Macrophages directly mediate diabetic renal injury

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1Division of Nephrology, Department of Medicine, Penn State University College of Medicine, Hershey, Pennsylvania; and 2Department of Comparative Medicine, Penn State University College of Medicine, Hershey, Pennsylvania

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You H, Gao T, Cooper TK, Reeves WB, Awad AS. Macrophages directly mediate diabetic renal injury. Am J Physiol Renal Physiol 305: F1719–F1727, 2013. First published October 30, 2013; doi:10.1152/ajprenal.00141.2013.—Monocyte/macrophage recruitment correlates strongly with the progression of renal impairment in diabetic nephropathy (DN), yet their direct role is not clear. We hypothesized that macrophages contribute to direct podocyte injury and/or an abnormal podocyte niche leading to DN. Experiments were conducted in CD11b-DTR mice treated with diphertheria toxin (DT) to deplete macrophages after streptozotocin-induced diabetes. Additional experiments were conducted in bone marrow chimeric (CD11b-DTR→C57BL/6/J) mice. Diabetes was associated with an increase in the M1-to-M2 ratio by 6 wk after the induction of diabetes. Macrophage depletion in diabetic CD11b-DTR mice significantly attenuated albuminuria, kidney macrophage recruitment, and glomerular histological changes and preserved kidney nephrin and podocin expression compared with diabetic CD11b-DTR mice treated with mutant DT. These data were confirmed in chimeric mice indicating a direct role of bone marrow-derived macrophages in DN. In vitro, podocytes grown in high-glucose media significantly increased macrophage migration compared with podocytes grown in normal glucose media. In addition, classically activated M1 macrophages, but not M2 macrophages, induced podocyte permeability. These findings provide evidence showing that macrophages directly contribute to kidney injury in DN, perhaps by altering podocyte integrity through the proinflammatory M1 subset of macrophages. Attenuating the deleterious effects of macrophages on podocytes could provide a new therapeutic approach to the treatment of DN.

Macrophages; diabetic nephropathy; podocytes

DIABETIC NEPHROPATHY (DN) is the leading cause of end-stage kidney disease, responsible for >40% of all cases in the United States, and this number is likely to increase unabated (6). Thus, it is important to identify the mechanisms involved in the development of diabetic kidney disease. Early alterations in diabetic kidneys include the development of glomerular hyperfiltration and hypertrophy followed by thickening of the glomerular basement membrane, mesangial matrix accumulation, an increased urinary albumin excretion (UAE) rate, and, ultimately, the progression to glomerular sclerosis and end-stage renal failure.

Macrophage heterogeneity plays a role in tissue injury and repair, mainly through classically activated (proinflammatory) M1 macrophages the alternatively activated (anti-inflammatory) M2 macrophages, respectively (19). Monocytes and/or macrophages, their adherence to endothelial cells, and overexpression of proinflammatory cytokines and chemokines contribute to the pathogenesis of DN (10–12, 35). However, whether macrophage recruitment is the cause or a consequence of chronic kidney injury is still debated. Infiltrating macrophages release lysosomal enzymes, nitric oxide, ROS, transforming growth factor-β, VEGF, and cytokines such as TNF-α, IL-1, and interferon (IFN)-γ (41), which could play a pivotal role in the development and progression of DN. Diminished macrophage infiltration associated with reduced urinary albumin excretion has been shown in chemokine (C-C motif) receptor 2 (CCR2) and monocyte chemoattractant protein (MCP)-1-deficient mice in both type 1 and type 2 diabetes (2, 11, 12, 37). In human studies, glomerular macrophage accumulation occurs in DN (4, 17, 22, 35) and correlates strongly with the progression of renal impairment (35). Furthermore, macrophage accumulation in diabetic kidneys correlates strongly with serum creatinine, interstitial myofibroblast accumulation, and interstitial fibrosis scores (2, 13, 14, 32, 36, 47). These data suggest a role for macrophages and potentially other bone marrow-derived cells in the genesis and maintenance of inflammation leading to diabetic renal complications. While a pathogenic role for macrophages and macrophage-produced mediators has been shown for a number of forms of kidney disease (2, 5, 13, 23, 30–32, 38, 39, 42, 43), their role in DN remains unclear.

In a recent report (2), we demonstrated that monocyte CCR2 mediates macrophage-induced diabetic renal injury. In the present work, we first characterized the macrophage phenotype (M1/M2) during the course of diabetes. We further used a CD11b-DTR transgenic model to chronically deplete monocytes/macrophages by the administration of diphertheria toxin (DT). We show that bone marrow-derived macrophages directly contribute to diabetic kidney injury. In vitro, we provide evidence for direct podocyte/macrophase interactions and that M1, but not M2, macrophages impair podocyte integrity.

MATERIALS AND METHODS

Diabetic mouse models. All animal experiments were approved by the Institutional Animal Care and Use Committee of Penn State University College of Medicine. In vivo experiments were performed in male 6-wk-old B6.FVB-Tg(TGAM-DTR/iEGFP)34Lan/J (CD11b-DTR) mice (stock no. 006000, Jackson Laboratory, Bar Harbor, MN). Multiple low doses of streptozotocin (STZ; 50 mg/kg body wt dissolved in lactated Ringers solution, Sigma, St. Louis, MO) were injected intraperitoneally for 5 consecutive days to induce type 1 diabetes. STZ is a widely used and accepted model of type 1 diabetes despite its nonspecific tissue toxicity (8, 9). DT or mutant DT (List Biological Laboratories) was injected intraperitoneally at the dose of 25 ng/g body wt weekly for 6 wk beginning 1 wk after STZ injection. Additional experiments were conducted in chimeric mice as previously described (49). Briefly, donor (male CD11b-DTR) mice were euthanized, and their femurs were removed and flushed with RPMI medium containing 10% FCS to obtain bone marrow cells. Unfractionated bone marrow cells were washed and resuspended in PBS at a concentration of 20 million cells/ml. Recipient (male C57BL/6J) mice were lethally irradiated using a γ-cell irradiator (two doses of
600 rads, 4 h apart). Eight hours after the irradiation, 10 million donor bone marrow cells were injected into the lateral tail vein of recipients. Mice were maintained in specific pathogen-free conditions for 8 wk followed by STZ injection as described above. After 6 wk of DT or mutant DT injection, mice were euthanized, and their kidneys were removed for further experiments. Confirmation of bone marrow chimerism was conducted using RT-PCR according to the protocol from Jackson Laboratory after 8 wk of bone marrow transplantation. Briefly, wild-type and chimeric mouse bone marrow were collected, and genomic DNA was isolated. The difference of the threshold cycle (Ct) value (ΔΔCT) between wild-type and CD11b-DTR → C57BL/6J mice was used to calculate the percentage of bone marrow of chimeric mice derived from CD11b-DTR mice. Our data (ΔΔCT = 4.68) indicate that >95% of bone marrow in chimeric mice was from CD11b-DTR mice.

Blood pressure measurement. Systolic blood pressure was measured using Coda blood pressure (Kent Scientific, Torrington, Connecticut) as we have previously described (2, 32). Mice were allowed to rest quietly for 10 min at 26°C. All measurements were performed at the same time for all groups to prevent any diurnal variations. Blood pressure measurement was performed using Coda blood pressure (Kent Scientific, Torrington, Connecticut) as we have previously described (2, 32). Mice were allowed to rest quietly for 10 min at 26°C. All measurements were performed at the same time for all groups to prevent any diurnal variations.

Histology and immunohistochemistry. Mice kidney sections were fixed in 4% paraformaldehyde and embedded in paraffin, and 3-μm sections were cut. Sections were stained with periodic acid-Schiff. All glomeruli were examined at ×40 magnification in a blinded manner. All images were taken with an Olympus BX51 microscope and DP71 digital camera using cellSens Standard 1.6 image software. Images were obtained with a ×100 (oil) objective with a total magnification of ×1,000. Semi-quantitative scores (0–4+) were assigned based on the masked readings. Mesangial matrix expansion or sclerosis scoring was performed as we have previously described (2, 32). For glomerular area measurements, individual glomeruli were imaged at ×600 total magnification using an Olympus BX51 microscope and DP71 digital camera with cellSens Standard 1.6 image software (Olympus America, Center Valley, PA). Glomeruli were traced using the closed polygon area feature and a Wacom Intuos 4 tablet. Fifteen glomeruli per mouse were analyzed in a blinded manner.

Immunohistochemistry for macrophages was performed using rat anti-mouse Mac-2 antibody (clone M3/38, Cedarlane, Burlington, NC) and rat anti-mouse F4/80 monoclonal antibody (Fitzgerald, Concord, MA) on paraffin sections as previously described (2, 32).

Flow cytometry. Kidney macrophage content was analyzed by fluorescence-activated cell sorting (FACS) in a LSR-II flow cytometer (BD, Franklin Lakes, NJ) as previously described (2, 32). In brief, kidneys were removed, minced, digested, and then passed through a 40-μm filter mesh. Kidney macrophages were defined as CD45+CD11b+F4/80+, and cells were gated on live cells. Counting beads (product no. PCB100, Invitrogen, Carlsbad, CA) to exclude dead cells. Counting beads (product no. PCB100, Invitrogen, Carlsbad, CA) to exclude dead cells. Counting beads (product no. PCB100, Invitrogen, Carlsbad, CA) to exclude dead cells. Counting beads (product no. PCB100, Invitrogen, Carlsbad, CA) to exclude dead cells.

Analytical methodology. The UAE rate was measured by ELISA using an Albuwell M kit (Exocell, Philadelphia, PA) as previously described (2, 32). Urine creatinine content was determined using a Creatinine Liquid Reagents assay kit (Diazyme Laboratories, Poway, CA) as previously described (2, 32). Blood urea nitrogen (BUN) was determined using Vitros DT6011 chemistry slides (Ortho-Clinical Diagnostics, Rochester, NY) as previously described (2, 32). Body composition was measured using LF90 Minispec Time Domain Nuclear Magnetic Resonance Spectrometer (Burker Optics, Billerica, MA) as previously described (2, 32).

Isolation of bone marrow-derived monocytes. Bone marrow cells were isolated from the mouse femur and tibia under sterile conditions as previously described (2). In brief, bones were flushed with RPMI-1640 (Invitrogen-Life Technologies) plus 10% FBS. Marrow cells were passed sequentially through a 22-gauge needle followed by three passages through a 25-gauge needle to obtain single cell suspensions of bone marrow cells. Monocytes were isolated from bone marrow cells using the STEMCELL (Vancouver, BC, Canada) mouse monocyte enrichment negative selection kit according to the manufacturer’s protocols. The enriched population comprised >90% monocytes (data not shown) and was further processed into M0, M1, or M2 macrophages. M0 macrophages were cultured in RPMI-1640 supplemented with 10% FBS, 1% glutamate, 20 μg/ml gentamycin, 55 μM 2-mercaptoethanol, and 10 ng/ml macrophage colony-stimulating factor (R&D Systems) for 7 days. To induce M1 macrophages, cells were stimulated with 1 μg/ml lipopolysaccharide (Sigma) for an additional 24 h. To induce M2 macrophages, cells were incubated with 10 ng/ml IL-4 (R&D Systems) and 10 ng/ml IL-13 (R&D Systems) for an additional 48 h.

Murine podocyte culture. Conditionally immortalized murine podocytes (kindly provided by Dr. John Sedor, Case Western Reserve University) were used in our in vitro experiments as we have previously described (3). Cells were grown under permissive conditions to propagate in RPMI-1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μ/ml IFN-γ (R&D Systems) in a collagen type I-coated flask at 33°C. Cells were then grown under restrictive conditions for 10–14 days in the absence of IFN-γ at 37°C to allow cells to differentiate in either high-glucose (33 mM) or normal glucose (11 mM) media.

Quantitative RT-PCR. Total RNA was isolated from kidney tissues using Tri Reagent (Molecular Research Center, Cincinnati, OH) per the manufacturer’s instructions. Single-strand cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) for two-step quantitative RT-PCR. Mouse nephrin and podocin were amplified in the Bio-Rad CFX96 Real-Time System. Data were analyzed using Bio-Rad CFX Manager software (version 2.0). Relative expression quantification was calculated using the 2−ΔΔCT equation after normalization to GAPDH as previously described (1, 3).

For M1 and M2 macrophage markers, quantitative PCR assays were performed using an ABI Prism 7900HT PCR system, TaqMan Fast Universal PCR master mixture (Applied Biosystems, Foster City, CA), and a TaqMan Gene Expression Assay Mix comprising Tnf (Mm00443260_g1) and mannose receptor (MR; Mm00485148_m1).

Western blot analysis. Fifteen micrograms of kidney lysates were electrophoresed on −4–12% NuPAGE Bis-Tris Mini Gels (Life Technologies) and electroblotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed with antibodies for nephrin (catalog no. 20R-NP002, Fitzgerald Industries, Acton, MA), podocin (catalog no. P0372, Sigma-Aldrich), and β-actin (catalog no. A5441, Sigma-Aldrich), and immunoblots were developed with the appropriate secondary antibodies and enhanced chemiluminescence. Densitometric analysis of autoradiograms was performed using ImageJ software (National Institutes of Health; http://rsweb.nih.gov/ij/index.html).

Macrophage migration assay. The mouse bone marrow-derived macrophage migration assay was performed in a Boyden chamber transwell system (Corning, New York, NY). Macrophages were plated and cultured in the upper chamber on a 5-μm porous membrane while the lower chamber was plated with podocytes cultured in medium as indicated. Mouse MCP-1 (R&D Systems) was used as a positive control. Anti-mouse MCP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a negative control. Sixteen hours after the upper chamber was placed in the lower chamber, cells were removed from the upper side of the membrane, and cells in the lower side of the membrane were stained with crystal violet and visualized under a microscope.

Podocyte permeability assay. Podocytes were plated at a density of 1 × 10^5 cells on collagen type I-coated Transwell-Col PTFE filters...
mice had significantly reduced numbers of macrophages kidneys of DT-treated nondiabetic and diabetic CD11b-DTR groups. Our results showed increased glomerular macrophages; Fig. 3A and tubulointerstitium (F4/80-positive cells, and neutrophils did not change between all groups C). We chose 6 wk after STZ-induced diabetes. Macrophage depletion reduces kidney hypertrophy in DN. To assess the possible significance of macrophage depletion in diabetic mice, we administered DT or mutant DT to diabetic CD11b-DTR mice for 6 wk. We chose 6 wk since this is the period in which M1/M2 macrophage markers predominate (Fig. 1). As shown in Table 1, mutant DT-treated diabetic mice had increased blood glucose levels, increased kidney weight-to-body weight ratios, and increased glomerular area compared with nondiabetic mice treated with or without DT (CD11b-DTR background). Macrophage depletion using DT in diabetic CD11b-DTR mice significantly reduced kidney weight-to-body weight ratios and glomerular areas compared with mutant DT-treated diabetic mice despite comparable blood glucose and blood pressure levels.

**RESULTS**

**M1 versus M2 kidney macrophages during the course of diabetes.** We first determined the distribution of kidney M1 and M2 markers during the course of diabetes. Kidneys were removed at baseline (normal) and 3, 6, and 12 weeks after STZ-induced diabetes (n = 6 animals/group) and subjected to RT-PCR analysis for total kidney M1 macrophage (TNF-α) and M2 macrophage (MR) markers. As shown in Fig. 1, DN was associated with an increase in both M1 and M2 macrophage markers after STZ-induced diabetes. Furthermore, the percentage of M1/M2 macrophage markers peaked at 6 wk after STZ-induced diabetes.

**DT administration chronically depletes kidney macrophages in CD11b-DTR mice.** Next, we determined the dose (12.5 or 25 ng/g body wt) of DT induced maximal kidney macrophage depletion compared with mutant DT (A) with no significant differences between intraperitoneal versus intravenous injection (B). Furthermore, the effect of kidney macrophage depletion was maintained for 7 days after DT administration (Fig. 2C). In contrast, B cells, T cells, and neutrophils did not change between all groups (data not shown). Therefore, we used a dose of 25 ng/g body wt DT in CD11b-DTR mice via intraperitoneal injection every 7 days in our experiments.

**DT injection decreases glomerular and tubulointerstitial macrophage recruitment in diabetic CD11b-DTR mice.** The distribution and number of macrophages were determined using immunohistochemistry in the glomeruli (Mac-2-positive macrophages; Fig. 3A) and tubulointerstititum (F4/80-positive macrophages; Fig. 3B). Our results showed increased glomerular and tubulointerstitial macrophage recruitment in the mutant DT-treated diabetic mouse group compared with the other groups.

Similar results were obtained using flow cytometry (FACS; CD11bhigh/F4/80low) analysis of total kidney macrophage recruitment. Kidneys of mutant DT-treated diabetic CD11b-DTR mice had significantly greater numbers of macrophages compared with normal nondiabetic mice (2.3 ± 0.2 × 10⁴ vs. 1.5 ± 0.5 × 10⁴ macrophages/g kidney tissue, P < 0.01). In contrast, kidneys of DT-treated nondiabetic and diabetic CD11b-DTR mice had significantly reduced numbers of macrophages (1.1 ± 0.1 and 1.4 ± 0.04 × 10⁴ macrophages/g kidney tissue, P < 0.01) compared with mutant DT-treated diabetic CD11b-DTR mice.
Macrophage depletion decreases renal histological changes of DN. Periodic acid-Schiff staining of kidney sections showed increased glomerular cellularity and mesangial expansion (score: 0.5 ± 0.05 vs. 0.2 ± 0.01, *P < 0.01) after 6 wk of diabetes in mutant DT-treated diabetic mice compared with nondiabetic mice (Fig. 3C). Nondiabetic or diabetic CD11b-DTR mice depleted of macrophages with DT displayed significantly fewer glomerular histological changes (score: 0.2 ± 0.05 vs. 0.2 ± 0.03, **P < 0.01) compared with mutant DT-treated diabetic CD11b-DTR mice (Fig. 3C).

Macrophage depletion reduces albuminuria and BUN in diabetic CD11b-DTR mice. To determine if macrophages directly contribute to diabetic renal injury, we measured 24-h UAE, the urine albumin-to-creatinine ratio, and BUN as indicators of renal injury in nondiabetic and diabetic CD11b-DTR mice treated with DT or mutant DT for 6 wk after STZ injection. Mutant DT-treated diabetic CD11b-DTR mice had a significant increase in albuminuria (Fig. 4A), the urine albumin-to-creatinine ratio (Fig. 4B), and BUN (Table 1) compared with nondiabetic mice treated with or without DT. In contrast, DT treatment of diabetic CD11b-DTR mice significantly reduced renal dysfunction as evidenced by a reduction in albuminuria, the urine albumin-to-creatinine ratio, and BUN.

Macrophage depletion preserves nephrin and podocin mRNA and protein expression in diabetic CD11b-DTR mice. Podocyte structural proteins (nephrin and podocin) play critical roles in the maintenance of the slit diaphragm. We and others (1, 15, 34) have previously shown that DN is associated with a downregulation of nephrin and podocin mRNA expression. Therefore, we assessed the effect of macrophage depletion on the expression of nephrin and podocin mRNA in diabetic kidneys (Fig. 5). Mutant DT-treated diabetic CD11b-DTR mice exhibited a significant reduction in nephrin and podocin expression, an effect significantly prevented by DT treatment. Similar results were obtained using Western blot analysis of total kidney homogenates for nephrin and podocin protein expression (Fig. 6).

Macrophage/podocyte interaction in vitro. To test for direct effects of macrophages on podocyte injury, we first hypothesized that podocytes express chemokines under pathological conditions (including high glucose) that lead to the recruitment of macrophages. Therefore, we used an in vitro macrophage

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Fig. 2. Dose, route, and time response curves of diphtheria toxin (DT) administration in CD11b-DTR mice. CD11b-DTR mice were administered DT at the indicated doses/frequencies (A–C). Mice were euthanized, and mouse kidney tissues were collected, minced, digested, and subjected to flow cytometry. Macrophages were defined as CD11b<sup>high</sup>F4/80<sup>low</sup>, and absolute macrophage numbers were calculated. Results are means ± SE. *P < 0.05 and **P < 0.01 compared with control (mutant DT).

Fig. 3. DT treatment reduces glomerular and tubulointerstitial macrophage infiltration and renal histological changes in diabetic CD11b-DTR mice. A: Mac-2-positive macrophages in glomeruli were identified by immunohistochemical staining at 6 wk after STZ-induced diabetes in CD11b-DTR mice. Images are representative of 40 fields from each group of mice. B: F4/80-positive macrophages in the tubulointerstitium were identified by immunohistochemical staining at 6 wk after STZ-induced diabetes in CD11b-DTR mice. Images are representative of 40 fields from each group of mice. C: CD11b-DTR mouse kidney tissues were paraformaldehyde fixed and paraffin embedded. Tissue sections were stained with periodic acid-Schiff (PAS) as indicated. All glomeruli were examined at ×25 magnification, graded at ×40 magnification, and pictured at ×100 magnification (oil).
The effect of M1 macrophages on podocyte permeability was blocked by the addition of anti-MCP-1 antibody (Fig. 8B). Confirmation of M1 and M2 macrophage differentiation was performed using RT-PCR (Fig. 9). M1 macrophages showed increased TNF-α expression, whereas M2 macrophages showed increased MR expression.

**Macrophage depletion reduces characteristics of DN in chimeric CD11b-DTR mice.** We next questioned whether the effect of DT was bone marrow or nonbone marrow dependent after diabetes. Toward this goal, we generated bone marrow chimeric (CD11b-DTR → wild-type) mice followed by STZ-induced diabetes. As shown in Table 2, both blood glucose and the kidney weight-to-body weight ratio were significantly increased in mutant DT-treated diabetic chimeric mice 6 wk after STZ injection. DT treatment in diabetic chimeric mice significantly reduced kidney weight-to-body weight ratios without affecting other parameters.

**Table 1. General characteristics of CD11b-DTR mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nondiabetes</th>
<th>Nondiabetes + DT Treatment</th>
<th>Diabetes + Mutant DT Treatment</th>
<th>Diabetes + DT Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice/group</td>
<td>10</td>
<td>5</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>171 ± 6a</td>
<td>169 ± 9a</td>
<td>487 ± 6b</td>
<td>433 ± 30b</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>119 ± 3</td>
<td>116 ± 3</td>
<td>116 ± 3</td>
<td>114 ± 6</td>
</tr>
<tr>
<td>Kidney weight/body weight, mg/g</td>
<td>0.60 ± 0.03</td>
<td>0.57 ± 0.02</td>
<td>0.81 ± 0.03b</td>
<td>0.61 ± 0.02</td>
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<tr>
<td>Glomerular area, µm²</td>
<td>2011 ± 69</td>
<td>2110 ± 87</td>
<td>2490 ± 122c</td>
<td>2131 ± 110</td>
</tr>
<tr>
<td>Percent fluid</td>
<td>6.6 ± 0.14</td>
<td>6.7 ± 0.10</td>
<td>5.9 ± 0.23</td>
<td>6.8 ± 0.18</td>
</tr>
<tr>
<td>Plasma BUN, mg/dl</td>
<td>25 ± 0.6</td>
<td>24 ± 1.1</td>
<td>35 ± 3.7c</td>
<td>19 ± 1.6</td>
</tr>
</tbody>
</table>

Data are means ± SE. SBP, systolic blood pressure; BUN, blood urea nitrogen. *P < 0.01 compared with diabetes + mutant diptheria toxin (DT) treatment and diabetes + DT treatment; **P < 0.01 compared with nondiabetes, nondiabetes + DT treatment, and diabetes + DT treatment; &P < 0.05 compared with nondiabetes, nondiabetes + DT treatment, and diabetes + DT treatment.

Fig. 4. DT treatment attenuates diabetic renal injury. CD11b-DTR mice were injected with STZ at the dose of 50 mg/kg body wt ip for 5 days to induce type 1 diabetes; vehicle (ve) solution was injected as the control. After STZ injection, DT was injected intraperitoneally weekly for 6 wk; mutant DT was used as the negative control. Twenty-four-hour urine was collected for the measurement of urinary albumin excretion rate (UAER; A) and albumin-to-creatinine ratio (B). Results are means ± SE. *P < 0.05 and **P < 0.01 compared with nondiabetic groups with or without DT; &P < 0.01 compared with the mutant DT-treated diabetic group.

Fig. 5. DT treatment restores nephrin and podocin mRNA expression in diabetic CD11b-DTR mice. Expression of kidney nephrin (A) and podocin (B) mRNA were normalized to GAPDH. Data are presented as relative fold changes between groups. Results are means ± SE. *P < 0.05 compared with nondiabetic groups with or without DT; &P < 0.05 compared with the mutant DT-treated diabetic group.
Macrophage depletion ameliorates renal dysfunction in diabetic chimeric mice. Mutant DT-treated diabetic chimeric mice had a significant increase in the urine albumin-to-creatinine ratio (Fig. 10) and BUN (Table 2) in a similar way to mutant DT-treated diabetic CD11b-DTR mice. In contrast, DT treatment of diabetic chimeric mice significantly ameliorated these changes in a similar manner to DT-treated diabetic CD11b-DTR mice.

DISCUSSION

Macrophage accumulation is closely associated with chronic renal injury, yet their direct role in diabetic kidney injury has not been directly established. This study shows that macrophage depletion mediates renal tissue protection as evidenced by a reduction in albuminuria, BUN, histopathological changes, and kidney macrophage recruitment during diabetes. This effect is mainly mediated via bone marrow-derived macrophage depletion as determined in our chimeric experiment. Furthermore, our data provide the first evidence for direct podocyte/macrophage interaction and that M1, but not M2, macrophages impair podocyte integrity, possibly through MCP-1. These findings reveal an important direct role for macrophages in the pathogenesis of DN and provide evidence for macrophage inhibition as a potential therapeutic modality for the primary prevention of DN. Additional studies are needed to test the role of macrophage depletion in secondary prevention in a later stage of DN. In addition,
role in inducing renal injuries through potent cytokines such as MCP-1 (18, 40, 44), TNF-α (42), and IL-1 (43). Furthermore, macrophage repletion can induce kidney dysfunction such as proteinuria and mesangial cell proliferation (25, 45). In contrast, macrophage abrogation attenuates renal dysfunction in rodent non-DN disease models using different approaches to deplete macrophages. Macrophage depletion significantly reduced albuminuria and decreased histological changes compared with mutant DT-treated diabetic mice. Since CD11b has been reported to be expressed in other non bone marrow-derived cells, such as rectal epithelial cells (24), and to exclude any unwanted sys-

**Table 2. General characteristics of chimeric mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nondiabetes</th>
<th>Diabetes + Mutant DT Treatment</th>
<th>Diabetes + DT Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice/group</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>140 ± 5a</td>
<td>446 ± 20</td>
<td>447 ± 33</td>
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<td>SBP, mmHg</td>
<td>115 ± 3</td>
<td>109 ± 4</td>
<td>105 ± 5</td>
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<tr>
<td>Kidney weight/body weight, mg/g</td>
<td>0.62 ± 0.01b</td>
<td>0.79 ± 0.02c</td>
<td>0.67 ± 0.04</td>
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<tr>
<td>Percent fluid</td>
<td>6.9 ± 0.13</td>
<td>6.4 ± 0.04</td>
<td>6.6 ± 0.06</td>
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<tr>
<td>Plasma BUN, mg/dl</td>
<td>35 ± 0.5a</td>
<td>43 ± 3a</td>
<td>35 ± 0.6</td>
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</tbody>
</table>

Data are means ± SE. *P < 0.01 compared with diabetes + mutant DT treatment and diabetes + DT treatment; bP < 0.01 compared with diabetes + mutant DT treatment; cP < 0.05 compared with diabetes + DT treatment; dP < 0.01 compared with diabetes + mutant DT treatment; eP < 0.05 compared with diabetes + DT treatment.

The contribution of M1 and M2 macrophages in the setting of DN is not known. Macrophage heterogeneity has been recently recognized, with M1 (classically activated) macrophages mediating renal injury and alternatively activated M2 macrophages mediating renal tissue repair (29). Our data show that DN is associated with an increase in the M1-to-M2 macrophage ratio at 6 wk of diabetes. These data indicate that depletion of macrophages and/or macrophage secretory products may be important in the early phase of DN.

To examine the direct role of macrophages in DN, we generated a diabetic mouse model in CD11b-DTR mice, in which human DTR expression is under the control of the CD11b promoter. This mouse model allowed us to chronically deplete macrophages conditionally through DT injection. This validated mouse model has been previously used to study acute kidney and liver injury (16, 20). We independently confirmed that, upon DT injection, kidney macrophages were successfully depleted chronically.

In our study, we show that DT injection during the initial 6 wk of diabetes is able to chronically deplete kidney macrophages. Macrophage depletion significantly reduced albuminuria and decreased histological changes compared with mutant DT-treated diabetic mice. Since CD11b has been reported to be expressed in other non bone marrow-derived cells, such as rectal epithelial cells (24), and to exclude any unwanted sys-

![Graph](image-url)
temic effect of DT injection, we generated bone marrow chimeric mice in which CD11b-DTR mice were used as donors and C57/BL/6J mice were used as recipients. Our results were consistent with the data generated from CD11b-DTR mice, indicating that it is bone marrow-derived macrophages, rather than nonhematopoietic cells expressing CD11b, that play a crucial role in DN. It is noteworthy that albuminuria was not attenuated in chimeric mice (Fig. 10) to the same degree as in regular CD11b-DTR mice (Fig. 4B). This finding can be explained by residual wild-type macrophages in chimeric mice that may still able to exacerbate albuminuria. In addition, it is possible that the radiation used to create the chimeras may have had a direct effect to impair renal function. This conclusion is supported by the observation that BUN levels in nondiabetic chimeric mice (Table 2) were higher than in nondiabetic CD11b-DTR mice (Table 1).

Being slit diaphragm molecules, nephrin and podocin are of great importance in the pathogenesis of proteinuria (48). In humans, nephrin and podocin expression are significantly decreased in diabetic individuals compared with nondiabetic individuals (27). Moreover, podocyte nephrin and podocin expression were inhibited by macrophage activation (26). In our study, kidney nephrin and podocin expression were significantly downregulated compared with normal mice. Interestingly, DT injection was able to restore nephrin and podocin expression, indicating that macrophage recruitment either directly or indirectly induces podocyte dysfunction in diabetes.

To answer this question directly, we first tested whether podocytes are able to promote macrophage migration. Our data clearly show that macrophage migration was significantly induced by podocytes cultured with high-glucose medium compared with normal glucose medium and that effect is mainly mediated through elevated secretion of MCP-1 by podocytes. These data are consistent with increased secretion of inflammatory cytokines and chemokines from activated podocytes (7).

To further study the direct role of macrophages on podocyte permeability, we cocultured podocytes and bone marrow-derived monocyte/macrophage subsets in a Transwell system. Our data show that M1 (classically activated) macrophages, but not M0 or M2 macrophages, induced podocyte injury, as evidenced by the increased permeability of BSA mainly through MCP-1. Taken together, our data indicate that MCP-1 may be a triggering mechanism for both macrophage infiltration and podocyte permeability (28). These findings are consistent with previous reports (45, 46) in which M1 macrophage infusion resulted in increased kidney damage. Classically activated M1 macrophages have been shown to cause injury in other organs (29, 33). In contrast, M2 macrophages have been shown to play a role in tissue repair (29, 33). In our in vivo experiments, macrophage ablation using DT did not discriminate between the different types of macrophage subsets, such as M1 versus M2. However, the improvement in kidney function and structure upon macrophage ablation suggests a role for M1 macrophages during the early phase of diabetes.

In conclusion, our data demonstrate that macrophages directly induce early stage diabetic kidney injury. The effect of macrophages is mediated, at least in part, via impairment of podocyte function, as evidenced by increased permeability and reduced expression of nephrin and podocin through classically activated (M1) macrophages but not alternatively activated (M2) macrophages. In addition, macrophage migration was promoted by podocytes grown in high-glucose medium. Taken together, our data provide the first direct evidence of macrophages as a critical player in DN. Attenuating the deleterious effects of macrophages and/or macrophage secretory products will provide a new therapeutic approach to the treatment of DN.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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