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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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Short title: MSC reduce renal injury in nephritis.

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ABSTRACT

Mesenchymal Stromal Cells (MSC) can differentiate into renal adult cells, and have anti-inflammatory and immune-modulating activity. We investigated whether MSC have protective/reparative effects in anti-Thy 1 disease, an antibody-induced mesangiolytic resulting in mesangioproliferative nephritis. We studied five groups of rats. A: rats injected with anti-Thy Ab on day 0. B: rats injected with anti-Thy Ab on day 0 + MSC on day 3. C: rats injected with anti-Thy Ab on day 0 + mesangial cells on day 3. D: rats injected with saline on day 0 + MSC on day 3. E: rats injected with saline on day 0. Rats were sacrificed on days 1, 3, 7, 14. MSC prevented the rise in serum creatinine, proteinuria, glomerular monocyte influx and glomerular histopathological injury. Furthermore, MSC suppressed release of interleukin-6 (IL-6) and transforming growth factor- β (TGF- β), modulated glomerular platelet-derived growth factor- β (PDGF- β), and reset the Scatter Factors and their receptors, potentiating hepatocyte growth factor (HGF)/Met and inactivating macrophage stimulating protein (MSP)/Ron. Rare 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.

Abbreviations

α -SMA: α -smooth muscle actin
BSA: bovin serum albumin
DMEM: Dulbecco's Modified Eagle Medium
EDTA: ethylenediaminetetraacetic acid
EGFP: enhanced green fluorescent protein
GPI: glycosylphosphatidylinositol
GFP: green fluorescent protein
HGF: hepatocyte growth factor
IL-6: interleukin-6
MSC: mesenchymal stromal cells
MSP: macrophage stimulating protein
PBS: Phosphate-buffered saline
PCNA: proliferating cell nuclear antigen
PDGF- β : platelet derived growth factor- β
SD: standard deviation
TGF- β : transforming growth factor- β

INTRODUCTION

Mesenchymal stromal cells (MSC) 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 has been tried to regenerate injured organs in experimental and human disease (3-4). In addition, MSC seem 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 antibody directed against the Thy 1 antigen (CD90) constitutively expressed in mesangial cells. The disease is characterized by an acute mesangiolytic followed by early inflammatory cells infiltration, repopulation of the mesangium by new mesangial cells and accumulation of mesangial matrix (9).

Kunter et al. (10) already investigated the effects of MSC in anti-Thy 1 nephritis and showed that MSC reduced the mesangiolytic when injected into the renal artery, but not when infused intravenously. However, the conclusions are jeopardized by relevant methodological limitations, the most important of which is that the MSC used in the 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 antibody 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 International Society for Cellular Therapy (ISCT) (11), and the loss of CD90 implies important functional modifications. In fact, CD90 is a glycosylphosphatidylinositol (GPI)-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 loss of immunosuppressive activity (13). Furthermore, the authors based their assumption of the paracrine action of MSC only on evidence *in vitro*. In this 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 we injected MSC 72 hours after the injection of the anti-Thy 1.1 antibody, i.e. at a time when no residual antibody was circulating. In addition, we investigated the effects of MSC on cytokines *in vivo*, and extended our investigation to the Scatter Factors, i.e. Hepatocyte Growth Factor/Scatter Factor (HGF) and its receptor Met, and Macrophage Stimulating Protein/HGF-like Scatter Factor (MSP) and its receptor Ron.

We used transgenic Enhanced Green Fluorescence Protein (EGFP) rats as MSC donors to track injected MSC (14), and studied five groups of rats, as described in figure 1.

MATERIALS AND METHODS

Animals.

Experiments were performed according to guidelines of our ethical committee for animal studies. Wild-type Sprague Dawley rats (Charles River, Calco, Italy) and transgenic Sprague-Dawley rats that express EGFP (Japan Slc, Hamamatsu, Japan) were used. The rats carrying the EGFP transgenic 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 sacrificed by CO₂ inhalation. The total marrow cell content of both femurs and tibias was flushed from bones and EGFP positive cells were plated at a concentration of 10⁶ cells/ml with Murine MesenCult medium (Stemcell Technologies, Vancouver, Canada) and incubated at 37°C with 5% humidified CO₂ 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, St Louis). Collected cells were replanted at a concentration ranging between 0.05-0.15x10⁵/ml of MesenCult medium for several passages.

Rat mesangial line (CRL-2573) was obtained by ATCC (American Type Culture Collection, Manassas, USA). 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 concentration of 64,000/cm² on a culture flask and cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose medium (Gibco, Invitrogen, Paisley, UK), containing Penicillin/Streptomycin 1%, Amphotericin B, G418 (0.4 mg/mL) (Gibco) and Fetal Bovine Serum 15% (Euroclone, Torquay, UK), at 37°C in 5% humidified CO₂ 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-ethylenediaminetetraacetic Acid (EDTA). Collected cells were replanted for several passages at a concentration ranging between 12,000 and 20,000/cm².

Immunocytochemical Characterization and Osteogenic and Adipogenic Differentiation of MSC.

Cultured EGFP MSC were evaluated by flow cytometry starting from the 3rd passage for CD90, CD73, and CD45, CD11b (Becton Dickinson, San José, CA) using FacsCalibur flow cytometer (BD Biosciences, San José, CA). The differentiation into osteocytes and adipocytes was evaluated as previously described (2). Histochemical analysis of the cell layer was performed evaluating calcium deposition (Alizarin red staining, Sigma-Aldrich, St Louis MO). For adipogenic induction, 2x10⁴ MSC/cm² were seeded in chamber slides with Mesencult. At confluence cells were stimulated with adipogenic medium (Cambrex, Walkersville, MD USA) for 3 weeks replacing the medium every 3 days. Cells were stained with Oil-Red O (Bioptica, Milan Italy).

In vivo experiments.

We studied a total of 5 groups of rats. Group A: 20 rats were injected at day 0 via tail vein (vtv) with 400 µl of saline that contained 400 µg of anti-Thy 1.1 IgG mAb (Cedarlane Laboratories, Toronto, Canada), as previously described (18). Group B: 25 rats were injected vtv at day 0 with 400 µg of anti-Thy 1.1 IgG mAb, followed after 3 days by injection vtv of 1 ml of phosphate-buffered saline (PBS) containing 3x10⁶ MSC isolated

from EGFP rats. Group C: 20 rats were injected vtv at day 0 with 400 μg of anti-Thy 1.1 IgG 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. Group E: 20 rats were injected vtv with 400 μl of saline. Five rats of each group were sacrificed at day 1, 7, 10, 14 and five rats of group B were sacrificed 12 hours after MSC infusion. Soon after sacrifice kidneys, lung and spleen were removed and half fixed in 10% neutral-buffered formalin, half frozen in liquid nitrogen.

All rats were male, weighed 180-200 g and were housed at constant temperature (20 $^{\circ}\text{C}$) and humidity (75%) under controlled light cycle, with free access to water and standard chow diet.

24-hour urine was obtained by housing individual rats in metabolic cages for up to 24 hours on days 0, 7, 10, 14; urine volume was measured and samples were stored at -20 $^{\circ}\text{C}$. Blood was drawn on day 0 from the caudal vein, and on days 7, 10, 14 by cardiac exsanguination and soon centrifuged, and serum was stored at -20 $^{\circ}\text{C}$.

In vivo tracking of circulating anti-Thy 1.1 Ab.

MSC (1×10^5) cultured in chamber slides were fixed with formalin-acetate 10% for 10 minutes, washed in PBS and incubated in dark-humid chamber at room temperature for 60 minutes with rat serum (100 μl) sampled immediately before and 1, 24, 48, 72 h after anti-Thy1.1 Ab injection. Moreover, we used anti-Thy1.1 Ab at concentration of 1 $\mu\text{g}/\mu\text{l}$ adding 20 μl of Ab in a total volume of 100 μl of normal rat serum, as positive control. After three washings in PBS the cells were treated with the secondary antibody (Universal Biotinylated Link, Dako, Glostrup, Denmark) and the streptavidin-biotin-peroxidase complex (Dako). Visualization was in 3, 3-diaminobenzidine (Dako). Harris hematoxylin was used to counter stain the nuclei lightly. Finally the cells were dehydrated in increasing alcohol scale (95 $^{\circ}\text{C}$ to 100 $^{\circ}\text{C}$, xylol) and the cover slip was mounted with synthetic non aqueous mounting media (Dako) for analysis with microscope Olympus IX8 connected with a computer system for cell imaging and CCD camera (Magnification X200) (Olympus Optical Co, Tokyo, Japan). All experiments were duplicated.

Deposition of anti-Thy1.1 Ab.

Anti-Thy 1.1 Ab deposition was detected by immunohistochemistry in formalin fixed kidney of rats of all groups sacrificed at day 1. Three- μm thick sections were collected on poly-L-lysine-coated slides (Dako), were dewaxed in xylol, passed in a decreasing series of alcohol, and finally rehydrated with distilled water. Endogenous peroxidase was blocked with H_2O_2 3.7% vol/vol followed by H_2O for 15 min. After 3 washings 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 ready-to-use anti-mouse secondary antibody EnVision Plus (Dako) and colour reaction was developed with 3, 3-diaminobenzidine (Dako) as the chromogen. The slides were then counterstained with Harris hematoxylin. The extent of anti-Thy 1.1 Ab deposited was evaluated in blinded manner in 40 random glomeruli in each kidney section by image analysis, using Olympus IX8 microscope connected with CCD camera and Cell-R software on image processing computer. We measured the ratio between the glomerular anti-Thy 1.1 Ab positive area and glomerular tuft area.

Deposition of C3 complement fraction.

Glomerular deposition of C3 was detected in frozen tissue of rats of all groups sacrificed at day 1. Five- μm thick sections were collected on poly-L-lysine-coated slides (Dako), were fixed in acetone for 5 minutes at room temperature, and then passed in distilled water. Subsequently the sections were exposed to polyclonal sheep anti-human C3 FITC Ab, 1:600 cross-reacting with rat C3 (ICN, Segrate, Italy) v/v PBS/Bovine Serum Albumin (BSA) 1% for one hour at room temperature. After 3 washings in PBS the cover slips were mounted with aqueous mounting media for analysis with fluorescence microscope. Negative controls included both omission of the primary Ab and substitution of IgG for primary antibodies.

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

Biochemical measurements.

Serum creatinine and urinary protein concentration were measured at days 0, 7, 10, 14 by an automated method (Abbott Laboratories, Abbott Park, IL), and the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA).

Interleukin-6 (IL-6) and transforming growth factor- β (TGF- β) were quantified in rat serum at days 7, 10, 14 by ELISA (R&D Systems, Minneapolis, USA). IL-6 was measured in 24 h urine at days 7, 10, 14 by ELISA.

All experiments were quadruplicated.

Renal Histopathology

Renal Morphology.

3- μm thick sections cut along the maximal sagittal plane of formalin-fixed kidney were stained with hematoxylin-eosin and were examined by two investigators in double blind fashion, using an Olympus IX8 microscope connected with a CCD camera and software imaging analysis Cell-R. 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, (v) necrosis. The damage score was established using the following criteria: glomeruli without obvious changes were factored with 0, glomeruli with mesangiolysis and/or ballooning involving <25% of the glomerular area were factored with 1, glomeruli with mesangiolysis and/or ballooning involving 26 to 50% of the glomerular area were graded with 2, glomeruli with mesangiolysis and/or ballooning involving 51 to 75% of the glomerular area were graded with 3, glomeruli with mesangiolysis and/or ballooning involving >75% of the glomerular area and/or microaneurysm and/or crescents and/or glomerular necrosis were graded with 4.

The results were 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 total glomerular area.

Expression of ED-1 Ag, PCNA, α -SMA, PDGF- β , HGF, Met, MSP and Ron.

The glomerular expression of ED-1 Ag, proliferating cell nuclear antigen (PCNA), α -smooth muscle actin (α -SMA), platelet derived growth factor- β (PDGF- β), HGF, Met, MSP, and Ron were studied by immunohistochemistry in formalin fixed tissue.

Three- μm thick sections of paraffin embedded tissue were collected on poly-L-lysine-coated slides (Dako), they were dewaxed in xylol, passed in a decreasing series of alcohol, and finally rehydrated with distilled water. Endogenous peroxidase was blocked with H_2O_2 3.7% vol/vol followed by H_2O for 15 min. After 3 washings in 150 mM of PBS the

sections underwent microwave antigen retrieval. Subsequently the sections were exposed overnight at 4 C° to the following antibodies: 1) monoclonal mouse anti-rat ED-1 (Serotec Ltd, Oxford, UK), 1:300; 2) monoclonal mouse anti-PCNA, mAb (Santa Cruz Biotechnology, CA), 1:200; 3) monoclonal mouse anti-human α -SMA), 1:100, (Dako); 4) polyclonal rabbit anti-human PDGF- β (Santa Cruz Biotechnology), 1:200; 5) polyclonal rabbit anti-human HGF (Santa Cruz Biotechnology, 1:50; 6) monoclonal mouse anti-human Met (Novocastra Laboratories Ltd, UK), 1:20; 7) polyclonal goat anti-mouse MSP (Santa Cruz Biotechnology), 1:600; 8) monoclonal mouse anti human RON (Transduction Laboratories, Lexington, KY), 1:750. After 3 washings in PBS the immunocomplex was visualized with the biotin-streptavidin-peroxidase complex and 3,3-diaminobenzidine (DAKO). Sections were faintly counterstained with Harris hematoxylin. Negative controls included both omission of the primary Ab and substitution of IgG for primary antibodies. In all glomeruli of three sections of each kidney we counted the cells positive for ED-1 Ag and PCNA and we measured the extent of PDGF- β , α -SMA, HGF, MSP and Ron expression as the ratio between the glomerular PDGF- β , α -SMA, HGF, MSP, 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 glomerular capillaries.

Tracking of EGFP positive cells.

We tracked by immunohistochemistry EGFP positive cells in kidney, spleen, lung excised 12 hours and at days 7, 10, 14 after injection of MSC. Three- μ m thick sections of paraffin embedded tissue were collected on poly-L-lysine-coated slides (Dako), they were dewaxed in xylol, passed in a decreasing series of alcohol, and finally rehydrated with distilled water. Endogenous peroxidase was blocked with H₂O₂ 3.7% vol/vol followed by H₂O for 15 min. After 3 washings in 150 mM of PBS the sections underwent microwave antigen retrieval. Subsequently the sections were exposed overnight at 4 C° to monoclonal mouse anti-green fluorescent protein (GFP) antibody IgG1, (Chemicon, Temecula, CA), diluted 1:1000 in PBS/BSA 1%. EGFP positive cells were visualized with the biotin-streptavidin-peroxidase complex and coloured in 3,3-diaminobenzidine (DAKO). Sections were faintly counterstained with Harris hematoxylin. Negative controls included omission of the primary Ab and substitution of IgG for primary antibodies. Positive controls were sections of kidney of EGFP rat. Ten sections of each organ were analyzed.

Statistical Analysis.

ANOVA followed by the Newman-Keuls test or Student t test were used for comparison 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 bone marrow of Sprague Dawley EGFP rats differentiated into osteogenic and adipogenic cells when cultured in appropriate conditions (figure 1 suppl) Flow cytometric analysis showed that MSC were positive for CD90 ($\geq 95\%$), CD73 ($\geq 95\%$) and were negative for CD45 ($< 5\%$), 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 hours after i.v. injection of anti-Thy 1.1 Ab and we detected MSC-binding antibody present in serum by immunocytochemistry. Figure 2 suppl shows no serum binding activity before antibody injection and intense staining of cell-bound antibody 1 hour after its injection. Progressively attenuated staining is visible after 24 and 48 hours and no residual antibody is detectable after 72 hours. These results exclude that residual circulating antibody can interfere with MSC infused after 72 hours.

In vivo experiments.

Throughout the study, no significant differences were observed in body weight, 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 as in rats treated with MSC, we evaluated the glomerular deposition of anti-Thy 1.1 and C3 complement fraction within 24 hours of antibody injection. As expected, no deposition of anti-Thy 1.1 Ab nor C3 was observed in rats of groups D and E. 24 hours after the injection of anti-Thy 1.1 both the antibody and C3 were deposited in glomeruli similarly in groups A, B and C (figure3 suppl).

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; group A from 0.41 ± 0.10 (mean \pm SD) to 0.62 ± 0.14 mg/dl ($P < 0.05$); group B from 0.51 ± 0.20 to 0.74 ± 0.20 mg/dl ($P < 0.05$); group C from 0.44 ± 0.30 to 0.64 ± 0.05 mg/dl ($P < 0.05$). 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 B than in A and C ($P < 0.001$ B vs A and C). At day 14 creatinine decreased to levels no longer different from baseline in all three groups: group A (0.51 ± 0.07 mg/dl), group B (0.44 ± 0.14 mg/dl), group C (0.57 ± 0.01 mg/dl). No change of creatinine occurred in rats not treated with anti-Thy 1.1 IgG (group E), even in those receiving MSC (group D).

Baseline urinary protein excretion was similar in the five groups. Proteinuria rose similarly in groups A, B and C on day 3, then proteinuria increased further in groups A and C, but not in group B. 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, while it remained higher than baseline in groups A and C. No change in proteinuria occurred in rats not treated with anti-Thy 1.1 IgG (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 (mesangiolytic and /or ballooning involving >75% of the glomerular area and /or microaneurysm and /or crescents and/ or glomerular necrosis). A representative picture of renal histology is shown in figure 3. No histological changes were observed in groups D and E. The damage scores of glomeruli in groups A and B are summarized in figure 4. In group A, a high percentage of glomeruli were factored 3-4, while a low percentage of glomeruli was normal. The proportion of normal vs severely damaged glomeruli was inverted in group B; a high percentage of glomeruli scored 0 and a minority scored 3-4. In group C, the injury scores were similar as in group A: a high percentage of glomeruli were factored 3-4 (68%, 60% and 47% on days 7, 10 and 14 respectively), while a low percentage of glomeruli was 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 we found that the extension of 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) (figure 4 suppl).

Glomerular monocytes infiltration and Ron expression

As shown in figure 5, the number of ED-1 positive cells was significantly lower in group B than in groups A and C at day 7 and day 10. As expected from the spontaneous course of the disease (9), the number of glomerular monocytes declined in all 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. Since in anti-Thy 1 disease the MSP/Ron system attracts monocytes into the glomeruli and stimulates mesangial cell proliferation (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 did not also change in nephritic rats receiving mesangial cells (group C).

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 by 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 release (figure 6). In fact, in nephritic rats injected with MSC a steady rise of endocapillary proliferation occurred at days 7, 10 and 14, while in rats not treated with MSC and rats treated with mesangial cells (A and C) a peak increase in proliferation occurred at day 7 followed by a decline at day 10 and by a further rise at day 14. Similar glomerular expression of α -SMA was steady in nephritic treated rats while it peaked at 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 day 7 and at day 10, while, in non nephritic rats (groups D) MSC did not affect α -SMA, PCNA, MSP and PDGF- β glomerular expression. There was no change in group E.

HGF/Met system.

In non-nephritic rats HGF (figure 5 suppl) and Met (figure 6 suppl) were diffusely expressed in tubules, while 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, while it declined on day 14 in untreated rats and rats treated with mesangial cells. It is notable that MSC injected in healthy rats (group D) did not upregulate HGF. Glomerular expression of Met increases slightly in rats with anti-Thy 1 disease that were not treated with MSC (group A and C). In contrast, treatment with MSC induced a striking increase in glomerular Met expression at day 10 and at day 14 (figure 7). Interestingly, MSC induced overexpression of Met in endothelial cells of glomerular capillaries, but only in nephritic rats (figure 6 suppl panel a and b).

MSC localization.

We examined ten kidney sections and we 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 tubule, in the interstitium or in the glomerular tuft. We found 3-4 cells/field in spleen sections at 12 h after MSC infusion and occasionally we detected MSC in lung sections at the same time (Figure 7 suppl).

IL-6 and TGF- β levels.

As shown in,figure 8 serum levels of IL-6 increased in all groups with anti-Thy 1 disease, but the rise was striking in groups not treated with MSC in comparison with the modest rise occurring in rats treated with MSC. The suppression of IL-6 induced by MSC was especially evident at day 10 when serum IL-6 peaked in untreated rats. The infusion of MSC induced modest, but significant increase in serum IL-6 in rats without nephritis (D $P < 0.001$ vs E). Also urinary IL-6 increased significantly in all groups with nephritis and also the rise in urinary IL-6 was significantly attenuated by treatment with MSC throughout the study period.

TGF- β serum levels rose significantly in all groups with anti-Thy 1 nephritis and the rise was significantly attenuated by treatment with MSC at days 10 and 14. MSC did not induce an increase of TGF β serum levels in non nephritic rats (group D) (figure 8).

DISCUSSION

The purpose of our study was to investigate the effects of treatment with MSC on the course of anti-Thy 1 nephritis. We used MSC expressing CD 90 in accordance with the minimal criteria for defining MSC provided by the ISCT (11). CD 90 is targeted by the anti-Thy 1.1 Ab that is injected to induce the disease, therefore residual circulating antibody may react with CD90 positive MSC and interfere with their viability and their physiological functions. Conversely, MSC may bind circulating antibody and prevent it from reaching and injuring mesangial cells. In order to obviate such interferences, we made preliminary experiments to determine when the injected antibody is completely cleared from the circulation. We found no residual MSC-binding antibody in serum three days after its injection and consequently we infused MSC three days after the induction of the disease.

The main finding of our study is that MSC infused intravenously mitigate anti-Thy 1 disease. In fact, treatment with MSC anticipated 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 cells infusion had striking effects on glomerular pathology. In rats treated with MSC about half of the glomeruli were spared from any lesion in 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 while normal glomeruli were a minority. MSC offered the most impressive protection at day 10, when MSC reduced the percentage of glomeruli scoring 4 from 60% (group A) and 56% (group C) to 18% (group B), while raising 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 suppression of complement activation. In fact, the amount of antibody and of 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 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 this study we show 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 of 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, 17, 18, 21, 22). Activated proliferating mesangial cells express α -SMA and PCNA (16, 17, 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 , MSP and serum and urinary IL-6). At day 10, in spite of further rise in growth factors, mesangial cell proliferation transiently fell in coincidence with the occurrence of peak necrosis, that reduced the number of alive 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 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. Altogether, 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 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 (Krampera et al) i.e. that MSC need an inflammatory environment to be committed to induce a reparative effect. We also found a little increase of 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 mesangial stalk causes 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 study, treatment with MSC induced a striking expression of 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 intravenously 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, 27, 28). We have tracked MSC 12 hours, 3, 7 and 14 days after their injection and we have found very rare 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 antiThy 1 nephritis. In particular, MSC reduced glomerular mononuclear infiltration by suppressing MSP and attenuated 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 into the reset of the two Scatter Factor systems, potentiating HGF/Met and disactivating MSP/Ron.

Our study does not clarify the mechanisms by which MSC operated such 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-b.

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, 30, 31). Furthermore, recent studies have demonstrated that MSC release soluble

microvesicles (MV). MV are a novel mechanism of cell-to-cell communication which can transfer to target cells mRNA and /microRNA sequences. In fact, Bruno et al. have shown that injection of MSC-derived MV protected against acute tubular injury by shuttling mRNA into tubular cells (32, 33). 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 MSC effect only in nephritic rats and not in non nephritic rats and also the inefficacy of mesangial cell infusion.

In conclusion, 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 intravenously after the disease had been established, thus reproducing conditions that make treatment with MSC easily feasible in the clinical setting.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

All authors state no conflict of interest. The manuscript has not been published previously and is not being considered concurrently by another journal.

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TITLES AND LEGENDS TO FIGURES

Figure 1

Experimental design.

We studied 5 groups of rats. Group A: rats in which anti-Thy 1 nephritis was induced by injecting anti-Thy 1.1 Ab intravenously 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, that were treated with MSC infused intravenously on day 3. These rats were studied to understand whether MSC modify the course of the disease and the mechanisms there of. Group C: rats in which anti-Thy 1 nephritis was induced as in groups A and B, that 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 kidney. Group E: control rats injected with saline on day 0.

Slashes indicate days in which rats were sacrificed.

Five rats for each group were sacrificed at day 1, 7, 10, 14.

Five rats of group B were sacrificed 12 hours after MSC infusion.

Figure 2

Urinary protein excretion rate.

Groups are defined in figure 1.

Symbols are mean daily (24 h) urinary protein excretion, bars are SD. # day 0 $P < 0.001$ vs day 7 and day 10 in groups A and C, °day 0 $P < 0.005$ vs day 7 and day 10 in group B, * $P < 0.0001$ vs B at days 7, 10, 14.

Figure 3

Renal morphology at day 10.

Groups are defined in figure 1.

Representative renal histology of groups A, B, C, D and E at day 10. Hematotoxylin-eosin. (Magnification X200).

Figure 4

Glomerular damage score.

Groups are defined in figure 1.

Numbers are the average percentage of glomeruli scoring 0, 1, 2, 3 and 4 at days 7, 10, and 14 (Kruskal Wallis test group A, $P < 0.0001$ vs group B).

Figure 5

Glomerular monocytes and Ron expression.

Groups are defined in figure 1.

Panel a. Columns are the mean number of monocytes/glomerulus. Bars are SD. ° $P < 0.0001$ vs A and C; * $P < 0.05$ vs A and C; § $P < 0.0001$ vs A, B and C.

Panel b. Columns are the mean percent glomerular area staining for Ron: * $P < 0.001$ vs A and C at days 7 and 10; ° $P < 0.05$ vs A, B and C at days 7,10.

Figure 6

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

Groups are defined in figure 1.

Top: columns are the mean percent glomerular area staining for α -SMA and the mean number of PCNA-positive cells/glomerulus, respectively.

Bottom: columns are the mean percent glomerular area staining for PDGF- β and MSP, respectively.

Bars are SD. Top panels: α -SMA: $^{\circ}P < 0.005$ vs A and C; $^{*}P < 0.0001$ vs D and E; PCNA: $^{\circ}P < 0.05$ vs A and C; $^{*}P < 0.01$ vs A and C; $\#P < 0.001$ vs D and E.

Bottom panels: PDGF-b: $^{\circ}P < 0.0001$ vs D and E; $\#P < 0.005$ vs A and C; MSP: $^{*}P < 0.05$ vs A and C; $^{\circ}P < 0.05$ vs A, B and C.

Figure 7

Glomerular expression of HGF and Met

Groups are defined in figure 1.

Panel a. Columns are the mean percent glomerular area staining for HGF:

$^{*}P < 0.05$ vs A and C at day 7; $^{\circ}P < 0.01$ vs A, B and C at days 7, 10, and 14; $\#P < 0.05$ group B day 7 vs group B days 10 and 14; $\S P < 0.05$ groups A and C day 10 vs groups A and C day 14.

Panel b. Columns show percent of glomerular capillaries stained for Met:

$^{*}P < 0.001$ vs A, C, D and E at days 10 and 14; and A, B, C, D, E at day 7; $^{\circ}P < 0.05$ vs A, and C at days 10, and 14.

Figure 8

IL-6 and TGF- β levels

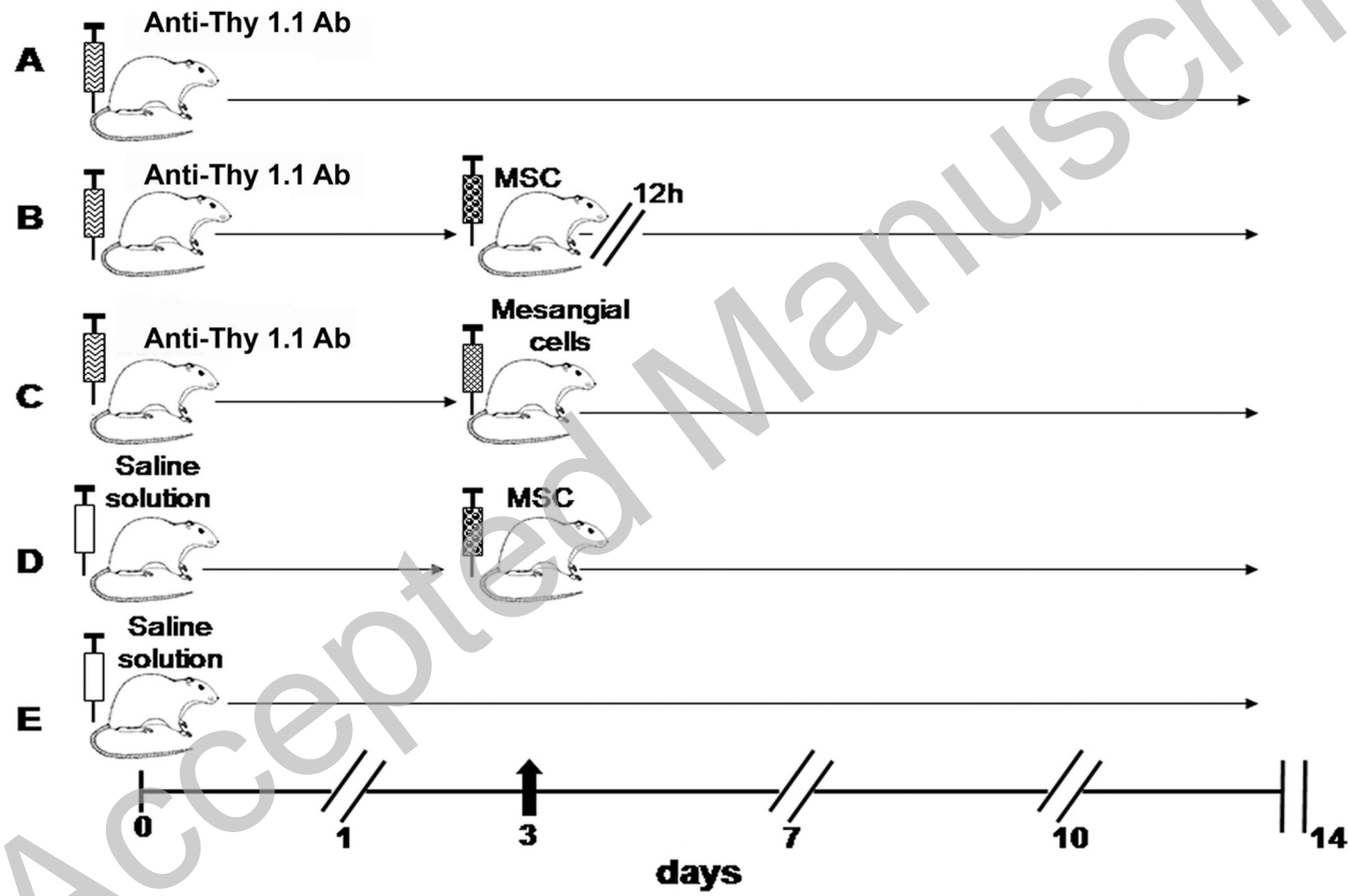
Groups are defined in figure 1.

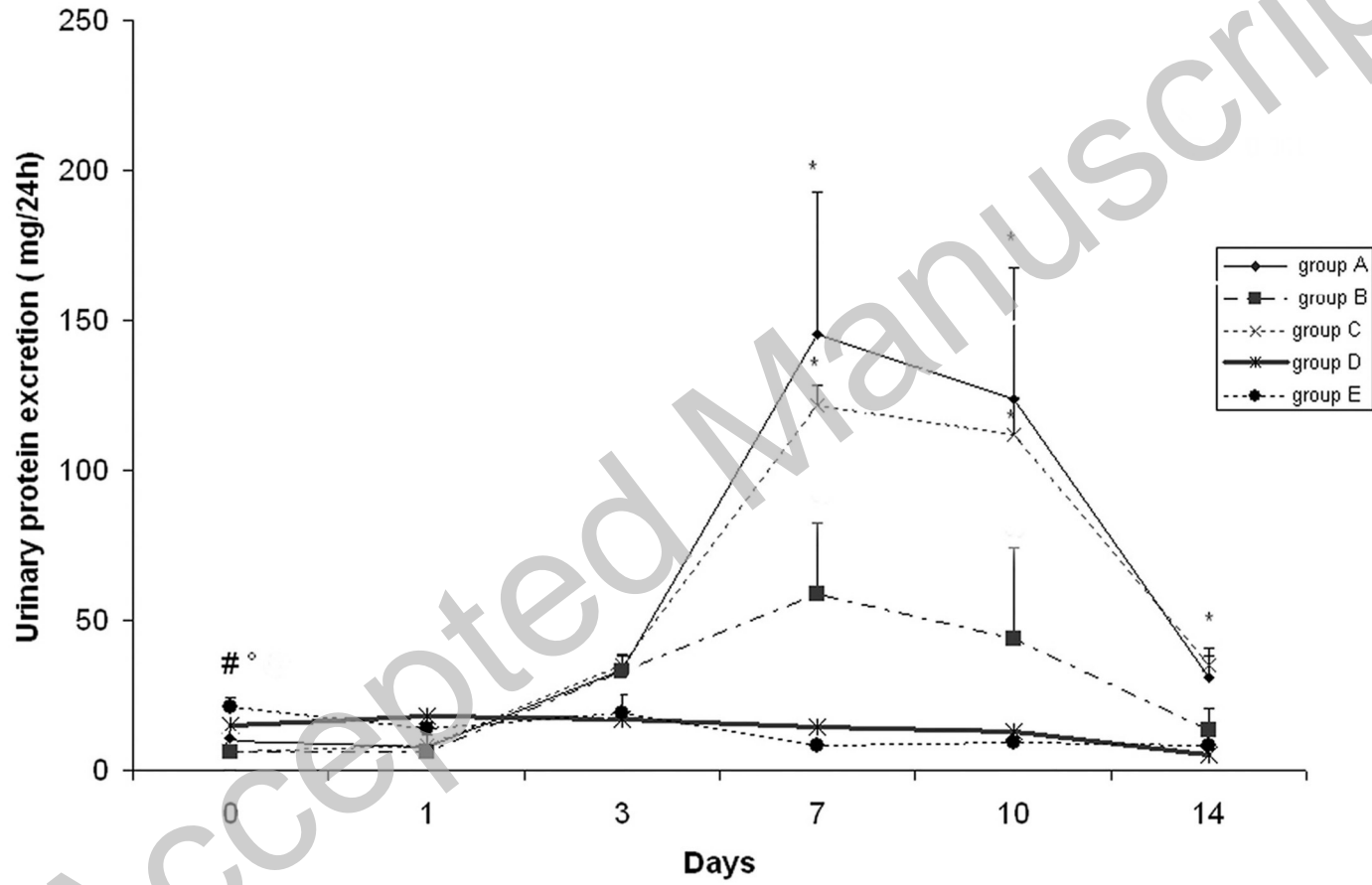
Panel A: serum IL-6 levels. Columns are means, bars are SD: $^{*}P < 0.05$ vs A and C. $^{\circ}P < 0.005$ vs E, $\#P < 0.001$ vs E

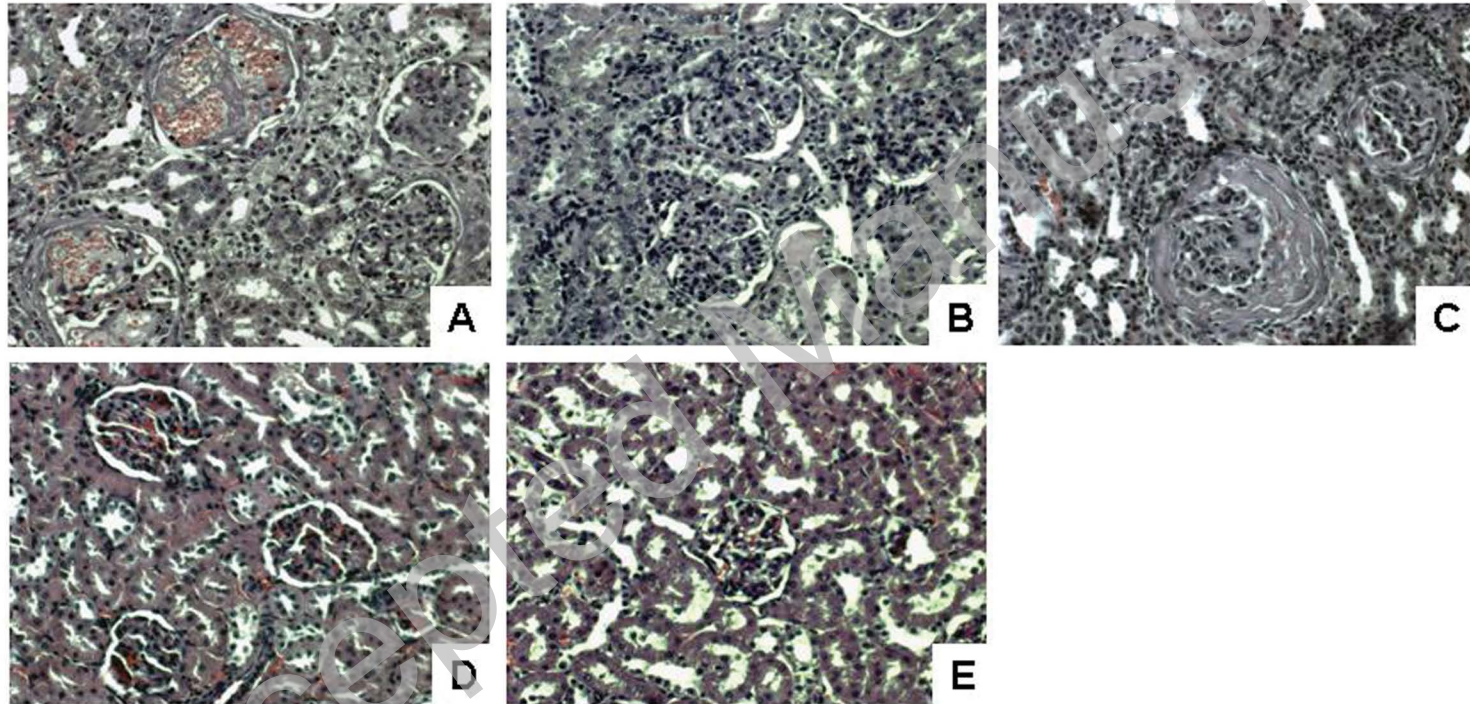
Panel B: urine IL-6/urine protein concentration (PTU). Columns are means, bars are SD: $^{*}P < 0.05$ vs A and C; $^{\circ}P < 0.05$ vs D and E.

Panel C: Columns are means, bars SD: $^{*}P < 0.05$ vs A and C; $^{\circ}P < 0.001$ vs D and E.

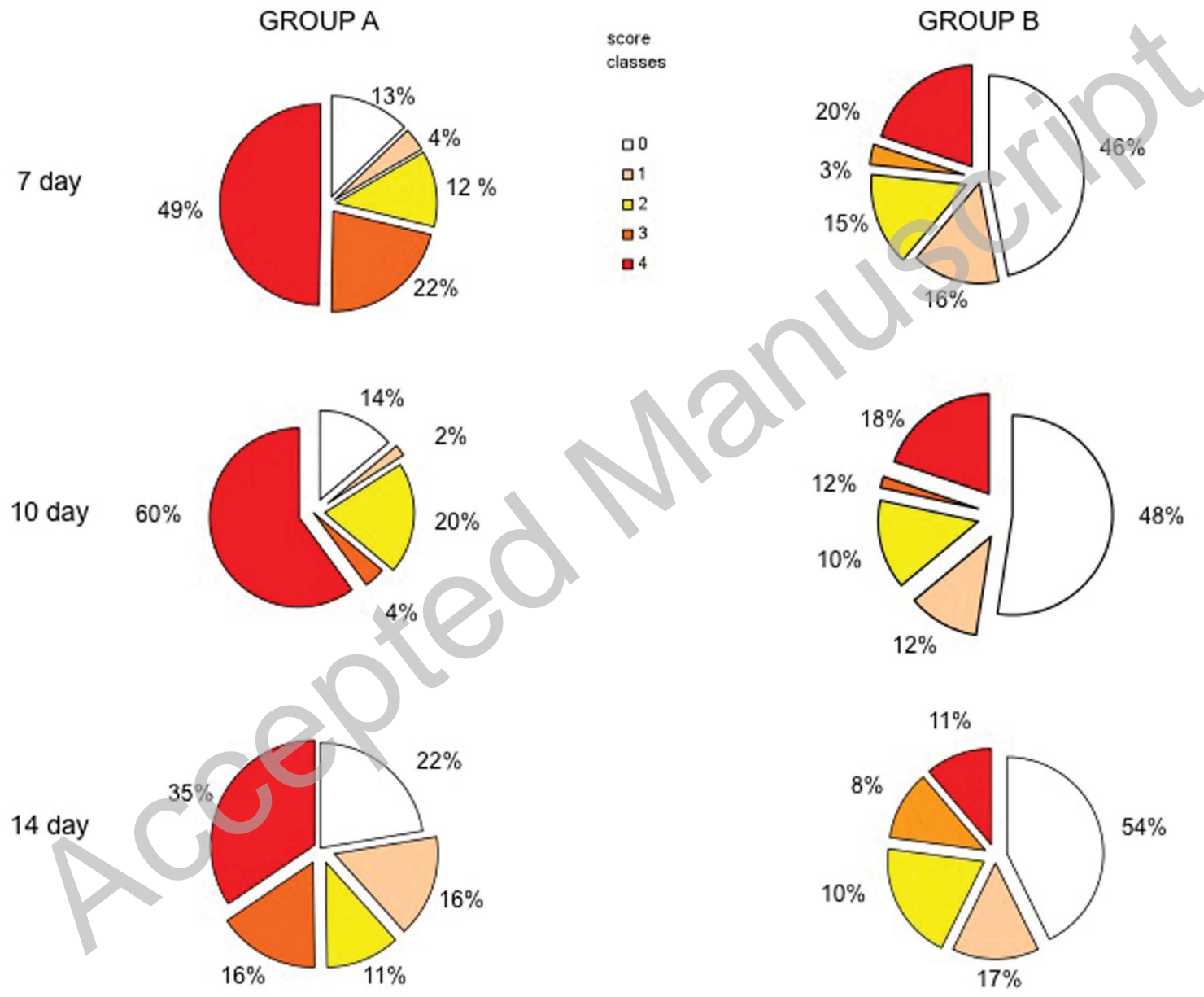
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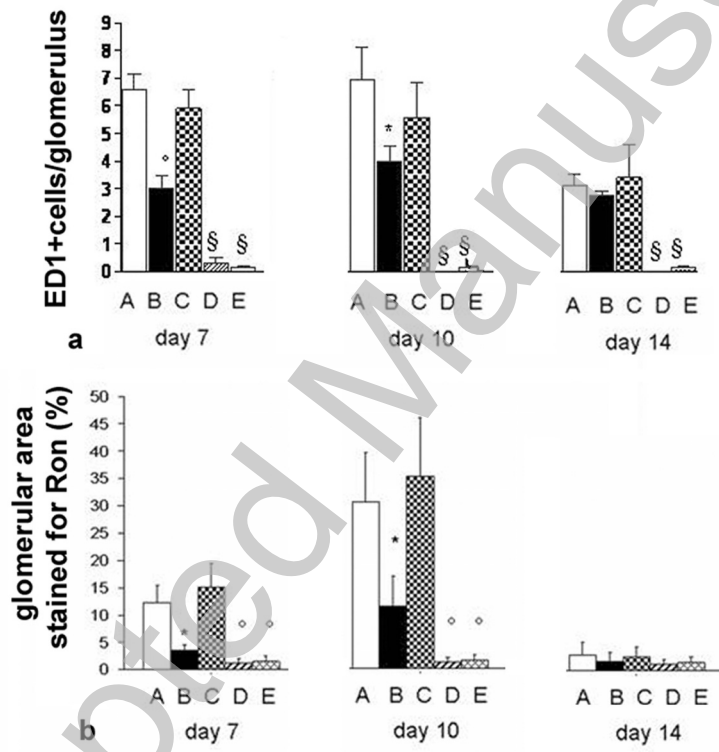


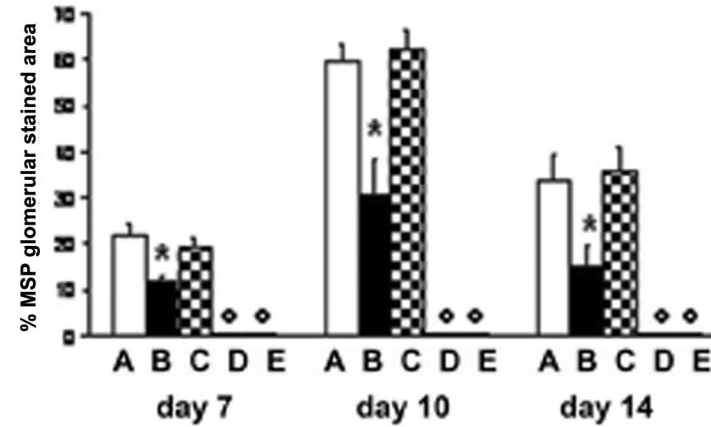
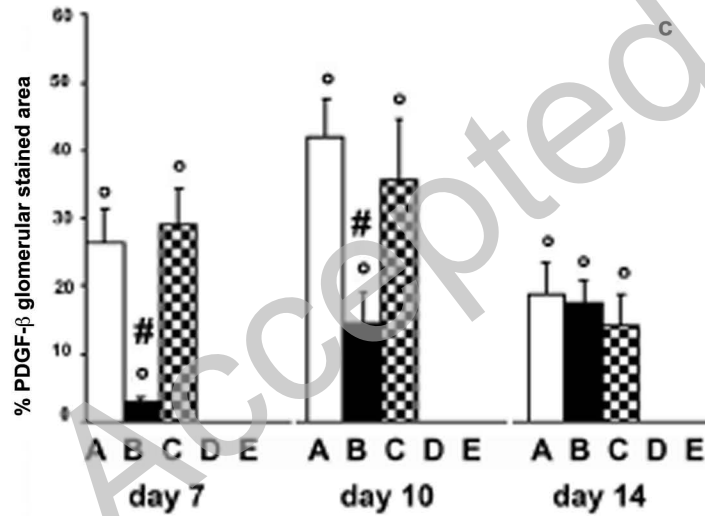
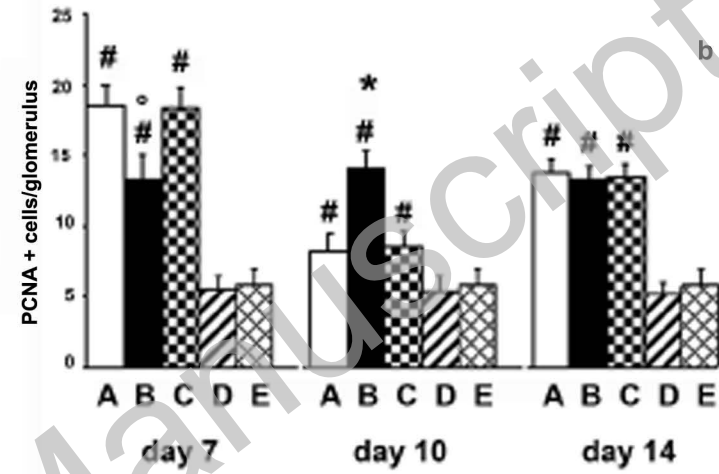
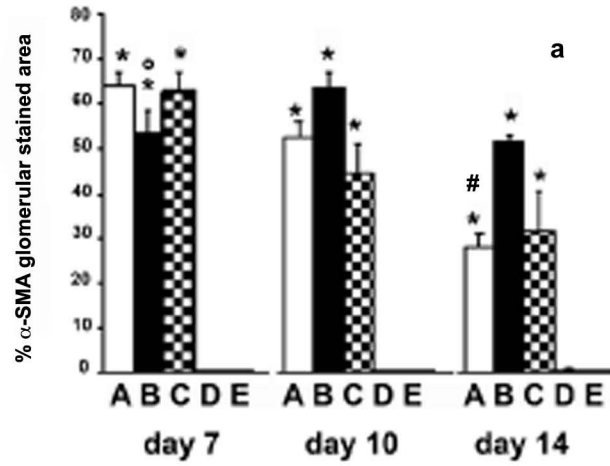


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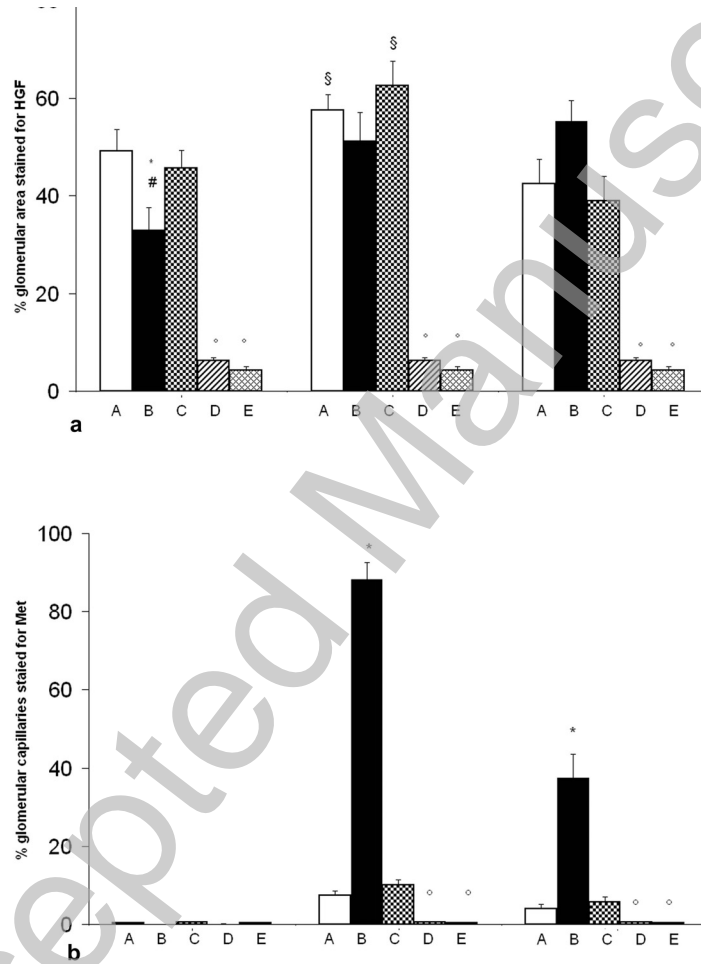


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