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The Timing of Immunomodulation Induced by Mesenchymal Stromal Cells Determines the Outcome of the Graft in Experimental Renal Allotransplantation

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The immunomodulatory characteristics of mesenchymal stromal cells (MSCs) may lead to multifaceted strategies in rejection of organ transplantation. This study was designed to investigate, first, the effect of the donor-type MSCs from Wistar rats on the immune system of immunocompetent Lewis rats and, second, the rejection responses in a renal transplantation model of Wistar to Lewis. In the first experimental model, MSCs from the bone marrow induced a systemic immune response in the immunocompetent Lewis rats, characterized by two different phases. In the initial phase (days 1–3 after MSCs infusion), the main findings were a decrease in the percentage of the main peripheral blood (PB) lymphocyte subpopulations [T cells, B cells, and natural killer (NK) cells], an increase in the FOXP3 MFI in Tregs, and an elevated concentration of circulating proinflammatory cytokines (IL-1β and TNF-α). In the late phase (days 4–6), the percentage of T cells, B cells, and NK cells returned to baseline levels; the concentration of circulating IL-1β and TNF-α decreased; and the level of anti-inflammatory cytokines (IL-10 and IL-4) increased with respect to the initial phase. In the allogeneic kidney transplantation model, rats were randomized into four groups: nontreated, cyclosporine oral administration, and two groups of rats treated with two different schedules of MSC infusion: 4 days (MSCs-4) and 7 days (MSCs-7) before kidney transplantation and in both a further infusion at the day of transplantation. Both MSC treatments decreased the percentage of T, B, and NK cells in PB. Creatinine levels, survival, and histological parameters were better in MSCs-7 than in MSCs-4. We can conclude that MSCs, by themselves, produce changes in the immune system; they do not need a pathological condition to produce immunomodulatory responses. In the renal allograft model, the optimal time schedule for MSC infusion before grafting was 7 days to prevent acute rejection.

Key words: Mesenchymal stromal cells (MSCs); Renal transplantation; In vivo immunomodulation

INTRODUCTION

Mesenchymal stromal cells (MSCs) are found in a variety of tissues, including bone marrow, skin, and adipose tissue[1]. They are of interest because of their potential therapeutic effects[2] that are mediated by multiple mechanisms such as immunomodulatory effects through the secretion of regulatory cytokines[3], activation of regulatory immune cells[4,5], and the capacity to increase tissue repair through the secretion of antiapoptotic[6], antifibrotic[7], and proangiogenic factors[8]. Extensive in vitro studies have demonstrated that MSCs are capable of suppressing T-cell proliferation, influencing dendritic cell maturation and function, suppressing B-cell proliferation and terminal differentiation, and modulating other immune cells such as natural killer (NK) cells and macrophages[9,10]. These complex characteristics indicate that MSCs have multiple therapeutic effects in various diseases and/or organs[11,12].

Organ transplantation is the only definite treatment for many critical diseases of the liver, kidney, heart, and other organs. Although it is the primary therapeutic option at present, organ transplantation is still burdened by the need for lifelong immunosuppression to prevent graft

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rejection. Drugs currently in use for immunosuppression induction and maintenance include a variety of immunosuppressants that frequently cause severe side effects. Recently, in vitro expanded MSCs have been applied in preclinical and clinical studies in the treatment of graft-versus-host disease (GVHD)[13], multiple sclerosis[14], systemic lupus erythematosus[15], and renal transplant[16,17], with varying levels of success.

The in vivo immunomodulatory properties of MSCs have been inferred mainly from the in vitro studies. However, in vivo models or even clinical trials may fall far from in vitro observations[18]. In this regard, several issues should be examined in the search for MSC-based optimal effective regimens.

The present study was designed to (1) analyze the peripheral immunomodulatory response induced upon intravenous (IV) infusion of MSCs in immunocompetent rats, (2) investigate the peripheral immune response and the MSC-mediated modulation of acute rejection in a rat model of allogeneic kidney transplantation, and (3) find the optimal timing for infusion of MSCs in this setting.

MATERIALS AND METHODS

Isolation and Culture of Bone Marrow Rat MSCs

All procedures and animal housing conditions were in accordance with the Guidelines of the Committee on the Care and Use of Laboratory Animals and Good Laboratory Practices at the University of Barcelona. Bone marrow cells were extracted from Wistar rats (250 g; Charles River Laboratories, Barcelona, Spain) and were flushed out of the tibias and femurs with phosphate-buffered saline (PBS; BD Biosciences, Madrid, Spain) and passed through a 70-µm mesh. After washing, cells were transferred to T-75 flasks at a density of 9×10^5 cells/cm^2 and maintained in modified Eagle's medium-α (MEM-α) supplemented with 2 mM L-glutamine, 1% penicillin and streptomycin, and 15% fetal bovine serum (FBS) (all from Lonza, Madrid, Spain). After 24 h, medium and nonadherent cells were removed, and fresh medium was added.

Characterization of MSCs

For immunophenotyping, MSCs were harvested and stained with CD90-allophycocyanin (APC) (Miltenyi Biotec, Madrid, Spain), CD73-phycoerythrin (PE) (Abd Serotec, Raleigh, NC, USA), CD45-fluorescein isothiocyanate (FITC), CD34-PERCP (BD Biosciences), major histocompatibility complex I (MHC-I)-FITC (BD Biosciences), and MHC-II-FITC (BD Biosciences). Samples were analyzed on a fluorescence-activated cell sorting (FACS) Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) using the FACSDiva software.

MSCs were proven to be multipotent with a rat mesenchymal stem cell functional identification kit, following the manufacturer's instructions (R&D Systems, Madrid, Spain).

Infusion of MSCs in Immunocompetent Rats

One million MSCs (passages 3–5) from Wistar rats in 300 µl of PBS were infused intravenously through the dorsal penis vein of inbred Lewis rats. Every 24 h for 6 days, blood was collected in heparin lithium tubes. The main subpopulations of peripheral blood (PB) cells were analyzed with flow cytometry. Two experiments were carried out, totaling three animals per experiment.

Quantification of Circulating Donor-Specific Antibodies in Immunocompetent Rats

The presence of circulating class I and class II donor-specific antibodies (DSAs) was quantified in immunocompetent rat serum samples that were incubated with donor spleen cells and measured with flow cytometry. After MSC infusion, serum samples were collected every 24 h for 6 days. Donor splenocytes were isolated from Wistar rat spleens by Ficoll® (GE Healthcare, Madrid, Spain) density gradient and were freshly used. Different controls were added: serum from nontransplanted Lewis rat as naive and serum from a transplanted Lewis rat with high anti-MHC antibody titer as a positive control.

Briefly, 5×10^5 splenocytes were incubated with 25 µl of serum from Lewis rats treated with MSCs for 30 min at room temperature, washed in PBS, incubated in the dark (30 min, 4°C) with a 1:25 mixture of anti-CD3 (eBioscience, Madrid, Spain) and anti-immunoglobulin G (IgG) Fc specific (Jackson ImmunoResearch, West Grove, PA, USA), and analyzed with flow cytometry. A fluorescence increase of 15% with regard to the negative control was considered positive.

Measurement of Soluble Cytokines in Immunocompetent Rats

Cytokines from the plasma were analyzed with cytometric bead array immunoassay. The technique was based on the binding of particles that were labeled with different fluorescence intensities to antibodies. The particles were bound by a covalent bond to an antibody against one of the cytokines: interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-1B, and IL-10 (BD Biosciences). This method allowed simultaneous determination of different cytokines in the same sample. Standard curves (0 to 5,000 pg/ml) were derived from a set of calibrators, and the same set was used for all assays. Then 50 µl of the sample or cytokine standard was added to the mixture of 50 µl of each of antibody-PE detector and antibody-bead reagent. The mixture (150 µl) was incubated for 160 min in the dark at room temperature and washed, and the test samples were acquired using the flow cytometer.

Surgical Transplantation Technique and MSC Infusion

Inbred male Lewis rats (250 g) received an allogeneic kidney from Wistar rats (250 g). This strain combination was described by Azuma et al.[19] as an acute allograft rejection model. The surgical technique has been described previously[20]. Briefly, kidneys were preserved for 40 min in Euro-Collins solution (Fagron Iberica, Barcelona, Spain) at 4°C. Kidneys were heterotopically anastomosed to the aorta artery and cava vein, respectively, and the ureter end to end. Recipient rats were binephrectomized at the moment of transplantation.

Treatments and Design of Transplantation Groups: Follow-Up

Rats were randomized into the four groups listed below and then followed for 21 days:

1. Nontreated: rats received infusion of PBS ($n=7$).
2. CsA: rats received a single daily dose of 5 mg/kg cyclosporine (Novartis) by oral gavage for 21 days ($n=7$).
3. MSCs-4: rats received two infusions of MSCs (1×10^6 cells), at day 4 before renal transplantation and at 1 h after surgery ($n=5$).
4. MSCs-7: rats received two infusions of MSCs (1×10^6 cells), at day 7 before renal transplantation and at 1 h after surgery ($n=5$).

PB samples were collected from the tail vein before surgery, on day 7 posttransplant (post-tx), and on the day of sacrifice in order to study cell subpopulations. The animals were euthanized with isoflurane when they appeared to be clinically ill, suggestive of renal failure, or at day 21 after surgery when the outcome was excellent. To monitor renal function, serum creatinine was determined with Jaffe reaction (Autoanalyzer; Olympus, Hamburg, Germany) at baseline and every 2 days, beginning the day before surgery. Upon sacrifice, grafted kidneys were processed for histological studies.

Flow Cytometry

PB samples were incubated with an antibody cocktail to analyze lymphocyte subsets: mouse anti-rat CD3-FITC, mouse anti-rat CD45ra-PE, and mouse anti-rat CD163-APC (BD Biosciences). Another sample was also incubated with a combination of PE-conjugated mouse anti-rat forkhead box P3 (FOXP3) antibody (eBioscience), CD3-PE-cyanine 7 (Cy7), CD4-FITC, and CD25-Alexa fluor 488. The cells were then analyzed with flow cytometry. For FOXP3 staining, an intracellular staining buffer set (eBioscience) was used following the manufacturer's instructions.

Histological Studies

Coronal 1- to 2-µm-thick slices of graft kidneys were fixed in buffered formalin (Sigma-Aldrich, Madrid, Spain), dehydrated, and embedded in paraffin. For light microscopy, 3- to 4-µm-thick tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS; Sigma-Aldrich). A blinded pathologist assessed all sections for tubulitis, interstitial infiltration, vasculitis, glomerulitis, tubular necrosis, glomerular necrosis, and interstitial fibrosis, following the Banff criteria for acute/active lesion scoring[21]. A global SCORE was defined as a summary of individual Banff parameters.

Paraffin-embedded tissue sections were immunoperoxidase-stained complement component 4 C4d (1:30; Biomedica Gruppe, Vienna, Austria). Negative controls were carried out by immunostaining-matched serial sections without the primary antibodies. C4d assessed in peritubular capillaries and glomeruli was semiquantitatively scored: 0 denoted negative staining; 1, positive staining in <25% of the sample; 2, positive staining in 25%–50%; 3, positive staining in 50%–75%; and 4, positive staining in 75%–100%.

Statistical Analysis

All results were expressed as mean ± standard deviation (SD). Differences between time points were evaluated using the Student's *t*-test, and values of $p<0.05$ were considered to be statistically significant. Statistical differences between treatment groups were evaluated using single-factor or two-factor, without replication, analysis of variance (ANOVA). Post hoc analysis was performed using the Tukey–Kramer method because group sizes varied between experimental groups. The software used was StatView (SAS, Cary, NC, USA).

RESULTS

Isolation, Characterization, and Labeling of MSCs

Adherent cells displayed typical MSC morphology, namely, a spindle shape or flat-polygonal appearance (Fig. 1A). Cells were used at passages 3–5. The multipotency of the expanded MSCs was assessed by differentiating the cells into adipocytes (Fig. 1B), osteoblasts (Fig. 1C), and chondroblasts (Fig. 1D). Flow cytometry (Fig. 1E) analysis demonstrated positive staining for CD90 (93.2 ± 5.1%) and CD73 (89.6 ± 10.1%), and low expression of MHC-I (Fig. 1F) (92.5 ± 8.4%) but negative for CD34, CD45, and MHC-II, indicating a mesenchymal rather than hematopoietic origin.

MSCs Alter the Percentage of the Main Lymphocyte Subsets in PB

In Figure 2A, a representative study of the distribution of the major lymphocyte subpopulation in the PB of naive Lewis rats is depicted. Lymphocyte population was defined using a forward scatter-height (FSC-height)/side scatter-height (SSC-height) dot plot (G1). Subsequently, T cells, B cells, NK cells, and NK cells derived from

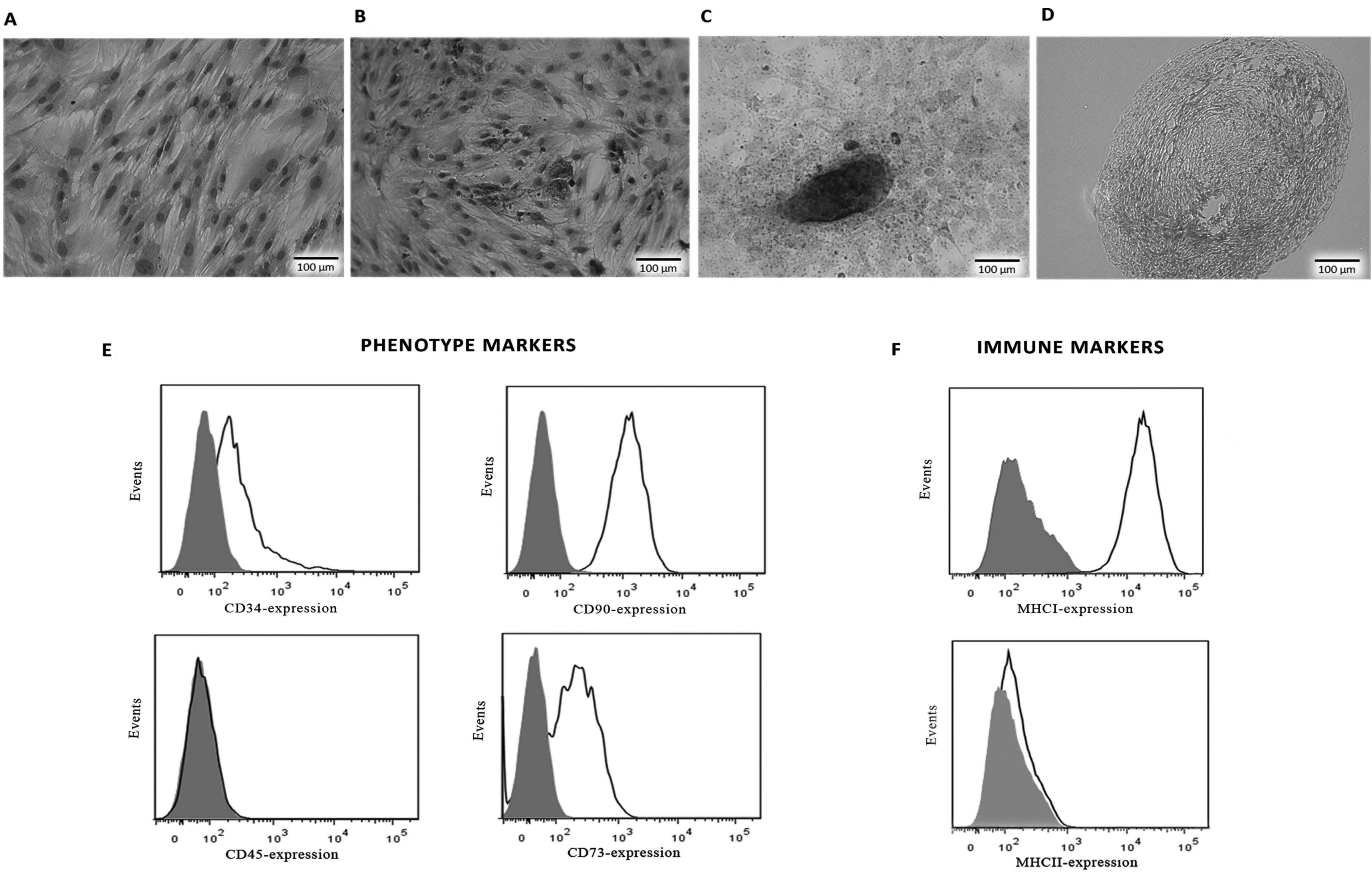

Figure 1. Characterization of mesenchymal stem cells (MSCs) isolated from the bone marrow of Wistar rats. (A) Bright-field image of cultured MSCs. (B) MSCs cultured under adipogenic conditions, stained with Oil red O. (C) MSCs cultured under osteogenic conditions, stained with von Kossa staining. Calcified nodules appear in dark gray/black. (D) MSCs cultured under chondrogenic condition, stained with thionin. (E) Phenotype markers of rat MSCs. (F) Immune markers of rat MSCs. The gray histograms signify staining with isotype controls, and the white histograms represent staining with the specified surface marker antibody. MHC, major histocompatibility.

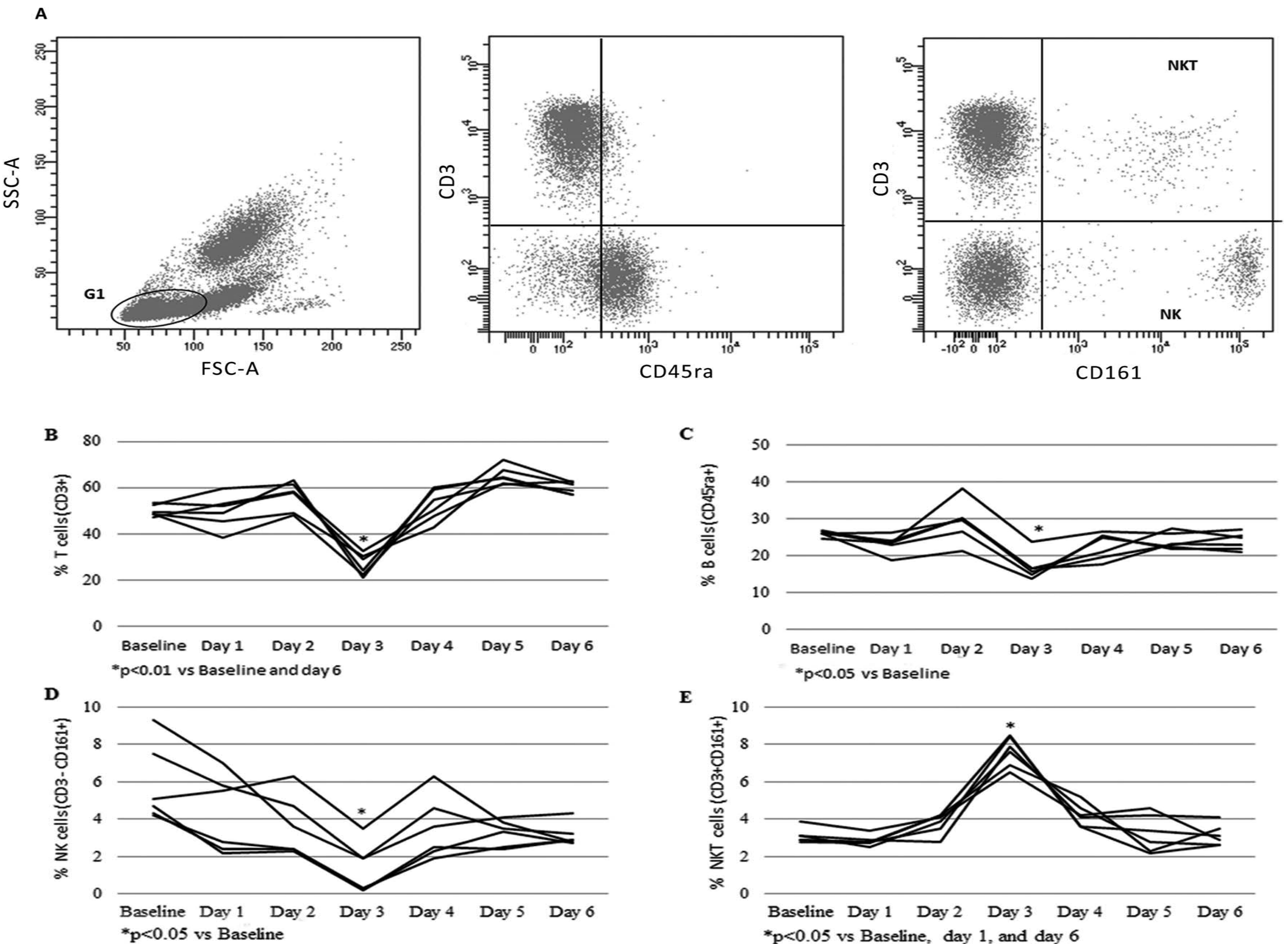

Figure 2. MSC administration induces a response of circulating lymphocyte subsets in peripheral blood (PB) of immunocompetent rats. (A) Representative density plots illustrating the gating strategy used to identify T cells, B cells, natural killer (NK) cells, and NK cells derived from T cell (NKT) subsets in PB by flow cytometry. (B–E) Values represent percentage of different lymphocyte subsets for all the immunocompetent rats at different time points. SSC, side scatter; FSC, forward scatter.

T cell (NKT) lymphocytes in the G1 population were assessed using a CD3-FITC/CD45ra-PE and CD3-FITC/CD161-APC dot plot.

At 3 days after infusion, the percentages of T and B cells (CD3: 26.6±4.7%; CD45ra: 16.9±3.6%) were both significantly reduced compared to baseline (CD3: 50±2.5%, $p<0.05$; CD45ra: 25.9±0.8%, $p<0.05$) (Fig. 2B and C). Rat NK cells were defined as $CD3^-CD161^+$, and NKTs as $CD3^+CD161^+$. Notably, NK cells showed an about fourfold drop at day 3 after infusion (CD161: 1.3±1.3%) with respect to baseline (5.9±2.1%, $p<0.05$) (Fig. 2D). In contrast, the percentage of NKTs increased at day 3 after infusion (7.6±0.8%) with respect to baseline (3.1±0.4%, $p<0.05$) (Fig. 2E).

MSCs Increase FOXP3 Expression in Regulatory T Cells

We next investigated whether MSCs might influence the regulatory T cells (Tregs) profile. To this end, the $CD4^+CD25^+FOXP3^+$ lymphocyte subpopulation was analyzed with flow cytometry (Fig. 3A). The percentage of Tregs with respect to the total number of $CD3^+$ cells remained stable after MSC infusion (range: 3.3%–3.7%) at all of the time points (Fig. 3B). Interestingly, in contrast to Treg frequencies, considerable differences in the mean fluorescence intensity (MFI) of FOXP3 in Tregs at different time points were observed (Fig. 3C). The MFI of FOXP3 in Tregs increased at day 1 (1,351.5±60.5 MFI) compared with baseline (743.5±100.7 MFI, $p<0.05$) and peaked at day 4 (1,831±124 MFI).

Circulating Donor-Specific Antibodies (DSAs)

Donor splenocytes obtained from spleens of naive Wistar rats were cultured with serum from MSC-treated immunocompetent Lewis rats. DSA assay was negative at all time points; the fluorescence increase was lower than 15% with regard to the negative control (data not shown).

Effects of IV Infusion of MSCs on the T Helper 1 (TH1)/TH2 Balance

To analyze whether the MSCs could modify the molecular circulating environment in healthy animals, some TH1/TH2 cytokines were measured in serum at various time points. After administration of MSCs, we observed a dynamic response, with two distinct phases (Fig. 4). An initial phase, between day 1 and day 3 after MSC infusion, was characterized by an increase in the TH1 cytokines IL-1B (1.8-fold) and TNF-α (2-fold) with respect to the baseline. A later phase, between day 5 and day 6, saw the concentration of proinflammatory cytokines decrease significantly and return to baseline levels (Fig. 4A). In contrast, in this later phase, TH2 cytokine concentrations

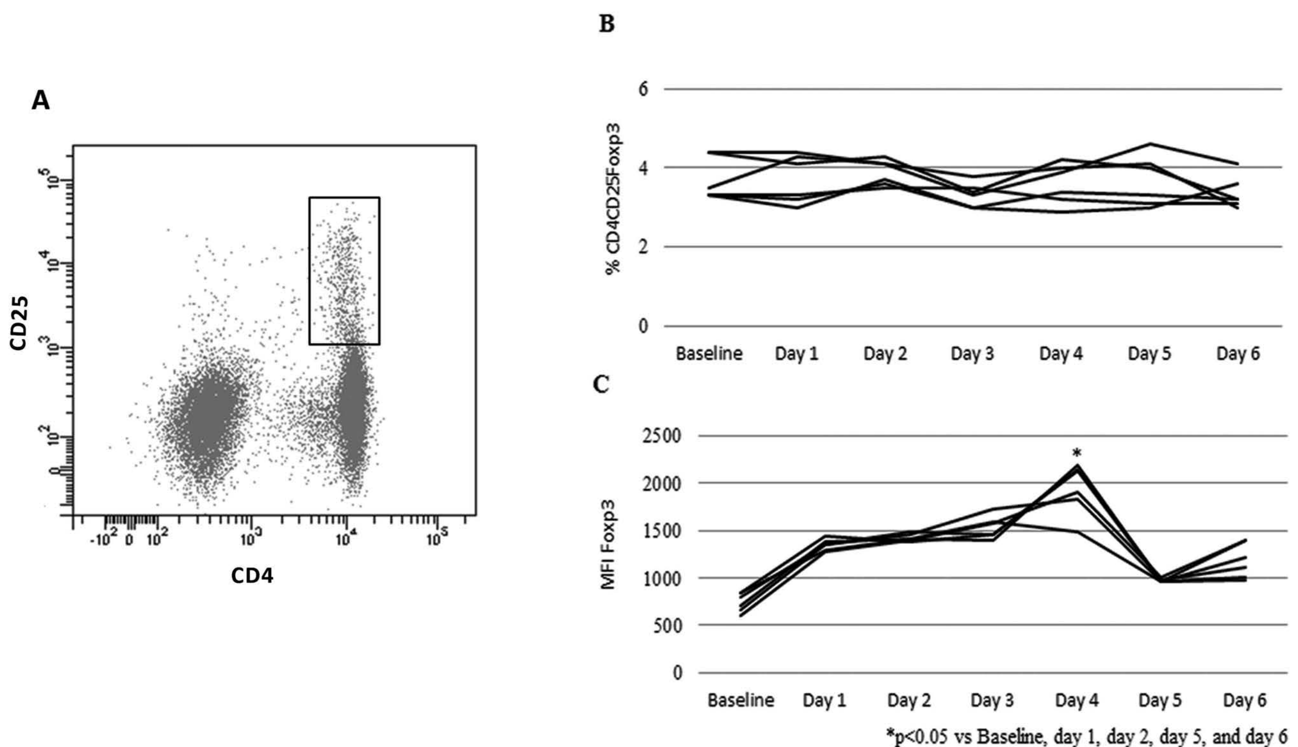

Figure 3. (A) Phenotypic analysis of regulatory T cells (Tregs) ($CD3^+CD4^+CD25^+FOXP3^+$) in PB by flow cytometry. (B) Kinetics of Treg percentage and (C) forkhead box P3 (FOXP3) expression by flow cytometry in Tregs. MFI, mean fluorescence intensity.

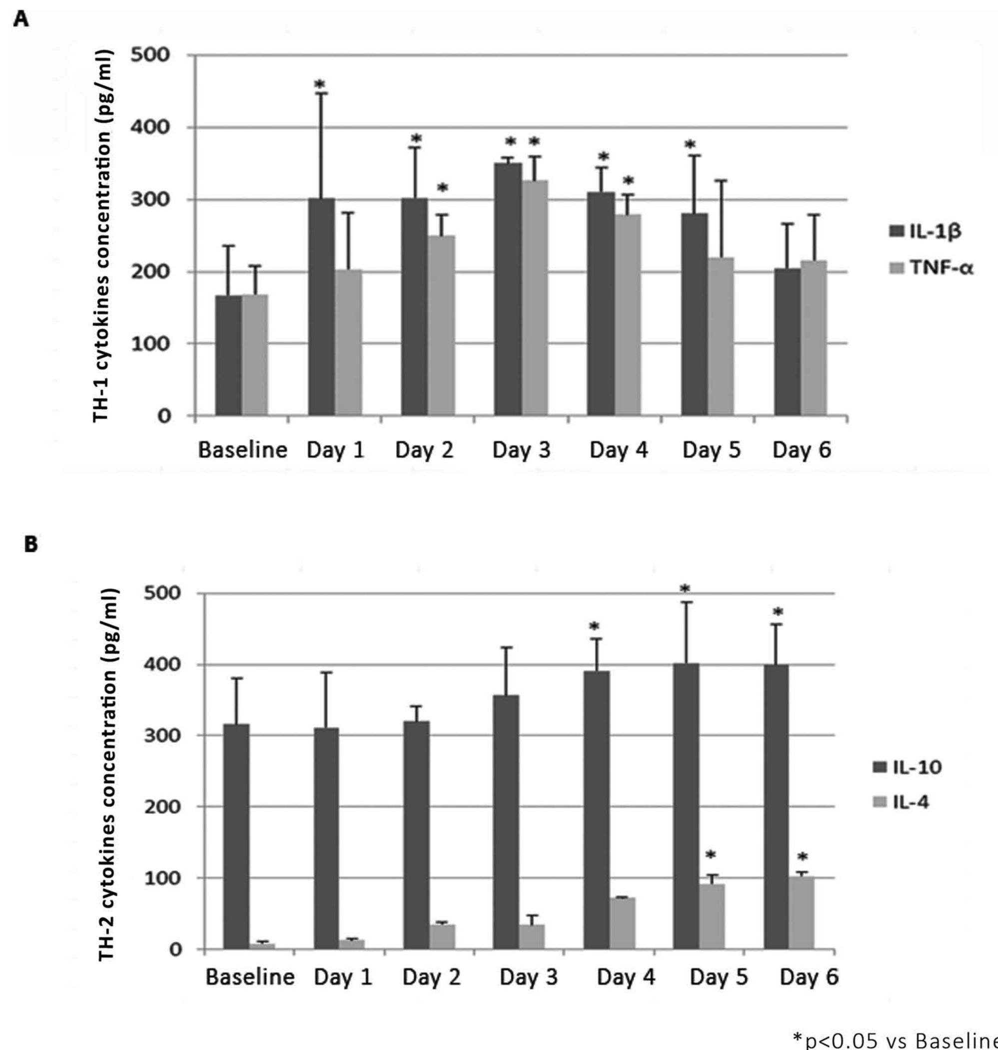

Figure 4. Serum concentrations of cytokines after infusion of MSCs. Serum was collected at several time points after administration of MSCs. Concentrations of interleukin-1B (IL-1B), tumor necrosis factor-α (TNF-α), IL-10, and IL-4 were determined with cytometric bead array immunoassay. (A, B) Measurements of six animals. Data represent the mean± standard deviation (SD). *$p<0.05$. TH, T helper.

started to increase at day 4 and picked up at day 6 (IL-10: 1.25-fold; IL-4: 6-fold) with respect to the baseline (Fig. 4B). No significant changes in serum concentration of IFN-γ or IL-2 were observed (data not shown).

Effects of MSC Administration on Lymphocyte Subsets in Renal Grafted Rats

No presurgical baseline levels of lymphocyte populations differed significantly between treatment groups. The analysis revealed that there were no statistical differences in the percentage of $CD3^+$ cells between groups at day 7 post-tx and sacrifice point (Fig. 5A).

Post-tx levels of circulating $CD45ra^+$ B cells were decreased in all groups at day 7 (CsA: 21.3±1.5, $p<0.05$; nontreated: 12.2±4.2, $p<0.01$; MSCs-4: 18.3±4.8, $p<0.05$; MSCs-7: 15.4±2.9, $p<0.05$) with respect to the baseline. At the point of sacrifice, $CD45ra^+$ B cells in the CsA group (27.7±3.5) returned to baseline levels, while in the other groups it continued to be clearly lower ($p<0.05$) (Fig. 5B).

The nontreated group showed an increase in the percentage of NK cells at day 7 post-tx (23.2±6.6%) compared with the CsA (4.1±1.3%, $p<0.01$), MSCs-4 (12±4.3%, $p<0.01$), and MSCs-7 (9±3.6%, $p<0.01$) groups. At the time of sacrifice, the percentage of NK cells in each group was similar to the day 7 post-tx percentage (Fig. 5C).

At day 7 post-tx, the increase in NKTs varied between groups (CsA: 3.6±0.9%; nontreated: 10.5±0.6%, $p<0.01$; MSCs-4: 9.2±2.5%, $p<0.01$; MSCs-7: 6.3±1.5%, $p<0.05$) compared to baseline. It is important to note that the percentage of NKTs in the MSCs-7 group was clearly lower than that in the MSCs-4 ($p<0.05$). At sacrifice day, there

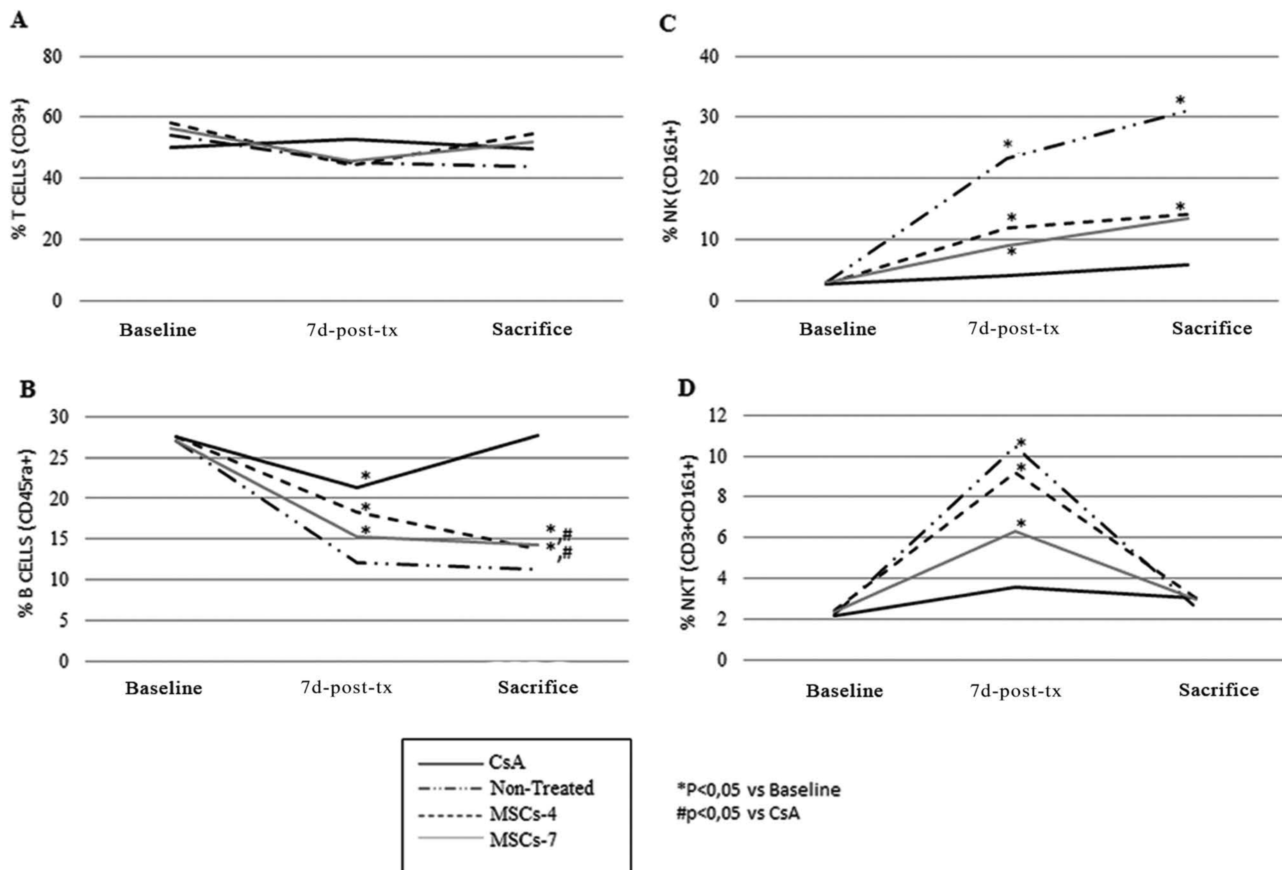

Figure 5. Percentage of lymphocyte subsets in PB after renal transplantation. Graphic values (mean ± SD) represent circulating levels of each lymphocyte subset averaged for all transplant recipients within each respective treatment group at the indicated time points. (A) T cells ($CD3^+$), (B) B cells ($CD45ra^+$), (C) NK cells ($CD3^-CD161^+$), and (D) NKT ($CD3^+CD161^+$). Differences between time points were evaluated using the Student's *t*-test, and values of $p < 0.05$ were considered to be statistically significant. MSCs, mesenchymal stem cells; CsA, rats that received a single daily dose of 5 mg/kg cyclosporine by oral gavage for 21 days; post-tx, posttransplant; d, day.

were no differences between groups; all had returned to baseline values (Fig. 5D).

MSC Treatment Increased Tregs After Kidney Transplantation

The percentage of Tregs was increased at day 7 post-tx in both MSC groups (MSCs-4: 3.9 ± 0.7%; MSCs-7: 3.8 ± 0.7%) and CsA (3.1 ± 0.3%) compared to the nontreated group (2.2 ± 0.6, $p < 0.05$) group. At sacrifice, Tregs in MSCs-7 increased (5 ± 0.7%) with respect to day 7 post-tx ($p < 0.05$) and other groups (Fig. 6A). We analyzed FOXP3 expression in Tregs to assess its functional status[22], and it was higher in both MSC groups (MSCs-4: 2,308.8 ± 486.4 MFI; MSCs-7: 3,203.2 ± 810.4 MFI) with respect to the nontreated group (1,137.8 ± 267.9 MFI, $p < 0.05$) and CsA groups (1,569.4 ± 295.3 MFI, $p < 0.05$) at day 7 post-tx and with respect to baseline levels. At the time of sacrifice, the differences between groups were similar to that at day 7 post-tx (Fig. 6B).

Prolongation of Graft Survival and Protection of Graft Function With MSC Treatment

Survival mean of the nontreated group was 14.4 ± 4.8 days (28.6% of survival). All the animals receiving a single daily dose of 5 mg/kg cyclosporine were alive at day 21 (100% of survival). The MSCs-4 showed a survival time of 17.6 ± 4.9 days (60% survival), and the MSCs-7 group 20.2 ± 1.8 days (80% survival). The results clearly show that the infusion of MSCs significantly prolonged animal survival, and differences between the two MSC administrations were observed ($p < 0.05$) (Fig. 7A).

Graft function was assessed serially by measuring the serum creatinine levels (Fig. 7B). In the nontreated rats, serum creatinine increased progressively from day 7 and remained high throughout follow-up. In contrast, the CsA group maintained stable low serum creatinine levels throughout follow-up. MSC-infused rats showed different renal function profiles from the nontreated animals. MSCs-4 rats had increased serum creatinine earlier,

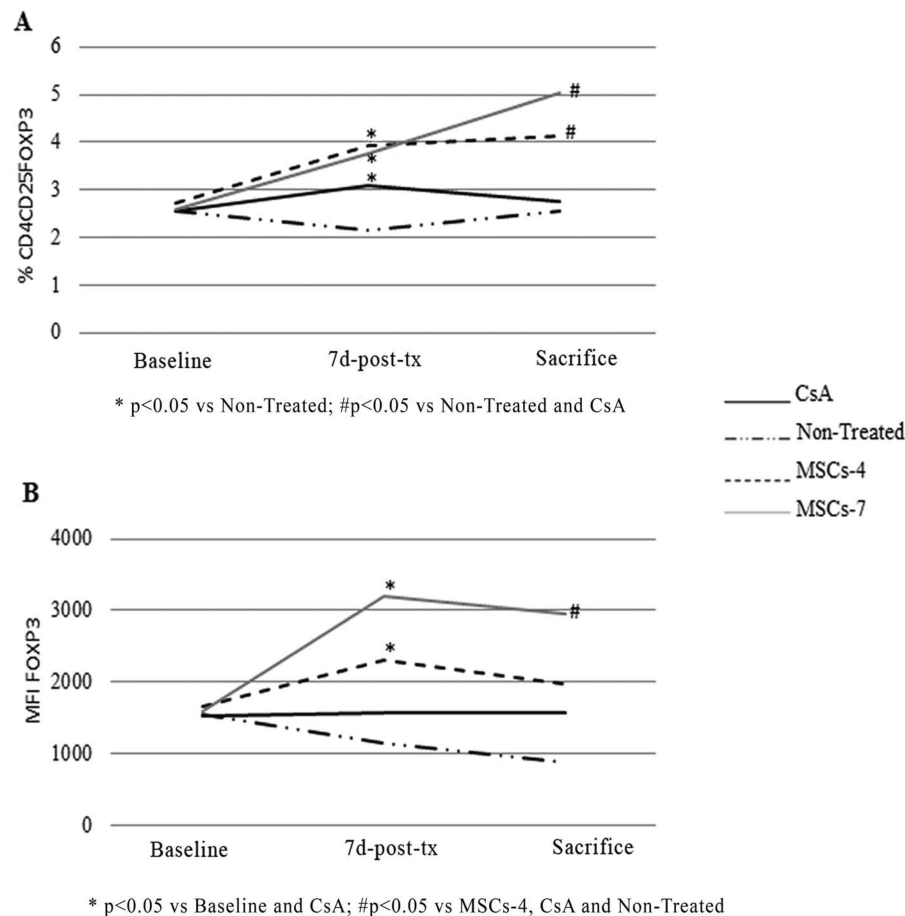

Figure 6. Effects of MSCs on Tregs after kidney transplantation. (A) Percentage of Tregs ($CD3^+CD4^+CD25^+foxp3^+$) in rat PB and (B) mean fluorescence intensity (MFI) of FOXP3. Differences between time points were evaluated using the Student's *t*-test, and values of $p < 0.05$ were considered to be statistically significant. CsA, rats that received a single daily dose of 5 mg/kg cyclosporine by oral gavage for 21 days; post-tx, posttransplant.

between day 3 and day 5, while the serum creatinine levels of MSCs-7 rats began increasing between day 5 and 7 but with less severe kidney failure. Both MSC groups showed lower serum creatinine than the nontreated group (peak 5.8±1.6 mg/dl); however, the MSCs-7 serum creatinine (peak 2.4±2.6 mg/dl) was significantly lower at all time points from day 3 than the MSCs-4 (peak 3.6±1.6 mg/dl, $p<0.05$).

Graft Inflammatory Histopathological Status Depends on Timing of MSC Infusion

Nontreated rats showed severe inflammatory cell infiltration in all kidney compartments. This was accompanied by extensive glomerular necrosis associated with glomeruli infiltration and a considerable degree of secondary acute tubular necrosis. Cyclosporine administration clearly reduced the inflammation in all compartments, though a nonnegligible degree of tubulitis and interstitial infiltration was seen. In accordance with the higher mortality and renal failure of MSCs-4, this group showed a higher global histological SCORE, even worse than nontreated animals (Fig. 8A). These differences were related in particular to a more severe degree of interstitial cell infiltration, but especially to a more severe vascular inflammation. This indicates an exacerbation of the graft inflammatory status. In contrast, when MSCs were infused 7 days before allotransplantation, a reduction in graft inflammation was observed, clearly better than both MSCs-4 and nontreated rats. Notably, this was accompanied by a lesser degree of secondary glomerular and tubular necrosis (Fig. 8B).

C4d deposition was completely absent in cyclosporine-treated kidneys. In contrast, in nontreated kidneys, an intense deposition was seen in both glomeruli and intertubular capillaries, completing the picture of humoral vascular rejection (Fig. 9A). In MSCs-7 kidneys, there was a clear reduction in C4d deposition, similar to that of cyclosporine kidneys. This was not the case with MSCs-4 kidneys that showed the same C4d deposition as nontreated kidneys (Fig. 9B).

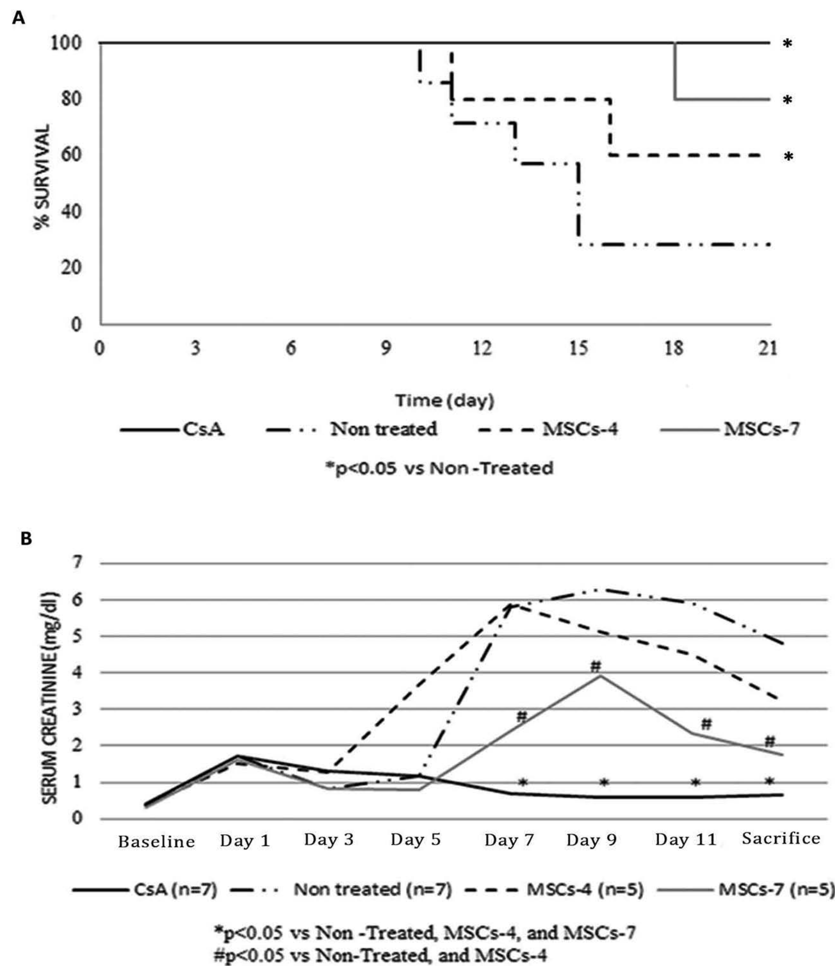

Figure 7. Effect of MSC infusion on animal survival and renal function in kidney transplant. (A) Animal survival was markedly prolonged when the animals were treated with CsA (rats that received a single daily dose of 5 mg/kg cyclosporine by oral gavage for 21 days) or double infusion of MSCs at 7 days before the transplant and the same day of the transplant. (B) Graft function evaluated with serum creatinine (mg/dl). The level of serum creatinine was in line with survival results.

DISCUSSION

In the present study, we investigated the effect of donor-type MSCs in two different scenarios: the immune system of immunocompetent rats and the rejection response modulation in a rat renal transplantation model. In the first experimental model, MSCs, after their IV administration, elicited a systemic immune response in the immunocompetent rats, characterized by two different phases. The initial phase, between day 1 and day 3 post-MSC infusions, was marked by a decrease in the percentage of T, B, and NK cells, an increase in the FOXP3 MFI in Tregs, and an elevated concentration of TH1 cytokines. Then there was a later phase, between day 5 and day 6, in which the percentage of the main PB cells returned to baseline percentages, the concentration of proinflammatory cytokines decreased, and, interestingly, the level of TH-2 cytokines increased with respect to the initial phase. The most interesting observation in our study is that MSCs do not need a pathological condition to produce immunomodulatory responses. They have, by themselves, the capacity to modulate the main cellular subsets of the immune system in the PB of healthy animals. These in vivo inherent characteristics of MSCs may offer beneficial effects in several disease models[14,15], but how the immune-activating effects of MSC administration are produced remains unclear. Hoogduijn et al. reported that human MSCs induced an inflammatory response in mice 2 h after their IV infusion[23]. Thus, it may be speculated that there is an immune response to foreign-like MSCs and/or to their unusual location, rather than an MSC-specific response.

After the first part of our study, we investigated the effectiveness of the immunomodulatory response produced by MSCs in a rat model of allogeneic kidney transplant. To this end, four groups of animals were studied: nontreated rats, rats receiving cyclosporine to prevent

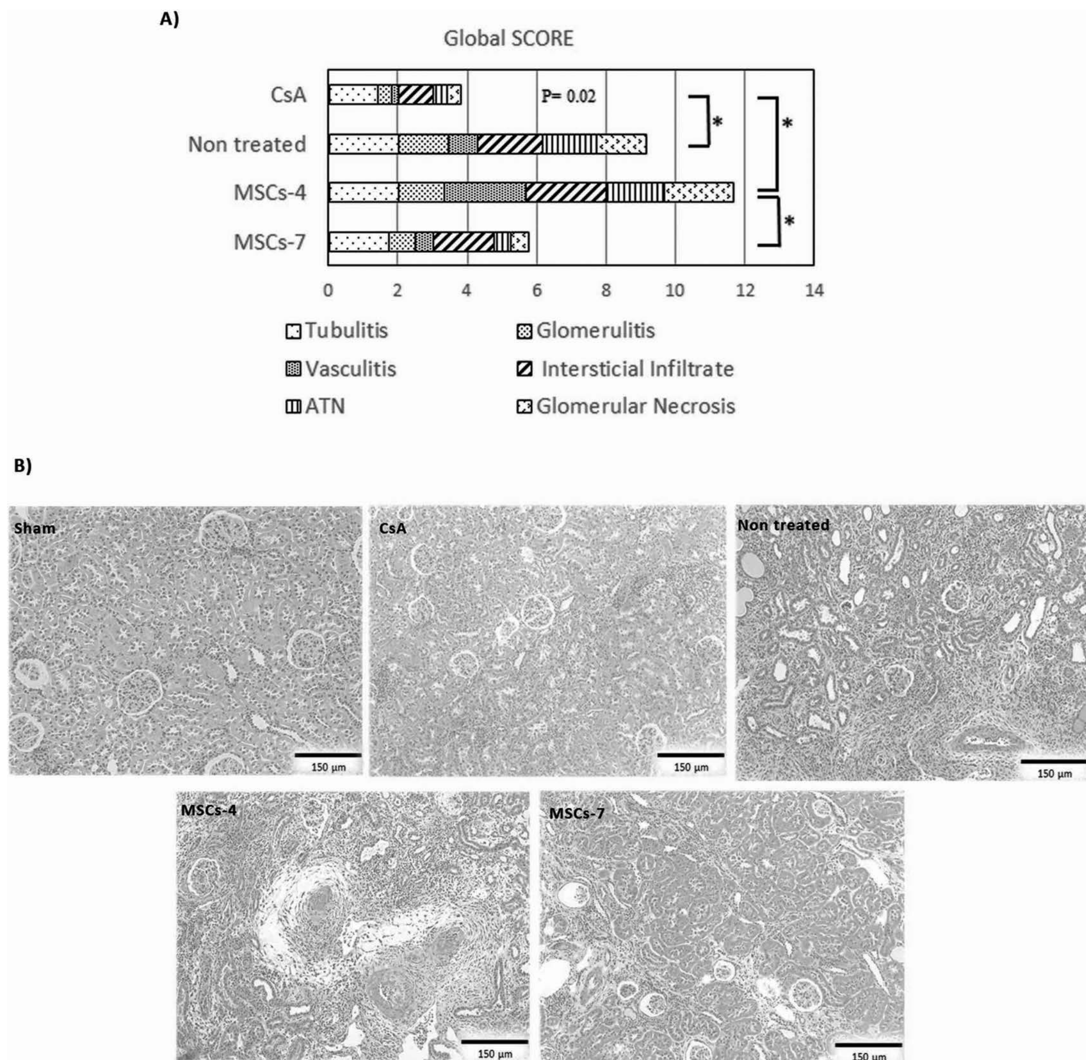

Figure 8. MSC infusion transforms graft inflammatory histopathological status. (A) The global SCORE, which includes inflammatory elemental lesions (tubulitis, glomerulitis, and vasculitis interstitial infiltrate) plus the subsequent tissue damage, shows the better histological picture in kidneys from the MSCs-7 group. (B) Representative microphotographs from each group: CsA (rats that received a single daily dose of 5 mg/kg cyclosporine by oral gavage for 21 days), little tubulitis and interstitial infiltrate; nontreated, severe exacerbation of inflammation in all renal compartments; MSCs-4, grade 3 vascular involvement and severe renal structural destruction with edema and inflammatory infiltration; MSCs-7, less severe vascular damage with well-preserved renal architecture. ATN, acute tubular necrosis.

acute rejection, and two groups receiving MSCs following two different infusion schedules. In the first MSC administration group (MSCs-4), cells were infused at 4 days pretransplantation (this time point was considered the early phase), and in the other group (MSCs-7), cells were infused 7 days pretransplantation (this time point was considered the later phase). Our results show that the percentages of the major lymphocyte subpopulations (T, B, and NK cells) after renal transplant, in the MSC groups, were closer to the CsA group than to the nontreated group. The lymphocyte subset most influenced by MSCs was NK cells. It has to be noted that the MSCs-7 group showed better results than the MSCs-4 group. NK cells are implicated in the early phase of innate immune responses, and consequently NK cell inhibition by MSCs could result in downregulation of the innate immune response potency, which could be beneficial for transplantation[24,25].

Looking at the regulatory arm of the immune response, the percentage of Tregs in the MSCs-7 group was higher than in the MSCs-4. These results are in line with previous studies[16,26] reporting that pretransplant infusion of MSCs induced tolerance, which was associated with Treg expansion and impaired TH1 activity. In agreement with the phases of our study of immunocompetent rats, the timing

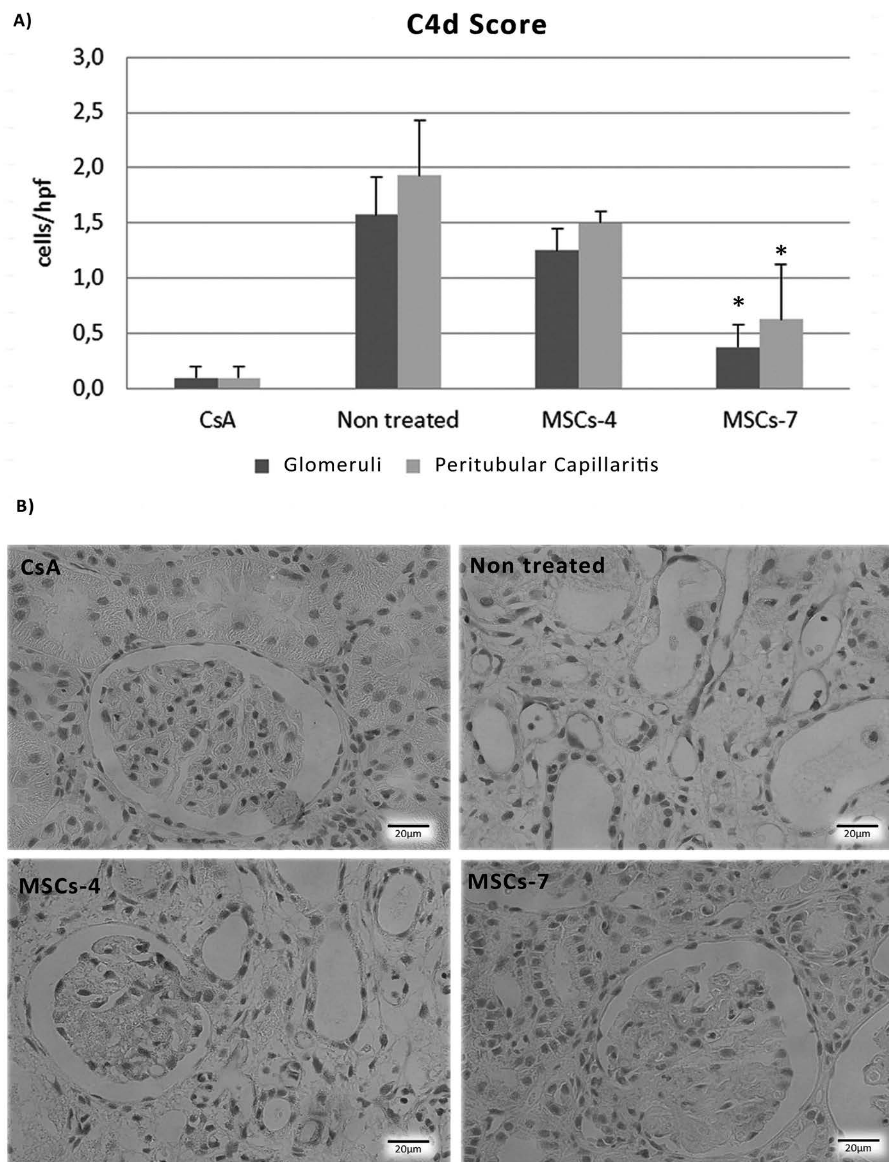

Figure 9. Pretransplant MSC infusion modulates graft C4d deposition. (A) Nontreated rats show an extensive C4d deposition both in glomeruli and in peritubular capillaries. CsA-treated rats (rats that received a single daily dose of 5 mg/kg cyclosporine by oral gavage for 21 days) showed no C4d deposition. The infusion of MSC 7 days before grafting clearly reduced the glomeruli and capillary C4d deposition. $*p<0.05$ versus nontreated and MSCs-4. (B) Representative microphotographs of C4d from each group.

of MSC infusion clearly determines optimal clinical immunomodulation in the transplant model. Thus, results showed that MSCs-7 treatment absolutely improved the renal function and animal survival compared to the MSCs-4 group. The histology of the nontreated group rounded out a picture of humoral rejection with severe inflammation, increased glomerulitis and vascular cell infiltration, and prominent deposits of C4d in microvessels. The administration of MSCs 4 days before kidney grafting did not improve the histological signs of damage compared to the nontreated animals. However, the infusion of MSCs 7 days before transplant improved the inflammatory graft status, with subsequently less acute tubular necrosis, glomerular necrosis, and C4d deposits.

Several authors have described different effects of MSCs in renal transplant. There are studies in which we find successful results[27] or results that have failed[28]. When considering these studies, we observe that the dosage and timing of the MSC infusion are completely different. Koch et al.[28] reported that in a model of acute kidney rejection, detrimental effects from MSC infusion 4 days before the renal transplant were observed. In contrast, Perico et al. obtained better results when they infused the cells 7 days before transplant[16]. In light of our

results, which in the later phase (4–6 days after infusion) saw an anti-inflammatory environment, we may speculate that 6–7 days is the time needed for MSCs to induce a tolerance status in the immune system. This is therefore the most suitable time for receiving a graft.

In conclusion, MSCs by themselves produce changes in the immune system; they do not need a pathological condition to produce immunomodulatory responses. More importantly, the fact that MSCs are not totally ignored by the immune system makes them potential candidates for alternative immunotherapy in organ transplantation. However, the optimal time schedule for MSC infusion needs to be considered in future studies.

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