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Mesenchymal stromal cells improve renal injury in anti-Thy 1 nephritis by modulating inflammatory cytokines and scatter factors

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ABSTRACT

MSC (mesenchymal stromal cells) can differentiate into renal adult cells, and have anti-inflammatory and immune-modulating activity. In the present study, we investigated whether MSC have protective/reparative effects in anti-Thy1 disease, an Ab (antibody)-induced mesangiolysis resulting in mesangioproliferative nephritis. We studied five groups of rats: (i) rats injected with anti-Thy1.1 Ab on day 0 (group A); (ii) rats injected with anti-Thy1.1 Ab on day 0+MSC on day 3 (group B); (iii) rats injected with anti-Thy1.1 Ab on day 0+mesangial cells on day 3 (group C); (iv) rats injected with saline on day 0+MSC on day 3 (group D); and (v) rats injected with saline on day 0 (group E). Rats were killed on days 1, 3, 7 and 14. MSC prevented the increase in serum creatinine, proteinuria, glomerular monocyte influx and glomerular histopathological injury. Furthermore, MSC suppressed the release of IL-6 (interleukin-6) and TGF-β (transforming growth factor-β), modulated glomerular PDGF-β (platelet-derived growth factor-β), and reset the scatter factors and their receptors, potentiating HGF (hepatocyte growth factor)/Met and inactivating MSP (macrophage-stimulating protein)/Ron (receptor origin nantaise). Few MSC were found in the kidney. These results indicate that MSC improve anti-Thy 1 disease not by replacing injured cells, but by preventing cytokine-driven inflammation and modulating PDGF-β and the scatter factors, i.e. systems that regulate movement and proliferation of monocytes and mesangial cells.

INTRODUCTION

MSC (mesenchymal stromal cells) are multipotent cells that can be isolated from bone marrow and fetal tissues [1]. Because of their differentiation into mature cells of various types and their easy expansion *in vitro* [2], MSC are a promising cell therapy that have been tried to regenerate injured organs in experimental and human disease [3,4]. In addition, MSC appear to have a distinct immunomodulatory action that results in a protective effect in immune-mediated diseases [5–8].

Anti-Thy 1 nephritis is an experimental model of immune-mediated glomerular disease, induced by an Ab (antibody) directed against the Thy 1 antigen (CD90)

Key words: anti-Thy 1 nephritis, hepatocyte growth factor, interleukin, mesangioproliferative nephritis, mesenchymal stromal cell, scatter factor.

Abbreviations: Ab, antibody; CCD, charge-coupled-device; DAB, diaminobenzidine; GFP, green fluorescent protein; EGFP, enhanced GFP; HGF, hepatocyte growth factor; IL-6, interleukin-6; ISCT, International Society for Cellular Therapy; i.v., intravenous(ly); mAb, monoclonal Ab; MSC, mesenchymal stromal cell(s); MSP, macrophage-stimulating protein; MV, microvesicles; PCNA, proliferating cell nuclear antigen; PDGF-β, platelet-derived growth factor-β; Ron, receptor origin nantaise; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; vtv, via a tail vein.

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constitutively expressed in mesangial cells. The disease is characterized by acute mesangiolysis followed by early inflammatory cell infiltration, repopulation of the mesangium by new mesangial cells and accumulation of mesangial matrix [9].

Kunter et al. [10] have previously investigated the effects of MSC in anti-Thy 1 nephritis and showed that MSC reduced mesangiolysis when injected into the renal artery, but not when infused i.v. (intravenously). However, the conclusions are jeopardized by relevant methodological limitations, the most important of which is that the MSC used in that study did not express the Thy 1 antigen CD90. The rationale for selecting CD90-negative MSC was to obviate binding of residual circulating anti-CD90 Ab to the injected MSC. However, CD90 is one of the minimal criteria for defining MSC according to the Mesenchymal and Tissue Stem Cell Committee of the ISCT (International Society for Cellular Therapy) [11], and the loss of CD90 implies important functional modifications. In fact, CD90 is a GPI (glycosylphoshatidylinositol)-anchored glycoprotein that affects numerous biological processes, including cell adhesion, cell–cell and cell–matrix interaction, cell migration, and cell death [12], and a low expression of CD90 in human MSC is associated with a loss of immunosuppressive activity [13]. Furthermore, the authors [10] based their assumption of the paracrine action of MSC only on *in vitro* evidence. In the present study, we treated rats with anti-Thy 1 nephritis with typical MSC expressing CD90. We administered MSC intravenously in order to reproduce the condition in which they might be easily used in the clinical setting, and MSC were injected 72 h after the injection of the anti-Thy 1.1 Ab, i.e. at a time when no residual Ab was circulating. In addition, we investigated the effects of MSC on cytokines *in vivo*, and extended our investigation to the scatter factors, i.e. HGF (hepatocyte growth factor)/scatter factor and its receptor Met, and MSP (macrophage-stimulating protein)/HGF-like scatter factor and its receptor Ron (receptor origin nantaise). We also used transgenic EGFP [enhanced GFP (green fluorescence protein)]-expressing rats as MSC donors to track injected MSC [14].

MATERIALS AND METHODS

Animals

Experiments were performed according to guidelines of our ethical committee for animal studies. Wild-type Sprague–Dawley rats (Charles River) and transgenic Sprague–Dawley rats that express EGFP (Japan Slc) were used. The rats carrying the EGFP transgene were established with the same construct and technique used to produce EGFP transgenic mice [14].

Isolation and culture of MSC and mesangial cells

Sprague–Dawley EGFP transgenic rats were killed by CO_2 inhalation. The total marrow cell content of both the femurs and tibias was flushed from bones and EGFP-positive cells were plated at a concentration of 10^6 cells/ml with Murine MesenCult medium (Stemcell Technologies) and incubated at 37 °C in a 5 % humidified CO_2 atmosphere. After 48 h of culture, non-adherent cells were discarded; fresh medium was added and replaced twice a week. When cultures reached sub-confluence, adherent cells were detached with 0.05 % trypsin (Sigma–Aldrich). Collected cells were replated at a concentration ranging from 0.05×10^5 to 0.15×10^5/ml of MesenCult medium for several passages.

The rat mesangial line CRL-2573 was obtained from the A.T.C.C. The cells were at passage 8, expressed normal genes of wild-type mesangial cells, and were positive for desmin and vimentin and negative for cytokeratin 8. The cells were seeded at a density of 64 000/cm^2 in a culture flask and were cultured in high-glucose DMEM (Dulbecco's Modified Eagle Medium) (Gibco), containing 1 % (v/v) penicillin/streptomycin, 1 % (v/v) amphotericin B, 0.4 mg/ml G418 (Gibco) and 15 % (v/v) FBS (fetal bovine serum) (Euroclone), at 37 °C in a 5 % humidified CO_2 atmosphere. After 48 h of culture, non-adherent cells were discarded; fresh medium was added and replaced twice a week. When cultures reached sub-confluence, adherent cells were detached with 0.25 % trypsin/EDTA. Collected cells were replanted for several passages at a concentration ranging from 12 000 to 20 000/cm^2.

Immunocytochemical characterization and osteogenic and adipogenic differentiation of MSC

Cultured EGFP MSC were evaluated by flow cytometry starting from passage 3 for CD90, CD73, CD45 and CD11b using a FacsCalibur flow cytometer (BD Biosciences). Differentiation into osteocytes and adipocytes was evaluated as described previously [2]. Histochemical analysis of the cell layer was performed by evaluating calcium deposition (Alizarin Red staining; Sigma–Aldrich). For adipogenic induction, 2×10^4 MSC/cm^2 were seeded into chamber slides with Mesencult. At confluence, cells were stimulated with adipogenic medium (Cambrex) for 3 weeks replacing the medium every 3 days. Cells were stained with Oil Red O (Bioptica).

In vivo experiments

We studied a total of five groups of rats (Figure 1): group A, 20 rats were injected i.v. at day 0 vtv (via a tail vein) with 400 μl of saline that contained 400 μg of anti-Thy 1.1 mAb (monoclonal Ab) (Cedarlane Laboratories), as

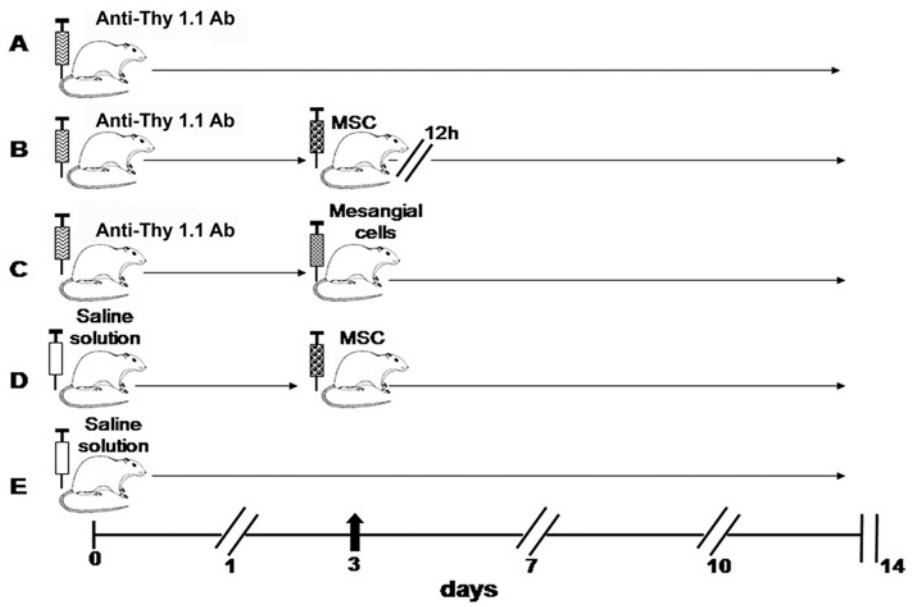

Figure 1 Experimental design of the study

A total of five groups of rats was studied. Group A, rats in which anti-Thy 1 nephritis was induced by injecting anti-Thy 1.1 Ab i.v. on day 0. These rats were studied to define the spontaneous course of the disease. Group B, rats in which anti-Thy 1 nephritis was induced as in group A and were treated with MSC infused i.v. on day 3. These rats were studied to understand whether MSC modify the course of the disease and the mechanisms thereof. Group C, rats in which anti-Thy 1 nephritis was induced as in groups A and B and were treated with mesangial cells on day 3. These rats were studied to investigate the systemic and renal effects of MSC-sized cells endowed with potential tropism to the kidney. Group D, rats injected with saline on day 0, followed by injection of MSC on day 3. These rats were studied to investigate the effects of MSC on normal kidneys. Group E, control rats injected with saline on day 0. Slashes indicate days in which rats were killed. Five rats in each group were killed on days 1, 7, 10 and 14. Five rats in group B were killed 12 h after MSC infusion.

described previously [18]; group B, 25 rats were injected vtv at day 0 with 400 μg of anti-Thy 1.1 mAb, followed after 3 days by injection vtv of 1 ml of PBS containing 3×10^6 MSC isolated from EGFP rats; group C, 20 rats were injected vtv at day 0 with 400 μg of anti-Thy 1.1 mAb, followed after 3 days by injection vtv of 3×10^6 rat mesangial cells; group D, 20 rats were injected vtv at day 0 with 400 μl of saline, followed at day 3 by injection vtv of 1 ml of PBS containing 3×10^6 MSC isolated from EGFP rats; and group E, 20 rats were injected vtv with 400 μl of saline. Five rats from each group were killed at day 1, 7, 10 and 14, and five rats from group B were killed 12 h after MSC infusion. Soon after killing, kidneys, lungs and spleen were removed, and half were fixed in 10% neutral-buffered formalin and half were frozen in liquid nitrogen.

All rats were male, weighed 180–200 g and were housed at a constant temperature (20 C°) and humidity (75%) under a controlled light cycle, with free access to water and standard chow diet.

24-h Urine was obtained by housing individual rats in metabolic cages for up to 24 h on days 0, 7, 10 and 14; urine volume was measured and samples were stored at −20 °C. Blood was drawn on day 0 from the caudal vein and on days 7, 10 and 14 by cardiac exsanguination. Samples were centrifuged, and serum was stored at −20 °C.

In vivo tracking of circulating anti-Thy 1.1 Ab

MSC (10^5) cultured in chamber slides were fixed with 10% formalin/acetate for 10 min, washed in PBS, incubated in a dark humid chamber at room temperature (25 °C) for 60 min with rat serum (100 μl) and sampled immediately before and 1, 24, 48 and 72 h after anti-Thy1.1 Ab injection. Moreover, we used anti-Th1.1 Ab at a concentration of 1 μg/μl adding 20 μl of Ab in a total volume of 100 μl of normal rat serum as a positive control. After three washes in PBS, the cells were treated with the secondary antibody (Universal Biotinylated Link; Dako) and the streptavidin–biotin–peroxidase complex (Dako). Visualization was with DAB (diaminobenzidine) (Dako). Harris haematoxylin was used to lightly counterstain the nuclei. Finally, the cells were dehydrated with an increasing alcohol concentration (95–100% xylol) and the coverslips were mounted with synthetic non-aqueous mounting medium (Dako) for analysis with an Olympus IX8 microscope connected to a computer system for cell imaging and a CCD (charge-coupled-device) camera

(magnification, × 200). All experiments were performed in duplicate.

Deposition of anti-Thy1.1 Ab

Anti-Thy 1.1 Ab deposition was detected by immunohistochemistry in formalin-fixed kidneys of rats from all of the groups killed on day 1. Sections (3-μm-thick) were collected on poly-(L-lysine)-coated slides (Dako), dewaxed in xylol, passed through a decreasing series of alcohol, and finally rehydrated with distilled water. Endogenous peroxidase was blocked with 3.7% (v/v) H_2O_2, followed by H_2O for 15 min. After three washes in 150 mM PBS, the sections underwent microwave antigen retrieval. Subsequently, in order to visualize anti-Thy 1.1 Ab deposition, the sections were incubated with the ready-to-use anti-mouse secondary antibody EnVision Plus (Dako) and the colour reaction was developed with DAB (Dako) as the chromogen. The slides were then counterstained with Harris haematoxylin. The extent of anti-Thy 1.1 Ab deposited was evaluated in a blinded manner in 40 random glomeruli in each kidney section by image analysis, using an Olympus IX8 microscope connected to a CCD camera and Cell-R software on an image processing computer. We measured the ratio between the glomerular anti-Thy 1.1 Ab-positive area and the glomerular tuft area.

Deposition of C3 complement fraction

Glomerular deposition of C3 was detected in frozen tissue of rats from all groups killed on day 1. Sections (5-μm-thick) were collected on poly-(L-lysine)-coated slides (Dako), fixed in acetone for 5 min at room temperature and then passed through distilled water. Subsequently, the sections were exposed to an FITC-conjugated polyclonal sheep anti-(human C3) Ab [1:600 dilution in PBS/1% (w/v) BSA; ICN]), which cross-reacts with rat C3, for 1 h at room temperature. After three washes in PBS, the coverslips were mounted with aqueous mounting medium for analysis with a fluorescence microscope. Negative controls included both the omission of the primary Ab and IgG substituted for primary antibodies.

The extent of C3 deposition was evaluated in a blinded manner in 40 random glomeruli in each kidney section, as described above.

Biochemical measurements

Serum creatinine and urinary protein concentrations were measured at days 0, 7, 10 and 14 using an automated method (Abbott Laboratories) and the Bio-Rad Laboratories Protein Assay.

IL-6 (interleukin-6) and TGF-β (transforming growth factor-β) were quantified in rat serum at days 7, 10 and 14 by ELISA (R&D Systems). IL-6 was also measured in 24-h urine samples at days 7, 10 and 14 by ELISA. All experiments were performed in quadruplicate.

Renal morphology

Sections (3-μm-thick) cut along the maximal sagittal plane of formalin-fixed kidneys were stained with haematoxylin/eosin and examined by two investigators in a double-blind fashion, using an Olympus IX8 microscope connected to a CCD camera and Cell-R software imaging analysis. A thorough analysis of glomerular lesions was performed in all glomeruli present in three sections of all kidneys of groups A, B and C. The following types of lesions were distinguished: (i) mesangiolysis, (ii) ballooning, (iii) microaneurysm, (iv) matrix or cellular crescent, and (v) necrosis. The damage score was established using the following criteria: glomeruli without obvious changes were scored as 0, glomeruli with mesangiolysis and/or ballooning involving <25% of the glomerular area were scored as 1, glomeruli with mesangiolysis and/or ballooning involving 26–50% of the glomerular area were scored as 2, glomeruli with mesangiolysis and/or ballooning involving 51–75% of the glomerular area were scored as 3, and glomeruli with mesangiolysis and/or ballooning involving >75% of the glomerular area and/or microaneurysm and/or crescents and/or glomerular necrosis were scored as 4.

The results are expressed as the percentage of glomeruli in each grade score. Furthermore, we evaluated the extent of ballooning and necrosis as the ratio between the glomerular area replaced by ballooning and necrosis and the total glomerular area.

Expression of ED-1 antigen, PCNA (proliferating cell nuclear antigen), α-SMA (α-smooth muscle actin), PDGF-β (platelet-derived growth factor-β), HGF, Met, MSP and Ron

The glomerular expression of ED-1 antigen, PCNA, α-SMA, PDGF-β, HGF, Met, MSP and Ron were studied by immunohistochemistry in formalin-fixed tissue.

Sections (3-μm-thick) of paraffin-embedded tissue were collected on poly-(L-lysine)-coated slides (Dako), dewaxed in xylol, passed through a series of decreasing alcohol concentrations, and finally rehydrated with distilled water. Endogenous peroxidase was blocked with 3.7% (v/v) H_2O_2 followed by H_2O for 15 min. After three washes in 150 mM PBS, the sections underwent microwave antigen retrieval. Subsequently the sections were exposed overnight at 4 °C to the following antibodies: (i) monoclonal mouse anti-(rat ED-1) (1:300 dilution; Serotec); (ii) monoclonal mouse anti-PCNA (1:200 dilution; Santa Cruz Biotechnology); (iii) monoclonal mouse anti-(human α-SMA) (1:100 dilution; Dako); (iv) polyclonal rabbit anti-(human PDGF-β) (1:200 dilution; Santa Cruz Biotechnology); (v) polyclonal rabbit anti-(human HGF) (1:50 dilution; Santa Cruz Biotechnology); (vi) monoclonal mouse anti-(human Met) (1:20 dilution;

Novocastra Laboratories); (vii) polyclonal goat anti-(mouse MSP) (1:600 dilution; Santa Cruz Biotechnology); and (viii) monoclonal mouse anti-(human Ron) (1:750 dilution; Transduction Laboratories). After three washes in PBS, the immunocomplex was visualized with the biotin–streptavidin–peroxidase complex and DAB (DAKO). Sections were faintly counterstained with Harris haematoxylin. Negative controls included both omission of the primary Ab and IgG was substituted for primary antibodies. In all glomeruli of three sections of each kidney, cells positive for ED-1 antigen and PCNA were counted, and the extent of PDGF-β, α-SMA, HGF, MSP and Ron expression was measured as the ratio between the glomerular PDGF-β, α-SMA, HGF, MSP and Ron-positive area and the whole glomerular area. Glomerular capillary Met expression was measured as the ratio between the number of Met-stained glomerular capillaries and total number of glomerular capillaries.

Tracking of EGFP-positive cells

We tracked EGFP-positive cells by immunohistochemistry in the kidney, spleen and lung excised 12 h and at days 7, 10 and 14 after injection of MSC. Sections (3-μm-thick) of paraffin-embedded tissue were collected on poly-(L-lysine)-coated slides (Dako), dewaxed in xylol, passed through a series of decreasing alcohol concentrations, and finally rehydrated with distilled water. Endogenous peroxidase was blocked with 3.7 (v/v) H_2O_2 followed by H_2O for 15 min. After three washes in 150 mM PBS, the sections underwent microwave antigen retrieval. Subsequently the sections were exposed overnight at 4 °C to monoclonal mouse anti-GFP (IgG_1; Chemicon), diluted 1:1000 in PBS/1 % (w/v) BSA. EGFP-positive cells were visualized with the biotin–streptavidin–peroxidase complex and stained with DAB (Dako). Sections were faintly counterstained with Harris haematoxylin. Negative controls included the omission of the primary Ab and IgG was substituted for primary antibodies. Positive controls were sections of kidney from EGFP rats. A total of ten sections from each organ were analysed.

Statistical analysis

ANOVA followed by the Newman–Keuls test or Student's *t* test were used for comparisons of the means. Differences in renal injury scores were evaluated with Mann–Whitney and Kruskal–Wallis tests.

RESULTS

Characterization of rat MSC

MSC isolated from the bone marrow of Sprague–Dawley EGFP rats differentiated into osteogenic and adipogenic cells when cultured in appropriate conditions (see Figure S1 at http://www.clinsci.org/cs/120/cs1200025add.htm). Flow cytometric analysis showed that MSC were positive for CD90 ($\geqslant$95 %) and CD73 ($\geqslant$95 %), and were negative for CD45 (<5 %) and CD11b (<5 %).

Tracking of circulating anti-Thy 1.1 Ab

In preliminary experiments, we incubated MSC *in vitro* with rat serum sampled 1, 24, 48 and 72 h after i.v. injection of anti-Thy 1.1 Ab and detected an MSC-binding Ab present in serum by immunocytochemistry. Figure S2 (at http://www.clinsci.org/cs/120/cs1200025add.htm) shows there was no serum binding activity before antibody injection and intense staining of cell-bound antibody 1 h after its injection. Progressively attenuated staining was visible after 24 and 48 h, and no residual antibody was detectable after 72 h. These results suggest that residual circulating Ab does not interfere with MSC infused after 72 h.

In vivo experiments

Throughout the study, no significant differences were observed in body weight, and water and food intake among the five groups of rats.

Glomerular deposition of anti-Thy1.1 Ab and C3 complement fraction

In order to demonstrate that the pathogenic steps that start anti-Thy 1 disease occurred similarly in untreated rats and in rats treated with MSC, we evaluated the glomerular deposition of anti-Thy 1.1 and C3 complement fraction within 24 h of Ab injection. As expected, no deposition of anti-Thy 1.1 Ab or C3 was observed in rats from groups D and E. At 24 h after the injection of anti-Thy 1.1 Ab, both the Ab and C3 were similarly deposited in glomeruli in groups A, B and C (Figure S3 at http://www.clinsci.org/cs/120/cs1200025add.htm).

Renal function

Baseline (day 0) serum creatinine was similar in the five groups of rats. In groups A, B and C, creatinine increased significantly on day 7 compared with day 0 [from 0.41 ± 0.10 to 0.62 ± 0.14 mg/dl in group A ($P < 0.05$); from 0.51 ± 0.20 to 0.74 ± 0.20 mg/dl in group B ($P < 0.05$); and from 0.44 ± 0.30 to 0.64 ± 0.05 mg/dl in group C ($P < 0.05$); values are means $\pm$ S.D.]. At day 10, creatinine remained higher than baseline in group A (0.59 ± 0.01 mg/dl) and group C (0.58 ± 0.03 mg/dl), but not in group B (0.45 ± 0.01 mg/dl); indeed, at day 10, creatinine was significantly lower in group B than in groups A and C ($P < 0.001$ compared with groups A and C). At day 14, creatinine decreased to levels no longer different from baseline in all three groups (0.51 ± 0.07, 0.44 ± 0.14 and 0.57 ± 0.01 mg/dl in groups A, B and C

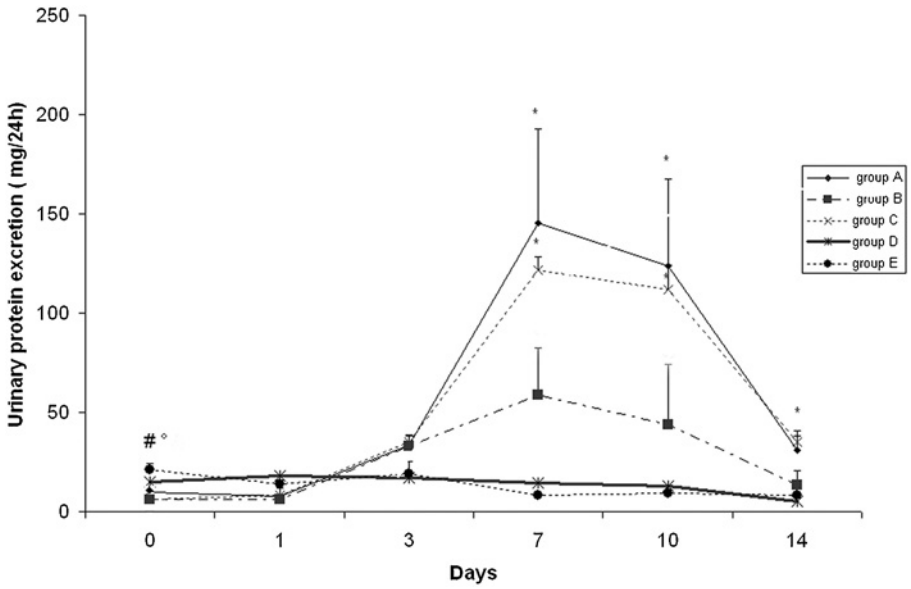

Figure 2 Urinary protein excretion rate in the experimental groups

Groups are defined as in Figure 1. Values are mean+S.D. daily (24 h) urinary protein excretion. #$P < 0.001$ compared with day 7 and day 10 in groups A and C; °$P < 0.005$ compared with day 7 and day 10 in group B; and *$P < 0.0001$ compared with group B at days 7, 10 and 14.

respectively). No change in creatinine occurred in rats not treated with the anti-Thy 1.1 Ab (group E), even in those receiving MSC (group D).

Baseline urinary protein excretion was similar in the five groups. Proteinuria increased similarly in groups A, B and C on day 3, and then increased further in groups A and C, but not in group B (Figure 2). In fact, proteinuria was significantly higher in groups A and C than in group B at days 7, 10 and 14. Furthermore, on day 14, proteinuria returned to levels not different from baseline in group B, whereas it remained higher than baseline in groups A and C. No change in proteinuria occurred in rats not treated with anti-Thy 1.1 Ab (group E), even in those receiving MSC (group D) (Figure 2).

Renal histopathology

Glomerular injury

A damage score was established ranging from 0 (no obvious change) to 4 (mesangiolysis and/or ballooning involving >75% of the glomerular area and/or microaneurysm and/or crescents and/or glomerular necrosis). A representative image from the renal histology is shown in Figure 3(a). No histological changes were observed in groups D and E. The damage scores of glomeruli in groups A and B are summarized in Figure 3(b). In group A, a high percentage of glomeruli had a score of 3–4, whereas a low percentage of glomeruli were normal. The proportion of normal compared with severely damaged glomeruli was reversed in group B, with a high percentage of glomeruli scoring 0 and a minority scoring 3–4. In group C, the injury scores were similar to those in group A, i.e. a high percentage of glomeruli scored 3–4 (68, 60 and 47% on days 7, 10 and 14 respectively), whereas a low percentage of glomeruli were normal (10, 19 and 20% on days 7, 10 and 14 respectively). Moreover, we quantified by image analysis the glomerular area occupied by ballooning and/or necrosis and found that the extent of the injured area was reduced in nephritic rats treated with MSC on days 7, 10 and 14, compared with untreated rats and rats receiving mesangial cells. MSC did not induce histological changes in healthy rats (group D) (see Figure S4 at http://www.clinsci.org/cs/120/cs1200025add.htm).

Glomerular monocytes infiltration and Ron expression

As shown in Figure 4, the number of ED-1-positive cells was significantly lower in group B than in groups A and C at days 7 and 10. As expected from the spontaneous course of the disease [9], the number of glomerular monocytes declined in all of the three groups at day 14. Mesangial cells did not affect monocyte infiltration in nephritic rats, whereas no inflammatory infiltration was found in healthy rats receiving MSC (group D) and in the control group E. As the MSP/Ron system attracts monocytes into the glomeruli and stimulates mesangial cell proliferation in anti-Thy 1 disease [15,16], we investigated the effects of MSC on glomerular expression of Ron. MSC decreased glomerular expression of Ron on days 7 and 10 in nephritic rats (group B), but did not modify Ron expression in healthy rats (group D). Ron expression also did not change in nephritic rats receiving mesangial cells (group C).

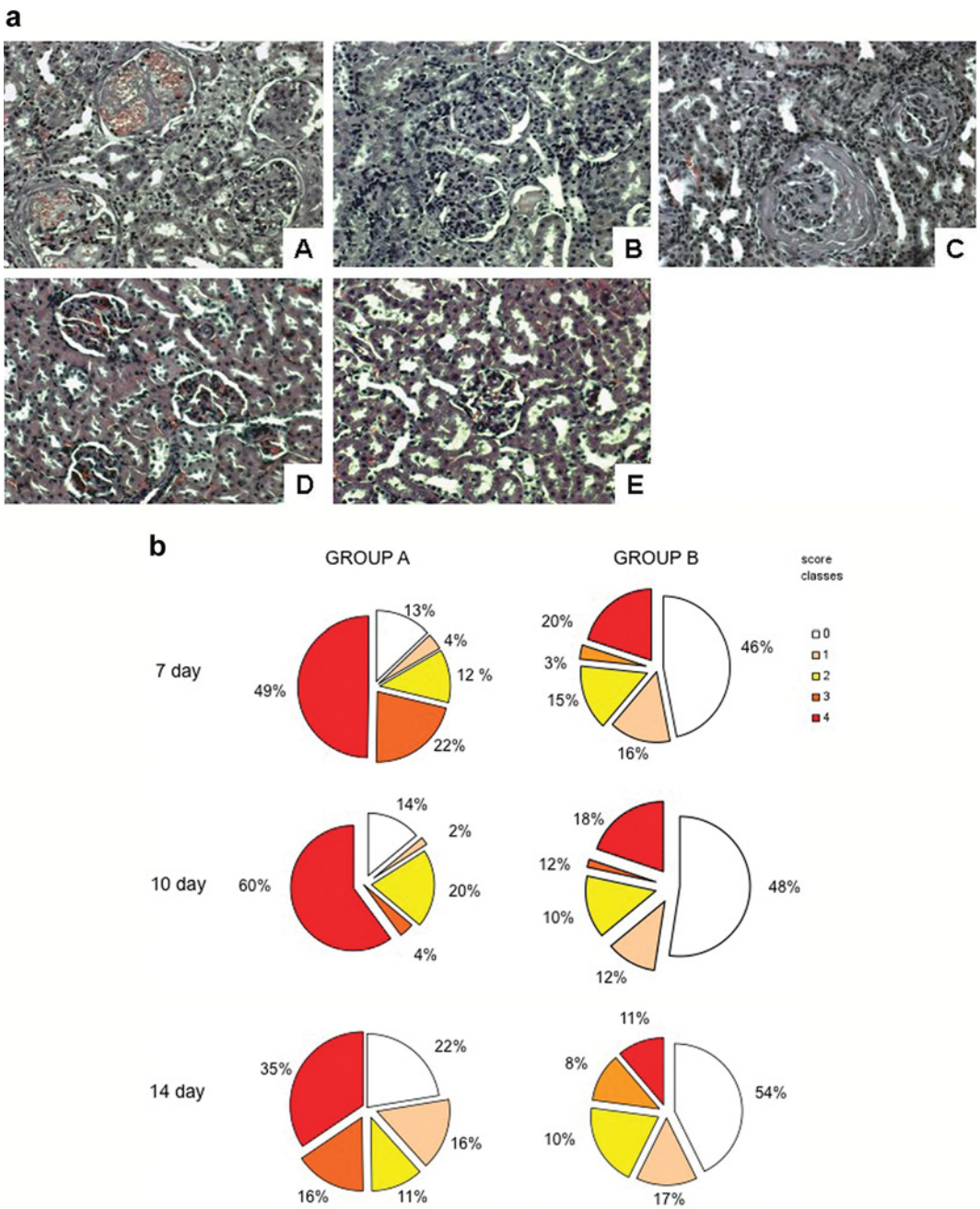

Figure 3 Representative renal morphology in the experimental groups at day 10 (a), and the glomerular damage score in groups A and B (b)

Groups are defined in Figure 1. In (a), sections were stained with haematotoxylin/eosin. Magnification, × 200. In (b), the mean percentage of glomeruli scoring 0–4 on days 7, 10 and 14 are shown ($P < 0.0001$ when group A is compared with group B, using the Kruskal–Wallis test).

Glomerular expression of α-SMA, PCNA, MSP and PDGF-β

Anti-Thy1 disease is a mesangioproliferative nephritis in which the proliferation of activated mesangial cells is stimulated by MSP released locally by mesangial cells themselves [15,16] as well as by PDGF-β released by mesangial cells and monocytes [17]. The injection of MSC modulated glomerular endocapillary proliferation (PCNA-positive cells), mesangial cell activation (α-SMA-positive cells) and the release of MSP and PDGF-β (Figure 5). In fact, in nephritic rats injected with MSC, a steady increase in endocapillary proliferation occurred at days 7, 10 and 14, whereas in rats not treated with MSC and rats treated with mesangial cells (groups A and C respectively) a peak increase in proliferation occurred at day 7, followed by a decline at day 10 and by a further increase at day 14. Similarly glomerular expression of

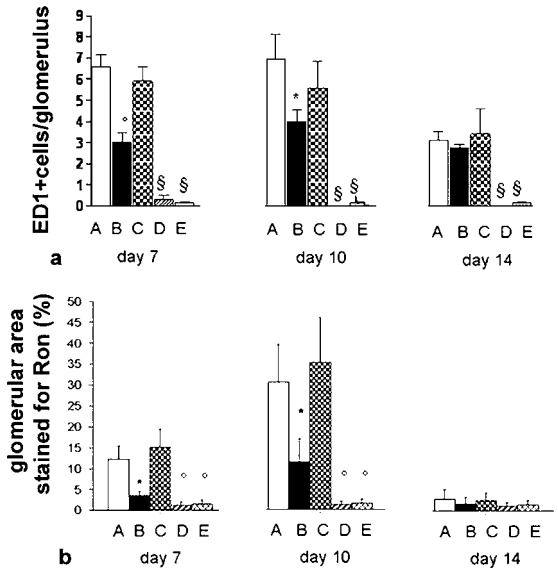

Figure 4 Glomerular monocytes and Ron expression in the experimental groups

Groups are defined in Figure 1. (a) Values are the mean + S.D. numbers of monocytes/glomerulus, as determined by ED-1 staining. $^{*}P < 0.05$ and $^{\circ}P < 0.0001$ compared with groups A and C; $\S P < 0.0001$ compared with groups A, B and C. (b) Values are mean+S.D. percentage glomerular area staining for Ron. $^{*}P < 0.001$ compared with groups A and C at days 7 and 10; and $^{\circ}P < 0.05$ compared with groups A, B and C at days 7 and 10.

α-SMA was steady in nephritic-treated rats, whereas it peaked on day 7 and fell at day 10 in rats not treated with MSC. It is noteworthy that the suppression of glomerular proliferation in nephritic rats not treated with MSC was synchronous at day 10 with the maximum extent of severe glomerular injury, as indicated by ballooning and necrosis. Moreover, in nephritic rats, MSP and PDGF-β expression was also significantly suppressed by MSC at days 7 and 10, whereas, in non-nephritic rats (group D), MSC did not affect the glomerular expression of α-SMA, PCNA, MSP and PDGF-β. There was no change in group E.

HGF/Met system

In non-nephritic rats, HGF (Figure S5 at http://www.clinsci.org/cs/120/cs1200025add.htm) and Met (Figure S6 at http://www.clinsci.org/cs/120/cs1200025add.htm) were diffusely expressed in tubules, whereas they were almost absent in glomeruli. *De novo* expression of HGF in glomeruli occurred in all rats with anti-Thy 1 disease, but the level of HGF was steadily high in MSC-treated rats, whereas it declined on day 14 in untreated rats and in rats treated with mesangial cells. It is notable that MSC injected into healthy rats (group D) did not result in the up-regulation of HGF. Glomerular expression of Met increased slightly in rats with anti-Thy 1 disease that were not treated with MSC (groups A and C). In contrast, treatment with MSC induced a striking increase in glomerular Met expression at days 10 and 14 (Figure 6). Interestingly, MSC induced overexpression of Met in endothelial cells of glomerular capillaries, but only in nephritic rats (Figures S6a and S6b).

MSC localization

We examined ten kidney sections and detected very few EGFP-positive cells (0–5 for total kidney/section) in the kidney of each group of rats at each time point. EGFP-positive cells were located in tubules, in the interstitium or in the glomerular tufts. We found 3–4 cells/field in spleen sections at 12 h after MSC infusion and, occasionally, MSC in lung sections were detected at the same time (Figure S7 at http://www.clinsci.org/cs/120/cs1200025add.htm).

IL-6 and TGF-β levels

As shown in Figure 7, serum levels of IL-6 increased in all of the groups with anti-Thy 1 disease (groups A, B and C), but the increase was striking in groups not treated with MSC (groups A and C) in comparison with the modest rise occurring in rats treated with MSC (group B). The suppression of IL-6 induced by MSC was particularly evident at day 10 when serum IL-6 peaked in untreated rats. The infusion of MSC induced a modest, but significant, increase in serum IL-6 in rats without nephritis ($P < 0.001$ when group D was compared with group E). In addition, urinary IL-6 increased significantly in all groups with nephritis, and the increase in urinary IL-6 was significantly attenuated by treatment with MSC throughout the study period.

Serum TGF-β levels increased significantly in all of the groups with anti-Thy 1 nephritis, and the increase was significantly attenuated by treatment with MSC at days 10 and 14. MSC did not induce an increase in serum TGF-β levels in the non-nephritic rats (group D) (Figure 7).

DISCUSSION

The aim of our present study was to investigate the effects of treatment with MSC on the course of anti-Thy 1 nephritis. We used MSC expressing CD90 in accordance with the minimal criteria for defining MSC provided by the ISCT [11]. CD90 is targeted by the anti-Thy 1.1 Ab that is injected to induce the disease, therefore residual circulating Ab may react with CD90-positive MSC and interfere with their viability and their physiological functions. Conversely, MSC may bind circulating Ab and prevent it from reaching and injuring mesangial cells. In order to obviate such interferences, we performed preliminary experiments to determine when the injected Ab was completely cleared from the circulation. We found no residual MSC-binding Ab in serum at 3 days

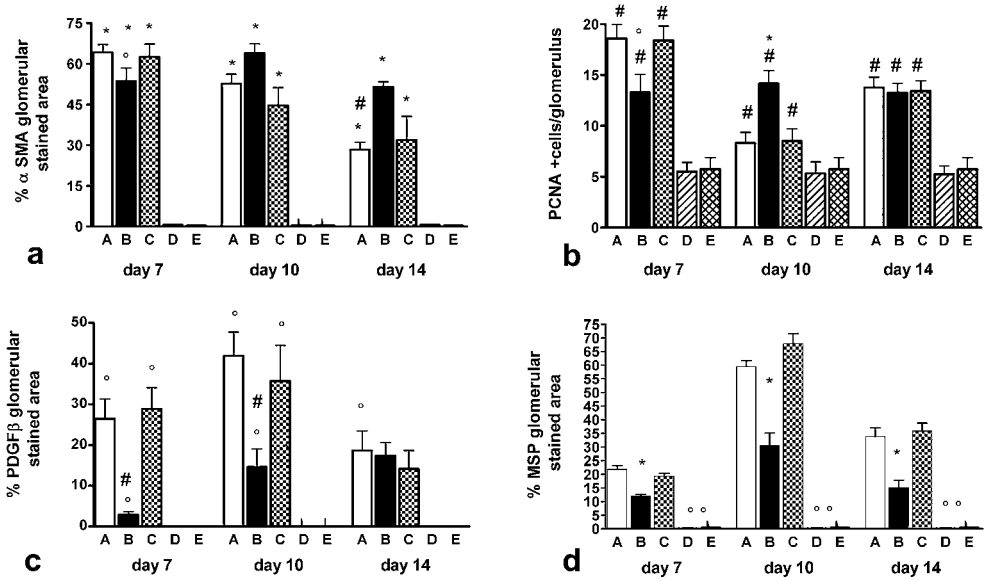

Figure 5 Glomerular expression of α-SMA (a), PCNA (b), PDGF-β (c) and MSP (d) in the experimental groups

Groups are defined in Figure 1. Values are means + S.D. In (a), $^{\circ}P < 0.005$ compared with groups A and C; $^{*}P < 0.0001$ compared with groups D and E. In (b), $^{\circ}P < 0.05$ and $^{*}P < 0.01$ compared with groups A and C; and $\#P < 0.001$ compared with groups D and E. In (c), $^{\circ}P < 0.0001$ compared with groups D and E; $\#P < 0.005$ compared with groups A and C. In (d), $^{*}P < 0.05$ compared with groups A and C; and $^{\circ}P < 0.05$ compared with groups A, B and C.

after its injection and, consequently, we infused MSC 3 days after the induction of the disease.

The main finding of our present study is that MSC infused i.v. mitigate anti-Thy 1 disease. In fact, treatment with MSC resulted in the recovery of serum creatinine to baseline levels at day 10 and reduced proteinuria in each of the days studied, returning it to baseline at day 14. Furthermore, MSC and not mesangial cell infusion had striking effects on glomerular pathology. In rats treated with MSC, approximately half of the glomeruli were spared from any lesions on each of the days studied and only a minority scored 4, i.e. had severe lesions. In contrast, in rats not treated with MSC, glomeruli scoring 4 were the prevalent population, whereas normal glomeruli were a minority. MSC offered the most impressive protection at day 10, with MSC reducing the percentage of glomeruli scoring 4 from 60 % (group A) and 56 % (group C) to 18 % (group B), while increasing the percentage of intact glomeruli from 14 % (group A) and 19 % (group C) to 48 % (group B). The less aggressive course of the disease in rats infused with MSC did not depend on a decrease in anti-Thy 1.1 Ab bound to mesangial cells or on the suppression of complement activation. In fact, the amount of Ab and the C3 complement fraction deposited in glomeruli was the same in rats treated with MSC as in control nephritic rats. In anti-Thy 1 nephritis, monocytes invade the glomerulus and cause glomerular injury by releasing ROS (reactive oxygen species) and inflammatory cytokines [15]. The activated monocytes that infiltrate the glomerulus express Ron and are recruited into the tuft by the powerful chemotactic effect of MSP [16–18].

PDGF-β and TGF-β are other powerful chemoattractants for monocytes, which are released in the glomerulus in anti-Thy 1 disease [19]. In the present study, we have shown that MSC suppress glomerular MSP and Ron expression. Furthermore, MSC in nephritic rats decrease the local levels of PDGF-β and circulating levels of TGF-β. Such inhibition of different chemotactic systems is a straightforward explanation for the reduction in infiltrating monocytes and for the attenuation of glomerular injury.

Anti-Thy 1 disease represents a nominal model of mesangioproliferative glomerulonephritis [9,19]. In fact, the disease is characterized by an initial mesangiolysis that is followed by reparative overgrowth of mesangial cells [9,20]. Excessive mesangial proliferation is driven by growth factors that include MSP, PDGF-β and IL-6 [16–18,21,22]. Activated proliferating mesangial cells express α-SMA and PCNA [16–18,23]. Accordingly, in untreated nephritic rats, at day 7 we found overexpressed markers of mesangial cell activation (α-SMA) and proliferation (PCNA), and high levels of growth factors (local PDGF-β and MSP, and serum and urinary IL-6). At day 10, in spite of a further increase in growth factors, mesangial cell proliferation transiently fell, coinciding with the occurrence of peak necrosis, which reduced the number of living cells responding to growth factors. Mesangial growth resumed at day 14, still sustained by growth factors, in particular by MSP and IL-6. Mesangial cell

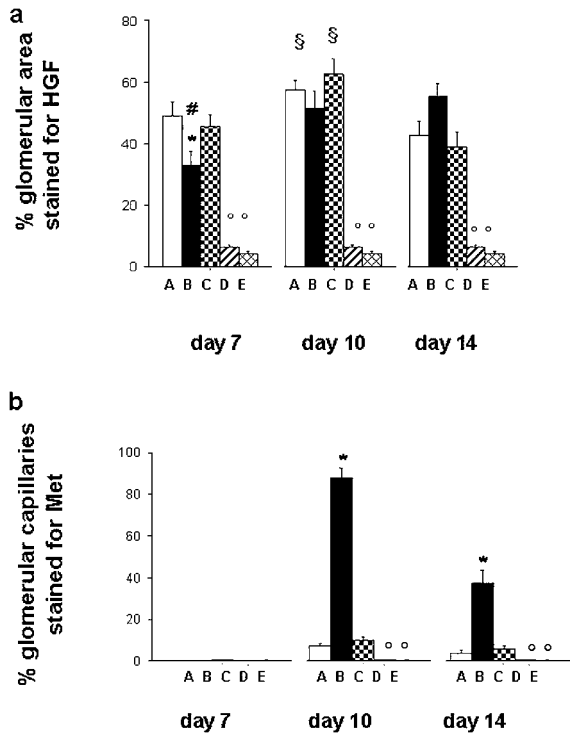

Figure 6 Glomerular expression of HGF (a) and Met (b) in the experimental groups

Groups are defined in Figure 1. Values are means + S.D. In (a), $^{*}P < 0.05$ compared with groups A and C at day 7; $^{\circ}P < 0.01$ compared with groups A, B and C at days 7, 10 and 14; $\#P < 0.05$ compared with group B at days 10 and 14; and $\S P < 0.05$ compared with groups A and C on day 14. In (b), $^{*}P < 0.001$ compared with the other groups at days 10 and 14, and all of the groups at day 7; $^{\circ}P < 0.05$ compared with groups A and C at days 10 and 14.

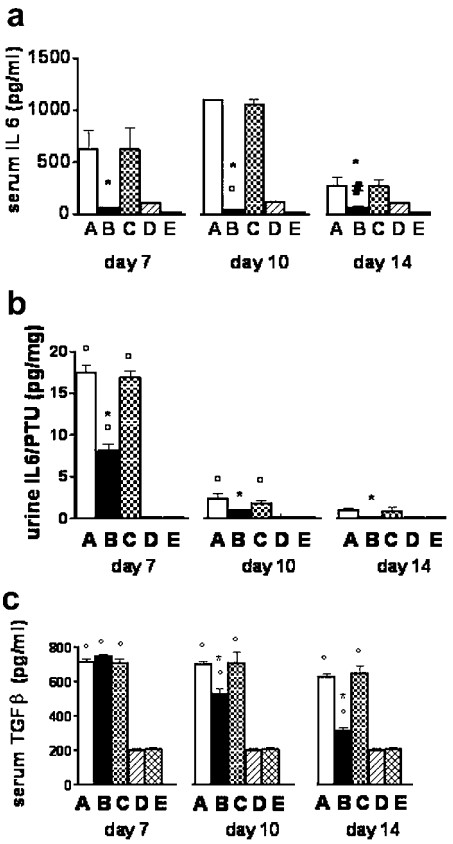

Figure 7 Serum IL-6 (a), urinary (b) IL-6 and serum TGF-β (c) levels in the experimental groups

Groups are defined in Figure 1. Values are means+S.D. In (a), $^{*}P < 0.05$ compared with groups A and C; $^{\circ}P < 0.005$ and $\#P < 0.001$ compared with group E. In (b), $^{*}P < 0.05$ compared with groups A and C; and $^{\circ}P < 0.05$ compared with groups D and E. PTU, urine protein concentration. In (c), $^{*}P < 0.05$ compared with groups A and C; and $^{\circ}P < 0.001$ compared with groups D and E.

therapy did not modify the course of nephritis. In contrast, treatment with MSC reduced the number of PCNA-positive cells at day 7, and maintained growth steady at days 10 and 14. Such stabilization was associated with levels of PDGF-β, MSP and IL-6 lower than in untreated rats. Taken together, these results suggest that MSC regulate the repair response driven by PDGF-β, MSP and IL-6, making glomerular proliferation less aggressive and more steady. We speculate that, by preventing mesangial overgrowth, MSC reduce the risk of a shift to the fibrogenic form of the disease. Accordingly, MSC inhibit the release of PDGF-β, one major inducer of collagen accumulation and glomerular scarring [17,19,21]. It is notable that MSC work in nephritic rats and not in healthy rats, in fact the number of PCNA- and α-SMA-positive cells, as well as the local levels of PDGF-β and MSP, did not change in non-nephritic rats. These findings confirm the hypothesis suggested by others [23a], i.e. that MSC need an inflammatory environment to be committed to induce a reparative effect. In the present study, we also found a small increase in IL-6 in healthy rats treated with MSC compared with untreated rats, but the range of IL-6 remained normal [24].

In anti-Thy 1 disease, the lysis of the mesangial stalk causes a loss of glomerular capillaries, rarefaction of the capillary network and formation of microaneurysms [9]; therefore the reconstruction of new glomerular capillaries is part of the healing process [20]. The HGF/Met system is a powerful inducer of neoangiogenesis and exogenous administration of HGF to rats with anti-Thy 1 disease has been shown to accelerate glomerular repair through proliferation of endothelial cells and regeneration of glomerular capillaries [25]. In our present study, treatment with MSC induced a striking expression of the Met receptor in glomerular capillaries only in nephritic rats at days 10 and 14. We suggest that MSC-induced Met expression made local HGF operative and that the HGF/Met system mediated the reconstruction of glomerular capillaries.

Studies in disease models have shown that i.v.-injected MSC home to injured areas and participate in tissue regeneration by differentiating into mature tissue cells [4] and/or inducing the release of soluble factors [3,26–28]. We have tracked MSC 12 h, and 3, 7 and 14 days after their injection and we have found very few MSC in the glomerular tuft, in the interstitium and in the tubules. These results indicate that MSC did not participate in glomerular repair by replacing injured glomerular cells, but they improved the course of anti-Thy 1 disease by modulating the principal cytokines and growth factors involved in the pathogenesis of anti-Thy 1 nephritis. In particular, MSC reduced glomerular mononuclear infiltration by suppressing MSP and attenuating mesangial activation and glomerular endocapillary proliferation by down-regulating inflammatory and fibrogenic cytokines, such as TGF-β, PDGF-β and IL-6. In this way, MSC promoted glomerular repair and prevented glomerular scarring.

Furthermore, MSC contributed to the regeneration of glomerular capillaries by inducing Met expression. Thus MSC injection resulted in the resetting of the two scatter factor systems, potentiating HGF/Met and deactivating MSP/Ron.

Our present study does not clarify the mechanisms by which MSC operated such a complex regulation. A renal paracrine action is suggested by the local changes induced by MSC in the glomerular expression of HGF/Met, MSP/Ron systems and PDGF-β. However, others have already shown that the protective effect of MSC is independent of their engraftment in the injured tissue. In fact, elegant studies have demonstrated in experimental models of acute tubular necrosis and myocardial infarction that the protective effect of MSC could be reproduced by injecting conditioned medium from cultured MSC [3,27]. Thus the effect of MSC is mediated by soluble factors that can be released by MSC wherever they are in the body and reach the site of injury through the circulation [29–31]. Furthermore, recent studies have demonstrated that MSC release soluble MV (microvescicles) [32,33]. MV are a novel mechanism of cell–cell communication which can transfer to target cells mRNA and/or microRNA sequences. In fact, Bruno et al. [32] have shown that injection of MSC-derived MV protected against acute tubular injury by shuttling mRNA into tubular cells. The transfer of transcripts can modify cell behaviour and may be bidirectional from stem cells to injured cells or vice versa. This hypothesis may explain why we found that MSC effect only nephritic rats and not in non-nephritic rats and also the inefficacy of mesangial cell infusion.

In conclusion, in the present study we have shown that MSC ameliorate the course of anti-Thy 1 nephritis by suppressing local and systemic release of inflammatory cytokines and growth factors and, for the first time, by modulating the activity of the scatter factor systems. We injected MSC i.v. after the disease had been established, thus reproducing conditions that make treatment with MSC easily feasible in the clinical setting.

AUTHOR CONTRIBUTION

Teresa Rampino contributed to the experimental design, renal histopathology studies, statistical analysis and writing of the manuscript; Marilena Gregorini contributed to the experimental design, renal histopathology studies and statistical analysis; Giulia Bedino contributed to the *in vivo* experiments, biochemical measurements and statistical analysis; Giovanni Piotti contributed to the *in vivo* experiments, biochemical measurements, statistical analysis and technical assistance; Elisa Gabanti contributed to the biochemical measurements, renal histopathology studies, and culture of mesangial cells; Adalberto Ibatici contributed to the isolation and culture of MSC, and immunocytochemical characterization and differentiation of MSC; Nadia Sessarego contributed to the isolation and culture of MSC, immunocytochemical characterization and differentiation of MSC; Cristina Piacenza contributed to the *in vivo* experiments; Chiara Teresa Balenzano contributed to the renal histopathology studies; Pasquale Esposito contributed to the biochemical measurements; Francesca Bosio contributed to the *in vivo* experiments and biochemical measurements; Grazia Soccio contributed technical assistance; Francesco Frassoni contributed to the experimental design, isolation and culture of MSC, and analysis of the results; Antonio Dal Canton contributed to the experimental design, analysis of the results, final writing and revision of the paper.

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SUPPLEMENTARY ONLINE DATA

Mesenchymal stromal cells improve renal injury in anti-Thy 1 nephritis by modulating inflammatory cytokines and scatter factors

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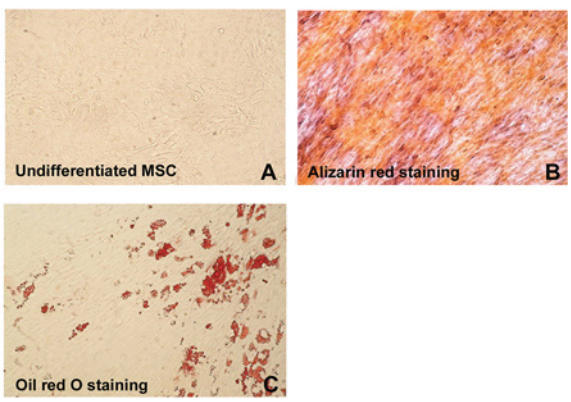

Figure S1 Osteogenic and adipogenic differentiation of MSC

MSC cultured with complete medium had the spindle-shaped morphology typical of undifferentiated MSC (A). Osteogenic differentiation of MSC is demonstrated by formation of calcium-hydroxyapatite-positive areas stained in red by Alizarin Red (B). Adipogenic differentiation is demonstrated by intracellular lipid vacuoles stained in red by Oil Red O (C). Magnification, ×100.

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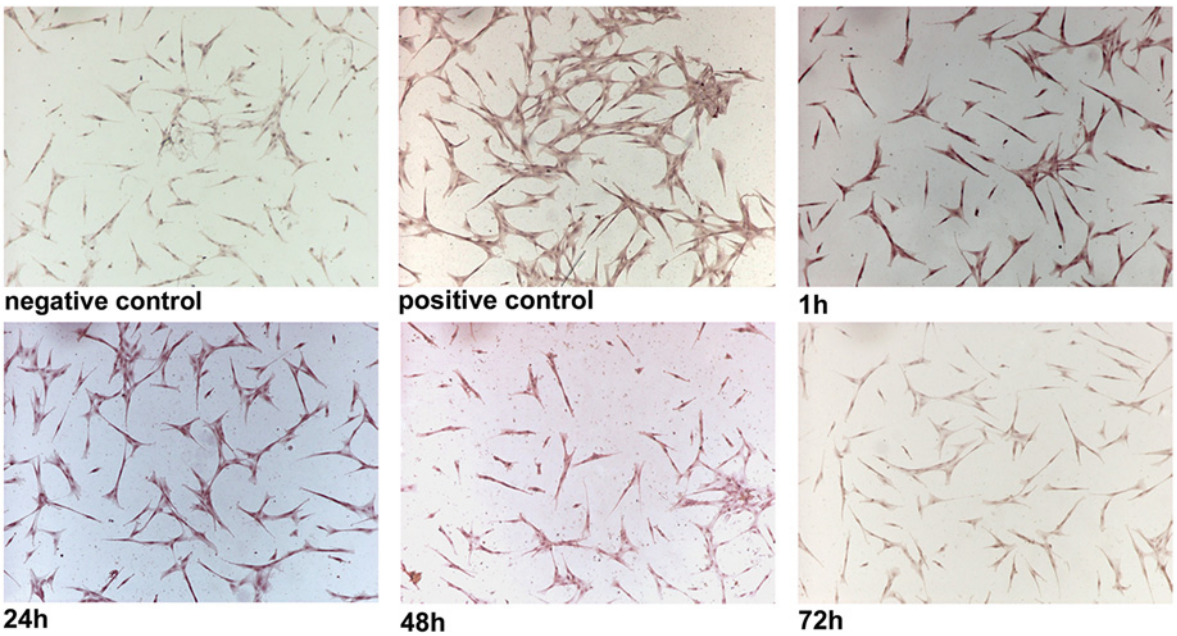

Figure S2 ***In vivo*** **tracking of circulating anti-Thy 1.1 Ab**

MSC, fixed with 10% (v/v) formalin/acetate, were incubated with rat serum and sampled immediately before and 1, 24, 48 and 72 h after anti-Thy1.1 Ab injection. Ab bound to MSC was detected by immunocytochemistry using the streptavidin–biotin–peroxidase complex and DAB, which stains positive cells brown. No cell-binding Ab was present in serum before the injection of anti-Thy 1.1 Ab (negative control). Anti-Thy 1.1 Ab added to normal rat serum stained the cells (positive control). Intense staining of cell-bound Ab was visible in MSC incubated with serum sampled 1 h after injection of anti-Thy 1.1 Ab. Progressively attenuated staining is visible in MSC incubated with serum sampled after 24 and 48 h of injection of anti-Thy 1.1 Ab, and no residual staining was detectable in MSC incubated with serum sampled 72 h after injection of anti-Thy 1.1 Ab. Magnification, $\times 200$.

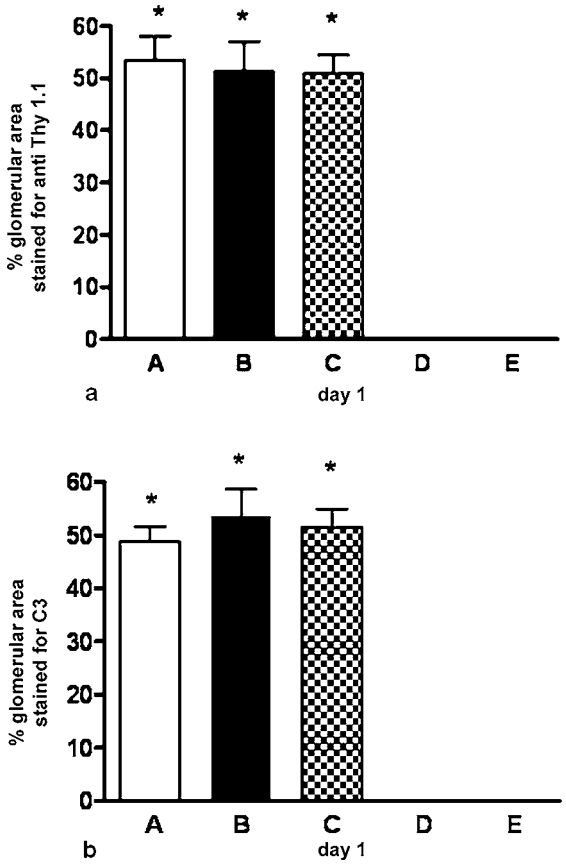

Figure S3 Glomerular deposition of anti-Thy1.1 Ab (a) and C3 complement (b)

Groups are defined as in Figure 1 of the main text. Values are means + S.D. (a) Mean percentage glomerular area staining for anti-Thy1.1 Ab at day 1 measured by quantitative image analysis. (b) Mean percentage glomerular area staining for C3 complement fraction at day 1 measured by quantitative image analysis. $^{*}P < 0.001$ compared with groups D and E.

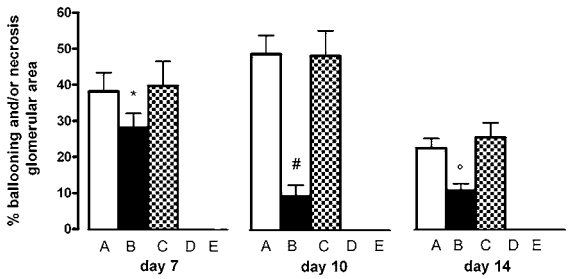

Figure S4 Glomerular ballooning and/or necrosis

Groups are defined as in Figure 1 of the main text. Values are means + S.D. The average percentage of glomerular area occupied by necrosis and/or ballooning is shown. $^{\circ}P < 0.05$, $^{*}P < 0.001$ and $\#P < 0.0001$ compared with groups A and C.

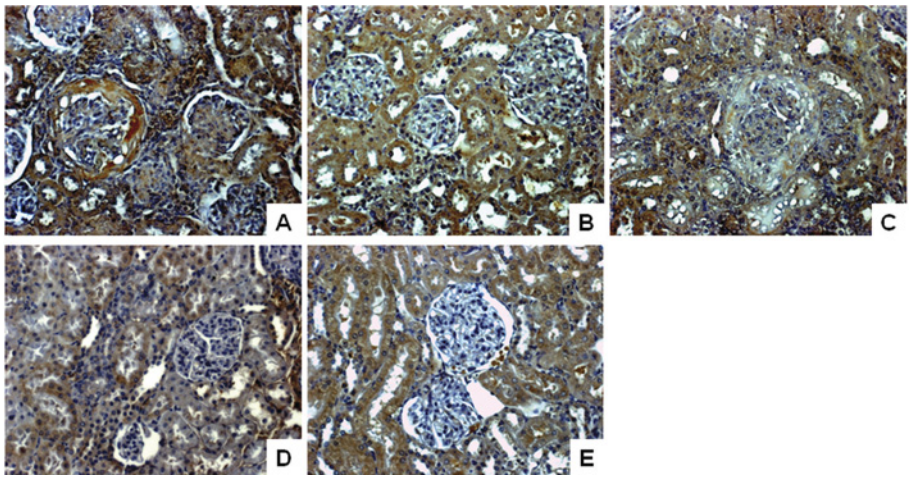

Figure S5 Glomerular expression of HGF

Groups are defined as in Figure 1 of the main text. HGF staining of representative renal sections at day 10 in groups A–E is shown. Magnification, ×200.

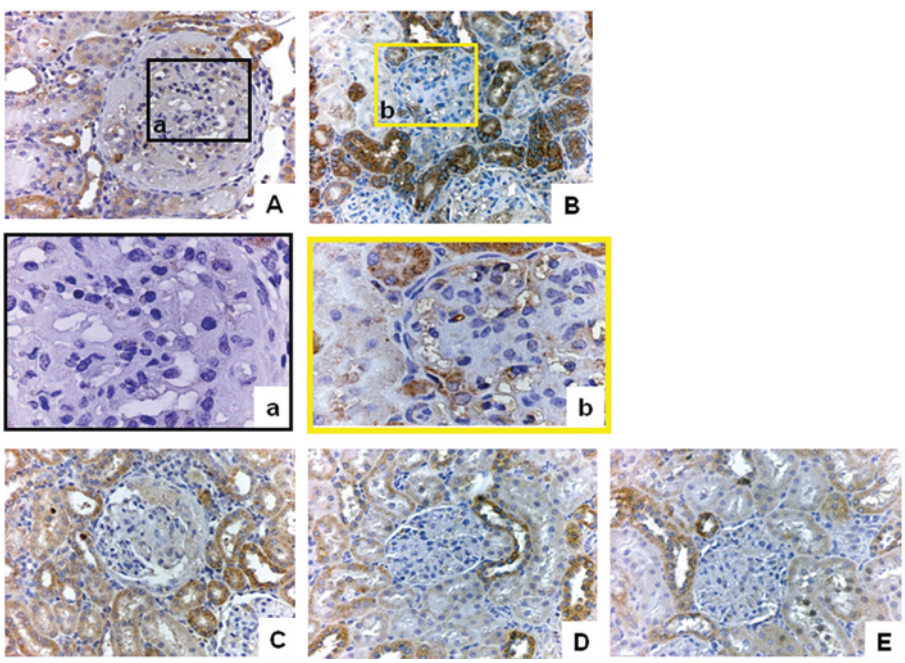

Figure S6 Glomerular expression of Met

Groups are defined as in Figure 1 of the main text. Met staining of representative renal sections at day 10 are shown. Magnification, ×200. The black and yellow rectangular areas are shown at higher magnification (×1000).

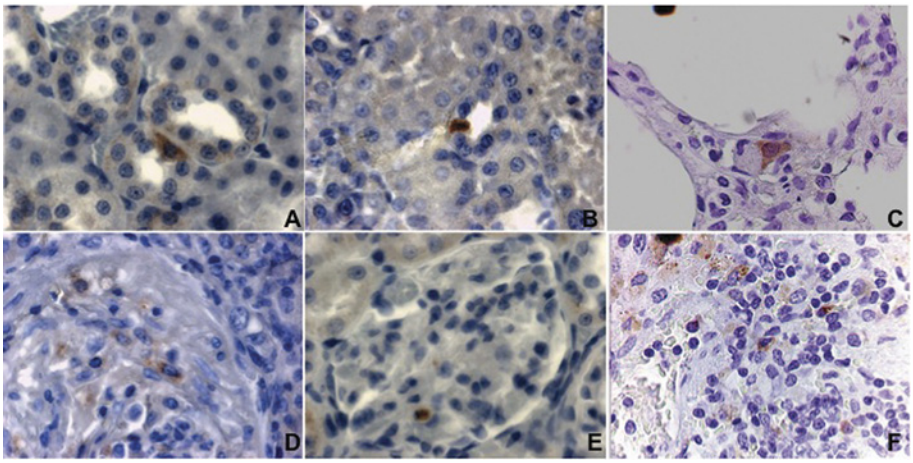

Figure S7 Localization of MSC

EGFP staining of representative kidney, lung and spleen sections. (A) An EGFP-positive cell apparently in the interstitium 12 h after administration of MSC in rats from group B. (B) An EGFP-positive cell in a tubule at day 7 after administration of MSC in rats from group B. (C) EGFP-positive cells in a lung section 12 h after administration of MSC in rats of group B. (D) EGFP-positive cells in the glomerulus at day 14 in rats from group B. (E) EGFP-positive cells in a glomerulus at day 10 in rats from group B. (F) EGFP-positive cells in a spleen section 12 h after administration of MSC in rats of group B. Magnification, $\times 400$.

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