IL-10/TGF-β-Modified Macrophages Induce Regulatory T Cells and Protect against Adriamycin Nephrosis

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ABSTRACT

IL-10/TGF-β-modified macrophages, a subset of activated macrophages, produce anti-inflammatory cytokines, suggesting that they may protect against inflammation-mediated injury. Here, macrophages modified ex vivo by IL-10/TGF-β (IL-10/TGF-β M2) significantly attenuated renal inflammation, structural injury, and functional decline in murine adriamycin nephrosis (AN). These cells deactivated effector macrophages and inhibited CD4+ T cell proliferation. IL-10/TGF-β M2 expressed high levels of the regulatory co-stimulatory molecule B7-H4, induced regulatory T cells from CD4+CD25+ T cells in vitro, and increased the number of regulatory T cells in lymph nodes draining the kidneys in AN. The phenotype of IL-10/TGF-β M2 did not switch to that of effector macrophages in the inflamed kidney, and these cells did not promote fibrosis. Taken together, these data demonstrate that IL-10/TGF-β-modified macrophages effectively protect against renal injury in AN and may become part of a therapeutic strategy for chronic inflammatory disease.


Macrophages are a diverse and dynamic population of cells that have the capacity to perform a wide range of critical functions.1,2 They can be classified into two major functional subsets: Classically activated macrophages (M1), which after stimulation by LPS or IFN-γ are defined by antimicrobial and cytotoxic properties, and alternatively activated macrophages (M2), which after incubation with Th2 cytokines such as IL-4 and IL-10 are characterized by anti-inflammatory and regulatory properties. M2 have been further subdivided into three groups: M2a, induced by IL-4 or IL-13; M2b, induced by immune complexes with IL-1 or LPS; and M2c, induced by IL-10, TGF-β, or glucocorticoids.3,4 In most types of human kidney disease, macrophage accumulation correlates closely with the degree of renal structural injury and renal dysfunction.5 Thus, macrophages have been considered to be important mediators of injury in both immune and nonimmune renal disease.6 Depletion of macrophages has been shown to reduce renal injury,7 and adoptive transfer of macrophages has been shown to worsen inflammation in animal models of renal injury.8 Recently, we demonstrated in macrophage transfer studies that marked renal injury can be caused by transfusion of small numbers of M1 macrophages.9 In contrast to M1, the role of M2 in chronic kidney disease is poorly defined. Although M2 macrophages have been studied extensively in regard to...
their membrane molecules, cytokine secretion, suppressive activity, and ability to modulate wound healing and angiogenesis, the role of M2 in vivo and their therapeutic potential have received little attention to date. Previously, we reported the polarization of macrophages to an IL-4/13 macrophage phenotype and their administration as an effective treatment for adriamycin-induced nephropathy in SCID mice, an inflammatory renal disease analogous to human FSGS. That study provided direct proof that IL-4/13 macrophages are able to protect against renal injury, suggesting ex vivo modulation of macrophages into M2 as a potential strategy for treating chronic inflammatory renal disease; however, the effects of macrophages modified by IL-10/TGF-β, another subset of M2, are unknown.

The aim of this study was to examine the effects of IL-10/TGF-β-modified macrophages in immunocompetent mice with adriamycin nephrosis (AN). The potential mechanisms underlying their protective role against renal injury were explored. The phenotypic stability of transfused IL-10/TGF-β macrophages was also examined.

RESULTS

Macrophage Phenotypes

In comparison with M0, M1 macrophages expressed high levels of inducible nitric oxide synthase (iNOS), TNF-α, CD86, B7-H1, and MHC class II (MHC-II). In contrast, both types of M2 macrophage expressed TGF-β, IL-10, mannose receptor (MR), and arginase. There was strong expression of B7-H4 in IL-10/TGF-β-modified macrophages (IL-10/TGF-β M2) but not in IL-4/13-modified macrophages (IL-4/13 M2) or M1. IL-10/TGF-β M2 expressed higher levels of IL-10 and TGF-β than did IL-4/13 M2. Conversely, IL-4/13 M2 expressed CCL17, FIZZ1, and YM1 at higher levels than did IL-10/TGF-β M2 (Figure 1).

Effects of IL-10/TGF-β M2 on Renal Functional and Structural Injury

In mice with AN, serum creatinine and urinary protein were significantly increased compared with that of normal mice and were significantly improved in mice that had AN and received a transfusion of IL-10/TGF-β M2 compared with that of mice that had AN and received a transfusion of M0. Similarly, creatinine clearance was significantly reduced in mice with AN compared with normal mice and was improved in mice that had AN and received a transfusion of IL-10/TGF-β M2 compared with that of mice that had AN and received a transfusion of M0 (Figure 2, A through C). There were no significant differences in renal structural and functional injury between mice that had AN and received a transfusion of M0 and untransfused mice with AN.

In AN, renal injury was characterized by glomerulosclerosis, tubular atrophy, and interstitial fibrosis. Glomerulosclerosis, interstitial volume, and interstitial fibrosis each were significantly reduced in mice that had AN and received a transfusion of IL-10/TGF-β M2 compared with mice that had AN and received a transfusion of M0. Tubular cell height was significantly improved in mice that had AN and received a transfusion of IL-10/TGF-β M2 compared with mice that had AN and received a transfusion of M0 (Figure 2, D through I).

Figure 1. Macrophages were polarized into M0, M1, IL-4/13 macrophages (IL-4/13 M2), and IL-10/TGF-β macrophages (IL-10/TGF-β M2), as described in the Concise Methods section. (A) MHC-I and CD86 were assessed by FACS. (B) The mRNA expression of IL-10, TGF-β, TNF-α, iNOS, arginase, MR, CCL17, YM1, and FIZZ1 was measured by quantitative PCR, relative to the control of each experiment. (C) B7 family members were examined using FACS. Data are means ± SEM of four experiments. *P < 0.05, **P < 0.01 versus M0; #P < 0.05 versus IL-4/13 M2.
expression of MR and arginase significantly but did not change M1 expression of IL-10 and TGF-β (Figure 3A). In comparison with mice that had AN and received a transfusion of M0, the expression of iNOS and TNF-α by endogenous renal macrophages (EM), which include both resident and infiltrating macrophages, in mice that had AN and received a transfusion of IL-10/TGF-β M2 was significantly reduced at day 28. Interestingly, the level of arginase in EM was increased, but that of TGF-β was reduced significantly in mice that had AN and received a transfusion of IL-10/TGF-β M2. The expression of IL-10 by EM in mice that had AN and received a transfusion of IL-10/TGF-β M2 was significantly increased (Figure 3, B and C).

**IL-10/TGF-β M2 Suppression of T Cell Proliferation**  
IL-10/TGF-β M2 significantly suppressed T cell proliferation in comparison with M0. In contrast, M1 significantly increased T cell proliferation in comparison with M0 (Figure 4, A through C). To study the effects of IL-10, TGF-β and B7-H4 induced by IL-10/TGF-β M2 on suppression of T cell proliferation, we added neutralizing antibodies against IL-10, TGF-β, B7-H4, or a mixture of the three antibodies to the co-culture of T cells and M2. Suppression of CD4+ T cell proliferation by IL-10/TGF-β M2 was blocked partially and independently by IL-10, TGF-β, and B7-H4 antibody. Combined blockade using anti–IL-10, –TGF-β, and –B7-H4 diminished completely the suppressive effects of IL-10/TGF-β M2 on CD4+ T cell proliferation (Figure 4D).

**Induction of Regulatory T Cells**  
IL-10/TGF-β M2 increased significantly the percentage of Foxp3+ T cells in co-culture with CD4+CD25+ T cells for 7 days (from 2 to 13%; \( P < 0.01 \)). In contrast, neither M0 nor M1 induced the transformation of CD4+CD25+ T cells into regulatory T cells (Tregs). Only blockade of B7-H4 partially eliminated induction of Tregs by IL-10/TGF-β M2, in contrast to IL-10 and TGF-β blockade, which had no effect. Transwell studies demonstrated that induction of Tregs by IL-10/TGF-β M2 requires cell-to-cell contact (Figure 5, A through D). Tregs induced by IL-10/TGF-β M2 exhibited suppressive effects on CD4+CD25+ T cell proliferation (data not shown).

The number of Foxp3+ cells was significantly increased in renal draining lymph nodes (RDLN) from mice that had AN and were treated with IL-10/TGF-β M2 compared with those that were treated with M0 (17.8 ± 4.3 versus 8.7 ± 2.6%;
There were no differences in the percentage of Foxp3 cells in PBMC between the groups (Figure 5E). Immunohistochemistry confirmed that Foxp3 T cells were significantly increased in RDLN in mice that had AN and were treated with IL-10/TGF-β M2 as compared with those that were treated with M0 (Figure 5F). The number of Foxp3 T cells in kidneys was very low, and there was no observable difference between mice that had AN alone and mice that had AN and received a transfusion of IL-10/TGF-β M2.

**Tracking of Macrophages**

At day 28, many fluorescently labeled cells were seen in kidney and RDLN of mice that had AN and received a transfusion of IL-10/TGF-β M2 but many fewer in mice that received a transfusion of M0 (Figure 6A). Transfused IL-10/TGF-β M2 were located mostly in interstitium. There were few in glomeruli. Their distribution was diffuse in the interstitium but also condensed in and around areas of injury (Figure 6B).

**Phenotypic Changes during the Course of AN**

Phenotypes of transfused IL-10/TGF-β M2 were examined by FACS sorting and quantitative PCR (Figure 7A). The mRNA expression of TGF-β and arginase in IL-10/TGF-β M2 gradually diminished, whereas the levels of IL-10 did not change. MR and B7-H4 expression in IL-10/TGF-β M2 was significantly reduced at week 1 and maintained at that level until week 3. In contrast, expression of TNF-α and iNOS mRNA was increased at week 1 and week 2 but returned at week 3 close to the level of fresh IL-10/TGF-β M2. Correspondingly, immunofluorescence staining for iNOS in IL-10/TGF-β M2 was increased at week 1 and week 2 but diminished at week 3 (Figure 7C); however, MR and B7-H4 staining in IL-10/TGF-β M2 was unchanged from week 1 to week 3. Many transfused IL-10/TGF-β M2 co-stained positively for iNOS, MR, and B7-H4 at week 1 and week 2 (Figure 7B).

**In Vivo Proliferation**

The proliferation of transfused macrophages was measured by flow cytometry. There was no proliferation of IL-10/TGF-β M2 throughout the disease course (Supplemental Figure S1).

**DISCUSSION**

In this study, we examined the effects of transfusion of alternatively activated macrophages in a murine model of chronic kidney disease, AN. We showed that IL-10/TGF-β–modified macrophages significantly reduced inflammatory infiltrates and renal structural and functional injury in mice with AN. The mechanisms underlying the protective effect of IL-10/TGF-β–modified macrophages against renal injury were related to their ability to deactivate endogenous renal macrophages and inhibit CD4 T cell proliferation. Of particular interest, IL-10/TGF-β–modified macrophages were able to induce Tregs from CD4 T cell proliferation. In contrast, IL-10/TGF-β–modified macrophages drifted with time but did not switch to M1 in inflamed kidney or induce profibrotic effects. These data demonstrate that macrophages polarized ex vivo with IL-10/TGF-β might be a useful therapeutic strategy for chronic renal inflammatory disease.
Interestingly, IL-10/TGF-β–modified macrophages were found to express high levels of B7-H4, whereas IL-4/13-modified macrophages did not. B7-H4 is a recently discovered inhibitory molecule of the B7 family.11,12 It has been reported that B7-H4 is expressed by tumor-associated macrophages in human ovarian carcinoma.13,14 It has also been shown that Tregs can convey suppressive activity to macrophages by stimulating B7-H4 expression through IL-10.15 Antigen-specific T cell responses are impaired in mice treated with a B7-H4 Ig fusion protein.11 Although mouse B7-H4 ligation of T cells has an inhibitory effect on T cell activation, the regulatory mechanisms of B7-H4 remain to be defined. A striking finding in our study was that IL-10/TGF-β–modified macrophages could convey suppressive activity to T cells via B7-H4, resulting in induction of Tregs. Thus, this study has identified a specific functional marker of IL-10/TGF-β–modified macrophages. Our study thus reveals two novel observations about B7-H4 and IL-10/TGF-β–modified macrophages: (1) IL-10/TGF-β–modified macrophages can be distinguished from IL-4/13-modified macrophages by their high level expression of B7-H4; and (2) B7-H4 on IL-10/TGF-β–modified macrophages not only suppresses T cell proliferation but also can induce Tregs in vitro and in vivo.

We found that IL-10/TGF-β–modified macrophages were able to deactivate proinflammatory macrophages (M1 phenotype). This ability of IL-10/TGF-β–modified macrophages was shown in vitro co-culture with M1 and further demonstrated in vivo in AN, where endogenous renal macrophages were deactivated. This is the first demonstration that deactivation of inflammatory macrophages in kidney by IL-10/TGF-β–modified macrophages could explain their ability to protect against renal injury.

A major consideration in evaluating M2 as a therapeutic approach is the possibility of phenotype switch in vivo. Numerous studies have examined the stability and longevity of activated macrophages within the host.16 Several studies have indicated that the phenotype of macrophages can change in vivo over time17,18; for example, it was reported that macrophages in the earliest stages of cancer resembled classically activated macrophages, yet, with tumor growth, macrophages developed a regulatory phenotype.19,20 Another example is the observation that macrophages in adipose tissue of nonobese humans have a wound-healing phenotype yet can switch to a proinflammatory phenotype in obese individuals.21,22 It is not clear whether phenotypic deviation can occur in transfused anti-inflammatory macrophages used to treat various diseases. This is very important, because transfused macrophages may do more harm than good if they switch from an anti-inflammatory to a proinflammatory phenotype. In this study, we examined transfused macrophages during the course of disease in an immunocompetent model of AN; the phenotype of transfused IL-10/TGF-β–modified macrophages did change but not toward a distinct M1 phenotype.23 The suppressive features of transfused IL-10/TGF-β–modified macrophages were partially lost, as shown by their reduced expression of anti-inflammatory genes such as TGF-β, arginase, and MR. The expression of proinflammatory genes by IL-10/TGF-β–modified macrophages was elevated at week 1 and week 2 but diminished at week 3. These observations indicate that transfused IL-10/TGF-β–modified macrophages do not convert to an M1 phenotype and that their phenotype varies across stages of disease.

Another potential concern in using IL-10/TGF-β–modified macrophages as a therapeutic approach is the possibility of phenotype switch in vivo. Numerous studies have examined the stability and longevity of activated macrophages within the host.16 Several studies have indicated that the phenotype of macrophages can change in vivo over time17,18; for example, it was reported that macrophages in the earliest stages of cancer resembled classically activated macrophages, yet, with tumor growth, macrophages developed a regulatory phenotype.19,20 Another example is the observation that macrophages in adipose tissue of nonobese humans have a wound-healing phenotype yet can switch to a proinflammatory phenotype in obese individuals.21,22 It is not clear whether phenotypic deviation can occur in transfused anti-inflammatory macrophages used to treat various diseases. This is very important, because transfused macrophages may do more harm than good if they switch from an anti-inflammatory to a proinflammatory phenotype. In this study, we examined transfused macrophages during the course of disease in an immunocompetent model of AN; the phenotype of transfused IL-10/TGF-β–modified macrophages did change but not toward a distinct M1 phenotype.23 The suppressive features of transfused IL-10/TGF-β–modified macrophages were partially lost, as shown by their reduced expression of anti-inflammatory genes such as TGF-β, arginase, and MR. The expression of proinflammatory genes by IL-10/TGF-β–modified macrophages was elevated at week 1 and week 2 but diminished at week 3. These observations indicate that transfused IL-10/TGF-β–modified macrophages do not convert to an M1 phenotype and that their phenotype varies across stages of disease.
TGF-β secreted by M2 also suppress immune responses, dampen inflammation, and promote tissue remodeling in early stages of disease and thereby could further reduce fibrosis. Another possible explanation arising from our study is that transfused M2, after executing their anti-inflammatory effects, decrease their secretion of TGF-β. TGF-β expression by transfused IL-10/TGF-β–modified macrophages in inflamed kidney did reduce progressively from week 1, toward levels seen in that of resting macrophages; however, IL-10 expression of transfused IL-10/TGF-β–modified macrophages remained at high levels throughout the observed course of disease. These results suggest that transfused M2 may have a greater ability to limit inflammation and a reduced profibrotic effect as the disease progresses.

In vivo proliferation is another potential concern in the therapeutic use of transfused M2. It has been reported that fetal macrophages have a high proliferative capacity, which declines once permanent hematopoiesis is established. It is unknown whether M2 macrophages are able to proliferate or whether proliferating M2 would maintain their M2 phenotype at sites of inflammation. We demonstrated that IL-10/TGF-β–modified macrophages separated from spleen do not proliferate in vitro culture for up to 3 weeks; neither do transfused IL-10/TGF-β–modified macrophages separated from kidneys of mice with AN.

In conclusion, we present evidence that IL-10/TGF-β–modified macrophages can protect against renal structural and functional injury in AN. The mechanisms underlying the protective effect of IL-10/TGF-β–modified macrophages on renal injury in AN include their B7-H4–dependent ability to transform naive T cells into Tregs. The polarization of macrophages toward an M2 phenotype with IL-10/TGF-β ex vivo may provide a novel and effective therapeutic approach for chronic renal inflammatory disease.

**CONCISE METHODS**

**Murine AN Model**

Six- to 8-wk-old male BALB/c mice obtained from the Animal Resources Centre (Perth, Australia) were used in this study. The Animal Ethics Committee of Westmead Hospital approved all procedures.
**Macrophage Isolation and Polarization**

BALB/c mouse splenocytes were harvested and washed in ice-cold RPMI 1640 medium. Tissue was triturated with sterile syringes, and the resulting cell suspension was filtered through 40-μm nylon mesh and purified by MACS CD11b+ MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). For further purification of CD11b+ macrophages, CD11b+ cells were incubated at 37°C for 40 minutes, and then the culture supernatant that contained floating cells (e.g., T cells, NK cells, dendritic cells) was discarded. The adherent spleen-derived macrophages were rinsed three times in RPMI 1640 medium for assessment of purity or further processed to become M0, M1, or M2. The adherent cells expressed 96% of CD11b+ (macrophage marker), 1.45 ± 0.42% of CD11c+ (dendritic marker), 0.82 ± 0.25% of CD49b+ (NK marker), and 0.38 ± 0.15% of CD19+ (B cell marker), similar to previous descriptions of such spleen-derived cells.10 Macrophages were cultured for 48 hours with the normal medium to become M0, with LPS (100 ng/ml) and IFN-γ (100 U/ml) to become M1, with IL-4/13 (10 ng/ml each) to become IL-10/TGF-β macrophages. LPS was purchased from Sigma, and IFN-γ, IL-4, IL-13, IL-10, and TGF-β were purchased from Invitrogen.

**Flow Cytometry Analysis**

For analysis of purity of isolated macrophages, cells were stained with FITC-conjugated anti-mouse CD11b, CD49b, and CD11c and PE-conjugated anti-mouse CD19. For analysis of macrophage surface antigen expression, PE-Cy5 anti-mouse CD86 and PE-conjugated anti-mouse MHC-II, B7-H1, B7-H2, B7-H3, and B7-H4 (eBioscience) were used. For intracellular staining of Foxp3, T cells were fixed and stained with PE-Cy5–conjugated anti-mouse Foxp3 (eBioscience). Control antibodies were included in the assays: Rat IgG2a for antibodies to CD19, B7-H1, B7-H2, and Foxp3; rat IgG2b for antibodies to CD11b, MHC-II, and B7-H4; and Armenian hamster IgG for antibody to CD11c. Flow cytometry was performed using a FACS Calibur cytometer (BD Biosciences). Percentage of positive cells was analyzed using Cellquest software (BD Biosciences) in comparison with fluorescence-labeled isotype controls.

**Co-culture Experiments**

For proliferation assays, CD4+ T cells were isolated from splenocytes and labeled with CFSE (Invitrogen). Cells (1 × 10⁵ per well) were added into 96-well plates in the presence of predherent M0, M1, or IL-10/TGF-β M2 and stimulated by anti-mouse CD3/CD28 (100 and 200 ng/ml) for 48 or 72 hours. Cell proliferation was examined with WST-1 and CFSE (Invitrogen). For some experiments, IL-10–, TGF-β–, and B7-H4–neutralizing antibodies (10 μg/ml each) were added in co-culture.

For Treg induction experiments, CD4+CD25– T cells were co-cultured with M0, M1, or IL-10/TGF-β M2 for 7 days. For some experiments, CD4+CD25– T cells and IL-10/TGF-β M2 were co-cultured in 24-well plates or placed separately in Transwell chambers in the presence or absence of IL-10–, TGF-β–, or B7-H4–neutralizing antibodies (10 μg/ml each) for 7 days. After incubation, CD4+ T cells were stained with Foxp3 antibody and analyzed by FACS.

**IL-10/TGF-β M2 Co-cultured with M1**

Co-culture of two monolayers of cells was as described previously.31 Splenic macrophages were grown on 13-mm-diameter plastic coverslips (Nunc, Australia) with various cytokines to become M0, or IL-10/TGF-β M2. Macrophages also were seeded onto 24-well plates and further processed to become M1. The coverslips seeded with monolayers of IL-10/TGF-β M2 were placed over confluent monolayers of M1 and co-cultured for 24 hours. After incubation, M1 were examined.
Macrophage Labeling and Adoptive Transfer to BALB/c Mice

For in vivo study, macrophages of various phenotypes were labeled with 1,1’di-octadecyl-3,3’,3’’,3’’’-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen).32 DiI has been used extensively as a long-term tracer for neuronal and other cells. Cells (1 × 10⁶) were transfected into treated BALB/c mice by a single tail-vein injection at day 5 after adriamycin administration. Mice were divided into three groups (n = 7 per group): AN + saline, AN + M0, and AN + IL-10/TGF-β M2. Mice were killed on day 28 after adriamycin administration. Kidney, spleen, and lymph node were examined. For examination of the phenotype changes of transfused macrophages, kidneys were collected at week 1, week 2, and week 3 after macrophage transfusion. Transfused macrophages were examined by real-time PCR and immunofluorescence staining.

Examination of Transfused and Endogenous Cells

Kidneys were perfused before removal. Mononuclear cells from kidney were separated using a step-gradient sucrose separation procedure and stained with FITC-conjugated anti-mouse CD11b. Transfused macrophages (CD11b⁺DiI⁻) and endogenous renal macrophages (CD11b⁺DiI⁺) were sorted by FACS. Sorted cells were used for real-time PCR or FACS analysis to detect phenotype changes of these macrophages.

A single-cell suspension from RDLN was used for FACS analysis.

Renal Function

All urine and blood specimens were examined by the Institute of Clinical Pathology and Medical Research (Westmead Hospital). Creatinine clearance (µl/min) = (urine creatinine/serum creatinine) × urine volume (µl)/[time (hours) × 60]

Histology and Immunohistochemistry

Coronal sections of renal tissue were stained with periodic acid-Schiff (PAS) or Masson trichrome. Glomerulosclerosis, tubular atrophy, interstitial volume, and interstitial fibrosis were evaluated using methods described previously.33 Briefly, images viewed under a light microscope were digitalized using a video camera and then transferred onto computer screen (Tang Computer, Sydney, Australia) using image analysis software (Optimas 5; Media Cybernetics, Seattle, WA). The outline of the glomerular capillary tuft was traced, and the computed area was used as a measure of total glomerular area. The area covered by PAS-positive staining in the same glomerulus was then determined.

The percentage of glomerulosclerosis for each glomerulus was calculated by dividing the total PAS-positive area by the total glomerular area. The mean value of 20 randomly selected glomeruli was determined for each section. The degree of interstitial expansion was determined by quantification of the relative interstitial volume. Random cortical fields were viewed at a magnification of ×200. The percentage of relative interstitial volume was calculated from the area occupied by trichrome-stained interstitium divided by the total area. The mean value of five cortical fields was determined for each section. For avoidance of selection bias, the areas to be viewed for morphometric analysis were anatomically identical for each section and positioned before microscopic visualization. Tubular atrophy was defined by the finding of a tubule with low cell height and absence of brush border.

For immunohistochemical staining, rat anti-mouse F4/80, CD4, CD8, or Foxp3 (eBiosciences) was used as the primary antibody and biotinylated rabbit anti-rat Ig as the secondary antibody. Control rat IgG to primary antibodies was included in staining. Standard immunohistochemical procedures were used.34 The number of interstitial F4/80⁺, CD4⁺, CD8⁺, and Foxp3⁺ cells was quantified in 10 nonoverlapping cortical fields (×400, measuring 0.075 mm² each).

Immunofluorescence Staining for iNOS, MR, and B7-H4

Consecutive sections were stained with rabbit anti-iNOS (1:100; BD Biosciences Pharmingen), rat anti-MR (1:50; BioLegend), or goat anti-
B7-H4 (1:50; R&D Systems) and then incubated with AF488 goat anti-rabbit IgG (1:500), AF488 goat anti-rat IgG (1:800), or AF488 donkey anti-goat IgG (1:500; Invitrogen), respectively. Isotype control IgGs to these rat, rabbit, and goat antibodies were included in immunofluorescence staining. Tissue sections were analyzed by inverted fluorescence microscopy. 10

Statistical Analysis
Renal functional data (serum creatinine, creatinine clearance, and urine protein) were log-transformed before analysis to stabilize the variance. Statistical tests included unpaired, two-tailed t test using Welch correction for unequal variances and one-way ANOVA with Tukey multiple comparison test. Statistical analyses were done using Prism 4 (GraphPad). Results are expressed as the mean ± SEM. P < 0.05 was considered statistically significant.

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DISCLOSURES
None.

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