Ferritin-Mediated Iron Sequestration Stabilizes Hypoxia-Inducible Factor-1α upon LPS Activation in the Presence of Ample Oxygen

Graphical Abstract

Authors
Isabel Siegert, Johannes Schödel, Manfred Nairz, ..., Günter Weiss, Carsten Willam, Jonathan Jantsch

Correspondence
jonathan.jantsch@ukr.de

In Brief
Siegert et al. find that the microbial cell wall component LPS reduces cytosolic free iron availability via induction of ferritin. Low free iron levels impair the prolyl hydroxylase domain enzyme (PHD) activity, thereby inhibiting hypoxia-inducible factor (HIF)-1α hydroxylation. This results in inflammatory HIF1α stabilization under normoxic conditions.

Highlights
- LPS blocks prolyl hydroxylase domain (PHD) enzyme activity and HIF1α degradation
- LPS induces ferritin expression and lowers free available iron levels
- This results in deprivation of an essential PHD cofactor and HIF1α stabilization
Ferritin-Mediated Iron Sequestration Stabilizes Hypoxia-Inducible Factor-1α upon LPS Activation in the Presence of Ample Oxygen

Isabel Siegert,1 Johannes Schödel,2 Manfred Nairz,3,13 Valentin Schatz,4 Katja Dettmer,8 Christopher Dick,4 Joanna Kalucka,2,14 Manfred Nairz,13 Valentin Schatz,4 Katja Dettmer,5 Christopher Dick,4 Joanna Kalucka,2,14 Kristin Franke,6 Martin Ehrenschwender,4 Gunnar Schley,2 Angelika Beneke,7 Jörg Sutter,8 Matthias Moll,6 Claus Hellerbrand,9 Ben Wielockx,10 Dörthe M. Katschinski,7 Roland Lang,1 Bruno Galy,10 Matthias W. Hentze,11 Peppi Koivunen,12 Peter J. Oefner,5 Christian Bogdan,1 Gunter Weiss,8 Carsten Willam,2 and Jonathan Jantsch1,4,*

1Mikrobiologisches Institut – Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen and Friedrich-Alexander Universität (FAU) Erlangen-Nürnberg, 91054 Erlangen, Germany
2Department of Nephrology and Hypertension, Universitätsklinikum Erlangen and Friedrich-Alexander Universität (FAU), 91054 Erlangen, Germany
3Department of Internal Medicine VI, Infectious Diseases, Immunology, Rheumatology, Pneumology, Medical University of Innsbruck, 6020 Innsbruck, Austria
4Institute of Clinical Microbiology and Hygiene, University Hospital of Regensburg and University of Regensburg, 93053 Regensburg, Germany
5Institute of Functional Genomics, University of Regensburg, 93053 Regensburg, Germany
6Heisenberg Research Group, Department of Clinical Pathobiocchemistry, Institute of Clinical Chemistry and Laboratory Medicine, University of Technology, 01307 Dresden, Germany
7Institute of Cardiovascular Physiology, University Medical Center, Georg-August-University Göttingen, 37073 Göttingen, Germany
8Department of Chemistry and Pharmacy, Inorganic Chemistry, Friedrich-Alexander Universität (FAU) Erlangen-Nürnberg, 91058 Erlangen, Germany
9Department of Internal Medicine I, University of Regensburg, 93053 Regensburg, Germany
10Division of Virus-Associated Carcinogenesis, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany
11European Molecular Biology Laboratory, 69120 Heidelberg, Germany
12Biocenter Oulu, Faculty of Biochemistry and Molecular Medicine, Oulu Center for Cell-Matrix Research, University of Oulu, 90014 Oulu, Finland
13Present address: Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
14Present address: Laboratory of Angiogenesis and Neurovascular Link, Department of Oncology, KU Leuven, 3000 Leuven, Belgium
*Correspondence: jonathan.jantsch@ukr.de
http://dx.doi.org/10.1016/j.celrep.2015.11.005
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SUMMARY

Both hypoxic and inflammatory conditions activate transcription factors such as hypoxia-inducible factor (HIF)-1α and nuclear factor (NF)-κB, which play a crucial role in adaptive responses to these challenges. In dendritic cells (DC), lipopolysaccharide (LPS)-induced HIF1α accumulation requires NF-κB signaling and promotes inflammatory DC function. The mechanisms that drive LPS-induced HIF1α accumulation under normoxia are unclear. Here, we demonstrate that LPS inhibits prolyl hydroxylase domain enzyme (PHD) activity and thereby blocks HIF1α degradation. Of note, LPS-induced PHD inhibition was neither due to cosubstrate depletion (oxygen or α-ketoglutarate) nor due to increased levels of reactive oxygen species, fumarate, and succinate. Instead, LPS inhibited PHD activity through NF-κB-mediated induction of the iron storage protein ferritin and subsequent decrease of intracellular available iron, a critical cofactor of PHD. Thus, hypoxia and LPS both induce HIF1α accumulation via PHD inhibition but deploy distinct molecular mechanisms (lack of cosubstrate oxygen versus deprivation of co-factor iron).

INTRODUCTION

Hypoxia-inducible transcription factors (HIFs) are essential for cellular adaptation to low oxygen microenvironments. The oxygen-dependent regulation of HIF-1α (HIF1α) stabilization involves hypoxic inhibition of prolyl hydroxylase domain enzyme (PHD) activity, leading to impaired post-translational HIF1α hydroxylation at proline 402 (P402) and proline 564 (P564) in the oxygen-dependent degradation (ODD) domain. This diminishes VHL (von Hippel-Lindau tumor suppressor)-dependent HIF1α ubiquitination and proteasomal degradation (reviewed in Kaelin and Ratcliffe, 2008; Semenza, 2012). In addition to hypoxia, a variety of pathogen-derived molecules and inflammatory mediators are able to stabilize HIF1α under normoxic conditions (reviewed in Palazon et al., 2014). In macrophages, HIF1α promotes...
glycolytic activity, motility, invasiveness, and bacterial killing under normoxia (Cramer et al., 2003). In dendritic cells (DC), HIF1α supports maturation, activation, migration, and antigen presentation (Bhandari et al., 2013; Jantsch et al., 2008; Köhler et al., 2012; Pantel et al., 2014). However, data on the molecular mechanisms that underlie inflammation-driven, normoxic HIF1α accumulation are sparse. In macrophages, lipopolysaccharide (LPS)-induced HIF1α accumulation requires nuclear factor (NF)-κB- and p42/44 MAPK-dependent transcriptional events (Frede et al., 2006; Rius et al., 2008). In addition, LPS-triggered reactive oxygen species (ROS) production and/or succinate accumulation is linked with PHD inhibition in macrophages (Nicholas and Sumbayev, 2010; Tannahill et al., 2013). However, the mechanism by which LPS stabilizes HIF1α in DC is unknown.

RESULTS

**LPS-Induced HIF1α Stabilization Requires Posttranslational Mechanisms**

As demonstrated earlier (Jantsch et al., 2011), stimulation of DC with Toll-like receptor (TLR) ligands 4 (LPS) and 9 (CpG) stabilized HIF1α (Figure 1A). This was paralleled by a weak Hif1a mRNA induction. In comparison, TLR 3 activation using poly(I:C) significantly augmented Hif1a mRNA levels but failed to stabilize HIF1α protein. Hence, multistep processes are likely to be involved in TLR-induced HIF1α accumulation. To examine the role of mRNA stability, we measured Hif1a mRNA levels following treatment with RNA-synthesis inhibitor actinomycin D (ActD). Hif1a mRNA stability in LPS-treated DC was not altered, compared to untreated DC (Figure 1B). In order to assess potential posttranslational mechanisms, we silenced Hif1a in LPS-stimulated DC (Figure 1C). In this situation, Hif1a mRNA was not stabilizing.

![Figure 1. LPS-Induced HIF1α Stabilization Requires Hif1a mRNA and Additional Posttranslational Processes](image)

(A) DC were left untreated (un) or stimulated for 17 hr with 10 ng/ml LPS, 1 μM CpG, or 10 μg/ml poly(I:C). Upper panel: Hif1a mRNA expression (data indicate mean ± SEM; N = 9; n = 10; Kruskal-Wallis test with Dunn’s multiple comparison test; *p < 0.05, versus for untreated). Lower panel: HIF1α and actin (representative of N = 3).

(B) After culture of DC with or without 10 ng/ml LPS for 17 hr, 5 μM ActD was added. Hif1a mRNA levels were determined relative to time 0 of ActD treatment (data indicate mean ± SEM; N = 8; n = 4–6).

(C) After culture of DC with or without 10 ng/ml LPS for 17 hr, DC were electroporated with either non-silencing (ns) siRNA or Hif1a-specific siRNA. Hif1a mRNA expression (data indicate mean ± SEM; N = 3; n = 4) and the HIF1α and actin levels were determined (representative of N = 4).

(D) DC were electroporated with ns siRNA or Hif1a-specific siRNA and subsequently cultured with or without 10 ng/ml LPS for 17 hr. Hif1a mRNA expression (data indicate mean ± SEM; N = 5; unpaired Student’s t test; *p < 0.05, HIF1α, and actin (representative of N = 4) levels are shown.

(E) DC were cultured with or without 10 ng/ml LPS for 17 hr under normoxic or hypoxic (0.5% O2) conditions. PHD1–3 and VHL protein levels are shown. Either actin or non-specific (n.s.) bands of the respective antibody were used to demonstrate equal loading (representative of N = 2).

(F) DC were left untreated or stimulated with either 10 ng/ml LPS or 100 μM DFO for 17 hr. Using the radiolabeled HIF1α-ODD domain, relative PHD activity of the lysates was determined with or without addition of cofactors. PHD activity is given in relation to the respective maximal enzymatic activity (in presence of all co-factors, including iron) after normalization to untreated controls (data indicate mean ± SEM; N = 4; n = 6; Kruskal-Wallis test with Dunn’s multiple comparison test; *p < 0.05).

(G) DC were treated with or without 10 ng/ml LPS for 20 hr under normoxic or hypoxic (0.5% O2) conditions. PHD1–3 and VHL protein levels are shown. Either actin or non-specific (n.s.) bands of the respective antibody were used to demonstrate equal loading (representative of N = 2).

(F) Luciferase activity (light units, L.U.) was determined in DC generated from ODD-Luc mice unstimulated (un) or stimulated with 10 ng/ml LPS for 17 hr (data indicate mean ± SEM; N = 6; n = 22; Mann-Whitney test; *p < 0.05).
required to maintain HIF1α on the protein level (Figure 1C). Vice versa, silencing of Hif1α before the LPS challenge interfered with HIF1α accumulation (Figure 1D). Hence, Hif1α mRNA expression is necessary but not sufficient for LPS-induced HIF1α stabilization, which requires additional posttranslational mechanisms. In DC, either LPS or hypoxia, or both, induced PHD2 and PHD3 on the protein level (Figure 1E). Furthermore, LPS did not negatively affect the abundance of PHD1 and VHL (Figure 1E). Hence, LPS-induced HIF1α accumulation is not the result of reduced PHD or VHL protein levels.

Given the central role of PHD in the posttranslational regulation of HIF1α, we hypothesized that LPS stimulation might interfere with enzymatic PHD activity. Hence, we compared the PHD activity of DC treated with LPS and the iron (Fe) chelator desferrioxamine (DFO). DFO impairs PHD activity by chelating the enzyme’s critical cofactor Fe (reviewed in Greer et al., 2012). Using the radiolabeled HIF1α-ODD domain, the percentages of the maximum relative PHD activities of lysates were determined by calculating the ratio of the respective PHD activity in the presence or absence of all required cofactors, including Fe. LPS and DFO were comparably effective in reducing PHD enzyme activity (Figure 1F; P_{DFO versus LPS} > 0.99). This was paralleled by impaired P402 and P564 hydroxylation of HIF1α (Figure 1G).

LPS-induced PHD inhibition was confirmed in DC derived from genetically engineered mice having the ODD domain fused to a luciferase gene (ODD-Luc; Safran et al., 2006). LPS treatment augmented light emission (Figure 1H), again suggesting reduced PHD activity. Importantly, we excluded exhaustion of PHD activity through endogenous LPS-induced HIF1α accumulation. Silencing Hif1α using small interfering RNA (siRNA) duplexes that do not target ODD-domain-encoding sequences reduced LPS-induced HIF1α accumulation, whereas increased light emission from ODD-Luc-derived DC following LPS challenge was detectable (Figure S1). Taken together, LPS stimulation reduced PHD activity and subsequent HIF1α hydroxylation.

**LPS-Induced PHD Inhibition Is Not Caused by Deficiency of PHD Cosubstrates or Inhibitory Endogenous Metabolites**

Next, we tried to identify the factor that impeded PHD activity in LPS-stimulated DC. PHD activity requires α-ketoglutarate (zKG) and O2 as substrates and Fe as a cofactor (reviewed in Greer et al., 2012) (Figure 2A). By competing with zKG, the tricarboxylic acid cycle (TCA) intermediates fumarate and succinate act as PHD inhibitors. Furthermore, ROS and reactive nitrogen species (RNS) negatively affect PHD activity (reviewed in Greer et al., 2012).

Given that monolayers of cells cultured under normoxic conditions can experience hypoxia, depending on the cell culture conditions (Doege et al., 2005), we assessed the O2 levels of our cell culture system by using luminescence optical O2 imaging. In contrast to hypoxic controls, LPS did not lower the oxygen concentration at the bottom of the cell culture dish excluding LPS-triggered O2 depletion (Figure 2B).

LPS stimulation did not deplete the PHD substrate zKG (Figure 2C), and supplementation of zKG (Figure S2A) did not affect LPS-induced HIF1α stabilization (Figure 2D). LPS increased fumarate and succinate levels in DC (Figures 2E and 2F). However, raising fumarate levels in DC with dimethylfumarate (Figure S2B) did not induce HIF1α accumulation (Figure 2G). Elevation of succinate levels brought about by a succinate dehydrogenase inhibitor (malonate; Figure S2C) did not enhance HIF1α levels in DC (Figure 2H).

Endogenous ROS and exogenous RNS are known to inhibit PHD activity (Metzen et al., 2003; Nicholas and Sumbayev, 2010). Notably, LPS stimulation resulted in ROS and RNS production by DC (Figures 2I and 2J). Pretreatment with the ROS scavenger N-acetyl cysteine (NAC) impaired LPS-induced ROS production (Figure 2I) but did not block HIF1α accumulation (Figure 2K). In line with earlier findings (Metzen et al., 2003), we observed that LPS-triggered HIF1α accumulation was partially reduced in DC treated with the inducible nitric oxide (NO) synthase 2 (Nos2) inhibitor L-NIL (Figures 2L and S2D). Nos2 ablation (Nos2^-/-) had the same effect, regardless of the presence or absence of the phagocyte NADPH oxidase (gp91 phox, cytochrome b-245 β chain; Cybb^-/-; Figure 2M). Importantly, LPS-induced HIF1α accumulation remained substantial in Nos2^-/- or L-NIL-treated DC, suggesting additional regulatory mechanisms.

**LPS Activation Depletes the Available Intracellular Iron Pools and Impairs PHD Activity**

Next, we assessed the levels of cellular Fe and its impact on LPS-induced HIF1α stabilization. Compared to controls, LPS stimulation did not reduce total cellular Fe levels (Figure 3A). However, gel retardation assays demonstrated that LPS stimulation increased binding of iron-regulatory proteins (IRPs) to a radiolabeled iron responsive element (IRE) RNA probe (Figure 3B), indicative of reduced cytosolic Fe availability (Hentze et al., 2010). This was confirmed by assays that use the ability of Fe to quench calcein fluorescence. LPS stimulation enhanced the quenchable iron pool and, hence, is linked to a depletion of intracellular available Fe (Figure 3C).

Accordingly, exogenous addition of Fe(II)Cl2 blunted LPS-induced HIF1α accumulation in a dose-dependent manner (Figure 3D). Similar results were obtained for Fe(III)Cl3 or ferric ammonium citrate (FAC) (Figure S3A). Besides abolishing LPS-induced HIF1α accumulation, Fe(II)Cl2 addition impaired LPS-induced upregulation of MHCII and co-stimulatory molecules (Figure S3B). This observation conforms to findings that HIF1α promotes LPS-induced maturation of DC (Bhandari et al., 2013; Jantsch et al., 2008). Of note, the addition of Fe(II)Cl2 also interfered with LPS-induced HIF1α accumulation in mouse macrophages or human monocytic THP-1 cells (Figures S3C and S3D).

Treatment with the lipophilic zKG-analog PHD inhibitor ICA or injection of LPS induced HIF1α accumulation in splenic DC in vivo (Figure 3E). In line with our in vitro findings, pretreatment of mice with Fe(III)-gluconate reduced LPS-induced HIF1α accumulation in splenic DC (Figure 3E).

The iron-mediated reduction of HIF1α accumulation was paralleled by restored hydroxylation of HIF1α at P402 and P564 (Figure 3F). Furthermore, the addition of Fe(II)Cl2 to LPS-stimulated ODD-Luc-derived DC blocked LPS-induced light emission, indicating restoration of PHD activity (Figure S3E). The PHD inhibitor ICA extinguished the effect of Fe(II)Cl2 on LPS-induced HIF1α
accumulation (Figure 3G). In untreated Phd2−/− and Phd2/3−/− DC, HIF1α was detectable and was enhanced by LPS stimulation (Figure S3F). The addition of Fe(II)Cl2 had only marginal effects on HIF1α levels in Phd2-deficient DC and was completely ineffective in Phd2/3-deficient DC (Figure S3F).

LPS-Induced Upregulation of FTH1 Triggers HIF1α Stabilization

Several molecules are involved in the regulation of intracellular available Fe in immune cells (reviewed in Nairz et al., 2014). In DC, LPS induced the expression of the antimicrobial Fe-scavenging molecule Lipocalin-2 (LCN2) (Figure 4A), which is also involved in reducing intracellular available Fe pools (reviewed in Nairz et al., 2014). However, in Lcn2−/− DC, LPS-induced HIF1α accumulation remained unaffected (Figure 4B).

Ferroportin-1 (SLC40a1) is the only known Fe export protein (reviewed in Nairz et al., 2014). In line with unaltered total cellular Fe content in LPS-treated DC, SLC40a1 levels were not affected by LPS stimulation in DC (Figure S4A). Hence, it is unlikely that LPS-induced HIF1α accumulation is based on SLC40a1-mediated Fe export. Nevertheless, the silencing of basal SLC40a1 protein expression slightly reduced LPS-triggered HIF1α levels, supporting our findings that available intracellular Fe content plays an important role in LPS-induced HIF1α accumulation (Figure S4B).

Natural-resistance-associated macrophage protein 1 (SLC11a1) is thought to deplete available Fe in cells (reviewed in Nairz et al., 2014). SLC11a1-dependent Fe depletion has been implicated in HIF1α accumulation in monocytes (Knowles et al., 2006). LPS stimulation induced expression of Slt1a1 in DC (Figure S4C). Nevertheless, a role for SLC11a1 in our model system is unlikely, because we generated DC from C57BL/6 mice harboring a homozygous mutation in both SLC11a1 alleles.

**Figure 2.** LPS-Induced PHD Inhibition Is Not Caused by Deficiency of PHD Cosubstrates or Accumulation of Inhibitory Endogenous Metabolites

(A) Schematic overview of PHD-dependent HIF1α regulation.

(B) DC were cultured with or without 10 ng/ml LPS under ∼20% O2 (N) or 0.5% O2 (H). O2 was detected at the bottom of plates (mean; N = 4; n = 5).

(C) DC were cultured with or without 10 ng/ml LPS for 17 hr, and αKG levels were determined (mean + SEM; N = 4; n = 17–18; unpaired Student’s t-test, un, untreated).

(D) DC were cultured as described in (C). Where indicated, αKG was added. HIF1α and actin levels are shown (representative of N = 3).

(E and F) DC were cultured as in (C), but fumarate levels (E) and succinate levels (F) were detected (mean + SEM; N = 4; n = 17–18; unpaired Student’s t-test with Welch correction; *p < 0.05).

(G and H) DC were cultured as in (C) with or without (G) 2 μM DMF (representative of N = 4) or (H) 1 mM malonate (representative of N = 2).

(I) 10 mM NAC (dashed line) or untreated DC (solid line) were cultured in the absence (black) or presence (red) of LPS (10 ng/ml). ROS levels were assessed by flow cytometry (N = 2). Gray filled area indicates DC without ROS dye.

(J) DC were cultured as in (C), but nitrite levels in the supernatant were assessed (data indicate mean + SEM; N = 10; n = 12; Mann-Whitney test; *p < 0.05). Triangle: data are not detectable.

(K) Same as in (I). HIF1α protein and actin are shown (N = 2).

(L and M) L-NIL-treated wild-type DC (N = 2) or DC from wild-type (WT), Nos2−/−, or Cybb−/−/Nos2−/− (N = 4) were cultured with or without 10 ng/ml LPS. All cells used in (L) are from wild-type animals. HIF1α and actin levels are shown.
after (dashed line) the addition of Fe(II)Cl2/8-hydroxyquinoline. The quench-mean fluorescence (given in brackets) was determined before (solid line) and LPS-stimulated (black line) DC were loaded with calcein-AM, and geometric (C) DC were treated with or without LPS for 17 hr. Untreated (gray line) or (F) DC cultured with or without 10 ng/ml LPS and with or without 160 mM Fe(III)-gluconate (Fe) followed by 30 min. Intracellular staining of HIF1α treated with or without 10 mg Fe(III)-gluconate (Fe) followed by 30 min.

Intracellular staining of HIF1α (E) Left panel: mice were treated with or without PHD inhibitor (ICA) for 3 hr. Right: mice were treated with or without 10 mg Fe(III)-gluconate (Fe) followed by 30 min.

3 hr. Intracellular staining of HIF1α treated with or without 10 ng Fe(III)-gluconate (Fe) followed by 30 min.

Figure 3. LPS-Induced Reduction of Intracellular Fe Availability Impairs PHD Activity and Allows for HIF1α Accumulation (A) Atomic absorption spectroscopy of lysates derived from DC cultured with or without 10 ng/ml LPS for 17 hr (data indicate mean ± SEM; N = 3; n = 6; unpaired Student’s t test), un, untreated. (B) IRE-binding activity was assessed in DC cultured with 160 μM Fe(II)Cl2, 100 μM DFO, or 10 ng/ml LPS (N = 4).

(C) DC were treated with or without LPS for 17 hr. Untreated (gray line) or LPS-stimulated (black line) DC were loaded with calcein-AM, and geometric mean fluorescence (given in brackets) was determined before (solid line) and after (dashed line) the addition of Fe(II)Cl2/8-hydroxyquinoline. The quenchable intracellular iron pool was calculated (data indicate mean ± SEM; N = 2; n = 9-10; unpaired Student’s t test with Welch correction; *p < 0.05).

(D) DC were cultured with or without 10 ng/ml LPS in the presence of increasing Fe(II)Cl2 concentrations (0.8–160 μM) for 17 hr (one of four similar experiments is displayed).

(E) Left panel: mice were treated with or without PHD inhibitor (ICA) for 3 hr. Intracellular staining of HIF1α in splenic DC is given (N = 2). Right: mice were treated with or without 10 ng/ml LPS and with or without 160 mM Fe(II) Cl2 for 20 hr. Where indicated, 100 μM MG132 was added for the final 4 hr.

(F) DC cultured with or without 10 ng/ml LPS and with or without 160 μM Fe(II) Cl2 for 20 hr. Where indicated, 100 μM MG132 was added for the final 4 hr.

(G) DC were cultured with or without 10 ng/ml LPS in the absence or presence of 100 μM ICA ± 80 μM Fe(II)Cl2 for 17 hr. HIF1α and actin levels are shown (representative of N = 2).

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(C) DC were treated with or without LPS for 17 hr. Untreated (gray line) or LPS-stimulated (black line) DC were loaded with calcein-AM, and geometric mean fluorescence (given in brackets) was determined before (solid line) and after (dashed line) the addition of Fe(II)Cl2/8-hydroxyquinoline. The quenchable intracellular iron pool was calculated (data indicate mean ± SEM; N = 2; n = 9-10; unpaired Student’s t test with Welch correction; *p < 0.05).

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(C) DC were treated with or without LPS for 17 hr. Untreated (gray line) or LPS-stimulated (black line) DC were loaded with calcein-AM, and geometric mean fluorescence (given in brackets) was determined before (solid line) and after (dashed line) the addition of Fe(II)Cl2/8-hydroxyquinoline. The quenchable intracellular iron pool was calculated (data indicate mean ± SEM; N = 2; n = 9-10; unpaired Student’s t test with Welch correction; *p < 0.05).

(D) DC were cultured with or without 10 ng/ml LPS in the presence of increasing Fe(II)Cl2 concentrations (0.8–160 μM) for 17 hr (one of four similar experiments is displayed).

(E) Left panel: mice were treated with or without PHD inhibitor (ICA) for 3 hr. Intracellular staining of HIF1α in splenic DC is given (N = 2). Right: mice were treated with or without 10 mg Fe(III)-gluconate (Fe) followed by 30 μg LPS for 3 hr. Intracellular staining of HIF1α in splenic DC is given (representative of N = 4).

(F) DC cultured with or without 10 ng/ml LPS and with or without 160 μM Fe(II) Cl2 for 20 hr. Where indicated, 100 μM MG132 was added for the final 4 hr.

(G) DC were cultured with or without 10 ng/ml LPS in the absence or presence of 100 μM ICA ± 80 μM Fe(II)Cl2 for 17 hr. HIF1α and actin levels are shown (representative of N = 2).

**DISCUSSION**

Regulation of Fe availability is crucial in innate immune defense (reviewed in Nairz et al., 2014). Withholding Fe from invading pathogens efficiently contributes to the control of infections by nutrient deprivation. Conversely, excess availability of Fe favors pathogen replication and impairs certain immune responses such as NO production (Weiss et al., 1994) and the capability of antigen presentation (Carrasco-Marín et al., 1996). In DC, LPS treatment enhanced the expression of Fth1 both at the mRNA level (Figure 4C) and protein level (Figure 4D), which was associated with commensurate increases in HIF1α protein levels (Figure 4D). FTH1 stimulation occurred in spite of a concomitant increase in IRP activity (Figure 3B). While IRP ablation in DC expectedly increased basal FTH1 protein levels, it did not prevent FTH1 stimulation by LPS (Figure 4D). Hence, LPS regulation of FTH1 in DC is largely independent of the IRP/IRE system and could reflect alternative IRP-independent regulation of FTH1 as, for instance, observed in fibrosarcoma cells (Daba et al., 2012). Pharmacological inhibition of NF-κB activity abolished LPS-triggered FTH1 induction, HIF1α accumulation, and LPS-induced NO production (Figure 4E). Silencing of Fth1 did not affect LPS-triggered increases in Hif1α mRNA levels but reduced HIF1α protein accumulation (Figure 4F). This was paralleled by reduced expression of inflammatory (Nos2) and glycolytic (Pgp1 and Slc2a1 [or Glut1]) HIF1α-target genes (Figures 4F and S4E). Upon Fth1 or Hif1a silencing, expression of an NF-κB response gene Tnfaip3 (A20) (Coornaert et al., 2009) and triacylglycerol scavenger receptor (CD36) linked to alternative activation of myeloid cells (Huang et al., 2014) remained unchanged (Figure S4E). Moreover, Hif1α silencing impaired Nos2 expression but did not affect LPS-triggered FTH1 induction, which excludes regulation of FTH1 by HIF1α (Figure 4F). These data demonstrate that FTH1 is required for LPS-induced HIF1α accumulation. Finally, we treated Fth1-silenced DC with the Fe chelator DFO. DFO mimicked the effect of FTH1 induction by LPS and restored HIF1α accumulation in Fth1-silenced DC (Figure 4G). Altogether, we conclude, that LPS-triggered, NF-κB-dependent FTH1 upregulation depletes available cellular Fe. Lack of this co-factor results in impaired PHD activity and allows for normoxic HIF1α stabilization.
Figure 4. LPS-Induced Upregulation of the Fe Storage Protein FTH1 Triggers HIF1α Stabilization

(A) DC were cultured with or without 10 ng/ml LPS. Lcn2 mRNA levels were measured at indicated time points (data indicate mean ± SEM; N = 2; n = 4–6), untreated.

(B) DC derived from Lcn2−/− and littermates were cultured with or without 10 ng/ml LPS. HIF1α and actin levels are shown (N = 3).

(C) DC were cultured as in (A). Fth1 mRNA expression levels are shown (data indicate mean ± SEM; N = 2; n = 2–6).

(D) DC were cultured with or without 10 ng/ml LPS, HIF1α, FTH1, and actin levels are shown (N = 3).

(E) DC were pretreated with 40 μM BAY11-7085 (BAY11) or left untreated. After 1 hr, DC were cultured with or without 10 ng/ml LPS for 17 hr. Upper panel: nitrite levels in the supernatant are shown (N = 2; n = 5). Lower panel: HIF1α, FTH1, and actin levels are shown (N = 2).

(F) DC electroporated with non-silencing (ns)-, Hif1a-, or Fth1-specific siRNA were cultured ± 10 ng/ml LPS for 17 hr. Upper panel: Hif1a mRNA expression levels are shown (data indicate mean ± SEM; N = 3; n = 2–3). Lower panels: HIF1α, NOS2, FTH1, and actin levels are shown (N = 4).

Iron availability via the induction of ferritin, resulting in subsequent PHD inhibition and HIF1α stabilization. In addition to HIF1α stabilization, LPS-triggered inhibition of PHD activity might have broader consequences in innate immune cells, because prolyl hydroxylation sites are not only present in HIF1α but also occur in several other proteins involved in NF-κB signal transduction and interleukin (IL)-1β signaling (Cummins et al., 2006; Scholz et al., 2013).

Inflammatory (LPS-triggered) and canonical (hypoxia-driven) HIF1α stabilization are both dependent on PHD inhibition. However, while hypoxia reduces PHD activity due to a lack of oxygen, LPS blocks PHD activity through deprivation of the essential cofactor Fe. HIF1α stabilization plays an important role in the proinflammatory activation of innate immune cells and their ability to combat infections and to regulate adaptive immune responses (Palazon et al., 2014). However, in contrast to infectious diseases, reduced HIF1α stabilization in innate immune cells might be desirable in inflammation-driven pathological conditions. Given that TLR4 signaling and inflammatory HIF1α are implicated in the pathology of autoimmune diseases such as rheumatoid arthritis (Cramer et al., 2003), our dissection of LPS-induced signaling pathways that result in HIF1α stabilization might be exploited for new therapeutic approaches that selectively target inflammatory HIF1α activation without affecting hypoxic HIF1α stabilization, which is critically required for adaption of cells to low-oxygen conditions.

EXPERIMENTAL PROCEDURES

Generation of DC, RNAi, Immunoblotting, qPCR, NO, and ROS Production

DC and macrophages were generated from the bone marrow of C57BL/6, ODS-Luc, Nos2−/−, Cybb−/−, Lcn2−/−, LysMCre−/−, LysMCre Phd2fl/fl, and LysMCre Phd2/3−/− mice. RNAi, immunoblotting, and qPCR were performed as described previously (Jantsch et al., 2011). Nitrite and ROS production were assessed by Griess reaction and after staining with CM-H2DCFDA, respectively.

Luciferase Activity

DC were lysed with a suitable lysis buffer and processed with luciferase substrate. Luminescence was detected with a TopCount NTX reader.

Determination of Total Intracellular Iron Content by Atomic Absorption Spectroscopy and Gel Retardation Assays

Total cellular iron content was quantified with a Shimadzu AA-7000 atomic absorption spectrophotometer. IRE/IRP complexes were analyzed by nondenaturing gel electrophoresis and autoradiography after incubation with an in vitro transcribed 32P-labeled IRE probe. The labile iron pool was quantified by using the property of Fe(II) to quench the fluorescence of calcein. The quenchable iron pool was calculated by subtracting the geometric mean fluorescence of (un)stimulated cells before and after incubation with Fe(II)Cl2/8-hydroxyquinoline. The greater the quenchable iron pool, the smaller the intracellular available Fe.

Oxygen Consumption with Oxodish and SDR SensorDish Reader

In order to quantify oxygen levels in cell culture plates, we used precalibrated Oxodish six-well plates. Signals were generated and detected by SDR SensorDish and SDR software.
Analysis of HIF1α in Spleen DC
C57BL/6 mice were injected intraperitoneally (i.p.) with either ICA in vehicle or vehicle alone, and with Fe(III)-gluconate in PBS or PBS alone, followed after 1 hr by injection of either LPS in PBS or PBS alone. 3 hr later, splenic single-cell suspensions were prepared, and intracellular HIF1α was analyzed by flow cytometry in DC. All animal experiments were carried out according to protocols approved by the Animal Welfare Committee of the local government (Regierung von Mittelfranken).

Statistical Analysis
Results are expressed as means ± SEM. If not indicated otherwise, n represents biological samples obtained from N independent experiments or mice. Statistical significance was calculated with Prism v6.0 (GraphPad Software).

Further detailed information is in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.005.

AUTHOR CONTRIBUTIONS

ACKNOWLEDGMENTS
J.J. and M.E. were funded by the DFG (JA1993/1-1 and HE465/2-1). C.B. was supported by funds from the Emerging Field Initiative of the FAU Erlangen-Nürnberg (consortium Metal Redox Inorganic Chemistry) and from the Interdisciplinary Center for Clinical Research (IZKF) of the Universitätsklinikum Erlangen (project A61). J.J., J. Schödel, G.S., and C.W. were supported by the Center for Kidney and Blood Pressure Research Regensburg-Erlangen-Nürnberg (REN). J. Schödel is a recipient of an Else Kröner-Fresenius Exzellenzstipendium (2014, EKES.11). A.B. is supported by the DFG-funded IRTG1816. We are grateful for the excellent technical assistance of Kirstin Castiglione and stipendium (2014_EKES.11). J. Scho¨del is a recipient of an Else Kro ¨ ner-Fresenius Exzellenz- Erlangen (project A61). J.J., J. Scho¨del, G.S., and C.W. were supported by the Interdisciplinary Center for Clinical Research (IZKF) of the Universita ¨ tsklinikum supported by funds from the Emerging Field Initiative of the FAU Erlangen-Nürnberg (consortium Metal Redox Inorganic Chemistry) and from the €[84x21]13, 2048–2055, December 15, 2015

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