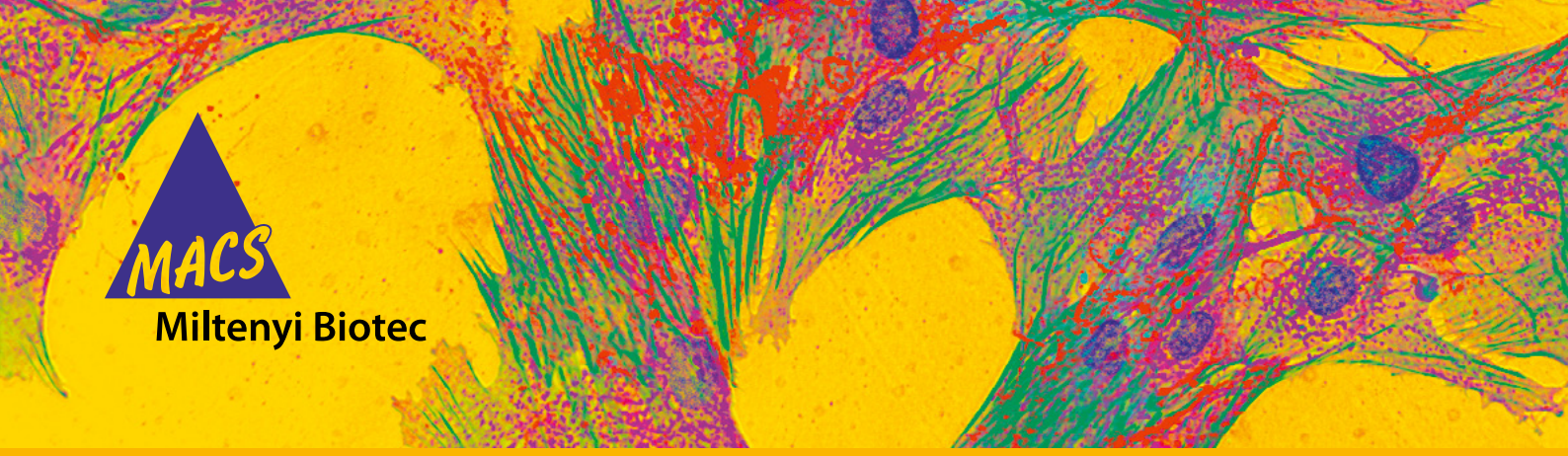

Miltenyi Biotec

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.[1,2] 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 IFN-γ and TNF-α to become more immunosuppressive and show a more homogeneous phenotype in this regard.[3] Licensing defines the multistep process that leads to the functional maturation of MSCs.[3] 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 $CD4^+CD25^+CD127^{dim/-}$ 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)
- CO_2 incubator, 37 °C with 5% CO_2 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.

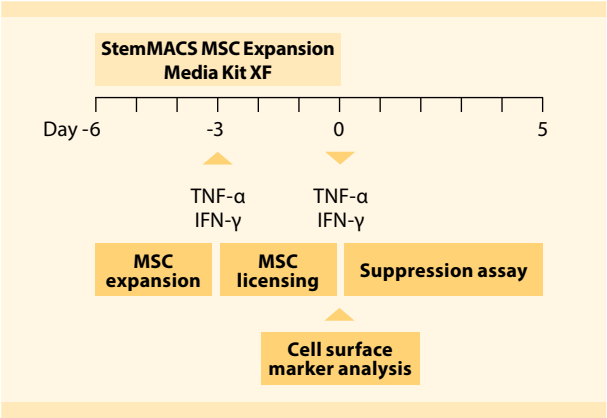

Figure 1: Outline of the licensing and suppression assay protocol. MSCs are maintained under xeno-free conditions in StemMACS MSC Expansion Media XF starting at day –6. Licensing of MSCs is initiated at day –3 using TNF-α and IFN-γ. The suppression assay, i.e., coculture with stimulated T cells starts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

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 MACSiBead™ 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 1×10^5 cells/well.
2. The 96-well plate is placed into an incubator at 37 °C and 5–7% CO_2 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% CO_2 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.

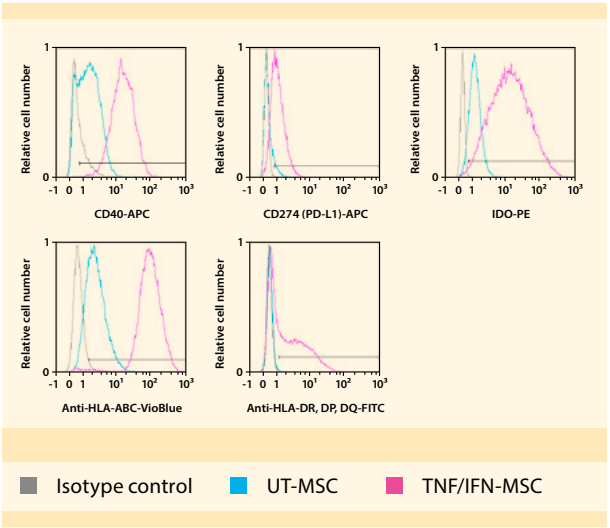

Figure 2: Expression of CD40, CD271 (PD-L1), HLA-ABC, HLA-DR, and IDO by MSCs under different culture conditions. MSCs were either left untreated (UT-MSC) or licensed (TNF/IFN-MSC) for 3 days. Cells were then labeled with the indicated antibodies and analyzed by flow cytometry. Results from a representative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

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).

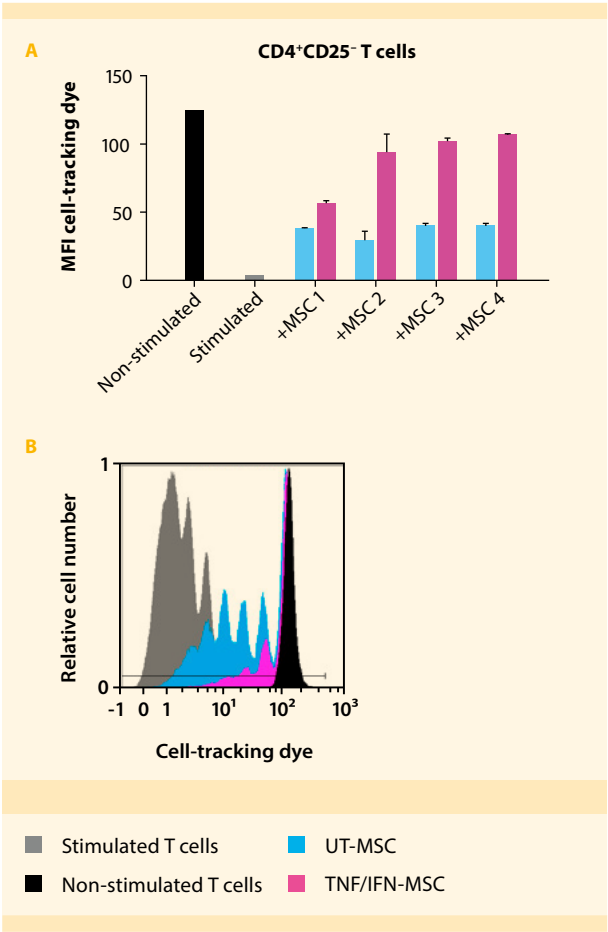

Figure 3: Effect of licensing on the capacity of MSCs to suppress T cell proliferation. Tresp cells alone were either cultured in the absence (non-stimulated) or presence (stimulated) of MSC Suppression Inspector. Stimulated Tresp cells were cocultured with four different preparations of untreated MSCs (UT-MSC) or licensed MSCs (TNF/IFN-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.

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

1. Di Nicola, M. *et al.* (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99: 3838–3843.
2. Bartholomew, A. *et al.* (2002) Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. Exp. Hematol. 30: 42–48.
3. Krampera, M. *et al.* (2011) Mesenchymal stromal cell 'licensing': a multistep process. Leukemia 25: 1408–1414.

MACS Product	Order no.
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 $CD4^+CD25^+CD127^{dim/-}$ 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

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