A Myeloid Hypoxia-inducible Factor 1α-Krüppel-like Factor 2 Pathway Regulates Gram-positive Endotoxin-mediated Sepsis*

Ganapati H. Mahabeleshwar1,2, Muhammad Awais Qureshi3, Yoichi Takami4, Nikunj Sharma5, Jerry B. Lingrel6, and Mukesh K. Jain7,8

From the 7,8Case Cardiovascular Research Institute, Department of Medicine, Harrington-McLaughlin Heart and Vascular Institute, Case Western Reserve University School of Medicine, University Hospitals Case Medical Center, Cleveland, Ohio 44106 and the 6Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524

Background: Gram-positive bacterial infections and sepsis are a significant cause of morbidity and mortality.

Results: KLF2 inhibits Gram-positive, bacterial endotoxin-induced HIF-1α expression and macrophage activation.

Conclusion: Transcription factors KLF2 and HIF-1α are critical regulators of Gram-positive sepsis.

Significance: Pharmacological agents that modulate the KLF2/HIF-1 pathway may allow for therapeutic gain in the treatment of bacterial infections and sepsis.

Although Gram-positive infections account for the majority of cases of sepsis, the molecular mechanisms underlying their effects remains poorly understood. We investigated how cell wall components of Gram-positive bacteria contribute to the development of sepsis. Experimental observations derived from cultured primary macrophages and the cell line indicate that Gram-positive bacterial endotoxins induce hypoxia-inducible factor 1α (HIF-1α) mRNA and protein expression. Inoculation of live or heat-inactivated Gram-positive bacteria with macrophages induced HIF-1α transcriptional activity in macrophages. Concordant with these results, myeloid deficiency of HIF-1α attenuated Gram-positive bacterial endotoxin-induced cellular motility and proinflammatory gene expression in macrophages. Conversely, Gram-positive bacteria and their endotoxins reduced expression of the myeloid anti-inflammatory transcription factor Krüppel-like factor 2 (KLF2). Sustained expression of KLF2 reduced and deficiency of KLF2 enhanced Gram-positive endotoxins induced HIF-1α mRNA and protein expression in macrophages. More importantly, KLF2 attenuated Gram-positive endotoxins induced cellular motility and proinflammatory gene expression in myeloid cells. Consistent with these results, mice deficient in myeloid HIF-1α were protected from Gram-positive endotoxin-induced sepsis mortality and clinical symptomatology. By contrast, myeloid KLF2-deficient mice were susceptible to Gram-positive sepsis induced mortality and clinical symptoms. Collectively, these observations identify HIF-1α and KLF2 as critical regulators of Gram-positive endotoxin-mediated sepsis.

Sepsis, the most feared complication of bacterial infection, is a major source of morbidity and mortality worldwide. Epidemiologic studies indicate that the incidence of sepsis has been increasing, perhaps because of numerous factors such as aging of the population, increased performance of complex procedures (e.g. organ transplantation), increased use of chemotherapy, and frequent use of indwelling lines and devices (1, 2). The most common causative agents for sepsis are Gram-negative and Gram-positive bacteria. Although much attention has focused on understanding how Gram-negative organisms induce sepsis, we note that over the past few decades the incidence of Gram-positive sepsis has increased (3–5). Thus, a greater understanding of the molecular mechanisms governing Gram-positive sepsis is required to combat this lethal disease. Akin to Gram-negative sepsis, where bacterial wall components such as LPS are causative, Gram-positive bacterial wall components such as teichoic acid (TA),3 lipoteichoic acid (LTA), and peptidoglycans (PGN) are thought to contribute to disease pathogenesis (6–9). Indeed, recent reports have indicated that isolated PGN and LTA can induce most of the clinical manifestation of septic shock syndrome (10). Several reports also support a cooperative effect of these agents on experimental disease. For example, De Kimpe et al. (11) indicated that LTA acts synergistically with PGN to elevate TNF-α release and iNOS expression/activity and cause multi organ failure and shock. Similarly, studies by Kengatharan et al. (12) indicated that coadministration of LTA and PGN induced

* This work was supported, in whole or in part, by National Institutes of Health Grants HL72952, HL75427, HL76754, HL086548, and HL084154 (to M. K. J.); HL097023 (to G. H. M.); and HL78806 (to J. B. L.). This work was also supported by American Heart Association Grants 11POST7200004 (to N. S.), Sankyo Foundation of Life Science grants, and a Kanae Foundation for the Promotion of Medical Science grant (to Y. T.).
1 To whom correspondence may be addressed. E-mail: ghm4@case.edu.
2 To whom correspondence may be addressed. University Hospitals Harrington-McLaughlin Heart and Vascular Institute and Case Cardiovascular Research Institute, Wolstein Research Building, 2103 Cornell Rd., Rm. 4-537, Cleveland, OH 44106-7290. Tel.: 216-368-3607; Fax: 216-368-0556. E-mail: mukesh.jain2@case.edu.
3 The abbreviations used are: TA, teichoic acid; LTA, lipoteichoic acid; PGN, peptidoglycan(s); iNOS, inductible nitric oxide synthase; HRE, hypoxic response element; KLF, Krüppel-like factor; m.o.i., multiplicity of infection; HIF, hypoxia-inducible factor.
iNOS in various organs, significantly elevated plasma levels of TNF-α, and caused multiorgan dysfunction syndrome.

Recent mechanistic efforts have also begun to shed light on the cellular events that mediate the effects of Gram-positive bacterial wall products. For example, studies from Dunne et al. (13) demonstrated that the type 1 macrophage scavenger receptor binds to LTA through polyanionic bonds. Studies by Schwandner et al. (14) indicated that LTA induced cellular activation via Toll-like receptors (e.g. TLR2) and NF-κB activation. Consistent with these observations, in vitro and in vivo studies indicated that Gram-positive bacterial products can induce expression of proinflammatory cytokines (e.g. TNF-α, INF-γ, IL-1β, IL-6) and iNOS in myeloid cells that are key contributors to the sepsis syndrome. In keeping with these observations, studies using a human whole blood model indicated that lipoteichoic acid can induce expression of TNF-α, IL-6, and IL-10 in a time- and dose-dependent manner (15). Collectively, these studies indicate that precise molecular mechanisms mediate the effects of Gram-positive bacterial endotoxins that contribute to the systemic inflammatory response syndrome.

In addition to bacterial wall components, sites of infection are typically characterized by hypoxia. The importance of hypoxia has been highlighted by studies focused on the role of the master regulator of hypoxic signaling, hypoxia-inducible factor 1α (HIF-1α). Studies by Johnson and co-workers (16–18), largely through loss of function approaches in vitro and in vivo, have revealed that myeloid HIF-1α augments proinflammatory cytokine expression and alters macrophage metabolic activity and bacterial killing. HIF-1 is a heterodimeric helix-loop-helix transcription factor whose expression is stringently regulated at mRNA and protein levels. Protein stability of the α subunit of HIF-1 is regulated by a family of enzymes termed prolylhydroxylases, whose action directs HIF-1α degradation by the ubiquitin-proteasome pathway in a process dependent on interaction with von Hippel-Lindau (VHL) tumor suppressor protein (19). Under hypoxic conditions, prolylhydroxylase activity is inhibited, and HIF-1α accumulates and translocates into the nucleus, where it binds the constitutively activated HIF-1β. The resultant heterodimer HIF-1 binds to the hypoxic response elements (HREs) of target genes (20). In addition to hypoxia, lipopolysaccharide can potentely induce HIF-1α transcription. Thus, the combination of bacterial products and hypoxia can, through both transcriptional and posttranscriptional mechanisms, augment HIF-1α levels (21).

Krüppel-like factors (KLFs) are a subclass of the zinc finger family of DNA-binding transcription factors implicated in a broad spectrum of biological processes. Krüppel-like factor 2 (KLF2) was initially identified by the Lingrel laboratory and termed lung Krüppel-like factor because of its high level in lung tissues (22). In the context of myeloid cell biology, our group identified KLF2 as a negative regulator of myeloid cell activation (23). KLF2 overexpression was found to attenuate LPS-induced expression of cytokines and chemokines through inhibiting transcriptional activity of NF-κB and activator protein 1 (AP-1). More recently we found that myeloid KLF2 regulates the host response to polymicrobial infection and lipopolysaccharide-induced septic shock (24). However, a role for this factor in Gram-positive infection and sepsis has not been elucidated. Herein, we identify HIF-1α as an important mediator of Gram-positive bacterial endotoxin-induced cellular functions and inflammatory gene expression. Further, we provide evidence that KLF2 modulate Gram-positive endotoxin-induced HIF-1α expression and myeloid cell response in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Lipopolysaccharide was purchased from Sigma-Aldrich (St. Louis, MO). Teichoic acid was obtained from GenWay Biotech, Inc. (San Diego, CA). Anti-HIF-1α antibody and anti-actin antibody were obtained from Novus Biologicals (Littleton, CO) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Transwell permeable supports with 8-µm pore size were obtained from Corning, Inc. (Lowell, MA). Escherichia coli (ATCC number 21149) and Staphylococcus aureus (strain Newman) bacterial strains were obtained from the ATCC. Lipoteichoic acid was obtained from Sigma-Aldrich or extracted as described previously (25). Ad-GFP (control) and Ad-KLF2 (KLF2) adenoviral constructs were generated by the Harvard Gene Therapy Group as described previously (26). All other chemicals and reagents used were of analytical grade and were obtained from commercial sources.

Cell Culture—The RAW264.7 cell line was purchased from the ATCC and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Primary mouse macrophages and neutrophils were obtained from the peritoneal cavity by inducing peritonitis with 3% thioglycolate broth in 8- to 12-week-old mice as described previously (16). These primary peritoneal macrophages and neutrophils were cultured in serum supplemented DMEM as described above.

Experimental Mouse Models—The mouse lines used in this study were generated as described previously (24). Briefly, a mouse line expressing lysozyme M promoter-driven Cre recombinase (LysMcrecre) was crossed to HIF-1α-floxed (HIF-1αL/JΔ) mice to generate a myeloid-specific HIF-1α-deficient mouse line. Similarly, KLF2-floxed (KLF2L/JΔ) mice were crossed with LysMcrecre mice to generate myeloid-specific KLF2-deficient mice. All mouse colonies were maintained in a clean animal facility, and all animal experimentation was approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Mice (8–12 weeks old) were injected intraperitoneally with a mixture of lipoteichoic acid (3 mg/kg) and peptidoglycan (1 mg/kg) or saline solution. Mice were monitored for 4 days following this injection and their rectal temperature, blood pressure, and heart rate were recorded. The shock index was calculated using the following formula: Shock index = heart rate/ systolic blood pressure. Survival data were analyzed by the construction of Kaplan-Meier plots and use of the log-rank test.

Real-time Quantitative RT-PCR, Luciferase Reporter Assay, and Western Blot Analysis—Total RNA was isolated from RAW 264.7 cells or mouse primary peritoneal macrophages and neutrophils following indicated treatment using TRIzol reagent (Invitrogen). 1 µg of total RNA was reverse-transcribed using M-MuLV reverse transcriptase in the presence of random primers.
**KLF2 Regulates Gram-positive Toxic Shock Syndrome**

hexamers and oligo(dT) primers. Real-time quantitative PCR was performed using Universal SYBR Green PCR Master Mix on an Applied Biosystems Step One plus Real-Time PCR system by applying gene-specific primers.

A luciferase reporter plasmid driven by three tandem copies of the HRE sequence were transfected or cotransfected with KLF2 plasmids in RAW264.7 cells using Lipofectamine 2000 reagent (Invitrogen). Twenty-four hours after transfection, these cells were exposed to live (5 m.o.i.) or heat-inactivated bacteria or bacterial endotoxins and luciferase activity was measured with a luciferase reporter assay system (Promega, Madison, WI). Results are presented as relative luciferase activity over the control group.

Primary mouse peritoneal macrophages or RAW264.7 cells were lysed using radioimmunoprecipitation lysis buffer (Sigma) supplemented with a protease inhibitor mixture tablet (Roche) following the indicated treatment. Equal quantities of total protein were separated by SDS-PAGE and detected by the indicated antibody by immunoblotting assay.

**Cell Migration and Invasion Assay**—Mouse primary peritoneal macrophages from the indicated genotype or RAW264.7 cells infected with Ad-GFP or Ad-KLF2 were induced with teichoic acid or lipoteichoic acid for 4 h. A cell suspension containing $2 \times 10^5$ cells was added to the upper chamber of the transwell inserts. The lower chambers of the transwell plates were filled with DMEM supplemented with 5% FBS. These transwell chambers were incubated at 37 °C for 18 h in a humidified incubator with an atmosphere of 5% CO$_2$ and 95% air. Following incubation, cells on the upper part of the wells were removed, and the migrated cells in the lower side of the filter were fixed and stained with Giemsa. The migrated cells on the filter were counted under an inverted microscope. To analyze macrophage-invasive properties, cells treated with teichoic acid or lipoteichoic acid were added to the upper wells of the transwell inserts coated with growth factor-reduced Martigel, and was performed as described previously (24).

**Antibiotic Protection Assay**—Antibiotic protection assays were performed as described previously (16). Briefly, *S. aureus* bacteria were grown in logarithmic phase in Luria broth medium. These bacterial cultures were centrifuged to sediment actively proliferating bacteria. These bacterial pellets were washed in sterile ice-cold 1× PBS and diluted with DMEM supplemented with 0.1% BSA to the required concentration. These actively proliferating bacteria were added to the top of the monolayer macrophage culture for 2 h. These cells were washed three times with 1× PBS and incubated with antibiotic-containing media for 20 min to eliminate cell surface-associated bacteria. These macrophage cultures were cultured for an additional 6 h and lysed with non-ionic detergent-containing buffer. These macrophage cell lysate-containing intracellular proliferating bacteria were serially diluted with sterile 1× PBS and spread on agar plates to enumerate bacterial colony-forming units.

**Statistical Analysis**—All data are presented as mean ± S.D. unless indicated otherwise. The statistical significance of differences between two groups was analyzed with Student’s *t* test. Values of *p* < 0.05 were considered statistically significant.

**RESULTS**

**Gram-positive Bacteria Wall Components Transcriptionally Induce HIF-1α Expression**—Previous studies from our group (24) and others (27, 28) indicated that Gram-negative bacterial cell wall components such as lipopolysaccharide induce HIF-1α expression in myeloid cells. To determine whether a parallel effect occurred with Gram-positive wall components, we examined whether HIF-1α transcriptional activity is modulated by exposure of macrophages to Gram-positive bacteria such as *S. aureus*. We found that transcriptional activity of HIF-1 was significantly elevated in macrophages following exposure to live (5 m.o.i.) or heat-inactivated, Gram-positive bacteria under normoxic condition (Fig. 1A). Indeed, induction of HIF-1 transcriptional activity by Gram-positive bacteria was as strong as Gram-negative bacteria (*E. coli*). Intriguingly, individual endotoxin components of Gram-positive bacteria (TA and LTA) robustly induced HIF-1-dependent transcriptional activity in the macrophage cell line (Fig. 1B). Taken together, these results indicated that Gram-positive bacteria and endotoxins derived from Gram-positive bacteria strongly activate HIF-1-dependent transcriptional activity in myeloid cells under normoxic condition.

We next sought to determine whether the induction of HIF-1 transcriptional activity by Gram-positive bacteria and their endotoxins led to an equitable increase in HIF-1α mRNA and protein expression. Accordingly, wild-type mouse peritoneal macrophages were exposed to live (5 m.o.i.) or heat-inactivated *E. coli* or *S. aureus* and expression of HIF-1α mRNA and protein were analyzed by quantitative PCR and immunoblot analysis. As shown in Fig. 1C, both live and heat-inactivated *S. aureus* induced HIF-1α mRNA expression. Importantly, inoculation of live or heat-inactivated *S. aureus* with wild-type macrophages also robustly induced HIF-1α at the protein level (Fig. 1E). Taken together, these results indicate that the heat-stable component of *S. aureus* induced HIF-1α expression at both mRNA and protein levels in macrophages. Therefore, we next evaluated the individual endotoxin component of Gram-positive bacteria such as TA and LTA on expression of HIF-1α mRNA and protein in wild-type mouse peritoneal macrophages. As shown in Fig. 1, D and F, compared to control treatment, both TA and LTA induced HIF-1α mRNA and protein expression.

**Kinetics of Teichoic and Lipoteichoic Acid Induced HIF-1α Expression**—Our previous experiments indicated that Gram-positive endotoxins induce HIF-1α at mRNA and protein levels. Next, we analyzed the kinetics of HIF-1α mRNA and protein induction by TA in wild-type mouse peritoneal macrophages. Teichoic acid induced HIF-1α mRNA expression in a dose-dependent manner (Fig. 2A) up to 4 μg/ml. Interestingly, a higher dose (8–10 μg/ml) of TA did not alter HIF-1α expression at both mRNA and protein levels. Furthermore, the time-dependent HIF-1α mRNA and protein expression analysis indicated that TA (4 μg/ml) induced HIF-1α mRNA and protein expression as early as 6 h, an effect that was sustained for up to 24 h (Fig. 2B). A similar pattern of HIF-1α mRNA and protein expression was observed in wild-type mouse peritoneal macrophages in response to LTA (Fig. 2, C and D). Collectively, these results indicate that Gram-positive endotoxins
such as TA and LTA induce HIF-1α mRNA and protein expression in a dose- and time-dependent manner.

**Gram-positive Endotoxins Induced Myeloid Inflammatory Gene Expression in a HIF-1α-dependent Manner**—The studies above identify HIF-1α as important in the response to Gram-positive infection. We next sought to examine whether Gram-positive bacterial products affected key myeloid cellular processes. Accordingly, mouse peritoneal macrophages derived from control (LysM<sup>Cre/Cre</sup>) and HIF-1α<sup>−/−</sup> mice were stimulated with TA or LTA and subjected to a cell migration or inva-
sion assay (Fig. 3, A and B). Although both agents robustly induced macrophage cell migration and invasion, this effect was significantly attenuated in HIF-1α/H9251-deficient macrophages. To further evaluate the functional consequences of HIF-1α activation by Gram-positive bacteria and TA/LTA, we performed an antibiotic protection assay to enumerate intracellular killing of \textit{S. aureus} by control (LysMCre/Cre) macrophages compared with intracellular bacterial killing by those derived from the HIF-1α-deficient macrophages (Fig. 3, C). Our results indicate that deficiency of HIF-1α significantly attenuates macrophage intracellular bacterial killing and results in a significantly higher number of colony-forming units in an antibiotic protection assay.

We next sought to determine whether the altered cellular response to Gram-positive toxins in HIF-1α-deficient macrophages was due to diminished expression of HIF-1α target genes that modulate these cellular processes. Accordingly, control (LysMCre/Cre) and HIF-1αΔ/Δ mice peritoneal macrophages and neutrophils were stimulated with TA or LTA, and expression of HIF-1α target genes that are involved in intracellular bacterial killing and cellular motility were analyzed (Fig. 3, D–H). Both TA and LTA robustly induced iNOS expression in control macrophages, an effect that was significantly attenuated in HIF-1α-deficient macrophages (Fig. 3D). This reduction in iNOS expression in HIF-1α-deficient macrophages corresponded with a decrease in macrophage intracellular bacterial killing ability. Further, TA and LTA treatment increased expression of COX-2, MMP-2, IL-1β, and IL-6 in macrophages and neutrophils derived from control mice; effects that were significantly diminished in HIF-1α-deficient myeloid cells (Fig. 3, E–H). Taken together, these results indicated that Gram-positive bacterial endotoxins mediate some of their key effects through activation of the HIF-1α signaling pathway.

**KLF2 Modulates Gram-positive Bacterial Endotoxin-induced HIF-1α Expression**—Previous work from our group and others has implicated KLF2 as a critical transcriptional regulator of myeloid cell activation and HIF-1α expression (23, 24,
29). Therefore, as a first step, we examined whether Gram-positive bacteria and bacterial products modulate KLF2 expression in macrophages. Accordingly, wild-type mouse peritoneal macrophages were exposed to heat-inactivated \( S.\) aureus, TA, and LTA separately, and expression of KLF2 at the mRNA and protein levels was examined by quantitative PCR and immunoblot analysis respectively (Fig. 4, \( A \) and \( B \)). Exposure of wild-type macrophages to heat-inactivated \( S.\) aureus, TA, or LTA significantly reduced KLF2 expression at both the mRNA and protein levels. Next, we evaluated whether KLF2 regulates the Gram-positive bacterial product-induced HIF-1 transcriptional activity in macrophages (Fig. 4, \( C \)). Overexpression of KLF2 alone significantly attenuated TA- or LTA-induced HIF-1 transcriptional activity in RAW264.7 cells. Next, RAW264.7 cells were infected with Ad-GFP/Ad-KLF2 or peritoneal macrophages from control (LysMcre/cre) and KLF2-deficient (KLF2\(^{-/-}\)) mice were stimulated with \( S.\) aureus, and intracellular bacterial killing was analyzed by antibiotic protection assay. \( D-H \), primary peritoneal macrophages and neutrophils from control (LysMcre/cre) and HIF-1\(^{-/-}\) mice were stimulated with 4 \( \mu \)g/ml teichoic or lipoteichoic acid for 6 h. Indicated target genes were analyzed by quantitative PCR and normalized to \( 36B4 \). Combined data of three experiments are shown in each case. Data represent mean ± S.D., \( * \) \( p < 0.05 \) versus indicated control.

**FIGURE 3.** Gram-positive, endotoxin-induced macrophage cell motility, bactericidal function, and inflammatory gene expression are HIF-1\(^{-/-}\)-dependent. \( A \) and \( B \), primary peritoneal macrophages from control (LysMcre/cre) and HIF-1\(^{-/-}\) mice were stimulated with 4 \( \mu \)g/ml teichoic or lipoteichoic acid and subjected to a migration or invasion assay. The number of cells migrated (\( A \)) or invaded (\( B \)) in unstimulated wells across the membrane were assigned as 100%, and fold changes over this are indicated. \( C \), primary peritoneal macrophages from control (LysMcre/cre) and HIF-1\(^{-/-}\) mice were inoculated with \( S.\) aureus, and intracellular bacterial killing was analyzed by antibiotic protection assay.

**KLF2 Regulates Gram-positive Toxic Shock Syndrome**
phages from control (LysM cre:cre) and KLF2-deficient (KLF2^+/−) mice were stimulated with TA or LTA and subjected to a cell migration assay (Fig. 5, A and B). Overexpression of KLF2 reduced and deficiency of KLF2 enhanced TA- or LTA-induced macrophage cell migration. Analysis of intracellular bacterial-killing ability of control (LysM cre:cre) and KLF2-deficient (KLF2^+/−) macrophages indicated that deficiency of KLF2 enhanced macrophage intracellular bacterial killing (Fig. 5, C).

Further, inflammatory gene expression analysis indicated that deficiency of KLF2 significantly enhanced Gram-positive, bacterial product-induced expression of iNOS in primary macrophages (Fig. 5, D). Concordant with this observation, overexpression of KLF2 suppressed and deficiency of KLF2 induced expression of IL-1β and COX2 in macrophages exposed to Gram-positive endotoxins (Fig. 5, E and F). Taken together, these results are consistent with increased expression of HIF-1α in KLF2-deficient macrophages (Fig. 4, D–G) and decreased cellular motility, intracellular bacterial killing, and inflammatory gene expression observed in HIF-1α-deficient macrophages (Fig. 3, A–H).

**HIF-1α and KLF2 Regulate the Sepsis Phenotype Induced by LTA/TA in Vivo**—We observed that Gram-positive bacterial endotoxins such as LTA/TA reduced KLF2 expression and induced HIF-1α activation and proinflammatory gene expression in myeloid cells. Given that the elaboration of excess proinflammatory factors can contribute to septic physiology, we hypothesized that HIF-1α deficiency may reduce and KLF2 deficiency may enhance susceptibility to sepsis. To test this...
KLF2 Regulates Gram-positive Toxic Shock Syndrome

The central findings of this study are that 1) Gram-positive bacterial endotoxins transcriptionally induce HIF-1α expression; 2) teichoic and lipoteichoic acid induced HIF-1α expression in a dose- and time-dependent manner; 3) Gram-positive endotoxins induced macrophage cell motility, bacterial killing, and inflammatory gene expression in an HIF-1α-dependent manner; 4) KLF2 modulates Gram-positive bacterial endotoxin-induced HIF-1α expression; 5) KLF2 suppresses TA/LTA-induced macrophage cellular functions and gene expression; and 6) HIF-1α and KLF2 regulate the sepsis phenotype induced by LTA/TA in vivo. Collectively, these observations identify HIF-1α as central regulator of Gram-positive bacterial endotoxin-mediated sepsis and that KLF2 modulates this pathophysiologic response by regulating expression of HIF-1α in myeloid cells.

The myeloid cell response to infection is a central component of host defense. These cells invade infected tissues to contain and combat the invading organism. If containment fails and bacteria/bacterial products leak out into the circulation, a vicious self-perpetuating cycle of inflammation can lead to septic shock (30). Our study provides insights regarding the molecular basis for these events in the context of Gram-positive infection. We note that tissue foci of infection are characterized by very low levels of oxygen and glucose in combination with high concentrations of lactate and free oxygen radicals. Therefore, the innate immune cells that respond to infection must adapt to these adverse conditions quickly to combat the invading organism.

hypothesis, we examined whether myeloid KLF2 or HIF-1α deficiency modulates LTA-induced sepsis syndrome in vivo. Control (LysMCre/Cre), KLF2-/-, and HIF-1α-/- mice were intraperitoneally injected with a combination of LTA and PGN. This challenge in KLF2-/- mice induced 100% mortality by 72 h, whereas control (LysMCre/Cre) mice experienced only 50% mortality. By contrast, myeloid deficiency of HIF-1α was protective against Gram-positive toxin-induced mortality (Fig. 6A). In addition, although myeloid-specific KLF2-deficient mice exhibited all the cardinal features of endotoxic shock, including hypothermia, hypotension, and elevated shock index (Fig. 6B–D), the myeloid-specific HIF-1α-deficient mice were protected from these effects. These in vivo observations are consistent with our in vitro studies that the Gram-positive bacterial endotoxins mediate their proinflammatory effects through modulating KLF2 and HIF-1α expression in myeloid cells.

**DISCUSSION**

The central findings of this study are that 1) Gram-positive bacterial endotoxins transcriptionally induce HIF-1α expression; 2) teichoic and lipoteichoic acid induced HIF-1α expression in a dose- and time-dependent manner; 3) Gram-positive endotoxins induced macrophage cell motility, bacterial killing, and inflammatory gene expression in an HIF-1α-dependent manner; 4) KLF2 modulates Gram-positive bacterial endotoxin-induced HIF-1α expression; 5) KLF2 suppresses TA/LTA-induced macrophage cellular functions and gene expression; and 6) HIF-1α and KLF2 regulate the sepsis phenotype induced by LTA/TA in vivo. Collectively, these observations identify HIF-1α as central regulator of Gram-positive bacterial endotoxin-mediated sepsis and that KLF2 modulates this pathophysiologic response by regulating expression of HIF-1α in myeloid cells.

The myeloid cell response to infection is a central component of host defense. These cells invade infected tissues to contain and combat the invading organism. If containment fails and bacteria/bacterial products leak out into the circulation, a vicious self-perpetuating cycle of inflammation can lead to septic shock (30). Our study provides insights regarding the molecular basis for these events in the context of Gram-positive infection. We note that tissue foci of infection are characterized by very low levels of oxygen and glucose in combination with high concentrations of lactate and free oxygen radicals. Therefore, the innate immune cells that respond to infection must adapt to these adverse conditions quickly to combat the invading organism (31). Our studies suggest that Gram-positive bacteria and endotoxins induce HIF-1α mRNA, protein expression, and transcriptional activity. This increase in HIF-1 signaling is beneficial to myeloid cell adaptation to the hypoxic condition by increasing the rate of glycolysis and offering protection from apoptosis (32). In addition, increased HIF-1α also enhances the production of antimicrobial agents such as iNOS from myeloid cells. The importance of HIF-1α in this response to Gram-positive infection is strongly supported by the observation that myeloid-specific deficiency of HIF-1α abrogates many of the cardinal cellular responses to infection including migration, inva-
sion, bacterial killing, and cytokine production. However, this response is truly a double-edged sword because if this induction of HIF-1 is not carefully controlled, excessive myeloid cell activation can ensue and lead to a systemic cytokine storm and endotoxic shock. Indeed, previous studies indicate that Gram-positive bacterial endotoxins can induce shock and multiple organ failure (10, 11). Consistent with this observation, we also observed dramatic mortality in response to LTA. Importantly, this effect on mortality was strongly attenuated in the absence of HIF-1. These findings are reminiscent of the protective effect of HIF-1 deficiency observed by Peyssoneux et al. (18) in the context of Gram-negative sepsis.

Given the importance of carefully titrating HIF-1 activation, stringent regulatory mechanisms must clearly exist. In this regard, the identification of KLF2 is particularly noteworthy. Previous studies from our group were first to implicate KLF2 as an inhibitor of myeloid proinflammatory activation (23, 24). More recently, we showed that myeloid-specific deletion of KLF2 led to spontaneous low-level myeloid activation even under basal conditions (24). Following stimulation with agents such as LPS, KLF2-deficient macrophages exhibited hyperinduction of HIF-1 and numerous cytokines/chemokines. In this work, we found that the same stimuli (LTA/TA) that induced HIF-1 also reduced KLF2 expression. This reduction of KLF2 is likely important, as it allows for HIF-1 induction and bactericidal activity. Consistent with this idea, overexpression of KLF2 inhibited and deficiency led to an exaggerated induction of HIF-1. Our in vivo studies also provide support for the importance of this KLF2-HIF-1 regulatory axis in vivo. Given that unbridled HIF-1 activity can be deleterious to the host, we observed that myeloid-KLF2-null mice succumbed more readily to LTA-induced sepsis.

We note that a previous study from Schwander et al. (14) indicated that lipoteichoic acid mediate their cellular effect through binding Toll-like receptor 2 (TLR2) and induction of NF-κB transcriptional activity. A recent study from our group and others indicated that Gram-negative endotoxins (e.g. LPS) bind TLR4 and induce HIF-1α expression through transcriptional activation of NF-κB (24, 28). Given these observations, it is likely that a parallel mechanism accounts for the TA/LTA-mediated induction of HIF-1. Intriguingly, NFκB activation has also been shown to inhibit KLF2 expression. Thus, the initial activation of NFκB by Gram-positive and Gram-negative endotoxins may account for the parallel induction of HIF-1 and reduction of KLF2. These observations also provide insights as to how KLF2 may prevent the transcriptional induction of HIF-1. Initial studies from our group showed that KLF2 can inhibit NFκB transcriptional activity in numerous cell types, including myeloid cells (23). More recently, we determined that this inhibition occurs through sequestration of key coactivators (e.g. p300/pCAF) required for optimal NF-κB transcriptional activity (24). Thus, a mutually antagonistic relationship exists between KLF2 and NFκB, and the balance of these two factors is ultimately important in determining HIF-1 levels and attendant downstream events.

In sum, the in vitro and in vivo observations presented here underscore the importance of a KLF2-HIF-1 pathway in regulating myeloid cell function in the context of Gram-positive

**FIGURE 6.** HIF-1α and KLF2 regulate the Gram-positive, endotoxin-mediated sepsis phenotype in vivo. A, age- and sex-matched control (LysMCre/Cre), HIF-1αlox/lox and KLF2lox/lox mice were challenged with lipoteichoic acid (3 mg/kg) supplemented with peptidoglycan by intraperitoneal injection. These mice were observed for 96 h for survival. B–D, age- and sex-matched control (LysMCre/Cre), HIF-1αlox/lox, and KLF2lox/lox mice were challenged with lipoteichoic acid (3 mg/kg) containing peptidoglycan by intraperitoneal injection and were monitored for changes in core body temperature (rectal probe), systolic blood pressure (tail cuff blood pressure monitor), and shock index (shock index = heart rate/systolic blood pressure). n = 10 mice per group in each experiment (A–D). Data represent mean ± S.D. *p < 0.05 versus the indicated control.
sepsis. These results, coupled with our recent findings in Gram-negative infections, identify this pathway as a major regulator of myeloid function in host defense. Consequently, agents targeting this pathway may allow for therapeutic gain in the treatment of bacterial infections and sepsis.

REFERENCES

1. Martin, G. S., Mannino, D. M., Eaton, S., and Moss, M. (2003) The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J. Med.* **348**, 1546–1554

2. Lever, A., and Mackenzie, I. (2007) Sepsis. Definition, epidemiology, and diagnosis. *BMJ* **335**, 879–883

3. Angus, D. C., and Wax, R. S. (2001) Epidemiology of sepsis. An update. *Crit. Care Med.* **29**, S109–S116

4. Friedman, G., Silva, E., and Vincent, J. L. (1998) Has the mortality of septic shock changed with time? *Crit. Care Med.* **26**, 2078–2086

5. Danai, P., and Martin, G. S. (2005) Epidemiology of sepsis. Recent advances. *Curr. Infect. Dis. Rep.* **7**, 329–334

6. Opal, S. M., and Cohen, I. (1999) Clinical gram-positive sepsis. Does it fundamentally differ from gram-negative bacterial sepsis? *Crit. Care Med.* **27**, 1608–1616

7. Lappin, E., and Ferguson, A. J. (2009) Gram-positive toxic shock syndromes. *Lancet Infect. Dis.* **9**, 281–290

8. van Zoonen, M. A., Verstege, M. I., Draing, C., de Beer, R., van’t Veer, C., Florquin, S., Bresser, P., van der Zee, J. S., te Velde, A. A., van Aulock, S., and van der Poll, T. (2011) Endogenous MCP-1 promotes lung inflammation induced by LPS and LTA. *Mol. Immunol.* **48**, 1468–1476

9. Campbell, J., Singh, A. K., Santa Maria, J. P., Jr., Kim, Y., Brown, S., Swooboda, J. G., Mylonakis, E., Wilkinson, B. J., and Walker, S. (2011) Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in Staphylococcus aureus. *ACS Chem. Biol.* **6**, 106–116

10. Kengatharan, K. M., De Kimpe, S., Robson, C., Foster, S. J., and Thiemermann, C. (1998) Mechanism of gram-positive shock. Identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. *J. Exp. Med.* **188**, 305–315

11. De Kimpe, S. J., Kengatharan, M., Thiemermann, C., and Vane, J. R. (1995) The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10359–10363

12. Kengatharan, M., De Kimpe, S. J., and Thiemermann, C. (1996) Analysis of the signal transduction in the induction of nitric oxide synthesis by lipoteichoic acid in macrophages. *Br. J. Pharmacol.* **117**, 1163–1170

13. Dunne, D. W., Resnick, D., Greenberg, J., and Joiner, K. A. (1994) The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1863–1867

14. Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., and Kirschning, C. J. (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J. Biol. Chem.* **274**, 17406–17409

15. Wang, J. E., Jørgensen, P. F., Almlof, M., Thiemermann, C., Foster, S. J., Asen, A. O., and Solberg, R. (2000) Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor α, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. *Infect. Immun.* **68**, 3965–3970

16. Cramer, T., Yamanishi, Y., Clausen, B. E., Förster, I., Pawlinski, R., Mackman, N., Haase, V. H., Jaenisch, R., Corr, M., Nizet, V., Firestein, G. S., Gerber, H. P., Ferrara, N., and Johnson, R. S. (2003) HIF-1α is essential for myeloid cell-mediated inflammation. *Cell* **112**, 645–657

17. Peyssonnaux, C., Datta, V., Cramer, T., Doedens, A., Theodorakis, E. A., Gallo, R. L., Hurtado-Ziola, N., Nizet, V., and Johnson, R. S. (2005) HIF-1α expression regulates the bactericidal capacity of phagocytes. *J. Clin. Invest.* **115**, 1806–1815

18. Peyssonnaux, C., Cejudo-Martin, P., Doedens, A., Zinkernagel, A. S., Johnson, R. S., and Nizet, V. (2007) Cutting edge. Essential role of hypoxia inducible factor-1α in development of lipopolysaccharide-induced sepsis. *J. Immunol.* **178**, 7516–7519

19. Semenza, G. L. (2001) HIF-1, O(2), and the 3 PHDs. How animal cells signal hypoxia to the nucleus. *Cell* **107**, 1–3

20. Semenza, G. L. (2011) Oxygen sensing, homeostasis, and disease. *N. Engl. J. Med.* **365**, 537–547

21. Nizet, V., and Johnson, R. S. (2009) Interdependence of hypoxic and innate immune responses. *Nat. Rev. Immunol.* **9**, 609–617

22. Anderson, K. P., Kern, C. B., Crable, S. C., and Lingrel, J. B. (1995) Isolation of a gene encoding a functional zinc finger protein homologous to erythroid Krüppel-like factor. Identification of a new multigene family. *Mol. Cell Biol.* **15**, 5957–5965

23. Das, H., Kumar, A., Lin, Z., Patino, W. D., Hwang, P. M., Feinberg, M. W., Majumder, P. K., and Jain, M. K. (2006) Krüppel-like factor 2 (KLF2) regulates proinflammatory activation of monocytes. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 6653–6658

24. Mahabaleshwar, G. H., Kawanami, D., Sharma, N., Takami, Y., Zhou, G., Shi, H., Nayak, L., Jeyaraj, D., Grealy, R., White, M., McManus, R., Ryan, T., Leahy, P., Lin, Z., Haldar, S. M., Atkins, G. B., Wong, H. R., Lingrel, J. B., and Jain, M. K. (2011) The myeloid transcription factor KLF2 regulates the host response to polymicrobial infection and endotoxic shock. *Immunity* **34**, 715–728

25. Behr, T., Fischer, W., Peter-Katalini, J., and Egge, H. (1992) The structure of pneumococcal lipoteichoic acid. Improved preparation, chemical and mass spectrometric studies. *Eur. J. Biochem.* **207**, 1063–1075

26. SenBanerjee, S., Lin, Z., Atkins, G. B., Greif, D. M., Rao, R. M., Kumar, A., Feinberg, M. W., Chen, Z., Simon, D. I., Luscin ska, F. W., Michel, T. M., Gimbrone, M. A., Jr., García-Cardeña, G., and Jain, M. K. (2004) KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. *J. Exp. Med.* **199**, 1305–1315

27. Blouin, C. C., Pagé, E. L., Soucy, G. M., and Richard, D. E. (2004) Hypoxic gene activation by lipopolysaccharide in macrophages. Implication of hypoxia-inducible factor 1α. *Blood* **103**, 1124–1130

28. Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A. S., Nizet, V., Johnson, R. S., Haddad, G. G., and Karin, M. (2008) NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. *Nature* **453**, 807–811

29. Humbert, M., Halter, V., Shan, D., Laedrach, J., Leibundgut, E. O., Baerlocher, G. M., Tobler, A., Fey, M. F., and Tschan, M. P. (2011) Deregulated expression of Krüppel-like factors in acute myeloid leukemia. *Leuk. Res.* **35**, 909–913

30. Serbin, N. V., Jia, T., Hohl, T. M., and Pamer, E. G. (2008) Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* **26**, 421–452

31. Semenza, G. L. (2001) Hypoxia-inducible factor 1. Control of oxygen homeostasis in health and disease. *Pediatr. Res.* **49**, 614–617

32. Walmsley, S. R., Print, C., Farahi, N., Peyssonnaux, C., Johnson, R. S., Cramer, T., Sobolewski, A., Condiffe, A. M., Cowburn, A. S., Johnson, N., and Chilvers, E. R. (2005) Hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity. *J. Exp. Med.* **201**, 105–115