

Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells

A. M. Madec · R. Mallone · G. Afonso · E. Abou Mrad · A. Mesnier · A. Eljaafari · C. Thivolet

Received: 26 January 2009 / Accepted: 25 March 2009 / Published online: 7 May 2009
© Springer-Verlag 2009

Abstract

Aims/hypothesis Displaying immunomodulatory capacities, mesenchymal stem cells (MSCs) are considered as beneficial agents for autoimmune diseases. The aim of this study was to examine the ability of MSCs to prevent autoimmune diabetes in the NOD mouse model.

Methods Prevention of spontaneous insulinitis or of diabetes was evaluated after a single i.v. injection of MSCs in 4-week-old female NOD mice, or following the co-injection of MSCs and diabetogenic T cells in irradiated male NOD recipients, respectively. The frequency of

CD4⁺FOXP3⁺ cells and *Foxp3* mRNA levels in the spleen of male NOD recipients were also quantified. In vivo cell homing was assessed by monitoring 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled T cells or MSCs. In vitro, cell proliferation and cytokine production were assessed by adding graded doses of irradiated MSCs to insulin B9–23 peptide-specific T cell lines in the presence of irradiated splenocytes pulsed with the peptide. **Results** MSCs reduced the capacity of diabetogenic T cells to infiltrate pancreatic islets and to transfer diabetes. This protective effect was not associated with the modification of diabetogenic T cell homing, but correlated with a preferential migration of MSCs to pancreatic lymph nodes. While injection of diabetogenic T cells resulted in a decrease in levels of FOXP3⁺ regulatory T cells, this decrease was inhibited by MSC co-transfer. Moreover, MSCs were able to suppress both allogeneic and insulin-specific proliferative responses in vitro. This suppressive effect was associated with the induction of IL10-secreting FOXP3⁺ T cells.

Conclusions/interpretation MSCs prevent autoimmune beta cell destruction and subsequent diabetes by inducing regulatory T cells. MSCs may thus offer a novel cell-based approach for the prevention of autoimmune diabetes and for islet cell transplantation.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1374-z) contains supplementary material, which is available to authorised users.

A. M. Madec (✉) · E. Abou Mrad · A. Mesnier · C. Thivolet
INSERM U870/INRA 1235, Faculté de Médecine Lyon-Sud,
165 chemin du Grand-Revoyet,
69600 Oullins, France
e-mail: madec@laennec.univ-lyon1.fr

A. M. Madec · E. Abou Mrad · A. Mesnier · C. Thivolet
INSA-Lyon,
Lyon, France

A. M. Madec · E. Abou Mrad · A. Mesnier · C. Thivolet
Université Lyon1,
Lyon, France

A. M. Madec · E. Abou Mrad · A. Mesnier · C. Thivolet
Hospices Civils de Lyon,
Lyon, France

R. Mallone · G. Afonso
INSERM U561, Hôpital Cochin St Vincent de Paul,
Paris, France

A. Eljaafari
Unité mixte HCL-Biomérieux,
Lyon, France

Keywords Cell therapy · Diabetes · Protection

Abbreviations

APC Antigen-presenting cell
CFSE 5,6-Carboxyfluorescein diacetate succinimidyl ester
IDO Indoleamine 2,3-dioxygenase
MSC Mesenchymal stem cell
PLN Pancreatic lymph node
Treg Regulatory T cell

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells, most of which reside within the bone marrow. These cells, first characterised by Friedenstein and colleagues more than 40 years ago [1], can be induced to undergo differentiation under appropriate conditions into several lineages, including cartilage, bone, adipose and stromal tissues, as reviewed recently [2, 3]. Studies conducted in both humans and animal models have demonstrated that MSCs are capable of long-term engraftment and multilineage differentiation *in vivo*.

In addition to these characteristics, which support the successful use of allogeneic MSCs in regenerative therapy, MSCs are able to modulate T cell responses and/or induce an immunosuppressive local milieu, which make them attractive potential therapeutic agents for various immunological disorders [4]. The *i.v.* administration of MSCs prolonged skin graft survival modestly but significantly [5], to an extent similar to that seen in response to the immunosuppressive agents currently in use. MSCs have also recently been demonstrated to suppress several T lymphocyte activities [6] and have been proposed as a possible treatment for acute graft-versus-host disease in humans [7]. Although the mechanisms mediating such effects are only partially understood, it is likely that both cell-to-cell contact and soluble factors are involved [8]. Another property unique to MSCs—a lack of expression of MHC class II and other surface markers such as CD11b, CD14, CD31, CD34 or CD45—results in their failure to initiate CD4⁺ T cell activation and likely explains how MSCs escape the normal process of alloantigen recognition.

Modulation of autoimmunity is therefore considered a potential novel target for MSC-based treatments. It has recently been reported that MSCs are capable of improving experimental autoimmune encephalomyelitis, the murine counterpart of human multiple sclerosis [9, 10]. In a murine model of rheumatoid arthritis, infusion of MSCs had no beneficial effects on collagen-induced arthritis [11], while another report demonstrated that allogeneic murine MSCs are able to prevent joint tissue damage in rheumatoid arthritis [12]. However, the therapeutic potential of MSCs in type 1 diabetes remains completely unexplored, as reviewed recently by Abdi et al. [13]. The NOD mouse is the foremost model used to assess preventive strategies in type 1 diabetes, as it develops a spontaneous form of autoimmune diabetes that closely resembles the human disease [14, 15]. Beta cell destruction results from progressive islet infiltration by autoreactive T cells and macrophages on a permissive genetic background. In this paper we report on the capacity of MSCs to inhibit autoimmune beta cell destruction in the NOD mouse by induction of regulatory T cells.

Methods

Isolation, culture and characterisation of mouse MSCs MSCs generated from BALB.B (H2^b) mice were a generous gift of E. Simpson (Department of Immunology and Transplantation Biology, Hammersmith Hospital, London, UK) and were cultured as previously described [8]. Before experimental use, the ability of the MSCs to differentiate into adipocytes was tested by adding 10⁻⁸ mol/l dexamethasone to the culture medium for 1 week. Oil Red O dye was used to identify adipocytes. The phenotypic properties of MSCs were also determined on the basis of CD105, CD73, CD29 and CD44 expression and the absence of any hematopoietic (*i.e.* CD45, CD14, CD11c) or endothelial (*i.e.* CD31) markers. All antibodies for these experiments were purchased from BD Biosciences Pharmingen (Le Pont-de-Claix, France). MSCs were maintained in culture for less than 20 passages in mouse Mesencult stem cell medium (StemCell Technologies, Meylan, France) before being used.

Mice NOD (H2^{g7}) mice (Taconic Europe, Laven, Denmark) were purchased and bred under specific pathogen-free conditions in our animal facility. Spontaneous diabetes starts at 12 weeks of age in females, and the incidence of diabetes in our colony reaches 76% in females and 15% in males by 30 weeks of age. Diabetes was assessed by measuring urine glucose with chemstrips (Bayer Diagnostics, Puteaux, France). C57BL/6 (H2^b, MSC-matched) mice were purchased from Charles River (L'Arbresle, France). The institutional review committee for animal experiments approved all the procedures for mouse care and animal killing.

Effects of MSCs on spontaneous and accelerated diabetes To examine the protective effects of MSCs against spontaneous diabetes, a single injection of 10⁵ MSCs was administered either *i.p.* or *i.v.* to 4-week-old NOD female mice; sham-injected mice served as controls. Accelerated diabetes experiments were performed as previously described, using the adult adoptive cell transfer model [15, 16]. Briefly, splenic CD3⁺ T cells from diabetic NOD female mice were purified using enrichment columns (R&D Systems Europe, Lille, France) and 5 × 10⁶ T cells from diabetic mice were co-injected with 10³ to 10⁶ MSCs *i.v.* into 8- to 12-week-old irradiated (7.5 Gy) male NOD mice. Control mice were injected with T cells alone. At the end of each experiment, the levels of insulinitis of five non-diabetic mice from each group were evaluated, as previously described [15, 16], using 5 μm frozen sections of pancreas as follows: 0, no infiltration; 1, peri-islet infiltration; 2, intra-islet infiltration <50%; 3, extensive infiltration (>50%). After scoring, a mean was calculated.

Flow cytometry analysis Single cell suspensions were obtained from the spleen and pancreatic lymph nodes

(PLNs) of each treated animal as previously described [16]. CD4⁺ and CD8⁺ cells were identified in the different experimental groups using appropriate fluorescent conjugated antibodies. To study cellular homing *in vivo*, either T cells or MSCs (10⁷ cells/ml) were labelled for 10 min at 37°C with 10 μmol/l 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) immediately before injection, as previously described [16]. CFSE-positive T cells in the thymus, spleen and PLNs at 3, 5 and 7 days after transfer were analysed by flow cytometry. The percentage of labelled cells was evaluated 1, 3, 5 and 7 days after cell transfer. At least 5 × 10⁵ cells were analysed for each sample.

Mononuclear cell culture and proliferation assays To examine the suppressive capacities of MSCs *in vitro*, we set up a series of allogeneic mixed lymphocyte cultures. MNCs for proliferation assays were obtained from the spleen of either C57BL/6 (H2^b) mice or NOD (H2^{g7}) mice. Cells were proliferated in round-bottomed 96 well plates (Dominique Dutscher, Brumath, France) in a total volume of 0.2 ml RPMI 1640 supplemented with 2 mmol/l glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (vol./vol.) heat-inactivated fetal calf serum (Invitrogen, Cergy-Pontoise, France). Spleen cells (2 × 10⁵/well) from NOD mice (H2^{g7}) were used as responder cells and primed with irradiated C57/BL6 (H2^b) spleen cells (5 × 10⁵/well). Cells harvested at day 7 from these primary cultures were then seeded at a concentration of 2 × 10⁵/well together with increasing numbers (10², 10³, 10⁴ per well) of irradiated (60 Gy) MSCs (H2^b) and irradiated C57/BL6 splenocytes (2 × 10⁵/well) as antigen-presenting cells (APCs). In some experiments, 100 U/ml IL-2 or 100 U/ml IFN-γ was added to cell cultures to test whether suppression corresponded with T cell anergy or defects in MHC Class II expression. Under each set of conditions, viability was assessed by the Trypan Blue exclusion test. A total of 0.0185 MBq (0.5 μCi) [³H]thymidine (PerkinElmer, Courtaboeuf, France) was added to each well 3 days later, and cells were harvested onto glass fibre Filtermat filters (Skatron Instruments, Lier, Norway) using a 96 well cell harvester (Titertek, Skatron Instruments) after an additional 24 h. A Packard Tricarb beta counter (Perkin-Elmer) was used to measure [³H]thymidine uptake. The results were expressed as the mean cpm of sextuplicates (SEs were routinely <10%).

To test whether MSCs could induce cell apoptosis, we determined indoleamine 2,3-dioxygenase (IDO) activity by measuring the conversion of tryptophan to kynurenine in culture supernatant fractions. We examined several culture conditions, including MSCs cultured alone, which were treated with IFN-γ or untreated, and MSCs co-cultured with MNCs. Tryptophan and kynurenine concentrations were determined by HPLC by H. Faure (Department of

Integrative Biology, Centre hospitalier universitaire Michalon, Grenoble, France).

Effects of MSCs on antigen-specific T cell proliferation Insulin B9–23-specific T cell lines were generated from male NOD mice. Animals were immunised subcutaneously with 100 μg insulin B9–23 peptide (amino acids 9–23 of the B chain; SHLVEALYLVCGERG) [17, 18] in complete Freund's adjuvant, together with 140 μg of a helper peptide consisting of amino acids 128–140 of hepatitis B virus core antigen (TPPAYRPPNAPIL), at the base of the tail. After 12–14 days, an *i.p.* boost was given at half dose in incomplete Freund's adjuvant without helper peptide. After another 12–14 days, mice were killed, then splenocytes were collected and pulsed for 3 h with 10 μmol/l of the same insulin peptide. Splenocytes were then washed, plated and cultured for 6–7 days with 5 U/ml recombinant human IL-2. T cell line specificity was tested with an in-house IFN-γ enzyme-linked immunoassay in the presence of fresh splenocytes pulsed with either insulin B9–23 peptide or DMSO.

For MSC suppression assays, male NOD splenocytes pulsed with 10 μmol/l insulin B9–23 peptide and irradiated at 35 Gy were used as APCs and plated at a concentration of 150,000 cells/well in 96 well round-bottomed plates. T cell lines were added at a concentration of 75,000 cells/well, together with different numbers of irradiated MSCs. After 24 h, 50 μl of the supernatant fractions were removed from each well and concentrations of IL-4, IL-10, IFN-γ were measured by a mouse Th1/Th2 ELISA Ready-SET-GO! Kit (eBiosciences, Clinisciences, Montrouge, France). After an additional 48 h, [³H]thymidine was added overnight and cells from each plate were harvested and counted the following morning.

To study the effects of MSCs on insulin-specific T cell responses, we performed CFSE dilution assays using flow cytometry. An optimal ratio of one MSC to 250 CFSE-labelled T cells was used in these experiments. To study the induction of regulatory T cells, cultures were set up as described above using CFSE-labelled T cell lines and CD4-depleted irradiated splenocytes, either in the presence or absence of 1B1 anti-IL10 receptor-blocking monoclonal antibody (10 μg/ml; kindly provided by A. Lehuen, INSERM U561, Paris, France). After 96 h, brefeldin A (5 μg/ml; Sigma, St Quentin Fallavier, France) was added overnight, after which time cells were harvested, stained with anti-CD4-PerCP-Cy5.5, anti-IL-10-R-PE (both from BD Biosciences Pharmingen) and anti-FOXP3-APC (Miltenyi Biotec, Paris, France) monoclonal antibodies and analysed on a BD FACSAria flow cytometer (BD Biosciences).

FOXP3 analysis Spleens were collected from male NOD mice, 5 days after transfer of 5 × 10⁶ T cells, either alone or

with 10^6 MSCs, as well as from untreated mice. Purified T cells were examined by flow cytometry to evaluate CD4 and FOXP3 expression. Total RNA was isolated from another set of T lymphocytes from the same experimental mice using the Trizol reagent method (Invitrogen). Total RNA (1 μ g) was reverse-transcribed in a final volume of 20 μ l containing 100 U Superscript II (Invitrogen), using 0.5 μ g random hexamer primers and 0.5 μ g oligo(dT)₁₅ primer (Promega, Charbonnières, France), 6.25 mmol/l dNTP (Promega), 0.1 mol/l dithiothreitol and 5' First-Strand Buffer (Invitrogen). The resultant cDNA was treated with 5000 U/ml RNase-H (New England BioLabs/Ozyme, Saint Quentin en Yvelines, France). Real-time RT-PCR using a LightCycler (Roche Diagnostics, Meylan, France) was performed in a final volume of 20 μ l containing 15 μ l of reaction buffer from the FastStart DNA Master Plus SYBR Green I kit (Roche Diagnostics), 10.5 pmol of each forward and reverse specific primer (Eurobio, Les Ulis, France) and 5 μ l of a 60-fold dilution of the reverse transcription product. Pairs of PCR primers were designed using the Beacon Designer software (<http://beaconsoftware.net/>, last accessed 8 April 2009), as follows. *Foxp3* primer sequences: Forward: 5'-CCCAG GAAAGACAGCAACCTT-3', Reverse: 5'-TTCTCAAC CAGGCCACTTG-3'. Samples were quantified using the LightCycler relative quantification software (Roche Diagnostics). Specific mRNA levels were expressed as a percentage relative to the level of mouse *Hprt1* mRNA.

Statistical analysis Cell transfers were analysed by comparison of survival curves using Wilcoxon's rank-sum test. Score of insulinitis, cell proliferation, cytokine analysis and *Foxp3* expression data were compared using the Student's *t* test for unpaired samples. A *p* value of less than 5% was considered statistically significant.

Results

MSCs protect NOD mice from diabetes MSCs from BALB. B (H2^b) mice constitutively expressed CD105, CD73, CD29, CD44 and MHC class I molecules, but not CD14, CD34, CD45 (haematopoietic markers), CD31 (endothelial marker) or MHC class II molecules (Electronic supplementary material [ESM] Fig. 1). The same cells exhibited multilineage differentiation potential as assessed by culturing in adipogenic medium (not shown).

To examine the protective effect of MSCs, we first conducted a series of co-transfer experiments that involved the co-injection of 5×10^6 diabetogenic T cells and an increasing number of MSCs (10^3 to 10^6) into irradiated NOD male mice. As a control, a series of 43 irradiated male NOD mice were only injected with 5×10^6 T cells from diabetic mice. The results from five independent experiments

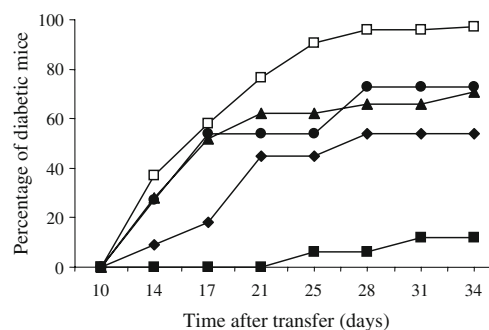


Fig. 1 MSCs confer protection against the transfer of diabetes by NOD splenic T cells. Splenic T cells (5×10^6) from NOD diabetic female mice were intravenously injected into irradiated syngeneic male recipients, either alone (white squares, $n=43$) or together with (black symbols) 10^3 (circles, $n=11$), 10^4 (triangles, $n=21$), 10^5 (diamonds, $n=11$) or 10^6 (squares, $n=15$) MSCs. Mice were subsequently followed to determine diabetes development. The results are the means of five independent experiments

are presented in Fig. 1. While the co-injection of 10^3 or 10^4 MSCs together with 5×10^6 diabetogenic T cells had no significant effect on diabetes prevalence (9/11 [81.2%] or 15/21 [71.4%] vs 42/43 [97.7%] for mice injected with 5×10^6 diabetogenic T cells alone, $p=NS$), higher concentrations of MSCs (10^5 or 10^6 per mouse) delayed the onset of diabetes and decreased the incidence of diabetes 34 days after cell transfer (6/11 [54.5%] and 2/16 [12.5%] diabetic mice, respectively, $p<0.01$ and $p<0.001$). As shown in Fig. 2, histological analysis of the pancreases of the mice at 34 days after cell transfer revealed that mice injected with MSCs showed a marked reduction in inflammation compared with those injected with diabetogenic T cells alone, and that the percentage of intact islets was higher in mice injected with 10^6 MSCs than in those injected with lower levels. The mice injected with diabetogenic T cells alone had significant insulinitis (i.e. stage 2–3) in 90% of islets. When co-injected intravenously with 5×10^6 diabetogenic T cells into irradiated NOD male mice, splenic T cells (5×10^6) from protected animals did not exert a protective effect (data not shown).

To examine the effect of MSCs on the incidence of spontaneous diabetes, we treated 4-week-old female mice with a single i.p. or i.v. injection of 10^5 MSCs. As shown in Fig. 3, the injection delayed the spontaneous onset of diabetes and decreased the incidence of the disease, with 16 out of 27 (59.3%) MSC-treated mice vs 26 out of 26 (100%) control animals becoming diabetic at 36 weeks of age ($p<0.05$).

Migration of CFSE-labelled cells To examine whether MSCs modify T cell homing to the pancreas and lymphoid organs, we performed a series of co-transfer experiments using CFSE-labelled, intravenously injected T cells to follow their migration in irradiated recipients (ESM Fig. 2 and Table 1). Although MSC-injected mice displayed

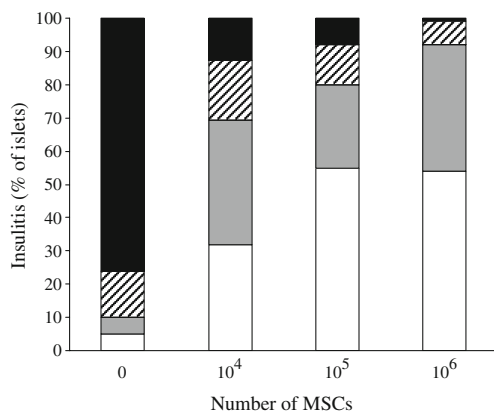


Fig. 2 MSC administration reduces the severity of insulinitis. Diabetogenic NOD spleen cells (5×10^6) were intravenously injected into irradiated syngeneic male recipients, either alone or together with 10^4 , 10^5 or 10^6 MSCs. Non-diabetic mice were killed 34 days after cell transfer and the insulinitis level scored on pancreatic sections. White fill, stage 0, no infiltration; grey fill, stage 1, peri-islet infiltration; hatched fill, stage 2, intra-islet infiltration $<50\%$; black fill, stage 3, extensive infiltration ($\geq 50\%$). A mean score was calculated from five mice per group

tendencies towards a decrease in the number of diabetogenic T cells in the spleen and an increase in the number in PLNs as compared with mice that received T cells alone, these differences were not statistically significant. We therefore conclude that the MSCs did not interfere with T cell homing.

Next, we followed the migration of MSCs in recipient mice, using CFSE-labelled i.v. injected MSCs (ESM Fig. 2). As illustrated in Fig. 4, the concentration of MSCs was significantly higher in the PLNs than in the spleen, with peak values achieved at day 5 ($4.40 \pm 0.72\%$ vs $1.86 \pm 0.14\%$, respectively, $p < 0.01$). Interestingly, these differences persisted 18 days later ($1.75 \pm 0.25\%$ vs $0.52 \pm 0.04\%$, respectively, $p < 0.01$). However, we were not able to detect any CFSE-labelled MSCs in the islets of recipient mice by direct immunofluorescence analysis of pancreatic sections from recipient mice at 5, 11 and 18 days after cell transfer (data not shown).

MSCs inhibit alloreactive NOD T cells in secondary H2-mismatched mixed lymphocyte reactions To test their ability to inhibit alloreactivity, MSCs were added to secondary allogeneic mixed lymphocyte reactions. As few as 10^2 MSCs were able to completely suppress alloreactive T cell proliferation (Fig. 5). The addition of exogenous IL-2 to cell cultures did not rescue T cells from MSC suppression (Fig. 5). Moreover, induction of MHC Class II expression by pre-treating MSCs with IFN- γ (ESM Fig. 1) prior to secondary mixed lymphocyte reactions did not result in the recovery of T cell proliferation (data not shown), which suggests that the defect in MHC class II expression cannot account for MSC suppression.

Furthermore, no IDO activity was detected under any of the mixed lymphocyte reaction co-culture conditions; the kynurenine level was <0.6 mmol/l and the tryptophan level was 4.9 mmol/l in all supernatant fractions, compared with 5.9 mmol/l and <0.6 mmol/l, respectively, in the supernatant fractions of positive controls. MSC suppression was not related to the subsequent reduction of cell number and viability measured by the Trypan Blue exclusion method.

MSCs suppress beta cell-specific T cell responses and induce IL-10-producing regulatory T cells in vitro Finally, we tested whether the MSCs could also suppress T cell responses relevant to autoimmune diabetes in vitro. We chose to study responses against the key epitope, insulin B9–23, as it has been shown to play a critical, and likely initiating, role in the autoimmune beta cell destruction [18]. As previously observed for alloreactive T cell responses, NOD insulin B9–23-specific proliferative responses were significantly suppressed by MSCs in a dose-dependent fashion, from 75% with 10^2 MSCs to 100% with 10^5 MSCs (Fig. 6a). As shown in Fig. 6b, this suppression was associated with a dose-dependent increase in IL-10 secretion ($p < 0.001$ vs no MSCs).

Insulin-specific T cell responses in the presence of MSCs were further investigated by flow cytometry (Fig. 7). Using CFSE dilution assays, we observed the inhibition of T cell proliferation in the presence of MSCs ($CD4^+CFSE^+$ T cells: 2.7% vs 0.6% in the absence and presence of MSCs, respectively) (Fig. 7a), and this effect was associated with the induction of IL-10-producing FOXP3 $^+$ regulatory T cells (Tregs) (Fig. 7b). Indeed, while some Treg induction was triggered by epitope stimulation alone (2.7% vs 6.4% in the absence and presence of peptide, respectively), the addition of MSCs to the culture significantly increased the proportion of Tregs. This MSC-mediated Treg induction

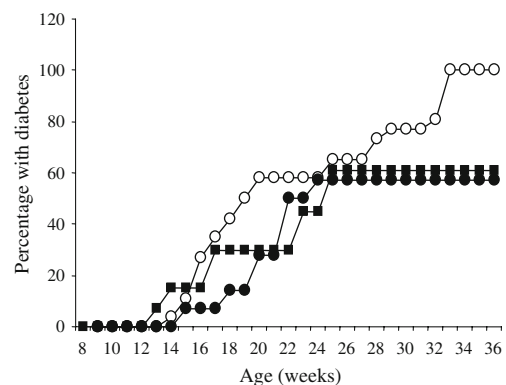


Fig. 3 MSCs protect female NOD mice from spontaneous diabetes. A single injection of 10^5 MSCs was administered either intraperitoneally (black circles, $n=14$) or intravenously (squares, $n=13$) to 4-week-old NOD female mice. The control mice were injected with PBS alone (open squares, $n=26$). Animals were subsequently followed to determine diabetes development

Table 1 Effects of MSCs on the migration of CFSE⁺ T cells

Time after transfer (days)	3		5		7	
	–	+	–	+	–	+
10 ⁶ MSCs	–	+	–	+	–	+
Percentage of CFSE-labelled T cells						
Spleen	1.79 ± 0.54	1.46 ± 0.35	1.58 ± 0.62	0.88 ± 0.30	0.79 ± 0.44	0.20 ± 0.12
PLNs	0.41 ± 0.14	0.91 ± 0.52	0.48 ± 0.02	0.71 ± 0.23	0.12 ± 0.08	0.14 ± 0.03
Thymus	0.16 ± 0.04	0.18 ± 0.08	0.04 ± 0.005	0.04 ± 0.006	0.01 ± 0.003	0.01 ± 0.002

The results are the mean±SD of three independent experiments. The percentage of CFSE-labelled T cells was determined by FACS analysis 3, 5 and 7 days after the transfer of 5×10^6 T cells. No significant difference was seen between the experimental groups

was not antigen specific (21.3% vs 20.2% in the absence and presence of the insulin B9–23 epitope, respectively). Thus, these results indicate that MSCs are able to promote the generation or expansion of Tregs producing both IL-10 and FOXP3. Importantly, however, MSC-mediated suppression was not IL-10-dependent, as blockade of the IL-10 receptor with a neutralising antibody did not prevent the inhibition of T cell proliferation (2.3% vs 0.5% in the absence and presence of MSCs, respectively) (Fig. 7c).

Tregs are induced in vivo following MSC administration To explore the possibility that the in vivo immunosuppressive action of MSCs was mediated by activation of Tregs in vivo, we analysed the frequency of CD4⁺FOXP3⁺ cells in the spleen of irradiated male NOD mice 5 days after i.v. cell transfer. In mice, at day 5, the frequency of CD4⁺FOXP3⁺ lymphocytes in the frequency in mice treated with diabetogenic T cells alone ($n=5$) was lower than the frequency in untreated mice ($2.9 \pm 0.3\%$ vs $4.9 \pm 1.2\%$, $p < 0.01$), while the

co-transfer of MSCs with diabetogenic T cells resulted in the recovery of the proportion of CD4⁺FOXP3⁺ cells (a representative experiment is shown on Fig. 8). *Foxp3* mRNA was also expressed at significantly higher levels in T lymphocytes isolated from the splenocytes of both untreated NOD mice ($n=5$) and mice receiving a mixture of diabetogenic T cell and MSCs ($n=7$), compared with the mice that received diabetogenic T cells alone ($n=5$) ($p < 0.001$) (Fig. 9). We also analysed the frequency of CD4⁺FOXP3⁺ cells in the PLNs of recipient animals. At day 5, the frequency of CD4⁺FOXP3⁺ lymphocytes was lower in mice treated with diabetogenic T cells alone ($n=3$) than in untreated mice ($p < 0.01$; $n=3$), but co-transfer of MSCs with diabetogenic T cells resulted in the recovery of the percentage of CD4⁺FOXP3⁺ cells in the PLNs ($7.6 \pm 0.8\%$; $n=3$).

Discussion

Although the immunosuppressive capacity of MSCs has been demonstrated in several medical conditions, few

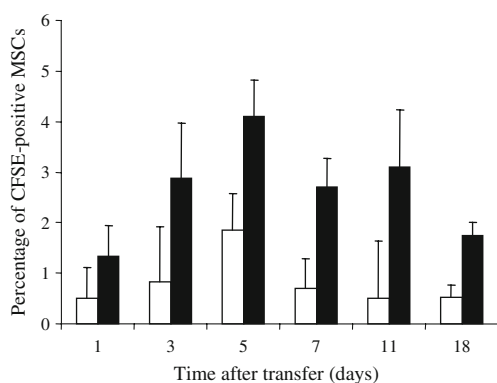


Fig. 4 Injected MSCs preferentially home to PLNs. CFSE-labelled MSCs were co-transferred with 5×10^6 diabetogenic T cells into irradiated male NOD recipients. Mice were subsequently killed at the indicated time points, and the number of CFSE⁺ MSCs in the spleen (white bars) and PLNs (black bars) were determined by flow cytometry. The graph shows the percentage of CFSE⁺ MSCs injected that were detected in the spleen and PLNs. All values are the mean±SD of measurements made in triplicate

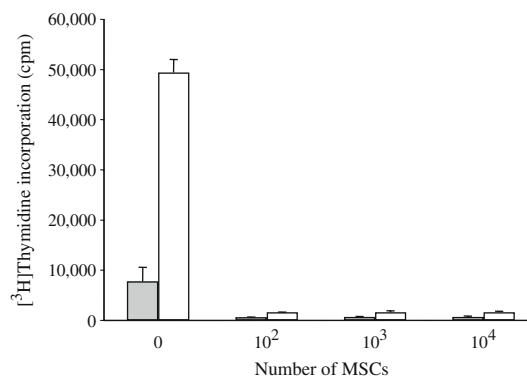


Fig. 5 MSCs suppress the proliferation of alloreactive NOD T cells. Increasing numbers of MSCs were added to secondary allogeneic mixed lymphocyte reactions. The graph shows [³H]thymidine incorporation over 25 h. Experiments were performed either in the absence (grey bars) or the presence (open bars) of IL-2. The results are the mean±SD of three independent experiments

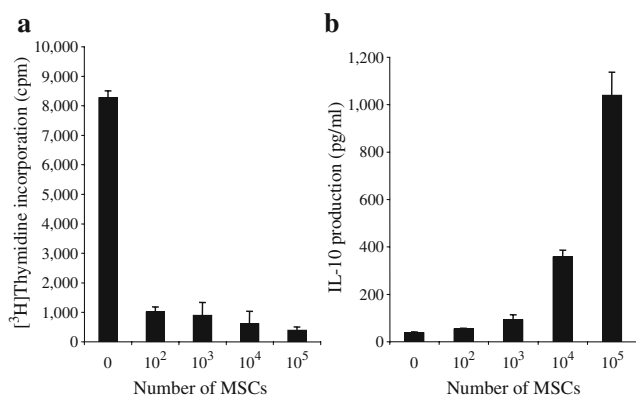


Fig. 6 MSCs suppress insulin-specific T cell responses in vitro. **a** Insulin B9–23-specific T cells were stimulated with B9–23-pulsed irradiated splenocytes in the absence or presence of MSCs. [³H] Thymidine incorporation after a culture period of 72 h is shown. Basal proliferation (i.e. on control-pulsed APCs) was <500 cpm. **b** The IL-10 level in 24 h culture supernatant fractions was measured by ELISA. All values are expressed as the mean±SD of measurements made in triplicate. The results in (a) and (b) are representative of three independent experiments. Irradiated splenocyte APCs alone did not display any proliferation or IL-10 secretion (data not shown)

studies have evaluated their capacity to inhibit T cell function in autoimmune diseases. MSCs exhibit the intriguing characteristics of being able to escape immune recognition and to inhibit immune responses. In mixed lymphocyte reactions, MSCs suppress T cell proliferation independently of MHC matching between T cells and MSCs [19, 20]. Murine MSCs have been shown to prevent experimental autoimmune encephalomyelitis in mice through the induction of peripheral tolerance against the pathogenic antigen [9, 10]. This protection was effective when the MSCs were administered at the onset of the disease or at the peak of the disease, but not after disease stabilisation. Controversial results were obtained in

collagen-induced arthritis, but recent reports have shown that MSC infusion prevented tissue damage and exerted immunomodulatory effects by educating antigen-specific Tregs [11, 12].

In the present study we examined the effects of the administration of a single dose MSCs in NOD mice, a mouse model of human type 1 diabetes. We provide strong evidence that MSCs confer significant protection against both T cell-transferred and spontaneous disease. This therapeutic effect correlates with decreased islet infiltration, MSC homing to the draining PLNs, and in vitro suppression of both allo- and beta cell-specific responses. The induction of FOXP3⁺CD4⁺ Tregs secreting IL-10 was also observed in vitro, although IL-10 did not prove to be involved in the suppressive mechanisms. Despite this apparent discrepancy, our results are in line with those of Thornton and Shevach [21] for mouse Tregs, and of Jonuleit et al. [22] for human Tregs. In both models, Tregs were induced and produced both IL-10 and TGFβ, but their suppressive activity was not dependent on either cytokine. Our results are also in line with other studies suggesting that MSC-mediated suppression may be contact-dependent in vitro but cytokine-mediated in vivo [4, 21, 22]. Indeed, this discrepancy between in vitro and in vivo MSC suppression studies is reminiscent of the conundrum regarding the mechanisms of suppression of murine Tregs, which also display distinct behaviours in vitro and in vivo [23]. Our results also highlight important differences between mouse and human MSCs [24, 25]. Glennie et al. [25] reported that human MSC-mediated inhibition induces an unresponsive T cell profile that can be reverted by the addition of exogenous IL-2. Interestingly, IL-2 did not restore T cell proliferation in our experiments, suggesting that the MSCs did not induce classical T cell anergy [25]. Moreover, since the number and viability of the cells were not affected, we conclude that

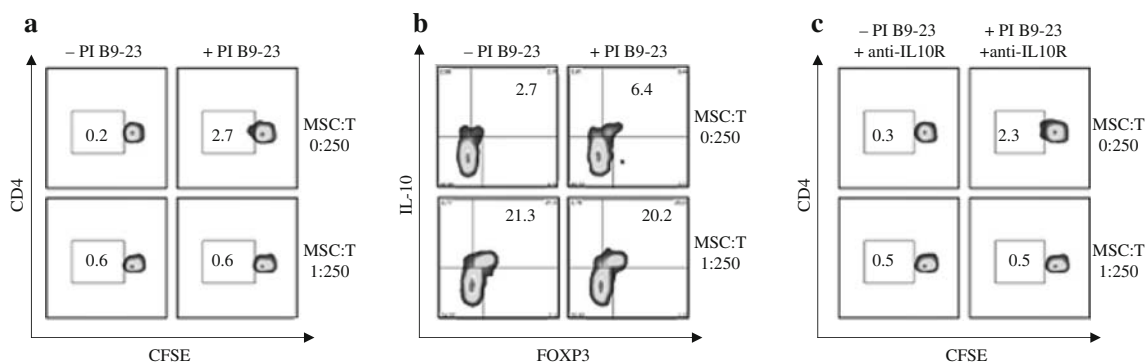
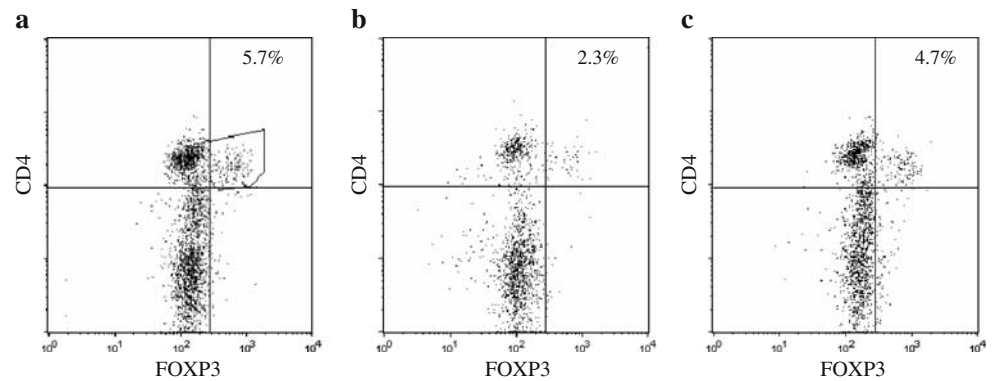


Fig. 7 MSC suppression of insulin-specific T cell responses is associated with induction of IL-10-producing Tregs. **a** Insulin B9–23 peptide (PI B9–23)-specific proliferation in the absence (upper panels) or presence (lower panels) of MSCs using CFSE-labelled insulin-specific T cells. Cells were gated on the CD4⁺CFSE⁺ fraction. **b** Induction

of IL-10⁺FOXP3⁺CD4⁺ T cells. These experiments were conducted under the same conditions as those in panel a, with cells gated on the CD4⁺CFSE⁺ fraction. **c** PI B9–23-specific proliferation in the presence of an anti-IL-10 receptor blocking monoclonal antibody

Fig. 8 MSCs induce Tregs in vivo. The frequency of CD4⁺FOXP3⁺ T cells isolated from the spleen of control NOD mice (a), mice injected with T cells from diabetic animals alone (b) or together with MSCs (c). Plots representative of five experiments are shown



MSCs did not induce T cell apoptosis. One other established mechanism of human MSC-mediated suppression is IDO activity upregulation. IDO activation is induced by IFN- γ , and IDO catalyses the conversion of tryptophan into kynurenine [26, 27]. Activation of IDO thus causes tryptophan depletion, resulting in reduced lymphocyte proliferation [28]. In contrast to previous findings in human MSCs stimulated by IFN- γ [29], we did not detect significant IDO activity in MSC-suppressed mixed lymphocyte reactions, even in the presence of IFN- γ (not shown). Thus, the mechanism of MSC-mediated suppression remains controversial. However, although we do not have direct evidence, our data are compatible with a contact-dependent mechanism of suppression, perhaps mediated by an intermediate mediator linking the MSCs to Treg induction. In this respect, Li et al. recently showed that Treg-inducing dendritic cells can be generated through direct cell-to-cell contact between haematopoietic stem cells and MSCs by activation of the Notch

signalling pathway [30]. Whether a similar mechanism is at play in our model requires further investigation.

The administration of a single dose of adult MSCs was sufficient to prevent the onset of diabetes. One possible explanation for this efficacy could be related to the regeneration of pancreatic islets by MSCs; however, we considered this hypothesis unlikely since protection was obtained during the early inflammatory phase of the disease. Moreover, Urban et al. [31] have recently reported in a streptozotocin-induced mouse model of diabetes that MSCs cannot induce pancreas regeneration by themselves, as they require co-injection of bone marrow stem cells. In addition, clinical efficacy in our model was correlated with greatly decreased insulinitis scores, further suggesting immune-mediated, rather than regenerative, mechanisms. In a recent paper, Tritt et al. [32] demonstrated that a decrease in the frequency of CD4⁺ Tregs contributes to the onset of autoimmune diabetes. We therefore measured the expression of FoxP3 on cells in the spleen and PLNs of MSC-treated or untreated mice and found that MSC treatment increased FOXP3 T cell frequency. These results correlate with the in vitro findings showing that the immunosuppressive activity of MSCs is correlated with the induction of FOXP3⁺ T cells. Following this model of Treg-mediated suppression by MSCs, it was surprising to observe that splenic T cells from protected animals were not able to transfer protection. This discrepancy may be due to the preferential homing of induced Tregs towards PLNs. Indeed, MSCs were found to preferentially home to PLNs rather than the spleen. It is therefore reasonable to hypothesise that MSC-induced Tregs may also preferentially reside in PLNs or in the islets themselves. Supporting this possibility, Tritt et al. [32] reported that Tregs preferentially localise in the pancreatic environment, where they suppress the accumulation and function of effector T cells.

In conclusion, we have demonstrated the therapeutic potential of the administration of a single dose of adult MSCs to prevent the development of type 1 diabetes beta cell autoimmunity. This clinical efficacy correlates with the

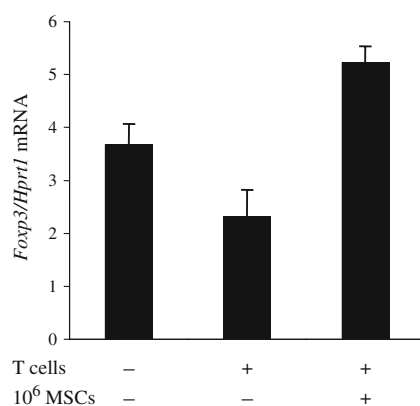


Fig. 9 *Foxp3* expression levels in T splenocytes by real-time PCR. The addition of MSCs to T lymphocytes from diabetic mice significantly increased the level of *Foxp3* expression ($p < 0.001$). Values are the mean \pm SD of relative expression ratio as compared with the *Hprt1* reference gene (untreated mice and mice treated with diabetogenic T cells, $n = 5$; mice treated with diabetogenic T cells and MSCs, $n = 7$)

suppressive effects of MSCs on both allogeneic and autoimmune responses in vitro, and with the induction of Tregs both in vivo and in vitro. MSC suppression does not require MHC matching or any additional immunosuppressive therapy. This may have important clinical implications for type 1 diabetes immune interventions.

Acknowledgements We are indebted to E. Simpson (Department of Immunology and Transplantation Biology, Hammersmith Hospital, London, UK) for the gift of the MSCs, to A. Durand (INSERM U870/INRA 1235/INSA/Université Lyon1/Hospices Civils de Lyon, Lyon, France) for excellent technical assistance and for assistance with the NOD colony, and to J. Plumas (Laboratoire R&R, EFS Rhône-Alpes, Grenoble, France) for analysis of the IDO results. This study was supported by INSERM grants to A. M. Madec and C. Thivolet, and by grants from the European Foundation for the Study of Diabetes (EFS-DJDRF-Novo Nordisk award), the Fondation Recherche Médicale (Installation Nouvelle Equipe) and the Programme National de Recherche sur le Diabète 2007 (to R. Mallone).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

- Friedenstein AJP, Petrokova KV (1966) Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 16:381–390
- Barry FP, Murphy JM, English K, Mahon BP (2005) Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem Cells Dev* 14:252–265
- Barry FP, Murphy JM (2004) Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 36:568–584
- Nauta AJ, Fibbe WE (2007) Immunomodulatory properties of mesenchymal stem cell. *Blood* 15:3499–3506
- Bartholomew A, Sturgeon C, Siatskas M et al (2002) Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 30:42–48
- Rasmusson I, Ringden O, Sundberg B, Le Blanc K (2003) Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation* 76:1208–1213
- Le Blanc K, Rasmusson I, Sundberg B et al (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 363:1439–1441
- Krampera M, Glennie S, Dyson J et al (2003) Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 101:3722–3729
- Zappia E, Casazza S, Pedemonte E et al (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T cell anergy. *Blood* 106:1755–1761
- Zhang J, Li Y, Chen J et al (2005) Human bone marrow stromal treatment improves neurological functional recovery in EAE mice. *Exp Neurol* 195:16–26
- Djouad F, Fritz V, Apparailly F et al (2005) Reversal of the immunosuppressive properties of mesenchymal stem cells by tumor necrosis factor α in collagen-induced arthritis. *Arthritis Rheum* 52:1595–1603
- Augello A, Tasso R, Negrini SM, Cancedda R, Pennesi G (2007) Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum* 56:1175–1186
- Abdi R, Fiorina P, Adra CH, Atkinson M, Sayegh MH (2008) Immunomodulation by mesenchymal stem cells. A potential therapeutic strategy for type 1 diabetes. *Diabetes* 57:1759–1767
- Kikutani H, Makino S (1992) The murine autoimmune diabetes model: NOD and related strains. *Adv Immunol* 51:285–322
- Thivolet C, Bendelac A, Bedossa P, Bach JF, Carnaud C (1991) CD8⁺ T cell homing to the pancreas in the nonobese diabetic mouse is CD4⁺ T cell-dependent. *J Immunol* 146:85–88
- Aspord C, Czerkinsky C, Durand A, Stefanutti A, Thivolet C (2002) α 4 Integrins and L-selectin differently orchestrate T-cell activity during diabetes prevention following oral administration of CTB-insulin. *J Autoimmun* 19:223–232
- Daniel D, Gill RG, Schoot N, Wegmann D (1995) Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. *Eur J Immunol* 25:1056–1062
- Nakayama M, Abiru N, Moriyama H et al (2005) Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 435:220–223
- Di Nicola M, Carlo-Stella C, Magni M et al (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 99:3838–3843
- Rasmusson I (2006) Immune modulation by mesenchymal stem cells. *Exp Cell Res* 312:2169–2179
- Thornton AM, Shevach EM (1998) CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287–296
- Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH (2000) Induction of interleukin 10-producing, non-proliferating CD4⁺ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 192:1213–1222
- Bach JF (2003) Regulatory T cells under scrutiny. *Nat Rev Immunol* 3:189–198
- Klyushenkova E, Mosca JD, Zernetkina V et al (2005) T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *J Biomed Sci* 12:45–57
- Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F (2005) Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 105:1815–1822
- Le Rond S, Gonzalez A, Gonzalez AS, Carosella ED, Rouas-Freiss N (2005) Indoleamine 2,3 dioxygenase and human leucocyte antigen-G inhibit the T-cell alloproliferative response through two independent pathways. *Immunology* 116:297–307
- Plumas J, Chaperot L, Richard MJ, Molens JP, Bensa JC, Favrot MC (2005) Mesenchymal stem cells induce apoptosis of activated T cells. *Leukemia* 19:1597–1604
- Terness P, Bauer TM, Rose L et al (2002) Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med* 196:447–457
- Fruento G, Rotondo R, Tonetti M, Ferrara GB (2001) T cell proliferation is blocked by indoleamine 2,3-dioxygenase. *Transplant Proc* 33:428–430
- Li YP, Paczesny S, Lauret E et al (2008) Human mesenchymal stem cells license adult CD34⁺ hematopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the NOTCH pathway. *J Immunol* 1:1598–1608
- Urban VS, Kiss J, Kovacs J et al (2008) Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes. *Stem Cells* 26:244–253
- Tritt M, Sgouroudis E, d’Hennezel E, Albanese A, Piccirillo CA (2008) Functional waning of naturally occurring CD4⁺ regulatory T cells contributes to the onset of autoimmune diabetes. *Diabetes* 57:113–123