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Macrophage Cyclooxygenase-2 Protects Against Development of Diabetic Nephropathy

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COMPLICATIONS

Diabetic nephropathy (DN) is characterized by increased macrophage infiltration, and proinflammatory M1 macrophages contribute to development of DN. Previous studies by us and others have reported that macrophage cyclooxygenase-2 (COX-2) plays a role in polarization and maintenance of a macrophage tissue-reparative M2 phenotype. We examined the effects of macrophage COX-2 on development of DN in type 1 diabetes. Cultured macrophages with COX-2 deletion exhibited an M1 phenotype, as demonstrated by higher inducible nitric oxide synthase and nuclear factor-κB levels but lower interleukin-4 receptor-α levels. Compared with corresponding wild-type diabetic mice, mice with COX-2 deletion in hematopoietic cells (COX-2 knockout bone marrow transplantation) or macrophages (CD11b-Cre $COX2^{f/f}$) developed severe DN, as indicated by increased albuminuria, fibrosis, and renal infiltration of T cells, neutrophils, and macrophages. Although diabetic kidneys with macrophage COX-2 deletion had more macrophage infiltration, they had fewer renal M2 macrophages. Diabetic kidneys with macrophage COX-2 deletion also had increased endoplasmic reticulum stress and decreased number of podocytes. Similar results were found in diabetic mice with macrophage PGE_2 receptor subtype 4 deletion. In summary, these studies have demonstrated an important but unexpected role for macrophage COX-2/prostaglandin E_2/PGE_2 receptor subtype 4 signaling to lessen progression of diabetic kidney disease, unlike the pathogenic effects of increased COX-2 expression in intrinsic renal cells.

In the U.S., >20 million people are currently affected by chronic kidney disease (1). Diabetes is the most common cause of chronic kidney disease and end-stage renal disease, accounting for about half of all cases. Diabetic nephropathy (DN) is characterized by infiltration of hematopoietic cells, especially macrophages, and there is an association between increased tubulointerstitial inflammatory cell infiltrate in human diabetic kidneys and loss of renal function (2,3).

The role of macrophages in development of DN is of particular interest because they can exhibit distinctly different functional phenotypes, broadly characterized as proinflammatory (M1 or classically activated) and tissue-reparative (M2 or alternatively activated) phenotypes (4). M1 macrophages increase in diabetic kidneys of rodents (5). Furthermore, our previous studies showed that interventions that decrease progression of DN are associated with inhibition of renal macrophage infiltration (6–8).

The cyclooxygenase (COX)/prostaglandin system contributes to development of DN. COX is the rate-limiting enzyme in metabolizing arachidonic acid to prostaglandin G_2 and subsequently to prostaglandin H_2, which serves as the precursor for subsequent metabolism by prostaglandin and thromboxane synthases. Prostanoid cellular responses are mediated by specific membrane-associated G-protein–coupled receptors (9–15). Two isoforms of COX exist in mammals, constitutive COX-1 and inducible COX-2. Previous studies focused on the role of intrinsic renal cortical COX-2 (macula densa and adjacent cortical

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thick ascending limb) in development of DN. Early diabetes is characterized by hyperfiltration, and COX-2–derived prostaglandin E2 (PGE_2) contributes to the altered hemodynamics. In this regard, selective COX-2 inhibitors have been reported to attenuate development of DN (16–18).

In addition to intrinsic kidney cells, COX-2 is also expressed in renal immune cells, particularly renal monocytes/macrophages. Macrophages express COX-2 and are a rich source of prostaglandins, and macrophage-dependent COX-2 expression has been shown to be important for macrophage polarization. Previous studies by us and others indicate that COX-2 inhibition or macrophage deletion of PGE_2 receptor subtype 4 (EP4), the major receptor for PGE_2 on macrophages, led to decreased expression of macrophage M2 markers in tumors (19–21). However, the potential role of the macrophage COX-2/PGE_2/EP4 pathway in development of DN has not been investigated. Therefore, in the current studies, we determined the role of macrophage COX-2–derived prostaglandin expression and activity in mediation of development of DN.

RESEARCH DESIGN AND METHODS

Animal Studies

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Vanderbilt University. COX-$2^{-/-}$ mice on the 129/Bl6 background were originally generated by Dinchuk et al. (22). Heterozygous breeding pairs were obtained from The Jackson Laboratory (002476; Bar Harbor, ME) and backcrossed onto 129/svj background for 12 generations (22). $EP_4^{flox/flox}$ mice were generated in Matthew Breyer's laboratory (23), COX-$2^{flox/flox}$ mice in which exons 6, 7, and 8 of Cox-2 gene are flanked by Lox P sites were originally generated in Dr. Fitzgerald's laboratory (24), and CD11b-Cre mice with transgene integration in the Y chromosome were generated in Dr. Vacher's laboratory (25), all of which were originally on the C57/Bl6 background and later backcrossed onto an FVB background for 12 generations. Macrophage COX-2 deletion mice (CD11b-Cre COX-$2^{flox/flox}$) and macrophage EP4 deletion mice (CD-11b-Cre $EP4^{flox/flox}$) and corresponding wild-type (WT) (COX-$2^{flox/flox}$ mice and $EP4^{flox/flox}$ mice, respectively) on the C57/Bl6 or FVB backgrounds were used for experiments. Male mice received daily intraperitoneal injections for 5 consecutive days of streptozotocin (STZ; 50 mg/kg) that was freshly prepared in 0.1 mol/L citrate buffer (pH 4.5) (8). The onset of diabetes was evaluated by measuring fasting blood glucose with a B-glucose analyzer (HemoCue, Lake Forest, CA) in conscious mice on saphenous vein samples at noon after fasting for 6 h initiated at 6:00 A.M. Urinary albumin and creatinine excretion was determined using Albuwell M kits (Exocell, Philadelphia, PA). Albuminuria is expressed as the urinary albumin-to-creatinine ratio (ACR; μg/mg). Periodic acid-Schiff (PAS)–stained slides were evaluated for glomerular injury without knowledge of the identity of various groups as described previously (7).

Creation of Chimeric Mice

Bone marrow transplantation (BMT) was performed as previously described (26). Briefly, recipient mice (WT female 129/svj mice) were lethally irradiated with 9 Gy using a cesium γ source. Bone marrow cells from donors (male 129/svj WT and COX-$2^{-/-}$ mice) were harvested from femurs and tibias. Recipient mouse received 5×10^6 bone marrow cells in 0.2 mL medium through tail vein injection. BMT from males to females made it easier to assess effective engraftment by identification of Y chromosomes in engrafted hematopoietic cells. Five weeks after transplantation, blood was sampled to determine chimerism by determination of COX-2 expression with PCR.

Cell Culture

Male 129/svj WT or COX-$2^{-/-}$ mice were intraperitoneally injected with 1 mL sterile thioglycollate (3%; Sigma-Aldrich). Four days later, peritoneal cells including macrophages were harvested. Peritoneal macrophages were cultured according to a previous report (27).

Isolation of Kidney Macrophages/Dendritic Cells

CD11b-expressing cells in kidney single-cell suspensions were enriched using mouse CD11b Microbeads and MACS.

Antibodies

Rat anti-mouse F4/80 (MCA497R), CD68 (MCA1957), CD11c (MCA1369), CD3 (MCA1477), CD4 (MCA2961), CD8α (MCA2694), and Ly-6G (Gr-1, MCA2387) were purchased from AbD Serotec; rabbit anti-human fibronectin (FN; F3648) and mouse anti–α-smooth muscle actin (α-SMA, a marker of myofibroblasts; A5228) were from Sigma-Aldrich; rabbit antimurine collagen type I (600-401-103-01) and collagen type IV (600-401-106-01) were from Rockland Immunochemicals; goat anti-human connective tissue growth factor (CTGF; SC-14939) was from Santa Cruz Biotechnology; mouse anti–mannose receptor (MR or CD206; MAB25341) was from R&D Systems; rabbit anti-human interleukin-4 receptor-α (IL-4Rα; or CD124, NBP1-00884) was from Novus Biologicals; rabbit anti–Wilms Tumor Protein (WT1; ab89901), inducible nitric oxide synthase (iNOS; ab3523), and tumor necrosis factor-α (ab6671) were from Abcam; and rabbit-anti–nuclear factor-κB p65 (8284) and mouse-anti–C/EBP homologous protein (CHOP; 2895) were from Cell Signaling Technology. Rabbit anti-renin antiserum (1:6,000 dilutions) was a gift from T. Inagami (Vanderbilt University).

RNA Isolation and Quantitative RT-PCR

Total RNA from tissues and cells were isolated using TRIzol reagents (Invitrogen). Quantitative RT-PCR was performed using TaqMan real-time PCR (7900HT; Applied Biosystems). The Master Mix and all gene probes were also purchased from Applied Biosystems. The probes used in the experiments included mouse S18 (Mm02601778), IL-4Rα (Mm01275139), MR (Mm01329362), chitinase 3–like protein 3 (Ym-1) (Mm00657889), nephrin (Nphs1;

Mm00497828), podocin (Nphs2, Mm01292252), chemokine (C-C motif) ligand 2 (CCL2; MCP-1; Mm00441242), COX-2 (PTGS2; Mm00478374), EP4 (PTGER4; Mm00436053), collagen I (Col1a1; Mm00801666), collagen IV (Col4a1; Mm01210125), α-SMA (acta2; Mm01546133), renin (Mm02342889), angiotensinogen (Mm00599662), ACE (ACE1; Mm00802048), ACE2 (Mm01159003), angiotensin II type I (AT1a; agtr1a; Mm01166161), AT1b (agtr1b; Mm01701115), AT2 (agtr2; Mm01341373), and Mas (Mas1; Mm00627134).

Immunohistochemistry Staining and Quantitative Image Analysis

The animals were anesthetized with Nembutal (70 mg/kg, i.p.; Abbot Laboratories) and given heparin (1,000 units/kg, i.p.) to minimize coagulation. One kidney was removed for immunoblotting and quantitative RT-PCR, and the animal was perfused with 3.7% formaldehyde, 10 mmol/L sodium *m*-periodate, 40 mmol/L phosphate buffer, and 1% acetic acid through the aortic trunk cannulated by means of the left ventricle. The fixed kidneys were dehydrated through a graded series of ethanols, embedded in paraffin, sectioned (4 μm), and mounted on glass slides. Immunostaining was carried out as in previous reports (28). For WT1 staining, antigen retrieval was achieved by boiling in citric acid buffer (100 mmol/L; pH 6) for 3 × 5 min. For staining with mouse monoclonal antibodies (α-SMA and MR), the tissues were blocked and primary and secondary antibodies diluted using reagents from the M.O.M. Kit (PK-2200; Vector Laboratories, Burlingame, CA). On the basis of the distinctive density and color of immunostaining in video images, the number, size, and position of stained area were quantified by using the BIOQUANT true-color windows system (R&M Biometrics, Nashville, TN). Four representative fields from each animal were quantified at ×160 magnification, and their average was used as data from one animal sample. Podocyte density is expressed as podocytes per glomerulus.

Immunoblotting

Cultured cells were lysed, and kidneys were homogenized with buffer containing 10 mmol/L Tris-HCl (pH 7.4), 50 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L EDTA, 0.5% Nonidet P-40, 0.1% SDS, 100 μmol/L Na_3VO_4, 100 mmol/L NaF, 0.5% sodium deoxycholate, 10 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. The homogenate was centrifuged at 15,000*g* for 20 min at 4°C. An aliquot of supernatant was taken for protein measurement with a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Immunoblotting was described in a recent report (29).

Statistics

All values are presented as means, with error bars representing ± SEM. Fisher exact test, ANOVA, and Bonferroni *t* tests were used for statistical analysis.

RESULTS

To determine potential effects of COX-2 expression on monocyte/macrophage phenotype, we isolated and cultured peritoneal macrophages from WT mice and mice with global deletion of COX-2 (COX-$2^{-/-}$). As indicated in Fig. 1, compared with WT mice, peritoneal macrophages from COX-$2^{-/-}$ mice had increased total expression of the proinflammatory, M1 marker iNOS. There was also increased NF-κB expression, indicative of an M1 phenotype. In contrast, there was decreased expression of IL-4Rα, an M2 marker.

As noted in the introductory paragraphs, there is increasing evidence for an important pathophysiologic

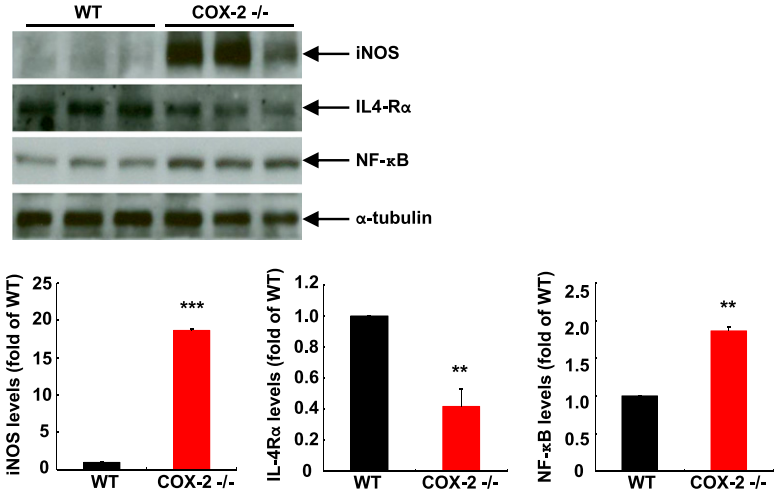

Figure 1—COX-2–deficient macrophages exhibited an M1 phenotype. Peritoneal macrophages from WT and COX-$2^{-/-}$ mice were cultured as described in RESEARCH DESIGN AND METHODS. iNOS (a marker of M1 macrophages) was minimal in WT macrophages but readily detected in COX-$2^{-/-}$ macrophages. In contrast, levels of IL-4Rα (a marker of M2 macrophages) were markedly lower in COX-$2^{-/-}$ macrophages. NF-κB levels were also significantly higher in COX-$2^{-/-}$ macrophages. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT; $n = 3$.

role for infiltrating inflammatory cells in progression of DN (2,3), so in further studies, we investigated the role of COX-2 in mediation of their effects. In our initial studies, we used BMT from either male COX-2$^{-/-}$ or male WT mice into female WT mice (all of them on the 129/svj background), as we have previously reported (30). Following successful transplantation, mice were then rendered diabetic with STZ. Although hyperglycemia was

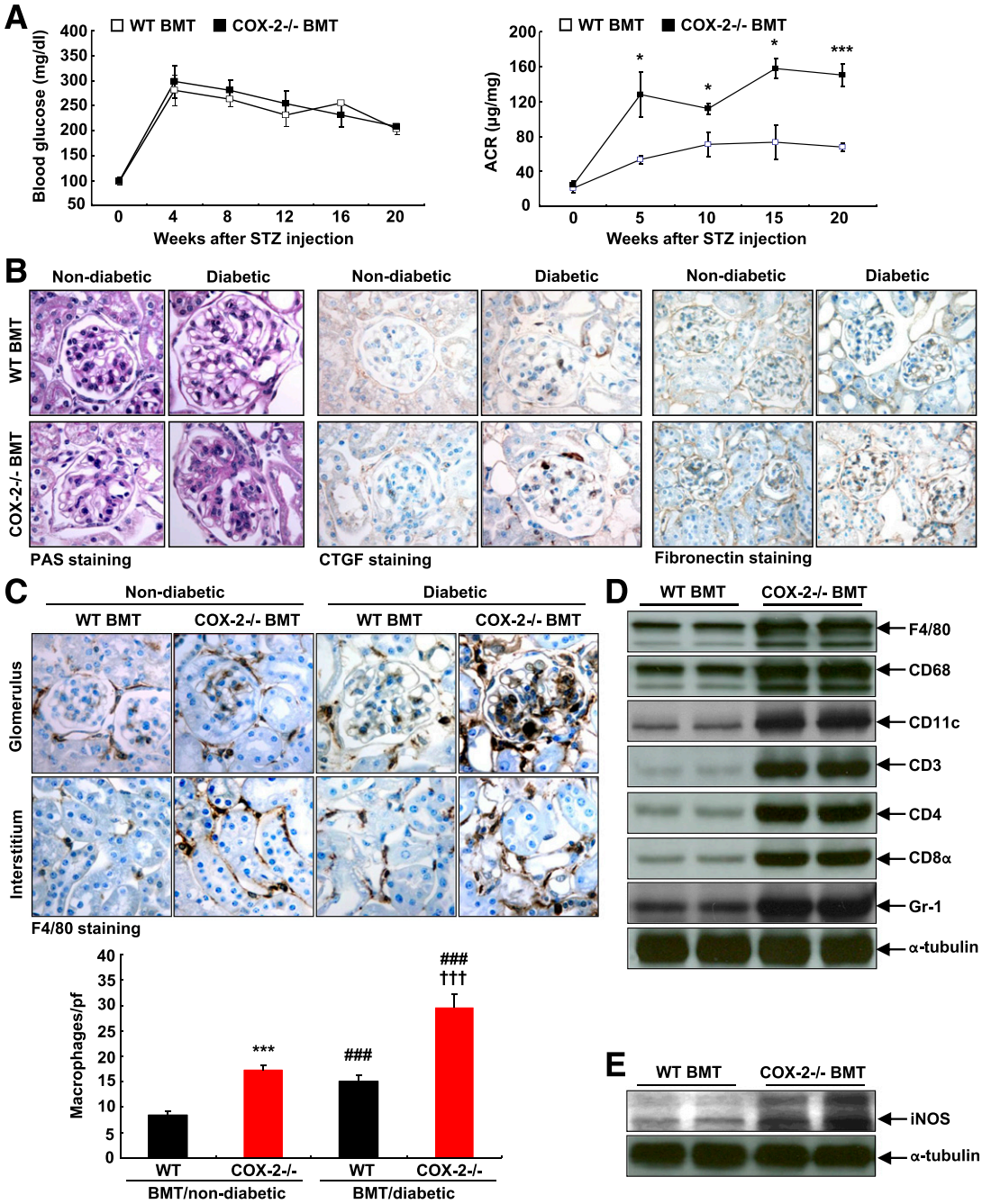

Figure 2—Hematopoietic cell COX-2 deficiency augmented renal infiltration of immune cells and progression of DN. *A*: Although blood sugars were comparable between WT BMT and COX-2$^{-/-}$ BMT diabetic mice, albuminuria (measured by urinary ACR) was markedly augmented in COX-2$^{-/-}$ BMT diabetic mice. **P* < 0.05, ****P* < 0.001 vs. corresponding WT BMT diabetic mice; *n* = 4–6. *B*: PAS staining indicated more mesangial expansion, and immunostaining showed higher levels of profibrotic and fibrotic components of CTGF and FN in COX-2$^{-/-}$ BMT diabetic mice. *C*: Increased renal macrophage infiltration was seen in COX-2$^{-/-}$ BMT nondiabetic kidney. Diabetes led to increases in renal macrophage infiltration in WT BMT mice and further increases in COX-2$^{-/-}$ BMT mice. ****P* < 0.001 vs. WT BMT nondiabetic mice, ###*P* < 0.001 vs. corresponding nondiabetic mice, †††*P* < 0.001 vs. WT BMT diabetic mice; *n* = 4 in each group. *D:* Diabetic kidneys of COX-2$^{-/-}$ BMT mice had higher expression levels of F4/80 (macrophages), CD68 (monocytes/macrophages), CD11c (dendritic cells), CD3 (T lymphocytes), CD4 (CD4 lymphocytes), CD8α (CD8 lymphocytes), and Gr-1 (neutrophils). *E*: iNOS levels were higher in diabetic COX-2$^{-/-}$ BMT kidney than in diabetic WT BMT kidney. Original magnification: FN and CTGF immunostaining: ×160; PAS and F4/80 staining: ×400.

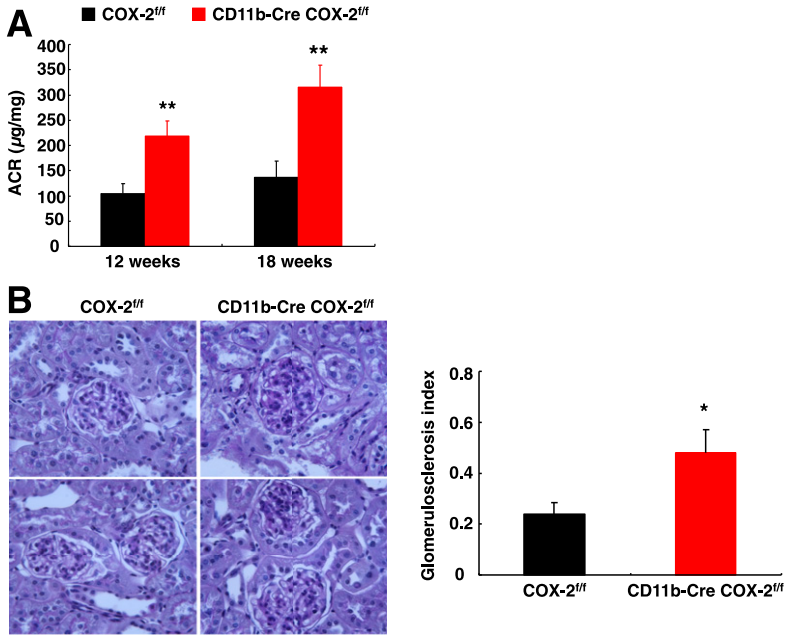

Figure 3—Macrophage COX-2 deficiency augmented development of DN. *A*: Urinary ACR was markedly higher in diabetic CD11b-Cre COX2$^{f/f}$ mice than diabetic WT mice at 12 and 18 weeks after initiation of hyperglycemia. $^{**}P < 0.01$; $n = 5$–9. *B*: PAS staining indicated more severe glomerulosclerosis in diabetic CD11b-Cre COX2$^{f/f}$ mice. $^{*}P < 0.05$; $n = 5$. Original magnification: ×160.

comparable in the two groups, albuminuria was significantly increased in the COX-2$^{-/-}$ BMT mice compared with the WT BMT mice (Fig. 2*A*). Nondiabetic kidneys from COX-2$^{-/-}$ BMT and WT BMT mice had similar normal histology and low levels of FN and CTGF, whereas diabetic kidneys of COX-2$^{-/-}$ BMT mice had increased mesangial expansion and increased expression of FN and CTGF compared with diabetic kidneys of WT BMT mice (Fig. 2*B*). There was increased macrophage infiltration in nondiabetic kidneys of COX-2$^{-/-}$ BMT mice. Although

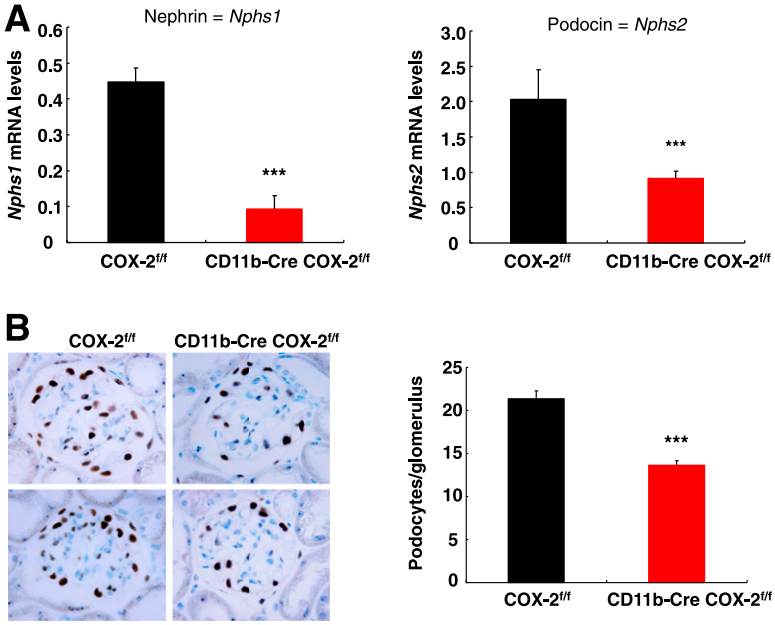

Figure 4—Macrophage COX-2 deficiency led to accelerated podocyte loss in diabetic mice. *A*: Both nephrin and podocin mRNA levels were significantly lower in diabetic CD11b-Cre COX2$^{f/f}$ mice than diabetic WT mice at 18 weeks after initiation of hyperglycemia. $^{***}P < 0.001$; $n = 6$. *B*: Podocyte number in each glomerulus (WT1-positive nuclei) was markedly lower in diabetic CD11b-Cre COX2$^{f/f}$ mice than diabetic wild-type mice. $^{***}P < 0.001$; $n = 4$–7. Original magnification ×400.

renal macrophage infiltration increased in both diabetic WT BMT and COX-2$^{-/-}$ BMT mice, it was still significantly higher in COX-2$^{-/-}$ BMT mice than in WT BMT mice (Fig. 2*C*). The increased renal macrophage infiltration of the COX-2$^{-/-}$ BMT mice was further confirmed by immunoblotting for F4/80 and CD68 (Fig. 2*D*). Furthermore, there was increased expression of the M1 marker iNOS (Fig. 2*E*). In addition to increased macrophage infiltration, kidneys from COX-2$^{-/-}$ BMT mice had increased expression of the dendritic cell marker CD11c, the neutrophil marker Gr-1, and the pan-T cell marker CD3 as well as both CD4 and CD8 (Fig. 2*D*).

To determine more specifically the role of macrophage COX-2 in DN, we backcrossed both COX2$^{f/f}$ mice and CD11b-Cre mice to a more kidney injury–prone strain, FVB. In preliminary studies, we found that macrophages from CD11b-Cre COX2$^{f/f}$ mice had essentially no COX-2 expression (Supplementary Fig. 1*A*). Diabetes was induced in WT (COX2$^{f/f}$) and CD11b-Cre COX2$^{f/f}$ mice with STZ. Compared with WT mice (COX2$^{f/f}$ mice), CD11b-Cre COX2$^{f/f}$ mice had increased albuminuria (Fig. 3*A*) and increased glomerulosclerosis (Fig. 3*B*). Similar relative increases in albuminuria were seen in diabetic CD11b-Cre COX2$^{f/f}$ mice on the nephropathy-resistant C57Bl/6 strain (Supplementary Table 1). Furthermore, CD11b-Cre COX2$^{f/f}$ mice had decreased expression of podocyte markers nephrin and podocin (Fig. 4*A*), and consistent with these decreases, there was a decreased number of podocytes, indicated by WT1 immunolocalization (Fig. 4*B*). Diabetic CD11b-Cre COX2$^{f/f}$ kidneys had increased mRNA and periglomerular and interstitial expression of the myofibroblast marker α-SMA, as well as increased mRNA and glomerular expression of collagens I and IV

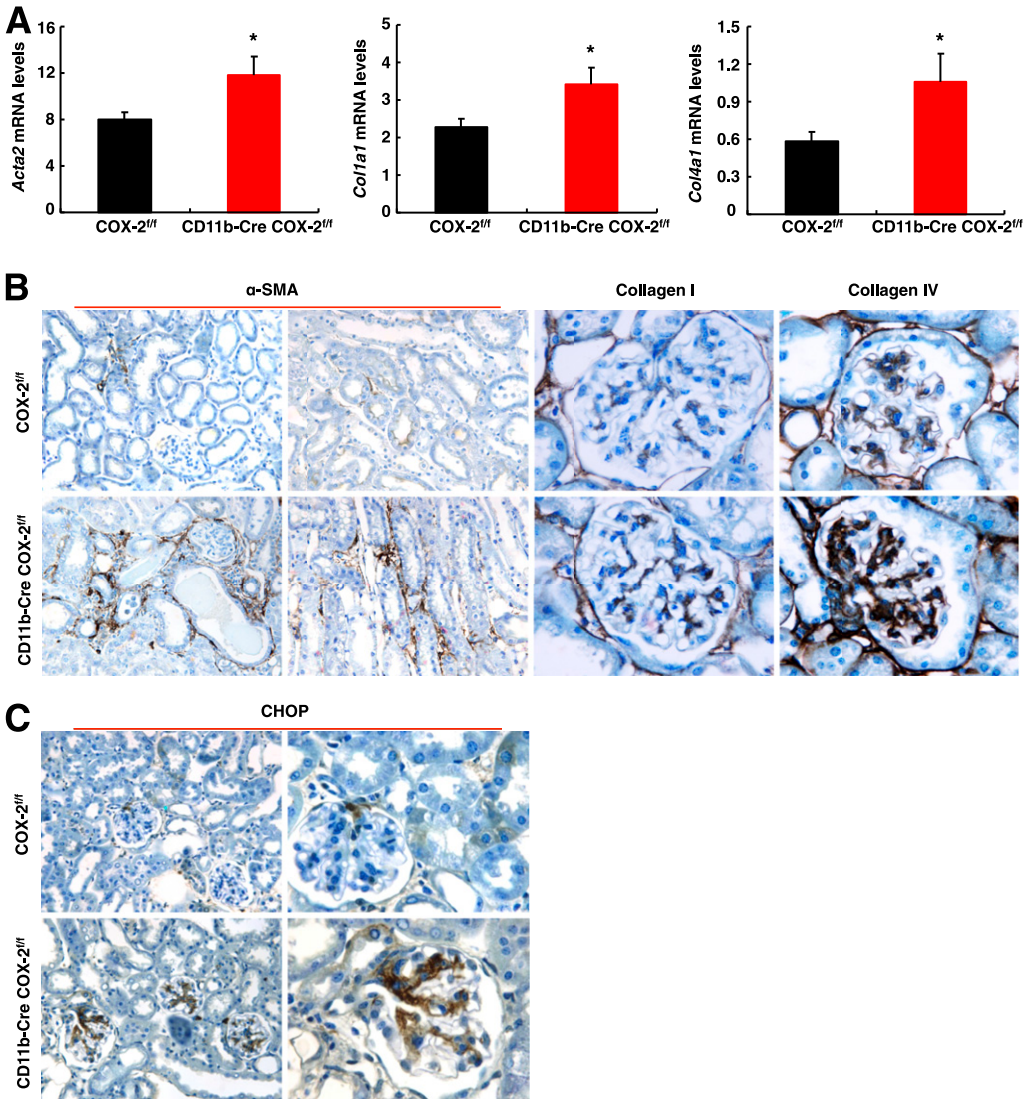

Figure 5—COX-2 deletion in macrophages led to increased renal fibrosis in diabetic mice. Both mRNA levels (*A*) and protein levels (*B*) of α-SMA (marker of myofibroblasts) and collagen I and collagen IV were markedly increased in diabetic CD11b-Cre COX2$^{f/f}$ mice, compared to diabetic WT mice. *$P < 0.05$; $n = 5–7$. *C*: Diabetic CD11b-Cre COX2$^{f/f}$ kidneys had increased ER stress, as indicated by CHOP. Original magnification: α-SMA and left panel of CHOP: ×160; collagen I and IV and right panel of CHOP: ×400.

(Fig. 5*A* and *B*). Of note, diabetic CD11b-Cre COX2$^{f/f}$ kidneys had increased expression of CHOP, a marker of endoplasmic reticulum (ER) stress, consistent with the development of more severe DN in the absence of macrophage COX-2 expression (Fig. 5*C*).

Diabetic CD11b-Cre COX2$^{f/f}$ kidneys had increased levels of the macrophage-attracting chemokine, CCL2 (MCP-1) (Fig. 6*A*) and increased F4/80-positive macrophage infiltration (Fig. 6*B*). Furthermore, there were increases in pan-T cell marker–positive CD3 cells as well as both CD4- and CD8-positive T cells and increased neutrophil infiltration in CD11b-Cre COX2$^{f/f}$ mice (Supplementary Fig. 2).

Diabetic CD11b-Cre COX2$^{f/f}$ kidneys had decreased mRNA for the M2 markers CD206 (MR) and Ym-1 (Fig. 7*A*). Although there were increased F4/80-positive cells in diabetic CD11b-Cre COX2$^{f/f}$ kidneys, there was a significant decrease in MR-positive infiltrating cells (Fig. 7*B*). In addition, macrophages isolated from diabetic CD11b-Cre COX2$^{f/f}$ kidneys had decreased mRNA expression of MR and IL-4Rα (Fig. 7*C*).

As noted in the introductory paragraphs, there is evidence that macrophage activation of EP4 promotes polarization to an M2 phenotype (19,30). We generated CD11b-Cre EP4$^{f/f}$ mice on an FVB background and made them diabetic with STZ. Macrophages from CD11b-Cre EP4$^{f/f}$ mice had markedly decreased expression of EP4 mRNA (Supplementary Fig. 1). Similar to what we observed in diabetic CD11b-Cre COX2$^{f/f}$ mice, macrophage-selective deletion of EP4 led to increased albuminuria (Fig. 8*A*), increased expression of α-SMA, collagen I, and collagen IV as well as CHOP, a marker of ER stress (Fig. 8*B*) and decreased kidney expression of the M2 markers MR and Ym-1 (Fig. 8*C*). Similar relative increases in albuminuria were seen in diabetic CD11b-Cre EP4$^{f/f}$ mice on the nephropathy-resistant C57Bl/6 strain (Supplementary Table 1).

Activation of renin-angiotensin system (RAS) plays an important role in development of DN, and inhibition of RAS attenuates kidney injury. We have previously shown that renal renin expression and activity is regulated by macula densa COX-2 (31). Therefore, we first investigated renal renin expression with immunohistochemistry. As indicated in Supplementary Fig. 3, renal renin expression was comparable between diabetic COX2$^{f/f}$ and CD11b-Cre COX2$^{f/f}$ mice and between diabetic EP4$^{f/f}$ and CD11b-Cre EP4$^{f/f}$ mice. Further analysis showed that renal mRNA levels of the major components of the RAS including renin, angiotensinogen, ACE, ACE2, AT1a and AT1b, AT2, and angiotensin 1–7 receptor (Mas) were comparable between diabetic COX2$^{f/f}$ and CD11b-Cre COX2$^{f/f}$ mice (Supplementary Fig. 4).

DISCUSSION

The current studies demonstrate an important role for the macrophage COX-2/PGE_2/EP4 axis to mitigate against development of DN. Both mice with BMT from global COX-2 knockout mice and mice with selective deletion of COX-2 in macrophages had increased structural and functional injury in response to STZ-induced diabetes. Similar acceleration of renal injury was seen in diabetic mice with selective macrophage deletion of the PGE_2 receptor subtype, EP4. The increased injury with macrophage COX-2 deletion was accompanied by increased infiltration of inflammatory cells, macrophages, neutrophils, and T cells. Although there was

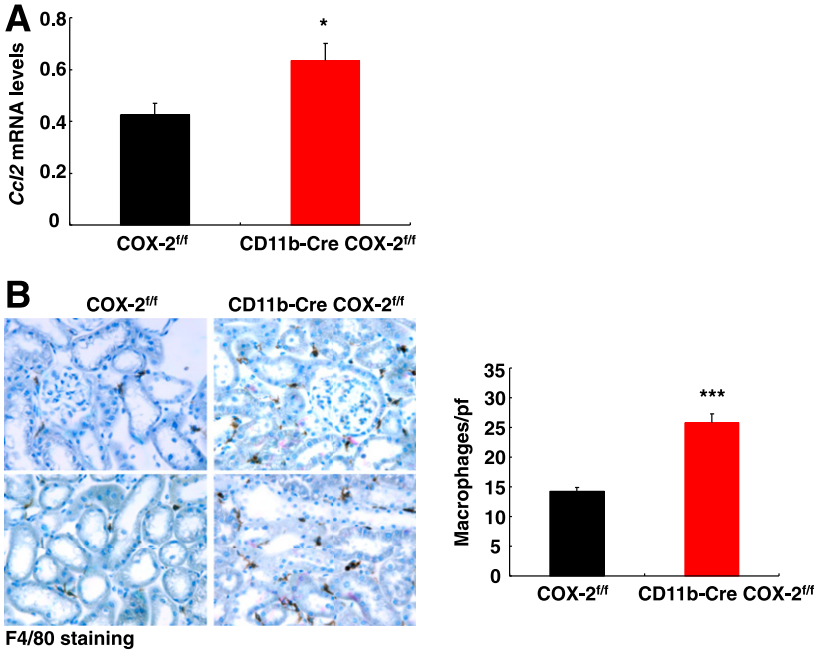

Figure 6—COX-2 deletion in macrophages led to increased renal macrophage infiltration in diabetic mice. *A*: CCL2 (or MCP-1) mRNA levels were markedly increased in diabetic CD11b-Cre COX2$^{f/f}$ mice. *$P < 0.05$; $n = 5–7$. *B*: Renal macrophage infiltration (F4/80-positive cells) was markedly increased in diabetic CD11b-Cre COX2$^{f/f}$ mice. ***$P < 0.001$; $n = 4$. Original magnification: ×250.

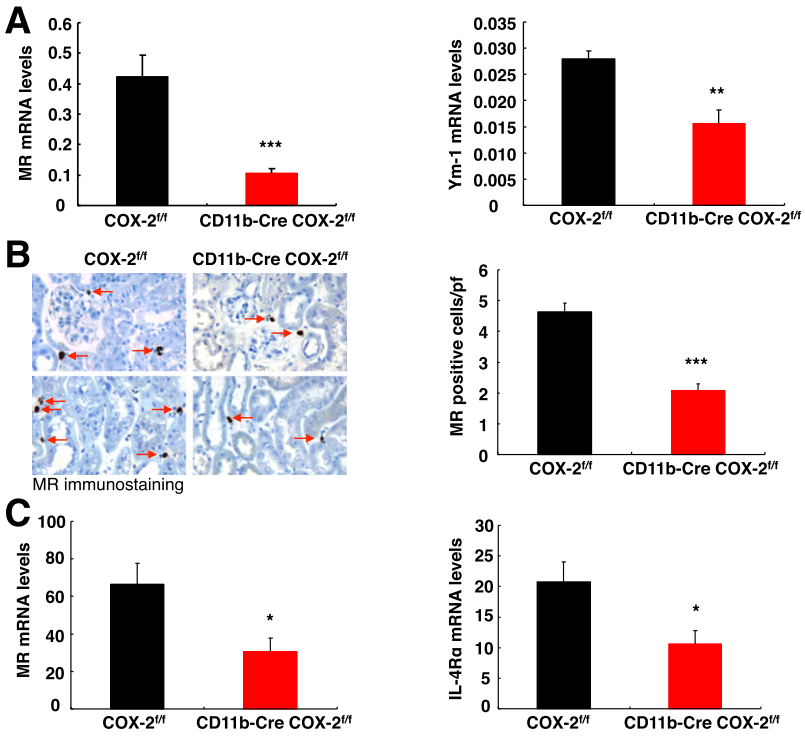

Figure 7—COX-2 deletion in macrophages led to decreased renal M2 macrophages in diabetic mice. *A*: The mRNA levels of MR (CD206) and Ym-1, markers of M2 macrophages, were markedly decreased in diabetic CD11b-Cre COX2$^{f/f}$ kidneys. $^{**}P < 0.01$, $^{***}P < 0.001$; $n = 5$–7. *B*: The number of renal M2 macrophages (MR-positive cells [arrows]) was significantly lower in diabetic CD11b-Cre COX2$^{f/f}$ mice than in diabetic WT mice. $^{***}P < 0.001$; $n = 4$. Original magnification: ×250. *C*: The mRNA levels for both MR and IL-4Rα (M2 markers) were markedly lower in isolated renal macrophages from CD11b-Cre COX2$^{f/f}$ mice than WT mice at 2 weeks after initiation of hyperglycemia. $^{*}P < 0.05$; $n = 4$.

an increase in total numbers of macrophages, the percentage that expressed M2 markers was significantly decreased.

Previous studies by us and others have demonstrated increased expression of intrinsic renal COX-2 localized to the macula densa in both experimental and human diabetes (16–18,32). Administration of selective COX-2 inhibitors decreased diabetes-induced hyperfiltration and decreased renal injury. In addition to mediating hyperfiltration, our previous studies have demonstrated that macula densa COX-2 is an important mediator of the RAS so inhibition of macula densa COX-2 may also inhibit the intrarenal RAS, which has been implicated in progression of DN (33). In addition, we have also previously shown that increased podocyte COX-2 expression accelerated diabetic glomerular injury through increased prorenin receptor expression (34).

However, in contrast to our previous studies that inhibited intrinsic renal COX-2 activity, the current studies indicate an opposing, protective role for macrophage-specific COX-2 activity. COX-2–derived PGE_2 acting through the EP4 receptor is important for macrophage polarization to an M2 phenotype (35). We have previously shown that macrophage EP4 deletion led to decreased macrophage M2 markers in tumors (19). In contrast, EP4 activation inhibits release of the cytokines tumor necrosis factor-α and IL-2 from mouse macrophages (36) and inhibits the NLRP3 inflammasome in human macrophages (37). EP4 is coupled to G_s, and receptor activation activates adenylate cyclase and increases cAMP production, which polarizes macrophages to an M2 phenotype (38).

In diabetic rats, administration of hemin (a heme-oxygenase inducer) suppressed increased M1 macrophages and restored decreased M2 macrophages in association with decreases in proinflammatory cytokine/chemokine, reduction of extracellular matrix/profibrotic protein, and improvement of kidney function and histology (5). Similarly, pentraxin-3 polarized macrophages to an M2 phenotype and ameliorated experimental diabetic renal injury (39). In addition, suppression of renal M1 macrophages by deletion of Toll-like receptors 2 or 4 also protected against development of DN in mice (40,41).

DN is known to be a proinflammatory state. Devaraj et al. (40) have shown that peritoneal and kidney macrophages from mice with STZ diabetes have greater expression of M1 (Ly6c, IL-6, and CCR2) than M2 markers (CD206 and CD163). Monocytes isolated from patients with both type 1 and type 2 diabetes expressed high levels of COX-2 mRNA compared with minimal levels from those isolated from normal nondiabetic volunteers. In human monocyte THP-1 cells, high glucose induced COX-2 mRNA and protein as well as COX-2–derived PGE_2, but had no effect on COX-1 expression (42). The kidney macrophages in the diabetic kidney may be similar to a phenotype described

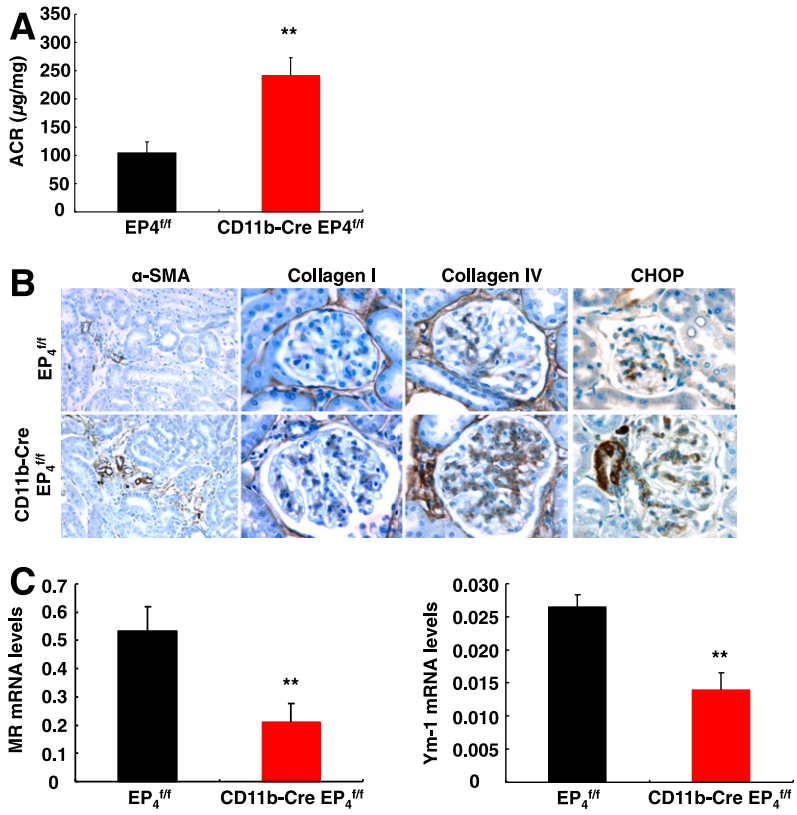

Figure 8—EP4 deletion in macrophages augmented development of DN. *A*: Macrophage EP4 deletion augmented albuminuria at 12 weeks after initiation of hyperglycemia. $^{**}P < 0.01$; $n = 5$. *B:* The levels of α-SMA (marker of myofibroblasts), collagen I, collagen IV, and CHOP (marker of ER stress) were markedly increased in diabetic CD11b-CreEP4$^{f/f}$ mice, compared to diabetic WT mice. Original magnification: α-SMA, ×160; and collagen I and IV and CHOP, ×400. *C*: The mRNA levels of MR and Ym-1, markers of M2 macrophages, were markedly decreased in diabetic CD11b-Cre EP4$^{f/f}$ kidneys. $^{**}P < 0.01$; $n = 5$.

as resolution macrophages. These resolution macrophages do not express markers that characterize them as either classically nor alternatively activated but are a hybrid of both phenotypes. Of note, they are characterized by increased COX-2 expression, and maintenance of this phenotype is dependent upon macrophage-derived cAMP production. They have been postulated to be dispensable for clearing polymorphonuclear neutrophils during self-limiting inflammation but are necessary for postresolution innate lymphocyte repopulation and restoring tissue homeostasis (43). Further studies will be necessary to characterize the functions of macrophages in the diabetic kidney.

The role of COX-2 in the development of DN and other kidney injury is complicated. COX-2 expression increases in both podocytes and macula densa in diabetic kidney (16–18). Overexpression of COX-2 predisposes to podocyte injury and macula densa COX-2 contributes to hyperfiltration in early diabetes (34). Therefore, systemic COX-2 inhibition has beneficial effects because of inhibition of podocyte COX-2 and attenuation of hyperfiltration. The present observations indicate that the beneficial effect of macrophage COX-2 is the result of a specific effect on immune cells. Recently, Nilsson et al. (44) reported that global COX-2 deficiency exacerbated unilateral ureteral obstruction (UUO)–induced kidney damage. Similarly, Kamata et al. (45) reported that inhibition of COX-2 activity with celecoxib exacerbated development of fibrosis in UUO kidneys. However, COX-2 knockdown in macrophages using a chitosan delivery system was reported to attenuate UUO-induced kidney damage in association with decreases in inflammation, oxidative stress, and apoptosis (46). Therefore, the role of COX-2 in kidney injury may depend on the sources of COX-2, the mechanisms of renal injury, the expression and subtype of PGE_2 receptors, and timing of COX-2 inhibition.

In summary, these studies have demonstrated an important but unexpected role for macrophage COX-2 signaling to lessen progression of diabetic kidney disease, unlike the pathogenic effects of increased COX-2 expression in intrinsic renal cells. Although increased COX-2 expression in macrophages is often cited as a characteristic of a proinflammatory, M1 phenotype, these studies indicate that its expression may actually mitigate against detrimental effects in DN.

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