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EDITOR'S CHOICE

A single administration of human adipose tissue-derived mesenchymal stromal cells (MSC) induces durable and sustained long-term regulation of inflammatory response in experimental colitis

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Summary

Current therapies for inflammatory bowel diseases (IBD) are aimed at controlling the exacerbated response in the gut, but no treatment is fully effective for many refractory patients. Mesenchymal stromal cells (MSC) are multi-potent cells with regulatory immunosuppressive activity that may control inflammatory diseases. In this study, we investigated the short- and especially the long-term protective effects of MSC on experimental colitis. We show that MSC elicited protection to acute intestinal inflammation with gain of weight, improvement in the clinical disease score and expressive reduction in the mortality rate of treated mice. MSC changed the population of neutrophils, eosinophils and augmented the frequency of CD4 T lymphocytes in the gut-draining lymph nodes, together with reduced accumulation of these cells in the colon intraepithelial compartment. Interestingly, there were increased levels of programmed death 1 (PD-1) and glucocorticoid-induced tumour necrosis factor receptor family-related receptor (GITR) in the spleen regulatory T cells of mice that received MSC treatment, which also presented a reversal in the pattern of immune response in the gut, with diminished inflammatory, T helper type 1 (Th1) and Th17 profile, in contrast to augmented Th2 responses. Most strikingly, this balanced response elicited by a single administration of MSC during the acute colitis persisted long-term, with restored goblet cells, eosinophils and maintenance of elevated gut interleukin (IL)-4, besides increased $CD4^+CD25^+PD\text{-}1^+$ cells in the spleen and reduced Th17 response in mesenteric lymph nodes (MLN) of treated mice on day 60. Taken together, our findings provided a significant contribution to translational immunology by pointing human adipose tissue-derived MSC as a novel therapeutic approach with long-term beneficial regulatory effects in experimental colitis.

Keywords: inflammatory bowel diseases, long-term remission, mesenchymal stromal cells, Th17 response

Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammation of intestinal mucosa affecting millions of people. These diseases are characterized by abdominal pain, diarrhea, rectal bleeding and bowel obstruction or fistulae [1]. Although IBD etiology is not yet fully understood, gut inflammation results from unregulated immune reactions in genetically susceptible individuals [2–4].

IBD response is driven by cytokines and leukocyte infiltrate in the gut [5–7], with histological features of crypt cell hyperplasia, interstitial edema and mucosa ulcerations [8]. Most importantly, there is an imbalance between effector and regulatory T cell (T_{reg}) responses in the gut that predispose to uncontrolled immunity against intestinal or microbiota antigens [9–12]. Accordingly, current available treatments are aimed at reducing inflammation in an attempt to avoid disease recurrence or to prolong clinical

remission periods. However, many patients are refractory, so that surgery remains their only alternative [13].

The partial inefficacy of pharmacological interventions and the unresponsiveness of some subjects has spurred clinical interest in developing other approaches for effectively controlling IBD. Recently, transplantation of hematopoietic stem cells (HSCT) has become a therapeutic alternative for autoimmune or inflammatory diseases [14,15]. Preclinical studies have shown that the infusion of bone marrow cells (BMC) has successfully controlled experimental colitis [16,17], with the pretransplant conditioning of immunosuppressive therapies such as cyclophosphamide or total body irradiation. The success of these therapies seem to be more correlated with immunosuppression than with transplantation *per se*, although prompt bone marrow reconstitution following immune ablation is essential to avoid receptor mortality [16,17].

Despite the importance of immunosuppression for IBD treatment with HSCT, its aggressive feature has prompted the use of more safe approaches. In this sense, mesenchymal stromal cells (MSC) have been applied to treat intestinal inflammation, considering their immunomodulatory properties, with reduced harmful effects [14]. The immunosuppressive capacity of MSC allows tolerance in transplantation, while their potential to induce regeneration of damaged tissues and cell differentiation make them an effective tool for treating IBD [18]. Indeed, several clinical trials have shown short-term positive results in more than 200 non-responsive patients with refractory IBD, in which MSC promoted a complete clinical remission in approximately 40% and an overall response in approximately 60% (for review, see Grégoire *et al.* [19]). These effects involve T_{reg} recruitment and T helper type 1 (Th1)/Th17 inhibition [19–25]. Nevertheless, despite these well-described short-term properties, little is known about long-term MSC effects on IBD. A few studies have showed that MSC injections persistently improved murine colitis by down-regulating Th1 inflammation [25], suppressing T cells [26], inducing T_{reg} differentiation [27] and up-regulating T_{reg} responses [28].

Taken together, in view of the known regulatory properties of MSC in the gut inflammatory responses, in this study we evaluated the persistent protective effects of an initial single dose of MSC infusion in the long-term modulation of experimental colitis, especially in the balance of the Th1–Th17–T_{reg} axis that controls gut homeostasis.

Materials and methods

Animals and experimental design

Colitis was induced in 6–8-week-old female BALB/c mice, which are the receptors for human MSC transplantation. Mice were maintained with food and water *ad libitum* in the animal housing facility of the Federal University of Triângulo Mineiro (UFTM), Brazil. The animals were divided into four groups (five animals/group), as follows: (1) the phosphate-buffered saline (PBS group), containing the control animals (without colitis induction) that received intrarectal enema of PBS; (2) the ethanol group, containing control animals that received the drug vehicle (ethanol 50%) used to induce colitis; (3) the colitis group, containing sick animals in which colitis was induced by an enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) diluted in ethanol 50%; and (4) the colitis + MSC group, containing sick animals with TNBS-induced colitis treated with MSC derived from human adipose tissue. All experiments were performed in accordance with protocols approved by the UFTM Animal Care and Use Committee under the protocol number 106.

Induction of experimental colitis by TNBS

For colitis induction, mice were anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg) followed by an intrarectal enema of TNBS (Sigma-Aldrich, Deisenhofen, Germany) diluted in 50% ethanol at a dose of 175 mg/kg in a total volume of 120 µl. The solution was administered through a 3·5-F catheter carefully inserted into the rectum until 4 cm proximal to the anal verge. To ensure TNBS distribution throughout the colon, mice were held in a vertical position for 2 min after the enema [16]. Control animals received PBS or 50% ethanol using the same technique. As there were no significant differences between both groups, we chose to show only ethanol data as the control group in the figures. The results shown in this paper are representative of two to five independent experiments, with five animals/group at each time-point evaluated, as stated in the figure legends. As the animals were killed on days 3, 7, 14 or 60, the samples were obtained from different mice at each time-point evaluated, except for Fig. 1, which shows the total number of mice still alive throughout the clinical disease evaluation period.

MSC isolation, culture and characterization

Samples of adipose tissue were harvested from healthy donors at Clinics Hospital from UFMT (Brazil) for MSC isolation, culture and characterization, as described previously [23,25]. For the entire study we used adipose tissue from three non-obese female subjects aged 25–55 years which were submitted to plastic surgery for aesthetic reasons. These donors were healthy and did not present any significant disease conditions. The collected adipose tissue was immersed in PBS containing antibiotic (penicillin G solid at 0·01 IU/ml plus streptomycin sulfate

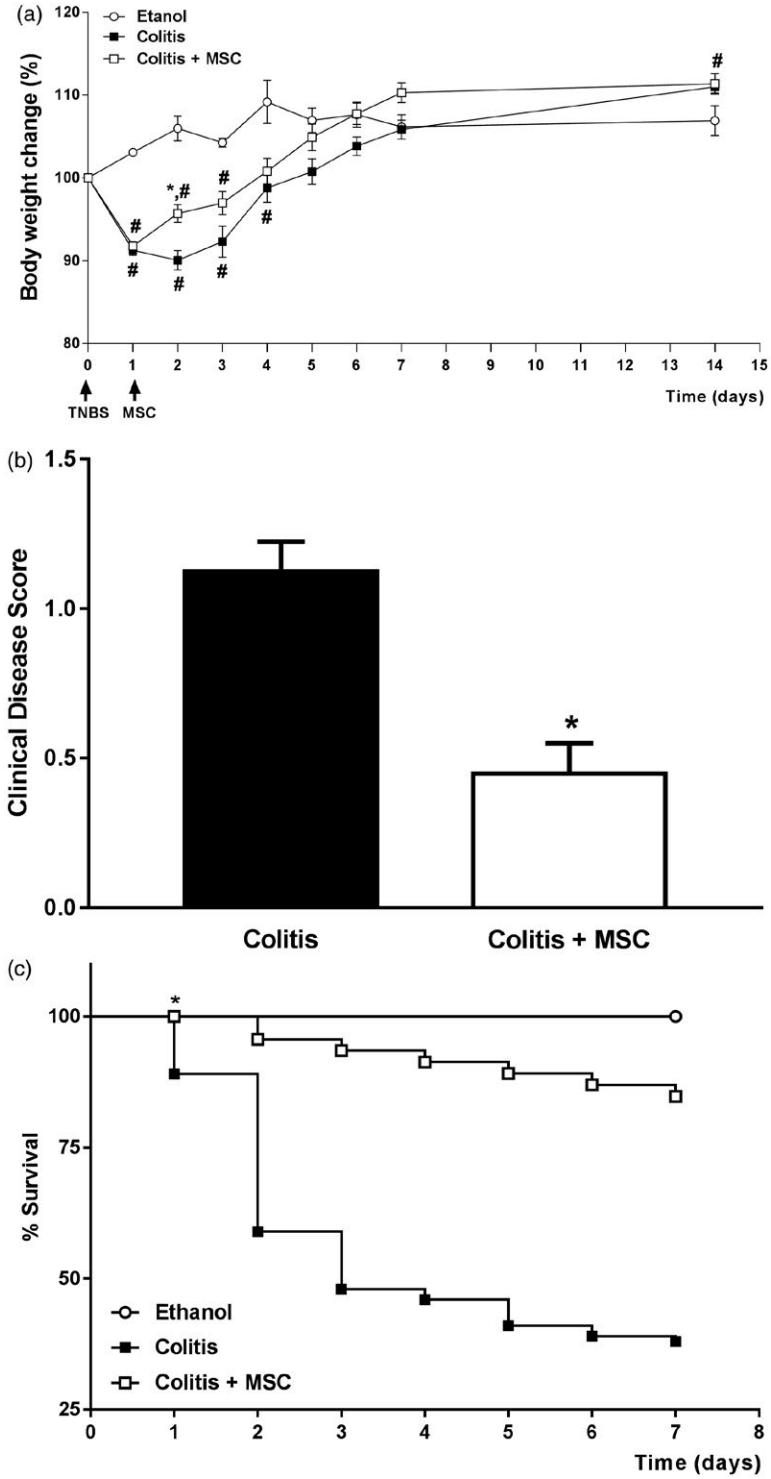

Fig. 1. Mesenchymal stromal cells (MSC) treatment improves mice survival and clinical signs of experimental colitis. Mice received intrarectal enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) for induction of colitis and were treated 24 h later with 1×10^6 human MSC cells. (a) Body mass change relative to the first day of colitis. (b) Sum of clinical disease score during the first 14 days, according to the presence of diarrhea, bleeding, blood plus diarrhea and severe weight loss (more than 5%). (c) Survival curve of colitis and MSC-treated mice during the first 7 experimental days. Results are representative of five independent experiments, with five animals per group. Symbols represent significant differences ($P < 0{\cdot}05$) from the respective ethanol (#) or colitis (*) groups.

at 0·01 g/ml) and anti-fungal (amphotericin B at 25 μg/ml) drugs for at least 4 h, and then incubated at 37ºC with collagenase type I (0·3 mg/ml in PBS) (Gibco BRL, Grand Island, NY, USA) to digest the tissue. The cells released by collagenase treatment were carefully collected and centrifuged twice at 4ºC for 10 min at 677 ***g*** with RPMI-1640 (Gibco BRL) containing 10% of fetal bovine serum (FBS) (Gibco BRL). Afterwards, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 15% of FBS (Hyclone Thermo Scientific, South Logan, CA, USA) and antibiotic drugs (0·01 mg/ml of penicillin + streptomycin). The cells were counted with 0·2% Trypan blue exclusion in a Neubauer chamber, plated in 75-cm^2 bottles and cultured at 37ºC with 5% of CO_2. After 24 h, the non-adherent cells and the culture medium were removed and 20 ml of fresh DMEM were added to the flasks. After the third passage, cell samples were separated for immunophenotyping, multi-potential characterization and infusion in mice with experimentally induced colitis. For immunophenotypical characterization, monoclonal antibodies against the markers CD45, CD14, CD90, CD73, CD31, CD166, CD34 and CD13 were used. For differentiation into adipocytes, we used 15% of FBS containing DMEM supplemented with 10 mmol/l of dexamethasone (Decadron injection; Prodome, Campinas, SP, Brazil), 10 g/ml of insulin (Sigma-Aldrich, St Louis, MO, USA) and 100 mmol/l of indomethacin (Sigma-Aldrich, USA). To differentiate into osteocytes, 7·5% of FBS containing DMEM supplemented with 0·10 mmol/l of dexamethasone (Decadron injection; Prodome), 100 mmol/l of ascorbic acid and 10 mmol/l of β-glycerolphosphate (React, Reagen, Colombo, Brazil) was used. All experiments with human adipose tissue were performed in accordance with protocols approved by the UFTM Human Committee under protocol number 1447.

Colitis treatment with MSC and sample collection

For determining the optimal cell concentration to treat colitis, MSC were injected by the intraperitoneal route 24 h after TNBS enema at various concentrations ($0{\cdot}25 \times 10^6$ cells, 1×10^6 cells or 2×10^6 cells per mouse; Supporting information, Fig. 1), as well as literature searching for similar studies with MSC infusion [23,25]. To avoid clumps, MSC were resuspended in an appropriate volume of saline solution supplemented with 1 mg/ml of a recombinant human DNase (Pulmozyme; Roche, South San Francisco, CA, USA) before infusion. The next experiments were all performed with 1×10^6 cells/mouse, according to the results from the dose–response preliminary studies (Supporting information). Colitis mice that did not receive MSC were injected with the same saline volume used to administer the cells in the treated group. Mice were then examined for the evaluation of colitis clinical score, weight and mortality, and the samples were collected for further assays performed at 3, 7, 14 or 60 days after colitis induction and treatments. The time-point of TNBS enema was considered as day 0 in all experiments. For the evaluation of circulating leukocytes, the animal blood was collected by retro-orbital puncture and cells were counted with 0·2% of Trypan blue exclusion in a Neubauer chamber. The differential counts were made in smears stained with Panotico LB (Laborclin, Pinhais, PR Brazil).

Clinical disease score evaluation

Mice with colitis treated or not with MSC transplantation were evaluated for the presence of clinical signs of disease, such as weight loss, diarrhea, rectal bleeding and bloody diarrhea, as well as for mortality during the first 7 days post-induction. This evaluation used a criterion score in which each signal exhibited by the animal corresponded to one point. Finally, the sum of all values for each animal was calculated and plotted in the respective graphics [16].

Histopathological analysis and morphometry

Tissue samples were fixed in formaldehyde 10% in PBS for 24 h followed by dehydration in 70% ethanol. The samples were then processed and embedded in paraffin, cut into 5-μm-thick sections and stained with hematoxylin and eosin (H&E). The histological images (at ×20 or ×40 magnification) were captured by a common optic microscope connected to a digital camera (Evolution MP 5.0-color; Media Cybernetic, Silver Spring, MD, USA). In each colon sample, a total area of 0·0093958 mm^2 was evaluated to quantify the lamina propria infiltrate using Image J software (http://rsb.info.nih.gov/ij/), as described previously [29]. The criteria used for evaluation of tissue infiltrate and diagnosis of inflammatory bowel disease were adapted from guidelines of the British Society of Gastroenterology, as described previously [30]. Furthermore, another set of slides was stained with periodic acid-Schiff (PAS) and the frequency of areas considered positive for this staining was quantified in the same total area described above in each colon sample evaluated. The results were depicted as percentage of area covered by goblet cells.

Tissue homogenization and cytokine quantification

For quantification of cytokines, colon segments were collected and resuspended in buffer containing protease inhibitors (cOmplete; Roche, South San Francisco, CA, USA). The cytokines presented in the homogenates were measured by enzyme-linked immunosorbent assay (ELISA) (Pharmingen™ BD, San Diego, CA, USA) or cytometric bead array (CBA) (Pharmingen™ BD), according to the manufacturer's instructions.

Eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activity assays

For neutrophils, MPO activity was evaluated in the colon samples. The reactions were developed by addition of TMB (3,3′,5,5′-tetramethylbenzidine (Pharmingen™ BD) and the readings were performed in a spectrophotometer at 450 nm after reaction stopping with 4 mol/l of H_2SO_4. For eosinophils, EPO enzyme activity was evaluated and the reactions were developed with o-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, USA) diluted in Tris/HCl and H_2O_2 for 30 min at room temperature. The reaction was stopped with 4 mol/l of H_2SO_4 and the absorbance read at 492 nm. In both cases, results were expressed as optical density (nm) per gram of tissue [16].

Flow cytometry

Spleen, mesenteric lymph nodes and colon were collected for flow cytometry assays for quantification and phenotyping of inflammatory cells. Colon was removed and separated into fragments for assessing lamina propria and intraepithelial lymphocytes. Lamina propria tissue was cut into small pieces, which were immersed in PBS containing ethylenediamine tetraacetic acid (EDTA) (3 mmol/l) then placed on a magnetic stirrer for 20 min. The samples were centrifuged at 470 ***g*** for 10 min at 4°C, the supernatant was discarded and RPMI-1640 containing FBS (1%), EDTA (1 mmol/l) and $MgCl_2$ (1·5 mmol/l) were added to the pellet that was stirred again for 30 min followed by centrifugation at 470 ***g*** for 10 min at 4°C. The samples were vortexed for 2 min and incubated with liberase at 50 μg/ml (Roche, Mannheim, Germany) diluted in incomplete RPMI (with no FBS) medium for 60 min at 37°C. Cells extracted from tissues were washed twice, counted in a Neubauer chamber and then used in culture or immunophenotyping assays. For detection of intracellular cytokines, cells were cultured in RPMI FBS (10%) and stimulated with phorbol myristate acetate (PMA, 50 ng/ml) plus ionomycin (1 μg/ml) for 4 h at 37°C in the presence of brefeldin. The selected antibodies against specific markers or isotype controls were conjugated with fluorochromes such as fluorescein isothiocyanate (FITC), Alexa Fluor 488, phycoerythrin (PE) or phycoerythrin/cyanin 5 (PerCy5) (BD Pharmingen™, San Diego, CA, USA). To obtain the percentage of positively stained cell populations, the samples were acquired in a fluorescence activated cell sorter (FACS)calibur (Becton Dickson®, San Jose, CA, USA) cytometer and analyzed in FlowJo version 7.6.5 software in accordance with the properties of size and granularity [forward- (FSC) and side-scatter (SSC)], followed by dot-plot or histogram evaluation.

Statistical analysis

Results were analyzed in GraphPad Prism version 7.0 by analysis of variance (ANOVA) followed by Tukey's *post-hoc* test for parametric data. In the case of non-Gaussian distribution (non-parametric data), Mann–Whitney or Kruskal–Wallis tests followed by Dunn's *post-hoc* analysis were used. The Kaplan–Meyer log-rank test was used to analyze the survival curve. $P < 0{\cdot}05$ was considered statistically significant.

Results

MSC treatment improves clinical signs of colitis

First, our preliminary results confirmed the phenotypical characterization of human MSC, their potential in differentiating into osteocytes and adipocytes as well as their *in-vitro* suppressive capacity, thus validating the subsequent studies (data not shown). Colitis was then induced in BALB/c mice, which were treated with 1×10^6 xenogeneic MSC/mice by intraperitoneal route 24 h afterwards. This dose was chosen based on literature findings and on preliminary studies performed with different numbers of MSC administration, as shown in Supporting information, Fig. S1. We show that MSC treatment elicited protective effects on acute intestinal inflammation, as treated sick mice gained weight during the first days after cell infusion when compared to the untreated animals (Fig. 1a). We also found a significant improvement in the clinical disease score (Fig. 1b), followed by an expressive reduction in the mortality rate exhibited by treated colitis mice (Fig. 1c).

MSC treatment reduces systemic and local inflammation in colitis

The peripheral changes induced by intestinal inflammation and the consequences of MSC treatment were evaluated by differential counts of leukocytes in mice blood. Our data show that intestinal inflammation led to an overall increase in the number of blood leukocytes, mainly neutrophils, as early as 3 days after TNBS enema. Such leukocytosis was transitorily reduced after MSC treatment and controlled within 7 days, followed by a reduction in the number of neutrophils and mononuclear cells at this time-point. On day 14, no significant difference was observed among the groups (Table 1).

Histopathological analysis showed a significant increase in the number of inflammatory cells in the colon from untreated sick animals at day 3 when compared to ethanol-infused mice (Fig. 2a). We also observed that goblet cells from both treated and untreated sick mice were decreased within 3 days when compared to ethanol animals, and at day 14 there was a complete restructuring of goblet

Table 1. Total and differential systemic leukocyte counts in mice with colitis and post-MSC treatment

Groups		Total blood leukocytes ($\times 10^4$/ml)	Eosinophils ($\times 10^4$/ml)	Neutrophils ($\times 10^4$/ml)	Mononuclear cells ($\times 10^4$/ml)
3 days	Ethanol	815 ± 334·54	17·82 ± 14·52	96·34 ± 21·66	700·84 ± 304·56
	Colitis	1381·25 ± 368·77	38·06 ± 29·15	493·71 ± 197·29$^{\#}$	846·14 ± 374·46
	Colitis + MSC	1450 ± 467·80	27·62 ± 22·09	511·17 ± 210·03$^{\#}$	897·25 ± 440·68
7 days	Ethanol	1121·25 ± 227·46	25·15 ± 37·91	347·96 ± 141·89	745·46 ± 81·96
	Colitis	1172·5 ± 475·93	36·31 ± 7·18	524·99 ± 229·17	611·2 ± 265·01
	Colitis + MSC	316·25 ± 128·35*	7·96 ± 8·62	65·95 ± 33·95*	242·34 ± 88·56$^{\#}$
14 days	Ethanol	1123·75 ± 129·57	7·82 ± 10·18	195·99 ± 43·07	919·94 ± 124·05
	Colitis	1425 ± 396·88	28·07 ± 24·38	428·67 ± 354·37	962 ± 256·96
	Colitis + MSC	1287·5 ± 387·59	29·19 ± 29·22	307·05 ± 103·94	949·15 ± 322·51

Results are expressed as mean ± standard deviation. (*)Significantly different ($P < 0·05$) from the respective ethanol (#) or colitis (*) groups. MAC = mesenchymal stromal cells.

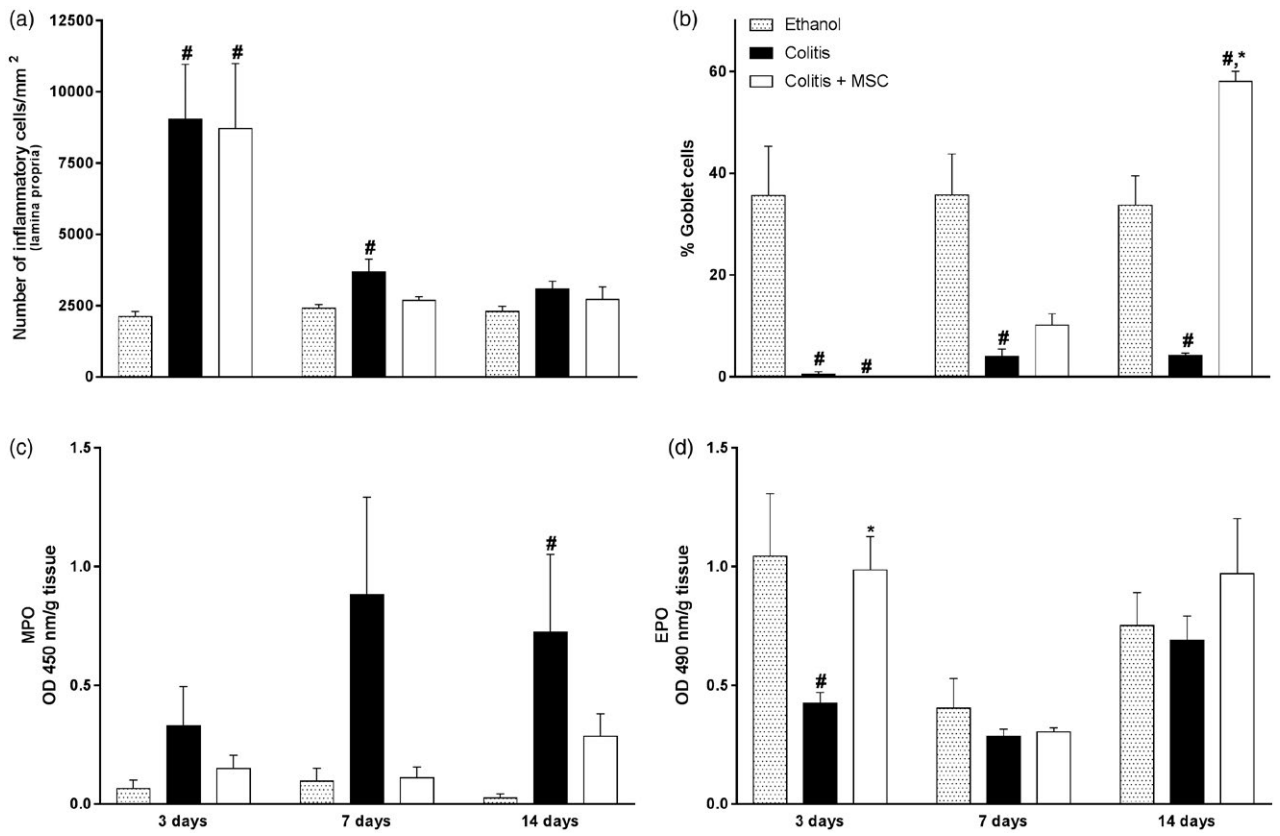

Fig. 2. Colitis treatment with mesenchymal stromal cells (MSC) restores intestinal homeostasis and gut architecture. On days 3, 7 and 14, mice had their colon removed, processed for paraffin, stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) and evaluated for quantification of inflammatory cells in the lamina propria (a) or goblet cells (b) by morphometry. Neutrophils [myeloperoxidase, MPO)] (c) and eosinophils [eosinophil peroxidase (EPO)] (d) activities were determined by enzymatic assays in the colon of colitis and MSC-treated mice throughout the experimental period. Results are representative of three independent experiments, with five animals/group, at each time-point evaluated. Symbols represent significant differences ($P < 0·05$) from the respective ethanol (#) or colitis (*) groups.

cells in mice which received MSC, but not in the untreated sick group (Fig. 2b).

In ethanol-treated mice, the mucosal epithelial surface was similar to the PBS group (data not shown), despite a mild and focal increase in lamina propria cellularity, composed of a mixed infiltrate with predominance of mononuclear cells on days 3, 7 and 14. There was no accumulation of polymorphonuclear cells on crypts or

intraepithelial lymphocytes. These ethanol mice did not present any abnormality, such as epithelial flattening, mucin depletion, focal loss of cells or erosion, except for a discrete infiltrate in submucosa, that was not different among the time-points evaluated (Supporting information, Fig. S2, upper panel). Conversely, the TNBS-colitis group presented large erosion areas with loss of surface epithelial and tubular glands, especially on the third day, along with deep ulcers that went beyond the muscularis mucosae. The lesion borders had irregular surface epithelia and dilated crypts, which accounted for abnormal tissue architecture. These structures were infiltrated with polymorphonuclear cells, thus characterizing cryptitis or crypt abscesses. There was epithelial flattening and mucin depletion. Furthermore, mixed and intense lamina propria inflammatory infiltrate were observed, especially in ulcer or erosion areas (Supporting information, Fig. S2, middle panel). Mice which had TNBS infusion and were treated with MSC presented similar histological alterations in the colon on the third day when compared to the non-treated group, except for an apparently increased frequency of re-epithelialization interleaved with lesion areas (Supporting information, Fig. S2, lower panel). On day 7, the TNBS-colitis mice had some re-epithelialization of the mucosa, interleaved with ulcers or erosion. The lesion borders still presented mucin depletion and lamina propria had edema, with fibroblasts and mixed leukocyte infiltrate of varied intensity. The submucosa also presented moderate to intense mixed inflammatory infiltrate (Supporting information, Fig. S2, middle panel). Conversely, mice treated with MSC maintained similar infiltrate, but with the absence of mucosal ulcers or erosion. Despite the presence of just a few crypts, the mucosal surface was re-epithelialized (Supporting information, Fig. S2, lower panel). On day 14, we observed crypt formation with a few goblet cells in colitis mice, in contrast to the well-structured crypts with columnar epithelium and increased number of goblet cells in the group treated with MSC. There was a mixed leukocyte accumulation on the lamina propria of TNBS mice with varied intensity and predominance of mononuclear cells, similar to that found in the MSC group. Furthermore, in the mice that received cellular therapy there was mucosal preservation in the areas apart from the lesions (Supporting information, Fig. S2). On day 60, the inflammatory infiltrate in the colon of TNBS-treated mice was similar to the control ethanol, despite the treatment with MSC (data not shown). The most remarkable alteration at this time-point concerned the reduced goblet cells in the group not treated with MSC, which were restored to normality in mice that received cell administration (Fig. 6).

Quantitative analysis of the accumulation of neutrophils and eosinophils in the inflamed gut and its relation to MSC treatment was performed by MPO and EPO assays, respectively. Overall, the results showed that mice undergoing intestinal inflammation had a higher accumulation of neutrophils in the colon when compared to the ethanol group, followed by a reduction after MSC treatment (Fig. 2c). Interestingly, while MPO activity tends to be reduced in treated sick animals throughout 3, 7 or 14 days when compared to the untreated sick group, EPO activity tends to be increased after MSC therapy, mainly on the first days after cellular infusion (day 3) (Fig. 2d).

MSC treatment changed lymphocyte frequency and induced T_{reg} cell response

We observed a significant increase in the frequency of $CD3^+$ cells in the gut-draining lymph nodes of treated sick mice compared to the untreated group at day 7 (Fig. 3a) that could be related to the elevated frequency of $CD3^+CD4^+$ cells at days 7 and 14 (Fig. 3b). These changes were followed by a reduced frequency of $CD3^+CD4^+$ cells in intraepithelial T lymphocytes from treated colitis animals, mainly at day 14 (Fig. 3b). No significant differences in any immune compartments were found in the population of $CD3^+CD8^+$ T cells or $CD3^+CD49b^+$ cells [supposedly natural killer T cells (NK T)] or NK $CD49b^+$ cells from spleen or mesenteric lymph nodes between untreated and treated colitis mice. Furthermore, there were no changes in $CD3^+\gamma\delta^+$ T cells from intraepithelial lymphocytes (data not shown). Nevertheless, by comparing specific markers of T_{reg} cells, we found increased levels of programmed cell death 1 (PD-1) in both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells of spleen, as well as augmented frequency of $CD4^+CD25^-$ leukocytes expressing glucocorticoid-induced tumour necrosis factor (TNF) receptor family-related receptor (GITR) in the spleen of mice that received MSC treatment on day 14 (Fig. 3c). There were no significant changes in the regulatory markers of MLN on day 14 or in spleen and MLN on day 7 (data not shown).

MSC treatment modulates uncontrolled production of inflammatory cytokines in colitis

A reduction of the proinflammatory cytokines IL-1β and TNF-α was observed in treated colitis animals within 3 days (Fig. 4a and 4b, respectively). At day 7, mice that received MSC exhibited elevated IL-1β and TNF-α levels, whereas TNF-α levels remained increased within 14 days of treatment when compared to the colitis non-treated group. Interestingly, the augmented TNF-α levels at days 7 and 14 was not higher than the values observed at day 3; on the contrary, a clear reduction on this cytokine was observed throughout the experimental period (Fig. 4b). Among the proinflammatory cytokines from the Th1 pattern, IL-12 was reduced in the treated colitis group after 14 days of MSC infusion (Fig. 4c). Otherwise, considering cytokines

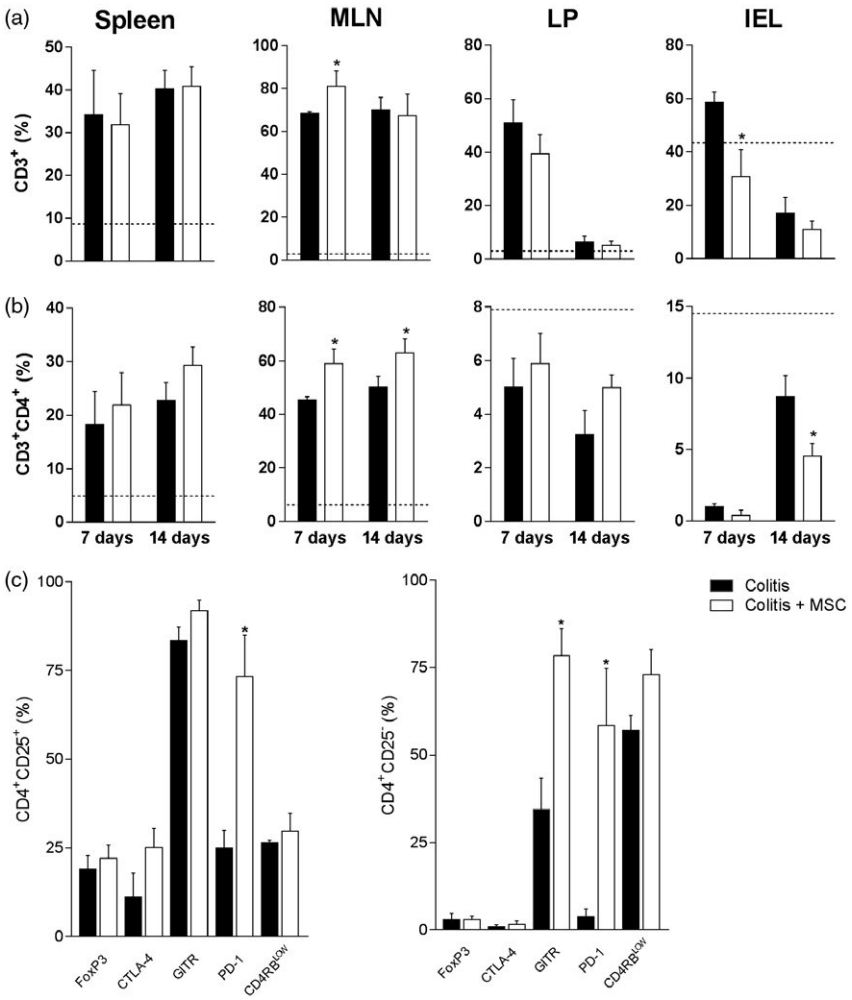

Fig. 3. Mesenchymal stromal cells (MSC) treatment modifies inflammatory infiltrate in the gut mucosa and draining lymphoid organs. Leukocytes from the spleen, mesenteric lymph nodes (MLN), colon lamina propria (LP) and intraepithelial (IEL) compartments of colitis and MSC-treated mice were isolated and stained with fluorochrome-conjugated antibodies for flow cytometry on days 7 or 14. After sample acquisition, the frequency of $CD3^+$ T cells (a), $CD3^+CD4^+$ (b) or regulatory T cells in the spleen (c, day 14) was determined after analysis by FlowJo software. Results are representative of two independent experiments, with five animals/group, at each time-point evaluated. Dashed line: control animals treated with ethanol [2,4,6-trinitrobenzene sulfonic acid (TNBS) (TNBS) vehicle] at day14. (*) Symbols represent significant differences ($P < 0{\cdot}05$) from the respective colitis groups.

associated with the Th17 response, only IL-23 and IL-17 were detected at reduced levels at days 3 and 7, respectively, in treated colitis mice when compared to untreated sick group (Fig. 4e and 4f, respectively). IL-6 levels did not alter significantly post-treatment (Fig. 4d). Cytokines from the Th2 profile, including IL-4 and IL-5, exhibited higher production in treated colitis mice at days 3 and 14 when compared to the untreated sick group (Fig. 4g and 4h, respectively). These data pointed to a reversal of the immune inflammatory profile in intestinal inflammation after MSC treatment.

MSC treatment reduces Th1 and Th17 response in colitis

The increase in regulatory markers previously described was followed by an altered functional profile of Th1 and Th17 cells, mainly by a reduced frequency of $CD4^+$ T cells producing IL-17 in spleen and MLN from treated mice at day 14 when compared to untreated sick mice (Fig. 5a), which was also observed at day 7 (data not shown). Furthermore, interferon (IFN)-γ production by T $CD4^+$ cells was reduced, especially in spleen from colitis animals that received MSC at day 14 (Fig. 5b). The simultaneous production of IL-17 and IFN-γ by $CD4^+$ T cells was also decreased in spleen, but not in MLN, from treated sick mice when compared to untreated colitis animals at day 14 (Fig. 5c).

Long-term persistence of the therapeutic efficacy of MSC treatment

To investigate whether the immune modulatory effects assigned to MSC treatment could persist long-term after

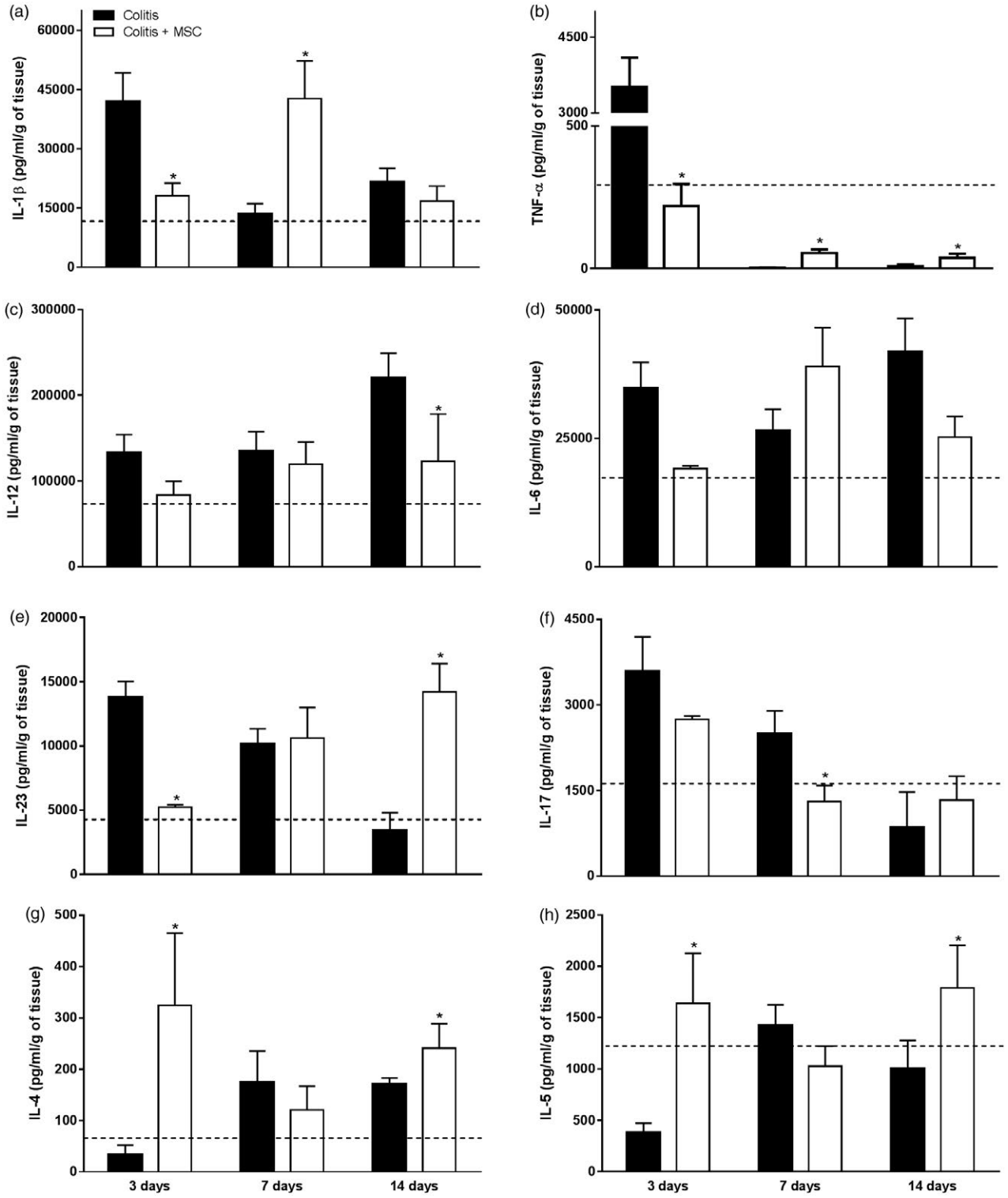

Fig. 4. Evaluation of the cytokine profile in the colon of mice with colitis and treated with mesenchymal stromal cells (MSC). Colon fragments of mice receiving ethanol, 2,4,6-trinitrobenzene sulfonic acid (TNBS) or treated with MSC were removed and homogenized in a solution containing protease inhibitors. The cytokines interleukin (IL)-1β (a), IL-12 (c), IL-23 (e), IL-17 (f), IL-4 (g) and IL-5 (h) were assessed by enzyme-linked immunosorbent assay (ELISA). The presence of the cytokines tumour necrosis factor (TNF)-α (b) and IL-6 (d) was quantified by cytometric bead array (CBA) in samples acquired on the flow cytometer. The data were expressed as pg/ml per gram of tissue. Results are representative of two independent experiments, with five animals/group, at each time-point evaluated. Dashed line: control animals treated with ethanol (TNBS vehicle) at day 14. (*) Symbols represent significant differences ($P < 0{\cdot}05$) from the respective colitis groups.

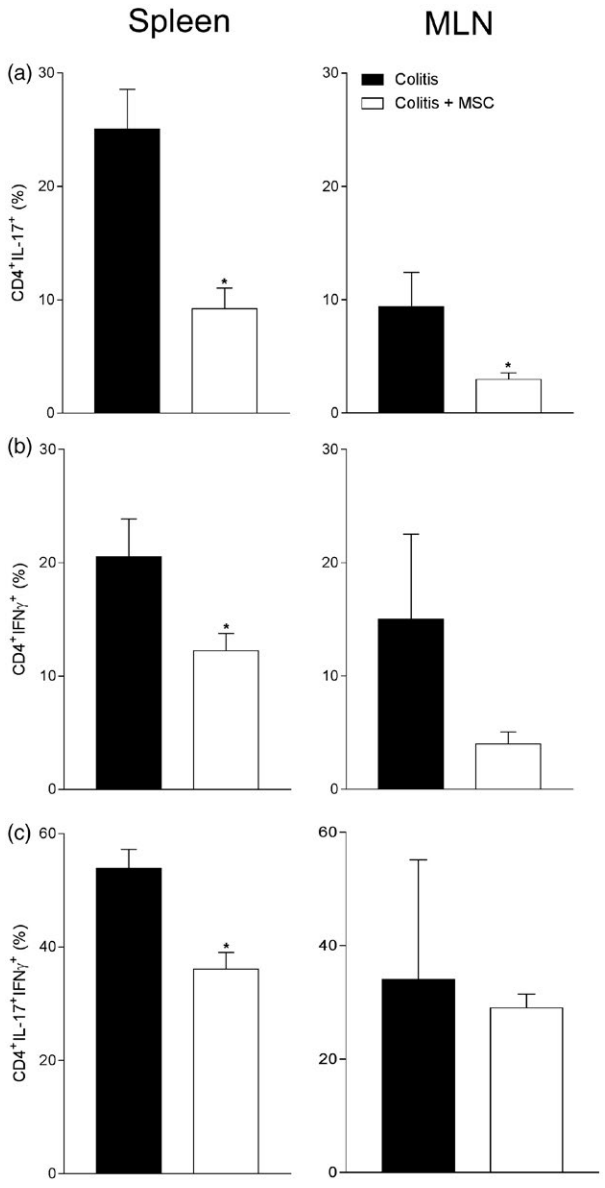

Fig. 5. Quantification of interleukin (IL)-17 and/or interferon (IFN)-γ production by CD4 cells after 14 days of induction of colitis and/or treatment with mesenchymal stromal cells (MSC). The spleen and mesenteric lymph nodes (MLN) from mice with colitis treated or not with MSC were removed and macerated for extracting leukocytes. After 4 h of *ex-vivo* re-stimulating culture, cells were collected and stained with specific antibodies against CD4 [phycoerythrin/cyanin 5 (PE/Cy5)], IL-17 (Alexa 488) and IFN-γ (PE) for flow cytometric detection of $CD4^+$ T lymphocytes producing IL-17 (a), $CD4^+$ T lymphocytes producing IFN-γ (b) and $CD4^+$ T lymphocytes double-positive for IFN-γ and IL-17 (c). Results are representative of two independent experiments, with five animals/group, at each time-point evaluated. (*) Symbols represent significant differences ($P < 0{\cdot}05$) from the respective colitis groups.

transplantation, we evaluated the experimental intestinal inflammation induced by a single TNBS infusion during a chronic period of 60 days, as previous studies of the group showed that immunological changes persisted long-term after breakdown of the mucosal tolerance by this hapten [17]. Indeed, even after 60 days of treatment, the potential immune modulatory effects of MSC could still be observed. Histopathological analyses showed regular colon tissue architecture with the re-establishment of goblet cell counts in treated colitis mice, while untreated animals were not able to restore normal mucus production in the gut (Fig. 6a). Regarding these local alterations, although EPO reached control levels at day 60 (Fig. 6c), neutrophil activity remained elevated at this time-point in colitis mice, indicating that a chronic barrier dysfunction may still exist in mice not treated with MSC (Fig. 6b). In agreement with these results, IL-4 levels were still elevated in mice that received cellular therapy, indicating the maintenance of the immunological shift of the local response (Fig. 6d).

Similarly to the acute colitis, the percentage of $CD4^+CD25^+$ cells in the spleen from treated sick mice was unchanged. However, a significant increase in the frequency of PD-1 levels by this putative regulatory population persisted to 60 days post-MSC treatment (Fig. 6e), pointing to an important role for this molecule in inflammation control. Interestingly, the production of IL-17 by $CD4^+$ cells from MLN was also constrained long-term by an early injection of MSC, indicating that this proposed therapy may lead to chronic beneficial effects in treated animals presenting an initial acute intestinal inflammation (Fig. 6g).

Discussion

Human adipose tissue has been described as an ideal source of MSC, which are easily isolated and whose proliferative and immunological properties have been widely explored in several inflammatory diseases [18,23–25,31–36]. Therefore, we used MSC for treating experimental colitis and especially showed the long-term effects of this immunomodulatory therapy, which is essential for chronic relapsing diseases such as IBD. Overall, MSC attenuate immune and inflammatory diseases by suppressing the mixed lymphocyte reaction, involving allogenic or autologous T cells by inhibiting autoimmune responses, by reducing infiltrate or proinflammatory cytokine production or by improving local tissue repair [18,20,32–36].

Our first result showed that MSC treatment promoted short-term protective effects on acute experimental colitis, as treated sick animals exhibited lower weight loss followed by an improved clinical disease score and an increased survival rate. In agreement with the macroscopic findings, we observed that the colon from treated sick animals presented a higher frequency of re-epithelized areas that were repaired earlier. Accordingly, these results corroborated previous studies that showed the short-term protective efficacy of MSC on macroscopic and

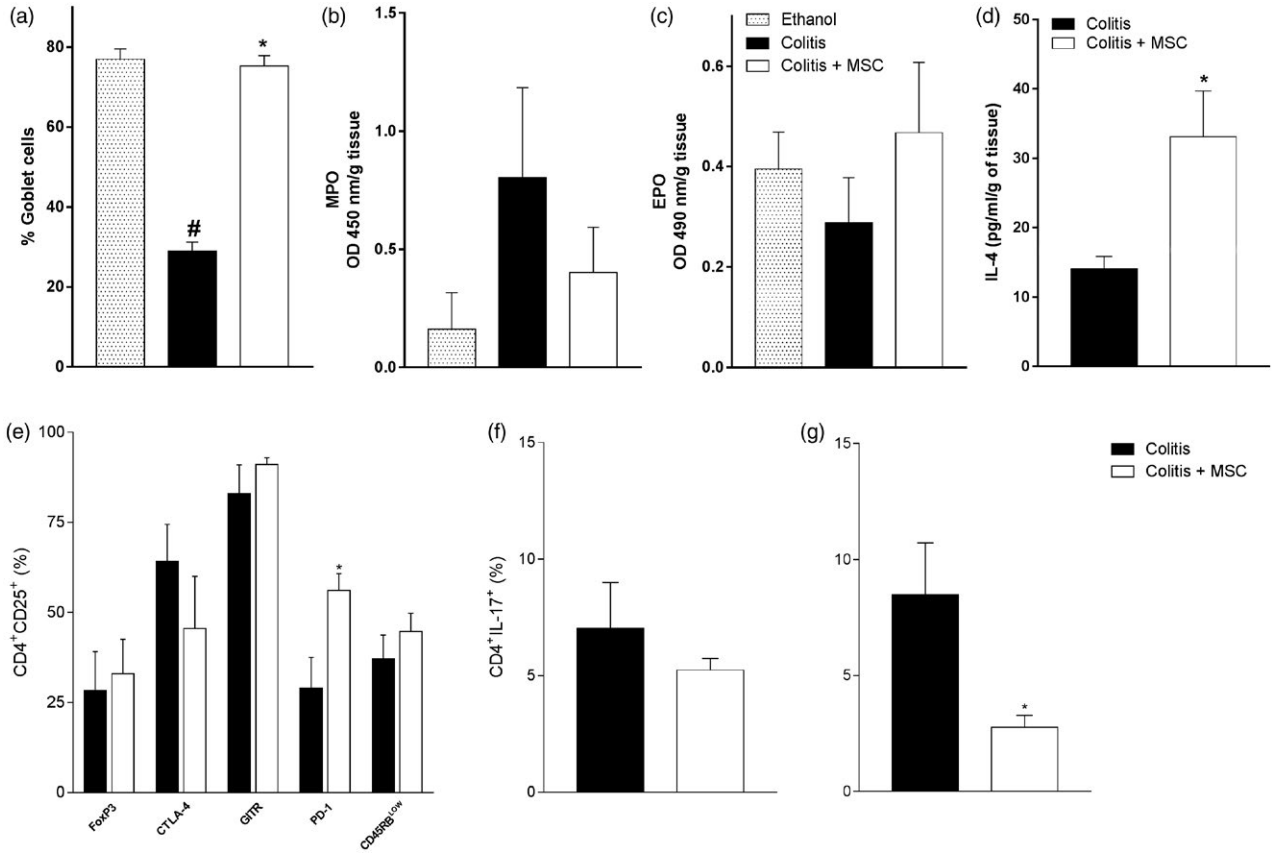

Fig. 6. Long-term effects of mesenchymal stromal cells (MSC) therapy. Mice received intrarectal 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce colitis on day zero and were treated with a single dose of MSC after 24 h. The colon was removed on day 60 and processed for goblet cell counting (a), determination of myeloperoxidase (MPO) (b), (c) eosinophil peroxidase (EPO) activity and (d) IL-4 quantification. In (e), evaluation of regulatory T lymphocytes in the spleen, (f) and (g) T helper type 17 (Th17) response on day 60 in the spleen and mesenteric lymph nodes (MLN), respectively, from treated or untreated sick mice. Results are representative of two to three independent experiments, with five animals/group, at each time-point evaluated. Symbols represent significant differences ($P < 0{\cdot}05$) from the respective ethanol (#) or colitis (*) groups.

histopathological severity of inflammatory diseases, which has been correlated with the down-regulation of Th1 inflammation [23–28,33,37] and the suppression of collagen-reactive T cell-driven production of matrix degrading enzymes [38].

Leukocytosis was observed to be temporally reduced after MSC treatment at day 7, with a reduced number of neutrophils and mononuclear cells at this time-point. As MSC suppress cell activation *in vivo*, this may be a way for the body to maintain homeostasis by inhibiting the activation of the immune system in different compartments. However, the molecular mechanism underlying this immunosuppressive effect of MSC is not yet completely understood [18,31,39].

IBD exhibit chronic inflammation with enhanced IFN-γ production in intestinal lamina propria, which is followed by an excess of TNF-α and neutrophil infiltration in CD [9,40–45], in contrast to the excess of IL-4, IL-5 and IL-13 in UC [9,40,42]. The experimental model of TNBS enema is immunologically similar to CD in humans [37]. Thus, when we analyzed the inflammatory infiltrate of neutrophils by MPO and its correlation with MSC treatment, we observed that these cells were directly related to the colon mucosa inflammation, corroborating previous studies that showed accumulation of neutrophils in the inflammatory site and its reduction after MSC injection [23–25,37]. In addition, the apparent decrease of MPO and increased EPO levels after MSC treatment suggested that this therapy interferes in the activity of neutrophils and eosinophils at the site of disease, leading to a possible reversal of immune response to a Th2 profile. In this sense, the recruitment of eosinophils may be directly related to the regression of intestinal inflammation. Furthermore, our results showed that MSC treatment led to an overall reduction in the gut proinflammatory cytokines such as IL-12, IL-1β, TNF-α, IL-6, IL-23 and IL-17, especially during the first days of treatment, while IL-4 and IL-5 were kept at increased levels, confirming

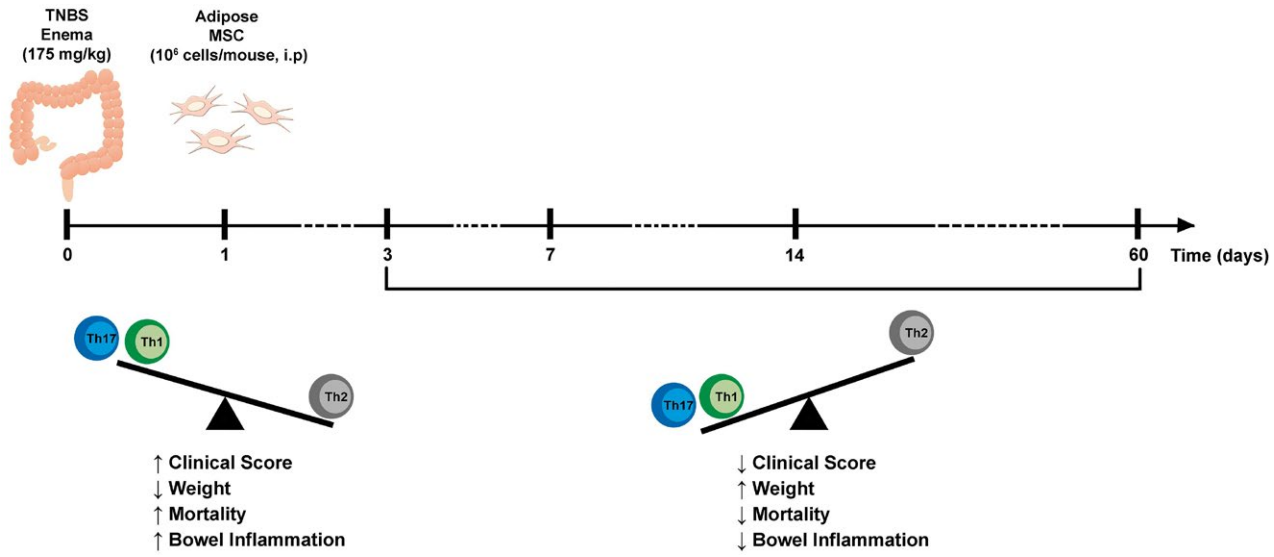

Fig. 7. Summary of the long-term effects of MSC on experimental colitis. TNBS (2,4,6-trinitrobenzene sulfonic acid) enema triggered intestinal inflammation in mice by enhancing Th1 and Th17 response and reducing Th2 cells. An early and unique intraperitoneal (i.p.) administration of adipose tissue-derived MSC (mesenchymal stromal cells) produced persistent protective effects on bowel inflammatory response by constraining inflammation and restoring Th1/Th17/Th2 balance.

the results obtained for EPO. These findings indicated that MSC treatment can revert the local profile of immune response and down-regulate cytokines responsible for the exacerbated inflammation of colon mucosa, in agreement with the reduction of IFN-γ or IL-17 production in the gut of treated mice [25–28].

A relative increase in T cells from MLN was observed after 7 days of MSC treatment, which could be explained by the higher frequency of $CD4^+$ T cells as effector or regulatory lymphocytes. However, the reduced accumulation of T lymphocytes in the intraepithelial compartment at day 14 after MSC infusion could be indicative of the presence of a potentially inflammatory or pathogenic population in non-treated colitis. In general, our results partially corroborate previous studies which showed that the treatment of experimental IBD with bone marrow transplantation associated with immunosuppression leads to the reduction of $CD4^+$ and $CD8^+$ cells in the colon, which seemingly contributes to the improvement of intestinal inflammation and the regression of the disease [16,17].

The regulation of intestinal inflammation depends on the presence and functionality of T_{reg} in the gut, where these cells may act by inhibiting excessive inflammatory responses such as Th1 and Th17 [45,46]. Here, we also showed that MSC treatment increased the frequency of T_{reg} in contrast to a reduction in Th17 and Th1 responses. In agreement, MSC administration alleviates TNBS-induced murine colitis by correcting Th1/Th17/T_{reg} imbalances [47]. Furthermore, MSC are able to induce the differentiation of T_{reg} from effector T cells so that these new T_{reg} are immunosuppressive [48,49]. Therefore, we believe that the colitis amelioration after MSC treatment may be correlated with the control of inflammatory responses upon direct or indirect modulation of the recipient's T_{reg}. Yamanishi *et al.* [50] have shown that the induction of forkhead box protein 3 $(FoxP3)^+CD4^+CD25^+$ cells improves experimental colitis, corroborating our findings. Moreover, although we have not observed higher expression of FoxP3 in mice treated with MSC, the increase of other markers of T_{reg} is in agreement with the hypothesis that infused MSC may have promoted immunoregulation and controlled the intestinal response in animals with colitis. Indeed, T_{reg} can also express other phenotypical molecules, including $CD45RB^{low}$ [51], CTLA-4 [52–54], PD-1 [55–58] and GITR [59].

PD-1 and its ligand PD-L1 are highly expressed in T_{reg} during colitis, where they act by limiting autoimmune response and attenuating inflammation. Indeed, the blockage of immune checkpoints by anti-PD-1 or anti-PD-L1 monoclonal antibodies, usually applied for reducing colorectal cancer malignancy, exacerbates gut inflammation during colitis [60]. Interestingly, in this study we found that MSC treatment led to increased PD-1 expression in T_{reg} after short- and long-term evaluation, which may contribute to the protective effects of MSC on gut inflammation. In fact, MSC present PD-L1, which enhances T_{reg}-mediated immunossupression by affecting PD-1 expression and the activation of PD-1 downstream signaling [61]. Conversely, GITR is constitutively expressed by

T_{reg} and upon exposure to alloantigens [62]. The interaction of these surface molecules has been reported as important factor that attenuates the inflammatory response in autoimmune diseases [63]. Accordingly, we show that MSC treatment led to an early increase in GITR expression, which may contribute to the protective effects of MSC on gut inflammation.

As discussed previously, our results indicate an important role for MSC in reducing the pathogenic Th17 response in experimental colitis, in agreement with Godoi *et al.* [16], who observed reduced levels of IL-17 after bone marrow transplantation for the treatment of experimental intestinal inflammation. Th17 cells and the cytokine IL-17 have been found at elevated levels in intestinal tissue and serum from patients with IBD, confirming the finding that this pattern of immune response contributes to the development of the disease [64]. Interestingly, we also show that MSC infusion reduced the frequency of IL-17/IFN-γ double-producing cells at spleen, which was followed by a decreased accumulation of neutrophils in the colon of treated mice. Taken together, these findings suggested that IL-17/IFN-γ double-producing cells may also be a pathogenic population that is constrained by MSC treatment. Accordingly, IL-17 recruits mononuclear cells and neutrophils to peripheral tissues for amplifying local inflammation [46,65–70]. Human Th17 cells in IBD can express IFN-γ, IL-23R and IL-12Rβ2 [66]. In turn, IL-12 can reduce the expression of IL-17 and retinoid-related orphan receptor gamma t (RORγt) [66,67], thus pointing to the interplay between Th17 and Th1 cell profiles in the pathogenesis of IBD. Indeed, both IFN-γ and IL-17 responses are prejudicial in CD [68].

Finally, our results showed that, even after a long period of time, MSC were able to keep the intestinal inflammation under control. This effect was observed in the histopathological results and was also represented by the maintenance of regulation and higher IL-4 levels, together with a reduction of Th17 response after treatment. Therefore, although previous studies have already shown the relevance of MSC treatment in variable periods after cellular therapy [25–28], our findings are pioneering in showing such long-term effects of this modulatory single therapy in colitis, as described previously in other models. This durable effect of cellular therapy is of great relevance for IBD, as CD and UC are chronic disabling diseases usually treated with complex and prolonged treatments that require patient compliance, despite the unpleasant adverse effects of medications. Therefore, a single long-term intervention (with MSC alone or even combined with biologicals or other drugs – to be tested) could encourage a higher engagement of the patients and then ameliorate their commitment to the therapy as well as the disease outcome. Furthermore, the patient's stabilization for a longer time could also contribute to fewer relapse episodes, improved mucosal healing, inflammation control and reduced complications.

In conclusion, our findings confirm the long-term protective efficacy assigned to MSC for treating IBD, thus supporting the use of MSC in the modulation of inflammatory and autoimmune diseases. Most importantly, we show, for the first time to our knowledge, that acute effects of MSC administration on correcting Th1/Th17/T_{reg} imbalance persist chronically and may be essential to the long-term maintenance of gut homeostasis in hosts treated with these cells (7). Finally, our results open new perspectives for more effective treatments for diseases of difficult control such as CD and UC, based on cellular therapy with human MSC.

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Disclosure

The authors declare that they have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Dose-response evaluation of body weight variation of mice treated with mesenchymal stromal cells (MSC). Mice received intrarectal enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) for induction of colitis and were treated 24h later with 0.25, 1 or 2x106 human MSC cells by intraperitoneal route. The body mass change (in percentage) was evaluated in relation to the first day of colitis

Fig. S2. Histopathological evaluation of colitis outcome in mice treated with mesenchymal stromal cells (MSC). Mice received intrarectal enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) for induction of colitis and were treated 24h later with 1x106 human MSC cells by intraperitoneal route. The histological alterations in colon tissues were observed after haemaoxilin-eosin staining of samples collected on days 3, 7, 14 and 60 post TNBS infusion and treatment. In the upper panel, image depicts control ethanol mice, on day 3. The middle panel shows images representative of TNBS colitis group on days 3, 7 and 14, while the lower panel depicts TNBS mice on the same days, treated with MSC infusion.