**TITLE:**

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

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**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](#_ENREF_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](#_ENREF_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](#_ENREF_3),[4](#_ENREF_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](#_ENREF_5). These studies have yielded mixed results, with the effectiveness of MSCs in ameliorating autoimmune symptoms varying between studies[6](#_ENREF_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](#_ENREF_7),[8](#_ENREF_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](#_ENREF_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](#_ENREF_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/cm2 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 37oC. Collect cells and wash with HBSS. Return MSCs to culture in T175 flasks at 60 cells/cm2.

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 106 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 -80oC 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](#_ENREF_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 107 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 106/ml in pre-warmed HBSS containing 0.5 µM CFSE (carboxyfluoroscein diacetate, succinimidyl ester). Incubate cells for 20 minutes at 37oC. 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 106 – 0.2 x 106 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 4oC. 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 4oC 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 4oC 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 106/ml in pre-warmed HBSS containing 0.5 µM CFSE. Incubate cells for 20 minutes at 37oC. 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 106/well), irradiated NOD splenocytes (4 x 106/well), MSCs (0.2 x 106 – 0.5 x 106/well), and IGRP206-2014 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 4oC 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](#_ENREF_6),[12](#_ENREF_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](#_ENREF_7),[13-16](#_ENREF_13).

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](#_ENREF_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.

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**DISCLOSURES:**

The authors declare no conflicts of interest.

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