Mesenchymal stem cells differentially mediate regulatory T cells and conventional effector T cells to protect fully allogeneic islet grafts in mice

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Abstract

Aims/hypothesis Limited information is available on the cellular interactions between regulatory T (T_{reg}) cells and mesenchymal stem cells (MSCs). In particular, a direct effect of MSCs on the survival and proliferation of T_{reg} cells has not been demonstrated.

Methods We investigated the effects of MSCs on effector T (T_{eff}) cells and T_{reg} cells, and the molecular mechanisms involved in the distinct regulation of these two cell populations by MSCs in vivo and in vitro.

Results We show that MSCs are capable of selectively suppressing T_{eff} cells and fostering the generation of T_{reg} cells. T_{eff} cells, but not T_{reg} cells, fail to respond to IL-2 and undergo profound apoptosis in the presence of MSCs. The differential regulations of these two T cell subsets by MSCs are associated with their distinct expressions of CD25, with MSCs specifically reducing the expression of CD25 on T_{eff} and sparing T_{reg} cells intact. In vivo, the administration of MSCs significantly delays the rejection of allogeneic islet grafts in adaptive transferred recipients by favouring the induction of T_{reg} cells. In this model, MSCs inhibit the

proliferation and development of alloreactive T_{eff} but potentially enhance the induction of T_{reg} cells.

Conclusions/interpretation We demonstrate that MSCs are capable of regulating T_{eff} and T_{reg} cells differentially in vitro. MSCs inhibit T_{eff} cells by inducing apoptosis and impairing the proliferative response to IL-2 in T_{eff} cells, but favour the survival and expansion of T_{reg} cells. This result is further demonstrated in mice that have undergone allogeneic islet transplantation, in which MSCs suppress alloreactive T_{eff} cells while favouring the induction of T_{reg} cells, thus protecting the islet allografts from rejection.

Keywords Immunosuppression · Islet transplantation · Mesenchymal stem cells

Abbreviations

BMFC	Bone marrow flushed cell
CFSE	5,6-Carboxyfluorescein diacetate succinimidyl ester
^3H -TdR	[^3H]Thymidine
FOXP3	Forkhead box P3
MFI	Mean fluorescent intensity
MSC	Mesenchymal stem cell
STZ	Streptozotocin
T_{eff}	Effector T cells
T_{reg}	Regulatory T cells

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Introduction

Mesenchymal stem cells (MSCs) are a rare subset of cells within the bone marrow cavity with proliferative capacity and an ability to differentiate into a variety of mesodermal tissues [1–3]. The suppressive capabilities of MSCs are

exploited to control several subsets of immune cells as well as to reduce inflammatory cytokine production [4, 5]. MSCs have been shown to inhibit effector T (T_{eff}) lymphocyte proliferation induced by alloantigens or mitogens [4, 6, 7]. Recently, we showed that MSCs exert potent immunosuppressive properties by reducing the surface expression of CD25, the alpha subunit of the IL-2 receptor, on T_{eff} cells, thus blocking the IL-2 cytokine signalling pathway required for T lymphocyte activation, expansion and differentiation [8]. By targeting the detrimental alloreactive T cell response, MSCs have been shown to exhibit therapeutic potential in controlling or preventing graft rejection in many experimental animal models of organ transplantation [9–11].

CD25⁺CD4⁺ regulatory T cells (T_{reg}), which account for 5–10% of CD4 T cells, are an important cellular subset with immunosuppressive activity that have been demonstrated to suppress immune responses directed against allografts as well as against pathogen antigens in mice and humans [12, 13]. Rather than being subjected to MSC-mediated immunosuppression like their CD4⁺CD25[−] T_{eff} counterparts, it has been reported that T_{reg} cells can be induced by MSCs in vitro and in patients following autologous MSC therapy [14–16]. However, the precise mechanisms mediating such differential effects remain elusive. This is an important question that needs to be addressed in order to develop MSC-based immunotherapy that allows a selective targeting of alloreactive T_{eff} cells while sparing T_{reg} cells with regulatory activities.

Islet transplantation offers an attractive and effective approach to the treatment of patients with type 1 diabetes, and can lead to a normal metabolic control of glucose levels and an independence from exogenous insulin [17]. Despite great success in terms of restoring normoglycaemia, the long-term efficacy of this approach after transplantation has been limited due to chronic graft rejection [18]. Immunosuppressive regimens used in the Edmonton protocol and its derivatives [17, 19], such as tacrolimus, have been found to affect the calcineurin-dependent IL-2 production that is critical for T_{reg} function and expansion in preventing graft rejection [20]. Thus, an optimal approach would involve an improved islet transplantation protocol that would prevent graft rejection without the need for potentially dangerous long-term and non-specific immune suppression. Previous reports [21], including our own results [8], have shown that the infusion of MSCs results in a reversal of rejection episodes and a prolongation of islet function in islet-transplanted animal models, cementing the rationale for the therapeutic application of MSCs in allogeneic islet transplantation. The precise mechanisms involved in the beneficial effects of MSCs in allogeneic islet transplantation, however, require further investigation.

In this study, we demonstrate the differential regulation by MSCs of T_{eff} and T_{reg} cells with regard to their

differences in expansion, survival and response to IL-2. Our results show that MSCs are capable of fostering the survival and expansion of T_{reg} cells while inducing potent apoptosis and an impaired proliferative response to IL-2 in T_{eff} cells. To explore the functionality of MSCs with regulatory activity in vivo, we used a mouse islet allograft transplantation model with BALB/c $Rag^{-/-}\gamma^{-/-}$ mice, deficient in T, B and natural killer cells, as recipients. Using these, we analysed the rejection response against MHC fully mismatched C57BL/6 islet allografts and assessed the impact of MSCs with regulatory activity on the modulation of this response in vivo.

Methods

Mice C57BL/6 (B6, H2b), BALB/c (H2d), and BALB/c $Rag^{-/-}\gamma^{-/-}$ mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were bred and housed under specific pathogen-free conditions at the Tongji Medical School Facilities for Animal Care and Housing, and were between 6 and 8 weeks of age at the time of the first procedure. All animal studies were carried out according to the guidelines of the Chinese Council on Animal Care and were approved by the Tongji Medical College Committees on Animal Experimentation.

MSCs Bone marrow cells were collected from the femurs and tibias of BALB/c mice by a standard flushing method, and cultured according to the protocol previously described [8]. The identification of all primary MSCs was confirmed by morphology, phenotypic analysis and their capability to differentiate into adipocytes and osteoblasts under appropriate culture conditions, as previously described [8]. All experiments were set up within passage 10 of the MSC cultures.

Selection of CD4⁺CD25[−] T cells CD4⁺CD25[−] T_{eff} and CD4⁺CD25⁺ T_{reg} cells were purified from the spleen of BALB/c mice using CD25 MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Each preparation used in this study was greater than 95% pure by FACS analysis.

Proliferation assay Standard 3-day cultures were set up in 96-well round-bottomed plates using 1×10^5 T_{eff} or T_{reg} cells stimulated by CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA, USA) in a 1:1 ratio with the number of T cells in the presence of 2×10^4 MSCs. Thymidine was added during the last 18 h of culture before measuring the incorporation of [³H]thymidine (³H-TdR) using a liquid scintillation counter.

Flow cytometry Single-cell suspensions were generated from spleen and lymph nodes, and red cell lysis was performed by brief exposure to deionised water. Aliquots were then divided for various panels of analysis. T_{reg} cells were

identified by co-expression of CD4 and CD25, and intracellular detection of forkhead box P3 (FOXP3). For cytokine analysis, cells were re-stimulated for 5 h with PMA (Sigma-Aldrich, St Louis, MO, USA) before being stained for intracellular IFN- γ , IL-10, IL-4 and TGF- β (BD Biosciences, San Diego, CA, USA).

Induction of diabetes BALB/c $Rag^{-/-}\gamma^{-/-}$ mice were rendered diabetic by a single intravenous injection of 200 mg/kg

streptozotocin (STZ; Sigma-Aldrich). The dose of STZ was titrated to give a level of about 20 mmol/l blood glucose at the day of islet transplantation.

Islet isolation Islets were isolated from mouse pancreata (C57BL/6) by a discontinuous Ficoll gradient centrifugation as previously described [8], and were counted under a microscope after dithizone staining.

Adoptive transfer of cells and islet transplantation A total of 500 IE C57BL/6 islets were transplanted into BALB/c $Rag^{-/-}\gamma^{-/-}$ mice. Islets were aggregated in a clot of recipient (BALB/c) strain blood, and inserted into a subcapsular pocket of the left kidney. The grafts were allowed to normalise for 2 days before the adoptive transfer of allogeneic splenocytes. BALB/c $Rag^{-/-}\gamma^{-/-}$ mice were reconstituted intraperitoneally with 1×10^6 splenocytes from naive BALB/c mice, or with 3×10^6 BALB/c MSCs or bone marrow flushed cells (BMFCs). Blood glucose levels were determined using a blood glucose meter (Accu-Chek Advantage Meter; Roche Diagnostics, Basel, Switzerland). Rejection was defined as a blood glucose level of more than 14.5 mmol/l for at least two consecutive days. In same experiments, graft function was determined by removing the kidney containing the transplanted islets.

Immunofluorescence To visualise the functional islets transplanted beneath the kidney capsule, kidneys were frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and 10 μ m sections were stained for insulin according to the protocols previously described [8]. Nuclear counterstaining was performed using Hoechst dye, and sections were viewed with a Leica epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Fig. 1 **a, b** A total of 2×10^5 CD4⁺CD25⁺ T_{eff} cells from naive BALB/c (H2d) mice were stimulated by anti-CD3/CD28 Dynabeads in a 1:1 bead:cell ratio alone (no MSCs) or in the presence of a graded number of MSCs in each well of a 96-well plate for 72 h. CFSE fluorescence intensities were analysed by flow cytometry. The percentage of cells proliferating was calculated by dividing the number of cells undergoing division by the total number of T cells present in the culture. Standard 3-day cultures were set up in 96-well round-bottomed plates using 1×10^5 CD4⁺CD25⁺ T_{eff} cells (**c**) or CD4⁺CD25⁺ T_{reg} cells (**d**) stimulated by CD3/CD28 Dynabeads in a 1:1 bead:cell ratio in the presence of 2×10^4 BALB/c MSCs. To obtain cell counts, a fixed number of Calibrite beads were added to the cells, and the ratio of 7-amino-actinomycin D (7-AAD)⁺ T cells to beads was determined by flow cytometry. Absolute numbers of viable T cells were calculated by multiplying this ratio by the absolute number of beads added to the cells. **e** The numbers of CD4⁺CD25⁺FOXP3⁺ cells were obtained by multiplying the percentage of these cells by the number of Calibrite beads added to the cultures prior to acquisition. Data are represented as mean $\pm$ SD. ** $p < 0.01$, T_{reg} cells co-cultured with MSCs (+MSCs) vs T_{reg} cells cultured alone (no MSC). Results are the average of at least three experiments of identical design

Statistical analysis Data are expressed as mean $\pm$ SE. Kaplan–Meier analysis was used for survival analysis with significance set at $p < 0.05$. The Student's *t* test was used for two-tailed comparison.

Results

MSCs suppress the proliferation of T_{eff} but not T_{reg} cells MSCs obtained from BALB/c bone marrow cells are spindle-shaped, with a typical MSC phenotype—positive for CD44, CD73, CD105, and negative for CD11b—distinct from that of fibroblasts (data not shown). Under the appropriate culture conditions, the MSCs can be differentiated into adipogenic and osteogenic lineages, as verified by oil red O and Alizarin red staining (for adipogenic and osteogenic induction) [8].

In order to examine the effect of MSCs on T_{eff} responses in vitro, T_{eff} cells were stimulated with anti-CD3/CD28

beads with or without a graded number of MSCs. As shown in Fig. 1a,b, T_{eff} cells proliferated vigorously in response to anti-CD3/CD28 stimulation, and 93.2% of T_{eff} cells lost 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescence intensity; however, MSCs profoundly prevented T_{eff} proliferation in a dose-dependent manner, resulting in a potent inhibition of cell proliferation at a ratio of 1:10 or 1:20 (MSC : T cell) respectively.

To compare the regulatory effects of MSCs on the T_{eff} and T_{reg} populations, $CD4^+CD25^-$ T_{eff} and $CD4^+CD25^+$ naive T_{reg} cells were isolated from single-cell suspensions of splenocytes, and were stimulated with anti-CD3/CD28 beads alone or in the presence of MSCs. Absolute cell numbers were quantified by flow cytometry after adding Calibrite beads (BD Biosciences) to the cultures. As shown in Fig. 1c, the presence of MSCs resulted in a significant reduction of the T_{eff} population. Interestingly, in a converse regulatory fashion, MSCs induced the expansion of T_{reg} cells, as demonstrated by a fourfold increase in the number

Fig. 2 **a** Exogenous IL-2 was added to the MSC– T_{eff} (white bars) or MSC– T_{reg} (black bars) co-cultures at concentrations of 5, 50 and 200 IU/ml. After 72 h, cell proliferation was measured from the incorporation of 3H -TdR added during the last 8 h of culture. Data are represented as mean $\pm$ SD. Results are the average of three experiments of identical design. **b** $CD4^+CD25^-$ T_{eff} (squares) or $CD4^+CD25^+$ T_{reg} (triangles) cells from naive BALB/c mice were stimulated with anti-CD3/CD28 beads in the presence of MSCs (MSC:T cell ratio 1:5) for 72 h. T cells were purified by anti-CD4 magnetic beads (Miltenyi Biotec) and cultured with 5, 50 and 200 IU/ml IL-2 for 72 h. Cell

proliferation was analysed by 3H -TdR incorporation. Data from three independent experiments, each performed in triplicate, are presented as mean $\pm$ SD. CPM, counts per minute. **c** $CD4^+CD25^-$ T_{eff} and $CD4^+CD25^+$ T_{reg} cells were cultured at a density of 1×10^5 per well in 96-well plates, and stimulated with anti-CD3/CD28 beads alone or in the presence of MSCs (2×10^4). Annexin V staining of T_{eff} and T_{reg} cells after 72 h of culture was analysed by flow cytometry. ** $p < 0.01$, NS= $p > 0.05$. The data are mean percentage $\pm$ SD of Annexin V-positive cells from three individual experiments

of T_{reg} cells co-cultured with MSCs compared with T_{reg} cells cultured alone ($5.44 \pm 1.80 \times 10^5$ vs $1.34 \pm 0.29 \times 10^5$, $p < 0.01$; Fig. 1d). The expanded cell populations induced by MSCs were defined as dominantly cells of the $CD4^+CD25^+FOXP3^+$ T_{reg} compartment ($p < 0.01$; Fig. 1e).

MSCs differentially regulate the survival and IL-2 response of T_{eff} and T_{reg} cells The addition of exogenous IL-2 has already been shown to restore T cell proliferation in many situations of in vitro cell hyporesponsiveness [22]. We examined the IL-2-driven proliferation of T_{eff} or T_{reg} cells

Fig. 3 a–g $CD4^+CD25^-$ T_{eff} and $CD4^+CD25^+$ T_{reg} cells purified from naive BALB/c mouse spleens using CD25 MicroBeads were stimulated with anti-CD3/CD28 beads for 72 h in the presence (blue line in a and b) or absence (red line in a and b) of MSCs. c Solid line, T_{reg} + MSCs; dotted line, T_{eff} + MSCs. Histograms are representative of three independent experiments. $CD4^+CD25^-$ T_{eff} (e; circles) or $CD4^+CD25^+$ T_{reg} (f; squares) cells were stimulated with anti-CD3/CD28 beads and co-cultured with MSCs (squares) for 24, 48 and 72 h, followed by surface staining for CD25. ** $p < 0.01$, T_{eff} cells co-

cultured with MSCs (T_{eff} + MSCs) vs T_{reg} cells cultured alone (T_{reg}). Data from at least three independent experiments are presented as mean $\pm$ SD. g A total of 1×10^5 T cells (BALB/c) were stimulated with anti-CD3/CD28 in a bead:T cell ratio of 1:1 in the presence of MSCs (2×10^4) for 72 h, followed by surface staining for CD4 and CD25, and intracellular staining for FOXP3. Cells were gated on 7-amino-actinomycin D (7-AAD)⁺ lymphocytes, and are represented as mean percentage $\pm$ SD from three independent experiments

when co-cultured with MSCs. As shown in Fig. 2a, T_{eff} cells in the presence of MSCs failed to respond to IL-2, and the proliferation of these cells could not be restored by the addition of exogenous IL-2. In contrast, T_{reg} cells in the presence of MSCs responded normally and proliferated in correlation with the amounts of IL-2 added into the cell cultures ($p < 0.01$; Fig. 2a).

We then considered whether the differential response to IL-2 of the T_{eff} and T_{reg} cells would remain after the MSCs had been removed. T_{reg} or T_{eff} cells co-cultured with MSCs in the primary cultures were re-isolated by positive selection and then incubated with exogenous IL-2 at different concentrations for another 72 h. As shown in Fig. 2b, T_{eff} cells stimulated with anti-CD3/CD28 in the primary cultures with

Fig. 4 **a** BALB/c $Rag^{-/-}\gamma^{-/-}$ mice were rendered diabetic by a single intravenous injection of 200 mg/kg STZ. Mice had an average blood glucose concentration of 20 mmol/l immediately prior to islet transplantation. Pancreatic islets (500 islet equivalents, IE) from C57BL/6 mice (H2b) were transplanted under the kidney capsules of STZ-induced diabetic BALB/c $Rag^{-/-}\gamma^{-/-}$ mice. The grafts were allowed to normalise for 2 days before the adoptive transfer of allogeneic splenocytes. Islet transplant recipients were reconstituted intraperitoneally with only 1×10^6 naive BALB/c splenocytes as effector populations, or with these together with 3×10^6 BALB/c MSC or BMFCs. Rejection was defined as a blood glucose level greater than 14.5 mmol/l for at least two consecutive days. Continued function of the islet allografts was confirmed by removal of the islet-bearing kidneys and a return to hyperglycaemia. Square, +MSC; diamond, control. **b** Islet allograft survival. Square, +MSC; diamond, control; triangle, +BMFC. **c** Kidneys transplanted with islet grafts were excised at the time of rejection or at the endpoint of this study. Immunofluorescence examination of islet grafts in MSC-treated recipients revealed an intense staining of insulin-producing islets beneath the kidney capsule (c; +MSCs), whereas in the BMFC-treated (BALB/c, +BMFC) and control (control) recipients, the insulin-positive islet tissue was not detected at the time of rejection. **d,e** At 7 days post-transplantation, the area of kidney containing the islet grafts was dissected. After haemolysis, a single-cell suspension of the islet graft was stained for CD3, CD4 and FOXP3. Cells were gated on a lymphocyte fraction for flow cytometric analysis. Data shown are representative of one individual experiment with $n=3$ for each group

MSCs responded poorly to exogenous IL-2. Intriguingly, T_{reg} cells co-cultured initially with MSCs proliferated vigorously in response to IL-2 in secondary cultures.

To determine whether the different responses to IL-2 of the T_{reg} and T_{eff} cells were related to the alteration in cell survival, we analysed the apoptosis of these two T cell subsets when co-cultured with MSCs. As shown in Fig. 2c, the presence of MSCs led to substantial apoptosis of the T_{eff} cells: 81.8% of T_{eff} cells co-cultured with MSCs were apoptotic, in clear contrast to a background level of apoptosis in T_{eff} cells alone. Intriguingly, the addition of MSCs led to no statistical difference in T_{reg} apoptosis (NS, $p>0.05$), and T_{reg} cells did not undergo apoptosis as T_{eff} cells did in response to MSC treatment ($10.8 \pm 1.4\%$ vs $81.8 \pm 6.9\%$, $p<0.01$; Fig. 2c). Taken together, these results demonstrate that MSCs mediated a selective inhibition of T_{eff} cells by inducing apoptosis and preventing their proliferation in response to IL-2, but failed to affect the survival and proliferation of the T_{reg} cells.

T_{eff} , but not T_{reg} , cells lose surface expression of CD25 in the presence of MSCs We and other groups have previously demonstrated that the MSC-mediated suppression of T cell proliferation is related to a significant reduction in CD25 expression on the activated T cells [8, 23, 24]. CD25, known as the alpha subunit of the IL-2 receptor, is constitutively expressed on the surface of T_{reg} cells and plays an important role in the survival and expansion of these cell populations [25–27]. We therefore sought to determine whether the different response to IL-2 of T_{eff} and T_{reg} cells induced by

MSCs was related to the expression of CD25 on these two T cell subsets.

T_{eff} and T_{reg} cells were stimulated with anti-CD3/CD28 beads (in a cell:bead ratio of 1:1) alone or co-cultured with MSCs for 72 h, and the expression of CD25 was evaluated by flow cytometry. As shown in Fig. 3a, the presence of MSCs led to a substantial reduction in CD25 expression on activated T_{eff} cells. CD25 expression (measured as mean fluorescent intensity [MFI]) of the T_{eff} cells was reduced from $2577 \pm 244 \times 10^3$ to $432 \pm 198 \times 10^3$ after the addition of MSCs (T_{eff} vs T_{eff} + MSCs, $p<0.01$; Fig. 3d). In contrast, MSCs barely affected the expression of CD25 on the T_{reg} cells. Bead-activated T_{reg} cells co-cultured with MSCs expressed CD25 to a similar extent as they did in the absence of MSCs (2614 ± 346 of T_{reg} vs 2812 ± 174 of T_{reg} + MSCs, N.S. $p>0.05$; Fig. 3b, d). This differential regulation of T_{eff} and T_{reg} cells by MSCs appeared to be specific to CD25 as the expression of CD69, another activation marker, was unaffected on T_{eff} cells compared with T_{reg} cells after co-culture (Fig. 3c).

We then examined the dynamic expression of CD25 on T_{eff} and T_{reg} cells when co-cultured with MSCs for 24, 48 and 72 h. Our results showed that, although the presence of MSCs had no effect on T_{reg} cells, the addition of MSCs resulted in a significant reduction in CD25 expression by T_{eff} after 2 days ($p<0.01$, Fig. 3e, f), suggesting the differential regulation by MSCs of the surface expression of CD25 in T_{eff} and T_{reg} cells. Additional evidence for this differential regulation is that the presence of MSCs resulted in a statistically significant reduction of CD25 expression only on $CD4^+CD25^+FOXP3^-$ T_{eff} cells, whereas there was no effect on $CD4^+CD25^+FOXP3^+$ T_{reg} cells when the total population of T cells containing both T cell compartments was stimulated with anti-CD3/CD28 beads in culture (Fig. 3g). Thus, these results indicate that MSCs specifically modulated the expression of CD25 on T_{eff} and spared T_{reg} cells intact.

MSCs protect islet allografts from rejection To investigate the function of MSCs in mice that had received an islet transplantation, 1×10^6 splenocytes from BALB/c mice alone (control) or together with 3×10^6 BALB/c MSCs or BMFCs were intraperitoneally injected into diabetic (STZ-induced) BALB/c $Rag^{-/-}\gamma^{-/-}$ mice that had already had C57BL/6 islets transplanted under their renal capsules. Implantation of 500 islets resulted in an immediate establishment of normoglycaemia within 24–48 h post-transplant in all three groups (Fig. 4a). Blood glucose levels were typically maintained in the normal human range, between 5 and 7 mmol/l.

Islet grafts were allowed to normalise for 2 days before the adoptive transfer of allogeneic splenocytes. As shown in Fig. 4a,b, acute rejection of the islet allografts was observed

in all recipients, with a median graft survival time (MST) of 16 days after the adoptive transfer of an effector population (control, $n=5$). However, when the MSCs were co-transferred along with whole splenocytes, islet allograft survival was dramatically prolonged, with a MST of postoperative day (POD) 38 ($n=5$, +MSC vs 16 POD in a control group, $p<0.01$; Fig. 4b). This effect was due solely to the survival of the transplanted islets and not to endogenous islet regeneration, as demonstrated by the fact that removal of the transplanted islets by nephrectomy in normoglycaemic recipients at day 38 resulted in an abrupt return to hyperglycaemia (Fig. 4a, arrow). Glucose homeostasis in BMFC co-transfer animals (+BMFC) was comparable to that in the untreated control group ($n=4$, +BMFC vs control, $p>0.05$; Fig. 4b), and the animals became profoundly diabetic within 15–22 days (MST=16.5 days) as a result of unmodified allograft rejection. MSC-protected grafts, which showed as insulin-positive mass, were readily detected in the kidneys (+MSC; Fig. 4c) but were completely absent from mice administered BMFCs and from the control group.

These results demonstrate the beneficial effects of MSCs on graft survival in the mouse islet allograft model, although MSCs appeared to be unable to alter the tempo of rejection in the more stringently immunocompetent recipients: both MSC-treated and control mice promptly rejected grafts post-transplantation (C57BL/6 to BALB/c, data not shown). Furthermore, the protective activities of MSCs on the survival of islet grafts correlated with a substantial reduction in T cell infiltration into the islet grafts (3.2% in the +MSC vs 11.4% in the control and 14.7% in +BMFC groups; Fig. 4d) and an induction of $CD4^+FOXP3^+T_{reg}$ cells (17.6% in the +MSC group vs 9.2% in controls; Fig. 4e).

MSCs suppress the proliferation of T_{eff} cells in vivo To explore the mechanism underlying the action of MSCs in this model, 1×10^6 CFSE-labelled splenocytes from BALB/c mice, alone or with 3×10^6 MSCs or BMFCs, were injected intraperitoneally into BALB/c $Rag^{-/-}\gamma^{-/-}$ mice that had previously been transplanted with islet allografts. Cells were recovered from the spleen, from the mesenteric lymph nodes and by peritoneal lavage of recipients at day 10 after adoptive transfer, and CFSE-labelled T cells were counted using flow cytometry. CFSE fluorescence is lost after cell proliferation, and maintenance of CFSE intensity is indicative of cells that have not undergone cycles of division. As shown in Fig. 5a, co-transfer of MSCs (+MSC) resulted in the profound suppression of T cell proliferation, with $2.0 \pm 0.3 \times 10^4$ CFSE-stained $CD3^+$ T cells detected in the spleen, compared with $2.1 \pm 0.4 \times 10^3$ in the group co-transferred with BMFCs (+BMFC) and $2.3 \pm 0.7 \times 10^3$ in the control group (Fig. 5a, b). Analysis of absolute cell numbers also showed a marked suppression of $CD3^+$ T cell proliferation in mesenteric lymph nodes and the peritoneal cavity

Fig. 5 MSCs suppress the proliferation and activation of T cells responding to alloantigens in vivo. Single-cell suspensions (BALB/c) were incubated for 10 min at 37°C with 10 μ mol/l CFSE, washed twice in ice-cold medium and resuspended in PBS for intraperitoneal injection. A total of 1×10^6 CFSE-labelled splenocytes were injected intraperitoneally alone (control, $n=3$), or together with 3×10^6 autologous MSCs (+MSC, $n=3$) or BMFCs (+BMFC, $n=3$) into mice that had been previously engrafted with B6 islet allografts. Cells were recovered 10 days later from the spleen (a,b), mesenteric lymph nodes (LN) (c) and peritoneum (d). The quantification of the CFSE-labelled T cells in vivo was measured by FACS by gating for the $CD3^+CFSE^+$ population. Data are represented as mean $\pm$ SD

(Fig. 5c, d). These results suggested that T cell proliferation in response to islet allografting was attenuated by a co-transfer of MSCs, whereas in the group with a co-transfer of BMFCs and in the control group, there was no effect.

MSCs affect T_{eff} cell differentiation in vivo and in vitro To determine the effect of MSCs on T cell differentiation in response to alloantigen stimulation, single-cell suspensions of spleens (BALB/c) were stimulated with irradiated C57BL/6 splenocytes for 5 days alone or in the presence of MSCs. Cells from co-cultures were then analysed for cytokine production by flow cytometry. As shown in Fig. 6a, the presence of MSCs led to a significant reduction in IFN- γ production and increased IL-10 production in T

Fig. 6 a CD4⁺CD25[−] T cells from the spleens of naive BALB/c mice were prepared using a CD4⁺ T cell isolation kit. A total of 2×10^5 CD4⁺CD25[−] T cells (BALB/c) were co-cultured with 5×10^5 irradiated (3,600 rad) C57BL/6 splenocytes in the presence of 1×10^4 MSCs for 5 days. The percentages of IFN- γ -, IL-10- and IL-4-producing cells were evaluated by intracellular staining. Flow cytometric data are representative of three independent experiments. IFN- γ (b), IL-10 (c), IL-4 (d) and TGF- β (e) production by CD4⁺ splenocytes isolated from mice that had been transplanted with islet allograft under the kidney capsule before reconstitution with either 1×10^6 BALB/c splenocytes alone or together with 3×10^6 MSCs or BMFCs was analysed by FACS (** $p < 0.01$, NS= $p > 0.05$; $n = 5$ for each group). Mice reconstituted with splenocytes alone or together with BMFCs were analysed at the time of rejection; mice reconstituted with splenocytes with MSCs were analysed at the end of the observation period

cells stimulated with alloantigens, whereas BMFCs had no effect.

We further investigated the development of T_{eff} cells in islet-transplanted recipients after the administration of MSCs or BMFCs, comparing these with the controls. At the time of rejection, or at the endpoint of the observation period, spleens from BALB/c *Rag*^{−/−}*γ*^{−/−} mice adoptively transferred with splenocytes were harvested and analysed by flow cytometry. In parallel with the observations *in vitro*, MSC co-transfer dramatically prevented the production of IFN- γ by CD4⁺ T cells (+MSCs vs control, $p < 0.01$; Fig. 6b); meanwhile, treatment with BMFCs led to little

change in IFN- γ production (+BMFCs vs control, NS [$p > 0.05$]; Fig. 6b). Conversely, IL-10 production was significantly elevated in the group given MSCs compared with the BMFC-treated and control groups ($p < 0.01$; Fig. 6c). MSC treatment, however, led to no effect on IL-4 and TGF- β production, and the frequency of CD4⁺IL-4⁺ and TGF- β ⁺ cells remained unchanged in comparison with the control group (+MSCs vs control; NS $p > 0.05$; Fig. 6d, e). Hence, our data demonstrate that MSCs were capable of modulating the allogeneic rejection response against islet allografts both *in vitro* and in an *in vivo* model of allogeneic islet transplantation.

MSCs suppress alloreactivity by inducing T_{reg} cells in vivo To address whether MSC administration induced the generation of T_{reg} cells, and thus contributed to the prolonged survival of islet allografts in vivo, mesenteric lymph nodes from recipients that had undergone an islet allograft were harvested at the time of graft rejection or the endpoint of the observation, and the absolute cell numbers of $CD4^+CD25^+FOXP3^+$ cells were determined by flow cytometry. As shown in Fig. 7a, elevated frequencies of $CD4^+CD25^+FOXP3^+$ T_{reg} cells were observed in mice administered MSCs in comparison with the BMFC-treated or

control groups (+MSCs vs +BMFC, $p<0.05$; +MSCs vs control, $p<0.01$).

Next, we tested the immunological tolerance induced in the MSC-treated recipients in standard mixed lymphocyte reactions (MLR). Splenocytes from mice that had been treated with MSCs were harvested at the endpoint of observation, and those from mice treated with MSCs and mice in the control group were harvested when the islet allograft was rejected. Irradiated splenocytes from naive BALB/c, C57BL/6 and CBA mice served as antigen-presenting cells. The MLR assay revealed that splenocytes from recipients administered MSCs displayed a low level of cell proliferation in response to donor antigen-presenting cells compared with cells from the BMFC-treated group ($p<0.05$; Fig. 7b), whereas the response to third-party CBA stimulation remained normal.

Discussion

To specifically modulate aberrant immune responses such as autoimmune disorders, allograft rejection or graft-vs-host disease by immunosuppressive therapy, while sparing T_{reg} activity, it is crucial to obtain a more detailed insight into the biological differences between T_{eff} and T_{reg} cells. It is well recognised that MSCs can inhibit T cell responses induced by mitogens or alloantigens [4, 28]. Recently, it has been shown that regulatory lymphocytes are generated in co-cultures of peripheral blood mononuclear cells with MSCs, which may potentiate the MSC-mediated immunosuppressive effect [14, 16].

Despite the far-reaching applications of MSCs in clinical situations, questions remain with regard to the mechanisms underlying the actions of MSC on T_{eff} and T_{reg} cells. In this paper, we characterised the impact of MSCs on the expansion and survival of $CD4^+CD25^+FOXP3^+$ T_{reg} cells, as well as their proliferative response to IL-2, and found important differences compared with their $CD4^+CD25^-$ T_{eff} counterparts. T_{eff} cells co-cultured with MSCs failed to respond to IL-2 stimulation and were more prone to apoptosis, in contrast to T_{reg} cells, which had a normal proliferative response to IL-2. The differential regulation of T_{eff} and T_{reg} cells by MSCs may provide novel insights into the development of successful immunotherapy in order to obtain a sufficient number of T_{reg} cells for clinical purposes, while reducing the risk of eliciting an unwanted alloreactive response in vivo.

The steadily growing interest in MSC-mediated immunosuppression has involved its potential in the activation, expansion and induction of T_{reg} cells from conventional $CD4^+$ T_{eff} cells both in vivo and in vitro [29–31]. We previously demonstrated that matrix metalloproteinases -2 and -9 secreted by MSCs play an important role in MSC-

Fig. 7 **a** Mesenteric lymph nodes were harvested from islet-transplanted recipients at the endpoint of observation. Absolute numbers of $CD4^+CD25^+FOXP3^+$ T cells were calculated by multiplying the percentages of the cell populations by the absolute number of beads added to the cells. Data from more than four animals per group are represented as mean $\pm$ SD. Student's *t* tests were performed to evaluate the statistical difference. * $p<0.05$, ** $p<0.01$. **b** Splenic cells from islet-transplanted recipients that were adoptively transferred with splenocytes only (black bars), or together with MSCs (white bars) or BMFCs (grey bars), were used as responder cells in the mixed lymphocyte reactions (MLR) (* $p<0.05$; $n=4$). A total of 2×10^5 responder cells were stimulated with 5×10^5 irradiated (3600 rad) splenocytes from naive C57BL/6 donor, syngeneic BALB/c mice or third-party CBA for 72 h. Proliferation was assessed by 3H -TdR incorporation and was represented as counts per minute (CPM) $\pm$ SD. Statistical comparisons were performed using one-way ANOVA

mediated suppressive activity by reducing the surface expression of CD25 on activated T_{eff} cells [8]. Regulatory $CD4^{+}$ T cells also constitutively express CD25. As an important T_{reg} marker, CD25 has been reported to be critical in the maintenance and expansion of T_{reg} populations [26, 32]. It is also required for sustained expression of FOXP3 and enhances the suppressive capacity of T_{reg} cells [33]. We therefore aimed to study the impact of MSCs on CD25 expression of T_{reg} cells and its functional importance on cell survival and proliferation.

Intriguingly, we found that MSCs differentially regulated CD25 expression on T_{reg} and T_{eff} cells. Despite being co-cultured with MSCs, CD25 expression on T_{reg} cells remained intact, in clear contrast to the reduced CD25 expression on T_{eff} cells. The differential regulation by MSCs of CD25 expression on different T cell compartments was functionally correlated with their survival and proliferative responses to IL-2. Taking all our results together, we have for the first time demonstrated that MSCs are capable of selectively fostering the survival and expansion of T_{reg} cells, while inhibiting T_{eff} cells by inducing apoptosis and an impaired response to IL-2.

Emerging evidence has suggested that MSCs may offer therapeutic opportunities in islet transplantation by blunting the effector arm of the alloreactive response [3, 34]. Our findings that MSCs preferentially inhibit the expansion of T_{eff} cells while sparing T_{reg} activity prompted us to analyse the therapeutic potential of MSCs in an islet-transplanted mouse model. Recently, the therapeutic effects of MSCs in islet transplantation have been described in a non-primate animal model in which MSC treatment significantly enhanced islet engraftment and resulted in a reversal of rejection episodes [21].

In alignment with this study, we showed that co-administration of MSCs with allogeneic splenocytes led to a profound inhibition of the alloreactive response, a dramatic reduction in IFN- γ and an augmented production of IL-10, and as a result favoured the induction of peripheral tolerance. Our results that treatment with MSCs resulted in an enhanced production of IL-10, with no effect on the level of TGF- β , suggest that IL-10 rather than TGF- β could be involved in the functionality of these induced T_{reg} cells. Moreover, our results provide evidence that the immunosuppressive activity of MSCs occurs in a donor-specific manner since the alloresponse against a third unrelated party was largely intact. Nevertheless, it is unlikely that MSCs could be used as a stand-alone therapy in islet transplantation as the administration of MSCs apparently has no substantial therapeutic effect in immunocompetent murine recipients (data not shown). Further studies are warranted in order to optimise MSC-based therapy, especially its dosage and route, as well as possible combinations with accepted immunosuppressants.

Taken together, our results suggest that MSCs may hold the potential not only for the suppression of conventional T_{eff} cells, but also in the development of applications that could benefit transplantation through inducing the proliferation of T_{reg} cells. These findings cement the rationale for their implication in clinical islet transplantation.

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