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Loss of the Oxygen Sensor PHD3 Enhances the Innate Immune Response to Abdominal Sepsis

Judit Kiss,*1 Martin Mollenhauer,*1 Sarah R. Walmsley,† Johanna Kirchberg,* Praveen Radhakrishnan,* Thomas Niemietz,* Johanna Dudda,* Gunnar Steinert,* Moira K. B. Whyte,‡ Peter Carmeliet,§ Massimiliano Mazzone,§ Jürgen Weitz,* and Martin Schneider*†

Hypoxia and HIFs (HIF-1α and HIF-2α) modulate innate immune responses in the setting of systemic inflammatory responses and sepsis. The HIF prolyl hydroxylase enzymes PHD1, PHD2 and PHD3 regulate the mammalian adaptive response to hypoxia; however, their significance in the innate immune response has not been elucidated. We demonstrate in this study that deficiency of PHD3 (PHD3−/−) specifically shortens the survival of mice subjected to various models of abdominal sepsis because of an overwhelming innate immune response, leading to premature organ dysfunction. By contrast, this phenotype was absent in mice deficient for PHD1 (PHD1−/−) or PHD2 (PHD2−/−). In vivo, plasma levels of proinflammatory cytokines were enhanced, and recruitment of macrophages to internal organs was increased in septic PHD3-deficient mice. Reciprocal bone marrow transplantation in sublethally irradiated mice revealed that enhanced susceptibility of PHD3-deficient mice to sepsis-related lethality was specifically caused by loss of PHD3 in myeloid cells. Several in vitro assays revealed enhanced cytokine production, migration, phagocytic capacity, and proinflammatory activation of PHD3-deficient macrophages. Increased proinflammatory activity of PHD3-deficient macrophages occurred concomitantly with enhanced HIF-1α protein stabilization and increased NF-κB activity, and interference with the expression of HIF-1α or the canonical NF-κB pathway blunted their proinflammatory phenotype. It is concluded that impairment of PHD3 enzyme function aggravates the clinical course of abdominal sepsis via HIF-1α− and NF-κB−mediated enhancement of the innate immune response.

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Abbreviations used in this article: BMC, bone marrow cell; BMDM, bone-marrow derived macrophage; CalX, carboxic-anhydrase 9; CLP, cecal ligation and puncture; PHD, prolyl hydroxylase domain; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; WT, wild-type.

*Department of General and Transplantation Surgery, University of Heidelberg, Heidelberg, D-69120 Germany; †Department of Infection and Immunity, The Medical School, University of Sheffield, Sheffield S10 2RX, United Kingdom; ‡Laboratory of Angiogenesis and the Neurovascular Link, Vesalius Research Center, KU Leuven, Leuven B-3000, Belgium; §Laboratory of Molecular Oncology and Angiogenesis, Vesalius Research Center, KU Leuven, Leuven B-3000, Belgium; ‖Department of Infection and Immunity, University of Sheffield, Sheffield S10 2RX, United Kingdom; ‡Laboratory of Molecular Oncology and Angiogenesis, Vesalius Research Center, KU Leuven, Leuven B-3000, Belgium; and †Laboratory of Molecular Oncology and Angiogenesis, Vesalius Research Center, KU Leuven, B-3000, Belgium

1J. Kiss and M. Mollenhauer contributed equally to this work.

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Address correspondence and reprint requests to Dr. Martin Schneider, University of Heidelberg, Department of General, Visceral and Transplantation Surgery, Im Neuenheimer Feld 110, D-69120 Heidelberg, Germany. E-mail address: m.schneider@uni-heidelberg.de

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Sepsis leads to generalized tissue hypoxia due to inadequate perfusion and hypoxemia (3). Hypoxia, in turn, represents a major stimulus for macrophages to mount an innate immune response (4, 5). This process relies on increased stabilization of hypoxia-inducible transcription factors HIF-1α and HIF-2α (4, 6, 7). Macrophage bactericidal activity and motility are significantly impaired in mice specifically lacking HIF-1α in myeloid cells (8, 9). Consequently, these animals are less susceptible to bacterial LPS-induced mortality (6). According to recent experimental evidence, HIF-2α likewise modulates macrophage proinflammatory activity during acute inflammation (4).

Prolyl hydroxylase domain (PHD)-containing enzymes (PHD1, PHD2, and PHD3) regulate the stability of HIF-1α and HIF-2α in an oxygen-dependent manner. In the presence of oxygen, PHD enzymes catalyze the hydroxylation of two proline residues within the oxygen-dependent degradation domain of HIF-1α and HIF-2α (10), thus preventing the activation of hypoxia-adaptive target genes (10, 11). Recent studies applying genetic loss-of-function approaches indicated that PHD enzymes carry out specific and nonredundant in vivo functions. Loss of PHD1 specifically alters the mitochondrial energy metabolism of skeletal muscle (12) or liver cells (13), thus increasing their hypoxic survival. Furthermore, loss of PHD1 increases the intestinal barrier function and is protective against experimental colitis (1, 14). PHD2 exerts a major physiologic function during placentaion and cardiac development (15, 16). Of pathophysiologic relevance, haplodeficiency of PHD2 can normalize the vasculature of expanding tumors, thereby improving tumor oxygenation and delaying distant metastasis (17). PHD3 has been assigned a physiologic function in normal development of the sympato-adrenal system.
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PHD2 and PHD3 are cooperatively involved in the development of hepatic steatosis and diluted cardiomyopathy, which occurs as a result of excessive HIF-1α stabilization (19). Recent studies revealed that PHD enzymes likewise exert specific functions in WBCs. In macrophages, loss of one PHD2 allele results in a proatherigenic phenotype that confers enhanced collateral vascularization and protection against ischemia (20). PHD3 selectively regulates the life span of neutrophils in hypoxic environments without however affecting neutrophil proinflammatory functions and metabolic activity (21).

Given the significance of hypoxia in the innate immune response, we hypothesized that PHD enzymes exert important functions in systemic inflammation and sepsis. In this study, we delineate a novel function of the PHD3 oxygen sensor in the innate immune response. We demonstrate that loss of PHD3 aggravates proinflammatory macrophage functions upon polymicrobial or LPS challenge. These changes are relevant in murine models of abdominal sepsis, where loss of PHD3 triggers an overwhelming innate immune response, leading to premature organ dysfunction and lethality. These findings extend our current insight into cell-specific functions of HIF prolyl hydroxylases and their implication in the systemic inflammatory response syndrome and sepsis.

Materials and Methods

Mouse models

The generation of PHD knockout mice has been described elsewhere (13, 17, 18). All animal experiments were approved by the ethical commission of the local government. To induce endotoxemia, 8- to 10-wk-old mice were injected i.p. with 12 mg/kg or 15 mg/kg Escherichia coli LPS serotype 0111:B4 (Sigma-Aldrich, Taufkirchen, Germany). Mice were continuously monitored to assess survival for a period of up to 96 h. For the assessment of clinical symptoms, disease activity was scored as described (22). Blood was collected by cardiac puncture 8 h after LPS challenge to assess serum creatinine values or for the measurement of cytokine levels.

For cecal ligation and puncture (CLP), animals were anesthetized and subjected to midline laparotomy. Subsequently, the cecum was ligated and punctured twice below the ileocecal valve (22-gauge needle). Sham-operated animals underwent the same procedure except ligation and puncture. Subsequently, mice were continuously monitored to assess survival for a period of 7 d. For assessment of bacterial spread to the peritoneal cavity and systemic circulation, mice were sacrificed 20 h after CLP for peritoneal lavage and blood sampling. Peritoneal fluid and peripheral blood were seeded on blood agar plates, and bacterial CFUs were quantified after incubation for 48 h at 37°C.

For bone marrow transplantation, 5-wk-old mice were sublethally irradiated and subsequently received bone marrow from syngenic animals via i.v. injection. Repopulation of the bone marrow was allowed for 5 wk.

Glomerular clearance was calculated according to a modified Cockroft-Gault formula as follows: 

\[ \text{CCR} = \frac{140 \times \text{mass (in kg)} / 72 \times \text{plasma creatinine (in mg/dl)}}{1} \]

Levels of murine TNF-α, IL-1β, and IL-6 protein were assayed using the Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany).

Histology and immunostaining

Internal organs from septic mice were dissected, paraffin embedded, and sectioned on 6-μm thickness. For histological assessment of organ damage, sections were stained with H&E. For immunostaining of leukocytes, macrophages, and neutrophils, sections were stained for CD45 (BD Pharmingen, San Diego, CA), F4/80 (AbD Serotec, Düsseldorf, Germany), or Ly6G (BD Pharmingen), respectively. Photomicrographs were taken with a Zeiss Axiosstar Plus microscope applying an Axioscam MRC camera and Axiovision software. Histomorphometric quantification was performed by two independent investigators applying 18 standardized microscopic fields.

Phagocytosis assay

Intraperitoneal zymosan A (0.1 mg) was used to generate a resolving model of sterile peritonitis (23) with cells harvested by lavage with 2 ml PBS at 24 h and total and differential cell counts determined by hemocytometer count and cytospin morphology. Quantification analysis and calculation of phagocytic index (ratio of ingesting cells to noningesting cells × average number of zymosan A particles per cell) was performed for both zymosan A particles and apoptotic bodies (efferocytosis) after 24 h of zymosan A injection.

Cell culture experiments

Bone marrow-derived or peritoneal macrophages were isolated as described (24). Differentiation of bone marrow cells was induced by the addition of M-CSF (unless indicated differently: 10 ng/ml; Sigma) in the media for 5 d. Differentiated bone-marrow derived macrophages (BMDMs) were fixed and stained with May–Grünwald Giemsa according to standard protocols or harvested for RNA isolation. For mRNA expression analyses, cells were treated with LPS (E. coli serotype 0111:B4, 1 μg/ml for 2 h; Sigma-Aldrich) prior to RNA isolation. Immunocytochemical and FACs stainings were performed applying the CD36 and F4/80 macrophage markers (AbD Serotec) according to standard protocols. Phalloidin–FITC and DAPI staining (DAKO, Hamburg, Germany) were applied to visualize cell shape and nuclei, respectively, on immunohistochemical slides.

Tandem murine macrophage cell line J774A.1 was obtained from American Type Culture Collection (Berlin, Germany). Cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin at 37°C in humidified air. J774A.1 cells were transfected with a small interfering RNA (siRNA) oligonucleotide targeting PHD3, HIF-1α, HIF-2α, the NF-κB p65 subunit, or a control siRNA oligonucleotide (sequences available on request). Transfection was performed using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany).

In vitro migration assay

Migration was assessed applying a modified Boyden chamber assay consisting of cell culture inserts and a polycarbonate filter (pore size 5 μM; Cell Biolabs, Heidelberg, Germany). One hundred microliters of cell suspension (5 × 10⁶ cells) was added to the upper wells. Chambers were incubated for 24 h at 37°C in a 5% CO₂ atmosphere. Cells on the lower side of the filter were quantified by dissolving cell-bound crystal violet in 10% acetic acid for 5 min and subsequent spectrophotometric analysis at 560 nm.

Quantitative real-time PCR

RNA of macrophages exposed to 1 μg/ml LPS (Sigma-Aldrich) for 2 h was isolated using an RNA isolation kit (Qiagen) according to the manufacturer’s instructions. First-strand synthesis and real-time PCR were performed with Fermentas products and a TaqMan Universal SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany) applying specific primers (sequences available on request). Rates were normalized to the expression level of 18S RNA.

Western blot studies

Transfected J774A.1 cells were matured for 3 d with 10 nM PMA (Sigma) and treated with LPS (1 μg/ml for 2 h). After harvesting, nuclear protein was isolated for the detection of HIF-1α and HIF-2α (Novus Biologicals, Cambridge, U.K.) or the p50 subunit of NF-κB (Santa Cruz, Heidelberg, Germany). Actin (Santa Cruz) or histone H3 (NEB, Frankfurt, Germany) was used as loading control. After development with an HRP-conjugated secondary Ab, semiquantitative analysis of the bands was performed applying Imagel software (National Institutes of Health, Bethesda, MD).

NF-κB activity assay

BMDMs were treated with LPS (1 μg/ml for 2 h). Subsequently, cells were harvested and nuclear protein isolated for the detection of NF-κB activity applying the TransAM Transcription Factor ELISA kit specific for the p65 subunit of eNF-κB (Active Motif, Rixensart, Belgium).

Statistical analysis

All values are represented as mean ± SEM. The significance of experimental differences was evaluated by the Student t test. Survival data were analyzed by the construction of Kaplan–Meier plots and application of the log-rank test.

Results

Loss of PHD3 aggravates sepsis-related disease symptoms and lethality

To investigate the function of HIF prolyl hydroxylase enzymes in generalized abdominal sepsis, mice deficient for PHD1, PHD2, or PHD3 and wild-type (WT) littermates were subjected to i.p.
jection of bacterial LPS. In WT mice, LPS injection (12 mg/kg) induced a transient systemic inflammatory response, causing death in a minority of 10% (Fig. 1A). Strikingly, survival of PHD3-deficient mice (PHD3<sup>−/−</sup>) injected with similar concentrations of LPS was significantly impaired by 86% compared with that of WT littermates (Fig. 1A, right panel). By contrast, survival of PHD1-deficient (PHD1<sup>−/−</sup>) or PHD2-haplodeficient (PHD2<sup>+/−</sup>) mice did not change significantly compared with that of their WT counterparts (Fig. 1A, left and middle panels). This phenotype was maintained upon treatment with higher concentrations of LPS (Supplemental Fig. 1A–D).

To determine further whether loss of PHD3 accelerated the course of LPS-induced endotoxemia, clinical disease symptoms were monitored and scored according to a disease activity index reflecting body temperature, posture, and alertness (25). Indeed, clinical disease symptoms were significantly aggravated in PHD3<sup>−/−</sup> (but not PHD2<sup>+/−</sup> or PHD3<sup>−/−</sup>) mice compared with WT mice (Fig. 1B).

As LPS-induced endotoxic shock only partly reproduces human abdominal sepsis, we also investigated whether PHD3 deficiency accelerated the susceptibility to polymicrobial peritonitis. For this purpose, mice were subjected to large bowel perforation (CLP). This procedure caused substantial release of enteric bacteria into the peritoneal cavity and blood circulation, which occurred to a similar extent in WT and PHD3<sup>−/−</sup> mice (Supplemental Fig. 1E). Strikingly, survival of PHD3<sup>−/−</sup> mice was shortened compared with that of WT animals when subjected to CLP (Fig. 1C). Survival of sham-operated animals was 100% in both genotypes (data not shown).

Thus, loss of PHD3 specifically enhanced the susceptibility of mice to sepsis-induced death in two alternative models mimicking human abdominal sepsis.

**Loss of PHD3 enhances septic organ damage**

To investigate whether accelerated septic death of PHD3<sup>−/−</sup> mice coincided with enhanced organ damage, internal organs were subjected to histopathological analysis 8 h after LPS treatment (15 mg/kg), when clinical symptoms of systemic inflammatory response first became apparent (Supplemental Fig. 1D). At healthy conditions, no morphologic alterations were observed in lungs or kidneys from PHD3<sup>−/−</sup> mice compared with those of WT animals (Fig. 2A, 2B, left panels). At 8 h after LPS treatment, however, alveolar septa were significantly thicker in LPS-treated PHD3<sup>−/−</sup> mice than in WT littermates (Fig. 2A, right panels), indicating that PHD3<sup>−/−</sup> mice suffered more severe interstitial pulmonary edema. Histological assessment of kidneys from LPS-treated animals revealed more extensive glomerular swelling in PHD3<sup>−/−</sup> than in WT mice (Fig. 2B, right panels). Histomorphometric analysis confirmed that thickness of alveolar septa and renal glomeruli was significantly increased in PHD3<sup>−/−</sup> compared with WT animals after LPS treatment (Fig. 2C, 2D).

To assess whether the observed difference in glomerular damage was functionally relevant, we measured serum creatinine levels and determined creatinine clearance. LPS-treatment impaired kidney function in both WT and in PHD3<sup>−/−</sup> mice; however, the impairment of creatinine clearance was significantly enhanced in PHD3<sup>−/−</sup> animals (Fig. 2E).

Taken together, PHD3<sup>−/−</sup> mice displayed enhanced structural damage and dysfunction of vital organs in conditions of sepsis.

**Enhanced activation of the innate immune response in septic PHD3<sup>−/−</sup> mice**

As septic organ dysfunction is boosted by the innate immune response, we sought to determine whether production of proinflammatory cytokines and accumulation of innate immune cells were enhanced in LPS-treated PHD3<sup>−/−</sup> mice.

ELISA assays were performed to determine the abundance of proinflammatory cytokines in the blood of septic mice after LPS administration. Expectedly, plasma levels of TNF-α and IL1-β were markedly increased between 2 and 8 h after LPS challenge, but this increase was significantly more pronounced in septic PHD3<sup>−/−</sup> than in WT animals (Fig. 3A, 3B). Plasma IL-6 levels were under the detection limit at baseline conditions but significantly increased in LPS-challenged PHD3<sup>−/−</sup> mice (IL-6 concentrations, pg/ml: 3021 ± 88 in septic WT mice versus 3144 ± 104 in septic PHD3<sup>−/−</sup> mice; p < 0.01, n = 6). Accordingly, ELISA analysis of tissue homogenates revealed that the LPS-induced increase of proinflammatory cytokines was significantly more pronounced in lungs or kidneys from PHD3<sup>−/−</sup> mice than in comparable WT organs (Supplemental Fig. 2A, 2B). Consistently, the abundance of proinflammatory cytokines was significantly increased in PHD3<sup>−/−</sup> mice compared with WT animals suffering polymicrobial sepsis induced by CLP (Supplemental Fig. 1F–H).

In an initial attempt to determine whether increased cytokine levels in LPS-challenged PHD3<sup>−/−</sup> mice were due to enhanced recruitment of innate immune cells, we assessed the accumulation of leukocytes and tissue-infiltrating macrophages in septic organs. Immunohistochemistry for the leukocyte common Ag CD45 and morphometric quantification revealed comparable numbers of
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CD45-positive leukocytes in internal organs such as the kidney of WT and PHD3"""" mice at baseline conditions (Fig. 3C [top panels], 3D). LPS treatment induced a substantial accumulation of leukocytes in internal organs, and this increase was significantly enhanced in PHD3"""" mice (Fig. 3C [bottom panels], 3D). Staining for the macrophage-specific F4/80 Ag revealed that the accumulation of macrophages was likewise comparable in kidneys from WT and PHD3"""" mice at baseline conditions (Fig. 3E [top panels], 3F) but significantly increased in organs from LPS-injected PHD3"""" mice compared with their WT littermates (Fig. 3E [bottom panels], 3F). Comparable results were obtained upon immunohistochemical analysis of other internal organs such as the lung or the liver (data not shown).

Taken together, enhanced septic organ dysfunction and lethality coincided with increased proinflammatory cytokine release and accumulation of innate immune cells in internal organs of septic PHD3"""" mice. Notably, immunohistochemical staining for the Ly6G Ag, which is expressed predominantly on neutrophil granulocytes, and quantification of leukocytes in bronchoalveolar lavage samples indicated comparable abundance of neutrophil granulocytes in organs of septic WT and PHD3"""" mice (Supplemental Fig. 2C, 2D). We therefore speculated that the increased presence of CD45-positive leukocytes in septic organs from PHD3"""" mice is predominantly related to increased invasion by macrophages (as suggested by immunohistochemical staining for the macrophage marker F4/80, Fig. 3E, 3F).

**Accelerated sepsis-related death in PHD3"""" mice is macrophage dependent**

Indeed, macrophages of the innate immune system are essential regulators of systemic inflammation, and their function is critically affected by hypoxia (26), altogether supporting the notion that increased susceptibility to sepsis progression in PHD3"""" mice is attributable to altered macrophage function. The following experiments were performed to address this hypothesis.

First, animals were treated with clodronate liposomes specifically to induce apoptosis of phagocytic cells, including macrophages (27). Immunohistochemical staining of spleens for the macrophage marker F4/80 revealed efficient depletion of F4/80-positive cells after clodronate liposome treatment, whereas control treatment with PBS liposomes had no such effect (Fig. 4A). Injection with PBS liposomes did not significantly affect the survival of WT or PHD3"""" mice subjected to i.p. LPS challenge (Fig. 4B, left panel; compare with Fig. 1A, right panel). Strikingly, clodronate treatment significantly prolonged the survival of LPS-injected PHD3"""" mice (Fig. 4B, right panel; compare with Fig. 1A, right panel), thus reverting the phenotype of enhanced sepsis susceptibility.

Second, to assess whether enhanced septic lethality was truly attributed to loss of PHD3 function in innate immune cells, mice were depleted of bone marrow by sublethal irradiation. Subsequently, bone marrow transplantation was performed to obtain chimeric WT mice harboring PHD3"""" bone marrow-derived leukocytes (WT/PHD3"""" BM) or PHD3"""" mice harboring genetically unaltered WT bone marrow (PHD3""""/WT BM). Strikingly, the presence of PHD3"""" bone marrow was sufficient to induce enhanced septic lethality in otherwise genetically unaltered mice. Indeed, WT mice carrying syngenic PHD3"""" bone marrow (WT/PHD3"""" BM) displayed worse septic survival than WT mice implanted with WT bone marrow (WT/WT BM) (Fig. 4C, left panel). Conversely, the presence of WT bone marrow reverted the
phenotype of accelerated septic lethality in PHD3−/− mice. Indeed, survival of LPS-exposed PHD3−/− mice carrying WT bone marrow (PHD3−/−/WT BM) was significantly improved compared with that of equally treated PHD3−/− mice implanted with PHD3−/− bone marrow (PHD3−/−/PHD3−/− BM) (Fig. 4C, right panel).

Altogether, these experiments suggested that enhanced sepsis progression in PHD3−/− mice was due to altered function of innate immune cells.

Enhanced proinflammatory macrophage activity upon loss of PHD3

We next sought to determine whether loss of PHD3 enhanced proinflammatory macrophage functions. To test whether PHD3 deficiency stimulated macrophage expression of proinflammatory cytokines, BMDMs were isolated from WT and PHD3−/− mice and subjected to LPS treatment in vitro. Expressional analyses revealed the presence of PHD3 transcript expression in BMDMs from WT mice (PHD3 mRNA copies/10^5 copies of 18S: 8.3 ± 0.3), whereas PHD3 transcript expression was expectedly absent in BMDMs isolated from PHD3−/− mice. Quantitative real-time PCR (qRT-PCR) analysis revealed that proinflammatory cytokine expression was expectedly upregulated in both WT and PHD3−/− macrophages 2 h after LPS challenge. Importantly, expression of IL-6 and IL-1β was significantly higher in LPS-treated PHD3−/− than in WT BMDMs (Fig. 5A, 5B; Supplemental Fig. 3A, 3B).

Consistently, ELISA analysis revealed that IL-6 cytokine production was markedly increased in LPS-treated PHD3−/− cells (IL-6 protein concentration, pg/mg total protein: 178 ± 14 in WT versus 609 ± 43 in PHD3−/− BMDMs; p = 0.001, n = 5).

To assess whether PHD3 deficiency affected macrophage migration, we screened for the expression of ICAM-1—a surface glycoprotein that is crucial for the transmigration of macrophages into inflamed tissues (28)—in BMDMs derived from WT or PHD3−/− mice. Indeed, ICAM-1 transcript expression was significantly increased by 3.7-fold in PHD3−/− BMDMs compared with WT cells upon LPS challenge (Fig. 5C), suggesting enhanced migration potential. As a functional readout of macrophage migration, we performed in vitro migration assays applying peritoneal macrophages (29) from WT or PHD3−/− mice. Transwell migration of these cells was significantly increased upon loss of PHD3 (Fig. 5D).

Finally, to assess effects of PHD3 deficiency on macrophage phagocytic activity, experimental peritonitis was induced by i.p. injection of the yeast-derived β-glucan zymosan A, and phagocytic
index was determined via assessment of zymosan A particles and apoptotic bodies ingested into peritoneal macrophages (23). Strikingly, PHD3−/− macrophages displayed significantly enhanced phagocytic capacity (Fig. 5E, 5F, left panel) compared with WT cells after i.p. instillation of zymosan A. Loss of PHD3 likewise enhanced the ingestion of apoptotic cells (efferocytosis (30)) by peritoneal macrophages (Fig. 5F, right panel).

Taken together, loss of PHD3 strikingly enhanced macrophage proinflammatory cytokine production, migration, and phagocytosis.

Altered macrophage maturation and polarization in PHD3−/− mice

We likewise sought to determine whether absence of PHD3 stimulated the maturation of macrophages from monocytes, which represents a key initiatory step of the innate immune response during septicemia (31). Mononuclear cells were harvested from the bone marrow of WT and PHD3−/− mice, and their differentiation into macrophages was initiated by the addition of M-CSF in vitro. By fluorescence microscopy, PHD3−/− bone marrow cells (BMCs) rapidly altered their morphology toward a macrophage-like phenotype with increased cytoplasmic dimensions, pseudopodia, and surface expression of the macrophage marker CD36 (Fig. 6A, right panel). By contrast, more round-shaped and smaller cells devoid of CD36 expression were observed in differentiating cultures of WT BMCs after M-CSF treatment (Fig. 6A, left panel). For indirect quantification of macrophage maturation in differentiating cultures of WT and PHD3−/− BMCs, we screened for the expression of the macrophage marker CD36. By qRT-PCR, CD36 transcript expression was almost undetectable in undifferentiated cultures of WT or PHD3−/− BMCs (Fig. 6B). However, upon 5 d of M-CSF treatment, CD36 transcript expression was strikingly increased by 3-fold in PHD3−/− compared with WT cells (Fig. 6B). Consistently, FACS analysis revealed that the fraction of CD36-expressing macrophages was significantly higher in M-CSF-treated cultures of PHD3−/− BMCs than in corresponding WT cultures (Fig. 6C).

To establish further whether absence of PHD3 affected the M1 (microbicidal) versus M2 (immunomodulatory) polarization of macrophages in septicemia, we assessed the expression of M1- and M2-associated genes in BMDMs isolated from WT or PHD3−/− mice. At baseline culture conditions, the expression of M1- and M2-associated genes was comparable in WT and PHD3−/− BMDMs (data not shown). LPS treatment in vitro expectedly upregulated the expression of M1-associated but not of M2-associated genes in BMDMs of both genotypes (Fig. 6D). However, upregulation of M1 genes was significantly increased by 2- to 14-fold in LPS-challenged PHD3−/− BMDMs compared with similarly treated WT cells. As expected, no such differences were observed in the expression of genes specific for the M2 phenotype (Fig. 6D).

Taken together, these findings indicate that loss of PHD3 promoted the maturation of macrophages from monocytes and boosted their proinflammatory M1 polarization upon LPS exposure.

Downstream effectors of PHD3 in macrophage-mediated inflammation

We finally aimed to delineate some of the molecular mechanisms that act downstream of PHD3 to increase macrophage proinflammatory responses. Focus was placed on the roles of NF-κB and HIFs, as these are well-described PHD3 targets with a key regulatory function in innate immunity (32, 33).

NF-κB activity assays were performed on primary BMDMs isolated from WT or PHD3−/− mice and revealed comparable NF-κB p65 activity in WT and PHD3−/− macrophages at baseline culture conditions (Fig. 7A). LPS treatment caused a marked increase of NF-κB p65 activity in both WT and PHD3−/− macrophages; however, LPS-induced activation of NF-κB p65 was

FIGURE 4. Enhanced lethality of septic mice is due to loss of PHD3 in macrophages. (A and B) Phagocyte depletion by liposomal clodronate. (A) Representative immunostainings of the spleen, revealing abundant F4/80-positive macrophages in PHD3−/− mice injected with PBS liposomes (left), but not in clodronate liposome-treated animals (right). Scale bars, 100 μm. (B) Survival curves of PBS-treated (left) and clodronate-treated (right) mice after injection of LPS (12 mg/kg, i.p.), revealing significantly impaired survival of PBS-treated PHD3−/− mice (left), but unaltered survival of clodronate-treated PHD3−/− mice (right). n = 8. Results are representative of two independent experiments. **p < 0.01. (C) Survival curves of sublethally irradiated and bone marrow-transplanted mice upon LPS-induced sepsis (12 mg/kg, i.p.). Left: Impaired septic survival of WT mice carrying PHD3−/− bone marrow (WT/PHD3−/− BM) compared with WT mice implanted with WT bone marrow (WT/WT BM). Right: Improved septic survival of PHD3−/− mice carrying WT bone marrow (PHD3−/−/WT BM) compared with PHD3−/− mice transplanted with PHD3−/− bone marrow (PHD3−/−/PHD3−/− BM). n = 9. Data are representative of two independent experiments. *p < 0.05.
HIF-1α detection limit (6, 34), we assessed mRNA expression levels of the baseline conditions, where HIF-1α revealed increased ingestion of zymosan particles (white arrows) by PHD3-deficient peritoneal macrophages. (FIGURE 5. Enhanced proinflammatory functions of PHD3-deficient macrophages. (A–C) qRT-PCR analysis of BMDMs isolated from WT or PHD3−/− mice, revealing significantly increased expression of IL-6 (A), IL-1β (B), and ICAM-1 (C) mRNA transcripts in LPS-treated (1 μg/ml) PHD3−/− BMDMs. (D) Transwell migration assay applying peritoneal macrophages isolated from WT or PHD3−/− mice, revealing enhanced migration of PHD3−/− cells. (E and F) Phagocytosis assays applying peritoneal macrophages. (E) Representative May–Grüwald Giemsa stainings reveal increased ingestion of zymosan particles (white arrows) by PHD3−/− macrophages (E) right compared with WT cells (E) left. Scale bar, 10 μm. (F) Quantification analysis reveals increased ingestion of zymosan particles [phagocytosis; (F) left] and of apoptotic bodies [efferocytosis; (F) right] by PHD3−/− cells. Bars represent mean ± SEM. n = 6. Data are representative of three independent experiments. *p < 0.05, **p < 0.01.

significantly more pronounced in PHD3−/− macrophages (Fig. 7A). Notably, further analysis revealed that interference with PHD3 expression did not affect nuclear translocation of the NF-κB p50 subunit (Supplemental Fig. 3C).

Stabilization of HIF-1α and HIF-2α was studied in murine J774A.1 macrophages subjected to siRNA-mediated knockdown of PHD3 expression. mRNA expression analysis via qRT-PCR demonstrated that RNAi transfer resulted in incomplete silencing of PHD3 mRNA levels by 76% versus siControl-transfected J774A.1 cells (PHD3 mRNA copies/106 copies of 18S: 2.1 ± 0.3 in siControl-transfected cells versus 0.3 ± 0.04 in siPHD3-transfected cells, n = 7; p = 0.001). Because of the difficulty of quantifying appropriate amounts of murine HIF-1α protein under baseline conditions, where HIF-1α protein is mostly below the detection limit (6, 34), we assessed mRNA expression levels of the HIF-1α target gene carbonic-anhydrase 9 (Cahx) (35). Indeed, expression of CahX was 2.3-fold upregulated in PHD3-silenced macrophages compared with control-transfected cells (CahX mRNA copies/106 copies 18S: 0.9 ± 0.3 in control-transfected cells versus 2.1 ± 1.2 in PHD3-silenced cells; p = 0.03, n = 6). After LPS exposure, HIF-1α protein levels were detectable by Western blotting and found to be significantly increased in PHD3-silenced compared with control-transfected macrophages (Fig. 7B). HIF-2α protein levels were slightly but not significantly increased in PHD3-silenced cells already at baseline conditions (data not shown). LPS treatment did not cause a significant further increase of HIF-2α protein in PHD3-silenced or control cells (Fig. 7C). We likewise investigated hypoxic stabilization of HIF-1α and HIF-2α in BMDMs isolated from WT and PHD3−/− mice. Western blotting revealed that hypoxic stabilization of HIF-1α was enhanced in BMDMs lacking PHD3 compared with WT cells (Supplemental Fig. 3D). By contrast, HIF-2α levels were extremely low in BMDMs at both normoxic and hypoxic conditions (Supplemental Fig. 3D). Taken together, interference with PHD3 expression increased the LPS-dependent induction of both NF-κB activity and HIF-1α stabilization in macrophages.

To investigate whether these factors are indeed mediators of the proinflammatory phenotype in PHD3-deficient mice, we assessed whether interference with the expression of HIF-1α, HIF-2α, or the p65 subunit of NF-κB reverted increased cytokine production and differentiation of PHD3-deficient macrophages in vitro. RNAi transfer resulted in incomplete and selective silencing of HIF-1α, HIF-2α, or NF-κB p65 mRNA levels by ~80% (Supplemental Table 1). In accordance with the findings outlined earlier, expression of IL-6 mRNA transcripts was increased by ~2-fold in PHD3-silenced cells compared with control-transfected cells after 2 h of LPS treatment (Fig. 7D). A comparable induction of IL-6 transcript expression was observed in PHD3 and HIF-2α double-silenced macrophages (Fig. 7D). By contrast, simultaneous downregulation of HIF-1α or NF-κB p65 dramatically blunted the LPS-induced increase of IL-6 expression in PHD3-silenced cells (Fig. 7D). Similarly, knockdown of HIF-1α or NF-κB p65 expression fully reverted the enhanced maturation of PHD3-silenced J774A.1 cells into macrophages, as indicated by a blunted increase of the macrophage differentiation marker CD36 (Fig. 7E). Of note, silencing of HIF-2α expression did not affect enhanced macrophage differentiation from PHD3-deficient BMCs. Altogether, these experiments suggest a prominent role for NF-κB and HIF-1α but not HIF-2α in mediating the increased inflammatory response that occurs upon macrophage loss of PHD3.

Discussion

Hypoxia and HIFs are crucial modulators of systemic inflammation (4, 32, 36); however, experimental insight into specific in vivo functions of PHD1, PHD2, and PHD3 in this context is scarce. All three HIF prolyl hydroxylases are reportedly expressed in leukocytes and macrophages of the innate immune system (6, 26), suggesting that interference with their activity might alter innate immune functions and, therefore, the inflammatory response to bacterial pathogens. In this study, we provide in vivo evidence that loss of PHD3 aggravates the clinical course of abdominal sepsis. This effect appeared to be specific for loss of PHD3, as deficiency of PHD1 (PHD1−/−) or PHD2 (PHD2−/−) did not result in a similar phenotype.

Several lines of in vivo evidence from this study indicate that premature lethality of septic PHD3−/− mice was due to hyperactivated innate immune responses. First, increased lethality of septic PHD3−/− mice occurred concomitantly with enhanced levels of leukocyte-derived cytokines and with enforced recruitment of macrophages to internal organs. Second, depletion of functional macrophages markedly attenuated the hypersensitivity of PHD3−/− mice to abdominal sepsis. Third, insertion of PHD3−/− bone marrow induced premature septic lethality of otherwise genetically unaltered mice, indicating that enhanced progression of abdominal sepsis was attributable to specific alterations in innate immune cells lacking PHD3.
Consistently, a series of functional in vitro assays demonstrated that inhibition of PHD3 led to a robust stabilization of diverse proinflammatory macrophage functions. For instance, PHD3−/− macrophages displayed increased phagocytic and migratory properties. Further experiments confirmed that differentiation and proinflammatory activation of macrophages was augmented in the absence of PHD3. By contrast, recent studies from our laboratories revealed that loss of PHD3 does not significantly affect proinflammatory functions of neutrophils (21), altogether indicating that the enhanced susceptibility of PHD3−/− mice to abdominal sepsis is specifically attributable to macrophage-mediated systemic inflammatory responses.

Hypoxia and inflammation are closely related. Indeed, inflammation can be caused by chronic hypoxia, such as in humans exposed to high altitudes (3). Vice versa, tissue inflammation commonly results in hypoxia, as indicated by frequent stabilization of HIF-1α and HIF-2α in inflamed tissues (3). The notion that immunomodulatory functions could be assigned to HIF prolyl hydroxylases is therefore not far-fetched. Macrophage proinflammatory functions, in particular, are crucially stimulated by hypoxia (5, 9, 26, 37, 38). For instance, macrophages produce higher levels of proinflammatory cytokines when exposed to hypoxia than under normoxic conditions, an effect that is mediated by HIF-1α (26). HIF-1α is likewise stabilized upon activation of macrophages by LPS treatment (7, 39) and is then indispensable to foster the potential of leukocytes to invade tissues and destroy bacterial pathogens (6, 8, 9, 37, 40). Because of the relevance of HIF-1α in inflammatory responses, its cell-specific deletion in macrophages is protective against LPS-induced lethality in mice (9). These findings are consistent with our observation that loss of PHD3, which is suspected positively to regulate HIF-1α-dependent transcriptional signaling in macrophages, promotes LPS-induced lethality.

On the molecular level, it therefore appears likely that HIF-1α is a major stimulator of proinflammatory functions in PHD3-deficient macrophages. Indeed, our protein expression analyses revealed that stabilization of HIF-1α in macrophages is strongly enhanced by the presence of LPS [which is consistent with other reports (6, 7)] but even further increased upon interference with PHD3 expression. By contrast, HIF-2α protein stabilization in macrophages was neither significantly affected by LPS treatment nor by knockdown of PHD3 expression. Notably, increased differentiation and proinflammatory function of PHD3-deficient macrophages was fully reverted by simultaneous interference with HIF-1α and HIF-2α in inflamed tissues (3). The notion that HIF-2α in myeloid cells display increased re-

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FIGURE 6. Enhanced macrophage differentiation and M1 polarization upon loss of PHD3. (A–C) Macrophage differentiation of BMCs isolated from WT or PHD3−/− cells and subjected to M-CSF (10 ng/ml) for 5 d. (A) Representative immunochemistry for CD36 (macrophage marker, red), actin (cytoplasm, green), and DAPI (cell nuclei, blue). Note predominantly CD36-negative cells (arrowheads) in differentiating cultures of WT BMCs [(A) left] and increased fraction of CD36-positive macrophages (arrows) in differentiating cultures of PHD3−/− cells [(A) right]. Scale bars, 20 μm. (B) qRT-PCR analysis, revealing increased CD36 transcript expression in M-CSF-treated PHD3−/− BMCs. (C) Representative histogram (left) and quantification of FACS analyses (right), revealing increased fractions of CD36+ macrophages in differentiating cultures of PHD3−/− BMCs. (D) qRT-PCR analysis, revealing increased mRNA transcript expression of M1 differentiation markers (iNOS, TNF-α, IL-23, CXCL10) but not M2 differentiation markers (Arg = arginase, CCR2, IL-10, RANTES) in LPS-treated (1 μg/ml) PHD3−/− BMDMs. Expression values were normalized to expression in non-LPS-treated cells. Bars represent mean ± SEM. n = 6. Data are representative of four independent experiments. *p < 0.05, **p < 0.01.
cytokines driving proinflammatory (M1) activation and Th2 cytokines driving immunomodulatory (M2) activation of macrophages, respectively (39). This is in keeping with our finding that increased HIF-1α protein stabilization in PHD3-deficient macrophages occurs concomitantly with enhanced proinflammatory (M1) activation.

It is worthwhile to speculate whether the hyperinflammation phenotype in PHD3<sup>−/−</sup> mice is at least partly mediated independently of HIF pathways. For instance, loss of PHD3 in macrophages might directly affect the transcriptional activity of the master regulator of innate immunity, NF-κB (29). It has been demonstrated that HIF prolyl hydroxylases are repressors of NF-κB activity, likely via their potential directly to hydroxylate the IκB kinase (IKKβ), which is responsible for phosphorylation-dependent degradation of IκB inhibitors, and, therefore, liberation and activation of NF-κB in response to inflammatory stimuli (41, 42). Furthermore, it has been documented that PHD3 can associate with IKKβ independently of its hydroxylase function, thereby blocking further interaction between IKKβ and the chaperone heat shock protein 90, which is required for IKKβ phosphorylation and release of NF-κB (43, 44). In this study, we found that NF-κB activity in macrophages was significantly increased upon loss of PHD3 and that interference with NF-κB p65 expression reverted the proinflammatory phenotype of PHD3-deficient macrophages. This might appear paradoxical in the light of previous findings that targeted inhibition of IKKβ, which blocks NF-κB activation, likewise causes enhanced susceptibility of mice to endotoxemia (45). However, unlike in our model, these effects importantly relied on excess recruitment of neutrophil granulocytes. Indeed, IKKβ-deficient mice develop massive granulocytosis, and the depletion of neutrophil granulocytes improves their survival in conditions of sepsis (45). By contrast, anti-inflammatory effects of IKKβ were also reported. In particular, myeloid-specific deletion of IKKβ can enhance the survival of mice during bacterial inflammation (46), likely via inhibiting classical “M1” activation of macrophages, thus preventing their excess proinflammatory activity during infection (46). Consistent with previous reports (47), it is therefore conceivable that proinflammatory effects in PHD3-deficient macrophages are partly due to HIF-independent induction of NF-κB activity in macrophages and that additional knockout of HIF-1α alone would therefore be insufficient fully to revert the phenotype of enhanced sepsis progression in PHD3<sup>−/−</sup> mice.

On the basis of our study, it can be concluded that both HIF-1α and NF-κB are required for boosting of the innate immune response in septic PHD3<sup>−/−</sup> mice. Recent work from our laboratories (21) has shown that PHD3 deficiency accelerates resolution of tissue inflammation, specifically by abrogating hypoxia-mediated neutrophil survival. Notably, there was no evidence of upregulation of HIF-1α or NF-κB targets in PHD3-deficient neutrophils (21). Further work will determine whether these differences in myeloid cell behavior reflect differences between monocyte-macrophages and neutrophils, specific responses to hypoxia, the local versus systemic nature of the insults, or distinct...
roles for PHD3 in the initiation compared with resolution of inflammation. It is in this context remarkable that HIF-1α itself does not solely provoke proinflammatory effects, but may likewise drive anti-inflammatory pathways in certain pathologic conditions. For instance, HIF-1α can attenuate hypoxia-induced inflammation of the intestine by increasing mucosal adenosine signaling (48) and by inducing the expression of netrin-1 in hypoxic epithelial cells (49). It will therefore become important further to delineate specific contributions of the individual components in the PHD–HIF regulatory axis, and their interplay with cellular effectors of innate immunity, in diverse pathologic settings that are crucially determined by the innate immune responses, such as organ injury caused by ischemia and reperfusion (50), asthma and chronic obstructive pulmonary disease (51, 52), viral infections (32), or granulomatous inflammation (53).

Regardless of the underlying molecular mechanisms, the finding that loss of PHD3 enzyme function augments the innate immune response might have consequences from a clinical perspective. On the one hand, it should be considered that interference with PHD3 function might lead to unfavorable effects in critically ill patients, a notion that might become of clinical relevance in the prospect of strong and immediate activation of the innate immune response, applying pharmaceutical PHD inhibitors to treat anemia (54) or a notion that might become of clinical relevance in the prospect of inducing arteriogenesis.

Disclosures

The authors have no financial conflicts of interest.

References


**Supplemental Figure 1.** **A-C**, Survival curves of mice suffering LPS-induced sepsis (15 mg/kg, i.p.), revealing comparable survival of WT, PHD1<sup>−/−</sup> and PHD2<sup>−/−</sup> mice (**A, B**), but impaired survival of PHD3<sup>−/−</sup> mice (**C**). **D**, Disease activity index, indicating increased clinical sepsis symptoms in PHD3<sup>−/−</sup> mice 8 and 20 hours following i.p. injection of LPS (15 mg/kg). **E**, Quantification of colony-forming units (CFUs) harvested 20 hours following CLP, revealing comparable abundance of enteric bacteria in peripheral blood and peritoneal lavage fluid from WT and PHD3<sup>−/−</sup> mice. **F-H**, Cytokine measurements after induction of polymicrobial sepsis (by caecal ligation and puncture), revealing elevated levels of pro-inflammatory cytokines IL-1β (**F**), IL-6 (**G**) and TNF-α (**H**) in PHD3-deficient mice. *P < 0.05, **P < 0.01, n = 8 in all groups. Results are representative of two independent experiments.
Supplemental Figure 2.

A, B, ELISA-based assessment of TNF-α in kidneys (A) and lungs (B) from baseline healthy or LPS-treated mice (15 mg/kg, i.p.), revealing enhanced TNF-α-concentrations in organs from septic PHD3⁻/⁻ animals after 4 and 8 hours of LPS-treatment. Comparable results were obtained when assaying for the pro-inflammatory cytokines IL1-β and IL-6 in lungs and kidneys of LPS-challenged mice (not shown). C, D, Ly6G immunohistochemistry of septic kidneys (C) and bronchoalveolar lavage (BAL) of septic lungs (D) to further delineate the contribution of accumulating neutrophil granulocytes to septic organ damage in WT and PHD3⁻/⁻ mice. Ly6G immunohistochemistry expectedly revealed enhanced neutrophil accumulation in kidneys (C) of septic mice 8 hours following LPS-treatment (15 mg/kg; arrowheads). However, sepsis-induced neutrophil-invasion was comparable in WT and PHD3⁻/⁻ mice (Ly6G⁺ cells/mm²: 12.4 ± 8.1 in WT versus 12.3 ± 6.4 in PHD3⁻/⁻ kidneys; P = N.S.; n = 5). Quantification of leukocytes in BAL samples (D) revealed increased numbers of macrophages, but unaltered numbers of neutrophils in lungs from septic PHD3⁻/⁻ mice compared to their WT littermates (% of neutrophils in BAL: 0.85 ± 0.01 in WT, 0.78 ± 0.01 in PHD3⁻/⁻ mice; P = N.S.; n = 5). Bars represent mean ± SEM, * P < 0.05, n = 6. Data are representative of 2 independent experiments. Scale bar = 50 μm in 2C,D.
Supplementary Figure 3.

**A**, **B**, qRT-PCR analysis of BMDM isolated from WT or PHD3−/− mice, and continuously treated with LPS (1μg/ml) for 24 hours. Expression of IL-6 (**A**) and IL-1β (**B**) was highly induced in WT and PHD3−/− BMDM at 2-4 hours following LPS-challenge, but then gradually decreased, suggesting that loss of PHD3 enhances macrophage cytokine expression in the initiation phase of acute systemic inflammation. Curves represent mean ± SEM, * P < 0.05, n = 6. **C**, Western blotting of nuclear extracts, revealing unaltered activation (nuclear translocation) of the of NF-κB p50 subunit in PHD3-silenced (siPHD3) or control-transfected (siCx) J774A.1 macrophages with or without LPS-treatment. Histone H3 expression was assessed as loading control. **D**, Western blotting of nuclear extracts, revealing enhanced hypoxic stabilization of HIF-1α in cells deficient for PHD1 (PHD1−/−), PHD2 (PHD2−/−) and PHD3 (PHD3−/−) compared to BMDM isolated from WT mice. HIF-2α was only weakly detectable in WT and PHD-deficient BMDM at both normoxic and hypoxic conditions. Data are representative of 3 independent experiments. N, normoxia; H, hypoxia.
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**Supplementary Table 1.** qRT-PCR analysis of HIF-1α, HIF-2α, revealing knockdown efficacies in J774A.1 cells. Residual expression levels of HIF-1α and HIF-2α in siControl-transfected-, siHIF-1α-transfected-, and siHIF-2α-transfected cells are expressed as mRNA copies/10⁴ copies of 18S. Knockdown of HIF-1α resulted in selective downregulation of HIF-1α transcripts, without affecting HIF-2α expression. Conversely, HIF-2α siRNA selectively downregulated HIF-2α-, but not HIF-1α expression. **P < 0.01 vs siControl, ##P < 0.01 vs siControl, n = 8.**