source of antigen-presenting cells to activate T cells from the transgenic animals.

5.3) Thaw and culture an aliquot of MSCs as described in steps 1.3 – 1.4. Wait until the MSCs are 80% confluent before preparing mouse cells as described below.

5.4) Euthanize several NOD 8.3 mice with carbon dioxide and remove spleens and lymph nodes. Crush lymph nodes and spleens with a syringe plunger in HBSS and pass through a cell strainer to remove debris. Wash pooled lymph node cells with HBSS and count using a hemocytometer. Spin down pooled splenocytes at 300 x g for 10 minutes and remove most of the HBSS.

5.4.1) Re-suspend splenocytes in the residual HBSS and add 1 ml of ACK lysis buffer for each spleen to the re-suspended pellet. After one minute, directly add HBSS to dilute the ACK buffer and spin down splenocytes at 300 x g for 10 minutes. Pass cells through a cell strainer and wash twice with HBSS.

5.5) Pool splenocytes and lymphocytes together for isolation of $CD8^+$ cells by negative selection. Follow the manufacturer's protocol for selection using magnetic cell sorting.

5.6) After separation, re-suspend NOD 8.3 $CD8^+$ T cells at 20 x $10^6$/ml in pre-warmed HBSS containing 0.5 µM CFSE. Incubate cells for 20 minutes at 37$^o$C. Wash cells with HBSS and re-suspend in an appropriate volume of standard RPMI medium.

5.7) Euthanize several NOD/ShiLtj mice with carbon dioxide and collect spleens. Process pooled spleens as described in step 5.4. Irradiate splenocytes after isolation with 4,000 rads. Wash twice with HBSS and re-suspend in an appropriate volume of standard RPMI medium.

5.8) Harvest MSCs from culture flasks as described in step 1.4. Wash with HBSS, count, and re-suspend in an appropriate volume of standard RPMI medium.

5.9) Dilute an aliquot of the relevant IGRP peptide (206-214) in standard RPMI medium.

5.10) Set up cultures in a 24-well flat-bottomed plate. Add NOD 8.3 $CD8^+$ T cells (2 x $10^6$/well), irradiated NOD splenocytes (4 x $10^6$/well), MSCs (0.2 x $10^6$ – 0.5 x $10^6$/well), and $IGRP^{206-2014}$ peptide (1 µg/ml final concentration) for a final volume of 2 ml/well.

**6) Assessing activation-associated surface markers and proliferation of murine T cells**

6.1) After 3-4 days in culture, remove non-adherent T cells from the 24-well plate and move to either a 96-well flat-bottomed plate or individual tubes for staining. Wash cells twice with staining buffer.

6.2) Prepare an antibody cocktail with fluorochrome-labeled antibodies against surface antigens in staining buffer according to manufacturer's specifications. Useful antibodies for comparing the activation status of T cells include anti-CD4, anti-CD8, anti-CD25, anti-CD69, anti-CD44, and anti-CD62L. T cells expressing the transgenic TCR can be identified by using an anti-Vβ4 TCR antibody.

6.3) Spin cells down at 300 x g for 10 minutes and remove excess staining buffer after final wash. Re-suspend with a vortex and add 50 µl of the antibody cocktail. Incubate cells at room temperature for 30 minutes while protecting them from light.

6.4) Wash cells twice with staining buffer, then re-suspend in 200 µl of staining buffer and read immediately on an appropriate flow cytometer. Alternatively, washed and stained cells can be fixed overnight at $4^{o}C$ in 2% formalin and analyzed by a flow cytometer within 5 days of fixation.

**REPRESENTATIVE RESULTS:**

Activation of T cells results in proliferation, production of cytokines, and changes in the expression of various surface markers. Representative results from co-culture of human PBMCs and human MSCs are presented in Figure 1 and Figure 2. Using multicolor flow cytometry, $CD4^+$ and $CD8^+$ cells can be analyzed from a single sample. MSCs inhibit proliferation of both $CD4^+$ and $CD8^+$ T cells as measured by dilution of CFSE (Figure 1A). Activation-induced expression of CD25 is also inhibited by MSCs (Figure 1B). Pro-inflammatory cytokines such as IFNγ and TNFα are produced by T cells upon activation, and the frequency of T cells producing these cytokines is reduced by co-culture with MSCs (Figure 2). Culture of purified NOD 8.3 transgenic T cells with antigen-presenting cells in the presence of IGRP peptide results in increased surface expression of the activation markers CD69 and CD25 (Figure 3A and 3B). When MSCs are present, these signs of activation are inhibited (Figure 3A and 3B).

**FIGURES LEGEND:**

**Figure 1. MSCs suppress proliferation and CD25 expression in activated human $CD4^+$ and $CD8^+$ T cells.**

Peripheral blood mononuclear cells were stained with CFSE and activated with beads coated with anti-CD3 and anti-CD28 in the presence of increasing numbers of human mesenchymal stem cells. Graphs represent mean values ± standard deviation. **(A)** Representative histograms of CFSE staining and quantitation of CFSE-diluted cells among $CD4^+$ and $CD8^+$ cells. **(B)** Representative histograms of CD25 staining and quantitation of $CD25^+$ cells among $CD4^+$ and $CD8^+$ cells. Significance was tested by a Student's t test comparing activation in the presence or absence of MSCs (***$p$<0.001).

**Figure 2. MSCs suppress secretion of TNFα in activated human $CD4^+$ and $CD8^+$ T cells.**

Peripheral blood mononuclear cells were activated with beads coated with anti-CD3 and anti-CD28 for three days. Brefeldin A was added for the final 12 hours in culture, and cells were stained for intracellular TNFα. **(A)** Representative dotplots of TNFα staining among $CD4^+$ and $CD8^+$ cells. **(B)** Quantitation of replicate samples from the experiment

presented in (A). Graphs represent mean values ± standard deviation. Significance was tested by a Student's t test comparing activation in the presence or absence of MSCs (***$p$<0.001).

**Figure 3. MSCs suppress expression of CD69 and CD25 in activated murine NOD 8.3 CD8$^+$ Vβ4$^+$ T cells.**
CD8$^+$ T cells from NOD 8.3 TCR transgenic mice were isolated and co-cultured for three days with irradiated NOD splenocytes and IGRP peptide in the presence or absence of MSCs at two T cell: MSC ratios. **(A)** Representative histogram overlays of CD69 and CD25 expression among CD8$^+$VB4$^+$ T cells. **(B)** Quantitation of median fluorescence intensity (MFI) and % positive for each marker from replicate samples from the experiment presented in (A). Graphs represent mean values ± standard deviation. Significance was tested by a Student's t test comparing activation in the presence or absence of MSCs (*$p$<0.05 and **$p$<0.01).

**DISCUSSION:**
The efficacy of human MSCs in treating inflammatory conditions such as type 1 diabetes, graft-versus-host disease, and Crohn's disease has been tested in several clinical trials with sometimes conflicting results[6,12]. Variability in immunosuppression due to varying donor sources, culture passages, tissue sources, and expansion protocols may be the source of these discrepant outcomes. To better understand the biology behind this variability, as well as predict which specific MSC lots are likely to be most effective clinically, a reliable and repeatable bioassay for the immunosuppressive capacity of MSCs is required.

In this work we have presented protocols for assessing the immunosuppressive capacity of MSCs *in vitro* using both human and murine target cells. Using human-derived PBMCs as the target cells allows for the full complement of immunosuppressive mechanisms used by MSCs to be tested. Unfortunately, inter-donor variability between PBMC donors can make reconciling discrepant results obtained on different days and from different laboratories difficult. By using murine target cells from TCR transgenic mice some of this inter-experiment variability can be reduced, but with the caveat that some suppressive mechanisms employed by human MSCs may not be effective on mouse target cells. There is strong evidence, however, that human MSCs can suppress murine cells *in vitro* and *in vivo*, and even home to tissues and survive upon transfer into mice or rats[7,13-16].

The assays presented herein can be used to compare the immunosuppressive function of MSCs from different donors, passage, tissues of origin, and expansion methods. Additionally, these techniques allow for the evaluation of candidate molecular mechanisms for MSC-induced immunosuppression. To this end, inhibitory molecules can be used in culture, or specific effector molecules can be knocked down in MSCs prior to co-culture. Using mouse target cells allows investigators to evaluate candidate molecules by taking advantage of the great number of transgenic and knockdown mice currently available, while such experiments are not possible using human-derived cells as targets. While the results presented here only describe experiments using CD8$^+$ T

cells, previous work in our lab has shown that these protocols also can be used to test immunosuppression in transgenic $CD4^+$ T cells, and it is likely these techniques could be used to test the effect of MSCs on other cell types as well[17].

In summary, we present here techniques for assessing the immunosuppressive activity of human MSCs *in vitro* using both human and murine target cells. Activation is evaluated by the presence of associated surface markers, proliferation as assessed by CFSE dilution, and the frequency of cytokine-producing cells among both $CD4^+$ and $CD8^+$ cells. The use of murine targets cells in addition to human targets cells allows for increased reliability of the bioassay by eliminating donor-to-donor variation in target cells.

**ACKNOWLEDGEMENTS**
This work was supported by the FDA Modernizing Science grant program, the FDA medical countermeasures initiative (MCMi) as well as the Division of Cellular and Gene Therapies. Matthew Klinker, Cristina Nazarov and Jean Manirarora were supported through fellowships administered by the Oak Ridge Institute for Science and Education. The authors would like to thank Drs. Deborah Hursh and Patrick Lynch in CBER, FDA for critically reviewing this manuscript.

**DISCLOSURES:**
The authors declare no conflicts of interest.

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Figure 1

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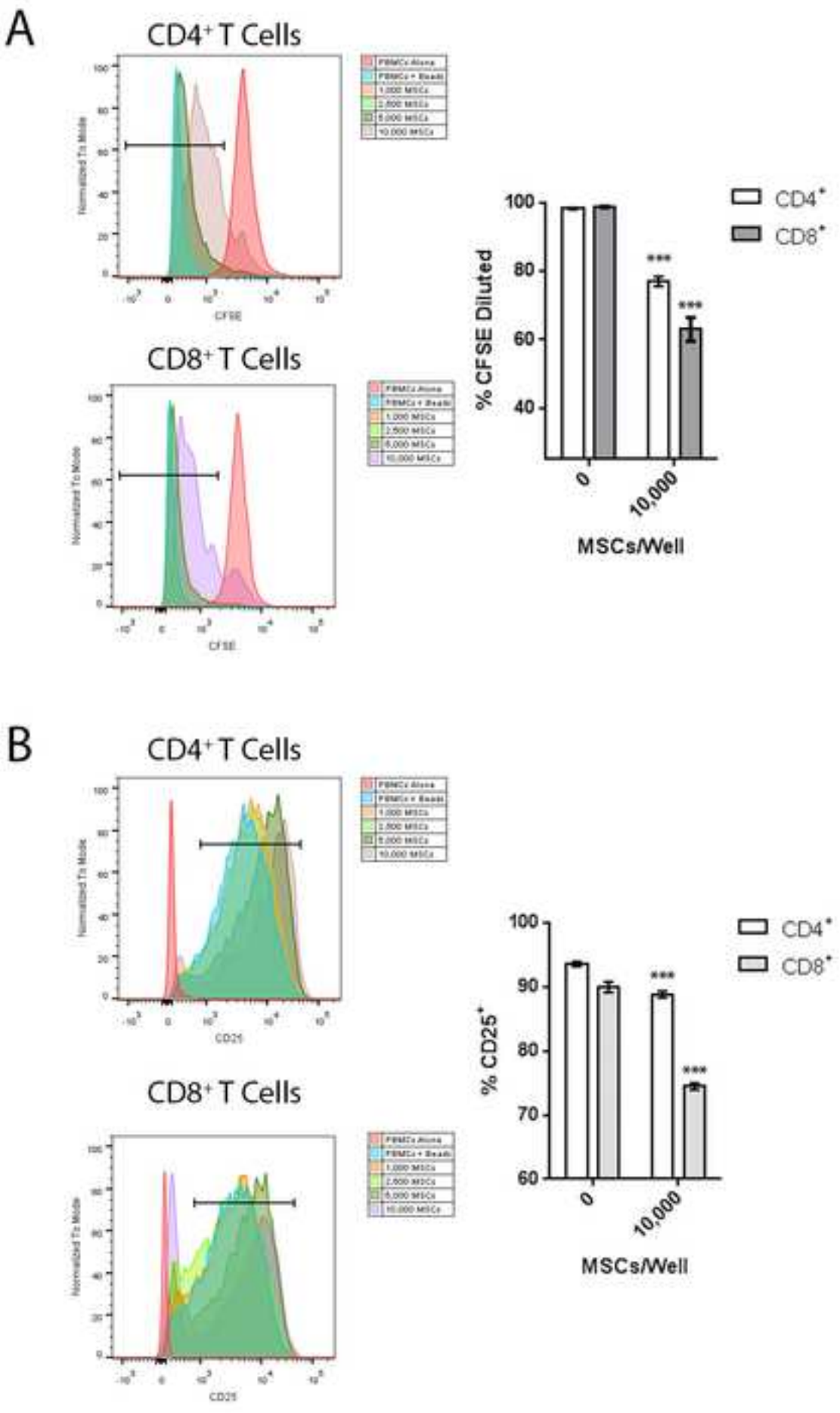

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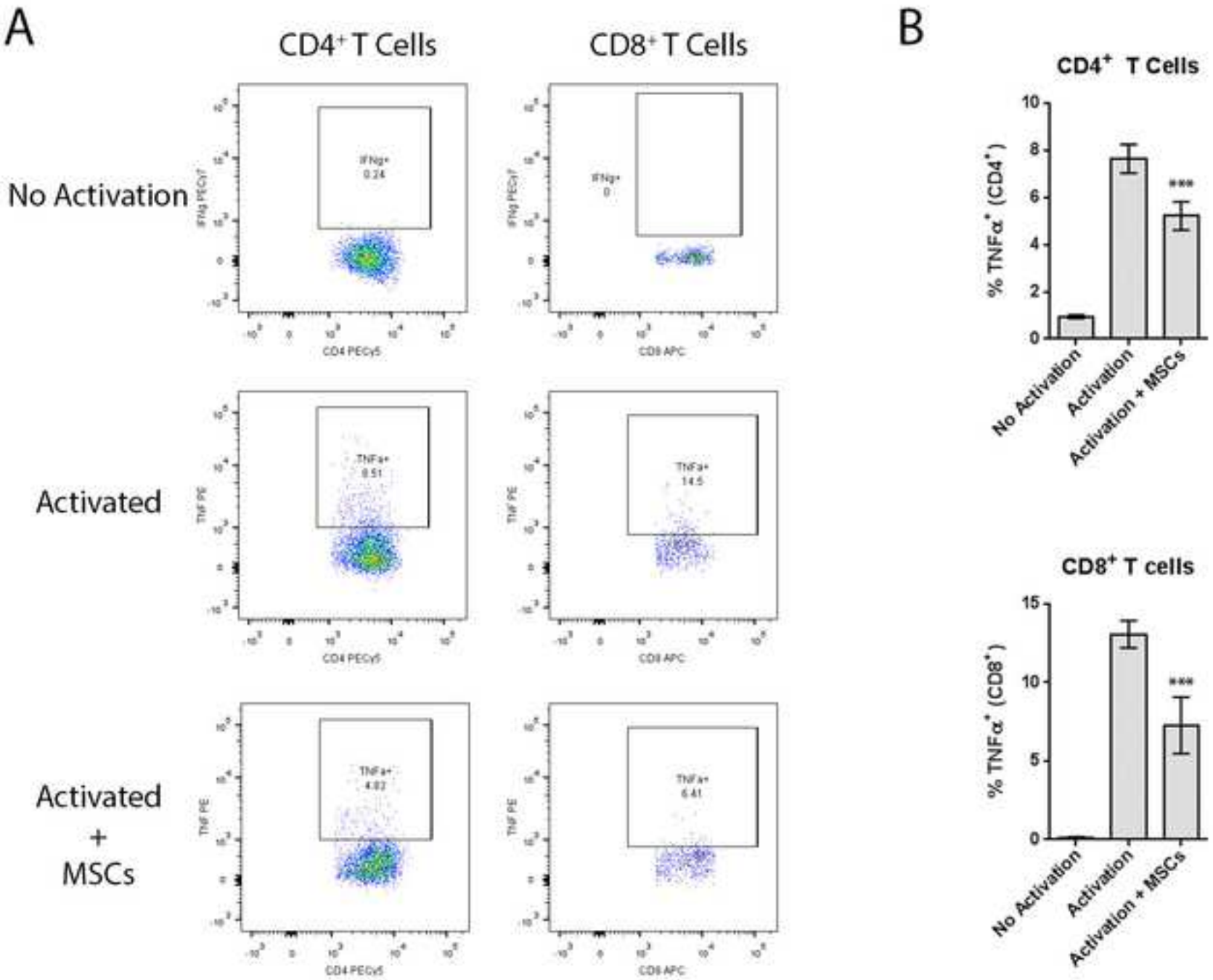

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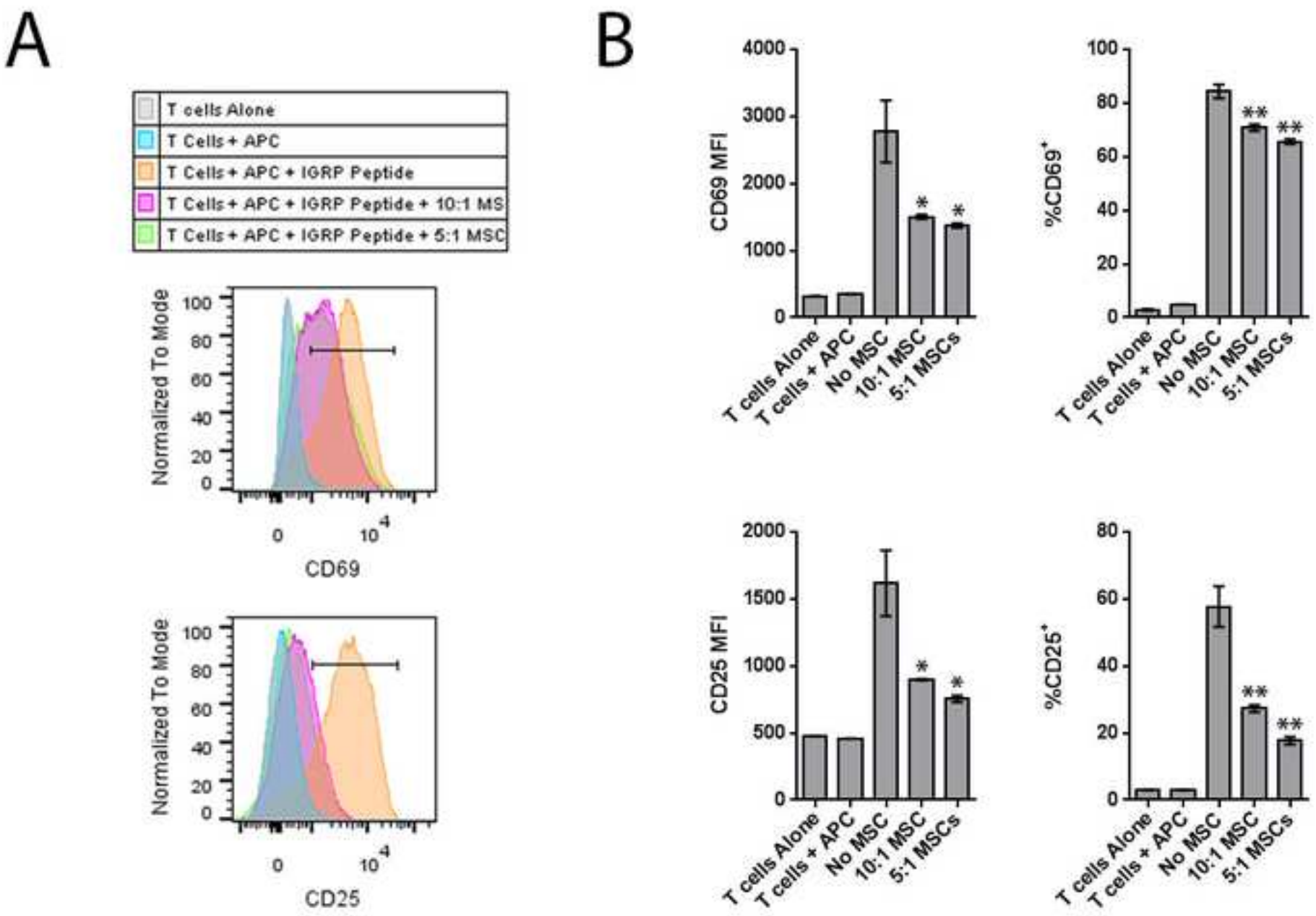

Excel Spreadsheet- Table of Materials/Equipment

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| Name of the Material/Equipment | Company |
|---|---|
| Human Mesenchymal Stem Cells | AllCells, LLC |
| MEM Alpha 1X (α-MEM) | Gibco/Life Technologies |
| Fetal Bovine Serum | JM Bioscience |
| Penicillin/streptomycin | Gibco/Life Technologies |
| L-Glutamine (200 mM) | Life Technologies |
| Trypsin/EDTA | Lonza |
| HBSS | Gibco/Life Technologies |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich |
| Peripheral Blood Mononuclear Cells | AllCells, LLC |
| RPMI 1640 (1X) + GlutaMAX | Gibco/Life Technologies |
| T Cell Activating Beads (Human) | Life Technologies |
| Anti-CD4-PECy5 (Human) | BD Biosciences |
| Anti-CD8-APC (Human) | BD Biosciences |
| Anti-CD25-V450 (Human) | BD Biosciences |
| Anti-CD69-PECy7 (Human) | BD Biosciences |
| Anti-CD44-PE (Human) | BD Biosciences |
| Anti-TNFα-PE (Human) | BD Biosciences |
| Anti-IFNγ-PECy7 (Human) | BD Biosciences |
| Formalin (10%, Buffered) | Sigma-Aldrich |
| Brefeldin A (GolgiPlug) | BD Biosciences |
| Cytofix/Cytoperm Kit | BD Biosciences |
| IGRP Peptide | FDA CBER core facility |
| CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry | Life Technologies |
| Anti-CD4 FITC (Mouse) | BD Biosciences |
| Anti-CD8α-APC (Mouse) | BD Biosciences |
| Anti-CD25-PECy5 (Mouse) | eBioscience |
| Anti-CD44-PE (Mouse) | eBioscience |
| Anti-CD62L-eFluor 450 | eBioscience |
| Anti-Vβ4 TCR-FITC (Mouse) | BD Biosciences |
| Anti-Vβ4 TCR-PE (Mouse) | BD Biosciences |
| NOD.Cg-Tg(TcraTcrbNY8.3)1Pesa/DvsJ (TCR transgenic mice) | The Jackson Laboratory |
| NOD/ShiLtJ (Control mice) | The Jackson Laboratory |
| ACK Lysis Buffer | Gibco/Life Technologies |
| EasySep Mouse CD8+ T Cell Isolation Kit | Stemcell Technologies |

| Catalog Number | Comments/Description |
|---|---|
| 12561-056 | |
| 15070-063 | |
| 11875093 | |
| CC-3232 | |
| 14175-095 | |
| D2650-100ML | |
| PB003F | |
| 72400-047 | |
| 111.31D | |
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Department: CBER

Institution: FDA

Article Title: Measuring the Immunosuppressive Activity of Human Mesenchymal Stem Cells in Vitro

Signature: Cheng-Hong Wei Date: 7/25/14

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**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.**

2. *Please include the removal of the activating beads at some point. (3.1?)*

   a. **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.**

3. *Steps 5.4/5.7 could use a reference or more detail.*

   a. **We have added a reference for these isolation techniques, and directed readers to this reference at the beginning of section 5.**

4. *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.*

5. *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

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