Bone marrow-derived cPLA2α contributes to renal fibrosis progression

John R. Montford, Allison M. B. Lehman, Colin D. Bauer, Jelena Klawitter, Jost Klawitter, Joanna M. Poczobutt, Micah Scobey, Mary Weiser-Evans, Raphael A. Nemenoff, Seth B. Furgeson

Department of Medicine, Renal Division, Department of Anesthesiology, and School of Medicine, Consortium for Fibrosis Research and Translation, University of Colorado Anschutz Medical Campus, Aurora, CO; Denver Veterans Affairs Medical Center, Denver, CO; and Denver Health and Hospitals, Denver, CO

ORCID IDs: 0000-0003-4824-1922 (J.R.M.); 0000-0003-1893-6141 (J.K.); 0000-0002-6413-4820 (J.K.); 0000-0002-5019-6158 (M.W.E.); 0000-0002-2369-2535 (R.A.N.)

Abstract The group IVA calcium-dependent cytosolic phospholipase A2 (cPLA2α) enzyme directs a complex “eicosanoid storm” that accompanies the tissue response to injury. cPLA2α and its downstream eicosanoid mediators are also implicated in the pathogenesis of fibrosis in many organs, including the kidney. We aimed to determine the role of cPLA2α in bone marrow-derived cells in a murine model of renal fibrosis, unilateral ureteral obstruction (UUO). WT C57BL/6j mice were irradiated and engrafted with donor bone marrow from either WT mice [WT-bone marrow transplant (BMT)] or mice deficient in cPLA2α (KO-BMT). After full engraftment, mice underwent UUO and kidneys were collected 3, 7, and 14 days after injury. Using picrosirius red, collagen-3, and smooth muscle α actin staining, we determined that renal fibrosis was significantly attenuated in KO-BMT animals as compared with WT-BMT animals. Lipidomic analysis of homogenized kidneys demonstrated a time-dependent upregulation of pro-inflammatory eicosanoids after UUO; KO-BMT animals had lower levels of many of these eicosanoids. KO-BMT animals also had fewer infiltrating pro-inflammatory CD45+CD11b+Ly6C hi macrophages and reduced message levels of pro-inflammatory cytokines.

Our results indicate that cPLA2α and/or its downstream mediators, produced by bone marrow-derived cells, play a major role in eicosanoid production after renal injury and in renal fibrinogenesis. Montford, J. R., A. M. B. Lehman, C. D. Bauer, J. Klawitter, J. Klawitter, J. M. Poczobutt, M. Scobey, M. Weiser-Evans, R. A. Nemenoff, and S. B. Furgeson. Bone marrow-derived cPLA2α contributes to renal fibrosis progression. J. Lipid Res. 2018. 59: 380–390.

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Chronic kidney disease (CKD) is a highly prevalent disorder that dramatically increases morbidity and mortality. Regardless of etiology, CKD is characterized by histologic renal fibrosis, a final common pathway of CKD progression. Renal fibrosis is driven through complex interactions between renal tubular epithelial cells, resident and recruited monocytes, and other interstitial cells. Published data implicate that renal tubular epithelial cell injury (1) and interstitial pericyte reprogramming (2) are crucial events that drive renal fibrinogenesis. Distinct populations of monocytes are also known to home and differentiate in the injured kidney, mediating both pro- and anti-fibrotic effects in different animal models (3, 4). Furthermore, depletion of specific bone marrow-derived monocyte (BMDM) populations has shown efficacy in reducing renal fibrosis in animal models of ischemia reperfusion injury (5) and unilateral ureteral obstruction (UUO) (6). While

Abbreviations: AA, arachidonic acid; AII, angiotensin II; Alox5, 5-lipoxygenase; BMDM, bone marrow-derived monocyte/macrophage; BMT, bone marrow transplant; CCL, chemokine (C motif) ligand; CKD, chronic kidney disease; cPLA2α, group IVA calcium-dependent cytosolic phospholipase A2α (Pla2g4a); CYP4A12A, cytochrome P450 subunit 4A12a; CYP2C44, cytochrome P450 family 2, subfamily C, polypeptide 23; d12, delta-12; EET, epoxyeicosatrienoic acid; FACS, fluorescence-activated cell sorting; Fwd, forward; IHC, immunohistochemistry; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α; PGJ2, prostaglandin J2; PSR, picrosirius red; qRT-PCR, quantitative RT-PCR; qRT-PCR, reverse; αSMA, smooth muscle α actin; UUO, unilateral ureteral obstruction.

1 To whom correspondence should be addressed.
e-mail: John.Montford@UCDenver.edu
these cells are clearly involved in the pathogenesis of renal fibrosis, much is still unknown regarding the relative roles of each cell type in vivo. For example, defining which cells secrete specific pro-fibrotic factors is still an area of active study.

The group IVA calcium-dependent cytosolic phospholipase A2 (cPLA2α, encoded by Pla2g4a) enzyme is the rate limiting step in the generation of arachidonic acid (AA) for downstream eicosanoid biosynthesis. In response to toll-like receptor ligands, angiotensin II (AII), and other stimuli, intracellular calcium influx activates cPLA2α releasing free AA from its esterified form in glycerophospholipids (7, 8). AA is then shunted through several downstream pathways to produce a family of bioactive lipid mediators that signal through autocrine and paracrine pathways in multiple tissues. In this fashion, cPLA2α is the primary director of a complex “eicosanoid storm” that directs the innate immune system during acute injury and is implicated in the development of fibrosis in multiple organs (8–11). Studies using pharmacologic agents or genetic KO mice suggest that cPLA2α affects the development of neonatal hydropnephrosis (12), cisplatin-induced acute kidney injury (13), and AII-induced renal fibrosis and inflammation (8). Furthermore, cPLA2α is implicated in the development of hepatic (11) and pulmonary fibrosis (10), AII-induced heart failure (9), collagen-induced arthritis (14), and autoimmune encephalitis (15).

cPLA2α provides substrate for metabolism to distinct eicosanoid species, which are secreted by specific cell types. The 5-lipoxygenase (Alox5)-derived leukotrienes are produced mainly by leukocytes, while metabolites derived from other lipoxygenases, such as 15-lipoxygenase (encoded by Alox15), cytochrome P450 enzymes, and cyclooxygenases-1 and -2 (COX-1 and -2, encoded by prostaglandin synthases-1 and-2) are produced by multiple cell types, including leukocytes, epithelial cells, vascular smooth muscle cells, mesangial cells, and endothelial cells (16–18). These enzymes are expressed throughout the nephron (19, 20) and their products mediate a host of physiologic and pathologic processes in animal models of renal disease (21–23). We have shown that cPLA2α expression in human renal tubular epithelial cells promotes epithelial dedifferentiation and proliferation (24), and others have implicated cPLA2α in the control of the G2-M cell cycle checkpoint (25). These biological effects could be sufficient to modify renal injury and fibrosis, given the importance of tubular epithelial damage in renal fibrogenesis (26, 27). However, because eicosanoids are major effectors produced by leukocytes, it stands to reason that manipulating pathways controlling eicosanoid expression in bone marrow-derived cells may alter the renal response to injury. The aim of this study was to investigate the role of cPLA2α and eicosanoids specifically produced by bone marrow-derived immune cells after renal injury using a UUO model in mice. We hypothesized that deletion of cPLA2α from the bone marrow alone would blunt pro-inflammatory eicosanoid production by leukocytes after UUO, and this might be associated with amelioration of renal fibrosis.

MATERIALS AND METHODS

Generation of chimeric mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. Six-week-old WT C5BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were lethally irradiated using 1,000 centi-gray (cGy) split dosing X-ray. Donor WT and cPLA2α-deficient (Pla2g4a−/−) mice were euthanized; femurs and tibias were aseptically removed; and bone marrow was obtained through irrigation with sterile HBSS. Cells were resuspended in HBSS at a concentration of 1 × 10⁷ cells/ml. One hour following the second dose of irradiation, isoflurane-aneasthetized WT recipients were injected with donor marrow from WT or Pla2g4a−/− animals via retro-orbital injection (2 × 10⁶ bone marrow-derived cells per recipient). Newly transplanted animals were given Uniprim chow (Envigo, Denver, CO) for 2 weeks after bone marrow transplantation. Mice were allowed to recover and engraft for a total of 5 weeks prior to further experimentation. Separate nontransplanted animals were used to confirm lethal radiation dose.

UUO

Nonirradiated Pla2g4a−/− mice were injured using UUO to determine cPLA2α expression in WT mice. Under isoflurane anesthesia, a retroperitoneal flank incision was made, the right kidney exteriorized, and the proximal ureter ligated with 4-0 silk suture. The abdominal cavity was then washed with 1.0 ml of sterile saline, the peritoneum closed with vicryl suture, and the skin stapled. After 14 days, the animals were terminally anesthetized and obstructed (UUO) and contralateral unobstructed (Ctrl.) kidneys were collected, snap-frozen in liquid nitrogen, and later homogenized for protein measurements as below.

Later, the fully engrafted chimeric mice generated above (11–12 weeks old) underwent UUO. At 3, 7, and 14 days after UUO, separate animal groups were euthanized. We chose animals obstructed for 14 days after UUO [N = 8 WT-bone marrow transplant (BMT) (WT-BMT), N = 8 KO-BMT animals] to determine histologic changes in fibrosis, RNA, and protein measurements. For eicosanoid analysis, WT-BMT (N = 10) and KO-BMT (N = 12) animals underwent UUO for a total of 7 days. For flow cytometry, UUO was performed for two time points: 3 days (N = 6 WT-BMT, N = 6 KO-BMT) and 7 days (N = 6 WT-BMT, N = 6 KO-BMT). These time points were picked specifically for these measurements based on published and unpublished data using the UUO model. Terminally anesthetized animals underwent exsanguination via right atrial puncture and the left ventricle was perfused with ice-cold PBS and heparin. Additionally, to confirm the degree of chimerism, femurs were isolated from a random sample of both animal groups within each experiment and monocytes were cultured as previously described (28, 29). Cultured monocytes were collected for RNA using buffer RLT (Qiagen, Germantown, MD) or protein using mammalian protein extraction reagent (Thermo Fisher, Waltham, MA) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and cPLA2α expression was determined as below.

Histology and immunofluorescence

At the time of collection, control and UUO kidneys were cut into serial transverse sections, which were placed in either 10% phosphate-buffered formalin (Fisher Chemical, Pittsburgh, PA) or OCT compound (Sakura Finetek, Torrance, CA) for light microscopy and immunofluorescence, respectively. Formalin-suspended tissues were fixed for a total of 24 h, processed, paraffin embedded, and cut in 4.0 μm sections. Tissues were then stained for picrosirius red (PSR) as previously described (30). For collagen III immunohistochemistry (IHC), antigen retrieval (Vector Laboratories, Burlingame, CA) was followed by block for...
endogenous peroxidase activity using 0.3% H2O2 in 100% methanol, with Dako protein block (Agilent Technologies, Santa Clara, CA) and application of goat anti-collagen type 3 (SouthernBiotech, Birmingham, AL) diluted in Dako antibody diluent (Agilent) (1:100). Slides were incubated overnight then washed with PBS followed by application of Dako rabbit anti-goat antibody (Agilent) (1:200) and DAB reagent (Vector). Substrate color reaction was optimized under light microscopy. The reaction was quenched with deionized water and slides were counterstained in Harris modified hematoxylin (Fisher). Slides were scanned using an Aperio ScanScope (Leica Biosystems, Buffalo Grove, IL) and analyzed using ImageScope software (Leica) for fibrosis with a color deconvolution algorithm created separately for PSR and collagen 3 IHC. For immunofluorescence, 5.0 μm frozen sections were cut from OCT blocks. Tissues were fixed in 4% paraformaldehyde for 20 min, incubated with a Cy3-conjugated smooth muscle α actin (αSMA) antibody (Sigma-Aldrich) (1:200) for 1 h, and then mounted in medium containing DAPI (Vector). Images were taken with a Zeiss LSM 780 confocal microscope. Ten random 20× cortical fields per slide were imaged and percent αSMA labeling was determined using ImageJ software.

**Quantitative RT-PCR**

Snap-frozen tissues were homogenized using a sterile head homogenizer (Qiagen) in buffer RLT and β-mercaptoethanol. RNA purification was performed using an RNeasy Plus kit (Qiagen) and concentration determined using a spectrophotometer (NanoDrop, Wilmington, DE). cDNA was made from RNA template using a commercially available system (QuanT蚴b, Beverly, MA). Quantitative (q)RT-PCR reaction was performed with SYBR Green PCR master mix (Applied Biosystems, Thermo Fisher). All PCR reactions included no template and no reverse transcriptase controls. Sequence-specific primers were as follows: GAPDH: forward (Fwd) 5′-CTGGAAGTCTCTGCTCGTCAG-3′, reverse (Rev) 5′-GAGGTAGTCAACTACAGGGA-3′; αSMA: Fwd 5′-GCTCTCCAGCTATCTGTCAG-3′, Rev 5′-CCATTCACCATATTCTAGTA-3′; fibronectin: Fwd 5′-AGACTGGATCGACCACTATCC-3′, Rev 5′-AATGTGTCTCTTGAGGCCATAG-3′; Pla2a: Fwd 5′-AGATGGACAATGGCAGGGCC-3′, Rev 5′-AGTGTCCAGCATAATGGC-3′; TNFα: Fwd 5′-GTCCTCCAAGAGATGGAA-3′, Rev 5′-GACCTGACTACAGGGCTT-3′; chemokine (CC motif) ligand (CCL)2: Fwd 5′-GCTGAGTATTCCCTGCTGACA-3′, Rev 5′-GTCGCTGAGACATCAAGGCA-3′; CCL12: Fwd 5′-GACACGTGTCTCTGCTCCT-3′, Rev 5′-CGATCTGGTGCACCAATA-3′; CXCL1: Fwd 5′-CTAAGTGGCTTCGCA-3′, Rev 5′-TGCTGCTGCTCAGGAGCTG-3′; COX1+: prostanoid synthase-1 (PTGS1): Fwd 5′-ATGATGTCCATGC-3′, Rev 5′-CTAGTGTGATAGTGCCAGG-3′; COX2-: prostanoid synthase-2 (PTGS2): Fwd 5′-TGACCAATCTTGAAACCCAGC-3′, Rev 5′-GACGCTATGCTCGTACTAC-3′; microsomal prostaglandin E2 synthase-1 (PTGES): Fwd 5′-GACA- CATCTGTTGCTAC-3′, Rev 5′-AATGGATCAGCAAAGCCG-3′; prostacyclin synthase (PTGS2): Fwd 5′-AGGGATGTTTTTCCACTCCT-3′, Rev 5′-CAGAGGCGTGGTTGGAC-3′; Alox5: Fwd 5′-ACTACATCATCCCGACTTCT-3′, Rev 5′-GGATGACGTAGAGGACG-3′; 15-lipoxygenase (Alox15): Fwd 5′-GGTTGAGAAGCTGACGGGC-3′, Rev 5′-GATTGGTGTGACTTCTGACG-3′; cytochrome P450 subunit 4a12a (CYP4A12A): Fwd 5′-GGGGACCTCCTATCCGGAATG-3′, Rev 5′-ACTTGGTACAGGAGGAGT-3′, and CYP2C4: Fwd 5′-CCCGCTTCTGTGTCCTCATCT-3′, Rev 5′-GTCTTGGATCAACACTTCTTGG-3′.

**Western blot**

Snap-frozen tissues were homogenized in mammalian protein extraction reagent buffer (Thermo Fisher) with protease inhibitor cocktail (Sigma-Aldrich). Solubilized proteins were centrifuged at 18,400 g in a microcentrifuge (4°C) for 25 min. The protein concentration of supernatants was determined using a Bradford assay (Bio-Rad, Hercules, CA). Protein was separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to ImmobilonP membranes (Millipore, Billerica, MA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline [10 mM Tris-HCl (pH 7.4), 140 mM NaCl] containing 0.1% Tween-20 (TTBS) and 5% BSA (Sigma), and then incubated with BSA in TTBS containing primary antibodies for 1 h at 4°C. Membranes were washed in TTBS and bound antibodies were visualized with horseradish peroxidase-coupled secondary antibodies and ECL reagent (Thermo Fisher) according to the manufacturer’s directions. The antibodies used were: rabbit anti-fibronectin (Abcam, Cambridge, MA) (1:20,000), rabbit αSMA (Abcam) (1:20,000), rabbit anti-cPla2a (Cell Signaling, Danvers, MA), and rabbit anti-GAPDH (Santa Cruz, Dallas, TX) (1:30,000). Goat anti-rabbit (Santa Cruz) (1:5,000) was used as a secondary antibody.

**Fluorescence-activated cell sorting analysis**

Kidneys were placed in DMEM/F12 medium (Mediatech, Manassas, VA), mechanically dissociated, and incubated in Liberase TL and DNase (both Sigma-Aldrich) for 30 min. The reaction was then terminated with ice-cold FA3 buffer (PBS, 10 mM HEPES, 2 mM EDTA, 1% FBS). Contralateral unobstructed kidneys and spleens were used for negative and positive fluorescence-activated cell sorting (FACS) controls, respectively. Samples were washed, passed through 70 μm and 40 μm cell strainers and then treated with RBC lysis buffer [4.01 g NH4Cl, 500.6 mg KCl/2, 2.5 ml 0.5 M NaEDTA, and deionized water (pH 7.2)] for 3 min. Cells were suspended at a final concentration of 1.0 × 106 cells/ml in FA3 buffer. Cells were stained for 1 h at 4°C with the following antibodies: CD45-PE (Clone 30-F11; BD Biosciences), CD11b-FITC (Clone M1/70; BD Biosciences), Ly6G-PE-Cy7 (Clone I-Ab; BD Biosciences), Ly6C-PerCP-Cy5.5 (Clone HK1.4; BioLegend, San Diego, CA), and CD11c-APC-Cy7 (Clone HL3, BD Biosciences). Cells were analyzed for flow cytometry at the University of Colorado Cancer Center Flow Cytometry Core using a Gallios flow cytometer (Beckman Coulter Life Sciences, Indianapolis, IN). Data were analyzed using Kaluza software (Beckman Coulter).

**Eicosanoid profiling**

Flash-frozen specimens were homogenized similarly to RNA and protein specimens using a 1:1 (v/v) mixture of methanol and homogenization buffer [0.1 M phosphate (pH 7.4), 1 mM EDTA, and 10 μM indomethacin], which was normalized to tissue weight (10 μl buffer per milligram tissue). Samples were centrifuged at 18,400 g for 10 min and transferred into HPLC vials. Upon addition of the internal standard solution (see below), 500 μl of the supernatants were injected onto a 3.0 × 5 mm guard column (Halo, C8, 2.7 μM; Advanced Materials Technology, Wilmington, DE) and back flushed with 100% acetonitrile/methanol (1:1, v/v) onto a 3.0 × 1.0 mm analytical column (Halo C8, 2.7 μM; Advanced Materials Technology). For HPLC separation, the starting mobile phase consisted of 40% water supplemented with 0.1% formic acid (buffer A) and 60% buffer B (acetonitrile/methanol, 1:1, v/v) with a flow of 0.8 ml/min for the first minute. After 2.5 min, the gradient increased to 53% B and further to 70% B within 8.5 min. At 11.5 min, buffer B was at 95% and was held for 1 min. The column was re-equilibrated for 2 min to starting conditions for a total of 14.5 minutes of analysis time. The API5500 mass spectrometer (AB Sciex, Concord, ON, Canada) was run in the negative ESI multiple reaction monitoring mode. The following hydroxy-fatty acids were quantified: prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), delta-12 (d12)-PGD2, prostaglandin J2 (PGJ2),...
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15-deoxy-PGJ2, prostaglandin F2α (PGF2α), 5-HETE, 11-HETE, 12-HETE, 15-HETE, 20-HETE, (±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid [(±)8(9)-EET], (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid [(±)11(12)-EET], and leukotriene (L)B4, LC4, LD4, and LE4. All quantifications were performed using freshly prepared calibration curves; the performance of the assay was monitored by inclusion of multiple quality control samples. The following internal standards were used: PGD2-d9, 13,14-dihydro-15-keto PGD2-d4, 15-deoxy-Δ12,14-PGJ2-d4, 11β-PGF2α-d4, PGE2-d9, PGF2α-d9, 12-HETE-d8 (for both 12-HETE and 20-HETE), (±)8(9)-EET-d11, (±)11(12)-EET-d11, (±)14(15)-EET-d11, LB-d4, LC-d5, LD-d5, and LE-d5. All compounds were purchased from Cayman Chemicals. No chiral analysis of hydroxylated fatty acids was performed, thus no information about the enzymatic source can be determined using this method.

RESULTS

cPLA2α expression is increased after UUO in mice

To evaluate changes in cPLA2α levels during renal injury, WT mice underwent UUO. After 14 days, kidneys were harvested and homogenized, and protein was analyzed for expression of cPLA2α by Western blot. Compared with contralateral unobstructed control specimens, cPLA2α was increased 4-fold in UUO-injured tissues (Fig. 1A). To analyze the contribution of bone marrow-derived cells to overall cPLA2α levels, we generated chimeric mice by transplanting Pla2g4a+/+ animals with bone marrow from either Pla2g4a−/− mice (KO-BMT) or Pla2g4a+/+ mice (WT-BMT). Following the generation of chimeric mice, we analyzed cPLA2α expression in primary cultured BMDMs derived from a random selection of animals during euthanization at 14 days. Protein levels of cPLA2α were virtually nonexistent in KO-BMT BMDMs (Fig. 1B), suggesting a low probability of mixed-chimerism in the transplanted animals. Using Western blot and qRT-PCR, cPLA2α expression was diminished, but not eliminated, in kidneys after UUO among mice receiving KO-BMT (37% relative protein reduction vs. WT-BMT; \( P \leq 0.0351 \); CI -0.1883 to -0.01137; Fig. 1C) (24% relative mRNA reduction vs. WT-BMT; \( P = 0.0344 \); CI 0.02491–0.7976; Fig. 1D).

Renal fibrosis is attenuated after UUO in mice chimeric for cPLA2α

To determine whether the presence or absence of cPLA2α from infiltrating bone marrow-derived cells influences renal fibrosis, we evaluated histologic specimens from WT-BMT and KO-BMT animals 14 days after UUO. We observed significantly more fibrosis among WT-BMT as compared with KO-BMT groups utilizing nonpolarized PSR staining (13.81% vs. 9.91%, respectively) \( P = 0.029 \); CI 0.3346–7.476), collagen 3 IHC (4.01% vs. 2.53%, respectively) \( P = 0.034 \); CI 0.0995–2.845), and αSMA immunofluorescence (23.53% vs. 11.51%, respectively) \( P = 0.002 \); CI 5.55–18.5) (Fig. 2A). Additionally, WT-BMT engrafted animals had significantly more protein levels of αSMA \( (P = 0.007 ; \text{CI} 0.1399–1.013) \) and fibronectin \( (P = 0.047 ; \text{CI} 0.0076–1.642) \) compared with KO-BMT obstructed animals, as determined by Western blot (Fig. 2B). Message levels for αSMA \( (P = 0.0351 ; \text{CI} 0.1813 \text{ to } -0.01137) \) and fibronectin \( (P = 0.0156 ; \text{CI} -0.1853 \text{ to } -0.343) \) were similarly elevated in obstructed WT-BMT compared with KO-BMT animals (Fig. 2C).

![Fig. 1.](image-url) cPLA2α expression is increased in mice after UUO and blunted by bone marrow chimerism. WT C57BL/6J mice were subjected to UUO and after 14 days, Ctrl. and UUO specimens were collected. A: Western blot demonstrating cPLA2α expression in both Ctrl. and UUO specimens with corresponding densitometry. Subsequently, engrafted WT-BMT and KO-BMT mouse groups were injured by UUO and euthanized after 14 days. B: Western blot and densitometry for cPLA2α in cultured BMDMs from both groups taken at the time of euthanization. cPLA2α protein expression (C) and mRNA expression (D) were also determined in homogenized renal tissues from these same animals. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), ****\( P < 0.0001 \).
Pro-inflammatory cytokines and major eicosanoid enzymes have altered expression after UUO in mice chimeric for cPLA2α

We next examined message levels of cytokines known to be involved in the tissue inflammatory response and recruitment of leukocytes during UUO progression. Compared with WT-engrafted animals, mice chimeric for cPLA2α had significantly diminished expression of TNFα, CCL2, CCL12, and chemokine (C-X3-C motif) ligand-1 (fractalkine or CX3CL1) (Fig. 3A). Major eicosanoid enzymes that metabolize AA downstream of cPLA2α were also analyzed to determine any changes after UUO between groups (Fig. 3B). Message levels of COX-1 (Ptgs1), prostacyclin synthetase (Ptgis), microsomal prostaglandin E synthase-1 (Ptges), and Alox5 were significantly increased to the same extent in obstructed kidneys from both WT-BMT- and KO-BMT-engrafted animals versus control specimens. Levels of Ptg2 and Alox15 were significantly increased in KO-BMT- relative to WT-BMT-engrafted animals subjected to UUO. Conversely, levels of Cyp4a12a and subunit 2c44 (Cyp4a12a) were reduced in obstructed versus nonobstructed specimens from both animal groups, with no differences observed between the bone marrow-transplanted groups.

Production of pro-inflammatory eicosanoid species is blunted 7 days after UUO in cPLA2α-chimeric mice

To define the contribution of cPLA2α in bone marrow-derived cells to eicosanoid production, we used LC-MS/MS to profile eicosanoids in kidney homogenates derived from control and UUO tissues from WT-BMT and KO-BMT animals euthanized at 7 days after UUO (Table 1, Fig. 4). We observed a robust increase in the pro-inflammatory AA-derived metabolites, PGE2, PGD2, PGJ2, d12-PGD2, PGF2α,
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and 11-, 12-, and 15-HETE, in obstructed kidneys from WT-BMT-engrafted animals versus unobstructed controls. Conversely, products of cytochrome P450 (CYP450) metabolism, such as 20-HETE and (±)14(15)-EET, appeared to decline in obstructed versus nonobstructed specimens. Relative to levels in WT-BMT-obstructed animals, KO-BMT mice had reduced levels of PGE2 (6,305 vs. 4,621 pg/ml, respectively; \(P = 0.035\); 95% CI 90.56–3,277), PGD2 (2,357 vs. 1,045 pg/ml, respectively; \(P = 0.009\); 95% CI 269.7–2,354), 11-HETE (178.5 vs. 102.4 pg/ml, respectively; \(P = 0.042\); 95% CI 19.22–150.2), 12-HETE (327.6 vs. 109.5 pg/ml, respectively; \(P = 0.016\); 95% CI 31.69–404.5), and 15-HETE (369.7 vs. 226.1 pg/ml, respectively; \(P = 0.028\); 95% CI 11.99–275.1). Levels of d12-PGD2 (275.9 vs. 202.4 pg/ml, respectively; \(P = 0.26\); 95% CI −33.05 to 180) and PGF2α (1,207 vs. 831.5 pg/ml, respectively; \(P = 0.06\); 95% CI −11.79 to 736.6) showed similar trends, but these results were not statistically significant. No significant differences in CYP450 products were observed between obstructed WT-BMT and KO-BMT specimens. Other eicosanoid species, such as 5-HETE, (±)8(9)-EET, (±)11(12)-EET, and LB4, LC4, LD4, and LE4 were measured in all samples, but the values were below the limits of detection.

Early influx of pro-inflammatory macrophages after UUO is significantly reduced in cPLA2α KO-chimeric mice

To profile inflammatory cell populations after UUO and define changes between WT-BMT and KO-BMT mice, we performed flow cytometry on single cell suspensions obtained from kidneys harvested 3 and 7 days after UUO. We observed statistically significant reductions in the percentage of total leukocytes (CD45+ cells) (WT-BMT 26.08% vs. KO-BMT 18.07% live; \(P = 0.0462\); CI 0.1356–15.48), and “M1-like” pro-inflammatory macrophages (CD45+Ly6G-CD11b+Ly6Chi cells) (WT-BMT 3.53% vs. KO-BMT 2.04% live; \(P = 0.298\); CI 0.139–2.838) at 3, but not 7, days after UUO (Fig. 5). No statistically significant differences were found in neutrophil (CD45+Ly6G+), dendritic cell (CD45+Ly6C-CD11c+), or “M2-like” pro-fibrotic macrophage (CD45+Ly6G-CD11b+Ly6Clow) at 3 or 7 days after UUO.

DISCUSSION

In this study, we have demonstrated that: 1) cPLA2α expression is increased during renal injury induced by UUO; 2) bone marrow-derived cells significantly contribute
to increased eicosanoid biosynthesis during UUO; and 3) silencing cPLA2α specifically from infiltrating bone marrow-derived cells is sufficient to blunt inflammation and fibrosis during progression of UUO. To our knowledge, this is the first study implicating that bone marrow-derived cPLA2α influences renal fibrinogenesis. Other investigators previously showed that bone marrow-derived cPLA2α influences endothelial dysfunction in L-NAME-induced hypertension using a similar irradiation/bone marrow transplantation approach (31). Recently published data has also shown that COX-2 expression, specifically in BMDMs, mediates salt-sensitive hypertension (32). Furthermore, in vitro studies have demonstrated that cPLA2α in leukocytes controls important biological functions, such as mast cell degranulation (33) and eicosanoid class switching during inflammasome formation (34). Our data contribute to this and other published work implicating that recruited bone marrow-derived cells and their eicosanoid products are key mediators of renal injury leading to fibrosis.

Our data raise interesting questions regarding eicosanoid regulation and expression in the injured kidney. The largest increase in production of acute pro-inflammatory eicosanoids after UUO was observed with PGE2, PGD2, PGF2α, and 12- and 15-HETEs (Table 1). The enzymes responsible for production of these lipid mediators are known to be expressed at various sites of the nephron (19, 20). However, production of many of these species, particularly PGD2 and 12- and 15-HETE during UUO was significantly blunted by bone marrow chimerism for cPLA2α (Fig. 4). These data suggest that bone-marrow-derived cells are key contributors of distinct pro-inflammatory eicosanoids at the tissue level during UUO progression. Obstructed KO-BMT mice did not completely lose expression of cPLA2α relative to WT-engrafted controls (Fig. 1B), which implies that a significant degree of upregulation in cPLA2α after UUO occurs from nonleukocytes. We noted upregulation in mRNA levels of Ptgs-2 and Alox15 in obstructed KO-BMT animals (Fig. 3B), which is consistent with compensation, potentially by resident cell types. Regarding PGE2, we noted significantly reduced levels in KO-BMT animals subjected to UUO. However, we detected that significant production occurs after UUO in these animals, suggesting that resident cell types can compensate in the absence of leukocyte-specific cPLA2α expression. Unfortunately, we were not able to generate chimeric Pla2g4a−/− mice to serve as recipients of WT or

### Table 1. Blunting of pro-inflammatory eicosanoids in obstructed mice chimeric for cPLA2α

| Product | WT-BMT Control (pg/ml) | WT-BMT UUO (pg/ml) | KO-BMT Control (pg/ml) | KO-BMT UUO (pg/ml) |
|---------|------------------------|--------------------|------------------------|--------------------|
| PGE2    | 2,847                  | 6,385              | 2,641                  | 4,629              |
| Median  | 2,847                  | 6,385              | 2,641                  | 4,629              |
| Mean (±SEM) | 2,847 ± 393.8        | 6,385 ± 592.8      | 2,641 ± 381.7         | 4,629 ± 392.4      |
| PGD2    | 377                    | 2,299              | 417                    | 957                |
| Median  | 479.3 ± 101.3          | 2,357 ± 565.6      | 477.3 ± 55.01         | 1045 ± 217         |
| Mean (±SEM) | 479.3 ± 101.3          | 2,357 ± 565.6      | 477.3 ± 55.01         | 1045 ± 217         |
| PGJ2    | 13.7                   | 27.25              | 15.15                  | 26.6               |
| Median  | 13.82 ± 1.06           | 28.52 ± 4.29       | 15.45 ± 1.44          | 25.63 ± 3.18       |
| Mean (±SEM) | 13.82 ± 1.06           | 28.52 ± 4.29       | 15.45 ± 1.44          | 25.63 ± 3.18       |
| d12-PGJ2 | 112                    | 289.5              | 156                    | 202                |
| Median  | 133.1 ± 24.6           | 275.9 ± 38.03      | 139.7 ± 14.26         | 202 ± 27.9         |
| Mean (±SEM) | 133.1 ± 24.6           | 275.9 ± 38.03      | 139.7 ± 14.26         | 202 ± 27.9         |
| PGF2α   | 664.5                  | 1,078              | 755                    | 890                |
| Median  | 662 ± 44.5             | 1,207 ± 128.1      | 721 ± 42.52           | 831.5 ± 122.1      |
| Mean (±SEM) | 662 ± 44.5             | 1,207 ± 128.1      | 721 ± 42.52           | 831.5 ± 122.1      |
| 11-HETE | 53.2                   | 175.7              | 67.5                   | 90.6               |
| Median  | 63.2 ± 12.3            | 178.5 ± 40.86      | 70.19 ± 7.96          | 102.4 ± 12.01      |
| Mean (±SEM) | 63.2 ± 12.3            | 178.5 ± 40.86      | 70.19 ± 7.96          | 102.4 ± 12.01      |
| 12-HETE | 56.8                   | 258.3              | 68.7                   | 87.75              |
| Median  | 67.7 ± 22.13           | 327.6 ± 78.57      | 64.27 ± 17.08         | 109.5 ± 22.87      |
| Mean (±SEM) | 67.7 ± 22.13           | 327.6 ± 78.57      | 64.27 ± 17.08         | 109.5 ± 22.87      |
| 15-HETE | 100.1                  | 353.9              | 123.9                  | 242.8              |
| Median  | 92.2 ± 11.51           | 369.7 ± 60.82      | 129.5 ± 9.03          | 226.1 ± 28.52      |
| Mean (±SEM) | 92.2 ± 11.51           | 369.7 ± 60.82      | 129.5 ± 9.03          | 226.1 ± 28.52      |
| 20-HETE | 121                    | 49.8               | 125.9                  | 51.6               |
| Median  | 121.7 ± 16.41          | 49.73 ± 11.67      | 124.5 ± 18.98         | 50.18 ± 6.02       |
| Mean (±SEM) | 121.7 ± 16.41          | 49.73 ± 11.67      | 124.5 ± 18.98         | 50.18 ± 6.02       |
| (±)14(15)-EET | 296.3                  | 122.7              | 192.4                  | 85.3               |
| Median  | 228.7 ± 31.12          | 118.7 ± 21.66      | 191.3 ± 22.95         | 99.91 ± 21.09      |

Median and mean ± SEM values are displayed for each product measured in control and injured (UUO) specimens from each animal group. 15d-PGJ2, 15-deoxy-PGJ2.

*P < 0.001 for control versus UUO.

iP < 0.05 for control versus UUO.

IP < 0.05 for UUO versus UUO.

dP < 0.01 for UUO versus UUO.

eP < 0.01 for control versus UUO.
KO bone marrow. We attempted to irradiate these mice using several strategies, but they were intolerant to irradiation, developing skin lesions, anorexia, and several deaths; thus, we presume that these mice have a lowered threshold for acute radiation sickness.

Our data indicate that eicosanoid production after renal injury influences renal fibrogenesis, but does not definitely address which eicosanoids exert pro- or anti-fibrotic effects or which cell types are the primary targets. Published data indicate that induction of COX-2 and PGE2-EP4...
receptor signaling are protective during UUO-induced murine injury (35, 36). Other data indicate that these and other prostanoid-derived signals worsen experimental renal disease (37–39). A recent study demonstrated that bone marrow-derived COX2, microsomal prostaglandin synthase-1 (mPGES-1), and monocyte/macrophage EP4 receptor silencing were sufficient to confer salt-sensitive hypertension in mice (32). Furthermore, the authors showed that these animals had an increase in M1 and Th1 signaling and increased staining for macrophages and T-cells. Therefore, it is possible that the timing and localization of PGE2 production may be crucial determinants of tissue injury. We also showed a significant blunting of 15-lipoxygenase-derived 12- and 15-HETE production after UUO in mice with bone marrow-silenced cPLA2α. 15-lipoxygenase and its products are known to be intimately involved in vascular disease, atherosclerosis, AT2-mediated hypertension, and glomerular disease (40). Furthermore, irradiation/transplantation studies of mice with 15-lipoxygeanse-deficient bone marrow have shown attenuation of APOE-deficient atherosclerosis (41) and LPS-induced acute lung injury (42). Whether these eicosanoids are major drivers of renal tubulointerstitial fibrosis warrants further study.

Surprisingly, we did not observe any detectable leukotrienes in homogenized renal tissue during UUO using LC-MS/MS, despite detecting elevated levels of Alox5 by message. Leukotrienes are detectable in the urine, serum, and tissue of animal models of renal injury by ELISA (43–45). LB4 has also been detected by radioimmunoassay in isolated glomeruli from obstructed rats (46). Leukotrienes are difficult to detect by LC-MS/MS (47), and failure to detect leukotrienes using this method was demonstrated in a broad lipidomic analysis of the spontaneously hypertensive rat kidney (48). It is hypothesized that intrinsic instability in leukotrienes ex vivo contributes to their difficult detection, even by LC-MS/MS, though investigators have reported success using this method in other tissues (49). Our own laboratory has detected leukotrienes in lung tissue from tumor-bearing mice (50, 51). Leukotriene detection after renal injury might also be model dependent.

In contrast to other AA-derived metabolites, we unexpectedly observed decreased levels of CYP450-derived products, such as 20-HETE and (±)14(15)-EET, by LC-MS/MS. These results are consistent with message levels showing similar trends for Cyp4a12a (which produces 20-HETE) and Cyp2c44 [which produces (±)8(9)-, (±)11(12)-, and (±)14(15)-EETs]. By LC-MS/MS, we were only able to detect (±)14(15)-EETs with (±)8(9)- and (±)10(12)-EET levels falling below the limits of quantification. These data are surprising given published data supporting CYP450-derived eicosanoids as major mediators of hypertension and renal injury (52). One potential explanation for these findings is that CYP450-derived HETEs and EETs are thought...
to be produced by endothelial cells of the vasculature; while the UUO model primarily involves renal tubular epithelial injury. Indeed, after UUO, degenerative “atubular glomeruli” are observed (55), suggesting a dropout of vascular endothelial cells in this model. Thus, decreased CYP450 message and products might simply be a consequence of loss of the specific cell populations producing these eicosanoids. An alternative hypothesis is that the failure to detect these EETs reflects soluble epoxide hydrolase activity with rapid conversion to dihydroxyeicosatrienoic acids (DHETs), which were not measured in our experiments. However, our data suggest that bone marrow chimerism for cPLA2α does not affect major CYP450 enzyme levels during UUO, and that these products are unlikely to be responsible for differences in fibrosis.

FACS analyses after UUO in our experiments are consistent with published data showing a robust increase in inflammatory cells as early as 3 days after UUO (3). Chimerism for cPLA2α appeared to blunt the early influx of total CD45+ populations, and pro-inflammatory CD11b+ Ly6C+ M1-like macrophages (Fig. 5B). These changes were statistically significant and normalized by 7 days after UUO, a time when eicosanoid expression was increased and altered by obstruction in the chimeric mice. We measured production of cytokines and chemokines known to influence pro-inflammatory leukocyte recruitment and differentiation, including TNF-α, fractalkine, CCL2, and CCL12 (Fig. 3A). These pro-inflammatory mediators were blunted in KO-BMT animals after UUO, consistent with our earlier FACS data. These results are also consistent with published data indicating that mice genetically silenced for cPLA2α expression experience reduced F4/80+ monocyte influx in the kidney after All-induced renal injury (8). Certain pro-inflammatory AA-derived eicosanoids, such as PGD2 and 12- and 15-HETE, are thought to participate in inflammatory cell recruitment (34, 39, 54–56) and reduction of these mediators individually, or in combination, could be responsible for blunting inflammation and fibrosis in the KO-BMT-obstructed animals. Another possibility is that silencing cPLA2α in the bone marrow causes intrinsic changes in the leukocytes that affect their ability to home to the injured kidney independent of downstream eicosanoid expression. cPLA2α is known to have several noncanonical functions most recently implicated in controlling proliferation and cell cycle turnover (24, 25). We did not examine cell proliferation or apoptosis in our current studies.

Our data adds to the growing body of literature supporting a central role for immune-derived cells in renal fibrosis initiation and progression. Given an expanding arsenal of enzymatic and receptor agonist/antagonists, our data identify several potential eicosanoids that might be targetable in a cell-specific fashion to halt renal injury, inflammation, and fibrosis. In summary, we have shown that silencing cPLA2α expression in bone marrow-derived inflammatory cells is sufficient to blunt the production of several known pro-inflammatory eicosanoids, alter acute inflammatory cell recruitment, and reduce the degree of fibrosis induced by UUO in irradiated/transplanted C57BL/6J mice.

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