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CUTTING EDGE

Cutting Edge: Essential Role of Hypoxia Inducible Factor-1α in Development of Lipopolysaccharide-Induced Sepsis[1]

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Sepsis, the leading cause of death in intensive care units, reflects a detrimental host response to infection in which bacteria or LPS act as potent activators of immune cells, including monocytes and macrophages. In this report, we show that LPS raises the level of the transcriptional regulator hypoxia-inducible factor-1α (HIF-1α) in macrophages, increasing HIF-1α and decreasing prolyl hydroxylase mRNA production in a TLR4-dependent fashion. Using murine conditional gene targeting of HIF-1α in the myeloid lineage, we demonstrate that HIF-1α is a critical determinant of the sepsis phenotype. HIF-1α promotes the production of inflammatory cytokines, including TNF-α, IL-1, IL-4, IL-6, and IL-12, that reach harmful levels in the host during early sepsis. HIF-1α deletion in macrophages is protective against LPS-induced mortality and blocks the development of clinical markers including hypotension and hypothermia. Inhibition of HIF-1α activity may thus represent a novel therapeutic target for LPS-induced sepsis. *The Journal of Immunology,* **2007, 178: 7516–7519.**

Sepsis is a major and increasing cause of mortality and morbidity throughout the world with an annual incidence of 2.4–3.0 cases per 1,000 in the population (1, 2). Important biological mediators of sepsis are inflammatory cytokines including TNF-α, IL-1, and IL-6, which are released by leukocytes in massive amounts in response to bacterial toxins such as LPS. The systemic effects of LPS are largely mediated by macrophages, because the transfer of LPS-sensitive macrophages renders previously LPS-resistant mice sensitive to sepsis (3). No single agent or treatment strategy has shown sufficient promise for use in routine clinical practice to block the aberrant cytokine activation patterns of sepsis. We recently identified novel and essential roles of the transcriptional regulator hypoxia-inducible factor (HIF)[3] 1α (HIF-1α) in controlling macrophage inflammatory responses in the skin and joints (4) and promoting their phagocytic function (5). In this study we hypothesized that HIF-1α could play an important role in mediating the inflammatory responses during LPS-induced sepsis. Using mice with a targeted deletion of HIF-1α in macrophages (4, 5), we examined this hypothesis in vitro and in vivo, demonstrating the potential relevance of HIF-1α as a target for sepsis therapy.

Materials and Methods

Mouse models

Transgenic HIF flox, VEGF flox, and LysM-cre alleles were individually backcrossed to >99% C57BL/6 before breeding to obtain the indicated genotypes. These mice and inbred mouse strains HIF$^{flox/flox}$/LysMcre and VEGF$^{flox/flox}$/LysMcre (backcrossed in a pure C57BL/6 genetic background) and inbred mouse strains CH3/HeOuJ and CH3/HeJ (obtained from The Jackson Laboratory) were handled by approved protocols of the University of California San Diego Institutional Animal Care and Use Committee (La Jolla, CA). For comparisons of targeted myeloid cell gene deletions driven by the LysM promoter, knockout mice were compared with the corresponding *flox/flox* littermates (designated wild type (WT)). Eight- to 10-wk-old male HIF$^{flox/flox}$/LysMcre or VEGF$^{flox/flox}$/LysMcre males were injected i.p. with 15 mg/kg *Escherichia coli* LPS serotype 0111:B4 (Sigma-Aldrich) or saline solution. Mice were monitored to assess survival for a period of up to 14 days. For the assessment of blood pressure and heart beat rate, tail cuff measurements were made by using the Kent Scientific XBP1000 system. Mean blood pressure was determined by the following method: diastolic pressure + [(systolic pressure − diastolic pressure) ÷ 3]. Surface temperatures were measured with a ThermoVision A20 thermal infrared camera (FLIR Systems).

Measurement of cytokine levels

Blood samples were drawn 90 min or 4 h after LPS injection by retro-orbital bleeding. Mouse TNF-α and IL-6 were assayed by using the ELISA kit from eBioscience and vascular endothelial growth factor (VEGF) was assayed with the Quantikine ELISA kit from R&D Systems. The other cytokines levels were measured by the ProteoPlex murine cytokine array kit (EMD Biosciences).

Western blot studies

Bone marrow-derived macrophages of CH3/HeOuJ and CH3/HeJ mice were isolated as described (5). LPS (100 ng/ml) or log-phase *Pseudomonas aeruginosa*

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[3] Abbreviations used in this paper: HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; VEGF, vascular endothelial growth factor; WT, wild type.

or *Salmonella typhimurium* were added (at a multiplicity of infection of five bacteria per cell) to bone marrow-derived macrophages for 4 h. Cells harvested and extracted with radioimmune precipitation assay buffer were probed using rabbit anti-mouse HIF (Novus Biological) at 1/1000.

Real-time quantitative RT-PCR

The RNA of macrophages exposed to 100 ng/ml LPS (Sigma-Aldrich) for 4 h was isolated using TRI Reagent (Molecular Research Center). First-strand synthesis and real-time PCR were performed as described (5) in a TaqMan Universal SYBR Green Master Mix (Applied Biosystems) using specific primers with the following sequences (rates normalized to the expression level of ribosomal RNA): HIF-1, 5′-GAAACGACCACTGCTAAGGCA-3′ (forward) and 5′-GGCAGACAGCTTAAGGCTCCT-3′ (reverse); prolyl hydroxylase (PHD) 1, 5′-GGCCAGTGGTAGCCAACATC-3′ (forward) and 5′-GTG GCATAGGCTGGCTTCAC-3′ (reverse); PHD2, 5′- TGACCACACCTC TCCAGCAA-3′ (forward) and 5′- CTGCCAACAATGCCAAACAG-3′ (reverse); and PHD3, 5′-GGTGGCTTGCTATCCAGGAA-3′ (forward) and 5′-ATACAGCGGCCATCACCATT-3′ (reverse).

Statistical analysis

The significance of experimental differences was evaluated by two-way ANOVA analysis followed by a Bonferroni posttest. Survival data were analyzed by the construction of Kaplan-Meier plots and use of the log-rank test.

Assurances

All animal experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee and performed using accepted veterinary standards.

Results and Discussion

HIF-1α is induced by LPS in a TLR4-dependent fashion

Cellular HIF-1α levels are controlled in part through PHD-mediated proteolytic degradation in which oxygen and iron serve as cofactors (6). Recently, we and others have demonstrated that bacteria or LPS induce HIF-1α stabilization even under normoxic conditions (5, 7–9). LPS is a microbial activator of TLR4, a pattern recognition molecule critical for initiating innate immune signaling cascades and proinflammatory responses (10). To determine whether the HIF-1α response to LPS was TLR4 dependent, we derived macrophages from the bone marrow of WT mice or those possessing a spontaneous mutation in TLR4 ($Tlr4^{Lps\text{-}d}$) (11). By Western blot analysis we found that the loss of TLR4 clearly reduced the level of macrophage HIF-1α induced following exposure to LPS and two Gram-negative bacterial species, *S. typhimurium* and *P. aeruginosa* (Fig. 1*A*). Real-time RT-PCR analysis strongly suggested that part of the TLR4-dependent increase in HIF-1α levels following LPS exposure occurs at the transcriptional level (Fig. 1*B*). Although differences in mRNA stability cannot be definitively excluded, a significant increase in the promoter activity of HIF-1α after LPS treatment has recently been described (7).

To further understand why LPS can raise HIF-1α levels even under normoxia, we explored a second mechanism for LPS-induced HIF-1α stabilization—expression of the three HIF-1α PHDs (PHD1–3) that catalyze degradation of the transcriptional regulator. LPS stimulation significantly decreased levels of PHD2 and PHD3 mRNA in WT macrophages but not in the TLR4-macrophages (Fig. 1*C*); PHD1 expression was not affected. Because these PHDs are non-equilibrium enzymes (i.e., they do not catalyze the reverse reaction), enzyme abundance is an important determinant of the rate of substrate hydroxylation and, thus, cellular HIF-1α levels (12). We conclude that the TLR4-dependent increase in HIF-1α after LPS stimulation reflects a combination of increased HIF-1α transcription and decreased HIF-1α degradation mediated by PHD2 and PHD3. The PHD2 isoform is known to play a key nonredundant role in HIF-1α regulation (13), and a similar HIF-1α stabilization mechanism that works through selective inhibition of PHD2 expression in response to TGF-β1 has recently been described (14).

Given our observation that macrophage HIF-1α levels are increased in response to LPS, we predicted that HIF-1α transcriptional regulation could influence the characteristic pattern of inflammatory gene expression attributed to LPS activation and TLR4 signaling. As a preliminary validation of this principle, we exposed isolated macrophages from WT and HIF1α myeloid null mice to LPS and measured the transcript levels for the signature cytokine IL-6 by real-time RT-PCR. HIF1α null macrophages showed a significant reduction in IL-6 mRNA compared with WT (Fig. 1*D*), suggesting that the induction of HIF1α could represent a functional intermediary in the LPS/TLR4 activation profile.

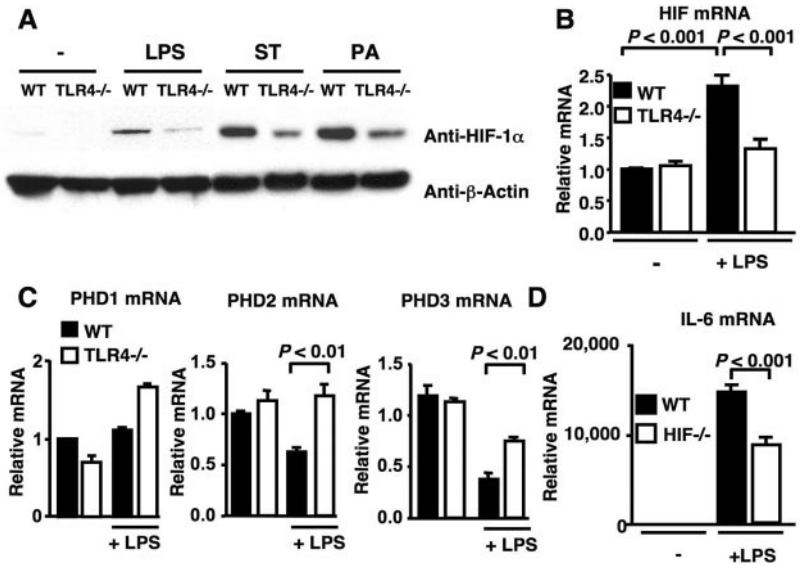

FIGURE 1. LPS induces HIF-1α expression through a TLR4-dependent pathway. *A*, Western blotting on protein extracts from WT and $Tlr4^{Lps\text{-}d}$ bone marrow-derived macrophages stimulated for 4 h with LPS, *S. typhimurium* (ST) or *P. aeruginosa* (PA). *B* and *C*, Real-time PCR for HIF-1α expression (*B*) or PHD-1, PHD-2, and PHD-3 (*C*) expressions on LPS-stimulated WT and $Tlr4^{Lps\text{-}d}$ bone marrow-derived macrophages; samples were normalized individually to each PHD isoform control. *D*, Real-time PCR for IL-6 expression on LPS-stimulated WT and HIF-1α myeloid null bone marrow-derived macrophages. Studies were performed in triplicate and repeated twice with similar results; a representative experiment shown.

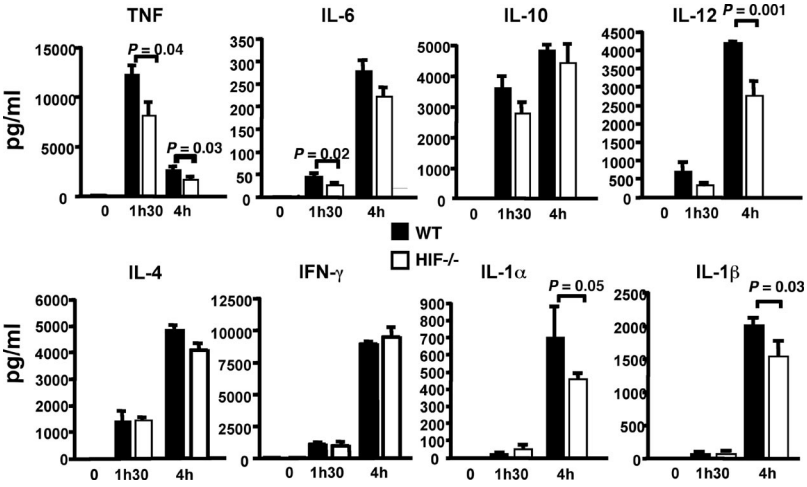

FIGURE 2. HIF-1α contributes to macrophage production of the inflammatory cytokines TNF-α, IL-6, IL-1α, IL-1β, IL-4, and IL-12. *A*, TNF-α and IL-6 levels measured by ELISA 90 min after LPS injection. *B*, Cytokine levels measured by the ProteoPlex murine cytokine array 1 h 30 min and 4 h after LPS injection; $n = 5$ animals per group. Studies were repeated twice with similar results; a representative experiment is shown.

LPS-induced cytokine release in vivo is influenced by HIF-1α

To confirm the significance of our observations in vivo, we injected WT and HIF-1α macrophage null mice with 15 mg/kg LPS i.p. and collected heparinized blood at 90 min or 4 h for the determination of several cytokine levels by ELISA (Fig. 2). Cytokines were undetectable at baseline and strongly up-regulated upon LPS challenge. The loss of macrophage expression of HIF-1α led to significant decreases in the production of TNF-α, IL-1α, IL-1β, and IL-12 but did not affect the production of IFN-γ or the anti-inflammatory cytokines IL-4 and IL-10. TNF-α and IL-1β are "proximal" cytokines well known to trigger many of the clinical manifestations of LPS-induced sepsis (reviewed in Ref. 15), whereas IL-6 levels correlate to poor outcome and organ system failure in human sepsis patients (16, 17). Neutralizing the Abs to IL-12 can reduce LPS-induced mortality in mice, indicating this less well studied cytokine also plays a significant role in the systemic LPS response (18, 19). We conclude that HIF-1α in macrophages contributes to the expression of several key cytokines implicated in the pathogenesis of LPS-induced sepsis.

Deletion of HIF-1α is protective against LPS-induced sepsis symptomatology and lethality

We hypothesized that the diminished responsiveness of HIF-1α null macrophages to LPS-induced inflammatory cytokine activation would be reflected in decreased LPS-induced sepsis symptomatology in the corresponding knockout mice. Two hallmark clinical manifestations of the LPS-induced systemic activation of cytokines, including TNF-α, in the mouse are hypothermia (20, 21) and hypotension (22). We found that the elimination of HIF-1α in the myeloid lineage reduced LPS-induced hypothermia, with mutant mice possessing an average surface temperature 2°C greater than that of WT mice (Fig. 3*A*). Significant ($p = 0.0005$) protection against LPS-induced hypotension was also observed in the HIF-1α myeloid null mice, with mean blood pressure at 24 h equaling 57 ± 1.9 compared with 41 ± 2 for the WT controls (Fig. 3*B*). With an improvement of this hemodynamic parameter, the shock index, defined as the ratio of the heart rate to systolic blood pressure, was significantly decreased in HIF-1α myeloid null mice compared with the WT controls ($p = 0.0004$) (Fig. 3*C*).

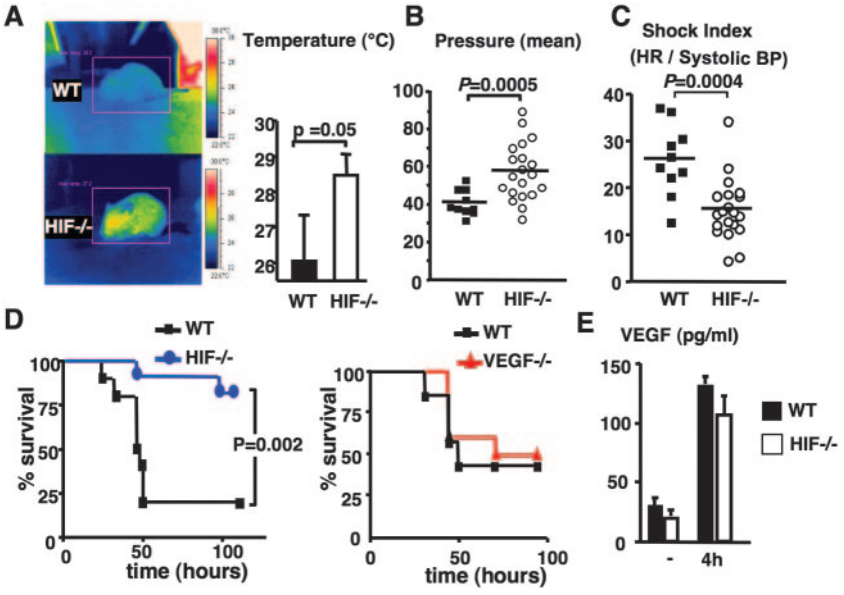

FIGURE 3. HIF-1α contributes to LPS-induced mortality and morbidity. *A–C*, Surface temperature (*A*), mean blood pressure (*B*), and shock index (*C*) of WT and HIF-1α myeloid null mice 24 h after LPS injection; mean blood pressure = diastolic pressure + [(systolic pressure − diastolic pressure) ÷3]. HR, Heart rate; BP, blood pressure. *D*, Survival following i.p. LPS injection in WT and HIF-1α myeloid null or VEGF myeloid null-mice ($n > 12$ per group). *E*, VEGF levels in serum of HIF-1α myeloid null mice 4 h after LPS challenge; $n > 5$ animals per group. The study was performed in triplicate and repeated twice with similar results; a representative experiment is shown.

The correlation of LPS-induced symptomatology with lethality was also established. Whereas LPS challenge of the WT mice produced 82% mortality by 48 h, HIF-1α myeloid null mice experienced only 14% mortality by day 5 ($p = 0.002$) (Fig. 3*D*). *VEGF*, a well-described HIF-1α target gene (23), has been recently shown to be an important mediator of sepsis morbidity and mortality (24). However, the deletion of *VEGF* in the myeloid lineage does not rescue LPS-induced mortality (Fig. 3*E*). Moreover, the loss of macrophage expression of HIF-1α did not by itself produce a significant decrease in the total production of VEGF by the mouse 4 h after LPS stimulation (Fig. 3*E*), with the understanding that VEGF production in other cell types (e.g., vascular smooth muscle, hepatocytes) remained unaffected in our targeted knockout mice. We conclude that the HIF-1α-dependent macrophage activation of inflammatory cytokines, and not VEGF, plays a more important function in influencing the sepsis phenotype.

An interplay of HIF-1α and NF-κB regulatory pathways in macrophage activation is revealed by our findings and other recent data. Frede et al. (7) found that LPS induced HIF-1α in human macrophages in vitro at normoxia through enhanced transcription of the *HIF-1*α gene. In this study, LPS induced the binding of NF-κB to a respective consensus element 130 bp upstream of the HIF-1α transcriptional start site, and the inhibition of NF-κB abolished LPS-induced HIF-1α target gene expression. Although NF-κB is an important downstream effector of the HIF-1α-dependent response in neutrophils to anoxia (25), we did not observe differences in NF-κB, IKKα, or IκBα expression in HIF$^{lox/lox}$/LysMcre macrophages vs WT after LPS stimulation (data not shown), indicating that the NF-κB pathway per se is not affected by HIF-1α deletion in macrophages. Thus, anoxia and infection/inflammation may induce HIF-dependent and HIF-independent NF-κB pathways, respectively. Lastly, it is possible that some proinflammatory cytokines could be directly activated by HIF; for example, we observe a hypoxia response element site GCGTG 5′ of the *IL-6* gene and two hypoxia response element sequences in tandem 3′ of the *TNF-*α gene in the mouse genome (hypoxia response elements in the classical HIF target gene erythropoietin are also located in the 3′ flanking region).

In conclusion, we have identified an additional global regulatory function of HIF-1α in the inflammatory function of macrophages. HIF-1α is activated by LPS in a TLR4-dependent fashion and contributes to the cytokine activation, symptomatology, and lethality of LPS-induced sepsis in vivo. To our knowledge, this represents the first linkage of the HIF-1α transcriptional regulator with TLR pattern recognition and should stimulate further exploration of the interactions of these important pathways in the regulation of macrophage activation. Previous work has prompted discussion of HIF-1α modulation as a pharmacologic approach for the treatment of chronic inflammatory disorders or the augmentation of innate immune function (26–28). Our studies suggest that HIF-1α may additionally represent a novel therapeutic target for ameliorating the aberrant cytokine activation pattern and poor prognosis of patients with LPS-induced sepsis.

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Disclosures

The authors have no financial conflict of interest.

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