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Macrophage Epoxygenase Determines a Profibrotic Transcriptional Signature

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Epoxygenases belong to the cytochrome P450 family. They generate epoxygenoic acid, which is known to have anti-inflammatory effects, but little is known about their role in macrophage function. By high-throughput sequencing of RNA in primary macrophages derived from rodents and humans, we establish the relative expression of epoxygenases in these cells. Zinc-finger nuclease-mediated targeted gene deletion of the major rat macrophage epoxygenase Cyp2j4 (ortholog of human CYP2J2) resulted in reduced epoxygenoic acid synthesis. Cyp2j4−/− macrophages have relatively increased peroxisome proliferator-activated receptor-γ levels and show a profibrotic transcriptional profile, displaying overexpression of a specific subset of genes (260 transcripts) primarily involved in extracellular matrix, with fibronectin being the most abundantly expressed transcript. Fibro-nectin expression is under the control of epoxygenase activity in human and rat primary macrophages. In keeping with the in vitro findings, Cyp2j4−/− rats show upregulation of type I collagen following unilateral ureter obstruction of the kidney, and quantitative proteomics analysis (liquid chromatography–tandem mass spectrometry) showed increased renal type I collagen and fibronectin protein abundance resulting from experimentally induced crescentic glomerulonephritis in these rats. Taken together, these results identify the rat epoxygenase Cyp2j4 as a determinant of a profibrotic macrophage transcriptional profile that could have implications in various inflammatory conditions, depending on macrophage function. The Journal of Immunology, 2015, 194: 000–000.

Macrophage-mediated innate immune response is associated with the synthesis of lipid mediators, such as PGs and leukotrienes, through the metabolism of arachidonic acid (AA) (1–4). The latter is metabolized by various enzymes, such as cyclooxygenases and lipoxygenases, where the activity/expression of these is tightly associated with macrophage function (3, 5).

AA is also metabolized by cytochrome P450 (CYP) enzyme isoforms to four cis-epoxygenoic acid (EET) regioisomers, with the CYP2 family (CYP2J2, CYP2C8, CYP2C9, and CYP2S1 in humans) being the main epoxygenases (6–8). Although considerable attention has been afforded to cyclooxygenases and lipoxygenases expression of CYP2J2 resulted in reduced inflammation character-

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Abbreviations used in this article: AA, arachidonic acid; BMDM, bone marrow–derived macrophage; CYP, cytochrome P450; ECM, extracellular matrix; EET, epoxygenoic acid; FDR, false discovery rate; FPKM, fragment/kb of transcript/ mRNA; GSEA, gene set enrichment analysis; GSE, Gene Expression Omnibus; HIF, hypoxia-inducible factor; IBD, inflammatory bowel disease; IFN, interferon; iNOS, inducible nitric oxide synthase; KEGG, Kyoto Encyclopedia of Genes and Genomes; KRT, keratinocyte rat xenograft; LPS, lipopolysaccharide; M2, alternatively activated macrophages; M1, classically activated macrophages; MFS, mouse fibroblast serum; MRC, Medical Research Council; MRE, mouse resident peritoneal macrophage; NLRP3, NOD-like receptor family, pyrin domain containing 3; NOD, NOD-like receptor; ovine, sheep; ORX, oxygen consumption rate; PPAR-γ, peroxisome proliferator-activated receptor-γ; qRT-PCR, quantitative RT-PCR; RNA-Seq, RNA sequencing; siRNA, small interfering RNA; UUO, unilateral ureter obstruction; WT, wild-type; ZFN, zinc-finger nuclease.
ized by reduced macrophage infiltration. More recently, an EET analog administered to salt-sensitive hypertensive rats resulted in reduced inflammation in the kidney and diminished renal and cardiac fibrotic responses (15). Although these studies clearly establish the anti-inflammatory effect of EETs associated with reduced macrophage infiltration, they focused primarily on the effect of either systemic delivery of EETs or endothelial-cell derived EET function. Macrophages equally express epoxygenases (6, 16, 17), and a recent genome-wide association mapping using macrophage infiltrates in the bronchoalveolar lavage fluid from 36 inbred mice strains exposed to hyperoxia identified the Cyp2j2 locus within a significant quantitative trait locus on chromosome 4 (18). This is in accordance with the soluble epoxide hydrolase (i.e., the enzyme metabolizing EETs to their corresponding diols) inhibition resulting in a significant decrease in total bronchoalveolar lavage cell number (by 37%) in tobacco smoke–exposed rats, with significant reductions in alveolar macrophages (19). These studies suggest a role for macrophage infiltration as a result of an increase in endogenous EET production. The specific role of macrophage-derived EET was investigated recently with the identification of CYP2S1 as a novel macrophage-derived epoxygenase localized in atherosclerotic plaques in humans (6, 20).

Allelic expansion of the Cyp2j locus in rodents has made targeted gene deletion experiments challenging, and a recent study generated mice deleted for the entire locus (21). Although multiple isoforms of epoxygenases may contribute to EET biosynthesis, tissue expression of specific isoforms may account for the majority of epoxygenase activity (22). In this article, we provide a comprehensive genome-wide expression profiling in rodent and human primary macrophages by RNA sequencing (RNA-seq). We show that rodent macrophages predominantly express the human CYP2J2 ortholog (Cyp2j2 in rats; Cyp2j2 in mice). By using zinc-finger nuclease (ZFN) technology in the rat, we generated an inbred rat strain deficient for functional Cyp2j2. We show that Cyp2j4+/− bone marrow–derived macrophages (BMDMs) have reduced EET production (11,12- and 14,15-EETs) and adopt a profibrotic phenotype with transcriptional activation of extracellular matrix (ECM)-related transcripts, among which fibronectin is the most abundant. Epoxygenase-derived EET regulates fibronectin production in rat and human macrophages. Cyp2j2+/− rats show enhanced renal Coll1a1, Coll1a2, and Coll3a1 expression and increased interstitial Coll1a following the unilateral ureter obstruction (UUO) of the left kidney. In addition, quantitative proteomics (liquid chromatography–tandem mass spectrometry [LC-MS/MS]) in control and nephrotoxic nephritis (NTN)-induced kidneys confirmed the relative increase in renal type I collagen and fibronectin protein abundance in Cyp2j4−/− animals compared with wild-type (WT) controls in a macrophage-dependent model of glomerular inflammation.

Materials and Methods

ZFN-mediated gene targeting and generation of Cyp2j4−/− rats

ZFN constructs specific for rat Cyp2j4 were designed, assembled, and validated by Sigma-Aldrich. Selected ZFNs were targeted to exon 4 of Cyp2j4 (target sequence GGAAGACTGGAA; see Supplemental Fig. 1). ZFN mRNAs were microinjected into single-cell Wistar-Kyoto (WKY/NCrI) rat embryos, and the injected embryos were transferred to the oviduct of day-0.5 pseudopregnant rats. The resulting offspring were screened for ZFN-induced gene alterations at the target site of Cyp2j4 by PCR on purified DNA samples derived from tail snips using specific primers flanking the ZFN target site (∼ 1 kb). PCR products were identified by agarose gel electrophoresis, and the ZFN-mediated 25-bp deletion was confirmed by direct automated fluorescent DNA sequencing in an ABI 3730xl (Applied Biosystems). Heterozygous animals carrying the 25-bp deletion in rat Cyp2j4 exon 4 (Cyp2j4+/−) were intercrossed, and phenotypic characterization was performed on WT Cyp2j4-knockout (Cyp2j4−/−) animals.

Measurement of cellular oxygen consumption by an XF Extracellular Flux Analyzer

BMDMs from WT and Cyp2j4−/− rats were plated in XF24 7 V cell culture plates (Seahorse Bioscience, North Billerica, MA) at 5× 10⁴ cells/well and incubated for 24 h in a 37°C5% CO₂ incubator. Cells were equilibrated with bicarbonate-free low buffered DMEM without any supplement or supplemented with glucose and/or glutamine, as indicated in each experiment, and incubated in a 37°C incubator without CO₂ for 45 min immediately before the XF assay. 11,12-EETs (Cayman Chemical) were prepared in the identical assay medium (1 µM) as in the corresponding well and were injected from the reagent ports automatically to the wells containing Cyp2j4−/− BMDMs. Oxygen consumption rate (OCR) measurements were taken every 10 min for a total duration of 3 h.

Evaluation of 11,12- and 14,15-EETs in macrophage lysates and supernatants by ELISA

To assess 11,12- and 14,15-EET production in macrophages, an ELISA kit (Detroit R&D, Detroit, MI) was used to determine concentrations of the stable 11,12- and 14,15-EET metabolites in the supernatants and BMDM lysates of WT and Cyp2j4−/− rats (n = 4 rats/group). Eicosanoids were extracted from the cell lysates and supernatants with ethyl acetate after acidification with acetic acid.

RNA-seq library preparation and data analysis in rodent and human macrophages

Total RNA was extracted from rat (WT Wistar-Kyoto strain, n = 3; Cyp2j4−/−, n = 3) and mice (commercially outbred mice; n = 4) BMDMs and human monocytic-derived macrophages (MDDMs; n = 3 healthy donors) using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions, with an additional purification step by on-column DNase treatment using the RNase-free DNase Kit (QIAGEN) to ensure elimination of any genomic DNA. Integrity and quantity of total RNA were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL) and an Agilent 2100 Bioanalyzer (Agilent Technologies). One microgram of total RNA from each species was used to generate RNA-seq libraries using the TrueSeq RNA sample preparation kit (Illumina), according to the manufacturer’s instructions. Briefly, RNA was purified and fragmented using poly-T oligo-attached magnetic beads using 5 rounds of purification followed by the first-strand synthesis. Next, cDNA 3’ ends were adenylated and adapters were ligated, followed by 10 cycles of library amplification. Finally, the libraries were selected by size using AMPure XP beads (Beckman Coulter) and purified, and their quality was checked using an Agilent 2100 Bioanalyzer. Samples were randomized to avoid batch effects, and libraries were run on a single lane/sample of the HiSeq 2500 platform (Illumina) to generate > 100 million-end reads. An average of 50, 81, and 60 million reads coverage per rat, mouse, and human samples was achieved, respectively. RNA-seq reads were aligned to the rat (m4), mouse (mm9), and human (hg19) reference genomes using TopHat2. The average mapping percentage for all species was > 80%. Sequencing and mapping were controlled for quality using standard tools provided in the FastQC software. Gene level read counts were computed using HT-Seq-count with “union” mode, and clustering of samples corresponding to different conditions was done using Ward’s methods based on the Euclidian distance of scaled sample gene expression profile. Differential expression analyses between WT and Cyp2j4−/− rat BMDMs were conducted using edgeR (23), with a false discovery rate (FDR) cutoff of 5%. Peroxisome proliferator-activated receptor-γ (PPARγ) target enrichment on the differentially expressed genes (5% FDR) was performed on the targets identified by chromatin immunoprecipitation, coupled with whole-genome tiling arrays, in T3T-L1 adipocytes (24). The coordinates were converted to the mouse reference genome mm10 using the liftOver utility from the UCSC genome browser; the identified peaks (± 50 kb of the transcription start site, as recommended in the original study (24)) resulted in 2109 mouse genes (1889 rat orthologs). The RNA-seq dataset in rat BMDMs showed ∼16,000 expressed transcripts with 1,117 PPARγ targets; the enrichment was calculated using the Fisher exact test.

Cell culture and RNA interference

Rat BMDMs were isolated from ferrets and differentiated using L929-conditioned media (25). Rat fibroblasts were isolated from skin and

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lungs, as previously detailed (26). Primary adult human dermal fibroblasts (Cellworks) were cultured in DMEM and F-12 medium containing 2 mm L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin supplemented with 10% FCS. The cells were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO₂, and fresh growth medium was added to the cells every 3–4 d until confluence. Cell growth was arrested with serum-free medium for 24 h before use in experiments, and all experiments were performed under serum-free conditions, unless otherwise stated. Human MDMs were differentiated from buffy coats from healthy donors using gradient separation (Histopaque 1077; Sigma-Aldrich) and adhesion purification. Following Histopaque separation, PBMCs were resuspended in RPMI 1640 (Life Technologies), and monocytes were purified by adherence for 1 h at 37°C, 5% CO₂. The monolayer was washed six times with HBSS to remove nonadherent cells, and the monocyte-enriched population matured for 5 d in RPMI 1640 containing 100 ng/ml M-CSF (PeproTech, London, U.K.). Macrophage purity was confirmed by immunohistochemical assessment of CD68, and >99% cells were CD68⁺.

For RNA interference, human MDMs were replated in six-well plates (1 × 10⁶ cells/well) in RPMI 1640 (Invitrogen) overnight and transfected for 48 h with ON-TARGET PLUS for human CYP2S1 (100 nM; Dharmacon SMART pool) or nontargeting small interfering RNA (siRNA) pool as the scrambled control siRNA using DharmaFECT 1 (1:50; Dharmacon) as a transfection reagent in Opti-MEM medium (Invitrogen). In some experiments, following 24 h of incubation with CYP2S1 or nontargeting siRNA, the culture medium was washed, and cells were cultured for an additional 24 h in the presence or absence of 11,12-EETs (1 μM). The siRNA sequences used in the siGENOME SMARTpool for all transcripts are available upon request.

**NTN and UUO**

NTN was induced in 10 wk-old male WT and Cyp24−/− rats by i.v. injection of 0.1 ml nephrectomy serum (NTS) (25). The animals were left for either 10 or 28 d to evaluate the potential effect of targeted deletion of Cyp24 on the progression of disease. At 9, 13, and 27 d later, urine was collected by placing rats in metabolic cages for 24 h with free access to food and water. Proteinuria was determined by the sulphosalicylic acid assay. At 10 and 28 d following NTN induction, rats were culled by asphyxiation and exsanguination. Following Histopaque separation, the urinary sediment was washed, and urine was cultured for 14 d in the presence of 5% CO₂. The urine was centrifuged, and 200 μl of the supernatant was used for the determination of proteinuria. A total of 10 ng cDNA/sample was used. ViiA 7 RUO Software was followed by the Tukey multiple-comparison test was used for comparison of three or more groups. Differences in relative cytokine quantities followed by the Tukey multiple-comparison test was used for comparison of three or more groups. Differences in relative cytokine quantities (TNF-α) were tested for significance using the nonparametric Wilcoxon signed-rank test.

**Quantitative proteomics by LC-MS/MS**

Kidneys from untreated and NTN-treated rats (n = 3/group) were ground to a powder using a liquid nitrogen–cooled mini mortar and pestle set (Fisher Scientific). The samples were lysed (8 M Urea in 20 mM HEPES) and sonicated, and 200 μg protein extracts was reduced with DTT (10 mM) before trypsin digestion. The differential expression of Cyp24 was assessed by tandem mass spectrometry (Thermo Scientific) with 18 h at 37°C. Peptides from each biological replicate were desalted by reversed-phase chromatographic cartridges (Oasis HLB; Waters), washed, dried, and analyzed by LC-MS/MS in technical duplicates. Samples were blocked with BSA, incubated with the Col1α1 primary Ab raised in goat (Southern Biotech: 1/40 dilution), washed, and reincubated with secondary Ab (donkey anti-goat–FITC, 1/100 dilution; Abcam).

**Western blot, ELISA, and quantitative RT-PCR**

The cells were lysed in Laemmli sample buffer supplemented with protease inhibitors and resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to immunoblotting with appropriate primary and secondary Abs. Proteinuria was determined by the sulphosalicylic acid assay. At 10 and 28 d following NTN induction, rats were culled by asphyxiation and exsanguination. Following Histopaque separation, the urinary sediment was washed, and urine was cultured for 14 d in the presence of 5% CO₂. The urine was centrifuged, and 200 μl of the supernatant was used for the determination of proteinuria. A total of 10 ng cDNA/sample was used. ViiA 7 RUO Software was followed by the Tukey multiple-comparison test was used for comparison of three or more groups. Differences in relative cytokine quantities (TNF-α) were tested for significance using the nonparametric Wilcoxon signed-rank test.
Targeted gene deletion of Cyp2j4 in the rat reduces macrophage EET production

Zinc-finger–mediated genome targeting of rat Cyp2j4 led to a deletion of 25 bp in exon 4. Genomic PCR of the ZFN target region of rat Cyp2j4 exon 4 showing the deletion in Cyp2j4+/− and Cyp2j4−/− animals (Fig. 2A) was confirmed with direct sequencing (Fig. 2B). The 25-bp frameshift deletion in rat Cyp2j4 causes a premature stop codon upstream of the putative heme binding site (Supplemental Fig. 1). Cyp2j4 deletion in the rat resulted in a markedly reduced expression of its transcript levels in different tissues and primary cells (Fig. 2C), suggesting a nonsense–mediated decay of the Cyp2j4 mRNA resulting from the ZFN-mediated frameshift deletion. To accurately establish Cyp2j4 transcript copies in macrophages, RNA-seq analysis was per-
formed in WT and Cyp2j4−/− BMDMs (Fig. 2D). Although WT BMDMs show average FPKM values of 15, Cyp2j4−/− BMDMs FPKM values were <1, reflecting the markedly reduced expression due to the frameshift deletion (Fig. 2D). We next measured the consequence of the disrupted rat Cyp2j4 on EET production in macrophage lysates and supernatants by EET ELISA. Targeted genomic deletion in Cyp2j4 resulted in reduced 11,12- and 14,15-EETs in BMDMs (Fig. 2E).

Cyp2j4 deletion in the rat generates a profibrotic macrophage transcriptome

BMDMs from Cyp2j4−/− and WT animals were subjected to RNA-seq. A total of 260 transcripts was found to be differentially expressed (5% FDR) between Cyp2j4−/− and WT BMDMs; interestingly, 92% of those (238 of 260) were upregulated in Cyp2j4−/− macrophages. Notably, the majority of the upregulated genes belong to the collagen, ECM, and collagen-associated gene families, together with genes involved in the TGF-β signaling pathway (Ctgf, Vegfa, Grem1, Cxcl12, Fig. 3A). The upregulated transcripts were significantly enriched for KEGG pathways for ECM-receptor interaction (p = 1.7 × 10−14) and focal adhesion (p = 1.5 × 10−13; Fig. 3B). Because of the previously described role of PPARγ as an effector for epoxygenase-derived EETs (28), we investigated PPARγ levels in WT and Cyp2j4−/− BMDMs and found relatively increased PPARγ protein levels in the absence of Cyp2j4 in primary macrophages (Fig. 3C). In keeping with the relatively increased PPARγ protein levels, we show that there was a significant enrichment for PPARγ targets among the differentially expressed genes between WT and Cyp2j4−/− BMDMs in the RNA-seq dataset (enrichment p = 2.5 × 10−17, Fig. 3D).
FIGURE 3. Cyp2j4 deletion results in a profibrotic macrophage transcriptome enriched for PPARγ targets. (A) Differential expression analysis of RNA-seq of WT and Cyp2j4<sup>−/−</sup> BMDMs (FDR < 0.05) revealed an upregulation of collagen family genes (upper left panel), TGF-β signaling genes (upper right panel), and ECM/collagen-associated genes (lower left panel). (B) KEGG pathway analysis showed markedly significant representation of ECM–receptor interaction and focal adhesion pathways. (C) Western blot analysis showing enhanced PPARγ protein levels in Cyp2j4<sup>−/−</sup> BMDMs. (D) Heat map showing the enrichment for PPARγ targets (59 of 259 differentially expressed genes, \( p = 2.5 \times 10^{-17} \)) among the differentially expressed genes (FDR < 0.05) between WT and Cyp2j4<sup>−/−</sup> BMDMs. *\( p < 0.01 \) versus WT.
together, these results suggest that Cyp2j4−/− BMDMs show a distinct profibrotic transcriptome signature associated with high PPARγ levels. Because fibroblasts are also primarily involved in ECM production, we tested, in parallel, whether the profibrotic transcriptome signature is a hallmark of Cyp2j4−/− fibroblasts. We isolated lung- and skin-derived primary fibroblasts from WT and Cyp2j4−/− rats and measured the mRNA levels of Coll1a1, Col1a2, Col3a1, and Fn1 in basal and TGF-β-stimulated cells (Supplemental Fig. 2). These results further demonstrated the enhanced ECM gene expression in Cyp2j4−/− dermal- and lung-derived fibroblasts (Supplemental Fig. 2).

Epoxigenase-derived EETs regulate fibronectin levels in macrophages

Among the significantly upregulated transcripts in Cyp2j4−/− BMDMs, the most abundant is fibronectin (Fn1; FPKM = 334). The relatively increased Fn1 expression levels are rescued and reversed by the addition of exogenous EETs to Cyp2j4−/− BMDMs (Fig. 4A), and human MDMs (Fig. 4B) downregulate Fn1 expression and protein levels in response to incubation with 11,12-EETs. In primary human MDMs, the addition of 11,12-EETs resulted in decreased PPARγ levels, suggesting that epoxigenase-mediated Fn1 expression is associated with PPARγ levels in the macrophages. We next performed siRNA-mediated knockdown of the most abundantly expressed human monocytic macrophage CYP2 epoxigenase (Fig. 1) (6), CYP2S1. This resulted in a significant increase in Fn1 expression (Fig. 4C) that was lost following the addition of 11,12-EETs (Fig. 4C), indicating that epoxigenase activity and intracellular EETs levels are partially responsible for Fn1 transcription in human MDMs.

In addition to macrophage Fn1 expression, we assessed whether targeted gene deletion of Cyp2j4 affected macrophage function, in general, by measuring oxygen consumption and LPS-mediated TNF-α secretion. OCR, as measured by an extracellular flux analyzer, was reduced in Cyp2j4−/− BMDMs but was rescued by the addition of 11,12-EETs (Supplemental Fig. 3A). Cyp2j4 deletion also resulted in diminished TNF-α secretion by BMDMs, and exogenous addition of 11,12-EETs had no effect (Supplemental Fig. 3B).

Cyp2j4 deletion and susceptibility to macrophage-dependent glomerulonephritis and UUO

We next investigated whether Cyp2j4 deletion resulted in altered disease outcome in the NTN model in the rat. WT and Cyp2j4−/− rats were injected with NTS, and glomerular crescents/scarring, proteinuria, and macrophage infiltration were measured at day 10 (the inflammatory phase) and at day 28 (the fibrotic phase of the disease). Targeted genetic deletion of Cyp2j4 did not result in a significant change in the inflammatory NTN phenotypes, with the exception of increased Coll1a1, Coll1a2, Col3a1, and Fn1 expression in the nephritic kidneys at day 10 (Fig. 5A, 5B). In addition, we induced UUO as a more fibrogenic model of kidney disease. Targeted genetic deletion of Cyp2j4−/− animals compared with WT controls (Fig. 5C). Coll1a2 was found to be markedly upregulated in Cyp2j4−/− UUO kidneys (Fig. 5C). Quantitative immunofluorescence for Coll1a1 in UUO and nephritic kidneys (day 28) showed a significant upregulation in Cyp2j4−/− rats, confirming the upregulation of Coll1a1 mRNA levels in NTN and UUO (Fig. 5D, 5E).

Quantitative proteomics shows relatively increased ECM peptide abundance in the nephritic kidneys of Cyp2j4−/− rats

To further confirm the relative increase in ECM peptides in macrophage-dependent kidney inflammation, we performed quantitative proteomics (LC-MS/MS) in control and NTN (day 10) WT and Cyp2j4−/− rats. Day 10 NTN corresponds to the peak of macrophage infiltration in the glomerulus of the Wistar-Kyoto rat (25, 29). This analysis led to the identification of 1902 proteins that were reliably detected (at least two peptides detected per protein) in all conditions for quantitative analysis of differential peptide abundance. At 5% FDR, we identified 170 annotated proteins for which their abundance was differentially detected in Cyp2j4−/− kidneys following NTN induction (peptides that are differentially expressed between Cyp2j4−/− and Cyp2j4−/− NTN but not between WT and WT NTN; peptides that are significantly expressed between Cyp2j4−/− NTN and WT NTN; Fig. 6). Gene Ontology analysis of the 170 differentially detected proteins showed the most significant enrichment for ECM organization (Benjamini corrected p value < 0.01) and confirmed the major effect of Cyp2j4 deletion on ECM production in nephritic kidneys. The results also confirmed that the expression of ECM genes upregulated by Cyp2j4−/− macrophages (i.e., Fn1, Coll1a1, and Lum, Fig. 3A) is mirrored by an increase in protein levels in Cyp2j4−/− kidneys compared with WT controls following NTN induction (Fig. 6).

Discussion

CYP epoxigenases can metabolize endogenous substrates, such as AA, to generate bioactive lipid mediators. The arachidonic epoxides or EETs have primarily been described as anti-inflammatory mediators with attenuating effects on inflammatory cell infiltration into different tissues (19, 30–32). Macrophages are effector innate immune cells in various inflammatory reactions; although the roles of the cyclooxygenase and lipoxygenase pathways were largely studied in relation to their activation levels, the effect of epoxigenase-derived EETs on macrophage function is an emerging field of research. In a comprehensive study, Frömel et al. (6) described human CYP2S1 as a novel macrophage epoxigenase regulating phagocytosis and colocalizing with CD68+ cells in human atherosclerotic plaques.

In this study, we established, by RNA-seq in rat, mouse, and human primary macrophages, the relative expression levels of CYP2 epoxigenases. We show that the most abundant CYP2 epoxigenases are Cyp2j4, Cyp2j6, and CYP2S1 in primary rat, mouse, and human macrophages, respectively. This is in accordance with the previous report describing the identification of human CYP2S1 by mass spectrometry as the main monocytic/macrophage epoxigenase (6). We then generated a Cyp2j4−/− rat strain by ZFN-mediated gene targeting to study the effect of targeted genomic deletion of Cyp2j4 on macrophage phenotype, as well as macrophage-dependent inflammatory kidney disease in comparison with Cyp2j4+/+ animals. BMDMs isolated from Cyp2j4−/− rats showed a specific transcriptome signature defined by upregulation of 260 genes primarily encoding proteins of ECM, collagen, and mediators of TGF-β signaling. Cyp2j4−/− BMDMs showed increased PPARγ levels, and there was a significant enrichment for known PPARγ targets among the upregulated transcripts, suggesting that the deletion of Cyp2j4 may have resulted in a PPARγ-mediated profibrotic macrophage phenotype. The nuclear orphan receptor PPARγ is known to regulate adipogenesis (33) and inflammatory responses (34), but previous studies also established a novel role for PPARγ signaling in the regulation of TGF-β-dependent fibrogenesis (35, 36). Importantly, a recent study showed the importance of endothelial-derived EETs in organ regeneration and wound healing (37), and an orally active EET analog was shown to attenuate kidney fibrosis in the rat (15); however, to our knowledge, our study is the first demonstration of a profibrotic macrophage transcriptome depending on epoxigenase level/activity. It should
be noted that epoxygenase products other than EETs (i.e., linoleic, eicosapentaenoic, and docosahexenoic acid products) could be affected in Cyp2j4−/− macrophages. This is the case for human macrophage epoxygenase CYP2S1-containing microsomes generating epoxides of linoleic and eicosapentaenoic acid (6), so further studies must focus on their potential profibrotic effect.

Tissue fibrosis is characterized by activated mesenchymal fibroblasts synthesizing elevated levels of matrix proteins, including collagen and fibronectin (38). To measure the effects of Cyp2j4 deletion in fibroblasts, we cultured dermal and lung fibroblasts from Cyp2j4−/− and WT animals. We found that, despite Cyp2j4 expression being 10-fold less in dermal and lung
FIGURE 5. Cyp2j4−/− rats show increased collagen expression in NTN and UUO. (A) NTN was induced by injection of NTS, and glomerular crescents, proteinuria, and glomerular ED1(CD68)+ macrophage infiltration were measured at day 10 in WT and Cyp2j4−/− rats. Col1a1, Col1a2, Col3a1, and Fn1 mRNA levels were measured in nephritic renal cortex of WT and Cyp2j4−/− animals. (B) NTN at day 28 following injection of NTS and measurement of proteinuria (days 14 and 28) and glomerular ED1(CD68)+ macrophage infiltration. (C) UUO was induced in WT and Cyp2j4−/− rats, and the expression of Col1a1, Col1a2, Col3a1, and Fn1 in the obstructed kidneys was measured by qRT-PCR 7 d later. At least five animals were used in each group in NTN and UUO experiments. Representative Col1a1 immunofluorescence images (left panel) and the quantification (right panel) for UUO (D) and NTN at day 28 (E). Original magnification ×20. ns, not significant.
fibroblasts compared with BMDMs, Cyp2j4−/− fibroblasts show relatively increased expression levels of collagen and fibronectin, suggesting that epoxygenases determine a profibrotic transcriptomic signature in major ECM-producing cells.

Among the upregulated transcripts in Cyp2j4−/− BMDMs, fibronectin (Fn1) had the highest expression levels (FPKM ~ 200). We also confirmed the relatively elevated levels of FN1 in human macrophages (FPKM > 200; data not shown). Macrophages are known as producers of fibronectin (39, 40), and our results show that endogenous EETs have an effect on the production of FN1 in human macrophages; however, a more generalized effect of epoxygenase-derived EETs on other ECM genes, including collagens, cannot be ruled out. Macrophage activation associated with collagen production was described previously (41), and, in breast cancer, macrophages regulate collagen fibrillogenesis (42). Our study underlines the importance of epoxygenase-mediated ECM production in pathologies where macrophages are likely to adopt a profibrotic phenotype. Given that epoxygenase mRNA

**FIGURE 6.** Quantitative proteomics (LC-MS/MS) confirms increased ECM peptide abundance in Cyp2j4−/− nephritic kidneys. (A) Heat map showing upregulated (left panel) and downregulated (right panel) peptides in Cyp2j4−/− specifically under NTN (day 10) conditions (differentially expressed between Cyp2j4−/− basal and Cyp2j4−/− NTN but not differentially expressed between WT and WT-NTN; differentially expressed between Cyp2j4−/− NTN and WT NTN). (B) Normalized abundance of unique peptides for Col1a1, Fn1, and Lum in WT and Cyp2j4−/− rat kidneys in basal (untreated) and under NTN conditions. Note that the expression of these three genes is upregulated in Cyp2j4−/− BMDMs (Fig. 3A).
levels are enhanced following M1-type activation of macrophages in vitro (6, 16), intracellular levels of EETs or other epoxygenase products could be responsible for a plastic macrophage phenotype between proinflammatory and profibrotic activation states, depending on the in vivo "immunological context" (43).

To test whether the epoxygenase-mediated profibrotic macrophage phenotype contributes to enhanced ECM production in vivo, we measured the susceptibility of Cyp2j2−/− rats to NTN and UUO. The latter is characterized by interstitial fibrosis, and depletion studies showed the importance of macrophage function in renal fibrosis (44). Our results showed increased Col1a1, Colla2, and Col3a1 in the UUO kidney of Cyp2j2−/− animals, suggesting that the profibrotic macrophage phenotype observed in vitro could translate into enhanced collagen expression in the fibrotic kidney. In keeping with this, type I collagen and fibronecin peptides measured by LC-MS/MS are increased in Cyp2j2−/− rat kidneys following NTN induction. Despite glomerular macrophage infiltration being unchanged between WT and Cyp2j2−/− kidneys following NTN, Cyp2j2−/− nephritic kidneys were characterized by enhanced ECM production, suggesting that the effect of Cyp2j2 is more likely to be on the fibrotic phase of experimental crescentic glomerulonephritis. Although these in vivo models of kidney inflammation do not establish the cellular origin of enhanced type I collagen and fibronecin after UUO and NTN, our data show that macrophages are not the only cell type responsible for enhanced ECM expression in the absence of Cyp2j2. It is noteworthy that NTN is characterized by a peak in macrophage infiltration at day 10 following the injection of NTS, the time point when the total kidney protein extracts were subjected to LC-MS/MS. Considered together, these findings suggest that the increased ECM production seen in NTN and UUO could have multiple cellular sources, with a combined contribution from stromal (fibroblasts) and innate immune cells (macrophages). Because we previously established the importance of genes expressed by macrophages in the pathophysiology of crescentic glomerulonephritis (45–47), the current study emphasizes the importance of factors influencing glomerular scarring. The effect of CYP2J2 overexpression on chronic kidney failure was investigated in a nephrectomy model in which adenovirus-mediated CYP2J2 gene delivery significantly lowered collagen I deposition by treatment with a soluble epoxide hydrolase inhibitor. Proc. Natl. Acad. Sci. USA 102: 2186–2191.

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