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Conditional Ablation of Macrophages Halts Progression of Crescentic Glomerulonephritis

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The presence of macrophages in inflamed glomeruli of rat kidney correlates with proliferation and apoptosis of resident glomerular mesangial cells. We assessed the contribution of inflammatory macrophages to progressive renal injury in murine crescentic glomerulonephritis (GN). Using a novel transgenic mouse (CD11b-DTR) in which tissue macrophages can be specifically and selectively ablated by minute injections of diphtheria toxin, we depleted renal inflammatory macrophages through days 15 and 20 of progressive crescentic GN. Macrophage depletion reduced the number of glomerular crescents, improved renal function, and reduced proteinuria. Morphometric analysis of renal tubules and interstitium revealed a marked attenuation of tubular injury that was associated with reduced proliferation and apoptosis of tubular cells. The population of interstitial myofibroblasts decreased after macrophage depletion and interstitial fibrosis also decreased. In the presence of macrophages, interstitial myofibroblasts exhibited increased levels of both proliferation and apoptosis, suggesting that macrophages act to support a population of renal myofibroblasts in a high turnover state and in matrix deposition. Finally, deletion of macrophages reduced CD4 T cells in the diseased kidney. This study demonstrates that macrophages are key effectors of disease progression in crescentic GN, acting to regulate parenchymal cell populations by modulating both cell proliferation and apoptosis. ***(Am J Pathol 2005, 167:1207–1219)***

The biology of macrophages in different tissue settings has been of broad scientific interest, at least in part because of the pleiotropic functions ascribed to macrophages. We have previously demonstrated that during development of the rodent eye, macrophages are responsible for deletion of capillary endothelial cells through induction of endothelial cell apoptosis.[1–3] This function of macrophages has been recapitulated in *ex vivo* modeling of inflammation in which activated inflammatory macrophages have been found to induce apoptosis of glomerular mesangial cells of the kidney.[4,5] In other model systems of inflammation, macrophages have the capacity to induce apoptotic cell death of epithelial cells during inflammation.[6–8] In fact, although macrophages are characterized by their capacity to phagocytose particles, phagocytes in the developing worm and fly also play a crucial role in active deletion of effete cells, in addition to phagocytic clearance, indicating that macrophage-directed killing of cells is common to all metazoans.[9–12] Rather confusingly, although macrophages have the capacity to induce apoptosis of parenchymal cells in inflammation and organ development, model systems of macrophages also indicate that they may induce proliferation of parenchymal cells.[6,13] Indeed, we have previously shown that the presence of macrophages in the inflamed glomerulus of the rat kidney is correlated with both proliferation and apoptosis of resident glomerular mesangial cells.[6,14]

When macrophages are cultured with fibroblasts/myofibroblasts, the net result is either deposition of matrix components, or lytic degradation of matrix, depending on the activation state of the macrophage.[15,16] These data suggest macrophages also play a role in the deposition of fibrous tissue, which so frequently heralds inflammatory scarring and organ

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dysfunction. In addition when activated macrophages are cultured with mesangial cells from the kidney, a cell with myofibroblast characteristics, the macrophages exhibit a cell-cycle-dependent killing by apoptosis of the mesangial cells, thereby showing the capacity to regulate survival of neighboring, scar-producing myofibroblast cells.[4] Further evidence for a role for inflammatory macrophages in the development of scarring can be seen in skin wounding because wounds undergoing repair occurring in the absence of macrophages exhibit no scarring.[17]

Macrophages have been studied previously using various methods to effect depletion including mustine hydrochloride, anti-macrophage serum, and more recently liposomal clodronate.[18–21] Many early studies pointed to macrophage involvement in disease initiation whereas few studied macrophages in disease progression.[22] Furthermore, many methods for depletion were not macrophage-specific, were highly toxic, were ineffective in some tissues or simultaneously depleted neutrophils, thereby sometimes rendering interpretation of the results difficult.[23]

We recently generated a transgenic mouse (CD11b-DTR) in which macrophages specifically express the human diphtheria toxin receptor (DTR) such that macrophages can be specifically ablated by a simple intravenous injection of diphtheria toxin (DT).[24,25] Mice are normally resistant to DT as the murine DTR binds DT poorly. Using this powerful new tool we have shown that inflammatory macrophages can be specifically depleted from solid organs and have begun to examine their biological effects *in vivo*.[24]

To understand the functions of macrophages in inflammation further, we ablated macrophages during a well-described model of progressive inflammatory injury in the kidney. The single injection of nephrotoxic serum into mice induces a crescentic glomerulonephritis (GN) that progresses throughout 4 weeks to organ failure and is analogous to human crescentic GN. It is characterized by inflammatory disease of the renal glomerulus resulting in characteristic crescents together with marked inflammatory macrophage infiltration. As disease progresses, inflammatory destruction of the whole nephron, interstitial scarring, organ failure, and death ensues. Although the importance of macrophages in disease initiation has been previously studied in this model,[26] the function of macrophages in progression of established disease remains unexplored. Furthermore, for the first time our transgenic mouse model allows highly selective ablation of macrophages.

Here we demonstrate macrophages are necessary for disease progression. Detailed tissue analysis indicates that inflammatory macrophages in the kidney induce both cell death and proliferation of tubular epithelial cell and interstitial myofibroblast populations. The outcome of persistent macrophage infiltration is progressive injury and loss of epithelial cells, and through maintenance of the population of interstitial myofibroblasts, the development of interstitial fibrosis.

Materials and Methods

Animals

CD11b-DTR mice were generated and characterized as previously described.[24,25] These mice are transgenic for the human diphtheria toxin (DT) receptor (DTR) under the control of the CD11b promoter. The murine DT receptor binds DT poorly such that expression of the human receptor confers toxin sensitivity. Administration of minute (ng) doses of DT results in rapid and marked macrophage ablation in the peritoneum, kidney, and ovary and does not affect murine neutrophil populations.[24,25] Strain match controls (FVB/N) were purchased from B and K Ltd., Hull, UK. Animals were maintained in a standard barrier facility and had free access to chow and water. All experiments were performed in accordance with Home Office (UK) guidelines.

Disease Model

Nephrotoxic serum was prepared by serial inoculation of sheep with crude insoluble protein fraction of mouse cortex as previously described.[26] In preliminary studies 10- to 14-week-old, 28 to 30 g, CD11b-DTR male mice were injected intravenously with 200 to 600 μl of nephrotoxic serum, divided in two doses 2 hours apart, to assess disease severity and progression. The 400-μl dose of nephrotoxic serum induced autologous proteinuric nephritis in all animals with ~40% glomerular crescents at 21 days. This regimen was selected for further study. Disease was induced in cohorts of 10- to 14-week-old male CD11b-DTR mice and dipstick proteinuria carefully monitored. At 15 days all mice with 3+ to 4+ proteinuria but without ascites were randomly assigned to macrophage depletion (DT) or control [phosphate-buffered saline (PBS)] injections (n = 11 per group). DT (10 ng/g body weight) or an equal volume of PBS was injected intravenously on days 15, 17, and 19 with tissue being harvested 24 hours later on day 20. To be sure that the groups were comparable we retrospectively assessed urine collected at 15 days in more detail. At day 15 the group assigned to DT had 145 ± 33 mg/L proteinuria with a Uprot/Cr ratio of 5.2 ± 1.1. The group assigned to PBS treatment had 114 ± 53 mg/L proteinuria with a Uprot/Cr of 3.2 ± 0.9. There were no statistical differences between the two groups. A small group of mice (n = 3) from each cohort had tissue harvested on day 15 before macrophage depletion while a further cohort (n = 3) of nephritic FVB/N wild-type mice completed the DT macrophage depletion regimen in parallel with CD11b-DTR mice. Two hours before harvesting of organs, bromodeoxyuridine (BrdU) (Boehringer Mannheim) (50 μg/g) was injected intraperitoneally.

Assessment of Disease

Urine was collected on day 15 and day 20, and protein and creatinine levels determined by the Bradford assay and picric acid method (Beckman Analyzer II), respec-

tively. Blood was collected (day 20) by cardiac puncture and creatinine concentration determined. Kidneys and spleen were harvested at day 20 and tissue was fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% glacial acetic acid), 10% neutral buffered formalin, or 2% paraformaldehyde, lysine, periodate (PLP) fixative.[26] The number of glomerular crescents, glomerular thrombonecrotic lesions, and tubulointerstitial disease was determined by blinded analysis of periodic acid-Schiff-stained formalin-fixed 3-μm sections using established scoring methods.[26] A semiquantitative tubular atrophy score was adapted from assessment of human biopsies:[27] 0, absence of atrophy; 1+, atrophy in 25% of biopsy; 2+, involving 25 to 50% of biopsy; and 3+, involvement of >50% of the biopsy. In addition, ~10 serial cortical images (magnification ×200) were digitally captured covering the whole cortex. The area of cortex occupied by tubular luminal space, a marker of tubulointerstitial disease,[28,29] was measured by quantitative morphometric analysis using image analysis software (Fovea Pro, Reindeer Graphics).[24,28] PLP-fixed, cryotome 5-μm sections were immunostained for macrophages using anti-CD68 antibody (Serotec, Oxford, UK) as described previously.[30] CD68 was selected over other macrophage markers because not all inflammatory macrophages in the murine glomerulus express other macrophage-specific markers such as F4/80.[30] The area of cortex occupied by macrophages was assessed by morphometric analysis of serially captured images (×200) of the whole cortex. In addition, the number of intraglomerular macrophages was counted in 50 sequentially observed glomeruli.[30] Sections were labeled with anti-CD4 or anti-CD8 antibodies (1:200) (eBioscience, San Diego, CA) in PBS containing rabbit and mouse serum. Specific labeling was detected using biotinylated anti-rat IgG and the ABC peroxidase system followed by incubation of sections with diaminobenzidine (DAKO, Carpinteria, CA). T cells were assessed in glomeruli as described for CD68-positive cells. Interstitial T cells were assessed by counting the number per high-power field (HPF). At least 50 HPFs were assessed serially covering the whole cortex in sagittal kidney sections, and data presented as T cells/HPF. Collagen III and α-smooth muscle actin (α-SMA) immunostaining was performed on methyl Carnoy's-fixed sections as previously described.[31] The area of collagen III deposition and α-SMA staining in tissue sections was assessed using the morphometric methods described above. Area of stain was expressed as a proportion of total cortical area. The number of α-SMA cells in glomerular crescents of 50 consecutive glomeruli was assessed (observer blinded) using the following scoring method: 0, no cells; 1, occasional periglomerular cells; 2, many but discontinuous cells; 3, a continuous layer of positive cells surrounding the glomerulus; 4, in addition to 3, multiple layers of positive cells.

Formalin-fixed sections were stained for BrdU incorporation using established antigen retrieval methods[24] and anti-BrdU antibody (Serotec). In addition, sections were stained for proliferating cell nuclear antigen (PCNA), another marker of proliferation, using anti-PCNA antibody (DAKO) as previously described.[32] To assess apoptosis, formalin-fixed sections were prepared for the terminal dUTP nick-end labeling (TUNEL) reaction and immunostained with diaminobenzidine using preprepared components (Oncor, Gaithersburg, MD).[14] BrdU- and PCNA-positive cells were counted within the glomeruli (50 per mouse), crescents (50 per mouse), tubules and interstitium (50 HPF per mouse). TUNEL-positive cells were counted similarly, but in addition to positive nuclear stain, the nucleus was required to exhibit morphological characteristics of apoptosis.[14] For quantification of proliferating interstitial macrophages, Methyl Carnoy's-fixed, 3-μm sections were initially immunostained with anti-F4/80 antibodies (Caltag, Burlingame, CA) using peroxidase-catalyzed diaminobenzidine stain.[24] Stained sections were acid-treated to retrieve nuclear antigens, then labeled with fluorescein isothiocyanate-conjugated anti-BrdU antibodies in the presence of Fc-block (Pharmingen, La Jolla, CA). Sections were viewed by bright field and by epifluorescence and combined (fluorescent and bright field) serial cortical images were scored positive if an interstitial nucleus was surrounded by F4/80 staining.

Peripheral blood leukocytes were prepared, immunolabeled, and assessed by flow cytometry as previously described.[25] Leukocytes were immunolabeled for neutrophils (anti-Gr-1-PE; eBioscience), monocytes (anti-CD11b-fluorescein isothiocyanate, eBioscience; and anti-F4/80-APC, Caltag), and T cells (anti-CD3-fluorescein isothiocyanate, Pharmingen). Monocytes were assessed as F4/80-positive or CD11b-positive, Gr-1-negative.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (PCR)

RNA was purified from nephritic kidneys and cDNA prepared as previously described.[24] cDNA was analyzed for content using a SYBR green-based, quantitative fluorescent PCR method (Qiagen, Valencia, CA). Fluorescence was detected with the iCycler system (Bio-Rad, Hercules, CA). The following primer pairs were used for detection of specific cDNAs: GAPDH, 5′-CTGAGAAACCTGCCAAGTA-3′ and 5′-AAGAGTGGGAGTTGCTGTTG-3′; tumor necrosis factor-α, 5′-CGCTCTTCTGTCTACTG AACTT-3′ and 5′-GATGAGAGGGAGGCCATT-3′; interleukin-10, 5′-CAGCCTTGCA GAAAAGAGAG-3′ and 5′-GGAAGTGGGTGCAGTTATTG-3′; transforming growth factor-β_1, 5′-CTCCACCTGCAAGACCAT-3′ and 5′-CTTAGTTTGGACAGGATCTGG-3′; matrix metalloproteinase (MMP)-13, 5′-TAAGGCAGAAATATTATGTAGAA-3′ and 5′-AAAGCAGAGAGGG ATTAACAA-3′; tissue inhibitor of matrix metalloproteinase (TIMP)-1, 5′-TTCCAGTAAGGCCTGTAG C-3′ and 5′-TTATGA CCAGGTCCGAGTT-3′. Each assay was performed in duplicate in a 25-μl reaction volume comprising 2× SYBR green mastermix, 0.5 μl of cDNA with primers included at 300 nmol/L. The following PCR conditions were used: 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds, 50°C for 30 seconds, 72°C for 30 seconds, followed by 72°C for a final 10 minutes. Each PCR reaction was also tested to assure a single product of the pre-

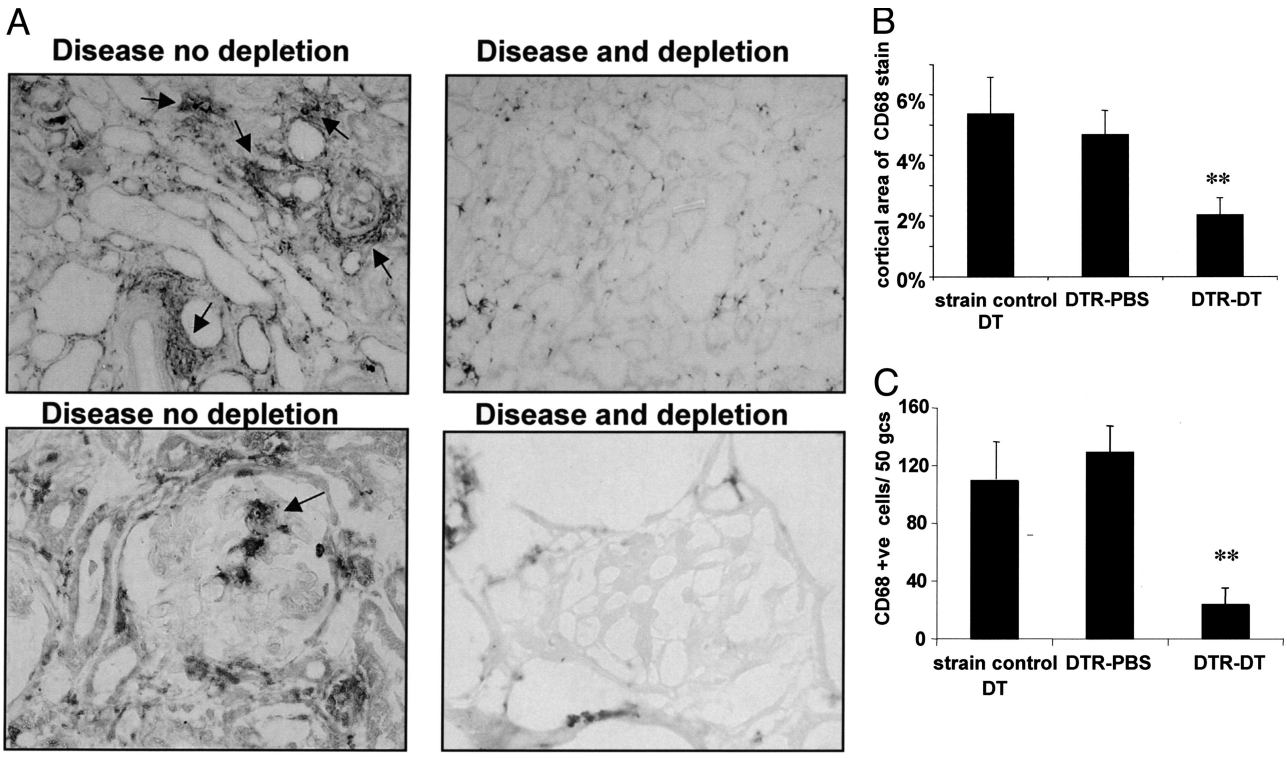

Figure 1. Diphtheria toxin successfully depletes glomerular and interstitial macrophages during nephrotoxic nephritis in the CD11b-DTR mouse. **A:** CD68 immunostaining of tissue sections from mice at 20 days after three doses of diphtheria toxin (DT) commencing at day 15 (**right**) or control treatment (**left**). Note areas of intense macrophage infiltration (**arrows**). Interstitial, crescentic, and intraglomerular macrophages are depleted. **B:** Morphometric analysis of the whole cortical area indicates that DT significantly reduces the area of tissue occupied by macrophages compared with PBS-treated diseased mice. Note, strain-matched, control diseased mice exhibit an interstitial macrophage infiltrate comparable to PBS-treated CD11b-DTR mice (**$P < 0.01$). **C:** The number of intraglomerular macrophages is significantly reduced after DT administration in the CD11b DTR mice compared with PBS-treated diseased mice and strain-matched controls (**$P <$ 0.01).

dicted size. Quantification of each PCR product was expressed relative to GAPDH.

Statistics

Continuous variables are expressed as mean ± SEM. Discontinuous variables as mean ± 95% CI. Differences among groups were assessed by analysis of variance and between groups assessed by Mann Whitney rank-sum test.

Results

Administration of Diphtheria Toxin Induces Conditional Macrophage Ablation in Advanced Crescentic GN

To determine the effects of macrophages on progressive disease we depleted macrophages for a period of 5 days during established crescentic GN between day 15 and day 20. Immunostaining of day 20 renal tissue for macrophages confirmed marked depletion of glomerular macrophages (Figure 1, A and C) and a 50% reduction in whole cortical staining for CD68 (Figure 1, A and B). In addition, the administration of DT to diseased strain-matched control mice had no effect on glomerular or interstitial macrophage infiltration confirming the specificity of macrophage ablation in this model of renal inflammation (Figure 1, B and C). We have previously demonstrated absence of the resident macrophage population in the normal kidneys of DTR mice 24 hours after intraperitoneal administration of DT.[25] Intravenous injection of 10 ng/g of DT into healthy DTR mice lead to a 97.4 ± 2.1% reduction in $CD68^+$ resident kidney macrophages 24 hours after the injection. Similar injection of DT led to an 87.4 ± 14.9% reduction in circulating monocytes (see Table 2 for details). It is therefore likely that the reduction in kidney macrophages during crescentic GN reflects a reduction in both resident and infiltrating populations of cells.

Macrophage Depletion Reduces Histological Injury

Macrophage depletion resulted in a striking and significant reduction in the number of cellular glomerular crescents (Figure 2, A and B). Glomerular crescents are associated with severe glomerular disease and signify poor outcome in many human studies. The proportion of glomeruli exhibiting focal areas of necrosis was also reduced after macrophage depletion but this was not significantly different (Figure 2, A and C). In addition to histological changes in the glomerulus, macrophage depletion resulted in a significant reduction in injury to ep-

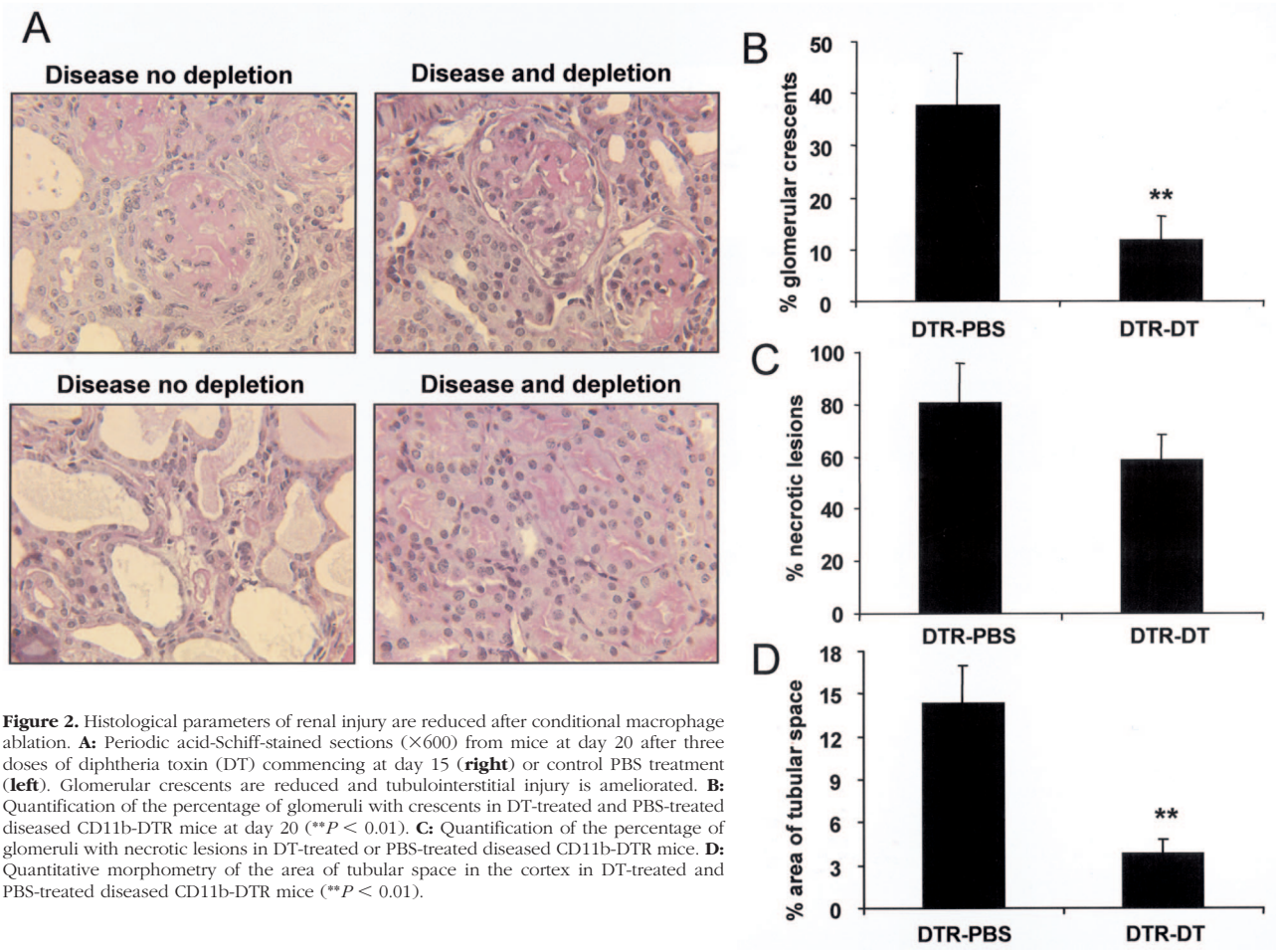

Figure 2. Histological parameters of renal injury are reduced after conditional macrophage ablation. **A:** Periodic acid-Schiff-stained sections (×600) from mice at day 20 after three doses of diphtheria toxin (DT) commencing at day 15 (**right**) or control PBS treatment (**left**). Glomerular crescents are reduced and tubulointerstitial injury is ameliorated. **B:** Quantification of the percentage of glomeruli with crescents in DT-treated and PBS-treated diseased CD11b-DTR mice at day 20 (**$P < 0.01$). **C:** Quantification of the percentage of glomeruli with necrotic lesions in DT-treated or PBS-treated diseased CD11b-DTR mice. **D:** Quantitative morphometry of the area of tubular space in the cortex in DT-treated and PBS-treated diseased CD11b-DTR mice (**$P < 0.01$).

ithelial tubules and reduction in interstitial disease. In macrophage-depleted mice, semiquantitative assessment of tubular injury using a tubular atrophy score (0 to 3) showed tubular atrophy to be reduced (2.4 ± 0.7 versus 1.1 ± 0.6; PBS versus DT treatment).[27] The degree of tubular injury was also assessed by quantifying tubular luminal space using computerized morphometric analysis expressed as a proportion of the area of the whole cortex.[28,31] Morphometric measurement of tubular luminal space, resultant on tubular dilatation and flattening of injured dedifferentiated epithelial cells, is a parameter of tubular injury and has recently been shown to be associated with a poor prognosis in lupus nephritis.[28] Using this automated objective method, this parameter of tubular injury was markedly attenuated in macrophage-depleted mice (Figure 2, A and D) indicating that infiltrating interstitial macrophages were important effectors of progressive tubulointerstitial injury. Importantly, our model of crescentic GN exhibited progressive histological changes during the third week of disease. At day 15 the percentage of glomeruli with crescents was 22 ± 6% (39 ± 10% at 20 days) and the percentage cortical area of tubular luminal space was 9.4 ± 2.9% (14 ± 3% at 20 days). By comparison the percentage of glomeruli with crescents at day 20 in DT-treated mice was 12 ± 6% and the area of tubular luminal space was 4 ± 1% (Figure 2D). The degree of reduction in crescents and tubular injury (as assessed by luminal space) might suggest that not only does macrophage depletion halt histological progression but may lead to reversal of histological disease.

Macrophage Depletion Improves Renal Function

This model of crescentic GN is associated with significant renal failure and plasma protein leak into the urine (proteinuria). Plasma creatinine level, a measure of renal dysfunction, was quantified in healthy mice, diseased mice at 15 days, before administration of DT or PBS, and at 20 days after macrophage depletion (Figure 3A). Compared with healthy mice, plasma creatinine was elevated in diseased mice at 15 days. At 20 days, the creatinine level remained elevated in both control diseased mice and DT-treated diseased mice. The macrophage-depleted diseased mice, however, exhibited less severe renal impairment. In addition, the degree of proteinuria expressed as the protein/Cr ratio was increased in diseased mice at day 15, compared with healthy mice. Although not reaching statistical significance, DT-treated mice at day 20 exhibited a reduced protein/Cr ratio compared with control dis-

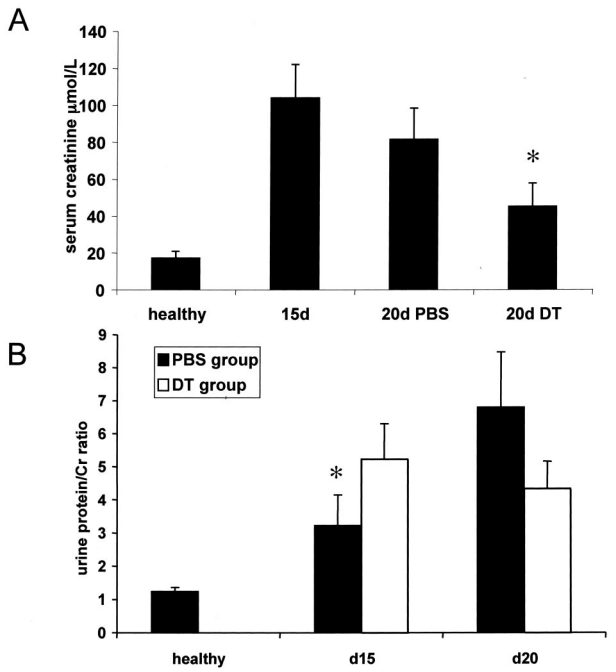

Figure 3. Renal function improves after conditional macrophage ablation. **A:** Plasma creatinine in healthy CD11b-DTR mice, diseased mice at 15 days, and diseased mice at 20 days after PBS treatment or DT treatment. Note significant reduction in creatinine after macrophage depletion ($^*P < 0.05$). **B:** Urinary protein to creatinine ratio in healthy CD11b-DTR mice, diseased mice at 15 days, and diseased mice at 20 days after PBS treatment or DT treatment. Note in the PBS-treated group proteinuria increases significantly from day 15 to day 20 ($^*P < 0.05$) whereas in the DT-treated group proteinuria does not increase.

eased mice in keeping with reduced glomerular injury (Figure 3B). Furthermore, between day 15 and day 20 the protein/Cr ratio increased significantly in the control diseased mice whereas in DT-treated mice the protein/Cr ratio did not rise.

Macrophage Depletion Reduces Interstitial and Crescent Myofibroblasts

We assessed periglomerular (crescent) and interstitial myofibroblasts because these cells are strongly implicated in crescent generation and the development of interstitial fibrosis.[33,34] Sections were immunostained for the cytoskeletal protein α-SMA expressed selectively by these myofibroblasts (Figure 4A) and the area of cortex occupied by α-SMA staining was assessed morphometrically as an indirect measure of the interstitial myofibroblast population. This analysis indicated that the population of interstitial myofibroblasts was significantly reduced after macrophage depletion (Figure 4C). In addition, using blinded, semiquantitative scoring of the periglomerular regions for the presence of myofibroblasts, macrophage depletion during disease resulted in a diminished population of periglomerular myofibroblasts that occupy the crescent (Figure 4B). These results suggest that macrophages are responsible for maintaining a population of myofibroblasts that are believed to be responsible for matrix deposition and fibrosis. To exclude the possibility that the loss of fibroblasts was due to death induced by DT, cultured bone marrow-derived macrophages and cultured embryonic fibroblasts from DTR mice were treated with DT. Cell viability was assessed by the reduction of MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to formazan dye by live cells. After 4 hours of exposure to DT bone marrow-derived macrophages lost viability at a dose of DT of 10 pg/ml whereas loss of viability of fibroblasts occurred at a dose 10^5 pg/ml (data not shown). This indicates that the loss of fibroblasts in the kidneys of DT-treated mice is secondary to macrophage depletion.

Macrophage Depletion Reduces Interstitial Deposition of Collagen III

Collagen III is a representative collagen of the injured and scarred renal interstitium and is deposited by fibroblasts during pathological disease.[35] We stained sections for collagen III and measured deposition of collagen III objectively in the renal cortex using morphometric methods. The area of collagen III in the cortex was significantly reduced after 5 days of macrophage depletion (Figure 5) despite the fact that macrophages do not synthesize this matrix protein. This suggests that macrophages may direct interstitial fibroblasts to synthesize interstitial matrix either by regulating the balance between synthesis and degradation of myofibroblast-generated matrix components, or regulating the size of the myofibroblast population.

Macrophage Depletion Modulates Both Proliferation and Apoptosis within Glomerular Crescents and the Tubulointerstitium

We have previously reported that macrophages may regulate glomerular mesangial cell number by inducing apoptosis and inhibiting mitosis in proliferating cells *in vitro*.[4,5] In addition, similar findings for apoptosis have been reported in co-cultures of macrophages and a proximal tubular cell line.[7] We therefore hypothesized that infiltrating renal macrophages may modulate the proliferation and apoptosis of resident renal cells *in vivo* during renal inflammation. Kidney sections were immunolabeled for markers of both proliferation and apoptosis. Compared with control diseased mice at 20 days, there was a marked reduction in BrdU-positive nuclei in both the glomerulus and the tubulointerstitium (Figure 6). Approximately half of the BrdU-positive tubulointerstitial cells were tubular epithelial cells and half were interstitial cells (Table 1). In both tissue compartments, macrophage depletion resulted in a marked reduction in BrdU positivity. PCNA staining of tissue sections revealed a very similar pattern [128 $\pm$ 8 (no macrophage depletion), 73 $\pm$ 12 (macrophage depletion) in the tubulointerstitium per 50 HPF, and 18 $\pm$ 3 (no macrophage depletion), 10 $\pm$ 2 (macrophage depletion) in the glomerulus per 50 gcs]. A significant correlation was evident between the area of cortical staining for macrophages and the number of BrdU-staining cells in each mouse (Table 1) thereby sup-

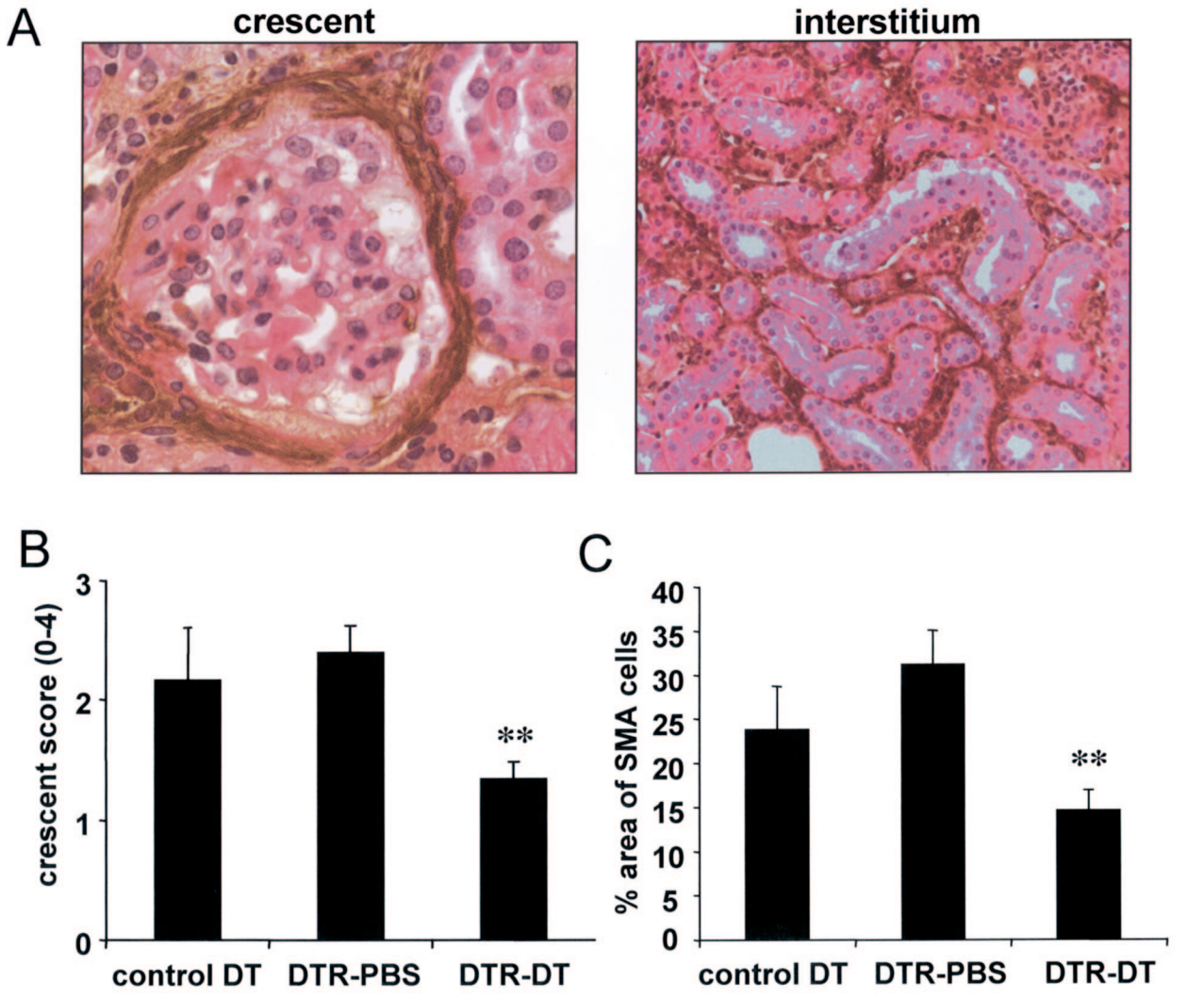

Figure 4. Crescentic and interstitial myofibroblasts are reduced after macrophage ablation. **A:** Immunolabeling of crescentic and interstitial α-SMA, which is restricted to myofibroblasts. **B:** Semiquantitative scoring of crescent fibroblasts. Note a significant reduction in DT-treated CD11b-DTR mice but not in strain matched (**$P < 0.01$). **C:** Quantitative morphometry of α-SMA-expressing myofibroblasts in the cortical interstitium. Note that myofibroblasts are reduced after DT treatment in the CD11b-DTR mice but not in strain matched control (**$P < 0.01$).

porting a link between macrophage infiltration and parenchymal cell proliferation. In addition, macrophage depletion resulted in reduced numbers of BrdU-positive cells in periglomerular areas (Table 1). It has been reported that macrophages in the interstitium of diseased kidneys can themselves proliferate.[36,37] To determine the proportion

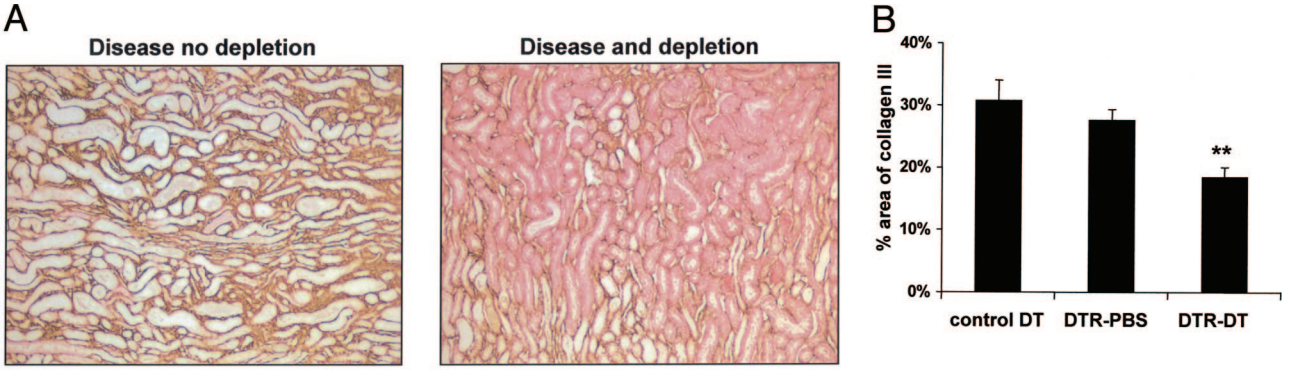

Figure 5. Interstitial collagen III deposition is reduced after macrophage ablation. **A:** Immunostaining (×100) for collagen III showing interstitial deposition in diseased mice, which is reduced after macrophage depletion (**right**). **B:** Quantitative morphometry of percentage area of cortical collagen III deposition in PBS-treated and DT-treated diseased CD11b-DTR mice and also strain-matched control DT-treated mice (**$P < 0.01$).

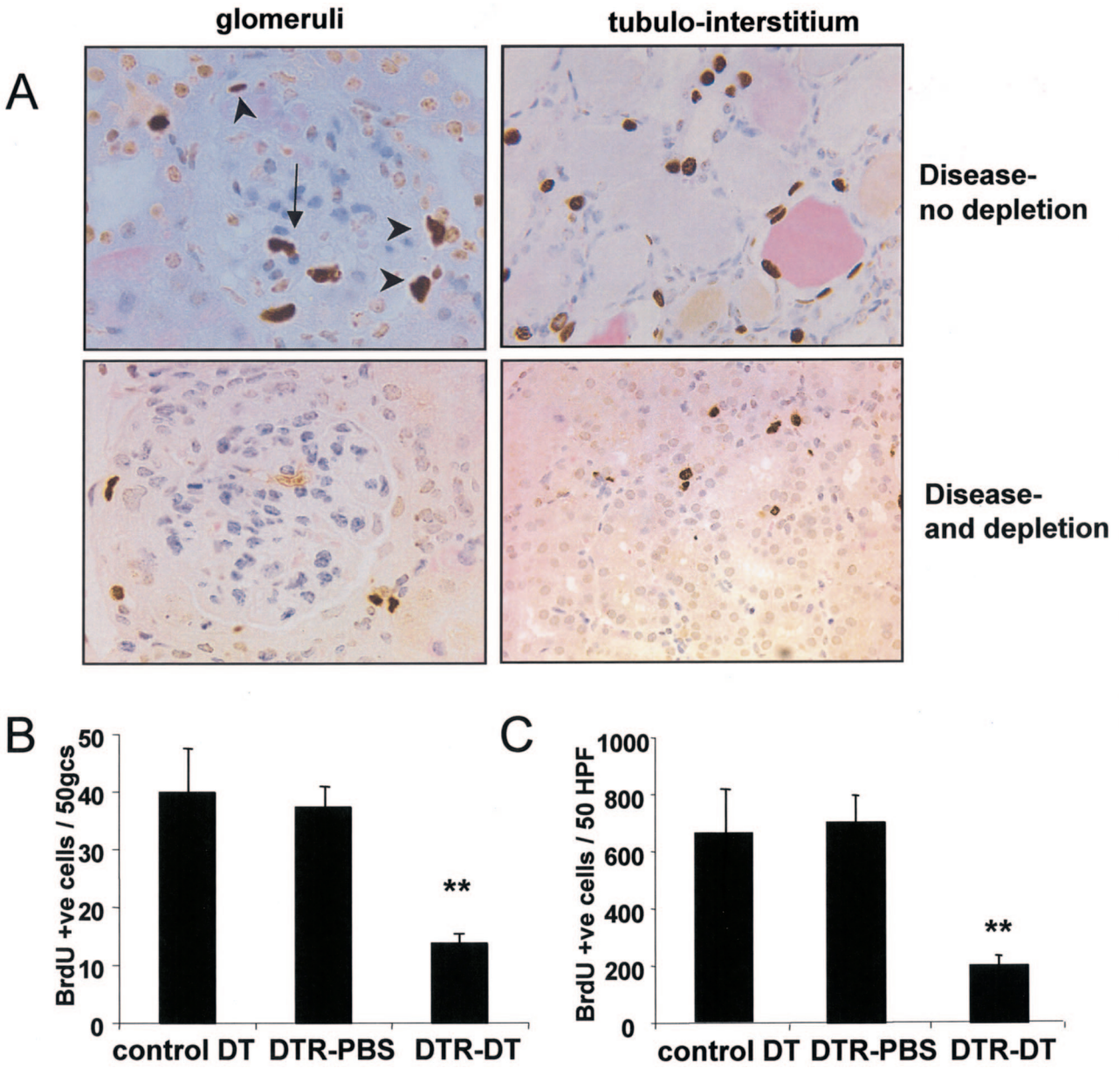

Figure 6. Glomerular and tubulointerstitial proliferation are reduced by macrophage ablation. **A:** Immunostaining (×600) showing BrdU-positive intraglomerular cells (**arrows**), periglomerular cells (**arrowheads**, **top left**), and (×400) BrdU-positive tubular epithelial and interstitial cells (**top right**). A reduction in glomerular (**bottom left**) and tubulointerstitial (**bottom right**) BrdU-positive cells is seen after macrophage depletion. **B:** The number of BrdU-positive cells per 50 glomerular cross sections in PBS-treated and DT-treated diseased CD11b-DTR mice and also strain control DT-treated mice (**$P < 0.01$). **C:** The number of BrdU-positive cells per 50 HPF of whole kidney in PBS-treated and DT-treated diseased CD11b-DTR mice and also strain control DT-treated mice (**$P < 0.01$).

Table 1. BrdU-Positive and TUNEL-Positive Cells per 50 HPFs Restricted to Renal Cortical Histological Compartments

	Periglomerular*	Tubular	Interstitial	Tubulointerstitial macrophage correlation†
BrdU				$r = 0.47$, $P < 0.01$
Depletion −	64 ± 4.6	497 ± 79	502 ± 39	
Depletion +	10 ± 7.6§	198 ± 8§	193 ± 28§	
TUNEL				$r = 0.47$, $P < 0.01$
Depletion −	47 ± 12	387 ± 94	356 ± 44	
Depletion +	17 ± 0.7§	221 ± 26‡	213 ± 10§	

*Per 50 glomeruli.
†Correlation between the number of BrdU-positive or TUNEL-positive tubulointerstitial cells and the area of macrophage staining.
Mean ± SEM. ‡$P < 0.05$ and §$P < 0.01$ compared with macrophage-depleted mice.

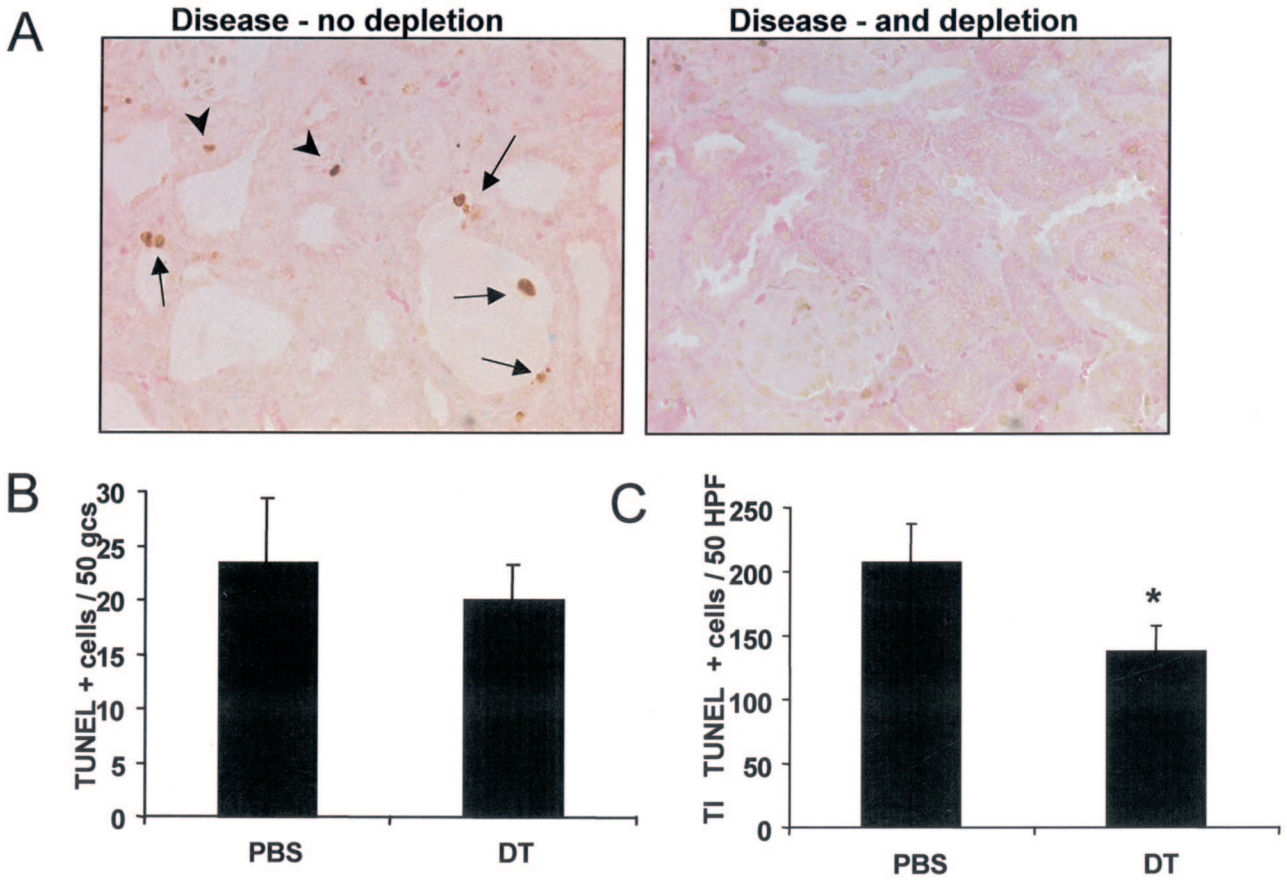

Figure 7. Tubulointerstitial apoptosis is reduced by macrophage ablation. **A:** Immunostaining for apoptotic nuclei using the TUNEL method (×400). Note interstitial apoptosis (**arrowhead**) and tubular cell apoptosis (**arrows**) in diseased mice (**left**) and a reduction in tubulointerstitial apoptosis after macrophage depletion (**right**). **B:** The number of intraglomerular apoptotic cells per 50 glomerular cross sections in CD11b-DTR diseased mice treated with PBS or DT. **C:** The number of tubulointerstitial apoptotic cells per 50 HPF in CD11b-DTR diseased mice treated with PBS or DT (*$P < 0.05$).

of interstitial macrophages that were proliferating, tissue sections were stained for the macrophage marker F4/80 and nuclear BrdU incorporation. In control-treated, diseased mice, 12.3 ± 1.6% of BrdU-positive interstitial cells were macrophages, representing 0.19 ± 0.13% of all macrophages. These results indicate that although macrophages can proliferate in the kidney, they represent relatively few proliferating interstitial cells, and therefore interstitial myofibroblasts are the major proliferating cell population in the injured interstitium. Thus, macrophages regulate proliferation of both tubular cells and interstitial cells, either directly or by indirect mechanisms.

Apoptosis was assessed in sections adjacent to sections used for BrdU staining. In the glomerulus the number of TdT uridine nick-end labeled (TUNEL)-positive cells (a marker of apoptosis) was similar in diseased mice irrespective of macrophage depletion (Figure 7, A and B). In the rest of the cortex, however, the number of TUNEL-positive cells was significantly reduced by macrophage depletion (Figure 7B, Table 1). Analysis of TUNEL-positive cells in separate tubular and interstitial compartments indicated that macrophage depletion was associated with reduced levels of tubular cell and interstitial cell apoptosis. Apoptosis of periglomerular cells was also decreased (Table 1). A significant correlation was evident between the area of cortical staining for macrophages and the number of TUNEL-positive cells in each mouse (Table 1) thereby providing further evidence for a link between macrophage infiltration and both tubular and interstitial cell death.

Recruitment of CD4$^+$ T Lymphocytes Is Diminished after Macrophage Depletion

We have previously reported this model of nephrotoxic nephritis to be dependent on activated CD4 T lymphocytes.[38] In some settings activated T-lymphocytes can activate monocytes/macrophages by both contact-dependent and paracrine mechanisms. For example, production of interferon-γ by activated CD4 + T_H1 lymphocytes may contribute to macrophage activation.[39] We now provide evidence that inflammatory macrophages in the kidney are partly responsible for the recruitment of lymphocytes to the kidney. The effect of intravenous DT injection on circulating T cells in the DTR mice was assessed by CD3 immunolabeling. Twenty-four hours after administration of DT, whole blood leukocytes were labeled with anti-CD3 antibodies. Simultaneously monocytes and neutrophils were assessed using CD11b and F4/80 antibodies and anti-GR-1 antibodies, respectively (Table 2). The number of circulating T cells was not

Table 2. Circulating Leukocytes 24 Hours after Administration of DT to CD11b-DTR and Renal T Cells at Day 20 of Nephrotoxic Nephritis with and without Macrophage Depletion

	CD3	CD4	CD8	Gr-1	F4/80
Blood*					
Depletion −	2042 ± 644			511 ± 59	261 ± 31
Depletion +	2226 ± 1061			487 ± 63	59 ± 8¶
Kidney					
Depletion −		13.3 ± 1.1†	3.3 ± 1.2		
Depletion +		6.3 ± 2.2§	2.0 ± 1.1		
Glomerulus‡					
Depletion −		12.7 ± 2.4	0.4 ± 0.4		
Depletion +		6.0 ± 3.0§	0.8 ± 0.7		

*Peripheral blood leukocyte counts expressed as cells (×1000) per ml of whole blood.
†Cortical kidney T-cell counts expressed as cells per high-power field.
‡Glomerular T-cell counts expressed as cells per 50 glomerular cross sections.
Mean ± SEM. §$P < 0.05$ and ¶$P < 0.01$ compared with macrophage-depleted mice.

significantly different in DT-treated DTR mice compared with PBS-treated DTR mice. By comparison the monocyte population was depleted by ~80% (Table 2). These data are in accordance with our previous work.[25] The number of T lymphocytes in the nephritic kidneys at day 20 of disease was assessed by immunostaining for CD4- and CD8-expressing cells. Compared with macrophages there were very few lymphocytes in both the interstitium and glomeruli (Table 2). The frequency of T cells was sufficiently low that greater accuracy was obtained by counting individual cells per HPF rather than by morphometry. The number of $CD4^+$ T cells was reduced in both the glomerular and interstitial compartments of the kidney after macrophage depletion (Table 2). The number of $CD8^+$ T cells was not reduced in nephritic kidneys of mice that underwent macrophage depletion. These results indicate that macrophages are likely to play an important role in the recruitment, or possibly survival, of $CD4^+$ T cells to the kidney and may represent an additional mechanism by which macrophage depletion halts progression of crescentic GN.

Depletion of Macrophages Alters the Balance of Enzymes Regulating Matrix Turnover and the Balance of Inflammatory Cytokines

To understand the molecular mechanisms by which inflammatory macrophages mediate the effects we have described, we measured the transcript levels in whole kidney of several candidate genes that might be modulated by macrophage depletion (Table 3). Although none of the candidate genes are exclusively expressed by macrophages, these genes are believed to play important roles in disease such as modulation of inflammatory processes, induction of cell death, and the deposition and degradation of matrix. Importantly, we did not identify any difference in the levels of *tnfα* or *tgfβ1* transcript in diseased kidneys after macrophage depletion. Transforming growth factor-β is widely expressed in both parenchymal cells of the kidney and inflammatory cells during disease.[31] Further, *tgfβ1* transcript levels have been reported not to correlate well with protein levels or cytokine activity.[40] Tumor necrosis factor-α may also be expressed by parenchymal cells of the kidney.[41] These observations may explain why transcript levels did not differ in our study. It is of interest that, although *il-10* transcript is generated at lower levels than *tnfα*, macrophage depletion resulted in a greater than sevenfold increase in *il-10* transcript expression. Interleukin-10 may be generated by macrophages as well as T_H2 cells and is a potent anti-inflammatory cytokine.[42] In addition we determined transcript levels for two major factors governing matrix turnover, *mmp-13* (collagenase 1) and *timp-1*. Although transcript levels of the major mouse collagenase, *mmp-13*, decreased approximately fourfold after macrophage depletion, transcript of its inhibitor *TIMP-1* decreased fivefold. TIMP-1 is a potent inhibitor of MMP activity such that there may be no collagenase activity in the presence of TIMP-1 expression even when tissue levels of MMP-13 are high.[43] Therefore these data suggest that regulation of TIMP-1 might represent a mechanism by which macrophage depletion ameliorates tissue fibrosis.

Discussion

In a murine model of severe crescentic GN we have successfully depleted macrophages from the renal cortex for 5 consecutive days during established progressive disease. Importantly, macrophage depletion had no effect on the amount of circulating anti-sheep immunoglobulin compared to control diseased mice (data not

Table 3. The Effect of Macrophage Depletion on Cytokine Message Levels within the Kidney

	TNF-α	TGF-β	IL-10	TIMP-1	MMP-13
Depletion −	1.36 ± 0.75	19.4 ± 6.6	0.046 ± 0.011*	0.0114 ± 0.0061*	0.0039 ± 0.0021*
Depletion +	1.62 ± 0.51	16.0 ± 3.4	0.365 ± 0.118	0.0029 ± 0.0011	0.00079 ± 0.00028

*$P < 0.05$ relative to depletion. All values are relative to GAPDH.

shown) indicating that the effects seen were directly related to macrophage depletion in the kidney. Histological and functional parameters demonstrate that macrophages are important mediators of disease progression and play a dominant role in the pathology of both glomerular crescents and tubulointerstitial inflammation and scarring.

A key finding of this study is that macrophage-depleted mice exhibited improved renal function and reduced levels of proteinuria indicating that the overall effect of macrophage infiltration is functionally detrimental in this model of progressive renal inflammation. Furthermore, although this model of renal inflammation is characterized by progressive disease between day 15 and day 20, histological analysis revealed a marked reduction in renal injury and fibrotic scarring after macrophage depletion. Our observations therefore suggest that macrophages represent a potential target for therapeutic intervention.

Our study has identified some of the mechanisms by which inflammatory macrophages mediate deleterious effects. Firstly, they target tubular epithelial cells such that macrophage depletion protects this tissue compartment from both injury and apoptotic cell death and the resultant histological changes of tubular atrophy and increased tubular luminal space. We and others have shown that morphometric measurement of tubular luminal space provides an objective measurement of tubular injury. Elevated tubular luminal space correlates with a poor renal prognosis in human nephritis.[28] Interestingly, interstitial macrophages may also trigger tubular cells into the cell cycle as evidenced by the significant reduction in both BrdU incorporation and PCNA expression after macrophage depletion. Secondly, macrophages target the myofibroblast populations of the interstitium and the periglomerular crescents. Macrophages may also trigger myofibroblasts to enter the cell cycle or undergo apoptosis. However, unlike tubular epithelial cells, the overall effect on the myofibroblast population is supportive. Whereas tubular cells are injured and are progressively lost, myofibroblasts survive and in the presence of macrophages the myofibroblast population undergoes net expansion. Thirdly, the deposition of interstitial collagens, associated with long-term scarring and a poor functional outcome, is also dependent on inflammatory macrophage infiltration.

A close relationship between the degree of inflammatory macrophage infiltration and tubular epithelial cell apoptosis has been reported in two recent studies.[8,44] Indeed, it has been proposed that macrophages might actively induce tubular cell apoptosis in both murine nephrotoxic nephritis and a murine model of Alport's GN.[45,46] Our findings would be in keeping with those observations. The fact that we actively and specifically depleted macrophages for the first time during kidney injury provides strong evidence that the correlations between epithelial cell death and macrophages previously reported are direct cause and effect.

We also report here that tubular cell proliferation was increased in the presence of macrophages. One interpretation of such findings is that macrophages not only trigger epithelial cells into cell cycle but they simultaneously trigger apoptosis. Selective killing of cells at checkpoints in the cell cycle has been recognized in tumor cell biology, inflammation, and development.[3,47,48] Indeed we have previously demonstrated that macrophages in the developing eye trigger cell cycle-dependent death of endothelial cells. In this scenario, macrophages trigger cells from G_0 into G_1, then by a second mechanism activate the apoptotic machinery at the G_1 cell-cycle checkpoint. It is plausible that inflammatory macrophages test tubular epithelial cell health, because there will be a delay at the G_1 checkpoint when cells enter the cell cycle if problems exist in the synthetic process. At this stage of the cell cycle, cells exhibit an enhanced susceptibility to proapoptotic stimuli that may be generated by inflammatory macrophages.

Our data suggest that macrophages mediate much of their deleterious effect in progressive renal inflammation by regulating the population of myofibroblasts both in the crescent and in the interstitium. Our data show that the populations of myofibroblasts are expanded in the presence of inflammatory macrophages. Furthermore, collagen III, a synthetic product of myofibroblasts and a component of interstitial fibrosis is deposited in greater quantities in the presence of macrophages. *In vitro* studies of the interaction between macrophages and myofibroblasts provide evidence that macrophages are capable of inducing myofibroblast proliferation,[49–51] apoptosis, abnormal cell matrix synthesis, and cell matrix degradation through production of nitric oxide, tumor necrosis factor-α, and up-regulation of collagenases and other metalloproteinases.[6] Our data also indicate that fibroblasts in the presence of macrophages are undergoing both cell proliferation and apoptosis. It is therefore likely that macrophages are also triggering fibroblasts to both proliferate and undergo apoptotic cell death. We have previously reported that classically activated macrophages induce cell death in proliferating mesangial myofibroblast cells *in vitro*.[4,5] This death is targeted to the G_1 to S phase of the cell cycle such that the greater the rate of proliferation, the greater the rate of macrophage-directed killing of mesangial cells. However, *in vivo* in the kidney, and unlike conditions for epithelial cells, the conditions favor successful proliferation, rather than excessive cell death, thereby resulting in an expanded myofibroblast population. In keeping with an expanded population of myofibroblasts, the deposition of collagen III is also increased in the presence of macrophages. Although increased deposition of matrix collagens might simply be a reflection of an increased population of myofibroblasts, *in vitro* studies indicate that myofibroblasts can result in a net loss of matrix collagens by expressing matrix metalloproteinases predominantly, or failing to express inhibitors of matrix metalloproteinases (TIMPs). Our study shows that whole kidney transcripts for both the major collagenase MMP-13 and its inhibitor TIMP-1 are decreased by macrophage depletion. Because TIMP-1 expression inhibits MMP activity potently one interpretation of these data are that MMP activity will rise after macrophage depletion due to down-regulation of TIMP-1. Thus one mechanism by which collagen III is reduced after macrophage depletion may be by down-regulation of TIMP-1. Both these data and the *in vitro* studies

suggest[15,16] that in addition to macrophage-myofibroblast cross talk in terms of cell cycle and cell death, macrophages also signal myofibroblasts to lay down matrix rather than remove it through activation of degradative enzymes.

Inflammatory macrophages in the kidney have been reported to proliferate.[37,52] Our data are in broad agreement with this finding. The proportion of macrophages that are actively proliferating is however low compared with tubular cells and myofibroblasts, and is comparable with recent reports of macrophage proliferation in mouse kidney tubulitis observed during transplant rejection.[37] In a rat model of GN as many as 62% of macrophages were reported to be proliferating,[52] and far exceeds the findings of this study. Whether this reflects differences between rat and mouse disease models, or disparity between PCNA-positive immunostaining (as reported in the rat model) and BrdU incorporation remains to be determined.

Although the number of T cells in the kidney was low compared with inflammatory macrophages, intrarenal $CD4^+$ T cells were reduced by macrophage depletion. Our DTR model has no effect on the number of circulating T cells. It is likely therefore that macrophages either contribute to the recruitment or survival of $CD4^+$ cells in the kidney. Thus another mechanism by which macrophages contribute to the pathogenesis of crescentic GN is through maintaining a population of $CD4^+$ T cells. We have recently demonstrated a pivotal role for resident peritoneal macrophages in recruiting neutrophils to the inflamed peritoneal cavity by release of the neutrophil chemokines MIP-2 and KC.[25] Macrophages express MIP-1α and MIP-1β, potent ligands for CCR5 that is the dominant chemokine receptor for $CD4^+$ T_H1 cells.[53] $CD8^+$ cells recruited to the kidney may rely more heavily on other receptors such as CCR1 or possibly CCR3, whose ligands include RANTES and eotaxin, chemokines expressed by many cells in the kidney including tubular cells.[54–56] It is possible therefore that the selective reduction in $CD4^+$ cells over $CD8^+$ cells reflects a reduction of macrophage-derived chemokines. In our study the transcript for *il-10* is increased after macrophage depletion. Further studies will be required to identify the source of interleukin-10, but one candidate is a population of T_H2 cells. It is tempting to suggest that because T_H2 $CD4^+$ cells represent a potent source of interleukin-10 and because they express different chemokine receptors (CCR4 and CCR8) from T_H1 cells, macrophage depletion selectively reduces recruitment or differentiation of T_H1 cells but not T_H2 cells.[57] Further studies will be required to dissect the mechanisms by which macrophages regulate T-cell recruitment.

The transgenic DTR conditional macrophage ablation system offers the possibility of dissecting the chronological role of macrophages in inflammation in more detail. It has been proposed that distinct functional subsets of macrophages exist within inflamed sites. One extreme of this dichotomy, the classically activated macrophage, would be expected to mediate tissue destruction and cell death, whereas the other extreme phenotype, the alternatively activated macrophage might be expected to promote cell proliferation, debridement of damaged tissue, and synthesis of new matrix.[6,58] Our data in nephrotoxic nephritis suggest that the *in vivo* situation is complicated because the infiltrating macrophage population appears to exhibit features of both subsets, ie, induction of cell death together with promoting cell proliferation. It might be that performing macrophage depletion at different time points in disease and subsequent recovery may well reveal functionally distinct macrophage subtypes as we have recently demonstrated in the liver.[24] Our work suggests that macrophages play a key role in determining the functional and pathological outcome of disease in an experimental model of glomerular and tubulointerstitial inflammation that has analogies with progressive human disease and that infiltrating macrophages may well merit specific targeting in human disease.

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