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ORIGINAL PAPER

Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model

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Abstract Recent studies indicate that mesenchymal stem cells (MSC) exhibit a degree of immune privilege due to their ability to suppress T cell mediated responses causing tissue rejection; however, the impact of allogeneic MSC in the setting of organ transplantation has been poorly investigated so far. The aim of our study was to evaluate the effect of intravenous donor MSC infusion for clinical tolerance induction in allogeneic skin graft transplantations in rats. MSC were isolated from Wistar rats and administered in Sprague-Dawley rats receiving Wistar skin graft with or without cyclosporine A (CsA). Graft biopsies were performed at day 10 post transplantation in all experimental groups for histological and gene expression studies. Intravenous infusion with donor MSC in CsA-treated transplanted rats resulted in prolongation of skin allograft survival compared to control animals. Unexpectedly, donor MSC infusion in immunocompetent rats resulted in a faster rejection as compared to control group. Cytokine expression analysis at the site of skin graft showed that CsA treatment significantly decreased pro-inflammatory cytokines IFN-γ and IL-2 and reduced TNF-α gene expression; however, the level of TNF-α is high in MSC-treated and not immunosuppressed rats. Results of our study in a rat tissue transplantation model demonstrated a possible immunogenic role for donor (allogeneic) MSC, confirming the need of adequate preclinical experimentation before clinical use.

Keywords Mesenchymal stem cells · Rat · Skin transplantation · CsA · Cytokine expression

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Introduction

The introduction of modern immunosuppressive therapies has improved the survival rates for solid organ allo-transplantation in humans. However, immunosuppressive drug toxicity and side effects lead to late allograft loss. Thus, the induction of a state of permanent tolerance to an allograft, defined as a state of unresponsiveness to donor antigens in the absence of long-term immunosuppressive therapy, is an elusive goal in human solid organ transplantation. Several approaches of cellular therapy have been used in an attempt to achieve immunological tolerance in non-human primate and human studies [11]. Stem cell mediated tolerance was experienced with the use of donor whole bone marrow cell (DBMC) infusions [2, 24, 26, 35, 39]. Results of published experiences suggest that donor bone marrow cells infusion

is safe and has a positive impact on chimerism and on the outcome after transplantation [32]. Patients who show a full chimerism after myeloablative therapy and bone marrow (BM) transplantation for treatment of haematopoietic malignancy will accept renal transplants and other tissue transplants from their specific BM donor without requirement for immunosuppressive therapy [6].

Mesenchymal stem cells (MSC) are cells derived from varying fetal and adult organs and have the capacity of self-renewal and of differentiating in several tissues including bone, cartilage, stroma, fat, muscle, and tendon [29]. Numerous in vitro studies demonstrate that MSC from various species can suppress T-cell proliferation, in autologous and alloreactive conditions, in response to various stimuli with cell contact dependent and independent mechanisms [1, 25, 31, 38].

In support of their in vivo immunosuppressive features are the observations that allogeneic MSC may prolong skin allograft survival in immunocompetent baboons [16], prevent the rejection of allogeneic B16 mouse melanoma cells in immunocompetent mice [7], attenuate graft versus host disease (GvHD) in mice and humans [4, 19], and have a role in the treatment of autoimmune disorders [40]. However, the impact of allogeneic MSC in the setting of organ transplantation has been poorly investigated to date. A recent work by Inoue et al. [16] on a rat organ transplant model confirms the MSC immunomodulatory properties in vitro but suggests caution for their use in vivo, as MSC injections were not effective in prolonging heart allograft survival inducing rejection instead of tolerance.

The immunogenicity of skin, the most antigenic tissue in the body, is the major obstacle for the skin allograft survival, resulting in rejection within 10–12 days without immunosuppressive therapy. Tolerance to skin allografts is very difficult to induce except under particular conditions. Recently, it has been demonstrated that skin allograft survival is prolonged by the injection of donor epidermal cells together with bone marrow cells [28]. The potential for the use of BM-derived MSC in prolonging skin graft survival was reported by the study of Bartholomew et al. [3]. The authors demonstrated that infusion of major histocompatibility complex (MHC)-mismatched MSC into baboons had been well tolerated and prolonged skin graft survival in vivo. Furthermore, baboon MSC suppress the proliferative activity of allogeneic peripheral blood lymphocytes (PBL) in vitro.

Based on the above-mentioned findings, our aim was to evaluate the role of donor MSC in tolerance induction in a rat model of skin transplantation. In particular, we evaluated the effects of intravenous donor MSC infusion on skin allograft survival in an experimental model using Wistar rats as donors and Sprague-Dawley (SD) rats as recipients; both as a comparison with, and in addition to, the conventional immunosuppressive treatment with cyclosporine A (CsA). In addition, we evaluated whether MSC infusion could affect the gene expression profile of some pro- and anti-inflammatory cytokines that could play a role in the modulation of the immune response for skin allograft tolerance or rejection.

Materials and methods

Animals

Wistar and Sprague-Dawley (CD) rats (Charles River Laboratories) were used in this study. Adult male Wistar rats weighing 300–350 g were used as skin graft and MSC donors. Adult male Sprague-Dawley rats weighing 300–350 g were used as skin graft recipients. For each experimental arm, we used three donors and at least 20 recipients.

Rats were housed in an accredited animal facility (Istituti Biologici-San Miniato Stabularium, Siena, Italy) and treated under conditions approved by the Local Ethical Committee of the University of Siena, Italy.

Study design

We performed a case–control study to assess the effect of intravenous administration of MSC in rats that had undergone skin allograft transplantation. The experimental design (Table 1) included four arms; in each arm we performed an autograft in two animals and an allograft in at

Table 1 Summary of graft rejection and survival after MSC injection

Group	Donor	Recipient	MSC injection (day 0 and +3)	CsA	Number of transplanted rats	Percent of not rejected grafts (%)	Percent of rejected grafts (%)	Graft survival* (days)
A	Wistar	Sprague-Dawley	No	No	13	0	100	17 ± 1.8
B	Wistar	Sprague-Dawley	No	Yes	14	43	57	15 ± 3.8
C	Wistar	Sprague-Dawley	Wistar MSC	Yes	14	57	43	19 ± 3
D	Wistar	Sprague-Dawley	Wistar MSC	No	21	0	100	13 ± 1

* Numbers represent mean ± SD days of graft survival among the animals that rejected the graft during the 30-day observation period. Immunosuppression was performed with intramuscular administration of CsA at 5 mg/kg per day. MSC were infused in the tail vein with a dosage of 5.7×10^6/kg at day 0 and 10.3×10^6/kg at day +3

least 20 animals. Group A is the control arm and comprises rats that received a skin transplantation only; rats of arm B received skin transplantation and immunosuppressive therapy with CsA; rats of arm C received skin transplantation, immunosuppressive therapy, and intravenous infusion of donor MSC; rats of arm D received skin transplantation and intravenous donor MSC infusion. For each arm, half the number of animals were inspected daily to evaluate skin graft survival (days between transplantation and rejection) and the others were sacrificed on day 10 post transplantation in order to perform two punch biopsies for histopathology and gene expression profile of the graft.

Skin graft placement and evaluation

Skin transplantation was performed according to the methods described by Taylor and Morris [37] with some modifications: in brief, after tiletamine–zolazepam sedation (Zoletil 20®) (30 mg/kg i.p.), 1.5 × 1.5 cm full-thickness skin grafts were harvested from the donor abdominal surface. After removal of the subcutaneous tissue, skin grafts were placed on the abdomen of the recipient rat, fixed with simple separate stitches, and covered with a wet buffered bandage. This procedure supports neo-vascularization and engraftment. The graft was then protected by a zinced bandage that was removed on day 7. Grafts that failed up to this point were considered as technical failure and excluded by the study. The graft was inspected daily until rejection or until the end of the experiment (30 days). Grafts were considered rejected when at least 90% of graft tissue had disappeared or had become necrotic.

MSC administration

Sprague-Dawley rats of arm C and D received Wistar MSC in the tail vein after completion of the skin graft procedure (day 0) and on day 3, post transplantation. The administered MSC dose was 5.7×10^6/kg in 200 µl of 0.9% NaCl solution at day 0 and 10.3×10^6/kg in the same conditions at day 3. Animals of arms A and B received intravenous 0.9% NaCl solution in the same volume and with the same timing.

CsA administration

Animals of arms B and C received intramuscular CsA (Sandimmun®; Novartis, Novartis Farma S.p.A., Varese, Italy) administration at a dose of 5 mg/kg per day from day 0, as previously described [5].

MSC isolation and culture expansion

Rat BM cells were collected from male Wistar rats following the Dobson procedure [9]. Briefly, once the femurs and tibiae were extracted, their proximal ends were removed, and the bones were then placed in microcentrifuge tubes supported by plastic inserts cut from 1 ml hypodermic needle casings and briefly centrifuged at 700*g* for 2 min. The marrow pellet was re-suspended in 10 ml of Hank's balanced salts solution (HBSS, w/o calcium and magnesium; Euroclone, Milan, Italy) + 1% fetal bovine serum (FBS, HyClone, South Logan, Utah, USA) and washed (300*g* for 7 min). After the cells were passed through a 22-G needle, they were re-suspended in culture medium (DMEM-Low Glucose, with L-glutamine, 25 mM HEPES and pyruvate, GIBCO™–Invitrogen, Milan, Italy, supplemented with 10% FBS), counted using a hemocytometer and seeded at 24×10^6/75 cm^2 flask. Cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Half of the complete medium was changed after 1 week and thereafter the whole medium every 3–4 days. When approximately 80% of the flask surface was covered, the adherent cells were incubated with 0.05% trypsin–0.02% EDTA (Eurobio, Courtaboeuf, Cedex B, France) for 5–10 min at 37°C, harvested, washed with HBSS and 10% FBS, and re-suspended in complete medium (primary culture, P0). Cells were then re-seeded at 10^4 cells/cm^2 in 100-mmØ dishes (P1): expansion of the cells was obtained with successive cycles of trypsinization and reseeding. MSC were identified and characterized according to published studies [20, 21].

CFU-F frequency

The number of colony forming units-fibroblasts (CFU-F) was used as a surrogate marker for MSC progenitors frequency: two 100-mmØ dishes were seeded with 1×10^6 total nucleated cells. After incubation for 14 days at 37°C in 5% CO_2 humidified atmosphere, the dishes were rinsed with HBSS, fixed with methanol and stained with Giemsa: visible colonies formed by 50 or more cells [12] were counted and reported as number of CFU-F/10^6 seeded total nucleated cells (TNC).

Osteogenic and adipogenic differentiation of MSC

MSC (10^4 cells/cm^2) were grown near confluence in 25 cm^2 flasks and then incubated in osteogenic medium (DMEM–LG with 10% FBS; 10 nM dexamethasone, 100 µg/ml ascorbic acid and 10 mM β-glycerophosphate (all from Sigma, St Louis, MO, USA), or in adipogenic medium (DMEM–LG with 10% FBS; 0.5 mM isobutyl methylxanthine, 10 µM dexamethasone, 10 µg/ml insulin, and 70 µM indomethacin, all from Sigma, St Louis, MO, USA). The medium was replaced every 3–4 days and the deposition of mineral nodules or the accumulation of lipid-containing vacuoles was revealed after

21 days with Alizarin Red-S or with Oil Red O staining, respectively.

Immunophenotyping

At the fourth or fifth passage, the morphologically homogeneous population of MSC was analysed for the expression of particular cell surface molecules using flow cytometric procedures: MSC recovered from flasks by trypsin–EDTA treatment and washed in HBSS and FBS 10% were re-suspended in flow cytometry buffer consisting of CellWASH (0.1% sodium azide in PBS; Becton Dickinson, San Jose, CA, USA) with 2% FBS. Aliquots (1.5×10^5 cells/100 μl) were incubated with the following conjugated monoclonal antibodies: CD45-CyChrome™, CD11b-FITC (in order to quantify hemopoietic-monocytic contamination), CD90-PE, CD106-PE, CD73-PE, CD54-FITC, CD44-FITC (BD Pharmingen, San Diego, CA, USA). Non-specific fluorescence and morphologic parameters of the cells were determined by incubation of the same cell aliquot with isotype-matched mouse monoclonal antibodies (Becton Dickinson, San Diego, CA, USA). All incubations were performed for 20 min and after incubation, cells were washed and re-suspended in 100 μl of CellWASH; 7-AAD (7-amino-actinomycin D) was added in order to exclude dead cells from the analysis. Flow cytometric acquisition was performed by collecting 10^4 events on a FACSort (488 nm argon laser equipped, Becton Dickinson, San Jose, CA, USA) instrument and data were analysed on DOT-PLOT bi-parametric diagrams using CELL QUEST software (Becton Dickinson, San Jose, CA, USA) on a Macintosh PC.

Histopathology

Skin graft punch biopsies (6 mmØ) were performed on day 10 post transplantation in ten animals for each group and skin samples were fixed with formalin and stained with haematoxylin–eosin for histological evaluation. Slides were analysed in a blinded fashion. Two parameters, inflammatory infiltrate (*F*) and epidermis thickness (*ET*), were analysed. Inflammatory infiltrate were scored as follows: 0 no inflammation, 1 focal and mild, 2 diffuse and moderate, 3 diffuse and severe. *ET* was calculated as the mean of five thickness measures (μm) of epidermis from granular to basal layer.

RNA extraction and real-time quantitative RT-PCR

Total RNA from rat skin was isolated by using the RNeasy midi kit (Qiagen, Germany) according to the manufacturer's protocol. Five micrograms of RNA were reverse transcribed separately using M-MLV transcriptase (Gibco BRL) and random hexamer primers (Amersham). In order to quantify the transcribed interleukin 2 (IL-2, M22899), interleukin 10 (IL-10, L02926), interferon gamma (IFN-γ, AF010466), transforming growth factor beta (TGF-β, AY550025), and tumor necrosis factor alpha (TNF-α, X66539) genes, we performed TaqMan RT-PCR (PE Applied Biosystems, Foster City, CA) in ABI-PE Prism 7700 sequence detection system. VIC-labeled rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay-on-demand #4308313) and FAM-labeled rattus IL-2 (assay-on-demand # Rn00587673_m1), IL-10 (assay-on-demand #Rn00563409_m1), IFN-γ (assay-on-demand #Rn00594078_m1), TGF-β (assay-on-demand #Rn00 579697_m1), and TNF-α (assay-on-demand # Rn01 525860_g1) TaqMan pre-developed assays (Applied Biosystems) were used. The threshold cycles (the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected) of each target product was determined and set in relation to the amplification plot of the housekeeping gene, GAPDH. All experiments were run in duplicate with 50 ng cDNA, and the same thermal cycling parameters were used. Fold change was calculated relative to control skin cycle threshold (Ct). The Ct value is defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. With the PCR efficiency of 100%, ΔCt values of the samples were determined by subtracting the average of the Ct values of the target genes from the average of the Ct values of the GAPDH gene. The relative gene expression levels were determined by subtracting the average ΔCt value of the target from the average ΔCt value of the calibrator. The amount of target (expressed as fold change), normalised to an endogenous reference and relative to a calibrator, was given by $2^{-\Delta\Delta Ct}$.

Statistical analysis

The survival allograft time in the groups was evaluated by the Kaplan–Meier method and the efficacy of treatment by the Logrank test. Differences in gene expression among different animal groups were tested by using the non-parametric Kruskal–Wallis test. Differences in gene expression between two groups were tested by using the non-parametric Mann–Whitney test. A *P* value of less than 0.05 was considered statistically significant.

Results

rMSC characterisation

Rat BM-derived MSC were successfully culture-expanded. Cells were particularly heterogeneous until the fourth–fifth passage in culture and also comprised numerous lipid

vacuoles. Haematopoietic cells were lost during the medium changes as shown by flow cytometric analysis. Primary culture cells were trypsinised and plated, reaching a cellular expansion up to a mean 10^9 factor in 3 months (Fig. 1a). After the fifth passage, the cells grew exponentially, requiring weekly passages (Fig. 1b). The CFU-F assay was used as a surrogate assay for MSC. In BM total nucleated cell population, the estimated CFU mean count resulted as 56/10^6 TNC. MSC treated with osteogenic medium formed small deposits of hydroxyapatite intensely red stained with Alizarin S (Fig. 1c). MSC treated with adipogenic medium were successfully differentiated towards adipogenic lineages: lipid vacuoles started to accumulate in the cytoplasm of the cells just after 2–3 days of stimulation and they were orange–red stained after 21 days (Fig. 1d). FACS analysis was used to assess the purity of our MSC and the existence of a homogeneous population of adherent cells (after 4–5 passages). After the exclusion of dead cells, (R1 on 7-AAD negative elements) cell population resulted uniformly positive for CD90, CD44, CD54, CD73, and CD106. There was no significant contamination of haematopoietic cells, as flow cytometry was negative for markers of haematopoietic lineage, including CD11b and CD45 (Fig. 1e).

Skin graft survival

The mean skin graft survival and percentage of rats with rejected or not rejected skin allograft is summarized in Table 1 and skin graft survival curves are shown in Fig. 2.

The graft from untreated animals (A; $n = 13$) was rejected with a mean ± standard deviation (SD) of 17 ± 1.8 days. Among CsA-treated rats (B; $n = 14$), 43% (6/14) did not reject and 57% (8/14) had a mean ± SD skin graft survival of 15 ± 3.8 days. The CsA concentration (5 mg/kg) used for the treatment of the transplanted rats significantly prolongs skin allograft survival compared to control animals (B vs. A $P < 0.01$) but it cannot induce complete engraftment in all animals. The administration of MSC in CsA-treated rats increased skin allograft survival in comparison with untreated animals (C vs. A $P < 0.001$) as 57% (8/14) rats receiving combined treatment of MSC and

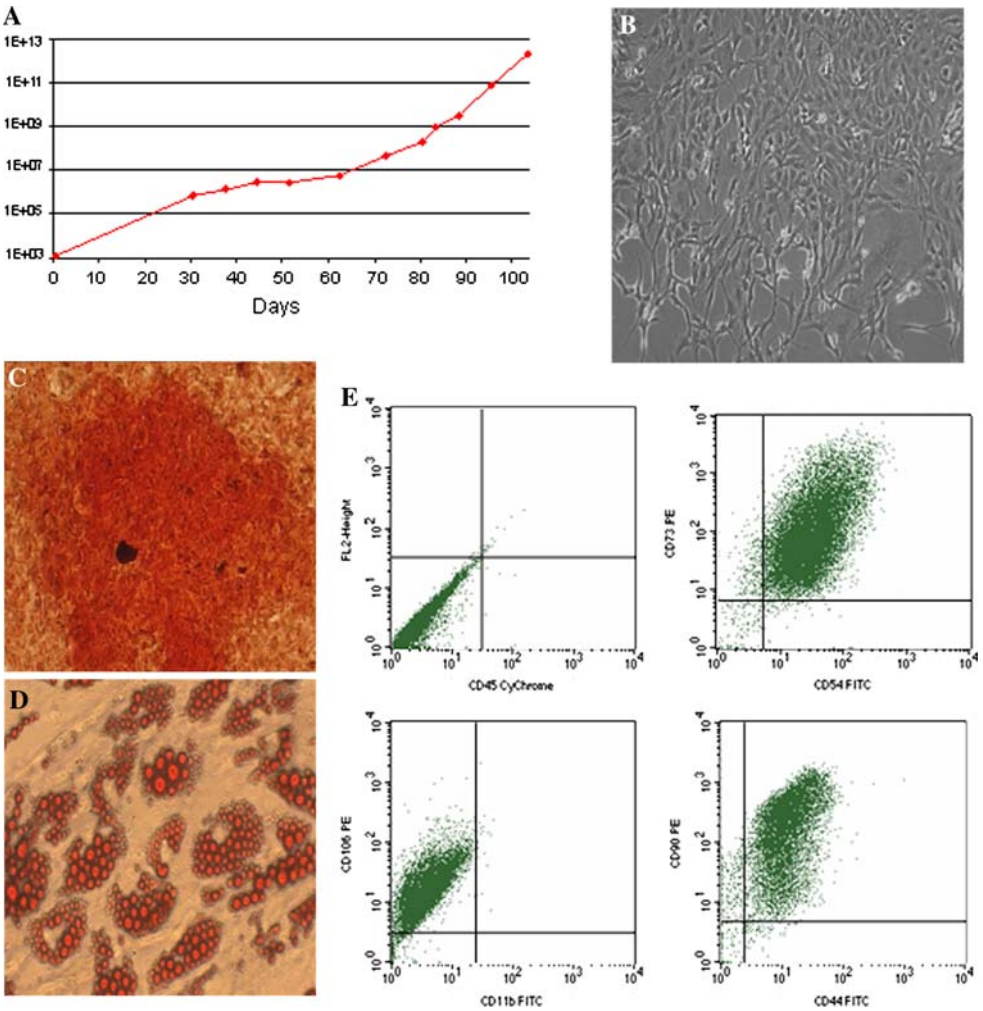

Fig. 1 Characteristics of MSC. Cells were enumerated using a haemocytometer at each passage and reached a cellular expansion up to a factor of 10^9 in 3 months: **a** Growth curve, **b** typical morphology. Differentiation into respective lineages was identified under specific conditions: **c** deposits of hydroxyapatite intensely red stained with Alizarin S after culture in osteogenic medium, **d** orange–red stained lipid vacuoles of the cytoplasm of MSC treated with adipogenic medium. Flow cytometric analysis including CD11b and CD45 antibodies showed no contamination of haematopoietic cells and positivity for classical mesenchymal markers CD44, CD54, CD73, CD90, CD106 (**e**)

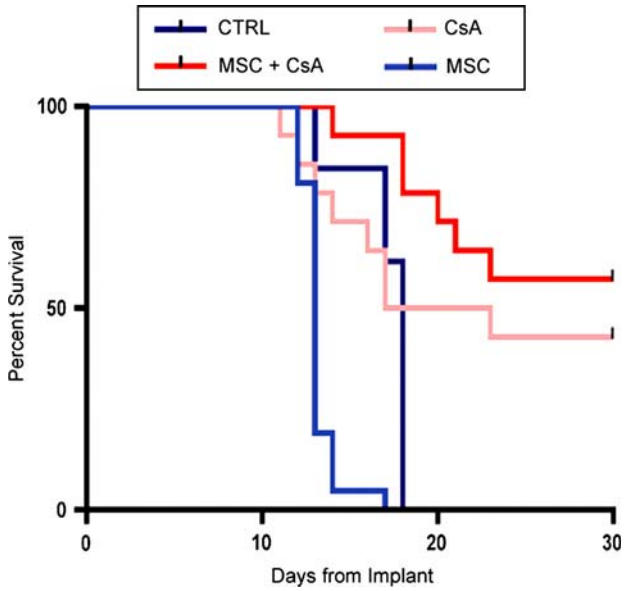

Fig. 2 Kaplan–Meier allograft survival curves for rats receiving skin allograft only (*CTRL* control, *blue line*, $n = 13$), skin allograft and CsA treatment (CsA, *pink line*, $n = 14$), skin allograft, CsA treatment and donor MSC infusion (MSC + CsA, *red line*, $n = 14$), skin allograft and donor MSC infusion (MSC, *azure line*, $n = 21$). Observation period: 30 days. Logrank test showed a significant difference among the groups ($P < 0.001$)

CsA (C, $n = 14$) had no allograft rejection and 43% (6/14) rejected the graft with a mean graft survival time of 19 ± 3.0 days. Some rats of this group were observed for 2 months and showed no allograft rejection. In contrast, rats treated with MSC only (D, $n = 21$) rejected the graft with a mean ± SD skin graft survival of 13 ± 1 days, indicating that donor MSC accelerate graft rejection (D vs. A $P < 0.001$). Kaplan–Meier curves (Fig. 2) indicate a detrimental effect of MSC administration on skin allograft survival in comparison with the other groups. Logrank analysis showed a significant difference among the four groups ($P < 0.001$). On the other hand, MSC infusion in immunosuppressed animals improved skin graft survival in comparison to the control (C vs. A $P < 0.001$) as well as MSC group (C vs. D $P < 0.001$). Combined treatment (MSC and CsA) decreased graft rejection compared with the group treated with CsA only, although the difference was not statistically significant ($P = 0.2$).

Histological evaluation

Histological analysis of skin biopsies from arm A rats showed a dense lymphocytic infiltrate in dermis, particularly in peri-follicular areas, with apoptotic keratinocytes, that is a typical graft rejection pattern (Fig. 3a). Inflammatory infiltrate (*F*) and epidermis thickness (*ET*) were analysed. Mean *F* value was 2.7 while mean *ET* value was 73.6 µm. In arm B, histological qualitative examination of skin graft showed mild-low dense lymphocytes dermal infiltrate and signs of re-epithelization in the epidermis. The graft rejection pattern was less evident and apoptotic keratinocytes were present almost exclusively in the follicular epithelium (Fig. 3b). Mean *F* value was 2.2, while mean *ET* value was 80.7 µm. In Group C, signs of mild-low graft rejection were evident, with a lichenoid infiltrate in the upper dermis and apoptotic keratinocytes in the epidermis (Fig. 3c). Signs of re-epithelization in the epidermis were also present. Mean *F* value was 2.2, and mean *ET* value was 77.6 µm. Group D was qualitatively very heterogeneus with processes of regeneration (fibroblastic activation) and with evident signs of epidermal degeneration and graft rejection only in some samples (Fig. 3d). In some fragments, the inflammatory infiltrate did not allow a clear evaluation of the type of reaction. Mean *F* value was 2.9 while mean *ET* value was 48 µm. Differences among groups were not statistically significant.

Histochemical stains were performed to exclude bacterial infections. No bacteria were identifiable in the tissue sections by Gram and Warthin–Starry stains. Neutrophils were attracted by the necrotic epidermis.

Cytokine expression analysis

Using a real-time quantitative RT-PCR method, we examined the mRNA expression of different genes in rat skin allograft on day 10 after transplantation. Figure 4 summarizes the data of mRNA levels expressed as fold changes in relation to the control group (untreated). We studied the expression of pro-inflammatory cytokine genes such as IFN-γ, IL-2 and TNF-α. IFN-γ and IL-2 gene expression were significantly different among the four groups ($P < 0.0001$ and $P = 0.023$, respectively). In particular, IFN-γ mRNA levels were significantly lower in CsA group (4.3-fold, $P = 0.004$) and MSC group (3.6-fold, $P = 0.002$) in comparison with the control group. Moreover, CsA + MSC group showed significantly higher IFN-γ mRNA levels in comparison to CsA and MSC groups ($P = 0.07$ and 0.008, respectively). IL-2 mRNA levels among the four groups showed an expression pattern similar to that observed for IFN-γ; the difference is statistically significant both in CsA and MSC groups in comparison with the untreated group ($P = 0.039$ and 0.010, respectively). The overall TNF-α mRNA levels among the four groups were statistically different ($P = 0.04$). TNF-α mRNA gene expression was lower in CsA and in CsA + MSC groups, where it reached statistical significance ($P = 0.023$) in comparison with the control group.

Analysis of anti-inflammatory molecules such as IL-10 showed no differences between MSC and untreated animals. IL-10 mRNA levels in all groups were not statistically different. Interestingly, in CsA + MSC group there

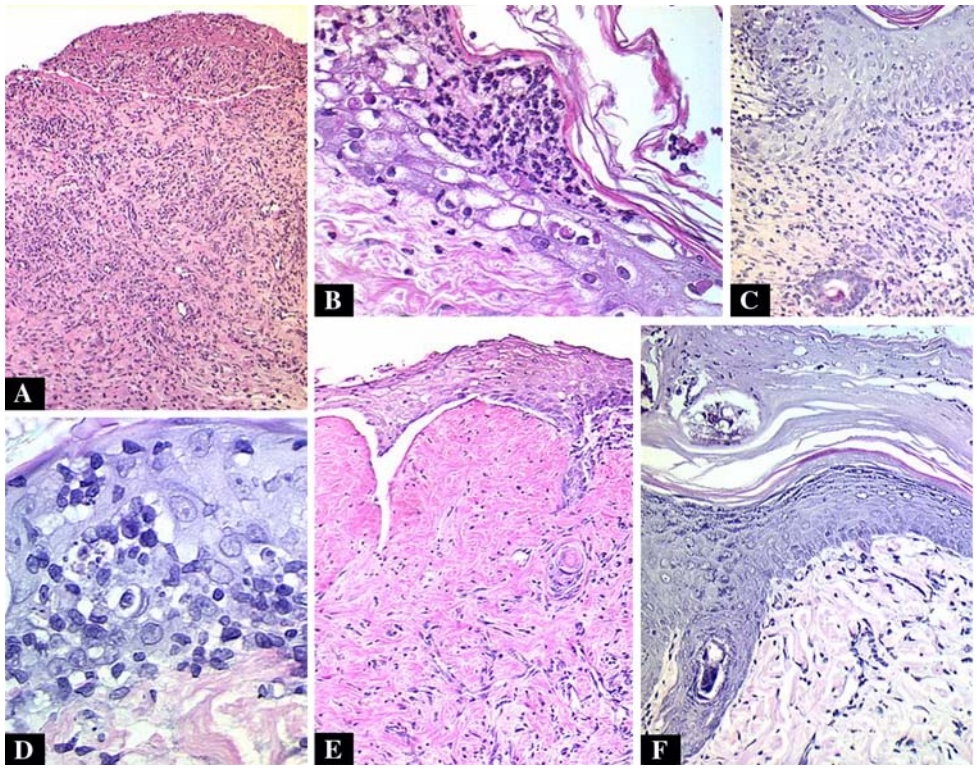

Fig. 3 **a** Haematoxylin–eosin ×50, **b** haematoxylin–eosin ×200; arm D (MSC): evident signs of epidermal degeneration and sometimes ulceration; necrotic apoptotic keratinocytes and neutrophil granulocytes recall are frequent findings; a dense lymphocytic infiltrate is present in the entire dermis. **c** Haematoxylin–eosin ×100; arm A (control): epidermis is conserved with lichenoid graft rejection and diffuse monocytic infiltrates in the dermis. **d** Haematoxylin–eosin ×400; particular of lymphocytic satellitosis. **e** Haematoxylin–eosin ×50, **f** haematoxylin–eosin ×200; arms B (CsA) and C (MSC + CsA) graft rejection pattern is less evident and apoptotic keratinocytes are present almost in the follicular epitelium, a mild–low dense lymphocytes dermal infiltrate is present and remnants of the pre-existing epidermis are detectable above a thickened corneum layer

was an up-regulation of IL-10 gene expression in relation to controls (1.6-fold), even if it did not reach statistical significance.

TGF-β gene is a well-known regulator of a number of cellular activities including tissue fibrogenesis, condrogenic differentiation and immunomodulation. The overall difference of TGF-β mRNA levels among the four groups was statistically different ($P = 0.002$). TGF-β mRNA levels were lower in CsA + MSC group than in CsA ($P = 0.002$), untreated ($P = 0.019$) and MSC ($P = 0.02$) groups.

Discussion

The demonstration of the in vitro immunosuppressive effect of MSC raised the issue of whether such property could also be reproduced in the setting of solid organs transplantation.

In this study we have examined the effects of MSC intravenous administration on skin allograft survival in an in vivo preclinical rat model. Results obtained from the present study showed that treatment with donor MSC without immunosuppressive therapy reduced the mean skin graft survival as compared to control animals, suggesting an immunogenic, rather than a tolerogenic role for these cells. CsA treatment resulted in graft survival in 43% of animals. Rats treated with combined therapy (CsA and MSC) showed a better pattern with 57% of graft survival, suggesting a synergistic effect of the two treatments, although the differences between the two groups was not statistically significant. The synergistic immunosuppressive effect of CsA and MSC for the inhibition of lymphocytes proliferation was demonstrated in vitro [22].

Accelerated graft rejection upon MSC administration was also recently demonstrated in a heart transplantation preclinical model [16]. The authors confirmed the in vitro immunomodulatory properties of MSC but do not support their tolerogenic properties in vivo, as MSC injection were ineffective in prolonging allograft survival irrespective of the dose, route of administration and origin (syngeneic or allogeneic). In addition, the authors reported that the administration of MSC, together with low dose delayed CsA administration, accelerates rejection. This discrepancy with our findings may either be due to different dosage and

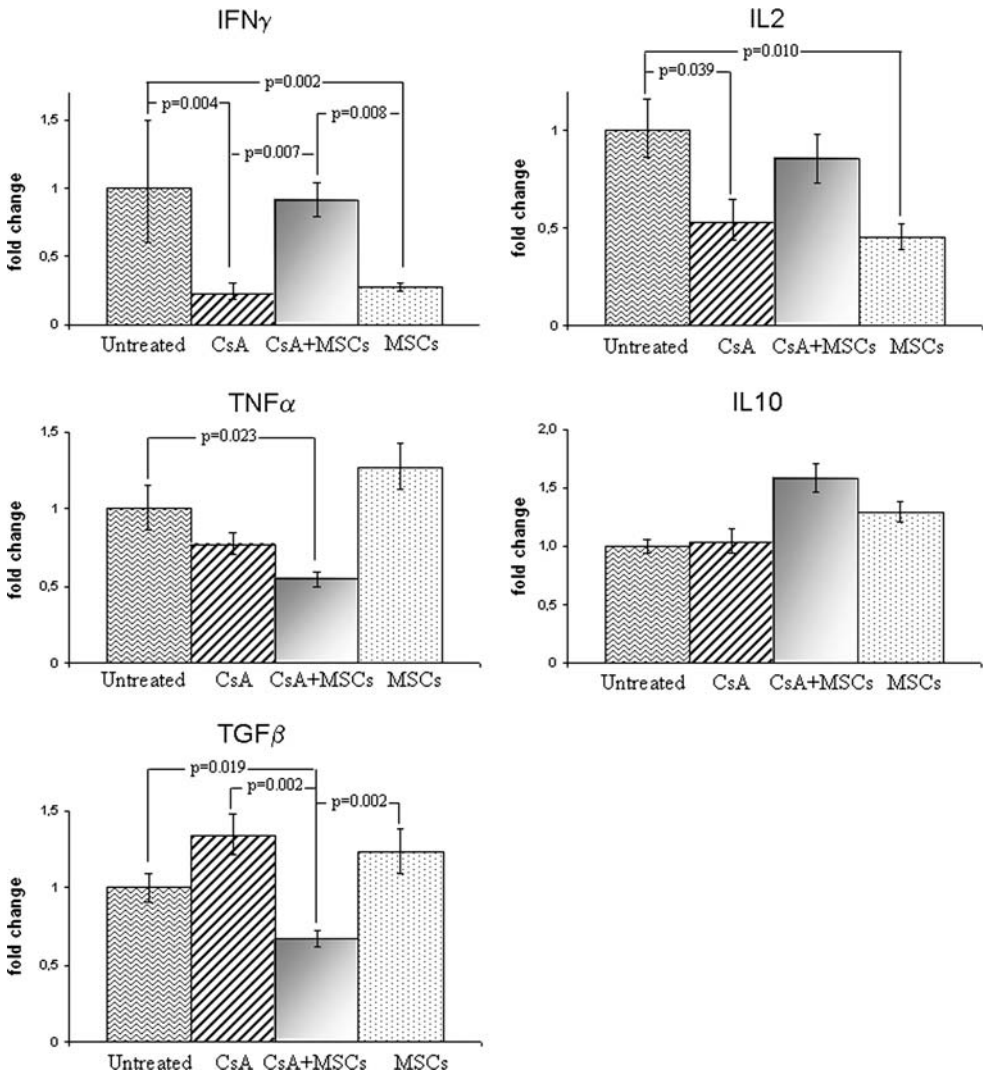

Fig. 4 mRNA expression of IFN-γ, IL-2, TNF-α, IL-10, and TGF-β genes. Data are expressed as fold changes in relation to the control group (untreated). Statistically significant differences among groups are reported *above the columns*

time of administration of CsA or due to different immunogenic properties of the organs. Moreover, our data are in agreement with another recent study [10] demonstrating that MHC-mismatched murine MSC led to a robust and specific cellular immune response in non-immunosuppressed allogeneic mice.

Results from histological analysis of skin biopsies correlated with skin graft survival data: inflammatory reaction was more intense in groups A and D which showed a rapid skin graft rejection. Although the epidermis thickness value did not reach statistical significance, in CsA-treated animals (groups B and C) the mean *ET* value was higher in comparison with the control group (A), indicating a correlation with reparative phenomena observed in the histopathological analysis. On the other hand, the MSC treated group (D) showed the lowest *ET* value in comparison to the control group that was in correlation with intense inflammatory infiltrate and sometimes with epidermal ulceration.

Our data on cytokines expression analysis at the site of skin graft showed that CsA treatment significantly decreased pro-inflammatory cytokines IFN-γ and IL-2 and reduced TNF-α gene expression. Under our conditions, CsA treatment did not affect IL-10 gene expression but the expression of TGF-β was enhanced, in agreement with studies showing that this cytokine is one of the most potent mediators of the immunosuppressive effect of CsA [17]. However, TGF-β is a polyfunctional molecule involved in several biological processes other than immunosuppression [13, 23, 33] TGF-β is a well-known regulator of tissue fibrogenesis by stimulating fibroblast proliferation, promoting collagen synthesis and inhibiting collagen degradation. Our data showed that TGF-β expression is downregulated in the combined treatment rat group C, where the fibrotic process seems less intense and there are signs of skin regeneration. In addition, cytokine gene expression profile results from a complex network of reactions. All investigated cytokines are synthesized, with peculiar timing, by different cell types present at skin graft biopsies sites and they play a pivotal role in several important processes including angiogenesis, fibrosis inflammatory

reaction and immune modulation. Unfortunately we cannot correlate the cytokine profile with histological examination between graft rejecting and graft accepting rats, as all rats used for these analyses were sacrificed at day 10 post transplantation.

Results obtained from gene expression analysis suggested a role of cytokine environment in determining the MSC effect on the outcome of solid organ transplantation. In CsA-treated rats, where the level of pro-inflammatory cytokines (TNF-α, IFN-γ) is lower than controls, MSC administration resulted in longer skin graft survival. This pattern is reverted when the MSC are administered in not immunosuppressed rats, where the level of the pro-inflammatory cytokine TNF-α is higher. This hypothesis is supported by a recent work demonstrating reversal of immunosuppressive properties of allogeneic MSC possibly mediated by TNF-α in vitro [8]. However, further investigation at systemic level is necessary. The finding that a high level of inflammation may overcome the immunosuppressive properties of MSC has also been observed in vivo in a preclinical model of allogeneic BM transplantation [36]. In this experimental setting, MSC treatment fails to prevent GvHD.

In our study, mRNA levels of the two pro-inflammatory cytokines, IFN-γ and IL-2, were increased in the combined (MSC + CsA) group with a better skin graft survival. Recently, cardiac tolerated grafts have been demonstrated to have a T cell and macrophage infiltrate with increased mRNA for Th1 cytokines, IL-2, and IFN-γ but not Th2 cytokines [30]. Moreover, in other preclinical models, gene expression of pro-inflammatory cytokines was observed in tolerated allograft [15].

Despite the large amount of data on the immunosuppressive properties of MSC in vitro [18], only few reports suggest that these immunomodulatory properties of allogeneic MSC may be translated to the in vivo setting, as little is known regarding host immune response to MSC. Discordant data are available in vivo on the use of syngeneic and allogeneic MSC in immunosuppressed or non-immunosuppressed animals [10, 14, 16, 27, 34, 36]. Varying results are probably due to different experimental animal models used, the different MSC source (human, rat, mouse) and to the different route of MSC administration (intravenously, in situ implant). In addition, controversy exists on the immunogenicity and immunomodulating potential of MSC in vivo. Few animal studies in vivo and in vitro reported that allogeneic/xenogenic MSC are rejected in an immuno-competent host [14, 16]. Recently Nauta et al. [27] demonstrated that allogeneic murine MSC are not intrinsically immunoprivileged as they can induce a memory T cell response in vivo resulting in graft rejection in a murine allogeneic BM transplantation model through a cellular immune response.

In conclusion, our study in a skin allograft transplantation model demonstrated a clear immunogenic role for donor (allogeneic) MSC as, when administered in immunocompetent rats, they stimulate graft rejection. It may be speculated that immunosuppressive effect of MSC can be fully elicited when the survival of these cells is prolonged by the simultaneous administration of CsA.

The reduction of immunosuppressive drug toxicity and side effects is an important goal in solid organ transplantation but further in-depth studies are necessary to demonstrate the efficacy of MSC treatment in immunosuppressed animals and to reach a clear definition of MSC therapeutic potential.

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