Effective licensing of human mesenchymal stem cells

Background
Mesenchymal stem cells (MSCs) are fibroblast-like plastic-adherent cells that can be isolated from a variety of tissues, such as bone marrow or adipose tissue. Bone marrow–derived MSCs are capable of suppressing T cell proliferation. However, the suppression potential of MSCs varies among in vitro expanded cells and shows donor-dependent differences. MSCs can be “licensed” by inflammatory cytokines such as IFN-γ and TNF-α to become more immunosuppressive and show a more homogeneous phenotype in this regard. Licensing defines the multistep process that leads to the functional maturation of MSCs. This application note describes the licensing of MSCs using IFN-γ and TNF-α and a procedure to analyze marker expression and the immunosuppressive characteristics of MSCs using the MSC Suppression Inspector.

Materials

Materials for cultivation and licensing of MSCs
- StemMACS™ MSC Expansion Media Kit XF (# 130-104-182)
- Human TNF-α, premium grade (# 130-094-014)
- Human IFN-γ1b, premium grade (# 130-096-481)

Materials for suppression assay
- MSC Suppression Inspector, human (# 130-096-207)
- CD4⁺CD25⁺ Regulatory T Cell Isolation Kit, human (# 130-091-301) or CD4⁺CD25⁺CD127⁻ Regulatory T Cell Isolation Kit II, human (# 130-094-775)
- Cell culture medium: RPMI 1640 supplemented with 10% AB serum
- Fluorescent cell-tracking dye for flow cytometry
- 96-well culture plates (flat bottom)

Instruments
- MACSQuant® Analyzer 10 (# 130-096-343)
- CO₂ incubator, 37 °C with 5% CO₂ in air and >95% humidity
- Centrifuge
- Microscope
- Water bath (37 °C)
- Orbital shaker with temperature control
- Laminar flow hood (biohazard containment hood)

Experimental procedure

General notes
The StemMACS MSC Expansion Media Kit XF is a serum- and xeno-free medium optimized and standardized for the reproducible and reliable expansion of human MSCs. A complete instruction of use as well as detailed protocols for primary culture, culture expansion, and passaging of MSCs from different tissues are available for download. Simply click on the hyperlinks in boldface.

This procedure involves a flow cytometry–based suppression assay. A detailed protocol for a tritium-based suppression assay is described in the data sheet of the MSC Suppression Inspector.

All steps in the protocol have to be performed under aseptic conditions.
Suppression assay

MSC expansion (day –6)
MSCs are expanded as indicated in the data sheet for StemMACS™MSC Expansion Media Kit XF.

MSC licensing (day –3)
When MSCs reach 70% confluency, medium is replaced with fresh StemMACS MSC Expansion Media XF with or without 1,200 IU/mL IFN-γ and 1,000 IU/mL TNF-α (both from Miltenyi Biotec; the lot-specific activity is provided in the corresponding certificate of analysis).

Cell surface marker analysis (day 0)
Licensed (TNF/IFN-MSC) or untreated (UT-MSC) MSCs are analyzed for cell surface marker expression involved in immunomodulation. To this end, MSCs are harvested using a trypsin-based method. Cells are stained with the antibodies CD40-APC, Anti-HLA-ABC-VioBlue®, Anti-HLA-DR, DP, DQ-FITC (all Miltenyi Biotec), CD274 (PD-L1)-APC (BD™ Biosciences), and IDO-PE (eBioscience) and analyzed on the MACSQuant® Analyzer 10 using MACSQuantify™ Software.

Suppression assay (day 0)
Harvested MSCs are analyzed for their capacity to suppress T cell proliferation. The suppression assay is performed by coculturing CD4+CD25+ or CD4+ responder T (Tresp) cells and MSCs at ratios of 1:1 to 10:1. T cell proliferation is induced optimally by adding the MSC Suppression Inspector. This stimulation reagent consists of cell-sized Anti-Biotin MACSISbead™ Particles that are pre-loaded with biotinylated CD2, CD3, and CD28 antibodies and thus mimic antigen-presenting cells. As a control, Tresp cells are cultured alone with or without MSC Suppression Inspector. Prior to the suppression assay, Tresp cells are labeled with a fluorescent cell-tracking dye, and after 5 days of coculture, Tresp cell proliferation is determined by flow cytometry measuring dye dilution.

1.MSCs are resuspended in cell culture medium and diluted into a flat-bottom 96-well culture plate at 1×10⁵ cells/well.
2. The 96-well plate is placed into an incubator at 37 °C and 5–7% CO₂ until Tresp cells are added.

3. CD4+CD25+ or CD4+ Tresp cells are isolated under aseptic conditions, e.g., with the CD4+CD25+ Regulatory T Cell Isolation Kit, human.
4. Tresp cells are labeled using a fluorescent cell-tracking dye.
5. Labeled Tresp cells are added to MSCs in the 96-well plate at different ratios (e.g. 1:1, 5:1, 10:1).
6. MSC Suppression Inspector is prepared as indicated in the data sheet.
7. The MSC Suppression Inspector is resuspended thoroughly and added to the wells (bead-to-Tresp cell ratio 1:1). A detailed pipetting scheme is provided in the data sheet.
8. The total volume in each well is adjusted to 210 μL with culture medium.
9. Plates are incubated at 37 °C and 5–7% CO₂ for 5 days.
10. After 5 days of MSC/Tresp cell coculture, the fluorescence intensity of the cell-tracking dye is measured by flow cytometry using an instrument equipped with a 405 nm laser, such as the MACSQuant Analyzer 10.

Results

Marker expression patterns of untreated and licensed MSCs
MSCs were licensed using TNF-α and IFN-γ according to this protocol. After 72 h, MSCs were analyzed for cell surface marker expression (fig. 2). Flow cytometry analysis confirmed the absence of HLA-DR (MHC class II) and PD-L1 on untreated MSCs. The corresponding isotype served as a control. Both markers were up-regulated upon treatment with IFN-γ and TNF-α (TNF/IFN-MSC). Untreated MSCs expressed low levels of the markers CD40, HLA-ABC (MHC class I), and IDO, all of which were induced after licensing. Up-regulation of IDO is one of the key events during MSC licensing as IDO is an important molecule contributing to suppression of T cell proliferation.

![Figure 1: Outline of the licensing and suppression assay protocol. MSCs are maintained under xeno-free conditions in StemMACS MSC Expansion Media XF starting at day –6. Licensing of MSCs is initiated at day –3 using TNF-α and IFN-γ. The suppression assay, i.e., coculture with stimulated T cells starts at day 0.](image1)

![Figure 2: Expression of CD40, CD271 (PD-L1), HLA-ABC, HLA-DR, and IDO by MSCs under different culture conditions. MSCs were either left untreated (UT-MSC) or licensed (TNF/IFN-MSC) for 3 days. Cells were then labeled with the indicated antibodies and analyzed by flow cytometry. Results from a representative experiment are shown.](image2)
**Conclusion**

This MSC licensing procedure, relying on a multitude of MACS® Products, supports:

- Optimal licensing of MSCs after expansion with StemMACS™ MSC Expansion Media Kit XF
- Comprehensive marker expression analysis by flow cytometry
- Functional characterization of human MSCs by *in vitro* suppression assay

**References**


**MACS Product** | **Order no.**
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StemMACS MSC Expansion Media Kit XF, human | 130-104-182
Human TNF-α, premium grade | 130-094-014
Human IFN-γ1b, premium grade | 130-096-481
MSC Suppression Inspector, human | 130-096-207
CD4+CD25+ Regulatory T Cell Isolation Kit, human or CD4+CD25+CD127dim/– Regulatory T Cell Isolation kit II, human | 130-091-301 or 130-094-775
CD40-APC | 130-094-137
Anti-HLA-ABC-VioBlue | 130-101-459
Anti-HLA-DR, DP, DQ-FITC | 130-104-826
Mouse IgG1-APC | 130-098-846
Mouse IgG1-PE | 130-098-845
REA Control (I)-FITC | 130-104-611
REA Control (I)-VioBlue | 130-108-346
MACSQuant Analyzer 10 | 130-096-343

**Capacity of untreated and licensed MSCs to suppress T cell proliferation**

In addition to phenotypic analysis, licensed MSCs were examined for their capacity to suppress proliferation of activated T cells after 5 days of coculture. Stimulation with the MSC Suppression Inspector induced effective T cell proliferation. Addition of untreated MSCs (UT-MSC; MSC 1–4) at a ratio of 1:1 suppressed T cell proliferation slightly. Suppression was enhanced after licensing MSCs (TNF/INF-MSC; MSC 1–4) with TNF-α and IFN-γ (fig. 3).

**Figure 3:** Effect of licensing on the capacity of MSCs to suppress T cell proliferation. Tresp cells alone were either cultured in the absence (non-stimulated) or presence (stimulated) of MSC Suppression Inspector. Stimulated Tresp cells were cocultured with four different preparations of untreated MSCs (UT-MSC) or licensed MSCs (TNF/INF-MSC). After 5 days the proliferation of Tresp cells was analyzed by measuring dye dilution through flow cytometry. (A) The median of the mean fluorescence intensity (MFI) of the cell-tracking dye is shown (n = 3). (B) Flow cytometry analysis is exemplified for a representative donor.