Original Paper

The Therapeutic Effect of ICAM-1-Overexpressing Mesenchymal Stem Cells on Acute Graft-Versus-Host Disease

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Key Words

Mesenchymal stem cells • Intercellular adhesion molecule-1 • Graft-versus-host disease • Bone marrow transplantation • Murine GVHD model

Abstract

Background/Aims: Mesenchymal stem cells (MSCs) do not readily migrate to appropriate sites, and this creates a major obstacle for their use in the treatment of graft-versus-host disease (GVHD). Intercellular adhesion molecule-1 (ICAM-1) can guide the homing of various immune cells to the proper anatomical location within secondary lymphoid organs (SLOs), which are the major niches for generating immune responses or tolerance. MSCs rarely migrate to SLOs after intravenous infusion, and are constitutively low expression of ICAM-1. So in our previous work, ICAM-1 was engineered into a murine MSC line C3H10T1/2 by retrovirus transfection system (ICAM-1MSCs). Here, we hypothesized that ICAM-1^{high}MSCs may significantly improve their immunomodulatory effect. **Methods:** We used different co-culture methods combined with real-time PCR and flow cytometry to evaluate ICAM-1^{high}MSCs immunomodulatory effect on dendritic cells (DCs) and T cells *in vitro* and *in vivo*. MSCs were labeled with carboxyfluorescein diacetate succinimidylester (CFSE) to detect its distribution in mouse model. **Results:** Our *in vitro* analyses revealed ICAM-1 MSCs could suppress DCs maturation according to co-culture methods and suppress the T cell immune response according to the mixed lymphocyte response (MLR) and lymphoblast transformation test (LTT) tests. We found that infusion of ICAM-1^{high}MSCs potently prolonged the survival of GVHD mouse model. The infused ICAM-1^{high}MSCs migrate to SLOs *in vivo*, and suppressed DCs maturation, suppressed CD4⁺ T cell differentiation to Th1 cells, and increased the ratios of Treg cells. **Conclusions:** Taken together, these data demonstrate that ICAM-1^{high}MSCs had an enhanced immunosuppressive effect on DCs and T cells, which may help explain the protective effect in a GVHD model. This exciting therapeutic strategy may improve the clinical efficacy of MSC-based therapy for GVHD.

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Published by S. Karger AG, Basel

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Introduction

Acute graft-versus-host disease (GVHD) is a major obstacle following allogeneic hematopoietic stem-cell transplantation (HSCT) [1], which is caused by alloreactive T cells that recognize alloantigens initially presented by host/donor antigen-presenting cells (APCs) [2-4]. Although GVHD can sometimes be constrained with rigorously T cell-depleted donor grafts or pharmacologic agents, such treatments predispose patients to relapses in malignancy and opportunistic infections. Mesenchymal stem cells (MSCs) are a heterogeneous population of plastic-adherent cells with the capacity to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts, which have a wide range of immunosuppressive effects on innate and adaptive immune cells [5, 6]. Nearly all immune cells, including T, B, natural killer (NK), and dendritic cells (DCs), are affected. Because of their relatively convenient isolation, *ex vivo* expansion, and low toxicity, the clinical utility of MSCs has rapidly expanded in recent years.

However, the immunosuppressive capacity of MSCs is not always as effective as originally hypothesized. Some researchers have claimed that MSCs have a protective effect against GVHD [7]. This effect has not been observed consistently in mouse models [8]. In addition, the results of clinical trials with MSCs are also confusing, as earlier trials showed substantial benefits, whereas two recent multicenter, prospective phase III trials with at least one source of MSCs did not show any benefit [9, 10]. Currently, it is recognized that the inefficient homing of systemically infused MSCs is a major barrier of existing MSC-based clinical GVHD therapy [11, 12].

Intercellular adhesion molecule-1 (ICAM-1) is a highly glycosylated protein that belongs to the immunoglobulin superfamily of cell adhesion molecules (CAMs), which were reported to play crucial roles in the specific and effective immune response to foreign pathogens [13, 14]. ICAM-1 is normally constitutively expressed on the surface of a wide variety of cell types, including leukocytes, endothelial cells, and fibroblasts [15]. It can guide the homing of various immune cells to the proper anatomical location within secondary lymphoid organs (SLOs). Normally, MSCs constitutively express a low level of ICAM-1 [16-18]. In our previous work, a recombinant retrovirus vector containing the mouse ICAM-1 gene was successfully constructed [19]. The overexpressing-ICAM-1^{high}MSCS cell line (ICAM-1MSCs) was obtained by the transfection of the MSC cell line C3H10T 1/2. Forced expression of ICAM-1 almost did not alter the immunophenotype. ICAM-1^{high}MSCs are positive for CD29, CD44, and Sac-1 and are negative for CD45, CD11b, CD31 and CD86. As for the multilineage differentiation potential, ICAM-1^{high}MSCs have the capacity to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts. Although other researchers have demonstrated that increased ICAM-1 is critical for MSC-mediated immunosuppression, the role of ICAM-1 in preventing GVHD has not been widely studied.

In this article, we show that overexpression of ICAM-1 enhances the immunomodulatory activity of MSCs and that they interact with DCs or T cells by making MSCs more adhesive to cells, which plays an important role in MSC-mediated immunosuppression. Therefore, this article uncovers a novel role of adhesion molecules in preventing GVHD in a mouse model, which will provide a potential future approach for the prevention or treatment of GVHD in patients.

Materials and Methods

Mice

Female C57BL/6 (B6, H2b) and BALB/c (BALB/c, H-2d) mice were purchased from Vital River (Charles River China). The age of the mice ranged from 8 to 10 weeks. All mice were kept under specific pathogen-free conditions and utilized in the animal center of the Academy of Military Medical Sciences. All experiments in this study were performed in accordance with the Academy of Military Medical Sciences Guide for Laboratory Animals (IACUC of AMMS-2013-021).

Cell preparations

Bone marrow cells (BMCs) were collected from the backbones and femurs. The spleens were crushed through 40- μ m screens, and then, cell suspensions were prepared by centrifugation with a Ficoll gradient at 1000x g for 20 min to separate leukocytes from the cell debris. The leukocytes were collected from the interface and washed before use. Primary MSCs (pMSCs) were isolated from newborn C57BL/6 murine compact bone and culture-expanded in α -minimal essential medium (α -MEM, Gibco) containing 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ at 37°C. The murine MSC line C3H10T1/2 (CCL-226) was obtained from ATCC. MSCs/MSCV (C3H10T1/2 cells transfected with the empty vector), and ICAM-1 MSCs were obtained as described in our previous report and passaged and expanded in MSC culture medium.

Preparation of DCs from bone marrow cells and DCs co-culture with MSCs *in vitro*.

BMCs from mouse (8 weeks old) femur were prepared by the depletion of red cells and were then cultured at a density of 2×10^6 cells/ml in 6-well plates in minimal essential medium (MEM, Gibco, Grand Island) with 10% fetal bovine serum (FBS), 10 ng/ml of recombinant mouse granulocyte-monocyte colony-stimulating factor (mGM-CSF, RD) and 1 ng/ml of recombinant mouse IL-4 (mIL-4, RD). On day 4, DCs were collected and co-cultured with pMSCs, MSCs/MSCV, or ICAM-1 MSCs at a ratio of 5:1 for 3 additional days in the presence of 1 μ g/ml LPS with the intent of stimulating DCs maturation. At day 7, the cell morphology of the

DCs and MSCs co-cultured DCs was observed under the microscope (Nikon TE2000-U), and the DCs were collected and CD11c, CD80, CD86 and Ia were used as indicators of fully mature DCs by flow cytometry.

Mixed lymphocyte response (MLR) and lymphoblast transformation test (LTT)

As for MLR testing, MSCs or ICAM-1 MSCs were preplaced in 96-well plates at 1×10^4 /100 μ l/well. The responder splenocytes from BALB/c mice (2×10^5 /well) were mixed with irritating stimulator splenocytes from C57BL/6 mice (15 Gy irradiated) at various densities (2×10^5 , 4×10^4 , 8×10^3 /well). Then, these cells were added and cocultured with MSCs or ICAM-1 MSCs to obtain a final volume of 300 μ l. Positive control wells contained responding and allogeneic irritating cells. As for the LTT, MSCs or ICAM-1 MSCs were preplaced in 96-well plates at various densities (1×10^4 , 2×10^4 and 4×10^4 /well) and irradiated 24 hours later at 15 Gy. Splenic T lymphocytes from BALB/c mice were added to the coculture with MSCs at a density of 2×10^5 /well in 96-well plates. Concanavalin A (5 mg/ml) (ConA; Sigma Aldrich Co.) were added to promote mitogen proliferation, and a negative control well contained only splenocytes from BALB/c mice. The plates were maintained at 37°C for 56 hours and then pulsed with 1 mCi of ³H thymidine deoxyribonucleoside per milliliter for another 16 hours. The cells were then harvested onto glass fiber filters, and their radioactivity was measured on a Wallac Microbeta Trilux 1450-02P. All experiments were performed in triplicate and repeated three times.

Murine GVHD Model

Mice underwent transplantation according to following protocol. Briefly, the recipients received a single dose of 800 cGy total body irradiation (TBI, Co⁶⁰ source). In the GVHD group, BMCs (10×10^6) and Spleen mononuclear cells (SPMNC) (10×10^6) from C57BL/6 mice were suspended in a total volume of 0.2 ml of PBS and injected intravenously into recipient animals on day 0. pMSCs (5×10^5), MSCs/MSCV (5×10^5), or ICAM-1MSCs (5×10^5) were injected into GVHD mice 24 hours after transplantation. The body weights and survival were monitored daily, and the GVHD clinical scores were measured weekly. The degree of systemic acute GVHD was assessed using a scoring system that incorporates five clinical parameters: weight loss, posture (hunching), activity, fur texture and skin integrity. Histopathology of GVHD was monitored according to Kaplan et al. [20]. Specimens from at least three recipient mice per group were collected for experiments except where otherwise described.

In vivo tracking of exogenously infused MSCs

To track MSCs in the GVHD models, MSCs (5×10^5) were labeled with carboxyfluorescein diacetate succinimidylester (CFSE, Life Technologies) to detect the distribution of the transplanted pMSCs, MSCs/MSCV, or ICAM-1 MSCs *in vivo* and then injected into the lateral tail vein of GVHD mice in a total volume of 0.2 ml of PBS. Forty-eight hours later, samples of the spleen, lung, liver, and intestine from the recipients were

collected for cryosectioning. Fluorescent cells in the sections were visualized under a fluorescence microscope equipped with a Nikon Eclipse 80i. For quantification of the CFSE+ cells, the average number of green fluorescent cells per 5 scope in triplicate mice was counted at 200× magnification.

Immune factor determination by real-time PCR and ELISA

RNase-free plastic and water were used throughout the assay. Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo). cDNAs were amplified by qRT-PCR using a SYBR Green PCR kit (Sigma). The qRT-PCR results were normalized using mouse HPRT as the housekeeping gene. All cDNA sequences were obtained from the GenBank database. The primer sequences used for the qRT-PCR are listed in Table 1. Meanwhile, Mouse IFN-γ and IL-10 concentrations were determined according to the reagent protocols of the quantitative determination kit (eBioscience).

Flow cytometry analysis

Antibodies, including PE-CY5 anti-mouse CD11c, PE anti-mouse CD86, FITC anti-mouse CD80, PE anti-mouse CD1A, FITC anti-mouse H2Kb, APC/Cy7 anti-mouse CD3, PerCP/Cy5.5 anti-mouse CD8, PE anti-mouse IFN-γ, PE anti-mouse IL4, and PE/Cy7 anti-mouse IL17A, were purchased from Biolegend. The procedure was performed as described elsewhere. Briefly, for surface staining, approximately one million cells were incubated with saturating concentrations of the antibody mixture at 4 °C for 30 minutes as illustrated in the instructions. For intracellular cytokine analysis, leukocytes isolated from spleen cells were stimulated with a Cell Stimulation Cocktail with protein transport inhibitors (500×, eBioscience), which could be directly added to the culture medium at 1X (2 μL/mL), for 4-18 h. For intracellular cytokine staining, cells were fixed and permeabilized using the BD IntraSure Kit.

Statistical analysis

The Kaplan-Meier product-limit method was used to calculate the survival curve. Differences between groups in the survival studies were determined using log-rank statistics. For all other data, Student's t test was used to analyze differences between groups, and the results were considered significant if the p value was less than or equal to 0.05. Unless otherwise specified, all error bars in this study are the S.E.M.

Results

Overexpressing ICAM-1 enhanced the suppression capacity of MSCs for DCs in vitro

DCs are specialized antigen-presenting cells that are pivotal in immune responses. It is generally accepted that immature DCs (iDC) in the periphery become mature and migrate to secondary lymphoid organs, where T cells are activated [21]. To investigate the enhanced expression of ICAM-1 on MSCs in the determination of DC fate, first, we found that numerous mature DCs (maDC) were generated from BMC using mIL4/mGM-CSF supplemented with an additional LPS incubation (Fig. 1 B). In terms of the cellular morphology, maDC were found to be relatively disperse and had long synapses (Fig. 1 A). After 3 days of co-culture with MSCs, numerous DCs were adhered to MSCs, and the long synapses could not be seen under a microscope, especially for ICAM-1 MSCs (Fig. 1A). Surface expression of costimulatory

Table 1. Primer sequences

Genes	Primer sequences	Annealing temperature
HPRT	forward, 5'- AGTCAAGGGCATATCCAACAACAA -3'	60 °C
	reverse, 5'- GCTGGTGAAGGACCTCTCG -3'	
TNF-α	forward, 5'-CACTTGCTGGTTGCTACGA-3'	
	reverse, 5'-GCCTCCCTCTCATCAGTTCTA-3'	
IL-4	forward, 5'- ACTCCATGCTTGAAGAAGAACTC -3'	
	reverse, 5'- ATGATGCTCTTTAGGCTTTCCA -3'	
IFN-γ	forward, 5'- TTACTACCTTCTTCAGCAACAGCAA -3'	
	reverse, 5'- CTGGTGGACCACTCGGATGA -3'	
Foxp3	forward, 5'- AGAGCCCTCACAACCAGCTA -3'	
	reverse, 5'- CCAGATGTTGTGGGTGAGTG -3'	

Fig. 1. ICAM-1^{high} MSCs suppressed the maturation and differentiation of DCs *in vitro*. (A) Morphologic characteristics of DCs: iDC (a); maDCs with longer synapses (b); DCs co-cultured with pMSCs (c); DCs co-cultured with MSCs (d); DCs co-cultured with MSCs/MSCV (e); DCs co-cultured with ICAM-1^{high}MSCs (f). After 3 days of coculture with MSCs, numerous DCs adhered to MSCs, and long synapses could not be seen under a microscope, especially in ICAM-1^{high} MSCs (f). (B) Phenotypic characteristics of DCs: expression of CD80, CD86 and Ia gated on CD11c were decreased by MSCs. The data are shown from one representative experiment of three replicates. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, n.s. not significant. Error bars indicate SEM). Abbreviations: iDC, immature DCs; maDC, mature DCs; pMSCs, primary MSCs; MSCs, a murine MSC line C3H10T1/2 cells; MSCs/MSCV, a murine MSC line C3H10T1/2 cells transfected with the empty vector; ICAM-1^{high}MSCs, overexpressing-ICAM-1 MSC cell line.

molecules and MHC II is critical for the function of DCs. We compared the expression of CD80, CD86 and Ia on DCs by flow cytometry. The expression of CD80, CD86 and Ia decreased in DCs co-cultured with MSCs, especially in ICAM-1^{high}MSCs (Fig. 1B).

Overexpressing ICAM-1 enhanced the immunosuppression capacity of MSCs

Next, studies were performed to assess the *in vitro* suppressive capacity of MSCs in MLR and LTT cultures. The results showed that pMSCs, MSCs, MSCs/MSCV, and ICAM-1^{high}MSCs all inhibited the proliferation of lymphocytes *in vitro* in a cell dose-dependent manner. Splenocytes co-cultured with ICAM-1^{high}MSCs had the lowest proliferative response compared with the MSC/MSCV group ($p < 0.05$; Fig. 2).

Fig. 2. Enhanced expression of ICAM-1 enhanced the immunomodulatory capacity of MSCs *in vitro*. Various doses of MSCs were added to the MLR and LTT system, and T-cell proliferation was analyzed via ³H-thymidine uptake. The data are shown from one representative experiment of three replicates. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, n.s. not significant. Error bars indicate SEM). Abbreviations: pMSCs, primary MSCs; MSCs/MSCV, a murine MSC line C3H10T1/2 cells transfected with the empty vector; ICAM-1^{high}MSCs, overexpressing-ICAM-1 MSC cell line.

Fig. 3. ICAM-1^{high} MSCs remarkably inhibits the development of lethal GVHD. (A) Survival curves of each group of mice (n=15). (B) Degree of the systemic GVHD scores of the GVHD, pMSCs, MSCs/MSCV and ICAM-1^{high} MSCs groups of mice. (C) Paraffin sections of liver, intestine and lung of three animals/treatment group were analyzed for histological signs of GVHD on the day mice were killed due to their moribund state or at the end of the experiment. The data are shown from one representative experiment of three replicates (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with the pMSCs or MSC/MSCV infusion group). Abbreviations: GVHD, graft-versus-host disease; pMSCs, primary MSCs; MSCs/MSCV, a murine MSC line C3H10T1/2 cells transfected with the empty vector; ICAM-1^{high} MSCs, overexpressing-ICAM-1 MSC cell line.

Fig. 4. ICAM-1^{high} MSCs conferred the capacity of targeted migration to secondary lymphoid organs in vivo. (A) pMSCs, MSCs/MSCV and ICAM-1^{high} MSCs were labeled with the green fluorescent dye CFSE and were then intravenously injected into GVHD mice. Five days later, the organ sections containing cells labeled with CFSE were observed by fluorescence microscopy (green staining). DAPI counterstaining was performed to distinguish recipient cells (blue staining). Bar=100 μ m. (B): Quantification of the CFSE+ cells: the average number of green fluorescent cells per 5 scope in triplicate mice was counted at 200 $\times$ magnification. The data are representative of three independent experiments. The data represent the mean value $\pm$ SD of at least 5 mice (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, n.s. not significant. Error bars indicate SEM, compared with the MSCs or MSC/MSCV infusion group). Abbreviations: GVHD, graft-versus-host disease; pMSCs, primary MSCs; MSCs/MSCV, a murine MSC line C3H10T1/2 cells transfected with the empty vector; ICAM-1^{high} MSCs, overexpressing-ICAM-1 MSC cell line; CFSE, carboxyfluorescein diacetate succinimidyl ester.

Enforced expression of ICAM-1 on MSCs significantly ameliorated the severity of acute GVHD

Since ICAM-1 MSCs gained enhanced adhesion and suppression abilities toward DCs and T cells *in vitro*, we hypothesized that overexpression of ICAM-1 on MSCs could improve their immunosuppressive activity *in vivo*. We tested this hypothesis in a murine GVHD model. The transplantation of allogeneic BM and spleen cells induced severe clinical GVHD with high GVHD scores and a lethality of 100% within 25 days after transplantation. The injection

Fig. 5. ICAM-1^{high} MSCs suppressed the maturation, differentiation and function of DCs in vivo. Seven days after ICAM-1^{high} MSC treatment, spleens of the model mice were harvested. (A) Phenotypic characteristics of DCs: expression of CD11c, CD80 and CD86 was detected by FCAS. ICAM-1^{high} MSCs down-regulated the percentage of cells positive for CD11c, CD80 and CD86. (B) The difference in percentage of CD11c, CD80 and CD86 was analyzed. The data are the mean of 5 independent experiments and a representative result is shown. The data represent the mean value $\pm$ SD of at least 5 mice (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Abbreviations: DCs, dendritic cells; GVHD, graft-versus-host disease; pMSCs, primary MSCs; MSCs/MSCV, a murine MSC line C3H10T1/2 cells transfected with the empty vector; ICAM-1^{high} MSCs, overexpressing-ICAM-1 MSC cell line.

of pMSCs reduce mortality to 20% (Fig. 3A), while ICAM-1 MSCs infusion could rescue mortality to approximately 50% (Fig. 3A). The lowest GVHD scores were detected with the infusion of 5×10^5 ICAM-1 MSCs (Fig. 3B). Consistent with the prolonged survival time, the GVHD + ICAM-1^{high}MSCs group of mice also had significantly alleviated clinical histological scores compared with the GVHD and GVHD + MSCs groups. A histological examination at the end of the experiment demonstrated that an ICAM-1^{high}MSCs infusion dramatically decreased lymphocyte infiltration in the GVHD target tissues, including the liver, intestine and lung (Fig. 3C).

Enhanced ICAM-1^{high}MSCs migration to SLOs in a GVHD mouse model

We further examined whether MSCs could migrate to target organs and SLOs *in vivo*. Forty-eight hours after the injection of 5×10^5 cells, there were

Fig. 6. ICAM-1^{high} MSCs regulated the differentiation of T cells in vivo. (A) The levels of the intracellular cytokines of donor T cells were detected by fluorescence-activated cell sorting (FACS) analysis at day 7 after transfusion. Gated on H2Kb, the frequencies of Th1 cells (IFN- γ producing CD4⁺ cells) are shown. In addition, the frequencies of Treg cells are also detected. Gated on CD4, the frequencies of Treg cells (CD25⁺ Foxp3⁺ cells) are shown. (B) The difference in percentage of Th1 and Treg cells was analyzed. The data are the mean of 5 independent experiments and a representative result is shown. The data represent the mean value $\pm$ SD of at least 5 mice (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the MSCs or MSC/MSCV infusion group). Abbreviations: GVHD, graft-versus-host disease; pMSCs, primary MSCs; MSCs/MSCV, a murine MSC line C3H10T1/2 cells transfected with the empty vector; ICAM-1^{high} MSCs, overexpressing-ICAM-1 MSC cell line.

almost an undetectable amount of MSCs in the liver and intestine of mice. CFSE⁺ MSCs and CFSE⁺ MSCs/MSCV appeared sporadically in the spleen and lymph nodes. In contrast, a significantly larger number of CFSE⁺ ICAM-1^{high}MSCs was dispersed in SLOs in model mice ($p < 0.05$; Fig. 4).

Overexpressing ICAM-1 enhanced the immunosuppression capacity of MSCs for DCs in vivo

Since ICAM-1 MSCs gained an immunosuppression capacity for DCs *in vitro*, we further examined whether they could suppress DCs function in the spleen after intravenous infusion. Seven days after infusion, surface expression of CD11c was slightly decreased in the ICAM-1^{high}MSCs group compared with MSCs/MSCV ($p = 0.035$). Expression of CD80 and CD86 also had an obvious reduction in the ICAM-1^{high}MSCs group compared with MSCs/MSCV ($p = 0.006$ and $p = 0.003$; Fig. 5).

Enhanced expression of ICAM-1 induced the induction of Treg cells and reduced the percentage of Th1 cells

In GVHD pathophysiology, the recruitment of donor-derived T cells to the tissue is an early and key event [1] [1]. GVHD is a result of naive T cell responses. Owing to their ability to differentiate into various effector T cell subsets — namely, Th1 cells, Th2 cells and Th17 cells — CD4⁺T cells are particularly important in the initiation of GVHD. At day 7 after BMT, PMA/iono stimulation of spleen cells showed that GVHD-associated Th1 cells strongly increased in mice developing GVHD. Importantly, co-transplantation of ICAM-1^{high}MSCs significantly decreased the percentage of Th1 cells (Fig. 6). Treg are defined by their expression of CD4, CD25 and the transcription factor forkhead box P3 (FOXP3), and control innate and adaptive immune responses [22]. Among these group, Treg cell skewing in ICAM-1MSC-treated mice was also observed (Fig. 6).

Enhanced expression of ICAM-1 affected the expression of T cell related cytokine

Th1 type cytokine, such as IFN- γ and TNF α have been shown to be aetiological factors in the induction of GVHD [23]. Th2-type cytokines, such as IL-4, can reduce acute GVHD [24]. The mRNA levels of the Th1 cytokines IFN- γ and TNF α were strongly decreased in mice co-

Fig. 7. ICAM-1^{high} MSCs decreased Th1-type cytokines and increased Treg-type cytokines. Seven days after MSC infusion, mice were euthanized and splenic cells were isolated from recipients. Then, q-PCR was performed to assess the expression of TNF α , IFN- γ and IL-4, and the expression relative to Hprt was calculated (A, B, C, and D). The IFN- γ and IL-10 concentrations were determined in the serum of transplanted animals 7 days after MSC infusion, according to the reagent protocols of the quantitative determination kit (E, F). The data represent the mean value $\pm$ SD of at least 5 mice. The data are representative of three independent experiments. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with the pMSCs or MSC/MSCV infusion group). Abbreviations: TNF- α , tumour necrosis factor α ; IFN- γ , interferon γ ; IL-4, interleukin-4; Foxp3, forkhead box P3; IL-10, interleukin-10.

transplanted with ICAM-1 MSCs (Fig. 7A and 7B), while the expression of IL4 displayed no obvious change among each group (Fig. 7C). Most importantly, Treg cell skewing in ICAM-1MSC-treated mice was also observed, and mRNA expression of *foxp3* was also up-regulated (Fig. 7D). Th1 serum cytokine concentration of IFN- γ obviously decreased in the group treated with ICAM-1 MSCs (Fig. 7E), while Treg related serum cytokine concentration of IL10 was elevated in mice treated with ICAM-1^{high}MSCs compared to GVHD group (Fig. 7F).

Discussion

GVHD presents a major complication after allogeneic bone marrow transplantation. Idiopathic pneumonia syndrome (IPS) may be considered as a pulmonary manifestation of GVHD. The immunosuppressive properties of MSCs have been established, and the treatment of GVHD/IPS with MSCs has been reported, which suggests that MSCs can inhibit undesirable immune responses [25, 26]. Recently, several clinical trials using MSCs infusions have been reported. However, these reports were confusing, as they did not show any benefit after MSCs infusion [27]. The experimental basis of MSCs is somewhat limiting the clinical use of MSCs. MSCs do not readily migrate into the appropriate target tissues, and this creates a major obstacle for the use of MSCs.

Some methods have been developed to enhance the migration of MSCs to target organs. Attaching a synthetic ^{high} affinity peptidomimetic ligand (LLP2A) to integrin $\alpha 4\beta 1$ on the MSCs surface can direct MSCs to the designated sites [28]. The ^{high} expression of CXCR4 enhanced the homing and engraftment of MSCs by increasing the response to SDF-1 [29, 30]. Additionally, CCR7 overexpression enhanced the homing of MSCs to secondary lymphoid organs and thus ^{highly} intensified their *in vivo* immunomodulatory effect [31].

To our knowledge, we show for the first time, that the positive therapeutic effects of ICAM-1 MSCs in GVHD animal models with improved survival, low GVHD scores and attenuated tissue injury. Of note, transplanted ICAM-1^{high}MSCs enhanced their homing to SLOs *in vivo*. SLOs are also a strategically positioned collecting station for initiating the adaptive immune response, where a naive T cell scan the surfaces of antigen-presenting cells for cognate antigens and activates and generates armed effectors under the signaling cues from the APC [32-35]. And it was also found that in the absence of secondary stimulation even ^{high} numbers of host-reactive T cells are hardly activated in GVHD [36]. DCs, one kind of professional APC, are the most potent initiator of *in vivo* immune responses. Overexpressing ICAM-1 enhanced the immunosuppression capacity of MSCs for DCs and T cells *in vitro*. Whether the increased quantity of ICAM-1^{high}MSCs counters DCs and T cells in SLOs, it can result in enhanced immunosuppression capacity for DCs and T cells. Our results here demonstrate that the presence of ICAM-1^{high}MSCs can significantly inhibit maturation and differentiation of DCs, and regulated CD4⁺Th cell differentiation *in vivo*. Therefore, the results indicated that ICAM-1 can alter the migratory capacity of MSCs to SLOs and thus, show the enhanced immunosuppression capacity to effector cells.

The fate of the murine GVHD model is determined at least partially by the types and numbers of effector T cells. It is generally understood that Th1 cells and pro-inflammatory molecules such as IFN- γ and TNF α have been shown to be aetiological factors in the induction of GVHD [37, 38]. These pro-inflammatory molecules contribute to a systemic syndrome with variable presentations of weight loss, diarrhea and skin changes, and increased mortality. ICAM-1^{high}MSCs infusion reduced the percentage of Th1, and the expression of IFN- γ and TNF α . The presence of Treg cells was recognized as one of the critical factors in attenuating GVHD. Natural Treg cells, which are defined by their expression of CD4, CD25 and FOXP3, regulate innate and adaptive immune response [39, 40]. Treg cell impairment is associated with a loss of tolerance, as well as with GVHD [41]. So our observations suggest that the interruption of ICAM-1^{high}MSCs may affect the balance of regulatory and effector T cells in SLOs.

Several papers have shown that MSCs suppress T-cell immunity *in vitro* by both direct cell-cell contact and the soluble substances that MSCs release in the supernatants [42-44]. Our results here showed that ICAM-1^{high} MSCs could suppress DC differentiation to mDCs, and suppress the T cell immune response as shown by the MLR and LTT tests *in vitro*. Additionally, when DCs were co-cultured with MSCs-conditioned medium *in vitro*, we observed that the ICAM-1^{high} MSCs supernatant had a greater inhibition efficiency for DCs compared with other groups (unpublished data), which clearly indicates that such an effect not only depends on the physical contact between MSCs and DCs, but also on soluble factors released by ICAM-1^{high} MSCs. These soluble factors maybe not constitutively secreted by MSCs. It is important to further explore which molecules secreted by ICAM-1^{high} MSCs plays a crucial role in immune regulation.

Conclusion

Taken together, our results indicate that ICAM-1^{high} MSCs had greater migration activity to the modulatory center (SLOs) of immune responses in a mouse model. Given that MSCs express a low level of ICAM-1, it is plausible to speculate that a novel strategy could be applied to human MSCs to achieve targeted organ trafficking to attenuate GVHD.

Acknowledgements

This work was supported by the National Key and Development Program of China (grant number 2016YFC1000305) and the Origin cell Technology Group grant.

Disclosure Statement

The authors have no competing interests.

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