Original Article

The Quality Control of Mesenchymal Stromal Cells by *in Vitro* Testing of Their Immunomodulatory Effect on Allogeneic Lymphocytes

(mesenchymal stromal cells / allogeneic / immunosuppression / GVHD)

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Abstract. Mesenchymal stromal cells (MSC) represent a promising treatment of graft-versus-host disease (GVHD) in patients after allogeneic haematopoietic stem cell transplantation. We performed co-cultivation experiments with non-specifically stimulated lymphocytes to characterize the immunosuppressive activity of MSC. MSC influenced expression of some activation antigens. CD25 expression was lower with MSC and reached 55.2 % vs. 84.9 % (CD4^{+}, P = 0.0006) and 38.8 % vs. 86.6 % (CD8^{+}, P = 0.0003) on day +4. Conversely, CD69 antigen expression remained higher with MSC (73.3 % vs. 56.8 %, P = 0.0009; 59.5 % vs. 49.7 %, ns) and its down-regulation along with the culture time was less pronounced. MSC reduced proliferation of the stimulated lymphocytes. The cell percentages detected in daughter generations were decreased (32.82 % vs. 10.68 % in generation 4, P = 0.0004 and 29.85 % vs. 10.09 % in generation 5, P = 0.0008), resulting in a lower proliferation index with MSC (1.84 vs. 3.65, P < 0.0001). The addition of MSC affected expression of some cytokines. Production of pro-inflammatory cytokines was decreased: IL-6 (19.5 vs. 16.3 MFI; P < 0.0001 in CD3^{+}/CD4^{+} and 14.5 vs. 13.2 MFI; P = 0.0128 in CD3^{+}/CD8^{+}), IFN-γ (13.5 vs. 12.0 MFI; P = 0.0096 in CD3^{+}/CD4^{+}). Expression of anti-inflammatory IL-10 was only slightly increased after the addition of MSC (ns). The analysis confirmed the immunomodulatory activity of MSC. The functional tests have proved to be an important part of the quality control of the advanced therapy cellular product intended for GVHD treatment. Future research should focus on the interaction between MSC and the patient immune environment more closely.

Abbreviations: ATMP – advanced therapy medicinal products, BM – bone marrow, CCM – complete culture medium, CFSE – carboxyfluorescein succinimidyl ester, GVHD – graft-versus-host disease, HBSS – Hank's balanced salt solution, LSM – lymphocyte separation medium, MEM – minimum essential medium, MFI – mean intensity of fluorescence, MHC – major histocompatibility complex, MSC – mesenchymal stromal cells, PBMC – peripheral blood mononuclear cells, PBS – phosphate-buffered saline, PHA – phytohaemagglutinin, pHPL – pooled human platelet lysate, STAT – signal transducer and activator of transcription.

Introduction

Human mesenchymal stromal cells (MSC) are a population of multilineage progenitor cells with the ability to differentiate into multiple mesenchymal lineages including chondrocytes, osteoblasts and adipocytes (Abdallah and Kassem, 2008). These non-haematopoietic stem cells can be found, isolated and expanded from many tissues including bone marrow, umbilical cord blood or adipose tissue. The immunophenotype of MSC is dependent on their source and cultivation conditions. MSC are distinguishable from haematopoietic cells by being negative for the surface leukocyte markers CD45, CD34, CD19, CD3, HLA-DR, but express CD105, CD73, CD90 (Dominici et al., 2006).

MSC do not express class II human histocompatibility antigens or costimulatory molecules (e.g. CD80, CD86). The effects of MSC on T cells are independent of HLA matching between MSC and lymphocytes; therefore, MSC can be administered repeatedly without

provoking an immunologic response in the HLA-incompatible recipient (Sundin et al., 2009).

MSC are able to interact with the cells of innate and adaptive immunity and influence secretion of certain cytokines. They can move the cytokine profile of dendritic cells, naive and activated T lymphocytes and NK cells to the anti-inflammatory or tolerant phenotype. MSC reduce secretion of IFN-γ in Th1 cells and increase expression of IL-4 in Th2 cells when cultured with these lymphocyte subpopulations. Immature dendritic cells and Tregs increase expression of IL-10 in the presence of MSC, whereas mature dendritic cells reduce production of TNF-α and IL-12 (Aggarwal and Pittenger, 2005; Le Blanc and Pittenger, 2005).

The MSC-mediated immunosuppression acts mainly through secretion of soluble molecules that are produced or upregulated following interaction between the immune cells and MSC. MSC can inhibit proliferation of T lymphocytes with production of indoleamine 2,3-dioxygenase, which catalyses conversion of tryptophan to kynurenin and reduces the T-cell answer through depletion of tryptophan and accumulation of its toxic metabolites. MSC express the enzyme cyclooxygenase, which increases production of prostaglandin E2 and induces regulatory T lymphocytes (Aggarwal and Pittenger, 2005; Le Blanc and Ringdén, 2007). Activated MSC can also produce other molecules with the capability to reduce the activity of immunocompetent cells, such as IL-6, nitric oxide, TGF-β. Therefore, immune regulation mediated by MSC is the result of the cumulative action displayed by several molecules and cell types.

Human MSC suppress lymphocyte alloreactivity *in vitro* in mixed lymphocyte reaction assays. The antigen-specific or mitogen-induced non-specific lymphocyte proliferation is significantly reduced in the presence of MSC. The reduction of reactivity of T lymphocytes is non-selective and touches naive and memory T cells as well as CD4+ and CD8+ subpopulations (Tse et al., 2003; Ramasamy et al., 2008). The suppression is independent of the major histocompatibility complex (MHC) and can be mediated through allogeneic or autologous MSC (Le Blanc et al., 2003). The inhibition of T-lymphocyte proliferation is mediated by arresting them in G0/G1 phase of the cell cycle (Glennie et al., 2005). The expression of early activation markers of T cells, notably CD25 and CD69, can be altered by MSC (Le Blanc et al., 2004). The immunosuppressive effect of MSC arises as a consequence of both anti-proliferative activity and the ability to affect T-cell activation.

A number of studies indicate that MSC possess an immunosuppressive function both *in vitro* and *in vivo*. The immunomodulative properties of MSC predetermine them for affecting the immune response in many diseases that are associated with alloreactive immunity or autoimmunity. MSC are an attractive candidate as a potential cellular therapy for the treatment of severe graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. GVHD represents a significant cause of morbidity and mortality after stem cell transplantation. This cellular therapy could be of great clinical importance as it may ameliorate the symptoms of the GVHD refractory to the standard corticosteroid-based immunosuppression (Le Blanc et al., 2008; Ringdén and Keating, 2011).

From a regulatory perspective, all MSC-based products in the European Union are classified as advanced therapy medicinal products (ATMP). The culture process corresponds to "substantial manipulation" and the derived cells are qualified as an active substance of a medicinal product. The cell characterization and the product release criteria cover a complex testing, including the potency analysis. The potency assay represents quantitative measurement of the biological activity based on the attribute of the product, which is linked to the relevant biological properties and expected clinical response.

In our study, we tested the immunomodulatory properties of MSC, which were prepared under a clinical study of GVHD treatment. The repeated co-cultivation experiments were used to observe the changes in the proliferation rate, activation and cytokine production in non-specifically stimulated allogeneic lymphocytes after the addition of MSC. The goal of the study was to assess the capacity of MSC to modulate activation and proliferation of T-cell subsets and to prove the functional activity of MSC, which is essential for their clinical application in the treatment of GVHD. We tried to validate this "functional testing" as a suitable method for the quality control of MSC products.

Material and Methods

MSC isolation and cultivation

MSC were isolated from bone marrow (BM) aspirates obtained from healthy voluntary donors of the Czech National Marrow Donor Registry. Donors were enrolled for the purpose of unrelated allogeneic stem cell transplantation. The BM grafts were collected from the posterior iliac crest under general anaesthesia. All donors provided written informed consent for MSC donation. About 10 to 20 ml of the BM aspirate was diluted 1 : 1 with HBSS (PAA, Linz, Austria) and layered over LSM 1077 solution (PAA). Mononuclear cells were isolated by gradient centrifugation, washed and resuspended in 1 ml of PBS (PAA). All cells were then placed into a 175 cm² culture flask (Corning, NY) containing 30 ml of pre-warmed complete culture medium – CCM (αMEM, PAA; heparin, Biochrom, Berlin, Germany; 10% pooled platelet lysate – pHPL, local source) and cultivated at 37 °C and 5% CO_2. After 48 h, the medium with non-adherent cells was removed. The remaining cells were washed with PBS and fed with fresh medium. The medium was changed every 3–4 days. After reaching 80 % confluence the cells were detached with TrypLE Select solution (Gibco, Grand Island, NY) and passaged at concentration $1 \times 10^6/175$

cm^2 flask. The MSC from the 2nd to 4th passage were used for the co-cultivation experiments. The MSC were tested for viability and for characteristic expression of positive or negative markers (CD45 – Becton Dickinson, San Diego, CA; CD34, HLA-DR, CD13, CD14 – Immunotech, Boston, MA; CD73, CD90 – Biolegend, San Diego, CA; CD19, CD105 – Exbio, Vestec, Czech Republic). The purity of MSC exceeded 90 %. Expression of all positive antigens (CD73, CD90, CD105, CD103) was over 90 % and all negative antigens (HLA-DR, CD14, CD19, CD34, CD45) below 10 %.

The differentiation capacity of MSC was tested during the validation of the cultivation protocol. The cells (from 4th passage) were seeded in 6-well plates and cultivated for two days in standard medium. Then the differentiation media for adipogenesis, chondrogenesis and osteogenesis were applied (StemPro, Invitrogen, Carlsbad, CA) and cells were further cultivated for 14 days. Finally, staining with Oil Red O, Toluidine Blue O and Alizarin Red S was performed (Sigma, St. Louis, MO).

Preparation of lymphocyte samples

Peripheral blood mononuclear cells (PBMC) used for co-cultivation experiments were isolated from healthy donors. Donors were not HLA compatible with the donors of MSC. The donors were typed at low resolution using commercial PCR-SSO kits (LIFECODES HLA-SSO Typing kits (Immucor, Norcross, GA) for use with Luminex® (Gen-Probe, Stamford, CT)). PCR-SSO typing was performed for HLA-A*, HLA-B* and HLA-DRB1* loci. PBMC were isolated by gradient centrifugation (Histopaque – 1077, Sigma), washed with the cultivation medium (RPMI 1640, Lonza, Verviers, Belgium) and diluted to the final concentration of 1×10^6 cells/ml.

Lymphocyte activation and proliferation analysis

Proliferation of the stimulated PBMC and expression of activation markers on the lymphocytes were evaluated in 20 co-cultivation experiments. The mixed cultures were carried out in 5 ml tissue culture tubes (TPP, Trasadingen, Switzerland), the final volume of cell suspension was 2 ml. Proliferation of PBMC was measured by the use of CFSE (carboxyfluorescein succinimidyl ester) tracking. After staining with CFSE (Molecular Probes, Inc., Eugene, OR), 2×10^6 cells were stimulated with 10 μl of phytohemagglutinin (PHA; 1 μg/μl; PAA) and cultivated with or without MSC (MSC/lymphocyte ratio 1 : 2) at 37 °C and 5% CO_2. The parent and successive populations were measured after four days of cultivation and analysed with ModFit LT software (Verity Software House, Topsham, ME).

The level of lymphocyte activation antigen expression was tested on days 2 (48 h), 3 (72 h) and 4 (96 h). Each test consisted of three tubes: 2×10^6 of stimulated PBMC only (activation control); 2×10^6 of non-stimulated PBMC plus 1×10^6 MSC (1 : 2, control of stimulation via MSC) and stimulated PBMC plus MSC. PBMC were stimulated non-specifically with phytohaemagglutinin (10 μg/2×10^6 lymphocytes). The cells were cultivated in RPMI 1640 medium (Lonza) supplemented with 10 % pHPL and cultivated at 37 °C and 5% CO_2 for four days. About 200 μl of the cell suspension was taken from the cultivation tube and mixed with the antibody cocktail (CD45 – Becton Dickinson; CD19, HLA-DR – Immunotech; CD3, CD4, CD25, CD69 – Exbio). The cells were washed after the incubation and analysed immediately. All analyses were performed in a FACS Canto II flow cytometer (Becton Dickinson) and with FlowJo software (Tree Star, Ashland, OR).

Evaluation of cytokine production

We further evaluated production of certain cytokines in another set of 25 co-cultivation experiments. PBMC were diluted to a final concentration of 1×10^6 cells/ml, and 10 μl of antibody-based stimulation reagent (Cyto-Stim, Miltenyi Biotec, Bergish Gladbach, Germany) and 4 μl of monensin (intracellular protein transport inhibitor, Cytodetect kit, IQ Product, Groningen, Netherlands) were added according to the manufacturer's instructions. Then, the cells were incubated with or without the addition of MSC (1 : 2 ratio) for 6 h at 37 °C and 5% CO_2. Fixation with 1% paraformaldehyde and staining for the surface markers CD45, CD4, CD8 followed. Finally, the cells were permeabilized with saponin (Cytodetect kit) and the intracellular cytokines were stained (IL-6, IL-10, IFN-γ; Becton Dickinson). Data were acquired in a FC 500 flow cytometer, with Kaluza software (Beckman Coulter, Brea, CA), and expressed as mean intensities of fluorescence (MFI).

Evaluation of phosphorylated proteins of the STAT family

The identical set of 25 co-cultivation experiments was used for detection of phosphorylated signal transducer and activator of transcription (STAT) proteins. PBMC at a concentration of 1×10^6 cells/ml were incubated with 10 μl of CytoStim (Miltenyi Biotec), again with or without MSC for 24 h at 37 °C and 5% CO_2. The staining was performed after cell membrane permeabilization and inhibition of phosphorylation enzymes. The cells were fixed with 1.5% paraformaldehyde for 10 min, centrifuged and resuspended in 100% cold methanol (Sigma-Aldrich, St. Louis, MO) and incubated for 30 min at 5 °C in the dark. Then the cell wash was repeated, cells were stained with monoclonal antibodies against antigens CD3, STAT1, STAT3, STAT4, STAT6 (Becton Dickinson) and incubated for 30 min in the dark. After one wash step the cells were analysed in a FC 500 flow cytometer, with Kaluza software (Beckman Coulter), and mean fluorescence intensities were acquired.

Statistical methods

The mean, median, SD, variations, minimum, maximum and other basic statistical measurements were

computed for all the parameters. The Wilcoxon paired test (non-parametric ANOVA) was used for comparison of daughter populations in the CFSE tracking assay, and for comparison of the expression of cytokines, STAT proteins and activation antigens on the cultured lymphocytes. The kinetics of activation antigens was analysed by Friedman ANOVA. A P value equal to or lower than 0.05 was taken as statistically significant. Software Statistica 98 Edition (StatSoft, Inc., Tulsa, OK) was used for the analysis.

Results

Activation of lymphocytes

Twenty experiments were performed in our study of activation markers. Non-specifically stimulated PBMC were co-cultivated with MSC for four days and the expression of activation antigens CD25, CD69 and HLA-DR on lymphocytes was observed on days +2 to +4.

The expression of CD25 increased during the observed period on both $CD4^+$ and $CD8^+$ stimulated lymphocytes (P = 0.00077 and P = 0.00001). Conversely, no significant changes of CD25 kinetics were observed in the tests with addition of MSC (ns). The CD25 expression was about 20–30 % lower with the MSC and reached 55.2 % vs. 84.9 % (CD4+ lymphocytes, P = 0.0006) and 38.8 % vs. 86.6 % (CD8+ lymphocytes, P = 0.0003) on day +4. The addition of MSC caused downregulation of CD25 in all tests, see Fig. 1A, C and Table 1.

The CD69 antigen expression showed a slightly different characteristic. The maximum expression was detected on day +2 followed by a decrease, irrespective of the MSC presence. The level of CD69 expression on $CD4^+$ cells was similar in the tests with and without MSC on day +2 (81.9 % vs. 87.6 %, ns). Thereafter, the lymphocytes co-cultivated with MSC showed a slower decline of CD69 expression, resulting in higher percentages on day +4 (73.3 % vs. 56.8 %, P = 0.0009), Fig. 1B. A similar trend was also observed for $CD8^+$ lympho-
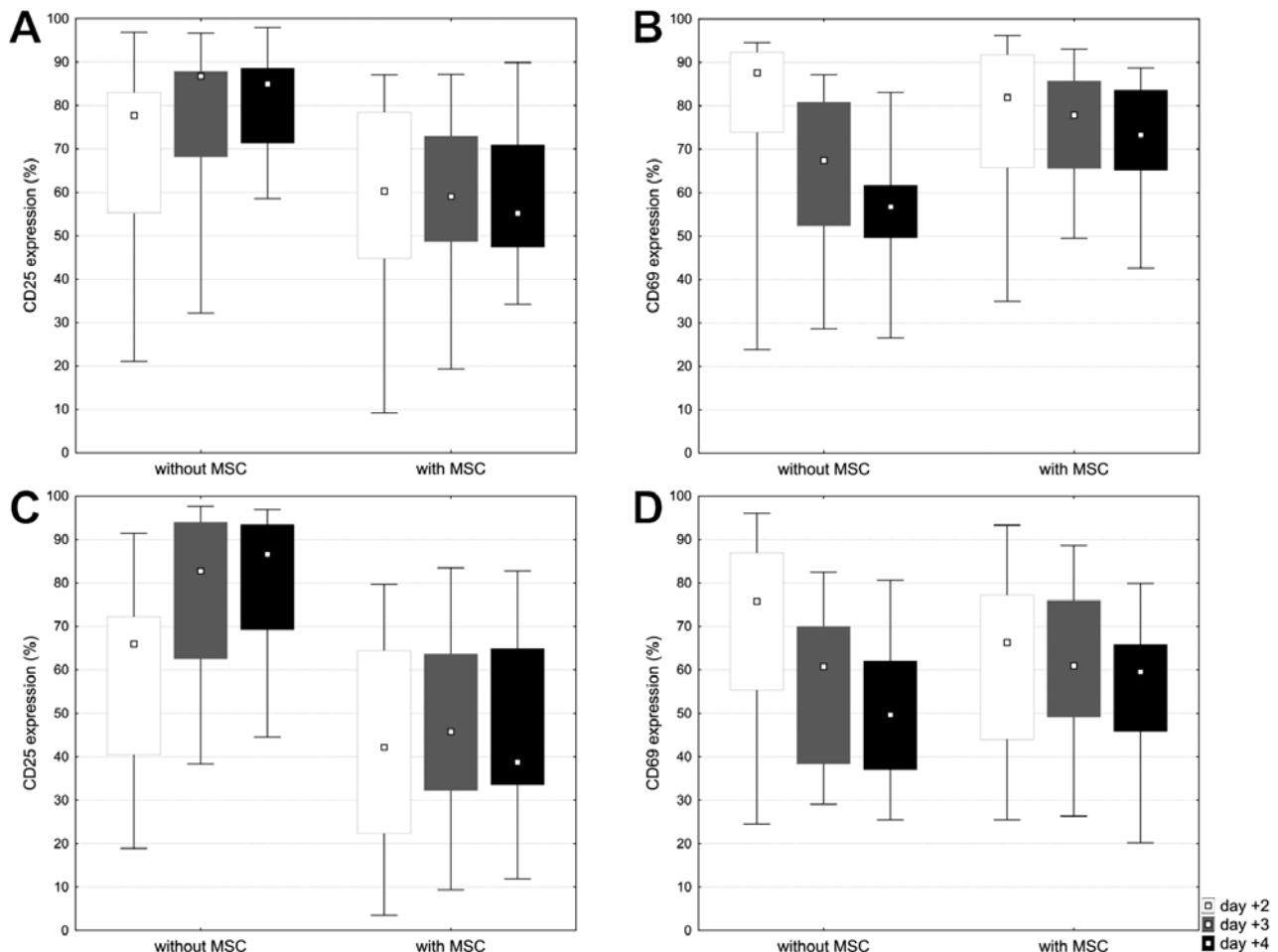

Fig. 1. Comparison of CD25 and CD69 kinetics on $CD3^+/CD4^+$ and $CD3^+/CD8^+$ lymphocytes
The lymphocytes stimulated non-specifically with phytohaemagglutinin were co-cultivated with MSC (MSC/lymphocyte ratio 1 : 2) for four days and the expression of activation antigens CD25, CD69 was observed on days +2 to +4. The addition of MSC to the culture resulted in different kinetics of activation antigen CD25 on both $CD3^+/CD4^+$ (**A**) and $CD3^+/CD8^+$ (**C**) lymphocytes as well as antigen CD69 (**B** for $CD3^+/4^+$ and **D** for $CD3^+/CD8^+$). See text for details (median; box: 25 %, 75 % quantiles; non-outlier min, non-outlier max).

Table 1. Expression of activation antigens on T lymphocytes

% (median)		CD3$^+$/CD8$^+$			CD3$^+$/CD4$^+$		
		no MSC	with MSC	P	no MSC	with MSC	P
CD25	Day +2	65.9	42.2	0.0441	77.7	60.3	ns
	Day +3	82.8	45.8	0.0009	86.8	59.0	0.0036
	Day +4	86.6	38.8	0.0003	84.9	55.2	0.0006
CD69	Day +2	75.8	66.3	ns	87.6	81.9	ns
	Day +3	60.7	60.9	ns	67.4	77.9	ns
	Day +4	49.7	59.5	ns	56.8	73.3	0.0009
HLA-DR	Day +2	10.7	5.1	0.0166	6.4	2.6	0.0118
	Day +3	13.1	6.0	0.0008	8.7	3.3	0.0028
	Day +4	11.1	6.2	0.0750	9.8	3.6	0.0003

cytes cultivated with and without the addition of MSC on day +2 (66.3 % vs. 75.8 %, ns) and day +4 (59.5 % vs. 49.7 %, ns). The down-regulation of the CD69 antigen was less pronounced with MSC as its expression remained above the level observed without MSC, see Fig. 1D and Table 1. An example of how MSC influence CD25 and CD69 expression is given in Fig. 2.

HLA-DR was another activation marker evaluated in our experiment. No statistically significant changes of the kinetics were recorded between day +2 and day +4. HLA-DR levels remained low in both CD4$^+$ and CD8$^+$ lymphocytes. However, the co-cultivation with MSC resulted into the reduction of HLA-DR expression in comparison to tests without MSC addition.

Control tests demonstrated no significant induction of CD25, CD69 or HLA-DR antigens by MSC. The ex-

pression was maintained between 0 % and 5 % (data not shown).

CFSE evaluation

We used the CFSE cell tracking assay for analysis of proliferation of non-specifically stimulated PBMC cultured with and without the addition of MSC. After four days of cultivation, the CD3$^+$/4$^+$ lymphocytes divided into four to seven generations. In the tests without MSC, only 17.4 % of all lymphocytes remained undivided. When the PBMC were co-cultivated with MSC, their proliferation rate was reduced and about 50 % of all cells were retained within the parent generation (P < 0.0001). Figure 3 shows an example of CFSE analysis. Most of the lymphocytes only reached three to four divisions. The addition of MSC caused a significant reduc-
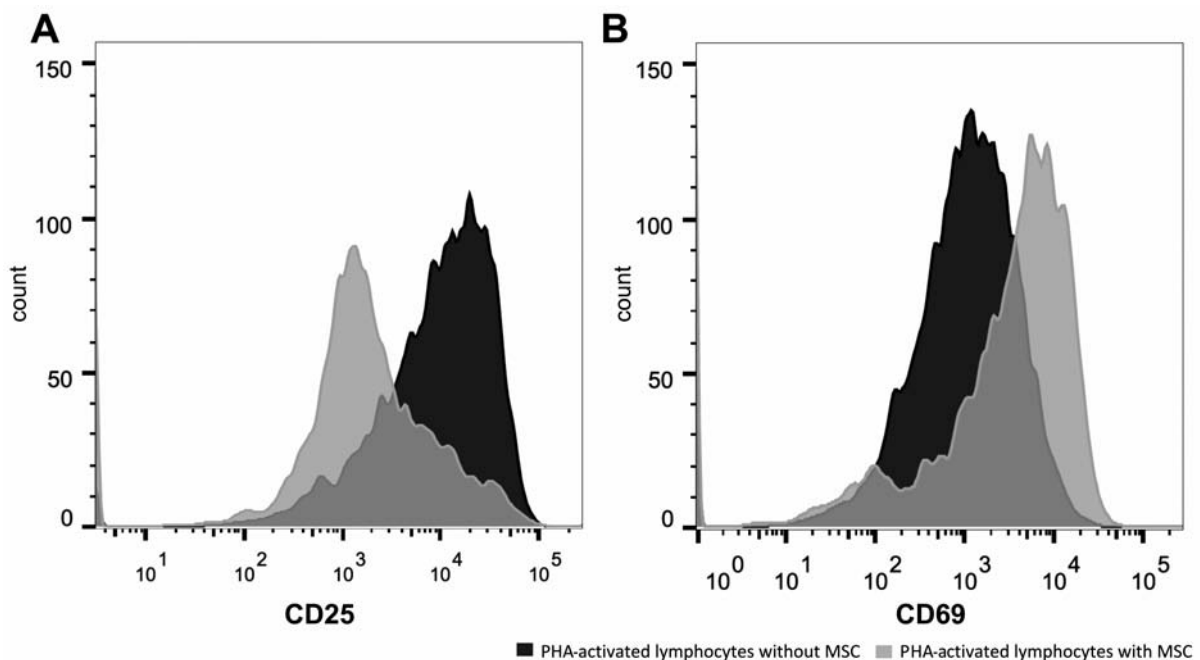

Fig. 2. Example of influence of MSC addition on the activation of antigen expression
Co-cultivation with MSC changed the expression of CD25 and CD69 antigens on stimulated lymphocytes (an example after 72 h of cultivation); overlay histograms; Flow-Jo software; dark: without MSC, grey: with MSC; see Results for details.
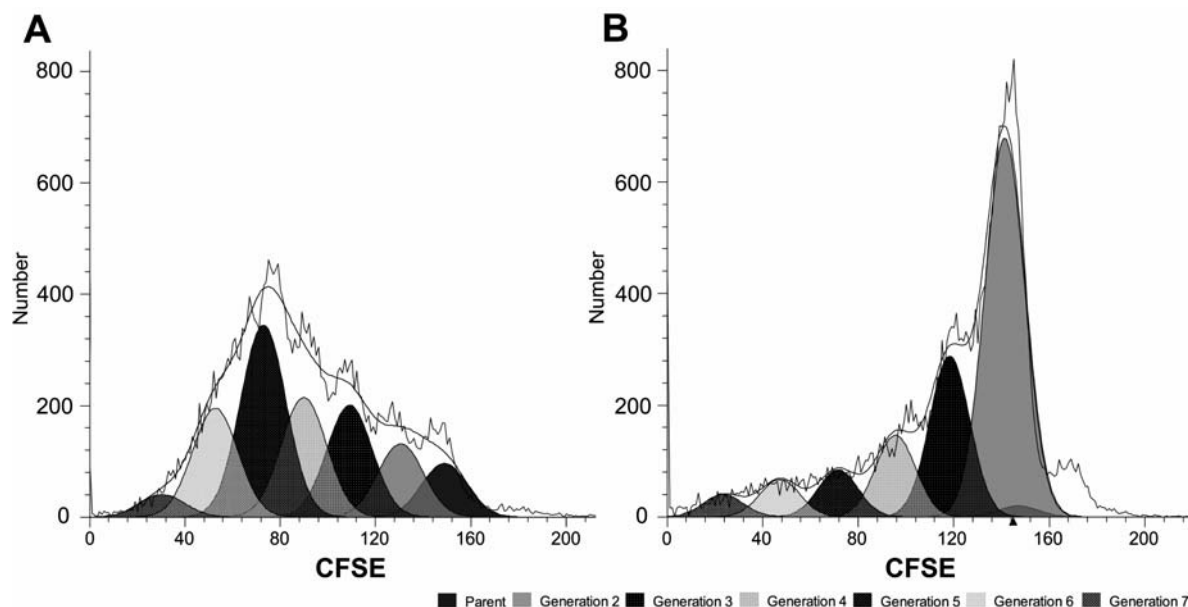
Fig. 3. Example of CFSE proliferation analysis
PHA-stimulated lymphocytes were cultivated for four days with or without MSC. The analysis of lymphocyte proliferation was performed with CFSE tracking and ModFit LT software. The addition of MSC reduced the numbers of lymphocytes dividing into daughter populations (**B**) in comparison to the culture without MSC (**A**); see Methods and Results for details; PARENT = parent generation, GEN2–8 = daughter generations.

Table 2. T-lymphocyte proliferation (CFSE tracking)

Generation[a]	no MSC	with MSC	P
Parental	17.41	50.95	< 0.0001
2nd	7.98	6.50	ns
3rd	12.92	13.01	ns
4th	32.82	10.68	0.0004
5th	29.85	10.09	0.0008
6th	12.52	10.05	ns
7th	4.58	8.90	0.0319

[a] percentage of cells in the generation (means)

tion of the percentage of cells detected in daughter generations (32.82 % vs. 10.68 % in generation 4, P = 0.0004 and 29.85 % vs. 10.09 % in generation 5, P = 0.0008). For details see Table 2 and Fig. 4A. Also the cumulative percentage of the lymphocyte population representation from parent to third generation was different (38.32 % without vs. 70.55 % with MSC, P < 0.0001) as shown in Fig. 4B.

The proliferation index, calculated as the sum of the cells in all generations divided by the number of parent cells, theoretically present at the start of the experiment, was decreased in the culture with MSC (1.84 vs. 3.65, P < 0.0001). The tests without MSC possessed a higher representation of the new daughter populations.

Cytokine and STAT protein expression analysis

The non-specific stimulation resulted in increased expression of all the tested cytokines (IL-6, IL-10 and IFN-γ). The addition of MSC lowered pro-inflammatory IL-6 and IFN-γ expression on both CD3$^+$/4$^+$ (from 19.5 to 16.3 MFI; P < 0.0001; from 13.5 to 12.0 MFI; P = 0.0096) and CD3$^+$/CD8$^+$ lymphocytes (from 14.5 to 13.2 MFI; P = 0.0128; from 12.5 to 12.4 MFI; ns). The opposite effect was observed for production of interleukin-10. IL-10 expression was slightly increased in the presence of MSC; however, these changes were not statistically significant. Details are given in Table 3 and Figure 5.

Furthermore, we tested expression of four phosphorylated proteins of the STAT family – STAT1, STAT3, STAT4 and STAT6. After mitogenic stimulation, the STAT expression increased in all tests. The MSC presence was associated with a significant effect on the phosphorylated STAT protein expression. STAT proteins STAT1, 4 and 6 were silenced and their expression decreased significantly with MSC. In contrast, there was no effect on STAT3. For details see Table 4 and Figure 6.

Discussion

Human MSC have generated considerable interest in the field of cellular therapy. Experimental evidence and preliminary clinical studies have demonstrated that MSC have an important immunomodulatory function. Several studies have been focused on their ability to treat acute or chronic graft-versus-host disease after allogeneic haematopoietic stem cell transplantation.

MSC-based products belong to advanced therapy medicinal products. ATMP reflect a complex and innovative class of biopharmaceuticals as these products are highly research-driven, characterized by innovative ma-
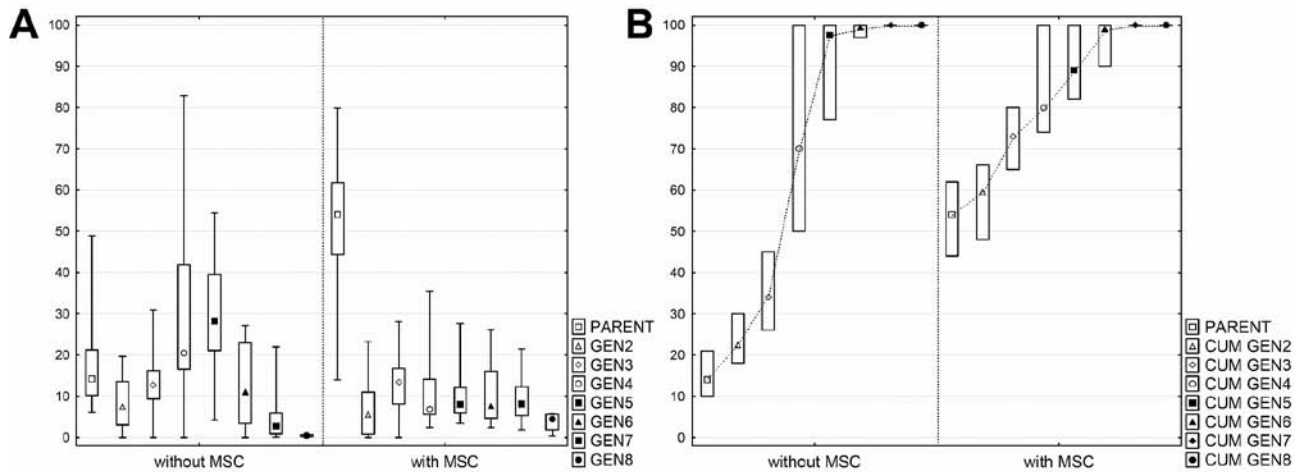
Fig. 4. Changes in stimulated lymphocyte proliferation due to MSC exposure

Lymphocytes labelled with CFSE were stimulated with PHA and cultivated with or without MSC (MSC/lymphocyte ratio 1 : 2). The presence of MSC reduced the proliferation rate of the stimulated lymphocytes, measured after four days of cultivation. **A. Parent and successive populations.** The number of cells retained within the parent generation was increased with MSC (50.95 % vs. 17.41 %, P < 0.001) **B. The cumulative percentage of lymphocyte populations.** Lymphocytes from parent to third generation accounted for 38.32 % (without MSC) vs. 70.55 % (with MSC) of all cells depending on the MSC addition to the culture (P < 0.0001). Median; box: 25 %, 75 % quantiles; non-outlier min, non-outlier max; PARENT = parent generation, GEN2-8 = daughter generations.

Table 3. Expression of cytokines on T lymphocytes

MFI	CD3⁺/CD4⁺					CD3⁺/CD8⁺				
	NK	PK	Pᵃ	MSC	Pᵇ	NK	PK	Pᵃ	MSC	Pᵇ
IL-6	3.5	19.5	< 0.0001	16.3	< 0.0001	1.0	14.5	< 0.0001	13.2	0.0128
IL-10	4.2	17.0	< 0.0001	17.4	ns	1.0	14.6	< 0.0001	15.2	ns
IF-γ	2.1	13.5	< 0.0001	12.0	0.0096	0.6	12.5	< 0.0001	12.4	ns

MFI – mean fluorescence intensity (median), NK – negative control (unstimulated lymphocytes), PK – positive control (stimulated lymphocytes without MSC), MSC (stimulated lymphocytes with MSC)
ᵃ negative vs. positive control
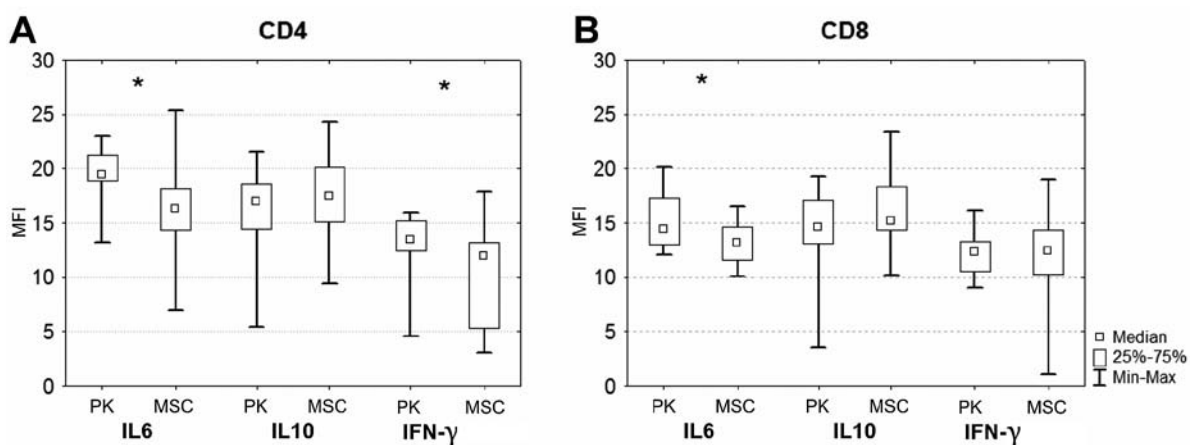ᵇ positive control vs. lymphocytes co-cultured with MSC

Fig. 5. Comparison of cytokine expressions

Lymphocytes were stimulated with Cytostim and monensin. The detection of intracellular cytokines was performed after 6 h of co-cultivation with or without MSC. The expression of pro-inflammatory IL-6 was reduced on both CD3⁺/CD4⁺ (19.5 to 16.3; P < 0.0001) and CD3⁺/CD8⁺ lymphocytes (14.5 to 13.2; P = 0.0128) in the cultures with MSC addition. IFN-γ expression was only significantly reduced on CD3⁺/4⁺ (13.5 to 12.0; P = 0.0096) lymphocytes. Wilcoxon test, median; box: 25 %, 75 % quantiles; non-outlier min, non-outlier max; PK = positive control.

Table 4. Expression of phosphorylated STAT proteins on T lymphocytes

MFI	NK	PK	Pa	MSC	Pb
STAT 1	0.5	33.4	< 0.0001	30.5	0.0335
STAT3	0.5	30.6	< 0.0001	30.5	ns
STAT4	0.4	31.6	< 0.0001	30.2	0.0058
STAT6	0.4	31.0	< 0.0001	30.5	0.0448

MFI – mean fluorescence intensity (median), NK – negative control (unstimulated lymphocytes), PK – positive control (stimulated lymphocytes without MSC), MSC (stimulated lymphocytes with MSC);
[a] negative vs. positive control
[b] positive control vs. lymphocytes co-cultured with MSC
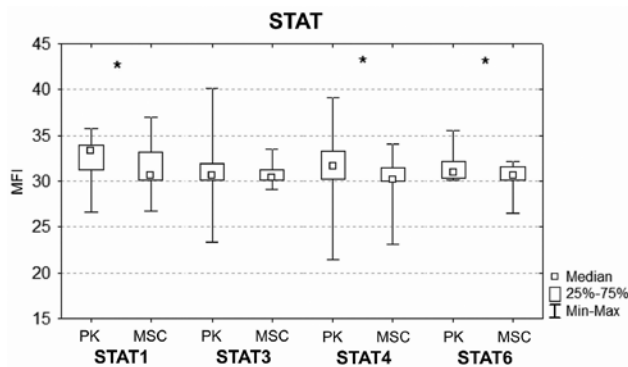

Fig. 6. Comparison of expression of phosphorylated STAT proteins

Lymphocytes were stimulated with Cytostim and cultivated with or without MSC for 24 h. Staining with antibodies against phosphorylated STAT proteins was performed after cell membrane permeabilization and inhibition of phosphorylation enzymes. The expression of STAT1 (P = 0.0335) STAT4 (P = 0.0058) and STAT6 (P = 0.0448) was significantly reduced with MSC. Wilcoxon test, median; box: 25 %, 75 % quantiles; non-outlier min, non-outlier max; PK = positive control.

nufacturing processes and high complexity. The manufacture of ATMP cannot be controlled as precisely as the chemically synthesized small-molecule products. The analysis and quality control of these products are more complex in comparison to standard stem cell grafts as they comprise some functional potency assays.

We evaluated the immunomodulatory effects of MSC on PBMC when isolated from peripheral blood and activated by mitogens. The activation of lymphocytes is a complex cascade of events that results in the expression of several surface molecules, production and secretion of cytokines, lymphocyte proliferation, etc. There are still conflicting data in the literature regarding the mechanisms by which MSC modulate immune cells. Assessing the changes of immunophenotype and the functions of lymphocytes following immunotherapy provides information about the immune response mediated by MSC.

The resting lymphocytes from healthy donors show low or minimal expression of CD69, CD25 and HLA-DR.

The treatment with PHA leads to an increased time-dependent expression of these markers (Reddy et al., 2004). CD69's density grows from 3 to 12 h, reaches its maximum at between 16 and 24 h, and declines thereafter. The expression of CD25 and HLA-DR increases especially in the first 24 h after simulation and further grows until 72 h (Gibbons and Evans, 1996; Werfel et al., 1997; Arvå and Andersson, 1999). The peak is reached between days 4 and 8 (Caruso et al., 1997). Our analysis started 48 h after stimulation and monitored the period of decreasing expression after the presumed peak. A large number of studies have compared the activation marker expression within certain days of cultivation, while we focused on the entire kinetics of early (CD69) and late (CD25 and HLA-DR) antigens on the lymphocytes. We intended to take into account the dynamic nature of the activation process, where the regulation of surface protein expression accompanies the transition to early, intermediate and later activated stages of the T-cell activation process.

CD25 antigen expression was down-regulated in our experiments on both CD4^{+} and CD8^{+} stimulated lymphocytes. This, similarly to the findings of Le Blanc et al. (2004), indicates significantly lower expression of CD25 when MSC are present in the culture (Zheng et al., 2008). On the other hand, Maccario et al. (2005) reported increased numbers of CD4^{+}CD25^{high} lymphocytes in mixed lymphocyte reaction after the addition of MSC. The induction of "true" regulatory (FoxP3^{+} or CD127^{neg}) T cells, a lymphocyte subset with a presumed regulatory function, may be possibly mediated by different pathways in alloantigen- and mitogen-stimulated cultures. Moreover, alloantigen-reactive CD4^{+}CD25^{high} T cells may not be the principal mechanism responsible for the reduction of lymphocyte proliferation and cytolytic activity; probably a complex of other mechanisms, including release of multiple soluble factors, indoleamine 2,3-dioxygenase activity, etc., is involved.

The lymphocytes co-cultured with MSC also displayed a different CD69 expression pattern compared to PHA-stimulated cells without MSC. The maximum activation was observed on day +2, and was further maintained up to day +4 with only a slow decline. There are conflicting reports in the literature about the effect of MSC on the expression of CD69 by activated lymphocytes. Some studies observed inhibition or no significant alteration of CD69 expression in the presence of MSC (Le Blanc et al., 2004; Groh et al., 2005; Ramasamy et al., 2008). In our analysis, the down-regulation of CD69 along with the culture time was less pronounced in the presence of MSC. The potential role of CD69 as a regulatory molecule has been reported by other authors, who even observed an increase in CD69 expression during co-cultivation experiments (Saldanha-Araujo et al., 2012). The receptor may modulate the inflammatory response by inducing TGF-β production, which is known to induce expression of the *Foxp3* gene and generation of regulatory T lymphocytes. This finding is consistent with CD69 being a marker of cells with a regulatory po-

tential, and a stable or late increase of CD69 expression could define cells with immunomodulatory properties.

MSC influenced the expression levels of lymphocyte activation markers, and this inhibitory effect of MSC was evident on both the CD4^{+} and CD8^{+} T-cell subsets. Although a direct comparison is not possible due to different culture conditions, cell types, nature of the stimulus or culture period, both CD4^{+} and CD8^{+} T cells were equally inhibited by MSC in other studies as well (Di Nicola et al., 2002; Le Blanc et al., 2004). Nevertheless, the inhibitory effect of MSC on the expression of the activation markers in response to PHA stimulation has not been documented in some similar studies.

Ramasamy et al. (2008) suggested that the immuno-suppressive effect of MSC is exclusively a consequence of anti-proliferative activity since the expression of CD25 and CD69 was not significantly altered by MSC in their study. We confirmed a strong effect of MSC on the reduction of proliferation of the stimulated lymphocytes. The observation is in agreement with those described in the literature, demonstrating that MSC inhibit lymphocyte growth and decrease the mean number of new generations (Ramasamy et al., 2008; Najar et al., 2009). MSC inhibit T-cell proliferation in a concentration-dependent manner. We used the MSC/lymphocyte ratio 1 : 2, and in other studies the ratio 1 : 1 to 1 : 10 also allowed most significant inhibition (Di Nicola et al., 2002; Najar et al., 2009).

The activation with PHA induces both Th1 and Th2 cytokines. MSC cause a cytokine profile shift in the Th1/Th2 balance towards the anti-inflammatory Th2 phenotype. We observed significant changes in the expression of factors proposed to be involved in GVHD development on the one hand and the immunomodulatory activity of MSC on the other hand – IFN-γ and IL-6. The activation of lymphocytes and IFN-γ secretion in mixed lymphocyte reaction or the ability of antigen-specific T cells to secrete IFN-γ against cognate antigen re-challenge is reduced when co-cultured with MSC (Groh et al., 2005; Ramasamy et al., 2008). The elevated serum levels of IL-6 have been found in patients with acute GVHD, and *IL6* gene polymorphism studies have shown an association with increased GVHD severity after allogeneic transplantation (Cavet et al., 2001; Morris and Hill, 2007). Co-cultures with MSC contain higher levels of IL-6, and neutralizing this interleukin-6 reverses their inhibitory effect (Melief et al., 2013). The inhibition of IL-6 in donor T cells in an experimental allogeneic bone marrow transplantation model led to a reduction in GVHD-induced mortality and prolonged survival (Tawara et al., 2011). There was a slight but statistically insignificant induction of IL-10 in the cocultures of MSC and lymphocytes compared to lymphocytes alone. The results concerning production of IL-10 by stimulated lymphocytes under co-culture with MSC differ between studies. Some authors confirmed the increased IL-10 levels after MSC addition (Groh et al., 2005; Jui et al., 2012); however, others recognized this effect only in mixed lymphocyte culture experiments

without any difference in IL-10 when stimulating lymphocytes with PHA (Rasmusson et al., 2005). The recent work by Melief et al. (2013) revealed that MSC do not produce IL-10, but that IL-10 detected in cell-free supernatants is exclusively produced by monocytes in the culture.

The STAT proteins are critical mediators of cytokine and growth factor signalling. These proteins transmit signals from a receptor complex to the nucleus and activate transcription of their target genes in response to the cell stimuli. STAT proteins play an important role in many cellular processes involved in cell proliferation, apoptosis, immune cell development, etc. The transcriptional activity is regulated mainly by STAT serine phosphorylation. We detected decreased expression of phosphorylated STAT1, which targets genes to promote inflammation and antagonize proliferation (Schindler et al., 2007). The expression of STAT4 and STAT6 was also reduced under MSC co-cultivation. STAT4 is important in the differentiation of naive T cells into IL-2/IFN-γ-producing Th1 cells (Ross et al., 2007). Both STAT3 and STAT4 regulate Th17 differentiation and expansion (Schindler et al., 2007; Durant et al., 2010).

STAT6 is critical for a number of responses in T cells, including cell proliferation and development of Th2 cells. When we observed the slightly increased expression of IL-10, we expected STAT6 up-regulation in the context of the anticipated shift to the Th2 phenotype. In contrast to previous views equating STAT6 with Th2 differentiation, it appears that this process probably involves more complex interactions of STAT3, STAT5 and STAT6 with relevant target genes (O´Shea and Plenge, 2012). The STAT proteins interact with numerous transcriptional regulators. Most cytokines activate more than just one STAT. Our simple *in vitro* test cannot describe the complexity of the JAK/STAT pathways. Despite this fact we have demonstrated that MSC affect the immune cell activity on a transcriptional basis.

We used a relatively low MSC/lymphocyte ratio (1 : 2) in our *in vitro* experiments. The inhibitory effect of MSC is known to be dose dependent. Based on the concentration, some authors observed that MSC possess two distinctive activities. MSC support lymphocyte proliferation at high MSC/lymphocyte ratios (1 : 40 and 1 : 80). The stimulatory activity only happens at these low MSC concentrations and is mediated, in particular, by soluble factors (Najar et al., 2009). MSC become suppressive at lower ratios and acquire an inhibitory profile responsible for T-cell suppression. The dual ability of MSC to either stimulate or suppress T-cell proliferation according to the ratio of cells should be considered in the context of their clinical utilization.

ATMP are used in clinical settings, targeting many conditions with unmet medical needs. Numerous challenges arise from the derivation and nature of ATMP products. Functional assays such as analysis of MSC impact on stimulated lymphocyte activation and proliferation represent a useful tool for the verification of the potency of manufactured MSC-based products.

Significant progress has been made in the understanding of MSC immunomodulatory functions. Their activity was confirmed in a broad range of *in vitro* series and preliminary clinical studies, as has been the case for GVHD. Some of these studies produced conflicting evidence regarding the mechanisms of the MSC functions or their ability to promote or inhibit immune responses. In the present study, we confirmed the inhibitory effect of *ex vivo* expanded MSC on T-cell proliferation and the activation triggered by mitogenic stimuli. We suggest a combination of tests suitable for *in vitro* confirmation of the immunomodulatory activity of MSC that are produced for clinical application. Future research should more closely focus on the interaction between MSC and the local host immune environment and other factors such as infections or relapses. These aspects can appear during the GVHD treatment and could play an important role in a real clinical scenario.

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