Video Article

Assessment of the Immunomodulatory Properties of Human Mesenchymal Stem Cells (MSCs)

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Abstract

The immunomodulatory properties of multilineage human mesenchymal stem cells (MSCs) appear to be highly relevant for clinical use towards a wide-range of immune-related diseases. Mechanisms involved are increasingly being elucidated and in this article, we describe the basic experiment to assess MSC immunomodulation by assaying for suppression of effector leukocyte proliferation. Representing activation, leukocyte proliferation can be assessed by a number of techniques, and we describe in this protocol the use of the fluorescent cellular dye carboxyfluorescein succinimidyl ester (CFSE) to label leukocytes with subsequent flow cytometric analyses. This technique can not only assess proliferation without radioactivity, but also the number of cell divisions that have occurred as well as allowing for identification of the specific population of proliferating cells and intracellular cytokine/factor expression. Moreover, the assay can be tailored to evaluate specific populations of effector leukocytes by magnetic bead surface marker selection of single peripheral blood mononuclear cell populations prior to co-culture with MSCs. The flexibility of this co-culture assay is useful for investigating cellular interactions between MSCs and leukocytes.

Video Link

The video component of this article can be found at https://www.jove.com/video/53265/

Introduction

Human mesenchymal stem cells (MSCs) are somatic progenitors that can differentiate into the paraxial mesodermal lineages of bone, cartilage, and adipose tissue[1-4], as well as to a few extramesodermal lineages[5]. First isolated from the adult bone marrow, these multilineage progenitors have now been found in numerous tissues[6-8] and, unexpectedly, shown to have strong immunomodulatory properties that appear highly amenable to clinical application[9-12]. Detailed mechanisms involved in the immunomodulatory effects are actively being investigated for effective application on specific disease entities. One of the most straightforward ways to evaluate immunomodulation is by assessing for the suppression of effector leukocyte proliferation[13]. Most effector leukocytes such as T lymphocytes and monocytes proliferate prodigiously when stimulated or activated. Immunomodulatory function can be assessed when suppression of proliferation is evidenced.

Traditionally, effector leukocyte proliferation has been evaluated by detection of [^{3}H] thymidine incorporation into DNA. However, this method has significant drawbacks due to the concerns of radiation and post-use disposal, as well as the complex equipment needed. While there are non-radioactive assays to assess cell proliferation, the carboxyfluorescein succinimidyl ester (CFSE) assay has other advantages such as allowing for identification of specific cellular populations, which is especially useful in co-culture experiments involving multiple cell types. CFSE is a fluorescent cellular dye which can be assessed by flow cytometric analysis. As cells divide, the intensity of this cellular label is decreased proportionally; this not only enables determination of overall cell proliferation but also allows for the assessment on the number of cell divisions up to 8 divisions before the fluorescence becomes difficult to detect against background signal. Moreover, the stability of the fluorescent CFSE allows for *in vivo* tracking of labeled cells such that cells can be visualized up to many months[14].

This assay can also be varied to evaluate specific types of effector leukocytes or the immunomodulatory function of specific populations of MSC-induced immunomodulatory leukocytes-such as interleukin-10 (IL-10) producing CD14$^+$ monocytes[15] by performing magnetic bead surface marker selection of cell populations of interest prior to or after co-culture as appropriate. Our protocol describes the basic assay of assessing the immunomodulatory effects of MSCs on effector leukocytes (flow chart shown in **Figure 1**) and a variation on this basic assay for evaluation of MSC-induced leukocyte immunomodulation on allogeneic CD4$^+$ effector T lymphocytes (flow chart shown in **Figure 4**).

Protocol

Patient informed consent as approved by the institutional review board must be obtained for use of human cells.

1. Density Gradient Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)

1. Add 25 ml heparinized whole blood into a 50 ml tube by a 25 ml pipette.
    1. Dilute cells with 25 ml of phosphate-buffered saline (PBS).
2. Add 15 ml of Ficoll-Paque density gradient into a new 50 ml tube, and while tilting the tube, very slowly and carefully add in 25 ml of the diluted cell suspension over the density gradient so that there is no mixing of the whole blood with the density gradient, *i.e.*, no disturbance of the blood-density gradient interface.
3. Centrifuge the suspension from step 1.2 at 400 × g for 30 to 40 min in a temperature-controlled swinging-bucket rotor without brake at 20 °C.
   Note: Three distinct layers should be apparent after centrifugation: the upper layer being plasma; the bottom clear layer being the Ficoll-Paque density gradient; and a thin, middle cellular layer being the PBMCs
4. Aspirate and discard the upper layer by suction with a Pasteur pipette, with care not to disturb the interphase layer of mononuclear cells (*i.e.,* lymphocytes, monocytes, and platelets).
5. Carefully collect this mononuclear cell layer with a 10 ml pipette to a new 50 ml centrifuge tube.
6. Fill the tube with 20 ml of PBS, mix well, and centrifuge at 300 × g for 10 min at 20°C. Remove the supernatant completely after centrifugation.
7. Resuspend the cell pellet in 20 ml of PBS and centrifuge at 200 × g for 10-15 min at 20 °C. Remove the supernatant completely after centrifugation.
   Note: This step removes the platelets-which are unwanted-within the PBMCs; this step can be repeated to ensure complete removal of the platelets.
8. If no selection of specific populations is desired, resuspend cell pellet in leukocyte complete medium (10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin in RPMI-1640 medium) for culturing after performing a cell count[16] to suspend 1 ml of medium per 10^7 PBMCs before proceeding to the next step.

2. Magnetic Labeling of Leukocyte Populations (Skip to STEP 4 if no Selection of Specific Populations is Required)

1. Centrifuge PBMC suspension at 300 × g for 10 min and aspirate supernatant completely.
2. Resuspend cell pellet in 80 µl of PBS per 10^7 total cells.
3. Add 20 µl of specified magnetic beads (*i.e.,* CD14 magnetic beads for isolation of monocytes, CD4 magnetic beads for isolation of T lymphocytes) per 10^7 total cells.
4. Mix well and incubate for 15 min at 2 to 8 °C in the refrigerator.
5. Add 1-2 ml of PBS per 10^7 cells to wash, and centrifuge at 300 × g for 10 min.
6. Aspirate supernatant completely and resuspend up to 10^8 cells in 500 µl of PBS.

3. Magnetic Selection of Leukocyte Subpopulations

Note: A variety of magnetic bead separators and columns are available for isolation of specific population of leukocytes by cell surface marker from a number of manufacturers. Isolation can be by positive selection based on one or a few positive markers or by negative selection after depletion of unwanted populations. A general approach of performing positive selection using one marker (*i.e.,* CD4 for T helper lymphocytes, or CD14 for monocytes) is outlined below.

1. Prepare and prime magnetic separator including separation column according to manufacturer's instructions.
2. Apply tube containing the sample of labeled PBMCs. Provide two 15 ml centrifuge tubes for collection of the positively labeled and unlabeled cell fractions, following manufacturer's instructions.
3. Count cell number[15] and resuspend the positively selected leukocytes (*i.e.,* $CD4^+$ T lymphocytes or $CD14^+$ cells) in leukocyte complete medium, using 1 ml of medium per 10^7 cells.

4. CFSE Staining of Leukocytes for Assessment of Proliferation

Note: CFSE has been widely used in immunological investigations, for both *in vivo* and *in vitro* studies. Our protocol has been optimized for in vitro study of MSC/PBMC (or other leukocyte) interactions. While the general steps involved in conducting CFSE in vitro labeling are similar, there may be differences in the specific dose and timing of the protocol[14]. This may be due to a number of factors, *i.e.,* the specific cell type used, cell numbers used-which can likely affect the intensity of the CFSE fluorescent signal.

1. Dilute the CFSE stock solution (10 mM) in PBS to the desired working concentration of 10 µM (CFSE-working solution). Prepare 10^7 cells (*i.e.,* PBMCs or T cells) for labeling with the CFSE-working solution.
2. Centrifuge at 300 × g for 10 min to obtain a cell pellet and aspirate the supernatant.
3. Resuspend the cells gently in 1 ml of pre-warmed (37 °C) CFSE-working solution and incubate the cells for 10 min at 37 °C.
4. To wash off excess CFSE, dilute the cell suspension with 10x (by volume) of precooled (4 °C) RPMI medium containing 10% FBS. Sediment the cells by centrifugation at 300 x g for 5 min and discard the supernatant. Wash the cell pellet in this manner twice more.

5. Re-pellet the cells by centrifugation and count cell number[15]. Resuspend 10^7 cells (either PBMCs or T cells) in 1 ml fresh prewarmed leukocyte complete medium.

5. Co-culture of MSCs with Leukocytes and Activation of Leukocytes

1. Pre-warm MSC complete medium (10% FBS (pretested for optimal MSC growth), 1% L-glutamine, and 1% penicillin/streptomycin in DMEM-low glucose medium) to 37 °C for no more than 30 min.
2. Seed MSCs at 50,000 cells in 1 ml of MSC complete medium in 24-well plates (MSC density: 25,000 cells/cm^2) for attachment O/N in a 37 °C incubator, allowing for the stem cells to reach 80% confluence.
3. Aspirate medium, and based on seeded MSC numbers, add CFSE-labeled PBMCs (previously labeled-please refer to protocol Step 4 above) to cultured MSCs (seeded the previous day in 24-well plates) in 1 ml of leukocyte complete medium at a 1:10 (cell ratio) co-culture ratio of MSCs to PBMCs.
4. Add the mitogen phytohemagglutinin (PHA), a non-specific leukocyte activator, to a final concentration of 10 µg/ml in a total volume of 1 ml leukocyte complete medium per well.
    1. Alternatively, to stimulate for the activation of T lymphocytes specifically: use α-CD3/28 microbeads and add to the two-cell co-culture to obtain a bead-to-T lymphocyte ratio of 1:1.
5. For negative control, plate 500,000 PBMCs/well (or a specific effector leukocyte population; cell density: 250,000 leukocytes/cm^2) in a 24-well plate with 1 ml leukocyte complete medium only; for positive control, in addition to plating the same number of PBMCs/well, add PHA to a final concentration of 10 µg/ml.
6. On the 3rd and 5th day of the co-culture experiment, assess proliferation of CFSE-labeled leukocytes (placed in round-bottom tubes) by flow cytometric analysis[17] with 488 nm excitation and emission filters appropriate for fluorescein.
   Note: Intracellular cytokine staining for flow cytometric analysis can also be performed to these CFSE-labeled leukocytes at this point to assess for changes in leukocyte cytokine expression profile as modulated by MSCs. Since CFSE is evaluated with a filter appropriate for fluorescein, the antibodies selected to assess various cytokines need to be conjugated to fluorochromes other than fluorescein or similar spectrum (*i.e.,* phycoerythrin, peridinin chlorophyll protein complex (PerCP)).

6. Variation: Effector Suppression Assay Magnetic Bead-selected, MSC-induced Immunomodulatory Leukocytes on Activated CFSE-labeled Effector $CD4^+$ T Cells

1. Seed MSCs at 250,000 cells in 3 ml of MSC complete medium in 6-well plates (MSC density: 25,000 cells/cm^2) for attachment O/N in a 37 °C incubator, allowing for the stem cells to reach 80% confluence. At least 3 6-well plates are necessary to ensure enough MSC-cocultured PBMCs for subpopulation selection.
2. Aspirate medium, and add PBMCs separated as per Step 1 but in 6-well plates at 2.5 x 10^6 cells/well (cell density: 250,000 leukocytes/cm^2) with 3 ml of leukocyte complete medium. Co-culture for 48-72 hr in a 37 °C incubator.
3. Magnetic bead-select specific population of MSC-induced immunomodulatory leukocytes (*i.e.,* $CD14^+$ cells) as per Sections 2-3.
   Notes: Intracellular cytokine staining for flow cytometric analysis can be performed at this point to assess for changes in leukocyte cytokine expression profile, *i.e.,* expression of interleukin-10-as modulated by MSCs.
4. Add CFSE-labeled allogeneic $CD4^+$ T cells generated as per Sections 2-4 in 24-well plates in 1 ml of leukocyte complete medium (T cell density: 250,000 cells/cm^2) to bead-selected MSC-induced leukocytes at various ratios, *i.e.,* 1:10, 1:5, 1:2, and 1:1 (cell to cell) ratios.
5. To stimulate $CD4^+$ T cells, add α-CD3/28 conjugated microbeads to obtain a bead-to-cell ratio of 1:1.
6. For a negative control, plate 500,000 $CD4^+$ T cells/well in a 24-well plate (T cell density: 250,000 cells/cm^2) with 1 ml leukocyte complete medium only; for positive control, in addition to plating the same number of $CD4^+$ T cells/well, add α-CD3/28 conjugated microbeads to obtain a bead-to-cell ratio of 1:1.
7. On the 3rd day of co-culture, assess proliferation of CFSE-labeled $CD4^+$ T cells (placed in round-bottom tubes) by flow cytometric analysis[16] with 488 nm excitation and emission filters appropriate for fluorescein.

Representative Results

Figure 1 denotes the overall schema of the experiment, and **Figure 2** demonstrates the appearance of the various cell culture conditions as visualized by phase-contrast inverted microscopy. MSCs are adherent cells with a fibroblastic, spindle-shaped morphology, whereas PBMCs and leukocytes are small round non-adherent cells. These two morphologically different cell types can be clearly seen in the co-culture. At the end of the assay, when the PBMCs (or leuckotyes) are aspirated for flow cytometric analyses, even if adherent MSCs are inadvertently included (*i.e.,* due to poor attachment or dislodgement by vigorous aspiration), there should be no problems with assessing the PBMC/leukocyte fraction since these cells would be labeled with CFSE. When there is no proliferation, histogram results for CFSE-labeled cells are seen as one highly positive and sharp peak; however, when activation has occurred, proliferation will be apparent as manifested by multiple smaller peaks with a loss and left-shift of the fluorescence intensity (**Figure 3**). When suppression of proliferation has occurred, *i.e.*, with the co-culture of MSCs, the multiple smaller peaks will decrease with a concomitant increase of fluorescent intensity as evidenced by shifting of the peaks to the right and increases in the sharpness of the right-most peak representing the non-dividing cells. **Figure 4** denotes the overall schema of one variation of the experiment: assessing the effector suppression of MSC-induced immunomodulatory leukocytes on activated allogenic effector $CD4^+$ T cells. The results of such as a variation on the experiment are similar to the original assay, with the additional information of dose-dependent suppression of proliferation seen (**Figure 5**).

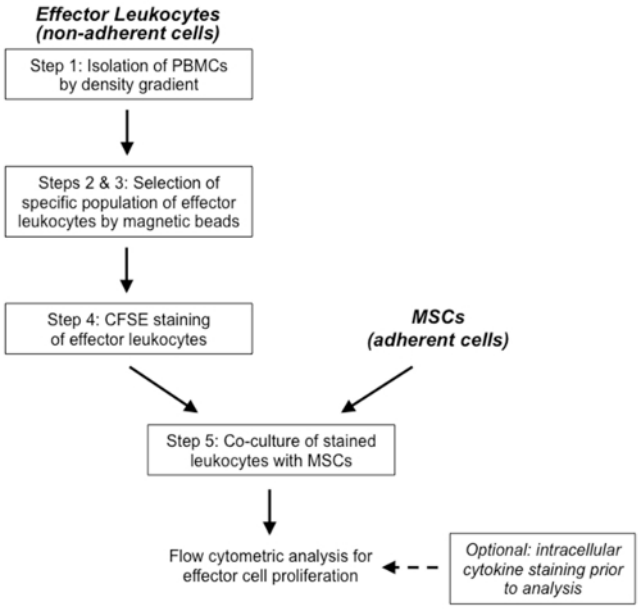

Figure 1. Flow chart for assessment of immunomodulatory effects of mesenchymal stem cells (MSCs) on allogeneic effector leukocytes. Please click here to view a larger version of this figure.

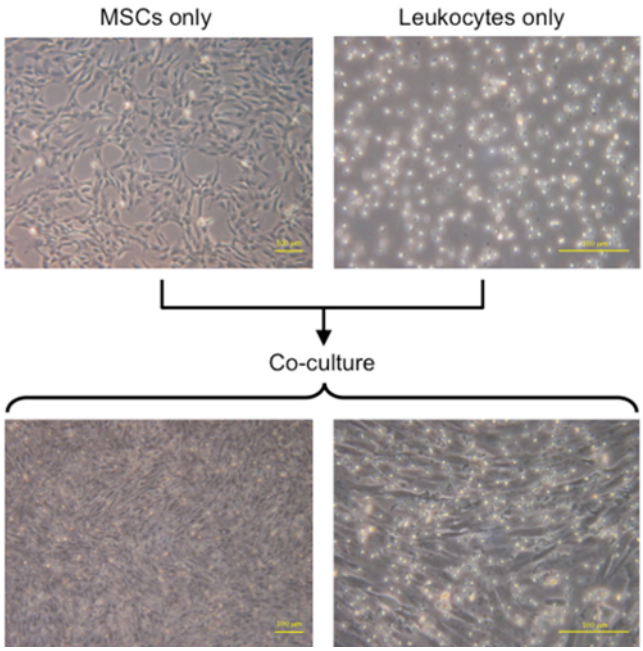

Figure 2. Cell morphology of MSCs, leukocytes, and co-culture of two cell populations. Phase-contrast microscopy photographs of single-cell culture of MSCs or leukocytes, and MSC-leukocyte co-culture. Scale bar, 100 µm. Please click here to view a larger version of this figure.

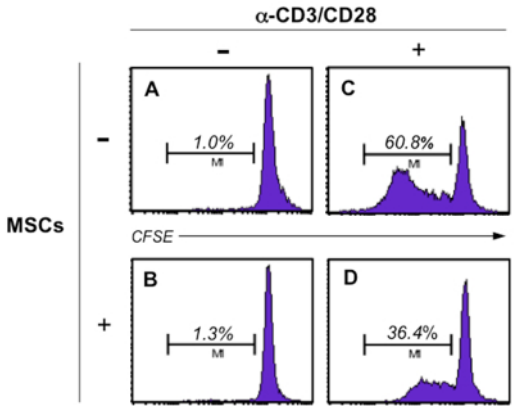

Figure 3. MSC suppressive effects on proliferating leukocytes. Flow cytometric histogram of carboxyfluorescein succinimidyl ester (CFSE)-labeled leukocyte proliferation (**A**) cultured alone unstimulated, (**B**) co-culture with MSCs, (**C**) cultured with anti-CD3/CD28 microbead stimulation (α-CD3/CD28) alone, (**D**) and with MSCs. Percentages shown in histograms denote proportion of proliferating leukocytes. Please click here to view a larger version of this figure.

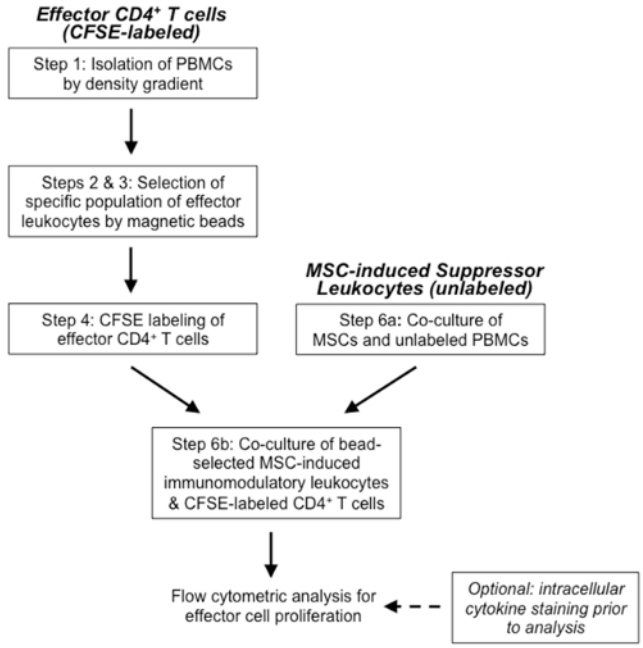

Figure 4. Flow chart for assessment of MSC-induced leukocyte immunomodulation on allogeneic CD4$^+$ effector T lymphocytes. Please click here to view a larger version of this figure.

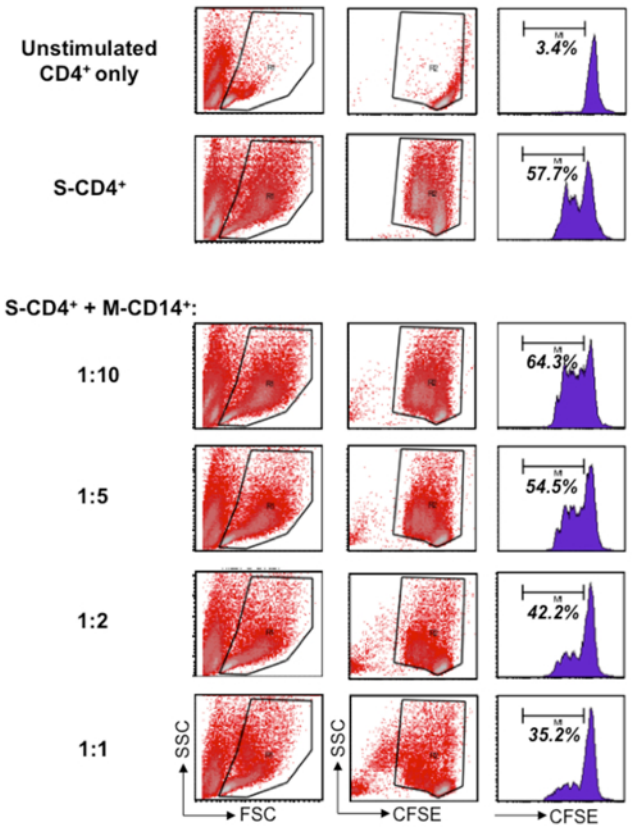

Figure 5. Suppressive capacity of MSC-co-cultured CD14 monocytes on proliferating CD4 lymphocytes. Scatter plot and flow cytometric histogram of the suppressive capacity of MSC-co-cultured $CD14^+$ monocytes (M-$CD14^+$) on anti-CD3/CD28 microbead-stimulated, CFSE-labeled $CD4^+$ effector T lymphocytes (S-$CD4^+$). Co-culture ratio of S-$CD4^+$ to M-$CD14^+$ is shown. $CD4^+$ T lymphocytes were gated (R1) and assessed for CFSE intensity. Percentages shown in histograms denote proportion of proliferating $CD4^+$ T cells. Please click here to view a larger version of this figure.

Discussion

Increasingly, the immunomodulatory properties of MSCs are being translated into clinical use more rapidly than the multilineage capacity of these stem cells[18-20]. Thus, co-culture techniques of MSCs with leukocytes and assays to evaluate immune function are important to further delineate the specific mechanisms involved in these properties for optimizing effective therapeutic application.

One of the most critical technical aspects for success in these assays is having adequate PBMC numbers, which is dependent on the starting cell number in the heparinized whole blood sample as well as PBMC purity after density gradient separation (Step 1.3). Careful layering of whole blood on to the density gradient to ensure that no mixing between the whole blood and density gradient is critical to ensure that erythrocytes and platelets are excluded. Another important consideration is the need for adequate activation of effector leukocytes for robust proliferation, otherwise suppression is difficult to discern. This is dependent not only on the purity but also the quality of starting human leukocytes, which can be affected by variables such as individual differences, preservation procedures, and time from blood draw to experimentation, in addition to technical expertise with the assays. Since the issue of individual differences cannot be resolved, sample numbers-both in terms of the leukocyte and MSC donors, since MSCs are primary isolated cells-must be adequate to ensure validity of results. The number of MSCs applied in the experiment is also critical because at very low MSC numbers, immune activation rather than immunmodulation has been reported to occur[21]. Because these assays involve living cells, all reagents used need to be within expiration dates to ensure full potency and optimal conditions.

One other important aspect which can affect the reproducibility of these assays is the passage number of the MSCs utilized. It has been noted that the mere act of removing MSCs from their physiologic *in vivo* environment and cryopreservation can compromise function, including immunomodulation[22,23]. Increasing reports demonstrate that *in vitro* expansion of MSCs result in senescence of these primary stem cells, and that the differentiation capacity of these stem cells is altered or compromised[22,24]. While currently there are only a few published report with regards to the effects of senescence on MSC immunomodulation[22], caution should be taken when using MSCs which have been passaged for a prolonged period of time based on our own experience as well (data not shown). Senescence is manifested as a lack of proliferation without cell death, and flattened, enlarged morphology (youthful MSCs are fibroblastic in shape). While β-galactosidase staining is the definitive test for establishing senescence, for our purposes performing this stain is not likely to be necessary since the phenotype of cessation of proliferation and morphologic change is very clear and easily assayed. There is no specific passage number by which senescence sets in, since different sources and developmental age of MSCs as well as culturing technique all can contribute to replicative senescence.

For the selection of specific leukocyte populations that can be identified by one positive marker, we have elected to use the magnetic bead selection technique as opposed to fluorescence-activated cell sorting for its relative low cost, simplicity, and speed. However, this technique has its limitations: larger starting cell numbers are necessary and cell loss can occur during the selection process. The magnetic bead selection

process is also not recommended if the leukocyte population to be selected is rare and/or require multiple positive markers for identification; these types of leukocytes would be more optimally selected by the fluorescence-activated cell sorting. One other important limitation to the protocol is its *in vitro* nature, which greatly simplifies the interaction between the two cell types used to allow for mechanistic study and cannot adequately represent *in vivo* process.

In summary, adaptation of leukocyte proliferation experiments to assess MSC immunomodulatory properties is an important "first assay" for such studies. This assay can be flexibly altered at various steps for interrogation of specific populations of leukocytes at both the effector end and the MSC-induction end. Assessment in the alteration of cytokine protein expression profile is also possible. Thus, this assay is a highly useful tool in the armamentarium to elucidate mechanisms of MSC-leukocyte interactions in an *in vitro* setting.

Disclosures

We have nothing to disclose.

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References

1. Friedenstein, A. J. Precursor cells of mechanocytes. *Int Rev Cytol.* **47**, 327-359 (1976).
2. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science.* **284**, 143-147 (1999).
3. Prockop, D. J. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science.* **276**, 71-74 (1997).
4. Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* **8**, 315-317 (2006).
5. Engler, A. J., Sen, S., Sweeney, H. L., & Discher, D. E. Matrix elasticity directs stem cell lineage specification. *Cell.* **126**, 677-689 (2006).
6. Zuk, P. A. *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* **7**, 211-228 (2001).
7. Yen, B. L. *et al.* Isolation of multipotent cells from human term placenta. *Stem Cells.* **23**, 3-9 (2005).
8. Erices, A., Conget, P., & Minguell, J. J. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol.* **109**, 235-242 (2000).
9. Bartholomew, A. *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol.* **30**, 42-48 (2002).
10. Chang, C. J. *et al.* Placenta-derived multipotent cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-gamma. *Stem Cells.* **24**, 2466-2477 (2006).
11. Uccelli, A., Moretta, L., & Pistoia, V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol.* **8**, 726-736, (2008).
12. Chen, P. M., Yen, M. L., Liu, K. J., Sytwu, H. K., & Yen, B. L. Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. *J Biomed Sci.* **18**, 49-59 (2011).
13. Muul, L. M. *et al.* Measurement of proliferative responses of cultured lymphocytes. *Curr Protoc Immunol.* Ch. 7, Unit 7.10, Wiley & Sons, Inc. (2011).
14. Quah, B.J., Warren, H.S., & Parish, C.R. Monitoring lymphocyte proliferation *in vitro* and *in vivo* with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc.* **2**, 2049-2056 (2007).
15. Chen, P. M. *et al.* Induction of immunomodulatory monocytes by human mesenchymal stem cell-derived hepatocyte growth factor through ERK1/2. *J Leukoc Biol.* **96**, 295-303 (2014).
16. Oughton, J. A., & Kerkvliet, N. I. UNIT 18.8 Immune cell phenotyping using flow cytometry. *Curr Protoc Toxicol.* (eds L.G. Costa, J.C. Davila, D.A. Lawrence, D.J. Reed, and Y.Will) Ch. 23:18.8:18.8.1-18.8.24 Wiley & Sons, Inc., (2005).
17. Phelan, M. C., & Lawler, G. APPENDIX 3A Cell counting. *Curr Protoc Cytometry.* (eds J.P. Robinson et al) Ch. 00:3A:A.3A.1-A.3A.4 Wiley & Sons, Inc., (2001).
18. Gebler, A., Zabel, O., & Seliger, B. The immunomodulatory capacity of mesenchymal stem cells. *Trends Mol Med.* **18**, 128-134 (2012).
19. Le Blanc, K. *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet.* **371**, 1579-1586 (2008).
20. Tan, J. *et al.* Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA.* **307**, 1169-1177 (2012).
21. Le Blanc, K. *et al.* Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol.* **57**, 11-20 (2003).
22. Li, X.Y. *et al.* Long-term culture *in vitro* impairs the immunosuppressive activity of mesenchymal stem cells on T cells. *Mol Med Rep.* **6**, 1183-1189. (2012).
23. Moll, G. *et al.* Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem Cells.* **32**, 2430-2442 (2012).
24. Ho, P. J., Yen, M. L., Tang, B. C., Chen, C. T., & Yen, B. L. H2O2 accumulation mediates differentiation capacity alteration, but not proliferative decline, in senescent human fetal mesenchymal stem cells. *Antioxid Redox Signal.* **18**, 1895-1905 (2013).

