Alterations between Effective and Ineffective Multipotent Mesenchymal Stromal Cells Used for Acute Graft Versus Host Disease Prophylaxis

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Abstract

Multipotent mesenchymal stromal cells (MSCs) are applied for prophylaxis of acute graft versus host disease (aGvHD) after allogeneic hematopoietic cell transplantation (allo-HCT). Not all samples of MSC used in the National Research Center for Hematology were efficient for aGvHD prevention. The suitability of MSCs for aGvHD prophylaxis was studied. MSCs derived from the bone marrow of a HCT donor were injected intravenously precisely at the moment of blood cell reconstitution. MSCs were cultivated for 3 passages. The characteristics of donor bone marrow samples including colony forming unit fibroblast (CFU-F) concentration, growth parameters of MSCs and the relative expression levels (REL) of different genes in them were analyzed. MSCs infusion induced a decrease in aGvHD development in patients with related and unrelated donors compared with the standard prophylaxis group. aGvHD prophylaxis with MSCs was ineffective in 13.5% of cases. In these MSC samples, a significant decrease in total cell production and the REL of *CFH, FGFR1, PDGFRa* and *ICAM1* were observed. This study showed that MSCs injection resulted in a significant 2-fold decrease in aGvHD development compared with patients in the standard prophylaxis group. The effective MSC samples are characterized by higher total cell production and REL of *CFH, FGFR1, PDGFRa* and *ICAM1* than the ineffective ones.

Running head: Characteristics of MSCs used for aGvHD prophylaxis

Key words: aGvHD prophylaxis, MSC, CFU-F, gene expression

1. Introduction

One of the major causes of morbidity and mortality after allogeneic hematopoietic cell transplantation (allo-HCT) is acute graft-versus-host disease (aGvHD) (Billingham, 1967). aGvHD develops due to the response of the donor's immune cells within the donated material (graft) to the transplant recipient's (host) antigens (Welniak, Blazar, & Murphy, 2007). Even with current methods of prophylaxis, 30%–60% of patients still develop aGvHD after receiving allo-HCT (Prasad et al., 2011; Servais et al., 2016). aGvHD pathogenesis actually starts before receiving allo-HCT. Damage to the recipient's tissues by the conditioning regimen, which is applied prior to allo-HCT, activates monocytes and tissue macrophages, which then secrete pro-inflammatory cytokines, including interleukin 1 beta (IL-1 beta) and interferon gamma (IFNγ) (Fong & Mosmann, 1990; Visentainer et al., 2003). These cytokines activate the donor's T cells, which in turn produce interleukin 2 (IL-2) and IFNγ (Visentainer et al., 2003). The release of cytokines stimulates the expression of adhesion molecules and increases the number of MHC class 2 molecules presented on cell surfaces that comprise different recipient tissues (Ferrara, Levy, & Chao, 1999). This process favors conditions for aGvHD development.

Multipotent mesenchymal stromal cells (MSCs) possess unique immunomodulating capacities (Jones & McTaggart, 2008). MSCs secrete various cytokines, growth factors, extracellular matrix molecules, and immunologically active exosomes (Deans & Moseley, 2000; Zhang et al., 2014). These findings have facilitated MSC application in clinical practice for aGvHD treatment (Maziarz et al., 2015; Prockop et al., 2010; Ringdén et al., 2006a; Samuelsson, Ringdén, Lönnies, & Le Blanc, 2009). A large number of studies investigated the possibility of using MSCs for aGvHD treatment and prophylaxis (Introna & Rambaldi, 2015; Kim et al., 2013; Rizk et al., 2016). *In vivo* studies demonstrated that MSC could be efficiently applied to prevent and treat aGvHD (Larisa A Kuzmina et al., 2012; Le Blanc et al., 2004; Ringdén et al., 2006b). As MSCs barely expresses MHC type 2, it was thought that MSCs would not induce a prominent immune response (Aggarwal & Pittenger, 2005; Vaes, Van't Hof, Deans, & Pinxteren,

2012). Commercial MSC samples (such as Multistem® and Prochymal®) derived under standard conditions from unknown, unrelated donors have been proposed for immunomodulatory therapy applications as a universal source of cells for any possible transplant patient (Maziarz et al., 2015; Vaes et al., 2012). Recent data, however, have revealed that the immune system may respond to allogeneic MSCs (Schu et al., 2012). Under cytokine influence, cell expression of HLA-DR increased; therefore, donor's cells could not be used for clinical applications without first conducting a histocompatibility investigation (Ankrum, Ong, & Karp, 2014). An attempt was made to develop the criteria for human MSC eligibility for therapeutic applications (Samsonraj et al., 2015). Despite significant advances in MSC applications, the therapy has not always proved effective (Prockop et al., 2010; Rizk et al., 2016; von Bahr et al., 2012). These failures could be attributed to the particular qualities of donor-recipient interactions, patient status, or the characteristics of the MSC samples. Usually, after allo-HCT, it is possible to choose MSC samples. A randomized clinical study of aGvHD prophylaxis after allo-HCT using MSCs from matched related and unrelated donors has been on-going since 2008 at the National Research Center for Hematology, Moscow, Russia. MSCs were isolated from the bone marrow of hematopoietic cell donors. The application of MSCs generally yielded good results, although prophylaxis failed in some cases (Larisa A Kuzmina et al., 2012, 2015).

The study of the main properties of MSCs can at least partially solve the basic problem of ineffective MSC samples for aGvHD prevention. This investigation aimed to analyze bone marrow stromal precursor cells (colony forming unit fibroblasts (CFU-F) and MSCs), their growth characteristics, the expression levels of selected genes, the connection between these parameters, and the prophylactic outcome.

2. Materials and Methods

2.1. GVHD prophylaxis with MSCs

A randomized clinical trial (ClinicalTrials.gov NCT01941394) has been ongoing at the National Research Center for Hematology since 2008. Until February 2016, allogeneic bone marrow from related donors was transplanted to 81 patients and from unrelated donors to 17 patients that

were in complete remission. Patients were randomly allocated to two groups as follows: (1) a group receiving the standard GVHD prophylaxis (52 patients) and (2) a group receiving the same prophylaxis combined with MSC infusion (46 patients). One patient from the second group was excluded from the analysis due to early aGvHD manifestation (prior to the scheduled MSC injection). In addition to the randomized groups, 8 patients with aplastic anemia (AA) and 5 patients with multiple myeloma (MM), who had been treated with MSCs, were included in the analysis. For each case, the MSCs were derived from a corresponding hematopoietic stem cell donor. The randomized patients' characteristics are presented in Table 1, and characteristics of patients with AA and MM are presented in Table 2. All work was conducted in accordance with the Declaration of Helsinki (1964). This study was approved by the local ethics committee, and the donors and patients provided written informed consent.

MSCs were intravenously injected precisely at the moment of blood cell reconstitution after allo-HCT (based on a leukocyte count of $1x10^9$/l, average days of infusion 25±1.0) into 46 patients from the second group, 8 patients with AA, and 5 patients with MM. This time point was selected because it is the beginning of active graft growth, which often coincides with aGvHD manifestation. Acute GvHD was graded according to internationally accepted criteria (Glucksberg et al., 1974). Short-term fever and chills occurred in half of patients during the first twenty-four hours after MSC injection. No other complications were evident.

The patients received either myeloablative or reduced-intensity conditioning (Table 1). Myeloablative conditioning (MAC) included cyclophosphamide (60 mg/kg/day for 2 days) combined mainly with busulfan (4 mg/kg/day for 4 days). Reduced-intensity conditioning (RIC) regimens included either fludarabine phosphate (30 mg/m^2/day for 6 days) combined with busulfan (4 mg/kg/day for 2 days) and anti-thymocitic globulin (ATG) (10 mg/kg/day for 4 days) or fludarabine phosphate (30 mg/m^2/day for 5 days) combined with BCNU (200 mg/m^2/day for 2 days), melphalan (140 mg/m^2/day for 1 day), and ATG (20 mg/kg/day for 2 days).

All patients received cyclosporine combined with methotrexate as standard aGvHD prophylaxis. Additionally, 9 patients with CML received prednisolone, and 25 patients after RIC received mycophenolate mofetil.

2.2. Culture and characterization of MSCs

MSCs were isolated from bone marrow samples used for allo-HCT performed at the National Research Center for Hematology from 2008 until 2016. In total, MSCs from 155 donors (83 male and 72 female) ranging in age from 13 to 62 years (median: 34) were analyzed.

For acute GVHD prophylaxis, MSCs were derived from 25–30 ml of bone marrow from donors. To separate mononuclear cells, the bone marrow was mixed with an equal volume of alpha-MEM medium (ICN) containing 0.2% methylcellulose (1500 cP, Sigma-Aldrich). After 40 min, the erythrocytes and granulocytes had mostly precipitated, while the mononuclear cells remained in suspension. The upper fraction (suspension) was aspirated and centrifuged for 10 minutes at 450 g. The sediment was suspended in a cultivation medium that consisted of alpha-MEM supplemented with 4% donor platelet lysate (Lange et al., 2007), 2 mM L-glutamine (ICN), 100 U/ml penicillin (Ferein), 50 μg/ml streptomycin (Ferein), and 2 U/ml heparin (Sigma). The cells were cultured at a density of 27-30 x 10^6 cells per T175 cm^2 culture flask (Corning-Costar). When a confluent monolayer of cells had formed, the cells were washed with 0.02% EDTA (ICN) in a physiological solution (Sigma-Aldrich). The cells were then detached from the surface by incubating them in 0.25% of trypsin solution (ICN) for 10–15 min at room temperature. The cells were then reseeded at a density 4 x 10^3 cells per cm^2 of flask area. The cultures were maintained in hypoxic conditions at 37°C in a 5% CO_2 and 5% O_2 atmosphere. The MSCs were harvested in 6% dextran (Biochimik) solution, diluted to 3–7 x 10^6 cells/ml, and then injected intravenously to the patient.

For all research assays, MSCs were derived from 5–10 ml donor bone marrow and processed from the same samples and in the same manner as for aGvHD prophylaxis except for the standard cultivation medium. This medium consisted of alpha-MEM supplemented with 10% fetal bovine serum

(Hyclone), 2 mM L-glutamine (ICN), 100 U/ml penicillin (Ferein), and 50 μg/ml streptomycin (Ferein). The cells were cultivated at a density of 3 x 10^6 cells per cm^2 in T25 culture flasks (Corning-Costar). These cultures were maintained at 37°C in 5% CO_2. The number of harvested cells was directly counted; cell viability was assessed using trypan blue dye exclusion staining.

The immunophenotype of the MSCs from the 2^{nd}–3^{rd} passage is shown in Table S1.

All MSCs were immunophenotyped using standard protocols with different markers, including CD105, CD73, CD45, CD34, CD14, and HLA-DR. The antibodies were purchased from BD Pharmingen (CD105, CD59, CD73, CD90, CD31, CD34, and CD14), Sigma (CD45, FSP), and DAKO (HLA-DR).

The differentiation capacity of these cells was estimated at passages 1 and 5 under standard conditions (Svinareva et al., 2009).

For the CFU-F analysis, $5x10^5$ and $1x10^6$ bone marrow cells were cultivated in alpha-MEM supplemented with 20% fetal bovine serum (Hyclone), 2 mM L-glutamine (ICN), 100 U/ml penicillin (Ferein), and 50 μg/ml streptomycin (Ferein). The CFU-Fs were measured on day 14 after staining with 4% crystal violet in 20% methanol.

2.3 RNA isolation and quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from MSCs at passage 1 using the standard method (Chomczynski & Sacchi, 1987), and cDNA was synthesized using a mixture of random hexamers and oligo(dT) primers. Gene expression levels were quantified by real-time quantitative PCR using hydrolysis probes (Taqman) and a Rotor-Gene 6000 machine (Corbett). Gene-specific primers and probes were designed by the authors and synthesized by Syntol R&D. All primers and probes are provided in Table S2. For each MSC sample, the relative gene expression levels (REL) were determined by normalizing the expression of each target gene to the levels of β-actin and GAPDH and calculated using the $\Delta\Delta C_t$ method (Schmittgen & Livak, 2008) for each MSC sample.

The REL of differentiation markers were analyzed from MSCs cultivated without any inducers.

2.4. An analysis of the ability of MSCs to inhibit lymphocyte proliferation

Peripheral blood from healthy donors was separated on a Lymphoprep gradient (density 1.077 g/cm^3) (MP Biomedicals), and a mononuclear cell fraction (PBMC) was obtained. PBMC was washed twice with RPMI-1640 medium without serum and adjusted to a concentration of 5x10^7/ml. Subsequently, 3 μM of the fluorescent dye CFSE (molecular probes) was added, the cells were incubated for 10 min at 37^{0}C and then washed twice with RPMI-1640 containing 10% fetal calf serum. Lymphocyte blast transformation was performed using phytohemagglutinin (PHA). PHA was added to the cell suspension to a final concentration of 5 μg/ml. The cells were plated at a density of 2x10^5 per well in 96-well plates onto MSCs, which had been seeded at a concentration of 1 x 10^3 cells per well 3 days earlier. PBMC with MSCs were incubated for 4 days at 37^{0}C and 5% CO_2. PBMC incubated without MSCs were used as controls. Using flow cytometry, the relative number of proliferating lymphocytes cultured on MSCs was compared with control samples on day 4. The cells were stained with 7-AAD (Sigma) prior to the analysis. Lymphocytes after blast transformation were gated using forward and side light scattering. Dead cells were excluded by 7-AAD staining. Peaks corresponding to proliferating and non-proliferating cells were determined on the histograms built for the CFSE fluorescence channel. The ratio of the proportion of non-dividing cells cultivated with MSCs to the proportion of non-dividing cells cultivated without MSC reflected the MSC-induced inhibition of lymphocyte proliferation.

2.5. Statistical analysis

All values are expressed as the means ± SEM. The data were analyzed using paired Student's t-tests in Excel. The chi-square test was used to compare the results of aGvHD prophylaxis in two randomized groups. Patient survival was assessed using the Kaplan-Meier method.

3. Results

3.1. Acute GVHD prophylaxis with MSC

MSCs were injected into 59 patients after allo-HCT at the moment of blood count recovery. The 100-day observation period (typical time of aGvHD manifestation) for all patients after allo-HCT revealed a

significant 2-fold decrease in aGvHD frequency in the group of patients who received MSCs (13.5% aGvHD grade II–IV) compared with the standard prophylaxis group (28.8% aGvHD grade II–IV), p=0.041. MSC injection did not affect the engraftment or the relapse frequency (Table 1).

Table 1. Characteristics of the randomized patients, treatments and outcomes

Group characteristics	First group (1) standard GVHD prophylaxis	Second group (2) standard GVHD prophylaxis + MSCs
Related donors/unrelated donors	42/10	39/7
Sex of patient, male/female	25/27	22/24
Median age, years (range)	33 (18-60)	37 (19-63)
Diagnosis, n		
AML/MDS	29	32
ALL	16	11
CML	7	3
Conditioning regimen, n		
RIC	25	25
MAC	27	21
Observation time, months	33 (3.5-90)	36 (7-90)
Outcomes		
aGvHD, n, (%)	13 (25.5%)	5 (10.9%)
Relapse rate, n, (%)	13 (25.5%)	10 (21.7%)
Graft rejection, n, (%)	3 (5.9%)	2 (4.3%)
Death, n, (%)	17 (32.6%)	10 (21.7%)

AML – acute myeloid leukemia, MDS – myelodysplastic syndrome, ALL – acute lymphoid leukemia, CML – chronic myeloid leukemia, RIC- reduced intensity conditioning, MAC – myeloablative conditioning.

Table 2. Characteristics of the patients with AA and MM injected with MSCs

Group characteristics	AA	MM
Sex of patient, male/female	6/2	3/2
Median age, years (range)	21.5 (17-30)	38 (27-43)
Conditioning regimen, n RIC MAC	8	5
Observation time, months	47-53	8-36
Outcomes		
aGvHD, n, (%)	1(12.5%)	2(40%)
Relapse rate, n, (%)	0	0
Graft rejection, n, (%)	1 (12.5%)	0
Death, n, (%)	0	0

Among the 59 patients who received MSCs, 8 developed grade II–IV aGvHD. According to published data, the frequency of aGvHD after allo-HCT of a fully matched sibling donor graft is 30%–45% (Appelbaum, 2003). The 2-fold reduction in aGvHD frequency after MSC injection was an important and essential achievement in the prophylaxis of the patients after allo-HCT, and it significantly improved patient survival.

The main features of stromal precursor cells were studied to reveal crucial differences in donors' MSCs and to determine which MSCs were effective or ineffective for aGvHD prophylaxis.

3.2. An analysis of stromal precursor cells from donor bone marrow that was effective and ineffective for aGvHD prophylaxis

Two categories of precursor cells (MSC and CFU-F) are currently being used

to characterize the stromal microenvironment. The CFU-F concentration was estimated in the bone marrow of healthy donors whose MSCs were effective for aGvHD prophylaxis and was found to be 23.4 ± 3.8 per 1 x 10^6 bone marrow mononuclear cells (Table 3). This concentration was approximately 2-fold higher than in the bone marrow of inefficient samples, but the data were not significant; this nonsignificance was probably due to insufficient numbers of inefficient samples and large variances in data. Therefore, the relationship between the CFU-F concentration and the efficiency of aGvHD prophylaxis with MSC derived from the same samples was not determined.

Table 3. Characteristics of efficient and inefficient MSC samples for aGvHD prophylaxis

	Efficient		Non efficient		p
Number of samples	52		8		
	M±SE*	Median	M±SE	Median	
Donors' age	34±1.8	34	40.1±5.4	45	
CFU-F concentration per 10^6 BM cells	23.4±3.9	11.4	13.5±6.0	5.7	
Cumulative MSCs production for 3 passages, x10^6	10.3±1.6	8.6	4.5±1.0	3.6	0.004

*M- mean, SE- standard error

Cumulative MSC production was 2-fold higher in efficient for aGvHD prophylaxis MSC samples (Table 3). The MSC populations are heterogeneous (Colter, Sekiya, & Prockop, 2001). Analyses of the whole populations have shown a wide range of REL for different genes (Menssen et al., 2011). Our investigation of the REL of 27 genes (Table S3) revealed 4 genes that were significantly different ($p<0.05$) in efficient and inefficient MSC samples (Fig. 1).

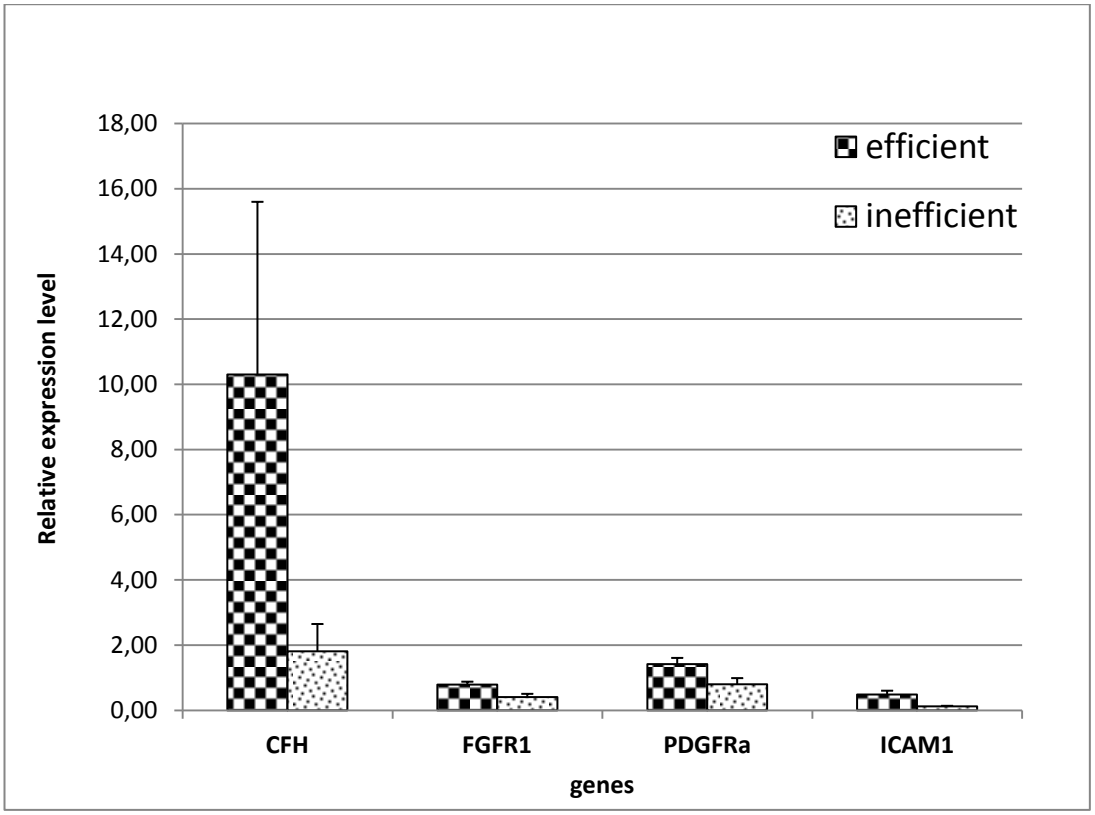

Figure 1. Significant alterations in REL in efficient and inefficient MSC samples

The REL of *CFH*, *FGFR1*, *PDGFRa*, and *ICAM1* increased 5.7, 2, 1.8, and 3.9-folds, respectively, in MSC samples that were effective for aGvHD prophylaxis in comparison with ineffective samples.

The REL of other studied immunomodulatory genes varied significantly, but these variations were not related to the patients' sex or age or efficiency of aGvHD prophylaxis. Significant correlations were identified between the REL of some genes (Larisa A Kuzmina et al., 2015).

The ability to inhibit lymphocyte proliferation did not differ between MSCs that succeeded and failed to prevent aGvHD. The relative number of non-proliferating lymphocytes doubled relative to the control (lymphocytes cultivated without MSCs) in both groups. In the successful prophylaxis group, this number increased 2.3 ± 0.23 fold, while in the failed prophylaxis group, this number increased 2.0 ± 0.32 fold.

4. Discussion

aGvHD prophylaxis using MSC led to a significant 2-fold decrease in the frequency of aGvHD. Not all MSC samples, however, proved to be effective. Recent studies describing a generation of antibodies

against allogeneic donor MSC and their immune rejection suggest that MSC may not be immune privileged (Ankrum et al., 2014). In this study, each patient received MSC derived from his own bone marrow donor, so no immune responses were expected. The goal of this study was to find differences between effective and ineffective MSC samples. Finding specific markers for effective MSC samples should provide more efficient prophylaxis. The MSC populations are heterogeneous (Colter et al., 2001) and vary between individual donors as well as among cells in the sample (Bigildeev et al., 2012). After studying donor age and CFU-F concentration in bone marrow, no significant differences were found between these factors that affected the MSC used for aGvHD prophylaxis. Nevertheless, there was a clear trend in donor characteristics involving ineffective MSC. First, the median age of ineffective MSCs' donors was 11 years more than that of effective MSC donors' ages. The age of hematopoietic stem cell donor is very important. Adjusting for patient disease and transplantation characteristics, survival was better after transplantation of grafts from young donors (18–32 years) who were HLA matched to recipients ($P < 0.001$). For every 10-year increment in donor age, there is a 5.5% increase in the hazard ratio for overall mortality (Kollman et al., 2016). A cohort study to compare the prognosis of unrelated bone marrow transplantation from younger and older donors showed the inferiority of older donors in aplastic anemia; thus, donor age should be considered when multiple donors are available (Arai et al., 2016). CFU-F concentration in donors' bone marrow was also different. The median CFU-F concentration in the bone marrow of effective MSC donors was 2 fold higher than that from the other donors. These data are in good agreement with the age differences among donors. The CFU-F concentration in the bone marrow of donors younger than 34 years was significantly higher than that of older donors (Irina N. Shipounova et al., 2013). The differences in age and CFU-F concentration in the bone marrow were not significant, most likely due to the small number of ineffective aGvHD prevention cases. Changes in MSC growth capacity were previously shown to correlate with colony forming efficiency and telomere length for sex- and age-matched donors (Samsonraj et al., 2015). We found

significant difference between growth capacity of efficient and inefficient MSC (p=0.004). Cumulative MSC production was 2.5-fold higher in the efficient samples than in the inefficient ones. Thus, with the possibility of selecting between the bone marrow and MSC donor, the preference should be given to a younger donor.

Analyses of whole populations have shown a wide range of REL for different genes in MSCs (L.A. Kuzmina et al., 2015; Menssen et al., 2011). We revealed 4 genes whose REL significantly differed between effective and ineffective MSC samples. In MSC samples that were effective for GvHD prophylaxis, the REL of *CFH* was 5.7-fold, *FGFR1* 2-fold, *PDGFRa* 1.8-fold, and *ICAM1* 3.9-fold higher than in ineffective MSC. The main mechanism by which MSCs inhibit the immune response has been shown to be via secretion of IDO1, CFH, IL6, PTGES, CSF1, LGALS1 and other cytokines (Jones & McTaggart, 2008). Despite the fact that IDO1 is considered to be one of the major players of MSC immunomodulatory action (Meisel et al., 2004; Sivanathan & Gronthos, 2014), no significant differences in its REL between effective and ineffective MSC were found. Among the genes directly involved in immunomodulation, significant differences were revealed only in the *CFH* REL. The production of CFH by MSCs inhibited complement activation, which could contribute to the immunosuppressive activities of MSCs (Tu, Li, Bu, & Lin, 2010)(Fallarino et al., 2002).

The growth capacity of MSCs depends on the expression of either growth factors (FGF2 and PDGF) or their receptors (FGFR1, FGFR2, and PDGFRa). The increased REL of *FGFR1* and *PDGFRa* in efficient MSC samples correlated with the cumulative MSC production. In the model for discrimination between effective and ineffective MSC samples that was previously described by Shipounova et al., a decrease in the expression level of the *FGFR1* combined with an increase in the expression levels of differentiation- (*PPARG*) and aging-related (*IGF1*) genes indicated that the MSCs that failed to prevent aGvHD consisted primarily of more mature cells (I. N. Shipounova et al., 2014).

ICAM-1 has been shown to mediate cellular interactions by binding to its counter-receptors, lymphocyte function-associated antigen (LFA-1, CD11a/CD18)

and Mac-1 (CD11b/CD18) (Staunton, Dustin, Erickson, & Springer, 1990). Recent studies have demonstrated that increased ICAM-1 plays an important role in the maintenance of MSC immunomodulation (Ren et al., 2010; Shi et al., 2010). Increased REL of *ICAM1* in effective MSC samples indicated improved intercellular interaction of lymphocytes with MSCs. It is known that the *ICAM1* expression levels decrease as differentiation of MSCs occurs (Xu et al., 2014). Thus, the decrease in the expression of this molecule may indirectly confirm that the less differentiated MSC have a stronger immunomodulatory potential.

Previously, we had attempted to identify factors influencing the effectiveness of MSC-induced aGvHD prevention (L.A. Kuzmina et al., 2015; I. N. Shipounova et al., 2014). Two different mathematical models have been proposed, both of which take into account the total MSC production and the REL of various genes. New data have revealed the main differences between effective and ineffective MSC samples. Additional data accumulation may provide additional or more specific eligibility criteria for effective MSC samples that are used for clinical application.

In summary, this study showed that MSC injections resulted in a significant 2-fold decrease in aGvHD development compared with the standard prophylaxis treatment. When compared with ineffective MSC, effective MSC samples are characterized by higher total cell production and REL of *CFH, FGFR1, PDGFRa,* and *ICAM1*.

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