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Myeloid Cell-Derived Hypoxia-Inducible Factor Attenuates Inflammation in Unilateral Ureteral Obstruction-Induced Kidney Injury

Hanako Kobayashi,* Victoria Gilbert,† Qingdu Liu,* Pinelopi P. Kapitsinou,* Travis L. Unger,‡ Jennifer Rha,‡ Stefano Rivella,‡ Detlef Schlöndorff,† and Volker H. Haase*‡

Renal fibrosis and inflammation are associated with hypoxia, and tissue pO2 plays a central role in modulating the progression of chronic kidney disease. Key mediators of cellular adaptation to hypoxia are hypoxia-inducible factor (HIF)-1 and -2. In the kidney, they are expressed in a cell type-specific manner; to what degree activation of each homolog modulates renal fibrogenesis and inflammation has not been established. To address this issue, we used Cre-loxP recombination to activate or to delete both Hif-1 and Hif-2 either globally or cell type specifically in myeloid cells. Global activation of Hif suppressed inflammation and fibrogenesis in mice subjected to unilateral ureteral obstruction, whereas activation of Hif in myeloid cells suppressed inflammation only. Suppression of inflammatory cell infiltration was associated with downregulation of CC chemokine receptors in renal macrophages. Conversely, global deletion or myeloid-specific inactivation of Hif promoted inflammation. Furthermore, prolonged hypoxia suppressed the expression of multiple inflammatory molecules in noninjured kidneys. Collectively, we provide experimental evidence that hypoxia and/or myeloid cell-specific HIF activation attenuates renal inflammation associated with chronic kidney injury. *The Journal of Immunology, 2012, 188: 5106–5115.

A discrepancy between oxygen availability and demand has been shown to associate with renal fibrosis and the progression of chronic kidney disease (CKD), and is thought to result from multiple causes, including capillary rarefaction, limited oxygen diffusion from extracellular matrix (ECM) expansion, atherosclerotic vascular disease, anemia, and alterations in oxygen consumption itself (1). Although this discrepancy

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Abbreviations used in this article: Arg1, arginase 1; CKD, chronic kidney disease; ECM, extracellular matrix; EPO, erythropoietin; HIF, hypoxia-inducible factor; HPF, high-power field; IHC, immunohistochemistry; iNos, inducible NO synthase; MØ, macrophage; Mcp1, monocyte chemotactic protein-1; mGFP, membrane-bound GFP; M/M, monocyte/macrophage; Mr, mannose receptor; mT, membrane-bound tomato-red; Ngal, neutrophil gelatinase-associated lipocalin; Pkg, phosphoglycerate kinase; PHD, prolyl hydroxylase domain; PTEC, proximal tubular epithelial cell; UOU, unilateral ureteral obstruction; Vegfa, vascular endothelial growth factor A; VHL, von Hippel-Lindau.

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To address the role of HIF in experimental CKD, we have used a genetic approach to dissect cell type-specific Hif functions in the context of renal fibrosis and inflammation. In this article, we show that global activation of Hif ameliorates inflammation and reduces ECM accumulation after UUO, whereas global deletion of Hif enhances renal inflammation. These alterations in inflammatory responses are phenocopied when Hif is either activated or deleted cell type specifically in myeloid cells. Hif signaling in myeloid cells may therefore contribute to the renoprotective effects of global Hif activation. Furthermore, we provide experimental evidence that hypoxia and/or activation of the Hif pathway in the kidney leads to a downregulation of chemokine and chemokine receptor expression. Taken together, our study establishes a critical role for myeloid cell-derived Hif in inflammatory cell recruitment associated with renal injury, and supports the notion that hypoxia and activation of the Hif system induce cell type-dependent molecular responses that result in different disease outcomes.

Materials and Methods

Generation of mice, genotyping, and surgical procedures

The generation and genotyping of Hif1a, Hif2a (Epas1), or Vhl conditional alleles has been described elsewhere (12, 13). To achieve either inducible global or myeloid-specific recombination, we crossed mice carrying the Hif1a, Hif2a, or Vhl conditional alleles to mice that either expressed a tamoxifen-inducible Cre-recombinase, which is under control of the ubiquitin c promoter, referred to as Ubc-cre/ERT2 (14), or LysM-cre, where Cre-recombinase is controlled by lysozyme M regulatory elements (15). Inducible conditional Vhl knockout mice, Vhl<sup>lox/lox</sup>;Ubc-cre/ERT2, are referred to as Ubc-Vhl<sup>lox/lox</sup> before tamoxifen treatment and Ubc-Vhl<sup>−/−</sup> after tamoxifen treatment. Inducible conditional Hif1a/Hif2a double-knockout mice, Hif1a<sup>lox/lox</sup>Hif2a<sup>lox/lox</sup>;Ubc-cre/ERT2, are referred to as Ubc-Hif1a<sup>lox/lox</sup>Hif2a<sup>lox/lox</sup> before tamoxifen injection and Ubc-Hif1a<sup>−/−</sup>Hif2a<sup>−/−</sup> after tamoxifen treatment. Vhl<sup>lox/lox</sup>LysoM-cre and Hif1a<sup>lox/lox</sup>Hif2a<sup>lox/lox</sup>;Ubc-cre/ERT2, LysoM-cre are referred to as LysoM-Vhl<sup>−/−</sup> or LysoM-Hif<sup>−/−</sup> mice. For activation of the Cre-ERT2 transgenic system, tamoxifen (Sigma-Aldrich, St. Louis, MO) was injected i.p. every other day for 10 d at a concentration of 10 mg/ml (1.5 mg/mouse), dissolved in a mixture of 90% sunflower oil and 10% ethanol. To assess recombination in renal macrophages (MØs), we bred LysoM-cre mice to a membrane-bound tomato-red/GFP (mT/ mGFP) double-fluorescent Cre reporter strain (16), generating LysoM-mT/mGFP mice, which were then subjected to UUO. A mouse model of β-thalassemia (Hbb<sup>−/−</sup>M34I) was generated by deletion of both β-globin genes, β<sup>target</sup> and β<sup>mutar</sup>, and has been described previously (17, 18). UUO was performed in 8- to 10-wk-old mice as previously described (5). Cre-negative (Cre−) littermates were always used as control mice. Mice were analyzed on day 8 after ureteral ligation. All procedures involving mice were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pennsylvania, Cornell University, and Vanderbilt University Institutional Animal Care and Use Committees.

DNA, RNA, and protein analysis

DNA and RNA were isolated using DNeasy and RNeasy kits, respectively, according to manufacturer’s protocols (Qiagen, Valencia, CA). For quantitative real-time PCR, cDNA was prepared from 1 μg total RNA and analyzed using SYBR green or TaqMan PCR master mix (Applied Biosystems, Carlsbad, CA). Primer sequences for vascular endothelial growth factor A (Vegfa), phosphoglycerate kinase (Pgk)-1, Epo, collagen-Ia, F4/ 80, inducible NO synthase (iNos), arginase 1 (Arg1), mannose receptor (Mr), TNF-α (Tnfa), and IL-10 (Il10) have been described previously (5, 19–22). Primer sequences for other genes analyzed are listed in Supple-

FIGURE 1. Global activation of Hif ameliorates renal fibrosis and inflammation. (A) Shown are representative images of paraffin-embedded tissue sections from Cre− and Ubc-Vhl<sup>−/−</sup> kidneys stained for collagen with Sirius red and for MØs with F4/80 immunohistochemistry. For statistical analysis, 10 measurements per individual mice were averaged for Sirius red (n = 7 for control; n = 4 for mutants) and for F4/80 staining (n = 6 for control; n = 4 for mutants). Original magnification ×200. (B) Quantitative RT-PCR analysis of F4/80, Ccr2, Ccl2, collagen Ia, Kim-1, and Vegfa mRNA levels in contralateral (CTL) and UUO kidneys 8 d after ligation. Relative expression values were normalized to 18S rRNA. Data points represent individual mice. Error bars represent mean values ± SEM. *p < 0.05, **p < 0.01.
mental Table 1. 18S rRNA was used to normalize mRNA. For the quantification of mRNA expression levels, the relative standard curve method was used according to the manufacturer’s instructions (Applied Biosystems).

The Whole Mouse Genome Oligo Microarray G4122A (Agilent Technologies, Santa Clara, CA) was used for global gene expression profiling. Total RNA prepared from H6klox/lox and control mice was quantified with, and purity and integrity verified by, Bioanalyzer (Agilent Technologies). All procedures were performed according to instructions provided by Agilent Technologies. Arrays were scanned with Model G2565BA (Agilent Technologies) and analyzed using the SAM statistical analysis package Version 3.1 (http://www-stat.stanford.edu/~tibs/SAM). Nuclear protein extracts were prepared and analyzed by Western blotting as previously described (13, 19).

Immunohistochemistry

To assess collagen deposition, we stained paraffin-embedded kidney sections with Sirius red (0.1% fast green FCF and 0.1% direct red 80 in saturated picric acid). A rat anti-F4/80 Ab was used for quantitative assessment of M0 dendritic cell infiltration of renal tissue (ab6640; Abcam, Cambridge, MA). Goat anti-rat secondary Ab (Vector Laboratories, Burlingame, CA) was used. Ten random high-power fields (HPFs) per kidney section were examined for both Sirius red and inflammatory cell analysis. Sirius red-positive area expressed as percentage of total area analyzed was determined with National Institutes of Health ImageJ software (rsweb.nih.gov/j). For statistical analysis, Sirius red-positive area percentages and F4/80* cell numbers for individual mice were averaged across control and mutant cohorts. UUO and contralateral kidneys from LysM-mt/mGFP mice were sacrificed at day 8 after ureteral ligation and stained for F4/80 to visualize recombination event. Tissue sections were visualized with confocal fluorescent microscopy (Zeiss LSM510 META; Carl Zeiss, Thornwood, NY).

Purification of renal MØs

CD11b+ cells were isolated by immunomagnetic separation (MACS beads; Miltenyi Biotech, Auburn, CA) according to the manufacturer’s instructions at day 8 after UUO. Kidneys were harvested, minced, and homogenized, followed by incubation with 0.1% collagenase A (Roche, Madison, WI) and 100 μg/ml DNase I (BioRad, Hercules, CA) for 45 min at 37˚C. To obtain sufficient quantities of cells, we pooled and then analyzed isolates from three to five littermates of the same genotype. Total RNAs isolated from the renal CD11b+ cells were subjected to real-time RT-PCR.

Primary cell culture and in vitro assays

Peritoneal exudates were collected by sterile lavage on day 3 after i.p. injection of 3% thioglycollate (BD Biosciences, Sparks, MD). Isolated cells were washed and plated in RPMI 1640 containing 10% FBS, which was changed 4 h after plating to remove nonadherent cells. Cells were grown for 24 h in normoxia (21% O2), hypoxia (1% O2), in the presence or absence of LPS (100 ng/ml; Sigma-Aldrich), or IL-13 (10 ng/ml; Invitrogen, Carlsbad, CA). Primary proximal renal tubule epithelial cells were isolated from tetracycline-inducible Hif1a2lox/2lox;R26-rtTA2;Lc-1-cre mice as previously described (5).

Statistical analysis

Statistical analysis was performed using two-tailed Student t test. The p values < 0.05 were considered significant.

Results

Global activation of Hif reduces renal fibrosis after UUO and is associated with a decrease in inflammation

We have previously demonstrated that proximal tubular Hif-1 promoted fibrogenesis in two experimental models of chronic renal injury (5, 10). In contrast, systemic Hif activation by pharmacologic prolyl-hydroxylase inhibition was found to ameliorate fibrosis and improved renal injury (23). These findings suggested that the effects of Hif activation with regard to renal fibrogenesis might be cell type dependent. To examine the combined effects of Hif stabilization in different renal cell types, we used a genetic approach and globally activated Hif-1α and Hif-2α in a model of rapidly progressing renal fibrosis induced by UUO. For this, we used a ubiquitously expressed tamoxifen-inducible Cre-recombinase (Ubc-Cre/ERT2) to generate mice with global inactivation of the Vhl tumor suppressor (Ubc-Vhlfl/fl), because Vhl germline deletion results in embryonic lethality (24). Vhlfl/fl cells are unable to degrade Hif-α and are characterized by the activation of Hif transcriptional programs (24). Induction of Ubc-cre/ERT2 by tamoxifen resulted in efficient recombination in the kidney and other organs as determined by genomic PCR (Supplemental Fig. 1). As a consequence of Hif-1α and Hif-2α stabilization in the kidney, the expression of Hif-regulated genes, Vegfa, Pgk-1, lactate dehydrogenase (Ldh), and Epo, were increased (Fig. 1, Supplemental Fig. 1).

To investigate the effects of global Vhl inactivation on renal fibrosis, we subjected Ubc-Vhi−/− and Cre− littermate controls to UUO and analyzed on day 8 after ureteral ligation. Tamoxifen was administered before surgery to both Cre− littermate controls and Ubc-Cre/ERT2-expressing animals to control its potential effects on fibrosis development (25, 26). Tamoxifen administration by itself did not induce fibrosis or inflammation in wild type mice (Supplemental Fig. 1F). We found a ~30% reduction in collagen accumulation in Ubc-Vhi−/− R26-rtTA2;Lc-1-cre kidneys by Sirius red staining (Fig. 1A). Attenuation in collagen deposition was associated with a marked decrease in F4/80* inflammatory cell infiltration as determined by F4/80 mRNA levels in whole kidney homogenates (~65% reduction) and by immunohistochemistry (IHC) (63 ± 8 versus 13 ± 4 cells/HPF) (Fig. 1A, 1B). The reduction in MØ numbers was furthermore associated with decreased expression of chemokine (CC motif) receptor-2 (Ccr2) and its ligand chemokine (CC motif) ligand-2 (Ccl2), also known as monocyte chemotactic protein-1 (Mcp1) (Fig. 1B). Consistent with ameliorated injury was a ~40% reduction in Kim-1 mRNA levels (Fig. 1B). The expression of Kim-1, a transmembrane tubular protein, correlates with the degree of tubular injury and is strongly induced in human and murine models of kidney injury (27, 28). These results suggest that Vhl loss leads to increased renal injury, possibly by activating Hif-2α, which differs from the effects of Hif-1α (5, 10). For this reason, we focused on the role of Hif-1α in renal fibrogenesis.

FIGURE 2. Global deletion of Hif exacerbates renal inflammation. (A) Shown are representative images of paraffin-embedded tissue sections from Cre and Ubc-Hif−/− kidneys stained with Sirius red. (B) Quantitative RT-PCR analysis of Vegfa mRNA level in contralateral (CTL) and UUO kidneys 8 d after ligation. Shown are the means of relative expression values normalized to 18S rRNA. Data points represent individual mice. Error bars represent mean ± SEM. **p < 0.01.
positively with renal damage in both acute and chronic injuries (27). mRNA levels of neutrophil gelatinase-associated lipocalin (Ngal), another renal injury marker (28, 29), were also reduced but did not reach statistical significance (reduction by ~40%; Supplemental Fig. 1E). Ngal expression levels, however, are difficult to interpret in our model, because Hif-1 has been shown to be involved in the regulation of Ngal (30).

Although our data suggest that global Hif activation suppresses inflammation and reduces collagen accumulation in UUO kidneys, we could not completely rule out Hif-independent effects of Vhl inactivation. We therefore hypothesized that global deletion of both Hif-1α and Hif-2α together would increase inflammation and fibrosis in this model. To test this hypothesis, we used the Ubc-cre/ERT2 transgenic system to ablate both Hif-1α and Hif-2α globally (Ubc-Hif−/−). Tamoxifen treatment resulted in highly efficient deletion of both Hif-1α and Hif-2α (Supplemental Fig. 2B), and was associated with a 40% reduction in Vegfa levels (Fig. 2B), as well as a reduction of Hif-targeted genes Pgk-1 and Epo (Supplemental Fig. 2C). In contrast with Ubc-Vhl−/− kidneys, the number of infiltrating F4/80+ cells was significantly increased in Ubc-Hif−/− mice compared to controls (98 ± 8 versus 57 ± 4 cells/HPF; Fig. 2A), whereas ECM accumulation was not different, dissociating renal inflammation from fibrosis (Fig. 2A). Taken together, our findings suggest that Hif functions as potent modulator of inflammatory responses in the kidney and, when activated, confers renoprotection by suppressing inflammation.  

**MØ-restricted Hif activation attenuates, whereas Hif elimination enhances, inflammation without altering the fibrotic response after UUO**  

Because we observed that global Hif activation suppressed inflammation, and thereby attenuated renal injury, we investigated the role of MØ-specific Hif activation in UUO-induced renal injury. For this study, we generated myeloid cell-specific Vhl−/− mice, in which Hif-1α and Hif-2α were stabilized in MØ, from hereon referred to as LysM-Vhl−/−, and mice that lacked both Hif-1α and Hif-2α in MØ, from hereon referred to as LysM-Hif−/−. LysM-mediated inactivation of Vhl or Hif did not affect the number of circulating leukocytes and monocyte or neutrophil differential counts, which is consistent with previous reports (31). In agreement with Cramer and colleagues (31), recombination of >90% was observed in peritoneal monocyte/MØs (M/M) isolated from LysM-Vhl−/− or LysM-Hif−/− mice (data not shown). Because Lysosome M promoter activity is maturation stage dependent (32), we assessed the degree of recombination in renal MØ with LysM-mT/mGFP reporter mice that were subjected to UUO. In this reporter strain, mT is ubiquitously expressed at baseline.

![FIGURE 3. Activation of myeloid Hif attenuates inflammation.](image)

**FIGURE 3.** Activation of myeloid Hif attenuates inflammation. (A) Shown are representative confocal laser microscopy images of contralateral (CTL) and UUO kidneys from LysM-mT/mGFP mice. Recombined cells express GFP (a), nonrecombined cells express tomato red (b); F4/80+ cells are depicted by blue fluorescence (c), and overlay of images (a)–(c) is shown in (d). Arrowheads point toward F4/80+GFP+ myeloid cells. Asterisks depict F4/80+ cells stained for collagen with Sirius red, and overlap of images (a)–(c) is shown in (d). (B) Representative images of paraffin-embedded sections from Cre− and LysM-Hif−/− kidneys stained for collagen with Sirius red, and for MØs with F4/80 immunohistochemistry. For statistical analysis, 10 measurements per individual mouse were averaged for Sirius red (n = 7 for control; n = 6 for mutants) and for F4/80 staining (n = 7 for control; n = 6 for mutants). Original magnification ×200. (C) Quantitative RT-PCR analysis of F4/80, collagen Iα, and Kim-1 mRNA levels in CTL and UUO kidneys 8 d after ligation. Relative expression values were normalized to 18S rRNA. Data points represent individual mice. Error bars represent mean ± SEM; *p < 0.05, **p < 0.01.

![FIGURE 4. Deletion of myeloid Hif enhances inflammation.](image)

**FIGURE 4.** Deletion of myeloid Hif enhances inflammation. (A) Shown are representative images of paraffin-embedded tissue sections from Cre− and LysM-Hif−/− kidneys stained with Sirius red to assess ECM expansion (upper panel) and analyzed by immunohistochemistry for MØ marker F4/80. For statistical analysis, 10 measurements per individual mouse were averaged for Sirius red (n = 7 for control; n = 6 for mutants) and for F4/80 staining (n = 3 for control; n = 5 for mutants). Original magnification ×200. (B) Quantitative RT-PCR analysis for F4/80, collagen Iα, and Kim-1 levels in contralateral kidney (CTL) and UUO kidneys 8 d after ligation. Shown are the means of relative expression values normalized to 18S rRNA. Data points represent individual mice. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01.
and switches to mGFP in cells that have undergone Cre-mediated excision (16). By day 8 after UUO, most F4/80+ cells were GFP+, indicating significant recombination in renal MØ (Fig. 3A). Using quantitative RT-PCR, we found a 40% reduction in the ratio of Hif exon 2/exon 8 (Hif exon 2 is flanked by loxP sites and excised upon Cre-mediated recombination) in CD11b+ cells isolated from LysM-Hif+/− UUO kidneys, indicating efficient recombination (Supplemental Fig. 3A). Furthermore, an increase and a decrease of Hif-targeted gene, Vegfa, were observed by Vhl+/− and Hif+/− peritoneal MØ, respectively (Supplemental Fig. 3B).

When LysM-Vhl−/− mice were subjected to UUO, we found a significant decrease in renal F4/80+ cell accumulation as determined by F4/80 mRNA levels (36% reduction) and by IHC (40% reduction; 119 ± 5 versus 71 ± 6 cells/HPF; Fig. 3B, 3C). However, despite the marked decrease in the F4/80+ cell numbers, no differences were observed in collagen-1a mRNA levels or ECM accumulation as determined by Sirius red staining (Fig. 3B, 3C). Kim-1 expression levels between LysM-Vhl−/− and Cre−/− littermate controls were not different, consistent with comparable tubular injury (Fig. 3C). Taken together, our data suggest that MØ-restricted Hif activation reduces the infiltration by F4/80+ cells but does not influence the degree of ECM accumulation or tubular injury. In contrast with LysM-Vhl−/− mice, renal F4/80+ cell infiltration was significantly enhanced in LysM-Hif−/− mice subjected to UUO as determined by F4/80 mRNA (2.5-fold increase) and F4/80 IHC (1.9-fold increase; 45 ± 6 versus 85 ± 7 cells/HPF; Fig. 4A, 4B). However, despite this increase in F4/80+ cell infiltration, collagen-1a expression, collagen accumulation, and Kim-1 levels were not different (Fig. 4A, 4B). Taken together, our findings demonstrate that genetic manipulation of Hif in myeloid cells alone is sufficient to reproduce the changes in F4/80+ cell infiltration that were observed in Ubc-Vhl−/− or Ubc-Hif−/− UUO kidneys, suggesting that myeloid cell-derived Hif regulates renal inflammation. Surprisingly, however, Hif-dependent regulation of F4/80+ cell infiltration did not affect tubular injury, as indicated by

### Table 1. Hif modulates immune responses in renal MØs

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<th>Gene</th>
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CD11b+ cells were isolated from UUO kidneys 8 d after ureteral ligation using the MACS beads system. For each experimental set, CD11b+ cells were pooled from three to five littermates (mutant and control mice each). Quantitative RT-PCR analysis was performed with pooled CD11b+ cells and gene expression was normalized to 18S rRNA. Alterations in gene expression are presented as fold changes over pooled littermate controls.
the absence of differences in \( \text{Kim-1} \) levels or collagen accumulation between mutants and control.

**HIF regulates MØ polarization**

Because MØ polarization (M1 versus M2 phenotype) has a major influence on the development and progression of renal disease (33), we next investigated the effects of Hif activation on MØ polarization. For this study, we isolated M/M from \( \text{LysM-Vhl}^{+/+} \) and \( \text{LysM-Hif}^{-/-} \) mice. We then examined the expression of M1-associated \( \text{iNos} \) and M2-associated Arg1 after stimulation with LPS or IL-13; LPS treatment of M/M induces the M1 phenotype and increases \( \text{iNos} \) expression, whereas IL-13 induces the M2 phenotype and increases Arg1 expression. We found that the induction of \( \text{iNos} \) mRNA by LPS was significantly enhanced in \( \text{Vhl}^{-/-} \) compared to LPS-treated \( \text{Cre}^{-/+} \) control cells (~45-fold), whereas Arg1 induction was further increased in IL-13–treated \( \text{Vhl}^{-/-} \) M/M compared to \( \text{Cre}^{-/-} \) controls (~3.5-fold; Fig. 5A). In line with these findings and Hif dependence is the decreased expression of \( \text{iNos} \) and Arg1 in stimulated \( \text{Hif}^{-/-} \) M/M (Fig. 5B). Furthermore, the hypoxic induction of \( \text{iNos} \) and Arg1 in \( \text{Cre}^{-/-} \) control M/M was almost completely abrogated in \( \text{Hif}^{-/-} \) M/M (Fig. 5B), suggesting Hif-dependent regulation of these markers under hypoxia. Another hallmark of M2-MØ is the expression of high levels of \( \text{Mr} \), which did not differ between \( \text{Vhl}^{-/-} \) M/M and \( \text{Hif}^{-/-} \) M/M (data not shown).

To determine the contribution of individual Hifs to the regulation of \( \text{iNos} \) and Arg1, we compared M/M isolated from \( \text{LysM-Vhl/Hif-1a} \) and from \( \text{LysM-Vhl/Hif-2a} \) double-deletion mice (activation of Hif-2a or Hif-1a alone, respectively) with \( \text{Cre}^{-/-} \) controls. We found that the enhanced induction of \( \text{iNos} \) after LPS treatment noted in \( \text{Vhl}^{-/-} \) M/M was blocked in \( \text{Vhl/Hif-1a}^{-/-} \) M/M, but persisted in \( \text{Vhl/Hif-2a}^{-/-} \) M/M, indicating Hif-1a regulation, whereas increased Arg1 induction by IL-13 was blocked in \( \text{Vhl/Hif-2a}^{-/-} \) M/M, but preserved in \( \text{Vhl/Hif-1a}^{-/-} \), indicating Hif-

### Table II. Differentially regulated inflammatory pathway genes in kidneys from chronically hypoxic mice

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Relative expression levels of genes assigned to inflammatory pathways that were at least ±1.5-fold upregulated or downregulated and had <10% false discovery rate (total of 1170 probes and 912 genes) in kidneys from mice with mutated \( \beta\)-hemoglobin (\( \text{Hbh}^{\text{modified}} \)) compared to wild type littermates. Inflammatory pathway genes were considered those genes with ontologies of inflammatory response, activation of plasma proteins involved in acute inflammatory response, cytokine activity, and chemokine activity (http://david.abcc.ncifcrf.gov/).
HIF modulates immune responses in renal MØs

To elucidate the mechanisms by which myeloid-derived Hif suppressed renal inflammation, we isolated MØ from UUO kidneys to gain further insight into underlying mechanisms. CD11b+ cells from kidneys of LysM-M-Hif−/− or LysM-Hif−/− mice were isolated using the MACS beads immunomagnetic purification system, and inflammatory gene expression was analyzed with quantitative RT-PCR. With regard to M1/M2-associated gene expression, we showed a downregulation of iNos was upregulated in Hif−/− MØ and downregulated in Vhl−/− MØ compared to control MØ from Cre− littermates. Although we observed a downregulation of Arg1, Mr, Tfα, and Il10 in Vhl−/− MØ compared to control, differential regulation was not found between Vhl−/− MØ and Hif−/− MØ (Table I). Because of their critical role in renal inflammation (34), we next examined the expression of chemokine receptors. We found that Ccr2 and Ccr5 mRNA levels were downregulated in CD11b+ cells from UUO kidneys of LysM-Vhl−/− mice compared to Cre− littermate controls (Table I). In contrast with renal Vhl−/− MØ, Ccr2 and Ccr5 were increased in renal Hif−/− MØ compared to controls (Table I), which is consistent with increased F4/80+ cell infiltration in UUO kidneys from LysM-Hif−/− mice. Taken together, gene expression analysis of renal MØ is consistent with the LysM-cre knockout phenotype and raises the possibility that Hif-mediated inhibition of inflammatory cell recruitment in UUO kidneys is mechanistically linked to a suppression of proinflammatory molecules in renal MØ.

Proinflammatory gene expression is suppressed in chronically hypoxic kidneys

Because we used conditional Vhl inactivation to stabilize Hif either globally or in myeloid cells specifically, we asked the question whether activation of Hif in the kidney under hypoxic conditions alone, that is, in the presence of wild type Vhl, would result in a suppression of proinflammatory genes. For this study, we used genome-wide RNA expression analysis and examined kidneys from mice with mutated β-hemoglobin (Hbbth3/th3), which develop severe anemia resulting in substantial Hif activation in the kidney (Fig. 6A). We found that a large number of genes, which were differentially expressed and encoded inflammatory molecules, were downregulated in Hbbth3/th3 kidneys compared to control (data accessible at National Center for Biotechnology Information Gene Expression Omnibus database, accession no. GSE36312, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36312), supporting our hypothesis that hypoxia and/or activation of Hif signaling suppresses inflammation in the kidney (Table II). Consistent with our findings in renal Vhl−/− MØ, we observed a suppression of Ccr2 and Ccr5 in Hbbth3/th3 kidneys (Fig. 6B). Furthermore, the main ligand for Ccr2, Ccl2/Mcp1, was downregulated in Hbbth3/th3 kidneys (Fig. 6B). Because Ccl2/Mcp1 increases in renal tubular cells upon injury (35) and initiates monocyte recruitment, we determined Ccl2/Mcp1 expression levels in primary proximal tubular epithelial cells (PTEC) grown under hypoxia (1% O2). Although Ccl2 was not differentially expressed in either anemic kidneys or hypoxic PTEC (Figs. 6B, 7), we found Hif-1α–dependent downregulation of Ccl2/Mcp1 in PTEC (Fig. 7), raising the possibility that Hif activation in proximal renal epitheial cells impinges on M/M recruitment. These data are consistent with in vivo findings from global and myeloid-specific Vhl knockout mice and support the notion that activation of Hif signaling in both myeloid and epithelial cells contributes to the renal inflammatory response.

Discussion

The role of cell type-specific Hif signaling in the pathogenesis of progression of CKD is not well understood and is controversial (1). In this report, we have used genetic means to investigate the effects of global and myeloid-cell specific Hif activation or inactivation in a model of rapidly progressing fibrosis and inflammation induced by UUO. Our studies demonstrate that global Hif activation ameliorates fibrosis and F4/80+ cell infiltration, which is associated with decreased expression of Ccl2 chemokine and its receptor Ccr2 in the injured kidney. Specifically, we have identified myeloid-derived Hif as a key factor in the suppression of renal inflammation.

The general role of Hif in the regulation of inflammatory responses is complex and appears to be context dependent. Although our studies indicate that Hif suppresses F4/80+ cell infiltration in a model of chronic renal disease, myeloid Hif-1 has been shown to promote inflammation in experimental models of dermatitis and rheumatoid arthritis (31). In these settings, Hif is thought to support inflammation by: 1) facilitating anaerobic ATP production, which enhances cellular survival and enables defensive immune functions at inflammatory sites that are often characterized by a very low pO2 (<1%); 2) by promoting motility and migration to sites of inflammation; and 3) by positively regulating cytokine production (31, 36, 37). Genetic studies have furthermore indicated that myeloid Hif-1 is required for efficient bacterial killing under hypoxic conditions, and that it promotes a septic phenotype (38). In contrast with these reports, hypoxia and Hif have also been shown to suppress immune responses by producing anti-inflammatory and antipapoptotic effects in inflamed tissues and by suppressing cytokine production in T cells (39–44). In support of the latter findings are recent studies that demonstrated that pharmacologic or genetic manipulation of Hif prolyl-hydroxylation is protective in experimental models of inflammatory bowel disease, where it inhibits inflammation (45, 46). This anti-inflammatory effect of Hif activation is likely to involve HIF-dependent and -independent, as well as NF-κB–mediated, signals,
as there is complex and bidirectional interplay between both pathways (47, 48). Although PHD1 and PHD2 inhibit IκB kinase-β, which keeps NF-κB inactive, transcription of Hif-1α is stimulated by NF-κB (48, 49). PHD3, in contrast, has been shown to promote neutrophil survival in an Hif-independent manner (50). In conjunction with these studies, our findings support further investigations into the role of pharmacologic PHD inhibition as a therapeutic strategy for the treatment of certain inflammatory conditions. However, careful characterization of the injured tissue’s microenvironment is required to better understand the molecular basis of Hif-induced proinflammatory or anti-inflammatory effects. It is plausible that differences in tissue-specific chemokine/cytokine production and/or in local pO2 may explain Hif’s opposing roles in the regulation of inflammatory responses.

In support of Hif’s suppressive role in renal inflammation, the expression of CC chemokine receptors Ccr2 and Ccr5 was decreased in renal CD11b⁺ cells. Suppression most likely occurred indirectly via Hif-dependent regulation of transcriptional repressors (51–53) that are furthermore modulated by disease microenvironment and cell type-specific signals. For example, Ccr5 and, to a lesser degree, Ccr2 levels were both suppressed in Vhl−/− peritoneal MØ treated with LPS in vitro but were not increased in LPS-treated Hif−/− peritoneal MØs (data not shown). Chemokine receptors and their ligands function as major regulators of inflammatory cell recruitment in renal injury (54). Inhibition of Ccr1 or Ccr2 reduces renal MØ infiltration and the development of interstitial fibrosis (55–57). In the setting of acute ischemia-reperfusion injury, deletion of Ccr2 was protective and decreased inflammation (58). In line with these observations are studies where blockade of the Ccr2 ligand Ccl2/Mcp1 decreased MØ recruitment in Col4a3-deficient Alport mice (59). In contrast with Ccr2, deletion of Ccr5 led to disease exacerbation in autoimmune nephritis (60), but improved the long-term outcome of renal allografts (61, 62). Although our data raise the possibility that Hif-mediated inhibition of inflammatory cell recruitment is mechanistically linked to a suppression of proinflammatory molecules in MØ, it is likely that Hif activation in other renal cell types, such as epithelial cells, has contributed to the attenuation of renal inflammation under certain conditions.

The notion that Hif could act as a more general suppressor of inflammation in the kidney is furthermore supported by our analysis of chronically hypoxic mice. In this study, we examined kidneys with high levels of Hif activity and found suppression of both Ccr2 and its ligand Ccl2/Mcp1. We also found that Ccl2/Mcp1 is expressed in renal epithelial cells and is negatively regulated by hypoxia in an Hif-1α−dependent fashion, as we have shown in primary cell culture with conditional Hif-1α deletion. These results are consistent with findings from Ubc-Vhl−/− URO kidneys and illustrate that the anti-inflammatory effects of renal hypoxia/Hif activation involve more than one cell type, in this case, both infiltrating MØ and renal epithelial cells, which initiate MØ recruitment during injury. The hypoxic regulation of Ccl2/Mcp1 appears to be cell type dependent, because Hif has been shown to increase CCL2/MCP1 in astrocytes but not in renal epithelial cells (63, 64). A more recent example of Hif-dependent anti-inflammatory signals in the kidney is axonal guidance molecule netrin-1, which is expressed in endothelial and renal epithelial cells, and attenuates inflammation by inhibiting myeloid cell migration (65).

We previously reported that renal epithelial Hif promoted fibrosis in a model of UUO and 5/6 nephrectomy (5, 10). Although these data appear to be in contrast with this study, where global Hif activation was beneficial, we believe that these are not contradictory findings, and rather support the notion that Hif has cell type-specific functions, which impact inflammation and fibrosis differentially. It is likely that the DNA binding sites, to which Hif is recruited, differ between MØs and epithelial cells, thus permitting activation of specialized transcriptional programs that could impact biological outcomes (66, 67).

We found that activation of both renal Hif-1α and Hif-2α in Ubc-Vhl−/− kidneys led to a suppression of UUO-associated fibrosis and inflammation. Further dissection of the Hif response in LysM-Cre mutant mice revealed that reduced MØ infiltration did not impact on indicators of fibrosis. This implies that the inhibition of inflammatory cell recruitment in this model is not simply a consequence of the degree of fibrotic injury, but rather represents a mechanistically independent change in the ability to recruit and/or retain MØ. Although it is well established that inflammatory cells contribute to and modulate renal fibrosis (34), differences in functional outcomes after experimental manipulations of MØ numbers have been reported. Depletion of MØ after UUO on day 4 reduced interstitial fibrosis in a diphertheria toxin-based transgenic model (68), whereas no correlation between the degree of MØ infiltration and interstitial fibrosis was observed after MØ blockade with a c-fms kinase inhibitor (69). These opposing effects can be explained by the high degree of cellular and functional heterogeneity in MØ populations (70), because ex vivo induction of different MØ phenotypes had substantial effects on disease outcome in Adriamycin-induced nephrosis (20, 71). Consistent with Takeda et al. (72) was our in vitro finding that Hif-1α promoted M1 marker expression, whereas Hif-2α enhanced M2 marker expression in normoxic peritoneal M/M. Although no polarization bias was found when MØ from UUO kidneys were examined, it should be noted that the use of CD11b magnetic beads will yield a mixed population of inflammatory cells; thus, a shift from an M1 to M2 phenotype in a subpopulation of CD11b⁺ cannot be completely excluded.

In summary, our study established a critical role for myeloid cell Hif in bone marrow-derived monocyte inflammatory cell recruitment and activation associated with renal injury, and suggests that Hif activation in the kidney suppresses renal inflammation. Whether pharmacological targeting of Hif can be used therapeutically to control inflammation in CKD warrants further investigation.

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Disclosures
The authors have no financial conflicts of interest.

References


