Figure S1. Kidney allograft survival in BL6 mice given syngeneic MSC infusion the day of transplant (n = 7) or the day before infusion (n = 5) or left untreated (n = 7). Survival data of pretransplant MSC infusion was included in previous publication (Casiraghi F et al, Am J Transplant 2012, 12:2373) and reported in this figure for ease of comparison. *P < 0.05 versus no MSC infusion.
Figure S2. Homing to the kidneys and spleen of untreated MSCs or MSCs pre-exposed to vehicle for C3aR-A and C5a-RA. Number (mean ± SD) of PKH26+ MSCs in kidney grafts (A) and spleens (B) from mice given posttransplant (day +2) infusion of untreated MSCs or MSCs pre-exposed to vehicle during the last 48 hours of culture. $P = \text{NS}$. 
**Figure S3.** Total counts of Tregs, CD8⁺TEM and CD4⁺TEM cells and Tregs/CD8⁺TEM ratio. Total counts of CD4⁺CD25⁺Foxp3⁺ T cells (A), CD44⁺CD62L⁺CD4⁺TEM (B), ratio between numbers of Treg and CD8⁺TEM (C), and total counts of CD44⁺CD62L⁺CD4⁺TEM of spleens from MSC-treated and control mice. Data are mean ± SD; *P < 0.05 versus the other groups, **P < 0.05 versus MSC day 0.
Supplementary methods

Infusing mesenchymal stromal cells on the day of transplant, but not after transplant, promotes t\textsubscript{reg} induction and prolongs kidney transplant survival

Federica Casiraghi, Marta Todeschini, Nadia Azzollini, Paolo Cravedi, Paola Cassis, Samantha Solini, Sonia Fiori, Cinzia Rota, Aida Karachi, Camillo Carrara, Marina Noris, Norberto Perico, and Giuseppe Remuzzi

MSC isolation

MSCs were isolated from bone marrow of 2-mo-old BL6 mice, as previously described.\textsuperscript{1} Primary MSC cultures were depleted of CD45\textsuperscript{+} and CD11b\textsuperscript{+} cells by incubating cells with rat anti-mouse CD45 and rat anti-mouse CD11b Abs (0.2 μg/10\textsuperscript{6} cells; Caltag Laboratories) and then with goat anti-rat IgG magnetic microbeads (Miltenyi Biotec). CD45\textsuperscript{-}CD11b\textsuperscript{-} cells were then isolated by a MACS system (Miltenyi Biotec). CD45\textsuperscript{-}CD11b\textsuperscript{-} MSCs expressed low levels of MHC class I and II were positive for CD44 expression and negative for CD86 expression and differentiate in vitro toward osteoblasts, adipocytes, and chondroblasts, as previously described.\textsuperscript{1} Independent MSC batches were used for in vitro and in vivo transplant experiments.

Kidney transplantation

Kidney transplantation was performed as previously described,\textsuperscript{2} with minor modifications. The donor left kidney was flushed with ice cold heparinized saline and removed along with the ureter and vessels. Recipient mice underwent left-sided nephrectomy and orthotopic implant. The ureter was inserted into the bladder and pulled through. The right native kidney was then removed, and the recipient became dependent on a functioning graft. Cold (organ preservation in cold solutions) and warm (time for completion of transplant surgical procedures) ischemia times were routinely 5 and 20 min, respectively. The technical success rate was 70%. Failure in surgical procedure (usually ureter stenosis) resulted in animal suffering within 36 h of the controlateral nephrectomy. Kidney graft function was monitored by measurement of blood urea nitrogen (BUN, by Reflotron test, Roche Molecular Biochemical). Two consecutive measurements of BUN >130 mg/dL were considered indication of severe graft rejection and recipients were euthanized.
Detection of infused MSCs in recipient organs
MSCs were labelled with the membrane dye PKH26 according to the manufacturer’s protocol (Red Fluorescence Cell Linker kit; Sigma-Aldrich) prior to intravenous infusion. Labelling efficacy was found to be >90% by FACS analysis. To evaluate MSC localization into kidneys and spleens, the organs were fixed in paraformaldehyde, impregnated with sucrose, and rapidly frozen. Tissues were then sectioned on a cryostat (8 μm), fixed with acetone, stained with 4’,6-diamidino-2-phenylindole (1 μg/mL; Sigma-Aldrich) and analyzed by fluorescence confocal microscopy. For each tissue, 3 nonconsecutive sections were analyzed and PKH26+ cells in 50 randomly selected high-power fields (HPF) were counted. Results are expressed as number of PKH26+ cells per mm².

Flow cytometry analysis for C3aR and C5aR expression
BL6 MSCs were labeled with anti-C3aR (clone 14D4, Hycult Biotech) and anti-C5aR (clone 20/70, Biolegend) or appropriate isotype matched monoclonal antibodies. Flow cytometric analysis was performed by FACSCanto flow cytometer (Becton Dickinson) with FlowJo software.

Immunohistochemistry
Intragraft neutrophils and C3b deposition were analyzed by immunofluorescence technique on frozen tissue previously fixed in PFA 4%. Air-dried and fixed sections (3μm) were incubated with either rat anti-mouse Gr1 (5 μg/mL, Biolegend) followed by FITC-conjugated goat anti-rat IgG for neutrophils or with FITC-conjugated goat anti-mouse C3 (2 μg/mL, Cappel). Neutrophils were counted in at least 10–15 randomly selected HPF (X400) and expressed as number of cells/mm². C3 deposition was scored (0 = absent; 1 = faint; 2 = moderate; 3 = intense staining), as previously described.³ Around 10 glomeruli and 10–15 randomly selected HPF (X400) with tubuli for each section were examined. Negative controls were carried out by omitting the primary antibody or with isotype antibody, usually on a second section on the same slide.

Ex vivo and in vitro immune assays
MLR was conducted using splenocytes from MSC-infused transplanted mice cultured with irradiated BALB/c splenocytes (50 000 each). MLR cultures proceeded for 48 hr at 37 °C, and then cultured cells were transferred to Enzyme-linked immunosorbent spot (ELISPOT) plates precoated with capture anti-IFNγ antibody. ELISPOT assay was then performed according to the manufacturer’s instructions (BD Bioscience).
PE-Cy7-conjugated anti-mouse CD3ε (clone 145-2C11), APC-Cy7-conjugated anti-mouse CD4 (clone RM4-5), PE-conjugated anti-mouse CD8 (clone 53-6.7), Alexa-647-conjugated anti-mouse CD44 (clone IM7), PerCP-conjugated anti-mouse CD62L (clone MEL-14), PE- or Alexa-488-conjugated anti-mouse Foxp3 (clone FJK-16S), APC-conjugated anti-mouse CD25 (clone PC61), PE Armenian Hamster Anti-Helios (clone 22F6), and respective isotype antibodies were used for splenocyte phenotypic analysis (FACSArray flow-cytometer, Becton Dickinson, using FlowJo software). For the evaluation of CD4+CD25+ T-cell suppressive function, CD4+ T cells were enriched from splenocytes by CD4+ T-Cell Isolation Kit (Miltenyi), labeled with FITC–anti-mouse CD4 and APC–anti-mouse CD25 antibodies and then FACS sorted to obtain CD4+CD25− effector T cells from splenocytes of BALB/c- sensitized untransplanted BL6 mice and CD4+CD25+ T cells from splenocytes of mice from MSC-infused transplanted mice (purity > 99%). CD4+CD25+ T cells were added on 25 000 CD4+CD25− effector T cells at 2:10 or 1:10 ratios and incubated in MLR with irradiated BALB/c splenocytes. IFNγ ELISPOT assay was then performed, as described above.

References

