

In vitro testing of immunosuppressive effects of mesenchymal stromal cells on lymphocytes stimulated with alloantigens

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Aims. Mesenchymal stromal cells (MSC) derived from adult bone marrow or adipose tissue offer the potential to open a new frontier in medicine. MSC are involved in modulating immune response and tissue repair *in vitro* and *in vivo*. Experimental evidence and preliminary clinical studies have demonstrated that MSC exhibit an important immunomodulatory function in patients with graft versus host disease (GVHD) following allogeneic hematopoietic stem cell transplantation. The immunosuppressive properties of MSC have already been exploited in the clinical setting. However the precise mechanisms are being still investigated.

Methods. We examined the immunosuppressive function of MSC by coculturing them with stimulated HLA incompatible allogeneic lymphocytes in a mixed lymphocyte culture test. The metabolic and proliferative activity of lymphocytes was determined by MTT test.

Results. After stimulation with alloantigens the presence of MSC caused significant decrease of absorbance levels by 62% ($P<0.01$), 26% ($P<0.01$) and 6% ($P=0.0437$) in comparison to positive control depending on the MSC/lymphocyte ratio (1:5, 1:50, 1:500). The mitogenic stimulation of lymphocytes with fMLP or PHA was also significantly reduced during MSC cocultivation. The absorbance was reduced by 42% ($P<0.001$) and 67% ($P<0.001$).

Conclusions. Allogeneic bone marrow is an ideal source of MSC for clinical application. The experiments confirmed the dose-dependent inhibitory effect of MSC on lymphocyte proliferation triggered by cellular or mitogenic stimulation. The mixed lymphocyte culture test offers a simple method for characterization and verification of the immunosuppressive potential of MSC, being prepared for clinical use.

Key words: mesenchymal stromal cells, allogeneic, immunosuppression, GVHD

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INTRODUCTION

Mesenchymal stromal cells (MSC) are multipotent non-hematopoietic progenitor cells of stromal origin that can be isolated from the bone marrow or other tissues (adipose tissue, cord blood). MSC can differentiate into cells of mesodermal origin (adipocytes, chondrocytes, osteoblasts) and likely into stromal fibroblasts, endothelial cells, neural cells and other cell types under certain condition¹. MSC are not characterized by one specific marker. The absence of hematopoietic antigens such as CD45, CD34, CD14, CD19 and presence of surface antigens CD105, CD90, CD73 is typical for their immunophenotype. The expression is dependent on the cell source and cultivation conditions².

Mesenchymal stromal cells are considered to be hypoinmunogenic. They have low expression of HLA class I antigens and no expression of co-stimulatory molecules like CD80, CD86 and CD40. They do not induce a proliferative response in allogeneic lymphocytes and can be used and transplanted without respecting the usual

transplant barrier represented by major histocompatibility complex^{3,4}. MSC offer considerable immunomodulatory capacity. They are able to interact with the cells of innate and adaptive immunity and modulate some of the functions of the immune system. When cultivated with different immune system cell subpopulations, they can shift the cytokine profile of dendritic cells, T-lymphocytes and NK cells to the anti-inflammatory phenotype. MSC reduce the secretion of INF- γ in Th1 cells and increase the expression of IL-4 in Th2 cells. Immature dendritic cells and Tregs increase in the presence of MSC expression of IL-10 whereas mature dendritic cells reduce the production of TNF- α and IL-12 (ref.^{5,6}). Antigen-specific or mitogen-induced non-specific lymphocyte proliferation is significantly reduced in the presence of mesenchymal stromal cells. Reduced reactivity of T-lymphocytes is non-selective and concerns both CD4+ and CD8+ subpopulations^{7,8}. The suppression is human leukocyte antigen (HLA) independent and can be mediated through allogeneic and autologous MSC. Conversely, the degree of suppression is related to the dose of MSC (ref.^{7,9}). T-lymphocyte pro-

liferation inhibition is mediated by arresting them in the G0/G1 phase of the cell cycle¹⁰. The biological relevance of these *in vitro* findings is not entirely clear and requires further studies.

MSC can affect the outcome of the immune reaction and change the inflammatory environment to immune tolerant or even anti-inflammatory. The immunomodulatory properties of MSC predetermine them for influencing the immune response in a number of diseases which originate in alloreactive immunity or autoimmunity. A number of studies target the issues of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation^{11,12}. There is little information on the mechanism of GVHD treatment by mesenchymal stromal cells. MSC probably suppress donor T-cell responses to recipient alloantigens. The suppression is induced by several mechanisms, including induction of regulatory T-cells, production of soluble cytokines and repair of damaged target tissues¹³.

In our study, we evaluated the inhibitory activity of MSC, which are being prepared as a part of a preclinical study of GVHD treatment. We used the one-way mixed lymphocyte culture (MLC) test and observed changes in metabolic activity of lymphocytes after stimulation with alloantigens (cocultivation of HLA incompatible lymphocytes) and after non-specific mitogenic stimulation, respectively. Our target was to create a simple *in vitro* model situation of GVHD and to confirm the functional activity and regulatory potential of MSC, which are essential for their clinical application in the treatment of severe GVHD.

METHODS

GVHD model

We used a one-way mixed lymphocyte culture for testing the immunomodulatory properties of MSC. Lymphocytes from healthy donors were stimulated with alloantigens (HLA incompatible inactivated lymphocytes), nonspecific mitogens and chemotaxis activators (PHA, fMLP) and cocultivated with MSC. The effect of the stimulation and immunomodulation was determined by the MTT test which corresponds to mitochondrial metabolic activity and cell viability. In total 32 tests were performed on lymphocytes obtained from 15 healthy donors. All donors provided written informed consent to the study.

Lymphocyte samples preparations

Lymphocytes' 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 for use with Luminex®, Gen-Probe). PCR-SSO typing was performed for HLA-A*, HLA-B* and HLA-DRB1* loci. Lymphocytes were isolated by gradient centrifugation (Histopaque - 1077, Sigma, USA), washed and diluted with cultivation medium RPMI 1640 (Lonza, Belgium) to a final concentration 1×10^6 cells/mL. Half of the prepared lymphocytes were inactivated

by cultivation in 1% solution of paraformaldehyde in PBS for 5 min.

Mesenchymal stromal cell cultivation

Mesenchymal stromal cells were derived from iliac crest bone marrow aspirate performed under general anaesthesia. All donors provided written informed consent for MSC donation. MSC were isolated by gradient centrifugation using separation solution. Briefly, 10 - 20 mL of BM aspirates were diluted 1:1 with HBSS (PAA, Austria) and layered over LSM 1077 solution (PAA, Austria). After centrifugation at 1000 g for 15 min, the mononuclear cells were collected into a new tube and resuspended in 20 mL of PBS (PAA, Austria). Cell suspension was washed by centrifugation at 1000 g for 10 min, the supernatant was discarded and pellet was resuspended in 1 mL PBS. All the cells were put into 175 cm² flask (Corning, USA) containing 30 mL of Complete Culture Medium (α -MEM, PAA, Austria; 10% pooled human platelet lysate, local source) and cultivated in humidified incubator at 37 °C and 5% CO₂. The non-adherent cells were removed after 48 h and the remaining cells were further cultivated (the medium was changed every 3-4 days). After reaching 80% confluence the cells were detached with TrypLE Select solution (Invitrogen, USA) and passaged in a concentration 1×10^6 /175 cm² flask. The MSC from 2nd to 4th passage were used for the cocultivation experiments.

Mitochondrial activity testing (MTT)

This method is based on the reduction of soluble 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) to insoluble formazan. The reaction takes place on the mitochondrial membrane of living cells. Formazan dissolves after adding strong solvent (DMSO, Sigma USA) and detergent (SDS, Sigma, USA). The arising color is detected by spectrophotometry with a wavelength 540 nm (MRXII, Dynex, CZ). The level of absorbance indicates the number of living and metabolic active cells. 200 μ L of the lymphocyte mixture with 50 μ L of MSC were incubated for 6 h in humidified incubator at 37 °C and 5% CO₂ in 96 well plates. The mixture was prepared from 100 μ L of living lymphocytes and 100 μ L of inactivated lymphocytes, always from different, HLA-incompatible donors. MSC were added to the culture in three different concentrations: 4×10^5 cells/mL (MSC/lymphocyte ratio 1:5), 4×10^4 cells/mL (1:50) and 4×10^3 cells/mL (1:500). Phytohemagglutinin (PHA) and N-formyl-Met-Leu-Phe (fMLP, both Sigma, USA) were used at concentrations of 0.05 mg/mL and 0.025 mg/mL. The MTT solution was added after 1 h incubation and the mixture was incubated in for 2 h. The spectrophotometric analysis was performed after this time.

Statistical methods

The Wilcoxon paired test was used for statistical evaluation. The analysis was performed in statistical software R project (The R Foundation for Statistical Computing). A *P* value equal to or lower than 0.05 was taken as statistically significant.

RESULTS

Thirty-two experiments were performed in our study. The lymphocytes from healthy donors were stimulated non-specifically with alloantigens or PHA and fMLP, respectively. Cocultivation with mesenchymal stromal cells enabled to analyze the immunosuppressive effect of MSC on the cultivated lymphocytes. The results were obtained through the determination of absorbance of formazan solution released from the cells in each test. The absorbance level represented the metabolic and proliferative activity of cocultivated lymphocytes.

The stimulation of lymphocytes with HLA incompatible inactivated lymphocytes (positive control) manifested as expressive increase in metabolic activity with an increment of absorbance by 0.24 ($P<0.001$) compared to the negative control (unstimulated lymphocytes). In contrast to this observation in cocultivation experiments in the presence of MSC, we observed an average decline of absorbance of 0.23 ($P<0.01$) in undiluted (MSC/lymphocyte ratio 1:5), by 0.10 ($P<0.01$) in 10 x diluted MSC (1:50) and by 0.02 in 100 x diluted MSC (1:500) ($P=0.0437$). The presence of mesenchymal stromal cells led to an absorbance decline in comparison with positive control in all tests. The absorbance levels decreased by 62%, 26% and 6%, respectively. The effect of mesenchymal stromal cells was dose-dependent. The higher amounts of mesenchymal stromal cells added to the lymphocytes at 1:5 or 1:50 ratios provided the best immunosuppressive response. Extreme dilution of MSC (1:500) weakened their effect on the lymphocytes. For details see Table 1. and Fig. 1.

Significant differences in absorbance were also found in tests with PHA or fMLP stimulated lymphocytes. The lymphocytes were cultivated without and with MSC (1:5 ratio). The presence of MSC decreased the lymphocyte activity. The absorbance was reduced by 0.17 ($P<0.001$) in samples stimulated with fMLP and by 0.31 ($P<0.001$) in samples treated with PHA, which corresponded to the decline by 42% and 67%. Details are provided in Table 1. and Fig. 2.

DISCUSSION

Currently, mesenchymal stromal cells (MSC) are a subject of a large number of studies in various fields of medicine. They represent a promising treatment method for severe chronic graft-versus-host disease refractory to corticosteroids in patients after allogeneic hematopoietic stem cell transplantation. Allogeneic bone marrow is an appropriate source of MSC for clinical application. The cells can be cryopreserved and cultivated on request in reasonable timeframe. MSC can avert rejection in recipients of solid organ transplants¹⁴. Another field of successful utilization of MSC is the treatment of autoimmune diseases such as Crohn's disease, multiple sclerosis and rheumatoid arthritis^{15,16}.

Mesenchymal stromal cells are able to interact with the immune system and have effective immunomodulative

Table 1. Lymphocyte cytotoxicity test (MTT absorbance values).

	mean	SD	<i>P</i>
negative control	0.1354	0.03	< 0.001
positive control	0.3746	0.06	
MSC 1:5	0.1427	0.02	< 0.01
MSC 1:50	0.2778	0.03	< 0.01
MSC 1:500	0.3505	0.05	0.0437
fMLP ^a	0.4136	0.05	< 0.001
fMLP/MSC	0.2405	0.08	
PHA ^b	0.4588	0.04	< 0.001
PHA/MSC	0.1506	0.04	

positive control – stimulation with inactivated lymphocytes; negative control – without stimulation; MSC 1:5, MSC 1:50 and MSC 1:500 – stimulation with inactivated HLA incompatible lymphocytes and MSC cocultivation at different MSC:lymphocyte ratios; ^afMLP stimulation with and without MSC addition; ^bPHA stimulation with and without MSC addition; abbreviations: MSC – mesenchymal stromal cells, fMLP – N-formyl-Met-Leu-Phe, PHA – phytohemagglutinin

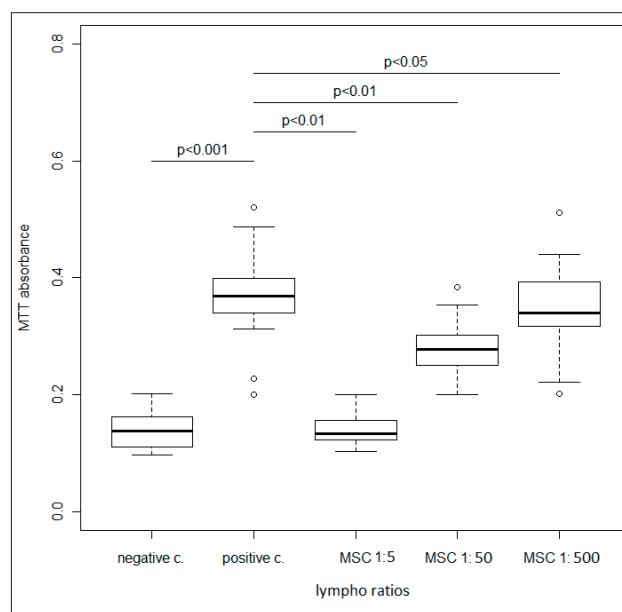


Fig. 1. Reduction of stimulated lymphocytes metabolic activity after MSC addition (stimulation with alloantigens). The cocultivation with MSC reduces the metabolic activity of stimulated lymphocytes in comparison to positive control (no MSC added). The MSC effect is dose dependent and higher MSC dilutions are less effective in lymphocyte suppression. See details in the text. MTT absorbance values showed, Wilcoxon pair test (median; box: 25%, 75% quantiles; non-outlier min, non-outlier max).

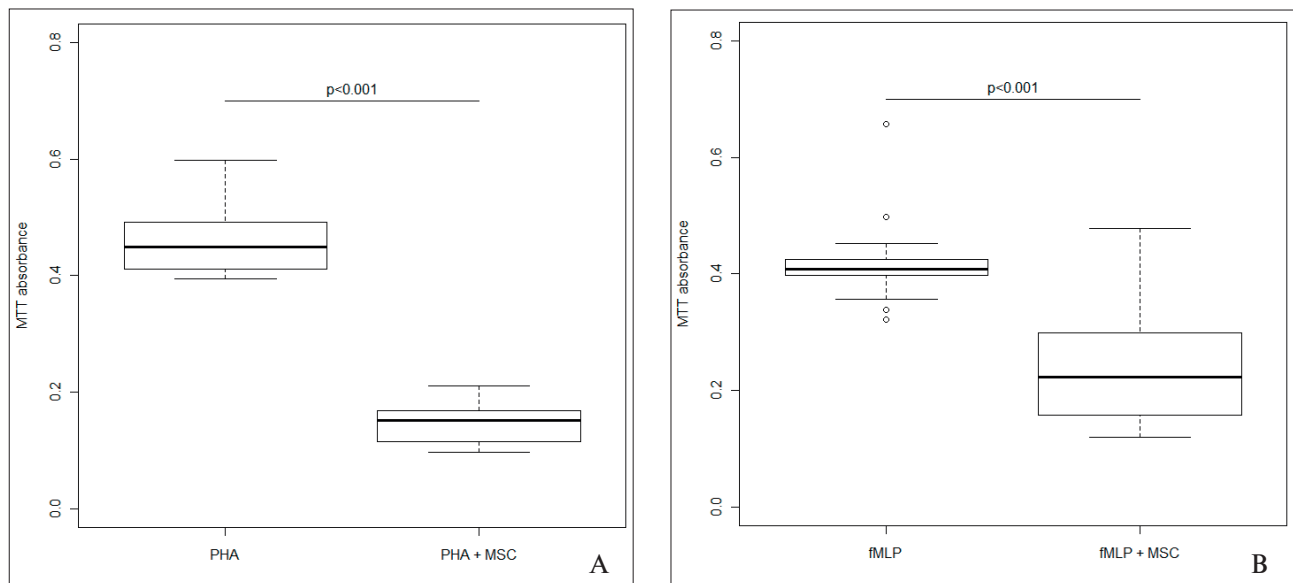


Fig. 2. Reduction of stimulated lymphocytes metabolic activity after MSC addition (nonspecific stimulation). The effect of MSC addition to the culture of lymphocytes stimulated with PHA (A.) and fMLP (B.). Cocultivation with MSC reduces significantly the metabolic activity of lymphocytes ($P < 0.001$). Abbreviations: fMLP – N-formyl-Met-Leu-Phe, PHA – phytohemagglutinin. MTT absorbance values showed, Wilcoxon pair test (median; box: 25%, 75% quantiles; non-outlier min, non-outlier max)

properties. They can affect the immune reaction by inhibition of inflammatory cytokines and by increase in expression of suppressive cytokines. Studies have confirmed that during the cocultivation, MSC can negatively influence T-lymphocytes proliferation^{8,17,18}. Our results are consistent with these data. We observed that MSC possess the capacity to significantly affect the nonspecific activation of lymphocytes. When lymphocytes were cocultivated with MSC, there was a marked decline in absorbance, measured by MTT test of almost 60 percent in comparison with the positive control. The lower absorbance levels corresponded to reduction in the metabolic and proliferative activity of stimulated lymphocytes.

The immunosuppressive effect was present both during cocultivation of lymphocytes stimulated with alloantigens (inactivated incompatible lymphocytes) and in cultures with lymphocytes stimulated with PHA and fMLP. The laboratory analysis confirms that mesenchymal stromal cells could regulate the lymphocyte activation *in vitro* when stimulated with HLA incompatible lymphocytes. MSC can therefore be used for the treatment of severe forms of chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. This clinical application of MSC has begun to be studied in the first clinical trials with encouraging results. The immunomodulatory effect of MSC is dose-dependent⁸. An MSC/lymphocyte ratio of 1:5 provided the most noticeable suppression of lymphocyte proliferation in our analysis. The dose dependent nature of MSC inhibitory effects emerged from a similar study showing statistically significant suppression at MSC/lymphocyte ratios ranging from 1:1 to 1:10 (ref.¹⁹). It appears that the higher doses of MSC produce stronger effect. However, the exact dose

and frequency of application optimal for the GVHD treatment must be determined in larger clinical studies. Lower numbers of MSC are unable to inhibit mitogen-induced T-cell response. Some studies have reported stimulatory effects at very low concentrations of MSC (1:100 – 1:10000) on allogeneic lymphocytes provoking speculation that surface structures on MSC can act synergistically with HLA-DR antigens after mitogenic stimulation^{9,20}.

Our data confirm the inhibitory effect of MSC on lymphocyte proliferation triggered by cellular or mitogenic stimulation. The mixed lymphocyte culture offers a simple way of confirming the immunosuppressive potential of MSC and validating the cell therapy medicinal product intended for clinical use. Cocultivation experiments of lymphocytes and MSC offer a simple *in vitro* method for simulating the MSC effect on alloreactive interactions of donor and recipient lymphocytes similar to the processes during the post-transplant period and GVHD reaction.

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